

**ISOLATION, PURIFICATION AND CHARACTERIZATION OF FREE AND
IMMOBILIZED ALPHA-AMYLASE FROM *BACILLUS LICHENIFORMIS***

BY

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**A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,
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MARCH, 2015

DECLARATION

I hereby declare that this thesis entitled: “ISOLATION, PURIFICATION AND CHARACTERIZATION OF FREE AND IMMOBILIZED ALPHA-AMYLASE FROM *BACILLUS LICHENIFORMIS*” was performed by me in the department of Biochemistry, Ahmadu Bello University, under the supervision of Prof. E. Onyike and Mr. G.C Njoku. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at this or any other institution.

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CERTIFICATION

This thesis entitled “ISOLATION, PURIFICATION AND CHARACTERIZATION OF FREE AND IMMOBILIZED ALPHA-AMYLASE FROM *BACILLUS LICHENIFORMIS*” by Abiodun AINA meets the regulations governing the award of Master of Science (M.Sc) degree of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to the Lord, God Almighty for the wisdom granted to me by him and for his guidance and protection throughout the period of doing this work.

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ABSTRACT

The bacteria *Bacillus licheniformis* was cultured in nutrient agar and then incubated for 15h at 35°C. The bacteria cells were harvested by centrifugation after incubation. The cell free supernatant was used to estimate *alpha-amylase* activity. The *alpha-amylase* obtained was isolated and purified using ammonium sulfate precipitation, gel filtration and ion exchange chromatography. It was purified up to 15.5 fold and a yield of 20.2% on DEAE- Sephadex column with a final specific activity of 12.14 u/mg. The *alpha-amylase* was immobilized by entrapment in calcium alginate beads. The free and immobilized enzyme had broad temperature ranges from 20°C to 70°C with optima of 60°C and 70°C respectively and optimum pH of 7.0 and 8.0 respectively. Initial velocity studies for the determination of kinetic constants with maltose as substrate revealed a K_M value of 2.5 mg/ml and 1.0 mg/ml for the free and immobilized enzyme respectively and a V_{max} value of 0.4unit/mg/min and 0.95unit/mg/min for the free and immobilized enzyme respectively. Both the free and immobilized enzyme activity were enhanced by Ca^{2+} , Mn^{2+} , and Na^+ while Hg^{2+} and Zn^{2+} were found to be strong inhibitors of both the free and immobilized enzyme.

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LIST OF ABBREVIATIONS

Spp	-	Specie
K_M	-	Michaelis Menten Constant
V_{max}	-	Maximal Velocity
PGA	-	Penicillin G Acylase
CM-Sephadex	-	Carboxymethyl Sephadex
DEAE- Sephadex	-	Diethylaminoethyl Sephadex
μ l	-	Microliter
ml	-	Milliliter
Mg	-	Milligram
nm	-	Nanometer
w/V	-	Weight per volume
g	-	Gram
CBRT	-	Center for Biotechnology and Research Training
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetracetic acid

- G.M.O. - Genetically modified organism
- SPR - Surface Plasmon Resonance
- ELISA - Enzyme – linked Immunosorbent assay

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background of Study

Amylase is a digestive enzyme classified as a saccharidase (an enzyme that cleaved polysaccharides). It is mainly a constituent of pancreatic juice and saliva, needed for the breakdown of long-chain carbohydrate (such as starch) into smaller units like disaccharides and trisaccharides.

Alpha-amylase is the major form of amylase found in humans and other mammals. It is also present in seeds containing starch as food reserve and it is secreted by many fungi. Although found in many tissues, alpha-amylase is most prominent in pancreatic juice and saliva. Alpha-amylase found in saliva breaks starch down to maltose and dextrin. It breaks large insoluble starch molecules into soluble forms e.g. amylopectin, erythropectin and achropectin producing successively smaller starches and ultimately maltose. The pancreas produces alpha-amylase which hydrolyses dietary starch into disaccharides and trisaccharides which are converted by other enzyme to glucose to supply the body with energy (Alistair *et al.*, 2006).

Although amylase can be derived from several sources such as plants, animals and microbes, the microbial amylase meet industrial needs and demands. Large numbers of microbial amylase have completely replaced chemical hydrolysis of starch in starch processing industries (Pandey *et al.*, 2000).

Nowadays the use of enzyme in industrial sector is increasing due to the increase of industries, especially in food, beverages, textile, leather and paper industries. Besides its uses in industry, it can also be used in treatment of industrial waste such as cellulase which is able

to convert cellulose of wood and paper wastes to ethanol (Vielle and Zeikus., 1996). One of the enzymes widely used in industrial sectors is alpha-amylase. Alpha-amylase from *Bacillus* species has found application in many industries such as pharmaceutical, textile, paper, detergent and chemical industries. Therefore, these enzymes account for about 39% of the world's enzymes production (Gomes and Steiner, 2004).

A biocatalyst is termed immobilized, if its mobility has been restricted by chemical means. Immobilized enzymes are used in food technology, biotechnology, biomedicine and analytical chemistry. Immobilized enzymes offers variety of advantages over free enzyme catalysis including increased stability of enzyme, easy recovery of enzyme, easy separation of reactant and product, repeated or continuous used of a single batch of enzyme which will ultimately save the enzyme, labor and overhead costs (Gerhartz, 1990).

Enzymes can be immobilized to a multitude of different carriers by entrapment, adsorption, ionic binding and covalent binding (Varavinit *et al.*, 2002). Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability and protects enzyme from microbial contamination (Kennedy and Cabral, 1987).

Physical entrapment of alpha-amylase in calcium alginate beads has shown to be a relatively easy, rapid and safe technique (Dey *et al.*, 2003) in comparison with other immobilization methods.

1.2 Statement of Research Problem

- a. Amylases possess important applications in the production of syrup with high glucose content, sweetener manufacture, detergent and ethanol (Pandey *et al.*, 2000).
- b. The annual sale of alpha-amylase in global market is estimated to be eleven million dollars (Kilara and Desai, 2002).
- c. There is a need to discover more bacterial sources of alpha-amylase that will produce alpha-amylase with better properties e.g. thermostability that will be of greater use to the industries.
- d. There is a need to discover more ways of producing alpha-amylase in bulk and that will be economically viable.
- e. Most of the enzymes used in the industrial sector in Nigeria including food industries are still imported enzymes and economically, this is not favorable to the nation because Nigeria is rich in natural resources especially the microbial which can be use as enzymes producer for example alpha-amylase enzyme.
- f. There is a need to immobilize alpha-amylase in order to explore the various benefits that can come from the immobilization process.

1.3 Justification

- a. Bacterial alpha-amylase is preferred for the application in starch processing and textile industries due to it's stability at higher temperature (75-105°C) and its neutral to alkaline pH (Shah and Kothari, 1991).
- b. *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. vulgarus* have been used for alpha amylase production in solid state fermentation (Babu and Satyanarayana, 1995).

- c. Due to the increase in the demand for these enzymes in various industries, there is therefore a need to discover more strains of bacteria that can produce alpha-amylase with better properties in terms of thermo-stability, mass production of the enzyme and consistency.
- d. There is also a need to compare the kinetics and physico-chemical properties of the free and immobilized alpha amylase to discover various benefits that immobilization of alpha amylase can offer to the industries.
- e. The strain of *B. licheniformis* used for this research work is a local strain isolated from Kaduna metropolis soil in Kaduna State, Nigeria and it is different from imported strains of the bacteria that are usually used for other research work.
- f. Also there is no documented work on the kinetic studies and effect of metal ions on immobilized alpha amylase.

1.4 Aim

To isolate, purify and characterize free and immobilized alpha-amylase from *Bacillus licheniformis*.

1.4.1 Objectives

- a. Isolation of the enzyme alpha-amylase in fermentation media from the cell of the Bacteria isolate of *Bacillus licheniformis*.
- b. Purification of alpha-amylase obtained from the *Bacillus licheniformis*
- c. Immobilization of the enzyme by entrapment in calcium alginate beads.
- d. Characterization of the free and immobilized alpha amylase.

1.5 Research Hypothesis (Null)

Immobilization of alpha-amylase does not affect the stability, kinetics and physico-chemical properties of the enzyme.

CHAPTER TWO

2.0 Literature Review

2.1 Enzymes

An enzyme is a biological catalyst which alters the rate of chemical reactions usually by speeding it up. An enzyme is a protein molecule with a specific shape to accommodate reactant molecules. One enzyme molecule can catalyze 10 million reactions in a single second.

Enzyme works at relatively low temperature (usually about 40⁰C) thereby saving energy and saving money. The exact function of enzymes is determined by their structure, which is a combination of the sequence of amino acids from which they are build up. The sequence will result in a certain three-dimensional structure that may be stabilized by moieties other than amino acids that are added to the protein backbone. Their complete structure determines their function. Factors that modify enzyme structure will often also affect the function. (Bugg, 2004).

2.2 Uses of Enzymes in the Industries

Enzymes are used in the industries in many areas. They are starting to replace petroleum based solvents used to make vegetable seed oil, replacing harsh acids used in the production of glucose products such as corn syrup, replacing sulphides used in the tanning industry and many other aspects. Today, enzymes are helping industry to manufacture products used by society in a way that is less harmful to the environment.

Some of the uses of enzymes in the industries are highlighted below.

Amylases is use in the production of high fructose corn syrup by converting starch to sugars. Trypsin is used for primary food processing for babies because it can predigest baby foods. Papain is used for meat tendering (Nagodawithana and Reed, 1993).

Amylase is used to split polysaccharides from malt during mash process. Proteases help to remove remaining protein from yeasts, which make beer clearer and easily filtrated. Glucose isomerase is used to convert glucose into fructose in production of high fructose syrups from starchy materials. Fungal enzymes are used to convert starch to glucose (Julio and Andrew 2007).

Amylases are used to degrade starch to lower viscosity thereby aiding sizing and coating of paper. Xylanases are used to reduce bleach required for decolorizing. Cellulases are used to smooth fibers, enhance water drainage and to promote ink removal. Amylases are used in detergents for machine dish washing to remove resistant starch residues Cellulases are used in biological fabric conditioners. Alpha-amylases are used to improve bread quality and to increase shelf life. Xylanases decrease the water absorption and thus reduces the amount of water needed in baking which leads to more stable dough. Proteinases are used to improve dough-handling properties. (Julio and Andrew 2007).

2.3 Microorganisms

A microorganism or microbe is a microscopic organism, which may be a single cell or multi-cellular organisms. The study of microorganisms is called microbiology. This subject began with Anlon Van Leeuwenhoek's discovery of microorganisms in 1675, using a microscope of his own design. Microorganisms are very diverse; they include all of the prokaryotes, namely the bacteria and archaea, and various forms of eukaryotes, comprising the protozoa, fungi, algae, microscopic plants (green algae) and animals such as rotifers and planarians. Some microbiologists also classify viruses as microorganisms, but others considered these as non-

living. Most microorganisms are microscopic but there are some like *Thiomargarita namibienses*, which are macroscopic and visible to the naked eye.

Microorganisms live in every part of the biosphere including soil, hot springs, on the ocean floor, high in the atmosphere and deep inside rocks within the earth's crust. Microorganisms are crucial to nutrient cycling in ecosystems because they act as decomposers. Some of them can fix nitrogen, they are a vital part of the nitrogen cycle, and recent studies indicate that airborne microbes may play a role in precipitation and weather (Madigan and Martinko, 2006).

2.3.1 Sources of Microorganism

The refrigerator, the supermarket are examples of places that provides safe sources for microorganisms.

Other examples of sources of microorganisms include

- a. Yeast: - Fresh or dried yeast can be used in either case, a small amount of yeast is dissolved in a little bit of water, until the liquid becomes turbid. This suspension is then observed under a microscope to see how yeast cells look like.
- b. Yogurt: - Yogurt contains many bacteria.
- c. Cheese: - Gorgonzola blue cheese is not only good for eating but also a valuable source for the fungi penicilium.

Microorganisms can also be found in the air, water and soil (Madigan and Martinko, 2006).

2.3.2 Uses of Microorganisms

Microorganisms are useful in the following areas

Food: - Microorganisms are involved in the production of food or may be directly edible. For example various types of yeast are used for the production of beer, bread and cheese. Spirulina is a blue-green algae used as single cell protein (SCP) in human beings as protein source.

Health: - There are several billion times more bacteria cells inside the body of man than there are human cells. Some of them live in mutualistic relationship with man. The presence of bacteria also inhibits the growth of potentially pathogenic bacteria through competitive exclusion.

Biotechnology: - Modern biotechnology is often associated with the use of genetically altered microorganisms such as *Escherichia coli* (*E. coli*) or yeast for the production of substances such as synthetic insulin or antibiotics. Using the same process, a multitude of drugs are manufactured relatively cheaply, including human growth hormone, clotting factors for hemophiliacs, fertility drugs, erythropoetin and other drugs (Rybicki, 1990).

2.4 Industrial Microorganisms

Industrial microbiology includes the use of microorganisms to manufacture food or industrial products in large quantities. Numerous microorganisms are used within industrial microbiology, these include naturally occurring organisms, laboratory selected mutants or even genetically modified organisms (GMOs). Currently, the debate in the use of genetically modified organism (GMOs) in food sources is gaining both momentum, with more and more supporters on both sides. However, the use of microorganisms at an industrial level is deeply rooted into today's society. The following is a brief overview of the various microorganisms that have industrial uses and the roles they play.

- a. The ability of specific microorganisms to produce specialized enzymes and proteins has been exploited for many purpose in industry.
- b. Industrial microorganisms are used to produce many things, including food, cosmetics, pharmaceuticals and construction materials.
- c. Microorganisms can be generally modified or engineered to aid in large-scale production (Rybicki, 1990).

2.5 Bacteria

Bacteria constitute a large domain of prokaryotic microorganisms. Typically a few micrometers in length, bacteria have a wide range of shapes, ranging from spheres to rods and spirals. Bacteria were among the first life forms to appear on Earth and are present in most habitats on the planet. Bacteria inhabit soil, water, acidic hot springs, radioactive waste and the deep portions of Earth's crust. Bacteria also live in plants, animals and have flourished in manned space vehicles (Fredrickson *et al.*, 2004).

There are typically 40 million bacterial cells in a gram of soil and a million bacteria cells in a millilitre of fresh water. There are approximately 5×10^{30} bacteria on earth (Whitman *et al.*, 1998). Bacteria are vital in recycling nutrients with many steps in nutrient cycles depending on this organism such as the fixation of nitrogen from the atmosphere and putrefaction. In the biological communities surrounding hydrothermal vents and cold seeps, bacteria provide the nutrient needed to sustain life by converting dissolved compound such as hydrogen sulfide and methane to energy. On 17 March 2013, researchers reported data that suggested bacteria life forms thrive in the Mariana Trench, the deepest spot on the Earth (Glud *et al.*, 2013). Other researchers reported related studies that microbes thrive inside rocks up to 1900 feet below the sea floor under 8500 feet of ocean off the coast of the north western united states (Oskin, 2013).

Microbes can be found everywhere and they are extremely adaptable to conditions, and survive wherever they are. Most bacteria have not been characterized, and only about half of the phyla of bacteria have species that can be grown in the laboratory (Rappe and Giovannoni, 2003).

There are approximately ten times as many bacteria cells in human flora as there are human cells in the body, with large numbers of bacteria on the skin and as gut flora (Sears, 2005). The vast majority of the bacteria in the body are rendered harmless by the protective effects of the immune system, and a few are beneficial. However, a few species of bacteria are pathogenic and cause infectious diseases including cholera, syphilis, anthrax, leprosy, and bubonic plague. In industries, bacteria are important in sewage treatment and break down of oil spills, the production of cheese and yogurt through fermentation, the recovery of gold, palladium, copper and other metals in the mining sector as well as in biotechnology and the manufacture of antibiotics and other chemicals (Madigan and Martinko, 2006).

2.6 Bacillus species

The genus *Bacillus* currently comprises in excess of 60 species, commonly found in the environment and as laboratory contaminants. *Bacillus* species are Gram positive rods often arranged in pairs or chains with rounded or square ends and usually have a single endospore. The endospores are generally oval and are very resistant to adverse conditions. Sporulation is not repressed by morphology of the spore and sporangium. The groups are:

Group 1 – Gram positive, produce central or terminal ellipsoidal or cylindrical spores that do not distend the sporangium.

Group 2 – Gram variable with ellipsoidal spores and swollen sporangia.

Group 3 – Gram variable, sporangia swollen with terminal or sub terminal spores (Beckerly, 1997).

Bacillus is a genus of gram-positive, rod-shaped bacteria and a member of the phylum *Firmicutes*. *Bacillus* specie can be obligate aerobes or Facultative anaerobes and test positive for the enzyme catalase (Turnbull, 1996). Ubiquitous in nature, *Bacillus* includes both free-living and pathogenic species. Under stressful environmental conditions the cells produce oral endospores that can stay dormant for extended period. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera (Madigan and Martinko, 2006).

