UNIVERSITY OF NAIROBI

ISOLATION AND CHARACTERIZATION OF BIOMASS MODIFYING ENZYMES FOR BIOREMEDIATION AND PRODUCTION OF "GREEN SPECIALTY PRODUCTS"

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work, or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirement

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DEDICATION

This work is dedicated to my dear parents for their steadfast support and sacrifice they made throughout my studies and always believing in my academic potential. You are my biggest pride.

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This task has been accomplished with the grace of God. I am grateful for the wisdom, blessings and perseverance that he has showered upon me during the course of this project and indeed, throughout my life.

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No one walks alone in this journey of life: to my family and friends thanks for walking beside me and urging me to move on even when I had no will to.

ABSTRACT

Environmental pollution has been a major problem and poses many challenges to sustainable development to the chemical-based industrial development in both developed and developing countries. Leather industry which extensively uses lime and sodium sulfide in dehairing hides and skins is currently facing serious environmental pollution problems. Lime and sodium sulfide heavily contribute to increased odour, effluent toxicity, health hazards to the tannery workers, production of poisonous sludge with disposal challenges and blockage to sewerage pipes. Similarly, use of organic dyes in industries pose pollution problems in the form of colored wastewater discharge into water bodies resulting into high turbidity, increased chemical oxygen demand (COD) and reduced light penetration. Furthermore, dyes are not only recalcitrant and refractory pollutants but also toxic, mutagenic and carcinogenic. To address these concerns, the present research affords the opportunity to isolate crude alkaline protease enzyme from extremophile bacteria from Lake Bogoria, Kenya and apply the isolated enzyme in processing leather and bioremediation of industrial wastewater containing organic dyes as part of the process of developing biotechnology for solving environmental problems.

Four (4) protease producing bacterial strains were isolated from Soil samples collected from different parts of Lake Bogoria in the Kenyan Rift Valley. Biochemical test and phylogenetic characterization indicated that the four isolates were all associated mainly with members of the *Bacillus Cereus*. The submerged fermentation process parameters such as pH, temperature, incubation time, different substrates and concentrations that influence production of protease enzyme were analyzed and optimized for large scale enzyme production.

Decolorization and biodegradation of Malachite Green (MG) dye and Reactive Black 5 (RB5) dye was studied using crude alkaline protease enzyme from isolate *1-p*. From the dynamic batch

experiments carried out for dye decolorization, over 98% decolorization efficiency was achieved within 24 hours for MG using an initial dye concentration of 1.0×10^{-5}M. Similarly, over 98% decolorization was achieved within 120 hours for RB5 using an initial dye concentration of 1.0×10^{-4}M. Experimental results revealed that the decolorization process was highly dependent on contact time, initial dye concentration, aqueous solution temperature and pH. Biodegradation of dyes was monitored by UV-VIS spectrophotometer and the resultant metabolites confirmed by Thin Layer Chromatography (TLC), Liquid Chromatography–Hybrid Quadrupole Time of Flight Mass Spectrometry (LC–QToF-MS) and Gas Chromatography/Mass Spectrometry (GC - MS). The results obtained revealed that the enzymatic degradation of MG and RB5 by crude alkaline protease enzyme from strain *1-p* resulted in complete mineralization and benzene ring-removal; the latter known to be responsible for the organic dye toxicity.

Kinetic study results revealed that first-order kinetic equation was the appropriate equation to describe decolorization of both MG and RB5 dye. Michaelis-Menten kinetics, Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the dye decolorization process. Lineweaver–Burk plot provided the best theoretical correlation of the experimental data for the decolorization of both dyes. The maximum rate (V_{max}) and Michaelis-Menten constant (K_m) were found to be 17.70 mg l^{-1} h^{-1} and 124 mg l^{-1}, respectively for MG dye and 3.975 mg l^{-1} h^{-1} and 548.06 mg l^{-1} for RB5, respectively using Lineweaver–Burk plot. The results provide evidence that the crude enzyme from *Bacillus cereus* strain *1-p* is an effective and potential candidate for industrial wastewater treatment

The efficacy of the isolated crude alkaline protease enzyme from *Bacillus cereus* strain *1-p* to dehair cow hide and descale Nile perch (*Lates niloticus*) skin was investigated. Factors affecting enzyme dehairing process such as temperature, pH and incubation time were determined and

optimized. Industrial demonstrations of large scale beam house dehairing process (trial) was successfully accomplished whereby a 21 kg full bull hide was 100% chemical free dehaired and degreased using the isolated crude alkaline protease enzyme within 8 hours compared to the conventional method which take between 2- 6 days. Furthermore, 10 pieces (9.5 kg) of Nile perch (*Lates niloticus*) skin were successfully descaled and degreased within 2 hours. 1.1 kg of quality hair and 0.98 kg of valuable scales removed during dehairing/descaling process were readily recovered while still in good condition suitable for further use. The envisaged recoveries of hair and scales should greatly contribute to the reduction of BOD, COD and TDS loads in effluents. Physical-mechanical characteristics of the resultant leather after full processing was analyzed and the results compared with the set Kenya Bureau of Standards (KEBS) standards. The results revealed that the quality of the final leather from bull hide was within the set KEBS standards.

In view of the results obtained and considerations of environment protection, application of crude alkaline protease enzyme from *Bacillus cereus* strain *1-p* is promising to yield a clean technology which completely eliminates the traditional use of sodium sulfide and lime in dehairing. These will greatly reducing the pungent smell which is characteristic of tanneries across the world without compromising the quality of leather. This can be seen as a means to producing "green" leather through a "green leather" process. Furthermore, the same enzyme can be utilized in bioremediation of organic dye in wastewater effluent.

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LIST OF ABBREVIATIONS

A	Adenine
BOD	Biological Oxygen Demand
bp	base pair
C	Cytosine
C_0	Dye concentration at the beginning of the incubation through decolorization (mgl^{-1})
COD	Chemical Oxygen Demand
C_t	Residual dye concentration at selected time (t) of batch test through decolorization (mg dye l^{-1})
DNA	Deoxyribonucleic Acid
G	Guanine
GC - MS	Gas Chromatography/Mass Spectrometry
k_0	Zero order rate constant through decolorization (mg l^{-1} h^{-1})
k_1	First order rate constant through decolorization (h−1)
k_2	Second order rate constant through decolorization (l mg^{-1} h^{-1})
Kb	Kilo base pair
KEBS	Kenya Bureau of Standards
k_m	Michaelis constant
kp	kilobase
LC-QToF-MS	Liquid Chromatography–Hybrid Quadrupole Time of Flight Mass Spectrometry
LD_{50}	Lethal Dose, 50%
MG	Malachite green dye
PCR	Polymerase Chain Reaction

R^2	Regression analysis coefficient
RB5	Reactive Black 5 dye
rpm	Revolutions per minute
T	Thymine
TDS	Total Dissolved Solids
TLC	Thin Layer Chromatography
V	velocity
V_{max}	Maximal velocity
SDS	Sodium dodecyl sulfate

CHAPTER ONE

1.0 INTRODUCTION

1.1   Background of the Study

Leather is recognized and valued as one of the most important ancient natural products still surviving in the industrial era, and is even more appreciated as it still brings in itself a degree of emotion that technology is hardly ever able to classify (Beghetto *et al*., 2013). This industry plays an important role in today's global economy by providing necessities such as leather clothing, body armor, boots, saddles, hunting accessories, weapons, tent coverings, containers, boat coverings, paper, dog chews, drum heads, book bindings, lacing, shoes, purses, gloves, luggage, coats, clothing accessories and other garments while using animal hides/skins a by-product of the meat industry. Developing and developed countries which depends on the agro-economy earn foreign currency and create employment from leather processing and production.

It has been estimated that about 18 billion ft^2 of leather are made annually around the world with a trade value estimated to be approximately US$ 70 billion (Rao *et al*., 2003). In this respect, the leather processing industries could be considered as an environmentally friendly industry which processes waste products from food industry into noble and highly valuable products which otherwise would have been disposed off. However, the leather industry has commonly been associated with high pollution due to the bad smell, organic wastes and high water consumption caused during traditional manufacturing processes (Taylor *et al.*, 1998). Different kinds of waste in quantity and quality emerge at different stages of hides and skins processing in thousands of leather factories; from primitive to modern all around the world, have negative impacts on the environment. For example, tanneries across the world are constantly concerned with the

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obnoxious odour and the pollution caused by the extremely toxic sodium sulfide used in the dehairing process. Conventional leather processes employs about 25-30 L of process water per kg of hide and subsequently discharges them as wastewater along with pollutants (Christopher *et al*., 2014). This leads to the generation of enormous amounts of liquid effluent. The high effluent volume requires huge investments for effluent treatment plants in order to meet the required specification for the discharge of liquid effluents to various water bodies (Rao *et al*., 2003).

Environmental pollution is currently the major setback to chemical-based industrial development in both developed and developing countries due to pollution problems. In leather processing, the first step in the beam house is to remove hair from hides and skins before tanning. The conventional dehairing method involves the use of high proportions of lime and sulfide, which contributes 80–90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan *et al.*, 2004), a serious hazard for both tannery and sewer workers. Deaths attributable to this toxic chemical process have also been reported (Balasubramanian and Pugalenthi, 2000; Gupta *et al*., 2002a). Moreover, conventional lime and sulfide process leads to the destruction of the valuable hairs uprooted from the skin which results in high Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD) and Total Dissolved Solids (TDS) loads in the effluents and the skin is also shrunken and distorted in shape (Saran *et al*., 2013).

The huge solid wastes are generated as by-products from various process steps during leather-making and manufacturing of garment. Chromium is one of the most important environmental concerns related to tanneries. Management of chrome-containing shavings is not easy and nowadays, chrome shavings and solid wastes are mainly disposed in landfills. In general, conventional dehairing method and leather processing causes huge air, water and soil pollution.

Considering the severe environmental pollution in dehairing of skin/hide process and high costs incurred in wastewater disposal, there is a need to develop alternative cleaner technologies in order to minimize the wastes generated from the industry in an effort to combat environmental pollution problem and comply with environmental norms. Enzymes are well known biocatalysts that perform a multitude of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Enzymatic dehairing in tanneries has been envisaged as an alternative to the use of lime and sulfide. The major advantages of enzymes in dehairing and fibre opening processes are: complete removal of lime and sodium sulfide, recovery of hair/wool with good quality (Jian *et al.*, 2008) and better leather strength characteristics. Furthermore, uses of enzymes in hide/skin dehairing can create an ecologically conducive and safe atmosphere for the tannery workers. Natural microbial isolates may be used to produce enzymes, and these natural sources may be obtained from different environments.

Similarly, synthetic dyes have been extensively used in a number of industries such as textile dyeing and paper printing. It was estimated that over 10,000 commercially available dyes exist and more than 7×10^5 tons of dyestuffs are produced annually (Ayed *et al.*, 2011). Dyes are widely used in these industries for coloring products to make them attractive to the user. As a result, a large volume of water containing processed dyes is released into the environment. The extensive use of dyes often poses pollution problems in the form of colored wastewater discharges into water bodies. Dyes are not only recalcitrant and refractory pollutants that constitute a significant burden on the environment, but are also toxic, mutagenic and carcinogenic. The strong colour of industrial wastewaters containing dyes even at a small concentration has a huge impact on the aquatic environment due to their turbidity, increased

COD and reducing light penetration, which has adverse effects on photosynthetic phenomena (Wanyonyi *et al*., 2014a).

In developing nations like Kenya, treatment of synthetic dyes in wastewaters is a matter of great concern. There is a need to eliminate these hazardous substances in a cost effective manner due to limited financial and budgetary allocation geared towards environmental remediation. Several physical and chemical methods have been employed for the removal of dyes (Wanyonyi *et al*., 2013, 2014a, 2014b). However, these procedures have not been widely used due to their high cost, formation of hazardous by-products and intensive energy requirement.

Chemical and chemical-based industries are the prime targets of the environmental activists for their crusade against pollution, and leather, textile, and paper industries have not been left out of such environmental protection campaigns. There is an enormous pressure from governments and various pollution control bodies and environmentalists to regulate and minimize the amount of pollution generated from leather processing as per the laid down standards. Moreover, in this scenario of global warming, limited resources and climate change, greener technologies with zero or little wastage or pollution are in high demand globally.

Extremophiles like alkaliphile bacteria exhibit the ability to grow at extremely harsh environmental conditions such as high pH and temperature, high levels of salinity or salt, and pressure which critically influence their growth (Wanyonyi *et al*., 2014a). Enzymes from Extremophile can be utilized in leather preparation processes to improve leather product quality and reduce pollution load in the industry. Alkaline proteases can be used as they enable the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair (Gupta *et al*., 2002). Although enzyme-assisted dehairing process reduces the pollution load to some extent, a technology based on enzyme alone, without the use

of sulfide and other chemical inputs, has yet to be explored (Thanikaivelan *et al*., 2004). Several after-treatment methods for industrial wastewater have been explored and some have been adopted commercially as the need to prevent pollution through in-plant measures is gaining importance. The most significant approach in preventing environmental pollution is the idea that prevention is better than reuse, reuse is better than recycling, and recycling is better than disposing of the wastes (Rao *et al*., 2003). In-plant control measures for mitigating tannery pollution should aim at the reduction or elimination of toxic wastes through process adjustments.

1.2   Statement of the Problem

Hides and skins come to the tanneries as a by-product of the meat industry. Conventional leather processing involves several unit operations, in which the use of chemicals like lime, sodium sulfide, chrome in different stages of leather processing especially in pre-tanning and tanning processes brings about almost 80-90% of the pollution. Besides sulfide liberating toxic hydrogen sulfide gas, a serious hazard for both tannery and sewer workers, leather processing produces large amounts of liquid, solid and hazardous wastes which are toxic to health and difficult to dispose. Use of large quantities of water in the dehairing process and careless disposal of solid wastes has come under severe scrutiny due to scarcity of water and the unavailability of landfills in Kenya. Currently, the leather industry is facing stringent regulation from pollution control boards to meet the effluent discharge limits (Christopher *et al*., 2014). In summary, the leather industry is a heavy pollution industry and efforts to develop new clean technologies are crucial for its survival. To address problems encountered in the leather industry, enzymes can be a better alternative to conventional chemicals in the context of cleaner leather processing as well as solid waste management.

Similarly, synthetic dyes used in various industries are common water pollutants due to their good solubility. The textile industry is considered as one of the largest water consumers in the world. It is rapidly expanding, and dyes are also continuously being upgraded and replaced by superior compounds that have enhanced fastness, stability, brightness and resistance to natural degradation such as sunlight. Due to the hazardous effects of organic dyes to the environment and human life, their presence in water is unwanted and it is desirable to remove them from the effluents before being discharged into the environment. Physical and chemical methods can be used for remediation, but these methods are costly and sometimes produce hazardous by-products (Zhang *et al.*, 2012). Therefore, development of new technologies which are economical, renewable and highly effective to cope with the treatment of dye-bearing effluents in textile wastewater cannot be over emphasized.

1.3 Objective

1.3.1 General Objective

The overall objective of this study is to isolate biomass-modifying enzymes from microorganisms of Kenyan origin and apply the isolated enzyme in bioremediation of organic dyes, leather processing and production of green specialty chemical products. This is part of a strategy to exploit biotechnology for solving environmental problems.

1.3.2 Specific Objectives

The specific objectives of this study are:

1. To isolate, identify and characterize biomass-modifying enzymes from micro-organism of Kenyan origin.

2. To investigate the efficacy of the isolated enzyme in dehairing of hide and descaling of fish skin and determine mechanical properties of leather products obtained.

3. To determine the efficacy of the isolated enzyme in bioremediation of Malachite Green dye and Reactive Black 5 dye - containing aqueous solution.

4. To determine the kinetics of decolorization of Malachite Green dye and Reactive Black 5 by the enzymes

1.4   Justification and Significance of the study

The leather industry has already marked a place of prominence in the Kenya economy and national planning in view of its massive potential for employment generation, growth and exports. The sector generates KES Sh10 billion per year and has the capacity to generate 10 times more if the country moved from export of wet blue (semi-processed) to finished leather. Due to its huge potential, the government through the ministry of Industrialization and Enterprise Development plans to establish about 15 tanneries; although there is potential to have even more. (*http://www.nation.co.ke/business/Machakos-to-host-planned-leather-city/-/996/2588514/-/8w6otp/-/index.htm*l). However, these plans are held back by the huge investment required in the establishment of the effluent treatment plant systems. Beside, over 60% of the land area for livestock production is located mostly in the arid and semi-arid lands (ASAL) of Kenya where there is scarcity of water.

Leather industry has commonly been associated with high pollution due to the bad smell, organic wastes and high water consumption caused during traditional manufacturing processes. Conventional leather processing involves use of toxic chemicals like lime, sulfide, and chrome in different stages of leather processing especially in pre-tanning and tanning processes accounts for about 80-90% of the pollution. Another source of environmental pollution in the leather

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industry is solid wastes generated as by-products from various process steps during leather-making and manufacturing of garments. Most of these wastes contain chromium and its management poses various challenges.

Similar problems are encountered in the textile industry that consumes huge quantities of organic dyes which are toxic, mutagenic and carcinogenic hence the prevalent problems with water quality. These problems are particularly more serious in a developing nation like Kenya where regulatory compliance is low and may not be fully enforced.

Identifying suitable pollution prevention techniques that will minimize or eliminate pollution in process streams, or end-pipe treatment techniques that allows reuse of treated wastewaters appear to be an ideal solution to the existing pollution problems from leather and textile industries. In this respect, biotechnological approaches can overcome the problems posed by use of chemicals by making use of natural capabilities of microorganisms and/or their enzymes. Use of microorganisms or their enzymes for industrial processes offers a viable option to decrease or eliminate environmental pollution. Microorganisms represent excellent sources of enzyme production owing to their broad biochemical diversity, rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. The use of enzyme-based products is currently being explored in many areas of leather making process, with increasing importance in the dehairing process, thus eliminating the use of hazardous sodium sulfide (Thanikaivelan *et al.*, 2004). However, the potential micro-organisms and their enzymes from Kenya's extremophile environments like Lake Bogoria have not been extensively exploited.

The research work described in this thesis seeks to address these pollution problems by evaluating the potential of utilizing protease enzymes from micro-organisms of Lake Bogoria in dehairing hide, descaling of fish skin and in the biodegradation of selected organic dyes. The research work is important because it aims at developing robust tools for the leather industry; providing an incentive for adopting eco-friendly and sustainable technology. Biotechnology has a high potential for improving various aspects of leather making processes with a view to reducing costs, raising product quality or lessening the environmental impact of an industry which has traditionally been deemed highly contaminating. The study is part of a concerted effort to reduce and/or eliminate threats of using toxic chemicals in industrial processing, rehabilitate uncontrolled hazardous waste sites and remove hazardous substance threats to public health and the environment in a more cost-effective manner.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Synthetic Dyes

Dyes are an important class of chemicals widely used in many industrial processes such as leather, pharmaceutical, plastics, rubber, coke, textile, petroleum, printing, food, cosmetics, paper and pulp industries. Synthetic dyestuffs have been increasingly used in industries because of their wide variety of color shades, affordability, high wet fastness and stability profiles, ease of application, brilliant colors, and minimal energy consumption compared with natural dyes. Although the exact number and amount of dyes produced in the world is not known, it is estimated that over 100,000 commercially available dyes with a production rate of 7×10^5 metric tons each year are consumed extensively in a wide range of industries (Kiran *et al*., 2009). It is estimated that textile industries alone generate about 4500 million kiloliters of wastewaters annually (Singh *et al*., 2013). As a result, a large volume of water-containing processed dyes and other industrial wastes are released into the environment. These dyes usually have a complex aromatic structure which makes them resistant to biodegradation by conventional biological treatment methods (Safa and Bhatti, 2011; Song *et al*., 2011). Furthermore, some dyes are either toxic or mutagenic and carcinogenic due to the presence of metals, chlorides, etc., in their structure (Aksu, 2003; Roibnson *et al*., 2001).

Environmental pollution due to large scale use of different varieties of organic dyes in industries is becoming increasingly alarming. Inefficiency of the dyeing processes, poor handling of spent effluent and insufficient treatment of wastes of the dyestuff industries lead to dye contamination of the environment such as soil and natural water bodies. Discharge of colored wastewater from

industries using organic dyes into natural streams causes many significant problems, such as increased toxicity and COD of the effluent, and also reducing light penetration, which adversely affects the photosynthetic phenomena (Bulut and Aydın, 2006).

Among the many classes of synthetic dyes used in the industries, triphenyl methane group of dyes such as malachite green and crystal violet constitute a major and versatile group that play a predominant role in almost every type of application (Moturi and Charya, 2009). Malachite oxalate green dye (MG) [(C$_{23}$H$_{25}$N$_2$)·(C$_2$HO$_4$)]2·C$_2$H$_2$O$_4$], (Bis[[4-[4-(dimethylamino) benzhydrylidene]cyclohexa-2,5-dien-1-ylidene]dimethylammonium] oxalate, dioxalate; N,N,N',N'-Tetramethyl-4,4'-diaminotriphenylcarbenium oxalate) MW 929.03g (Figure 2.1) has been found to be highly toxic to mammalian cells; promote hepatic tumor formation in rodents and cause reproductive abnormalities in rabbits and fish (Cha *et al*., 2001). It is absorbed and all tissues accumulate significant and fairly persistent residues of the dye, which shows high levels of toxicity to cells in fish as well as in other animals (Jang *et al*., 2009).
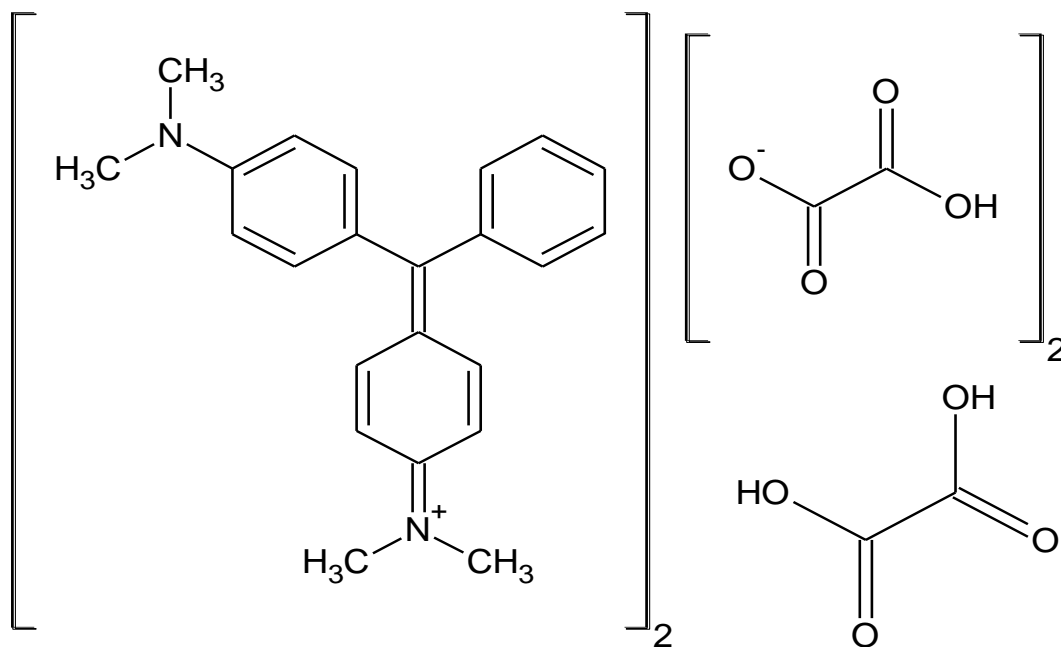
Figure 2.1: Structure of Malachite Green Oxalate (MG) dye

Malachite green dye was identified by the Food and Drug Administration as a priority chemical for carcinogenicity testing by the National Toxicology Programme of the USA (Culp and Beland, 1996). Despite its high toxicity, MG is currently used extensively for dyeing silk, paper, wool, jute, leather, ceramic and cotton due to its relatively low cost, ready availability, and efficiency. Therefore, potential human exposure to MG could result either from the consumption of treated fish, or from working in the dye and aquaculture industries.

Azo dyes is another group of synthetic organic dyes widely used in different industries such as textile dyeing, paper printing, manufacture of pharmaceutical drugs and toys. Azo dyes are usually designed to resist biodegradation under aerobic conditions. This chemical class of dyes, which is characterized by the presence of at least one azo bond (—N=N—) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70% (Soares *et al*., 2002). They are extensively used in the textile and leather industries due to their favorable characteristics of brilliant color, water-fast, simple application techniques with low energy consumption. However, they are characterized by high water solubility, non-biodegradability and low adsorption ability onto the biomass (Nabil *et al*., 2014). They differ from all other classes of dyes in that they bind to the cotton fibers by addition or substitution mechanisms under alkaline conditions and high temperatures (Aksu *et al*., 2007). In the leather industry, Azo dyes and chromium are common pollutants from tannery effluents (Ng *et al*., 2010). Reactive azo dyes released from dyeing industies are highly recalcitrant to conventional wastewater treatment processes (Lucas *et al*., 2006).

The Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) was inaugurated in 1974 with the goals of minimizing environmental damage, protecting users and consumers and cooperating with government and public concerns in relation to the

toxicological impact of their products (Robinson *et al.*, 2001). ETAD reported that the highest rates of toxicity (LD_{50}) were found amongst basic and diazo direct dyes. Azo dyes can be absorbed after skin exposure, and such dermal exposure to azo dyes can occur as an occupational hazard or from the use of cosmetic products (Chequer *et al.*, 2011). One of the major concerns on effluent toxicity is the possibility that some of its components could cause damage to genetic material of plants, animals or humans that come into contact with these molecules (genotoxicity)(Salas-Veizaga *et al.*, 2013)

Reactive Black 5 dye (RB5) also referred to as remazol black B ($C_{26}H_{21}N_5Na_4O_{19}S_6$, molecular weight of 991.8g/ mol) shown in Figure 2.2 is a tetrasulphonated disazo dye extensively used in the textile industry as the largest group of dying materials for cellulose and cotton fibers. Reactive dyeing is the most important method for the coloration of cellulosic fibres where a lot of water is applied. As a result, large quantities of colored wastewater is generated and discharged into the environment.
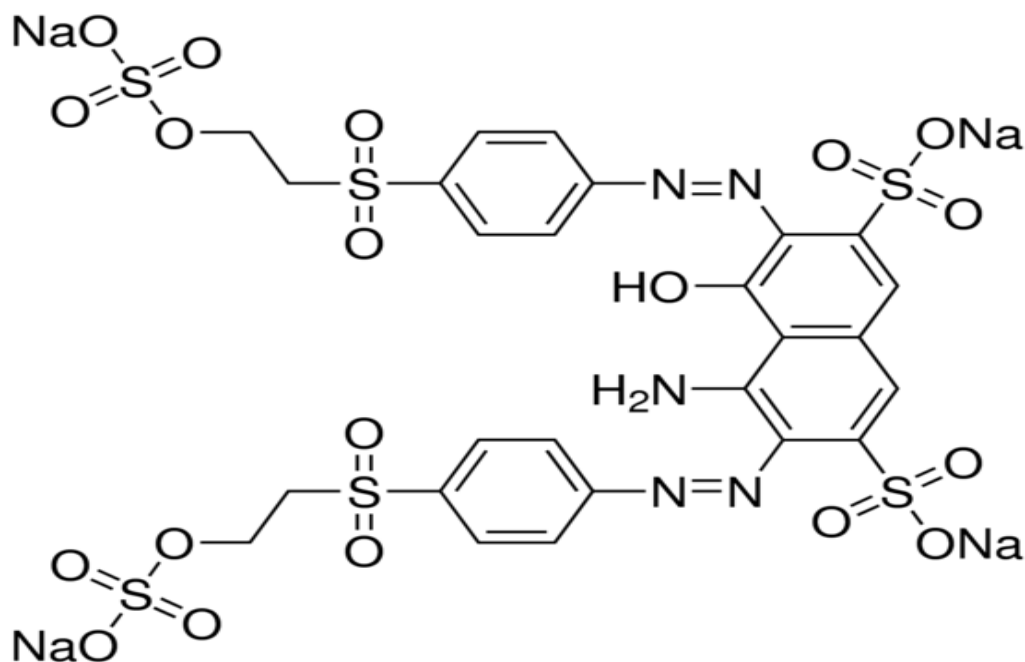
Figure 2.2. Chemical structure of Reactive Black 5 Dye.

The need to maintain a cleaner environment for the survival of both aquatic and terrestrial lives has forced many governments to establish environmental restrictions with regard to the quality of colored effluents discharged into the environment. Dying houses are currently being forced to decolorize/detoxify their effluents before discharging. Treatment of industrial wastewater-containing dyes is an essential, but difficult process because dyes usually have a complex aromatic structure which makes them resistant to biodegradation by conventional biological treatments. Conventional physicochemical methods such as adsorption with activated carbon, coagulation, precipitation, solvent extraction, membrane filtration, chemical oxidation, ozonation, and flocculation have been used to treat dye-containing effluents, however, most of these methods are expensive or require chemicals (Deepa *et al*., 2013)

Decolorization and degradation of dye in wastewater using microbial enzymes has been a subject of many studies in recent years due to their low cost of production and efficient application. Compared to physicochemical treatment methods, the enzymatic treatments of dyes have low energy cost and are more ecofriendly processes although not commonly used in the textile industries (Pengthamkeerati *et al*., 2010). Extremophile like alkaliphiles bacteria exhibit the ability to grow and survive in extremely harsh environmental conditions such as high pH and temperature, high levels of salinity or salt, and pressure which critically influence their growth. Products of industrial importance from alkaliphiles have been commercialized; the most successful of which have been in the detergent and food industries (Sarethy *et al*., 2011). However, relatively few studies have been reported on the application of alkaline enzyme in decolorization of organic dyes. The present study aims at investigating the ability of crude alkaline protease enzyme in decolorizing and degrading MG and RB5 dye.

2.1.1   Metabolites analysis

Analyzing degraded dye metabolite can provide an insight on how enzyme attacks the parent dye molecule at different positions thus providing information on degradation pathway. The efficacy of the isolated enzyme to biodegrade, mineralize and detoxify organic dye can only be determined by analyzing the resultant metabolites. Analytical procedures are characterized by a variety of technical operating parameters, such as accuracy, bias, precision, percent recovery, and dynamic linear range (Armbruster *et al*., 1994). Metabolomic studies make use mostly of hyphenated techniques which rely on chromatographic separation of metabolites using either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) to analyze complex mixtures of extracted metabolites (Farag *et al*., 2012). These methods provide a detailed chromatographic profile of the sample at different retention times and consequently measurements of the relative or absolute amounts of the components using a high resolution mass spectrometer.

2.1.1.1   Gas Chromatography/Mass Spectrometry (GC - MS)

GC–MS has been proven to be a robust, powerful and suitable tool for the determination of volatile compounds because of its high separation efficiency and sensitive detection (Kopka, 2006). GC-MS-based metabolomics requires a high-throughput technology to handle a large volume of samples and accurate peak identification through the standard retention times and mass spectra (Zhang et al., 2012a). Metabolite profiling with GC-MS involves six general steps as described by Kopka (2006)

i)   Metabolites Extraction from the biological sample or solution mixture.

ii)   Derivatisation of metabolites making them amenable to gas chromatography for metabolites which are not volatile per se require chemical modification prior to GC analysis.

iii)   Separation by GC. High resolution GC can also be highly reproducible as it involves automated sample injection robotics, highly standardized conditions of gas-flow, temperature programming, and standardized capillary column material.

iv)   Ionisation of compounds as they are eluted from the GC. Electron impact (EI) ionisation is most widely used, as it is the technology which is least susceptible to suppression effects and produces reproducible fragmentation patterns.

v)   Time resolved detection of molecular and fragment ions

vi)   Acquisition and evaluation of GC-MS data files. All GC-MS system manufacturers provide software which is tuned for targeted, quantitative metabolite analysis. Recent software developments support the non-targeted analysis of GC-MS patterns, and the full evaluation of all resolved compounds.

One of the drawbacks of Gas Chromatography/Mass Spectrometry technique is the fact that it requires metabolites to be derivatized to create volatile compounds. Non-volatile compounds which are not derivatized cannot be detected limiting the applicability of this technique thus complicating identification process.

2.1.1.2 Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

Mass Spectrometry (MS) and chromatography have been extensively developed in the past few decades and hold a distinguished position in qualification and separation science (Zhang *et al*., 2012a). HPLC coupled to MS does not need compounds to be derivatize prior to analysis which solves the shortcoming of GC-MS. LC coupled to TOF/MS for routine analyses has been

developed in the last few years. One of the main advantages of LC-ToF instruments is that atmospheric pressure ionization (API) interfaces are used to couple LC with (Q)ToF-MS, similarly to other types of analyzers, with the possibility to perform ionization and in-source fragmentation of target compounds (Lacorte and Fernandez-Alba, 2006). The resolving power enables mass-measurement accuracy for small molecules, charge-state identification of multiply-charged ions, and greater differentiation of isobaric species (two different compounds with the same integer mass but different elemental compositions and, therefore, different exact masses (Ferrer and Thurman, 2003). The following are the main advantages of TOF/MS instrument as described by Ferrer and Thurman, (2003)

   i)   The ability to collect data across a wide mass range without a decrease in sensitivity, so
        that a full spectral sensitivity is achieved;

   ii) The possibility of resolving interferences away from signals of interest with high
        resolving power;

   iii) The achievement of mass-measurement accuracy for the estimation of elemental
        composition.

Despite its inherent high sensitivity for whole-spectrum recording, TOF-MS cannot yet compete with triple-quadrupole - MS operated in MRM mode in both sensitivity of detection and dynamic range (~2 orders of magnitude distance) (Reemtsma and Jekel, 2006). Therefore, the main field of TOF and Q-TOF MS is qualitative analysis rather than the quantitation of known target compounds. Even though this technique provides high mass accuracy and greatly reduces the potential molecular formulas corresponding to one metabolic peak, there may be several molecular formulas that are appropriate for the accurate mass data (depending on the resolution of the instrument), and numerous potential isomers for each molecular formula (Schultz *et al*., 2013).

2.1.2 Kinetics of dye decolorization

Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction investigated. In order to develop an efficient enzymatic decolorization process, knowledge regarding the kinetics of decolorization and the environmental factors affecting the rate of decolorization need to be well identified (Chang and Kuo, 2000). Studying an enzyme's kinetics can reveal the catalytic mechanism of the enzyme, its role in metabolism, how its activity is controlled, and how an agonist might inhibit the enzyme. A kinetic representation often adopted for the description of the rate of consumption of substrate in a bioreaction is the Michaelis-Menten model (Michaelis and Menten, 1913).

2.1.2.1 Michaelis-Menten kinetics

As a starting point, it is assumed that the enzyme and substrate combine to form a complex, which then dissociates into product and free enzyme as follows:

$$E + S \overset{K_{Ms}}{\longleftrightarrow} ES \overset{k_2}{\rightarrow} E + P \qquad (1)$$

Where E, S, ES, P are enzyme, substrate, enzyme–substrate complex and product, respectively. K_{Ms} and k_2 represent the Michaelis-Menten and the catalytic rate constants, respectively. This approach has been successfully used to model the initial rates of several enzymatic reactions. However, the Michaelis-Menten approach to modeling the kinetics over an extended period seems to be rather limited. In terms of reduced errors in estimating the specificity constant and a more accurate representation of the kinetics of enzyme-catalyzed reactions, a better form of the Michaelis-Menten equation would be given as follows (Michaelis and Menten, 1913):

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad (2)$$

Where V_{max} is the maximum substrate (i.e. dye) consumption rate in mg l^{-1} h^{-1}; V is the substrate consumption rate in mg l^{-1} h^{-1}; S is the substrate concentration in mgl^{-1}; K_m is the Michaelis-Menten constant in mg l^{-1}. K_m is equal to the concentration of the substrate when the reaction rate is half of the maximum velocity. The Michaelis-Menten constant, K_m and the maximal rate, V_{max} can be obtained from rates of catalysis measured at various substrate (dye) concentrations.

2.1.2.2 Lineweaver-Burk plot

When Michaelis-Menten equation is transformed by a double reciprocal we obtain Lineweaver-Burk (Lineweaver and Burk, 1934) equation below:

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \tag{3}$$

A plot of 1/V versus 1/[S] gives a straight line with 1/V_{max} as intercept on the ordinate when 1/S approaches zero, and an intercept of (1/K_m) on the abscissa as V approaches zero.

2.1.2.3 Eadie-Hofstee plot

Solving Michaelis-Menten equation (2) for V_{max} and rearranging the resulting expression, results to Eadie-Hofstee equation below:

$$V = K_m \left(V/S \right) + V_{max} \tag{4}$$

A plot of V against (V/S) gives a straight line with a slope of K_m and intercept $V_{max.}$.

2.1.3 Kinetics Order Models

The most common kinetics models used to express the rate of biodegradation reactions include Zero order, first-order and second-order kinetic models (Shah *et al*., 2012).

2.1.3.1 Zero order kinetic model

The standard equation for a Zero-order reaction is given by Equation (5).

$$C_t = C_0 - k_0 t \tag{5}$$

Where k_0 is the zero order degradation rate constant ($mgl^{-1}min^{-1}$) and it can be estimated from the slope by plotting C_t vs t, where C_t is dye concentration in the solution (mgl^{-1}) at any time t and C_0 is the initial concentration of the dye in the solution (mg/l).

2.1.3.2 First order kinetic model

The first order kinetic model is given by Eq. (6).

$$\frac{dC_t}{d_t} = k_1 C_t \tag{6}$$

In the linear form, equation (6) becomes:

$$\ln(C_t) = k_1 t + \ln(C_0) \tag{7}$$

Where k_1 is the first order degradation rate constant (min^{-1}) and it can be estimated from the slope by plotting $\ln(C_t)$ vs t. dC_t is the change in dye concentration in the solution (mgl^{-1}) at any time t and d_t is the change in time t.

2.1.3.3 Second order kinetics model

The second order reaction kinetic model is given by Equation (8).

$$-\frac{dC_t}{dt} = k_2 C^2 \tag{8}$$

In the linear form, equation (8) becomes:

$$\frac{1}{C_t} = \frac{1}{C_0} + k_2 t \tag{9}$$

Where k_2 is the second order degradation rate constant (mg/l min) and it can be estimated from plotting $1/C_t$ vs. t. the slope with best fit will give the rate constant k_2, and the intercept will give $1/C_o$.

2.2 Conventional leather processing method

Animal hides and skins have hair attached to them which is often removed during leather processes. Similarly, fish skins have scales attached to them and are removed in leather

processing. Conventional leather processing method is associated with discharges of significant amounts of environmental contaminants owing to the use of various toxic chemicals. The conventional dehairing method involves use of high proportions of lime and metal sulfide, which contributes 80–90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan *et al.*, 2004). Furthermore, use of lime and sodium sulfide in dehairing results in environmental pollution problems such as increased effluent toxicity, health hazards to the tannery workers and poisonous sludge which blocks sewerage pipes.

Conventional leather processing involves about 14 to 15 steps comprising soaking, liming, deliming, bating, pickling, chrome tanning, basification, rechroming, basification, neutralization, washing, retanning, dyeing, fat-liquoring and fixing. These steps are generally categorized into three main parts i.e., A) Pre-tanning, B) Tanning and C) Post tanning. Prior to pre-tanning, the raw animal skin and hides are preserved by application of salt that restrains microbial attack. Soaking rehydrates the skin and also opens up the contracted fibre structure of the skin. Beam house processes (liming and re-liming which takes a period of between 12 hours to 6 days) employ lime and sodium sulfide and purifies the skin matrix by the removal of hair, flesh and other unwanted materials. The process of dehairing is taken to completion during the liming process, and there is appreciable modification of the collagen due to the action of alkali. Reaction of skin protein (collagen) with alkali (lime) lead to; 1) Hydrolysis of amide groups, 2) Modification of guanide groups, 3) Hydrolysis of keto-imide links in protein chains, 4) Swelling and 5) Removal of unwanted material. That is;

1. Hydrolysis of amide groups

$$R-C(=O)-NH_2 + OH^- \longrightarrow R-C(=O)-O^- + NH_3$$
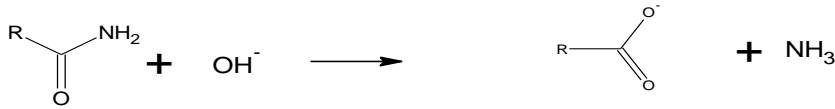
2. Modification of guanide groups (arginine residue)

$$H_3C-(CH_2)_3-NH-C(NH_2)=NH + H_2O + OH^- \longrightarrow H_3C-(CH_2)_3-NH-C(=O)-O^- + 2NH_3$$

3. Hydrolysis of keto-imide links in protein chains

$$R-CH_2-C(=O)-NH-CH_2-R + OH^- \longrightarrow R-CH_2-C(=O)-O^- + H_2N-CH_2-R$$
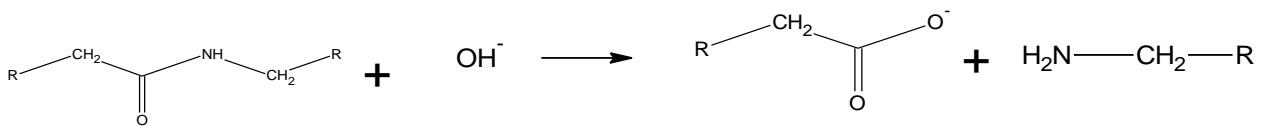
4. Swelling

In addition to the chemical action, in the presence of an alkali, swelling of the fibrous structure takes place. This is due to an osmotic pressure effect. The result is the separation of the fibres and the fibrils from one another and an opening up of the whole structure.

5. Removal of unwanted material

Unwanted materials in the collagen structure include globular proteins and other interfibrillary substances. These are hydrolyzed and removed in the subsequent washing and bating processes. The unwanted fats are saponified.

Pre-tanning operations uses toxic chemicals in large amounts which makes leather processing industry one of the world's worst offenders with regard to environment pollution. Dehairing of hide and skin is one of the most important steps which define removal of hair, fat, soluble proteins from the raw hide or skins.

Deliming removes lime, bating split the fibre into fibril, degreasing removes the fat and pickling reduces the pH of the skin from 8 to 2.8-5.0 to prepare the skin for subsequent tanning. The

tanning or stabilizing process can be carried out by means of vegetable, mineral or synthetic tanning materials. The product of this process is generally known as wet-blue. Tanned skin matrix are further re-tanned to gain substance, fat liquored to attain required softness and dyed to preferred shades. Tanning renders permanent stability to the skin/hide.

Conventional dehairing process uses lime blended with sodium sulfide to remove wool and hair and dissolve these into a pulp. Additionally, this process opens the fiber structure and plumps the hide due to alkalinity. The duration of the process may vary from 18 hours to 7 days depending upon the method employed (Rose *et al*., 2004). This process is responsible for the major parts of the COD load from a tannery due to the use of lime and sodium sulfide. The water polluted with these chemicals and the solubilized hair leads to an increase in alkalinity, organic nitrogen, high COD, BOD, TDS loads while the skin is left shrunken and distorted in shape. Moreover, these chemicals results in pollution due to production of hydrogen sulfide and the solid wastes with hair pulp, lime and organic matter forming sludge. The major public concern over tanneries has traditionally been about odors due to generation of noxious gases, water pollution from untreated discharges and problems of solid waste disposal.

2.2.1 Fish skin leather

The rivers, lakes, seas and oceans of the world are the source of fish which supplies valuable protein and other essential nutrients. Fish is widely eaten as sliced raw fresh sashimi or fish fillet prepared by removal of skin, bones and fins. Processing of fish leads to enormous amounts of waste. It is estimated that fish processing waste after filleting accounts for approximately 75% of the total fish weight (Shahidi, 1994) and 30% of the waste is in the form of bones and skins (Gómez-Guillén *et al*., 2002). Despite the presence of several valuable components, fish processing by-products are usually dumped in landfills or into the oceans having potentially

harmful environmental effects or end up as low commercial value products, such as fish meal, silage, and fertilizer (Gómez-Guillén *et al.*, 2002; Muyonga *et al.*, 2004a; Rustad *et al.*, 2011; Shahidi, 1994)

Nile perch (*Lates niloticus*), like tilapia species (eg *Oreochromis eaculentus, Oreochromis niloticus*), are fresh-water fish species. It is the most important commercial fish species in East Africa from Lake Victoria. Approximately 100,000 tons of Nile perch are processed in Uganda alone, annually (Muyonga *et al.*, 2004b). The fish landed vary greatly in size. It is the largest apex fish predator of Lake Nasser and the Nile River ecosystems reaching sizes over 2 m in length and up to 200 kg in weight. About 30% of the total fish weight remains as waste in the form of skins and bones during preparation of fish fillets (Shahidi, 1994). The fish skins can be processed into fish leather, thus contributing to solving the problem of waste disposal and in addition creating a value-added product.

The main raw materials for making leather products generally include cattle hides, goatskins and sheepskins. In addition, reptile skin such as crocodile and snakes are used for making leather goods. Although crocodile and snake skins leather goods have good reception, their share of the leather market is still limited because they are not easily available and relatively costly to process and manufacture. Hence, some manufacturers have started looking for alternative materials like fish skins for making leather since fish skins are readily available as waste.

The consumption of fish leather in the leather and fashion industry is currently low, probably due to the fact that the quality of the fish leather is devalued by descaling methods currently used. Conventional fish skin processing methods generally include pretreatment of the fish skin pelt by preservation, drying, and softening in a water bath. Just like hair removal in hide, de-scaling is achieved by means of calcium hydroxide and sodium sulfide before deliming and tanning. This
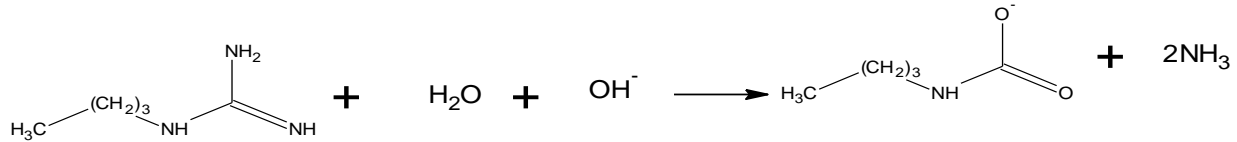
processing method is associated with discharges of significant amount of environmental contaminants owing to the use of the toxic chemicals. In some cases, de-scaling of fish skin is performed in a manual operation by scraping off the scales with the aid of knives or knife-like implements. This is not only a time consuming operation but also frequently causes harm and wounds to the hands of persons scraping. The scale pockets and the natural structural patterns are permanently destroyed during descaling processing.

The need to maintain a clean environment and produce highly valued fish skin leather with unique fish-scale pocket patterns while maintaining the soft texture of leather has baffled many researchers. Since the discovery of the enzymatic dehairing process, considerable amount of work has been done in an effort to eliminate or reduce environmental pollution in leather processing industry. However, no enzymes from bacteria originating from Kenyan extremophile environment have been reported so far for dehairing or descaling of skin in the absence of lime and/or lime and sulfide system. Use of protease enzyme can completely eliminate the use of sodium sulfide and lime in descaling of fish skin as well as greatly reduce the pungent smell which is the characteristic smell of tanneries across the world. In addition, scales and hair can be recovered from the effluent system thus greatly helping in reduction of sludge concentration and pollution load.

2.3   Environmental pollution associated with leather industry

Leather-manufacturing industries or tanneries are highly polluting and generate enormous solid and liquid wastes and obnoxious smell due to the degradation of proteinaceous components of waste and the release of gases like H_2S, NH_3 and CO_2 (Thanikaivelan *et al*., 2004). Deaths because of this toxic chemical process have also been reported (Balasubramanian and Pugalenthi, 2000; Gupta *et al*., 2002). The main chemicals used during the liming and dehairing

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steps include sodium sulfide and lime. The use of large amounts of these chemicals results in high concentrations of S_2^- and suspended solids (SS) in wastewater (Li *et al.*, 2010). The processes are also responsible for 60–70% of BOD and COD of pollution load (Li *et al.*, 2010).

Generation of large quantities of solid wastes related to hide processing in beam house operations are closely linked to the characteristics of the raw material. Worldwide, it is estimated that 315 million bovine leathers are produced per year (Arunachalam and Saritha, 2009). Considering a waste treatment cost of $0.30 per m^2 of leather produced more than $1 million is spent per day to treat the waste from tanneries around the world (Masih and Singh, 2014). Although the tanning industry is environmentally important as a principal user of meat industry waste, the industry is perceived as a consumer of resources and a producer of pollutants. Processing one metric ton of raw hide generates 200 kg of final leather product (containing 3 kg of chromium), 250 kg of non-tanned solid waste, 200 kg of tanned waste (containing 3 kg of chromium), and 50,000 kg of wastewater (containing 5 kg of chromium) (Hüffer and Taeger, 2004). Thus, only 20% of the raw material is converted into leather, and more than 60% of the chromium is in the solid and liquid waste. Figure 2.3 shows some of the wet-blue leather waste at a local tannery in Kenya. During the production of leather goods, especially shoes, manipulation waste is produced, which makes about 15–20% of the entry material leather. The last kinds of waste are used leather products which have lost their utility value (Kolomaznik *et al.*, 2008). Alternative methods employ more environmentally friendly technologies in which some of the solid waste constituents can be used as by-products or as inputs for other industries *(Andrioli et al.*, 2015).

Figure 2.3. Wet-blue leather waste at a local tannery in Kenya.

2.4   Alkaline Proteases Enzyme

Enzymes are the biological substance or biological macromolecules in the form of proteins that are produced by a living organism which acts as a catalyst to bring about a specific biochemical reaction. As such, they have evolved – along with cells – under the conditions found on planet earth to satisfy the metabolic requirements of an extensive range of cell types. Though the enzymes were discovered from microorganisms in the 20th century, studies on their isolation, characterization of properties, production on bench-scale to pilot-scale and their application in bio-industry have continuously progressed, and the knowledge has regularly been updated (Nigam, 2013). Microorganisms such as bacteria, fungi, yeast and actinomycetes represent

excellent sources of enzyme production owing to their broad biochemical diversity, rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various industrial applications. Proteases are the enzymes that occupy a pivotal position both physiological and commercial fields. The detergent industry is the largest user of industrial enzymes, which accounts for more than one third (1/3rd) of the global market and is considered to be total of 1.6 billion US dollars (Kumari and Rani, 2013).

According to the recommendation of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/), the term protease equivalent to peptidase is used for a class of enzymes hydrolyzing peptide bonds (CO–NH) (Enzyme Commission number (EC 3.4)) in a protein molecule. Among various proteases enzymes, alkaline protease enzyme is the most important enzymatic group which play a vital role in industrial field such as leather, detergent, silk degumming, pharmaceutical, photography, meat tenderization, cosmetics, medicinal and peptide synthesis due to their robustness, catalytic activity, high degree of substrate specificity and production capacities (Kumar and Takagi, 1999). In addition, alkaline protease are the main enzymes among known proteases which constitute 60-65% of the global industrial enzyme market (Amoozegar *et al.*, 2007) because of their capability to work under high pH, temperature and in presence of inhibitory compounds. With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields such as clinical, medicinal and analytical chemistry (Kalaiarasi and Sunitha, 2009). To meet the current largely increased industrial demand for enzymes, studies on the cost-effective production of industrially important enzymes have received continued interest. Protein engineering

techniques are likely to become a powerful tool in the improvement of existing enzymes, but continued screening and studies of new enzymes is also a useful means of obtaining typical isolates with great potential for new applications or for existing processes (Kalaiarasi and Sunitha, 2009).

2.5 Application of enzyme in Industrial and leather processing

Many chemical reactions in various industries have inherent drawbacks from a commercial and environmental point of view. Harsh and hazardous processes involving high temperatures, pH, pressures, salinity, acidity, or alkalinity need high capital investment especially in the designed equipment and control systems. Furthermore, high chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. In general, most of chemical reactions are not only costly but results in air, water and soil pollution.

Chemists have always been inspired by nature, not only due to the vast diversity of compounds that the living organisms are capable of creating, but also due to the extraordinary synthetic strategies deployed (Oroz-Guinea and García-Junceda, 2013). Inside a cell, different biochemical reactions catalyzed by enzymes take place to support cellular growth and survival. Extremophiles like alkaliphile bacteria exhibit the ability to grow at extremely harsh environmental conditions such as high pH and temperature, high levels of salinity or salt, and pressure which critically influence their growth (Wanyonyi *et al.*, 2014a). They may represent an attractive source of proteases enzyme as they can be cultured in large quantities in a relatively short time by established fermentation methods which mimic the harsh natural environment but represent perfect and optimum condition under which most chemical reactions in industries occur. Microbial proteases are extracellular in nature and are directly secreted into the

fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals.

Enzymes, the catalysts used by living organisms, have evolved to perform diverse chemical transformations with high selectivity and specificity. This extraordinary synthetic effectiveness that enzymes display is due in part to their work in multistep reactions, or cascade processes, which give rise to the complex metabolic networks found in biological systems (Oroz-Guinea and García-Junceda, 2013). Microbial enzymes are known to be superior enzymes obtained from different microorganisms, particularly for applications in industries on commercial scales.

Biotechnology is utilizing a wide range of these synthesized enzymes on a commercial scale taking advantage of their special characteristics such as tolerance to a varied range of pH, stability of enzyme activity over a wide range of temperature and pH and other harsh reaction conditions. Most of the commercially applicable proteases are alkaline and are bio-synthesized mainly by bacteria such as *Pseudomona*s, *Bacillus*, and *Clostridium*, and some fungi are also reported to produce these enzymes (Kumar and Takagi, 1999). Several thousand enzymes possessing different substrate specificities are known. However, only comparatively few enzymes have been isolated in a pure form and crystallized, and little has been known about their structure and function (Hasan *et al.*, 2006). These enzymes have numerous applications in the industrial production of different items including detergents, foods, pharmaceuticals, leathers and diagnostic reagents (Amoozegar *et al*., 2007). In addition, enzymes have also been used for waste management and silver recovery (Gupta *et al*., 2002).

In the leather industry, biotechnology has a high potential for improving various aspects of leather making processes with a view to reducing costs, raising product quality, reduce waste and lessening the environmental impact of an industry which has traditionally been deemed highly

polluting. This industry, like many others, is facing tougher and tougher environmental regulations in many parts of the world. Consumption of chemicals and the impact on the environment can be minimized with the use of enzymes in soaking, dehairing and liming, bating, degreasing and waste disposal process.

Enzymatic dehairing and descaling in tanneries has been envisaged as an alternative to the use of lime and sulfide. Proteases are considered valuable in these processes due to their potential to act selectively against specific unwanted proteins without affecting the major leather forming protein. Furthermore, Alkaline proteases can be used and enables the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair (Gupta *et al.*, 2002).

Use of enzymes in hide/skin dehairing have the following advantages: **1)** there is a significant reduction or even complete elimination of the use of sodium sulfide, **2)** total recovery of hair resulting in good quality with good saleable value, and **3)** creation of an ecologically conducive and safe atmosphere for the workers (Arunachalam and Saritha, 2009). The dehairing process which contributes a lot to the environmental pollution can provide an opportunity whereby the recovered hair is used in felt manufacture, as organic fertilizer, or as feed for poultry. In addition, the recovered hair and other protein material can be hydrolyzed enzymatically and applied to generate biogas or recover keratins for cosmetics or pharmaceutical purposes. The main problem related to the hair removal process using lime and sodium sulfide is the toxicity of these elements (Andrioli *et al.*, 2015). Although enzyme-assisted dehairing process reduces the pollution load to some extent, a technology based on enzyme alone, without the use of sulfide and other chemical inputs, has yet to be explored (Thanikaivelan *et al.*, 2004).

Despite all these advantages of applying enzymes in leather processing, there has been few studies done in screening, isolating and characterizing microorganism with high application potential. Only about 2% of the world's microorganisms have been tested as enzyme sources (Hasan *et al*., 2006). In addition, relatively few studies have been reported on the application of alkaline enzyme from extremely harsh environmental conditions such as high pH and temperature, high levels of salinity or salt, and pressure for industrial application.

