**ALPHA AMYLASE PRODUCTION FROM LOCALLY ISOLATED *Aspergillus sp.* USING SELECTED AGRO WASTE AS SUBSTRATE**



BY

**HAUWA IBRAHIM FARI**

1122

July, 2022

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Under the Guidance of  
MR. ABDULSALAM MUSTAPHA  
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A Project Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Bachelor of Science in Microbiology, At the Department of Biological Science, School of Sciences and Information Technology, Skyline University Kano, Nigeria.

July, 2022

**DECLARATION**

I hereby declare that this work is the product of research efforts undertaken under the supervision of Mr. Abdulsalam Mustapha and has not been presented and will not be presented elsewhere for the award of a degree or certificate. All the sources have been duly acknowledged.

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Hauwa Ibrahim Fari Sign/Date

1122

**CERTIFICATION**

This is to certify that this study was carried out by Hauwa Ibrahim Fari 1122 in the Department of Biological Science, School of Sciences and Information Technology, Skyline University Nigeria, under my supervision.

Mr. Abdulsalam Mustapha \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Supervisor Sign/Date

**APPROVAL**

The panel of examiners recommends the candidate HAUWA IBRAHIM FARI1122 for the award of the Degree of Bachelor of Science in Microbiology subject to effecting all the corrections pointed out during the oral examination.

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Internal Examiner Date

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Project Supervisor Date

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Head of Department Date

**DEDICATION**

I dedicate this study to my parents and families who patiently bear my academic and non-academic burdens and also provide me with parental guidance throughout my lives.

**ACKNOWLEDGEMENTS**

My heartfelt thanks and gratitude go to Allah (SWT) for sparing my life and to those who, in their infinite mercy, made this work possible; may His peace and blessings be upon our beloved prophet Muhammad (SAW).

    I also want to express my deepest appreciation to my wonderful supervisor, Mr. Abdulsalam Mustapha, for sparing his time to review, correct, and guide me at various stages up to the completion of this project.

Special thanks to my parents, Alhaji and Hajia Ibrahim Fari, May Almighty Allah bless you all.

Finally, to my lovely siblings, colleagues, and friends, thank you all for your understanding and prayers; I will live to appreciate you all AMIN.

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# **ABSTRACT**

This study was conducted to investigate the potential of producing α-amylase from selected agricultural waste (groundnut shell). Firstly, fugal isolates were isolated from soil sample using serial dilution method. A total of six (6) fungal isolates were isolated and their microscopic and morphological characteristics were described, and the isolates were screened for α-amylase production by utilizing amylase agar. The isolate (A1) which was identified tentatively as *Aspergillus* sp. had the highest zone of clearance (1.6 cm) and it was then employed in fermentation studies. This study demonstrates that groundnut shell which is a cheap and readily available waste could be a perfect substrate for the production of value-added products.

**CHAPTER 1**

**1.0 INTRODUCTION**

Enzymes are chemical motivating factors that control specific biochemical reactions. The potential for using microorganisms as biotechnological sources of industrially relevant enzymes has sparked interest in the study of extracellular enzymatic activity in a variety of microorganisms in recent years. Proteases and amylases are the most prominent industrially important enzymes because they are widely used in the brewing, detergent, and food industries (Doss and Anand, 2012). Amylases are used in the starch processing industries to hydrolyze polysaccharides such as starch into simple sugar constituents. With the advent of new biotechnology frontiers, the spectrum of amylase applications has expanded into many new fields such as clinical, medicinal, and analytical chemistry (Khan and Yadav, 2011). The first step in starch processing is polysaccharide liquefaction using bacterial α-amylase, followed by saccharification catalyzed by fungal glucoamylase.

α-Amylases (E.C.3.2.1.1) are enzymes that catalyze the hydrolysis of internal α-1,4-glycosidic linkages in starch to produce low molecular weight products such as glucose, maltose, and maltotriose (Rajagopalan and Krishnan, 2008). Amylases are among the most important enzymes and have a significant impact on biotechnology, constituting a class of industrial enzymes that accounts for approximately 25% of the global enzyme market (Reddy *et al*., 2003). They can be derived from a variety of sources, including plants, animals, and microorganisms. Today, a large n umber of microbial amylases commercially available, and they have nearly completely replaced chemical starch hydrolysis in the starch processing industry. Microorganism amylases have a wide range of industrial applications because they are more stable than plant and animal -amylases (Tanyildizi *et al*., 2005). The main advantage of using microorganisms for amylase production is the low cost of bulk production and the ease with which microbes can be manipulated to obtain enzymes with desired properties.

α-amylase has been derived from a variety of fungi, yeasts, and bacteria. However, enzymes derived from fungi and bacteria have dominated industrial applications (Gupta *et al*., 2003). α-amylases have the potential to be used in a wide range of industrial processes, including food, fermentation, textile, paper, detergent, and pharmaceutical industries. Fungal and bacterial amylases have the potential to be useful in the pharmaceutical and fine-chemical industries. However, Amylase applications have expanded with advances in biotechnology, including clinical, medicinal, and analytical chemistry, as well as their widespread use in starch saccharification and the textile, food, brewing, and distilling industries (Gupta *et al*., 2003). -amylases are one of the most common and important types of industrial amylases, and this important enzyme can be produced by a variety of microorganisms.