Species differentiation of the genus is complex and, in some instances around a laboratory, a combination of Gram stain and colonial appearance may be regarded as sufficient indication of a *Bacillus* species being present in a clinical specimen (Koneman *et al.*, 1997)

2.6.1 Industrial Significance

Many *Bacillus* species are able to secrete large quantities of enzymes. *Bacillus amyloliquefaciens* is the source of natural antibiotic proteins bamase, and also the enzyme alpha-amylase use in starch hydrolysis. It also produces the protease subtilisin used with detergents and the Bam HI restriction enzyme used in DNA research.

A portion of the *Bacillus thuringiensis* genome was incorporated into corn crops. The resulting GMOs, are therefore resistant to some insect pests (Madigan and Martinko, 2006).

2.6.2 Clinical Significance

Two *Bacillus* species are considered medically significant. *B. anthracis*, which causes anthrax, and *B. cereus* which causes a food borne illness similar to that of staphylococcus. A third species, *B. thuringiensis*, is an important insect pathogen, and is sometimes used to

control insect pests. The species *B. subtilis* is an important model organism. It is also a notable food spoiler, causing ropiness in bread and related food. Some environmental and commercial strains of *B. coagulans* may play a role in food spoilage of highly acidic tomato based products. An easy way to isolate *Bacillus* is by placing non-sterile soil in a test tube with water, shaking and placing in melted manitol salt agar, and incubating at room temperature for at least a day. Colonies are usually large, spreading and irregularly shaped. Under the microscope, the *Bacillus* cells appear as rods, and a substantial portion usually contain an oval endospore at one end, making it bulge (Ryan and Ray, 2004).

2.6.3 Cell Wall

The cell wall of *Bacillus* is a structure on the outside of the cell that forms the second barrier between the bacterium and the environment, and at the same time maintains the rod shape and withstand the pressure generated by the cell's turgor. The cell wall is composed of teichoic and teichuronic acids. *B. subtilis* is the first bacterium for which the role of an actin-like cytoskeleton in cell shape determination and peptidoglycan synthesis was identified, and for which the entire set of peptidoglycan synthesising enzymes was localized. The role of the cytoskeleton in shape generation and maintenance is important (Scheffers, 2012).

2.6.4 Phylogeny

The genus *Bacillus* was coined in 1835 by Christian Gottfried Ehrenberg to contain rod-shaped bacteria, later amended by Ferdinand Cohn to spore-forming Gram-positive/variable, rod-shaped bacteria. Like other genera associated with the early history of microbiology such as *Pseudomonas* or *Vibrio*, members of the *Bacillus* genus (226 species) (Euzéby, 1977) are found ubiquitously, and it is one of the genera with the largest 16S diversity and environmental diversity (Alcaraz *et al.*, 2010).

Several studies have tried to construct the phylogeny of the genus. The *Bacillus*-specific study with the most diversity covered is by Xu and Cote using 16S and the ITS region, where they divide the genus into 10 groups, which includes the nested genera *Paenibacillus*, *Brevibacillus*, *Geobacillus*, *Marinibacillus* and *Virgibacillus* (Xu and Cote, 2003).

One clade, formed by *B. anthracis*, *B. cereus*, *B. nycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis* under current classification standards, should be a single species (within 97% 16S identity), but due to medical reasons, they are considered separate species, an issue also present for four species of *Shigela* and *Escherichia Coli* (Brenner, 1984).

Bacillus Phylogenetics

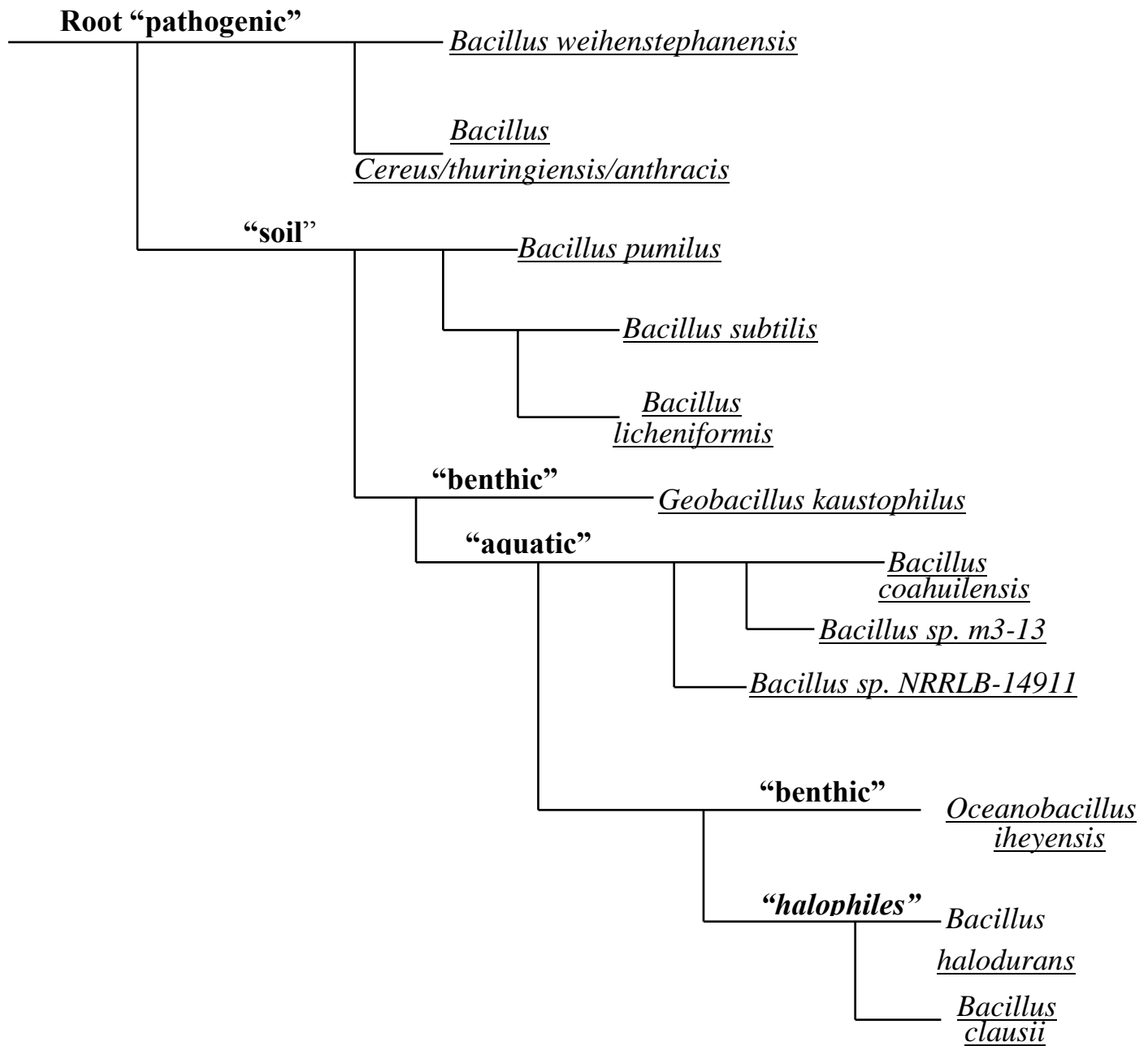


Figure 2.1: Phylogeny of the genus *Bacillus* according to Alcaraz *et al* 2010

2.7 *Bacillus licheniformis*

Bacillus licheniformis is a bacteria commonly found in the soil. It is found on bird feathers, especially chest and back plumage, and most often in ground-dwelling birds like sparrows and aquatic species like ducks. It is a gram-positive, thermophilic bacterium. Its optimal growth temperature is around 30⁰C, though it can survive at much higher temperature. The optimal temperature for enzyme secretion is 37⁰C. It can exist in spore form to resist harsh environments, or in a vegetative state when conditions are good. Scientists are currently exploring its ability to degrade feathers for agricultural purpose. Feathers contain high amounts of non-digestible proteins, but researchers hope that, through fermentation with *B. licheniformis*, they can use waste feather to produce cheap and nutritious feather meal to feed livestock. Ecological research is also been done looking at the interactions between plumage colors and *B. licheniformis* activity, and the consequences thereof (Edward *et al.*, 2010).

2.7.1 Uses of *Bacillus licheniformis*

- a. Feather protection through *psittacofulvin*: *Bacillus licheniformis* degrades feathers of parrots especially white feathers. Red feathers with high levels of *psittacofulvin* are more resistant. Feather degrading bacteria may have played an important role in the evolution of molting and patterns in feather coloration (Edward *et al.*, 2010).
- b. Biological laundry detergent: *Bacillus licheniformis* is cultured in order to obtain protease for use in biological laundry detergent. The bacterium is well adapted to grow in alkaline conditions, so the protease it produces can withstand high pH levels, making it ideal for this use. The protease has a pH optimum of between 9 and 10 and is added to laundry detergents in order to digest, and hence remove dirt made of proteins. This allows for much lower temperatures to be used, resulting in lower

energy use and a reduced risk of shrinkage of garments or loss of colored dyes (Julio and Andrew, 2007).

- c. Nanotechnology applications: *Bacillus licheniformis* can be used in synthesis of gold nanocubes. Researchers have synthesized gold nanoparticles with sizes between 10 to 100 nanometres. Gold nanoparticles are usually synthesized at high temperatures, in organic solvents and using toxic reagents. The bacteria produce them in much milder conditions (Kalishwaralal *et al.*, 2009).

2.8 Alpha-Amylase

As diastase, amylase was the first enzyme to be discovered and isolated by French chemists Anselme Payen and Jean-Franconis Peroz from germinating barley in 1833. In 1862 Alexander Danilewsky (1838 - 1923) separated pancreatic amylase from trypsin (Robert and Joseph, 1970). All amylase are glycosidic hydrolases and act on α -1,4-glycosidic bonds (Jochanah, 1998).

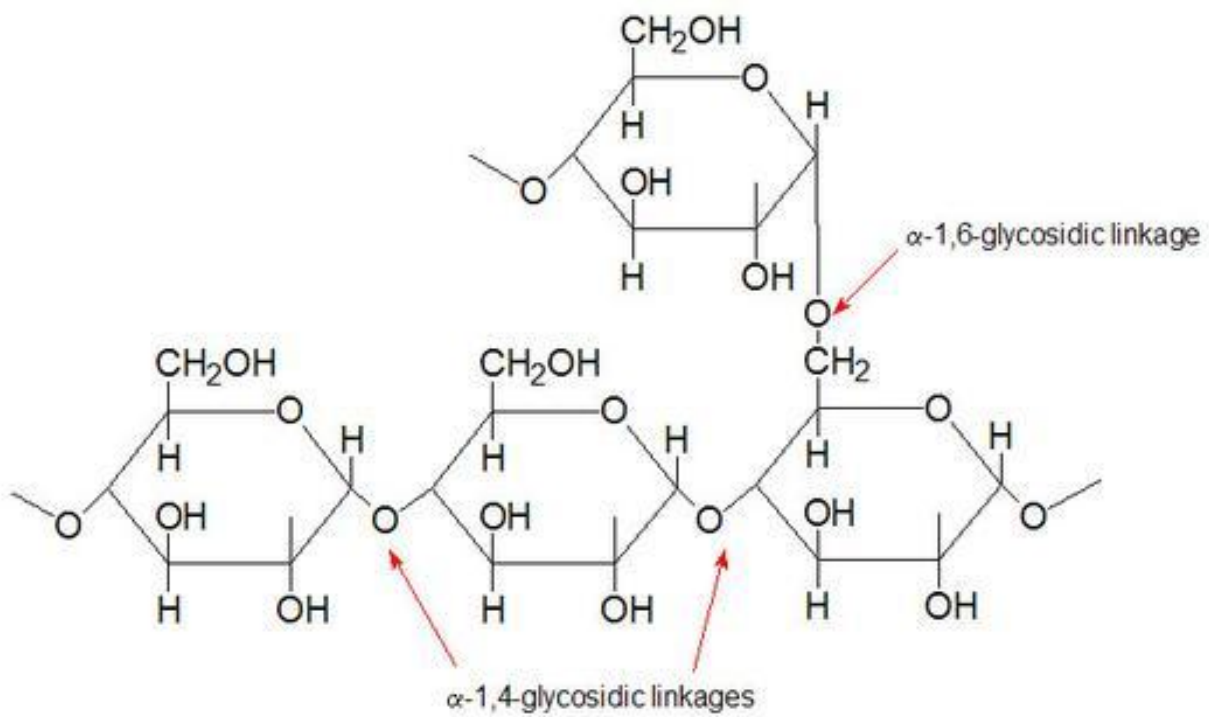


Figure 2.2: A section of starch molecule showing α -1,4 and α -1,6- branching point (Jochanah, 1998).

2.8.1 Types of Amylases

α -amylase (EC 3.2.1.1) (alternate names: 1,4- α -D-glucanohydrolase glycogenase).

The α -amylases are calcium metallo-enzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α – amylase breaks down long-chain carbohydrate leading to the formation of soluble maltodextrins, maltose and glucose. Because it can act anywhere on the substrate, α – amylase tends to be faster-acting than β -amylase. In animals, it is a major digestive enzyme and its optimum pH is 6.7 – 7.0. In human physiology, both the salivary and pancreatic amylase are α - amylases. It is also found adequately in plants, fungi (*ascomycetes* and *basidiomycetes*) and bacteria (*Bacillus*).

β -Amylases (EC 3.2.1.2) (alternate names 1,4- α -D-glucan maltohydrolase; glycogenase or saccharogen amylase). β -amylase is also synthesized by bacteria, fungi and plants. Working from the non-reducing end, β -amylase catalyzes the hydrolysis of the second α -1,4-glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruits, β -amylase breaks starch into maltose resulting in the sweet flavor of ripe fruit. Both α -amylase and β -amylase are present in seeds. Animal tissue does not contain β -amylase but it may be present in microorganisms contained within the digestive tract.

γ - amylase (E.C 3.2.1.3) (alternate names: glucan 1,4 – α –D- glucosidase, amyloglucosidase or Exo-1,4 – α –D – glucosidase; glucoamylase or 1,4 - α – D –glucan glucohydrolase). In addition to cleaving the last α -1,4 – glycosidic linkages at the non-reducing end of amylose and amylopectin yielding glucose, γ – amylase will cleave α – 1,6 – glycosidic linkages. Unlike the other forms of amylase, γ – amylase is most efficient in acidic environments and has an optimum pH of 3. (Jochanah, 1998).

2.8.2 Sources of Alpha-Amylase

α -amylase have been derived from several fungi, yeast, bacteria and actinomycetes, however, enzymes from fungi and bacteria source have dominated applications in industrial sectors (Pandey *et al.*, 2000). α -amylase may be derived from several bacteria, yeasts, and fungi. However, bacterial amylases are generally preferred over fungal amylases due to several characteristic advantages that it offers. Strains of *Bacillus* sp., mainly *Bacillus subtilis*, *Bacillus megaterium* are employed (Gayal and Khandeparker, 1979).

2.8.3 Uses of Alpha-Amylases

α -amylase is a key enzyme in the production of starch derivatives and also widely used in food, textile, paper, detergent, chemical, pharmaceutical and other industrial fields (Bhat, 2000; Forgyat and Kelly; 1990; Kandrah, 2003; Kirk *et al.*, 2002).

α -amylase is used in the food industry for the production of glucose syrups, crystalline glucose, high fructose corn syrups, maltose syrups, reduction of viscosity of sugar syrups, reduction of haze formation in juices (Sivaramakrishnan *et al.*, 2006).

α -amylase is also used as an additive to remove starch based dirt in the detergent industry. It is used in paper industry to reduce viscosity of starch for appropriate coating of paper. It is also used in the textile industry for warp sizing of textile fibers while it is used as digestive aid in pharmaceutical industry (Sivaramakrishnan *et al.*, 2006).

Bacterial α -amylase is widely used in pharmaceutical industry in various digestive aid preparations. Due to the presence of Bacterial α -amylase, starch in the consumed food is better digested, it increase overall digestibility of the food. Digestive aid preparations made from α -amylase are used for treatment of patient whose digestive power is reduced due to illness. Many such a commercial formulation of digestive aid either as syrup or tablet are

seen in many drug stores. Amylase is also used as a food additive. It is also used in clothing and dishwasher detergents to dissolve starches from fabrics and dishes (Sousa *et al.*, 2010).

2.9 Isolation and Purification of Protein/Enzymes

Most of the enzymes properties are clearly and reliably revealed only with purified enzymes. Prerequisites for the isolation of a pure enzyme are selected protein chemical separation methods carried out at 0-4°C since enzymes are often not stable at higher temperatures (Ehle and Horn, 1990)

2.9.1 Tissue Disintegration and Extraction

Disintegration and homogenization of biological tissue requires special precautions: procedures should be designed to rupture the majority of the cells in order to release their contents so that they become accessible for extraction.

