DECOLOURISATION AND BIODEGRADATION OF TEXTILE REACTIVE AZO DYES BY A STRAIN OF *PROTEUS MIRABILIS*

A thesis submitted to the School of Postgraduate Studies, University of Lagos, Nigeria in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry

By

OLUKANNI, OLUMIDE DAVID

B.Sc. (O.O.U.) M.Sc. (Lagos)

OCTOBER, 2011

DECLARATION

DECOLOURISATION AND BIODEGRADATION OF TEXTILE REACTIVE AZO DYES BY A STRAIN OF *PROTEUS MIRABILIS*

THIS IS TO CERTIFY THAT THIS RESEARCH WORK WAS CARRIED OUT BY

OLUKANNI, OLUMIDE DAVID

UNDER OUR SUPERVISION

1. SUPERVISOR

A.A. Osuntoki
.....

Dr. Akinniyi A. Osuntoki
Department of Biochemistry,
College of Medicine,
University of Lagos,
Idi-Araba, Lagos, Nigeria.

2. SUPERVISOR

.....

Prof. George O. Gbenle
Department of Biochemistry,
College of Medicine,
University of Lagos,
Idi-Araba, Lagos, Nigeria.

UNIVERSITY OF LAGOS
SCHOOL OF POSTGRADUATE STUDIES

CERTIFICATION

This is to certify that the thesis:

"Decolourisation and Biodegradation of Textile Reactive Azo Dyes by a Strain of *Proteus mirabilis*.

Submitted to the School of Postgraduate Studies, University of Lagos

for the award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

is a record of original research work carried out

By

OLUKANNI, OLUMIDE DAVID
In the Department of Biochemistry

MR. O.D. OLUKANNI AUTHOR'S NAME	SIGNATURE	26/10/2011 DATE
DR. A.A. OSUNTOKI SUPERVISOR'S NAME	SIGNATURE	26/10/2011 DATE
Prof. G.O. GBENLE SUPERVISOR'S NAME	SIGNATURE	26/10/2011 DATE
Dr (Mrs) K.O. Olayinka INTERNAL EXAMINER	SIGNATURE	26/10/11 DATE
Dr. O.S. Odesanmi INTERNAL EXAMINER	SIGNATURE	26/10/11 DATE
Prof. T.A. Arowolo EXTERNAL EXAMINER	SIGNATURE	26/10/2011 DATE
PROF. M.O. ILORI SPGS REPRESENTATIVE	SIGNATURE	26/10/2011 DATE

DEDICATION

To:

The Weeping Earth and **The Researchers** whose works bring succor;

also to:

Pa Oluwole Olukanni (1936-2007) and **The Redeemer** who will soon make a perfect earth.

And God shall wipe away all tears from their eyes; and there shall be no more death…. for the former things are passed away. (Revelations 21: 4)

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisors, Dr A.A. Osuntoki and Prof 'Bode Gbenle for their supports and invaluable advice with a continuous stream of suggestions, feedback, and encouragement that have guided me throughout the whole period of this research.

I would also like to thank all the departmental PG committee members, Profs Magbagbeola, Okochi, Ebuehi and Dr Odesanmi for their constructive comments and suggestions particularly during the seminars. Many thanks to my colleagues: Deji Olasore, Deji Awotula, Mr Ojokuku, Olumide Adeyemi, Dr. Okunowo and others, for creating a stimulating and supportive environment.

Prof Govindwar and his team (Jadhav, Dayanand, Telke, Ristri and Swapnil), of the Department of Biochemistry, Shivaji University, India are acknowledged for being my surrogate supervisors and allowing me to use their laboratories free for the final phase of this research.

The technologists, staff and management staff of the Redeemer's University are also acknowledged for their invaluable supports while I was yet to get my compass, during the research progress and particularly when I was in far away India. I specially want to appreciate Prof Emerole, Dr Adeyemi, Mama Elutidoye, Mr Popoola, Dr. Eseola, Dr. Sojinu, Mr. Abarikwu and Owoyokun all of the Department of Chemical sciences. Mr. Scott of the department of Biological Sciences and Dr. Akpa of Mathematical Sciences are also appreciated.

The contributions of my brother – Benedict is appreciated so also are those of Donatus, Pastor (Mrs) Adeboye and all the brethren of the RCCG Redeemer's House Parish, Holy Nations Parish and God is Able Parish: The Olunloyos, the Salius, the Oyenugas, the Ikpenkos, Adebanjos, Oyenues, Ewulos, Tanimolas, Deacon Oyetunde and the rest that are so numerous to mention.

I sincerely appreciate the sacrifices of love of the following: My mum (Mrs Olukanni) who was deprived of some token; my in laws (Mr and Mrs Adefeso) who I deprived some visits; my sweet wife (Dedayo) who I deprived affection; particularly my son - Moboluwarin (a.k.a my daddy) and his sister Oluwasemilore who was barely one month old when I had to travel to India.

Finally, my sincere gratitude goes to the Only One who can bring something out of nothing, who calls forth what are not as if they are, who made another part of my life full of joy and excitement. This is your doing and it is marvelous in our sight. THANK YOU JESUS.

TABLE OF CONTENTS

CHAPTER 3: MATERIALS AND METHODS

LIST OF FIGURES

LIST OF TABLES

No. **Title** **Page**

LIST OF APPENDICES

ABSTRACT

The discharge of coloured wastewaters into water-bodies has serious implications for the environmental and public health. Furthermore, the usual colour removal methods often result in aromatic amines which are toxic, mutagenic and carcinogenic. Developing a method that can both decolourise azo dyes and degrade the corresponding aromatic amines formed is expedient. In this study, the ability of newly isolated indigenous bacteria to decolourise textile/non-textile azo dyes and degrade the aromatic amines formed was investigated. An estimated 0.05 g of the organism was inoculated into triplicates flasks containing 100 ml of a simulated effluent prepared from reactive blue 13 (RB 13), reactive yellow 42 (RY 42) and reactive red 58 (RR 58). The effect of media, oxygen, pH and temperature on the decolourisation activity of an effective strain was also investigated. The formation of aromatic amines and the mineralization of same were investigated by subjecting the metabolites of RB 13 to UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR) and gas chromatography/ mass spectroscopy (GCMS) analyses. The enzyme systems responsible for the decolourisation/ biodegradation were also studied. Three of the fourteen bacterial strains isolated from various environmental sources showed >95 % decolourisation of the simulated effluent, within 24 hours. One of these effective strains, which has been identified as a stain of *Proteus mirabilis* using 16S rDNA, decolourised methyl red (a standard azo dye) effectively at optimal pH and temperature of 7 and $37^{O}C$ respectively. The strain preferred nutrient broth to minimal media of Mills *et al* and 0.02 g dry mass decolourised 50 ml, 56 mg/L solution of methyl red within 6 hours under adequate oxygen supply. UV-visible analyses of aniline sulphate (an aromatic amine) and those of the metabolic products of methyl red revealed that methyl red was first converted to aromatic amines

and that the aromatic amines were probably mineralized. The Fourier transform infra-red (FTIR) spectra of the metabolites of RB13, when compared to that of the dye, revealed the disappearance of certain peaks, which were noticeably those of the aromatic C – H bending around 600 – 800 cm^{-1}. Similar results were obtained using high performance liquid chromatography (HPLC). Gas chromatography- mass spectrophotometry (GCMS) analysis of the dye metabolite showed (among other metabolites) the presence of Sodium 2(2-formyl-2hydroxyvinyl) benzoate, with a tropylium cation (m/z 91) as its base peak. This suggested the breakage of naphthalene rings in RB13, indicating mineralization of the aromatic amines. The presence of azoreductase and laccase activities suggested both symmetrical and non-symmetrical enzymatic reduction of azo bonds prior to mineralization. Based on the FTIR, GCMS and enzymatic studies a pathway for the biodegradation of RB13 was proposed. Partial characterization of the laccase revealed that it is a protein with molecular weight of about 60kD. It has an optimum temperature of about 40 °C and acts best between pH 4 and 5. In conclusion, an indigenous strain of *Proteus mirabilis* that can decolourise, degrade and detoxify azo dyes has been isolated. This organism is thus a candidate for ecofriendly treatment of coloured effluents.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Textile industries release an estimated 10-15% of their dyes as effluent during dyeing processes. The treatment of such effluent is difficult since it contains various types of chemicals including residual dyes with complex structures and other pollutants such as dispersants, leveling agents, acids, alkalis and carrier agents (Cooper, 1993).

Reactive azo dyes are the most important class of dyes used by the textile industries, accounting for 50-65% of residual dyes in industrial effluent (Chung and Stevens, 1993). This group of dyes forms the largest class of water-soluble synthetic dyes with the greatest variety of colours and structures, and they are generally recalcitrant to oxidative decolourisations (Maier *et al.*, 2004). Thus, the recalcitrant nature of the azo dyes results in persistent colouration of water bodies and colour in water reduces light penetration, hence, making aquatic life more difficult (Goncalves *et al.*, 2000). In addition, aromatic amines formed during decolourisation of coloured wastewaters have been found to be toxic and/or mutagenic (Chung and Stevens, 1993; Field *et al.*, 1995).

Several methods are used in the treatment of textile effluents to achieve decolourisation. These include physiochemical methods such as filtration, specific chemical oxidation, reverse osmosis, coagulation-flocculation, adsorption and photo-degradation (Van der Zee, 2002). Some of these methods are effective but quite expensive (Maier *et al.*, 2004).

Biotreatment, however, offers a cheaper and environmentally friendlier alternative for colour removal in textile effluents.

However, studies have shown that optimizing the culture medium could enhance the biodegradation of xenobiotics. For instance, a positive impact on biodegradation of some aliphatic chlorinated xenobiotics has been observed when the culture medium is supplemented with minerals (Hennery and Girbic-Garlic, 1999). Also, biodegradation using whole organisms are often less effective due to transport of substrates across the membranes. This makes the use of enzymes an attractive alternative.

Enzymatic treatment is very useful when pollutants are present in much diluted solution and recalcitrant to the action of various microbes participating in the degradation of dyes (Husain, 2006). Other benefits of enzymatic treatments include fast and effective degradation, lack of biomass accumulation reduction of odour, and the elimination of the need of media. Majority of these enzymes are oxidoreductive enzymes such as Mn^{+} peroxidases, laccases, lignin peroxidases, plant polyphenol oxidases, micro peroxidases and azoreductases (Husain, 2006). Major challenges in enzymatic treatment of effluent are stability and catalytic ability of enzymes, which decrease with the complexity of the effluent (Zille *et al.*, 2003).

1.2 Statement of the Problem

Pollution of communal water bodies by dyestuffs released from textile industries and dye houses is a major environmental concern. In many Nigerian cities, the textile factories daily discharge millions of liters of untreated effluents in forms of wastewater into drains

that eventually empty into rivers. This alters the pH, increases the biochemical oxygen demand (BOD) and Chemical oxygen demand (COD), and gives water intense colouration (Olayinka and Alo, 2004).

In addition, methods that have been used in the treatment of textile effluents include physico-chemical methods such as filtration, specific chemical oxidation, reverse osmosis, coagulation-flocculation, adsorption and photo-degradation (Young and Yu, 1997; Do *et al*., 2002). Some of these methods are effective but quite expensive (Maier *et al.*, 2004). Although, biotreatment offers a cheaper and environmentally friendlier alternative for textile effluents treatments, there are still demands for better biodegradation procedures that are faster and more effective. Biotreatment like every other colour removal procedure has its own demerits. One example is the fact that anaerobic decolourisation of azo dyes produces aromatic amines which are toxic to aquatic life (Chung and Stevens, 1993), mutagenic to humans and cannot be mineralized under same condition (Chung and Cerniglia, 1992, Goncalves *et al.*, 2000). Studies that have reported the mineralization of aromatic amines under aerobic conditions argued that aerobic decolourisation of azo dyes which is the first step in azo dyes biodegradation, is not possible (Shaul *et al.*, 1991; Van der Zee and Villaverde, 2005). There is, therefore, the need to investigate organisms that can both decolourise and degrade azo dyes effectively.

1.3 Overall Aim

The aim of this research is to investigate indigenous bacterial strains for their ability to decolourise azo dyes and to degrade aromatic amines which are toxic products of such decolourisation.

1.4 Specific Objectives

The following are the objectives of this research:

i. To isolate and characterize an aerobic bacterium that can decolourise azo dyes effectively.
ii. To determine the effect of media, temperature, oxygen and pH on the decolourisation activities of the particular bacterium.
iii. To elucidate the pathway for the biodegradation of azo dyes by this organism using reactive blue 13 as a model dye.
iv. To study the enzyme systems responsible for the decolourisation / biodegradation and to characterize the prominent enzyme.

1.5 Significance of the Study

Bacterial degradation is a viable treatment option for wastewater containing azo dyes. However, a great drawback is the generation of potentially toxic and mutagenic end products (aromatic amines) by anaerobic bacteria. This study is part of efforts to develop textile effluent bio-treatment processes that would not only decolourise but also mineralize the toxic metabolites generated during such treatments. In addition, organisms isolated in this study will help in the development of a continuous-flow, textile wastewater decolourisation process and the possible reuse of the treated wastewater in the dyeing operation thereby minimizing water consumption, wastewater volume and concentration of textile pollutants.

1.6 Definition of Terms

- **Aerobic –** a reaction or process taking place in or characterized by the presence of free oxygen.
- **Anaerobic -** a reaction or process taking place in the absence of free oxygen; lacking free oxygen.
- **Aromatic amine –** a chemical compound that consists of one or more benzene rings with at least an amino group attached.
- **Azo dyes –** a group of dyes characterized by the presence of at least an azo (–N=N-) bond.
- **Biodegradation –** decomposition of large molecules (to smaller ones) brought about by living organisms.
- **Biomonitor –** an organism or component of organisms designed to detect and / or determine the presence or extent of pollution.
- **Bioremediation –** the use of any biological means to remove undesirable substance in waste; it includes but not limited to biodecolourisation, biodegradation, and biosorption.
- **Biotreatment –** the removal of pollutants by the action of biological agents…
- **Decolourisation –** the act of removing colour from wastewaters; this is not necessarily biodegradation.
- **Effluent –** liquid waste flowing out of a factory, a commercial establishment or household into a water body.
- **Metabolites –** Substances formed during the degradation of large molecules by biological agents.
- **Mineralization –** the complete degradation of large organic molecules into small inorganic molecules like nitrates, ammonia, carbon dioxide and water.
- **Reactive dyes –** are dyes with reactive groups that form covalent bonds with the OH-, NH- and SH- groups of fibres.

1.7 Abbreviations

APHA	American Public Health Association
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
DMP	2, 3-Dimethoxylphenol
FTIR	Fourier transform infrared spectroscopy
GCMS	Gas Chromatography with mass spectrometer as detector
HPLC	High performance liquid chromatography
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
RB13	Reactive blue 13
RR58	Reactive red 58
RY42	Reactive yellow 42
VAO	Veratryl Alcohol Oxidase

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Dyes

The history of dyes has been traced to the primitive age; for instance, the Egyptian mummies were found wrapped in dyed cloths and natural matters have been used to stain hides, decorate shells and feathers within the living memory. In a similar way, stories have been painted on walls and tattooed on bodies using natural dyes. The British were discovered by the Romans to have tattoos painted with indigo dyes, thus, the Latin name "Briton" meaning painted men. The dyes used in those days were extracted mainly from plants and sometimes from animal sources. Inorganic pigments such as soot, manganese oxide, haematite and ochre were also used. Dyes were so important then that certain dyes like Tyrian purple were part of the local economy and world trade (Ali, 2006).

Synthetic dyes were not discovered until in the 19th century: precisely in 1856, when the English chemist W.H. Perkin in an attempt to synthesize quinine, obtained a bluish substance later identified as Mauveine. Impressed by the excellent dyeing properties of the substance, Perkin patented his work and started the manufacturing of the dye. Subsequently, the discovery of the molecular structure of benzene by Kékulé in 1865 further enhanced synthetic dyes research and manufacturing such that by the end of the century, synthetic dyes had almost replaced natural dyes in application. By the last century, synthetic dyes have been prepared in bewildering number and variety (Van der Zee, 2002).

Generally, dyes are a broad spectrum of chemical compounds characterized structurally by substituted aromatic or heterocyclic groups linked to a chromophore and an auxochrome. They are used for colouration of textile, paper, leather, plastics and in

specialized applications such as in food, drugs, cosmetics and photochemicals productions (Van der Zee, 2002).

2.2. Classification of Dyes

A comprehensive classification of dyes is found in *Colour Index*. The *Colour Index* is a book first edited in 1924 and revised every three months by the Society of Dyers and Colourists and the American Association of Textile Chemists and Colourists (AATCC). In the book, dyes are classified using either their chemical structure (Chromophore) or their application method (Zille, 2005).

The basic structure of dyes consists of an aromatic compound linked to a chromophore and auxochrome. Usually, the aromatic compounds possess extensive conjugation, which absorb electromagnetic wave in the visible region (380-780 nm). While the presence of chromophore is responsible for the colour of the transmitted light, the auxochrome, either by donating or withdrawing electrons from the chromophore, enhances the colour.

Common molecular fragments that characterizes chromophores that are usually encountered in dyes include azo (–N=N–), carbonyl (>C=O), alternating carbon-carbon double bonds (–C=C–), oximes (>C=NH), imines (–CH=N–), nitroso (–NO), nitro (–NO_2), and sulphur (>C=S, and other carbon-sulphur groups). However, usual auxochromes possess ammonium (–NH_3^+), carboxyl (-COOH), sulphonate (–SO_3H), and hydroxyl (–OH) groups (Van der Zee, 2002; Ali, 2006).

2.2.1. Classification based on chemical structures

Based on chemical structures, dyes have been classified into 20-30 groups. Prominent families among these groups are azo (monoazo, diazo, triazo or polyazo), anthraquinone, phythalocyanine and triarylmethane dyes.

2.2.1.1 Azo dyes

Azo dyes are the largest group of water soluble dyes commonly used in the textile industries (Chung and Stevens, 1993). They are characterized by -N=N- chromophore between aromatic rings. Azo dyes could be monoazo, diazo, triazo, and polyazo dyes based on the number of azo-groups present. They are usually produced by diazotisation of a primary amine.

Azo reactive dyes form the largest class of water-soluble synthetic dyes with the greatest variety of colours and structures, and are generally recalcitrant to oxidative decolourisations (Maeir *et al.*, 2004). They are used to dye various materials such as textiles, leather, plastics, cosmetics and food.

2.2.1.2 Anthraquinone dyes

Anthraquinone dyes constitute the second largest class of textile dyes, after azo dyes. They are used extensively in the textile industry due to their wide variety of colour shades and ease of application (Baughman and Weber, 1994). They are used in colouration of cotton and cellulose fibres as well as hydrophobic synthetic materials.

Anthraquinone, the basic unit of this class of dyes, is faint yellow in colour. Although anthraquinone can be used as a dye, it is not classified as one. The presence of hydroxyl

and amino groups in anthraquinone results in a wide range of colours. Most mordant, disperse and vat dyes contain anthraquinone units. Its quinonoid system acts as a chromophore. Anthraquinone dyes have excellent fastness properties.

2.2.1.3 Phythalocyanine dyes

A phthalocyanine is a macrocyclic compound having an alternating conjugated system of C=N units (both inside and outside five-membered rings) linked together in molecular cycle. The isoindo nitrogen atoms of phthalocyanine enable the molecule to form coordinate bonds with hydrogen and metal cations in its center. Several phthalocyanine compounds exist because the cationic metal core readily undergoes further complexation with various other ligands. Therefore, a variety of phthalocyanine complexes exist.

Like most dyes, phthalocyanine dyes were accidentally synthesized by two researchers from Switzerland as copper complexes of phthalocyanine, naphthalocyanine and octamethylphthalocyanine when they tried to convert *o*-dibromobenzene into phthalonitrile. They remarked the enormous stability of these complexes but failed to appreciate their discovery and to fully characterize these blue complexes. The *real discovery* also started as an accident when a blue product was found in a reaction flask where only white product was expected. However, this accident occurred in a dye company, Scottish Dyes Ltd., Grangemouth, Scotland (later ICI) and the discovery was followed on (Lillie, 1969). The structure of a phthalocyanine molecule is closely related to that of the naturally occurring porphyrin systems.

2.2.1.4 Arylmethane dyes

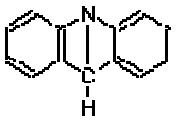
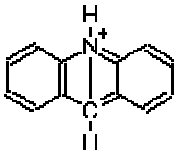
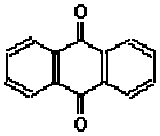
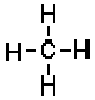
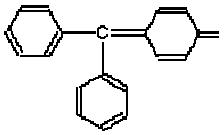
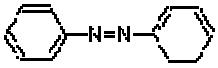
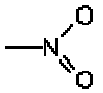
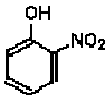
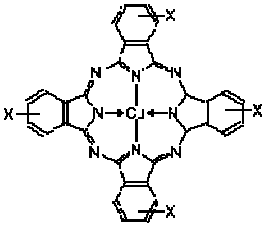
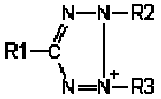
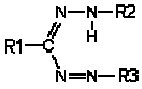
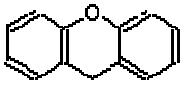
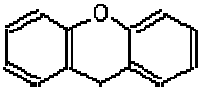
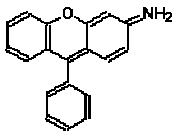
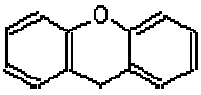
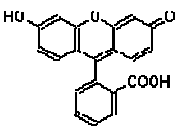
Arylmethane dyes are so called because they are derived from methane, which some of its hydrogen atoms are replaced with aryl rings. Since a synonym for aryl is phenyl, they are also called phenylmethane dyes. Aryl rings are often referred to as benzene rings. However, benzene is a specific chemical compound and this usage is technically incorrect, although very common (Zille, 2005). The arylmethane dyes are further classified based on the numbers of aryl groups.

Diarylmethane (or diphenylmethane) dyes have two aryl rings. Only one dye in this subgroup has gained much use, and that is auramine O, a fluorescent dye used for the demonstration of acid fast bacteria and to make a fluorescent Schiff reagent. Sometimes alkylamino groups are attached to the aryl rings resulting in an arylmethane subclassified as an aminodiarylmethane. The chromophore is the C=N double bond (Suwanruji, 2004).

Triarylmethane (or triphenylmethane) dyes contain three aryl rings. These are much more common and numerous examples can be given. The pararosanin homologues are good examples. These dyes also contain amino groups, and for that reason are sometimes identified as aminotriarylmethane dyes, which are often basic. In pararosanilin, each of the three aryl rings has an amino group (Zille, 2005).

Not all aminotriarylmethane dyes are basic; some are acid dyes. These have strongly anionic sulphonic groups as well, which makes the overall charge on the molecule negative. Examples are acid fuchsin and methyl blue. Another subgroup of the arylmethanes bears hydroxyl groups instead of the amino groups. These dyes are termed hydroxytriarylmethane dyes and are acid dyes. An example is chromoxane cyanin R, which has gained some use as a hematoxylin substitute. Dyes in this subgroup are not widely used (Zille, 2005).

Table 2.1 Chemical structures of some classes of dyes

Class	Derived from	General formula
Acridine		
Anthraquinone		
Triarylmethane		
Azo		
Nitro		
Phthalocyanine	Not applicable	
Tetrazolium		
Xanthene Fluorene - Pyronin		
Xanthene Fluorene – Rhodamine		
Xanthene Fluorone		

Source*:* **(Lillie, 1969)**

2.2.2. Classification based on application

Based on the mode of applications dyes have been classified into the following groups:

2.2.2.1 Acid dyes

Acid dyes are water-soluble anionic dyes that are applied to fibres such as silk, wool, nylon and modified acrylic fibres using neutral to acid dye baths. Attachment to the fibre is attributed partially to salt formation between anionic groups in the dyes and cationic groups in the fibre. Acid dyes are not substantive to cellulosic fibres. Most synthetic colours used in food fall into this category (Van der Zee, 2002).

Some acid dyes are used for colouring animal fibres; this involves the actions of acidified solution (containing sulphuric acid, acetic acid, sodium sulphate, and surfactants) on amphoteric protein.

2.2.2.2 Basic dyes

Basic dyes are water-soluble cationic dyes. They are mainly applied on acrylic fibres, but find some use for wool and silk. Usually acetic acid is added to the dyebath to help the uptake of the dye onto the fibre. Amino derivatives of basic dyes are also used in the colouration of paper (Van der Zee, 2002).

2.2.2.3 Direct dyes

Direct dyes are usually azo dyes that require sodium salts and metallic chrome such as copper, cobalt and chromium. They are water-soluble anionic dyes that have high affinity to cellulose fibres when dyed from aqueous solution. Substantive dyeing is normally

carried out in a neutral or slightly alkaline dyebath, at or near boiling point in the presence of either sodium chloride (NaCl) or sodium sulphate (Na_2SO_4). Direct dyes are used on cotton, paper, leather, wool, silk and nylon. They are also used as pH indicators and as biological stains (Van der Zee, 2002).

2.2.2.4 Disperse dyes

These are water insoluble, nonionic dyes used for dyeing hydrophobic fibres from aqueous dispersion. Usually they are small azo or nitro compounds (yellow to red), anthraquinones (blue and green) or metal complex. They require dispersing agent such as cellitoin and were originally developed for the dyeing of cellulose acetate. The dyes are finely ground in the presence of a dispersing agent and then sold as a paste, or spray-dried and sold as a powder. Their main use is towards dyeing polyesters, but they can also be used to dye nylon, cellulose triacetate, and acrylic fibres. In some cases, a dyeing temperature of 130°C and a pressurized dyebath are required (Zille, 2005).

2.2.2.5 Mordant dyes

This class of dyes requires a mordant, a compound that improves the fastness of the dye against water, light and perspiration. Mordant dyeing is probably one of the oldest ways of dyeing, but the use of mordant dyes is gradually decreasing. The final colour of the fabric depends greatly on the choice of mordant use. Therefore, mordant must be carefully chosen. The most important mordant dyes are the synthetic mordant dyes (or chrome dyes) used for wool. These comprise some 30% of dyes used for wool, and are especially useful for black and navy shades. It is important to note that many mordants, particularly those in the heavy metal category, can be hazardous to health and extreme

care must be taken in using them. Most mordant dyes are azo, oxazine or triarylmethane compounds. Dichromate or chromium complexes mordant are usually applied as an after-treatment (Van der Zee, 2002).

2.2.2.6 Sulphur dyes

Sulphur dyes are complex polymeric aromatics with heterocyclic S-containing rings. These are two part "developed" dyes: reduction and oxidation. The initial bath imparts a yellow or pale chartreuse colour, which is then treated with a sulphur compound to produce the dark colours. They are mainly used for dyeing cellulose fibres. Such fabrics have dull shades with good fastness to fibres, washing, and acids but susceptible to chlorine and light.

2.2.2.7 Solvent dyes

Solvent dyes, also known as lysochromes, are non-ionic dyes that are used for dyeing substrates in which they can dissolve; e.g. plastics, varnish, ink, waxes and fats. They are not often used for textile-processing, but their application is increasing. Most solvent dyes are diazo compounds that undergo some molecular rearrangement, which leads to losing their ionic nature. Common examples include Sudan III (solvent red 23), Sudan IV (solvent red 24) and oil red O (solvent red 27). These are dyes commonly used for demonstrating fat staining (Lillie, 1969).

2.2.2.8 Vat dyes

Vat dyes are water insoluble dyes applied mainly to cellulosic fibres. They are ordinarily insoluble, but reduction in an alkaline bath produces a water soluble alkali metal salt of the dye referred to as its leuco (colourless) form. Sodium dithionite is usually the reducing agent. The reduced dye is impregnated into fibre where it is subsequently reoxidized to the original insoluble dye. Almost all vat dyes are anthraquinones or indigoids. The dyes obtained their name 'Vat' from the vats which are used for the reduction of indigo plants through fermentation (Van der Zee, 2002).

2.2.2.9 Reactive dyes

Reactive dyes are dyes with reactive groups that form covalent bonds with the OH-, NH- and SH- groups of fibres. The covalent bonds that attach reactive dye to natural fibres make them among the most permanent of dyes. Reactive dyes are commonly used on cellulose fibres such as cotton, but can be used on nylon and wool also (Van der Zee, 2002). Some of these dyes are very easy to use and can be applied at room temperature. These are known as "Cold" reactive dyes. Due to their low cost and easy methods of application, reactive dyes are now preferred for dyeing cotton and other cellulose fibres at home, art studio and dye-houses.

2.3 Reactive Dyes: Structure and Importance

Reactive dyes react with the textile fibre to produce both hydroxyl and oxygen linkages. The chlorine (Figure 2.1) combines with the hydroxyl to form a strong ether linkage. This reaction results in a fast and brilliant colour.

2.3.1 General structure

The molecular structure of reactive dyes (Figure 2.1) consists of a chromogen (C) with solubilizing groups (S), a bridging group (B), a reactive group (R) and a leaving group (X). The reactive groups are capable of reacting with nucleophilic groups (NH2, -SH, and –OH) in textile fibres by addition or substitution reactions. The chromogen, a conjugated system containing one or more chromophores, provides the colour. The bridge separates the reactive group from the chromogen and prevents the colour generated by the chromogen from changing after the chromogen is attached to the reactive group (Suwanruji, 2004).

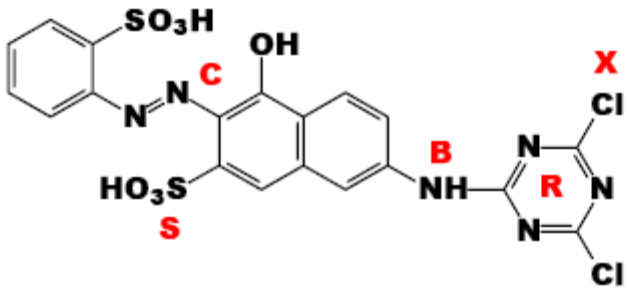


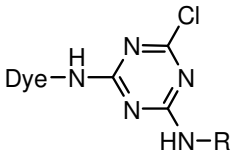
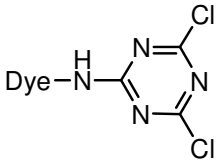
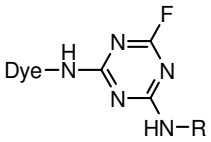
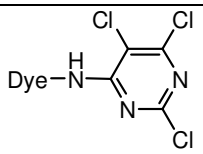
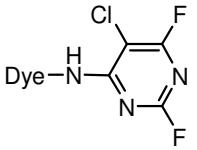
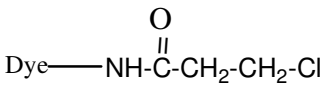
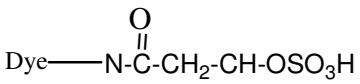
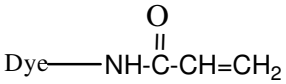
Figure 2.1 Generic structure of a fibre reactive dye chromogen (C) with solubilizing groups (S), a bridging group (B), a reactive group (R) and a leaving group (X).

2.3.2 Reactive groups

The incorporation of reactive groups to dyes has been traced to 1890s. The drive behind the manufacturing of reactive dyes was probably the presence of nucleophilic groups in cotton and wool, the then two major clothing materials. Prior to this period, cotton and wools were forced to react with acylating reagents under nearly anhydrous condition. This treatment caused shrinkage and degradation of the fibre. This method was later replaced by the use of strong caustic soda in 1895 to compell certain dyes to be more reactive. The discovery of reactions such as benzolylation, nitration, and diazotization soon made the reactive dyes research lucrative (Suwanruji, 2004).

The first commercialized reactive dyes appeared in 1953, when ICI introduced Procion $^{(R)}$ into the market. These dyes contain dichlorotriazine reactive groups, which were capable of reacting with the OH groups on cellulose (CellOH) at low temperatures (20-40^{O}C)**.** The success story of Procion soon led to the emergence of other reactive groups. The reactive groups are usually heterocyclic aromatic rings substituted with chlorine or fluorine. An example is the monochlorotriazine reactive dye (Cibacron) first produced by Ciba in 1957. Another common reactive group is the vinyl sulphone first manufactured by Hoechst in 1957. Several reactive groups are currently used in dyes manufacturing, which include triazine, vinylsulphone, quinoxaline, and pyrimidine base dyes (Van der Zee, 2002).

Table 2.2 Some Reactive Groups commonly use in Manufacturing of Reactive Dyes

Group	Specific Examples	Structure
Triazine	Monochlorotriazine	
	Dichlorotriazine	
	Monofluorotriazine	
Pyrimidine	Trichloropyrimidine	
	Difluoropyrimidine	
Vinyl Sulphone	β-sulphatoethylsulphone	Dye- SO_2 - CH_2 - CH_2 - OSO_3H
	β-sulphatoethylsulphone	Dye- SO_2 - NH - CH_2 - CH_2 - OSO_3H
Amido	Chloropropylamido	
	Sulphatopropylamido	
	Acrylamido	

Sources: Van der Zee (2002), Abadulla *et al.* (2000)

2.3.3 Advantages and disadvantages of reactive dyes

The introduction of reactive dyes has resulted in extensive and fundamental changes in the procedures of the dyeing industries. This is probably because of their ability to form stable covalent bonds with fibres. Reactive dyes provide bright shades that are unachievable with most other classes of dyes (Sekar and Sivakumar, 1993). Full spectrums of colours are also possible; since their chromophores can be changed easily (Sekar and Sivakumar, 1993). Furthermore, because of the stable covalent bonds, reactive dyes have good wash-fastness, i.e they hardly bleed with water. They have high solubility and reactivity, which enable them to be used in variety of applications and for cold dyeing (Sekar and Sivakumar, 1993). Above all, the cost is moderate, which is a serious advantage over the more expensive, but better fastness dyes like the Vat dyes.

One major disadvantage of the reactive dyes is the inactivation of the reactive groups (hydrolysis) during dyeing processes, which lowers the degree of fixation. To overcome this, dyers often add 60 and 200 g/l of salt and ureum to the dyeing bath. In spite of this, an estimated 10-50% dye will not react with the fabric and remains in the water phase (Van der Zee, 2002). In addition, reactive dyeing process always produce high volumes of wastewater of varied composition, often containing salt, urea, metal ions and dye (Do *et al.*, 2002) . Bleeding may also occur if dyed fabrics are stored in areas with acidic gases such as carbon dioxide, sulphur oxides or when bleaching agents like chlorine (in tap water), sodium percarbonate and sodium perborate (in detergents) are used on them (Suwanruji, 2004).

2.3.4 Typical dyeing process

Normally, to obtain a desired fabric colour shade, a mixture of dyes, especially red, yellow and blue, is used in textile dyebaths. These dyes consist of many different chromophores (dye groups) including azo, anthraquinone and phthalocyanine (Hao *et al.*, 2000; Van der Zee, 2002). Among the textile dyes, reactive dye is an important class used not only to colour cellulosic fabric (mainly cotton), but also wool and polyamide fibres. A steady increase in reactive dye usage has been observed as a result of the increase in cotton and wool use worldwide.

A typical dyeing process is a water consuming and effluent generating process (figure 2.2). The effluent from sizing, desizing, scouring, bleaching, dyeing, rinsing, and finishing wastes contains unfixed dyes with a high intensity of colour (Babuna *et al.*, 1998; Hao *et al.*, 2000). It has been estimated that approximately 50% of applied reactive dyes is wasted because of dye hydrolysis in the alkaline dyebath. This results in intensely coloured wastewater of environmental concern. In most countries the state and federal environmental regulations have become more stringent (requiring lower effluent colour limits) and reduction or total elimination of colours in wastewater is mandated (Hao *et al.*, 2000).

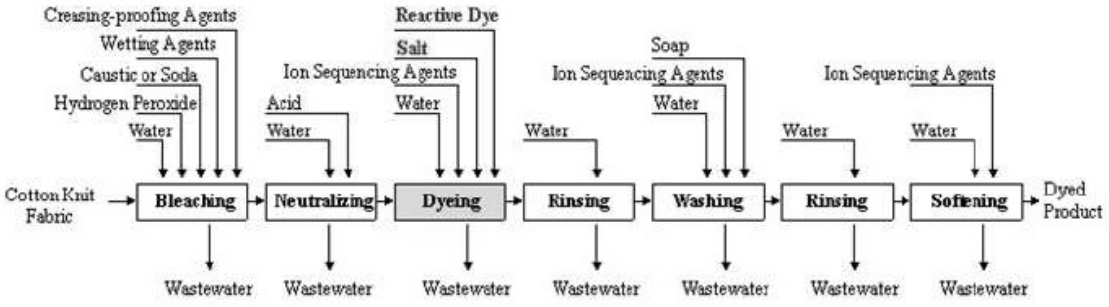


Figure 2.2: A typical flow diagram for a textile cotton dyeing process (Babuna *et al.*, 1998).

2.4 Environmental Considerations

Pollution of communal water bodies by dyestuffs released from textile industries and dye houses is a major environmental concern. An estimated 10-15% of the industrial dyes are released as effluent during dyeing processes. The complex nature of the effluent makes its treatment difficult. Worse still is the presence of reactive dyes, which are preferred by the industries because of their chemical stability (Cooper, 1993).

Reactive dyes have environmental implications because up to 50% of the initial dye mass (up to or exceeding 800 mg/L) used in the dyeing process remains in the spent dyebath in its hydrolyzed form which no longer has an affinity for the fabric, and thus cannot be reused in the dyeing process (Van der Zee, 2002). Reactive dyes are not readily removed by typical wastewater treatment processes due to their inherent properties, such as stability and resistance towards light or oxidizing agents (Banat *et al.*, 1996). In addition to the presence of dye, the high salt concentrations as well as high pH values under typical reactive dyeing conditions further complicate the management of spent reactive dyebaths.

2.4.1 Temperature and pH

Temperature control is crucial to dyeing processes. Generally increase in temperature increases reactivity, resulting in efficient dye process. For instance, while dichlorotriazinyl dyes can be applied at room temperature because of their high reactivities, the monochlorotriazines require hot dyeing. Discharge of wastewater during hot dyeing processes results in thermal pollution which is injurious to aquatic lives.

Dyeing process is usually done in alkaline pH. Alkali is added to dyebath to achieve the fixation of dye on the fibre (Van der Zee, 2002). This altered pH will reflect in wastewater and subsequently in water bodies to which untreated wastewaters are discharged.

2.4.2 Colouration

Generally, synthetic dyes are chemically and photolytically stable. They are highly visible – can be detected at concentration as low as 1 mg/L, and are persistent in natural environments (Nigam *et al.* 2000, Rieger *et al.* 2002). Colour in water bodies reduces light penetration, alters the pH, increases the biochemical oxygen demand (BOD) and Chemical oxygen demand (COD), and thereby makes aquatic life difficult (Goncalves *et al.*, 2000). In addition to the negative consequences by which colour prevention of light penetration interferes with the photosynthesis of aquatic plants, coloration also impacts on the aesthetic appearance of water bodies (dos Santos *et al.*, 2005).

2.4.3 Bioaccumulation

Accumulation of dyes, like other toxins, is of great concern in aquatic food chain, since the accumulated toxicants can be transfer to the consumer species. The propensity of various organisms to accumulate azo dyes has been studied accross a wide range of living systems involving vertebrate and invertebrate, aquatic and terrestrial, mammals and non mammals. Benthic microinvertebrates that inhabit the sediments of rivers have been observed to absorb, metabolize or accumulate toxic compounds such as dyes and poly-

aromatic hydrocarbons (PAHs) (Chen and White, 2004). Studies on the tendency of azo dyes to bioaccumulate in fish have shown that several factors influence the rates of dyes uptake. These factors include partition coefficient, diffusion resistance, molecular size, respiratory volume and gill perfusion (Erickson and McKim, 1990). The possibility of fish metabolizing the dyes is reduced by the hydrophobic nature of the dyes. The elimination rates for hydrophobic chemicals are low since the uptake and elimination of hydrophobic chemicals by fish have often been shown to be a first-order exchange process (Joshi *et al.*, 2001). Water-soluble dyes like acid, reactive and basic dyes are generally not bioaccumulated. So also are the poorly – soluble, disperse dyes for which bioaccumulation potential is much lower than expected (Van der Zee, 2002). In general, ionic dyes do not have significant bioaccumulation potentials, but some acid dyes may bioaccumulate. Non-ionic dyes and pigments, on the other hand, have a high bioaccumulation potential (Van der Zee, 2002).

2.4.4 Toxicity of dyestuffs

Toxicity of azo dyes on aquatic organisms has been studied using algae (Greene and Bauughman, 1996; Hu and Wu, 2001) and fishes (Joshi *et al.*, 2001; Sharma *et al.*, 2004). Results from these literatures showed that algae growth and fish mortality are not affected by dye concentrations below 1 mg/L. Basic and acidic dyes were adjudged the most toxic dyes for algae and fishes. In a study conducted with rats, only few dyes (1% of 4461 dyestuff tested) showed LD_{50} values below 250 mg/kg body weight of rat. Therefore the chance of human mortality for acute dyestuff toxicity is low (Van der Zee, 2002). Low toxicity of azo dyes has been associated with the presence of sulphonate

group, which enhances the urinary excretion of the dye and its metabolites (Brown and DeVito, 1993). Brown and DeVito (1993) also observed that the cleavage of azo bonds of azo dyes often results in more toxic aromatic amines and that the toxicity of these metabolites depends greatly on the spatial arrangement of the atoms/group in the molecules, particularly the amino groups. Other substituents that increase toxicity are nitro, methyl or methoxyl group; while carboxyl or sulphonate groups lower toxicity (Chung and Cerniglia, 1992). Other toxicity studies that have been investigated are cytotoxicity, mutagenicity, carcinogenicity and genotoxicity (Khehra *et al.*, 2006).

2.4.4.1 Cytotoxicity

Azo dyes toxicity to anaerobic biomass has been reported (Sponza and Isik, 2002). This toxicity was associated with high dye concentration, presence of heavy metals (metal complex dyes) and/or presence of non-hydrolyzed reactive groups of reactive dyes. In addition, azo dyes have been reported to delay the onset of reproduction in *Moina macropopa* , a fresh water cladoceran (Wong *et al.*, 2006).

