BIOETHANOL PRODUCTION FROM SUGARCANE BAGASSE AND RICE STALK USING LOCAL STRAINS OF ASPERGILLUS NIGERANDSACCHAROMYCES CEREVISIAE AS CO-CULTURES

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ZARIA

OCTOBER, 2017

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[B.Sc. (Hons) MICROBIOLOGY, A.B.U. - 2012]

P13SCMC8024

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY,

ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

OCTOBER, 2017

DECLARATION

I declare that the work in this dissertation entitled "Bioethanol production from sugarcane bagasse and rice stalkusing local strains of *Aspergillus niger*and*Saccharomyces cerevisiae* as co-cultures" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

_____ _____

ABUBAKAR, Salim Charanchi Date

CERTIFICATION

This dissertation entitled "Bioethanol production from sugarcane bagasse and rice stalk using local strains of *Aspergillus niger*and*Saccharomyces cerevisiae* as co-cultures" by ABUBAKARSALIM CHARANCHI meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENTS

I am grateful to Almighty ALLAH, the Mighty and Majestic, Who granted me the wisdom and good health to carry out the research.

Words are not adequate enough to express my deep and sincere appreciation to my supervisors Prof.S.A. Ado and Prof.J.B. Ameh for dedicating so much time and energy in guiding, instructing and correcting me in order to ensure a successful completion of this work.

My appreciation also goes to the entire academic and non-academic staff of Department of Microbiology especially Dr. M.S. Aliyu, Dr. M.B. Tijjani, Dr. (Mrs) H. M. Rajiand MalanShituAdamuand also, to Dr.Salihu and MalanAliyu of Department of Biochemistry A.B.U., Zaria.

I also have to express my deep and profound gratitude to my parents (AlhajiAbubakar Ibrahim and Hajiya Halima Yusuf), my guardian Prof.Rabi'u Nasir of Department of Physics A. B. U. Zaria, my brothers (Eng. Ibrahim, Dr.Abdurrahman, Pharm. Mustapha, DrMuhammad and DrAhmad) and my only sister (Sis. Bilkisu) for their moral, prayerful and financial support toward a successful completion of this research and the study as a whole. May Allah (S.W.T.) reward them all and make paradiseto be their final abode (AmeenThumma Ameen).

In addition, I am also indebted to recognise the support and contributions I received from Mal. Bishir Musa, Muhammad Husssaini Ibrahim andAbdulhakeem Belloof Department of Microbiology A.B.U., Zaria for their advices and contributions. So also to MalanYahuzaMaitala of Department of Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

Finally, I would like to use this window to thank all my colleagues, who in one way or the other contributed to the success of this work thank you all.

DEDICATION

This piece of dissertation is dedicated to Almighty ALLAH(Mighty and Supreme) for His countlessfavours and mercies upon me.

ABSTRACT

This study was aimed at producing an alternative energy source(bioethanol)from sulphuric acid and alkaline hydrogen peroxide treated sugarcane bagasse and rice stalkemploying simultaneous saccharification and fermentation process using co-cultures of isolated and characterized strains of *Aspergillus niger* and *Saccharomyces cerevisiae*. The proximate composition of the substrates was determine. The composition of sugarcane bagasse and rice stalk treated with sulphuric acid were determined to be (6.30% ash), (3.05% protein), (84.15% carbohydrates) and (4.75 ash), (5.25 protein), (80.55 carbohydrates) respectively, while that of sugarcane bagasse and rice stalk treated with alkaline peroxide were (7.95% ash), (1.75% protein), (83.20% carbohydrates) and (5.10% ash), (7.00% protein), (77.65% carbohydrates) respectively.The characterization scheme used were cultural, morphological and microscopic for *A.niger*and cultural, microscopic and physiological for *S. cerevisiae*. Garden Soil(GS02) isolateof *A. niger*that possess 6.2mm and 17.1mmdiameter of zone of starch and cellulose hydrolysis respectively and palm wine (PW02) isolateof *S. cerevisiae*that tolerates up to 15% ethanol where selected for the production. At optimal fermentation conditions of 35ºC temperature, 5.0 pH, 4% substrate concentration, 300rpm agitation rate and 4 days fermentation period, a maximum of 6.20g/100ml and 5.06 g/100ml of ethanol were obtained from sulphuric acid-treated sugarcane bagasse and rice stalk respectively.After determining the quantity of the ethanol produced using specific gravity method, qualitative analysis using FTIR-Spectrophotometrywhich showed the absorbance peaks of the ethanol functional groups of all the ethanol samples produced and the functional groups had their absorption peaks within their normal ranges of 3100-3600cm^{-1}, 2800-3000cm^{-1} and 1600-1675cm^{-1} for hydroxyl functional group, saturated and unsaturated parts of the compound respectively.

In conclusion, 84.15% was the highest carbohydrate content observed, the isolates GS02 and PW02 were isolated, identified and employed for bioethanol production. The maximum quantity of ethanol obtained was 6.20g/100ml from sulphuric acid treated sugarcane bagasse and the absorption peaks of the functional groups were within their normal ranges.

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List of Abbreviations and Symbols

GS	Garden Soil
PW	Palm Wine
FTIR	Fourier Transform Infra-Red Spectrophotometry
CB-H_2SO_4	Sulphuric Acid-Treated Cane Bagasse
CB-H_2O_2	Hydrogen Peroxide-Treated Cane Bagasse
RS-H_2SO_4	Sulphuric Acid-Treated Rice Stalk
RS-H_2O_2	Hydrogen Peroxide-Treated Rice Stalk
NAOH	Sodium Hydroxide
LCB	Lignocellulosic Biomass
β	Beta
α	Alpha
µm	Micrometre
df	Degree of freedom
p	P Value
t	T Value
R^2	Coefficient of Determination
SDA	Saburoud Dextrose Agar
SSF	Simultaneous Saccharification and Fermentation
NAPRI	National Animal Production Research Institute
GDP	Gross Domestic Product

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Increase in world's energy demand and the progressive depletion of oil reserves motivate the search for alternative energy resources, especially for those derived from renewable materials such as biomass (Saxena*et al*., 2009). Global concern about climate change and the consequent need to diminish greenhouse gas emissions have encouraged the use of bioethanol as a gasoline replacement or additive (Balat*et al*., 2008). This is because cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gas emissions by 86% (Wang *et al.,* 2007).

The current estimated sugarcane production of Nigeria as at 2008 was put over 1.4m tones. This figure represents the combined production of both industrial and domestic consumption. Sugarcane for domestic consumption is produced more than that produced for industrial use. Thus, chewing cane accounts for between 55 – 65% of the total cane production, the bulk of whichis consumed raw for the sweetness of it's juice but some of it is processed into a variety of products such as sugar, molasses, bagasse, sweets and left-over leaves/stalks (Busar and Misari, 2007). Although there are vast potential for the commercial production of this crop, its processing industry did not come into existence in Nigeria until the early 1960s (Abdullahi, 2000).

*Aspergillus niger*can be used in the industry in so many ways such as production of citric and gluconic acids (Ojumu, 2003).It can also be used in biotechnology industry for production of magnetic isotope-containing variants of biological molecules (Kanso*et al.,* 1999).Furthermore, Solomon *et al.*,(1999) reported that *A. niger*can be cultured for extraction of enzyme glucose

oxidase which can be used in design of glucose biosensors due to its high affinity for beta-D glucose.

*Saccharomyces cerevisiae*is a species of yeast whose cells are round to ovoid, 5–10 μm in diameter. It reproduces by a process known as budding. It is perhaps the most useful yeast, having been instrumental in baking, and brewing since ancient times. It is believed to have been originally isolated from the skin of grapes (one can see the yeast as a component of the thin white film on the skin to winemaking of some dark-colour fruits such as plums; it exists among the waxes of the cuticle). It is one of the most intensively studied eukaryoticmodel organism in molecular and cell biology, just like *Escherichia coli*. It is the microorganism behind the most common types of fermentation (Feldmann*et al.*,2010).

Lignocellulosics in both forms of either dedicated energy crops such as sorghum, switch grass or agricultural residues such as sugarcane bagasse and corn stover or from forestry residues, serve as a renewable energy resources with energy value of about 300 x 10^8 J worldwide (Huber *et al.*, 2006). The extraction of sucrose from sugarcane stems produces a biomass residue known as bagasse. Bagasse, containing 40% - 45% cellulose, 25% – 30% hemicelluloses and 25% – 30% lignin, is an ideal candidate for the production of ethanol and biopolymers (Rein, 2007). With world energy demand predicted to increase in the near future (EIA, 2009), and the fossil fuel reserves being depleted and non-renewable, biomass resources have drawn much attention as renewable feed stocks for alternative fuels and chemicals. This attention is further driven by issues such as the need to reduce carbon dioxide emissions, the need to rely on local, renewable and sustainable fuel sources and the need to reduce dependency on remote and unsustainable fuel sources (e.g. petroleum imports). Bioethanol fuel and other products of fermentation can be manufactured from sugars and high value polymers can be synthesized from lignin. When these

are derived from lignocellulosic biomass (a non-food renewable resource), they represent a promising sustainable alternative to petroleum based fuels and chemicals (Sergios, 2011).

The characteristics of native lignocellulosic biomass such as its crystallinity, presence of lignin and hemicellulose, inaccessible surface area, degree of cellulose polymerization, and degree of acetylation of hemicelluloses make it resistant to enzymatic degradation.Therefore, to economically convert carbohydrates in lignocellulosic biomass into fermentable sugars, a pretreatment step is necessary to render the cellulose fraction accessible to hydrolysing enzymes (Wyman, 1996).

Pretreatment is amongst the most costly steps in biochemical conversion of lignocellulosic biomass, accounting for up to 40% of the total processing cost (Zheng *et al*., 2009). Thus, cost-effective pretreatment of lignocellulosic biomass is a major challenge of cellulosic biofuels and bioproducts technology research and development in recent years (Hamelinck*et al*., 2005).

The goal of pretreatment is to decrease the crystallinity of cellulose, increase biomass surface area, remove hemicellulose, and break the lignin seal (Taherzadeh and Niklasson, 2004). This process changes the biomass structure and improves downstream processing. Various pretreatment methods had been proposed to make lignocellulosic biomass susceptible to enzymatic and microbial conversion (Hendriks and Zeeman, 2009), such as wet oxidation (Schmidt and Thomsen, 1998), dilute acid hydrolysis (Saha*et al*., 2005), and steam explosion (Ballesteros *et al*., 2006), and many more.

1.2 Statement of the Research Problems

The release of greenhouse gases mainly CO_2 from automobiles and heavy duty machines fromindustries(as a result of combustion of fossil fuel)lead to global warming with a

consequence of changes in weather and climatic conditions which is a serious threat to the environment, health, economy and to humanity in general (Ballesteros *et al.,* 2006).

With the expansion ofhuman population and increase of industrial prosperity, globalenergy consumption also has increased gradually. Import oftransport fuel is affected by limited reserves of fossil fuel indicating depletion of natural fossil fuel reserves.Thus, annualglobal oil production will begin to decline within the near future. Hence, there is a serious and urgent need for an alternative fuelsource (Campbell, 1998).

Moreover, ‘‘first generation’’ approach of ethanol production led to the‘‘food versus fuel’’ conflict/competition. Thus, a search for an alternative sources for the ‘‘next generation biofuels’’ mostly based on cellulose is needed (Valentine *et al*., 2012).

The practice of indiscriminate bush burning causes environmental pollution. As such, the development of alternative agricultural menace/waste disposal which is environmentally friendly will be significantly appreciated.

In Nigeria, the exploration, drilling and transportation, and refining of petroleum has caused a lot of negative impacts including deforestation to pave way for oil exploration, soil and water pollution during oil spills (Ogri, 2001).

Oil spills are common in the Niger Delta inwhich biological entities in the spilled environment as fisheries; farms, forests and other ecosystems are impacted upon which the indigenous people rely on for their survival, thus affecting the livelihood of the people. This is principally due to the activities of vandals and illegal bunkers (World Bank, 1995).

In addition, dredging, which is typically done to create navigable accesses for exploration, drilling, pipeline, and to support oil field logistics including the transportation of crude oil, result in environmental and social impacts (Ohimain, 2008) and alteration in the topography and

hydrology of the wetland ecosystem causing the death of mangroves vegetation and other associated flora and fauna including fisheries (Ohimain, 2004).

Though, these impacts are localized within the areas of oil exploration, the socio-economic effects as wide spread agitation, illegal oil bunking, armed struggle, kidnapping and other social problems are much wider and frequent in those areas.

1.3 Justification of the Study

Use of bioethanol as a gasoline replacement or additive is encouraged to diminish greenhouse gases emissions and solve the problem of global concern about climatic change and the consequent global warming(Balat*et al*.,2008). This is because cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gases emissions by 86% (Wang *et al.*, 2007).

Furthermore, increase on world's energy demand and the progressive depletion of oil reserves motivates the search for alternative energy resource among which is ethanol from agricultural residues (field and processing residues) i.e.,lignocellulosic biomass (Saxena*et al*., 2009).

Plant cell walls are the most abundant renewable resource on our planet with 150–170 x 10^9 tons produced annually (Pauly and Keegstra, 2008). Thus, the production of ethanol from the cell walls of non-crop plants or non-edible parts of plants like sugarcane bagasse and rice stalk (i.e., lignocellulosic biomass) is considered as a sustainable solution for biofuel production without competing with food supply.

Moreover, converting such agricultural waste as agricultural field and processing residues (including cane bagasse and other substrates as papers) into valuable products like glucose and ethanol serves as a perfect alternative for treatment and disposal of such agricultural menace in an environmentally friendly and economically advantageous way.

Ogabohet *al*. (2010) reported that the anticipated benefits of biofuel industry to rural communities in Nigeria include employment and wealth creation, rural infrastructure development, expansion of rural market, rural poverty reduction and skill acquisition, increase in school enrolment, good health services and other social infrastructures/facilities including road, water, sanitation, communication, and recreation for the sustenance of the rural dwellers.

The aim is to gradually reducethe nation's dependency on imported gasoline, reduceenvironmental impacts while creating a commerciallyviable industry that can precipitate sustainable domesticjobs (NNPC, 2007).

The biomass resources are generated from agriculture, construction and municipal waste. There are great potentials in rural areas to support the production of biofuels in Nigeria. About 70% of the country's labour force resides in rural areas. This labour force can be trained and retrained to implement innovations arising from the biofuel industry (Bugajeand Mohammed 2008).

1.4 Aim of the Study

The aim of this research work was to produce and compare the yield of bioethanol from sugarcane bagasse and rice stalk using local strains of *A. niger* and *S. cerevisiae* as co-cultures.

1.5 Objectives of the Study

The specific objectives of this study were to:

1. Determine the proximate composition of sugarcane bagasse and rice stalk substrates.
2. Isolate and characterize *Aspergillus niger*and *Saccharomyces cerevisiae* from soil and palm wine respectively using conventional techniques.
3. Produce bioethanol from pretreatedsugarcane bagasse and rice stalk using simultaneous saccharification and fermentation.
4. Determine the quantitative and qualitative yield of the ethanol produced.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is ethanol that is been produced from biological materials by biological systems (microorganisms). It is water-free alcohol produced as a result of fermentation of sugar or converted starch. It is colourless clear liquid with mild characteristic odour, volatile, miscible in both water and non-polar solvents at ordinary conditions. Ethanol is less hygroscopic, has a reasonable heat of combustion, and a lower evapouration heat. It is biodegradable by nature, has low toxicity and causes little environmental pollution if spilled. Ethanol has a high octane fuel and replaces lead in octane enhancement in petrol (Appiah, 2013).

