OPTIMIZATION AND PURIFICATION OF ALPHA AMYLASE PRODUCED BY *ASPERGILLUS NIGER* USING YAM (DIOSCOREA SPP) PEEL AS SUBSTRATE IN SUBMERGED FERMENTATION

BY

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CERTIFICATION

This is to certify that this research was carried out by Ibukun Celestina FABIYI with Matric Number 16/27/MMI008 under my supervision and it is a fair reflection of the student's input.

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DECLARATION

I, Ibukun Celestina FABIYI hereby declare that this research titled optimization and purification of alpha amylase produced by *Aspergillus niger* using yam (Dioscorea spp) peel as substrate in submerged fermentation was researched and carried out by me under the supervision of Prof. O. Adedayo and Dr. A. O. Sulyman. All sources used were adequately referenced.

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16/27/MMI008

DEDICATION

This work is dedicated to Almighty God, the King of kings, my parents Late Mr Olufemi Fabiyi and Mrs Mojisola Fabiyi and the best siblings in the world; Adetokunbo Fabiyi, Omolola Fabiyi Ogunsuada, Oluwamayowa Fabiyi Agbabiaka and Oluwaseun Fabiyi.

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I also appreciate my friends for their love and care. May God bless you always, Amen. I love you all.

ABSTRACT

Amylases are important enzymes employed in starch processing industries for hydrolysis of polysaccharides into simple sugar constituents. Amylases have a wide range of applications in food, textiles, pharmaceuticals, bakery, detergent and paper industries. Despite the enormous usefulness of amylases in the industries, the high cost of amylase production in Nigeria is a big challenge. Consequently, there is a need to look for a cheaper and eco-friendly way to produce amylase. This research was designed to evaluate the utilization of yam peel as substrate in a submerged fermentation for the production of α-amylase using *Aspergillus niger*. Operational parameters optimized to improve α-amylase production by *Aspergillus niger* using yam peel in submerged fermentation included temperature, pH, inoculums size, incubation period and carbon source. The α-amylase was purified by subjecting it to ammonium sulphate precipitation, dialysis, and gel filtration chromatography. The optimal production of the enzyme was achieved in three days, at pH 5, temperature of 50^0C, inoculum size of 9×10^5 spores ml^{-1}, sucrose was the best carbon source. Therefore, it may be rational to employ local *Aspergillus niger* in the environment to ferment yam peel, the agro-waste littering our communities for the cost effective production of industrially valuable α-amylase.

Key word: α amylase, *Aspergillus niger,* yam peel, submerged fermentation.

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CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Agricultural wastes refer to wastes produced from agricultural operations, including waste from farms, poultry houses, and slaughter houses. They are also referred to as agro- wastes, they are the non-products outputs of production and processing of agricultural products that may contain material that can benefit man whose economic values are less than the cost of collection, transportation and processing for beneficial use. Agriculture and industrial wastes are among the causes of environmental pollution and converting these wastes into useful products may ameliorate the problems they cause. These untreated wastes create different problems with climate change by increasing a number of greenhouse gases (Bos and Hamelinck, 2014). Therefore, it is a worldwide concern to dictating the improvement of alternative cleaner and renewable bioenergy resources (Sadh *et al.,* 2018). The composition of wastes depends on the type of agricultural activities and the wastes can be in form of liquid, slurries or solids. These wastes can be taken up by microorganisms to produce other metabolites. Examples of agro wastes that are used as substrates for the production of enzymes includes rice bran, wheat bran, grape stalk, grape seeds, groundnut shell, banana peel, corn stalks, sugarcane bagasse, drops and null from fruits and vegetables, rice straws, cereal straws, cassava peel, potato peel, yam peel e.t.c enzymes such as cellulase, protease, lipase, amylase e.t.c can be produced on microorganisms when cultured on agro-waste.

Enzymes are biological catalysts which are an indispensible component of biological reactions. The use of chemical catalysts has been pursued for a very long time. The disadvantages that this method poses include need for high temperature and pressure for catalysis and the moderate specificity (Rajendra *et al*., 2016). These limitations were overcome by the use of enzymes. Enzymes are produced from animal and plant sources but

the microbial sources are generally the most suitable for commercial applications as a result of its bulk production capacity and easy manipulation for better product yield (Sivakumar *et al*., 2012; Shinde and Soni, 2014). Interestingly, the first commercially produced enzyme of a microbial source was an amylase of fungal origin in 1894 and, it used as a therapeutic aid to cure digestive disorder are a group of commercial enzymes that share nearly 25% of the global enzyme production (Mojsov, 2012; Oseni *et al*., 2014).

α-amylase enzymes account for about 30 % of the world's enzyme production (Akcan 2011; Malle *et al*., 2012; Deb *et al*., 2013). The world market for enzymes remains in excess of $4500 million (Sivakumar *et al*., 2012) and was about USD 8.18 billion in 2015 with an increase by 4% annually (Deb *et al*., 2013). The global alpha-amylase baking enzyme market is expected to reach USD 320.1 million by 2024, according to a new report by Grand View Research, Inc. (2016).

Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan *et al.*, 1999; Fogarty and Kelly, 1980; Nigam and Singh, 1995). Potential of using microorganism as biotechnological sources of industrially relevant enzymes has stimulated interest in exploration of extra cellular enzymatic activities in several microorganisms (Akpan *et al.*, 1999; Bilinski and Stewart, 1995; Buzzini and Martini, 2002).

These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria and molds (Abu *et al.*, 2005). Sources of amylases in yeast, bacteria and molds have been reported and their properties have been described (Akpan *et al.*, 1999; Buzzini and Martini, 2002). Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial scale; many attempts have been made to optimize culture conditions and suitable strains of fungi (Abu *et al.*, 2005).

Among microbial, plant and animal enzymes, microbial amylases have immense applications in various fields in world market because of their wide application in starch based industries especially food, textile, paper, detergent, pharmaceutical and baking industries (Anupama and Jayaraman 2011; Amutha and Priya 2011; Akcan 2011; Kaur *et al*., 2012).

Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefit. They are among the most important enzymes and are of great significance in many areas. There are various types of amylases, namely α, β, and glucoamylases. α-amylases (endo-1,4-α-Dglucan glucohydrolase, EC 3.2.1.1) are extra cellular enzymes that randomly cleave the 1,4-α-D-glycosidic bonds between adjacent glucose units in the linear amylase chain and are classified according to their action and properties. β-amylases (β-1,4-glucan maltohydrolase, EC 3.2.1.2) are usually of plant origin, but a few microbial strains are also known to produce them. It is an exoacting enzyme that cleaves nonreducing ends of amylose, amylopectin, and glycogen molecule.

Glucoamylase (amyloglucosidase, glucanogenic enzymes, starch glucogenase, and exo-1, 4-α-D-glucan glucanohydrolase (EC 3.1.2.3)) hydrolyses single glucose units from the nonreducing ends of amylose and amylopectin in stepwise manner. Amylases can be derived from various sources, such as plants, animals, and microorganisms, but enzymes of microbial origin have immensely exploited in many industries owing to their low production cost, higher yield, chemical and thermal stability, eco-friendly nature, flexibility, and gigantic accessibility (Mishra and Behera, 2008). Also, the possibility of increasing the levels of microbial enzyme synthesized by classical genetic techniques, continuous culture selection, induction, or optimization of growth conditions for the enzyme of interest (Konsoula *et al*., 2007).

Among microbial production of amylases, fungal amylases are superior over the bacterial amylases mainly because of their easy cultivation, low nutritional need, facilitated product recovery, higher yield, and stability (Prakash *et al*., 2009).

The fungal amylases are preferred over other microbial sources because of their more acceptable generally regarded as safe status (GRAS), the hyphal mode of growth, and good tolerance to low water activity (a_w), and high osmotic pressure conditions make fungi most efficient for bioconversion of solid substrates (Raimbault, 1998) and thus attracting increasing attention as source of amylolytic enzymes suitable for industrial applications (Mishra and Maheshwari, 1996).

Filamentous fungi have been well known for secreting starch degrading enzymes. The capability of filamentous fungi to produce large quantities of extracellular protein has made them appropriate for the industrial enzyme production. Different spp. of *Aspergillus* including *Aspergillus niger*, *Aspergilus tamarii*, *Aspergillus awamori* and *Aspergillus oryzae* have attain attention to obtain several types of hydrolytic enzymes like lipase, alpha amylase, amyloglucosidase and protease. However, *A. oryzae* is the organism of choice because of its ubiquitous nature, non-fastidious nutritional requirements and high productivity of alpha amylase (Abe *et al.*, 1988; Archer & Wood, 1995; Agger *et al*., 2001; Zangirolami *et al*., 2002; Shafique *et al*., 2009, Malik *et al.*, 2011). Few microorganisms have been reported to possess ability to produce raw starch degrading a-amylases (Da silva, 2009).

However, studies on fungal α-amylase, lipase, pectinase and proteases especially in developing countries are primarily focused on *Aspergillus* spp. and *Rhizopus* spp., probably due to their ubiquity and non- retentious nutritional requirements. Substrates like vegetable waste, rice husk, banana peels (Khan and Yadav, 2011), wheat bran (Negi and Banerjee, 2010), date wastes (Acourene and Ammouche, 2012), sugarcane baggase (Roses and Guerra,

2009), wheat straw, rye straw, corncob leaf, oil cakes, and many others (Bhargav *et al*., 2008).

Although most researchers have used liquid culture, which allows greater control of culture conditions such as temperature and pH, Cost of substrates on which enzyme-producing microbes can be cultivated has always been an important factor in production. Complex lignocelluloytic residues are generally considered the best substrates for the SSF processes (Singh *et al*., 2009). The nature of the moistening agent and the ratio of substrate to the moistening agent are crucial factors in enzyme production (Babu and Satyanarayana, 1995). With the advent of new frontiers in biotechnology, the spectrum of amylase application has widened in many other fields, such as clinical, medicinal (Giri *et al.*, 1990), and analytical chemistry as well as their widespread application in starch saccharification, food and brewing industries, baking, and preparation of digestive aids.

Increasing utility of alpha amylase in several industries pose a greater pressure on increasing enzyme production on local scale and exploration of new rapid processes (Carlsen *et al*., 1996; Ramachandran *et al*., 2004; Kathireasan and Manivanan, 2006; Gupta *et al*., 2008).

The industrial production of enzymes for biotechnological exploitation, isolation and its characterization, searching of novel efficient strains is an assiduous exercise (Kumar *et al*., 2002). To reiterate, the enzyme biosynthesis by filamentous fungi is reported to be influenced by numerous factors, such as pH, temperature (Ferreira Costa and Peralta, 1999), carbon and nitrogen sources (George *et al*., 1999). The activity of enzyme mostly relies on the availability of huge surface area and requires intense mild conditions.

Microorganisms usually produce commercially important metabolites in very low concentrations by their inherent control system. Although the yield may be increased by optimizing the cultural conditions, ultimately the productivity is controlled by the organism's genome (Stanbury *et al*., 1995). In the last few decades, the exponential increase in the

application of amylases in various fields has placed stress and demand in both qualitative improvement and quantitative enhancement through strain improvement. Such improved strains reduce the cost of the process

Keeping in view the importance of cheap and efficient production of amylases for industrial processes and the fact that the potential of amylases in textile industry needs to be extensively explored, the present study was carried out to screen out indigenously isolated fungal strains for amylase production for possible application in textile industry.