Several researchers have reported that cultivating microorganisms on lignocellulosic materials is a promising approach to producing enzyme, which will lead to a reduction in the cost of producing enzyme. Lignocellulosic materials are abundant in the environment, accounting for 50% of terrestrial biomass (Fernanda *et al*., 2013), which consists primarily of agribusiness materials, urban waste, and angiosperm and gymnosperm wood (Castro and Pereira, 2010). Lignocellulosic wastes have a complex structure composed primarily of cellulose, hemicellulose, and lignin, which are joined together by covalent bonds to form a complex network resistant to microbial attack (Priyanka *et al*., 2017). To prevent lignocellulosic waste from becoming a nuisance in the environment, it has been used to produce many value-added products such as enzymes.

Agricultural and industrial activities generate lignocellulosic waste. Sugarcane bark, bagasse and straw, rice straw and rice bran, cassava peel, corn cobs and straw, chaff and bran from wheat, banana straw, cassava peel, wood scraps, and groundnut shell are all examples of lignocellulosic wastes produced in Nigeria. It may surprise you to learn that these plentiful wastes are underutilized, contributing to environmental pollution problems. These wastes are typically composed of 20-60% cellulose, 20-30% hemicellulose, and 15-30% lignin (Fernanda *et al*., 2013). Because of their high nutrient content, lignocellulosic wastes have been identified as potential substrates for the production of a wide range of value-added products, including bioethanol, enzymes, organic acids, biosurfactants, biogas, biohydrogen, and biofertilizers (Kumar *et al*., 2016). Lignocellulosic waste is a low-cost source of enzymes.

Groundnut (*Arachis hypogea)* originated from Latin America and was introduced into West Africa by Portuguese traders in the 16th century (Taru *et al*., 2008). Groundnut has been ranked as the world's 13th most important food crop. It is the world's fourth largest source of edible oil and third largest source of vegetable protein (Taru *et al.,* 2008). Groundnut is planted on 26.4 million hectares worldwide, with a total production of 37.1 million metric tonnes and an average productivity of 1.4 metric tonnes/ha. Developing countries account for 97 percent of the global area and 94 percent of global production of this crop (FAO, 2011). Groundnut is primarily grown in Asia and Africa by small-holder farmers under rain-fed conditions with limited inputs. Groundnut is one of the most common commercial crops in Nigeria, accounting for 70% of total Nigeria export earnings between 1956 and 1967 but declining between 1955 and mid 1980s due to the combined effect of drought and disease (Taru *et al.,* 2008). During this time, Nigeria's groundnut area fell to nearly half of its current level of 1.7 million hectares. In 2002, Nigeria produced 23390000mt of groundnuts (Taru *et al.,* 2008). Apart from riverine and swampy areas, the crop is currently grown throughout Nigeria. It is a crop that requires rain in order to grow. During the crop's flowering and pegging, rainfall should be evenly distributed. The total amount of rainfall required for pre-sowing operations is 100 mm, 150 mm for sowing, and 400-500 mm evenly distributed rainfall is required for flowering and pod development. Apart from Nigeria, the crop is currently grown throughout the country. Groundnut cannot withstand frost, severe drought, or water stagnation. The crop grows best in sandy loam, loamy, and black soils with good drainage. Heavy and sticky clays are not suitable for groundnut cultivation because pod development is hampered in these soils (Taru *et al.,* 2008). One to five seeds are found in each groundnut pod. Groundnut has a tap root system with numerous nodules in the root and lateral roots. Rhizobium bacteria, which are symbiotic in nature and focus atmospheric nitrogen, are found in these nodules. The groundnut shell is the outer layer of the groundnut: The shell makes up about 25-35 percent of the pod. The remainder is accounted for by the seed (65-75%) (Sada *et al*., 2013).

# **1.1 Justification of the study**

# So the cost of producing α-amylase is very high, there is a need to establish cheaper methods of producing the enzyme. This can be accomplished by utilizing readily available and abundant wastes such as groundnut shell, which is a common solid waste in developing countries. Its potential for producing α-amylase will promote waste management at a low cost, reduce pollution caused by this waste, and expand the country's economic base.

# **1.2 Research aim and objectives**

The aim of this research is to produce α-amylase from cheap and readily available waste (groundnut shell). The enzyme will be produced by locally isolated *Aspergillus* sp. isolated from soil samples.

The specific objectives of the research are:

* To isolate, screen and characterize *Aspergillus* sp. from soil samples.
* To evaluate the capacity of the isolate to produce α-amylase when grown on groundnut shell by solid state fermentation process.

**CHAPTER 2**

# **2.0 LITERATURE REVIEW**

Groundnut shell can be found in large quantities as agricultural farm waste in Northern Nigeria.

The shells are the mature pods' dry pericarp, which contains cellulose, carbohydrate, protein, minerals, and lipids (Nautiyal, 2002). According to Masenda (2004), groundnut shell contains cellulose (65.7 %), carbohydrates (21.2 %), protein (7.3 %), minerals (4.5 %), and lipids (1.2 %), making it suitable for amylase production. Groundnut shell is a common solid waste, particularly in developing countries. As a result, the use of groundnut shell will promote waste management at a low cost, reduce pollution caused by this waste, and increase the farmer's economic base when such waste is sold. (Sada *et al*., 2013).