2.4.4.2 Mutagenicity

Although poor correlation exists between results of mutagenicity /carcinogenicity of azo dyes in *in vitro* studies and animal studies, metabolic activation of azo dyes have resulted in aromatic amines, which have been reported to be mutagenic in *in vitro* tests. Aromatic amines formed after degradation of azo dyes have been found to be cytotoxic and/or mutagenic (Chung and Stevens, 1993; Field *et al.*, 1995). Highly purified azo dyes are not mutagenic; but many of the commercially available azo dyes may, due to impurities,

show metabolic activation and mutagenic activity *in vitro*. The presence of sulphonated groups also contribute to the low genotoxic potential of azo dyes (Jung *et al.* 1992).

2.4.4.3 Carcinogenicity

Carcinogenicity of anthraquinone dyes has been reported. Some of them have been implicated in bladder tumor in rats. A higher number of bladder cancer deaths were reported for men manufacturing azo dyes; 127 deaths compared to the 4 deaths expected in those not working with azo dyes (Atkins 2000). The possibility of genotoxicity mechanism of cancer induction cannot be excluded, since aromatic amines, which are raw materials for azo dyes synthesis are potent carcinogen. Cartwright (1983) has reported that there is correlation between the exposures to aromatic amines and human cancer.

2.4.4.4 Other effects

Sensitization to azo dyes is a well known phenomenon in the textile industry. Occupational eczema has been reported in about 20% of the workers dyeing cotton with red azoic dyes. The explanation is probably that the attachment of molecules from disperse dyes is weak and, as such, they are more easily available for skin contact (Seidenari *et al.,* 2002). Respiratory and nasal symptoms have also been reported in 15% of workers handling reactive dyes. Symptoms of asthma, rhinitis and dermatitis were also reported in workers exposed to reactive dyes (Nilsson *et al.*, 1993). Dyes containing anthraquinone or azo structures are also known to cause contact dermatitis (Platzek *et al.*, 1999).

2.5 Assessment of Pollution Effect of Textile Effluents

Over the years, the extent of pollution and effectiveness of treatment of wastewaters have been measured with the use of conventional methods such as colour intensity, chemical oxygen demand (COD) and biochemical oxygen demand (BOD). Recently, certain analytical methods involving sophisticated instruments are being employed not only to detect the presence of the pollutants but also to determine their chemical nature. Some of the conventional methods and the analytical methods are discussed in this section.

2.5.1. Biochemical oxygen demand

Biochemical oxygen demand is usually determined after five days (BOD_5). It is the amount of oxygen required by bacteria to break down all biodegradable compounds present in the water sample. It corresponds to the phases of assimilation of synthesis by microorganisms present in the water sample. Standard Methods by American Public Health Association (APHA) is the generally accepted method for BOD determination. The ultimate BOD is measured after 21 days and it includes the auto-oxidation phase of the bacteria (Ademoroti, 1996).

2.5.2. Chemical oxygen demand

Chemical oxygen demand (COD) is the amount of oxygen required to completely oxidize all oxidizable matters present in a sample of water to carbon dioxide and water. Certain minerals such as sulphides and sulphites are also oxidized in this process. COD gives information on biodegradability of wastewaters and the extent of biodegradation, which is important in complying with the regulated discharge limit (Eremektar *et al.*, 2007).

2.5.3. Dissolved oxygen

Dissolved oxygen (DO) is a measure of the amount of oxygen in the water, wastewater or effluent at the time of collection of such water. The determination of DO is a key test in water pollution and treatment monitoring (Ademoroti, 1996).

2.5.4. Biomonitor

Microorganisms or their mutants are often used to determine the extent of pollution or biotreatment of wastewaters. The use of fresh water organisms such as *Ceriodaphina dubia* (Dennis, 2001) and *Moina macropopa* (Wong *et al.,* 2006) has been reported. Ames' test with *Salmonella typhimurium*, a form of mutagenicty test has been widely used to study toxicity of wastewater, contaminated soil, sewage sludge and sediments (Donelly *et al.*, 1991; Malachova, 1999; Novotny *et al.,* 2006).

2.5.5 Analytical techniques

Conventional methods of assessment of textile effluent treatments/ biodegradation of dyes, such as % Decolourisation, COD and BOD determination are only quantitative in a sense. Determination of the nature of the pollutants and their metabolic products must be done using analytical instruments such as UV-visible, Fourier transform infrared spectroscopy (FTIR), High performance liquid chromatography (HPLC) and Gas chromatography/ mass spectrometry (GC-MS). The inherent complexity of dye structures and their not well-understood biotransformation mechanisms make the identification of unknown metabolites a difficult task and a combination of these techniques have to be used to tackle the problems.

2.5.5.1 Ultraviolet-visible light spectroscopy

The major advantage of this type of spectrophotometry is that the facility can scan the wavelength range over both ultraviolet and visible light and obtain absorption spectra. In this way the absorbance of dyes which are generally in the visible region can be compared to that of their metabolites which are usually in the UV region. The decrease or disappearance of the peak (s) in the visible region indicates decolourisation, while decrease or disappearance of those in the UV region suggests biodegradation (Shaw *et al.*, 2002). This instrumentation has been used to prove both decolourisation and biodegradation (Isik and Sponza, 2004; Parshetti *et al.*, 2006; Jadhav *et al.*, 2007; Telke *et al.*, 2008).

2.5.5.2 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is used for identification of intermediate-sized molecules such as drugs, metabolic intermediates and substrates. It is an ideal and rapid method for measuring certain contaminants in foodstuff and environmental pollutants (Wilson and Walker, 1995). Infrared spectroscopy is generally used to predict the functional groups in a sample using the type of peaks formed and the wave-number at which such peaks appear. Alterations of the spectral of the metabolites when compared to that of the undegraded dyes are indication of biodegradation. The nature and positions of the peaks, distorted or disappearance can also be used to predict degradation of dyes. In a similar way, formation of aromatic amines can be detected (Isik and Sponza, 2004; Parshetti *et al.*, 2006; Jadhav *et al.*, 2007; Telke *et al.*, 2008).

2.5.5.3 High performance liquid chromatography

High performance liquid chromatography (HPLC) has been used for analysis of various dyes in wastewater and metabolites from various degradation procedures (Baiocchi *et al.*, 2002; Pielesz *et al.*., 2002; Nachiyar and Rajkumar, 2003; Plum *et al.*, 2003; Jadhav *et al.*, 2007; Telke *et al.*, 2008). The changes in the retention time and/or formation of more peaks in the chromatograms of the metabolites are important differences commonly used to indicate biodegradation. The products formed can be identified by comparing such chromatograms to that of standard aromatic amines. HPLC is advantageous over GC because it does not need the sample to be volatile or stable to elevated temperatures. The most popular detector used for HPLC is the UV-systems.

2.5.5.4 Gas chromatography - mass spectrometry

Gas chromatography (GC) is a widely used method for qualitative and quantitative analysis of thermally stable, volatile and semi-volatile organic compounds in a mixture. Individual compounds are characterized by their retention time. Currently, GC is often coupled with mass spectrometry (MS) to form a hyphenated instrument known as GC-MS. GC-MS is a powerful instrument that supplies unambiguous mass information for the unknown compounds separated from the injected sample. The instrument is therefore suitable for analysis of unknown metabolites resulting from the biodegradation of dyes. GC-MS has been widely used to identify products of dyes degraded with fungi, bacteria or enzymes (Chivukula and Renganathan, 1995; Adosinda *et al.*, 2003; Telke *et al.*, 2008).

2.6. Dye Removal Processes

Several methods are used in the treatment of dye containing wastewaters. These include physico-chemical methods such as filtration, specific chemical oxidation, specific coagulation, use of activated carbon, reverse osmosis, chemical coagulation-flocculation, adsorption and photo-degradation etc (Young and Yu, 1997; Van der Zee, 2002). Some of these methods are effective but quite expensive (Do *et al.,* 2002, Maier *et al.*, 2004). The biological methods include bacteria and fungi biosorption and biodegradation, which could be aerobic, anaerobic or combined anaerobic/aerobic treatment processes.

2.6.1. Physical processes

Physical processes used in the treatment of textile dyes include membrane filtration, coagulation and sorption. Several advanced treatment such as colour adsorption by activated carbon, has also been suggested. This is, however, not commonly used because of high cost (He *et al.*, 2004). Sometimes physical processes are combined with chemical process in what is known as Physiochemical processes. Some of these processes are discussed below.

2.6.1.1. Membrane filtrations.

Common techniques used in membrane filtration are ultrafiltration, microfiltration, nanofiltration and reverse osmosis. The use of membrane processes for the treatment of wastewater and reuse has been proved effective from a technical point of view. Utrafiltration and microfitration are generally not suitable for dye removal in wastewaters because the membrane pore size is too large to prevent dye molecules, but can be used as

pretreatments for nanofiltration and reverse osmosis (Rozzi *et al.*, 1999). Nanofiltration and reverse osmosis can serve as either main or post – treatment process for dye containing wastewaters (Koyuncu *et al.*, 2004). Both techniques require membranes of molecular weight cut off (MWCO) of below 10,000 Dalton; in reverse osmosis, however, moderate pressure is used to force the effluent across a semi-permeable membrane. The major drawbacks are high capital, management and energy cost, since the membranes have to be cleaned on a regular basis (Hao *et al.,* 2000). Also filtered dyes are not degraded but are concentrated and would subsequently need to be disposed (Soares *et al.*, 2006).

2.6.1.2. Coagulation and flocculation.

Coagulation and flocculation are commonly used to partly remove coloids, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) prior to further treatment of wastewater (Altinbas *et al.*, 1995). Inorganic coagulants such as lime, magnesium, iron, and aluminum salts have been used to treat textile effluent for many years (Semerjian and Ayoub 2003; Allegre *et al.,* 2004; Peres *et al.,* 2004; Aguilar *et al.,* 2005; Golob *et al.,* 2005).

Coagulation requires the use of inorganic coagulants, which coagulate the pollutants to form precipitates that are easily removed by filtration. In flocculation, the chemical use (flocculants) flocks the pollutants causing them to float. The major setback of these processes is that considerable volumes of sometimes toxic sludge are generated. This sludge often requires further treatment to reduce its water content (Semerjian and Ayoub, 2003; Papiç *et al.,* 2004; Aguilar *et al.,* 2005; Mishra and Bajpai, 2005). Another serious

disadvantage is that most coagulants are not suitable for the removal of highly soluble dyes. Recently, organic coagulant polymers have been developed with advantages, such as low sludge production and improved colour removal ability, over the inorganic coagulants (Al-Mutairi *et al.,* 2004).. There are, however, concerns for the toxicity of the coagulants and flocculants.

2.6.1.3. Adsorption.

Sorption is generally used for adsorption and absorption. An ordinary adsorbent is suitable for anionic dyes while those with ion exchange facilities can be used for effective removal of cationic dyes. The surface area and nature of adsorbent/dyes are important in selecting adsorbent. Activated charcoal is a common adsorbent that has been successfully used in removing dyes from waste waters, (Slokar and Le Marechal, 1998; Robison *et al.*, 2001). The need for reactivation and cost are major disadvantages facing the use of activated charcoal. Similar problems are associated with the use of other synthetic adsorbent such as synthetic resins and cellulose based adsorbents. As alternatives, low-cost adsorbents are currently being employed. These include:

- Soil materials such as silica, cinder ash, clays, bentonite, diatonite clay, synthetic clays *etc.* (Ahmed and Ram, 1992)
- Agro-materials/waste such as corn, wheat, rice husks, wood chips, sawdust, bark, cellulose, cotton waste *etc.* (McKay *et al.*, 1987; Boussahel *et al.*, 2009)
- Mineral ore e.g. activated bauxite and activated alumina (Lambert *et al.*, 1997)
- Biomass such as fungal biomass, bacterial biomass.

- Chitin, deacylated chitin and chitosan have also been used as adsorbent (Jocic *et al.,* 2004; Cestari *et al.,* 2005).

Although there are several publications on adsorption technology as an effective ways of removing dyes in waste waters, its industrial application are often coupled with other methods such as biodegradation. High cost of solvent/electrolyte to regenerate the ion-exchanger, and large amount of sludge generated during the process make adsorption an unattractive method for effluent treatment (Slokar and Le Marechal, 1998; Robinson *et al.,* 2001). Another serious setback is that they do not degrade the dyes but they simply transfer the pollutant from the liquid phase (water) to a solid matrix, (adsorbent). This normally requires expensive reactivation operations of the adsorbent materials and post-treatment of the solid waste is required (Shaw *et al*., 2002; Liu *et al*., 2005).

2.6.2. Chemical methods

Most chemical methods used in dye removal processes are quite effective, but cost and the production of more toxic effluent are great concern. Some of these processes include electrophoresis, advanced oxidation processes and photocatalysis.

2.6.2.1 Electrolysis.

The electrochemical technique is a very efficient method that has been used to achieve decolourisation of various dye solutions and dye containing wastewaters in the laboratory. Its application in the industries is however not pronounced because of the cost of producing energy and of replacing spent electrodes (Do and Chen, 1994). The process is based on the application of electric current through the wastewater using a 'sacrificial

metal' usually an Iron metal as the anode and a hydrogen electrode as the cathode. As the process proceeds the dyes removals are achieved in several ways: oxidation of dyes by $Fe(OH)_2$, O_2, O_3 and Cl_2 produced at the anode (electrochemical oxidation); coagulation by metallic iron at the anode or in the solution (elctrocoagulation); reductions by electrons produced at the cathode (electrochemical reduction), which is followed by flotation by bubbles of H_2 gas produced at the cathode (electroflotation). Electrochemical removal of pollutant has an estimated efficiency of about 90%; the process is, however, expensive due to large energy requirements, short lifetime of the sacrificial electrode and uncontrolled radical reactions (Hao *et al.,* 2000; Van der Zee, 2002; Cerón-Rivera *et al.,* 2004).

2.6.2.2. Advanced oxidation processes

Advanced oxidation processes (AOPs) are chemical degradation of pollutants that is currently being given attention in waste treatment (Soares *et al.*, 2006). Common chemical used for this purpose include ozone (O_3) a process known as ozonation; peroxidation, which is usually coupled with ultraviolet treatment (UV/H_2O_2); use of Fenton's reagent (Fe^{2+}/H_2O_2); and combined ultraviolet Titanium oxide (UV/TiO_2). The mechanism of actions of AOPs is their ability to generate very reactive and oxidizing free radicals involved in aromatic cleavage of dye molecules (Raghavacharya, 1997). Many studies have demonstrated that AOPs effectively removed colour but partially removed the organic content of dyes containing wastewaters (Chen *et al.*, 1997; Galindo *et al.*, 2001).

Addition of ozone into wastewaters results in the oxidation of water to more reactive hydroxyl radicals (HO*). At alkaline pH of >10, ozone reacts almost indiscriminately with all compounds present in the reacting medium converting organic compounds into smaller and biodegradable molecules (Peralta-Zamora *et al.,* 1999). There is therefore need for further treatment to achieve complete mineralization (Krull and Hempel 2001). Ozone is effective with water-soluble dyes but more dosage of ozone is usually required with non-soluble dyes like vat dyes and disperse dyes, since they react much slower. A major limitation of the ozonation process is the relatively high cost of ozone generation process coupled with its very short half-life (Gogate and Pandit, 2004).

Peroxidation is a chemical oxidation using ultraviolet radiation (UV) in the presence of hydrogen peroxide (H_2O_2). This process is usually done using UV radiation at 254 nm to disassociate H_2O_2 progressively. The UV/H_2O_2 systems generate hydroxyl radicals (OH•), which are highly powerful oxidizing species. Hydroxyl radicals can oxidize organic compounds (RH) producing organic radicals (R•), which are highly reactive and can be further oxidized (Camel and Bermond, 1998; Catalkaya *et al.*, 2003).

Fenton's reagent consists of hydrogen peroxide and a ferrous salt (Fe^{2+}/H_2O_2). This oxidation process can decolourise a wide range of dyes and it relatively cheaper and more effective in COD removal than ozonation (Park *et al.,* 1999). Higher oxidised iron species like $[Fe(OH)_2(H_2O_2)_5]^{2+}$ are believed to be the main oxidant in fenton oxidation processes (Hsueh *et al.,* 2005). There is, however, the formation of hydroxyl radicals HO* from the reduction of H_2O_2 by Fe^{2+}. Certain feton-like reagents, such as Fe^{3+}/H_2O_2 and

$Fe_{(s)}/H_2O_2$, have also been reported to decolourised dyes in wastewaters, these however require high temperature and/or UV-light as catalysts (Van der Zee, 2002). This oxidation system can be effectively used for the destruction of non-biodegradable toxic waste effluents and render them more suitable for a secondary biological treatment (Nesheiwat and Swanson, 2000). Fenton oxidation is limited to the fact the textile process wastewaters usually have high pH, while the Fenton process requires low pH. At higher pH, large volumes of waste sludge are generated by the precipitation of ferric iron salts and the process loses its effectiveness (Van der Zee, 2002).

2.6.2.3 Photocatalytic methods

Recently, attention has been shifted to combined UV irradiation/metallic semiconductors (catalysts) such as ZnO and TiO_2 as wastewaters treatment systems; this might not be unconnected with their effectiveness in mineralization of organic compounds (Soares *et al.,* 2006). They have also been reported to remove colours in dye wastewaters (Goncalves *et al.,* 1999). In certain experiments, complete oxidation of organic compounds to CO_2 has been achieved by photocatalysis. This quantitative formation of CO_2 provided unequivocal evidence of the total destruction of organic pollutants present in wastewater. Photocatalytic reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the molecules of chemical species present in the solution (Gogate and Pandit, 2004). One major set back of this process in the treatment of dye wastewaters is that UV cannot penetrate the depth of the wastewater; also such chemical treatment methods pose serious threat of introducing secondary pollution due to excessive chemical use (Pearce *et al.*, 2003).

2.6.3. Biological processes

Biological treatment (or biotreatment) of textile dyes offers low-cost and environmentally friendly solutions to textile effluent management. Various organisms or enzymes isolated from such organisms have been used in biodegradation of dyes containing wastewaters (Swamy and Ramsay, 1999; Olukanni *et al.,* 2006).

2.6.3.1 Fungal biodegradation

The presence of lignolytic enzymes confers on fungi the ability to degrade diverse kinds of recalcitrant organic compounds including various azo, heterocyclic and polymeric dyes (Bumpus and Brock, 1988; Abadulla *et al.,* 2000; Wesenberg *et al.,* 2003). The most widely used fungi are the white rot fungi (WRF); they are known to produce extracellular lignolytic enzymes such as lignin peroxidase, manganese peroxidase, manganese-independent versatile peroxidase and laccase (Nagai *et al.,* 2002; Boer *et al.,* 2004). Catalytic power and dye selectivity vary with fungal species. Fungal degradation of aromatic structures is a secondary metabolic event that occurs in limiting nutrient, when C, N and S become limiting (Van de Zee, 2002). Industrial application of fungal biotreatment has however been limited by:

(i) requirement for extensive acidification since optimum pH of fungi is between 4.5 – 5 (Zhang and Yu, 2000);

(ii) stunted growth and low production of enzymes: water activity required for growth of fungi is generally low; fungi grow well on solid or moist substance and not in water.

(iii) formation of mycelia aggregates, fouling and clogging often emerged after a short time, there is thus need for periodical removal of fungal biomass (Hai *et al.,* 2006).

(iv) long growth cycle and moderate decolourisation are often encounter in such biodegradation (Banat *et al.*, 1996).

2.6.3.2. Algal biodegradtion

The role of algae in biodegradation process is scarcely reported (Semple *et al.*, 1999; Lima *et al.*, 2004). Only a few algae like *Chlorella sp* were found to decolourise certain azo dyes and use them as carbon and nitrogen sources (Jinqi and Houtian, 1992). The mechanism of the degradation is thought to be reductive cleavage of the azo linkages prior to mineralization of aromatic amines (Luther and Soeder, 1991). Lima *et al.* (2004) reported that micro-algae isolated from a waste discharge container were able to remove some aromatic pollutants.

2.6.3.3 Bacterial biodegradation

Bacteria are ubiquitous in nature, and are often exposed to polluted environment from where they develop various resistance and catabolic potentials. These potentials are advantageous to mankind in various ways including bioremediation. Bacteria could reduce the colour intensity of dyes more satisfactorily than other biological treatment and chemical treatment (Banat *et al.*, 1996). Textile dyes degrading activities of *Pseudomonas sps, Baccilus sps, Halomona sps., and Orthobacter sps*. have been documented (Davies and Burns, 1990). Generally, bacterial treatments are classified as either anaerobic or aerobic.

2.6.3.3.1 Anaerobic biodegradation

Various bacteria have been reported to decolourise azo dyes under anaerobic condition. Anaerobic processes are usually less expensive than aerobic. They are simpler to set up and can be applied to treatment of more complex components (Goncalves *et al.*, 2000). Usually anaerobic decolourisation requires non-specific enzymatic reduction of highly electrophilic azo bond in the dye molecule (Stolz, 2001; Pearce *et al*., 2003). It allows the decolourisation of a wide range of textile dyes thus making this process more suitable for application on a commercial scale. The major disadvantage of anaerobic azo dye reduction is that the aromatic amines formed from reductive cleavage cannot be further mineralized (Rafii *et al*., 1990; McMullan *et al.,* 2001; Van der Zee, 2002). The accumulation of these toxic aromatic compounds (e.g. naphthylamine or benzene derivatives) is a serious concern since they are presumed carcinogenic (Novotny *et al.,* 2001; Kapdan and Kargi, 2002).

2.6.3.3.2 Aerobic biodegradation

Over the years, microbial removal of colours from textile wastewaters has focused on anaerobic sludge systems. The end products of such treatments, aromatic amines, have been reported to be more toxic than the dyes themselves (Chung and Stevens, 1993; Field *et al.*, 1995). Although aerobic treatment is a safe method for the biodegradation of aromatic amines, aerobic decolourisation of azo dyes has been reported as an ineffective process (Shaul *et al.*, 1991; Pagga and Taeger, 1994). According Van der Zee and Villaverde (2005), aerobic biodegradation of aromatic amines requires specific microorganisms; it has also been reported that degradation of sulphanilic acid (aromatic

amine) was only achieved by bioaugementation with a proper bacterial culture (Van der Zee, 2002). Specialized peroxidase producing bacterial strains of the genera *Streptomyces* and *Sphingomonas* were shown to decolourise azo dyes aerobically (Paszczynski, 1992). Also an aerobic azoreductase, a tetrameric NADPH dependent flavoprotein, has been isolated from a strain of *Staphylococcus aureus* (Chen *et al.*, 2005). These suggested that if metabolic versatile bacteria are employed complete mineralization of azo dyes could be achieved under aerobic condition; more also that the effectiveness of microbial treatment depends on the survival, adaptability and activity of the selected organism (Cripps *et al.,* 1990; Pasti-Grigsby *et al.,* 1992).

2.6.3.3.3 Combined anaerobic/aerobic biodegradation

The inability of anaerobic organisms to further degrade the aromatic amines formed during azo dyes reduction and the ability of their aerobic counterparts to mineralize these aromatic amines make combined anaerobic-aerobic treatment a logical concept (Field *et al.*, 1995; Rajaguru *et al.*, 2000). There are two approaches to the combined process: sequential treatment and simultaneous/integrated treatment. During sequential treatment, the anaerobic and aerobic treatments are done in separate reactors or in phases in the same reactor (Sponza and Isik, 2002). The simultaneous approach, anaerobic zones are created within an aerobic reactor using bio-films, granular sludge or biomass immobilized in other matrices with little oxygen diffusion, such that anaerobic and aerobic conditions coexist in the same environment (Kudlich *et al*., 1996). Both treatments require auxiliary substrates to feed the bacteria in the anaerobic zones with carbon and energy sources as well as reducing equivalents for azo bond cleavage.

Summarily, two phases of treatments; a decolourisation and a detoxification phase, are required for combined anaerobic/aerobic treatments. This facilitates complete decolourisation of dyes and significant reduction in BOD and COD levels. In the subsequent aerobic phase the remaining BOD (which includes the aromatic amines) from the auxiliary substrates may be completely mineralized (O'Neill *et al*., 2000). Combine anaerobic – aerobic biological treatment has two major disadvantages (i) anaerobic azo dye reduction is a time consuming process and (ii) the fate of aromatic amines during treatment is not conclusively elucidated (Van de Zee, 2002).

2.6.4. Comparison of various methods

The state of the art treatments for the decolourisation of wastewaters include the physiochemical methods such as adsorption, precipitation, chemical oxidation, photodegradation or membrane filtration (Van der Zee and Villaverde, 2005). All of these have serious restriction as economically feasible methods for the decolourisation of textile wastewaters. Such restrictions include high cost, formation of hazardous by-products or intensive energy requirements. Although anaerobic processes are less expensive and can be carried out in simple and relatively inexpensive reactors, the generation of aromatic amines which cannot be degraded further under this condition is a serious setback. Reduction of the azo bond is the serious concern in aerobic biotreatment. Some of these short-comings have been compared (Mutambanengwe, 2006), and are shown in table 2.3.

Table 2.3: Advantages and disadvantages of physical and chemical textile effluent treatment

Physical/ Chemical treatments	Advantages	Disadvantages
Fenton's reagent	Effective decolourisation of both soluble and insoluble dye	Sludge generation
Ozonation	Applied in gaseous state; no alteration of volume	Short half life
NaOCl	Initiates and accelerates azo bond cleavage	Release of aromatic amines
Photochemical degradation	No sludge production	Formation of bi – products
Cucurbituril	Good sorption capacity for various dyes	High operating cost
Electrochemical destruction	Breakdown compounds are non hazardous	High cost of electricity
Activated carbon (Adsorption)	Good removal of a variety of dyes	Very expensive to operate
Peat (Adsorption)	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips (Adsorption)	Good sorption capacity for acid dyes	Requires long retention times
Silica gel (Adsorption)	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration; no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O2
Electro kinetic coagulation	Economically feasible	High sludge production

Source: Mutambanengwe, 2006

2.7. Mechanism of Azo Dye Reduction

Several mechanism of azo dyes reduction has been proposed: enzymatic (Rafii *et al.*, 1990; Haug *et al.*, 1991), non – enzymatic (Van der Zee, 2002) and mediated (Kudlich *et al.*, 1996). These processes can also be extracellular or intracellular. For instance, there are some non enzyme dependent reduction mechanisms (chemical method) and those due to aromatic ring opening by enzymes such as laccase (Van der Zee, 2002).

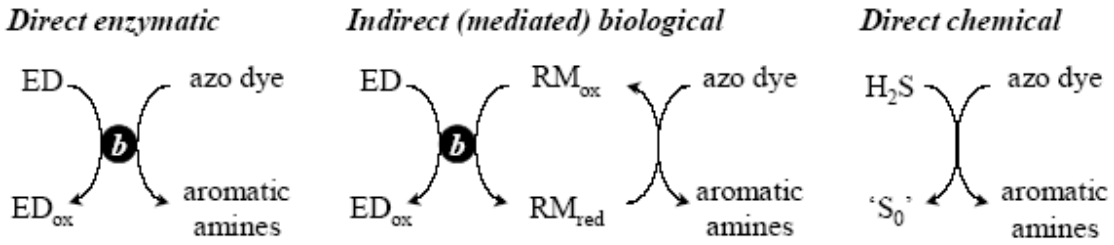


Figure 2.3 Schematic representations of the different mechanisms of anaerobic azo dye reduction (RM = redox mediator; ED = electron donor; b = bacteria /enzymes)

Source: Van der Zee, 2002.

2.7.1 Direct enzymatic reduction.

In direct enzymatic reduction, an enzyme transfers the reducing equivalents resulting from the oxidation of organic substrates to the azo dyes. These enzymes could be specific catalyzing azo bonds reduction only or non-specific catalyzing wide range of compounds. The specific enzymes are commonly referred to as azoreductases, while the hydrogenase and perosidase are good examples of the non-specific azo bonds reducing enzymes.

2.7.2 Indirect (mediated) biological mechanism.

The mechanism of anaerobic azo dye reduction is generally believed to be the passage of electrons down the respiratory electron transport chain to an azo dye, which serves as an alternative final electron acceptor under anaerobic conditions. The electrons react with dye by reducing the azo bonds, thereby resulting in decolourisation (Van der Zee, 2002). This is a type of mediated reduction. Another type of mediated mechanism involves the use of redox mediator.

2.7.3 Direct chemical method

The addition of certain reducing agents to dye containing effluent often results in considerable decolourisation. Common among these reductants are dithionite, zero-valent iron, and biogenic reductants like sulphide (Mutambanengwe, 2006). Dye houses effluent often contain sulphate, either as additive or product of oxidation of more reduced sulphur species used in dyeing processes, such as sulphide, hydrosulphite and dithionite. Sulphate is also produced from the neutralization of alkaline dye effluents with sulphuric acid. Sulphide reducing bacteria can easily convert the sulphate to sulphide during treatment of these wastewaters in anaerobic bioreactors (Van der Zee, 2002).

2.8 Dye Decolourisation Kinetics

Several organisms have been employed in industrial colour removal. Some of these that were found effective were not able to do it at desired rate, it is therefore important for the rate of decolourisation of the isolated organisms to be determined. Decolourisation of dyes is a microbial fermentation that can be described using different kinetics parameters.

First order kinetics (equation 2.1) has been observed for biological decolourisation of monoazo dyes.

$$A_t = A_o e^{-kt} \quad \text{.....} \quad \textbf{2.1}$$

where A_o is initial absorbance, A_t is final absorbance, t is the incubation time and k is a constant.

However chemical azo dyes reduction was found to deviate from first order kinetics, in the same study (Van der Zee, 2002). Specific degradation rate of dye effluent and specific decolourisation rate has been described as an analogue of Michaelis – Menten equation (Rajamohan and Karthikeyan, 2006),

$$V = \frac{d[S]}{dt} = \frac{V_m[S]}{K_m + [S]} \quad \text{.....} \textbf{2.2}$$

Where V_m is the maximum COD removal rate, [S] is initial initial COD, Km is the specific COD removal rate.

In another report He *et al.* (2004) described specific decolourisation rate (r_{dye}) as:

$$r_{dye} = \frac{r_{dye\max} \times [4BS]}{Km + [4BS]} \quad \text{.....} \textbf{2.3}$$

$r_{dye,max}$ is the maximum decolourisation rate, K_m is Michaelis constant, [4BS] is the initial concentration of the dye

2.9. Important Enzymes in Dyes Biodegradation

Several enzymes have been employed in decolourisation and detoxification of synthetic dyes. Their uses stem from the ubiquitous nature of the organisms from which they are isolated. Different microbial populations (i.e. fungi, bacteria, and algae) with different

chemical and physiological characteristics have been investigated for their potential to produce dye degrading enzymes. Majority of these are oxidoreductive enzymes such as Mn^{+} peroxidase, laccases, lignin peroxidase, plant polyphenol oxidases, micro peroxidases and azoreductases (Husain, 2006). Over the years, lignin modifying enzymes are believed to be sole products of fungi, particularly the white rot fungi. These enzymes are capable of transforming aromatic dyes, polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, pesticides and even explosives (Rodriguez *et al.*, 1999).

2.9.1. Azoreductases

Azoreductases are enzymes that catalyze the reductive cleavage of azo (N=N) linkages to produce aromatic amines that are potentially carcinogenic (Platzek *et al.*, 1999). Based on the current literature regarding azoreductase activity and amino acid sequence data, it appears different azoreductases exist in different bacteria (Abraham and John, 2007). The physiology and mechanism of azoreductase is not well understood. Broadly azoreductases have been classified into two groups: true azoreductases, which are produced in aerobic condition and anaerobic azoreductases. While anaerobic azoreductases are still subject of discussions, few aerobic azoreductases have been characterized from obligate aerobic bacteria (Chen *et al.*, 2004).

Generally, azoreductase require biochemical electron equivalent, NAD(P)H, for their activities (Stolz, 2001). Most were described as cytoplasmic flavin bound protein (Rafii and Cerniglia, 2005; Chen *et al.*, 2005). Those described by Zimmermann *et al.* (1982 and 1984) were however monomeric flavin-free enzymes that preferred NADPH to NADH as co-factors. Azoreductases are bacterial enzymes isolated mainly from aerobic

bacillus strains; Hu (2001) has reported the decolourisation of azo dye by an azoreductase from *Pseudomonas luteola*, a facultative aerobe.

Aerobic azoreductases catalyze reductive metabolism of azo dyes in the presence of molecular oxygen, while the reductive cleavage of azo dyes under anaerobic conditions is still unclear and has generated several theories. Previous studies have indicated that anaerobic azo bonds reduction is enzymatically catalyzed by an 'azoreductase' (Zimmermann et al., 1984), but it still remains uncertain whether a single enzyme or composite system of enzymes is responsible for these reactions.

Although azoreductase can decolourise azo dye containing effluent effectively, their requirement for biochemical electron equivalent is a major set back for their possibly application in the industries. Also the end-products of the catalysis are aromatic amines (figure 2.4), which are toxic and/or mutagenic (Chung and Stevens, 1993; Field *et al.*, 1995).

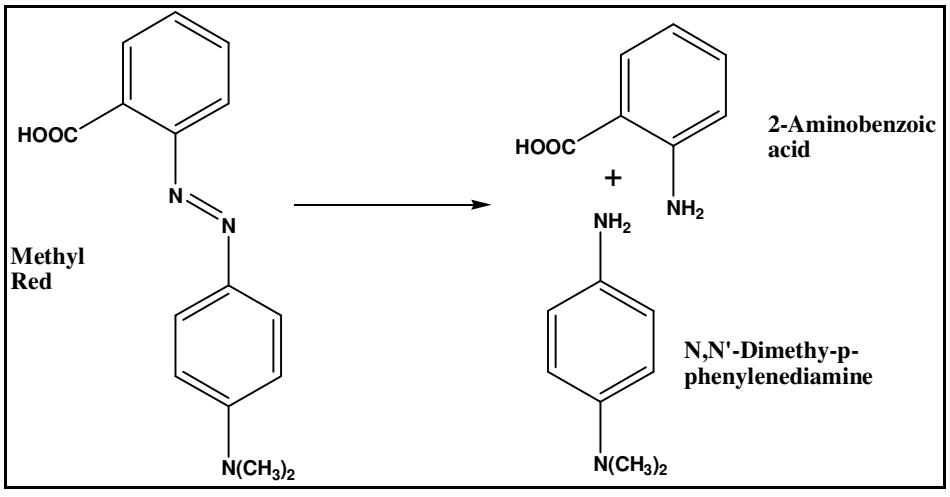


Figure 2.4: Reductive cleavage of the azo dye methyl red (MR) (Wong and Yuen 1996)

2.9.2. Peroxidases (EC.1.11.1.X)

Peroxidase is a group of enzymes that catalyze the oxidation of certain organic substrates in the presence of hydrogen peroxide. Most are glyco-proteins containing one iron protoporphyrin IX (heme) prosthetic group. They exist in multiple isoforms with molecular weights ranging between 32 – 62.5 kDa (Wesenberg *et al.*, 2003). The ability of various peroxidases to mineralize and/or oxidize a variety of recalcitrant aromatic and phenolic compounds has been reported (Karam and Nicell, 2000). Peroxidases are similar to laccase in their mode of actions, except that they have lower oxidation potential.

Lignin peroxidase (LiP) (diarylpropane: oxygen H_2O_2 oxidoreductases EC.1.11.1.14) degrades azo dyes by oxidation of the phenolic group to produce a radical at the carbon bearing the azo linkage. The phenolic carbon is then attacked by a water molecule resulting in its break down to produce phenyldiazene which is easily oxidised by a one electron reaction to generate nitrogen (Rodriguez *et al*., 1999). The ability of LiP to degrade other non-dye pollutants such as phenethrene (Hammel *et al.*, 1992), polycyclic aromatic hydrocarbons (Bogan and Lamar, 1995), phenolic polutants and benzo (a) pyrene have been reported (Husain, 2006).

Manganese peroxidase (manganese (II) H_2O_2 oxidoreductases EC.1.11.1.13) is the most commonly produced enzyme by white-rot basidiomycetes. This enzyme has high potential for biodegradation. Unlike lignin peroxidase, it does not require mediators such as veratryl alcohol for its activities. Also it is easily produced on solid – state lignocellucosic waste, such as wood shavings (Rodriguez – Couto *et al.*, 2002). The enzyme acts by specific oxidation of Mn^{2+} to highly reactive Mn^{3+} ions (Ziegenhagen and Hofrichter, 2000). The formation Mn^{3+} ions makes the activities of Mn-Peroxidase to be

apparently unspecific, since Mn^{3+} chelates with organic acids such as oxalate, malonate, malate or lactate which are produced and excreted into the microenvironment by fungi under natural conditions. The chelated Mn^{3+} ions act as redox mediators which oxidize a wide range of aromatic compounds including lignin, humic substances and organo-pollutants: dyes and pesticides inclusive (Ziegenhagen and Hofrichter, 2000). MnP production is regulated by the concentration of Mn^{2+} ions; a 24 –fold increase in enzyme activity has been reported when fungi are grown in the presence of $MnSO_4$ or $MnCl_2$ (Perez and Jeffries, 1992).

2.9.3. Polyphenol oxidase (1.14.18.1)

Phenol oxidase refers to a group of enzymes that oxidize phenolic compounds to quinones. They are responsible for browning in plants: monophenol oxidase in mushrooms, polyphenol oxidases in potato and apple. These two enzymes are confusingly referred to as tyrosinase. Tyrosinase is a bifunctional, copper-containing oxidase having both catecholase and cresolase activity (Husain, 2006).

Tyrosinase is of biotreatment importance because it is oxygen and 4-electron-transferring phenol oxidase, which converts phenols to their quinoinoid forms that are susceptible to ring cleavage (figure 2.5). Polyphenol oxidase uses O_2 to catalyze the dehydrogenation of catechols to orthoquinones and the orthohydroxylation of phenols to catechols. Khan and Husain (2007) have reported effective decolourisation of textile and industrial dyes using salt fractionated plant polyphenol oxidase, at pH 3. The decolourisation is accompanied with the formation of an insoluble precipitate product which could be easily removed by centrifugation; this suggested possibly detoxification.

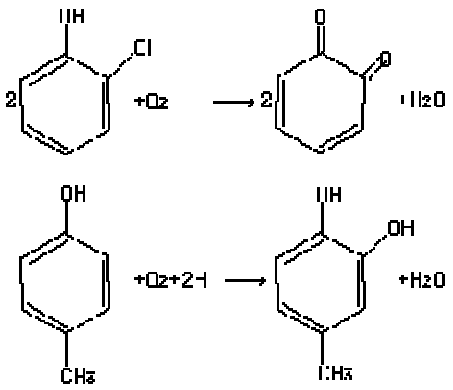


Figure 2.5: Catalytic actions of tyrosinase

2.9.4. Veratryl alcohol oxidase

Veratryl alcohol oxidase (VAO) is a type of aryl-alcohol oxidase (EC 1.1.3.7), a group of enzymes that catalyzes the oxidation of aromatic primary alcohols to their respective aldehyde in the presence of molecular oxygen. Thus, the two substrates of this enzyme are aromatic primary alcohol and O_2, whereas its two products are aromatic aldehyde and H_2O_2. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. The systematic name of this enzyme class is aryl-alcohol: oxygen oxidoreductase. Other names in common use include aryl alcohol oxidase, veratryl alcohol oxidase, and arom alcohol oxidase.

Although only little is known about the role of VAO in biotreatment of effluent, the enzyme has, however, been reported to assist laccase in biodegradation. In the same research Marzullo *et al.* (1995) concluded that VAO from *Pleurorotus ostreatus* is able to reduce synthetic quinones, laccase – generated quinonoids, and phenoxyl radicals with concomitant oxidation of veratryl alcohol to veratryl aldehyde. They also explained that

the cooperative actions of laccase and VAO prevent polymerization of reactive rings produced by laccase thereby making laccase products susceptible to ring cleavage (figure 2.6). There was a recent report that this enzyme can decolourised azo dyes in the absence of laccase (Jadhav *et al.*, 2009), the mechanism of such decolourisation is however not yet understood.

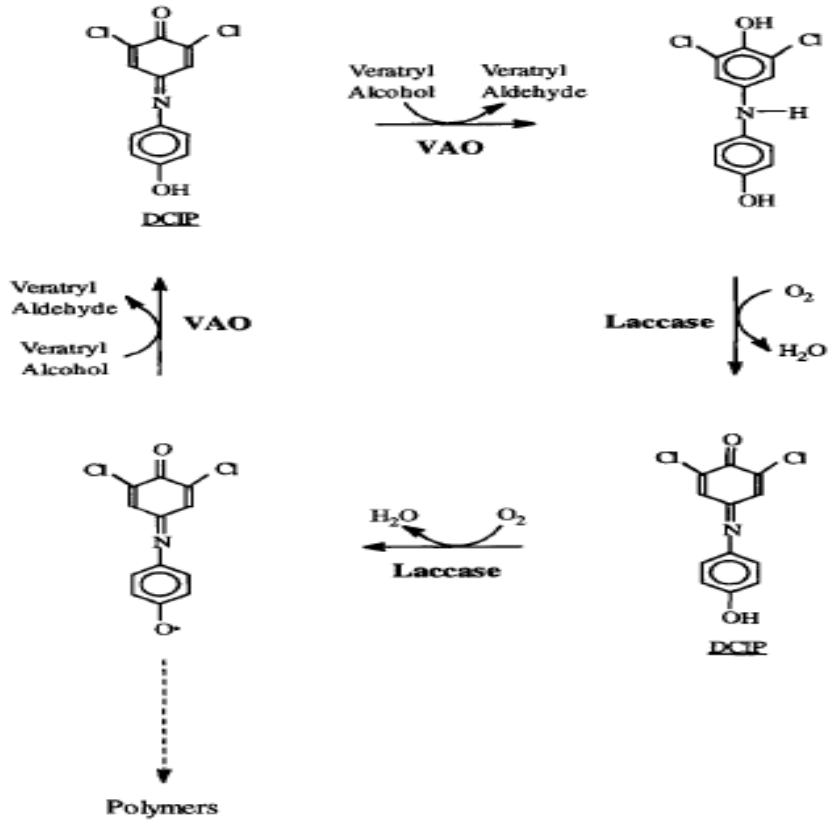


Figure 2.6: Schematic diagram of Laccase-VAO cycle and its implications in the prevention of lignol substrate polymerization (Marzullo *et al.*, 1995).

