PARTIAL PURIFICATION AND CHARACTERIZATION OF AMYLASE FROM GERMINATED AFRICAN YAM BEAN SEEDS (*Sphenostylis stenocarpa)*

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

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JANUARY, 2015

CERTIFICATION

Ugwu, Christopher Ejike a post graduate student with Registration Number PG/M.Sc/12/61850 in the Department of Biochemistry has satisfactorily completed the requirement for the course work and research for the award of 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.

…….
……..

Prof. F. C. Chilaka
(Supervisor)

Prof. O.F.C Nwodo
(Head of Department)

…….
External Examiner

DEDICATION

This work is dedicated to the Holy Spirit.

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ABSTRACT

The activity of Ŭ-amylase and protein concentration in African yam bean seeds increases as germination progresses up to day 8 of germination where it exhibited its highest level, followed by sequenced decreased activity and protein concentration till day 12. Starch from African yam bean seeds, corn and cassava were used for the hydrolysis experiment. There was a significant activity of Ŭ-amylase in each of the substrate used, however, the starch from African yam bean seeds had higher Ŭ-amylase activity (369.55µmol/min at pH 5.5 and 369.55µmol/min at pH 9.0) followed by starch from corn (367.08µmol/min and 360.49µmol/min at pH 5.5 and 9.0 respectively), while cassava had the least with activity of 353.50µmol/min and 351.03µmol/min at pH 5.5 and 9.0 respectively. The crude enzyme was purified to the level of gel filtration (sephadex G-25) via 80% ammonium sulphate precipitation. The purification fold of 1.36 with specific activity 226.44εmol/min/mg protein and 1.62 with specific activity 367.65εmol/min /mg protein were observed for 80% ammonium sulphate precipitation and gel filtration, respectively. The enzyme displayed optimum activity at pH 5.5 and temperature 45°C in all the three substrates (African yam bean, corn and cassava). The Michaelis menten constant (Km) and maximum velocity (Vmax) obtained from Lineweaver-Burk plot of initial velocity data at different concentrations of starch from African yam bean seeds as substrate were found to be 0.588mg/ml and 588.24εmol/min, respectively. Similarly, 0.625 mg/ml and 625εmol/min were obtained using starch from corn, respectively, while 0.733mg/ml and 666.7εmol/min were also observed using starch from cassava, respectively. The enzyme activities were enhanced in the presence of some metal ions like Ca^{2+}, Co^{2+} and Fe^{2+}. Zn^{2+} and Na^{2+} neither increased nor decreased enzyme activity while Pb^{2+} completely inactivated the enzyme.

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CHAPTER ONE
INTRODUCTION

African yam bean (*Sphenostylis stenocarpa*), belongs to the legume family. It originated in Ethiopia, both wild and cultivated types now occur in tropical Africa as far north as Egypt and also throughout West Africa from Guinea to Southern Africa (Assefa and kliener, 1997). It is cultivated in Nigeria mainly for its seed. The African yam bean (AYB) is a climbing legume adapted to lowland tropical conditions. There are seven species in the genus *Sphenostylis* (Potter and Doyle 1994). African yam bean is the most valuable. Some species in the genus *Sphenostylis* provide two consumable products, the tuber which grows as the root source and the actual yam beans which develop in pods above ground (Daniel, 2010). African yam beans seed is classified as a neglected under-utilized species (Mentreddy, 2007) due to its low esteem and lack of detailed information on its compositional analysis (Adewale *et al*., 2010).

Starch is a storage polysaccharide present in seeds and it consist of two components; a linear glucose polymer, amylose, which contain Ŭ-1,4 linkage chains and a branch polymer, amylopectin in which linear chains of Ŭ-1,4 glucose residue are inter-linked by Ŭ-1,6 linkages. Starch is hydrolyzed into smaller oligossaccharides by Ŭ-amylase, which is one of the most important commercial enzyme processes.

Ŭ-Amylases catalyze the hydrolysis of Ŭ-1,4-glycosidic linkages in starch to produce low molecular weight products, such as glucose, maltose and maltotriose units (Tangphatsornruang *et al*., 2005). Amylases can be obtained from several sources, such as plants, animals and microorganisms. Funke and Melzing, (2006) reported that amylases of plant origin have the highest hydrolytic potential followed by that of fungi, while amylases from bacteria have relatively less hydrolytic potential. A number of plant amylases have been identified and plants are one of the abundant sources of Ŭ-amylase (Conforti *et al*., 2005). The enzyme has been extracted from plants sources like barley, millets, wheat, sorghum and maize among others. However, no study was carried out on activity of Ŭ-amylase from germinated African yam bean seeds. Ŭ-amylases from beans have gained importance due to their suitability for biotechnological applications in supplementary foods, breweries and starch saccharification (Muralikrishna and Nirmala, 2005).
Recently, interest and demand for enzymes with novel properties are very high in various industries and it leads to the discovery of various types of Ŭ-amylase with unique properties. Therefore, the present investigation was initiated to African yam bean seeds for Ŭ-amylase activity, to determine the relative abundance and activity of this enzyme during germination and to establish some characteristics for their action

1.1 African Yam Beans

African yam bean is an underutilized tropical African tuberous legume (figure 1A). It belongs to the class *Magnoliopsida;* order *Fabales;* family *Fabaceae;* subfamily *Papilionoideaea;* and genus *Sphenostylis.* There are seven species in the genus *Sphenostylis* (Potter and Doyle, 1994). African yam bean (AYB) is the most valuable. It is a vigorously climbing herbaceous vine whose height can reach 1.5ï 3 m or more. The main vine/stem produces many branches which also twine strongly on available stakes. The vegetative growing stage is characterized with the profuse production of trifoliate leaves (Utter, 2007).

From four to ten flowers are arranged in racemes on long peduncles, usually on the primary and secondary branches. The large and attractive flowers blend pink with purple; the standard

petals twist slightly backwards on themselves at anthesis. The flowers seem to exhibit self-pollination; up to six pods/peduncle may result after fertilization

Figure 1: African yam bean plant showing mature pods ready for harvest (Daniel, 2010).

They are usually linear and long unicarpel pods turn brown when mature (Hutchinson and Dalziel, 1958; Dukes, 1981). The pods (figure 1) which may sometimes be flat or raised in a ridge-like form on both margins are usually prone to shattering; they dehisce along the dorsal and ventral sutures when dry. Each pod can yield up to 20 seeds which may be round, oval, oblong or rhomboid (Figure 2B). There are varieties of seed colour (Oshodi et al., 1995) and size (Adewale et al., 2010) with mono-coloured or mosaic types. Mono-coloured seeds are white, grey, cream, light or dark brown purple, or black. *Sphenostylis sternocarpa* is native to tropical west and central Africa and is cultivated in southern and eastern Africa. It thrives on deep, loose sandy and loamy soils with good organic content and good drainage. It grows better in regions where annual rainfalls range between 800-1400 mm and where temperatures are comprised between 19-27°C. The plant flowers after 90 days and the pods mature in 140 to 210 days while the tubers can be harvested 150 to 240 days after sowing (Utter, 2007). African yam bean is usually grown in mixtures with yam and cassava. Protein content is up to 19% in the tubers and 29% in the seed grain.

(A)

(B)

Figure 2: (A) Tuber yield per stand of AYB (Daniel, 2010) and (B) raw seeds of African yam bean

1.1.2 Seed germination

Seed germination is a very important phase in the growth of any plant. Seed coat which may be thick and hard or thin and soft is the outer covering of seed which protects embryo from mechanical injury , entry of parasites and prevents it from drying. Endosperm is a temporary food supply which is packed around the embryo in the form of cotyledons or seed leaves. Processes involved in germinating occur in different stages:

Absorption of water and bursting of seed coat is the first sign of germination. In this stage, there is an activation of enzymes, increase in respiration, and plant cells get duplicated. A chain of chemical changes starts which leads to development of plant embryo.
Chemical energy stored in the form of starch is converted to sugar which is used during germination process. This leads to enlargement and bursting of seed coat.
Growing plant emerges out tip of root first emerges and help to anchor the seed in a place. It also allows embryo to absorb mineral and water from the soil.

During germination, the principal enzyme involved in carbohydrate breakdown is Ŭ-amylase which hydrolyses Ŭ(1-4) bond in amylose and amylopectin releasing fragments that can be further broken down by ɓ-amylase, Ŭ-glucosidase and debranching enzymes. Ŭ-amylase is synthesized de novo in two specific tissue of seeds, the scutella epithelium of the embryo and the aleurone layer of the endosperm (Ball *et al*., 2003). In the seed, enzyme syntheses begins initially in the scutellum after imbibition and then in the aleurone layer after few days (Okano *et al*., 2009). Secretion of amylase from the cells of the aleurone layers is well established in cereal grains and there is evidence that a similar process takes place in at least some dicotyledonous seeds (Niittyla *et al*., 2004).

Starch in the endosperm of cereals is the most abundant reserve synthesized during seed development. Degradation of starch into soluble sugar is important to support seedling growth

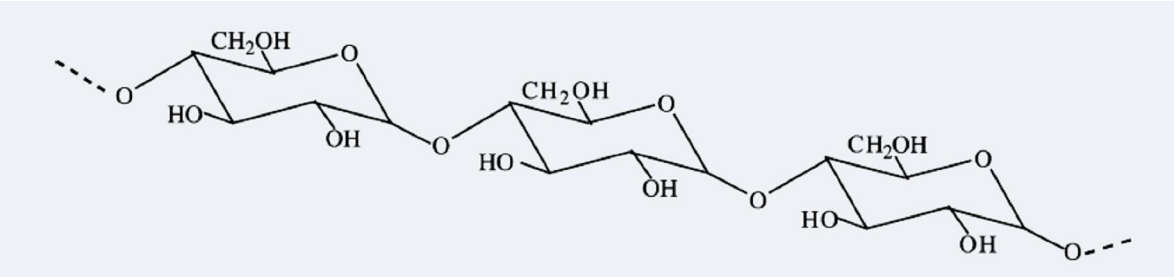
during seed germination. Starch can be degraded either by hydrolysis with amylase or phospholysis with starch phosphorylase. In germinating seeds, hydrolysis but not phospholysis is the major process to breakdown starch molecules. Ŭ-amylase and ɓ-amylase are the major amylolytic enzymes found during seed germination and it was suggested that both enzymes are involved in the degradation of endospermic starch (Marc *et al*., 2002). However, ɓ-amylase is synthesized and accumulated as a latent form in the starchy endosperm during seed development (Hang *et al*., 1996).

1.2 Starch

In the green leaves, carbon dioxide and water are transformed into glucose and oxygen under the influence of sunlight and with the help of chlorophyll. This process is known as photosynthesis. During the day this starch is deposited as grains in the leaf, the so-called leaf-transition starch. During the night this starch is partially broken down again into sugars which are transported to other areas of the plant. From these sugars the starch arises which is won in the familiar grain shape. The forming of starch is a process which has by far not been clarified yet and during which a number of enzymes play a role.

Starch or amylum is a carbohydrate consisting of a large number of glucose units joined by glycosidic bonds. 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 (Van der Maarel *et a.l.* 2002., Goyal *et al,.*2005).With the help of a microscope the grain shape reveals from which plant species the starch derives. Native starch, the starch as it occurs in the plant, cannot be dissolved in cold water. When we scatter starch, while stirring, into water we get a milky white suspension which can be stirred without much difficulty. When the stirring is stopped the starch sinks to the bottom (sedimentation), during which a transparent upper layer is formed. When the suspension is heated the white colour disappears at a temperature characteristic for starch. The starch dissolves into an almost transparent solution. This is what we call gelatinized starch. In comparison with the ungelatinized suspension, stirring takes considerably more difficulty. The temperature at which the resistance during stirring noticeably increases, is called the gelatinization temperature. Gelatinizing starch into viscous substances is one of the most, if not the most important characteristic(s) of starch. This phenomenon lies at the basis of the successful application of starch in a large number of sectors. 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 (Fig. 3). Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units with Ŭ-1,4glycosidic 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-1,4glycosidic bonds present in the inner part of the amylose or amylopectin chain (Muralikrishna and Nirmala, 2005; Van der Maarel *et al*., 2002).

A. Structure of amylose



B. Structure of amylopectin

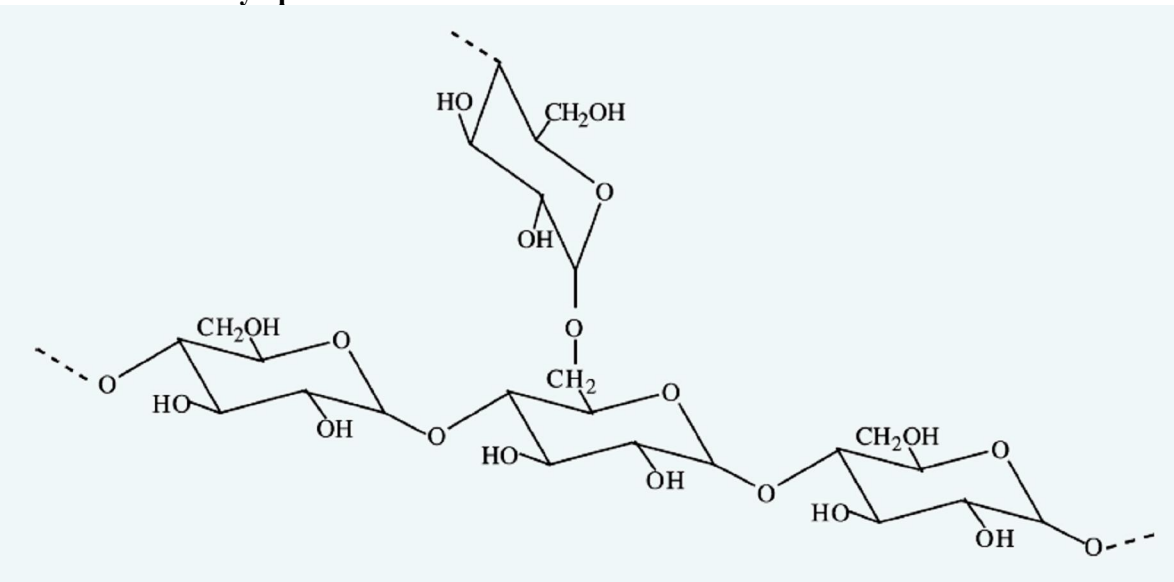


Figure 3**:** Two types of glucose polymers are present in starch: amylose (A) is a linear polymer consisting of up to 6000 glucose units with Ŭ-1,4glycosidic bonds (56) and amylopectin (B) consists of short Ŭ-1,4 linked to linear chains of 10ï 60 glucose units and Ŭ-1,6 linked to side chains with 15ï 45 glucose units (Muralikrishna and Nirmala 2005).

Endoamylases are able to cleave Ŭ,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. Ŭ-Amylase (EC3.2.1.1) is a well-known endoamylase. It is found in a wide variety of microorganisms, plant and animal (Pandey *et al*., 2000). The end products of Ŭ-amylase action are oligosaccharides with varying length with an Ŭ-configuration and Ŭ-limit dextrins, which constitute branched oligosaccharides, which is one of the most important commercial enzyme processes. Saccharide composition obtained after hydrolyze 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). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and Ŭ-glucosidase), or maltose and ɓ-limit dextrin (ɓ-*amylase*).

1.2.1 Sources and utilization of starch

Starch occurs mainly in the seeds, roots and tubers of higher plants. Some algae produce a similar reserve polysaccharide called phytoglycogen. Plants synthesize starch via photosynthesis. The shape and diameter of these granules depend on the botanical origin. Regarding to commercial starch sources, the granule sizes range from 2ï 30 ɛm (maize starch) to 5ï 100 ɛm (potato starch) (Robyt and Whelan, 1998). A variety of different enzymes are involved in the synthesis of starch. Sucrose is the starting point of starch synthesis. It is

converted into the nucleotide sugar ADP-glucose that forms the actual starter molecule for starch formation. Subsequently, enzymes such as soluble starch synthase and branching enzyme synthesize the amylopectin and amylose molecules (Smith, 2001). Starch-containing crops form an important constituent of the human diet. Besides the direct use of starch-containing plant parts as a food source, starch is harvested and chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. 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.

1.2.2 Biosynthesis of Starch

Plants produce starch by first converting glucose 1-phosphate to ADP-glucose using the enzyme glucose-1-phosphate adenylyltransferase. This step requires energy in the form of ATP. The enzyme starch synthase then adds the ADP-glucose via a 1,4-alpha glycosidic bond to a growing chain of glucose residues, liberating ADP and creating amylose. Starch branching enzyme introduces 1,6-alpha glycosidic bonds between these chains, creating the branched amylopectin. The starch debranching enzyme isoamylase removes some of these branches. Several isoforms of these enzymes exist, leading to a highly complex synthesis process (Smith, 2001). Glycogen and amylopectin have the same structure, but the former has about one branch point per ten 1,4-alpha bonds, compared to about one branch point per thirty 1,4-alpha bonds in amylopectin (Shinke *et al.,* 1974). Amylopectin is synthesized from ADP-glucose while mammals and fungi synthesize glycogen from UDP-glucose; for most cases, bacteria synthesize glycogen from ADP-glucose (analogous to starch) (Ball *et al.,* 2003).

1.2.3 Enzymatic degradation of starch

The effective hydrolysis of starch demands the action of many enzymes due to its complexity, although a prolonged incubation with one particular enzyme can lead to (almost) complete hydrolysis. There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferase. Endoamylases are able to cleave Ǔ,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and Ǔ-glucosidase), or maltose and ɓ-limit dextrin (ɓ-amylase). The third group of starch-converting enzymes is the debranching enzymes that exclusively hydrolyze Ǔ,1-6 glycosidic bonds: isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41). These enzymes exclusively degrade amylopectin, thus leaving long linear polysaccharides. There are also a number of pullulanase type enzymes that hydrolyze both Ǔ, 1-4 and Ǔ,1-6 glycosidic bonds. These belong to the group II pullulanase and are referred to as Ǔ-amylaseï pullulanase or amylopullulanase. The main degradation products are maltose and maltotriose. The fourth group of starch-converting enzymes are transferases that cleave an Ǔ,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (EC 2.4.1.25) and cyclodextringlycosyltransferase (EC 2.4.1.19) form a new Ǔ, 1-4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new Ǔ,1-6 glycosidic bond. Cyclodextringlycosyltransferases have a very low hydrolytic activity and make cyclic oligosaccharides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrins. Amylomaltases are very similar to cyclodextringlycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextringlycosyltransferase gives a cyclic product. Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose,

maltose, and glucose, etc. Most of the enzymes that convert starch belong to one family based on the amino acid sequence homology: the Ŭ-amylase family or family 13 glycosyl hydrolases according to the classification by Henrissat (1991). Other little enzymes that convert starch don't belong to family 13 glycosyl hydrolases like ɓ-amylases that belong to family 14 glycosyl hydrolases (Henrissat and Bairoch, 1993); and glucoamylases which belong to family 15 glycosyl hydrolases (Aleshin *et al*., 1994).

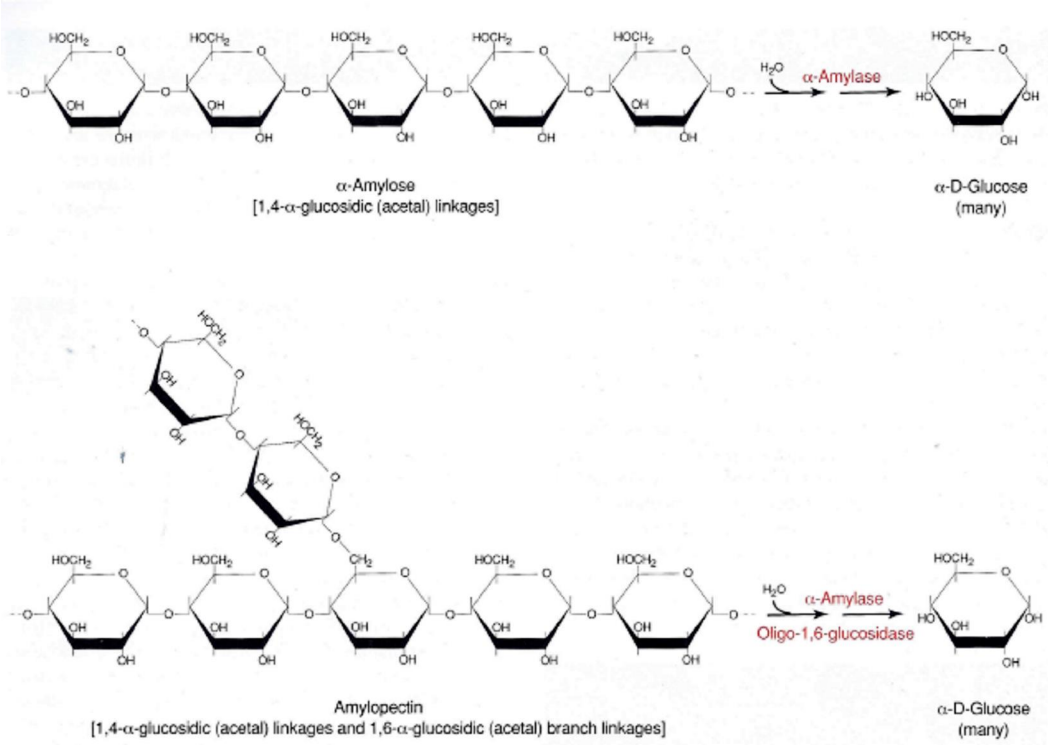


Figure 4: Action of amylase on amylose and amylopectin (Obi and Odibo, 1984).

1.2.4 Test for starch

The Iodine test is used to test for the presence of starch. Iodine solution (iodine dissolved in an aqueous solution of potassium iodide) reacts with the starch producing a purple black colour. The colour can be detected visually with concentrations of iodine as low as 0.00002M at 20°C. However the intensity of the colour decreases with increasing temperature and with the presence of water-miscible, organic solvents such as ethanol. Also the test cannot be done at very low pHs due to the hydrolysis of the starch under these conditions.

1.2.5 Cassava starch

Cassava (*Manihot esculenta*), also called manioc, tapioca or yuca, is one of the most important food crops in the humid tropics, being particularly suited to conditions of low nutrient availability and able to survive drought (Burrell, 2003). The plant grows to a height of 1 to 3 m and several roots may be found on each plant. Although, cassava leaves are sometimes consumed, the major harvested organ is the tuber, which is actually a swollen root. The plant is propagated mostly from stem cuttings. Starch is one of the most important plant products to man. It is an essential component of food providing a large proportion of the daily calorific intake. In West Africa, cassava flour and garri (a processed cassava product) are consumed in large quantities. Cassava starch is recommended for use in extruded snacks for improved expansion. It is also used as a thickener in foods that are not subject to rigorous processing

conditions. Cassava starch, which is very bland in flavor, is used in processed baby foods as a filler material and bonding agent in confectionary and biscuit industries (Nyerhovwo, 2004). The separation of the starch granules from the tuber in as pure a form as possible is essential in the manufacture of cassava flour. The granules are locked in cells together with all the other constituents of the protoplasm (proteins, soluble carbohydrates, fats and so on), which can only be removed by a purification process in the watery phase. Processing the starch can therefore be divided into the following stages:

1. Preparation and extraction. Crushing of the cells and separation of the granules from other insoluble matter (i.e. adhering dirt and cell-wall material) including the preparatory operations of washing and peeling the roots, rasping them and straining the pulp with the addition of water.

2. Purification. Substitution of pure water for the aqueous solution surrounding the starch granules in the mash obtained in the first stage, as well as the operations of sedimentation and the washing of the starch in tanks and on flour tables, silting, centrifuging, etc.

3. Removal of water by centrifuging and drying.

4. Finishing, grinding, bolting and other finishing operations.

This method of processing is essential in the preparation of any kind of starch. For cassava, however, because of the relatively small amount of secondary substances, the separation at each stage is performed with great ease. Whereas with maize and other cereals the grinding of the seed and the mechanical separation of the germ and the pericarp from the grain present special problems in stage 1, and the separation of protein and other constituents in stage 2 can only be accomplished with the aid of chemicals, these operations can be reduced to a minimum in cassava preparation. It is indeed possible to obtain a first-rate flour from the cassava root without special equipment by using only pure water. This makes the processing of cassava flour particularly suitable for rural industries

1.2.6 Corn starch

Corn-starch, corn-flour or maize-starch is the starch derived from the corn (maize) grain. The starch is obtained from the endosperm of the corn-kernel. Corn-starch is a popular food ingredient used in thickening sauces or soups, and is used in making corn syrup and other sugars.

1.3 Amylase

Enzymes that participate in the hydrolytic degradation of starch are collectively referred to as amylolytic enzymes or amylases. Specific enzymes classified within this group include Ŭ-amylase, ɓ-amylase, gluco-amylase (also known as amyloglucosidase), pullulanase and inso-amylase. Amylases are, classified into two categories, endoamylases and exoamylases (Gupta *et al*., 2003). Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successfully resulting in short end products. A large array of amylases, are involved in the complete breakdown of starch. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars. Also, enzyme - mediated isomerization of glucose yields high-fructose syrups.

Abundant supplies of starch may be obtained from seeds and tubers, such as corn, wheat, rice tapioca and potato. The widespread availability of starch from such inexpensive sources, coupled with large-scale production of amylolytic enzymes, facilitates the production of syrups containing glucose, fructose or maltose, which are of considerable importance in the food and confectionery industry. Furthermore, they may be produced quite competitively when compared with the production of sucrose, which is obtained directly from traditional sources such as sugar-beet or sugar-cane. Starch may be hydrolyzed by chemical or enzymatic means. Chemical hydrolysis was used formerly and involves heating in the presence of acid. However, enzymatic hydrolysis generates fewer byproducts and produces higher yields of end product compared to the chemical method. The initial step in starch hydrolysis entails disruption of the starch granule (Gupta *et al*., 2003). Solubilisation of the granules, (the process of ñgelatinisationò), facilitates subsequent catalytic degradation. Gelatinisation is normally achieved by heating the starch to temperatures often in excess of 100ºC for several minutes. Ŭ-Amylase may be added immediately prior to the heating step, in order to render more efficient the process of granule disruption. Once the granules have been disrupted, additional Ŭ-amylase is added in order to liquefy the starch slurry. This process reduces the viscosity of the starch solution (Pandey *et al.*, 2000),

1.3.1 Sources of amylase

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 for the 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).

1.3.2 Microbial amylase

The microbial enzyme meets the industrial demands and a large number of them are available commercially and have almost replaced chemical hydrolysis of starch processing industries (Pandey *et al*., 2000). The major advantage of using microorganisms for the amylase production is economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). Alpha amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Among bacteria, *Bacillus* sp. is widely used for thermostable Ŭ-amylase production to meet industrial needs. *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis* and *Bacillus 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 a-amylase. Furthermore, a variety of ruminal bacteria exhibit the ability to utilize starch as a growth substrate and arepresent in the rumen in sufficient numbers to be of quantitative significance in the fermentation of this substrate. These species include *Bacteroides ruminicola*, *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, and *Streptococcus bovis*. Genes encoding intracellular Ŭ-amylases have been reported for Escherichia coli and Streptococcus bovis (Satoh *et al*., 1997). Although there has been some characterization of these activities, no clear

physiological role for intracellular Ŭ-amylase has been established for either E. coli or Streptococcus bovis. However, it is postulated that it plays an important role in rapid cell growth in Streptococcus bovis (Brooker and McCarthy, 1997).

Many hyperthermophilic microorganisms possess starch-hydrolyzing enzymes in their genomes even though they live in environments where starch is rare (Sambrook *et al*., 1989). Among the polysaccharide-degrading enzymes of Thermotoga maritime described so far are two Ŭ-amylases, one is an extracellular putative lipoprotein (AmyA) (Liebl *et al*., 1997) and the other is located in the cytoplasm (AmyB) (Lim *et al*., 2003). Geobacillus thermoleovorans has been found to produce hyperthermostable, high maltose-forming and Ca^{2+} independent Ŭ-amylase (Malhotra *et al*. 2000; Narang and Satyanarayana 2001). Numerous hyperthermophilic Archaea, especially deep-sea Thermococcale and Sulfolobus species have been reported to produce Ŭ-amylases (Sunna *et al.,* 1997). The industrial potential of high-maltose forming Ŭ-amylases from Thermomonospora curvata (Collins *et al*., 1993) is limited by their moderate thermostability and Ca^{2+} requirement. Ŭ-amylases are secreted by several species of Streptomyces, for example S. albus (Andrews and Ward, 1987), S. griseus IMRU3570 (Vigal *et al.*, 1991), S. thermocyaneoviolaceus (Hang *et al.,* 1996). Gene encoding extracellular Ŭ-amylase has been cloned from many Streptomyces species (Virolle *et al*., 1988; Bahri and Ward, 1990). In addition, Ŭ-amylase activity of Thermoactinomyces species was first reported by Kuo and Hartman (1966). Several Ŭ- amylases with different characters were also found in other studies (Shimizu *et al*., 1978; Obi and Odibo, 1984; Uguru *et al*., 1997). Within actinomycetes, available reports on ɓ-amylase production are scanty and refer mainly to non-thermostable enzyme (Shinke *et al*., 1974).

1.3.3 Plant amylase

Plant amylase is the amylase found in plant (seed). Both Ŭ-amylase and ɓ-amylase are present in seeds; ɓ-amylase is present in an inactive form prior to germination, whereas Ŭ-amylase and proteases appear once germination has begun.

1.3.4 Animal amylase

Two kinds of Ŭ-amylase are produced by various mammals i.e. salivary Ŭ-amylase from the parotide gland and pancreatic Ŭ-amyalse from the pancrease. The digestion of food starch begins with salivary Ŭ-amylase in the mouth and reaction of this enzyme can be stop by low pH of gastric juice of stomach. When the food boluses from stomach pass into small intestine, the pH of gastric juice is neutralized by pancreatic juice secreted from pancreas in which the digestion of the starch is completed by the reaction of a pancreatic Ŭ-amyalase.

1.4 Classification of amylase

The most known amylolytic enzymes are Ŭ-amylase (EC 3.2.1.1), ɓ-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3) that are, however, quite different from each other. They differ not only in their primary and tertiary structures, but also in their catalytic machineries and reaction mechanisms employed (Coutinho and Reilly, 1997).

1.4.1 α-amylase

Ŭ-amylase activity is widely distributed in nature. Ŭ-Amylase is an endo-acting enzyme, catalyzing the random hydrolysis of internal Ŭ-1,4 glycosidic linkages present in starch. This enzyme is incapable of hydrolyzing Ŭ-1,6 glycosidic linkages present at branch points of amylopectin chains. One exception to this is the Ŭ-amylase produced by *Thermactinomyces vulgaris*, which can hydrolyse both Ŭ-1-6 and Ŭ-1-4 glycosidic linkages. The Ŭ-amylase family consists of a large group of starch hydrolases and related enzymes, currently known as glycosyl

hydrolases family 13 (Henrissat, 1991). Thermostable Ŭ-amylases have been characterised from *Pyrococcus woesei, Pyrococcus furiosus* (Koch *et al.,* 1991) and *Thermococcus profundus* (Chung *et al*., 1995; Kwak *et al*., 1998; and Lee *et al*., 1996). The optimum activity is 100ºC and 80ºC, respectively. The gene encoding an extracellular Ŭ-amylase from *P. Furiosus* has been recently cloned and the recombinant enzyme expressed in *Bacillus subtilis* and in *E. coli* (Dong *et al*., 1997; Jorgenesn *et al*., 1997). The high thermostability of the pyrococcal extracellular Ŭ-amylase (thermal activity) even at 130^0C in the absence of metal ions, together with its unique product pattern and substrate specificity, makes this enzyme an interesting candidate for industrial application (Niehaus *et al.,* 1999). Two of the more commonly used bacterial Ŭ-amylases are those isolated from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. *Bacillus* amylases exhibit a pH optimum close to neutrality and are stabilized by the presence of calcium ions. Ŭ-amylase produced by *Bacillus licheniformis* is particularly suited to industrial applications because of its thermal stability. This enzyme consists of 483 amino acids and has a molecular weight of 55.2 kDa (figure 5). Its pH optimum is 6.0 and its temperature optimum is 90^0C. Most other Ŭ-amylases, including those produced by *B. amyloliquefaciens*, are rapidly inactivated at temperatures above 40^0C (Niehaus *et al.,* 1999). Several thermostable Ŭ-amylases have already been characterized (Koch *et al*., 1991). The most thermostable Ŭ-amylase to date is from *Pyrococcus woesei*. It remained active after autoclaving for 4 h at 120^0C (Antranikian *et al.,* 1987).