Plate 1: Groundnut shell

# **2.2 Classification of amylases**

Amylases are classified based on their catalytic properties, which include substrate and product specificities. These enzymes are classified into three EC classes: transferases (EC 2), hydrolases (EC 3), and isomerases (EC 5), with the majority of them belonging to the EC 3 class. Enzymes are classified into two types based on their action mechanism: retaining enzymes and inverting enzymes. The anomeric configuration in the substrate is retained after the catalytic action of retaining enzymes, whereas it is inverted after the catalytic action of inverting enzymes. Amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3) are inverting enzymes, whereas all other enzymes containing amylase (EC 3.2.1.1) are retaining enzymes.

The third criterion used to classify amylase enzymes is their glucan chain specificity. Enzymes are classified as either exo-acting or endo-acting. Amylase and glucoamylase are exoenzymes that release maltose or glucose from the nonreducing end of glucans. Amylase, on the other hand, is an endo-acting enzyme because it attacks the inner glucosidic bonds of glucans to produce oligosaccharides (Tiwari *et al*., 2015).

## **2.2.1 α-amylase (EC 3.2.1.1)**

The enzyme is essential for starch hydrolysis in both nature and the starch industry (Tiwari *et al*., 2015). Because starch is the most important energy source for all known living organisms, it can be found in a wide variety of living organisms. This group's enzymes have accumulated a large amount of data from animals, plants, and microorganisms. As a microbial amylase, the enzyme from *Aspergillus oryzae* has received the most attention, and the information on this enzyme accounts for the majority of our understanding of amylase (Tiwari *et al*., 2015).

The α-1,4 linkages in glucan are hydrolyzed by α-amylase, but the α-1,6 linkages are not. Dextrins with relatively higher molecular weights are produced early in the hydrolysis process, resulting in a rapid decrease in the viscosity of starch solution. The average DP of dextrins gradually decreases as the hydrolysis proceeds, and the hydrolysis products in the final stage are large amounts of maltose, maltotriose, glucose, and oligosaccharides (limit dextrins) with the -1,6 linkage. The -configuration is shared by all hydrolysis products. The composition of the product during hydrolysis varies greatly depending on the origin of the enzyme. Some exhibit a sharp decrease in viscosity early in the hydrolysis process, whereas others exhibit a relatively slow decrease at the same stage. This group's enzymes have been purified and characterized from a diverse range of microorganisms, including archaea like *Sulfolobus*, prokaryotes like *Bacillus*, and eukaryotes like *Aspergillus.* The majority of them have maximum activity at 30-37oC at neutral pH; however, some have maximum activity at pH as low as 3 or as high as 10, and at temperatures above 100oC (Tiwari *et al*., 2015).

Enzymes from *Aspergillus niger, Aspergillus oryzae*, *Bacillus amyloliquefaciens, Bacillus circulans, Bacillus licheniformis, Bacillus stearothermophilus,* and *Bacillus subtilis* are particularly important for basic research and industrial application. All enzymes in this group are members of the GH 13 family (Tiwari *et al*., 2015).

### **2.2.1.1 Structural and functional characteristics of α-amylase**

Microorganisms, plants, and higher organisms all contain α-amylase (-1,4-glucan-4-glucanohydrolase) (Kandra, 2003). The α-amylase is an endo-amylase that catalyzes the initial hydrolysis of starch into shorter oligosaccharides by cleaving -D-(1-4) glycosidic bonds. (Kandra, 2003). α-amylase cannot cleave terminal glucose residues or -1,6-linkages (Whitcomb and Lowe, 2007). The end products of α-amylase action are oligosaccharides of varying length with a -configuration and -limit dextrins (van der Maarel *et al.,* 2002), which are a mixture of maltose, maltotriose, and branched oligosaccharides of 6-8 glucose units with both -1,4 and -1,6 linkages (van der Maarel *et al*., 2002). (Whitcomb and Lowe, 2007). Other amylolytic enzymes participate in the starch breakdown process, but -amylase is the most important for the process's initiation (Tangphatsornruang *et al*., 2005).

The amylase has a three-dimensional structure that allows it to bind to substrate and promote the breakage of glycoside links through the action of highly specific catalytic groups (Iulek *et al.,* 2000). Human α-amylase is a calcium-containing enzyme with a molecular weight of 57.6 kDa that is composed of 512 amino acids in a single oligosaccharide chain (Whitcomb and Lowe, 2007). The protein has three domains: A, B, and C. (Figure 1). The largest domain is A, which has a typical barrel-shaped (/)8 superstructure. The B domain is inserted between the A and C domains and is disulphide-bonded to the A domain. The C domain has a sheet structure and appears to be an independent domain with unknown function, linked to the A domain by a simple polypeptide chain. The α-amylase active site (substrate-binding) is located in a long cleft between the carboxyl ends of the A and B domains. Calcium (Ca2+) is located between the A and B domains and may act as an allosteric activator and in the stabilization of the three-dimensional structure. Figure 1 shows the structure of α-amylase.