Furthermore the use of enzymes in leather manufacturing process particularly for dehairing has not been accepted by the industry to the desired level. This is mainly because: a) enzymes are not effective enough to eliminate the sulfide completely, b) there is an apprehension that the enzyme assisted process needs stringent process control, and c) the cost of enzymes is not encouraging (Arunachalam and Saritha, 2009). Hence, this present work has focused on screening for proteolytic enzyme from a suitable microorganism from extremophile environment, which is economically viable and effective enough to eliminate the use of sodium sulfide completely.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1   Methodology 1: Isolation, Identification, Characterization and Production of Modifying Enzymes.

3.1.1   Sample collection

Soil samples were collected by scooping off 10g of soil sample 2 to 5 cm below mud water surface with a sterile spatula on the shores of Lake Bogoria. These samples were collected from diverse locations, transported to the laboratory in zip lock bags and stored in a refrigerator at $4°C$ for subsequent isolation and screening.

3.1.2   Screening for protease producing bacterial strains

The bacterial strains were isolated from the collected soil samples. One gram of the soil sample was weighed aseptically into a 100ml conical flask containing 10ml distilled water, agitated for 45 min on a thermo shaker (Gallenkamp, London, England) at $45°C$. 0.2 ml of this mixture was spread on nutrient agar with 1% (w/v) casein and incubated at $37°C$ for 48 hours. Sub-culturing was done until pure isolates were obtained which were later stored on a class petri dish in the refrigerator at $4°C$ for identification. Protease producing strains were selected by spotting the bacterial cultures on a casein culturing medium. The strain that exhibited maximum clear zone was selected for protease production and identification.

3.1.3   Proteases assay on ager plates

Proteases activity of the bacterial isolates was screened on a solid medium containing Reese agar medium (Reese *et al*., 1950). The plates were incubated at $37°C$ to allow the growth of test bacteria for 12 hours. They were then examined for the formation of zone of clearance around

the colony. The zone was made clearer, by flooding the plates with a solution of 5% Trichloroacetic acid (TCA) and kept for 30 min to allow the precipitation of residual proteins in the medium. The diameter of the bacteria colony and the total zone of enzyme activity including the growth diameter were measured in each case. On the basis of this 'Relative Enzyme Activity' (REA) was calculated.

Glycerol stocks of the isolated strains were prepared by mixing 2.0 ml of an overnight culture with 2.0ml of 60% sterile glycerol (Sigma, St. Louis, USA) in sterile micro centrifuge tubes and the cells dispersed by vortexing briefly before being stored at -20ºC for future use.

3.1.4 Biochemical Characteristics of the isolated micro-organism

The pure culture colonies of the isolates were subjected to a number of biochemical tests. Gram stain test and Sugar fermentation pattern was carried out on the bacterial isolates as described in the Manual of Veterinary Laboratory Techniques in Kenya (Lamb, 1981). Catalase and oxidase activities were determined as described by Smibert and Krieg (1994). Gas production in the medium supplemented with different sugars was examined using Durham tubes.

3.1.5 Molecular identification

3.1.5.1 Genomic DNA extraction

The molecular identification of the isolate was based on partial sequence analysis of 16S r DNA. Bacteria cells were cultured in nutrient broth (Sigma, St. Louis, USA) containing meat extract 1 g/L, yeast extract 2 g/L, peptone 5 g/L and sodium chloride 5 g/L and the final pH 8.±0.2 at 25° C. Nutrient broth was prepared by adding distilled water to 3.25 g of the powder to make 250 ml and sterilization done by autoclaving at 121°C for 15 minutes. Incubation was done overnight at

$45^{°}$C and shaking at150 rpm. Genomic DNA was isolated from the bacterial isolate following the procedure described by Chachaty and Saulnier (2000) method.

3.1.5.2 DNA analysis by TAE/agarose/ ethidium bromide (EtBr) gel electrophoresis

The quality of the extracted genomic DNA was analyzed on 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1× TAE buffer. A 1% TAE /agarose/EtBr gel was prepared by boiling 0.70 g agarose in 70 mL of 1× TAE. The hot agarose solution was allowed to cool down to about 50ºC prior to addition of ethidium bromide to a final concentration of 0.5µg/mL. Caution was taken not to inhale ethidium bromide fumes. The solution was then poured into the gel casting chamber and a comb placed in position. After polymerization for 30 minutes, the gel was transferred into the electrophoresis chamber with the slots facing the cathode and covered with a running buffer (1× TAE buffer). 6× orange DNA loading dye (Fermentas, Pittsburgh, USA) was premixed with the DNA samples in the ratio 1:5 µL of sample (final concentration: 1×) prior to loading of the samples into the wells in the gel.

The sample was electrophoresed at 5 V/cm (80V) for genomic DNA and 10 V/cm (120 V) for Polymerase Chain Reaction (PCR) products for 45 minutes. The current was supplied by an electrophoresis power supply (Consort EV265, Holliston, USA). A 1 kb DNA ladder (GeneRuler 1kb DNA Ladder) was run alongside DNA samples. The DNA bands were visualized under a UV trans illuminator (Herolab,Wiesloch, Germany).

3.1.5.3 Polymerase Chain Reaction (PCR) amplification of 16S rDNA

An aliquot (2 µL) of genomic DNA extracted from each isolate was used as a template to amplify 16S rDNA gene. Polymerase chain reaction was performed in a TProfessional thermocycler (Biometra,Gottingen, Germany). The 16s rDNA gene was amplified using two bacterial specific primers: 16S F27, forward 5'…AGA GTT TGA TC(AC) TGG CTC AG…3'

and 16S R 1492, reverse 5'…TAC GG(CT) TAC CTT GTT ACG ACT T…3'. The PCR reaction was performed in a total volume of 50 μl thin-walled PCR reaction tube (Simport,Quebec,Canada) using 36.5 μL PCR grade water, 5 μL 10× Dream Taq PCR buffer (Fermentas, Pittsburgh, USA), 4 μL MgCl$_2$ (25 mM) 1 μL dNTP mix (10 mM), 1 μL of each primer (200 μM), 0.5 μL Dream Taq DNA polymerase (Fermentas, Pittsburgh, USA), (5 U/μL) and 1 μL of isolated DNA. The thermocycler (Biometra, Göttingen, Germany) conditions for the PCR were as the following program: 94°C for 5min, followed by 40 cycles of 94°C for 20sec, 58°C for 20sec, and 72°C for 1 min, followed by 72°C for 10 min. The amplified PCR products were run on a 2% pre-stained ethidium bromide agarose gel to confirm amplification.

The PCR products were then gel purified using MinElute Gel Extraction Kit (QIAGEN, Venlo, Netherlands) according to manufacturer's protocol and sent to International Livestock Research Institute, Kenya (ILRI) for sequencing. The sequencing trace files for isolates *1-p, 2-p 3-p* and *4-p* were aligned into contigs with slight modifications of the default conditions using Sequencher software version 4.6 (Gene Codes Corporation, USA). All chromatograms and sequences were visually inspected and sequences edited, and only high quality sequences were further used. A consensus sequence for each of the isolate's 16S rDNA was generated using Chromas Lite and sequences deposited in Genebank.

3.1.5.4 Phylogenetic analysis

The 16S rDNA sequences obtained were compared with known 16S rDNA sequences at National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) algorithm obtained from; http://www.ncbi.nlm.nih.gov/BLAST . All the sequences were then aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). Phylogenetic trees constructed based on the nucleotide

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sequences with the Bayesian phylogenetic method in MrBayes software obtained at http://mrbayes.net. The trees were then visualized using fig tree software was obtained at http://tree.bio.ed.ac.uk/ . The identified 16S rDNA sequences of isolated strains were submitted to NCBI and assigned accession codes KM201428, KM201429 and KM201430 for isolates 1-*p*, 2-*p*, and 3-*p*, respectively.

3.1.6   Enzyme Assays

3.1.6.1   Determination of Protease Activity

Protease activity was determined by measuring the amount of tyrosine liberated when crude alkaline protease enzyme was reacted with casein. Protease activity in the crude enzyme was assayed by the modified procedure (Tsuchida *et al*., 1986) using 1% Casein as substrate at 45˚C for 10 min. The reaction was terminated by the addition of 4 ml of 10% (w/v) chilled trichloroacetic acid and the reaction mixture allowed to stand in ice for 20 min to precipitate the insoluble proteins. The mixture was centrifuged to obtain the supernatant. 5ml of 0.4M Na_2CO_3 and 1 ml of one fold diluted Folin ciocalteau reagent was added to the supernatant which was further incubated for 30 minutes to develop the color. The absorbance was measured against an appropriate blank at 660 nm using a digital UV-VIS spectrophotometer (Digilab Hitachi, Tokyo, Japan). Using the established tyrosine standard curve, the amount of tyrosine produced was calculated and values obtained used to determine enzyme activity.

3.1.7   Process optimization for maximum protease production

3.1.7.1   Determination of Optimum pH for Protease Production by Strains *1-p* and *2-p*
The effect of initial pH of the medium on protease production was studied by adjusting the pH of the production medium in the range of 3 to 12 using 1M HCl or 1M NaOH. The broth production

medium consisted of 1% Casein, in 250-ml conical flasks containing 50 ml of medium on a rotary shaker (140 rpm) for 48 h at 37°C. The culture was centrifuged at 5,000 rpm for 15 min at 25 °C and tyrosine content in the supernatant determined by measuring absorbance at 660 nm. Using a tyrosine standard curve, the amount of tyrosine produced was calculated.

3.1.7.2   Determination of Optimum Time for Protease Production

The effect of incubation time on protease production was carried out using 1% casein at pH 8±0.2 and pH 9±0.2 for strains *1-p* and *2-p,* respectively by incubating the culture medium at different time intervals ranging from 12 to 156 hrs. The Tyrosine content in the supernatant was determined after a time interval of 12 hours by centrifuging the culture media at 5,000 rpm for 15 min at 25 °C and reading the absorbance at 660nm using a UV–Vis spectrophotometer.

3.1.7.3   Determination of Optimum Temperature for Protease Production for strains *1-p* and *2-p*

The effect of temperature on protease production was carried out at a pre- determined pH 8±0.2 and pH 9±0.2 for strains *1-p* and *2-p*, respectively by incubating the culture medium at different temperatures ranging from 27°C - 57°C. The broth production medium consisted of 1% Casein. Protease activity at each temperature was determined by measuring tyrosine content in the supernatant after 48 hours.

3.1.7.4   Effect of Nitrogen Source on Protease Production

The effect of nitrogen source on protease production was investigated by incorporating various nitrogenous sources which included peptone, Trypeptone, casein, Beans, green gram, soybean

meal feathers, and wool incorporated at 1% in each production medium. The experiment was carried out at 37°C and pH 8±0.2 and pH 9±0.2 for strains *1-p* and *2-p,* respectively.

3.1.7.5 Large Scale Production of Crude Alkaline Protease Enzyme

After optimizing the fermentation condition, strain *1-p* was selected for large scale production of crude alkaline protease enzyme at the optimum condition using 1% casein under Submerged Fermentation (SmF) for subsequent application. The pH of the media was adjusted to pH 8.5. Ten 1000mL Erlenmeyer flasks containing 500mL of media plugged in cotton and aluminum foil was sterilized in an autoclave at 121ºC (15 lb) for 15 min and after cooling the flask was inoculated with 5% overnight grown seed bacterial culture. The inoculated medium was on a rotary shaker (140 rpm) incubator for 72 hours. The cultures were centrifuged at 4000 rpm (Thermo Scientific, Pittsburgh, Germany) at 4˚C for 15 minutes to obtain the crude extract which served as the crude enzyme source. The crude enzyme extract were stored at -20˚ C for subsequent use.

3.1.8 Determination of Protease Activity at Various Environmental Conditions

After optimizing the fermentation condition, strain *1-p was* selected for large scale enzyme production, protease activity at various environmental conditions such as pH and temperature was determined.

These were to be taken as the standard optimum conditions for operation.

3.1.8.1 Effect of pH on enzyme activity

The protease activity of the crude enzyme was measured at different pH values (4 − 12). The pH was adjusted using the following buffers (0.2 M) of acetate (pH 4.0), phosphate (pH5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0). Casein solution (1.0 ml) was mixed with an

equal volume of crude enzyme solution and incubated at 45°C for 10 min. 4 ml of chilled trichloro acetic acid was added and the reaction mixture allowed to stand in ice for 20 min to precipitate the insoluble proteins. The mixture was centrifuged and to the supernatant was added to 5ml of 0.4M Na_2CO_3 and 1 ml of one fold diluted Folin ciocalteau reagent which was further incubated for 30 min. The Uv/Vis absorbance was measured against an appropriate blank at 660 nm using a UV−Vis spectrophotometer and the activity of the enzyme calculated.

3.1.8.2   Effect of temperature on enzyme activity

Determination of optimum temperature for protease activity was investigated at optimum pH (pH11) and varying the temperatures from 30, 40, 50, 60, 70 to 80°C. Casein solution (1.0 ml) was mixed with an equal volume of crude enzyme solution and incubated at the appropriate temperature for 10 min. 4 ml of chilled trichloroacetic acid was added and the reaction mixture allowed to stand in ice for 20 min to precipitate the insoluble proteins. The mixture was centrifuged and to the supernatant was added to 5ml of 0.4M Na_2CO_3 and 1.0 ml of one fold diluted Folin ciocalteau reagent which was further incubated for 30 min. The UV/Vis absorbance was measured against an appropriate blank at 660 nm using a UV−Vis spectrophotometer and the activity of the enzyme calculated.

3.2   Methodology II: Dehairing of Hide and Descaling of Fish Skin

3.2.1   Materials

Fresh wet cowhide of compact thickness range of 0.4 - 0.5 cm and average weight of 15 kg was sourced from Kayole slaughter house, Nairobi Kenya. Similarly, fresh Nile perch skins were sourced from Nairobi city market. The hides and skins were washed with tap water to remove cow dung, blood remains, soil and other impurities and then frozen at -20°C to restrain microbial

attack before processing. Prior to processing, the hides and skins were thawed at 4˚C for two hours. Crude alkaline protease enzyme were locally produced in our laboratory in large quantities by a newly isolated bacterial species labeled as *1-p.* HCl and NaOH for pH adjustment were obtained from KOBIAN Limited, Nairobi, Kenya, and used without further purification.

3.2.2   Dehairing of hide assays

3.2.2.1   Effect of pH on dehairing by crude enzyme

Effect of pH on enzymatic dehairing of hide was investigated at a pH range of 2.5 to 12.5 using crude alkaline protease enzyme. A negative control using distilled water instead of crude enzyme at various pH 12 was used. Hydrochloric acid of 0.1M concentration was prepared from a stock HCl (12M) by appropriate dilution using distilled water and also 0.1M NaOH was prepared by dissolving 4 g of NaOH pellets into 1 liter of distilled water. Pieces of hide with average weight of 40g and measuring 10 by 10 cm were cut from wet cowhide, immersed in the crude enzyme solution and incubated at 37°C. After every 30 minutes, samples from each conical flask and a corresponding negative control were removed and visually analyzed for:

1. Ease and complete removal of the hair.

2. Ease of removal of the hair, with incomplete removal of the hair on the hide.

3. Absence of difficulty in removal of the hair.

The dehairing efficacy was assessed according to the depleted area of the skin at various time intervals and the quality of the dehaired hide was estimated according to the appearance observed by the naked eye during and after treatment process. Also, the smell produced during the dehairing process was monitored at all pH values. When all hair had loosened out, the skin was dehaired manually by gently scraping with a blunt knife and the extent of removal of melanin visually assessed and photographs taken.

3.2.2.2   Effect of temperature on dehairing by crude enzyme

Effect of temperature on enzymatic dehairing of hide was investigated at a temperature range of 27˚C to 77˚C and pH 12 using crude alkaline protease enzyme. A negative control using distilled water instead of crude enzyme at various temperatures and pH 12 was used. Pieces of hide with average weight of about 40g and measuring 10 by 10 cm was cut from the wet cowhide. Dehairing at 27°C was accomplished in two conical flasks labeled T27 containing 40ml of crude enzyme and two pieces of cow hide. They were separately placed in a thermo shaker (Gallenkamp, London, England) set at 27 °C for 30 minutes to attain equilibrium temperature before mixing. After 30 minutes, the temperature of crude alkaline protease enzyme and the pieces of hide were measured to ensure that the temperature was at 27 °C before mixing, thoroughly shaken to ensure uniform enzyme coverage on the hide and incubated at 27 °C. Similar experiments were done at temperatures 37 ° C, 47 °C 57 °C, 67 °C and 77 °C. When all hair had loosened, the skins were dehaired manually by gently scraping with a blunt knife and the extent of removal of melanin was visually assessed and photographs taken.

3.2.3   Full bull hide Dehairing

Fresh bull hide measuring about 2.0 m by 1.8 m (21.75 square feet) with compact thickness range of 0.4 - 0.5 cm and weighing 15 kg was sourced from Kayole slaughter house and stored in a frozen state at -20 °C. On the dehairing day, the frozen bull hide was thawed for 5 hours at room temperature to remove the solidified ice and bring it in its natural state. The hide was soaked in water at 25˚C to remove impurities such as dirt, salt contents, blood remains, and soluble proteins on the surface of the fish skin and halt any activities of bacteria and viruses. The solution was drained and then the hide subjected to main soaking in an alkaline solution

maintained at a pH of between pH 11 to 12 and temperature of 35°C for three hours with constant agitation and stirring at frequent intervals.

3.2.3.1   Dehairing of hide

The cleaned hide was placed in a 20 liter plastic bucket, filled with 4 liters of the crude alkaline protease enzyme at pH 12.0 and a temperature of 37°C. A constant agitation and stirring at frequent intervals ensures uniform temperatures and pH and thus accelerating rate of uniform dehairing. After every one hour, the ease of hair depilation was analyzed and the pH of the crude enzyme monitored. When all hair had loosened, the hair was removed manually by gently scraping with a blunt knife. The extent of removal of melanin was assessed visually and photographs taken. The recovered hair was washed with tap water to remove enzyme residues and then dried in the sun. The weight of dried hair was taken and its quality examined. The dehaired hide was also was thoroughly washed with tap water to remove any enzyme residues and also to stop the action of enzyme on the skin. Physical testing on the enzymatically dehaired hide was determined before further processing. The physical tests included hide thickness, tensile strength, tear stress, elongation, lactometer grain crack, lactometer-burst, stretch /shrinkage and degree of swelling. The procedures for these tests are described in details in physical testing and hand evaluation of leathers (Section 3.3.6)

3.2.4   Post Dehairing Processing

The dehaired hide was taken to a tannery at Leather Industries of Kenya Ltd (LIK) in Thika Kenya for post dehairing processing. Post dehairing processing operations subjected to the enzymatically dehaired hide included pickling, tanning, retanning, dyeing, and splitting.

3.2.4.1 Pickling

Pickling was done to lower the pH of the dehaired hide to pH 2.8 in preparation for chrome tanning. The enzymatically dehaired hide were soaked for 10 minutes in a solution at 25 °C prepared by mixing water and NaCl in the ratio of 150: 4.0 (wt/wt). The hide were then soaked for 20 minutes in a solution at 25 °C prepared by mixing water, sodium chloride, and then formic acid (HCOOH) (85%) in the ratio of 150: 4.0: 1.0 (wt/wt/wt). Thereafter, the solution having the hide therein was left as it was for 30 minutes. A mixture containing Sulfuric acid (H_2SO_4) and water in the ratio of 1.3: 150 (wt/wt) was further added to the solution, and then left for two hours before confirming that pH had reduced to 2.8. The solution was drained.

3.2.4.2 Tanning

Basic chrome (Cr_2O_3) and water mixture in the ratio of 3.0:150 (wt/wt) was added and the hide soaked for 60 minutes. Next, chrome and water mixture in the ratio of 4.0:150 (wt/wt) was further added to the solution containing hide and left for 60 minutes. Thereafter, sodium bicarbonate ($NaHCO_3$) and water mixture in the ratio of 0.7:150 was added to the solution, and the solution having the hide therein was stirred six times at intervals of 10 minute for 1 hour. Again, sodium bicarbonate and water mixture in the ratio of 1.0:150 (wt/wt) was added to the solution, stirred for 10 minutes and was left for 50 minutes. The above procedure was repeated eight times with addition of sodium bicarbonate to increase the pH to 4.0-4.3. This increase induces cross-linking between the chromium and the collagen. After dehydration, the hide were matured for 5 hours

3.2.4.3 Retanning and Dyeing

The hide matured by the process described above was soaked at 40 ˚C for an hour in a solution prepared by mixing water, basic chrome and sodium formate in the ratio of 200: 4.0: 1.3

(wt/wt/wt) respectively. The hide was then left for an hour in a solution of 40 °C prepared by mixing water, sodium formate, and sodium bicarbonate ($NaHCO_3$) in the ratio of 150 : 2.0 : 2.5 (wt/wt/wt). Hot water at 70 °C and synthetic oil in the ratio of 200: 140 (wt/wt) were added into the solution and the mixture was left as it was for an hour. Thereafter in the same bath containing hide, f water and Baygenol Black TD 01 in the ratio of 200: 02 (wt/wt) was added and stirred for 30 minutes. 1% granalan Sp (1:4) was dissolved with warm water and then added and stirred for 20 minutes. 3% garoval Ap (liq) was also added, run for 20 minutes before adding 6% dark mimosa powder which run for 40 minutes. The solution was drained and then 50% water with respect to weight of hide at 70˚C through the axel added, run for 10 minutes before adding Granalan SP 4% over a one hour period. Fixation was done by adding 0.3% formic acid (10%) for 20 mins and the procedure repeated twice before draining and washing the hide with cold water for 10 minutes. The leather was folded with the grain side kept inside and the flesh outside to avoid dirt on the grain side.

3.2.4.4 Drying

The hide that had been subjected to the processes described above was put on a drying hanger to dehumidify at 60°C.

3.2.4.5 Splitting

The hide that had been subjected to the processes described above was split to produce leather with uniform thickness.

3.2.5 Descaling of Nile Perch (*Lates niloticus*) skin

Fresh fish skin of Nile Perch (*Lates niloticus*) was sourced from a local fish processing industry (City market) in Nairobi and stored in frozen state at -20 °C. On the descaling day, the frozen

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Nile perch skins were thawed for 2 hours at room temperature to remove the solidified ice. Eight pieces of Nile Perch skins weighing a total of 6.25 kg were soaked in water at 25˚C and at pH 10 to remove impurities such as dirt, salt contents, blood remains, and soluble proteins on the surface of the fish skin. The solution was drained and then the Nile Perch skins subjected to the main soaking in an alkaline solution maintained at a pH of between 11-12 and temperature of 35˚C for two hours with constant agitation and stirring.

3.2.5.1 Enzymatic Descaling of Nile Perch skin

The Nile Perch skins from the process described above was soaked for three hours in a bucket filled with a solution of the crude alkaline protease enzyme from *Bacillus Cereus* Strain *1-p* at pH 12.0 and incubated at temperature of 47˚C in orbital shaker at 150rpm. The Nile Perch skin weight to enzyme ratio was 1:1 w/w basis. The pH was adjusted at regular interval to pH12 to maintain optimum conditions and eliminate the pungent smell. After 3 hours, the loosened scales were removed by exposure to mild mechanical action. Subsequently, the small scales under the skin were removed by rubbing the skin on each other in the opposite direction (from the tail to the head). The scale free -Nile Perch skins were thoroughly washed with clean tap water to remove the enzyme solution, solubilized proteins and fats. The scales were filtered from the enzyme liquor and also washed.

3.2.5.2 Post Descaling Processing

The descaled Nile Perch skins were taken to a tannery at Leather Industries of Kenya Ltd (LIK) at Thika, Kenya for post descaling processing. Post descaling processing operations subjected to enzymatically descaled nile perch skin included pickling, tanning, retanning, dyeing, and

splitting. The procedure followed in undertaking this procedures were similar as the one described in Post dehairing processing section above.

3.2.6 Physical Testing and Evaluation of Leathers

After conditioning the crust leather at room temperature and at above 65% relative humidity over a period of 48 hours, two pieces were cut from each enzymatically-processed leathers from which test pieces were subsequently cut as shown in Figure 3.1 for various test. SATRA, ISO and Kenyan standards (KS) test methods were used to determine physical and mechanical properties of leather
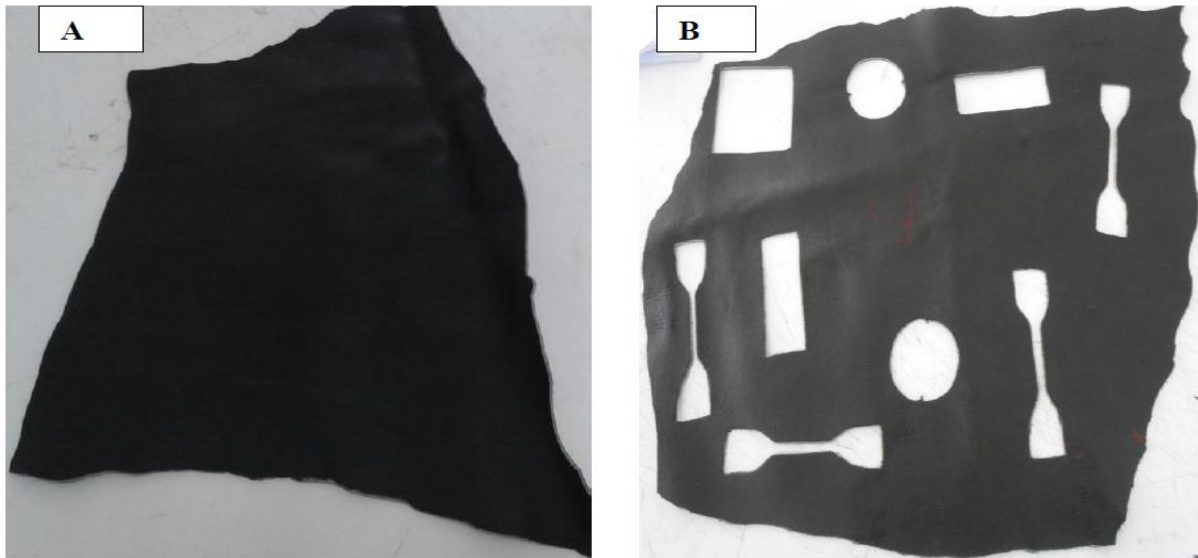
Figure 3.1. Pieces of enzymatically-processed leather cut for physical testing and evaluation. B shows void left after chopping of test pieces for various tests.

3.2.6.1 Tensile Strength

A test piece was prepared by cut the skin from the molded dumb-bell (SATRA TM43) along the grain direction as shown in Figure 3.2 below. The thickness of the test piece at the middle was 1.20 - 1.34 mm. and width (0.6 ± 0.04 mm) cm. Only sharply cut test pieces were used. The test pieces were marked sharply and clearly with vertical reference lines 2 cm from the center.
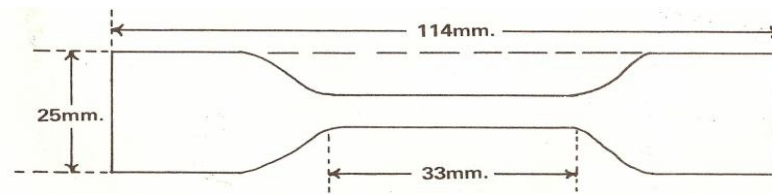
Figure 3.2. Dimensions of Dumb-bell knife for Tensile Test

The thickness of the test piece was measured at the middle in three different places and the average taken. The width of the test pieces of the narrow central part was checked by a pair of dividers before starting the motor on the tensile test machine (Hounsfield rubber testing machine by Tensometer Ltd - England). The dumb-bell shaped leather was inserted into the grips of the machine, taking care to adjust symmetrically to distribute the stress over the cross section to ensure that the reference lines remain parallel during stress. The machine was switched on and the elongation of the skin checked on the scale. The load at 100% elongation was recorded, i.e. the reference marks separate to 4 cm. Replicate readings were taken and the mean determined from which the Tensile strength and % Elongation were calculated using the equation below:

$$\text{Tensile strength Mpa} = \frac{\text{Breaking load x 100}}{\text{Width (mm) x Thickness (mm)}} \qquad (10)$$

$$\text{Elongation \%} = \frac{\text{Final length — (distance between ref. line 2 cm) x 100}}{2} \qquad (11)$$

3.2.6.2 Tear Resistance

SATRA TM65 test method was used for tear resistance test. Test pieces were cut out of the slab prepared as described in section 3.3.6.2. The test piece was clamped in the jaws of the testing machine (Hounsfield rubber testing machine by Tensometer Ltd - England) so that the bite of the jaws is at the center of the slab and in line with the direction of load application. The load was applied with a jaw speed of 30±5 cm/min. After rupture of the test piece, the breaking load in kg was noted from the dial and recorded with the average thickness of the specimen. The tear resistance was calculated from the maximum load registered by the testing machine and the

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thickness of the skin. This was expressed as the pull in kg required to tear the skin 1 cm in thickness.

Tear Strength = (L) Kg/cm   (12)

Where L = Maximum load in Kg

3.2.6.3   Thickness

Thickness of leather was measured by a dial micrometer type of gauge standing on a firm base (SATRA TM1). A square piece of test leather was placed on the upper part in the gauge. The load was gently applied and readings taken five seconds when the full load is reached

3.2.6.4   Lastometer-grain crack and burst

ISO 3378 and SATRA TM24 test method were used to determine Lastometer-grain crack and burst respectively. Two test specimens of test leather measuring 4.45 cm diameter were cut from the leather. The test machine (Lastometer) was reset with the central plunger retracted to zero or minimum distension. Test specimens were tightly clamped round its edge with the grain side up into the test machine. The test was started by forcing a small metal ball attached to a plunger through the specimen at a controlled speed. During the test, the displacement of the ball plunger and the surface of leather were continually observed at the center of the specimen where the maximum distension was taking place. At the first sign of surface cracking, the force on the plunger and the distension of the specimen were recorded. The plunger was retracted and the test specimen removed. The average results of distension for the two pieces were calculated to the nearest 0.1 mm and load to the nearest 10 N.

3.3 Methodology III: Decolorization and Bioremediation of Organic dye.

3.3.1 Determination of wavelength of maximum absorbance (λ_{max}) for Malachite green (MG) and Reactive Black 5 (RB5) dye

A cationic basic dye, malachite green oxalate, was obtained from Loba-Austria and used without further purification while Reactive Black 5 dye (CAS Number 17095-24-8) was obtained from Sigma-Aldrich and used without further purification. MG solution was prepared from stock solution of 1.0×10^{-4}M (92.903 mg/L) by dilution while RB5 solution was prepared from stock solution of 1.0×10^{-3}M (991.82 mg/L) by dilution. All solutions were prepared in double distilled water and pH adjusted by adding either 0.1 M HCl or NaOH. All the experiments in this section were performed in triplicates and the mean value used to plot the graphs.

1.0×10^{-5}M solution of MG and 1.0×10^{-4}M solution of RB5 dye were prepared from the stock solutions. A sample of dye solution was placed in a cuvette and placed in the UV-VIS spectrophotometer that had been set to the absorbance mode. Starting from the lower wavelength (400 nm), the wavelength was adjusted upward sequentially and the wavelength together with the corresponding absorbance recorded over the entire wavelength range of the spectrophotometer. The spectrophotometer was then set to the wavelength of maximum absorbance value for all the absorbance measurements thereafter.

3.3.2 Calibration Plot

Malachite green dye solution of concentrations ranging from 1.0×10^{-6}M to 1.0×10^{-5}M were prepared from the stock solution and used to obtain the calibration curve of MG dye solution against absorbance at maximum wavelength. Similarly, RB5 dye solutions of concentrations ranging 6.25×10^{-6}M to 1.0×10^{-4}M were prepared from the stock solution and used to obtain the calibration curve of RB5 dye solution against absorbance at maximum wavelength.

3.3.3   Determination of the effect of contact time on MG dye decolorization

10ml of crude protease enzyme at pH 8 was mixed with 40 ml of 1.0×10^{-5}M MG dye solution at room temperature (25˚C) in 100 ml conical flasks with constant agitation. After an interval time of 10 minutes, aliquot amounts from the reaction mixture were analyzed for residual MG concentrations using a UV−Vis spectrophotometer set at λ_{max} 617 nm. The solution from the cuvette (after absorbance measurements) was returned to the original solution mixture to maintain the initial volume. The absorbance measurements were taken in each case and the mean value used to plot a calibration graph.

For RB5, 10ml of crude protease enzyme at pH 9 was mixed with 40 ml of 1.0×10^{-4}M RB5 dye solution at 36˚C in 100 ml conical flask with constant agitation. After an interval time of 12 hours, aliquot amounts from the reaction mixture were analyzed for determination of the residual RB5 concentrations using a UV−Vis spectrophotometer at λ_{max} 597 nm. The solutions from the cuvette (after absorbance measurements) were returned to the original solution mixture to maintain the initial volume. The absorbance measurements were taken in each case and the mean value used to plot a calibration graph.

3.3.4   Determination of the effect of pH on MG dye decolorization

The effect of pH on decolorization of MG dye was investigated at room temperature (25˚C) over a pH range of 4-11. 10 ml of free crude enzyme at appropriate pH was mixed with 40 ml of 1.0×10^{-5}M MG dye solution at room temperature in 100ml conical flask. The pH was adjusted by adding either 0.1 M HCl or NaOH as required. The corresponding absorbance measurements were taken λ_{max} 617 in each case and the mean value used to plot a graph.

Similarly, for RB5 dye, effect of pH was investigated at 36°C and over a pH range of 4-12. 10 ml of free crude enzyme at appropriate pH was mixed with 40 ml of 1.0×10^{-4}M RB5 dye solution in 100ml conical flask. The pH was adjusted by adding either 0.1 M HCl or NaOH as required. The corresponding absorbance measurements were taken in each case and the mean value used to plot a line graph.

3.3.5 Determination of the effect of temperature on MG dye decolorization

The effect of temperature on MG dye decolorization was investigated over temperature range of 25 to 70°C on a water bath incubator. The pH of both dye solution and crude enzyme was adjusted to a pre- determined optimum pH of 8. 40ml of MG dye of initial concentration of 1.0×10^{-5}M was mixed with 10 ml of crude protease enzyme both pre equilibrated at a study temperature and before mixing in 100ml conical flask. The temperature of the water bath and reacting solution was closely monitored by a thermometer. Absorbance readings were taken at intervals of 10 minutes till equilibrium was attained at the maximum wavelength using UV-Vis spectrophotometer. From the absorbance measurements, mean value used to plot a graph.

3.3.6 Determination of the effect of initial MG dye concentration on decolorization

The effect of initial concentration on dye concentration was investigated at room temperature and pH 8. 40 ml of MG dye of concentrations :1.0×10^{-6}M, 2.0×10^{-6}M , 4.0×10^{-6}M, 6.0×10^{-6}M, 8.0×10^{-6}M and 1.0×10^{-5}M were placed in 100ml conical flask and 10 ml of crude protease enzyme added. Absorbance readings were taken after every interval of 30 minutes for 6 hours.

Similar experiments were performed using RB5 dye at 36°C and pH 9. 40 ml of MG dye of concentrations : 1.0×10^{-5}M, 2.0×10^{-5}M , 4.0×10^{-5}M, 5.0×10^{-5}M, 6.0×10^{-5}M, 8.0×10^{-5}M

and 1.0 x 10^{-4}M were placed in 100ml conical flask and 10 ml of crude protease enzyme added. Absorbance readings were taken at an interval of 30 minutes for 6 hours and the mean value used to plot a graph.

3.3.7   Determination of Dye Decolorization Rate

Starting absorbance at characteristic max for each dye (control) was designated as 100%. The extent of decolorization rate was defined by the following formula:

$$\text{Decolorization percentage (\%)} = \frac{A_o - A}{A_o} \times 100 \qquad (13)$$

Where A_o is the absorbance of the untreated dye and A is the absorbance after treatment

3.3.8   Assay of metabolites formed from the biodegradation of the dyes

3.3.8.1   Thin Layer Chromatography (TLC)

After complete decolorization of MG dye, the metabolites from biodegraded products were extracted with equal volume of ethyl acetate (25: 25 ml). The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The dry crystals were placed in 25 ml conical flask and 5ml of methanol added to dissolve the metabolites for TLC analysis. Metabolite formation was examined by thin layer chromatography (TLC) using silica gel activated in chloroform. The solvent system used was n-propanol: ethyl acetate: acetic acid: distilled water (6:1:1:2 v/v). The separated products were visualized in iodine chamber.

3.3.8.2 Analysis   of   Degraded   MG   and   RB5   Metabolites   by   Gas Chromatography/Mass Spectrometry (GC/MS)

Malachite Green dye and Reactive Black 5 plus their degraded metabolites and crude enzyme were extracted into organic phase using toluene and dichloromethane, respectively. For MG dye, two 100 ml conical flasks; one containing 25ml of 1.0 x 10^{-4} Malachite Green dye and another

containing 25ml of decolorized malachite green metabolite was each mixed with 5mls of toluene. The mixture was stirred at 25˚C before adding 3ml of 50 percent calcium carbonate. The mixture was stirred for one hour at 140 rpm at 25˚C and then allowed to settle for one hour after which the lower aqueous layer was drawn off and discarded, and the toluene layer containing malachite green dye and metabolite was removed for analysis.

Similarly, for RB5 dye, two 100 ml conical flasks; one containing 25ml of 1.0×10^{-3} RB5 dye and another containing 25ml of degraded RB5 metabolite were each mixed with 10mls of dichloromethane. The mixture was stirred for one hour at 140 rpm at 25˚C and then allowed to settle for one hour after which the lower organic layer was drawn for analysis.

The GC−MS analyses of the MG and RB5 dye and their degraded metabolites fractions were carried out analyzed by split/splitless injection using a model using Hewlett Packard Agilent GC/Mass Spec, Model 6890 coupled to a 5975C inert XL EI/CI mass spectrometer (Agilent Technologies, Palo Alto, CA) (GC−MS), equipped with integrated gas chromatograph with a DB-5 column (30 m long, 0.25 mm internal diameter). Helium was used as carrier gas at a flow rate of 1.3 mL/min. The injector temperature was maintained at 300°C with oven conditions. The initial column temperature was held at 40°C for 3 min, then increased linearly at 10°C/min to 325°C, and held for 2 min. The ionization was carried out in the electron impact mode. The electron multiplier voltage (El 70 eV) and automatic gain control target were set automatically. Degraded products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the National Institute of Standards and Technology (NIST) spectral library stored in the computer software of the GC-MS.

3.3.8.3 Analysis of Degraded MG Metabolites, Using Liquid Chromatography– Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

After complete decolorization of MG and RB5 dyes, 15ml degraded products containing the metabolites, crude enzyme and dye solution were centrifuged at 14000 rpm for 10 minutes to remove solid particles. The clear solution containing degraded metabolite and non-degraded dye and crude enzyme were analyzed. The extracts were concentrated in vacuo to dryness then re-dissolved in 3 mL of LC−MS grade CHROMASOLV methanol (Sigma-Aldrich) before centrifuging at 14,000 rpm for 10 min; after which 0.5 μL was automatically injected into LC−QToF−MS. The chromatographic separation was achieved on a Waters ACQUITY UPLC (ultra- performance liquid chromatography) I-class system (Waters Corpo- ration, Maple Street, MA) fitted with a 2.1 mm × 100 mm, 1.7-μm particle size Waters ACQUITY UPLC BEH C18 column (Waters Corporation, Dublin, Ireland) heated to 40 °C and an auto sampler tray cooled to 15 °C. Mobile phases of water (A) and acetonitrile (B), each with 0.01% formic acid were employed.

The following gradient was used: 0−1.5 min, 10% B; 1.5−2 min, 10−50% B; 2−6 min, 50−100% B; 6−9 min, 100% B; 9−10 min, 90−10% B; 10−12 min, 10% B. The flow rate was held constant at 0.4 mL/min. The UPLC system was interfaced by electrospray ionization (ESI) to a Waters Xevo QToF−MS operated in full scan MS^E in positive mode. Data were acquired in resolution mode over the m/z range of 100−1200 with a scan time of 1 s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature of 100 °C, and desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/ h.

For the high-energy scan function, a collision energy ramp of 25−45 eV was applied in the T-wave collision cell using ultrahigh purity argon (≥99.999%) as the collision gas. A continuous

lock spray reference compound (leucine enkephalin; $[M + H]^+ = 556.2766$) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50−1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 2-propanol/water (v/v). MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using monoisotopic masses with specifications of a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms were set as follows: carbon, ≤ 100; hydrogen, ≤ 100; oxygen, ≤ 50; nitrogen, ≤ 6; sulfur, ≤ 6. The LC−QToF−MS data acquisition and analysis were based on the following defined parameters: mass accuracy (ppm) = $1{,}000{,}000 \times$ (calculated mass − accurate mass)/ calculated mass; fit conf % is the confidence with which accurate mass (measured data) matches the theoretical isotope models of the elemental composition in the list; elemental composition is a suggested formula for the specified mass. This is a summation of the quantities of elements, isotopes, or superatoms that can compose the measured data, calculated using the following atomic masses of the most abundant isotope of the elements: C = 12.0000000, H = 1.0078250, N = 14.0030740, O = 15.9949146, and S = 31.9720718. The empirical formula generated was used to predict structures that were proposed based on the online database, fragmentation pattern, and literature.

3.3.9 Determination of Maximum Dye Consumption Rate (V_{max}), Decolorization Rate Constant (K_m) and Reaction Kinetics Order Models.

Determination of maximum MG dye consumption rate (V_{max}), decolorization rate constant (K_m) and reaction kinetics order models was investigated at 25˚C and pH 8. 40 ml of MG dye of concentrations of 0.93mg/L, 1.86mg/L, 3.72mg/L, 5.57mg/L, 7.43mg/L and 9.30mg/L were

placed in 100ml different conical flasks and 10 ml of crude protease enzyme added to each flask. Absorbance reading was taken in duplicates at intervals of 30 minutes for 6 hours and the mean values calculated. The results obtained were fitted to various kinetic model equations from which the appropriateness of the model determined and values for V_{max}, K_m, k_0, k_1 and k_2 were calculated.

For RB5 dye, consumption rate (V_{max}), decolorization rate constant (K_m) and reaction kinetics order models were investigated at 36˚C and pH 9. 40 ml of RB5 dye of concentrations of 9.92mg/L, 19.84mg/L, 39.67mg/L, 49.59mg/L, 59.51mg/L, 79.35mg/L and 99.18 were placed in separate 100ml conical flasks and 10 ml of crude protease enzyme at pH 9 added to each flask. Absorbance readings were taken in duplicates at intervals of 12 hours for 120 hours and the mean value calculated. The results obtained were fitted to various kinetic model equations from which the appropriateness of the model determined and values for V_{max}, K_m, k_0, k_1 and k_2 were calculated.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1   Isolation of bacteria strains that produce protease enzyme

A total of fourteen pure bacterial species were isolated from the soil samples collected from Lake Bogoria and among the isolates, 4 strains showed good zone of clearance around the colony. The four isolates were treated as different strains and denoted as *1-p, 2-p, 3-p and 4-p*. Figure.4.1 shows a photograph of the four bacteria colonies isolated from soil with clear zones around their colonies indication that they are good protease enzyme producers. The isolate which showed higher protease activities were maintained on fresh nutrient agar plates and sub cultured after every thirty days.
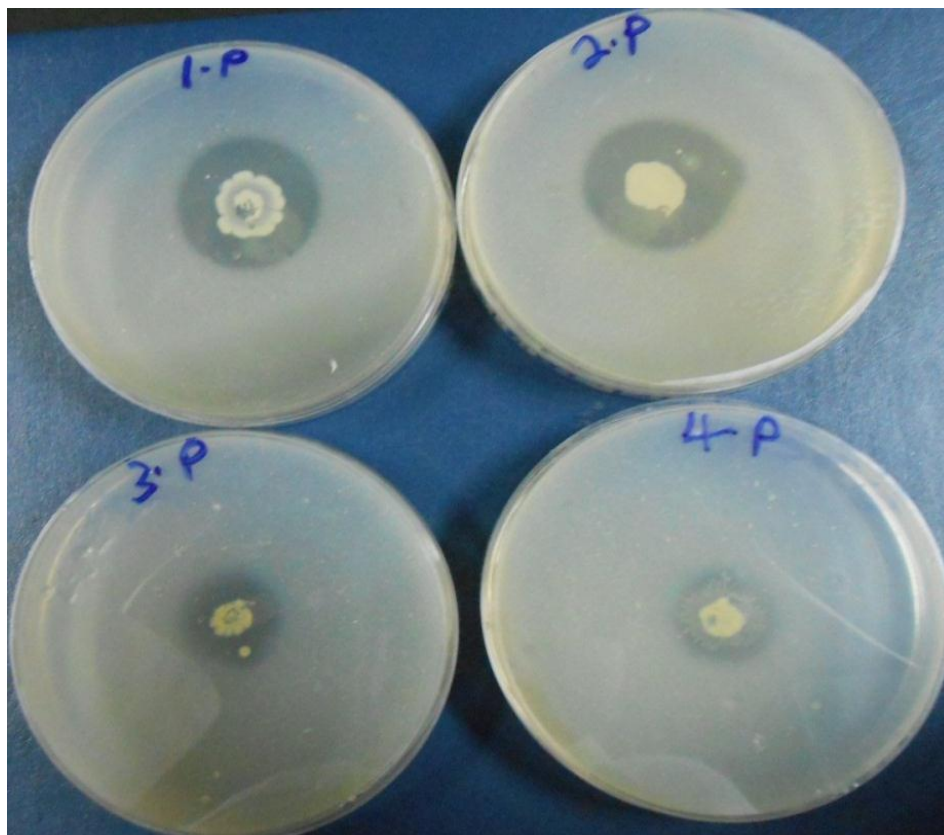

Figure 4.1.   Four pure bacteria isolates from soils samples obtained from Lake Bogoria (within the Kenyan Rift Valley) with proteases activity.

4.2   Proteases assay on agar plates

Protease activity on agar plates by the 4 bacteria isolates was determined using 0.5% casein as protein substrate in solid Reese media (Reese *et al.*, 1950). Degradation of protein in casein was indicated by the presence of a clear zone around the bacterial colonies. All the four strains were found to have proteolytic activity as shown in Figure 4.1. On the basis of clear zones around the colony 'Relative Enzyme Activity' (REA) was calculated using equation 14.

$$REA = \frac{\text{Total zone of enzyme activity and colony (cm)} - \text{Diameter colony(cm)}}{\text{Diameter colony(cm)}} \qquad (14)$$

The relative enzyme activities of the four bacteria were calculated and the results summarized in Figure 4.2. The results show that strains *1-p* and *2-p* had a higher relative enzyme activity of 2.0 compared to strains *3-p* and *4-p* which had a relative enzyme activity of 1.5 and 0.19, respectively. On this basis, Strains *1-p* and *2-p* were selected for further studies.
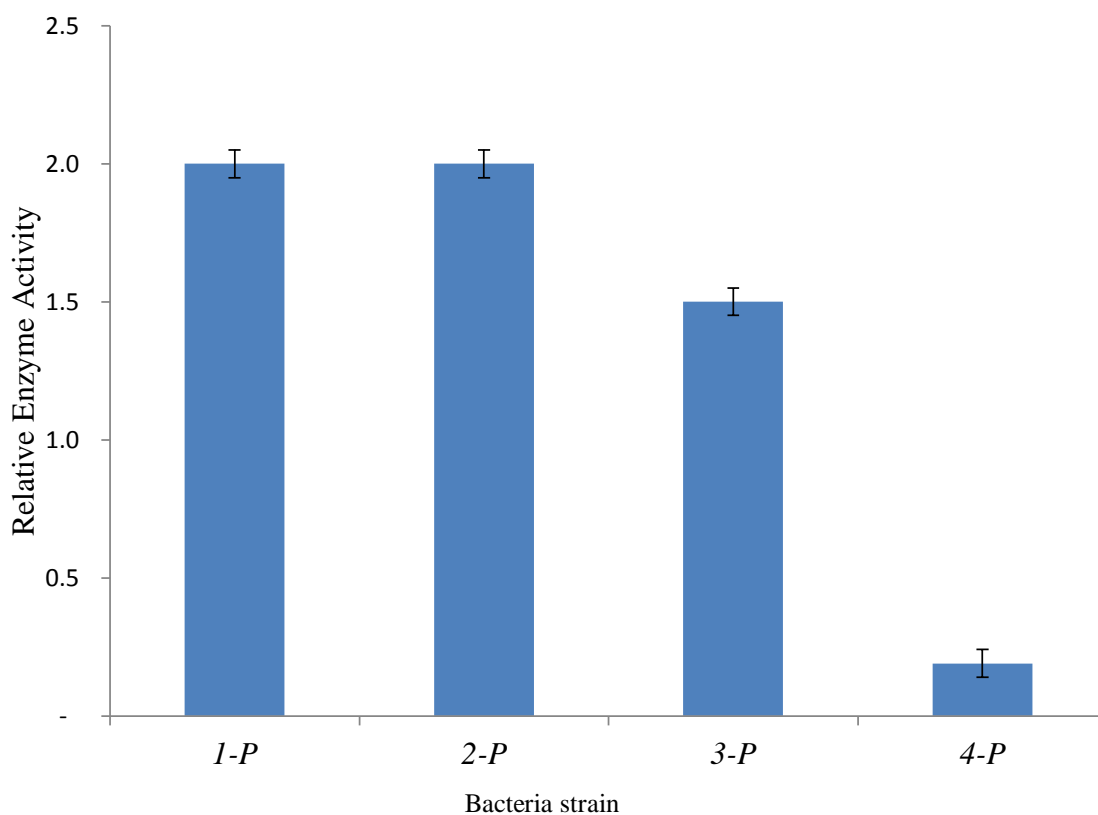

Figure 4.2. Relative Enzyme Activity' (REA) for four bacterial isolates.

4.3   Characterization of bacteria isolates

4.3.1   Morphological characterization

Gram staining allows most bacteria to be divided into two groups; Gram positive and Gram negative bacteria. The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram positive cells have a thick cell wall comprising of 50-90% peptidoglycan, whereas in case of Gram negative it is much thinner and surrounded by outer lipid containing layers. Gram positive cell wall has a stronger attraction for crystal violet when gram's iodine is applied than the gram negative cell wall. Gram's iodine is a mordant that forms a complex with the crystal violet that is attached more tightly to the Gram positive cell wall than to the Gram negative cell wall. This complex can easily be washed from the Gram negative cell wall with ethyl alcohol. Gram negative bacteria, however, are able to retain the crystal violet and therefore remain purple after decolorizing with alcohol. Since Gram negative bacteria will be colorless after decolorizing with alcohol (95% ethanol), counterstaining with counterstain safranin will make them appear pink.

The isolated bacteria strain morphological features were determined by Gram stain test. All the four strains isolates retained the purple color of crystal violet stain implying that they were Gram positive and rod shaped hence likely to belong to the *Bacilli* genus (Figure 4.3). Similar findings have previously been reported (Ngow and Wan Khairina, 2013).
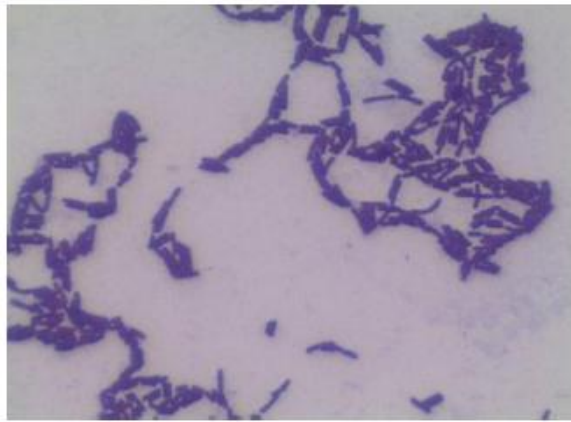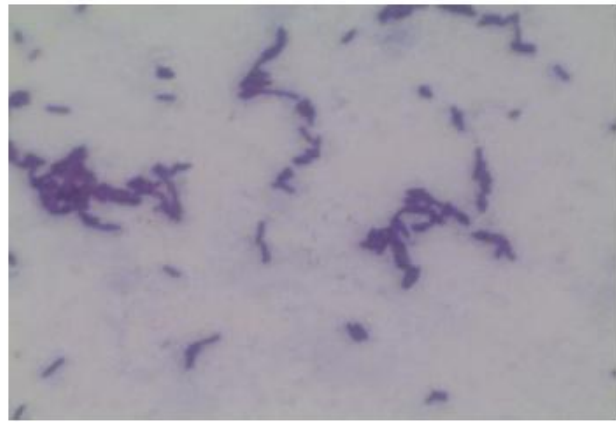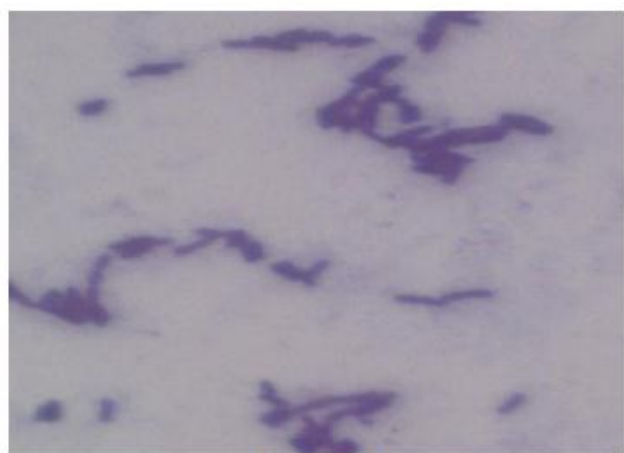
Isolate 1-p   Isolate 2-p
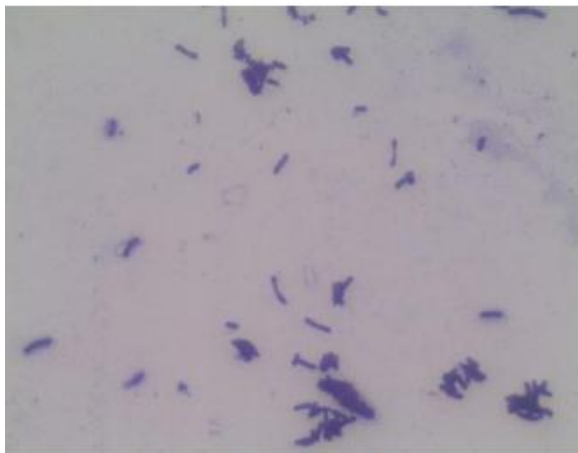
Isolate 3-p   Isolate 4-p

Figure 4.3. Photograph of bacteria *1-p, 2-p, 3-p* and *4-p* under electronic microscope (X 100) showing that they are Gram-positive Rod Shaped Bacillus strain.

4.3.2 Biochemical characterization

Biochemical tests are important tests in microbiology because they can be used to identify bacteria. The results of biochemical tests of the bacteria strains with high relative enzyme activity (*1-P* and *2-p*) are summarized in Table 4.1. These isolated strains showed that they could ferment sucrose and glucose, but not lactose. No bubble was detected from the glucose inserted with Durham tube indicating that no gas production could be associated with the growth. The results suggest that the isolates are likely to belong to the *Bacilli* genus.

Table 4.1. Biochemical tests of bacteria strain *1-p* and *2-p*.

