EFFECT OF *ASPERGILLUS NIGER* AMYLASES ON CASSAVA STARCH HYDROLYSIS

By

NELSON AKEME-ESUOMINI OSARIBIE

DEPARTMENT OF CHEMICAL ENGINEERING
FACULTY OF ENGINEERING
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA.

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By

Nelson Akeme-Esuomini OSARIBIE, B.ENG (NDU) 2006
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DEPARTMENT OF CHEMICAL ENGINEERING,
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NIGERIA

FEBRUARY, 2013

DECLARATION

I declare that the work in this Thesis entitled Effect of *Aspergillus niger* amylases on cassava starch hydrolysis has been carried out by me in the Department of Chemical Engineering. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Nelson Akeme-Esuomini Osaribie		
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled EFFECT OF *ASPERGILLUS NIGER* AMYLASES ON CASSAVA STARCH HYDROLYSIS by NELSON AKEME-ESUOMINI OSARIBIE meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Dr. B. O. Aderemi — Signature _____

Date _____

Chairman, Supervisory Committee

Engr. Dr. P. C. Okonkwo — Signature _____

Date _____

Member, Supervisory Committee

Dr. I. A. Mohammed-Dabo — Signature _____

Date _____

Head of Department

Professor A. A. Joshua — Signature _____

Date _____

Dean, School of Postgraduate Studies

DEDICATION

This research work is dedicated to Almighty God. Glory and honour be to Him, Who is able to keep me from falling.

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ABSTRACT

The enzymatic hydrolysis of cassava starch to glucose has been studied. *Aspergillus niger* was isolated from deteriorated groundnut seeds and used as the amylase producer source. The effect of various process variables such as culture age, mycelia mass loading, pH, temperature and substrate (cassava starch) concentration were investigated in order to optimize the hydrolysis process. The *A. niger* amylases exhibited optimum activity at pH and temperature of 4.8 and 60^{o}C respectively. The cells appear most viable on the fifth day. Results obtained from this study indicated that increase in cassava starch concentration from 0.3 to 1.5 g/l was accompanied with a proportionate increase in glucose concentration from 0.239 to 0.310 g/l and decrease in glucose yield, falling from 74% to 18%. In addition, increase in mycelia mass loading resulted to increase in both the concentration and yield of glucose. An initial rate method was employed in estimating the kinetic parameters using Michealis-Menten model. The Langmuir form of the Michealis-Menten model gave a high regression coefficient (0.9939) and the values of the maximum reaction velocity (r_{max}) and Michaelis constant (K_m) were found to be $2.42*10^{-3}$ g/l.min and 0.221 g/l respectively.

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ABBREVIATIONS AND SYMBOLS

$[E]_t$	Total Enzyme Concentration
[P]	Product Concentration (g/l)
[S]	Substrate Concentration (g/l)
AMG	Amyloglucosidase
CSTR	Continuous Stirred Tank Reactor
DE	Dextrose Equivalent
FWS	Freshly Prepared Wheat Starch
K_m	Michaelis constant (g/l)
PDAS	Potato Dextrose Agar Streptomycin
PIY	Commercial Wheat Starch, PIYALE
R^2	Linear regression coefficient
r_{max}	Maximum reaction velocity (g/l.min)
RME	Rice Malt Enzyme
r_o	Initial rate of reaction (g/l.min)
r_p	Rate of product formation (g/l.min)
$-r_s$	Rate of substrate depletion (g/l.min)

CHAPTER 1

INTRODUCTION

1.1 Background Information

There has been a great emphasis on plant biomass as a source of fermentable sugars. Starch is considered as one of the world most abundant polymer, believed to be the renewable energy source that can provide liquid fuel and other bulk chemicals on a sustainable basis (Gomez *et al.,* 2008).

The world cassava production was estimated to be 230 million tons in 2010 (Leen *et al*., 2007; Hermiati *et al.,* 2012; FAO, 2012). Nigeria has been recognised as the largest producer of cassava, a viable source of starch with an aggregate annual production rate of about 49 million tonnes (Aderi *et al*., 2010). However, despite its abundance in Nigeria, it has remained relatively underutilized.

A large proportion of this tuber starch is lost yearly due to inadequate and ineffective storage facilities (Omemu *et al*., 2005; Zainab *et al*., 2011), non-availability of appropriate processing technology and industry to convert these raw materials into various value-added products such as glucose syrup, maltose syrup, high fructose syrup and maltodextrins, which are industrial products of economic relevance.

Processing starch into finished products at industrial scale has involved both chemical and enzymatic approaches. Owing, to the short comings associated with the chemical method of starch hydrolysis such as formation of undesirable coloured and off-flavoured by-products, which consequently corrode processing equipment (Shambe *et al*., 1989). In addition, the process appears to be totally random which is not influenced

by the presence of α-1,6 glucosidic linkages (Haki and Rakshit, 2003; Zainab *et al*., 20011). The use of enzyme has become a method of choice to industrialists and others alike.

Conventionally, conversion of starch to glucose is accomplished on a large scale with the use of two commercially purified enzymes such as α-amylase and amyloglucosidase (glucoamylase) from different sources (Kyriakides *et al.,* 2001; Azmi *et al*., 2012). With the view of minimising the cost of operation, this study is aimed at using a micro-organism that can secret the two enzymes under favourable conditions, thereby hoping to mitigate the high cost of glucose production.

1.2 Research Problem Statement

Although cassava production capacity exceeds 49 million tons in Nigeria, a large proportion of the produce is wasted annually (Nwagu and Okolo, 2011), which means it remained underutilized. Based on research evidence, the use of whole cell for cassava starch degradation has not been sufficiently explored and there is little information in the literature about kinetic parameters for cassava starch hydrolysis using whole cell of *Aspergillus niger*.

1.3 Research Aim

The purpose of this research was to study the effect of *Aspergillus niger* amylases on cassava starch hydrolysis.

1.4 Research Objectives

Therefore, the research focuses on:-

1. To study the effect of process variables on cassava starch hydrolysis using *Aspergillus niger* amylases.

2. To evaluate kinetic parameters based on initial rate method.

1.5 Justification

Starch is an industrial raw material which can be explored to produce many products of value for human consumption. It can be sourced from cassava which is produced in large amounts in Nigeria. The quality of products obtained from raw starch depends to a very large extent on the method of hydrolysis employed. Starch hydrolysing enzymes from different sources have performed at different capacities, though yielding better results when compared to acid hydrolysis. Thus, exploring microbial sources for optimum performance becomes imperative as that can be translated into quality finished products, reduced cost of production, availability of job and also reduced environmental problems since the by-products are less hazardous.

1.6 Scope

This research work was designed to investigate the effect of culture age, mycelia mass loading, operational pH, operational temperature, substrate (cassava starch) concentration on *Aspergillus niger* amylases activity as well as selecting appropriate kinetic model for estimating kinetic parameters.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Starch is the second most abundant polysaccharide in green plants, after cellulose. It is synthesised in chloroplasts and stored as energetic reserve substance in roots, seeds and tubers as small particles known as granules (Whistler and Daniel, 1990). It is a polysaccharide in which D-glucopyranose, cyclised form of glucose, units are bonded with α-1,4 and α-1,6 glucosidic linkages. It is synthesised through a process known as photosynthesis utilizing carbon (IV) oxide, water and sunlight as represented by the Chemical Equation (2.1), (Storz and Steffens, 2004).

$$6nCO_2 + 12nH_2O \xrightarrow[\text{Chlorophyll}]{\text{Sunlight}} (C_6H_{10}O_5)_n + 6nO_2 + 7nH_2O \qquad (2.1)$$

Starch constitutes an important source of raw material for the production of syrups like glucose, fructose or maltose which are widely used in food industries. In addition to that, the sugars produced can be fermented to produce bio-ethanol (Storz and Steffens, 2004).

In spite of the large number of plants producing starch, very few are commercially grown for starch processing industry; they include root (tapioca and arrowroot), stem (sago), legume (mainly pea); tuber (sweet potato); and cereal (wheat, corn, rice and sorghum) crops. Starch occurs as minute granules in nature, but the size, shape and molecular arrangement inside the granule differ and depend on the species as well as crop variety (Ratnayake and Jackson, 2008), as presented in Table 2.1.

Table 2.1: Sources and granule size distribution of various starches

Starch species	Extracted from	Granule shape	Granule size Range (μm)
Corn (Maize)	Seed	Round or Polygonal	5-25
Cassava	Root	Round or Oval	2-25
Waxy sorghum	Seed	Round or Polygonal	6-30
Sweet Potato	Tuber	Polygonal	10-25
Wheat	Seed	Round or Elliptical	2-10 or 20-35
Arrowroot	Root	Oval	15-70
Irish Potato	Tuber	Egg-shaped	15-100
Sago	Stem	Oval or Egg-shaped	20-60
Barley	Seed	Round or Elliptical	2-6 or 20-35
Rice	Seed	Polygonal	3-8
Waxy rice	Seed	Polygonal	2-13
Waxy maize	Seed	Round	5-25

Source: Storz and Steffens (2004)

2.2 Chemistry of Starch

Regardless of the botanical source of starch, it is a biopolymer of two high molecular weight glucans, namely amylose and the amylopectin (Kearsly and Dziedzic, 1995; Nwagu and Okolo, 2011). The amylose is linear and consists of α-1, 4 linked glucose units, with molecular weight of about 10^5-10^6 $gmol^{-1}$. It is insoluble in water and constitutes 25-30% w/w of the starch molecule. Figure 2.1 is a typical structure of amylose.

Amylopectin (Figure 2.2) is soluble in water and has a fraction of about 70-75% w/w of starch molecule with α-1, 4 linear and α-1, 6 branched glucosidic bonds. The degree of branching varies depending on the species, and the molecular weight varies from $50x10^6$ to $500x10^6 gmol^{-1}$ (Kearsly and Dziedzic, 1995; Ellis *et al.*, 1998).

Amylose:

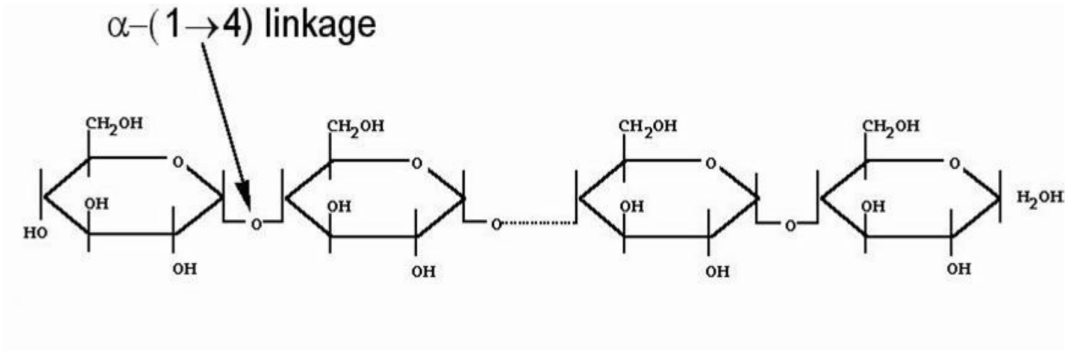


Figure 2.1 Structure of amylose (Parker and Ring, 2001)

Amylopectin:

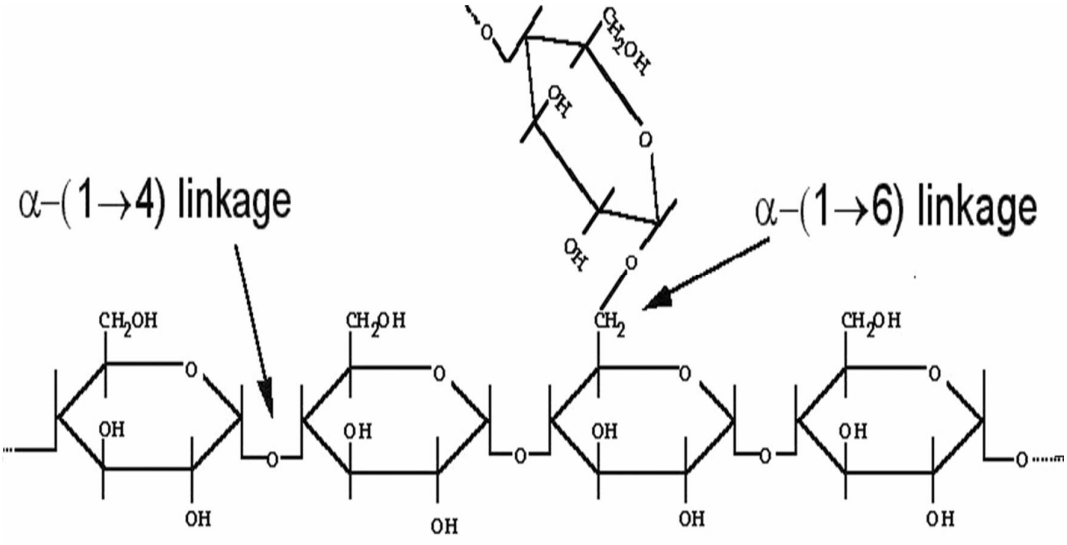


Figure 2.2 Structure of amylopectin (Parker and Ring, 2001).

2.3 General Characteristics of Starch

Regardless of the sources of starch, it is white, tasteless and odourless. In hot water, it is insoluble and appears jelly. At room temperature, it is not soluble in cold water and form a blue complex compound in a reaction with iodine solution. Its hydrolysis is brought about by either acid or amylolytic enzymes to yield glucose.

