KINETIC STUDY OF PINEAPPLE (*Ananas comosus*) PEEL CONVERSION TO GLUCOSE BY *Aspergillus niger*

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NIGERIA

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DECEMBER, 2012

DECLARATION

I hereby declare that the work in this thesis titled "Kinetic Study of Pineapple (*Ananas comosus*) Peel Conversion to Glucose by *Aspergillus niger"* was performed by me in Chemical Engineering Department under the supervision of Dr. B.O Aderemi and Dr. C.S. Ajinomoh.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been presented for another degree or diploma at any institution.

…… ……..……

Ososanya, Akinola Philip **Date**

CERTIFICATION

This thesis entitled "KINETIC STUDY OF PINEAPPLE (*Ananas comosus*) PEEL CONVERSION TO GLUCOSE BY *Aspergillus niger"* meets the regulations governing the award of Master Degree in Chemical Engineering of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This research work is dedicated to Almighty God who gave me the strength, grace and favour throughout this work. I am also dedicating this work to the memory of my Late father, Chief Peter Atanda Ososanya for the legacy he bequeathed me and to my mother, Deaconess Funmilayo Grace Ososanya for her care and love after he passed on.

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ABSTRACT

The kinetic of the production of glucose from pineapple using *Aspergillus niger* species isolated from fresh groundnut seed has been investigated. Soluble sugar was extracted from the pineapple peel, the peel was then dilignified and subsequently enzymatic hydrolysis of the polysaccharides to glucose was conducted. Effect of the operational parameters such as enzyme loading, substrate concentration, pH of buffer, particle size of substrate and incubation temperature on the hydrolysis of pineapple peel to glucose was studied in a batch process. Within the limits of experimental conditions, the results show that an optimum temperature of 45 ^{o}C, optimum pH range of 3.8-4.2 and optimum particle size of 100-150 μm were best conditions for the process. Furthermore, as substrate concentration increases from 10 g/l to 35 g/l, glucose concentration increases from 0.813 g/l to 2.32 g/l and yield was 8.13% and 6.63% respectively after an incubation time of two hours. Michaelis-Menten kinetic model was found to adequately represent the experimental data having a standard deviation of 0.001. The corresponding kinetic parameters are: maximum rate of reaction, r_{max} = 0.021 g/l.s and Michaelis-Menten constant, K_M =44.91 g/l at a process condition of 45 ^{o}C and pH 4.0.

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LIST OF SYMBOLS

K_M Rate constant (g/l)

r_{max} Maximum reaction rate (g/l.s)

r Production rate (g/l.s)

[S] Substrate concentration

β-1-4-endoglucanase - (EC 3.2.1.4.)

β-1-4-exoglucanase or cellobiohydrolase - (EC 3.2.1.91.)

β-glucosidase - (EC 3.2.1.21.)

PDA Potato Dextrose Agar

CHAPTER 1

1.0 INTRODUCTION

Continuous growth in the world population and consequent increase in the level of urbanization have grossly influenced the global annual energy demand (Akpan *et al*., 2005). Furthermore, as the world population grows, production of waste materials also increases proportionately. A perspective aimed at promoting greater sustainable development and resource recovery has influenced solid waste management and practices, and is gradually becoming implemented through policy guidelines at national levels in a number of industrialized and even developing countries. Guidelines and directives to reduce waste generation and promote waste recovery are laid down according to "waste management hierarchy", in which waste prevention, reuse, recycling and energy recovery are designed to minimize the amount of waste left for final disposal (Taherzadeh and Keikhosro, 2008)

Lignocelluloses are often a major components of different waste streams from various industries, forestry, agriculture and municipalities. Lignocellulosic wastes (LCW) refer to plant biomass wastes that are composed of cellulose, hemi-cellulose and lignin.

They may be grouped into different categories such as wood residues (including saw dust and paper mill discards); agricultural residues (including straw, peelings, stover, cobs, nutshells, bagasse, non food seeds); domestic waste (lignocellulosic garbage and sewages); food industry residues and municipal solid wastes (Mtui, 2009).

Currently, the second generation bio-products such as bio-ethanol, bio-diesel, bio-hydrogen and methane from lignocelluloses' biomass are increasingly been produced from wastes rather than from energy crops because the latter competes for land and water with food crops that are already in high demand. The use of food crops such as corn and sugar cane to produce bio-fuels is increasingly being discouraged due to the worldwide rise in food prices in order to minimize Food-Feed-Fuel conflicts. It is necessary to integrate all kinds of bio-waste into a biomass economy (Mtui, 2009).

The lignocellulosic biomass, which represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Mtui, 2009) is mostly wasted in the form of pre-harvest and post-harvest agricultural losses and are inexpensive. Due to their large abundance and renewability, there has been a great deal of interest in utilizing LCW for the production and recovery of many value–added products. Among the main recovery products include fermentable sugar, furfural, ethanol, enzyme, protein and amino acid, activated carbon, surfactants, resins, disorbent etc. Alongside producing these products, the process removes wastes from the environment.

The barrier to the production and recovery of valuable materials from LCW is the structure of lignocelluloses which has evolved to resist degradation due to cross-linking between the polysaccharides (cellulose and hemi-cellulose) and the lignin via ester and ether linkages. The main goal of any pretreatment, therefore, is to alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of

intended products. These methods cause mechanical, physical, chemical or biological changes in plant biomass in order to achieve the desired product(s).

Studies on enzymatic hydrolysis of rice straw using *Trichoderma reseei* cellulase and effect of cross-linked *Aspergillus niger* cellulase on the hydrolysis of rice hull have been reported (Kaur *et al., 1*998; Sharma *et al.*, 2001). Aderemi *et al.* (2008) studied the kinetics of glucose production from rice straw by *Aspergillus niger*. Highina *et al.* (2012) investigated production of glucose from wheat straw using *Aspergillus niger*.

Pineapple fruit (*Ananas comosus*) ranks second most widely consumed fruit. It takes roughly 18 months to grow and picked at maturity. Approximately 75% of the world's pineapple supply comes from the following countries: Thailand, Philippines, Brazil, China, Costa Rica, Nigeria, India and Kenya. Nigeria is the 6th largest producer and exporter of pineapple as at 2010. Estimated global pineapple fruits production in 2010 was 15.8 million tons (http:www.Biomassgenesis.com/summary.htm).

About 20% of a typical pineapple fruit are discarded as peels. Pineapple peels has been identified as waste resulting from the consumption of pineapple fruit but this portion discarded is rich in fermentable nutrients and lignocellulosic (Avril, 2008).

Hence, it is the goal of this study to produce glucose from pineapple peels, using crude enzyme (cellulase from *Aspergillus niger*) in a batch reactor in order to evaluate the kinetic parameters which are inevitable tools in reactor design and analysis of the process. Furthermore, the kinetic data obtained from the research will serve as a guide to evaluate

the optimum conditions for the performance(s) of the crude enzyme during the production of glucose from pineapple peels.

1.1 Research Problems

While much efforts have been expended on the conversion of the common food-feed-energy crops and some agricultural farm wastes such as straws, stovers and cobs, there exist few reports on the use of pineapple peel conversion to glucose by enzymatic hydrolysis.

Pineapple peel, which constitutes a considerable quantity of wastes being generated daily both in urban and rural settings of our country cannot be exploited effectively and efficiently, without readily available processing parameters such as processing temperatures, pH, substrate concentration etc. and kinetic constants i.e. Michaelis-Menten constant (K_M) and maximum reaction rate (r_{max}), which are presently absent in conventional literatures.

1.2 Research Justification

1. It is obvious that Nigeria is experiencing a problem in waste management. Million tons of garbage are being thrown away by Nigerians everyday and the accumulated waste occupies valuable real estate and is a source of land pollution. Pineapple fruit and its peels are part of these wastes. Therefore, pineapple peels could be made useful by transforming it into glucose thereby reducing environmental pollution and getting other value added products into Nigeria economy.

2. It is important to discover other sources of renewable raw materials for glucose production.
3. It is also necessary to develop the non-oil sector of the Nigerian economy.
4. Further more, the kinetic parameters obtained from the production of glucose from pineapple peel are vital to reactor design and process analysis.
5. The crude enzyme will serve as an alternative to commercially purified enzymes which are very expensive.
6. The research work aims to utilize pineapple peels to obtain glucose, which will significantly contribute to the reduction of Nigerian's total waste count.

1.3 Aim and Objectives

This project is aimed at evaluating the kinetic parameters associated with glucose production from pineapple peel by employing *Aspergillus niger* as cellulase source.