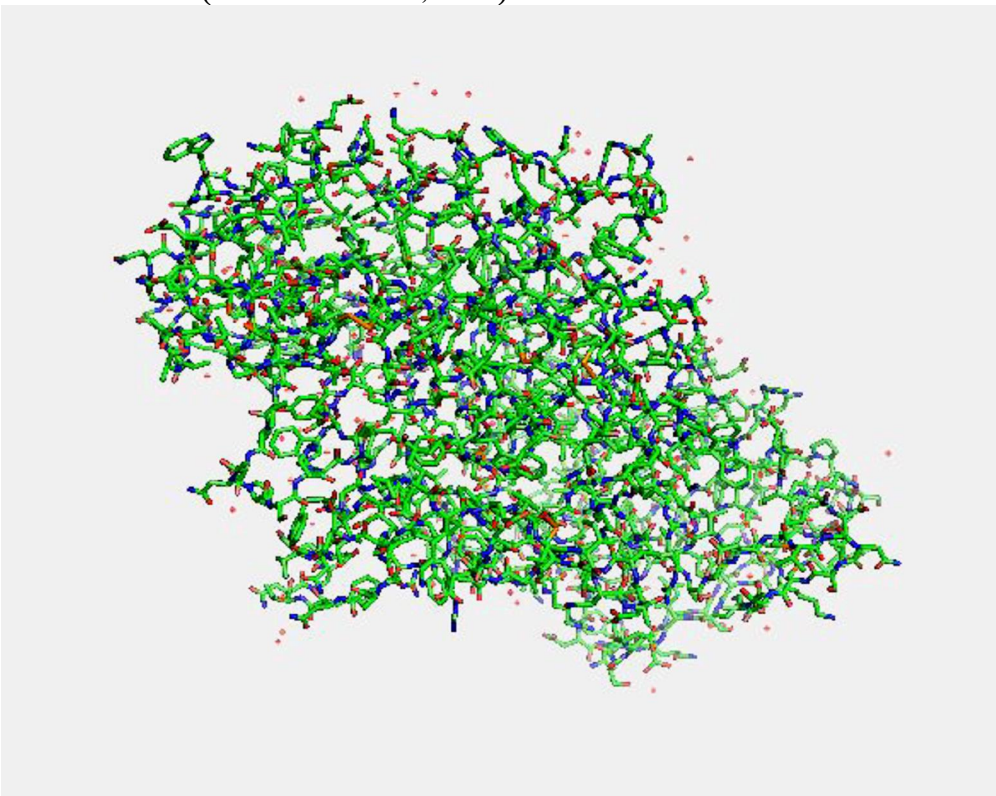


Figure 5: 3-Dimensional representation of the molecular structure of Ŭ-amylase (Payan and Qian, 2003).

1.4.2 β-amylase

Another form of amylase, ɓ-amylase (EC3.2.1.2) also known as 1,4-Ŭ-D-glucanmaltohydrolase; glycogenase or saccharogen amylase is also synthesized by bacteria, fungi, and plants. Working from the non-reducing end, ɓ-amylase catalyzes the hydrolysis of the second Ŭ-1,4glycosidic bond, cleaving off two glucose units (maltose) at a time. During the

ripening of fruit, ϐ-amylase breaks starch into maltose, resulting in the sweet flavor and taste of ripe fruit. ϐ-amylase is found primarily in the seeds of higher plants and sweet potatoes. It yields a single product: maltose. The tuberous root of sweet potato is unusually rich in the enzyme, accounting for approximately 5% of the total soluble proteins. In contrast, other tuberous roots only contain trace amounts of ϐ-amylase activity (Malhotra *et al.*, 2000). ϐ-amylase is present in an inactive form prior to germination, whereas Ŭ-amylase and proteases appear once germination has begun. Animal tissues do not contain ϐ-amylase, although it may be present in microorganisms contained within the digestive tract. The optimum pH for ϐ-amylase is 4.0-5.0. Most ϐ-amylases are monomeric enzymes (Sorensen *et al.,* 2004). However, that of sweet potato is tetrameric consisting of four identical subunits (Sorensen *et al.* 2004). The gene that encodes for ϐ-amylase is *amyB*. A 1404 bp open reading frame encodes for the 499 amino acid precursor to the ϐ-amylase subunit. After methionine processing, the mature form of the protein contains 498 residues (Potter and Doyle, 1994).

1.4.3 γ-Amylase

ɔ-Amylase (EC3.2.1.3) also known as Glucan 1,4-Ŭglucosidase; amyloglucosidase; Exo-1,4-Ŭ-glucosidase; glucoamylase; lysosomal Ŭ-glucosidase or 1,4-Ŭ-D-glucanglucohydrolase cleave Ŭ(1-6) glycosidic linkages, as well as the last Ŭ(1-4)glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. The ɔ-amylase has most acidic pH optimum because it is most active around pH 3.

1.5 Structural and functional characteristics of α-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 (Whitcomb and Lowe, 2007). A classification system for glycoside hydrolases, based on sequence similarity, has led to the definition of 85 different families. Most of the starch hydrolyzing enzymes belongs to the Ŭ-*amylase* family or family 13 glycoside hydrolases based on amino acid sequence homology according to the classification of Henrissat (1991). The Ŭ-*amylase* family of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (Henrissat 1991). A large variety of enzymes are able to act on starch. These enzymes are listed in Table 1. These enzymes can be divide basically into four groups:endoamylases, exoamylases, debranching enzymes and transferases (Van der Maarel *et al*. 2002):

1. Endoamylases: cleave internal Ŭ-1,4 bonds resulting in Ŭ-anomeric products,
2. Exoamylases: cleave Ŭ-1,4 or Ŭ-1,6 bonds of the external glucose residues resulting in Ŭ- or ϐ-anomeric products,
3. Debranching enzymes: hydrolyze Ŭ-1,6 bonds exclusively leaving longlinear polysaccharides, and
4. Transferases: cleave Ŭ-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond.

Table 1: Known activities of glycoside hydrolase family 13 enzymes

Enzyme	EC number	Main substrate

Amylosucrase	EC: 2.4.1.4	Sucrose
Sucrose phosphorylase	EC: 2.4.1.7	Sucrose
Glucan branching enzyme	EC: 2.4.1.18	Starch, glycogen
Cyclomaltodextrin glycosyltransferase	EC: 2.4.1.19	Starch
Amylomaltase	EC: 2.4.1.25	Starch, glycogen
Maltopentaose-forming alpha-amylase	EC: 3.2.1.-	Starch
Alpha-amylase	EC: 3.2.1.1	Starch
Oligo-1,6-glucosidase	EC: 3.2.1.10	1,6-alpha-D-glucosidic linkages in some oligosaccharides
Alpha-glucosidase	EC: 3.2.1.20	Starch
Amylopullulanase	EC: 3.2.1.41	Pullulan
Cyclomaltodextrinase	EC: 3.2.1.54	linear and cyclomaltodextrin
Isopullulanase	EC: 3.2.1.57	Pullulan
Isoamylase	EC: 3.2.1.68	Amylopectin
Maltotetraose-forming alpha-amylase	EC: 3.2.1.60	Starch
Glucodextranase	EC: 3.2.1.70	Starch
Trehalose-6-phosphate hydrolase	EC: 3.2.1.93	Trehalose
Maltohexaose-forming alpha-amylase	EC: 3.2.1.98	Starch
Maltogenic amylase	EC: 3.2.1.133	Starch
Neopullulanase	EC: 3.2.1.135	Pullulan
Malto-oligosyl trehalase hydrolase	EC: 3.2.1.141	Trehalose
Malto-oligosyl trehalose synthase	EC: 5.4.99.15	Maltose

Source: Mojsov, (2012)

The Ŭ-glycosidic bond is very stable having a spontaneous rate of hydrolysis at room temperature (Wolfenden *et al*., 1998). The catalytic mechanism of the Ŭ-amylase family is that of the Ŭ-retaining double displacement (Van der Maarel *et al*., 2002). Ŭ-Retaining mechanism is the characteristic feature of the enzymes from the Ŭ-amylase family. They vary widely in their reaction specificities. The attachment of different domains to the catalytic site or to extra sugar binding subsites around the catalytic site is the prime reason for these differences (Van der Maarel *et al*., 2002). The amylase has a three-dimensional structure capable of binding to substrate. The action of highly specific catalytic groups, promote the breakage of the glycoside links (Iim *et al*., 2003). 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). The protein contains 3 domains, namely: A, B, and C. The A domain is the largest, presenting a typical barrel shaped (ƃ/Ŭ) super structure. The B domain is inserted between the A and C domains and is attached to the A domain by disulphide bond. The C domain has Ŭ-sheet structure linked to the A domain by a simple polypeptide chain and seems to be an independent domain with unknown function. The active site (substrate-binding) of the Ŭ-amylase is situated in a long cleft located between the carboxyl end of the A and B domains (figure 6). The calcium (Ca^{2+}) is situated between the A and B domains and may act in the stabilization of the three-dimensional structure and as allosteric activator. Binding of substrate analogs suggest that Asp206, Glu230 and Asp297 participate in catalysis (Muralikrishna and Nirmala, 2005). The substrate-binding site contains 5 subsites with the catalytic site positioned at subsite 3. Substrate can bind to the first glucose residue in subsite 1 or 2, allowing cleavage to occur between the first and second or second and third glucose residues (Whitcomb and Lowe, 2007).

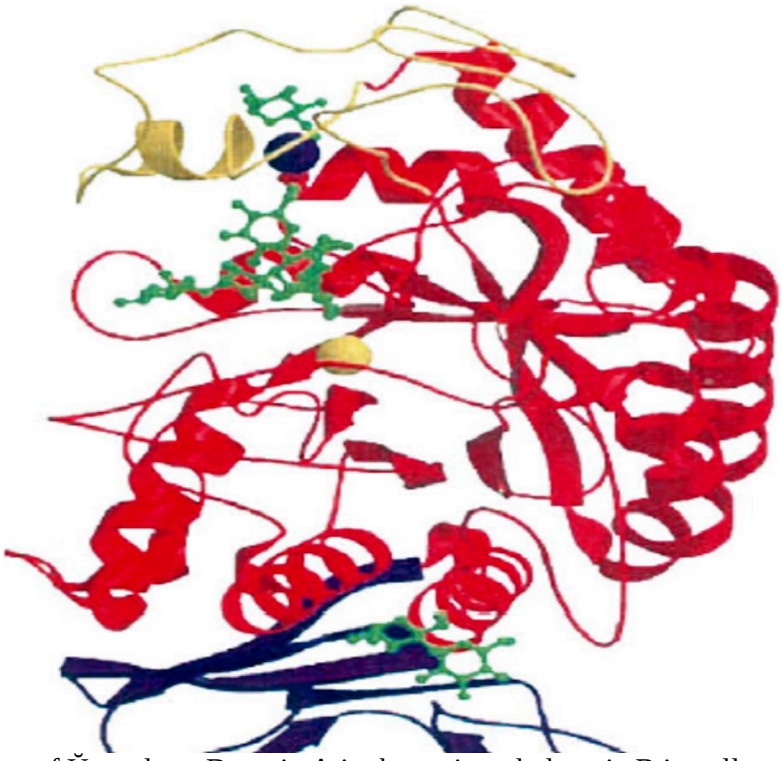


Figure 6: Structure of Ŭ-amylase. Domain A is shown in red, domain B in yellow and domain C in purple. In the catalytic center, the calcium ion is shown in the blue sphere and the chloride ion in the yellow sphere. The green structures are bound to the active site and to the surface binding sites (Payan, 2004).

1.6 Determination of α-amylase activity

Ŭ-Amylases are generally assayed using soluble starch or modified starch as the substrate. Ŭ-Amylase catalyses the hydrolysis of Ŭ-1,4 glycosidic linkages in starch to produce glucose, dextrins and limit dextrins. The reaction is monitored by an increase in the reducing sugar levels or decrease in the iodine colour of the treated substrate. Various methods are available for the determination of Ŭ-amylase activity (Priest, 1977). These are based on decrease in starch/iodine colour intensity, increase in reducing sugars, degradation of colour-complexed substrate and decrease in viscosity of the starch suspension.

1.6.1 Decrease in starch / iodine colour intensity

Starch forms a deep blue complex with iodine (Hollo and Szeitl, 1968) and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on this property. This method determines the dextrinising activity of Ŭ-amylase in terms of decrease in the iodine colour reaction.

1.6.1.1 Determination of dextrinising activity

The dextrinising activity of Ŭ-amylases employs soluble starch as substrate and after terminating the reaction with dilute HCl, iodine solution is added. The decrease in absorbance at 620 nm is then measured against a substrate control. One percent decline in absorbance is considered as one unit of enzyme (Fuwa, 1954). The major limitation of this assay is

interference of media components including Luria broth, tryptone, peptone, corn steep liquor (CSL), etc. and thiol compounds with starch iodine complex. Copper sulphate and hydrogen peroxide protect the starch/iodine colour in the case of interference by these media components (Manonmani and Kunhi, 1999). Further, zinc sulphate was found to be best for counteracting the interference of various metal ions. Various workers (Hansen, 1984; Carlsen *et al*., 1994) have successfully used the original assay procedure in combination with flow injection analysis (FIA). The flow system comprised of an injection valve, a peristaltic pump, a photometer with a flow cell and 570 nm filter and a pen recorder. Samples are allowed to react with starch in a coil before iodine was added. Absorbance is then read at 570 nm. This method has many advantages including high sampling rates, fast response, flexibility and simple apparatus.

1.6.1.2 Sandstedt Kneen and Blish (SKB) method

The SKB method (Sandstedt *et al*., 1939) is one of the most widely adopted methods for determination of amylases used in the baking industry. The potency of most commercial amylases is described in terms of SKB (Sandstedt *et al*., 1939) units. This method is used generally to express the diastatic strength of the malt and not for expressing Ŭ-amylase activity alone (Kulp, 1993).

1.6.1.3 Indian pharmacopoeia method

As described in the Indian pharmacopoeia, this method is used to calculate Ŭ-amylase activity in terms of grams of starch digested by a given volume of enzyme (Indian Standard, 1982). This procedure involves incubation of the enzyme preparation in a range of dilutions in buffered starch substrate at 40ºC for 1 h. The solutions are then treated with iodine solution. The tube, which does not show any blue colour, is then used to calculate activity in terms of grams of starch digested. This method is usually employed for estimating a-amylase activity in cereals.

1.6.2 Increase in reducing sugars or dinitrosalicyclic acid (DNSA) method

This method determines the increase in reducing sugars as a result of amylase action on starch (Bernfeld, 1955). The major defect in this assay is a slow loss in colour produced and destruction of glucose by constituents of the DNSA reagent. To overcome these limitations, a modified method for the estimation of reducing sugars was developed (Miller, 1959). Rochelle salts were excluded and 0.05% sodium sulphite was added to prevent the oxidation of the reagent. Since then the modified method has been used extensively to measure reducing sugars without any further modifications in the procedure. Alternate methods, which also rely on the estimation of the reducing sugars are also, employed (Mishra and Maheshwari, 1996). For some years, groups have been working on the development of a specific a-amylase determination method based on the use of new types of substrates. These methods employ starch covalently complexed with blue dye such as Remazol brilliant Blue R (Ceska and Birath, 1969) or Cibacron Blue F3 G-A as an alternative substrate. The synthesis of these substrates involves two major steps. Soluble starch is coloured under alkaline conditions using the dye. This is the result of formation of covalent bonds between starch and dye molecules. The coloured starch is subsequently cross-linked by the addition of 1,4-butanediol diglycide ether. This gives an insoluble network, which swells in water. The enzymic hydrolysis of such insoluble starch derivatives yields soluble starch hydrolysates carrying the coloured marker. This method is simple and sensitive for aamylase determination, but even minute quantities of glucose might lead to erroneous results due to starch contamination by dextrin substrate (Dhawale*, et al.,* 1982). Recently, a rapid and sensitive microassay based on dye cross linked

starch for a-amylase detection has been reported. It can successfully detect as low as 0.50 mg of enzyme (Wong *et al.*, 2000). Other novel substrates such as nitrophenyl derivatives of maltosaccharides have also been employed. The assay measures the release of free p-nitrophenyl groups. The use of nitrophenyl-maltosaccharides in conjunction with a specific yeast a-glucosidase can be used but these substrates are rapidly cleaved by glucoamylases commonly present in the culture broths. The use of nonreducing end blocked p-nitrophenyl maltoheptoside (BPNPG7) has also been described (Sheehan and McCleary, 1988). The blocking group (4,6-O-benzylidene) prevents the hydrolysis of the substrate by the exo-acting enzymes and is thus specific for Ŭ-amylase. The assay is simple, reliable and accurate but is expensive as it involves the use of a synthetic substrate and specific enzymes. Thus the use of this method is restricted only to very specific tests and not for routine analysis. A comparison was made for the use of end blocked p-nitrophenyl maltoheptoside (BPNPG7) with a number of accepted procedures that employ starch as the substrate. The reaction was monitored using the starch-/iodine colour. There was an excellent correlation between each of the assay procedures employed. This indicates that all the methods give an accurate and reliable measure of a-amylase activity and can be used as per the requirement.

1.6.3 Decrease in viscosity of the starch suspension

These methods are generally used in the bakery industry to assess the quality of the flour and not for estimating Ŭ-amylase activity which are based on the determination of the rheological properties of the dough. Methods, which fall into this category, are the falling number test and the Amylograph or Farinograph test.

1.6.3.1 Falling number (FN) method

The falling number (FN) method, internationally standardized (International Association for Cereal Chemistry) is accepted for assessing cereal Ŭ-amylase activity in flour/enzyme preparations at 100^0C. Both cereal and fungal Ŭ-amylases are used to improve the fermentation of flour deficient in amylase activities. Because fungal Ŭ-amylases have low thermostability, they cannot be detected by the standard FN method at 100^0C (Perten, 1984). This method has been modified and standardised (Perten, 1984) for measuring both cereal and fungal a-amylase activity at 300ºC, by replacing a part of the flour with pre gelatinized starch. A falling number of about 400 indicates a normally malted flour.

1.6.3.2. Amylograph/Farinograph test

The milling and baking industries generally assess the diastatic activity of flours by means of an amylograph. This method is also based on the relationship of peak viscosity of starch slurry and the enzyme activity level (American Association of Cereal Chemists, 1972). The higher the enzyme activity, the thinner is the hot paste viscosity. When the amylograph is used, values of 400/600 Brabender units of the Farinograph are considered optimal for bread baking flours (higher values indicate a lack and lower values indicate an excess of activity).

1.7 Purification and characterization of amylase

Purification is a key step in the enzymes production where residual cell proteins and other contaminants are removed. Different techniques have been developed for purification of enzymes based on their properties, prior to their characterization or use in biotechnological and industrial processes. The commercial use of Ŭ-amylase generally does not require purification of the enzyme but enzyme applications in food industries, pharmaceutical and clinical sectors require high purified amylases. The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. The used methods to purify

amylases can vary considerably, but most purification protocols involve a series of steps (Somers *et al*., 1995). The choice of purification protocol naturally depends on the intended use, the highest purity usually being required for basic purposes in which even separation of isozymes may be important. The purity and the yield attained depend on the number of steps and separation techniques employed. The purification of Ŭ-amylases from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from the fermentation broth, selective concentration by precipitation using ammonium sulphate or organic solvents. The crude enzyme is then subjected to chromatography. The most commonly used techniques are usually affinity chromatography, ion exchange, and/or gel filtration. Cross-linked starch or starch derivatives are useful affinity adsorbents for the isolation of bacterial Ŭ-amylases (Somers *et al*., 1995). Ŭ-amylase from *Bacillus licheniformis* has been purified 6-fold with a yield of 38% using two gel filtration chromatography steps on Sephadex G-100 and Superose 12 column (Bozic *et al*., 2011). In addition to the classical chromatographic techniques, immune-affinity chromatography has been applied for the preparation of highly purified amylases (Jang *et al*., 1994). Recent advances in the understanding of the physical and functional properties of amylases and the selectivity and capacity of the adsorbents have led to greater rationality in the design of separation methods. However, the potential of the methods for the separation of amylases has not been fully exploited.

1.8 Industrial application of amylase

The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes. Recent developments in biotechnology, particularly in areas such as protein engineering and directed evolution, have provided important tools for the efficient development of new enzymes. This has resulted in the development of enzymes with improved properties for established technical application and in the production of new enzymes tailor-made for entirely new areas of application where enzymes have not previously been used (Kirk *et al*., 2002). The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represent the second largest group (Godfrey and West, 1996). The fastest growth over the past decade has been seen in the baking and animal feed industries, but growth is also being generated from applications established in a wealth of other industries spanning organic synthesis to paper and pulp and personal care (Figure 7) (Kirk *et al*., 2002). Natural microorganisms have over the years been a great source of enzyme diversity. The developments in bioinformatics and the availability of sequence data have increased immensely the efficiency of isolating an interesting gene from nature (Kirk *et al*., 2002). Rational protein engineering and the possibility of introducing small changes to proteins, on the basis of their structure and the related biochemical and biophysical properties, introduced a new valuable tool to enzyme optimisation in the 1980's. Directed evolution is the latest addition to the toolbox (Tobin *et al*., 2000). New exciting technology is predicted to out-compete the existing technologies, but we expect that time will demonstrate how the combined use of rational design, directed evolution and natureôs diversity will be far superior to any technology (Kirk *et al*., 2002).

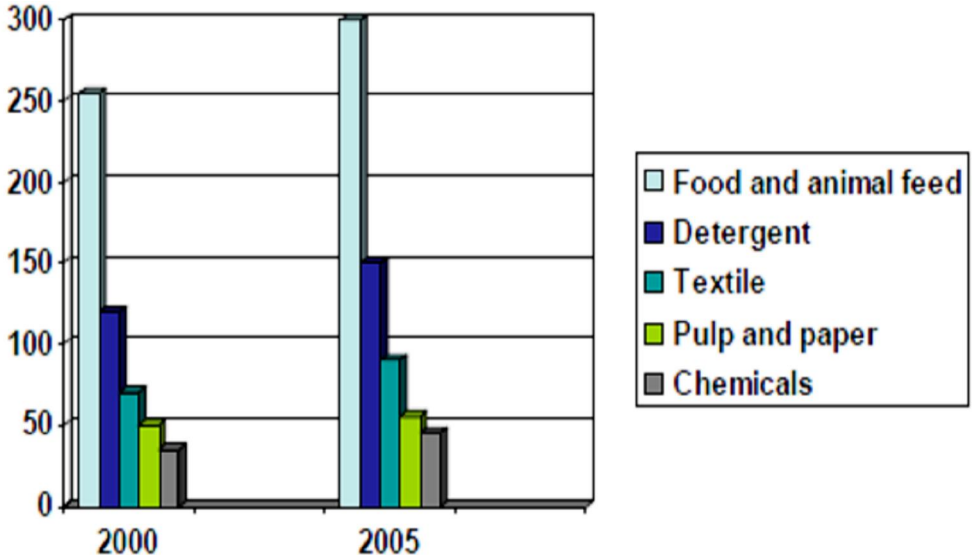


Figure 7: worldwide increases in the use of enzymes in industrial applications in the 2000 and 2005 (Vector research ï Global Technologies, Inc. 2005)

1.8.1 Bakery industry

The quantities, taste, aroma and porosity of the bread are improved by using the enzyme in the flour. More than 70 % bread in U.S.A, Russia and European countries contain alpha amylase. Amylases play important role in bakery products (Marc *et al*., 2002). For decades, enzymes such as malt and fungal alpha-amylases have been used in bread-making. The significance of enzymes is likely to rise as consumers insist more on natural products free of chemical additives. Enzymes can be employed to replace potassium bromate; a chemical additive that is used in bread baking that has been prohibited in a number of countries. Flour consists of gluten, starch, non-starch polysaccharides, lipids and trace amounts of minerals. As soon as the dough is made, the yeast starts to work on the fermentable sugars, transforming them into alcohol and carbon dioxide, which makes the dough rise. The major component of wheat flour is starch. Amylases can degrade starch and produce small dextrins for the yeast to act upon. The alpha-amylases degrade the damaged starch in wheat flour into small dextrins, which allows yeast to work continuously during dough fermentation, proofing and the early stage of baking. The result is improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavor (Loizzo *et al*., 2008).

1.8.2 Textile industry

Textile industries are extensively using alpha amylases to hydrolyze and solubilize the starch, which then wash out of the cloth for increasing the stiffness of the finished products. In textile weaving, starch paste is applied for warping. This gives strength to the textile at weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp. After weaving the cloth, the starch is removed and the cloth goes to scouring and dyeing (Becks *et al*., 1995). Fabrics are sized with starch. Alpha amylase is used as desizing agent for removing starch from the grey cloth before its further processing in bleaching and dyeing. Many garments especially the ubiquitous 8Jeanô are desized after mashing.

1.8.3 Sugar and glucose industries

Alpha amylase plays a very important role in the production of starch conversion products of low fermentability. The presence of starch and other polysaccharides in sugarcane creates problem throughout the sugar manufacturing which is minimized or eliminated by the action of alpha amylase. The high quality products depends upon the efficiency of the enzyme (Ŭ-amylase) which lead to low production and thus increased cost of starch processor (Marc *et al*., 2002). Many industries used alpha amylases for the production of glucose. Enzyme hydrolyzed the starch and converted it into glucose. They hydrolyze Ŭ-1,4glucosidic linkage in the starch polymer in a random manner to yield glucose and maltose (Becks *et al*., 1995). Therefore, Ŭ-amylase is extensively used in many industries for the production of glucose. This process also gives the water-soluble dextrin.

1.8.4 Alcohol Industry

Alpha amylases convert starch into fermentable sugars. Starches such as grain; potatoes etc. are used as a raw material that helps to manufacture ethyl alcohol. In the presence of amylases, the starch is first converted in to fermentable sugars. The use of bacterial enzyme partly replaces malt in brewing industry, thus making the process more economically significant. Alpha amylase can also carry out the reactions of alcohol hydrolysis by using methanol as a substrate (Van der Maarel *et al*., 2002). The amylolytic activity rate and amount of starch utilization and ethanol yields increase in several fold in cocultures (Van Lenen and Smith, 1968). Moulds amylases are used in alcohol production and brewing industries. The advantages of such system are uniform enzyme action in mashes, increase rate of saccharification, alcohol yield and yeast growth (Van Lenen and Smith, 1968).

1.8.5 Paper industry

Starch paste when used as a mounting adhesive modified with additives such as protein glue or alum, frequently, causes damage to paper as a result of its embrittlement. Starch digesting enzymes, e.g. alpha amylase, in immersion or as a gel poultice are applied to facilitate its removal. Alpha amylase hydrolyzed the raw starch that is used for sizing and coating the paper instead of expensive chemically modified starches. So, starch is extensively used for some paper size press publications (Obi and Odibo, 1984).

1.8.6 Chocolate Industry

Amylases are treated with cocoa slurries to produce chocolate syrup, in which chocolate starch is dextrinizing and thus syrup does not become thick. Cocoa flavored syrups having a high cocoa content and excellent stability and flow properties at room temperature may be produced by using an amylolytic enzyme and a sufficient proportion of Dutch process cocoa to provide a syrup pH of 5.5 to 7.5. The syrup is made by alternate addition of cocoa and sweetener to sufficient water to achieve a solids content of about 58 to 65 weight percent, adding an amylolytic enzyme, heating to a temperature of about 175 -185°F for at least 10 to 15 min, raising the temperature to about 200° F. and cooling. The stabilized cocoa flavored syrups may be added at room temperature to conventional non-acid confection mixes for use in the production of quiescently frozen chocolate flavored confections (Kandra, 2003)

1.8.7 Detergent, building product and feed industries

In detergent industries, the enzyme alpha amylase plays a vital role. It is widely used for improvement of detergency of laundry bleach composition and bleaching without color darkening (Burgess-Cassler *et al*., 1991). The addition of enzyme stabilizes the bleach agent and preserves effectiveness of the bleach in laundry detergent bar composition (Prasanna, 2005) Modified starch is used in the manufacture of gypsum board for dry wall construction. Enzyme modified the starch for the industry use. Many starches or barely material are present

in the feed. So, the nutritional value of the feed can be improved by the addition of alpha amylase.

1.8.8 Analysis in medical and clinical chemistry

With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Gupta *et al.*, 2003). There are several processes in the medicinal and clinical areas that involve the application of amylases. The application of a liquid stable reagent, based on Ŭ-amylase for the Ciba Corning Express clinical chemistry system has been described (Becks *et al*., 1995). Some other processes that have been developed using amylases are for example, detection of higher oligosaccharides (Giri *et al*., 1990) and biosensors with an electrolyte isolator semiconductor capacitor transducer for process monitoring (Menzel *et al*., 1998).

1.8.8 Miscellaneous applications

Besides amylases' use in the saccharification or liquefaction of starch, these are also used for the clarification of formed haze in fruit juices, the pretreatment of animal feed to improve the digestibility (Marc *et al*., 2002). Ŭ-amylase is used for the production of low viscosity, high molecular weight starch for coating of paper (Bruinenberg *et al*., 2004). Starch is a good sizing agent for the finishing of paper. It is added to the paper in the size press and paper picks up the starch by passing through two rollers that transfer the starch slurry. The temperature of this process lies in the range of 45ï 60^{0}C. A constant viscosity of the starch is required for reproducible results at this stage. The mill also has the flexibility of varying the starch viscosity for different paper grades. The viscosity of the natural starch is too high for paper sizing and is adjusted by partially degrading the polymer with Ŭ amylases in a batch or continuous processes. Also, good desizing of starch sized textiles is achieved by the application of Ŭ-amylases, which selectively remove the size and do not attack the fibers. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing. Furthermore, high molecular weights amylases were found in culture supernatants of an environmentally derived microbial mixed culture selected for its ability to utilize starch containing plastic films as sole carbon sources (Burgess-Cassler *et al*., 1991). This suggests a new application for amylases in biodegradation. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Becks *et al*., 1995).

