CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Environmental pollution is one of the most dangerous hazards affecting both developing and developed countries, as a result of industrial growth, urbanization and man-made problems resulting from population growth (Adewumi and Oguntuase, 2016). The main sources of environmental pollution are agricultural effluents, industrial effluents, sewage, fossil fuel plants and nuclear power plants. Starchy and lingo-cellulosic agro-industrial wastes and crop residues are main agricultural wastes in many tropical countries, which include Nigeria (Pothiraj, *et al*., 2006).

Cassava (*Manihot esculenta* Crantz), is a tropical crop consumed on daily basis in Nigeria. It has a wide extent of use in the country, ranging from fufu to gari, which are mainly taken with soup, to cassava flour and starch which are used for a variety of other purposes. Due to the diverse advantages of cassava, there is constant economic use for it in Nigeria, which leads to constant cassava processing going on in several cottage industries around the country. Cassava wastes generated from these industries are one of the most frequent agricultural wastes found in the country, whether in form of pulp, bark or cassava waste water. Out of these three major wastes generated from cassava processing, the pulp and bark are quite decomposable when disposed into the environment, but the waste water introduces harmful substances such as nitrogenous

compounds and cyano-glycosides which are hydrolyzed into hydrogen cyanide which is toxic and pose serious threat to the environment (Abiona, 2005). Cassava bioprocessing involves various other aspects which introduce environmental pollutants such as: milling which introduces Carbon (iv) oxide which is a greenhouse gas; fermentation which releases unpleasant odour to the environment; and lastly, effluent discharges which contaminate the soil and surrounding water bodies (Adewumi *et al*., 2016). Improper effluent discharge experienced around the country is bordered on two main factors which are; lack of adequate and workable drainage systems, as well as lack of technical expertise on drainage methods by the operators of these industries. In other words, most industries are sited across the nation, with little or no emphasis made on proper drainage systems to aid efficient effluent discharge. Thus, effluent discharge becomes the chief culprit in environmental pollution from cassava bio-processing industries. One of the ways to manage cassava effluents, in order to reduce environmental contamination with the toxins they contain is by converting the effluents to useful substrates for culturing microorganisms that possess the capacity to utilize or degrade the toxins contained in them.

A lot of microorganisms have been employed in the microbiology of bio-degradation of toxic substances from different environmental problems creating substrates. Microbes such as *Aspergillus, Pseudomonas, Microsporium* amongst others have been reported to have good bio-degradation ability. Amongst the species of *Aspergillus*, *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus* have been extensively

used for environmental bio-remediation. *Aspergillus* species are molds that are found to be naturally occurring in the environment. Some of the species are known to be toxigenic while some are not. Amongst the toxigenic strains is the popularly known *Aspergillus flavus* and its strains which produce aflatoxin which is a neurotoxin. However, both toxin producing and non-toxin producing species of *Aspergillus*, possess the ability for bio-remediation and bio-sorption activities in the environment. Abdulmajeed *et al*. (2016) demonstrated the efficiency of *A. niger* and *A. flavus* biomass in the removal of metals from refinery effluent. Malaviya and Sharma (2016) published work on the bioremediation of tannery waste water by *A. flavus* biomass, thus, proving that biomass of *Aspergillus* species are of great advantage to man when adopted in solving environmental pollution problems. They are also capable of producing different enzymes that can be used in achieving degradation purposes. One of the most important degrading enzymes produced by *Aspergillus* species is amylase.

Amylases are enzymes that catalyze the hydrolysis of glycosidic linkages in starch components or related carbohydrates, releasing malto-oligosaccharides and glucose in the anomeric form (Nazmi *et al*. 2006). Amylolytic enzymes are the most important industrial enzymes which can be used in a number of industrial processes including brewing, baking, textile, detergent, conversion of starch to sugar syrups, production of cyclodextrins, preparation of digestive aids, production of chocolates, cakes, fruit juice *inter alia*. Besides their use in saccharification, they also find application in paper and distillery industries (Ramachandran *et al*. 2004). Amylase has been derived

from several fungi, bacteria and actinomycetes. The major advantages of using microorganisms for the production of amylase are because of economical bulk production and the ease of manipulating microbes to obtain enzymes of desired characteristics.

1.2: Statement of the Problem

Cassava processing is an almost daily activity performed at different parts of Nigeria, to obtain useful finished products such as gari, cassava flour, starch, ethanol, amongst many others. Since the establishment of cassava mills is fairly cost-effective, there has been proliferation of cottage industries for cassava processing around many parts of Nigeria with the aim of running the business and making profits. In as much as this activity is beneficial to the economy, as well as provides food availability and sustenance, it poses a main disadvantage which is environmental pollution through improper cassava effluent discharge. According to Etinosa and Ozede (2015), cassava effluent contains harmful cyanides, copper and nickel, which have capacity to affect native biota of the soil, physico-chemical parameters of the soil and subsequently lead to reduction in soil fertility; hence there exists the need to device means of tackling this environmental pollution issue, of which the most environmental friendly method is the use of biological pollution control. Bioremediation is the use of microorganisms to breakdown complex environmental pollutants to their simplest non-toxic state. Bio-remediation of agro-industrial effluents using fungal cultures is a biological practice that most researchers have done and recommended. *Aspergillus* species have been

extensively used in the control of environmental pollution problems. Their roles in bioremediation and biosorption and transformation of environmental pollutants have made them become one of the preferred microbial populations in tackling environmental pollution issues. Thus in this work, *Aspergillus* sp. biomass would be used for amylase production from optimized cassava waste water, hence, converting the waste water from an environmental pollution source to a useful industrial raw material.

1.3: Aim of the Research

The aim of the research is to purify and characterize an amylase of *Aspergillus nomius* grown in cassava waste water.

1.4: Objectives of Research

The objectives of this research are to;

1 Isolate and characterize *Aspergillus* species from cassava waste water.

2 Assess the physico-chemical parameters of the cassava waste water.

3 Screen the *Aspergillus* isolates for amylase production.

4 Optimize conditions for amylase production using cassava waste water.

5 Purify and characterize the amylase produced from the fungal biomass.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cassava Importance in the 21st century Africa

Cassava (*Manihot esculenta* Crantz., Family: Euphorbiaceae) also known as tapioca or manioc is one of the major root crops grown in more than 100 countries of the humid tropics and sub-tropics. Globally cassava is grown in an area of 18.51 million ha producing 202.65 million tons with a productivity of 10.95 t/ha (FAO, 2009). It is one of the richest sources of starch. The roots contain up to 35% starch (on fresh weight basis), and are low in proteins, soluble carbohydrates and fats that make starch extraction from cassava comparatively easier.

Unlike other tropical roots crops such as sweet potato, yams and aroids, 75% of cassava in Africa, 75-80% in Asia and 65% in Latin America are processed either as fermented foods and feeds or as industrial fermented products such as starch, sour starch, starch-based sweeteners (glucose, fructose and maltose syrups), ethanol, acetone, butanol, lactic and other organic acids, mono-sodium glutamate, microbial polysaccharides (xanthan, pullulan, scleroglucan, etc.). Cassava is the third largest source of food carbohydrates in the tropics, after rice and maize (FAO, 2009). It is one of the most drought-tolerant crops, capable of growing on marginal soils. Nigeria is the world's largest producer of cassava, while Thailand is the largest exporting country of dried cassava.In 2014, global production of cassava root was 268 million

tons, with Nigeria as the world's largest producer of nearly 55 million tons or 21% of the world total.

(2019).

Plate 1: Photograph of Cassava Roots by the Researcher Umeoduagu, N.D. (2019).

Cassava was introduced to Africa by Portuguese traders from Brazil in the 16th century. Maize and cassava are now important staple foods, replacing native African crops (FAO, 2009). Cassava is sometimes described as the "bread of the tropics" however, it is not same with the tropical and equatorial bread tree (*Encephalartos*), the breadfruit (*Artocarpus altilis*) or the African breadfruit (*Treculia africana*).

The cassava root is long and tapered, with a firm, homogeneous flesh encased in a detachable rind, about 1 mm thick, rough and brown on the outside. Commercial cultivars can be 5 to 10 cm (2.0 to 3.9 in) in diameter at the top, and around 15 to 30 cm (5.9 to 11.8 in) long. A woody vascular bundle runs along the root's axis. The flesh can be chalk-white or yellowish. Cassava roots are very rich in starch and contain small amounts of calcium (16 mg/100g), phosphorus (27 mg/100g), and vitamin C (20.6 mg/100g). The cassava plant gives the third-highest yield of carbohydrates per cultivated area among crop plants, after sugarcane and sugar beets. Cassava plays a particularly important role in agriculture in developing countries, especially in sub-Saharan Africa, because it does well on poor soils and with low rainfall, and because it is a perennial that can be harvested as required. Its wide harvesting window allows it to act as a famine reserve and is invaluable in managing labor schedules. It offers flexibility to resource-poor farmers because it serves as either a subsistence or a cash crop (Stone, 2002).

Worldwide, 800 million people depend on cassava as their primary food staple (UNFAO, 2013). No continent depends as much on root and tuber crops in feeding its population as does Africa. In the humid and sub-humid areas of tropical Africa, it is either a primary staple food or a secondary co-staple. In Ghana, for example, cassava and yams occupy an important position in the agricultural economy and contribute about 46 percent of the agricultural gross domestic product. Cassava accounts for a daily caloric intake of 30 percent in Ghana and is grown by nearly every farming family. Cassava-based dishes are widely consumed wherever the plant is cultivated; some have regional, national, or ethnic importance (Frederick *et al.,* 2008).Cassava must be cooked properly to detoxify it before it is eaten.

Cassava can be cooked in many ways. The root of the sweet variety has a delicate flavor and can replace potatoes. It can be made into a flour that is used in breads, cakes and cookies. In Brazil, detoxified manioc is ground and cooked to a dry, often hard or crunchy meal known as *farofa* used as a condiment, toasted in butter, or eaten alone as a side dish. In Nigeria, cassava is consumed as fermented products such as gari, lafun, tapioca and cassava gablek; and as unfermented product, mainly cassava flour, which is used as thickener for making maize flour fufu. Aside the nutritional advantages of cassava, there exists other industrial advantages which are derived from cassava bio-processing.

2.2: Cassava Bio-processing

2.2.1: Biofuel

In many countries, significant research has begun to evaluate the use of cassava as an ethanol biofuel feedstock. Under the Development Plan for Renewable Energy in the Eleventh Five-Year Plan in the People's Republic of China, the target is to increase the production of ethanol fuel from non-grain feedstock to two million tons, and that of biodiesel to 200 thousand tons by 2010. This is equivalent to the replacement of 10 million tons of petroleum. As a result, cassava (tapioca) chips have gradually become a major source of ethanol production. On 22 December 2007, the largest cassava ethanol fuel production facility was completed in Beihai, with annual output of 200 thousand tons, which would need an average of 1.5 million tons of cassava. In November 2008, China-based Hainan Yedao Group reportedly invested US$51.5m (£31.8m) in a new biofuel facility that is expected to produce 33 million US gallons (120,000 m^3) a year of bioethanol from cassava plants (Lunsin *et al.,* 2012).

Bioethanol is a microbiological way of converting simple sugar into ethanol and carbon dioxide (CO_2) (Damaso *et al.,*2004). Bioethanol is a principal fuel that can be used as petrol substitute for vehicle (Aro *et al.,* 2005). It is a renewable energy source produced mainly by sugar fermentation process, although it can also be manufactured by the chemical process of reacting ethylene with steam (Anuj *et al.,* 2007).The main sources of sugar required to produce ethanol come from fuel or energy crops (Kim *et*

al 2005). These crops include maize, cassava and cassava products, wheat crops, waste straw, guinea corn husk, rice husk, millet husk, sawdust and sorghum plant. Ethanol is a high octane fuel and has replaced lead as an octane enhancer in petrol (Oghgren *et al* 2006). By blending ethanol with gasoline the fuel mixture can be oxygenated so it burns more completely and reduces pollution emission. Ethanol fuel trends are widely sold in the United States. Ethanol has been produced in batch fermentation with fungal strains such as *Aspergillus niger*, *Mucor mucedo*, *Saccharomyces cerevisiae* that cannot tolerate high concentration of ethanol (Ledward *et al.,* 2003; Oyeleke *et al.,* 2008; Seema *et al*., 2007). Biofuels can be produced by many different types of substrates. Among these, cassava being a plant with high starch content, is considered a cheap, abundant and renewable resource for production of fermentable glucose syrups and dextrins. Moreover, it is easily produced in tropical and sub-tropical zones, mainly in Asia, South-America and South-Africa. The technological availability and awareness of Africans especially local farmers to the economic potential of utilizing cassava waste in bio-ethanol production poses a great problem.

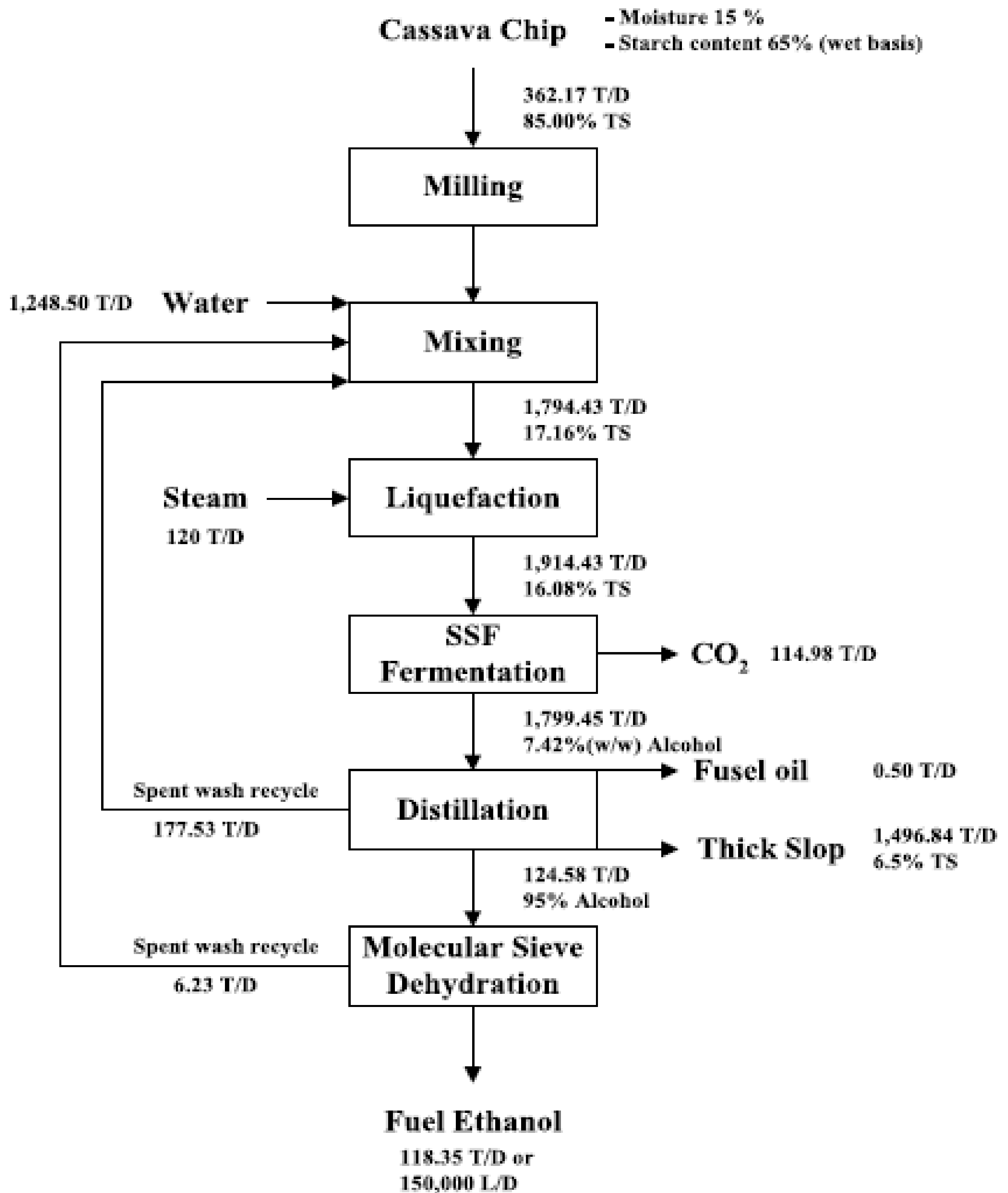

Plate 2: Flow Chart of Ethanol Production from Cassava. Source; (FAO 2000)

The step-wise processing of ethanol from cassava is given at the previous page as;

1. Milling cassava chips or flour through sieve of 0.4 mm.

2. Cooking Process with enzymatic liquefaction;

- Gelatinization
- Liquefaction and dextrinization (using dilute inorganic acid or thermostable α-amylase)
- Saccharification (using amyloglucosidase).

3. Fermentation, and

4. Distillation and Dehydration

2.2.1.1: Milling

Crushing and milling of cassava chips are carried out by the following methods:

(1) Dry milling - grinding of big solid particles into smaller ones (0.4 – 0.6mm), and

(2) Wet milling - soaking in water. The dry milling has several advantages such as -

· less capital investment in plant and equipment

· fewer control loops and simpler processing

· shorter time from construction to operation

· minimal loss of starch

2.2.1.2: Gelatinization

The crushed and milled flours are made into mash by steam cooking, above starch gelatinization temperature (68-74^{0}C). The process is marked by melting of starch

crystals, loss of birefringence, and starch solubilization. Granules absorb large amount of water, swell to many times their original size, and open up enough for α-amylase to hydrolyze long chains into shorter dextrin (Ward *et al*., 2006).

2.2.1.3: Liquefaction and Saccharification

The first step is gelatinization and liquefaction of starch by heating and use of heat stable amylase. This is then followed by conversion to simple sugars by another cocktail of amylolytic enzymes or acid hydrolysis, a process known as saccharification. The hydrolysis process and the utilization of an efficient low-cost saccharifying agent are important factors in the production of ethanol from starch.

The starch is cooked first to release the starch granules, which are bound to the lingocellulosic compound of roots. These will also facilitate the reaction between the saccharifying agents (acids, enzyme, etc.) and the lower substrate. Cassava starch, having a lower swelling and generalization temperature can be easily saccharified to simple sugars with the help of acids or enzymes. The main advantages of cassava over any other energy crop are the presence of high fermentable sugar after saccharification. The use of a dilute acid solution helps to recover approximately 98.8% of the reducing sugars from the stanch.

The formation of secondary revision reaction which sets a limit to the yield of glucose that may be obtained, the formation of high inorganic salts due to pH adjutant and the corrosion of the machinery are some of the disadvantages of the use of acids in the

saccharification process. The enzyme amyloglucosidase (AMG) (glucoamylase or glycoamylase) derived commercially from strains of the molds *Aspergillus* and *Rhizopus* can hydrolyze gelatinized starch completely to glucose unit. It can also hydrolyze the α-1, 4 glycosidic linkage of the non-reducing terminal glucose unit. It can also be hydrolyze the α-1-6 linkage from the amylopectin. Malt contains the three most important enzymes for the starch breakdown, namely, α- amylase, β- amylase, and amyloglucosidase. The α- amylase split the α- 1-4- link randomly within the molecules, forming dextrins, which are small chain of glucose. This makes the generalized starch slurry more fluid and supplies more chain ends for the action of saccharifying enzymes (Paolucci *et al*., 2000).

