"IDENTIFICATION OF A NOVEL BACTERIAL STRAIN CAPABLE OF DEGRADING TEXTILE AZO DYES"

Thesis submitted to Kuvempu University for the Degree of

DOCTOR OF PHILOSOPHY

In

BIOCHEMISTRY

Submitted by

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Declaration

I hereby declare that the thesis entitled **"IDENTIFICATION OF A NOVEL BACTERIAL STRAIN CAPABLE OF DEGRADING TEXTILE AZO DYES"** submitted to **Kuvempu University**, for the award of the degree of **Doctor of Philosophy in Biochemistry** is based on the results of research work carried out by me in the department of Post Graduate Studies and Research in Biochemistry, Kuvempu University, Shankaragatta-577451, Shivamogga, Karnataka, India, under the guidance of **Prof. Rajeshwara Achur** and **Prof. G. J. Sathisha**, Department of Post Graduate studies and Research in Biochemistry, Kuvempu University, Jnana Sahyadri, Shankaraghatta-577451, Shivamogga, Karnataka, India.

I further declare that the results presented in this thesis or any part thereof has not been submitted elsewhere for any other degree, diploma or similar title in this university or another.

Place: Shankaragatta
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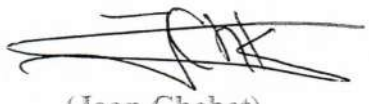

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Dedicated to God Almighty, My Supportive Family, Teachers and Friends

LIST OF ABBREVIATIONS

/	Slash
±	plus or minus
%	Per cent
h	Hour/s
°C	Degrees Celsius
e.g	for example
etc	et cetera
w/v	Wavelength
et al	and colleagues
g	Grams
mg	Milligrams
µg	Micrograms
M	Molar
mM	Millimolar
µmol	Micromoles
ml	Milliliter
L	Liter
MS	Mineral salts
OD	Optical density
NADH	Nicotine Amine Dinucleotide Hydrochloride
MSM	Mineral salt media
rpm	Revolutions per minute
UV-Vis	Ultraviolet-Visible spectrophotometry
LCMS	Liquid Chromatography Mass spectrophotometry
BBR	Bismarck Brown
OG	Orange G
CFU	Colony forming units
Decol'n	Decolorization

CONTENTS

Chapter I

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The introduction of hazardous substances into the ecosystem, endangers the lives of living organisms. The urbanization, industrialization and population growth which consequently increasing the demand for industrial products are a major source of environmental pollution globally. Transportation, agriculture, mining and oil industries are the leading sources of land, water and soil pollution. The increased release of pollutants has slowed down the rate of disposal significantly or rendered it impossible. Though some pollutants are introduced by environmental phenomena such as volcanic eruptions, floods and wild fires among others, majority are caused by human activities. Presently, the existence of pollutants in our environment is much higher compared to any other period in history making the fight against pollution is of global concern (Schell et al., 2006, Fereidoun et al., 2007).

Dye-stuff pollution has its source in dye-manufacturing and dye-consuming industries as most industries avoids the expensive effluent treatment plants. Dying industry is an important aspect of human life and plays a major role in textile, artistry, cosmetics, science, food, leather, paper and pharmaceutical industries among others. There are about 10,000 different pigments utilized in various coloring industries globally and three hundred tons of these finds use in textile sectors annually (Anjaneya et al., 2011). Textile is among the vast and economically significant industrial plants globally. However, this beautiful and treasured discovery in human history did not come without cost.

Dye related industries utilizes lots of water in wet processing and the resulting colored and contaminated waste water is deliberately or accidentally disposed into the environment (Pandey et al., 2007). Approximately 50,000 tons of synthetic colorants ends up in the eco-system worldwide every year (Chavan, 2013). The dying process is the main fount of textile pollution generating up to 80% of the sector's total waste water as large volumes of additives and technical complexity is employed in this process (Jiang et al., 2010).

Therefore, it is evident that this industry is accompanied by dreadful environmental hazards (Arora et al., 2018). Textile plants releases about 10-15% of all the dyes consumed into the surrounding water bodies (Gita et al., 2017). Azo dyes make up the largest group of all the produced dyes and the global production of azo dyes is

approximately 1000,000 tons (Anjaneya et al., 2011). Synthetic dyes are poorly biodegradable, toxic, mutagenic, alters metabolism, carcinogenic and leads to bioaccumulation. Therefore, proper treatment of waste water is essential before disposal to the environment.

1.2 History of Dyes

Pigments have been present in human life dating prehistoric periods of ancient civilizations where they played important roles in communication, painting, symbolism, ritual skin marking beauty and iron supply in the case of ochre (Gilbert et al., 2017, Robert et al., 2017, Duarte et al., 2014). Reports shows that the earlier colors were obtained from soil, water, animal fat, among other liquids (Barnett et al., 2006, Hodgskiss et al., 2017, Morriss-Kay et al., 2010). Among these early colorings was woad (natural Indigo) derived from Indigo tinctorial, Alizarine derived from madder, Campeachi an extract from wood and Tyrian purple extracted from the gland of the purple snails and processed by Phoenicians. Different colors were later derived from insects, vegetables and minerals with Medieval period marked by distinct use of dyes (Barnett et al., 2006). Red Lake was a dye synthesized in 1704 by Diesbach and this provided insight into synthesis of other dyes.

Early use of colors dates back to middle stone age periods where they were used in both Africa and Europe around 300,000 BC (Morriss-Kay, 2010). The prehistoric use of dyes included the red and black pigments obtained from rocks were used during the Palaeolithic period around 35, 000 to 10,000 BC (Hovers et al., 2003). Moreover, ochre paints have been discovered in 165,000 years old cave (Marean et al., 2007). Blue color was first produced by Egyptians around 2900 to 2750 BC and became known as the Egyptian Blue (Abel et al., 2012) followed by Han Blue developed by Chinese around 500 BC.

The purple color was so unique that it strongly influenced various cultures since only the rich and powerful could be clothed in raiment of this color. This purple pigment was later processed by snails by the Phoenicians and commercialized it around 1550-1200 BC (Stieglitz, 1994). Ochre which is an iron oxide were found as constituents of the orange, red and orange pigments while black color was mostly derived from carbon (Behrmann et al., 2009). Hence, these pigments were more available as compared to the rest of the colors such as blue which were more expensive and less available (Berke et al.,

2013). The components of the pigments produced was determined majorly by the intended use of the pigment. Owing to time, population growth, civilization and sedentary life styles there was increased need for more pigments to meet the high demand. Similarly, the use of dyes spread into various parts of the world over time (Chekalin et al., 2018).

Aniline was first prepared from Indigo pigment by the chemist, Otto Unverdorben. The first dye developed by William Henry Perkin in 1854 was named Mauveine produced from coal. Production was achieved through oxidation processes of aniline bases to produce a Violet dye cationic in nature. The picric acid produced in 1771 was used in dyeing silk to produce yellow color but this dye did not earn any notable attention. In 1859, Verguin was successful to synthesize fuchsine and Griess came up with the diazo compounds which opened doors for the production of the many classes of dyes we have today. The first true azo dye was manufactured in 1863 by Martius and was named Bismarck Brown. This marked the beginning of mass production of various new and cheap dyes (Johnston, 2008). Presently, over 100,000 synthetic dyes are available commercially (Paz et al., 2017). Globally, China is a top dye manufacturer and exporter followed by India, US, Brazil, UK and Germany, respectively. With high rates of dye production and usage, handling and proper disposal of resultant pollutants became a great challenge and an issue of environmental concern.

1.3 Classification of Colorants

i) ***Pigments*** are fine and highly insoluble solids made up of larger particles about 1-2 μm (Esteves Torres et al., 2016). During the application process, carriers such as the substrates deliver these molecules into the intended materials and maintain a crystalline or particulate state (Aspland,1993). Soluble compounds in the pigment molecule are avoided so as to maintain the insolubility aspect of the pigment which can as well be achieved by designing insoluble structures (Herbst et al., 2016; Gürses et al., 2016).

ii) **Dyes** are soluble in water and are made up of smaller particles around 0.025-1.0 μm while their appearance can be altered by selective absorption and/or dispersed light. They are known for the presence of chromophore and auxochrome in their structure. Chromophores are compounds consisting of a cluster of atoms responsible for the distinct color of the dye through the absorption of light in the near UV region. This is achieved through the fact that the group is able to absorb light in the near ultraviolet

region. Examples of chromophores are ─C═O, ─N═N─, N═O, (CH─CH)n, ─NO_2, ─C═N, C═S. Auxochrome is essential for the enhancement and attachment of the dye onto the fiber through chemical bonds. This bonding is achieved through the substitution of electrons via oxidation or reduction involving acidic or basic groups called auxochromes which includes groups such as, ─COOH, ─OH, ─SO_3H (Acidic) and ─NHR, ─NH_2, ─NR_2 (basic) (Rai et al., 2005).

1.3.1 Classification of dyes based on the nature of existence

i) Natural dyes

Natural dyes are derived from certain animals and plants which have been identified as excellent sources of dyes. The dye content in plants can be found in seeds, flowers, leaves, stems, barks or roots and the intensity of color depends on the soil, season, plant part and the age of the plant among others. Dyes have also been found in dry bodies of some insects and minerals. These dyes are further classified based on the structure and shade.

- ***Based on structure*** - Natural colorants are structurally classified into indigoids, flavones, anthocyanidin, carotenoids, anthraquinones, dihydropyrans, alpha naphthoquinones (Johnston, 2008).
- ***Based on shade*** - Based on the color index, dyes are classified by the chemical composition and major application categories. Based on the application, dyes are classified based hues. Each type is represented by a number as shown in ***Table 1-1***.

Color index of various natural dyes

Table 1-1: Dye color index

Color index	Number of dyes	Percentage (%)
Red	32	34.8
Green	5	5.5
Yellow	28	30.4
Orange	6	6.5
Brown	12	13.0
Blue	3	3.3
Black	6	6.5

ii) Synthetic dyes

These are man-made colorants and can be derived from organic or inorganic sources. Synthetic dyes are cheap, stick fast, and provide a larger gamut of colors (Paz et al., 2017). Natural dyes were limited in stability which was resolved by the discovery of synthetic dyes. Moreover, the synthetic dyes showed low production cost (Yamjala et al., 2016).

iii) Mordants

Natural dyes require the use of mordants for fixation/binding of the dye to the intended fabric through complex formation. This is due to the fact that natural dyes show low substantivity to the fibers. This enhances the resistance of the natural dye to light, detergents, water and pressure and therefore, increases fastness and prevents colors from fading. Types of mordants includes metallic, tannic acids (hydrolysable tannins and condensed tannins) and oil mordants (Johnston, 2008).

1.3.2 Classification of dyes based on the chemistry structure and application

a) Nitro dyes

These categories of dyes are made up of nitro (─NO_2) group(s) in their structure. N─O and N═O in this structure are equal due to resonance. A resonating C─C and C═C of the aromatic ring structure are also present.

b) Nitroso dyes

These structures result from reacting phenol with nitrous acid hence transferring the (─NO) group to a carbon atom either to the *para* or *ortho* position to phenolic hydroxyl group (─OH). In presence of oxygen at the *ortho* position of the nitroso, oxygen will bond with the metallic atom resulting in the formation of a pigment or a dye in case a solubilizing substituent, basically a sulphonate (─SO_3).

c) Arylmethane dyes

In this structure, all bonds are interconnected to one central carbon while the delocalized electrons spread across the conjugated system. All bonds attached to the carbon atom are identical but different from the C─H bond in methane, hence, called arylmethane (Zollinger, 1991). Some common substituents on the ring structures that dictate the structural properties of the dye are hydroxyl and amine giving rise to the

different categories of arylmethane such as hydroxytriarylmethane, diarylmethane and aminotriarylmethane.

d) Xanthene dyes

The chromophore consists of heterocyclic compound xanthene which contains oxygen atom. This structure resembles triphenylmethane with oxygen atom linking both phenyl groups. Hydrogens in this structure can be replaced by halogens. These dyes are used in textile (blue or violate) and as biological stain (Yellow or red).

e) Acridine dyes

The structure of this chromophore looks like xanthene structure with oxygen replaced with nitrogen as heteroatom. They are yellow in color, cationic and highly fluorescent. Phenanthridines are compounds whose chromophore is an acridine isomer. These groups of compounds include ethidium bromide, propidium iodide among others.

f) Oxazine dyes

In this, single atoms of oxygen and nitrogen forms a bridge between two six-atom carbon rings. Majority of oxazine dyes are metal-binding phenolic compounds or colored cations. Most pH indicators such are litmus and biological stains fall into this category.

g) Azine dyes

This chromophore is composed of phenazine, a heterocyclic aromatic group with a couple of benzene rings linked with two nitrogen atoms. The chromophore is made up of a delocalized positive charge and alternated aromatic and quinoid structures. The chromophore is basically cationic in nature, however, there some anionic azine dyes that result when sulfonate substituent groups are more than the cationic nitrogen atoms.

h) Thiazine dyes

"Thia" represents the sulfur atom in the structure. These dyes are made up of three-ring chromophore which consist of nitrogen and sulphur forming a linkage between two six-carbon rings that can be represented as quinoid or benzenoid structure. Thiazine dyes are cationic and delocalization of electrons results in partial positive charges on sulphur and on both nitrogen atoms.

i) Styryl dyes

These dyes contain styryl group linked with a tertiary amine group. The structure of the chromophore shares some similarities with polymethines and hemicyanines but in the case of this dye, the chromophore does not end in heterocyclic ring and hence, categorized separately. Few of these dyes consist of small molecules that can pass into the hydrophobic zone of polyester. In microbial staining, styryls of high molecular sizes are used as probes that penetrate in to the organelles and retained.

j) Indamine and indophenol dyes

Indamine (─N═) group forms a link between quinonoid and aromatic groups. Indamine structure involves the termination of both chains in the rings. Indophenol dyes, however, have the linkage broken by either quinoid, hydroxyl or phenolic groups.

k) Quinophthalone dyes

They are also called quinoline dyes and contain quinoline linked to phthalic anhydride with methine and azamethine at the centre of the structure. These dyes are relatively hydrophobic hence used in dyeing of plastics and polyester. They also show high affinity for cell membranes hence used for staining purposes.

l) Thiazole dyes

They are apocyanine dyes where the benzothiazole partially contain the methine that joins both rings. Ar is aromatic ring which can either be another benzothiazole or benzene. The positive charge of the chromophore results from either protonation or methylenation of the thiazole nitrogen.

m) Flavonoid dyes

The majority of the colored compounds found in plants are flavone ketones. They are referred to as neutrocyanines as the positioning of the quinonoid and aromatic rings vary among these dyes. Brazilin and hematoxylin are subjected to oxidation reaction to form flavone dyes, brazilein and hematein, respectively. These dyes alongside naphthoquinones and anthraquinones are sometimes grouped as hydroxyketone dyes (Allen, 1971).

n) Naphthoquinone dyes

Naphthols are oxidised to form naphthoquinones as phenols can be oxidized to quinones. Naphthoquinones are used in textile industry as well as in research.

o) Carbonyl dyes

Carbonyl dyes consist of carbonyl groups. Carbonyl structure is seen in ketones (R─CO─R') and aldehydes (R─CHO). In the case of quinones the C═O double bond is linked with C─C bonds giving rise to these cyclic diketones.

p) Anthraquinone dyes

In the anthraquinone dyes rings structure, the rings can be substituted or fused with other rings making these dyes. Anthraquinone dyes is the largest group of carbonyl dyes with vat and disperse dyes applied in textile industry and mordants such as carmin and alizarine. Examples of artificial anthraquinones are solvents, anionic, mordant reactive dyes.

q) Indigo and thioindigoid dyes

These are colored, insoluble compounds basically vat dyes and hence formed through oxidation of the soluble and colorless predecessors. Solubility in some cases can be achieved by adding sulphonate groups to indigoid structure. They are sometimes used as biological stains.

r) Coumarine dyes and fluorescent brighteners

These compounds are similar to flavonoid in structure. They are fluorescent and contain coumarine which can be replaced with a number of groups. This compound can be referred to as neutrocyanines or hemicyanines depending on the substituting group.

s) Stilbene dyes and fluorescent brighteners

These are synthetic compounds whose contains stilbene and aromatic substituents Ar and AR' in 4 and 4' positions, respectively. These groups find use in cotton dyeing and also applied as fluorescent brighteners used in botanical microscopy as fluorochromes.

t) Polyne dyes and fluorochromes

The chromophoric structure of these dyes consist of methine group (s) (─CH═) with an electron donor and electron acceptor at the respective ends which are mostly nitrogen atoms. In the conjugate chain, these dyes have one or more azamethine or indamine (─N═) (Gregory, 1990). Based on chemical characteristics these dyes are grouped into cyanines azomethines and cyanine-like fluorochromes.

1.3.3 Classification of dyes based on industrial application

a) Cationic or basic azo dyes

These dyes might contain either amine side chains protonated in acidic solutions or positively charged quaternary nitrogen atoms. This charged group might be part of chromogens or linked by non-conjugated carbon atoms.

b) Acid or anionic azo dyes

These dyes have their aromatic rings linked to sulphonic or carboxylic acid groups. Sulphonic acids are ionic at all pH. Hence, these colored ions mainly carry a negative charge and are drawn to proton carrying amino groups in protein substrates such as silk and wool at acidic media. On the other hand, carboxylic acids are not necessarily ionized at the same pH as the dying solution.

c) Direct azo dyes

These dyes are anionic in nature made up of larger molecules containing two or more azo linkages. These dyes have high affinity for cellulose hence used on cotton and linen. The binding to these materials is facilitated by non-ionic and non-covalent bonds.

d) Reactive dyes

This group of dyes portrays a characteristic pendant-like side chain which binds to the material of interest through covalent bonding and attach to hydroxyl group of the substrate. The most important advantage of these dyes is the wash-fastness which is more excellent.

e) Solvent azo dyes

These dyes do not dissolve in water owing to their lack of sulfonic acid and ionized amino groups. They are mostly utilized in the coloring of hydrophobic substrates such as wax, ball pen inks and some plastics.

f) Azoic dyes

Azoic dyes are insoluble in water and are developed either inside or on the surface of the receiving material through the action of the diazonium salts along with azo agents which are applied separately. Commonly used diazonium salts include azo diazo groups which are more stable. Presence of two groups in a molecule forms tetrazonium salts. These dyes are also used in histochemical procedures.

g) Vat dyes

Vat dyes are a class of dyes intended to be used on cellulosic fibres owing to their water solubility. These dyes bind the fabric and show strong fastness to photolysis, detergents, water or pressure. This group of dyes are known for the presence of at least two carbonyl group content (C=O) in the structure. This facilitates the reduction process which converts the dye into the subsequent water soluble 'leuco compounds' under alkaline conditions. Studies shows that indigo derivatives, especially halogenated derivatives produce other classes of vat such as indigoid, anthraquinone and thioindigoid. Indigo is a major natural vat dye which exist as a glucoside called indican found in different Indigofera species (Preston, 1986).

h) Reactive dyes

These dyes intended to react covalently with the nucleophilic sites of the intended fibrous material (substrate) via covalent bonds during the application. Reactive dyes produce a wide range of quality hue resistant to photolysis and washing on the fibres. These features place the reactive dyes at the high standard in markets and industries (Renfrew and Taylor, 1990). The dyes are further classified into:

- Monochloro-s-triazine dyes
- Bis(Monochloro-s-triazine) dyes
- Dichloroquinoxaline dyes
- Difluoro-5-chloro-pyrimidine dyes

- Vinyl Sulphone dyes
- Bifuctional reactive dyes

i) Sulphur dyes

Sulphur dyes are made by heating either aromatic amines or phenols or nitro groups within presence of sulfur. They consist of large molecular structures the type of phenothiazonethianthrone where Sulphur is contained as sulfide bridges and as thiazine site (Waring et al., 2013). These dyes are widely used and produce medium to heavy depths of brown, blue, black and green shades when applied to cellulose fibres. This black dye shows poor binding to fibres such as cellulose and hence, to enhance the dying ability, the black dye in presence of reducing agents is converted to leuco form which is more water soluble. Sulphur dyes can further be categorized as Sulphur, Leuco, solubilized and condensed sulphur dyes.

j) Acid dyes

These dyes are soluble in water and include a thraquinone, azo chromophores, copper phthalocyanine, and triphenylmethane. They are mostly used on nylon, leather or wool at a pH range of 3.0-7.0. It shows average wet and light fastness which is observed generally at a blue range of 5.0 to 6.0. Basically, acid dyes are applied under cationic environment which can be done using acetic acid or formic acid whose acidity varies with the properties of the particular dye. These dyes include non-metallized dyes and metal complex dyes.

k) Direct dyes

Direct dyes are cheaper, easy to apply, and further shows a broad shade spectrum. The major disadvantage of these dyes is the only moderate wash-fastness which has led to the relative replacement by reactive dyes. Direct dyes are further categorized based on chromophore, nature of application as well as fastness. Based on chromophore, these dyes fall into phthalocyanine, dioxazine, stilbene and azo among other smaller groups such as anthraquinone, formazan and thiazole groups (Burkinshaw et al., 2018).

l) Basic dyes

These dyes are normally used on nylon, paper, acrylic and modified forms of polyester. They are soluble in water and forms colored positively charged ions which are drawn electrostatically to the negatively charged anions on the fabric (substrate). The cations are localized on the ammonium group or dispersed as in the case of acridine, xanthenes and triarylmethane. Basic dyes show poor migratory properties at the boil and hence, applied with retarders.

m) Disperse dyes

Majority of these dyes contain azo groups in their structure which are easily cleaved to two amino groups by treatment with reducing groups (Aspland, 1992). Disperse dyes are less soluble or insoluble and are hence applied on hydrophobic substrates mainly polyester. They are also used on cellulose acetate and nylon though the wet-fastness on these fibers is very low.

n) Azo dyes

Azo group is represented by R1─N═N─R2 chromophore (where R1 and R2 are aromatic groups which can be substituted to generate the corresponding aromatic amines which can be further catabolized (Gholami-Borujeni et al., 2011). The azo group is generated when diazonium ion reacts with either phenol or amine with a coupling component such as phenolic or amino component. This process occurs at the *para* or *ortho* position to the amine or hydroxyl component. The reaction normally favours the *para* side, if empty. Azo dyes are the vast group of synthetic dyes consumed making up to about 70% of all industrial dyes. Studies shows that about one million tons of azo dyes are synthesized annually and presently, over two thousand diverse azo dyes are used for various purposes including textile, food, paper, leather, pharmaceutical and cosmetic industries (Luca, 2007; Fatima et al., 2021; Aracagök and Cihangir, 2013). Azo dyes are known for the characteristic azo bond (─N═N─) content in their structure. The azo compound is insoluble in water and depending on the number of the azo bonds, these dyes are referred to as mono azo, diazo and triazo dyes, if the structure contains one, two or three azo bonds, respectively. The use of azo dyes gained more popularity since their manufacturing process is simple and cheaper. Azo dyes also show numerous potentials for production of diverse structures and portrays a wide range of industrial applications.

Azoic dyes are applied to produce bright shades of color mainly orange to scarlet red shades and a few navy and back hues. The actual resultant shade developed with these azoic colorants is directly based on the specific combination of the diazo compounds which are primary aromatic amines, and the coupling agents. The azo bond develops *in situ* within the fabric during application. This occurs through the interaction of a diazonium compound with a coupling agent.

1.4 Structure and Properties of Azo Dyes

Azo chromophore consists of nitrogen atoms connected to aromatic rings in an angular pattern forming *cis* and *trans* isomers consisting of Ar─N═N─Ar. The hydrogen bond positioned between the nitrogen and hydrogen in the azo group of an adjacent polar substituting group results in the formation of trans isomers of the dyes. Dye color, among other characteristics can be significantly altered by changing the position of the nitrogen in the azo group and hydrogen in the phenolic group so as to enhance their bonding with a metal ion. Color and other characteristics of the dye are determined by different substituents in the aromatic ring.

1.5 Hazardous Effects of Dyes

Dyes are toxic, recalcitrant and visible even in low concentrations. Their long-term existence in the environment is contributed by their stability and resistance to sunlight and chemicals. Textile among other dye consuming industries utilizes large volumes of water and contrastingly, discharges contaminated colored waste water in to the environment which in turn pollutes the soil, surface water and ground water (Suteu et al., 2009). Studies have shown that about 10 to 15 percent of textile dyes remain unbound to the fibers and therefore find their way into the environment through the waste water (Khehra et al., 2006). There are up to 100,000 commercial dyes available and up to 30,000 to 150,000 tons ends up in water bodies every year. The color might not impart any significant load on the water, however, renders it unfit for both plant and animal consumption (Andleeb et al., 2010). Utilization of this contaminated water for agricultural purposes is harmful to plants and animals as the toxic chemicals enters the food chain and cause health problems to (Anjaneya et al., 2013). The recalcitrance of the azo dyes in the surrounding and the ─N═N─ group contributes significantly to their resistance to degradation/decolorization.

Careless and inconsiderate disposal of un-treated dyed effluents into the water bodies results in:

- The colored effluents alter the aesthetic nature of water resources
- Poor light penetration in to the water hence, disruption of photosynthetic activities of aquatic plants (Vijaykumar et al., 2007; Vandevivere et al., 1998).
- Presence of dyes in water bodies causes the depletion of dissolved oxygen, resulting in the death of aquatic lives.
- Inhibition of solubility of gases in the water.
- The organic and inorganic compounds which are known carcinogens and mutagens.
- Synthetic dyes alter the total organic carbon (TOC), biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids and salinity.
- Dyes causes changes in water parameters such as temperature and pH (Saratale et al., 2004).
- Azo dyes show toxicity, mutagenicity and carcinogenic effects (Pinheiro et al., 2004).

1.6 Conventional Methods of Treatment of Colored Effluents

Reclamation of water bodies is among the major concerns in the scientific field therefore, wastewater treatment plants were implemented to reduce the pollutant load on the environment. The nature, type and concentration of the dyes, location and extend of pollution determine the method of remediation to be applied. The conventional methods used in waste water treatment are physical, chemical, or biological. There are various physicochemical methods employed such as flocculation, reduction, oxidation and electro-chemical treatment (Rajeshkannan et al., 2010). However, these methods have proved ineffective, inefficient, expensive and leads to productions of much sludge and secondary products which might be more toxic. These methods are also more specific to particular dyes and might not work on the dyed effluents which is a compound of different dyes with different structures and chemicals (Bhatt et al., 2005).

1.6.1 Physical methods

These involves the elimination of contaminants using methods such as electrical attraction, gravity, Van der Waals forces and other manual filters.

1.6.1.1 Coagulation and flocculation

These techniques are effective and commonly used means of treatment of waste water treatment. The technique employs the utilization of inorganic materials like iron salts, alum and lime and organic substances such as non-ionic or anionic polymers either individually or as a mixture of other substances (Łukasiewicz et al., 2016; Hassan et al., 2009). This method is used in the elimination of color, turbidity causing agents, manganese, organic carbons and iron among others (Teh et al., 2016). Most particles found suspended in the waste water carries a negative charge.

The introduction of inorganic coagulants at isoelectric points hydrolyses the metal salts resulting in the formation of cationic groups which reacts with the negatively charged particles in the waste water leading to formation of floccules (Suopajärvi et al., 2013). To achieve the formation of larger, solid and stronger flocs, anionic or non-anionic polymeric flocculants are used and consequently, elimination through floatation, filtration or sedimentation is facilitated (Lee et al., 2012). Direct flocculation employs the use of cationic polymers with higher molecular sizes so as to cut on costs and time used in the process. These flocculants cancel the negative charge of the particles in the contaminated water and clumps the particles to form strong, solid and big floccules (Chong et al., 2012).

