STUDIES ON THE ACTIVITY OF α-AMYLASE PRODUCED FROM *Fusarium spp.* USING SWEET POTATO (*Ipomoea batatas*) STARCH.

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE (M.Sc) IN INDUSTRIAL BIOCHEMISTRY AND BIOTECHNOLOGY UNIVERSITY OF NIGERIA, NSUKKA

BY

EZEORAH, KELECHI ERNEST
PG/M.Sc./14/67709

DEPARTMENT OF BIOCHEMISTRY,
UNIVERSITY OF NIGERIA,
NSUKKA

SUPERVISORS: DR. V.N. OGUGUA

DR. (MRS.) C.A. ANOSIKE

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CERTIFICATION

Ezeorah, Kelechi Ernest, a postgraduate student of the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka with Registration Number: PG/M.Sc./14/67709 has satisfactorily completed the requirements for the research work for the degree of Master of Science (M.Sc.) in Industrial Biochemistry and Biotechnology. The work embodied in this report is original and has not been submitted, in part or full, for any other diploma or degree of this or any other University.

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DR. V.N. OGUGUA **DR. (MRS.) C.A. ANOSIKE**

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PROF.O.F.C NWODO External Examiner
(Head of Department)

DEDICATION

This work is dedicated to the Almighty God and to my ever supportive parents; Mr. and Mrs. Boniface Ezeorah.

ACKNOWLEDGEMENTS

My heartfelt appreciation goes to the Almighty God my lord and my king for being my ñall in allò during this research project.A big thank you goes to my supervisors DR. V.N. Ogugua and DR. (MRS.) C.A. Anosike for their supervision and moral support. Thank you very much sir/madam for impacting so much in me for the period I spent with you. Special thanks to my parents Mr. and Mrs. Boniface Ezeorah for financing me all through my academic life. Thank you for caring so much for me. My heartfelt regard and appreciation also goes to my wonderful sister Cynthia and brother Gabriel for believing in me and supporting my dream.

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Finally, to all those who have contributed to this success story in one way or the other but not mentioned above, I say thanks and wish you all Godôs blessing now and forever. Amen.

ABSTRACT

After a seven day pilot studies, day 6 was found suitable for enzyme production from *Fusarium species* using starch from *Ipomoea batatas* (sweet potato) tubers as the carbon source. The specific activity of the crude enzyme was 55.45µ/mg. After ammonium sulphate precipitation and gel filtration, the specific activities were found to be 35.93µ/mg and 119.61µ/mg, respectively which corresponds to 3.33 fold purification. The optimum pH and temperature of the partially purified enzyme were 6.0 and 50^{o}C, respectively. The enzyme activity was strongly activated by Mn^{2+}, Ca^{2+}, and Mg^{2+} but inhibited by Co^{2+}. The Michaelis constant (Km) and maximum velocity (Vmax) obtained from the Lineweaver-Burk plot of initial velocity data at different substrate concentrations were 5.44mg/ml and 12.57µmol/min, respectively.

Table of Contents

List of Figures

List of Tables

List of Plates

List of Abbreviations

$CaCl_2$	Calcium chloride
MgS04	Magnesium sulphate.
$CoCl_2$	Cobalt (II) chloride.
$MnCl_2$	Manganese II chloride.
H_20	Water
GRAS	Generally Recognized As Safe
NS	Nelson-Somogyi
FN	Falling Number
BSA	Bovine Serum Albumin.
DNSA	DiNitroSalicyclic acid.
SMF	Submerge fermentation.
SSF	Solid-state fermentation
Mg/ml	Milli gram per milli liter.
NH_4S0_4	Ammonium sulphate.

KH_2P0_4Di hydrogen potassium phosphate.

Tris-HClTris (hydroxymethyl)aminoethane hydrochloride

EC Enzyme Commission

CHAPTER ONE

1.0 INTRODUCTION

The Ŭ-Amylase (1,4-Ŭ-D-glucan glucano hydrolase, EC 3.2.1.1) is the most important carbohydrate degrading enzyme for all starch based industries viz. food, paper, detergent, pharmaceutical, textile, baking and brewing industries (Gupta *et al*., 2003). It belongs to the family 13 of glycoside hydrolases (GH13) which randomly cleaves the Ŭ-1,4 linkages between adjacent glucose units in starch and related polysaccharides to produce mainly maltodextrins and maltose retaining Ŭ-anomeric configuration in the products. According to the vast majority of known Ŭ-amylase structure, it composed of a single polypeptide chain folded into two large domains: N-terminal (A and B) and C-terminal (C) domains. Catalytic domain A contains $(ɓ/Ŭ)_8$ or TIM barrel structure, domain B consists of three-stranded antiparallel ɓ-sheet structures and protrudes between $ɓ_3$ and $Ŭ_3$ of domain A, whereas domain C with a ɓ sheet structure is located in the C-terminal part of the polypeptide chain. Domain B is concerned for the substrate specificity and stability of the enzyme (Kuriki and Imanaka, 1999; Singh and Kayastha, 2014). Amylases are widespread in animals, fungi, plants and are also found in the unicellular eukaryotes, bacteria, and archaea (Da lagea *et al*., 2007). Though plants and animals produce amylases, enzymes from microbial sources are generally used in industrial processes. This is due to a number of factors including productivity, thermo stability of the enzyme as well as ease of cultivating microorganisms (Reddy *et al*., 1999). Amylases have been reported to be produced by a number of fungi, including *Aspergillus*, *Rhizopus*, *Fusarium*, *Candida*, *Penicillium*, *Thermomucor*, *Basidiomycete, Fomitopsis*, and *Thermomyces* (Kunamneni *et al*., 2005;Balkan and Ertan 2005;Yoon *et al*., 2006; Kumar *et al*., 2007; Mohamed *et al*., 2007). Majority of the studies on fungal amylases are based on mesophiles, rarely on facultative thermophiles (Maheswari *et al*., 2000). Current researches focus on thermo tolerant enzymes from thermophilic microbial strains. Bhatti *et al*. (2007) and Figueira and Hirooka (2000) reported amylases from mesophilic *Fusarium* species. *Fusarium* is a large genus of filamentousfungi, part of a group often referred to as hyphomycetes, widely distributed in soil and associated with plants. Most species are harmless saprobes, and are relatively abundant members of the soil microbial community. Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for growth and metabolism of organisms. These nutrient sources include orange waste, pearl millet starch, potato, corn, tapioca, wheat, and rice as flours (Djekrif-Dakhmouche *et al*., 2005; Haq *et al*., 2005). In this work, the tubers of sweet potato will be used for the

production of alpha amylase. Ŭ-amylases with characteristics suitable for the industrially relevant process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of metal salts, surfactants and other stabilizing agents should be appropriately selected as per the demand (Sivaramakrishnan *et al*., 2006).

1.1TYPES OF AMYLASE

1.1.1 α-AMYLASE

Alpha amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal Ŭ-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium dependent enzyme i.e. it depends on the presence of a metal co factor (Ca^{2+}) for its activity. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase. Endo-hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends (Gupta *et al*., 2003). Hence, terminal glucose residues and Ŭ-1, 6-linkages cannot be cleaved by Ŭ-amylase. Starch is a polysaccharide composed of two types of polymers ï amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by Ŭ-1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by Ŭ-1, 4-glycosidic linkage while branching occurs every 15-45 glucose units where Ŭ-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for Ŭ-amylase activity is found to be 7.0. Ŭ-amylase has become an enzyme of crucial importance due to its starch hydrolysis activity. The use of enzymes in detergent formulations has increased dramatically with growing awareness about environment protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of the enzyme which is the driving force behind the research to produce this enzyme in an optimum, safe and convenient manner (Gupta *et al*., 2003)

1.1.2 β – AMYLASE

Beta Amylase (EC 3.2.1.2) is an exo-hydrolase enzyme that acts from the non-reducing end of a polysaccharide chain by hydrolysis of Ŭ-1, 4-glucan linkages to yield successive maltose

units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary sources of ß-amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, ß-amylase breaks down starch into maltose resulting in the sweetness of ripened fruit. The optimal pH of the enzyme ranges from 4.0 to 5.5. ßeta amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. It is used during fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups (Sivaramakrishnan *et al.*, 2006).

1.1.3γ – AMYLASE

Gamma Amylase (EC 3.2.1.3) cleaves Ŭ(1-6)glycosidic linkages, in addition to cleaving the last Ŭ(1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, unlike the other forms of amylase, yielding glucose. Gamma amylase is most efficient in acidic environments and has an optimum pH of 3 (Sivaramakrishnan *et al*., 2006).

1.2ALPHA AMYLASE FAMILY

The Ŭ-amylase family, i.e. the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (Van Der Maarel *et al.*, 2002). A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes, and transferases (Henrissat, 1991; Van Der Maarel *etal.*,2002).

1.2.1 Endo Amylase

The endo amylase otherwise known as α-amylase group are those amylase that catalyzes the internal hydrolysis of Ŭ- 1,4-o-glycosidic bonds resulting in the formation of alpha anomeric products (Swetha *etal*.,2006).

1.2.2 Exo Amylases

The exo amylase are those amylase that catalysis the cleavage of either Ŭ- 1, 4 or Ŭ- 1,6 linkages of external glucose residues resulting in alpha or beta anomeric products (Van Der Maarel *etal*.,2002).

1.2.3 Debranching Amylase

The debranching amylase catalysis the hydrolysis of Ŭ- 1, 6 bonds exclusively leaving long chain polysaccharides (Van Der Maarel *etal*.,2002).

1.2.4 The Transferases

They are those group of amylases that catalyze the cleavage of Ŭ-1,4 glycosidic bond of the donor molecule or compound and still catalyze the transfer of the donor to a glycosidic acceptor forming a new glycosidic bonds mostly 1,6 glycosidic bond (Swetha *etal*.,2006).
Generally, the glycoside hydrolases are able to metabolize large varieties of saccharides. They have been divided into classes based on their mode of reaction and families based on their amino acids sequence compositions and its similarities with membered families. Most of the starch converting enzymes belongs to the glycoside hydrolase thirteen families (GH13). The GH13 family can be further classified based on a larger unit called clan, which is the three dimensional structure of catalytic module. A clan may consist of two or more families with the same three dimensional structures of catalytic domain but with limited sequence similarities, indicating that protein structure is best preserved by evolution than amino acid sequences. Among the fourteen clans ranging from A-N defined for glycosidase and trans glycosidase, Ŭ-amylase family (GH13) belongs to the eight clan i.e GH-H (MacGregor,2005).This concept was proposed by Takata *etal*. (1992) members of this family satisfy the following requirements which are:

- They must act on an Ŭ- 1,4 glycoside linkages and hydrolyse them to produce an Ŭ-anomeric monosaccharide and oligosaccharide or form an α-glucosidic linkages by trans glycosylation.
- They have four highly conserved regions in their primary structures consisting of catalytic and substrate binding sites (Allosteric).
- Comprises of the following amino acids: Aspartate (Asp 206), Glutamate (Glu 230) and Aspartate (ASP 297) at corresponding positions in their catalytic domain.
- They should possess a $(Beta/alpha)_8$ or Tim barrel catalytic domain.

1.3STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF ?-AMYLASE

The Ŭ-amylase (Ŭ-1,4-glucan-4-glucanohydrolase) can be found in microorganisms, plants and higher organisms (Kandra, 2003). The Ŭ-amylase belongs to a family of endo-amylases that catalyses the initial hydrolysis of starch into shorter oligosaccharides through the

cleavage of Ŭ-D-(1-4) glycosidic bonds (Brayer *et al*., 1995; Iulek *et al*., 2000; Kandra, 2003; Tangphatsornruang *et al*., 2005). Neither terminal glucose residues nor Ŭ-1,6-linkages can be cleaved by Ŭ-amylase (Whitcomb and Lowe, 2007). The end products of Ŭ-amylase action are oligosaccharides with varying length with an Ŭ-configuration and Ŭ-limit dextrins (Van Der Maarel *et al*., 2002), which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6ï 8 glucose units that contain both Ŭ-1,4 and Ŭ-1,6 linkages (Whitcom and Lowe, 2007). Other amylolytic enzymes participate in the process of starch breakdown, but the contribution of Ŭ-amylase is the most important for the initiation of this process(Tangphatsornruang *et al*., 2005). The amylase has a three-dimensional structure capable of binding to substrate and, by the action of highly specific catalytic groups, promote the breakage of the glycoside links (Iulek *et al*., 2000). The human Ŭ-amylase is a classical calcium-containing enzyme composed of 512 amino acids in a single oligosaccharide chain with a molecular weight of 57.6 kDa (Whitcomb and Lowe, 2007).

1.4STARCH

Starch is an important constituent of the human diet and, for this purpose, is used chemically and enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, maltodextrin derivatives or cyclodextrins, used in food industry. In addition to that, the sugars produced can be fermented to produce ethanol. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat, but limitations such as low shear resistance, thermal resistance, thermal decomposition and high tendency towards retro gradation limit its use in some industrial food applications (Agrawal *etal*., 2005; Goyal *et al*., 2005; Muralikrishna and Nirmala, 2005). Among carbohydrate polymers, starch is currently enjoying increased attention due to its usefulness in different food products. Starch contributes greatly to the textural properties of many foods and is widely used in food and industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent (Jaspreet *et al*., 2007). Starch is a polymer of glucose linked to another one through the glycosidic bond. Two types of glucose polymers are present in starch: amylose and amylopectin (Figures 1a and 1b). Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units with Ŭ-1,4 glycosidic bonds. Amylopectin consists of short Ŭ-1,4 linked to linear chains of 10ï 60 glucose units and Ŭ-1,6 linked to side chains with 15ï 45 glucose units. Granule bound starch synthase can elongate malto-oligosaccharides to form

amylose and is considered to be responsible for the synthesis of this polymer. Soluble starch synthase is considered to be responsible for the synthesis of unit chains of amylopectin. Ŭ-amylase is able to cleave Ŭ-1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chain (Van Der Maarel *et al*., 2002; Sorensen *et al*., 2004; Tester *et al*., 2004; Muralikrishna and Nirmala, 2005).