Since all the α- amylase cannot split the α-1-6- links of starch and dextrin's, all the branch points remain intact after α - amylase action. The amylase also breaks the α-1-4 link of dextrin and starch, but only from the non-reducing ends of the molecules, resulting to maltose formation. Since neither of these enzymes attack α -1-6 linkage, their combined action converts only up to 85% of the starch into reducing sugars. Amyloglucosidase splits the α- 1, 6 linkage of the maltose and short chain of carbohydrates, thus completing the hydrolysis of starch into fermentable sugars. The continued action of the above enzymes produced from the microorganisms can also split the cassava starch to glucose units. Since amylase enzymes do not tolerate temperature above 55°C, they must be added after generalization has taken place. The

use of heat-stable bacteria amylase has received considerable attention at least for effective conversion of starch to sugar.

Commercial enzymes i.e. thermostable α-amylase (Termamyl) and amyloglucosidase are consistently used to liquefy and saccharify the cassava starch to fermentable sugars, achieving an ethanol yield of 400-450 litre/ton of the cassava starch, thus processing a total of 3,969 million litres of ethanol per annum which is 88% efficiency of the installed capacity (Toyin, 2000).

2.2.1.4: Fermentation

Traditionally, the yeast *Saccharomyces cerevisiae* is used for ethanol production. In recent years, however, research is being focused in processes involving Gram negative anaerobic bacterium, *Zymomonas mobilis* as it has several advantages (Nellaiah and Gunasekaran, 2002). Ethanol production from fresh cassava roots, flour or starch using *S. cerevisiae* showed 90-95% fermentation efficiency when the slurry consisting 20% solids was hydrolyzed by liquefaction- saccharification processes. Saccharification of the mash resulted in 10-12% reducing sugar and inoculation at this stage with yeast led to simultaneous saccharification and fermentation and hence curtails the total time of the fermentation process (Ray *et al*., 2004). Direct ethanol production from raw sago starch was investigated using a mixture of strains of *A. niger* and ethanol yeast, *S. cerevisiae*. Ethanol yield from raw sago starch, using the fed-batch mixture, was 50 ml ethanol/100g starch (Ray *et al*.,2004).

Large volumes of the saccharified starch are fed into fermentation vessels and inoculated with actively growing *S. cerevisiae*. Many strains of yeasts with varying capabilities and tolerances have been reported. The yeast inoculation is usually 5 to 10% of the total volume and is growing aerobically in stages, from a laboratory pure culture. The optimum concentration of n-sugars for ethanol fermentation is 12 to 18 %. The pH of the mash for fermentation is optimally 4 to 4.5 and the temperature of fermentation is 28 to 32°C. Sugar is converted to ethanol, carbon dioxide and yeast/bacterial biomass as well as much smaller quantities of minor end products such as glycerol, fusel oils, aldehydes and ketones (Laopaiboon *et al*., 2007).

2.2.1.5: Distillation and Dehydration

Alcohol is recovered from the fermented mash after 48 to 72 hours at the end of fermentation; the yeast is separated from the mash by centrifugation or sedimentation and used for the next batch of fermentation. The resulting liquid is distilled for the recovery of ethanol. Alcohol distilled from fermented mash is concentrated up to 95% v/v. This is further concentrated to produce ethanol with 99.6% v/v (minimum) concentration (Panesar *et al*., 2001). The treatment of vinasse generated in the distillation section can be done by the concentration of part of vinasse to 20-25% solids followed by composting using press mud available and concentration of rest of the vinasse to 55% solids and can be used as liquid fertilizer.

2.2.2: Production of Industrial Starch

Starch is a textile material which can be produced from cassava. Cassava roots are peeled, washed and grated. The grated pulp is steeped for 2-3 days in a large quantity of water, stirred and filtered through a piece of cloth. The filtrate stands overnight and the supernatant is then decanted. The starch sediments are air-dried under shade. Cassava starch is being produced primarily by the wet milling of fresh cassava. When cassava roots are harvested for starch extraction, age and root quality are critical factors that determines the yield (Akpa and Dagde 2012). According to Akpa and Dagde (2012), cassava roots need to be processed almost immediately after harvest, as the roots are highly perishable and enzymatic processes accelerate deterioration within 1-2 days. Substantial amount of cassava will be peeled, washed and grated to finer particles. The starch will then be extracted from the grated pulp by sieving while the fiber will be retained. The fiber retained will then be washed repeatedly for at least three to four times with distilled water on the screen. The starch thus extracted will be allowed to sediment after which the fiber will be decanted off and the starch rewashed with distilled water to remove the remaining fiber. The starch will then be dried in an oven at a temperature of (45ºC) for six hours to reduce the amount of moisture content and finally dried under the sun for four hours. The powdery starch produced can then be stored in an air-tight container to prevent contamination and moisture.

The advantages of cassava for starch production over other grains or root crop include: high purity level, excellent thickening characteristics, a neutral (bland) taste, desirable textural characteristics, is relatively cheap and it contains a high concentration of starch (dry matter basis) (Massamba *et al*., 2001). Cassava starch has many remarkable characteristics, including high paste viscosity, high paste clarity and high freeze-thaw stability which are advantageous to many industries. Cassava is a renewable, and an almost unlimited resource and one of the most abundant substances in nature. It is one of the most important starchy root crops of the tropics used for food and industrial purposes (Akpa and Dagde 2012).

2.2.3: Production of Microbial Raw Starch Digesting Enzymes

Raw starch digesting enzymes refer to enzymes that can act directly on raw starch granules below the gelatinization temperature of starch. With the view of energy-saving, a worldwide interest has been focused on raw starch digesting enzymes in recent years, especially since the oil crisis of 1973. Raw starch-digesting enzymes are ubiquitous and produced by plants, animals and microorganisms. However, microbial sources are the most preferred for large-scale production. During the past few years, the production of raw starch digesting enzymes by various microorganisms has been studied extensively. According to Sun *et al*., (2009), various raw starches and soluble starches are preferred choices for most microorganisms; however, cassava starch is

more preferable due to its low cost and better induction of raw starch degrading enzyme production.

2.2.4: Production of Animal Feed

This section discusses the processing of cassava into animal feed in the form of chips, pellets and feed grade single cell protein. The cassava plant, made up of the roots, leaves and stem, is a good source of carbohydrate and protein. The different parts of the plant can be used as animal feed. The leaves can be used as silage, dried for feed supplementation and as leaf meal for feed concentrates. The stem can be mixed with leaves and used as ruminant feed, or dried for feed concentrates. The roots can be chipped or pelletized and used as feed, while the root peel, broken roots, fiber and baggase from starch extraction and *garri* processing can be dried and used directly as animal feed or as substrate for single cell protein production. The use of cassava root as animal feed is increasing in importance in the developing countries of Latin America and Asia where an export market for this commodity has developed.

There is very little difference in the technologies used at different scales of chip and pellet production. The main difference is in sun-drying and mechanical drying. Chips can be produced by very simple techniques in the household or village as well as on a large mechanized scale. About 2.5-3.0 tonnes of fresh roots are required for 1 tonne of pellets giving a conversion rate of 33-40%. The first step can be washing and peeling, depending on the quality of the harvested roots.

Dried cassava leaves and stems can be fed to pigs, poultry, and dairy cattle. The meal produced from them has a nutritive value similar to that of alfalfa though deficient in methionine, isoleucine and threonine. Cassava leaves are a good source of about 20% protein. The amount of protein depends on the stage of growth.. For the extraction of cassava leaf protein, the leaves and the stem are interacted in a chopper or grinder and the juice pressed out. The extracted juice is then coagulated with injection of steam. The pressed cake is sent to the dehydrator. The coagulated juice is then sent to a separator where the soluble fraction is separated from the green curd and moved to the evaporator where it is concentrated to 50% by volume. The curd is sent to the drier to produce the cassava protein concentrate which is 50% protein.

The use of cassava as a substrate for single cell protein has been investigated since the mid- 1960s. Gray and Abou-El-Seoud (2000) grew some filamentous fungi on ground cassava roots, supplemented with ammonium chloride and corn steep liquor, to obtain biomass containing 13-24% crude protein. Shrassen *et al*. (2004) described a process in which the yeast *Candida utilis* fermented enzymatically hydrolyzed cassava in a submerged culture to produce a product containing 35% crude protein on a dry weight basis. They also reported using *Aspergillus fumigatus*1-21 to ferment whole cassava in a non-aseptic continuous fermentation system to produce single cell protein containing 37% crude and 27% true proteins. The fungus was a non-reversible sporogonous mutant of *A.fumigatus*1-21. This product was fed to rats and it produced

good growth responses. Single cell protein can be produced by two types of fermentation processes, namely submerged fermentation and semi-solid state fermentation.

2.3: Waste Management and Pollution- Case Study of Cassava Cottage Industries

Management of waste is demanding and challenging undertaking in African countries, with important implications for human health and well-being, environmental preservation, sustainability and economy. Wastes from agro-allied industries could be generated from farms, dairies, orchards, vine yards and feed lots. Agro-allied wastes could be solid e.g spoiled foods; and liquids e.g waste water from food processing. Agricultural wastes are included as one of the environmental waste streams, amongst others which are industrial, commercial, residential and institutional waste sources. Some of the agro-allied wastes are regarded as hazardous (especially when they pose threat to man) and non-hazardous. Agro-allied wastes can be generated from food processing, food farming and also from the machines used for these processes.

According to FAO (2009), small-scale cassava processing could affect the environment more than large-scale. Environmental pollution from medium scale cassava processors may be more difficult to deal with. Significant amounts of wastes are generated during cassava processing. The traditional processing of cassava into

garri generates more wastes. Cassava waste waters have suspended solid levels of about 15,000 mg/L, and they are mostly not treated before disposal; thus, they freely get into the environment, thereby, contaminating nearby water sources. The release of waste products and contaminants into surface run-off, get into rivers through drainage systems, leaching into ground water, liquid spills, waste water discharges and littering. Cassava processing effluents have serious environmental impacts, causing acidification due to hydrolysis of cassava cyanogenic glucosides, thus, producing hydrogen cyanide which is toxic to household animals, fisheries and other organisms.Waste water from cassava processing cottage industries, released directly into the environment without treatment, causes pollution and foul odour leading to contamination of soil, surface waters and ground waters.

Suspended solids contained in cassava waste waters play a major role in pollution in the sense that they serve as attachment points for pathogens. Soil pollution from cassava effluent discharge leads to increased microbial density, acidity, temperature, electrical conductivity, low clay content and organic carbon content of the soil. Soils receiving cassava effluents have some levels of heavy metal enrichment; metals such as zinc, copper and manganese are mainly seen in cassava effluent contaminated soil (Ubalua, 2007).

Gaseous emissions are other important source of environmental pollution, emanating from cassava cottage industries. Gaseous pollutants are mainly derived from machines

used in cassava processing into other desired finished products. Carbon (IV) oxide emission from fire and internal combustion engines is a major environmental pollutant from the cottage industries. Addition of carbon (IV) oxide to the existing one in the atmosphere, raises the average temperature, thus, contributing to climate change. Other common gaseous pollutants from cassava processing industries are nitrogen oxides, sulphur (IV) oxide and chloro-fluoro carbons. All these pollution issues call for the need to manage these wastes and possibly convert them to sources of revenue generation.

2.4 Cassava Waste Management Options

The problems of pollution from cassava processing are more social and economic in nature than technological. Interventions, usually from the government, are required. Most governments recognize the need to control waste produced by cassava factories, but they are equally aware of the economic risk involved in such a strategy. Accessible technologies for most scales of processing are available; however, the cost of implementing the technology is, in many cases, prohibitive. According to starch processors, the installation of pollution control devices can be 20 - 50% of the total investment cost of a large-scale factory. Full implementation of strict environmental controls too quickly can have negative consequences, forcing the industry to forfeit its competitiveness. Dealing with environmental problems resulting from processing is generally regarded as a necessary expense with no direct return.

Waste materials from cassava processing (e.g. starch) are divided into four categories:

a) Peelings from initial processing

b) Fibrous by-products from crushing and sieving (pulp waste)

c) Starch residues after starch settling and

d) Waste water (effluent).

Ensiling of solid residues

A residue is a substance resulting from the processing of a product. It becomes a co-product or a by-product when profitable use is made of it. If this is not the case, the residue becomes a waste, which is defined as a material with no apparent market, social, or environmental value, that constitutes an environmental nuisance and a source of pollution. In starch processing, pulp waste is the main problem, especially for the bigger factories, which produce massive quantities. Dealing with this waste is difficult, as it is not easily dried, due to its high moisture and starch contents (Sriroth *et al.*, 2000). On a dry weight basis, pulp constitutes about 20% of the original roots. Ensiling of cassava residues is done by washing, drying and grinding of the residues. Ensiling is followed immediately after addition of 0.5% salt (of the fresh weight of the residue) before placing them either in a pit dug out of the ground, in a cement container or in plastic bags. These are filled with the residues as quickly as possible and compacted properly to eliminate air, so as to minimize the loss of nutrients by oxidation. Usually a polyethylene sheet is used to cover the ensiled material to create

anaerobic conditions for fermentation. During ensiling, anaerobic bacteria multiply rapidly, accelerating fermentation. Ideally the microorganisms which grow most rapidly will be predominantly *Lactobacillus* species which produce lactic acid from the fermented residues. The produced lactic acid lowers the pH and fermentation ceases after 3 to 4 weeks when the pH becomes so low that all microbial growth is inhibited. If ensiling procedures are such that lactic acid producing bacteria are not favoured, *Clostridium* strains of microorganisms will grow. These organisms utilize water-soluble carbohydrates, lactic acid and protein for growth and produce butyric acid. However, the quality of silage is greatly reduced if a *Clostridium* type of fermentation predominates. In addition to Lactobacilli and *Clostridium* microorganisms, silage also contains yeasts, molds, coliforms, bacilli and propionic acid producing bacteria. Apart from utilization of sugars as energy sources, silage microorganisms degrade protein to amino acids, amines and ammonia during fermentation. Literally hundreds of fermentation products are formed in addition to lactic and butyric acids. In case of cellulosic materials with high lignin components, digestion by animals is minimal. In such cases, a small amount of fermentable carbohydrates such as corn meal or molasses is added to produce silage, thus ensuring rapid fermentation. Silage making normally serves as one of the effective means of conserving high-moisture products for animal feed. Many crop residues are deficient in protein and minerals. It is therefore not uncommon for farmers to add products such as urea and minerals at the time of ensiling.

Ensiling lowers the cyanide level to non-toxic level leads to a reduction in pH to 4.0 and allows lactic acid to build-up. Increased ensiling time decreased the levels of organic acids (acetic and butyric acids) while that of lactic acid is increased. The product can be subsequently used as animal feed (Siroth *et al.*, 2000).

Fermentation of cassava peels

Cassava peel wastes are generated in the production of farihna, garri and chikwangue. Inappropriate storage of the peels for long periods is the main issue especially with heavy rainfall. Utilization of the peel is limited by its low digestibility and toxicity from extremely high levels of hydrocyanic acid. Fermentation not only reduces toxicity, but the enzyme-resistant ligno-cellulose material is converted into a more digestible substrate. Following fermentation, cassava peel can be formulated into pig and poultry feed. Oboh (2006) studied the nutrient enrichment of cassava peels using a mixed culture of *Saccharomyces cerevisae* and *Lactobacillus* spp. solid media fermentation techniques. Three treatments (mixing of 150 ml of waste water from fermented cassava pulp with 200 g of washed, dried and ground cassava peels and mixing of 150 ml of wastewater from the fermented inoculated pulp with 200 g of washed, dried and ground cassava peels) were observed for fermentation. The unfermented cassava peels served as control. The result of the analysis of the fermented cassava peels revealed that there was an increase in the protein content of the cassava peels fermented with wastewater from fermented cassava pulp when

compared to unfermented peels (8.2%) (Oboh,2006). This increase was highest in the peel fermented with wastewater from the inoculated cassava pulp (211.1%). The increase in the protein content of the cassava peels fermented with wastewater from the inoculated fermented cassava pulp could be attributed to the possible secretion of some extracellular enzymes (proteins) such as amylases, linamarase and cellulose (Oboh and Akindahunsi 2003) into the cassava mash by the fermenting organisms, as well as increase in the growth and proliferation of the fungi/bacterial complex in the form of single cell proteins (Oboh *et al*., 2002). Conversely, a decrease in the carbohydrate content of the cassava peels fermented with wastewater from the inoculated cassava pulp (51.1%) was observed when compared to the unfermented cassava peels (64.6%). The decrease could be attributed to the ability of the fungi/bacteria complex to hydrolyze starch into glucose and ultimately the glucose will be used by the same organisms as a carbon source to synthesize fungi/bacteria biomass rich in protein (Oboh *et al*., 2002). However, Oboh (2006) observed that there was no discernable trend in the fat, crude fibre, ash and the mineral content of the cassava peels. Cassava peels normally have higher concentration of cyanogenic glucosides than the parenchyma (pulp) and this makes the peel unsuitable for animal feed. Its fermentation with wastewater from the fermented cassava pulp reduced the cyanide content of the peels when compared with the unfermented cassava peels (Oboh, 2006). However, the cassava peels fermented with wastewater from cassava pulp fermented with a mixture of *S. cerevisiae*, *Lactobacillus delbruckii* and

Lactobacillus coryneformis had a lower cyanide content (6.2 mg/kg) than those cassava peels fermented with wastewater from the naturally fermented cassava pulp(23.5 mg/kg). Results showed that wastewater from the inoculated cassava pulp were very efficient in cyanide detoxification than that of naturally fermented cassava.

The fermented cassava peels could be considered safe in terms of cyanide poisoning in view of the fact that the cyanide was below the deleterious level of 30 mg/kg (Tweyngyere *et al*., 2002). A decrease in phytate content of the fermented cassava peels (705.1 – 789.7 mg/100 g) was reported by Oboh (2006) and this decrease was more in cassava peels fermented with wastewater from naturally fermented cassava pulp (705.13 mg/100 g),while the unfermented cassava peels had 1043.56 mg/100 g phytate content. Such a decrease could be as a result of possible secretion of the enzyme phytase by the microorganisms in the wastewater. This enzyme is capable of hydrolyzing phytate thereby decreasing the phytate content of the fermented cassava peels (Oboh *et al*., 2000). In view of the increase in protein content of the cassava peels fermented with wastewater from fermented cassava products (inoculated and natural) and the significant decrease ($p < 0.05$) in the anti-nutrients (residual cyanide and phytate), this by-product could be a good supplement in compounding animal feed provided that it is acceptable and highly digestible.

Composting of cassava solid wastes

Recycling through composting is a largely neglected form of management. Yet, for something to be compostable, it must be biodegradable. Composting is the accelerated degradation of heterogenous organic matters by a mixed microbial population in a moist, warm, oxygenated environment under controlled conditions. Composting of biodegradable products like cassava wastes and paper wastes along with other organic compostable materials such as food and agricultural wastes can help to generate much-needed stable profitable carbon-rich compost that can be used for soil rejuvenation and conditioning.