1.6.1.2 Adsorption

This method is by far the most preferred among other physical methods of industrial treatment colored waste water. Adsorption makes use of natural and synthetic adsorbents such as alumina, silica, clay, laumontite and ferrierite among others. Activated carbon is commonly used in removal of organic compounds based on the cost efficacy and high level of adsorption. Other adsorbents include waste red mud, fire clay, silica gels, zeolites, montmorillonite, kaolin and fullers earth (Zhou et al., 2011). Most adsorbents such as polymeric substances and synthetic cellulose are natural and environmentally friendly. Some important materials used as adsorbents includes siliceous substances (Crini et al., 2002), natural clay (Shichi et al., 2000) and zeolites (Armağan et al., 2004). Commonly used bio-sorbents include peats, chitosan and chitin (Chao et al., 2004; Wu et al., 2013), activated solid wastes (Garg et al., 2004; Anjaneyulu et al., 2005).

1.6.1.3 Membrane filtration processes

This technique uses membrane pressure in the treatment and the choice of the technique depends on the type of the waste water. Filtration processes are widely classified based on the sizes of the particles it can retain. The common classes of membrane filtration include reverse osmosis (RO), ultrafiltration, nanofiltration and microfiltration. These methods are able to retain the ions present in the dye containing water (Tahri et al., 2012).

1.6.1.4 Reverse osmosis

This method involves running water forcefully at a pressure around 7 to 100 bars through a semi-permeable membrane making this technique a form of hyperfiltration. This technique removes both organic and inorganic substances (Wiesner et al., 1992). The energy required in the separation process as well as the osmotic stress determines the efficiency of the separation. This method is efficient and retains over 90% ionic compounds (Dasgupta et al., 2015)

1.6.1.5 Nanofiltration

This technique can be used either before or after the main treatment or after to remove toxic compounds. This method uses membranes of 0.5 to 2nm pore sizes and pressures between 5 to 40 bars (Abid et al., 2012). Nanofiltration can eliminate dyes and chemical oxygen demand at a rate of 99 and 87%, respectively (Lopes et al., 2005).

1.6.1.6 Ultra-Filtration

This method facilitates the efficient separation and as well as the recovery of the colorants from the contaminated water. It involves the removal of particles of large molecular sizes. This provides a partial removal of dyes and the rate of color removal is minimal ranging from 31 to 76% and the rate at which the molecules move through the membrane directly depends on the thickness of the membrane. Ultrafiltration can be applied before or after the main treatment such as reverse osmosis (Ciardelli et al., 2001).

1.6.1.7 Microfiltration

This technique is mostly used in removing pigments from the colored waste water. It is also applied before reverse osmosis, ultrafiltration or nanofiltration as it removes most colloidal substances and suspended solids (Ghayeni et al., 1998).

1.6.1.8 Ion exchange

Ion exchange works by removing of dyes and heavy metals in the treatment of textile waste water. Ion exchange removes dyes through the interaction and bonding between the charged particles of the dye and the exchanger which can be anionic or cationic (Ahmad et al., 2015).

1.6.1.9 Floatation

This is a technique that combines water, gas and solid phases where the dye particles are subject ted to reaction with air under pressure. This combination sediments owing to its low density and heavy metals, having high density are eliminated.

1.6.1.10 Electrocoagulation

This method of waste water treatment employs redox reactions processes to eliminate metal ions under the influence of electricity. The technique is versatile and can be applied in the treatment of liquids, gases and even solids. The technique is clean due to the fact that it utilizes electrons as the main reagent (Rajeshwar et al., 1994).

1.6.2 Chemical methods

These methods are used to detoxify the waste water which is commonly achieved by the use of oxidizing agents and radiations such as ultraviolet (Gulzar et al., 2017).

1.6.2.1 Electrochemical treatment

This method of waste water treatment employs the use of redox reactions to remove metals under the influence of electric current. The technique is versatile and can be applied in the treatment of liquids, gases and even solids. The technique is clean due to the fact that it utilizes electrons as the main reagent (Rajeshwar et al., 1994). This method has been proved to be effective and includes electro-oxidation, electrocoagulation, electro-floatation and electro disinfection among others (Martínez-Huitle et al., 2009). However, the energy cost is too high.

1.6.2.2 Ozonation

Ozone is a strong oxidizing agent made up of three atoms of oxygen. The use of ozone in color removal from the colored effluents mainly depends on the dye concentration and the structure of the dye in the waste water. It has been proved as effective means of color removal (Zaharia et al., 2009). This method is clean; however, it is limited by its chemical instability which in turn requires electric intervention thereby increasing the cost of application. Ozone is also hazardous and a special ozone destruction unit should be used to prevent the entrance of the ozone into the environment which causing further deleterious effects (Davis et al., 1994).

1.6.2.3 Sodium Hypochlorite

This is a form of treatment by oxidation which uses the active form of chlorine sodium hypochlorite. This chemical decolorizes soluble dyes including reactive dyes, direct dyes and acid dyes while ineffective with insoluble dyes. Many dyes can be decolorized using this technique which is also relatively cheaper. However, dechlorination is necessary to prevent toxicity to biological activities. Sodium hypochlorite is also an oxidant with high redox potential characterized by a foul smell. It is also corrosive and shows stability at high concentrations and pH (Uygur, 1997).

1.6.2.4 Oxidative processes

This technique is applied in the treatment of waste water in the textile industries. The method employs the use of hydrogen peroxide which is activated by the ultraviolet light (Slokar and Majcen, 1994). This method works by cleaving the aromatic ring of the dye structure (Anjaneyulu et al., 2005). In this process, sodium hypochlorite is degraded to produce chlorine which in turn breaks down the amino group.

1.6.2.5 Fenton's reagents (Hydrogen peroxide)

This method is used in decolorization of colored waste water by removing a wide range of dyes such as vat, chrome and sulphur dyes among others. The technique does not generate harmful secondary products, however, the set up requires a large space and the time needed for decolorization is long. Further the dye removal rate is less effective. The hydroxyl radicals formed from this technique also shows high oxidation potential compared to those generated by ozone or Fenton reagent (Uygur, 1997).

1.6.3 Photocatalysis

This method is facilitated by the photolytic characteristics of the dyes in the waste water. Semiconductor photocatalysis can degrade dyes into simpler molecules such as carbon (IV) oxide and water and hence avoids the formation of toxic metabolites and other harmful sludge (Mamba et al., 2015). Oxidation and photocatalytic degradation with hydrogen peroxide is an area vastly explored by scientists. In the case of homogenous systems, the technique is applied alongside ozone, peroxidases and ultraviolet or visible light (Jadhav et al., 2013).

1.6.3.1 Photochemical decolorization of dyes

This method works by degrading the molecules using ultraviolet radiation in the presence of hydrogen peroxide in the waste water (Pelegrini et al., 1999).

1.6.3.2 Decolorization by ultraviolet radiation

This technique uses ultraviolet radiation alongside an oxidant in decolorization of dyed waste water. UV is used alongside catalysts like TiO_2 so as to enhance the potentiality since UV do not generate high energy. This method is considered clean as the materials needed are air, water and the radiation (Davis et al., 1994). The efficacy of this technique to remove dyes and metals depends on the temperature, pH, catalyst, illumination, design of the reactor catalysts among others (Ollis et al., 1991). This technique however produces a lot of toxic slurry which are difficult to dispose.

1.6.3.3 Decolorization by gamma radiation

This technique is effective removing up to 90% of dyes from the waste water. Further, it removes the targeted molecules even at low concentrations. However, it is expensive, leads to incomplete decolorization and results in generation of secondary products. It also leads to oxidation or reduction processes which might result in other consequent reaction with carbon (IV) oxide, salts or water (Haberl et al., 1991). Below is a summary of the various methods of the treatment of colored waste water alongside their merits and demerits **(*Table 1-2*).**

Table 1-2: Physico-chemical methods of effluent treatment

Method		Merits	Demerits	References
	Adsorption	Cheap, simple and easy to apply The adsorbent can be regenerated Removes a wide range of dyes	Adsorbents wears off with time Adsorbents are expensive	(Samsami et al., 2020)
	Ion exchange	Can be regenerated It is effective	Do not work on many dyes and molecules	(Monteagudo et al. 2000)
	Membrane filtration	No chemicals required It is effective	Membrane fouling Frequent replacement of the membrane	(Huotari et al., 1999)
	Reverse osmosis	Effective, no additional chemicals required.	High cost of technological and operational maintenance	(Ul-Islam and Shahid, 2017)
	Coagulation and flocculation	Cheap (uses natural coagulants) Reduces COD and BOD Removes most dyes	Inefficiency Large amount of sludge Utilizes non-reusable Materials and requires monitoring of parameters	(Zajda et al., 20019) (Crini et al.,2006)
	Electrochemical techniques	No other chemicals required No formation of sludge	The cost of electricity is high Less effective compared to other methods	(Lin et al., 1994)
	Oxidation	Removes a variety of dyes No sludge is formed	Formation of unwanted sludge Expensive Strict pH regulation is required	(Samsami et al.,2020)
	Sodium hypochlorite	Effective and cheaper compared to oxidants	Toxicity to biological processes, applicable on small scale, high risk of AOX	(Lee et al., 2013)
	Hydrogen peroxide	Eco-friendly	Not effective on dyes due to high oxidation potential	(Zaharia et al., 2009)
	Ozonation	Less time required, about 15 to 20 mins with less sludge formation	Expensive	(Zajda et al., 20019)
	Photocatalysis	Highly effective Cost effective Wide range of use	Utilizes UV spectrum only due the high band gap property	(Sasikala et al., 2016) (Bora et al., 2017)
	Ultraviolet Radiation	Heavy metal elimination	Sludge formation Risk of UV light scattering	(Davis et al., 1994; (Ollis et al., 1991)
	Gamma radiation	Expensive	Expensive, incomplete decolorization, generates undesired by-products	(Haberl et al., 1991

Method		Merits	Demerits	References
	Fungal treatment	Eco friendly Can be used over a wide range of pollutants over a large area	pH dependent Long lag-phase	(Samsami et al.,2020) (Solis et al., 2012)
	Enzymatic decolorizations	No toxic by-products generated Re-usable Effective	Difficult to produce large quantities of enzymes	(Singh, et al., 2015)
	Algal degradation of dyes	Eco-friendly, cheap-continuous growth in presence of sunlight	Unstable Sensitive to the surrounding factors	(Rathod and Harshad, 2014; (Rajasulochana et al., 2016)
	Bacterial decolorization	Cheap, easy to grow, Short lag phase of growth, eco-friendly	pH dependent and sensitive to shock load	(Parmar et al., 2019)
	Yeast degradation	Grows and survive under harsh environment Rapid growth	Sensitive to temperature and pH	(Dias et al., 2010) (Martorell et al., 2012)

1.6.4 Biological methods (Bioremediation)

Bioremediation refers to a process of reclaiming the polluted environment by the use of biological methods. This technique is;

- Cost effective
- Eco-friendly
- Re-usable
- Versatile
- Results in no or less sludge formation which are non-toxic.
- Organisms grow and multiply continuously on their own up on application.

Biological methods such as plants, bacteria, yeast, enzymes and fungi are embraced by scientists as this method is environmentally friendly, cost saving, efficient and results in less sludge production (Yang et al., 2003; Bhatia et al., 2017). Heavy metals inhibit biological processes and enzymatic activities and hence, it hinders the biological decolorization of waste water. However, some microbes show tolerance to these heavy metals (Saranya et al., 2017; Roan et al., 2001). These merits and pros therefore prove that biological methods are a better alternative method in reclamation of dyed-water in textile industries (Salar et al., 2012). Biodegradation refers to the process of decomposing organic compounds into simpler and smaller intermediates through different enzymatic reaction while utilizing energy (Kaushik et al., 2009).

Commonly used terms in decolorization of dye contaminated waste water include:

Mineralization is a complete degradation of dyes results and results in generation of non-toxic by-products such as ammonia, methane, water, phosphates and CO_2.

Bioaccumulation refers to agglomeration of contaminants by the growing microbial cells through metabolic processes (Aksu et al., 2003).

Biotransformation similarly refers to incomplete mineralization of organic compounds by the microbes.

Biosorption refers to the binding of the dye solute molecules to the cell biomass (living or dead) in an energy-dependent process (Martorell et al., 2018).

1.6.5 Dye decolorization by fungi (Mycoremediation)

Fungi can survive harsh environments by changing their metabolism to adapt to the new environmental conditions. In presence of dyes, fungi employ their extracellular or intracellular enzymes to facilitate a rapid metabolism and hence, degradation of the different dyes in the surrounding. Fungi secretes non-ligninolytic enzymes which include lignin peroxidases, laccases, manganese peroxidases involved in degradation of dyes (Lopez et al., 2004; Chen et al., 2015; Glenn et al., 1980). The white rot fungi and *Pharnerochaete chrysosporium* (Capalash and Sharma, 1992) are the commonly in dye decolorization and are applied in the treatment of wastes in paper, textile and pulp industries which contain polyclic aromatic hydrocarbon (PAH). White-rot fungi are the prominently used fungus either live or dead. There are other fungal strains identified with decolorization capability such as *Aspergillus niger and Trametes versicolor* as studied earlier (Singh et al., 2010; Amaral et al., 2004). The studies carried out by Yesilada on the decolorization of Astrazone Black, Blue and Red by fungus *Fuinalia trogii, Coriolus versicolor, Pleurotus florida, Pleurotus ostreatus, Pleurotus sajorcaju and P.chrysosporium* and all the fungus tested were able to remove over 75% of the dyes (Yesilada et al., 2003).

Further, *Penicillium* sp. QQ was able to decolorize the selected dyes Methanil Yellow G, Reactive Brilliant Red, Acid Orange and Red B, Direct Fast Black through adsorption and it was observed that the fungus took the color of the azo dye at the beginning of the of decolorization which is then followed by complete mineralization of Acid Red B, Direct Fast Black G, Acid Orange G and Reactive Brilliant Red X-3B (Gou et al., 2009). Decolorization of methylene blue by inviable fungi *Aspergillus fumigatus* at different parameters like, concentration, pH, temperature and time showed that decolorization was the

highest at pH 7 with up to 70% decolorization at ambient temperature, with a decreased particle size for 120 min contact time under shaking conditions (Kabbout and Samir., 2014). Studies carried out by Singh and Singh on decolorization of Basic Blue Acid Red, Congo Red, Direct Green and Bromophenol Blue by *Trichoderma harzianum* which is used in the treatment of textile waste water clearly showed the accumulation of the dye as observed from the change in the color of the mycelium (Singh and Ved, 2010). These was accompanied by the retarded fungal growth where 43, 29, 18, 13 and 9% growth inhibition by Bromophenol Blue, Congo Red, Acid Red, Basic Blue and Direct Green respectively was observed which further confirmed the toxic effects of dyes. The fungal strains *Ulva lactuca, Caulerpa sp., Ulva reticulate, Ulva clathrate broth were investigated for decolorization and Ulva lactuca* showed excellent results while *Caulerpa sp., Ulva reticulate, Ulva clathrate* broth showed good results of up to 65-85% decolorization of the effluents. The rate and efficiency of decolorization also increased with time from 16 h to 24 h (Singh and Ved, 2010).

1.6.6 Dye decolorization by yeast

Various species of yeast are known dye adsorbents which can decolorize colored waste water by taking up high dye concentrations from the dyed-effluents. Yeast species are selected their fast growth rate and the capacity to tolerate various unfavourable environmental factors including temperature and pH, aeration and temperature (Khan et al., 2013). The commonly used yeast species in removal of colorants in the textile waste water are *Ascomycetous* such as *Saccharomyces*, *Candida, Debaryomyces* and *Kluyveromyces*. There are various studies on identification of potential yeast species for the decolorization of colored waste water as reported by Martorell et al., (2018) where basidiomycetous yeast *Trichosporon* and *Rhodototorula* was used to decolorize Reactive Black 5 dye.

Decolorization of Reactive Brilliant Red K-2BP by 44 strains of yeast and 16 strains among them are *Saccharomyces italicus*, *P. rugulosa, C. krusei* and *Saccharomyces chevalieri* expressed effects on the color of the medium containing the dye by removing the color from the media after the experiment whereas other strains could not decolorize the dye (Yu and Xianghua, 2005). Six of these strains were able to remove up to 70% of the dye after 70% and G-1 and Y-48 strains were able to remove up to 90% deodorization of Reactive Brilliant Red after 18 h. Immobilized yeasts have also been employed in the treatment of colored waste water as applied by (Kakuta et al in 1992) where *Candida*

curvata was used in waste water treatment (Danouche et al., 2021). A study conducted by Kim et al., (1995) also showed the decolorization of a wide range of dyes by *G. candidum* present in both liquid and solid states where the rate of decolorization was dependent on oxygen and energy source (Kim et al., 1995).

1.6.7 Dye decolorization by plants (Phytoremediation)

Some members of the kingdom plantae have been identified with dye eliminating properties. The most prominent in this group if algae though few other plants also possess the same capability.

1.6.8 Dye decolorization by algae (Algal remediation)

Algae are classified as macroalgae or microalgae. Algae requires no maintenance or storage as their growth depend on the sunlight, CO_2 do not generate any sludge, cheap and easy to grow. The algal treatment of dye effluents is easy, eco-friendly and cheap (Pandya et al., 2017). Algae is often chosen for its high surface area and binding capacity, moreover, algal biomass also provides a good sorbent in decontamination of colored effluents (Gupta et al., 2014).

Algal removal of dyes involves the consumption of the dye by algae utilizing through biosorption into its surface to facilitate the algal growth and in this process, decolorizes the dyes (Khataee et al., 2013). In the initial step of dyes removal, the algae utilize the dye chromophores to its biomass that is, water and CO_2 (Bhatia et al., 2017). Followed by an algae facilitated conversion of the chromophore to a non-chromophore material. Finally, this is followed by the absorption of the resultant chromophore into the algal biomass follows (Samsami et al., 2020).

Microalgae can be easily grown Microalgae inhabit and survives in polluted waste water and genera such as *Chlorella, Scenedesmus* and *Nitzschia* are known to show high tolerance to pollutants in the waste water (Muñoz and Guieysse, 2006). Chlorella vulgaris also had potential to remove nitrates and COD while *Scnendesmus quadricauda* showed removal of Amido Black, BOD and reduction of phosphates (Pandya et al., 2017). Cyanobacterial species which includes *Oscillatoria* sp. *Nostoc* sp. *Synechococcus, Nodularia* sp and *Cyanothece* sp. have proved to be highly potent in biodegradation and biosorption of dyes with varying capability.

Algal removal of dyes has also been investigated by Daneshvar et al., (2005) where *Cosmarium* sp. removed malachite green dye and they noted that decolorization was

inversely proportional to the algal biomass, that is, 87% decolorization at 4.5×10^6 cell/ ml. There are seaweeds along the shore lines and coastal areas which have been identified with capability to decolorize colored waste water. Macro algae have also shown potential to decolorize synthetic dye-effluents collected from textile mills (Pandya et al., 2017).

1.6.9 Decolorization by higher plants

Some plants take up the dyes thereby decolorizing the colored effluents. Tolerance to the target pollutants is a key factor as contaminants is a key factor in the choice of dye eliminating plants as dyes can inhibit plant growth or seed germination. Plants releases photosynthetic carbon which promotes the growth of various enzymes like laccase and peroxidase. These in turn stimulate dye decolorization (Vyas and Hans-Peter, 1995). Their roots also pump oxygen into the rhizosphere to form anaerobic and aerobic environment which is the vital microenvironment for biological degradation of azo dyes (Ong et al., 2009).

Studies conducted by Mbuligwe and Stephen, (2005) showed a successful decolorization of colored waste water using the Engineered Wetland Systems which comprised of cocoyam and cattail plants (Mbuligwe, 2000). The results obtained indicates that the plants Engineered Wetland Systems could eliminate up to 72-77% of the dyes, 68-73% of COD and 53-59% of sulphates. The high percentage of COD removal indicates that the removal of the color was accompanied by complete dye mineralization. They also compared the efficiency of the unplanted and vegetative EWS beds and found that the vegetative beds were twice as efficient compared to unplanted beds. Less research has been done on decolorization by higher plants and therefore detailed mode of action is not conclusive and the exact mechanism of dye biodegradation is not properly understood.

1.6.10 Enzymatic degradation/decolorization of dyes

Enzymatic degradation/decolorization has recently become a subject of scientific interest. These enzymes include peroxidases, oxidases, polyphenol oxidases, oxidoreductases among others. Enzymes are able to decolorize the dyed-waste water through interaction with a vast spectrum of aromatic compounds in aqueous medium (Liu et al., 2017). Enzymes act on contaminants by converting complex pollutants into simpler intermediates that are less toxic or non-toxic through reductive or oxidative reactions (Gholami-Borujeni et al., 2011).

Azoreductase an enzyme derived from microorganisms catalyses the breaking of azo bonds reductively to form simpler intermediates (Punj and Gilbert, 2009). The use of pure enzymes is not the best option in the treatment of colored waste water owing to high cost of production, enzyme turnover and minimal availability in their pure forms. However, crude enzymes have also been used (Gholami-Borujeni et al., 2011). Enzymes are also specific to the substrates and sensitive to various parameters such as pH, temperature, substrate concentration and salts.

Laccases (EC 1.10.3.2)

Some laccases have been identified with dye decolorizing ability (Rodriguez et al., 1999). Decolorization of various dyes by laccase in an enzyme-reactor was studied by Kandelbauer et al., (2004) with an enzyme derived from *Trametes modesta*. Among the dyes decolorized were, Indigo Carmine, anthrachinoid dyes such as Lanaset Blue2R and Terasil Pink 2GLA and azo dyes such as Diamond Fast Brown and Diamond Black PV. According to Erkurt et al., (2007) decolorization of Drimaren Blue CL-BR and Remazol Brilliant Blue Roya was studied with the selected fungi that is, *Copriolus versicolor, Funalia trogii* and *Pleurotus, ostreatus*. From the findings obtained, the activity of laccase increased with the addition of the dyes and decolorizing ability increased with the increase in laccase activity. Moreover, Hadibarata et al., (2012) reported the complete decolorization of 200mg/L of anthraquinone RBBR by a purified laccase (UL-1) at pH 5 and 50°C temperature.

Peroxidases (1.11.1.X) and oxidases (1.1.3.X)

Peroxidases and oxidases are a group of enzymes called oxido-reductases containing hydrogen peroxide and a dioxygen respectively as an electron acceptor. The end products of the reactions are water and hydrogen peroxide (Phale et al., 2020).

Lignin peroxidases (EC 1.11.1.14)

Lignin peroxidase are topical enzymes belonging to the group of ligninolytic enzymes. According to Verma and Madamwar neem hull wastes were used in the release of this enzyme by *Phanerochaete chrysosporum* through fermentation. The enzyme was used in decolorization of Porocion Brilliant Blue HGR, Ranocid Fast Blue, Acid 119 and Navidol Fast Black MSRL where a maximum of 80, 83, 70, and 61% decolorization was achieved respectively (Verma and Madamwar, 2002).

Lignin peroxidase derived from *K. rosea* MTCC 1532 was able to decolorize 60, 90, 100, 70, 80, 90, 100, 100, 60, 80 and 60% of Amido Black, Orange HE2R, Reactive Blue 25, Direct Blue 6, Direct Blue 6, Reactive Yellow 81, Red HE4B, Green HE4B, Methyl Orange, Cotton Blue, Methyl Violet and Reactive Green 19 A dyes respectively where the difference in decolorization percentage is attributed to variation in the structure of dyes involved (Parshetti et al., 2012). Rodriguez and colleagues reported that the lignin peroxidase derived from *P. chrysosporium* was able to breakdown a number of with different structures such as azo dyes, heterocyclic, triphenylmethane like crystal violet among other dyes through a series of N-demethylations (Rodriguez et al., 1999).

Manganese peroxidases (MnP, EC 1.11.1.13)

Manganese peroxidase is an extracellular peroxidase enzyme and is reportedly responsible in oxidation of lignin compounds. MnP extracts an electron from aromatics nucleus and therefore leads to formation of unstable cation radical which consequently undergo multiple breakdown and transformation resulting in degradation of the substrate (Kersten et al., 1985). Manganese peroxidase contains an easily dissociable "heme" and requires Mn^{2+} for activity. This enzyme oxidizes Manganese (II) to Manganese (III) in presence of "heme" and Mn^{2+} to facilitate the oxidation of various compounds and produce hydrogen peroxide (Paszczyński et al., 1988; Glenn et al., 1980).

Manganese peroxidase has been investigated for decolorization of textile effluents for the purpose of treatment of colored waste water and has proved to be a good decolorizer. MnP1 and MnP2 enzymes purified from *B. adusta* were able to decolorize the selected anthraquinone and azo dyes following Mn^{2+} independent reactions. MnPL1 and MnPL2 derived from *P.eryngii* were also able to decolorize the same dyes through the independent oxidation of 2,6-dimethoxyphenol and veratryl alcohol by Mn^{2+} (Heinfling et al., 1998).

Kariminiaae-Hamedaani et al., (2007) reported the use of white rot fungus (L-25) in the decolorization of 12 dyes consisting of azo, diazo and anthraquinone dyes. A high percentage decolorizations of 84.9-99.6% was achieved within 14 days of cultivation at an initial dye concentration of 40mg/L. The strain L-25 secretes MnP as its major ligninolytic enzyme and plays an active and major role in dye decolorization as evidenced by the fact that the previously adsorbed dye disappeared from the cells immediately after the release of MnP by L-25 (Kariminiaae-Hamedaani et al., 2007).

Polyphenol oxidases (PPOs EC. 1. 14.18.1)

Polyphenol oxidases are enzyme responsible for browning of vegetables and fruits. These are copper protein groups found widely distributed in living organisms. These enzymes catalyze the oxidation of hydroxy phenols to quinones which polymerizes subsequently (Shi et al., 2001). Polyphenol oxidases catalyses a wide range of reactions and acts on a vast spectrum of substrates including dyes. However, the large-scale use of this enzyme is limited by its low enzymatic activity, catalytic efficiency and high purification costs.

Decolorization of dyes by polyphenols has been investigated in the search for a clean and environmentally friendly method of textile waste water treatment. According to the study conducted by Jadhav and co-workers, dyes were incubated with banana PPO in phosphate buffer at 30°C and they observed that the PPO derived from banana could degrade the seven dyes tested that is, Direct Yellow 5GL, Direct Blue GLL, Reactive Navy Blue, Direct Red 5B, DK Red 2B, Reactive Blue HERD and Blue 2RNL (Jadhav et al., 2011). The rate and efficiency of decolorization also varied depending on the dye structure.

Tyrosinase (EC1.14.18.1)

Tyrosinase is a type III tetrameric oxidoreductase enzyme with four copper atoms in each molecule. The structure also has sites for binding two aromatic groups and oxygen. In the treatment of waste water, crude tyrosinase is preferred due to its cost-effectiveness over purified enzyme (Suryamathi et al., 2021). Tyrosinases exhibits the capability to remove organic pollutants from the environment which is demonstrated by decolorization of dyes such as Direct Red 5B, Yellow 5G, Direct Blue GLL, Brown R, Reactive Black and Reactive Red 141 (Singh et al., 2015).

Decolorization efficacy of Tyrosinase enzyme has been illustrated by Wang and co-workers with Tyrosinase rTYR isolated from a bacterial strain *B. aryabhattai* TCCC 111983 (TYR). This enzyme could decolorize erythrosine and carmine effectively. In the absence of mediators, rTYR was able to decolorize over 88% these dyes at pH 5.0. Moreover, it was able to decolorize 93 and 78% of carmine and erythrosine dyes respectively at pH 7.0 while 59 and 77% decolorization was achieved at pH 9.0 (Wang et al., 2021).

Azoreductases (EC 1.7.1.6)

These enzymes are responsible for the decolorization azo dyes by bacteria, microbiota and fungi which take place either inside the cell or outside (Chen et al., 2006). Azoreductases facilitates azo dye decolorization by catalyzing the reductive cleavage of azo bonds either aerobically or anaerobically to form colorless aromatic amines. This is an enzymatic reaction involving the transfer of the reducing equivalents generated from substrates oxidation into the dye. Based on the co-factor requirement, azoreductases are categorized as flavin dependent or flavin independent. Flavin dependent azoreductases are further classified as NADH or NADPH dependent (Matsumoto et al., 2010) and catalyze the reactions in presence of NADH or NADPH or $FADH_2$ as cofactors (Zimmermann et al., 1982).