A modern trend is to use starch for production of a more efficient and specific degradation products through a particular combination of activities. Amylase from *Aspergillus niger*, a saccharifying enzyme which produces maltose, maltotriose and some glucose, is capable of alcoholysis for the synthesis of methyl-glucosides from starch in the presence of methanol. As these products are a series of methyloligosaccharides, from methyl-glucoside to methylhexomaltoside, the biotechnological applications of using starch as substrate for the production of alkyl-glucosides is analyzed. Moreover, it becomes possible to produce lactic acid directly from starch by an efficient simultaneous saccharification and fermentation from soluble starch by recombinant *Lactobacillus* strains (Okano *et al*., 2009). Finally, Ŭ-amylase is suggested as an enzyme that contributes to the reduction of AuCl4- to gold nanoparticles (Au-NPs) which makes it ideal for the production of Au-NPs (Kalishwaralal *et al*., 2010).

1.9 Factor affecting amylase activity.

Within normal range, temperature, pH, concentration of substrate and enzyme affect the rate of reaction in accordance with predictable interaction between enzymes and substrate molecules.

1.9.1 Effect of temperature of amylase Activity.

Every enzyme exhibits its catalytic function maximally at particular optimum temperature depending on it source. Enzyme activity is changed by variation in temperature. As temperature rises, the rate of chemical reactions increases because temperature increases the rate of motion of molecules. This leads to more interactions between an enzyme and its substrate (Morton *et al.*, 2000) above or below the temperature optimal. The tertiary and / or quaternary structures of the enzyme will be altered, affecting the enzymeôs binding ability, active sites, the binding of substrate (S) and subsequent catalysis to yield product.

1.9.2 Effect of pH on amylase activity.

The pH optima of Ŭ-amylases vary from 2 to 12 (Burhan *et al.*, 2003). Every enzyme has its optimum pH at which it exhibit maximum activity. The pH of a solution can have several effects on the structure and activity of enzymes (Anosike, 2001). For example; pH can have an effect on the state of ionization of acidic or basic amino acids. Acidic amino acids have carboxyl functional group in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered then the ionization bonds that help to determine the 3-D shape of the protein / enzyme may be altered and this can inactivate the enzyme (Anosike, 2001). Changes in pH may not only affect the shape of an enzyme but it may also change the shape or charge properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis. In general enzymes have a pH optimum. However the optimum pH is not the same for all enzymes. Amylases have different pH optima depending on the sources.

1.9.3 Effect of metal on amylase activity

Heavy metal affect the tertiary structure of enzymes by catalyzing protein-destroying reactions or interfering with the sulphur-sulphur cross bridges. In nature enzymes need not only have specificity and reactivity towards there substrate but also resilience towards inhibitors or in-activator derived from other coexisting microbial or abiotic processes (Baldrian and Valaskova, 2008). Metal ions (Ca^{2+}, Al^{3+}, Fe^{2+}, Fe^{3+}, Mn^{2+}) could be effectors since they exist universally in micro-biota habitat and are soluble in water. They often serve as structural/ functional cofactors (e.g. Ca^{2+}, Zn^{2+}, Fe^{2+},Mn^{2+}, and Cu^{2+}) or inhibitors (e.g. Ag^{+}, Hg^{2+} and Pb^{2+}) of many enzymes including amylase (Kim *et al.*, 2001). Among the divalent metal ions, most often Cu^{2+} and Hg^{2+} are found to exert strong inhibitory effect on amylase. Metallic cofactors like Ba^{2+}, Ca^{2+}, Co^{2+}, K^{+}, Ni^{2+}, $A\,l^{3+}$, Mn^{2+} Hg^{2+}, Li^{2+}, Cu^{2+}, Mg^{2+}, and Pb^{2+} are important because their presence or absence regula]tes enzyme activity. The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, and therefore the rate of digestion.

1.9.4 Effect of substrate concentration on Amylase Activity.

Enzymatic activity is dependent upon the concentration of the enzyme and the substrate. A higher concentration of enzyme will increase the reaction rate although not in direct proportion to substrate availability. The amount of time the enzyme and substrate are together directly affects the extent of reaction.

1.10 Aim of the research study

There is a dearth of information on amylase from African yam bean seed. Therefore, the present study is aimed at extraction, partial purification and characterization of Ŭ- amylases from germinated African yam bean seeds (*Sphenostylis stenocarpa)*

This research work is therefore set out to achieve the following objectives:

a) Extraction of starch from African yam bean seeds

b) Isolation of Ŭ-amylases from germinated African yam bean seeds
c) To partially purify the enzyme by ammonium sulphate saturation and gel filtration chromatography.
d) To compare the hydrolysis of corn starch, cassava starch and African yam beans starch using the isolated and partially purified Ŭ-amylase.
e) To examine temperature and pH dependence of the partially purified Ŭ-amylase
f) To examine some kinetic parameters of the partially purified Ŭ-amylase and
g) To examine effect of some metal ion on the activity of the partially purified Ŭ-amylase

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 African yam beans

African yam bean seeds were obtained from local market, at Orba, in Udenu Local Government Area of Enugu State, Nigeria.

2.1.2 Chemicals/Reagents/

ŏ 3,5-dinitrosalicyclic acid (DNS) (Sigma,USA)
ŏ Bovine Serum Albumin (BSA) (BDH, England)
ŏ Folin-Ciocalteau reagent (BDH, England)
ŏ Glucose,sodium hydroxide, and sodium carbonate (Fluka, England)
ŏ Sephadex G-25 (Sigma USA)
ŏ Tris hydroxymethyl (Merck, England)
- Sodium Hypochlorite and copper sulphide (BDH, England)

ŏ Ammonium sulphate. British Drug House (BDH) Chem. Limited (England)

All chemical used for this research were of analytical grade. Distilled water was used for all preparations of solutions and all pH measurements were made at room temperature using a pH meter.

2.1.3 Apparatus

ŏ Centrifuge Finland Nigeria 80-2B

ð Glass wares Pyrex(England)
ð Magnetic stirrer AM-3250B Surgi Friend Medicals, England.
ð pH-Meter Eciscan pH meter made in England
ð UV-Spectrophotometer UV/visible spectrophotometer: Jenway 6405
ð Water bath Grant Instrument, Ltd Barrington, Cambridge
ð Weighing balance Ohause Dial-O-Gram Ohaus Cooperation N.J.(USA)

2.2 Methods

2.2.1 Experimental Design

Extraction of starch from African yam bean seeds and cassava tubers.
Ŭ-amylase in germinating African yam bean seeds were monitored.
The enzyme extracted from the day of germination with highest amylase activity was used for hydrolysis of starch solutions

2.2.2 Collection of African yam beans

African yam bean seeds were obtained from local market, at Orba, in Udenu Local Government Area of Enugu State, Nigeria.

2.2.3 Seed identification

The plant seeds used for this experiment were identified as African yam bean seed by Mr. C. U. Eze, Department of Crop Sciences, University of Nigeria Nsukka, Nigeria.

2.2.4 Preparation of African yam bean starch

The African yam bean starch was processed using the method described by Eneche (2003) with the following modifications. African yam bean seeds were dehulled to recover the kernel. The kernels were sun dried and ground into powder. Two hundred grams of the powder were suspended in 2L of distilled water for 24hr. The suspended African yam bean seed powder was sieved using a muslin cloth and allowed to sediment for 5hrs at room temperature. The supernatant was decanted and the starch washed with 2L of distilled water twice and finally allowed to stand for another 5hr. The supernatant was decanted and the resulting wet starch was sun-dried. The dry powdered starch was further grinded using mortar and pestle and packed in an air-tight container.

2.2.5 Preparation of cassava starch

Starch from cassava was processed using the method described by Corbishley and Miller (1984). Cassava tubers (freshly harvested) were peeled, washed and grated. The grated cassava (1kg) was soaked in 5 L of distilled water for an hour. The suspended cassava flour was sieved with muslin cloth and allowed to sediment for 4h at room temperature. The supernatant was decanted and the isolated wet starch was sun dried. The dried cassava starch was further grinded using mortar and pestle and packed in an air-tight container.
Corn starch was bought from Ogige market in Nsukka LGA.

2.2.6 Germination of African yam bean seeds.

The viable seeds were washed with 1% NaOCl, rinsed twice with distilled water and imbibed (i.e. taking up of water resulting in swelling) for 24 hr. At the end of imbibition, the seeds were germinated on already prepared sand beds for 12 days. The beds were watered at every 48hr.

2.2.7 Extraction of α-amylase

Germinated seeds (100g) were homogenized using pestle and mortar in 200mls of 0.05M cold sodium acetate buffer pH 5.5. The un-imbibed seeds were also treated as above. Germination of seeds was done for a period of 12days while homogenization of seeds took place every 24hr during germination. The homogenate was filtered using whatman No 1 filter paper and centrifuged at 4000rpm for 10min. The supernatant was stored at -18^0C as crude extract.

2.2.8 Partial purification of the α-amylase

The crude extract was purified using ammonium sulphate precipitation and gel filtration column chromatography using sephadex G25.

2.2.8.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a method used to purify protein by altering their solubility. Ammonium sulphate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Different concentrations of ammonium sulphate were used for this study (10%, 20%, 30%, 40%,é .100%) using standard ammonium sulphate table from Data for Biochemical Research. The precipitates were recovered by centrifugation (4000rpm for 10min), and dissolved in 1ml of 0.05M sodium acetate buffer pH 5.5. Ŭ-amylase activity was assayed on the supernatant and precipitate using starch from African yam bean seeds, cassava and corn.

2.2.8.2 Gel filtration column chromatography

2.2.8.3 Principle of gel column chromatography

Gel filtration chromatography separates protein based on size. Protein mixtures are applied to a gel filtration column containing a chromatographic material of defined pore sizes. Proteins are eluted with an aqueous buffer, collected as an individual chromatographic fraction and analyzed separately. Gel filtration can be used to separate protein based on differences in their molecular sizes or weight to desalt proteins (remove low molecular weight contaminants such as amino acids and protein). Porath and Foldin (1959) method with little modification was used.

Swelling of gel: 60g of the gel (sephadex G-25) was mixed with 1000ml of distilled water, stirred gently using a glass rod and allowed to stand for three days.

Packing/ filling of the column: The column was mounted vertically on a retort stand. The swollen gel was introduced gently via the sides of the column after which, 500ml of 0.05M sodium acetate buffer pH 5.5 was carefully introduced to equilibrate the gel. A column bed height of 60.4 was achieved.

Application of sample: 20ml of the enzyme precipitate was applied to the packed column using Pasteur pipette. After the sample was applied, 5ml of buffer was layered gently on top of the gel and allowed to elute. The elution flow rate was 2ml/min.

Collection of column fraction: the eluent was collected using tube collector. The absorbance of the fractions were measured at 280nm for protein determination. Amylase activity was assayed in each fraction.

2.2.9 Assay of amylase activity

2.2.9.1 Glucose standard curve

Glucose standard curve was prepared as described by Miller (1959) using 10mM glucose solution. Ten test tubes were arranged in duplicate containing 0.0-1.0ml of 10mM glucose solution. Each tube was made up to 1ml using freshly prepared 0.05M sodium acetate buffer pH 5.5. Then 1ml of DNS (3,5-dinitrosalicyclic acid) reagent was added to each of the test tubes and placed in boiling water bath for 10mins. The tubes were then removed and 1ml of potassium tarterate solution was added to stabilize the color, the tubes were allowed to cool at room temperature, and the absorbance (optical density) read at 540nm.(Figure 47). A plot of absorbance at 540nm against glucose concentration in (µmole/min) was constructed.

α-amylase activity

Ŭ-Amylase activity was assayed in duplicate using the method described by Miller (1959). Each of the test tubes containing 0.5 ml of 1% starch solution, 0.5ml of the enzyme and 1ml of 0.05M sodium acetate buffer pH 5.5, was incubated for 30min at room temperature, after which 1ml of DNS was added. The test tubes were immersed in boiling water bath for 10mins. 1ml sodium potassium tarterate was added to each of the test tubes and were allowed to cool at room temperature and the absorbance read at 540nm using UV-VIS (Jenway 6405). Amylase activity was estimated by determination of the concentration of glucose produced per unit time from a standard glucose curve (Figure 47). One unit of Ŭ-amylase activity was defined as the amount of enzyme that released 1µmole of reducing sugar as glucose per min under assay conditions.

2.2.9.2 Protein estimation of the crude enzyme extract

Protein concentration in the crude was determined using Lowry *et al*, (1951) method. The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids

Protein standard curve:

Nine test tubes were arranged in duplicate each containing 0.1ml of protein. The volume was made up to 1ml by adding 0.9ml of 0.05M sodium acetate buffer pH 5.5. Then, 2ml of alkaline solution (solution E) was added to the reaction mixture and allowed to stand for 10min at room temperature. 0.2ml of Solution C (Folin-ciocalteau reagent) was added rapidly with immediate mixing and allowed to stand for 10min and absorbance taken at 750nm. Protein standard curve was obtained using Lowry *et al*, (1951) method and bovine serum albumin (1g BSA/5mls of distilled water) as the standard (Figure 46).

2.2.9.3 Determination of the specific activity of α-amylase enzyme

The specific activity of the Ŭ-amylase enzyme protein was expressed in terms of units/mg protein according to the following equation:

Specific activity = enzyme activity / protein concentration (mg/ml)

2.2.10 Studies on purified enzyme

2.2.10.1 Effect of pH on α-amylase activity

Optimal pH of Ŭ-amylase activity was determined as described by Thoomati and Peramachi, (2012) using the following buffer system: 0.05M sodium acetate buffer (pH 3.5-5.5), 0.05M disodium hydrogen phosphate buffer (pH 6.0-7.5) and 0.05M Tris-HCl buffer (pH 8.0-10.0). The assay was carried out as described above (2.5) but varying the buffer concentration.

2.2.10.2 Effect of temperature on α-amylase activity

Amylase activity was determined at different temperatures ranging from 25- 75ºC at 5ºC interval in a water bath using 0.05M phosphate buffer pH 6.0. The activity was assayed as using the method described by Thoomati and Peramachi, (2012).

2.2.10.3 Effect of substrate concentration on α-amylase activity:

To determine the optimum concentration of substrate for maximum enzyme activity using DNS method of Miller (1959), starch solution of various concentrations (0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5%) were used for the assay while enzyme concentration remained constant.

2.2.10.4 Effect of metal ions on the enzyme activity

This was also carried out using the method described by Varalakshmi *et al.* (2009) with the following modifications. The partial purified enzyme was incubated for 30min in different concentrations of metal ions (0.02 to 0.05M) with each of the metal ions as chloride salts. Two test tubes each was used for the metal ion concentration. The enzyme activity was assayed as described in 2.5 using the determined optimum temperature and pH. The amylolitic activity in each case was compared with the activity obtained in the absence of metal ion (control).

2.2.10.5 Effect of incubation time on total α-amylase activity.

The effect of incubation time on Ŭ-amylase activity was determined by measuring the total Ŭ-amylase activity at difference period of incubation (0, 5, 10, 20, 30, 40, 60, 90, and 120 min) under the above optimum assayed conditions of pH and temperature using the method of Bernifield, P (1955).

CHAPTER THREE

RESULTS

3.1 Preliminary studies on the seeds after imbibition

The results of the activity of Ŭ-amylase from African yam bean seeds at different pH after 12hrs imbibition using starch from African yam bean seeds is represented in figure 8.

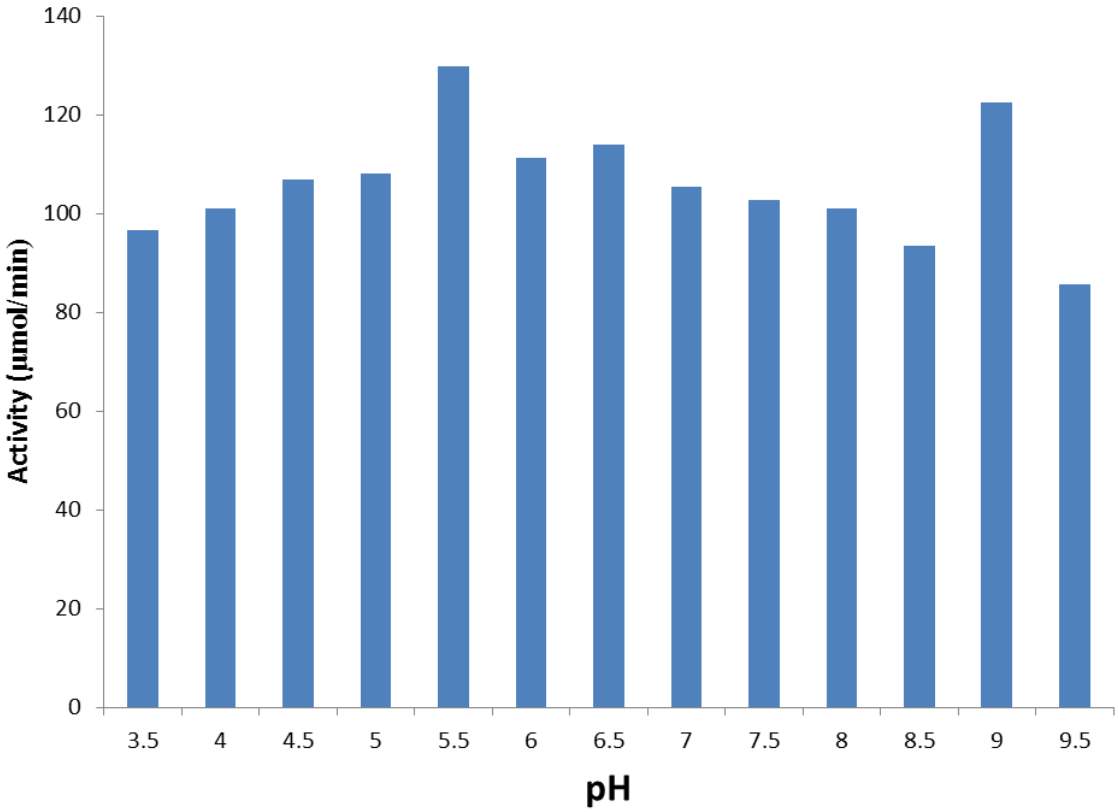


Figure 8: Activity of Ŭ-amylase from African yam bean seeds at different pH after imbibition, using starch from African yam bean seeds as substrate

Figure 8 shows the effect of pH on Ŭ-amylase activity in freshly processed African yam bean seeds after imbibition at room temperature using starch from African yam bean seeds as the substrate. The enzyme as shown in the result had a maximum activity at pH 5.5 and 9.0.

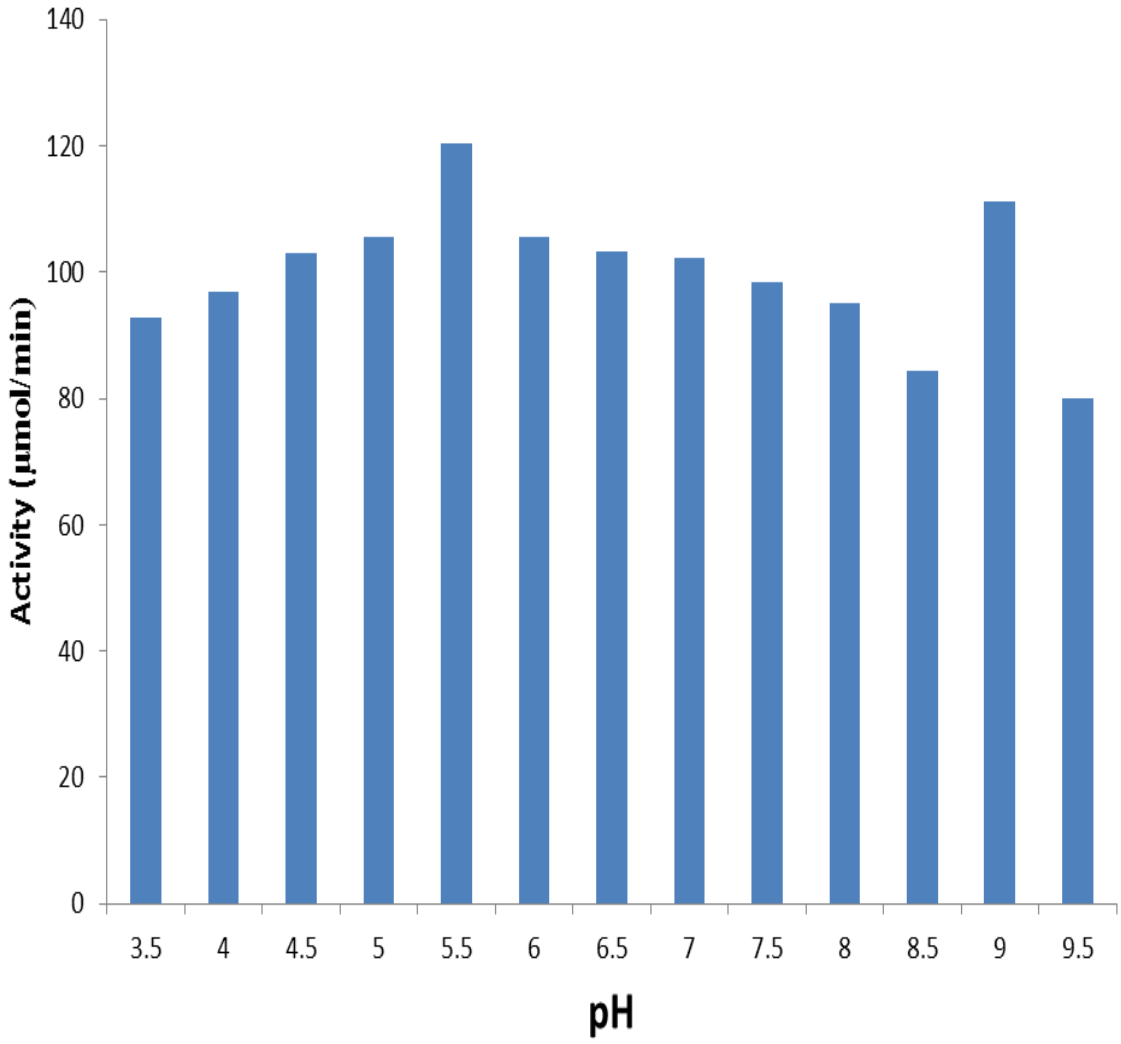


Figure 9: Activity of Ŭ-amylase from African yam seeds at different pH after imbibition, using starch from corn as substrate

Figure 9 shows the effect of pH on Ŭ-amylase activity in freshly processed African yam bean seeds after imbibition at room temperature using corn starch as the substrate. Result obtained show the enzyme had a maximum activity at pH 5.5 and 9.0. The result is similar to the result obtained when starch from African yam bean seeds was used as substrate, but lower in activity when compare to starch from African yam bean seeds.

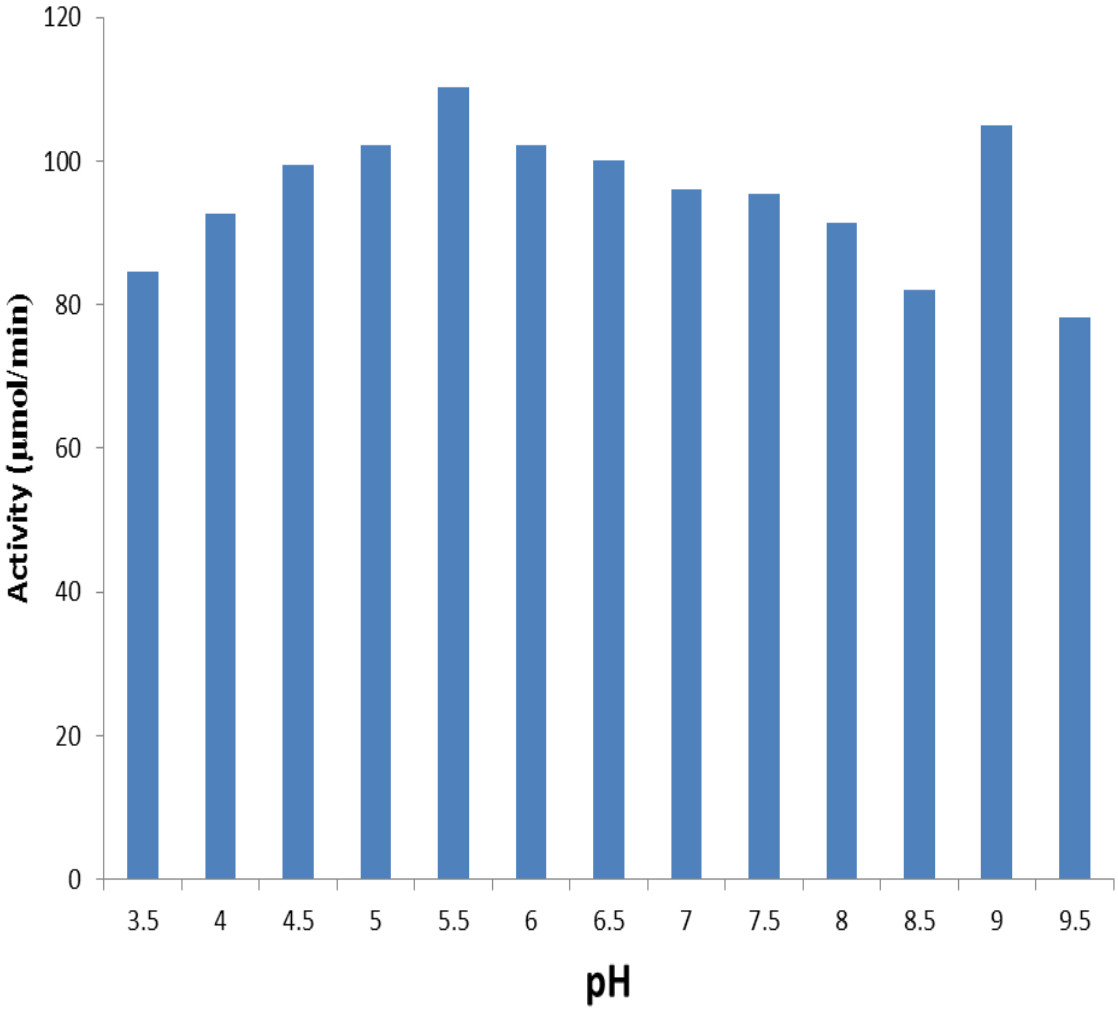


Figure 10: Activity of Ŭ-amylase from African yam seeds at different pH after imbibition using starch from cassava as substrate.

Figure 10 shows the effect of pH on Ŭ-amylase activity in freshly processed African yam bean seeds after imbibition at room temperature using starch from cassava as the substrate. The enzyme had a maximum activity at pH 5.5 and 9.0. The result is similar to the result obtained when starch from African yam bean seeds and corn were used as substrates, but lower in activity when compare to starch from African yam bean seeds and corn.

3.2 Pre-germination studies

Result of the activity of Ŭ-amylase in the dry seeds and after 24hr imbibition using 0.05M sodium acetate buffer pH 5.5

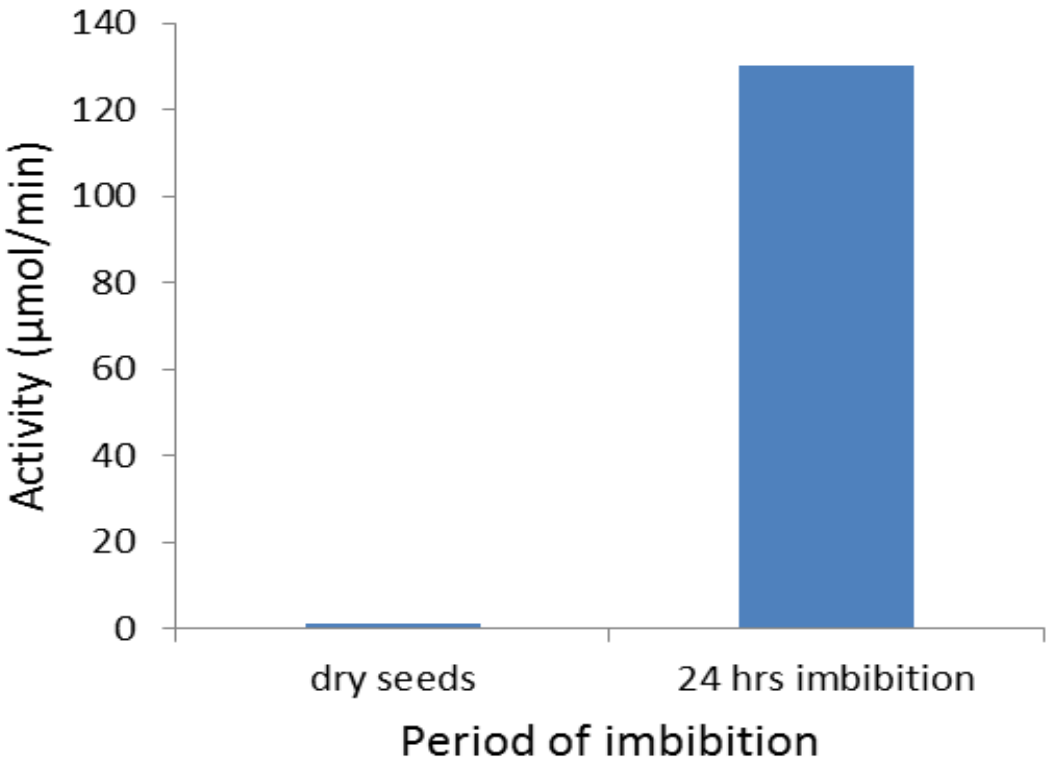


Figure 11: Pre-germination studies on the activity of Ŭ-amylase using starch from African yam bean seeds as substrate.

This result (Figure 11) shows a considerable high activity of Ŭ-amylase after 24hr imbibition, but no activity of Ŭ-amylase was found in dry seeds of African yam bean seeds when assayed at the same conditions of temperature and pH.

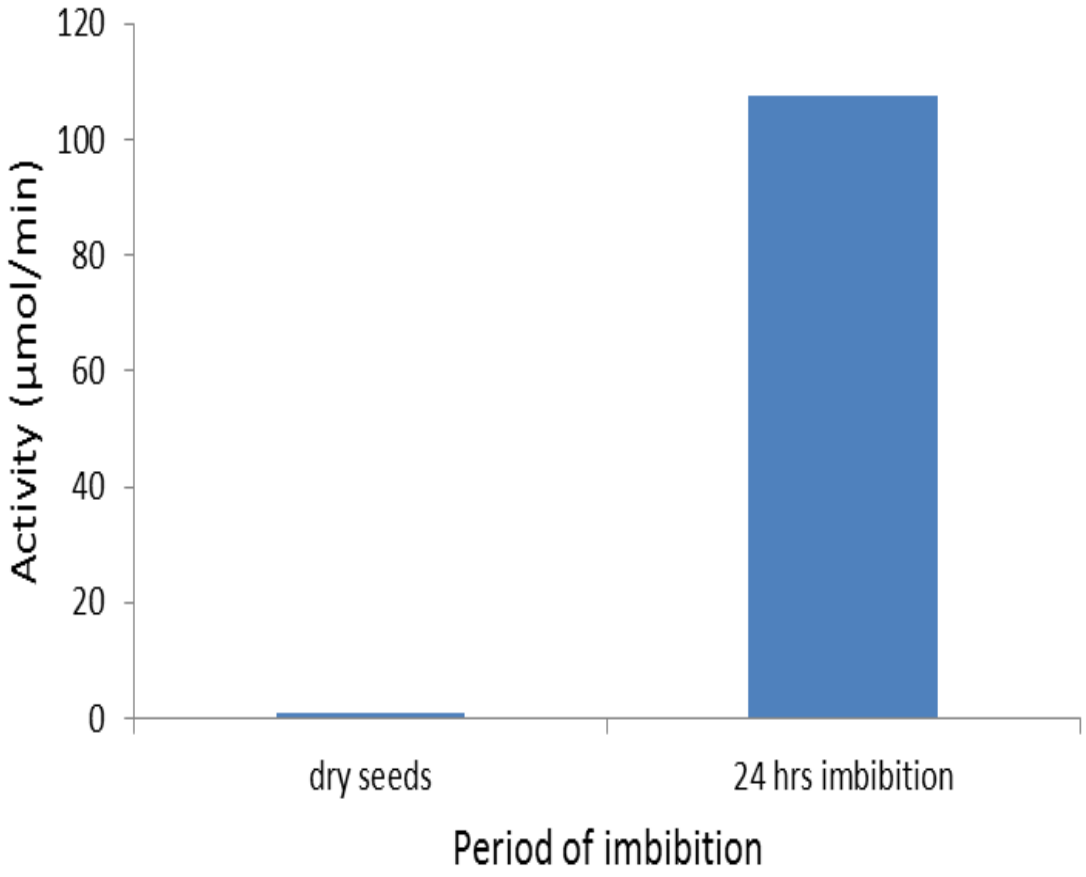


Figure 12: Pre-germination studies on the activity of Ŭ-amylase using corn starch as substrate.
This result (Figure 12) shows a considerable high activity of Ŭ-amylase after 24hr imbibition, but no activity of Ŭ-amylase was found in dry seeds of African yam bean seeds when assayed at the same conditions of temperature and pH.