2.4 Economic Importance of Starch

The properties of starch from various species differ considerably not only in the relative proportions of amylose and amylopectin, but also in the amount of non-starch components of the starch granules, such as fibre, lipids, proteins and phosphate groups. Numerous industrial applications of starch exist, either in its native form or after modification. In the construction industry, starch is applied as an additive in cement to improve the setting time (Ellis *et al.*, 1998; Eliasson, 2004).

Its use in geosciences cannot be over emphasized as it is used to improve the viscosity of drilling mud in oil wells. It is also used to seal the walls of bore holes to prevent fluid loss.

In paper production, starch is currently used as glue to achieve brightness and strength. It is also an important raw material for making powder in the cosmetics industries. It is used to get better recovery and to improve the shelf life of detergents. In the rubber and foam industries, starch is employed for getting better foaming and colour (Muntean *et al.,* 2010).

While in the pharmaceutical industry, it serves as a filler material and binding agent for making tablets. Starch is utilized in sizing and dyeing in the textiles industries to increase brightness and weight of cloth. It is also used in processing baby foods as a filler material and binding agent in confectionary and biscuit industries and to make glucose, fructose syrups and bioethanol (Ellis *et al.*, 1998; Eliasson, 2004).

Furthermore, being a biodegradable polymer, materials based on starch does not pose environmental threat and thus, has been considered part of the new generation materials contributing to the reduction of environmental problems such as green-house effect as compared to nonbiodegradable materials (Storz and Steffens, 2004).

2.5 Modification of Starch

The modification of starch to products with low molecular weight is of great interest in the field of biotechnology (Wang *et al*., 1993). Native starch is unsuitable for industrial applications due to poor characteristics exhibited under some processing conditions, such as high temperature, high shear pressure and varying pH (Wang *et al*., 1993). In order to enhance their industrial applications, starch can be modified by chemical, physical and biological methods (Ellis *et al.*, 1998; Tharanathan, 2005). Modified starch has generally better paste clarity, gel stability, increased resistance to retrogradation, increased solubility and improved freeze–thaw stability, which improves their application as stabilizers, fillers, binders and adhesives (Ellis *et al.*, 1998; Tharanathan, 2005).

2.5.1 Chemical treatment method

It entails treating native starch with specific chemical reagents. For instance, various oxidizing agents such as sodium hypochlorite, hydrogen peroxide, periodate, permanganate, dichromate, persulfate, and chlorite are introduced into the starch molecule. This is called oxidation. Oxidation of starch mainly causes the scission of the glucosidic linkages and hydroxyl groups in starch (Sirroth *et al*., 2002; Tharanathan, 2005).

Another important chemical approach is esterification, in which the hydroxyl group of starch is replaced by an esterifying agents like acetic anhydride, propionic anhydride, vinyl acetate, succinic anhydride and sodium tripolyphosphate (Sirroth *et al*., 2002). Etherification another method of starch modification involves the use of etherifying agents like ethylene oxide, propylene oxide to replace some of the hydroxyl groups in the starch molecule. In all, the most important method of starch modification is cross linking which involves the replacement of the hydrogen bonds between starch chains by stronger, more permanent covalent bonds. In this manner, the swelling of the starch granule is inhibited, pre-empting disintegration either by chemical attack, mechanical attrition (shear) or cooking (Wurzurg, 1986; Ferris *et al*., 2001; Sirroth *et al*., 2002; Tharanathan, 2005).

2.5.2 Physical treatment method

This method includes annealing, a process in which starch granules are heated in excess amount of water at a temperature slightly below gelatinization temperature for a relatively long time. Annealing decreases the swelling power and solubility of starch molecules (Sirroth *et al*., 2002). Starch can also be pre-gelatinised, a method in which

cooked starch is dried on a drum dryer or in an extruder making the starch soluble in cold water.

Another physical approach widely employed in starch modification is the heat moisture treatment in which starch is treated at varying moisture levels (<35%) for a certain period of time at a temperature above the glass transition temperature but below the gelatinization temperature (Tester and Debon, 2000; Sirroth *et al*., 2002).

In all, these methods are characterised by shortcomings such as high time-consumption of the processes, it is expensive more so, chemicals used in some of the methods can be hazardous and be a source of environmental pollution and consequently health problems.

The most important process in starch modification is the enzymatic treatment method, this consists of treatment of starch with hydrolytic enzymes during which glucosidic bonds are broken (Guzman-Maldonado and Paredes-Lopez, 1995; Sirroth *et al*., 2002). The processing methods and undesired products generated pose no serious harm to the environment and human health.

2.6 Starch Processing Enzymes

Starch processing enzymes are classified as amylases, which is a generic name for the group of enzymes that catalyse the hydrolysis of starch and other related carbohydrate derivatives. Amylases are sourced from a variety of organisms like bacteria, fungi, plants and animals and are grouped into endo-acting amylase (α-amylase) and exo-

acting amylase (glucoamylase) based on their mode of action (Windish *et al*., 1965; Godon, 1994; van der Maarel *et al*., 2002; Aehle, 2004; Rodriguez *et al*., 2006).

The endo-acting α-amylase (α-1,4 glucan-4-glucanohydrolase), hydrolyzes α-1,4 bonds present in the inner part of amylose or amylopectin chain in a random manner, for the formation of oligosacchrides with varying length (Gupta *et al*., 2003).

The exo-acting amylase includes β-amylase, glucoamylase that cleaves both α-1,4 and α-1,6 bonds on the external glucose residues from the non-reducing end, producing glucose molecules (Godon, 1994; Gupta *et al*., 2003; Aehle, 2004).

Pullulanase, the de-branching enzyme is involved exclusively in hydrolyzing α-1,6 linkages in pullulan and other branched polysaccharides, producing maltotriose, and sometimes, it is used in combination with other amylolytic enzymes in starch saccharification process (Nigam and Singh, 1995; Pandey *et al*., 2000; Gupta *et al*., 2003). Figure 2.3 and Table 2.2 show the scheme of the mode of action of amylase on starch and the various uses of enzymes in starch processing industry respectively.

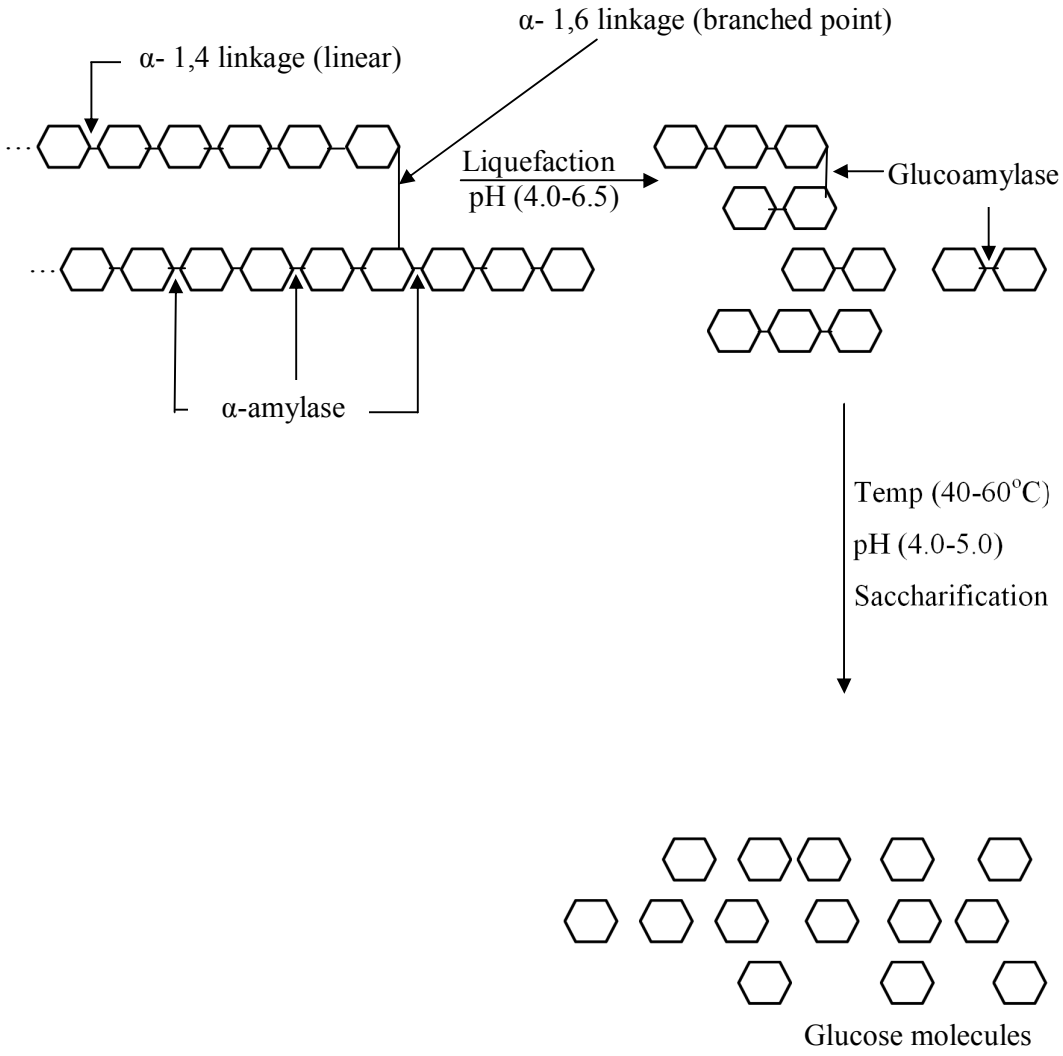


Figure 2.3: The scheme of action of amylases on α-(1, 4) and α-(1, 6) glucosidic bonds of starch

Table 2.2: Uses of enzymes in starch processing industry

Enzyme	EC Number	Source	Action
α-amylase	3.2.1.1	*Bacillus amyloliquefaciens*	Only α-1,4-oligosaccharide links are broken down to give dextrin, maltose (G_2), G_3, G_6 and G_7 oligosaccharides
		B. licheniformis	Only α-1,4-oligosaccharide links are cleaved to give dextrin and predominantly maltose, G_3, G_4, and G_5 oligosaccharides
		A.oryzae, A.niger	Only α-1,4-oligosaccharide links cleaved to give dextrin, maltose and G_3 oligosaccharides
Glucoamylase	3.2.1.3	*A. niger*	α-1,4 and α-1,6 links are cleaved from the non-reducing ends, to give glucose
Pullulanase	3.2.1.41	*Klebsiella*	Exclusively breaks α-1,6 glucosidic linkages
β – amylase	3.2.1.2	*Bacillus spp.*	Only α-1,4 links are cleaved from non-reducing ends, to give dextrin and maltose

Source: Crabb and Mitchinson (1997)

Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable amylases are available from the mesophile *Bacillus licheniformis* (Morgan and Priest, 1981), *Bacillus sp*. ANT-6 (Burhan *et al*., 2003) and *Bacillus sp.* ASMIA-2 (Teodoro and Martin, 2000). Also some hyper thermophilic bacteria and archaea species of the amylase family such as *Bacillus stearothermophilus*, *Clostridium thermosulfurogenes*, *Clostridium thermocellum*, *Pyrococcus woesei*, *Thermococcus*

celer and *Thermo anaerobacter sp* have also been reported (Wind *et al*., 1994; Bertoldo and Antranikian, 2002; Leveque *et al*., 2002).

2.7 Industrial Starch Hydrolysis

Conversion of starch into sugar syrups and dextrin forms the major part of the starch processing industry. Sugar syrups obtained from starch are sweet edible products that are widely used in beverages and other food products. Also, solid glucose can be prepared by crystallization from completely hydrolyzed liquors. In United States, these syrups are known as corn syrups since they are produced by acidic or enzymatic hydrolysis of corn starch. Starch from wheat, potato, cassava, pea and rice, can also be used to manufacture such products (Whistler *et al*., 1984).

The major steps in the conversion of starch into glucose syrup are gelatinisation, liquefaction and saccharification (Slominska, 1993; Godon, 1994; Kearsly and Dziedzic, 1995; Akberg *et al*., 2000; Ayernor *et al*., 2002; Storz and Steffens, 2004).

In some industries, gelatinisation and liquefaction are often carried out simultaneously by adding a thermostable α-amylase to the starch slurry in the jet cooker. The starch concentration during this process is limited to 30–35 w/w % dry solids, because higher concentrations cannot be processed in a jet-cooker. However, increasing the starch concentration during gelatinisation, enzymatic hydrolysis can potentially yield a higher volumetric productivity and enzyme stability (Klibanov, 1983; De Cordt *et al*., 1994).

During liquefaction, the α-1,4 linkages in amylose and amylopectin of the exposed starch molecules are broken down by the action of the endoenzyme α-amylase, resulting in shorter chains (oligosaccharides) called dextrin. Usually, maltose ($C_{12}H_{22}O_{11}$) is

produced during this stage as shown in Equation (2.2), but other dextrin such as maltotriose ($C_{18}H_{32}O_{16}$) can also be produced as in Equation (2.3).

$$2(C_6H_{10}O_5)_n + nH_2O \xrightarrow{\alpha\text{-amylase}} nC_{12}H_{22}O_{11} \quad (2.2)$$

$$3(C_6H_{10}O_5)_n + nH_2O \xrightarrow{\alpha\text{-amylase}} nC_{18}H_{32}O_{16} \quad (2.3)$$

Liquefaction can be done by Acid-enzyme liquefaction and Single stage enzyme liquefaction.