A. Structure of amylase

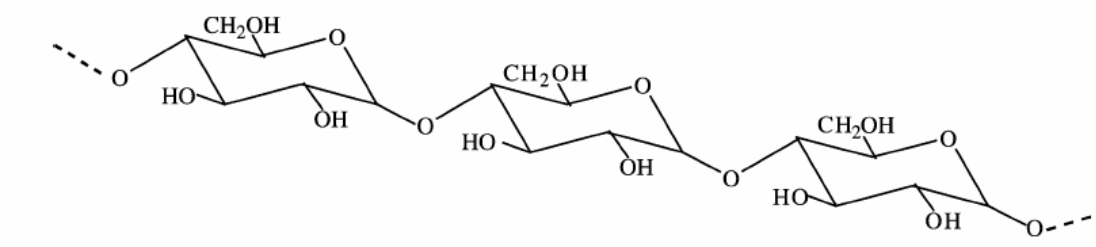

Figure 1a: Structure of amylase

Source: (Van Der Maarel *et al*., 2002; Sorensen *et al*., 2004; Tester *et al*., 2004; Muralikrishna and Nirmala, 2005).

B. Structure of amylopectin

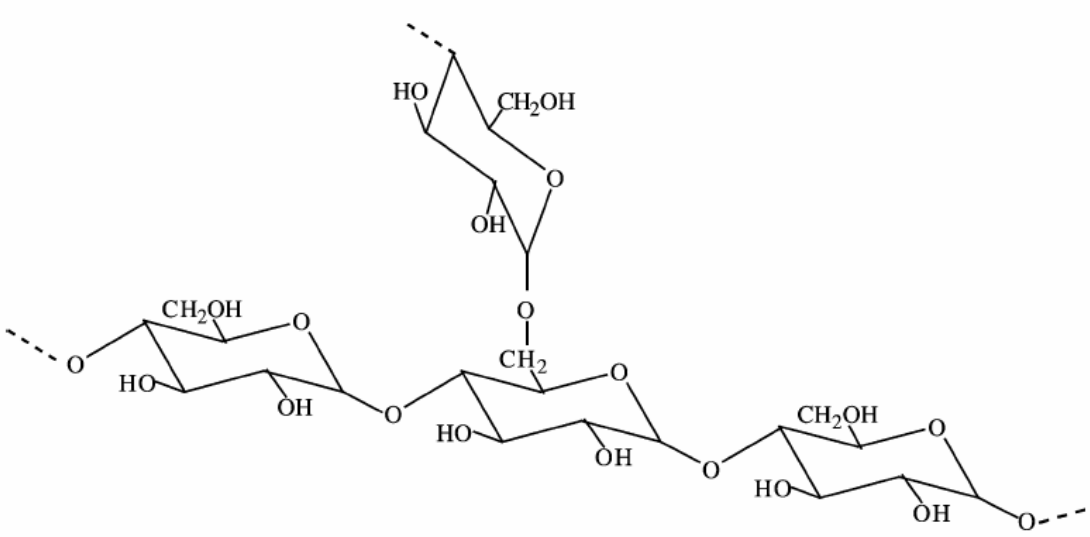

Fig: 1b. Structure of amylopectin

Source: (Muralikrishna and Nirmala, 2005).

Two types of glucose polymers are present in starch: amylose (A) is a linear polymer consisting of up to 6000 glucose units with Ŭ-1,4 glycosidic bonds (Muralikrishna and Nirmala, 2005) and amylopectin (B) consists of short Ŭ-1,4 linked to linear chains of 10ï 60 glucose units and Ŭ-1,6 linked to side chains with 15ï 45 glucose units (Muralikrishna and Nirmala, 2005).

Starch is hydrolyzed into smaller oligosaccharides by Ŭ-amylase, which is one of the most important commercial enzyme processes. Amylases find application in all the industrial processes such as in food, detergents, textiles and in paper industry, for the hydrolysis of starch (Gupta *et al*., 2003; Konsula and Liakopoulou-Kyriakides, 2004; Tanyildizi *et al*., 2005). Saccharide composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. Specificity, thermo stability, and pH response of the enzymes are critical properties for industrial use (Kandra, 2003).

1.5SOURCES OF α-AMYLASE

Alpha amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases from plant and microbial

sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used in preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan *et al*., 2003). Among bacteria, *Bacillus sp*. is widely used for thermostable Ŭ-amylase production to meet industrial needs. *B. subtilis*, *B.stearothermophilus*, *B. licheniformis,* and *B. amyloliquefaciens* are known to be good producers of Ŭ-amylase and these have been widely used for commercial production of the enzyme for various applications. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including Ŭ-amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of Ŭ-amylase. Production of enzymes by solid-state fermentation (SSF) using these moulds turned a cost-effective production technique. Detailed literature is available on various microbial sources for the production of amylases (Vihinen and Mantasala, 1989; Pandey *et al*., 2000).

1.5.1 BACTERIAL AMYLASES

Alpha Amylase can be produced by different species of microorganisms, but for commercial applications Ŭ-amylase is mainly derived from the genus *Bacillus*. Ŭ-amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles, and paper industries (Pandey *et al*., 2000; Konsula and Liakopoulou-Kyriakides, 2007). Thermostability is a desired characteristic of most of the industrial enzymes. Thermal stable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100ï 110^{o}C), thermal stable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose, and maltodextrins (Stamford *et al*., 2001; Gomes *et al*., 2003; Asgher *et al*., 2007). *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermal stable Ŭ-amylase, and these have been widely used for commercial production of the enzyme for various

applications. Thermal stable Ŭ-amylases have been reported from several bacterial strains and have been produced using submerged fermentation(SmF) as well as solid state fermentation (SSF) (Teodoro and Martins, 2000). However, the use of SSF has been found to be more advantageous than SmF and allows a cheaper production of enzymes (Sodhi *et al*., 2005). The production of Ŭ-amylase by SSF is limited to the genus *Bacillus*, and *B. subtilis*, *B.polymyxia*,*B.mesentericus*, *B. vulgarus*, *B. megaterium*, and *B.licheniformis* have been used for Ŭ-amylase production in SSF (Baysal *et al*., 2003). Currently, thermal stable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are being used in starch processing industries (Gomes *et al*., 2003). Enzymes produced by some halophilic microorganisms have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions (Amoozegar *et al*., 2003; Prakash *et al*., 2009). In addition, most halobacterial enzymes are considerably thermotolerant and remain stable at room temperature over long periods (Mohapatra *et al*., 1998). Halophilic amylases have been characterized from halophilic bacteria such as *Chromohalobacter sp*. (Prakash *et al*., 2009), *Halobacillus sp*. (Amoozegar *et al*., 2003), *Haloarcula hispanica* (Hutcheon *et al*., 2005), *Halomonas meridiana* (Coronado *et al*., 2000), and *Bacillus dipsosauri* (Deutch, 2002).

1.5.2 FUNGAL AMYLASES

Most reports about fungi that produce Ŭ-amylase have been limited to a few species of mesophilic fungi, and attempts have been made to specify the cultural conditions and to select superior strains of the fungus to produce on a commercial scale (Gupta *et al*., 2003). Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* (Kathiresan and Manivanna, 2006). The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance (Hernandez *et al*., 2006). Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favourable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. Ŭ-amylase (Jin *et al*., 1998). *Aspergillus oryzae* has been largely used in the production of food such as soy sauce, organic acid such as citric and acetic acids and commercial enzymes including Ŭ-amylase (Kammoun *et al*., 2008). *Aspergillus niger* has important hydrolytic capacities in the Ŭ-amylase production and, due to its tolerance of acidity (pH <3), it allows the avoidance of bacterial contamination (Djekrif-Dakhmouche *et*

al., 2005). Filamentous fungi are suitable microorganisms for solid-state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate (Rahardjo *et al*., 2005). The fungal Ŭ-amylases are preferred over other microbial sources due to their more accepted status GRAS (Generally Recognized As Safe) (Gupta *et al*., 2003). The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase. Jensen *et al*. (2002) and Kunamneni *et al*. (2005) purified the Ŭ-amylase, proving its thermostability.

1.6PRODUCTION METHODS FOR α-AMYLASE

There are mainly two methods which are used for production of Ŭ-amylase on a commercial scale. These are; Submerged and solid state fermentation.

The latter is a fairly new method while the former is a traditional method of enzyme production from microbes which has been in use for a longer period of time.

1.6.1 SUBMERGED FERMENTATION (SmF)

Employs free flowing liquid substrates, such as molasses and broths. The products yielded in fermentation are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence the substrates need to be constantly replenished. This fermentation technique is suitable for microorganisms such as bacteria that require high moisture content for their growth. SmF is primarily used for the extraction of secondary metabolites that need to be used in liquid form (Couto and Sanroman, 2006). This method has several advantages. SmF allows the utilization of genetically modified organisms to a greater extent than SSF. The sterilization of the medium and purification process of the end products can be done easily. Also the control of process parameters like temperature, pH, aeration, oxygen transfer and moisture can be done conveniently (Kunamneni *et al.*, 2005).

1.6.2SOLID STATE FERMENTATION

This is a method which require less moisture content for their growth.The solid substrates commonly used in this method are, bran, bagasse, and paper pulp. The main advantage is that nutrient-rich waste materials can be easily recycled and used as substrates in this method. Unlike SmF, in this fermentation technique, the substrates are utilized very slowly and steadily. Hence the same substrate can be used for a longer duration, thereby eliminating the need to constantly supply substrate to the process (Kunamneni *et al*., 2005). Other advantages

that SSF offers over SmF are simpler equipment, higher volumetric productivity, higher concentration of products and lesser effluent generation (Couto and Sanroman, 2006). For several such reasons SSF is considered as a promising method for commercial production of enzymes.

Table 1: Microbial sources and the mode of fermentation used for production

Bacterial source	Method
Bacillus amyloliquefaciens	Solid-state fermentation
Bacillus licheniformis	Solid-state fermentation
Halomonas meridian	Submerged fermentation
Rhodothermus marinus	Submerged fermentation
Bacillus cereus	Solid-state fermentation
Fungal source	Method
Aspergillus oryzae	Solid-state fermentation
Penicillium Fellutanum	Submerged fermentation
Streptomyces rimosus	Solid-state fermentation, Submerged fermentation
Aspergillus kawachii	Solid-state fermentation, Submerged fermentation
Pycnoporus sanguineus	Solid-state fermentation

Source: (Kunamneni *et al*., 2005; Couto and Sanroman, 2006).

Fungal sources have been investigated for Ŭ-amylase production through submerged and solid state fermentation. However, studies reveal that SSF is the most appropriate process in developing countries due to the advantages it offers which make it a cost effective production process (Kunamneni *et al*., 2005). Also SSF provides a medium that resembles the natural

habitat of fungal species, unlike Smf which is considered a violation of their habitat (Kenneth *et al*., 1993).

1.7PURIFICATION OF α-AMYLASE

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of Ŭ-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in the purified form is also a prerequisite in studies of structure-function relationships and biochemical properties (Gupta *et al*., 2003). Different strategies for purification of enzymes have been investigated, exploiting specific characteristics of the target biomolecule. Laboratory scale purification for Ŭ-amylase includes various combinations of ion exchange, gel filtration, hydrophobicity interactions, and reverse phase chromatography. Alternatively, Ŭ-amylase extraction protocols using organic solvents such as ethanol, acetone and ammonium sulphate precipitation (Glymph and Stutzenberger, 1977; Hamilton *et al*., 1999; Khoo *et al*., 1994) and ultrafiltration have been proposed (Moraes *et al*., 1999). These conventional multi-step methods requires expensive equipment at each step, making them laborious, time consuming, barely reproducible and may result in increasing loss of the desired product (Arauza *et al*., 2009). However, liquidï liquid extractions consist of an interesting purification alternative since several features of the early processing steps can be combined into a single operation. Liquidï liquid extraction is the transfer of certain component from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. This process is widely employed in the chemical industry due to its simplicity, low costs, and ease of scale up. Purification of biomolecules using liquidï liquid extraction has been successfully carried out on a large scale for more than a decade. Advantages of using this system are lower viscosity, lower cost of chemicals and shorter phase separation time. The dynamic behaviour of these systems has to be investigated and understood to enhance plant-wide control of continuous liquidï liquid extraction and to assess safety and environmental risks at the earliest possible design stage (Mazzola *et al*., 2008).

Table 2: Methods of one-step purification of Ŭ-amylases

Method	Adsorbent	Yield/%	Purificationfolds
Affinity adsorption chromatography	b-cyclodextrin-iminodiacetic acid-Cu^{2+}	95	_
Expanded bed chromatography	Alginic acid-cellulose cell beads	69	51
High speed counter current chromatography	PEG4000-aqueous two-phase system	73.1	_
Magnetic affinity adsorption	Magnetic alginate microparticles	88	9
Substitute affinity method	Insoluble corn starch at 4 °C	78	163

Source: (Swetha *et al.*, 2006).