Compost soil increases organic carbon, water and nutrient retention, with the consequent reduction in chemical inputs, and suppression of plant diseases. The disposal of various solid wastes is an ever increasing problem, both financially and environmentally. A typical composting cycle usually includes the following processes: the cassava wastes are piled into heaps of approximately 500 $m^3$and left to 'age', thereafter water and nitrogen (usually in the form of urea) are added to initiate composting. Temperatures within the heaps rise dramatically with peak sometimes as high as 80^0C.Subsequently the heaps are watered and turned or mixed weekly for aeration, for a period of about three months as composting continues (stabilization). The finished product is then screened to obtain particles of a uniform size. Composting processes are generally developed empirically, and the biological component is often regarded as a simple chemical reaction rather than the complex set

of interactions that actually occur. Key factors that affect the rate of composting, such as temperature, pH, moisture and nutrient availability are all factors that profoundly influence microbial growth and activity. It is generally accepted that composting occurs in three stages: readily degradable organic compounds are utilized; followed by the thermophilic stage when temperature rises and the stabilization period when temperature drops. The self-heating that takes place is due to heat liberation from microbial metabolic activities. The optimal moisture content for most composting processes is 50 - 70% (w/w), and the oxygen concentration should be maintained at greater than 0.1% preferably 5 -12%. Waste substances for composting that have high C:N ratios generally require the addition of a nitrogen source to initiate the intense microbial activities associated with composting. Other organic wastes such as chicken and piggery manure, and soy waste may be added. If a suitable nitrogen source is not added, the high temperatures associated with composting will not occur. At all stages of composting, bacterial populations usually outnumber fungal propagules. Mesophilic and thermophilic fungi are killed off as the temperature of the organic waste increases to a maximum. On the other hand, thermophilic bacteria and actinomycete populations out-number mesophilic populations during the latter part of composting. Bacteria are more important particularly in the initial stages and bacterial metabolism is responsible for the dramatic temperature increases that occur in composting while fungi may as well be important in the later stages.

The numbers of mesophiles and thermophiles may be similar at the start of composting but there may be subtle changes in the microbial species making up the populations during the aging period or changes due to degradation of organic constituents. Cassava solid wastes degradation is generally initiated by mesophilic heterotrophs, which, as the temperature rises, are replaced by thermophilic microorganisms. Thermophilic microorganisms that are prominent in the composting process includes *Bacillus stearothermophilus*, *Thermomonospora* spp., *Thermoactinomyces* spp and *Clostridium thermocellum* while *Geotrichum candidum, Aspergillus fumigatus, Mucorpusillus, Chaetomium thermophile, Thermoascus aurantiacus* and *Torula* spp. are among the group of fungi implicated in the process. 'Sour' composting may result from anaerobic decompositions which liberate less heat and generate different end products from their aerobic equivalents. Composting has the advantage of being suppressive to many pathogens, production of a well balanced fertilizer, destruction of weed seeds and disease germs during the heating phase, pH increase in acid soils, reduction in waste volume, increase in soil organic matter content and also can reduce the phytotoxic properties (e.g. high C:N ratio).A major drawback of composting is the necessity to separate organic material from other wastes. Therefore, it is probably economically advantageous only when organic material is collected separately from other wastes.

2.5 Amylase

Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristics of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4-glycosidic bonds of amylase, amyl pectin, glycogen and their degradation products (Damien *et al*., 2010). They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved (Dhanya *et al*., 2012).

In recent years a number of new enzymes associated with degradation of starch and related polysaccharides structures have been detected and studied. The enzymes having potential commercial importance of microbial origin that split α-1, 4 or α-1, 4 and/or α-1,6 bonds in these structures, may be divided in the following six classes (Mohammad *et al*., 2010).

1. Enzymes that hydrolyze alpha-1,4 bonds and bypass alpha -1,6 linkages e.g. α-amylase (endo-acting amylases).
2. Enzymes that hydrolyze α-1, 4 and cannot bypass α-1, 6 linkages e.g. β-amylase (exo-acting amylase producing maltose as a major end product).
3. Enzymes that hydrolyse α-1,4 and α-1,6 linkages e.g. amylo glucosidase (gluco amylase) and exo-acting amylase.

4 Enzymes that hydrolyse only α-1,6 linkages e.g. pullulanase and other debranching enzymes.

5 Enzymes that hydrolyse preferentially α-1,4 linkages in short chain oligosaccharides produced by the action of other enzymes on amylase and amylopectin e.g. α- glucosidases.

6 Enzymes that hydrolyze starch to a series of non-reducing cyclic D-glucosyl polymers called cyclodextrins or sachardinger dextrins e.g. *Bacillus macerans* amylase (cyclodextrin producing enzyme) (Archana *et al*., 2015).

2.5.1 Types of Amylase

α-Amylase

α-Amylase is a hydrolase enzyme that catalyses the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products like glucose and metalloenzyme i.e. it depends on the presence of a metal co-factor for its activity. There are 2 types of hydrolases: endo-hydrolase and exo-hydrolase (Drauz *et al*., 2016).

Endo-hydolases act on the interior of the substrate molecule, whereas exo-hydrolases act on the terminal non-reducing ends. Hence, terminal glucose residues and α-1, 6-linkages cannot be cleaved by α-amylase. The substrate that α-amylase acts upon is starch. Starch is a polysaccharides composed of two types of polymers – amylose and amylopectin. Amylose constitutes 20-25% of starch molecule. It is a linear chain

consisting of repetitive glucose units linked by α-1, 4-glcosidic linkage while branching occurs every 15-45 glucose units where α-1,6 glycosidic bonds are present. The hydrolysate composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. The optimum pH for activity is found to be 7.0. α- Amylase has become an enzyme of crucial importance due to its starch hydrolysis activity and the activities that can be carried out owing to the hydrolysis. One such activity is the production of glucose and fructose syrup from starch α- Amylase catalyses the first step in this process.Previously, starch was hydrolyzed into glucose by acid hydrolysis. But this method has drawback like the operating conditions are of highly acidic nature and high temperature. These limitations are overcome by enzyme hydrolysis of starch to yield high fructose syrup (Drauz *et al*., 2016).

The use of enzymes in detergent formulations has also increased dramatically with growing awareness about environmental protection. Enzymes are environmentally safe and enhance the detergents ability to remove tough stains. They are biodegradable and work at milder conditions than chemical catalysts and hence preferred to the latter. There are many such applications of the enzyme which is the driving force behind the research to produce this enzyme in an optimum, safe and convenient manner (Gupta *et al*., 2013).

β - Amylase

β – Amylase is an exo-hydrolase enzyme that acts from the non-reducing end polysaccharide chain by hydrolysis of α- 1, 4-glucan linkages to yield successive maltose units. Since it is unable to cleave branched linkages in branched polysaccharides such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. Primary source of β-amylase are the seeds of higher plants and sweet potatoes. During ripening of fruits, β-amylase break down starch into maltose resulting in the sweetness of ripened fruits. The optimal pH of the enzyme ranges from 4.0 to 5.5. β-amylase can be used for different applications on the research as well as industrial front. It can be used for structural studies of starch and glycogen molecules produced by various methods. In the industry it is used for fermentation in brewing and distilling industry. Also, it is used to produce high maltose syrups (Drauz *et al*., 2016)

γ- Amylase

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, unlike the other forms of amylase, yielding glucose. γ- amylase is most efficient in acidic environments and has an optimum pH of 3 (Archana *et al*., 2015).

Fungal Amylases: Bacteria and fungi secret amylase to the outside of the cell to carry out extra-cellular digestion. Ellaiah *et al*. (2014) identified amylolytic activity from

several fungal species isolated from soil and *Aspergillus* sp. It was found to possess the highest amylase activity produced extracellular amylase using brand (wheat bran, rice brand, blank grain bran) as carbon source in shake flask cultures of a hemophilic strains of *Aspergillius niger*.

Fungal amylases are used for hydrolyzing carbohydrate, protein and other constituents of soybeans, wheat into peptides, amino acid, sugars and other low molecular weight compounds. The amylase producing strains of *Aspergillus niger* have spore bearing heads which are large, tightly packed, globular and may be black or brownish black. They are considered to be mesophilic with optimal temperature for growth between 25^0C and 35^0C. They are aerobic in nature and can grow over a wide range of hydrogen ion concentrations. These organisms can utilize different kinds of agricultural wastes from simple to complex ones, which make them easy to cultivate and maintain in the laboratory (Ellaiah *et al*.,2014).

Filamentous fungi are microorganisms that secret large amounts of protein in culture medium. *Aspergillus niger* has been described as secreting an alpha-amylase and glucoamylase of a number of different molecular weights in submerged culture. Filamentous fungi have been used for the industrial production of a wide variety of native products, such as antibiotics (e.g. penicillin and cephalosporin), organic acids (Citric and acetic acid) and commercial enzymes (e.g., protease, catalase, amylase) (Gupta *et al.,* 2013).

2.5.2 Fermentative Production of Amylase

To meet the demand of industries, low cost medium is required for the production of alpha-amylase (Aliyu *et al.,* 2011). Both solid state fermentation (SSF) and submerged fermentation (SMF) could be used for the production of amylase, although traditionally these have been obtained from submerged cultures because of easy handling and greater control of environmental factors such as temperature and pH (Xusheng *et al.,* 2011). Mostly synthetic media have been used for the production of bacterial amylase through SMF (Ajay *et al.,* 2018). The contents of synthetic media such as nutrient broth, soluble starch, as well as other components are very expensive and these could be replaced with cheaper agricultural by products for the reduction of the cost of the medium (Solange *et al.,* 2016). The solid substrate may provide only support and nutrition (Hashemi *et al.,* 2011). SSF is considered as an interesting alternative since the metabolites so produced are concentrated and purification is of less quality (Nasrin *et al.,* 2010). SSF is preferred to SMF because of simple technique, low capital investment, lower levels of catabolite repression and end product inhibition, low waste water output, better product recovery and high quality has been reported to produce promising results (Ajay *et al.,* 2018). Other substrates such as sunflower meal, rice husk,cotton seed meal, soybean meal, and pearl millet and rice bran have been tried for SSF (Maryam *et al.,* 2010).

SSF technique is generally confined to the process involving fungi. However, successful aerial bacterial growth in SSF is known much in natural fermentation. The production of alpha amylase by SSF is limited to the genus *Bacillus* like *B.subtilis, B. polymaxa, B. mesentiricus, B. vulgarus. B.coagulans, B. megaterium and B. licheniformis* have been used for alpha amylase production in SSF (Natasa *et al.,* 2017).The production of bacterial amylase using alpha amylase technique requires less fermentation time which leads to considerable reduction in the capital and recurring expenditure (Li Zhuang *et al.,* 2014). Research on the selection of suitable substrates for SSF has mainly been centred around agro industrial residues due to their potential advantages for filamentous fungi which are capable of penetrating into the hardest of these solid substrates aided by the presence of turgor pressure at the tip of the mycelium. In addition, the utilization of these agro industrial wastes, not only provides alternative substrates but also on the other hand helps in solving pollution problems (Kiran *et al.,* 2013).

Solid state fermentation

Alpha amylase production by a strain of *Bacillus subtillis* has been demonstrated in solid state fermentation utilizing rice bran as substrate. A maximum amylase production of 2311.1 U/g was showed with pH 7, when SSF was carried out at 37°C for 48 hr using a substrate with 75% initial moisture. The effect of different carbon and nitrogen sources were also analyzed. The results showed a 1% increase in

amylase production with soluble starch and glucose (Rameshkumar and Sivasudha,2005).

Optimization of thermostable alpha-amylase production by *Streptomyces erumpens* MTCC 7317 in solid-state fermentation using cassava fibrous residue have been reported. Response surface methodology (RSM) was used to evaluate the effect of incubation period (60 h), moisture holding capacity (60%) and temperature (50°C) on enzyme production. Varying the inoculum concentration (5-25%) of *S. erumpens* showed that 15% inoculum (v/w. 2.5 x 10 6 CFU/ml) was optimum for alpha-amylase production. The results also showed that, beef extract was most suitable nitrogen source for enzyme production. The maximum hydrolysis of soluble starch 85%) and cassava starch (70%) was obtained with the application of 5ml crude enzyme (17185 units) after 5 h of incubation (Kar *et al.,* 2010).

Production of alpha-amylase from *Penicillium chrysogenum* under solid state fermentation using agricultural by-products such as corncob leaf (CL), rye straw (RS), wheat straw (WS) and wheat bran (WB) was investigated. Optimal moisture levels of substrates were 75. 65. 65 and 55 % for CL,WS, WB and RS substrates, respectively. Optimal particle size and inoculum concentration for the production of alpha-amylase were: >1 mm, 20 %; >1 mm, 20 %; 1 mm, 20 % and >1 mm. 30% for CL, WS, WB and RS, respectively. Maximum enzyme production of 160 U/ml was reported under the optimum conditions with wheat bran as substrate (Solange *et al.,* 2016).

Submerged fermentation

Optimization of the growth of an alpha-amylase production of *Bacillus subtilis* IP 5832 in shake-flask and laboratory fermentor batch cultures were examined. The results showed that 0.5 % starch was necessary for maximum alpha-amylase production, inducing 1.55 lU/ml of amylase to be secreted after 8 h of cultivation in shaking flasks and a 60 % higher activity (2.5 lU/ml) was obtained, when fermented in 2L laboratory fermentor (Bozici *et al.,* 2011).

Process Optimization

Optimization of the various parameters and manipulations of media are one of the most important techniques used for the over production of amylase in large quantities (Balasubramanien *et al.,* 2011). To meet industrial demands, production of alpha amylase in fungi is known to depend on both morphological and metabolic state of the culture (Juliana *et al.,*2011). Growth of mycelium is crucial for extracellular enzyme like alpha amylase (Sangeeta *et al*., 2009).

The classical method for medium optimization involves changing one independent variable, keeping the other factors constant OVAT (one-variable-at-a-time). This method is time-consuming and incapable of detecting the true optimum, due to the interactions among the factors and this limitation of a single factor optimization process can be .eliminated by different techniques (Abdel-Fattah *et al*..2013) unlike conventional optimization, statistical optimization methods present a more balanced

alternative to the OVAT approach, since it takes into account the interaction of variables in generating the process response. Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response. Recently, the results analyzed by a statistically planned experiment are better acknowledged than those carried out by the traditional OVAT method. Some of the popular choices, applying statistical designs to bio-processing, include the Plackett-Burman design (El-Sharouny *et al.,* 2015).

Physico-chcmical Parameters

The role of various physico-chemical parameters, including carbon and nitrogen source, surface acting agents, phosphate, metal ions, temperature, pH and agitation have been studied. Various physical and chemical factors have been known to effect the production of alpha amylase such as temperature, pH, incubation period, carbon, nitrogen sources, surfactants, phosphate, different metal ions, moisture and agitation with respect to SSF and SmF (Ellaiah *et al.,* 2014).

Temperature

The influence of temperature on amylase production is related to the growth of the organism. Hence the optimum temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi most amylase production studies have been done with mesophilic fungi within the temperature range of 25-37^{0}C (Takahiro

*et al.,*2011). A raw starch degrading amylase was produced by *Aspergillus ficum* at 30°C by Ahmad *et al.,* 2010. Yeasts such as *Saccharomyces kluyveri* and *S. cerevisiae* were reported to produce alpha amylase at 30^0C. Amylase production at optimum level has been reported between 50- 55^0C for the thermophilic fungal cultures such as *Talaromyces emersonni*, *Thermomonospora fusca* etc. (Ahmad *et al.,* 2010).

Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25 to 35^0C. Optimum yields of α-amylase were achieved at 30-37°C for *Aspergillus oryzae*. α-Amylase production has also been reported at 55°C by the thermophilic fungus *Thermomonospora fusca* and at 50°C by *Thermomonospora lanuginosus***.** α-Amylase has been produced at a much wider range of temperature among the bacteria. Continuous production of amylase from *Bacillus amyloliquefaciens* at 36°C has been reported. However, temperatures as high as 80°C have been used for amylase production from the hyperthermophile *Thermococcus profundus* (Ellaiah *et al.,* 2014).

pH

pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium (Ellaiah *et al.,* 2014). Earlier studies have revealed that fungi require slightly acidic pH and bacteria require neutral pH for optimum growth. pH is known to affect the synthesis and secretion of alpha-amylase just like its stability (Yakup *et*

al., 2012). Fungi of *Aspergillus* sp. such as *A. oryzae, A. ficuum* and *A. niger* were found to give significant yields of alpha amylase at pH equal to 5.0 to 6.0 in SMF. Alpha-amylase producing yeast strains such as *S. cerevisiae* and *S. kluyveri* exhibited maximum enzyme production at pH 5.0 (Samrat *et al.,* 2014).

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the *Bacillus* strains used commercially for the production of bacterial a-amylases by SMF have an optimum pH between 6.0 and 7.0 for growth and enzyme production. This is also true of strains used in the production of the enzyme by SSF. In most cases the pH used is 4.2-8.0 in the case of *Aspergillus oryzae*, and 6.8 for *Bacillus amyloliquefaciens* (Takahiro *et al.,* 2011).

In fungal processes, the buffering capacity of some media constituents sometimes eliminates the need for pH control. The pH value also serves as a valuable indicator of the initiation and end of enzyme synthesis. It is reported that *Aspergillus oryzae* accumulated amylase in the mycelia when grown in phosphate or sulphate deficient medium and was released when the mycelia were replaced in a medium with alkaline pH (Li Zhuang *et al.,* 2014).

Substrate Sources

α- Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose. Most reports available on the induction of α-amylase in different strains of *Aspergillus oryzae* suggest that the general inducer molecule is maltose. There is a report of a 20-fold increase in enzyme activity when maltose and starch were used as inducers in Asp*ergillus oryzae* (Kiran *et al.,* 2013). Similarly, strong α-amylase induction by starch and maltose in the case of *Aspergillus oryzae* has been reported (Takahiro *et al*., 2011). Apart from maltose, in some strains, other carbon sources as lactose, trehalose, α-methyl-D-glycoside also served as inducers of amylase. Not only the carbon source, but also the mycelial condition/age affects the synthesis of α-amylase by *Aspergillus oryzae.* There are reports that 5-days starved non-growing mycelia were the most appropriate for optimal induction by maltose, α - Amylase production is also subjected to catabolite repression by glucose and other sugars, like most other inducible enzymes. However, the role of glucose in the production of α-amylase in certain cases is controversial. α - Amylase production by *Aspergillus oryzae* was not repressed by glucose rather; a minimal level of the enzyme was induced in its presence. However, xylose or fructose has been classified as strongly repressive although they supported good growth in *Aspergillus nidulans* (Samrat *et al.,* 2014)

Carbon sources such as glucose and maltose have been utilized for the production of α-amylase. However, the use of starch remains promising and ubiquitous. A number

of other non-conventional substrates such as lactose, casitone, fructose, oilseed cakes and starch processing waste water have also been used for the production of α-amylase while the agro-processing by-product, wheat bran has been used for the economic production of a-amylase by SSF. The use of wheat in brain liquid surface fermentation (LSF) for the production of α-amylase from *Aspergillus fumigatus* and from *Clavatia gigantea*, respectively, has also been reported. High α-amylase activities from *Aspergillus fumigatus* have also been reported using α-methyl-D-glycoside (a synthetic analogue of maltose) as substrate (Ajay *et al.,* 2018).

Use of low molecular weight dextran in combination with either Tween 80 or Triton X100 for α-amylase production in the thermophilic fungus *Thermomyces lanuginosus* has been reported. Triton X-100 had no effect, whereas Tween 80 increases the a-amylase activity 27- fold.

Carbon Sources

Carbon sources such as galactose, glycogen and inulin have been reported as suitable substrates for the production of amylases by *B. licheniformis* and *Bacillus* sp. (Xusheng *et al.*.2011). Starch and glycerol were known to increase enzyme production in *B. subtilis* 1MG22, *Bacillus* sp. PS-7 and *Bacillus* sp.1-3. Soluble starch has been found as the best substrate for the production of alpha amylase by *B. stearothermophilus*. *Bacillus* sp. was noted to give a maximum raw starch digesting

amylase in a medium containing lactose (1%) and yeast extract (15%). *Thermomyces lanuginosus* was reported to give maximum alpha amylase yield when maltodextrin was supplemented to the medium. Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. The waste consists of carbon and nitrogen sources necessary for the growth and metabolism of organisms. These nutrient sources include orange waste, peer millet starch, potato, corn, tapioca, wheat and rice as flours (Archana *et al.,* 2015).