This aim will be achieved through the realization of the following objectives:

1. Studying the effects of operational parameters, such as: temperature, substrate concentration, enzyme loading, pH and substrate particle size during the production of glucose from pineapple peels through enzymatic hydrolysis. This is to establish the optimal or the most favorable process conditions.
2. Evaluation of kinetic parameters based on Michaelis-Menten kinetic model using the initial rate method of analysis for the reaction

1.4 Scope of the Study

This project is divided into four stages: proximate analysis of the pineapple peels, pretreatment of the pineapple peels, monitoring the effects of various operation conditions on glucose production from pineapple peel by enzymatic hydrolysis and evaluation of kinetic parameters.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 OVERVIEW OF LIGNOCELLULOSIC BIOMASS

Lignocellosic materials and wastes are great feed stocks for producing fuels and chemicals (Lee, 2002). They are renewable, inexpensive, widely available and present in ample quantities. Large amount of lignocellulosic materials are generated by commercial and agricultural processes. The best method and conditions of pretreatment depend greatly on the type of lignocelluloses. For instance, pretreatment of bark from popular 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 stalks.

2.2 COMPOSITION AND STRUCTURE OF LIGNOCELLULOSIC MATERIALS

Lignocellulosic materials have a common basic structure, but vary greatly in chemical composition and physical structure. Typically, these materials contain 30 percent to 60 percent cellulose, 10 percent to 30 percent hemicelluloses (polyoses) and 10 percent to 20 percent lignin (Lee, 2002). Cellulose provides strength and flexibility, while lignin supports and protects the cellulose from biological and chemical attack. Hemi-cellulose bonds lignin to cellulose.

2.2.1 Cellulose

Cellulose (figure 2.1 shows the structure of cellulose) is a high molecular weight linear homopolymer of repeated units of cellobiose (two anhydrous glucose rings joined via a β-1, 4 glycosidic linkages). The long-chain cellulose polymers are linked together by hydrogen and van der Walls bonds, which cause the cellulose to be packed into micro-fibrils. By forming these hydrogen bonds, the chains tend to arrange in parallel and form a crystalline structure. Therefore, cellulose micro-fibrils have both highly crystalline regions (around 2/3 of the total cellulose) and less-ordered amorphous regions. More ordered or crystalline cellulose is less soluble and less degradable ((Mussatto and Teixera, 2010).

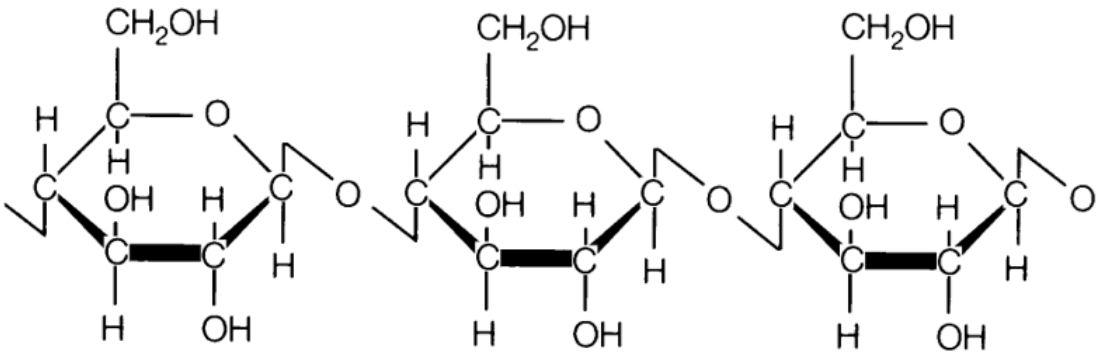


Figure 2.1: Representation of cellulose structure, Alexander *et al*., 2009

2.2.2 Hemi-cellulose

Hemi-cellulose is a linear and branched heterogeneous polymer typically made up of five different sugars – L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose as well as other components such as acetic, glucuronic, and ferulic acids. The backbone of the chains of hemicelluloses can be a homo-polymer (generally consisting of single sugar

repeat unit) or a hetero-polymer (mixture of different sugars). According to the main sugar residue in the backbone, hemi-cellulose has different classifications e.g., xylans, mannans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, alactoglucomannans, β-glucans, and xyloglucans. When compared to cellulose, hemicelluloses differ thus by composition of sugar units, by presence of shorter chains, by a branching of main chain molecules, and amorphous, which made its structure easier to hydrolyze than cellulose (Mussatto and Teixera, 2010).

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2.2.3 Lignin

Lignin is a very complex molecule constructed of phenylpropane units linked in large three-dimensional structure. Three phenyl propionic alcohols exist as monomers of lignin: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is closely bound to cellulose and hemi-cellulose and its function is to provide rigidity and cohesion to the material cell wall, to confer water impermeability to xylem vessels, and to form a physic–chemical barrier against microbial attack. Due to its molecular configuration, lignins are extremely resistant to chemical and enzymatic degradation (Mussatto and Teixera, 2010).

2.3 CELLULOSE HYDROLYSIS

Chemical and enzymatic methods are the most common techniques for hydrolyzing cellulose. The chemical method, also known as concentrated acid hydrolysis, is conducted with mineral acids such as H_2SO_4 or HCl (in the range of 10-30%), at temperatures of about 160 ^{o}C and pressures of about 10 atm. These harsh conditions (high temperature and acid

concentration) are needed to liberate glucose from the tightly associated chains, because most celluloses are crystalline. In this process, acid concentration, temperature and time are crucial factors, and must be controlled to avoid the sugars and lignin degradation to by-products (Mussatto and Teixera, 2010).

Enzymatic hydrolysis has attracted increasing attention as an alternative to concentrated acid hydrolysis because the process is highly specific, can be performed under milder reaction conditions (pH around 5 and temperature less than 50 ^{o}C) with lower energy consumption and lower environmental impact. In addition, it does not present corrosion problems, and gives high yield of pure glucose with low formation of by-products that is favorable for the subsequent hydrolysate use in fermentation processes (Mussatto and Teixera, 2010).

However, the conversion of cellulosic biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugars, still pose severe problems that are economic and technical in nature. The economic problems consist exclusively of the cost of the production of cellulase and the pretreatment of native cellulose substances to enhance their susceptibility to enzymatic attack. Besides, enzyme production is the most expensive factor, which has been estimated to be 50% of the total cost of the glucose production from cellulosic materials. Consequently, any means that is capable of reducing this cost significantly would have a dramatic effect on the cost of production of useful substances from renewable resources (Highina *et al*., 2005).

2.4 CELLULASES REACTION ON CELLULOSE

Microorganisms such as white, brown and soft rot-fungi are capable of producing extra cellular enzymes that can degrade cellulose, include *Trichoderma reesei;* *Trichoderma viride, Aspergillus niger*, etc. Bacteria such as *cellulomonas* along with *Clostridium thermacellum* can also produce cellulases. Typical proportions of the endoglucanase and exoglucanase in the extra cellular protein from *Trichoderma reseei* grown on cellulose are 15-25% and 35-55% respectively. The endoglucanase hydrolyses cellulose molecule randomly within the molecule and are more effective in combination with exoglucanase.

Enzymatic hydrolysis of cellulose is a reaction carried out by cellulase enzymes, which correspond to a mixture of several enzymes, among which at least three major groups are involved in the hydrolysis of cellulose: (1) β-1-4-endoglucanase (EC 3.2.1.4.), which attacks regions of low crystallinity in the cellulose fiber creating free chain ends;

(2) β-1-4-exoglucanase or cellobiohydrolase (EC 3.2.1.91.), which degrades the molecule further by removing cellobiose units from the free chain ends; (3) β-glucosidase (EC 3.2.1.21.), which hydrolyzes cellobiose to produce glucose (Taherzadeh and Keikhosro, 2008; Mussatto and Teixera 2010).