Ethanol has many uses as a solvent thus; it is potable in beer, saki, perry and variety of fermented fruit juices and in such distilled beverages as whisky, gin, brandy and other liquors. It is also used in the pharmaceutical industry in such preparations as tonics and cough syrups, as solvent for hop constituents, and as well as in cosmetics. Ethanol is also used as co-surfactant in oil-water micro-emulsions. Its other uses may involve use as antiseptic and sterilant. Lastly, Ethanol is now used as fuel and fuel additive in automobiles (Appiah, 2013).

Production of ethanol from lignocellulosic substrates instead of food crops assuresthe preventionof diversion of food crops to ethanol production. Corn-based ethanol has been blamed to be the cause of higher food prices and shortages. This is because of the factthat food producers were forced to compete with energy companies for grain, the demand for such crops is also responsible for indirect land-use change and the destruction of rain forest and wetlands to create room for more farmland. Recently, cellulosic ethanol is seen as the best alternative for reducing oil dependences at the same time protecting food crops (Otulugbu, 2012).

2.1.1 History of ethanol

Ethanol or ethyl alcohol has existed since the beginning of recorded history. As a case study, the ancient Egyptians have naturally produced alcohol by fermentation of vegetative materials. At the same ancient times, the Chinese discovered the art of distillation, which is a manoeuver that helps in increasing the concentration of alcohol in fermented mixtures. Ethanol was first prepared synthetically in 1826. This is as a result of the independent effort of Henry Hennel in Britain and S.G in France. Michael Faraday prepared ethanol by the acid-catalysed hydration of ethylene in 1828, in a similar passion to that used for industrial synthesis of ethanol nowadays (Otulugbu, 2012).

As early as 1840 in the United States, ethanol was used as fuel for lamps, but a tax levied on industrial alcohol during the civil war made this use economically discouraging which (the tax) was repelled in 1906. In 1907, Henry Ford re-introduced ethanol for use in automobile by producing his first vehicle to run on ethanol and this was made known publically. The first Ford motor company automobile was configured to run on corn-based ethanol (the most common substrate used for nearly 99% of ethanol production in the United States) (Otulugbu, 2012).

The first fuel ethanol plant was built in the U.S. in 1940s by army and operated an ethanol plant in Omaha, Nebraska, in order to provide fuel for the army and for regional fuel blending. Major quantities were not manufactured until the 1970s due to low cost of gasoline between 1940s and 1970s, however the dawn of advancement of ethanol industry has been witnessed when ethanol was used as a fuel extender during gasoline shortages caused by the OPEC oil embargoes in 1980s(Walker, 2006).

Brazil was able to keep the program alive during the 1980s as a result of a serious investment in renewable fuels since 1970s. With its robust ethanol programs, Brazil was able to develop a

comprehensive ethanol industry. By the mid-1980s, cars that runs on pure ethanol accounts for almost 90% of all new-auto sales in Brazil, thus, making the country the biggest alternative fuel market in the world (Ranese*et al*., 1998; Lundberg*et al*., 2015).For the first time in 1988, reduction of carbon dioxide emissions was begun by adding ethanol to gasoline. By 2000, Brazil deregulated the ethanol market and removed its subsidies. But still, as an order, all fuels are required to be blended with 20 to 25% of ethanol(Ranese*et al*., 1998; Lundberg*et al*., 2015).

2.1.2 Properties of ethanol

2.1.2.1 Physical properties of ethanol

Ethanol at standard temperature and pressure is volatile, flammable, clear, colourless liquid. Its odour is pleasant, familiar and has a characteristic sweet taste when diluted with water. The physical and chemical properties of ethanol are solemnly dependent upon the hydroxyl group. These two properties are to a greater extent responsible for the abnormal physical behaviour of lower molecular weight alcohols as compared to hydrocarbons of equivalent weight((Li *et al*., 2005; Torres-Jimenez*et al*., 2010).

Infrared spectrographic studies have shown that, in the liquid state, hydrogen bonds are formed by the attraction of the hydroxyl hydrogen of one molecule and the hydroxyl oxygen of a second molecule. The net effect of this bonding is to make liquid alcohol behave as though it were largely dimerized. This behaviour is analogous to the behaviour of water, which however is more strongly bonded and appears to exist in liquid clusters of more than two molecules (Tangka *et al*., 2011).

Trouton's constant for ethyl alcohol is 26.9 as compared to 21 for unassociated liquids. This constant is the entropy of vaporisation at atmospheric pressure and is obtained by dividing the molecular heat of vaporisation by the absolute temperature of the atmospheric boiling point.

Ethyl alcohol's polarity and association also manifest themselves in the non-ideal behaviour of many ethyl alcohol solutions and in the fact that ethyl alcohol can form a large number of azeotropes. A summary of physical properties of ethyl alcohol is given in Table 1 below.

2.1.2.2 Chemical properties

The chemical properties of ethyl alcohol are predominantly the function of the hydroxyl group and the reactions involved includes dehydration, dehydrogenation, esterification and oxidation. Active metals, such as sodium, potassium and calcium can be used to replace the H_2 atom of the hydroxyl group with resulting metal ethoxide (ethylate) formation and H_2 gas evolution. Sodium ethoxide is produced by the reaction of absolute ethyl alcohol and sodium or by refluxing absolute ethyl alcohol with anhydrous sodium hydroxide which addition of anhydrous acetone leads to the precipitates of the sodium ethoxide. This strong base submissively hydrolyses to give ethyl alcohol and sodium and hydroxyl ions (Chen, *et al*., 2009).

Furthermore in a large scale, water is removed by azeotropic type of distillation with benzene. Another way of producing sodium ethoxide is by reacting sodium amalgam with ethyl alcohol. Sodium ethoxide is used in organic synthesis as a condensing and reducing agent. Moreover, diethyl thiosulphate is obtained as a result of the reaction between sodium ethoxide and sulphur monochloride(Heysiattalab*et al*., 2011).

In addition to that, large scale production of barbiturates (amytal, barbital, luminal and vernal), ethyl orthoformate and other chemicals is partly pendent upon the use of sodium ethoxide. Aluminium and magnesium also react to form ethoxides, but the reaction must be catalysed by amalgamating the metal with a small amount of mercury (Kwanchareon*et al*., 2007).

However, ethyl alcohol also reacts with acid anhydrides or acid halides to give corresponding esters while the direct conversion of ethyl alcohol to ethyl acetate is believed to take place

viaacetaldehyde and its condensation to ethyl acetatea reaction also known as Tischenko reaction (Li *et al*., 2005).

Similarly, the conditions of formation of both ethylene and ethyl ether that are formed to some extent at the same time may be altered to favour one reaction or the other. The dehydrogenation of ethyl alcohol to acetaldehyde may be obtained by a vapour phase reaction over various catalysts. So also,ethyl alcohol reacts with sodium hypochlorite to give chloroform (the haloform reaction). Generally, ethyl alcohol is the only primary alcohol that undergoes a reaction in which bromoform and iodoform are obtained from sodium hypobromite and hypoiodite respectively(Tangka*et al*.,2011; Reactions ofAlcohols.doc).

2.1.3 Production of ethanol

Ethanol can be produced via two main methods; chemical synthesis and biological production (Appiah, 2013).

2.1.3.1 Chemical synthesis of ethanol

This is an option through which ethanol is been produced by hydration of ethylene an approach employed mostly by petrochemical industries. Ethanol can be produced from a reaction of ethane with steam in a reversible exothermic reaction. For each run, about 5% of ethane can be recovered. An overall 95% conversion rate can be achieved by removing the ethanol from the recycling mixture (Sergeui*et al.*, 2005).

Table 2.1: Physical Properties of Ethyl Alcohol.

Property	Value
Freezing point,	^{0}C = -114.1
Normal boiling point,	^{0}C = 78.32
Critical temperature,	^{0}C = 243.1
Critical pressure,	atm = 63.0
Critical volume,	1/mole = 0.167
Solubility in water at 20	^{0}C = miscible
Flammable limits in air	
Lower % by volume	= 4
Upper % by volume	= 19
Auto-ignition temperature,	^{0}C = 793
Flash point, open cup,	^{0}F = 70.0
Specific heat, at 20^{0}C,	cal/kg^{0}C = 0.579
Thermal conductivity, at 20^{0}C	= 0.00170J/sec.cm2 (^{0}C/cm)
Density at 15.5^{0}C	= 0.792 g/cm^3.

Sources: (NIOSH;Li *et al*., 2005 and Appiah, 2013).

2.1.3.2 Biological production of ethanol (fermentation)

The techniques, approaches and steps involved in biological means of ethanol production of ethanol depend on the starting materials. Nevertheless, the yeast *Saccharomyces cerevisiae*, through Calvin cycle, converts the sugars to carbon dioxide and alcohol by fermentation.

Sugar cane, citrus fruits, punk, and juice of trees contain simple sugars like glucose and fructose thatcan befermented direct by microorganisms.While for starch and glycogen containing materials such as rye, corn, wheat and sorghum,saccharification of the carbohydrate before the subsequent microbial conversion to ethanol is needed. Both cellulosic and lignocellulosic substrates require pretreatmentbefore fermentation (Appiah, 2013).

Ethanol production from lignocellulosic biomass is still developing, even though many technologies for that are established (Appiah, 2013). The standard ethanol production approach in the U.S. today is the conversion of corn starch to ethanol at a significant rate of 2.7 gallons of ethanol per bushel of corn, a three-step procedure consisting of a wet and/or dry milling procedure in series with a hydrolysis and fermentation step. The milling step (wet or dry) in ethanol production is the most costly step. The main difference between wet and dry milling is the treatment of the grain prior to milling (Cotter, 2006).

In dry milling, the entire corn kernel is grinded into a ‘meal’ which is then treated with enzymes in order to release the simple sugars (dextrose) that are subsequently fermented by *Saccharomyces cerevisiae*.Wet milling in the other hand uses water and acid pretreatment or ‘steeping’ technique before the milling step. This technique provides a space for extraction of co-products before the fermentation step. In all respects of wet and dry milling of ethanol production, such valuable co-products as Distiller’s Dried Grains with Solubles (DDGS), used as an animal feedstock and carbon dioxidecan be obtained (Cotter, 2006).

2.1.4 Ethanol testing

Alcohol concentrations are mostly measured by two methods in biofuel plants and concerned industries. These are:

2.1.4.1 The density dependent method

This method has a long history in the brewing industries. It puts into consideration, the density of the starting material and that of the product using hydrometer, where the change in specific gravity during fermentation represents the alcohol content (Lachenmeier*et al*.,2005).

2.1.4.2 Infrared ethanol sensor method

This is where the vibration of the frequency of the dissolved ethanol is measured using the CH band at 2900cm^{-1}. The method compares the CH band with a reference band in order to ascertain the ethanol content using Beer-Lambert's law (Yi, 2017).

2.1.5 Uses of ethanol

Ethanol has a higher compression ratio, therefore in an only ethanol engine, this will allow for increase in power output and better fuel economy than gasoline (Appiah, 2013).

The following are some of the various uses of ethanol:

1. Raw materials: It is a raw material in the manufacturing of acetaldehyde, acetic acid, ethylene, glycol, dyes, detergents and cleaning solution.
2. Pharmaceutical preparation: Ethanol has a wide application in the pharmaceutics industries. It is used in the preparation of cough syrups as well as in the preparation of antiseptics.
3. Fuel: Ethanol can be blended with gasoline to produce an oxygenated motor fuel. Has advantage of being renewable, can as also be added to gasoline to add oxygen to meet clean air act requirement and it is as well used to improve octane number in conventional

fuel. Fuel blended with very high ethanol concentration has lower hydrocarbon emission than 100% gasoline.

4. Miscellaneous: It is used as anti-freeze in automobile radiator. It is also used in preparation of alcoholic beverages (Otulugbu, 2012).

2.1.6 Economic impacts of ethanol production

Ethanol production from renewable sources is expected to boost the economy of the society concerned by creating new job opportunities especially to the local communities where the production facilities will be located (Appiah, 2013).

Adding value to farm products locally instead of selling them raw will spice up the economic status of the concerned community. As additional income to farmers and workers in the area, the wastes from their crops can also be sold to the ethanol production companies.

For a particular country, ethanol production industry will stimulate capital investment. It was said that in 2007, the ethanol industry is said to have provided employment for 238,000 workers in all sectors of the U.S economy, improved the nation's GDP by $47.6billion and added additional $12.3 billion into the pockets of American consumers. The increase in job opportunities as a result of the facility can boost a local household income by more than $100 million (Appiah, 2013).

2.2 Biofuels

2.2.1 Biofuels in developing countries

Biomass are substantially used for heat and cooking i.e. wood, animal dung and crop residues are usually burned in the rural populations of developing countries. According to the international energy agency, biomass energy accounts for around 30% of the total primary energy supply in

developing countries and over two billion people depend on biomass fuels as their domestic energy source (World Energy Outlook, 2006).

The biomass resources are generated from agriculture, construction and municipal waste. There are great potentials in rural areas to support the production of biofuels in Nigeria. About 70% of the country's labour forces reside in rural areas. These labour forces can be trained and retrained for the biofuel industry. Many developing countries have extensive biomass resources that are valuable and at same time, the demand for these biomass and biofuel increases worldwide. (Bugajeand Mohammed, 2008).

Ogabohet *al*. (2010) reported that the anticipated benefits of biofuel industry to rural communities in Nigeria encloses employment and wealth creation, rural infrastructure development, expansion of rural market, rural poverty reduction and skill acquisition, increase in school enrolment, good health services and other social infrastructures/facility including road, water, sanitation, communication, and recreation etc. for the sustenance of the rural dwellers.

2.2.2 Generations of biofuels

This refers to the different approaches of bioethanol production over the centuries worldwide. Currently, there are two generations of bioethanol production i.e. first and second generations and a third/next generation still under development.

2.2.2.1 First generation biofuels

First generation biofuels refers to biofuels produced primarily from food crops such as grain, sugar beet, starch, vegetable oil and oil seeds or animal fats. These 1st-generation biofuels have received considerable criticism as a result of rising food prices, relatively low greenhouse gas abatement, and high marginal carbon abatement cost, the continuing need for significant government support and subsidies or indirect impacts on land use and change and related

greenhouse gas emissions. Thus, these with the exception of ethanol from sugar, have limitations in their ability to achieve targets for oil-product substitution, climate change mitigation and economic growth (Sims *et al*., 2008).

Examples of such biofuels are biobutanol, bioethanol, biodiesel, biogas, methanol and vegetable oil. The three main types of first generation biofuels used commercially are biodiesel (bio-esters), ethanol, and biogas of which worldwide large quantities have been produced so far and for which the production process is considered 'established technology' (Sims *et al*., 2008).

Bio-butanol is formed by Acetone, Butanol and Ethanol fermentation (ABE fermentation) and experimental modification of the process demonstrates promising high net energy gains with butanol as the only liquid product. It can produce more energy and fortunately can be used by gasoline engines without obligation of engine modifications (Marshall, 2007).

Bioethanol derived from sugar or starch through fermentation is a substitute for gasoline even in the so called flexi-fuel vehicles. It can also serve as feedstock for the production ofEthyl Tertiary Butyl Ether (ETBE) which blends more readily with gasoline (Naik*et al*., 2010).