2.7.1 Acid-enzyme liquefaction

Starch slurry containing 30-40% dry solids is cooked at a temperature range between 105-175^{o}C for about 5 minutes. A jet cooker is used for mechanical thinning due to shearing. The pH range is 2-5, but if it is too low, by-product formation will be significant however too high pH values result in increased colour formation. After cooking, the slurry is flash cooled to about 100°C before the addition of enzyme; pH is set to between 6.0 and 6.5. Enzyme consumption is slightly reduced and since better fat/protein separation is achieved, filtration properties are also improved. On the other hand, due to high temperature of cooking; there is an increase in steam consumption, so increase in fuel costs (Prasanna, 2005).

2.7.2 Single stage enzyme liquefaction

It is carried out in stirred tank reactors, continuous stirred reactors (CSTR) or a jet cooker (Kearsly and Dziedzic, 1995). Single dose jet cooking process is mostly used in many plants. In this process, α-amylase is metered into starch slurry in feed tank after pH adjustment, the slurry is pumped through a jet cooker of which temperature is raised

to 105°C by steam injection, and a subsequent passage through a series of holding tubes, provides a 5 minutes residence time (Kearsly and Dziedzic, 1995).

The applications of the simultaneous action of α-amylase and glucoamylase on starch, known as synergism have been studied by various researchers. Fujii and Kawamura (1985) and Fujii *et al.* (1988) reported the synergistic hydrolysis pattern of α-amylase and glucoamylase, α-amylase hydrolyses glucosidic bonds at the interior of starch granules, producing non-reducing sugars that remain on the surface of the granule, inhibiting α-amylase from proceeding into the granule. These sugars are further hydrolysed by glucoamylase in a way that it peels off the granule surface, exposing new substrate to α-amylase, the synergistic action results in enhanced reaction yields and increased activity toward crude starch granules. Franco *et al.* (1989) further described the use of a synergistic scheme of the two enzymes for the determination of enzyme activity. They also reported increased glucose and maltose levels in the products depending on the ratio of the enzymes used.

Arasaratham and Balasubramaniam (1993) studied and reported increased efficiency of the synergistic hydrolysis of dry milled corn starch compared to wet-milled corn starch. Shevel'kova and Sinitsyn (1993) also reported dependence of the synergistic action on substrate characteristics and concentration, enzyme concentration, time duration, and the mode of α-amylase action.

In the saccharification stage, the release of monosaccharides from the liquefied mixture of dextrin occurs. Saccharification is carried out by the action of the exo-enzyme glucoamylase, which releases the single glucose ($C_6H_{12}O_6$) molecules. Dextrin (e.g.

maltose or maltotriose) conversion to glucose during saccharification is expressed in Equation (2.4) and (2.5).

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{\text{glucoamylase}} 2C_6H_{12}O_6 \quad (2.4)$$

$$C_{18}H_{32}O_{16} + 2H_2O \xrightarrow{\text{glucoamylase}} 3C_6H_{12}O_6 \quad (2.5)$$

In general, depolymerisation of starch into glucose can be expressed by Equation

$$(C_6H_{10}O_5)_n + nH20 \xrightarrow{\text{Enzymes}} nC_6H_{12}O_6 \quad (2.6)$$

Sakintuna (2002) studied the use of two glucoamylase obtained from Orba and Sigma companies in a saccharification studies in batch system. The liquefied starch was saccharified by Orba glucoamylase and Sigma glucoamylase in separate batch runs and reported that Sigma glucoamylase hydrolyse the starch faster than Orba glucoamylase at 60^{o}C. Benvides *et al*. (1985) carried out primary and secondary hydrolysis of rice flour with novo bacterial amylase and glucoamylase, and reported 94.6 and 87.5 percent yield for liquefaction and saccharification steps. Glucoamylase from *Aspergillus terreus* (*A. terreus*) showed strong capacity for digesting starches (62 per cent of a 4 percent wheat starch suspension in 10 minutes and 100 percent of this starch precooked for 20 minutes) and was considered promising for the first stage of industrial starch saccharification (Ghosh *et al*., 1991).

Keating *et al*. (1998) reported that α-amylase of *Bacillus coagulans* was a saccharifying glucoamylase which hydrolyses the disaccharide maltose, and maximum starch saccharification occurs at pH 6.0. Karakatsanis *et al.* (1997) studied the conversion of corn starch, rice starch and soluble starch to glucose using combination of α-amylase

and glucoamylase and reported 87, 79.27 and 36.92 percent, for the various conversions.

Arasaratnam *et al.* (1998) hydrolysed starch in corn flour by synergistic action of α-amylase (Termamyl 60 L) and glucoamylase at 70°C and obtained 76 percent hydrolysis at 16 percent suspension of corn flour. Niagam and Singh (1995) stated that a careful balance of the ratio of amyloglucosidase (AMG) to α-amylase produces high glucose syrup (30-50% glucose, 30-40% maltose) or high maltose syrup (30-50% maltose, 6-10% glucose). On the simultaneous consideration of temperature, pH, and time on yield of sugars (DE 60-80), highest yields occurred at 60^{o}C, pH 4.5 in 5 hours of liquefaction and saccharification using rice malt enzyme (RME) and AMG.

Lee (2002) studied the enzymatic treatment of corn starch using α-amylase and fungal glucoamylase. Glucose was produced with an optimal temperature range of 55-60^{o}C and pH of 4-4.5. Sarbatly (2007) investigated simultaneous hydrolysis of tapioca starch to glucose at two reaction temperatures of 55 and 60^{o}C. It was reported that at 55^{o}C, the conversion of tapioca starch to glucose was 65%.

Linko and Javanainen (1996) reported the simultaneous liquefaction, saccharification and lactic acid fermentation on barley starch at two reaction temperatures of 37^{o}C and 60^{o}C. The concentration of glucose produced over 25 hours hydrolysis time was the same, however, when the reaction time was reduced to 5 hours, glucose produced at 60^{o}C was twice that at 37^{o}C.

Kyriakides *et al.* (2001) also investigated the synergistic hydrolysis of starch using amylase and glucoamylase from *Aspergillus niger* (*A. niger*) and found that at temperature 60^{o}C and pH 4-5 about 76% starch conversion to glucose was obtained in 5 hours of reaction time.

2.8 Enzyme Kinetics

Enzyme kinetics describes the rate at which substrate is converted to product in an enzyme catalysed reaction. The conversion of substrate [S] into product [P] catalysed by the enzyme [E] is expressed in Equation (2.7), (Guilbault, 1976).

$$[E]+[S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [P]+[E] \qquad (2.7)$$

Binding Catalysis

In Equation (2.7), the Michaelis-Menten model for enzyme kinetics presumes reversible 2-step reaction i.e. the binding step, where the substrate binds to the enzyme and the catalytic step, the substrate is converted to product and enzyme released. When the enzyme E and substrate S are mixed, very shortly a steady state is established in which the concentration of enzyme-substrate complex [ES] remains essentially constant with time.

In order to achieve a rational process design, the kinetic study of enzymatic hydrolysis is very important. Usually, the determination of Michaelis-Menten constant (K_m) and maximum reaction velocity (r_{max}) is essential in such study. Presently, there are a number of procedures that can be used to determine the values of K_m and r_{max} such as linearization and non-linearization methods. However, the direct estimation of K_m and r_{max} is difficult from the Michaelis-Menten plot due to the hyperbolic nature of rate (r) against substrate concentration [S]. Therefore, the determination of the kinetic

parameters is usually done by transforming the Michaelis-Menten model equation into linearised plots like the Lineweaver-Burk, Langmuir and Eadie-Hofstee (Guilbault, 1976; Lee, 2002; Inamdar, 2009; Rajaram and Kuriacose, 2009).

Resorting to the initial rate method facilitates the interpretation of experimental results and avoids the influence on the reaction rate of factors such as loss of enzyme activity through changes in pH, thermal deactivation at very low and high temperatures and likely inhibition caused by substrate or reaction products. Nevertheless, the kinetic model obtained in this way is only valid for the initial periods of the reaction and may not be so at later times (Rajaram and Kuriacose, 2009).

2.9 Evaluation of Steady State Parameters (K_m and r_{max})

Several simplifying assumptions allow for the derivation of the Michaelis-Menten Equation (Guilbault, 1976; Lee, 2002; Inamdar, 2009):

(1) The binding step ($E + S \rightleftharpoons ES$) is fast, allowing the reaction to quickly reach equilibrium ratios of [E], [S] and [ES].

The catalytic step ($ES \rightleftharpoons P + E$) is slower, and thus rate-limiting.

(2) At early time points, where initial velocity (r_o) is measured, the product concentration [P] is so low that product inhibition is considered negligible.

(3) ES immediately comes to steady state, so [ES] is constant (throughout the measured portion of the reaction) that is, pseudo-steady-state or quasi-steady-state assumption

(4) $[S] >> [E_T]$, so the fraction of S that binds to E to form ES complex is negligible, and [S] is constant at early time points.

(5) The enzyme exists in only two forms: free (E), and substrate-bound (ES). Thus, the total enzyme concentration (E_T) is the sum of the free and substrate-bound concentrations: $[E_T] = [E] + [ES]$.

Considering Equation (2.7), the rate of product formation is proportional to the difference between the enzyme-substrate complex formation and dissociation step, which implies that

$$r_p = k_3 [ES] - k_4 [E] [P] \tag{2.12}$$

Now from assumption (2), the rate of product formation can be expressed as

$$r_p = k_3 [ES] \tag{2.13}$$

Based on Equation (2.13) and the stated assumptions, the Michaelis-Menten kinetic model can be represented as

$$r_p = \frac{k_3 C_{Et} C_s}{K_m + C_s} \tag{2.14}$$

$$r_p = \frac{r_{max} C_s}{K_m + C_s} \tag{2.15}$$

where K_m and r_{max} in Equation (2.15), denotes Michaelis constant and maximum reaction velocity, linearizing Equation (2.15) gives

$$\frac{1}{r} = \frac{1}{r_{max}} + \frac{K_m}{r_{max}} \frac{1}{C_s} \tag{2.16}$$

$$\frac{C_s}{r} = \frac{K_m}{r_{max}} + \frac{C_s}{r_{max}} \tag{2.17}$$

$$r = r_{max} - k_m \frac{r}{C_s} \tag{2.18}$$

Which are the Lineweaver-Burk, Langmuir and Eadie-Hofstee plots that gives straight line with various slopes and intercepts to evaluate the kinetic parameters K_m and r_{max}.

CHAPTER 3

MATERIALS AND METHODS

This chapter considers the materials, process equipment/apparatus and the methodology that was used in the research work.

3.1 Materials

3.1.1 Sample collection

Cassava starch was procured from Samaru market in Zaria, Kaduna State, Nigeria. The starch was sun dried for seven days and stored in an air-tight plastic container at room temperature.

3.1.2 Chemicals

All reagents and chemicals used were of analytical grade and were obtained from: Merck Darmstadt Germany, BDH Chemicals Ltd, BEMA Scientific and Chemical Co. Ltd Nig., Guanghua Chemical Co. Ltd China., Chemelex S.A Pol Ind. Can, Spain.

3.1.3 Equipment and apparatus

The followings are the equipment and apparatus used in this investigation: autoclave (Griffin and George 31-417), weighing balance (GF-2000 EC), Water bath (Techmel and Techmel TT420), Colorimeter (AE11D), pH metre (Jensway 3150), Incubator (Gallenkamp 1EF097 XX2.5), Hotbox oven (Gallenkamp 7B1759), Light microscope (Olympus CH-2), Micropipette, Conical flask and measuring cylinder (Pyrex) and Glucose labkit (Chemelex).

3.2 Methods

3.2.1 Media preparation

Aspergillus niger mycelia that were used throughout the study were cultured in the Laboratory of the Department of Crop Protection/Institute for Agricultural Research (I.A.R), ABU- Zaria, Kaduna State of Nigeria.

Procedure:

Peeled Irish potato (200 g) was chopped into small pieces and added to 1000 ml of sterilized distilled water and boiled gently for 60 minutes, then filtered through a muslin cloth into a 1000 ml conical flask. Then 29 g of dextrose and 25 g of agar were added to the extract and the volume brought to 1000 ml with distilled water before being boiled for 20 minutes to dissolve the agar completely. After adjusting the pH to 6.5 with 0.1 M NaOH, portion of 500 ml of the potato dextrose agar (PDA) mixture was dispensed into two conical flasks. The mouth of the flasks were wrapped with aluminium foil properly and sterilized by autoclaving for 20 minutes at a pressure of 15 psi and temperature of 121^{o}C. The media was allowed to cool to 45^{o}C and then supplemented with 5 ml of streptomycin sulphate antibiotic stock solution to discourage the growth of bacteria (Difco, 1960; Salako and Anjorin, 2012).

3.2.2 Plate preparation

Procedure:

Petri dishes with cover were put into a basin containing Izal water solution for 5 minutes then washed with detergent and rinsed under running tap water to remove dirt and spread on table to dry, and then they were arranged in a canister and sterilized in a hotbox oven at 150^{o}C for 90 minutes. The mouth of the conical flask containing the molten Potato Dextrose Agar supplied with streptomycin was flame sterilized to destroy

surface micro-organisms and then carefully poured into the sterilized Petri plates and covered, and allowed to solidify.