2.9.5. Laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2)

Laccases belong to a family of enzymes commonly referred to as the blue copper oxidases with molecular weight ranging from 60 to 390kDa (Call and Mucke, 1997). They have been reported to oxidise many recalcitrant substrates, such as chlorophenols,

PAHs, lignin-related structures, organo phosphorus compounds, non phenol lignin model compounds, phenols and aromatic dyes (Bourbonnais *et al.*, 1996; Abadulla *et al.*, 2000). Laccases require molecular oxygen to oxidise both their phenolic and non-phenolic substrates (Hublik and Schinner, 2000; Husain, 2006; Madhavi and Lele, 2009). This gives the enzyme an advantage over other lignin modifying enzymes such as peroxidase that requires H_2O_2 and azoreductases that require redox mediators such as NADH and NADPH. Laccases are widely distributed in nature and have been reported in fungi (Baldrian, 2006; Dantan-Gonzalez *et al.*, 2008), plants (Husain, 2006), insects (Dittmer *et al.*, 2004), and in bacteria (Arias *et al.*, 2003).

The mechanism of laccase action involves the removal of an H^+ atom from the hydroxyl and amino groups of the *ortho-* and *para-* substituted mono and polyphenolic substrates, and aromatic amines. This is achieved through one electron step abstraction to form free radicals capable of undergoing further re-polymerization, and demethylation or quinone formation (Abadulla *et al*., 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

All chemicals used are of analytical grade. The reactive dyes used: Reactive Blue 13 **(RB 13)**, Reactive yellow 42 **(RY 42)**, and Reactive Red 58 **(RR 58)**, were obtained from Atlantic Textile Mill (ATM), Oshodi Lagos, Nigeria. Methyl red was supplied by Merck, South Africa, while nutrient broth and nutrient agar were obtained from *Lab M*, UK.

3.2 Isolation and Identification of Bacterial Strains

3.2.1 Sample sources

Bacteria were isolated from four sources as shown in Table 3.1.

Table 3.1: Sources of samples for bacteria isolation.

S/N	Sample Collected	Location	Coordinate
1	**D**- **D**yes	Atlantic Textile Mill , Osodi Lagos State.	(6°34'17"N, 3°21'59"E)
2	**E**- **E**ffluent samples	Discharge pipes and drainage of Atlantic Textile Mill, Oshodi Lagos State.	(6°34'17"N, 3°21'59"E)
3	**P**- Pond water	Redemption City, Mowe, Ogun State.	(6°48'29"N, 3°27'41"E)
4	**R**- soil from a **r**efuse dump	Redemption City, Mowe, Ogun State.	(6°34'10"N, 3°21'59"E)

3.2.2 Isolation from textile dyes

Approximately 20 mg of each of the three dyes mix were added to nutrient broth (LAB M) previously prepared with distilled water. The solution was incubated in triplicate at 25°C overnight.

3.2.3 Isolation from textile effluents/ pond water

Textile effluent/pond water (0.5 mL) was added to about 10 mL of dye solution prepared above, and incubated overnight.

3.2.4 Isolation from refuse dump

Water (0.5 mL) obtained by washing the site's soil sample in distilled water were added to about 10 mL of dye solution.

All strains were then isolated on separate nutrient agar plate using sterile inoculating loop dipped into the broths. The strains were stored on nutrient agar slants at 4^{o}C.

3.3 Morphological and Biochemical Characterization

Bacteria show characteristic type of growth on solid media under appropriate cultural conditions and the colony morphology can be used in presumptive identification.

3.3.1 Morphological characterization of isolated strains

The morphological characterization was done by examining 24 h culture of the organisms on nutrient agar. Colony size, diameter, outline (circular, wavy, rhizoid *etc.*) elevation (flat, raised, convex, *etc.*) and translucency (transparent, opaque, and translucent) were used for the classification. The colours of the colonies and the changes that they brought about in their surroundings were also used as diagnostic tools in the tentative identification of the bacteria.

3.3.2 Biochemical characterization of isolated strains

The following tests were done on the isolated strains: Gram's staining, Shape, endospores formation, growth on McConkey, lactose fermentation, motility, catalase and oxidase test. Sugar fermentation tests were also done to confirm certain strains.

3.3.2.1 Gram's staining

This was done using Gram's method (Barrow and Feltham, 1993). Smears of the strains were prepared on glass slides, air-dried, and heat fixed. The slides were flooded with crystal violet for 30 s, rinsed in water and Lugol's iodine. Solutions were drained off and smears were decolourised with few drops of acetone for 2-3 s. The decolourised slides were washed thoroughly in water and counterstained with 0.5% safranin for 30 s, slides were again rinsed in water and drained. Gram-positive organisms were blue or purple while gram-negative were red under oil immersion microscope.

3.3.2.2 Acid-fast staining

This was done using Ziehl-Neelsen's method: prepared slides were flooded with strong carbol fuchsin and steam for 5 min. Slides were rinsed in water and decolourised with 3 to 5 changes of acid-alcohol solution. Slides were rinsed and counterstained with Loeffler's methylene blue for 1 min. Acid fast organisms were red while non acid fast organisms were blue or green.

3.3.2.3 Spore staining

Spore staining was done with the method of Schaetter and Fulton (Barrow and Feltham, 1993). Slides were flooded with 5% aqueous malachite green for 10 min., washed under running water, and counter-stained with 0.5% aqueous safranin for 25 s. The slides were rinsed again and drained. Bacterial bodies stained red while spores were stained green in this method.

3.3.2.4 Motility

Motility was observed by stabbing semi-solid media (motility medium) in test tube with inoculating needles. Turbidity around the stabs was recorded as motility (See appendix I for constituents of motility medium).

3.3.2.5 Aerobic growth

Streaks were made on clear nutrient agar plate, which were then incubated at 37^{o}C in an aerobic incubator. Growth after 24 h or 48 h was observed as positive result.

3.3.2.6 Anaerobic growth

Stricken nutrient agar plates of strains were put in anaerobic jar (Eqiutron, UK), the two valves were opened and CO_2 was blow into the jar from one end to replace the air in the jar. The valves were quickly closed and jar was incubated at 37^{o}C. Plates were observed after 24 h and 48 h, for growth.

3.3.2.7 Catalase

Few drops of 3% H_2O_2 were put in clean Petri dish, a loop of overnight culture of strain were transferred into the solution, evolution of gas was recorded as positive result.

3.3.2.8 Oxidase (cytochrome C oxidase) activity

This was done by the method of Kovacs, modified by Cowan and steel (Barrow and Feltham, 1993). Fresh aqueous solution of oxidase reagent (tetramethol-p-phenylenedamine dihydrochloride) was prepared (a loopful of reagent in 3 mL sterile water). A filter paper was placed on Petri dish and wet with few drops of the solution. Smear of test culture were made on the moist paper with clean glass rod. Dark purple colour on the paper within 30 s denotes a positive reaction.

3.3.2.9 Glucose (acid)

10 mL of sterile nutrient broth sugar (see appendix I) were poured into test tubes. The test tubes were inoculated with the individual strains; colour changes from orange to yellow were recorded as positive reaction.

3.3.2.10 Carbohydrates oxidation/fermentation (O/F) test

OF medium (see appendix I) was poured into sets of test tubes. Each strain was stab-inoculated into two test tubes with a straight wire. One of the two test tubes was covered with paraffin while the other was left opened.

Results:

	Open tube	**Sealed tube**
Oxidation (O)	Yellow	Green
Fermentation (F)	Yellow	Yellow
No reaction (-)	Blue or Green	Green

3.3.2.11 Pigment production

Nutrient agar plates were inoculated with a light suspension of organisms. The plates were incubated for 24 h at 37^{o}C, after which they were transferred to room temperature and observed for 5 days. Pigments colours were recorded as **R**-red, **Y**- yellow, **O**-orange, **G**-green, **V**-violet or negative (-).

The results of these tests were compared to Cowan and Steel's identification scheme; with the same scheme, the organisms were identified to the generic level (Barrow and Feltham, 1993).

3.3.3 16S rDNA characterization

The 16S rDNA sequence of one of the organisms that demonstrated high decolourisation activities during initial screening was done by BAC Full-length service from Laragen inc., Los Angeles USA. The 16S rDNA sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences were downloaded and evolutionary history was inferred using the Neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki *et al*., 2004). The clock calibration to convert distance to time was 0.01 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

3.4 Decolourisation of Reactive Azo Dyes

3.4.1 Choice of dyes

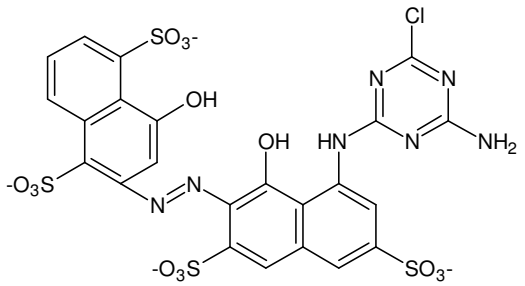
Three dyes: Reactive Black, Reactive Red and Reactive Yellow were chosen for the purpose of this research. This is because reactive azo dyes are the most widely used dyes accounting for 85% of textile dyeing process and they are known to be recalcitrant (Soares *et al.*, 2006) .

The dyes were collected from Atlantic Textile Mill (ATM), Oshodi Lagos. Their chemical characteristics are shown in Table 3.2:

Table 3.2: Chemical nature of the azo dyes used in this research

	Chemical Class	**Colour Index Name**	**Molecular formula**
Blue H5R	Monoazo (Cu complex)	Reactive Blue 13	$C_{29}H_{16}ClN_7Na_4O_{14}S_4$
Red P8B	Azo	Reactive Red 58	*
Yellow FG	Monoazo (pyrazolone)	Reactive Yellow 42	*

* The structures of these two dyes have been disclosed to the Colour Index on confidential basis.



5–[(4–amino– 6–chloro–1, 3, 5–triazin–2–yl)amino]–2–[N'–(4–hyrdoxy–1,6–disulphonato–2–naphthyl)hydrazino]naphthalene–1,7–disulphonate.

Figure 3.1: Molecular structure and IUPAC name of reactive blue 13.

Sulphonated azo compounds are usually used as dyes in textiles, foods and cosmetics. They resist biodegradation in conventional sewage system and are precipitated with the sewage sludge or released by the effluent into the rivers (Shaul *et al.*, 1991).

3.4.2 Sterilization techniques

All glasswares were washed with detergent, rinsed thoroughly with distilled water and oven sterilized at 80^{o}C for 2½ h. All polypropylene tubes and tips used as well as media and solutions prepared were sterilized by autoclaving at 121^{o}C for 15 – 25 min. Inoculations were done with flame sterilized loops and all experiments were performed wearing sterile disposable hand gloves.

3.4.3 Solutions preparation

The bacterial decolourisation assays were conducted in either 150 mL or 250 mL conical flasks. The basal nutrient was nutrient broth containing per litre: 1 g beef extract, 2 g yeast extract, 5 g peptone and 5 g NaCl buffered to pH 7.2. The nutrient broth was either from *Lab M* Uk or Himedia, India. The solutions' maximum absorbances (λmax) were determined by scanning through 200-1000 nm using Shimadzu UV-visible spectrophotometer (UV-1650PC).

3.4.4 Biomass estimation

The dry cell mass of the strain was constantly estimated using the turbidity of its 24h culture at 600 nm (OD_{600}).

$$OD_{600} = OD_{sample} - OD_{\sup ernan\tan t}$$ (Dong *et al.*, 2003).

One mL of the culture was dried to constant weight in three pre-weighed small vessels (1.5 mL eppendorf tubes). The average change in weight between the vessels and the

vessels with cells was regarded as the dry cell mass (DCM) of the organism/mL. The relationship between the DCM of *Proteus mirabilis* and OD_{600} was found to be **1.0 OD_{600} = 10.7678 g DCM/L**

An analogue of beer-lamberts law **(OD600= εCl)** was used to determine an extinction factor, this showed that the organism is related to biomass by the equation:

$$Biomass(gDCM/L) = \frac{OD_{600}}{0.09286}$$

The biomass concentration was constantly determined using this equation.

3.4.5 Decolourisation under limited supply of oxygen

50 mL of the solution were transferred into 150 mL conical flask; 3 flasks were inoculated aseptically with ~0.1 g DCM of each of the 14 strains, the flasks were plugged with sterile cotton wool, and incubated at 37°C.

Decolourisation was measured spectrophotometrically at the simulated effluent pre-determined maximum absorbance (549λ_{max}). 3 mL of sample were collected at 1 h intervals and centrifuged at 4000xg for 15 minutes to remove biomass. Decolourisation was recorded as %:

$$\%Decolourisation = \frac{A_o - A_t}{A_o} \times 100$$

Where A_o - Absorbance of the dyes solution

A_t - Absorbance of the treated dyes solution at specific time, t.

3.4.6 Decolourisation under normal supply of oxygen

Under limited supply of oxygen, it was observed that certain strains grew above solution (around the flask); it was predicted that those strains require more oxygen for their growth and decolourisation activity. To investigate this, the experiment was repeated for strain D_2, without plugging the flasks with cotton wool; the abiotic control was not plugged also. Decolourisation of methyl red solution, a well known azo dye, was done with the same strain in a similar experiment. Strain D_2 showed the highest decolourisation under limited supply of oxygen and was among those that grew around the flask, above the decolourised dye solution.

3.4.7 Decolourisation in shaking and static conditions

The decolourisation ability of strain D_2 was tested in shaking condition to further substantiate the requirement of the organism for oxygen. 0.04 g of the strain was inoculated into 250 mL flask containing 100 mL nutrient broth-dye solution (60 mg/L) of RB 13 the reaction was incubated in a rotary shaker (120 rpm) at 37^{O}C. Samples (6 mL) were withdrawn at 1 h intervals for 6 h. 3 mL of samples were centrifuged at 5000 rpm for 15 min to remove biomass. Biomass estimation and decolourisation were determined as described in sections 3.4.4 and 3.4.5 respectively.

3.4.8 Effect of pH and incubation temperature on decolourisation

The pH of the methyl red-nutrient broth solution was adjusted to pH 4, 5, 6, 7, 8, 9 and 10 using dilute solutions of HCl and NaOH as appropriate. Approximately 0.01 g DCM of the bacteria was added to 50 mL of the solution in 150 mL conical flasks. All

experiments were done in triplicates and abiotic controls were used as 0% decolourisation. The extents of decolourisation were determined after 6 h at the maximum absorbance of the solution (482 nm).

Decolourisation of methyl red in nutrient broth solution was investigated at various temperatures: 25, 30, 37, 42 and 45^{o}C. 25^{o}C was achieved with the aid of a monitored ice-bath, 30^{o}C was the room temperature, while 37^{o}C, 42^{o}C and 45^{o}C incubation temperature were achieved with the aid of water bath. % Decolourisation was determined as described in section 3.2.5.

3.4.9 Effect of media composition on decolourisation

To determine the effect of media composition on decolourisation, five media were designed: Distilled water, **DW**; Nutrient Broth, **NB**; Minimal Media (Mills *et al.*, 1978), **MM**; Minimal Media with Yeast extract and Starch (200 and 250 mg/L respectively), **MM+YS**; Minimal Media with Starch only, **MM+S**. All media were adjusted to pH 7.2.

3.5 Kinetic Studies of RB13 Decolourisation

Kinetic studies are important to determine the relationship or dependence of decolourisation on dye concentration, substrate concentration and cell mass concentration. It also gives clear evidence of applicability of the strain in effluent decolourisation or degradation.

3.5.1 Dependence of specific decolourisation rate on initial concentration of RB13

Experiments with different concentrations (30 – 2500 mg/L) of the Reactive Blue 13 were performed. Approximately 0.11 g DCM of the cell mass was added to 100 mL of each dye solution made with nutrient broth and decolourisation was studied at the strain's optimal temperature of 37^{o}C. Natural logarithm of the specific decolourisation rates ($V_{dye,M}$) was plotted against that of the dye concentrations. ($V_{dye,M}$) was described as

$$V_{dye,M} = \frac{(A_o - A_t)[RB13]}{A_o tM} \quad \ldots\ldots\ldots\ldots\ldots\ldots 3.1$$

Where $V_{dye,M}$ is the specific decolourisation rate (mg dye$L^{-1}h^{-1}$g DCM^{-1}), , [RB13] is the initial concentration of RB13 (mg dye L^{-1}), t is incubation period (h) and Where M is cell mass concentration (g DCM/L).

The order of the decolourisation with respect to concentration of RB13 was determined as the gradient of the equation:

$$\ln V_{dye,M} = a \ln[RB13] + \ln k \ldots\ldots\ldots\ldots\ldots 3.2$$

Where 'a' is the order of the reaction and k is the rate constant.

3.5.2 Dependence of specific decolourisation rate on initial cell concentration

Effect of biomass on the rate of decolourisation was investigated in a 250 mL flask containing 100 mL, 100 mg/L RB13. Different flasks were inoculated with different concentrations of inoculums ranging from 0.4 – 3.02 g/L. The flasks were plugged and samples were drawn hourly. ln $V_{dye,M}$ was plotted against ln M and the order of reaction was determined using an equation similar to equation 3.2.

$$\ln V_{dye,M} = b \ln M + \ln k \ldots\ldots\ldots\ldots\ldots 3.3$$

Where 'b' is the order of the reaction and k is the rate constant.

3.5.3 Determination of maximum decolourisation rate

To determine the maximum decolourisation rate and maximum concentration tolerance of the organism (*Proteus* strain D2), experiments with different initial concentrations of R13 was conducted from 25 to 2500 mg/L of the dye. Lineweaver – Burk model was used to determine the maximum specific decolourisation rate, while the tolerance level was estimated from Michaelis – Meten plot.

3.6 Biodegradation of Reactive Azo Dyes

As an investigation of the ability of the strain to degrade and not just decolourise azo dyes, reactive blue 13, the azo dye whose structure was available was used. Biodegradation was determined using change in peaks profiles in HPLC and FTIR of the metabolite of reactive blue 13 and those of its abiotic control. Attempt was also made to identify the metabolites of RB13 degradation using GCMS spectra of the dyes metabolites.

3.6.1 Preparation of metabolite extract

The supernatant of 24 h or 48 h decolourised RB13 dye, centrifuged at 10,000 rpm for 10 min, was extracted using ethyl acetate. The extract was dried in rotary evaporator and re-dissolved in HPLC grade methanol. The solution was used for HPLC and GCMS analyses. Solid extracts were used for FTIR analyses.

3.6.2 UV-visible analyses

The uv-visible spectral analyses for both the biotic and abiotic solutions of the reactive dyes mixture and the methyl red were recorded at specific intervals using Shimadzu UV-visible spectrophotometer (UV-1650PC) and changes in their absorption spectra (200-1000 nm) were noted.

To investigate the formation of aromatic amine, a similar scan was done for 1% aniline sulphate, and compared with those of the metabolites of the reactive dyes and methyl red.

3.6.3 HPLC analyses

The HPLC analyses were done with a dual λ UV-visible detector HPLC (Waters, Austria) on a C18 column (symmetry, 4.6 X 250 mm). Sample (10 µl of dye or its metabolite) was injected and allowed to separate for 10 min at the flow rate of 1µl/min. A methanol isocratic mobile phase was used. Both reactive blue 13 and its metabolites were used in this study.

3.6.4 FTIR analyses

FTIR spectra of the reactive blue 13, and its metabolites recorded using FTIR model 800 (Shimadzu, Japan). The scans were done in the mid IR region of 400-4000 cm^{-1} with 16 scan repeat. The samples were previously mixed with IR grade KBr in the ratio 10:90, fixed in sample holder before the scans were done.

3.6.5 GCMS analyses

The GCMS scans were done with a QP2010 mass spectrometer (Shimadzu, Japan) at ionization voltage of 70ev with a temperature programmed mode (Resket column 0.25 mm – 60 mm; XTI-5). The initial column temperature was 80^{o}C for 2 min; the temperature was then increased linearly at 10^{o}C min^{-1} till 280^{o}C temperature was attained, the final temperature was held for 7 min. the temperature of the injection port was 280^{o}C and the GCMS interface was maintained at 290^{o}C. The carrier gas (helium) was maintained at a flow rate of 1.0 mLmin^{-1}. Degradation products were identified using the NIST spectral library software (version 1.10 beta, Shimadzu) and by comparing the structure of the dye (RB 13) with m/z values of the molecular ions/base peaks of its metabolites with the aid of ChemDraw Ultra 8.0®.

3.7 Toxicity Studies of Azo Dyes and their Metabolites

The end products of certain biotreatment of dyes, particularly the anaerobic reduction, have been reported to be more toxic than the dyes themselves (Chung and Stevens, 1993). This phase of the research was conducted to investigate whether the products formed by the degradation of reactive blue 13 were toxic to bacterial and/or plants.

3.7.1 Bacterial zone of inhibition

Microbial toxicity of control RB13 and its degradation product (the ethyl acetate extracted products of RB13, dried and dissolved in 5 mL of sterile distilled water to make a final concentration of 500 and 1000 ppm) was carried out with *Rhizobium rhodobacter*

and *Kocuria rosea*; zone of inhibition (diameter in cm) was recorded after 24 h of incubation at 37^{o}C. The experiments were done in triplicates.

3.7.2 Phytotoxicity

Reactive blue 13 degradation products extracted in ethyl acetate were dried and dissolved in water to form the final concentration (1000 ppm) for phytotoxicity studies. The phytotoxicity study was performed on seeds of *Zea mays* and *Phaseolus vulgaris*. The seeds (10 each) were watered separately in Petri dishes with 5 mL of Reactive blue13 solution and its degradation product (1000 ppm) per day. Seeds wet with tap water were included as control. Length of plumule (shoot), radical (root) and % germination were recorded after 7 days.

Data were analyzed by One-way analysis of variance (ANOVA) with Tukey-kramer multiple comparisons test. Readings were considered significant when P was $\leq$ 0.05

3.8 Production of Enzymes of Biodegradation Importance by Strain D2

Activities of azoreductase, lignin peroxidase, laccase and tyrosinase were assayed spectrophotometrically in the cell free extract. All enzyme assays were carried out at room temperature (~30^{o}C). The reference blank contained all components except the enzyme.

3.8.1 Preparation of extracellular crude extract

To obtain the extracellular cell free extracts, 200 mL nutrient broth with 100 ppm reactive blue 13 (induced) and without dye (Control) were inoculated with 10mL (~0.1g) 24 h culture of the organism. The 24 h culture of both the induced and control were

centrifuged separately at 10,000 rpm at 4^{o}C, and the supernatant was used as crude source of extracellular enzymes.

3.8.2 Preparation of intracellular crude extract

The harvested cells (pellets) obtained in section 3.6.1 were rinsed twice in distilled water, and suspended in 50 mM of either potassium of sodium phosphate buffer (20 mL). The suspension was homogenized, placed on ice-bath and ultrasonic waves were applied at 15 W (40-60 mA) for 30s. Five strokes were given at 2 minutes intervals at 4^{O}C. The cell lysate was centrifuged at 10,000 rpm for 15 min at 4^{O}C. The clear supernatant obtained was used as crude source of intracellular enzymes.

3.8.3 Enzyme assays

3.8.3.1 Azoreductase

Azoreductase activity was assayed by monitoring the removal of methyl red at 430 nm. The reaction mixture contained 0.6mL 50mM phosphate buffer (pH 7.4); 0.25 mM methyl red, 0.1 2mM $NADH_2$ (Maier *et al.*, 2004) or $NADPH_2$ (Chen *et al.*, 2005); different isozymes of this enzyme have often reported in different organisms/strains. Appropriate volume of distilled water was added to give a final volume of 2 mL.

3.8.3.2 Veratryl oxidase

Veratryl alcohol oxidase activity was determined as described by Tien and Kirk (1984). The oxidation of veratryl alcohol to veratryl aldehyde at 310 nm was monitored in 2 mL reaction mixture of containing 0.2 mL veratryl alcohol (10 mM), 1.6 mL citrate buffer

(pH 3.0) and 0.2 mL of the crude enzyme; to give a final concentration of 1 mM veratryl alcohol.

3.8.3.3 Lignin peroxidase

Lignin peroxidase activity was determined as the H_2O_2- dependent oxidation of veratryl alcohol to veratrylaldehyde. The increase in absorbance at 310 nm due to the oxidation of veratryl alcohol to veratraldehyde was monitored (Have *et al*., 1997). The reaction mixture contained in 2 mL, 0.2 mL veratryl alcohol (10 mM), 1.6 mL citrate buffer (pH 3.0) and 0.2 mL enzyme. The reaction was initiated with the addition of 100 µl of 0.5 mM H_2O_2, and monitored over 3-5 min.

3.8.3.4 Laccase

Laccase activity was determined spectrophotometrically as the oxidation of 4,4' -diamino-3,3' -dimethylbiphenyl (o-tolidine) to its aldehyde form at 310 nm (Miller *et al.*, 1997). The reaction mixture of 2 mL contains 1 mM o-tolidine (ethanolic solution) in 1.6 mL 0.1 M acetate buffer (pH 4.0). The reaction was initiated with the addition of 0.2 mL crude enzyme and monitored for 2 minutes.

3.8.3.5 Tyrosinase

Tyrosinase activity was determined in a reaction mixture of 2 mL, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) by measuring liberated catechol quinone at 495 nm (Zhang and Flurkey, 1997).

One unit of enzyme activity was defined as a change in absorbance unit/min/mg of protein. Specific activity was expressed in units per mg protein. All values reported are means of triplicates.

3.9 Purification and Partial Characterization of Azo Dyes Degrading Enzyme

The presence of lignin-modifying enzymes which are useful in textile and industrial effluent treatment prompted the isolation and characterization of the major enzyme of biodegradation interest in this organism – laccase.

3.9.1 Time course assay

A litre nutrient broth-dye solution (100 mg/L) was inoculated with 50 mL overnight culture of the strain. Crude extracellular and intracellular enzymes were obtained as previously described in sections 3.8.1 and 3.8.2. Samples of 100 mL were withdrawn from the bioreactor, at 6 h intervals, and assayed for protein concentration, laccase and veratryl alcohol oxidase activities. The enzyme activities were measured in triplicates

3.9.2 Culture

A litre of nutrient broth-RB13 (100 mg/L) was inoculated with 50 mL of a 24 h -culture of *Proteus mirabilis* (OD_{600} = 1.27) and incubated for 36 h at 37^{o}C.

3.9.3 Preparation of intracellular extract

Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4^{o}C, rinsed twice in distilled water, and suspended in 20 mL, 50 mM of either potassium or sodium phosphate buffer (~ 200 mg mL^{-1}). The suspension was homogenized, placed on ice-bath and

ultrasonic waves were applied at 15 W (40-60 mA) for 30s. Eight strokes were given at 1 min interval at 4^{o}C. The cell lysate obtained was centrifuged at 10,000 rpm for 15 min at 4^{o}C. The clear supernatant obtained was used as crude source of enzymes.

3.9.4 Ion exchange chromatography

The crude samples (20 mL) was applied unto a DEAE-cellulose (15 mm by 100 mm) pre-equilibrated with 50mM phosphate buffer, pH 7.4. The columm was previously equilibrated with the same buffer using twice the column volume until A_{280} of eluent had reached baseline. The sample was applied and eluted using an ion exchange gradient established by using 0-0.5 M NaCl. Fractions (3 mL) eluted at 0.75 mLmin^{-1}, were assayed for protein concentration using A_{280}. Laccase and veratryl alcohol oxidase were assayed for in the fractions that contained protein as described in sections 3.6.3.4 and 3.6.3.2 respectively. The fraction with enzyme activities were pooled, dialysed in distilled water and concentrated with sucrose.

3.9.5 Size exclusion chromatography

The concentrated sample (1 mL) was applied unto a Biogel P-100 column (10 by 450 mm) pre-equilibrated with phosphate buffer, and eluted with the same buffer at the column maximum flow rate of ~6 mL/h. Fractions (2 mL) were assayed for protein concentration. Laccase and veratryl alcohol oxidase were assayed for in those fractions that containing protein as described in sections 3.6.3.4 and 3.6.3.2 respectively. The fraction with enzyme activities were pooled, dialysed and concentrated with sucrose.

3.9.6 SDS-PAGE analysis

The extent of the purification was determined by running SDS-PAGE on samples exhibiting enzymes activity. Samples from each purification step (1000 µl) and 30 µl standard molecular weight markers (16– 98 kDa, Genei, Bangalore) were electrophoresed on 10 % SDS-PAGE (appendix II) at 120 V. The gels were stained with coomassie brilliant blue R-250 staining solution, then destained in methanol: acetic acid: water (1:1:8 v/v/v) destaining solution (appendix II).

3.9.7 Molecular weight determination

The molecular weight of the partially purified enzymes was determined by analysis of the gel picture with AlphaView software (AlphaInnotech).

3.9.8 Determination of optimal pH

The optimal pH for the enzyme was established by determining the enzyme activities in different pH buffers, Potassium-HCl (pH 1-2, 100mM), sodium acetate (pH 3 – 5, 100 mM); sodium phosphate buffer (pH 6 – 8, 100 mM); and bicarbonate buffer (pH 9-10, 100 mM). Laccase activity was determined in each of the samples at the different pH levels.

3.9.9 Determination of optimal temperature

The optimal temperature for the oxidation of o-tolidine by the partially purified enzyme was determined over a temperature range of 10 – 70°C. The reaction mixture was prepared as previously described (Section 3.8.3.4).

3.9.10 Determination of enzyme's V_{max} and K_m

The kinetic properties of the partially purified enzyme were determined by varying the substrate (veratryl alcohol, 2, 6-dimethoxyphenol and guaiacol) concentration between the ranges 01 – 1.0 mM. The enzymes activities were determined at each substrate concentration at 25^{o}C for 3 min. the rates of substrate utilization were determined by measuring the change in absorbance values at the respective wavelengths, and the molar extinction coefficients ($\boldsymbol{\varepsilon}$) were obtained from literature (Saito *et al.*, 2003; Lu *et al.*, 2007). V_{max} and K_m were determined for each of the substrates by plotting V against V/[S}, according to Eadie-Hofstee equation:

$$V = -k_m \frac{V}{[S]} + V_{\max} \quad \text{.....3.4}$$

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation and Identification of Bacterial Strains

A total of fourteen strains were isolated from the four environmental sources: 4 from dry dyes; 4 from textile effluent; 3 from pond water and 3 from refuse dump. Three of these strains were gram positive rods, two gram positive spheres, six gram negative rods and three gram negative spheres. None of the organisms was acid-fast, none had spores but all were catalase positive.

The identities of the organisms when their biochemical characteristics were compared to Cowan and Steel's scheme (Barrow and Feltham, 1993) are shown in **Table 4.1**. The fourteen newly isolated bacteria were identified tentatively as 3 strains of *Acinetobacter* sp, 3 strains of *Bacillus* sp, 6 strains of *Pseudomonas* sp and 1 strain each of *Staphylococcus* and *Micrococcus* sps.

Further characterization of the strain with the highest decolourisation using 16S rDNA showed irregularity, thus the strain with second-highest decolourisation (Strain D2) was used in this research. Strain D2 was identified tentatively as a strain of *Pseudomonas* (Tables 4.1 and 4.2).

Table 4.1: Sources and identities of bacterial isolates

LAB. #	Grams reaction	Shape	Acid fast	spores-	motility	aerobic	anaerobic	catalase	oxidase	Glucose (acid)	CHO (F/O/-)	Identity (Genus)
D1	-	R	-	-	+	+	-	+	-	+	*F*	*Pseudomonas*
D2	-	R	-	-	+	+	-	+	+	+	*O*	*Pseudomonas*
D3	+	R	-	-	+	+	-	+	+	+	*F*	*Bacillus*
D4	+	S	-	-	-	+	-	+	-	+	*F*	*Staphylococcus*
E1	-	S	-	-	-	+	-	+	+	+	-	*Acinetobacter*
E2	-	R	-	-	+	+	-	+	+	+	*F*	*Pseudomonas*
E3	-	S	-	-	+	+	+	+	-	+	*F*	*Acinetobacter*
E4	-	R	-	-	+	+	-	+	+	+	*F*	*Pseudomonas*
P1	-	R	-	-	-	+	+	+	+	+	-	*Pseudomonas*
P2	+	R	-	-	+	+	+	+	+	+	-	*Bacillus*
P3	+	R	-	-	+	+	-	+	w	+	*F*	*Bacillus*
R1	-	R	-	-	-	+	+	+	+	+	*O*	*Pseudomonas*
R2	-	S	-	-	-	+	-	+	+	+	-	*Acinetobacter*
R3	+	S	-	-	-	+	-	+	+	+	*F*	*Micrococcus*

Legend: + **Positive** **-** **Negative**

w **weak reaction** ***ND*** **Not determined**

R **Rod shape** **S** **Spherical**

D **Dyes isolates** **E** **Textile Effluent isolates**

P **Pond water Isolates** **R** **Refuse Soil isolates**

Table 4.2: Further characterization of Strain D2

Characteristics	Strain D2
Colony Morphology	
Margin	Entire
Elevation	Slightly raised
Surface	Circular
Opacity	Transluscent
Gram's reaction	Negative
Cell shape	Short rods
Cell size	~1µm
Motility	Negative
Biochemical Test	
Growth on MacConkey	Positive (LF*)
Citrate Utilization	Positive
H_2S Production	
Catalase	Positive
Oxidase	Positive
Nitrate reduction	
Argenine hydrolysis	Negative
Lysozyme	Resistance
VP Test	Positive
Pigment formation	Yellow
Identity	***Pseudomonas*** **

Legend:

* LF – Lactose Fermenter

** Based on Cowan and Steel's scheme (Barrow and Feltham, 1993)

4.1.1 16S rDNA Characterization

The result of 16S rDNA for strain D2 however showed that the organism is a strain of *Proteus mirabilis* and not a strain of *Pseudomonas* as initially identified. It is important to note that the two strains are gram negative rods. The 16S rDNA sequence (appendix III) was subsequently deposited at GenBank as that of *Proteus mirabilis* strain LAG.

Table 4.3: Genetic match and distance of strain D2 and related organisms

Closest Genetic Match	Genetic Distance
P.mirabilis	**0**
P.vulgaris	**0.003**
P.penneri	**0.006**
P.hauseri	**0.007**
X.hominickii	**0.028**
X.koppenhoeferi	**0.038**
X.ehlersii	**0.04**
X.stockiae	**0.041**
X.budapestensis	**0.046**

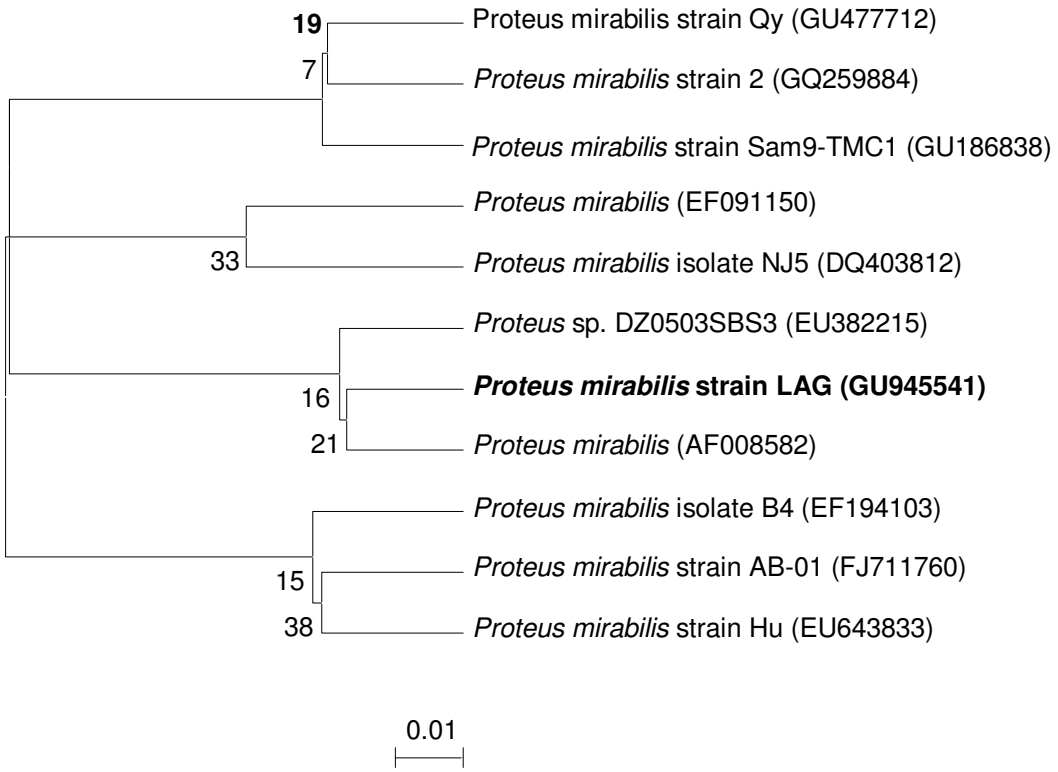


Figure 4.1: Phylogenetic analysis of 16S rDNA sequence of bacterial isolate *Proteus mirabilis* strain LAG. Distance tree constructed using neighbor joining method by using MEGA 4, showing the phylogenetic relationships of *Proteus mirabilis* strain LAG and other species of genus *Proteus*. Numbers at nodes shows the level of bootstrap support based on data for 1000 replication. Bar, 0.01 substitutions per nucleotide position.

4.2.1 Decolourisation under limited supply of oxygen

All the fourteen newly isolated strains decolourised the reactive dyes solution but to various percentages **(Table 4.4).** Under limited supply of oxygen, 3 strains (**D1, D2 and R3**) showed > 95% decolourisation within 24 h, two of these were isolated from textile dyes, while the third, which showed the highest decolourisation of 97.50 ± 0.83 was of refuse dump origin**.** Strains of textile dyes origin appear to perform the best on the average. The strain used for this research was strain D2, which was the second best and of textile dye origin.

Table 4.4: Initial decolourisation of reactive dyes mix under limited supply of oxygen

Lab No.	% Decolourisation[a]	
	12 h	24 h
D1	45.83 ± 3.00	95.00 ± 1.67
D2	61.67 ± 1.44	96.94 ± 2.10
D3	35.28 ± 4.88	52.22 ± 4.28
D4	20.56 ± 1.27	27.78 ± 1.73
E1	52.22 ± 3.94	72.50 ± 3.63
E2	19.72 ± 2.55	40.56 ± 3.37
E3	43.61± 4.88	65.83 ± 2.20
E4	27.78 ± 7.92	57.50 ± 3.63
P1	36.67 ± 3.33	39.72 ± 3.15
P2	41.94 ± 8.67	51.39 ± 3.37
P3	26.11 ± 2.41	30.28 ± 1.27
R1	29.17 ± 8.21	43.61 ± 2.93
R2	29.72 ± 3.94	41.39 ± 2.68
R3	71.67 ± 7.50	97.50 ± 1.83

Legend:

Sources of isolate: D – Dye E – Effluent P –Pond R – Refuse soil

Values are mean of triplicates ± SD

4.2.2 Decolourisation of azo dyes by strain D2

4.2.2 Decolourisation under normal supply of oxygen

Decolourisation under normal supply of oxygen reduced the duration of decolourisation by the *Proteus mirabilis* from 24 h to about 6 h. The plot of % decolourisation against incubation period (**Figure 4.2**) is similar to that of Michaelis – Menten.

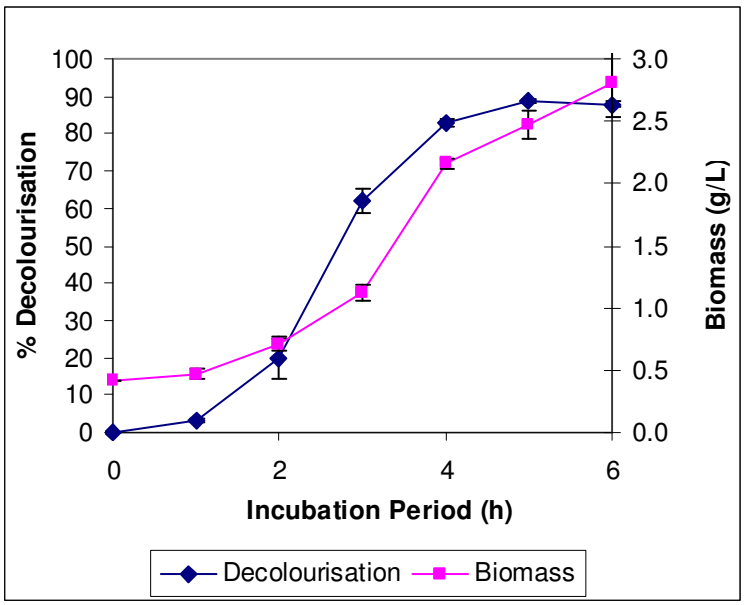


Figure 4.2: Decolourisation of reactive blue 13 under static anoxic condition

4.2.3 Effect of static and shaking conditions on decolourisation

Shaking condition seems to increase the lag phase of the microbial growth from about 1 h (under anoxic condition) to 3 h. This resulted in poor decolourisation of the dye (22.67 ± 2.60%) under shaking condition. Despise the prolonged lag phase experienced by the organism in shaking condition, cell growth was higher than in static condition at the end of the 6 h incubation period. The organism however showed 87.91 ± 0.62% decolourisation within 5 h, under static anoxic condition **(Figure 4.3).**

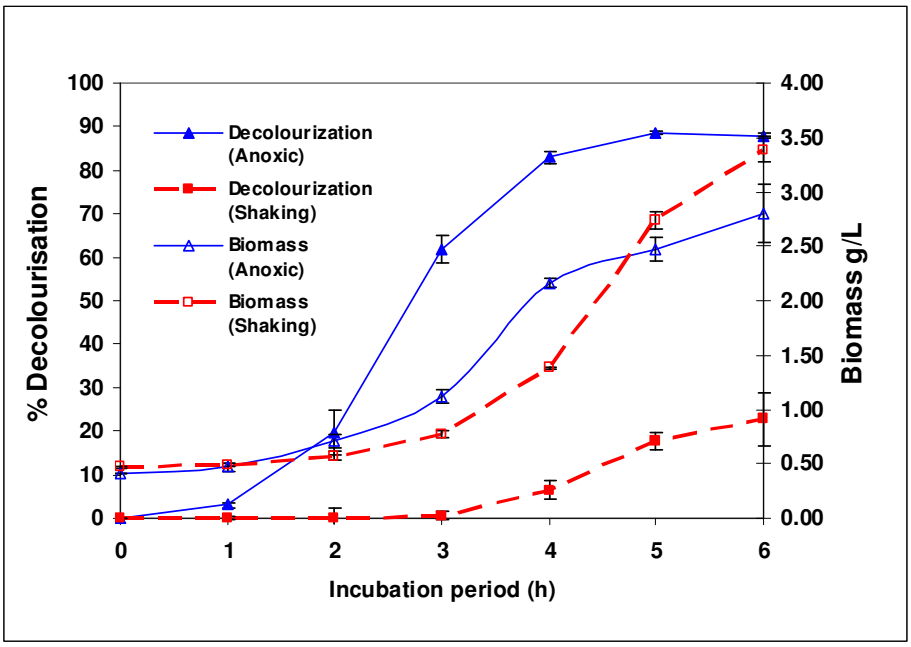


Figure 4.3: Comparison of decolourisation of Reactive Blue 13 under static anoxic and shaking condition. Error bars indicate standard deviation of three values.

