PRETREATMENT OF CASSAVA STARCH USING TRONA (AKANWU) FOR ENZYMATIC HYDROLYSIS AND BIOETHANOL PRODUCTION

BY

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TITLE PAGE

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CERTIFICATION

Aniamalu Blessing O., a postgraduate student in the Department of Microbiology, majoring in Industrial Microbiology has satisfactorily completed the requirements of course work and research for the degree of Master of Science (M.Sc.) in Microbiology. The work embodied in this dissertation is original, has not been submitted in part or full for other diploma or degree of this or any other University.

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DEDICATION

I dedicate this research work to the Most Blessed Trinity: Father, Son and Holy Spirit, and also to the Blessed Virgin Mary, whose intercession helped for the accomplishment of my desire.

ACKNOWLEDGEMENT

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ABSTRACT

Alkaline pre-treatment is one of the major chemical pretreatment technologies receiving studies. Pretreatment of cellulose and other fermentable substrates is important because it reduces acetyl and removes uronic acid substitution on hemicelluloses that reduce the accessibility of hemicellulase and cellulase enzymes. The mechanism of action is believed to be the speciation of the intermolecular ester bond, cross linking the xylanhemicelullose and other components e.g. lignin. Cassava waste can be utilised to produce ethanol due to its high cellulose and hemicellulose contents. In this study, cassava pretreatment using potash and enzymatic hydrolysis to convert starch into fermentable sugars was investigated. Optimization of the fermentation conditions for the production of bioethanol from cassava was carried out by simultaneous hydrolysis and fermentation using *Aspergillus niger*and *Saccharomyces cerevisiae* respectively. The starch content of the cassava, starch concentration, glucose and ethanol concentration were 32%, 62g/l, 0.5g/l and 37.32% respectively. The results showed that the variation of the following parameters: potash concentration, duration of gelatinization, pHand inoculum sizes of yeast cell has significant effects on bioethanol production. Under optimized conditions, the maximum yield of bioethanol was 37.32% obtained from 10% starch at pH 4, 10 minutes of gelatinization, while the control experiment gave 12.7% of bioethanol. The implication of this result therefore, is that a relative measure of potash is required to enhance the maximum production of ethanol. Pretreatment of cassava starch using potash for enzymatic hydrolysis and bioethanol is a preferable alternative to acidic pretreatment because potash is relatively cheap, readily available and environmental friendly.

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CHAPTER ONE

1.0 INTRODUCTION

With increasing demand for energy, continuous depletion of the world fossil energy reserves, instability in the global oil price, and various environmental problems such as climate change, global warming, soil, water, and air pollution associated with the use of fossil fuel, there is an increasing pressure for the development of renewable and sustainable alternative sources of energy. Bio-energy (energy from biomass materials) has very good potential as a substitute for the fossil fuels because they are renewable, sustainable and environmental friendly. Bioethanol is a good alternative to fossil fuel. It is now the most important renewable fuel in terms of volume and market value (Lincht, 2006). The raw material used for the production of bio-ethanoldepends on the region/country. One of the ways of producing ethanol is through fermentation of crops which are rich in sugars or starch such as sugar cane, sugar beet, sweet sorghum, corn and cassava (Abouzeid *et al*., 1983). Whether food crops or non-food crops are used, the net result is that there will be competition for farm land and agricultural input. It is widely acknowledged that the rate and extent of bioconversion of substrates is influenced not only by the effectiveness of the enzymes but also by the chemical, physical and morphological characteristics of the heterogeneous lignocellulosic substrate. The initial characteristics of the woody biomass and the effect of substrate pretreatment play a significant role in the development of substrate properties, which in turn govern the efficacy of enzymatic hydrolysis (Mohammed *et al*., 2008).

Lignocellulosic material pretreatment method is applied for improved production of ethanol from cassava which has high fibre content. Enzymatic hydrolysis of cassava fibre without pretreatment is usually not so effective because of high stability of the material to enzymatic or bacterial attacks (Sewalt *et al*., 1997). Pretreatment refers

to the solubilisation and separation of one or more of the four major components of biomass (hemicelluloses, cellulose, lignin and extractives) to make the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). Pretreatment results in enrichment of the difficult biodegradable materials, and improves the yield of ethanol and biogas from the wastes.

1.1 Statement of Problems

With the depletion of world energy reserves, instability of global oil prices and environmental related issues, bioethanol is considered to be among the most promising alternative to petroleum. Presently separating the carbohydrate and lignin components of substrates as well as disrupting the plant cell wall barriers constitutes a serious challenge to enzymatic hydrolysis during production of bioethanol.

1.2 Aim of Study

The aim of study is to determine the quantity of ethanol produced per unit time after pretreating the cassava with trona.

1.3 Research objectives

The objectives of this study are:

1. To evaluate the use of traditionally processed potash in cassava hydrolysis as a pre-step towards easy utilization for industrial scale production of bioethanol.
2. To study the effect of potash concentration, cassava starch concentration, pH, duration of gelatinization and inoculums concentration on ethanol production.

CHAPTER TWO

2.0 LITERATURE REVIEW

Tubers are part of the rhizome thickened for use as a storage organ. They are equally referred to as swollen region of an underground stem or root usually modified for storing food (Onwueme, 1978). Thus tubers are grouped into two; the stem tubers (e.g. potato) and root tubers (e.g. cassava). Cassava (*Manihotesculanta*) is a starch containing root crops of worldwide importance as food, feed, and non-food product. It is the shrubby, tropical, perennial plant that is not well known in temperate zone. For most people, cassava is most commonly associated with tapioca. It is the second most important staple. The edible parts are the tuberous root and leaves. The tuber (root) is somewhat dark brown in colour and grows up to two feet long. Cassava is a heat-loving plant that requires a minimum temperature of 26.67^{o}C to grow (Onwueme, 1978). Around the world, cassava is a vital staple for about 500 million people. Cassava's starchy roots produce more food energy per unit of land than any other staple crop. Its leaves, commonly eaten as a vegetable in parts of Asia and Africa, provide vitamins and proteins. Nutritionally, cassava is comparable to potatoes, except that it has twice the fibre content and high level of potassium (Hguyen *et al*., 2007). Cassava can be used as vegetables in dishes, grated to make pancakes, dried and ground into tapioca flour, or sliced and made into snack chips.

2.1 Cassava as a Bio-fuel Crop

Many characteristics of cassava such as high drought and heat tolerance, little requirement for agricultural fertilizers and high starch content make it one of the most attractive plants for starch production in the future. Apart from its traditional role as food crop, cassava is likely to increase its value by becoming an important bio-fuel crop. The high yield of starch and total dry matter in spite of drought

conditions and poor soil, together with low agro-chemical requirements, result in an energy input that represents only 5-6% of the final energy content of the total cassava biomass. This translates to an energy content in the total biomass (Hguyen*et al*., 2007). A direct comparison of bio-ethanol production from different energy crops was reviewed by (Wang, 2002). The conclusion was that cassava compared favourably to other crops such as maize, sugar cane and sweet sorghum. The energy conversion efficiency from cassava starch to bioethanol is still low. The average content of starch in cassava fresh storage is 24% - 32%. With current technology about 6.6 tonnes of fresh storage root may be converted into 1 tonne of ethanol (Song *et al*., 2008).

2.2 Starch and its Uses

Starch or amylum is a carbohydrate consisting of a large number of glucose units joined together by glucosidic bonds. This polysaccharide is produced by all green plants as an energy store. Most common carbohydrate in the pure human diet is contained in large amounts in such staple foods such as potatoes, wheat, maize (corn), rice and cassava. Pure starch is white, tasteless and odourless powder that is insoluble in cold water or alcohol. It consists of two types of molecules; the linear and helical amylose and the branched amylopectin.

Depending on the plant, starch generally contains 20 to 25% amylose and 75 to 80% amylopectin (Brown *et al*., 2005). Glycogen, the glucose store of animals, is a more branched version of amylopectin. Starch is processed to produce many of the sugars in processed foods. Dissolving starch in warm water gives white paste that can be used as a thickening, stiffening or gluing agent. The biggest industrial non-food use of starch is as adhesive in papermaking process. The word starch is derived from Middle English Steichen, meaning to stiffen. “Amylum” is Latin word for starch,

from the Greek word “amylon” which means not ground at a mill. The word amyl is used in biochemistry for several compounds related to starch (Brown, and Poon, 2005).

Starch molecules arrange themselves in the plant in semi crystalline granules. Each plant species has a unique starch granular size: rice starch is relatively small (about 2µm) while potato starches have larger granules (up to 100µm). Although in absolute mass only about one quarter of the starch granules in plants consists of amylose, there are about 150 times more amylose molecules than amylopectin molecules. Amylose is a much smaller molecule than amylopectin molecules (Anne- Charlotte, 2004). Starch becomes soluble in water when heated. The granules swell and burst, the semi-crystalline structure is lost and the smaller amylose molecules leaching out of the granules, forming a network that holds water and increasing the mixture viscosity. This process is called starch gelatinization. During cooking, the starch becomes a paste and increases further in viscosity. During cooling or prolonged storage of the paste, the semi-crystalline structure partially recovers and the starch paste thickens, expelling water. This is mainly caused by the retro gradation of the amylose. This process is responsible for the hardening of bread or staling, and form the water layer on top of a starch gel (syneresis). Some cultivated plant varieties have pure amylopectin starch without amylose, known as waxy starches. The most used is waxy maize; others are glutinous rice and waxy potato starch. Waxy starches have less retro gradation, resulting in a more stable paste. High amylose starch, amylomaize, is cultivated for the use of its gel strength. If starch is subjected to dry heat, it breaks down to form pyrodextrins, a process known as dextrinization. Pyrodextrins are brown in colour. This is partially responsible for the browning of toasted bread.

Paper making is the largest non-food application of starches globally, consuming millions of metric tonnes annually (Codex Alimentarius, 2001). In a typical sheet of copy paper for instance, the starch content may be as high as 8%. Both chemically modified and unmodified starches are used in paper making. In the wet part of the paper making process, generally called the "wet end",the starches used are cationic and have a positive charge bound to the starch polymer. These starch derivatives associate with the anionic or negative charged paper fibres/cellulose and inorganic fillers. Cationic starches together with other retention internal sizing agents help to give the necessary strength properties for the paper web to be formed in the paper making process (wet strength), and to provide strength to the final paper sheet (dry strength) (Chen *et al.*, 2008). In the dry end of the paper making process, the paper web is rewetted with a starch base solution. The process is called surface sizing. Starch used has been chemically or enzymatically depolymerised at the paper mill or by the starch industry. Starch is also used in paper coating as one of the binders for the coating formulation, a mixture of pigments, binders and thickeners. Coated paper has improved smoothness, hardness, whiteness and gloss, and thus improves printing characteristics. Clothing or laundry starch is a liquid that is prepared by mixing a vegetable starch in hot water, and is used in the laundering of clothes. Starch was widely used in Europe in the 16^{th} – 17^{th} centuries to stiffen the wide collars and ruffs of fine linen which surrounded the neck of the well-todo.

Another large non-food starch application is in the construction industry, where starch is used in the gypsum wall board manufacturing process; chemically modified or unmodified starches are added to the stucco containing primarily gypsum. The starches act as glue for the cured gypsum rock with the paper covering, and also provide rigidity to the board. Starch is used in the manufacturing of various adhesives or glues for bookbinding, wall paper adhesives, school glues and bottle

labelling. Starch derivatives, such as yellow dextrin can be modified by addition of some chemicals to form hard glue for paper work; some use borax or soda ash, which are mixed with the starch solution at 50ºC – 70ºC to create a very good adhesive. Sodium silicate can be added to reinforce those formulae.

2.3 The Importance of Amylase.

Amylase refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) sugars and starch. Amylasedigests carbohydrates (poly-saccharides)into smaller disaccharide units, eventually converting them into monosaccharide such as glucose. People who are fat intolerant (can't digest fats) often eat sugar and carbohydrates to make up for the lack of fat in their diet. All amylases are glycoside hydrolases and act on α-1,4- glucosidic bonds. Amylase enzyme is used in bread making, breaking down complex sugars such as starch (found in flour) into simple sugars. Yeast then feeds on these simple sugars and converts it into the waste product of alcohol and carbon dioxide. This imparts flavour and causes bread to rise. While amylase enzymes are found naturally in yeast cells, it takes time for the yeast to produce enough of these enzymes to break down significant quantities of starch in the bread. This is the reason for long fermented dough such as sour dough.

2.4 Pretreatment

Pretreatment refers to the solubilisation and separation of one or more of the four major components of biomass (hemicelluloses, cellulose, lignin and extractives) to make the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). According to Hu *et al*., 2008,pretreatment constitutes the means to separate the carbohydrate and lignin and, disrupt the crystalline region of these materials. Pretreatment is required for delignification, because lignin in

plant cell walls form a barrier against enzymatic attack (Sewalt*et a*l., 1997).An ideal pretreatment would reduce the lignin content and crystallinity of the cellulose and increase the surface area (Krishna and Chowdary, 2000). Lignin is degraded in nature by various organisms but the mechanism of natural degradation is not known.

2.4.1 Effective parameters in pretreatment of lignocelluloses

The inherent properties of native lignocellulosic materials make them resistant to enzymatic attack. The aim of pretreatment is to change these properties in order to prepare the materials for enzymatic degradation. Since lignocellulosic materials are very complicated, their pretreatment is not simple either. The best method and conditions of pretreatment depend greatly on the type of lignocellulose. For instance, pretreatment of bark from poplar trees or corn leaf with a dilute-acid process seems to be promising, but this method is not effective for treating the bark from sweet gum or corn stalk. The crystallinity of cellulose, its accessible surface area and protection by lignin and hemicellulose, degree of cellulose polymerization, and degree of acetylation of hemicelluloses are the main factors considered as affecting the rate of biological degradation of lignocelluloses by the enzymes. These factors will be discussed briefly below.