3.2.3 Isolation of *A. niger*

Procedure:

Nine deteriorated groundnut seeds were soaked in 10% Sodium hypochlorite for 2 minutes in a glass Petri dish to surface sterilize them and then were rinsed thrice with sterile distilled water (Salako and Anjorin, 2012). The seeds were blot dried on a sterile paper towel, and then a flame sterilized forceps was used to pick the seeds and plated on sterilized Petri dishes. The Petri plates were incubated at a temperature of 28^{o}C in an incubator for 2 days to observed growth of the fungi.

3.2.4 Lactophenol cotton blue staining of *A. niger*

The *Aspergillus niger* was identified with Lactophenol cotton blue staining. It stains the fungal cytoplasm and provides a light blue background against which the wall of hyphae was seen clearly.

Procedure:

A drop of Lactophenol cotton blue was placed on a clean glass slide and a small tuft of the mycelium from the fungal culture was removed and placed in the drop of Lactophenol using a flame sterilized teasing needle; the mycelium was spread very well on the slide with the aid of the inoculating needle. Then over the preparation, a cover slip was gently applied with little pressure to avoid the trapping of air bubbles in the stain. The slide was then mounted and examined under a light microscope (x10 and x40), and the colony morphological characteristics of *Aspergillus niger* were identified

by their sporulating structures, colour, size and shape and compared to standard *Aspergillus niger* structure (Barnett and Hunter, 2006; Salako and Anjorin, 2012).

3.2.5 Sub-culturing of *A. niger*

Procedure:

Inoculating needle was sterilized by flaming in the blue portion of spirit lamp until it is red and dipped in alcohol to cool, and was used to carefully transfer a tuft of the isolated *Aspergillus niger* on to a sterile PDAS plates by point inoculation. Pure cultures were maintained by sub-culturing onto freshly prepared PDAS routinely, and kept at 28^{o}C in an incubator as a stock culture.

3.3 Proximate Analysis of Cassava Starch

Proximate composition of the cassava starch used for the study was performed using standard methods described by AOAC (2010), in Biochemical Laboratory Unit of the Department of Animal Science, Faculty of Agriculture, ABU-Zaria.

3.3.1 Determination of moisture content

The moisture of the sample was lost by volatilisation caused by heat. The amount of material left after oven drying the sample to a constant weight, is the dry matter.

Procedure:

A clean aluminium dish was dried in an oven at 105^{o}C for one hour, and placed into desiccators to cool and weighed (W_1). The dish was handled with metal tongs, and then about 40 grams of the sample was weighed and put into the weighed dish and reweighed (W_2), then placed in an oven at 105^{o}C overnight to dry to a constant weight. The dish

and sample was cooled in a desiccator and weighed (W_3) quickly as possible to prevent moisture absorption.

The moisture content was calculated as

$$\%\text{Moisture} = \frac{(\text{wt of dish + sample before drying}) - (\text{wt of dish + sample after drying}) \times 100}{\text{Wt sample before drying (g)}}$$

$$= \frac{\text{Loss in weight on drying (g) x 100}}{\text{Wt sample before drying (g)}} = \frac{(W_2 - W_3) \times 100}{W_2 - W_1}$$

Triplicate determinations were carried out on the sample.

The %Dry Matter was calculated as 100 – % Moisture content.

3.3.2 Determination of ash

Principle:

The sample was ignited at 600^{o}C in a Muffle Furnace to turn off all organic materials. The inorganic material which does not volatilise at that temperature is called ash.

Procedure:

A clean crucible was placed into a Muffle Furnace at 600^{o}C for one hour. The crucible was transferred from the furnace to a desiccator and cooled to room temperature and weighed (W_1). A 2.0 g portion of the sample was put into the crucible and reweighed (W_2). Then the crucible and its content were placed into a muffle furnace at a temperature of 600^{o}C overnight to obtain whitish-grey ash. Thereafter, the crucible was transferred into a desiccator and cools to room temperature and weighed as quickly as possible to prevent moisture absorption (W_3).

The %Ash was calculated as

$$\%\text{Ash} = \frac{\text{Wt of Ash} \times 100}{\text{Wt of sample}} = \frac{(W_3 - W_1) \times 100}{W_2 - W_1}$$

Triplicate determinations were performed on the sample and the mean value recorded.

3.3.3 Determination of ether extract (oil)

Principle:

The sample was extracted with light Petroleum, boiling range of 40-60^{o}C, the solvent was distilled off and the dried extract was weighed.

Procedure:

Approximately 0.5 g of the dried sample was weighed and wrapped in a filter paper and placed into the extraction thimble. The thimble was placed inside the Soxhlet extractor; dried, tarred solvent flask was placed in position beneath, with about 180 cm^3 of solvent and connected to the condenser. The heating rate was adjusted to give a condensation rate of 3 drops/second and extraction was carried out for 6 hours. The thimble was removed and the ether reclaimed using the apparatus. The completion of the removal of ether was on a boiling water bath and dry flask at 105^{o}C for 30 minutes, allowed to cool in a desiccator before weighing to constant weight. Triplicate determinations were carried out on the sample.

The extractible lipid was calculated as:

$$\% \text{ lipid} = \frac{\text{Wt of lipid extracted (g)} \times 100}{\text{Wt of sample (g)}}$$

3.3.4 Determination of crude protein

Principle:

The sample was digested in sulphuric acid (H_2SO_4) to breakdown organic matter and reduces nitrogenous compounds to ammonium compounds. Ammonia was liberated by boiling with sodium hydroxide; the steam was distilled into boric acid solution and determined titrimetrically.

During the digestion phase, tablets containing potassium sulphate and mercuric oxide were added. The potassium sulphate raises and controls the temperature of the reaction, while the mercuric oxide acted as a catalyst. During the digestion process, the following reaction occurs

$$CH_3NH_2 + 4H_2SO_4 \longrightarrow CO_2 + 3SO_2 + 4H_2O + NH_4HSO_4$$

For the distillation phase, the following reaction occurs

$$NH_4HSO_4 + 2NaOH \longrightarrow NH_3 + Na_2SO + 2H_2O$$

Procedure:

About 0.5 g of the sample was weighed and put into 100 cm^3 Kjeldahl digestion flask. 30 cm^3 of concentrated sulphuric acid and two kjeltabs were added into the flask. The content in the Kjeldahl digestion flask was heated gently by means of electric element and then vigorously for 2 hours with intermittent swirling of the flask to ensure even digestion and prevent over heating of the content. At this stage of digestion, the reaction mixture turns black due to the dehydrating action of the sulphuric acid and formation of free carbon. Thereafter, it was allowed to cool and 10 cm^3 of distilled water was added to dissolve the crystals that were formed and poured into a 100 cm^3 volumetric flask and the volume was brought to 100 cm^3 with distilled water. A 10 cm^3 aliquot of the diluted solution was pipetted into a Markham distiller and 10 cm^3 of 40% Sodium hydroxide (NaOH) solution added.

The solution was distilled and the liberated ammonia trapped in a 100 cm^3 conical flask containing 10 cm^3 of 4% boric acid and 2 drops of methyl red indicator, distillation was continued until the indicator colour changes from pink to green. When about 50 cm^3 of the distillate was collected in the green boric acid and indicator solution, the distillate

was then titrated with 0.02 N Sulphuric acid which gave a grey-mauve end point. The volume of the acid used as well as the blank was noted.

%Total nitrogen was calculated as

$$\%N_2 = \frac{(\text{Sample titre} - \text{blank titre}) \times \text{Normality of acid} \times 1402 \times 10 \times 100}{\text{Weight of Sample (g)} \times \text{aliquot}}$$

%Crude Protein: $\%N_2$ x 6.25, where 6.25 is a conversion factor and triplicate determinations were carried out on the sample.

3.3.5 Determination of crude fibre

Principle:

It involves sequential treatment of the sample with boiling solutions of Sulphuric acid and Sodium hydroxide. The residue was separated by filtration, washed, dried, weighed and ached. The loss of weight resulting from aching corresponds to the crude fibre present in the sample.

Procedure:

Approximately 2.0 g of the sample was weighed (W_1) and put into a 600 cm^3 beaker and 200 cm^3 of 0.128 N Sulphuric acid solution was added and the mixture boiled for 30 minutes with the use of labconco heating mantle. The hot solution was quickly filtered and the insoluble matter washed with hot water several times to ensure that it was free of acid. The residue was transferred into a beaker and 200 cm^3 of hot Sodium hydroxide solution was added, and the mixture boiled for another 30 minutes and the solution was filtered quickly, washed with hot water until it was free of base. The insoluble residue obtained was then washed with acetone and collected into a clean crucible and dried in an oven at 105^oC to constant weight.

The dried residue was weighed in a crucible (W_2) as quickly as possible, after cooling in a desiccator to avoid moisture absorption. The crucible and its content were incinerated in a Muffle Furnace to ash at 600^{o}C for 3 hours. The crucible and ash content was allowed to cool in a desiccator and reweighed (W_3). The fibre content was calculated as

$$\%\text{Crude Fibre} = \frac{(\text{Dry wt of residue before ashing} - \text{wt of residue after ashing})(\text{g}) \times 100}{\text{Wt of Sample (g)}}$$

$$= \frac{(W_2 - W_3)\text{x } 100}{W_1}$$

3.3.6 Determination of nitrogen free extract

The nitrogen free extract (NFE) was calculated by difference as:

%NFE = 100 – (%CP + %CF + %EE + %Ash)

where,

CP = Crude Protein, CF = Crude Fibre, EE = Ether Extract (Oil)

3.4 Acetate Buffer Preparation

The acetate buffer used in this study was made of acetic acid and sodium acetate. It was prepared according to the method well spelled out in Chandra (2003). Solution A (sodium acetate trihydrate) was prepared by adding 13.60 g of sodium acetate trihydrate ($CH_3COONa.3H_2O$) into 1000 ml of deionized water and solution B (acetic acid) was prepared by adding 5.80 ml of acetic acid (CH_3COOH) into 100 ml of deionized water and then the solution was brought to 1000 ml. Each of the solution is of 0.1 M concentration. The various desired pH values were obtained by adjusting with either 0.1 M of solution A or B.

3.5 Sample (Cassava Starch) Slurry Preparation

The cassava starch was sun dried for seven days and ball milled into fine powder, then sieved with 0.05 mm mesh size to remove undesired particles. A 0.5 gram portion of the cassava starch powder was weighed into a 250 ml conical flask, and then a 100 ml of 0.1 M sodium acetate buffer solution was put into the flask, and swirl to form the slurry. To confirm the presence of starch, few drops of the solution was placed on a glass slide and a drop of 0.01 M iodine reagent was placed on the glass slide. There was a deep blue colouration which infers the presence of starch.

3.6 Enzymatic Treatment of Cassava Starch

A 0.4 g portion of finely ground cassava starch was suspended in 100 ml of 0.1 M acetate buffer of pH 4.4 in a conical flask and swirled for 2 minutes to form starch slurry. The mixture (slurry) was heated in a water bath at a temperature of 67±0.2^{o}C with intermittent swirling for 20 minutes. This was to enable the cassava starch become soluble in the buffer solution and also to destroy the crystalline structure of the starch molecules for ease access by the amylases.

Care was taken not to allow the solution to boil, and thereafter, it was cooled to room temperature under running tap water. Then mycelia mass was scraped aseptically, weighed and added to the mixture and incubated in a water bath at 40±0.2^{o}C. Aliquots of the reaction mixture were withdrawn at regular interval of time to analyze the fermentable sugar (glucose) concentration formed.

3.7 Assays for Glucose Concentration

The reducing sugar (glucose) concentration analysis in all the experimental runs was carried out using a glucose oxidase-peroxidase labkit 'GOD-PAP' following the method described by Trinder (1969). The glucose analysis was carried out using the sample, blank reagent (control), and glucose reagent standard. A 10 µL portion, each of the sample and standard solutions were withdrawn and mixed with 1000 µL of the glucose reagent in separate 1cm path length cuvette, the solutions were kept for 20 minutes at room temperature and absorbance of both the sample and standard were measured after blanking the colorimeter with the buffer at wavelength of 530 nm.

Calculation of glucose concentration (mmol/l):

$$\text{Glucose}\left(\frac{\text{mmol}}{\text{L}}\right) = \frac{A_{\text{Sample}}}{A_{\text{Stardand}}} \times 5.55 \tag{3.1}$$

where,

A_{sample} = Sample absorbance

$A_{standard}$ = Standard absorbance

Standard concentration of glucose (mmol/l) = 5.55

Calculation of glucose yield ($Y_{p/s}$):

$$\text{Glucose Yield } (Y_{p/s})\ \% = \frac{G_t - G_{to}\ (g/l)}{\text{Substrate } (g/l)} \times 100 \tag{3.2}$$

where,

G_t = Glucose concentration at time t

G_{to} = Initial glucose concentration

3.8 Optimisation of Process Conditions

Optimization of various parameters was carried out after the conventional unifactorial approach. In this method, all the process parameters were kept constant except the one under investigation which was varied within a range of values. The unifactorial way of

experimenting, may be very pragmatic and yield satisfactory results, though the effects or interactions of the various parameters were not considered at the same time.

3.8.1 Effect of culture age (*Aspergillus niger*) on cassava starch hydrolysis

The process parameters namely: temperature, pH, substrate concentration and mycelia mass were maintained at (40±0.2^{o}C), 4.4, 0.4 g/l and 0.1 g respectively. The hydrolysis reaction was performed by varying the culture age from 2 to 6 days. The plot of product formed (glucose) concentration against time (in days) was used to determine the most viable age of the fungi, which was used for further study.

