

**PRODUCTION AND OPTIMIZATION OF GLUCOAMYLASE
USING *CANDIDA FAMATA* ISOLATED FROM FERMENTED MASHED
PINEAPPLE MUST UNDER SOLID STATE FERMENTATION**

BY

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AUGUST, 2017

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BY

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE
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AUGUST, 2017

DECLARATION

I declare that the work in this dissertation entitled “Production and optimization of glucoamylase using *Candida famata* isolated from fermented mashed pineapple must under solid state fermentation” has been carried out by me in the Department of Microbiology. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Grace Michael AKPAN

Signature

Date

CERTIFICATION

This dissertation entitled PRODUCTION AND OPTIMIZATION OF GLUCOAMYLASE USING *CANDIDA FAMATA* ISOLATED FROM FERMENTED MASHED PINEAPPLE MUST UNDER SOLID STATE FERMENTATION by Grace Michael AKPAN meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Mr. and Mrs. Akpan

ABSTRACT

The present study was an investigation on comparative glucoamylase production by *Candida famata* isolated from spontaneously fermented pineapple must, using wheat bran, maize bran and the combination of both substrates in equal ratios under solid state fermentation conditions. Various cultural parameters were monitored and subsequently optimized for the production of glucoamylase using different agroindustrial residues and processing mills. The pretreatment of substrates was milling to 2 - 3mm particle size. Mixed bran of maize and wheat gave the highest glucoamylase yield of 25.34 U/ml under predetermined optimum fermentation conditions (30°C, pH 5.0, 10⁶ cells/ml inoculum concentration, 1:3 substrate to nutrient solution ratio and 3 days of fermentation). In contrast, lower activity of 17.93 and 13.28 U/ml were obtained in wheat bran and maize bran respectively. The mixed bran served as a better substrate in the production of glucoamylase. Highlighting the potential of this approach as an alternative strategy for waste management and sustainable production of enzymes could perhaps act as a springboard for applicable source in many biotechnological processes.

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CHAPTER ONE

1.0

INTRODUCTION

1.1 Background

Glucoamylases also known as (α -1,4-glucan glucohydrolase, amyloglucosidase) are important enzymes that allow the hydrolysis of starch and related polymers to glucose, they can be obtained from microbial as well as other sources. The major application of glucoamylase is the saccharification of partially processed starch/dextrin to glucose, which is an essential substrate for numerous fermentation processes and a range of food and beverage industries. Glucoamylase consecutively hydrolyzes alpha 1,4-glycosidic bonds from the non-reducing ends of starch and alpha 1,6-glucosidic linkages in polysaccharides yielding glucose as the end-product, which in turn serves as a feedstock for biological fermentations (Norouzian *et al.*, 2006; Siddhartha *et al.*, 2012). A more technical name is (1,4) glucan glycohydrolase where glucan is the name for a series of glucose units attached and glycohydrolase describes the breaking of the bond between two glucose units. Glucoamylase has a wide range of use among industrial enzymes. This enzyme is used in the baking industry, the brewing process, and the most important application of glucoamylase is the production of high glucose syrups, it also has various applications in major areas of food processing, animal nutrition, fermentation biotechnology, paper making, fabric industries and in whole grain hydrolysis for the alcohol industry (Selvakumar *et al.*, 1996; Zambare, 2010). Glucoamylases of microbial origin are divided into exo-acting, endo-acting, debranching and cyclodextrin producing enzymes. Glucoamylases hydrolyze α -1,4 and α -1,6 linkages and produce glucose as the sole end product from starch and related polymers (Svensson *et al.*, 2000; Parbat and Singhal, 2011).

Glucoamylases are industrially important hydrolytic enzymes of biotechnological significance and are currently used for dextrose production, confectionary, baking and in pharmaceuticals. Glucoamylase is an economically important enzyme because of its capacity to hydrolyse starch and related polymers into β -D-glucose as the sole end product, the principal industrial use of glucoamylase is therefore, the production of glucose, which in turn serves as a feedstock for biological fermentations in the production of ethanol or high fructose syrups, it is a key enzyme in the production of saké and soy sauce. Glucoamylase is also used to improve barley mash for beer production. (Pandey *et al.*, 2000; Pavezzi *et al.*, 2008; Zambare, 2010).

Enzymes are produced by various microorganisms including bacteria, fungi and yeast and are considered as important products obtained for human needs through microbial sources. The advantage of using microorganisms for the production of enzymes is that bulk production is economical and microbes are easy to manipulate to obtain enzymes with desired characteristics. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status (Sindhu *et al.*, 2009).

Solid State Fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free water. The concept of using solid substrates is probably the oldest method used by man to make microorganisms work for him. In recent years, SSF has shown much promise in development of several bioprocesses and products. Solid state offers greatest possibilities when fungi are used. Unlike other microorganisms, fungi typically grow in nature on solid substrates such as pieces of wood, seeds, stems, roots and dried parts of animals such as skin, bones and fecal matter that is low in moisture. In SSF, the moisture necessary for microbial growth exists in an absorbed state or in complex with solid matrix. However, SSF differs from solid substrate fermentation, since in solid substrate fermentation

the substrate itself acts as a carbon source and occurs in the absence or near absence of free water by employing a natural substrate or inert substrate as solid support (Kumar *et al.*, 2003).

The aim of SSF is to bring cultivated fungi or bacteria in tight contact with the insoluble substrate and to achieve the highest nutrient concentration from the substrate for fermentation. This technology so far is run only on a small scale, but has an advantage over submerged fermentation. Two types of SSF systems have been distinguished depending on the type of solid phase used. The most commonly used system involves cultivation on a natural material and less frequently on an inert support impregnated with liquid medium (Ooijkaas *et al.*, 2000). The use of agroindustrial wastes for enzymes production can become economically viable for the application of these biocatalysts in large scale, considering that one of the major problems in the enzymes utilization in industrial processes is the high cost of the microbial culture media, about 30 - 40% of the cost enzyme production (García-Martínez *et al.*, 2010)

1.2 Statement of Research Problem

A recent focus of research and development effort is the application of glucoamylases in the enzymatic degradation of carbohydrate-rich polysaccharides for the production of energy syrup. The development of microbial strains, media composition and process control all have contributed to the achievement of high levels of extracellular glucoamylases.

However, the cost of glucoamylase are still too high for the establishment of a cost effective production of energy syrup using it. One approach to overcome this obstacle is to employ Solid State Fermentation (SSF). This process (SSF) has the potential to significantly reduce the enzyme production costs because of lower energy requirements, increased productivity, smaller effluent volumes and simpler fermentation equipment. Agroindustrial residues are

generally considered the best substrates for SSF processes and use of SSF for the production of enzymes is no exception to that (Ellaiah *et al.*, 2002).

Commonly used carbon sources are dextrin, fructose, glucose, lactose, maltose and starch which are very expensive for commercial production of these enzymes. Various agricultural byproducts like wheat bran, rice husk, sugarcane bagasse, potato residue, rice bran, green gram bran, black gram bran, maize bran, can be abundantly used (Siddhartha *et al.*, 2012).

The food, beverage and agroindustries produce large quantities of residues that pose serious problems of disposal, inspite of them being sources of biomass and nutrients. These substrates can be used for the production of valuable compounds such as enzymes and various secondary metabolites. There is an environmental concern that most of the agroindustrial wastes contain phenolic compounds and/or other compounds of toxic potential; which may cause deterioration of the environment when the waste is discharged to nature (Soccol *et al.*, 2003; Solange *et al.*, 2012).

1.3 Justification of the Study

The production of amylolytic enzymes, particularly glucoamylase on solid substrate is more advantageous for the fermentation industry. In the Solid State Fermentation process, the solid substrate not only supplies the nutrients to the culture, but also serves as a thriving environment for the microbial cells (Joshi *et al.*, 1999).

The use of yeast to produce enzymes offer certain advantages, such as a moderate temperature for microbial growth, high metabolic diversity and rapid cell growth, which results in shorter fermentation cycles and easy adaptation to different cultivation conditions (Kato *et al.*, 2007). The use of agroindustrial wastes as substrate in solid state fermentation reduces enzyme production cost and contributes to minimize environmental problems caused

by the agroindustry. They are also inexpensive, readily available, renewable resource, has lower energy requirement and produce lesser waste water (Singhania *et al.*, 2009).

Large amount of wastes is generated every year from the industrial processing of agricultural raw materials, most of these wastes are used as animal feed or burned as alternative for elimination. However, such wastes usually have a composition rich in sugars, minerals and proteins, and therefore, they should not be considered “wastes” but raw materials for other industrial processes. The economics of enzyme production using inexpensive raw materials can make an industrial enzyme process competitive (Solange *et al.*, 2012; Shubhang and Vinod, 2014). Wheat bran, paddy husk, rice processing waste and other starch containing wastes have gained importance as supports for growth during enzyme production (Anto *et al.*, 2006). Microbial enzymes are preferred to plant enzymes due to their short growth period, higher productivity and thermostability (Mishra and Behera, 2008). Yeasts from several genera, such as *Ambrosiozyma*, *Arxula*, *Aureobasidium*, *Candida*, *Debaryomyces*, *Lipomyces*, *Saccharomyces*, *Saccharomycopsis* (Endomycopsis) and *Schwanniomyces*, rank among the best producers of glucoamylases (Chi *et al.*, 2009).

1.4 Aim of the Study

The aim of this study was to produce and optimize glucoamylase using *Candida famata* under solid state fermentation conditions.

1.5

Specific Objectives

The specific objectives of this study were to:

1. Isolate and characterize *Candida famata* from fermented pineapple must.
2. Determine the proximate composition of the substrates (wheat bran and maize bran).
3. Screen *Candida famata* for the production of glucoamylase and determine its glucoamylase-producing potential under solid state fermentation conditions.
4. Optimize cultural parameters such as moisture, pH, incubation temperature, inoculum concentration and incubation time for the production of glucoamylase.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Amylases

Enzymes are single chain or multiple chain proteins (molecular weight of 15,000 to several million daltons) that act as biocatalyst with the ability to promote specific chemical reaction under mild condition in most living organisms. The use of enzymes date from much longer than their ability to catalyze reactions was recognized and their chemical nature was known. The first completely enzymatic industrial process was developed in the year 1960 (Shuler and Kargi, 2008).

Enzymes responsible for degradation of starch and related saccharides are produced either by prokaryotic or eukaryotic organisms. Starch is not hydrolysed completely by a single enzyme but is attacked by a variety of enzymes, generally called amylolytic enzymes, produced by a large number of microorganisms including bacteria, fungi, yeast, algae and actinomycetes which have the ability to utilise starch as energy and carbon source (Antranikian, 1992). There are two scenarios regarding the use of enzymes, either the enzymes are used to convert the raw material into the main product, or the enzymes are used as additives to alter a functional characteristic of the product. In the first case, the enzymatic process is undertaken in optimized and controlled conditions to enhance the catalytic potential of the enzyme, whereas in the second situation it is more difficult to assure optimal conditions and to control the enzymatic reaction. An example of the first case is the use of immobilized glucose isomerase for the production of high-fructose syrups (HFS), and an example of the second scenario is the use of fungal proteases in dough making (Salleh *et al.*, 2006; Illanes, 2008).

Starch processing, which is undertaken in two steps, involves liquefaction of the polysaccharide using bacterial α -amylase, followed by saccharification catalyzed by fungal glucoamylase. Enzymes are used in the production of over 500 commercial products, they have a broad range of applications from food to detergents. Most enzymes are commercially available and used to enhance the product quality in food, detergents, leather, paper, cosmetics, and pharmaceuticals.

Commercial enzymes include lipase, amylases, proteases, pectic enzymes, and milk clotting enzymes (rennet). Enzymes are products of living organisms and have been used in the industry for many years due to their catalytic activities.