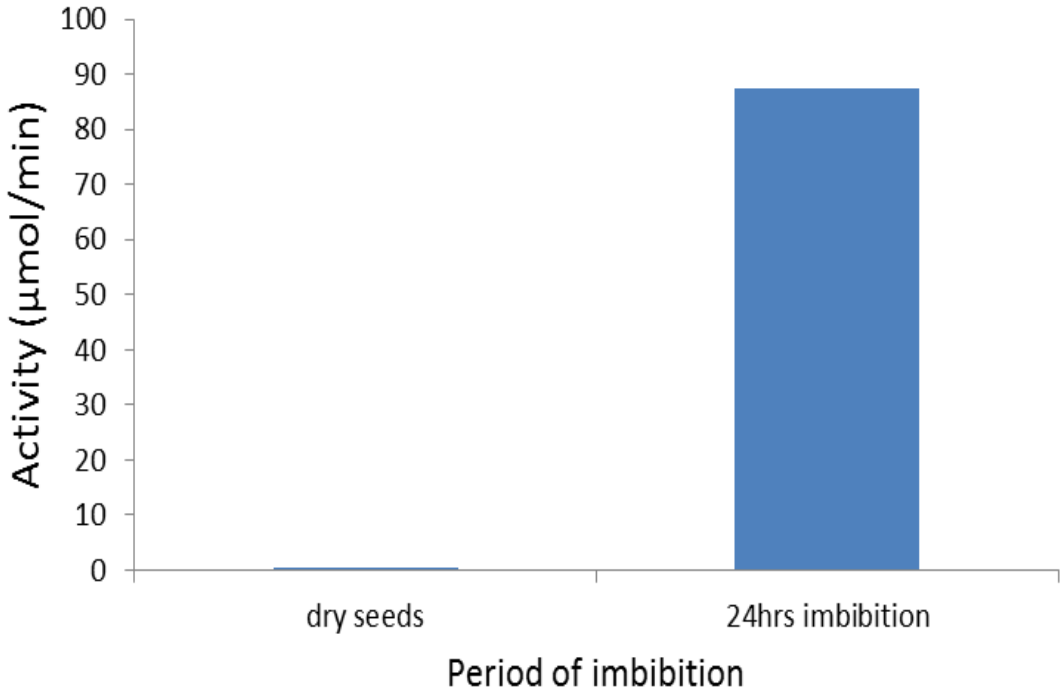


Figure 13: Pre-germination studies on the activity of Ŭ-amylase using cassava starch as substrate.

Results of the pre-germination studies of amylase activity on dry seeds and after 24hr imbibition using 0.05M sodium acetate buffer pH 5.5 at room temperature (Figures 11, 12 and 13) showed that, Ŭ-amylase activity after 24hr imbibition is considerably high when compared with dry seeds. However, there was no activity of Ŭ-amylase in dry seeds for each of the substrate (African yam bean seeds, corn and cassava starch) used. It was also observed that the activity of Ŭ-amylase (130.04ε mol/min) after 24hr imbibition using starch from African yam bean seeds is higher than starch from corn (107.41ε mol/min), while starch from cassava had the least amylase activity (87.45ε mol/min).

3.3 Germination Studies

A preliminary study of the effect of pH on Ŭ-amylase activity from un-germinated African yam bean seeds (after 24hrs imbibition) showed two pH optima (Figures 8, 9 and 10).

Figures 14, 15 and 16 showed Ŭ-amylase activity on each day of germination at pH 5.5 and 9.0.

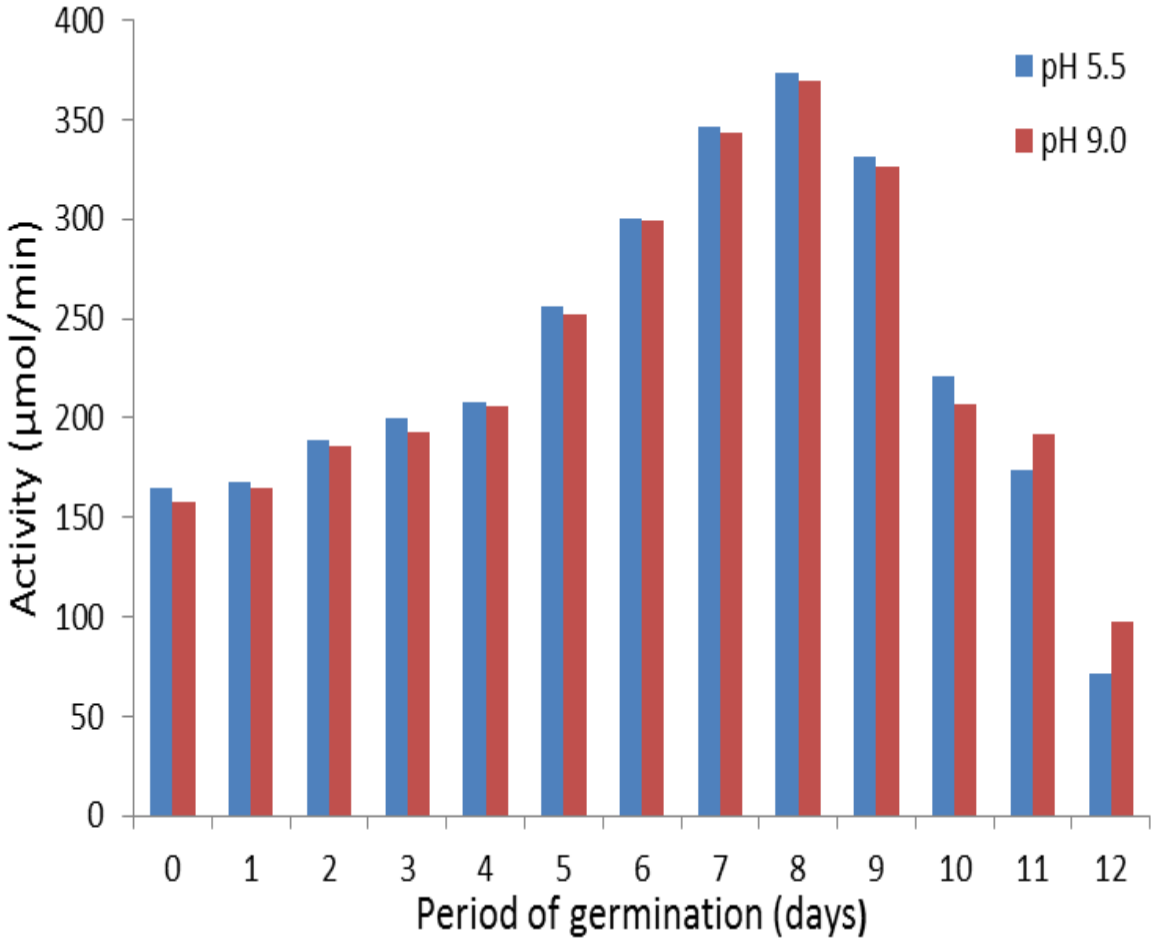


Figure 14: Determination of Ŭ-amylase activity during germination of African yam beans seeds (*Sphenostylis stenocarpa*), using starch from African yam bean seeds as substrate.

The result of the study (Figure 14) shows steady increase in Ŭ-amylase activity from day zero through day 8 of germination at the different pH (5.5 and 9.0) used for the study. Highest amylase activity was observed on the 8th day with an activity of 373.25µmol/min and 369.55µmol/min at pH 5.5 and 9.0, respectively. The Ŭ-amylase activity started decreasing from day 9 through day 12 during germination.

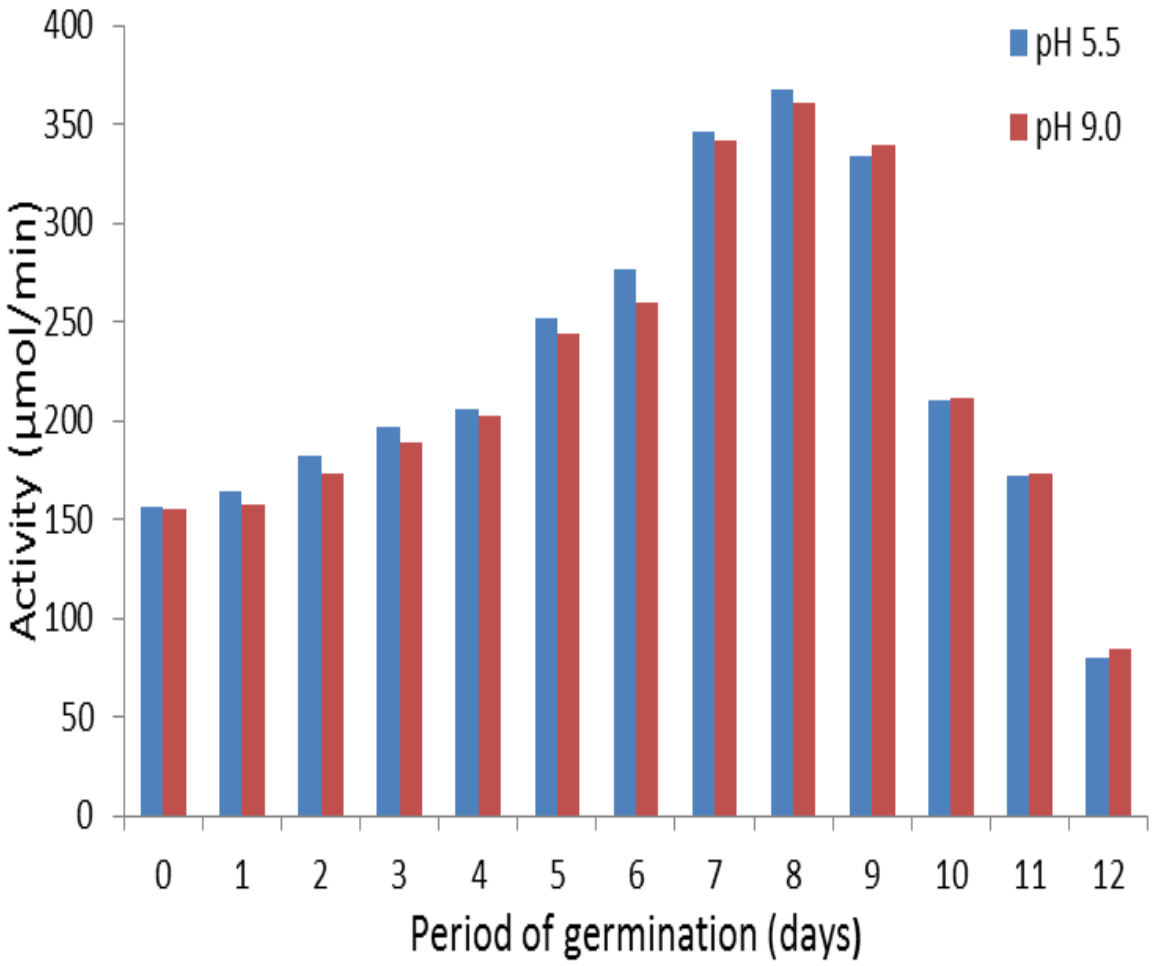


Figure 15: Determination of Ŭ-amylase activity during germination of African yam beans seeds (*Sphenostylis stenocarpa*), using starch from corn as substrate.

The result of this study (Figure 15) shows steady increase in Ŭ-amylase activity from day zero through day 8 of germination at the different pH (5.5 and 9.0) used for the study. Highest Ŭ-amylase activity was also observed on the 8th day with an activity of 367.08µmol/min and 360.49µmol/min at pH 5.5 and 9.0, respectively. The Ŭ-amylase activity also declined from day 9 through day 12 during germination.

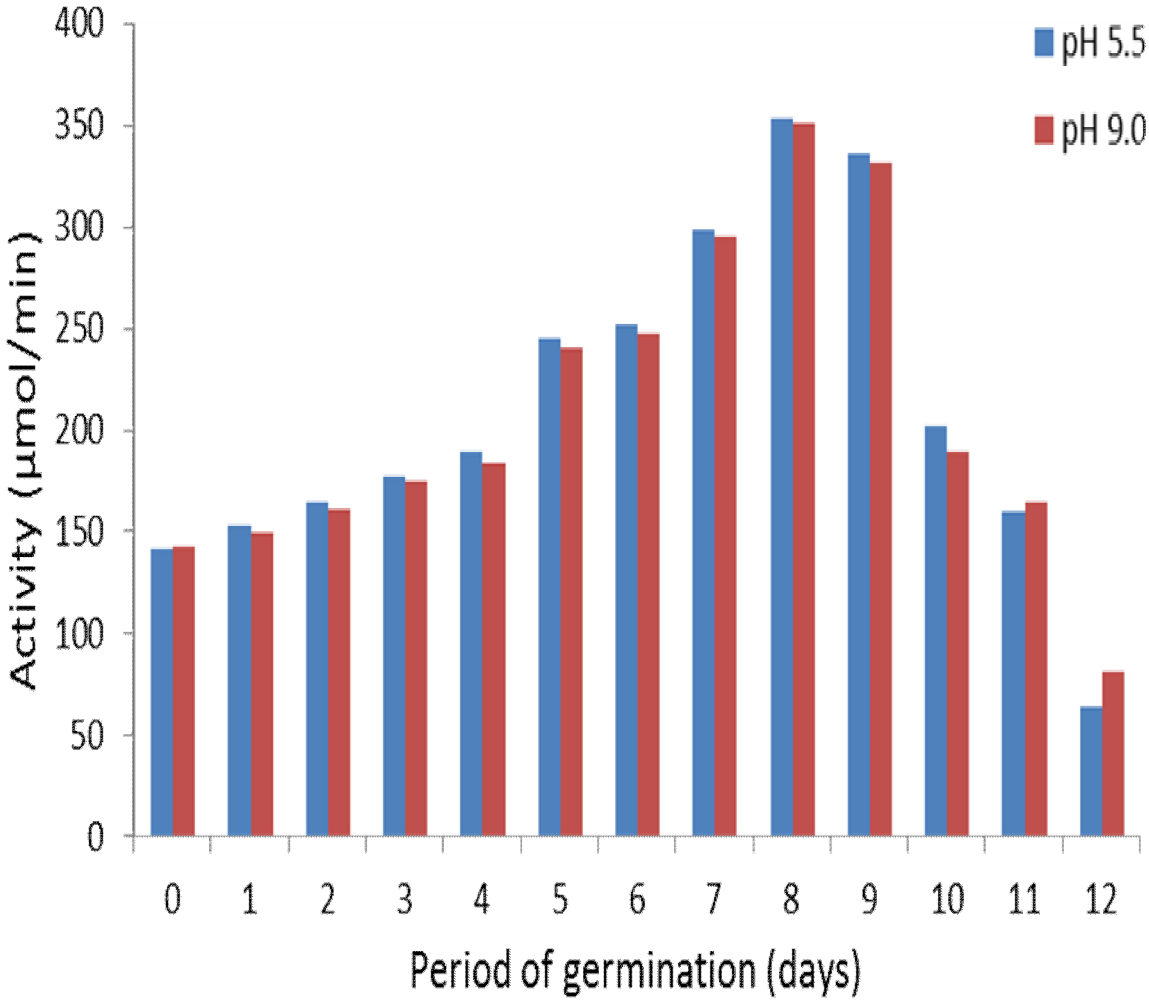


Figure 16: Determination of Ŭ-amylase activity during germination of African yam beans seeds (*Sphenostylis stenocarpa*), using starch from cassava as substrate.

The result of this study (Figure 16) shows steady increase in Ŭ-amylase activity from day zero through day 8th of germination at the different pH (5.5 and 9.0) used for the study. Highest Ŭ-amylase activity was also observed on the 8th day with an activity of 353.50µmol/min and 351.03µmol/min at pH 5.5 and 9.0, respectively. The Ŭ-amylase activity also declined from day 9 through day 12 after imbibition.

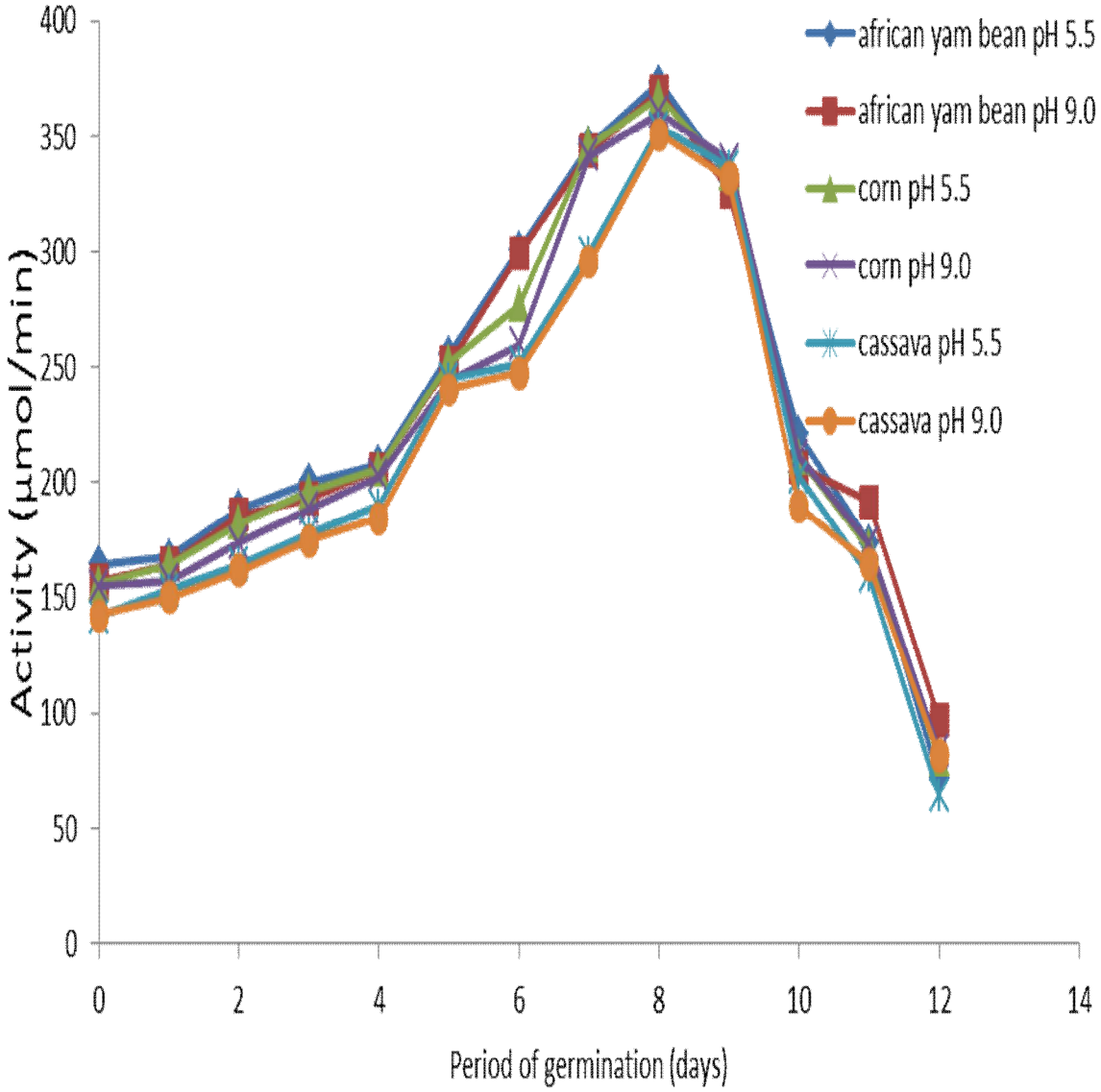


Figure 17: Summery of the activity of Ŭ-amylase during period of germination of African yam bean seeds using three substrate -African yam bean, corn and cassava starch.

It was observed that the Ŭ-amylase activity was higher at the different pH value used when starch from African yam bean seeds was used than when starch from corn and cassava were used as substrate.

3.4 Determination of protein concentration during germination of African yam bean seeds (*Sphenostylis stenocarcarpa*)

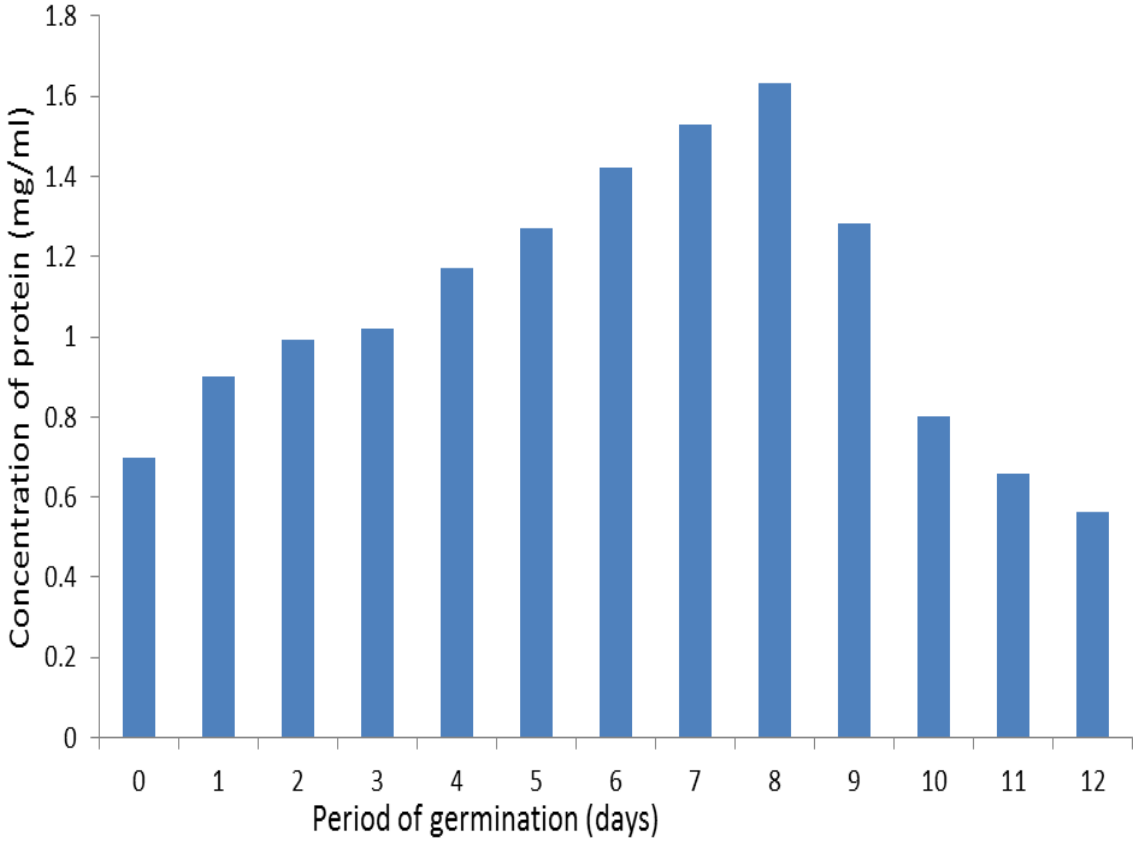


Figure 18: Determination of protein concentration during germination of African yam bean seeds (*Sphenostylis stenocarpa*).

Figure 18 shows a steady increase in protein concentration from day zero through day 8th of germination. Maximum protein concentration was observed on the 8th day with concentration of 1.63 mg/ml. The protein concentration started decreasing from day 9 through day 12 after imbibition. This result is similar to the observation made during the determination of Ŭ-amylase activity at the different days of germination using starch from African yam bean seeds, corn and cassava as substrates.

3.5 Purification studies

3.5.1 Percentage (%) ammonium sulphate saturation profile

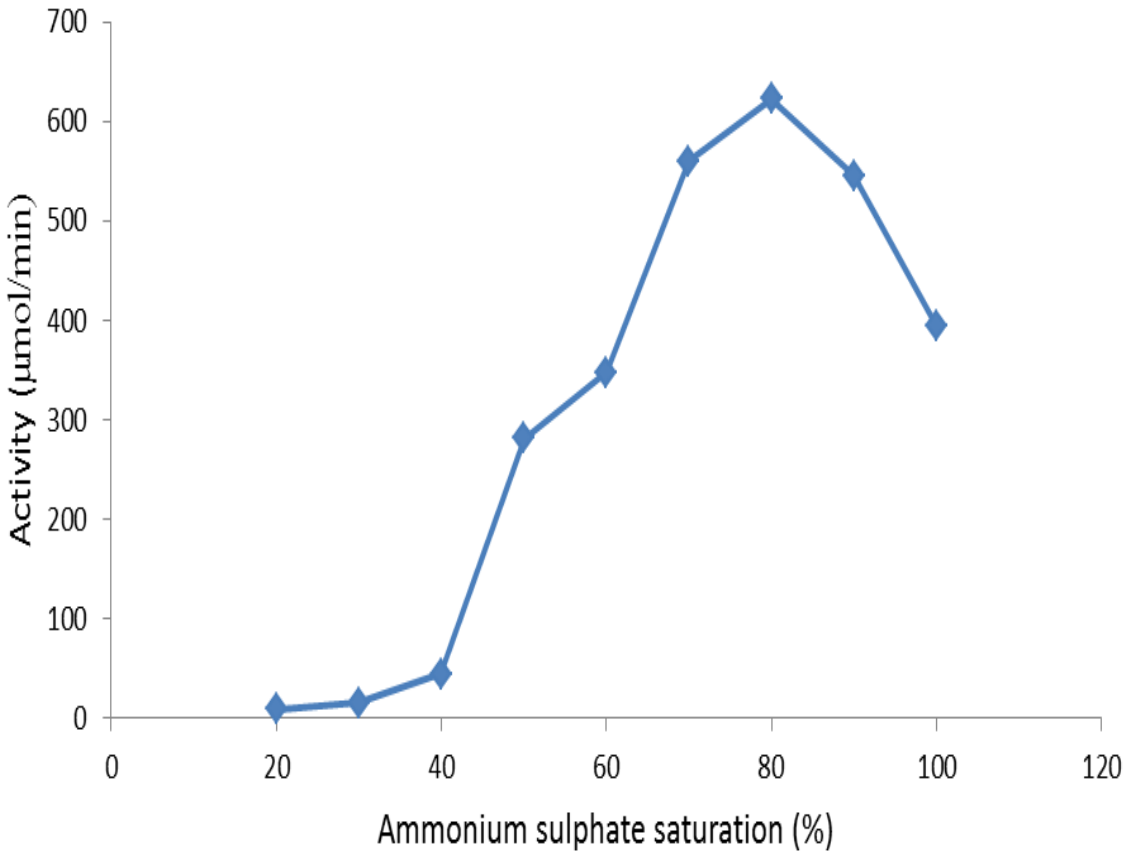


Figure 19: Ammonium sulphate precipitation profile using starch from African yam bean seeds as substrate.

Precipitation of Ŭ-amylase with solid ammonium sulphate was carried out between 20% - 100% saturation. 80% ammonium sulphate saturation was observed to have the highest Ŭ-amylase activity of 622.84µmol/min. Hence, 80% ammonium sulphate saturation was chosen for mass precipitation of our crude Ŭ-amylase (Figure 19).

3.5.2 Gel filtration column chromatography

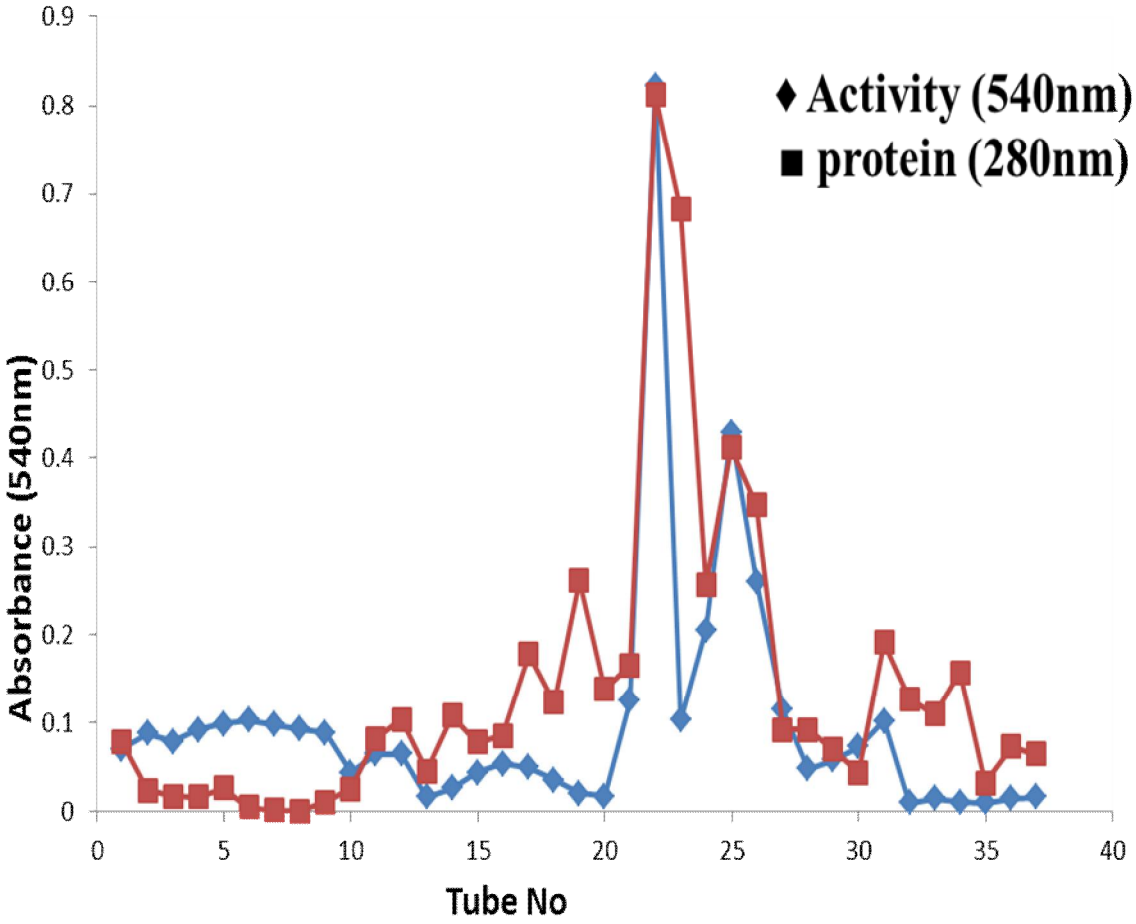


Fig. 20: Elution profile of gel filtration column chromatography using starch from African yam bean seeds as substrate.