4.3 Kinetics Studies of Dyes Decolourisation

Figures 4.4 and 4.5 showed the dependence of specific decolourisation rate on the concentration of RB13 and cell mass concentrations respectively. From the gradients of figures 4.4b and 4.5B, the order of reactions were found to be 0.5498 and 1.0977 with respect to dye concentrations and cell mass concentrations respectively.

When the reciprocal of the dependence of the specific rate of decolourisation on cell mass concentration was plotted (Lineweaver – Burk model), the specific decolourisation rate estimated from the experimental data was 36.23 $mgL^{-1}h^{-1}$, the value of the apparent K_m was 278.83mgL^{-1} (figure 4.6); and the inhibitory concentration was about 400mg/L (Figure 4.4A).

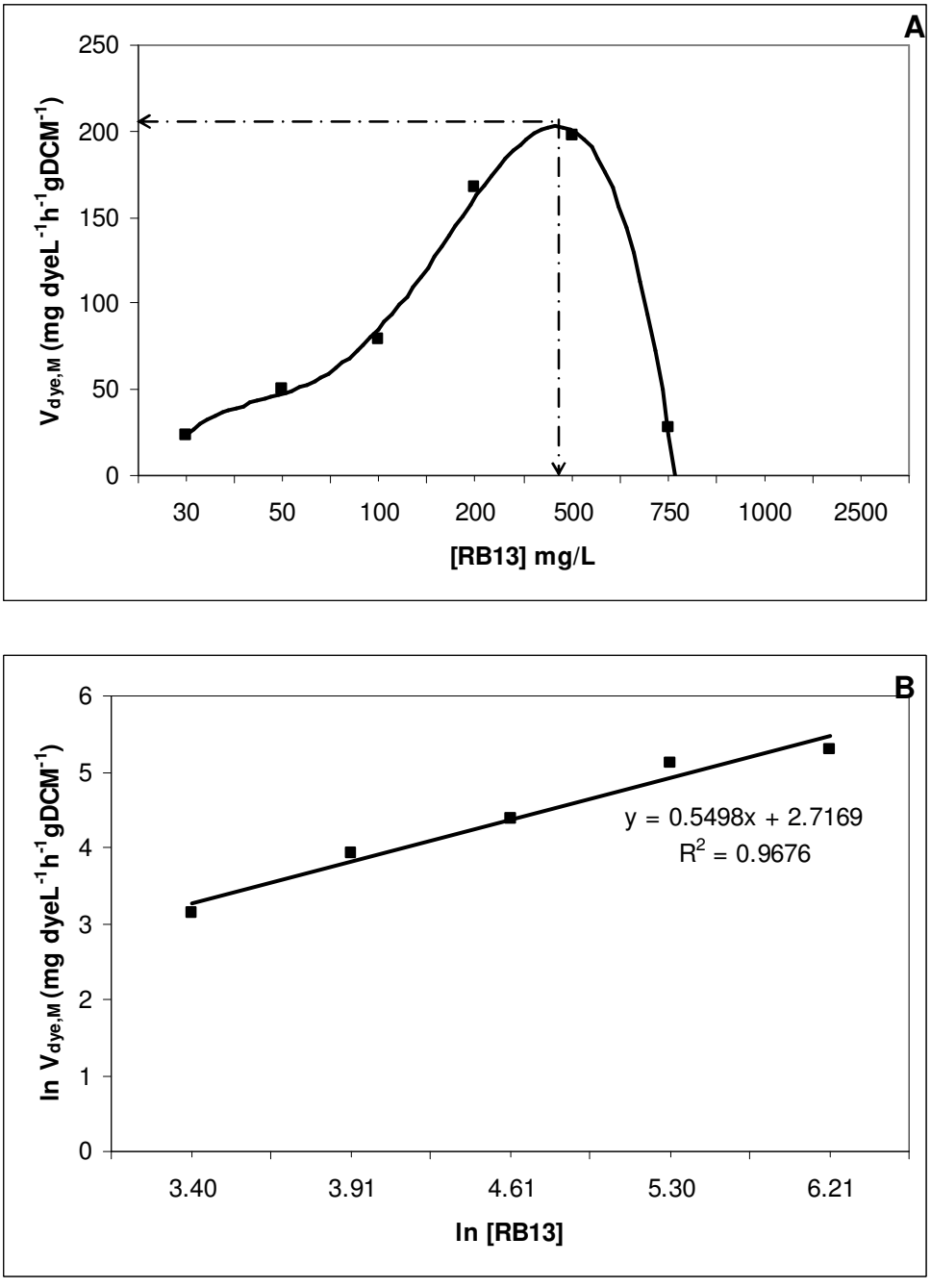


Figure 4.4. A: Dependence of specific decolourisation rate on the concentration of RB13: t = 6 h and 1.108 g dry cell mass/L. B: Data fitting and order of reaction determination with respect to dye concentration (equation 3.2).

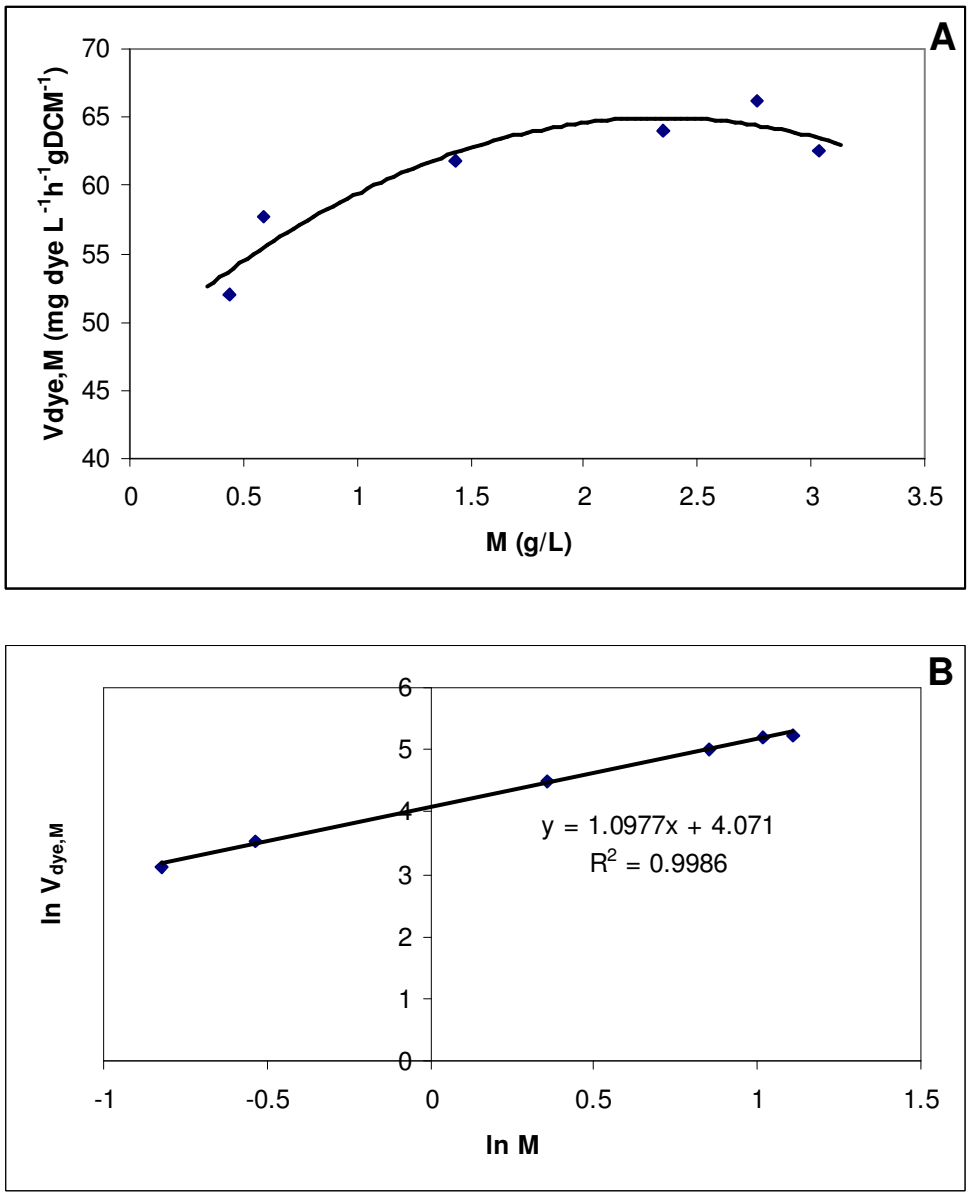


Figure 4.5. A: Dependence of specific decolourisation rate on biomass concentration: 100 mg dye/L and t = 6 h. B: Data fitting and order of reaction determination with respect to biomass (equation 3.3).

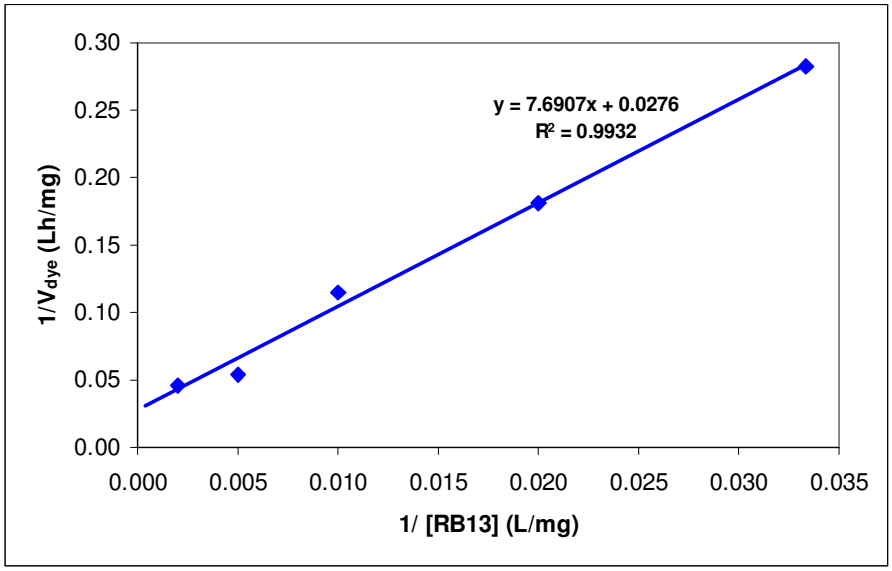


Figure 4.6: Reciprocal plot of dependence of specific decolourisation rate on dye concentration.

4.4 Effect of Temperature, pH and Media on Decolourisation

4.4.1 Optimal temperature and pH

The results of the experiments conducted at different incubation temperature showed that the optimal incubation temperature for the decolourisation of methyl red by the isolated *Proteus mirabilis,* is at 37^{o}C (94.89±4.79% decolourisation in 7 h). It was also observed **(figure 4.7a)** that the strain decolourised the solution well at the ambient environmental temperature of 30^{o}C (86.11±4.5% in 7 h).

Figure 4.7b shows the effect of initial pH on decolourisation of the azo dye methyl red at optimal temperature of 37^{o}C. The *Proteus mirabilis* effectively decolourised the methyl red solution at pH 6, 7 and 8, with % decolourisation of 84.60±1.16, 94.19±1.75 and 93.94±2.00 respectively. The optimal pH appears to be between 7 and 8.

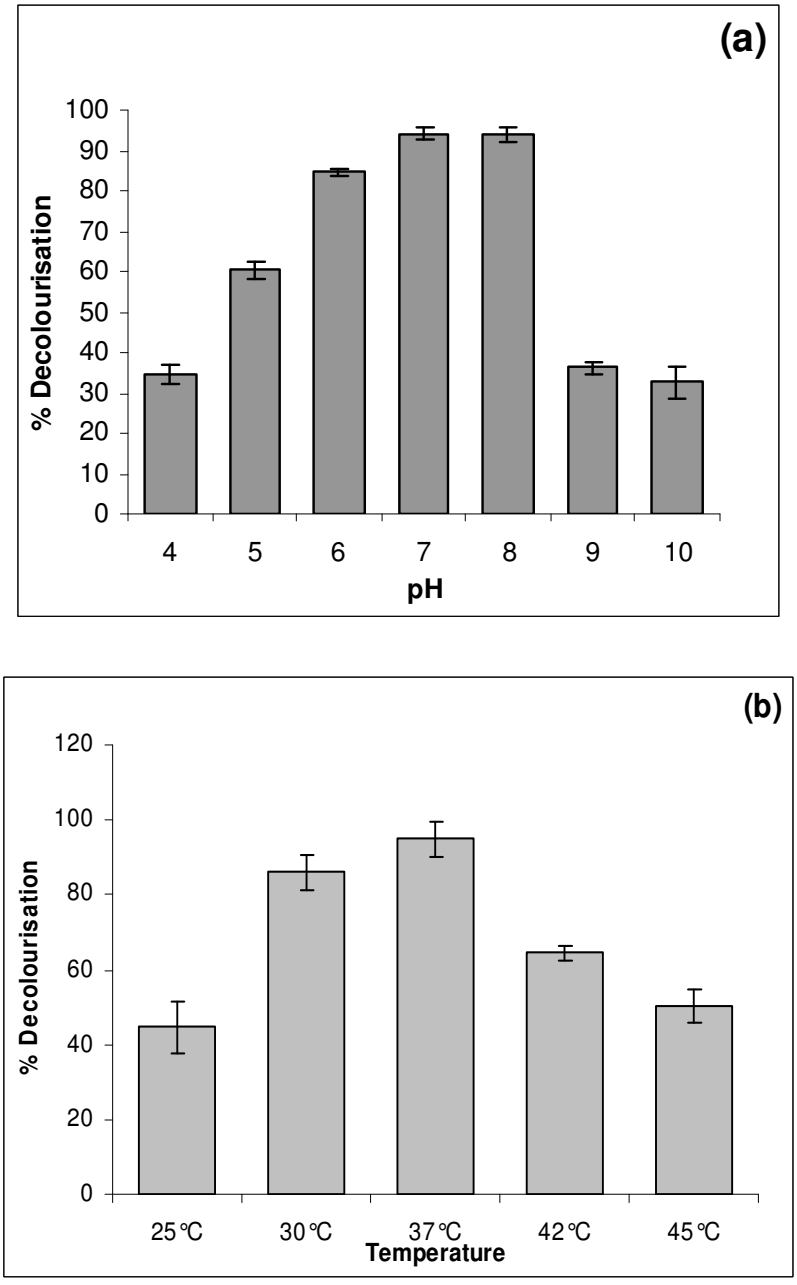


Figure 4.7: Effect of pH (a) and incubation temperature (b), on decolourisation of azo dyes

4.4.2 Media Preference

Figure 4.8 shows the effect of different media types on decolourisation of methyl red. Among the media types, the most effective decolourisation was achieved using nutrient broth with 97.39±0.38% decolourisation within 6 h.

The strain showed high level of decolourisation when Yeast (2 g/L) and Starch (0.25 g/L) were used as co-substrate in the minimal media (MM); 76.72±1.72% decolourisation in 6 h. The absence of yeast in a similar medium (MS) however reduced the decolourisation to almost zero (0.13±0.14%).

Within 6 h only 0.83±0.37% decolourisation was obtained with distilled water (DW), but when 2 g/L yeast was added (DW+Y) the decolourisation increased to 2.69±0.47%.

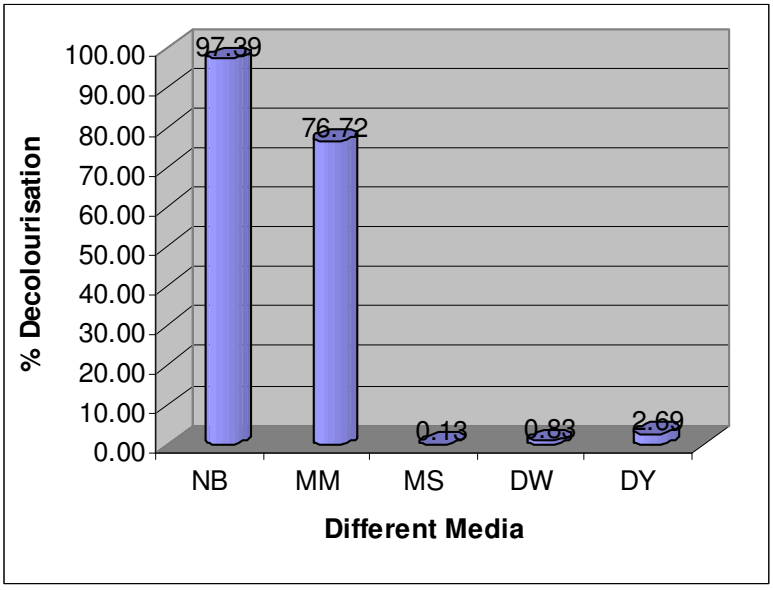


Figure 4.8: Effect of media composition on azo dye decolourisation

(NB - Methyl red in nutrient broth; MM- Methyl red in minimal medium with Yeast extract and Starch as co-substrate; MS- Methyl red in minimal medium with Starch only as co-substrate; DW - Methyl red in distilled water; DY - Methyl red in distilled water and yeast extract)

4.5 Biodegradation of Reactive Azo Dyes

The major difference between decolourisation and biodegradation is that decolourisation may occur with the removal of just an atom, without necessarily degrading the molecule. In azo dye decolourisation, for instance, only the N=N needed to be cleaved; this sometimes results in the formation of more toxic aromatic amine. It is therefore important to study biodegradation as against decolourisation which is the interest of most textile industries.

4.5.1 UV –visible analyses

The initial UV- Visible spectrum of the mixture of reactive dyes (0 h) revealed two peaks in the visible region (412 and 549 nm) and one in the near UV region (291 nm); the 412 nm peak showed the maximum absorbance. After 24 h of decolourisation experiment, the peaks in the visible region had been removed, while the one in the near UV remained **(Figure 4.9a).**

Figure 4.9b showed the spectra of methyl red solution at 0 h and after 3 h and 6 h decolourisation. The spectrum of methyl red at 0 h showed two major peaks: one in the visible region (482 nm) and the other in the near visible region (312 nm) after 6 h the peak at the visible region as been removed. It was also observed that the absorbance values of the peak at 0 h, 3 h and 6 h were 1.154, 2.749 and 2.255 respectively.

The aniline sulphate UV-visible spectrum revealed a single absorbance peak at 291 nm **(Figure 4.10)**

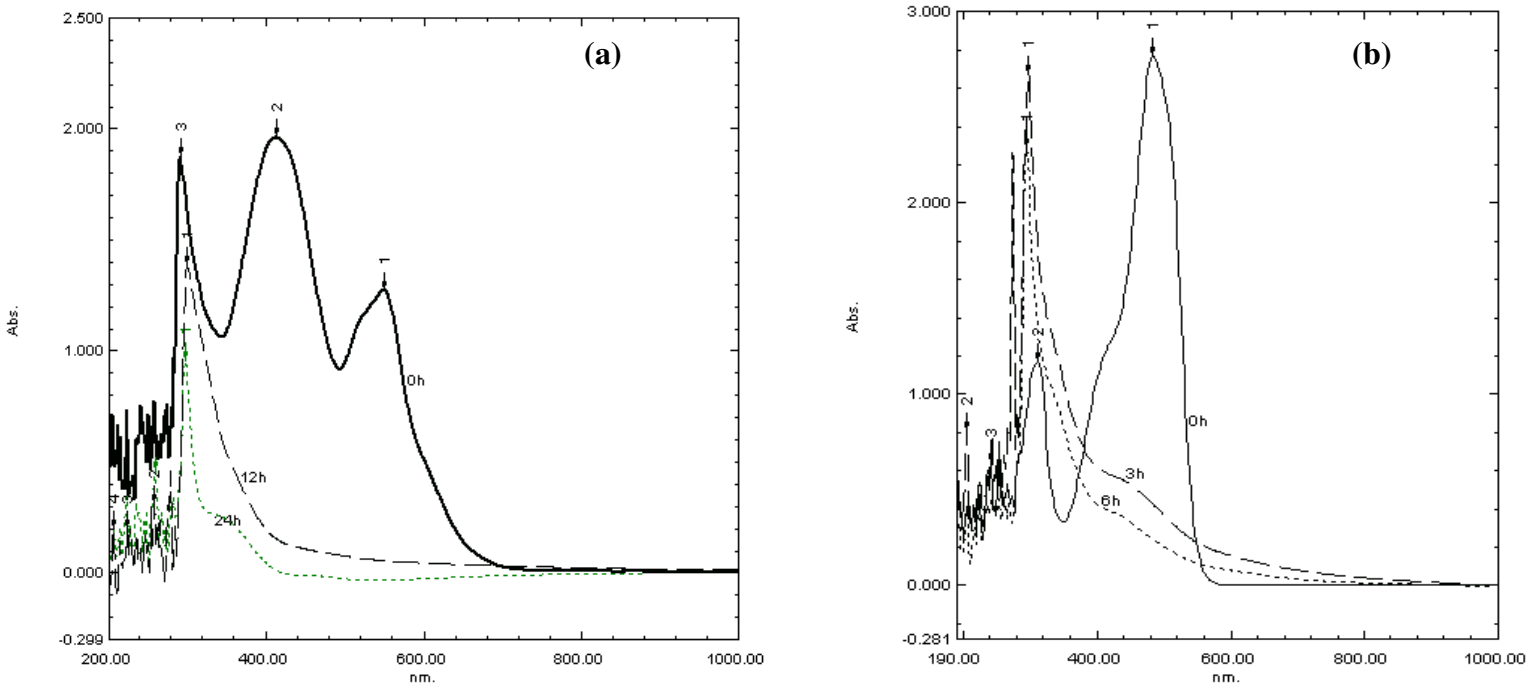


Figure 4.9: UV-visible Spectra of (a) reactive dyes mix (b) methyl red solutions; and their metabolites

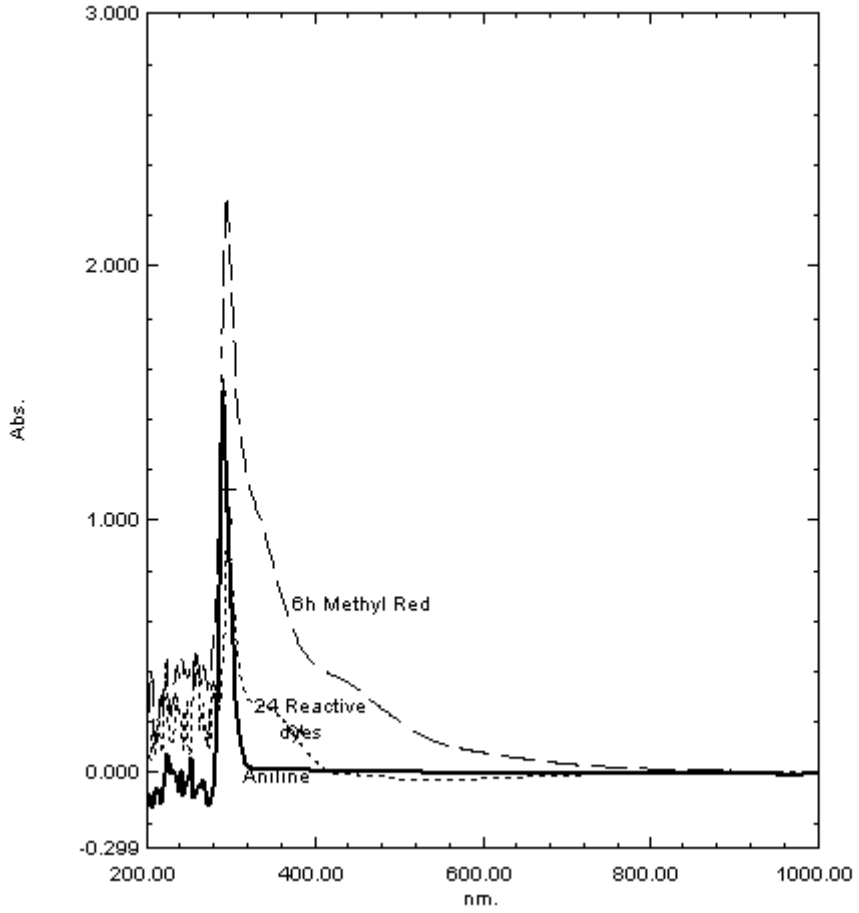
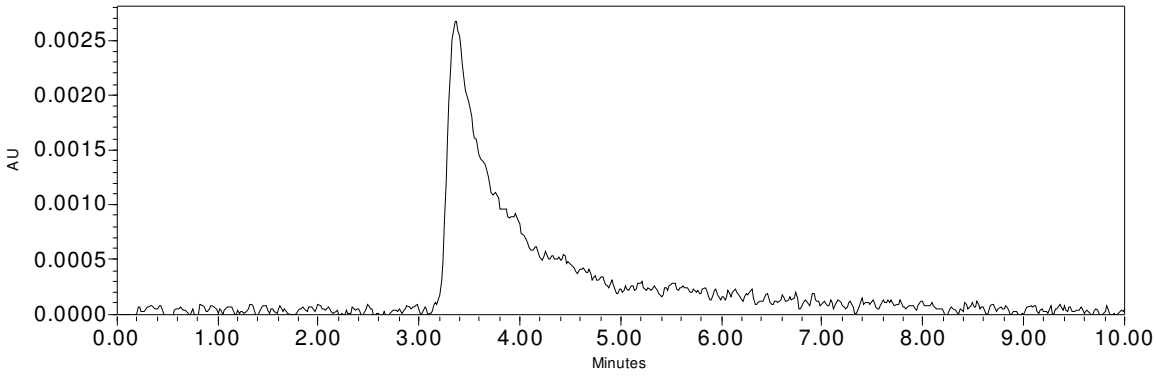


Figure 4.10: Overlaid spectra of aniline sulphate and metabolites of methyl red and reactive azo dyes

4.5.2 HPLC analyses

The HPLC of the reactive blue 13 showed a single peak at 13.362 minutes, the degradation products has a major peak at 5.408 minutes, a medium peak at 4.219 minutes and several minor peaks (Figure 4.11).

(a)



(b)

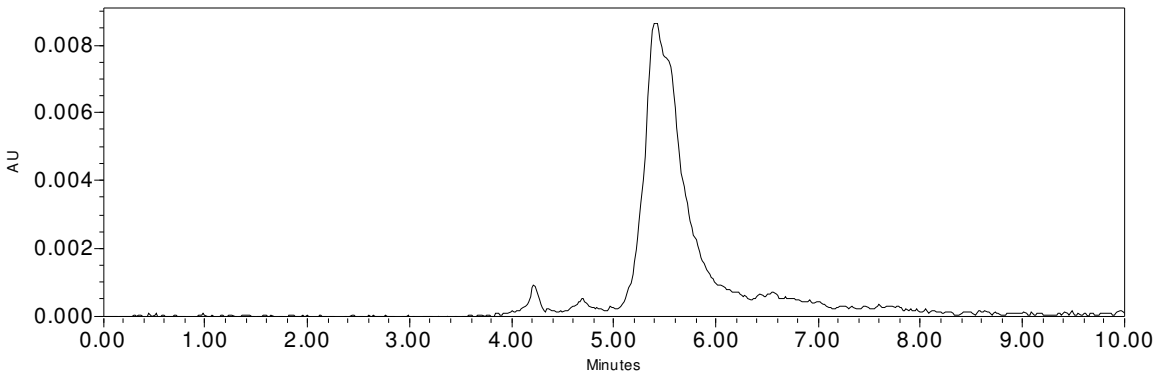


Figure 4.11: HPLC elution profile of Reactive blue 13 (a) and its metabolites (b)

4.5.3 FTIR analyses

An obvious difference was noticed between the FTIR spectra of Reactive blue 13 and its metabolite (Figure 4.12). The spectrum of the control showed specific peaks in the fingerprint region for substituted aromatic compounds (617, 704 and 834 cm^{-1}). S=O streaching vibration was noticed as a sharp peak at 1047 cm^{-1}, while the C–H stretching was reported at 2922 cm^{-1}.

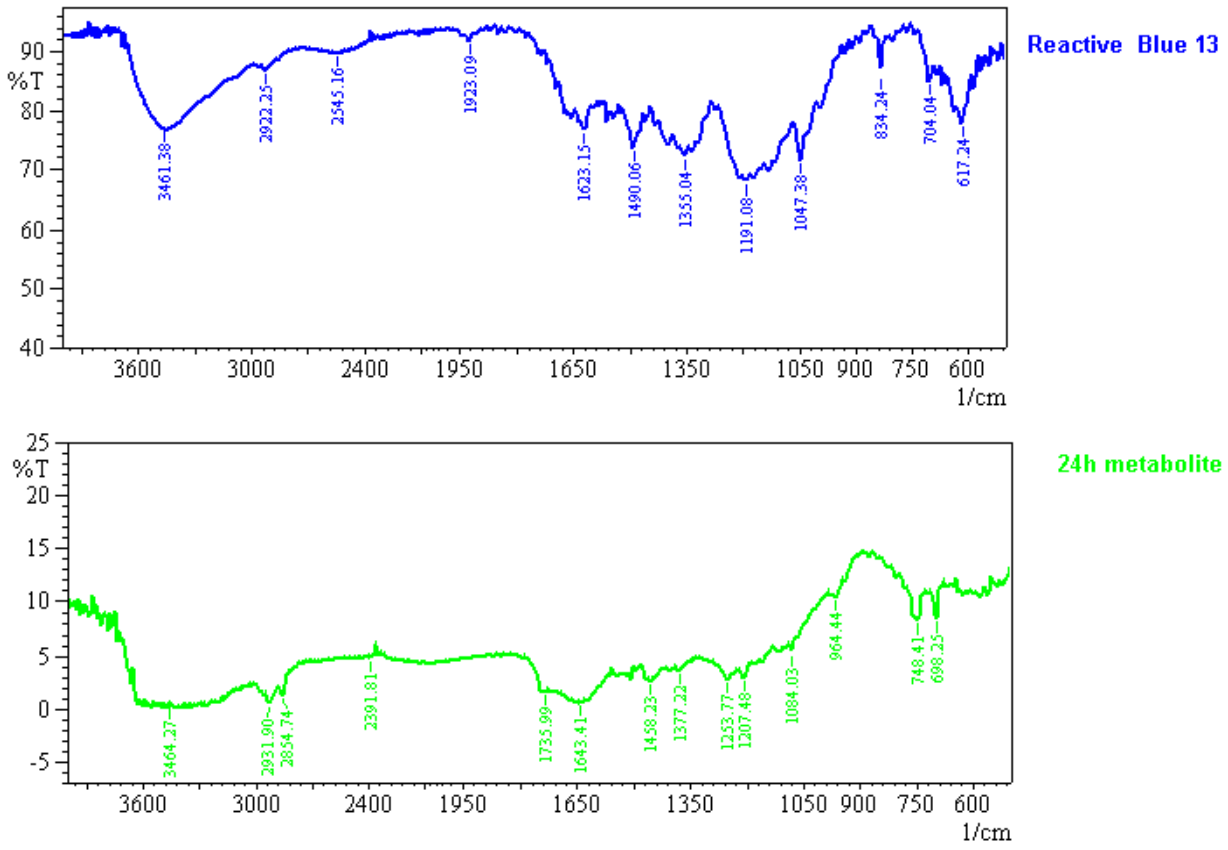


Figure 4.12: FTIR of RB13 and its metabolite (on different scale)

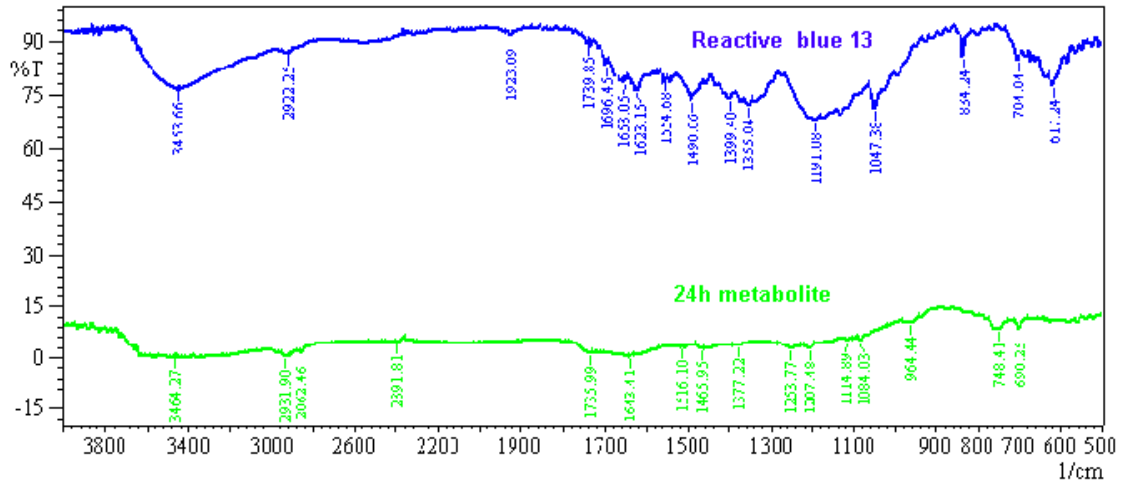


Figure 4.13: FTIR of RB13 and its metabolite (on same scale)

4.5.4 GCMS analyses

The results of the GCMS revealed thirteen different peaks (**Figure 4.14)** for the dye metabolites; their retention times, molecular ions and base peaks are shown in **Table 4.5**.

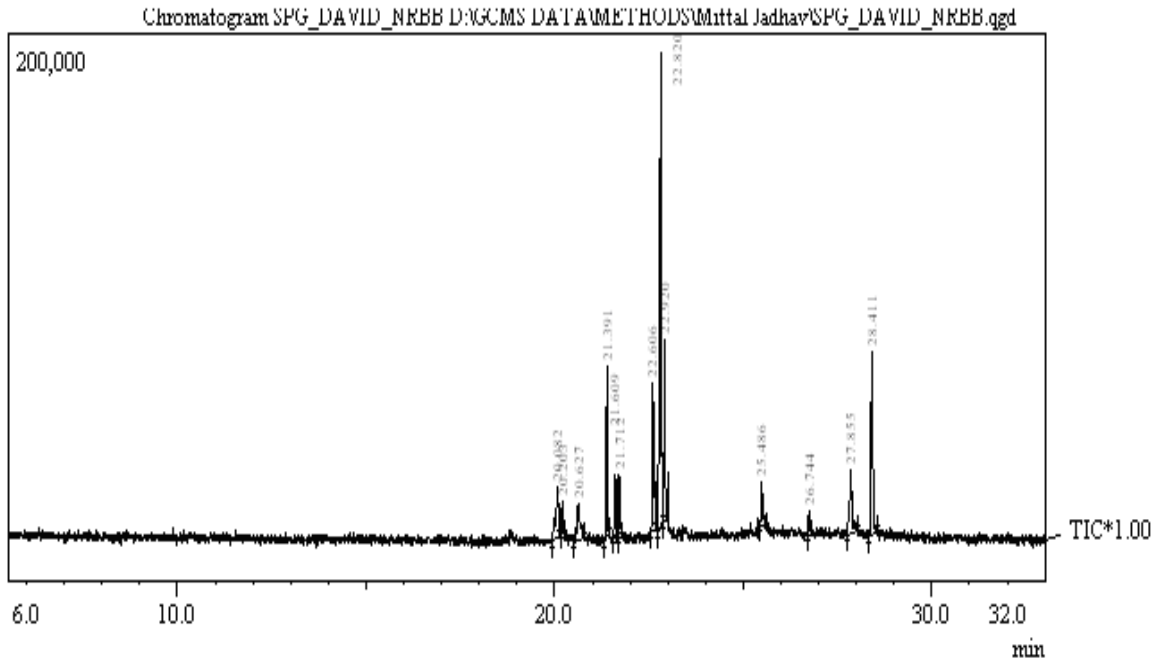


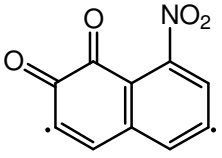
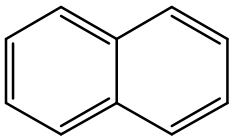
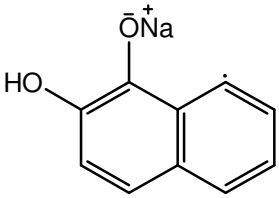
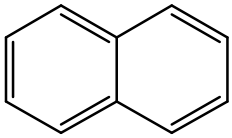
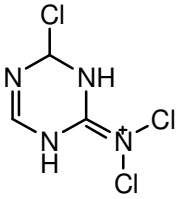
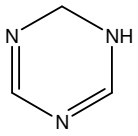
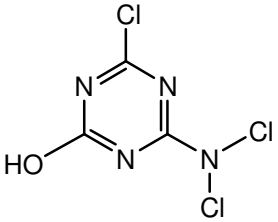
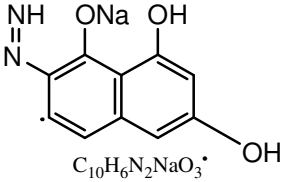
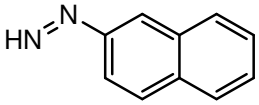
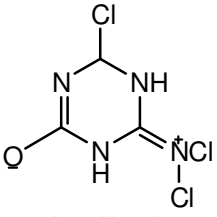
Figure 4.14: Gas Chromatogram of Reactive Blue 13 metabolites

Table 4.5: GCMS Parameters of the metabolites of RB 13 biodegradation

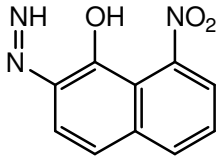
Peak #	Retention time	Area %	m/z	Base Peak
I	20.082	5.03	201	128
II	20.203	1.61	181	128
III	20.627	4.29	202	83.10
IV	21.391	8.61	217	70.10
V	21.609	3.24	225	156.15
VI	21.712	3.46	214	70.10
VII	22.606	9.55	217	154.15
VIII	22.820	32.24	215	154.10
IX	22.920	9.03	238	154.15
X	25.486	3.46	206	170.10
XI	26.744	1.58	214	91.10
XII	27.855	5.79	224	125.10
XIII	28.411	12.10	239	125.10

The interpretation of the result of GCMS analysis, in which the molecular ions of the GC peaks and their respective base peaks where accounted for showed the conversion of reactive blue 13 to various products most of which are substituted naphthalene and 1-3-5 triazine (Tables 4.6 and 4.7). Worth-noting is the 26.744 minutes peak with base peak of 91.10 identified as either Sodium-2(2-formyl-2hydroxyvinyl) benzoate or Sodium-2(2-carboxyvinyl) benzoate.