Biochemical test	Bacterial colony *1-p*	Bacterial colony *2-P*
Growth at 4°C	Good	Good
Catalase test	Positive	Positive
D-Glucose fermentation	Positive, no gas produced	Positive, no gas produced
Lactose fermentation	Negative	Negative
Sucrose fermentation	Positive, no gas produced	Positive, no gas produced
Methyl Red Test	Positive	Positive
Citrate Utilization	Negative	Negative
Oxidation Fermentation	Negative	Negative

4.3.3 Molecular characterization and identification of bacterial isolates

4.3.3.1 Genomic DNA and 16S rDNA gel analysis

Systematic studies coupled with molecular study are well established to reveal the true identity of the organisms (Saxena *et al*., 2014). This is because arbitrary morphological or biochemical characteristics as used in classical taxonomy (Lynd *et al*., 2002) may not give accurate results due to a large diversity and abundance of microorganisms exhibiting different characteristics at various growth stages. In this study, all the 4 bacterial isolates were checked for variability at the molecular level with respect to 16S rDNA sequence analysis. Genomic DNA from each isolate was extracted by the procedure described by Chachaty and Saulnier (2000) method. A good quality genomic DNA was isolated without any signs of smearing when observed under UV transilluminator. The quality of genomic DNA extracted from the bacterial isolates and their corresponding 16S ribosomal DNA PCR amplifications were analyzed on a 1% agarose gel stained with ethidium bromide. Figure 4.4 show the gel photographs of the Genomic DNA and purified PCR product of all the 4 isolates for 16S rDNA. The bands visualized on 1.0 % agarose gel showed the sequence size of about 1.5kp.
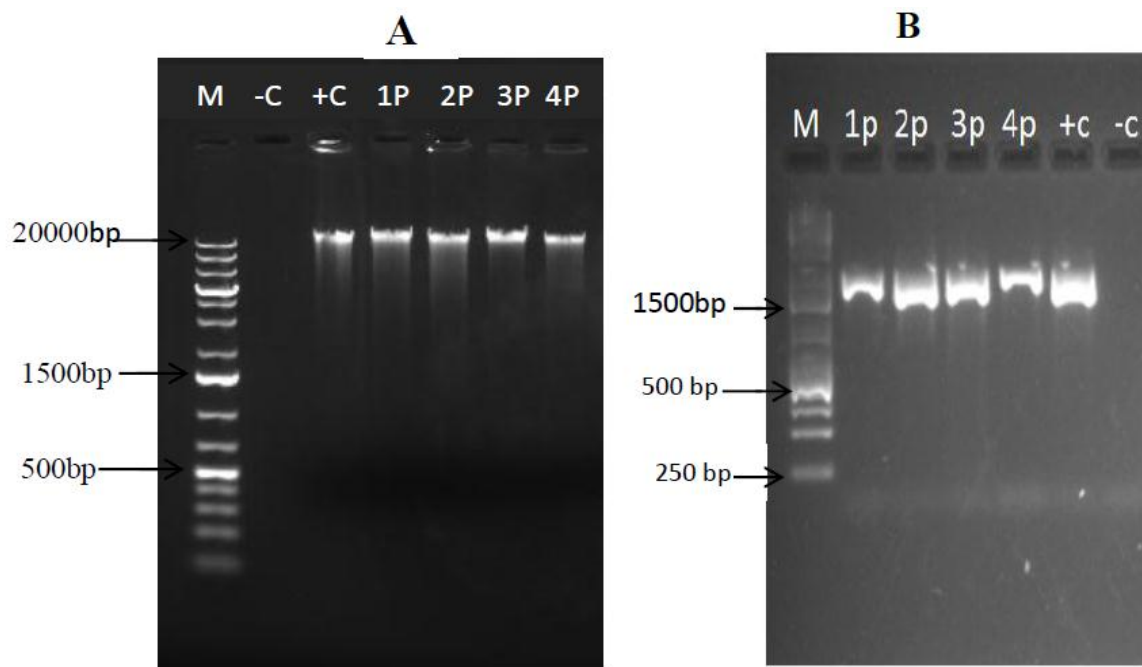
Figure 4.4: Agarose gel electrophoresis of genomic DNA (A) and 16S rDNA-PCR amplification (B) of alkaline protease producing-bacteria isolates 1-*p, 2 -p, 3-p* and 4-*p*. Lane M is the molecular ladder (1Kb ladder Generuler for genomic DNA and 1Kb ladder Fermentas for 16S rDNA-PCR), Lanes +C and –C are positive and negative control respectively.

4.3.3.2 Phylogenetic analysis

Classical taxonomy relies on a set of morphological and or biochemical characteristics (Lynd *et al*., 2002). Due to the limitation of this method, currently, inferring relatedness between organisms is based on phylogenetic trees that are based on measurements of sequence diversity of chronometers such as 16S ribosomal DNA (16S rDNA).

A phylogenetic tree based on the blast search was constructed using the 16S rDNA sequences from the four isolate and the topological robustness of the tree was evaluated using percentages of the posterior probabilities. Figure 4.5 shows the phylogenetic tree for 16S rDNA gene sequences. The tree was constructed in MrBayes, a program for the Bayesian inference of

phylogeny that is based on the Markov Chain Monte Carlo (MCMC) method. The four bacterial isolates clustered with *Bacillus cereus* strains suggesting they were likely to be *Bacillus cereus*. Furthermore, a BLASTN analysis carried out through GenBank (http://www.ncbi.nlm.nih.gov) revealed that all the isolates were members of *Bacillus cereus*.
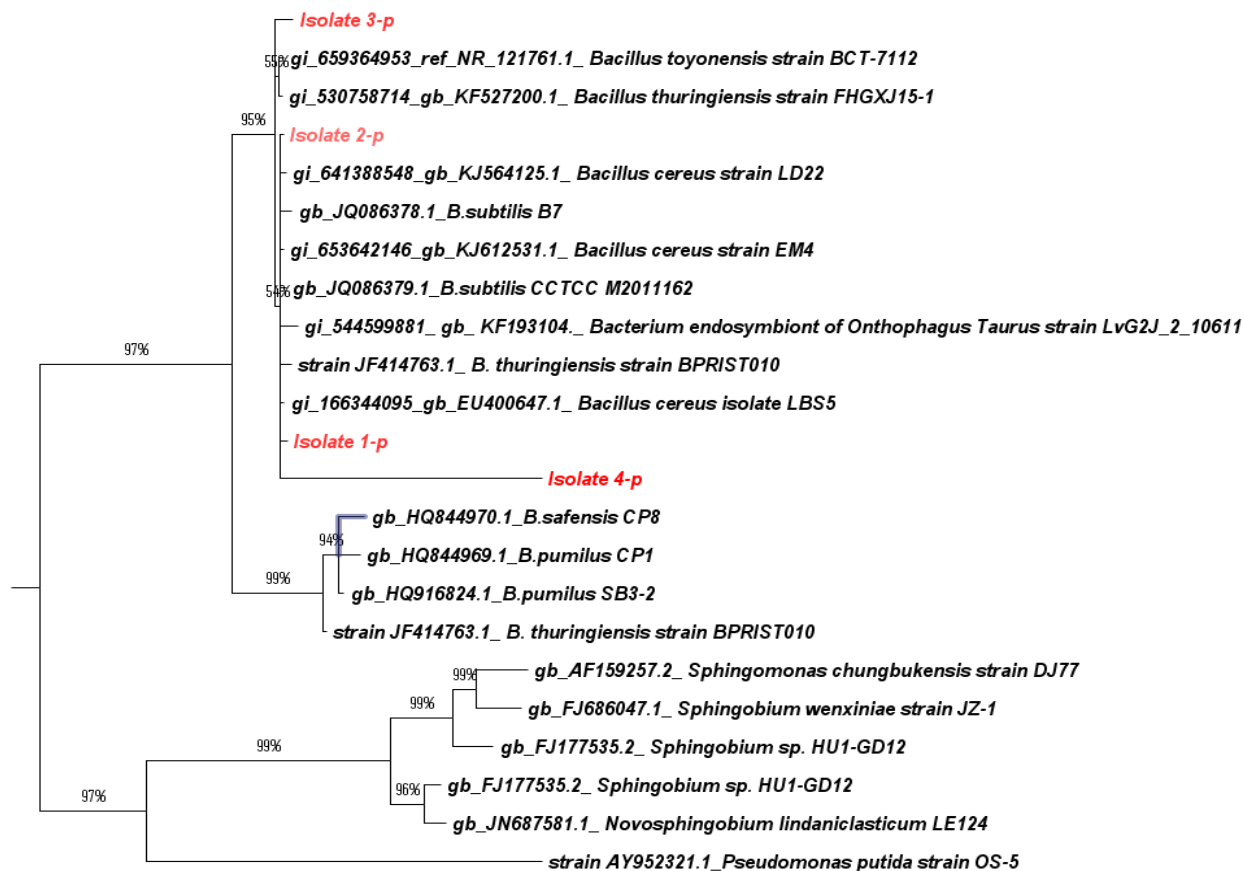
Figure 4.5: Phylogenetic tree constructed based on 16S rDNA gene sequence analysis from the isolates obtained from lake Bogoria, Kenya with reference sequences available at NCBI through BLAST analysis. Sequences highlighted in Red color are from the current study.

4.4 Enzyme assays

4.4.1 Generation of Tyrosine Standard Curve

A standard curve was generated using the Tyrosine Standard Solution thereby facilitating quantitation of the protease activity. Protease activity is directly proportional to the tyrosine

concentration/liberated which is directly proportional to absorbance at 660nm. Figure 4.6 shows Tyrosine Standard Curve generated using standard tyrosine concentrations. The high correlation coefficients (R^2 = 0.991) indicates how well data points fit a line.
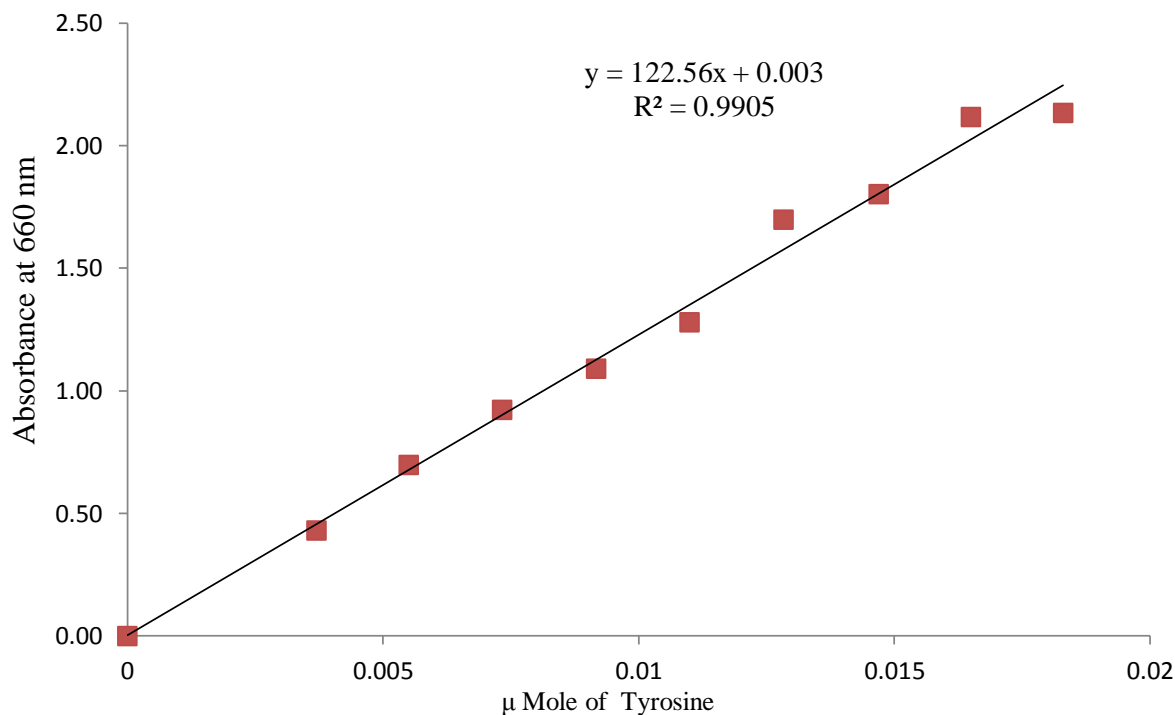

Figure 4.6. Tyrosine Standard Curve.

4.5 Process optimization for maximum protease production

Optimization of the process parameters was conducted in order to evaluate the effect of each individual parameter separately (i.e univariate optimization procedure) and to incorporate it as a standard level before optimizing the next parameter.

4.5.1 Determination of optimum pH for protease production

The pH of the culturing media strongly influences enzymatic processes and transport of compounds across the cell membrane. Many fermenting organisms exhibit satisfactory growth over a broad range of pH with maximum growth at an optimum point (Zwietering *et al.*, 1990). The effect of initial pH of the production medium on protease production by strains *1-p* and *2-p*

in broth was studied over a pH range of 3 to 12 and the results presented in Figures 4.7 and Figure 4.8, respectively.
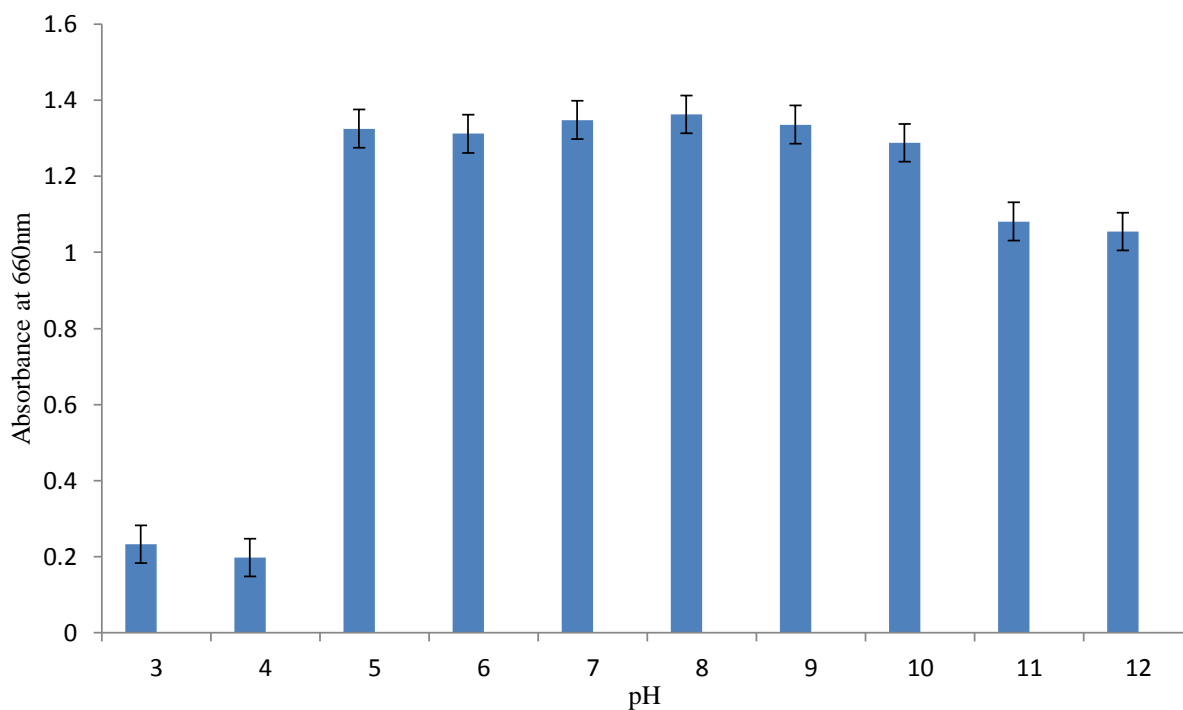

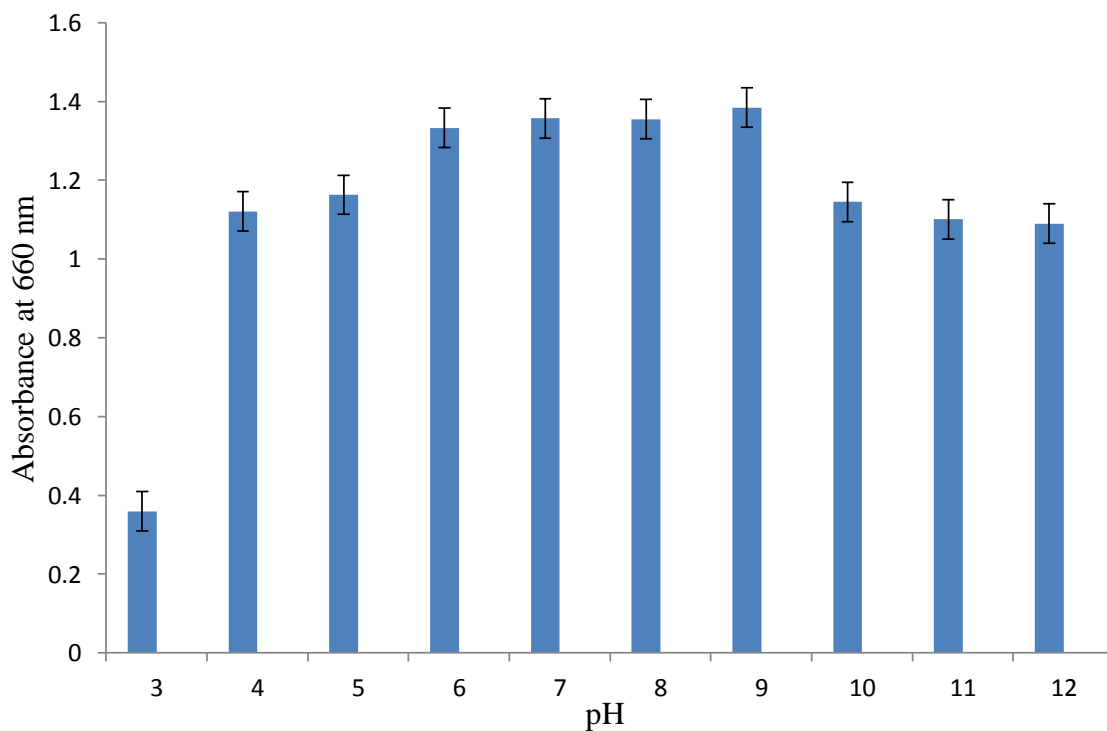Figure 4.7. Effect of pH on Tyrosine production by strain *1-p*.

Figure 4.8. Effect of pH on Tyrosine production by strain *2-p*.

The results show that at pH 3 and pH 4, there was low protease production for strain *1-p*. As the pH increased from 5 to 8 there was an increase in tyrosine concentration. Beyond pH 8, the concentration of tyrosine produced started to gradually decrease implying low protease production. Maximum tyrosine production for strain *1-p* was achieved at pH 8.0. However, the best range for protease production is between pH 5 and 10. The obtained results corresponds well with those obtained by Shivasharanappa *et al*.,(2014) who was investigating optimization and production of alkaline proteases from agro by product using a novel *Trichoderma Viridiae* strain VPG 12, isolated from agro soil.

Similarly, strain *2-p* had a low protease production at pH 3 but as the pH increased, there was an increase in tyrosine production till pH 9 beyond which tyrosine concentration started to gradually decrease with increase in pH. The maximum tyrosine production was achieved at pH 9.0 and the best pH range for protease production between pH 6 and 10. The results suggest that there is a stimulation of protease production under alkaline pH condition by both strains whereas extreme acidic pH inhibits protease production.

4.5.2 Determination of optimum time for protease production

The period of fermentation depends on the nature of media, fermenting organisms and physiological environment (Shivasharanappa *et al*., 2014). The effect of incubation time on protease production in broth was determined by incubating the culture medium at different time intervals (12 − 156 h) with intervals of 12 hours and the results are presented in Figure 4.9. Maximum Protease production was obtained after 48 hours for strain *1-p* and the tyrosine concentration gradually decreased from 48 to 156 hours. Strain *2-p* had maximum protease production after 60 hours and there after tyrosine concentration remained almost constant.
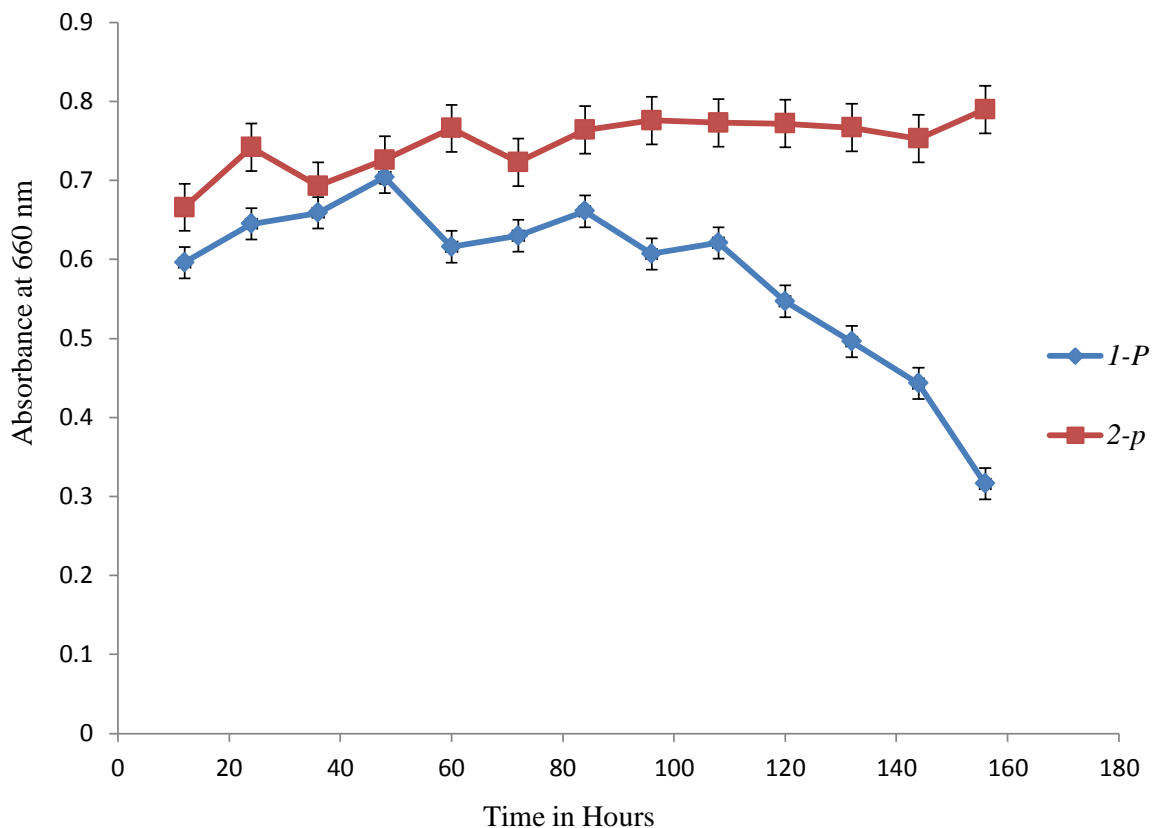
67

Figure 4.9. Effect of incubation time on Tyrosine production by bacteria strains 1-*P* and 2-*P*.

4.5.3   Determination of optimum temperature for protease production

Temperature is the most critical parameter to be controlled in any bio-process (Chi and Zhao, 2003) because it affects the growth and production of enzyme by microorganisms. The effect of initial temperature on protease production in broth was determined by incubating the production media at the optimum pH and different temperatures ranging from 27°C - 57°C for 48 h. Protease production was determined after 48 hours and the results are presented in Figure 4.10. It was generally observed that the protease production increased for both strains as temperature increased till 47°C when the protease production started to decline. The results indicate that both strains were sensitive to higher temperature. The optimum temperature for both bacterial strains for high level production of protease enzyme was found to be 47°C.
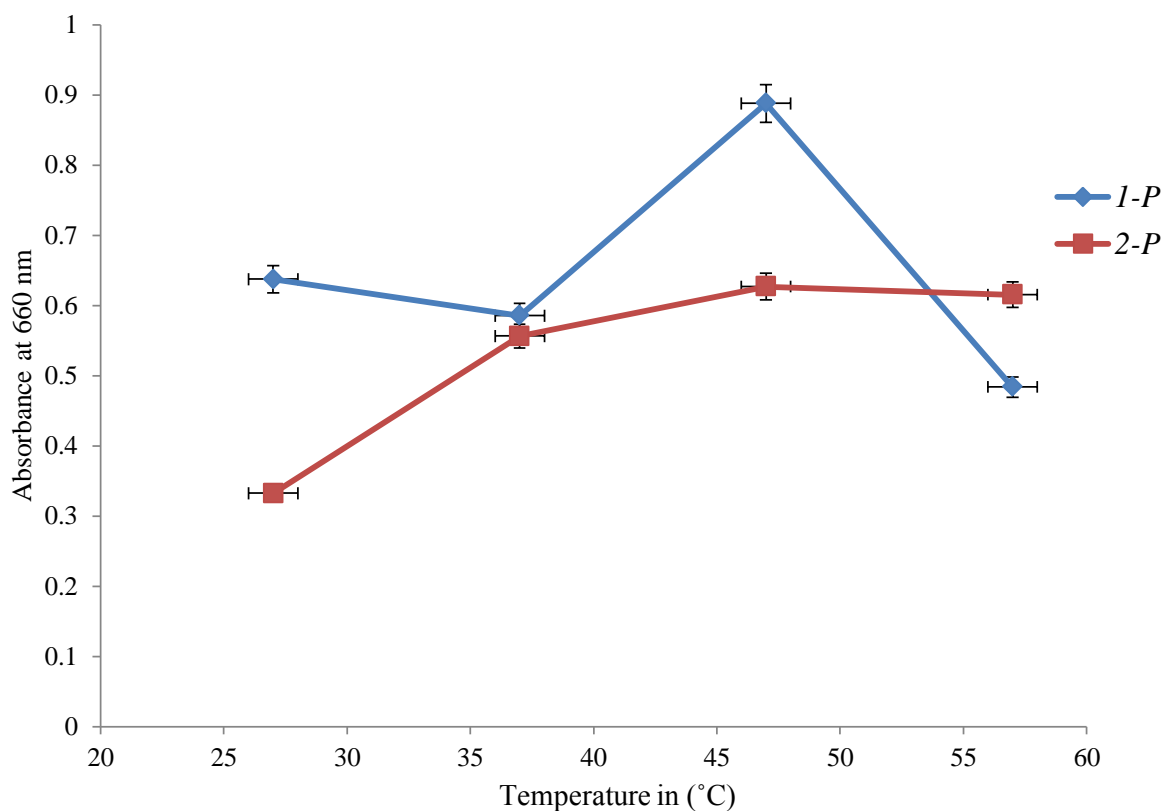
Figure 4.10. Effect of temperature on protease production by strains *1-P* and *2-P*.

4.5.4 Effect of nitrogen source on Protease production

Nitrogen either from organic or inorganic sources is an important factor in the growth of microorganism and production of enzyme. It should be noted that proteolysis enzyme producers are helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land (Choudhary and Jain, 2012). The effect of nitrogen source on protease production was investigated by incorporating various nitrogenous sources which included casein, beans, green gram, soybean, peptone, trypeptone, feathers, and wool in the production media and the results are presented in Figure 4.11 and 4.12 for strains *1-p and 2-p,* respectively. The highest tyrosine production was achieved by casein followed by trypeptone, soybean, wool and groundnuts for strains *1-p*. Similar results were obtained by strain *2-p* with wool liberating higher tyrosine.
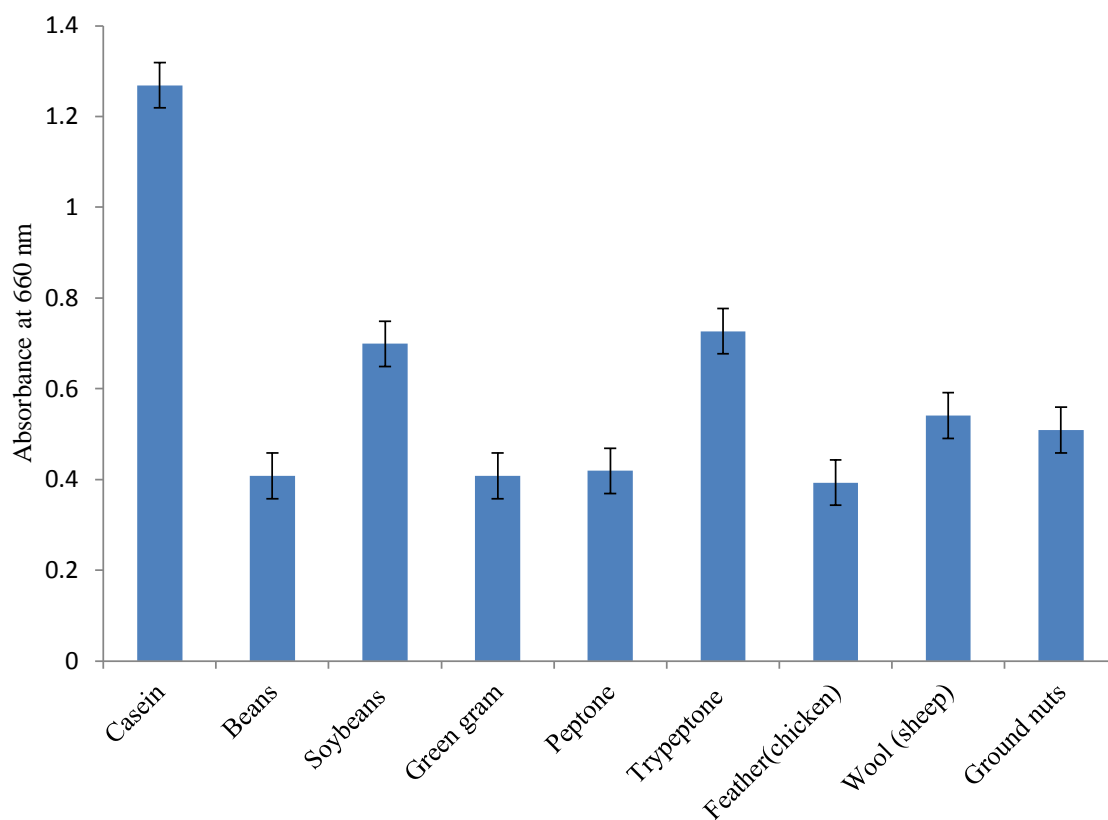
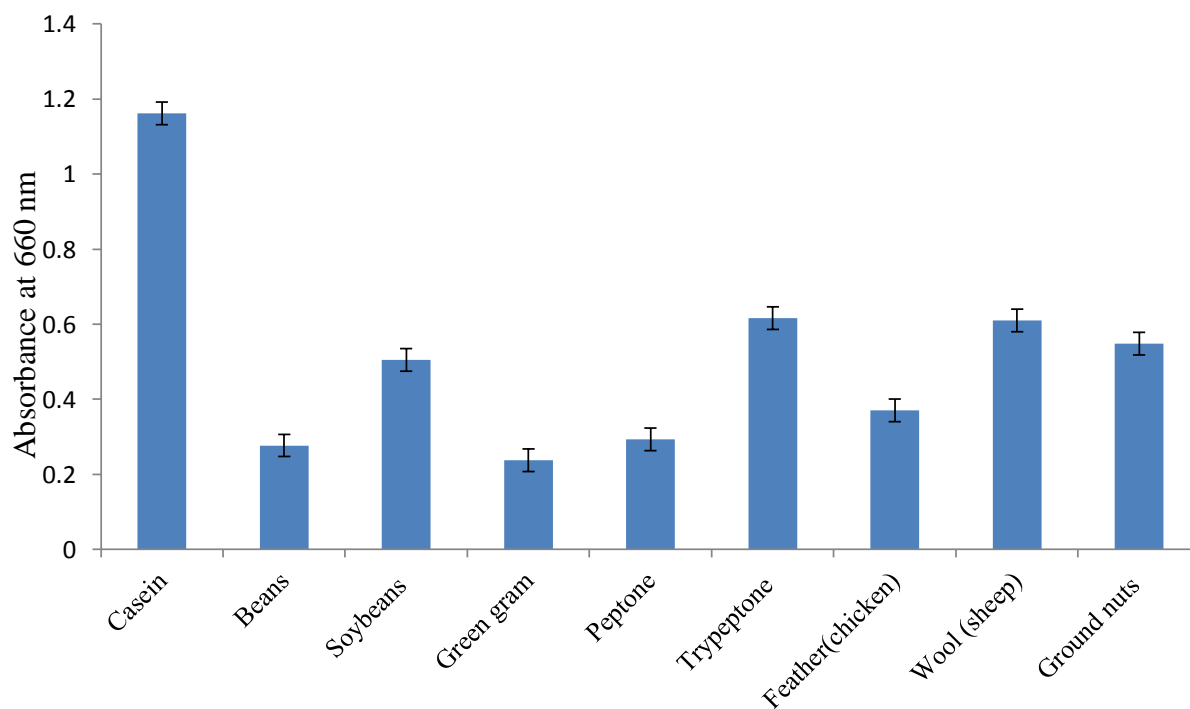Figure 4.11. Effect of nitrogen source on tyrosine production by strain *1-p*.

Figure 4.12. Effect of nitrogen source on tyrosine production by strain *2-p*.

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4.5.5 Large Scale Production of Crude Alkaline Protease Enzyme

The optimal fermentation conditions for crude alkaline protease production by strain *1-p* in shake flask were found to be at 47˚C at pH 8 for 48 hours while using casein as a nitrogen source. These conditions were used as standard conditions for large scale production of crude protease enzyme. About 5 liters of alkaline protease enzyme were produced under the above optimum conditions.

4.5.6 Protease Activity at Various Environmental Conditions

4.5.6.1 Effect of pH on crude proteases enzyme activity

Activity of the crude proteases enzyme from Strain *1-p* was investigated at different pH values ranging from 4 to 12. The enzyme showed good protease activity over a broader pH range in alkaline media. The optimum pH was found to be 11 (Figure. 4.13), indicating that the enzyme is an alkaline protease. The activity of the enzyme under alkaline pH conditions can present an advantage from industrial application point of view since reactions in tanneries and dye application in textile industry best take place in an alkaline environment.
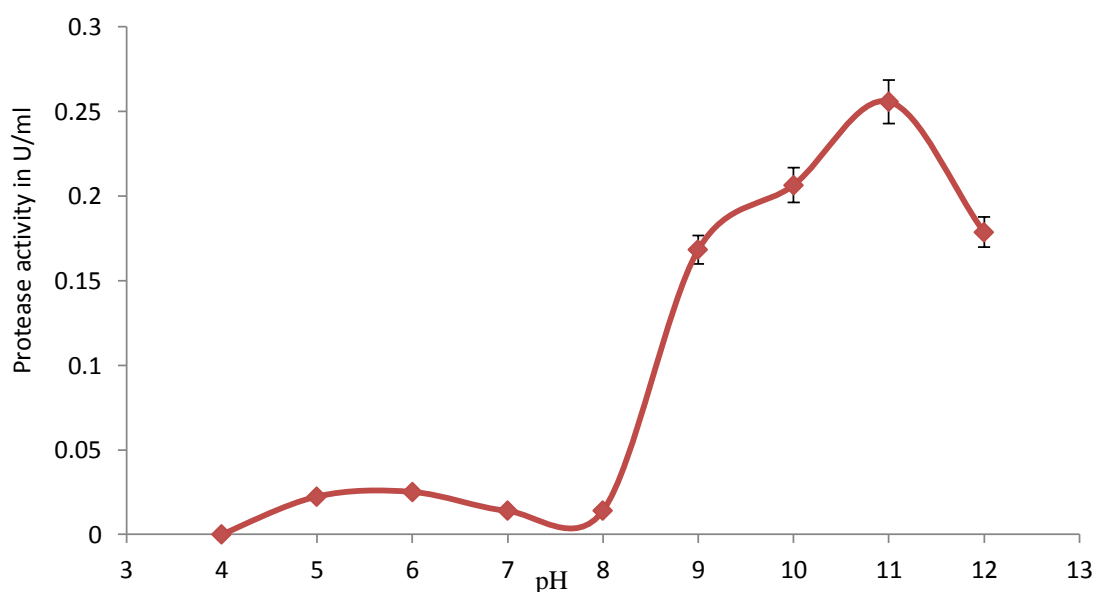

Figure. 4.13 Effect of pH on *Bacillus Cereus* Strain *1-p* protease activity.

4.5.6.2 Effect of temperature on crude proteases enzyme activity

Enzyme activity was marked over the entire temperature range of 20° C. to 90° C and the results are shown in Figure 4.14. The enzyme remained active over a wide range of temperatures but maximum enzyme activity was achieved at 45°C. The enzyme activity however, reduced drastically with temperatures beyond 45°C indicating that high temperature may denature the enzyme.
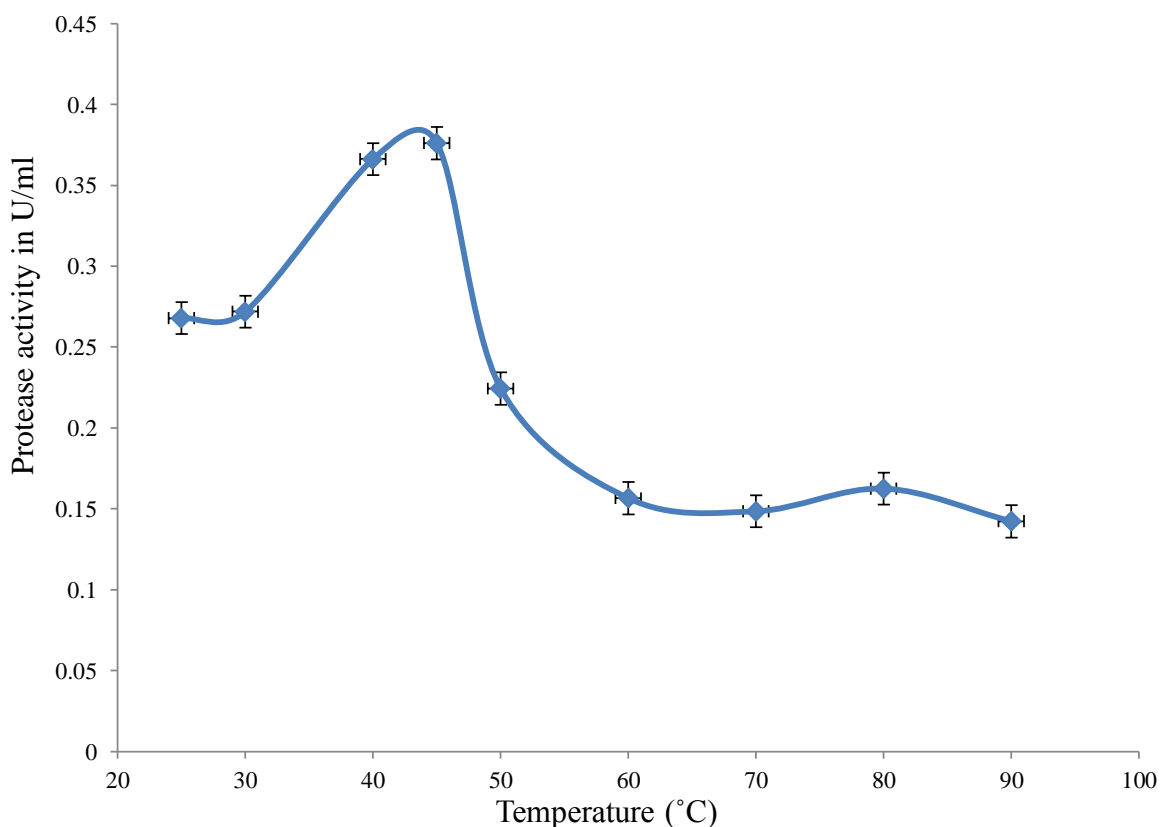

Figure 4.14 Effect of temperature on *Bacillus Cereus* Strain *1-p* protease activity.

4.6 Dehairing of Hide and Descaling of Fish Skin

4.6.1 Effect of pH on dehairing using crude protease enzyme

Enzymatic reaction is typically affected by changes in pH which is an important parameter in setting up a dehairing plant. The conventional liming method results in clean and white pelt due

to the operational pH being greater than 10 during lime-based processing (Punitha *et al.*, 2008). Effect of pH on the dehairing of cowhide using crude enzyme was investigated at 37°C within the pH range of 2.5 - 12.5 and the results are summarized in Table.4.2.

Table. 4.2 Effect of pH on enzymatic dehairing of cowhide at 37°C

pH	2.5	3	4	5	6	7	8	9	10	11	12	12.5
Time (hrs) required complete removal of the hairs	No hair removal after 24 hours	No hair removal after 24 hours	No hair removal after 24 hours	partial hair removal after 24 hours	partial hair removal after 24 hours	partial hair removal after 24 hours	18	5	5	4.5	3.5	3.0
Smell produced	none	none	none	Bad smell	Pungent smell	Pungent smell	Pungent smell	Bad smell	none	none	none	none

The results show that dehairing efficiency increased with increase in pH. At lower pHs of 3 and 4, there was no hair removal but as the pH was increased from 5 to 7, there was partial hair removal after 24 hours. At pH 8, there was difficulty in hair removal and complete dehairing was only achieved after 18 hours. When the pH was increased to 9 and 10 there was a drastic reduction in the time required for complete dehairing to 5 hours. About 4.5 hours was sufficient for complete hair removal at pH 11 and as the pH increased to 12 and 12.5, the time for easy and complete hair removal reduced to 3.5 and 3 hours, respectively. Difficulty, in hair removal was observed in negative control at all pH values tested indicating that crude alkaline protease enzyme was responsible for hair removal. Therefore, it can be concluded that the optimum pH condition for using alkaline protease in dehairing hide is about pH 12.5 and the general optimum pH range is from 11 to 12.5.

Figure 4.15a shows the photograph of the hide at pH 12 and 12.5 in a conical flask during the dehairing process while Figure 4.15b shows a photograph of a piece of hide completely de-haired at pH 12 within 3 hours as compared to a negative control which remained with hair when distilled water at pH 12 was used instead of crude alkaline protease enzyme. Increasing pH from

12 to 12.5 affects the physical characteristics of the dehaired hide. At pH 12, the hide is soft, flexible and bright grey in color while at pH 12.5, the hide is rigid, swollen and brown in color. It was noted that altering the pH of the enzyme does not only affect the time taken to loosen the hair but also changes the physical characteristics of the hide. It was also observed that at optimal pH, the pungent smell which is normally characteristic of many tanneries is eliminated. This is a positive result because crude alkaline protease enzyme does not only eliminate the use of sodium sulfide and lime but also eliminates the pungent smell which is a major air pollutant and of great health concern.
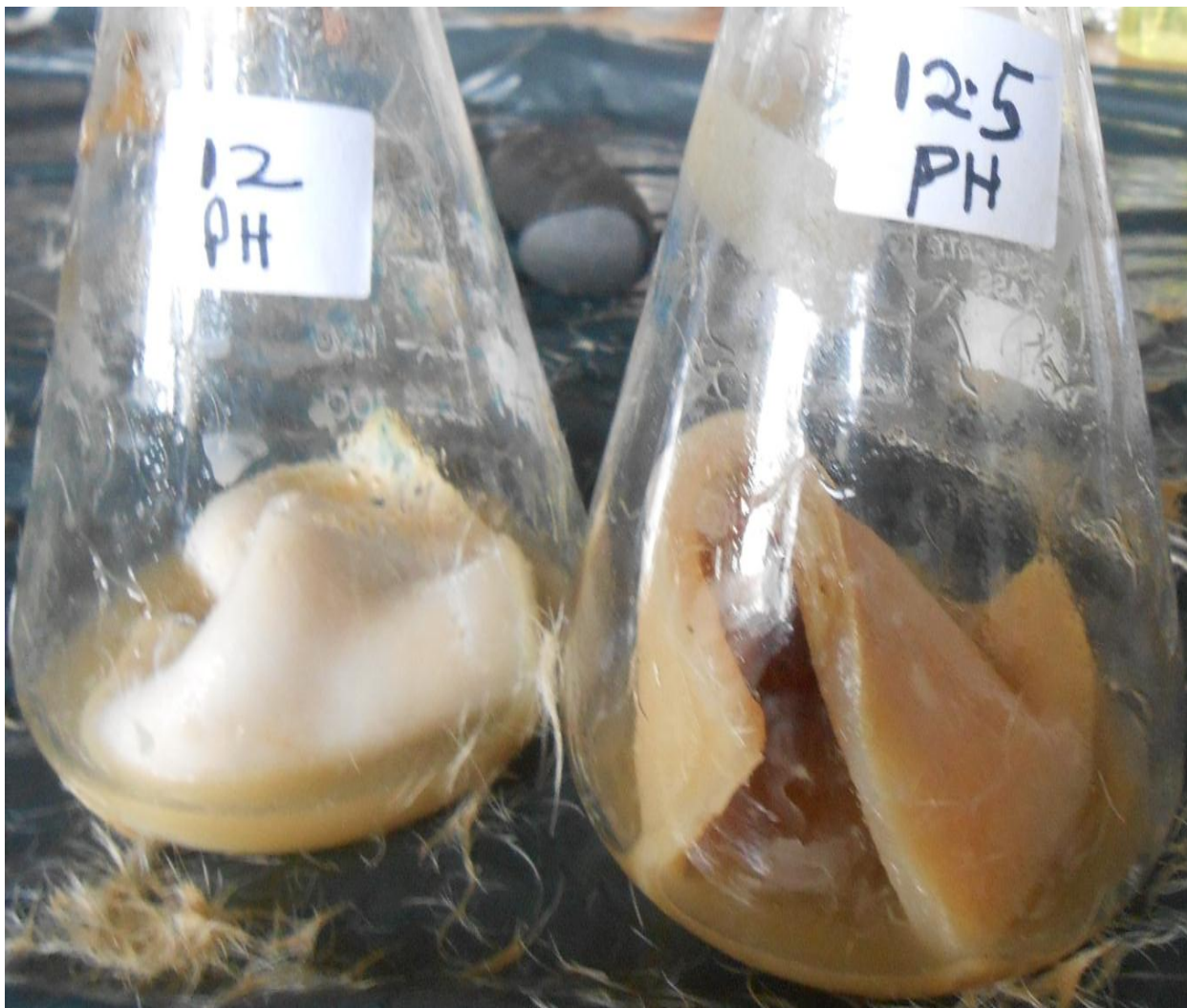

Figure.4.15 a. Photograph of the hide at pH 12 and 12.5 in a conical flask during the dehairing process.
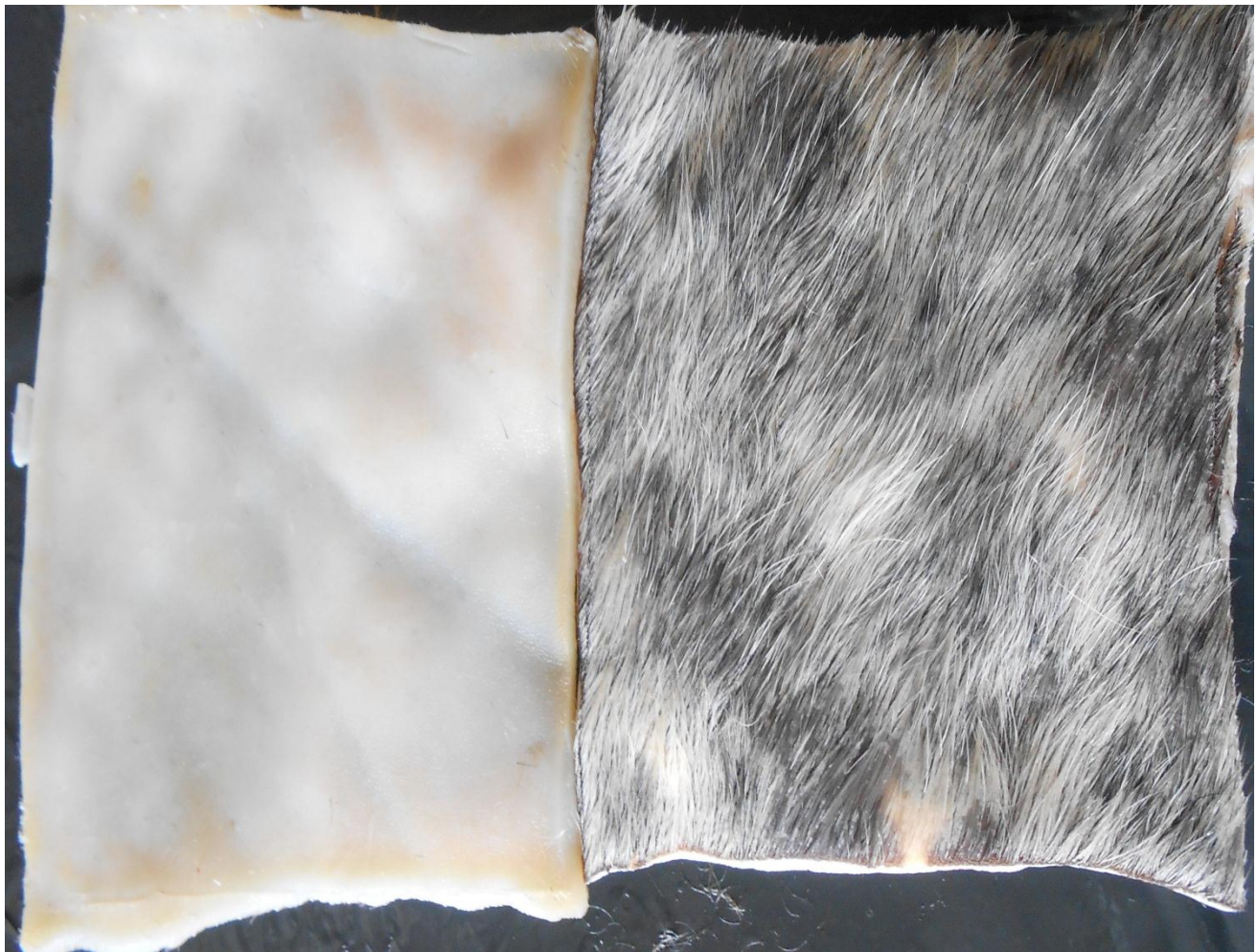
Figure. 4.15 b. Photograph of hide completely de-haired by crude alkaline protease enzyme within 3 hours at pH 12 and 37˚C; Negative control where distilled water at pH 12 was used remained with hair.

4.6.2 Effect of temperature on dehairing using crude protease enzyme

Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases with increase in temperature. A 10°C rise in temperature typically may increase the activity of most enzymes by 50 to 100% and variations in reaction temperature with as small as 1°C or 2°C rise in temperature may introduce changes of up to 10 to 20% in the reaction rate. Enzymatic reaction is complicated by high temperatures where many enzymes become denatured. The effect of temperature on dehairing of cowhide using crude protease enzyme was investigated at pH 12 within the temperature range of 27°C to 77 °C and the results are summarized in Figure 4.16.

The results show that increase in temperature lead to a general reduction in time required for dehairing. For instance, increasing temperature from 27°C to 57°C resulted in the reduction of time required for complete hair removal from 4 hours to 1 hour. Difficultly in hair removal was observed with the negative control at all temperatures.
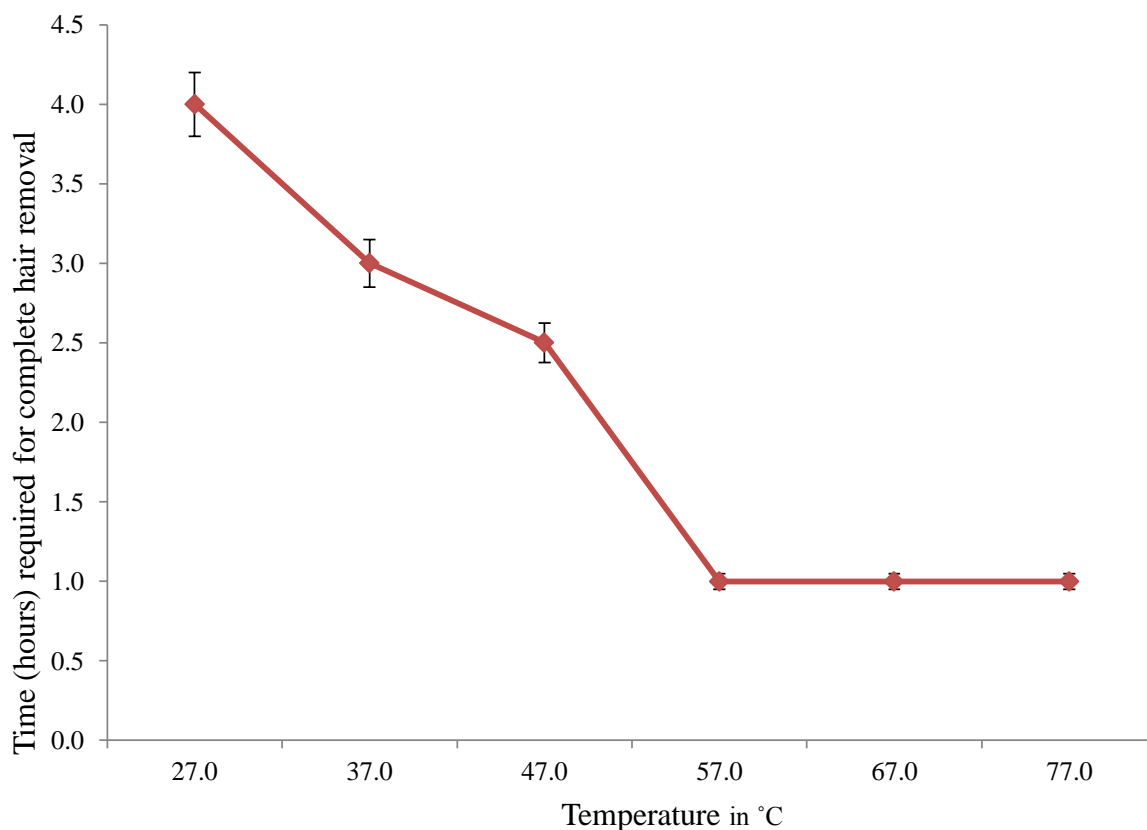

Figure 4.16. Effect of temperature on the time for dehairing of cowhide using crude protease enzyme at pH 12.

Figure 4.17 shows photographs of the dehaired hide at pH 12 and temperatures 27 °C, 37 °C, 47 °C, 57 °C, 67 °C and 77 °C. The results show that increasing temperature not only catalyzes the rate of hair removal but also affects the physical characteristics of the dehaired hide. Within the temperature range of 27 to 67°C, the skin is found to be soft and flexible but as the temperature increases to 77°C the hide becomes rigid, swollen and brittle. There was also gradual color change from bright grey to golden brown as the temperature is increased.
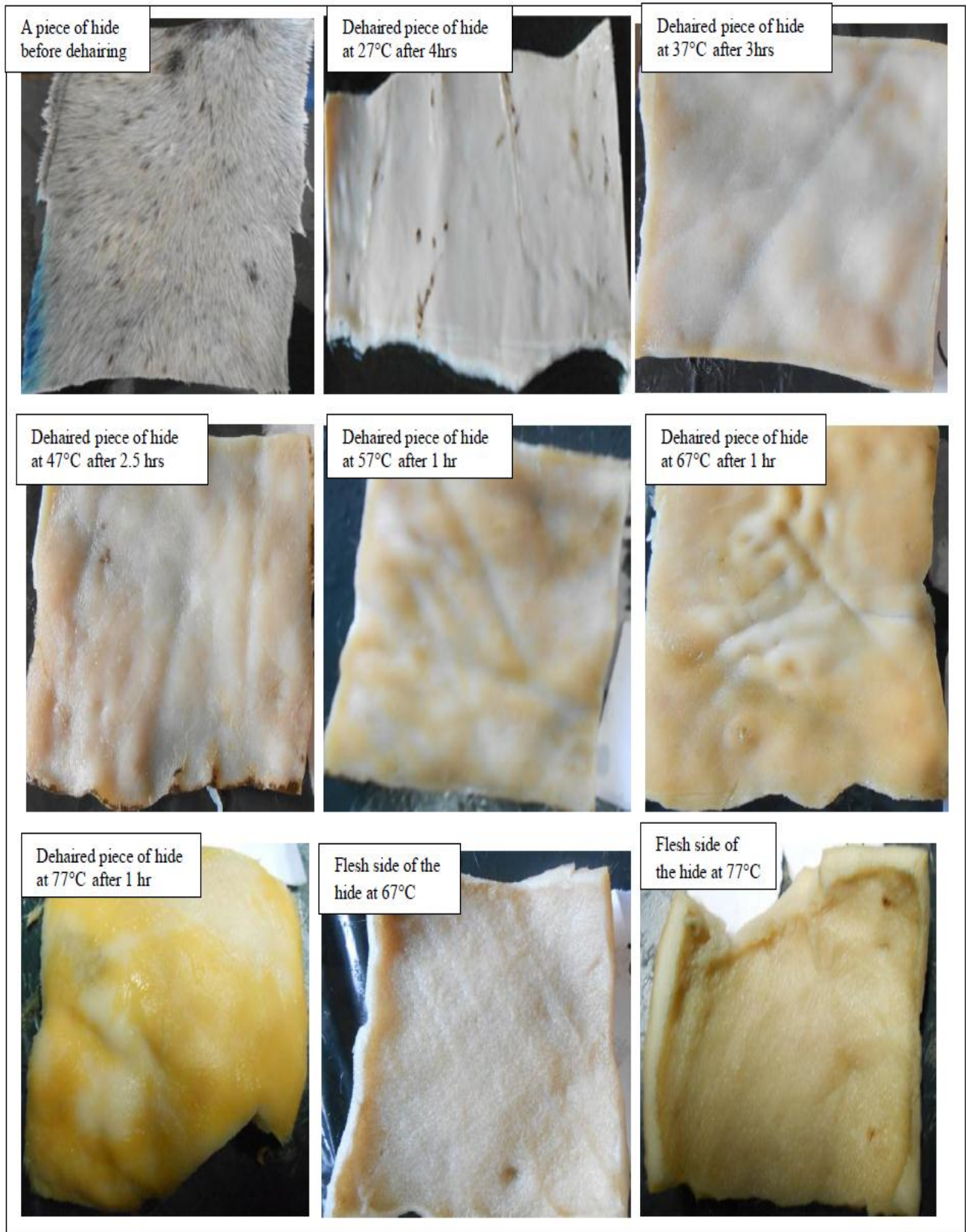
Figure 4.17. Photographs of 100% dehaired pieces of hide at pH 12 and temperatures of 27 °C, 37 °C, 47 °C, 57 °C, 67 °C and 77 °C.