1.2. Statement of Research Problem

Nowadays the use of enzyme in industrial sector is increasing due to the increase of industries, especially in food, beverages, textile, leather and paper industries. One of the enzymes widely used in industrial sectors is α-amylase. Most of the enzymes used in the industrial sector in Nigeria including food industries are still imported and economically, this is not favorable to the nation. Nigeria is rich in natural resources especially the microbial which can be used as enzymes producer for example α-amylase enzyme.

Global enzymes market is expected to rise to USD millions by 2020. Due to the potential and bright future prospects of these microbial enzymes in biotechnological applications, there is a need to discover more fungal sources of α-amylase that will produce α-amylase with better properties e.g. thermostability that will be of greater use to the industries.

Also, most industrial microorganisms are patented and may not be available (or must be purchased) for use outside their country of origin. This is of serious economic concern as it does not allow for rapid expansion of fermentation industries, hence the need to source for indigenous and suitable microorganisms from local substrates for sustainable Amylase production. After the feedstock and suitable microorganisms have been identified, it is also necessary to increase the product yield and rate of production of biomass hydrolysates to minimize production costs.

Also, strain improvement techniques remain the most valuable process for enhancement of enzymes productivity and profitability besides minimizing cost and time of production

1.3. Justification

a) In the Africa region, there is a huge potential for the production of starch based products for food and nonfood applications. Cassava, sweet potato, yam, and other root crops widely grown in the region can be used as starting material for the production of starch hydrolysates using amylase enzymes. For example, starch from cassava can be converted to glucose and fructose syrups, maltose syrups, maltodextrins, e.t.c. with huge potential application in the food and non-food industries.

b) Very little attention has been given to amylase production from fungi and as a result, the potential of such fungi to serve as source of amylase has remained largely neglected. Undoubtedly, the search for potent amylase producers from among the fungi would be of practical value in alleviating the shortage of amylase supply in our cottage industries and thereby boosting the Nigerian enzyme-based industrial biotechnology.

c) Due to the increase in the demand for these enzymes in various industries, there is therefore the need to discover more strains of fungi that can produce α-amylase with better properties in terms of thermo-stability, mass production of the enzyme and consistency. The α-amylase will be produced from a fungal isolate of *Aspergillus* spp cultivated on yam wastes as substrates under submerged fermentation (SmF) conditions. Further, the crude enzyme will be purified and characterized. This novel approach may facilitate in developing an eco-friendly, industrially suitable protocol for ease of extraction, purification, and characterization of low-cost high-valued

biotechnologically relevant amylase for numerous industrial applications to produce potent amylases of various industrial and non-industrial applications.

1.4. AIM

- To optimize and purify α-amylase produced by *Aspergillus niger* cultured on yam peel.

1.5. SPECIFIC OBJECTIVES

Specific objectives are to:

i. Carry out the proximate analysis of yam peel.

ii. Optimize α-amylase production by varying fermentation parameters, and

iii. Purify α-amylase from yam peel culture by precipitation, dialysis and chromatography**.**

CHAPTER TWO

LITERATURE REVIEW

2.1. YAM (DIOSCOREA SPP)

Yam is the common name for some plant species in the genus *Dioscorea* (family Dioscoreaceae) that form edible tubers. The tubers themselves are also called "yams", having numerous cultivars and related species.

Yam is herbaceous annual or perennials with trailing or climbing vines cultivated for the consumption of their starchy tubers in many temperate and tropical world regions. The origin of yam is uncertain and genetic information suggests that there may be more than one point of origin. The name, yam, appears to derive from Portuguese “inhame” or Canarian (Spain) “name”, which derived from West African languages during trade. The main derivations borrow from verbs meaning "to eat". They are cylindrical and vary in size. Some of the largest yams have weighed more than 100 pounds and have been several feet long. Yams have a dense white, purple, or red flesh and scaly brown skin with dark spots. In African culture, it is typically boiled or roasted. They have a shelf-life of six months, but you will have a hard time finding a true yam on a shelf in an American grocery store.

Yams of African species must be cooked to be safely eaten, because various natural substances in yams can cause illness if consumed raw (Felix *et al*., 2013). The most common cooking methods in Western and Central Africa are by boiling, frying or roasting (Felix *et al*., 2013).

Among the Akan of Ghana, boiled yam can be mashed with palm oil into eto in a similar manner to the plantain dish matoke, and is served with eggs. The boiled yam can also be pounded with a traditional mortar and pestle to create a thick, starchy paste known as iyan (pounded yam) which is eaten with traditional sauces such as egusi soup.

Another method of consumption is to leave the raw yam pieces to dry in the sun. When dry, the pieces turn a dark brown color. These are then milled to create a brown powder known in Nigeria as elubo. The powder can be mixed with boiling water to create a thick starchy paste, a kind of pudding known as amala, which is then eaten with local soups and sauces.

Yams are a primary agricultural and culturally important commodity in West Africa, (Felix *et al*., 2013) where over 95% of the world's yam crop is harvested. Yams are still important for survival in these regions. Some varieties of these tubers can be stored up to six months without refrigeration, which makes them a valuable resource for the yearly period of food scarcity at the beginning of the wet season.

Yam is the main staple crop of the Igbos in south eastern Nigeria where for centuries it played a dominant role in both their agricultural and cultural life. It is celebrated with annual yam festivals. Igbos call yam "ji", Yorubas call yams "isu" and hausa call yam "doya"

Yam is a multispecies (approximately 600 species) tuber crop grown in Africa, Asia, parts of South America, the Caribbean, and South Pacific islands (Asiedu *et al*., 2010). Yam (*Dioscorea* spp.) is one of the most important food crops in tropical climates, especially in areas with moderate rainfall. The cultivation of yam complements food security (Scott *et al*., 2000). Yam is a major source of calories for millions of the world's tropical and subtropical populations (Degras, 1993) and provides some nutritional benefits in the form of protein and micronutrients (Asiedu *et al*., 2010), while contributing to shaping the cultural fabric and social relations of most African societies (Nortey, 2012). The Guinea yam, which consists of white yam (*Dioscorea rotundata*) and yellow yam (*Dioscorea cayenensis*), is considered indigenous and most important to the people of West Africa except for the inhabitants of the Cóte d'Ivore, who consider *Dioscorea alata* [an Asian species] more important.

The edible tuber has a rough skin difficult to peel, but softens after heating. The skins vary in color from dark brown to light pink. The majority of the vegetable is composed of a much

softer substance known as the "meat". This substance ranges in color from white or yellow to purple or pink in mature yams.

Most times, after peeling yam for consumption, the peels are discarded because they were regarded as wastes. These peels accumulate in large quantity causing pollution to the environment. To prevent environmental pollution, it is therefore important to discover cheap and eco-friendly alternative used in the conversion of these wastes to wealth. This has led to the use of yam peel in large scale production of enzymes such as protease, lipase, cellulase and amylase.

2.2. ENZYMES

Enzymes are biological catalysts which are indispensible component of biological reactions. The use of chemical catalysts has been followed for a very long time. Chemical catalysis though widely used was very cumbersome. The disadvantages that this method poses include need for high temperature and pressure for catalysis and the moderate specificity. These limitations were overcome by the use of enzymes. Enzymes work at milder conditions when compared to that required by chemical catalysts for operation. Also enzymes are highly specific and catalyze reactions faster than chemical catalysts (Prasad, 2011). Enzymes are now being used in various sectors of industry. They are used in detergents, paper industry, textile industry, food industry and many other industrial applications. Enzymes have been in use since ancient times (Gupta *et al*., 2003) and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk (Drauz, 2010).

2.2.1. AMYLASES

Amylase is (E.C.3.2.1.1-1,4-alpha D-glucanohydrolase) an extracellular enzyme, which is involved in the starch processing industries where it breaks starch into simple sugar constituents. Alpha amylase has extensive application in starch processing, brewing and

sugar production, in textile industries and in detergent manufacturing processes. Interestingly, the first enzyme produced industrially was an amylase from fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Pandey *et al*., 2000). Amylases are among the most important enzyme and account for about 30% of the world's enzyme production (Neelam *et al.*, 2013). These enzymes are found in animal, plant, bacteria, and fungi. The different types of microorganisms like bacteria, fungi and yeast have been reported as the source of amylase and their properties have been described (Gupta *et al*., 2008).

Amylases, in most simple terms, are a group of enzymes that hydrolyze glucosidic bonds present in starch. However, recent findings of new enzymes and new effects of known enzymes on starch and the accumulated knowledge on primary and tertiary structures of enzymes have expanded the concept of amylase. In this article, amylases are defined as a group of enzymes that act on α-1,4- and α-1,6-glucosidic bonds present in starch and glycogen, which is a starch-related polysaccharide. It is sometimes difficult to discriminate certain enzymes from amylase, depending on their action specificity. Here, enzymes that act only on oligosaccharides derived from starch were excluded from amylase. Although some phosphorylases act on starch or glycogen, they are not included in amylase.

2.2.2 PRODUCTION OF AMYLASE

2.2.2.1. Solid state fermentation

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

(Kunamneni *et al.,* 2005). Other advantages that SSF offers over SmF are simpler equipments, higher volumetric productivity, higher concentration of products and lesser effluent generation (Couto and Sanroman, 2006). For several such reasons SSF is considered as a promising method for commercial production of enzymes.

2.2.2.2. Submerged fermentation

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

2.2.3. IMPORTANCE OF AMYLASE

α-Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potential and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier. The industrial applications of α-Amylase and the microbial sources used for its production are described in Table 1.

Table 1: Industrial applications of α-Amylase and the microbial source used

Industrial Applications	**Microbial sources**
Starch conversion	*Bacillus amyloliquefaciens, Bacillus licheniformis*
Food industry	*Aspergillus niger*
Medicine	*Penicillium sp*
Bakery industry	*Bacillus stearothermophilus*
Detergent industry	*Bacillus lichenformis*
Textile industry	*Bacillus sp*
Fuel alcohol production	*Escherichia coli, Bacillus subtilis*

SOURCE: Gupta *et al.,* (2003).

2.2.3.1. Production of Fructose and Glucose by Enzymatic Conversion of Starch

Starch is used in the production of fructose and glucose syrups (Van Der Maarel *et al*., 2002). This process involves three steps: Gelatinization, Liquefaction, and Saccharification. Gelatinization involves the dissolving of starch granules in water to form a viscous starch suspension. The amylase and amylopectin are dispersed into the water on dissolution. Liquefaction of starch is it's partial hydrolysis into short chain dextrins by α-Amylase resulting in reduction of the viscosity of the starch suspension. Saccharification is the production of glucose and fructose syrup by further hydrolysis. This is carried out by glucoamylase which acts as an exo-amylase by cleaving the α-1, 4 glycosidic linkages from the non-reducing terminal. The action of pullulanase along with glucoamylase yields high glucose syrup. This high glucose syrup can then be converted into high fructose syrup by isomerization catalysed by glucose isomerase. The fructose syrup obtained is used as a sweetener, especially in the beverage industry.