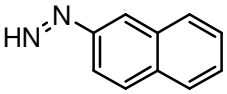
Table 4.6 Deduced structures of the metabolites of RB13

	Molecular ion	Base Peak
I	 $C_{10}H_3NO_4^{\bullet\bullet}$ Mol. Wt.: 201.14	 Mol. Wt.: 128.17
II	 Mol. Wt.: 181.14	 Mol. Wt.: 128.17
III	 Mol. Wt.: 202.45	 Mol. Wt.: 83.09
IV	 $C_3HCl_3N_4O$ Mol. Wt.: 215.43	Cl_2 Mol. Wt.: 70.91
V	 $C_{10}H_6N_2NaO_3^{\bullet}$ Mol. Wt.: 225.16	 $C_{10}H_8N_2$ Mol. Wt.: 156.18
VI	 $C_3H_3Cl_3N_4O$ Mol. Wt.: 217.44	Cl_2 Mol. Wt.: 70.91

VII

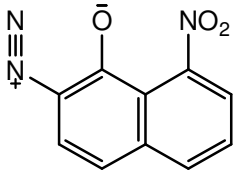


$C_{10}H_7N_3O_3$
Mol. Wt.: 217.18

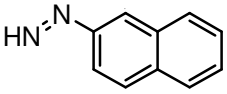


$C_{10}H_6N_2^{\bullet\bullet}$
Mol. Wt.: 154.17

VIII

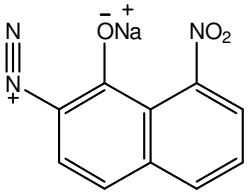


Mol. Wt.: 215.17

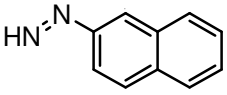


$C_{10}H_6N_2^{\bullet\bullet}$
Mol. Wt.: 154.17

IX

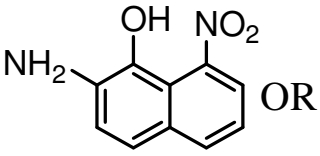


Mol. Wt.: 238.16



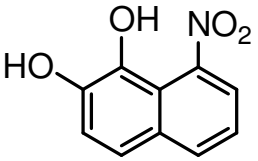
$C_{10}H_6N_2^{\bullet\bullet}$
Mol. Wt.: 154.17

X

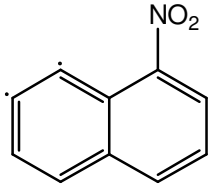


Mol. Wt.: 204.18

OR

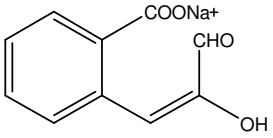


Mol. Wt.: 205.17

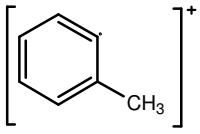


Mol. Wt.: 171.15

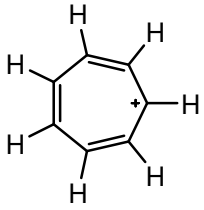
XI



Mol. Wt.: 214.15



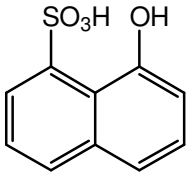
OR



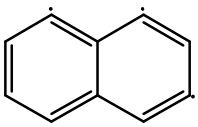
Mol. Wt.: 91.13

Tropylium cation

XII

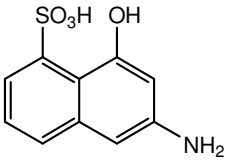


$C_{10}H_8O_4S$
Mol. Wt.: 224.23

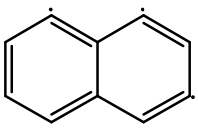


$C_{10}H_5^{\bullet\bullet\bullet}$
Mol. Wt.: 125.15

XIII

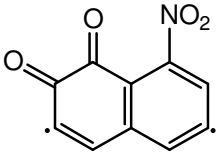
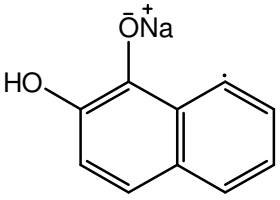
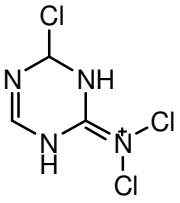
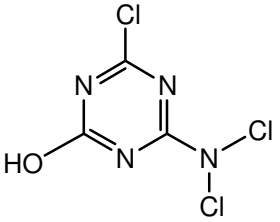
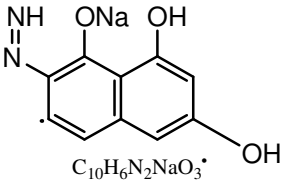
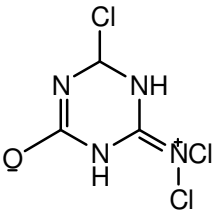


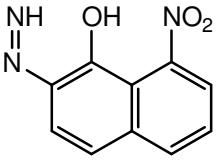
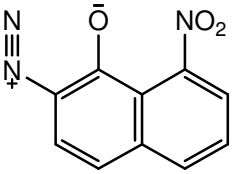
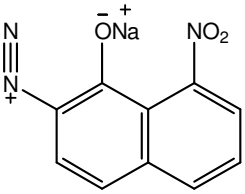
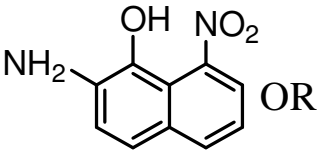
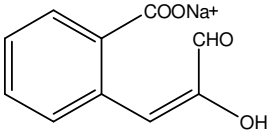
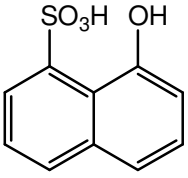
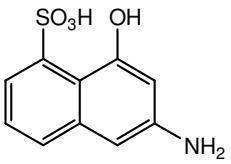
$C_{10}H_9NO_4S$
Mol. Wt.: 239.25



$C_{10}H_5^{\bullet\bullet\bullet}$
Mol. Wt.: 125.15

Table 4.7: The structures and identities of the metabolites of reactive blue 13 degradation by *Proteus mirabilis*

	Molecular ion	Name
I	 $C_{10}H_3NO_4^{\bullet\bullet}$ Mol. Wt.: 201.14	8-nitronaphthalene-1,2,-dione (20.082 minute, m/z 201, base peak 128.10)
II	 Mol. Wt.: 181.14	Sodium 8-hydroxylnaphtoxide (20.203 minute, m/z 181, base peak 128.10)
III	 Mol. Wt.: 202.45	6Chloro-4-(dichloroamino)-1-3-5-triazinoxide (20.627 minute, m/z 202, base peak 83.10)
IV	 $C_3HCl_3N_4O$ Mol. Wt.: 215.43	6Chloro-4-(dichloroamino)-1-3-5-triazin-2-ol (21.391 minute, m/z 217, base peak 70.10)
V	 $C_{10}H_6N_2NaO_3^{\bullet}$ Mol. Wt.: 225.16	Sodium 2-diazenyl 6,8-dihydroxylnaphthoxide (21.609 minute, m/z 225, base peak 156.15)
VI	 $C_3H_3Cl_3N_4O$ Mol. Wt.: 217.44	6-Chloro-4-(chloroamino)-1-2-dihydro-1-3-5-triazin-2-ol (21.712 minute, m/z 214, base peak 70.10)

VII	 $C_{10}H_7N_3O_3$ Mol. Wt.: 217.18	2-diazenyl-8-nitronaphthalen -1-ol (22.606 minute, m/z 217, base peak 154.15)
VIII	 Mol. Wt.: 215.17	2-diazonium-8-nitronaphthoxide (22.820 minute, m/z 215, base peak 154.10)
IX	 Mol. Wt.: 238.16	Sodium 2-diazonium-8-nitronaphthoxide (22.920 minute, m/z 238, base peak 154.15)
X	 Mol. Wt.: 204.18 OR Mol. Wt.: 205.17	8-nitronaphthalene-1,2,-diol (25.486 minute, m/z 206, base peak 170.10)
XI	 Mol. Wt.: 214.15	Sodium-2(2-formyl-2hydroxyvinyl)benzoate (26.744 minute, m/z 214, base peak 91.10)
XII	 $C_{10}H_8O_4S$ Mol. Wt.: 224.23	8-hydroxylnaphthalene-1-sulphonic acid (27.855 minute, m/z 224, base peak 125.10)
XIII	 $C_{10}H_9NO_4S$ Mol. Wt.: 239.25	6-amino, 8-hydroxylnaphthalene-1-sulphonic acid (28.411 minute, m/z 239, base peak 125.10).

4.6 Toxicity Studies of Azo dyes and their Metabolites

Some metabolites of dyes decolourisation are reported to be more toxic than the undegraded dye, it is therefore important to investigate the toxicity of the metabolite. This was done using Bacterial zone of inhibition and phytotoxicity tests.

4.6.1 Bacterial zone of inhibition

Only little zone of inhibition was observed with reactive blue 13 and its metabolite. Interestingly the control (tap water) showed some amount of zone of inhibition. At both concentrations and for both organisms, the dye showed higher but not significant zone of inhibition (Table 4.8).

Table 4.8: Microbial toxicity study of Reactive Blue 13 and its degradation products

	Diameter of zone of inhibition (cm)				
Bacteria	Control (tap water)	Reactive Blue 13 (500 ppm)	Degradtion Product (500ppm)	Reactive Blue 13 (1000 ppm)	Degradtion Product (1000ppm)
Rhodobacter legumes	0.23	0.30	0.13	0.53	0.18
Kocuria rosea	0.15	0.23	0.05	0.38	0.06

4.6.2 Phytotoxicity

Solution of Reactive Blue 13 appears not to affect the % germination, but it showed significant effect on the length of plumule and radicals (Figure 4.15). The germination percentage of both Zea *mays* and *Phageolus vulgaris* seeds were less with RB13 solution wetted seeds when compared to those wetted with the degradation products solution and those of tap water. The RB13 treatments affected the length of plumule and radical

significantly, while there were no significant effects with the degradation products (Table 4.9).

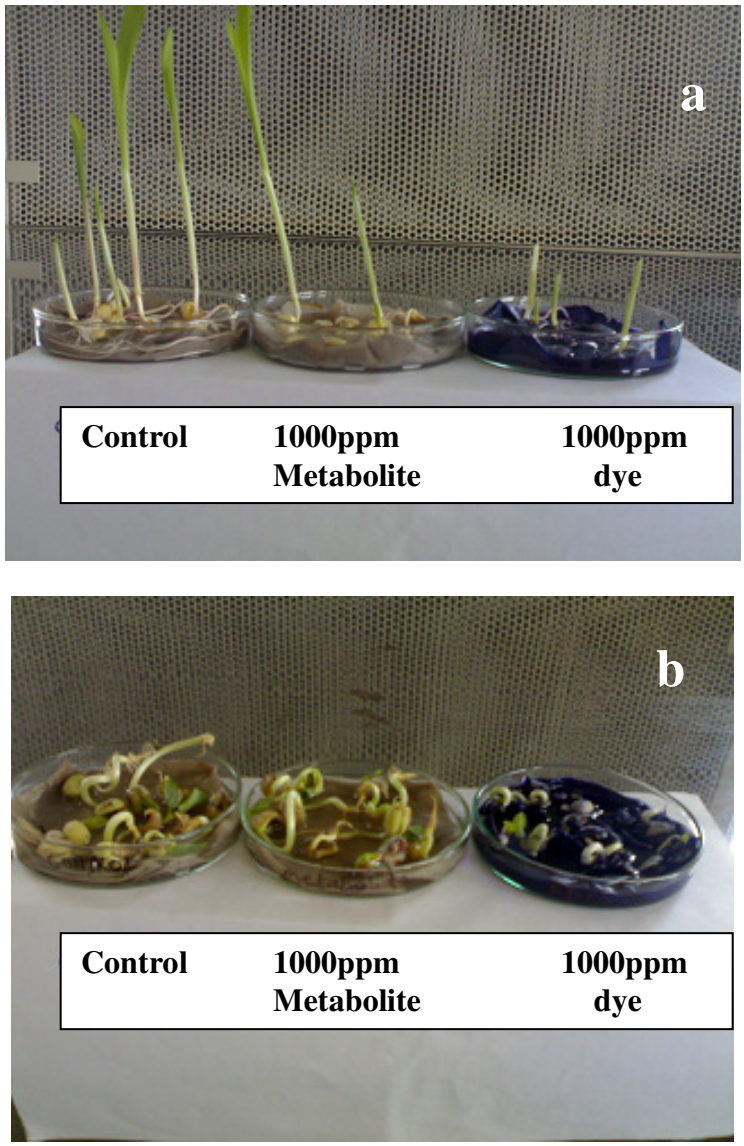


Figure 4.15: Pictures showing effect of RB13 dye and its metabolites on *Zea mays* (a) and *Phaseolus vulgaris* (b)

Table 4.9: Phytotoxicity study of Reactive blue 13 and its degradation products

Parameters	*Zea mays*			*Phaseolus vulgaris*		
	Water	Reactive blue 13 [a]	Extracted metabolite [a]	Water	Reactive Blue13 [a]	Extracted metabolite [a]
Germination (%)	100	70	90	100	90	90
Plumule (cm)	8.15 ± 3.12	3.62 ± 1.25[#]	7.26 ± 2.50*	6.56 ± 0.39	3.30 ± 0.60*	4.96 ± 1.09*
Radicle (cm)	7.45 ± 2.59	2.43 ± 0.68[#]	6.69 ± 1.97*	4.74 ± 0.79	5.70 ± 1.21*	4.92 ± 0.97*

Legend: Values represent mean of 10 seeds ± SEM

[a] Aqueous solution of 1000 ppm concentration.

* Not significantly different from the control (seeds germinated in water) at $P < 0.05$,

[#] Significantly different from the control (seeds germinated in water) at $P < 0.05$, using One-way analysis of variance (ANOVA) with Turkey Kramer comparison test.

4.7 Purification and Partial Characterization of Azo Dyes Degrading Enzyme

4.7.1 Enzyme assay

In control state, the organism showed the presence of both intracellular and extracellular laccase and veratryl alcohol oxidase activities, but only intracellur tyrosinase activity was noticed (Table 4.10). In the presence of reactive blue 13 as inducer, the tyrosinase activity was demonstrated in the extracellular fluid. While a reduction in the activities of the intracellular laccase was noticed (2.50 ± 0.40 to 2.17 ± 0.11) in the presence of the dye, a significant increase in the veratryl alcohol oxidase was recorded (2.19 ± 0.27 to 3.20 ± 0.55). In addition, the organism showed an induced NADPH dependent azoreductase activity.

Table 4.10: Enzyme Activities in control and induced (24 h decolourisation) state

Enzyme Assays		Control[a]	Induced[a]
Azoreductase (NADH)	Extracellular	ND	ND
	Intracellular	ND	ND
Azoreductase (NADPH)	Extracellular	ND	0.05 ± 0.02*
	Intracellular	ND	0.09 ± 0.02*
Laccase	Extracellular	0.07 ± 0.06	0.06 ± 0.03
	Intracellular	2.50 ± 0.40	2.17 ± 0.11
Veratryl Alcohol Oxidase	Extracellular	0.20 ± 0.07	0.10 ± 0.01
	Intracellular	2.19 ± 0.27	3.20 ± 0.55*
Lignin Peroxidase	Extracellular	ND	ND
	Intracellular	ND	ND
Tyrosinase	Extracellular	ND	0.03 ± 0.02*
	Intracellular	0.08 ± 0.05	ND

Legend: Values are mean of triplicate readings ± SEM

* Significantly different from control at $p<0.05$.

[a]Values in change in absorbance units min^{-1} mg $protein^{-1}$.

4.7.2 Time course assay

The result of the time course assays (figure 4.18 and 4.19) showed an apparent highest activity for extracellular laccase at 18 h but at 36 h for the intracellular enzyme. The isolation of the laccase enzymes were subsequently done at 36 h after incubation.

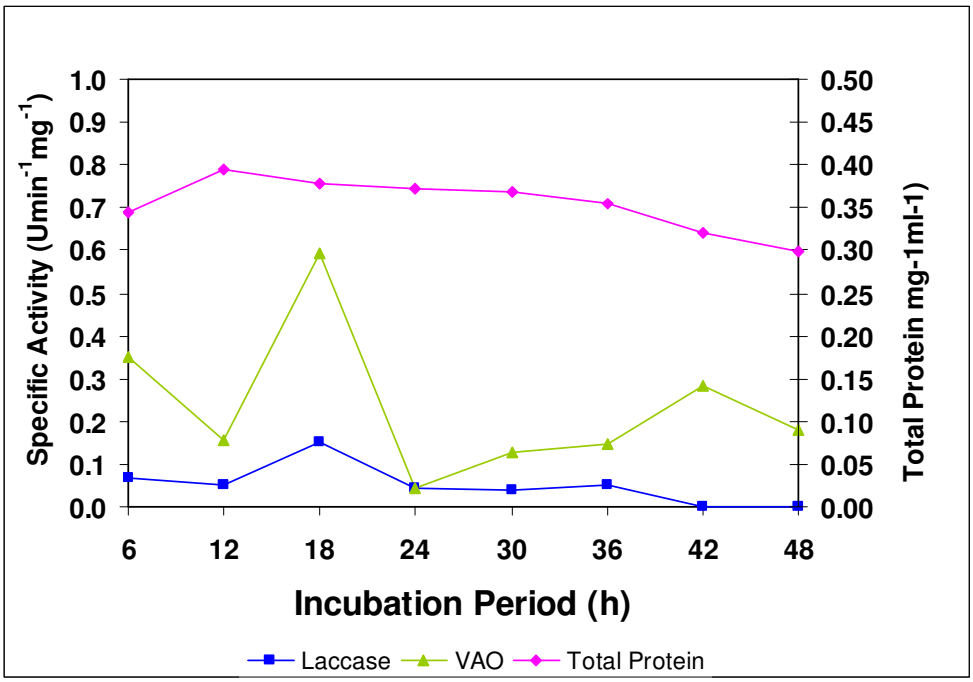


Figure 4.16: Time Course Assay of Extracellular laccase and VAO

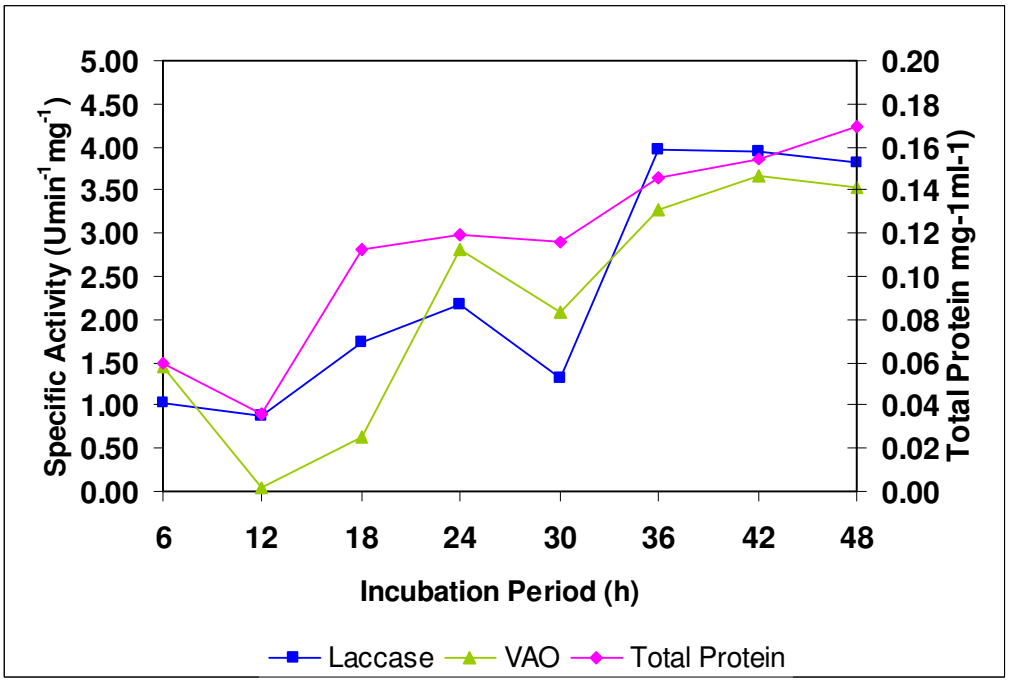


Figure 4.17: Time Course Assay of Intracellular Laccase and VAO

4.7.3 Enzyme purification

A summary of the laccase purification with DEAE-ion exchange and biogel P-100 size exclusion-ion exchange chromatography is shown in Table 4.11. The first purification step, ion exchange increased the specific activity from 0.23 to 0.44 absorbance unit mg^{-1}. A further increase to 0.94 Umg^{-1} was noticed after size exclusion chromatography using Biogel P-100. Similar changes were noticed in fold purification: from 1 to 1.91 to 8.08 respectively.

Unlike the specific activity and purification fold, the % yield dropped from 100 to 38.02 after DEAE and further to 7.50 after Biogel. So did the total protein from 70.40 mg to 14.04 mg and to 1.31 mg respectively.

Table 4.11: Purification table for Laccase using DEAE and Biogel P-100

	Total Protein (mg)	**Total Activity (U)**	**Specific Activity (U/mg protein)**	**Purification Fold**	**% Yield**
Crude (20 ml)	70.40	16.20	0.23	1	100.00
DEAE (18 ml)	14.04	6.16	0.44	1.91	38.02
Biogel (6 ml)	1.31	1.22	0.94	8.08	7.50

The DEAE elution profile for the purification of laccase (figure 4.18) revealed four protein peaks, two of which have prominent laccase activities: fractions 33-40 and fractions 46-48 corresponding to the 0.3 M and 0.4 M NaCl gradients respectively. The maximum activity was however recorded in the 0.3 M fractions. When veratry alcohol oxidase activities were assayed in the same DEAE fractions (figure 4.19), in a similar manner, prominent peaks were obtained at 0.3 M and 0.4 M; but as a kind of reversal the maximum peak was noticed at 0.4 M.

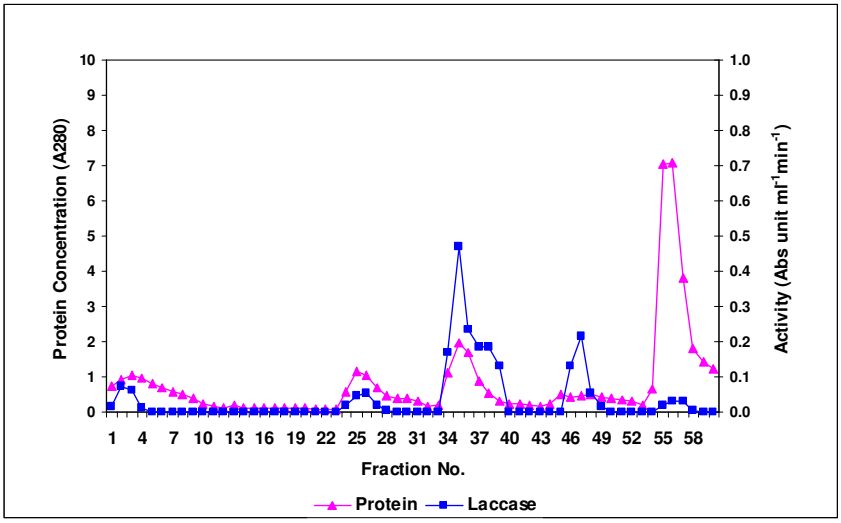


Figure 4.18: DEAE-cellulose ion exchange profile for laccase enzyme. Column dimension 15 X 100 mm; Flow rate, 0.75 ml/min. The Proteins were eluted by gradient addition of NaCl (0-0.5 M) with Sodium phosphate buffer (pH 7.2, 50 mM).

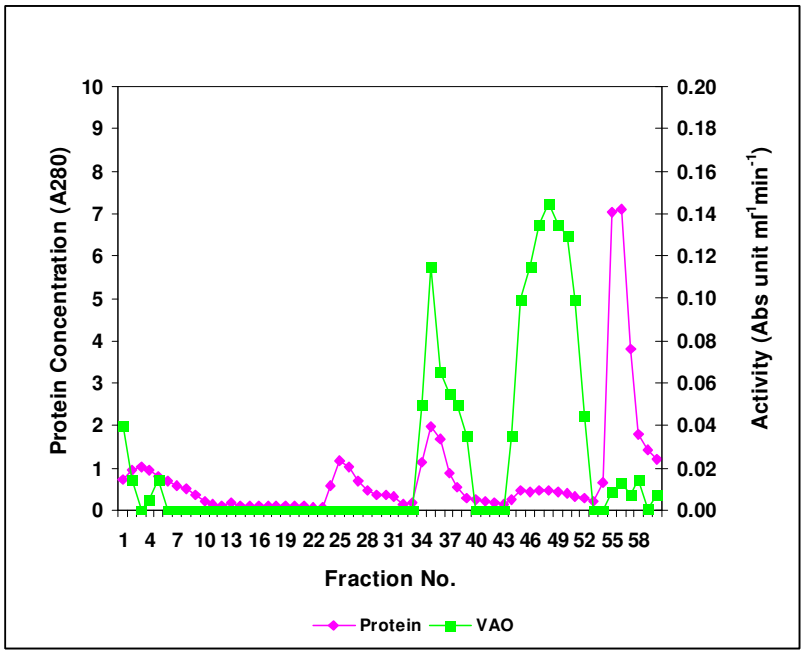


Figure 4.19: DEAE-cellulose ion exchange profile for Veratryl alcohol oxidase. Column dimension 15 X 100 mm; Flow rate 0.75 ml/min. The Proteins were eluted by gradient addition of NaCl (0-0.5 M) in Sodium phosphate buffer (pH 7.2, 50 mM).

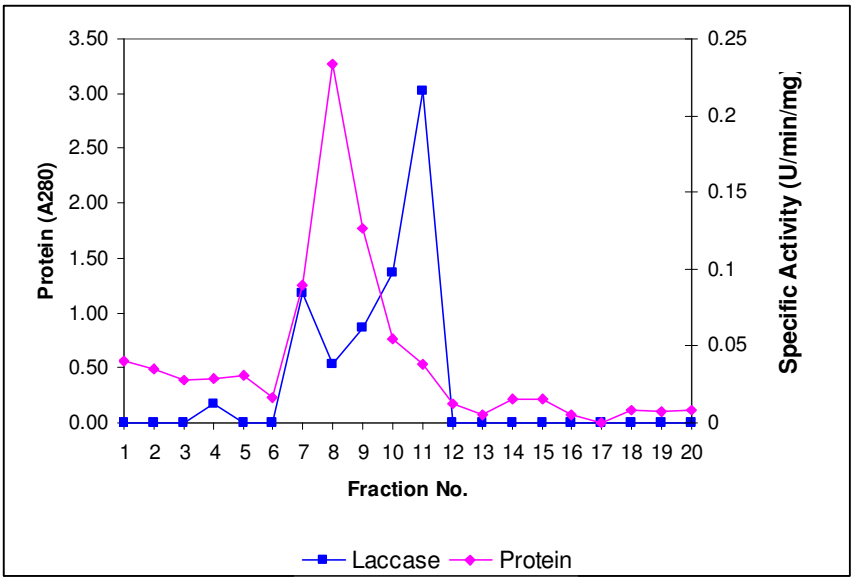


Figure 4.20: Molecular sieve with Biogel P-100 for laccase. Column Dimesion, 100 X 450 mm; flow rate 6 mL/h. The Proteins were eluted with Sodium phosphate buffer (pH 7.2, 50 mM).

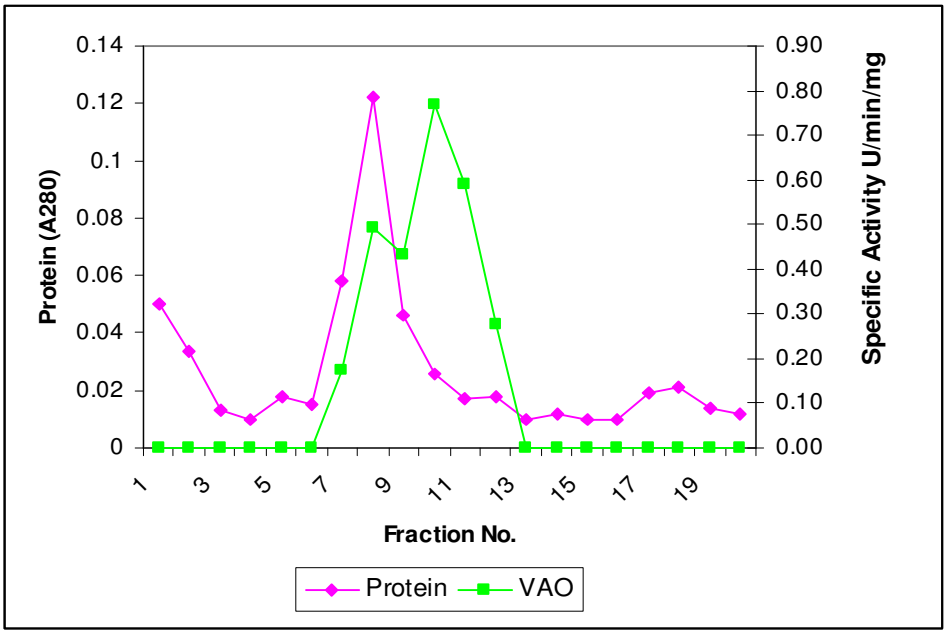


Figure 4.21: Molecular sieve with Biogel P-100 for VAO. Column Dimesion, 100 X 450 mm; flow rate, 6 ml/h. The Proteins were eluted with Sodium phosphate buffer (pH 7.2, 50 mM).

4.7.4 Molecular weight determination

Polyacrylamide gel electrophoresis (PAGE) is one of the most effective methods that are capable of separating molecules on the basis of their physical differences both in molecular size and net charge (Wilson and Walker, 1995). The results obtained showed that the DEAE coupled with biogel size exclusion chromatography step was unable to purify the enzyme. The molecular weight of the protein fraction exhibiting laccase activity was however estimated to be around 60 kDa (figure 4. 22).

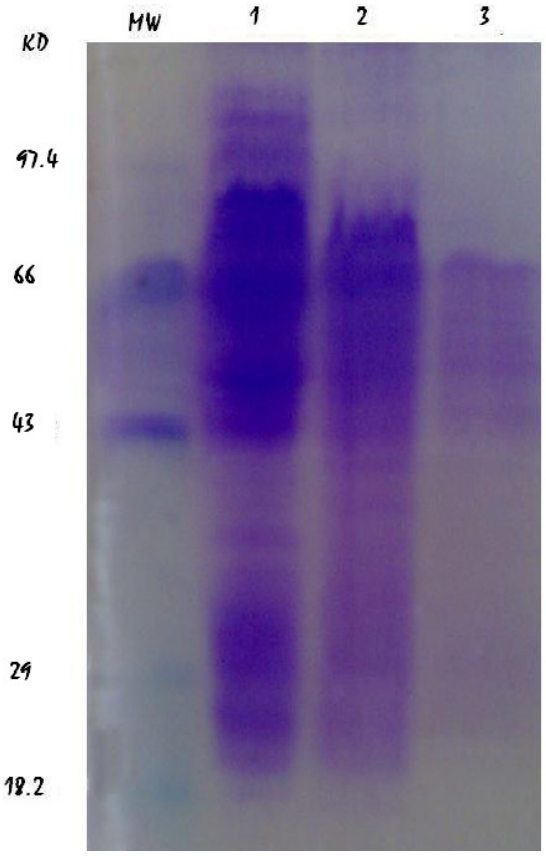


Figure 4.22: 10 % SDS-PAGE analysis of purified laccase. MW: Molecular weight markers. Lane 1: Crude extract. Lane 2 : Concentrated sample after DEAE-cellulose-ionic exchange chromatography. Lane 3: Sample after Biogel P-100 size exclusion chromatography.

Literature also suggests that the molecular weights of laccase are diverse and quite dependent on the particular culture of the bacteria as well as the culturing conditions (Zhang *et al.*, 2006). The laccase purified from this study was derived from a strain of *Proteus mirabilis.*

4.7.5 Determination of optimal pH and temperature

4.7.5.1 pH profile

Since laccase has been found in a wide range of organisms including bacteria, fungi, and algae, and in different environmental conditions, the pH profile was investigated over a range of 1–10, using different buffers coherent with the pH level. An optimum pH of 4 was observed for the laccase in this study (Figure 4.23); this is however different from the optimum pH for growth of the organism (pH 7).

4.7.5.2 Temperature profile

A maximum laccase activity was observed at 40 °C, which is within the growth temperature of mesophilic bacteria. The temperature curve follows a characteristic bell shape in which at lower temperatures, the activity is slower but gradually increases as temperature increases (Figure 4.24). This is typical of enzyme kinetics in which rate of enzymatic reaction increases as temperature increases, this being achieved by increase in the rate at which enzyme and substrate 'collide' or become in contact with one another (Wilson and Walker, 1995).

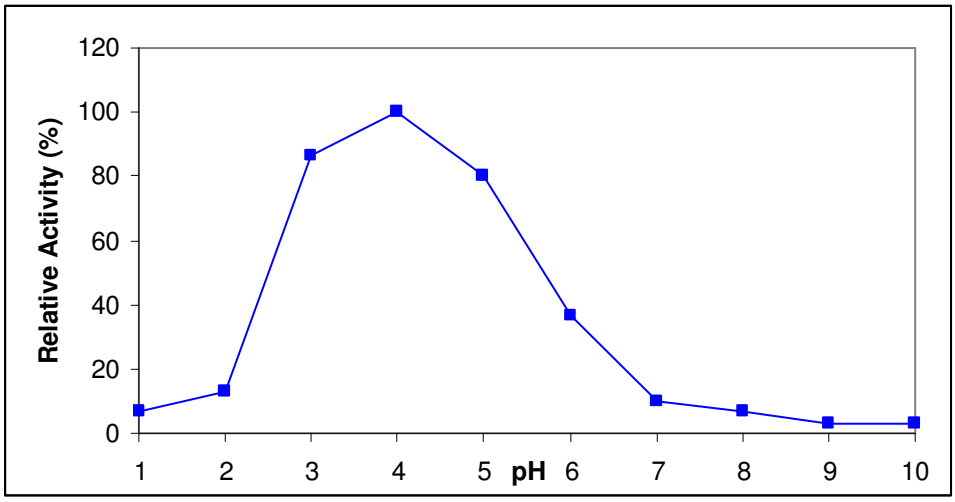


Figure 4.23: pH profile on laccase activity.

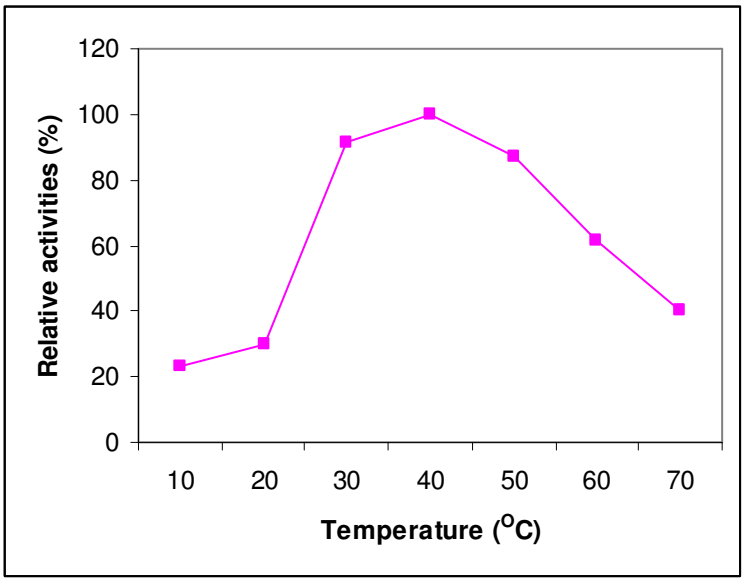


Figure 4.24: Effect of temperature on laccase activity.

4.7.6 Determination of enzyme's V_{max} and K_m

The K_m of the enzyme for 2, 6-dimethoxylphenol, veratryl alcohol and guaiacol were determined using Eadie-Hofstee diagram (appendix IV). The substrate specificity showed highest Vmax to veratryl alcohol followed by 2, 6-dimethoxylphenol, and guaiacol (table 4.12). The enzyme can also catalyzed various laccase substrates like 2,2'-azino-bis(3-ethylthiazoline-6-sulphonic acid) (ABTS), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), and o-tolidine (4,4'-diamino, 3,3'-dimethyl biphenyl).

Table 4.12: Kinetic parameters of laccase purified from *Proteus mirabilis* using selected substrates.

	ε_{max} (M^{-1} cm^{-2})	Wavelegth (nm)	K_m (mmol)	V_{max} (mmol/min)
Veratryl Alcohol	9,300	310	0.0504	0.002
Guaiacol	12,000	465	0.1376	0.0009
DMP	34,645	460	0.1623	0.0003

CHAPTER FIVE

5.0 DISCUSSION

Proteus mirabilis is a gram negative, facultative anaerobic bacterium. Along side, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*, the organism has been reported to degrade organosilicone compounds (Rosciszweewski *et al.* 1998). While the ability of the organism to decolourise azo dyes is scanty (Chen *et al.*, 1999), no report to the best of this researcher's knowledge has been given on its ability to mineralize those dyes. Mohana *et al.* (2007) has also reported a strain of the organism that can decolourise distillery spent waste.

Bacterial Strains Isolation and Decolourisation of dyes

A total of fourteen aerobic bacteria able to decolourise azo dyes with varied efficiency were isolated. One of the isolates identified using 16S rDNA as a strain of *Proteus mirabilis,* showed 96.94 ± 2.10% decolourisation within 24 h under limited oxygen availability and was able to achieve virtually the same level of activities within 6 h under normal supply of oxygen. This showed that the azo dyes were not only decolourised aerobically but that oxygen is required for the activities of the strain. This may be because *Proteus mirabilis* is a facultative anaerobe.

The *Proteus mirabilis* strain showed optimal decolourisation activity between pH 7 and 8. This is not strange to bacteria; a strain of *Pseudomonas* has been reported with optimal pH of 8 (Yu *et al*, 2001). High decolourisation (>80%) of the dyes at the ambient environmental temperature, 30^{o}C, indicates that the organism may reduce the cost of biotreatment by eliminating any associated cost with temperature regulation. The findings also point to the

influence of nutrient composition on decolourisation. This is in agreement with Hennery and Girbic-Garlic (1999) who reported that choice of mineral media affects the oxidation of certain pollutants.

Kinetic Studies of Decolourisation

In a previous study, Yu *et al*. (2001) showed that the decolourisation rate of certain *Pseudomonas* strains were first order with respect to cell mass and half order to dye concentration. This compares effectively with the findings of this research with rate order values of 1.0977 and 0.5498 for cell mass concentration and dye concentrations, respectively. It was also observed that the dependence of specific decolourisation rate on dye concentration could be interpreted by Lineweaver-Burk model.

$$\frac{1}{V_{dye}} = \frac{k_m}{V_{dye}} \times \frac{1}{[RB13]} + \frac{1}{V_{dye}} \quad \text{.....5.1}$$

The high inhibition concentration of about 400 mg/L indicated that the organism has a potential for practical application in the decolourisation of textile effluent, since most effluent have been reported to have dye concentration of about 60 mg/L (Do *et al*., 2002). The Michaelis constant (K_m) of 278.83 mgl^{-1}, maximum specific decolourisation rate of 36.23 $mgL^{-1}h^{-1}$ and the inhibitory concentration of 400 mg/L, showed that the organism has good toxic tolerance for the azo dye. Much higher specific decolourisation rate of 81.2 $mgL^{-1}h^{-1}$ and the inhibitory concentration of >1000 mg/L have been reported for azo dye 4BS (He *et al., 2004*), immobilized microbial consortium was however used, and not a single strain.

Biodegradation of Reactive Azo Dyes

The observed disappearance of the peaks in the visible region of the UV-visible spectra for both the reactive dyes and methyl red showed that the dyes were actually decolourised. The initial increase in the absorbance value of the near-UV peak of the methyl red between 0 h and 3 h followed by a decrease at 6 h suggests that the azo dyes were converted to aromatic amine(s) prior to mineralization. The UV-visible spectrum of aniline sulphate agreed with the report of Van der Zee & Villaverde (2005), that aromatic amines absorb around 260-300 nm. The presence of similar peaks in the spectra of the metabolites of the reactive dyes and methyl red further substantiated the formation of aromatic amines as products of the decolourisation.

The HPLC analysis of dye sample collected at the beginning showed one major peak at retention time 13.40 minutes. The change in the retention time of metabolite to 5.42 minutes and the formation of minor peaks are indicators of biodegradation. In a similar way, the FTIR of the reactive blue 13 showed many peaks most of which were removed in that of the metabolite. The peaks at 617, 704 and 834 cm^{-1} indicated the presence of aromatic C–H bending, while the stretching was reported at 2922 cm^{-1}. Stretching vibration of S=O was noticed as a sharp peak at 1047 cm^{-1}. The –N=N– was expected between 1500 and 1650. Several peaks around this region suggested the presence of different groups with –N=N– bond. The peaks at 3466 cm^{-1} were those of the stretching of multiple aromatic amines (Chowdhury *et al.*, 1998). The disappearance of the peak at 834 cm^{-1} and the distortion of those at 617, 704 cm^{-1} suggested removal of the aromatic

rings of the dyes. Similar changes in the peaks around 1500 and 1650 support earlier observations that the azo bond is reduced by the strain. Jadhav *et al*., (2007) have reported a similar peak removal as an indication of biodegradation.

The GCMS analysis revealed the presence of several peaks with molecular weight lower than that of the dye; indicating that the dye was degraded. The GCMS revealed among other peaks, the presence of Sodium 2(2-formyl-2hydroxyvinyl) benzoate, with a tropylium cation (m/z 91) as its base peak; tropylium cation is a major peak of substituted benzene fragmentation during mass spectrometry (Brown, 1995), and this suggested the opening of the naphthalene rings of RB13 by the organism.

Based on the GCMS findings coupled with the presence of laccase and veratryl alcohol oxidase, a pathway for the degradation of reactive blue 13 by the strain of *Proteus mirabilis* was proposed (figure 5.1). The presence of laccase suggested a two step oxidation of the dye in the presence of Cu^{2+}. The presence of veratryl alcohol oxidase is evidence that the products were not polymerized by the laccase, so that products with high molecular weight were not noticed in the GCMS results.

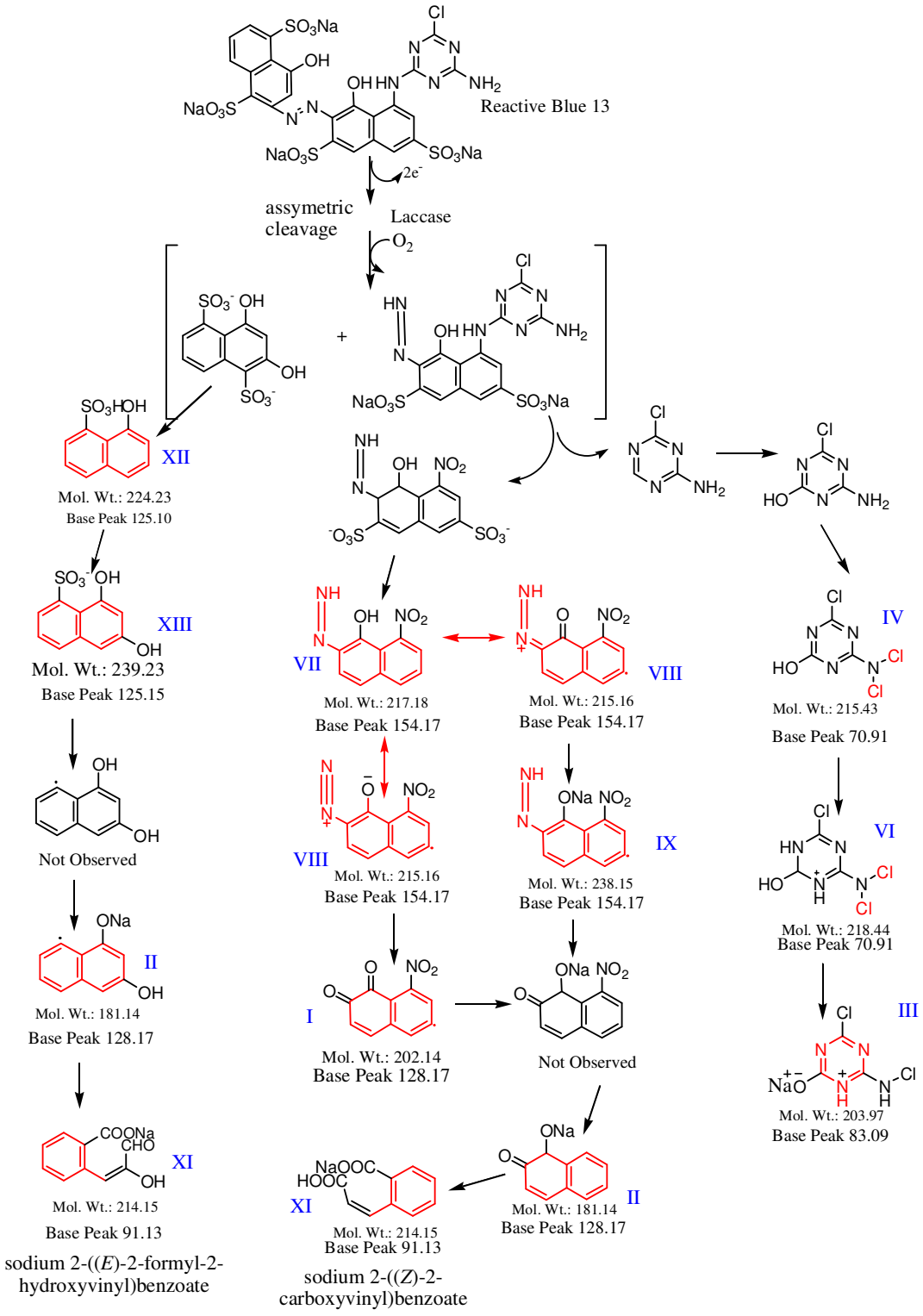


Figure 5.1: Proposed pathway for the biodegradation of Reactive Blue 13 by *Proteus mirabilis*; numbering are peak numbers as eluted by the GCMS.