3.8.2 Effect of mycelia mass loading on cassava starch hydrolysis

The cassava starch slurry was hydrolyzed by using different mycelia concentrations ranging from 0.05 to 0.30 g/l, while other process parameters were maintained at pH 4.4, temperature (40±0.2^{o}C), substrate (cassava starch) concentration 0.65 g/l and culture age of 5 days old, and the product concentration (glucose formed) was determined.

3.8.3 Effect of pH on *A. niger* amylases activity

Effect of pH on the *A. niger* amylases activity was investigated at different pH values ranging from 3.6 to 5.6 using sodium acetate buffer solution, with all other process parameters such as cassava starch concentration, mycelia mass, temperature and culture age held constant at 0.4 g/l, 0.1 g, 40±0.2^{o}C and 5 days old, and the amount of sugar (glucose) formed was evaluated.

3.8.4 Effect of temperature on *A. niger* amylases activity

The effect of temperature on the rate of enzymatic hydrolysis of cassava starch was assayed at different incubation temperatures ranging from 30 to 70^{o}C, while other process variables such as pH, mycelia mass, culture age and cassava starch concentration were kept constant at 4.4, 0.1 g, 5 days old and 0.4 g/l, and the reducing sugar (glucose) formed was determined.

3.8.5 Effect of substrate concentration on *A. niger* amylases activity

The effect of substrate concentration on the rate of enzymatic hydrolysis of cassava starch was studied at different concentrations ranging from 0.3 to 1.5 g/l while temperature, pH, mycelia mass and culture age were kept constant. The plot of product (glucose) formed against time was subsequently used to determine the initial reaction velocity, $r_{o.}$

CHAPTER 4

RESULTS AND DISCUSSION

In this chapter, results obtained from the isolation of the fungi and investigation of the effect of *Aspergillus niger* amylases on cassava starch hydrolysis under different process variables such as culture age, mycelia mass loading, pH, temperature and substrate concentration are presented and discussed.

4.1 Macroscopic Observation of *A. niger*

The growth of *Aspergillus niger* was monitored on a PDAS at 28^{o}C. The isolate grew rapidly on the PDAS, producing white floccose mycelia which turned yellow first and then black with profuse production of black conidia (spores). The reverse colour changed from white to cream, and then pale yellow and growth produce radial fissures in the agar. Plate I is a photomicrograph of matured colonies of the isolate.

4.2 Microscopic Observation of *A. niger*

The morphological characteristics of *Aspergillus niger* was observed as shown in Plate II based on unique characteristics of the organism such as the conidial head, presence of hyphae, conidia ornamentation, conidia and conodiophores. Confirmation of the fungi (*A. niger*) was made following a close comparison with the standard on Plate III.

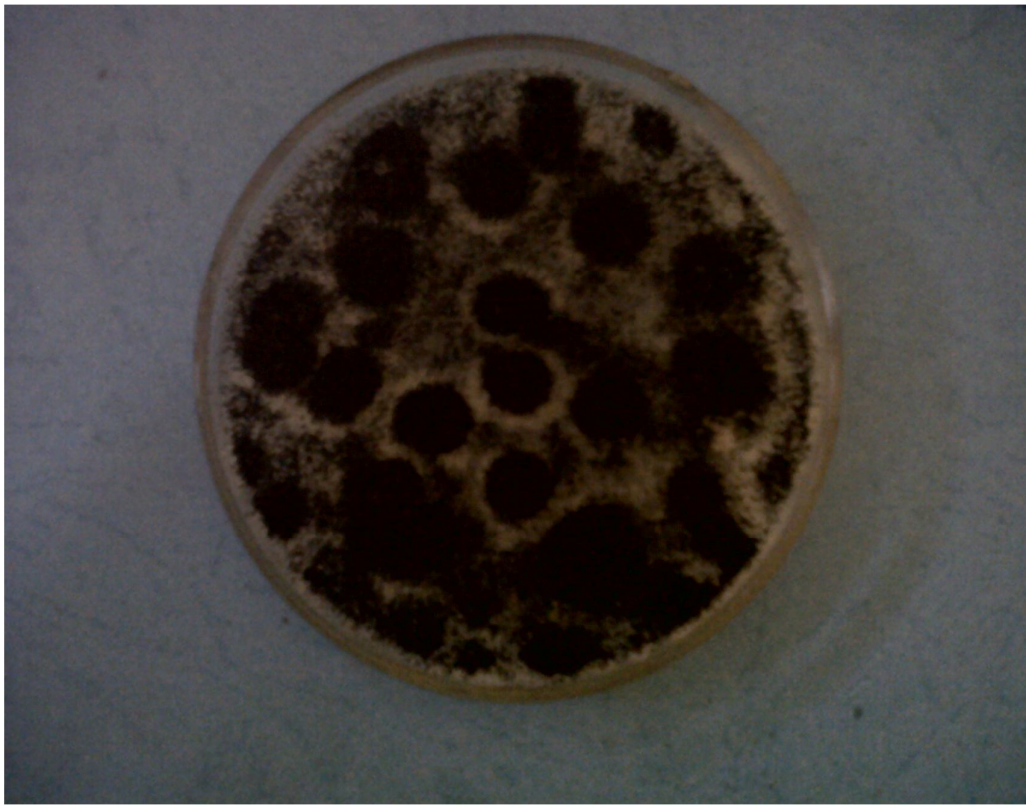


Plate I: Photograph of *Aspergillus niger* colonies

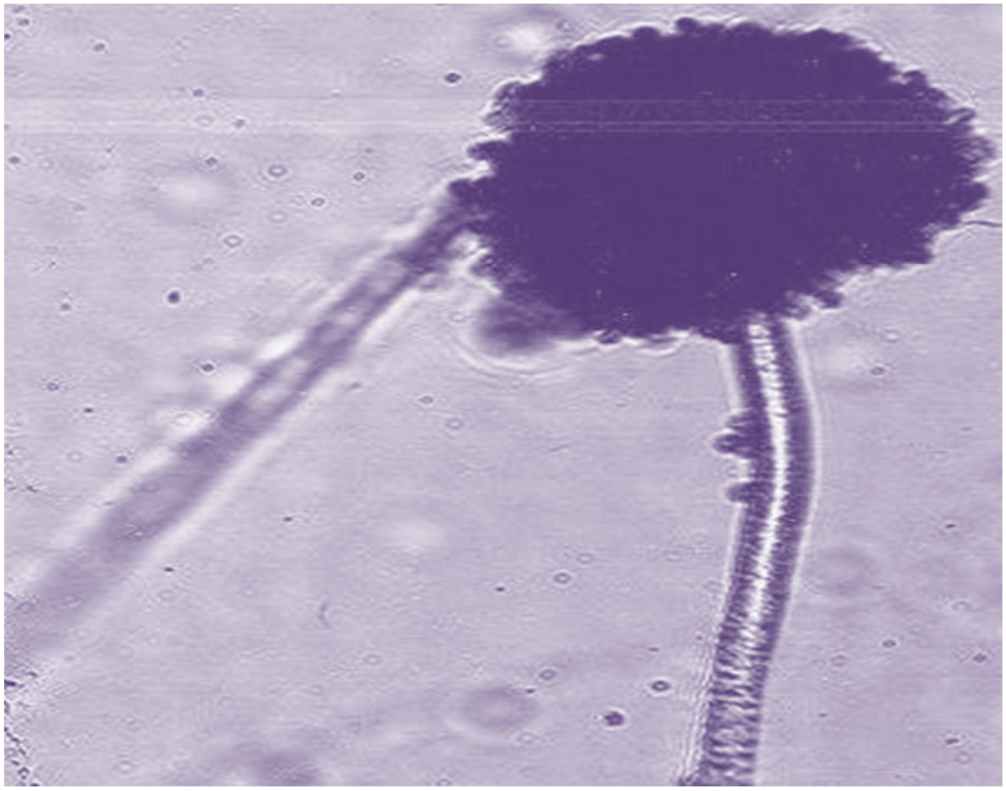


Plate II: Photomicrograph of *A. niger,* (x400), Lactophenol cotton blue staining



Plate III: Standard structure of *A. niger* **(**Barnett and Hunter, 2006)

4.3 Proximate Composition of Cassava Starch

The percentage proximate composition of the sample is shown in Table 4.1. The Nitrogen Free Extract (NFE) was found to be (96.10±0.35%) while crude fibre was (0.00±0.00%). Crude protein, oil and ash were found to be (0.64±0.01%), (0.04±0.00%) and (3.21±0.32%) respectively.

4.4 Optimisation of Process Parameters

4.4.1 Effect of culture age on cassava starch hydrolysis

The profile depicted in Figure 4.1, describes the effect of culture age on cassava starch hydrolysis. The hydrolysis was carried out with different culture ages ranging from 2 - 6 days old, while the other process parameters were fixed. From the Figure, it was observed that glucose concentration increases progressively with increase in age of the fungi and attain optimum value for 5 days old culture after 4 hours of reaction time. Further increase in culture age resulted to a decline in the rate of hydrolysis, which suggests that the microbial cultures of the sixth day and above are already suffering from activity lose due to accumulation of toxins or autolysis due to exhaustion of growth nutrients from the PDA. This observation is in agreement with results reported by Chinedu *et al.* (2008); Silva *et al*. (2009) and Prasanna and Raju (2012).

4.4.2 Effect of mycelia mass loading on cassava starch hydrolysis

The effect of mycelia mass loading on glucose production was studied using cassava starch as substrate by carrying out the experiment at different mycelia concentrations ranging from 0.05 to 0.30 g/l, keeping the other hydrolysis conditions constant. The result is shown in Figure 4.2. From the Figure, it was observed that increase in mycelia

Table 4.1: Proximate composition of cassava starch (dry basis)

Component	Content (%w/w)
Crude Protein	0.64 ± 0.01
Crude Fibre	0.00 ± 0.00
Oil	0.04 ± 0.00
Ash	3.21 ± 0.32
NFE	96.10 ± 0.35