1.8 DETERMINATION OF ALPHA AMYLASE ACTIVITY

The enzyme activity is determined by measuring the reducing sugars released as a result of the action of Ŭ-amylase on starch. Another method is to measure the extent of hydrolysis by reading the absorbance of starch-iodine complex. Few of the commonly used methods for enzyme assay are discussed below;

1.8.1 DINITROSALICYLIC ACID(DNSA) METHOD

In the dinitrosalicylic acid method, aliquots of the substrate stock solution are mixed with the enzyme solution followed by incubation for 10 min at 50^{o}C, dinitrosalicyclic acid solution is then added to the mixture and boiled in a boiling water bath for 5 min. After cooling to room temperature, the absorbance is read using a spectrophotometer at 540 nm. The blank will contain all the components except the enzyme solution (Kobayashi *et al*., 1992; Feller *et al*., 1998). In a study on alkalophilic Ŭ-amylase from *Bacillus* strain GM8901, the enzyme assay

was done by measuring the reducing sugars by dinitrosalicyclic acid method and the activity was found to be of 0.75 U/ml after incubation for 24 h (Gusakov *et al*., 2011).

1.8.2 NELSON – SOMOGYI (NS) METHOD

In the NS method, an aliquot of stock solution of substrate is heated at 50°C for 5min. The enzyme solution is added to the mixture and allowed to stand for 10min at 50°C. After incubation Somogyi copper reagent is added to terminate the reaction. This is then incubated in boiling water bath for 40 min and cooled to room temperature. In the next step Nelson arsenomolybdate reagent is added and incubated for 10 min at room temperature. Finally water is added and the mixture is centrifuged at 13,000 rpm for 1 min and absorbance of supernatant is read at 610 nm (Kobayashi *et al*., 1992). Haloalkaliphilic Ŭ-amylase isolated from archaebacterium *Natronococcus sp*. Strain Ah-36 was assayed using NS mettod. The maximum activity was found to be 0.12 U/ml after 90 h and was retained even after 110 h (El-safey and Ammar, 2004).

1.8.3 DETERMINATION OF ACTIVITY USING IODINE

The hydrolytic activity of Ŭ-Amylase can be determined based on the principle that starch and iodine react to form a blue coloured complex. On hydrolysis of starch this complex changes to a reddish brown coloured one. The absorbance can be read after the enzyme substrate reaction has been terminated. This gives a measure of the extent of hydrolysis of starch by Ŭ-Amylase (Gupta *et al*., 2003).

1.8.4 DEXTRINIZING ACTIVITY

In a study involving characterization of thermophilic Ŭ-Amylase from *P. furiosus*, an adaptation of the assay was employed to carry out the enzyme assay. The crude enzyme sample is incubated at 92°C with soluble starch to carry out the reaction for 10 min. The reaction is terminated by cooling the reaction mixture in ice water. Iodine solution (KI, Iodine) is added to form a coloured complex with the starch in the reaction mixture. This complex is diluted with water in order to bring the colour to a measurable range that can be read at 600 nm (Haki and Rakshit, 2003).

1.8.5 REDUCTION IN VISCOSITY OF STARCH SUSPENSION

This method is used to determine the quality of flour in the baking industry. Two methods used to measure enzyme activity in terms of decreasing viscosity of starch suspension are:

falling number (FN) method and amylograph/farinograph test. In the falling number method, the enzyme substrate preparations are assayed at 100°C. Normally malted flour has a falling number of around 400. The amylograph test employs the principle of the relationship between peak viscosity of starch slurry and the enzyme activity. The lesser the viscosity of the starch slurry, more is the activity of the enzyme. Values of 400-/600 Brabender units of the Farinograph are found optimal for flour used to bake bread (Gupta *et al*., 2003).

1.9INDUSTRIAL APPLICATIONS OF α-AMYLASE

Table 3: Uses of amylases in various sectors of industry

Sector	Uses
Food industry	Production of glucose syrups, crystalline glucose
	Production of high fructose corn syrups
	Production of maltose syrups
	Reduction of viscosity of sugar syrups
	Reduction of haze formation in juices
	Solubilization and saccharification of starch for alcohol fermentation in brewing industries
Detergent industry	Used as an additive to remove starch based dirts
Paper industry	Reduction of viscosity of starch for appropriate coating of paper
Textile industry	Warp sizing of textile fibers
Pharmaceutical industry	Used as a digestive aid

Source: (Van Der Maarel *et al*., 2002; Gupta *et al*., 2003; Riegal and Bissinger, 2003).

1.9.1 STARCH CONVERSION

The most widespread applications of Ŭ-amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen and Borchert, 2000). The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a

viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further hydrolysis (Gupta *et al*., 2003). Initially, the Ŭ-amylase of *Bacillus amyloliquefaciens* was used but it has been replaced by the Ŭ-amylase of *Bacillus stearothermophilus* or *Bacillus licheniformis* (Van Der Maarel *et al*., 2002). The enzymes from the *Bacillus* species are of special interest for large-scale biotechnological processes due to their remarkable thermo stability and because efficient expression systems are available for these enzymes.

1.9.2 DETERGENT INDUSTRY

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (Gupta *et al*., 2003;Mitidieri *et al*., 2006; Hmidet *et al*., 2009). These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate, etc. to dextrins and other smaller oligosaccharides (Olsen and Falholt, 1998; Mukherjee *et al*., 2009). Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing (Kirk *et al*., 2002; Chi *et al*., 2009). Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. Amylases used in the detergent industry are derived majorly from *Bacillus* or *Aspergillus* (Mitidieri *et al*., 2009).

1.9.3 FUEL ALCOHOL PRODUCTION

Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Chi *et al*., 2009). In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as Ŭ-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* (Moraes *et al*., 1999; Oner, 2006). The production of ethanol

by yeast fermentation plays an important role in the economy of Brazil (De Moraes *et al*., 1995). In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* (Chi *et al*., 2009). Among bacteria, Ŭ-amylase obtained from thermo resistant bacteria like *Bacilluslicheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions (Sanchez and Cardona, 2008).

1.9.4 FOOD INDUSTRY

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanroman, 2006). The Ŭ-amylases have been widely used in the baking industry. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of Ŭ-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread. Besides generating fermentable compounds, Ŭ-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products (Van Der Maarel, 2002; Gupta *et al*., 2003). Currently, a thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry (Van Der Maarel, 2002). Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber (Van Der Maarel, 2002; Gavrilescu and Chisti, 2005; Ghorai *et al*., 2009).

1.9.5 TEXTILE INDUSTRY

Amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry. Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. The Ŭ-amylases remove selectively the size and do not attack the fibres

(Feitkenhauer, 2003; Gupta *et al*., 2003; Ahlawat *et al*., 2009). Amylase from *Bacillus* stain was employed in textile industries for quite a long time.

1.9.6 PAPER INDUSTRY

The use of Ŭ-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (Van Der Maarel, 2002; Gupta *et al*., 2003). The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with Ŭ-amylases in a batch or continuous processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasebility, besides being a good coating for the paper. The size enhances the stiffness and strength in paper (Gupta *et al*., 2003). Examples of amylases obtained from microorganisms used in paper industry includes Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and Ŭ-amylase G9995® (Enzyme Biosystems, USA) (Saxena *et al*., 2004).

1.10POTATO

The sweet potato (*Ipomoea batatas*) is a starchy, tuberouscrop from the perennialnightshade The word "potato" may refer either to the plant itself or to the edible tuber.It is the world's fourth-largest food crop, following maize, wheat, and rice.

Table: 4. scientific classification of sweet potato

Kingdom	Plantae
(unranked)	Angiosperm
(unranked)	Eudicots
(unranked)	Asterids
Order	Solanales
Family	Convolvulaceae
Genus	*Ipomoea*
Species	*Ipomoea batatas*

Source: (Yen, 1963).

1.10.1 CHARACTERISTICS

Potato plants are herbaceous perennials that grow about 60 cm (24 in) high, depending on variety, with the leaves dying back after flowering, fruiting and tuber formation. They bear white, pink, red, blue, or purple flowers with yellow stamens. In general, the tubers of varieties with white flowers have white skins, while those of varieties with coloured flowers tend to have pinkish skins. Potatoes are mostly cross-pollinated by insects such as bumblebees, which carry pollen from other potato plants, though a substantial amount of self-fertilizing occurs as well. Tubers form in response to decreasing day length, although this tendency has been minimized in commercial varieties (Virginia *et al*., 2001).

1.10.2 NUTRITION

The potato contains vitamins and minerals, as well as an assortment of phytochemicals, such as carotenoids and natural phenols. Chlorogenic acid constitutes up to 90% of the potato tuber natural phenols. Others found in potatoes are 4-O-caffeoylquinic acid (crypto-chlorogenic acid), 5-O-caffeoylquinic (neo-chlorogenic acid), 3,4-dicaffeoylquinic and 3,5-dicaffeoylquinic acids (Ferretti, 2011). A medium-size 150 g (5.3 oz) potato with the skin provides 27 mg of vitamin C (45% of the Daily Value (DV)), 620 mg of potassium (18% of DV), 0.2 mg vitamin B6 (10% of DV) and trace amounts of thiamin, riboflavin, folate, niacin, magnesium, phosphorus, Iron, and zinc. The potato is best known for its carbohydrate content (approximately 26 grams in a medium potato). The predominant form of this carbohydrate is starch.

1.10.3 NUTRITIONAL VALUE

The nutritional value of sweet potato includes; Energy 321 kJ (77 kcal), Carbohydrates 17.47 g, Starch 15.44 g, and Dietary fiber 2.2 g (Raben *et al*., 1994; Cummings *et al*., 1996; Hylla *et al*., 1998).

1.10.4 USES

- Potatoes are used to brew alcoholic beverages such as vodka, potcheen, or akvavit.
- They are also used as food for domestic animals.
- Potato starch is used in the food industry as, for example, thickeners and binders of soups and sauces, in the textile industry, as adhesives, and for the manufacturing of papers and boards.

- Maine companies are exploring the possibilities of using waste potatoes to obtain polylactic acid for use in plastic products; other research projects seek ways to use the starch as a base for biodegradable packaging.
- Potato skins, along with honey, are a folk remedy for burns in India. Burn centers in India have experimented with the use of the thin outer skin layer to protect burns while healing.
- Potatoes (mainly Russets) are commonly used in plant research. The consistent parenchyma tissue, the clonal nature of the plant and the low metabolic activity provide a very nice "model tissue" for experimentation. Wound-response studies are often done on potato tuber tissue, as are electron transport experiments. In this respect, potato tuber tissue is similar to *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Escherichia coli*: they are all "standard" research organisms.

1.10.5 HEALTH BENEFITS OF SWEET POTATOES

1. They are high in vitamin B6. Vitamin B6 helps reduce the chemical homocysteine in our bodies. Homocysteine has been linked with degenerative diseases, including heart attacks.

2. They are a good source of vitamin C. While most people know that vitamin C is important to help ward off cold and flu viruses, few people are aware that this crucial vitamin plays an important role in bone and tooth formation, digestion, and blood cell formation. It helps accelerate wound healing, produces collagen which helps maintain skinôs youthful elasticity, and is essential to helping us cope with stress. It even appears to help protect our body against toxins that may be linked to cancer.

3. They contain vitamin D which is critical for immune system and overall health. Both a vitamin and a hormone, vitamin D is primarily made in our bodies as a result of getting adequate sunlight. You may have heard about seasonal affective disorder (or SAD, as it is also called), which is linked to inadequate sunlight and therefore a vitamin D deficiency. vitamin D plays an important role in our energy levels, moods, and helps to build healthy bones, heart, nerves, skin, and teeth, and it supports the thyroid gland.

4. Sweet potatoes contain iron. Most people are aware that we need the mineral iron to have adequate energy, but iron plays other important roles in our body, including red and white blood cell production, resistance to stress, proper immune functioning, and the metabolizing of protein, among other things.

5. Sweet potatoes are a good source of magnesium, which is the relaxation and anti-stress mineral. Magnesium is necessary for healthy artery, blood, bone, heart, muscle, and nerve function, yet experts estimate that approximately 80 percent of the population in North America may be deficient in this important mineral.

6. They are a source of potassium, one of the important electrolytes that help regulate heartbeat and nerve signals. Like the other electrolytes, potassium performs many essential functions, some of which include relaxing muscle contractions, reducing swelling, and protecting and controlling the activity of the kidneys.

7. Sweet potatoes are naturally sweet-tasting but their natural sugars are slowly released into the bloodstream, helping to ensure a balanced and regular source of energy, without the blood sugar spikes linked to fatigue and weight gain.

8. Their rich orange colour indicates that they are high in carotenoids like beta carotene and other carotenoids, which is the precursor to vitamin A in your body. Carotenoids help strengthen our eyesight and boost our immunity to disease, they are powerful antioxidants that help ward off cancer and protect against the effects of aging.

The health benefits stated above were gotten from (www.care2.com/greenliving).

1.11 *Fusarium*

Fusarium is a large genus of filamentous fungi, part of a group often referred to as hyphomycetes, widely distributed in soil and associated with plants. Most species are harmless saprobes, and are relatively abundant members of the soil microbial community (Nelson *et al*., 1994; Moretti, 2009; Watanabe *et al*., 2011).

Fig:2. *Fusarium verticillioides*

Source: (Nelson *et al*., 1994; Moretti, 2009; Watanabe *et al*., 2011)

Table: 5. Scientific classification of *Fusarium species*

Kingdom	Fungi
Division	Ascomycota
Class	Sordarimycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*

Source: (Watanabe *et al*., 2011).

1.11.2 SPECIES

Selected species includes; *F. avenaceum, F. bubigeum, F. culmorum, F. graminearum,F. langsethiae,F. oxysporum, F. poae, F. sporotrichioides, F. tricinctum, F. verticillioides, andF. virguliforme* (Watanabe *et al*., 2011).

1.12 α-AMYLASE CHARACTERISTICS

The properties of Ŭ-amylases such as thermostability and pH profile should match its application. Hence, the diversity of the applications creates the need for Ŭ-amylases with novel and improved properties. The rate of hydrolysis of starch by Ŭ-amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} ions and other stabilizing agents. Alpha amylases with characteristics suitable for the industrially relevant process conditions and applications have to be appropriately selected as per the demand.