A schematic representation of the cellulase enzymes over the cellulose structure is shown in Figure 2.2

MECHANISM OF REACTION

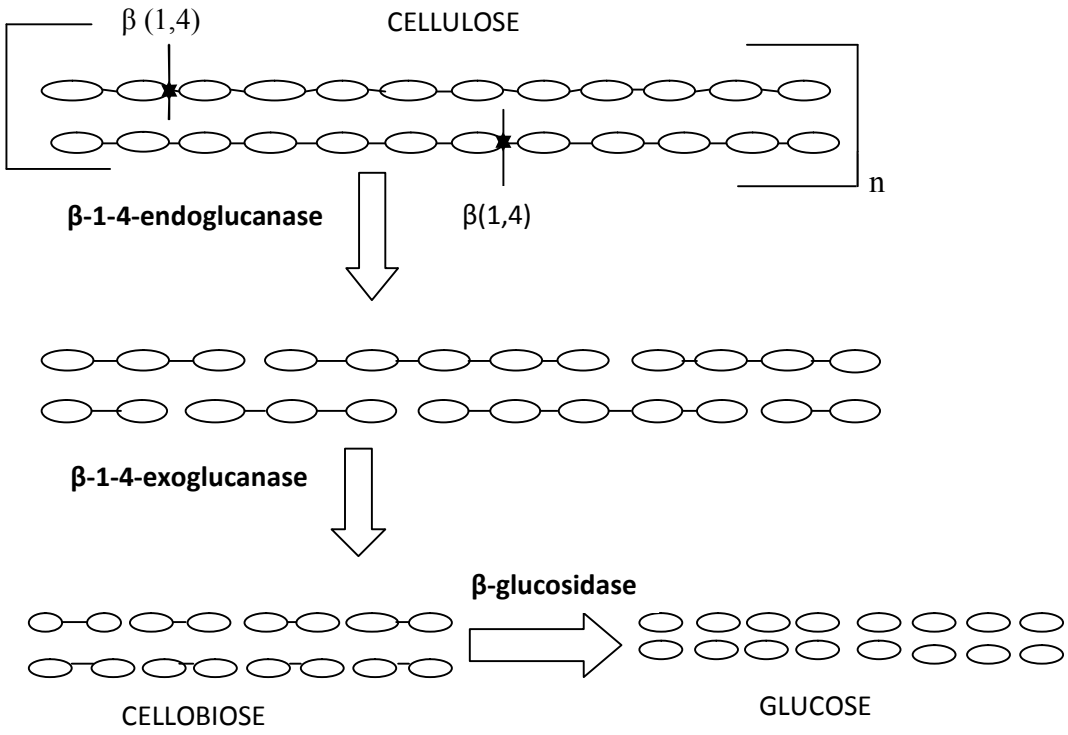


Figure 2.2: Schematic representation of cellulose enzyme over the cellulose structure (Mussatto and Teixera, 2010)

A wide variety of cellulolytic fungi and bacteria have been reported (Mtui, 2009). Among them, the enzyme system secreted by the filamentous fungus *Trichoderma reesei* has been intensively studied. Usually, lignocellulosic materials must be pretreated prior to the enzymatic hydrolysis in order to make the cellulose more accessible to enzymes.

2.5 EFFECTIVE PARAMETERS IN PRETREATMENT OF LIGNOCELLULOSES

Pretreatment is a crucial process step for the biological conversion of lignocellulosic biomass into glucose, bioethanol etc. The inherent properties of native lignocellulosic

materials make them resistant to enzyme attack. It is therefore required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrates polymers into fermentable sugars (Harmsen *et al.,* 2010).

Pretreatment has been recognized as one of the expensive processing steps in cellulosic biomass-to-fermentable sugars conversion and the best method and conditions of pretreatment depend greatly on the type of lignocelluloses.

Possible goals of effective pretreatment include the removal of lignin and disruption of the crystalline structure of the cellulose. The following criteria lead to an improvement in enzymatic hydrolysis of lignocellulosic materials:

- Increasing of the surface area and porosity
- Modification of lignin structure
- Removal of lignin
- (Partial) depolymerization of hemicelluloses
- Removal of hemicelluloses
- Reducing the crystallinity of cellulose

Ideal pretreatment process should achieve high yields of fermentable sugars, avoid degradation or loss of yielded sugars and the formation of inhibitors to the subsequent process, and require minimal energy, chemicals and equipment.

Due to the structural differences among these fractions, separation of cellulose, hemicelluloses, and lignin from lignocellulosic biomass requires the use of specific processes, which may be physical, physio-chemical, chemical or biological. There are a variety of methods that have been proved to be efficient for lignocellulosic hydrolysis. The most used are:

Mechanical Pretreatment Milling

Chemical Pretreatment

Physicochemical Pretreatment

Biological Pretreatment

2.5.1 Mechanical Pretreatment Milling

Prior to hydrolysis of lignocellulose biomass, reduction of particle size is often needed to make material handling easier and to increase the surface/volume ratio. This can be done by chipping, milling or grinding. 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 fibres.

2.5.2 Chemical Pretreatment

Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicelluloses and cellulose from LCW. Powerful oxidizing agents such as ozone and hydrogen peroxide effectively remove lignin; does not produce toxic residues for the downstream processes; and the reactions are carried out at room temperature and pressure (Sun and Cheng, 2002).

Alkali treatments refer to the application of alkaline solutions such as NaOH, $Ca(OH)_2$ or ammonia. Among these, treatment with NH_4OH and NaOH is one of the most

used for delignification of lignocellulosic materials. The alkali treatment causes swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. As a consequence, the lignin is dissolved from the raw material, being separated in the form of a liquor rich in phenolic compounds that represents the process effluent. The inconvenient of this technique is that it also degrades part of the hemicelluloses (Mussatto and Teixeira, 2010). The cost of ammonia and ammonia recovery determines the overall cost of this pre-treatment method (Harmsen *et al.,* 2010).

2.5.3 Physicochemical Pretreatment

Combined chemical and physical treatment systems are of importance in dissolving hemicellulose and alteration of lignin structure, providing an improved accessibility of the cellulose for hydrolytic enzymes (Hendriks and Zeeman, 2009). The most successful physicochemical pretreatments include thermo-chemical treatments such as steam explosion or (steam disruption), liquid hot water (LHW), and ammonia fiber explosion (AFEX) and CO_2 explosion (Sun and Cheng, 2002). In these processes, chipped biomass is treated with high-pressure saturated steam, liquid ammonia or CO_2 and then the pressure is swiftly reduced, making the materials to undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160 – 260 ^{o}C (corresponding pressure of 0.69 – 4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The processes cause hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Mtui, 2009).

2.5.4 Biological Pretreatment

This involves the use of whole organisms or enzymes in pretreatment of lignocelluloses. Both fungi and bacteria are used for bio-treatment of lignocelluloses. Commercial preparations of fungal and bacterial hydrolytic and oxidative enzymes are also widely used instead of these micro-organisms. Enzymatic hydrolysis has attracted increasing attention as an alternative to concentrated acid hydrolysis because the process is highly specific, can be performed under milder reaction conditions (pH around 5 and temperature less than 50 ^{0}C) with lower energy consumption and lower environmental impact. In addition, it does not present corrosion problems, and gives high yield of pure glucose with low formation of by-products that is favorable for the subsequent hydrolysate use in fermentation processes (Mussatto and Teixera, 2010).

2.6 FACTORS AFFECTING ENZYMATIC HYDROLYSIS OF CELLULOSE

Past techno-economic modeling works have identified the relatively large contribution that enzymatic hydrolysis adds to the cost of producing ethanol from lignocellulosic substrate. This cost was primarily due to high concentration of enzyme and long incubation time that was required to obtain a complete hydrolysis. Hydrolysis of cellulose in a batch fashion is generally characterized by a limited logarithmic phase, associated with the rapid release of soluble sugars, followed by declining rate of sugar production as the reaction proceeds. Several studies have shown that the specific hydrolysis rates declines rapidly with increased conversion of the substrate. There are several proposed explanations for the diminishing rate of hydrolysis. One proposal is that as hydrolysis proceeds, the substrate becomes enriched in the more recalcitrant cellulose as the less recalcitrant amorphous cellulose is hydrolysed. Other possible contributing factors

to the declining hydrolysis rate include enzyme denaturation through thermal, mechanical, and chemical actions (Sharma *et al.*, 2001). However, the complete mechanism of cellulose hydrolysis has not been determined, primarily due to the complexity of both the substrate and enzymatic system required to hydrolyze crystalline cellulose. Consequently, the characteristics of typical batch hydrolysis reaction, such as denaturation or inhibition, the intrinsic structural features of the substrate as the reaction progresses are yet to be properly understood. Only some of these factors are readily available for manipulation within the design or operation of the enzymatic hydrolysis step (Highina, 2005).

CHAPTER 3

3.0 MATERIALS AND METHODS

This chapter gives the description of the major materials, instruments and equipment used in the study. The experimental procedures are also presented.

3.1 PREPARATION OF CULTURE MEDIA (Potato Dextrose Agar, PDA)

The PDA used for culturing (plating) the micro-organism was composed of 200 g peeled Irish potato, 20 g of dextrose (analytical grade) and 20 g of Agar-agar powder (analytical grade) and 1000 ml of distilled water. The fresh Irish potato was sliced into small pieces and boiled in 1000 ml of distilled water for about 25-30 min. Then filtered through a muslin cloth into a 1000 ml flask, after which dextrose and agar were added and re-boiled for 15 min. to obtain complete dissolution. The pH of the medium was adjusted to 6.5 using sodium hydroxide solution in order to enhance the growth of fungi and wrapped with aluminium foil properly and sterilized by autoclaving (Griffing and George 31-417 model) for 20 min at a pressure of 15 psi and121 ^{o}C to prevent contamination. The content of the flask was allowed to cool to 45-50 ^{o}C and 5 ml of streptomycin sulphate solution was added to inhibit the growth of bacteria (Alexoponlus and Benke, 1984)

3.2 POUR PLATING

Petri dishes with cover were put into a basin containing Izal water solution for 5 min, then washed with detergent and rinsed under running tap water and spread on a table

to dry, thereafter were arranged in a canister and sterilized in an hotbox oven (Gallenkamp model) at 150 ^{o}C for 90 min. The mouth of the conical flask containing the medium was flame-sterilised to destroy surface micro-organisms and then the medium was poured into petri-dishes and covered, and left to solidify (Alexoponlus and Benke, 1984)

3.3 ISOLATION OF ORGANISMS

Fresh groundnuts were surface sterilized with sodium hypochlorite (Milton solution) in a petri dish and rinsed with distilled water. This was done to destroy secondary pathogens, then flame sterilized forcep was used to pick the groundnuts and transferred to the PDA medium in an aseptic working environment achieved by swabbing the work bench with alcohol. The petri plates were incubated at a temperature between 28-30 ^{o}C for 72 h, and the growth of micro-organism was monitored (DIFCO MANUAL, 1960) .