2.4.1.1 Crystallinity

The cellulose micro fibrils have both crystalline and amorphous regions, and the crystallinity is given by the relative amounts of these two regions. The major part of cellulose (around 2/3 of the total cellulose) is in the crystalline form. It was shown that cellulase readily hydrolyzes the more accessible amorphous portion of cellulose, while the enzyme is not so effective in degrading the less accessible crystalline portion. It is therefore expected that high-crystallinity cellulose will be more

resistant to enzymatic hydrolysis, and it is widely accepted that decreasing the crystallinity increases the digestibility of lignocelluloses.

On contrary, there are some studies that show more digestibilities of more crystalline lignocelluloses. This conflict in the reports might appear, while the effects of other factors are ignored. Grethlein (1984) pretreated hard-and softwoods by mild acid hydrolysis and determined their pore size distribution. Regardless of the substrate, the initial rate of hydrolysis was shown to be linearly correlated with the pore volume of the substrate accessible to the size of the cellulase. However, it was also shown that the crystallinity index has no relationship to the rate of hydrolysis. Kim and Holtzapple (2006) found that the degree of crystallinity of corn stoves slightly increased from 43% to 60% through delignification with calcium hydroxide, which was related to removal of amorphous components (lignin, hemicellulose). However, an increase in crystallinity of pretreated material did not negatively affect the yield of enzymatic hydrolysis. Fan *et al. (2001)* studied the effect of ball milling on surface area and crystallinity ofcellulose, and they observed an increase in crystallinity of cellulose by reducing the size of cellulose by milling. It is believed that recrystallization during water swelling may increase the crystallinity of highly ball-milled cellulose.

This discussion may indicate that the crystallinity is an important factor in digestibility of lignocelluloses. However, it is not the only factor in effective enzymatic hydrolysis of these materials, due to the heterogeneous nature of celluloses and the contribution of other components such as lignin and hemicellulose.

2.4.1.2 Effect of accessible surface area

Several studies have shown a good correlation between the pore volume or population (accessible surface area for cellulase) and the enzymatic digestibility of lignocellulosic materials. The main reason for improvement in enzymatic hydrolysis

by removing lignin and hemicellulose is related to the cellulose accessible surface area. The effect of this area may correlate with crystallinity or lignin protection or hemicellulose presentation or all of them. Therefore, many researchers have not considered the accessible surface area as an individual factor that affects the enzymatic hydrolysis. The first part of enzymatic hydrolysis consists of: (I) adsorption of cellulase enzymes from liquid phase onto the surface of cellulose (solid), (II) biodegradation of cellulose to simple sugars, mainly cellobiose and oligomers, and (III) desorption of cellulase to the liquid phase(Chang *et al*., 2000). Thus, the reaction is a heterogeneous catalytic reaction and direct physical contact between the cellulytic enzymes' molecules and cellulose is a prerequisite for enzymatic hydrolysis. As a result, the accessible surface area in lignocellulosic material and its interaction with the enzymes can be limiting in enzymatic hydrolysis.

Lignocellulosic materials have two different types of surface area: external and internal. The external surface area is related to the size and shape of the particles, while the internal surface area depends on the capillary structure of cellulosic fibers (Hguyen *et al*., 2007). Typically, dry cellulosic fibers have small size, about 15 to 40 μm, and therefore they possess a considerable external specific surface area, e.g. 0.6–1.6 m^2/g. However, the internal surface area of dried cellulosic fibers is smaller than the external surface area. Swelling of lignocelluloses with water and polar solvents creates a very large internal surface area. Drying of fibers can result in irreversible collapse and shrinking of the capillary and thus reduce the accessible surface area. Presence of water has a significant effect on the specific surface area of natural cellulose. The specific surface area is known to increase with wetting. Water is known to increase the crystallinity of cellulose, due to a re-crystallization of highly amorphous cellulose.

The accessible surface area changes during enzymatic hydrolysis. The rate of hydrolysis is usually very high at first, and then decreases in the later stages. The specific surface area, or accessible surface area per gram of substrate (m^2/g), sharply increases during the initial stage (Abouzied *et al*., 1983). However, it was shown that the cellulose surface area is not a major limiting factor for hydrolysis of pure cellulose. In other words, the slowdown of hydrolysis in the later stages is not due to a lack of associable surface area, but to the difficulty in hydrolysis of crystalline part of cellulose. Therefore, one may expect a lower rate of hydrolysis after hydrolysis of the amorphous cellulose.

2.4.1.3 Effect of lignin

The cellulose and hemicellulose are cemented together by lignin. Lignin is responsible for integrity, structural rigidity, and prevention of swelling of lignocelluloses. Thus, lignin content and distribution constitute the most recognized factor which is responsible for recalcitrance of lignocellulosic materials to enzymatic degradation by limiting the enzyme accessibility; therefore the delignification processes can improve the rate and extent of enzymatic hydrolysis. However, in most delignification methods, part of the hemicellulose is also hydrolyzed, and hence the delignification does not show the sole effect of lignin. Dissolved lignin due to pretreatment of lignocelluloses is also an inhibitor for cellulase, xylanase, and glucosidase. Various cellulases differ in their inhibition by lignin, while the xylanases and glucosidase are less affected by lignin.

The composition and distribution of lignin might also be as important as the concentration of lignin. Some soft woods are more recalcitrant than hardwoods. This might be related to the lignin type, since softwoods have mainly guaiacyl lignin while hardwoods have a mix of guaiacyl and syringyl lignin. It has been suggested

that guaiacyl lignin restricts fibre swelling and enzyme accessibility more than syringyl lignin (Sewalt *et al*., 1997).

In some investigations the inhibitory role of lignin has been related to its effect on cellulose swelling. On the other hand, the swelling can be achieved without removal of lignin, and it does not increase the pore size or the extent of hydrolysis. However, it was shown that lignin still has a significant effect on enzymatic digestibility, even in cases where it no longer prevents fibre swelling. The reason for improved rate of hydrolysis by removal of lignin might be related to a better surface accessibility for enzymes by increasing the population of pores after removing of lignin.

2.4.1.4 Effect of hemicellulose

Hemicellulose is a physical barrier which surrounds the cellulose fibres and can protect the cellulose from enzymatic attack. Many pretreatment methods were shown to be able to remove hemicelluloses and consequently improve the enzymatic hydrolysis. But most of these processes partly remove the lignin as well, so the improvement is not the result of removal of hemicellulose alone. The accessible surface for enzymatic attack may be related to cellulose crystallinity, lignin, and hemicellulose content. Hemicellulose can be hydrolyzed by enzymatic hydrolysis by hemicellulase. However, a suitable pretreatment, e.g. dilute-acid treatment which removes the hemicellulose, eliminates or reduces the need for use of hemicellulase enzyme mixtures for degrading of biomass (Mosier *et al*., 2005).

2.4.2 Pretreatment Methods for Lignocellulosic Wastes.

2.4.2.1 Physical Pretreatment

Physical pretreatment includesuncatalyzed steam explosion, liquid hot water pretreatment (LHW), mechanical and high energy radiation.

2.4.2.1.1 Uncatalyzed Steam Explosion.

This is also called autohydrolysis in which only steam is used. Extensive work has been done on auto hydrolysis and it is one of the commonest pretreatment methods for lignocellulosic biomass (Chandra*et al*., 2008). During steam pretreatment, the biomass is put in a large vessel and steam with a high temperature (up to $24^{O}C$) and pressure is applied for a few minutes. After a set time, the steam is released and biomass is quickly cooled down. The objective of a steam explosion is to solubilise the hemicelluloses to make the celluloses easily accessible for enzymatic hydrolysis and to avoid the formation of inhibitors.

2.4.2.1.2 Liquid Hot Water (LHW) Pretreatment

Hence pressure is utilized to maintain water in the liquid state at elevated temperatures (Rogalinsiki *et al*., 2008). The objective of the LHW is to solubilise mainly the hemicelluloses to make the cellulose better accessible and to avoid the formation of inhibitors. To avoid the formation of inhibitors, the pH is kept between 4 and 7 during the pretreatment. At this pH, formation of monosaccharide are minimized and the formation of degradation products that can further catalyze hydrolysis of the cellulose material during pretreatment (Mosier *et al.*, 2005).

2.4.2.1.3 Mechanical pretreatment

The mode of action is by milling (cutting the lignocellulosic biomass into smaller pieces). The objective of a mechanical pretreatment is primarily to disrupt cellulose crystallinity, decrease the degree of polymerization and increase the specific enzyme surface area of cellulosic biomass by breaking down the biomass into smaller particles, rending the substrate more amenable to subsequent enzymatic hydrolysis (Zheng *et al.*, 2009).

2.4.3 Chemical pretreatment

Chemical pretreatment include catalyzed steam explosion, acid pretreatment, alkaline pretreatment, Ammonia fibre/freeze explosion, (AFEX), organ solvent, pH-chemical pretreatment that have been studied to date with the primary goal of improving the biodegradability of cellulose, by removing the lining and/or hemicelluloses, and to a lesser degree decreasing the degree of polymerization (DP) and crystallinity of the cellulose component (Sewalt *et al*., 1997).

2.4.3.1 Acid pretreatment

The pretreatment can be done with dilute or strong acids. The main objective is to solubilise the hemicelluloses and by this, making the cellulose better accessible. The main reaction that occurs during acid pretreatment is the hydrolysis of hemicelluloses especially xylan as glucomannan, is relatively acid stable (Hu *et al*., 2008).

2.4.3.2 Alkaline pretreatment

This is one of the major chemical pretreatment technologies receiving studies. It employs various bases including sodium hydroxide, calcium hydroxide (lime), potassium hydroxide (KOH), ammonia ($NH_{3(aq)}$) and ammonium hydroxide (NH_3OH) in combination with hydrogen perioxide (H_2O_2) or other bases (Saha and Cotta, 2007). Alkaline pretreatment is basically a delignification process in which a significant amount of hemicelluloses is solubilised as well. The mechanism of action is believed to be specification of intermolecular ester bonds, cross linking Xylan hemicelluloses and other components, e.g. lignin and other hemicelluloses. Alkaline pretreatment also removes acetyl and reduce various uronic acid substitutions on hemicelluloses that reduce the accessibility of hemicelluloses and cellulose enzyme (Chang and Holtzapple, 2000).

2.4.3.3 Ammonic fibre/freeze explosion (AFEX)

AFEX was developed to pretreat biomass with concentrated ammonia. It makes the breakdown of cellulose more efficient. Using enzyme alone, about 15 percent of cellulose is broken down into simple sugars but when AFEX is used before adding enzymes, more than 90 present of the cellulose is broken down. In Ammonia Fibre explosion, the biomass is exposed to hot liquid ammonia under high pressure for a period of time, before the pressure is suddenly released. The swift reduction of pressure opens up the structure of lignocelluloses biomass leading to increased digestibility of biomass. AFEX pretreatment simultaneously delignifies and solubilises some hemicelluloses while decrystallizing cellulose but does not significantly remove hemicelluloses and acid catalysed steam explosion pretreatment (Mes – Harte *et al.*, 1988). Both micro and macro accessibilities of cellulose to the cellulose are affected.

2.4.3.4 pH controlled liquid hot water.

During liquid hot water pretreatment, the pH value of contents in the reactors can usually drop to below it, leading to the inhibitor formation due to the carbohydrate degradation. In order to control pH value of the LHW between 5 and 7, some bases such as KOH are usually added into LHW pretreatment process with its role to maintaining the pH value not as catalyst in alkaline pretreatment. In this method, the base differs in function from chemicals added as catalysts with its role of maintaining the pH value constant above 5 and below 7 in order to minimize hydrolysis to monosaccharide (Zheng *et al*., 2009).

2.4.3.5 Organosolv pretreatment

This is a delignification process with varying simultaneous hemicelluloses solubilisation. In this process, an organic or aqueous organic solvent mixture with or without acid or alkali catalyst is used to break the internal lignin and hemicelluloses bonds. The organic solvents include ethylene glycol, triethylene glycol, tetrahydrofarfuryl alcohol, glycerol, aqueous phenol and aqueous n-butanol. The usual operation temperature of organsolv is in the range of 150^{o}C– 200^{o}C (Zheng *et al*., 2009).

2.4.3.6 Biological pretreatment

This involves wood degrading microorganism including white – brown – soft rot fungi and bacteria to modify the chemical composition or structure of hydro-cellulose biomass (Zheng *et al*., 2009).

2.5 Trona (Potash): Occurrence and Composition

Trona- a sesquicarbonate has the formulae: Na_2CO_3-$NaHCO_3.2H_2O$ or $Na_3H(CO_3)_2.2H_2O$.The major natural evaporate deposits containing the alkali are found in Owens lake, New Searle Lake, Kenya Lake, Califonia, Magad, Texaco, Mexico, and Laqunillas. In Nigeria, trona is deposited along some minor lakes in Borno State, Kano State, and Plateau State. The main constituents of trona are Na_2CO_3, $NaHCO_3$ and H_2O. Upon analysis, trona was shown to contain 95% sodium sesquicarbonate and insolubles – mainly dolomite, shale and clays.

Table 1: Constituents of Trona

Constituent	Percentage
Na_2CO_3	45.11
Na_2HCO_3	35.85
H_2O	15.32
NaCl	0.03
Fe_2SO_4	0.11
Na_2SO_4	0.01
Insoluble	3.75

Source: Okoro U.C, (2004).