Nitrogen Sources

Organic nitrogen sources have been preferred for the production of α-amylase. Yeast extract has been used in the production of a-amylase from *Streptomycin* sp. *Bacillus* sp. and *Halomomas meridian*. Yeast extract has also been used in conjunction with other nitrogen sources such as bactopeptone in the case of *Bacillus* sp., ammonium sulphate in the case of *Bacillus sublilis,* ammonium sulphate and casein for *C. gigantea* and soybean flour and meat extract for As*pergillus oryzae.* Yeast extract increased the productivity of α-amylase by 156% in *Aspergillus oryzae* when used as an additional nitrogen source than when ammonia was used as sole source (Samrat *et al.,* 2014).

Various other organic nitrogen sources have also been reported to support maximum a-amylase production by various bacteria and fungi. However, organic nitrogen sources viz. beef extract, peptone and corn steep liquor supported maximum α-amylase production by bacterial strains, soybean meal and amino acids by *Aspergillus*

oryzae. Various inorganic salts such as ammonium sulphate for *Aspergillus oryzae* and *Aspergillus nidulans,* ammonium nitrate for *Aspergillus oryzae* and Vogel salts for *Aspergillus fumigatus* have been reported to support better α-amylase production in fungi (Solange *et al.,* 2016).

Amino acids in conjunction with vitamins have also been reported to affect α-amylase production. However, no conclusion can be drawn about the role of amino acids and vitamins in enhancing the α-amylase production in different microorganisms as the reports are highly variable. α-Amylase production by *Bacillus amyloliquefaciens* increased by a factor of 300 in the presence of glycine. The effect of glycine was not only as a nitrogen source rather it affected α-amylase production by controlling pH and subsequently amylase production increased. Alanine, DL -nor valine and D-methionine were effective for the production of alkaline amylase by Bac*illus* sp. However, the role of amino compounds was considered to be neither as nitrogen nor as a carbon source, but as stimulators of amylase synthesis and excretion. It has been reported that only asparagine gave good enzyme yields while the importance of arginine for α-amylase production from *Bacillus subtilis* has also been well documented (Solange *et al.,* 2016).

Soybean meal was found as the best nitrogen source for alpha amylase by *Bacillus* sp. Arpana *et al*. (2011) reported that peptone increased enzyme activity while yeast extract exhibited no effect on alpha-amylase production. Strains of *Bacillus*

stearothermophilus and *B. amylolyticus* secreted maximum alpha amylase in a medium supplemented with 1% peptone, 0.5% yeast extract and 0.5% maltose under vigorous shaking conditions, reported peptone to be a belter nitrogen source for enzyme production by *B. licheniformis* SPT 278 than ammonium phosphate, the best among inorganic nitrogen sources. L-asparagine was reported to be one of the most promising nitrogen sources for alpha-amylase production by *Thermomyces lanuginosus*. Yeast extract also resulted in a significant alpha-amylase yield. Supplementation of Casein hydrolysate to the medium resulted in 143% increase in alpha-amylase productivity by *A. oryzae* 1560 compared to ammonia (Adinarayana *et al.,* 2015).

Surfactants

Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability. Therefore addition of these surfactants is used for the production of extracellular enzymes (Samrat *et al.,* 2011). Addition of tween 80 (1.3%) to the fermentation medium increased alpha-amylase production by 2-fold in *Thermomyces lanuginosus*. A study on the effect of supplementation of Polyethylene glycols (PEG) (molecular mass of 600,3000,4000,8000 and 20,000) in fermentation medium for alpha-amylase production by two *Bacillus* spp. Researchers indicated that 5% PEG's 600 and PEG

3000 yielded 31% increase in enzyme production by *B. amyloliquefaciens* and 21% increase by *B. subtilis* (Drauz *et al*., 2016).

Role of Phosphate

Phosphate plays an important regulatory role in the synthesis of primary and secondary metabolites in microorganisms and likewise it affects the growth of the organism and production of α-amylase. A significant increase in enzyme production and condition in *Aspergillus oryzae* above 0.2 M phosphate levels has been reported . Similar findings were corroborated in *Bacillus amyloliquefaciens* where low levels of phosphate resulted in severe low cell density and no α-amylase production. In contrast, high phosphate concentrations were inhibitory to enzyme production by *Bacillus amyloliquefaciens* (Solange *et al.,* 2016)

Metal Ions

Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production as most alpha-amylases are known to be metalloenzymes. Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of Calcium chloride to the fermentation media increased the enzyme production (Arthur *el al*..2016). Positive results of the influence of $CaC1_2$ (0.1%) and NaCI (0.1%) on alpha-amylase production in SSF using *Amaranthus* grains as substrates were recorded- $LiSO_4$ (25 mM) and $MgSO_4$ (1 mM) increased

alpha-amylase production by *Bacillus* sp. 1-3 but $FeC1_3$ and $MgSO_4$ exhibited negative influence on alpha-amylase production (Vishwanathan *et al.,* 2016)

Moisture Content

Moisture is one of the most important parameters in SSF that influences the growth of the organism and thereby enzyme production (Pandey *et al.,* 2012). Low and high moisture levels of the substrate affect the growth of the microorganisms resulting in lower enzyme production (Ellaiah *et al.*, 2014). High moisture content leads to reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume. Bacteria are generally known to require initial moisture of 70-80%. Alpha-amylase production by *Bacillus licheniformis* M27 was highest with 65% initial moisture content in an SSF system (Namita *et al.,* 2007). Significant decrease in enzyme production was observed with high increase in moisture content which was due 'to the decrease in the rate of oxygen transfer. Studies indicated that enzyme litres could be increased significantly by agitation of the medium with high moisture content. A thermotolerant *B. subtilis* requires initial moisture of 30% for its growth and maximum enzyme production (Ahmad *et al*.,2010).

Particle Size of the Substrate

In SSF, particle size of the substrate affects growth of the organism and thereby influences the enzyme production (Ellaiah *et al.,* 2014). The adherence and penetration of microorganisms as well as the enzyme action on the substrate clearly

depends upon the physical properties of the substrate such as the crystalline or amorphous nature, the accessible area, surface area, porosity, particle size etc. In all the above parameters, particle size plays a major role because all these factors depend on it. Smaller substrate particles have greater substrate surface area for growth but inter particle porosity is lower. For larger particle sizes, the porosity is greater but the saturated surface area is smaller hence determination of particle size corresponding to optimum growth and enzyme production is necessary (Reeta *et al.,* 2015).

Agitation

Agitation intensity influences the mixing and oxygen transfer rates in much fungal fermentation and thus influences mycelial morphology and product formation. It has been reported that a higher agitation speed is sometimes detrimental to mycelial growth and thus may decrease enzyme production. However, it is reported that the variations in mycelial morphology as a consequence of changes in agitation rate do not affect enzyme production at a constant specific growth rate. Agitation intensities of up to 300 rpm have normally been employed for the production of amylase from various microorganisms (Ahmad *et al.,* 2010).

2.5.3 Applications of Amylase

The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat brankoji culture of *Aspergillus oryzae* in 1894. Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase began in 1959 (Pandey *et al.,* 2012). Since then amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major part of the starch processing industry (Noda e*t al..*2001). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch to products containing glucose, maltose etc, is brought about by controlled degradation (Hans *et al.,* 2009). Some of the applications of amylase are as follows:

Liquefaction

Liquefaction is a process of dispersion of insoluble starch granules in aqueous solution followed by partial hydrolysis using thermostable amylases. In industrial processes, the starch suspension for liquefaction is generally in excess of 35% (w/v) (Damien *et al.,* 2010). Therefore the viscosity is extremely high following gelatinization. Thermostable a-amylase is used as a thinning agent, which brings about reduction in viscosity and partial hydrolysis of starch, retrogradation of starch thus avoided during subsequent cooling (Vander *et al*., 2002).

The traditional thinning agent used in starch technology was acid (hydrochloric or oxalic acids). The introduction of thermostable α-amylases has meant milder processing conditions. The formation of by products is reduced and refining and recovery costs are lowered (Dhanya *et al., 2*009). In the enzymatic process the hydrolytic action is terminated when the average degree of polymerization is about 10-12. Two distinct types of thermostable α-amylases are commercially available and used extensively in starch processing technology (Tomasz *et al.*.2011). The amylase of *Bacillus amyloliquefaciens* was the first liquefying a-amylase used on a large scale, later a more heat-stable enzyme from *Bacillus licheniformis* was introduced commercially (T*omasz et al.,* 2011).

Manufacturing of Maltose

Maltose is a naturally occurring disaccharide. Its chemical structure has 4-0-a-D-iicopyronosil-D-glucopyranose, It is the main component of maltose sugar syrup (Yakup *et al., 20*10). Maltose is widely used as sweetener and also as intravenous sugar supplement. It is used in food industry because of low tendency to be crystallized and is relatively non-hygroscopic, Corn, potato, sweet potato and cassava starches are used for maltose manufacture. The concentration of starch slurry is adjusted to be 10-20% for production of medical grade maltose and 20-40% for food grade. Thermostable alpha amylase from *B. lichemformis* and B. *amyloliquefaciens* are used (Archana *et al.,* 2015).

Manufacture of High Fructose Containing Syrup

High fructose containing syrups (HFCS) 42 F (Fructose content equal to 42%) is prepared by enzymic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzyme liquefaction and saccharification (Shekufeh *et al.,* 2010).

Manufacture of High Molecular Weight Branched Dextrins

Branched dextrins of high molecular weight are prepared by hydrolysis of corn starch with alpha-amylase. The extent of starch degradation depends on the type of starch and physical properties desired (Dhanya *et al.,*2012). They are obtained as powder after chromatography and spray drying. These are used as extender and a glozing agent for production of powdery foods and rice cakes respectively (Harmeet and Chen 2005).

Removal of Starch Sizer from Textiles (Desizing)

In textile weaving, starch paste is applied for wraping. This gives strength to textiles at weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of the string due to laid down wrap. After weaving the cloth, the starch is removed and the cloth goes to scouring and dyeing. The starch on cloth is usually removed by application of alpha-amylase (Querong *et al.,* 2008).

Direct Starch Fermentation to Ethanol

The amylolytic activity rate and amount of starch utilization and ethanol yields increase several folds in co-cultures (Reela *et al.,* 2009). Mould amylases are used in alcohol production and brewing industries. The advantages of such systems are uniform enzyme action in mashes, increased rate of saccharification, alcohol yield and yeast growth (Maria and Swammy 2011).

2.5.4 Determination of Amylase Activity

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

Dinitrosalicylic Acid Method (DNS)

In the dinitrosalicylic acid method, aliquots of the substrate stock solution are mixed with the enzyme solution. Followed by 10 min of incubation at 50^0C, DNS reagent is added to the test tube and the mixture is incubated in a boiling water bath for 5 min (Maria and Swammy 2011). After cooling to room temperature, the absorbance of the supernatant at 540 nm is measured. The A540 values for the substrate and enzyme blanks are subtracted from the A540 value for the analyzed sample. In a study on alkalophilic α-Amylase from *Bacillus strain* GM8901, the enzyme assay was done by

measuring the reducing sugars by DNS method and the activity was found to be a maximum of 0.75 U ml"[1] after incubation of 24 h (Borge *et al*., 2015).

Nelson - Somogyi (NS) Method

In the NS method, an aliquot of stock solution of substrate is heated al 50°C for 5min. Preheated (50°C for 5 min) enzyme solution is added to the substrate. This reaction mixture is incubated at 50°C and the reaction is carried out for l0 min. After incubation Somogyi copper reagent is added to terminate the reaction. This is then incubated in boiling water bath for 40 min and cooled to room temperature. In the next step Nelson arsenomolybdate reagent is added and incubated for 10 min at room temperature. Finally water is added and the mixture is centrifuged at 13,000 rpm for 1 min and absorbance of supernatant is read at 610 nm. Haloalkaliphiiic α-amylase isolated from archaebacterium *Natronococcus* sp. strain Ah-36 was subjected to NS method of enzyme assay and activity of 0.01 U/ml was recorded in the end of 35 h of incubation. The maximum activity was found to be 0.12 U/ml after 90 h and was retained even after 110 h (Juge *et al*., 2016).

Determination of Activity Using Iodine

The hydrolytic activity of α-amylase can be determined based on the principle that starch and iodine react to form a blue colored complex; on hydrolysis of starch this complex changes to a reddish brown colored one. The absorbance can be read after the enzyme substrate reaction has been terminated. This gives a measure of the extent of hydrolysis of starch by α-amylase (Juge *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Four litres of cassava waste water was collected with a sterile plastic container, from local garri processing plants at Ifite road, Amaenyi-Awka.

3.2 Isolation and Phenotypic Identification of Fungi from Cassava Effluent

A 1 ml aliquot of cassava waste water was serially diluted in 9 ml of Sabouraud's dextrose broth, and 0.1 ml aliquot was aseptically pipetted from 10^{-3} tube and spread on sterile Sabouraud's dextrose agar plate, incubated for 48 h. Fungal mixed culture growths observed were separated into pure cultures. In order to examine the morphology of the fungal isolates, 2 ml aliquot of molten Sabouraud's Dextrose Agar was aspirated with a sterile syringe and the aliquot was carefully discharged in a block-like pattern on a clean grease-free slide, placed on a sterile u-shaped glass rod positioned on a sterile wet filter paper in a sterile Petri dish. Point inoculation of each mold sample from mixed culture plate was done on the SDA when it got solidified, thereafter, a sterile cover slip was placed on the inoculated region of the slide and the Petri dish was covered. The Petri dish was incubated for 24-48 hours at room temperature, before the culture and cover slip were separately viewed with x40 objective lens (Oyeleke and Okasanmi, 2008).

3.3 Physico-Chemical Analyses of Cassava Waste Water

3.3.1Determination of total solids in the waste water

This was carried out using the method described by APHA (1998); The initial dry weight of empty evaporating dish was taken, 50 ml of waste water sample was added and kept in water bath until dry, this was then kept in an oven (105^0C) for at least 1 hour for dryness, and thereafter cooled in desiccators, prior to final weight determination.

$$\textbf{Total solids (mg/L)} = \frac{\text{Final weight} - \text{initial weight}}{\text{Volume of water used}} \times 1000$$

3.3.2 Determination of total suspended solids

Total dissolved solid was first determined using an automated TDS Tester model 480. The tester probe was dipped into 50 ml of sample and the result was read from the display unit of the machine. Thereafter, the difference between the total solids and total dissolved solids was calculated to be the total suspended solids i.e

TSS = TS – TDS. Where,

$$\text{Total dissolved solids (mg/L)} = \frac{(A-B) \times 1000}{\text{Volume of water used}}$$

A= weight of solids + filter paper
B= weight of filter paper

3.3.3 Determination of pH

This was done using the method as described by APHA (1998); the pH meter was switched on for 15 mins and thereafter caliberated with standard buffers of pH 4 and pH 9. The pH of the waste water sample was taken by dipping the electrode of pH meter into sample until a steady reading was obtained.

3.3.4 Determination of Reducing Sugar

A 10 g portion of the sample was weighed and transferred to a 200 ml Kohlrausch flask. The sample was diluted with water and shaken for 30 minutes and filtered with Whatman no 1. filter paper. The filterate was transferred to a clean dry flask. A 10 ml aliquot each of Fehling's solution A and B was aspirated into a 20 ml Erlenmeyer flask. Twenty millilitre of the filterate and 10 ml of sterile water were added to same flask to make the total volume of 50 ml; and the flask mixture was gently swirled. Two small glass beads were added to the mixture and boiled for 5 minutes, cooled immediately in an ice bath at room temperature. A 10 ml aliquot of 30 % potassium iodide solution and 10 ml of 28 % sulphuric acid were added to the mixture and titrated with 0.1 N sodium thiosulphate solution, with 2 drops of indicator, until the blue black colourdiasppeared. Control experiment was also performed in same manner with sterile water substituted for sample filterate. Reducing sugars present were determined thus;

$$\frac{\text{Titer value} \times 200 \text{ ml} \times 100}{\text{Sample (g)} \times 20 \text{ ml} \times 1000 \text{ mg/g}}$$ (APHA, 1998).

3.3.5 Determination of Starch Content

A 2.5 ml of waste water sample was mixed with 50 ml water and allowed to stand for 1 h. Twenty milliltre (20 ml) conc. HCl and 150 ml distilled water were added to the mixture and refluxed for 2 h in a 250 ml round bottomed flask. The mixture was cooled and neutralized with NaOH and made up to 250 ml mark with distilled water. Varying concentrations (0-1mg/ml) of glucose were prepared to serve as standard glucose curve. Five milliliter of Anthrone reagent was added to each standard solution and boiled in water bath for 20 minutes. The tubes were cooled and their absorbances were read at 620 nm against a blank containing only 1 ml water and 5 ml Anthrone reagent (APHA, 1998).

3.3.6 Determination of Protein Content

One gram of waste water sample was weighed into a 30 ml Kjeldahl digestion flask, the flask was stoppered and shaken; thereafter 1 ml of Kjeldahl catalyst mixture was added. The mixture was heated cautiously in a digestion rack under fire until a clear solution appeared. The clear solution was then allowed to stand for 30 minutes and allowed to cool. After cooling, about 100 ml of distilled water was added to avoid caking and then 50 ml was transferred to the Kjeldahl distillation apparatus. A 100 ml receiver flask containing 5 ml of 2 % boric acid and indicator mixture containing 5 drops of bromocresol blue and 1 drop of methylene blue was placed under a condenser of the distillation apparatus so that the tap was about 20 cm inside the

solution. Then 5 ml of 40 % NaOH was added to the digested sample in the apparatus and distillation commenced immediately until 50 drops got into the receiver flask, after which it was titrated to pink colour using 0.01 N HCl. Protein content was determined thus;

% Nitrogen = titre value x normality of acid x atomic number of nitrogen x 4

% Protein = % Nitrogen x 6.25 (APHA, 1998).

3.4 Screening of Fungal Isolates for Amylase Production and Choice of Working Strain

Point inoculation of the isolates were made on starch medium containing 2 g soluble starch. The plate was incubated at 30 ^{0}C for 4 days. At the end of incubation, the plates were flooded with iodine solution. Clear zones in the media indicated a positive reaction while blue-black colouration on the plates showed negative reaction. Isolate with the highest diameter clear zone was selected as the working strain (Umeh and Odibo, 2014).

3.5 Molecular Characterization of Best Amylase Producing Isolate

The fungal isolate D which had the highest diameter of clear zone was tentatively identified as *Aspergillus* sp and was subjected to a purity check. Molecular assays were carried out on each sample using nucleic acid as a standard. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain

Reaction (PCR) was employed to amplify copies of the rDNA*in vitro*. The quality of the PCR product was assessed by undertaking gel electrophoresis.

PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing.

Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing pre-hydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing Big Dye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation.

Sample was loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available from the European

Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI). The strains were identified using Inter specific region sequencing analyses (Macrogen, 2014).

3.6 Enrichment of Cassava Waste Water for Biomass and Enzyme Production

Cassava waste water was enriched by the addition of 1.5 % peptone, 0.3 % NaCl, 0.3 % KH_2PO_4, 0.1 % $MgSO_4.7H_2O$ for biomass growth, while 0.3 % yeast extract was added in place of 1.5 % peptone in 500 ml cassava waste water for enzyme production, in Erlenmeyer flask and autoclaved. Agar plugs of the fungi were introduced into the enriched medium and were incubated at 30 ^{0}C in a rotary shaker for ten days. The contents of the Erlenmeyer flasks were filtered using pre-weighed Whatman No. 1 filter paper. The fungal biomass was gently washed with distilled water and transferred to pre-weighed Whatman No. 1 filter paper. The filter paper content was dried and total biomass produced was determined by the calculation;

Biomass = $W_2 - W_1$

Where W_1 = weight of empty filter paper

W_2 = weight of dried biomass and filter paper.