Enzyme activity depends on temperature, substrate, pH, inhibitors among others, and should be optimized for each process. Enzymes can be isolated from plants and mammalian tissues, or can be produced by microorganisms. However, microbial enzymes are preferred due to their availability and specificity (Johannes and Zhao, 2006). Many enzymes are conjugated proteins containing specific prosthetic groups. The complete enzyme is called a holoenzyme; the protein part is called the apoenzyme, and the non-protein moiety the prosthetic group. The prosthetic group is often only loosely attached to the protein and can be separated by dialysis. In this case, the protein moiety is termed the apoenzyme and the prosthetic group the co-enzyme or co-factor (deMan, 1996). At the first stage of enzyme mechanism, the substrate combines with the catalyst into a substrate-enzyme complex. The complex is unstable under reaction conditions and is quickly decomposed, the enzyme is regenerated. The substrate, which emerges from the reaction, can be in the final, changed form or in a highly activated state. When an enzyme reacts with its substrate, only the “active site,” which is certain regions of the protein molecule, participates in the process. Special groups of amino acid residues produced by the sequence and particular folding of the enzyme protein form active

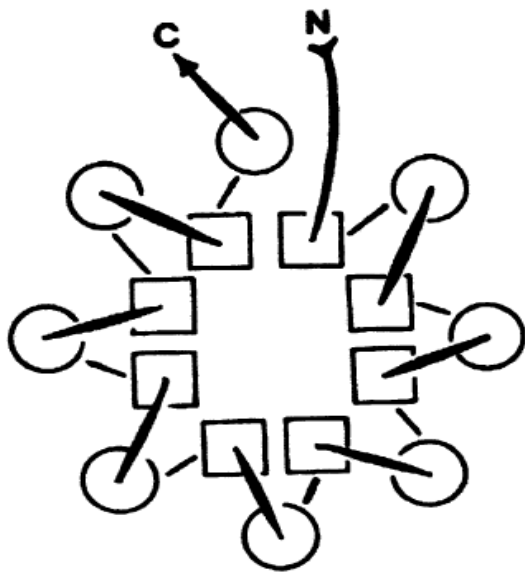
sites. The portion of the protein molecule outside the active regions provides the backbone and support for the active sites (Ali *et al.*, 2014).

Amylases are enzymes which are capable of hydrolyzing the α -1,4-glucosidic linkages of starch. Amylases are mainly used in the food processing industry such as for baking, brewing, high-fructose corn syrup, alcoholic beverages, cakes, and fruit juices. Although amylases are found in plants and animals, microbial amylases are the most common in industries, α -amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) are two major amylases that industry has taken advantage of their starch-degrading properties. Starch is produced mainly in higher plants, and is water insoluble (Bigelis, 1993; Pandey *et al.*, 2000; Aiyer, 2005; Couto and Sanroman, 2006). It is composed of two components: amylose and amylopectin. Amylose is a mainly linear polysaccharide which is formed by α -1,4 linked D-glucose residues and some α -1,6-branching points. Amylopectin has a highly branched tree-like structure, the proportion of branches is an important property of the substrate because enzymes hydrolyze different substrates with differing specificities. Several amylolytic enzymes hydrolyze starch or its degradation products and the actions of these enzymes can be divided into two categories; endoamylases which break down linkages randomly in the interior of the starch molecule and exoamylases which hydrolyze the polysaccharide from the non-reducing end thus producing short end-products. α -Amylase (endo- α -1,4-D-glucan glucohydrolase) hydrolyzes the α -1,4-D-glucosidic linkages in the linear amylose chain, randomly. However, glucoamylase (exo- α -1,4-D-glucan glucohydrolase) cleaves the α -1,6-linkages at the branching points of amylopectin as well as α -1,4-linkages (Pandey *et al.*, 2000). Glucoamylase has got a wide application in the enzymatic production of glucose and fructose from starch for food and fermentation industries. A broad variety of organisms, among them yeasts, are producers of this enzyme.

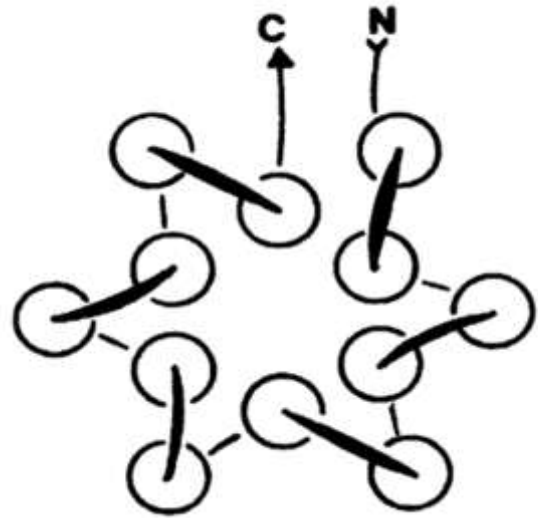
Yeasts from several genera, such as *Ambrosiozyma*, *Arxula*, *Aureobasidium*, *Candida*, *Debaryomyces*, *Lipomyces*, *Saccharomyces*, *Saccharomycopsis* (*Endomycopsis*) and *Schwanniomyces*, rank among the best producers of glucoamylases (Chi *et al.*, 2009).

2.2 Structure of Glucoamylase

It adopts a helical (α/α) α_6 -barrel fold (Fig 2.1), which consists of six mutually parallel α -helices forming an inner core (helical barrel mimicking the inner β -barrel of α -amylase and β -amylase), which is covered by a peripheral set of six further α -helices. The peripheral α -helices are parallel to each other, but antiparallel to the inner core of α -helices. Two Glu residues (gene encoding for glucoamylase) are responsible for catalysis (Aleshin *et al.*, 1992; Ševčík *et al.*, 1998).



a



b

Figure 2.1: Schematic representation of secondary structure of glucoamylase. The squares and circles represent the β -strands and α -helices respectively. C; Carbon; N; Nitrogen.

Adapted from Aleshin *et al.* (1992).

2.2.1 Modes of Action of Amyolytic Enzymes

Degradation of starch is essentially performed by the four groups of enzymes; endo and exo-amylases acting primarily on α -1,4-linkages, debranching enzymes attacking mainly the α -1,6-linkages, and cyclodextrin glycosyltransferases that degrade starch by catalysing mainly cyclisation and disproportionation reactions. Endoamylases cleave only the α -1,4-bonds in starch in the inner regions of the starch molecule by passing the α -1,6-branching points of amylopectin. The α -amylase (EC 3.2.1.1) is the best known endoamylase. It causes a rapid loss of viscosity of the starch solution. These enzymes are often divided according to their degree of hydrolysis of substrate into two categories: liquefying (30 - 40%) and saccharifying (50 - 60%). This division is widely used to describe the properties of α -amylases, thus the products of endoamylases are oligosaccharides of varying lengths.

Exoamylases also cleave the α -1,4-bonds, e.g. β -amylase (EC 3.2.1.2), but some of them are able to attack the α -1,6-bonds, e.g. glucoamylase (EC 3.2.1.3). These enzymes act externally on substrate bonds from the non-reducing end of starch and hence produce only low molecular weight products from starch, e.g. maltose and glucose respectively.

Pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) may be the examples of debranching enzymes. Both are specific for α -1,6-bonds in starch (amylopectin), related polysaccharides and branched limit dextrins. According to their inability or ability to degrade also the α -1,4-glucosidic bonds, pullulanases are classified into two categories pullulanase I and pullulanase II, respectively. Pullulanase type II is usually referred to as α -amylase-pullulanase or amylopullulanase. However, to make it clear the specificity should be proved which enzyme it refers to (Wind, 1997).

Cyclodextrin glycosyltransferases (CGTases, EC 2.4.1.19), produce cyclodextrins from starch, the rings are composed of 6, 7 or 8 glucose units bound by α -1,4-bonds. The CGTases catalyse intra and intermolecular reaction of glycosyl transfer (Pócsi, 1999).

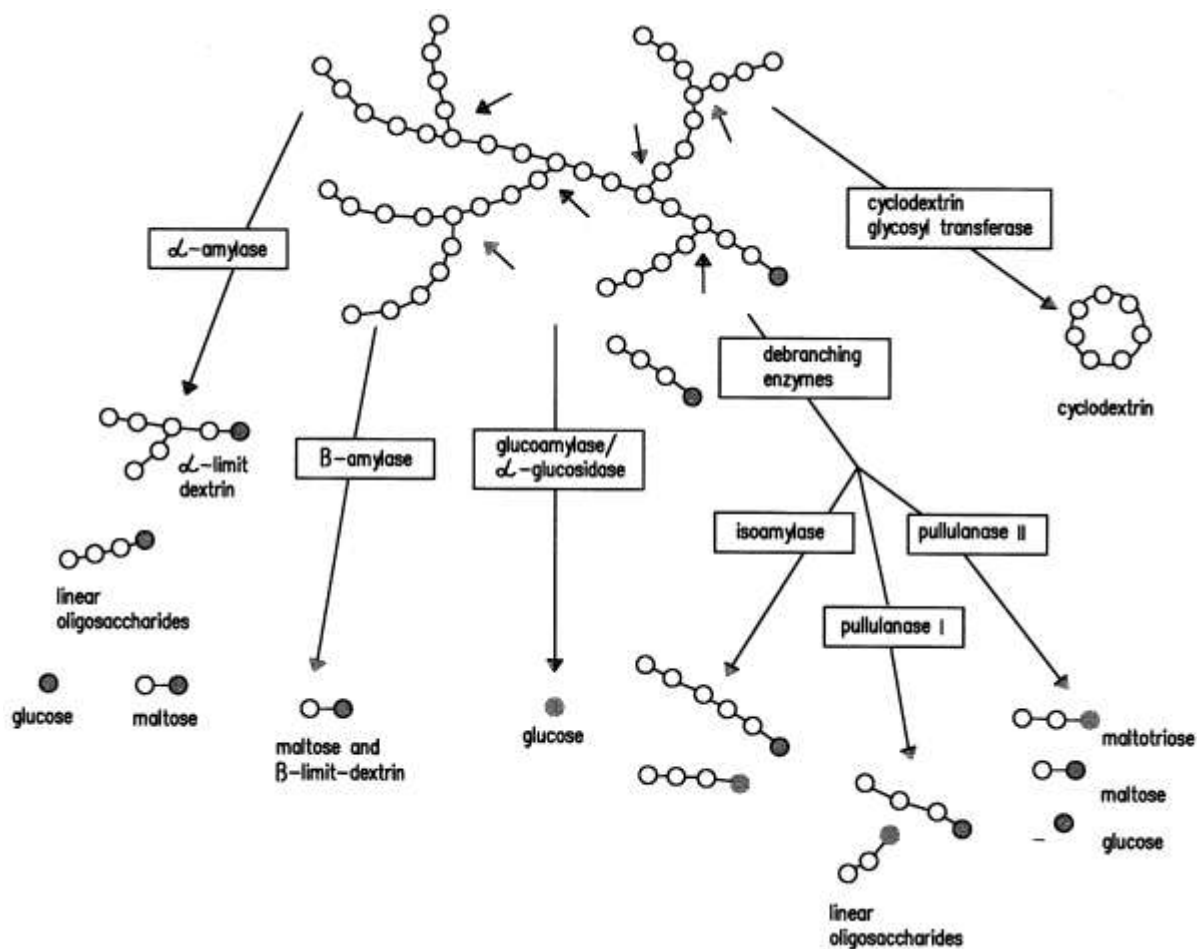


Figure 2.2: Modes of action of amylolytic enzymes. ●; Reducing α -D-glucose residue; ○ ; non-reducing α -D-glucose residue. Arrows indicate the α -1,6-branching points in the starch molecule. (Bertoldo and Antranikian, 2002).

Microorganisms play a prominent role in generating variety of products having applications in different areas, such as energy, food, chemicals, diagnostics, pharmaceuticals among others. They are the source of enzymes, organic acids, amino acids, antibiotics, vitamins, single cell proteins and other commodity chemicals having commercial importance. The advances in microbial fermentation techniques have led to the development of ecofriendly processes replacing some of the conventional chemical processes.

Microbial enzymes are used to catalyse reactions which otherwise would require very harsh chemical and physical conditions, that results in generating undesired byproducts and toxic effluents. They are mainly used in food processing, chemical transformations, therapeutics and molecular biology techniques.

Enzymatic hydrolysis of starch first produces short-chain polymers of glucose called dextrans, then the disaccharide maltose, and finally glucose. The enzymes are biocatalysts, which act in relatively mild conditions of temperature, pH, and pressure. The importance of enzymes in industrial processes can be assessed by the fact that they perform very specific reactions/modification of the substrate without formation of unwanted byproducts in comparison to the chemical catalyst. Processes based on enzymes are ecofriendly, require less energy input and do not require reaction vessels made up of expensive corrosion-resistant materials.

In enzymatic processes, the need for extensive refining of the target product is greatly reduced since enzymes catalyze both stereo- and regio-selective modification of substrate in comparison to the chemical catalysts in which side reactions are very common. Microbes are excellent source of enzymes because of their rapid growth, easy cultivation in large fermenters and genetic manipulation (Tek *et al.*, 2006).

2.2.2 Applications of glucoamylase

The glucoamylase preparation is widely utilized for the industrial production of crystalline D-glucose, high fructose corn syrups, wine, ethanol, beer, and in other fermentation processes.

Starch Hydrolysis

Starch is hydrolysed by a two stage process: liquefaction then saccharification using a mixture of amylolytic enzymes; thermostable amylase followed by glucoamylase (Lee, 1991). The first step called liquefaction, involves treatment with amylase at 85 - 100°C. The dextrin is soluble and so can be passed through a bed of immobilized glucoamylase which accomplishes further hydrolysis to glucose units. High glucose syrups contain 96 - 98% D-glucose. These are used either for production of crystalline D-glucose or as a starting material for the production of high fructose syrups.