The decomposition of trona at temperature above 150^0C yields soda.

$$2Na_2CO_3, Na_2HCO_3, 2H_2O \xrightarrow[(\text{above}150^0\text{C})]{\Delta} 3Na_2CO_3 + CO_2 + 3H_2O$$

2.6 Hydrolysis

This is a chemical reaction of a compound with water usually resulting in the formation of one or more new compounds. The most common hydrolysis occurs when a salt of a weak acid or weak base (or both) is dissolved in water. Water ionizes into negatively hydroxyl ions (OH) and positive hydrogen ion(H^+), which becomes hydrated to form positive hydronium ions (H_3O^+). The salt breaks up into positive and negative ions. For example, when sodium acetate is dissolved in water, it readily dissociates into sodium and acetate ions. Because sodium hydroxide is a strong base, the Na^+ ions react only slightly with the hydroxyl ions already present in the water to form sodium hydroxide molecules. Acetic acid is a weak acid, so the acetate ions react readily with the H^+ ions present in the water to form neutral acetic acid molecules. The net result of these reactions is the relative excess of OH ions,

causing an alkaline solution. The addition of strong acids or bases or the use of steam will often bring about hydrolysis where ordinary water has no effect (US patent). Some industrially important hydrolytic reactions are the synthesis of alcohol from olefins e.g. ethanol (CH_3COOH), from ethane (CH_2CH_2) in the presence of a strong catalyst, the conversion of starches to sugars in the presence of a strong acid catalyst, and the conversion of animal fats or vegetable oil to glycerol and fatty acids by reacting with steam. Hydrolysis is an important reaction in plants and animals. The catalytic action of certain enzymes allows the hydrolysis of proteins, fats, oil and carbohydrates. Hydrolysis can be classified into two groups: Chemical and enzymatic hydrolysis.

2.6.1 Chemical Hydrolysis

This involves the exposure or the use of acid (eg HCL or H_2SO_4) to hydrolyze the glucosidic bond ∝ -(1,4) and ∝ -(1,6) linkage that are binding the glucose unit of the starch polymers; first to dextrins, maltose and finally to glucose (Onwueme, 1978).

2.6.2 Enzymatic Hydrolysis

This involves the use of enzymes (hydrolytic enzymes) that are specific for ∝-1,4 and ∝-,6 – glucosidic linkages that bound the large polymers of starch. Disbranching enzymes are usually useful for complete hydrolysis of starch polymers to glucose (starch monomers) during enzymatic hydrolysis. Nevertheless, some of the hydrolytic enzymes produced by some organisms are capable of hydrolysing ∝ -(1,6) glucosidic linkage which poses a major problem in the use of enzymes in hydrolysis during biotechnological processes. For example glucoamylase from *Aspergillus niger* is capable of hydrolysing the ∝-(1,4) and∝-(1,6) glucosidic linkage of cassava starch and other starch from different sources of glucose (Saykaka

et al., 2002). Some hydrolytic enzymes produced by microorganisms and their site of action on starch are shown in the table below.

Table 2: Hydrolytic enzymes produced by some microorganisms and their hydrolytic site of action on starch.

Enzymes	Source	Action
∝ - amylase	*Bacillus amyloliquefaciens* *B. licheniformis*	Cleaves only ∝-1,4 – oligosaccharide linkage giving dextrin and predominantly maltose.
Glucoamylase	*Aspergillus niger*	Cleaves ∝-1,4 and ∝-1,6 linkage from the non-reducing end to give glucose unit.
Pulludanase	*B. acidopallylyticus*	Cleaves only ∝-1,6 linkage to give straight chain maltodextrins.

Source: Okolo *et al*. (1995).

The enzymatic conversion of cassava starch to glucose involves three stages: gelatinization**- t**his involves the dissolution of the monogram sized starch granules to form a viscous suspension; liquefaction involves the partial hydrolysis of starch and saccharification which involves the production of maltose and glucose by further hydrolysis.

2.7 Classification of Starch Hydrolysate

A very common method of classifying starch hydrolysate is to measure the degree of hydrolysis in terms of Dextrose Equivalent (DE) which is a measure of the reducing sugar content of the hydrolysate calculated as dextrose and expressed as a percent of the total dry substance. The dextrose equivalent value a starch hydrolysate is determined by the method of Smogyi, M described in the journal of Biological chemistry (1945). Starch hydrolysis having a dextrose equivalent (DE) value below 40 are generally considered low conversion products whereas those having a DE value higher than 60 are considered high conversion products with products having classified as regular or intermediate conversion products.

In addition to the above classification, starch hydrolysates are also characterized or classified by the method used to accomplish the hydrolysis i.e. whether the hydrolysates are converted by means of acids or enzymes. The acids and enzymes which are satisfactory for this purpose are well known in the art. The properties or characteristic of the starch hydrolysate depend upon the degree of conversion to sugars as well as the means (acid or enzymes eat) employed for hydrolysis. Generally speaking, high D.E starch hydrolysate products are preferred with respect to fermentability, flavour enhancement, hydroscopicity and sweetening properties whereas low D.E starch hydrolysate products exhibit higher viscosities and as are preferred with respect to cohesive and foam stabilizing properties.

Low conversion starch hydrolysate products having dextrose equivalent values not substantially above 18 are characterized by a unique polysaccharide composition, good clarity and little tendency of retrogradation in solution. Methods for preparing low conversion starch hydrolysate involve a two-stage hydrolysis; hydrolysis in the first stage being carried out with acids or enzymes at elevated temperatures for short periods to achieve liquefaction of the starch with very little dextrinization or saccharification and the second stage of hydrolysis being carried out at the alkaline

pH with bacterial alpha-amylase to achieve a desired dextrose equivalent value (Armbruster, 1971).

Starch is a naturally occurring polymer of α –D glucose connected by acetal linkages. The acetal linkages are susceptible to both acid or enzyme hydrolysis and both catalyst are employed in preparing starch hydrolysates.

The acid hydrolysis of starch has been shown to be a random process and at a given time. An acid hydrolyzed starch product comprises a mixture of many different molecular species which may range from the monomer glucose all the way up to polymers approaching the size of starch. Because of the wide range of molecular sizes obtained in acid hydrolysis, it is customary to conduct the conversion to an extent that the long polymers will no longer react with iodine. Accordingly, acid hydrolysis is not well suited for making low D.E products (Armbruster, 1971). Although the degree of hydrolysis can be reduced to obtain low D.E products, the long polymers present in such products result to rapid retrogradation with an accompanying loss of solubility and clarity. Another disadvantage of hydrolyzing starch with acid is that significant amounts of glucose are always present in the products even when the degree of conversion is held to a low level. Because of the presence of glucose and other low molecular weight saccharides, even low D.E acid-hydrolyzed products tend to be hygroscopic, sticky, and provide a sweetening effect which is desired for many applications.

The use of enzymes for hydrolyzing starch has gained widespread application in recent years and enzymes are employed commercially for manufacturing certain products. Enzymes have an advantage over acid catalysts in that they exhibit specificity for certain linkage (Taherzadeh *et al*., 2007). One type of microbial enzyme which is commonly employed is α -amylase. Alpha-amylase has the property of splitting 1-4 linkages more or less at random throughout the starch molecule with little effect on the 1-6 linkages. Moreover, α -amylase does not readily

hydrolyze or split the 1-4 linkage in maltose and maltotriose. Thus, it has been reported that when substantially complete conversion of starch is effected with &-amylase, maltose and small amounts of trisaccharides and other low molecular weight polysaccharides especially those containing the 1-6 linkages, are present in the final hydrolysate (Yu *et al*., 2004).

Another factor apart from acid or enzyme is the manner in which the starch is gelatinized when heated in water. The molecules of native starch are closely bound in the starch granule to a varying degree and those that are closely bound are not particularly susceptible to the action of enzymes. It is not until the starch molecules have been dispersed by swelling and gelatinization in water that significant hydrolytic cleavage will take place (Armbruster, 1971). In a conversion procedure where the starch is heated slowly the molecules which are closely bound are dispersed or gelatinized more slowly and therefore are available for attack by the acid or enzymes at a low rate. The result of this non-uniform rate of gelatinization is that by the time that all of the resistant molecules have been made available for attack the more easily dispersed molecules will already have been reduced to a relatively small molecular range. However, when preparing a low D.E hydrolysate product, the non-uniformity of gelatinization is particularly undesirable because a high proportion of very large molecules including some intact starch molecules will still be present when the desired low D.E. level is reached.

Another undesirable characteristic of low D.E. starch hydrolysate produced by known methods, which is related to the non-uniformity of degradation, is the tendency for certain of the large linear molecules to reassociate with other molecular fragments of starch to form large relatively insoluble aggregates. The rate and extent to which linear starch molecules reassociate into insoluble aggregates is a function of chain length since below a certain length the aggregation tendency is not great.

The occurrence of reassociation in a liquid hydrolysate is evidenced by the appearance of haze and or a change to a get or paste having poor solubility in cold water. Although the reassociation or retro gradation of starch hydrolysis occurs primarily in cooled hydrolysate where it is especially objectionable it may occur to a slight extent during cooking process if the rate of heating is slow. When this happens these molecular aggregates tend to remain intact during subsequent processing and add to the difficulty of filtering the hydrolysate.
(United States patent 3663369. Hydrolysis of starch,www.freepatentsonline. Com/3663369.html).

2.8 Bio-energy production

With increasing demand for energy, continuous depletion of the world fossil energy reserves, instability in the global oil prices, and various environmental problems such as climate change, global warming, soil, water and air pollution associated with the use of fossil fuel, there is an increasing pressure, for development of renewable and sustainable alternative sources of energies. The world fossil fuel reserves have been estimated to last for less than 50 years from now and there is urgent need to develop alternatives. The concept of “green development” vigorously pursued by various countries and organization is promising. Bio energy (energy from biomass materials) has very good potentials as a substitute for the fossil fuels because they are renewable, sustainable and environmentally friendly. It was initially used as an octane enhancer but since the oil-shock of the 1970s, bio-ethanol has been commercially produced as liquid fuel for various engines. Most car engines can run on gasohols (blends of gasoline and ethanol in various proportions) but some engines that can run on 100% ethanol have been developed. The raw materials used for production of bio-ethanol depend on the region/country. For example while most of the bio-ethanol produced in Brazil is from sugar cane, in united states, most of the

bio-ethanol is produced from corn, India uses molasses, and sugar cane, some parts of Europe use sugar beet, while china uses corn and cassava (Jansson *et al*., 2009). There is a general concern that food crops should not be used for fuel production because of its possible effects on food security. However, since the technology for bio-ethanol production from non-food crops as the sub bio-ethanol production, whether food crops or non-food crops are used, and the net results are that there will be competition for farm land and agricultural input. Furthermore, the choice of the crops will depend on the region and socio-economic conditions of the region. If our fossil oil is exhausted, we will not have any alternative but to produce energy from what we have.

2.9 Myths and Realities about Biofuels

The age of oil is ending. Even if we could afford to borrow over 51 billion per day indefinitely to import oil, we just cannot afford the cost of our oil addiction in terms of national security dangers, environmental damage and economic losses. Biofuels are among a small handful of petroleum alternatives that can simultaneously provide enhanced national security, environmental improvements and opportunities for broad based economic growth.

Unfortunately, many myths and misconceptions exist about biofuels and these include: (myth, 1) the “people are going to go hungry because of ethanol”,(myth, 2) the “there is not enough land to produce enough ethanol anyway”,(myth, 3) the “it takes more energy to make ethanol than you get from the ethanol”, (myth, 4) the “ethanol will always be more costly than gasoline” myth.

All fuel ethanol is the same. It is made by fermentation of sugars. The sugars can come from sugar cane (Brazil), or from corn grain (U.S.).“Second generation” ethanol, called cellulosic ethanol, will be produced form the sugars in plant cell walls. Cellulosic ethanol can be made from virtually anything that is or even was a

plant including: wood chips, urban waste, straw, crop wastes, hay and yard trimmings, and on and on. We can even grow energy crops, trees (willow and popular are promising species). Highly productive grasses such as switch grasses are grown for their energy content and then converted to ethanol (Michigan State University Alumni Magazine, winter 2008). Maybe a more picturesque and accurate name for cellulosic ethanol is “grassoline”.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Preparation of Cassava Starch

The cassava starch was prepared as shown in the flow diagram below:

Cassava root → peeled → washed well with clean water → cut into chips → sun dried → milled into powder using GX200 Delmar machine. The powder was kept in an air tighten container.

3.2 Microorganisms

The fungi isolate used (*Aspergillus niger*) was cultured for the production of amylase enzyme. It was obtained from pure stock culture of Prof. J.C. Ogbonna, from the department of microbiology, University of Nigeria, Nsukka. The *Aspergillus niger* was used for starch hydrolysis while the yeast *Saccharomyces cerevisiae* used for fermentation and for ethanol production was obtained from palm wine inoculated into yeast peptone Glucose (YPG) agar slant prepared according to manufacturers instruction. The yeast peptone Glucose agar suspension was then heated to get a clear solution. This was followed by addition of 100mg of Chloramphenicol to the medium shaked vigorously before dispensing into test tubes. Each test tube was capped with cotton wool and covered with aluminium foils and autoclaved at one hundred and twenty degree Celsius for 5 minutes. The content of each test tube was poured into autoclavedpetri dishes which were then allowed to solidify. Afterwards 1ml of palm wine was introduced into 9 ml of distilled water and shaken vigorously for proper mixing. The yeast cells formed from the agar slant were subcultured into cooled agar broth. The mixture was incubated for 48 hours.