Approximately 37 fractions (5mls/fraction) were collected at the flow rate of 2ml/min. Each of the fractions were assayed for enzyme activity using starch from African yam bean seeds, however, the fractions with relatively high amylase activity were pooled together and again assayed for enzyme activity using starch from African yam bean seeds as substrate. Also protein content of each fraction was also determined at 280nm. The plot of the absorbance against the tube numbers showed two peaks.

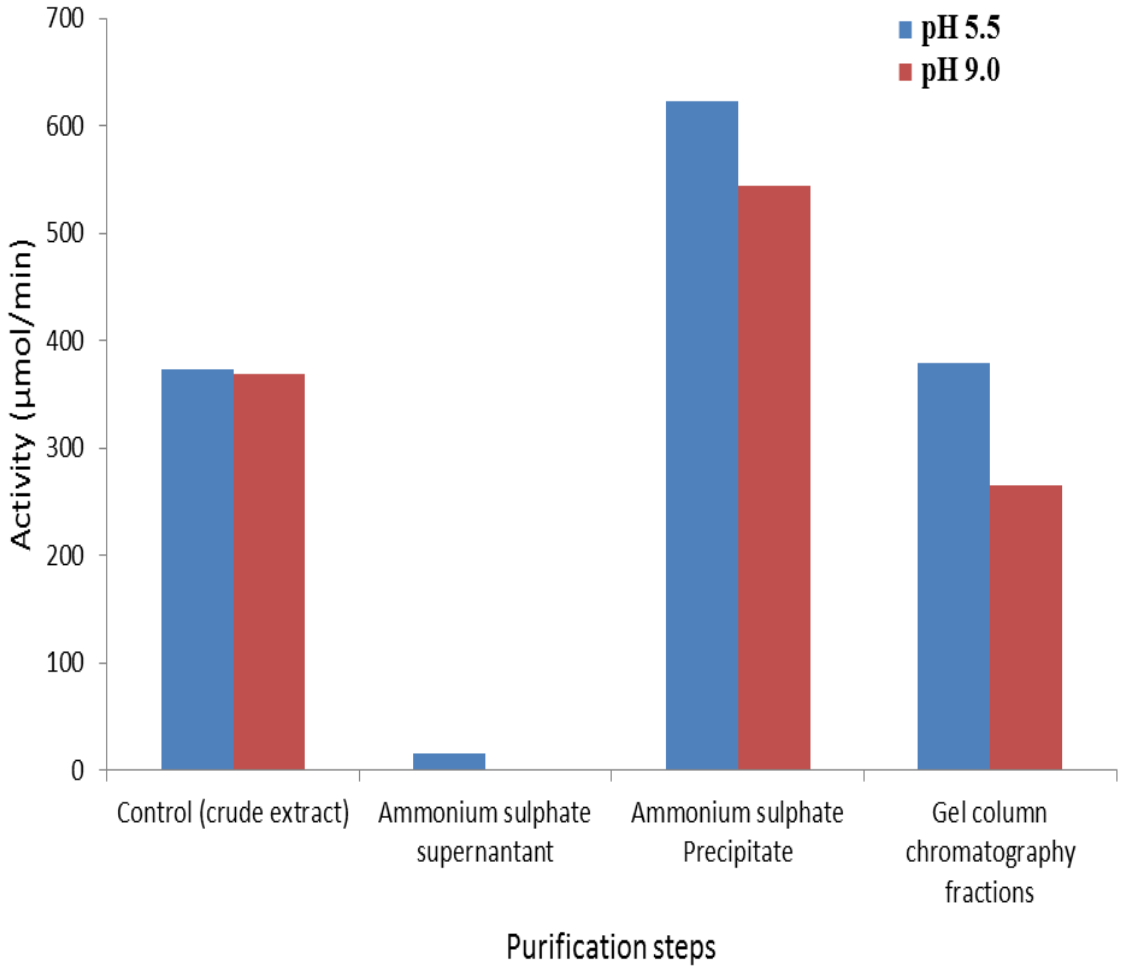


Figure 21: Change in Ŭ-amylase activity after purification step using starch from African yam bean seeds as substrate

Result of the study (Figure 21) showed that, precipitation of the enzyme at 80% ammonium sulphate saturation had highest enzyme activity when compared with other purification step (gel filteration column chromatography) used in the research.

Table 2: Purification profile of α-amylase from germinated African yam bean seeds

Purification steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	326.4	73910	226.44	1	100
$(NH_4)SO_4$ Precipitation	100.8	31142	308.96	1.36	42.14

Chromatography on sephadex G-25	20.64	7588	367.65	1.62	10.26

Note: ε mole/min = Unit (U).

Table 2 shows the partial purification of Ŭ-amylase from germinated African yam bean seeds. The crude enzyme gave a specific activity of 226.44U/mg protein. After ammonium sulphate precipitation, the purification fold and specific activity were found to be 1.36 and 308.96U/mg protein, respectively. The protein collected after gel filtration chromatography had specific activity of 367.65U/mg protein and a purification fold of 1.62. The percentage yields were found to be 100%, 42% and 10% for crude, precipitate and gel chromatography respectively.

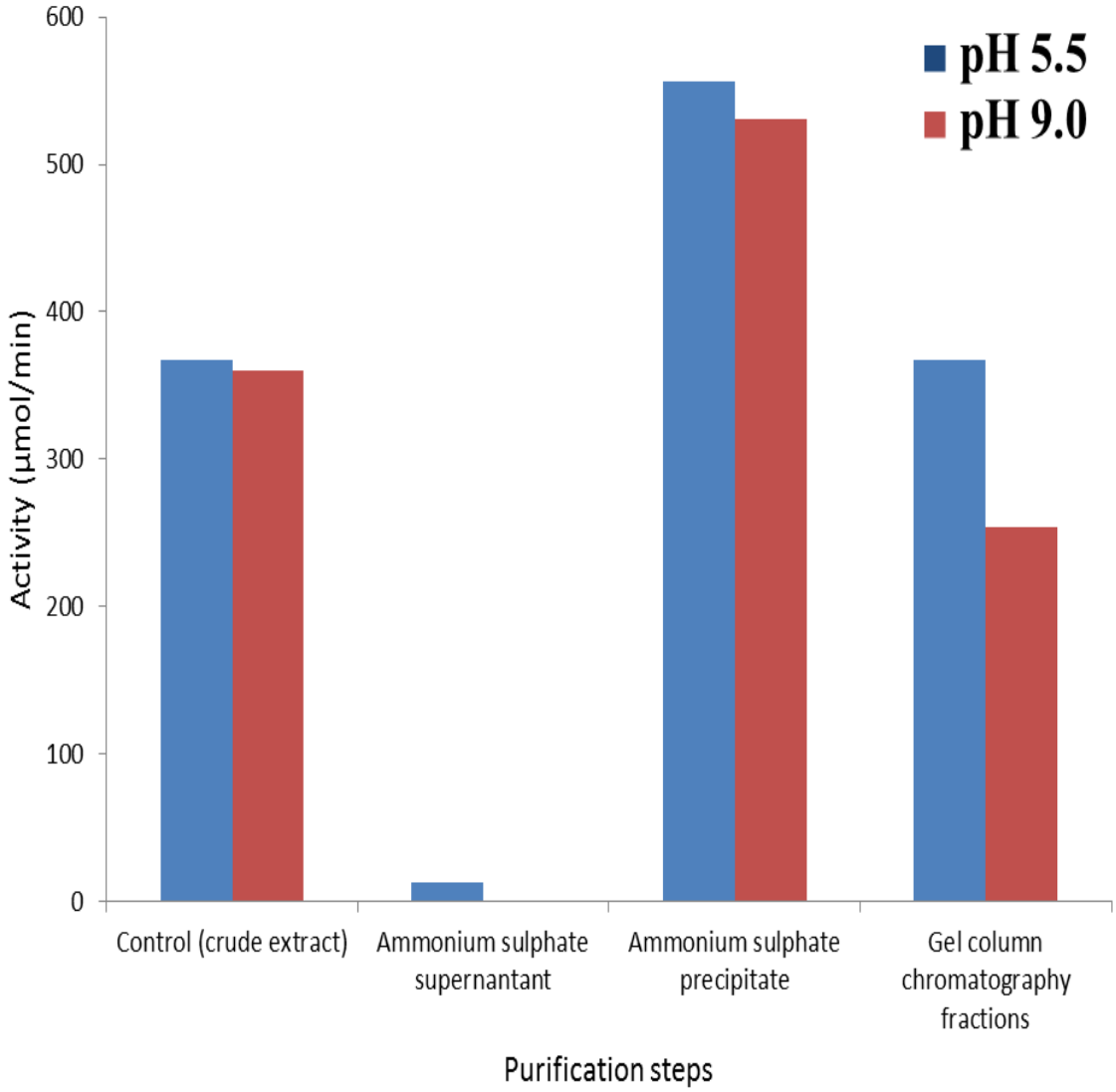


Figure 22: Change in Ŭ-amylase activity after purification step using corn starch as substrate.
The result obtained from the study (Figure 22) shows that the Ŭ-amylase activity was highest after precipitation (using ammonium sulphate) followed by gel filtration column chromatography with the supernatant as the least when compared with the control at the different pH value used for study.

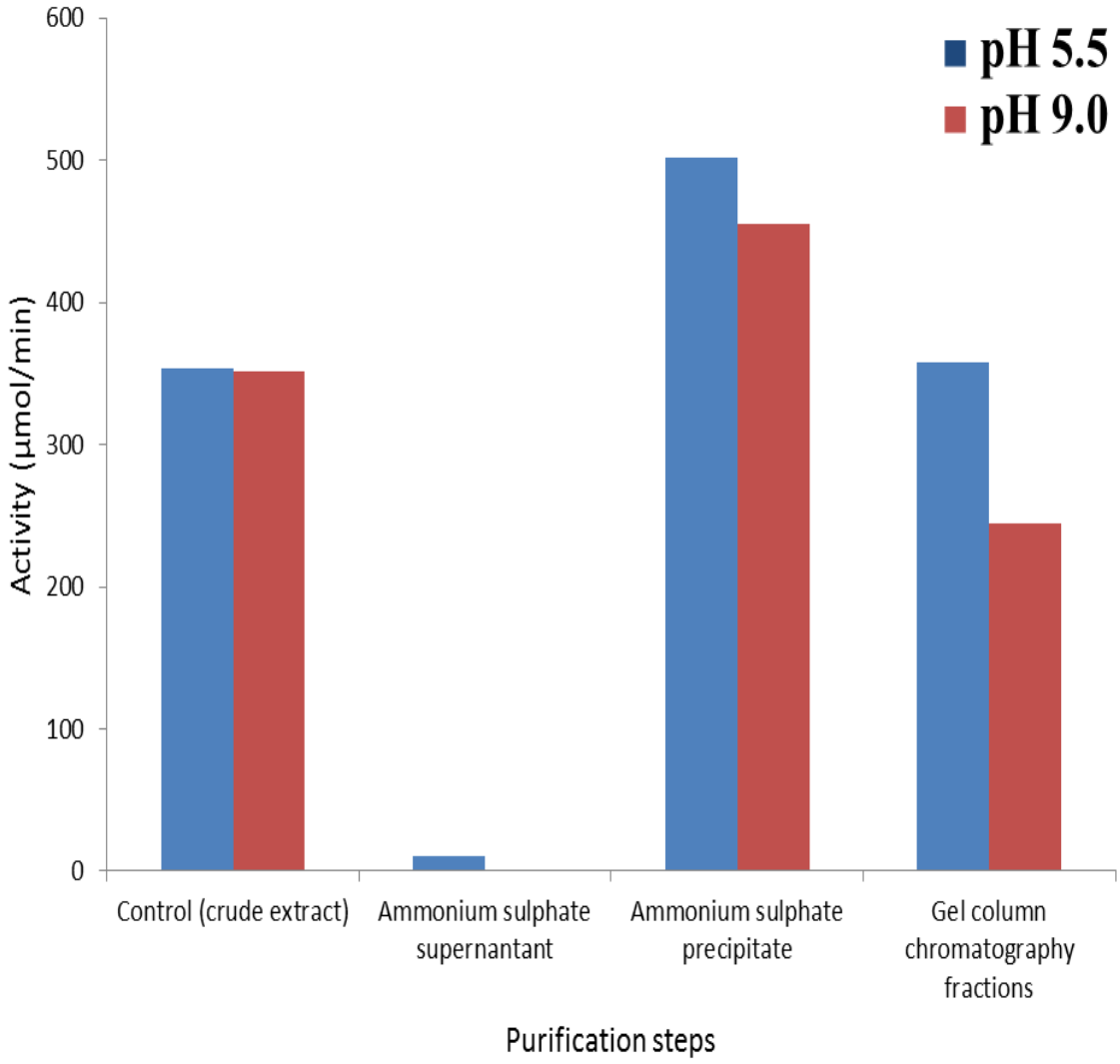


Figure 23: Changes in Ŭ-amylase activity after purification step using cassava starch as substrate
The result obtained (Figure 23) shows that the alpha amylase activity was highest after precipitation (using ammonium sulphate) followed by gel filtration column chromatography with the supernatant as the least when compared with the control at the different pH value used study. The result also showed higher Ŭ-amylase activity when starch from African yam bean seeds was used as substrate, followed by corn while cassava starch had the least Ŭ-amylase activity.

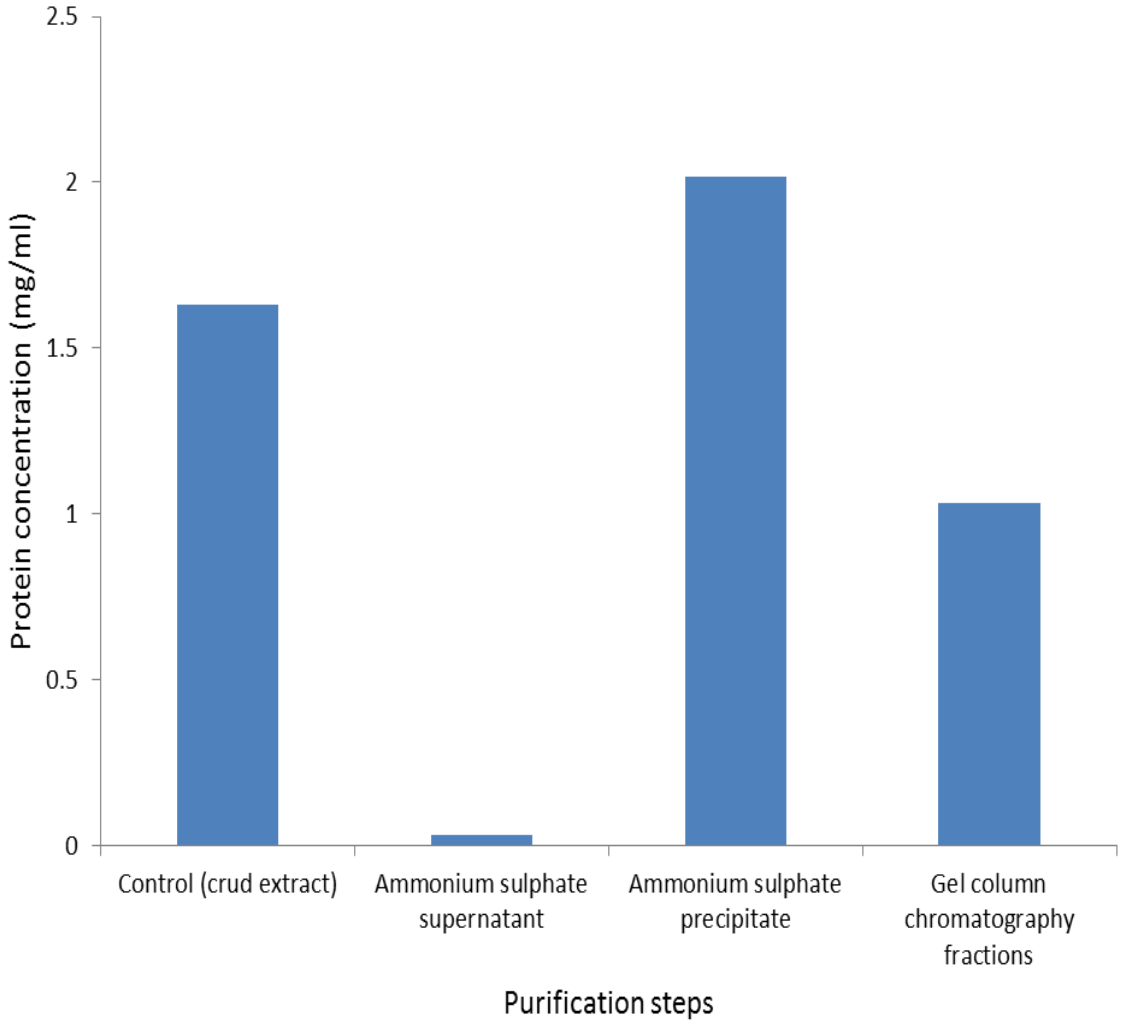


Figure 24: Changes in protein concentration after purification step

Figure 24 above shows that the protein concentration after purification using ammonium sulphate precipitation is relatively higher when compare to gel filtration column chromatography. The crude extract had a significant Ŭ-amylase activity while the supernatant has the least protein concentration.

3.6 Effect of incubation time on α-amylase activity

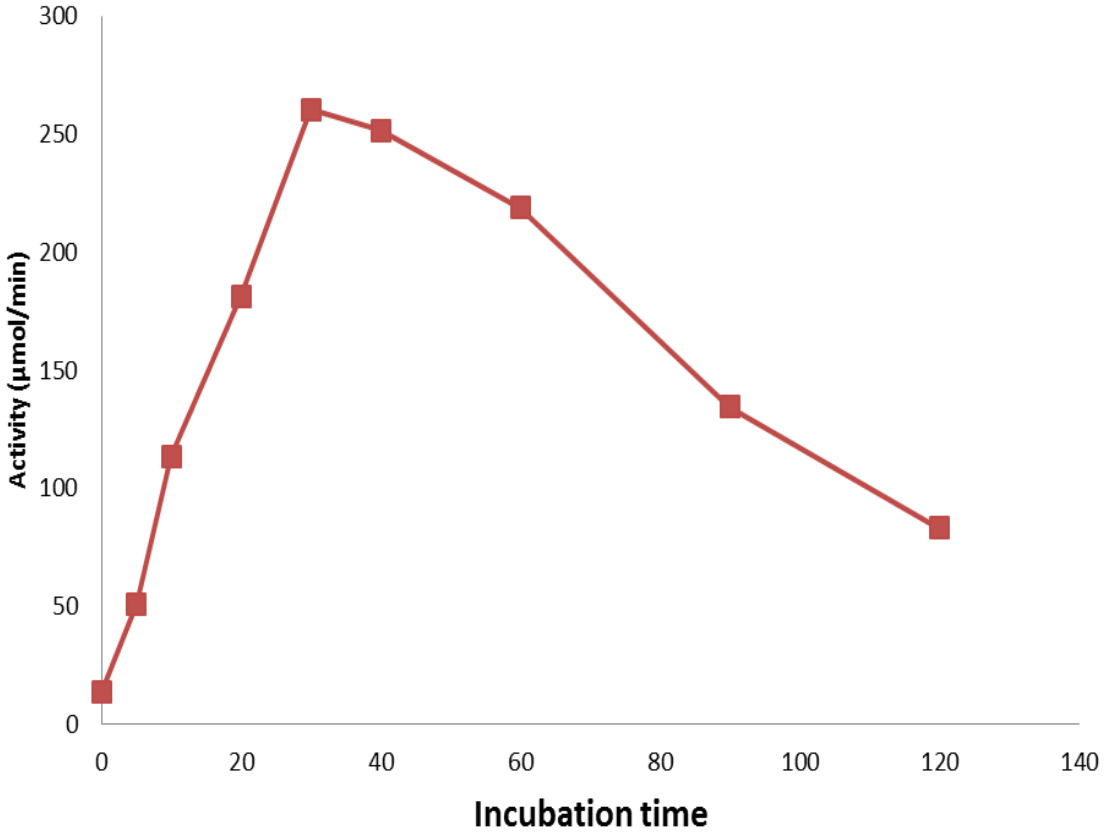


Figure 25: Effect of incubation time on Ŭ-amylase activity using starch from Africa yam bean seeds in 0.05M Na Acetate buffer pH 5.5

Figure 25 shows the plot of Ŭ-amylase activity against incubation time of Ŭ-amylase at pH 5.5. Result obtained showed a steady increase of enzyme activity with increase in incubation time, but above 30 minutes of incubation, the activity started declining. However 30 minutes of incubation was found to have highest enzyme activity (260.50µmol/min)

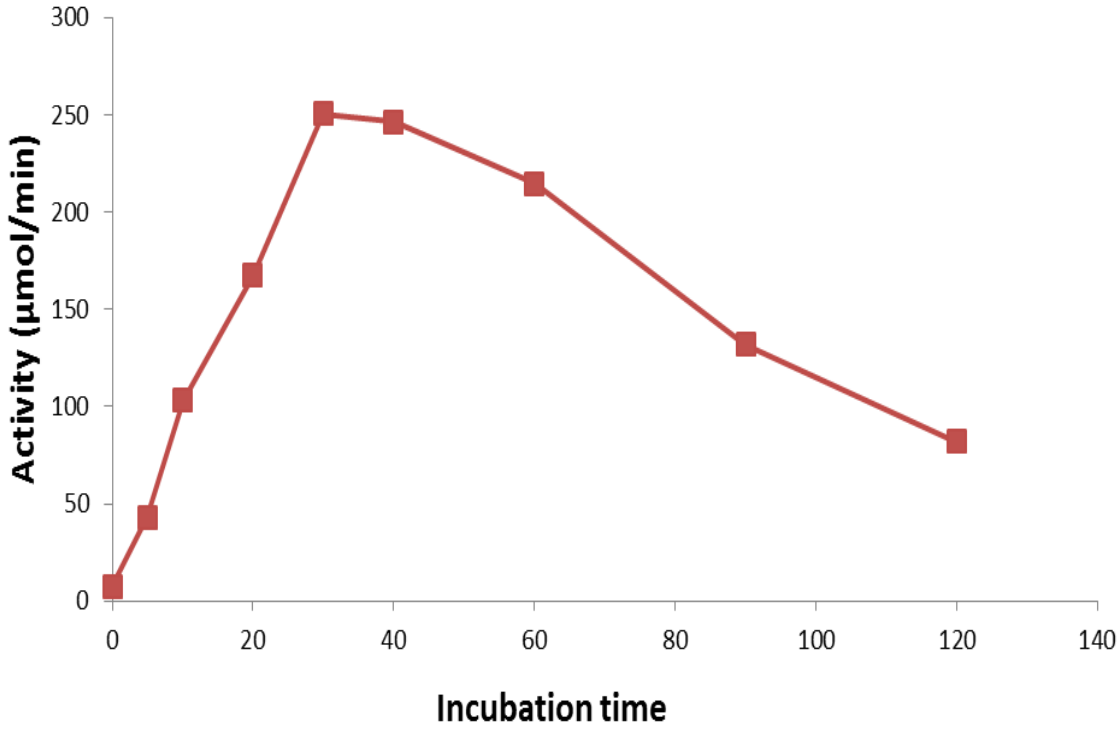


Fig. 26: Effect of incubation time on Ŭ-amylase activity using corn starch as substrate in 0.05M Na Acetate buffer pH 5.5

The shape of the graph showed that the activity of amylase at constant pH increases as the incubation time increases, but above 30 minutes of incubation, the activity started declining. Also the highest activity (250.62ε mol/min) was at 30 minutes of incubation.

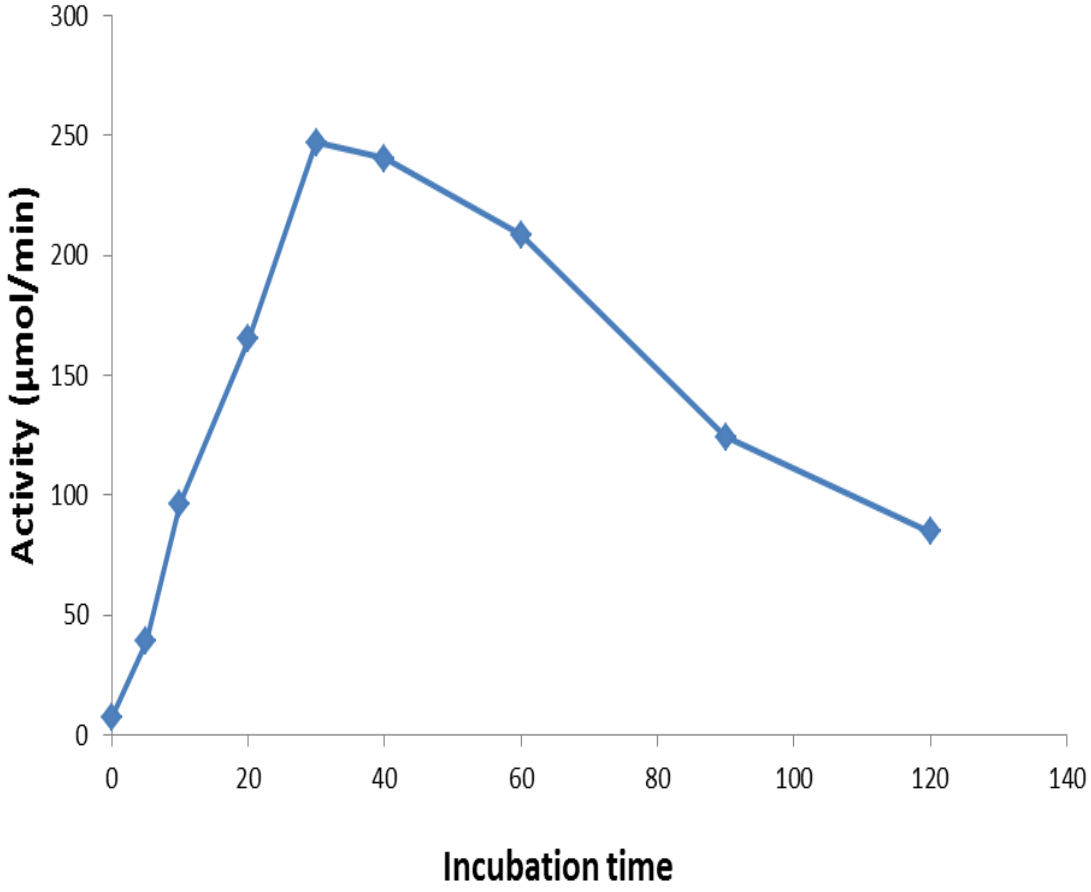


Figure 27: Effect of incubation time on Ŭ-amylase activity using cassava starch as substrate.

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Figure 25 shows the plot of Ŭ-amylase activity against incubation time of Ŭ-amylase at pH 5.5. Result obtained showed a steady increase of enzyme activity with increase in incubation time, but above 30 minutes of incubation, the activity started declining. However 30 minutes of incubation was found to have highest enzyme activity.

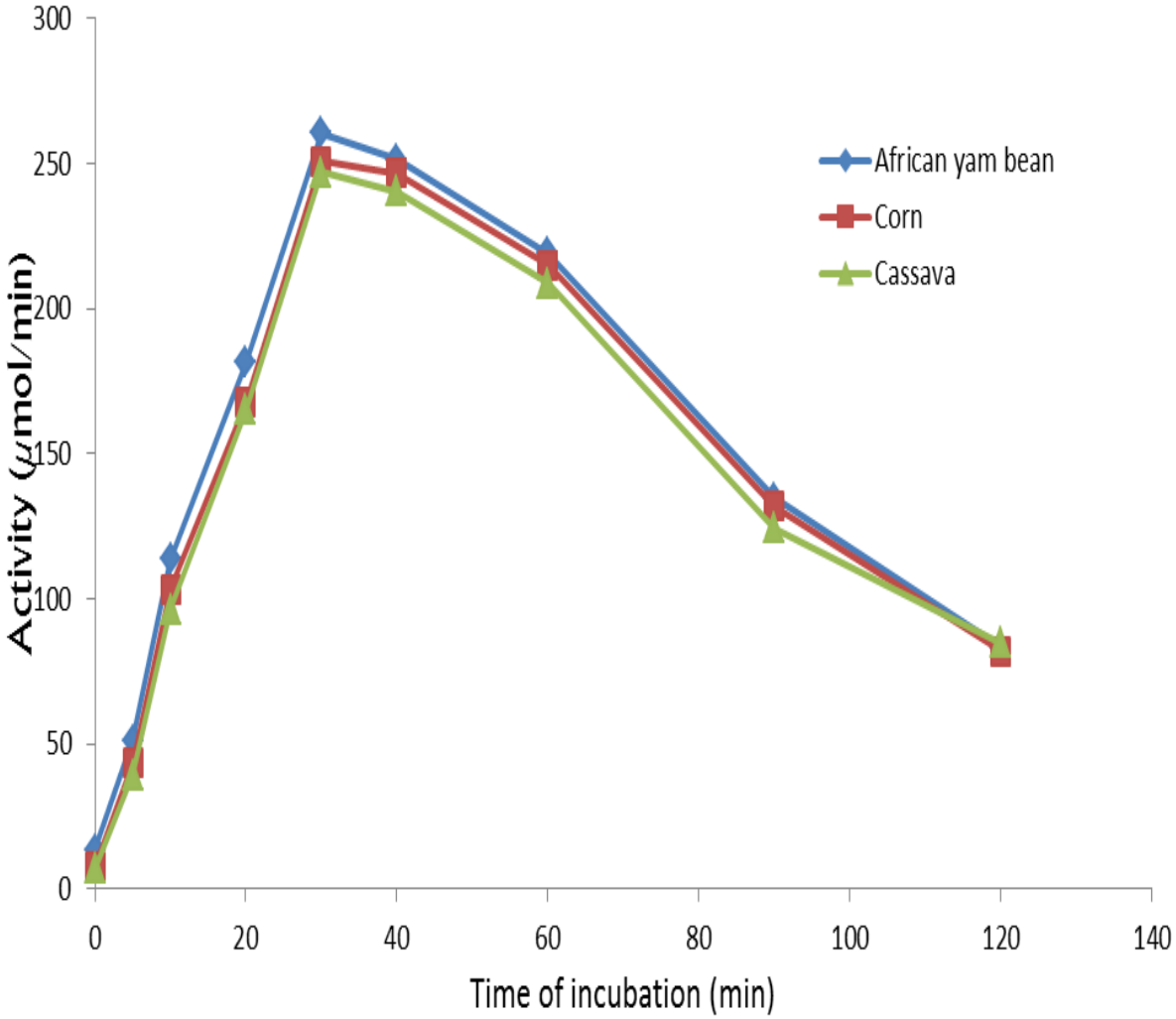

Figure 28: Summary of the effect of incubation time on the activity of Ŭ-amylase using starch from African yam bean, corn and cassava as substrate in 0.5m sodium acetate buffer pH 5.5

Assay of amylase activity was carried out at different incubation time (0, 5, 10, 20, 30, 40, 60, 90, and 120) as showed in figure 28. Each of the three substrates used (African yam bean seeds, corn and cassava starch) showed a steady increase in Ŭ-amylase activity as the incubation time increases up to 30 minutes after which it started declining. This result shows that the optimum incubation time for Ŭ-amylase is 30 minutes (figure 28).