The hydrolysis of starch for this purpose was previously carried by acid hydrolysis followed by saccharification using enzymes. This method had many drawbacks. The acidic nature of the process required corrosion resistant material to be used for the equipment and the high temperatures would inactivate the thermolabile enzymes if the hot starch hydrolysate passes into subsequent steps. Hence, enzymatic hydrolysis is a preferred method. The α-Amylase used in the liquefaction step can be produced from various microbial sources. Thermostable amylases can be employed for hydrolysis at a high temperature. *Bacillus Stearothermophilus, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Pyrococcus furiosus* are few of the many microbial sources used to produce α-Amylase that is used in starch conversion (Gupta *et al.,* 2003).

2.2.3.2. Bakery Industry

α-Amylase is added to the dough in bread baking process. This causes the starch to hydrolyze into small dextrins which can further be fermented by yeast. This increases the rate of fermentation. Also the starch hydrolysis decreases the viscosity of the dough, thus improving its texture and increasing loaf volume by rising of dough.

Once the baking is done, there may be changes during storage of baked products. All undesirable changes like increase of crumb firmness, loss of crispness of the crust, decrease in moisture content of the crumb and loss of bread flavor together are called staling. The enzyme is also used as an anti-staling agent to improve the shelf life and softness retention of baked goods (Gupta *et al.,* 2003). Though it has an anti-staling effect, a slight overdose may result in gummyness of the bread. This is caused due to production of branched dextrins (Chi *et al.,* 2009). In such cases pullulanase is used in combination with amylase resulting in specific hydrolysis of compounds responsible for the gummy nature of amylase treated bread.

2.2.3.3. Detergent Industry

The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Consumers prefer to use cold water and mild conditions which requires the detergent to work in those imitations. Earlier the chemicals used in detergents caused harm when ingested and the conditions of dishwashing were very harsh. Hence enzymes showed the industry an alternative path. The enzymes are environmentally safe and work at mild conditions. α-Amylase is used to digest the starch containing food particles into smaller water soluble oligosaccharides. Starch can attract soil particles on to the clothes. Hence removal of starch is also important to maintain the whiteness of clothes. The stability of α-Amylase at low temperature and alkaline pH contributes to its extensive use in detergents.

The disadvantage of using α-Amylase is the calcium dependency and oxidant sensitivity of the enzyme. This limitation can be overcome by using α-Amylase from genetically modified organisms. Scientists from Novozymes and Genencore International, two major suppliers of detergent enzymes, have worked on this. They replaced the oxidant sensitive amino acid residue *met* at position 197 by *leu* in *B. licheniformis* amylase which resulted in an amylase with improved resistance against oxidative compounds (Gupta *et al.*, 2003).

2.2.3.4. Desizing of Textiles

Modern production processes in the textile industry can cause breaking of the warp thread. To strengthen the thread, sizing agents are used which strengthen the thread by forming a layer on it and can be removed after the fabric is woven. Starch is a preferred sizing agent as it is easily available, cheaper and can be easily removed from the fabric. The layer of starch is subjected to hydrolysis in the desizing process where α-Amylase is employed to cleave starch particles randomly into water soluble components that can be removed by washing. The enzyme acts specifically on the starch molecules alone, leaving the fibers unaffected (Gupta *et al.,* 2003).

2.2.3.5. Paper Industry

Like textiles, paper is also treated with sizing agents to protect it from mechanical strain during processing. The sizing also contributes to better quality of the paper in terms of strength, smoothness, writing and erasebility. Starch is commonly used as the sizing agent. The role of α-Amylase in the paper industry is the partial hydrolysis of starch to make it less viscous in a batch or a continuous process. This is owing to the highly viscous nature of natural starch making it unsuitable for coating on paper (Gupta *et al.,* 2003).

2.2.3.6. Alcohol Industry

Fermentable sugars are produced by the conversion of starches with the help of α-amylase. Starches such as grain, potatoes and so on are required for the manufacturing of ethyl alcohol, a major chemical having essential role in most of the biological and chemical reactions (Juge *et al*., 2006). Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world. In this production, starch has to be solubilized and then subjected to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae*. The production of ethanol by yeast fermentation plays an important role in the economy of Brazil. In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae*. Among bacteria, α-amylase obtained from thermo-resistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions (Sanchez and Cadona, 2008).

2.2.3.7. Bio fuel Industry

Among biofuels, ethanol is most widely used. For the bioethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world. In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars (Amoozegar *et al*., 2003; Hutcheon *et al*., 2005). The conventional process for the bioconversion of starch into ethanol is first preceded by liquefaction to form a viscous starch suspension. This is followed by the saccharification process where the starch is hydrolyzed by α-Amylase to yield fermentable sugars. These sugars are then fermented by yeast to produce alcohol. As an improvisation of this process, protoplast fusion between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* was performed to obtain a new yeast strain that can directly produce the biofuel from starch, eliminating the need for a saccharification step (Saini *et al*., 2016).

2.2.3.8. Application in Medicine

A higher than normal concentration of amylases may predict one of several medical conditions, including acute inflammation of the pancreas, perforated peptic ulcer, strangulation ileus, torsion of an ovarian cyst, macroamylasemia, and mumps. Amylase can be measured in body fluids including urine and peritoneal fluid. In various human body fluids, the level of *α*- amylase activity is of clinical importance, for example, in diabetes, pancreatitis, and cancer research (Das *et al* ., 2011).

2.2.3.9. Feed industry

It has been reported that the use of amylase as an additive in animal feed industry has improved the digestion of starch, the body weight gain and feed conversion ratio. It readily hydrolyzes the starch polymers into fructose and glucose, which increases the digestibility of carbohydrates (Iji *et al*., 2003; Silva *et al*., 2006; Sidkey *et al*., 2011). Consequently, herbal feed which mainly consist of long chain polysaccharide can be digested effectively.

2.2.3.10. Food Industry

There is an extensive use of amylase in processed food industry such as baking, brewing, production of cakes, preparation of digestive aids, fruit juices, and starch syrups. The α-amylases have been used in the baking industry widely (Kumar, 2015). These enzymes are generally added to the dough of bread in order to degrade the starch into smaller dextrins, which are further fermented by the yeast. The α-amylase enhances the fermentation rate and the reduction of the viscosity of dough, which results in improvements in the volume and texture of the product.

2.2.4. CLASSIFICATION OF AMYLASE

2.2.4.1. CLASSIFICATION OF AMYLASE BASED ON TYPES

2.2.4.1.1. α-Amylase

α-Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and maltose. It is a calcium metalloenzyme i.e. it depends on the presence of a metal co factor for its activity. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase. Endo- hydrolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non reducing ends (Gupta *et al.,* 2003). Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharide composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of the starch molecule. It is a linear chain consisting of repetitive glucose units linked by α-1, 4-glycosidic linkage. Amylopectin constitutes 75-80% of starch and is characterized by branched chains of glucose units. The linear successive glucose units are linked by α-1, 4-glycosidic linkage while branching occurs every 15-45 glucose units where α-1, 6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme.

The optimum pH for activity is found to be 7.0. α-Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch. α-Amylase catalyses the first step in this process. Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawbacks like the operating conditions are of highly acidic nature and high temperatures. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup.

The use of enzymes in detergents formulations has also increased dramatically with growing awareness about environment protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of the enzyme which is the driving force behind the research to produce this enzyme in an optimum, safe and convenient manner (Gupta *et al.,* 2003).

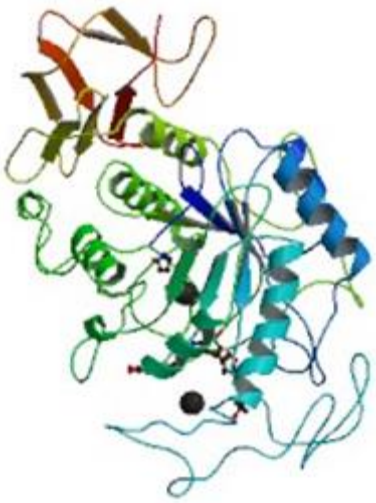

Figure 1. Structure of α-amylase (Das *et al*., 2011)

2.2.4.1.2. β – Amylase

β-Amylase (EC 3.2.1.2) is an exo-hydrolase enzyme that acts from the nonreducing end of a polysaccharide chain by hydrolysis of α-1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as

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

2.2.4.1.3. γ – Amylase

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

2.2.4.2 CLASSIFICATION BASED ON PRODUCER ORGANISM

2.2.4.2.1. Fungi Amylase

Ellaiah *et al.* (2003) identified amylolytic activity from several fungal species isolated from soil and *Aspergillus* sp. was found to possess the highest amylase activity. Most of the mesophilic fungi are reported to produce α-amylase, and many researches have been done for specific cultural conditions and to choose the best strains to produce commercially. Fungal enzymes are limited to terrestrial isolates, mostly to *Aspergillus and Penicillium* (Kathiresan and Manivannan 2006; Sundarram *et al*., 2014). The *Aspergillus* species usually produces a variety of extracellular enzymes, and amylases are the ones with the most significant industrial value. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce large quantities of enzymes that can be used extensively in the industry. *A. oryzae* is considered to be the favorable host for the production of heterologous proteins as it has

ability to secrete a vast amount of high value proteins and industrial enzymes, for example, *α*-amylase (Gomes *et al*., 2003). *Aspergillus oryzae* has been extensively used in the production of food such as soy sauce and organic acid such as citric and acetic and commercial enzymes including *α*-amylase (Jin *et al*., 1998). *Aspergillus niger* is acid tolerant (pH <3) and hence has important hydrolytic capacities in the *α*-amylase production, and it also avoids bacterial contamination (Djekrif-Dakhmouche *et al*., 2006), The fungal *α*-amylase is usually preferred over other microbial sources because of their more accepted Generally Recognized as Safe (GRAS) status (Gupta *et al*., 2003). The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase. Jensen *et al*., (2002) and Kunamneni *et al.,* (2005) purified the *α*-amylase, proving its thermostability.

2.2.4.2.2. Bacteria Amylase

For commercial applications, α-amylase is mainly derived from the genus *Bacillus*. α-Amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* find potential application in a number of industrial processes such as in food, fermentation, textiles and paper industries (Konsoula and Liakopoulou-kyrialades, 2007; Hussain *et al*., 2013). As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110 ^{o} C), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins (Asgher *et al*., 2007). *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications (Dash *et al*., 2015)

2.3. Aspergillus niger.

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mould on certain fruits and vegetables such as grapes, apricots, onions, and peanuts, and is a common contaminant of food (Pel *et al.,* 2007). It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mould"). Some strains of *A. niger* have been reported to produce potent mycotoxins called ochratoxins; other sources disagree, claiming this report is based upon misidentification of the fungal species. Some true *A. niger* strains have been reported to produce ochratoxin A and it also produces the isoflavone orobol (Mogensen *et al.,* 2007).

A. niger is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organisation. *A. niger* fermentation is "generally recognized as safe" (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act (Mogensen *et al.,* 2007).

Many useful enzymes are produced using industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high-fructose corn syrup, and pectinases are used in cider and wine clarification. Alpha-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano and other products that decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for NMR analysis (Pel *et al.,* 2007).