3.3 Preparation of Enzyme for Hydrolysis

A 223g of rice was steamed to cook and was allowed to cool. Then, 200g was weighed out of the cooked rice and poured onto a sterile clean cloth. Then 10ml of sterilized water was poured into the slant of *Aspergillus niger* and wire loop was used to harvest (scup) the spores. The spore suspension was poured into the 200g of cooked rice and mixed very well to ensure even distribution of spores in the rice. The mixture was so wrapped and incubated for 24hours after which it was thoroughly mixed again with a sterile spoon to ensure that the spores were well distributed. It was wrapped again and left for another 24 hours.

Heavy production of black spores of *Aspergillus niger* was observed at the end of 3days. The wrapped clean cloth containing the production enzyme was then stored in the refrigerator for hydrolysis.

3.4 Preparation of Yeast Cells

A broth of the Potato Dextrose Agar (3.9g of PDA in 100ml of distilled water) containing 250mg of chloramphenicol was prepared in a 500ml conical flask and autoclaved at 120^{o}C for 15 minutes. The prepared broth was allowed to cool and three loopful of the yeast from the slants that were subculture from the stock culture of yeast cells were added using a wire loop and then incubated for 48 hours. The medium was decanted, leaving the cells in the flask. The decantation was done only when the cells are about to be used for fermentation.

3.5 Determination of Starch Content

A 1g of cassava flour was weighed, homogenized in 100ml of distilled water, boiled for 30 minutes and allowed to cool. Then the volume was made up to 100ml with

distilled water and filtered. A 0.1ml of the filtrate was added in a test tube, mixed with 0.5ml of K1-1, made up to 10ml with distilled water. The absorbance was taken at 680nm. The percentage starch content was calculated by dividing the absorbance value of 10g of starch solution with the slope from the standard curve.

3.6 Gelatinization of the Starch

Various quantities of cassava starch (5, 10, 15, 20, 25, 30g) were weighed into different conical flasks and 100ml of distilled water added to each and mixed. These flasks were allowed to stand in inside boiling water for the starch to form gel, the flasks were vigorously shaken while heating The duration of heating was varied from 0 -20 minutes.

3.7 Pretreatment of Cassava Starch with potash.

Different grams of trona (akanwu) were added into five different flasks containing 10g of cassava starch each, mixed with 100ml of distilled water. The flasks were shaked vigorously. The following measures (gram) of potash were used: 5.0, 7.5, 10.0, 12.5, and 15.0 and the treatment effects of potash on ethanol production were noted.

3.8 Hydrolysis

A 10g of gelatinized starch was mixed with 40g of Koji (crude enzyme). The pretreatment effect on cassava starch was checked during which the pH, duration of gelatinization and inoculum size were varied. The variation was as followed:

10 grams of cassava starch was pretreated with the following quantities of potash: 5.0g, 7.5g, 10.0g, 12.5g, and 15g.

12.5g of potash was observed to be the best concentration. Then 12.5g of potash mixed in 10g of starch was hydrolysed atpH of 3 and 4.

12.5g of potash in 10g of starch at pH 4 but with various durations of gelatinization: 0, 5, 10, 15, 20.

12.5g of potash, 10g of starch, pH 4, 10 minutes gelatinization with various inoculum sizes.

3.9 Fermentation

A 10g of the gelatinized starch (hydrolysate), potash and enzyme were incubated with cells from the decantation of PDA broth prepared earlier. Samples were taken, and the starch, glucose and ethanol concentrations determined at every 24 hours for 5 days at room temperature.

3.10 Determination of Starch Concentration

A 0.5ml of the sample was taken into test tube and then made up to 5ml with water. 0.5ml of iodine was added and finally made up to 10ml with water. The absorbance was read at 680nm.

$$\text{Concentration (\%)} = \frac{\text{mean value}}{\text{slope}} \times \frac{100}{1}$$

3.11 Determination of Glucose concentration

A 0.3 ml of the reaction sample was added to 0.3ml of dinitrosalicyclic acid (DNSA) in a test tube. The test tube was allowed to stand in boiling water for 10 minutes. 3 ml of water was added, and the absorbance was read at 540nm.

$$\text{Concentration (\%)} = \frac{\text{mean value}}{\text{slope}} \times \frac{100}{1}$$

3.12 Determination of Ethanol Concentration in Aqueous Solution

A 1ml of the sample from fermentation vessel was diluted with 1ml of distilled water. Then, 10ml of acid dichromate solution was transferred into a 250ml conical flask with matching rubber stopper and was stored overnight at 25-30^{0}C.

The flask was allowed to come to room temperature and the stopper was carefully removed. The walls of the flask were rinsed with 100ml of distilled water, 1ml potassium iodide solution added and mixed thoroughly. Three blank titrations were prepared by adding 10ml of acid dichromate solution to conical flasks, 100ml of water and 1ml of potassium iodide solution and swirled to mix. This served as control. Sodium thiosulfate solution was filled into a burette for titration of each flask. The titration continued until a colour change from brown to yellow was observed. Then, 1ml of starch solution was added and titrated until the blue colour disappeared. The volume of thiosulfate used to obtain the end point was recorded.

Concentration (%) = $\frac{\text{mean value}}{\text{slope}} \times \frac{100}{1}$

3.13 Determination of Enzyme Activities in Koji

3.13.1 Gluco-amylase Assay

The gluco-amylase assay was carried out by incubating a reaction mixture containing 0.5ml of 1% soluble starch 0.2ml of 0.1M sodium acetate buffer (pH 5.6) and 0.3 ml koji enzyme solution at room temperature for 30mins. This was done in duplicate after which 1ml of DNSA was added to terminate the reaction, and the mixture boiled for 10 minutes. After cooling, 4ml of distilled H_2O was added and absorbance taken at 540nm. Gluco-amylase activity is defined as the amount of enzyme that releases one micromole of sugar in 1 minute under the assay condition.

3.13.2 Alpha (∝) – Amylase Assay

This assay was carried out by incubating a reaction mixture containing 0.5ml of 1% soluble starch, 0.2ml of 0.1M sodium acetate buffer (pH5.6) and 0.3ml koji enzyme solution at room temperature for 30 minutes. This was done in duplicate. 1ml of 1M acetic acid was added to stop the reaction followed by addition of 8ml distilled H_2O and finally, 1 ml iodine solution and reading taken at 600nm. Alpha ($\propto$) – amylase activity is defined as the amount of enzyme that degrades one micromole of starch in 1 minute under the conditions of assay.

3.14 Statistical Analysis

The data in this study were subjected to one way analysis of variance (ANOVA).Relationship between variables and comparison of the different treatments were tested for level of significant difference at $p<0.05$ using Least Significant Difference (LSD) and post hoc multiple comparison tests. The data analysis was performed using software (Statistical Package for Social Science (SPSS) version 20.0.

CHAPTER FOUR

4.0 RESULTS

4.1 Starch Content of Cassava Used in the Study.

The starch content obtained from the cassava starch used for the experiment was 32%. Theoretically the average content of starch in cassava fresh storage is 24% - 32% (Wang, 2002).The high yield of starch content in the experiment could result to good yield of bioethanol and other end products.

4.2 Fermentation of Gelatinized Starch.

The ethanol yield was at its peak at 72 hours after incubation but declined progressively. This was because about 70% of the substrate had been used up by the enzymes, and in the production of inhibitors or by products. Maximum ethanol yield obtained from fig 1 was 20.43%.

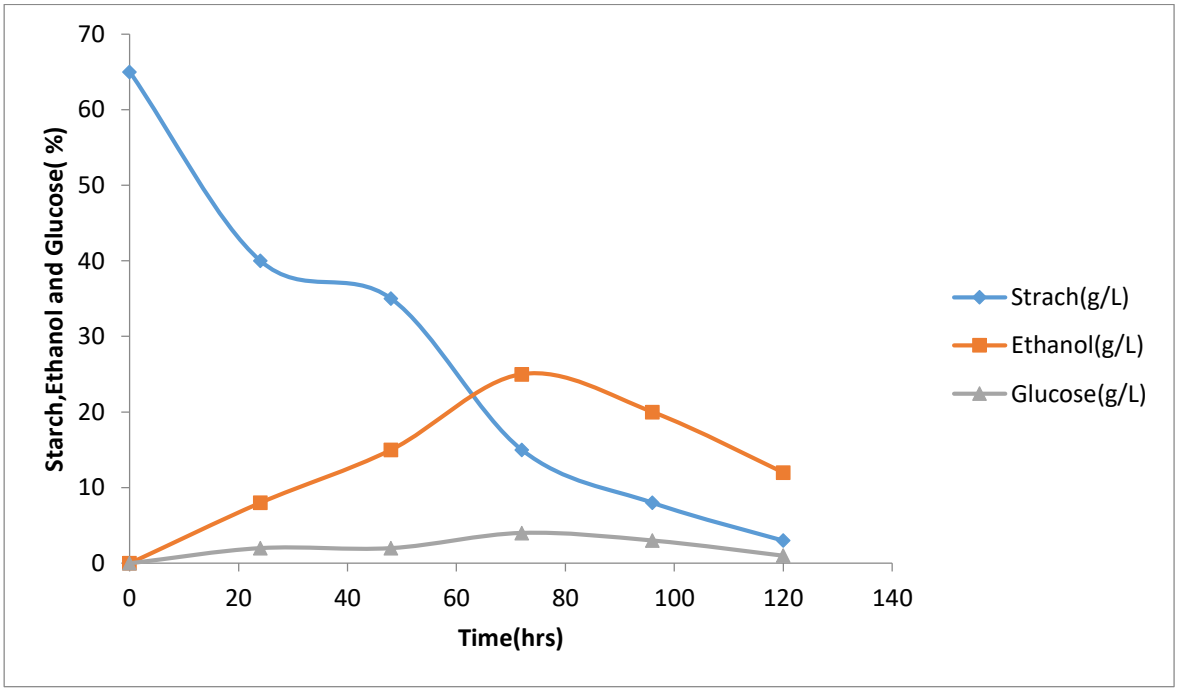

Figure 1: Simultaneous hydrolysis and fermentation of cassava flour using 5% potash, 2.58x10^6 cells of *Saccharomyces cerevisiae*, at pH 3 and gelatinized for 10 minutes.

In fig 2, 7.5g of potash was used for the pretreatment of cassava starch prior to enzymatic hydrolysis. This subsequently affected the energy conversion efficiency of the starch by enzyme. From this graph, the ethanol concentration increased from 0.1% to 20% from the first day to the third day progressively attaining its peak on the third day.

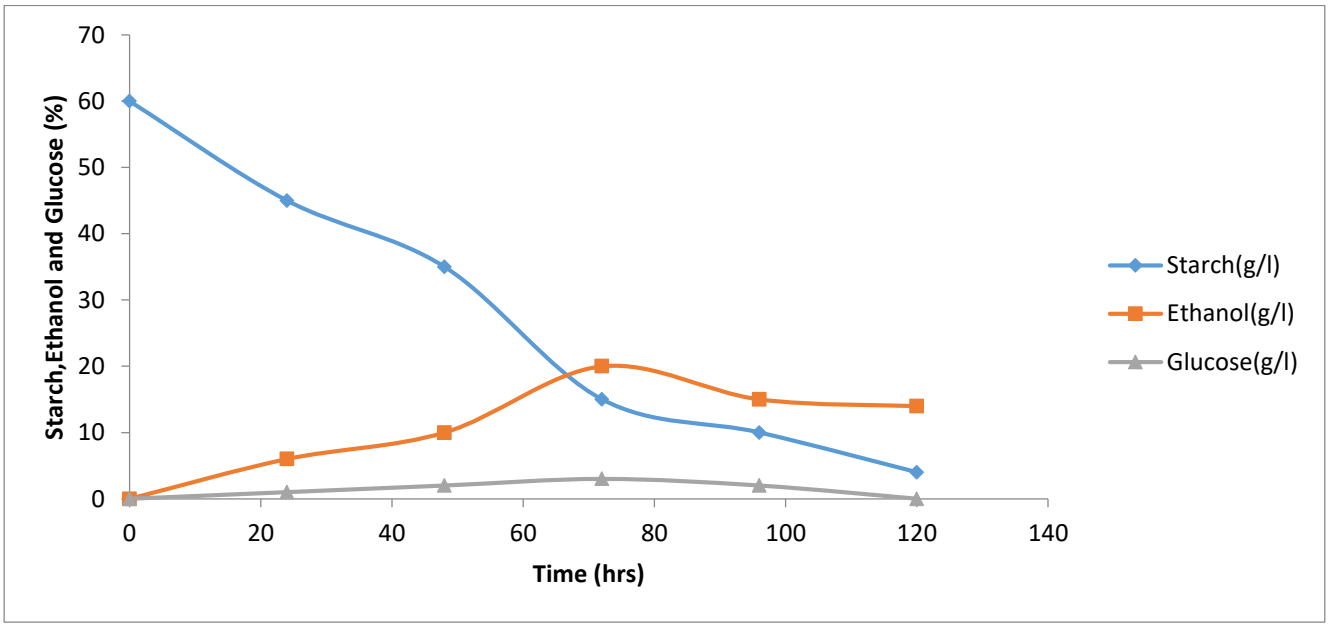

Figure 2: Simultaneous hydrolysis and fermentation of cassava flour using 7.5% potash, 2.58x10^6 cells of *Saccharomyces cerevisiae*, at pH 3and gelatinized for 10 minutes.

The concentration of potash was increased from 7.5g to10g/l. The ethanol obtained from this experiment increased to 24.8% at the same conditions of reaction.