Azoreductases can be substrate specific based on the number of dyes the it can decolorize. An enzyme decolorizing a broad-spectrum dye is more efficient decontamination of industrial and textile dyed-effluents with mixed dye content (Pandya et al., 2017). Oxygen sensitive azoreductases do not catalyze dye decolorization under aerobic/shaking or agitated conditions. Oxygen competes with the dye for the redox mediator and hence blocks the reduction of the dye (Kudlich et al 1997). Studies have been done to determine the decolorization efficiency of azoreductases in dye degradation/decolorization. Dong et al., (2019) reported that the azoreductase AzoR2 derived from *Streptomyces* sp. S27 was able to decolorize azo dye waste water with high stability to pH and organic solvents and Methyl Orange indicating that it can remain active and decolorize the colored waste water which are known for organic matter, organic solvents and mixed dye content. This enzyme was able to decolorize Methyl Red removing up to 99% of the dye within 120 min at a concentration of 250µM (Dong et al., 2019).

1.7 Microbial degradation of azo dyes

Microbial degradation of synthetic dyes involves a complex process where various factors such as temperature, pH, dye concentration, microbial load, oxygen concentration, microorganism, enzyme activity among other factors. Microorganisms decolorizes colored effluents under facultative, aerobic, anaerobic or combination of all.

Bacterial degradation of azo dyes

Diverse groups of bacteria have been investigated to determine their role in azo dye decolorization (Pandey et al., 2007). Isolation of pure bacterial cultures with dye decolorizing abilities begun as early as 1970 where species like *Bacillus subtilis, Aeromonas hydrophila* and *Bacillus cereus* exhibits dye decolorizing capabilities. Presently, there are significant amount of research on decolorization or even degradation of azo dyes by bacterial species such as, *Pseudomonas* sp, *Proteus mirabilis* and *Pseudomonas luteola* among others which have exhibited promising results on azo dye decolorization under anaerobic conditions (Saratale et al., 2011). The use of microbes especially bacteria in degradation/decolorization of azo dyes has proved effective various with merits:

- Environmentally friendly,
- Low cost,
- Leads to complete mineralization of the dye
- Leads to generation of less toxic end products
- Less water consumption
- No electricity cost
- Applicable to a wide spectrum of dye variety
- Less sludge generation (Rai et al., 2005; Khehra et al., 2005).

The use various bacterial strains in decontamination of colored effluents has gained much popularity in the recent times. This treatment begins with the microbes acclimatizing themselves to the toxic contaminants in the waste water leading to natural development of new and resistant strains which are able to convert the harmful pollutants into less toxic products. The use of bacteria in dye decolorization/degradation processes came into use about 20 years ago and has proved to be normally much faster, more effective and convenient to apply compared to fungal systems. Decolorization of azo dyes is a process initiated by azoreductase an enzyme which catalyzes the breakdown of the azo bonds in absence or limited oxygen supply. Azoreductase enzymes catalyses the aerobic or anaerobic reduction of the azo linkage to generate intermediates, that is, aromatic amines (Singh et al., 2015). The resultant aromatic amines can further be degraded aerobically to simpler products (Joshi et al., 2008; Van der Zee et al., 2005). Decolorization of dyes by bacteria mainly occurs under anoxic conditions by species such as *Proteus mirabilis, Aeromonas*

hydrophilia, Pseudomonas luteola, Bacillus subtilis among others (Sandhya et al., 2005).

Azo dyes are decolorized by different bacterial strains under aerobic, anaerobic and anoxic conditions. Bacteria are chosen for remediation of dye contaminated water owing to their fast growth rate as compared to other organisms. Studies have shown that bacteria are able to contaminants using these materials as energy source (Yang et al., 2014).

Research has been done to identify a competent bacterial strain which can be employed in decolorization/degradation of azo dye contaminated waste water efficiently and rapidly. The rate and efficacy of microbial color removal depends the activity and adaptability of the selected microbe. There are diverse Gram-positive bacterial strains with the ability to decolorize a wide spectrum of dyes with diverse structures and they include *Bacillus subtitlis, Proteus* sp., *Pseudomonas aeruginosa, Clostridium perfringens* and *Pseudomonas putida* among others.

Also, Gram negative bacteria such as *Enterococcus* sp. and *Klebsiella pneumoniae*, *Escherichia coli* have proved effective in decolorization of dyes. Bhatia et al., (2017); Liu et al., (2006) studied the decolorization of four selected azo dyes (B, Acid Red G, RBR X-3B, Acid Red and Reactive Blue GL by *Rhodopseudomonas palustris* AS1.2352 at a dye concentration of 50mg/L. This strain was able to decolorize these dyes anaerobically at a temperature range of 30 to 35°C and pH 8.0. The optimum pH was 8.0 where the strain could remove 90% of the dye. 90% decolorization was realized at a temperature between 30 to 35°C after 24h of incubation. A decolorization of up to 70% was also achieved over a wide range of dye concentration within 30 h (Liu et al., 2006).

Dye decolorization efficacy has also been investigated with *Aeromonas hydrophila* by this strain shows excellence in elimination a larger number of dyes and exhibits rapid growth under aerobic conditions. Highest decolorizations ability was observed under anaerobic conditions at the pH 5.5-10.0 and 20-35°C temperature. Further, this strain was able to remove over 90% of RED RBN dye at 3000mg/L concentration after 8-days of incubation period (Chen et al., 2003).

Earlier investigations on bacterial decolorization of dyes

Decolorization of dyes by bacteria has been investigated from as early as 1970s, however, the search for a more potent, effective and efficient strain is ongoing. Research also deals with the identification of bacterial strains that can decolorize a wide range of

dyes at higher concentrations and tolerate adverse environmental conditions. Below is a list of some strains alongside the decolorized dye, physicochemical parameters and references (***Table 1-3***).

Table 1-3: Bacterial decolorization of dyes

Bacterial Strain	Dye	Conc.	pH & Temp. (°C)	Time	% Decolori-zation	References
Bacillus fusiformis, KMK5	Disperse Blue 79 Acid Orange 10	1.5g/L each	pH 9.0 37°C	48h	100%	(Kolekar et al., 2008)
Pseudomonas sp. SUK1	Reactive Red 2	5g/L	pH 6.2-7.5 30°C	6h	96%	(Kalyani et al., 2008)
Micrococcus glutamicus, NCIM 2168	Reactive Green 19 A	50mg/L	pH 6.8 37°C	42h	100%	(Saratale et al., 2009)
Klebsilella pneumoniae R5-13	Methyl Red	100mg/ L	pH 6.0-8.0 30°C	168h	100%	(Wong et al 1996)
Pseudomonas sp. SU-EBT	Congo Red	1g/L	pH 8.0 40°C	12h	97%	(Telke et al., 2009)
Rhizobium radiobacter, MTCC 8161	Reactive Red 141	50g/L	pH 7.0 30°C	48h	90%	(Telke., 2008)
Pseudomonas sp.	Reactive Blue 13	200mg/ L	pH 7.0 35°C	70h	83.2%	(Lin et al., 2010)
Proteus mirabilis	RED RBN	1g/L	pH 6.5-7.5 30-35°C	20h	95%	(Chen et al., 1999)
Pseudomonas aeruginosa, NBAR12	Reactive Blue 172	500mg/ L	pH7.0-8.0 40°C	43h	83%	(Bhatt et al., 2005)
Exiguobacterium sp. RD3	Navy Blue HE2R	50mg/L	pH 7.0 30°C	48h	91%	(Dhanve et al., 2008)
Citrobacter sp. CK3	Reactive Red 180	200mg/ L	pH 7.0 32°C	36h	96%	(Wang et al., 2009)
Rhodopseudo-monas palustris AS1.2352	Reactive Brilliant Red; X-3B	50mg/L	pH 8.0 30-35°C	24h	90%	(Liu et al., 2006)
Acinetobacter Calcoaceticus NCIM-2890	Direct Brown	50mg/L	pH 7.0 30°C	48h	91.3%	(Ghodake et al., 2009)

1.8 Decolorization of dyes by bacterial consortium

Individual bacterial strains are effective at eliminating dyes from colored effluents. However, they are limited in complete mineralization of dyes due to the structural differences of various dyes. This leads to generation of secondary by-products that will require further degradation (Tan et al., 1999). Removal of dyes and other xenobiotics using a single bacterial strain often lead to incomplete mineralization and hence the secondary products accumulate (Joshi et al., 2008; Kudlich et al 1997). Investigation into the breakdown Mordant Yellow 3 by a bacterial consortium under low supply of oxygen supply resulted in the reduction of the dye. This reduction led to generation of two aromatic amines that is, 6-aminonaphthalene-2-sulfonate and also 5-aminosalicylate. Moreover, re-aeration of the culture led to further mineralization of these metabolites by different members on the consortium resulting in complete degradation of the dye. This proves that complete bacterial mineralization of dyes can be reached by alternating aerobic and anaerobic processes using a mixed culture (Haug et al., 1991). It is evident that degradation/decolorization of dyes and other xenobiotics by bacterial consortia ifs fast, effective and efficient. This is achieved as different catabolic reaction in the bacterial mixture complement one another leading to complete mineralization of even complex xenobiotic compounds (Knackmuss and Hans-Joachim, 1996). This suggests that in the mixed cultures, the single strains utilize the intermediate products generated by the other strains in the consortium, further degrading them to simpler forms. Another pathway involves the attack on the dye molecule from different positions the structure by the single strains involved.

Generally, the intermediate products (aromatic amines) which are mostly harmful are further broken down by the complementary communities in the consortium thereby achieving high efficacy (Saratale et al, 2011). The advantages of using bacterial consortia in effluent treatment over individual strains are further expounded in the report by (Joshi et al., 2008) on decolorization of structurally different azo dyes by a novel bacterial consortium (TJ-1). This consortium showed high efficacy to decolorize Acid Orange 7 among others as compared to individual strains which further explains the significance of complementary interactions among different strains in elimination of xenobiotics. These outcomes therefore clearly indicate that the treatment of waste water using mixed bacterial communities leads to high degree of biomineralization/decolorizations/biodegradation

compared to pure cultures. This efficiency might result from synergistic activities of the diverse bacterial communities involved (Khehra, et al., 2006).

1.9 Factors affecting bacterial decolorization of dyes

Agitation

Shaking can be applied as a means to enhance the even distribution of supplements such as carbon and nitrogen sources as well as the circulation of oxygen and therefore promote cell growth and consequently, the biodegradation process. The same effect is also observed in fungal decolorization as explained by Hadibarata and colleagues on the breakdown of Reactive Black 5 by *P. eryngii* where 93.57% decolorization was observed with agitation of 120rpm. This percentage decreased to 35.03% with an increase in agitation to 160 rpm. (Hadibarata et al., 2012).

Aeration

Oxygen supply is a vital factor in bacterial dye degradation. Decolorization of Fast Red in static as well as shaking conditions was investigated and it was observed that the decolorization rate was lower in the shaken cultures compared to the static ones. The maximum percentage of dye decolorization was 85.4%, achieved within 12 h incubation period in a flask containing 40 ml media and under static conditions. These results clearly indicate that the dye decolorization by *B. subtilis* HM was higher under anaerobic conditions. This implies that oxygen supply does not favour decolorization rates (Mabrouk et al., 2008).

Anaerobic decolorization

Anaerobic decolorization has also been analyzed by Knapp and Newby, (1995). It has been mostly established that oxygen inhibits the microbial decolorization based on the fact that decolorization efficiency is higher under static conditions and less or absent under shaking conditions. This is because shaking promotes the supply of oxygen (Kalme et al., 2007) whereas low oxygen concentration is essential for the degrading/decolorization enzymes. Studies have shown that the effects of this oxygenation process are irreversible (Pearce et al., 2003).

Concentration of the dye

Decolorization rate is inversely proportional to dye concentration. This is might result from dye toxicity to individual bacteria and/ or inadequate biomass concentrations. Excess dyes molecules can also block the actives sites on the respective enzymes as well as the unproportionable enzyme-dye ratio (Hadibarata et al., 2012; Sani et al., 1999). As explained by Parshetti et al., (2006), the decolorization of malachite green at a concentration range of 10, 30, 50, 70 and 100mg/L by *Kocuria rosea* MTCC 1532 decreased with an increase in the concentration of the dye. The strain was able to decolorize up to 100% of the dye at a concentration of 10, 30 and 50mg/L after 2h, 3h and 5h incubation respectively. The rate of decolorization decreased at a concentration beyond 50mg/L as 13% and 6% decolorization only was exhibited at 70 and 100mg/L of the dye respectively which infers that Malachite Green was toxic to the strain at high concentrations.

Effect of dye structures

Dyes with diverse structures also affect decolorization as some inhibits the microbial growth at higher concentrations. This is commonly observed with some reactive groups such as sulfonic acid groups (Chen et al., 2003). It is generally established dyes possessing simple structures and low molecular weights exhibits high rates of decolorization. Further, the presence of substitution electron withdrawing groups like SO_3H and $-SO_2NH_2$ located on the para side phenyl ring structure in azo dyes can be easily displaced in monoazo dyes compared to diazo, triazo and others with more than one azo bond (Hsueh et al., 2009; Hu *et a., 2001).*

Effect of temperature on decolorization

Temperature is a vital factor in microbial decolorization of dyes and generation of activation energy. Temperature also affects the microbial growth rates reaction mechanism and biomass yield (Angelova et al., 2008). The effect of temperature on decolorization of Reactive Red 22 by *Pseudomonas luteola* was investigated at a range of 20-45°C. In this study, the decolorization rate increased with the increase in temperature and up to 98% was exhibited at 45°C beyond which the rate decolorization decreased sharply. In fungi, the effect of temperature has been investigated at a temperature range of 15-40°C by (Tony et al., 2013).

It was found that the fungal community *P.eryngii* F032 could decolorize the dye Reactive Black 5 with an increase in decolorization from 46.37 to 96.77% as the temperature rose from 15-40°C which could be attributed to the increased solubility of laccase enzyme at high temperatures. The decreased decolorization with increased temperature beyond the optimum this might result from the loss of cell or the denaturation of the enzymes involved such as azoreductase by the excess heat (Chang et al., 2001., Khan et al., 2013).

Effect of pH on bacterial degradation/decolorization of dyes

pH plays a vital role in bacterial decolorization of dyes a process which is mostly enzymatic and therefore pH sensitivity is expected. Previous studies conducted by Chang et al., (2001) on decolorization of the dye Reactive Red 22 carried out at a pH range of 5-9 shows that *Pseudomonas luteola* could decolorize the dye at higher pH. Similar reports were made by Modi et al., (2010) on the influence of pH on decolorization of azo dyes by the isolated strains M1 (*B.cereus*). The results showed that *B.cereus* decolorized RR195 at pH range of 7-7.5 with an almost similar decolorization percentage of about 80% and beyond pH7.5 the decolorization decreased. M6 on the other hand showed good decolorization at a pH range of 6.5-7.5 and a maximum decolorization of 87% was achieved at pH 7.0 (Modi et al., 2010).

Redox mediator

Redox mediators play a key role in microbial decolorization of dyes under anaerobic conditions. Compounds of Quinone origin have proved their efficacy as redox mediators and works. These compounds promote the transfer of electrons from a primary donor to a terminal acceptor in both chemical and microbial reactions (Khan et al., 2013). Earlier studies have proved this as demonstrated by Guo et al., (2012) and the results showed that decolorization of Acid Red B dye by the *Halomonas* sp. was increased by 2.68, 2.58, 1.91 and 1.49 times by adding 1,5-dichloroanthraquinone, 1,8-dichloroanthraquinone, anthraquinone and 1,4,5,8-tetrachloroanthraquinone respectively (Guo et al., 2012).

Effect of electron donor

In some cases, the contaminants of the textile waste water appear in low but toxic concentrations. Therefore, they are insufficient substrate sources for bacterial growth and complete decolorization cannot be achieved. It is therefore important to provide an

external substrate for complete decolorization of dyes. This fact is supported by the results obtained by Telke and colleagues on the study which explains the decolorization of the dye Reactive Orange using a combination of phenol oxidase and NADH-DCIP reductase enzymes of *Bacillus* sp. ADR. In this process, decolorization is achieved as ADR detoxifies the sulfonated azo dye in a reaction that releases the azo bonds as molecular nitrogen which in turn inhibits the generates aromatic amines (Telke et al. 2009).

Supplementation (Carbon and Nitrogen)

Azo dyes are deficit of carbon and nitrogen sources making decolorization difficult in absence of these substrates. Therefore, a supply of organic carbon and nitrogen in the form of yeast extract, peptone or carbohydrates is essential (Khan et al., 2013). Azo dye reduction involves the transfer of the reducing equivalents from different carbon sources in to the dye. However, it is observed that this carbon transfer is less effective in enhancing the azo dye decolorization. This can be attributed to preference of the bacterial cell to assimilate an extra carbon source while utilizing the dye molecule as a carbon source (Saratale et al., 2009).

The supply of nitrogen sources such as urea, yeast extract, beef extract, peptone however can regenerate NADH which in turn acts as an electron donor in microbial reduction of dyes (Khan et al., 2013). According to Modi et al the isolated strain, M1 (*B.cereus*) exhibited the highest decolorization in presence of maltose, sucrose, glucose and glycogen respectively while the strain M6 showed decolorization above 50% in presence of carbon sources with highest decolorization efficiency of 92% in presence of sucrose. Peptone was found as an excellent source of nitrogen in the reduction of Reactive Red 195 by M1 (*B.cereus*) (Modi et al., 2010).

1.10 The plot of the Current Study

The current study involves the studies on decolorization/degradation of various synthetic dyes using a newly some selected bacterial strains some selected bacterial strains. This study involves the investigations on the decolorization of eight azo dyes that is Fast Red, Bismarck Brown, Ponceau 4R, Amaranth RI, Orange G, Amido Black, Methyl Orange and Tartrazine using a newly isolated strain (*Pseudomonas aeruginosa*), JKAK and other strains Bacillus sp. AK1, *Lysinibacillus* sp. AK2, *Kerstersia* sp. VKY1.

The initial step of this work deals with the isolation, screening and characterization of the bacterial strain capable of decolorizing various textile azo dyes. Further, enzyme assay was performed to analyze the enzymatic activity of the responsible enzymes. The decolorization pathways was then elucidated with UV-Vis Spectrophotometry and LCMS analysis. Additionally, phytotoxic activity of the dyes was investigated using the decolorized samples. This thesis therefore, reports the first-hand results of our studies based on these objectives:

1.10.1 Objectives of the Study

- Isolation, screening and identification of the bacterial strain capable of decolorizing some selected textile dyes.
- Studies on the decolorization of azo dyes using isolated bacterial strains and identification of dye degradation products by LC-MS.
- Effect of dye concentration, pH, temperature, NaCl and metal ions on the decolorization efficiency of the azo dyes.
- Isolation, purification and characterization of azoreductase from *Pseudomonas aeruginosa.*

This thesis is composed of four chapters where the **first chapter** provides an introduction and review of literature on the history and evolution of synthetic dyes, classifications and their use. It also provides insights to various biological means of dye decolorization and their application at industrial level.

The **second chapter** consist of details illustrating the various materials and methods employed in this study. These includes media preparation, bacterial culturing, various morphological and biochemical methods techniques employed in this study such as spectrophotometry and 16S rDNA analysis.

The **third chapter** provides an elaborate decolorization studies on azo dye Orange G by the selected bacterial strains. It also contains the identification of intermediate products of dye metabolism and enzyme assay. Further, the effect of various parameters on the dye decolorization was analyzed, that is, temperature (30-55°C), pH (6.5-10.5), salts (NaCl 5-30mg/L), initial dye concentration (200-800 mg/L) and metal ions that is, Pb^{2+}, Zn^{2+} and

Co^{2+} (200-100mg/L) on the decolorization of Orange G by *Pseudomonas aeruginosa*, JKAK and other strains, *Bacillus* sp. AK1, *Lysinibacillus* sp. AK2, *Kerstersia* sp. VKY1 was studied. The chapter also contains preparation of bacterial consortia by combining different strains (JKAK, AK1, AK2 and VKY1) prepared to form a total of seven consortia and its subsequent use in decolorization of Orange G. A crude enzyme extract prepared from the cells of *Pseudomonas aeruginosa*, JKAK, was used as a source of enzyme in the assay of enzyme activity. The extraction of the enzyme was carried out during the log phase period of bacterial growth, a phase characterized by rapid increase in bacterial cells as well as increased enzyme secretion. The LCMS investigation of the dye decolorized products revealed that 8-amino-7-hydroxy naphthalene-1,3-disulfonic acid and aniline were the intermediate products formed in the decolorization process of Orange G is also provided in this chapter.

The second part of this chapter illustrates the study on the decolorization of azo dye Bismarck Brown the newly isolated bacterial strain JKAK and other strains (AK1, AK2 and VKY1). The effects of various physical factors including temperature (5.5-10.5), pH (35-50 °C), dye concentration (200-800 mg/L), NaCl (5-30g/L) and metal ions Pb^{2+}, Zn^{2+} and Co^{2+} (200-100mg/L) on the decolorization of Bismarck Brown by JKAK is described. This section also explains the decolorization of Bismarck Brown by the consortia consisting of JKAK, AK1, AK2 and VKY1. Further, the assay of azoreductase enzyme which was carried out using the cell-free extract as a source of the enzyme is also included herein. LCMS analysis of the decolorized dye samples revealed that 4-methylbenzene-1-3-diamine and 5-methylbenzene-1,2,4-triamine were the intermediate products generated in the process of Bismarck Brown metabolism. Phytotoxic effect of Bismarck Brown is also included here.

The third part begins with the decolorization of eight individual dyes (Fast Red, Amaranth RI, Ponceau 4R, Orange G, Bismarck Brown, Tartrazine, Methyl Orange and Amido Black) to ensure they are all decolorizable. This is followed by preparation of the dye mixture using the afore mentioned eight dyes containing 25mg/L concentration of each. The influence of dye concentration on decolorization of the dye mixture was analyzed in two phases; decolorization by JKAK at an initial total concentration range of 200-1000mg/L and the second phase was decolorization of the same dye mixture by AK1, AK2 and VKY1 at an initial total concentration range of 200-600mg/L. The effect of pH was studied in the range of 6.5-10.5 whereas the effect of temperature was analyzed in the

range of 30-50°C. This part also includes an experimental treatment of real textile effluents using JKAK at 40°C temperature followed by phytotoxicity studies Green gram (*Vigna radiata*) seeds.

The **fourth chapter** of this thesis explains the purification of azoreductase enzyme using ion exchange chromatography, gel filtration chromatography and the purity was determined by SDS-PAGE analysis. The last part of this thesis provides the summary of our investigations as a conclusion on the study and the results obtained. This is followed by a list of all references cited in this thesis.

Chapter II

MATERIALS AND METHODS

2.1 Chemicals and Reagents

All dyes used in this study, that is, Fast Red E, Amaranth RI, Ponceau 4R, Orange G, Bismarck Brown R, Tartrazine, Methyl Orange and Amido Black were procured from S.D Fine Chemicals, Mumbai, India. The media supplements such as Yeast extracts, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $MnSO_4$ and Na_2MoO_4 were purchased from Hi-Media Mumbai, India. Inorganic chemicals used in the media preparation were procured from Hi-Media and SD Fine chemicals Mumbai, India. NADH was purchased from Sigma, Steinem, Germany, whereas, all the organic solvents were obtained from Spectrochem, India. The reagents used in this study, that is, NaOH, DEAE, NaCl, TEMED, Acrylamide, SDS, Ammonium persulphate, BSA, yeast extract, dyes were procured from Hi-Media and SD Fine chemicals Mumbai, India. All the chemicals used in this study were of analytical grade.

- **Azoreductase purification reagents**

Separating gel

Distilled Water	-	4.0ml
30% Acrylamide	-	3.3ml
Separating buffer (pH 8.8)	-	2.5ml
10% SDS	-	100µl
10% Ammonium persulfate	-	100µl
TEMED-N, N, N', N'-tetramethlethylene	-	10µl

Stacking gel

Distilled water	-	2.7ml
30% Acrylamide	-	670µl
Stacking buffer- 1M Tris-HCl (pH 6.8)	-	500µl
10% SDS	-	100µl
10% Ammonium persulfate	-	100µl
TEMED-N, N, N', N'-tetramethlethylene	-	10µl

Coomassie brilliant silver blue stain

2.2 Experimental techniques

This section illustrates the various methods employed in maintaining the bacterial culture, analysis of dye decolorization efficiency, enzyme assay, dye intermediate products and enzyme purification, among others.

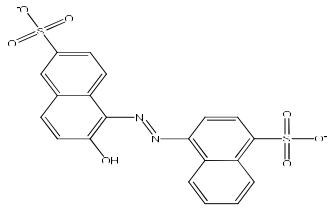
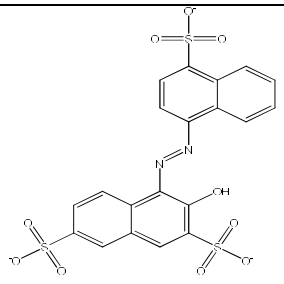
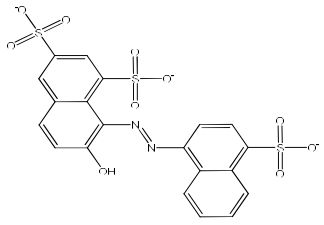
2.3 Cultivation and sub-culturing of the bacterial cultures

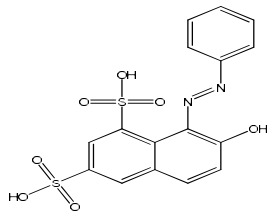
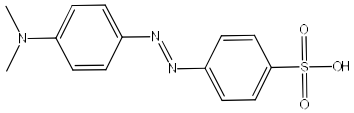
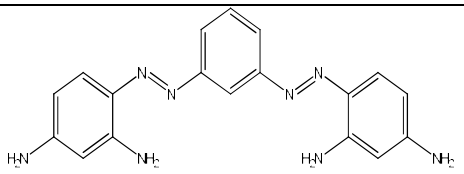
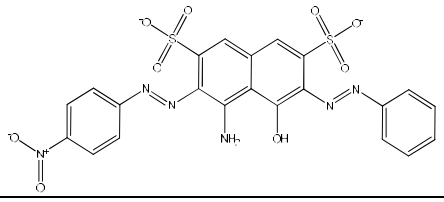
Bacterial cultures were grown in nutrient agar plates which also facilitated the colony counting. The cultures were also grown in phosphate mineral salt medium buffered in the absence of ammonium nitrate.

2.4 Dyes used in the present study

The selected eight azo-dyes that were used in the present bacterial decolorization study are given in ***Table 2-1*** alongside their respective molecular structure, molecular formula, λmax and uses.

Table 2-1: Selected azo dyes used in this study

Dye Name	Molecular Formula	Molecular structure	λmax max	Uses
Fast Red E	$C_{20}H_{12}N_2Na_2O_7S_2$		505nm	Used in drugs, cocktails, tinned fruits, biscuits, chocolates, bakeries, snacks industries and textile industries.
Amaranth RI	$C_{20}H_{11}N_2Na_3O_{10}S_3$		521nm	Dying natural and synthetic fibres, leather, paper, phenol-formaldehyde resins, food color, medicine color, chemical indicator.
Ponceau 4R	$C_{20}H_{11}N_2Na_3O_{10}S_3$		510nm	Coloring of alcoholic and non-alcoholic beverages, coloring of food stuff.