A. niger growing from gold-mining solution contained cyano-metal complexes, such as gold, silver, copper, iron, and zinc. The fungus also plays a role in the solubilization of heavy-metal sulphides (Varga *et al.,* 2007). Alkali-treated *A. niger* binds to silver to 10% of dry

weight. Silver biosorbtion occurs by stoichiometric exchange with Ca(II) and Mg(II) of the sorbent (Hong *et al.*, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Yam Peel

The substrate Dioscorea spp. (yam) was obtained from a local market in Ilorin, Kwara state.

3.1.2. *Aspergillus niger*

Aspergillus niger was obtained from microbial culture bank, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara state.

3.1.3 Chemicals and Reagents

Potato dextrose agar (PDA), $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4$, lactose, maltose, fructose, sucrose, dinitrosalicylic acid (DNS), phosphate buffer and bovine serum albumin are products of Sigma-Aldrich Chemical Co., USA.

3.2 METHODS

3.2.1 Identification and Preparation of substrate (Yam Peel)

The yam peels were collected from Oja tuntun market and washed to remove contaminants. The washed substrates were air dried for a week. The yam peel was identified as white yam (Dioscorea rotundata) peels at the Herbarium unit, Department of Plant Biology, University of Ilorin, Ilorin, Kwara State.

The dried substrates were chopped into small pieces and were pulverized into powdery form using a Philips blender HR 2815 model. The pulverized substrates were collected in a sterile container and stored for further use.

3.2.2 Maintenance of Microorganism

The organism (*Aspergillus niger*) was cultured on a potato dextrose agar (PDA) slant. The slant was grown at 28°C for three days and stored at 4°C. The organism was later sub

cultured on potato dextrose agar plates and was used as the inoculum during fermentation for optimization parameters of this research work.

3.2.3. Pretreatment of Substrate

The raw substrate was air dried individually and then crushed into bits. Then, the substrate was soaked individually in 1% sodium hydroxide (NaOH) solution in the ratio 1:10 (substrate: solution) for two hours at room temperature and autoclaved at 121°C for 1 hour. The treated substrate was then filtered and washed with distilled water until the wash water became neutral. It was then dried overnight at 60°C. The dried substrate was packed in polypropylene bags until use.

3.2.4. Proximate Analysis

Proximate analysis was carried out on the substrate using the method described by AOAC (2016) to determine some parameters. Parameters such as moisture, ash, crude protein, crude lipid, crude fibre and nitrogen-free extracts (digestible carbohydrates) were determined.

3.2.4.1. Moisture Contents:

This was done by method describe by (AOAC, 2000).

3.2.4.2 Total Ash

Total ash was carried out by method describe by (AOAC, 2000) using furnaces incineration. Two (2) gram of the sample was weighed into a pre-weighed crucible and incinerated in a muffle furnace at 600 °C. The sample was burnt to ashes, after it has completely ashed , it was cooled and weighed. Ash content was calculated according to the following formula: Ash

$$= \frac{W2-W1}{\text{sample weight}} \times 100$$

Where, w2 = weight in gram of crucible with ash and w1 = weight in gram of empty crucible.

3.2.4.3. Crude Lipid

Five gram of samples was wrapped in a Whatman filter paper and put in a thimble. The thimble was put in a soxlet reflux flask and mounted into a weighed extraction flask containing 40-60 ml of petroleum ether. The upper of the reflux flask was connected to a water condenser. The solvent (petroleum ether) was heated, boiled and vaporized and condensed into the reflux flask filled. Soon the sample in the thimble was covered with the solvent until the reflux flask filled upon and siphoned over, caring its oil extract down to the boiling flask. Thus process was allowed to go on repeatedly for 4hours before the defatted sample was removed, the solvent recovered and oil was left in the flask. The flask (containing the oil extract) was dried in the oven at 60^0C for 30min to remove any residual solvent. It was cooled in the desiccator and weighed. The weight of oil (fat) extract was calculated as a percentage of the weight of sample analyzed thus Fat (%) = $\frac{\text{Digested sample (W2)} - \text{Ashed sample (W1)}}{\text{weight of sample}} \times 100$

3.2.4.4. Crude Fiber

Crude fiber was determined by the method describe by (AOAC, 2000). One (1) gram of the defatted sample (fermentation waste) was placed in a glass crucible and attached to extraction unit. The sample was digested with 1.25% of boiling 150 ml sulphuric acid for 30min, drained and washed with boiling distilled water. It was returned to the flask and boiled again with 1.25% of 150ml NaOH for 30min , drained and washed with boiling distilled water and also with HCl and then again with boiling water. After washing in several portion of hot water the sample was allowed to drain dry before being transfer to a weighed crucible where it was dried in the oven at 105^0C and allowed to cool in a desiccator and weighed (W1). It was thereafter taken to the muffle furnace where it was burnt to ash; it was cooled in a

desiccator and reweighed (W2). The percentage fibre was calculated by the formula: crude fiber % = $\frac{\text{Digested sample (W1)} - \text{Ashed sample (W2)}}{\text{weight of sample}} \times 100.$

3.2.4.5. Crude Protein

This was done by Kjedhal method described by Chang (2003). Two (2) grams of the sample was mixed with 25 ml of sulphuric acid in the digestion flasks. Five grams of sodium sulphate and 1g of copper sulphate was also added. Little amount of selenium powder was also added before it was heated under a fume cup board until a clear solution was obtained (the digest). The digest was diluted to 100ml in a volumetric flask and used for the analysis. 10ml of the digest was mixed with10ml of 40 % NaOH was dispensed into the conical flask and a conical flask containing 10ml of boric acid was attached to the condenser outlet and distillation carried out for 4 minutes. A total of 5ml of the distillates was collected and titrated with 0.1M HCl to a purplish – grey end point.

Percentage Nitrogen = $\frac{0.14 \times \text{A}}{\text{weight of substrate in gram.}}$

A = volume (ml) of 0.1M HCl used in the titration.

3.2.4.6. Carbohydrate

This was done by adding total ash, crude lipid, moisture, crude fat and protein together.

3.2.5. Preparation of Enriched Medium

The basal salt solution was prepared by mixing 1g of $(NH_2)SO_4$, 0.5g of KH_2PO_4, 0.5g of $MgSO_4.7H_2O$ and 0.05g of $ZnSO_4$ into 500mls of distilled water in a beaker and kept for further use.

3.2.6. Optimization Parameters for α-amylase Production

Submerged fermentation was done to determine the effect of various parameters required for the production of α-amylase optimally by *A. niger*. The parameters were effect of incubation period, pH, temperature, inoculum size and carbon sources.

3.2.6.1 Effect of Incubation Period on α-amylase Production

The fermentation process was carried out in 250 mL Erlenmeyer flasks containing 2 grams of yam peel moistened with 100 ml of mineral salt solution containing (g/l) $(NH_4)_2SO_4$, KH_2PO_4 , $ZnSo_4$, $MgSO_4.7H_2O$. The medium was autoclaved at 121°C for 15 minutes, It was then allowed to cool and inoculated with 10 x 10^5 spores ml^{-1}. The contents of the flasks were mixed thoroughly using a rotary shaker at 150 rpm, to ensure uniform distribution of the inoculum and incubated at room temperature for 5 days. Samples were withdrawn every 24hrs and α-amylase activity was determined. (Bertrand *et al*., 2004).

3.2.6.2 Effect of pH on α-amylase Production

The basal salt solution was adjusted to different pH (5.0, 6.0, 7.0, 8.0, 9.0 and 10.0). The initial pH 7.6 was adjusted by using 0.1M HCl to decrease the pH and 0.1M NaOH to increase the pH. Submerged fermentation was carried out and the activity of enzyme was determined on the third day by using the standard assay conditions (Millern, 2010).

3.2.6.3 Effect of Temperature on α-amylase Production

The fermentation process was carried out in 250 mL Erlenmeyer flasks containing 2g of yam peel moistened with 100ml of mineral salt solution containing (g/l) $(NH_4)_2SO_4$, KH_2PO_4 , $ZnSo_4$, $MgSO_4.7H_2O$. After sterilization by autoclaving at 121°C for 15 min, the flask was cooled and inoculated with 10 x 10^5 spores ml^{-1}. The contents of the flasks was mixed thoroughly using a rotary shaker at 150 rpm, to ensure uniform distribution of the inoculum The fermentation process was incubated at different temperatures of 20°C, 30°C, 40°C, 50°C, 60°C and 70°C at optimum pH of 5 for 72 hours. The activity of α-amylase produced was determined by standard assay method (Millern, 2010).

3.2.6.4 Effect of Inoculum Size on α-amylase Production

The effect of inoculum size on growth and enzyme production was determined by inoculating different sizes of the test organism in submerged fermentation medium. The fermented

process was subjected to inoculum sizes of 8, 9, 10, 11 and 12cfu/ml. Incubating at optimum incubation period (3 days), optimum pH (5) and optimum temperature of 50°C. Then, the assay of α-amylase produced was determined.

3.2.6.5 Effect of Carbon Sources on α-amylase Production

The effect of carbon sources on growth and enzyme production was determined by growing the organism in submerged fermentation medium in which 2 grams of the following carbon sources: glucose, sucrose, fructose, maltose was used as a sole carbon source at optimal incubation period, pH, temperature and using the optimal inoculum size . After harvesting the enzymes, standard assay was carried out to determine the effect of carbon sources on enzyme production.

3.2.7 α-amylase Production

Fermentation process was carried out in a 250 ml Erlenmeyer flask. Two gram of the substrate was added to 100 ml basal salt solution containing $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, ZnSO4, KH_2PO_4. The mixture was sterilized using an autoclave at 121°C for 15min, the flask was cooled and the content was inoculated with10×10^5 spores ml^{-1}. The content was mixed with the aid of a rotary shaker at 150rpm at room temperature to ensure uniformity in the distribution of inoculum. After fermentation, the content was filtered using whatman filter paper. The filtrate was centrifuged at 4000rpm for 10mins, the clear supernatant was used as a source of extracellular enzyme.

3.2.8. Determination of α-amylase Activity

The enzyme assay was performed using the method described by Sindiri *et al.* (2013). The enzyme solution (0.5ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at 60°C for 10min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube to stop the reaction. The tubes were placed in boiling water for 5mins and were allowed to cool to room temperature. The contents of the

tube were diluted up to 3ml with distilled water. The absorbance were then determined at 540nm using a spectrophotometer.

3.2.9 Protein Content Determination

The concentration of protein present in the enzymes was estimated by following the method described by Lowry *et al*. (1951) using bovine serum albumin as the standard.

3.3. Purification of α-amylase

Keeping all the optimal optimization parameters constant, the fermentation process was carried out for three days. The crude enzyme was filtered using a 90mm diameter Whatman filter paper, the filtrate was centrifuged at 4000rpm for 10 minutes. The supernatant (crude enzyme) was kept in a sterile container at 4°C.