The tissue is usually homogenized in the presence of an extraction buffer which often contains an ingredient to protect the enzymes/proteins from oxidation and traces of heavy metal ions. Particular difficulty is encountered during the isolation of enzymes/proteins which are bound tenaciously to membranes which are not readily solubilized. Extraction in the presence of tensides may help to isolate such enzymes. As a rule, large amounts of tissue have to be homogenized because the enzymes content in proportion to the total protein isolated is low and is usually further diminished by the additional purification of the crude enzyme isolate (Ehle and Horn, 1990).

2.9.2 Protein Purification

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture. Protein purification is vital for the characterization of the structure and interactions of the protein of interest. The starting material is usually a biological tissue

or a microbial culture. The various steps in the purification process mainly free the protein from a matrix that confines it, separate the protein and non-protein parts of the mixture, and finally separate the desired protein from all other proteins. Separation of one protein from all others is typically the most laborious aspect of protein purification. Separation steps may exploit differences in protein size, physico-chemical properties, binding affinity and biological activity (Ehle and Horn, 1990).

2.9.3 Purpose of Protein Purification

Purification may be preparative or analytical. Preparative purifications aim to produce a relatively large quantity of purified proteins for subsequent use. Examples include the preparation of commercial products such as enzymes, nutritional protein like soy protein isolate, and certain biopharmaceuticals like insulin. Analytical purification produces a relatively small amount of a protein for a variety of research or analytical purpose, including identification, quantification, and studies of the protein's structure, post-translational modifications and function. Pepsin and Urease were the first proteins purified to the point that they could be crystallized.

Choice of a starting material is key to the design of purification process. In a plant or animal, a particular protein usually isn't distributed homogeneously throughout the body, different organs or tissues have higher or lower concentrations of the protein. Use of only the tissue or organs with the highest concentration decreases the volume needed to produce a given amount of purified protein. If the protein is present in low abundance, or if it has a high value, scientists may use recombinant DNA technology to develop cells that will produce large quantities of the desired protein (Keneddy, 1990).

An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column.

Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis. This is very useful for scientific purpose and the detection limits for protein are nowadays very low and nanogram amounts of protein are sufficient for their analysis. Thirdly, proteins can be separated by polarity/hydrophobicity via high performance liquid chromatography or reversed-phase chromatography (Ehle and Horn, 1990).

2.9.4 Evaluation of Purification Yield

The most general method to monitor the purification process is by running a SDS-PAGE of the different steps. This method only gives a rough measure of the amounts of different proteins in the mixture, and it is not able to distinguish between proteins with similar apparent molecular weight.

If the protein has a distinguishing spectroscopic feature or an enzymatic activity, this property can be used to detect and quantify the specific protein, and thus to select the fractions of the separation, that contains the protein. If antibodies against the protein are available then western blotting and ELISA can specifically detect and quantify the amount of desired protein. Some proteins function as receptors and can be detected during purification steps by a ligand-binding assay, often using a radioactive ligand.

In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280nm, however some reagents used

during the purification process may interfere with the quantification. For example imidazole is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

Another method is Surface Plasmon Resonance (SPR). SPR can detect binding of label free molecules on the surface of a chip. If the desired protein is an antibody, binding can be translated directly to the activity of the protein. The active concentration of the protein can be expressed as the percent of the total protein. SPR can be a powerful method for quickly determining protein activity and overall yield. It is a powerful technology that requires an instrument to perform (Ehle and Horn, 1990).

2.9.5 Methods of Protein Purification

The methods used in protein purification can roughly be divided into analytical and preparative methods. The distinction is not exact, but the deciding factor is the amount of protein that can be practically purified with that method. Analytical methods aim to detect and identify a protein in a mixture, whereas preparative methods aim to produce large quantities of the protein for other purposes, such as structural biology or industrial use. In general, the preparative methods can be used in analytical applications, but not the other way round (Regnier, 1983).

2.9.5 .1 Extraction

Depending on the source, the protein has to be brought into solution by breaking the tissue or cells containing it. There are several methods to achieve this. Repeated freezing and thawing, sonication, homogenization by high pressure, filtration, or permeabilization by organic solvents.

The method of choice depends on how fragile the protein is and how sturdy the cells are. After this extraction process, soluble proteins will be in the solvent, and can be separated from cell membranes, DNA and other components of the cell by centrifugation. The extraction process also extracts proteases, which will start digesting the protein in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly, and keep the extract cooled to slow down proteolysis (Regnier, 1983).

2.9.5 .2 Precipitation

It is recognized that among the different precipitants the most widespread is ammonium sulfate (Bodzon-Kulakowska *et al.*, 2007). The addition of high amounts of this salt or other such as sodium chloride into a protein solution provokes an increase of protein interactions followed by protein aggregation and finally precipitation. This is known as salting-out process and, as the salt concentration needed for protein precipitation varies from one protein to another, it allows selective protein separation. An alternative salting-out method using decreasing solutions of salt can also be used to enrich previously precipitated protein fractions. This salting-out approach has been used to separate the main storage soybean proteins, *glucinin* and β -conglycinin (Deak *et al.*, 2006).

Another type of protein enrichment is immunoprecipitation, based upon the binding of the antigen to its specific antibody to form the antigen-antibody complex. In general it offers high recoveries of the proteins and its widely used for food allergens (Pastorello and Trambaioli, 2001).

2.9.5.3 Centrifugation

The use of centrifugation is one of the simplest methods used for isolation and enrichment/fractionation of proteins. Centrifugation can be used for different purposes. It can be a first step to separate different cell substructures where proteins of interest are locally concentrated, for instance, mitochondria, membrane, or nucleus. This process involves multiple centrifugation steps, as a result, the cellular homogenate is separated into different layers based on the molecular weight, size and shape of each component. Afterwards, solubilization steps, enrichment and fractionation steps should be carried out to isolate the protein fraction from the selected layer prior to mass spectrometry analysis.

Apart from its use separating crude mixtures of cell components, centrifugation is also commonly used to fractionate a protein mixture into different fractions. The separation takes place based on the coefficient of sedimentation of the proteins. This coefficient is usually expressed in *Svedberg units* (S), and the smaller the S value, the slower a molecule moves in a centrifugal field. Separation will depend on the mass, the shape, and the protein density. Numerous examples are found in the literature using the different coefficient of sedimentation of the proteins to carry out fractionation (Jiang et al., 2011).

2.9.5.4 Chromatographic Methods

Usually a protein purification protocol contains one or more chromatographic steps. The basic procedure in chromatography is to flow the solution containing the protein through a column packed with various materials. Different proteins interact differently with the column material, and can thus be separated by the time required to pass the column, or the conditions required to elute the protein from the column usually proteins are detected as they are coming off the column by their absorbance at 280nm. Many different chromatographic methods exist which include the following (Regnier, 1983).

2.9.5.4.1 Size Exclusion Chromatography

Size-Exclusion Chromatography (SEC) separate proteins according to their molecular mass, as the second dimension of 2-Dimensional Polyacrylamide Gel Electrophoresis (2-D-PAGE). However, unlike 2 D-PAGE this chromatography can be used under nondenaturing condition allowing the study of protein complexes. As an example, SEC has been used for the evaluation of the bread-making quality of hard spring wheat flours (Ohm *et al.*, 2009). Additional methods based on the use of chip-based arrays are gaining importance recently, with surface-enhanced laser desorption/ionization (SELDI) one of their maximum exponents (Righetti *et al.*, 2005).

2.9.5.4.2 Ion Exchange Chromatography

Ion-exchange chromatography is probably the most used, with proteins being separated according to their isoelectric point. Acidic proteins are usually fractionated by anion-exchange chromatography whereas basic proteins are usually fractionated by cation-exchange chromatography. Ion-Exchange chromatography has been often used to separate

milk proteins as reported by Gomez-Ruiz *et al.*, (2007), who used cation-exchange chromatography to separate sheep milk caseins. Before the separation begins in an Ion-exchange chromatography, a buffer is pumped through the column to equilibrate the opposing charged ions. Upon injection of the sample, solute molecules will exchange with the buffer ions as each competes for the binding sites of the resin. The length of retention for solute depends upon the strength of its charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, buffer type, buffer concentration and temperature all play important roles in controlling the separation. Ion exchange chromatography is a very powerful tool for use in protein purification and is frequently used in both analytical and preparative separations (Regnier, 1983).

2.9.5.4.3 Reverse Phase Liquid Chromatography

Reverse phase Liquid Chromatography (RP-LC) separates proteins according to their hydrophobicity. Proteins are adsorbed on a stationary phase carrying hydrophobic groups, and are eluted with increasing concentration of an organic solvent, generally acetonitrile. RP-LC is widely used in proteomics in combination with Ion-exchange chromatography and mass spectrometry analysis, usually in shotgun multidimensional strategies that are used as an alternative to 2-Dimensional-Polyacrylamide Gel technology. A special case of chromatography based on hydrophobic interactions uses a high concentration of lyotropic salts (frequently ammonium sulphate) to expose the hydrophobic parts of proteins towards the hydrophobic patches of solid-phase sorbents. Desorption is promoted by using a decreasing concentration of the lyotropic salts (Martinez-Maqueda *et al.*, 2013).

2.10. Properties of Enzymes

There is a need is to characterize an enzyme in order to understand it's function. In order to understand the function of an enzyme, there is a need to know something about it's structure. There are some properties of the enzyme that can be use to characterize it and these include:-

2.10.1 pH Optimum and Stability

Each enzyme is catalytically active only in a narrow pH range and each has a pH optimum which is often between pH5.5 and 7.5. The optimum pH is affected by the type and ionic strength of the buffer used in the assay.

The reasons for the sensitivity of enzymes to changes in pH are because

- a. sensitivity is associated with a change in protein structure leading to irreversible denaturation.
- b. the catalytic activity depends on the quantity of the electrostatic charges on the enzymes active site generated by the prototropic group of the enzyme (Lai, 2012).

2.10.2 Temperature Optimum and Stability

The thermal stability of enzymes is varied. Some lose activity at low temperatures while others can withstand high temperatures at least for a short period of time. Enzymes behave differently below freezing point. The activity is positively influenced by increasing the enzyme and substrate concentration due to formation of ice crystals. In completely frozen samples, the catalytic activity stops temporarily. Relatively few enzymes are irreversibly destroyed by freezing (Lai, 2012).

2.10.3 K_M and V_{max} Values

The Michaeli's Menten equation has two parameters: V_{max} , the maximal rate of reaction, which occurs when the substrate concentration is saturating and the K_M the Michaeli's constant, which has the value of the substrate concentration at the half maximal reaction rate. This means that if an enzyme has a small K_M value, it achieves maximal catalytic efficiency at low substrate concentrate. K_M describes enzyme affinity for the substrate, a low K_M value indicates a high affinity of enzyme for the substrate while a high K_M value indicates a low affinity (Lily, 2007)

2. 10. 4 Substrate Specificity

Substrate specificity of enzyme shows the occurrence of a distinct functional group in the substrate. Many enzymes activate only one single substrate or preferentially catalyze the conversion of one substrate while the other substrates are converted into products at lower reaction rates.

2.10.5 Effects of Metal ions for example Ca^{2+} , Mg^{2+} , Zn^{2+} and Hg^{2+}

Besides proteins, enzyme consists of metal ions and or low molecular weight non protein organic molecules. These are known as cofactors and are essential for enzymes activity. Metal ions are cofactors and stabilizers of the conformation of many enzymes (Lai, 2012).

2.10.2 Enzyme Assay

The method of determining the activity of the enzyme is known as enzyme assay. The component of an assay includes substrate, enzyme extract and diluents.

The reaction mixture is incubated at a set of temperature and time to allowed reaction to take place. The reaction is stopped usually when the activity is still proportional to the time of reaction by changing the pH, application of heat, addition of protein denaturing agent or any other means. Once the reaction is stopped the amount of substrate remaining or product formed is determined (Lai, 2012). Several techniques can be used which include titrimetry, colorimeter, ultraviolet spectrophotometry and so on.

2.11 Immobilization of Enzymes

Enzyme immobilization can be defined as the attachment of free or soluble enzymes to different types of supports resulting in the reduction or loss of mobility of the enzyme. Selection of an immobilization strategy greatly influences the properties of biocatalyst. The varying levels in activity and diffusion limitations occurring with immobilization are mainly dependent on the properties of support material and the immobilization method.

(Amjad and Mohammed, 2010).

Support materials play an important role in the usefulness of an immobilized enzyme as it should be low-cost and provide adequate large surface area together with the least diffusion limitation in the transport of substrate and product for enzymatic reactions (Krajewska, 2004). In order to fully retain the biological activity, enzymes should be attached onto surface without affecting their conformational and functional properties (Amjad and Mohammad, 2010).

Generally, the choice of a suitable immobilization strategy is determined by the physico-chemical properties of both supporting surface and the enzyme of interest (Khan *et al.*, 2006).

The use of free enzymes as compared to their immobilized forms show some significant drawbacks such as thermal instability, susceptibility to attack by protease, activity inhibition, high sensitivity to several denaturing agents and the difficulty of separating or reusing the free catalyst at the end of the reaction from the reaction mixture. Many of these restrictions can be resolved by using enzymes in immobilized forms. (Khan *et al.*, 2006).

2.11.1 Background of Enzyme Immobilization

Enzymes are biological catalyst that promotes the transformation of chemical species in living systems. These molecules consisting of thousands of atoms in precise arrangements are able to catalyse the multitude of different chemical reactions occurring in biological cells. Their role in biological processes and in health and disease has been extensively investigated. They have also been a key component in many ancient human activities, especially food processing, well before their nature or function was known. Enzymes have the ability to catalyze reactions under very mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalyzed are a number of very complex chemical transformations between biological macro molecules, which are not accessible to ordinary methods of organic chemistry. This makes them very interesting for biotechnological use. At the beginning of the 20th century, enzymes were shown to be responsible for fermentation processes and their structure and chemical composition started to come under screening. The resulting knowledge led to widespread technological use of biological catalyst in a variety of other fields such as the textile, pharmaceutical and chemical industries. (Brena and Batista-Viera, 1997).

Enzymes can catalyze reactions in different states: as individual molecules in solutions in aggregate with other entities, and as attached to surfaces. The attached or “immobilized” state has been of particular interest to those wishing to exploit enzymes for technical purpose. The term immobilized enzymes refers to “enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Katchalski-Katzir, 1993).

The introduction of immobilized catalyst has in some cases greatly improved both the technical performance of the industrial processes and their economy. The first industrial use of immobilized enzymes was reported in 1997 by Chibata and co-workers, who developed the immobilization of *Aspergillus Oryzae Aminoacylase* for the resolution of synthetic racemic D-L amino acids (Tosa *et al.*, 1966). Other major applications of immobilized enzymes are the industrial production of sugars, amino acids and pharmaceuticals. In some industrial processes, whole microbial cells containing the desired enzyme are immobilized and used as catalysts. Aside from the application in industrial processes, the immobilization technologies are the basis for making a number of biotechnological products with applications in diagnostics, bioaffinity chromatography, and biosensors. Therapeutic applications are also foreseen, such as the use of enzymes in extra-corporeal shunts. In the past three or four decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design, but there is still the need for further development. Extension of the use of immobilized enzymes to other practical processes will require new methodologies and a better understanding of current techniques (Brena and Batista-Viera, 1997).

2.11.2 History of Enzyme Immobilization

It is possible to visualize three steps in the development of immobilized biocatalysts. In the first step at the beginning of the 19th century, immobilized microorganisms were being employed industrially on an empirical basis. This was the case with both the microbial production of vinegars (by letting alcohol containing solution trickle over wood shavings overgrown with bacteria) and the development of the trickling filter or percolating process for waste water clarification (Brena and Batista-Viera, 1997).

The modern history of enzyme immobilization goes back to the late 1940's but much of the early work was largely ignored by biochemists because it was primarily published in journals of other disciplines (Trevan, 1980). The basis of the present technologies was developed in the 1960's and there was an explosive increase in publications (Tosa *et al.*, 1966). In the second step, only immobilized, single enzymes were used but by the 1970, more complex systems, including two-enzymes reactions with co-factor regeneration and living cells, were developed (Brodelius and Mosbach, 1987).

2.11.3 The Biology of Enzyme Immobilization

Although the science of enzyme immobilization has developed as a consequence of its technical utility, the advantages of having enzymes attached to surfaces have been exploited by living cells for as long as life has existed. An inquiry into the biological role of enzyme immobilization may provide some lessons for biotechnologists and serve as a second point of departure, in addition to purely chemical one. There is experimental evidence that the immobilized state might be the most common state for enzymes in their natural environment. The attachment of enzymes to the appropriate surface ensures that they stay at the site where

their activity is required. Thus immobilization enhances the concentration at the proper location and it may also protect the enzyme from being destroyed. Multimolecular assembly depends normally on weak non-covalent forces and hydrophobic interactions, but sometimes on covalent bonds as well. All these have been exploited in the development of immobilized enzymes, (Brena and Batista-Viera, 1997).