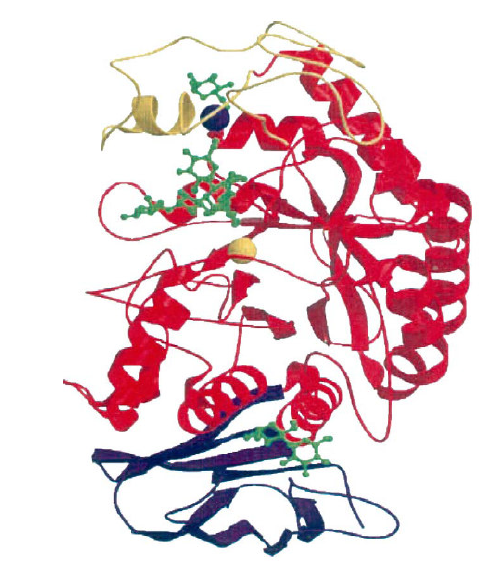


Figure 1: α-amylase structure. Domain A is represented by red, Domain B by yellow, and Domain C by purple. The calcium ion is shown in the blue sphere and the chloride ion is shown in the yellow sphere in the catalytic center. Green structures are linked to the active site as well as the surface binding sites (Payan, 2004).

### **2.2.1.2 α-amylase production**

A variety of physicochemical factors influence the production of α-amylase via submerged fermentation (SmF) and solid-state fermentation (SSF). Because of the ease with which different parameters such as pH, temperature, aeration and oxygen transfer, and moisture can be controlled, SmF has traditionally been used to produce industrially important enzymes (Gangadharan *et al*., 2008). SSF systems appear promising due to their inherent potential and benefits. SSF resembles microorganisms' natural habitat and is thus the preferred choice for microorganisms to grow and produce useful value-added products. SmF can be regarded as a violation of their natural habitat, particularly for fungi (Singhania *et al.,* 2009).

According to the theoretical concept of water activity, fungi and yeast were deemed suitable microorganisms for SSF, whereas bacteria were deemed unsuitable. However, past experience has demonstrated that bacterial cultures can be effectively managed and manipulated for SSF processes (Pandey, 2003). Other benefits of SSF over SmF include

* Superior productivity
* Simpler technique
* Lower capital investment
* Lower energy requirement and less water output
* Better product recovery, and
* Lack of foam build up.

Furthermore, it is said to be the best process for developing countries. Recently, researchers investigated whether SSF is the best system for enzyme production. They discovered that SSF is suitable for the production of enzymes and other thermolabile products, especially when yields are higher than with SmF (Couto and Sanromán, 2006).

Because of their impact on the economy and practicability of the process, the optimization of fermentation conditions, particularly physical and chemical parameters, is critical in the development of fermentation processes (Francis *et al*., 2003). For -amylase production, various factors such as pH, temperature, metal ions, carbon and nitrogen sources, surface acting agents, phosphate, and agitation have been studied. The thermostability, pH profile, pH stability, and Ca-independence of each -amylase must be matched to its application. α-amylases, for example, must be active and stable at low pH values in the starch industry but not at high pH values in the detergent industry. The composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source, and nitrogen source are the most notable of these (Couto and Sanromán, 2006). Physical and chemical properties of -amylases from bacteria and fungi have been extensively researched and described (Gupta *et al*., 2003). The properties of some microorganism amylases are shown in Table 1.

Table 1: Properties of amylases produced by various microorganisms

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Microorganism | Type of fermentation | Optimal pH | Optimal temperature | References |
| *Aspergillus niger* | SSF | 5.5 | 70 oC | Uguru *et al.* (1997) |
| *Penicillium fellutanum* | SmF | 6.5 | 30 oC | Kathiresan and Manivannan (2006) |
| *Bacillus amyloliquefaciens* | SmF | 7.0 | 33 oC | Tanyildizi *et al*. (2007) |
| *Thermomyces lanuginosus* ATCC 58160 | SSF | 6.0 | 50 oC | Kunamneni *et al*. (2005) |

## **2.2.2 β-amylase (EC 3.2.1.2)**

Enzymes belonging to this group, the exoamylases, either exclusively cleave α,1-4 glycosidic bonds such as β-amylase or cleave both α,1-4 and α,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (E.C. 3.2.1.3) and α-glucosidase (E.C. 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α-glucosidase), or maltose and β-limit dextrin. β-amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α to β. Glucoamylase and -glucosidase have different substrate preferences; -glucosidase prefers short maltooligosaccharides and liberates glucose with a -configuration, whereas glucoamylase prefers long-chain polysaccharides. α-amylases and glucoamylases have also been discovered in a wide range of microorganisms. (Couto and Sanromán, 2006).

## **2.2.3 γ-amylase (EC 3.2.1.3)**

γ-amylase cleaves α-(1-6) glycosidic linkages, in addition to cleaving the last α-(1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. Unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3 (Tiwari *et al*., 2015).

# **2.3 Substrate of amylases**

Starch is a polysaccharide that is photosynthesized and accumulated in green plants, and it is used as an energy source by nearly all living organisms on the planet. Starch is made up of two components: amylose and amylopectin. Amylose is a polyglucan with a degree of polymerization (DP) of 700–4000, consists essentially of α-1,4-linked glucans, and usually accounts for 20–25 % of starch. On the other hand, amylopectin is a much larger molecule (DP 104–105) in which α-1,4-linked glucans connect through a α-1,6 linkage to form a highly branched structure. Branching occurs on a regular basis in specific regions of the amylopectin molecule, allowing for the formation of crystalline structure. Amylopectin accounts for 75-85% of total starch. Amylopectin is the only component of waxy starch found in some waxy plants such as waxy corn and waxy rice. Starch is found in nature as insoluble starch granules (raw starch) with a crystalline structure. The majority of amylase research, whether basic or applied, has been done on gelatinized starch, which is a starch solution obtained by heating starch granules in water.