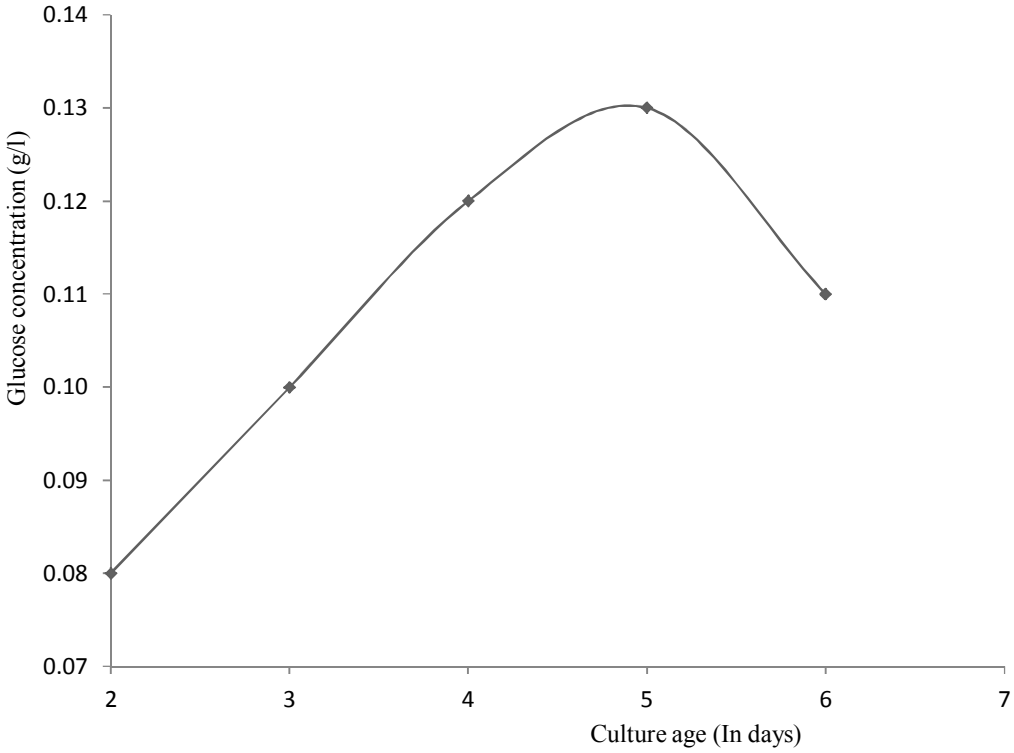


Figure 4.1: Effect of culture age on cassava starch hydrolysis

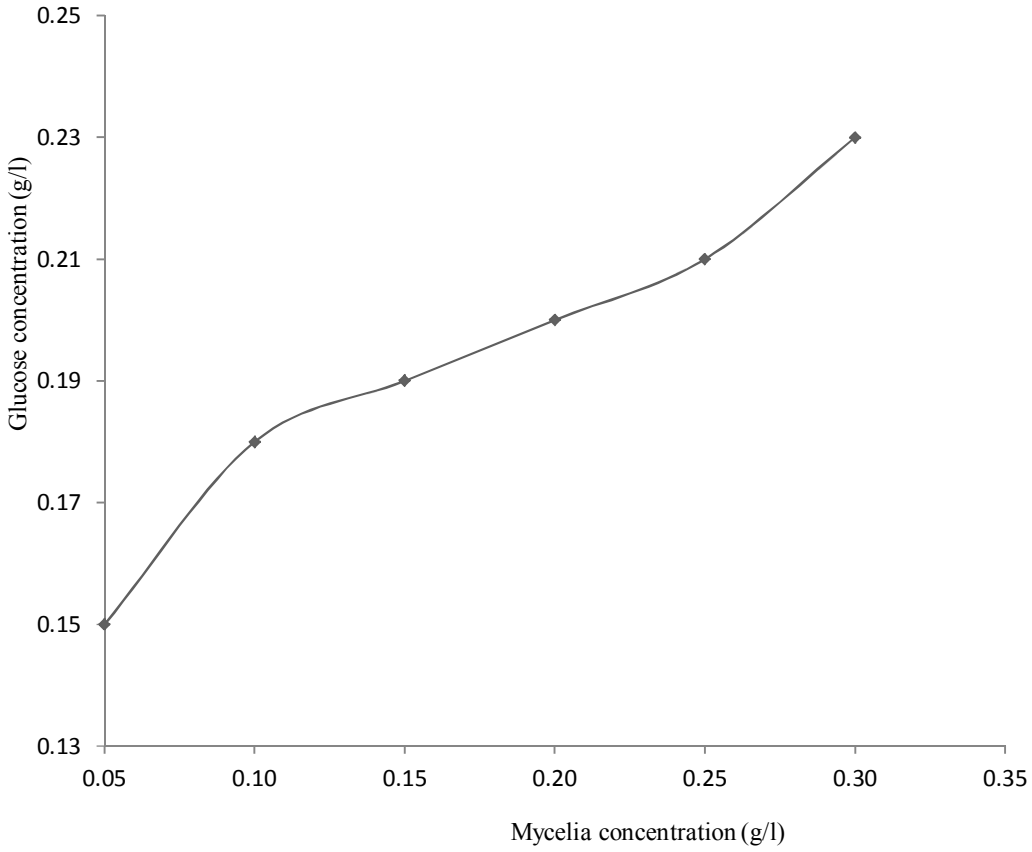


Figure 4.2: Effect of mycelia mass loading on cassava starch hydrolysis

concentration was accompanied by a proportionate increase in glucose concentration within the range of mycelia mass investigated; the reaction was monitored for 5 hours. The increase in glucose concentration observed may be as a result of the continuous secretion of amylases by the fungi into solution for the hydrolysis process. Similar profiles were reported by other researchers for hydrolysis of cassava starch using *Aspergillus niger* (Sarbatly, 2007; Baskar *et al.,* 2008).

4.4.3 Effect of pH on *A. niger* activity

The effect of pH on the activity of *Aspergillus niger* for cassava starch hydrolysis was investigated, and the result is shown in Figure 4.3. Figure 4.3 clearly describes the pH profile, which indicates that the cells perform better in a slightly acidic than basic medium. From the Figure, the best pH for the cells was achieved at 4.8 which gave the highest concentration of glucose (0.2188 g/l) after 5 hours of hydrolysis. Further increase in pH resulted to a decrease in the rate of hydrolysis because of a decline in the activity of the cells this may be due to a distortion of the residual ionic charges on the active site of the amylases. Similar observations were reported (Chotineeranat *et al.,* 2004; Baskar *et al.,* 2008; Ruiz *et al.,* 2011; Yabefa, 2012) at which the highest glucose concentration was obtained between pH 4 and 5.

4.4.4 Effect of temperature on *A. niger* activity

Cassava starch hydrolysis was carried out at various temperatures ranging from 30 to 70^{o}C to study their effect on *Aspergillus niger* activity while other process parameters were fixed. The result is shown in Figure 4.4, it was observed from the Figure that the activity of the cells increase gradually with respect to increase in temperature. The best temperature for the cells activity was achieved at 60^{o}C, which corresponds to maximum

glucose concentration of (0.1893 g/l) after 5 hours of hydrolysis. However, beyond 60^{o}C the temperature profile changed gradually leading to a decrease in the rate of hydrolysis; this may be as a result of the unfolding or disruption of the tertiary structure of the amylases leading to deactivation or denaturation, which is an irreversible loss of the active sites of the amylase. This observation is in agreement with results reported by Chotineeranat *et al.* (2004), Omemu *et al.* (2005) and Baskar *et al.* (2008) at which their highest glucose concentration was obtained between 50 and 60^{o}C.

4.4.5 Effect of cassava starch concentration on *A. niger* amylases activity

The influence of substrate (cassava starch) concentration on the activity of *Aspergillus niger* is depicted in Figure 4.5. The investigation was carried out at various initial substrate (cassava starch) concentrations ranging from 0.3 to 1.5 g/l. The profile of the plot in Figure 4.5 revealed that glucose concentration increases proportionally with increase in initial substrate (cassava starch) concentration. However, it was observed that glucose yield per unit starch experiences a reverse trend (Figure 4.6), falling from a value of about 74 wt% to 18 wt% as initial substrate (cassava starch) concentration increased from 0.3 g/l to 1.5 g/l respectively. This probably explains why most bioprocessing runs at low solid to liquid ratio, apart from special cases of substrate-enzyme inhibition phenomena at high substrate concentration. The trade off for high yield at low substrate concentration is increase in product separation cost. Hence, the overall economy is dictated by the comparative cost between substrate procurement and product post production treatment costs. Similar results were reported from previous works on the effect of substrate on enzyme activity (Nichols and Cholewiak, 1991; Lee, 2002; Chotineeranat *et al.,* 2004; Ghosh and Ray, 2010; Ruiz *et al.*, 2011; Highina *et al.,* 2012).

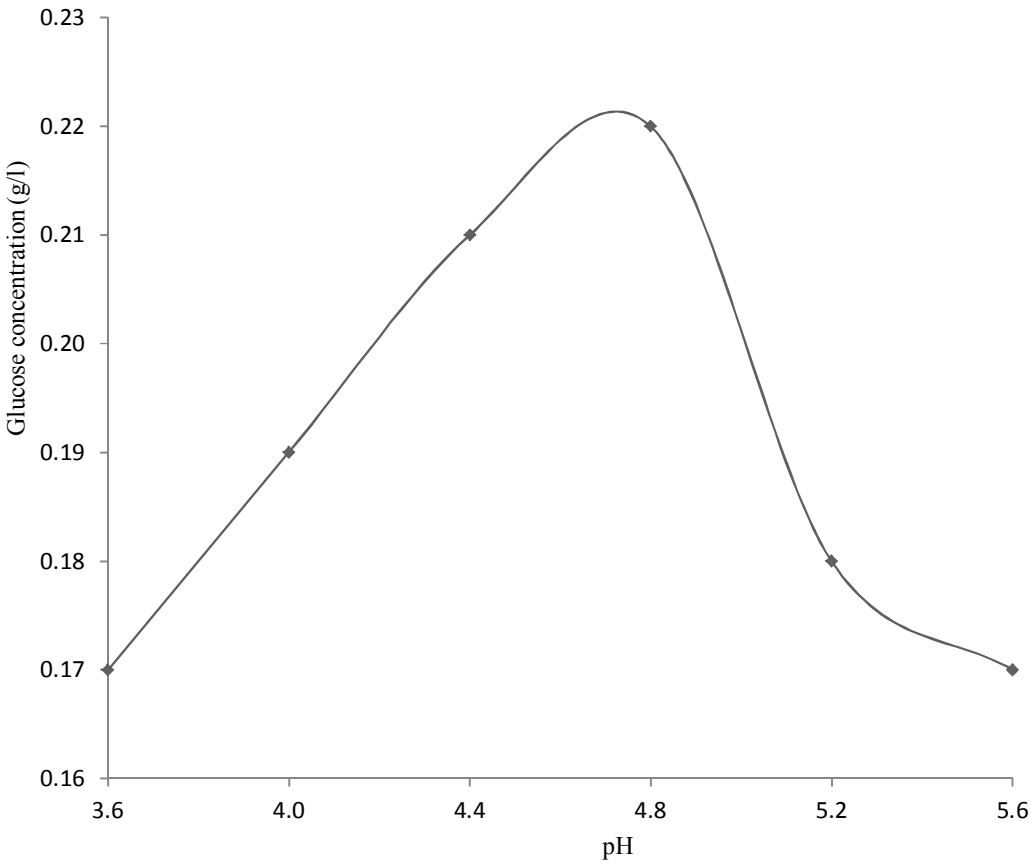


Figure 4.3: Effect of pH on *A. niger* amylases activity

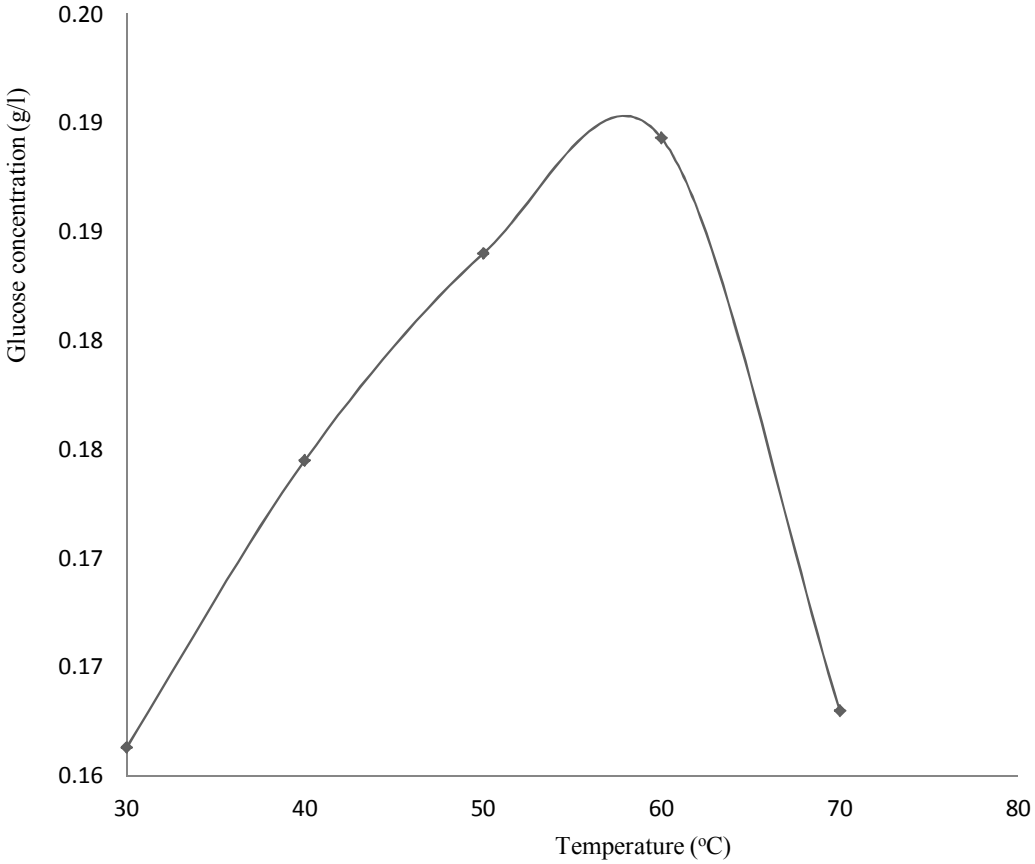


Figure 4.4**:** Effect of temperature on *A. niger* amylases activity

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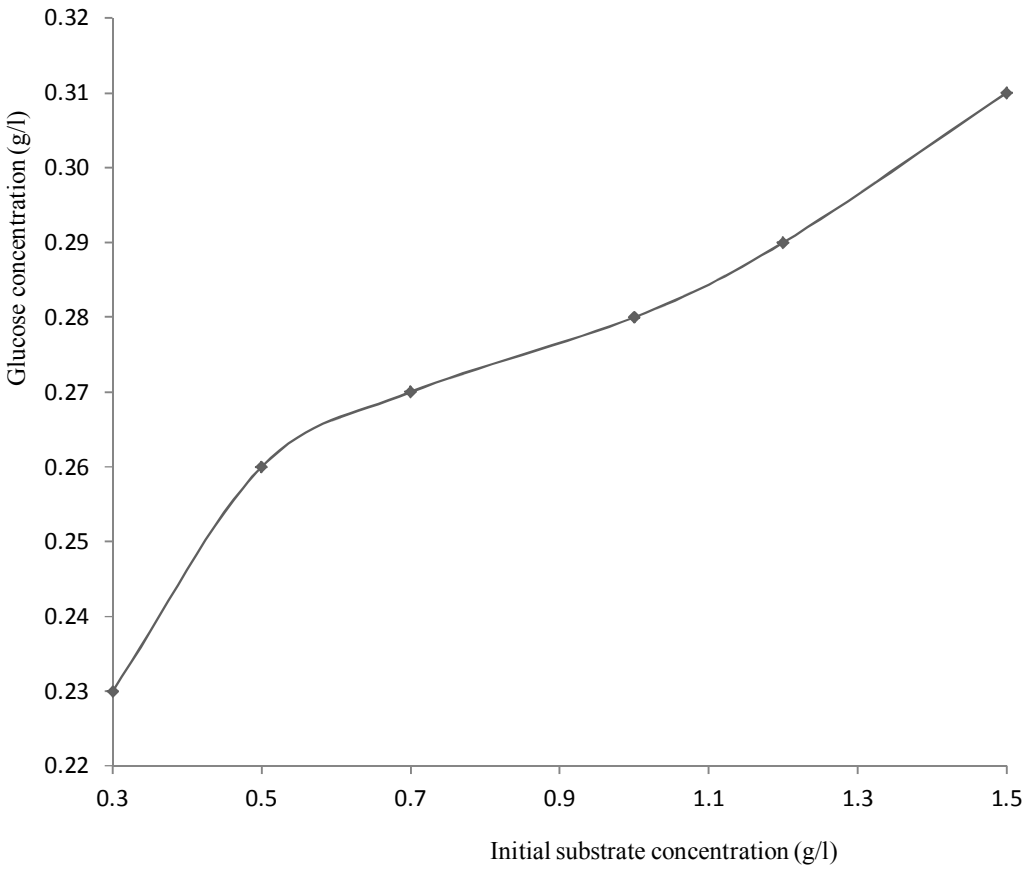