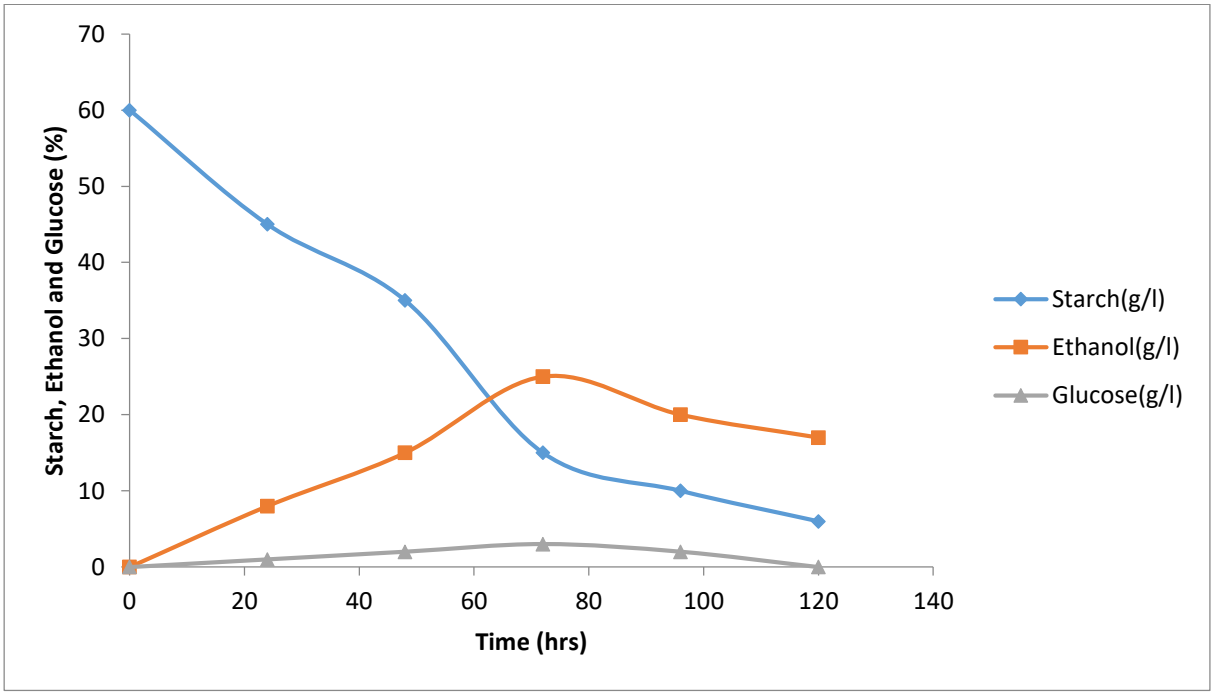

Figure 3: Simultaneous hydrolysis and fermentation of cassava flour using 10.0% of potash, 2.58x10^{6}cells of *Saccharomyces cerevisiae*, *pH* 3 and gelatinized for 10 minutes.

As the starch concentration decreased, during the course of bioconversion of substrate to product, the ethanol concentration increased, with the highest yield of 20.8% but decreased progressively with time.

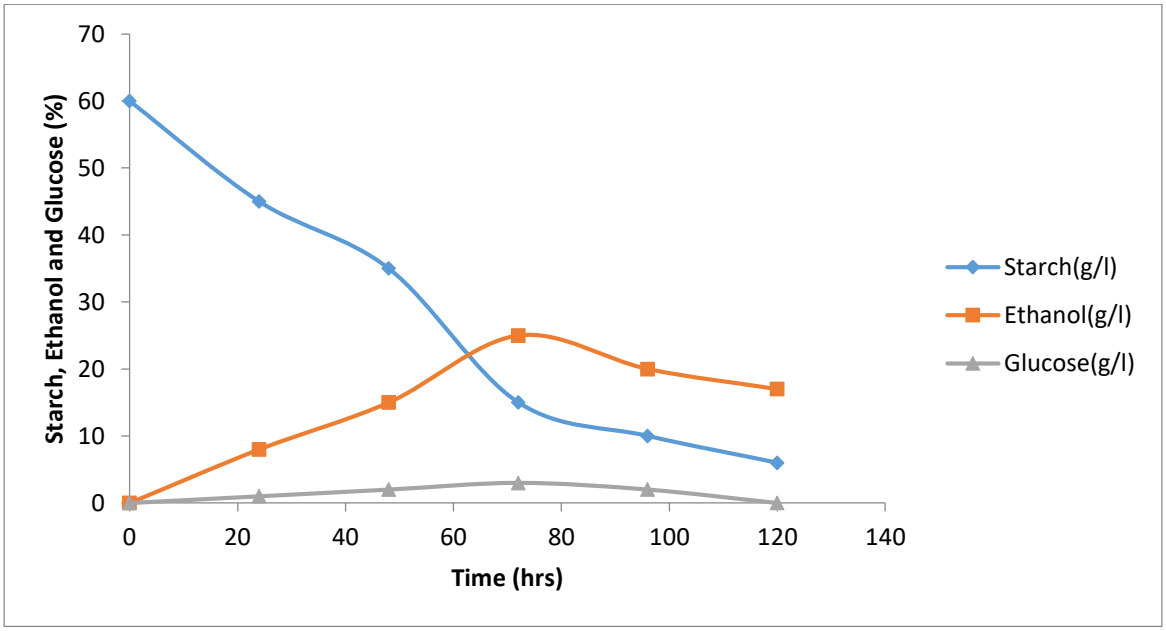

Figure 4: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash with 2.58 x10^6 cells of *Saccharomyces cerevisiae*, at pH 3 and gelatinized for 10 minutes.

During the fermentation process, it was observed that the ethanol yield increased steadily, reaching its peak after 72 hours of fermentation and then declined progressively as the residual sugar decreased with increase in time. The highest ethanol obtained was 28.0% at the above mentioned reaction conditions.

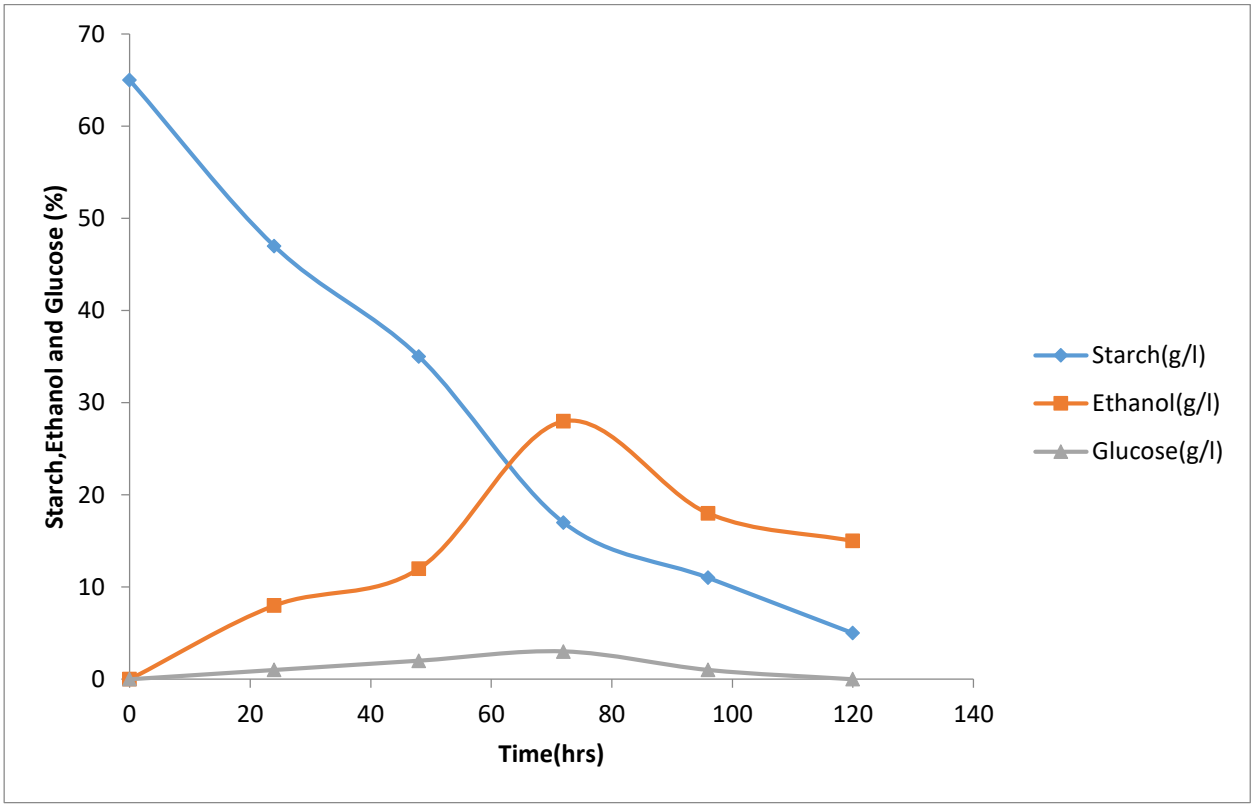

Figure 5: Simultaneous hydrolysis and fermentation of cassava flour using 15% of potash, $2.58x10^6$ cells of *Saccharomyces cerevisiae*, at pH 3 and gelatinized for 10 minutes.

During the simultaneous hydrolysis and fermentation of cassava flour, the quantity ethanol increased with its peak of 28.0% at 96 hours instead of at 72 hours.

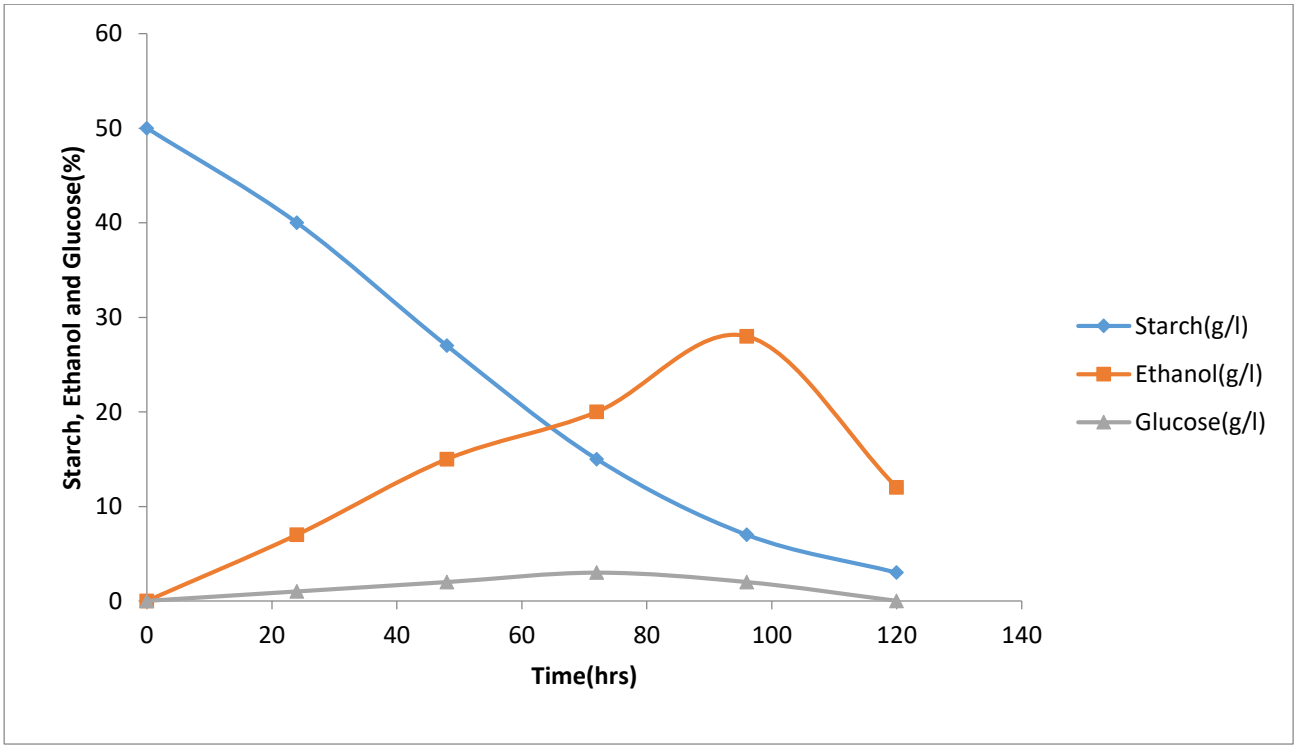

Figure 6: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 5.16 x10^6 cells of *Saccharomyces cerevisiae* at pH 4 and gelatinized for 10 minutes.