Biodiesel is a substitute of diesel and is produced through trans-esterification of vegetable oils, and residual oils and fats, with minor engine modifications; it can serve as a full substitute as well (Naik*et al*., 2010).

Biogas or biomethane, is a fuel that can be used in gasoline vehicles with slight modifications. It can be produced through anaerobic digestion of liquid manure and other digestible feedstock (Naik*et al*., 2010).

2.2.2.2 Second generation biofuels

The cumulative effects of the concerns of first generation approach of biofuels production have created the awareness and make it mandatory to develop biofuels from non-food biomass i.e. the

lingo-cellulosic materials and such purpose grown energy crops as vegetative grasses and short term rotation forests. These are called “the second generation biofuels” which could avoid many of the concerns faced by the first generation approach and potentially offer greater cost reduction potential in the long run (Rethabile, 2011).

2.2.2.3 Third/Next generation biofuels

Next generation biofuels involves the use of algae. Microalgal biomass provides a promising alternative as a result of its high (annual) surface biomass productivity, efficient conversion of solar energy into chemical energy and the ability to grow on non-agricultural land. In spite of these considerably eminent advantages, microalgal approach of biofuels production is not yet commercially sustainable. Challenges in improving both cultivation technologies and microalgal strains are of great concern in this regard (Li-Beisson and Peltier, 2013).

Algae cultures can be processed into lipids, otherwise known as Fatty Acid MethylEsters (FAME) and residual biomass made up of an important plant fibres. These lipids can further be processed into biodiesel while the residualbiomass can be used in pharmaceuticals, biochar, or other applications.

Algae biomass is a good sourceof next-generation biofuels that has the potential of surpassing all othersbecause of its rapid growth and extremely highyield potential. In contrast to other traditional biofuel feedstock that slowly grow, get developed or can only beharvested at a particular periods of the year, Algae can double in volume overnightand can be continuouslyharvested on a daily basis.

The production process mainly demands for some sunlight or photo-bioreactor,lots of carbon dioxide with some nitrogen, phosphorus, and other required nutrients. Algae canhelp produce

biodiesel, ethanol, hydrogen, methanol, and biopower, along with other by-products used inthe pharmaceutical industry and for bioplastics (Kanes, 2009).

2.2.2.4 Merits of cellulosic bioethanol over starch ethanol

The advantages of cellulosic ethanol over starch ethanol are as follows:

1. A 1% increase in food prices (as a result of starch approach of ethanol production) causes and average 0.75% decline in food consumption in developing countries. In addition to reducing caloric intake as food prices increase, people of low economic status also switch to less nutritious food.
2. Cellulosic ethanol is a viable alternative for reducing oil dependence while preventing food versus fuel conflict.
3. Cellulosic ethanol could help reduce air pollution. Cellulosic ethanol not only emits less greenhouse gas than gasoline but emits fewer fine particles into the air.
4. From the analysis carried out, it is apparent that a shorter residence time is required for the production of ethanol from cellulose.
5. Lignocellulosic ethanol reduces the amount of fertilizer and pesticide runoff into rivers and lakes.

A report from the University of Minnesota gives a cost analysis of both climate change and health that; For each billion-equivalent gallons of fuel produced and combusted in the US, the combined costs are $469 million, $472-952 million, but only $123-208 million for gasoline, corn ethanol and cellulosic ethanol respectively (Otulugbu, 2012).

2.2.2.5 Demerits of cellulosic bioethanol over starch ethanol production

1. It involves more complex methods of production.
2. It is very expensive to produce depending on the approach employed (Otulugbu, 2012).

3. Emissions of aldehyde, predominantly acetaldehydes which are higher in cellulosic ethanol than those of gasoline. However, acetaldehydes emissions generate relatively less adverse health concerns when compared to formaldehydes emitted as a result of gasoline combustion (Appiah, 2013).

2.2.2.6 Environmental impact of bioethanol production

The toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources. In addition, the released carbon dioxide following ethanol combustion is recycled into plant tissues because plants use CO_2 to synthesize cellulose during photosynthesis thus, ethanol production is therefore a closed carbon dioxide cycle thus, there is no net carbon dioxide addition to the atmosphere, making ethanol an environmentally beneficial energy source(Appiah, 2013).

As energy demand increases, the global supply of fossil fuels causes more harm to human health by contributing to the greenhouse gas (GHG) emission. Therefore, in this regard, cellulosic ethanol is as well aimed at reducing the GHG pollution of the atmosphere as a result of combustion of fossil fuel in automobiles.

It's been found that combustion of ethanol blended diesel (E10 and E30) increases octane number, high heating value, aromatics fractions,changes the kinematic viscosity as well as distillation temperatures. These factors lead to the complete burning of ethanol with less emission(Appiah, 2013).

Bioethanol can also play a significant role in reducing the harmful gasses in the cities. Bioethanol has the ability to reduce ozone precursors by 20-30%. Ethanol blended diesel (E-15) causes 41% reduction in particulate matter and 5% nitrous oxide emission (Appiah, 2013).

2.3 Fungi

Fungi is among the eukaryotic organisms i.e. those organisms that are spore-bearing, have absorptive nutrition, lack chlorophyll and reproduce both sexually and asexually.

They are widely distributed and are found wherever moisture is present. They are of great importance to human in both beneficial and detrimental approaches. They exist mostly as filamentous hyphae a mass of which is called mycelium with the exception of yeast that exist like a bacterial cell i.e. unicellular cells. (Nester *et al*., 2001).

Like some bacteria and protists, fungi digest insoluble organic matter by secreting exoenzymes that solubilizes the attacked matter then absorbing the solubilized nutrients. Two reproductive structures occur in fungi; sporangia forms asexual spores and gametangia forms gamets (Willey *et al*.,2009).

2.3.1 Importance of fungi

Estimates of fungal species are around 90,000 though up to 1.5 million species may exist. With bacteria and a few other chemoorganotrophic groups of organisms, fungi act as decomposers, a role of enormous significance as far as human life is concerned.

They decompose complex organic materials in the environment to simple organic and inorganic molecules. In this way, carbon, nitrogen and phosphorus and other significant constituents of dead organisms are released and made available for the benefit of other living organisms including human being (TalaroandTalaro,2002).

Fungi, considerably the yeasts, are instrumental in many industrial processes involving fermentation. Fungi also play a significant role in commercial production of many antibiotics, certain drugs and organic acids.

Furthermore, the contribution of fungi as a research tool cannot be over emphasized. It is used in the study of such fundamental biological process as biophysics, biochemistry, cytology, genetics and microbiology (Willey *et al*.,2009).

Lastly and on the other way round, fungi are the major causes of plant diseases because of them being the most vulnerable. This is because fungi can penetrate the plants through their stomates. Quit a considerable number of species attacks economically valuable crops, garden plants and many other wild plants. At the same time, pathogenic species also cause both human and animal diseases (Talaroand Talaro, 2002).

2.3.2 Characteristics of fungi

The long evolutionary history of fungi is full of examples of converging and diverging evolutions. Many structurally and functionally similar structures evolved independently (converging) while other structures became dissimilar over time (diverging).

Without a comprehensive fossil record, the use of morphology in phylogenetic analysis is deficient. However, sequence analysis of 18S rRNA and certain protein-coding genes has shown that fungi comprises of a monophyletic group with eight subdivisions. Four of which have been recognized as separate group over some time and the other four have only been proposed as a group just recently(Willey*et al*., 2009).

2.3.3 Structure of fungi

The vegetative structure of a single fungus is termed a thallus. It varies in complexity and size from the single cell microscopic yeast to multicellular moulds, macroscopic puffballs and mushrooms. Yeast is a unicellular fungus that has a single nucleus and reproduces either

asexually by budding and transverse division or sexually through spore bearing. A mould consists of long branched threadlike filaments of cells called the hyphae (Lansing, 2005a).

With the exception of yeast, fungi consist of masses of intertwined filaments of cells and each cell of single hyphae is eukaryotic with one or more nuclei and other organelles. The cell wall is composed of small amounts of cellulose and a large amount of chitin. Chitin which gives the cell wall it's rigidity, is a polymer of acetyl-glucosamine units that is glucose molecules containing amino and acetyl groups (Nester *et al*., 2001).

In quite a considerable number of species of fungi, individual cells are separated by cross sectional walls or septa and such fungi are described as septate, the septations are upon incomplete thus the pores allow adjacent cytoplasms to mix in some fungal species, the cytoplasm and organelles of neighbouring cells mingle freely. These fungi are referred to as coenocytic(Willey*et al*., 2009).

2.3.4 Classification of fungi

Fungi are classified into four divisions (phyla) based on their type of reproduction.

- Zygomycota: Members of this division are called zygomycetes. Sexually produced spores are referred to as zygospores.
- Ascomycota: Consist of a group called ascomycetes and produce ascospores.
- Basidiomycota: members of this phylum are commonly known as basidiomycetes. Its sexually produced spores are known as basidiospores.
- Deuteromycota:they are imperfect fungi and reproduces only by asexually means of reproduction.

2.4 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae belongs to the Kingdom fungi; Phylum Ascomycota*;* subphylum Saccharomycotina; Class Saccharomycete; Order Saccharomycetales; Family Saccharomycetaceae and Genus *Saccharomyces* (Feldmann, 2010).

Yeast in general is a unicellular fungus. The precise classification is a field that uses the characteristics of the cell, ascospore and a colony. Physiological characteristics are also used to identify species. The most important characteristic is their ability to ferment sugars for the production of ethanol. Budding yeast are true fungi of the phylum Ascomycetes and class Saccharomycetes orHemiascomycetes (Willey *et al*., 2009).

It can disperse widely in natural habitats. They are common on plant leaves, flowers, soil and salt water. It is also found on the skin surfaces and in the intestinal tract of warm blooded animals symbiotically or as parasites. The common yeast infection is typically candidiasis caused by the yeast like fungus called *Candida albicans. Candida* is also a cause of diaper rash and thrust of the mouth and throat (Talaroand Talaro,2002).

Moreover, yeast in general as single cell can multiply or divide by budding e.g., *Saccharomyces* or direct division (fission) e.g., *Schizosaccharomyces* or may grow as simple irregular filarments i.e., mycelium. In sexual reproduction, most yeast forms asci, which can contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or as with certain yeasts, fuse with other ascospores (Talaro and Talaro,2002).

2.4.1 History of *Saccharomyces cerevisiae*

The word "*Saccharomyces*" was derived from Greek word which means "sugar mold" while *"cerevisiae*" was derived from Latin word and it means "of beer" *Saccharomyces cerevisiae* is otherwise known as brewer's yeast or ale yeasts or top fermenting yeast or baker's yeast.

Itscells are round to ovoid, 5-10µm in diameter, reproduce by a division process known as budding and are the microorganisms behind most common types of fermentations.It is a species of budding yeast which is about the most important yeast because of its instrumental importance in baking and brewing. It is believed that it was originally isolated from the skins of grapes. It is about one of the most intensively studied eukaryotic model organism in molecular and cell biology much like *Escherichia coli* as the model prokaryote.

2.4.2 Growth metabolism and life cycle of *Saccharomyces cerevisiae*

The sizes of haploid and diploid cells vary with the phase of growth from one strain to another. Typically, diploid cells are 5-6µm ellipsoids while the haploids are 4µm spheroids in diameter respectively. During exponential growth phase, haploid cultures tend to have higher numbers of cells per cluster compared to diploid cultures. Also, haploid cells have buds that appear adjacent to the previous once, whereas diploid cells have buds that appear to be at the opposite pole. Each mother cell usually forms no more than 20-30 buds, and its age can be determined by the number of bud scars left on the cell wall (Nester *et al*., 2001).

Strains reach a maximum density of 2 x 10^8 cells/ml in YPD medium and titres that are 10 times this value can be achieved under such special conditions as pH control, continuous addition of balanced nutrients, filter-sterilized media and extreme aeration during fermentation (Willey *et al*., 2009).

An integral characteristic of *S. cerevisiae* is itsability to utilize two different metabolic pathways: aerobic respiration and anaerobic fermentation. *S. cerevisiae* is considered a heterotrophic organism because it breaks down carbohydrates to use as energy (Otterstedt*et al*., 2011). *S. cerevisiae* performs respiration or fermentation depending on the presence or absence of oxygen (Madigan and Michael, 2009). When undergoing respiration, *S. cerevisiae* completely reduces pyruvate to carbon dioxide, utilizing oxygen as the final electron acceptor. This provides the organism with the largest amount of ATP. When *S. cerevisiae* undergoes anaerobic fermentation, glucose is fermented to produce ethanol (Otterstedt*et al*., 2011). Fermentation will only occur under anoxic conditions because respiration yields much more energy per glucose than fermentation (Madigan and Michael, 2009). Fermentation produces carbon dioxide and ethanol, which explain the carbonation and alcohol found in beer (Otterstedt*et al*., 2011).

The life cycle of *S. cerevisiae* begins as a unicellular diploid that reproduces through budding. Under times of environmental stress, meiosis occurs and results in four haploid spores. These spores are called ascospores. The haploid spores can be one of two "mating types", a or α. The combination of two asci of different mating types will result in a diploid vegetative cell. *S. cerevisiae* also has a HO gene, which allows asci to switch mating types. Strains with this gene are referred to as homothallic. As a result of this, a mother cell can switch mating types and fuse with the daughter cell after division. This inbreeding can result in a loss of heterozygosity (Landry *et al*., 2011).

2.4.3 Industrial uses of *Saccharomyces cerevisiae*

S. cerevisiae is known as top fermenting yeast. It is one of the major types of yeast used in the brewing of beer along with *S. carlsbergensis*. During the fermentation process, it rises to the top of the fermentation vessel (Barnette, 2003).

This organism has long been utilized in fermentation of the sugars contained in rice, wheat, barley and corn to produce alcoholic beverages and in the baking industry, it is commonly used as baker's yeast and often taken as vitamin supplement because it is 50% protein and it is a rich sources of B vitamins, niacin and folic acid. The yeast's function in baking is to ferment sugars present in the flour or dough. This fermentation produce a significant rise in the quantity of carbon dioxide which is trapped within tiny bubbles and results in the dough rising and ethanol (Jean-luc*et al*., 2007).

Itis a widely used model organism in science. Yeast are comparatively similar in structure to human cells both being eukaryotic. Many proteins important in human biology were first discovered by studying their homologues in yeast (Barnett, 2003).

2.5 *Aspergillus niger*

Aspergillus niger is a fungus and one of the most common species of the genus Aspergillus. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions and peanuts and is common contaminant of foods. It is ubiquitous in soil and it is commonly reported from indoor environments. Some strains of *A. niger* have been reported to produce potent mycotoxins called Ochratoxins (Abarca*et al.*, 1994).

2.5.1 Taxonomy of *Aspergillus niger*

A. niger belongs to the domain Eukaryota, kindomfungi, phylum Ascomycota, subphylum Pezizomycotina, classEurotiomycetes, orderErotiales and family Trichocomacea. *Aspergillus niger* is included in Aspergilus subgenus circumdati, section nigri.This section includes 15 related black spored species that may be confused with *A. niger*including *A. tubigensis, A. foetidus, A. carbonarius A. awamori* (Samson *et al.*, 2004).