3.7 Characterization of partially purified α-amylase

3.7.1 Effect of pH on Ŭ-amylase activity

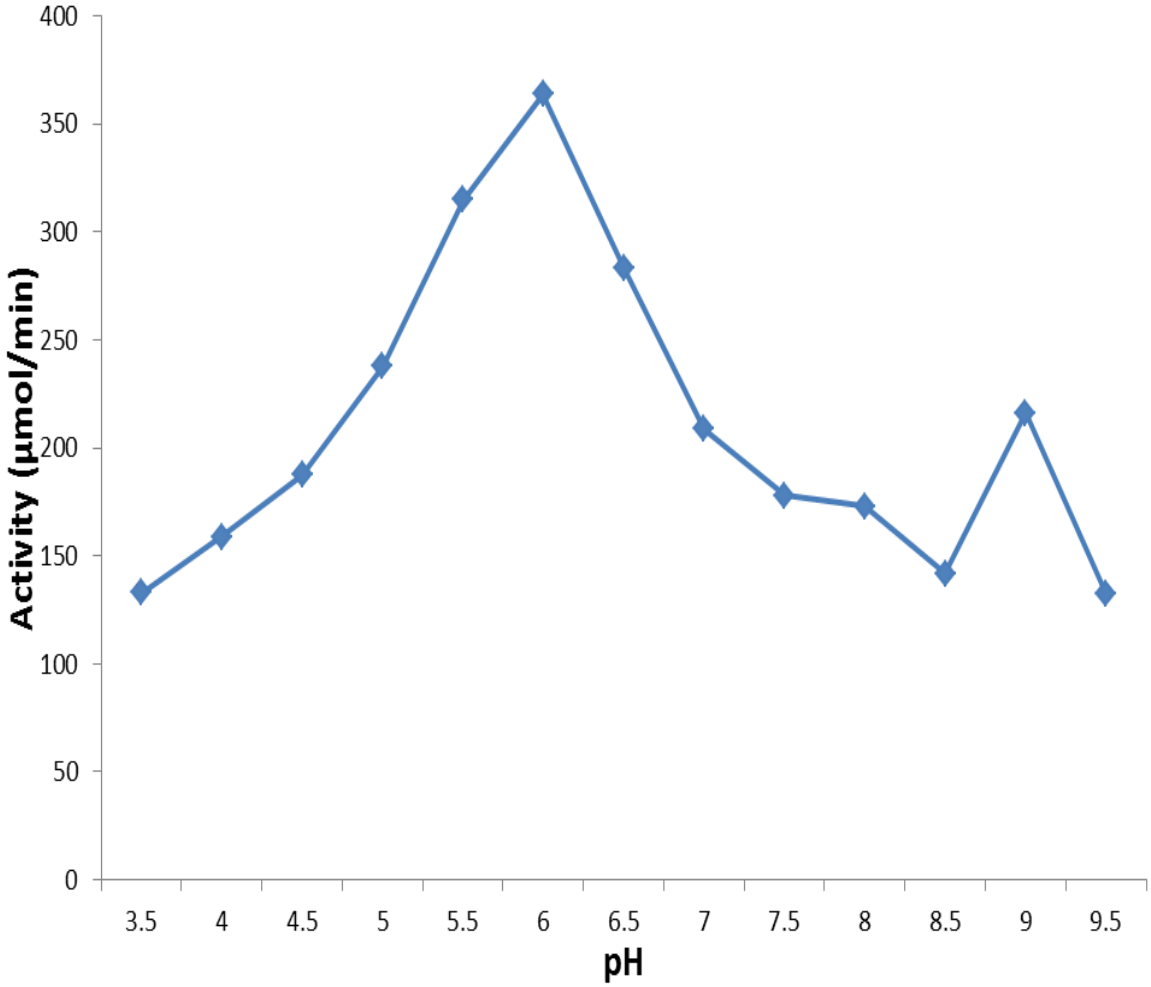

Figure 29: Effect of pH on Ŭ-amylase activity using starch from African yam bean seeds as substrate.

Effect of pH on Ŭ-amylase activity using starch from African yam bean seeds as substrate was assayed using 0.05M sodium acetate buffer (pH 3.5-5.5), sodium phosphate buffer (pH 6-7.5) and Tris-HCl buffer (pH 8.0-9.5) at interval of 5. The results (Figure 29) showed a steady increase in Ŭ-amylase activity from pH 3.5- 6.0 after which it decline up to pH 8.5. Also, the Ŭ-amylase activity slightly increases at pH 9.0 and decreases at pH 9.5. The result as demonstrated above (Figure 29) showed that, the Ŭ-amylase activity was highest at pH 6.0.

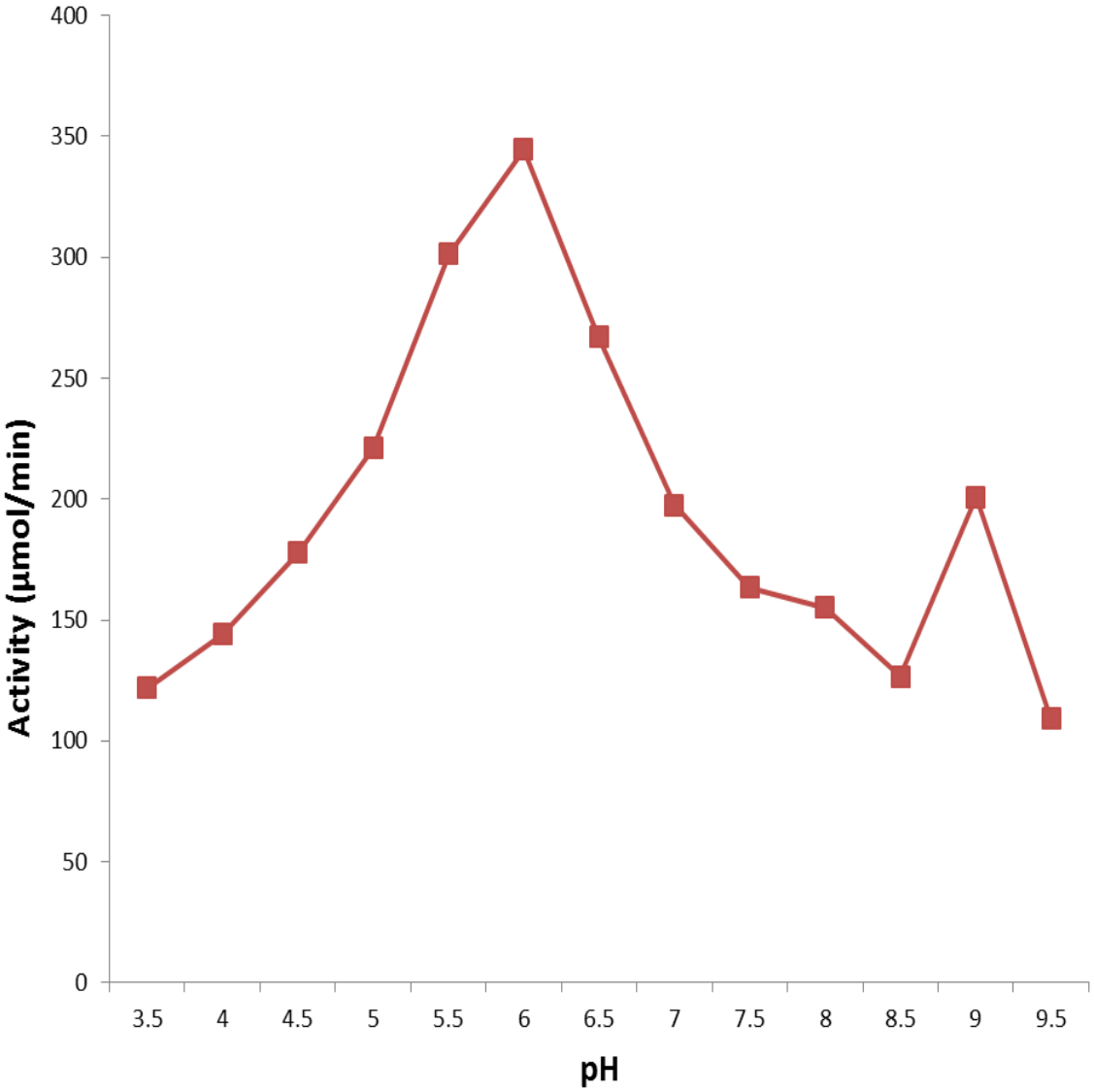

Figure 30: Effect of pH on Ŭ-amylase activity using corn starch as substrate.

Effect of pH on Ŭ-amylase activity using starch from corn as substrate was assayed using 0.05M sodium acetate buffer (pH 3.5-5.5), sodium phosphate buffer (pH 6-7.5) and Tris-HCl buffer (pH 8.0-9.5) at interval of 5. The results (Figure 30) showed a steady increase in Ŭ-amylase activity from pH 3.5- 6.0 after which it decline up to pH 8.5. Also, the Ŭ-amylase activity slightly increases at pH 9.0 and decreases at pH 9.5. The result as demonstrated above (Figure 30) showed that, the Ŭ-amylase activity was highest at pH 6.0

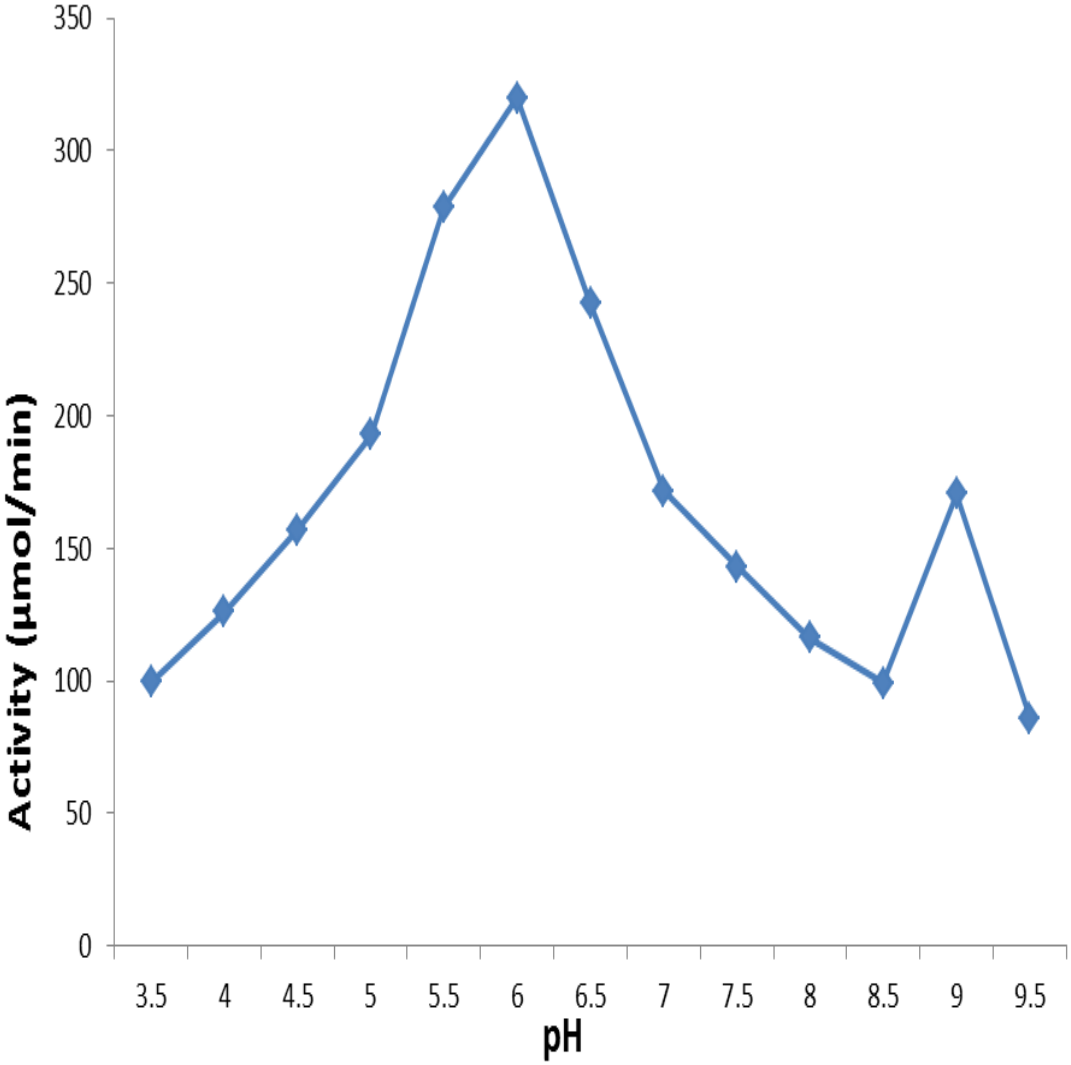

Figure 31: Effect of pH on Ŭ-amylase activity using cassava as substrate.

Effect of pH on Ŭ-amylase activity using starch from cassava as substrate was assayed using 0.05M sodium acetate buffer (pH 3.5-5.5), sodium phosphate buffer (pH 6-7.5) and Tris-HCl buffer (pH 8.0-9.5) at interval of 5. The results (Figure 31) showed a steady increase in Ŭ-amylase activity from pH 3.5- 6.0 after which it decline up to pH 8.5. Also, the Ŭ-amylase activity slightly increases at pH 9.0 and decreases at pH 9.5. The result as demonstrated above (Figure 31) showed that, the Ŭ-amylase activity was highest at pH 6.0

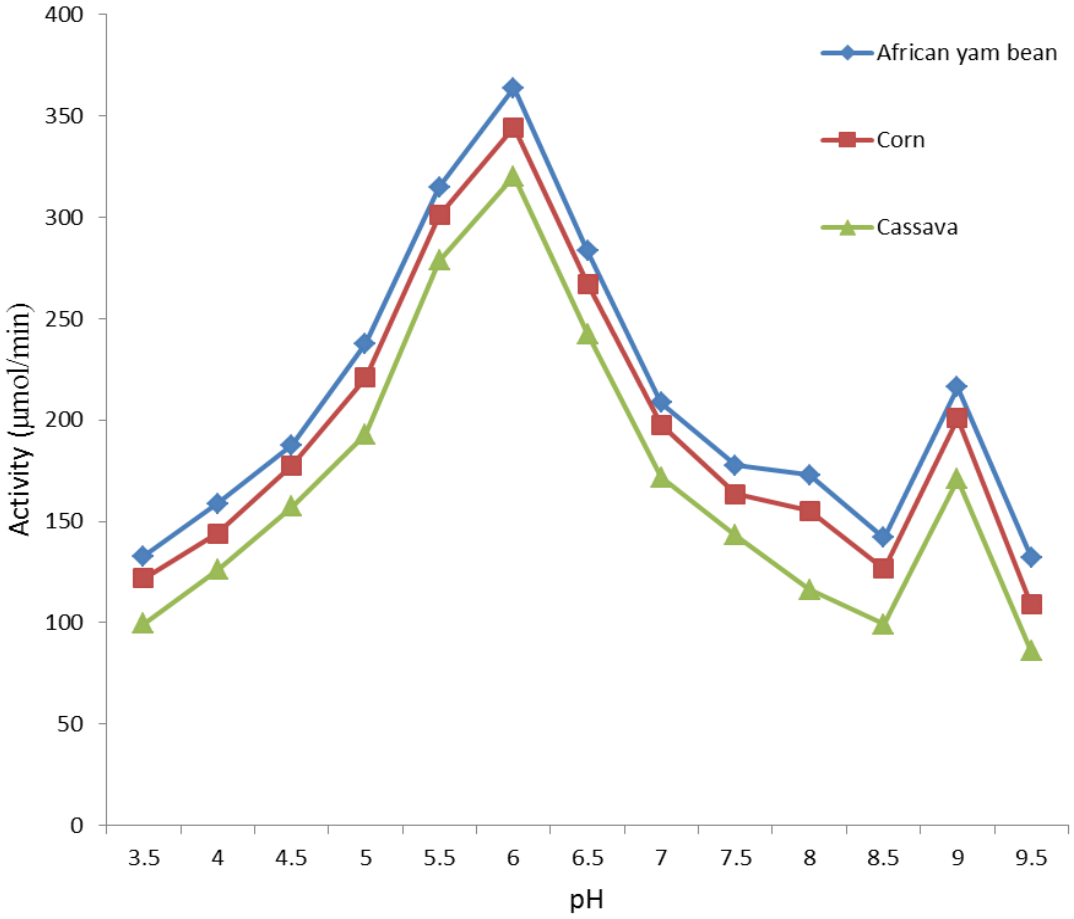

Figure 32: Compering the effect of pH on Ŭ-amylase activity using starch from African yam bean seeds, corn and cassava as substrate.

Effect of pH on Ŭ-amylase activity using starch from African yam bean seeds, corn and cassava as substrate was assayed using 0.05M sodium acetate buffer (pH 3.5-5.5), sodium phosphate buffer (pH 6-7.5) and Tris-HCl buffer (pH 8.0-9.5) at interval of 5. The results (Figure 32) showed a steady increase in Ŭ-amylase activity from pH 3.5- 6.0 after which it declined up to pH 8.5. Also, the Ŭ-amylase activity slightly increased at pH 9.0 and decreased at pH 9.5. The result as demonstrated above (Figure 32) showed that, the Ŭ-amylase activity was highest at pH 5.5 in each of the three substrates used (African yam bean seed, corn and cassava seed) with activities 363.79ε mol/min, 344.44ε mol/min and 320.16ε mol/min respectively.

3.7.2 Effect of temperature change on α-amylase activity

At pH 6.0, an increase in temperature was accompanied by an increase in Ŭ- amylase activity up to the optimum temperature of 45 ºC in each of the three substrates (African yam bean seeds, corn and cassava starch).

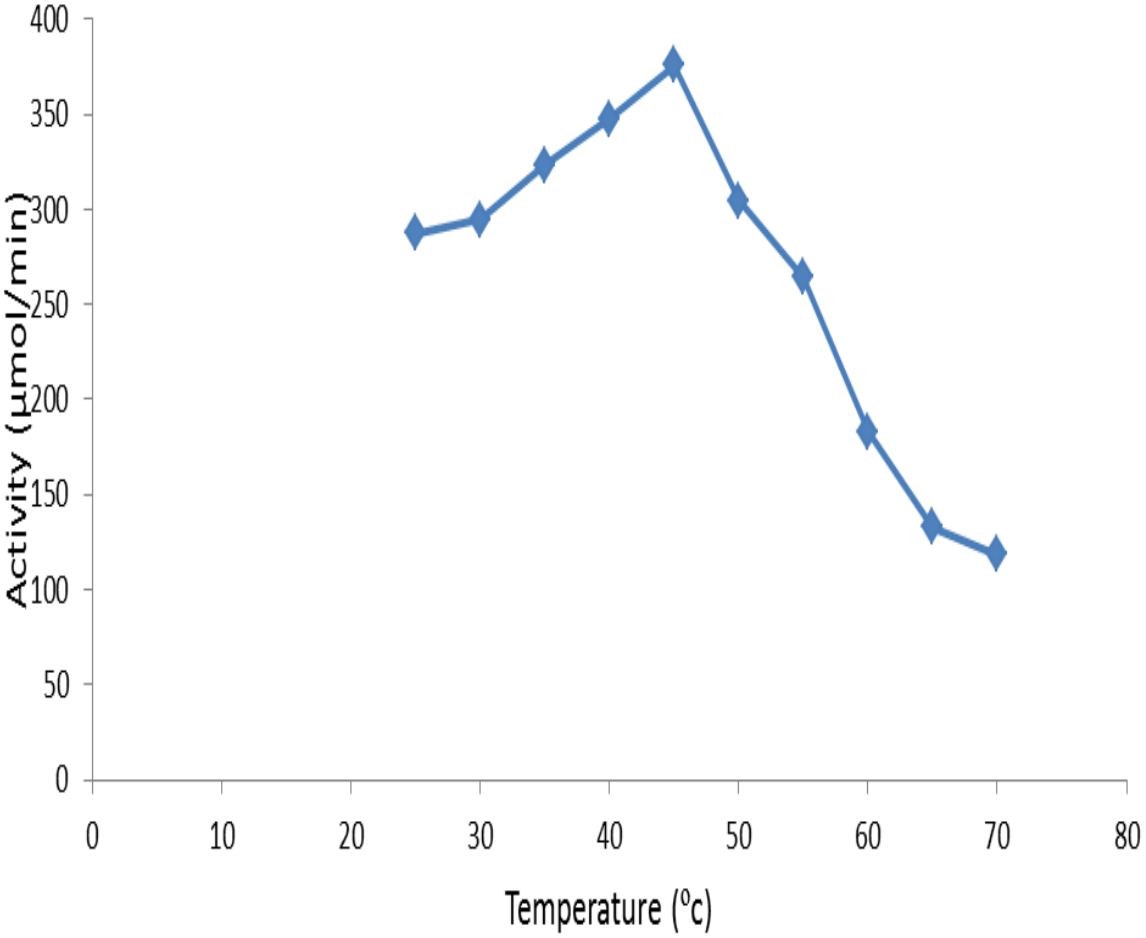

Figure 33: Effect of temperature change on Ŭ-amylase activity using starch from Africa yam bean seeds in 0.05M phosphate buffer pH 6.0

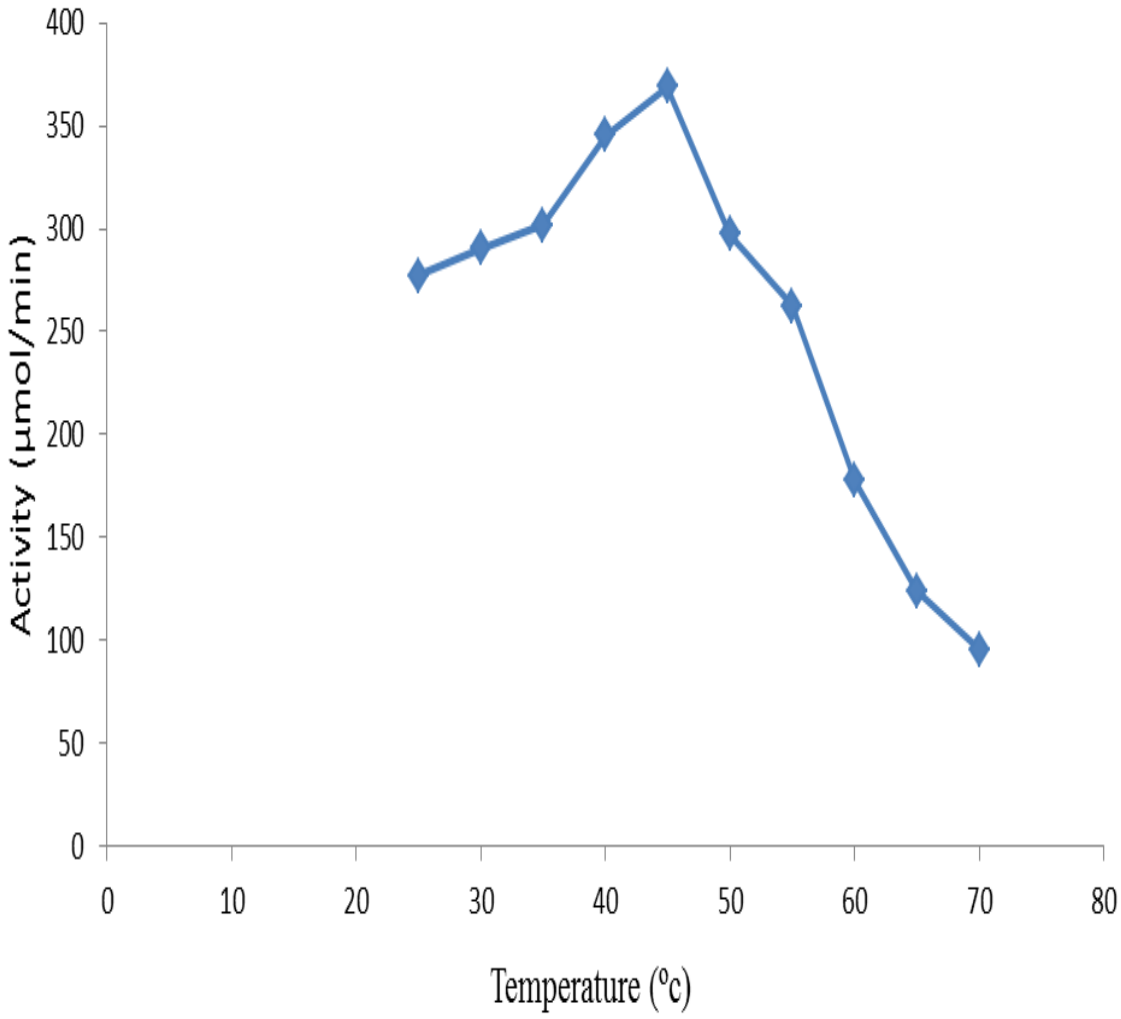

Figure 34: Effect of temperature change on Ŭ-amylase activity using corn starch as substrate in 0.05M phosphate buffer pH 6.0

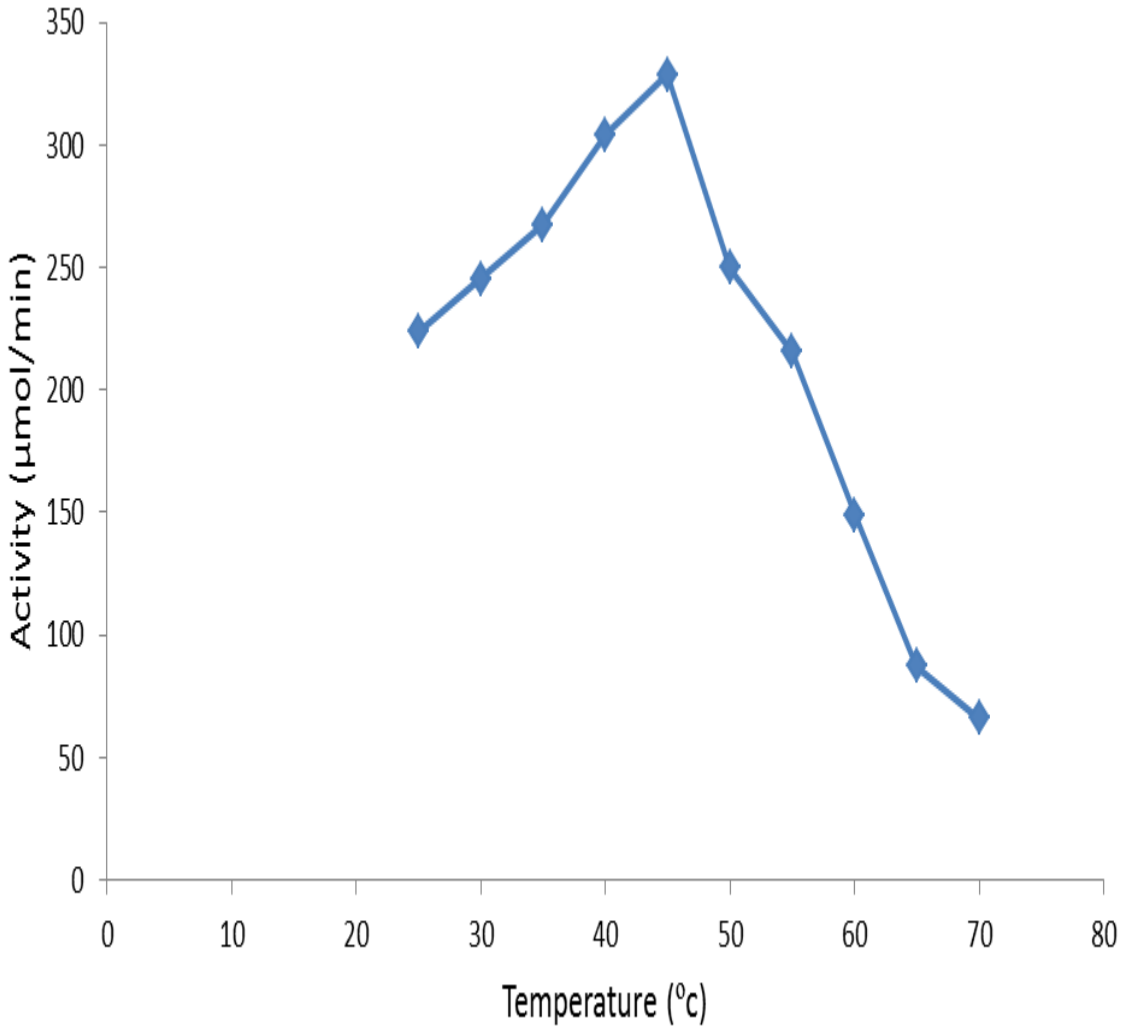

Figure 35: Effect of temperature change on Ŭ-amylase activity using cassava starch as substrate in 0.05M phosphate buffer pH 6.0.

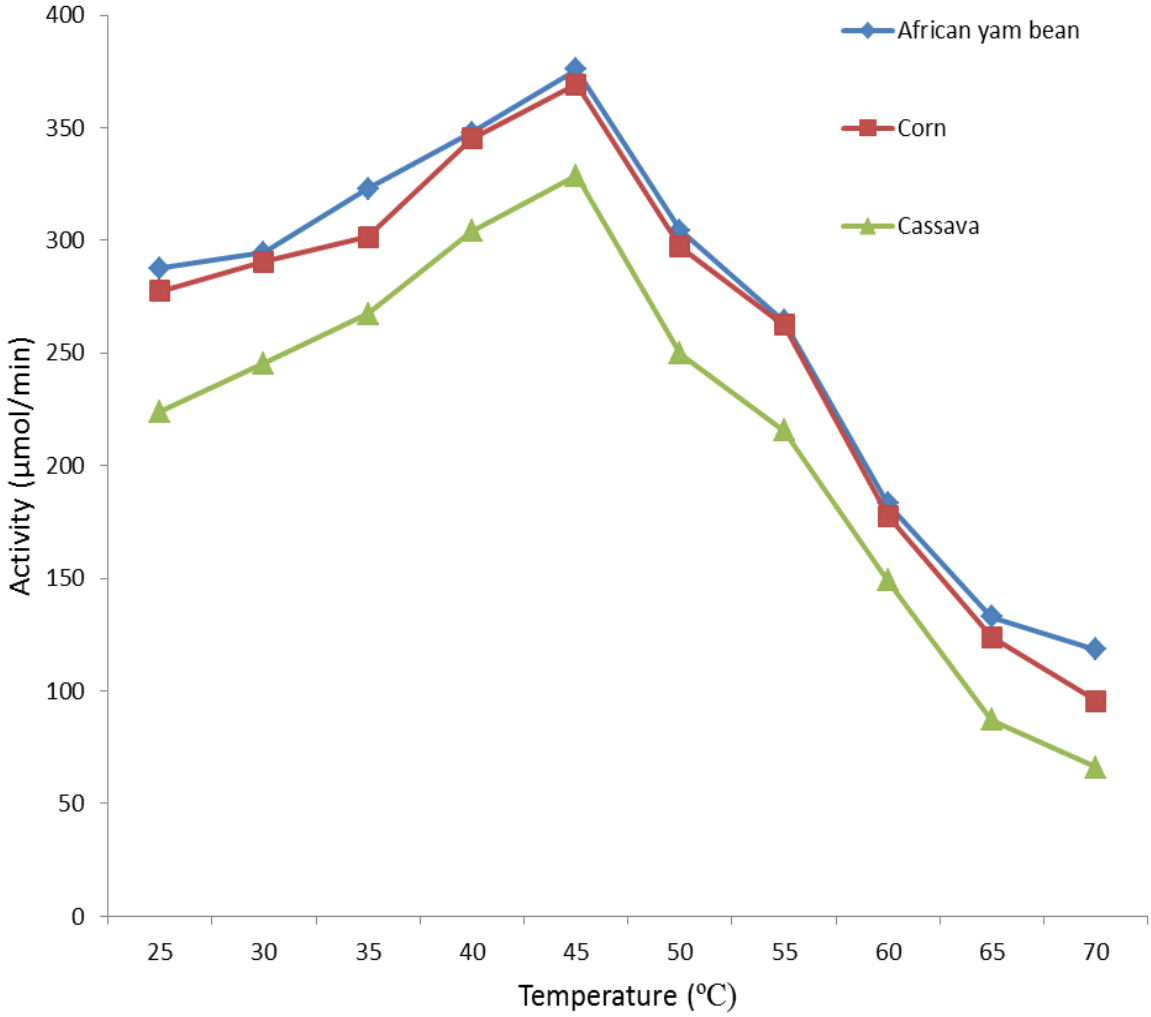

Figure 36: Comparing the effect of temperature change on Ŭ-amylase activity in Africa yam bean seed, corn and cassava starch as substrates in 0.05M phosphate buffer pH 6.0.