2.11.4 Choice of Supports for Enzyme Immobilization

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness towards enzymes, ease of derivatization, biocompatibility, resistance to microbial attack and availability at low cost (Trevan, 1980).

Supports can be classified as inorganic and organic according to their chemical composition. The organic support can be subdivided into natural and synthetic polymers (Cabral and Kennedy, 1991).

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions (that is, stirred tank, fluidized, and fixed beds). In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Non porous supports show few diffusional limitations but have a low loading capacity. Therefore porous supports are generally preferred because the high surface area allows for a higher enzyme loading and the immobilized enzyme receives greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers like high stability against physical, chemical and microbial degradation,

most of the industrial applications are performed with organic matrices (Brena and Batista-Viera, 1997). An excellent matrix that has been extensively used is agarose. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages of using agarose as a matrix are hydrophilic character, ease of derivatization, absence of a charged group which prevents adsorption of substrate and products, and commercial availability. However, an important limitation is the high cost. This problem can be circumvented by employing reversible methods that allow matrix regeneration and re-used. The enzymes can be attached to the support via interactions ranging from reversible physical adsorption and have linkages to stable covalent bonds. One way of classifying the various approaches to immobilizing enzymes is in two broad categories, that is irreversible and reversible methods (Kim *et al.*, 2008).

2.11.5 Methods of Irreversible Enzyme Immobilization

The concept of irreversible immobilization means that once the biocatalyst is attached to the support it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or micro-encapsulation, and cross linking (Brena and Batista-Viera, 1997).

2.11.5.1 Formation of Covalent bonds

Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage (Wong *et al.*,

2008). The covalent linkage is strong and stable and the support material of enzymes includes polyacrylamide, porous glass, agarose and porous silica (Ghous, 2001). Covalent method of immobilization is mainly used when a reaction process does not require enzyme in the product, this is the criteria to choose covalent immobilization method. This covalent binding of the enzyme with the support material involves two main steps such as, the activation of the support material by the addition of the reactive compound and the second one is the modification of the polymer backbone to activate the matrix. The activation step produces the electrophilic group on the support material, so that the support material couples reacts with the strong nucleophiles on the proteins. For example if glutaraldehyde is the activation method, in this reaction the amine group reacts with the activated matrix (Porath and Axen, 1953). The covalent binding is normally formed between the functional group in the support matrix and the enzyme surface that contains the amino acid residues. The amino acid residues involved in the covalent binding are the sulfhydryl group of cysteine, hydroxyl group of serine and threonine (Chae *et al.*, 1998). The attachment between the enzyme and the support material can be achieved either through direct linkage or through the spacer arm. The potentiality of using the spacer arm is that it provides the greater degree of the mobility to the enzymes hence the enzymes show the higher activity when compared to the direct attachment (Nisha *et al.*, 2012).

2.11.5.2 Entrapment

Enzymes are occluded in the synthetic or natural polymeric networks, it is a permeable membrane which allows the substrates and the products to pass, but it retains the enzyme inside the network, the entrapment can be achieved by the gel, fiber entrapping and microencapsulation (Bernfield and Wang, 1963). The advantage of entrapment is that it is

fast, cheap and mild conditions are required for reaction process. The disadvantage is that there is limitation in mass transfer. The support matrix protects the enzymes from microbial contamination, proteins and enzymes in the micro environment. Microencapsulation method is that the enzyme molecules are capsulated within spherical semi-permeable membranes with a selective controlled permeability. This method provides the large surface area between polymeric material and the enzyme. The drawback of this method is inactivation of enzyme during encapsulation (Rosevear *et al.*, 1987).

2.11.6 Methods of Reversible Immobilization

Reversibly immobilized enzymes can be detached from the support under gentle conditions because of the type of the enzyme-support binding. The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. The cost of the support is often a primary factor in the overall cost of immobilized catalyst (Brena and Batista-Viera, 1997).

2.11.6.1 Adsorption

This is a simple method of preparing an immobilized enzyme and the materials used for adsorption are activated charcoal, Alumina and Ion exchange resins. This method is cheap and easy for use and the disadvantage is a weak binding force between the carrier and the enzyme. This method comes under carrier bound immobilization and the process of immobilization is reversible. Adsorption is the easiest and oldest immobilization technique (Tanyolac *et al.*, 1998). The interaction between the enzyme and the surface of the matrix is

through weak forces by salt linkage, hydrogen bonds, hydrophobic bonds, ionic bonds and Van der waals forces. Based on the charges of the matrix and the protein arrangements the strongly bound, but not distorted enzyme will be formed. The advantage of enzyme adsorption is minimum activation step and as a result of minimum activation, no reagents is required. It is cheap and easy way of immobilization (Tosa *et al.*, 1966).

2.11.6.2 Ionic Binding

The bonding involved between the enzyme and the support material is salt linkages. The nature of this non covalent immobilization method is that the process will be reversed by changing the temperature, polarity and ionic strength conditions. This principle is similar to protein-ligand interactions principles used in chromatography (Guisan, 1997)

2.11.6.3 Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. It relies on well-known experimental variables such as pH, salt concentrations, and temperature. The strength of the interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule. The successful reversible immobilization of β -amylase and amyloglucosidase to hexyl-agarose carriers has been reported (Brena and Batista-Viera, 1997).

2.11.6.4 Chelation or Metal Binding

In metal linked enzyme immobilization, the metal salts are precipitated over the surface of the carriers and it has the potential to bind with the nucleophilic groups on the matrix. The precipitation of the ion on the carrier can be achieved by heating. This method is simple and the activity of the immobilized enzymes is relatively high (30-80%). The carrier and the enzyme can be separated by decreasing the pH , hence it is a reversible process. The matrix and the enzyme can be regenerated (Yusel, 2011).

2.11.6.5 Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, it can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, because the reactivity of the thiol groups can be modulated via pH alteration, the activity yield of the methods involving disulfide bond formation is usually high, provided that an appropriate thiol-reactive adsorbent with high specificity is used (Brena and Batista-Viera, 1997).

2.11.7 Properties of Immobilized Enzymes

As a consequence of enzyme immobilization, some properties of the enzyme molecules, such as its catalytic activity or thermal stability become altered with respect to those of its soluble counterpart. This modification of the properties may be caused either by changes in the intrinsic activity of the immobilized enzymes or by the fact that the interaction between the immobilized enzymes and the substrate takes place in a microenvironment that is different from the bulk solution. The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked

by the binding of the enzyme to the matrix. These effects have been demonstrated and to a lesser extent, exploited for a limited number of enzyme systems (Nisha *et al.*, 2012).

Quite often when an enzyme is immobilized, its operational stability is improved. The concept of stabilization has thus been an important driving force for immobilizing enzymes. In many cases the observed operational stabilization is usually the result of loading an excess of enzyme, which in turn makes the process diffusion controlled. However, true stabilization at the molecular level has also been demonstrated, such as the case of proteins immobilized through multipoint covalent binding. One of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. Because of the limited access of the substrate to the active site of the enzyme, the activity may be reduced to accessible surface groups of the substrate only. This steric restriction may in turn change the characteristic pattern of products derived from the macromolecular substrate. There are several strategies to avoid these steric problems such as selection of supports composed by networks of isolated macromolecular chains, careful choice of the enzyme residues involved in the immobilization, and use of hydrophilic and inert spacer arms (Trevan, 1980).

2.11.8 Uses of Immobilized Enzyme

During the initial years of the development of the field of immobilized enzymology, researchers used to find only the advantage of the immobilized enzymes in comparison to their soluble/free counterparts. Advantages of immobilized versus soluble enzymes include comparative studies in pH profile, various denaturing agents, organic solvents, temperature and others. Now recently during the last couple of decades, immobilized enzyme technology has advanced into an ever-expanding and multidisciplinary fields to analyze chemical, industrial and environmental samples (Amjad and Mohammad, 2010).

2.11.8.1 Use of Immobilized Enzyme as Biosensors

Biosensors are electrical, optical, chemical or mechanical devices with the capability to detect biological species selectively. They are often modified with biological entities to enhance their selectivity. Examples of biological recognition molecules include enzymes, antibodies and oligonucleotides. Biosensors have wide applications including biomarker detection for medical diagnostics, pathogen and toxin detection in food and water. The development of biosensors based on immobilized enzymes came out to solve several problems such as loss of enzyme, maintenance of enzyme stability and shelf life of biosensors and additionally to reduce the time of enzymatic response and offer disposable devices which can be easily used in stationary or in flow system (Amjad and Mohammad, 2010).

2.11.8.2 Uses of Immobilized Enzymes in Medicine

Immobilized enzymes are used in medicine from 1990, immobilized enzymes are used for diagnosis and treatment of diseases in the medical field. The inborn metabolic deficiency can be overcome by replacing the encapsulated enzymes (i.e, enzymes encapsulated by erythrocytes) instead of waste metabolites, the red blood cell acts as a carrier for the exogenous enzyme drugs and the enzymes are biocompatible in nature, hence there is no immune response (Johnson *et al.*, 1998). The enzyme encapsulation through electroporation is the easiest way of immobilization in the biomedical field and it is a reversible process for which enzyme can be regenerated. The enzyme when combined with the biomaterials provides biological and functional systems. The biomaterials are used in tissue engineering application for repair of the defect. The advantage of the enzyme immobilization in biomedical is that the free enzymes are consumed by the cells and not active for prolonged use, hence the immobilized enzymes remains stable, to stimulate the growth and to repair the defect. The cancer therapy which involve the delivery of enzymes to the oncogenic sites have

been improved with new methods. The nanoparticles and nanospheres are often used as enzyme carriers for the delivery of therapeutic agents (Nisha *et al.*, 2012).

2.11.8.3 Use of immobilized Enzyme for Antibiotic Production

Competition with well established, fine tuned chemical processes for antibiotics production is a major challenge for the industrial implementation of the enzyme synthesis of biologically important antibiotics such as β -lactam. Enzyme based routes are acknowledged as an environment friendly approach avoiding organic chloride solvents and working at room temperature. Among different alternatives, the kinetically controlled synthesis using immobilized penicillin G Acylase (PGA) in aqueous environment with the simultaneous crystallization of the product is the most promising one (Giordano *et al.*, 2006).

2.11.8.4 Uses of Immobilized Enzyme in Food Industry

In food industry, the purified enzymes are used but during the purification the enzyme will be denature. Hence the immobilization technique makes the enzymes stable. The immobilized enzymes are used for the production of syrups. Immobilized beta-galactosidase is used for lactose hydrolysis in whey for the production of bakers yeast. The enzyme is linked to porous silica matrix through covalent linkage. This method is not preferably used due to its cost and the other technique developed. This method was used for various purposes such as confectionaries and ice creams (Bozoglu *et al.*, 1984).

2.11.8.5 Use of Immobilized Enzyme for Biodiesel Production

Biodiesel is monoalkyl esters of long chain fatty acids. Biodiesel is produced through triglycerides (vegetable oil, animal fat) with esterification of alcohol (methanol, ethanol) in the presence of the catalyst. The production of catalyst is a drawback of high energy requirements, recovery of glycerol and side reaction which may affect the pollution. Hence

the biological production of liquid fuel with lipases nowadays has a great consideration with rapid improvement. Lipase catalyses the reaction with less energy requirements and mild conditions required. But the production of lipase is of high cost, hence the immobilization of lipases which results in repeated use and stability (Salis *et al.*, 2008). The immobilization of lipase includes several methods such as entrapment, encapsulation, cross linking, adsorption and covalent bonding. Adsorption method of immobilization is widely used in recent years when compared to covalent bond, entrapment and cross linking. In the biological production of biodiesel the methanol inactivates the lipase, hence the immobilization method is an advantage for the biodiesel production (Shimada *et al.*, 2002).

2.11.8.6 Use of Immobilized Enzyme for Bioremediation

Lot of water bodies today are mixed up with polluted industrial waste water and waste water treatments are necessary at present. The sources of dye effluents are textile industry, paper industry, leather industry and the effluents are rich in dye colorants. These effluents are threat to the environment and even in low concentration it is carcinogenic. Nowadays enzymes used in the waste water treatments are peroxidases, laccase and azo reductases. These enzymes due to harsh conditions like extreme temperature, low or high pH and high ionic strength may lose their activity; to overcome this problem immobilized enzymes are used. The Horse radish Peroxidases are entrapped in calcium alginate beads, this method is still in laboratory scale research. The immobilized laccase enzyme has the ability to degrade anthracinoid dye, Lancet blue and Ponceau red. Adsorption method is widely used because of its easy regeneration. The immobilized lipase is of high interest for the hydrolysis of oils and fats for treating the waste water from the food industry. The drawback of the conventional treatment

method is slow biodegradability and this leads to oil and fats being absorbed on the surface of sludge (Nisha *et al.*, 2012).

2.11.8.7 Use of Immobilized Enzyme in Textile Industry

The enzymes derived from microbial origin are of great interest in textile industry. Laccase, pectinase and cutinase are used for various textile application such as scouring, biopolishing, desizing, treating wools etc. Among these enzymes cellulase has been widely used from the older period till now. The textile industries now turned to enzyme process instead of using harsh chemical which causes pollution and damage to the fabrics (Rita *et al.*, 2008).

The processing of fabrics with enzymes requires high temperatures and increased pH, the free enzymes are not able to withstand the extreme conditions. Hence, immobilized enzymes used for this process are able to withstand extreme conditions and maintain their activity for more than 5-6 cycles (Nisha *et al.*, 2012).

2.11.8.8 Use of Immobilized Enzyme in Detergent Industry

The detergent industry also employs enzymes for removal of stains. The enzymes used in detergent industry are protease which is used to remove the stains of blood, egg, grass and human sweat. Amylase used to remove the starch based stains like potatoes and chocolate. Lipase is used to remove the stains of oil and fats and also used to remove the stains in cuffs and collars. Cellulase is used for cotton based fabrics in order to improve softening, color brightening and to remove soil stains (Trevan, 1980). Nowadays biotechnology cleaning agents are widely used in the detergent industries. When compared to synthetic detergents, the biobased detergents have good cleaning property (Fariha *et al.*, 2010).

The enzymes based detergents can be used in low quantity when compared to the synthetic detergent, it has increased biodegradability, does not affect the environment and works well at low temperature.

When immobilized protease was treated with wool for 72hrs with 100U at 40⁰C, the free enzyme was degraded by wool but the immobilized enzyme retained 76% of the tensile strength of wool (Nisha *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism Sample

The microorganism sample *Bacillus licheniformis* which was isolated from Kaduna metropolis soil was obtained from the microbiology department of Ahmadu Bello University, Zaria.

3.1.2 Chemicals and Reagents

Coomasie Blue G-250, Sephadex G-25, DEAE-Sephadex, bacteriological peptone, dinitrosalicylic acid (DNS), sodium alginate were purchased from representatives of Sigma Chemical Co. St Louis, England. Sodium dihydrogen phosphate, hydrogen disodium phosphate, sodium chloride, maltose, starch, glycerol, copper(II) tetraoxosulphate (VI), mercury(II) chloride, manganese(II) chloride, cobalt(II) chloride, zinc chloride, aluminium chloride, tris, glycine and ammonium sulphate are of analytical grade.

3.1.3 Equipment

UV-visible spectrophotometer, weighing balance, pH meter, incubator, micropipette, quartz cuvettes, syringes (5ml, 10ml); ultracentrifuge were used. Most of these equipments were obtained in Biochemistry Department, Multi-user Science Research Laboratory and Center for Biotechnology and Research Training (CBRT).

3.2 Methods

3.2.1 Production of Alpha-Amylase

The inoculum was prepared by the addition of about 5ml of sterile distilled water into the freshly grown nutrient agar slants. 0.5ml of the cell suspension was then inoculated into 100ml of sterilized fermentation medium and incubated at 35⁰C for 15hrs. The composition of the fermentation medium in g/l was 6.0g bacteriological peptone, 0.5g MgSO₄. 7H₂O, 0.5g KCl, 1.0g starch, pH 7 (Vidyalakshim *et al.*, 2009).

3.2.2 Extraction of Alpha-Amylase from the Fermentation Media

After incubation, the fermentation medium was harvested by centrifugation at 7,000×g for 30minutes at 4⁰C. The cell-free supernatant was collected and it was used to estimate amylase activity (Vidyalakshim *et al.*, 2009).

3.2.3 Partial Purification of Alpha-Amylase

3.2.3.1 Purification with Ammonium Sulfate

Ammonium sulfate was added slowly to the crude enzyme with constant stirring starting from 30% until 70% saturation was achieved.

The sample was allowed to stand overnight at 4⁰C, thereafter, it was centrifuged at 12,000×g for 20minutes, the supernatant was then discarded and the pellet was suspended in the assay buffer and tested for the enzyme activity as described by Rick and Stegbauer, (1974).