Dye Name	Molecular Formula	Molecular structure	λmax max	Uses
Orange G	$C_{16}H_{10}N_2Na_2O_7S_2$		476nm	Maker in agarose gel electrophoresis, biological stain, Microbiology stain, Textile (wool and silk), paper, leather.
Methyl Orange	$C_{14}H_{14}N_3NaO_3S$		465nm	Titration indicator, textile industry, printing, paper, food, pharmaceutical industries.
Bismarck Brown	$C_{21}H_{24}N_8.2HCl$		468nm	Biological stain (stains acid mucin to yellow and mast cell granules to brown) textile, leather
Amido Black 10B	$C_{22}H_{14}N_6Na_2O_9S_2$		620nm	Stain for total protein on transferred membrane blots, criminal investigation (detect blood with latent fingerprints)

2.5 Media preparation

The mineral salt media employed in all the experiments involving the bacterial decolorization of dyes along with their preparation is discussed below.

2.5.1 Preparation of MSM

To prepare this media, a 2000 ml beaker is half-filled with distilled water and mounted on a magnetic stirrer with a magnetic bead. The reagents are then added one at a time beginning with Na_2MoO_4 to K_2HPO_4 while the stirring continues to provide thorough mixing and dissolution. The stirring is continued for few minutes after the addition of all the reagents and the pH was adjusted to 7.0 using 4N NaOH and HCl. The volume is made up to 1000 ml using distilled water and this aqueous solution is boiled mildly to facilitate further dissolution of the constituents. This concoction was allowed to cool at room temperature where the solutes settles at the bottom of the beaker followed by careful decantation and filtration with filter paper to obtain a clear colorless solution. This was supplemented with 0.5% yeast extract for decolorization experiments in broth **(*Table 2-2*).**

Table 2-2: Composition of mineral salt media

Composition/Reagents	Quantity (g/L)
K_2HPO_4	6.3
KH_2PO_4	1.8
$MgSO_4$	0.1
$CaCl_2$	0.1
$FeSO_4$	0.1
$MnSO_4$	0.1
Na_2MoO_4	0.006

2.5.2 Nutrient agar medium

Nutrient agar medium was used in isolation, screening, purification and growth of the strain. It was also used in counting the number of viable bacterial cells on plates. The agar media was prepared in 250 ml Erlenmeyer flask containing 200 ml of MSM, 0.5% yeast extract and 200mg/L of the dye, in case of decolorization studies. The pH was adjusted to 7.0 using 4N NaOH and HCl. To this mixture, 2% Agar-Agar was added and sterilized by autoclaving at 121°C for 15mins. The molten agar containing media was aseptically poured onto sterilized agar plates and allowed to cool and solidify and room temperature before inoculation.

2.6 Decolorization of Orange G, Bismarck Brown and mixture of dyes by the bacterial strains (JKAK, AK2, AK1 AND VKY1)

Studies on the decolorization of Orange G, Bismarck Brown and the dye mixture was carried out using the newly isolated strain as well and the strains already existing in the laboratory.

2.6.1 *Pseudomonas aeruginosa,* JKAK

The bacterial strain with remarkable decolorizing ability was isolated from the soil contaminated with textile effluents discharged from textile industry located at Tirupur and Pallipalayam, Tamil Nadu, India. The soil sample was collected by taking the surface soil (0-15 cm deep) using stainless steel spade. The sample was mixed thoroughly and stored in polythene bags for subsequent studies (Sunitha Rangasamy et al., 2015). One litre of the effluent was collected into sterile specimen bottles. The effluent was stored at 4°C degrees to arrest any possible biological activities (Thirumurugan et al., 2017). 100g of the soil

sample was suspended in 50 ml of MS medium and stored in the refrigerator. Two ml of the sample was then suspended in 100 ml MSM and used as an inoculum source. The bacterial strain was grown for 72 h at 40 °C under static condition in 250 ml Erlenmeyer flasks containing 50 ml MSM, 0.5% yeast extract and 0.5% glucose and 0.5% peptone (Mutafov et al., 2007).

2.6.1.1 Isolation, Screening and characterisation of the bacterial strain

i) Decolorization on agar plate

The screening of dye decolorizing bacterial cultures was done by spread plate techniques on nutrient agar plate surface which was also employed in determining the number of viable cells. The determination of dye decolorizing ability of the strain was studied on petri plates. In this study, 200 ml of MSM was taken in 250 ml Erlenmeyer flasks. To this, 0.5% yeast extract and 200mg/L of the dye was added and the pH was adjusted to 7.0 using 4N NaOH and HCl. Further, 2% Agar-Agar was added and sterilized by autoclaving at 121°C for 15mins. The dyed-nutrient containing agar was poured in the previously sterilized petri plates and allowed to cool at ambient temperature in a sterilized inoculation chamber. This was followed by inoculating the nutrient agar plate by spread plate technique and the decolorization was observed after 28 h. The strains that were able to form a clear zone on agar plate containing the dyes were selected for further tests by aseptically picking the colonies with a sterilized loop and transferring into broth.

ii) Decolorization on broth culture

To study the decolorizing ability of the strain in broth culture, the above strains surrounded by a clear zone were transferred aseptically into 250 ml Erlenmeyer flask which contained 50 ml MSM, 0.5% yeast extract and 200mg/L of the respective dye. The pH of this mixture was adjusted to 7.0 using 4N NaOH and 4N HCl and sterilized thoroughly by autoclaving at 121°C for 15mins. The sterilized nutrient media was allowed to cool at ambient temperature before inoculation and decolorization was analyzed after 28 h. The decolorization potential was evident with an observable color change from the original to a nearly colorless solution. It was inferred that the flasks with complete decolorization contained the dye decolorizing bacteria and these cultures were selected for further studies. Purification of the strain involved serial dilutions followed by streak plate and spread techniques on nutrient agar and the potential colonies were subsequently picked after 12 h and transferred in to broth media.

2.6.1.2 Identification, 16S rDNA Analysis and phylogenetic position of the bacterial strain

The identification of the strain was performed based on morphological and biochemical characteristics as well as 16S rDNA sequencing to enable the analysis of phylogenetic position of this strain. The evolutionary history was interpreted using the Neighbor-Joining method (Chen et al., 2004). The phylogenetic relationship of JKAK with other related strains was analyzed and the data is available in GenBank database.

2.6.1.3 Determination of decolorization efficiency of the strain

These experiments were performed in 100 ml Erlenmeyer flasks containing (50 ml MSM, 0.5% yeast extract, 200mg/L dye and 1 ml inoculum ($13x10^5$ CFU/ml). The flasks were incubated at 40ºC under static conditions and the samples were withdrawn aseptically after every 4h intervals for analysis of decolorization efficiency. This was analyzed spectrophotometrically using UV-Vis Spectrophotometer (Specord 50 BU., Germany) by recording the decrease in absorbance at the respective λmax of the dye in nanometers. The control flasks were also analyzed to provide comparison and to determine the possibility of non-bacterial related decolorization of dyes. Each decolorization value is a mean of three parallel experiments. Decolorization percentage was calculated by employing the formula as used earlier (Shah et al., 2021).

$$\% \text{ Decolorization} = \frac{(i-f)}{i} \times 100$$

where i = initial absorbance and f = absorbance of decolorized medium

2.6.2 *Bacillus* sp (AK1), *Lysinibacillus* sp (AK2) and *Kerstersia* sp (VKY1)

These strains were from previously screened, isolated and stored in the laboratory, Department of Biochemistry, Gulbarga University, India. The above strains were isolated from dye contaminated soils collected from Atul Dying industry, Bellary, Karnataka, India, and from coconut coir samples which were collected from Tiptur, Karnataka, India. The strains AK1, AK2 and VKY1 have also been deposited the National Collection of Industrial Microorganisms (NCIM), Pune, India, with an accession number NCIM5332, 5333, 5312, respectively, for public accessibility (Vijaykumar et al., 2007).

2.7 Effect of various physico-chemical parameters on decolorization Orange

The studies on decolorization of dyes by the bacterial stains was carried out in 250 ml Erlenmeyer flask containing 50 ml MSM, 0.5% yeast extract and 200mg/L of the dye. Decolorization ability of dyes by the newly isolated strain, JKAK, and the other strains, AK1, AK2 and VKY1 was studied under various physico-chemical conditions such as initial dye concentration (200-800mg/L) and 200-600mg/L with mixture of dyes, pH (5.5-10.5), temperature (30-50°C), NaCl (5-30g/L) and metal ions (200-2000mg/L). Decolorization efficiency was analyzed by recording the decrease in the absorbance of broth supernatant at 4 h intervals until decolorization was 100% completed. Optimal parameters were taken as the point where the microbe showed the highest decolorization percentage.

2.8 Development of bacterial consortia

The need for increased decolorization efficiency and rates over a shortened period of time led us to investigate the effect of synergistic activity of the strains on the decolorization of individual dyes and mixture. This investigation was carried out in 250 ml Erlenmeyer flask containing 100 ml MSM (pH 8.5), 0.5% yeast extract and 200mg/L of the dye. This content was autoclaved at 121°C for 15mins for thorough sterilization and allowed to cool down at ambient temperature. The development of the bacterial consortia was performed by aseptically taking 0.25 ml of each pure culture (JKAK, AK1, AK2 and VKY1) at their log phase (18 h growth) to make a final volume of 1 ml mixed culture. This phase ensured that the same number of cells was maintained in the pure cultures as well as consortia. This mixed culture was aseptically inoculated into the cool nutrient media and incubated at 40°C under static conditions.

2.9 Preparation of cell free extract

The assay of azoreductase enzyme was carried out by following the method described earlier with slight modifications (Anjaneya et al., 2011). In this study, *Pseudomonas aeruginosa*, JKAK was grown in MSM containing 0.5% yeast extract and 200mg/L Fast Red E dye. Cells were harvested during the log phase of cell, growth (18 h) and subjected to centrifugation at 4,125x g for 10 min at 4°C. The supernatant was discarded while cell pellet was washed with 50mM phosphate buffer (pH 7.0) and

resuspended with the same buffer. To release the intracellular azoreductase, the cells were disrupted using Vibra Cell UIltrasonicator (VC 375, USA) at 70% output, 5x for 30 seconds. This was followed by centrifugation at 4,500x g for 20 min at 4°C and the supernatant obtained was used as a source of azoreductase. Assay of azoreductase was performed by taking 1 mM dye (40 µL) and crude enzyme (100 µL) in 50 mM phosphate buffer (650 µL, pH 7.0). The reaction mixture was pre-incubated for 3 min followed by the addition of 1 mM NADH (40 µL). The activity of azoreductase enzyme was analyzed spectrophotometrically at room temperature by monitoring the decrease in absorbance of NADH at 340nm. One enzyme unit was taken as the quantity of the enzyme that catalyzed 1µmole of the substrate in one minute. All the assays were carried out in triplicates (Ayed et al., 2010; Du et al., 2011).

2.10 Estimation of the concentration of proteins

Estimation of the protein content of the crude enzyme was carried out by following the standard method with slight modifications (Lowry et al., 1951). In this study, 1 ml of the crude enzyme was precipitated with equal volume of 10% w/v of TCA. The precipitate was recovered by centrifugation at 4,500x g for 15 min at 4°C. The pellet was dissolved in 0.1M NaOH and used as the protein source to determine the protein content of the enzyme. Bovine Serum Albumin (BSA) was used as the standard protein.

2.11 Analysis of the dye decolorized intermediate products

2.11.1 Extraction of the intermediate products

The broth culture was centrifuged at 4,125x g for 20 min after a complete decolorization of the dye to remove the bacterial cells. The supernatant was then passed through 0.45µ filter. The filtrate was extracted with equal volume of *n*-butanol and the organic phase was dried by vacuum. The residue obtained was dissolved in *n*-butanol (Supaka et al., 2004).

2.11.2 Analysis of the intermediate products by HPLC

The metabolic products were analyzed by HPLC (Waters, reverse phase C-18 column) at 25 °C, with an isocratic condition eluent of methanol-water (50:50, v/v) at an injection volume of 100 µL and a flow rate of 1 ml/min (Anjaneya et al., 2011).

2.11.3 Analysis of the intermediate products by LCMS

The LCMS analysis of the sample was performed at SAIF-IIT Bombay, India, using 410 Prostar Binary LC (Varian Inc., Palo Alto, CA) with C18 column as well as direct infusion mass with positive mode ionization. Ammonium acetate (10mM, pH 4) and methanol (1:1 v/v) mixture was used as an eluent.

2.12 Phytotoxicity studies

In this study, the phytotoxicity effect of dyes were analyzed before and after decolorization. In this experiment, green gram and cowpea seeds were used owing to their wide spread occurrence across most parts of the world. In the case of mixture of dyes, only green gram (*Vigna radiata*) was investigated. Phyto-toxicity studies were performed to determine the toxic effect of the selected dyes on seed germination and plant growth. The seeds were soaked overnight in water and planted the following day in glass cups containing loam soil. The concentrations of sample (dye and decolorized derivatives) were made up to 1000mg/L with distilled water.

The experiments were performed in three sets named as control set, test set 1 and test set 2, each containing five healthy, viable and certified seeds. The control set was watered with distilled water, the test 1was watered with dye decolorized products and the test 2 was watered with dye solution (2000 ppm). The seeds were watered with 10 ml of the respective sample twice a day and allowed to germinate and grow undisturbed for 72 h (Kabra et al., 2012). The saplings were then uprooted and the lengths of plumule and radical were measured. The percentage germination was also calculated (Araújo et al., 2005).

$$\text{Relative seed germination (\%)} = \frac{\text{Number of seeds germinated in the extract}}{\text{Number of seeds germinated in the control}} \text{X100}$$

$$\text{Root elongation (\%)} = \frac{\text{Mean root elongation in extract}}{\text{Mean root elongation in control}} \text{X100}$$

$$\text{Germination index GI (\%)} = \frac{\text{Mean root elongation in extract}}{\text{(\% Seed germination) X (\% Root elongation)}} \text{X100}$$

Statistical analysis

All the values presented in this study are means of three independent analyzes and plus/minus sign (±) indicating the standard deviation. Statistical calculation was performed using parametric statistics program, version 1.01 (Lundon Software Inc. Chargrin Falls, OH, USA).

2.13 Experimental treatment of textile effluent by JKAK

Along with the soil samples, textile effluents were also collected from the same site around textile areas in Tirupur, Tamil Nadu, India. One liter of the effluent was collected into sterile specimen bottles and stored at 4^{o}C to arrest any possible biological activities. Naturally, textile effluents discharged consists of different types of dyes with diverse molecular and structural formulas as well as various chemicals. The investigation on the effect of JKAK on real textile effluents was also performed in this study. In this case, the control flask contained 100 ml of the effluent sample while the test sample contained 100 ml of the effluent sample and 1 ml of JKAK inoculum. The two flasks were supplied with 0.5% yeast extract and incubated at 40 °C under static conditions.

2.14 Purification and partial characterization of azoreductase from *Pseudomonas aeruginosa,* JKAK

Azoreductase was extracted from *Pseudomonas aeruginosa*, JKAK strain which was isolated from dye contaminated soil. The ion exchange and gel filtration techniques were employed for the purification of this enzyme as described later in the sections.

2.14.1 Cultivation of *Pseudomonas aeruginosa*, JKAK and enzyme extraction

The azoreductase producing bacterium, *Pseudomonas aeruginosa*, JKAK, was isolated from dye-contaminated areas was grown in the laboratory to provide a source of azoreductase. The bacterial strain was sub-cultured in two 1000 ml Erlenmeyer flasks containing MS media (pH 8.5), 0.5% yeast extract and 200mg/L of the dye. This was inoculated with 1 ml (13x10^{5} CFU/ml) of the culture and incubated at 40°C.

2.14.2 Centrifugation and sonication

To separate the cells, centrifugation was performed at 4,125x g for 15 min at 4°C temperature. The supernatant was discarded but the pellet was washed twice with phosphate buffer (50mM, pH 7.0). The cells obtained were resuspended in 2 ml of the

same buffer and subjected to sonication using QSonica Sonicator at an output of 70%, 5x for 30 seconds. To separate the crude enzyme from the cell residues, centrifugation was carried out 4,125x g for 20 min at 4°C (Anjaneya et al., 2011). The cell pellet was discarded but the supernatant was used as source of enzyme azoreductase.

2.14.3 Azoreductase Enzyme assay

The assay of azoreductase was carried out following the earlier method (Anjenaya et al., 2011). In this analysis, 40 µL of the 1 mM dye and 100 µL of the crude enzyme was taken in 50 mM phosphate buffer (650 µL, pH 7.0). This mixture content was pre-incubated for 3 min after which 40 µL of 1 mM NADH was added. Enzyme activity was analyzed spectrophotometrically at ambient temperature by tracking the decrease in absorbance of NADH at 340nm (Ayed et al., 2010; Du et al., 2011).

$$C = \frac{A}{\mathrm{E} \times \mathrm{L}}$$

where C is the concentration of NADH utilized, A is Absorbance, E is Molar extinction coefficient of NADH (6220), L is the path length (1 cm).

$$Activity = \frac{C}{\mathrm{Mwt} \times \mathrm{time} \times \mathrm{vol.\,of\,enzyme\,(ml)}}$$

where C is the concentration of NADH utilized, A is absorbance, E is molar extinction coefficient of NADH (6220), L is the path length (1cm).

2.14.4 Estimation of the concentration of proteins

This study was carried out by following the method of Lowry et al., (1951) with slight modifications. Bovine serum albumin (1mg/ml) standard was prepared in phosphate buffer. One ml of the crude enzyme extract was taken and precipitated with an equal amount of 10% (w/v) TCA. The precipitated enzyme was recovered through centrifugation at 4,500x g for 15 min at 4°C. The recovered pellet was dissolved in 0.1M NaOH and used as the source of protein in determination of the protein content of this enzyme while using bovine serum albumin as the standard protein and the optical density was measured at 660nm.

Purification of azoreductase

This process was carried out by ion exchange chromatography and gel filtration chromatography. The molecular weight of this enzyme was determined by SDS-PAGE.

2.14.5 Ion exchange chromatography

The crude enzyme was extracted, concentrated and introduced into DEAE cellulose column of length and diameter of 10cm x 1.5cm, respectively. Equilibration of the column was performed in 0.1 Tris-HCl buffer (pH 7.0) and the column was washed with the same buffer. The protein sample was eluted with linear gradient of NaCl in the range of 0 to 0.6M NaCl at a flow rate of 25 ml per hour. The protein containing fractions were pooled and subjected to the analysis of enzyme activities and the fractions showing the activity were subjected to dialysis at 4°C.

2.14.6 Gel filtration chromatography

The enzyme was purified by using Sephadex G-25 gel filtration chromatography by employing a column with a dimension of 50 x 2cm (length x diameter). The column was packed and equilibrated using 50 mM phosphate buffer, pH 7.0 and three ml fractions were collected in capped vials. The protein containing fractions were pooled and analyzed for the activity of azorerductase and the eluents were subjected to dialysis at 4°C.

2.14.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel-Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulphate-Polyacrylamide Gel-Electrophoresis was carried out by following the method of Laemmli et al., (1970). The purified enzyme was subjected to electrophoretic process using 10% acrylamide gel. The gel was run alongside standard molecular weight markers. After the run, the gel was submerged in Coomassie brilliant Blue stain and destaining was performed for better visualization under white light transilluminator.

2.14.8 Biochemical characterization of azoreductase enzyme

This analysis was performed to determine the influence of various parameters on the activity of azoreductase. To calculate the final activity, the control enzyme activity prior to subjecting to these conditions was considered 100%.

2.14.8.1 The effect of pH on azoreductase

The activity of azoreductase was assayed in buffers of different pH ranging from 5.5-9.5. Buffers employed in this study were sodium acetate buffer (pH 5.5), phosphate buffer (pH 7.5) and Tris-HCl (pH 8.5-10.5). The residual enzyme activity was calculated by taking the activity at pH 9.5 as the control.

2.14.8.2 Effect of temperature on azoreductase

The optimal temperature for the maximum activity of azoreductase was determined in the range of 15-60°C. The stability of this enzyme at these temperatures was analyzed by pre-incubating the enzyme for 60 min at 15-60°C. The enzyme samples were withdrawn after every 10 min intervals and assayed for activity.

2.14.8.3 Effect of different metal ions on azoreductase

The effect of the selected metal ions, that is, $Mg^{2+,}$ Zn^{2+}, Pb^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+} on the activity of azoreductase was studied with a metal in the concentration range of 20-100mM. This was carried out by pre-incubating the enzyme in the ionic solution for 20 min, pH 9.5 at 40°C followed by the assay of the residual enzyme activity.

2.14.8.4 Effects of textile reagents on azoreductase

Reagents commonly used in textile industries were investigated for their influence on the activity of azoreductase. The selected chemicals include sodium carbonate, caustic powder (NaOH), Fenton's reagent (H_2O_2), NaCl, acetic acid, sodium nitrate, Aerial soap and formic acid were incubated at a concentration of 10-30mM. This was followed by the assay of the residual azoreductase activity.

2.14.8.5 Effect of organic solvents on azoreductase

The effect of selected organic solvents, that is, acetone, glycerol, ethyl acetate, ethanol, propanol and pyridine at a concentration of 15, 30, 45 and 60% on the activity of azoreductase enzyme was investigated. This was done by pre-incubating the enzyme in the solvents for 60 min followed by the assay of residual azoreductase activity.

Chapter III

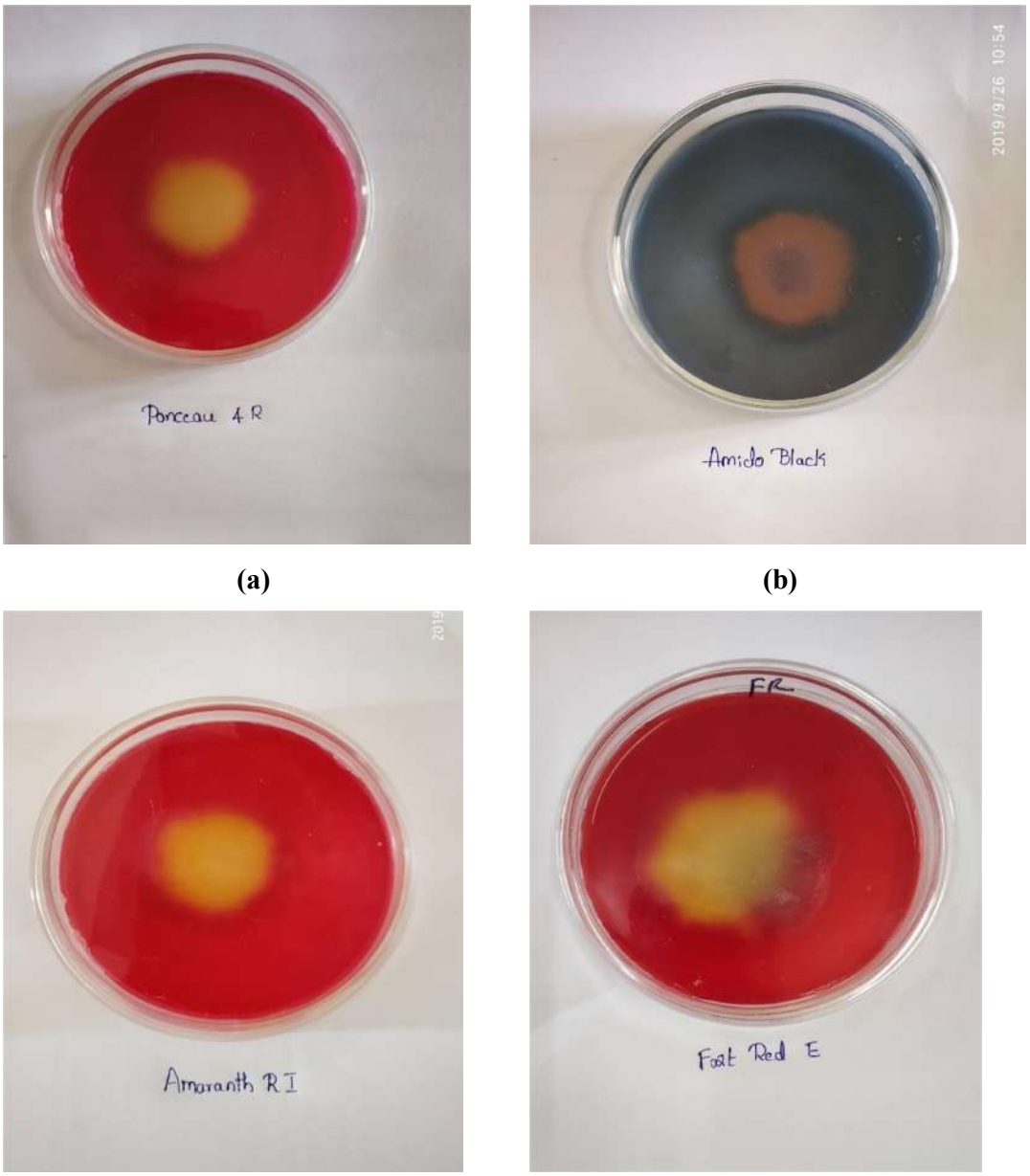
RESULTS AND DISCUSSION

The data presented herein provides an elaborative results and discussions on the proposed objectives. The findings on isolation, identification and characterization of the bacterial strain with the ability to decolorize some selected textile dyes as well as the subsequent phylogenetic positioning of this strain has also been discussed. Further, this chapter presents the outcome on decolorization of azo dyes such as Orange G, Bismarck Brown R and a mixture of eight selected dyes by the newly isolated strain, *Pseudomonas aeruginosa*, JKAK and other previously isolated and stored strains such as *Lycinibacillus*, (AK1), *Bacillus* sp., (AK2) and *Kerstersia* sp., (VKY1) (Anjaneya et al., 2013). In this chapter, the influence of various parameters such as dye concentration, temperature, pH, salt (NaCl) and metal ions on decolorization of the afore mentioned dyes has also been discussed. These experiments were performed to determine the optimal and economically effective environmental conditions over which the rate and efficiency of decolorization is maximum. This is vital for efficient and effective application of this technique at an industrial level of effluent treatment. Additionally, the extraction and identification of metabolite products of dye decolorization, enzyme assay and phytotoxicity studies are also elaborated in this chapter.