4.6.3 Dehairing of full bull hide

The ability of crude alkaline protease enzyme to dehair a full bull hide was put to test at room temperature (25˚C) and pH 12. Figures 4.18 a, b and c shows the photographs of full hide before dehairing, during dehairing and after completion of dehairing, respectively.

Figures 4.18 a. Photograph of full hide before dehairing.

Figures 4.18 b. Photograph of full hide during dehairing (after 4 hours).

Figures 4.18 c. Photograph of full bull hide after 100% dehairing (after 12 hours)

Complete dehairing of bull hide was achieved within 12 hours. However, Four hours was sufficient to loosen hair in most parts of the skin except areas with healed scars, hump area and around the shoulder and neck. In addition, the pH of crude alkaline protease enzyme solution had drastically dropped to pH 7.5; a possible explanation why the hair was partially removed. This is attributable to possible solubilization of fatty acid from the skin. The non-contaminated and dissolved fatty acids can be used in the production of chemicals, biodegradable detergents, pesticides, and flotation agents (Gavrilescu and Chisti, 2005). The pH of the crude enzyme was re-adjusted to pH12 and then incubated for the next 6 hours where all the hair loosened out and

were manually and gently scraped with a blunt knife. The removed hair was collected, dried and its quality checked.

The hair which was removed from the hide during the enzyme treatment was readily recovered from the enzyme liquor by decantation and filtration techniques. Figure 4.19 shows the photograph of the removed hair after enzymatic dehairing. About 1.1 kg of hair was collected and the hair was of good quality without any breakage or signs of fragileness when pulled apart. Evidently, this is an advantage of using crude alkaline protease enzyme in comparison with the conventional lime sulfide-dehairing system which would otherwise lead to the destruction of the hair leading to increased COD, BOD and TDS loads in the effluent (Dayanandan *et al*., 2003). About 40% of BOD, and 50–60% COD is contributed by these methods (Dayanandan *et al*., 2003).

The recovered hair was in good condition suitable for further use in making brushes or other textile products. Recovery of good hair from the effluent system greatly helps in the reduction of sludge concentration and ultimate pollution load. Moreover, after removal of the hair, the clear liquor may be re-used alone, or in combination with fresh enzyme solution for the treatment of subsequent batches of hides or skins. In conclusion, the present invention has surprisingly succeeded in finding an environmentally friendly method for dehairing hides or skins by means of enzymes, which results in completely dehaired hides or skins with an undamaged gram. The use of crude alkaline protease enzyme from *Bacillus Cereus* Strain *1-p* in leather processing has shown huge potential to eliminate the pollution-causing chemicals such as sodium, lime and solvents.

Figure 4.19. Photograph of the removed hair after enzymatic dehairing.

Physical and mechanical properties of enzymatically dehaired hide were determined prior to tanning and the results compared with specifications provided by The Kenya Bureau of Standards (KEBS) as summarized in Table 4.3. As shown, the results of various parameters obtained for enzymatically dehaired hide revealed that the quality of the hide was within KEBS specifications and therefore recommended as suitable for tanning process.

Table. 4.3. Physical test results of enzymatically dehaired hide

Parameter	Unit	T/Method	KEBS Specifications	Actual Measurement (mean)	Remarks
Thickness	mm	Micrometer gauge	0.8-2.0	1.8	pass
Tensile strength	Mpa	Tensometer	>10	11.1	pass
Tear stress	N	Tensometer	35	35	pass
Elongation	%	Tensometer	10-20	12	pass
Lastometer grain crack	mm	Lastometer	4.0 min	3	fair
Lastometer-burst	mm	Lastometer	6 .0 min	5	fair
Fat content	%	soxhlet extraction system	6 max	5	pass
Pressure folds	%	Physical/ Visual	No pressure folds	None	pass
Putrefactions marks		Physical/ Visual	No Putrefactions marks	None	pass
Degree of swelling	%	Physical/ Visual	No Swelling	None	pass
Stretch /Shrinkage	%	Physical/ Visual	97	98	pass
Enlarged flanks /loose grain or cracked grain		Physical/ Visual	No Enlarged flanks /loose grain or cracked grain	None	pass
Swelling and plumping		Physical/ Visual	No Swelling plumping	None	pass
Staining		Physical/ Visual	No Staining	None	pass
Hair removal	%	Physical/ Visual	97	98	pass
Short hair and scud removal	%	Physical/ Visual	97	85	fair
Growth marks and wrinkled grain		Physical/ Visual	No Growth marks and wrinkled grain	None	pass
Recommendation: HIDE SUITABLE FOR TANNING					

4.6.4 Descaling of Nile perch skin

The isolated crude alkaline protease enzyme was applied in the descaling of Nile perch skin at 47˚C and pH 12. Figures 4.20a and 4.20b shows photographs of Nile perch skin before and after 100% enzymatic descaling, respectively. Complete descaling of Nile perch skin was achieved within 1 hour at pH12 and 47˚C. It was observed that at pH 12 the characteristic fish smell is

greatly reduced or eliminated. This effectively solves the major public concern over tanneries and fish processing industries which has traditionally been odour due to the generation of noxious gases, water pollution from untreated discharges and solid waste pollution.

Figure 4.20a. Photograph of Nile Perch (*Lates niloticus*) skin before descaling

Figure. 4.20 b. Photograph of Nile perch skin (*Lates niloticus*) descaled within one hour using crude alkaline protease enzyme from *Bacillus Cereus* Strain *1-p*

Crude alkaline protease enzyme at high pH and temperatures aided in the swelling of the fiber structure of the Nile Perch skin which also enabled the enzyme to hydrolyze unnecessary water-soluble proteins (such as collagen), grease or fat matter between its layer and fibers of the skin. The natural grease and fatty acids recovered are highly valued and can be used for commercial value. Agitation or stirring of the enzyme solution with the Nile Perch skin provides for more rapid and uniform descaling process. The loosened scales were removed by exposure to mechanical agitation.

The descaled Nile perch skins were then thoroughly washed with clean tap water to remove enzyme remnants, solubilized proteins and fatty acid. The scales were easily recovered by filtering from the enzyme liquor before being washed thoroughly by clean water and dried in the sun (Figure 4.21). Recovery of valuable scales from the effluent system greatly helps in the reduction of sludge concentration which highly contributes in blockage of sewerage pipes, increased COD, BOD, TDS and ultimate increase in pollution load of the receiving waters. Moreover, after removal of the scales, the clear liquor may be re-used alone, or in combination with fresh enzyme solution for the treatment of subsequent batches of Nile Perch skins. This process of enzymatic descaling of fish skin eliminates processes such as liming, deliming and bating which consumes and emit most toxic chemicals in conventional leather manufacturing processes.

Figure. 4.21 Photograph of the recovered scales after descaling Nile perch skin using crude alkaline protease enzyme from *Bacillus Cereus Strain 1-p.*

4.6.5 Post dehairing and descaling Leather processing

The enzymatically dehaired hide and descaled Nile perch skin were taken to Thika Leather Industries of Kenya Ltd (LIK) tannery for tanning and post tanning processing. At the tannery, prior to chrome tanning, the pH of both the hide and Nile perch skins were reduced from pH 10 to pH 2.8 in a process called pickling. This process was particularly important with regards to grain tightness and improved chrome uptake, thus reducing the input of chrome. It also gives the skin appropriate pH for long term preservation, so that it can be protected from mold, fungus and bacteria against deterioration.

After undergoing pickling, the hide and Nile perch skins were subjected to chrome tanning by reacting with basic chrome (Cr_2O_3). This process was meant to impart thermal resistance, corrosion resistance, anti-perishability and flexibility by transforming the collagen proteins of

unstable structure into the property of mineral leather with the use of chrome through a cross-linking action. After tanning, the leathers were drained, rinsed by hanging up to age and subsequently sammed (brought to a uniformly semi-dry state) to reduce the moisture content before further mechanical action. Figure 4.22a and b below shows photographs of tanned hide and Nile perch skin, respectively after drying. The tanned hides or skins are tradable intermediate products as wet-blue leather (Afshar *et al.*, 2011).

Figure. 4.22a. Photograph of tanned hide

Figure. 4.22b. Photograph of tanned Nile perch skin (*Lates niloticus*)

Re-tanning process was performed on the wet blue leather to improve its characteristic and re-wetting properties of the hides/skin necessary to facilitate and optimize the subsequent dyeing process. After re-tanning, the hide and Nile perch skin were dyed by dark mimosa imparting basic color and flexibility to the hide and skin. The resultant leather were subjected to fat liquoring where the leathers were lubricated to achieve product-specific characteristics and to reestablish the fat content lost in the previous procedures before air drying to eliminating water. Unnecessary tissues of the leather were physically adjusted to obtain the desired thickness of raw hides using a High-Tech Leather Splitter.

Finishing operations were subjected to the leather to enhance the appearance and provide the performance characteristics with respect to color, gloss, feel, flex, and adhesion. In addition, other properties including stretch-ability, break, light- and perspiration fastness, water vapor permeability, and water resistance were enhanced in the finishing process. Figures 4.23a and b provides photographs of the resultant leather from the bull hide and Nile perch skin after post tanning processing.

Figure 4.23 a. Photograph of the resultant leather after post-tanning processing of bull hide

Figure 4.23b. Photograph of the resultant leather after post-tanning processing of Nile perch skin

In order to determine the quality of leather produced after full processing, physical and mechanical properties were evaluated and from the results, possible commercial applications would be recommended.

4.6.6 Physical and Mechanical properties Testing and Evaluation of Enzymatically-Processed Leathers

Effective dehairing system should take care of the leather qualities as well as the environment. Mechanical properties have generally been given the greatest consideration in the evaluation of leather quality (El-Monem *et al*., 2014). The properties investigated included tensile strength, elongation at break, tear strength, water absorption and grain crack. The results were compared against specifications provided by Kenya Bureau of Standard (KEBS) as tabulated in Table 4.4 for the bull hide leather.

Table 4.4. Physical test results of leather produced from enzymatically-dehaired bull hide

Parameter	Unit	Test Apparatus	KEBS Specifications (Hide Leather)	Actual Measurement (mean)	Remarks
Thickness	mm	Micrometer gauge	1.6 - 1.8	1.8	pass
Tensile strength	MPa	Tensometer	>20	25.83	pass
Tear stress	N	Tensometer	60 - 120	117.72	pass
Elongation at Break	%	Tensometer	40 - 70	43	pass
Lastometer – Grain Crack	mm	Lastometer	≥ 7.0	9.95	pass
Lastometer – Burst	mm	Lastometer	≥ 10.0	13.4	pass
Moisture content	%		< 60	48.57	pass
Recommendation: Leather suitable for production of Military Boot Uppers					

Considering results in Table 4, it can be stated that enzymatically-dehaired hide yield good quality leather which meets the requirements set by Kenyan Bureau of Standards. The strength properties of enzymatically dehaired leather was significantly stronger compared to strength properties of the leathers dyed with and without ultrasound using 4% acid red dye (Sivakumar

and Rao, 2003). Further interpretation of results showed that the resultant leather was best suitable for production of Military Boot Uppers; which require leather of great strength to withstand the hard task performed by army personnel. Enzymatically descaled Nile perch skins were analyzed and the result presented in Table 4.5.

Table 4.5. Physical test results of leather produced from enzymatically descaled Nile perch skin

Parameter	Unit	Test Apparatus	KEBS Specifications (Hide Leather)	Actual Measurement (mean)	Remarks
Thickness	mm	Micrometer gauge	1.6 - 1.8	2	Fail
Tensile strength	MPa	Tensometer	>20	14.85	Fail
Tear stress	N	Tensometer	60 - 120	32.5	Fail
Elongation at Break	%	Tensometer	40 - 70	34	Fail
Lastometer – Grain Crack	mm	Lastometer	≥ 7.0	11.8	pass
Lastometer – Burst	mm	Lastometer	≥ 10.0	13.7	pass
Moisture content	%		< 60	49.7	pass
Recommendation: Leather suitable for production of Ladies hand bags, Sofa set Lining and Making of vehicle mats					

The results were compared with Specifications set for hide leather since KEBS doesn't have specification for Nile perch skin. Parameters such as thickness, lastometer – grain crack & burst and moisture content meet KEBS Specifications set for Hide Leather while Tensile strength, Elongation at Break and Tear stress failed. Generally, the leather obtained exhibit better qualities such as softness, toughness and pliability. The resultant fish leather exhibited moiré patterns unique to fish skin created through the descaling process, which in turn provide beauty totally different from that of general leather. From these results, the Nile perch skin leather was best suited for production of ladies hand bags, sofa set lining and making of vehicle mats. The result from this analysis does not only make Enzymatic dehairing/descaling a clean green technology which reduces pollution load but produces leather of high quality within a short time. In

summary, the results obtained in this research provide overwhelming advantages of enzymatic dehairing/descaling which can be summarized as follows:

(i) Complete elimination of the use of sodium sulfide and lime which cause environmental pollution

(ii) Production of high quality leather which is soft, tough, pliable and supple

(iii) Recovery of hair of good quality and strength with a good saleable value thus reducing dissolved hair in wastewater. This also ensures an additional profit of the value of the processed skin.

(iv) Recovery of scales/hair which significantly reduces total solids, dry sludge and COD in the effluent.

(v) Creation of an ecologically conducive atmosphere for the workers because toxic chemicals and pungent smell are eliminated.

(vi) Simplification of pre-tanning processes by cutting down some step, viz. liming, deliming, bating and the costs associated with these steps are eliminated.

(vii) Enzymatic dehairing/descaling saves time. The lime-sulfide process takes over 36 hrs, whereas the enzymatic dehairing would be completed between 1 to 12 hours thus cutting down cost.

In view of these aspects, the enzymatic dehairing method is advantageous from the practical point of view in that in addition to reducing the load of waste water disposal, a leather of good quality is obtained and all hair recovered. However, the main problem which hinders the utilization of enzymatic dehairing is the difficulty in the control of the degree of enzyme action (Jian *et al.*, 2011). This is because protease can digest collagen in the process of dehairing/descaling and must be carefully controlled to avoid reducing the quality of the leather

or digesting the skin. Enzymatic dehairing/ descaling must be carried out by experienced and skillful technicians to optimize on profit.

In view of the resultant leather products; both from the bull hide and Nile perch skins, It can now be authoritatively stated that crude alkaline protease enzyme from *Bacillus Cereus* Strain *1-p* can be used to process hides and skins to yield good leather.

4.7   Decolorization and Bioremediation of Malachite Green Dye

4.7.1   Wavelength of Maximum Absorbance (λ_{max}) for Malachite Green Dye

The wavelength for maximum absorbance for malachite green dye was determined to be 617nm. This wavelength is the one at which the samples absorb the most light and is called lambda max and the Greek letter λ is used to symbolize wavelength. Figure 4.24 below is a plot of absorbance against wavelength for Malachite green dye solution. It is evident from the plot that the wavelength at which there is a maximum absorption is 617nm. These results corresponds well with those obtained by Afshar *et al.*, (2011) . The UV-VIS spectrophotometer was set to this wavelength (λ_{max} = 617nm) and used for the rest of the experiments involving the use of Malachite green dye.
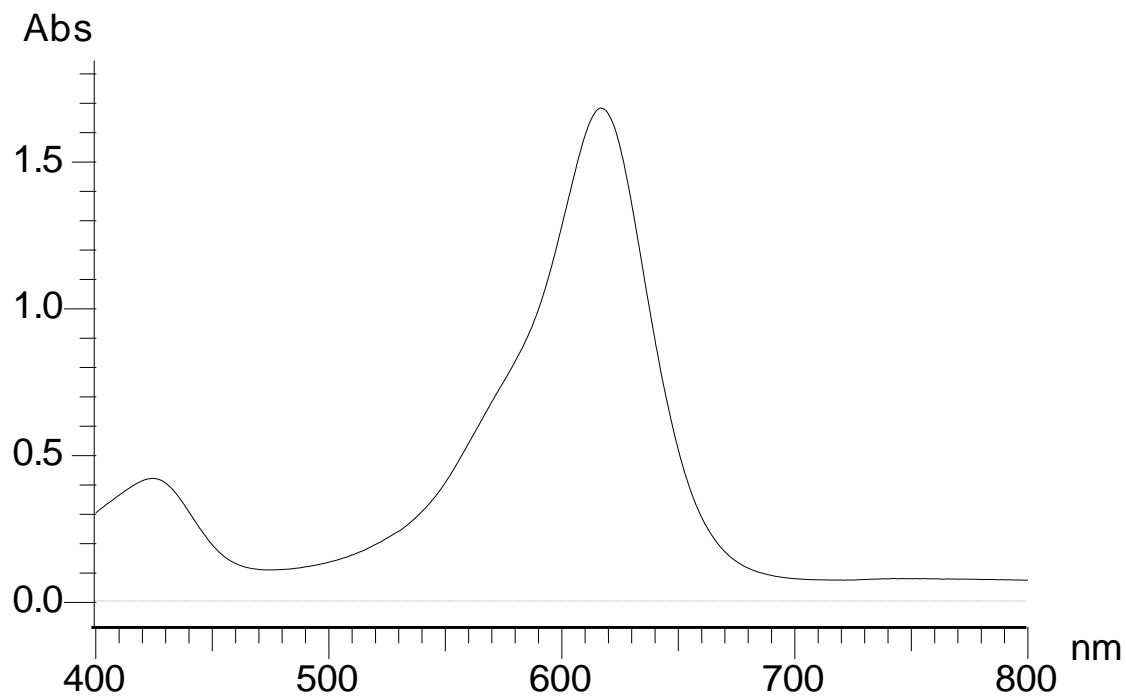
Figure 4.24. UV/VIS spectrum of Malachite Green Dye.

4.7.2   Calibration Plot

Decolorization of MG dye by crude enzyme was followed by monitoring changes in absorbance
of the solution mixture at 617 nm using a UV–Vis spectrophotometer. In order to determine the
absolute concentration of MG in the residue solution, a calibration curve (absorbance versus
concentration) of known MG dye solution was constructed as shown in Figure 4.25 and used to
determine the concentration of MG in the unknown. The high correlation coefficient ($R^2 = 0.999$)
indicates how well data points fit a line.
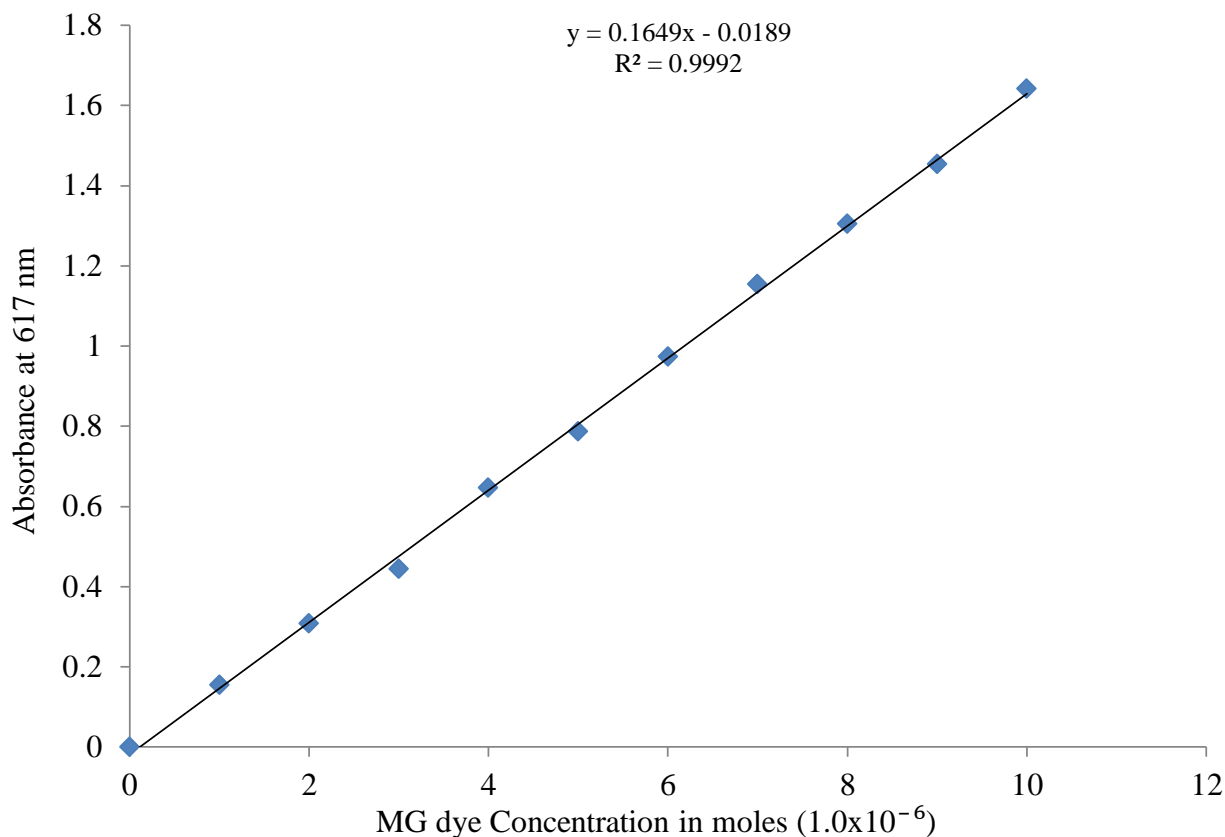
Figure 4.25. Calibration Plot of malachite green dye.

4.7.3 Effect of Contact Time on Decolorization

As part of the optimization process, it was important to establish the optimum (or minimum) time required for optimum dye decolorization. The effect of contact time on MG dye decolorization by crude enzyme was determined by varying the time of incubation and the results are presented in Figure 4.26. The results show that dye decolorization was rapid within the first 4 hours with approximately 82 % dye decolorization. The change in percentage decolorization after the 4th hour became relatively gradual attaining equilibrium at the 20th hour with 97.5 % dye decolorization. This observation suggests that the initial four hours is significant for dye decolorization but quite slow beyond the fourth hour possible due to inhibition by products. These results are in agreement with earlier published work for decolorization of textile dyes (Arabaci and Usluoglu, 2014).
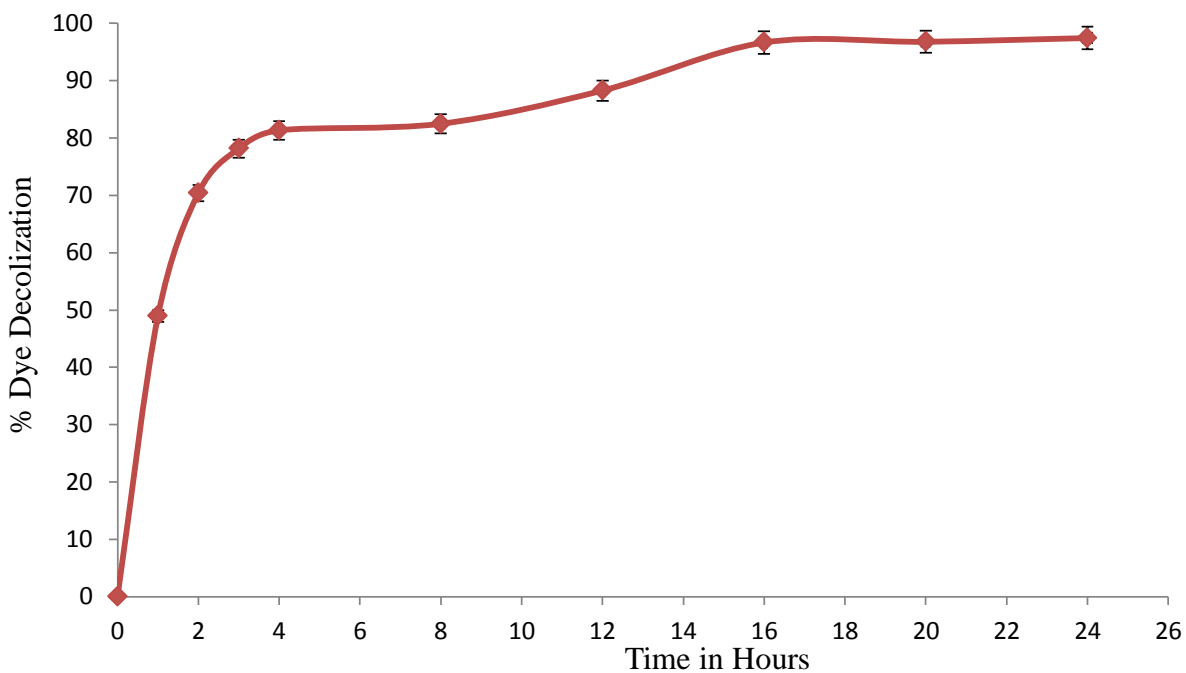
Figure 4.26. Effect of contact time on decolorization of Malachite Green dye.

4.7.4 Effect of pH on Malachite Green dye Decolorization

Enzymes are greatly affected by variation in pH. The effect of pH on the decolorization of MG dye by crude protease enzyme was investigated over the pH range of 4 to 11 and the results presented in Figure 4.27.
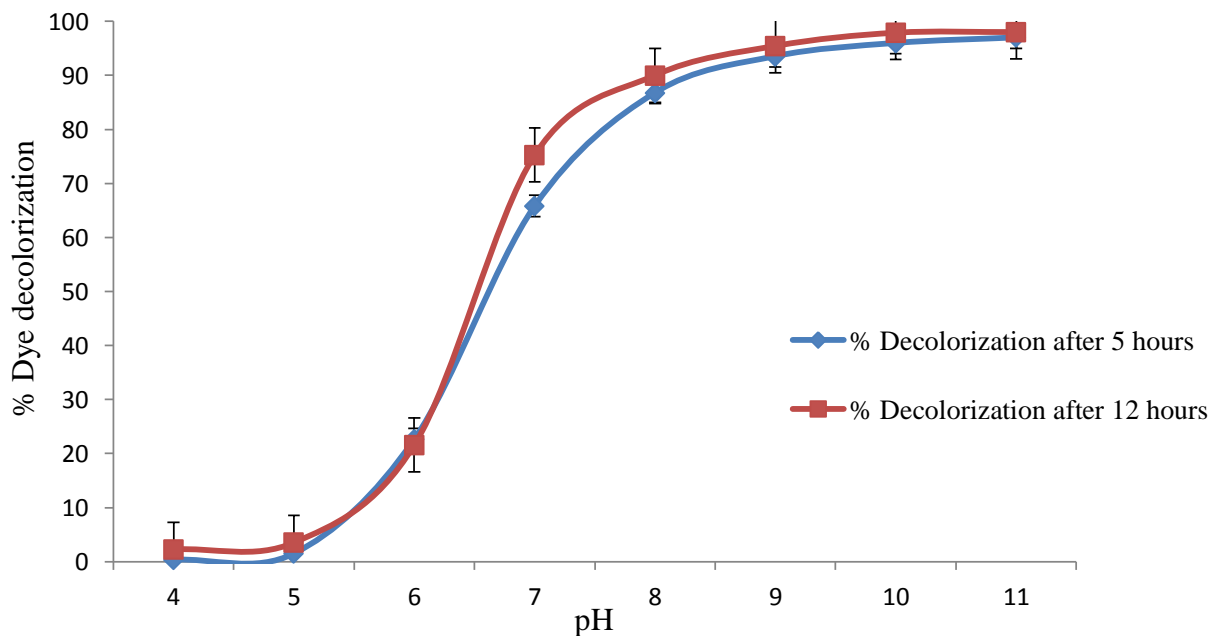
Figure 4.27. Effect of pH on decolorization of Malachite Green dye.

From figure 4.27, it can be inferred that at pHs below 5, there was significantly very low decolorization but when the pH was increased above 6.0, the rate of decolorization rapidly increased from 21% at pH 6 to 98% at pH 11. The result shows that the optimum pH for efficient MG dye decolorization is between pH 9 and 11. These results obviously present an advantage from industrial application point of view since most of dye effluents are characterized by alkaline pH under which crude protease enzyme works optimally. These findings are consistent with related studies done on decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria (Amoozegar *et al*., 2007). Figure 4.28 shows a photograph of MG dye at pH 4, 5, 6, 7, 8, 9, 10 and 11 taken after two hours during the decolorization process.
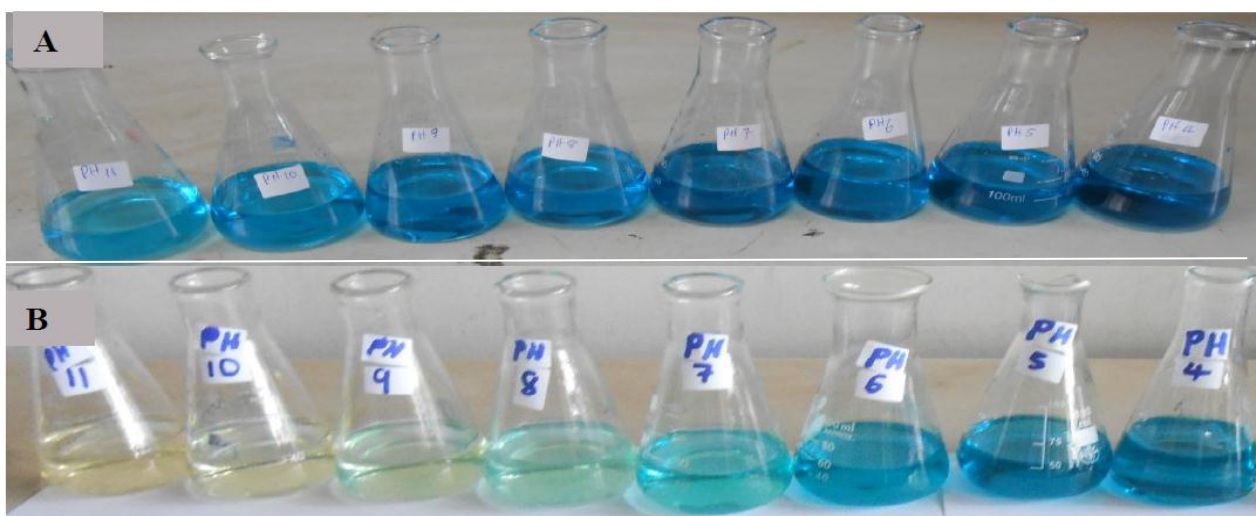
Figure 4.28. Photograph of MG dye at pH 4, 5, 6, 7, 8, 9, 10 and 11 taken after two hours during decolorization process. (A- Represent negative control consisting of MG dye alone without the enzyme while B- represents a mixture of MG dye and crude alkaline protease enzyme).

4.7.5   Effect of Temperature on Malachite Green dye Decolorization

Temperature plays an important role in enzyme activity. Effect of temperature on decolorization of MG dyes was investigated over the temperature range of 25 to 70˚C and the results are presented in Figure 4.29.
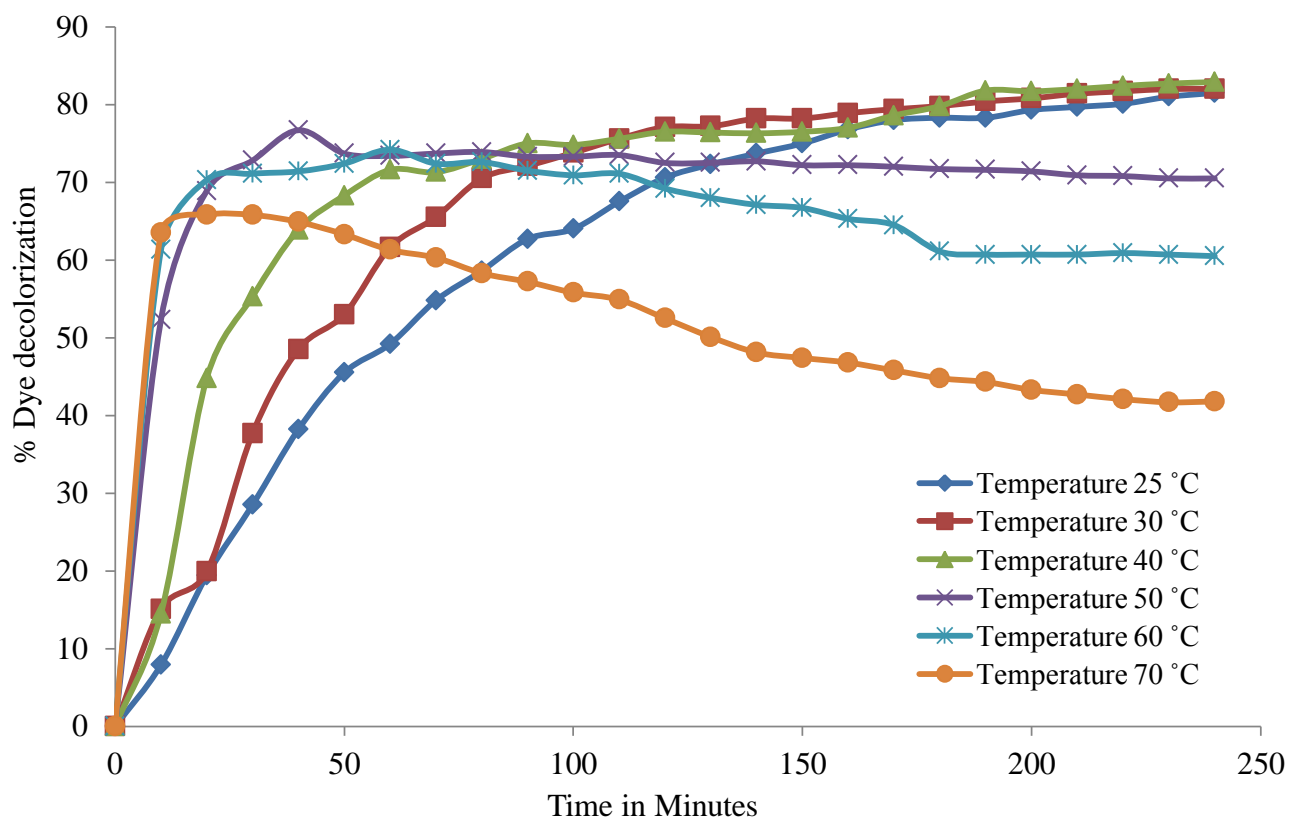
Figure 4.29: Effect of temperature on the decolorization of MG dye.

The results show that the rate of MG dye decolorization increased with increase in temperature between 25 ˚C, and 40˚C with decolorization efficiency increasing with increase of incubation time. The optimum temperature for crude protease enzymes was found to be 40˚C with 83% decolorization efficiency after 4 hours. At elevated temperature above 50°C, the rate of dye decolorization increased during the first 20 minutes but after one hour, percent dye decolorization remarkably decreased. This can be attributed to the denaturization of enzyme and thermal inactivation of enzyme above the optimum operating temperature.

4.7.6   Effect of initial Malachite Green dye concentration on decolorization

The decolorization of MG was studied at various increasing concentration of dye ranging from 1.0×10^{-6}M, to 1.0×10^{-5}M and the results are presented in Figure 4.30. It can be inferred from

the results that the rate of decolorization decreased with increase in the initial MG dye concentration. However, the crude protease enzyme was able to decolorize higher concentration in the range of 83−100% during 24 h incubation period. The decrease in decolorization efficiency at high concentration might be due to substrate (dye) being in large excess relative to the active sites on the enzyme. Similar results were observed by Murugesan *et al.*, (2007) who investigated the decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture and indicated that decolorization of RB-5 and Remazol Brilliant Blue (RRBBR) dyes decreased with increasing dye concentration.
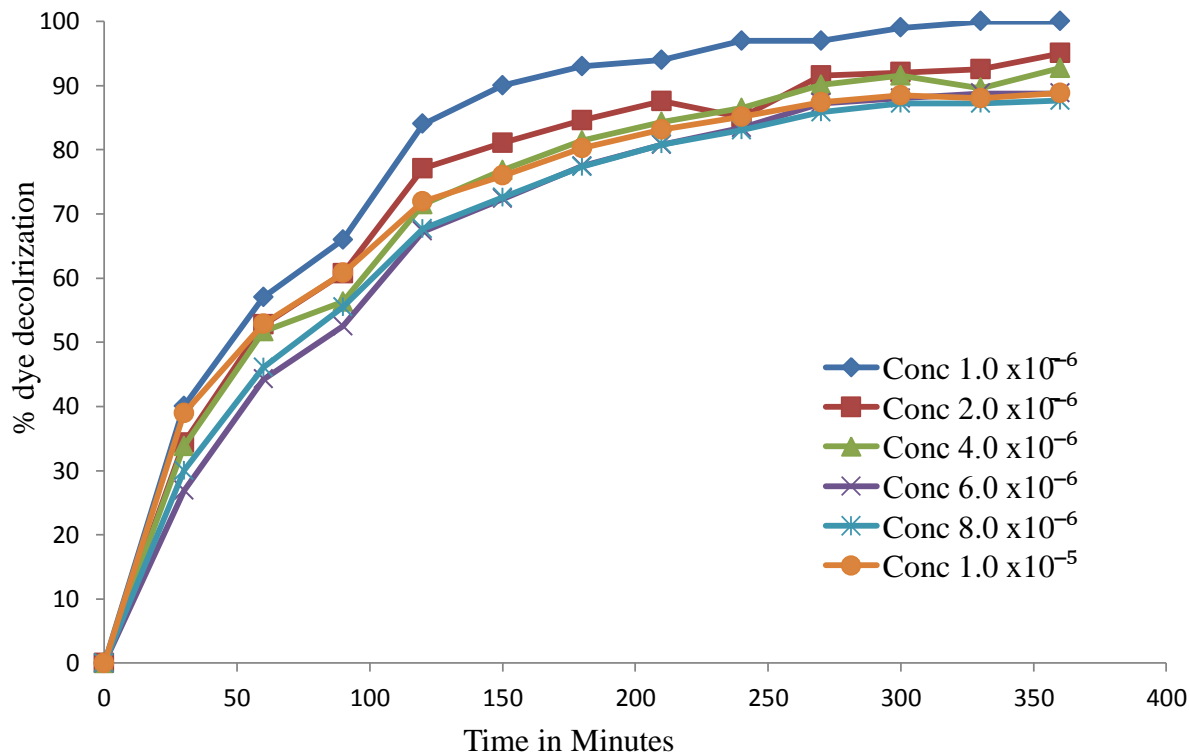
Figure 4.30. Effect of Malachite Green dye concentration on enzyme Decolorization.

4.7.7   Analysis of Biodegradation of the MG Dye by UV−VIS Spectrophotometer

The biodegradation of the MG dye was monitored by UV−Vis Spectrophotometer analysis. Figure 4.31 illustrates the typical UV−Vis spectra of the control MG (0 minute) and biodegraded

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sample mixture at various time periods (5 minutes, 10 minutes, 1, 1.5, 2, 3, 6 and 12 hrs). The peaks observed (428 and 620 nm) at initial time (0 minute) decreased without any shift in λ_{max} up to complete degradation of the dye. The spectrum of MG in the visible region exhibits a main peak with maximum absorbance at 617 nm and its complete disappearance after 12 hours as well as the minor peak at 428 nm clearly suggests that crude alkaline protease enzyme decolorized MG dye through biodegradation. To be sure that the decrease in absorbance was due to the biodegradation and not caused by pH change, the effect of pH over the range pH 4 to10 of the reaction mixture was determined. It was noticed that there was no intense green color formation or any other color in the samples treated with the crude alkaline protease enzyme indicating complete degradation. This observation is consistent with previous reports by Du *et al.*, (2011) who investigated biodegradation of malachite green by *Pseudomonas sp. strain* DY1 under aerobic conditions.
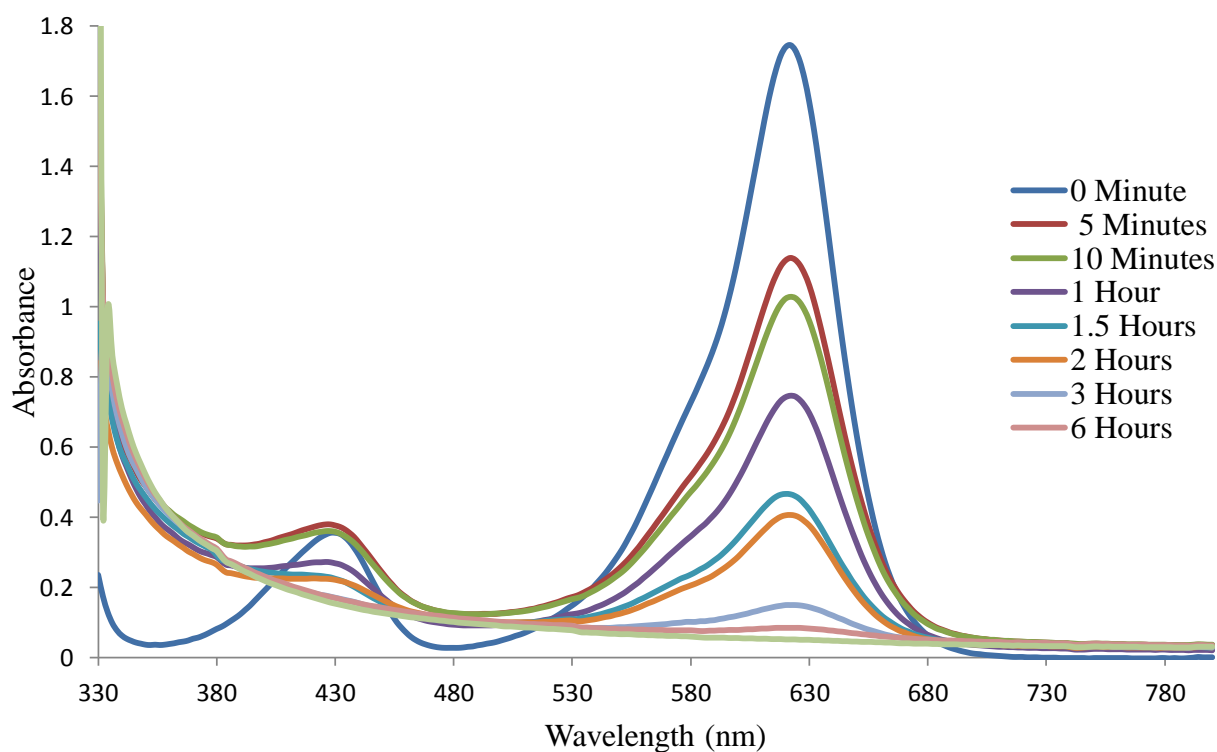
Figure 4.31. UV–Vis spectra scans of MG (1.0x 10^{-5}M) biodegraded by crude alkaline protease enzyme from *Bacillus cereus* strains *1-p* at different time period, pH 8 and temperature 25˚C

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4.7.8 Analysis of Degraded MG Metabolites by Thin Layer Chromatography (TLC)

The metabolites produced during the biodegradation of the malachite green dye were analyzed by thin layer chromatography and the results are presented in Figures 4.32a and 4.32b. The comparison of TLC chromatograms before and after decolorization by crude protease enzyme in iodine chamber showed the appearance of three additional bands (M1, M2, and M3) of retardation factor (Rf) values of 0.96, 0.92 and 0.90, respectively (Figure 4.32b) as compared to control Rf value of 0.87 which can be attributed to the degraded dye metabolites. The TLC results suggested that crude protease enzyme was able to degrade MG dye giving rise to three main metabolites which accounted for the color disappearance. Due to the limitations of TLC, further analytical techniques were desirable in order to identify the metabolites obtained in the biodegradation reaction. The results are given in the next section 4.7.9.
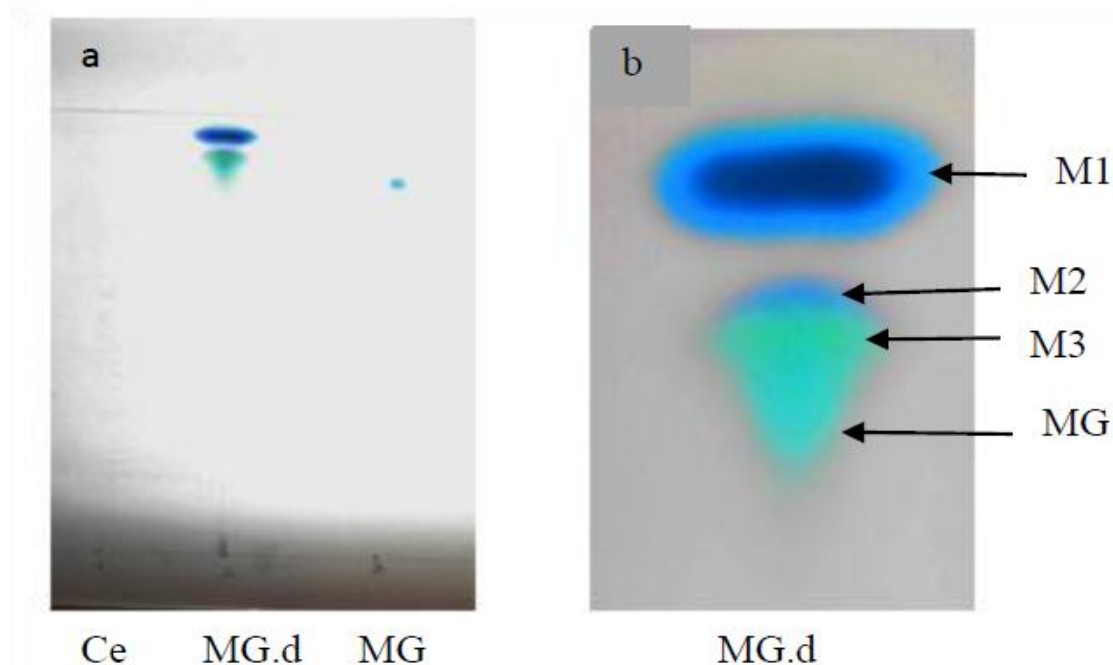
Figure 4.32. TLC Chromatograms of MG dye before and after degradation by crude protease enzyme **(a)** column 1,2 and 3 represent negative control with enzyme alone (Ce), degraded Malachite green dye (MG.d) and positive control with untreated Malachite green dye (MG) respectively; **(b)** Three metabolites (M1, M2, & M3) of degraded Malachite green dye.

4.7.9 Analysis of Degraded MG Metabolites by Liquid Chromatography–Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

Structures of the metabolites resulting from the enzymatic degradation of malachite green dye were successfully identified by LC−QToF−MS. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), identification and quantification were accomplished. From the results of LC-QToF-MS analysis, twelve peaks of specific intermediate products that were clearly distinguished compared with the controls of MG without crude alkaline protease enzyme or only alkaline protease enzyme without MG (Figure 4.33).
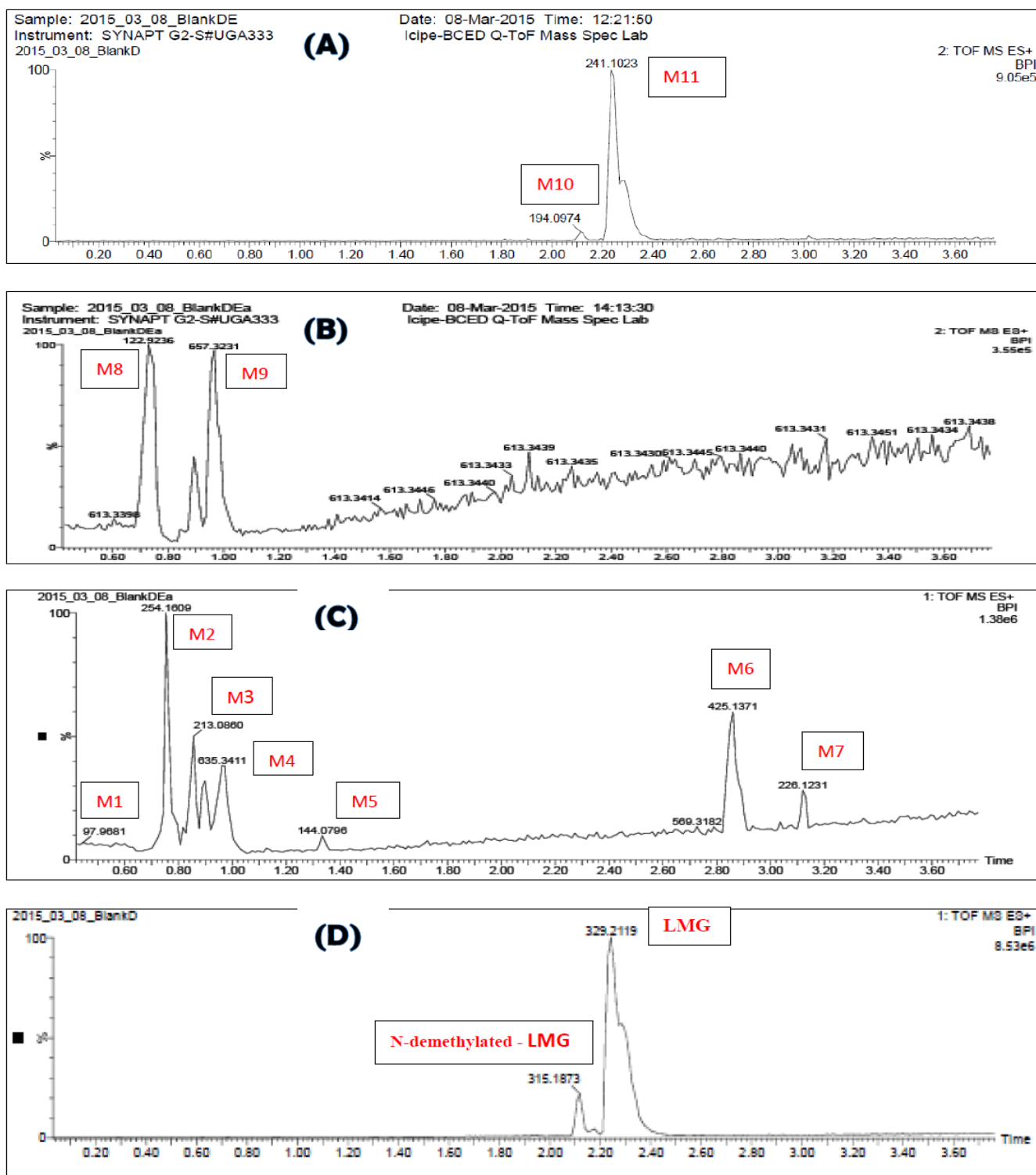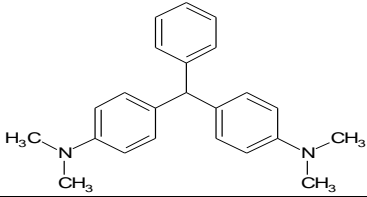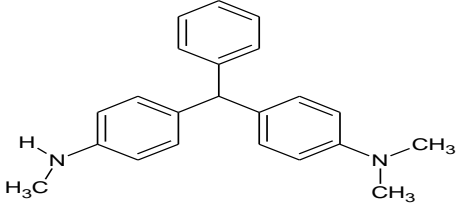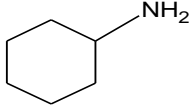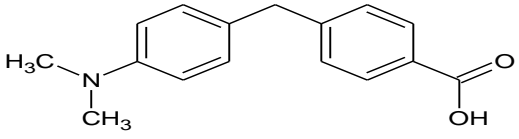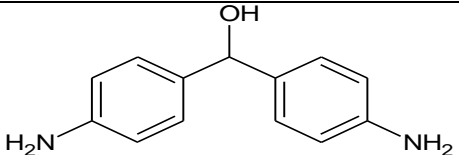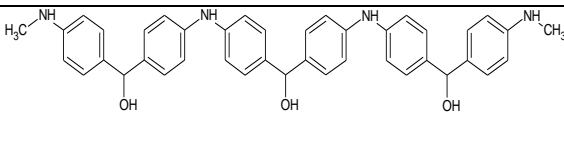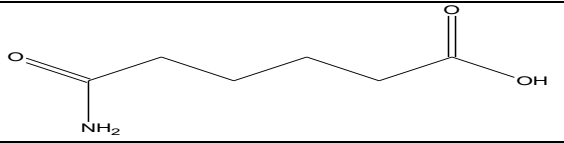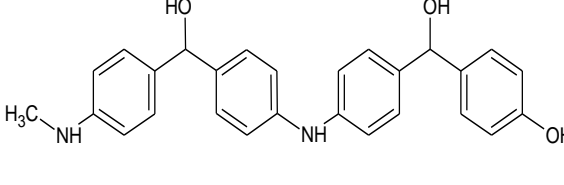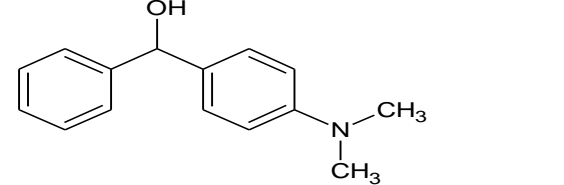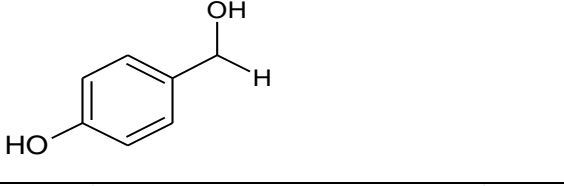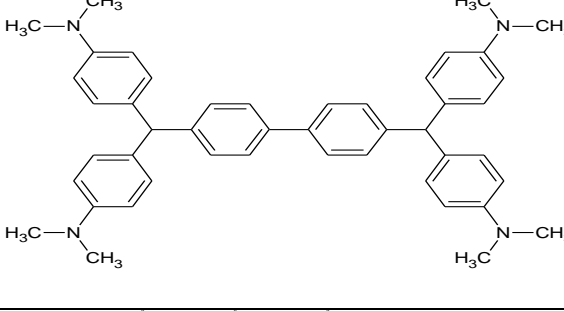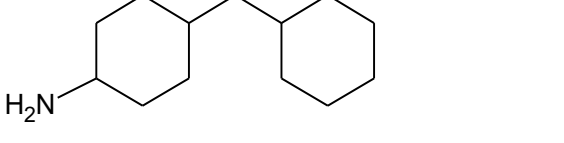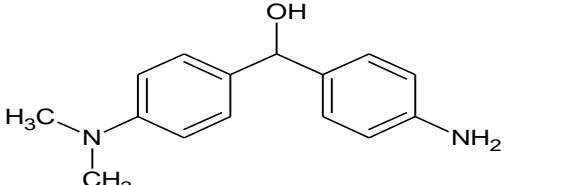
Figure 4.33. Extracted ion chromatograms of MG dye metabolites detected by LC–QTOF-MS in crude alkaline protease enzyme supernatant. (A), (B) & (C) represent chromatograms of degraded MG metabolites numbered M1-M12 and (D) represent chromatograms of leuco malachite green (LMG) resonance structure detected MG dye dissolved in water without crude alkaline protease enzyme.