3.2.3.2 Gel Filtration on CM-Sephadex G-25 Column

Gel filtration was carried out using CM-Sephadex G-25 column. The pooled fractions from the ammonium sulfate precipitation were loaded onto CM-Sephadex G-25 column (1.6cmx90cm) previously equilibrated with 5mM sodium phosphate buffer, pH 7.5. The

column was eluted with the same buffer and 30 fractions of 2ml each were collected at a flow rate of 5minutes. Alpha-amylase activity was then determined in each of the fractions. Fractions containing the enzyme activity were pooled together and the protein concentration was estimated by the method of Bradford, (1976).

3.2.3.3 Ion-Exchange on DEAE-Sephadex Column

Ion-exchange was carried out using DEAE-Sephadex column. The pooled fractions from the gel filtration column were loaded unto DEAE-Sephadex column (1.6cmx90cm) previously equilibrated with 5mM sodium phosphate buffer (pH 7.5). The column was eluted with a linear gradient of NaCl (0.5M, 1M, 1.5M, 2.0M, 2.5M and 3.0M) in the buffer and 30 fractions of 2ml each were collected at a flow rate of 14minutes. (Ikram-ul-Haq *et al*; 2010). Alpha-amylase activity was then determined in each of the fractions. Fractions containing the enzyme activity were pooled together and the protein concentration was estimated by the method of Bradford, (1976).

3.2.4 Determination of Total Protein

The protein concentration was quantified according to the method described by Bradford, (1976). The assay is based on the principle that the maximum absorbance of an acidic solution of coomasie blue G-250 shift from 365nm to 595nm when binding of the dye to protein occurs. Both the hydrophobic and ionic interactions stabilize the ionic form of the dye, causing a visible color change.

Coomasie Brilliant Blue G-250 (25mg) was dissolved in 12.5ml 95% ethanol. To this solution, 25ml 85% (w/V) phosphoric acid was added. The resulting solution was diluted to a final volume of 250ml. Protein solution (0.1ml) was pipetted into test tubes. Three milliliters of protein reagent was added to the text tube and the contents mixed by shaking.

The absorbance at 595nm was measured after 5min and before 1hr against a reagent blank prepared from 0.1ml of the appropriate buffer and 3ml of the protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in the unknown samples.

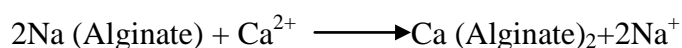
3.2.5 Alpha-Amylase Assay

The enzyme assay was performed using the Rick and Stegbauer, (1974) method. The enzyme solution (0.5ml) was transferred to a test tube containing 0.5ml of 1.0% soluble starch solution. The mixture was incubated at 60⁰C for 10min. Then 1.0ml of dinitrosalicylic acid reagent was added to each test tube. The tubes were placed in boiling water for 5min to stop the reaction and it was then cooled at room temperature. The contents of the tube was diluted up to 10ml with distilled water. The absorbance was then determined at 540nm using a spectrophotometer and it was converted to micromole of maltose from the standard. One enzyme unit was taken as being equivalent to that amount of enzyme which catalyses the hydrolysis of soluble starch into one micromole of maltose per milligram of protein per minute.

3.2.6 Enzyme Immobilization

The method of James, (1996) was used for the immobilization. 30.63g of sodium alginate was dissolved in 87.5ml of distilled water and autoclaved at 121⁰c for 15minutes. It was then left to cool to room temperature after which 17.5ml of the partially purified alpha amylase enzyme was added and the mixture was then carefully pumped through a sterile syringe dropwise into a beaker containing 250ml of 0.12M calcium chloride solution to form beads. The sodium- alginate enzyme mixture when added to calcium chloride solution gelled to form beads thereby entrapping the enzyme. The beads were then left in solution for 1hr at

4⁰C to ensure complete precipitation. The process of gelation was the exchange of calcium ions for sodium ions as represented in the equation.



3.2.7 Characterization of the Partially Purified Alpha-Amylase

3.2.7.1 Determination of Optimum Temperature

The optimum temperature of the free and immobilized enzyme was determined in 50mM sodium phosphate buffer (pH 7.0) over a temperature range of 20 to 70⁰C for 30min. 0.5ml and 0.5g of the free and immobilized enzyme was used for the test respectively (Jianguo *et al.*, 2011).

3.2.7.2 Determination of Optimum pH

The optimum pH for the free and immobilized enzyme was determined at 50⁰C in 50mM citrate buffer (pH 4.0 to 5.0), 50mM sodium phosphate buffer (pH 6.0 to 7.0), 50mM Tris-HCl (pH 8.0 to 9.0) and 50mM Glycine-NaOH (pH 10.0 to 11.0). All pH values was adjusted at room temperature. 0.5ml and 0.5g of the free and immobilized enzyme was used for the test respectively. (Jianguo *et al.*, 2011).

3.2.7.3 Determination of K_M and V_{max}

To obtain K_M and V_{max} for soluble starch, 2ml of 0.2, 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 7% w/v soluble starch in 50mM phosphate buffer (pH 7.0) were mixed with 2ml and 2g of the free and immobilized enzyme respectively and the mixture were incubated at 50⁰C for 20min and the reaction were stopped thereafter by heating at 100⁰C for 10min. K_M and V_{max} values were obtained from Lineweaver-Burk plot. (Jianguo *et al.*, 2011).

3.2.7.4 Determination of Effect of Metal Ions

The effect of metal ions on the activity of the free and immobilized enzyme was examined by determining their activities after 1h incubation at 4⁰C in 50mM phosphate buffer (pH 7.0), containing various metal ions at 5mM. The activity assayed in the absence of metal ions was defined as the control. The metal ions tested include Na⁺, Cu⁺⁺, Ca⁺⁺, Mn⁺⁺, Co⁺⁺, Zn⁺⁺, and Al⁺⁺⁺, (Jianguo *et al.*, 2011).

3.3 Data Analysis

The graphs were plotted using excel programming and the tabular data were analysed by using one-way analysis of variance (ANOVA). The results were expressed as mean ± standard error of mean (SEM). P values of less than 0.05 were taken as significant (P<0.05).

CHAPTER FOUR

RESULTS

Incubation Time for Alpha-amylase Production

The production of alpha-amylase was lowest at 3h incubation period and this was 0.05 unit/mg/min while the maximum production was observed at 15h incubation period and this was 0.41 unit/mg/min. Further increase in the incubation period did not show any significant increase in enzyme production rather it was decreased. The enzyme production continue to decrease gradually until 24h incubation period where it was 0.06 unit/mg/min (Figure 4.1).

Purification Profile of the Alpha-amylase from *B.licheniformis*.

Maximum enzyme activity was obtained in the 70% fraction with a high yield of 42.9% and specific activity of 3.09. The collected fraction was subjected to gel filtration on CM-sephadex G.25 column and a yield of 23% and specific activity of 10.16 was observed. Finally the fraction obtained from the second step of the purification process was subjected to ion exchange chromatography on DEAE-sephadex column and this yielded a partially purified enzyme with a specific activity of 12.14 unit/mg of protein. Overall, the specific activity increased about 15 fold with about 20.2% yield of activity and a 15.6% yield of protein (Table 4.1).

Protein Pattern and Activity of Alpha-amylase from CM-Sephadex G-25 Column

From Figure 4.2, fraction 8 contains the first alpha-amylase peak with an activity of 1.941 unit/mg/min while fraction 18 contains the second peak with an activity of 2.077 unit/mg/min. The first protein peak is at fraction 8 with a value of 0.19mg/ml while the second protein peak is at fraction 18 with a value of 0.13mg/ml (Figure 4.2).

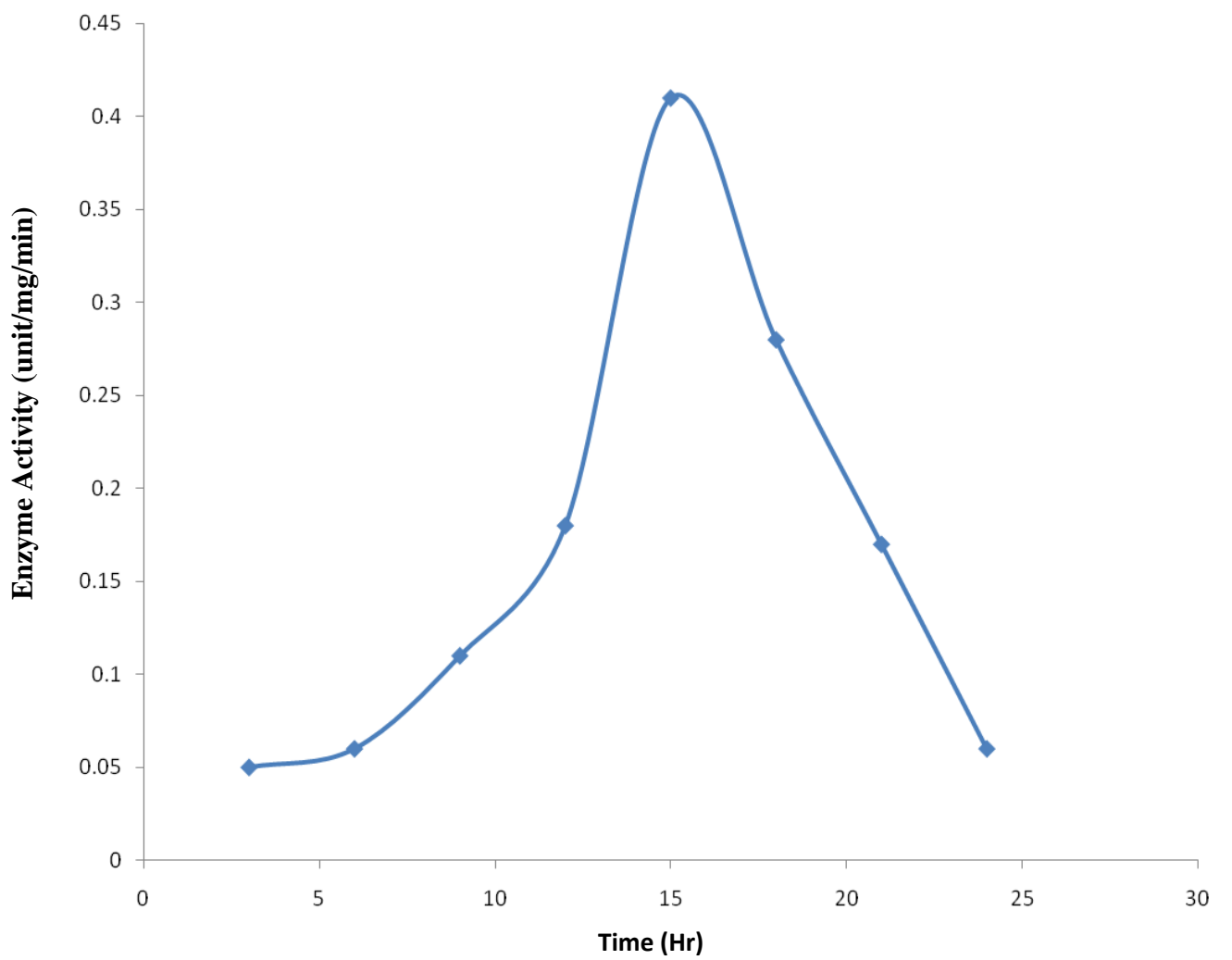


Figure 4.1: Incubation time for Alpha amylase Production.

TABLE 4.1: Purification profile of the alpha-amylase from *B. licheniformis*

Purification Step	Volume (ml)	Total Activity (unit/mg/min)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg)	Percentage Yield	Purification Fold
Crude	70	25.2	0.46	32.2	0.78	100	1
Ammonium Sulphate Precipitation	10	10.8	0.35	3.5	3.09	42.9	4.0
Gel Filtration on CM- Sephadex G-25 Column	3	5.79	0.19	0.57	10.16	23.0	13.0
Ion exchange Chromatography on DEAE-Sephadex Column	3	5.1	0.14	0.42	12.14	20.2	15.6

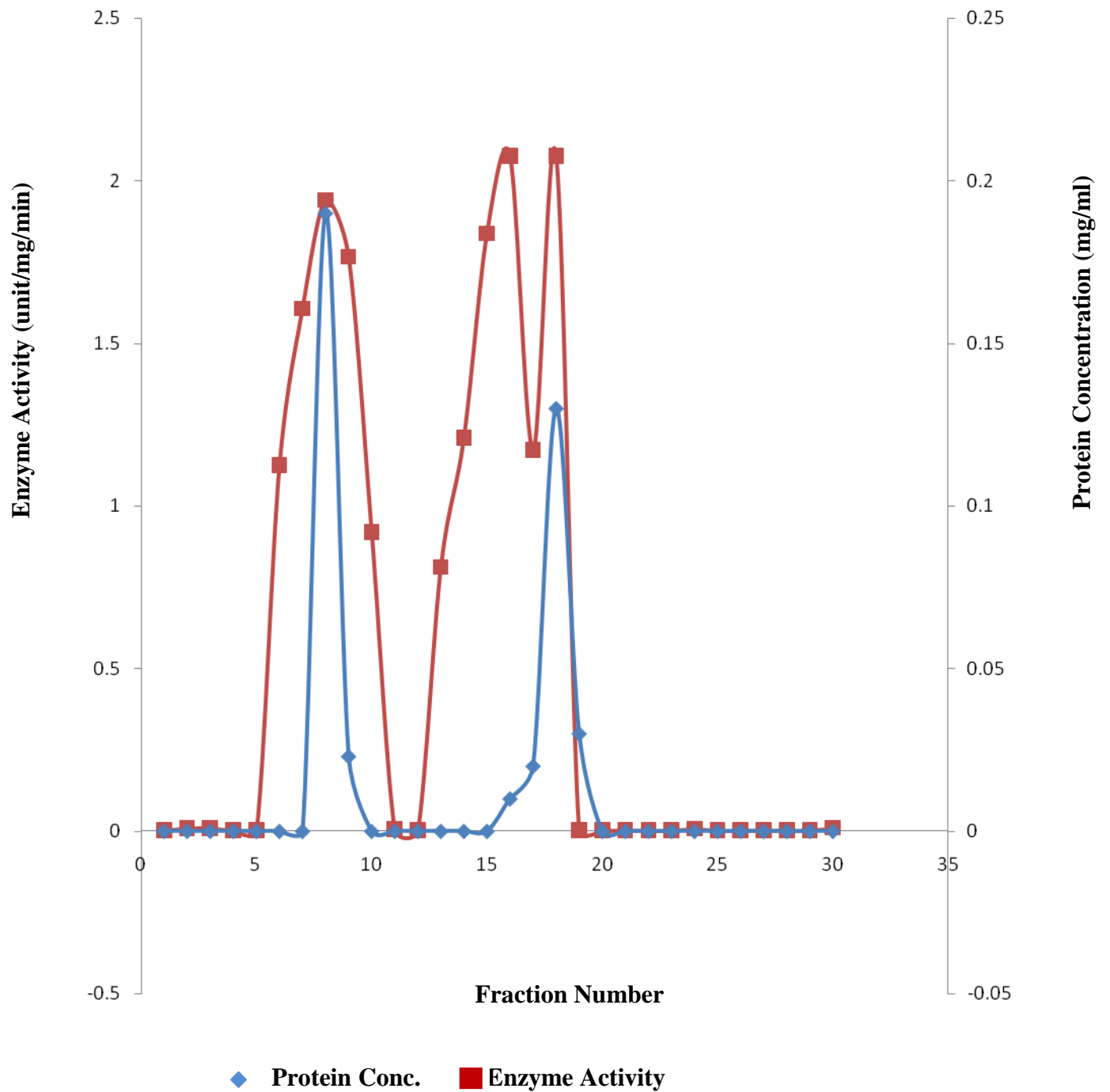


Figure 4.2: Protein pattern and activity of Alpha Amylase from CM-Sephadex G-25 Column

Protein Pattern and Activity of Alpha-amylase from DEAE-Sephadex Column

From Figure 4.3, fraction 15 contains the first alpha-amylase peak with an activity of 1.705 unit/mg/min while fraction 19 contains the second peak with an activity of 0.716 unit/mg/min. The first protein peak is at fraction 15 with a value of 0.14 mg/ml while the second peak is at fraction 19 with a value of 0.04 mg/ml (Figure 4.3).

Optimum Temperature of the Free and Immobilized Alpha-Amylase

The optimum temperature of the free enzyme was observed to be 60⁰C with activity of 0.926 unit/mg/min while the optimum temperature of the immobilized enzyme was 70⁰C with enzyme activity of 1.02 unit/mg/min (Figure 4.4).

Optimum pH of the Free and Immobilized Alpha-Amylase

The optimum pH of the free enzyme was observed to be pH 7 with an activity of 0.549 unit/mg/min while the optimum pH of the immobilized enzyme was pH 8 with activity of 0.849 unit/mg/min (Figure 4.5).