3.7Assay of Amylase

This was determined using a modified version of the methods described by Umeh and Odibo (2014). A 1 ml aliquot of crude enzyme was added to 1ml of standard starch solution (1% w/v soluble starch and 0.006M NaCl in 0.2M Phosphate buffer pH 6.9)

and incubated at 40^0C for 30 minutes. Reducing sugars released by the enzyme were determined by adding 2 ml of dinitrosalicyclic acid (DNS) reagent, boiled for 10 mins and then cooled under running tap water. Ten millilitres of distilled water was then added and allowed to stabilize for about 5mins. The absorbance of the resulting solution was determined at 540 nm with a spectrophotometer against a reagent blank. One unit of amylase activity is taken as the amount of enzyme in 1ml of crude amylase that produced 1.0mg of reducing sugar from starch under the assay conditions.

3.8 Protein Assay

Bovine serum albumin (BSA) was serially diluted to five concentrations of 5-100 µg protein. A 30 µl aliquot each of each dilution and unknown protein sample were labeled appropriately. A 30 µl aliquot of water and same concentration for protein buffer were added to the sample tubes. A 1.5 ml aliquot of Bradford reagent was added to each tube and mixed. The tubes were allowed to stand at room temperature (28^0C) for 5 mins and absorbance were measured at 595 nm (Bradford, 1976).

3.9 Effect of Nitrogenous Sources on Amylase Production Using Cassava Waste Water

The effect of the following nitrogen sources on amylase production was investigated: soybean meal, bacteriological peptone, ammonium sulphate, yeast extract and groundnut cake. The cassava waste water was fortified with 1% of each nitrogen

source and the following mineral salts: 0.3% NaCl, 0.3% KH_2PO_4 and 0.1% $MgSO_4.7H_2O$. Flasks containing 50 ml of medium each were inoculated with 1 ml of spore suspension of *A. nomius* and incubated at 30^0C in a rotary shaker at 200 rpm. Amylase activity (u/ml broth) was determined 24 hourly until 96 h.

3.10 Effect of Yeast Extract Concentrations on Amylase Production

The effect of different concentrations (0.5-4%) yeast extract on amylase production in cassava waste water was determined. Fermentation was carried out as described but for 48 h and the amylase activity determined using DNS.

3.11 Time Course of Amylase Production in Fortified Cassava Waste water

The time course for amylase production was studied by growing *Aspergillus nomius* in a medium consisting of cassava waste water (87 mg/ml starch content), 1.5% yeast extract, 0.3% NaCl, 0.3% KH_2PO_4 and 0.1% $MgSO_4.7H_2O$. Fermentation was carried out in a shaker at 30^0C and 300 rpm in 250 ml Erlenmeyer flasks each containing 50 ml of broth. Enzyme activity (u/ml broth), culture pH and biomass were monitored daily until maximum enzyme yield was obtained.

3.12 Amylase Production System

Six 250 ml Erlenmeyer flasks each containing 50 ml of fermentation broth of the following composition: cassava waste water (87 mg/L starch content), 1.5% yeast extract, 0.3% NaCl, 0.3% KH_2PO_4 and 0.1% $MgSO_4.7H_2O$, pH 7.0 were used.

Inoculum consisted of 1 ml of spore suspension of slant culture of *Aspergillus nomius*. Cultures were inoculated at 30^0C in an orbital incubator at 200 rpm for 96 h, and subsequently subjected to purification steps.

3.13 Amylase Purification

Purification was carried out at room temperature (28-30^0C). Following the termination of the enzyme fermentation, the broth cultures were cooled to 8^0C and then centrifuged at 5000 rpm for 30 minutes in an Eppendorf refrigerated centrifuge. Protein content and amylase activity of the cell-free supernatant were estimated. Protein content was determined according to Bradford (1976) using egg albumin as a protein standard and by measuring the absorbance at 280 nm. The crude supernatant was brought to 30% saturation with ammonium sulphate. The precipitate was removed by centrifugation and the resultant supernatant was further saturated up to 60% with ammonium sulphate. The precipitate was re-suspended in 162 ml 0.1 M phosphate buffer, pH 6.8. The protein content and enzyme activity of this precipitate were estimated. The enzyme fraction was further desalted by dialysis against phosphate buffer (pH 6.8) and then subjected to ion-exchange chromatography on CM-Cepharose column (1.6 x 26 cm), previously equilibrated in 0.1 M phosphate buffer pH 6.8. After a wash with 100 ml of this buffer, proteins were eluted with the same buffer (pH 6.8) with a linear gradient of NaCl (0-0.5 M) at a flow rate of 10 ml in 5 minutes. A total of 45 fractions (each tube containing 10 ml) were collected. Fractions were each analysed for amylase activity and protein was estimated by

measuring the absorbance at 280 nm. Fractions (36-42) which showed high amylase activity were pooled and then concentrated by dialysis against 5 M sucrose solution. The dialysate was analysed for enzyme activity and protein by Bradford (1976). It was next subjected to hydrophobic interaction chromatography on a Phenyl-Sepharose-CL-4B column (2.0 x 5.0 cm) equilibrated with 4 M sodium chloride. The enzyme solution was also made hydrophobic with 4 M NaCl prior to its addition to the column. The enzyme was eluted with 0.1 M phosphate buffer pH 6.8 containing decreasing concentrations of NaCl (4-0 M) at a flow rate of 10 ml in 25 minutes. Ten milliliters fractions were collected and those fractions (5-8) with high amylase activity were pooled and further dialysed against phosphate buffer to remove NaCl, and further concentrated by dialysis against 6 M sucrose solution. Protein content and amylase activity were measured as described above. The enzyme thus purified was stored frozen and used for subsequent experiments.

3.14 Characterization of Partially Purified Amylase

3.14.1 Effect of Temperature on Enzyme Activity

A 0.5 ml aliquot of 1% starch solution was reacted with 0.5 ml of purified enzyme at various temperatures (40, 50, 60, 70, 80 and 90^0C) for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. Distilled water (10 ml) was added to the mixture and the optical

density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.14.2 Effect of Temperature on Enzyme Stability

A 0.5 ml aliquot of enzyme was incubated in a thin-walled test tube at different temperatures (40, 50, 60, 70, 80 and 90^0C) for 30 minutes. The enzyme was promptly chilled in ice and the remaining activity was assayed by adding 0.5 ml of 1% (w/v) starch solution and incubated at optimal temperature. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. 10 ml of distilled water was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.14.3 Effect of pH on Enzyme Activity

A 0.5 ml aliquot of the enzyme was incubated with 0.5 ml of 1% (w/v) soluble starch prepared in buffers of different pH values and incubated at optimum temperature for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. 10 ml of distilled water was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.14.4 Effect of pH on Enzyme Stability

A 0.5 ml aliquot of enzyme in 1 ml of buffers with different pH values were incubated for 24 h at room temperature. After incubation, 0.5 ml of 1% (w/v) soluble starch was added to the mixtures and incubated at optimum temperatures for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. 10 ml of distilled water was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.14.5 Effect of Metal Ions on Enzyme Activity

A 0.001M solution of salts of $Ca^{2+,}$ Ba^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} were prepared. Reaction mixtures containing 0.5 ml of enzyme in 1 ml of the metal salt solutions and 0.5 ml of 1% soluble starch (prepared in buffer of optimum pH) were set up. The set up was incubated at optimum temperature for 30 minutes. A control was set up in same manner but without the metals. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. 10 ml of distilled water was added to the mixture and the optical density was measured at 540 nm.

3.14.6 Effect of Some Inhibitors on Enzyme Activity

The effect of EDTA, lead acetate and sodium dodecyl sulphate (SDS) on amylase activity was examined following the method of Raza and Rehman (2016). About 0.5

ml of enzyme was pre-incubated with 0.5 ml of the inhibitors (0.001 M) for 10 minutes at 40^0C. Thereafter, 0.5 ml of 1% starch solution was added to each mixture and incubated further at 40^0C for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. Thereafter, 10 ml of distilled water was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.14.7 Effect of Concentrations of Substrates on Enzyme Activity

Different concentrations (0-1 mg/ml) of starch, amylopectin, glycogen and glycogen-β-limit dextrin were prepared in buffer of optimum pH. Aliquots of 0.5 ml of enzyme was added to 0.5 ml of each concentration of polysaccharide and incubated at optimum temperature for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. Distilled water (10 ml) was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

The K_m values of the enzyme for the different substrates were derived by the Lineweaver-Burk linear transformation of the Michaelis-Menten equation.

3.14.8 Effect of Best Metal Ion on Heat Stability of Amylase

The effect of the best metal ion (in terms of enhancement of enzyme heat stability) was investigated by incubating 0.5 ml of enzyme with 0.5 ml of best metal ion solution at various temperatures (40, 50, 60, 70, 80 and 90^0C). Residual enzyme activity was assayed with 0.5 ml soluble starch at 40^0C for 30 minutes. The reaction was stopped with 1 ml DNS and heated for 10 minutes in boiling water and cooled under running tap water. Distilled water (10 ml) was added to the mixture and the optical density was measured at 540 nm using blank prepared in same manner without enzyme being added. Water was added in place of enzyme for the blank.

3.15 Hydrolysis of Gelatinized and Raw Starch

Wild cocoyam (*Colocasia esculenta*)(*Ede mmuo*), cassava, millet, and *Dioscorea bulbifera* (*JiAdu*) were processed. The method described by Obi and Odibo (1984) was used. Briefly, wild cocoyam, cassava and Ji Adu were first peeled and reduced to pulps using a hand grater. Subsequently, the pulps after soaking for 1 h in water were separately homogenized in a waring blender. From each homogenate contained in a bag of fine white cloth, starch was leached into a plastic vat by churning with excess water. The crude starch suspension in the vat was allowed to settle overnight, after which the sediment was separated from the supernatant by decantation and dried at 50^0C for 48 h. The resultant flakes were ground to a fine powder and used as native starches. To assess the hydrolysis of the starches by the amylase, one set of each

starch was gelatinized by heating a suspension of each starch (1% w/v) in 0.1 M phosphate buffer pH 6.8, while the other set was suspended in buffer without heating. Aliquots (0.5 ml) of both the gelatinized and raw starches were separately hydrolyzed with 0.5 ml of amylase at 40^0C for 10 minutes and the reaction stopped with 1 ml of DNS. The optical density of the reaction mixture was determined at 540 nm.

3.16 Thin Layer Chromatography of Products of Amylolysis

The products resulting from the action of the amylase on β-cyclodextrin, amylopectin, amylose, glycogen-β-limit dextrin,soluble starch, wild cocoyam starch, *Dioscorea bulbifera* starch, and millet starch were examined using thin layer chromatography. Each polysaccharide was prepared as a 1% suspension in 0.1 M phosphate buffer (pH 6.8) and heated to gelatinize. A 0.15 ml amount of enzyme was reacted with 0.15 ml of each starch separately at 40^0C for 5 h. The reaction was stopped by heating at boiling temperature for 5 minutes after which thin layer silica gel 60F (Merck) chromatography plate (10 cm by 12 cm) was spotted 1 cm apart with 1 μl of the digests. Glucose, maltose, maltotriose and maltotetraose at 1% (w/v) concentration was spotted alongside the tests as standard. The chromatogram was irrigated with a solvent system consisting of butanol-acetone-water (5:4:1) in one ascent. Reducing sugars were visualized by spraying the plate with urea-phosphoric acid reagent. The sprayed and air-dried chromatogram was placed in an oven at 110^0C for 10 minutes

for colour development. The resolution factor of the separated components was calculated in relation with standard sugars (St. John *et al*., 1996).

3.17 Statistical Analyses

One sample t-test was used to analyse means using SPSS version 17.

CHAPTER FOUR

RESULTS

4.1: Characterization and Identification of the Fungal Isolates

Four fungal isolates labeled A, B, C and D, were obtained from cassava waste water and characterized. All the four isolates were tentatively identified as *Aspergillus* sp. Isolate D which gave the highest amylase activity was selected for further studies and was identified as *Aspergillusnomius* after molecular typing as shown in Table 1 and Plate 3.

4.2: Physico-chemical Assessment of Cassava Waste Water.

Physico-chemical parameters of cassava waste water revealed a pH of 3.81±1.33, starch content of 87.857±3.03 mg/L , reducing sugar of 12.93% and protein content of 11.20±2.52mg/L, total solids of 109.34±0.69, total dissolved solids of 42.90±1.05mg/L and total suspended solids of 66.44±1.28mg/L as shown in Table 2.

4.3: Screening of Isolates for Enzyme Production

Aspergillus isolate D, gave the highest diameter clear zone value of 11.4±0.25 mm while isolate A had the least diameter clear zone of 8.8±1.02 as shown in Table 3.

Table 4.1: Identification of *Aspergillus* Isolates from Cassava Waste Water

Fungi Codes	Morphological Identification	Microscopic Identification	Presumptive Identification
A	Black fluffy colony with white coloured edge	Branched septate hyphae with blackish brown exine conidia and biseriate phialides	*Aspergillus* sp
B	Grey coloured cottony colony with black coloured center and white coloured edge	Branched septate, blue green rough conidia and uniseriate phialides	*Aspergillus* sp
C	Light brown coloured cottony colony with white coloured center and white coloured edges	Branched septate, cinnamon brown, fairly smooth conidia with uniseriate phialides	*Aspergillus* sp
D	Light green colony with grey coloured center and white coloured edge	Branched septate greyish smooth conidia with biseriate phialides	*Aspergillus* sp

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3'	ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3'
ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3'	ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3'

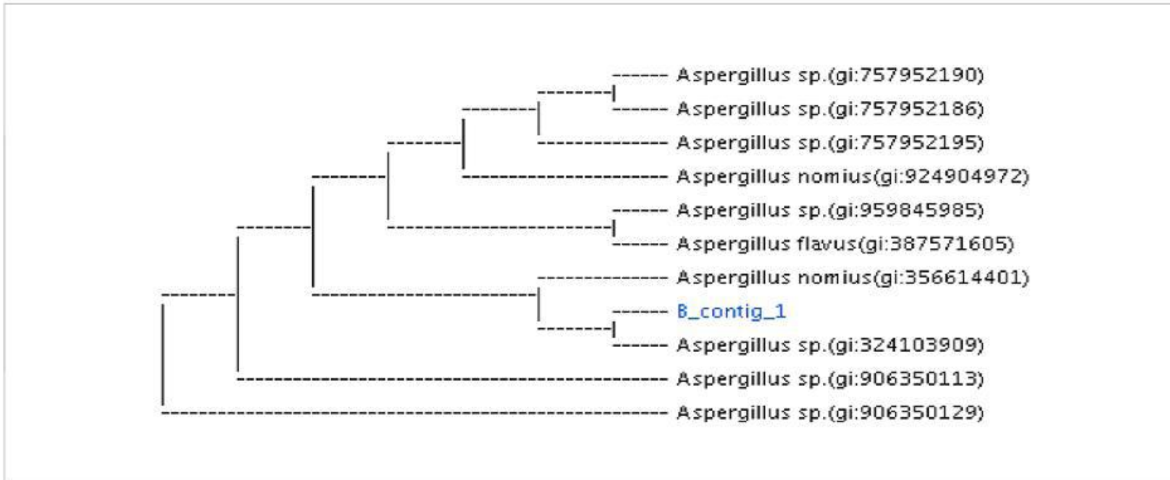

Plate 3: Phylogenetic Tree of *Aspergillusnomius*

Table4. 2: Physico-chemical Parameters of Cassava Waste Water

Parameters	Concentration
pH	3.81±1.33
Total Solids (mg/L)	109.34±0.69
Total Dissolved Solids (mg/L)	42.90±1.05
Total Suspended Solids (mg/L)	66.44±1.28
Reducing Sugar (%)	12.93
Starch Content (mg/L)	87.857±3.03
Protein Content (mg/L)	11.20±2.82

Table 4.3: Amylase Production Screening of Isolates

Aspergillus Isolates	Diameter Clear Zone (mm)
A	8.8±1.02
B	9.4±1.33
C	9.6±0.33
D	11.4±0.25

4.4: *Aspergillus nomius* Biomass Production Using Optimized Cassava waste Water Medium

Biomass production using optimized waste water medium gave a highest wet biomass value of 3.9 g by day 6 of cultivation at pH 8.2 as seen on Figure 1. Dry biomass weight measured gave the highest value of 1.9 g at same pH and day of cultivation as shown on Figure 2. The percentage biomass yield was calculated to be 40.91 % as shown on Table 5. Assay of amylase activity from the crude enzyme gave highest value of 4.9 u/ml at pH 8.2 at day 4 as shown on Figure 3. Likewise assay for betaglucosidase activity of the crude enzyme gave highest value of 5.1 u/ml at pH 7.2 on day 2 as shown on Figure 8.

4.5: Effect of Cassava Waste Water Fortification with Different Nitrogenous Sources on Amylase Production

Incorporating 1% of different nitrogenous bases to the cassava waste water showed that yeast extract gave the highest amylase activity out of them all (Fig. 4). Precisely 1.5 % of yeast extract gave the highest amylase production from *A. nomius* at 48 h (Fig. 5).

4.6: Time Course of Enzyme Production

The time course of the production of amylase of *Aspergillus nomius* is shown in Figure 6. Amylase production followed exponential growth of the organism with maximum enzyme occurring during the exponential (48 h) phase corresponding with the culture pH of 8.0.