Toxicity Studies of Azo dyes and their Metabolites

The reduction in the radii of the zone of inhibition of *Rhizobium rhodobacter* and *Kocuria rosea* when the matabolite was used (compared to the dye) showed that the product of the biodegradation were less toxic. The non inhibition of germination with viable plumule and radicle formation in the toxicity experiments of reactive blue 13 (at 1000 ppm) suggested the dyes were not only decolourised but also detoxified. This may be due to removal of aromatic amine by the organism.

Enzyme assay

The result of the enzyme assays showed that a consortium of enzymes was involved in the biodegradation. Laccase appeared to be the prominent enzyme. The presence of an induced intracellular NADPH-dependent azoreductase suggested desulphonation of the azo dye RB13 prior to transport across the cell membrane. Since sulphonated compounds cannot cross the membrane (Russ *et al*., 2000). NADPH-dependent azoreductase has been reported in *Stahphylococcus aureus* (Chen *et al.*, 2005); and aerobic azoreductase are known to require NADPH instead of NADH (Husain, 2006). The azoreductase properly contributed just little to the decolourisation when the activities of laccase and veratryl alcohol oxidase were put into consideration. The presence of laccase and veratryl alcohol oxidase activities as both extracellular and intracellular fluid suggested a non-specific reduction of azo bond as shown in the proposed pathway **(Figure 5.1).** This is substantiated by the decolourisation of dyes, when dyes were added to cell free culture (supernatant) of the bacterium. The possibility of more than one mechanisms of azo bonds reduction in this bacterium is then not overruled. Laccase is known to attack

aromatic rings via two steps electron transfer in the presence of molecular oxygen (Bourbonnais *et al.*, 1996), while veratryl alcohol has been reported to prevent polymerization of reactive rings produced by laccase, thereby, making laccase products susceptible to ring cleavage (Marzullo *et al.*, 1995).

Enzyme purification and characterization

A salient observation in this phase of the research is that both the laccase fraction of the biogel and the veratryl alcohol oxidase fraction seem to have similar catalytic activities. This is probably because the two enzymes catalyse molecules that are similar in structures.

The result of the SDS-PAGE showed that the use of DEAE-cellulose and biogel was not good enough for the purification of this particular laccase, since several attempts with the protocol did not give a single band. Mishra and Bisaria (2006) first used ultrafitration before using DEAE-Sepharose and sephacryl S-200. However, the result showed that the molecular weight of the enzyme was between 43 and 65 kDa. Previous studies on laccase have shown that the enzyme is a protein with molecular mass of between 55 and 65 kDa, few exceptions with molecular masses ranging between 60 and 80 kDa have also been reported (Kiiskinen *et al.*, 2002, Telke *et al.*, 2009).

The optimal enzymatic activity was found at pH 4.0 for o-tolidine. Previous studies on laccase characterization have reported optimal pH of between 3.0 and 5.0 for the enzyme (Chen *et al.*, 2005; Molina-Guijarro *et al.*, 2009). It can therefore, be deduced that laccase

enzymes function optimally in an acidic environment, since it has been postulated that pH dependence of enzymatic reactions is the consequence of the changing degrees of ionization of functional groups in the enzyme as well as the substrate (Wilson and Walker, 1995). Laccases usually have optimal temperatures for activity between 30 and 60°C; the optimal temperature for activity of the laccase produced from this strain of *Proteus mirabilis* was 40°C. The difference between the optimal pH for the growth of the organism and that of the partially purified laccase suggested that the use of the enzyme may require acidification of the effluent.

The laccase activity of the partially purified enzyme compared fairly with those of other laccase isolated from bacteria. The maximum activity (V_{max}) of 2.0 mmol for veratyl alcohol, 0.9 mM for guaicol and 0.3 mM for dimethoxy phenol, is similar to those obtained for a salt resistance laccase isolated from *Streptomyces ipomea* (Molina-Guijarro *et al*., 2009). Michaelis constant (K_m) values of 0.0504, 0.1376, and 0.1623 mM were recorded for the same substrates respectively. The lowest K_m for veratryl alcohol suggested that the enzyme has high affinity for the substrate. It is important to note that laccase with better affinity have been reportedly isolated from fungi (Mishra and Bisaria, 2006). The relatively higher K_m, suggested that the laccase isolated from this organism will be able to catalyze a broad range of substrates and may be useful as a catalyst for treatment of textile effluent.

Summary of Findings

This research work investigated possible decolourisation and biodegradation of azo dyes by a newly isolated aerobic bacterium, *Proteus mirabilis*. The probable pathway for the biodegradation of a referenced textile reactive azo dye (reactive blue 13) was elucidated. The mechanism of decolourisation and biodegradation was studied using the GCMS and enzyme assays; attempt was also made to purify laccase, the prominent enzyme. The findings are summarized below.

(i) Implementing the first objective of the research, fourteen bacterial strains able to decolourised azo dyes were isolated from various environmental sources. Three strains showed >95 % decolourisation of the reactive dyes solution, within 24 h. One of these bacteria that effectively decolourised the azo dyes was further identified using 16S rDNA as a strain of *Proteus mirabilis*.

(ii) The effects of incubation temperature, initial pH, shaking condition (agitation) and media preference on decolourisation efficiency of the *Proteus mirabilis* showed optimal pH and temperature values of about 7 and 37^{o}C respectively. The strain preferred nutrient broth to minimal media; and aeriation as against agitation increased the rate of decolourisation by up to four-fold.

(iii) The third objective of the research verified the mineralization of the dyes, as against ordinary decolourisation. The result showed that the model dye, reactive blue 13, was degraded to several metabolites of lower molecular weights than the

dye. One of the metabolites, 2(2-formyl-2 hydroxyvinyl) benzoate, showed that the naphthalene ring of the dye was cleaved. A degradative pathway was then proposed using the identified metabolites.

(iii) Investigating the mechanism of decolourisation cum biodegradation, enzymes that are of decolourisation and biodegradation importance were assayed (fourth objective). The results showed the presence of NADPH-dependent azoreductase and laccase activities. This suggested enzymatic reduction of azo bonds prior to mineralization and that there is possibility of more than one mechanisms of azo bonds reduction in this bacterium. Partial characterization of the laccase revealed that it is a protein with molecular weight of about 60 kDa. It has an optimum temperature of about 50^{o}C and it acts best between pH 4 and 5. The enzyme showed highest affinity for veratryl alcohol when compared with guaiacol and dimethoxyphenol.

Contributions to Knowledge

(i) Isolation of an indigenous strain of *Proteus mirabilis* that can both decolourise and degrade textile reactive azo dyes.

(ii) Proposition of a pathway for the biodegradation of reactive blue 13.

(iii) Discovery of more than one azo dye decolourisation enzyme systems (laccase enzyme and NADPH dependent azoreductase) in a single bacterial strain.

REFERENCES

Abadulla, E., Tzanof, T., Costa, S., Robra, K.H., Cavaco-Paulo, A. and Gubitz G.M (2000). Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Appl. Environ. Microbiol.,* 66(8): 3357-3362.

Abraham, J.K. and John, G.H. (2007). Development of a classification scheme using a secondary and tertiaary amino acids analysis of azoreductase gene.*J. Med. Biol. Sci.*1:1-5.

Ademoroti, C.M.A. (1996). Standard methods for water and effluent analysis. Foludex Press, Ibadan.

Adosinda, M., Martins, M., Lima, N., Silvestre, A. and Queiroz, M. (2003). Comparative studies of fungal degradation of single or mixed bioaccessible reactive azo dyes. *Chemosphere*, 52, 967.

Aguilar, M. I., Sáez, J., Lloréns, M., Soler, A., Ortuño, J. F., Meseguer, V. and Fuentes A. (2005). Improvement of coagulation–flocculation process using anionic polyacrylamide as coagulant aid. *Chemosphere,* 58:47-56.

Ahmed, M.N. and Ram, R.N. (1992). Removal of basic dye from waste-water using silica as adsorbent. *Environ. pollution*, 77: 79-86.

Ali, N. (2006). Biotechnological approaches for the treatment of textile dyes by indigenous organisms. PhD Thesis. Quaid-i-Azam University, Islamabad, Pakistan.

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

Al-Mutairi, N. Z., Hamoda, M. F. and Al-Ghusain, I. (2004). Coagulant selection and sludge conditioning in a slaughterhouse wastewater treatment plant. *Bioresour. Technol.,* 95:115-119.

Altinbas, U., Dokmeci, S. and Baristiran, A. (1995). Treatability study of wastewater from textile industry. *Environ. Technol.*, 16: 389-394.

Arias, M.E., Arenas, M., Rodriguez, J., Soliveri, J., Ball, A.S., and Hernandez, M. (2003). Kraft pulp biobleaching and mediated oxidation of a non-phenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Appl. Environ. Microbial.,* 69: 1953-1958.

Atkins, W.S. (2000). Assessment of the risks to human health posed by certain chemicals in textiles. Final report, 17th CSTEE Plenary meeting, Brussels, September 5, 2000.

Babuna, F. G, Orhon, D., Ubay Çokgör, E., Insel, G. and Yaprakli, B. (1998). Modelling of activated sludge for textile wastewaters. *Water Sci. Technol.*, 38 (4): 9–17.

Baiocchi, C., Brussino, M. C., Pramauro, E., Prevot, A. B., Palmisano, L. and Marci, G. (2002). Characterization of methyl orange and its photocatalytic degradation products by HPLC/UV-VIS diode array and atmospheric pressure ionization quadrupole ion trap mass spectrometry. *Intl. J. Mass Spectro.*, 214: 247-256.

Baldrian, P. (2006). Fungal laccases – occurrence and properties. *FEMS Microbiol. Rev.,* 30: 215-242.

Banat, I.M., Nigam, P., Singh, D. and Marchant, R. (1996). Microbial decolorization of textiledye-containing effluents: a review. *Biores. Technol.*, 58: 217-227.

Barrow, G. I. and Feltham, R.K.A. (1993). Cowan and Steel's Manual for the identification of Medical Bacteria (3rd Ed.). Cambridge University Press.

Baughman, G. L. and Weber, E. J. (1994). Transformation of dyes and related compounds in anoxic sediment: Kinetics and products. *Environ. Sci. Technol.,* 28: 267-276.

Boer, C.G., Obici, L., de Souza, C.G.M. and Peralta, R.M. (2004). Decolorisation of Synthetic dyes by solid-state cultures of *Lentinula (Lentinus) edodes* producing manganese peroxidase as the main ligninolytic enzyme. *Biores. Technol*., 94: 107-112.

Bogan, B.W. and Lamar, R.T. (1995). One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.,* 61: 2631-2635.

Bourbonnais, R., Paice, M.G., Freiermuth, B., Bodie, E. and Bornemann, S. (1996). Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.*, 63: 4627-4632.

Boussahel R., Irinislimane H., Harik D., and Moussaoui K. M. (2009). Adsorption, Kinetics, and Equilibrium Studies on Removal of 4,4-DDT from Aqueous Solutions Using Low-Cost Adsorbents. *Chem. Eng. Comm.*, 196:1547–1558.

Brown, M.A. and DeVito, S.C. (1993). Predicting azo dye toxicity. *Crit. Rev. Env. Sci. Tec.*, 23: 249-324.

Brown, W.H. (1995). Organic Chemistry. Saunders College Publishing, Orlando, Florida pp568-569.

Bumpus, J.A, and Brock, B.J (1988). Biodegradation of crystal violet by white rot fungus *Phanerochaete chysosporium*. *Appl. Environ. Microbiol.*, 54:1143-1150.

Camel, V. and Bermond, A. (1998). The Use of Ozone and Associated Oxidation Processes in Drinking Water Treatment. *Water Res.,* 32: 3208-3222.

Catalkaya, E., Bali, U. and Sengul, F. (2003). Photochemical Degradation and Mineralization of 4-chlorophenol. *Env. Sci. Pollut. Res.,* 10 (2): 113-118.

Call, H. P. and Mucke, I. (1997). History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems. *J. Biotechnol.,* 53:163-202.

Cartwright, R.A. (1983). Historical and modern epidemiological studies on populations exposed to N-substituted aryl compounds. *Environ. Health Persp.*, 1: 4913-9.

Cerón-Rivera, M., Davila-Jimenez, M. M. and Elizalde-Gonzalez, M. P. (2004). Degradation of the textile dyes Basic yellow 28 and Reactive black 5 using diamond and metal alloys electrodes. *Chemosphere,* 55:1-10.

Cestari, A. R., Vieira, F. S. E., dos Santos A. G. P., Mota, J. A. and de Almeida, V. P. (2005). Adsorption of anionic dyes on chitosan beads: The influence of the chemical structures of dyes and temperature on the adsorption kinetics. *J. Coll. Interf. Sci.,* 280:380-386.

Chen, G., and White, P.A. (2004). The mutagenic hazards of aquatic sediments: a review. *Mutat. Res.*, 567: 151-225.

Chen, H, Hopper, S. L. and Cerniglia, C. E. (2005). Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. *Microbiology,* 151: 1433-1441.

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

Chen, J., Rulkens, W. H. and Bruning, H. (1997). Photochemical Elimination of Phenols and COD in Industrial Wastewaters. *Wat. Sci. Tech.*, 35 (4): 231-238.

Chen, K.C., Huang, W.T., Wu, J.Y. and Haung J.Y. (1999). Microbial decolourisation of azo dyes by *Proteus mirabilis*. *J. Indus. Microbiol. Biotechol.*, 22: 686-690.

Chivukula, M. and Renganathan, V. (1995). Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. *Appl. Environ. Microbiol.,* 61 (12): 4374 – 4377.

Chowdhury, P. K., Sugawara, K., Nakanaga, T. and Takeo, H., (1998). NH stretching bands of the hydrogen-bonded $C_6H_5NH_2$--$N(C_2H_5)_3$ cluster *J. Mol. Struct.*, 447: 7-12

Chung, K.T. and Cerniglia, C.E. (1992). Mutagenicity of azo dyes: Structure-activity relationships. *Mut. Res.*, 277: 201-220.

Chung, K.T. and Stevens, S.E.J. (1993). Degradation of azo dyes by environmental microorganisms and helminthes. *Environ. Toxico. Chem.*, 12: 2121–2132.

Cooper, P. (1993). Removing colour from dyehouse waste waters - a critical review of technology available. *J. Soc. Dyers Col.*, 109: 97-100.

Cripps, C., Bumpus, J. and Aust, S. (1990). Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol*., 56: 1114-1118.

Dantán-González, E., Vite-Vallejo, O., Martínez-Anaya, C., Méndez-Sánchez, M., González, M.C., Palomares, L.A. and Folch-Mallol, J. (2008). Production of two novel laccase isoforms by a thermotolerant strain of *Pycnoporus sanguineus* isolated from an oil-polluted tropical habitat. *Int. Microbiol.,* 11:163-169.

Davies, S. and Burns, R.G. (1990). Decolourisation of phenolic effluent. *Biotech.*, 32 (6): 721-726.

Dennis, L.H. (2001). Green chemistry and the global water crisis. *Pure Appl. Chem.,* 73 (8): 1237–1241.

Dittmer, N.T., Suderman, R.J., Jiang, H., Zhu, Y.C., Gorman, M.J., Kramer, K.J. and Kanost, M.R. (2004). Characterization of cDNAs encoding putative laccase-like

multicopper oxidases and developmental expression in the tobacco hornworm, Manduca sexta, and the malaria mosquito, *Anopheles gambiae. Insect Biochem. Mol. Biol.*, 34: 29 - 41.

Do, J.S. and Chen, M.L. (1994). Decolorization of dye-containing solutions by electrocoagulation. *J. Appl. Electrochem.*, 24: 785-790.

Do, T., Shen, J., Carwood, G. and Jenkins, R., (2002). Biotreatment of textile effluent using *Pseudomonas* spp. Imobilised on polymer support, in *Advanced Biotechnology for Textile processes*. The University of Georgia.

Donelly, K.C., Brown K.W., Anderson C.S., Thomas C.J. and Scott B.R. (1991). Bacterial mutagenity and acute toxicity of solvents and aqueous extracts of soil samples from a chemical manufacturing site. *Toxicol. Chem.*, 10: 1123-1131.

Dong, J., Kaufmann, R.K., Myneni, R.B., Tucker, C.J., Kauppi, P.E., Liski, J., Buermann, W., Alexeyev, V. and Hughes, M.K. (2003). Remote sensing estimates of boreal and temperate forest woody biomass: carbon pools, sources and sinks. *Remote Sensing Environ.*, 84: 393-410.

Dos Santos, A.B., Traverse, J., Carvantes, F.J. and Van Lier, J.B. (2005). Enhancing the electron transfer capacity and subsequent color removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators. *Biotech. Bioeng.,* 89(1): 42-52

Eremektar, G., Selcuk, H. and Meric, S. (2007). Investigation of the relationship between COD fractions and the toxicity in a textile finishing industry wastewater: effect of preozidation. *Desalination*, 211: 314-320.

Erickson, R. J. and McKim, J. M. (1990). A simple flow-limited model for exchange of organic chemicals at fish gills. *Environ. Toxicol. Chem.,* 9: 159-165.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* 39: 783-791.

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

Galindo, C., Jacques, P. and Kalt, A. (2001). Photochemical and Photocatalytic Degradation of an Indigoid Dye: a Case Study of Acid Blue 74 (AB74). *J. Photochem. Photobiol. A*. 141 (1): 47-56.

Gogate, P. R. and Pandit, A. B. (2004). A review of imperative technologies for wastewater treatment I: oxidation technologies at ambient conditions. *Adv. Environ. Res.,* 8: 501-551.

Golob, V., Vinder, A., and Simoniã, M. (2005). Efficiency of the coagulation/flocculation method for the treatment of dyebath effluents. *Dyes and Pigments.* 67: 93-97.

Gonçalves, M.S.T., Oliveira Campos, A.M.F., Pinto, E.M.M.S., Plasencia, P.M.S. and Queiroz Maria, J.R.P. (1999). Photochemical treatment of solutions of azo dyes containing TiO2. *Chemosphere*, 39: 781-786.

Goncalves, I.M.C., Gomes, A., Bras, R., Ferra, M.I.A., Amorin, M.T.P. and Porter, R.S. (2000). Biological treatment of effluent containing textile dyes. *J. Soc. Dyers. Color.,* 116: 393–397.

Greene, J.C. and Baughman, G.l. (1996). Effects of 46 dyes on population growth of freshwater green alga *Selenastrum capricornutum. Text. Chem. Color.*, 28: 23-30.

Hai F.I., Yamamoto K. and Fukushi K. (2006). Development of a submerged membrane fungi reactor for textile wastewater treatment. *Desalination* 192: 315 – 322.

Hammel, K.E., Gai, W.Z., Green, B., and Moen, M.A. (1992). Oxidative degradation of phenethrene by the ligninolytic fungus *Phanerochaete chysosporium. Appl. Environ. Microbiol.* 58: 1832-1838.

Hao, O.J., Kim H. and Chang, P.C. (2000). Decolorization of wastewater. *Crit. Rev. Env. Sci. Tec.*, 30: 449-505.

Haug, W., Schmidt, A., Nortemann, B., Hempel, D.C., Stolz, A. and Knackmuss, H.J. (1991). Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6 aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.*, 57: 3144-3149.

Have R. T., Hartmans S., Teunissen P.J.M., Field Y. A. (1997). Purification and characterisation of two peroxidase isozymes produced by *Bjerkandera* sp. Strain BOS55. *FEBS Letters* 422: 391 – 394.

He, F., Hu, W., and Li, Y. (2004). Biodegradation mechanisms and kinetics of azo dye 4Bs by microbial consurtium. *Chemosphere,* 57: 293-301.

Hennery, S. and Girbic-Garlic., D (1999). Effect of mineral media on trichloroethylene oxidation by aquifer methanothrophs. *Micro. Ecol.,* 20: 151-169.

Hsueh, C. L., Huang ,Y. H., Wang, C. C. and Chen, C. Y. (2005). Degradation of azo dyes using low iron concentration of Fenton and Fenton-like system. *Chemosphere*, 58: 1409-1414.

Hu, T. L. (2001). Kinetics of azoreductase and assessment of toxicity of metabolic products from azo dyes by *Pseudomonas luteola. Water Sci. Technol.*, 43: 261–269.

Hu, T.L and Wu, S. C. (2001). Assessment of the effect of azo dye RP_2B on the growth of a nitrogen fixing cyanobacterium – *Anabaena* sp. *Biores. Technol.*, 77(1): 93-95.

Hublik, G. and Schinner, F. (2000). Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. *Enz. Microb. Technol.*, 27:330-336.

Husain, Q. (2006). Potential Applications of the Oxidoreductive Enzymes in the Decolorization and Detoxification of Textile and Other Synthetic Dyes from Polluted Water: A Review. *Crit. Rev. Biotech.* 26: 201-221.

Isik, M. and Sponza, D. (2004). Monitoring of toxicity and intermediates of C.I. Direct Black 38 azo dye through decolorization in an anaerobic/aerobic sequential reactor system. *J. Hazard. Mater.*,114: 29-39.

Jadhav, J.P., Parshetti, G.K., Kalme, S.D. and Govindwar, S.P (2007). Decolourisation of azo dye methyl red by *Saccharomyces cerevisiae* MTCC 463. *Chemosphere*, 68: 394-400.

Jadhav, U. U, Dawkar, V. V, Tamboli, D. P and Govindwar, S. P (2009). Purification and Characterization of Veratryl Alcohol Oxidase From *Comamonas* Sp. UVS And Its Role In Decolorization of Textile Dyes . *Biotech. Biopros. Eng.*, 14 (3): 369-376.

Jinqi, I. and Houtian, O. (1992). Degradation of azo dyes by algae. *Environ Pollut.* 75: 273-278.

Jocic, D., Vílchez, S., Topalovic, T., Navarro, A., Jovancic, P., Julià, M. R. and Erra, P. (2004). Chitosan/acid dye interactions in wool dyeing system. *Carbohydrate Polymers*. 60:51-59.

Joshi, S, Nemawewarkar, S, and Birla, S. (2001). Static bioassay and toxicity of azo dye stuff to the air breathing fish *Heteropneustes fossilis* and its possible rectification. *Asian J. Chem.,* 12: 439-43.

Jung, R., Steinle, D. and Anliker, R. (1992). A compilation of genotoxicity and carcinogenicity data on aromatic aminosulphonic acids. *Food Chem. Toxicol.*, 30: 635-660.

Kapdan, I. K. and Kargi, F. (2002). Biological decolorization of textile dyestuff containing wastewater by *Coriolus versicolor* in a rotating biological contactor. *Enz. Microb. Technol.*, 30:195-199.

Karam, J. and Necell, J.A. (2000). Potential applications of enzymes in waste treatment. *J. Chem. Technol. Biotechnol*., 69: 141-147.

Khan, A.A., and Husain, Q. (2007). Potential of plant polyphenol oxidases in the decolorization and removal of textile and non-textile dyes *J. Environ. Sci.,* 19 (4): 396-402

Khehra, M.S., Saini, H.S., Shamar, D.K., Chadha, B.S., and Chimni, S.S. (2006). Decolourisation of various azo dyes by bacterial consortium. *Dyes and Pigments* 67: 55-61.

Kiiskinen L.L., Viikari L. and Kruus K. (2002). Purification and characterisation of a novel laccase from the ascomycete Melanocarpus albomyces. *Appl. Microbiol. Biotechnol*. 59:198–204

Koyuncu, I., Topacik, D. and Yuksel, E. (2004). Reuse of reactive dyehouse wastewater by nanofiltration: process water quality and economical implications. *Sep. Pur. Technol.,* 36:77-85.

Krull, R. and Hempel, D. C. (2001). Treatment of dyehouse liquors in a biological sequencing batch reactor with recursive chemical oxidation. *Water Sci. Technol.,* 44:85-92.

Kudlich, M., Bishop, P., Knackmuss, H-J., and Stolz, A. (1996). Synchronous anaerobic and aerobic degradation of the sulfonated azo dye Mordant Yellow 3 by immobilized cells from a naphthalenesulfonate- degrading mixed culture. *Appl. Microbiol. Biotechnol.,* l46: 597–603.

Lambert, S.D., Graham, N.J.D., Sollars, C.J. and Fowler, G.D. (1997). Evaluation of inorganic adsorbents for the removal of problematic textile dyes and pesticides. *Water Sci. Technol.*, 36:173-180.

Lillie, R. D. (1969). *Conn's Biological Stains.* Williams & Wilkins, Baltimore, MD., U.S.A also available at http://stainsfile.info/StainsFile/dyes/class/clsarylm.htm

Lima, S.A.C., Filomena, M, Raposo, J, Castro, P.M.L and Morais, R.M. (2004). Biodegradation of chlorophenol by a microalgae consortium. *Water Res.* 38: 97-102.

Liu, R., Chiu, H.M., Shiau, C-S., Yeh, R. Y-L., and Hung, Y.T., (2005). Degradation and sludge production of textile dyes by Fenton and photo-Fenton processes. *Dyes and Pigments*, 1 – 6.

Lu L., Zhao M., Zhang B.B., Yu S.Y., Bian X.J., Wang W. and Wang Y. (2007). Purification and characterization of Laccase from Pycnoporus sanguineus and decolourization of an anthraquinone dye by the enzyme. *Appl. Microbiol. Biotechnol.,* 74:1232-1239.

Luther, M. and Soeder, C.J. (1991). 1-Naphthalenesulfonic acid and sulfate as sulfur sources for the green alga Scenedesmus obliquus. *Water Res.*, 25: 299-308.

Madhavi V. and Lele S.S. (2009). "Laccase properties, use," ***BioResources*** 4(4), 1 -24 .

Malachova K. (1999). Using short term mutagenicity tests for the evaluation of genotoxicity of contaminated soil. *J. Soil Contam.,* 8: 667-680.

Maier, J., Kandelbauer, A., Erlacher, A., Cavaco – Paulo, A. and Gubits, G.M. (2004). A new alkali – thermostable azoreductase from *Bacillus* sp. Strain SF. *Appl. Environ. Microbiol.,* 70: 837-844.

Marzullo, L., Cannio, R., Giardian, P., Santini, M.T. and Sannia, G. (1995). Veratryl alcohol oxidase from Pleurotus ostreatus participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. *J. biol. Chem*., 270: 3823-3827.

McKay, G., Ramprasad, G. and Mowli, P. (1987). Desorption and regeneration of dye colours from low-cost materials. *Water Res.*, 21: 375-377.

McMullan, G., Meehan, C., Conneely, A., Nirby, N., Robinson, T., Nigam, P., Banat, I.M., Marchant, R. and Smyth, W.F. (2001). Mini Review: Microbial decolourisation and degradation of textile dyes. *Appl. Microbiol. Biotechnol.. 56*, 81–87.

Mills, A.L., Breuil, C. and Colwell, R.R. (1978). Enumeration of petroleum degrading marine and estuarine microorganisms by the most probable number method. *Can. J. Microbial*. 24: 552-557.

Miller R., Kuglin J., Gallagher S. and Flurkey W.H. and (1997). A spectrophotometric assay for laccase using o-tolidine, J. Food Biochem. 21 445–459.

Mishra, A. and Bajpai, M. (2005). Flocculation behaviour of model textile wastewater treated with a food grade polysaccharide. *J. Hazard. Mater.*, 118: 213-217.

Mishra, S.S. and Bisaria, V.S. (2006). Production and characterization of laccase from Cyathus bulleri and its use in decolourisation of recalcitrant textile dyes. *Appl. Microbiol. Biotechnol.,* 71: 646-653.

Mohana S., Desai C. and Madamwar D. (2007). Biodegradation and decolorization of anaerobically treated distillery spent wash by a novel bacterial consortia. *Bioresour. Technol*. 98: 333-339.

Molina-Guijarro, J.M., Pérez, J., Muñoz-Dorado, J., Guillén, F., Moya, R., Hernández, M. and Arias, M.E.. (2009). Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from *Streptomyces ipomoea*. *Int. Microbiol.*, 12(1):13-21.

Mutambanengwe, C.C.Z. (2006). Hydrogenases from sulphate reducing bacteria (Unpublished thesis), Rhodes University, South Africa.

Nachiyar, C. V. and Rajkumar, G. S. (2003). Degradation of a tannery and textile dye, Navitan Fast Blue S5R by *Pseudomonas aeruginosa*. *W. J. Microbiol. Biotechnol.*, 19: 609-614.

Nagai, M., Sato, T., Watanabe, H., Saito, K., Kawata, M., and Enei, H. (2002). Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolourisation of chemically different dyes. *Appl. Microbiol. Biotechnol.* 60: 327-335.

Nesheiwat, F. K. and Swanson, A.G. (2000). Clean contaminated sites using Fenton's reagent. *Chem. Eng. Prog.*, 96:61-66.

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

Nilsson, R., Nordlinder, R. and Wass, U. (1993). Asthma, Rhinitis, and Dermatitis in workers exposed to reactive dyes. *Br. J. Ind. Med.*, 50: 65–70.

Novotny, C., Rawal, B., Bhatt, M., Patel, M., Sasek, V., and Molitoris, H.P (2001). Capacity of *Ipex lacteus* and *Pleurtus ostreatus* for decolourisation of chemically different dyes. *J. Biotechnol.*, 89: 113-122.

Novotny, C., Dias, N., Kapanen, A., Malachova A., Vandrocova, M., Itavaara, M., and Lima, N. (2006). Comparative use of bacterial, algal and protozoan tests to study toxicity of azo and anthraquinone dyes. *Chemosphere*, 63: 1436-1442.

Olayinka, K.O. and Alo, B.I. (2004). Studies on industrial pollution in Nigeria: The effect of Textile effluent on the quality of ground waters in some parts of Lagos. *Nig. J. Health. Biomed. Sci.,* 3(1): 44-50.

Olukanni, O. D., Osuntoki, A. A. and Gbenle, G. O. (2006). Textile effluent biodegradation potentials of textile effluent-adapted and non-adapted bacteria. *Afri. J. Biotech.*, 5 (20): 1980-1984.

O'Neill, C., Lopez, A., Esteves, S., Hawkes, F.R., Hawkes, D.L. and Wilcox, S. (2000). Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. *Appl. Microbiol. Biotechnol.*, 53: 249-254.

Pagga, U. and Taeger, K. (1994). Development of a method for adsorption of dyestuffs on activated sludge. *Water Res.*, 28: 1051-1057.

Papić, S. , Koprivanac, N., Loncaric Bozic, A. and Metes, A. (2004). Removal of some reactive dyes from synthetic wastewater by combined Al(III) coagulation/carbon adsorption process. *Dyes and Pigments*, 62:291-298.

Park, T.J., Lee, K.H., Jung, E.J. and Kim, C.W. (1999). Removal of refractory organics and color in pigment wastewater with Fenton oxidation. *Water Sci. Technol.*, 39: 189-192.

Parshetti, G., Kalme, S., Saratale, G. and Govindwar, S. (2006). Biodegradation of Malachite Green by *Kocuria rosea* MTCC 1532. *Acta Chim. Slov.*, 53: 492-498.

Pasti-Grigsby, M. B., Paszczynski, A., Goszczynski, S., Crawford, D. L. and Crawford, R. L. (1992). Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 3605-3613.

Paszczynski, A., Pasti-Grigsby, M.B., Goszcznski, S., and Crawford, R.L., (1992). Mineralisation of sulfonated azo dyes and sulphanilic acid by *Phanaerochaete chrysosporium* and *Streptomyces chromofocus. Appl. Environ. Microbiol.,* 58: 3598-3604.

Pearce, C.I., Lloyd, J.T. and Guthrie, J.T. (2003). The removal of colour from textile waste water using whole bacteria cells: A review. *Dyes and Pigments* 58: 179-196.

Peralta-Zamora, P., Kunz, A., de Moraes, S.G., Pelegrini, R., Moleiro, P.D., Reyes, J. and Duran, N. (1999). Degradation of reactive dyes - I. A comparative study of ozonation, enzymatic and photochemical processes. *Chemosphere*, 38: 835-852.

Perez, J., and Jeffries, T.W. (1992). Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanaerochaete chrysosporium. Appl. Environ. Microbiol.,* 58: 2402-2409.

Peres, J. A., Beltrán de Heredia, J., Joaquín, B. and Domínguez, R. (2004). Integrated Fenton's reagent: coagulation/flocculation process for the treatment of cork processing wastewaters. *J. Hazard. Mater.*, 107:115-121.

Pielesz, A., Baranowska, I., Rybak, A. and Wlochowicz, A. (2002). Detection and determination of aromatic amines as products of reductive splitting from selected azo dyes. *Ecotoxicol. Environ. Safety*, 53: 42-47.

Platzek, T., Lang, C., Grohmann, G., Gi, U-S. and Battes, W. (1999). Formation of carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. *Human Exp. Toxicol.*, 18: 552-559.

Plum, A., Braun, G. and Rehorek, A. (2003). Process monitoring of anaerobic azo dye degradation by high-performance liquid chromatography-diode array detection continuously coupled to membrane filtration sampling modules. *J. Chromato., A,* 987: 395-402.

Rafii, F., Franklin, W. and Cerniglia, C.E. (1990). Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. *Appl. Environ. Microbiol.*, 56: 2146-2151.

Rafii, F. and Cerniglia, C.E. (2005). Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from Human intestinal track. *Environ. Health Perspect.* 103 (Suppl. 5): 17 -19.

Raghavacharya, C. (1997). Colour removal from industrial effluents – A comparative review of available technologies. *Chem. Eng. World,* 32: 53-64.

Rajaguru, P., Kalaiselvi, K., Palanivel, M. and Subburam, V. (2000). Biodegradation of azo dyes in a sequential anaerobic-aerobic system. *Appl. Microbiol. Biotechnol.*, 54: 268-273.

Rajamohan, N. and Karthikeyan, C. (2006). Kinetics of dye effluent degradation by *Pseudomonas stutzeri.* ECO services international. Available at http://www.eco-web.com/editorial/061103.html.

Rieger, P. G., Meier, H. M., Gerle, M., Vogt, U., Groth, T. and Knackmuss, H. J. (2002). Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. *J. Biotechnol.*, 94:101-123.

Robinson, T., McMullan, G., Marchant, R. and Nigam, P. (2001). Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Biores. Technol.*, 77: 247-255.

Rosciszweewski P., Lukasiak J., Dorosz A., Galinski J. and Szponar M. (1998). Biodegradation of polyorganosiloxanes. *Macromol Symp* 130: 337-346.

Rodriguez, E., Pickard, M.A. and Duhalt, R.V. (1999). Industrial dye decolourisation by laccases from lignolytic fungi. *Current Microbiol.,* 38: 27-32.

Rodriguez – Couto, S., Dominguez, A. and Sanromán, A. (2002). Production of maganese dependent peroxidase in a new solid state bioreactor by *Phanerochaete chrysosporium* grown on wood shavings. Application to the decolourisation of synthetic dyes. *Folia Microbiol.*, 47: 417-421.

Rozzi, A., Malpei, F., Bonomo, L. and Bianchi, R. (1999). Textile wastewater reuse in Northern Italy (COMO). *Water Sci. Technol.*, 39: 122-128.

Russ R., Rau R. and Stolz A. (2000). The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. *Appl. Environ. Microbiol*. 66:1429-1434.

Saito, T., Hong, P., Kato, K., Okazaki, M., Inagaki, H., Maeda, S. and Yokogawa, Y. (2003). Purification and characterization of extracellular Laccase of a fungus (family Chaetomiaceae) isolated from soil *Enz. Microbiol. Biotechnol.* 74:1232-1239.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* 4: 406-425.

Seidenari, S., Giusti, F., Massone, F. and Mantovani, L. (2002). Sensitization to disperse dyes in a patch test population over a five-year period. *Am. J. Contact. Dermat.*, 13:101-107.

Sekar, N. and Sivakumar, K. (1993). Bifunctional reactive dyes for cottons. *Colourage*, 40(7): 41.

Semerjian, L. and Ayoub, G. M. (2003). High-pH-magnesium coagulationflocculation in wastewater treatment. *Adv. Environ. Res.*, 7:389-403.

Semple, K.T., Cain, R.B. and Schmidt, S. (1999). Biodegradation of aromatic compounds by microalgae. *FEMS Microbiol. Lett.*, 170: 291-300.

Sharma, D.K., Saini, H.S., Singh, M., Chimni, S.S. and Chadha, B.S. (2004). Isolation and characterization of microorganisms capable of decolourising various triphenylmethane dyes, *J. Basic Microbiol.,* 44 (1): 59–65.

Shaul, G.M., Holdsworth, T.J., Dempsey, C.R. and Dostal, K.A. (1991). Fate of water soluble azo dyes in the activated sludge process. *Chemosphere*, 22: 107-119.

Shaw, C.B., Carliell, C.M., and Wheatley, A.D. (2002). Anaerobic/aerobic treatment of coloured textile effluents using sequencing batch reactors. *Water Res.,* 36: 1993 – 2001.

Slokar, Y.M., and Le Marechal, M.L. (1998). Methods of decolourisation of textile wastewater. *Dyes and Pigments,* 37 (4): 335 – 356.

Soares, G.M.B., Amorin, T.P., Lageiro, M. and Costa-Ferreira, M. (2006). Pilot scale enzymatic decolorization of industrial dyeing process wastewater. *Textile Res. Journ.*, 76(1): 4-11.

Sponza, D.T., and Isik, M. (2002). Decolourisation and azo dye degradation by anaerobic/aerobic sequential process. *Enzy. Microbial Technol.,* 31: 102-110.

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

Suwanruji, P. P. (2004). The Design, Synthesis and Application of Easy Wash Off Reactive Dyes. PhD thesis submitted to the North Carolina State University.
Swamy, J. and Ramsay, J.A. (1999). The evaluation of white rot fungi in the decoloration of textile dyes. *Enzy. Microbiol. Technol.*, 24: 130-137.

Takezaki, N., Rzhetsky, A. and Nei, M. (2004). Phylogenetic test of the molecular clock and linearized trees. *Mol. Biol. Evol.,* 12: 823-833.

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA*, 101: 11030-11035.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.,* 24: 1596-1599.

Telke, A., Kalyani, D., Jadhav, J. and Govindwar, S. (2008). Kinetics and mechanism of reactive red 141 degradation by a bacterial isolate *Rhizobium radiobacter* MTCC 8161. *Acta Chim. Slov.*, 55: 320-329.

Telke A. A., Kalyani D. C., Jadhav U. U., Parshetti G. K. and Govindwar S. P. (2009). Purification and characterization of an extracellular laccase from a *Pseudomonas sp.* LBC1 and its application for the removal of bisphenol A, *J. Mol. Catal. B: Enz.* 61: 252–260.

Tien, M. and Kirk T.K. (1984). Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H202-requiring oxygenase. *Proc. Natl. Acad. Sci. USA*. 81: 2280-2284.

Van Der Zee, F. (2002). Anaerobic azo dye reduction. PhD Thesis. Wageningen University, The Netherlands.

Van der Zee, F.P. and Villaverde, S. (2005). Combined anaerobic – aerobic treatment of azo dyes – a short review of bioreactor studies. *Water res.* 39: 1425-1440.

Wesenberg, D., Kyriakioles, and, I, Agathos, S.N. (2003). White Rot Fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Advances,* 22: 161-187.

Wilson, K., and Walker, J. (1995). Practical Biochemistry: Principles and techniques. Fourth (low price) Ed. Cambridge University Press, Britain.p17 – 18, 498 – 507.

Wong, P.K. and Yuen P.V. (1996). Decolorisation and biodegradation of methyl red by *Klebsiella pneumoniae* RS-13. *Water Res.,* 30: 1736-1744.

Wong, C. K., Liu, X. J., Lee, A. O. K. and Wong, P. K. (2006). Effect of Azo Dyes on Survivorship, Oxygen Consumption Rate, and Filtration Rate of the Freshwater Cladoceran Moina macrocopa *Human Ecol Risk Assess.* 12 (2): 289-300.

Young, L. and Yu, J. (1997). Liginase-catalysed decolourisation of synthetic dyes. *Water Res.*, 31:1187-1193.

Yu, J., Xiaoweiwang G. and Yue, P.L. (2001). Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas* strains. *Water Res.,* 35 (15): 3579-3586.

Zhang, F. and Yu, J. (2000). Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon. *Bioprocess Eng.*, 23: 295-301.

Zhang M., Wu F., Wei Z., Xiao Y., Gong W. (2006) Characterization and decolorization ability of a laccase from *Panus rudis*. *Enz. Microbial Technol.,* 39: 92–97.

Zhang, X. and Flurkey, W. (1997). Phenol oxidases in Portabella Mushrooms. *J. Food Sci.*, 62: 97-100.

Zille, A. (2005). Laccase reactions for Textile application. PhD Thesis. University of Minho, Portugal.

Zille, A., Tzanov, T., Guebitz, G. M., and Cavaco-Paulo, A. (2003). Immobilized laccase for decolorization of Reactive Black 5 dyeing effluent. *Biotechol. Lett., 25*: 1473–1477.

Zimmermann, T., Kulla, H.G. and Leisinger, T., (1982). Properties of purified orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.*, 129: 197-203.

Zimmermann, T., Gasser, F., Kulla, H. and Leisinger, T. (1984). Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.*, 138: 37-43.

Ziegenhagen, D. and Hofrichter, M. (2000). A simple and rapid method to gain high amounts of manganese peroxidase with immobilized mycelium of the agric white-rot fungus *Clitocybula dusenii. Appl. Microbiol. Biotechnol.,* 53: 553-557.

Appendix I

Composition of Media Used in the Research

Nutrient Agar

The nutrient agar contains the following (per litre):

Beef extract	3g
Peptone	5g
Agar No.2	12g
NaCl	8g

Nutrient broth

The nutrient broth contains the following (per litre):

Beef extract	1g
Yeast extract	2g
Peptone	5g
NaCl	5g

Mills medium (Mills et al, 1978)

Composition	**(g/300ml)**
NaCl	3.000
MgSO4.7H2O	0.216
KCl	0.087
KH2PO4	0.249
NaHPO4	0.375
CaCl2	0.015
NaNO3	0.420
FeSO4.7H2O	0.003

Semi solid medium (motility medium)

Peptone	10 g
Meat extract	3 g
NaCl	5 g
Agar	2 g
Gelatin	80 g
Distilled water	1000 ml

Soak the gelatin in water for 30 min, add the other ingredients, heat to dissolve, and sterilize at 115^{O}C for 20 min.

Nutrient broth sugar

Peptone	10 g
Meat extract	3 g
NaCl	5 g
Distilled water	1000 ml
Phenol red aq. soln	10 ml

Dissolve solids in water, add the indicator and adjust to pH 7.1-7.2. Sterilize at 115^{O}C for 20 min.