High Fructose Corn Syrup (HFCS) Production

High Fructose Corn Syrup (HFCS) is used as a sweetener in beverages and foods. It has fewer calories and is cheaper than sucrose. An aqueous slurry of starch (35 - 45% DS) is liquefied and partially hydrolysed using a bacterial thermostable α -amylase. After liquefaction, the slurry is adjusted to pH 4.5 and the temperature is lowered to about 60°C, glucoamylase, the saccharifying enzyme, is then added to produce glucose. Several stages of purification follow to reach the required glucose concentration (96 - 98%), further purification steps follow, to obtain a highly refined high fructose corn syrup. Tapioca starch from cassava, could be treated similarly with α -amylase for liquefaction and glucoamylase for saccharification, to form glucose. Whole potato has also been successfully converted to glucose, maltose and soluble oligosaccharides by multiple enzyme treatment (Lee, 1991).

Baking Industry

Glucoamylases are also used in commercial baking to improve flour quality, retard dough staling and improve dough, giving more efficient machinability. They have also been used to enhance bread crust color, bleach flour, improve the quality of high-fibre baked products and reduce the phytate content in whole grain formulations (James and Simpson, 1996). Fungal and bacterial enzymes which are commercially available for use in bakery processing include amylase, proteases, glucoamylases, pentosanases among others.

Starch Hydrolysis for Alcohol Production

Processes are in operation to convert starch waste enzymatically into mono and disaccharides which are then fermented by yeast or *Zymomonas* bacteria into alcohol. The first step in the process is the liquefaction of starch with α -amylase, followed by saccharification with glucoamylase to form a high yield of glucose. Debranching enzymes like isoamylase and pullulanase could be added to improve glucose yields (Linko and Wu, 1993).

2.3

Yeast

Yeast are the simplest eukaryotic organism of our days, they are unicellular microorganisms classified in the kingdom Fungi. Nevertheless, yeasts were probably the first microorganism to be domesticated and since early in human history. Nowadays, yeast has become a key microorganism for many types of industrial and food processing manufactures. Yeasts are found in diverse natural environments; colonizing from terrestrial, to aerial and aquatic environments.

They can be found on decomposing fruits, on soils, as opportunistic pathogens in human beings, in the gut of the fish and free living in the sea. In general they contribute to the decay of organic materials, but their successful colonization is intimately related to their ability to

physiologically adapt to diverse milieu. Hitherto, approximately 1500 species has been described (Kurtzman *et al.*, 2011; Fábio *et al.*, 2013). Yeasts are widely distributed in nature and have been isolated from soil, air and plant surfaces. The population of microflora on the substrate always depends upon the pH of the substrate. Since, fruits are acidic in nature they are predominantly inhabited by yeasts (Deepak, 1994).

Yeasts have been used by humans for thousands of years with wide application, both fundamental and industrial, in science, food, medicine and agriculture. They are traditionally involved in many food fermentations and the manufacture of products such as beers, ciders, wines, saké, baked goods, cheese, sausages and other fermented foods. Industrial processes long involved yeast in the production of fuel ethanol from Single Cell Protein (SCP) for animal feed or industrial enzymes, vaccine production and carotenoids (Buzzini, 2000). Enzymes from yeast are increasingly being used in industries to facilitate the production processes and reduce the energy cost of the finished product particularly in the food industry. The search for new yeast enzymes having potentials for industrial applications continues to grow. Yeasts such as *Pichia pastoris*, *Saccharomyces cerevisiae* and *Hansenula polymorpha* are currently used for the industrial production of proteins and enzymes, including pharmaceutical proteins (Johnson and Echavarri, 2011). Fungi and yeasts seem to be favorable than bacteria because of their ability to grow on the substrates which are low in water content. Due to high water activity requirement, bacterial cultures might not be suitable for Solid State Fermentation. However, Pandey in 1992 reported that bacterial culture can be well managed and manipulated for SSF process. It seems that the high yield in SSF as compared to Submerged Liquid Fermentation SLF is due to the growth of the microorganisms in an environment similar to their natural habitat, resulting in higher metabolic activities.

Scragg (1991) observed that yeast growth is affected by a number of factors. These include composition of medium commonly sugar source, aeration (oxygen), agitation of the medium, pH, temperature and period of propagation among others. The main carbon and energy source for most yeast is glucose which is converted via the glycolytic pathway to pyruvate and by the Krebs cycle to anabolites and energy in the form of ATP.

Yeasts are further classified according to their modes of further energy production from pyruvate: respiration and fermentation. These processes are regulated by environmental factors, mainly glucose and oxygen concentrations.

Prescott and Dunn (1959) observed that yeasts grow well at acidic pH (acidophilic organisms). For industrial propagation low pH is helpful in restricting the development of many bacterial contaminations; however, the color of the yeast may be affected at low pH. The pH of the media is commonly adjusted by the addition of H₂SO₄, NH₃, Na₂CO₃ or NaHCO₃ to the substrate.

Blackwell *et al.* (1997) observed that varying concentrations of magnesium sulfate were supplemented under the above optimized levels of urea, diammonium phosphate (DAP) and orthophosphoric acid (OPA) for yeast growth. Results showed that cell growth, ethanol yield and fermentation efficiency increased with magnesium sulfate addition and 10kg concentration gave maximum ethanol content of 9.6% (v/v) ethanol with remaining sugars, 1.32, final cell count 3.6×10^8 /ml and yeast yield 12.4 g/L.

Deficiencies and imbalances in minerals and cations serving as cofactors for glycolytic and other enzymatic reactions can result in fermentation arrest. Magnesium plays a key role in metabolic control, growth and cell proliferation, glycolytic pathway, and subsequently ethanol production.

2.4

Classification and Identification of Yeasts

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features. The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50 - 60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application. Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. They are distinguished from most fungi by their usual existence as single ovoid cells about 8 μm long and 5 μm in diameter, doubling every 1-3 hours in favorable media, yeasts may reproduce asexually or sexually (Glazer and Nikido, 1995).

2.4.1 Yeast reproduction

a) Asexual reproduction

Yeast can be classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. Buds may arise either on yeast cells or on hyphal cells. Budding is initiated by the formation of a small evagination or outgrowth at some point on the surface of the cell. The parent cell remains more or less constant in size during subsequent development, while the bud (blastoconidium) increases in size to form a new cell, which usually after some time separates from the parent (mother) cell. Reproduction by fission is the duplication of an asexual cell by means of a septum growing inward from the cell wall to bisect the long axis of the cell. The newly formed fission cells, which are termed

arthroconidia (arthrospores), elongate and the process is repeated. Recurrent fission by a cell may give rise to transverse multiple scars or annellations (Kurtzman *et al.*, 2011).

b) Sexual reproduction

Sexual union in the yeasts take place either between two somatic cells or between two ascospores which assume the function of copulating gametangia, unite and form a zygote cell. Eventually an ascus forms which contains ascospores, their number depending on the number of nuclear divisions which took place and on the subsequent development of the nuclei. Four or eight ascospores per ascus are the usual number, but other numbers may also be encountered. The reproduction of yeast, proceeding by the formation of buds on the cell surface, but sexual reproduction can be induced under special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and α . Each type can develop by budding into other haploid cells. The mating of an a haploid cell and an α haploid cell yields a normal $a\alpha$ diploid cell. Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or α/α) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer and Nikido, 1995).

2.5

Pineapple

Pineapple (*Ananas comosus*) belonging to the Bromeliaceae family is grown in different parts of Nigeria either for export or for the local market. Pineapples are sliced and eaten fresh in the homes, or processed into fruit juices for consumption or concentrates for future use. Pineapple as a fruit crop has a lot of economic, nutritional, medicinal, and industrial importance. Pineapple as food for human consumption contains about 81.2 - 86.2% moisture, 13 - 19% total solids of which sucrose, glucose, and fructose are the main components, in

addition to 2 - 3% fibre and rich source of vitamin C. About 0.6 - 1.2% of pineapple is acid of which 87% is citric acid and 13% is malic acid. The pH of pineapple is acidic, which is 3.71 (Sarah *et al.*, 1997).

Lipids and nitrogenous compounds constitute 0.1% of which 25 - 30% of the nitrogenous compounds are true proteins. The fruit is also rich in calcium (Ca) and also has proteolytic activity due to the presence of an enzyme bromelin (used as a meat tenderizing agent and for medicinal purposes). The consumption of pineapple enhances the detoxification of the human body and prevents blood clotting. It also prevents kidney problems, protects the heart, regulates stomach acidity and helps prevent constipation. Its origin has been traced to Brazil and Paraguay in the Amazonic basin where the fruit was domesticated. Thailand, Philippines, Brazil and China are the main pineapple producers in the world supplying nearly 50% of the total output (FAO, 2004). Other important producers include India, Nigeria, Kenya, Indonesia, México and Costa Rica and these countries provide most of the remaining fruit available (50%), (Medina and Garcia, 2005).

2.5.1 Morphology of pineapple

The pineapple plant is a short herbaceous perennial with 30 - 80 cm trough-shaped and pointed leaves 30 - 100 cm long, surrounding a thick stem. This shape of the plant has to drive water onto the stem, this water might be absorbed by axils. The early inflorescences has about 100 - 200 flowers. Flowers of pineapple are spirally placed and each is supported by bracteas. Each flower consists of 3 calyxes, 3 bluish corollas, 6 filaments and a carpel with three parts of stigma. Inflorescence goes to bloom about 3 weeks and it blooms from down to up. Pineapples are auto-sterile and fruits developed are parthenocarpic (COVECA, 2002). A temperature range between 23 to 24°C is optimal for growing pineapple (FAO, 2002).

2.6

Agroindustrial Wastes

Agroindustrial wastes are generated during the industrial processing of agricultural or animal products. Those derived from agricultural activities include materials such as straw, stem, stalk, leaves, husk, shell, peel, lint, seed/stones, pulp or stubble from fruits, legumes or cereals (rice, wheat, corn, sorghum, barley), bagasses generated from sugarcane or sweet sorghum milling, spent coffee grounds, brewer's spent grains, and many agroindustrial residues.

These wastes are generated in large amounts throughout the year, and are the most abundant renewable resources on earth. They are mainly composed of sugars, fibres, proteins, and minerals, which are compounds of industrial interest. Due to the large availability and composition rich in compounds that could be used in other processes, there is a great interest on the reuse of these wastes, both from economical and environmental view points. The economical aspect is based on the fact that such wastes may be used as low-cost raw materials for the production of other value-added compounds, with the expectancy of reducing the production costs. The environmental concern is because most of the agroindustrial wastes contain phenolic compounds and/or other compounds of toxic potential which may cause deterioration of the environment when the waste is discharged to nature. Large amount of the agroindustrial wastes are mainly composed of cellulose, hemicelluloses and lignin, being called "lignocellulosic materials".

In lignocellulosic materials, these three fractions are closely associated with each other thus constituting the cellular complex of the vegetal biomass, and forming a complex structure that act as a protective barrier to cell destruction by bacteria and fungi. Basically, cellulose forms a skeleton which is surrounded by hemicellulose and lignin (Fig 2.3).

The cellulose structure is composed only of glucose units, i.e., it is a homopolymer where units of cellobiose (two anhydrous glucose rings joined via a β -1,4 glycosidic linkage) are sequentially repeated (Klemm *et al.*, 1998). On the contrary of the cellulose, hemicellulose is a heterogeneous polymer usually composed of five different sugars (L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose) and some organic acids (acetic and glucuronic acids, among others). The structure of the hemicellulose is linear and branched. The backbone of the hemicellulose chain can be formed by repeated units of the same sugar (homopolymer) or by a mixture of different sugars (heteropolymer). According to the main sugar in the backbone, hemicellulose has different classifications e.g. xylans, glucans, mannans, arabinans, xyloglucans, arabinoxylans, glucuronoxylans, glucomannans, galactomannans, galactoglucomannans and β -glucans.

The percentage of cellulose, hemicellulose and lignin is different to each waste since it varies from one plant species to another, and also according to the process that the agricultural material was submitted. In addition, the ratios between various constituents in a single plant may also vary with age, stage of growth, and other conditions.

Usually, cellulose is the dominant fraction in the plant cell wall (35 - 50%), followed by hemicellulose (20 - 35%) and lignin (10 - 25%). The presence of sugars, proteins, minerals and water make the agroindustrial wastes a suitable environment for the development of microorganisms, mainly fungal strains, which are able to quickly grow in these wastes. If the cultivation conditions are controlled, different products of industrial interest may be produced, avoiding the loss of potential energy sources (Solange *et al.*, 2012).