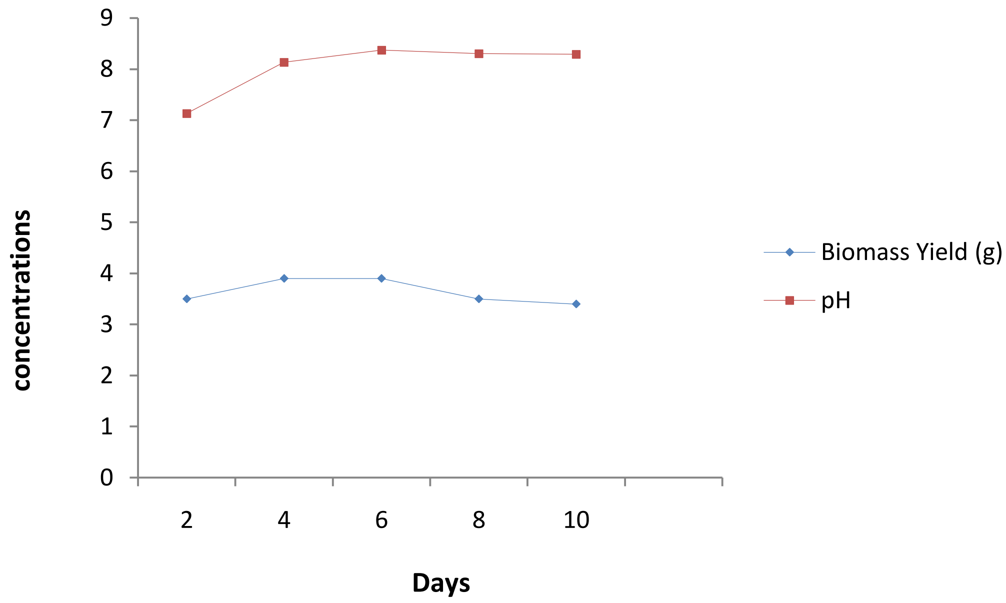

Fig 4.1: Mean Wet Biomass Yield of *A. nomius* from Fortified Cassava waste Water

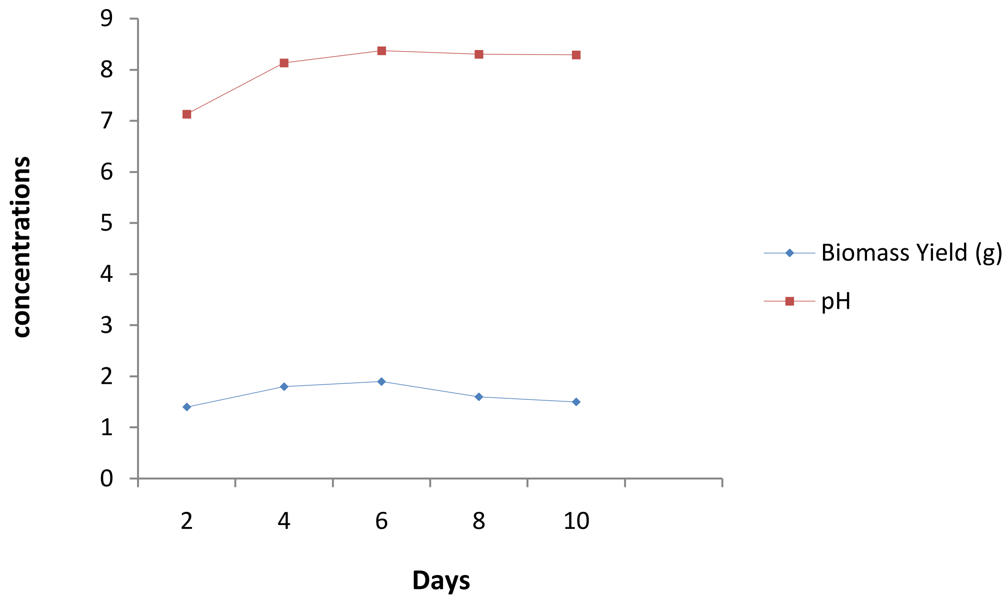

Fig 4.2: Mean Dry Biomass Yield of *A. nomius* in Fortified Cassava Waste Water

Table 4.4:Percentage Dry Biomass of *A. nomius* on Soluble Starch Substrate

Cultivation Days	Dry Biomass (%)
2	32.35
4	37.51
6	40.91
8	27.91
10	27.27

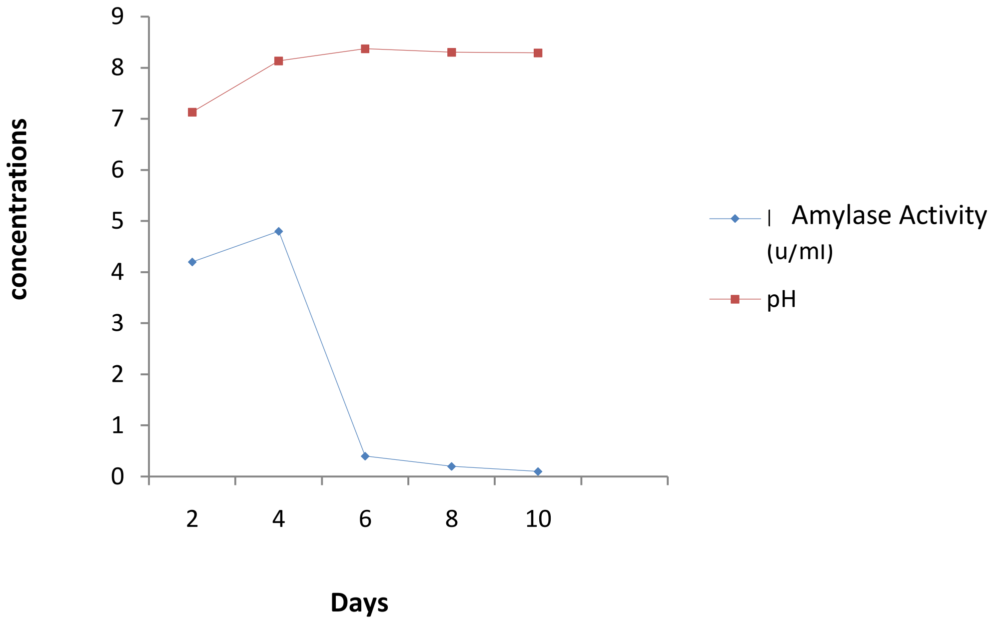

Fig 4.3: Amylase Production of *A. nomius* from Enriched Cassava waste Water

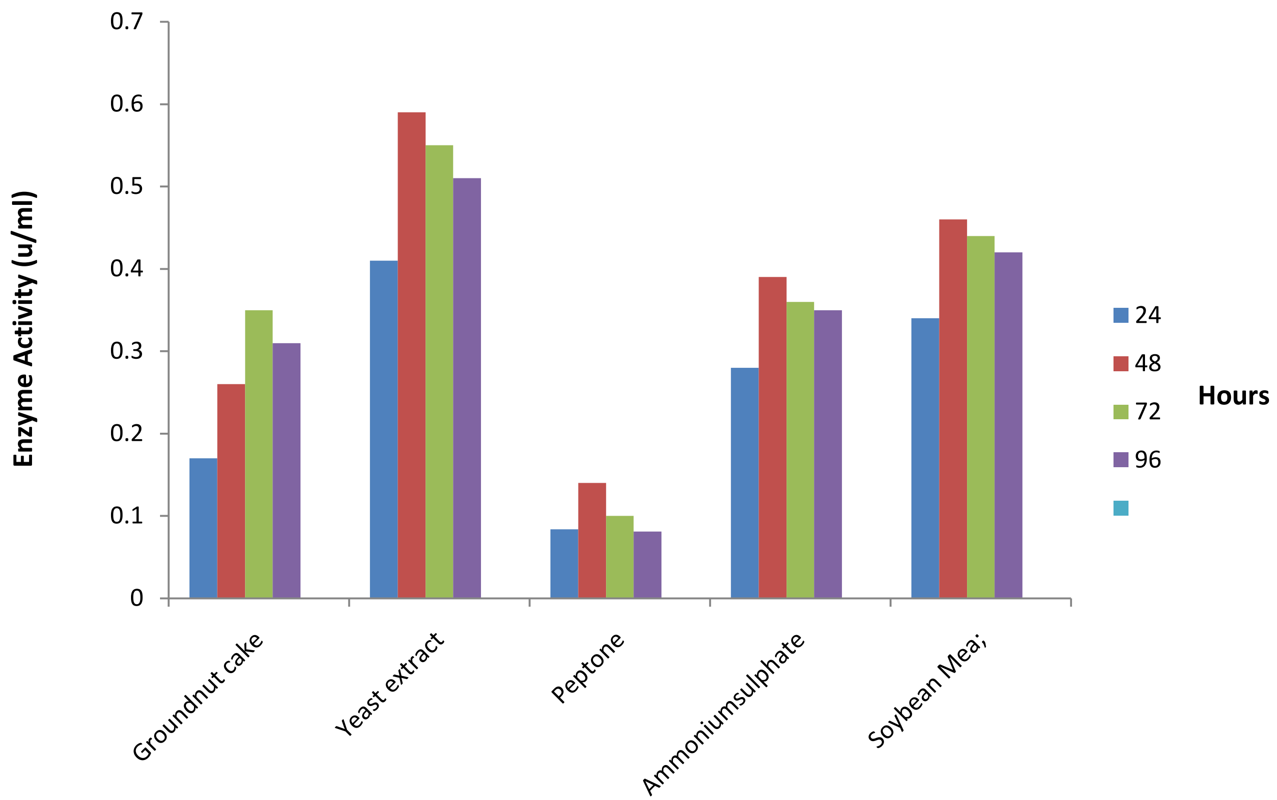

Figure 4.4: Effect of 1 % Nitrogenous Substrate Fortification on Amylase Production

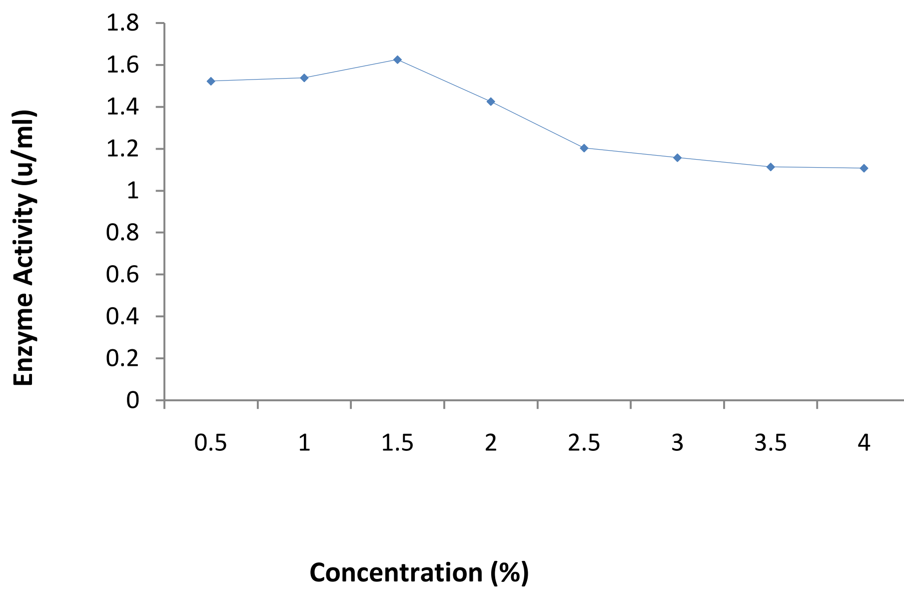

Figure 4.5: Effect of Yeast Extract Concentrations (%) on Amylase Production at 48 hours

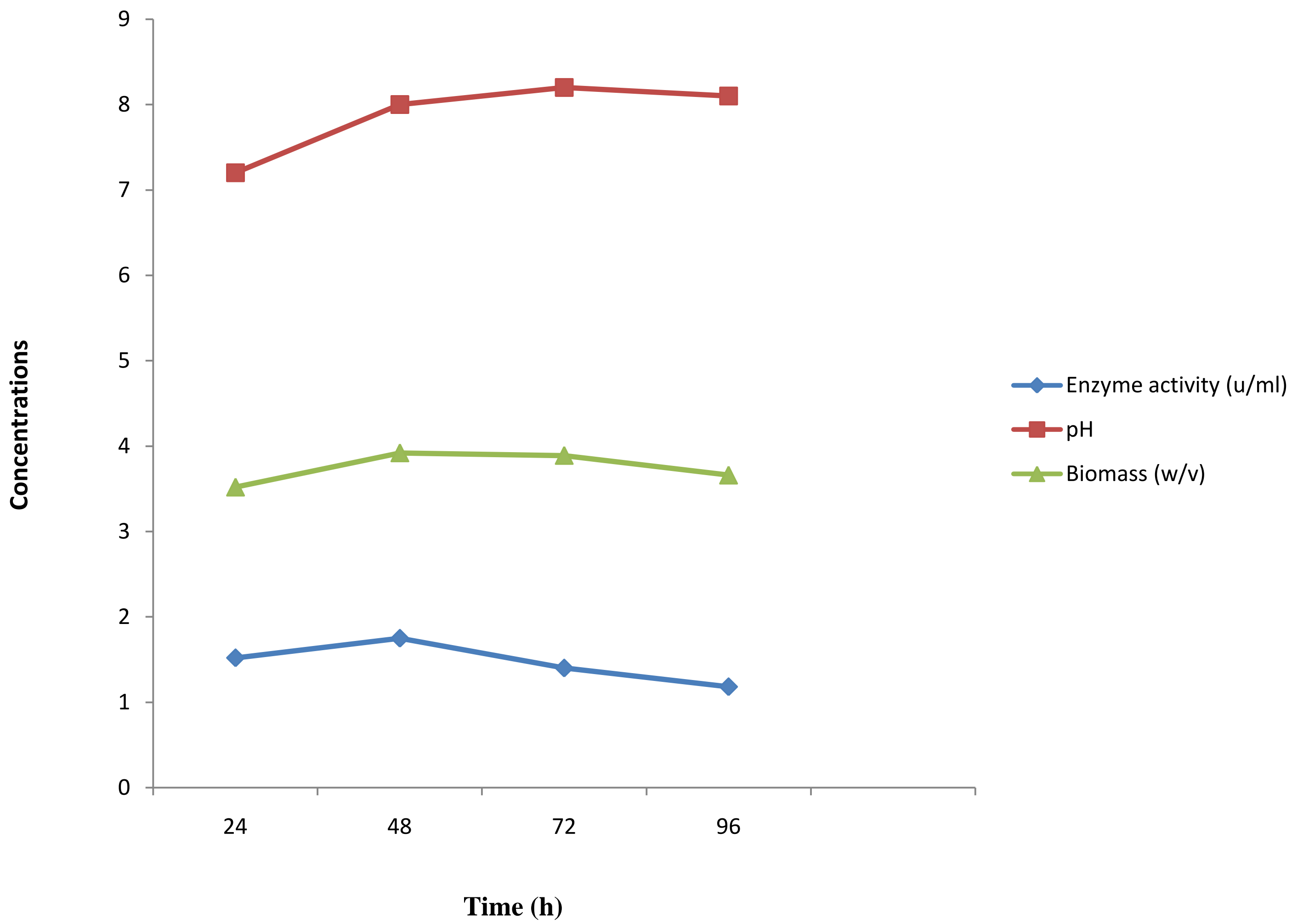

Figure4. 6: Time Course of Enzyme Production

4.7: Elution and Purification of *A. nomius* Crude Enzyme

Elution profile of *A. nomius* enzyme on CM-Sepharose Ion exchange chromatography showed a major peak of amylase activity between fractions 36-42 (Figure 7)at a flow rate of 10 ml in five minutes. Recovery of these fractions led to a 7.25% retention of overall activity and a 7.3 fold purification (Table 5). Similarly, fractions 5-8 (Figure 8) showed high enzyme activities on hydrophobic interaction chromatography. The enzyme was purified 0.88 fold to give a 1.1% yield relative to the total activity in the crude broth (Table 5) and a specific activity of 0.52 u/mg protein.

4.8: Effect of Temperature on Purified Enzyme Activity and Stability

Figure 9 shows the effect of temperature on enzyme activity. The enzyme is optimally active at 40^0C. It was also maximally stable at 40^0C but retained about 50% of its activity at 60^0C (Figure 10).

4.9: Effect of pH on Purified Enzyme Activity and Stability

The effects of pH on enzyme activity and stability are shown in Figures 11 and12, respectively. The enzyme was optimally active at pH 7.0. It was maximally stable at pH 7 but retained over 80% of its original activity between pH 6-9 for 24 h.

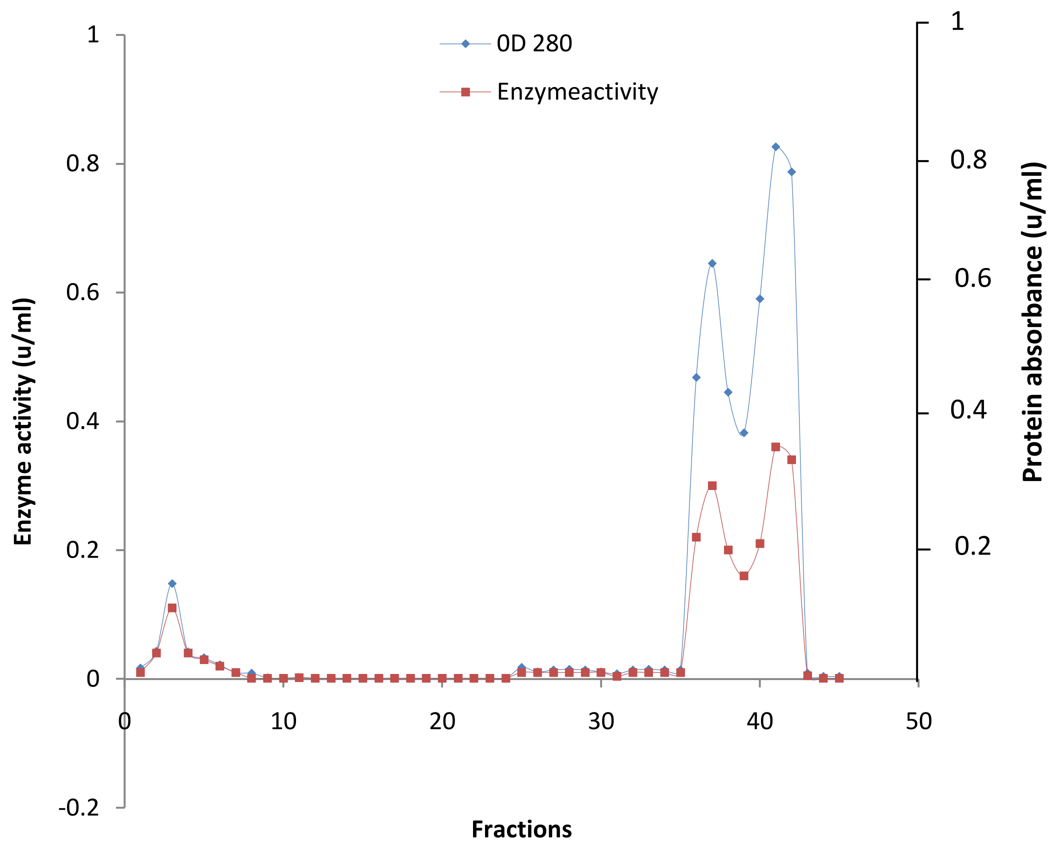

Figure 4.7: Elution Profile of *A. nomius* Amylase on CM-Sepharose Ion Exchange Chromatography

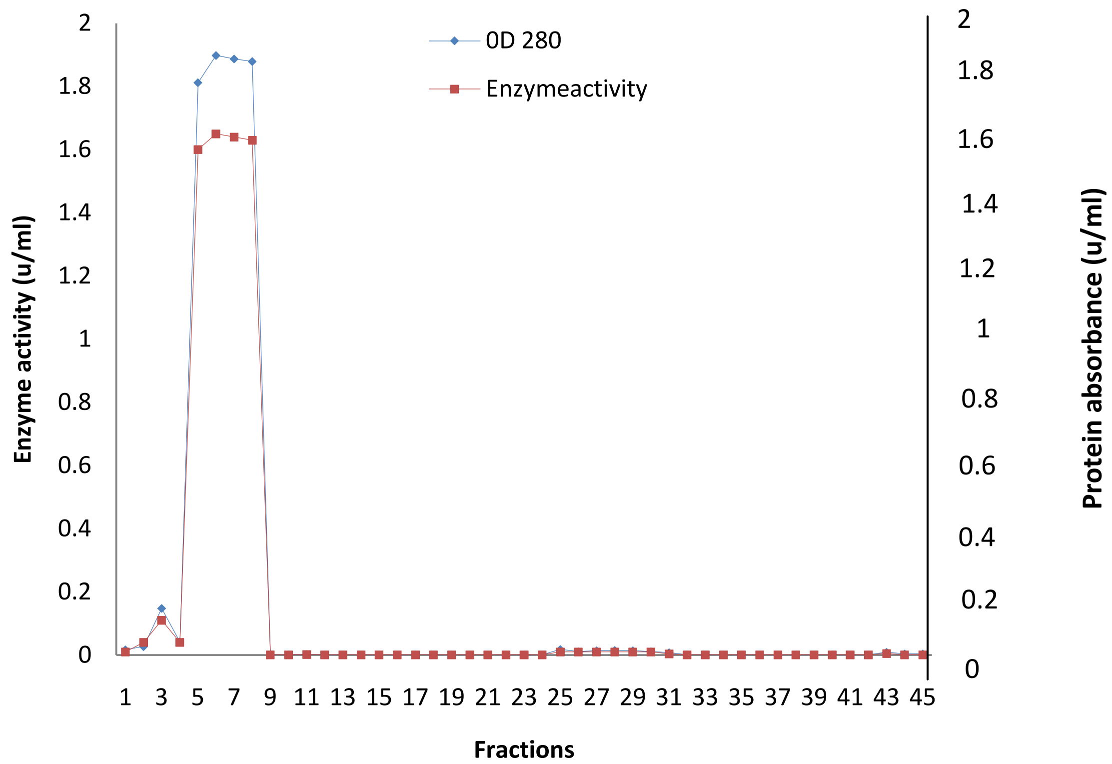

Figure4. 8: Elution Profile of *A. nomius* Amylase on Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography