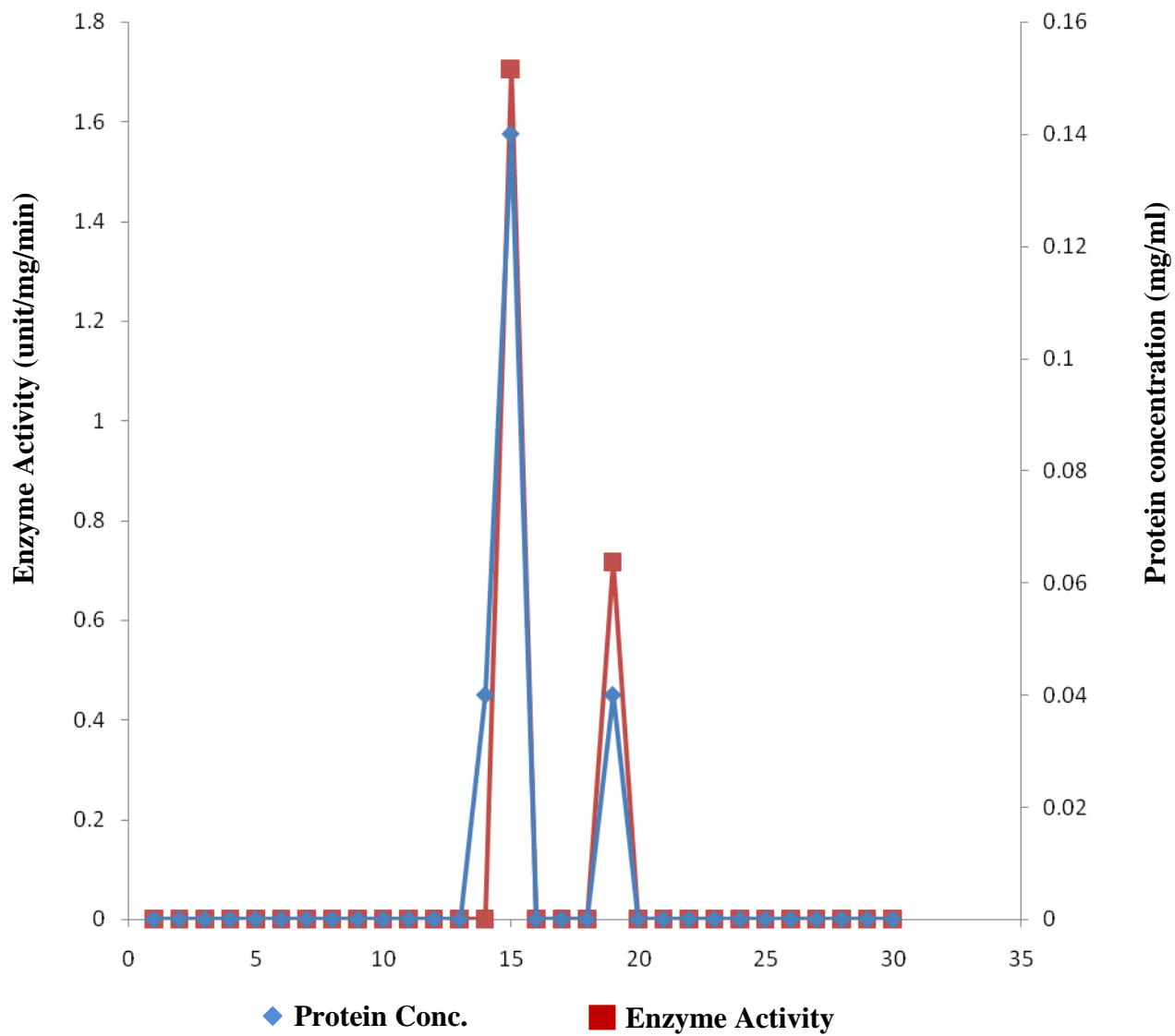


Figure 4.3: Protein pattern and activity of Alpha Amylase from DEAE – Sephadex Column

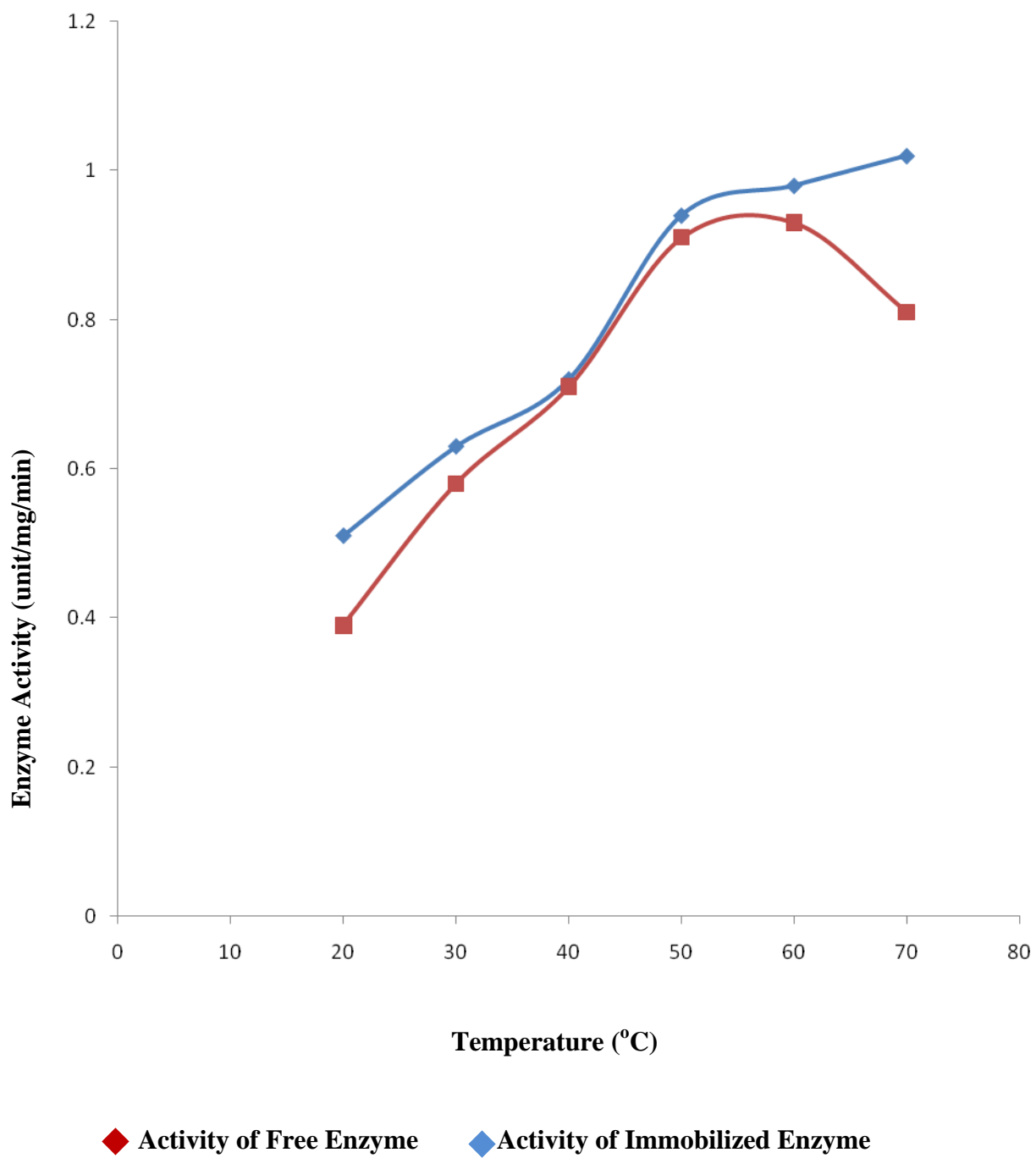


Figure 4.4 Optimum Temperature of the Free and Immobilized Alpha Amylase

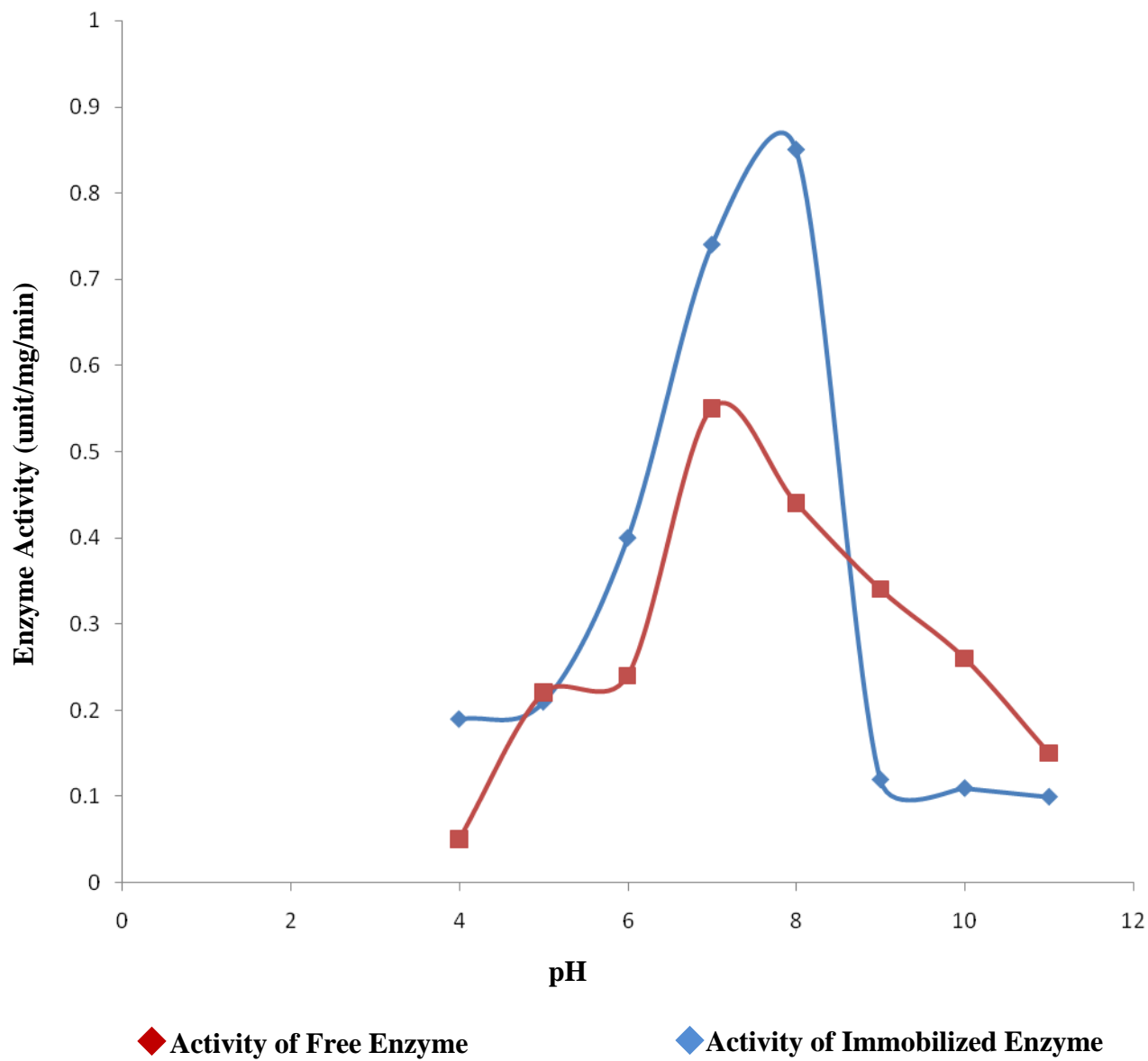


Figure 4.5: Optimum pH of the Free and Immobilized Alpha Amylase

Lineweaver-Burkplot for Determination of the k_M and V_{max} of the Free and Immobilized Alpha-Amylase

Based on the Lineweaver -Burk equation, the V_{max} value obtained for the free enzyme was 0.4unit/mg/min where as it's k_M value was 2.5 mg/ml while the V_{max} value obtained for the immobilized enzyme was 0.95 unit/mg/min and k_M is 1.0mg/ml (Figure 4.6).

Effect of Metal Ions on Alpha-amylase Activity

Among the cations tested Ca^{2+} , Mn^{2+} , Co^{2+} and Na^+ were found to have activating effect on the free enzyme compared with the control with enzyme activity of 0.353, 0.242, 0.195 unit/mg/min. Cu^{2+} has no significant effect on the free enzyme activity. It has an activity of 0.184 unit/mg/min. Hg^{2+} , Zn^{2+} and Al^{3+} were found to be strong inhibitors of the free enzyme compared with the control with enzyme activity of 0.049, 0.085, 0.163 unit/mg/min (Figure 4.7).

For the immobilized enzyme, Ca^{2+} , Mn^{2+} , and Na^+ were found to have stimulating effect on the immobilized enzyme compared with the control with enzyme activity of 0.434, 0.413, 0.388 unit/mg/min respectively. Cu^{2+} , Co^{2+} and Al^{3+} have no much significant effect on the immobilized enzyme with enzyme activity of 0.365, 0.369, 0.35 unit/mg/min.

Hg^{2+} , Zn^{2+} were found to be strong inhibitors of the immobilized enzyme with enzyme activity of 0.309 and 0.314 unit/mg/min (Figure 4.7).

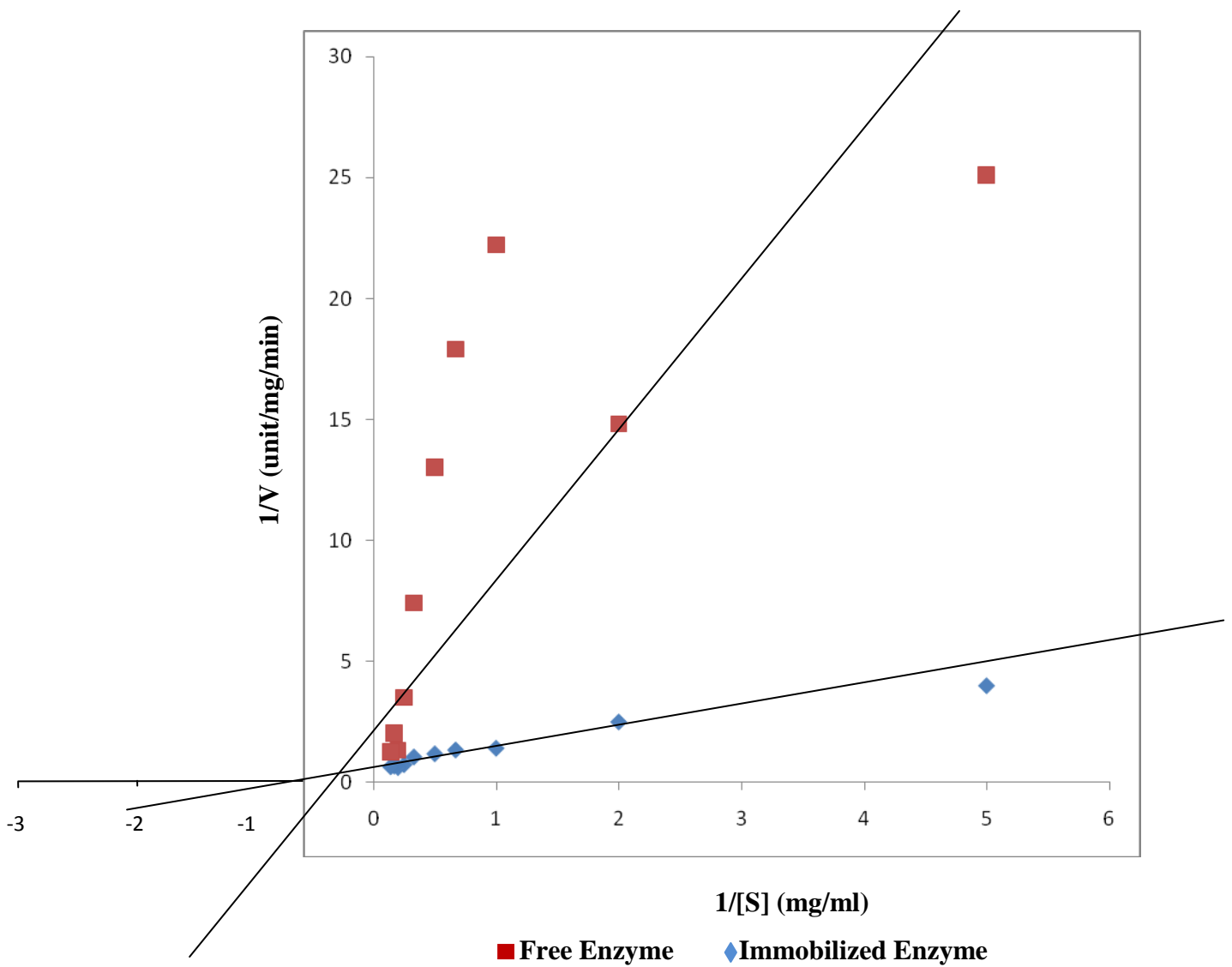


Figure 4.6: Lineweaver-Burk plot for determination of the K_M and V_{max} of the Free and Immobilized Alpha Amylase