1.12.1 EFFECT OF TEMPERATURE

It is desirable that Ŭ-amylases should be active at high temperatures of gelatinization (100ï 110 °C) and liquefaction (80ï 90 °C) to economize the process; therefore, there has been a need and continual search for more thermophilic and thermostable Ŭ-amylase. Most of the Ŭ-amylases are reported to have one, or two intrinsic Ca^{2+} ions, present near the active centre

formed by the two domains, A and B. The Ca^{2+} is necessary for enzyme folding and enzyme stability. Secondary calcium binding sites have also been reported, which enhanced the thermostability. Saboury (2002) reported the presence of 17 different secondary binding sites for calcium in Ŭ-amylase of *B. amyloliquefaciens*, which were responsible for stabilization of the enzyme against thermal and surfactant denaturation. Weemaes *et al*. (1996) studied the stability of Ŭ-amylases produced by *B. amyloliquefaciens*, *B. licheniformis* and,*B.stearothermophilus* under combined high temperature and pressure and the results indicated that Ŭ-amylase produced by *B. licheniformis* was the most stable enzyme among the three (Table 6).

Table 6. Ŭ-amylases exhibiting considerable temperature stability

Organism	**Temperature range (°C)**	**Residual activity (%)**	**Temperature optimum (°C)**
Lactobacillus manihotivorans	50ï 60	70 (50 °C for 1.0 h)	55
Bacillus sp. I-3	65ï 100	50 (80 °C for 2.5 h)	70
Pyrococcus furiosus	80ï 100	50 (98 °C for 13 h)	100
Thermobifida fusca NTU22	50ï 60	70 (60 °C for 3 h)	60
Cryptococcus flavus	50ï 60	60 (60 °C for 60 min)	50

Source: (Singh *et al*., 2014).

1.12.2 EFFECT OF pH

Natural pH of starch slurry is generally around 4.5. It is known that gelatinized starch is more susceptible to degradation than non-gelatinized starch. The extreme conditions required for such pre-treatment necessitate the use of an enzyme that is resistant to high temperatures and low pH. Acid hydrolysis of peptide bonds at low pH has been reported to occur most often at the C-terminal side of Asp residues, with the Asp-Pro bond being the most susceptible. This

may be due to the facts that the nitrogen of proline is more basic than that of other residues, and Asp has an increased propensity for Ŭi ɓ isomerization when linked on the N side of a proline (Vieille and Zeikus, 2001). Peptide bond hydrolysis never occurs in the helical and beta structures. Thus, it appears that Asp residues, or Asp-Pro bonds, occurring in regions except helical and beta structures, are susceptible to hydrolysis at low pH. Studies on influence of polyols indicated that stability towards high temperature and proteolytic digestion of enzymes was markedly enhanced in the presence of additives such as sorbitol, glycerol and trehalose. Moreover, enzymes were resistant to acidic digestion (Table 7) (Khajeh *et al.*, 2006).

Table 7: Ŭ-Amylases having different optimal pH and stability

Organism	**pH range**	**Residual activity%**	**Optimum pH**
Bacillus spp 1-3	5.0-5.5	80 stable	7.0
Aspergilluskawachai IFO 4308	2.0-6.5	90(pH=2.0 for 30 min)	5.0
Rhizomucorpussilus	3-6	85(pH=6 for 1hr)	5.0
Rhizopusmicrospores	4-9	75(pH=4-6 for 1hr)	5
Aspergillusoryzae	3.5-5.5		5
Fusarium spp	2.5-8.5	60(pH=4.5-7 for 24hr)	6.5

Source: (Singh *et al*., 2014).

1.12.3 EFFECT OF METAL IONS

Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc. Calcium (ii) ion was reported to increase Ŭ-amylase activity of an alkaliphilic *Bacillus sp.* ANT-6. The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure (Goyal*et al*., 2005). Ca^{2+}independent enzymes have also been reported. Malhotra *et al*. (2000) reported the

presence of a thermostable Ŭ-amylase from *B. thermooleovorans* NP54, which did not require calcium ions for its activity or production. Zn^{2+} has varied effect on different amylases. Zn^{2+} was reported to inhibit thermostable Ŭ-amylases from a thermophilic *Bacillus sp*. suggesting that the inhibition with the ion determined the thermostability of enzyme. The presence of Zn^{2+} decreased the activity of ANT-6 enzyme indicating its high thermostability (Table 8) (Burhan *et al*., 2003).

Table 8: Lists of metal ions showing either positive or negative effects on amylase activity from different Organisms.

Metal ion	**Concentration Effect/mM**	**Organism**
Ca^{2+}	5 promoting	*Lipomyces starkeyi*
		Rhizopus microsporus
		Fusarium spp
		Rhizomucor pusillus
		Bacillus spp ANT6
K^{+}	10 promoting	*L. manihotivorans* LMG 18010
Na^{+}	5 promoting	*Bacillus* sp. L1711 (*89*)
	5 inhibitory	*Bacillus* sp. ANT-6
Co^{2+}	5 promoting	*Bacillus* sp. L1711
		Aspergillusniger
Mg^{2+}	5 promoting	*Fusariumspp*
		Vibro spp
		Rhizopus spp
	5 inhibitory	*Aspergillus oryzae*
Ba^{2+}	10 promoting	*L. manihotivorans* LMG 18010
Mn^{2+}	1 promoting	*Bacillus* sp. I-3
	1 inhibitory	*Bacillus* KSM-K38
Zn^{2+}	10 promoting	*L. manihotivorans* LMG 18010
		Aspergillusniger

		Fusarium spp
Zn^{2+}	5 inhibitory	*Bacillus* sp. ANT-6
Fe^{2+}	1 promoting	*Bacillus* sp. I-3
	20 inhibitory	*Rhizopus spp*
	inhibitory	*Aspergillus niger*
		Cryptococcus flavus Aspergillus oryzae
Cu^{2+}	1 promoting	*Bacillus* sp.1-3
	10 inhibitory *L*.	*manihotivorans* LMG 18010

Source: (Sudha *et al.,* 2012).

1.12.4 EFFECT OF SUBSTRATE

Amylases show substrate specificity. The substrate specificity of the Ŭ-amylase was evaluated on soluble starch, amylose, amylopectin, glycogen, maltodextrins, and Ŭ- and ɓ-cyclodextrins. Amylose was found to be the best substrate for *L. manihotivorans* LMG 18010T Ŭ-amylase. The relative activity observed on amylose was 1.6 times higher than that obtained on starch. The hydrolysis of amylopectin was 90% in relation to starch. Glycogen and dextrins with 10 and 20 DP were hydrolyzed at low rate reaching 6.7, 7.3, and 8.9 %, respectively, and no activity was found on Ŭ- and ɓ-cyclodextrins (Aguilar *et al*., 2000). Natural starches such as maize starch (Kunamneni and Singh, 2005), raw sago starch (Yang and Liu, 2004), corn starch (Shigechi *et al*., 2004; Najafi and Kembhavi, 2005) and wheat starch (Iefuji *et al*., 1996) increased Ŭ-amylase activities.

1.12.5 RAW STARCH DIGESTING PROPERTY

Gelatinization of starch requires a high-energy input resulting in increased production cost of starch-based products. Hydrolysis of raw starch below gelatinization temperatures has gained importance in view of energy costs, effective utilization of natural resources and viscosity problems (Goyal*et al*., 2005). It turns out to be an economically superior alternative to the conventional process, which uses pregelatinised starch as substrate (Okolo *et al*., 1995). Among microorganisms, fungi such as *Aspergillus sp*., *Rhizopus sp*., and,*Corticium rolfsi* were reported to be good producers of raw starch digesting amylases. *Bacillus sp.* (e.g. *B. stearothermophilus*) was also reported to produce amylases capable of digesting raw starch

granules. A thermostable Ŭ-amylase from *Bacillus sp.* I-3 hydrolyzed raw potato starch at a concentration of 12.5% within 12 h (Goyal*et al*., 2005). The digestibility of Ŭ-amylase reported from *A. niger* from rotting cassava bagasse were of the order maize starch>cassava starch>sorghum starch>soluble potato starch (Okolo *et al*., 1995). Raw starch degrading Ŭ-amylase from a protease-negative *A. ficuum* mutant dissolved raw corn starch granules (Hayashida and Teramoto, 1986). Raw starch digesting Ŭ-amylases was reported to lose raw starch binding domain by the action of protease present in the culture filtrate. A liquefying Ŭ-amylase (RBLA) from *Bacillus sp*. was reported to act on raw starches over a wide range of pH=5.0ï 9.0 (Hayashida and Teramoto, 1986; Mitsuiki *et al*., 2005). Iefuji *et al*. (1996) compared raw starch digesting abilities of a thermostable Ŭ-amylase from the yeast *Cryptococcus sp*. S-2 and Taka amylase. Alpha Amylase from *Cryptococcus sp*. S-2 exhibited a stronger digestibility towards raw potato starch and weaker activity for wheat, corn,rice, and sweet potato starches while Taka amylase was reported to exhibit negligible raw starch digesting activity.

1.13AIM AND OBJECTIVES OF THE STUDY

1.13.1 Aim of the Study

The study was aimed at production, partial purification, and characterization of Ŭ-amylase from *Fusarium sp*. in a submerge fermentation system using *Ipomoea batatas* (sweet potato) starch as the only carbon source.

1.13.2 The specific objectives of the study include:

- Isolation and identification of strains of *Fusarium sp*.
- Production of Ŭ-amylase from the *Fusarium sp*. in submerge fermentation system using starch from *Ipomoea batatas* (sweet potato) as the only carbon source.
- Partial purification of extracellular Ŭ-amylase using ammonium sulphate precipitation and gel filtration.
- Characterization of the partially purified Ŭ-amylase with respect to pH, metal ions, temperature, and substrate concentration.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Soil Samples

Soils samples were collected from debries located at Zoological garden, site behind Odili post graduate hostel in University of Nigeria, Nsukka, Enugu State.

2.1.2 Collection of Potato Tubers

Potato tubers commonly known as sweet variety were purchased from Old-Park Market, Enugu, Enugu State.

2.1.3 Equipment and Instruments

The equipment used during the course of the experiment includes;conical flasks, test tubes, centrifuge (Fin lab-80-2B), electronic weighing balance(B2404-5 Mettle Toledo, Switzerland), hot plate, autoclave (280-A, China),wire loop, glass rod spreader, glass slide, scapel blade, A v-shaped glass rod, cover slip, 40x microscope (WESO microscope), bursen gas cylinder with stand, water bath (Model SSY-H,Shangai), pH meter (model PHS-3C, Search Tech. instrument), refrigerator (Haier Thermocool, China), UV/VIS spectrophotometer (Jenway 6405), petri dishes, thermometer, test tube racks, spatula, beakers (250ml), measuring cylinder, EDTA bottles, weighing balance (Ohans-Dial-O-Gram. Ohans-co-operation N J USA.), retort stand with clamp, and gel chromatography column (50 by 2.5 cm).

2.1.4Reagents

The reagents used during the course of the experiment includes;sabouraud dexterous agar (life Biotech, USA), sephadex G-100 (Pharmacy fine chemicals), acetic acid (May and Baker,Dagenhan, England), ammoninm sulphate (Burgoyne, India), magnesium sulphate(Anhydrous), ferrous chloride(Unhydrated), zinc chloride (powdered), starch solution, Bovine serum albumin (BSA) (Merck, England),sodium carbonate (Merck, England), Sodium hydroxide (Merck, Germany),Sodium potassium tartarate (Merck, Germany),folin-ciocalteau phenol reagent (Sigma-Aldrich, Germany.), dinitrosalicyclic acid (DNS) (Sigma-Aldrich,Germany), ethanol (Sigma-Aldrich Germany), hydrochloric acid (Sigma-Aldrich,Germany), phosphoric acid (BDH, pools England), sodium acetate (Vickers Laboratories Ltd, West Yorkshire, London.), sodium dihydrogen phosphate (BDH, Pools,

England.), sodium hydroxide (Avondale Laboratories), tris (hydroxymethyl) aminomethane (May & Baker Dagenhan, England).Whatman No 1 filter paper (Whatman International Ltd.,Maldstone England), distilled water (Pure and Industrial Chemistry, UNN). All the chemicals used in this experiment are of analytical grades and the reagents were freshly prepared at each use.

2.2 METHODS

2.2.1 Processing of Starch from Potato

Starch from potato was processed using the method described by Corbishley and Miller (1984). The tubers were bought, peeled, grated, rasped, and soaked in 1000ml of distilled water and allowed to stand for 24hr. The mixture was sieved and washed 3 times using 1000ml of distilled water. The starch was allowed to settle, then sun dried and stored in an air tight container.

2.2.2 Isolation of Amylolytic Fungi from Soil

Soil samples containing debries were pooled together from the site of collections into clean plastic cans and was taken to the laboratory for fungi isolation according to the method described by Ezeonu *etal*. (2013). Soil sample (20g) was weighed and mixed with 40ml of distilled water in a clean conical flask. The mixture was mixed vigorously and was used as the stock culture. From the stock preparation, ten folds serial dilution was carried out and the 10^{-4} to 10^{-6} dilutions were plated in a media plate.

2.2.2.1 Preparation of Media and Plate Pouring

Sabouraund dexterous agar was prepared according to the manufacturer description which involves dissolving 65g in 1000ml of distilled water. Five petri dishes were displayed and each contained 6.5g of sabouraund dexterous agar (SDA) dissolved in 100ml of distilled water. Chloramphenicol (250 mg) was added to the liquid broth and was mixed vigorously. The mixtures were autoclaved together with the petri dishes at 121^{o}C, 15 psi for 15min and was allowed to cool. The media was aseptically poured in each of the displayed petri dishes with gentle rocking of the plates to ensure even distribution of the media in the plate and was allowed to gel.