As the fermentation process progressed, the highest ethanol concentration formed ethanol concentration formed was 30.0% on the third day. On the fifth day, it declined to the quantity close to that on the first day**.**

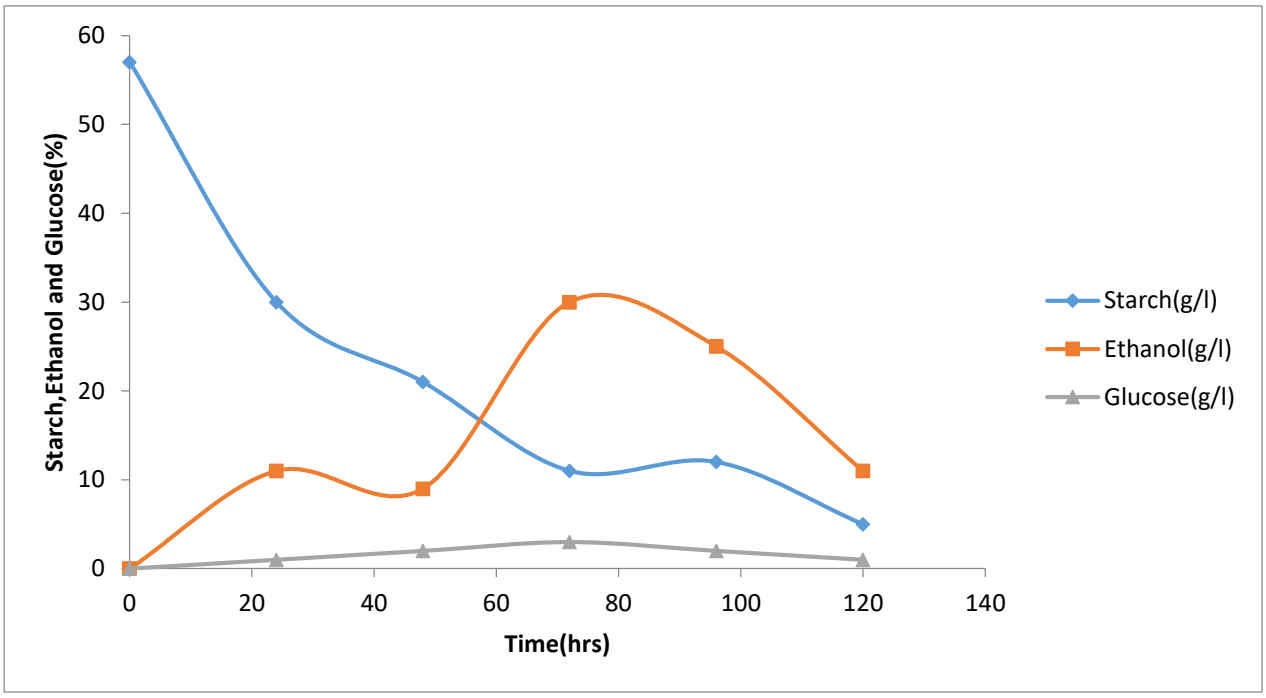

Figure 7: Simultaneous hydrolysis and fermentation of cassava flour suing 12.5% of potash, 3.87 x10^{6} cells of *Saccharomyces cerevisiae*, at pH 4 and gelatinized for 10 minutes.

There was an increase in the ethanol yield of this figure when compared the figure 7 above. The increase in pH in the yeast cell concentration could be the reason for the 37.0% of ethanol produced.

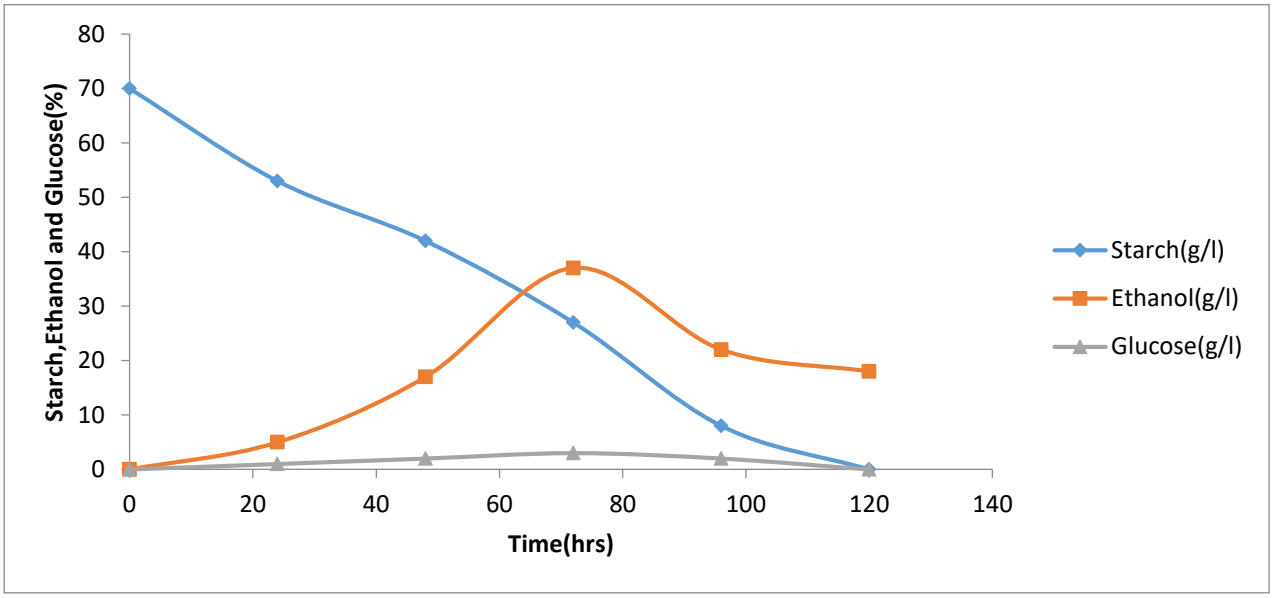

Figure 8: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 2.58 x 10^6 cells of *Saccharomyces cerevisiae*, at pH 4 and gelatinized for 10 minutes.

The number of cells used in this figure was 1.29 x 10^6 cells at pH 4. The quantity of the substrate used here was the same as that used in figure 8 above but the highest ethanol concentration obtained here was 35.0%.

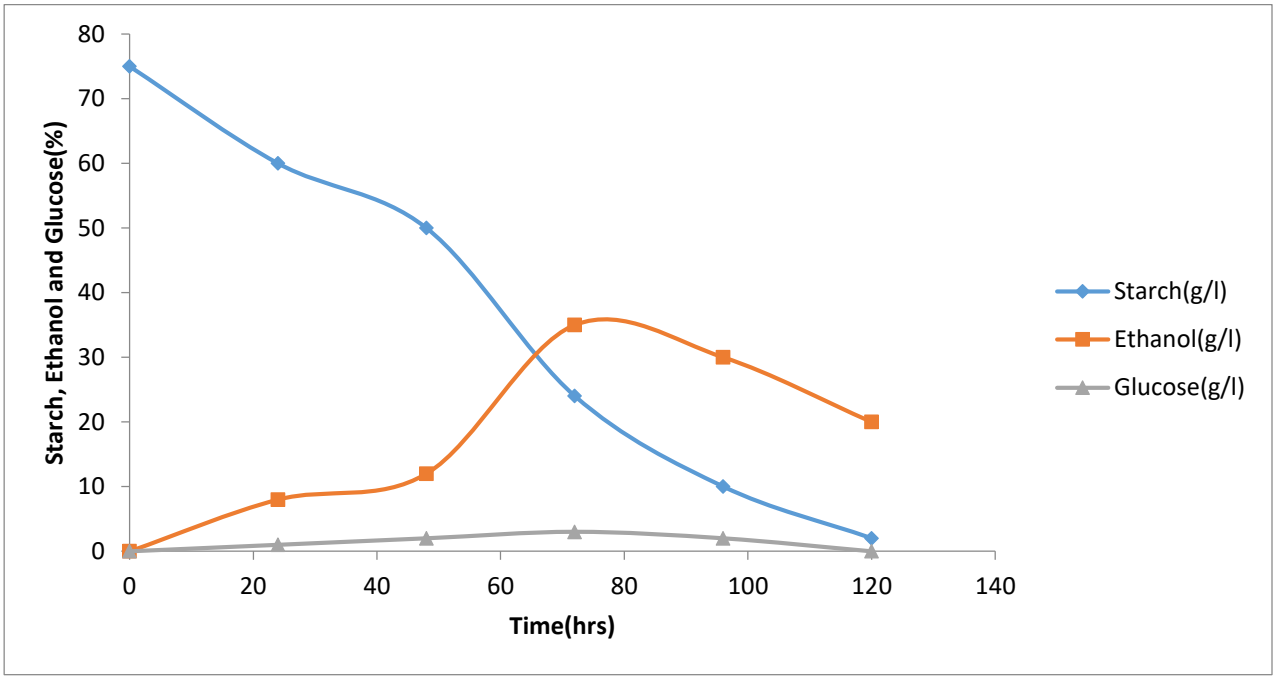

Figure 9: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 1.29 x 10^6 cells of *Saccharomyces cerevisiae*, at pH 4 and gelatinized for 10 minutes.

In order to determine the pretreatment effect of potash and provide adequate sugar release in fermentation broth, fig. 10 experiment was done in comparison with fig.8. The maximum ethanol yield gotten from fig. 8 was 30.0% while in figure 10; 25.0 % of ethanol was formed.

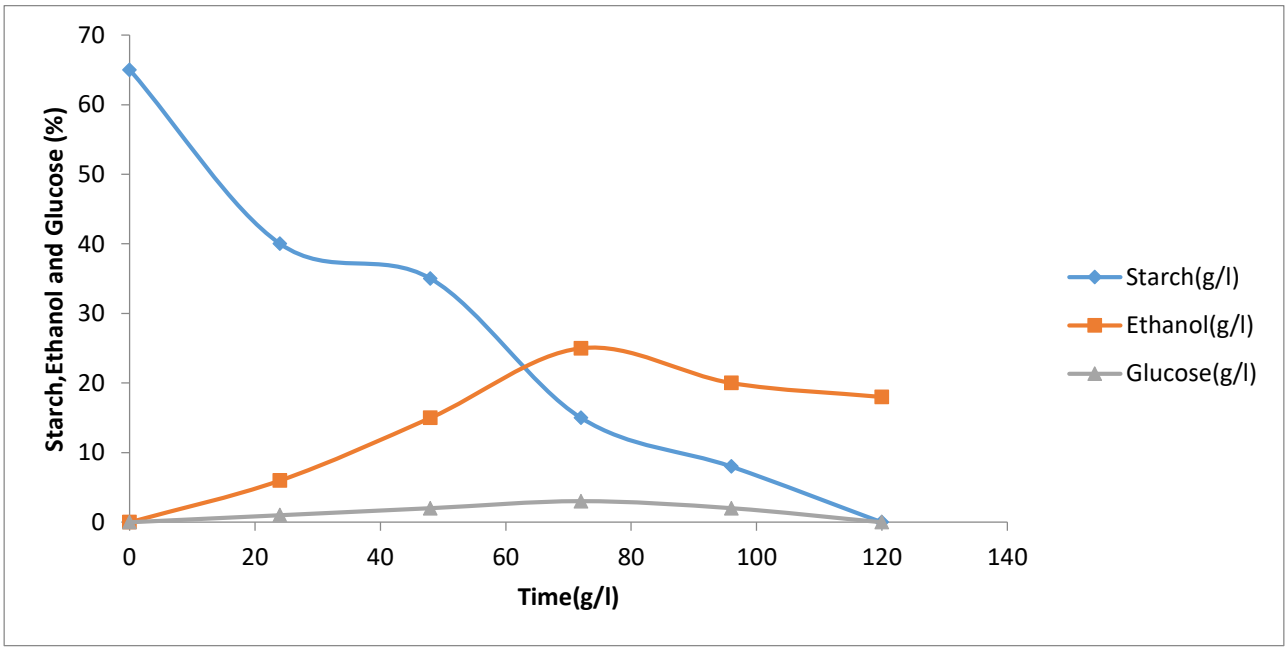

Figure 10: Simultaneous hydrolysis and fermentation of cassava flour using no potash, 2.58 x10^6 cells of *Saccharomyces cererisiae*at pH 4 and gelatinized for 10 minutes (control).

The increase of ethanol yield as the fermentation time increased was little with its maximum of 12.0%. The residual sugar bioconversion to ethanol was not efficient.

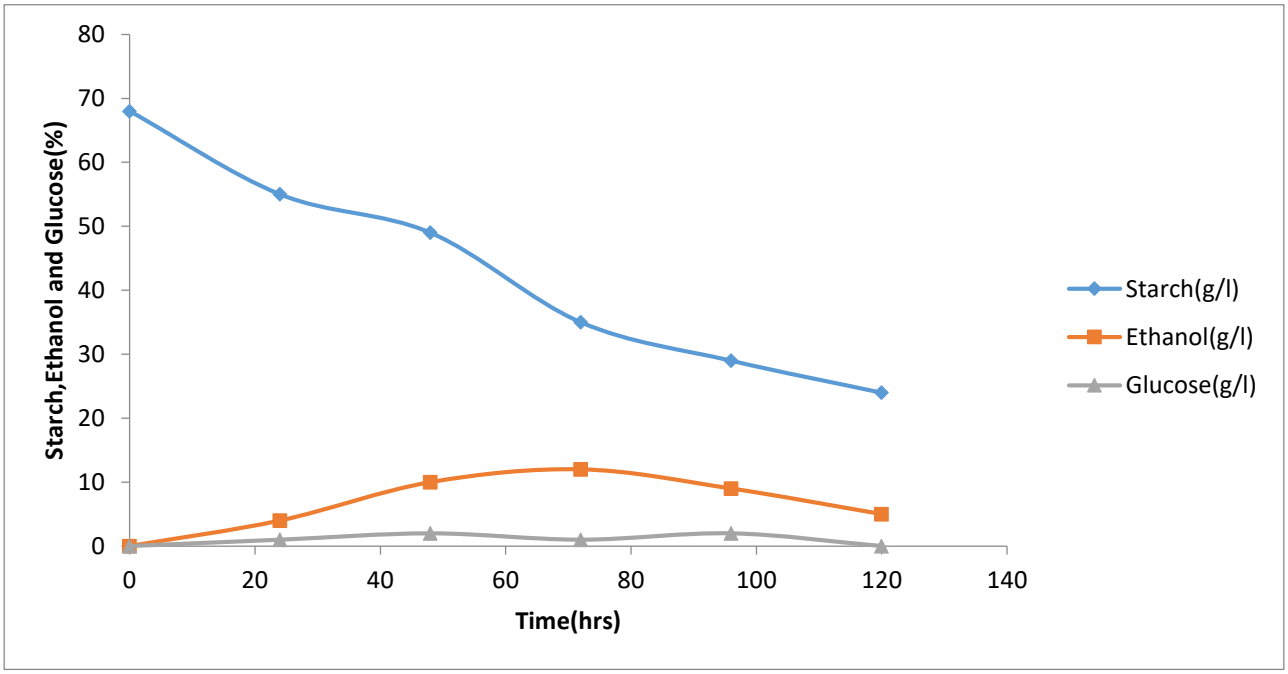

Figure 11: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 3.87 x 10^6 cells of *Saccharomyces cervisiae*, at pH 4 and gelatinized for 20 minutes.