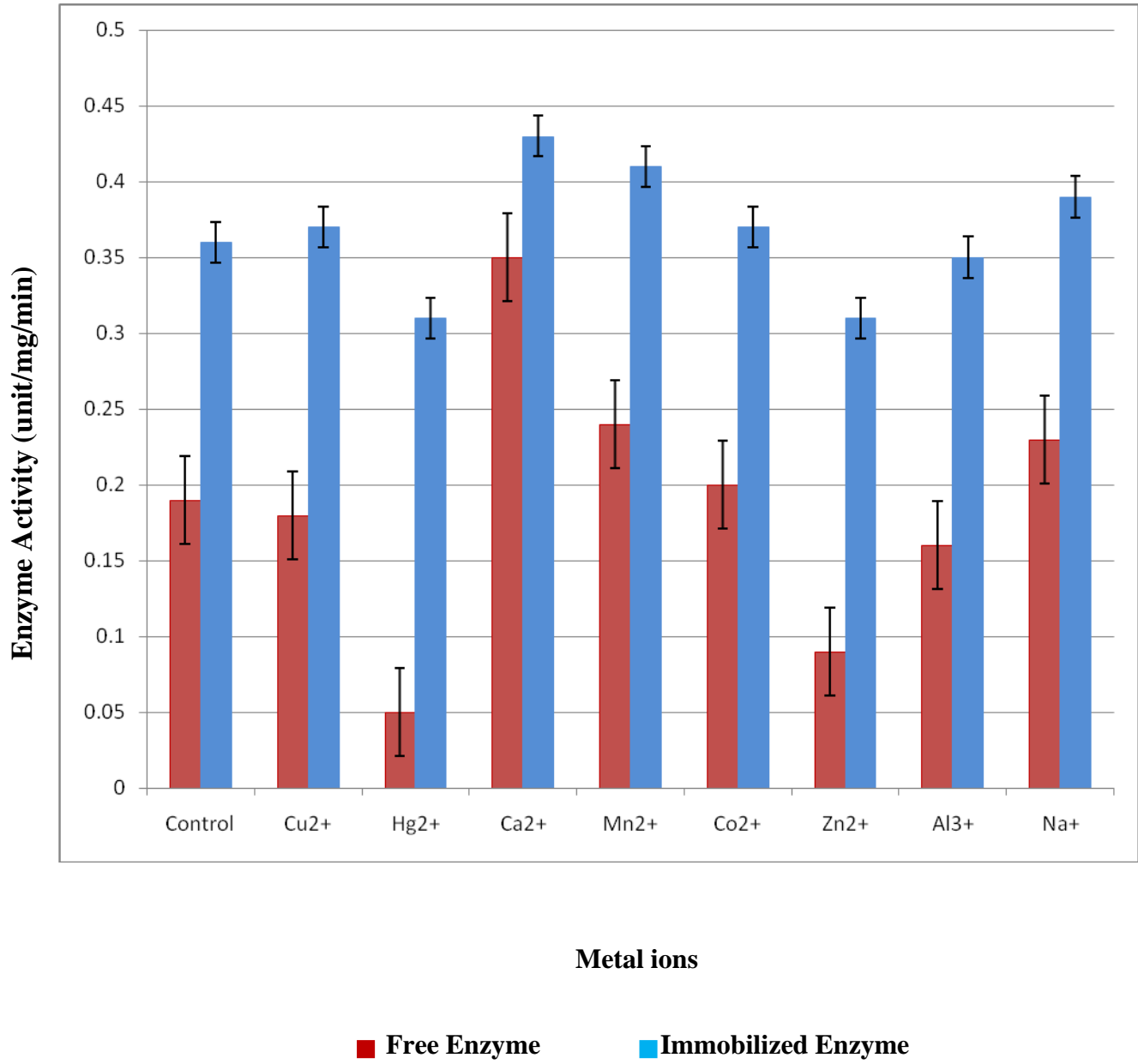


Figure 4.7: Effect of Metal ions on the Activity of the Free and Immobilized Alpha Amylase

CHAPTER FIVE

5.0 DISCUSSION

The optimum incubation period for *B. licheniformis* for alpha amylase production was 15h, with an enzyme activity of 0.41unit/mg/min. Incubation beyond the optimum time course was followed by a decrease in the enzyme activity which gradually declined to 0.06 unit/mg/min after 24hr.

The decreased activity in the later phase of growth was probably due to the repression of the catabolite by the readily metabolizable substrate glucose (Lim *et al.*, 1998). Catabolite repression is an important part of global control system of various bacteria and other micro-organisms. Catabolite repression allows bacteria to adapt quickly to a preferred carbon and energy source first. This is usually achieved through inhibition of synthesis of enzymes involved in catabolism of carbon sources other than the preferred one. Catabolite repression can be initiated by glucose and other carbon sources (Deutscher, 2008). Similar results have been reported in *B. flovothermus* after 24h (Tourney *et al.*, 2007) and in *B. amyloliquefaciens* after 72h (Sarıkaya and Gurgun, 2000).

Vidyalakshim *et al.*, [2012] also reported optimum growth for alpha amylase from *B.subtilis* KC3 to be 48h with activity of 16 U/ml. The short incubation period for *Bacillus spp.* compared with other bacteria and fungi offers unique potential for inexpensive enzyme production (Saad, 2010). The short incubation time observed for *B.licheniformis* (15h) in this research work is an added advantage and also a new finding.

The disparity in the time observed for optimum production of alpha-amylase in this research work compared with other researchers may be due to the fact that the *B. licheniformis* used for this research work belong to a different strain of *B. licheniformis*. Total amylase activity from the culture fluid of *B. licheniformis* was 25.2 unit/mg/min. The ammonium sulfate precipitation reduced the

total enzyme activity by 57.1% but only gave an improvement of specific enzyme activity of 4.0. The effect of gel filtration chromatography decreases the total protein significantly from 0.35 to 0.19mg but only to decrease its total enzyme activity by 46.4%. The effect of ion exchange decreases the total protein from 0.19 to 0.14mg and decreases the total Enzyme activity by 12.0%. The overall purification fold of 15.6 was obtained. All these results indicate that the ammonium sulfate precipitation and gel filtration chromatography are the main reason for the total enzyme activity loss in the purification processes.

The loss in the enzyme activity observed in the two steps of ammonium sulfate precipitation and gel filtration chromatography may be as a result of linking of reactants to separation matrices, insolubilities or losses in to the fringe fractions during separation procedures (Etienne *et al.*,2012).

The loss in enzyme activity observed in these two steps could also be as a result of the presence of contaminants which interfere with the purification steps or with enzyme activity assay (Etienne *et al.*, 2012).

At the end of the purification processes, the overall specific activity increased about 15.6 fold and enzyme activity recovery was 20.2%. This result was similar to that reported by Lichko *et al*, (2000) who did purification and characterization of a polyphosphate enzyme from mitochondrion of *Saccaromyces cerevisiae*. The increase of specific activity was not too high (only 17 times) with recovery of 11.7%. Liu *et al*, (2011) who did purification on alpha amylase from marine *Pseudomonas sp.* K6-28-040 reported an increase in specific activity of about 5 fold and enzyme recovery yield of about 44%. The protein pattern and activity of the alpha-amylase obtained from the CM-Sephadex G-25 column chromatography shows alpha-amylase first peak activity at fraction 8 and the second peak at fraction 18 with activity of 1.941 and 2.077 unit/mg/min respectively. Also

there are two protein peaks with the first peak at fraction 8 and second peak at fraction 18 with values of 0.19 and 0.13 mg/ml respectively.

Yandri *et al*, (2010) using Sephadex G-100 to purify extracellular alpha-amylase from locale bacteria isolate *B.subtilis* ITBCCB148 reported, only one protein peak showed alpha-amylase activity and that fraction was at fraction 18 with activity of 115 U/ml. The protein pattern and activity of the alpha-amylase obtained from the ion-exchange chromatography on DEAE-Sephadex shows two peaks. The first peak is at fraction 15 while the second one is at fraction 19 with activity of 1.705 and 0.716 unit/mg/min. There are two protein peaks. The first and second peak is at fraction 15 and 19 with values of 0.14 and 0.04 mg/ml. Yandri *et al*, (2010) using DEAE-Cellulose ion-exchange column to purify extracellular alpha-amylase from locale bacteria isolate *B. subtilis* ITBCCB148 reported 7 protein peaks with alpha-amylase activity in peaks 6 and with the highest amylase activity at peak with activity of 190U/ml. 0.0021mg of protein was entrapped per bead in this research work. Aliya *et al*, 2009 reported that 1.2ml of enzyme was trapped per one gram of bead when he immobilized alpha-amylase from *B. subtilis* by entrapment in calcium alginate beads.

The result for the optimum temperature of the free and immobilized enzyme shows that the immobilized enzyme is more stable than the free enzyme with an optimum temperature of 70⁰c while that of the free enzyme is 60⁰c. The higher temperature profile of the immobilized alpha amylase could result from a lower temperature in the gel microenvironment compared to the bulk solution. (Kennedy and Cabral, 1987). Immobilization of a protein may produce some distortion on the active site, thereby reducing the overall mobility of the protein groups. Moreover, enzymes that suffer great conformational changes during catalysis could have these changes distorted when immobilized thereby producing enzymes with fully altered catalytic activities (Cesar *et al.*, 2007).

Thermal stability during immobilization generally results from the molecular rigidity introduced by attachment to a rigid support and creation of a protected microenvironment (Padma and Laxmi, 2008). Dhanya *et al*, (2009) reported an increase in the activity of the immobilized enzyme compared with that of the free enzyme at different temperatures when alpha amylase from *B. amyloliquefaciens* was immobilized in calcium alginate beads by entrapment which is in agreement with the results obtained in this research work.

The catalytic activity of the free and immobilized enzyme in a covalent bound system was compared at different temperatures and the immobilized enzyme had higher activity due to their higher resistance to heat than the free form (Dhanya *et al.*, 2009). Ikram-ul-Haq *et al*, (2010) reported an optimum temperature range of 60⁰ C-70⁰C for purified alpha amylase from *B. licheniformis* EMS-6 with enzyme activity of 120 U/ml/min.

The free and immobilized enzyme work best in neutral to alkaline pH. Therefore this enzyme can be useful in starch and textile industries. The enzyme activity for the immobilized enzyme is about 2 times that of the free enzyme at pH 8. Yandri *et al*, (2010) reported optimum pH of 6.0 for the purified enzyme obtained from *B. subtilis* with relative enzyme activity of 97% while Shafaat *et al*, (2011) reported an optimum pH of 7 for purified alpha amylase from *B. subtilis* with enzyme activity of 199 U/ml which is in agreement with our result.

Where the enzyme is immobilized within a porous solid such as a protein aggregate or crystal, an inert porous support, some sources of enzyme inactivation such as aggregation, adsorption onto hydrophobic surface and auto-proteolysis are minimized (Don and Roberto, 2011). The deformation and reduced conformational mobility of protein structures induced by immobilization can greatly alter (and in certain cases improve) enzyme specificity and enantioselectivity and has been shown to

reduce substrate inhibition (Don and Roberto, 2011). Immobilization of enzymes inside a porous structure of a solid may permit to have the enzyme molecule fully dispersed and without the possibility of interacting with any external interface. Thus, immobilization will stabilize the enzyme against interaction with molecules from the enzymatic extract, preventing aggregation, autolysis or proteolysis by proteases from the extract (that will also dispersed and immobilized). Moreover the immobilized enzyme molecules will not be in contact with any external hydrophobic interface such as air bubbles. These bubbles may produce enzyme inactivation of soluble proteins but cannot inactivate the enzymes immobilized on a porous solid (Cesar *et al.*, 2007).

The enzyme follows the Michaelis-Menten kinetic of catalysis. The results of the V_{\max} for the free and immobilized enzyme showed that the V_{\max} of the immobilized enzyme is far higher than that of the free enzyme, which is about 2 times higher. This shows that the immobilized enzyme operate at higher activity than the free enzyme. The immobilized enzymes have a lower K_M value compared with the free enzyme. Low values of K_M indicates high affinity of the enzyme for the substrate (Hamilton *et al.*, 1998). Therefore the results showed that the immobilized enzyme has better affinity towards the starch substrate than the free enzyme. Yandri *et al.*, (2010) reported a K_M value of 2.5mg/ml and a V_{\max} value of 192.3 μ mol/ml/min for a purified alpha amylase from *B.subtilis* ITCCB148. Liu *et al.*, (2011) reported a K_M value of 1.73 ± 0.3 mg/ml and V_{\max} value of 1.24 ± 0.02 mg/ml/min respectively for purified alpha amylase from marine *Pseudomonas sp.* k6-28-040. The disparity in the values of the K_M and V_{\max} obtained in this research work compared with the values obtained by other researchers could be because the K_M and V_{\max} values of different enzyme are difficult to compare because they depend on the substrate used and the reactions conditions (Ikram ul-Haq *et al.*, 2010).

Five millimolar CaCl_2 , MnCl_2 , and NaCl increases the production of alpha amylase by *B. licheniformis* in both free and immobilized enzyme. In addition Co^{2+} have an activating effect on the free enzyme but not on the immobilized enzyme (Figure 4.10). Vidyalakshim *et al*, (2012) reported that 0.1% CaCl_2 increases the production of alpha amylase by *B. subtilis* KC3 with enzyme activity of 30 U/ml compared with control having enzyme activity of 22 U/ml which is in agreement with our result.

Liu *et al*, (2011) reported a similar result in which five millimolar Ca^{2+} , Mn^{2+} and Co^{2+} have a stimulating effect on the alpha amylase from marine *Pseudomonas sp.* K6-28-040 with relative enzyme activity of 117, 118, 143.7% compared with control having enzyme activity of 100%. Ca^{2+} had significant effects on the metabolism and physiology of bacteria and was also found to be effective on enzyme activity (Deshpande and Cheryan, 1984). Cu^{2+} has no significant effect on the activity of both the free and immobilized enzyme. In addition Co^{2+} and Al^{3+} have no significant effect on the activity of the immobilized enzyme. Hg^{2+} and Zn^{2+} were found to have strong inhibiting effect on the activity of both the free and immobilized enzyme. In addition Al^{3+} has an inhibitory effect on the free enzyme but not on the immobilized enzyme. Liu *et al*, (2011) reported that five millimolar Hg^{2+} and Al^{3+} strongly inhibit the alpha amylase from marine *Pseudomonas Sp.* K6-28-040 with relative enzyme activity of 35.6 and 46.2% respectively compared with control having relative activity of 100% which agrees with our results. Vidyalakshims *et al*, (2012) reported 0.1% ZnSO_4 completely inhibited alpha amylase production which agrees with our result. There is no documented work on the effect of metal ions on immobilized alpha amylase.

CHAPTER SIX

6.0 SUMMARY AND CONCLUSIONS

6.1 Summary

The local strain of *B.licheniformis* used in this research work is a good producer of the enzyme alpha-amylase. The alpha-amylase produced by this bacterium is a thermostable alpha-amylase which can be used in the textile and starch processing industries.

The immobilization of this enzyme in calcium alginate beads increased its optimum temperature which will in turn affects its shelf life and this will help in the repeated use of the enzyme.

The immobilized alpha-amylase from the local strain of *B.licheniformis* used in this research work can also be used commercially as a replacement of free enzyme system because the immobilized enzyme has shown greater operational flexibility and higher enzymatic activity than the free enzyme.

6.2 Conclusions

- a) Immobilization of alpha-amylase from *B. licheniformis* by entrapment in calcium alginate beads affects its optimum temperature, pH and kinetics.
- b) The bacteria, *Bacillus licheniformis* produces a thermostable alpha-amylase with optimum temperature of 60⁰C and 70⁰C for the free and immobilized enzymes respectively and therefore can be used in starch processing and textile industries.

- c) The immobilization of this enzyme in calcium alginate beads increased its optimum temperature and this will also increase its shelf life.
- d) The enzyme can work best in neutral to alkaline pH and therefore can be useful in starch and textile industries.

RECOMMENDATIONS

- a) Further studies should test the reusability of this alpha amylase immobilized by entrapment in calcium alginate beads.
- b) There is a need to develop more thermotolerant and pH tolerant alpha amylases from the local strain of *B.licheniformis* used in this research work, modify it genetically to acquire desired properties in the enzyme.