3.3.1. Ammonium Sulphate $(NH_4)_2SO_4$ Precipitation

One hundred ml of the crude enzyme was treated with ammonium sulphate at 80% saturation. The content was refrigerated at 4°C for 24hrs. The precipitated protein was centrifuged at 4000rpm for 10 minutes by using a high speed cold centrifuge and the supernatant was discarded. The protein precipitate was reconstituted in small quantity of phosphate buffer solution (pH 7.6). The α-amylase activity and the protein content of the enzyme precipitate were determined.

3.3.2. Dialysis

The ammonium sulphate precipitated enzyme was dialysed using a 12kb molecular weight cut off dialysis bag under several changes of buffer (pH 7.5) to ensure complete removal of impurities at 4°C for 24 hours. After the dialysis, the final volume was noted. Thereafter, the α-amylase activity and the protein content were determined.

3.3.3. Gel Filtration Chromatography

The dialysed enzyme was subjected to gel filtration chromatography technique to separate the protein based on size. Column was filled with gel (sephadex G-100) and left for 24 hours to

be well packed. After 24 hours, 10 ml of the dialyzed enzyme was added and buffer was added as a mobile phase. Sixteen fractions were collected from the column in sterile sample bottles (2ml/bottle) and the eluted fractions were analysed for α-amylase activity and protein contents were determined.

3.4. Statistical analysis

The graphs were plotted using Microsoft excel programming. All experiments were carried out in triplicates and results were expressed as mean ± standard error of mean (SEM).

CHAPTER FOUR

RESULTS

4.1 Proximate Analysis

The proximate analysis of treated and untreated yam peel is presented in Table 2. The alkaline pre-treated yam peel has the highest carbohydrate content as compared to the untreated yam peel. Also, there was an increase in the ash content of the treated yam peel when compared to the untreated yam peel. The % crude fibre and lipid was higher in untreated than treated yam peel. There was no difference in the % protein of both untreated and treated yam peel. There was an increase in the % moisture content of treated yam peel when compared to the untreated yam peel.

Table 2: Proximate Analysis of Treated and Untreated Yam Peel.

Composition (%)	**Untreated Yam (%)**	**Treated Yam (%)**
Moisture	10.41	10.73
Ash	3.35	11.33
Protein	2.84	2.84
Lipids	4.53	2.81
Crude fiber	37.86	25.26
Carbohydrate	41.01	47.03

4.2 Optimization of α-amylase Production

4.2.1. Effect of incubation Period on α-amylase Production

The α-amylase activity produced from *A. niger* cultured on yam (dioscorea) peel was 2.36 U/mL on the first day of incubation, 2.35U/mL on the second day, it was at its peak on the third day with activity of 2.94U/mL. After the third day, there was a gradual decrease in the activity of α-amylase with activities 2.70U/mL and 2.57U/mL on the fourth and fifth days respectively. The optimum α-amylase production was obtained on the 3rd day (Figure 2).

4.2.2 Effect of pH on α-amylase Production

The optimum pH was obtained at 5 with activity of 5.48 U/mL. The α-amylase activity decreased gradually as the pH increases. This resulted to about 39% loss of activity. (Figure 3).

4.2.3 Effect of Temperature on α-amylase Production

The optimum temperature for α-amylase produced by *A. niger* cultured on yam peel was recorded at 50°C with activity of 3.18 U/mL. At temperature above 50°C, the α-amylase activities decreased gradually with the loss of activity of about 78%. (Figure 4).

4.2.4 Effect of Inoculum Size on α-amylase Production

The optimal α-amylase activity was obtained at inoculum of size 9×10^5 spores/ml with an activity of 3.69U/mL. There was no difference in the activities of sizes 8×10^5 spores/ml and 9×10^5 spores/ml (Figure 5).

4.2.5 Effect of Carbon Sources on α-amylase Production

The effect on different carbon source was determined and sucrose was found to be the best carbon source for α-amylase production with an activity of 4.38U/mL. There was no much difference between the activities of α-amylase in glucose and maltose (Figure 6).

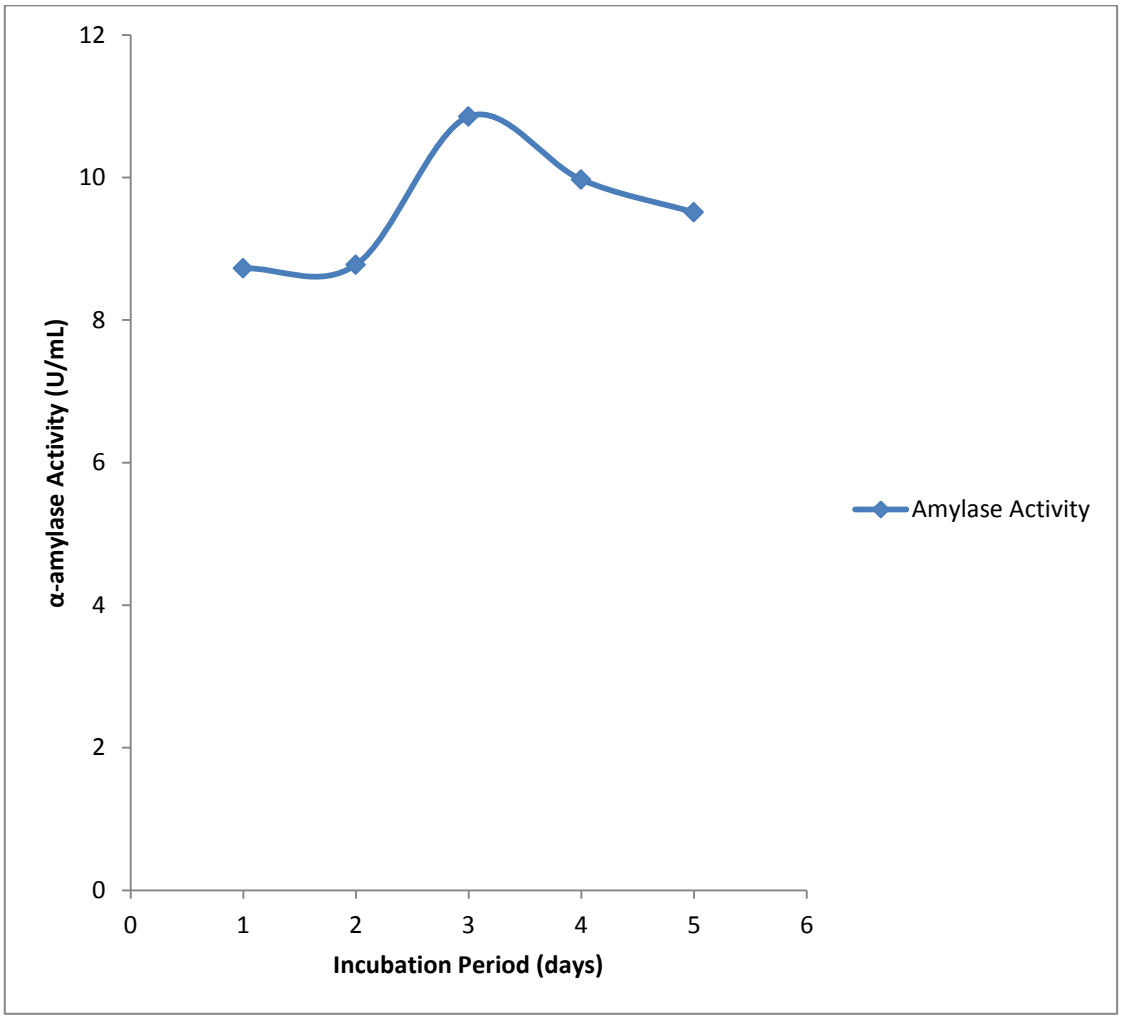

FIGURE 2: Effect of incubation period on the activity of α-amylase produced by *Aspergillus niger* cultured on yam (Dioscorea) peel.

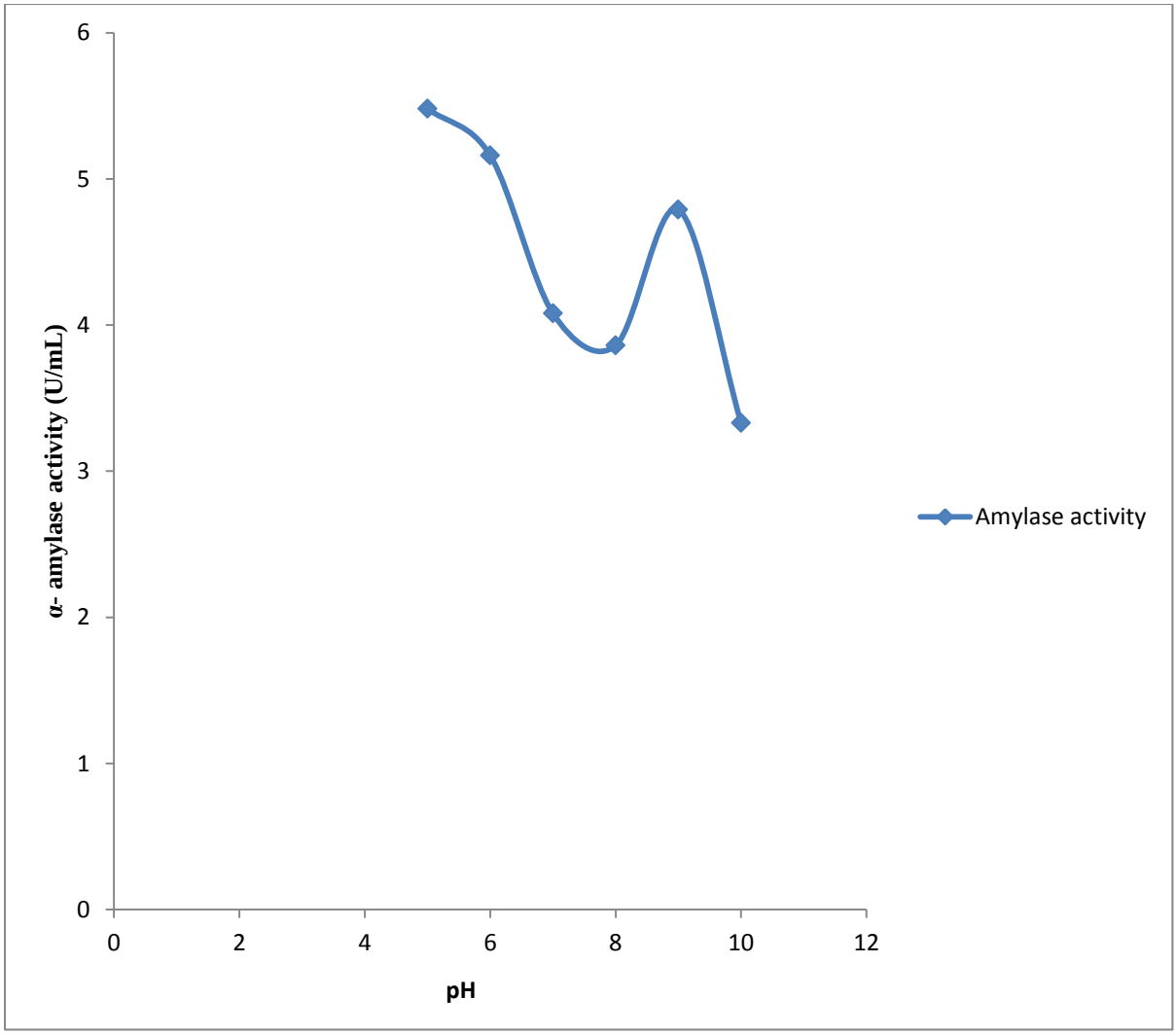

FIGURE 3: Effect of pH on the activity of α-amylase produced by *Aspergillus niger* cultured on Dioscorea (yam) peel.