3.4 IDENTIFICATION OF FUNGUS

A small amount of the growth colonies were taken and smeared on a glass slide. The slide was covered with a slip and heated slightly to remove air bubbles. This was viewed under microscope (Sharmond Surgical model). The organism was identified as *Aspergillus niger* by observing it morphological structure through Microscope (Olympus Venox-T Microscope Model) and compared with the standard structure of *Aspergillus niger* given by Robert and Ellen, (1988).

3.5 SUB CULTURING

Sub Culturing from the parent culture was done to obtain pure colonies. A wire loop was sterilized using flame sterilization to kill surface bacteria in order to avoid contamination. The wire loop was used to take a portion of the growing fungal cultures from the edge of the culture plates. This was transferred to a sterilized bottle containing fresh PDA medium. Sub Culturing was repeated for 10 times so as to obtain fairly pure colony and stored in a refrigerator for subsequent exploitation.

3.6 SAMPLE COLLECTION

The fresh pineapple peel wastes were obtained from an open market in railway station market in Kaduna, see Figure 3.1.



Figure 3.1: Fresh Pineapple Peel Waste

3.7 PROXIMATE ANALYSIS

The proximate analysis of the unextracted pineapple peel was carried out by Weende's system of feed analysis (Galyean, 2010) after milling the pineapple peel to a particle size of 100-150 μm.

Dry Matter was based on the difference between the net weight and the weight after drying the pineapple peel to constant weight in an oven at 105 ^{o}C (Greenfield model) for 24 h. Using nitrogen as an index, Kjeldahl digestion method of crude protein analysis was used to estimate the crude protein present in the pineapple peel and the ether extract was obtained by extraction using petroleum ether (boiling point 40-60 ^{o}C) in a Soxhlet extractor. Ash content was determined from the loss in weight that occur by burning the pineapple peel at 550 ^{o}C in a muffle furnace (Greenfield model). The carbohydrate portion was determined using estimation by difference (i.e. the total protein, moisture content, ash content and lipid subtracted from 100).

3.8 SUBSTRATE PRETREATMENT

One kilogram of pineapple peels was added to 20 liters of distilled water and allowed to boil at 100 ^{o}C for 30 min in order to remove the soluble sugar from the pineapple peels. The extracted peels were then rinsed twice in order to remove the residual sugar. The pineapple peel was dried in an oven (Heraeus Hanau model; Type T50) at 50 ^{o}C to a constant weight and milled using the domestic grinding machine and sieved through various sieve sizes. Soaking in aqueous ammonia as described by Cao *et al.* (1996) was employed in order to delignify the substrate. This was done by mixing 20 g milled

pineapple peel with 100 ml of 2.9 M NH_4OH in a 250 ml Erlenmeyer flask. The NH_4OH solution was filtered using the muslin cloth to separate the pineapple peel from mixture. Then, the ammonia treated pineapple peel was washed repeatedly with distilled water to remove residual ammonia from the pineapple peel. It was then dried at 50 ^{o}C to constant weight in an oven.

3.9 ENZYMATIC HYDROLYSIS OF PINEAPPLE PEEL

In a typical run, the temperature of the water bath (Gallenkamp model) attached with a temperature controller (Jumo D.B.P model-Type STE-D) was set at 45 ^{o}C.

One hundred milliliters (100 ml) of 0.1 M sodium acetate buffer solution (pH 4.5) was poured into an Erlenmeyer flask, 0.1 g of *Aspergillus niger* specie and 2.0 g of untreated or treated pineapple peel were added. Samples were withdrawn on hourly basis for 5 h reaction time for analysis. The glucose concentration in the sample was determined via Randox glucose kit and Colorimeter (Jenway model 6030) at 520 nm according to Lee and Fan (1982) and Lee (2002). In separate runs, the effect of substrate pretreatment, concentration, particle size, cell loading, reaction temperature and medium pH on the extent of hydrolysis were studied as follows:

3.9.1 Effect of substrate particle sizes

The particle sizes were selected by passing the untreated pineapple peels through different sizes of standard sieves. Fractions of particle sizes of $\leq$ 50 µm, 50-100 µm, 100-150 µm, 150-250 µm, 250- 500 µm were then hydrolyzed and analyzed for glucose concentration produced.

3.9.2 Effect of temperature and pH

Enzymatic hydrolysis of the pineapple peel was carried out at different temperatures of 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C and the corresponding glucose produced was determined. Medium of different pH was used for the enzymatic hydrolysis of pineapple peels which was varied from 3 to 5.0 using sodium acetate buffer. The optimal pH for the hydrolysis was evaluated by analyzing glucose concentration produced at different pH level.

3.9.3 Effect of substrate concentration and cell loading

A series of enzymatic hydrolysis was carried out at different substrate concentrations of 10 g/l, 15 g/l, 20 g/l, 25 g/l, 30 g/l, and 35 g/l while other reaction conditions were kept constant.

The series of enzymatic hydrolysis were carried out at different cell loading of 1 g/l, 2 g/l, 3 g/l, 4 g/l, 5 g/l at constant Substrate concentration and other conditions were identical to the normal hydrolysis conditions. The glucose and concentrations were measured with time.

The flow diagram for the production of glucose from pineapple peel is shown in Figure 3.2