Based on the time for decolorizing, the parent compound and intermediate compounds formed were identified. Malachite green dye could not be detected by LC−QToF−MS suggesting that MG dye split into Leuco Malachite Green structure (LMG) (mw 330) before fragmentation. Similar results have been previously reported in malachite green biodegradation by *Exiguobacterium sp.* MG2 (Wang *et al.*, 2012). From the LC−QToF−MS results, it was possible to assign unique elemental compositions to each peak observed during the course of the analysis and probable molecular structure drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com) as shown in Table 4.6.

Table 4.6. Intermediate product analysis after decoloration and degradation of the malachite green dye using LC–QTOF-MS (Probable Intermediates molecular structure drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com))

LC–QTOF-MS proton-ionization mass	Metabolite Peak; Molecular Weight & Molecular Formula	Intermediates Structure
329.2119	**LMG** Leuco Malachite Green (LMG) Also known as 4,4'-(Phenylmethylene)bis(N,N-dimethylaniline) MW = 330.209595 $C_{23}H_{26}N_2$	
315.1837	**N-demethylated - LMG** *N*-[(1Z,4Z)-4-{[4-(dimethylamino)phenyl](phenyl)methylidene}cyclohexa-2,5-dien-1-ylidene]methanaminium $C_{22}H_{24}N_2$ MW = 316.193939	
97.9681	**M1** Cyclohexanamine $C_6H_{13}N$ MW = 99.1741	
254.1609	**M2** 4-[4-(dimethylamino) benzyl]benzoic acid $C_{16}H_{17}NO_2$ MW = 255.3117	
213.086	**M3** bis(4-aminophenyl) methanol MW = 214.2631 $C_{13}H_{14}N_2O$	

635.3411	**M4** MW = 636.310059 $C_{41}H_{40}N_4O_3$	
144.0796	**M5** 6-amino-6-oxohexanoic acid MW - 145.073 $C_6H_{11}NO_3$	
425.1371	**M6** MW = 426.194336 $C_{27}H_{26}N_2O_3$	
226.1231	**M7** [4-(dimethylamino) phenyl](phenyl)methanol MW = 227.30158 $C_{15}H_{17}NO$	
122.9236	**M8** 4-(hydroxymethyl) phenol MW - 124.052429 $C_7H_8O_2$	
657.3231	**M9** $C_{46}H_{50}N_4$ MW - 658.403564	
194.0974	**M10** 4-(cyclohexylmethyl) cyclohexanamine MW = 195.198700 $C_{13}H_{25}N$	
241.1023	**M11** (4-aminophenyl) [4-(dimethylamino)phenyl]methanol MW= 242.141907 $C_{15}H_{18}N2O$	

An interesting observation noted during the analysis was the detection of metabolites with molecular weight than leuco malachite green structure (LMG) (mw 330). This could be due to the polymerization of degraded metabolite products by the action of any residual enzyme. The

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end products in the degradation of MG or LMG were found to be Cyclohexanamine, 4-(cyclohexylmethyl) cyclohexanamine and 6-amino-6-oxohexanoic acid as shown in Table 7. Due to the limitation of the analysis method, it was not possible to ascertain if these products were further degraded into CO, H_2O and NH_3. However, it is clear from the degraded metabolites that the triphenylmethane structure was cleaved off followed by benzene ring-removal together into cyclohexane. These observations differ with earlier results reported by Cha *et al*., (2001) which indicated that either tridesmethyl MG or LMG, the end-products in MG degradation by the *Fungus Cunninghamella elegans*, keeps the intact triphenylmethane structure. From these results, it be can inferred that the enzymatic degradation of MG using alkaline protease enzyme from strain *1-p* comprised not only the decolorization reaction but also the more significant mineralization and benzene ring-removal which are very important for the efficient removal of organic toxic pollutants. Considering the potential biases for detecting compounds among assays, the metabolites were further identified by GC-MS. These latter results are described in section 4.7.10.

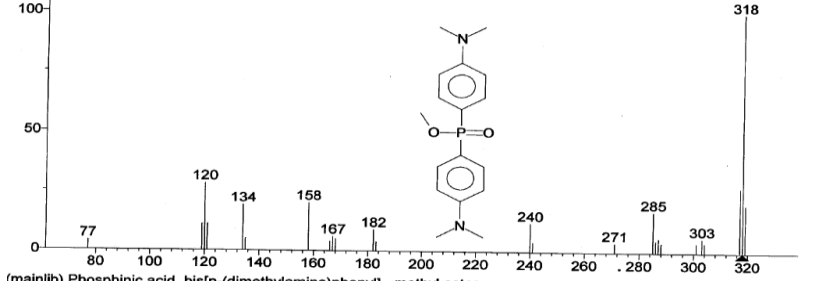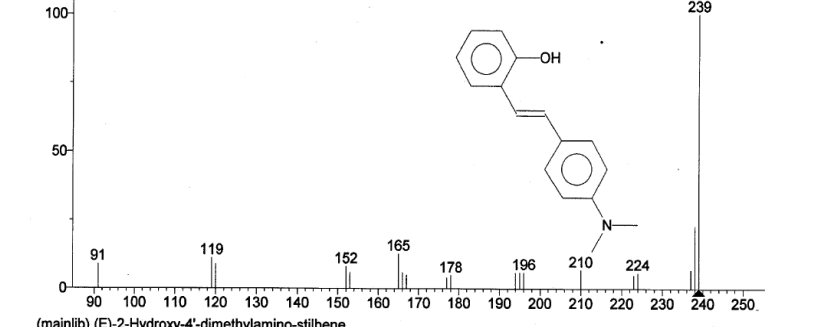### 4.7.10 Analysis of Degraded MG Metabolites by Gas Chromatography/Mass Spectrometry (GC - MS)
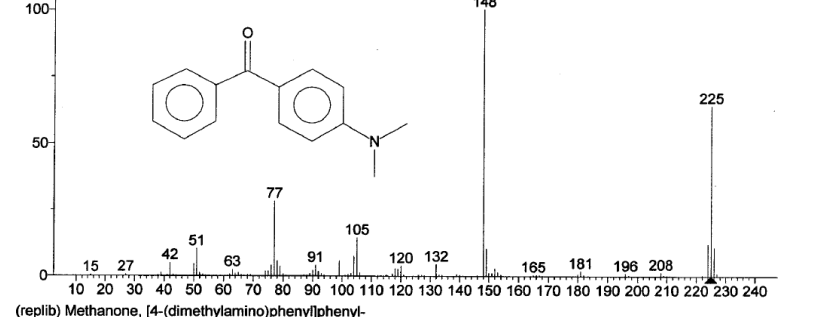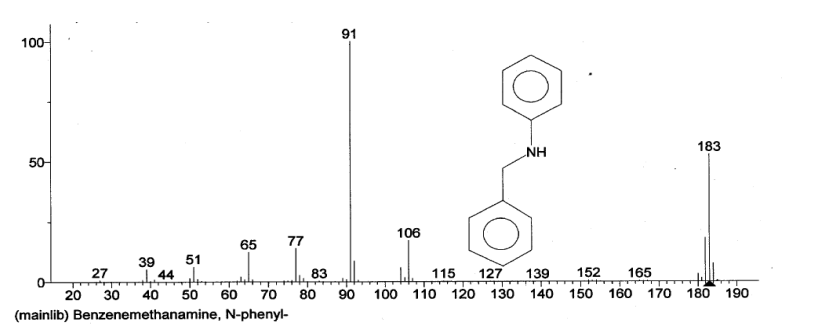
To identify metabolites resulting from the enzymatic degradaion of MG, GC-MS analysis was carried out on the resultant biodegradation products. The components that eluted having different retention times were subjected to mass spectrometry and identified by matching their spectra with those recorded in the National Institute of Standards and Technology (NIST) spectral library stored in the computer software of the GC-MS. The retention times (Total ion chromatograph) of various degradation products after 12 hrs of degradation are summarized in Table 4.7 suggesting the high biodegradation efficiency of MG by crude alkaline protease

enzyme. To eliminate the possibility of dye degradation during GC-MS analysis, the standard of MG not treated with enzyme was analyzed and Leuco Malachite Green (LMG) was detected. It is widely known that LMG is very toxic to aquatic organisms as it is deposited in fatty tissues and remain for more than ten months after treatment (Jian *et al.*, 2008). Hence its further degradation is very desirable and critical.

The GC-MS results obtained reveal that LMG was further degraded to Methanone [4-(dimethylamino) phenyl] phenyl (m/z 225); Phosphinic acid, bis[p-(dimethylamino)phenyl], methyl ester (m/z 318), (E)-2-Hydroxy-4'-dimethylamino-stilbene(m/z 239) and Benzylaniline (m/z 183). Overall, combining the results obtained from TLC, LC–QTOF-MS and GC-MS, it can be concluded that biodegradation of MG by crude alkaline protease enzyme from strain *1-p* involved a series of reactions of N-demethylation, reduction, benzene ring-removal, and oxidation which lead to detoxicification MG. This finding point out that enzyme from strain *1-p* is a very useful and promising candidate for degrading recalcitrant chemicals in industrial wastewaters.

Table 4.7. Chromatogram of metabolite obtained from GC–MS analysis and the corresponding chemicals structure and name obtained after 12 h of degradation

Metabolite name and chemical formula	R_t. time (min)	Mw (m/z)	Mass spectrum and corresponding chemicals structure
Leuco Malachite Green (LMG) $C_{23}H_{26}N_2$	27.746	330	

Compound	Retention	m/z	Mass spectrum
Methanone, [4-(dimethylamino)phenyl] phenyl $C_{15}H_{15}NO$	26.809	225	 (replib) Methanone, [4-(dimethylamino)phenyl]phenyl-
Phosphinic acid, bis[p-(dimethylamino)phenyl]-, methyl ester $C_{17}H_{23}N_2O_2P$	25.214	318	 (mainlib) Phosphinic acid, bis[p-(dimethylamino)phenyl]-, methyl ester
(E)-2-Hydroxy-4'-dimethylamino-stilbene $C_{16}H_{17}NO$	27.291	239	 (mainlib) (E)-2-Hydroxy-4'-dimethylamino-stilbene
Benzenemethanamine, N-phenyl $C_{13}H_{13}N$	15.426	183	 (mainlib) Benzenemethanamine, N-phenyl-

4.8 Kinetic study of Malachite green dye decolorization

In order to measure the maximum decolorization rate of MG dye by crude alkaline protease enzyme, test solutions containing 40 ml of MG dye at different concentrations and 10ml crude alkaline protease enzyme were mixed at 25°C and pH 8. Michaelis-Menten kinetics,

Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the MG decolorization process.

4.8.1 Michaelis-Menten kinetics

The experimental results at various MG dye concentration were analyzed based on the interpretation of the Michaelis-Menten kinetics Equation (2). Figure 4.34 shows plots of S (MG dye concentration in mgl^{-1}) verses V ($mgl^{-1}h^{-1}$) for the enzymatic decolorization of MG dye. The experimental data fitted quite well with the Michaelis-Menten equation with a high R^2 value of 0.991 indicating that decolorization of MG dye obeyed Michaelis-Menten kinetics equation and also suggesting a first-order reaction order. The corresponding V_{max} and K_m values were calculated and are given in Table 4.8.
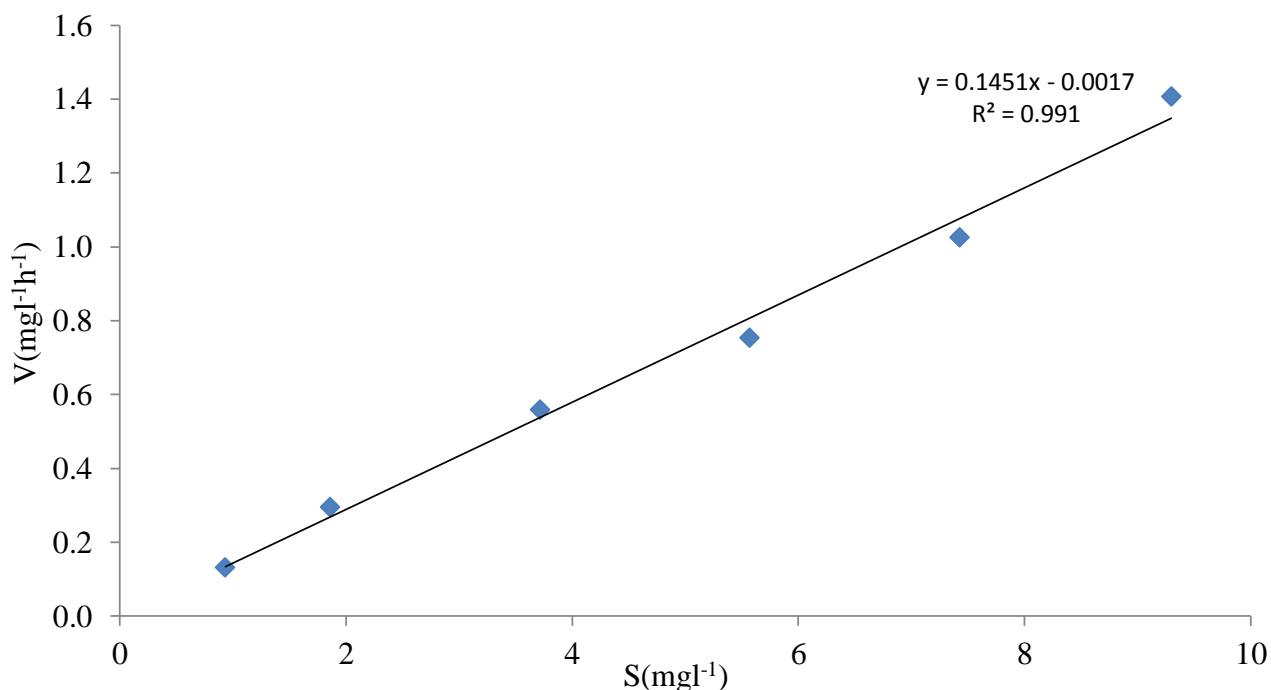
Figure 4.34. Michaelis-Menten plot for the enzymatic decolorization of MG dye

Since the Michaelis-Menten approach to kinetic modeling over an extended period seems to be rather limited, further analysis of data were tested against the Lineweaver–Burk plot and Eadie-Hofstee plot models

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4.8.2 Lineweaver-Burk plot

When Michaelis-Menten equation is transformed by a double reciprocal approach, we obtain Lineweaver-Burk equation (3). The experimental results obtained at various MG dye concentration were subjected to the Lineweaver-Burk equation. The Lineweaver-Burk plot is shown in Figure 4.35. The figure demonstrates a good fit with a straight line and having R^2 value of 0.996 indicating that Lineweaver-Burk plot model was best suited for describing the biodegradation of MG dye by crude alkaline protease enzyme from strain *1-p*. K_m and V_{max} values were calculated and summarized in Table 4.8.
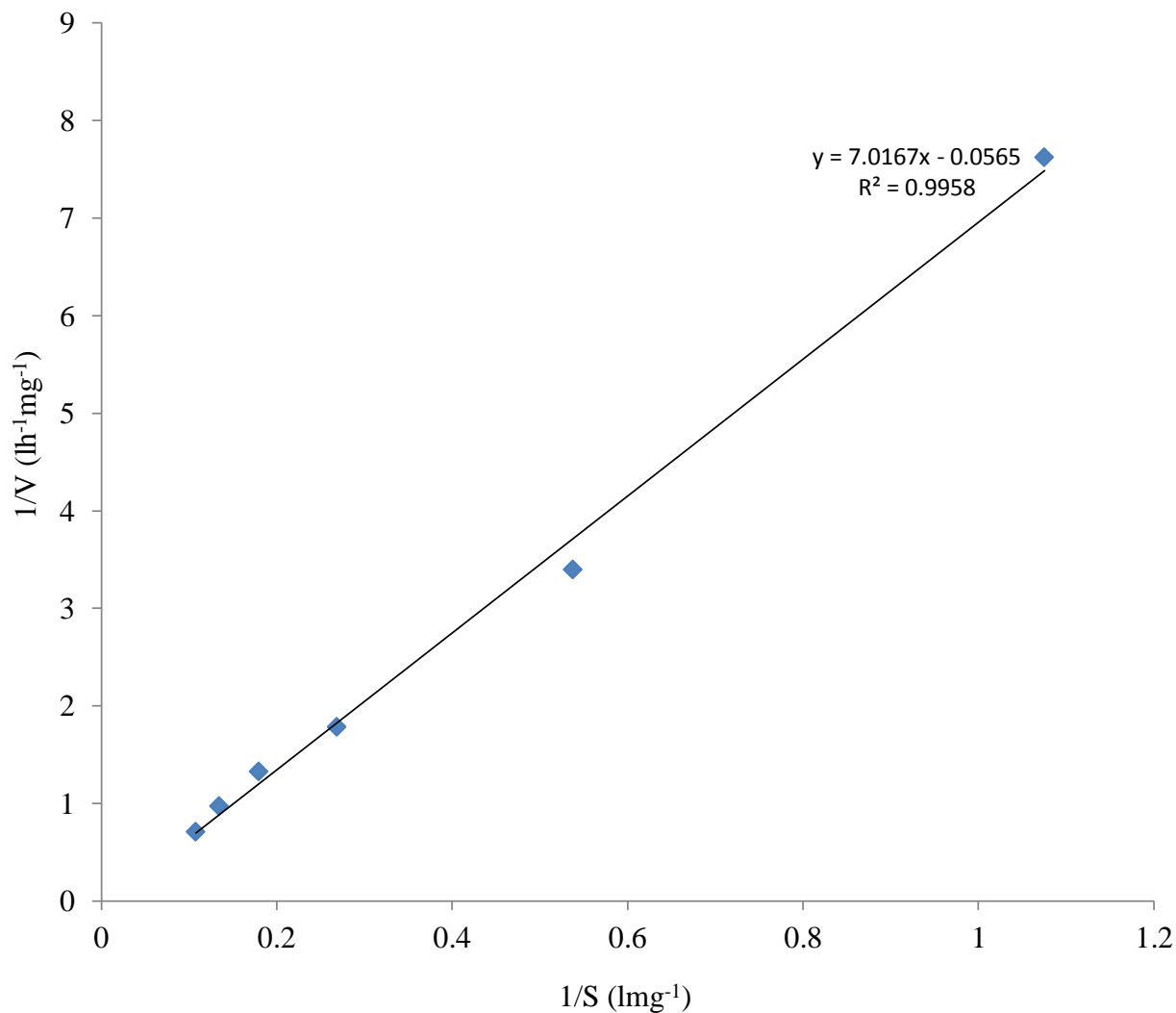
Figure 4.35. Lineweaver-Burk plot for the enzymatic decolorization of MG dye

Similar results have previously been reported for the enzymatic degradation of textile dye Reactive Orange 13 by newly isolated bacterial strain *Alcaligenes faecalis* PMS-1 (Shah *et al.*, 2012). Even though the data obtained fitted quite well with the Lineweaver-Burk plot, the data was further fitted to Eadie-Hofstee plot

4.8.3 Eadie-Hofstee plot

Another way to determine K_m and V_{max} is the use of Eadie-Hofstee Plot represented by equation (4). The Eadie-Hofstee plot is shown in Figure 4.36. The correlation coefficients (R^2) were not close to 1.0 ($R^2 = 0.0104$) indicating that Eadie-Hofstee plot was not appropriate in describing the biodegradation of MG dye by crude alkaline protease enzyme from strain *1-p*.
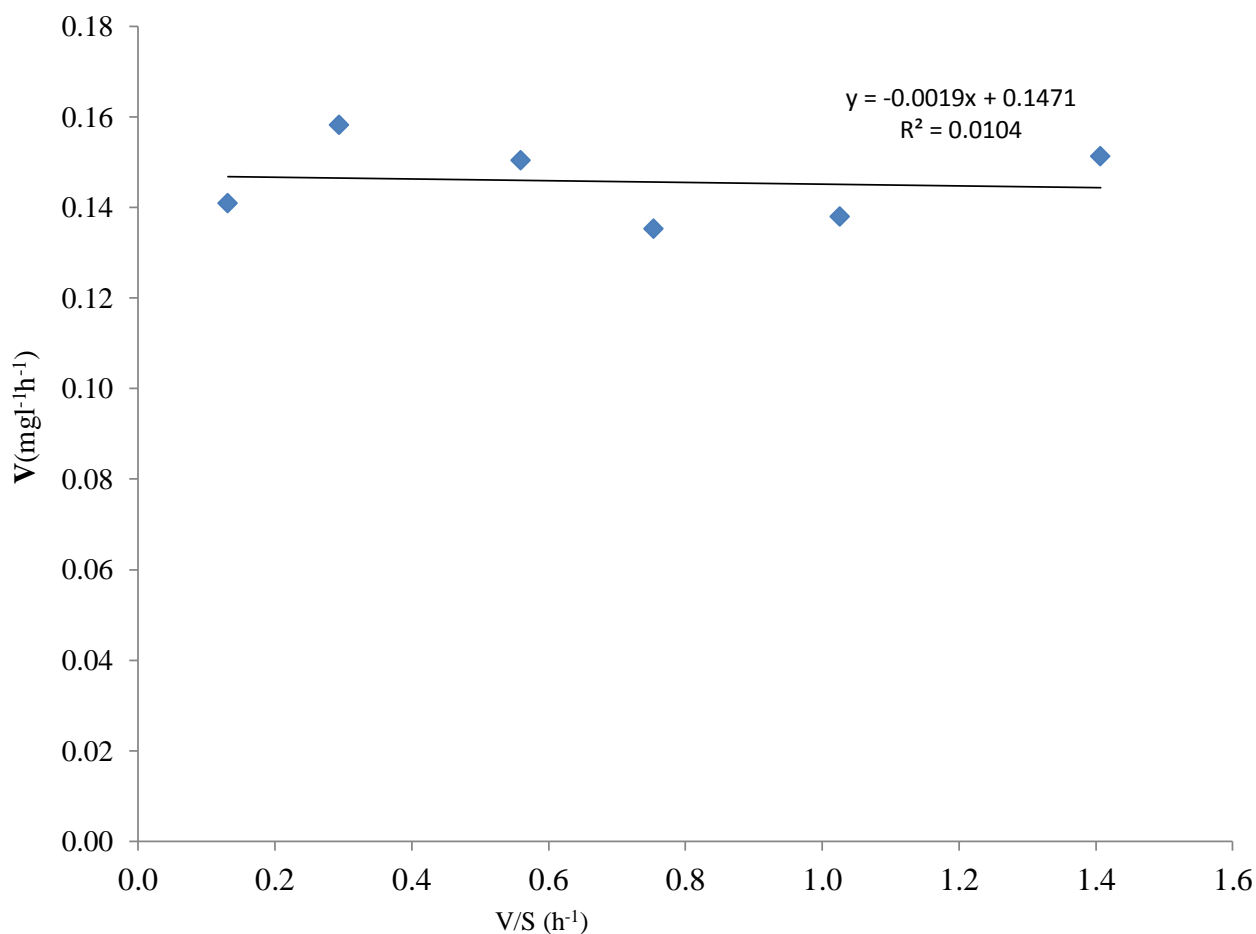
Figure 4.36. Eadie-Hofstee plot for the enzymatic decolorization of MG dye.

A comparison of K_m and V_{max} values obtained from Michaelis-Menten kinetics and Lineweaver-Burk plot is shown in Table 4.8. It shows that these two results are not comparable. However, since Lineweaver-Burk plot had the highest R^2 value, it can be concluded that decolorization of MG by crude alkaline protease enzyme from strain *1-p* can be best described by the Lineweaver-Burk plot.

Table 4.8. Comparison of K_m and V_{max} values for the enzymatic decolorization of MG dye

Plot type	V_{max} (mg l^{-1} h^{-1})	K_m (mg l^{-1})	R^2
Michaelis-Menten kinetics	0.145	7.702	0.991
Lineweaver-Burk plot	17.700	124.196	0.996

4.8.4   Determination of reaction order

MG decolorization data from the batch tests were used to determine the order of the decolorization reaction. The kinetic equations used for Zero order, first order and second order kinetic study are given in equations 5 to 9. Zero order kinetic plot was obtained by plotting dye concentration (C_t) versus time as shown in Figure. 4.37.

First-order kinetic model graph was also obtained by plotting $\ln C_t$ against time (Figure. 4.38) while second order kinetic model shows a plot of ($1/C_t$) versus time as shown in Figure 4.39. The rate constants of decolorization reaction and coefficients of least square method analysis are tabulated in Table 4.9.
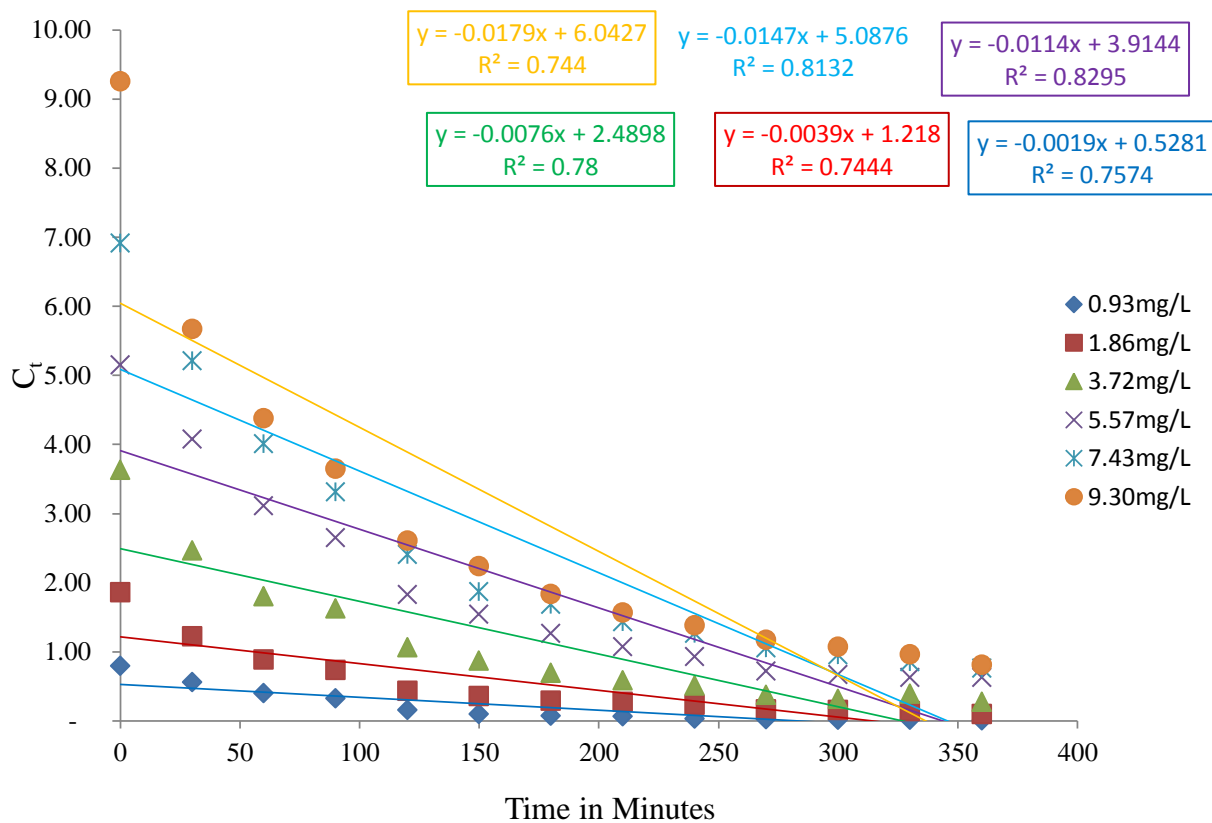
Figure. 4.37. Zero order kinetic at different initial concentration of Malachite green dye.
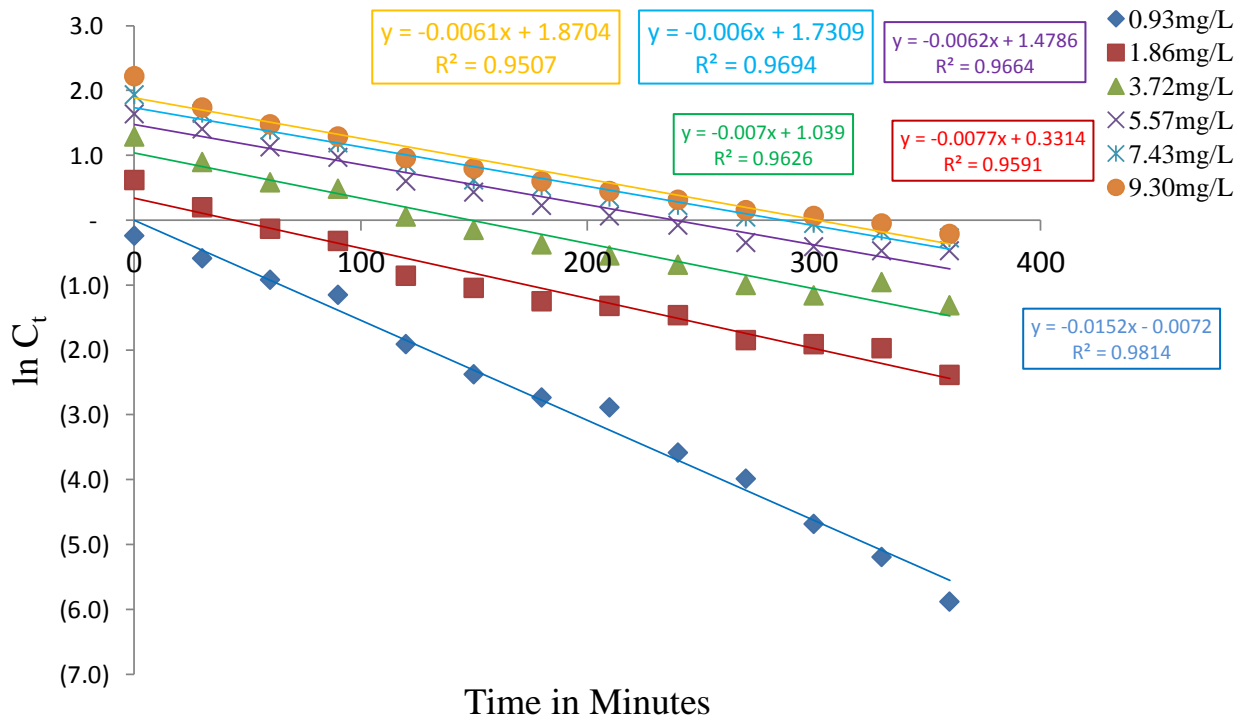

Figure 4.38. First order kinetic at different initial concentration of Malachite green dye
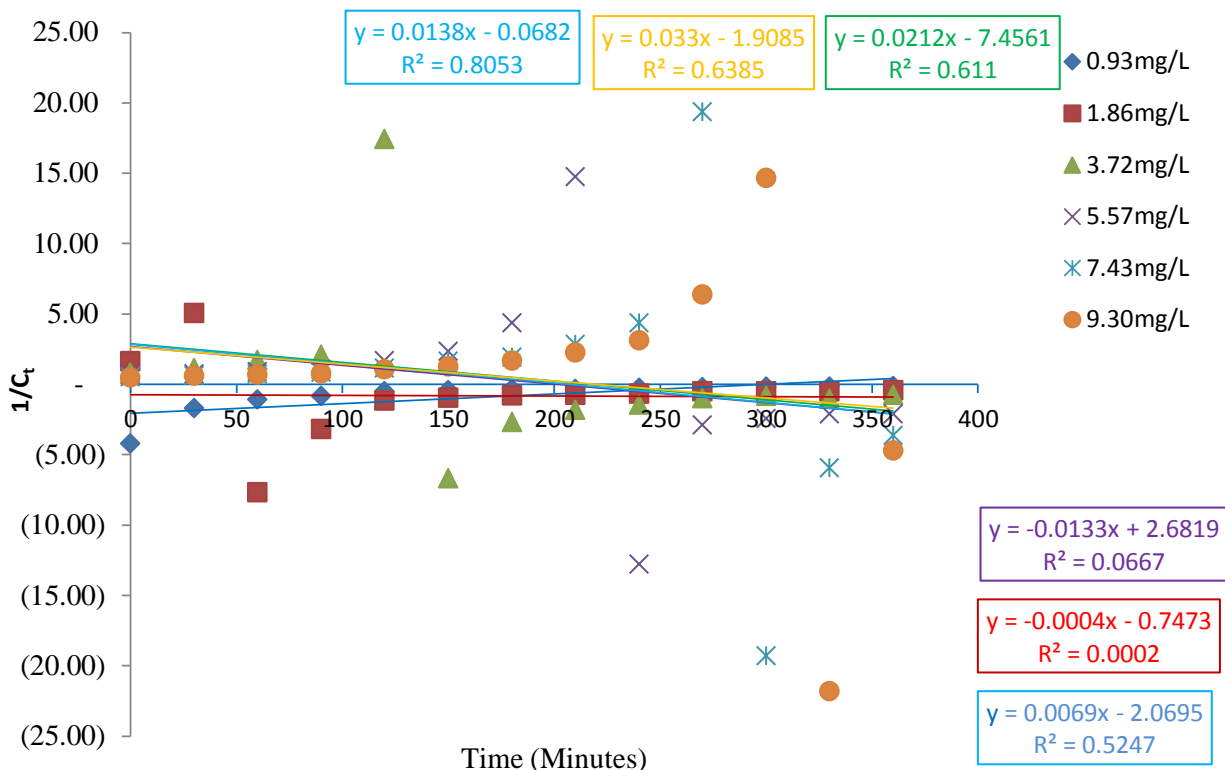
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Figure. 4.39. Second order kinetic at different initial concentrations of Malachite green dye.

Table 4.9. Zero, first and second order kinetic constants obtained in enzymatic degradation of Malachite green dye

Kinetics model	Constant	0.93mg/L	1.86mg/L	3.72mg/L	5.57mg/L	7.43mg/L	9.30mg/L
Zero order	$k_0(mgl^{-1}min^{-1})$	0.0019	0.0039	0.0076	0.0114	0.0147	0.0179
	$C_o(cal)$	0.5281	1.218	2.4898	3.9144	5.0876	6.0427
	R^2	0.7574	0.7444	0.780	0.8295	0.8132	0.744
First order	$k_1(min^{-1})$	0.0152	0.0077	0.007	0.0062	0.0060	0.0061
	$C_o(cal)$	1.0072	1.392	2.826	4.387	5.646	6.491
	R^2	0.9814	0.9591	0.9626	0.9664	0.9694	0.9507
Second order	$k_2 (l\ mg{-}1min^{-1})$	0.0069	0.0004	0.0212	0.0133	0.0138	0.033
	$C_o(cal)$	14.66	1.3381	0.1341	0.3729	0.4832	0.5240
	R^2	0.8053	0.0002	0.611	0.0677	0.5247	0.6385

The high degree of linearity (R^2 >0.96) was obtained with the first-order kinetic model for all MG dye concentrations. This gives a good indication that the degradation reaction follows a first-order kinetics model. Additionally, experimental data showed good compliance with the

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experimental data proving that first order kinetic model was the best applicable for describing the kinetics of the enzymatic degradation of MG dye using crude alkaline protease enzyme from strain *1-p*. It is clear from these results that decolorization process depends on MG dye concentration. This finding is consistent with earlier reports (Shah *et al*., 2012). The results for zero and second order kinetic models showed poor correlation coefficients (R^2 < 0.83) and therefore could not be used in describing the enzymatic degradation of MG dye.

4.9   Decolorization and Bioremediation of Reactive Black 5 (RB5) Dye

4.9.1   Determination of Wavelength of Maximum Absorbance (λ_{max}) for Reactive Black 5 Dye

Enzymatic dye decolorization was monitored by use of a UV–Vis spectrophotometer at different times. In order to determine the wavelength of maximum absorbance (λ_{max}) for RB5 dye, spectra scans were performed on freshly prepared 1.0 x 10^{-4} M reactive black 5 dye in the UV region between 340 and 800 nm. Figure 4.40 shows a plot of absorbance against wavelength for reactive black 5 dye solution. It is evident that the wavelength of maximum absorption is 597nm. The UV-VIS spectrophotometer was set to this wavelength (λ_{max} =597 nm) and used for the rest of the experiments involving the use of Reactive Black 5 dye.
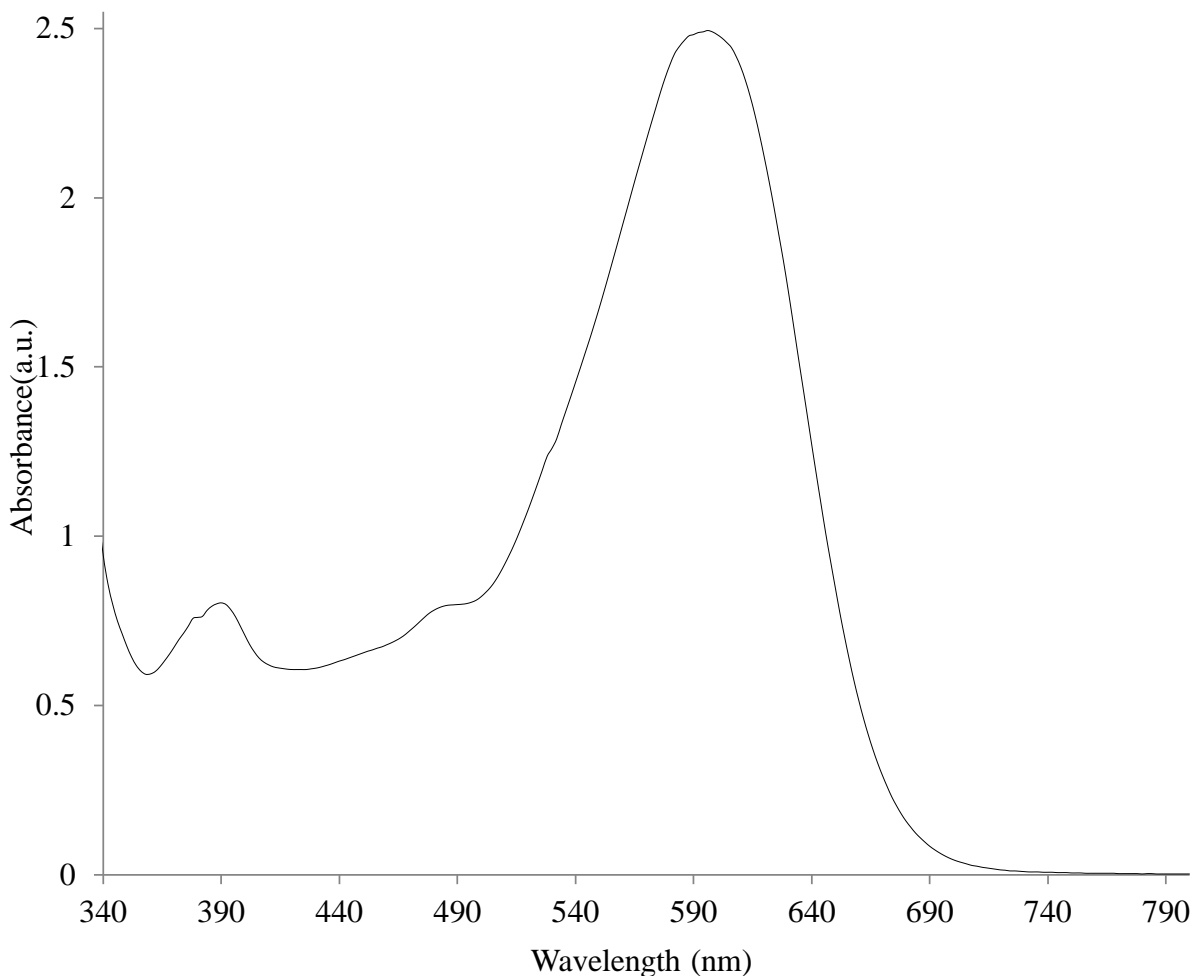
Figure 4.40. UV-Vis spectrum of Reactive Black 5 dye

4.9.2　Calibration Plot for Reactive Black 5 dye (RB5)

Decolorization of RB5 dye by crude enzyme was followed by monitoring changes in absorbance at 597 nm using a UV−Vis spectrophotometer. To determine the absolute concentration of RB5 in the residue solution, a calibration curve (absorbance versus concentration) of known RB5 dye solution was done as shown in Figure 4.41 and used to determine the concentration of RB5 in the unknown. The high correlation coefficients (R^2 = 0.999) indicates how well data points fit a line. Similar results were obtained in the plot of calibration curve of malachite green dye.
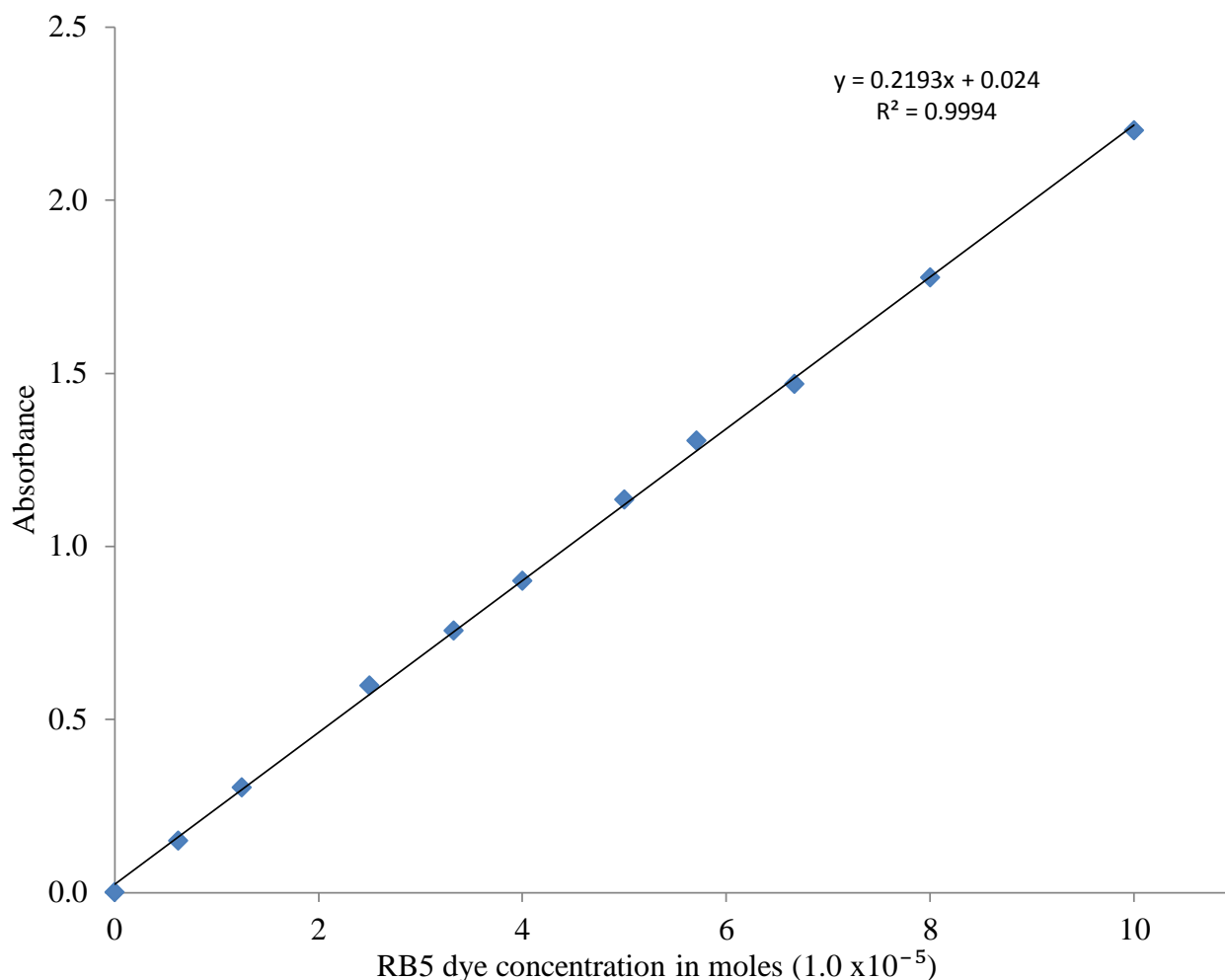
Figure 4.41. Calibration Plot of Reactive Black 5 dye.

4.9.3   Effect of Contact Time on Reactive Black 5 dye Decolorization

The maximum time required for decolorization varies with the nature of individual dyes. The effect of contact time on RB5 dye decolorization by crude alkaline protease enzyme was examined by varying the time of incubation and the results are presented in Figure 4.42. The results show that dye decolorization was rapid within the first 60 hours with approximately 87 % dye decolorization. The average time required to decolorize RB5 dye was about 120 hours with over 97% dye decolorization. This indicates that crude alkaline protease enzyme can be used to effectively treat the textile effluent containing RB5 within five days at pH 9.
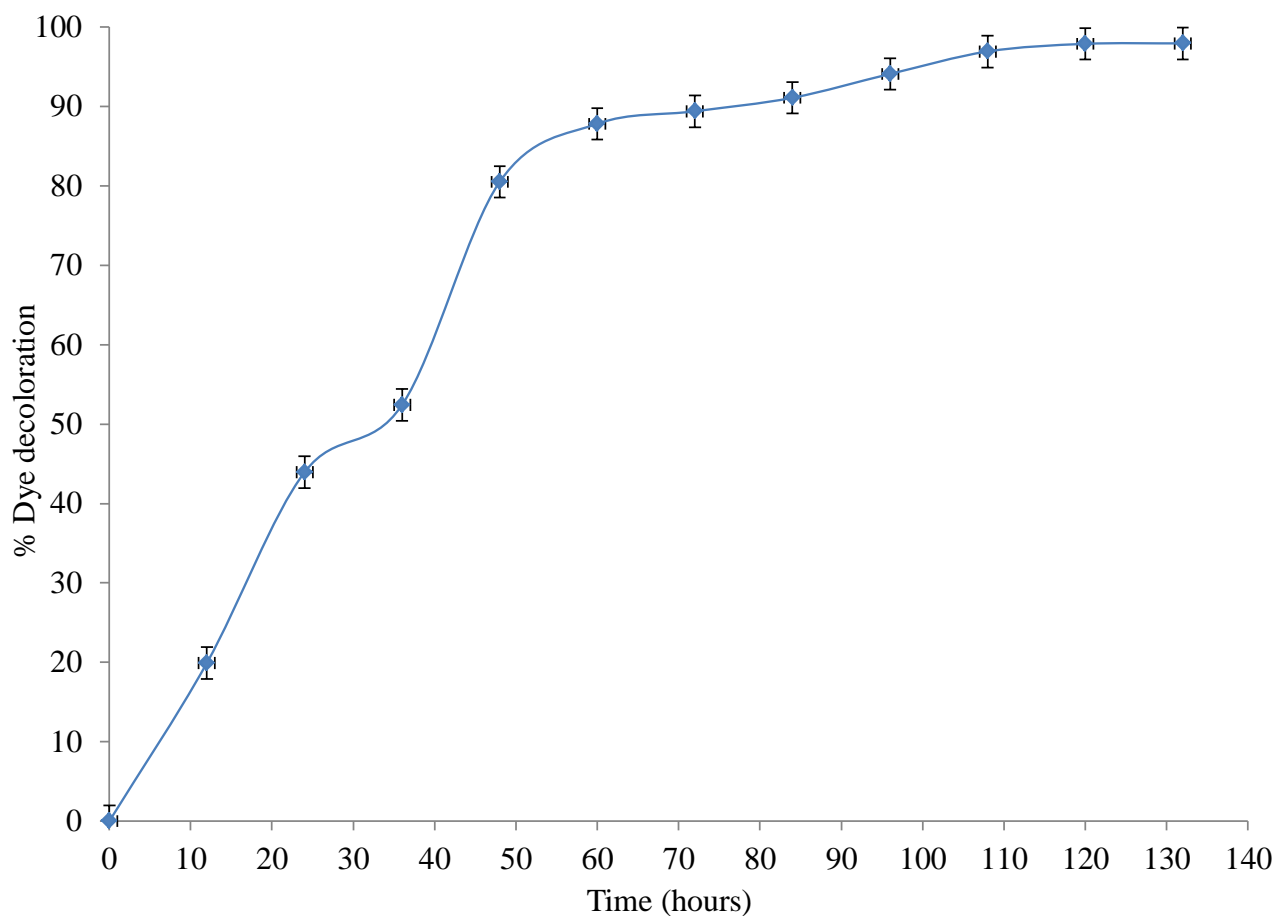
Figure 4.42. Effect of contact time on decolorization of Reactive Black 5 dye.

4.9.4   Effect of pH on Reactive Black 5 dye Decolorization

The pH tolerance of decolorizing enzyme is important because Reactive Black 5 dye (Remazol Black B reactive dye) binds to cotton fibers under alkaline conditions and hence the effluent is usually alkaline with pH greater than eight (Aksu *et al*., 2007). The effect of pH on decolorization of RB5 dye by crude protease enzyme was investigated over the pH range of 4 to 12. The pH profile results at equilibrium are presented in Figure 4.43. Figure 4.44 shows a photograph of RB5 dye at pH 4, 5, 6, 7, 8, 9, 10, 11 and 12 taken after 120 hours when incubated with crude alkaline protease enzyme.

The pH profile show that maximum decolorization efficiency was around pH 9 at 98%. At pH values below 5, there was significantly very low decolorization but when the pH increased above 6.0, decolorization rapidly increased from 50% at pH 5 to 98% at pH 9. Increase in pH beyond 11 resulted in a sharp reduction in dye decolorization. The optimum decolorization activity was observed in a narrow pH range (7 to 9). Figure 4.44 clearly shows that within a pH range of 7 to 9, the deep black color of RB5 was decolorized to assume the enzyme color while at pH 6 and 10 the color of RB5 changed from black to purple. As observed earlier in the decolorization of malachite green dye, these findings present an advantage from the industrial application point of view since most dye effluents, especially those containing Azo functional groups are characterized by alkaline pH under which crude protease enzyme works optimally.
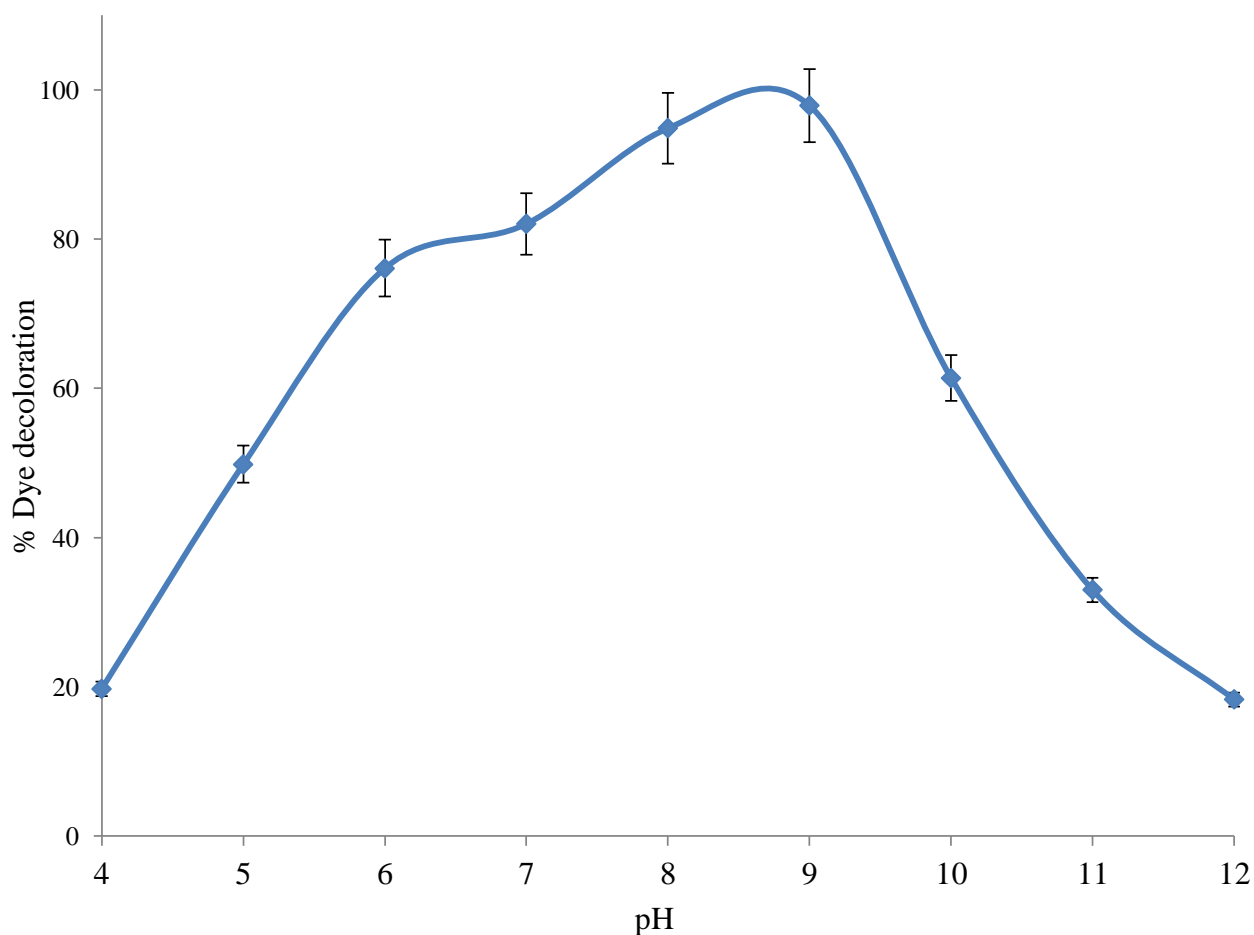
Figure 4.43. Effect of pH on decolorization Reactive Black 5 dye at equilibrium.

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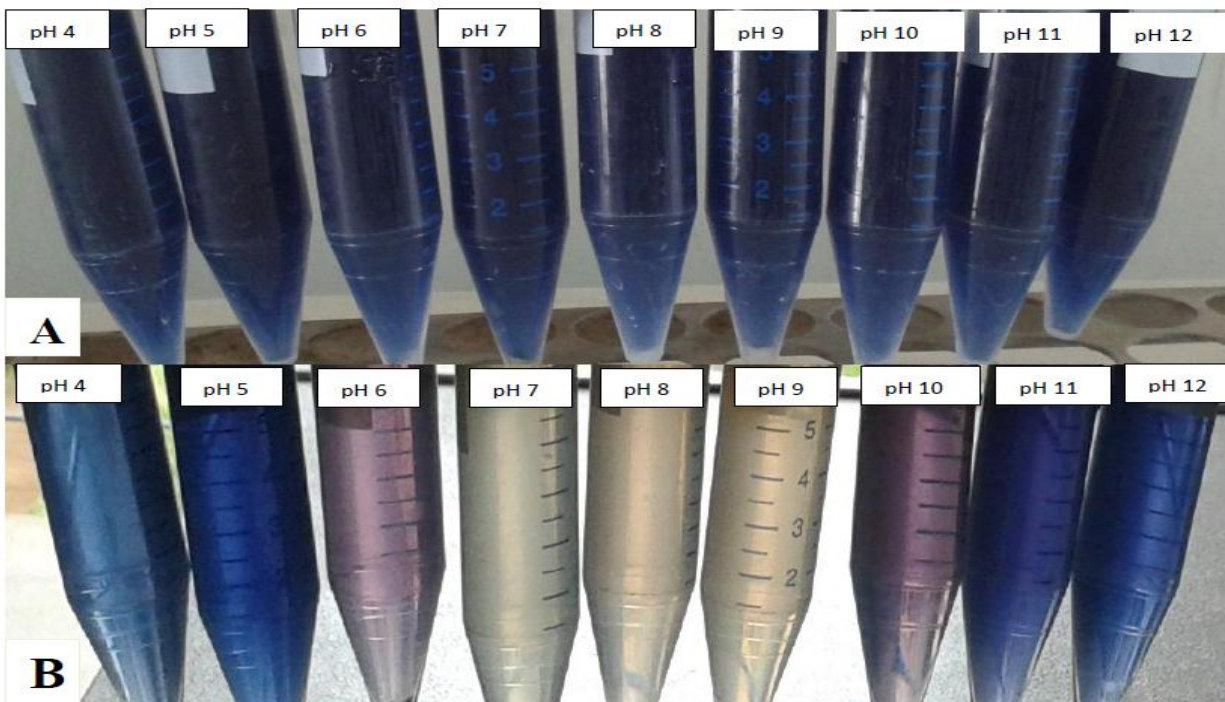
Figure 4.44. Photograph of RB5 dye at pH 4, 5, 6, 7, 8, 9, 10 11and 12 taken at time zero (**A**) and time 120 hours (**B**) during enzymatic decolonization process.