Glycogen is a storage α-glucan found in animals and microorganisms. Like amylopectin, it consists of α-1,4-linked glucans connected via the α-1,6 linkage (branching). This branching occurs frequently and is distributed evenly throughout the glycogen molecule and makes glycogen soluble in water. Amylase acts on both α-polyglucans.

Pullulan is a glucan produced by *Aureobasidium pullulans*. It is a linear polysaccharide consisting of α-1,6-linked maltotrioyl units and is often used to determine the substrate specificities of amylase (Whitcomb and Lowe, 2007).

# **2.4 Sources of α-amylases**

α-amylase can be acquired from plants, animals and microbes.

## **2.4.1 Plant sources**

Amylases are found in animals, fungi, and plants, as well as unicellular eukaryotes, bacteria, and archaea (Da Lagea *et al*., 2007). Although both plants and animals produce amylases, microbial enzymes are more commonly used in industrial processes. This is due to a variety of factors, including productivity, the thermostability of the enzyme, and the ease with which microorganisms can be cultured. The selectivity with its associated high yield and exclusivity toward the desired product are the primary advantages of the enzymatic route (Kim and Dale, 2004). Bacillus spp. are the bacteria used in commercial production. Others, including *Escherichia* sp., *Pseudomonas,* *Proteus, Serratia*, and *Rhizobium,* produce a significant amount of the enzyme (Oliviera et al., 2007). Fungi that produce commercially valuable extracellular amylases include *Aspergillus, Rhizopus, Mucor, Neurospora, Penicillium,* and *Candida* (Gupta *et al*., 2003).

Plant sources had not previously been considered as a significant source of these enzymes (Azad *et al*., 2009). The use of agricultural waste materials serves two purposes: pollution reduction and material upgrading. To reduce the cost of fermentation media, agricultural wastes are being used for both liquid and solid fermentation. These wastes contain carbon and nitrogen sources that organisms require for growth and metabolism. Pearl millet starch, orange waste, potato, corn, tapioca, wheat, and rice flours were used as nutrient sources for -amylase production (Azad *et al*., 2009).

## **2.4.2 Animal sources**

One of the most important enzymes in saliva is ptyalin, a salivary α-amylase (-1,4--D-glucan-4-glucanohydrolase; E.C. 3.2.1.1). Leuchs described the enzyme in saliva for the first time in 1831. (Zakowski and Bruns, 1985). It is made up of two families of isoenzymes, one of which is glycosylated and the other without carbohydrate. The glycosylated form has a molecular weight of about 57 kDa, while the non-glycosylated form has a molecular weight of about 54 kDa. Salivary amylases account for 40% to 50% of total salivary protein, with the majority of the enzyme produced in the parotid gland (80 percent of the total)(Zakowski and Bruns, 1985). It is a calcium-containing metalloenzyme that hydrolyzes the -1,4 linkages of starch to glucose and maltose. It is thought to be primarily involved in the initiation of starch digestion in the oral cavity

**2.4.3 Microbial sources**

Despite the wide abundance of amylases, microbial sources, primarily fungal and bacterial amylases, are used for industrial production due to benefits such as economic feasibility, consistency, shorter processing times, and convenience of process modification and optimization (Burhan *et al*., 2003).

Fungal amylases are extensively used in the preparation of oriental foods. Bacillus sp. is a widely used bacteria for thermostable -amylase production capabilities market demand. *B. subtilis*, *B. stearothermophilus, B. licheniformis, and B. amyloliquefaciens* are well-known -amylase producers, and have been widely used for commercial production of the enzyme for a variety of applications (Burhan *et al*., 2003). Correspondingly, filamentous fungi have been extensively used for centuries to produce amylases. Because these moulds are recognized to be prolific producers of extracellular proteins, they are commonly used to produce a variety of enzymes, including α-amylase.

**2.4.3.1 Fungal amylases**

Fungi from the genus *Aspergillus* are most commonly used to produce α-amylase. *Bacillus subtilis* is becoming a more appealing host for cloning as genetic engineering advances. Because of the advantages of B. subtilis, such as its high secretion level and non-pathogenic safe (GRAS-generally recognized as safe) status for nonantibiotic strains, it is well suited for the production of heterologous enzymes. Most reports on fungi that produce α-amylase have been constrained to the few species of mesophilic fungi, and efforts have been made to specify the circumstances and select superlative strains of the fungus for commercial production (Gupta *et al*., 2003). The fungal sources are limited to terrestrial isolates, primarily *Aspergillus* and *Penicillium* (Kathiresan and Manivannan, 2006). The *Aspergillus* species produce a wide range of extracellular enzymes, with amylases being the most important industrially (Hernandez *et al*., 2006). Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce lots of enzymes that are widely used in industry. Because of its ability to secrete a large number of high-value proteins and industrial enzymes, such as α-amylase, *A. oryzae* has gained significant attention as a desirable host for the development of recombinant proteins (Kathiresan and Manivannan, 2006). *Aspergillus oryzae* is widely used in the production of soy sauce, organic acids such as citric and acetic acids, and commercial enzymes such as α-amylase. *Aspergillus niger* has significant hydrolytic aptitudes in the production of α-amylase and, due to its acid tolerance (pH < 3), it allows for the obfuscation of bacterial contamination.