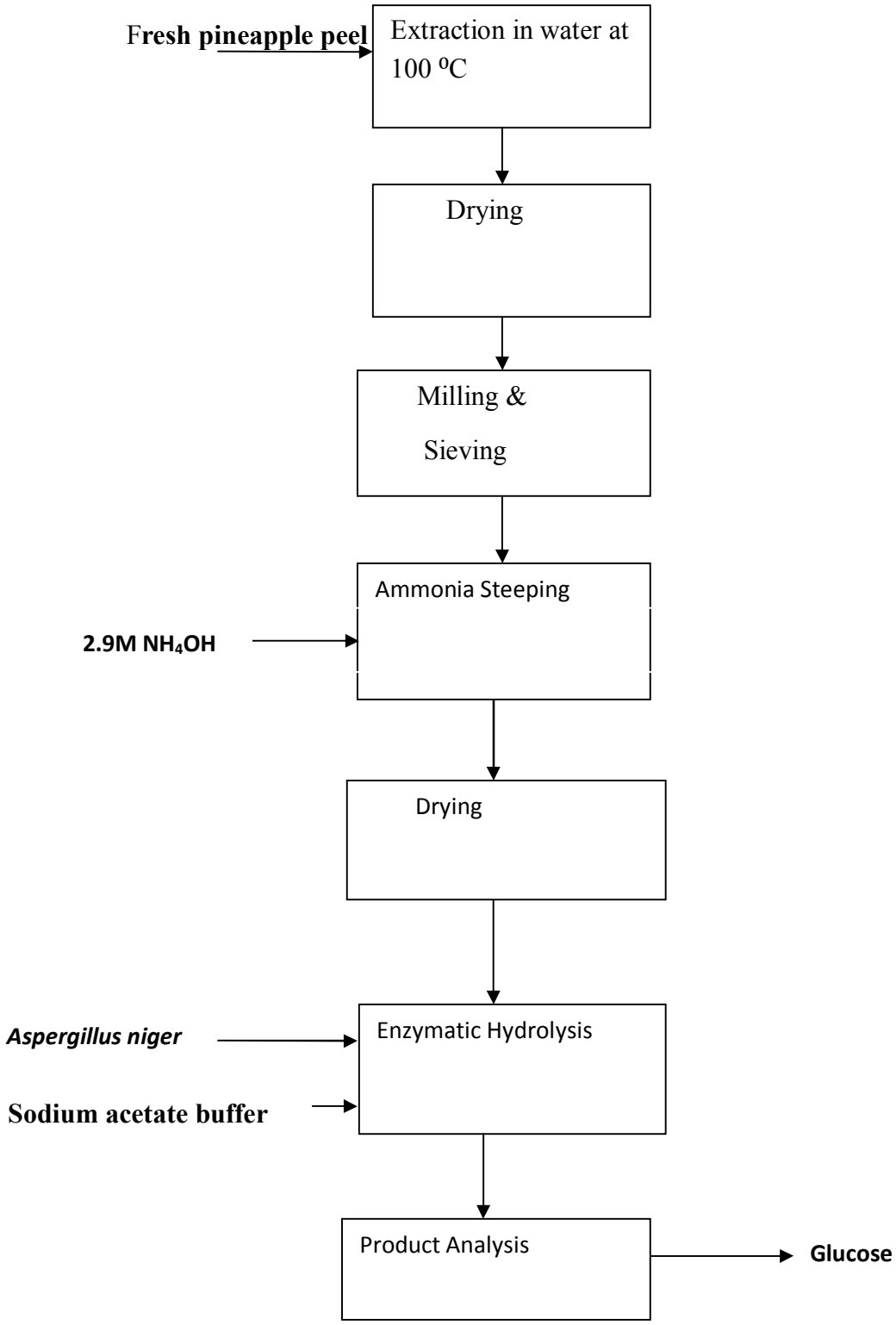


Figure 3.2: Flow diagram showing production of glucose from pineapple peel

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION OF RESULTS

4.1 ISOLATION OF MICRO- ORGNANISM

In the course of growing the organism on the PDA as a medium for isolation according to Alexophonlus and Beneke, (1984) method, black colonies, consisting of a compact white and dark colonies were obtained on a plate. This is presented in Figure 4.1a. The black colony was inferred to be *Aspergillus niger* colony (Robert and Ellen, 1988).

A sample of the black colony was sub cultured ten times; this produced a fairly homogenous black colony as shown in figure 4.1(b). The results of the morphological analysis in Figure 4.1(c) compared favourably with that of standard *Aspergillus niger* morphological shown in Figure 4.1(d). It consists of a compact white basal felt with a dense layer (head) of black condiophores typical of *Aspergillus niger* (Robert and Ellen, 1988).

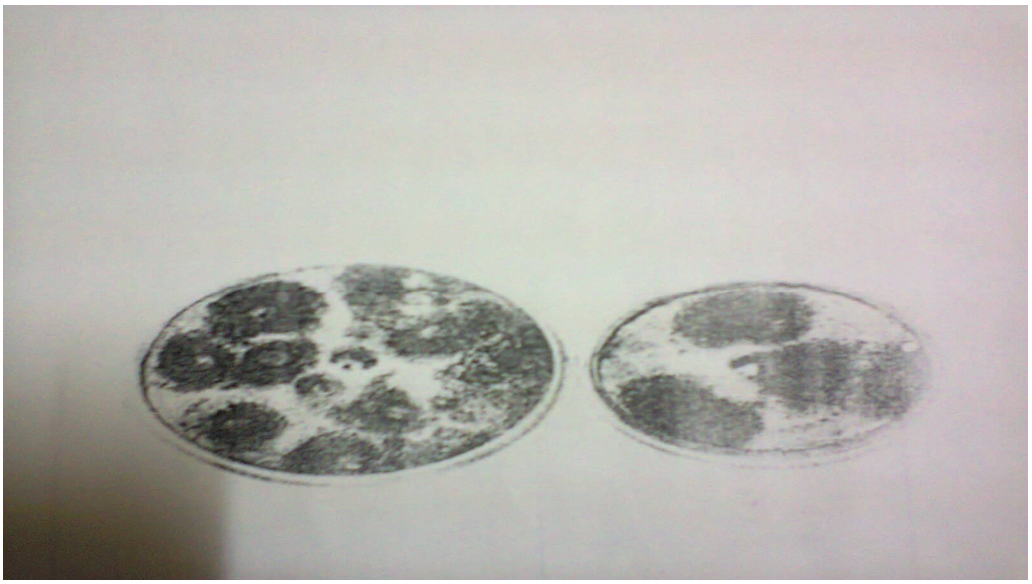


Figure 4.1a: Parent culture plate of *Aspergillus niger*



Figure 4.1b: Sub-cultured plate of *Aspergillus niger*



Figure 4.1c: Morphological structure of *Aspergillus niger * 480*

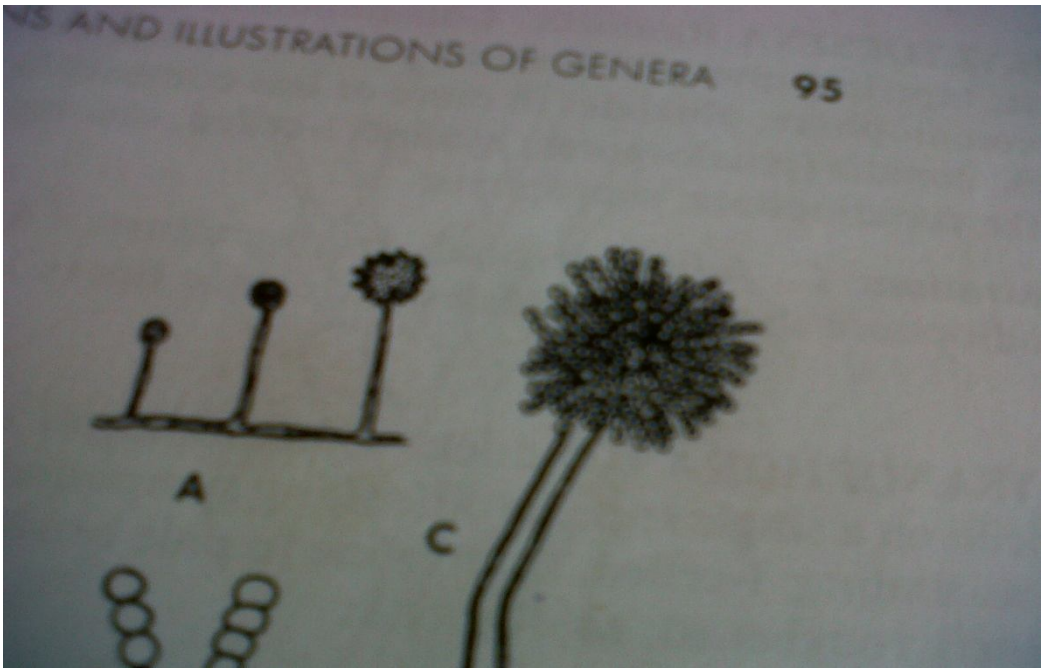


Figure 4.1d: A standard morphological structure of *Aspergillus niger at * 450 magnification (Robert and Ellen, 1988)*

4.2 PROXIMATE ANALYSIS

The proximate analysis of pineapple peel used throughout this study is as shown in Table 4.1. The result shows that pineapple peel is a suitable medium for glucose production as it contains the degradable nutrients; cellulose and hemicellulose with very low lignin (Table 4.2)

Table 4.1: Proximate Test on Pineapple peel

Component	Ash	Lipid	Moisture	Protein	Carbohydrate
Composition, %	4.3	8.85	5.12	9.64	72.09

Table 4.2: Secondary Fibre Determination

Component	Cellulose	Hemicellulose	Lignin
Composition, %	24.02	16.14	2.80

4.3 EFFECT OF PRETREATMENT

With the aim of improving the rate of hydrolysis, pineapple peel was pretreated in alkaline solution. Figure 4.2 shows the effect of pretreatment (ammonia steeping) on glucose yield. It can be inferred from Figure 4.2 that for the treated pineapple peel, part of the polysaccharide went into reaction during ammonia steeping process unlike the untreated peels whose glucose concentration increased with reaction time.

For the treated sample, parts of the polysaccharides (cellulose and hemicelluloses) were converted to by-products there-by reducing the amount of polysaccharides available for the enzyme to hydrolyse. Hence, alkaline pretreatment of pineapple peel before enzyme hydrolysis does not favour the production of glucose because of the low lignin present in the peel as revealed by the proximate analysis in Table 4.2. Similar result has been reported by Chinedu *et al.* (2008) during hydrolysis of sugar cane pulp.