Oxidation Fermentation (OF) medium (Hugh and Leifson, 1953)

Peptone	2 g
NaCl	5 g
K_2HPO_3	0.3 g
Agar	3 g
Distilled water	1000 ml
Bromothymol blue	15 ml (0.2% aq. soln)

Dissolve the solids by heating in water, adjust to pH 7.1-7.2 and add the indicator. Sterilize at 115^{O}C for 20 min.

Appendix II

Preparation of Buffers and Gels for SDS PAGE

(a)	**8% resolving gel (30ml)**	**Vol. (ml)**
	H_2O	15.9
	30% acryl-bisacrylamide mix	6.0
	1.5M Tris (pH 8.8)	7.5
	10% SDS	0.3
	10 % ammonium persulphate	0.3
	TEMED	0.024

30% acryl-bisacrylamide mix

- Dissolve 29g Acrylamide and 1g bisacrylamide in 60ml distilled water
- Heat the solution to 37^{O}C and adjust volume to 100ml
- Strore at 4^{O}C away from light.

10% ammonium persulphate

- Dissolve 1g APS in 8ml H2O
- Adjust to 10ml
- Stable at 4OC for 2 weeks

(b)	**5% stacking gel (10ml)**	**Vol. (ml)**
	H_2O	6.8
	30% acryl-bisacrylamide mix	1.7
	1.5M Tris (pH 8.8)	1.25
	10% SDS	0.1
	10 % ammonium persulphate	0.1
	TEMED	0.01

(c)	**2 X sample buffer (100ml)**	**Vol. (ml)**
	1.5M Tris (pH 8.8)	10
	10% SDS	12
	Glycerol	30
	β-mercaptoethanol	15
	Bromophenol blue	1.8mg

- adjust volume to 100ml with distilled H_2O
- divide into 10ml and store at -20^{O}C
- store working solution at 4^{O}C

(d) 10X Running Buffer (1L)

SDS	10g
Tris	30.3g
Glycine	144.1g

- Dissolve solids in 800ml distilled water
- Adjust volume to 1L with water
- Store at room temperature

(e) Staining Solution

Coomassie Brilliant Blue R-250	2.5g
Methanol	450ml
Acetic acid	100ml
Distilled H2O	400ml

- mix solvents, dissolve the dye, and adjust to 1L with distilled water
- store at room temperature

(f) Destaining solution

Methanol	450ml
Acetic acid	100ml
Distilled H2O	400ml

- mix solvents, and adjust to 1L with distilled water
- store at room temperature

(g) 1x Resolving buffer (Same as Tris buffer pH 8.8)

- add 45.35g tris-base into 850ml distilled water
- adjust to pH 8.8 at room temperature with HCl
- adjust to 1L with distilled water
- store at $4^{O}C$

(h) 1x Stacking buffer (Same as Tris buffer pH 6.8)

- add 15.13g Tris-base into 850ml distilled water
- adjust to pH 6.8 at room temperature with HCl
- adjust to 1L with distilled water
- store at $4^{O}C$

Appendix III

16S rDNA Sequence of Proteus mirabilis LAG and its GenBank parameters

GenBank Direct Submission Staff
gb-admin@ncbi.nlm.nih.gov

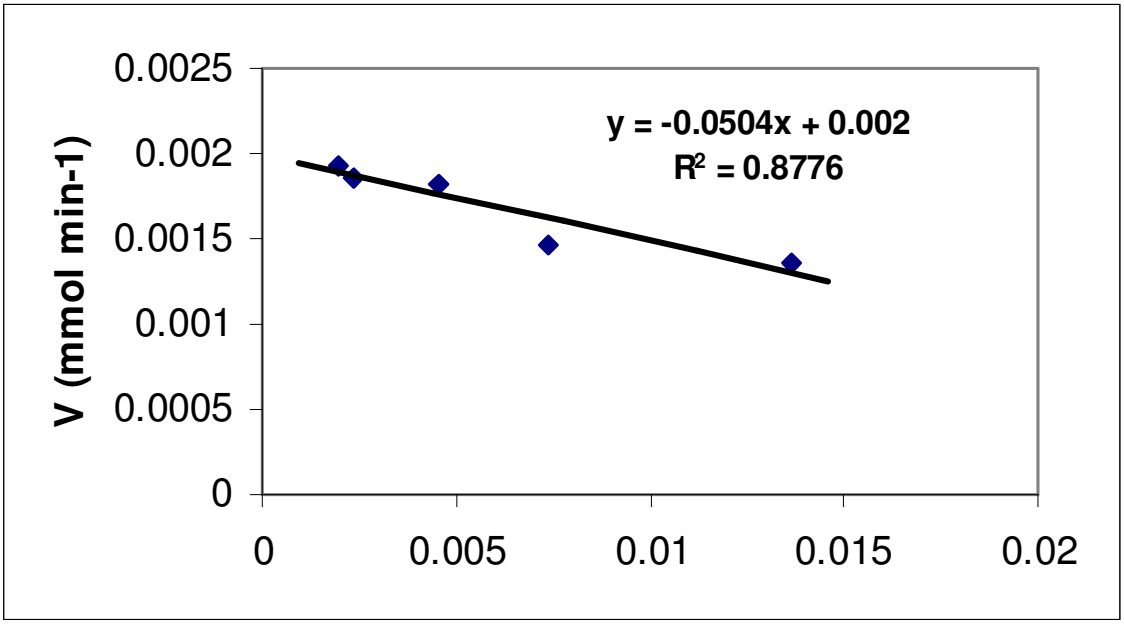
GenBank flat file:

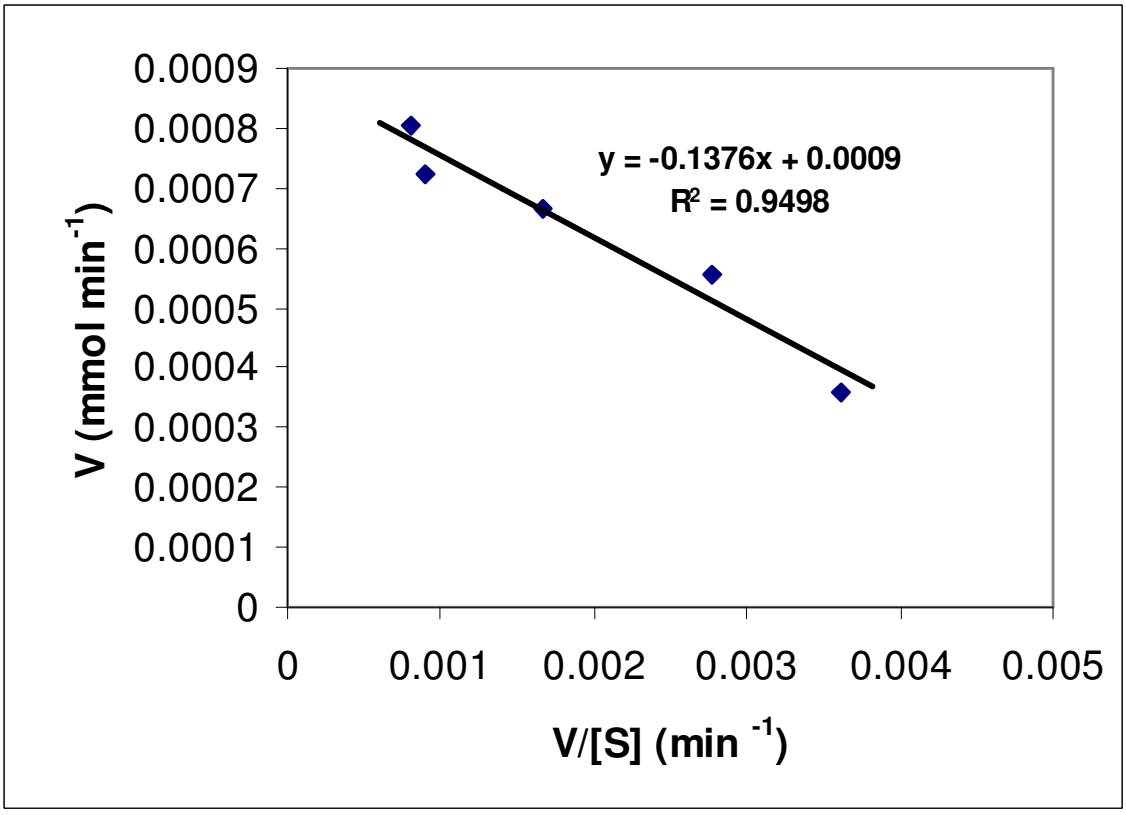
```
LOCUS       GU945541                 983 bp    DNA
   linear   BCT 19-MAR-2010
DEFINITION  Proteus mirabilis strain LAG 16S ribosomal RNA
gene, partial sequence.
ACCESSION   GU945541.
ORGANISM  Proteus mirabilis
          Bacteria; Proteobacteria; Gammaproteobacteria;
Enterobacteriales; Enterobacteriaceae; Proteus.
REFERENCE   1  (bases 1 to 983)
  AUTHORS   Olukanni,O.D., Osuntoki,A. and Gbenle,G.O.
  TITLE     Decolourization and biodegradation of textile
dyes
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 983)
  AUTHORS   Olukanni,O.D., Osuntoki,A. and Gbenle,G.O.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2010) Biochemistry
Department, University of
            Lagos, College of Medicine, Idi-Araba, Lagos
23401, Nigeria
FEATURES             Location/Qualifiers
     source          1..983
                     /organism="Proteus mirabilis"
                     /mol_type="genomic DNA"
                     /strain="LAG"
                     /db_xref="taxon:584"
     rRNA            <1..>983
                     /product="16S ribosomal RNA"
ORIGIN
        1 gcagcctgat gcagccatgc cgcgtgtatg aagaaggcct
tagggttgta aagtactttc
       61 agcggggagg aaggtgataa ggttaatacc cttatcaatt
gacgttaccc gcagaagaag
      121 caccggctaa ctccgtgcca gcagccgcgg taatacggag
ggtgcaagcg ttaatcggaa
      181 ttactgggcg taaagcgcac gcaggcggtc aattaagtca
gatgtgaaag ccccgagctt
      241 aacttgggaa ttgcatctga aactggttgg ctagagtctt
```

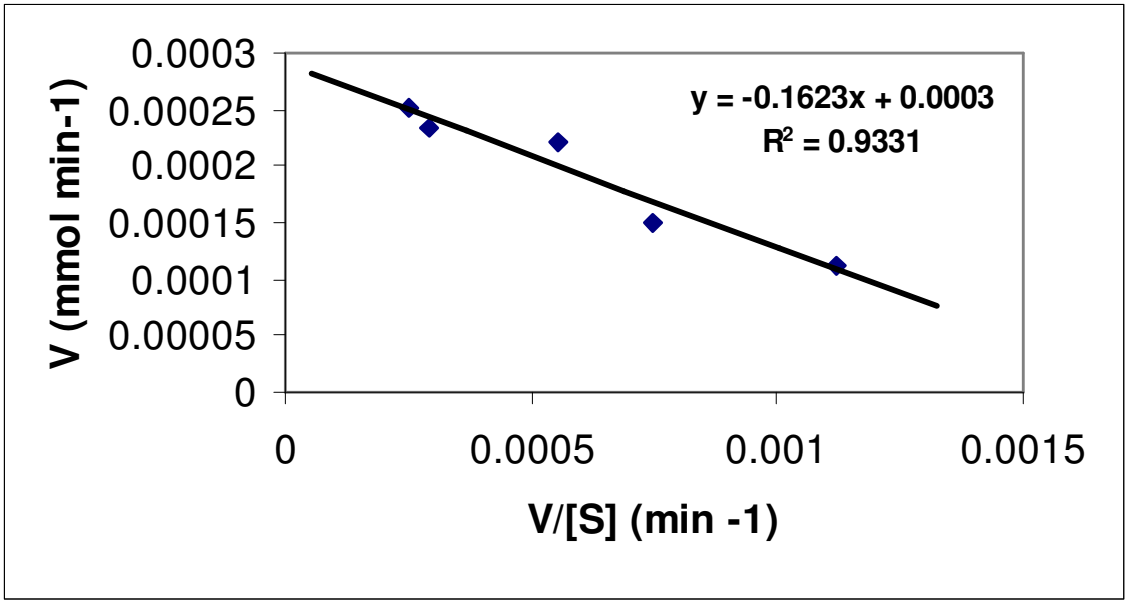
```
gtagaggggg gtagaattcc
      301 atgtgtagcg gtgaaatgcg tagagatgtg gaggaatacc
ggtggcgaag gcggcccccc
      361 ggacaaagac tgacgctcag gtgcgaaagc gtggggagca
aacaggatta gataccctgg
      421 tagtccacgc tgtaaacgat gtcgatttag aggttgtggt
cttgaaccgt ggcttctgga
      481 gctaacgcgt taaatcgacc gcctggggag tacggccgca
aggttaaaac tcaaatgaat
      541 tgacgggggc ccgcacaagc ggtggagcat gtggtttaat
tcgatgcaac gcgaagaacc
      601 ttacctactc ttgacatcca gcgaatcctt tagagataga
ggagtgcctt cgggaacgct
      661 gagacaggtg ctgcatggct gtcgtcagct cgtgttgtga
aatgttgggt taagtcccgc
      721 aacgagcgca acccttatcc tttgttgcca gcacgtgatg
gtgggaactc aaaggagact
      781 gccggtgata aaccggagga aggtggggat gacgtcaagt
catcatggcc cttacgagta
      841 gggctacaca cgtgctacaa tggcagatac aaagagaagc
gacctcgcga gagcaagcgg
      901 aactcataaa gtctgtcgta gtccggattg gagtctgcaa
ctcgactcca tgaagtcgga
      961 atcgctagta atcgtagatc aga
//
```

Appendix IV

Eadie-Hofstee diagram for the determination of enzyme's V_{max} and K_m

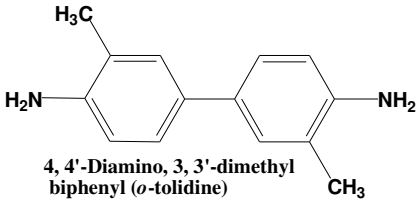




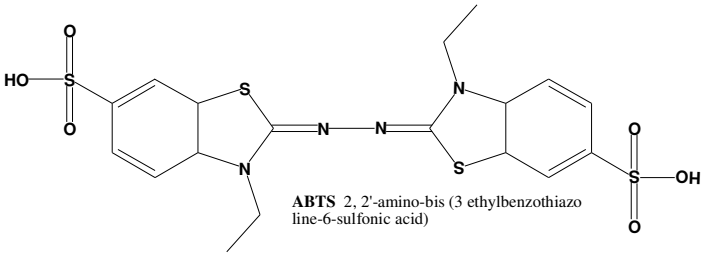

0.0003
0.00025
0.0002
0.00015
0.0001
0.00005
0
V (mmol min-1)
y = -0.1623x + 0.0003
R2 = 0.9331
0
0.0005
0.001
0.0015
V/[S] (min -1)

Appendix V

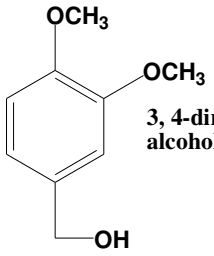
Substrates used in Enzyme Studies



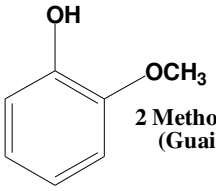
4, 4'-Diamino, 3, 3'-dimethyl biphenyl (*o*-tolidine)



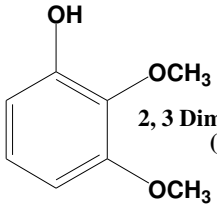
ABTS 2, 2'-amino-bis (3 ethylbenzothiazo line-6-sulfonic acid)



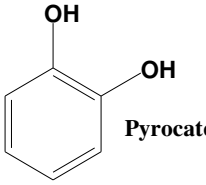
3, 4-dimethoxyl benzyl alcohol (Veratryl alcohol)



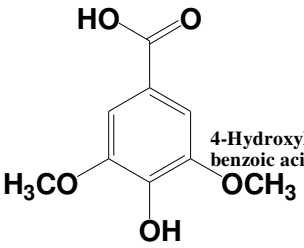
2 Methoxylphenol (Guaiacol)



2, 3 Dimethoxylphenol (DMP)



Pyrocatechol



4-Hydroxyl -3, 5-dimethoxyl benzoic acid (Syringic acid)

Appendix VI

Raw data of GCMS Analysis

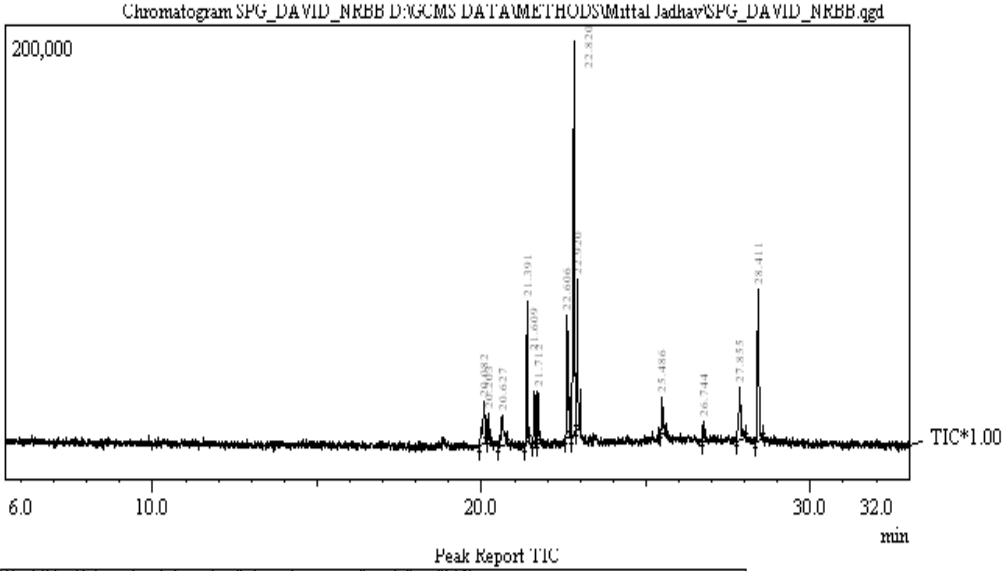
SHIVAJI UNIVERSITY, Kolhapur

Common Facility Center

Sample Information

Analyzed by	: Admin
Analyzed	: 12/29/2008 2:38:40 PM
Sample Name	: SPG_DAVID_NRBB
Sample ID	: SPG_DAVID_NRBB
Injection Volume	: 1.000
Data File	: D:\GCMS DATA\METHODS\Mittal Jadhav\SPG_DAVID_NRBB.qgd
Method File	: D:\GCMS DATA\METHODS\Mittal Jadhav\Mittal Jadhav.qgm
Tuning File	: D:\GCMS DATA\TUNING FILES\18 DEC 08.qgt

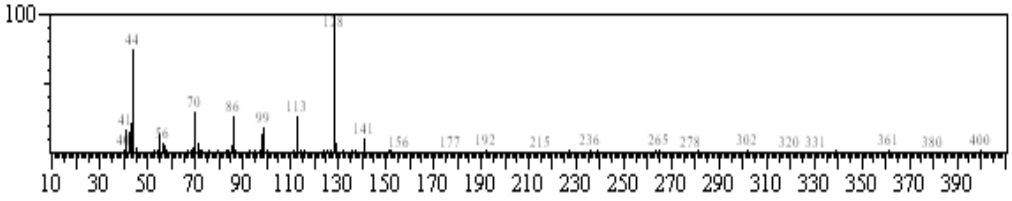
Chromatogram SPG_DAVID_NRBB D:\GCMS DATA\METHODS\Mittal Jadhav\SPG_DAVID_NRBB.qgd



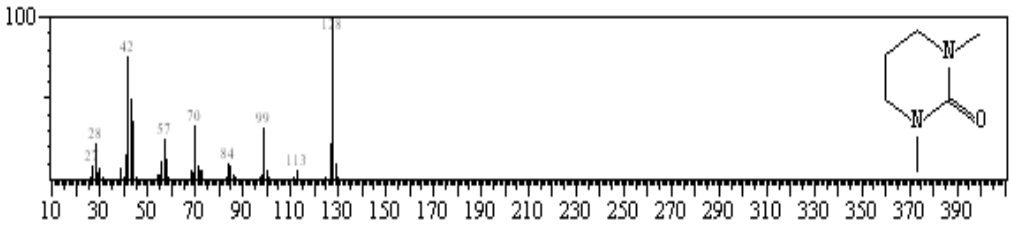
Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Name
1	20.082	19.925	20.142	92005	5.03	
2	20.203	20.158	20.267	29457	1.61	
3	20.627	20.525	20.783	78464	4.29	
4	21.391	21.317	21.450	157553	8.61	
5	21.609	21.533	21.650	59323	3.24	
6	21.712	21.667	21.783	63293	3.46	
7	22.606	22.550	22.700	174729	9.55	
8	22.820	22.733	22.867	589667	32.24	
9	22.920	22.875	22.958	165210	9.03	
10	25.486	25.433	25.608	63290	3.46	
11	26.744	26.700	26.817	28898	1.58	
12	27.855	27.742	28.033	105903	5.79	
13	28.411	28.300	28.575	221378	12.10	
				1829170	100.00	

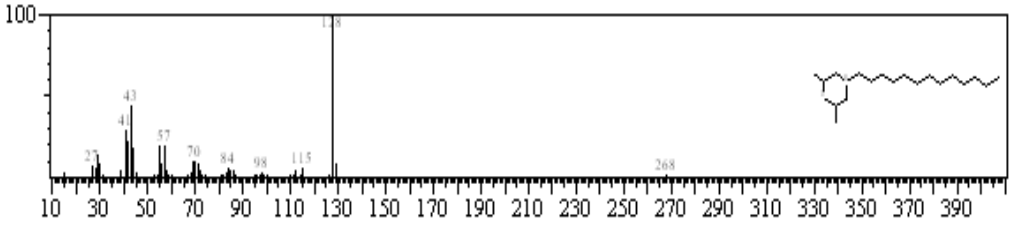
<< Target >>
Line#:1 R.Time:20.083(Scan#:1751) MassPeaks:201
RawMode:Averaged 20.075-20.092(1750-1752) BasePeak:128.10(4060)
BG Mode:Calc. from Peak



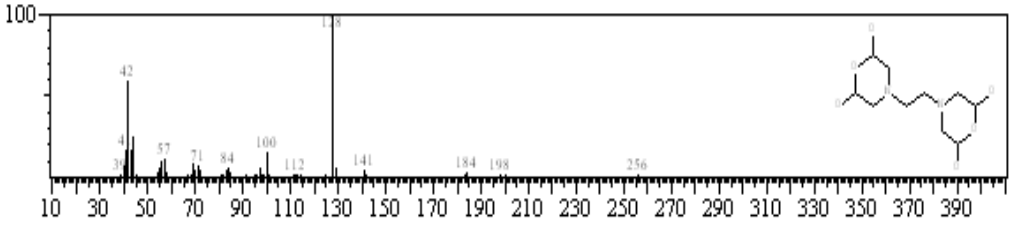
Hit#:1 Entry:6337 Library:NIST107.LIB
SI:80 Formula:C6H12N2O CAS:7226-23-5 MolWeight:128 RetIndex:0
CompName:1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone $$ 1,3-Dimethyltetrahydro-2(1H)-pyrimidinone $$ 2(.



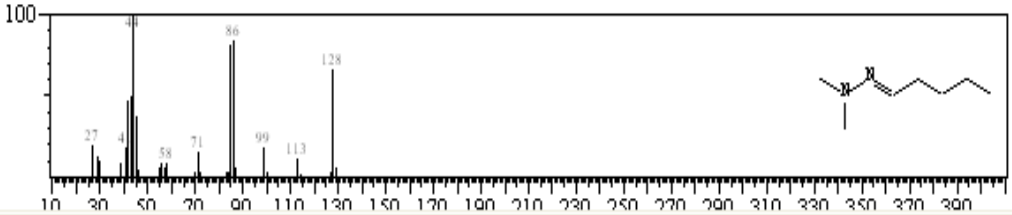
Hit#:2 Entry:70755 Library:NIST107.LIB
SI:78 Formula:C19H39NO CAS:24602-86-6 MolWeight:297 RetIndex:0
CompName:Tridemorph $$ Morpholine, 2,6-dimethyl-4-tridecyl- $$ Bas 22053f $$ Basf 22053f $$ Calixin $$ Calixine $$ T



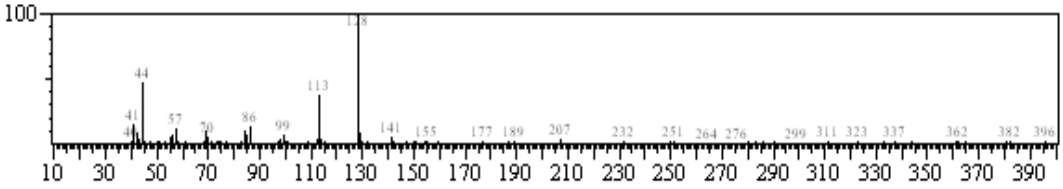
Hit#:3 Entry:56759 Library:NIST107.LIB
SI:77 Formula:C10H12N2O6 CAS:0-0-0 MolWeight:256 RetIndex:0
CompName:4,4'-Ethylenebis(2,6-morpholinedione) $$



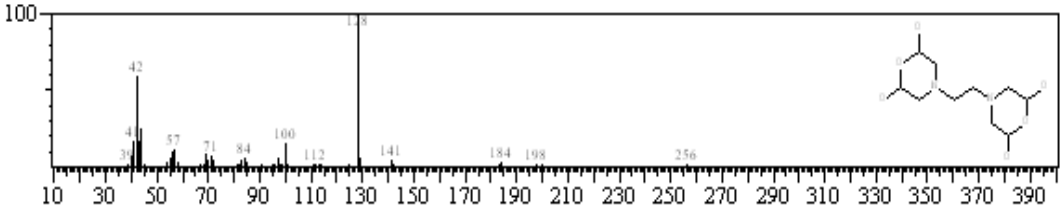
Hit#:4 Entry:6486 Library:NIST107.LIB
SI:76 Formula:C7H16N2 CAS:14090-57-4 MolWeight:128 RetIndex:0
CompName:Valeraldehyde, dimethylhydrazone $$



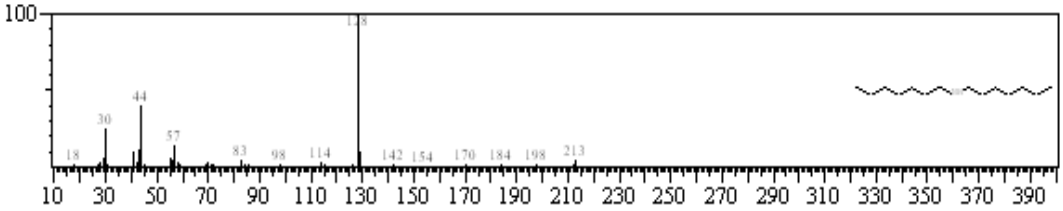
<< Target >>
Line#:2 R.Time:20.200(Scan#:1765) MassPeaks:181
RawMode:Averaged 20.192-20.208(1764-1766) BasePeak:128.10(32677)
BG Mode:Calc. from Peak



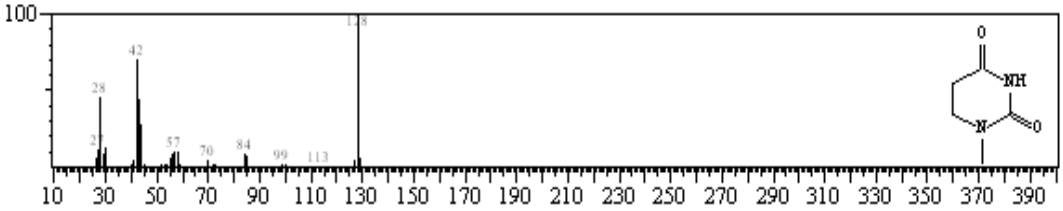
Hit#:1 Entry:56759 Library:NIST107.LIB
SI:78 Formula:C10H12N2O6 CAS:0-0-0 MolWeight:256 RetIndex:0
CompName:4,4'-Ethylenebis(2,6-morpholinedione) $$



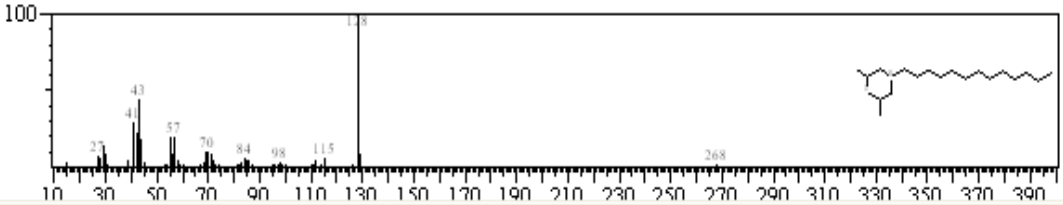
Hit#:2 Entry:40339 Library:NIST107.LIB
SI:77 Formula:C14H31N CAS:2470-68-0 MolWeight:213 RetIndex:0
CompName:1-Heptanamine, N-heptyl- $$ Diheptylamine $$ Di-n-heptylamine $$



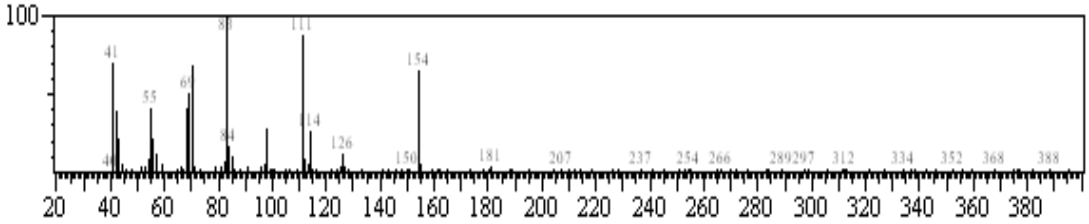
Hit#:3 Entry:62773 Library:NIST107.LIB
SI:76 Formula:C5H8N2O2 CAS:696-11-7 MolWeight:128 RetIndex:0
CompName:Hydrouracil, 1-methyl- $$ 2,4(1H,3H)-Pyrimidinedione, dihydro-1-methyl- $$



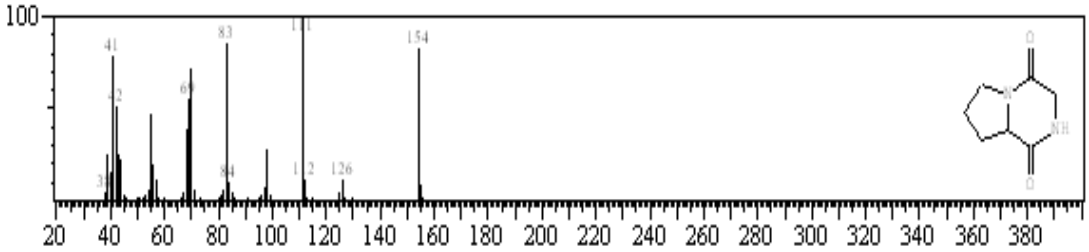
Hit#:4 Entry:70755 Library:NIST107.LIB
SI:76 Formula:C19H39NO CAS:24602-86-6 MolWeight:297 RetIndex:0
CompName:Tridemorph $$ Morpholine, 2,6-dimethyl-4-tridecyl- $$ Bas 22037 $$ Basf 22037 $$ Calixin $$ Calixine $$ F



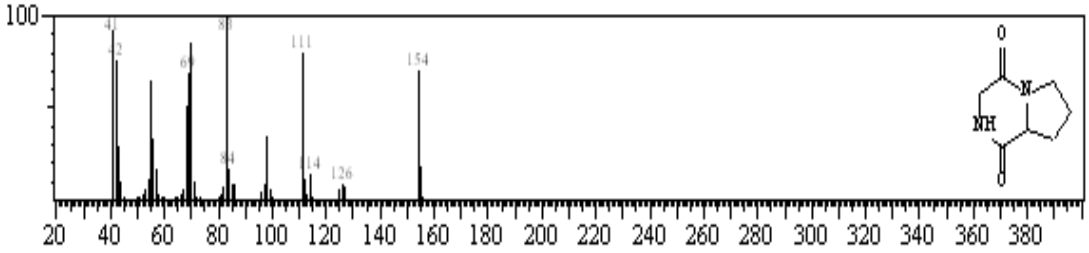
<< Target >>
Line#:3 R.Time:20.625(Scan#:1816) MassPeaks:202
RawMode:Averaged 20.617-20.633(1815-1817) BasePeak:83.10(1677)
BG Mode:Calc. from Peak



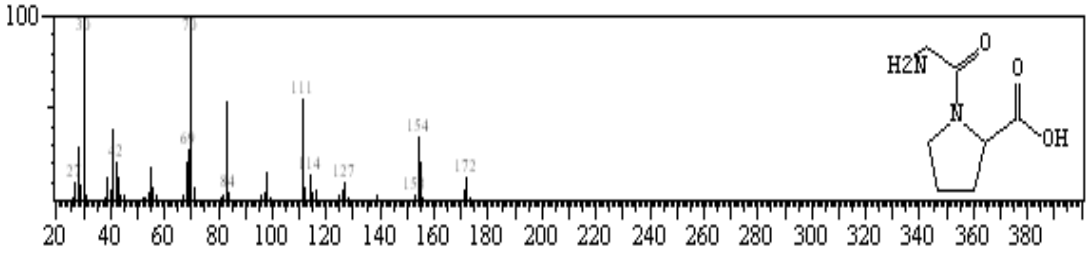
Hit#:1 Entry:14761 Library:NIST107.LIB
SI:91 Formula:C7H10N2O2 CAS:19179-12-5 MolWeight:154 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- $$



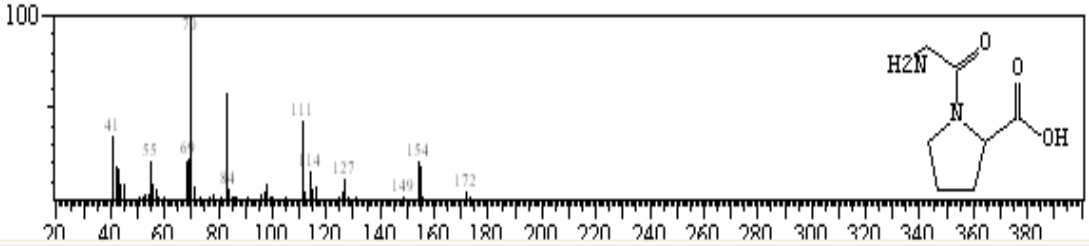
Hit#:2 Entry:14747 Library:NIST107.LIB
SI:90 Formula:C7H10N2O2 CAS:0-0-0 MolWeight:154 RetIndex:0
CompName:Pyrrolo[1,2-a]piperazine-3,6-dione $$



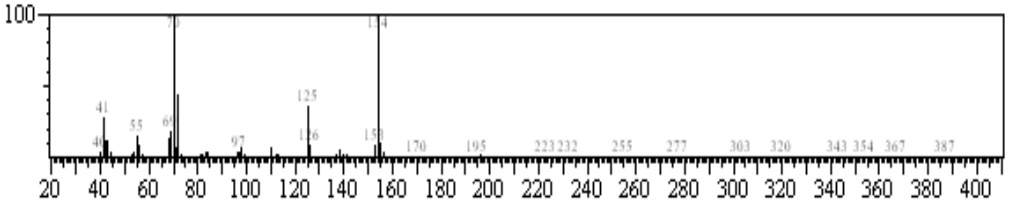
Hit#:3 Entry:22409 Library:NIST107.LIB
SI:79 Formula:C7H12N2O3 CAS:704-15-4 MolWeight:172 RetIndex:0
CompName:Glycyl-L-proline $$ L-Proline, 1-glycyl- $$ Glycylproline $$ N-Glycyl-L-proline $$ Proline, 1-glycyl-, L- $$



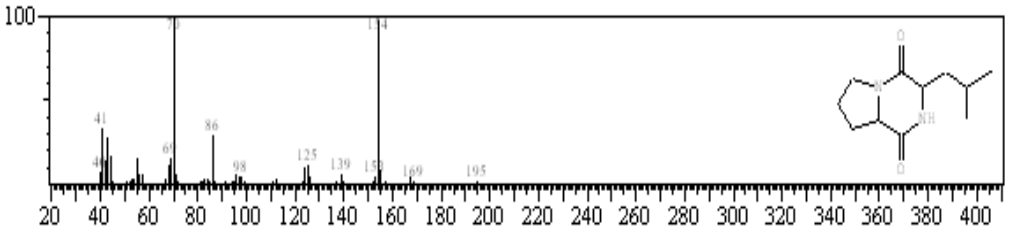
Hit#:4 Entry:9848 Library:NIST21.LIB
SI:78 Formula:C7H12N2O3 CAS:704-15-4 MolWeight:172 RetIndex:0
CompName:Glycyl-L-proline



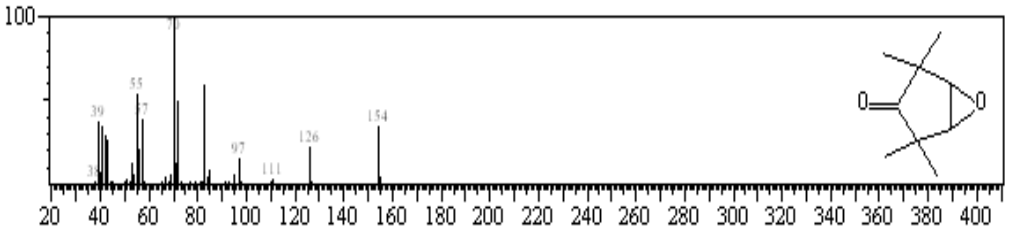
<< Target >>
Line#:4 R.Time:21.392(Scan#:1908) MassPeaks:217
RawMode:Averaged 21.383-21.400(1907-1909) BasePeak:70(122U6)
BG Mode:Calc. from Peak



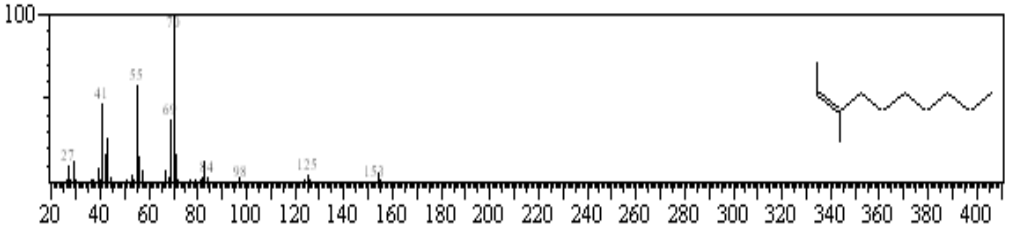
Hit#:1 Entry:38896 Library:NIST107.LIB
SI:84 Formula:C11H18N2O2 CAS:5654-86-4 MolWeight:210 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- $$



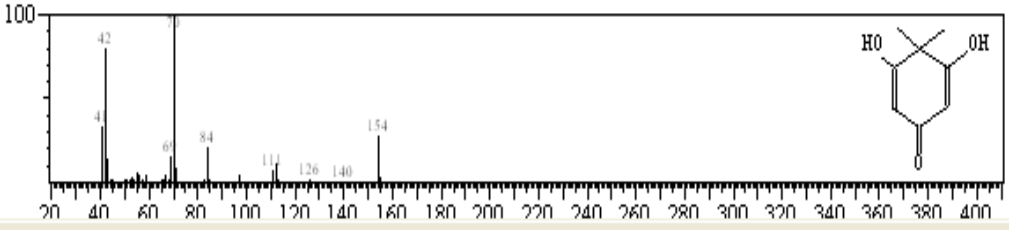
Hit#:2 Entry:14915 Library:NIST107.LIB
SI:76 Formula:C9H14O2 CAS:97455-13-5 MolWeight:154 RetIndex:0
CompName:6-Oxabicyclo[3.1.0]hexan-3-one, 2,2,4,4-tetramethyl- $$



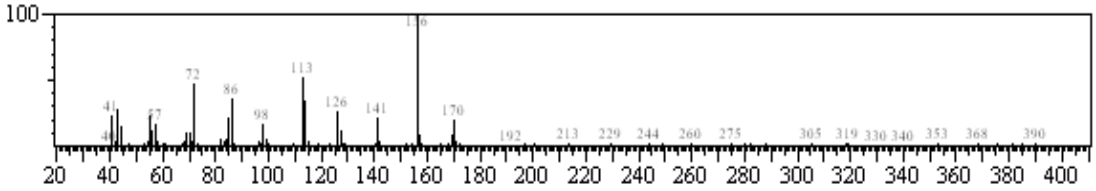
Hit#:3 Entry:15349 Library:NIST107.LIB
SI:75 Formula:C11H22 CAS:74630-26-5 MolWeight:154 RetIndex:0
CompName:2-Decene, 3-methyl-, (Z)- $$



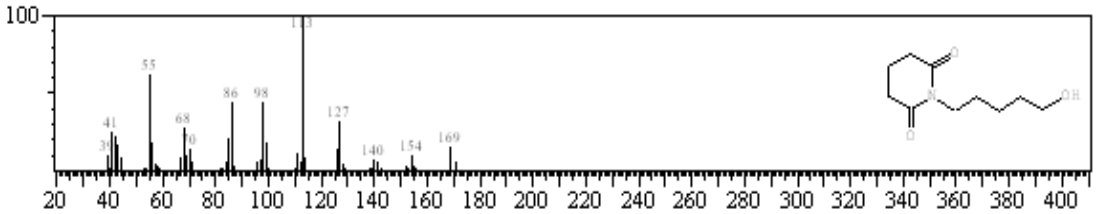
Hit#:4 Entry:14828 Library:NIST107.LIB
SI:75 Formula:C8H10O3 CAS:2065-0-1 MolWeight:154 RetIndex:0
CompName:2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,4-dimethyl- $$ Fihcmic acid $$