3.1 Isolation, identification and test for the dye decolorizing capability of the bacterium

3.1.1 Test for decolorization on nutrient agar plate

The bacterial strain was isolated from dye contaminated soils as explained previously. The soil sample was mixed with distilled water, filtered, and used as a source of inoculums to inoculate a dye containing nutrient agar plate and dye decolorizing strain was characterized by the formation of a colorless or clear zone around it. This strain was picked and transferred aseptically into MS media containing the dye and to further purify and confirm the decolorizing potential. The strain was spread once again on four agar plates containing model dyes, that is, Fast Red E, Ponceau 4R, Amido Black and Amaranth RI **(Figure 3-1).**

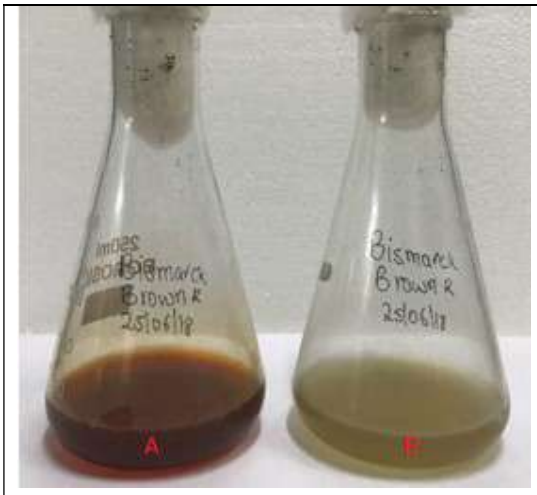


(a) **(b)**

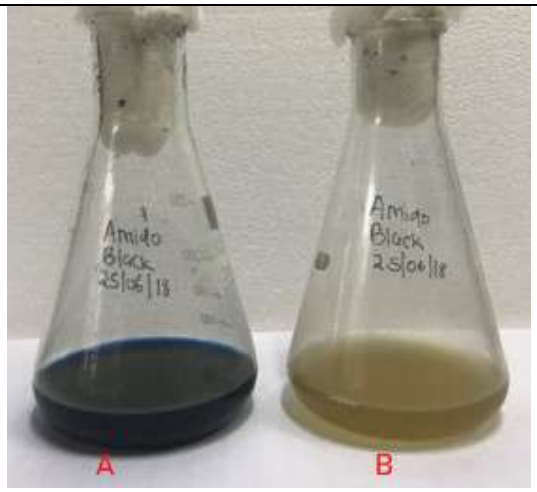
(c) **(d)**

Figure 3-1:Decolorization zones on agar plates exhibited by JKAK for selected dyes. a) Ponceau 4R, b) Amido black, c) Amaranth RI and d) Fast Red E

This experiment was carried out in 250 ml Erlenmeyer flask containing 50 ml MSM, 0.5% yeast extract, 200mg/L of the respective dye and inoculated with 1 ml ($13x10^5$ CFU/ml) of the culture. The time taken by the newly isolated strain, JKAK to decolorizes eight individual dyes employed in this study was investigated so as to understand the ease and difficulty in breaking down the various dyes by the selected strain **(Figure 3-2).**

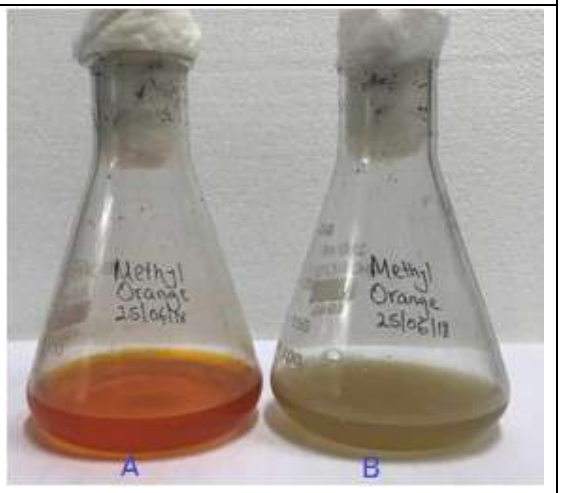


Bismarck Brown

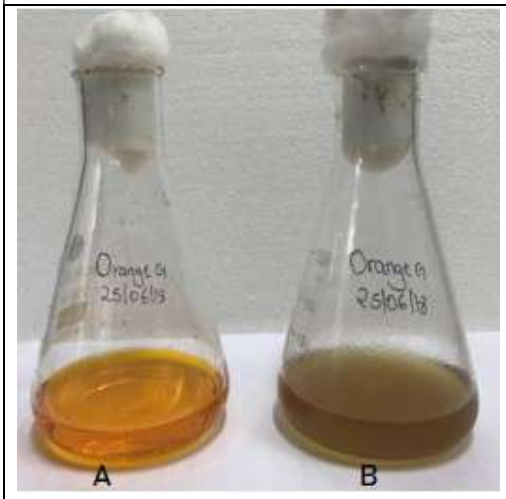


Amido Black

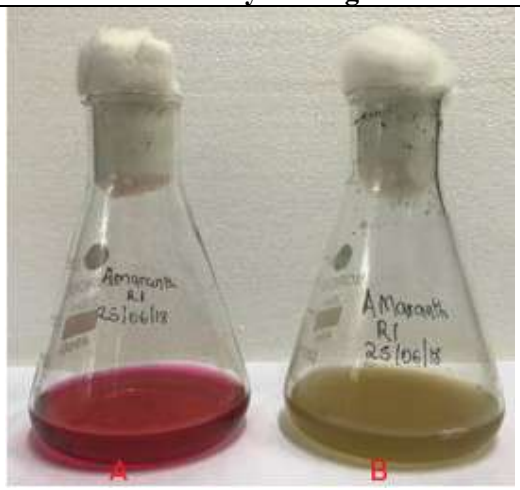
Ponceau 4R



Methyl Orange



Orange G



Amaranth RI

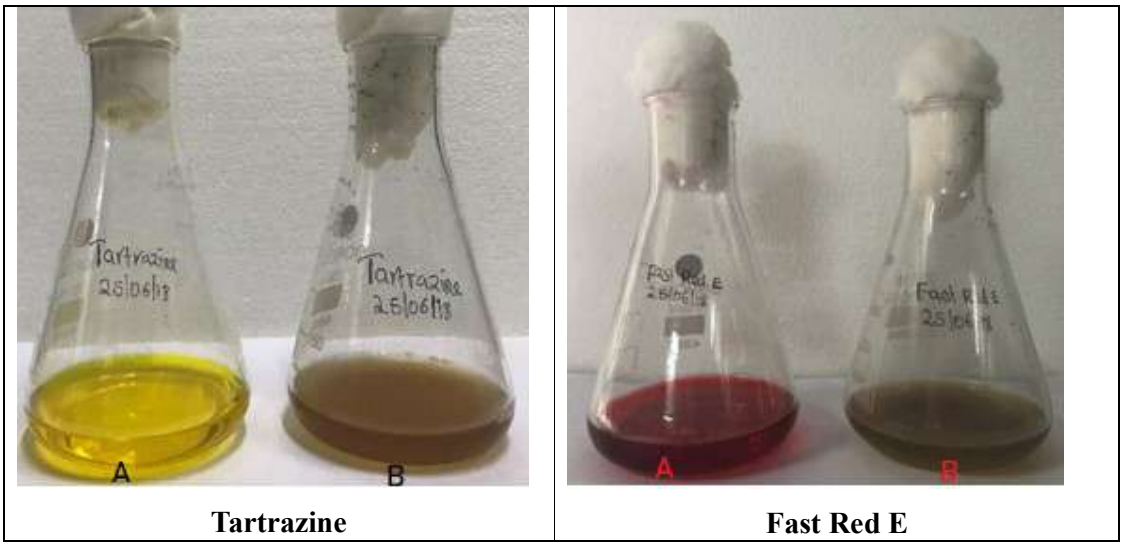


Figure 3-2: Decolorization of the selected dyes in MS media where a is the dye solution before bacterial decolorization and b is the decolorized dye
Note: (A) the dye solution before bacterial decolorization and (B) the decolorized dye.

The time period taken by *Pseudomonas aeruginosa*, JKAK, to eliminate 100% of eight selected individual dyes was also determined. It was observed that the rate at which these dyes decolorized increased in the order of Ponceau 4R (P4R), Amaranth RI, (AMRI), Fast Red E (FRE), Tartrazine (TZ), Orange G (OG). Methyl Orange (MO), Bismarck Brown R (BBR) and Amido Black (AB) was the last dye to exhibit 100% decolorization **(Figure 3-3).**

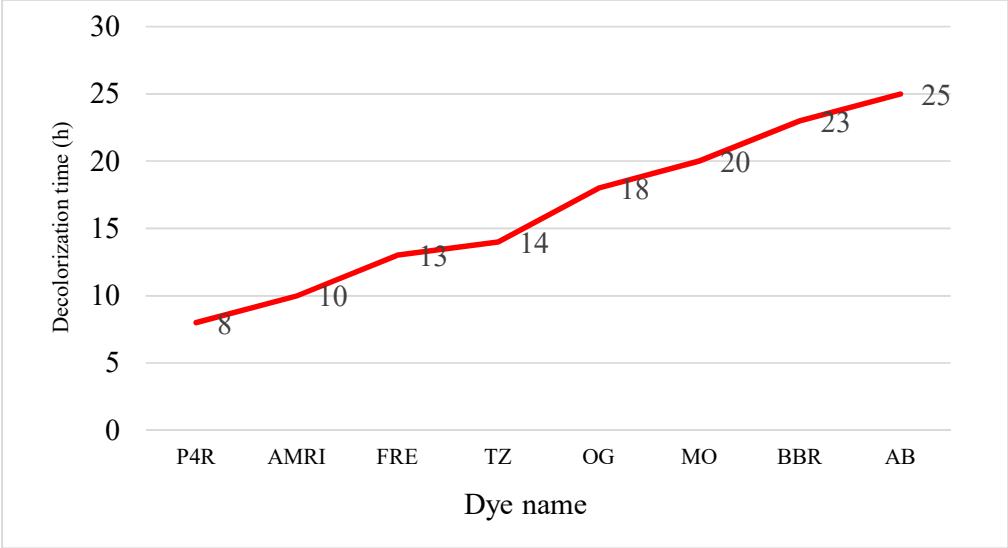


Figure 3-3: Incubation time over which the strain decolorized 100% of the individual dyes

3.1.2 Identification and characterization of the dye decolorizing bacterial

Morphological as well as biochemical characterization was performed to identify the newly isolated strain based on their appearance and biochemical reactions **(*Table 3-1*).**

Table 3-1:Morphological and Biochemical characterization of the isolated strain, JKAK

Sl. No	Test	Observation	Inference
		MORPHOLOGICAL STUDIES	
1.	Gram Staining	Thread-like pink cells observed	Gram Negative bacteria
2.	Endospore Staining	Pink colored cells. No spores observed	Non-spore formers
3.	Motility test	Random cell movement observed	Motile cells
		BIOCHEMICAL ANALYSIS	
1.	Urea Hydrolysis	Color changed from light orange to magenta	Positive
2.	Starch hydrolysis	No clear zone formation	Negative
3.	Catalase test	Rapid bubble production within 10 seconds	Positive
4.	Hydrogen Sulphide production	No black color formed. No FeS production	Negative
5.	Citrate utilization test	No blue color formed	Negative
6.	Gelatin liquification test	Solid control tubes after 30 mins refrigeration Liquid test tubes after 30 min refrigeration	Positive
7.	Methyl Red test	No color changes	Negative
8.	Voges Proskauer test	Color changed from yellow to red	Positive

3.1.3 16S rDNA Analysis and Phylogenetic studies

In order to further characterize the strain, 16S rDNA sequencing was performed to enable the analysis of phylogenetic positioning. The phylogeny of the newly isolated strain was compared with other related strains which are already available in GenBank database. Homologically, this newly isolated strain was in same phylogenetic branch as *Pseudomonas aeruginosa* (**Figure 3-4**). This strain has been deposited in the National Collection of Industrial Microorganisms (NCIM), Pune, India with an accession No. NC161118B for access to the public. 16S rDNA gene sequence was also deposited in GenBank database, with an accession No. MK281338.1 and was assigned the strain name JKAK in the NCBI data base. The evolutionary history herein was interpreted using the Neighbor-Joining method.

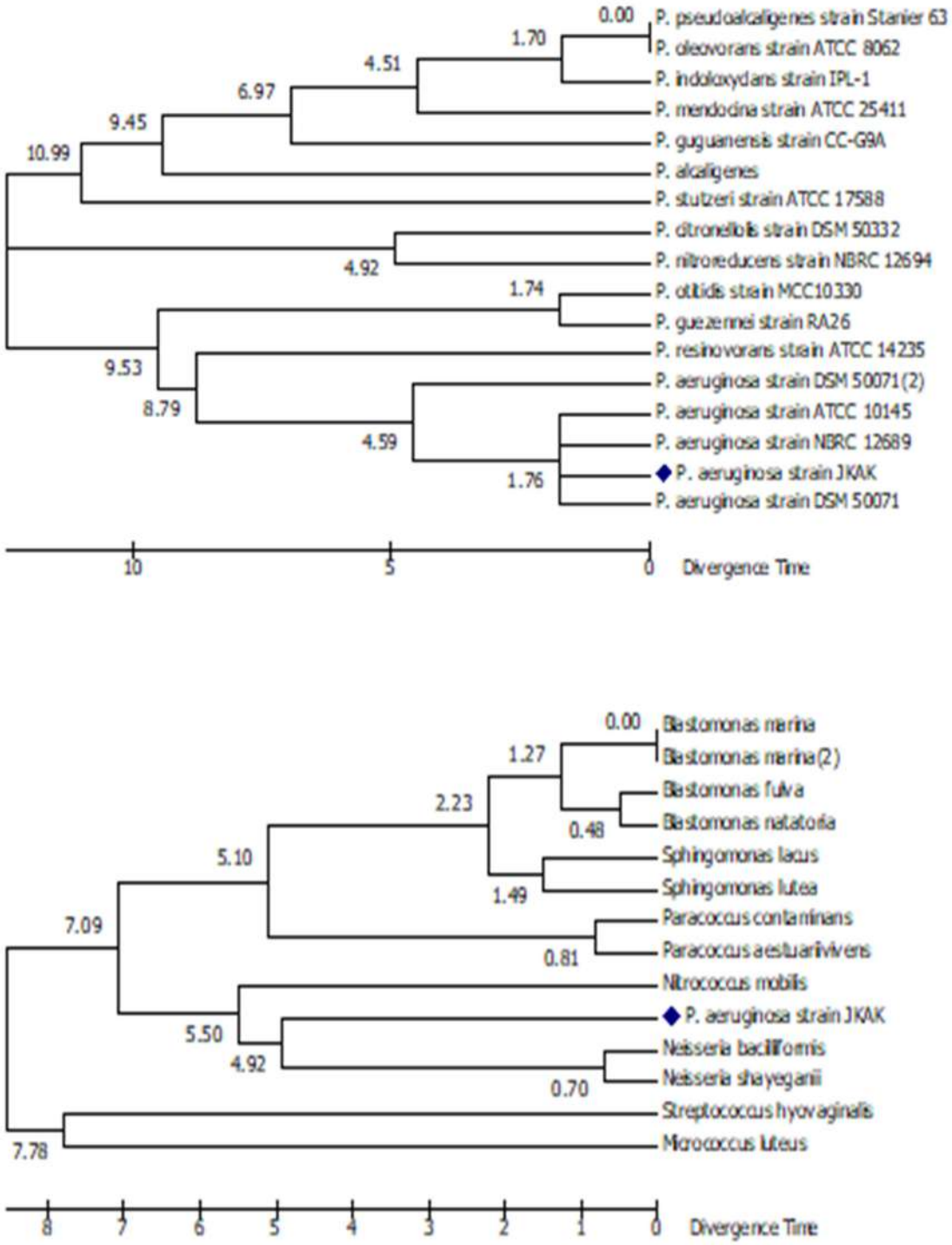


Figure 3-4: Intra-species phylogenetic analysis of the newly isolated strain

3.2 Studies on decolorization of Orange G, Bismarck Brown and a mixture of dyes by bacterial strains

Orange G and Bismarck Brown are the dyes that have been found to be useful in scientific research as a maker in agarose gel electrophoresis and staining in microbiology as well as in large industries such as leather and textile. Orange G is an acidic monoazo dye with the formula $C_{16}H_{10}N_2Na2O7S_2$ (***Table 3-2)***. Bismarck Brown, on the other hand, is a basic diazo dye with the formula $C_{21}H_{24}N_8.2HCl_2$ (***Table 3-3***). Decolorization of the dye mixture was carried out to determine the potential of the newly isolated bacterial strain, *Pseudomonas aeruginosa*, JKAK and the other previously isolated strains. The dye mixture was made up of eight dyes all with different molecular and structural formulae.

Table 3-2: Details on Bismarck Brown dye

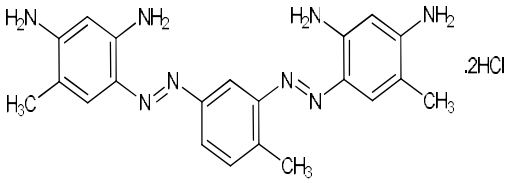
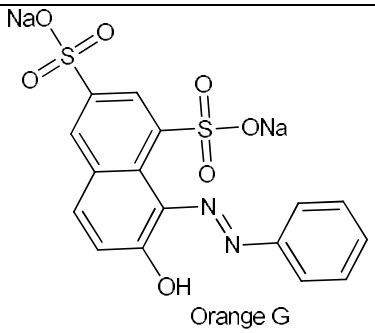
Name Dye	Chemical formula	Chemical structure	Uses
Bismarck Brown	$C_{21}H_{24}N_8.2HCl_2$	 Bismark Brown	Biological stain, Textile and leather industry

Table 3-3: Details on Orange G dye

Name Dye	Chemical formula	Chemical structure	Uses
Orange G	$C_{16}H_{10}N_2Na_2O7S_2$	 Orange G	Maker in agarose gel electrophoresis, biological stain, Microbiology stain, leather and textile color.

3.2.1 Influence of initial dye concentration on bacterial decolorization of OG and BBR

The selected bacterial strains, that is, *Pseudomonas aeruginosa*, JKAK, *Lycinibacillus* sp. AK1, *Bacillus* sp. AK2 and *Kerstersia* sp. VKY1 were subjected to a time-dependent decolorization of Orange G and Bismarck Brown, individually. This experiment was conducted to find out the influence of increasing dye concentration on bacterial decolorization of the newly isolated strain, JKAK, as well to compare this efficiency with other previously isolated strains AK1, AK2 and VKY1 at a dye concentration range of (200-800mg/L). Decolorization of Orange G dye by the above listed strains at 200mg/L was analyzed at every 4 h interval.

In this study, all the strains showed significant decolorization abilities of up to 95% decolorization of 200mg/L of Orange G after 16 h incubation period. The strains JKAK, AK1, AK2 and VKY1 exhibited 99.2, 98.62, 99.3 and 96.45% decolorization of the dye, respectively, after 16 h incubation under static conditions (***Table 3-4*****).** However, JKAK and AK2 expressed the highest decolorization percentage, as compared to AK1 and VKY1. Additionally, the dye concentration was increased from 400-800mg/L to further determine the effects of dye concentration. At the highest maximum concentration of 800mg/L, the strains JKAK, AK1, AK2 and VKY1 showed 76.8, 63.4, 52.6 and 49.1% decolorization, respectively, at 800 mg/L of the dye after 24 h of incubation (***Table 3-4*****).**

Table 3-4: Decolorization of Orange G by individual strains at increasing dye concentrations.

Strain	Conc. (mg/L)	Percentage decolorization at different time intervals					
		4 h	8 h	12 h	16 h	20 h	24 h
JKAK	200	32.40±0.41	56.29±0.23	87.40±0.62	99.12±0.34	99.12±0.85	99.12±0.43
	400	28.44±0.72	47.90±0.45	68.41±0.24	79.00±0.51	87.44±0.56	95.43±0.35
	600	24.87±0.63	36.67±0.54	59.13±0.84	70.13±0.74	77.54±0.79	86.32±0.27
	800	19.65±0.25	30.85±0.84	41.92±0.93	52.07±0.64	68.05±0.84	76.78±0.45
AK2	200	32.80±0.58	59.20±0.45	88.24±0.45	99.26±0.75	99.26±0.76	99.26±0.75
	400	25.28±0.15	45.57±0.84	64.22±0.64	76.17±0.65	85.32±0.34	93.73±0.23
	600	19.83±0.75	27.28±0.25	38.48±0.84	50.92±0.47	76.72±0.91	85.14±0.24
	800	15.92±0.62	23.11±0.78	31.13±0.87	44.14±0.81	57.21±0.81	63.42±0.46

AK1	200	31.20±0.34	55.21±0.56	86.23±0.52	98.62±1.14	98.62±1.23	98.62±1.31
	400	22.23±0.64	39.45±0.27	55.11±0.39	68.71±1.21	80.91±0.87	89.62±0.83
	600	15.87±0.61	32.41±0.62	47.89±0.37	61.82±0.79	72.88±0.76	89.02±0.59
	800	12.33±0.58	21.99±0.74	28.92±1.21	34.90±0.56	43.61±0.58	52.55±0.48
VKY1	200	30.11±0.68	54.80±0.43	84.48±0.25	96.45±1.12	96.45±1.25	96.45±1.34
	400	18.66±0.21	37.55±0.52	50.00±0.85	66.70±0.83	77.92±0.79	84.13±0.87
	600	13.14±0.35	26.43±0.49	38.11±0.68	47.21±0.67	59.47±0.82	71.42±0.58
	800	9.97±0.28	14.80±0.26	22.21±0.72	32.15±0.58	41.17±0.63	49.05±0.38

Similarly, Bismarck Brown was also subjected to the decolorization at the dye concentration as above with the same bacterial strains. At 200mg/L, all the strains showed a good decolorizing capability and eliminated over 96% of the dye after 16 h of incubation. JKAK, AK1, AK2 and VKY1 exhibited 98.00, 97.14, 100.0, and 96.00% decolorization of Bismarck Brown dye, respectively, after 16 h of incubation under static conditions. The strain AK2 showed the highest decolorization percentage, followed by JKAK, AK1 and VKY1. However, VKY1 exhibited the lowest decolorization ability of this dye (***Table 3-5*****).** At 400 mg/L of the dye, JKAK, AK1, AK2 and VKY1 showed a maximum of 92.50, 86.66, 89.16, and 80.00% decolorization, respectively, and at 800 mg/L, JKAK, AK1, AK2 and VKY1 showed a maximum of 61.16, 58.43, 65.00, and 53.75% decolorization, respectively, after 24 h of incubation (***Table 3-5***).

Table 3-5: Decolorization of Bismarck Brown by individual strains at different dye ncentrations.

Strain	**Conc. (mg/L)**	**Percentage decolorization at different time intervals**					
		4 h	**8 h**	**12 h**	**16 h**	**20 h**	**24 h**
JKAK	200	47.86±0.52	68.10±0.36	79.20±1.1	98.00±0.85	98.00±0.57	98.17±0.89
	400	26.25±0.81	40.83±0.70	53.75±0.74	68.33±0.68	84.58±0.79	92.50±0.70
	600	24.16±0.53	37.08±0.52	52.06±0.32	67.07±0.98	79.16±0.57	86.25±0.92
	800	13.33±0.28	19.50±0.44	31.25±0.65	44.58±0.33	56.25±0.44	61.16±0.74
AK2	200	45.61±0.46	62.00±0.80	78.00±0.51	100±1.41	100±1.25	100±1.42
	400	21.25±0.31	32.50±0.40	45.83±0.39	61.25±0.66	78.75±0.37	89.16±0.96
	600	15.83±0.28	25.00±0.62	41.66±0.43	54.16±0.33	70.83±0.89	81.25±0.62
	800	10.41±0.34	19.16±0.53	30.00±0.61	45.83±0.68	56.66±0.60	65.42±0.67

AK1	200	42.35±0.30	60.20±0.82	76.31±0.93	98.14±0.98	98.14±0.83	97.14±0.73
	400	18.00±0.24	27.01±0.27	42.90±0.63	58.33±0.83	73.32±00.46	86.66±0.56
	600	11.66±0.22	22.55±0.62	37.50±0.66	53.33±0.67	67.50±0.29	79.16±0.41
	800	8.31±0.26	14.16±0.77	22.90±0.30	34.68±0.29	49.60±0.37	58.43±0.43
VKY1	200	38.80±0.17	59.82±0.47	74.56±0.56	96.00±1.29	96.00±1.13	95.23±1.22
	400	14.58±0.38	21.66±0.61	36.63±0.82	52.90±0.69	65.00±0.28	80.31±0.52
	600	9.16±0.41	18.33±0.55	32.90±0.30	48.30±0.28	63.53±.54	72.50±0.75
	800	7.50±0.43	15.11±0.22	22.08±0.55	34.16±0.48	46.66±0.80	53.75±0.72

These results showed that the newly isolated strain *Pseudomonas aeruginosa*, JKAK as well as AK1, AK2 and VKY1 could completely decolorize a higher concentration of these dyes over a shorter period of time as compared to other strains previously studied. At 800mg/L, all the strains, JKAK, AK1, AK2, and VKY1 showed over 49-76% decolorization of BBR and OG after 24 h of incubation. These results show that our strains are more efficient as compared to the earlier reported results where a culture of *Kocuria rosea* removed 40% of Methyl Orange dye from a concentration of 100mg/L after 120 h of incubation (Parshetti et a., 2010). A decreased decolorization percentage with an increase in the concentration of the dye has also been reported where the percentage removal of Remazol Black B (RBB) dye by a mixed culture was 98, 94, 90, 88, 85, 81 and 75% at 25, 50, 100,150 200, 250 and 300 ppm initial dye concentration, respectively (Kumar et al., 2009).

Further studies by Sarayu and colleagues found that the decolorization of Remazol Orange by *Pseudomonas aeruginosa* after 24h of incubation resulted in 90% of decolorization at only 50mg/L of the dye (Sarayu et al., 2010). Similarly, *Pseudomonas desmolyticum* could efficiently decolorize 100, 92, 80, 40 and 15% of Direct Blue-6 dye at a concentration of 50, 100, 150, 200 and 250 mg/L after 72h of incubation at 50-100mg/L and 4-6 days at 150-200mg/L, above which cell toxicity was evident (Kalme et al., 2007).

3.2.2 Decolorization of a mixture of dyes by JKAK, AK2, AK1, VKY1 at different concentrations.

Textile effluents consists of varied dye composition with different molecular and structural formulae. Therefore, bacterial decolorization of a mixture of eight dyes studied so

as to determine the efficacy of the selected bacterial strains (JKAK, AK2, AK1, VKY1) to decolorize this mixture of dyes. The effect of dye concentration on decolorization of the dye-mixture was investigated at a final concentration range of 200-600 mg/L. The process was monitored over 40 h period of incubation.

The results from this study revealed that JKAK was able to eliminate a maximum of 88.04, 74.03 and 57.20% of the dye mixture at a concentration of 200, 400 and 600 mg/L, respectively, after 40 h. AK1 decolorized a maximum of 70.56, 64.11 and 48.00% of 200, 400 and 600 mg/L of the dye mixture, respectively, after 40 h. On the other hand, AK2 decolorized a maximum of 76.20, 62.82 and 56.60% of 200, 400, 600 mg/L of the dye mixture, respectively, after 40 h. The fourth strain, VKY1 could decolorize 68.45, 52.08 and 44.00% of the 200, 400, 600 mg/L of dye mixture, respectively, after 40h incubation period **(*Table 3-6*).**

The current study showed that higher decolorization percentage as compared to reports recorded earlier by Tony and co-workers where only 32-46% decolorization was observed at a concentration of 20 mg/L (Tony et al., 2009). At 10 mg /L, 48 - 57% of the dye was removed after 120 h of incubation. At 30 mg/L of the dye mixture, only 10–16.3% of the dye was removed (Tony et al., 2009).

Table 3-6: Decolorization of the dye mixture by JKAK, AK1, AK2, VKY1 at different dye concentrations.

Strain	Conc. (mg/L)	Percent decolorization				
		8 h	16 h	24 h	32 h	40 h
JKAK	200	13.51±0.7	28.07±0.9	48.24±0.7	65.78±0.8	88.04±0.3
	400	9.40±0.4	23.00±0.6	36.84±0.9	51.75±0.5	74.03±0.8
	600	5.6±0.2	13.60±0.1	28.80±0.2	38.80±0.7	57.20±0.6
AK2	200	16.20±0.9	27.19±0.22	43.42±0.10	66.66±0.23	76.20±0.64
	400	12.82±0.6	26.49±0.14	40.59±0.12	53.84±0.63	62.82±0.15
	600	9.60±0.13	20.00±0.21	32.88±0.31	45.20±0.7	56.60±0.4
AK1	200	14.56±0.7	35.52±0.2	55.00±0.63	70.61±0.41	70.56±0.42
	400	11.11±0.2	21.79±0.10	31.62±0.25	42.73±0.22	64.11±0.3
	600	8.00±0.31	13.21±0.35	21.61±0.17	33.60±0.36	48.00±0.21
VKY1	200	11.45±0.12	27.19±0.13	42.90±0.11	60.96±0.21	68.45±0.3
	400	8.08±0.2	21.36±0.8	33.33±0.1	46.15±0.8	52.08±0.32
	600	4.00±0.15	13.60±0.62	24.00±0.56	34.00±0.41	44.00±0.16

Dye concentration affects the decolorization of dyes significantly as shown in this study. The rate of decolorization decreased significantly with an increase in concentration of the dye (200-600 mg/L). It was also observed that the decolorization process was also slowed down by the elevated dye concentration. This agrees with results reported earlier by Çetin and Dönmez., (2006) where 90% of only 206.3 mg/L Remazol Blue was decolorized after 30h incubation time.