4.9.5 Effect of initial Reactive Black 5 dye concentration on decolorization

The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye at higher concentrations and the ability of the enzyme to recognize the substrate (dye) efficiently at very low concentrations (Holkar *et al.*, 2014). The decolorization of RB5 was studied at various increasing dye concentration and the results are presented in Figure 4.45. It is evident that for all the initial concentrations studied, the quantity of RB5 dye decolorization was rapid over the initial period of contact time (40 hours) after which the rate of decolorization gradually decreased with increasing contact time until equilibrium was established. It was also observed that the amount of dye decolorized in mg/l increased with increasing initial RB5 dye concentration. For instance the amount of RB5 dye decolorized at equilibrium increased from 8.51 mg/L to 93.52mg/L with an increase in RB5 dye concentration from 9.918 mg/l to 99.182 mg/l.
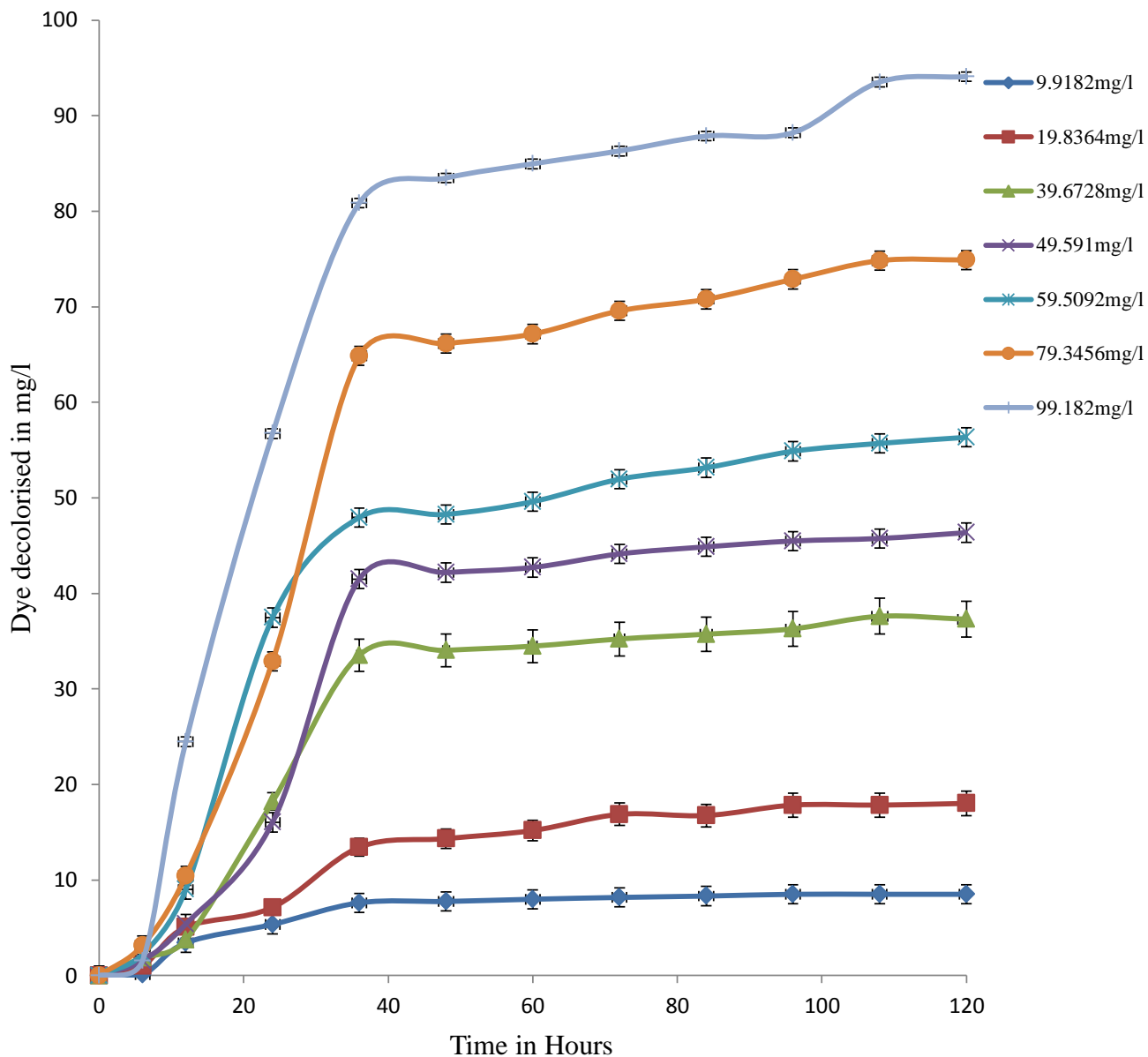
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Figure 4.45. Effect of initial Reactive Black 5 dye concentration on enzyme decolorization.

4.9.6 Analysis of Biodegradation of the RB5 Dye by UV−VIS Spectrophotometer

The biodegradation of the RB5 dye was monitored by a UV−Vis Spectrophotometer. Figure 4.46 illustrates the typical UV−Vis spectra of the control RB5 (0 minute) and biodegraded RB5 sample mixture at various time periods (6, 12, 24, 60 and 120 hours) and crude enzyme as negative control.
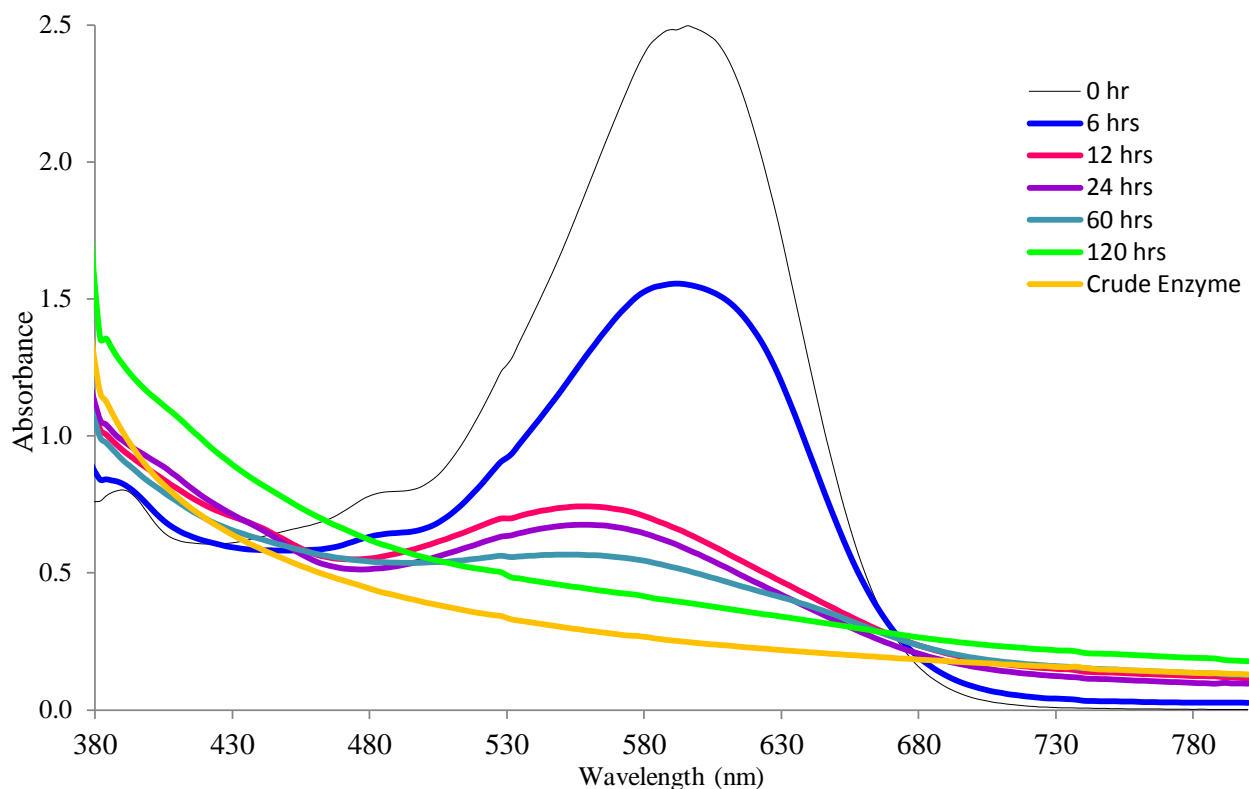
Figure 4.46. UV–Vis spectra scans of RB5 (1.0×10^{-4}M) biodegraded by crude alkaline protease enzyme from *Bacillus cereus* strains *1-p* at different time period, pH 9 and temperature 36˚C.

The overall spectra and the peaks in the spectra decreased gradually with increase in time indicating decolorization and decrease in RB5 dye concentration in the batch solution. It can be clearly observed that peak at zero hour decreased with shift of λ_{max} value of RB5 (597 nm) to lower wavelength (552 nm) indicating that new metabolite were formed in the media due to degradation of the original dye. The absorbance peak in the UV spectra disappeared completely at the end of decolorization (120 hours) and also the intense black color of RB5 turned to colorless in the samples treated with the crude alkaline protease enzyme indicated that RB5 was completely mineralized with formation of new metabolites. This observation is consistent with previous reports by Younes and Sayadi, (2013) who investigated the detoxification of Indigo carmine using a combined treatment via a novel trimeric thermo stable *laccase* and microbial consortium.

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4.9.7 Analysis of Degraded Reactive Black 5 Dye Metabolites by Liquid Chromatography –Hybrid Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

LC−QToF−MS was employed to analyze metabolites resulting from enzymatic degradation of Reactive Black 5 dye. Using positive mode electrospray ionization (ESI+) and multiple reaction monitoring (MRM), qualification and quantification were accomplished.
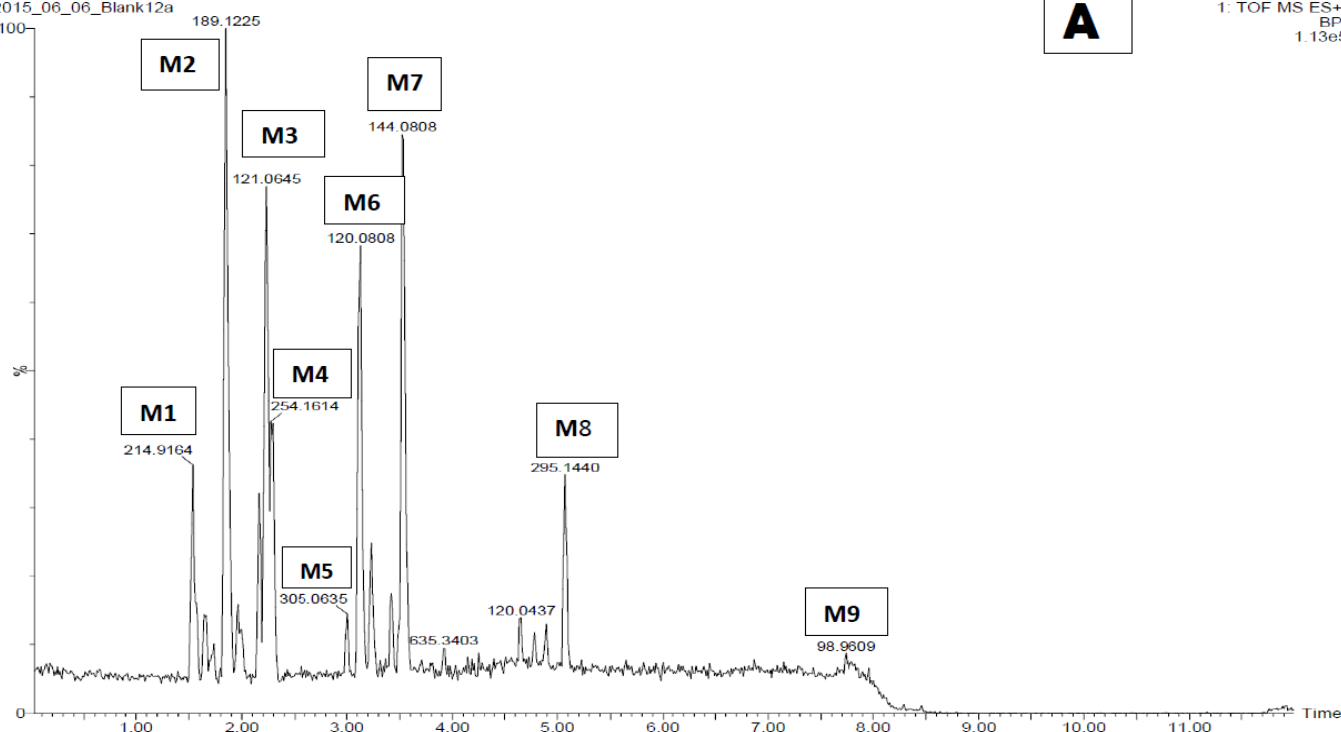
LC-QToF-MS analysis yielded 9 peaks of specific intermediate products that were clearly distinguished compared with the controls of RB5 without crude alkaline protease enzyme or only alkaline protease enzyme without RB5. Figure 4.47 shows extracted chromatogram of a specific ion obtained from LC-QToF-MS.
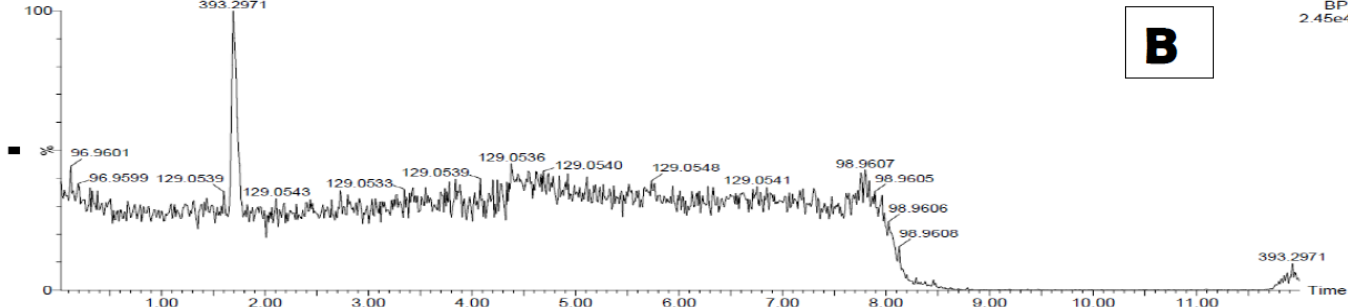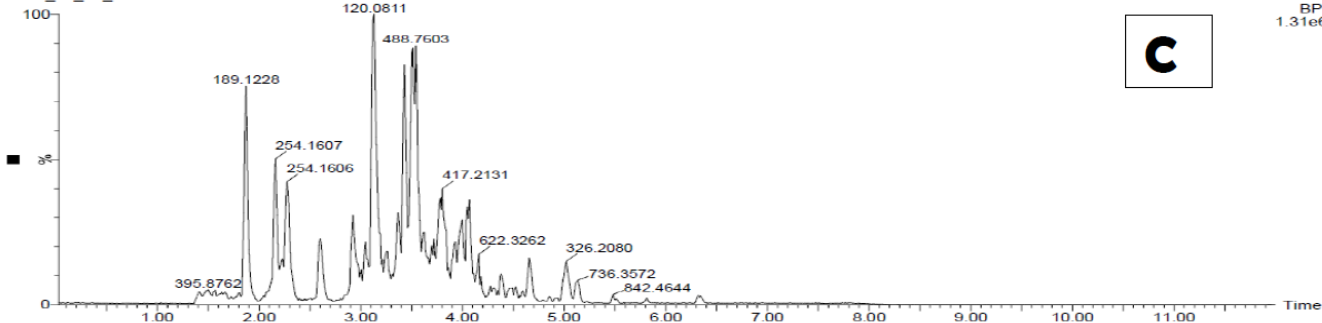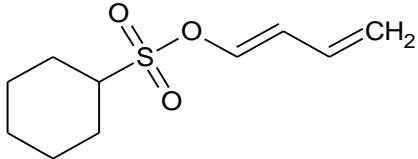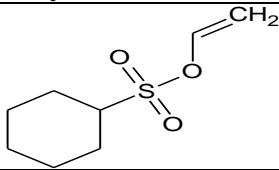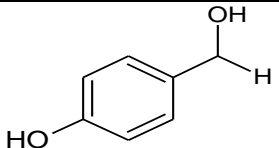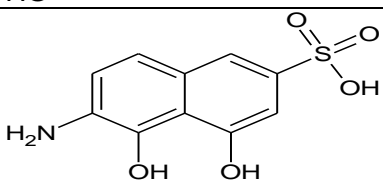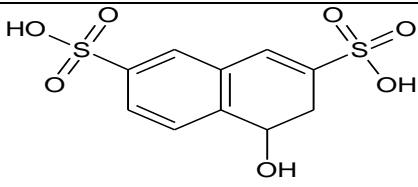
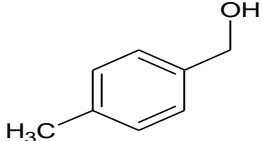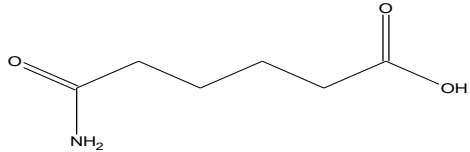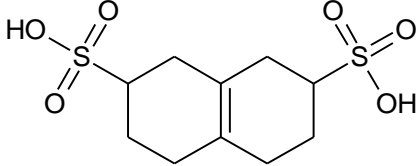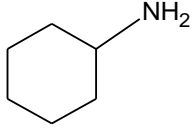Figure 4.47. Extracted ion chromatograms of RB5 dye metabolites detected by LC–QTOF-MS in crude alkaline protease enzyme supernatant. (A) Represents chromatogram of degraded RB5 metabolites numbered M1-M9; (B) chromatogram of non-degraded RB5 in distilled water and (C) chromatogram of crude alkaline protease enzyme.

Based on the parent compound structure, intermediate compounds formed were positively identified. Reactive Black 5 dye could not be detected by LC−QToF−MS; which suggest that RB5 dye dissociated when dissolved in water to form 4-amino-3,6-dihydrazinyl-5-hydroperoxynaphthalene-2,7-disulfonic acid (m/z = 393.29) before fragmentation. Similar results were observed in the enzymatic biodegradation of malachite green dye. From the LC−QToF−MS results, it was possible to assign unique elemental compositions to each peak observed during the course of an analysis and the most probable molecular structure determined drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com) as shown in Table 4.10.

Table 4.10. LC–QTOF-MS analysis and Proposed Intermediate metabolites after enzymatic decoloration and degradation of the Reactive black 5 dye (Probable Intermediates molecular structure drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com))

LC–QTOF-MS proton-ionization mass	Metabolite Peak ; Molecular Weight & Molecular Formula	Intermediates Structure
214.9164	**M1** Butyl cyclohexane sulfonate MW - 216.082 $C_{10}H_{16}O_3S$	
189.1225	**M2** Ethenyl cyclohexanesulfonate MW - 190.066 $C_8H_{14}O_3S$	
122.9236	**M3** 4-(hydroxymethyl) phenol MW - 124.052429 $C_7H_8O_2$	
254.1614	**M4** 6-amino-4,5-dihydroxynaphthalene-2-sulfonic acid MW - 255.02014 $C_{10}H_9O_5SN$	
305.0635	**M5** 4-hydroxy-3,4-dihydronaphthalene-2,7-disulfonic acid MW - 306.312 $C_{10}H_{10}O_7S_2$	

121.0645	**M6** (4-methylphenyl)methanol MW - 122.073166 $C_8H_{10}O$	
144.0808	**M7** 6-amino-6-oxohexanoic acid MW - 145.073 $C_6H_{11}NO_3$	
295.1440	**M8** 1,2,3,4,5,6,7,8-octahydro naphthalene - 2, 7-disulfonic acid MW - 296.038 $C_{10}H_{16}O_6S_2$	
98.9609	**M9** Cyclohexanamine MW - 99.1741 $C_6H_{13}N$	

Metabolic product analysis revealed that the tetrasulphonated structure of RB5 dye was cleaved off followed by benzene ring-removal into cyclohexane and 6-amino-6-oxohexanoic acid. This result should be of great importance because RB5 diazo dye decolorization and mineralization was achieved in a single step raising hopes of using enzyme from *Bacillus cereus* strain *1-p* for industrial application in wastewater treatment. The results obtained are consistent with the earlier results reported by Xingzu *et al*., (2008) who investigated the bio-decolorization and partial mineralization of reactive black 5 dye by a strain of *rhodopseudomonas palustris.*
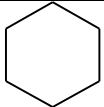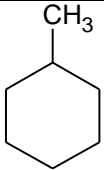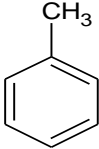
It can therefore be deduced that enzymatic degradation of RB5 using alkaline protease enzyme from strain *1-p* comprised not only the decolorization reaction but also the more significant mineralization and benzene ring-removal. These processes are very important for the efficient removal of toxic organic pollutants. Similar observations were made in the enzymatic degradation of MG dye. Further analysis of the metabolites were determined and identified by GC-MS.
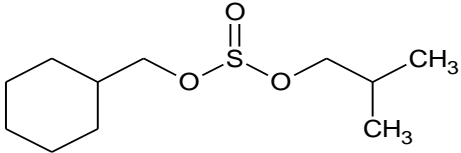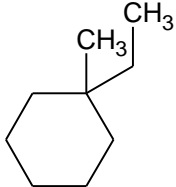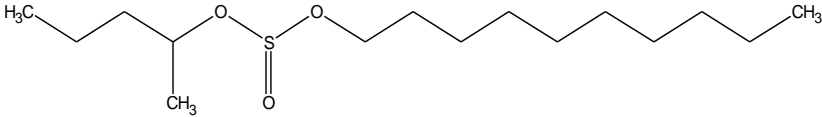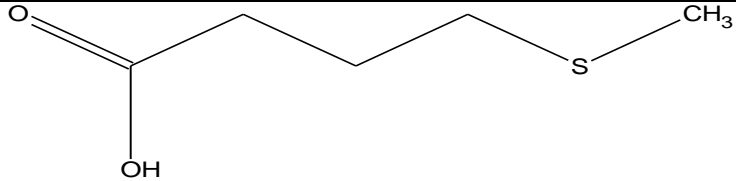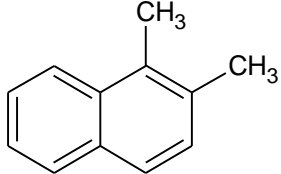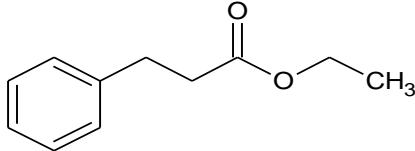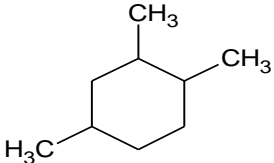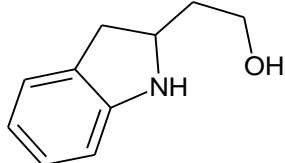
4.9.8 Analysis of Degraded Reactive Black 5 Dye Metabolites by Gas Chromatography - Mass Spectrometry (GC - MS)

GC-MS analysis was employed to identify the presence of relatively hydrophobic and semi-volatile compounds resulting from enzymatic degradation of RB5 dye. The components eluted having different retention times were subjected to mass spectrometry and identified by matching their spectra with those recorded in the National Institute of Standards and Technology (NIST) spectral library stored in the computer software of the GC-MS. The metabolites obtained from GC-MS analysis and the corresponding chemical structures are summarized in Table 4.11.

Overall, the GC-MS analysis revealed that RB5 was completely degraded into less toxic compounds such as Cyclohexane; methyl Cyclohexane, and 4-(methylthio) Butanoic acid. Combining the results obtained from LC–QTOF-MS and GC-MS, it can be concluded that RB5 was completely degraded by crude alkaline protease enzyme from strain *1-P* through series of reactions which included demethylation, reduction, benzene ring-removal, and oxidation.

Table 4.11. Intermediate metabolites after enzymatic decoloration and degradation of the Reactive black 5 dye as identified by GC-MS (molecular structure drawn using ACD/ChemSketch ver. 12.0 (www.acdlabs.com))

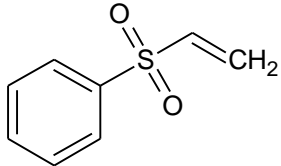| Metabolite name and chemical formula	R_t. time (min)	Mw (m/z)	Corresponding chemicals structure
Cyclohexane	3.858	84.15	
Cyclohexane, methyl-	4.999	98.10	
Toluene	6.151	92.06	

Sulfurous acid, cyclohexylmethyl isobutyl ester	9.907	234.12	
Cyclohexane, 1-ethyl-1-methyl	10.428	126.23	
Sulfurous acid, decyl 2-pentyl ester	12.832	292.47	
Butanoic acid, 4-(methylthio)-	14.751	134.04	
Naphthalene, 1,2-dimethyl	17.758	156.09	
Benzene, (ethenylsulfonyl)-	18.366	168.02	
Benzenepropanoic acid, ethyl ester	18.489	178.09	
Cyclohexane, 1,2,4-trimethyl-	19.402	126.23	
1H-Indole-3-ethanol	22.139	161.08	

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4.10 Kinetic study of Reactive black 5 dye decolorization

In order to determine the decolorization rate of RB5 dye by crude alkaline protease enzyme, test solution containing 40 ml of RB5 dye at different concentrations and 10ml crude alkaline protease enzyme were mixed at 36˚C and pH 9. Michaelis-Menten kinetics, Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the RB5 decolorization process.

4.10.1 Michaelis-Menten kinetics

The experimental results at various RB5 dye concentration were analyzed based on the interpretation of the Michaelis-Menten kinetics Equation (2). Figure 4.48 show a plot of V (mgl^{-1}h^{-1}) verses S (RB5 dye concentration in mgl^{-1}) for the enzymatic decolorization of RB5 dye. The experimental data fitted quite well with the Michaelis-Menten equation with a very high correlation coefficient R^2 value of 1.00; indicating that the model was best applicable for describing the biodegradation of MG dye by crude alkaline protease enzyme from strain *1-p*. Good fit to Michaelis-Menten kinetics equation also suggests first-order reaction kinetics. The corresponding V$_{max}$ and K$_m$ values were calculated and summarized in Table 4.12.
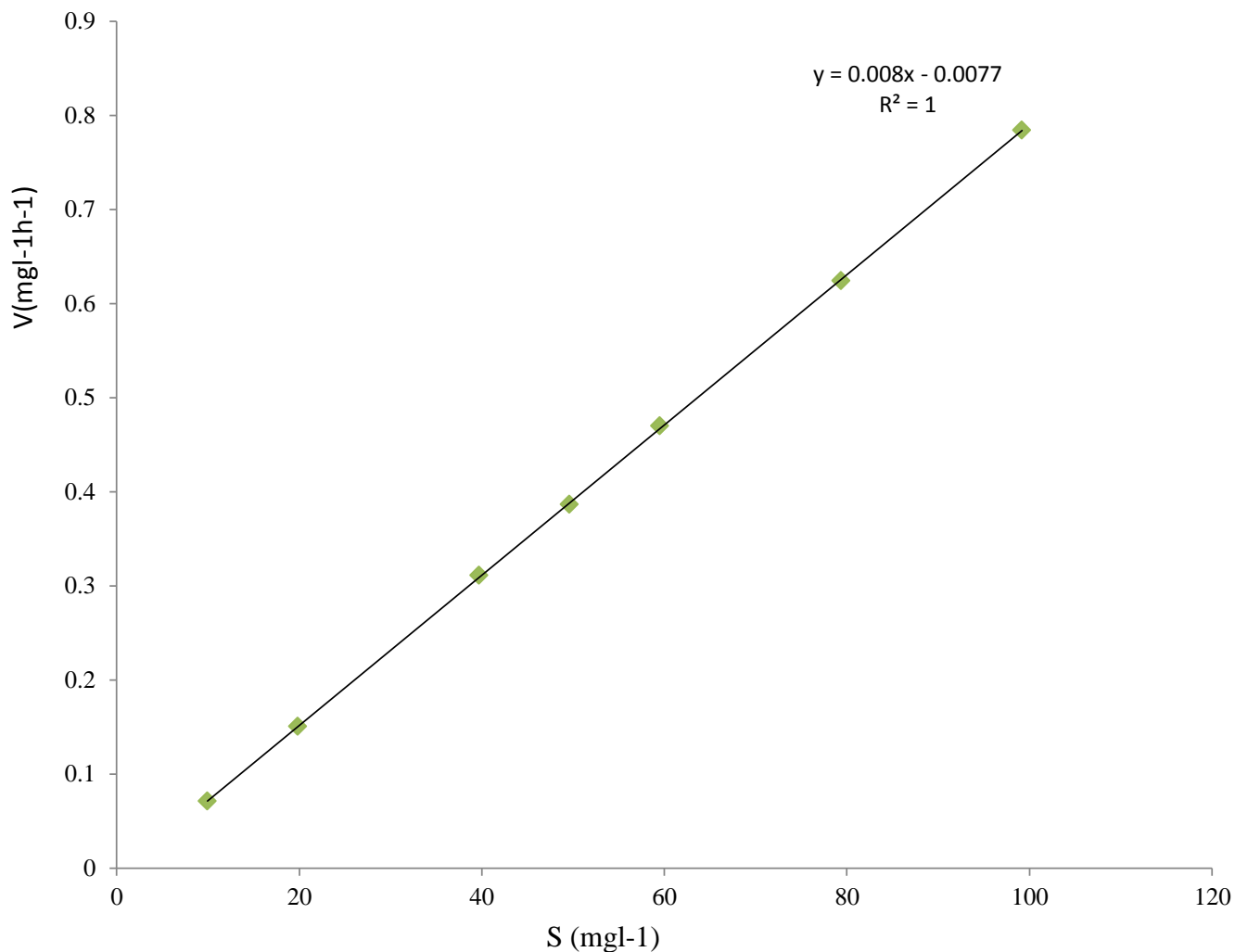
Figure 4.48. Michaelis–Menten plot for the enzymatic decoloration of RB5 dye.

4.10.2 Lineweaver-Burk plot

The experimental data obtained at various RB5 dye concentration were also subjected to the Lineweaver-Burk equation. Figure 4.49 shows the Lineweaver-Burk plot. The plot demonstrates a perfect fit with an R^2 value of 0.9994 indicating that Lineweaver-Burk plot model can also be applied in describing the biodegradation of RB5 dye by crude alkaline protease enzyme from strain *1-p*. K_m and V_{max} values were calculated and summarized in Table 4.12. Similar results were observed in enzymatic degradation of MG dye.
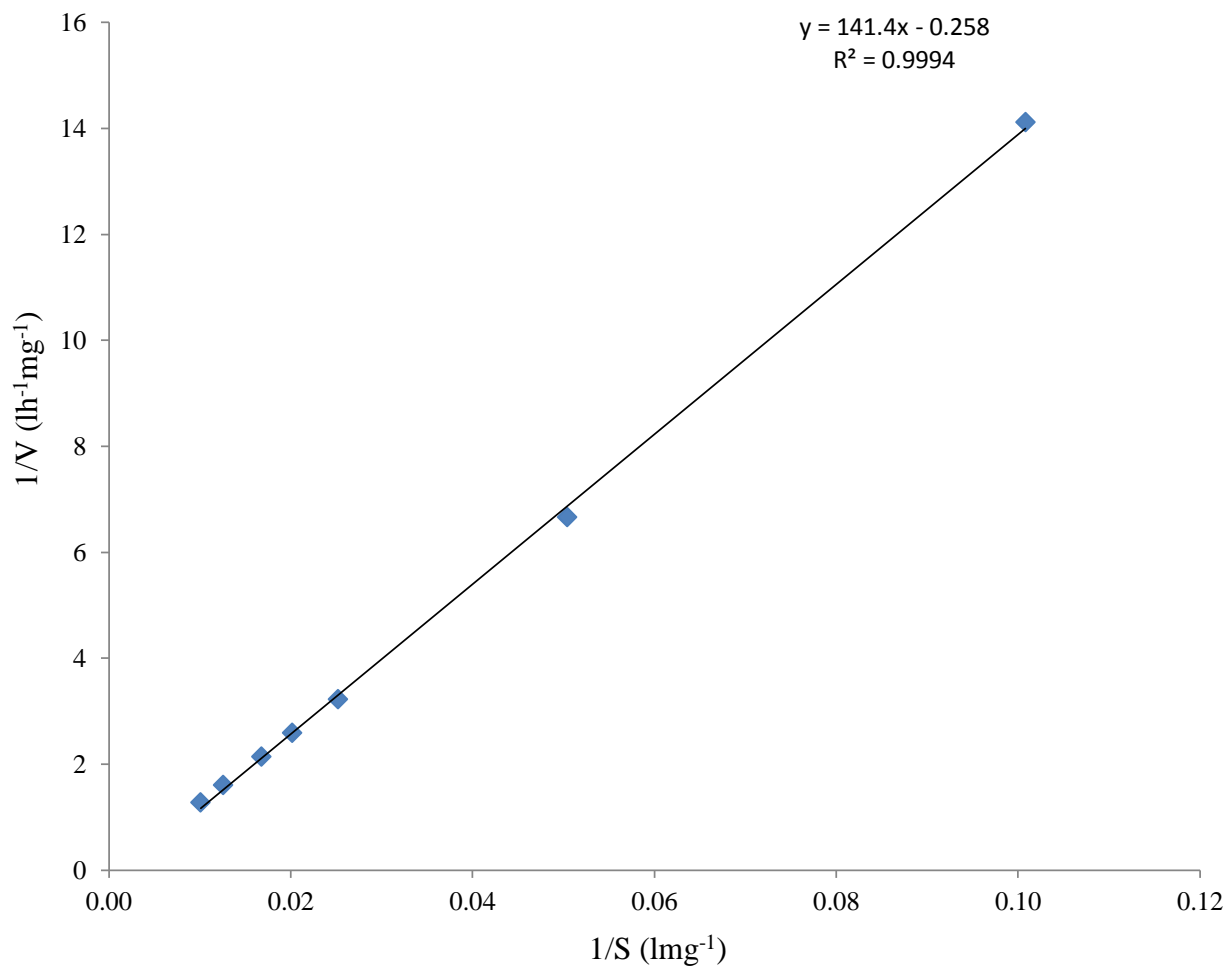
Figure 4.49. Lineweaver-Burk plot for the enzymatic decoloration of RB5 dye.

4.10.3 Eadie-Hofstee plot

Eadie-Hofstee plot was also used to determine K_m and V_{max} from the experimental data. Figure 4.50 shows the Eadie-Hofstee plot; of a poor correlation coefficient ($R^2 = 0.6328$). This indicates that the biodegradation of RB5 dye by crude alkaline protease enzyme from strain *1-p* cannot be described well by Eadie-Hofstee plot. The corresponding V_{max} and K_m values were calculated and summarized in Table 4.12
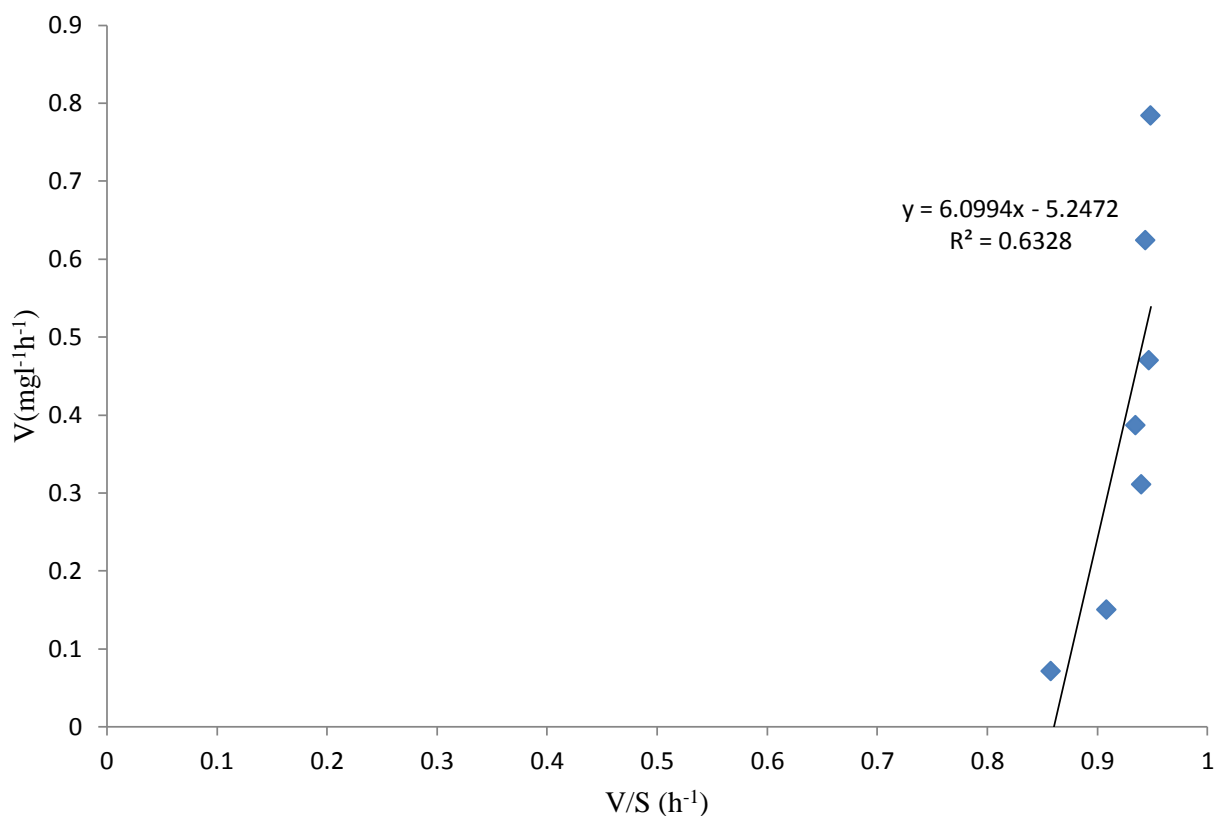
Figure 4.50. Eadie-Hofstee plot for the enzymatic decolorization of RB5 dye.

A comparison of K_m and V_{max} values obtained from above three approaches is shown in Table 4.12. It shows that these three results are not comparable. However, since Lineweaver-Burk plot and Michaelis-Menten kinetics had the highest R^2 value, it can be concluded that decolorization of RB5 by crude alkaline protease enzyme from strain 1-*p* can be best described by Michaelis-Menten kinetics and Lineweaver-Burk plot.

Table 4.12. Comparison of K_m and V_{max} values for the enzymatic decolorization of RB5 dye

Plot type	V_{max} (mg l^{-1} h^{-1})	K_m (mg l^{-1})	R^2
Michaelis-Menten kinetics	0.008	98.17	1.0
Lineweaver-Burk plot	3.875	548.06	0.9994
Eadie-Hofstee plot	5.2472	6.0994	0.6328

4.10.4 Determination of Reaction Order for Reactive Black 5 Decolorization

Reactive black 5 dye decolorization data from the batch tests were used to determine the order of dye decolorization reaction. The kinetic equation models used for Zero-order, first-order and second-order kinetic study are given in equations 5 to 9.

Zero order kinetic plots were obtained by plotting dye concentration (C_t) versus time as shown in Figure. 4.51. First-order kinetic model graph was also obtained by plotting $\ln C_t$ against time (Figure. 4.52) while second-order kinetic model shows a plot of ($1/C_t$) versus time as shown in Figure 4.53. The rate constants of decolorization reaction and coefficients of least square method analysis are tabulated in Table 4.13.
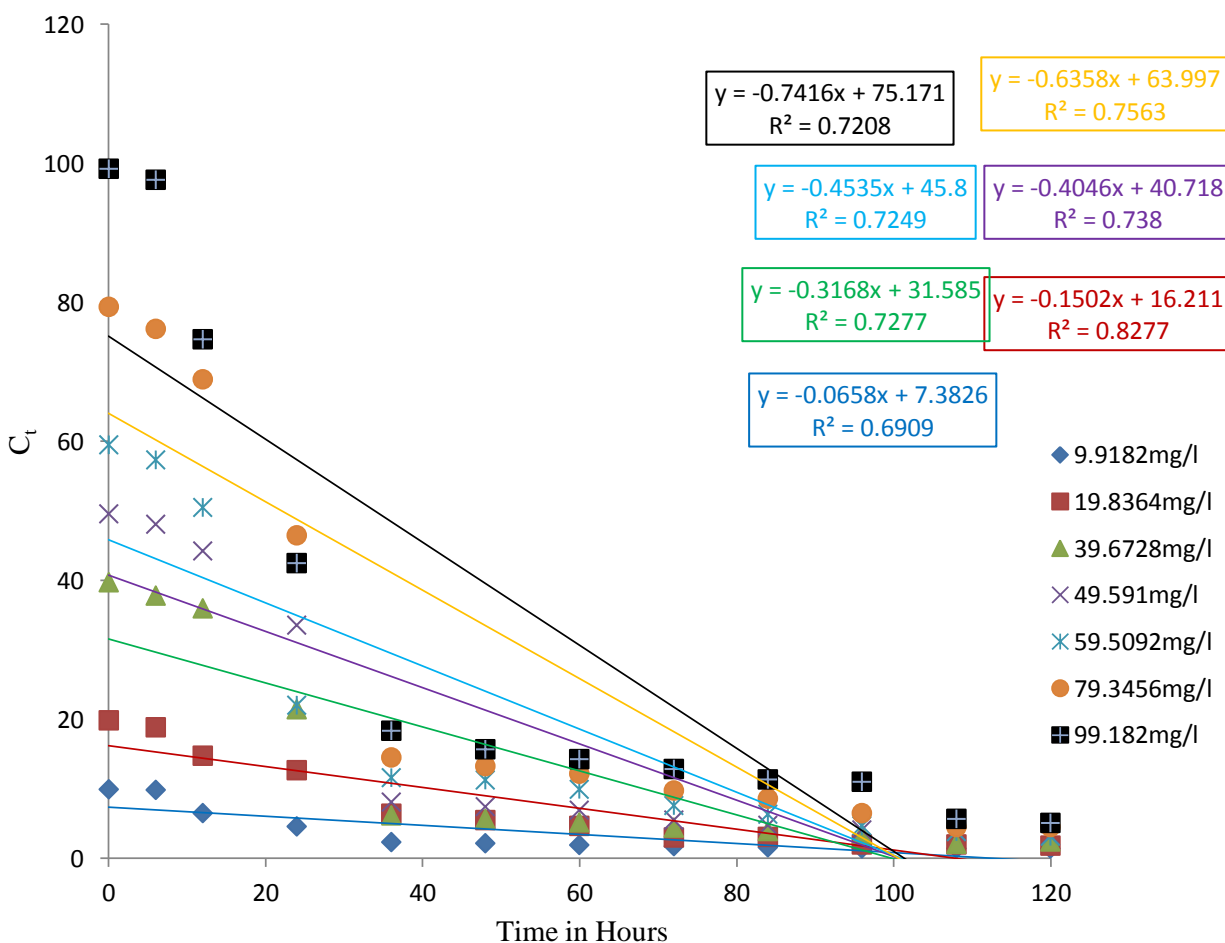
Figure. 4.51. Zero-order kinetic model fits at different initial concentration of Reactive black 5 dye.
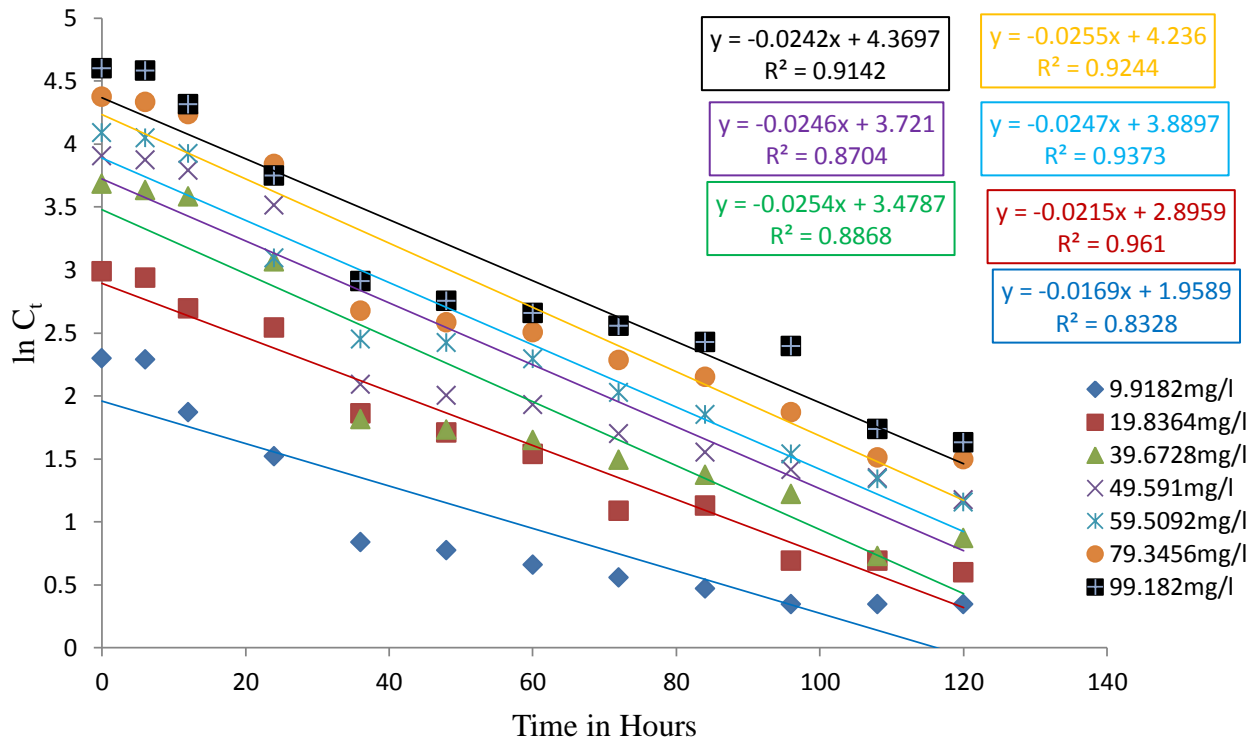
Figure 4.52. First-order kinetic model fits at different initial concentration of Reactive black 5 dye.

$y = -0.0242x + 4.3697$
$R^2 = 0.9142$

$y = -0.0255x + 4.236$
$R^2 = 0.9244$

$y = -0.0246x + 3.721$
$R^2 = 0.8704$

$y = -0.0247x + 3.8897$
$R^2 = 0.9373$

$y = -0.0254x + 3.4787$
$R^2 = 0.8868$

$y = -0.0215x + 2.8959$
$R^2 = 0.961$

$y = -0.0169x + 1.9589$
$R^2 = 0.8328$

9.9182mg/l
19.8364mg/l
39.6728mg/l
49.591mg/l
59.5092mg/l
79.3456mg/l
99.182mg/l
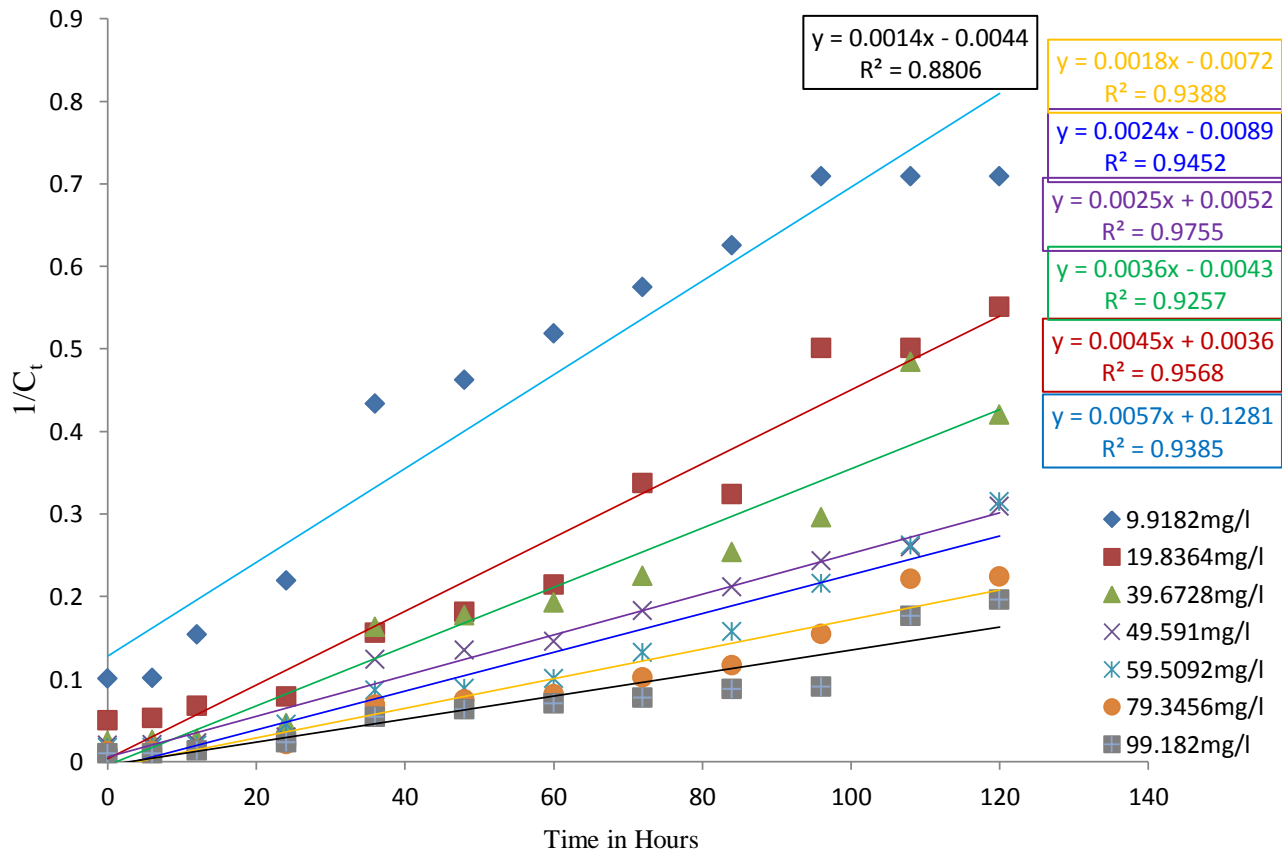
Figure. 4.53. Second order kinetic at different initial concentration of Reactive black 5 dye.

$y = 0.0014x - 0.0044$
$R^2 = 0.8806$

$y = 0.0018x - 0.0072$
$R^2 = 0.9388$

$y = 0.0024x - 0.0089$
$R^2 = 0.9452$

$y = 0.0025x + 0.0052$
$R^2 = 0.9755$

$y = 0.0036x - 0.0043$
$R^2 = 0.9257$

$y = 0.0045x + 0.0036$
$R^2 = 0.9568$

$y = 0.0057x + 0.1281$
$R^2 = 0.9385$

9.9182mg/l
19.8364mg/l
39.6728mg/l
49.591mg/l
59.5092mg/l
79.3456mg/l
99.182mg/l

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Table 4.13. Zero, first and second order kinetic constants obtained in Enzymatic degradation of Reactive black 5 dye

Kinetics model	Constant	9.91mg/l	19.83mg/l	39.67mg/l	49.591mg/l	59.50mg/l	79.34mg/l	99.18mg/l
Zero order	k_0(mgl^{-1}min^{-1})	0.0658	0.1502	0.3168	0.4048	0.4535	0.6388	0.7416
	C_o(cal)	7.386	16.211	31.585	40.718	45.800	63.997	75.171
	R^2	0.6709	0.8277	0.7277	0.7380	0.7249	0.7563	0.7208
First order	k_1(min^{-1})	0.0169	0.0215	0.0254	0.0246	0.0247	0.0255	0.0242
	C_o(cal)	7.092	18.100	32.424	41.306	48.896	69.131	79.020
	R^2	0.8328	0.961	0.8868	0.8704	0.9373	0.9244	0.9142
Second order	k_2 (l mg−1min^{-1})	0.0057	0.0045	0.0036	0.0025	0.0024	0.0018	0.0014
	C_o(cal)	7.81	277.78	232.56	192.31	112.36	138.89	227.27
	R^2	0.9385	0.9568	0.9257	0.9755	0.9455	0.9385	0.8806

As shown in Table 4.13, it is evident that first and second order reaction had a relatively high correlation coefficients (R^2). However, when experimental data is compared with calculated data (C_o(cal)) only first-order model showed good compliance as second order kinetic model deviated. Zero-order kinetic model had a low correlation coefficients (R^2) in the range of 0.67-0.83 showing that it cannot be applied in describing the decolorization of RB5 dye. It can be concluded that first-order kinetic model best describes the enzymatic degradation of RB5 dye using crude alkaline protease enzyme from strain *1-p*. The rate of RB5 decolorization reaction is inversely proportional to the initial dye concentration i.e as the dye concentration increases decolorization rate decreases. This finding is consistent with earlier studies reported in the literature (Matouq *et al*., 2014).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1.1 Conclusions

Environmental pollution has been a major irritant and setback to chemical-based industrial development in both developed and developing countries. Leather industries which extensively use lime and sodium sulfide in dehairing hides and skins face serious environmental pollution problems. This is because lime and sodium sulfide heavily contribute to increased odour, effluent toxicity, health hazards to the tannery workers, production of poisonous sludge with disposal challenges and blockage to sewerage pipes. Similarly, use of organic dyes in leather, textile and many other industries often pose pollution problems in the form of colored wastewater discharge into water bodies resulting into high turbidity, increased chemical oxygen demand (COD) and reduced light penetration. Furthermore, dyes are not only recalcitrant and refractory pollutants that constitute a significant burden on the environment but are also toxic, mutagenic and carcinogenic. This study has opened up the potential of utilizing enzyme from extremophile micro-organism in industrial bioprocessing and bioremediation of organic dyes.

Four protease producing bacteria were isolated from the shores of Lake Bogoria, Kenya. Biochemical test and phylogenetic characterization indicated that the four isolates were all associated mainly with members of the *Bacillus Cereus*. Submerged fermentation process parameters such as pH, temperature, incubation time, different substrates and concentrations that influence production of protease enzyme were analyzed and optimized. After optimizing the fermentation condition, strain 1-*p* was selected for large scale production of crude alkaline protease enzyme at the optimum condition using 1% casein under Submerged Fermentation

(SmF). Optimum temperature for crude protease enzyme activity measured over 30 minutes was found to be 45°C with relatively high enzyme activity in the range of 30°C to 50°C. The optimum pH was found to be 11 indicating that the enzyme is an alkaline protease. The activity of the enzyme at alkaline pH can present an advantage from industrial application point of view since reactions in tanneries and dye applications in the textile industry best takes place in an alkaline environment. Industrial applications of the isolated enzymes in bioremediation of organic dyes and leather processing were investigated.