2.5.2 Uses of *Aspergillus niger* in bioethanol production

Simultaneous saccharification and fermentation (SSF) process has been extensively studied to reduce the inhibition of end product of hydrolysis (Saxena*et al.*, 1992). In the process, reducing sugars are produced from cellulose hydrolysis by the cellulolytic and amylolytic activity of *A. niger* and simultaneously fermented to ethanol by *S. cerevisiae*. This greatly reduces the effect of product inhibition on the enzyme cellulase produccer(*A. niger*) (Kadar *et al.,* 2004).

Biomass fermentation processes either make use of commercial enzymes or biocatalyst (enzyme producing organisms) that are capable of partial hydrolysis of the cellulosic content of the biomass involved (Kadar *et al.,* 2004). Other fungi that have been studied are *Trichodermaressei* and *S. cerevisiae* (Xin *et al.,* 1993),*Candida* and *Kluveromyces* strains (Ballesteros *et al.,* 1991) and freshly isolated baker's yeast (Kadar *et al.,* 2004).

2.5.3 Industrial uses of *Aspergillus niger*

Various strains of *A. niger* are used in the industrial preparation of citric acid and gluconic acid (Ojumu*et al.,* 2003).

Many useful enzymes are produced through industrial fermentation of *A. niger* for example, *A. niger* glucoamylase is used in the production of high fructose corn syrup and pectinases are used in cider and wine clarification. Alpha galactosidase, an enzyme that breaks down certain complex sugars is a component of beano drug and other medications for flatulence reduction (Solomon *et al.,* 1999).

It is used in the biotechnology industry for the production of magnetic isotope-containing variants of biological macromolecules (Kanso*et al.,* 1999) as well as for the extraction of the enzyme glucose oxidase that is used in the design of glucose biosensors due to its high affinity for beta-D glucose (Solomon *et al.,* 1999).

2.6 Enzymes Produced by Lignocelluloses Degrading Fungi

The myriad of biomass-active, lignocellulose-degrading enzymes may be classified in ways emphasizing catalysed reaction (specificity), structural/evolutionary relation, or other aspects. Based on the International Union of Biochemistry and Molecular Biology's Enzyme Nomenclature and Classification, these enzymes belong to glycosidases, lyases, esterases, peroxidases, carbohydrate oxidases, phenol oxidase and other classes according to their main reactions (Sweeney and Xu, 2012).

Based on carbohydrate-active enzymes (Cantarel*et al.*, 2009) and fungal oxidative lignin enzymes (Levasseur*et al.*,2008) databases, lignocellulose-degrading enzymes belong to Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), Lignin Oxidases (LO), and Lignin Degrading Auxiliary enzymes (LDA) according to their sequence and structural homology.Becausethesefamilies has shared three-dimensional structure and catalytic mechanism(a feature that may facilitate bioinformatics analysis of metagenomic data), yet enzymes from different families may catalyse the same reaction (Sweeney and Xu, 2012).

A distinct structural feature of lignocellulose-degrading enzymes is their modularity. In addition to the catalytic core, many of these enzymes also possess non-catalytic but functionally important domains, including carbohydrate-binding modules (CBM), fibronectin 3-like modules, dockerins and immunoglobulin-like domains (Sweeney and Xu, 2012).

Having affinity to bundled, individual polysaccharide chains or single carbohydrate molecules, CBM anchors host enzymes to targeted carbohydrate substrates (Guillen *et al.*,2010), and in some cases even disrupts crystalline cellulose microfibrils to assist the reaction ofcellulase enzymes (Sweeney and Xu, 2012).

Many lignocellulose-degrading enzymes employ hydrolytic reactions (mainly acting on hemicellulose), while others employ oxidoreductive ones (mainly acting on lignin), to convert lignocellulose (Vocadlo and Davies,2008).

Almost all cellulases and hemicellulases are carbohydrate hydrolases relying on either a "retaining" mechanism which yields product of the same anomeric configuration after breaking a glycosidic bond with a "double-displacement" hydrolysis or an "inverting" mechanism which yields product of the opposite anomeric configuration after breaking a glycosidic bond with a "single nucleophilic-displacement" hydrolysis, both involving either of the two acidic amino acid residues (Glutamic or Aspartic) as a proton donor or general acid and as a nucleophile or base. In addition, "retaining" hydrolases might also act as glycosyltransferase (Vocadlo and Davies,2008).

All lignin-active peroxidases are heme-containing, some with manganese co-active center while phenol oxidases are copper-containing oxidoreductases relying on electron-transfer from lignin to high valence Fe(V/VI)-oxo, Mn(III) or Cu(II), which leads to subsequent radicalization, bond scission, or derivatization in lignin (Sweeney and Xu, 2012).

2.6.1 Cellulases

Hydrolytic scission of the β(1→4) glucosidic bond in cellulose, leading to the formation of glucose (Glc) and short cellodextrins, is carried out mainly by cellulases, a group of enzymes comprising cellobiohydrolase (CBH), *endo*-1,4-β-D-glucanase (EG), and β-glucosidase (BG). Although cellulose is relatively simple in terms of composition (anhydro-Glc units only) and morphology (mainly amorphous, monoclinic, triclinic and crystalline), there is a vast natural diversity of cellulases with catalytic modules (Duncan and Schilling, 2010).

2.6.2Hemicellulases

In plant cell walls, cellulose is shielded by hemicellulose, a group of complex polysaccharides made by different glyco-units and glycosidic bonds. Degradation of hemicellulose(which not only liberates cellulose for cellulases but also converts hemicellulose into valuable saccharides) is carried out mainly by an array of interdependent and synergistic hemicellulases(Sweeney and Xu, 2012).

Different plants have different hemicelluloses: acetylated glucomannan(galacto)(as well as arabinoglucuronoxylan), glucuronoxylan, and arabinoxylan are major hemicellulose in softwood, hardwood, and grass, respectively (Scheller and Ulvskov, 2010). Hence different hemicellulase combinations are needed for different biomass feedstocks in industrial biomass conversion. Synergism of hemicellulases is found both amongst hemicellulases themselves and between hemicellulases and cellulases(Gao *et al.*, 2010).

Common hemicelluloses include β-glucan, xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan, galactan, polygalacturonan, *etc*., which are targets of β-glucanase, xylanase, xyloglucanase, mannanase, arabinase, galactanase, polygalacturonase, glucuronidase, acetyl xylan esterase respectively and other enzymes (Van den Brink and de Vries, 2011).

Among hemicellulases, glycoside hydrolases (belonging to about 29 GH families) hydrolyseglycosidic bonds, carbohydrate esterases (belonging to about 9 CE families) hydrolyse ester bonds, polysaccharide lyases (belonging to about 5 PL families) cleave glycosidic bonds,*Endo*-Hemicellulases cleave internal/backbone of glycosidic bonds, whereas other glycosidases remove mainly the chain's substituents or side chains (Chundawat*et al*.,2011).

Cellulolytic microbes produce many hemicellulases such as *endo*-β-Xylanases and β-Xylosidase; Acetyl Xylan Esterase, Feruloyl Esterase and Glucuronoyl Esterase; α-L-Arabinofuranosidase,

α-Galactosidase and α-Glucuronidase; Glucanase, Mannanase, Xyloglucan Hydrolase and Pectinase along with cellulases for effective lignocellulose degradation (Chundawat*et al*.,2011).

2.6.3 Lignocellulose oxidoreductases

The secretomes of most cellulolytic microbes (particularly white and brown rots) contain oxidoreductases, in some cases at quite significant levels, whose co-presence with hydrolytic or lytic enzymes indicates the importance of having an oxidoreductive system as part of effective biological lignocellulose degradation (Lundell*et al.*,2010).

The main task of these oxidoreductases is likely aimed at degradation of lignin that is entangled with hemicellulose or cellulose and upon inhibitory to hemicellulases. Lignin degradation is imperative for industrial enzymatic biomass-conversion, because it does not only increases hemicellulose accessibility for hemicellulase but also diminishes hemicellulase inactivation caused by lignin adsorption.

Lignin peroxidase, Mn peroxidase and versatile peroxidase are extracellular fungal heme peroxidases with high potency to oxidatively degrade lignin. Upon interaction with H_2O_2, these enzymes form highly reactive Fe(V) or Fe(IV)-oxo species, which extract electrons from lignin (to cause oxidation or radicalization) either directly or via Mn(III) species (Sweeney and Xu, 2012).

Laccase is a multi-copper oxidase secreted by numerous lignocellulolytic fungi. This enzyme can directly oxidize phenolic parts of lignin, or indirectly oxidize non-phenolic lignin parts with the aid of suitable redox-active mediator (Sweeney and Xu, 2012).

Aryl-alcohol oxidase, glyoxal oxidase and various carbohydrate oxidases are also involved in natural lignocellulose degradation. These enzymes can generate H_2O_2 from O_2 with concomitant oxidation of aromatic alcohol, glyoxal, or reducing carbohydrates. The generated H_2O_2 may

support lignin-degrading peroxidases or non-enzymatic lignin degradation (Sweeney and Xu, 2012).

Cellobiose dehydrogenase (CDH) is produced by many lignocellulotic fungi. The flavoheme enzyme may dehydrogenate or oxidize cellobiose or other cellodextrins to corresponding aldonolactones, with concomitant quinone reduction to phenol or oxygen reduction to hydrogen peroxide(Sweeney and Xu, 2012).

Until recently, the role of CDH in enzymatic degradation of lignocellulose was largely considered only from a Fenton chemistry perspective (Zamocky*et al.*, 2006) or mitigation of cellulases' product inhibition by cellobiose(Bey*et al.*, 2011); however, the recent discovery of the CDH stimulation of GH61 enzyme activity may shed new light upon its function (Langston *et al.*, 2011).

The lignin-degrading oxidative species generated by these oxidoreductases may attack inhibitors of industrial sugar-to-fuel/chemical microbial conversion, making the process more effective (Parawira and Tekere, 2011). However, they may also attack hemicellulose or even hemicellulases, potentially hampering industrial enzymatic hemicellulose conversion (Bendl*et al.*,2008). For the peroxidases, autooxidation or inactivation may severely limit their performance. Applying such enzymes to industrial enzymatic biomass conversion needs further research for overall benefit (Sweeney and Xu, 2012).

2.7 The Lignocellulosic Materials

Ethanol can be produced from different kinds of raw materials. The raw materials are classified into three categories: cellulose, simple sugars and starch (Demirbas, 2005).

Cellulosic biomass can be grouped into four groups depending on the type of the source: wood, municipal solid waste, waste-paper and crop residue resources. The lignocellulosic biomass

comprises of cellulose, hemicelluloses and lignin and it is a rigid structure and harsh treatment is required to break it down. Cellulose is a remarkable crystalline organic homopolymer consisting solely of repeating units of glucose held together by beta-glucosidic linkages in a giant straight chain molecule. In contrary, hemi-cellulose consists of short, linear and highly branched chains of sugars thus, a hetero-polymer of D-xylose, D-glucose, D-galactose, D-mannose and L-arabinose (Appiah, 2013).

One of the primary functions of lignin is to provide structural support for the plant. Thus, in general, trees have higher lignin contents than grasses. Unfortunately, lignin which contains no sugars encloses the cellulose and hemicellulose molecules, making them difficult to reach. Cellulose molecules consist of long chains of glucose molecules as do starch molecules, but have a different structural configuration. These structural characteristics plus the encapsulation by lignin makes cellulosic materials more difficult to hydrolyse than starchy materials.Hemicellulose also comprises long chains of sugar molecules; but in addition to glucose (a 6-carbon or hexose sugar), it also contains pentose (5-carbon sugars). To complicate matters, the exact sugar composition of hemicellulose can vary depending on the type of plant. Since 5-carbon sugars comprise a high percentage of the available sugars, the ability to recover and ferment them into ethanol is important for the efficiency and economics of the process (Kuloyo, 2012).

2.8 Technology of Lignocellulosic Conversion to Fuel Ethanol

This encompasses such techniques involved in lignocellulosics conversion to ethanol as pretreatment, hydrolysis and fermentation of the substrate and distillation of the product.

2.8.1Pretreatment of lignocellulosic materials

The most important processing challenge in the production of biofuel is pretreatment of the biomass. Lignocellulosic biomass is composed of three main constituents namely lignin, hemicellulose and cellulose. Pretreatment refer to the solubilisation and separation of one or more of these components of the biomass so as to make the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). The lignocellulosic complex is made up of a matrix of cellulose and lignin bound by hemicelluloses chains. The pretreatment is done to break the matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Sanchez and Cardona, 2008).

Pretreatment is undertaken to bring about a change in the macroscopic and microscopic size and structure of biomass as well as submicroscopic structure and chemical composition. It makes the lignocellulosic biomass susceptible to quick hydrolysis with increased yields of monomeric sugars (Mosier *et al.*, 2005b).

Goals of an effective pretreatmentprocesses are (i) Formation of sugars directly or subsequently by hydrolysis (ii) To avoid loss and/ or degradation of sugars formed (iii) To limit formation of inhibitory products (iv)To reduce energy demands and (v) To minimize costs.

Physical, chemical, physicochemical and biological treatments are the four fundamental types of pretreatment techniques employed. In general a combination of these processes is used in the pretreatment step.

2.8.1.1 Physical pretreatment

a. Mechanical size reduction

The first step for ethanol production from agricultural solid wastes is comminution through milling, grinding or chipping. This reduces cellulose crystallinity (Sun and Cheng, 2002) and improves the efficiency of downstream processing. Wet milling, dry milling, vibratory ball milling and compression milling are usually done.

The power input for mechanical comminution of agricultural materials depends on the initial and final particle sizes, moisture content and on the nature of waste (hardwood, softwood, fibrous, etc) being handled (Sun and Cheng, 2002).

Size reduction may provide better results (Pandey, 2009) but very fine particle size may impose negative effects on the subsequent processing such as pretreatment and enzymatic hydrolysis. It may generate clumps during the subsequent steps involving liquid and may lead to channellingand specific energy consumption can also be increased.

It is advisable to use hammer mill or ball mill for hardwood and cutter mill for softwood. Ball milling (BM) and wet disk milling (WDM) are other processes which can be used for comminution (Da Silva *et al*., 2010).

b. Pyrolysis

Pyrolysis is an endothermic process where more energy input is required. In this process the materials are treated at a temperature greater than 300°C, whereby cellulose rapidly decomposes to produce gaseous products such as H_2 and CO_2 and residual char. The decomposition is much slower and less volatile products are formed at lower temperatures (Mtui, 2009).

The residual char is further treated by leaching with water or with mild acid. The water leachate contains enough carbon to support microbial growth for bioethanol production. Glucose is the

main component of water leachate. An average of 55% of total weight of biomass is lost during water leaching. Fan *et al*. (1987) have shown 80-85% conversion of cellulose to reducing sugars with more than 50% glucose through mild acid leaching (1N H_2SO_4, 95°C, 1h).

c. Microwave oven and electron beam irradiation pretreatment

Pretreatment of lignocellulosic biomass in a microwave oven is also a feasible method which uses the high heating efficiency of a microwave oven and it is also easy to operate (Bjerre, 1996). Microwave treatment utilizes thermal and non-thermal effects generated by microwaves in aqueous environments.