### Filamentous fungi are ideal microorganisms for solid state fermentation (SSF), owing to their ability to colonize and infiltrate the solid substrate (Kathiresan and Manivannan, 2006). Because of their more widely accepted GRAS status, fungal α-amylase are recommended over other microbial sources.

### **2.4.3.2 Bacterial amylases**

α-amylase can be produced by a variety of microorganisms, but for commercial reasons, α-amylase is primarily derived from the genus Bacillus. α-amylase derived from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* have the potential to be used in a variety of industrial processes, including food, fermentation, textile, and paper manufacturing (Konsoula and Liakopoulou-Kyriakides, 2007).

Thermostability is a desirable property in the majority of industrial enzymes. Because of their stability, thermophilic enzymes sequestered from thermophilic organisms have found a variety of commercial applications. Because enzymatic liquefaction and saccharification of starch are accomplished at high temperatures (100-110oC), thermostable amylolytic enzymes are being researched to improve industrial starch degradation processes and are of great interest for the production of valuable products such as glucose, crystalline dextrose, dextrose syrup, maltose, and maltodextrins (Asgher *et al.,* 2007). *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are well-known thermostable-amylase producers, and have been widely used for commercial production of the enzyme for a variety of applications (Asgher *et al*., 2007). Several bacterial strains have been reported to produce thermostable -amylases using both SmF and SSF (Asgher *et al.,* 2007). However, SSF has been found to be more beneﬁcial than SmF and allows for cheaper enzyme production. SSF can only produce α-amylase from the genus *Bacillus*, and *B. subtilis, B. polymyxia, B.mesentericus, B. vulgarus, B. megaterium,* and *B.licheniformis* have all been used (Asgher et al., 2007). In the starch processing industry, thermostable amylases from *Bacillus stearothermophilus* or *Bacillus licheniformis* are currently used.

**2.5 Industrial application of α-amylases**

## **2.5.1 Starch conversion**

It has been reported that several bacterial strains can produce thermostable α-amylase using both SmF and SSF (Asgher *et al*., 2007). SSF, on the other hand, has been found to be more valuable than SmF and allows for less expensive enzyme production. *Bacillus subtilis, B. polymyxia, B.mesentericus, B. vulgaris, B. megaterium,* and *B.licheniformis* have all been used to produce -amylase by SSF (Asgher *et al.,* 2007). Thermostable amylases from *Bacillus stearothermophilus* or *Bacillus licheniformis* are as of now used in the starch processing industry (Prakash and Jaiswal, 2009). Initially, the α-amylase of *Bacillus amyloliquefaciens* was used but it has been replaced by the α-amylase of *Bacillus* *stearothermophilus* or *Bacillus licheniformis*. The enzymes from the *Bacillus* species are of special interest for large-scale biotechnological processes due to their remarkable thermostability and because efficient expression systems are available for these enzymes (Prakash and Jaiswal, 2009).

## **2.5.2 Detergent industry**

In terms of both volume and value, the detergent industry is the primary consumer of enzymes. The addition of enzymes to detergent formulations improves the detergent's ability to remove tough stains while also making the detergent environmentally friendly. Amylases are the second key enzyme used in the conceptualization of enzymatic detergent, and they are found in 90% of all liquid detergents (Gupta *et al*., 2003). These enzymes are used in laundry detergents and automatic dishwashing machines to degrade starchy food residues such as potatoes, gravies, custard, chocolate, and so on to dextrins and other smaller oligosaccharides (Mukherjee *et al*., 2009). Amylases have activity at lower temperatures and alkaline pH, allowing them to maintain the necessary stability under detergent conditions. Amylases' oxidative stability is one of the most significant priority for their use in detergents where the washing environment is highly oxidizing. Because starch is an attractant for many types of particulate soils, removing starch from surfaces is also important for providing a whiteness benefit. *Bacillus* or *Aspergillus* amylases are examples of amylases used in the detergent industry (Mitidieri *et al*., 2006).

## **2.5.3 Fuel alcohol production**

Ethanol is the most commonly used liquid biofuel. Because of its low cost and ease of availability in most parts of the world, starch is the most commonly used substrate for ethanol production (Chi *et al*., 2009). To obtain fermentable sugars, starch must be solubilized and then subjected to two enzymatic steps in this process. Liquidification and saccharification, in which starch is converted into sugar using an amylolytic microorganism or enzymes such as α-amylase are followed by fermentation, in which sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast Saccharomyces cerevisiae (Oner, 2006). The production of ethanol through yeast fermentation is critical to the economies of many countries around the world.

**2.5.4 Food industry**

Amylases are widely used in the processed-food industry, including baking, brewing, the preparation of digestive aids, the manufacture of cakes, fruit juices, and starch syrups (Couto and Sanromán, 2006). α-amylases are commonly used in the baking industry. These enzymes can be added to bread dough to degrade starch in flour into smaller dextrins, which are then fermented by yeast. The addition of -amylase to the dough increases the rate of fermentation and decreases the viscosity of the dough, resulting in increased volume and texture of the product. Furthermore, it produces additional sugar in the dough, which enhance the bread's flavor, crust color, and toasting qualities. In addition to producing fermentable compounds, -amylases have an anti-staling effect in bread baking and improve the softness longevity of baked goods, extending their shelf life (Gupta *et al*., 2003).