Table 4.5: Purification Summary of Amylase of *A. nomius*

Step	Volume (ml)	Total activity (u/ml)	Total protein (mg)	Specific activity (u.mg/protein)	Yield (%)	Purification fold
Crude culture broth	270	210.6	521.64	0.404	100	1
$(NH_4)_2SO_4$ precipitation	162	59.84	17.82	3.36	3.52	4.61
Ion-exchange chromatograpy	10	8.25	18.12	0.46	7.25	7.30
Hydrophobic interaction chromatography	5	7.5	14.38	0.52	1.10	0.88

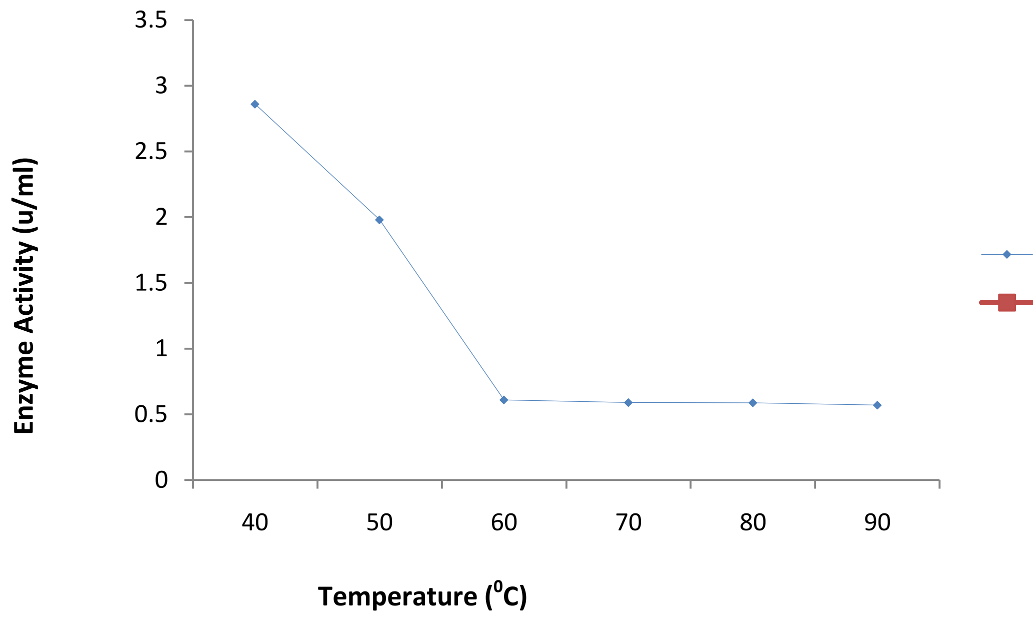

Figure 4.9: Effect of Temperature on Purified Enzyme Activity

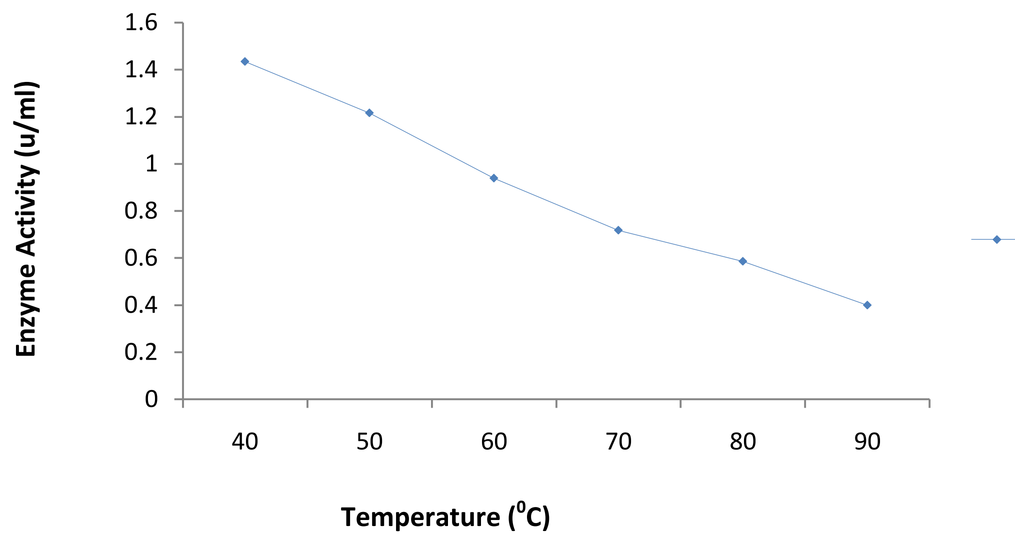

Figure 4.10: Effect of Temperature on Enzyme Stability

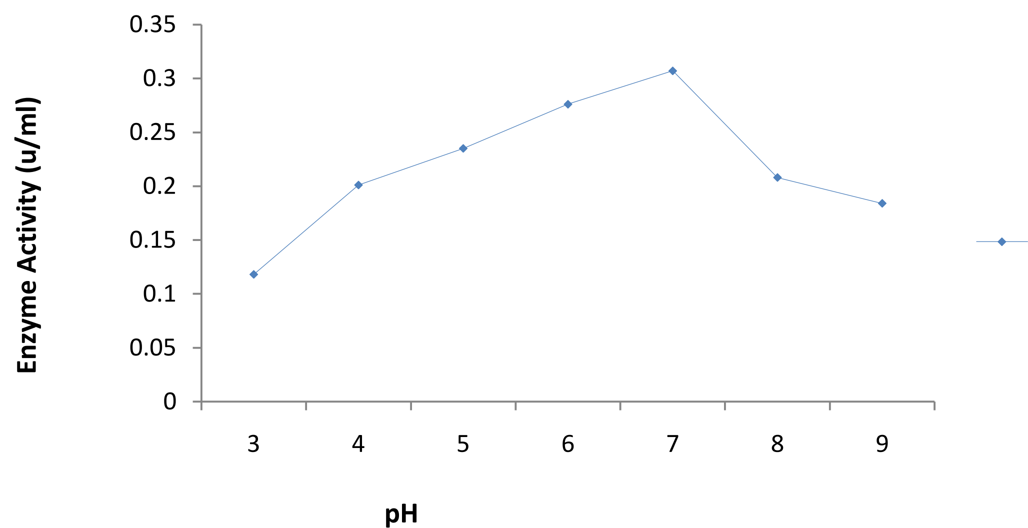

Figure 4.11: Effect of pH on Purified Enzyme Activity

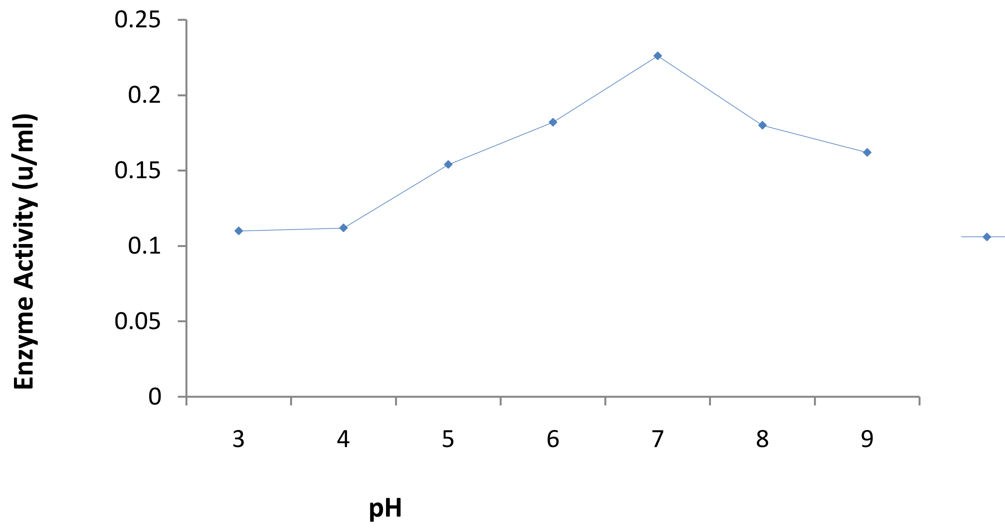

Figure 4.12: Effect of pH on Purified Enzyme Stability

4.10 Effect of Metal Ions and Inhibitors on Enzyme Activity

Figure 13 shows the effects of some metal ions on amylase activity. Mild stimulation of amylase activity was obtained with Fe^{2+} and lead acetate while $Mg^{2+}$and EDTA were found to exert some levels of inhibition on amylase activity.

4.11 Effect of Different Concentrations of Substrates on Enzyme Activity

Figure 14 illustrates the effects of various concentrations of soluble starch, amylopectin, glycogen and glycogen β-limit dextrin on amylase activity. The reaction rate and concentration did not show typical hyperbolic relationship indicating possible allosteric nature of the enzyme.The K_m and V_{max} of the substrates are as follows: starch (112.5 and 7.9), amylopectin (99 and 3.9), glycogen (0 and 4.7) and glycogen β-limit dextrin (150 and 4.1) respectively.

4.12 Effect of Metal Ion on Heat Stability of Enzyme

The effect of Fe^{2+}on enzyme activity is shown on Figure 16. There was appreciable stimulation of enzyme activity with increase in temperature, in the presence of Fe^{2+}.

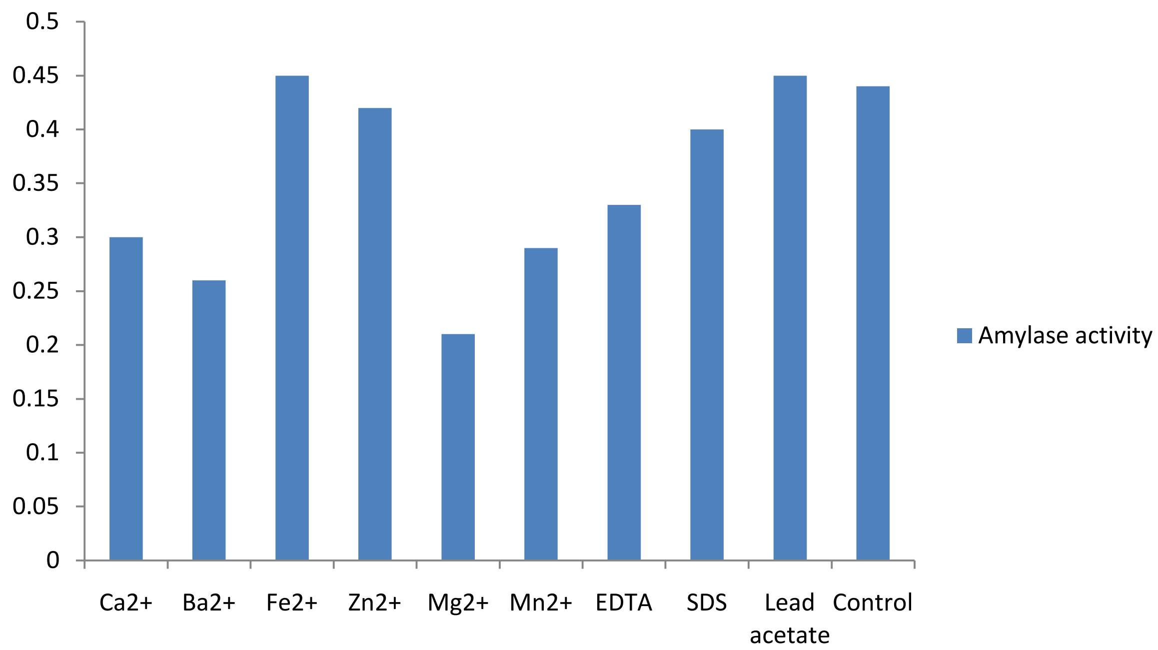

Figure 4.13: Effects of Metals and Inhibitors on Purified Enzyme Activity

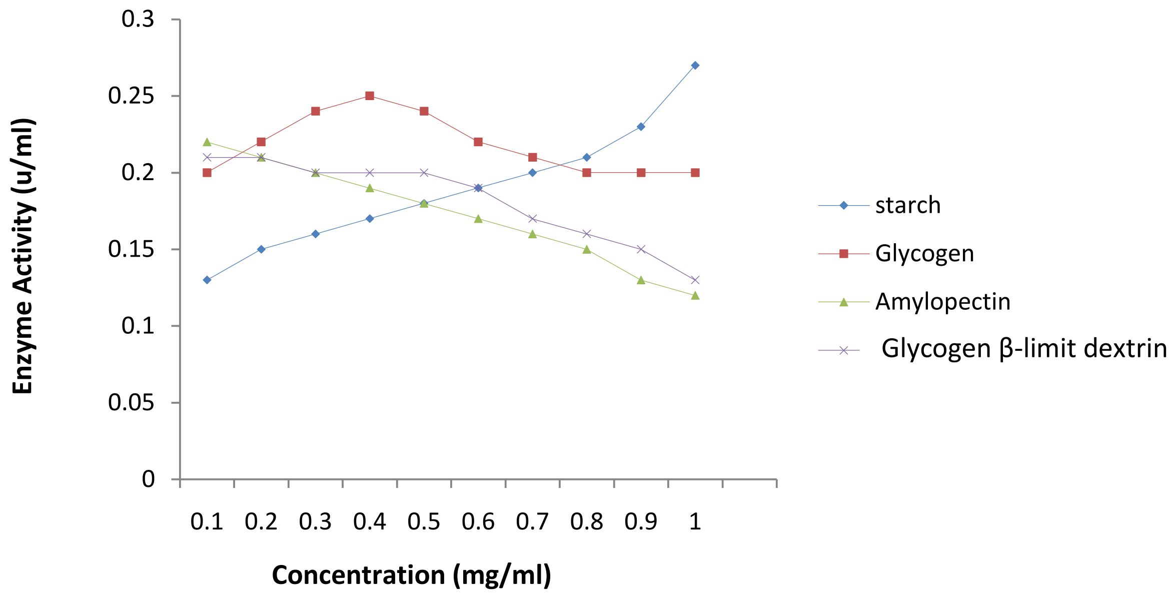

Figure 4.14: Effects of Different Substrate Concentrations on Enzyme Activity

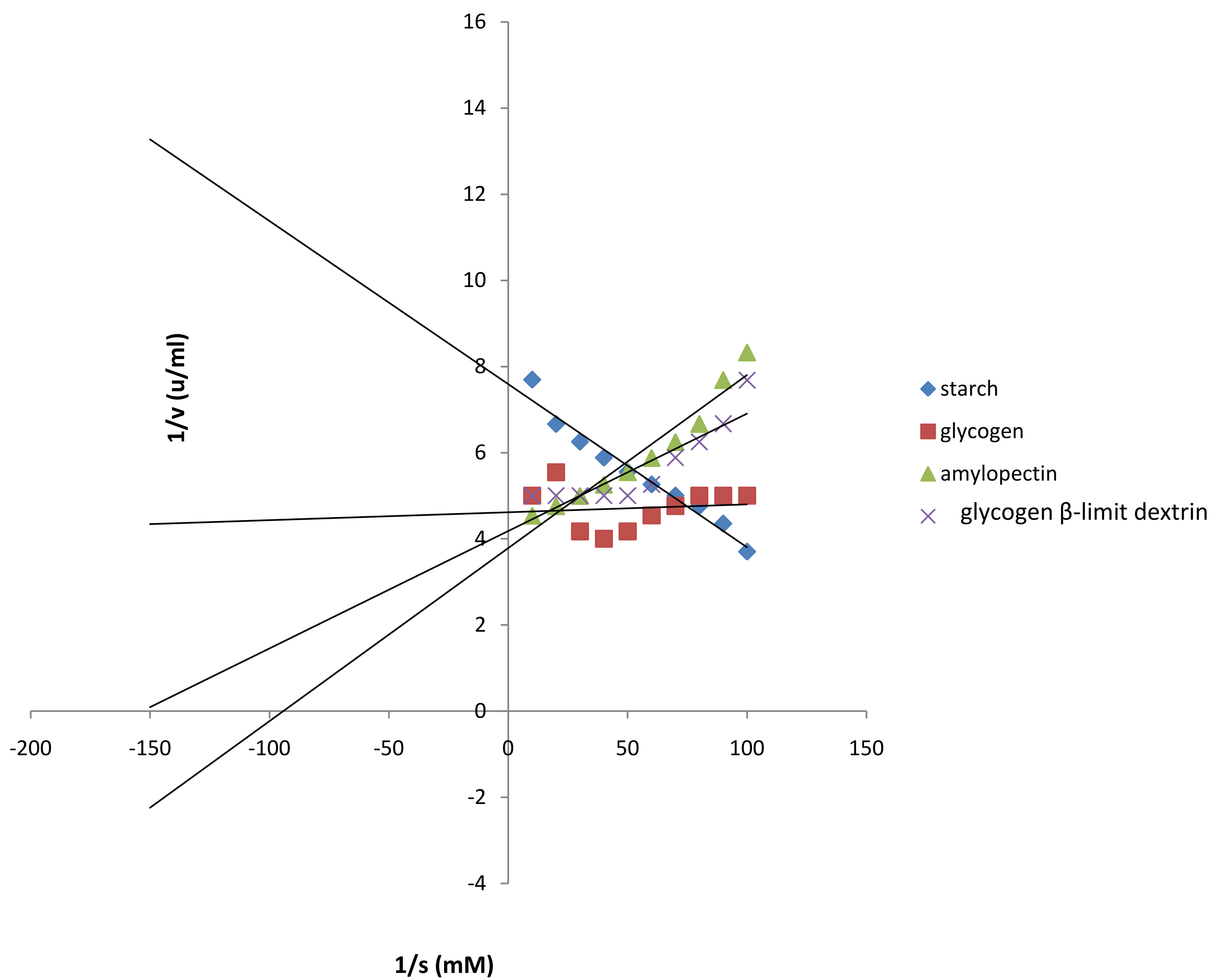

Figure 4.15:Lineweaver-Burk Plot for the Determination of K_m Values

Table 4.6: Effect of Substrate Concentrations on Amylase Activity

Substrates	**V_{max} (mg/ml/min)**	**K_m (mg/ml)**
Soluble starch	7.9	112.9
Glycogen	4.7	0
Amylopectin	3.9	99
Glycogen β-limit dextrin	4.1	150

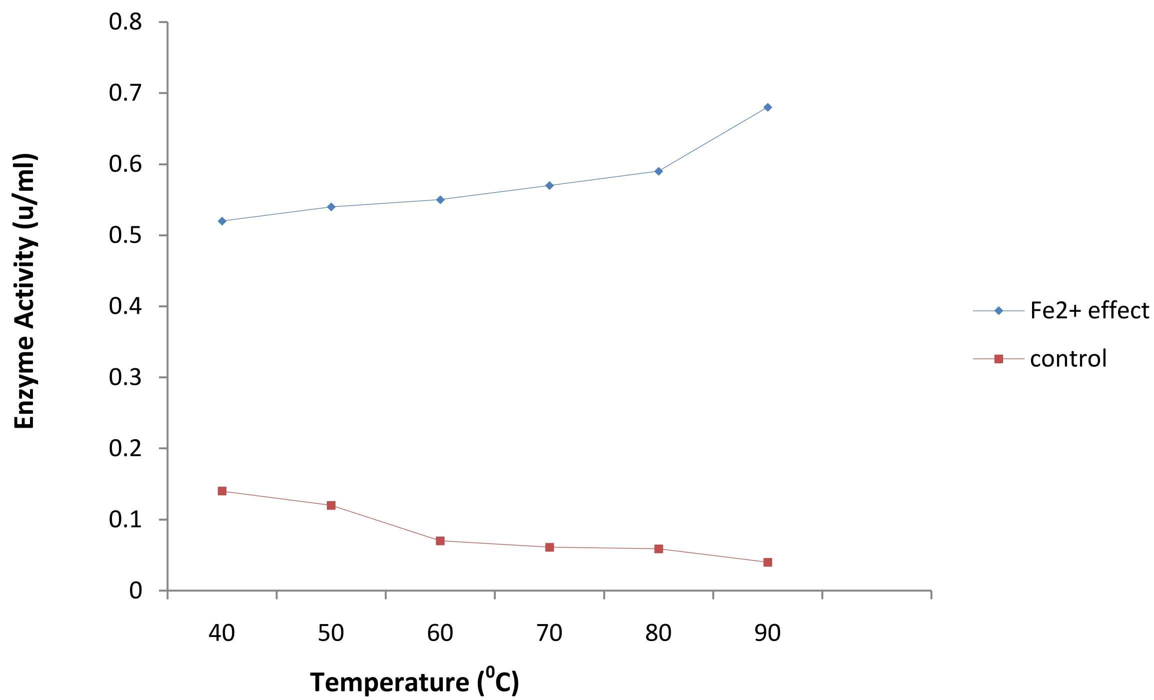

Figure 4.16: Effects of Fe^{2+} on Heat Stability of Amylase

4.13 Amylase Activity in Raw and Gelatinized Starch Digestion

The purified enzyme had highest amylase activity on raw millet starch and the least activity on raw wild cocoyam ('Ede mmuo') starch while there was highest amylase activity on gelatinized soluble starch and the least activity on *Dioscorea bulbifera* ('Ji Adu') starch. The ability of the amylase to hydrolyze raw and gelatinized starches is shown in Figure 17 and 18respectively. Percentage relative rate of hydrolysis in table 6 shows that raw millet starch had 100 % hydrolysis while gelatinized soluble starch also had 100% hydrolysis. Wild cocoyam had the lowest raw starch relative hydrolysis of 75% while *Dioscorea bulbifera* gelatinized starch had the lowest relative hydrolysis of 73.9%.