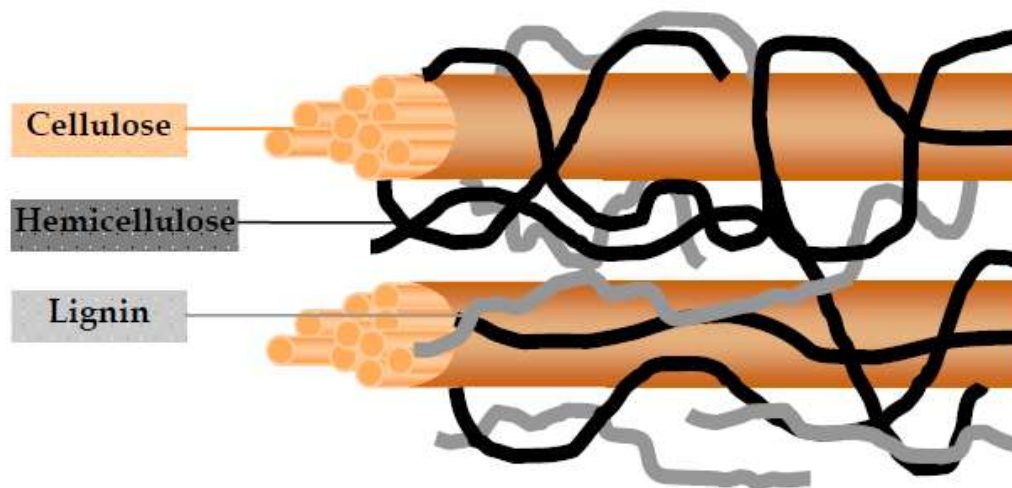


Figure 2.3: Schematic representation of composition of lignocellulosic materials. Adapted from Solange *et al.* (2012).

2.6.1 Wheat bran

The milling process of wheat produces large amount of wheat bran as a byproduct. During milling, the endosperm is broken down into fine particles (flour) and bran and germ are removed. In general, wheat kernel contains about 83% endosperm, 2.5% germ, and 14.5% bran. Bran fraction constitutes approximately 11% of total milling byproducts and only 10% of bran is used as fibre supplement in breakfast cereals and bakeries while the remaining 90% is sold as animal feed at an extremely low price. Wheat bran is often disposed off as waste by millers as the cost of transportation is more than its market value thus causing potential environmental concern (Xie *et al.*, 2008). Wheat is a composite material made up of three discrete layers that are formed from numerous histological tissues (Fig 2.4). These tissue layers are divided into outer and inner pericarp (tube cells and cross cells), testa or seed coat, pphyaline layer and aleurone layer (Antoine *et al.*, 2002).

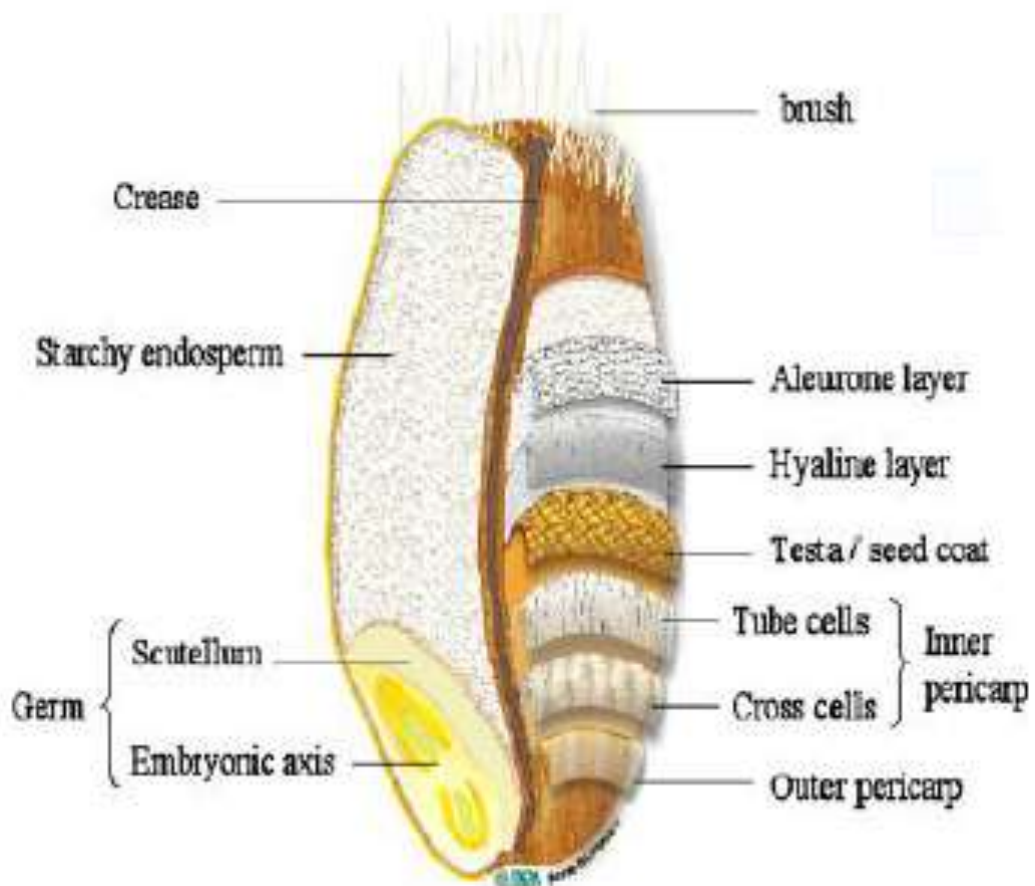


Figure 2.4: Histological composition of wheat grain. Adopted from Surget and Barron (2005).

Wheat bran contains several important compounds such as phenolic acids, carotenoids, lignans, phytosterols, flavonoids, α -tocopherol and phytic acid which are distributed unequally in different wheat bran tissues, isolated proteins from wheat bran may be used as ingredients in food formulations or special feeds, and contain superior nutritional value. Precise composition of macro and micronutrients may vary from cultivar to cultivar and the extraction technique of these compounds from bran. Wheat ash content is usually measured for quantification of bran. Amount of ash (mineral content) present is a true reflection of the bran quantity in wheat (Safdar *et al.*, 2009).

2.6.2 Wheat bran in the fermentation industry

Many agroindustrial byproducts are replacing the synthetic and expensive substrates for the production of biotechnological products. Among the agroindustrial substrates, wheat bran is one of the most attractive alternatives to synthetic medium in fermentation processes. The coarse variety of wheat bran is an efficient substrate due to its heat dissipation, better air circulation, loose particle binding and efficient penetration by mycelia and it is cheaper than fine bran so it is a better prospect economically in fermentation industry. Almost every type of enzyme can be produced by fermentation of wheat bran both by utilizing solid state fermentation (SSF) and submerged fermentation (SmF) systems.

Nowadays, wheat bran is widely used in solid state fermentation for the production of secondary metabolites and other industrial products because it reduces pollution effects (Malathi and Chakraborty, 1991; Pandey, 1992; Pandey *et al.*, 1999).

2.7 Maize Bran

The maize kernel or grain is made up of four main structures: the pericarp (bran), endosperm, germ (embryo), and the tip (pedicel). The bran is a byproduct from maize starch processing and constitutes a readily available agroindustrial residue.

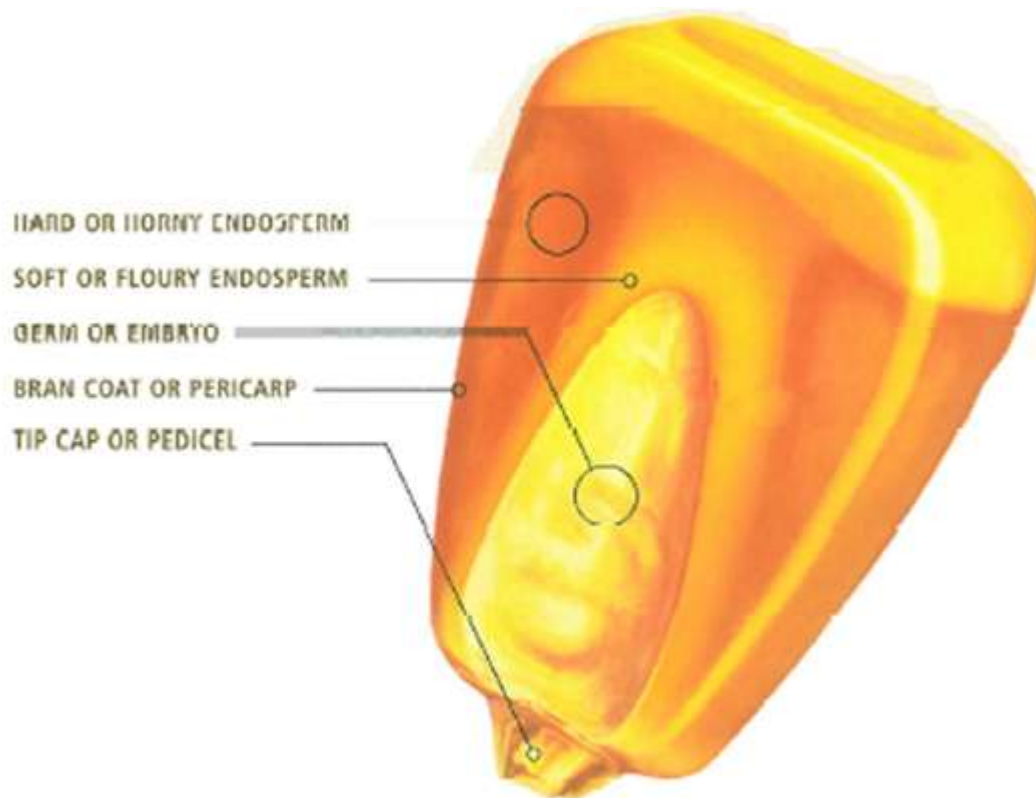


Figure 2.5: Typical composition of maize grain. Retrieved from <https://www.bungeservices.com/irj/go/>, (2016).

Maize bran represents the tough and resistant outer layer of maize kernels and is rich in C-5, C-6 carbohydrates that are potentially interesting substrates for upgrading to be used in food and fuel products.

Milled maize bran mainly consists of primary cell walls from the pericarp of maize kernels and possibly also the pedicel tips of the kernels, testa and some residual starch. Arabinoxylan, cellulose and starch make up the main constituents of maize bran. Milled maize bran mainly consist of primary cell walls from the pericarp and kernels, testa and some residual starch. The Arabinoxylan polysaccharide of maize bran are very complex in respect to structure and composition, and maize bran is exceptionally insubmissive to enzymatic degradation (Saulnier *et al.*, 1999; Agger *et al.*, 2010; Appeldoorn, *et al.*, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of Samples

Wheat bran and maize bran were obtained from agro-processing mills in Kaduna metropolis for this study. The two substrates were sundried separately for a period of 4 days to prevent microbial activity and pest infestation and milled to 2 - 3 mm particle sizes (Ana *et al.*, 2015).

Whole ripe pineapple fruits were obtained from Samaru market, Zaria, Kaduna state and fruits were taken to the Department of Botany herbarium, Ahmadu Bello University for voucher number identification prior to analysis.

3.2 Isolation of Yeasts from Spontaneous Fermentation of pineapple must

Intact pineapple fruits were washed with distilled water and peeled, then crushed in a sterile stomacher bag. The homogenized pulp was filtered through sterile cheesecloth and collected in 1000 ml sterile Erlenmeyer flasks, these steps were handled under aseptic conditions. The juice was incubated at 25°C for 6 days to initiate natural fermentation. Yeasts were routinely isolated everyday during the fermentation from an aseptically removed aliquot and then it was serially diluted in 0.1% peptone water in 6 fold dilution (10^{-6}). Aliquot of 0.1ml of 10^{-4} , 10^{-5} and 10^{-6} dilutions were cultured by spread inoculation onto plates of Malt Extract Agar (MEA) and incubated at 25°C for 3 days. Representative colonies of the different yeasts were purified by restreaking on Malt Extract Agar and incubated at 30°C for 48h. Purified cultures were routinely maintained on Malt Extract slants and kept at 4°C until further confirmation (Chanprasartsuk *et al.*, 2013).

3.2.1 Identification and characterization of isolates

Isolates were identified and characterized using cultural, morphological and biochemical methods.

3.2.1.1 Cultural identification

Cultural characteristics such as texture (mucoid, butyrous, moist), colour (creamy, white, creamy-white), surface (smooth), elevation (convex, raised, flat), and margin (entire, ridged) were considered and included in comprehensive colony description.

3.2.1.2 Morphological identification

A drop of methylene blue dye was placed on a clean slide. Using a sterile wire loop, a single colony of each of the yeast isolates was placed in the drop of methylene blue dye and teased. A clean cover slip was gently placed over it and viewed under the light microscope using the 100x objective with oil immersion.