2.2.2.2 Inoculations of Folds of the Prepared Plate and Sub Culturing

From the 10^{-5} to 10^{-7} fold dilutions inoculations were done on the prepared sabouraund dexterous agar plates using a 1ml insulin syringe and a glass rod spreader around a bursen flame. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. After the inoculation, the inoculated plates were incubated for 3-4 days at room temperature until colonies appeared on the medium. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued until pure fungal cultures were obtained.

2.2.2.3 Microscopic Features of the Isolated Fungi

The colour, texture, nature of mycelia or spores and growth patterns of the pure cultures were observed.

2.2.2.4 Fungal Identification

The three day old pure cultures were used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of lactophenol blue was added to it. A cover slip was placed over it and examination was performed under the light microscope at X400 magnification. Identification was carried out by relating features and the micrographs to ñAtlas of mycologyò by Barnett and Hunters (1972).

2.2.2.5 Storage of Pure Fungal Isolates

Pure fungal isolates were maintained on sabouraund dextrose agar (SDA) slants as stock cultures in a bijou bottle. SDA medium was prepared according to the manufacturerôs description as described in section 2.2.1 and poured inside the bijou bottle. The medium was autoclaved at 121^{o}C, 15 psi for 15mins. It was allowed to cool to 45^{o}C and the bijou bottle were slanted slightly and allowed to gel. The bijou bottles were then incubated at room temperature for 3-4days to check for sterility.

2.2.3 Fermentation Experiment

2.2.3.1 Fermentation Broth

Submerged fermentation (SmF) technique was employed using a 250ml Erlenmeyer flask containing 100ml of sterile cultivation medium optimized for Ŭ-amylase with 1.4% NH_4SO_4, 2% Na_2HPO_4,0.3% $MgS0_4.7H_2O$, 0.1% $FeSO_4$, 0.3%$CaCl_2$, 1.6% $MnSO_4$, 2% $CoCl_2$, 1.4%

$ZnSO_4$,and 1% potato starch. The flask was stoppered with aluminum foil and autoclaved at 121^oC, 15 psi for 15min.

2.2.3.2 Inoculation of the Broth

From the SDA slants, fresh plates were prepared as described in Section 2.2.2.1 and inoculated. Three day old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10mm and then plugged properly. A seven day pilot study was carried out at room temperature (30^oC).

2.2.3.3 Harvest of the Fermented Broth

At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration. The filtrate was analyzed for Ŭ-amylase activity and extracellular protein concentration till the7th dayof fermentation.

2.2.3.4 Mass Production of Enzyme

After the 7 days pilot SmF studies, 6th day was chosen for mass production of enzyme from the respective fungal isolates. Five Erlenmeyer flasks (250ml) were used to produce 1litre of the enzyme using the method described in Sections 2.2.3.1 and 2.2.3.2.

2.2.4 Protein Determination

Protein content of the enzyme was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.2.4.1 Procedure for Protein Determination

For protein standard curve preparation, the reaction mixture contained 0.0-1.0ml of protein stock solution (2mg/ml BSA) in test tubes arranged in triplicates. The volume was made up to 1ml with distilled water. But for the test mixture, 0.1ml of sample enzyme was mixed with 0.9ml of distilled water. In either case, 5ml of solution E was added and allowed to stand at room temperature for 10mins. Then 0.5ml of solution C (dilute Folin-Ciocalteau phenol reagent) was added with rapid mixing. After standing for 30mins, absorbance was read at 750nm using UV spectrophotometer. The protein concentration by extrapolation from the protein standard curve.

2.2.5Assay for α-Amylase Activity

Ŭ-amylase activity was assayed bymeasuring the release of glucose from potato starch using 3, 5-dinitrosalicylic acid (DNSA) reagent assay method described by Miller (1959).The

reaction mixture containing 0.5ml of 1% potato starch in 0.02M phosphate buffer of pH 7.0 and 0.5ml of enzyme solution were incubated for 1hr. 1ml of DNSA reagent was added and the mixture was boiled for 10mins. To stop the reaction, 1 ml of Rochelleôs salt solution was added to stabilize the colour. The reaction mixture was allowed to cool and theabsorbance was read using spectrophotometer at 540nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the release of one micromole of glucose per min under assay conditions.

2.2.6Purification of α-amylase from *Fusarium spp.*

2.2.6.1 Determination of Percentage Ammonium Sulphate Saturation Suitable for α-Amylase Precipitation

Nine test tubes were used to perform an ammonium sulphate precipitation profile. Alpha amylases were precipitated with gentle stirring at 20-100% saturation of solid ammonium sulphate at intervals of 10% in each test tube. The ammonium sulphate-crude enzyme solutions were allowed to stand at4^{o}C for 30hr till the supernatant could be gently decanted off. The test tubes were centrifuged at 3500 rpm for 20mins. Precipitates from the individual percentage ammonium sulphate saturations were re-dissolved, respectively, in equal volumes of 0.02M phosphate buffer pH 7.0. Alpha amylases activities of the precipitates were assayed as described in section 2.2.5.

2.2.6.2 Mass Precipitation of α-αmylase

Twenty percent (20%) ammonium sulphate saturation was found suitable to precipitate protein with highest Ŭ-amylase activity. Crude enzyme (1 litre) was used for mass precipitation as described in section 2.2.6.1.

2.2.6.3 Gel Filtrations

A volume (30ml) of precipitated protein was introduced into a (50× 2.5cm) gel chromatographic column pre-equilibrated with 0.02M phosphate buffer pH 7.0. Fractions were collected at a flow rate of 5ml/20min. The protein concentration of each fraction was monitored using spectrophotometer at 280nm. The amylase activity of each fraction was assayed as earlier discussed with the active fractions pooled and stored at -10^{O}C.

2.2.7 Characterization of the partially purified enzyme

2.2.7.1 Effect of pH on α-amylase Activity

The optimum pH was determined using 0.02M sodium acetate buffer of pH ranging from 3.5 ï 6.0, phosphate buffer of pH 6.0 - 7.5 and Tris-HCl buffer of pH 8.0 - 9.0 at intervals of 0.5 units.The enzyme assay was carried out as discussed in section 2.2.4.

2.2.7.2 Effect of Temperature on α-amylase Activity

The optimum temperature was determined by incubating the enzyme with starch solution at temperature ranging from 30-70^{o}C using a thermo regulator (water bath) at interval of 10^{o}C for 30mins using 0.03M of $MnCl_2$ at optimum pH 6.0. The activity was then assayed as described in section 2.2.4

2.2.7.3. Effect of Metal Ions on α-amylase Isolated from *Fusarium spp.*

The effect of various divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}) on enzyme activity was evaluated. The reaction mixture was pre-incubated with varying concentrations (0.01-0.03M) of the divalent ions at pH 6.0 for 60mins. The Ŭ-amylase activity was assayed as described in section 2.2.4 and was compared with the control (i.e one without a metal ion).

2.2.7.4. Effect of Substrate Concentration on α-amylase Activity

The effect of substrate concentration on Ŭ-amylase activity was carried out by incubating the enzyme with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1mg/ml of starch solutions at pH 6.0 and temperature 50^{o}C, respectively. The maximum velocity(Vmax) and Michaelis constant(Km) were determined from the double reciprocal plot of initial velocity data at different substrate concentration.

2.2.8 Percentage yield of Starch from Potato

[% yield = $\frac{\square\square\square}{\square\square\square\square}$ x 100 = 85.9%]

CHAPTER THREE
RESULTS

3.1 The Isolate of *Fusarium sp*.

The plate 1 below shows the pure isolate of*Fusarium sp.* from soil. This culture was used to prepare the working cultures used to carry out the fermentation experiments.

3.2 Percentage Yield of Starch from Potato

Starch was extracted from 1kg of potato with extraction yield of 85.9%

3.3 Effect of Incubation Time on α-amylase Production

The potato starch was fermented by *Fusarium spp* for seven days to determine the day(s) of highest Ŭ-amylase production (Figures 3 and 4). Maximum Ŭ-amylase activity was observed on the 6^{th} day. There was also corresponding increase in protein concentration with respect to increased Ŭ-amylase activity (Figures 3and 4).

Plate 1: Pure culture of *Fusarium sp*.

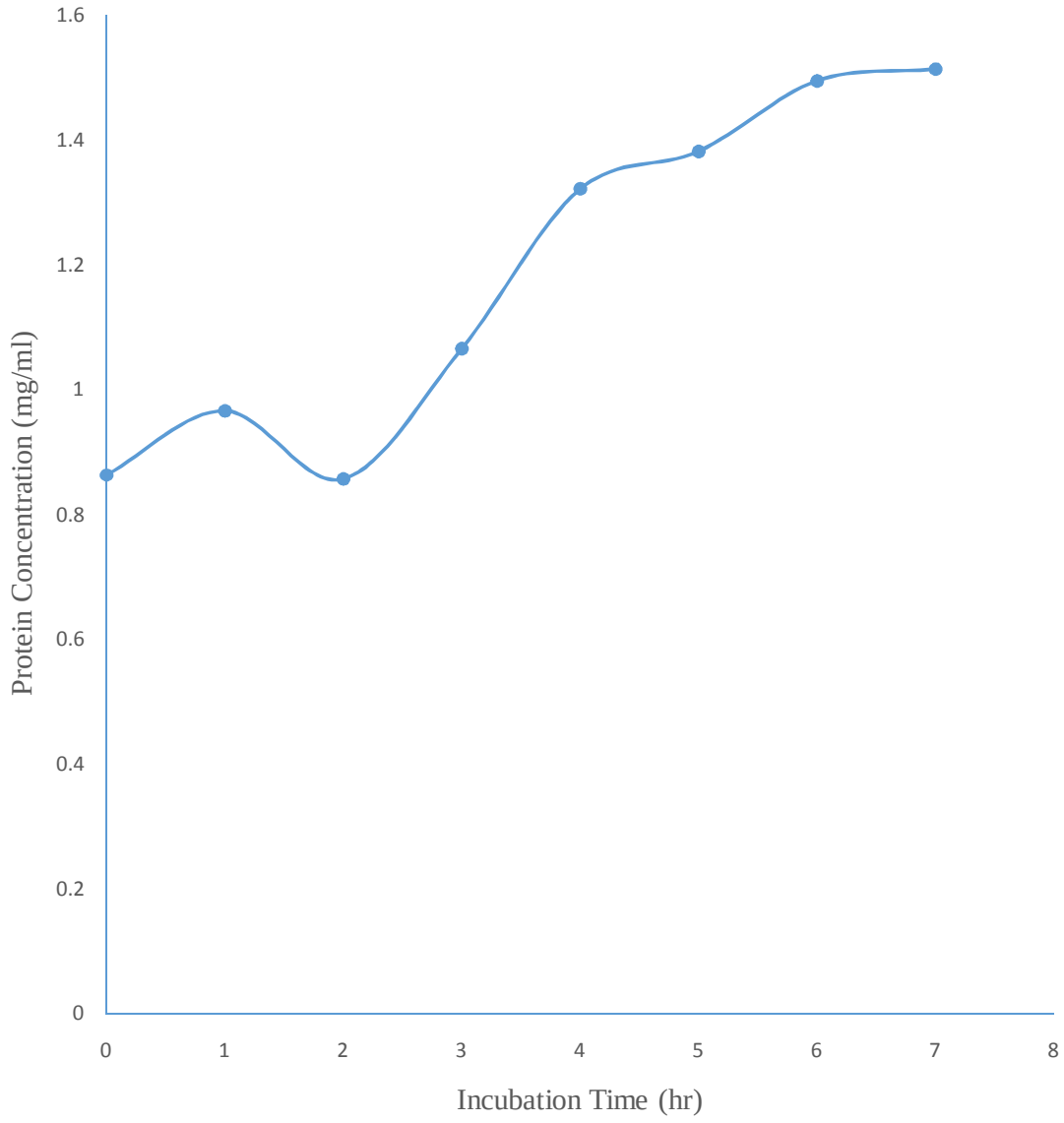

Figure 3: Effect of incubation time on the protein concentration in liquid broth using starch from potato tuber as the only carbon source.