In the thermal method, internal heat is generated in the biomass by microwave radiation, resulting from the vibrations of the polar bonds in the biomass and the surrounding aqueous medium. Thus a hot spot is created within the nonhomogeneous material. This unique heating feature results in an explosion effect among the particles and improves the disruption of recalcitrant structures of lignocellulose (Hu and Wen, 2008). Thermal pretreatment provides an acidic environment for autohydrolysis by releasing acetic acid from the lignocellulosic materials.In the non-thermal method, i.e., the electron beam irradiation method, polar bonds vibrate, as they are aligned with a continuously changing magnetic field and the disruption and shock to the polar bonds accelerates chemical, biological and physical processes (Sridar, 1998). High energy radiation results in more changes in cellulosic biomass including increase of specific surface area, decrease of degree of polymerization and crystallinity of cellulose, hydrolysis of hemicellulose and partial depolymerisation of lignin.

Ooshima*et al*. (1984) reported an improvement in total reducing sugar production by a factor of 1.6 and 3.2 for microwave radiated rice straw and bagasse respectively. Microwave

pretreatmentof rice straw and bagasse followed by lignin extraction has been reported to give a yield of 43-55% of total available reducing sugars (Kitchaiya, 2003).

2.8.1.2Physicochemical pretreatment

a. Steam explosion or autohydrolysis

Steam explosion is a promising method of pretreatment which makes biomass more accessible to cellulase attack (Neves *et al*., 2007). In this method, the biomass fractionates to yield levulinic acid, xylitol and alcohols (Balat*et al*., 2008). In this method the biomass is heated using high pressure steam (20-50 bar, 160-290°C) for a few minutes; the reaction is then stopped by sudden decompression to atmospheric pressure (Sanchez and Cardona, 2008).

When steam is allowed to expand within the lignocellulosic matrix, it separates the individual fibres (Balat*et al*., 2008). The high recovery of xylose (45-65%) makes steam explosion pretreatment economically attractive (Neves *et al*., 2007).

b. Liquid hot water method

The liquid hot water method uses compressed hot liquid water (at pressure above saturation point) to hydrolyse the hemicelluloses (Neves*et al*., 2007). It is a hydrothermal pretreatment method which releases high fraction of hemicellulosic sugars in the form of oligomers.

The treatment generally occurs at temperatures of 170-230°C and pressures above 5 MPa for 20 min. It however, also contributes to the production of small amounts of undesired degrading compounds such as furfural, carboxylic acid, that are very toxic to ethanol fermentation as they inhibit microbial growth (Talebnia*et al*., 2010). As xylose recovery is relatively high (88-98%), and no acid or chemical is required, it is an environmentally attractive and economically interesting method (Neves *et al*., 2007).

(Yu *et al*., 2010) studied two step liquid hot water treatment of *Eucalyptus grandis* and obtained a xylose recovery of 86.4%. Maximal glucose yield of 70-76% corresponding to 80% of xylan removal from soybean straw was obtained through combined liquid hot water and alkaline treatment (Wana*et al*., 2011).

c. Ammonia fiber explosion

Ammonia fibre explosion (AFEX) pretreatment involves liquid ammonia and steam explosion (Balat*et al*., 2008). AFEX is an alkaline thermal pretreatment which exposes the lignocellulosic materials by high temperature and pressure treatment followed by rapid pressure release.

This method does not produce inhibitors of the downstream processes and small particle size is not required for efficacy (Mosier *et al.,* 2005a). This pretreatment has the drawbacks of being less efficient for biomass containing higher lignin contents (e.g. softwood newspaper) as well as of causing solubilisation of only a very small fraction of solid material particularly hemicellulose (Talebnia*et al*., 2010).

The advantages are that it is simple and has a short process time. It is more effective for the treatment of substrates with less content of lignin compared to sugarcane. This system does not directly liberate any sugars, but allows the polymers (hemicellulose and cellulose) to be attacked enzymatically which break down to sugars.

The AFEX process requires efficient ammonia recovery to be economical due to the high cost of ammonia. A possible approach is to recover the ammonia after the pretreatment by evapouration. The major parameters influencing the AFEX process are ammonia loading, temperature, high pressure, moisture content of biomass, and residence time (Talebnia*et al*., 2010).

Moderate temperatures of 60-100^{0}C are used and residence time may vary from low (5-10 min) to intermediate (30 min) depending on the degree of saturation of the biomass. Ammonia loading

is done at 1kg ammonia per kg of dry substrates. The process optimization of AFEX pretreatment can yield better conversion rates. At optimal conditions, 90% cellulose and hemicellulose conversions have been achieved. In AFEX treatment, low enzyme loading is required compared to other pretreatment methods (Wyman *et al*., 2005).

d. Carbon dioxide explosion

CO_2 explosion acts in a manner similar to that of the steam and ammonia explosion techniques. However, CO_2 explosion is more cost effective than ammonia explosion and does not cause the formation of inhibitors as in steam explosion. Conversion yields are higher compared to the steam explosion method (Hamelinck*et al*., 2005).

2.8.1.3 Chemical pretreatment

Chemical pretreatment methods involve the usage of dilute acid, alkali, ammonia, organic solvent, SO_2, CO_2 or other chemicals. These methods are easy in operation and have good conversion yields in short span of time.

a. Acid pretreatment

Acid pretreatment is considered as one of the most important techniques and aims for high yields of sugars from lignocellulosics. It is usually carried out by concentrated or diluted acids (usually between 0.2% and 2.5% w/w) at temperatures between 130°C and 210°C.

Sulphuric acid is widely used for acid pretreatment among various types of acid such as hydrochloric, nitric and phosphoric acids (Cardona *et al*., 2009). Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis (Balat*et al*., 2008).

The acid medium attacks the polysaccharides, especially hemicelluloses which are easier to hydrolyse than cellulose (Cardona *et al*., 2009). However, acid pretreatment results in the production of various inhibitors like acetic acid, furfural and 5-hydroxymethylfurfural. These

products are growth inhibitors of microorganisms. Hydrolysates to be used for fermentation therefore need to be detoxified.

Moiser*et al*., (2005b) reported higher hydrolysis yield from lignocellulose pretreated with diluted H_2SO_4 compared to other acids. A saccharification yield of 74% was obtained from wheat straw when subjected to 0.75%v/v of H_2SO_4 at 121°C for 1hr. (Saha*et al*., 2005).

b. Alkaline pretreatment

Alkaline pretreatment of lignocellulosics digests the lignin matrix and makes cellulose and hemicellulose available for enzymatic degradation (Pandey *et al*., 2000). Alkaline pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier *et al.,* 2005b). Alkali treatment of lignocellulose disrupts the cell wall by dissolving hemicelluloses, lignin, and silica, by hydrolysinguronic and acetic esters, and by swelling cellulose.

Crystallinity of cellulose is decreased due to swelling. By this process, the substrates can be fractionated into alkali-soluble lignin, hemicelluloses and residue, which make it easy to utilize them for more valuable products. The end residue (mainly cellulose) can be used to produce either paper or cellulose derivatives (Cardona *et al*., 2009).

Hydroxides of sodium, potassium, calcium and ammonium can be used in this process. Sun *et al*., (1995) studied the effectiveness of different alkaline solutions by analysing the delignification and dissolution of hemicellulose in wheat straw. They found that the optimal process condition was that using 1.5% NaOH for 144 hr. at 20°C, releasing 60% and 80% lignin and hemicellulose respectively.

c. Wet oxidation

In wet oxidation, the feedstock material is treated with water and either by air or oxygen at temperatures above 120°C. The water is added to the biomass at a ratio of 1L per 6g of biomass (Martín *et al*., 2007).

The transfer of hemicelluloses from solid phase to the liquid phase is promoted in this technique. It does not hydrolyse the liberated hemicellulose molecules. The products of hemicellulose hydrolysis during wet oxidation are sugar oligomers (Cardona *et al*., 2009).

There have been several studies on wet oxidation as a pretreatment strategy using different substrates. Pedersen and Meyer (2009) obtained yields of 400 and 200g/kg of wet oxidation treated wheat straw for glucose and xylose respectively after 24 hr. at 50°C.

d. Organosolvpretreatmen

Organic solvent or organosolv pulping processes are alternative methods for the delignification of lignocellulosic materials. The utilization of organic solvent/water mixtures eliminates the need to burn the liquor and allows the isolation of the lignin (by distillation of the organic solvent).

Examples of such pretreatments include the use of 90% formic acid and that of pressurized carbon dioxide in combination (50% alcohol/water mixture and 50% carbon dioxide) (Cardona *et al*., 2009). Other various organic solvents which can be used for delignification are methanol, ethanol, acetic acid, formic acid and acetic acid and acetone etc. (Zhao *et al*., 2009).

A combination of ammonia and ionic liquid pretreatments of rice straw resulted in 97% conversion of cellulose to glucose (Nguyen *et al*., 2010).

2.8.1.4 Biological pretreatment

Degradation of the lignocellulosic complex to liberate cellulose can be brought about with the help of microorganisms like brown rot, white rot and soft rot fungi. Biological

pretreatmentrenders the degradation of lignin and hemicellulose (Talebnia*et al*., 2010) and white rot fungi seem to be the most effective microorganism. Brown rot fungi attacks cellulose while white rot fungi and soft rot fungi attacks both cellulose and lignin (Prasad *et al*., 2007).

Cellulase-less mutant was developed for the selective degradation of lignin and to prevent the loss of cellulose but in most cases of biological pretreatment the rate of hydrolysis is very low. This method is safe and energy saving due to less mechanical support (Talebnia*et al*., 2010). It needs no chemicals but low hydrolysis rates and low yields impede its implementation (Balat*et al*., 2008).

In the case of a marine microorganism *Phlebia sp*. MG-60, it was seen that when the substrate was supplemented with a nutrient medium such as Kirk's Medium, better delignification was observed compared to sterilized water (Cardona *et al*., 2009). Bio-delignification generally needs long periods of time.

In a biological pretreatment study with an aim to release the sugars from the lignocellulosic matrix of sugarcane trash using a number of microorganisms it was observed that both cellulose and lignin contents of the raw material can be drastically reduced. Reduction in the cellulose content by *Aspergillus terreus* was about 55.2% while delignification was found to be about 92% (Singh *et al*., 2008).

2.8.2 Hydrolysis process

The cellulose molecules are composed of long chains of glucose molecules. In the hydrolysis process, these chains are broken down to "free" the sugar, before it is fermented for alcohol production. There are two major hydrolysis processes: a chemical reaction using acids, or an enzymatic reaction.

2.8.2.1 Acid hydrolysis

There are two types of acid hydrolysis; dilute and concentrated acid hydrolysis. Dilute acid is conducted at high temperatures and pressure and has short reaction time. The hemicellulose component is depolymerized at lower temperatures than the cellulosic fraction (Chandel*etal*., 2007).

The biomass is mixed with dilute sulphuric acid and held at temperatures ranging from 120-220^{o}C for short period of time to hydrolyse the hemicellulose to xylose and other sugars. Depolymerisation of the hemicellulose fraction of the cell wall enhances cellulose digestibility in the residual solids (Appiah, 2013).

While concentrated acid hydrolysis uses higher concentrations of acid followed by dilution with water to dissolve the hydrolysed sugar, the process allows for complete and rapid conversion of both hemicellulose and cellulose to glucose and other sugars with a little degradation (Chandel*et al*., 2007). The concentrated acid process uses 70% sulphuric acid at between 40–50°C for 2 to 4 hrs. The low temperature and pressure aims to minimize the sugar degradation (Appiah, 2013).

Acid hydrolysis has been investigated as a possible process for treating lignocellulosic materials such as wood chips, rice husk, sugar beet pulp, wheat straw and sugarcane bagasse. Sugarcane bagasse can be hydrolysed using dilute acid to obtain a mixture of sugars with xylose as the major component.

However, the disadvantage with acid hydrolysis is that at high temperature or long residence time the monosaccharides produced degrades and gives rise to fermentation inhibitors like furan compounds, weak carboxylic acids, phenolic compounds, acetic acid and furfural or lignin degradation products. These inhibitors affect the performance of the ethanol producing microorganisms (Chandel*et al*., 2007).

To remove these inhibitors and increase the hydrolysatefermentability, several chemicals and biological methods have been used. These methods include overliming, charcoal adsorption, ion exchange, detoxification with laccase (Chandel*et al*., 2007), and biological detoxification. The cost is however higher than the benefits achieved (Appiah, 2013).

Processes such as two-stage acid hydrolysis can be employed to produce xylose and glucose. Treatment with dilute sulphuric acid at moderate temperatures (the first stage of acid hydrolysis) has proven to be an efficient means of producing xylose from hemicellulose. In the second stage more drastic reaction conditions are employed and glucose can be produced from cellulose hydrolysis(Appiah, 2013).

2.8.2.2 Enzymatic hydrolysis of cellulose

Enzymatic hydrolysis (saccharification) of cellulose is carried out by cellulase enzymes, which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50°C) and does not have a corrosion problem.

Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic However, only a few of these microorganisms are known to produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose *in vitro.* Fungi of the genera *Trichoderma* and *Aspergillus* are thought to be prominent cellulase producers, and crude enzymes produced by these microorganisms are commercially available for various industrial applications (Appiah, 2013).

Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases. *Cellulomonasfimi*and *Thermomonosporafusca*have been extensively studied for cellulase production.Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum*and *Bacteroidescellulosolvens*produce cellulases with high specific activity, they do not produce high enzyme titres. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi(Appiah, 2013).

For fungi, members of the genera that have received considerable attention under aerobic conditions are *Chaetomium*, and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum*and *Serpula* (Basidiomycetes); *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium,Paecilomyces, Penicillium*, and *Trichoderma* (Deuteromycetes) and *Mucor*(Zygomycetes). The genera with prominent cellulolytic activity within anaerobic fungal division are Chytridiomycetes are *Neocallimastix, Piromyces, Caecomyces, Orpimomyces and Anaeromyces*(Appiah, 2013).

Hydrolysis involves the use of an enzymatic cocktail consisting of different hydrolytic enzymes such as cellulases, xylanases and mannanases (Chandel*et al*., 2007). Cellulases are usually a mixture of several enzymes. At least three major groups ofcellulases are involved in the hydrolysis process:

- Endoglucanase (EG, endo-1,4-β-glucanohydrolase) which attacks regions of low crystallinity in thecellulose fiber, creating free chain-ends
- Exoglucanase or cellobiohydrolase (CBH,1,4-β-glucan cellobiohydrolase) which degrades the molecule furtherby removing cellobiose units from the free chain-ends

- β-Glucosidasehydrolyses cellobiose to produce glucose.

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (Sa´nchez and Cardona 2008).

2.8.2.3 Separate hydrolysis and fermentation (SHF)

When sequential process is utilized, solid fraction of pretreated lignocellulosic material that contains the cellulose is accessible to acids or enzymes and thuscan be hydrolysed and subsequently fermented. These last two steps of bioconversion of pretreated lignocellulosic residues to ethanol can be performed separately (SHF) or simultaneously (SSF). Once hydrolysis is completed, the resulting cellulose hydrolysate is fermented and converted into ethanol (Sa´nchez and Cardona 2008).

In the separate hydrolysis and fermentation (SHF), the hydrolysate products will be fermented to ethanol in a separate process. The advantage of this method is that both processes can be optimized individually (e.g. optimal temperature is 45-50°C for hydrolysis, whereas it is 30°C for fermentation). However, its main drawback is the accumulation of enzyme-inhibiting end-products (cellobiose and glucose) during the hydrolysis. This makes the process inefficient and the costly addition of β-glucosidase is needed to overcome the end-product inhibition (Dashtban*et al*., 2009).