Similar findings have also been reported by Wang and co-workers where the decolorization of the dye Reactive Black 5 by bacterial strain *Enterobacter* sp. EC3 was inversely proportional to the dye concentration (Wang et al., 2009). The decrease in decolorization with increase in the dye concentration can be attributed to the toxic effect of excess dyes on microbial cells as well as the blockage of enzyme active sites of the concerned enzyme by dye molecules (Singh et al., 2014).

3.2.3 Effect of pH on decolorization of Orange G and Bismarck Brown by JKAK

The pH demonstrates significant influence on bacterial decolorization of dyes as most decolorization studies reports high decolorization efficiency at neutral to low basic pH (Junnarkar et al., 2006). The influence of pH on decolorization of Orange G by JKAK was investigated in the pH range of 5.5–10.5 and the optimum pH was 8.5 where the strain was able to decolorize 100% of the dye after incubation for 12 h. The JKAK strain was able to decolorize a maximum of 97.51, 98.33, 100, 100, 98.12 and 91.03% at pH 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 after 20h (pH 5.5-6.5), 12h (pH 7.5-8.5), 16h (9.5-10.5) of incubation, respectively **(Figure 3-5).**

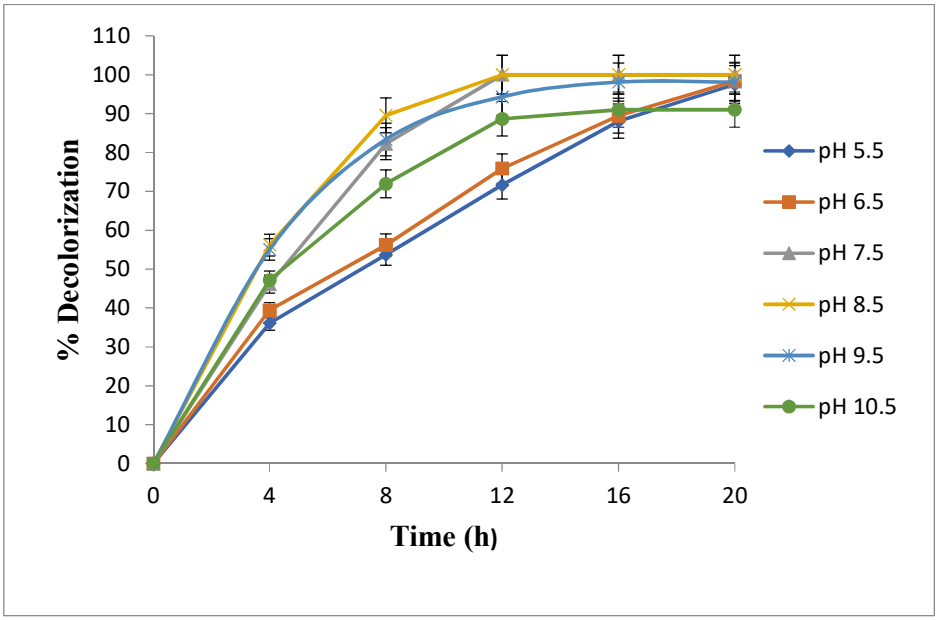


Figure 3-5: Effect of pH on decolorization of Orange G (200 mg/L) by JKAK.

Effect of pH on decolorization of Bismarck Brown by JKAK was also studied in the same range as above (pH 5.5- 10.5). The results obtained showed that the strain exhibited a maximum of 100% decolorization at pH 8.5 after 12 h of incubation. In this case, decolorization capacity was significantly affected by alkaline pH of up to 10. The pH below 6, however, could not favor the growth of the strains. After incubation, analysis was carried out to compare the decolorizations at various pH values (5.5-10.5). From these results, it was concluded that the strain showed a maximum of 92.45, 95.45, 98.36, 100, 94.21 and 92.90% decolorization after 20, 20, 12, 12, 16 and 16h of incubation, respectively, under static conditions (**Figure 3-6)**.

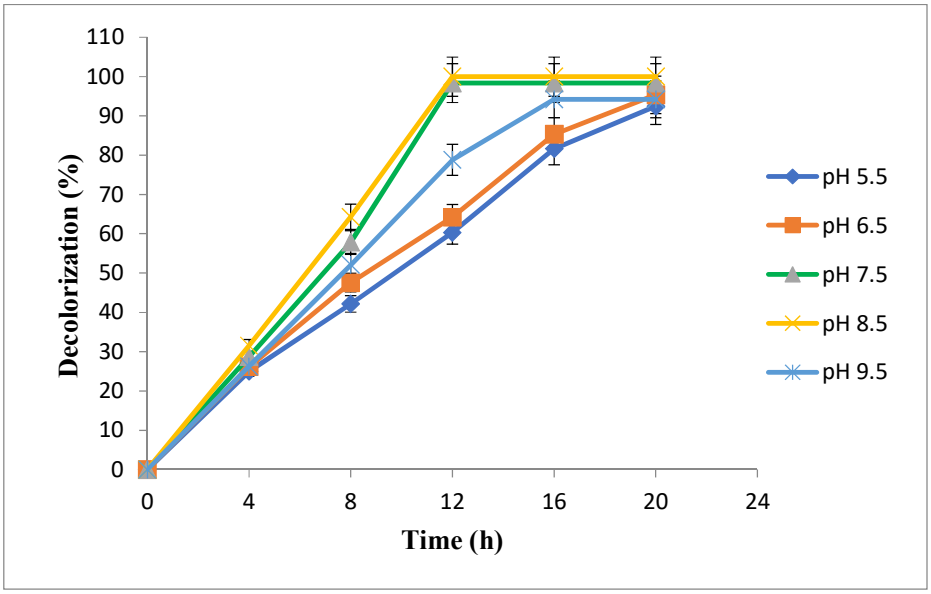


Figure 3-6: Effect of pH on decolorization of Bismarck Brown by JKAK.

Tolerance to changing pH is an important aspect of dye decolorizing bacteria as the reactive azo dyes bind to cotton fibers (substrate) through either addition or substitution reactions under higher temperature and pH conditions. The initial pH significantly influences the optimal growth, biosorption, biochemical, enzymatic and microbial metabolic processes which include the transfer of dye molecule across the cell membrane and therefore, pH greatly affects the decolorization processes (Ponraj et al., 2011). An optimum pH of 6.0-10.0 has been established as the common pH over which bacterial decolorization is optimal. It is commonly believed that pH influences the transfer of dye molecules across the cell membrane, and this is the rate limiting steps for decolorization (Prasad et al., 2014). Further increase or decrease in pH beyond the optimal (pH 8.5) automatically decreased the rate and percentage of decolorization.

The pH of the medium shows a great influence on decolorization potential and, as observed earlier, the bacterial strain *Enterobacter* sp. SXCR was able to decolorize 96% of sulphonated azo dye Congo Red at a maximum pH of 7.0 and only 24% decolorization was evident at pH 10 (Prasad et al., 2014). According to Ponraj and co-workers, *Pseudomonas* sp. showed an optimum decolorization of 89.06 and 86.72% at pH 6 and pH 8, respectively, while *Bacillus* sp. showed an optimum decolorization of 86.72% at pH 7.0 (Ponraj et al., 2011). An earlier report by Modi and coworkers showed a maximum of 80% decolorization of RR195 by M1 strain while the strain M6 was able to remove 87%

of the same dye (Modi et al., 2010). Any decrease or increase in pH from the optimal led to the decreased decolorization ability (Modi et al., 2010).

In another study, 60% decolorization of Reactive Black 5 by *Bacillus* species was observed after 24 h incubation (Wang., 2013). This pH sensitivity is an essential aspect in industrial biodecolorization and biotreatment of textile waste water as the contents of this water are varied in pH from acidic to alkali which is contributed by the various chemicals used in the process of dyeing.

3.2.4 Effect of pH on decolorization of the dye mixture by JKAK

Investigation on the effect of pH on decolorization of the mixture of dyes was performed and the results obtained showed that the optimum pH over which a highest decolorization percentage observed was in slightly alkaline conditions. At pH 8.5-9.5, JKAK decolorized a maximum of 91.36 and 88.24% of the dye, respectively, after 40 h of incubation. This newly isolated strain is slightly tolerant to pH changes as shown by the presence of decolorization at weakly acidic pH of 6.5 and strongly alkaline pH of 10.5, where the strain removed 37.02 and 56.49% of the dye after 40 h of incubation **(Figure 3-7).** Though these percentages are of course far less compared to decolorization efficiency at the optimum pH of 8.5, it is still clear that the enzyme is not completely inhibited at these pH conditions.

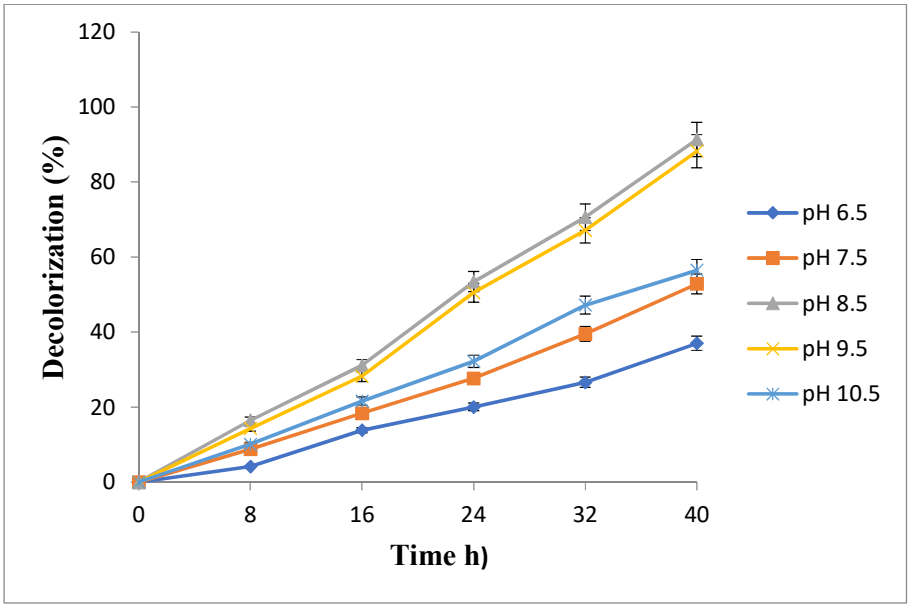


Figure 3-7: Decolorization of the dye mixture at different pH values by JKAK.

The results obtained in this study are in accordance with the previous reports on decolorization of RB-5, DR-81 and DO-3 by *Shewanella putrefaciens*, AS96 which showed high tolerance to pH in the pH range of 6-10 (Khalid et al., 2008). The optimum pH for efficient decolorization of RB-5, DR-81 and AR88 was at pH 7 and 9 while the optimum for decolorization of DO-3 was at pH 7-8 (Khalid et al., 2008). Increased decolorization at slightly basic media might result from elevated bacterial growth and enzyme activity at alkaline pH (Asad et al., 2007). The studies carried out on decolorization of a mixture of two dyes, that is, Methyl orange and Naphthol Green by *S. oneidensis* strain MR-1 showed a sharp decrease in decolorization at a pH below 6.5 (Cao et al., 2013). Dye decolorization through adsorption is reported to increase the pH inside the cell which results in a significant decrease in decolorization process which eventually hinders the bacterial growth and inhibits the dye decolorization process (Malakootian et al., 2018). Generally, an increase or decrease from the optimal pH results in a decrease in dye decolorization efficiency.

3.2.5 Effect of temperature on the decolorization of Orange G and Bismarck Brown, by JKAK

Temperature plays a key role in bacterial decolorization of dyes as it directly affects the rate of chemical reactions in biological systems. Optimal temperature is therefore essential for growth, metabolism and enzymatic activities of bacterial strains, resulting in optimized dye decolorization. In this study, the influence of temperature on the decolorization of Orange G by JKAK was studied at 30, 35, 37, 40 and 50°C. Our results showed that there is an increase in dye decolorization with an increase in temperature up to an optimum of 40°C where the decolorization was maximum. At 30, 35, 37, 40 and 50°C, JKAK was able to decolorize a maximum of 74.25, 94.40, 96.80, 100 and 69.25% decolorization over 20h (30-35°C), 16h (35-40°C) and 20h (50°C), respectively. However, any further increase or decrease in temperature beyond the optimum temperature resulted in a drastic decline in decolorization rates **(Figure 3-8).**

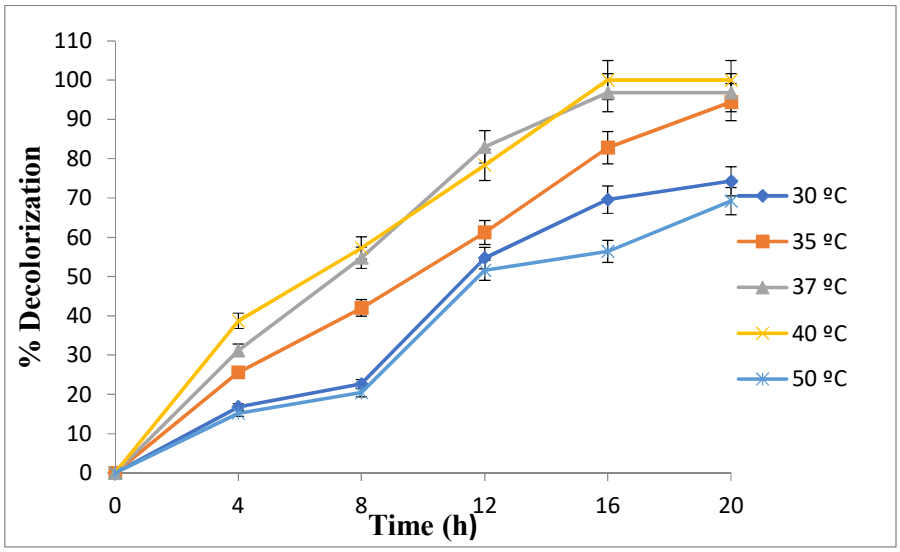


Figure 3-8: Effect of temperature on the decolorization of Orange G.

This analysis of effect of temperature on the decolorization of Bismarck brown by JKAK was also investigated in the temperature range of 30-50 °C. From the results obtained, it is clear that the rate of decolorization increased with a concomitant increase in temperature of up to 40 °C which is the optimum indicated by the highest decolorization percentage of 98.23% after 16 h of incubation. Any increase in temperature beyond the optimal point resulted in a drastic decease in decolorization efficiency. In this study, JKAK was able to eliminate a maximum of 71.12, 73.60, 92.00, 98 and 70.60% of the dye at 30, 35, 37, 40 and 50°C temperature after 20, 20, 20, 16 and 20h incubation periods, respectively (***Figure 3-9)***.

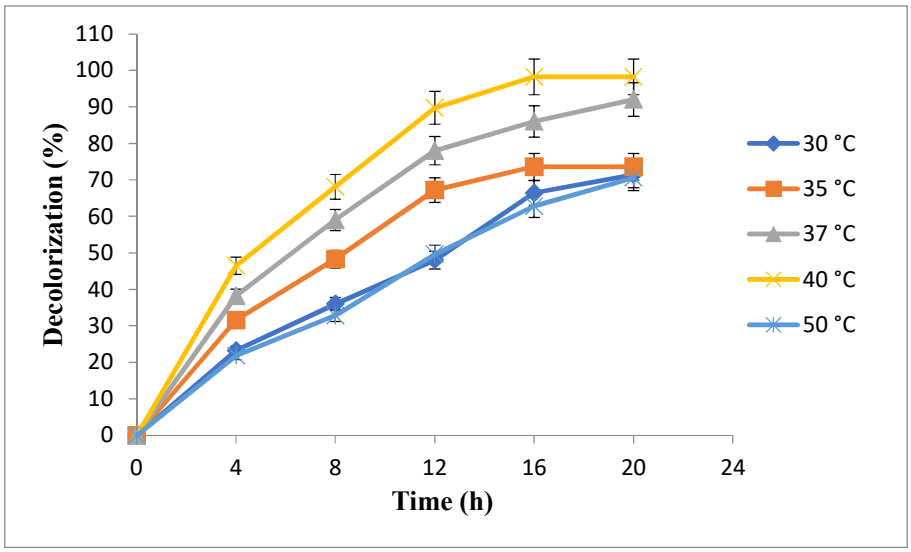


Figure 3-9: Effect of temperature on the decolorization of Bismarck Brown.

Regarding the influence of temperature on bacterial decolorization of dyes, Chang and colleagues had the similar observation of increased decolorization of rDye with an increase in temperature from 20 to 45°C with about 100% decolorization between 23 and 37°C (Chang et al., 2001). However, there was a drastic drop in decolorization rate at a temperature beyond 45°C (Chang et al., 2001). The rate of decolorization by JKAK at the optimum temperature of 40°C was much higher as compared to the strain, *Sphingomonas paucimobilis,* in the decolorization of Methyl Red where a maximum of 92% decolorization was recorded at an optimum temperature of 30°C. This might have resulted from the thermo-denaturation of cells by heat. A maximum of 93% decolorization of Reactive Violet 5R dye by a bacterial consortium JW-2 was observed at an optimum temperature of 37 °C (Moosvi et al., (2007). It was also observed that the rate of decolorization by *Pseudomonas luteola* dropped drastically beyond 40 °C (Chang et al., 2001). A maximum of 79.36, 52.38, 42.06 and 73.02% decolorization of Orange 3 dye by *Bacillus sp, Klebsiella sp, Salmonella* sp and *Pseudomonas* sp, respectively, at an optimum temperature of 37°C has also been reported (Ponraj et al., 2011). Similarly, a maximum of 71.72, 91.21, 99.26 and 88.23% decolorization of RR198 dye by a consortium of *Enterococcus faecalis-Klebsiella variicola* at 25, 30, 37 and 45°C, respectively, was observed after 72 h of incubation (Eslami et al., 2019). In dye decolorization experiments, it is mostly noted that decolorization increases with an increase in temperature up to an optimum temperature beyond which the decolorization rate and percentage reduces drastically.

3.2.6 Effect of temperature on decolorization of the dye mixture by JKAK

Just like the previous studies on Orange G and Bismarck Brown, an increased rate of decolorization by JKAK was realized with the rising temperature from 30 to 40°C beyond which there was a drastic decline. At 30, 35, 37 and 40°C, JKAK strain was able to remove 48.62, 70.44, 87.33 and 94.20% of the dye mixture, respectively, after 40 h of incubation. In this case, the optimum temperature was 40°C with decolorization percentage of 94.20%. Any change in temperature on either side of 30 and 40°C resulted in a drastic decline in decolorization. Accordingly, a decline was observed at 50 °C with decolorization efficiency of 56.11% after 40 h of incubation.

These results show that JKAK can tolerate moderately high temperatures as depicted by 56.11% decolorization at 50°C. This proves that JKAK has more thermo-tolerance and could decolorize a detectable percentage of the dye over a wide range of temperatures (30-

50 °C) (***Figure 3-10***). From these studies, it was deduced that the optimal pH and temperature for the decolorization activity of JKAK strain was 8.5 and 40°C, respectively. Hence, these two parameters were subsequently kept constant in the entire studies. AK1, AK2 and VKY1 were also studied under same conditions as that of newly isolated strain. All the experiments were carried out under static conditions.

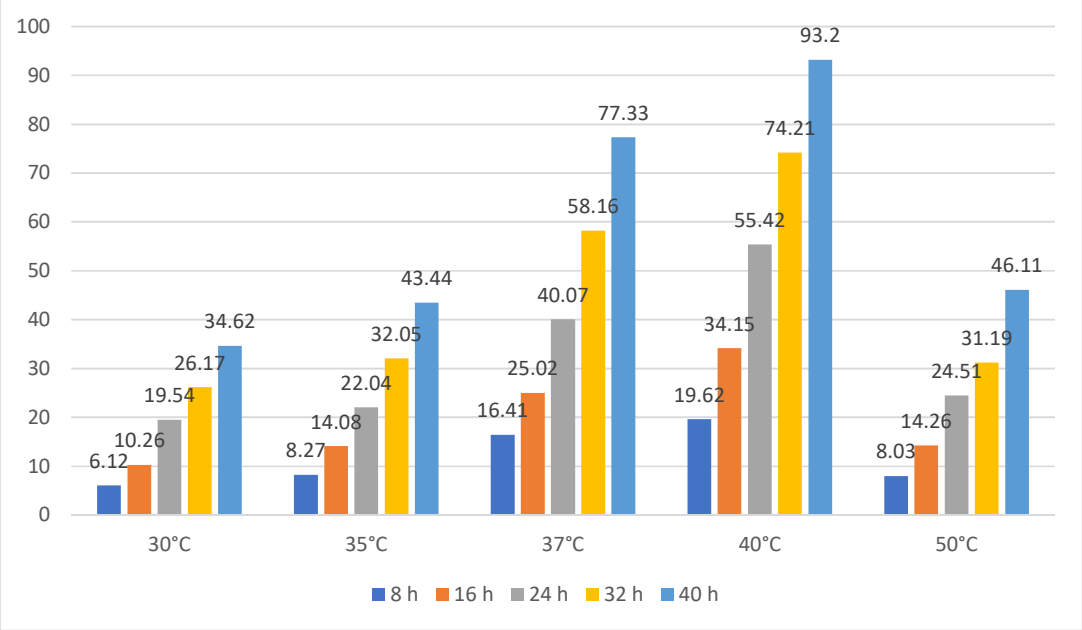


Figure 3-10: Decolorization of the dye mixture by JKAK at different temperature

The thermo-tolerance of this JKAK strain is evident when compared to *Pseudomonas aeruginosa*, NBAR12 which showed 3.8 times reduction in decolorization of the individual dye, Reactive Blue 172 at 50 °C as per the previous study (Bhatt et al., 2005). *Pseudomonas aeruginosa*, JKAK is also thermo-tolerant when compared to the *Alcaligenes faecalis* which was able to remove only 14.75 to 25.0 mg/L of Reactive Orange 13 per hour at 25-37°C (Shah et al., 2016). The rate of decolorization was directly proportional to the increase in temperature to the optimum level and this can be due to the increased bacterial growth rate as well as enzymatic activities of the strain at a favorable temperature (Zhuang et al., 2020). On the other hand, inhibited decolorization at low temperatures might be attributed to inhibition of major cell processes such as growth and enzymatic activities. Further, the drastic decrease in decolorization percentage at 50°C might result from the loss of cell viability and enzyme inactivation caused by excess heat decreased metabolic activity of the microorganism (Zhuang et al., 2020).

3.2.7 Decolorization of Orange G and Bismarck Brown in presence of NaCl

Salts greatly influences the efficiency of dye decolorization/degradation as discussed here. They are frequently used in the textile industries for salting the dyes out. These salts are eventually discharged into the environment alongside the unutilized dyes. To investigate the effect of salts on decolorization of Orange G and Bismarck Brown by JKAK, the media was supplemented with sodium chloride in the concentration range of 5–30 g/L. Decolorization was monitored spectrophotometrically at 476nm and 468nm, respectively, as explained earlier. Increased NaCl concentration from 5 to 30g/L did not exhibit significant inhibition on decolorization but slowed down the decolorization rate as the salt concentration increased. From the study of effect of NaCl on decolorization of Orange G at a concentration of 5, 10, 15, 20, 25 and 30 g/L NaCl, the rate of dye decolorization recorded was 100, 97, 95, 93, 92 and 90%, respectively, after 20 h of incubation **(*Table 3-7*).**

Table 3-7: Percentage decolorization of Orange G in presence of NaCl

Concentration (mg/L)	Percentage decolorization at different time points		
	4 h	12 h	20 h
5	52.94±0.4	84.40±0.3	100±10
10	51.57±0.6	75.74±0.7	97.50±0.9
15	49.38±0.2	74.29±0.8	95.93±0.6
20	48.91±0.8	72.98±0.2	93.52±0.1
25	46.31±0.6	71.67±0.9	92.33±0.4
30	44.86±0.1	69.00±0.2	90.82±0.9

Decolorization of Bismarck Brown by JKAK in the presence of same concentration of NaCl (5-30 g/L) also showed high tolerance to NaCl as indicated by 90% decolorization of the dye in presence of 30g/L after 20 h of incubation. As detailed in (***Table 3-8***), the strain was able to decolorize up to 100% of the dye in presence of 5, 10, 15 and 20g/L of NaCl while 92.72 and 90.90% decolorization was observed in the presence of 25 and 30g/L of NaCl, respectively (***Table 3-8*).**

Table 3-8: Decolorization of Bismarck Brown in presence of NaCl.

Conc. (mg L^{-1})	Decolourisation percent at different time		
	4 h	12 h	20 h
5	52.27±0.74	80.00±0.82	100±1.32
10	49.09±0.39	78.18±0.54	100±1.42
15	47.72±0.66	77.20±0.76	100±1.28
20	46.36±0.51	76.36±0.81	100±0.79
25	45.45±0.37	62.27±0.96	92.72±0.83
30	44.01±0.40	60.00±0.84	90.90±0.93

It has been observed that the rate and percentage decolorization decreased gradually with an increase in concentration of NaCl. There was no significant inhibition of decolorization up to a concentration of 30g/L, however, above this concentration there was no detectable decolorization. In this study, the newly isolated strain, JKAK could decolorize the dyes in presence of up to 30g/L NaCl concentration. These results showed that the strain was more halotolerant as compared to that reported earlier where 41% and 44% decolorization was observed by AK1 and AK2, respectively, in the presence of 20g/L NaCl (Anjenaya et al., 2011).

A decrease in decolorization with an increase in salt concentration and the consequent inhibition by excessive salts has also been reported earlier (Murugesan et al., 2009). In a study by Uddin and co-workers, about 100% of Acid Red B was decolorized by a culture grown in presence of 10-15% NaCl after 96 h of incubation and the inhibition was observed in presence of 25% NaCl (Uddin et al., 2007). In a study conducted with 5% NaCl, about 98.2% of AR27 dye and 79.1% of Methyl Orange was eliminated after 11 h incubation, respectively, by *Shewanella aquimarina*. It was also recorded that a low decolorization percentage of only 58.0, 28.5 and 47.9% of DB71, RR120 and AO7, respectively, has been observed after 11 h incubation (Meng et a., 2012).

3.2.8 Decolorization of Orange G and Bismarck Brown in presence of metal ions

Heavy metals are components of the dye molecules and since dyes are disposed into

the environment at various stages of the dying processes, these metals find their way to the ecosystem. Metal ions are important in microbial decolorization/degradation of dyes as they influence various biological pathways.

In this study, the effect of metal ions on decolorization of Orange G and Bismarck Brown was studied using Lead Nitrate, Zinc Chloride and Cobalt Chloride at a concentration of 200-1000mg/L. In the decolorization of Orange G in the presence of highest concentration (1000mg/L) of Pb^{2+}, Zn^{2+} and Co^{2+}, the JKAK strain was able to eliminate 92.05, 91.66 and 34.62% of Orange G after 20, 28 and 52 h of incubation, respectively **(*Table 3-9*).**

Table 3-9: Decolorization of Orange G in presence of metal ions

Concentration of ions (mg/L)	Percentage decolorization in presence of metal ions		
	Lead ions (20 h)	**Zinc ions (28 h)**	**Cobalt ions (52 h)**
200	100±0.9	100±0.5	100±0.3
400	100±0.7	100±0.6	100±0.1
600	100±0.5	100±0.4	91.04±0.3
800	100±0.4	94.29±0.2	65.11±0.2
1000	92.05±0.2	91.66±0.8	34.62±0.2

In the case of Bismarck Brown, there was no doubt that JKAK tolerates a high concentration of metal ions as expressed by 100±1.3 and 100±0.5 decolorization in presence of 200mg/L of Pb^{2+} and Zn^{2+} after 20h and 28h incubation, respectively. At a concentration of 1000mg/L of Pb^{2+} and Zn^{2+}, the strain was able to decolorize up to 86.40±0.9 and 92.0±0.4 of the dye after 20h and 28h of incubation, respectively. Cobalt, however, slowed down the decolorization efficiency as evidenced by 91.40±10 and 23.41±0.7 decolorization at a concentration in presence of 200mg/L and 1000mgh/L of the metal, respectively, after 48h of incubation (***Table 3-10***).