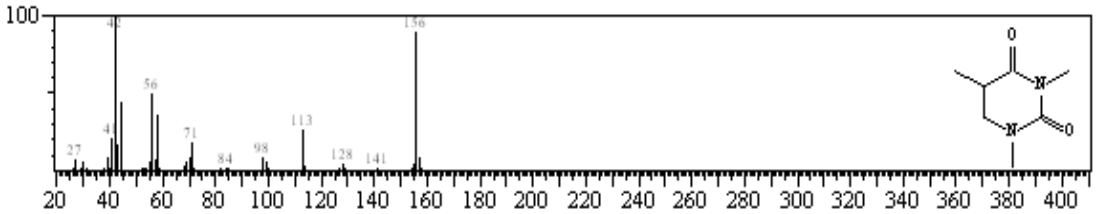
<< Target >>
Line#:5 R.Time:21.608(Scan#:1934) MassPeaks:225
RawMode:Averaged 21.600-21.617(1933-1935) BasePeak:156.15(3471)
BG Mode:Calc. from Peak



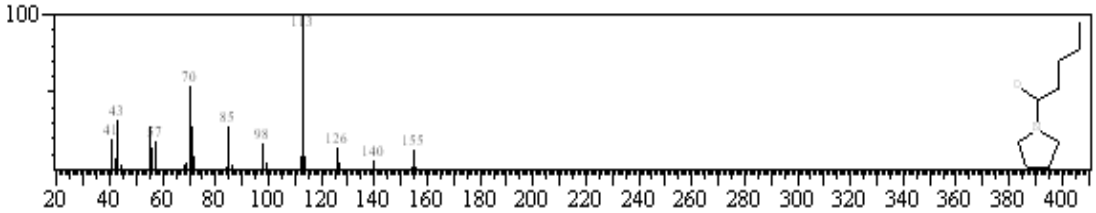
Hit#:1 Entry:34183 Library:NIST107.LIB
SI:71 Formula:C10H17NO3 CAS:0-0-0 MolWeight:199 RetIndex:0
CompName:2,6-Piperidinedione, 1-(5-hydroxypentyl)- $$



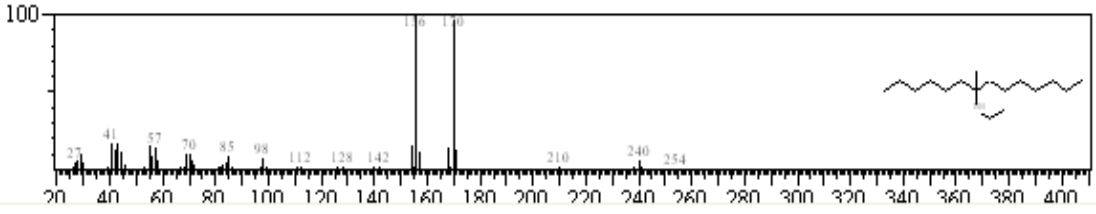
Hit#:2 Entry:15755 Library:NIST107.LIB
SI:70 Formula:C7H12N2O2 CAS:19274-22-7 MolWeight:156 RetIndex:0
CompName:2,4(1H,3H)-Pyrimidinedione, dihydro-1,3,5-trimethyl- $$



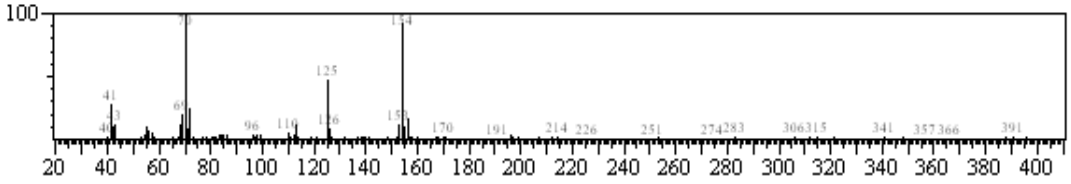
Hit#:3 Entry:15587 Library:NIST107.LIB
SI:69 Formula:C9H17NO CAS:4419-57-2 MolWeight:155 RetIndex:0
CompName:Pyrrolidine, 1-(1-oxopentyl)- $$ Pyrrolidine, 1-valeryl- $$



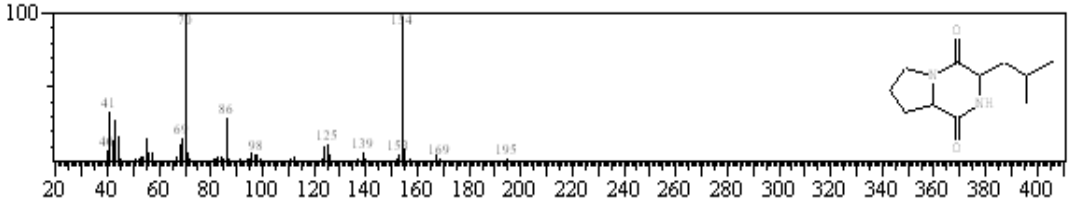
Hit#:4 Entry:56684 Library:NIST107.LIB
SI:69 Formula:C17H37N CAS:71275-6-4 MolWeight:255 RetIndex:0
CompName:N-Ethyl-7-methyl-7-tetradecanamine $$



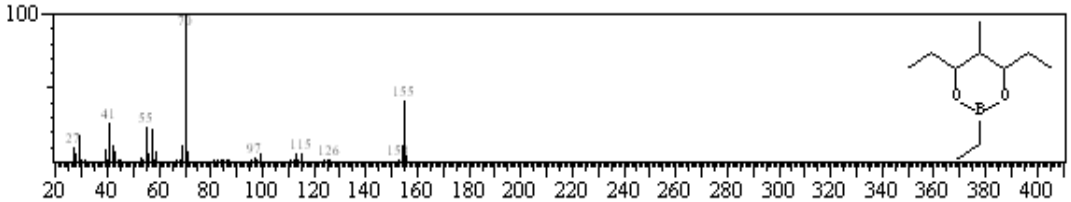
<< Target >>
Line#:6 R.Time:21.708(Scan#:1946) MassPeaks:214
RawMode:Averaged 21.700-21.717(1945-1947) BasePeak:70.1U(3929)
BG Mode:Calc. from Peak



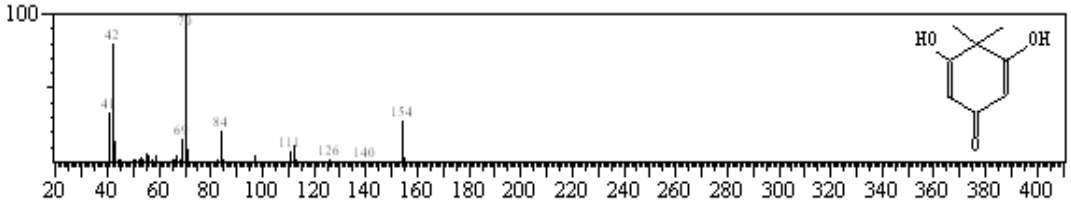
Hit#:1 Entry:38896 Library:NIST107.LIB
SI:82 Formula:C11H18N2O2 CAS:5654-86-4 MolWeight:210 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- $$



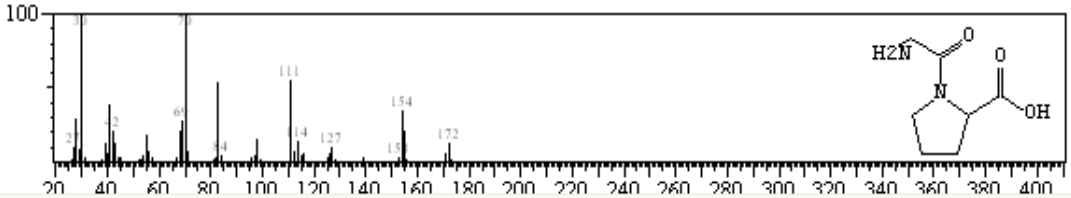
Hit#:2 Entry:27736 Library:NIST107.LIB
SI:75 Formula:C10H21BO2 CAS:74744-58-4 MolWeight:184 RetIndex:0
CompName:1,3,2-Dioxaborinane, 2,4,6-triethyl-5-methyl- $$



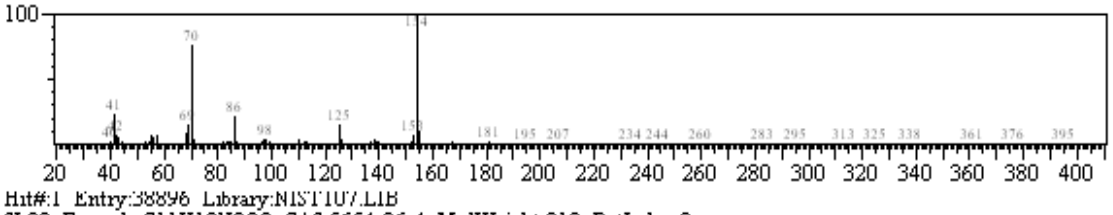
Hit#:3 Entry:14828 Library:NIST107.LIB
SI:74 Formula:C8H10O3 CAS:2065-0-1 MolWeight:154 RetIndex:0
CompName:2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,4-dimethyl- $$ Filicinic acid $$



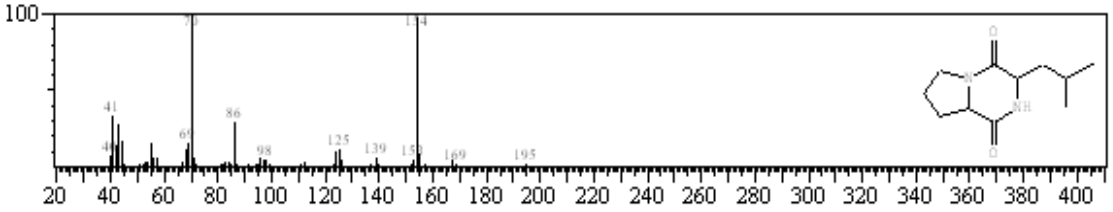
Hit#:4 Entry:22409 Library:NIST107.LIB
SI:73 Formula:C7H12N2O3 CAS:704-15-4 MolWeight:172 RetIndex:0
CompName:Glycyl-L-proline $$ L-Proline, 1-glycyl- $$ Glycylproline $$ N-Glycyl-L-proline $$ Proline, 1-glycyl-, L- $$



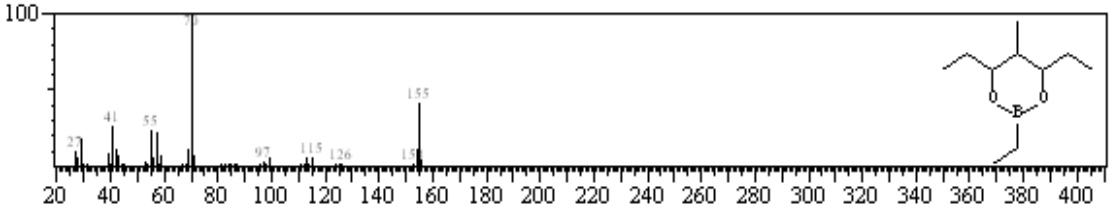
<< Target >>
Line#:7 R.Time:22.608(Scan#:2054) MassPeaks:217
RawMode:Averaged 22.600-22.617(2053-2055) BasePeak:154.15(14364)
BG Mode:Calc. from Peak



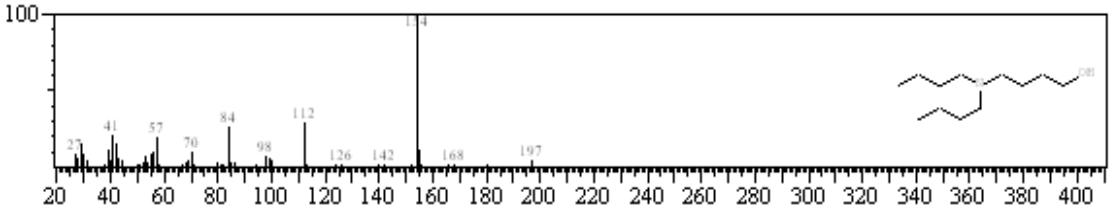
Hit#:1 Entry:38896 Library:NIST107.LIB
SI:88 Formula:C11H18N2O2 CAS:5654-86-4 MolWeight:210 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- $$



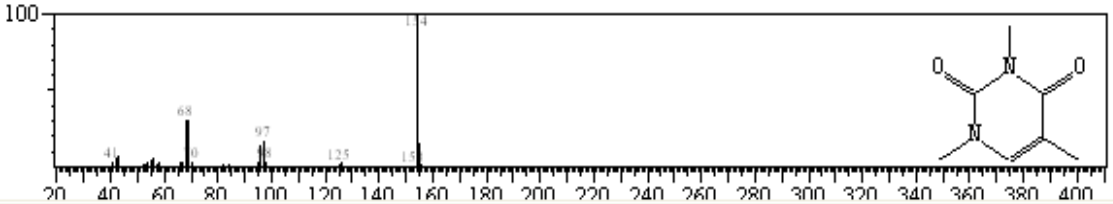
Hit#:2 Entry:27736 Library:NIST107.LIB
SI:75 Formula:C10H21BO2 CAS:74744-58-4 MolWeight:184 RetIndex:0
CompName:1,3,2-Dioxaborinane, 2,4,6-triethyl-5-methyl- $$



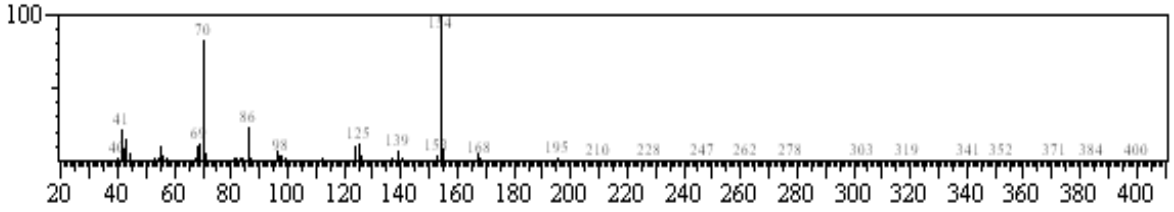
Hit#:3 Entry:33382 Library:NIST107.LIB
SI:75 Formula:C12H23NO CAS:0-0-0 MolWeight:197 RetIndex:0
CompName:4-Dibutylamunobut-2-yn-1-ol $$



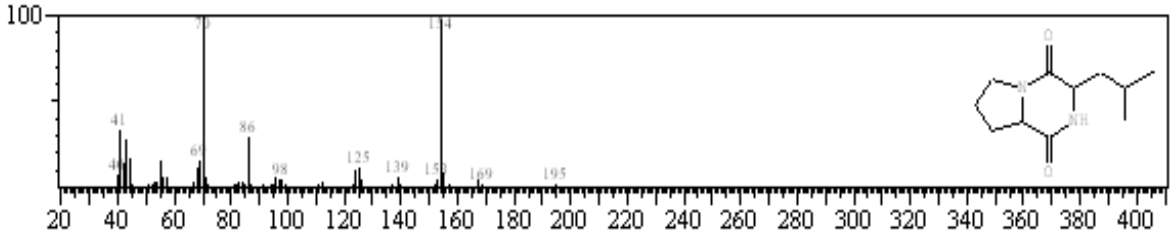
Hit#:4 Entry:7613 Library:NIST21.LIB
SI:75 Formula:C7H10N2O2 CAS:4401-71-2 MolWeight:154 RetIndex:0
CompName:2,4(1H,3H)-Pyrmudinedione, 1,3,5-trimethyl-



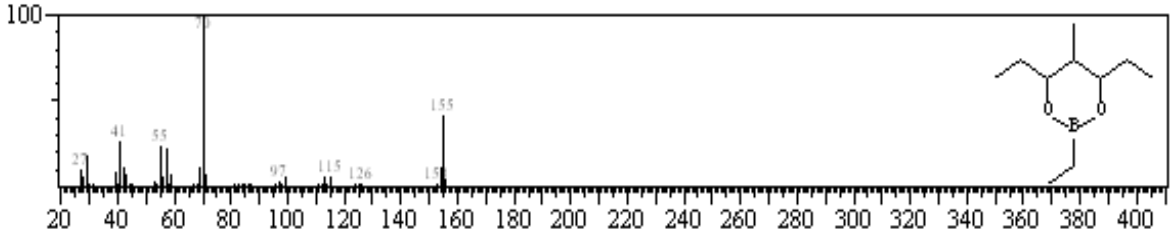
<< Target >>
Line#:8 R.Time:22.817(Scan#:2079) MassPeaks:215
RawMode:Averaged 22.808-22.825(2078-2080) BasePeak:154.10(44652)
BG Mode:Calc. from Peak



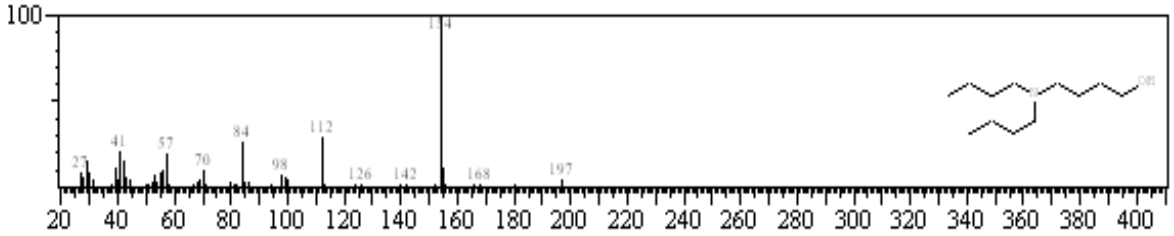
Hit#:1 Entry:38896 Library:NIST107.LIB
SI:92 Formula:C11H18N2O2 CAS:5654-86-4 MolWeight:210 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- $$



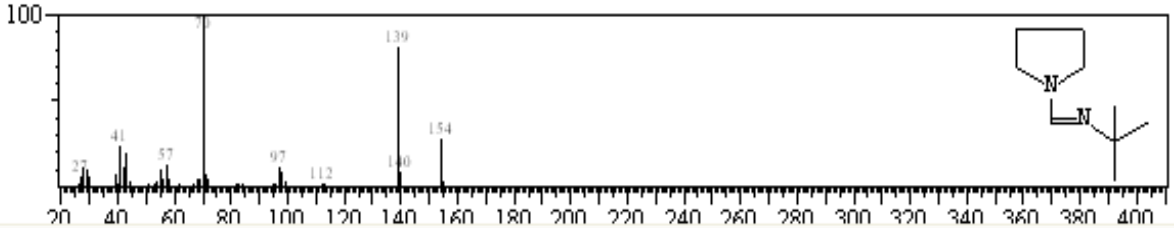
Hit#:2 Entry:27736 Library:NIST107.LIB
SI:75 Formula:C10H21BO2 CAS:74744-58-4 MolWeight:184 RetIndex:0
CompName:1,3,2-Dioxaborinane, 2,4,6-trethyl-5-methyl- $$



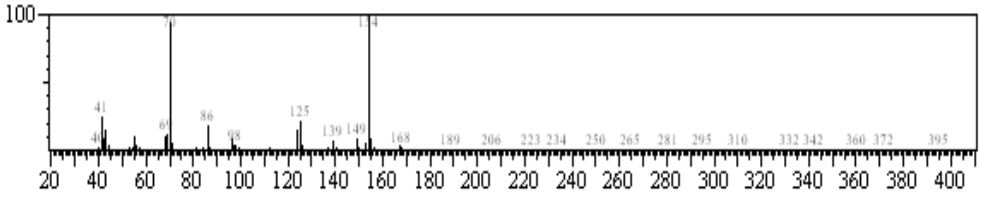
Hit#:3 Entry:33382 Library:NIST107.LIB
SI:75 Formula:C12H23NO CAS:0-0-0 MolWeight:197 RetIndex:0
CompName:4-Dibutylamunobut-2-yn-1-ol $$



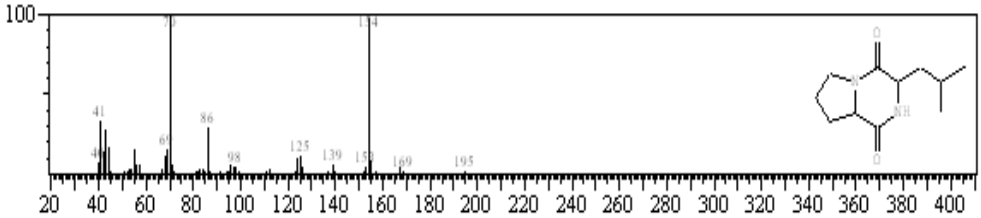
Hit#:4 Entry:15033 Library:NIST107.LIB
SI:74 Formula:C9H18N2 CAS:0-0-0 MolWeight:154 RetIndex:0
CompName:2-Propanamine, 2-methyl-N2-[1-tetrahydro-1H-1-pyrrolylmethylidene] $$



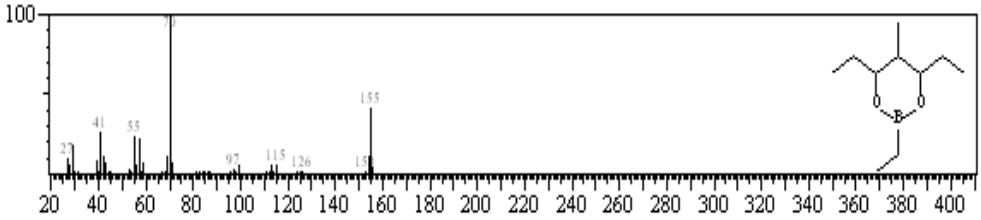
<< Target >>
Line#:9 R.Time:22.917(Scan#:2091) MassPeaks:238
RawMode:Averaged 22.908-22.925(2090-2092) BasePeak:154.15(15262)
BG Mode:Calc. from Peak



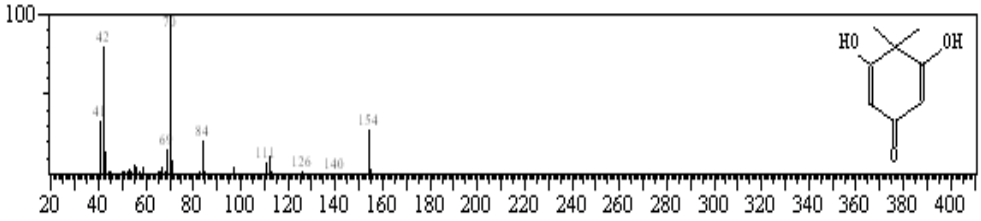
Hit#:1 Entry:38896 Library:NIST107.LIB
SI:90 Formula:C11H18N2O2 CAS:5654-86-4 MolWeight:210 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- $$



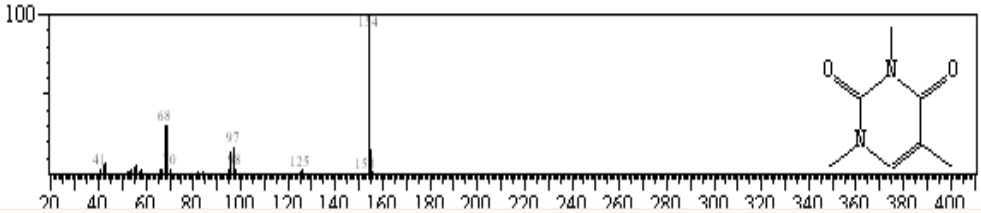
Hit#:2 Entry:27736 Library:NIST107.LIB
SI:75 Formula:C10H21BO2 CAS:74744-58-4 MolWeight:184 RetIndex:0
CompName:1,3,2-Dioxaborinane, 2,4,6-trimethyl-5-methyl- $$



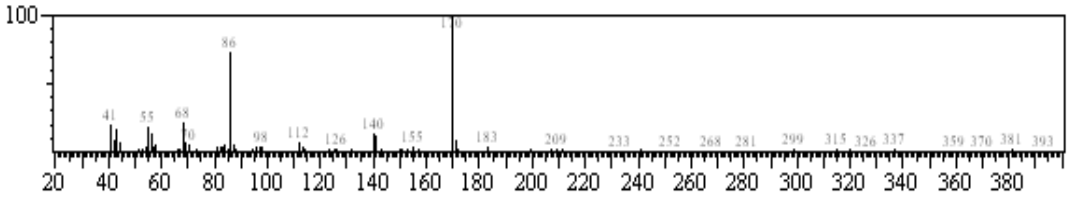
Hit#:3 Entry:14828 Library:NIST107.LIB
SI:74 Formula:C8H10O3 CAS:2065-0-1 MolWeight:154 RetIndex:0
CompName:2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,4-dimethyl- $$ Fihcmic acid $$



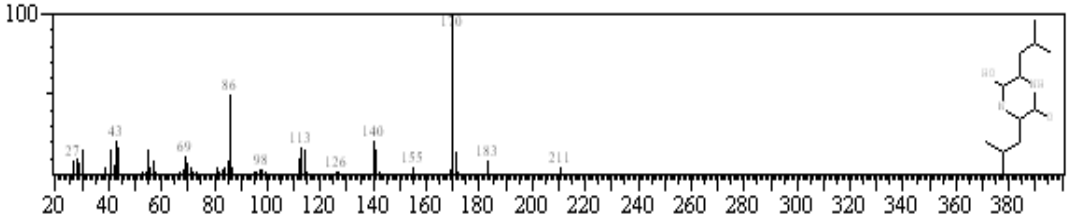
Hit#:4 Entry:7613 Library:NIST21.LIB
SI:73 Formula:C7H10N2O2 CAS:4401-71-2 MolWeight:154 RetIndex:0
CompName:2,4(1H,3H)-Pyrimidinedione, 1,3,5-trimethyl-



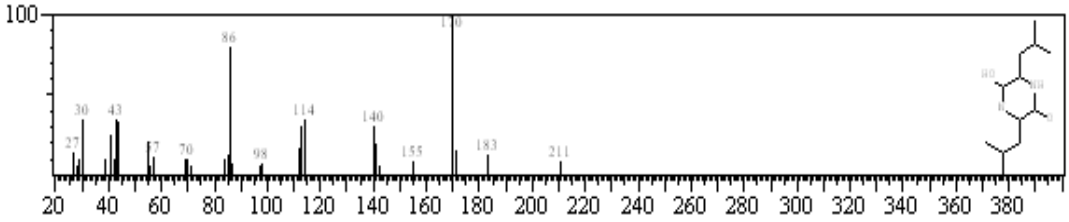
<< Target >>
Line#:10 R.Time:25.483(Scan#:2399) MassPeaks:206
RawMode:Averaged 25.475-25.492(2398-2400) BasePeak:170.10(4234)
BG Mode:Calc. from Peak



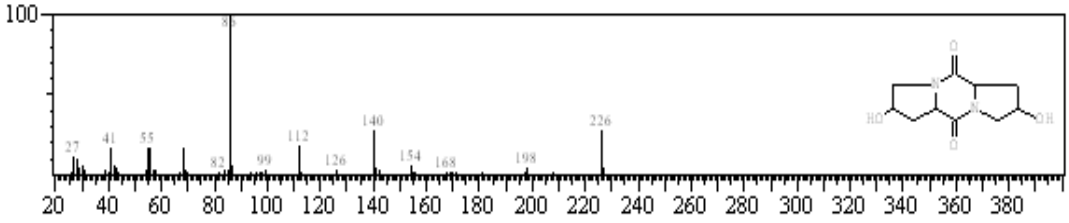
Hit#:1 Entry:45734 Library:NIST107.LIB
SI:86 Formula:C12H22N2O2 CAS:1436-27-7 MolWeight:226 RetIndex:0
CompName:2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- $$



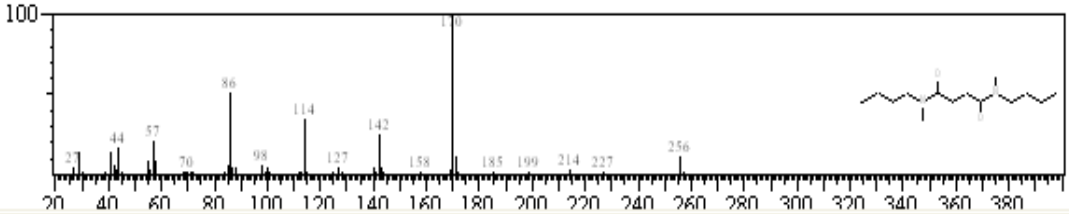
Hit#:2 Entry:14530 Library:NIST21.LIB
SI:81 Formula:C12H22N2O2 CAS:1436-27-7 MolWeight:226 RetIndex:0
CompName:2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-



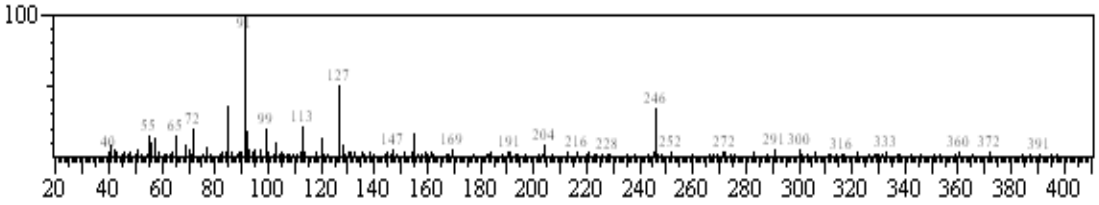
Hit#:3 Entry:45591 Library:NIST107.LIB
SI:73 Formula:C10H14N2O4 CAS:52363-44-7 MolWeight:226 RetIndex:0
CompName:Dipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione, octahydro-2,7-dihydroxy- $$



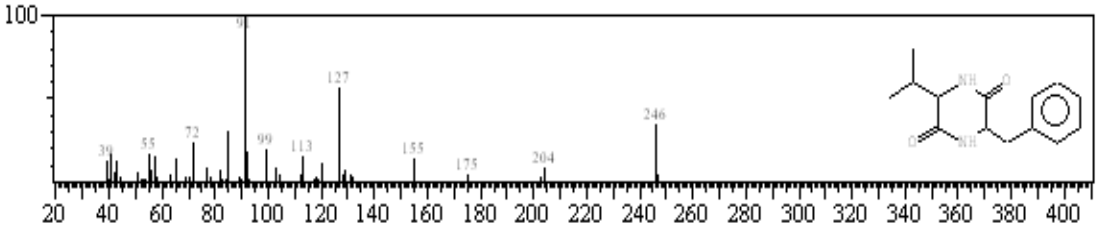
Hit#:4 Entry:56967 Library:NIST107.LIB
SI:73 Formula:C14H28N2O2 CAS:0-0-0 MolWeight:256 RetIndex:0
CompName:N,N'-Dibutyl-N,N'-dimethyl-succinamide $$



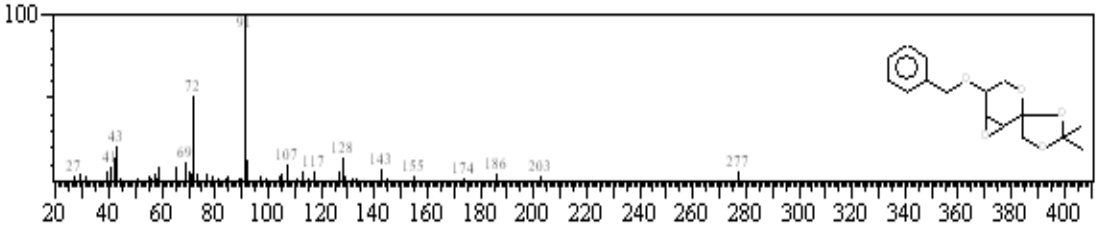
<< Target >>
Line#:11 R.Time:26.742(Scan#:2550) MassPeaks:214
RawMode:Averaged 26.733-26.750(2549-2551) BasePeak:91.10(1304)
BG Mode:Calc. from Peak



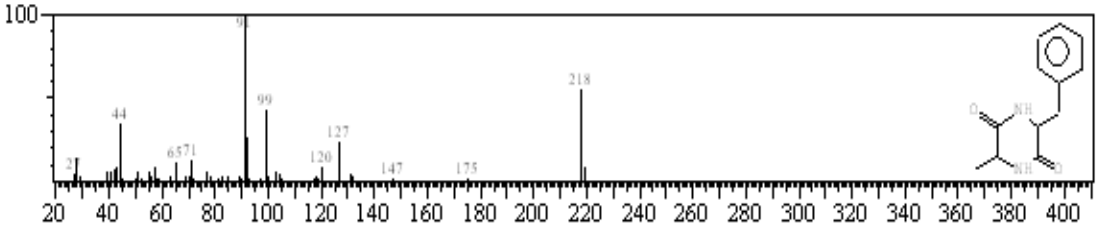
Hit#:1 Entry:53271 Library:NIST107.LIB
SI:83 Formula:C14H18N2O2 CAS:14474-71-6 MolWeight:246 RetIndex:0
CompName:2,5-Piperazinedione, 3-benzyl-6-isopropyl- $$



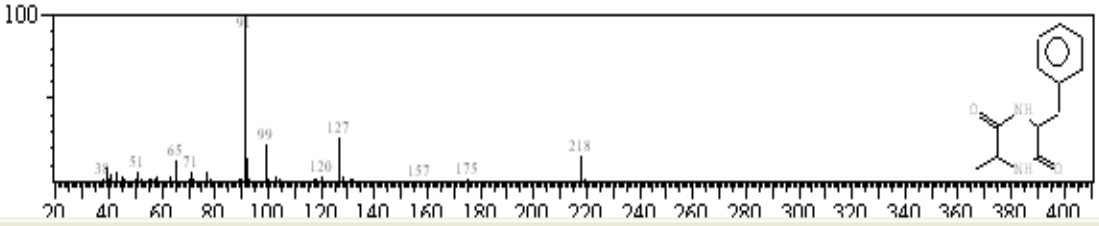
Hit#:2 Entry:69166 Library:NIST107.LIB
SI:61 Formula:C16H20O5 CAS:0-0-0 MolWeight:292 RetIndex:0
CompName:9,10-Epoxy-1,3,6-trioxaspiro[4.5]decane, 2,2-dimethyl-8-phenylmethoxy- $$



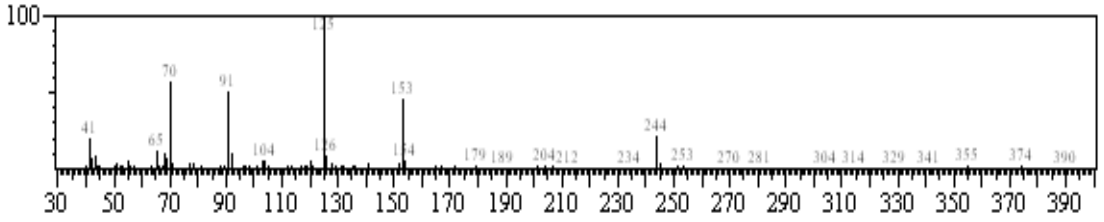
Hit#:3 Entry:13982 Library:NIST21.LIB
SI:60 Formula:C12H14N2O2 CAS:14474-78-3 MolWeight:218 RetIndex:0
CompName:2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-



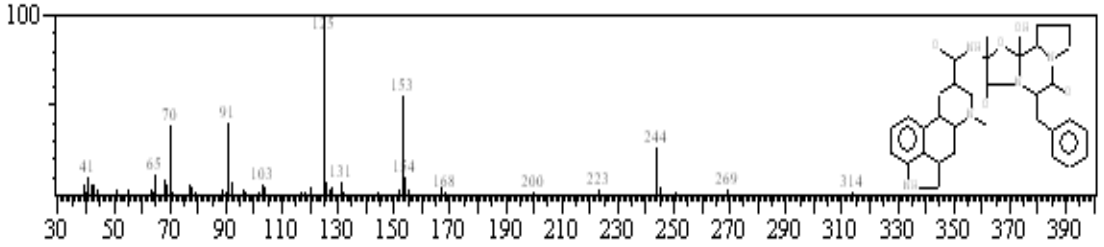
Hit#:4 Entry:42174 Library:NIST107.LIB
SI:60 Formula:C12H14N2O2 CAS:14474-78-3 MolWeight:218 RetIndex:0
CompName:2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)- $$



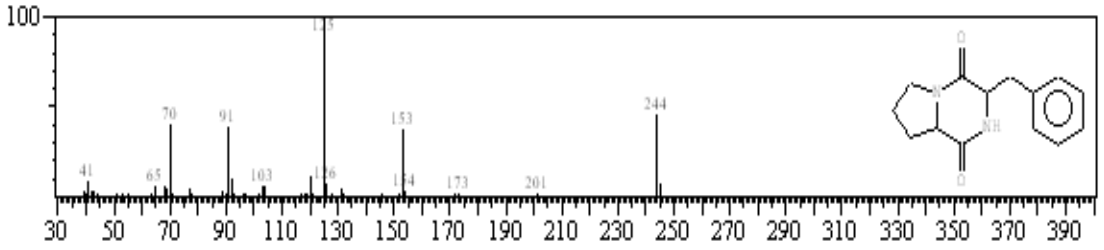
<< Target >>
Line#:12 R.Time:27.858(Scan#:2684) MassPeaks:224
RawMode:Averaged 27.850-27.867(2683-2685) BasePeak:125.10(4618)
BG Mode:Calc. from Peak



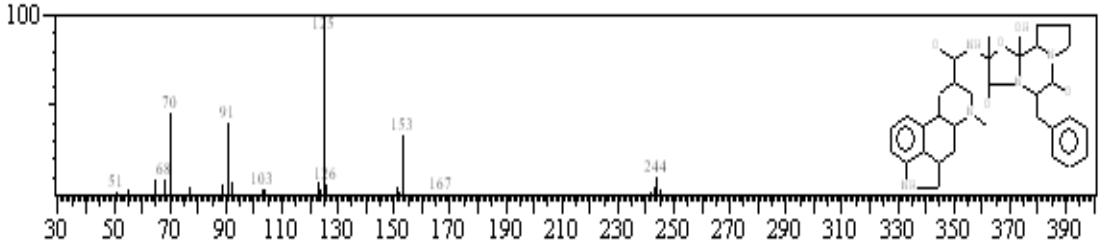
Hit#:1 Entry:21087 Library:NIST21.LIB
SI:89 Formula:C33H37N5O5 CAS:511-12-6 MolWeight:583 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)-



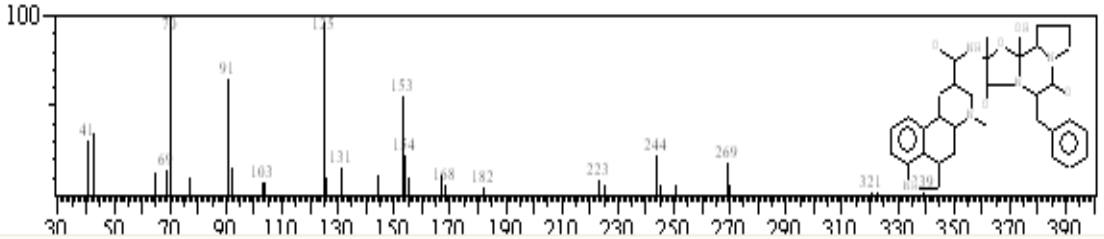
Hit#:2 Entry:52598 Library:NIST107.LIB
SI:89 Formula:C14H16N2O2 CAS:14705-60-3 MolWeight:244 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- $$



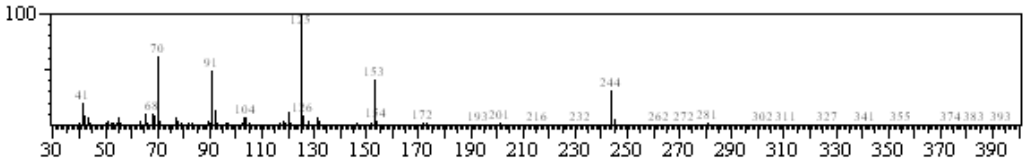
Hit#:3 Entry:21086 Library:NIST21.LIB
SI:80 Formula:C33H35N5O5 CAS:113-15-5 MolWeight:581 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.)-



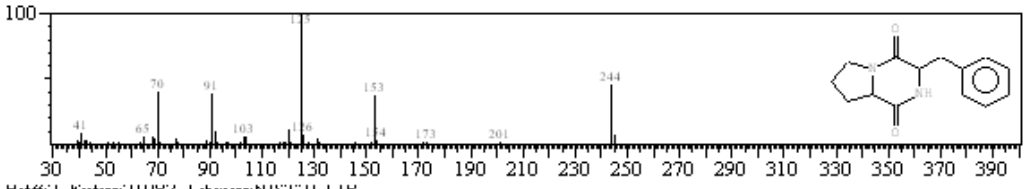
Hit#:4 Entry:105330 Library:NIST107.LIB
SI:77 Formula:C33H37N5O5 CAS:511-12-6 MolWeight:583 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)- $$



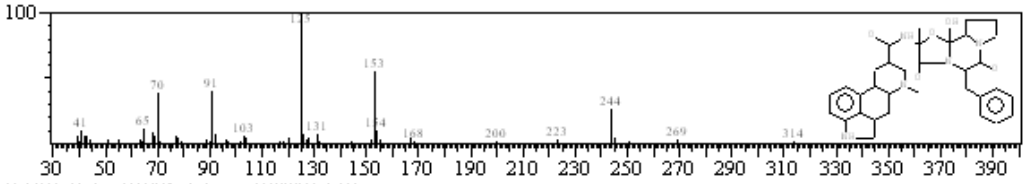
<< Target >>
Line#:13 R.Time:28.408(Scan#:2750) MassPeaks:239
RawMode:Averaged 28.400-28.417(2749-2751) BasePeak:125.10(13126)
BG Mode:Calc. from Peak



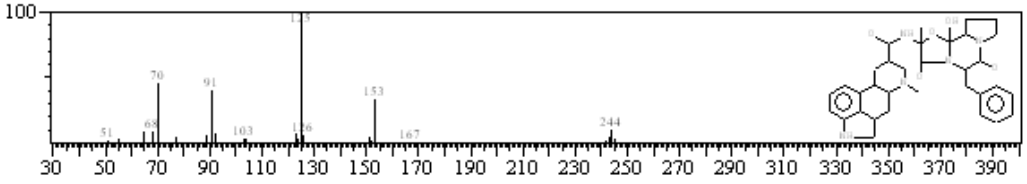
Hit#:1 Entry:52598 Library:NIST107.LIB
SI:93 Formula:C14H16N2O2 CAS:14705-60-3 MolWeight:244 RetIndex:0
CompName:Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- $$



Hit#:2 Entry:21087 Library:NIST21.LIB
SI:89 Formula:C33H37N5O5 CAS:511-12-6 MolWeight:583 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)-



Hit#:3 Entry:21086 Library:NIST21.LIB
SI:78 Formula:C33H35N5O5 CAS:113-15-5 MolWeight:581 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.)-



Hit#:4 Entry:105330 Library:NIST107.LIB
SI:76 Formula:C33H37N5O5 CAS:511-12-6 MolWeight:583 RetIndex:0
CompName:Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)- $$