The efficacy of the isolated crude alkaline protease enzyme from *Bacillus cereus* strain *1-p* to dehair cow hide and descale Nile perch (*Lates niloticus*) skin was investigated. Factors affecting enzyme dehairing process such as temperature, pH and incubation time were determined and optimized. Industrial demonstrations of large scale beam house dehairing process (trial) was successfully accomplished whereby 21 kg full bull hide was 100% chemical free dehaired and degreased using the isolated crude alkaline protease enzyme within 12 hours compared to the conventional method which take between 2- 6 days. Furthermore, 10 pieces (9.5 kg) of Nile perch (*Lates niloticus*) skin were successfully descaled and degreased within 2 hours. 1.1kg of quality hair and 0.98 kg of valuable scales removed during dehairing/descaling process were readily recovered while still in good condition suitable for further use. Recoveries of hair and scales greatly contribute in the reduction of BOD, COD and TDS load in the effluent. It was observed that at optimal pH, the pungent smell which is normally characteristic of many tanneries is eliminated. This is a positive result because crude alkaline protease enzyme does not only eliminate the use of sodium sulfide and lime but also eliminates the pungent smell which is a major air pollutant and of great health concern. The results show that use of enzymes in beam house operations is economically feasible and environmentally benign.

Physical-mechanical characteristics of the resultant leather after full processing was analyzed and the results compared with the set Kenya Bureau of Standards (KEBS) standards. Parameters tested included tensile strength, elongation at break, tear strength, water absorption and grain crack. The results obtained revealed that the quality of the final leather from bull hide was within the set KEBS standards for animal leather. Since KEBS doesn't have standard set for Nile perch skin, comparison was not possible. Generally, enzymatic dehairing/descaling is a clean green technology which reduces pollution load in the effluent, improve industrial hygiene; shorten the dehairing/descaling process without compromising the quality of leather. The leather obtained exhibit good qualities such as softness, toughness and pliability.

The ability of the isolated enzymes to decolorize and degrade the organic dyes namely; Malachite Green (MG) dye and Reactive Black 5 (RB5) dye was extensively studied using crude alkaline protease enzyme from isolate *1-p*. Dynamic batch experiments were carried out for the decolorization of dye by enzyme. Over 98% decolorization efficiency was achieved within 24 hours for MG using an initial dye concentration of 1.0×10^{-5}M. Similarly, over 98% decolorization was achieved within 120 hours for RB5 using an initial dye concentration of 1.0×10^{-4}M. Experimental results revealed that the decolorization process was highly dependent on contact time, initial dye concentration, aqueous solution temperature and pH. Kinetic study of the decolorization experiments approximates first-order reaction kinetics for both dyes. Michaelis-Menten kinetics, Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the enzymatic dye decolorization process. Lineweaver-Burk plot model was best applicable for describing the biodegradation of both MG and RB5 dye by crude alkaline protease enzyme.

Biodegradation of dyes was monitored by UV-VIS spectrophotometer and resultant metabolites confirmed by Thin Layer Chromatography (TLC), Liquid Chromatography–Hybrid Quadrupole Time of Flight Mass Spectrometry (LC–QToF-MS) and Gas Chromatography/Mass Spectrometry (GC - MS). The results obtained revealed that the enzymatic degradation of MG and RB5 by crude alkaline protease enzyme from strain *1-p* resulted in complete mineralization and benzene ring-removal, the latter being responsible for organic dye toxicity. The result of this study has revealed that crude protease enzyme isolated from *Bacillus Cereus* Strain *p-1* exhibit novel alkaline protease properties with ability to decolorize and degrade MG and RB5 dyes.

Kinetic study results revealed that first-order kinetic equation was the appropriate equation to describe decolorization of both MG and RB5 dye. The results show that decolorization process depends on dye concentration. Michaelis-Menten kinetics, Lineweaver–Burk plot and Eadie-Hofstee plot models were used to establish the kinetic parameters for the dye decolorization process. Lineweaver–Burk plot provided the best theoretical correlation of the experimental data for the decolorization of both dyes. The maximum rate (V_{max}) and Michaelis-Menten constant (K_m) were found to be 17.70 mg l^{-1} h^{-1} and 124 mg l^{-1}, respectively for MG dye and 3.975 mg l^{-1} h^{-1} and 548.06 mg l^{-1} for RB5, respectively using Lineweaver–Burk plot. The results provide evidence that the crude enzyme from *Bacillus cereus* strain *1-p* is an effective and potential candidate for industrial wastewater treatment.

In view of the results obtained and the need for environment protection, application of crude alkaline protease enzyme from *Bacillus cereus* strain *1-p* is a promising clean technology which will completely eliminate use of sodium sulfide and lime in the dehairing as well as greatly reduce the pungent smell which is the normally associated with tanneries across the world.

Kenya is a developing country which faces environmental pollution challenges due to extensive use of toxic chemicals in industries such as leather and textile. Environmental pollution and high cost of these toxic chemicals pose to be a major challenge in industrial development and achievement of Kenya's vision 2030 and the Millennium Development Goals. However, Government of Kenya and its policy makers can embrace such green technology in leather processing, bioprocessing of green product and bioremediation of industrial waste to quickly achieve the set developmental goals.

5.1.2   Recommendations

Crude alkaline protease enzyme from *Bacillus cereus* strain *1-p* has proved that it can be effectively utilized in industries to reduce pollution load, simplify leather processing, and bioremediation of organic dyes. In view of these results, I recommend the following:

1. I strongly recommended that the Government of Kenya and its policy makers embrace this green technology in leather processing, and bioremediation of organic dyes.
2. Further studies need to be done to compare the activity of purified enzyme and the crude enzyme to gain a complete understanding of the enzyme activity. This will be of great importance in establishing which one is cost efficient in industrial application.
3. More studies need to be done to test the ability of the isolated enzyme to degrade other groups/types of dyes since textile and leather industries use many different varieties of dyes.
4. The efficacy of other isolates (*2-p, 3-p and 4-p*) to biodegrade organic dyes either as single culture or mixed cultures or crude enzyme need to be determined with the objective of increasing the efficiency as a cost effective alternative technology for dye removal in wastewater treatment processes.

REFERENCES

Afshar, S., Samari Jahromi, H., Jafari, N., Ahmadi, Z., and Hakamizadeh, M. (2011). Degradation of malachite green oxalate by UV and visible lights irradiation using nanophotocatalyst. Sci. Iran. *18*, 772–779.

Aksu, Z. (2003). Reactive dye bioaccumulation by Saccharomyces cerevisiae. Process Biochem. *38*, 1437–1444.

Aksu, Z., Kılıç, N.K., Ertuğrul, S., and Dönmez, G. (2007). Inhibitory effects of chromium(VI) and Remazol Black B on chromium(VI) and dyestuff removals by Trametes versicolor. Enzyme Microb. Technol. *40*, 1167–1174.

Amoozegar, A.M., Zahra Fatemi, A., Reza Karbalaei-Heidari, H., and Reza Razavi, M. (2007). Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, Salinivibrio sp. strain AF-2004. Microbiol. Res. *162*, 369–377.

Andrioli, E., Petry, L., and Gutterres, M. (2015). Environmentally friendly hide unhairing: Enzymatic-oxidative unhairing as an alternative to use of lime and sodium sulfide. Process Saf. Environ. Prot. *93*, 9–17.

Arabaci, G., and Usluoglu, A. (2014). The Enzymatic Decolorization of Textile Dyes by the Immobilized Polyphenol Oxidase from Quince Leaves. Sci. World J. *2014*, 1–5.

Armbruster, D.A., Tillman, M.D., and Hubbs, L.M. (1994). Limit of detection (LQD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. Clin. Chem. *40*, 1233–1238.

Arunachalam, C., and Saritha, K. (2009). Protease enzyme: An eco-friendly alternative for leather industry. Indian J. Sci. Technol. *2*, 29–32.

Ayed, L., Mahdhi, A., Cheref, A., and Bakhrouf, A. (2011). Decolorization and degradation of azo dye Methyl Red by an isolated Sphingomonas paucimobilis: Biotoxicity and metabolites characterization. Desalination *274*, 272–277.

Balasubramanian, S., and Pugalenthi, V. (2000). A comparative study of the determination of sulphide in tannery waste water by ion selective electrode (ISE) and iodimetry. Water Res. *34*, 4201–4206.

Beghetto, V., Zancanaro, A., Scrivanti, A., Matteoli, U., and Pozza, G. (2013). The Leather Industry: A Chemistry Insight Part I: an Overview of the Industrial Process. Sci. CaFoscari *1*, 13–22.

Bulut, Y., and Aydın, H. (2006). A kinetics and thermodynamics study of methylene blue adsorption on wheat shells. Desalination *194*, 259–267.

Cha, C.J., Doerge, D.R., and Cerniglia, C.E. (2001). Biotransformation of Malachite Green by the Fungus Cunninghamella elegans. Appl. Environ. Microbiol. *67*, 4358–4360.

Chachaty, E., and Saulnier, P. (2000). "Isolating chromosomal DNA from bacteria." The nucleic acid protocols handbook. (Totowa, NJ: Humana Press).

Chang, J.-S., and Kuo, T.-S. (2000). Kinetics of bacterial decolorization of azo dye with Escherichia coli NO3. Bioresour. Technol. *75*, 107–111.

Chequer, F.M.D., Dorta, D.J., and de Oliveira, D.P. (2011). Azo Dyes and Their Metabolites: Does the Discharge of the Azo Dye into Water Bodies Represent Human and Ecological Risks? (INTECH Open Access Publisher).

Chi, Z., and Zhao, S. (2003). Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast strain. Enzyme Microb. Technol. *33*, 206–211.

Choudhary, V., and Jain, P.C. (2012). Optimization of process parameters for alkaline protease production by Aspergillus versicolor PF/F/107. J Acad Indus Res *1*, 8–14.

Christopher, J.G., Ganesh, S., Palanivel, S., Ranganathan, M., and Jonnalagadda, R.R. (2014). Cohesive system for enzymatic unhairing and fibre opening: an architecture towards eco-benign pretanning operation. J. Clean. Prod. *83*, 428–436.

Culp, S., J., and Beland, F., A. (1996). Malachite Green: A Toxicological Review. Int. J. Toxicol. *15*, 219–238.

Dayanandan, A., Kanagaraj, J., Sounderraj, L., Govindaraju, R., and Rajkumar, G.S. (2003). Application of an alkaline protease in leather processing: an ecofriendly approach. J. Clean. Prod. *11*, 533–536.

Deepa, K., Chandran, P., and Sudheer Khan, S. (2013). Bioremoval of Direct Red from aqueous solution by Pseudomonas putida and its adsorption isotherms and kinetics. Ecol. Eng. *58*, 207–213.

Du, L.-N., Wang, S., Li, G., Wang, B., Jia, X.-M., Zhao, Y.-H., and Chen, Y.-L. (2011). Biodegradation of malachite green by Pseudomonas sp. strain DY1 under aerobic condition: characteristics, degradation products, enzyme analysis and phytotoxicity. Ecotoxicology *20*, 438–446.

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. *32*, 1792–1797.

El-Monem, F.A., Hussain, A.I., Nashy, E.-S.H.A., El-Wahhab, H.A., and Naser, A.E.-R.M. (2014). Nano-emulsion based on acrylic acid ester co-polymer derivatives as an efficient pre-tanning agent for buffalo hide. Arab. J. Chem.

Farag, M.A., Porzel, A., and Wessjohann, L.A. (2012). Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC–MS, LC–MS and 1D NMR techniques. Phytochemistry *76*, 60–72.

Ferrer, I., and Thurman, E.M. (2003). Liquid chromatography/time-of-flight/mass spectrometry (LC/TOF/MS) for the analysis of emerging contaminants. TrAC Trends Anal. Chem. *22*, 750–756.

Gavrilescu, M., and Chisti, Y. (2005). Biotechnology—a sustainable alternative for chemical industry. Biotechnol. Adv. *23*, 471–499.

Gómez-Guillén, M.C., Turnay, J., Fernández-Dıaz, M.., Ulmo, N., Lizarbe, M.A., and Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. Food Hydrocoll. *16*, 25–34.

Gupta, R., Beg, Q.K., and Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. *59*, 15–32.

Hasan, F., Shah, A.A., and Hameed, A. (2006). Industrial applications of microbial lipases. Enzyme Microb. Technol. *39*, 235–251.

Holkar, C.R., Pandit, A.B., and Pinjari, D.V. (2014). Kinetics of biological decolorisation of anthraquinone based Reactive Blue 19 using an isolated strain of Enterobacter sp.F NCIM 5545. Bioresour. Technol. *173*, 342–351.

Hüffer, S., and Taeger, T. (2004). Sustainable leather manufacturing: A topic with growing importance. J. Am. Leather Chem. Assoc. *99*, 424–428.

Jang, G.H., Park, I.-S., Lee, S.H., Huh, T.-L., and Lee, Y.M. (2009). Malachite green induces cardiovascular defects in developing zebrafish (Danio rerio) embryos by blocking VEGFR-2 signaling. Biochem. Biophys. Res. Commun. *382*, 486–491.

Jian, S., Wenyi, T., and Wuyong, C. (2008). Ultrasound-accelerated enzymatic hydrolysis of solid leather waste. J. Clean. Prod. *16*, 591–597.

Jian, S., Wenyi, T., and Wuyong, C. (2011). Kinetics of enzymatic unhairing by protease in leather industry. J. Clean. Prod. *19*, 325–331.

Kalaiarasi, K., and Sunitha, P.U. (2009). Optimization of alkaline protease production from Pseudomonas fluorescens isolated from meat waste contaminated soil. Afr. J. Biotechnol. *8*.

Kiran, I., Ilhan, S., Caner, N., Iscen, C.F., and Yildiz, Z. (2009). Biosorption properties of dried Neurospora crassa for the removal of Burazol Blue ED dye. Desalination *249*, 273–278.

Kolomaznik, K., Adamek, M., Andel, I., and Uhlirova, M. (2008). Leather waste—Potential threat to human health, and a new technology of its treatment. J. Hazard. Mater. *160*, 514–520.

Kopka, J. (2006). "Gas ChromatographyMass Spectrometry" in Plant Metabolomics (Biotechnology in Agriculture and Forestry).

Kumar, C.G., and Takagi, H. (1999). Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv. *17*, 561–594.

Kumari, B.L., and Rani, M.R. (2013). Characterization Studies on caseinolytic extracellular alkaline protease from a mutant bacillus licheniformis. Int J LifeSc Bt Pharm Res *2*, 284–289.

Lacorte, S., and Fernandez-Alba, A.R. (2006). Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food. Mass Spectrom. Rev. *25*, 866–880.

Lamb, G.N. (1981). Manual of veterinary laboratory technique. CIBA-Geigy Kenya 96–107.

Li, S., Li, J., Yi, J., and Shan, Z. (2010). Cleaner beam house processes trial on cattle sofa leather. J. Clean. Prod. *18*, 471–477.

Lineweaver, H., and Burk, D. (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. *56*, 658–666.

Lucas, M.S., Amaral, C., Sampaio, A., Peres, J.A., and Dias, A.A. (2006). Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of Candida oleophila. Enzyme Microb. Technol. *39*, 51–55.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiol. Mol. Biol. Rev. *66*, 506–577.

Masih, H., and Singh, S. (2014). Degradation of Keratinous Waste Products by Keratinolytic Bacteria Isolated from soil. Int. J. Eng. Comput. Sci. *3*, 7588–7595.

Matouq, M., Al-Anber, Z., Susumu, N., Tagawa, T., and Karapanagioti, H. (2014). The kinetic of dyes degradation resulted from food industry in wastewater using high frequency of ultrasound. Sep. Purif. Technol. *135*, 42–47.

Michaelis, L., and Menten, M.L. (1913). Die kinetik der invertinwirkung. Biochem Z *49*, 333–369.

Moturi, B., and Charya, M.S. (2009). Decolourisation of Crystal Violet and Malachite Green By Fungi. Sci. World J. *4*.

Murugesan, K., Nam, I.-H., Kim, Y.-M., and Chang, Y.-S. (2007). Decolorization of reactive dyes by a thermostable laccase produced by Ganoderma lucidum in solid state culture. Enzyme Microb. Technol. *40*, 1662–1672.

Muyonga, J.., Cole, C.G.., and Duodu, K.. (2004a). Extraction and physico-chemical characterisation of Nile perch (Lates niloticus) skin and bone gelatin. Food Hydrocoll. *18*, 581–592.

Muyonga, J.H., Cole, C.G.B., and Duodu, K.G. (2004b). Characterisation of acid soluble collagen from skins of young and adult Nile perch (Lates niloticus). Food Chem. *85*, 81–89.

Nabil, G.M., El-Mallah, N.M., and Mahmoud, M.E. (2014). Enhanced decolorization of reactive black 5 dye by active carbon sorbent-immobilized-cationic surfactant (AC-CS). J. Ind. Eng. Chem. *20*, 994–1002.

Ng, T.W., Cai, Q., Wong, C.-K., Chow, A.T., and Wong, P.-K. (2010). Simultaneous chromate reduction and azo dye decolourization by Brevibacterium casei: Azo dye as electron donor for chromate reduction. J. Hazard. Mater. *182*, 792–800.

Ngow, H.A., and Wan Khairina, W.M.N. (2013). Bacillus cereus endocarditis in native aortic valve. J. Infect. Chemother. *19*, 154–157.

Nigam, P. (2013). Microbial Enzymes with Special Characteristics for Biotechnological Applications. Biomolecules *3*, 597–611.

Oroz-Guinea, I., and García-Junceda, E. (2013). Enzyme catalysed tandem reactions. Curr. Opin. Chem. Biol. *17*, 236–249.

Pengthamkeerati, P., Satapanajaru, T., Chatsatapattayakul, N., Chairattanamanokorn, P., and Sananwai, N. (2010). Alkaline treatment of biomass fly ash for reactive dye removal from aqueous solution. Desalination *261*, 34–40.

Punitha, V., Kannan, P., Saravanabhaven, S., Thanikaivelan, P., Rao, J., Saravanan, P., and Nair, B. (2008). Enzymatic removal of melanin in enzyme based dehairing and fiber opening. J. Am. Leather Chem. Assoc. *103*, 203–208.

Rao, J.R., Chandrababu, N.K., Muralidharan, C., Nair, B.U., Rao, P.G., and Ramasami, T. (2003). Recouping the wastewater: a way forward for cleaner leather processing. J. Clean. Prod. *11*, 591–599.

Reemtsma, T., and Jekel, M. (2006). Organic pollutants in the water cycle: properties, occurrence, analysis and environmental relevance of polar compounds. (John Wiley & Sons).

Reese, E.T., Siu, R.G.., and Levinson, H.S. (1950). The biological degradation of cellulose derivatives and its regulation to the mechanism of cellular hydrolysis. J Bacteriol *59*, 485–489.

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

Rose, C., Suguna, L., Rajini, R., Samivelu, N., Rathinasamy, V., Ramalingam, S., Iyappan, K., Parvathaleswara, T., and Ramasami, T. (2004). Process for lime and sulfide free unhairing of skins or hides using animal and/or plant enzymes.

Rustad, T., Storrø, I., and Slizyte, R. (2011). Possibilities for the utilisation of marine by-products: Utilisation of marine by-products. Int. J. Food Sci. Technol. *46*, 2001–2014.

Safa, Y., and Bhatti, H.N. (2011). Biosorption of Direct Red-31 and Direct Orange-26 dyes by rice husk: Application of factorial design analysis. Chem. Eng. Res. Des. *89*, 2566–2574.

Salas-Veizaga, D.M., Morales-Belpaire, I., and Terrazas-Siles, E. (2013). Evaluation of the genotoxic potential of reactive black 5 solutions subjected to decolorizing treatments by three fungal strains. Ecotoxicol. Environ. Saf. *89*, 125–129.

Saran, S., Mahajan, R.V., Kaushik, R., Isar, J., and Saxena, R.K. (2013). Enzyme mediated beam house operations of leather industry: a needed step towards greener technology. J. Clean. Prod. *54*, 315–322.

Sarethy, I.P., Saxena, Y., Kapoor, A., Sharma, M., Sharma, S.K., Gupta, V., and Gupta, S. (2011). Alkaliphilic bacteria: applications in industrial biotechnology. J. Ind. Microbiol. Biotechnol. *38*, 769–790.

Saxena, S., Verma, J., Shikha, and Raj Modi, D. (2014). RAPD-PCR and 16S rDNA phylogenetic analysis of alkaline protease producing bacteria isolated from soil of India: Identification and detection of genetic variability. J. Genet. Eng. Biotechnol. *12*, 27–35.

Schultz, A.W., Wang, J., Johnson, C.H., Yannone, S.M., Patti, G.J., and Siuzdak, G. (2013). Liquid chromatography quadrupole time-of-flight mass spectrometry characterization of metabolites guided by the METLIN database. Nat. Protoc. *8*, 451–460.

Shah, P.D., Dave, S.R., and Rao, M.S. (2012). Enzymatic degradation of textile dye Reactive Orange 13 by newly isolated bacterial strain Alcaligenes faecalis PMS-1. Int. Biodeterior. Biodegrad. *69*, 41–50.

Shahidi, F. (1994). Seafood processing by-products. In F. Shahidi, & J. R. Botta (Eds.), Seafoods chemistry, processing, technology and quality (Springer US).

Shivasharanappa, K., Hanchinalmath, J.V., Sundeep, Y.S., Borah, D., and Talluri, V.P. (2014). Optimization and production of Alkaline Proteases from Agro byproducts using a novel Trichoderma Viridiae strain VPG 12, isolated from agro soil. Int. Lett. Nat. Sci. *14*, 77–84.

Singh, M.P., Vishwakarma, S.K., and Srivastava, A.K. (2013). Bioremediation of Direct Blue 14 and Extracellular Ligninolytic Enzyme Production by White Rot Fungi: Pleurotus Spp. BioMed Res. Int. *2013*, 1–4.

Sivakumar, V., and Rao, P.G. (2003). Studies on the use of power ultrasound in leather dyeing. Ultrason. Sonochem. *10*, 85–94.

Smibert, R.M., and Krieg, N.R. (1994). Chapter 25, Phenotypic characterization. In Methods for General and Molecular Bacteriology., (American Society for Microbiology Press, Washington DC), pp. 611–651.

Soares, G.M., essoa Amorim, M.T.P., Hrdina, R., and Costa-Ferreira, M. (2002). Studies on the biotransformation of novel disazo dyes by laccase. Process Biochem. *37*, 581–587.

Song, J., Zou, W., Bian, Y., Su, F., and Han, R. (2011). Adsorption characteristics of methylene blue by peanut husk in batch and column modes. Desalination *265*, 119–125.

Taylor, M.M., Cabeza, L.F., Dimaio, G.L., Brown, E.M., Marmer, W.N., Carrio, R., Celma, P.J., and Cot, J. (1998). Processing of leather waste: pilot scale studies on chrome shavings. 1. isolation and characterization of protein products and separation of chrome cake. J Am Leather Chem Assoc *93*, 61–82.

Thanikaivelan, P., Rao, J.R., Nair, B.U., and Ramasami, T. (2004). Progress and recent trends in biotechnological methods for leather processing. Trends Biotechnol. *22*, 181–188.

Tsuchida, O., Yamagata, Y., Ishizuka, T., Arai, T., Yamada, J.-I., Takeuchi, M., and Ichishima, D.E. (1986). An alkaline proteinase of an alkalophilicBacillus sp. Curr. Microbiol. *14*, 7–12.

Wang, J., Gao, F., Liu, Z., Qiao, M., Niu, X., Zhang, K.-Q., and Huang, X. (2012). Pathway and Molecular Mechanisms for Malachite Green Biodegradation in Exiguobacterium sp. MG2. PLoS ONE *7*, 1–10.

Wanyonyi, W.C., Onyari, J.M., and Shiundu, P.M. (2013). Adsorption of Methylene Blue Dye from Aqueous Solutions Using Eichhornia crassipes. Bull. Environ. Contam. Toxicol. *91*, 362–366.

Wanyonyi, W.C., Onyari, J.M., Shiundu, P.M., and Mulaa, J.F. (2014a). Enzymatic Decolorization of Malachite Green Dye by a Newly Isolated Bacillus Cereus Strain wwcp1. J. Environ. Sci. Toxicol. Food Technol. *8*, 58–64.

Wanyonyi, W.C., Onyari, J.M., and Shiundu, P.M. (2014b). Adsorption of Congo Red Dye from Aqueous Solutions Using Roots of Eichhornia Crassipes: Kinetic and Equilibrium Studies. Energy Procedia *50*, 862–869.

Xingzu, W., CHENG, X., Dezhi, S., and Qi, H. (2008). Biodecolorization and partial mineralization of Reactive Black 5 by a strain of Rhodopseudomonas palustris. J. Environ. Sci. *20*, 1218–1225.

Younes, S.B., and Sayadi, S. (2013). Detoxification of Indigo carmine using a combined treatment via a novel trimeric thermostable laccase and microbial consortium. J. Mol. Catal. B Enzym. *87*, 62–68.

Zhang, A., Sun, H., Wang, P., Han, Y., and Wang, X. (2012a). Modern analytical techniques in metabolomics analysis. The Analyst *137*, 293–300.

Zhang, C., Diao, H., Lu, F., Bie, X., Wang, Y., and Lu, Z. (2012b). Degradation of triphenylmethane dyes using a temperature and pH stable spore laccase from a novel strain of Bacillus vallismortis. Bioresour. Technol. *126*, 80–86.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and Van't Riet, K. (1990). Modeling of the bacterial growth curve. Appl. Environ. Microbiol. *56*, 1875–1881.

INTERNET REFERENCES

http://mrbayes.net. Retrieved on 10 June 2014

http://tree.bio.ed.ac.uk/. Retrieved on 16 January 2014

http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/ Retrieved on 14 July 2014

*http://www.nation.co.ke/business/Machakos-to-host-planned-leather-city/-/996/2588514/-/8w6otp/-/index.htm*l. Retrieved on 14 January 2015

http://www.ncbi.nlm.nih.gov. Retrieved on 10 June 2014

http://www.ncbi.nlm.nih.gov/BLAST. Retrieved on 10 June 2014

http://www.acdlabs.com. Retrieved on 08 June 2015

APPENDICES

Appendix I : 16S rDNA sequences for the bacteria isolates *1-p, 2-p, 3-p* and *4-p*.

>Isolate *1-p*. Gene bank accession number KM201428

```
GCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCG
AAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAAC
GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCG
TGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTGAGGGAAGAACAAGTGCTAGTTGAATAAGCTG
GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATG
TGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAAGGCGACTTTCTGGTCTGTAACTG
ACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA
GTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTC
GGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAG
GCTGCAACTCGCCTACAAGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAA
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>Isolate *2-p*. Gene bank accession number KM201429

```
CTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTT
CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGC
TGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCA
TGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAAGGCGACTTTCTGGTCTGTAA
CTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCGTAAACG
ATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCT
CCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC
AATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGAT
TGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGG
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>Isolate *3-p*. Gene bank accession number KM201430

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CCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGA
AATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACG
GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGCTTTCGGGTCGTAAAACTTTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGG
CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCA
CGGCTCAACCGTGGAGGGTCATTTGGAAACTGGGAGACTTGAGTGCAGAAGAGGGAAAGTGGAATTCCAT
GTGTAGCGGGGAAATGCGTTAGAGATTATGGAAGGAACACCAGTGGCGAAAGGCGACTTTCTGGTTCTG
TAAACTGACACTGAGGCGCGAAAAGCGTGGGGAGCAAACGGATTTAGATTCCCTGGTAGTCCACGCCGT
AAAACGATGAGTGCTAAAGTGTTAGAGGGTTTCCGCCCCTTAAGTGCTGAAGTTTAACGCATTAAGCACT
CCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGAT
AGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCAATCATTAAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTC
AGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCG
CGGTGAATACGT
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>isolate *4-p.*

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AAGATGTAGGTGAGTTCCACTTATGTGAGTACCTTAGATCGCAGAGTCGCGAAGTAACCAAGAATAGGGC
CGAAACGGAGCTGGTTATTAACACCGAGACCTACGGCAAATCTTAAACTAGAGAAAGTTAAATGGACAAG
GCAGTGGGCAGGTGTCGAAGTGATCGTGAATTTGGTAATGGGAACGAGAACACCCTGATCTTACGCAAGG
ATTAAAGAGCCTTAGCCTCTTATGTAACTAACGGGCGGAGGGTGAGGAACACGTCGAATCATCTGCCCAT
AAGACCTGGGATAACTCCGGGAAACCGGGCGTACTACCGGATAACATTTGAAACGGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCGGCTGCGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGCTTTCGGGACGTAAAACTCTGTTGTTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCAC
CTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTTCTTAAGTCTGATGTGAAAAGCCCAC
GGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAGACTTGAGTGCAGAAGAAGGAAAGTGGAATTCCATG
TTGTAGCGGTGAAATGCGTAAAGATATGGGAGGAACAACCAGTGGCGAAGGCGACTTTCTGGTCTTGTAA
ACTGACACTGAAGGCGCGAAAGCGTGGTGAAGCAAACAGGGATTTAAGAATACCCTGGTAGTCCACGCCG
TAAAACGATGAAGTGCTAAGTGTTAGAAGGGGGTTTCCCGCCCCTTTTAGTGCCTTGAAAGTTAAACGCGC
ATTTAAAGCAACTTCCCGCCCCTGGGGGAAGTACACCGGCCGCCAAAGGCCTGAAAAACCTCTAAAAAGGA
AATTTTGAAAACGGCC
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Appendix II: GC-MS chromatograms for Malachite green dyeMetabolites

Appendix IIa : Total GC-MS chromatogram for Malachite green dyeMetabolites

le   : C:\HPCHEM\1\DATA\14MA-MG2.D
erator   : MADADI
quired   : 15 Mar 2015   5:10   using AcqMethod TPH-MA15
strument :   GC/MS Ins
nple Name: WICKLIFFE MG METABOLITE 1 TPH-MA15
sc Info   : madadi
al Number: 6
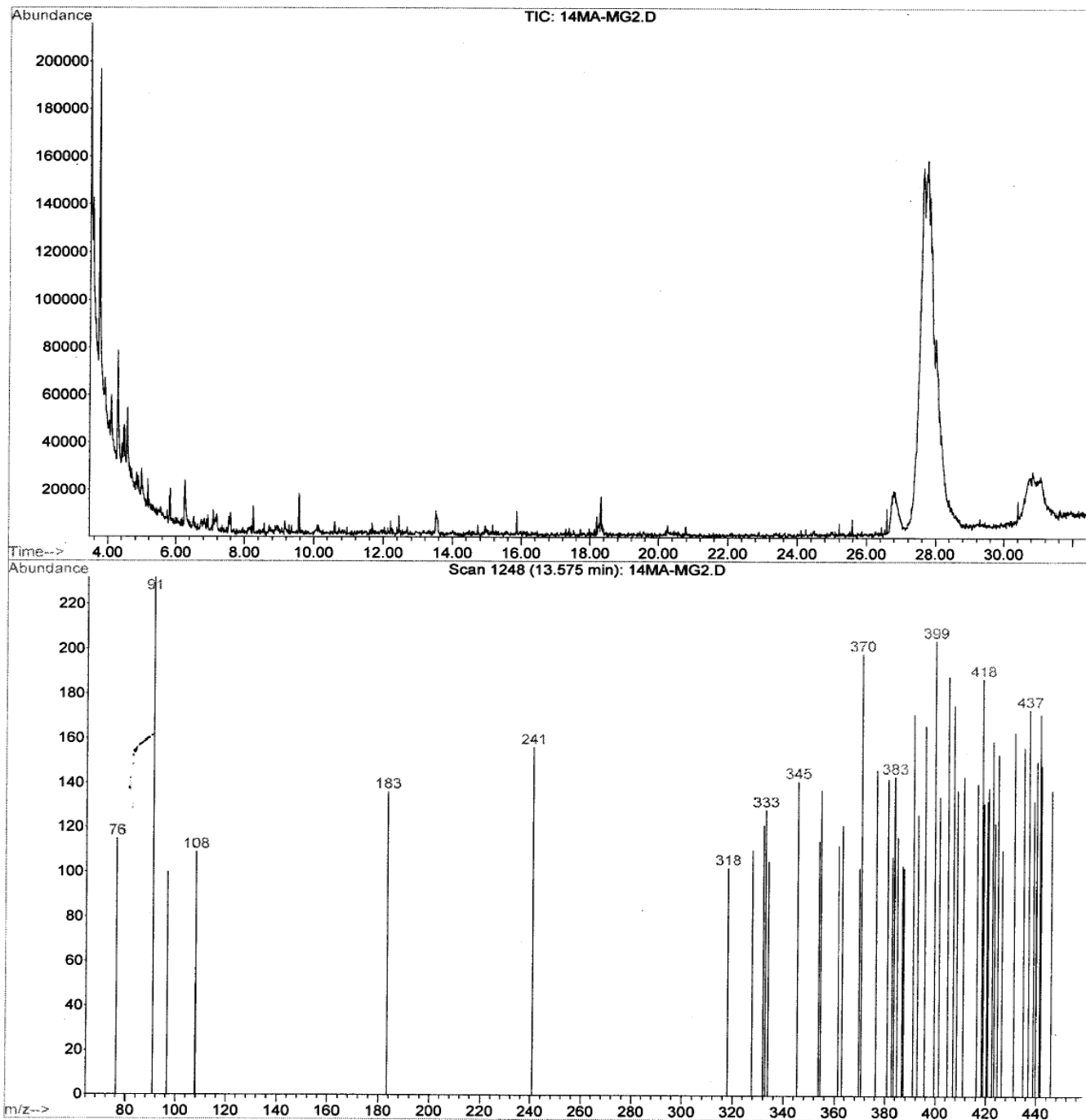
Appendix IIb: Leuco Malachite Green (LMG) also known as p,p'-Benzylidenebis(N,N-dimethylaniline)

Unknown; InLib=387
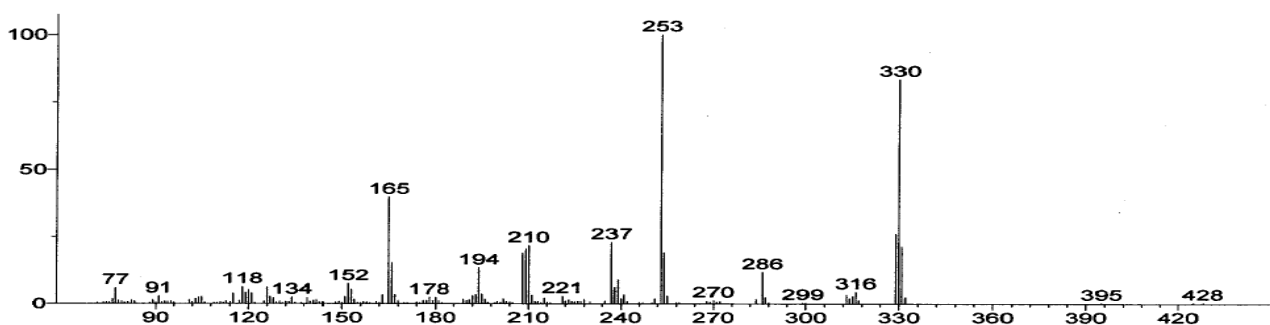
(Text File) Scan 2927 (27.746 min): 14MA-MG2.D

Name: Scan 2927 (27.746 min): 14MA-MG2.D
MW: N/A ID#: 6092 DB: Text File
Comment: WICKLIFFE MG METABOLITE 1 TPH-MA15
10 largest peaks:
253 999 | 330 833 | 165 398 | 329 259 | 237 230 | 210 217 | 331 213 | 209 204 | 254 190 | 208 188 |

Hit 1 : p,p'-Benzylidenebis(N,N-dimethylaniline)
C23H26N2; MF: 873; RMF: 882; Prob 97.2%; CAS: 129-73-7; Lib: mainlib; ID: 166434.
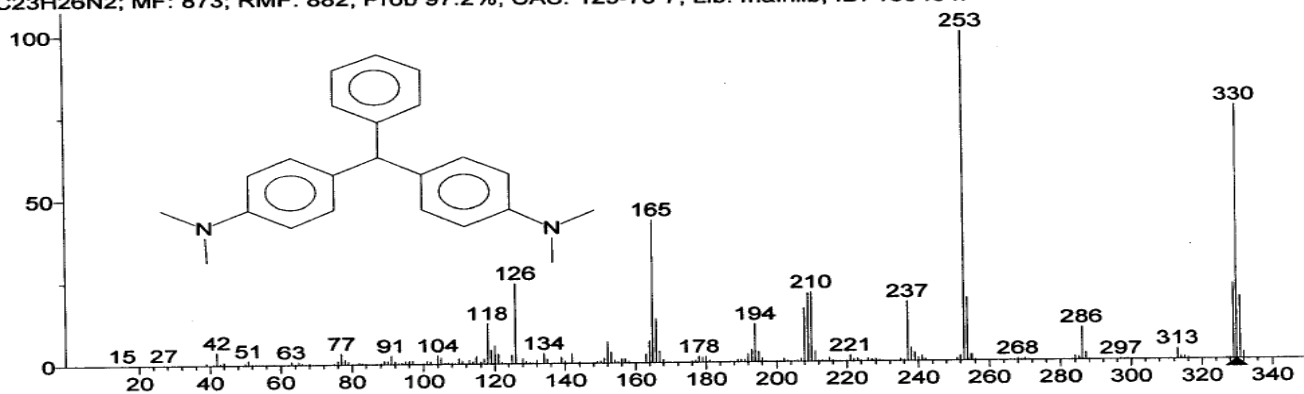
(mainlib) p,p'-Benzylidenebis(N,N-dimethylaniline)
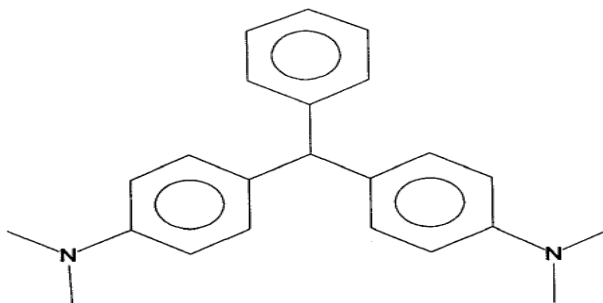
Name: p,p'-Benzylidenebis(N,N-dimethylaniline)
Formula: C23H26N2
MW: 330 CAS#: 129-73-7 NIST#: 341216 ID#: 166434 DB: mainlib
Other DBs: Fine, TSCA, RTECS, HODOC, NIH, EINECS
Contributor: NIST Mass Spectrometry Data Center
10 largest peaks:
253 999 | 330 769 | 165 432 | 126 242 | 329 228 | 210 210 | 209 206 | 254 190 | 331 189 | 237 180 |

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Appendix IIc: Methanone, [4-(dimethylamino)phenyl] phenyl

Unknown; InLib=-464
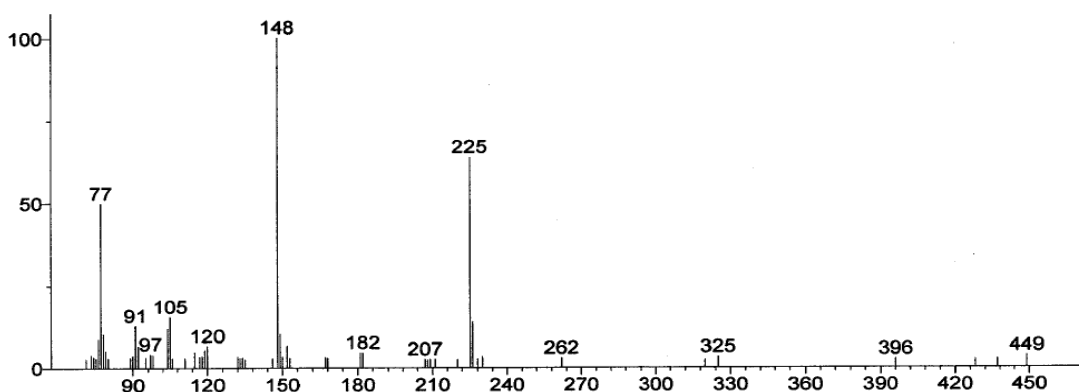
(Text File) Scan 2816 (26.809 min): 14MA-MG2.D

Name: Scan 2816 (26.809 min): 14MA-MG2.D
MW: N/A ID#: 6093 DB: Text File
Comment: WICKLIFFE MG METABOLITE 1 TPH-MA15
10 largest peaks:
148 999 | 225 637 | 77 498 | 105 155 | 226 138 | 91 128 | 104 119 | 78 103 | 149 102 | 76 87 |

Hit 1 : Methanone, [4-(dimethylamino)phenyl]phenyl-
C15H15NO; MF: 688; RMF: 772; Prob 76.8%; CAS: 530-44-9; Lib: replib; ID: 19952.
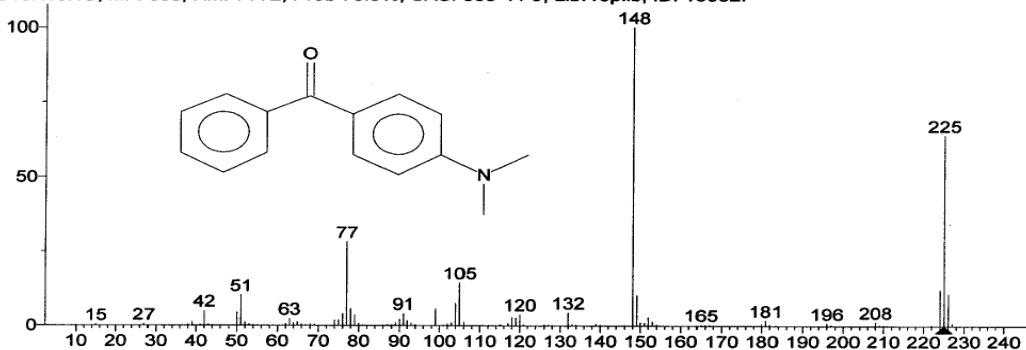
(replib) Methanone, [4-(dimethylamino)phenyl]phenyl-
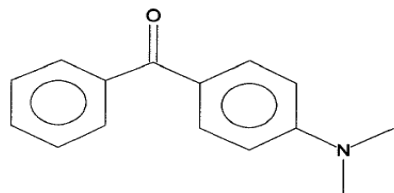
Name: Methanone, [4-(dimethylamino)phenyl]phenyl-
Formula: $C_{15}H_{15}NO$
MW: 225 CAS#: 530-44-9 NIST#: 70342 ID#: 19952 DB: replib
Other DBs: Fine, TSCA, HODOC, NIH, EINECS
Contributor: RADIAN CORP
10 largest peaks:
148 999 | 225 640 | 77 281 | 105 144 | 224 120 | 226 107 | 51 102 | 149 101 | 104 73 | 78 57 |

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Appendix IId: Phosphinic acid, bis[p-(dimethylamino)phenyl], methyl ester

(Text File) Scan 2627 (25.214 min): 14MA-MG2.D
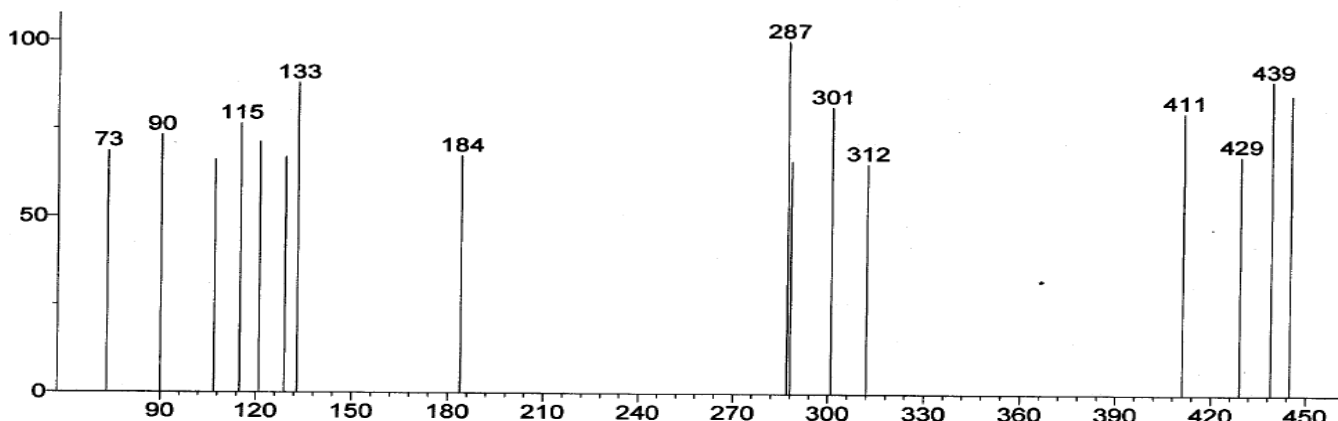
10 largest peaks:
287 999 | 439 890 | 133 877 | 445 851 | 301 813 | 411 800 | 115 762 | 90 730 | 121 710 | 73 685 |
Synonyms:
no synonyms.

Hit 1 : Phosphinic acid, bis[p-(dimethylamino)phenyl]-, methyl ester
C17H23N2O2P; MF: 327; RMF: 357; Prob 53.6%; CAS: 18593-23-2; Lib: mainlib; ID: 180951.

(mainlib) Phosphinic acid, bis[p-(dimethylamino)phenyl]-, methyl ester
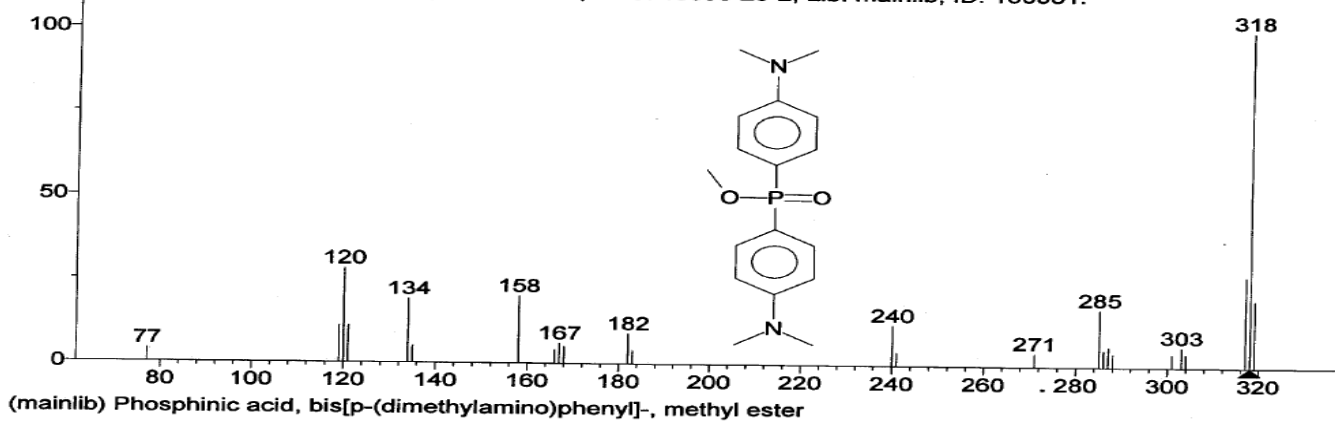
10 largest peaks:
318 999 | 120 280 | 317 270 | 158 200 | 319 200 | 134 190 | 285 170 | 240 120 | 119 110 | 121 110 |
Synonyms:
1.Methyl bis[4-(dimethylamino)phenyl]phosphinate #

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Appendix IIe: (E)-2-Hydroxy-4'-dimethylamino-stilbene

Unknown; InLib=-1682
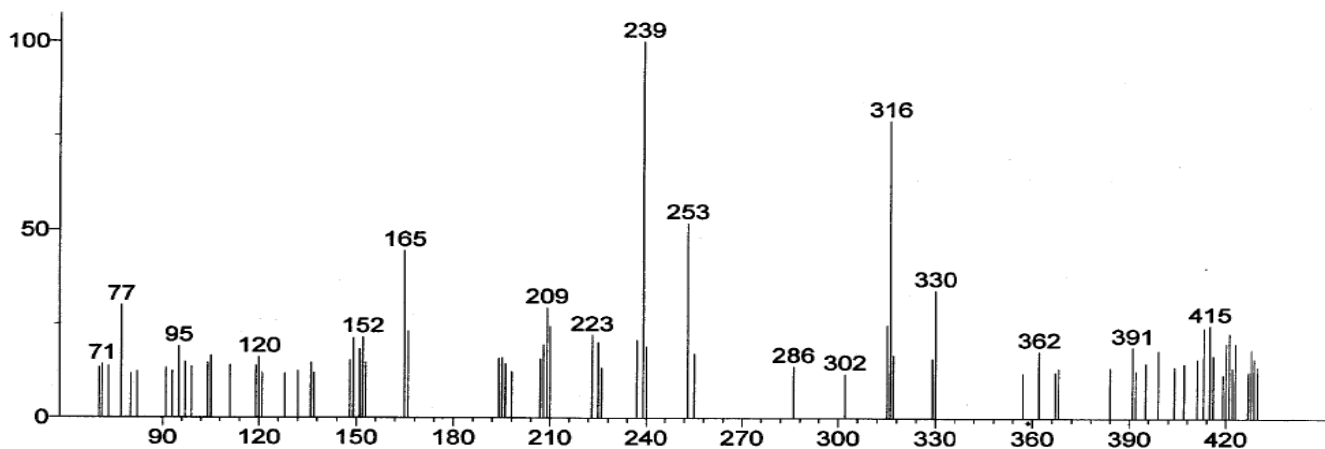
(Text File) Average of 27.291 to 27.291 min.: 14MA-MG2.D

10 largest peaks:
239 999 | 316 789 | 253 518 | 165 444 | 330 340 | 77 299 | 209 293 | 415 248 | 315 247 | 210 242 |
Synonyms:
no synonyms.

Hit 1 : (E)-2-Hydroxy-4'-dimethylamino-stilbene
C16H17NO; MF: 401; RMF: 806; Prob 23.3%; CAS: 110983-42-1; Lib: mainlib; ID: 161711.
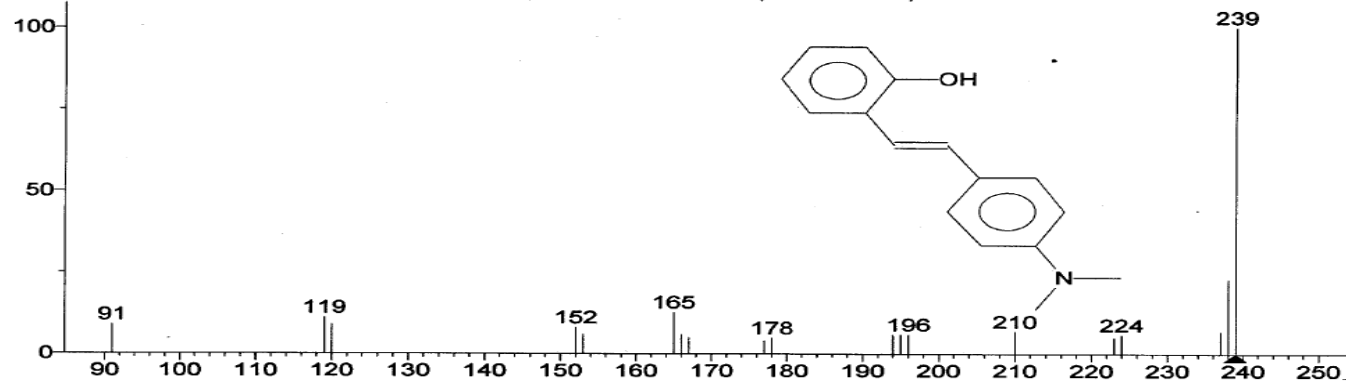
(mainlib) (E)-2-Hydroxy-4'-dimethylamino-stilbene
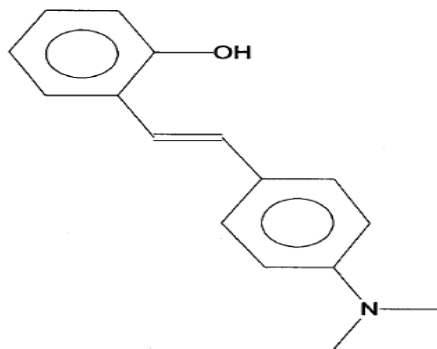
10 largest peaks:
239 999 | 238 229 | 165 128 | 119 111 | 91 89 | 120 89 | 152 81 | 210 69 | 237 69 | 196 60 |
Synonyms:
1.2-((E)-2-[4-(Dimethylamino)phenyl]ethenyl)phenol #

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Appendix IIf: Benzenemethanamine, N-phenyl

Unknown; InLib=-2085
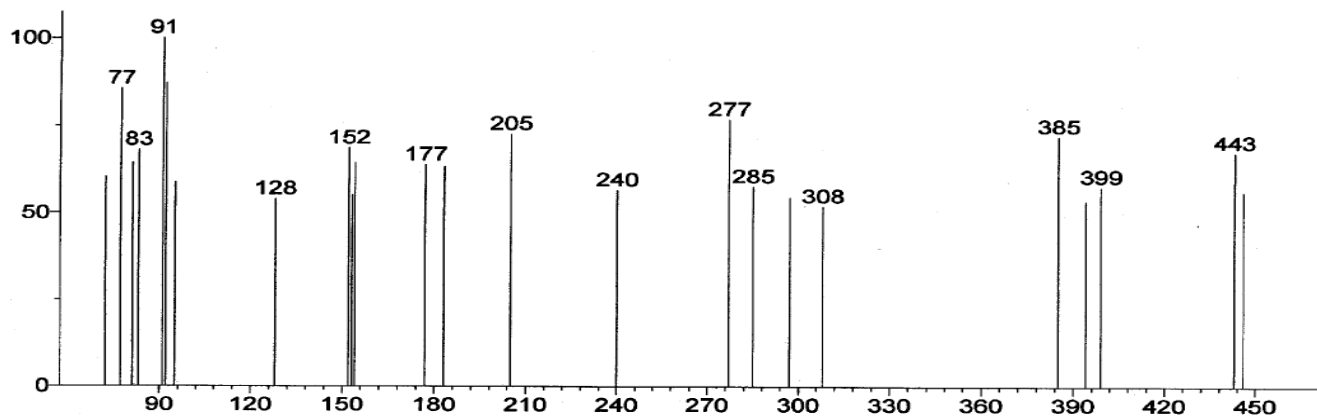
(Text File) Scan 1468 (15.426 min): 14MA-MG3.D

10 largest peaks:
    91 999 | 92 870 | 77 854 | 277 767 | 205 726 | 385 720 | 152 685 | 83 679 | 443 674 | 81 643 |
Synonyms:
no synonyms.