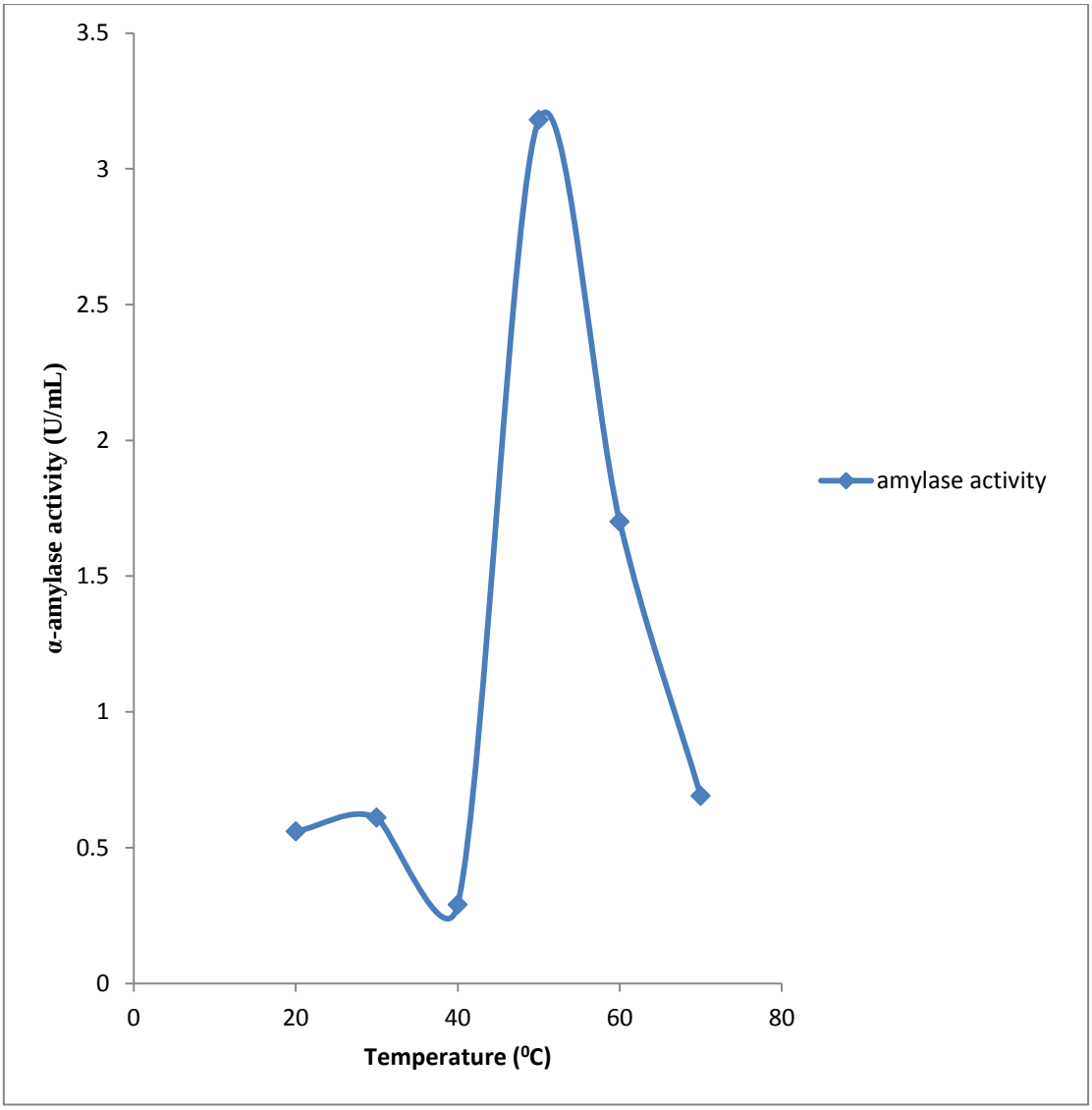

FIGURE 4: Effect of temperature on the activity of α-amylase produced by *Aspergillus niger* cultured on Dioscorea (yam) peel.

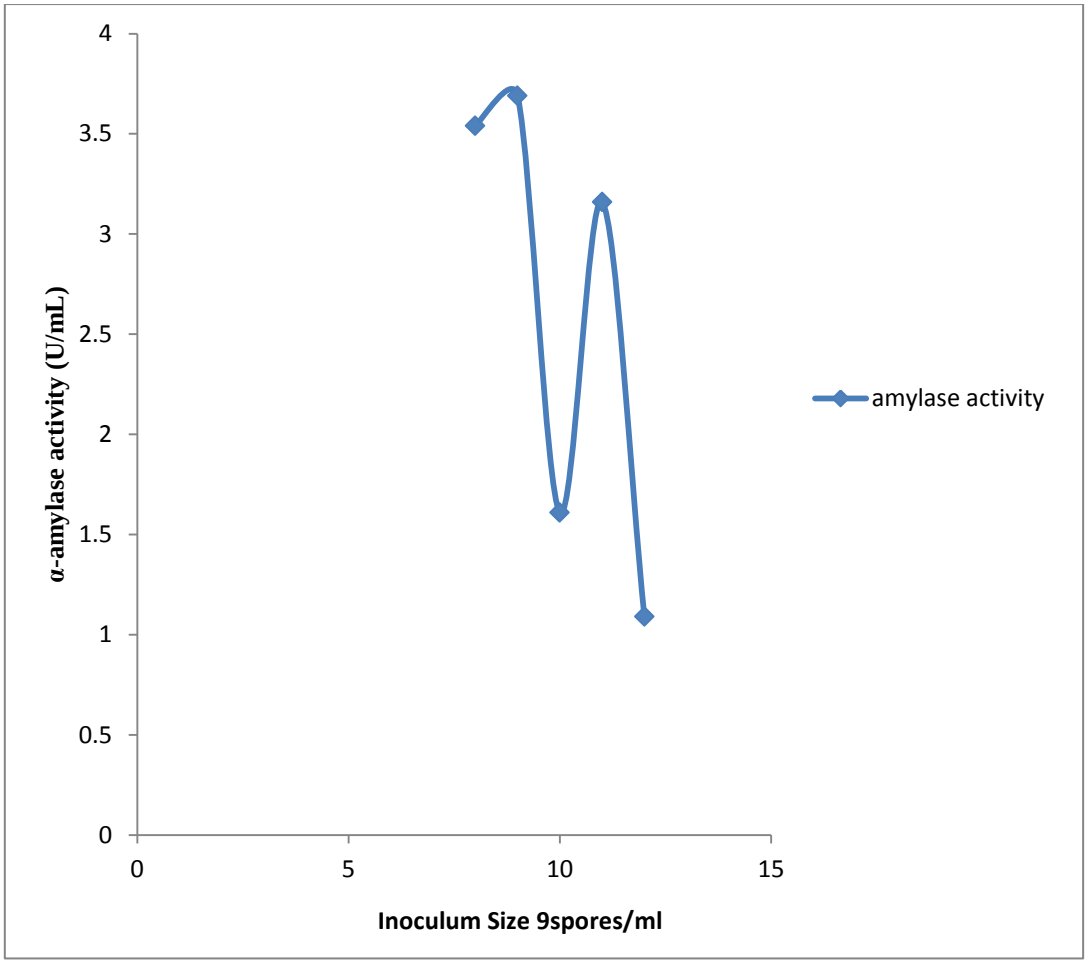

FIGURE 5: Effect of inoculum size on α-amylase produced by *Aspergillus niger* cultured on Dioscorea (yam) peel.

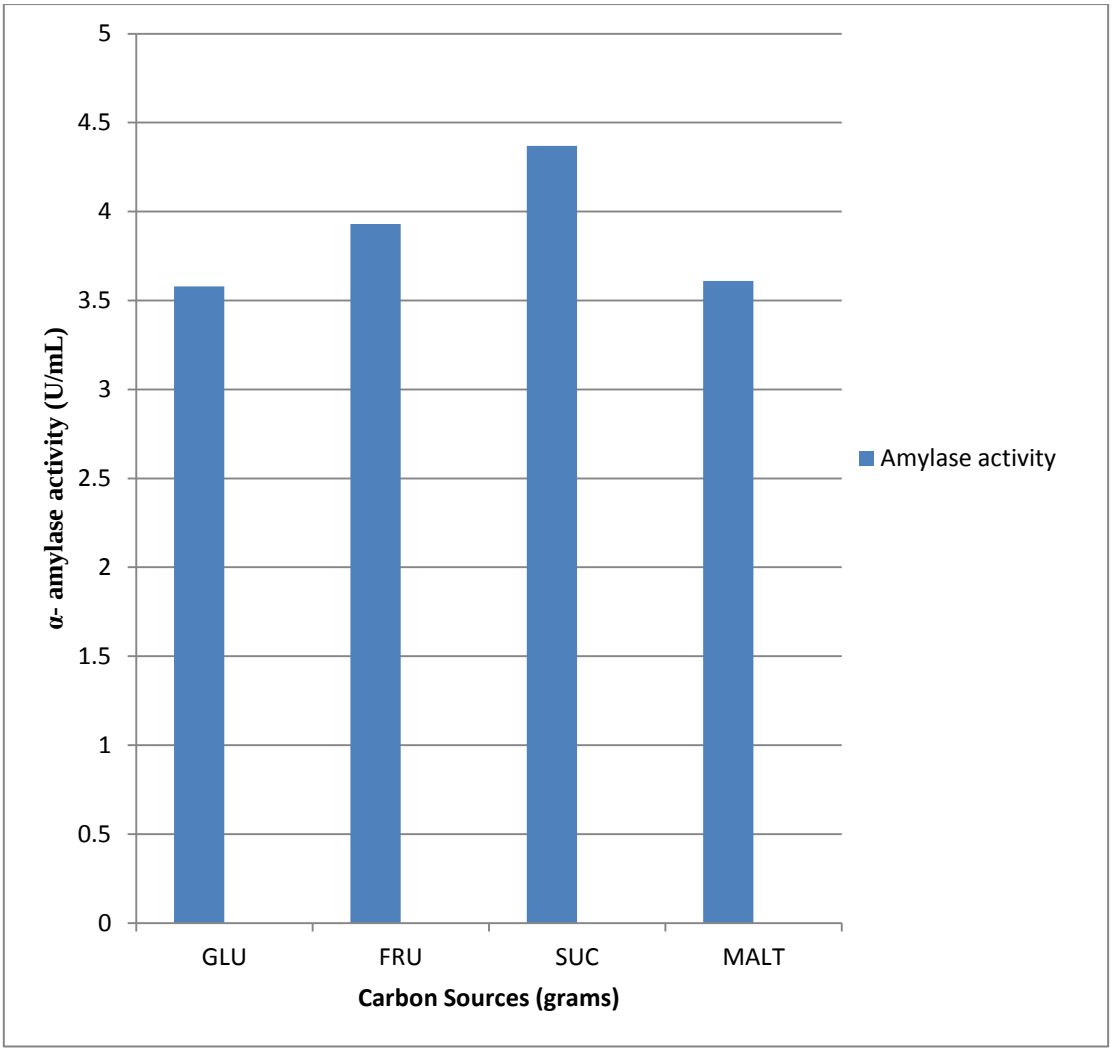

FIGURE 6: Effect of carbon sources on the activity of α-amylase produced by *Aspergillus niger* cultured on Dioscorea (yam) peel.