## **2.5.5 Textile industry**

Amylases are used in the textile industry to help with the desizing process. To verify a safe and reliable weaving process, sizing agents such as starch are decided to apply to yarn prior to fabric production. Starch is a very appealing size because it is inexpensive, widely available in most parts of the world, and easily removed. In the textile finishing industry, starch is subsequently removed from the synthetic material in a wet process. Desizing is the process of removing starch from fabric, which acts as a strengthening agent to keep the warp thread from breaking during the weaving process. The α-amylases remove disingenuously the size and do not intrusion the fibres (Ahlawat *et al.,* 2009). (Ahlawat *et al.,* 2009). Amylase from the Bacillus strain has long been used in the textile industry.

**2.5.6 Paper industry**

In the pulp and paper industry, -amylases are used to modify the starch of coated paper, resulting in low-viscosity, high molecular weight starch (Gupta *et al.,* 2003). The coating treatment improves the writing quality of the paper by making the surface of the paper suitably smooth and strong. The natural starch's viscosity is too high for paper sizing in this application, but this can be changed by partially diminishing the polymer with -amylases in batch or continuous processes. Starch is a good optimized agent for paper finishing, which improves quality, as well as a good paper coating. The size increases the stiffness and strength of the paper (Gupta *et al*., 2003).

# **CHAPTER THREE**

# **3.0 MATERIALS AND METHODS**

# **3.1 Substrate collection and processing**

The groundnut shells were gathered from local farmers in Kano State, Nigeria, they were then taken to a milling machine and milled into fine powder.

# **3.2 Sterilization of materials**

The glassware which include test tubes, MacCartney bottle, beakers, conical flask and measuring cylinder were washed with detergent and rinsed thoroughly with water. They were allowed to dry, wrapped in aluminum foil and then sterilized at temperature of 160 °C for 1 hour. The syringes and petri dishes were purchased sterile. The inoculating loop and cork-borer were sterilized by dipping in flame until they were red hot. The spatula was sterilized using alcohol and bent glass rod was sterilized with alcohol and flame.

# **3.3 Preparation of media**

Potato Dextrose Agar was prepared following the manufacturer’s specification while starch agar was prepared following the method described by Saha and Mazumdar (2019). The media were sterilized at 121oC for 15 minutes.

# **3.4 Isolation of microorganisms**

Amylase producing fungi were isolated from soil samples. The soil samples were collected from site where cassava wastes are discarded. The samples were serially diluted up to 10-4 and they were then plated on Potato Dextrose Agar using pour plate method. For 3-7 days, the plates were incubated at 30oC. After incubation, isolates with distinct colonies were carefully picked and sub-cultured on fresh media to obtain pure culture. Pure cultures were then stored in agar slants at 4oC.

# **3.5 Fungal isolates' Characterization**

The fungal isolates were described using their colonial morphology on the plates. Cultural and morphological characteristics such as nature of the hyphae, color of the colonies, appearance of the colonies and the growth rates were considered for proper characterization of the isolates. Microscopic examination of the isolates was also carried out as described by Fawole and Oso (2004). This involved picking a little amount of mycelial mat with a sterilized needle and putting it on clean glass slide, staining with lacto-phenol cotton-blue and covering with cover slip. Vegetative and reproductive structures were observed. Nature of sporangia, nature of spores, presence of septa and branching of the hyphae were reflected during microscopy. The isolates were identified using fungal atlas.

# **3.6 Screening of amylase producing microorganisms**

The isolated organisms were subjected to screening following the method described by Saha and Mazumdar (2019). The amylase agar contained (g/L): peptone, 0.90; (NH4)2HPO4, 0.40; KCl, 0.10; MgSO4,7H2O, 0.10; starch soluble, 10 and 2 % (w/v) agar-agar. The isolates were speckled on the amylase agar and incubated at 30oC for 7 days. After incubation the plates were flooded with iodine solution measured by 30 minutes of incubation. Plates were then wash away with duple distilled water and observed for starch hydrolysis zone of clearance about the colony growth. Microbial colonies showing the highest zone of clearance was selected as amylase producer.

# **CHAPTER FOUR**

# **4.0 RESULTS**

The results of this work aimed at optimizing alpha amylase production from locally isolated *Aspergillus* sp. using agricultural waste (groundnut shell) as substrate are documented in the subsections below:

# **4.1 Isolation and characterization of fungal isolates**

A total of six fungi were isolated from the various samples and they were coded as A1, A2, A3, A4, A5 and A6. The macroscopic and microscopic characteristics of the isolated fungi are presented in Table 2 while the microscopic view of their vegetative structure are shown in Plates 1-6.