Table 3-10: Decolorization of Bismarck Brown in presence of metal ions

Conc. of metal ions (mg/L)	Percentage decolorization in presence of metal ions		
	Lead ions (20 h)	Zinc ions (28 h)	Cobalt ions (48 h)
200	100±1.3	100±0.5	91.40±10
400	100±1.1	95.21±1	83.00±10
600	100±0.5	94.0±0.1	66.11±10
800	90.68±0.7	93.40±0.6	31.0±0.8
1000	86.40±0.9	92.0±0.4	23.41±0.7

From the study, it was established that the rate of dye decolorization was inversely proportional to the concentration metal ion, however, there was no significant inhibition of decolorization. Although, increased metal ion concentration decreased the rate of decolorization, no significant inhibition was observed up to 600 mg/L of Co^{2+} and up to 1000mg/L of Pb^{2+} and Zn^{2+}. The results from this analysis further explains the ability of JKAK to tolerate a wider range of environmental factors as proved from the tolerance to high concentrations of metals.

JKAK strain is increasingly tolerant to high concentrations of metal ions as compared to other previously investigated strains. This is evident when compared to *Shewanella* sp. strain which was able to decolorize a maximum of 49, 87 and 89% of Reactive Black-5 in the presence of 2.5mg/L Cu^{2+}, Zn^{2+} and Co^{2+}, respectively, after 4 h of incubation. An increase in the concentration of Cu^{2+}, Zn^{2+} and Co^{2+} to 10mg/L resulted in only 6.6, 46 and 86% decolorization of the dye after 4 h incubation time. Further, intolerance of other strains to metal ions is seen in the investigation on decolorization of Congo Red by *Pseudomonas* sp where a drastic declined microbial growth was observed when the concentration of Zn^{2+} and Cu^{2+} was increased beyond 0.2g/L. The decrease in decolorization with increase in the concentration of the metal ions can be attributed to suppression of bacterial growth by the presence of increased metal ions (Gopinath et al., 2011).

3.2.9 Decolorization of Orange G and Bismarck Brown by bacterial consortia

Dyes are structurally and chemically varied and for this reason, all dyes might not be biodecolorized following the same pathway. Different bacterial strains are characterized by difference metabolic activities and enzymatic secretions. Biodecolorization is a

metabolic process and is therefore directly influenced by the metabolic activities of the microbe. Some dye decolorizing bacteria can initiate the first step of decolorization, that is, the formation of aromatic amines but unable to completely mineralize the dye into simpler forms such as CO_2 and water. Some bacteria, however, can degrade the aromatic amines but cannot initiate the first step of decolorization.

Therefore, we studied the effect of synergistic activity of some selected bacterial strains, that is, JKAK, AK1, AK2 and VKY1. Bacterial consortia made of two to four strains of JKAK, AK1, AK2 and VKY1 was developed by mixing the individual cultures and used for the decolorization of Orange G and Bismarck Brown dyes. The strains selected for the development of the consortia can individually remove up to 70% of various dyes. Consortia consisting of either two, three or four strains were developed by mixing the JKAK, AK1, AK2 and VKY1 strains and the consortia showed more decolorization potentiality than the individual strains.

As shown previously, individual strains were able to decolorize over 96% of the respective dyes after 16 h of incubation. In the investigation of decolorization of Orange G by bacterial consortia, it was observed that JKAK+AK1, JKAK+AK2, JKAK+VKY1, JKAK+AK1+AK2, JKAK+VKY1+AK1, JKAK+VKY1+AK2 and JKAK+ AK1+A K2+ VKY1, decolorized to an extent of 100%, however, JKAK+VKY1 decolorized only 97.33% of the dye after16 h. On the other hand, individual strains, that is JKAK, AK1, AK2 and VKY1 decolorized 99.10, 99.20 and 98.6 and 96.45% of Orange G, respectively, after 16 h incubation **(Table 3-11).**

Table 3-11: Decolorization of Orange G by bacterial consortia

Consortia	Percentage decolorization at different Time (h)			
	4	8	12	16
JKAK+AK1	43.60±0.5	59.20±0.5	90.80±0.5	100±0.3
JKAK+AK2	40.40±0.8	55.60±0.6	89.60±0.2	100±0.4
JKAK+VKY1	38.56±0.3	49.23±0.8	87.08±0.7	97.33±0.2
JKAK+AK1+AK2	42.00±0.4	78.49±0.4	100±0.2	100±0.2
JKAK+VKY1+AK1	33.33±0.3	64.35±0.2	100±0.4	100±0.4
JKAK+VKY1+AK2	40.39±0.9	68.79±0.6	100±0.7	100±0.7
JKAK+AK1+AK2+VKY1	58.40±0.6	91.75±0.4	100± 0.3	100± 0.6 `

In the case of Bismarck Brown, the consortia consisting of two and three strains were able to decolorize over 98% and 99% of the dye respectively after 12 h incubation. We observed that JKAK+AK1, JKAK+AK2, JKAK+VKY1, JKAK+AK1+AK2, JKAK+ VKY1+AK1, JKAK+VKY1+AK2, JKAK+AK1+AK2+VKY1 showed a maximum decolorization of 100±1.06, 99.11±0.49, 98.42±0.53, 99.0±1.13, 99.78±0.75, 99.72±0.67 and 100±0.88% after 12h of incubation **(Table 3-12).**

Table 3-12: Decolorization of Bismarck Brown by bacterial consortia

Consortia	Percentage decolorization at different Time (h)		
	4	8	12
JKAK+AK1	35.20±0.45	64.13±0.58	100±1.06
JKAK+AK2	32.80±0.87	63.20±0.72	99.11±0.49
JKAK+VKY1	28.07±0.18	59.31±0.26	98.42±0.53
JKAK+AK1+AK2	45.60±0.38	78.40±0.54	99.0±1.13
JKAK+VKY1+AK1	43.97±0.61	77.12±0.47	99.78±0.75
JKAK+VKY1+AK2	42.81±0.39	75.60±0.74	99.72±0.67
JKAK+AK1+AK2+VKY1	58.40±0.81	82.00±0.96	100±0.88

In this study, we found that time taken by the consortia to decolorize 100% of the dyes was shorter compared to individual strains. Moreover, real textile effluents are made up of a mixture of dyes and therefore are biodecolorized differently and therefore a single strain might not decolorize the dyes effectively. Bacterial consortia were therefore prepared by mixing different selected strains so as to determine their efficacy in decolorizing more complex dyes or treating effluents composed of different dyes, a process which might be infective with a monoculture.

Studies on dye decolorization by bacterial consortia has also been carried out by Hussain and colleagues, where the consortium named BMP1/SDSC-01, BMP1/SDSC-2 BMP1/SDSC-03 and BMP4/SDSC-04, were developed from varied combination of these individual strains, that is, *Bacillus subtilus, Bacillus cereus, Bacillus* sp., *Micrococcus* sp. and *Pseudomonas* sp., which was able to decolorize efficiently. In this experiment, the consortia BMP1/SDSC-01 and BMP1/SDSC-2 showed a decolorization of 84 and 81% for green dye, 85 and 80% for yellow dye, 84 and 79% for black dye, and 85 and 80% for the yellow dyes, respectively. Similarly, BMP1/SDSC-03 and BMP4/SDSC-04 exhibited a decolorization rate of 81 and 82% for red, 82 and 83% for green, 80 and 83% for black, 81

and 84% for yellow dyes, respectively (Hussain et al., 2012). The decolorization efficiency of a consortia is based on synergistic metabolic activities of the strains (Moosvi et al., 2007).

3.3 Assay of azoreductase

Bacterial decolorization of dyes mainly involves the breakdown of the azo bonds through an azoreductase catalyzed reaction. Assay of azo-reductase enzyme was analyzed using a method by with slight modification (Ooi et al., 2007). Azoreductase utilizes NADH or NADPH as a cofactor and was therefore added to the preincubated mixture of phosphate buffer, dye (substrate) and the freshly prepared cell free extracts enzyme source). In this experiment, 1mM dye (40 µL) and crude enzyme (100µL) added to 650 ml of 50 mM phosphate buffer at pH 7.0. This reaction mixture was pre-incubated for 3 min before the assay. The reaction between the enzyme and substrate which is the dye was initiated with the addition of 1 mM (40 µL) NADH the enzyme and the substrate (dye), a reaction which is monitored spectrophotometrically by recording the decrease in the absorbance of NADH at 340nm.

The change in the absorbance value was correlated to the decolorization of dye by azoreductase. While using Orange G and Bismarck Brown dyes as substrates, azoreductase activities of 0.9 mM/min/mg and 0.56 mM/min/mg of proteins were recorded. In this experiment, both dyes were able to induce secretion of the enzyme azoreductase indicating that the dye is degraded through enzymatic processes. The capacity to induce azo-reductase secretion is realized from the cleavage of the azo bond. The literature presents evidence that ability of a dye to induce the secretion of an enzyme was an indicator of enzymatic activity being a role player in dye decolorization/degradation. This is explained in the decolorization of Acid Black 210 to induce the secretion of the enzyme tyrosinase results in breakage of the azo bond (Agrawal et al., 2014).

3.4 The identification of dye degradation products of Orange G by HPLC and LCMS

The decolorized medium was subjected to HPLC analysis so as to identify the metabolites formed in the breaking down of Orange G, a dye which shows a UV-Vis spectrum at 476nm. After the dye decolorization, a shift was observed in the absorption

maxima of the spent medium where the peak at 476nm declined and new peaked emerged in the UV region. This proves that the aromatic amines generated are of low molecular weight. It was found, with the analysis of HPLC with Orange G dye along with its decolorized products, that the presence of three distinct peaks corresponds to aniline and 8-amino-6- hydroxynaphthaline-1,3-disulfonate where corresponding standards were used to compare such metabolites **(*Figure 3-11*).**

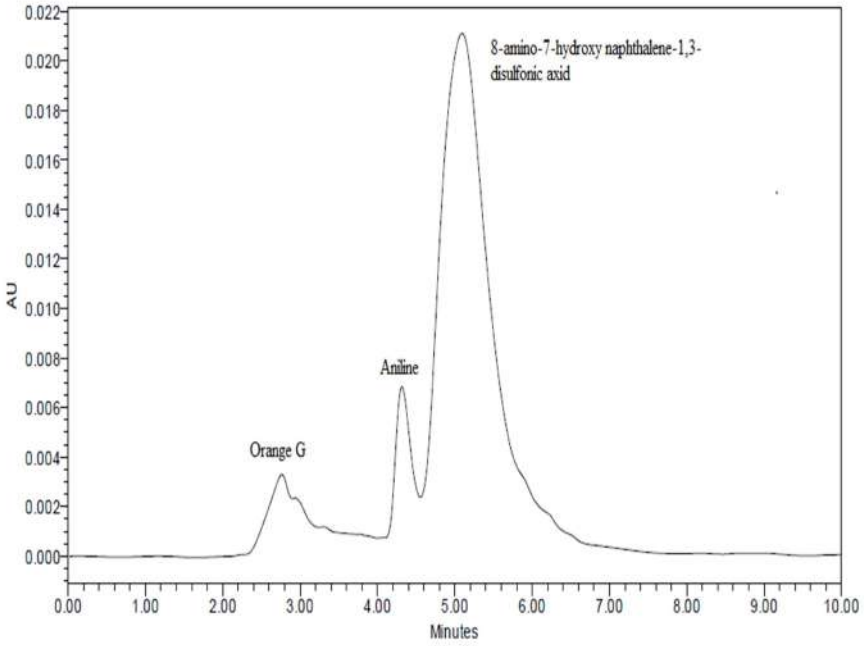


Figure 3-11: HPLC analysis of intermediate products of Orange G decolorization

To further establish the presence of these products, the spent medium from the breakdown of Orange G was subjected to LCMS analysis (Masarbo et al., 2019). The LCMS analysis revealed the presence of 8-amino-6- hydroxynaphthaline-1,3-disulfonate and Aniline which are the azo bond cleavage products. The metabolite I showed the peak at m/z 321 followed by peaks at 302 (M^+-NH_2), 222 (M^+-SO_3, -NH_2), 162 (M^+-$2SO_3$), 141 (M^+-$2SO_3$,-NH_2) which matches with the structure of 8-amino-6-hydroxynaphthaline-1,3, disulfonate. The peak at m/z 95 followed by another peak at 77 (M^+-NH_2) which matches with the structure of aniline consequently corresponds to the metabolite II **(*Table 3-13*).** Based on these results the degradation pathway of Orange G has been proposed (***Figure 3-12***).

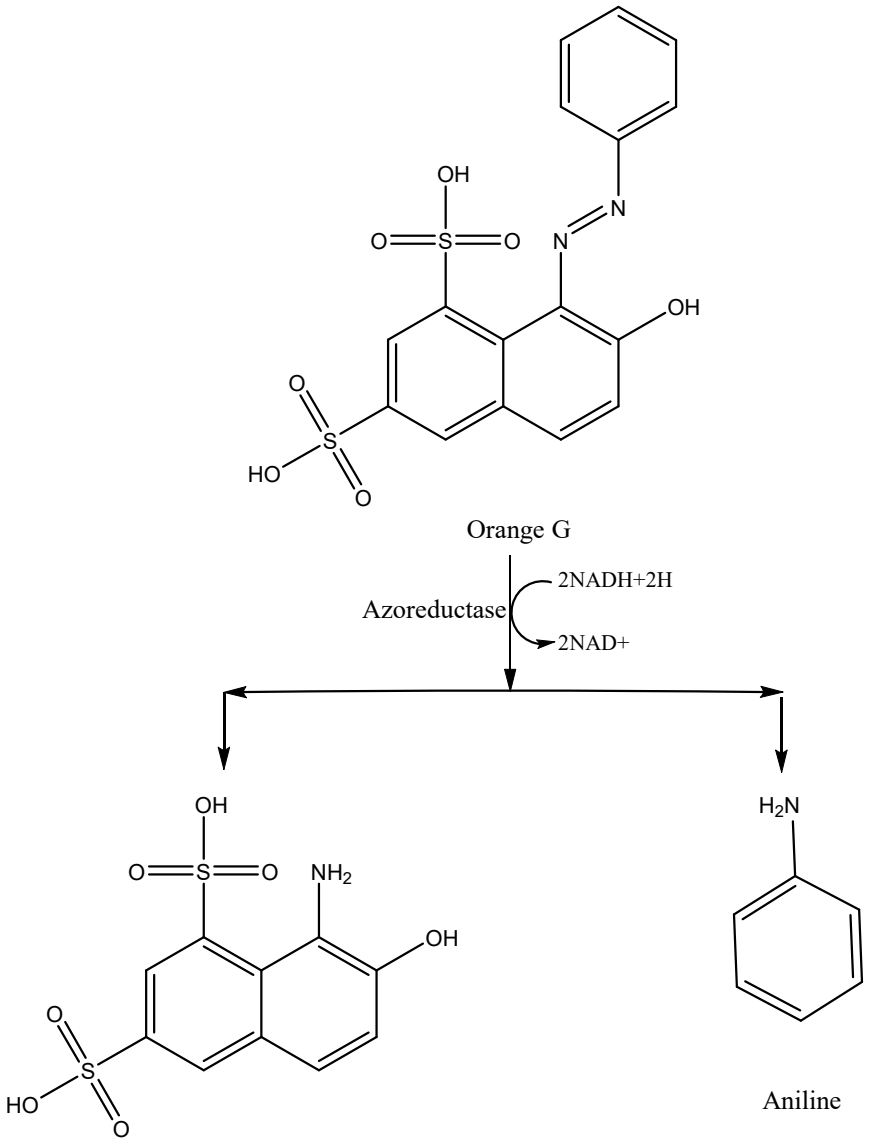
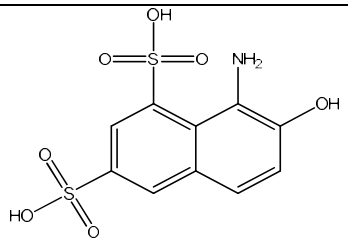
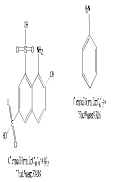


Figure 3-12: Degradation pathway of Orange G

Table 3-13: Mass fragmentation properties of metabolites isolated from the decolorized medium of Orange **G**

Metabolites	Structure	Molecular weight	m/z (%)	Identification
I		321	302,222, 162, 141	8-amino-7-hydroxy naphthalene-1,3-disulfonic acid
II		95	77	Aniline

3.5 LCMS analysis of the degradation products of Bismarck Brown

The decolorized products of Bismarck Brown dye were also subjected to LCMS analysis which revealed the presence of two distinct metabolites **(Table 3-14).** The metabolite I exhibited the peak at m/z 137 followed by peaks at 121 (M^+-NH_2/-CH_3), 105 (M^+-$2NH_2$/-NH_2,-CH_3), 90 (M^+-$3NH_2$/-$2NH_2$,-CH_3), 75 (M^+-$3NH_2$,-CH_3) which matches with the structure of 5-methyl-1,2,4-triaminobenzene. Metabolite II exhibited the peak at m/z 122 followed by peaks at 107 (M^+-NH_2/-CH_3), 91 (M^+-$2NH_2$/-NH_2,-CH_3), 75 (M^+-$2NH_2$,-CH_3) that matches the structure of 4-methyl-1,3-diaminobenzene (***Table 3-14***). Based on these results the degradation pathway of Bismarck Brown has been proposed (***Figure 3-13***).

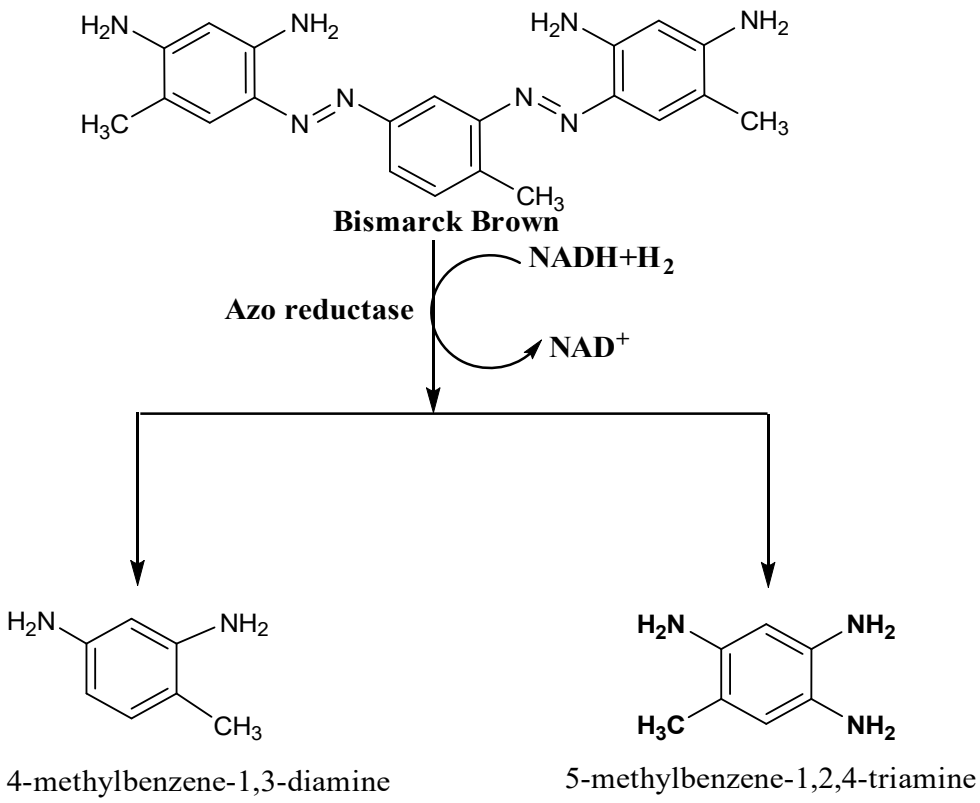
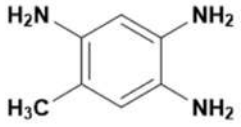
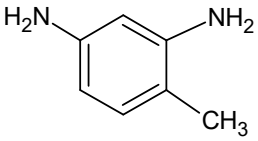


Figure 3-13: Degradation pathway of Bismarck Brown

Table 3-14: Mass fragmentation properties of metabolites isolated from the decolorized medium of Bismarck Brown

Metabolites	Structure	Molecular weight	m/z (%)	Identification
I		140	124, 107, 91	5-methylbenzene-1,2,4-triamine
II		126	110, 94, 80	4-methylbenzene-1,3-diamine

3.6 Phytotoxicity studies on Orange G and Bismarck Brown

We also determined the toxic effects of Orange G on the growth of green grams (*Vigna radiata*) and cowpeas (*Vigna unguiculate*). The dye solution and its decolorized products which were decolorized by the bacterial consortium (JKAK+AK1+AK2+VKY1) were used for the irrigation of the seeds and the saplings. The saplings were uprooted after 72 h and the root and shoot measurements were taken in centimeters.

3.6.1 Phytotoxicity studies of Orange G dye

The phytotoxic analysis of **Orange G** dye exhibited the results as presented below. In the case of green grams, 12 cm & 6.5 cm shoot and root lengths were recorded in the **control** set which was watered using distilled water. Investigations on **Test-I** where dye decolorized medium was used for irrigation, 8 cm and 4 cm shoot and root lengths were recorded. **Test II** unveils that there was 3 cm shoot and 1.2 cm root length when the saplings were watered with untreated dye.

In the same vein, similar tests were carried out on cowpeas, and it was observed that there was less shoot and root lengths as compared to the **control set** which exhibited 9 cm & 4.8 cm shoot and root lengths, respectively. However, when performing **Test-I** using dye decolorized medium, it was observed that the root and root lengths was 6 cm & 3.5 cm shoot length, respectively. **Test-II**, a set watered with untreated dye showed that the shoot length was 2.5 cm and 0.8 cm root and shoot length **(*Table 3-15; Figure 3-14*).** From the results recorded above, it can be concluded that, there is a significant decline in the shoot and root lengths when saplings are irrigated with untreated dye solutions. Moreover, the level of toxicity reduced significantly when seeds watered with dye decolorized samples.

Table 3-15: Phytotoxic studies on Orange G dye

Parameter	Green gram			Cow peas		
	Water	Dye	Decolorized sample	Water	Dye	Decolorized sample
Germination (%)	100	36	77	100	31	73
Shoot length (cm)	12±10	3±10	8±1.5	9±30	2.5±0.5	6±20
Root length (cm)	6.5±0.5	1.2±0.7	4±10	4.8±0.2	0.8±0.5	3.5±0.5

Figure 3-14: Phytotoxicity studies on seeds of Green gram watered with (a) Distilled water (b) Decolorized solution (c) Dye solution

3.6.2 Phytotoxicity studies of Bismarck Brown dye

The investigation on phytotoxic effects of **Bismarck Brown** showed that, in the case of the ***Vigna radiata*** **(green grams),** the **control set** which is watered with distilled water exhibited 10.8cm shoot & 5.8 cm root lengths. **Test-I,** which is watered with dye decolorized medium exhibited 7.8 cm shoot & 3.6 cm root lengths. **Test-II,** watered with untreated dye on the other hand exhibited 3.0 cm & 1.0 cm shoot and root lengths, respectively. There was no much difference in germination percentage between the control and Test I, that is 100% and 74% respectively while only 32% germination was recorded in the case of untreated dye.

Similarly, phytotoxic effect of Bismarck Brown dye was studied with cowpeas and it was found that the **control set** (watered with distilled water) exhibited 8 and 4.6 cm shoot and root and lengths. The **Test-I**, a set watered with dye decolorized medium showed 5.4 cm & 2.1 cm shoot and root lengths while the **Test-II,** which is watered with untreated dye solution exhibited 2.2 cm & 0.6 cm shoot and root lengths, respectively. The germination percentage in the case of test and control was 100 and 70%, respectively, while only 28% germination was recorded in the case of untreated dye **(*Table 3-16*).**

Table 3-16: Phytotoxicity studies on Bismarck Brown

Parameter	Green gram			Cow peas		
	Water	Dye	Decolorized sample	Water	Dye	Decolorized sample
Germination (%)	100	33	75	100	29	68
Shoot length (cm)	10.8±2	3±0.1	7.8±0.7	8±10	2.2±0.8	5.4±0.4
Root length (cm)	5.8±0.5	1.0±0.7	3.6±0.3	4.6±0.2	0.6±0.2	2.1±0.4

As portrayed in these results, it is evident that greatly reduced toxicity has been observed in the degraded dye products as compared to the untreated dye solution. These results are in line with that reported by Telke and colleagues on phytotoxic effect of Congo Red dye on plant species *Vigna radiata*, *Oryza sativa*, Sorghum bicolor and *Lens culinaris* after decolorization (Telke et al., 2010). Similar results have also been reported earlier on the decreased toxicity of Red BLI dye on *Sorghum vulgare* and *Phaseolus mungo* after the decolorization of the dye (Kalyani et al., 2008).

It can therefore be concluded that the treated dyed-water can be preferably utilized in various agricultural activities due to the decreased toxicity as compared to the untreated which is extremely toxic. This is in agreement with the toxicity studies of Malachite green on Lucerne and cabbage analyzed before and after decolorization which confirmed that the reduction in toxicity of dyes to plants after decolorization when *V. radiate*, *O. sativa, T. aestivum* and *S. bicolor* plants were exposed to Reactive Orange 13 dye before and after decolorization by *Alcaligenes faecalis* (Shah et al., 2012).

3.7 Experimental treatment of real dye effluent

Textile industries utilizes a wide range of dyes proving that the effluent is of a mixed dye content. The objective of this study is to determine the action of JKAK on real textile effluents. In this experiment, 100 ml of the sample was inoculated and incubated at 40°C under static condition. After 9 h of incubation, no change was observed on the control flask which contained the effluent and 0.5% yeast extract. On the other hand, sedimentation and coagulation was observed in the test flask containing the effluent sample, 0.5% yeast extract and JKAK inoculum. After 9 h of incubation, floccules were observed while the upper layer gets clearer and fades.

The floccules formed settled at the bottom of the flask to form a thick sediment. As suggested in earlier reports, this might prove that JKAK possesses both decolorization and flocculation abilities (Xu et al., 2006) **(*Table 3-17*).**

Table 3-17:Experimental treatment of original textile effluents

Effluent (100 ml)	**Observation (After 9 hrs)**	**Inference**
Test sample (Effluent+JKAK)	Coagulation/Sedimentation observed.	JKAK is able to coagulate/sediment the contaminants in the effluent
Control Sample (Effluent only)	No observable change	

Chapter IV

PURIFICATION AND PARTIAL CHARACTERIZATION OF AZOREDUCTASE

4.1 Introduction

This chapter presents the steps employed in the purification of azoreductase enzyme. This enzyme is responsible for catalyzing the reductive cleavage of azo bond, a process which renders the dye colorless. This aims at retrieving a highly purified and biologically active azoreductase enzyme so as to effectively determine the structure-function relationship. In this process, it is essential to remove other contaminating proteins from the crude extract of azoreductase. The purification process presented herein involves ion exchange and gel filtration chromatography.