Hit 1 : Benzenemethanamine, N-phenyl-
C13H13N; MF: 288; RMF: 471; Prob 6.73%; CAS: 103-32-2; Lib: mainlib; ID: 54092.
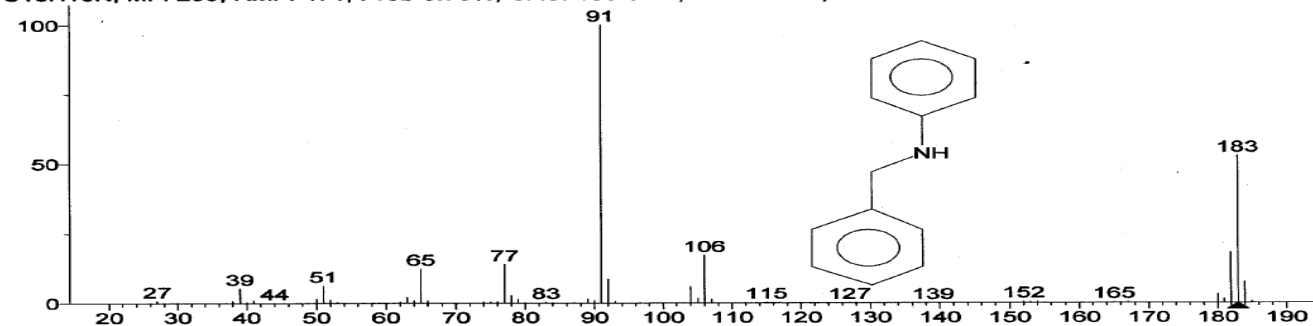
(mainlib) Benzenemethanamine, N-phenyl-
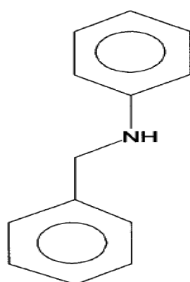
10 largest peaks:
    91 999 | 183 527 | 182 181 | 106 172 | 77 140 | 65 124 | 92 87 | 184 75 | 51 63 | 104 60 |
Synonyms:
1.Benzylamine, N-phenyl-
2.Aniline, N-benzyl-
3.Benzenamine, N-(phenylmethyl)-
4.Benzylaniline
5.Benzylphenylamine
6.N-Benzylaniline
7.N-Monobenzylaniline
8.N-Phenylbenzylamine
9.Phenylbenzylamine
10.N-Phenylbenzenemethanamine
11.N-Benzyl-N-phenylamine #

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Appendix III: GC-MS chromatograms for Reactive Black 5 Metabolites

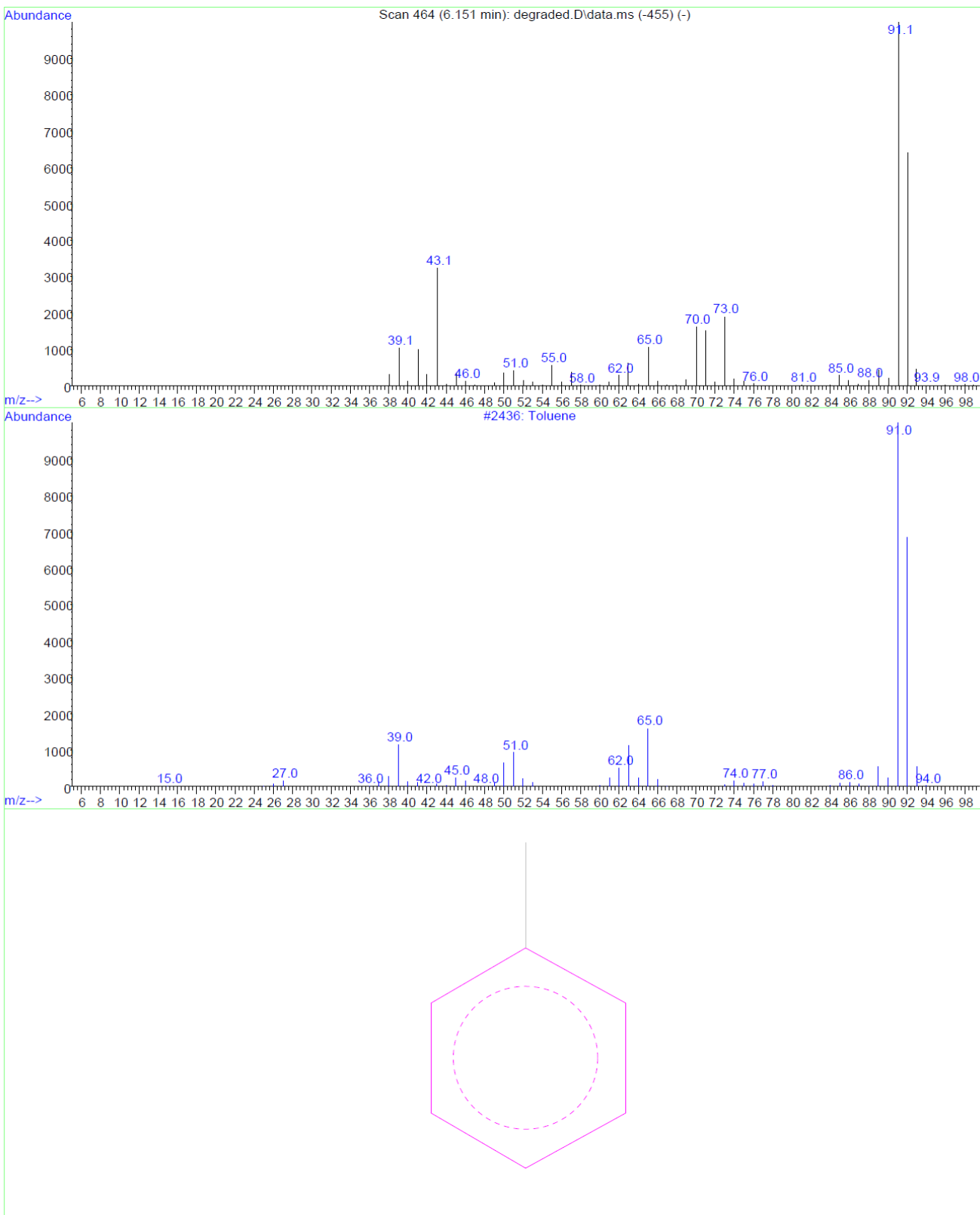**Appendix IIIa:** Total Reactive Black 5 Metabolites Chromatogram
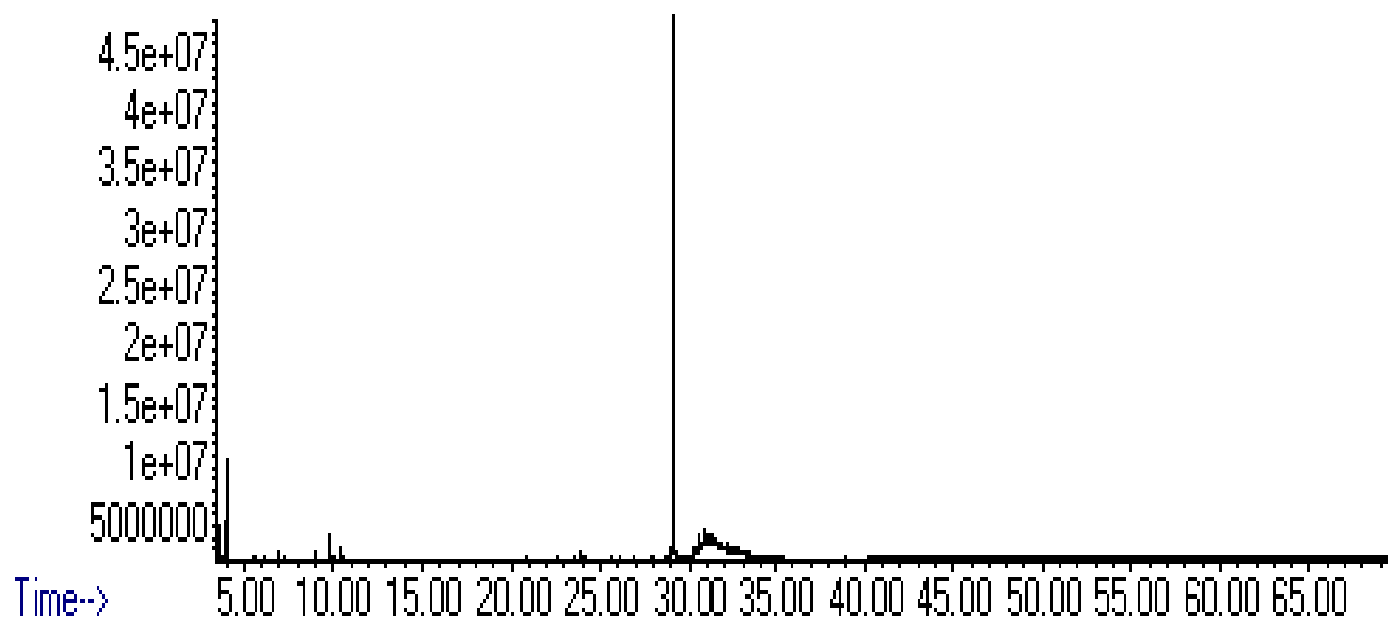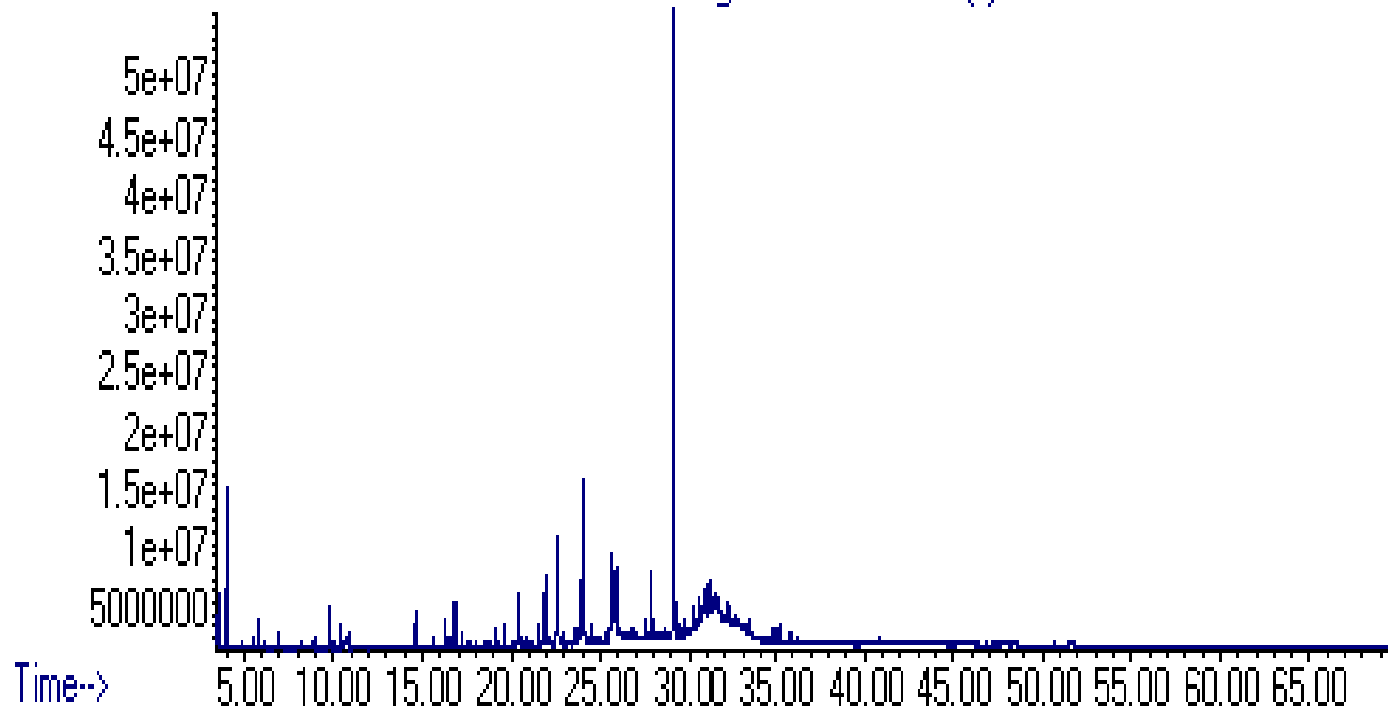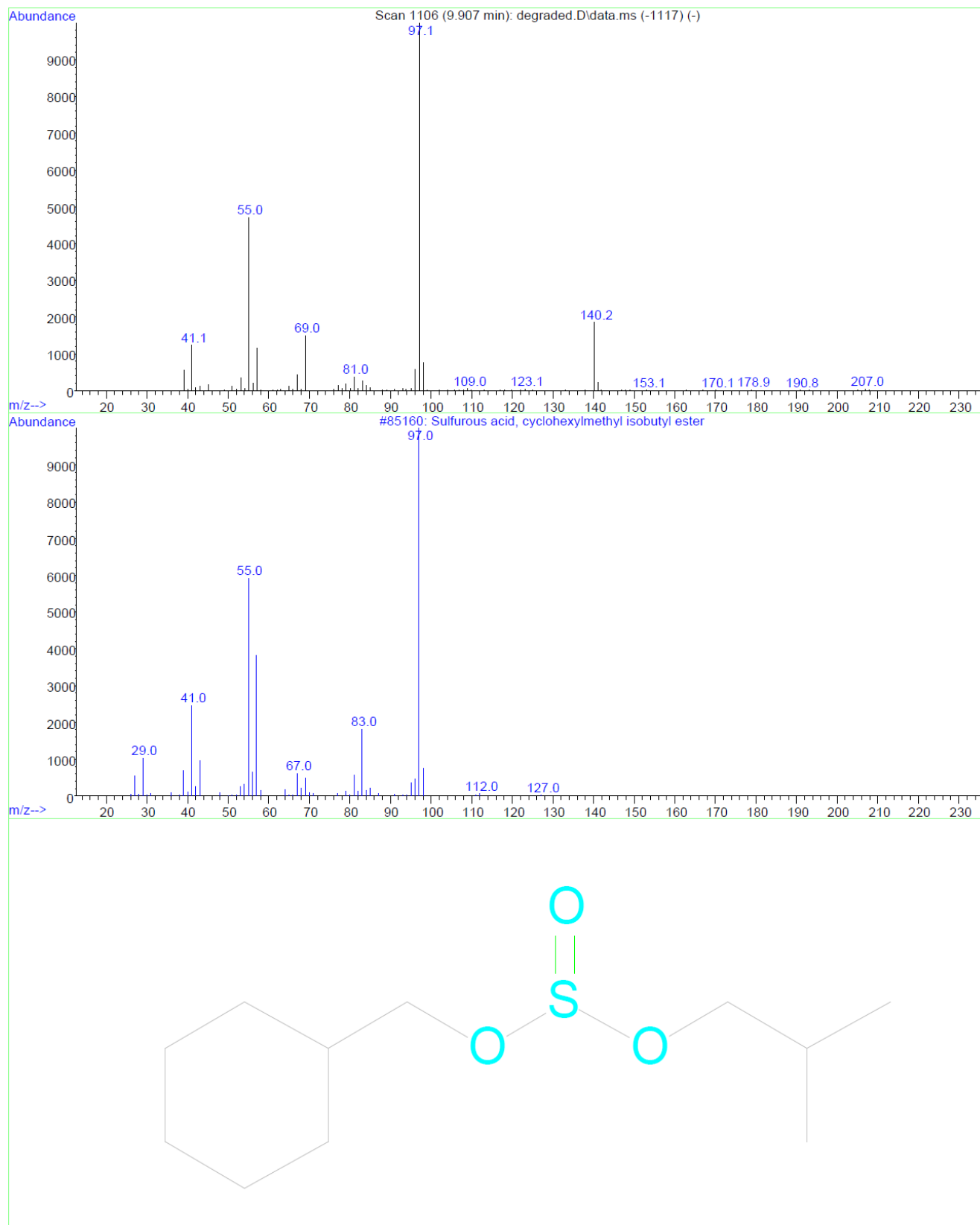
Appendix IIIb: Cyclohexane
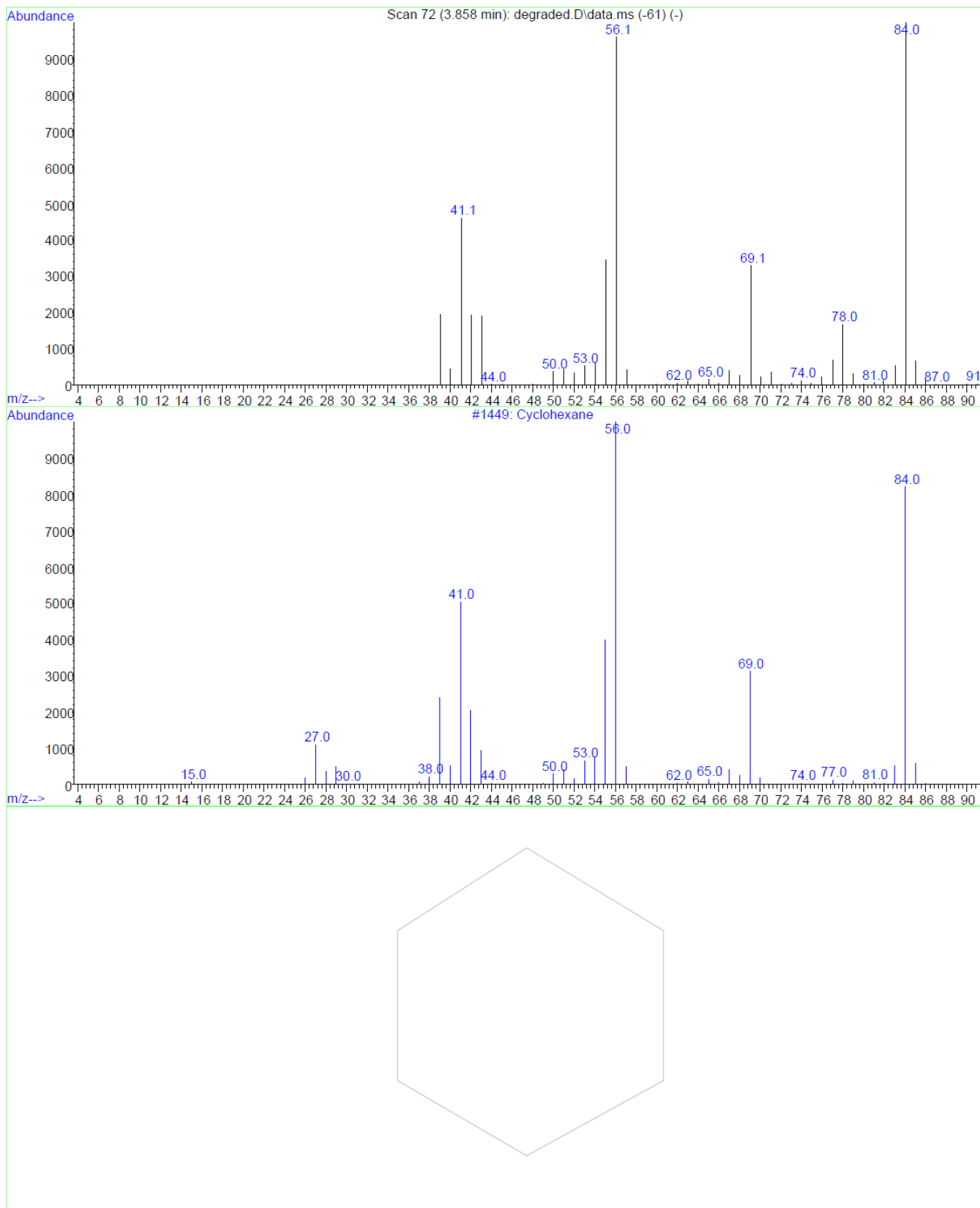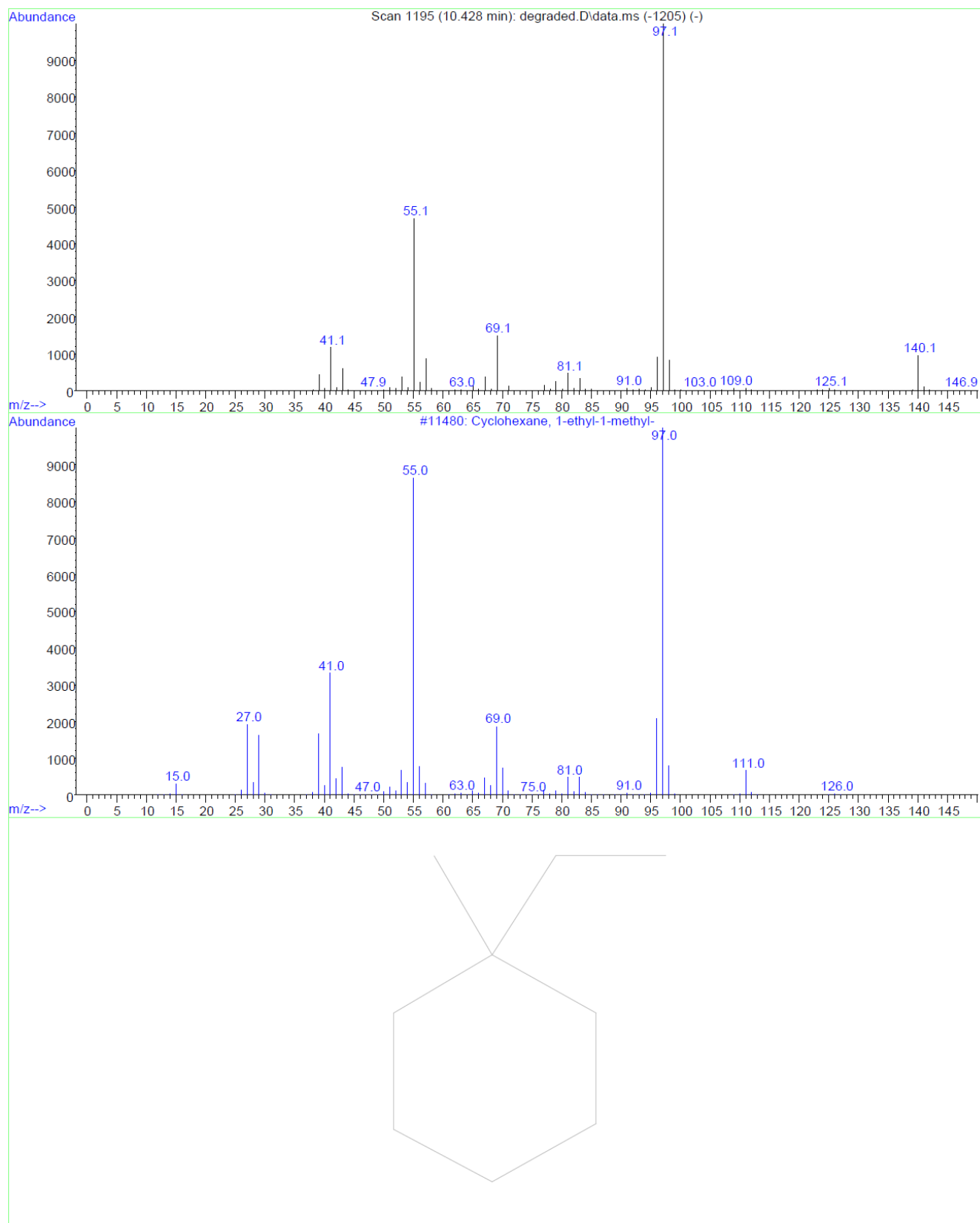
Appendix IIIc: Methyl Cyclohexane
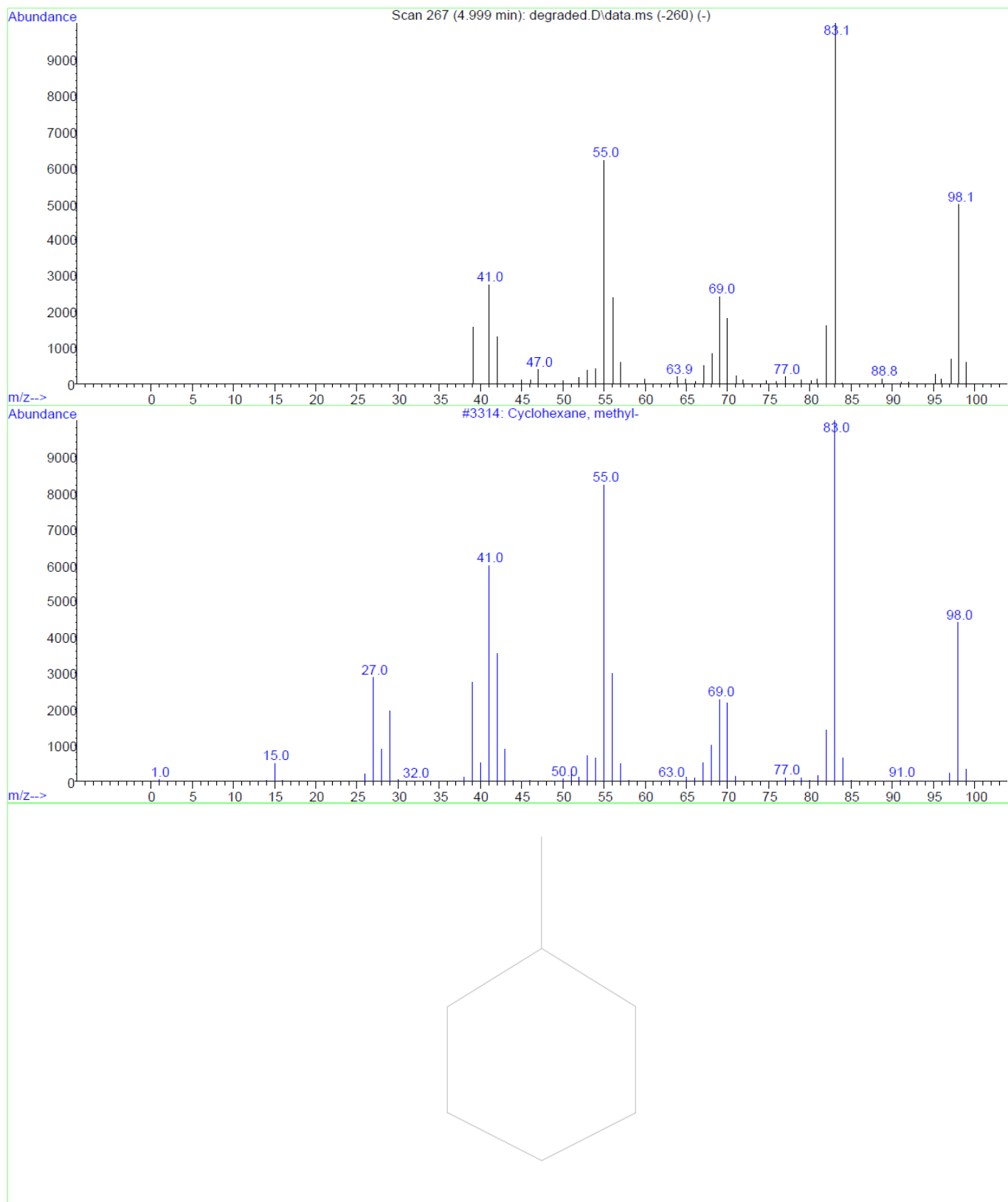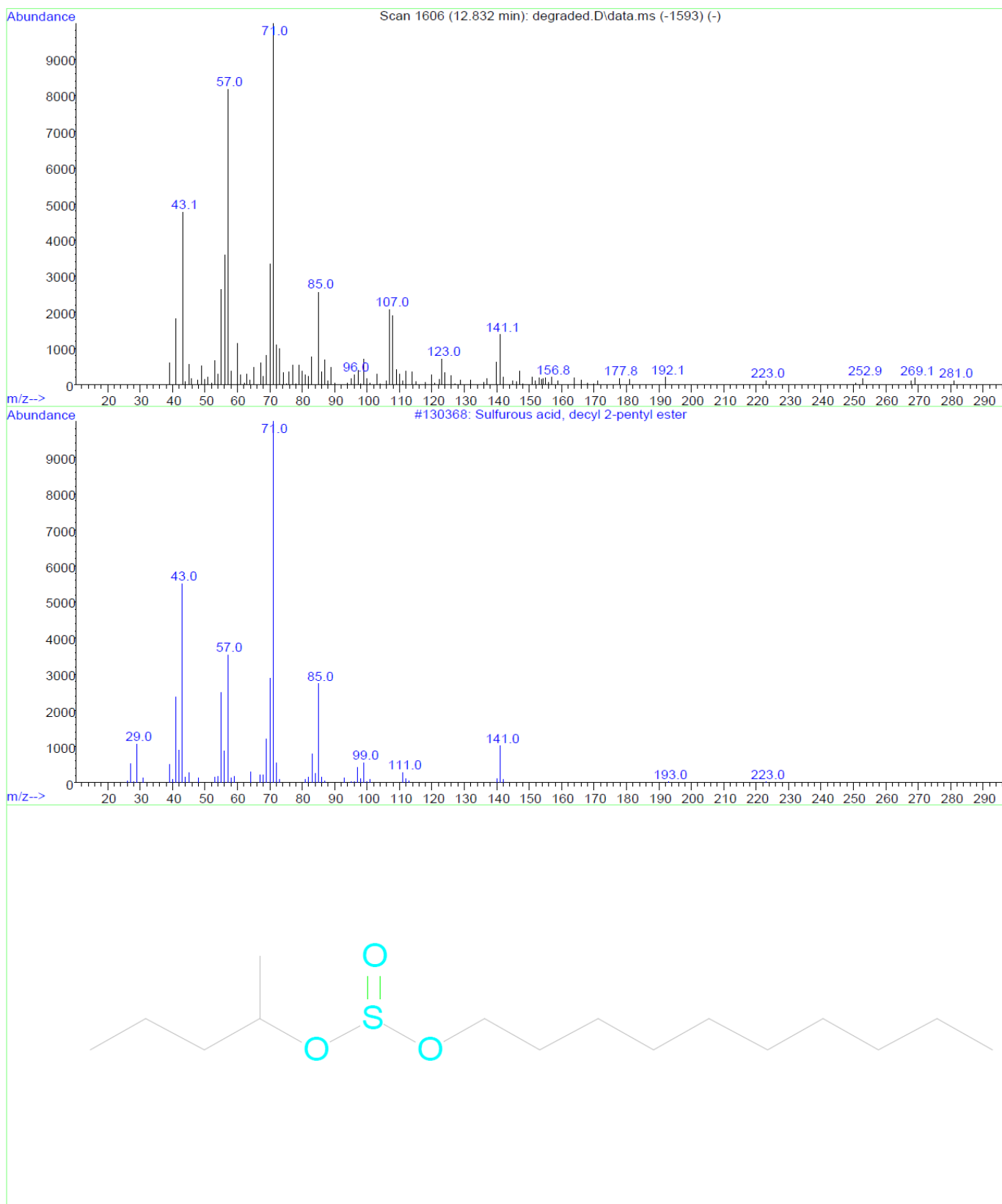
Appendix IIId: Toluene

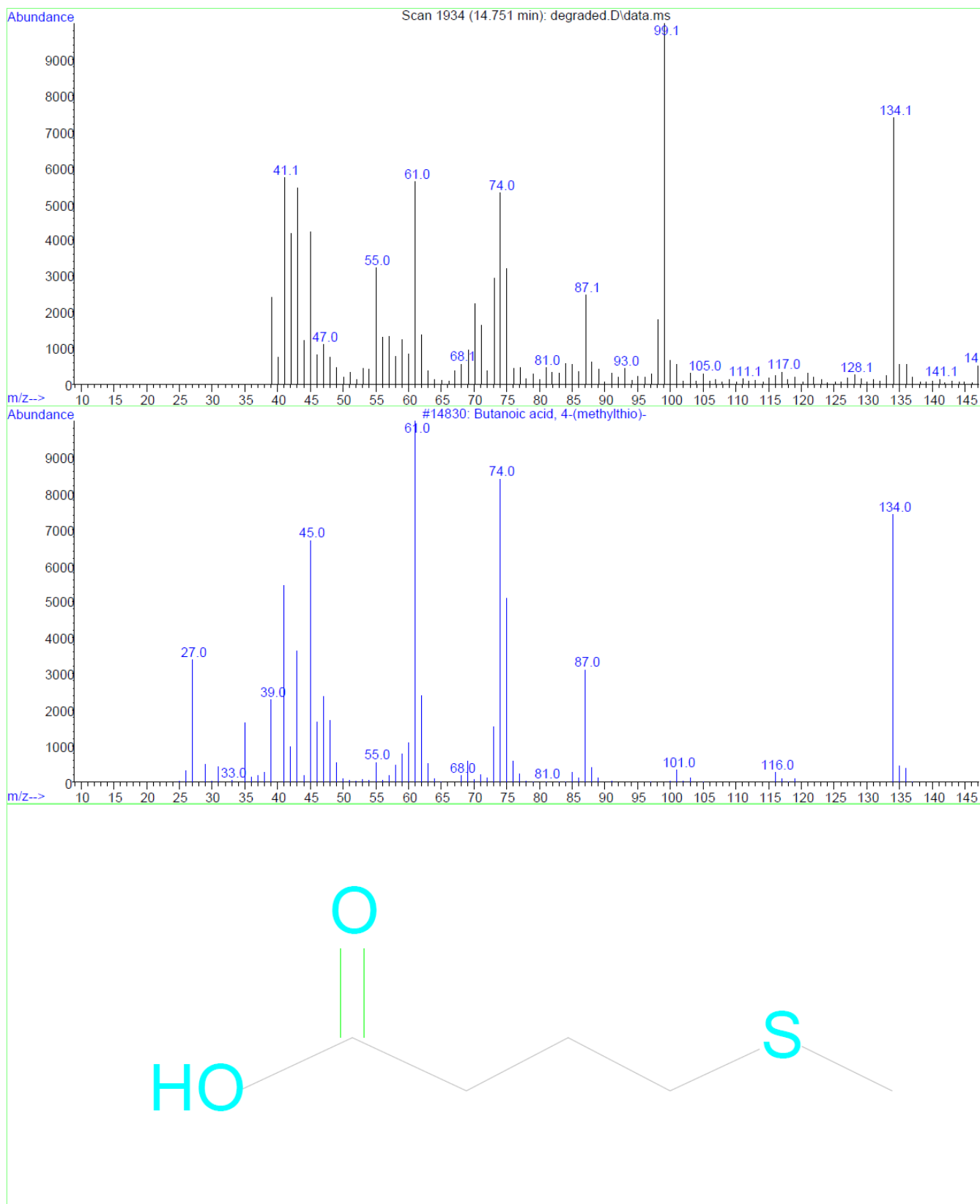**Appendix IIIe**: Sulfurous acid, cyclohexyl methyl isobutyl ester

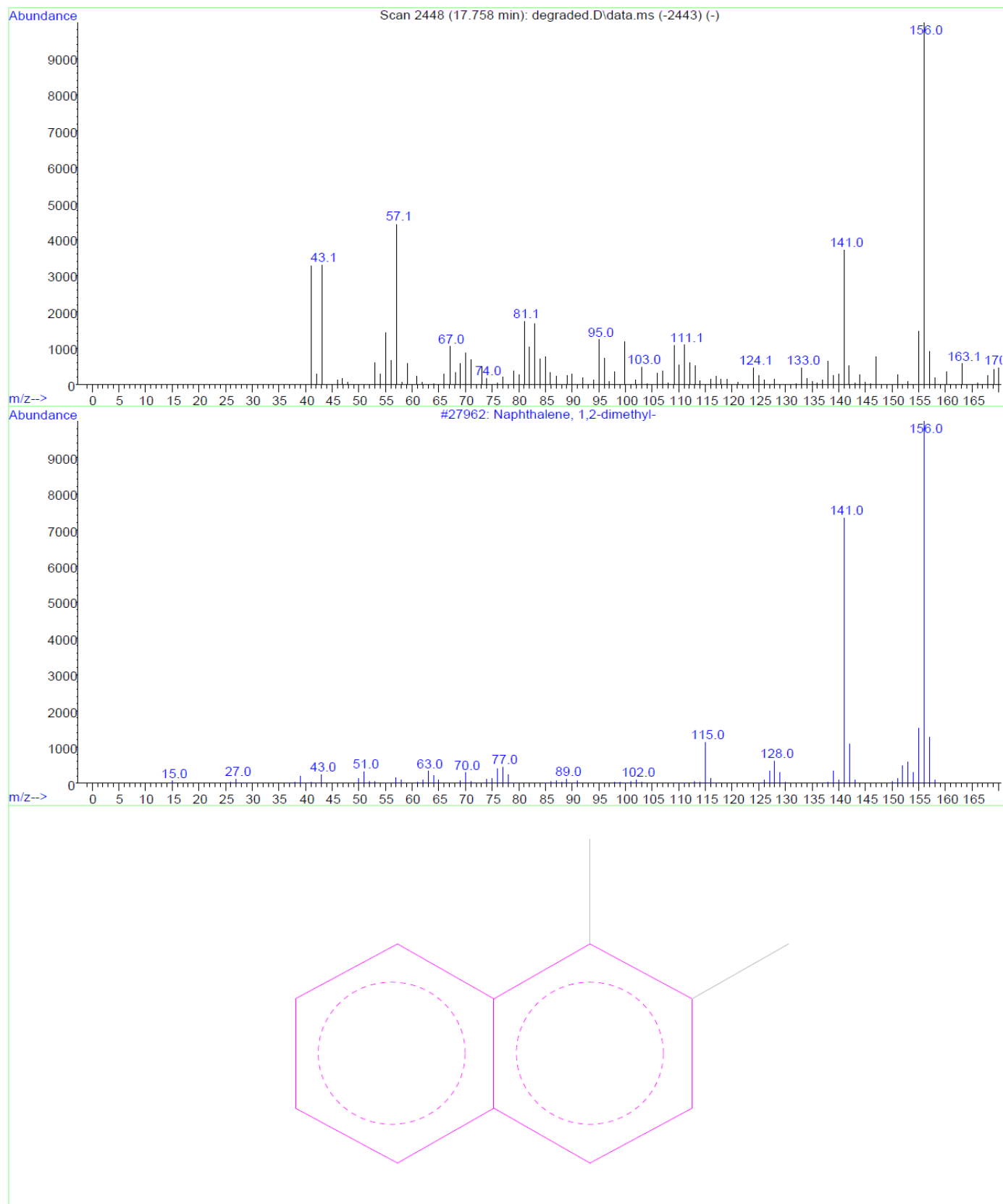**Appendix IIIf**: Cyclohexane, 1-ethyl-1-methyl

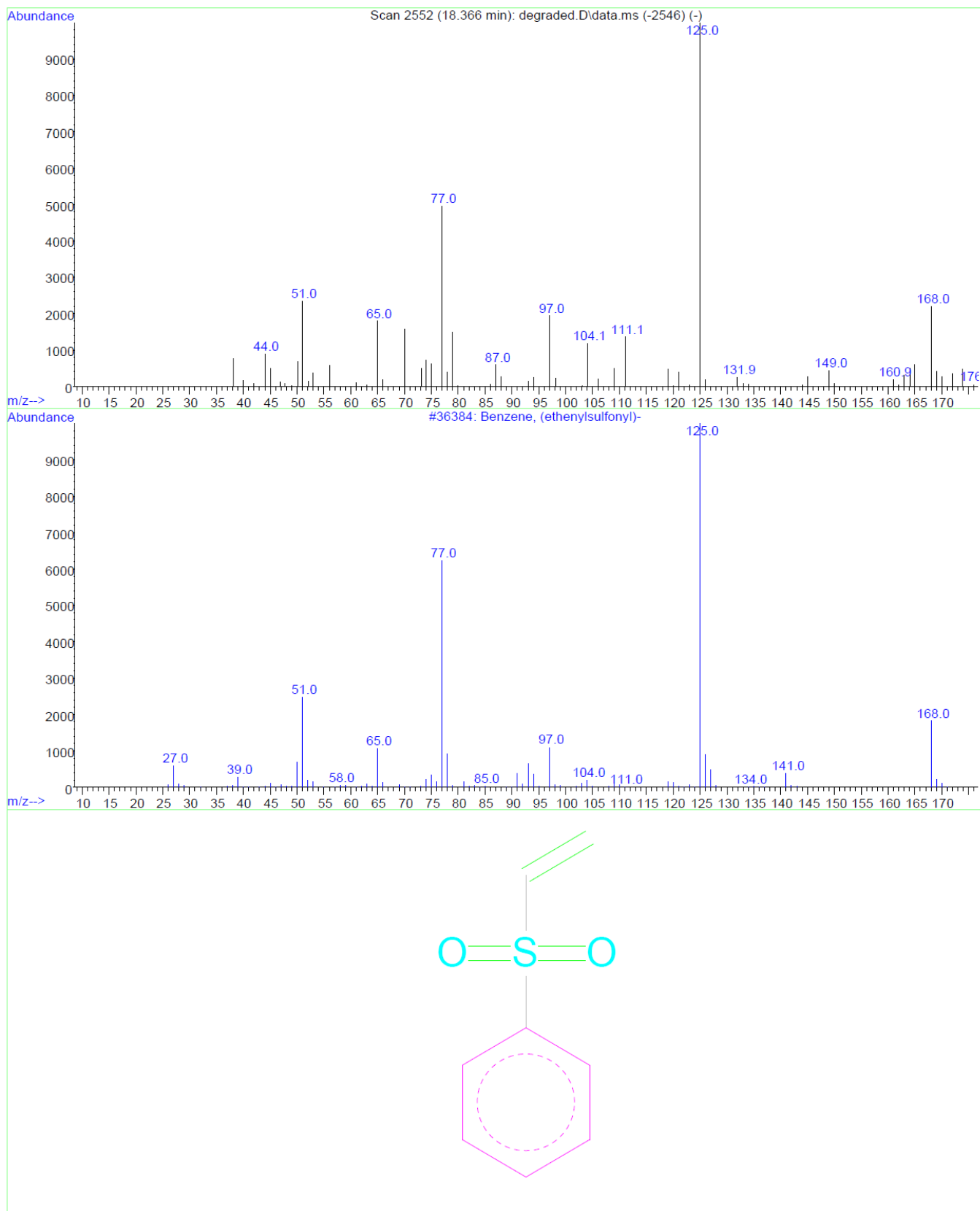**Appendix IIIg**: Sulfurous acid, decyl 2-pentyl ester
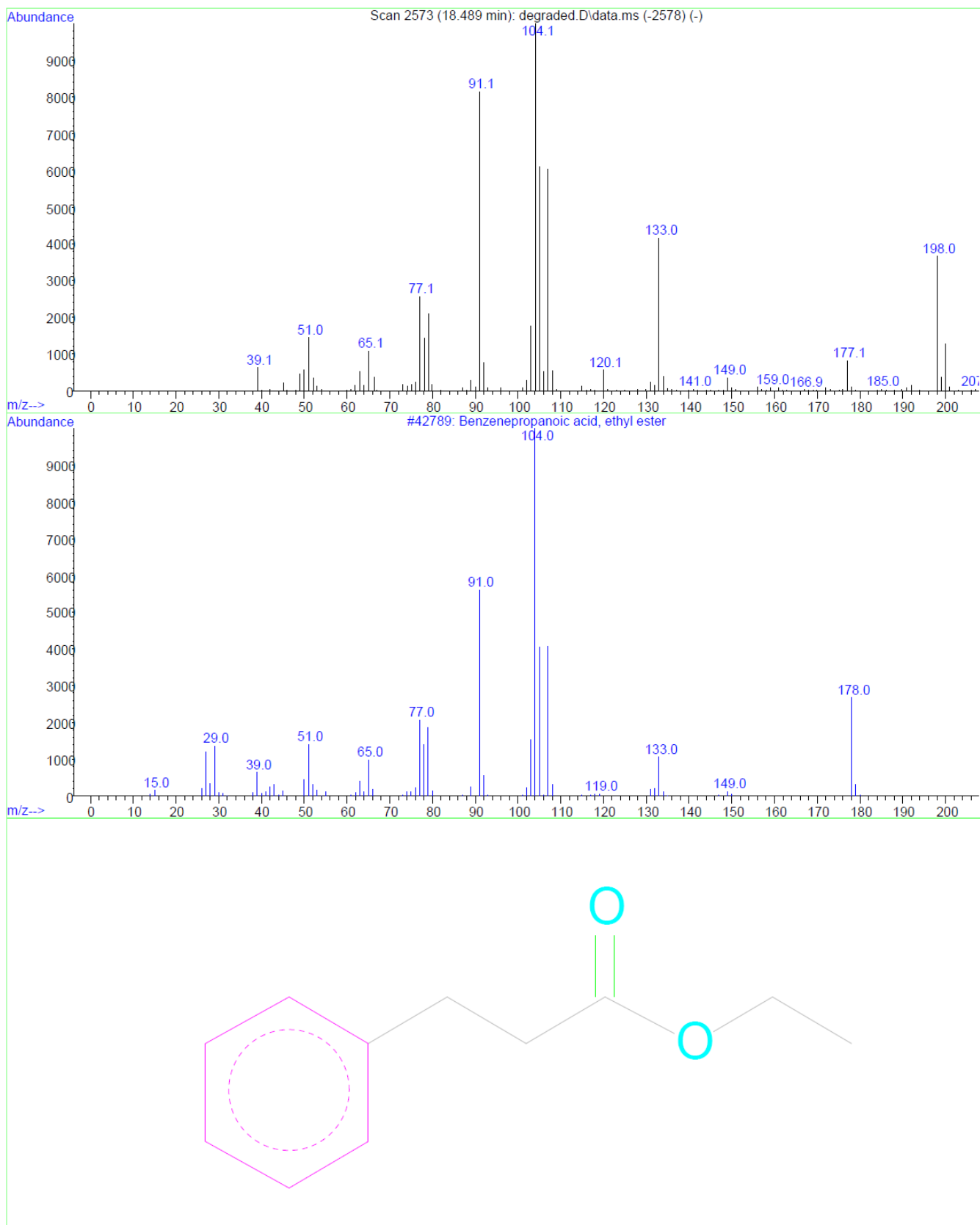
Appendix IIIh: Butanoic acid, 4-(methylthio)

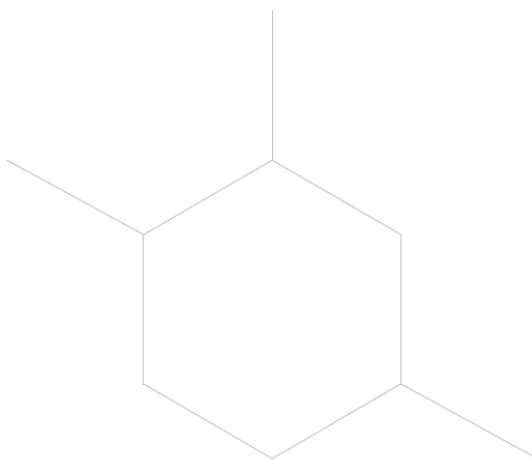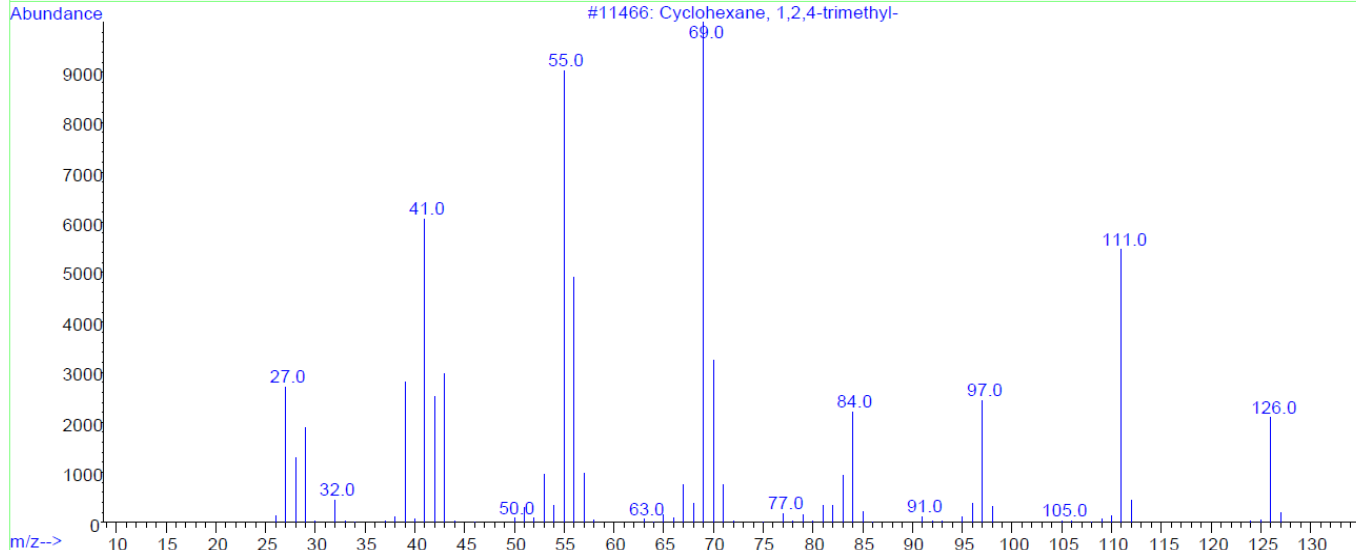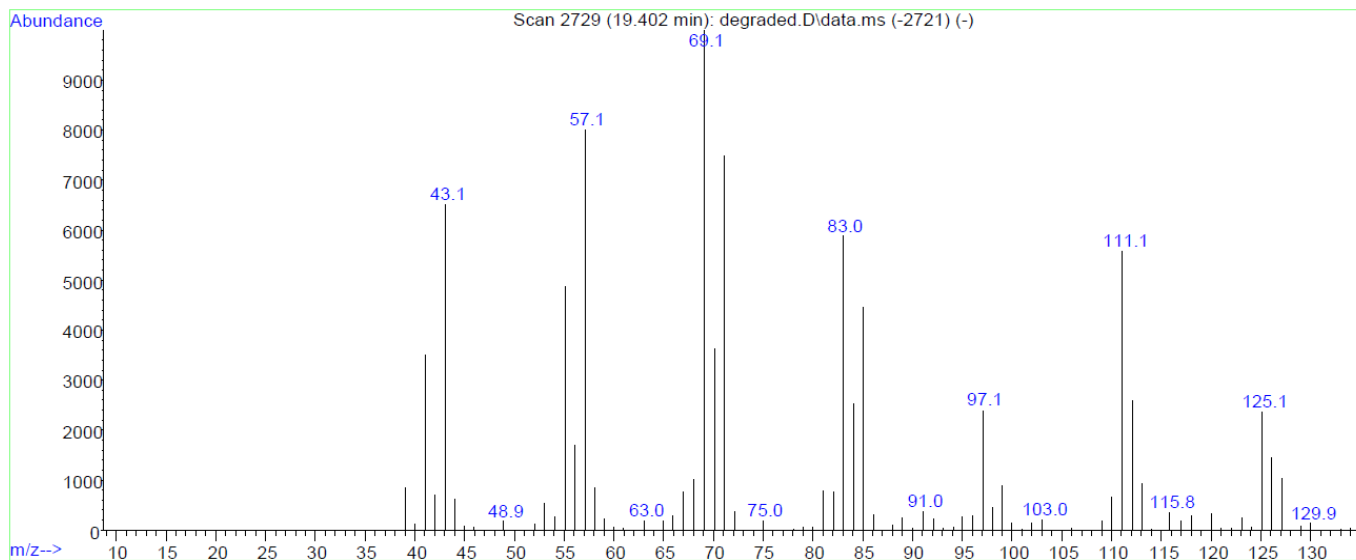**Appendix IIIi**: Naphthalene, 1,2-dimethyl
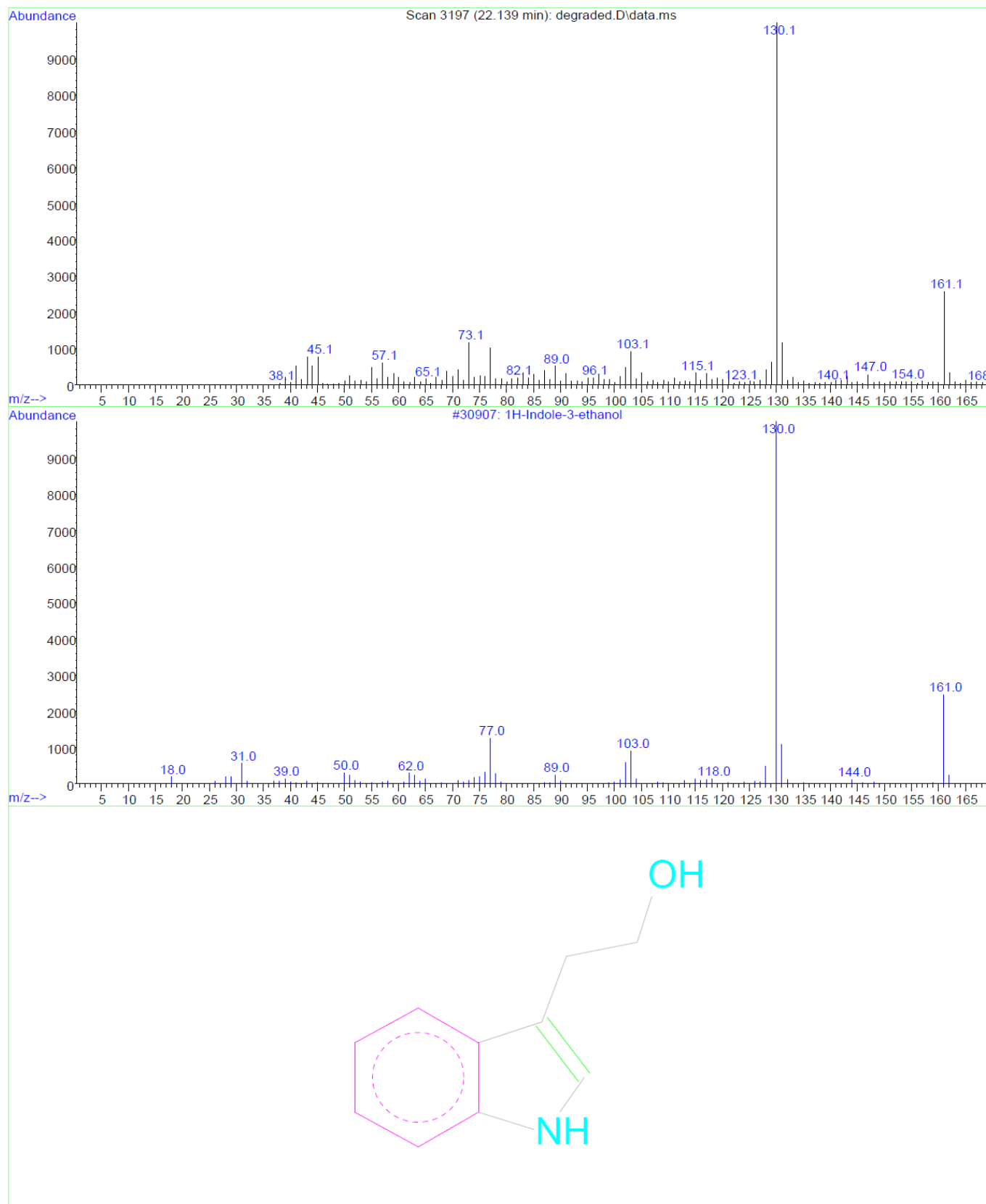
Appendix IIIj: Benzene, (ethenylsulfonyl)

Appendix IIIk: Benzenepropanoic acid, ethyl ester

Appendix IIIl: Cyclohexane, 1,2,4-trimethyl

Appendix IIIm: 1H-Indole-3-ethanol

Appendix IV: Determination of maximum Malachite Green dye consumption rate (V_{max}), decolorization rate constant (K_m) and reaction kinetics order models

Time (minutes)	Absorbance					
	Malachite Green dye Concentration					
	0.93 mg/l	1.86 mg/l	3.72 mg/l	5.57 mg/l	7.43 mg/l	9.30 mg/l
0	0.085	0.201	0.404	0.601	0.801	1.201
30	0.06	0.132	0.274	0.476	0.603	0.736
60	0.043	0.095	0.2	0.363	0.464	0.568
90	0.034	0.079	0.181	0.309	0.383	0.473
120	0.016	0.046	0.118	0.213	0.278	0.338
150	0.01	0.038	0.096	0.18	0.216	0.29
180	0.007	0.031	0.077	0.147	0.195	0.238
210	0.006	0.029	0.065	0.125	0.165	0.203
240	0.003	0.025	0.056	0.108	0.146	0.179
270	0.002	0.017	0.041	0.083	0.122	0.152
300	0.001	0.016	0.035	0.078	0.11	0.139
330	0.0006	0.015	0.043	0.073	0.098	0.124
360	0.0003	0.01	0.03	0.073	0.088	0.105

Appendix V: Determination of maximum Reactive Black 5 dye consumption rate (V_{max}), decolorization rate constant (K_m) and reaction kinetics order models

	Absorbance						
	Reactive Black 5 dye Concentration						
Time (Hours)	9.9182mg/l	19.8364mg/l	39.6728mg/l	49.591mg/l	59.5092mg/l	79.3456mg/l	99.182mg/l
0	0.211	0.328	0.634	0.736	0.937	1.177	1.402
6	0.209	0.311	0.604	0.713	0.902	1.13	1.38
12	0.138	0.244	0.574	0.656	0.795	1.022	1.056
24	0.097	0.21	0.343	0.498	0.347	0.689	0.6
36	0.049	0.106	0.098	0.12	0.182	0.215	0.259
48	0.046	0.091	0.09	0.11	0.177	0.196	0.222
60	0.041	0.077	0.083	0.102	0.156	0.181	0.201
72	0.037	0.049	0.071	0.081	0.119	0.145	0.182
84	0.034	0.051	0.063	0.07	0.1	0.127	0.16
96	0.03	0.033	0.054	0.061	0.073	0.096	0.155
108	0.03	0.033	0.033	0.057	0.06	0.067	0.08
120	0.03	0.03	0.038	0.048	0.05	0.066	0.072