2.8.2.4 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) involves the concurrent saccharification and fermentation of the hydrolysate product in the same reactor by *A. niger*and *S. cerevisiae* respectively (Dashtban*et al*., 2009). The rapid fermentation of the yeast *S. cerevisiae* helps in

maintaining low sugar concentration to prevent the effect of feedback inhibition on the cellulolytic activity of *A. niger*. By doing so, biomass production will be favoured instead of the ethanol production.

Despite the increase in the ethanol yield by impeding the inhibitory effects, the process also reduces cost of production by eliminating the need for other separate reactors (Ado *et al.*, 2010). Therefore, addition of high amounts of β-glucosidase is not necessary and this reduces the ethanol production costs. However, the main drawback of SSF is the need to compromise processing conditions such that temperature and pH are suboptimal for each individual step. Although, the development of recombinant yeast strains (i.e. improved thermotolerance) is expected to enhance the performance of SSF (Dashtban*et al*., 2009).

Consolidated bioprocessing (CBP) aims to minimize all bioconversion steps into one step in a single reactor using one or more microorganisms. CBP operation featuring cellulase production, cellulose/hemicellulose hydrolysis and fermentation of 5- and 6- carbon sugars in one step have shown the potential to provide the lowest cost for biological conversion of cellulosic biomass to fuels, when processes relying on hydrolysis by enzymes and/or microorganisms are used (Dashtban*et al*., 2009).

Apart from its enzyme production ability, *Pencilliumchrysogenum* also showed high levels of reducing sugars (glucose and xylose) in SSF. It suggested an integral process for the production of ethanol, furfural, and mixture of bagasse and wheat bran (4:1) for the production of cellulases.

2.9 Fermentation

Fermentation is the conversion of an organic material from one chemical form to another using enzymes produced by a variety of microorganisms generally, either bacteria, yeast or fungi.The breakdown of glucose to ethanol involves a complex sequence of chemical reactions. According

to the reactions, the theoretical maximum yield is 0.51 kg ethanol and 0.49 kg carbon dioxide per kg of sugar fermented. The actual yields of ethanol generally fall short of predicted theoretical yields.This is because about 5% of the sugar is used by the yeast for biomass production and minor products such as glycerols, acetic acid, lactic acid, and fuel oils. The dominant organisms used in ethanol production are members of the genus *Saccharomyces.*They are single-celled widely distributed in nature, commonly found in wood, dirt, plant matter, and on the surface of fruits and flowers. They are spread by wind and insects **(**U.S. Department Of Energy, 1982**)**.

These yeasts are sensitive to a wide variety of variables affecting ethanol production. However, pH and temperature are the most influential of these variables. *Saccharomyces* are most effective in pH ranges between 3.0 and 5.0 and temperatures between 27^0C and 35^0C (U.S. Department Of Energy, 1982). The length of time for the fermentation is dependent on the number of yeast cells per quantity of sugar. The higher the number initially added, the faster the process. However, there is a point of diminishing returns. Yeast strains, sugar concentration, temperature, infections, and pH are the factors among others that influence the efficiency of the yeast in fermentation (U.S. Department Of Energy, 1982).

Bacteria (*Zymomonasmobilis*) have drawn special attention from researchers because of their speed of fermentation. In general, bacteria can ferment in minutes as compared to hours for yeast. All microorganisms have limitations either in their inability to ferment both pentoses and hexoses, the low yields of ethanol, their speeds of fermentation or the co-production of cell mass at the cost of ethanol.*Saccharomyces cerevisiae* has few advantages such as its wide public acceptance, high fermentation rate and high ethanol tolerance that make it a good candidate for fermentation processes. However, it is unable to efficiently utilize xylose as the sole carbon source or ferment it to ethanol (Dashtban*et al*., 2009).In addition to xylose, *S. cerevisiae* is also

unable to ferment arabinose, unless supplemented with rich media. Thus, the latest recombinant *S. cerevisiae* (TMB 3400) has been shown to successfully ferment both xylose and arabinose in addition to glucose to ethanol at efficiency close to its theoretical value of $0.51g^{-1}$ (Chu and Lee, 2007, Dashtban*et al*., 2009). Methods used to improve the fermentation efficiencies thus includes mutagenesis of the fermenter, co-culturing and heterologous expression of cellulases(Dashtban*et al*., 2009).

Therefore, genetic engineering and new screening technologies will bring bacteria and yeast that are capable of fermenting all the five major biomass sugars–glucose, xylose, mannose, galactose and arabinose. Mid- to long-term technology will improve the fermentation efficiency of the organism (yielding more ethanol in less time), as well as its resistance, requiring less detoxification of the hydrolysate. Bioethanol can be produced from feed stocks that contains sucrose (e.g. sugar cane, sugar beet, sweet sorghum and fruits), starch (e.g. corn, wheat, rice, potatoes, cassava, sweet potatoes and barley) and lignocellulose (e.g. wood, straw, and grasses) (Goh *et al*., 2009). Most industrial ethanol production uses sugarcane molasses or enzymatically hydrolysed starch (from corn or other grains), and yeast (*Saccharomyces cerevisiae*) (Saratale and Oh, 2012).

In the fermentation process, the hydrolytic products including monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose) will be fermented to valuable products such as ethanol. Among these hydrolytic products, glucose is generally the most abundant, followed by xylose or mannose and other lower concentration sugars (Dashtban*et al*., 2009). By-products of this process are carbon dioxide, low amounts of methanol, glycerol, etc. Yeast fermentation of glucose syrups to ethanol has been well explored in recent years but found

economically infeasible. Thus, abundant and renewable lignocellulosic biomass feedstock has been considered as the low-cost feedstock for bioethanol production (Saratale and Oh, 2012).

The classical configurations employed for fermenting biomass hydrolysates currently involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out separately termed as separate hydrolysis and fermentation (SHF) and alternatively, simultaneous saccharification and fermentation (SSF) where the hydrolysis and fermentation are performed in a single unit (Sa´nchez and Cardona 2008).

2.9.1 Types of fermentation

2.9.1.1 Lactic acid fermentation

This is a type of fermentation mostly carried out by Bacteria such as lactic acid bacteria in which the end product is lactic acid, and also animals at times of strenuous exercise when glycolysis is taking place as a result of oxygen deficiency thus manifesting as muscle cramps. During this condition, the six-carbon glucose molecule is broken down to two molecules of three-carbon compound known as lactic acid. Curdling of milk to lactic acid is also as a result of lactose fermentation in the milk by bacteria (Lansing, 2005).

2.9.1.2 Alcoholic fermentation

This type of fermentation is common in such unicellular organisms as bacteria and Baker's yeast. It is a type of fermentation in which ethyl alcohol is the main product of fermentation. Yeast cells release zymase that brings about the degradation of glucose molecule to two molecules of two-carbon alcohol and two molecules of carbon dioxide (Lansing, 2005).

2.9.2 Fermentation parameters

2.9.2.1 Fermentation time

Duration or period of fermentation has a profound effect on the efficiency of ethanol production depending on the feed stock been used, method or approach and the organisms involved. This is because some feed stocks are too easy to be hydrolysed and converted to the product of choice e.g starch while others e.g lignocellulosic substrate are too complex. Likewise, approaches of the production varies in their speed of operation for example simultaneous saccharification and fermentation is faster than separate hydrolysis and fermentation. Moreover, the organisms involved also differs as bacteria are generally faster than yeast (Appiah, 2013).

2.9.2.2 Effect of pH

Most bacteria grow in the range of pH 6.5 to 7.5. Yeast and fungi tolerate a range of 3.5 to 5.0 pH. The ability to lower pH below 4.0 offers a way for operators using yeast in aseptic equipment to minimize loss due to bacterial contaminants. Srivastava *et al.*,(1997) showed that the optimum initial pH of guava pulp medium for the production of ethanol was 5.0 for all three strains of *S. cerevisiae* employed and obtained maximum yield of 5.8 percent during 36 hour fermentation (Appiah, 2013).

Simultaneous saccharification and fermentation with *Aspergillus niger* and *Zymomonasmobilis*was carried out at pH of 4.5 using guinea corn and millet husks. Ethanol yield from guinea corn husk (26.83g/l) and millet husk (18.31g/l) was maximum at 120th hour and with ethanol concentrations of 67.7 and 63.8%, respectively (Appiah, 2013).

2.9.2.3 Effect of temperature

All organisms function best at a particular optimum temperature. The optimum temperature depend on whether the organism is psychropilic, mesophilic or thermophilic. Mesophilic

organisms function between 30-38°C while thermophilic function between 60-65°C. Operating at higher temperatures is desirable for a number of reasons: high fermentation temperature increases growth rate and productivity exponentially when the ethanol producing organism can thrive at that high temperature. The hydrolysis and fermentation steps are mostly performed at the optimum temperature of 55ºC and 30ºC respectively and optimum ethanol productivity of 19 to 25/g/l were obtained (Appiah, 2013).

2.9.2.4 Osmotic tolerance

The semi-permeable membrane surrounding the microbial cell must be able to withstand wide osmotic pressure changes in extracellular fluids that impact the relative osmotic pressure difference. If not, the cells may be severely damaged or even killed. The cells may burst in a hypotonic solution, when the solution becomes more dilute than the intracellular fluid. If hypertonic, the cells will shrink from the osmotic pressure difference. Osmotic pressure limits can be one of the factors that restrict maximum substrate concentration (Appiah, 2013).

2.9.2.5 Alcohol tolerance

The majority of organisms cannot tolerate ethanol concentrations above 10-15% (w/v). Enzymatic proteins become denatured. Higher temperature lowers the tolerance of the organism. High alcohol concentration disrupts the structure of the lipid bilayer membrane and makes it less stable. Although ***S. cerevisiae*** and related species produce ethanol with apparent ease, it is toxic to the cell at levels ranging between 8 and 18% (w/v) ethanol depending on the strain of yeast and the metabolic state of the culture. One of the groups of chemicals that have been known to affect the tolerance of yeast to ethanol has been unsaturated fatty acids (Appiah, 2013).

Susan *et al.,* (1978) have shown that cells grown in the presence of linoleic acid are more tolerant to added ethanol than those grown in the presence of oleic acid. Moreover, the cell

viability was higher when the cells were cultured in a medium supplemented with ergosterol which has an unsaturation in the side chain at C28, than with campesterol which has a saturated side chain at C17.

*Zymomonasmobilis*is more tolerant to ethanol, as the cell-free system of *Z.mobilis*can rapidly consume glucose and produce ethanol more than 15% (w/v). The cell membrane of *Z. mobilis*has fatty acid content to counteract the adverse effects of ethanol. The major fatty acids occurring in *Z. mobilis*are myristic acid, palmitic acid, and *cis*-vaccenic acid (Appiah, 2013). Among the phospholipids, phosphotidyl ethanolamine is the most abundantly present. The high concentrations of cis-vaccenic acid and unusual hopanoids in the membrane are responsible for the high ethanol tolerance (Appiah, 2013).

2.9.3 Commercial applications of fermentation

Nowadays, vast types of fermentations are explored and carried out by different microorganisms. This important microbial activity is harnessed in large industrial scales to obtain useful end products for the sole benefit of mankind. The products produced include such important end products as dairy products, butyric acid, alcohol, acetic acid, tanning of leather, bakery products, curing tea & coffee and pharmaceutical industries that take advantage of microbial fermentation in production of wide variety of products including hormones and some specialized proteins to mention but few. Genetic engineering is of paramount importance here as it helps in producing the desired and specific product at certain desired quantity and quality and time interval.

2.10 Distillation

Distillation is the separation of mixtures based on the volatilities (boiling points) of the individual components that make up the mixture. Distillation is often used only if one product is required. The product which is of low volatility is called distillate while the substances of high

boiling point that remain in the flask is called residue or bottoms. In acid hydrolysis, part of the acid and water are recovered in distillation (Otulugbu, 2012).

At atmospheric pressure, water boils around 212°F (100°C) and ethanol boils around 173°F (78.2°C). It is this difference in boiling temperature that allows for distillative separation of ethanol-water mixtures.If a mixture of ethanol and water solution is heated, more ethanol molecules leave the mixture than water molecules. If the vapour leaving the mixture is caught and condensed, the concentration of ethanol in the condensed liquid will be higher than in the original mixture, and the mixture left will be lower in ethanol concentration. If the condensate from this step is again heated and the vapours condensed, the concentration of ethanol in the condensate will again be higher. This process could be repeated until most of the ethanol was concentrated in one phase **(**U.S. Department of Energy, 1982**)**.

Unfortunately, a constant boiling mixture (azeotrope) form at about 96% ethanol. This means that when a pan containing a 96%-ethanol solution is heated, the ratio of ethanol molecules to water molecules in the condensate remains constant. Therefore, no concentration enhancement is achieved beyond this point by the distillation method so, to break the azeotrope, benzene or the likes are added to the mixture in that it interferes with the boiling point of one of the liquids involved hence, there separation will be achieved **(**U.S. Department of Energy, 1982**)**.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Collection of soil sample

Ten grams of the soil sample (for the isolation of *A. niger*) was collected from 10cm soil depth in botanical garden of Department of Biological SciencesAhmadu Bello University Zaria, carefully scooped intoclean polythene bags using sterile hand trowel and transported to Food/Industrial laboratory of the Department of Microbiology, Ahmadu Bello University Zaria for further analysis (Lakshmi*et al*., 2014).

3.1.2 Collection of palm wine sample

Three hundred and fifty millilitre of palm wine (from which*Saccharomyces cerevisiae* was isolated) was obtained from market where it was been sold, properly packaged in a sterile glass bottle, properly labelled, placed in an ice pack and brought to Food/Industrial laboratory of the Department of Microbiology, Ahmadu Bello University Zaria for further studies. The sample was kept refrigerated until required.

3.1.3 Collection of substrate sample

Sugarcane (*Saccharumofficinarum*) bagasse (1.5kg) was obtained from National Animal Production Research Institute(NAPRI) ShikaSamaru, Zaria while rice stalk substrate was collected locally from rice processing plants in Basawa of Sabongari Local Government Zaria, Kaduna State.

All of the above samples were collected, carefully packaged in clean polythene bagsbrought and dried at ambient temperature and after drying, the samples were repackaged in clean plastic

containers and stored in storage cupboards for further analysis in the Food/Industrial laboratory of the Department of Microbiology, Ahmadu Bello University Zaria.

3.2 Media Preparation

For the purpose of isolation in this study, Sabouraud Dextrose Agar (SDA) and its slant was prepared by suspending 65g of the medium in one litre of distilled water. The flask was then heated with frequent agitation and boiling in order to homogenize the mixture and completely dissolve the medium. The flask was then Autoclaved at 15 lbs 121^0C for 15 minutes after which it was allowed to cool down to about 50^0C before it was carefully dispensed into sterile petri dishes to solidify.

3.3 Isolation of *Aspergillusniger* and *Saccharomyces cerevisiae*

3.3.1 Isolation of *A. niger*

One gram of the soil sample was suspended in 9.0ml sterile distilled water thus having a 1:10 dilution. One (1ml) of this stock solutionwas serially diluted up to 10^{-5} dilution by aseptically transferring 1ml from the1:10 dilution tube in to another tube of 9ml distilled water thus having a $1:10^2$ dilution, the same procedure was followed until $1:10^5$dilution was achieved (Harley, 2002). One millilitre from the last dilution of 10^{-5} suspension was spread on sterile solidified SDA plates that contains 0.03g/l chloramphenicol, a broad spectrum antibiotic for bacterial growth inhibition. The plates were incubated at 30^0C for 5 days (Warcup, 1950;Ratnasri*et al*., 2014). The grown colony with the best promising potential based on the colonial morphologywas sub-cultured on sterile SDA slants for further studies (Ratnasri*et al*., 2014).