Orange G and Fast Red E were used as model dyes to induce the production of azoreductase enzyme. In this study, the intracellular azoreductase was extracted from the newly isolated strain of *Pseudomonas aeruginosa*, JKAK, and subjected to purification and characterization. This chapter also deals with the studies on the effect of various conditions and reagents such as pH (5.5-9.5), temperature (15-60°C), and metal ions ($Fe^{2+,}$ Pb^{2+}, Ca^{2+}, Cu^{2+} and Mn^{2+}) at a concentration of 20-100mg/L on the residual activity of the enzyme was investigated. Further, the influence of organic solvents was analyzed in presence of ethanol, ethyl acetate, acetone, *n*-heptane, glycerol and propanol. The effect of selected textile reagents such as sodium carbonate, caustic powder (NaOH), Fenton's reagent (H_2O_2), acetic acid, sodium chloride, sodium nitrate, Aerial soap, formic acid, sulphuric acid, HCl and sodium carbonate was also examined for their influence on enzyme activity.

4.2 Ion exchange Chromatography

Ion exchange is a chromatographic technique based on the electrostatic interactions between the charged protein groups which bear either positive or negative charge and the matrix which is basically a solid support material. The matrix is loaded with ions of opposite charge to the protein to be separated. The proteins bound to the column were separated by either changing the concentration of the salts in the buffer or by a change in pH of the buffering solution (Karlsson et al., 1998). Matrixes are described on the basis of the charge it bears. A positively charged matrix is anion exchanger and binds to the negatively charged proteins and a negatively charged matrix binds to positively charged proteins are called cation exchangers. This technique was introduced early during 1940s and is designed to separate ionizable molecules based on differences in the charge (Fritz et al., 2004). This technique is commonly employed in purification of proteins, enzymes,

peptide, nucleic acids, amino acids and antibodies, among others. The technique is cost-effective and is made up of mobile and stationary phase where the mobile prophase is the buffer into which the sample to be separated is introduced. The phase is made up of an inert organic material with ionizable functional groups which carries a displaceable counterion of an opposite charge. The counter ions can be H^+, OH^-, Na^+, K^+, Cl^-, SO_4^{2-}, PO_4^{2-}, Ca^{2+}, Mg^{2+} etc. Since various proteins have different pI values, it means that these proteins can take up different net charges at different pH values. Therefore, a particular pH can be selected so as to bring the net charge of the intended protein to the opposite as that of functional group in the matrix. This way, the protein of interest displaces the functional group and binds to matrix through the process of adsorption. A reagent is used to elute the bound protein by changing the ionic strength or pH of the mobile phase.

4.3 Gel filtration chromatography

This protein separation technique is a form of partition chromatography which is used to separate proteins based on the differences in molecular sizes and can also be influenced by hydrodynamic diameter of the molecules. It is otherwise called size exclusion, gel-permeation, gel exclusion or molecular sieve chromatography. The technique is based on the separation of the molecules between the mobile and the stationary phases which consist of a porous matrix which functions to separate the molecules of various sizes. The column consists of beads within external liquid which is between the beads and the internal liquid is inside the beads. After the application of the sample, the molecules of higher molecular sizes than the bead pore of the stationary phase are separated by not entering the beads whereas the small molecules enter within the beads and elute later down the column. The molecules within the beads are trapped and migrate at a lower rate. Therefore, the molecules are separated in their order of decreasing molecular sizes. This technique can be carried out by following the conditions that are designed specifically to maintain the activity as well as the stability of the molecules.

4.4 Dialysis

During the purification of macromolecules such as proteins and nucleic acids, it is often necessary to eliminate small molecular weight substances that might interfere with subsequent steps in the experimental procedure. Similarly, it is often used to exchange the protein sample into a different buffer system for downstream application. Dialysis is often the method of choice for accomplishing both contaminant removal and buffer exchange

for proteins by placing the sample inside the suitable dialysis bags. The principle of dialysis is based on diffusion of solutes along a concentration gradient through partially permeable membrane.

4.5 Growth of the bacteria and extraction of crude azoreductase

One ml ($13x10^5$ CFU/ml) of JKAK strain was sub-cultured in MS media (pH 8.5) containing the necessary supplementation as described earlier. The yeast extract is an excellent nitrogen source in this study while the azo dye stimulates the production of azoreductase enzyme. The culture was incubated at 40°C under static conditions, the cells were harvested after 18 h. This incubation time is the log phase of the strain which is characterized by rapid cell growth, maximum enzyme secretion and a complete decolorization of the dye. Optimum parameters are essential to ensure maximum growth of the bacterium and the consequent secretion of the enzyme **(*Figure 4 -1*).**

4.6 Centrifugation and sonication

The centrifugation separates the enzyme containing cells from the media. After centrifugation, the supernatant was discarded and the pellet which contains the cells was washed with 50mM phosphate buffer, pH 7.0 to remove the yeast extract and the cells are then suspended in the same buffer. To extract the azoreductase, which in this case is an intracellular enzyme, the cells were lysed through sonication process using QSonica Sonicator and to separate the crude enzyme from the cell residues, centrifugation was carried out. The cell pellet was discarded but the supernatant was used as source of azoreductase enzyme **(*Figure 4-1*)**.

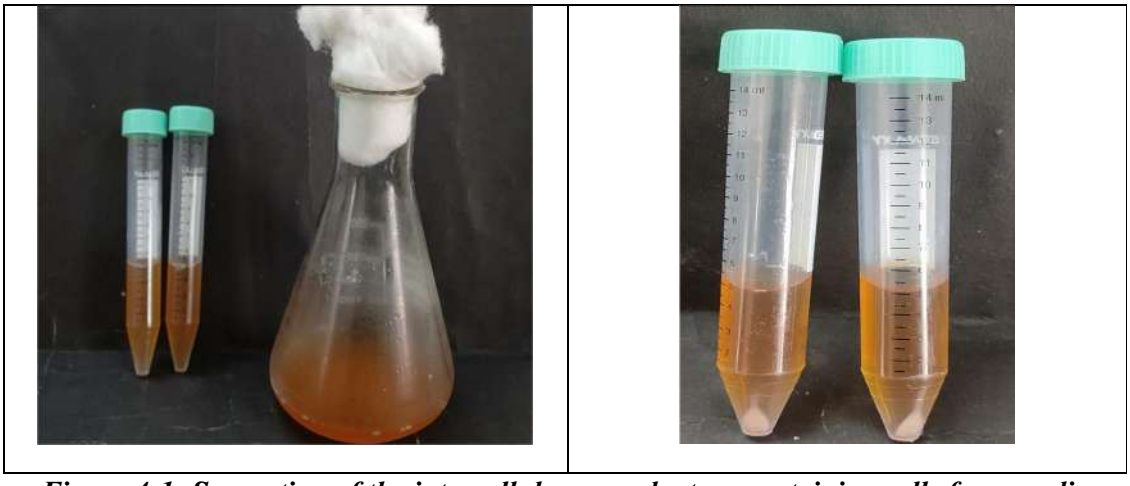


Figure 4-1: Separation of the intracellular azoreductase containing cells from media through centrifugation

4.7 Purification of azoreductase

The azoreductase, an enzyme derived from *Pseudomonas aeruginosa*, JKAK was purified by anion exchange chromatography using DEAE cellulose and gradient elution was carried out with 0 to 0.6M NaCl. The fractions were collected and investigated for enzyme activity and protein content. The results show that the enzyme was eluted at 0.2M NaCl concentration which was further subjected to dialysis and used for purification by Sephadex G-25 gel column chromatography**.** At this point, the protein content and enzyme activity of azoreductase were 30mg/ml and 161.818 (µm/min/ml), respectively.

The enzyme specific activity of 29.55U/mg was obtained after anion exchange chromatography at a protein concentration of 1.1mg. The 100% enzymatic activity of the enzyme before purification was considered as one-fold. Initial DEAE ion exchange purification and elution were carried out with 50mM Tris HCl (pH 7.0). The enzyme sample was further purified with Sephadex G-25 gel chromatography and analyzed for enzyme activity and protein concentration (***Table 4-1***).

Table 4-1: Activity profile of the azoreductase enzyme

Purification technique	Protein Concentration (mg)	Enzyme activity (µm/min/ml)	Specific Activity U/mg	Yield (%)
Cell-free extract	30	161.818	5.39	100
DEAE cellulose	1.1	32.506	29.55	20
Sephadex G-25	0.276	18.70	67.75	12

4.8 Determination of molecular weight of azoreductase

The protein sample purified through ion exchange and gel filtration chromatography was subjected to 10% SDS-PAGE so as to develop a protein purification profile of this enzyme. SDS-PAGE was run to completion and the gel was stained with Coomassie brilliant blue. A molecular weight marker was compared so as to determine the molecular weight of the partially purified enzyme which was found to be 20 kDa. Various literature review has shown that the molecular weight of azoreductase ranges between 20-30kDa with some exceptions (Shanmugapriya et al., 2012). An azoreductase produced by *Pseudomonas aeruginosa* has earlier been purified through ion exchange and gel filtration chromatography and the pure enzyme exhibited a molecular weight of 29 kDa (Nachiyar et al., 2005). However, another study showed that a pure azoreductase produced by an azo

dye decolorizing *Bacillus* sp. exhibited a molecular weight of 61.6 kDa with an optimum pH in the range of 8-9 (Maier et al., 2004). Molecular weight determination of the azoreductase is vital as it provides the necessary specific information essential for its application in real test conditions. From the ***Figure 4-2,*** the evident major band corresponds to molecular weight of azoreductase, that is ≈20kDa.

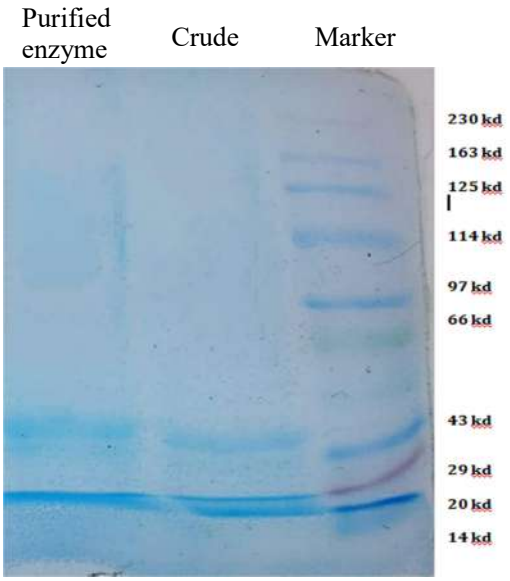


Figure 4-2: Molecular weight determination by SDS-PAGE (Lane 1, after Sephadex G-25 chromatography; Lane 2, after DEAE column chromatography; Lane 3, molecular weight markers

4.9 Effect of various parameters on the activity and stability of azoreductase

To determine the effect of various conditions and reagents on the activity of azoreductase, investigation was carried out at different pH values (5.5-9.5), temperature (30-50°C), metal ions (20-100mM), organic solvents (15-60%) and textile reagents (10% to 30%.)

4.9.1 Effect of pH on the activity of azoreductase

The analysis of effect of a range of pH on the enzymatic activity was performed by using respective buffers. The various pH buffers at 0.1 M concentration were prepared with sodium acetate buffer (pH 5.5-6.5), phosphate buffer (pH 7.5) and Tris-HCl (pH 8.5-10.5). The enzyme was pre-incubated in these buffers for 60 min before the residual activity was assayed. The purified azoreductase exhibited the highest residual enzyme

activity at a pH of 7.5 at which the enzyme activity was taken as 100% (***Figure 4-3***). Maximum enzymatic activity at this pH has also been demonstrated by azoreductase derived from *Enterobacter agglomerans* (Moutaouakkil et al., 2003). Moreover, it is established that the activity of azoreductase is optimal at neutral to basic pH (Zahran et al., 2019; Nachiyar et al., 2005).

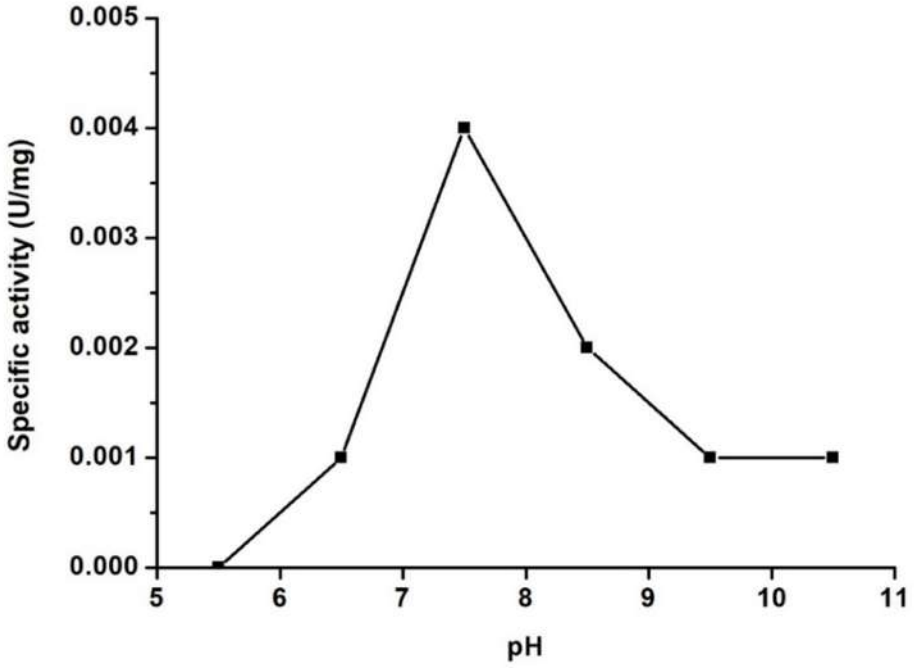


Figure 4-3: Effect of pH on enzyme activity

4.9.2 Effect of temperature on the activity/stability of azoreductase

Temperature plays a vital role on the enzymatic activities and therefore the influence of different temperature on the activity of azoreductase was investigated. The effect of temperature on the activity of azoreductase was studied at 20, 25, 35 and 40°C while maintaining a constant pH of 7.5 (***Figure 4-4*).** In this study, the enzyme was pre-incubated at different temperatures for 60 min. Among these temperatures, it was realized that the enzyme activity was optimal at 30°C. The thermo-tolerance of this enzyme to various temperatures has also been assessed earlier at pH 7.5 (Amin et al., 2010). To determine the residual enzyme activity, the activity at 30°C was taken as the control (100% activity).

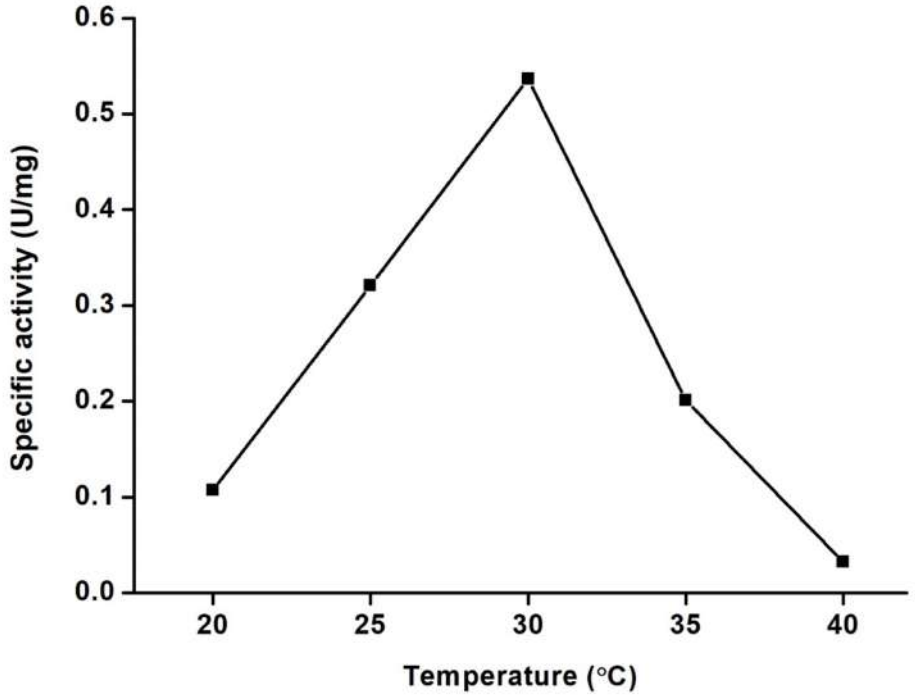


Figure 4-4: Effect of temperature on the residual activity of azoreductase

4.9.3 Effect of metal ions on the activity of azoreductase

The influence of different metal ions on the activity of azoreductase was investigated because the real textile effluent is rich in metal ions. In this study, the assay was performed by incubating the enzyme with selected metal ions which included, Mg^{2+}, Zn^{2+}, Pb^{2+}, Fe^{2+} and Mn^{2+} at a concentration of 20-100 mM/L. 50mM Tris-HCl buffer, pH 9.5 was employed in this analysis. This data clearly indicates that the presence of these metal ions (Mg^{2+}, Zn^{2+}, Pb^{2+}) had minimal effects on the activity of azoreductase, however, the presence of Fe^{2+} at a concentration beyond 60mg/L completely abolished the activity ***(Table 4-2).*** Further, the presence of Mg^{2+} favored the enzymatic activity as presented in ***Table 4-2).***

Table 4-2: Effect of metal ions on enzyme activity

Conc. (20-100mM)	Specific activity				
	Magnesium ions	Zinc ions	Lead ions	Iron ions	Manganese ions
0	0.8	0.6	0.4	0.3	0.4
20	0.0375	0.0163	0.0042	0.021	0.0033
40	0.0426	0.0037	0.0214	0.0106	0.0107
60	0.0643	0.0069	0.016	0.0108	0.0267
80	0.0214	0.0036	0.016	-0.0106	0.0321
100	0.0106	-0.0318	0.0107	-0.048	0.0376

4.9.4 Effect of organic solvents on the activity of azoreductase

Organic solvents are among the chemicals that are widely applied in dying industries. Selected organic solvents employed in this study includes acetone, glycerol, ethyl acetate, ethanol, propanol and pyridine at a concentration of 15, 30, 45 and 60%. Among the selected solvents investigated, ethanol exhibited less inhibition of enzymatic activity. The enzyme was incubated in these solvents for 60 min and assayed for enzyme activity. In this study, it was realized that ethanol had minimal effects on enzyme activity followed by glycerol and acetone. On the other hand, propanol, ethyl acetate and pyridine showed more inhibitions in that order **(*Figure 4-6*).**

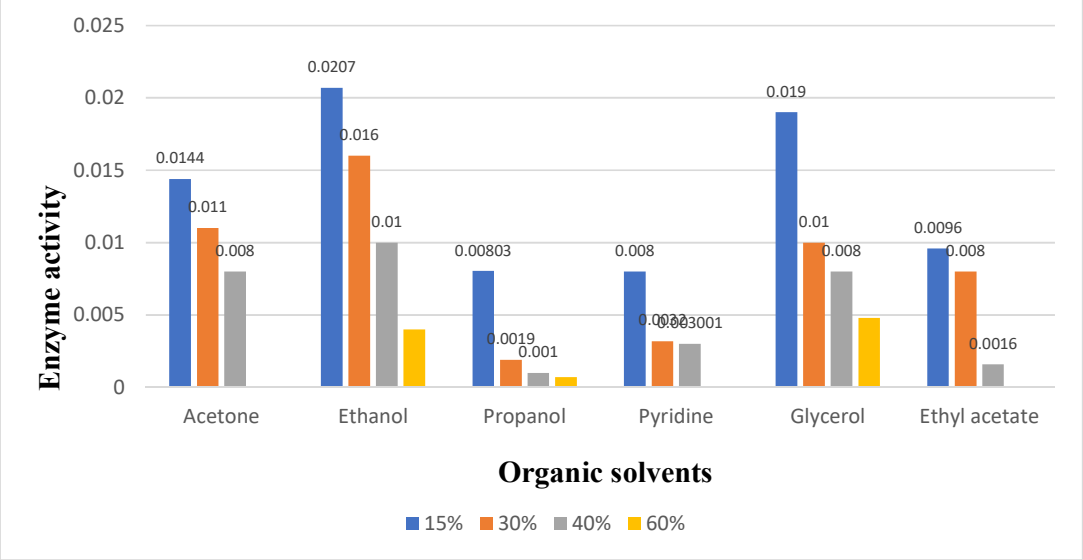


Figure 4-5: Effect of organic solvents on the activity of azoreductase

4.9.5 Effect of textile reagents on the activity of azoreductase

This analysis was carried out based on the fact that the real textile effluents are rich in diverse spectrum of chemicals apart from dye (Kant, 2011). The capability of bacterial strains to eliminate the dyes is based on the activity of this enzyme. Textile reagents selected for this study includes sodium carbonate, caustic powder (NaOH), Fenton's reagent (H_2O_2), acetic acid, sodium chloride, sodium nitrate, and Aerial soap at a concentration of 10, 20 and 30%. The reagents such as formic acid, sulphuric acid, HCl and sodium carbonate completely abolished the enzyme activity at 10% concentration itself. From the results obtained, only NaOH and NaCl showed less inhibitory effects as compared to other reagents which showed inhibition at a concentration beyond 20% as shown in the Table below **(*Table 4-3*)**

Table 4-3:: Effects of textile reagent on the activity of azoreductase

Textile reagent	**Enzyme activity in presence of varied concentrations (%) of organic solvent**		
	10	**20**	**30**
Fenton's reagent	0.00643	-	-
Caustic powder	0.00803	0.00803	0.0064
Acetic acid	0.00160	-	-
Aerial soap	0.00321	-	-
Sodium Chloride	0.0113	0.0145	0.0113

The findings from this study has proved that the azoreductase enzyme derived from the newly isolated strain *Pseudomonas aeruginosa* is tolerant to a wide range of pH, metal ions, organic solvents and relatively thermo-tolerant. This therefore show this enzyme as a good candidate for industrial application on the treatment of colored effluents.

SUMMARY AND CONCLUSION

Dye pollution is on the rise as seen from the fact that around 17-20% of all the industrial wastes is composed of textile effluents and 50% of these dyes ends up in the environment either directly or indirectly. Azo dyes make up to 70% of the approximately 900,000 tons of dyes produced annually (Carmen, et al., 2012; Rawat et al., 2016). These dyes are consumed industrially and locally in textile, leather, plastic, food, paper, pharmaceutical and cosmetic industries. In the context of rising dye pollution, research on dye especially azo dye elimination is on the rise owing to their massive employment in various industrious for various purposes. Consequently, a search for the best method based on efficiency, cost-efficacy and eco-friendliness has narrowed down to bacteria. Therefore, the search for a bacterial strain that meets the afore mentioned characteristics is on the rise. Presently, there's not much research data is not available on the elimination of dyes by some bacteria such as *Pseudomonas aeruginosa*, a species which is found widely distributed worldwide. Therefore, isolation, characterization and identification of a bacteria with potential to decolorize a wide range of textile azo dyes was performed in this study. This was carried out to so as to develop efficient, environmentally friendly and a cheaper method of dye elimination from our environment.

This doctoral thesis focuses mainly on the characterization of the newly isolated strain which was identified as *Pseudomonas aeruginosa* for which the strain name given was, JKAK This was additionally confirmed and identified by 16S rDNA analysis. Further, the time-dependent decolorization of Orange G, Bismarck Brown and a mixture of eighth dyes by the newly isolated strain and the others already existing in the laboratory (*Bacillus* sp. AK1, *Lysinibacillus* sp. AK2, *Kerstersia* sp. VKY1) was performed. The effect of various physico-chemical parameters such as pH (5.5-10.5), temperature (30°-50°C), NaCl concentration (5-30g/L), metal ions (Pb^{2+}, Zn^{2+}, Co^{2+} at 600-1000mg/L) on the decolorization of the selected dyes was also studied. The optimum pH at which decolorization was maximum was found to be pH 8.5 where JKAK decolorized 100% of Orange G, 91.36% of Bismarck Brown and 91.36 % of the dye mixture after 12, 12 and 40h of incubation, respectively. At an optimum temperature of 40°C, the strain was able to decolorize 100, 98 and 93.20% of Orange G, Bismarck Brown and the dye mixture after 16h (OG and BBR) and 40h (dye mixture), respectively. The subsequent investigations were carried out by maintaining the optimum pH of 8.5 and a temperature of 40°C as constant.

The effect of dye concentration (200-600mg/L) was analyzed with all the four

strains used in this study. At 800mg/L of the Orange G dye, JKAK, AK2, and VKY1 decolorized 76.78, 63.42, 52.55 and 49.05% of the dye while the study with Bismarck Brown showed that JKAK, AK2, AK1 and VKY1 decolorized 61.16, 65.00, 58.43 and 53.75% of the same concentration after 24h of incubation. At 600mg/L of the dye mixture, JKAK, AK2, AK1 and VKY1 were able to eliminate 57.20, 56.60, 48.00 and 44.00% of the dye after 40h of incubation. These results clearly indicate that these strains could tolerate a higher dye concentration. JKAK was also able to tolerate a high concentration of NaCl of 30mg/L where 90% decolorization of both Orange G and Bismarck Brown was observed after 12h of incubation. The metal ions such as Pb^{2+}, Zn^{2+} and Co^{2+} did not show any significant inhibitory effect on decolorization of Orange G and Bismarck Brown as evident by the fact that the presence of 1000mg/L of these metal ions, the strain was able to decolorize 92.05, 91.66 and 34.62% of Orange G after 20, 28 and 52h of incubation, respectively, whereas 86.40, 92.0 and 23.41% of Bismarck Brown was decolorized after 20, 28 and 48h of incubation, respectively. Generally, the increased concentration of metal ions lowered the rate of the decolorization process while no significant inhibitory effect was realized. The activity of azoreductase using Orange G and Bismarck Brown as the substrates were 0.9 mM/min/mg and 0.56 mM/min/mg of proteins, respectively. The strain also tolerated over 600mg/L of the respective dyes, 30g/L of NaCl, 1000mg/L of Pb^{2+} and Zn^{2+} ions and 600mg/L Co^{2+} ions.

We observed that decolorization efficiency by JKAK was maximum which was followed closely by AK2 while AK1 and VKY1 took the third position and fourth position, respectively, in terms of decolorizing capability. The consortia developed from these selected strains were used in decolorization of Orange G and Bismarck Brown and all the consortia were able to decolorize over 97% of these dyes after 16h of incubation. We also observed that the bacterial consortia developed from these strains did not significantly increase the decolorization percentage but rather increased the rate of decolorization, However, this depended on the strain combination and the efficiency of these strains individually.

The investigation of the dye decolorized products of Orange G and Bismarck Brown was conducted by using LCMS. Analysis of Orange G decolorized products revealed the presence of aniline and 8-amino-6- hydroxynaphthaline-1,3-disulfonate while studies on the products of Bismarck Brown revealed the presence of methylbenzene-1,2,4-triamine and 4-methylbenzene-1,3-diamine. Phytotoxicity studies of Orange G and Bismarck

Brown showed a reduced phytotoxic effect of these dyes after decolorization. This experimental study which was conducted with *Vigna radiata and Vigna unguiculate* showed that the control set watered with distilled water showed the highest germination percentages and longest shoot-root lengths followed by the set watered with decolorized medium and the poorest growth was observed in the experimental set watered with the untreated dye solution.

Colored waste water is composed of dyes of different molecular and structural formulas as well as salts, metal ions, bleach, and organic solvents, among others. Therefore, the biotreatment of textile effluents requires a proper choice of suitable biological system capable of tolerating various physico-chemical parameters such as pH, temperature and presence of metal ions. This include particularly the bacterial communities that are highly tolerant to metal ions, salts among other chemicals and with high decolorizing efficiency of a wider range of dyes. Bacterial consortia can also be more effective in a mixed effluent since the various individual communities might be specific to particular colorants or initiate the decolorization process by cleaving the dye molecules at particular sites allowing next step of breakdown to be completed by another strain. This synergistic activity of the strains facilitates the decolorization process. The most effective consortia of two were JKAK+AK1 and JKAK+VKY1, and consortia of three strains were JKAK+VKY1+AK1 and JKAK+VKY1+AK2. From the results obtained from this study, we can conclude that the strain JKAK is a potential novel candidate for the treatment of industrial colored waste water and can be used individually or alongside with other strains to promote faster and efficient decolorization.

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