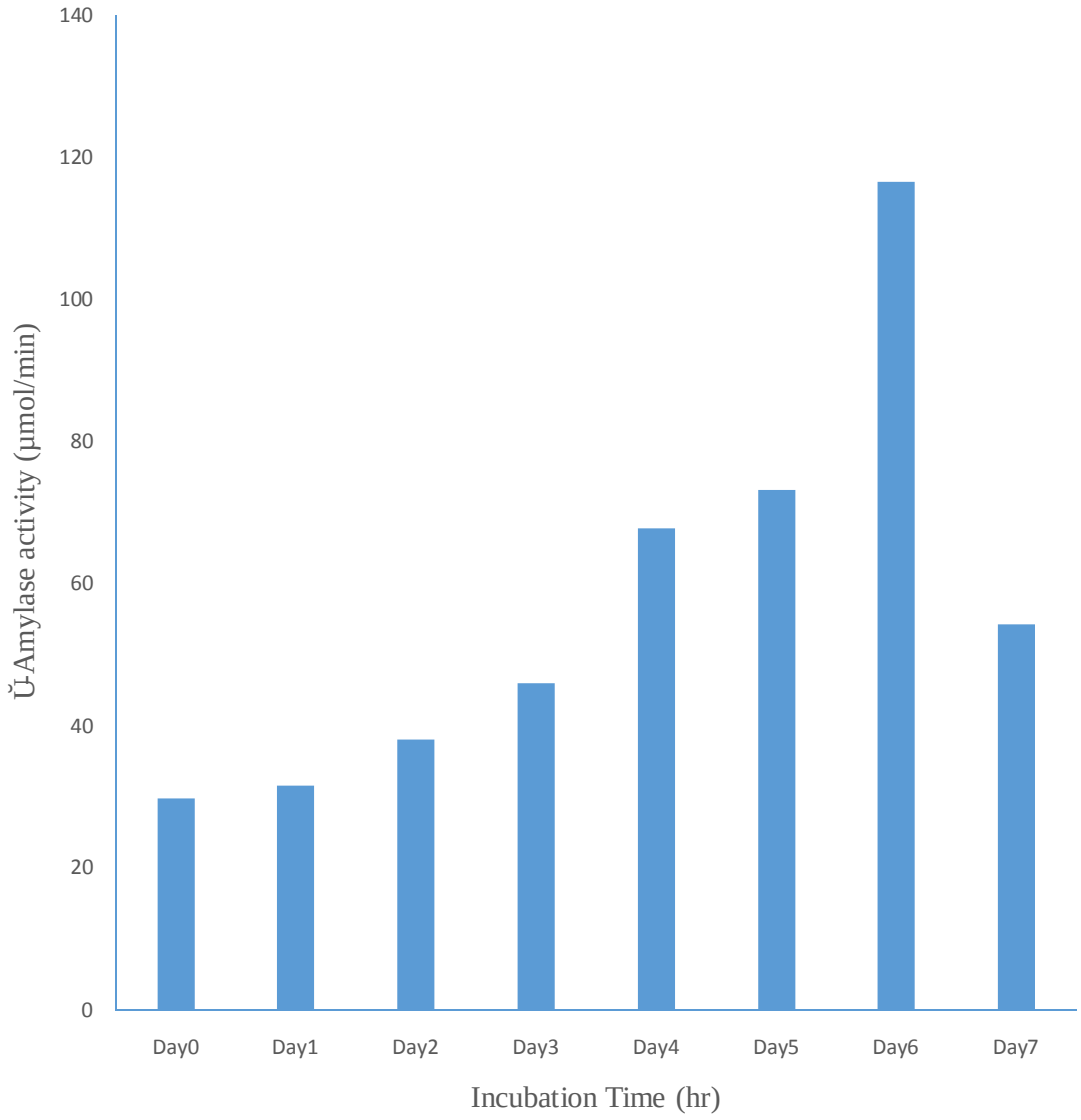

Figure 4: Effect of incubation time on Ŭ-amylase activity in liquid broth using starch from potato tuber as the only carbon source.

3.4 Purification of Crude α-Amylase

The harvested crude enzyme was subjected to ammonium sulphate precipitation and gel filtration respectively as outlined in Sections 2.2.6.2 and 2.2.6.3

3.4.1 Ammonium Sulphate Precipitation

The studies show that 20% ammonium sulphate saturation gave the highest Ŭ-amylase activity as shown in Figure 5.

3.4.2 Gel filteration

Figure 6 shows the elution profile of Ŭ-amylase obtained from *Fusarium sp.*

3.4.3α-amylase Purification

After partial purification of the Ŭ-amylase up to the level of gel filtration, it was found that the specific activity of crude enzyme was 55.45µmol/min/mg. After ammonium sulphate precipitation and gel filtration, the specific activities werefound to be 335.93 and 34.60µmol/min/mg, respectively (Table 7).

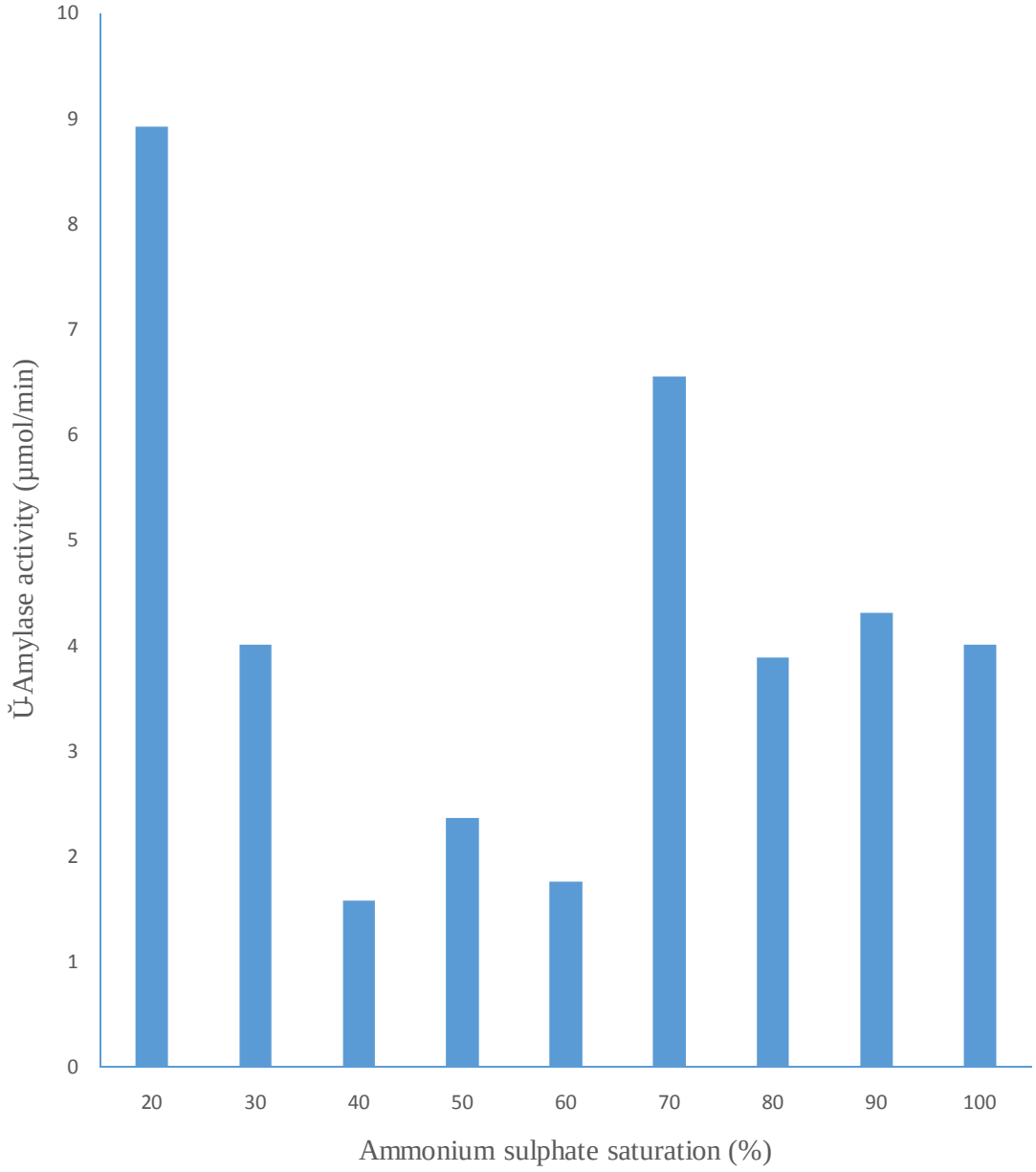

Figure 5: Ammonium sulphate precipitation profile ofŬ-amylase from *Fusarium sp*. cultured for six days using starch from Potato tuber as the carbon source.

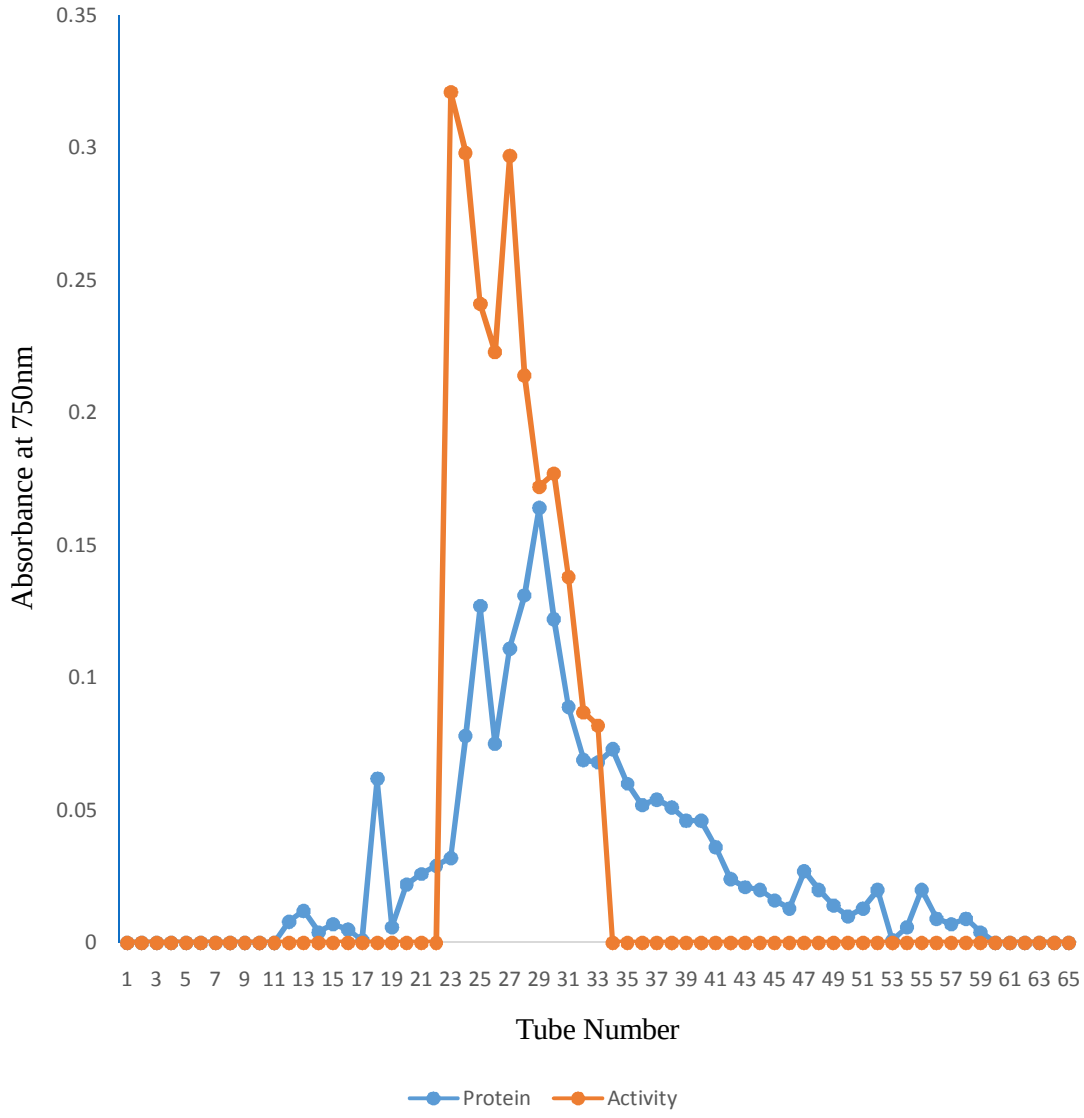

Figure 6: Elution profile of Ŭ-amylase from *Fusarium sp*. cultured for six days using starch from potato tuber.

Table 9: Purification table of Ŭ-amylase from *Fusarium sp*. harvested after 6days of fermentation using starch from potato tuber as the carbon source

Enzyme	Volume (ml)	Protein(mg/ml)	Total protein(g/dL)	Activity (µmol/min)	Total activity	Specific activity (U/mg)	Purification fold	Percentage yield
Crude	500	1.775	887.5	98.423	49211.5	55.45	1	100
$(NH_4)_2S04$ Gel	60	0.583	34.98	20.95	1257	35.93	0.65	2.55
Filtration	30	0.163	4.89	19.497	584.91	119.61	3.33	46.53

3.5Characterization of Partially Purified α-amylase

3.5.1 Effect of pH

Increase in pH from 3.5 to 6.0 was accompanied by an increase in Ŭ-amylase activity after which the enzyme activity decreased making 6.0 the optimum pH as shown in Figure 7.

3.5.2 Effect of Temperature

Increase in temperature from 30^{o}C to 50^{o}C was accompanied with an increase in Ŭ-amylase activity beyond which the enzyme activity decreased making 50^{o}C the optimum temperature as shown in Figure 8.