4.3 Purification of α-amylase Produced by *A. niger* Cultured on Dioscorea (yam) Peel

The α-amylase was purified by subjecting to ammonium sulphate precipitation, dialysis, and gel filtration chromatography. The crude enzyme had a total α-amylase activity of 17.31U/mL with a specific activity of 2.34u/mg, purification fold of 1 at 100% yield.

After ammonium sulphate precipitation, the crude enzyme had a total α-amylase activity of 7.32U/mL with a specific α-amylase activity of 3.59u/mg, purification fold increased to 1.53 at a reduced yield of 42.29%.

The crude enzyme had a total α-amylase activity of 4.92U/mL with a specific α-amylase activity 6.30u/mg after purification by dialysis with an increased purification fold of 2.70 at 28.42% yield.

After gel filtration chromatography, the crude enzyme had a total α-amylase activity of 1.66u/ml with a specific activity of 83.00, purification fold of 35.47 at 9.59% yield.

The percentage yield step wisely reduced as the crude enzyme was purified further from 100% to 9.59%. The reduction in the percentage yield of the enzyme as we purified step wisely is as a result of the reduction of contaminants. The elution profile curve is presented in Figure 6, a total of 16 fractions were collected and alpha amylase activity as well as the protein contents was determined. The summary is shown in Table 3.

TABLE 3: Summary of α-amylase Purification

S/N	purification steps	volume of enzyme (mls)	total α-amylase activity (U/mL)	total protein (mg/protein)	Specific activity of α-amylase (u/mg)	Purification fold	% Yield
1	Crude	150	17.31	7.40	2.34	1	100
2	$(NH_4)_2SO_4$ Precipitation	20	7.32	2.04	3.59	1.53	42.29
3	Dialysis	10	4.92	0.78	6.30	2.70	28.42
4	Gel filtration chromatography	2	1.66	0.02	83.00	35.47	9.59

Elution Profile Diagram

The elution profile curve/diagram represents the activity of α-amylase and the concentration of proteins present in each of the fractions collected after gel filtration chromatography.

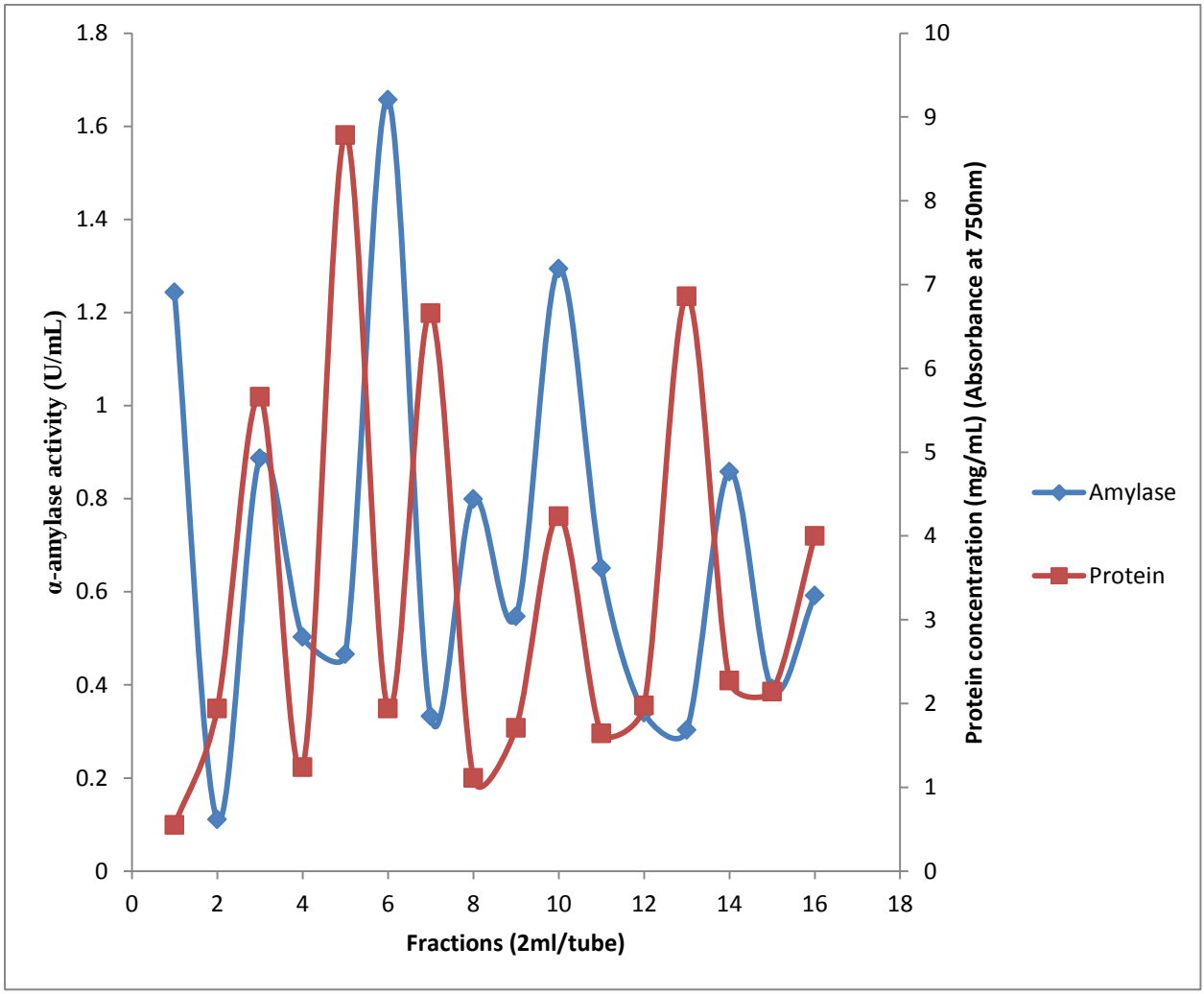

FIGURE 7: Elution Profile diagram of α-amylase produced by *A. niger* cultured on yam peel. X-axis: Fractions (2ml/tube); Y-axis: α-amylase activity(u/l) and protein concentration.

CHAPTER FIVE

DISCUSSION

5.1 Proximate analysis

The proximate analysis of untreated and treated yam peel was carried out in order to determine the percentage moisture, ash, lipid, fiber and carbohydrate. Moisture content which is the amount of water (in any form) in a material or substance. The result obtained from this study revealed that the moisture content was high in treated yam than the untreated yam which enables microorganisms to thrive well in it. However, the values are lower than 22.03% reported by Mulualem *et al.,* (2018).

In the present study treated yam has higher values of ash than the untreated yam which may indicate high mineral content of the peel. The ash content obtained was higher than the value reported by Mulualem *et al.,* (2018).

Proteins are large bio molecules or macromolecules consisting of one or more long chain of amino acids. It is an essential need for the survival of human being and animal. In this study, the % protein of the untreated and the treated yam peel were the same.

Lipid is a diverse group of organic compounds which includes fats and oils that are soluble in nonpolar solvents. The result obtained from the study indicates that lipid was high in untreated than in the treated yam peel which may serve as a good source of fat and soluble vitamin.

Fiber is made up of the undigested parts or compounds of plants. The main role of fiber is to keep the digestive system healthy. Untreated sample had the highest crude fibre which could help improve general health and well-being. The fiber content obtained from this study were higher than the value 1.68% reported by Abera, (2011) and 1.98% reported by Princewill and Ibeji, (2015).

The treated yam peel had high % carbohydrate than the untreated yam peel which implies the treated yam peel is a good source of carbohydrate when properly processed.

5.2 Optimization of α-amylase production

Yam can be classified as one of the important carbohydrate based agricultural products used as a staple food in most parts of the world. As a high carbohydrate food, yam is a good source of starch necessary for the production of α-amylase enzymes.

The effect of incubation period showed that the optimum α-amylase production was at 72 hours (Day 3). There was no significant difference in the activities on the day 1 and day 2, the α-amylase declined gradually before and after the day 3. This is in agreement with Kareem *et al.* (2009) who reported that the maximum α-amylase production by *Aspergillus oryzae* and cowpea wastes was recorded at 72 hours of incubation. Further increase in the incubation period led to the reduction in enzyme production due to depletion of nutrients in the medium.

pH of the growth medium plays an important role in enzyme production by inducing morphological changes in microbes. From the results obtained, the maximum α-amylase activity was at pH 5. There was a decline in the activity of the α-amylase produced at higher pH and this is in agreement with Sivaramakrishnan *et al., (*2007) who reported that α-amylase production occurred at pH range 3- 9 with an optimum at pH 5 by *A. oryzae* on wheat bran.

Temperature is a physical quantity expressing hot and cold and it plays an important role in the metabolic activities of microorganisms. At temperature 50°C, *A. niger* produced α-amylase maximally. The activity declined at lower and higher temperature. This implies that the enzymes are more active at 50°C and less active at temperature below and above 50°C. As the temperature increases, the rate of reaction increases but very high temperature denatures the enzymes.

Inoculums concentration is also an important factor reported in enzymes production by fermentation. From this result, inoculum size 9 x 10^5 Spores/mL had the best yield of enzyme production with an activity of 3.69U/mL. At above optimum inoculum size, α-amylase activity decreased as a result of competition for nutrients amongst microorganism in the medium.

The composition of media plays an important role in the production of the enzyme. Growth and enzyme production of any organism are greatly influenced by both environmental condition as well as the nutrients available in the medium (Singh *et al*., 2011). Supplementation of the culture medium with different carbon sources for α-amylase production showed increased production of the enzyme with sucrose (4.38U/mL). However, it has also been noticed that the absence of a proper carbohydrate (carbon source) in the medium results in a dramatic decrease in enzyme production. The addition of other carbon sources (glucose, fructose and maltose) had no significant difference in their α-amylase activities.

5.3 Purification of α-amylase

From the result obtained after purification, it was discovered that the specific activity increased from 2.34u/mg to 83.00u/mg and purification fold of α-amylase produced also increased from 1 to 35.47 as the purification went further but the percentage yield step wisely reduced from 100% to 9.59%. The reduction in the % yield of the enzyme is as a result of the reduction of contaminants in the medium.

CONCLUSION

From the result obtained in this study, it appeared that optimization of fermentation parameters resulted in high yield of pure amylase from yam peel. Amylase from this fungus has the potential to be utilized in various industrial processes. Agro wastes which are often carelessly discarded into the environment causing pollution and health hazards can be utilized

as readily available substrate for the production of α-amylase. Therefore, this method can be applied to produce enzyme locally for industries to convert wastes to wealth and reduce the challenges associated with enzyme importation to the country.