Figure 4.5: Effect of initial substrate concentration on *A. niger* amylases activity

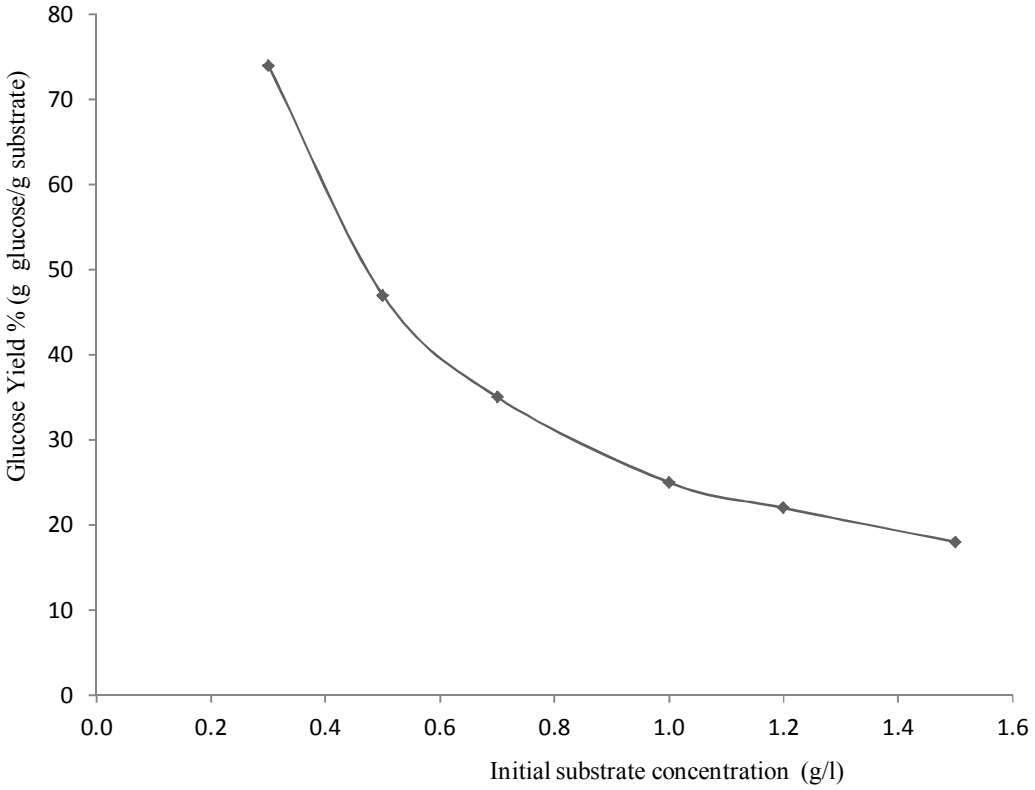


Figure 4.6: Effect of initial substrate concentrations on glucose yield

4.5 Evaluation of Cassava Starch Enzymatic Hydrolysis Kinetic Parameters Based on Michaelis-Menten Kinetic Model

The initial reaction rates (r_o) were determined by running a series of batch experiments with varying initial substrate concentrations [S_o] and the initial rate of product formation was determined for each initial concentration. The initial rates were obtained by drawing a straight line tangent to the concentration-time curves shown in Figure 4.7. The tangent of each curve was calculated (dp/dt) and values of r_o recorded as presented in Table 4.3.

In evaluating the kinetic parameters from the generated experimental data, the following assumptions were made:

- The whole cell (*Aspergillus niger*) produces/secrets α-amylase and glucoamylase as extra cellular components into the reaction media.
- The lump model was adopted.
- The density is assumed to be constant (liquid phase reaction).
- There is no thermal deactivation of the enzymes secreted by the cells within the operating temperature.
- The enzymatic hydrolysis of cassava starch leads to the production of glucose only.
- The temperature and pH in the process vessel is assumed to be constant at optimal values determined previously.
- The exponential growth phase of the *Aspergillus niger* cells were targeted and used throughout the study.
- Liquefaction and saccharification takes place in the same vessel i.e. one stage enzymatic hydrolysis process.

The initial rates evaluated and the initial substrate concentrations were plotted accordingly as shown in Figure 4.8. A typical Michealis-Menten kinetics was fairly depicted i.e. the reaction rate is first order at low substrate concentration, which plateau gradually toward zero-order as the initial substrate concentrations become high.

4.6 Estimation of K_m and r_{max} under Optimal Condition by Linear Methods

The kinetic parameters K_m and r_{max} were evaluated based on the Langmuir form of Equation (2.15) represented as Equation (2.17), because of its best fit correlation (R^2 =0.9939) when compared to that of Lineweaver-Burk or Eadie-Hofstee correlations (shown in Figures 4.10 and 4.11 respectively). Table 4.1 shows the comparison between the three linearized methods employed.

$$\frac{C_{so}}{r_o} = \frac{K_m}{r_{max}} + \frac{C_{so}}{r_{max}} \qquad (2.17)$$

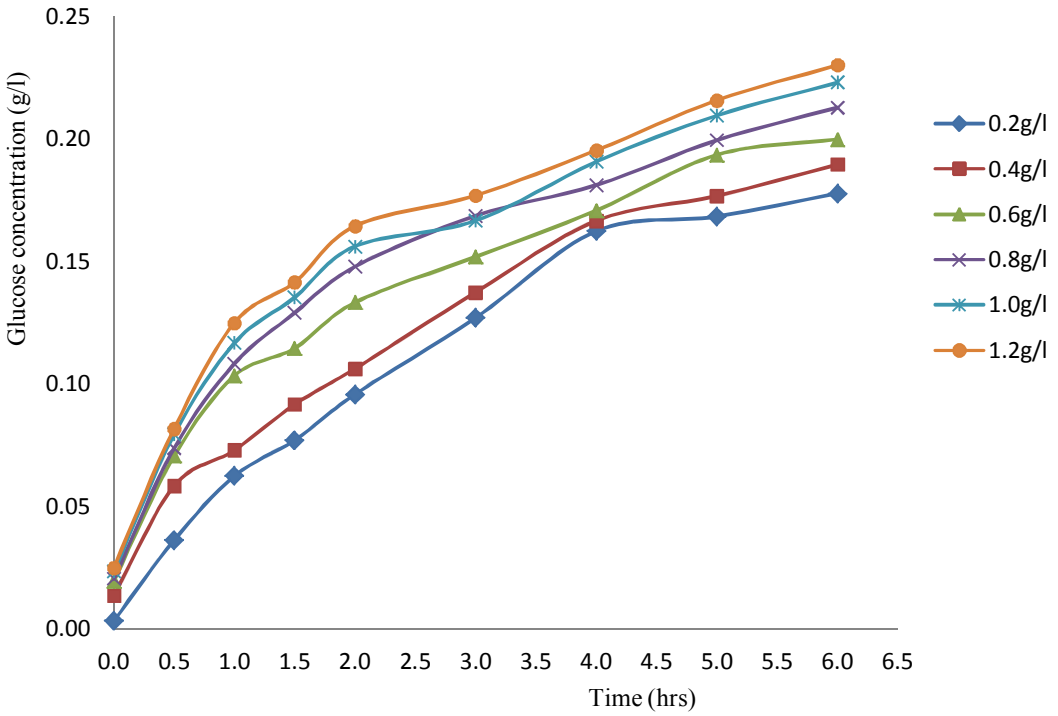


Figure 4.7: Effect of initial substrate concentration on glucose production

Table 4.2: Initial rates (r_o) at different initial cassava starch concentrations (C_{so})

C_{so} (g/l)	$r_o * 10^3$ (g/l.min)
0.2	1.214
0.4	1.524
0.6	1.738
0.8	1.857
1.0	2.071
1.2	2.024

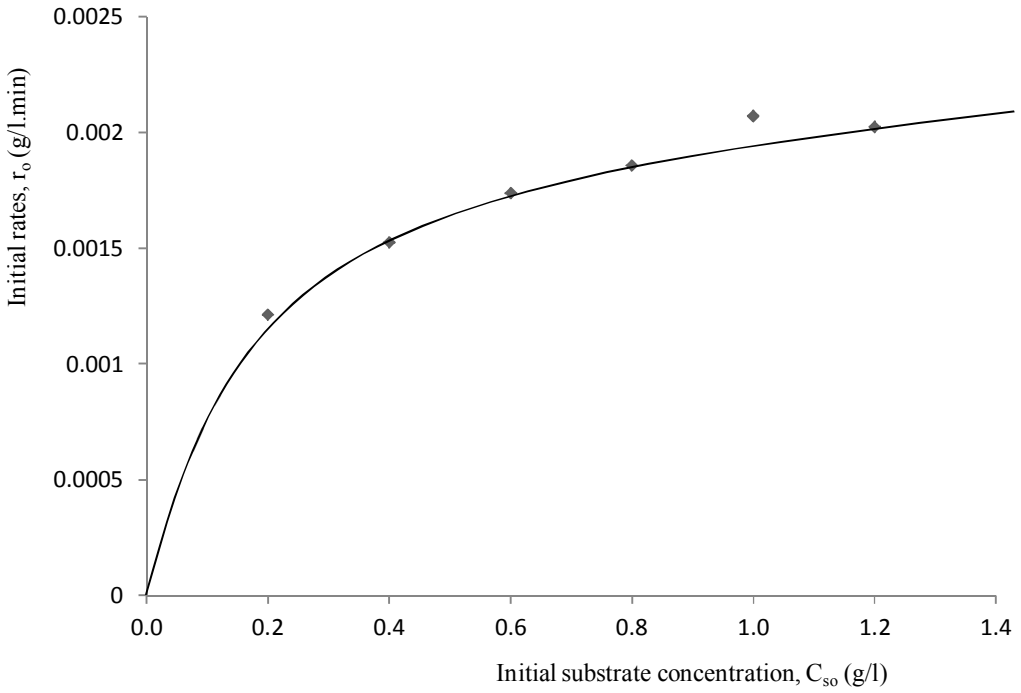


Figure 4.8: Plot of initial rates of reaction versus initial substrate concentrations