3.5.3 Effect of divalent metal ion concentration

Catalytic function of the partially purified Ŭ-amylase was stimulated by Ca^{2+}, Mg^{2+}, and Mn^{2+} while it was slightly inhibited by Co^{2+}. The effect of metal ion on Ŭ-amylase activity was found to be concentration dependent (Figure 9)

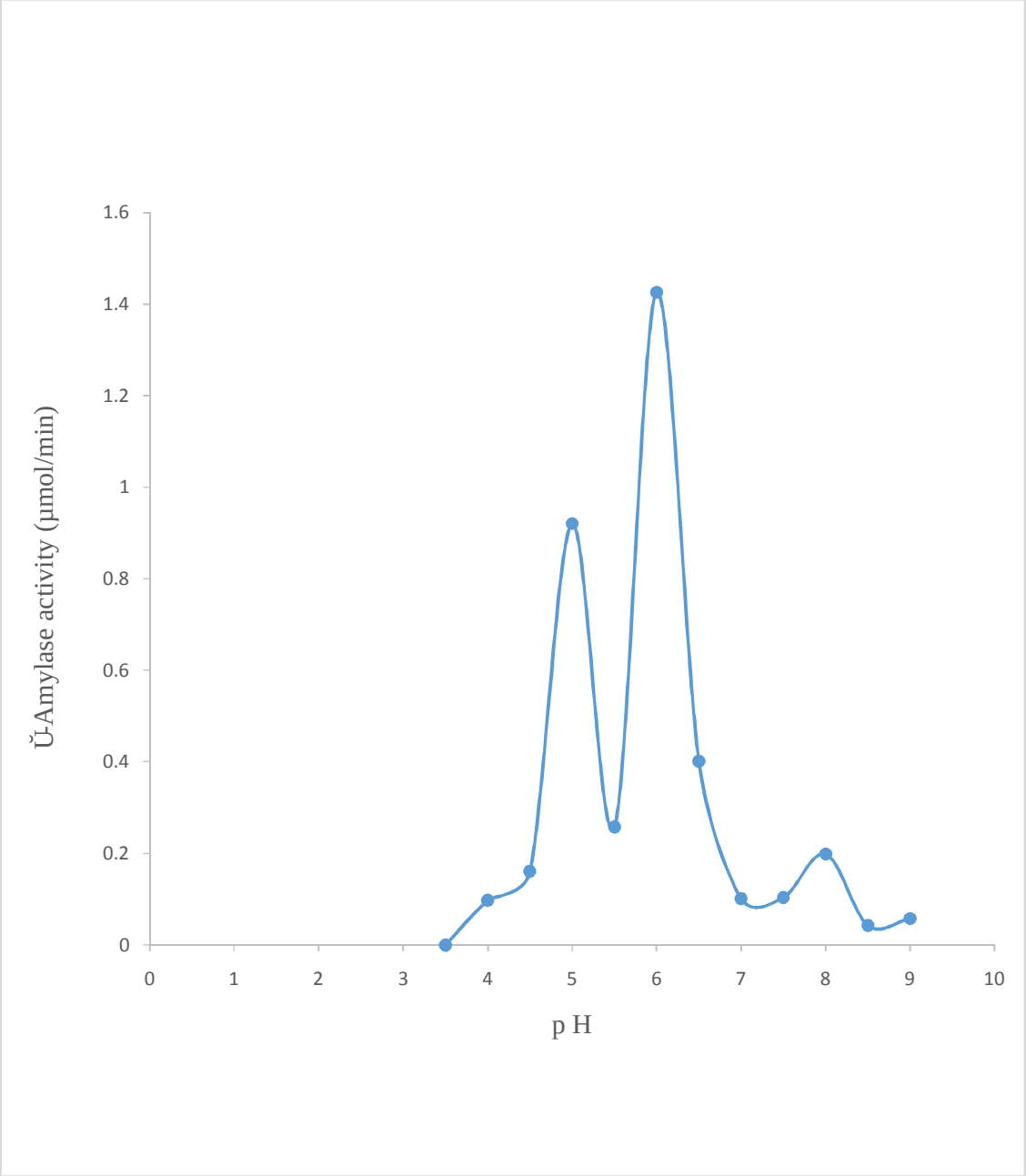

Figure 7: Effect of pH on Ŭ-amylase activity produced from *Fusarium sp*. cultured for six days using starch from potato tuber.

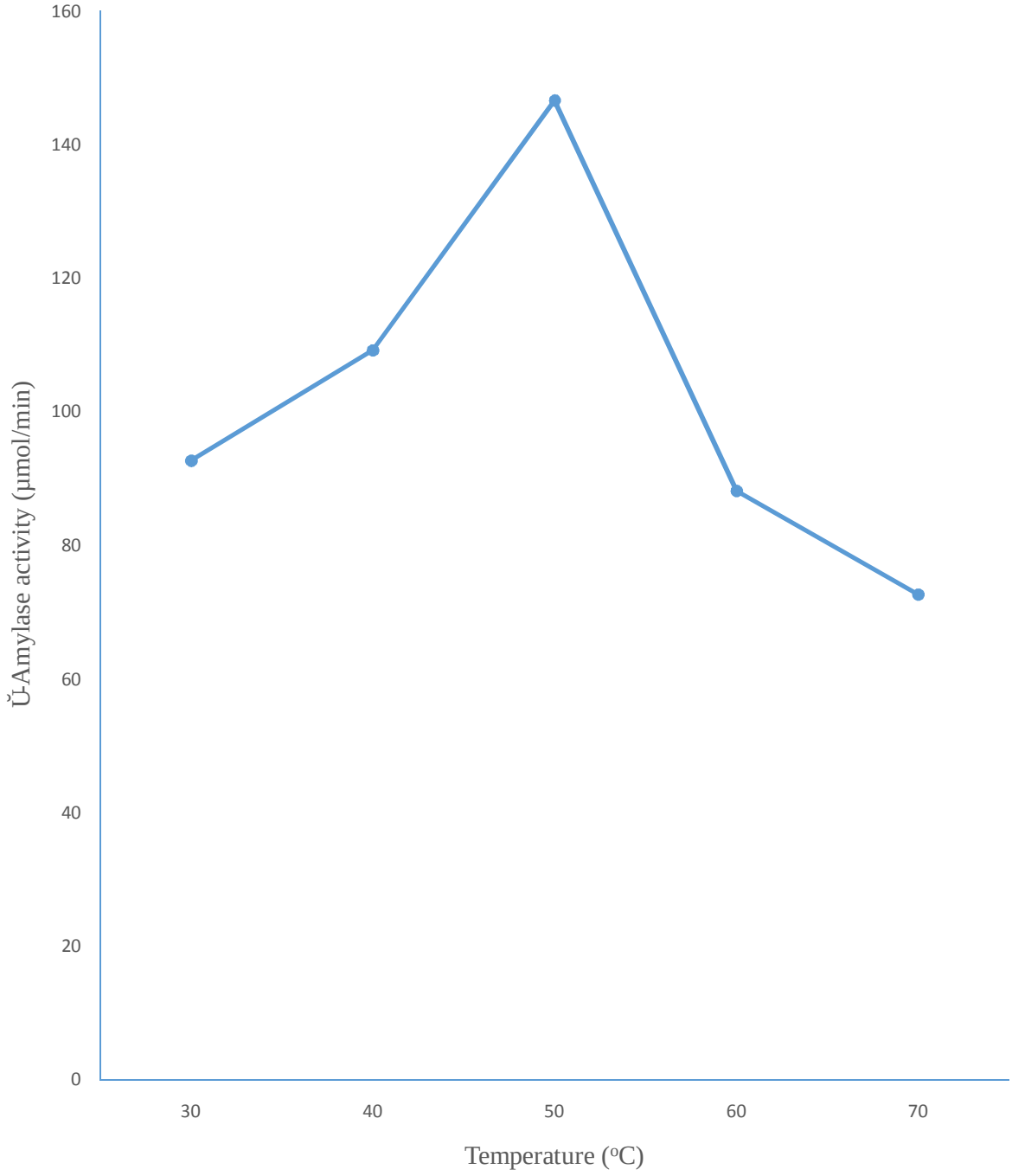

Figure 8: Effect of temperature on Ŭ-amylase activity produced from *Fusarium sp*. cultured for six days using starch from potato tuber.

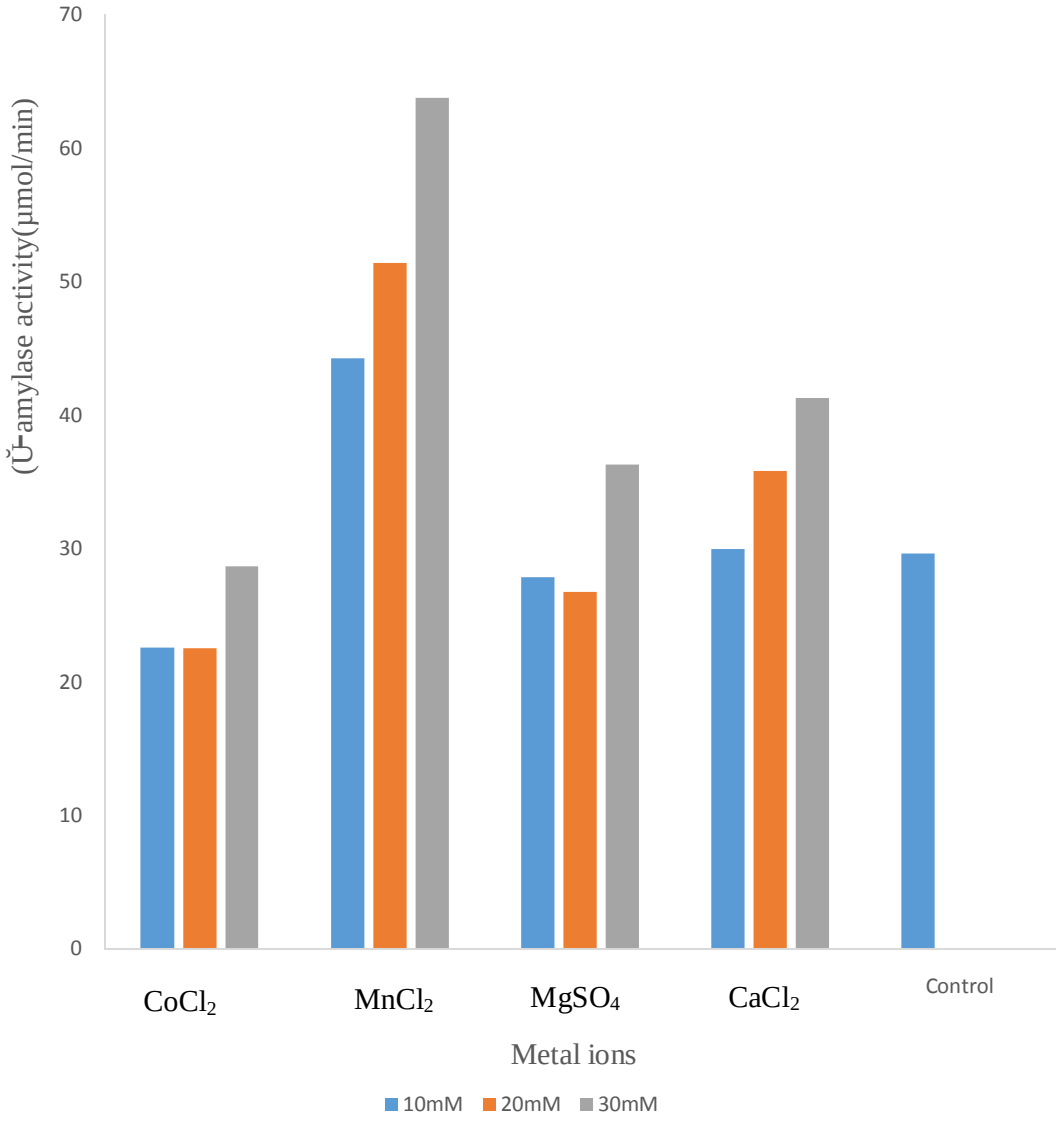

Figure 9: Effect of different metal ion concentrationson Ŭ-amylase activity

3.6 Determination of Kinetic Parameters

The Michaelis constant (Km) and maximum velocity (Vmax) obtained from the Lineweaver-Burk plot of initial velocity data at different substrate concentration were 5.44mg/ml and 12.57µmol/min, respectively as shown in Figure 10.

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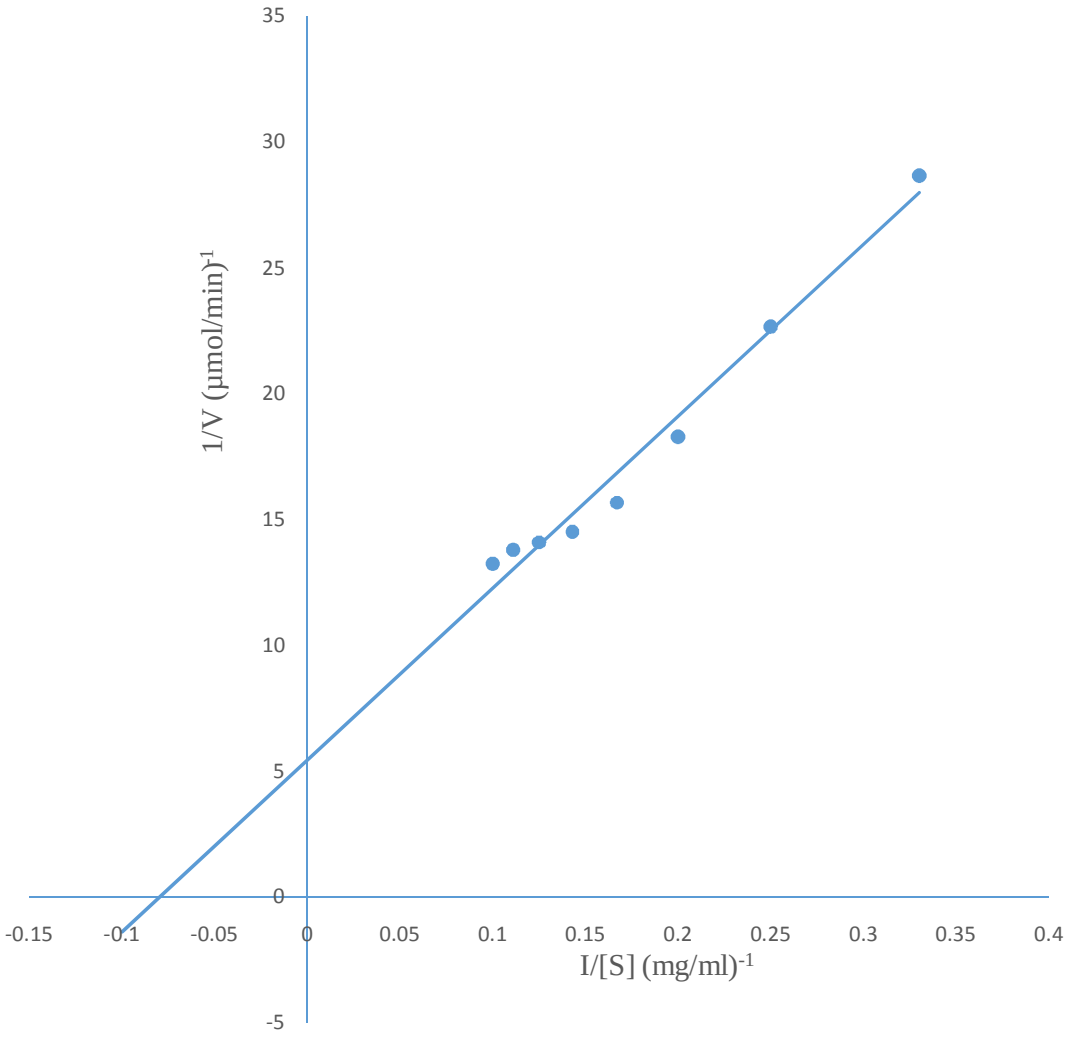

Figure 10: Lineweaver-burk plot for Ŭ-amylase from *Fusarium sp*. cultured for six days using different concentrations of starch from potato tuber as substrate and carbon source.