RECOMMENDATION

Amylases have enormous usefulness in industries. However, the high cost of amylase production is a limitation and there is a need to look for a cheaper and eco-friendly way to produce amylase. To address this problem for a developing economy like Nigeria, agro industrial wastes that are generated in Nigeria could be utilized for the cost effective production of amylase.

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APPENDIX

Preparation of glucose standard curve

A 20 miller of stock solution of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10mg of glucose was dissolved in 1ml of citrate buffer to give the concentration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10mg per ml of glucose. Then preparation of a blank of 0.5ml of distilled water was also prepared, Incubate at 60°C for 10min, add 1ml of DNS then heat the test tubes exactly for 5min in boiling water bath and observe for a color change. Cool in tap water for 5min and add 3mls of distilled water to each test tube. Finally, the density was measured at 540 nm using spectrophotometer and the standard curve was plotted. The standard formula, Y= 0.135x was developed based on the result and the following standard curve may be obtained. X= the amount of glucose equivalent; Y= absorbance val.

Preparation of Potato Dextrose Agar

An amount of 9.75g of potato dextrose agar was diluted in 250ml distilled water. Solution was autoclaved at 121°C and 15psi for 15 minutes until no colloid is seen to ensure the solution is completely diluted. Then the solution was poured into a petri dish until the solution is 1/3 height of the bottles. Lastly, the agar was left to solidify.

Preparation of 3, 5-Dinitrosalicylic Reagent (DNS)

In preparing 3, 5-Dinitrosalicylic Reagent (DNS), the reagent containing 3.74g of DNS acid, 6.99g of NaOH, 108g of Na-K (sodium tatrate), 2.9g of Na- metabisulphate (sodium metabisulphate) and 2.68ml of phenol was melted at 500C and was dissolved in 500ml of distilled water. This method was described by Miller (1959) was used.

Preparation of Reagent A

Dissolved 20g of anhydrous sodium carbonate and 4g of sodium hydroxide pellet in a little quantity of distilled water in 1 Liter standard volumetric flask and make it up to the mark with distilled water.

Preparation of Reagent B

20g of sodium potassium tatrate was dissolve in 1 Liter standard volumetric flask and then mark with distilled water.

Preparation of Reagent C

Dissolved 10g of copper sulphate in distilled water in 1 liter standard volumetric flask and make up to the mark.

Preparation of Reagent D

This should be prepared fresh, just before use by mixing 0.5ml of reagent B with 50ml of reagent A and 0.5ml of reagent C.

Table 4: Standard Protein Determination

Reagent	Blank	1	2	3	4	5
BSA(mg/ml)	0.00	0.02	0.04	0.06	0.08	0.10
Distilled water (ml)	0.60	0.58	0.56	0.54	0.52	0.50
Reagent D (ml)	3.00	3.00	3.00	3.00	3.00	3.00
Reagent E (ml)	0.30	0.30	0.30	0.300	0.30	0.30
Total vol. of Rxn mixture (ml)	3.90	3.90	3.90	3.90	3.90	3.90

BSA- Bovine Serum Albumin; Reagent D- Alkaline copper solution; Reagent E- Follin-ciocalteu

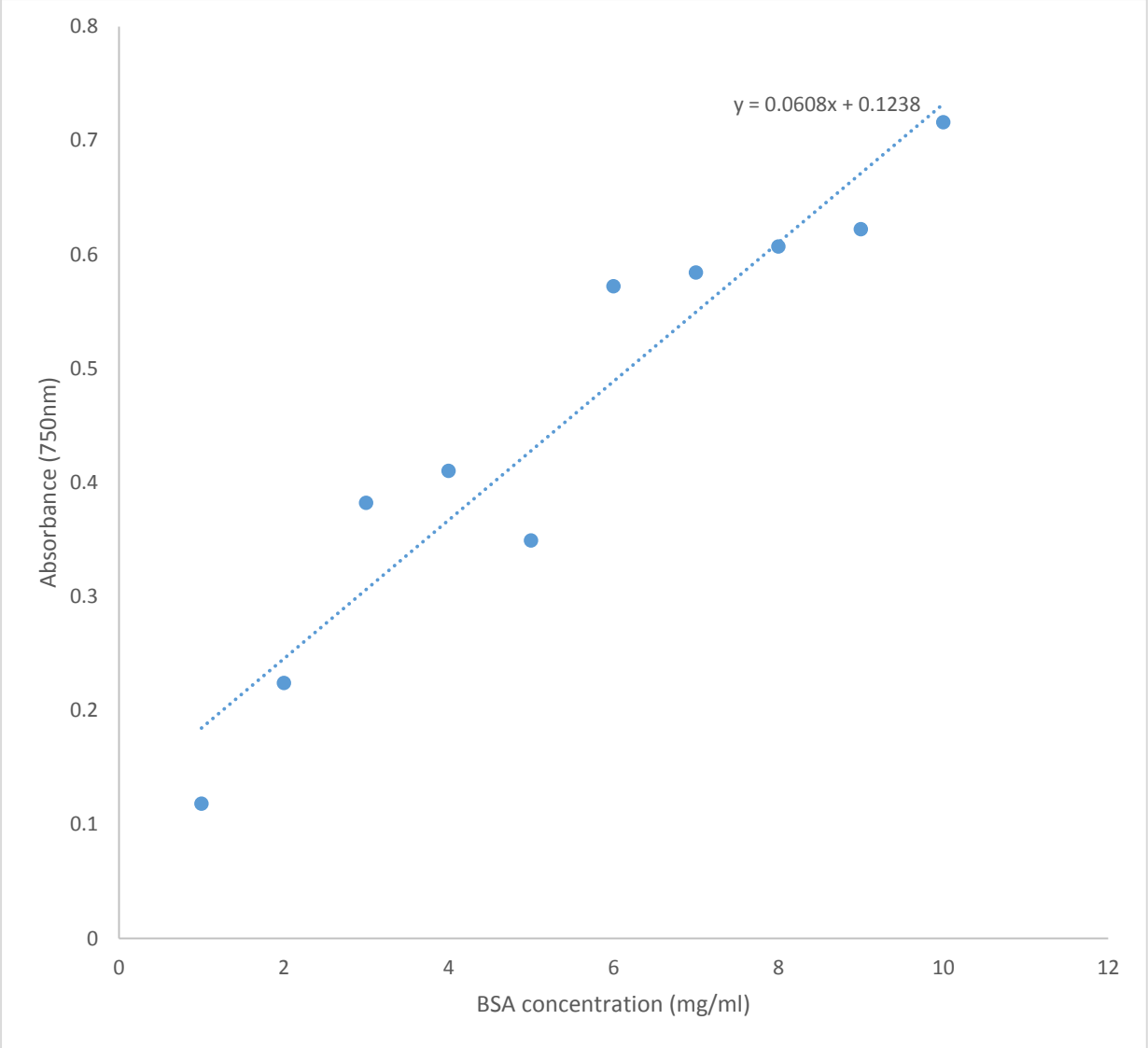

Figure 8: BSA concentration Curve

Table 5: Protocol for Protein Determination

	Test	Blank
Test sample (ml)	0.5	_____
Distilled water (ml)	_____	0.5
Reagent D (ml)	5.0	5.0

Mix well

Reagent E (ml)	0.5	0.5

Mix well

Read the absorbance at 750nm after 30mins.

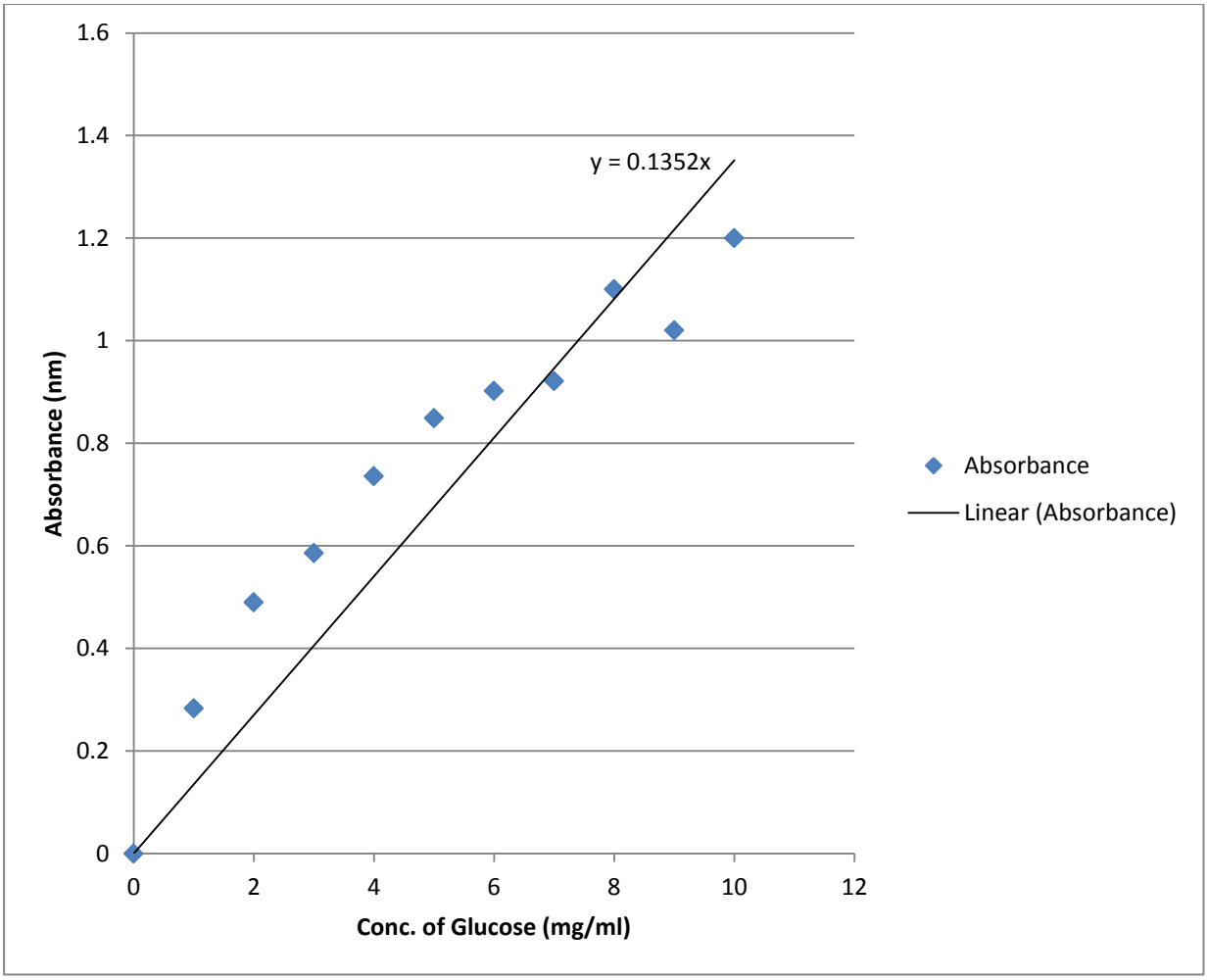

Figure 9: Glucose Standard