In each of the three substrates (Africa yam bean seeds, corn and cassava starch) used for the assay Ŭ-amylase activity was carried out at different temperatures (25 ï 70ºC) at 5ºC intervals. The result obtained showed a steady increase in Ŭ-amylase activity as the temperature increases up to 45ºC, after which there was decline in activity in the respective case.

3.7.3 Effect of substrate concentration on α-amylase activity

The effect of substrate concentration on Ŭ-amylase activity using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5%) in 0.05M phosphate buffer pH 6.0 at 45ºC were investigated in all the three substrates (Africa yam bean seeds, corn and cassava starch).

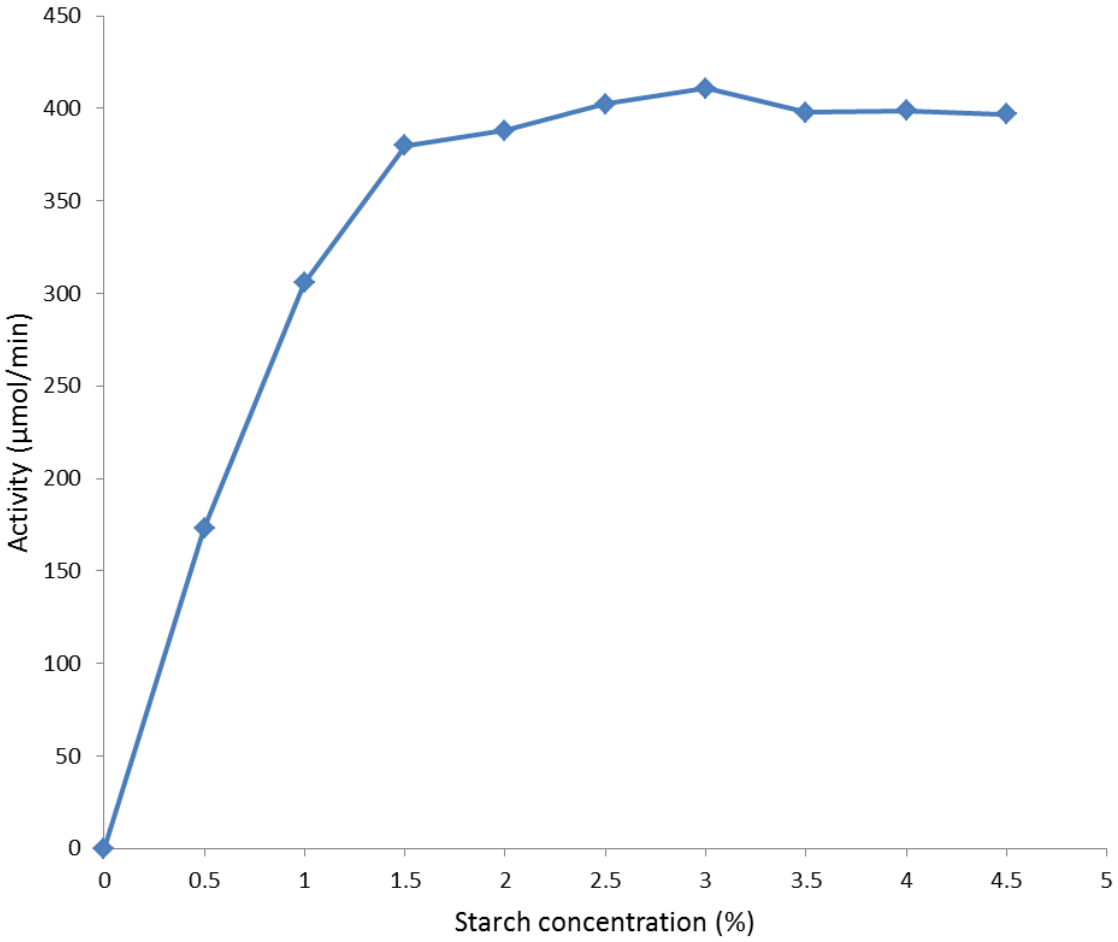

Figure 37: Michealis ï Menten plot of Ŭ-amylase activity from germinated Africa yam bean seeds using starch from Africa yam bean seeds as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45 ºC.

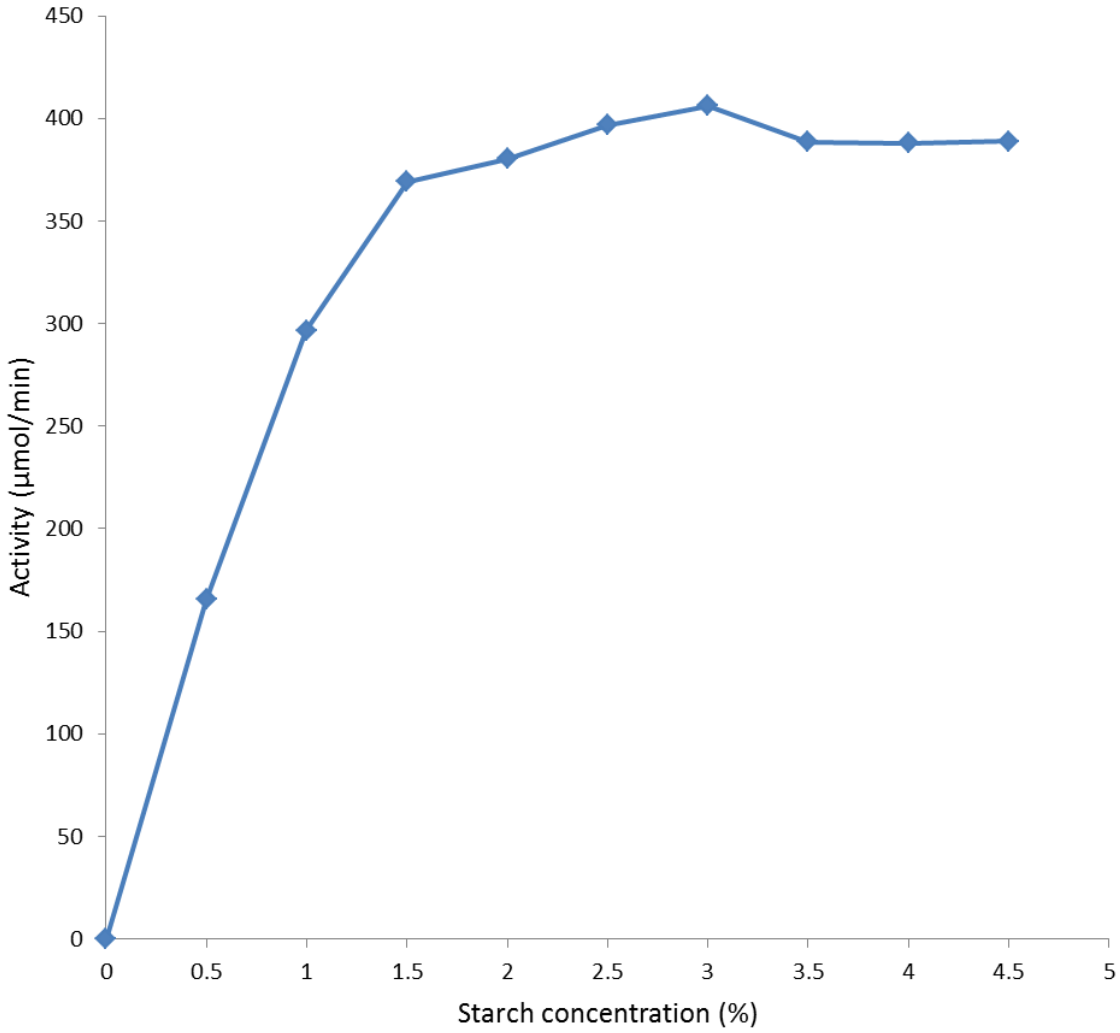

Figure 38: Michealis ï Menten plot of Ŭ-amylase activity from germinated Africa yam bean seeds using starch from corn as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45 ºC.

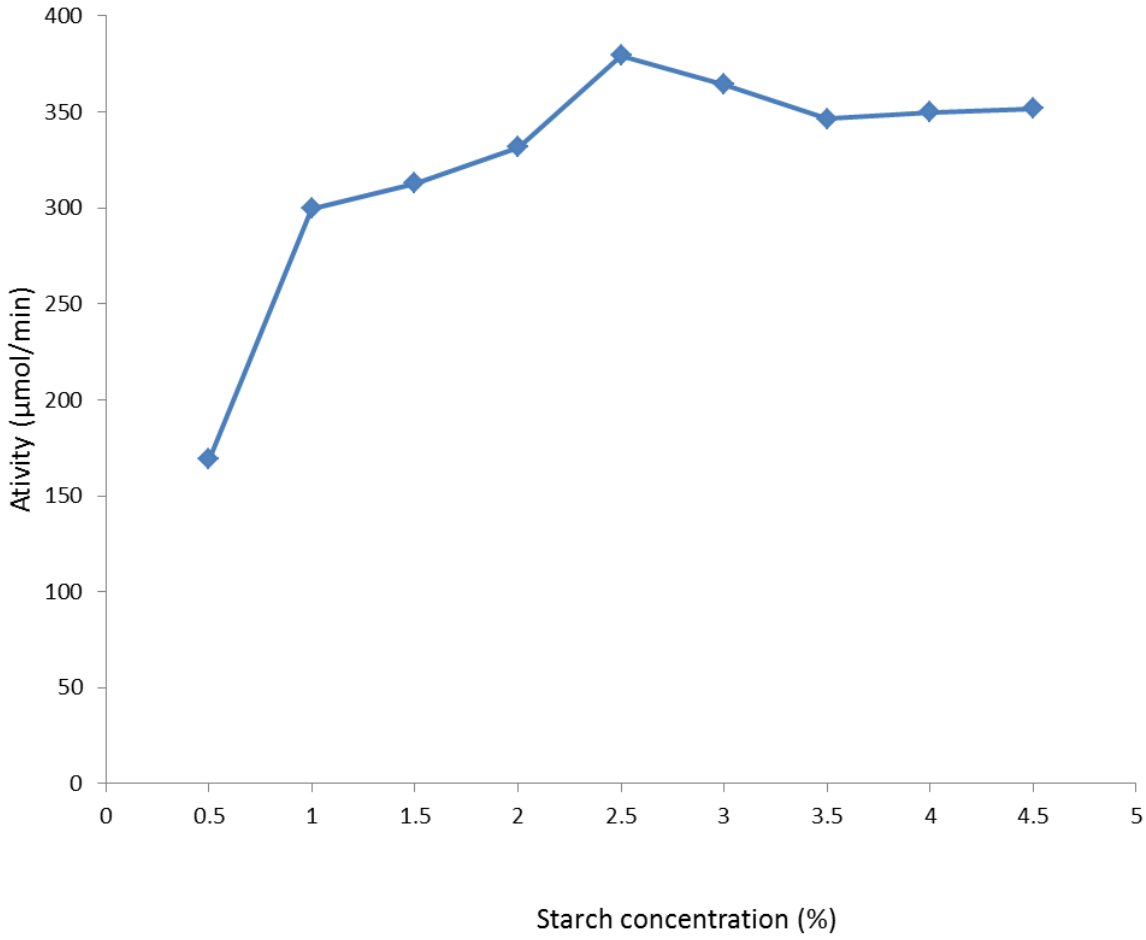

Figure 39: Michealis ï Menten plot of Ŭ-amylase activity from germinated Africa yam bean seeds using starch from cassava as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45 ºC.

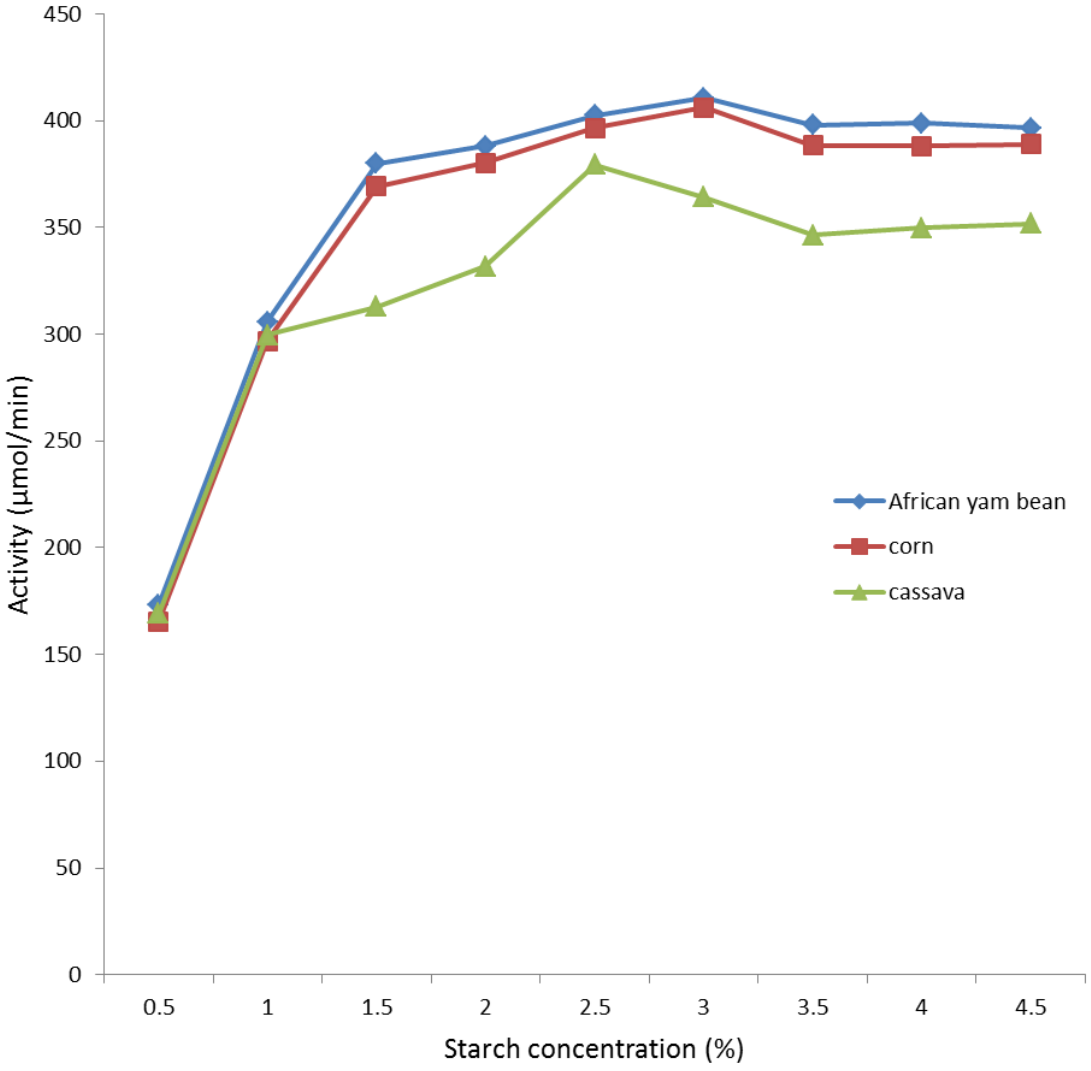

Figure 40: Effect of substrate concentration on Ŭ-amylase activity using starch from African yam bean seeds, corn and cassava as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45ºC.

Figure 40 shows the Michealis ï Menten plot of the initial velocity data using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5) from (a) Africa yam bean seeds (b) corn and (c) cassava in 0.05M sodium phosphate buffer pH 6.0 at 45 ºC. The plot showed a typical hyperbolic curve. The result obtained indicates that as the substrate concentration was increased above 0.5%, rate of reaction increased progressively up to 3% substrate concentration, thereafter, the rate of reaction decreases and progressively remained almost constant.

3.8 Determination of kinetic parameters

The results obtained from the effect of substrate concentration on Ŭ-amylase activity were used for the Lineweaver-Burk plot. Kinetic parameters (Vmax and Km) of the enzyme were calculated from the Lineweaver Burk plot (Figure 41, 42 and 43).

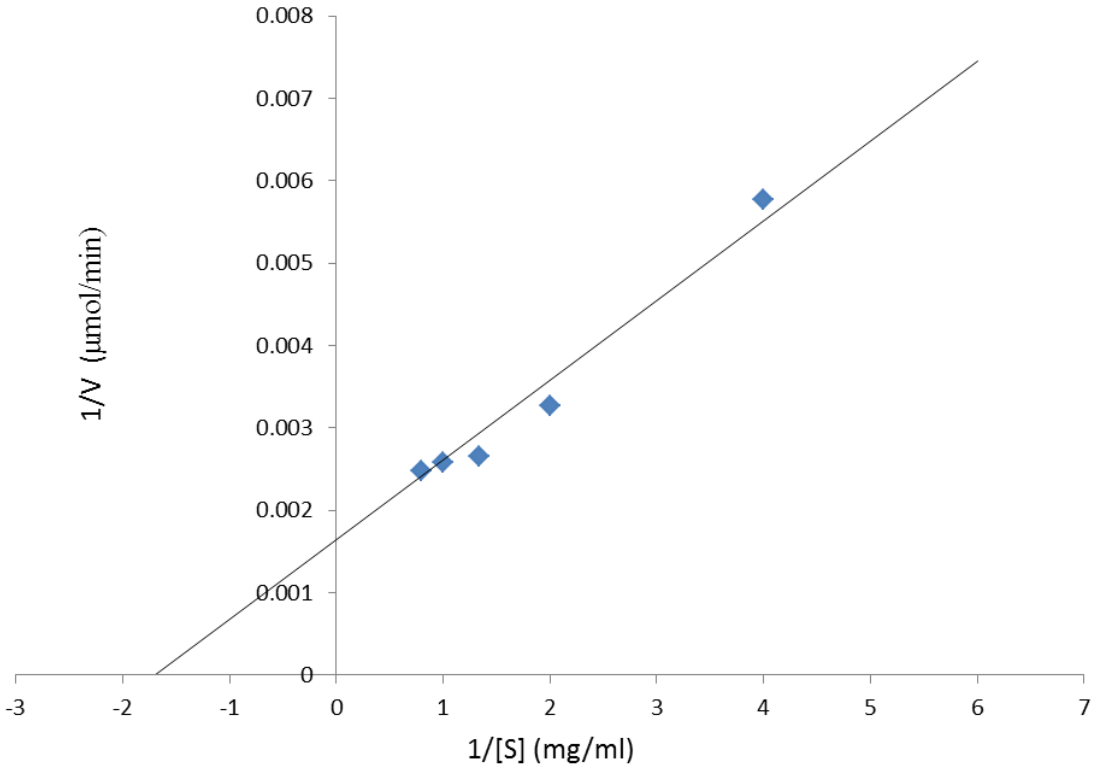

Figure 41: Lineweaver ï Burk plot of Ŭ-amylase from germinated Africa yam bean seeds using starch from Africa yam bean seeds as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45ºC.

The result (Figure 41) shows the lineweaver-Burk plot of the initial velocity data using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 %) from Africa yam bean seeds in 0.05M sodium phosphate buffer pH 6.0 at 45ºC. The Vmax and Km were observed to be 588.24ɛmol/min and 0.588mg/ml respectively

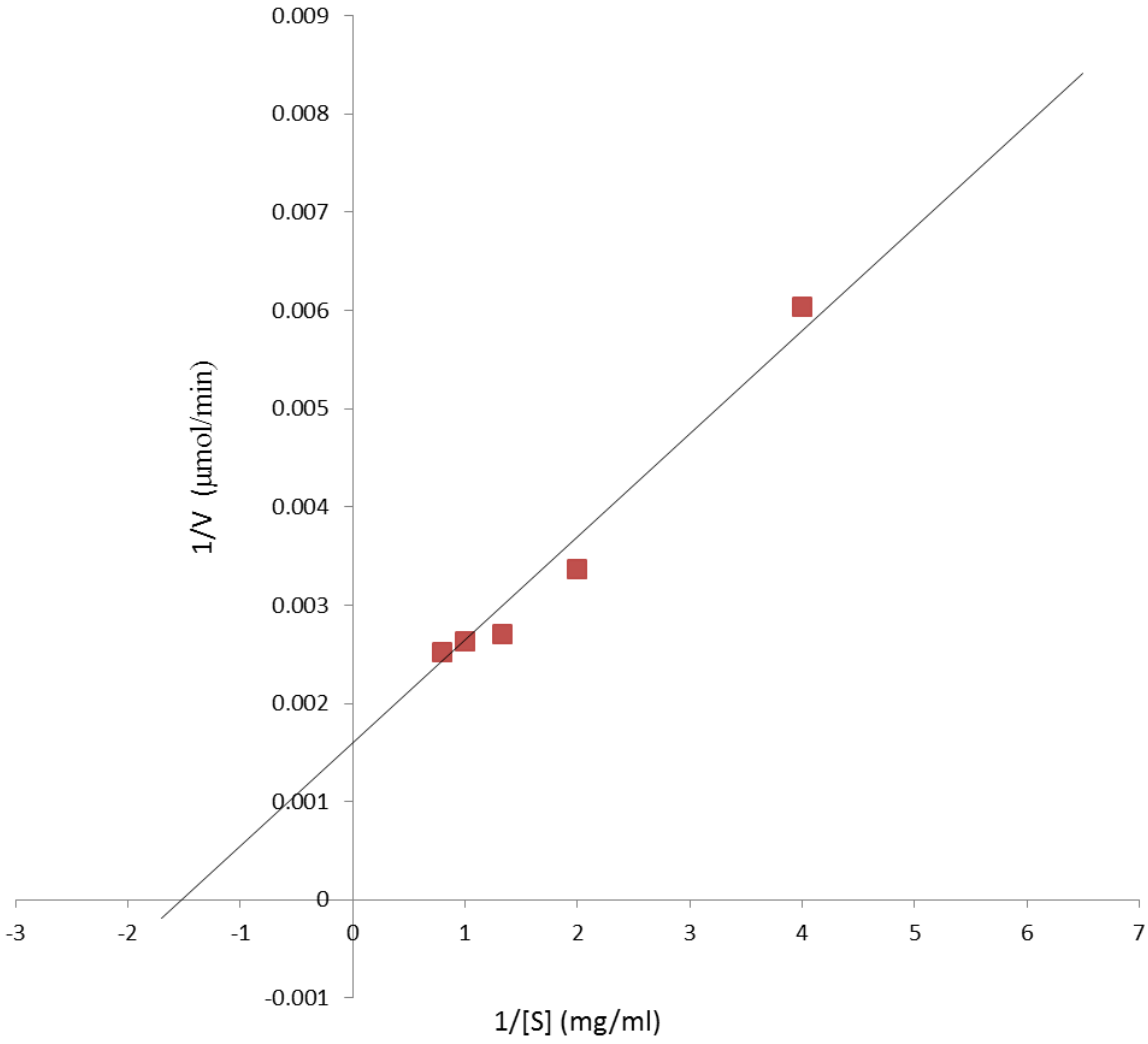

Figure 42: Lineweaver ï Burk plot of Ŭ-amylase from germinated Africa yam bean seeds using corn starch as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45ºC.

The result (Figure 42) shows the lineweaver-Burk plot of the initial velocity data using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 %) from corn in 0.05M sodium phosphate buffer pH 6.0 at 45ºC. The Vmax and Km were observed to be 625ε mol/min and 0.625 mg/ml respectively.

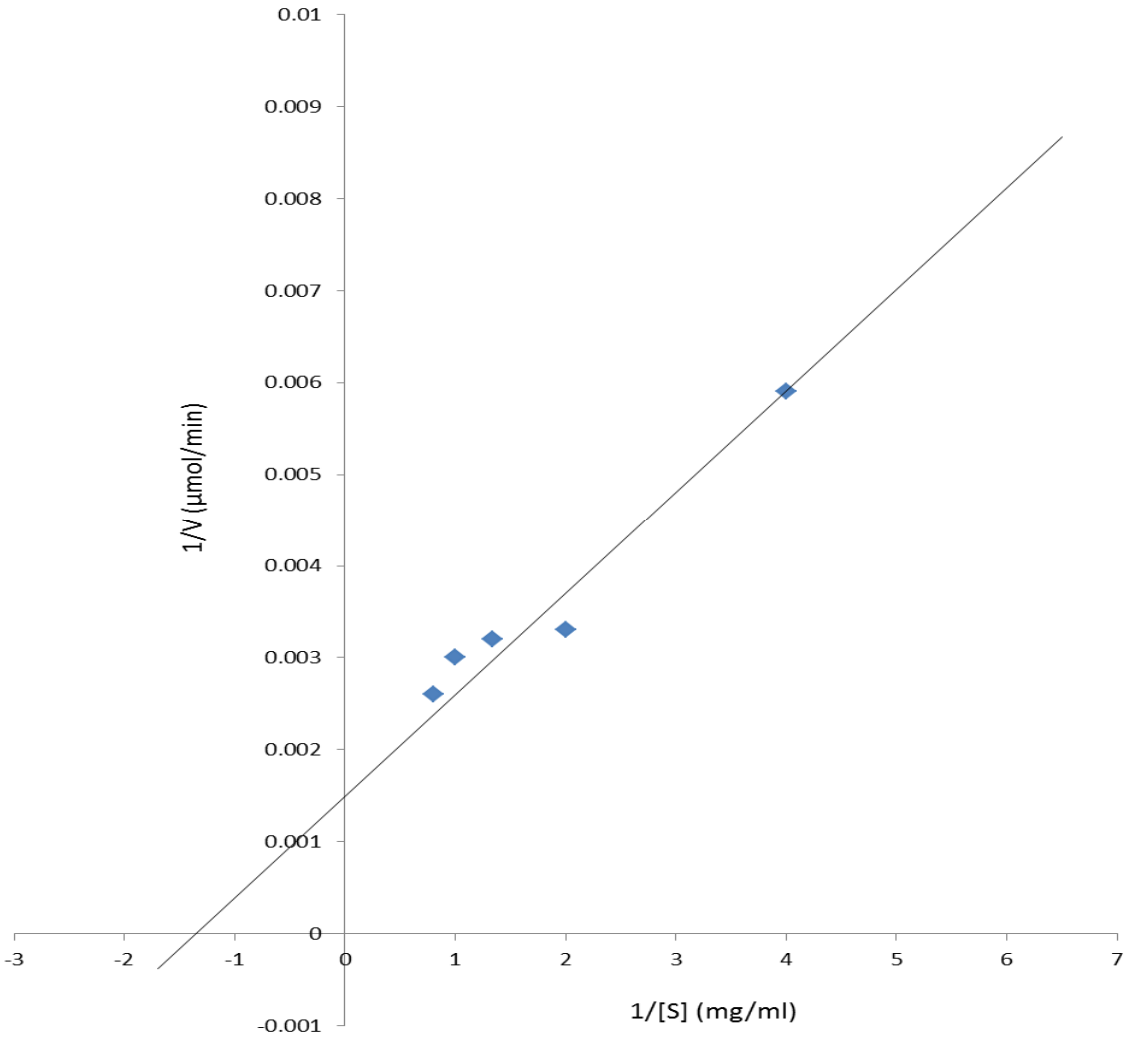

Figure 43: Lineweaver ï Burk plot of Ŭ-amylase from germinated Africa yam bean seeds using cassava starch as substrate in 0.05M sodium phosphate buffer pH 6.0 at 45ºC.

The result (Figure 44) shows the lineweaver-Burk plot of the initial velocity data using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 %) from cassava in 0.05M sodium phosphate buffer pH 6.0 at 45ºC. The Vmax and Km were observed to 666.7ε mol/min for Vmax and 0.733 mg/ml for Km

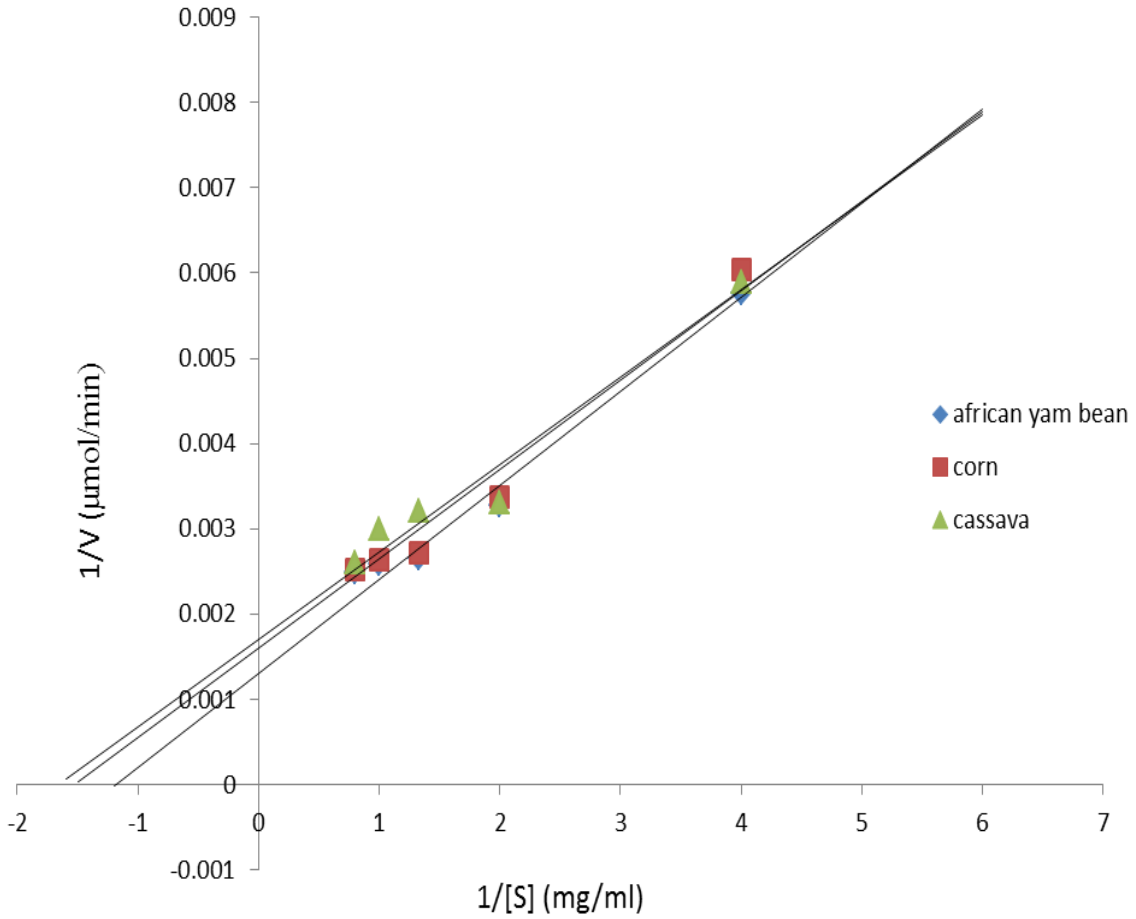

Figure 44: Lineweaver ï Burk plot of Ŭ-amylase from germinated Africa yam bean seeds using African yam bean seeds, corn and cassava starch as substrate.