3.2.1.3 Biochemical characterization

The fermentation basal medium in which the sugars were dissolved was that of Wickerham, (1951). Four and half (4.5g) of yeast extract powder and 7.5g of peptone were dissolved in 1000ml of demineralized water. A stock solution of bromothymol blue (indicator) of 50mg/75ml distilled water was prepared. To 100ml of the fermentation basal medium, 4ml of bromothymol blue was added to give it a sufficiently dark green colour. Two (2ml) of the basal medium was dispensed in test tubes containing Durham tubes of 12×150 mm in size (inverted tubes) which were sterilized at 121°C for 15 minutes. To this 1ml each of concentrated filter-sterilized sugar solution (D-glucose, D-galactose, Sucrose, Maltose, Lactose and Raffinose) was aseptically added to give a final sugar concentration of 2% (w/v) except for Raffinose at 4% (w/v).

The procedure for carrying out the fermentation was done according to the method of Kurtzman *et al.* (2011). One (1ml) of sterile distilled water was added to a 24 - 48h Yeast Malt slant culture (yeast extract 3g, malt extract 3g, peptone 5g, glucose 10g in 1000ml of water) and the cells were suspended by stirring with the pipette tip. This pipette was used to inoculate each tube of test media, including a sugar-free control, with 0.1 ml of cell suspension, the inoculated tubes were shaken gently to mix the cells. The tubes were incubated at 28°C for up to 28 days. The tubes were shaken and inspected at frequent intervals for accumulation of gas in the insert, and if the sugars were consumed, the indicator changes color from green to yellow, but if the sugars were not consumed and the amino acids present were utilized as a carbon source, the medium turns blue. The results were scored as follows, depending on the time taken to fill the insert with gas and the amount accumulating:

+ strongly positive, insert rapidly filled within 7 days;

I, delayed positive (latent), insert filled, but only after more than 7 days;

S, slowly positive, insert slowly filled after more than 7 days;

W, weakly positive, the insert is not fully filled with gas (e.g., less than one-third full is often considered weak, whereas greater than one-third full is positive);

- negative, no accumulation of gas in the insert;

V, variable, some strains are positive, others are negative.

3.2.1.4 Biochemical characterization using API Test Kit

This was carried out using the API 20C AUX system (bioMèrieux). Identification was accomplished as directed by the manufacturer. (This API 20 C AUX strip consisted of 20 cupules containing dehydrated substrates which enabled the performance of 19 assimilation tests. The cupules were inoculated with a semi-solid minimal medium from the manufacturer, yeast were only able to grow if they were capable of utilizing each substrate as the sole carbon source. The incubation box (tray and lid) was prepared by distributing about 5ml of sterile distilled water into the honey comb wells of the tray to create a humid atmosphere. Yeast cells picked from individual distinct colonies of 18 - 24h old culture were aseptically transferred in sterile distilled water to prepare a suspension with a final turbidity equivalent to McFarland standard number 2. Approximately 100 µl of this suspension was then dispensed to an ampule of API C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. After homogenizing, the inoculum suspension obtained in the ampule of API C medium was used to fill the cupules, until surface was slightly convex and without bubbles, after which the lid on the tray was replaced, and incubated at 30°C for 72h. A cupule more turbid than the control indicated a positive reaction to be recorded on the result sheet. Identification was made by generating a numerical profile (7-digits number) and using the API 20C Analytical Profile index. Morphology on cornmeal agar was also evaluated as suggested by the manufacturer. Dalmau plate method was used where corn meal agar was poured into petri plates, which were then put aside for a day or two to allow the surface to dry. The yeast were inoculated as a single streak near one side of the plate (for example from the ten to the two o'clock positions), and as two points near the other side of the plate (for example at the four and eight o'clock positions). A sterile cover glass was placed over the center of the streak and another over one of the point inoculations. The cultures were incubated and examined microscopically for formation of hyphae and

pseudohyphae along the edges of the streak and under the cover glass in the same way as slide cultures (Kurtzman *et al.*, 2011).

3.3 Proximate Analysis of Wheat Bran and Maize Bran

Eight (8g) of each of these substrates were used to carry out proximate analysis at the Food Science and Technology Research Programme (laboratory), Institute for Agricultural Research, Ahmadu Bello University, Zaria. The percentage compositions of moisture, ash, protein, lipid, crude fibre, and carbohydrate content were determined.

The methods are described below:

3.3.1 Moisture content

The moisture content of the sample was determined according to the standard of Association of Analytical Chemists, AOAC (2010). The crucibles were washed and dried in an oven at 100°C for 1 h.

The weight of the crucible was noted as W_1 , then 2g of each of the samples was separately weighed into the crucibles and their weights taken and noted as (W_2) before and during drying at 100°C to constant weight (W_3).

% moisture content was calculated as:
$$\frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where;

W_1 = weight of empty crucible

W_2 = weight of crucible and sample before drying

W_3 = weight of sample after drying to a constant weight

3.3.2 Ash content

The ash content of the samples were determined according the method of AOAC (2010). Crucibles were cleaned and dried in an oven, and then weighed (W_1), after which 2g of the samples were placed separately in the crucibles and weighed (W_2). The samples were charred on a Bunsen flame inside a fume cupboard. The charred samples were then placed in a muffle furnace set at 550°C for 2h until a white or light grey ash was obtained. The samples were then removed and cooled in desiccators and weighed (W_3).

$$\% \text{ ash content was calculated as: } \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Where;

W_1 = weight of empty crucible

W_2 = weight of crucible + weight of sample

W_3 = weight of crucible + weight of sample after ashing

3.3.3 Crude protein

The crude protein content of the samples were determined according to the method of Sule *et al.* (2014). One (1g) of each sample was weighed into a digestion flask. Ten (10g) of potassium sulphate, 0.7g mercuric oxide and 20cm³ concentrated sulphuric acid was added to the sample in the digestion flask. The flask was heated gently at an inclined angle until frothing subsided and boiled until the solution became clear. This was continued for half an hour. When the frothing was excessive, a small amount of paraffin wax was added. On cooling, 90ml of distilled water was added and mixed and a small piece of pumice was added to prevent bumping. Eighty (80ml) of 2M sodium hydroxide solution was added while the flask was tilted so that two layers were formed. The condenser unit was rapidly connected,

heated and the distilled ammonia collected in 50 ml boric acid / methyl red indicator. Fifty (50ml) of the distillate was collected and titrated against 0.1M hydrochloric acid solution. The percentage nitrogen content percent was calculated thus:

$$\% \text{ Nitrogen} = \frac{(\text{Volume of acid} \times \text{Molarity of standard acid}) \times 0.014 \times 100}{\text{Weight of sample (g)}}$$

Then crude protein content was calculated thus:

$$\% \text{ Crude protein content} = \% \text{ nitrogen content} \times 6.25 \text{ (protein factor)}$$

3.3.4 Lipid content

The lipid content was determined using the standard AOAC (2010). A soxhlet extractor with a reflux condenser and a 500ml round bottom flask was set up. About 300ml of petroleum ether was poured into the round bottom flask. The samples (2g) each was weighed into labelled thimble and sealed with cotton wool, then fitted into the extraction tube of the soxhlet extractor. The soxhlets extractor after assembly was allowed to reflux for about 6h, after which the thimble was removed carefully and the petroleum ether collected on top and drained into a container for re-use. The flask being free of ether was removed and dried in desiccators then weighed.

$$\% \text{ fat content was calculated as: } \frac{W_2 - W_1}{W} \times 100$$

Where;

W = weight of sample used

W₁ = weight of empty extracting flask

W₂ = weight of flask and extracted oil

3.3.5 Crude fibre

Two (2g) of each sample was placed separately in a beaker containing 1.3ml of H₂SO₄ per 100ml of solution and boiled for about 30minutes, the residue was filtered and washed with hot water, it was later transferred to another beaker containing 1.2g of NaOH per 100ml of solution and boiled again for another 30minutes, the residue was washed with hot water and dried in an oven and weighed (**C2**), the weighed sample was then incinerated in a muffled furnace set at 550°c for 2h, removed and allowed to cool, then weighed as **C3**.

% Crude Fibre was calculated as thus; $\frac{C2 - C3}{W} \times 100$

W

3.3.6 Carbohydrate (CHO)

The carbohydrate content was determined following the method of AOAC (2010). Total carbohydrate content was calculated by difference

CHO = 100 – (% Moisture + % Ash + % Protein + % Fat)

3.4 Screening Medium

This was carried out based on the modified method of Khan and Briscoe (2011); The amyolytic activity of the test isolate was determined by using the starch agar plate method, by inoculating the identified *Candida famata* into Nutrient Agar medium which was supplemented with 1g of starch. The agar plates were then incubated at 30°C for 72h. After the incubation period, freshly prepared iodine solution was added to the culture plate. Starch degradation was detected by the disappearance of the blue colour of the medium around microbial colonies after addition of iodine solution. Evaluation of the clear zones was

estimated as diameter (mm) of the clear zone, and measured to represent the amylolytic activity.

3.5 Preparation of Inoculum

The yeast isolate was cultivated in test tubes containing 5 ml of Yeast extract Peptone Dextrose medium (yeast extract 1%, peptone 2%, glucose 2% and agar 1.5%) for 48h at 28°C. The yeast suspension was obtained by scraping off the surface of the medium using 3 ml of Nutrient solution (0.5% ammonium sulfate, 0.5% magnesium sulfate heptahydrate and 0.5% ammonium nitrate). Yeast cells were counted with the aid of a hemacytometre. Yeast was inoculated in the substrate by transferring 3ml of the microbial suspension (at 10^6 cells/ml) to Erlenmeyer flasks to carry out solid state fermentation of the various substrates viz: wheat bran, maize bran wheat bran (1:1) and maize bran (Ana *et al.*, 2015).

3.6 Solid state fermentation for enzyme production

The yeasts was cultivated in solid state in Erlenmeyer flasks (250 ml) containing 5g of wheat bran, maize bran, wheat bran (1:1) and maize bran (ground to 2 - 3 mm size) with 70% moisture content (mass of dry substrate per volume), using the above described Nutrient solution to moisten the substrate. Prior to the inoculation of microorganisms, all the materials were autoclaved at 121°C for 15 minutes at pressure of 15 pounds/sq.inch. After inoculation, the Erlenmeyer flasks were incubated at 28°C for 5 days (Ana *et al.*, 2015).

3.6.1 Extraction of enzyme

Enzyme was extracted by adding 50ml of distilled water to the flasks containing the fermented medium. The flasks were kept under agitation at 150 rpm for 1h, after which they were filtered through Whatman paper No.1 and centrifuged at 3000x g for 10 minutes at 25°C. The supernatant were used for the enzyme assays (Ana *et al.*, 2015).

3.6.2 Enzyme assays

The enzyme activity was determined by adding 0.1ml of enzymatic extract to 0.9 ml of sodium acetate buffer (0.1M, pH 5.0, 1% corn starch). After 10 minutes of reaction at 50°C, the reducing sugar released was quantified by the 3,5-dinitrosalicylic acid method (DNS) (Miller, 1959). One unit of glucoamylase (U) was defined as the amount of enzyme required to release 1 μ mol of glucose per minute under the assay conditions and activity was expressed as U/ml. The enzyme reaction was stopped by the addition of 3 ml of 3,5- dinitrosalicylic acid reagent. Two (2g) of buffer (0.1M sodium acetate buffer) was used as reference blank. All the tubes containing 3,5-DNS treated reaction products were heated for 15 minutes in boiling water bath. One (1ml) of 40% solution of Rochelle salt was added to each tube prior to cooling to room temperature so as to maintain the color. The final volume in each case was made to 7ml by adding distilled water. Absorbance was measured at 575 nm using UV-Visible spectrophotometer and compared with standard curve using 0.10 to 1.0mg of glucose/ml.

3.7 Parameters Optimized for Glucoamylase Production

The following parameters were used to obtain the optimum conditions for the production of glucoamylase: moisture, pH, temperature, inoculum concentration, and incubation time. The parameter selected in each step was used for further cultivation, in an iterative strategy designed to optimize the fermentation processes for glucoamylase production.

3.7.1 Effect of initial moisture on enzyme production

The effect of initial moisture level on enzyme production was tested by varying the substrate to Nutrient solution ratio (w/v) in the range of 1:2, 1:3, 1:4, 1:5 and 1:6. Thus; 5g of substrate to 10 ml, 15ml, 20ml, 25ml and 30ml of medium respectively.

3.7.2 Effect of initial pH on enzyme production

The effect of initial pH on enzyme production was investigated by adjusting the pH of Nutrient solution to 3.0, 4.0, 5.0, 6.0 and 7.0 for glucoamylase production.

3.7.3 Effect of incubation temperature on enzyme production

The effect of incubation temperature on enzyme production was examined by varying temperatures ranging from 20 - 40°C with 5°C intervals i.e. 20, 25, 30, 35 and 40°C.