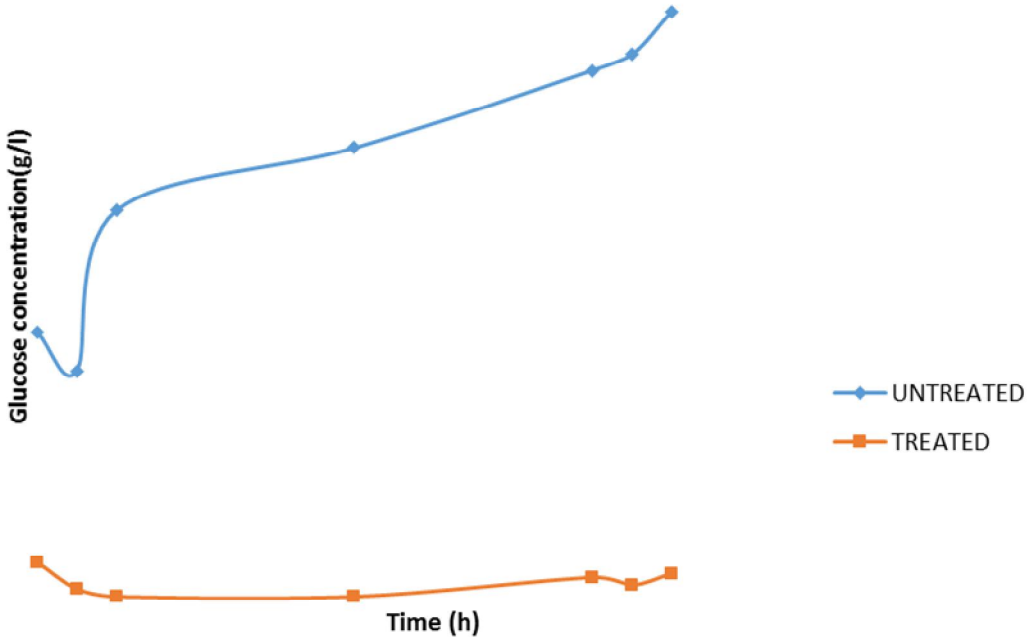


Figure 4.2: Effect of ammonia pretreatment of pineapple peel on glucose concentration

4.4 EFFECT OF PARTICLE SIZE

The effect of particle size on the hydrolysis of pineapple peel is as shown in Figure 4.3. As the particle size of the pineapple peel is reduced from 500 µm to 150 µm, the glucose concentration increased from 1.829 g/l to 1.892 g/l. This increase in glucose concentration can be justified by the reduction of crystallinity of the cellulose present in the peel and an increase in the surface area available for the enzyme to hydrolyse the β-1-4 glucosidic bonds present in the pineapple peel. Thereafter, decreasing the particle size below 150 µm has no significant effect on glucose concentration.

Similar work reported by Wen *et al.,* (2004) on hydrolysis of animal manure (lignocellulosics) for sugar production using particles sizes ranging from 350-590 µm showed similar pattern.

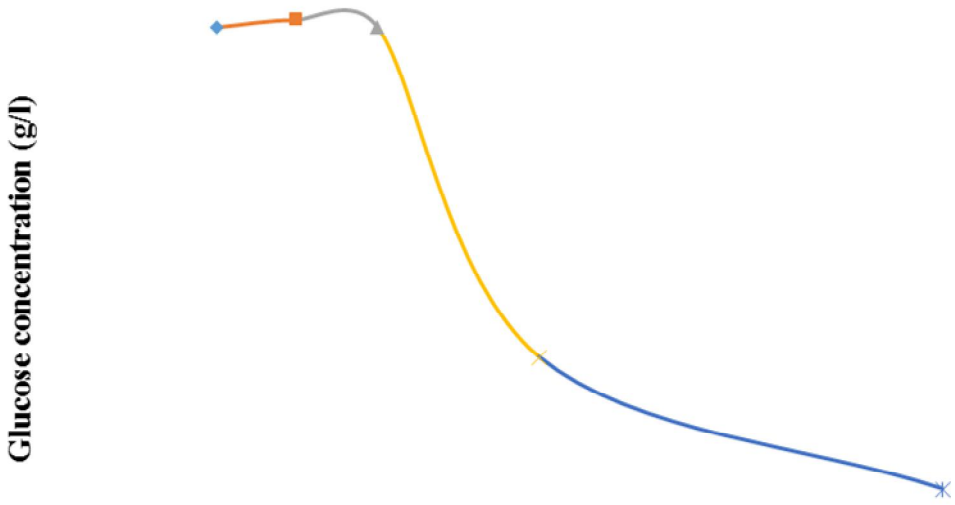


Figure 4.3 : Effect of particle size on glucose concentration

4.5 EFFECT OF TEMPERATURE

The enzyme activity was investigated at various temperatures: 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C in order to estimate the optimum temperature for glucose production. This is shown in Figure 4.4. The glucose concentration increased from 1.243 g/l to 2.427 g/l for a temperature range of 35-45 ^{o}C which signifies increase in enzymatic activity. Furthermore, increase in temperature beyond 45 ^{o}C resulted in a decrease in glucose production as it is evident in figure 4.4. As the temperature was increased to 50 ^{o}C, the glucose concentration reduced to 1.856 g/l and further increase in temperature to 55 ^{o}C further reduced the glucose concentration. This shows that 45 ^{o}C is the optimum temperature for the production of glucose from pineapple peel within the limits of the experimental conditions in this work. The observed trend could not be dissociated from lose of enzymes activity at higher temperature due to denaturing of the structure of the enzyme since they are proteineous in structure. Similar result has been reported by Highina *et al.* (2012) during the hydrolysis of wheat straw to glucose syrup which showed optimum range between 40-45 ^{o}C.

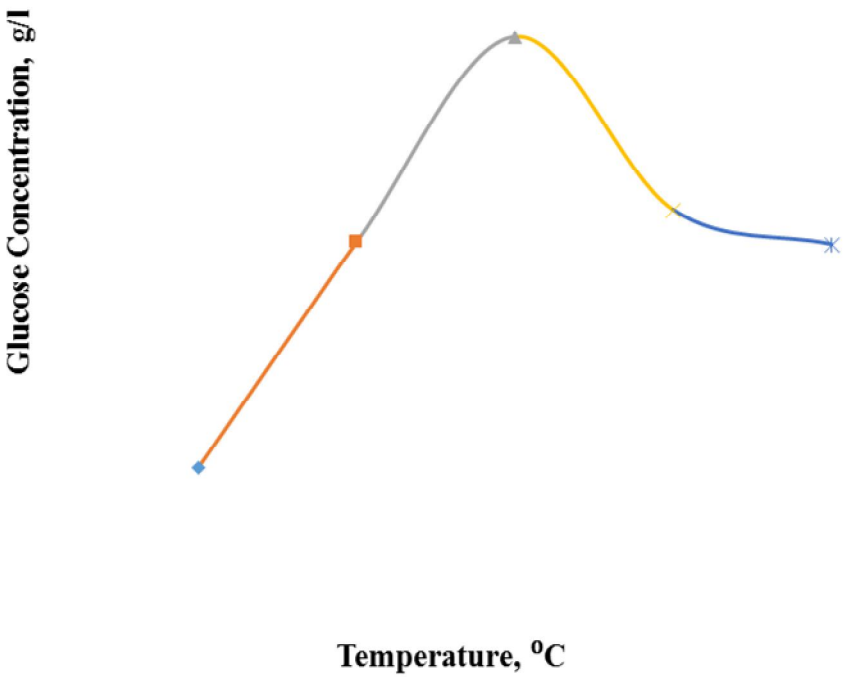


Figure 4.4 : Effect of temperature on glucose concentration

4.6 EFFECT OF pH

The influence of pH on enzyme activity is presented in Figure 4.5. The enzyme from the *Aspergillus niger* cell becomes inactive as the pH of the buffer approaches a value of 5.5. It showed optimum activity at a pH 4.0 (which is in the acidic medium) during the pineapple peel hydrolysis. The pH range which gives the optimum glucose concentration is between 3.8- 4.2.

The rate of enzyme reaction is influenced by pH because enzymes are protein, which consist of amino acids residues (i.e. amino acids minus water). These residues possess basic, neutral or acid side groups which can be positively or negatively charged at any given pH. Also, an enzyme is catalytically active when the amino acid residue at the active site possesses a particular charge. Therefore, the fraction of the catalytically active enzyme depends on the pH (Lee, 2002).

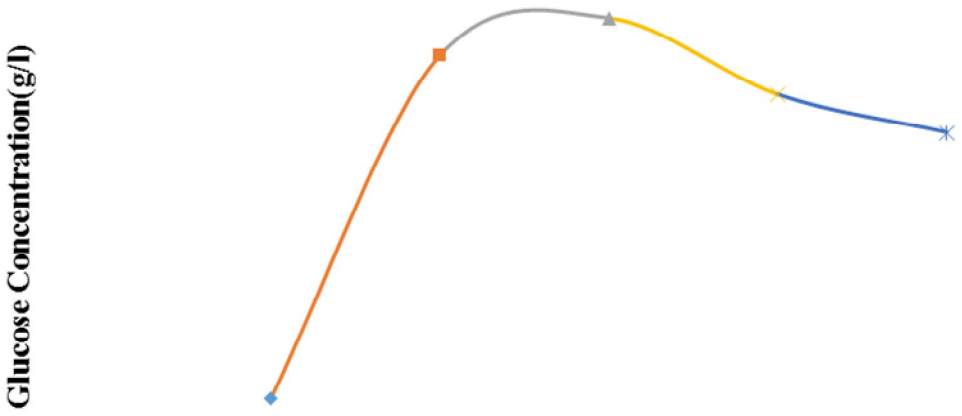


Figure 4.5 Effect of pH on *Aspergillus niger* activity on pineapple peel hydrolysis

4.7 EFFECT OF CELL LOADING

Concentration of glucose during pineapple peel hydrolysis was investigated by varying the cell loading at a fixed substrate concentration of 20 g/l and the results are as presented in Figure 4.6. From this Figure, it is seen that glucose concentration increases with increase in cell loading. This is in agreement with simple Michaelis-Menten equation in literature which describes the behaviour of an enzyme-substrate reaction (Lee, 2002).