CHAPTER FOUR

DISCUSSION

The properties of Ŭ-amylases such as thermostability and pH profile should match its application. Hence, the diversity of industrial applications creates the need to search for novel Ŭ-amylases with novel and improved properties. The activities of enzymes are generally affected or regulated by a number of biochemical and physico-chemical factors (Elias *et al*., 2014; Hu *et al.*, 2014), some of which may interact with the enzyme molecule in a non-covalent manner inducing a temporaryeffect (decrease or increase in activity) (Jadhav *et al*., 2014) or in a covalent interaction causing a permanent or irreversible change in the enzymeôs conformation (protein denaturation) (Filtz *et al*., 2014). Hence, enzymes can be characterized based on their responses to the influence(s) of effector molecules and or physico-chemical factors such as change in temperature, pH, presence of a ligand, cofactors (metal ions), affinity for substrate/substrate specificity, thermal stability, salt tolerance, values of Michaelis-Menten constants (Vmax, Km).

The percentage yield of starch from sweet potato (*Ipomoea batatas*) was 85.9%. After seven day pilot study, day 6 was found suitable for Ŭ-amylase production. This is in accordance with the report of Iieasanmi and Oluwaseun.(2012), thatmaximum amylase production by *Aspergillus flavus*was on the 6^{th} day of incubation. This is also very similar to reports of Mukherjee and Majumdar (1973) and Olama and Sabry (1989). The authors both reported maximum amylase production by *Aspergillus flavus* on the 7^{th} day. Decrease in amylase production beyond the optimum incubation period could be attributed to catabolite repression caused by glucose released into the medium. It could also be as a result of the secretion of proteolytic proteins which are known to cause the denaturation of proteins (Gupta *et al*., 2008).Short incubation period offers potential forinexpensive production of enzyme (Somjoy *et al*.,1995).

The crude enzyme was purified by gel filtration via ammonium sulphate precipitation. 20% ammonium sulphate saturation was found suitable to precipitate protein with highest Ŭ-amylase activity. This precipitation occurs in a way that, proteins in aqueous solution are heavily hydrated because of their hydrophilic interaction with water molecules and with the addition of ammonium sulphate, the water molecules become more attracted to the salt than to the protein due to the higher charge or ionic strength. This competition for hydration is usually more favourable towards the salt, which leads to interaction between the proteins,

resulting in aggregation and finally precipitation or salting out (Markus and Aaron, 2007). After ammonium sulphate precipitation and gel filtration, the specific activity was found to increase from 35.93 µmol/min/mg to 119.61µmol/min/mg with 3.33 purification fold. The increase in activity after gel filtration suggest increase in purity.Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of Ŭ-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in the purified form is also a prerequisite in studies of structure-function relationships and biochemical properties (Gupta *et al*., 2003). Different strategies for purification of enzymes have been investigated, exploiting specific characteristics of the target biomolecule. Adefila *et al*. (2012) reported a purification fold of 4 and a specific activity of 11,500 on a three purification steps using ammonium sulphate precipitation, ion exchange chromatography and CM- sepharose.

pH is known to affect the synthesis and secretion of Ŭ-amylase just like its stability (Fogarty, 1983). The optimum pH for Ŭ-amylase activity was 6.0. This is in accordance with Iieasanmi and Oluawseun.(2012), who reported the optimum pH of Ŭ-amylase from *Aspergillus flavus* as 6.0. Also, Iefuji *et al*. (1996) reported pH of 6.0 for Ŭ-amylase from *Cryptococcus sp*. The result is in close range with the report of Nwagu and Okolo (2011) that reported an optimum pH of 6.5 for Ŭ-amylase isolated from *Fusarium sp.*

It is desirable that Ŭ-amylases should be active at high temperatures of gelatinization and liquefaction to economize the process; therefore, there has been a need and continual search for more thermophilic and thermostable Ŭ-amylase.The result showed that the amylase was optimally active at 50°C. Afterward, the temperature decreased. Decrease in enzyme activity at higher temperature may be due to inactivation of amylase or suppression of cell viability. This report is in agreement with Nwagu and Okolo (2011) who reported a temperature of 50^{o}C for amylase from *Fusarium sp*. and also Wanderley *et al*. (2004), that reported the same temperature for amylase isolated from *Cryptococcus flavus.* The report iscontrary to the report of Yang and Liu (2004) who reported a temperature of 60^{o}C for amylase isolated from *Thermobifida fusca.*

Stability of an enzyme in presence of metal salts plays a vital role in their application in industries. More than 75% of enzymes require the presence of metal ion activators to express their full catalytic activity (Tunga *et al*., 1999).Ca^{2+}ions are reported to be present in majority

of these enzymes. The hydrolytic activity of the partially purified enzyme was highly activated by Mn^{2+}, Ca^{2+}, and Mg^{2+} and inhibited by Co^{2+}. The inhibition of amylase activity by Co^{2+}could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity (Leveque *et al*., 2000).

The Michaelis constant (Km) and maximum velocity(Vmax) obtained from the Lineweaver-burk plot of initial velocity at different substrate concentrations were 5.44mg/ml and 12.57µmol/min, respectively. Devendra *et al*. (2013) reported 3.7mg/ml and 42.39µmol/min for Km and Vmax using Ŭ-amylase isolated from *Fusarium solani.* Turk (2012) reported4.0mg/ml and 5000U/mg for Km and Vmax using Ŭ-amylase isolated from *Bacillus subtilis*. Also, Tapati and Rintu (2015), reported 0.5mg/ml and 1000U/mg for Km and Vmax respectively for Ŭ-amylase isolated from *Aspergillus oryzae.*

CONCLUSION

The study has shown that potato starch can be a very good carbon source for Ŭ-amylase production from *Fusarium sp*. Also, the enzyme has very high affinity for the substrate. The optimum conditions obtained suggest that the enzyme could have biological applications in industry.

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APPENDICES

Appendix 1:

Procedure for Glucose Determination

For glucose standard curve preparation, the reaction mixture contained 0.0-1.0ml of glucose solution (5mM) in test tubes arranged in triplicates. The volume was made up to 1ml with distilled water. It was mixed with 1ml of DNSA reagent, boiled for 10minutes and the reaction was stopped with rochelles salts and the reading was taken at the wavelength of 540nm. The final concentration of the stock glucose solution (5mM) was determined using the dilution equation $C_1V_1=C_2V_2$. Glucose concentration from the test samples were extrapolated from the glucose standard curve.

Appendix 2:

Preparation of Buffer Solutions

Sodium acetate buffer (stock solution)

In preparing 0.02M of sodium acetate buffer of pH 6.0, 1.64g of sodium acetate salt was dissolved in 1000ml of distilled water. The pH was adjusted to 6.0 with the conjugate acid.

Preparation of working Acetate buffer solution

The working acetate buffer (0.02M, pH 6.0) was freshly prepared when needed, measured and mixed with distilled water and refrigerated.

Sodium phosphate buffer (stock solution)

To prepare 0.02M of acetate buffer, 2.82g of sodium phosphate salts was dissolved in 1000ml of distilled water. The pH was adjusted with phosphoric acid.

Tris-HCl buffer (stock solution)

Tris buffer (0.02M) was prepared by weighing 2.422g of Tris (hydroxmethyl) aminomethane and dissolving some in 1000ml of distilled water, the pH was adjusted with hydrochloric acid

Appendix 3: Preparation of the Component Reagents for Protein Determination

Solution A: An alkaline sodium carbonate (Na_2CO_3) was prepared by dissolving 2g of Na_2CO_3 in 100ml of 0.1M NaOH (0.4g of sodium hydroxide pellets were dissolved in 100ml of distilled water).

Solution B: A copper tetraoxosulphate VI - sodium potassium tartarate solution was prepared by dissolving 0.5g of $CuSO_4$ in 1g of sodium potassium tatarate, all in 100ml of

distilled water. It was prepared fresh by mixing stock solution, and so was done whenever required.

Solution C: Folin-Ciocalteau phenol reagent was made by diluting the commercial reagent with water in a ratio of 1:1 on the day of use.

Solution D: Standard protein (Bovine Serum Albumin, BSA) solution.

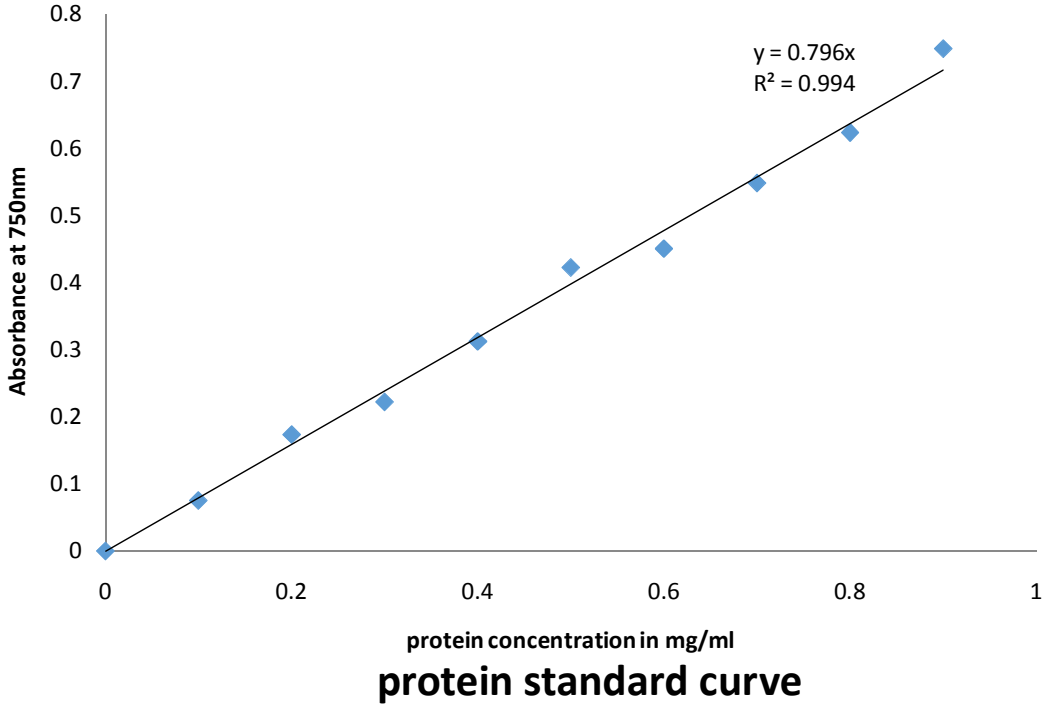
Solution E: Freshly prepared alkaline solution was made by mixing 50ml of solutions A and 1ml of solution B.

1.7 Preparation of 2mg/ml Bovine Serum Albumin (BSA) Standard Protein

BSA (0.2 g) was dissolved in 100ml of distilled water and then used as a protein stock solution.

Appendix 4: Values for Protein standard curve

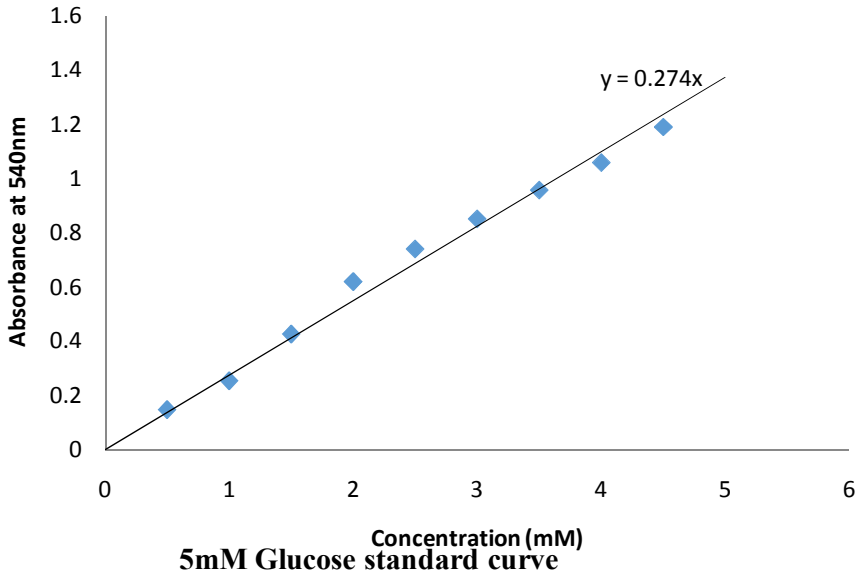
Protein conc	Absorbance
0	0
0.1	0.075
0.2	0.173
0.3	0.222
0.4	0.312
0.5	0.422
0.6	0.45
0.7	0.548
0.8	0.623
0.9	0.748



Appendix 5: Protein standard curve

Appendix 6: values for Glucose standard curve

0.5	0.147
1	0.254
1.5	0.4265
2	0.6195
2.5	0.7405
3	0.8515
3.5	0.957
4	1.0585
4.5	1.19



5mM Glucose standard curve

Appendix 7: Glucose standard curve