3.7.4 Effect of inoculum concentration on enzyme production

The effect of inoculum size based on the number of yeast was examined using the yeast cells concentration of 1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 cells ml^{-1} of distilled water for making the yeast suspension. The flasks containing the substrates having the sterile basal medium was inoculated with the different yeast cell suspensions for an optimal production of glucoamylase.

3.7.5 Effect of incubation time on enzyme production

The effect of incubation time on enzyme production was examined by varying incubation periods at 1, 2, 3, 4, 5 and 6 days interval and enzyme was extracted and assayed. All experiments were conducted in duplicate and values were averaged.

3.8 Data Analysis

Results were presented in tables, graphs and charts, data were analysed using one-way Analysis of Variance and Duncan Multiple Range Test and significance of variations was tested at 95% confidence limit.

CHAPTER FOUR

4.0

RESULTS

4.1

Identification and characterization of isolates

Five isolates of yeasts were identified; one of the isolates was confirmed to be *Candida famata* based on cultural, biochemical and microscopic characterization.

The cultural characteristics of the yeast isolates on Malt Extract Agar plate revealed their colony texture, colour, surface, elevation and margin as shown in Table 4.1.

Biochemical characterization of the yeast isolates using API 20C AUX system revealed their carbon assimilation patterns as seen in Table 4.2.

The morphology of *C. famata* was seen under the 100x objective as ovoid budding cells, this is shown in Plate 4.I.

Candida famata was isolated on the third day of the pineapple must fermentation. The pineapple voucher number was 2446 (*Ananas comosus*; Family – Bromeliaceae).

4.2

Proximate Composition of the Wheat Bran and Maize Bran

Proximate analysis of both substrates used revealed higher protein content in wheat bran which was easily digested to stimulate enzyme production than in maize bran, other compositions determined were; moisture content which increased the biodegradation rate of the substrates and this was observed to be higher in wheat bran, ash content which is usually measured for quantification of bran, the lipid content on the substrate surface had influence on the hydrophobicity and surface tension, forming a thin film, which was observed in maize bran, probably leading to the lower yield of glucoamylase in maize bran, and the crude fibre

contains hydroxyl and other oxygen containing groups that attract moisture through hydrogen bonding, details of this result is result shown in Table 4.3

4.3 Screening for Glucoamylase Production

This was revealed by the clear zone around the yeast colonies against the blue background of iodine flooded medium and this was 1.2 mm in diameter as seen in plate 4.2

Table 4.1: Cultural, Biochemical and Morphological Characteristics of the Isolates

Isolates code	Texture	Colour	Surface	Elevation	Margin	Sugar fermentation						Morphology	Tentative identity
						Suc	Glu	Gal	Mal	Lac	Raf		
D3d5	Butyrous	Creamy- white	Smooth	Convex	Entire	W	+	W	W	-	+	Ovoid budding cells, pseudohyphae absent	<i>C. famata</i>
D1d3	Mucoid	Creamy	Smooth	Raised	Entire	-	+	-	W	-	-	Ovoid, Hyphae present	<i>C. humicola</i>
D1d2	Moist	White	Smooth	Flat	Ridged	-	-	-	-	-	-	Ovoid budding cells, hyphae present	<i>T. mucoides</i>
D5d5	Moist	Creamy	Smooth	Flat	Ridged	-	-	-	-	-	-	Ovoid budding cells, Hyphae present	<i>T. mucoides</i>
D3d4	Mucoid	Creamy	Smooth	Raised	Entire	-	+	-	W	-	W	Ovoid, Hyphae present	<i>C. humicola</i>

KEY: , **Suc**; Sucrose, **Glu**; Glucose, **Gal**; Galactose, **Mal**; Maltose, **Lac**; Lactose, **Raf**; Raffinose, **W**; Weak, +; Positive -; Negative

Table 4.2: Biochemical Characterization of the Isolates by Use of API 20C AUX system

Carbon sources	Isolates Code				
	D3D5	D1D2	D1D3	D3D4	D5D5
D-glucose	+	+	+	+	+
Glycerol	-	+	+	+	+
Calcium 2-keto-gluconate	+	+	+	+	+
L –arabinose	-	+	+	+	+
D –xylose	-	+	+	+	+
Adonitol	+	+	+	+	+
Xylitol	-	+	+	+	+
D –galactose	+	+	+	+	+
Inositol	-	+	+	+	+
D-sorbitol	+	+	+	+	+
Methyl – α D-glucopyranoside	+	+	+	+	+
N –Acetyl-Glucosamine	+	+	+	+	+
D-celiobiose	-	+	+	+	+
D-lactose	+	+	+	+	+
D-maltose	+	+	+	+	+
D-saccharose (sucrose)	+	+	+	+	+
D- trehalose	+	+	+	+	+
D-melezitose	+	+	+	+	+
D-raffinose	+	+	+	+	+
Inference	<i>C. famata</i>	<i>T. mucoides</i>	<i>C.humicola</i>	<i>C.humicola</i>	<i>T. mucoides</i>

Key: +; Positive; -; Negative, *C. famata*; *Candida famata*, *T. mucoides*; *Trichosporon mucoides*, *C. humicola*; *Cryptococcus humicola*.

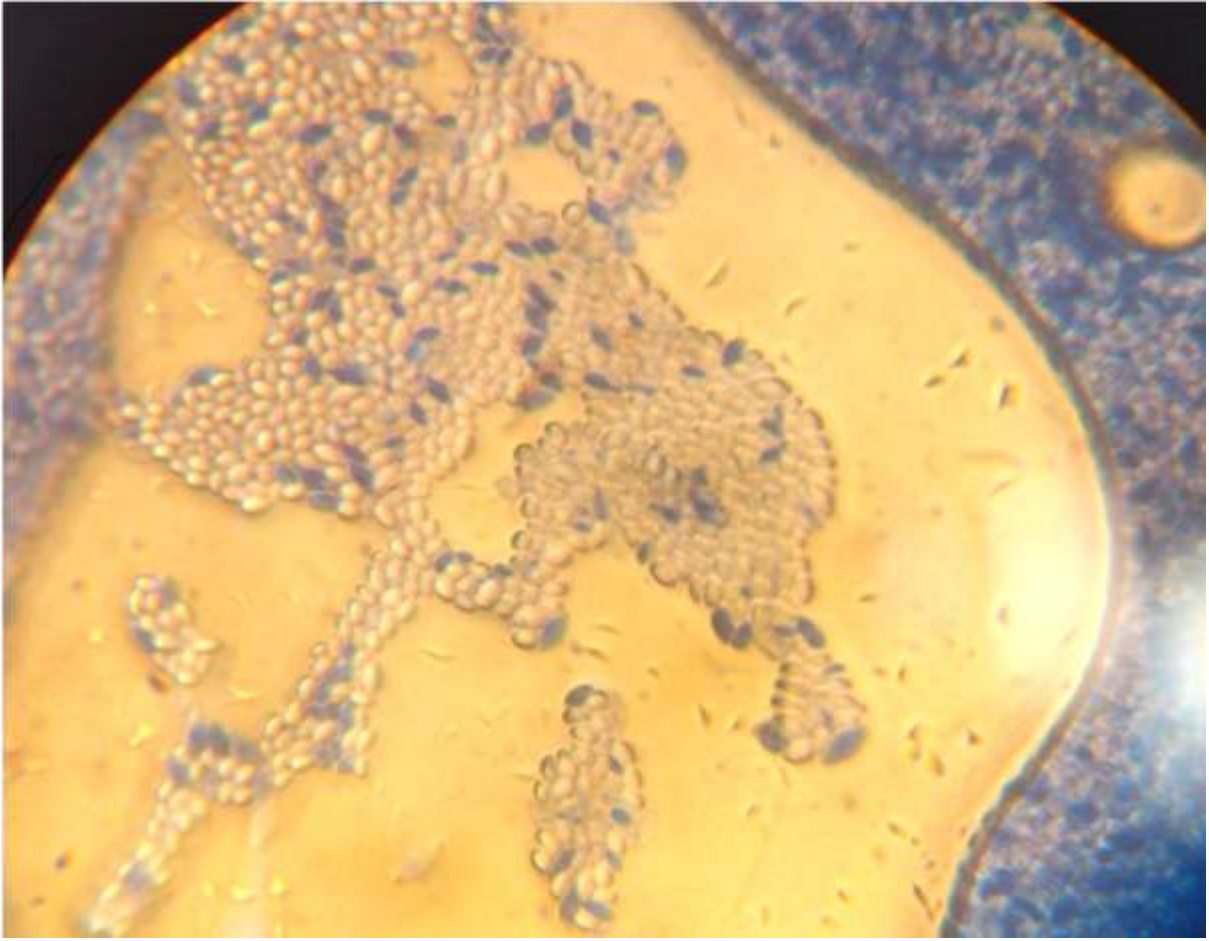


Plate 4.I: Microscopic view of *Candida famata* (100× objective)

Table 4.3: Mean Proximate Composition of the Wheat Bran and Maize Bran

Proximate composition (%)	Samples Analysed	
	Wheat bran	Maize bran
Moisture	8.65	8.35
Ash	3.54	3.91
Protein	10.5	7.00
Lipid	13.85	14.80
Crude fibre	2.46	2.23
Carbohydrate	63.47	65.94

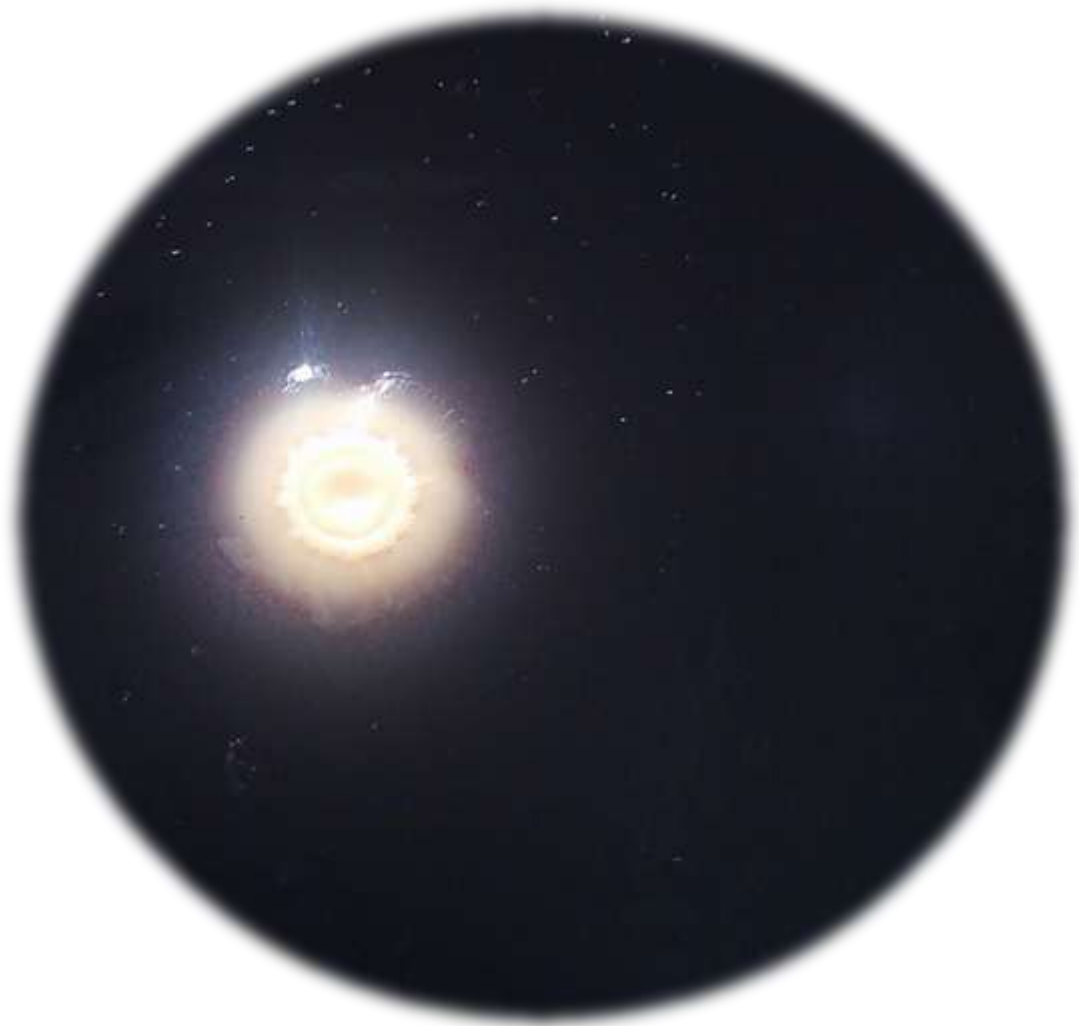


Plate 4.2: Clear zone showing amyolytic activity by *Candida famata* on starch agar plate.