**Table 2: Macroscopic and Microscopic characteristics of isolated fungi**

|  |  |  |  |
| --- | --- | --- | --- |
| Isolate Code | Colonial Characteristics | Morphological Characteristic Under Microscope | Tentative Identity |
| A1 | Filamentous with white hyphae, production of black spores was observed on the plate after 72hrs. The reverse of the plate was brown. | Conidiophores were hyaline, erect, simple, thick-walled, inflated at the apex forming globose vesicles, bearing conidial heads composed of catenulate conidia borne. | *Aspergillus niger* |
| A2 | Growth was rapid and filled the plate completely within a few days. Colonies were whitish. Dense and cottony which became greyish-brown with age, due to brownish sporangio-spores and brown black sporangia. Mycelia were interwoven | Well-developed hyphae, branched freely, coenocytic. Brown colored, smooth walled. Non-septate and erect sporangiophores developed from the hyphae | *Rhizopus stolonifer* |
| A3 | Pin-like green growth. | Non-branched conidiophore with rhizome end carried conidia. | *Aspergillus flavus* |
| A4 | Colonies with restricted growth, yellow-orange, ochraceous or buff. | Non-dense colonies, sporulated, amber-colored, flaky texture, white mycelium with yellow to pale orange or gray gold reverse. Strong presence of light brown sclerotia | *Aspergillus ochraceus* |
| A5 | Colonies showed slow growth, typically lilaceous-brown to blackish brown but also sometimes grey, buff or brown, suede-like to floccose, often pleasant powdery due to the production of ample conidia. | Conidiophores were more or less distinct from the vegetative hyphae, erect, straight, unbranched or branched only in the apical region, with geniculate sympodial elongation in some species. | *Cladosporium* sp*.* |
| A6 | Colonies were fast budding, flat, white to cream, dry and finely suede-like with no converse pigment. | Chains of hyaline, plane, one-celled, sub-globose to cylinder-shaped, sycophantic arthroconidia (ameroconidia) by the holoarthric fragmentation of uniform hyphae. | *Geotrichum candidum* |



Plate 1: Microscopic outlook of the vegetative structure of *Aspergillus niger* (x 40)

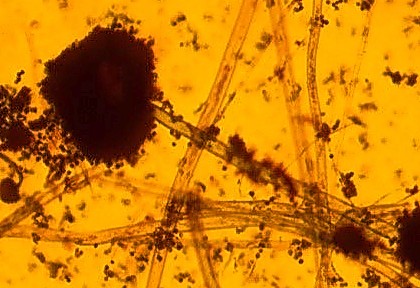


Plate 2: Microscopic outlook of the structure of *Rhizopus stolonifer* (x 40)



Conidium

Conidiophore

Plate 3: Microscopic outlook of the vegetative structure of *Aspergillus ochraceus* (x 40)



Plate 4: Microscopic outlook of the vegetative structure of *Aspergillus flavus* (x 40)



Plate 5: Microscopic outlook of the structure *Cladosporium* sp. (x 40)



Arthroconidium

Plate 6: Microscopic outlook of *Geotrichum candidum* (x 40)

# **4.2 Screening of isolated fungi for α-amylase activity**

Three out of the six isolates exhibited obvious zones of clearance on the amylase agar with the highest (greater than 1 cm) occurring in isolates A1 (1.6 cm) and A4 (1.2 cm) which significantly differ from others, as shown in Table 3. The isolate (A1) with the highest zone of clearance was therefore selected for further studies.

**Table 3: Zone of clearance (cm) of different fungal isolates**

|  |  |  |
| --- | --- | --- |
| Isolate code | Tentative name | Zone of clearance (cm) |
| A1 | *Aspergillus niger* | 1.6 |
| A2 | *Rhizopus stolonifer* | 0.5 |
| A3 | *Aspergillus ochraceus* | 1.0 |
| A4 | *Aspergillus flavus* | 1.2 |
| A5 | *Cladosporium* sp. | 0.7 |
| A6 | *Geotrichum candidum* | - |

# **CHAPTER FIVE**

# **5.0 DISCUSSION**

The present study aims at optimizing α-amylase production from groundnut shell using locally isolated *Aspergillus* sp. The results of this research work revealed that *Aspergillus* sp. could produce α-amylase from the substrate (groundnut shell). A total of six (6) fungal isolates were isolated from different samples (Table 2). The isolates' macroscopic and microscopic characteristics were investigated and reported on (Table 2). The isolates were then screened for α-amylase activity to ascertain if they possess the ability of producing the enzyme. This was done using amylase agar as reported in the materials and methods section and the zone of clearance exhibited by the different isolates were observed. The observed zone of clearance during hydrolysis test showed that isolate A1 had the highest diameter of 1.6 cm, which was higher than the other isolates (Table 3). The zones of clearance produced around the colonies indicate that the fungal isolates have the potential to produce extracellular amylase. The research findings are consistent with the work of Olakusehin and Oyedeji (2021), who asserted that *Aspergillus flavus* S2-OY possessed amylolytic properties. Sahnoun *et al*. (2015) also reported that *Aspergillus oryzae* S2 possess amylolytic properties. The findings of this study show that groundnut shell, a cheap and readily available substrate, can be used to produce value-added products such as -amylase.**5.1 CONCLUSION**

*Aspergillus* sp. was proficient to grow and yield good levels of α-amylase using groundnut shell, which is a no-cost agricultural substrate. Further studies should be conducted using the fungal strain and substrate for wide utilization in the biotechnological industries. The use of groundnut shell as the enzyme substrate was very promising as reasonable yields were recorded. Therefore, these findings suggest that groundnut shell, which is largely considered a waste, can be used as a source of cheap agro-industrial substrate for the enzyme production.

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