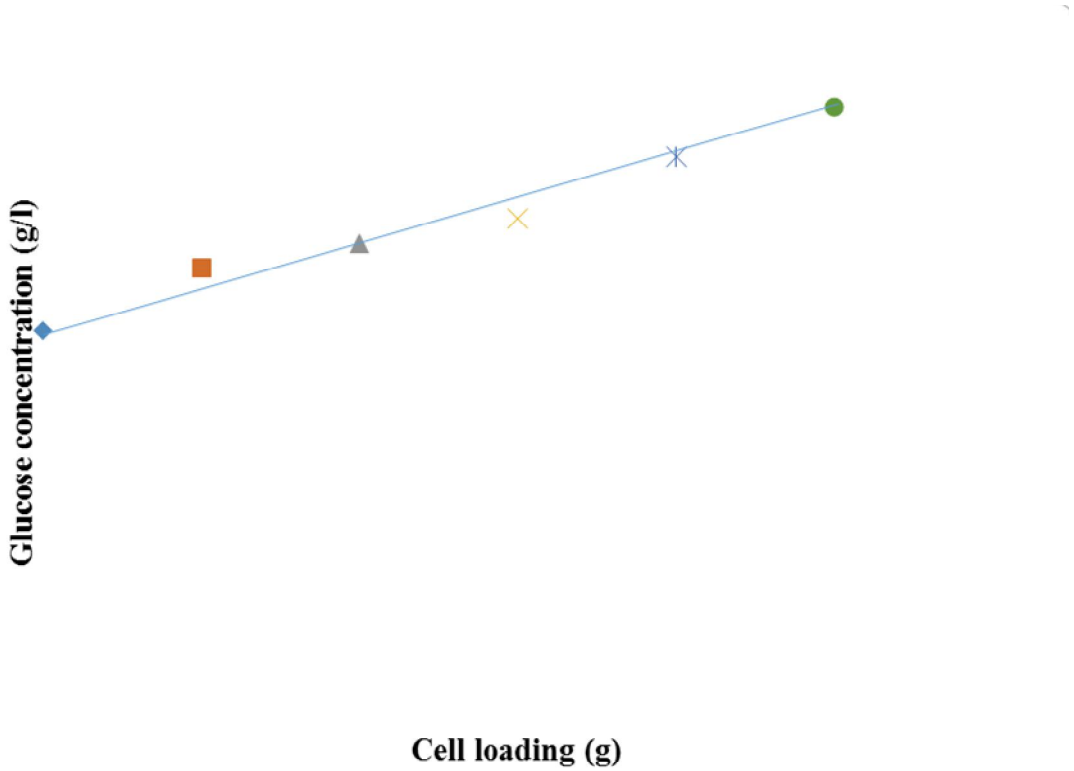


Figure 4.6 : Effect of cell loading on glucose concentration

4.8 EFFECT OF SUBSTRATE CONCENTRATION

The effect of substrate concentration on hydrolysis of pineapple peel was investigated for 2 h at a fixed cell loading of 0.1 g and the results are as presented in Figure 4.7. The results show that as the substrate concentration increases from 10 g/l to 35 g/l, there is a corresponding increase in glucose concentration while the glucose yield decreases from 8.13% to 6.62% respectively. Also end product and the initial glucose concentration present in the high substrate concentration are possible factors which inhibit the activity of the enzyme thereby resulting in lower yield in the higher substrate concentration. Furthermore, since the quantity of cell was not proportionally increased with substrate concentration, hence the low yield of glucose observed. Similar study has been reported on wheat straw hydrolyses to glucose (Highina *et al.,* 2012).

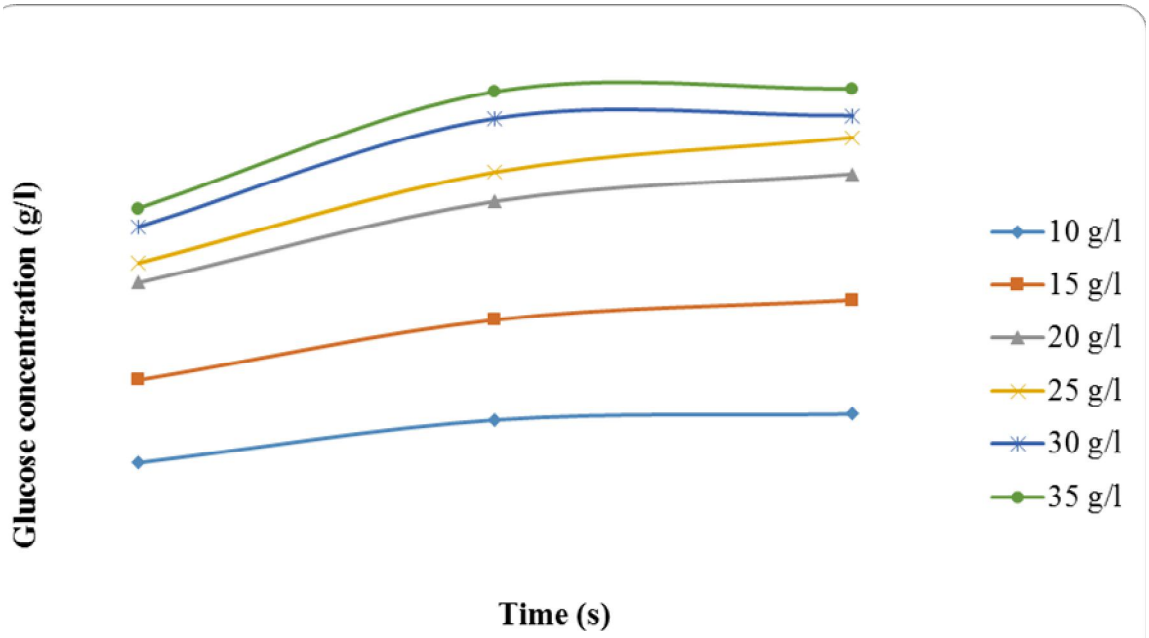


Figure 4.7: Effect of initial substrate concentration on glucose concentration at 0.1 g cell loading

4.9 DEVELOPMENT OF KINETIC MODEL

This involves the mathematical description of the functional dependencies observed in the enzymatic hydrolysis of the pineapple peel using simple Michaelis-Menten model of analysis and graphical differentiation of concentration-time curves, adopting the initial rates method as described by Lee (2002).

The modeling in this research work is based on the following observations and assumptions:

1. The cells were introduced while at their exponential growth phase
2. Glucose is the major product formed
3. The model is considering only the early stages (initial rate) of the concentration- time curve, before possible reversible reactions start to become significant.
4. The reaction was viewed as enzymes been excreted by the cells into the solution, enabling the overall system to be treated as that of enzyme-substrate kinetics.
5. A pseudo homogeneous system is assumed throughout based on solid concentration of ≤10g/l.

Table A6 in Appendices shows the results obtained from graphical differentiation of Figure 4.7 and Figure 4.8 shows the plot of initial rate against initial substrate concentration. From this curve (Figure 4.8), it can be observed that the reaction rate is proportional to the concentration when the substrate concentration is below 30 g/l. Also from the curve, it can be seen that the rate of reaction approaches a constant value as the substrate concentration exceed 30 g/l. That is the reaction rate changes gradually from first order to zero order as the substrate concentration was increased above 30 g/l.