4.4 Determination of glucoamylase - producing potential of *Candida famata*

At 70% moisture content, glucoamylase activity was observed in all substrates used. Maize:wheat bran composite (9.72 U/ml), wheat bran (7.33 U/ml) and maize bran (4.77 U/ml) The values obtained showed that there was a significant difference, i.e. ($p < 0.05$) between maize bran, wheat bran and the mixed bran, as seen on Table 4.4.

4.5 Determination of Optimum Conditions for Glucoamylase Production by *Candida famata*

4.5.1 Effect of initial moisture on glucoamylase production

The substrate to nutrient solution ratio was varied and result showed that at 15ml of nutrient solution (1:3 substrate to nutrient solution ratio), high enzyme activity was observed as 15.29 U/ml for maize:wheat bran composite, wheat bran gave an enzyme activity of 10.96 U/ml and for maize bran enzyme activity as 10.63 U/ml, further increase in mineral medium lead to a decrease in enzyme activity as seen on Table 4.5.

4.5.2 Effect of initial pH on glucoamylase production

The effect of various initial pH (3.0, 4.0, 5.0, 6.0, and 7.0) were investigated by using 1:3 substrate to nutrient solution ratio and the highest enzyme activity was observed at pH 5.0. Maize:wheat bran composite had the highest glucoamylase activity of 16.18 U/ml, wheat bran had glucoamylase activity of 12.31 U/ml and maize bran had glucoamylase activity of 11.29 U/ml as shown in Figure 4.1.

Table 4.4: Glucoamylase - producing Potential of *Candida famata*

Substrates	Glucoamylase activity (U/ml)
Maize bran	4.77±0.25 ^a
Wheat bran	7.33±0.62 ^b
Maize and Wheat bran	9.72±0.1 ^c

Values are mean ±SD, values with different superscript within the column are significantly different (p<0.05) by Duncan Multiple Range test.

Table 4.5: Effect of Initial Moisture on Glucoamylase Production

Substrates	Nutrient Solution				
	10ml	15ml	20ml	25ml	30ml
Maize bran	7.76±0.33 ^a	10.63±0.17 ^a	4.42±1.03 ^a	0.52±0.45 ^a	0.08±0.02 ^a
Wheat bran	10.26±1.15 ^a	10.96±2.06 ^a	10.09±0.03 ^b	8.20±0.21 ^b	2.28±0.35 ^b
Maize and Wheat bran	10.17±2.35 ^a	15.29±0.37 ^b	9.46±1.38 ^b	8.21±0.60 ^b	1.56±1.10 ^{ab}

Values are mean ±SD, values with different superscript within the column are significantly different (p<0.05) by Duncan Multiple Range test.

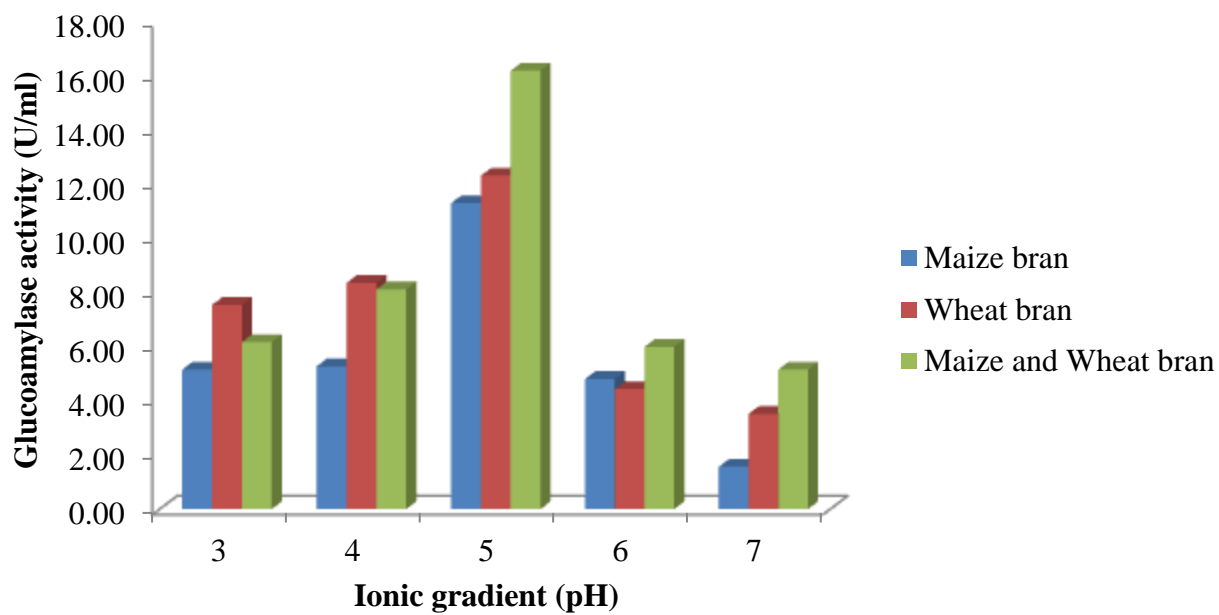


Figure 4.1: Effect of initial pH on glucoamylase production

4.5.3 Effect of incubation temperature on glucoamylase production

At different incubation temperatures, it was observed that 30°C was optimum for high glucoamylase activity with values of 21.85 U/ml, 16.96 U/ml and 12.00 U/ml for maize:wheat bran composite, wheat bran and maize bran respectively. With increasing temperature, a decrease was observed in the enzyme activity as shown in Table 4.6.

4.5.4 Effect of inoculum concentration on glucoamylase production

At inoculum concentration of 10^6 , high glucoamylase activity was observed in maize:wheat bran as 24.18 U/ml, wheat bran as 17.35 U/ml and maize bran 12.48 U/ml. Thereafter, increasing the inoculum concentration resulted in a decreased glucoamylase activity as seen in Figure 4.2.

4.5.5 Effect of incubation time on glucoamylase production

At 3 days of incubation, high glucoamylase activity was observed. Wheat bran (17.93U/ml), maize:wheat bran composite (25.34 U/ml) and maize bran (13.28 U/ml). Further incubation period lead to a decrease in enzyme activity as seen in Figure 4.3.

Table 4.6: Effect of Incubation Temperature on Glucoamylase Production.

Substrates	20°C	25°C	30°C	35°C	40°C
Maize bran	6.87±1.80 ^b	9.96±1.53 ^a	12.00±0.99 ^a	0.49±0.41 ^a	0.54±0.27 ^a
Wheat bran	10.38±0.49 ^c	16.03±0.81 ^b	16.96±0.14 ^{ab}	1.23±0.21 ^a	0.64±0.21 ^a
Maize and Wheat bran	2.15±0.22 ^a	16.24±0.93 ^b	21.85±4.31 ^b	7.15±0.86 ^b	1.74±0.93 ^a

Values are mean ±SD, values with different superscript within the column are significantly different (p<0.05) by Duncan Multiple Range test.

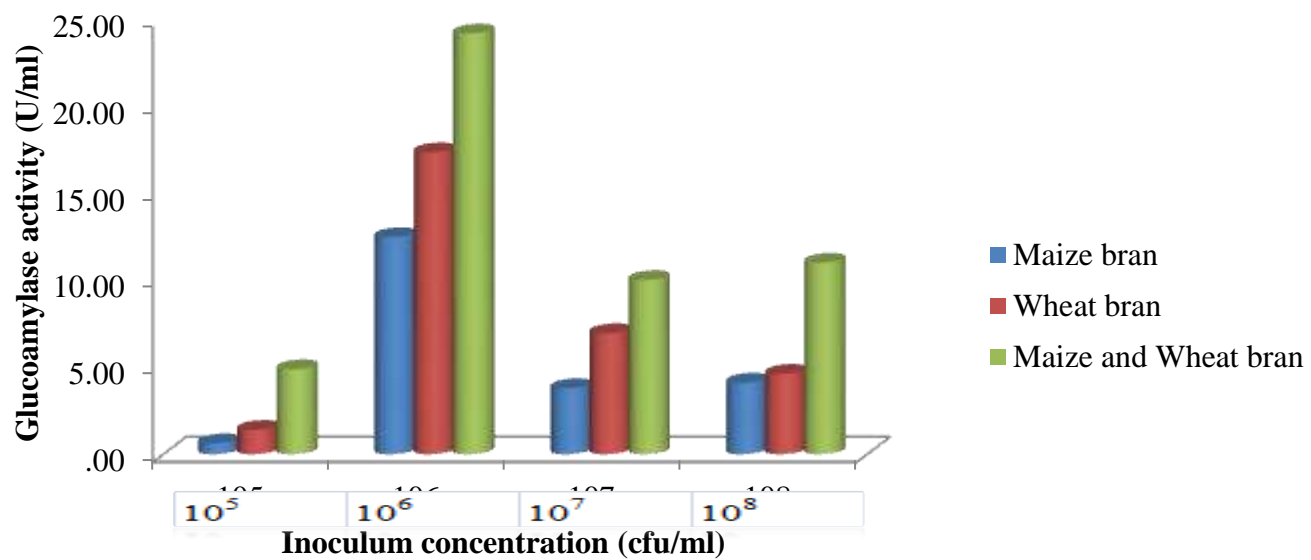


Figure 4.2: Effect of inoculum concentration on glucoamylase production

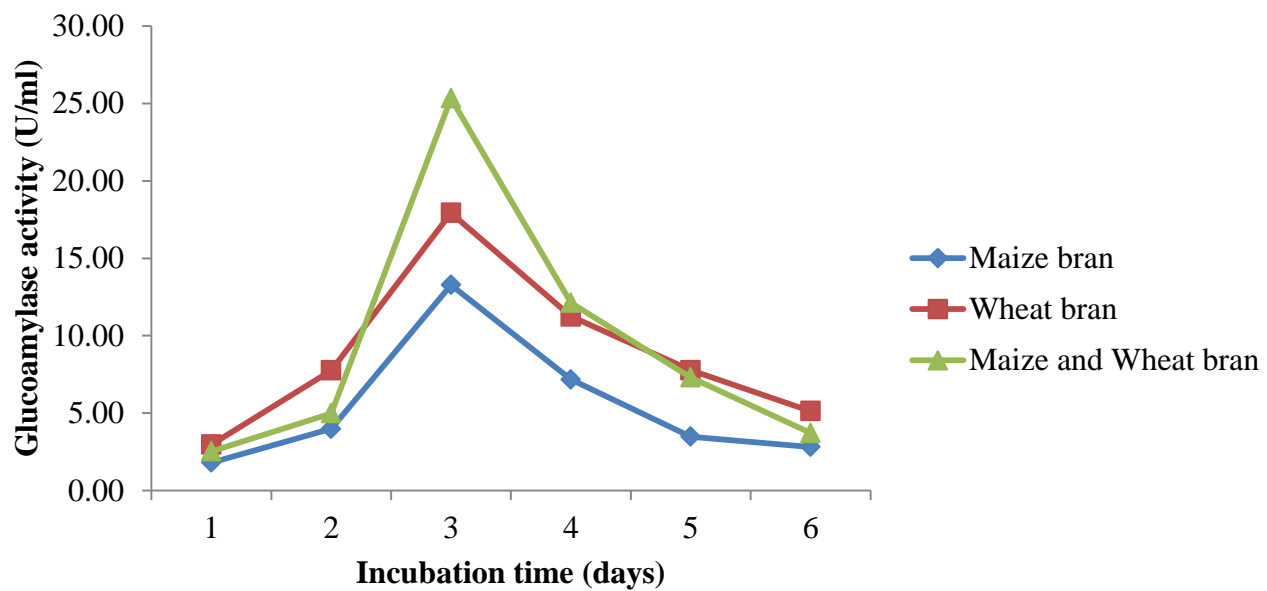


Figure 4.3: Effect of incubation time on glucoamylase production

CHAPTER FIVE

DISCUSSION

Isolation and characterization of *Candida famata*

In this study, all five isolates were yeasts, one of which was confirmed by cultural, microscopic and biochemical tests to be *Candida famata* at 99.5% identity, it was isolated on the third day of spontaneous fermentation of pineapple must. Pineapple is a sweet acidic fruit of pH 3.71. Deepak (1994) reported that yeast occur generally in sugary substances like palm wine, maple syrup, flower parts, and cane sugar, and that they are responsible for spoilage in pineapple. The population of microflora on the substrate always depends upon the pH of the substrate, since fruits are acidic in nature they are predominantly inhabited by yeasts. *Candida famata* was observed as creamy-white colonies on malt extract agar plate, it had a smooth surface, entire margin and a butyrous texture. Pseudohyphae was absent and cells morphology were ovoid. Carbon assimilation patterns are shown in Table 4.2.