4.14 Thin layer Chromatography of Products of Starch Hydrolysis by *A. nomius* Amylase

Thin layer chromatography of the starch hydrolysates shows the presence of released maltodextrins with wild cocoyam, *Dioscorea bulbifera* and soluble starch hydrolysates containing more maltodextrins than other polysaccharides as shown in plate 4.

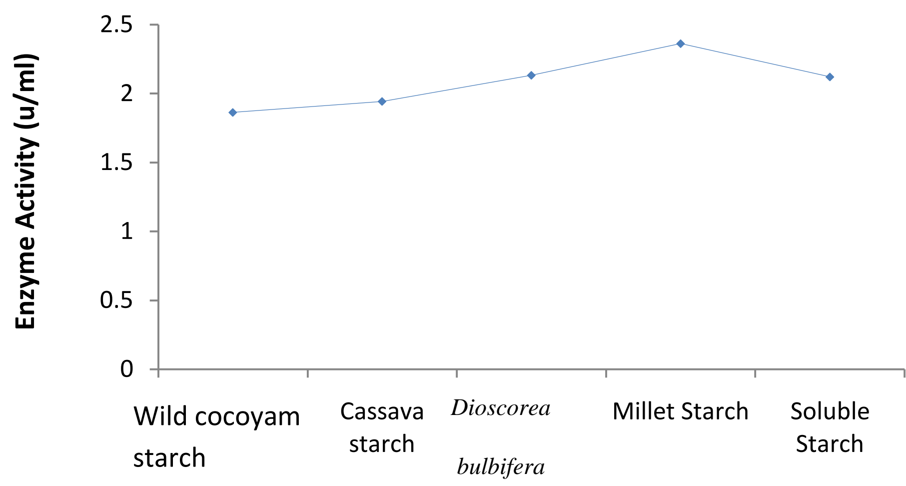

Figure 4.17: Raw Starch Digestion Activities of Amylase

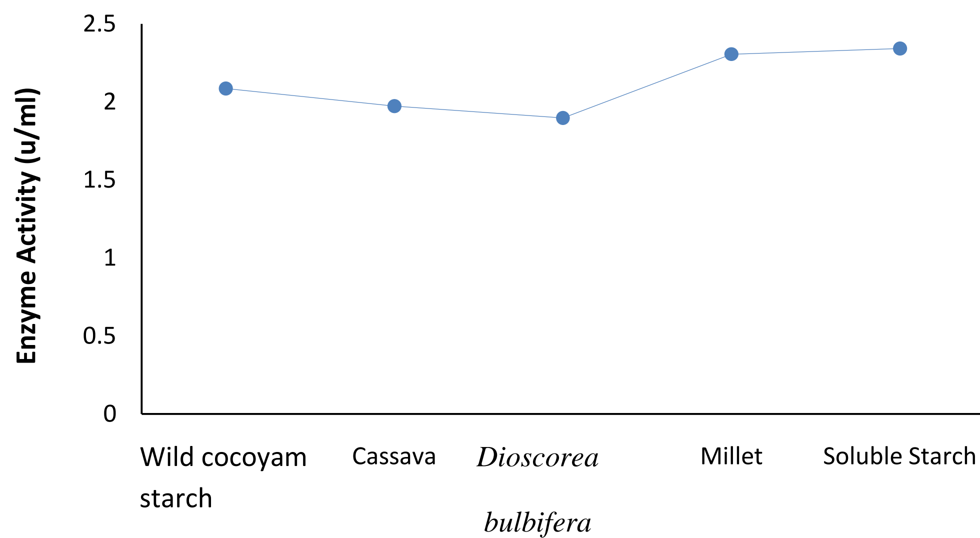

Figure 4.18: Gelatinized Starch Digestion Activities of Amylase

Table4. 7: Percentage Relative Rate of Hydrolysis of Starch Substrates

Starch substrates	Gelatinized starch (%)	Raw starch (%)
Soluble starch	100	79.1
Millet	95.7	100
Cassava	78.3	87.5
Wild cocoyam	89.1	75
Dioscorea bulbifera	73.9	91.6

Key: A: β-cyclodextrin, B: Amylopectin, C: Amylose, D: Wild cocoyam, E: *Dioscorea bulbifera*, F: Soluble starch, G: Glycogen-β-limit dextrin, H: sugar standards, I: Millet

Plate 4: Photograph of Thin Layer Chromatography of Amylase activity on Different Starch Sources

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Cassava waste water from garri processing plants was chosen as a source of carbon for the production of amylase from a mold because of its abundance and availability, cheapness and high starch content.

The physico-chemical properties of the waste water shows that it has a reducing sugar content of 12% (w/v), starch 87.7 mg/L and protein 1.2 mg/L (Table 2) and thus can serve as a nutrient medium for the growth of microorganisms for the production of value added products. Adetunji *et al*. (2015) explained this to be as a result of residual nutrients from cassava roots during retting and washing. Umeh and Odibo (2014) stated that retting periods have effect on the nutrients that get into the retting water from the peeled cassava roots, which when eventually the retted roots are removed, residual nutrients abide in the waste water.

Caasava waste water is a reperttoire of different microorganisms with industrial advantages. Different *Aspergillus* species isolated from the waste water possessed the ability to produce microbial enzyme amylase (Table 3). This explains their ability to be involved in cassava retting and to also survive in waste water, since cassava is made up of starch. Perveen *et al*. (2013) stated that ability to produce substrate

specific enzymes by *Aspergillus* species is essential to their ease of adaptation and biodegradation of the substrate. This is to say that microbes that can thrive in cassava waste water are to be equipped with enzymes that can hydrolyze the complex polysaccharides present in the waste water. Isolate D which had the highest amylase activities was identified with inter-specific (ITS) region sequencing as *Aspergillus nomius*. *A. nomius* is a species related to *Aspergillus flavus* and *Aspergillus tamarii* (Kurtzman *et al.*, 1987). Despite the fact that *A. flavus* and its strains have been considered to produce aflatoxin which is a neurotoxin, they have also shown to be useful to man through their ability to produce different microbial enzymes that have been used for industrial purposes, of which amylase is among (Sun *et al*., 2009).

Optimization of cassava waste water has been shown to be beneficial in the biomass growth of molds with industrial advantages. Oshoma *et al*. (2009) has reported that optimization of cassava waste water gave a biomass yield of 1.63 g/l for *Aspergillus niger* with amylase activity of 495 u/ml at 5 days of cultivation. While this research work has a biomass yield of 3.9 g (40.91% yield), amylase activity of 4.9 u/ml at 6 days of cultivation for *A. nomius*. Oshoma *et al*. (2009) reported yeast extract as the most favourable nitrogenous base to be supplemented with cassava waste water because it gave the highest biomass and amylase yield for *A. niger*. This report corresponds with the findings in this research where yeast extract also gave the highest amylase activity for *A. nomius* (Figure 4). Kalairasi and Palvartham (2013).also reported yeast extract as the best nitrogenous base for amylase production

by *Bacillus cereus*. $NaNO_3$ gave the least amylase activity for *A. niger* in the works of Oshoma *et al*. (2009) while peptone gave the least amylase activity for *A. nomius* as reported in this study (Figure 4). Kalairasi and Palvartham (2013) also reported that $NaNO_3$, urea and peptone gave the least amylase yield for *Bacillus cereus*. 1.5 % yeast extract was the best concentration that gave optimal amylase yield (Figure 5) and this corresponds with the works of Kalairasi *et al*. (2013). Time course for amylase production by *A. nomius* using yeast extract as nitrogenous base was optimal at 96 h and this corresponds with the reports of Oshoma *et al*. (2009) for *A. niger* while that of Kalairasi and Palvartham (2013) was 72 h for *Bacillus cereus* perhaps due to the fact that they made use of solid state cultivation while the other works reported made use of submerged cultivation.

The amylase was purified by ammonium sulphate precipitation, ion exchange chromatography on CM- Sepharose and hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. The enzyme was eluted as a single peak at the hydrophobic region (4 M NaCl) of the elution profile, indicating its tentative purity and hydrophobic nature. However, confirmation of the purity by electrophoresis could not be carried because of constraints of facilities. Purified amylase obtained from *A. nomius* had optimal activity and stability at 40^0C (Figures 7 - 12). This falls within the temperature range reported by Paula and Perola (2010) that most amylases from *Aspergillus* species have optimal activity and stability at temperatures of between 30^0C- 50^0C. They also stated that amylases that have activity and stability at lower

temperatures are good for use in detergent formulations because they can maintain stability under detergent conditions especially where the washing environment is very oxidizing.

Amylase of *A. nomius* exhibited optimum activity at pH 7.0 and was maximally stable between pH 6-9. The pH activity profile resembles that reported by Amutha and Priya (2011) for *Bacillus subtilis* KCX 006. Paula and Perola (2010) also gave same pH ranges for optimal amylase activity and stability with 6.5 pH being the best for a wide range of fungal amylases. They went further to state that generally, fungal amylases are preferred over other microbial amylases due to their more GRAS (Generally Recognized as Safe) status.

The metal ions $Fe^{2+}$and Pb^{2+} slightly stimulated amylase activity while Ca^{2+}, Mg^{2+} and other tested metal ions were inhibitory. This observation corresponds with the report of Raza and Rehman (2016) in respect of commercially available amylase, in which Fe^{2+} stimulated the enzyme activity. It, however, contrasts with the observation of Amutha and Priya (2011) in which Mg^{2+} enhanced the amylase of *Bacillus subtilis*. The observed inhibition of amylase by Ca^{2+} and its enhancement of activity by Pb^{2+} is unusual. Amylase and other enzymes are known to be activated and stabilized by Ca^{2+}. However, Odibo and Obi (1988) reported the inability of Ca^{2+} to stimulate the pullulanase of *Thermoactinomyces thalpophilus*. EDTA acted as a strong inhibitor to *A. nomius* amylase when compared to SDS and Lead actate (Figure 13). More *et al.*

(2012) reported that EDTA enhanced the amylase activity of *Aspergillus oryzae* and Aygan *et al*. (2014) reported EDTA enhancement of amylase activity for *Bacillus subtilis* A 10.

Fe^{2+} is a suitable co-factor that enhanced amylase activity of *A. nomius*. It was observed that amylase activity remained enhanced with increased temperature when Fe^{2+} was incorporated into the reaction mixture as shown on Figure 16. It implies that Fe^{2+} has the ability to maintain amylase stability at application of high temperatures of up to 90^0C. Aygan *et al*. (2014) reported that Ca^{2+} gave amylase heat stability and stated that implies that the amylase is Calcium-dependent.

The effect of substrate concentration on amylase activity is shown in Figure 14. From the figure, it is clear that the amylase of *A. nomius* is not a simple enzyme since it did not show hyperbolic curves with respect to the tested polysaccharides. The amylase of *A. nomius* could be an allosteric enzyme as double reciprocal plots of Lineweaver and Burk (1934), though showed straight line for some substrates but does not seem to obey Michaelis-Menten equation. Figures 17 and 18 show the amylase activity raw starch and gelatinized starch from different sources. It was observed that millet raw starch and heated soluble starch were the most hydrolyzed by *A. nomius* amylase. This implies that covalent bonds in millet raw starch are easily broken by the amylase and likewise, application of heat before introduction of enzyme enhanced the loosening of the Carbon covalent bonds that existed in millet and soluble starch. However, same

was not the case with *Dioscorea bulbifera*. Heat application on *Dioscorea bulbifera* starch led to decreased amylase activity compared to the unheated starch. This could be explained from the works of Omemu *et al*. (2005) who studied amylase activity of *Aspergillus niger* AM07 on yam and cocoyam raw starch. They stated that yam and cocoyam starch are usually not quick to hydrolyze by amylase except the incubation time is prolonged. Likewise, heat application leads to gelatinization of these starches and gelatinization decreases their susceptibility to hydrolysis by *A. niger* amylase.

The products of hydrolysis of the various polysaccharides were examined by thin layer chromatography (Plate 4). The amylase of *Aspergillus nomius* produced predominantly maltooligosaccharides from amylaceous substrates indicating random cleavage of the α-1,4-glycosidic bonds of the substrates and this corresponds with the works of Dutta *et al*. (2013) and Aygan *et al*. (2014).. Random cleavage of the α-1,4-glycosidic bonds of the substrates is peculiar to α-amylases which usually are liquefying and dextrinizing rather than saccharifying. From this observation, the amylase of *A. nomius* could be tentatively classified as an α-amylase, though further tests are needed to confirm this.

This study shows that an amylase can be produced from *Aspergilus nomius* using cassava waste water from a garri processing plant. From the literature, there is dearth of information on amylase production from this organism. Thus we believe that this is the first report on amylase production and characterization from *Aspergillus nomius*.

5.2 Conclusion

This study has shown that:

1. A starch degrading mold of *Aspergillus* species can be isolated from cassava waste water.
2. An amylase that produces maltooligosaccharides was produced and purified from the culture broth of *Aspergillus nomius* grown in cassava waste water.
3. The amylase was tentatively classified as an α-amylase and appears to be the first report of amylase production from *Aspergillus nomius*.

It has been observed that cassava waste water has more benefits that it is being considered a waste product.

5.3 Recommendations

It is recommended that further works be done on the production of other classes of amylase from cassava waste water by *A. nomius*. Likewise, such works should be done using other fungal strains.

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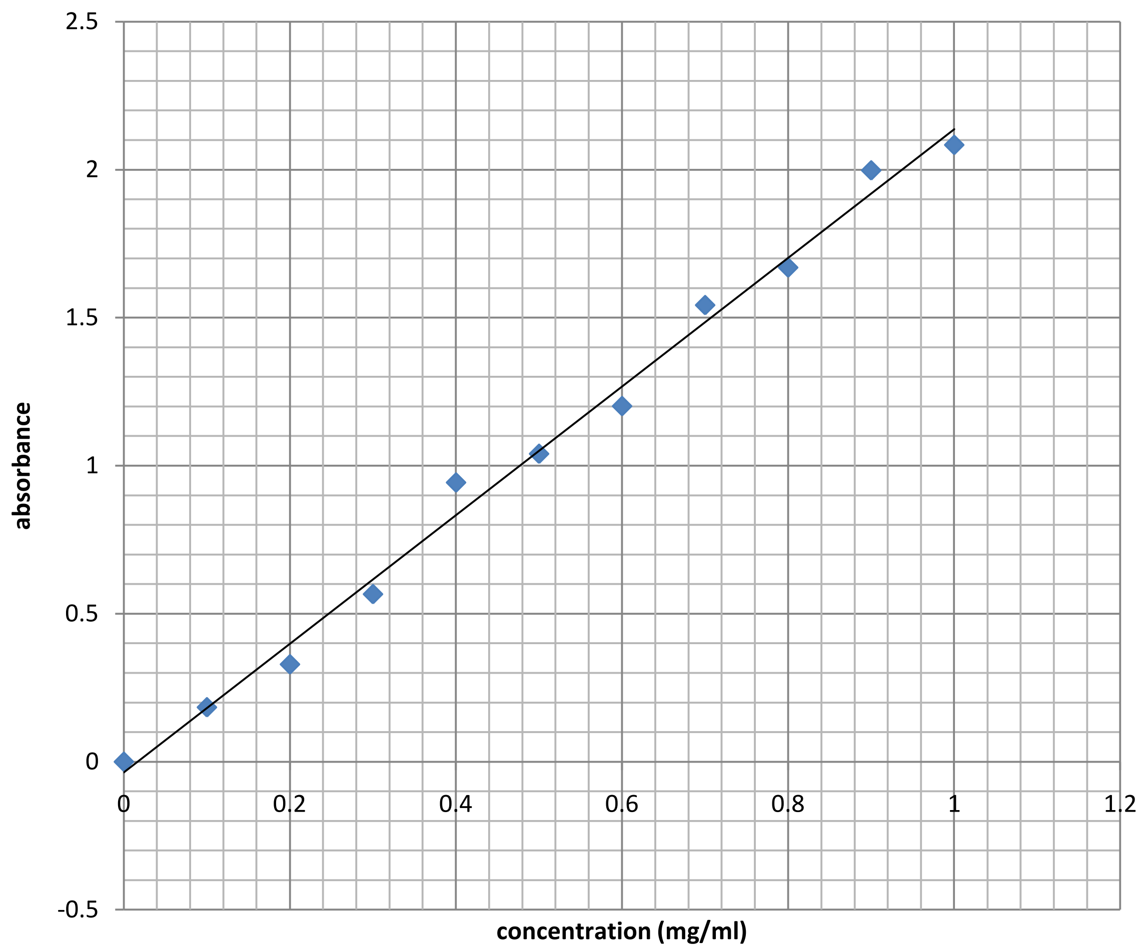

Figure 19: Glucose Standard Curve

Table 8: Resolution Factor (R_f) Values of Separated Sugar Groups on the TLC Plate

Sugar Groups	R_f Values
A	0.214
B	0.251
C	0.267
D	0.270
E	0.578
F	0.286
G	0.455
H	0.448
I	0.148