In this experiment, the starch was not gelatinized prior to hydrolysis. The maximum ethanol obtained was 22.0%. However, this finding disputed the fact that not until the starch molecules have been dispersed by swelling and gelatinization in water, that significant hydrolytic cleavage will take place.

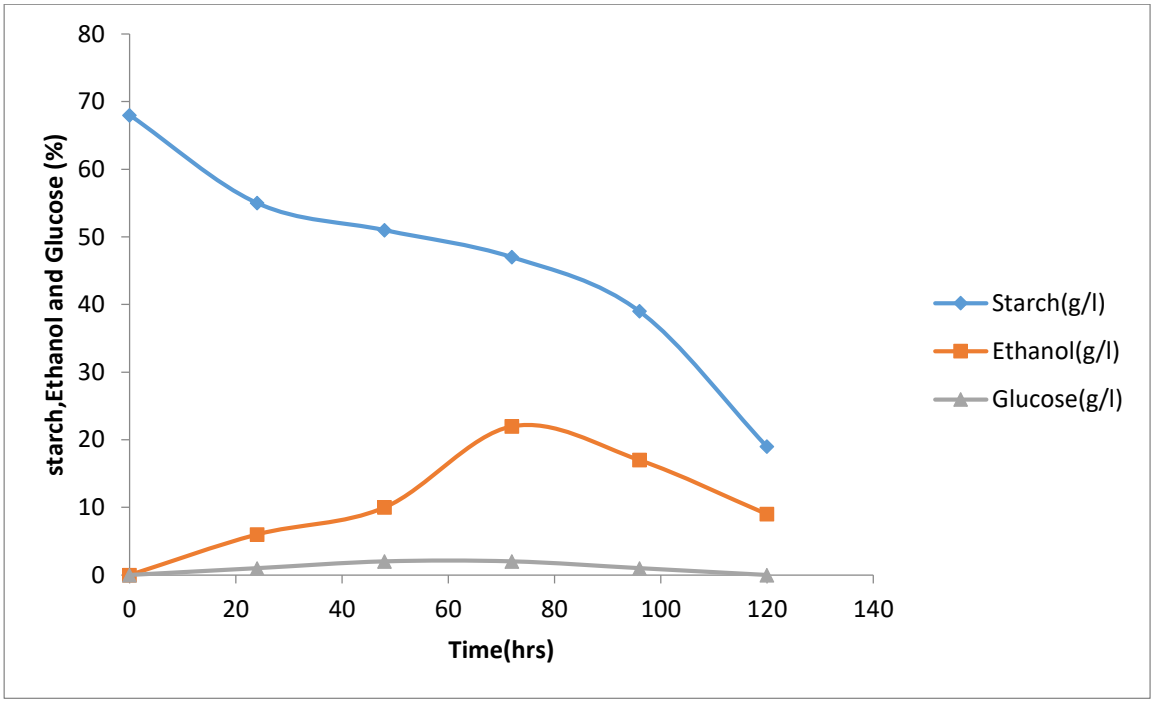

Figure 12: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 3.87 x 10^6 cells of *Saccharomyces cerevisiae*, at pH 4 and gelatinized for 0 minute.

The objective of this experiment was to assess the effect of gelatinization on cassava starch prior to enzymatic hydrolysis. From the graph above**,** it was observed that the ethanol concentration increased sharply from the first day of fermentation and got to the peak at 27% after 72 hours of fermentation. This yield was higher when compared with the yield from the non- gelatinized starch on fig. 12 above.

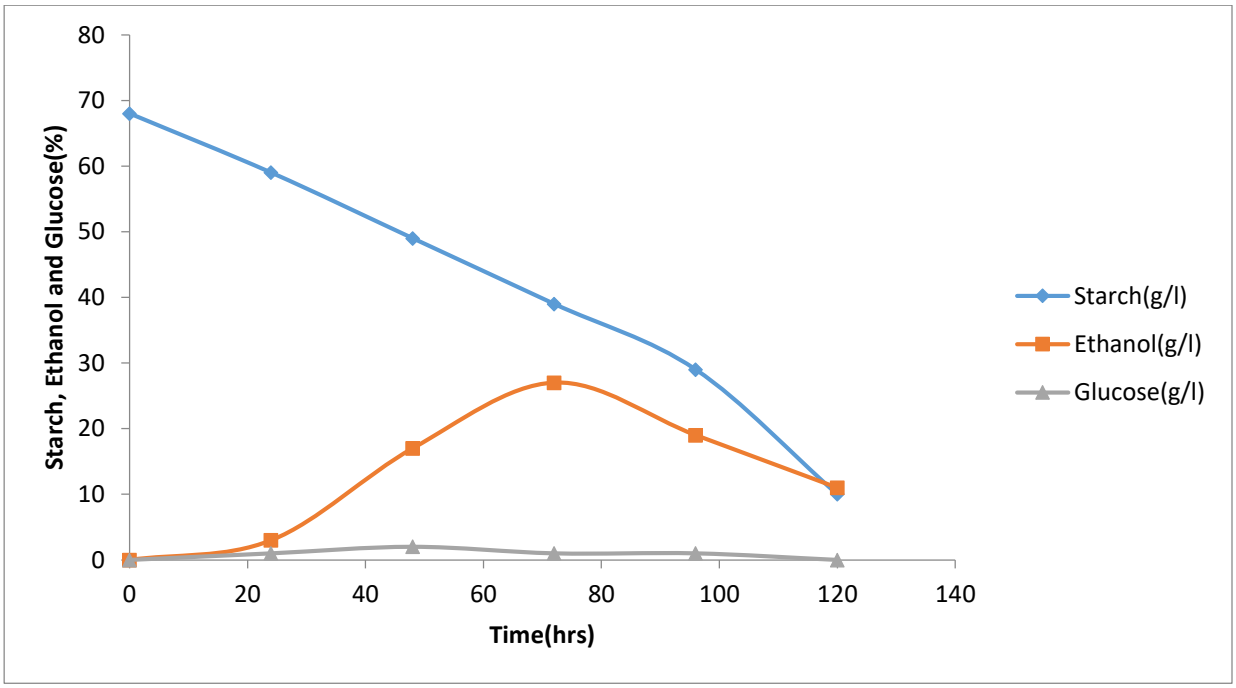

Figure 13: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 3.87× 10^6 cells of *Saccharomyces cerevisia*, at pH 4 and gelatinized for 15 minutes.

This experiment was conducted to ascertain the effect of the duration of gelatinization on ethanol production. It can be deduced that the maximum yield of bioethanol on the graph above was not as high as the yield obtained when the cassava starch was gelatinized for 10 minutes.

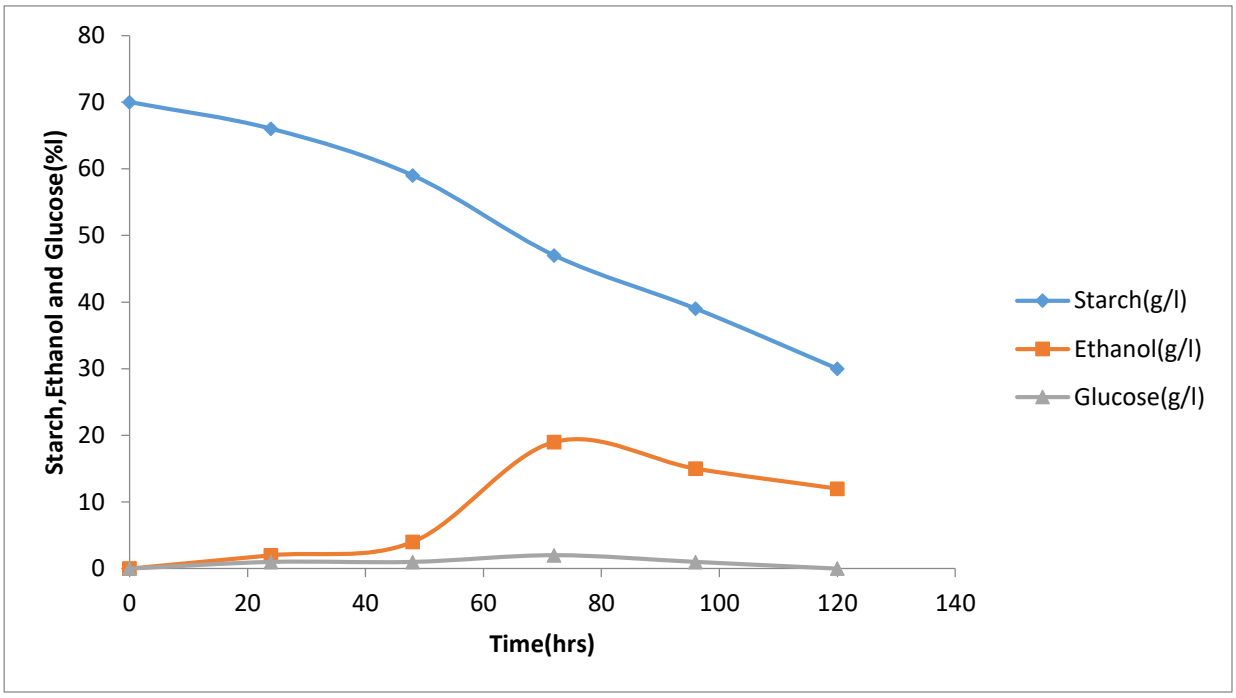

Figure 14: Simultaneous hydrolysis and fermentation of cassava flour using 12.5% of potash, 3.87×10^{6} cells of *Saccharomyces cerevisiae*, at pH 4 and gelatinized for 5 minutes.

Gluco – amylase enzyme had its highest activity on the 4th day of the assay. The gluco-amylase from *Aspergillusniger* was assayed for six days. This was to determine the rate of its activity on hydrolysing the glucosidic linkage of cassava starch to liberate glucose unit. The first day represented the zero hour of the incubation of *Aspergillus niger*. The enzyme expressed was 109mg/ml/day. The rate of the enzyme activity increased with time, with its peak of 800mg/ml/day obtained on the fourth day of the assay.

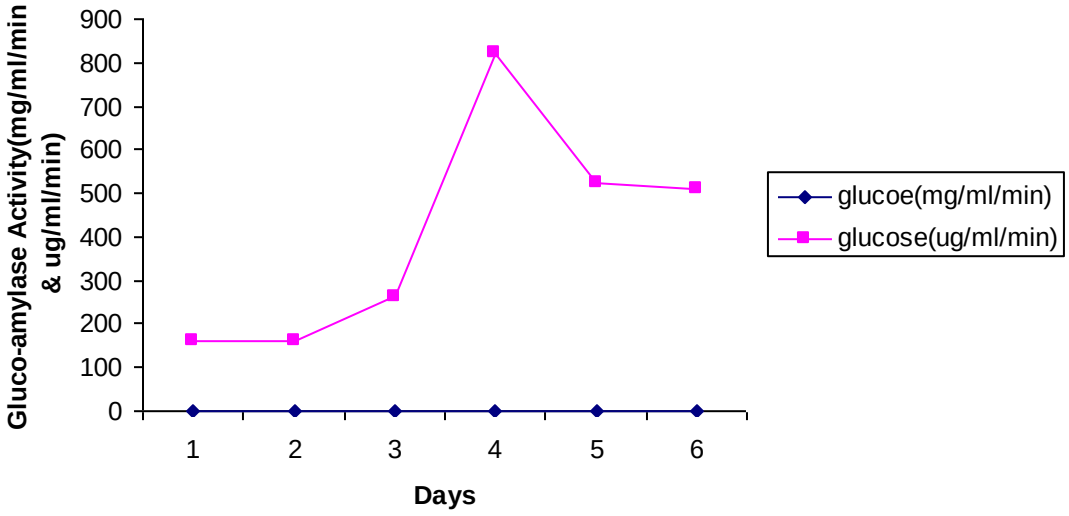

Figure 15: The rate of gluco-amylase activities on starch during fermentation.

On this assay, it was observed that α-amylase exhibited the highest activity on the 4th day during the fermentation process. This affirmed the reason why the maximum production of ethanol occurred after 4 days of starch fermentation.