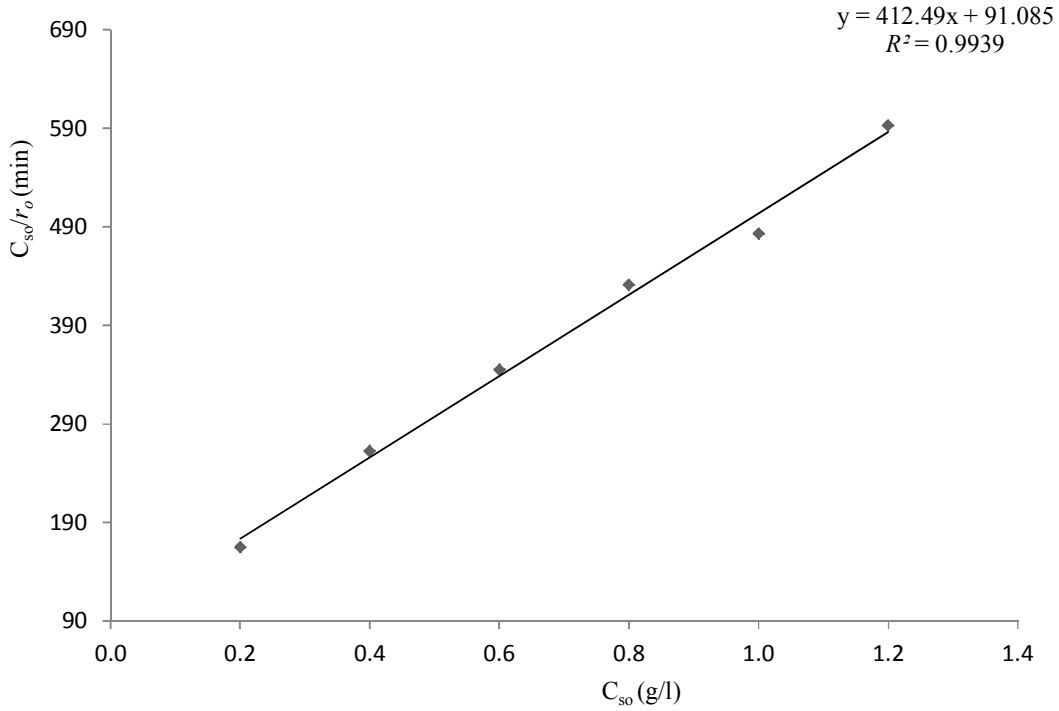


Figure 4.9: Langmuir form of the linearised Michealis-Menten kinetics

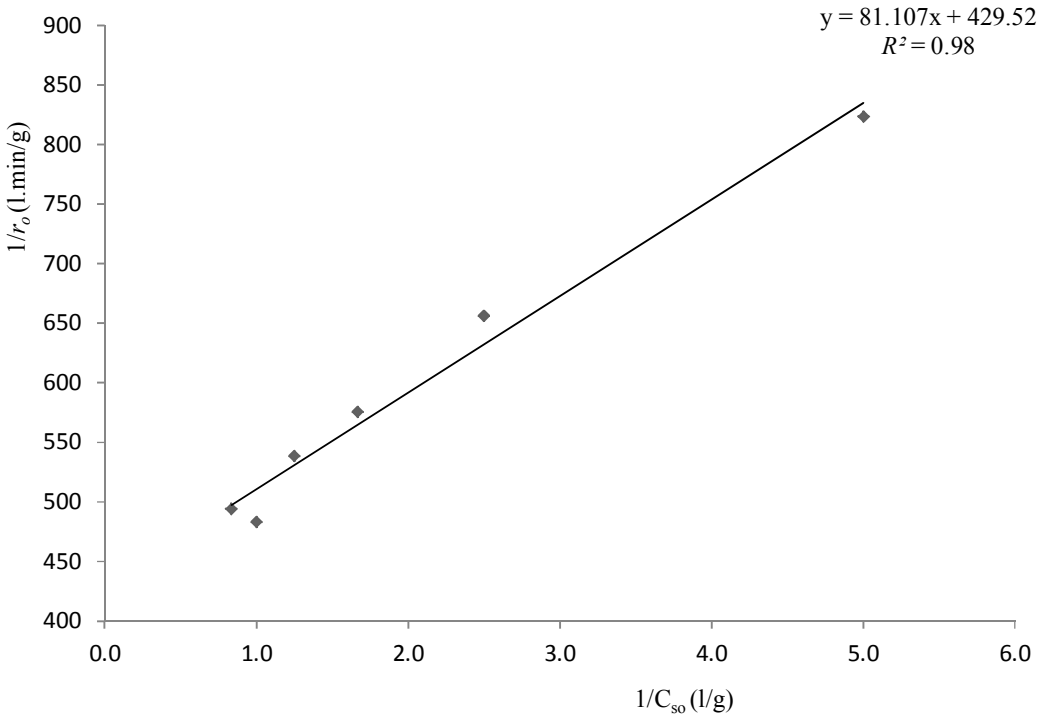


Figure 4.10: Lineweaver-Burk form of the linearised Michealis-Menten kinetics

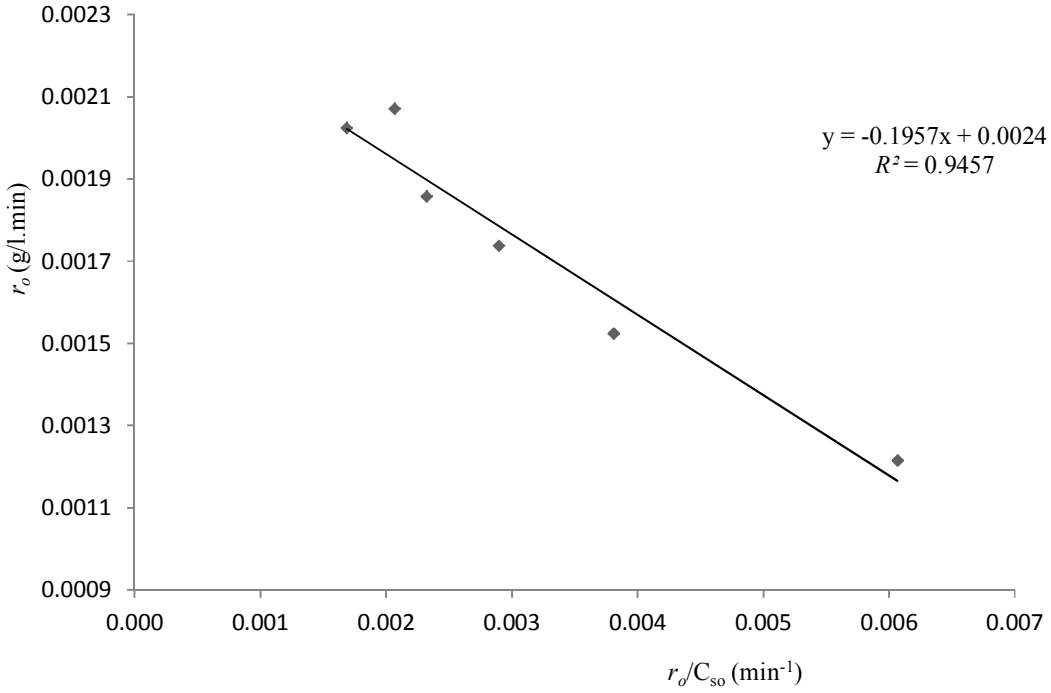


Figure 4.11: Eadie-Hofstee form of the linearised Michealis-Menten kinetics

Table 4.3: Summary of estimated values of K_m and r_{max} by employing linear methods

Methods	R^2	K_m (g/l)	r_{max*10^3} (g/l.min)
Lineweaver-Burk plot	0.9800	0.1888	2.30
Eadie-Hofstee plot	0.9457	0.1957	2.40
Langmuir plot	0.9939	0.2208	2.42

A plot of C_{so}/r_o versus C_{so} yields a straight line with a regression coefficient, R^2 = 0.9939 as shown in Figure 4.9, confirming the appropriateness of Michaelis-Menten kinetics to the study. The K_m and r_{max} values were calculated from the Linear Equation y = 412.49x + 91.085, equating the intercept to K_m/r_{max} and slope to $1/r_{max}$ respectively. The corresponding values obtained for the kinetic parameters (K_m and r_{max}) are 0.2208 g/l and $2.42*10^{-3}$ g/l.min respectively. Obviously, the mycelia used in this study showed better substrate-enzyme binding, K_m = 0.2208 g/l when compared to that of partially purified enzymes employed by Silva *et al*. (2009), acting on a similar substrate (cassava starch) with a corresponding value of 0.48 g/l. However, it is instructive to note that the activity of the mycelia ($r_{max} = 2.42*10^{-3}$ g/l.min) is much lower than that of the partially purified enzymes (1.54 g/l.min). By implication, it will require more mycelia mass to achieve comparative rate of conversion, especially at high substrate concentrations. Nonetheless, knowing that about 80% of enzyme production cost goes to enzyme separation and purification, it is reasonable to assert that using about five hundred fold whole cell quantities in place of purified enzymes is economically beneficial. In addition, the natural environment provided by the presence of the cells (*Aspergillus niger*) to the enzymes, and the possibility of continuous enzyme secretion to ameliorate susceptible enzyme-product inhibition in conventional route cannot be ignored (Aderemi *et al*., 2008). Comparing the findings from this work with other processes which employed amylase from *Aspergillus niger* showed the supremacy of corn starch process (Kyriakides *et al*., 2001) to that of cassava starch, when viewed from kinetic considerations alone (r_{max} = 2.83 g/l.min, K_m = 0.51 g/l to $2.42*10^{-3}$ g/l.min, 0.2208 g/l respectively), however, cassava starch conversion process is of great value to Nigeria economy, considering her relative advantageous position in cassava production over many countries that use corn starch as substrate for glucose production.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The results presented in this work showed a potential use of cassava starch in Nigeria for the production of fermentable sugar (glucose) in single stage enzymatic process. *Aspergillus niger* locally sourced showed desirable biochemical properties such as optimum temperature and pH of 60^{o}C and 4.8 and culture age of 5 days old that can be useful for industrial purposes. It was also observed that glucose concentration increases while the yield decreases with increase in initial substrate concentration. Furthermore, results showed that at a fixed mycelia concentration, the rate of hydrolysis increases proportionally with respect to substrate concentration at low levels and gradually plateau to a constant at high substrate concentration, which is in agreement with typical Michealis-Menten kinetic model for enzyme catalyzed reactions. The values of the kinetic parameters, K_m and r_{max} were found to be 0.2208 g/l and $2.42*10^{-3}$ g/l.min respectively.

5.2 Recommendations

1. Further studies should be done on the use of mycelia dry weight for cassava starch hydrolysis.

2. Multifactorial approach should be employed to study the interactive effects of the various process parameters using the results from this work as default range.

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APPENDICES

Appendix I: Effect of culture age on glucose production

	Culture Age/Glucose Concentration (g/l)				
Reaction Time(hr)	Day 2	Day 3	Day 4	Day 5	Day 6
0.0	0.0204	0.0204	0.0204	0.0204	0.0204
0.5	0.0438	0.0659	0.0734	0.0811	0.0701
1.0	0.0674	0.0723	0.0912	0.0952	0.0835
2.0	0.0742	0.0928	0.1014	0.1073	0.0961
3.0	0.0804	0.1029	0.1112	0.1151	0.1089
4.0	0.0870	0.1064	0.1213	0.1234	0.1193

Appendix II: Effect of mycelia mass loading on glucose production

	Mycelia Mass Loading/Glucose Concentration (g/l)					
Reaction Time (hr)	0.05	0.10	0.15	0.20	0.25	0.30
0.0	0.0209	0.0209	0.0209	0.0209	0.0209	0.0209
0.5	0.0526	0.0736	0.0921	0.1104	0.1315	0.1505
1.0	0.0789	0.0973	0.1081	0.1341	0.1446	0.1630
1.5	0.0922	0.1131	0.1183	0.1262	0.1420	0.1762
2.0	0.1052	0.1288	0.1394	0.1552	0.1624	0.1844
3.0	0.1215	0.1535	0.1656	0.1788	0.1893	0.2025
4.0	0.1396	0.1683	0.1840	0.1998	0.2130	0.2261
5.0	0.1572	0.1814	0.1919	0.1972	0.2103	0.2314

Appendix III: Effect of pH on glucose production

	pH/Glucose Concentration (g/l)					
Reaction Time(hr)	3.6	4.0	4.4	4.8	5.2	5.6
0.0	0.0267	0.0267	0.0267	0.0249	0.0267	0.0267
0.5	0.0471	0.0692	0.0823	0.1148	0.0924	0.0872
1.0	0.0898	0.1134	0.1345	0.1397	0.1187	0.1082
1.5	0.1234	0.1462	0.1656	0.1736	0.1603	0.1393
2.0	0.1420	0.1762	0.1849	0.1919	0.1814	0.1624
3.0	0.1577	0.1893	0.1998	0.2108	0.1788	0.1719
4.0	0.1599	0.1840	0.2051	0.2127	0.1866	0.1745
5.0	0.1724	0.1949	0.2113	0.2188	0.1851	0.1771

Appendix IV: Effect of temperature on glucose production

	Temperature(^{o}C)/Glucose Concentration (g/l)				
Reaction Time(hr)	30	40	50	60	70
0.0	0.0243	0.0243	0.0243	0.0243	0.0243
0.5	0.0789	0.0921	0.1025	0.1052	0.0868
1.0	0.1043	0.1079	0.1183	0.1315	0.0924
1.5	0.1104	0.1262	0.1341	0.1578	0.1131
2.0	0.1201	0.1367	0.1446	0.1595	0.1405
3.0	0.1338	0.1472	0.1656	0.1710	0.1451
4.0	0.1499	0.1581	0.1727	0.1762	0.1604
5.0	0.1613	0.1745	0.1841	0.1893	0.1632

Appendix V: Effect of initial cassava starch concentrations on glucose production

	Substrate/Glucose Concentrations (g/l)					
Reaction Time(hr)	0.3	0.5	0.7	1.0	1.2	1.5
0.0	0.0175	0.0263	0.0316	0.0345	0.0394	0.0473
0.5	0.0421	0.0526	0.0605	0.0657	0.0815	0.0946
1.0	0.0736	0.0868	0.0999	0.1052	0.1104	0.1236
1.5	0.0920	0.1028	0.1130	0.1183	0.1288	0.1367
2.0	0.0973	0.1157	0.1262	0.1341	0.1446	0.1604
3.0	0.1315	0.1499	0.1551	0.1656	0.1709	0.1788
4.0	0.1577	0.1630	0.1735	0.1840	0.1919	0.1893
5.0	0.1683	0.1762	0.1867	0.1972	0.2024	0.2123
6.0	0.1851	0.1945	0.1998	0.2156	0.2287	0.2366
7.0	0.2103	0.2208	0.2340	0.2438	0.2524	0.2712
8.0	0.2261	0.2323	0.2546	0.2600	0.2639	0.2864
9.0	0.2392	0.2615	0.2730	0.2892	0.2995	0.3101

Appendix VI: Effect of initial cassava starch concentrations on glucose yield

	Substrate Concentrations (g/l)/Percentage Glucose Yield					
Reaction Time (hr)	0.3	0.5	0.7	1.0	1.2	1.5
0.0	5.82	5.26	4.52	3.45	3.28	3.06
0.5	8.35	5.60	4.12	3.12	3.50	3.15
1.0	18.71	12.09	9.75	7.07	5.92	5.08
1.5	24.84	15.47	11.65	8.36	7.48	5.88
2.0	26.61	17.87	13.51	9.97	8.76	7.53
3.0	38.01	24.72	17.64	13.10	10.96	8.76
4.0	46.75	27.34	20.27	14.95	12.71	9.58
5.0	50.28	29.98	22.15	16.26	13.57	10.99
6.0	55.87	33.64	24.02	18.12	15.76	12.62
7.0	64.27	38.89	28.89	20.91	17.90	14.94
8.0	69.55	41.20	31.86	21.55	1871	15.93
9.0	73.92	47.03	35.28	25.47	21.67	17.52