3.3.2 Isolation of *S. cerevisiae.*

Saccharomyces cerevisiae for the fermentation process was isolated from palm wine. Aliquots fromserially diluted (as described above) 10^{-5} dilutions of the beverage was spread on the

solidified SDA plates and were incubated at 37^0C for 3days. Colonies suspected to be *S. cerevisiae* based on colonial characteristics were sub-cultured on SDA slants for subsequent identification. A smear of the isolate was stained with methylene blue and examined microscopically (Ameh*et al*., 1989).

3.4 Characterization of the Isolates

3.4.1 Characterization of *Aspergillus niger*

This was achieved by observingthe cultural and microscopic characteristics though slide culture techniqueand comparing them with atlas for fungal identification (Klitch, 2012) and as well, amylase and cellulase production assays.

3.4.1.1 Slide culture technique

The materials that were used included sterile Petri dish, sterile glass slide, sterile cotton wool, sterile distilled water and sterile solidified nutrient agar.

A moist cotton wool was aseptically placed in a sterile petridish and a sterile glass slide was placed on the sterile moist cotton wool. An SDA agar block was also aseptically cut and placed on top of the sterile glass slide and then point inoculated with a pinch of a 3 days old growing *A. niger* colony. The whole plate was then incubated for another 3 days after which the whole set up was viewed under 10x and 40x objectives (Wijedasa and Liyanapathirana, 2012).

3.4.1.2 Screening for amylase production

The centre of the prepared sterile solidified starch medium plates were inoculated with 0.1ml of the standardized fungal cultures. The plates were flooded with iodine solution following incubation at 30^0C for 5 days after which zones of starchhydrolysis around the grown colonies were observed and recorded(Ratnasri*et al*., 2014). The medium consist of the following per

100ml: soluble starch(1% (w/v), peptone (1% (w/v), yeast extracts (1% (w/v) and agar- agar (1.5% (w/v)(Ratnasri*et al*., 2014).

3.4.1.3 Screening for cellulaseproduction

Cellulase production ability of the fungal isolates were also determined using carboxymethyl cellulose (CMC) enriched mineral salt medium (Mendel's mineral medium) containing (g/L): Urea-0.3g, $(NH_4)_2SO_4$ -1.4g, KH_2PO_4- 2.0g, $CaCl_2$-0.3g, $MgSO_4$ -0.3g, yeast extract- 0.25g and proteose peptone -0.75g with 10g of CMC and 17.5g agar. The centre of the CMC medium plates were inoculated with 0.1ml of the standardized fungal cultures. The plates were then incubated at 30ºC for 5days.

After the incubation period,the plates were then flooded with 1% Congo red dye for 30 min, followed by destaining the plates with 1M NaCl solution for 20 min. clear zones of hydrolysis wherethen observed and recorded (Khokhar*et al*., 2011).

3.4.2 Characterization of *Saccharomyces cerevisiae*

Cultural and microscopic observations werecarried out on the isolates. These weredone by staining with methylene bluestaining which was achieved by preparing a smear of the isolate on a grease free slide, air dried and heat fixed. The prepared slide was then flooded with the methylene blue stain for 1min. and was gently rinsed with water, air dried and viewed under oil immersion objective. While Gram's stainwas carried out by staining the prepared slides with crystal violet for 1min., the slides were rinsed with water and then Gram's iodine was then added for 1min. the slides were rinsed with water then with 95% ethanol for until no colour drops from the slide. The slides were then rinsed with water and counter stained with safranin for 30sec. the slides were then rinsed with water and viewed under oil immersion objective after they

dried(Harley, 2002). Thermotolerance, ethanol tolerance and sugar utilization tests were also carried out on the yeast isolates (Klitch, 2002).

3.4.2.1 Thermotolerance test

This was achieved by inoculating five prepared SDA plates with the test organisms and the plates were incubated at different temperature of 25, 30, 35, 40and 45^0C for 72Hrs and the plates were observed and recorded for evidences of growth after the incubation period (Ali *et al*., 2014).

3.4.2.2 Ethanol tolerance test

An aliquots of the 24 hours washed yeast cells were inoculated into yeast extract broth containing 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20% ethanol. The tubes were incubated for 72 hours after which the optical densities of the content of the respective tubes were then obtained usingspectrophotometerin order to ascertain the various ethanol tolerances of the respective test isolates involved in the study (Ali *et al*., 2014).

3.4.2.3 Sugar utilization test

Three (3) milliliter of Andrade peptone water that contains Andrade indicator was used. One percent solution of each of the sugars (galactose, glucose, fructose, sucrose, maltose, arabinose and lactose)that has been tested were preparedand then, 2ml of the 1% sugar solution prepared was added to the 3ml Andrade peptone water in a test tube making it to a total of 5ml solution and then sterilized. The *S. cerevisiae*isolates wereasepticallyinoculated into the test tubes and incubated at 37^0C for a period of 5 days (Ali *et al*., 2014).

3.5 Sample Preparation and Pretreatment

3.5.1 Sample preparation

The substrate samples collected (sugarcane bagasse and rice stalk) weredried at ambient temperature, groundwith mortar and pistil and after drying, the samples were repackaged in clean plastic containers and stored at ambient temperature in storage cupboards for further analysis in the Food/Industrial laboratory of the Department of Microbiology, Ahmadu Bello University Zaria before subsequent analysis.

3.5.2 Sample pretreatments

All of the substrates were subjected to alkaline hydrogen peroxide and dilute acid pretreatments during the course of this work.

3.5.2.1 Alkaline hydrogen peroxide pretreatment (AHP)

A hydrogen peroxide solution was prepared by dissolving 7.36% (v/v) of H_2O_2 in 100.0 ml distilled water and adjusting the pH to 11.5 with sodium hydroxide.The substrates werethen added to the pretreatment solution prepared and the flasks wereautoclaved at 121ºC for 1 hr. (Rivera *et al*., 2010).

3.5.2.2 Dilute acid pretreatment

Dilute acid pretreatment was carried out by dissolving 0.75% (v/v) of H_2SO_4 in 100 ml distilled water and the substrates were added and autoclaved at 121ºC for 1hr. (Saha*et al.,* 2005).

All the pretreated substrates were washed with distilled water and sieved with a muslin cloth until their pH reaches neutrality.

3.6 Determination of Proximate Compositions of the Substrates

3.6.1 Determination of moisture content

Aluminium dishes were washed and dried to a constant weight in an oven at 100^0C. They were later removed and cooled in a desiccator and weighed (W1). Two grams of the ground (powdered) sample was placed on the dishes and reweighed as (W2). The dishes containing the samples were kept in an oven for about 3hours, the samples were removed and cooled in the desiccator and reweighed as (W3).

The % of moisture was calculated as: $\frac{W2-W3}{W2-W1} \times 100$ (A.O.A.C., 2010).

3.6.2 Determination of ash content

Crucibles were cleansed and dried in the oven and after drying, they were cooled in the desiccator and weighed (W1). Two grams of the ground (powdered) samples were placed in the crucibles and weighed (W2). They were transferred into the Muffle Furnace and heated at 550^oC, then removed and cooled in the desiccator and weighed (W3).

The % of Ash was calculated as: $\frac{W3-W1}{W2-W1} \times 100$ (A.O.A.C., 2010).

3.6.3 Determination of fibre

Two grams of the samples were placed in a beaker containing 1.2ml of H_2SO_4 per 100ml of solution and boiled for about 30minutes, the residues were filtered and washed with hot water, the residues werethen transferred to beakers containing 1.2gram of NaOH per 100ml of solution and boiled for about 30minutes, the residues werethen washed with hot water and dried in an oven and weighed (C2), the weighed samples were incinerated in a Furnace at about 550^OC, removed and allowedto cool and reweighed (C3).

The % of Fibre was calculated as: $\frac{C2-C3}{W} \times 100$ (A.O.A.C., 2010).

3.6.4 Determination of lipids (fat)

A clean 250ml capacity boiling flaskswere dried in an oven and thentransferred into a desiccator and allowed to cool. An empty filter paper was weighed and labelled W1.

Two grams of the sampleswere weighed onto labelled thimbles (filter papers) and labelled W2. The boiling flaskswere filled with N-hexane and the soxhlet apparatus was assembled and refluxing was allowed for 8hours.

The contentswere removed and transferred to an oven for drying and then later transferredfrom the oven into a desiccator and allowedto cooland then weighed and labelled as W3.

The % of Fat was calculated as: $\frac{W2-W3}{W2-W1} \times 100$ (A.O.A.C., 2010).

3.6.5 Determination of protein

3.6.5.1 Digestion:

Two grams of the sampleswere weighed into a kjeldahlflasks and anhydrous sodium sulphate of 5g was added. Twenty five (25ml) concentratedsulphuric acid (H_2SO_4) was then added to the mixtures above with some few boiling chips.

The flask contents were then heatedin the fume cupboard until the solutionsassumes a green colour.Thesolutions were then cooled and washed with distilled water in a 250mlvolumetric flasks.After cooling, the digestswerethen transferred into Erlenmeyer flask containing 100ml distilled water.

3.6.5.2 Distillation:

Before use, the Markham distillation apparatus was steamed through for about 15minutes.Under the condenser, a 100ml Erlenmeyer flasks containing 10ml of boric indicator were placed.

Ten (10ml) of the digestswere pipetted into the body of the apparatus via the small funnel aperture and then washed down with distilled water followed by 10ml of 40% NaOH solution. Steaming was again done through for about 5-7 minutes to collect ammonium sulphate (about 30-40ml) after which the receiving flaskswere removed and washed down the tip of the condenser into the flask.

3.6.5.3 Titration:

The solutionswere titrated into the receiving flasks using N/100 (0.01N) hydrochloric acid and the Nitrogen content was calculated and hence the Protein content of the sample.

NB: blank was always run along with the sample.

The % of protein was calculated as: Final reading –Initial reading-blank (0.2) x standard number of Nitrogen (1.4) divide by initial weight (0.5) x standard number of protein (6.25) (A.O.A.C. 2010).

3.6.6 Determination of carbohydrate by difference

By difference, in this method, carbohydrate contentswere obtained by calculations having estimated all other fractions by proximate analysis i.e.

100- (% of moisture + %Ash + %Protein + %Fat) =Carbohydrates. (A.O.A.C. 2010).

3.7 Ethanol Production

3.7.1 Fermentation medium composition

The medium used for the simultaneous saccharification and fermentation process comprised the following per 100ml; Peptone, 0.1g; Malt Extract, 0.1g; Yeast Extract, 0.2g; Magnesium Chloride, 0.1g; Calcium Carbonate, 0.2g; Ammonium Phosphate, 0.2g; and Ferrous Sulphate, 0.001g (Ado *et al*., 2010).

3.7.2 Inocula preparation and inoculation

The inoculum of*A. niger* was prepared from the stored slant cultures of SDA by the spore suspension of the organism i.e., *A. niger* was washed by transferring about 10ml of 0.25% sterile tween 80 into the stored culture slant of the organism. It was shaken to wash the spores, and then the spore suspension was decanted into sterile bottle after which the spores were counted under the microscope with a haemocytometer in order to obtain the density in spores/ml. This washed spore suspension was then used as the inoculum from which the fermentation medium was inoculated (Ado *et al*., 2010).

S. cerevisiae inoculum was prepared by transferring the yeast cells from the stored slants using sterile wire loop to an Erlenmeyer flasks containing 10ml sterile growth medium and then incubated at 30^{o}C, 300 rpm for 24 hours from which 1ml wasre-transferred to another 50ml growth medium and thenre-incubated at same conditions as above. The content of the flask was then collected, centrifuged and the residue was washed with sterile distilled water and compared with McFarland's standard scale one. This standardized content was then used as the inoculum for the fermentation medium (Ado *et al*., 2010).

The fermentation medium was inoculated with both of the inocula at a concentration of 5% v/v. *A. niger*corresponding to 2.5 x 10^{6} spores/ml and *S. cerevisiae* 3.0 x 10^{8} cells/ml were used as the inocula for Simultaneous Saccharification and Fermentation (SSF).

3.7.3 Optimal culture conditions

For optimum yield, it had been established that temperature of 35ºC, pH of 5.0, agitation rate of 300rpm(Ado *et al.,* 2010) and substrate concentration of 4% (Aiyejagbara, 2015)gave a better result thus, these conditions were maintained in this work.

3.7.4 Ethanol production

Simultaneous saccharification and fermentation method of ethanol production was employed in this research work.

Ten (10g) of each substrate and 250ml medium in 500ml Erlenmeyer flask were used. ThepH of the content of the flasks wasthen buffered to 5.0 using NaOH and then plugged with cotton wool and autoclaved at 121ºC for 15 minutes. After sterilization, the flasks were allowed to cool down at room temperature and to each flask, 5% v/v *A. niger* and *S. cerevisiae* inoculum corresponding to 2.5 x 10^6 spores/ml and3.0 x 10^8 cells/ml respectively were added. The flasks were then incubated with shaking at 300rpm and 35ºC for 7 days (Ado *et al.*, 2010).

3.7.5 Distillation

This was performed using a distillation set-up. The fermented product was transferred into a round bottom flask and placed on a heating mantle. Another flask was fixed to the other end of the distillation column to collect the distillate at 78ºC.

Thus, ethanol concentrations and quality were determined using the distillate by measuring its specific gravity after distillation using specific gravity bottle and FTIR analysis respectively (Ado *et al*., 2010).

3.7.5.1Determination of specific gravity

This was carried out by filling a 30ml capacity specific gravity bottle with distilled water of equal volume of the distillate to be measured. The bottle was then emptied, dried, filled with the produced sample and then re-weighed and the specific gravity was measured as follows:

$$\text{Specific gravity} = \frac{\text{Weight of unit volume of the sample}}{\text{Weight of unit volume of water}}$$

The specific gravity values for the respective produced ethanol samples obtained using the formula above were used to determine the ethanol concentration from a standard curve prepared using known concentrations of pure ethanol (Emeka, 2001).

3.8 Assays for Ethanol Yield

3.8.1 Quantitative analysis of the ethanol samples produced

The concentration of ethanol was determined by measuring its specific gravity after distillation. This was done by taking 30ml from each flask and centrifuging it at 1500rpm for 3minutes after 24hrs intervals (Ado *et al.*, 2010). The supernatant obtained was distilled (Emeka, 2001).

3.8.1.1 Preparation of ethanol specific gravity standard curve

Eight stocks of pure ethanol of varying concentrations 2%, 4% to 16% wereprepared. Five (5ml) of each of these concentrations was taken into specific gravity bottle and weighed against equalvolume of distilled water. The values obtained were then used to plot a standard curve against the eight known concentrations (Emeka, 2001).

3.8.2Qualitative analysis of ethanol samples produced

This was achieved by infrared determination test that was carried out on all the produced samples using Fourier Transform Infra Redspectrophotometry. The infrared spectra of an absolute ethanol were used as a gold standard (Ado *et al*., 2010).