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APPENDICES
APPENDIX 1.0

CALCULATION FORMULA FOR THE PURIFICATION PROFILE

$$\text{Total Protein} = \frac{\text{Total Activity}}{\text{Specific Activity}}$$

$$\text{Total Activity} = \text{Specific Activity} * \text{Total Protein}$$

$$\text{Specific Activity} = \frac{\text{Enzyme Activity (Total Activity)}}{\text{Total Protein}}$$

$$\text{Purification Fold} = \frac{\text{Specific Activity of Protein at a Purification Step}}{\text{Original Specific Activity (Crude)}}$$

$$\text{Percentage Yield} = \frac{\text{Total Activity of Protein at step}}{\text{Total Activity of Original Purification (Crude)}} * 100$$

$$\text{Enzyme activity} = \frac{\text{Conc. Extrapolated from Std curve} * \text{Vol. of Rxn mixture}}{\text{Time in mins}}$$

APPENDIX 2.0

PREPARATION OF STANDARD REAGENTS

0.005M CuSO₄ exactly 0.8g of CuSO₄ was weighed and dissolved in one liter of deionized water

0.005M HgCl₂ exactly 1.36g of HgCl₂ was weighed and dissolved in one liter of deionized water

0.005M CaCl₂ exactly 0.55g of CaCl₂ was weighed and dissolved in one liter of deionized water

0.005M MnCl₂ exactly 0.63g of MnCl₂ was weighed and dissolved in one liter of deionized water

0.005M CoCl₂ exactly 0.65g of CoCl₂ was weighed and dissolved in one liter of deionized water

0.005M ZnCl₂ exactly 0.68g of ZnCl₂ was weighed and dissolved in one liter of deionized water

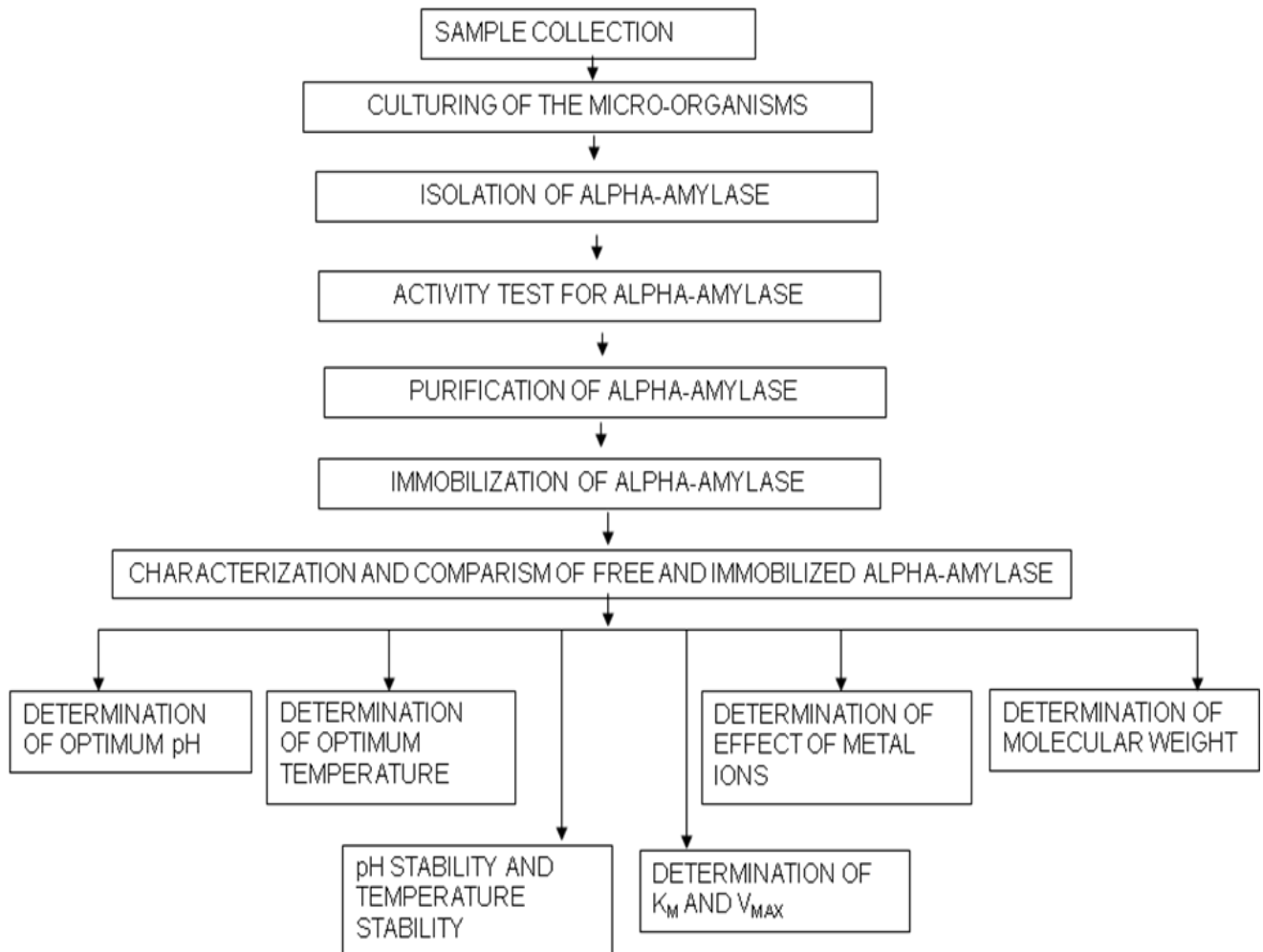
0.005M AlCl₃ exactly 0.67g of AlCl₃ was weighed and dissolved in one liter of deionized water

0.005M NaCl exactly 0.23g of NaCl was weighed and dissolved in one liter of deionized water

0.2M CaCl₂ exactly 22.2g of CaCl₂ was weighed and dissolved in one liter of deionized water

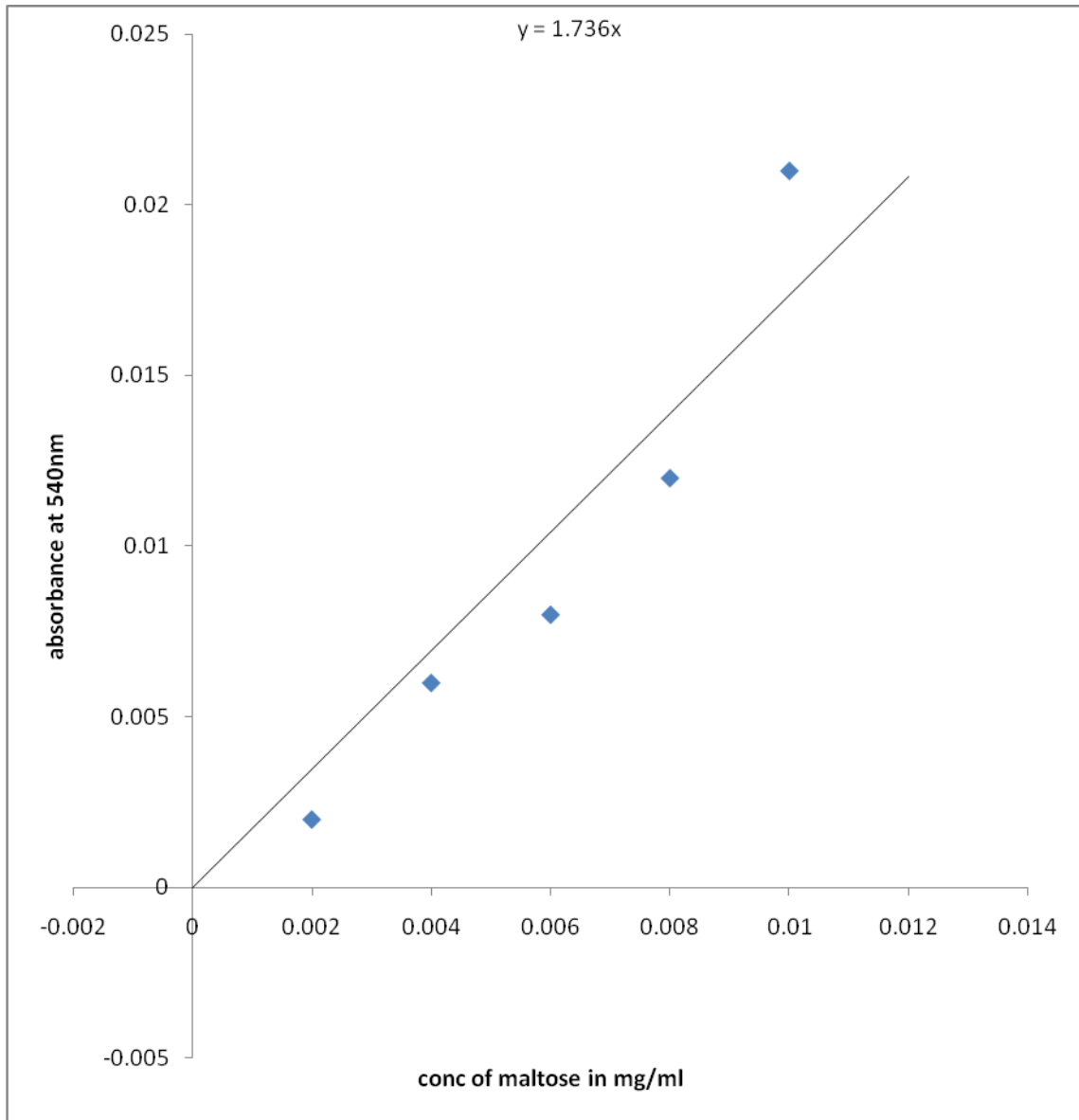
APPENDIX 3.0

Experimental design for the isolation, purification and characterization of alpha-amylase.



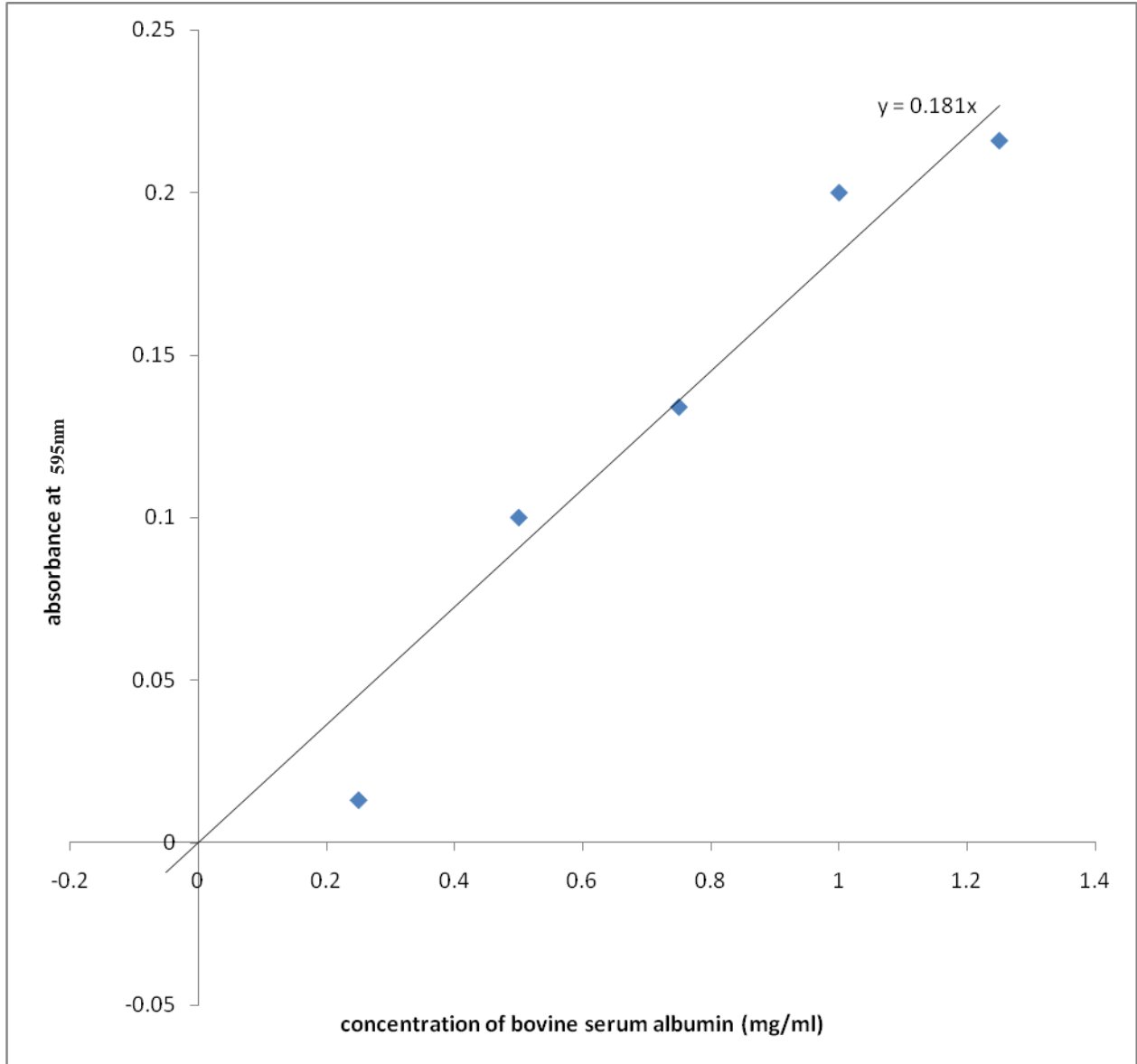
APPENDIX 4.0

STANDARD CURVE FOR ENZYME ACTIVITY USING MALTOSE



APPENDIX 5.0

STANDARD CURVE FOR PROTEIN ESTIMATION USING BSA



APPENDIX 6.0

CALCULATION OF THE AMOUNT OF PROTEIN ENTRAPPED PER BEAD.

8.75ml of the enzyme extract was immobilized in 40.96g of beads

Total amount of protein in 8.75ml

$$= 0.14\text{mg} \times 8.75\text{ml}$$

$$= 1.225\text{mg of protein}$$

$$1.225\text{mg of protein} \equiv 40.96\text{g}$$

Total number of beads = 572 beads

$$1.225\text{mg of protein} \equiv 40.96\text{g} \equiv 572 \text{ bead}$$

$$1.225\text{mg of protein} \equiv 572 \text{ beads}$$

therefore Xmg of protein \equiv 1 bead

$$X = \frac{1.225 \times 1}{572}$$

$$= \mathbf{0.0021\text{mg}}$$
 of protein per bead.

Amount of protein = 0.14mg/ml

Therefore Amount of protein in 0.5ml

$$= \frac{0.14}{2} = 0.07\text{mg}$$

0.07mg of protein was present in 0.5ml of the extract 1.225mg is present in 40.96g of bead

Therefore Xmg is present in 0.5g of beads

$$X = \frac{1.225 \times 0.5}{40.96} = \frac{0.6125}{40.96}$$

$$= 0.014954\text{mg}$$

$$= 0.0150\text{mg}$$

Therefore **0.015mg** of protein was present in 0.5g of beads

APPENDIX 7.0

CHARACTERIZATION OF THE ENZYME ALPHA-AMYLASE

TABLE 7.1: Determination of Optimum Temperature of the Free and Immobilized Alpha-Amylase

TEMP(⁰ C)	Free Enzyme Activity	Immobilized Enzyme Activity
	(Unit/mg/min)	(Unit/mg/min)
20	0.39±.0100 ^c	0.51±.0030 ^c
30	0.58 ±.0175 ^d	0.63±.0300 ^{bc}
40	0.71 ±.0025 ^c	0.72±.0055 ^b
50	0.91 ±.0110 ^a	0.94±.0045 ^a
60	0.93 ±.0050 ^a	0.98±.0145 ^a
70	0.81 ±.0125 ^b	1.02±.0720 ^a

Table 7.2: Determination of Optimum pH of the Free and Immobilized Alpha-Amylase

pH	Free Enzyme Activity (Unit/mg/min)	Immobilized Enzyme Activity (Unit/mg/min)
4	0.05±.0100 ^f	0.19±.0020 ^e
5	0.22±.0100 ^d	0.21±.0020 ^d
6	0.24±.0100 ^d	0.40±.0015 ^e
7	0.55±.0001 ^a	0.74±.0025 ^b
8	0.44±.0100 ^b	0.85±.0010 ^a
9	0.34±.0050 ^e	0.12±.0050 ^f
10	0.26±.0300 ^d	0.11±.0005 ^g
11	0.15±.0200 ^e	1.10±.0020 ^h

TABLE 7.3: Effect of Metal ions on the Activity of the Free and Immobilized

Alpha-Amylase		
Metal ions	Free Enzyme Activity (Unit/mg/min)	Immobilized Enzyme Activity (Unit/mg/min)
Control	0.187±.0002 ^d	0.363±.0002 ^{de}
Cu ²⁺	0.184±.0020 ^d	0.365±.0010 ^d
Hg ²⁺	0.049±.0015 ^g	0.309±.0015 ^f
Ca ²⁺	0.353±.0015 ^a	0.434±.0020 ^a
Mn ²⁺	0.242±.0010 ^b	0.413±.0015 ^b
Co ²⁺	0.195±.0015 ^e	0.369±.0045 ^d
Zn ²⁺	0.085±.0025 ^f	0.314±.0040 ^f
Al ³⁺	0.163±.0030 ^e	0.351±.0085 ^e
Na ⁺	0.229±.0015 ^c	0.388±.0010 ^e