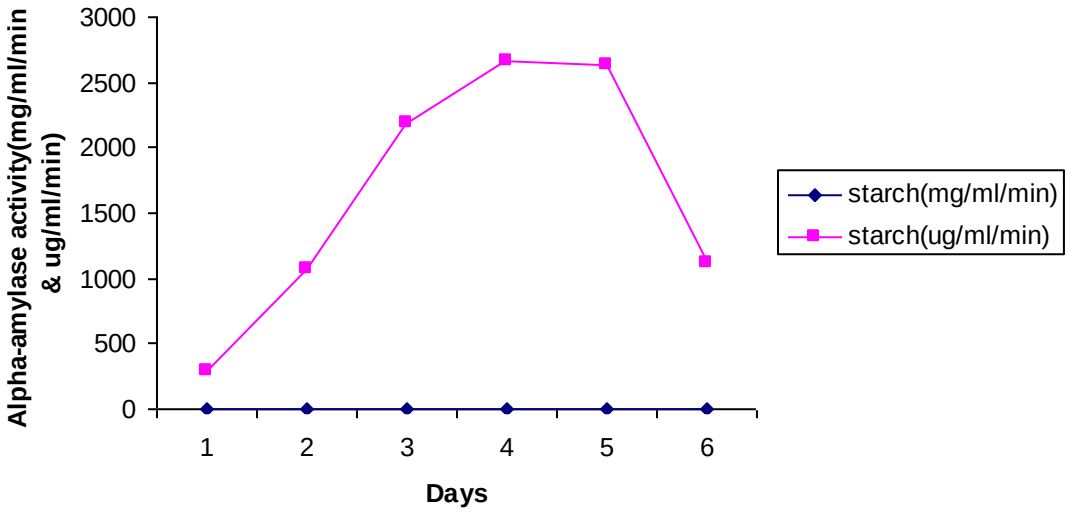

Figure 16: The rate of alpha-amylase enzyme activities on starch during fermentation.

Table 1: Determination of cell concentration

	P 1	P 2	P 3	P 4
Day 1	1.48×10^7	1.48×10^7	1.48	1.48×10^7
Day 2	1.58×10^7	2.1×10^7	1.98	1.7×10^7
Day 3	1.85×10^7	2.45×10^7	2.1×10^7	2.1×10^7
Day 4	1.64×10^7	1.64×10^7	1.3×10^7	1.17×10^7
Day 5	1.35×10^6	1.50×10^6	0.4×10^6	0.95×10^6

Samples of palm wine were assed to determine cell population at different days. The results were determined through cell count using haemocytometer. P1 – P4 are different samples of palm wine. It can be noted that cell density of various sample increased initially but decreased after some days. This experiment was done to determine which sample of palm wine was to be used in fermenting glucose to ethanol. Sample p4 of the palm wine was used to isolate the *Saccharomycete cerevisiae* used for fermentation.

CHAPTER FIVE

5.1 DISCUSSION

Ethanol production from wastes and food crops can be enhanced by pretreatment of substrate prior to hydrolysis and subsequent fermentation. During the course of this research, it was observed that pH, potash concentration, enzyme concentration and duration of gelatinization play important role in optimization of bioethanol yield. The sugar that was produced at various time courses during starch hydrolysis by fungal enzyme was used for growth by yeast cells and the rest was detected as reducing sugar liberated(Bandaru *et al.,* 2006). The volume of reducing sugar detected was always small because of the short time lag between its liberation and conversion to alcohol. The results showed that the highest alcohol was usually obtained on day 3 of fermentation and it sharply declined until the ethanol yield equals the yield on days 1 and 2 in the fermentation process. This could be because enzymes fed initially on the substrate as their carbon source and as well had to adapt to the reaction condition. This usually happened on the first 48 hours, before the conversion of the residual sugar to ethanol. Ethanol yield tends to decline after 72 hours of fermentation as most of the simple sugars have been converted to bioethanol and other by-products.

The enzymatic activities on starch at pH ranging from 1 to 5 were tested and the best pH was 4. Deviation from this either left or right decreases the yield of ethanol. This is because enzymes are protein in nature and are denatured and deactivated by abnormal acidity or alkalinity of the reaction mixture. Various potash concentrations used for the pretreatment of cassava starch were 5.0%, 7.5%, 10.0%, 12.5% and 15.0%. Pretreatment of cassava with alkaline substance before hydrolysis solubilizes the starch, increases the surface area and as well makes the substrate easily accessible by enzymes (Zheng *et al*., 2009).

The comparison of the quantity of ethanol produced when the cassava starch was pretreated with 5.0g and 7.5g showed no significant difference.

When the potash concentration used to pretreat cassava starch was increase to 10g, 12.5g, 15.0g and 20.0g there were significant differences in bioethanol production. The statistical analysis of the result showed significant difference

Upon analysis, it was observed that 12.5% of potash used gave a significant yield of ethanol when compared with other concentrations of potash and the control experiment. However, it was also observed that the pH equilibrium of the reaction medium shifted to the right when potash was added but acetate buffer was added to maintain a balance between enzymes activity and bioethanol production (Bandaru *et al*., 2006). This discussion may indicate that alkaline pretreatment is an important factor in digestibility of lignocellulosis. However, it is not the only factor in effective enzymatic hydrolysis of biomass due to the heterogeneous nature of cellulose and the contribution of other factors.

The molecules of native starch are closely bound in the starch granule to a varying degree and those that are closely bound are not particularly susceptible to the action of enzymes. Starch granules swell and burst, the semi-crystalline structure is lost and the smaller amylase molecules leaching out of the granules, forming a network that holds water and increasing the mixture viscosity. It is not until the starch molecules have been dispersed by swelling and gelatinization in water that significant hydrolytic cleavage will take place. The objective of steaming is to solubilize the hemicelluloses, to make the cellulose easily accessible for enzymatic hydrolysis and to avoid the formation of inhibitors (Chandra et al., 2006). In a conversion procedure where the starch is heated slowly, the molecules which are closely bound are dispersed or gelatinized more slowly and therefore are available for attack by acid or enzyme at a low rate. The result of this non-uniform rate of gelatinization is that by the time all of the resistant molecules have been made available for attack; the

more easily dispersed ones have already been reduced to a relatively small molecular range. The duration of gelatinization was varied at 0-20 minutes. The values of ethanol concentration produced were statistically tested. Among the intervals of time of gelatination tested, there were significant difference at ($p<0.05$). The reason for this could be that too much heating results in disorganization of the starch structure and as well as the reduction of water activity required by enzymes. Enzymes used for the experiment when assayed gave the activity of 2.413mg/ml/min which is significantly high when compared with the theoretical enzyme assay. More also, the effect of yeast cell concentration in optimizing the bioconversion of simple sugars to alcohol was also determined. Enzyme concentration was varied. All these parameters discussed were equally varied with different concentrations of cassava starch and the best results from each testing factor are pulled together. The starch content used for experiment is 32 percent. This result is in accordance with (Wang *et al*., 2002).

Under these conditions of optimization of fermentation process for the production of bioethanol from pretreated cassava starch, the maximum yield was 37.32%. Also statistical analysis of the results obtained affirmed that there are significant differences between the optimum conditions: pH 4, potash concentration of 12.5 percent, fermentation periods of 72h, duration of gelatinization of 10 minutes, enzyme concentration of 2.58 x 10^6 cells and others, with maximum ethanol concentration occurring at the optimum conditions. In comparison, acid hydrolysis of waste cotton using 0.2 mol H_2SO_4 produced 14.2g/l (Yu and Zang, 2004), which is less efficient than alkaline hydrolysis.

5.2 Conclusion

In conclusion therefore, alkaline pretreatment of cassava or any other fermentable starch for ethanol production is recommendable, since bioenergy has very good

potential as a sustainable source. It is relatively cheap, readily available and also environmentally friendly. Bioethanol is now the most important renewable fuel in terms of volume and market value (Lincht, 2006).

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APPENDIX

REAGENTS AND MEDIA COMPOSITION

Potato Dextrose Agar (PDA) medium.

Yeast Peptone Glucose (YPG).

Nutrient Broth.

Yeast peptone Glucose broth (YPG).

Dinitro salicylic acid (DNSA) reagent.

Sodium acetate buffer.

Acid dichromate solution (0.01mol L^{-1}): Add 125ml of water to a 500ml conical flask. Carefully add 70ml of concentrated sulphuric acid with constant swirling. Cool flask under cold tap water and add 0.75g of potassium dichromate. Dilute to 250ml with distilled water.

Starch Indicator Solution($1.0^0/_0$ solute ion): Dissolve 1.0g of soluble starch in 100ml of recently boiled water, Stir until it dissolves.

Sodium thiosulphate solution (0.03mol $^{-1}$): Add 7.44g of $Na_2\ S_2O_3.5H_2O$ to a 1 litre volumetric flask. Dissolve in distilled water and dilute up to the mark.

Potash Iodide Solution (1.2molL^{-1}): Dissolve 5g of potassium iodide (KI) in 25ml of water.

EFFECT OF POTASH CONCENTRATION

ONE WAY ANOVA TABLE

% Ethanol concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	107.697	4	26.924	254.003	.000
Within Groups	1.060	10	.106		
Total	108.757	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: % Ethanol concentrations

LSD

(I) Potash_conc	(J) Potash_conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
5 % potash	7.5 % potash	.06667	.26583	.807	-.5256
	10.0 % potash	-4.16667*	.26583	.000	-4.7590
	12.5 % potash	-1.70000*	.26583	.000	-2.2923
	15.0 % potash	-6.93333*	.26583	.000	-7.5256
7.5 % potash	5 % potash	-.06667	.26583	.807	-.6590
	10.0 % potash	-4.23333*	.26583	.000	-4.8256
	12.5 % potash	-1.76667*	.26583	.000	-2.3590
	15.0 % potash	-7.00000*	.26583	.000	-7.5923
10.0 % potash	5 % potash	4.16667*	.26583	.000	3.5744
	7.5 % potash	4.23333*	.26583	.000	3.6410
	12.5 % potash	2.46667*	.26583	.000	1.8744
	15.0 % potash	-2.76667*	.26583	.000	-3.3590
12.5 % potash	5 % potash	1.70000*	.26583	.000	1.1077
	7.5 % potash	1.76667*	.26583	.000	1.1744
	10.0 % potash	-2.46667*	.26583	.000	-3.0590
	15.0 % potash	-5.23333*	.26583	.000	-5.8256
15.0 % potash	5 % potash	6.93333*	.26583	.000	6.3410
	7.5 % potash	7.00000*	.26583	.000	6.4077
	10.0 % potash	2.76667*	.26583	.000	2.1744
	12.5 % potash	5.23333*	.26583	.000	4.6410

*. The mean difference is significant at the 0.05 level.

EFFECT OF CELL CONCENTRATION

ONE WAY ANOVA TABLE

% Ethanol concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	152.383	3	50.794	136.360	.000
Within Groups	2.980	8	.372		
Total	155.363	11			

Post Hoc Test

Multiple Comparisons

Dependent Variable: % Ethanol concentrations

LSD

(I) Cell_conc	(J) Cell_conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	3.87 x 10	-1.63333*	.49833	.011	-2.7825	-.4842
5.16 x 10	2.58 x 10	-8.63333*	.49833	.000	-9.7825	-7.4842
	1.29 x 10	-6.83333*	.49833	.000	-7.9825	-5.6842
	5.16 x 10	1.63333*	.49833	.011	.4842	2.7825
3.87 x 10	2.58 x 10	-7.00000*	.49833	.000	-8.1492	-5.8508
	1.29 x 10	-5.20000*	.49833	.000	-6.3492	-4.0508
	5.16 x 10	8.63333*	.49833	.000	7.4842	9.7825
2.58 x 10	3.87 x 10	7.00000*	.49833	.000	5.8508	8.1492
	1.29 x 10	1.80000*	.49833	.007	.6508	2.9492
	5.16 x 10	6.83333*	.49833	.000	5.6842	7.9825
1.29 x 10	3.87 x 10	5.20000*	.49833	.000	4.0508	6.3492
	2.58 x 10	-1.80000*	.49833	.007	-2.9492	-.6508

*. The mean difference is significant at the 0.05 level.

EFFECT OF DURATION FO GELATINIZATION

ONE WAY ANOVA TABLE

% Ethanol concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	547.431	4	136.858	801.900	.000
Within Groups	1.707	10	.171		
Total	549.137	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: % Ethanol concentrations

LSD

(I) Gelatinized_time	(J) Gelatinized_time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
					Lower Bound
5.0 minutes	10.0 minutes	-9.10000*	.33731	.000	-9.8516
	15.0 minutes	-6.90000*	.33731	.000	-7.6516
	20.0 minutes	8.20000*	.33731	.000	7.4484
	control (0.0 minute)	-1.76667*	.33731	.000	-2.5182
10.0 minutes	5.0 minutes	9.10000*	.33731	.000	8.3484
	15.0 minutes	2.20000*	.33731	.000	1.4484
	20.0 minutes	17.30000*	.33731	.000	16.5484
	control (0.0 minute)	7.33333*	.33731	.000	6.5818
15.0 minutes	5.0 minutes	6.90000*	.33731	.000	6.1484
	10.0 minutes	-2.20000*	.33731	.000	-2.9516
	20.0 minutes	15.10000*	.33731	.000	14.3484
	control (0.0 minute)	5.13333*	.33731	.000	4.3818
20.0 minutes	5.0 minutes	-8.20000*	.33731	.000	-8.9516
	10.0 minutes	-17.30000*	.33731	.000	-18.0516
	15.0 minutes	-15.10000*	.33731	.000	-15.8516
	control (0.0 minute)	-9.96667*	.33731	.000	-10.7182
control (0.0 minute)	5.0 minutes	1.76667*	.33731	.000	1.0151
	10.0 minutes	-7.33333*	.33731	.000	-8.0849
	15.0 minutes	-5.13333*	.33731	.000	-5.8849
	20.0 minutes	9.96667*	.33731	.000	9.2151

*. The mean difference is significant at the 0.05 level.