Pretreatment of substrates (particle size)

Particle size of the substrate is a critical factor for enzyme production by solid state fermentation. The milling of the maize and wheat bran was a pretreatment process which was necessary to reduce their particle sizes, increase the accessibility of the interior of the particles to the yeast (*Candida famata*) that acted on them, expose these substrates to a greater enzymatic accessibility and a higher susceptibility of macromolecules within these brans to attack by microbial enzymes in the fermentation medium. This is in line with the report made by Mitchell *et al.* (2000), that chopping or grinding, thermal pretreatment or even chemical hydrolysis may be necessary to create higher enzymatic accessibility to the

substrate and susceptibility of macromolecules within the solid to attack by microbial enzymes. Enzymatic hydrolysis yields increase with reduction in substrate particle especially for maize bran. Particle size affects the extent and rate of microbial colonization, air penetration, CO₂ removal and downstream extraction, maximum production of enzyme was noted with small particle sizes as it increases the surface area of substrates (Foda *et al.*, 2013).

Proximate composition

Proximate analyses of the maize and wheat bran showed that wheat bran was a better substrate for the production of glucoamylase, the protein content in wheat bran (10.50%) was higher than the protein content of maize bran (7.00%), probably due to the easy digestion of wheat bran proteins as compared to that of the endosperm proteins present in maize bran as stated by Cavalcante *et al.* (2008). Other compositions determined were; moisture content which increased the biodegradation rate of the substrates, this was also stated by Pommier *et al.* (2008) that the moisture content provides a medium for the transport of dissolved nutrients required for the metabolic and physiological activities of microorganisms in the solid fuels and an increase in moisture content will increase the biodegradation rate of organisms, ash content which is usually measured for quantification of bran, the lipid content on the substrate surface had influence on the hydrophobicity and surface tension, forming a thin film, this agrees with the work of Andrzej *et al.* (2010) that cereal fibre surface contains lipid and proteinaceous compound and the lipid molecule is usually bonded to the protein molecule by ester or thioester bond. The amount of lipid on the cereal surface has influence on hydrophobicity and surface tension. The more amount of lipid on the surface means the more hydrophobic and more surface tension as well as smoother the fibre surface forming a thin film as observed in maize bran, probably leading to the lower yield of glucoamylase in maize bran, and the crude fibre contains hydroxyl and other oxygen containing groups that

attract moisture through hydrogen bonding. It can be said that wheat bran may be more suitable for enhancing enzyme production than maize bran as evident in the glucoamylase activity observed in each of the cultural parameters used for the investigation, this could be as a result of suitable nutrient and high oxygen transfer that can stimulate enzyme secretion and also this agrees with reports from several researchers such as Kaur *et al.* (2003); Anto *et al.* (2006); Sivaramakrishnan *et al.* (2007); that wheat bran is the most promising substrate for glucoamylase production in solid state fermentation because of its high ability to retain moisture, which promotes fungal growth just as in the natural environmental conditions, its sufficient nutrients and its ability to remain loose even in moist conditions and a larger surface area, these factors is said to promote aeration and easier mycelial penetration.

***Candida famata* for glucoamylase production**

The clear zones around the colony of *Candida famata* on the nutrient agar medium incorporated with starch flooded with iodine was an indication that it had amylolytic ability of degrading starch and were able to grow in the presence of starch as the major carbon source. This agrees with work done by Ouédraogo *et al.* (2012) that the amylolytic system of yeast is very diversified: the major enzymes for this system are the α -amylase, glucoamylase, pullulanase and cyclodextrinase and the secretion of amylases depend on the composition of the culture medium.

The glucoamylase - producing potential of *Candida famata* was found to be significantly different statistically ($p < 0.05$) between the two substrates used individually and the mixed brans. Values for glucoamylase activity were (4.77 U/ml), (7.33 U/ml) and (9.72 U/ml) for maize bran, wheat bran and maize:wheat bran composite respectively.

Optimum cultural parametres

The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the fermentation medium is an important factor in enzyme production. For optimum glucoamylase activity, intermediate moisture content is required for an efficient solid state fermentation process, at a low moisture level, the volume of medium per unit weight of substrate is low, and this affected the microbial activities as there could have been a limited nutrient solubilization and low degree of substrate swelling. Therefore, the enzyme activity is usually very high (Arpana *et al.*, 2013). In determining the optimum moisture content for the production of glucoamylase in these substrates, high enzyme activity was observed in substrate to nutrient solution ratio 1:3 (5g/15ml). A low enzyme activity was observed especially in maize bran (0.08 U/ml), this could have been as a result of the physical structure of maize bran, its inability to retain high moisture level thereby making the fermentation environment water-logged which in turn reduces the porosity of the substrate, limiting oxygen transfer to the yeast. The result of this finding is in line with the work of Norouzian *et al.* (2006) that the critical importance of moisture level in solid state fermentation media and its influence on the biosynthesis and secretion of the enzyme can be attributed to the interference of moisture with the physical properties of the solid particles. High substrate moisture results in decreased substrate porosity which in turn prevents oxygen penetration and decreased substrate degradation.

pH is one of the important factors that determine the enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Enzyme production is sensitive to changes in pH, therefore to get the highest glucoamylase activity, initial pH of the fermentation medium was varied. And this was found to be pH 5, Eva and Juraj (2010), reported that the pH optimum of yeast glucoamylases lie between 4.5 and 6.0,

varying little among the enzymes and species. However at a pH higher than the optimum, the activity decreased, there could have been an inactivation of the enzymes of the organisms which could have hindered more saccharification of the substrate. This corroborates with the work of (Mohamed *et al.*, 2007) who reported that optimization of growth and production of glucoamylase by *Candida famata* isolate exhibited maximum glucoamylase activity at pH 5 in liquid medium, but disagrees with the work of Ana *et al.* (2015), who reported a maximum activity at pH range of neutrality 7.0 for yeasts.

Optimum glucoamylase activity was observed at 30°C for maize bran (12.00 U/ml) and wheat bran (16.96 U/ml), and maize:wheat bran composite (21.85 U/ml). At temperatures above 30°C, it resulted in very low glucoamylase activity in wheat bran and particularly maize bran (0.54 U/ml) however when the two substrates were combined, there was just a slight improvement in glucoamylase activity, the probable explanation for this is that the high temperature in the fermentation environment led to a loss of moisture in the substrates individually and growth slowed down, also thermal death may have occurred so the yeast cells could not survive for the entire fermentation duration, therefore there was no or very little metabolic activities, but the synergy between both substrate was able to cushion that high temperature effect as the yeast cells would have adapted a way of utilizing the nutrients in both substrates such that, its metabolic rate was active but extremely reduced. Also, enzymes that would have been present before exceeding the optimum temperature ranges would have been denatured afterwards. At higher temperature, large amount of metabolic heat is produced thus the fermenting substrate temperature is increased, thereby inhibiting microbial growth and enzyme formation The result of Rajoka *et al.* (2006) showed that at high temperatures there is collapse of membranous structures and denaturation of structural proteins and enzymes while Zambare (2010), also reported that higher temperature

inactivation may be attributed to incorrect conformation of enzyme molecules due to hydrolysis of the peptide chain, destruction of amino acid or aggregation.

Inoculum concentration was an important factor for the production of glucoamylase in this research, inoculum concentration of 10^6 cells/ml was optimal for glucoamylase activity in maize bran (12.48 U/ml), wheat bran (17.35 U/ml) and maize:wheat bran composite (24.18U/ml). It was observed that at a lower inoculum concentration of 10^5 cells/ml, maize bran gave a very low enzyme activity which could have resulted from the high level of arabinoxylan polysaccharide in maize bran which is generally complex and exceptionally resistant to enzymatic degradation. Also in general, maize bran has proven a more difficult substrate for enzymatic degradation compared with others such as wheat bran. Since the inoculum concentration was low, it took longer for the cells to multiply and yield glucoamylase thus a longer time period for fermentation to have occurred. It was observed that at higher inoculum concentration, the wheat:maize composite remained fairly high, while a decrease was observed in maize and wheat bran. The higher inoculum size may have increased moisture content and the free excess liquid present in an unabsorbed form therefore gave rise to an additional diffusion barrier together with that imposed by the solid nature of the substrate thereby leading to a decrease in growth and enzyme production, while lower inoculum size may require a longer time for fermentation to form the desired product, this observation agrees with Baysol *et al.* (2003).

High glucoamylase activity was observed on day 3 of fermentation, wheat bran had 17.93U/ml, maize:wheat bran composite had 25.34 U/ml and maize bran had 13.28 U/ml, a decline on the enzyme activity was observed above this period and the likely reason for this would probably be that nutrients in the fermentation medium had depleted and secondary

metabolites produced may have inhibited the production of glucoamylase. Other researchers like (Soni *et al.* (2003) and Melikoglu *et al.* (2013) have confirmed that maximum glucoamylase production normally occurs after 2-5 days of incubation with solid state cultures involving bacteria and fungi. Further increase in incubation period beyond the optimum incubation time resulted in decrease in the enzyme activity, this decline in the productivity may be due to depletion of one or more essential nutrients in the medium or due to accumulation of toxic byproducts which may interfere with protein synthesis as well as enzymatic activity as reported by Ominyi, (2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From this research, the following conclusions could be drawn;

That *Candida famata* was isolated from fermented pineapple must after a three (3) day spontaneous fermentation period.

That the proximate composition of wheat bran and maize bran showed that wheat bran was richer in proteins than maize bran.

That *Candida famata* had amylolytic activity and had glucoamylase - producing potential under solid state fermentation conditions, it was observed that maize:wheat bran composite had the best yields with enzyme activity value of (25.34 U/ml), followed by wheat bran (17.93 U/ml) and the least enzyme activity was observed in maize bran (13.28 U/ml).

The yield of glucoamylase could be controlled by various cultural optimization parameters such as moisture, temperature, inoculum concentration, pH and incubation time.

6.2 Recommendations

1. Strain development technologies, such as mutation or recombinant DNA should be encouraged to enhance the metabolic capabilities of wild yeast strains for utilization of agroindustrial residues in industrial application, as production levels of these wild strains may be too low for economical production.
2. Mutants can be developed through further research to make the strain have the ability to synthesize one component, as wild strains may produce a mixture of substances which are chemically related, this can simplify product recovery.

3. Identification of more productive strains of yeast that are capable of converting wastes of agricultural origin to potentially useful biotechnological products should be encouraged.

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APPENDIX

Appendix I: Effect of pH on glucoamylase production

	pH3	pH4	pH5	pH6	pH7
Maize bran	5.14±0.41 ^a	5.27±0.77 ^a	11.29±0.06 ^a	4.80±0.24 ^b	1.56±0.23 ^a
Wheat bran	7.53±0.21 ^b	8.34±2.05 ^a	12.31±0.56 ^b	4.43±0.16 ^a	3.52±0.29 ^b
Maize and Wheat bran	6.16±0.69 ^{ab}	8.11±0.01 ^a	16.18±0.41 ^c	5.99±0.12 ^c	5.14±0.41 ^c

Values are mean ±SD, values with different superscript within the column are significantly different ($p < 0.05$) by Duncan Multiple Range test.

Appendix II: Effect of inoculum concentration on glucoamylase production

Substrates	10^5	10^6	10^7	10^8
Maize bran	0.63±0.30 ^a	12.48±1.67 ^a	3.79±1.58 ^a	4.11±0.41 ^a
Wheat bran	1.40±0.73 ^b	17.35±0.41 ^a	6.95±1.36 ^{ab}	4.62±0.45 ^b
Maize and Wheat bran	4.88±0.37 ^c	24.18±2.25 ^b	10.01±0.59 ^b	11.01±0.11 ^c

Values are mean ±SD, values with different superscript within the column are significantly different ($p < 0.05$) by Duncan Multiple Range test.

Appendix III: Effect of incubation time on glucoamylase production

Substrates	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Maize bran	1.81±1.03 ^a	3.99±4.11 ^a	13.28±2.05 ^a	7.18±3.29 ^a	3.49±2.06 ^a	2.82±0.41 ^a
Wheat bran	2.97±1.44 ^a	7.76±1.64 ^a	17.93±0.41 ^b	11.25±1.65 ^a	7.78±0.39 ^b	5.14±0.41 ^a
Maize and wheat bran	2.53±2.05 ^a	4.99±2.66 ^a	25.34±1.03 ^c	12.12±0.41 ^a	7.31±0.64 ^{ab}	3.72±1.36 ^a

Values are mean ±SD, values with different superscript within the column are significantly different ($p < 0.05$) by Duncan Multiple Range test.

Appendix IV: Filtration of fermentation medium to obtain enzyme extract



Appendix V: Agitation of fermentation medium



Appendix VI: Fermentation medium after inoculation with yeast cells



Appendix VII: Plate of *C. famata* on malt extract agar plates