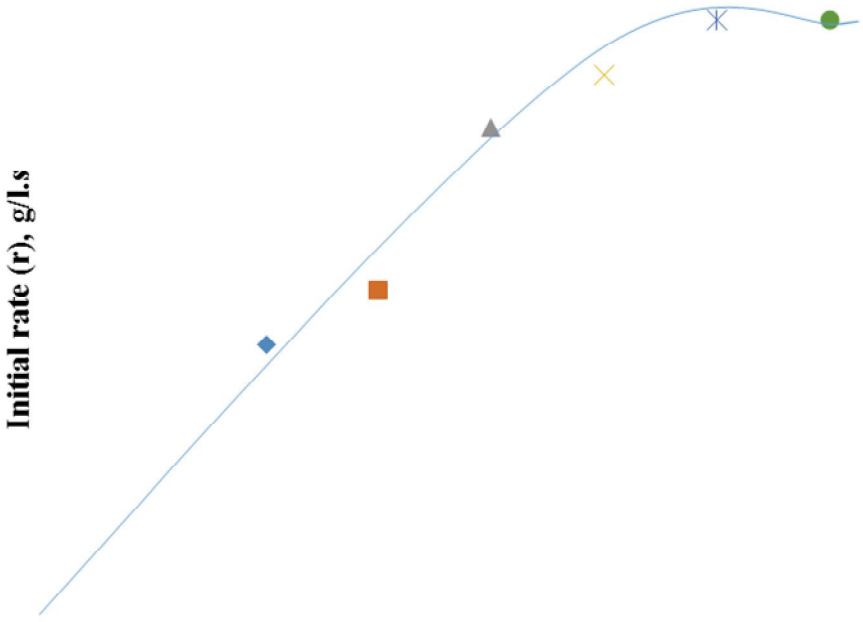


Figure 4.8 : Initial rate vs Initial substrate concentration

This form of behavior (where the product concentration will increase and reach a maximum) is commonly described by Michaelis-Menten's kinetic (equation 4.1):

$$\mathbf{r} = \frac{r_{max}[S]}{K_M + [S]} \qquad 4.1$$

Where r_{max} (maximum reaction rate) and K_M (Michaelis-Menten constant) are the kinetic parameters, which need to be experimentally evaluated.

Applying the Lineweaver – Burk method to linearize the rate expression by inverting equation (4.1), it becomes:

$$1/r = 1/r_{max} + K_M/r_{max}[S] \qquad 4.2$$

A plot of the reciprocal of initial rate, versus the reciprocal of initial substrate concentration, is expected to give a straight line with an intercept $-1/r_{max}$ and the

slope- K_M/r_{max}. A plot of this using the generated experimental data in this work is shown in Figure 4.9 from which the kinetic parameters (r_{max} and K_M) were estimated to be 0.021 g/l.s and 44.91 g/l respectively. Therefore based on the research findings and these kinetic parameters, the model equation can be represented as

$$r = \frac{0.021[S]}{44.91 + [S]} \qquad 4.3$$

4.10 EVALUATION OF MODEL PARAMETERS

In order to compare and validate the consistency of the model equation with the experimental data, a plot of the reciprocal of the rates against the initial substrate concentrations were generated as shown in Figure 4.9. It can be seen that the generated model data follows the trend of the experimental data as shown in Figure 4.8 with a standard deviation of 0.001.

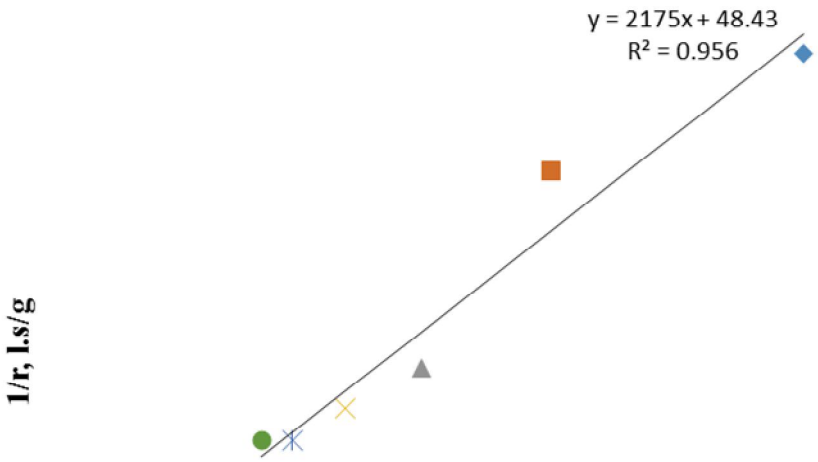


Figure 4.9: 1/r vs 1/[S]

CONCLUSIONS

From the results of this study, the following conclusions were arrived at:

1. Pre-treatment of pineapple peel with ammonia steeping has negative impact on the glucose yield.
2. The enzyme was most active for pineapple peel hydrolysis at this working conditions; temperature range of 43-47 ^{o}C with optimum at 45 ^{o}C, pH peak of 4.0, and particle size of 100-150 μm.
3. It is possible to hydrolyse pineapple peel.
4. Within the limits of experimental conditions in this work, at a fixed enzyme concentration increase in substrate concentration attracted a corresponding increase in glucose while the glucose yield decreased.
5. The kinetic parameters of the hydrolysis of pineapple peel using cell of *Aspergillus niger* at 45 ^{o}C and pH 4.0 with 0.1M sodium acetate buffer were obtained as

   r_{max}= 0.021 g/l.s and K_M = 44.91 g/l and the model equation is:

$$r = \frac{0.021[S]}{44.91+[S]}$$

6.0 RECOMMENDATIONS

The following measures are suggested in future studies aimed at exhaustive analysis of the reaction under study:

1. The possibility of using other chemicals such as hydrogen peroxide, sodium hydroxide solution for delignification should be explored.
2. The age/activity of the micro-organism (fungi) should be investigated.
3. A pilot scale based on the parameters obtained in this work should be given adequate attention to translate the benefit of this work to the domain of the public.

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APPENDICES

Table A1: Effect of Particle Size on Glucose Concentration

Particle size (µm)	**Glucose concentration (g/l)**
≤50	1.892
50-100	1.893
100-150	1.892
150-250	1.847
≥500	1.829

Cell loading- 1 g/l

Incubation Temperature – 45 ^{o}C

[Pineapple peel] - 20 g/l

pH - 4.5

Table A2: Effect of Temperature on Glucose Concentration

Temperature (^{0}C)	**Glucose concentration (g/l)**
35	1.243
40	1.861
45	2.427
50	1.951
55	1.856

Cell loading- 1 g/l

[Pineapple peel] - 20 g/l

pH - 4.0

Particle size – 100-150 µm

Table A3: Effect of pH on Glucose Concentration

pH	**Glucose concentration (g/l)**
3.0	1.816
3.5	2.180
4.0	2.220
4.5	2.139
5.0	2.099

Cell loading- 1 g/l

Incubation Temperature – 45 ^{o}C

[Pineapple peel] - 20 g/l

Particle size – 100-150 µm

Table A4: Effect of Cell Loading on Glucose Concentration

Cell loading (g)	Glucose concentration (g/l)
0.1	1.333
0.2	1.405
0.3	1.477
0.4	1.658
0.5	1.802

Incubation Temperature – 45 ^{o}C

[Pineapple peel] - 20 g/l

pH - 4.0

Particle size – 100-150 µm

Table A5: Effect of Substrate concentration on glucose concentration at 0.1g cell loading

Time (s)/ [S]	**Glucose concentrations (g/l)**					
	10	15	20	25	30	35
0	0.5893	0.9713	1.42	1.51	1.676	1.762
60	0.7857	1.2506	1.795	1.93	2.18	2.303
120	0.8127	1.341	1.921	2.092	2.193	2.316

Temperature- 40 ^{o}C

Particle size – 100-150 µm

Cell loading – 0.1 g

pH - 4.0

Yield Calculation

For Substrate concentration of 10 g/l

Glucose concentration was 0.8127g/l in 100 ml of buffer after 2 h

Therefore weight of glucose produced in 100 ml of buffer = 0.8127 g/l * 0.1 l

= <u>0.0813</u> g

Yield = Weight of glucose produced
Weight of feedstock (pineapple) used

Yield = $\frac{0.0813g * 100}{1g}$

Yield = 8.13%

For Substrate concentration of 35 g/l

Glucose concentration was 2.316 g/l in 100 ml of buffer after 2 h

Therefore weight of glucose produced in 100 ml of buffer = 2.316 g/l * 0.1 l

= 0.2316 g

Yield = $\frac{0.2316g * 100}{3.5g}$

Yield = 6.62%

Table A6: The initial rates (r) at various initial substrate concentrations [S]

[S] (g/l)	r x 10^{3} (g/l.s)	1/[S] (l/g)	1/r (l.s/g)
10.0	3.858	0.100	259.202
15.0	4.630	0.067	215.983
20.0	6.944	0.050	144.009
25.0	7.716	0.040	129.601
30.0	8.487	0.033	117.827
35.0	8.487	0.029	117.827

Graphical Differentiation to obtain the initial rate (r)

$$\textbf{Initial rate (r)} = \frac{\textbf{Change in glucose concentration}}{\textbf{Change in time}}$$

For substrate concentration of 10 g/l

Change in glucose concentration was calculated as 0.125 g/l

Change in time was calculated as 32.4 s

$$r = \frac{0.125 \text{ g/l}}{32.4 \text{ s}}$$

r = 0.003858 g/l.s

$r = 3.858 \times 10^{-3}$ g/l.s