The result (Figure 44) shows the lineweaver-Burk plot of the initial velocity data using different concentrations of starch (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 %) from (a) Africa yam bean seeds (b) corn and (c) cassava in 0.05M sodium phosphate buffer pH 6.0 at 45ºC. The Vmax and Km were observed to be 588.24ε mol/min and 0.588mg/ml respectively with starch from African yam bean seeds, 625ε mol/min and 0.625 mg/ml with starch from corn and 666.7ε mol/min for Vmax and 0.733 mg/ml for Km with starch from cassava.

3.9 Effect of some metal ions on the activity of α-amylase.

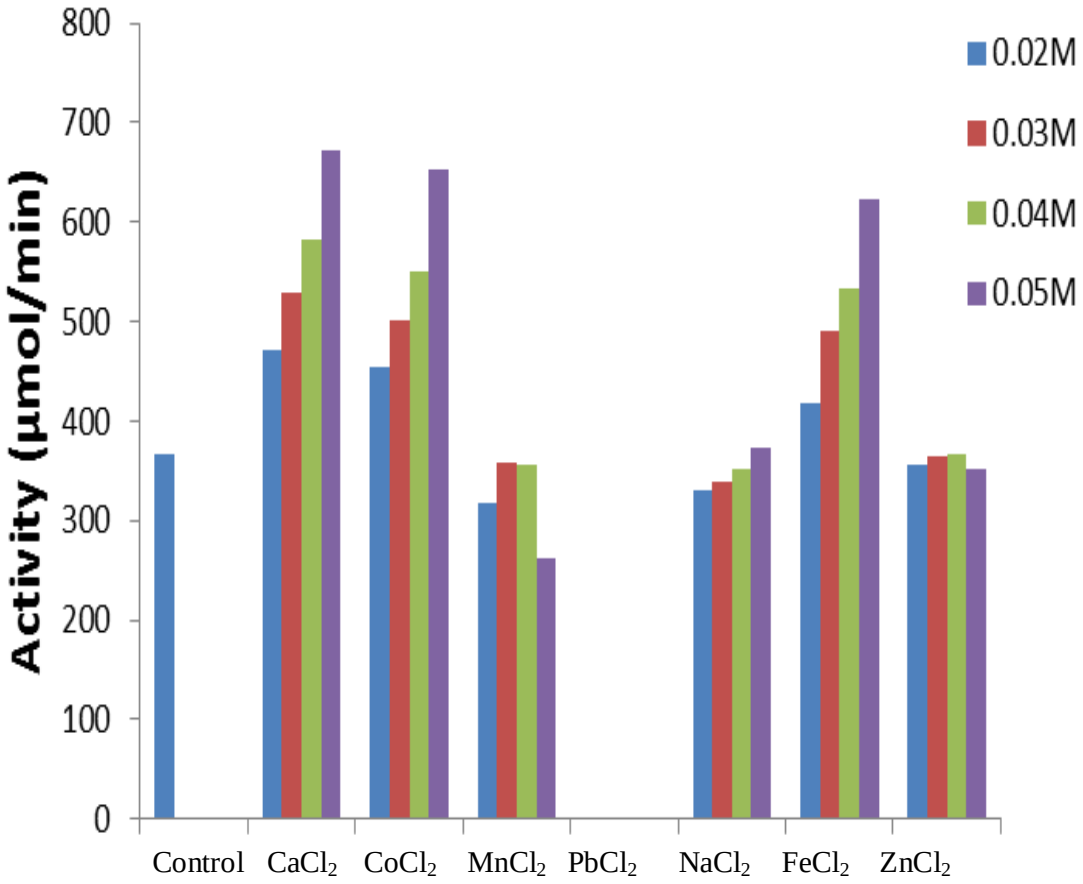

Figure 45: Effect of metal ion on the activity of Ŭ-amylase using starch from African yam bean seeds using phosphate buffer pH 6.0

Figure 45 shows the effect of different concentrations of metal ions (0.02M to 0.05M) when incubated for 30mins with the partially purified Ŭ-amylase extracted from germinated African yam bean seeds at 45ºC and pH 5.5 or 6.0 . There was progressive increase of Ŭ-amylase activity from 0.02M to 0.05M for calcium ion. The same trend was observed with Co^{2+} and Fe^{2+} while Pb^{2+} inhibited amylase activity even at 0.02M concentration. Zinc and sodium were observed as not having any effect on the enzyme activity.

CHAPTER FOUR
DISCUSSION

An adequate carbohydrate supply contributes to the survival of seeds under conditions of limited oxygen availability. The amount of soluble, readily fermentable carbohydrates in dry legume seeds is usually very limited, with starch representing the main storage macro molecule. Starch breakdown during the germination of seeds is as a result of the actions of hydrolytic enzymes. It is through the concerted action of Ŭ-amylase (EC 3.2.1 .l), ɓ-amylase (EC 3.2.1.2), debranching enzyme (EC 3.2.1.41 1), and Ŭ-glucosidase (EC 3.2.1.20) can starch be hydrolyzed completely. Ŭ-amylase and ɓ-amylase are the major amylolytic enzymes found during seed germination and it has been suggested that both enzymes are involved in the degradation of endospermic starch (MacGregor, 1983). However, during germination, the principal enzyme involved in carbohydrate breakdown is Ŭ-amylase which hydrolyses Ŭ (1-4)

bond in amylose and amylopectin releasing fragments that can be further broken down by ß-amylase, Ŭ-glucosidase and debranching enzymes.

Our pre-germination studies on the activity of Ŭ-amylase showed that, dry seeds of African yam bean seeds had no amylase activity when the gelatinized starch from African yam bean seeds, corn and cassava were used as substrates respectively. Lorenzo *et al* (1995) also reported absence of activity of Ŭ-amylase in dry seeds of rice, wheat, and barley. The absence of activity of Ŭ-amylase on the dry seeds of African yam bean seeds could be due to the fact that, dry seeds are characterized by a remarkably low rate of metabolism that is undoubtedly attributable to their low moisture content, but activation of amylase after 24hr of imbibition resulted in hydrolysis of starch from African yam bean seeds, corn and cassava (Figures 11, 12 and 13). Imbibition of seeds leads to mark change in metabolism resulting to activation of hydrolytic enzymes, which breaks down stored food resources into metabolically useful chemicals. Our studies also showed that the activity of Ŭ-amylase was considerably higher when gelatinized starch from African yam bean seeds was used as substrate than when corn and cassava starch were used.

The germination of African yam bean seeds started 3 days after imbibition. These results agree with the finding of Beckley and Joseph (2012), who reported emergence of high percentage seedling after 3 days of planting. There was a steady increase in activity of Ŭ-amylase from day zero through the 8th day after imbibition (where it exhibited its highest level of activity) in each of the three substrates used in this study (Figure 17). However, the Ŭ-amylase activity started decreasing from day 8 through day 12 after germination. Increase in Ŭ-amylase activity with corresponding increase in germination period is possible, because Ŭ-amylase is expressed and secreted out of the cells of the aleurone layer and scutella epithelium in the germinating seeds, to hydrolyze the endosperm starch to glucose as germination progresses (Higgin *et al*., 1982). Activities of Ŭ-amylase during germination have been reported by several authors. According to Okamoto and Akazawa (1979), Ŭ amylase is synthesized *de novo* and its level increased several hundred folds over a 4 or 5 day period of germination. In barley aleurone, it is approximately 30% of the protein synthesized during germination (Jones and Jacobsen, 1991). Ŭ amylase from germinated safflower seeds showed maximum activity after 5 days of growth (Elabri *et al.,* 2009), while Ŭ amylase from wheat local Verity (balady) showed maximum activity at 6th day of germination (Mohamed *et al*., 2009). Our confirmation test to determine the presence of Ŭ-amylase in germinating African yam bean seeds was based on change in the yellow colour of DNS to its orange-red derivatives, by the modified method of Miller (1959).

It was observed that the protein concentration increased as the germination time increased as shown in Figure 18. This could be attributed to the liberation of bound proteins during germination. During seed germination, protease enzyme increases and is involved in the degradation of peptide component to amino acids and this increases the amount of protein. Inyang and Zakari (2008) also noted that germination may increase the protein concentration. In cereals and legumes, this increase is due to the presence of protein hydrolysis as well as the results of protease enzyme activity during germination of the seeds. Hsu *et al.* (1980) observed that protein content of legumes generally increase during germination as a result of biochemical changes induced by sprouting leading to an increase in free amino acids. Nzelibe and Nwasike

(1995) also noted that protease activities during germination of ñachaò increases protein content. Similar observations on increase in protein concentration during germination have been cited by other authors (Akpapunam and Achinewhu, 1985; Giami, 1993; Obatolu *et al*., 2001).

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 (Gupta *et al*., 2003). Enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties. Several amylases have been purified from different plant source using conventional as well as classical methods. Ŭ amylase extracted from African yam bean seeds was purified by conventional steps of protein purification such as ammonium sulphate saturation and gel filtration. 80% ammonium sulphate saturation (Figure 19) gave highest Ŭ-amylase activity (622.84µmol/min.) with specific activity of 308.95U/mg protein when starch from African yam bean seeds was used as substrate. Maher *et al.,* (2011) also reported 80% ammonium sulphate saturation for Ŭ-amylase extracted from yemeni bean (*Dolichos lablab*) seeds. The precipitate occurs in that, protein in a buffer solution is highly hydrated because of their hydrophobic 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 density. This competition for hydration is usually more favorable towards the salt, which leads to interaction between the proteins, resulting in aggregation and finally salting out. (Markus and Aaron, 2007).

Figure 20 shows the elution profile of the partially purified amylase from germinating African yam bean seeds. From the elution profile during chromatography on sephadex G-25 (Figure 20), the fractions with highest Ŭ-amylase activity as well as protein concentration were pooled and assayed for enzyme activity. The two (Figure 20) peaks could possibly indicate that the Ŭ-amylase exists in two isoforms in African yam bean seeds. This assertion could be attributed to the fact that both Ŭ- and ɓ- amylase are present during germination of seeds, but the former becomes by far the predominant amylolytic enzyme. These results agreed with that of Eke and Oguntimehin, (1992) which showed two types of Ŭ-amylases from *Bacillus polymyxa* ɓ-amylase with only a slight difference in their isoelectric points.

The purified Ŭ-amylase was characterized based on the effects of optimum pH, optimum temperature, and optimum substrate concentration as well as incubation period on Ŭ-amylase activity.

The effect of incubation time (min) on purified Ŭ-amylase extracted from germinating African yam bean seeds was shown in Figure 28. The result showed that, at room temperature and pH 5.5, Ŭ-amylase exhibited highest activity at 30 minutes of incubation in all the substrates (African yam bean seeds, corn and cassava starch) used in this research. However beyond 30 minutes of incubation the activity started decreasing. This result is inconsistent with observation made by Kirti, (2012) that the optimum incubation period for bacteria Ŭ-amylase is 30 minutes while fungi showed 50 minutes optimum incubation time. This result is contrary to the earlier report by Kirti, (2012) that the optimum time of incubation for *Vigna mungo, Glycine max and Phaseolus vulgaris* amylases were 20, 25 and 15 minutes respectively (Kirti, 2012). The longer an enzyme is incubated with its substrate, the greater the amount of product that will be formed. All proteins suffer denaturation, and hence loss of catalytic activity with

time. Some enzymes, especially in partially purified preparations, may be noticeably unstable, losing a significant amount of activity over the period of incubation

The effect of pH change on the activity of alpha amylase using starch from African yam bean seeds, corn and cassava is shown in Figure 32. The hydrolytic action of Ŭ-amylase is greatly affected by pH. In this present study, Ŭ-amylase was found to have pH optima at 6.0 which is acidic in all the substrate used for this research; however the activity of the purified alpha amylase at pH optimal of 6 is higher in starch from African yam bean seeds than in corn with cassava having the least activity. The decrease in enzyme activity at higher pH (Figure 32) may be due to the amphoteric nature of protein molecules which containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. The increase in pH will affect the total net charge of the enzymes and the distribution of charge on their exterior surfaces, in addition to the reactivity of the catalytically active groups. This effect of pH on charge distribution on the ionizable groups interrupts the tertiary structure of the enzyme and thus courses its denaturation (Reena *et al..,* 2005; Eleonora *et al*.., 2009; Ezugwu *et al*., 2012). Acidic pH optima of a range 4.5 to 6.5 was reported for alpha amylase from wheat Sakha 69 (Fahmy *et al*., 2000) finger millet (Nirmala and Murlikrishna, 2000), shoots and cotyledons of pea (*Pisum sativam* L.) seedlings (Beers and Duke, 1990), mung beans(Tripathi, 2007) and wheat isoenzymes AI to AIV (Mohamed *et al.,* 2009). Khoo *et al*. (1994) also reported that the Ŭ-amylase enzyme was found to have maximum activity at pH 6.0.
ɓ-amylase in seed are in inactive form before germination, but are active during germination. The germinating embryo produces gibberellins which stimulate the aleruone layer to produce enzymes including Ŭ-amylase, ɓ-amylase and limit-dextrin during germination (Bewle and Black, 1985). Zeiglier (1999) reported that the optimum pH for plant ɓ-amylase is between 8.0 and 9.0 and its isoelectric point is between 5.0 and 6.0. Plant Ŭ-amylase has a pH optimum of 6.0 (Chotineeranat *et al*., 1998) while limit dextrinase has a pH optimum of 7.0 (Lewis and Young, 1998). This suggests that during amylase extraction, Ŭ-amylase, ɓ-amylase or limit dextrinase may be part of the extracted enzyme since they are present in an active form in the seed during germination. This also suggest that, the peak for pH 5.5 indicate the presence of Ŭ-amylase and the peak for 9.0 indicates the presence of ɓ-amylase and that accounts for the two peak in the pH profile as shown in Figures 8, 9,and 10. Lauriere *et al*., (1992), reported that during germination, both Ŭ-amylase and ɓ-amylase activities increases with increase in the endosperm but the former becomes by far the predominant amylolitic enzyme.

Enzyme activity is affected by variation in temperature. As temperature rises the rate of chemical reactions increases because temperature increases the rate of motion of molecules. This leads to more interactions between an enzyme and its substrate (Morgan 2000). The effect of temperature on the activity of the purified Ŭ-amylase extracted from African yam bean seeds using starch from African yam bean seeds, corn and cassava is shown in Figure 36. The production and stability of Ŭ-amylase depend on its temperature. In this study, the optimum incubation temperature for the partially purified Ŭ-amylase was found to be 45ºC. The partially purified Ŭ-amylase activity reached up to 375.72ε mol/min, 369.14ε mol/min and 328.60ε mol/min when starch from African yam bean seeds, corn and cassava were used as substrate, respectively. While the temperature below or above 45 ºC exhibited lower activities of Ŭ-amylase. This result agrees with the report by Chakraborty *et al,* (2009), which showed a similar temperature optimal of 45ºC for amylase from strain of marine Streptomyce specie D1. The optimum temperature for maximum purified Ŭ-amylase activity was 30ºC as reported by Strumeyer and Fisher (1982). The same finding was reported by El-Safey, (1994) who indicated that, the purified MM-Ŭ-amylase displayed maximal activity at 30ºC. On the other

hand, Abd El-Rahman (1990) and Lin et al., (1998), concluded that, the optimum temperature for purified Ŭ-amylase ranges from 30 to 85 ºC. Moreover, Khoo *et al*. (1994) reported that, the optimum temperature for purified Ŭ-amylase was 55ºC. Chakraborty *et al*. (2000) found that the maximum activity of a thermostable purified Ŭ-amylase was observed at 50ºC. While, Odibo and Ulbrich-Hofmann, (2001) concluded that the optimum temperature for the enzymes were 60 ºC for Ŭ-amylase and 70ºC for glucoamylase, respectively. 45 ºC optimum temperatures obtained from this research was an indication that the enzyme could be used at moderate temperature.

The Ŭ-amylase activity reached the maximum with an optimum substrate (starch) concentration of 3% with enzyme activity 410.70εmol/min, 406.17εmol/min, and 364.28εmol/min for African yam bean seeds, corn and cassava starch respectively. Any increase or decrease in substrate concentration gave a corresponding decrease in Ŭ-amylase activity (Figure 29). Kuiper *et al*. (1978) reported that the maximum activity of Ŭ-amylase enzyme was obtained at 1.67 % of substrate (starch) concentrations. In addition to that, Abd El-Rahman, (1990) concluded that, the optimal concentration of starch for maximum Ŭ-amylase activity was between 2ï 3%. Moreover, El-Safey, (1994) reported that, the optimal substrate (starch) concentration in reaction mixture of the MM-Ŭ-amylase enzyme was found to be 0.1 % (w/v) corresponding to 2% (w/v) for RH-Ŭ-amylase enzyme.

Double reciprocal plot from the effect of different starch concentration on amylase activity was shown in Figure 42. The maximum velocity, Vmax and Michealis constant, Km determined from Lineweaver-Burk plots of initial velocity data of different concentration of starch from African yam bean seeds as substrate were found to be 0.588 mg/ml and 588.24 εmol/min, respectively. Similarly, 0.625 mg/ml and 625 εmol/min were obtained using starch from corn, respectively, while 0.733mg/ml and 666.7εmol/min were also observed using starch from cassava, respectively. Km is a true characteristic of the enzyme under defined condition of temperature, pH and etc. it establishes a relationship between enzyme and its affinity with the substrate. The firmly an enzyme binds to its substrate, the smaller will be the value for Km. Therefore a small Km indicates that the enzyme require only a small amount of substrate to become saturated; hence, the maximum velocity is reach at relatively low substrate concentration while a large Km indicates the need for high substrate concentration to achieve reaction velocity. The implication of low Km to our research work is that partially purified amylase extracted from germinating African yam bean seeds has more affinity for starch from African yam bean seeds than from corn and cassava and can be applied in biotechnology. Also the Vmax or maximum velocity gives information on the turnover number of an enzyme (Anosike, 2001). The turnover number of an enzyme is the number of moles of substrate converted into product per active site of the enzyme per second, when then enzyme is fully saturated with substrate. This implies that Ŭ-amylase convert 0.588mg/ml, 0.625mg/ml and 0.733mg/ml into product per minute when starch from African yam bean seeds, corn and cassava were used as substrate respectively.

The effect of different concentrations (0.02M to 0.05M) of metallic chloride ($CaCl_2$, $CoCl_2$, $MnCl_2$, $FeCl_2$, $PbCl_2$, $NaCl_2$, and $ZnCl_2$) on the activity of Ŭ-amylase at 45ºC of pH 6.0 was studied because their presence or absence regulates enzyme activity. Our results as shown in figure 45 indicates that, calcium chloride had almost similar trend of effect on all the substrates (African yam bean seed, corn and cassava starch) used for the study with considerable increase in the activity of purified Ŭ-amylase. It is well known that plant and animal Ŭ-amylases are metalloenzymes that contain a Ca^{2+}-binding domain which is important for the stabilization of the tertiary structure (Vallee *et al*., 1959; Greenwood and MacGregor, 1964; Berbezy *et al*.,

1996). All plant Ŭ-amylases appear to contain loosely bound Ca^{2+} compared to microbial enzymes and its removal results in both irreversible as well as reversible inactivation resulting in the loss of thermal stability (Thoma *et al.,* 1971). Amylase activity of Ca^{2+} ions enhancement is based on its ability to interact with negatively charged amino acid residues such as aspartic and glutamic acids, which resulted in stabilization as well as maintenance of enzyme conformation. In addition, calcium is known to have a role in substrate binding (Sprinz, 1999). It has also been documented that binding of Ca^{2+} to amylase is preferred over other cations such as Mg^{2+} (Bush *et al*., 1989). A significant stimulation in amylase activity was observed with increasing concentration of $CoCl_2$ and $FeCl_2$ (from 0.02M to 0.05 M, Figure 45). $MnCl_2$ neither increased nor decreased enzyme activity in all three substrates used for this research. Dahot *et al*. (2001) found an increase in Ŭ-amylase activity with CO^{2+} and Mn^{2+} with *Moringa oleifera* seeds. Similarly, Dutta *et al*. (2006) obtained an enhanced Ŭ-amylase activity in *Heliodiaptomus viduus* with these ions (at 5 mM). In contrast, an inhibitory effect of CO^{2+} on Ŭ-amylase from *B. subtilis* JS-2004 was observed (Asgher *et al*., 2007). *Penicillium olsonii* Ŭ-amylase showed an enhanced enzyme activity with Mn^{2+} (1 mM) while no effects in enzyme activity was found with Co^{2+} (Afifi *et al*., 2008). In this present study, $PbCl_2$ leads to complete loss of enzyme activity while $NaCl_2$ and $ZnCl_2$ had no effect on the enzyme activity. The inactivation by metal ions may be due to their binding to either catalytic residues or by replacing the Ca^{2+} from the substrate binding site of the enzyme (Nirmala and Muralikrishna, 2003). It appears that the actions of metallic ions on Ŭ-amylase vary from one species to the other.

4.2 Conclusion

The use of Ŭ-amylase in starch based industries has been prevalent for many decades and a number of plant sources exist for the efficient production of this enzyme. Our result suggests that Ŭ-amylase can be extracted from African yam bean seeds (*Sphenostylis stenocarpa*). The ability of this enzyme to show high affinity (low Km) for native starches from corn, cassava and African yam bean seeds meets the prerequisites for industrial application. The study has demonstrated that the Ŭ-amylase purified from germinated African yam bean seeds was found to be suitable for industrial use with respect to pH and temperature optima, incubation time and resistance to inactivation by some metal ions. The economic feasibility of plant enzyme production applications depends on the cost of its production processes. In order to achieve maximum yield, it is pertinent to ascertain the best germination condition that will give higher activity on a particular substrate. Also, it is vital to note that the optimal parameters for Ŭ-amylase production from plant origin vary greatly with the variation of the environment and plant type.

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APPENDICES

Appendix 1: **Preparation of buffers**

The standard buffers used in this study were pH 4.0, pH 7.0 and pH 9.2. These buffers were used to standardize the pH meter. The working buffers were prepared as thus: 0.05M sodium acetate and 0.05M Tris-HCl buffers were prepared by dissolving 4.10g sodium acetate salt and 6.01g Tris base, respectively in 1000ml of distilled water and stirred with a magnetic stirrer till a homogenous solution was formed. The solutions were titrated against acetic acid and HCl, respectively till the required pHs were obtained. Also 0.05M phosphate buffer was prepared by dissolving 7.10g disodium hydrogen phosphate salt in 1000ml of distilled water and stirred and then tritrated against the solution of its conjugate acid (sodium dihydrogen phosphate) till the required pHs were obtained.

Preparation of dinitrosalicylic acid (DNS) reagent

A modification of DNS reagent method of Miller (1959) as contained in wang *et al.* (1997) was used in the assay. The reagent contains 44mM dinitrosalicylic acid, 4mM sodium sulphite, and 375mM sodium hydroxide.

Preparation of 50mM glucose

A known volume, 50mM solution of glucose was prepared by weighing 0.9g of industrial grade glucose and dissolving in 100ml of distilled water.

Calibration curve for glucose

A method described by miller 1959 with little modifications by Wang *et al*. (1997) was used. Ten test tubes were arranged in duplicate containing 0.0-1.0ml of 50mM glucose. Each tube was made up to 1ml using 0.05M sodium acetate buffer of pH 5.5. 1ml DNS reagent was added to each of the tubes and placed in boiling water bath for 10mins. The tubes were then removed and allowed to cool to room temperature. Na-K tartarate was added to the different tubes to stabilize the colour, after which the absorbance was read at 540nm. The concentration of reducing sugar in each of them was extrapolated using the formula ñ$C_1V_1=C_2V_2$ò where:

$C_{1=}$ initial concentration of reducing sugar (mM)

$V_{1=}$ initial volume of the 50mM preparation measured into the tubes

C2= final concentration of reducing sugar (mM)

V2= final volume of the preparation measured in the tube

Using the value obtained from the table described above, the plot of optical density against concentration was constructed.

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 IV - 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.

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

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

1.6 Preparation of sodium potassium tartarate

75g of sodium potassium tartarate was dissolved in 125mls of distilled water.

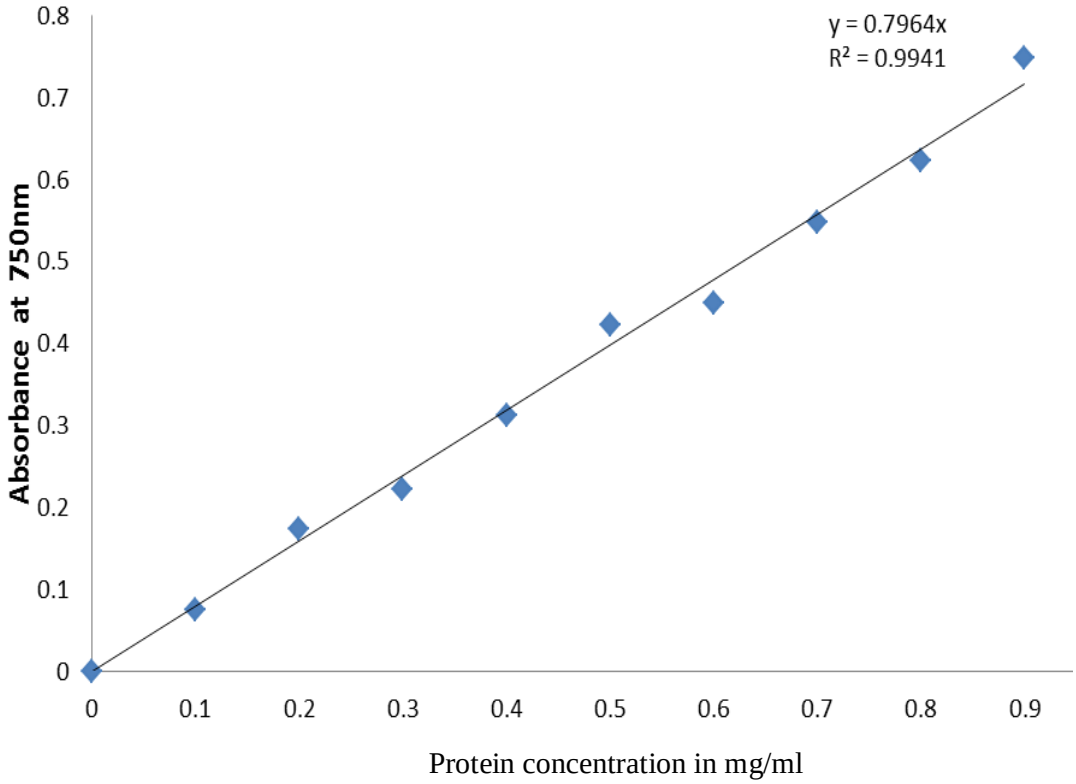

Figure 46: Protein Standard Curve using bovine serum albumen (BSA)

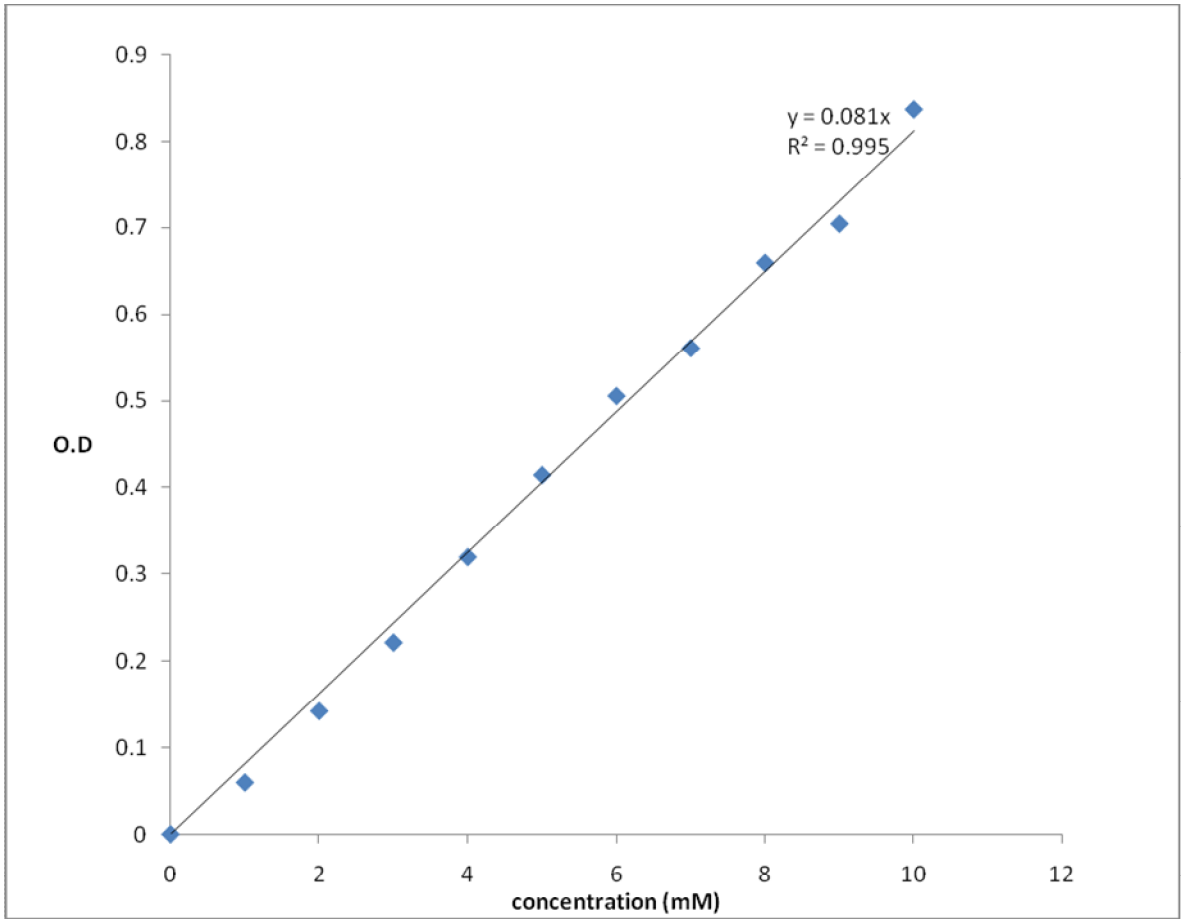

Figure 47: Glucose standard curve