3.9 Analysis of Data

The data obtained were analysed statistically using GraphpadQuickCals (www.graphpad.com). ANOVA was employed in order to determine the effect of the various ethanol concentrations on the yeast isolates tested statistically and also, paired T-test was used to ascertain the statistical relationship between the different samples of ethanol been produced.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition of the Substrates

Table 4.1 is the proximate composition of sugarcane bagasse and rice stalk treated with sulphuric acid were determined to be (6.30% ash), (3.05% protein), (84.15% carbohydrates) and (4.75 ash), (5.25 protein), (80.55 carbohydrates) respectively while that of sugarcane bagasse and rice stalk treated with alkaline peroxide were (7.95% ash), (1.75% protein), (83.20% carbohydrates) and (5.10% ash), (7.00% protein), (77.65% carbohydrates) respectively.

4.2 Isolation and Characterization of *A. niger* from Soil

The identificationof *A. niger* isolates through slide culture technique and both macroscopic and microscopic features revealed densely packed dark brown colonies on SDA plates whose reverse side of the plate was yellow in colour and very dark brown and densely packed irregular roughened conidial heads with inconspicuous white non-septate hyphae as presented in Table 4.2

4.3 Screening of *A. niger* Isolates for Amylase Production

Figure 4.1 shows the result of the screening of amylase production by the *A. niger* isolates. The isolate GS2 had the highest observed zone of starch hydrolysis of 6.3mm in contrast to GS1 which was 5.7mm whereas the isolate GS3 had the least zone of clearance of 3.8mm.

4.4 Screening of *A. niger* Isolates for Cellulase Production

Figure 4.2 shows the result of the screening of cellulase production by the *A. niger* isolates. The highest zone of cellulose hydrolysis was produced by the isolate GS01 with 17.4mm diameterof zone of cellulose hydrolysis in contrast to GS02 which had17.1mm whereas GS03 had the least zone of halo of 9.3mm.

Table 4.1: Proximate composition of the treated and untreated substrates

	Proximate Compositions (%)					
Sample Code	**Moisture**	**Ash**	**CF**	**CP**	**CFb**	**CHO**
N-T SB	3.95	12.70	5.60	3.50	62.35	74.25
H_2O_2-T SB	3.80	7.95	3.30	1.75	40.20	83.20
H_2SO_4-T SB	3.30	6.30	3.20	3.05	59.55	84.15
N-T RS	5.00	6.80	6.90	5.25	33.90	76.05
H_2O_2-T RS	3.65	5.10	6.60	7.00	38.65	77.65
H_2SO_4-T RS	4.95	4.75	4.50	5.25	50.90	80.55

KEY:

N-T SB: Non-treated sugarcane bagasse.
H_2O_2-T SB: Hydrogen peroxide-treated sugarcane bagasse.
H_2SO_4-T SB: Sulphuric acid-treated sugarcane bagasse.
N-T RS: Non-treated rice stalk.
H_2O_2-T RS: Hydrogen peroxide-treated rice stalk.
H_2SO_4-T RS: Sulphuric acid-treated sugarcane bagasse.

CF: Crude fat.
CP: Crude protein.
CFb: Crude fibre.
CHO: Carbohydrates

Table 4.2: Cultural and microscopic characteristics of *Aspergillus niger*isolates

	Macroscopic/Cultural Characteristic			Microscopic Characteristics				
Isolate	**SDA Growth**	**Colour**	**Revers**	**Con. head**	**Con. shape**	**Hyp. Sep.**	**Hyp. Opac**	**T. Identity**
GS01	+	DBC	Y	R	GR	-	T	*A. niger*
GS02	+	BC	Y	R	GR	-	T	*A. niger*
GS03	+	DBC	Y	R	GR	-	T	*A. niger*

Key: Con. Head = Conidial head, Con. Shape = Conidial shape,Hyp. Sep. = Hyphal septation, Hyp. Opac = Hyphal opacity, + = Positive, DBC = Dark brown colonies, BC = Black colonies, Y = Yellow, R = Radiate, GR = Globose and Rough, - = Negative, T = Transparent and GS = Garden soil isolates.

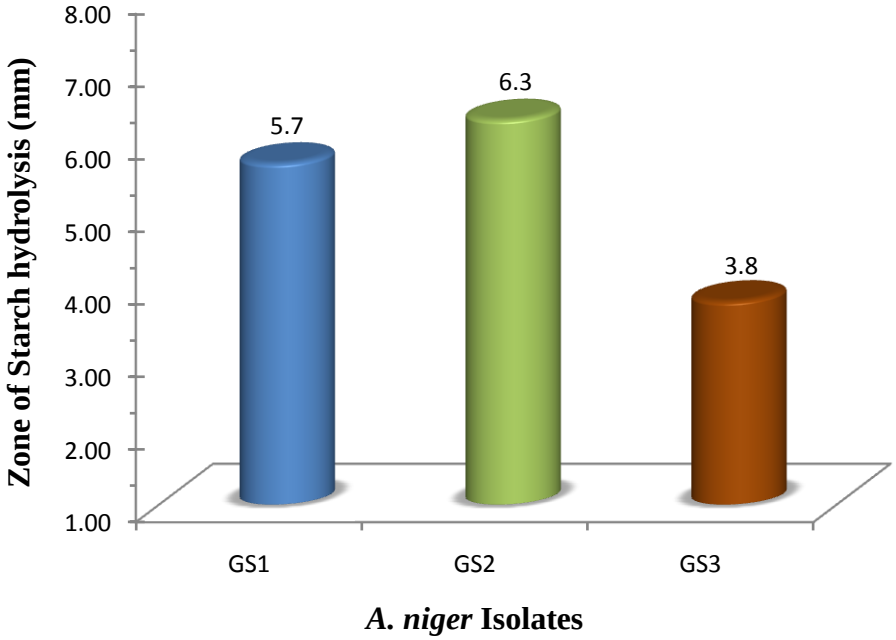


Figure 4.1: Assay for amylase production by *A. niger*isolates

Key:
GS1- Garden Soil isolate 1.
GS2- Garden Soil isolate 2.
GS3- Garden Soil isolate 3.

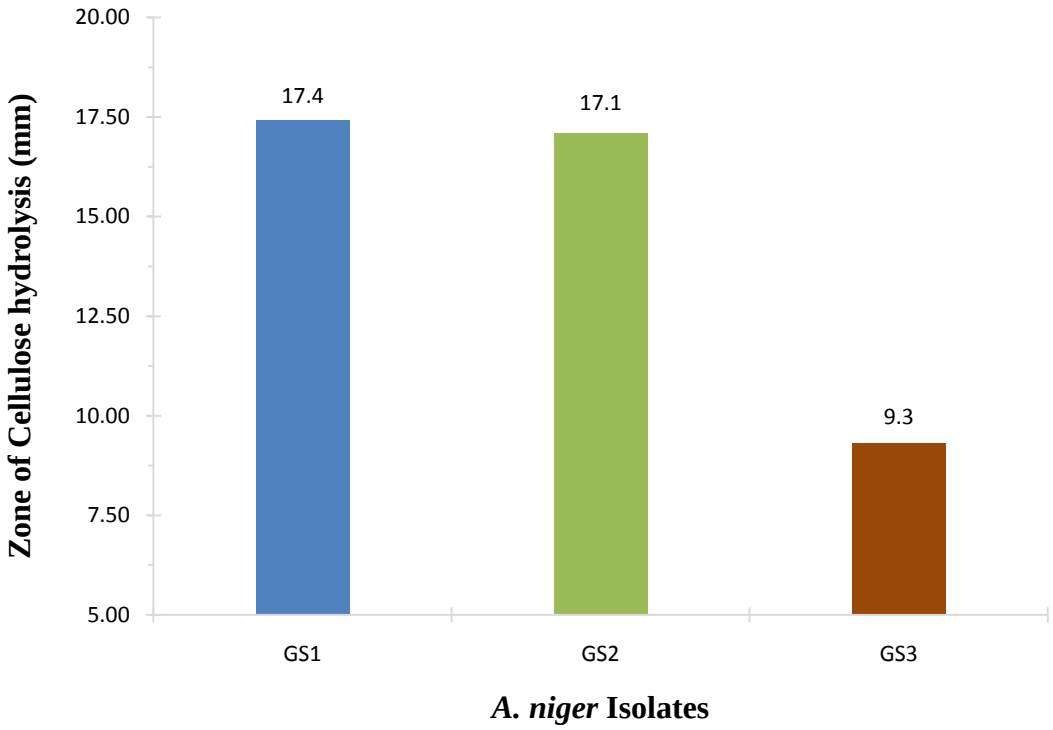


Figure 4.2: Assay for cellulase production by *A. niger*isolates

Key:
GS1- Garden Soil isolate 1.
GS2- Garden Soil isolate 2.
GS3- Garden Soil isolate 3.

4.5 Isolation and Characterization of *S. cerevisiae*

Table 4.3 shows the cultural, microscopic and sugar fermentation properties of the *S. cerevisiae* isolates. For *Saccharomyces cerevisiae* identification, two of the isolates appeared to be cream coloured with smooth moist and shiny colonies and the other isolate was observed to have smooth whitish colonies on SDA culture plates. All the isolates were able to assimilate and ferment galactose, glucose, fructose and sucrose with gas production except PW02 that did not produce gas following galactose fermentation. All the isolates did not produce gas after maltose fermentation and all the isolates were not able to assimilate and ferment arabinose and lactose.

4.6 Thermotolerance Property of *Saccharomyces cerevisiae* Isolates

The thermotolerance test result is presented in Table 4.4. All the isolates were able to grow well on all the plates incubated at all temperatures except 45ºC. Even though, PW02 showed feeble or weak growth at temperatures of 40^0C.

4.7 Ethanol Tolerance Property of *Saccharomyces cerevisiae* Isolates

The ethanol tolerance test carried out on the isolates revealed the tolerance limits of the strains to the physiologic stress of ethanol and it was taken as a concentration of ethanol after which there was a sharp decrease in growth. The result is shown in Figure 4.3. PW02 had the highest tolerance of 15% ethanol while the other isolates(PW01 and PW03)onlytolerates 10% ethanol.

Table 4.3: Cultural, microscopic and sugar fermentation properties of *Saccharomyces cerevisiae* isolates

	Cultural and Microscopic Characteristics						Sugar fermentation/Gas production						
Isolates	**Colour**	**Nature**	**App.**	**Elev.**	**Marg.**	**Bud**	**Arab**	**Gal**	**Glu**	**Fruc**	**Lac**	**Mal**	**Suc**
PW01	Cream	SS	YL	C	E	+	-	+G	+G	+G	-	+	+G
PW02	Cream	SS	Gl	C	E	+	-	+	+G	+G	-	+	+G
PW03	Whitish	S	Gl	R	W	+	-	+G	+G	+G	-	+	+G

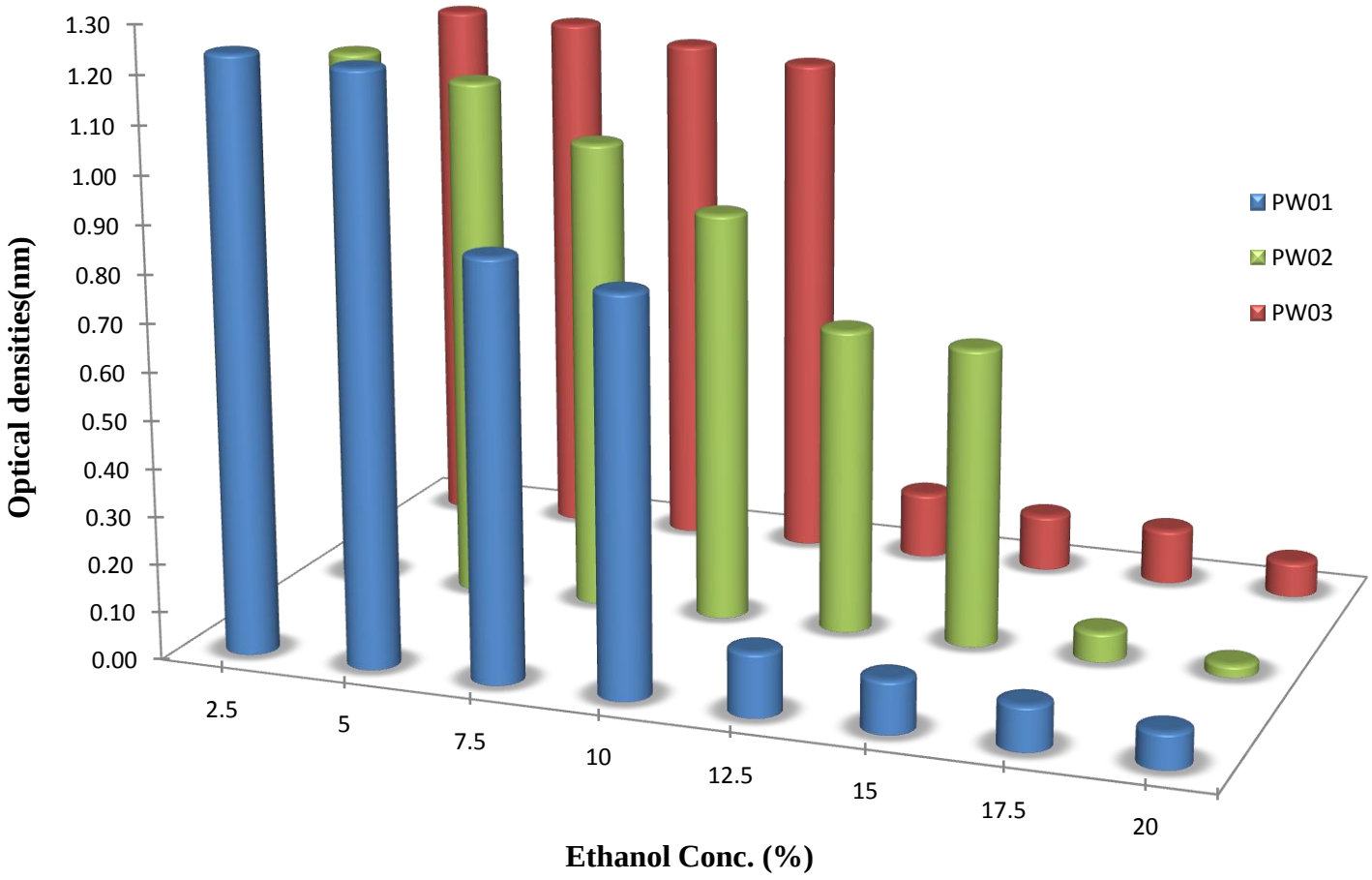
KEY: App. = Colony appearance, Elev. = Colony elevation, Marg. = Colony margin, Arab = Arabinose, Gal = Galactose, Glu = Glucose, Fruc = Fructose, Lac = Lactose, Mal = Maltose, Suc = Sucrose, SS = Smooth and shiny, S = Smooth, YL = Yeast like, Gl = Globose, C = Convex, R = Raised, E = Entire, W = Wavy, + = Positive, - = Negative, G = Gas and PW = Palm wine isolates.

Table 4.4: Thermo-tolerance properties of *Saccharomyces cerevisiae* isolates at various temperatures

Temperatures (ºC)					
Isolates	**25**	**30**	**35**	**40**	**45**
PW01	++	++	++	++	-
PW02	++	++	++	+	-
PW03	++	++	++	++	-

KEY:

+: There is weak or feeble growth
++: There is strong growth
-: No growth
PW: Palm wine isolates.



f = 16.1218 *p = 0.0001*

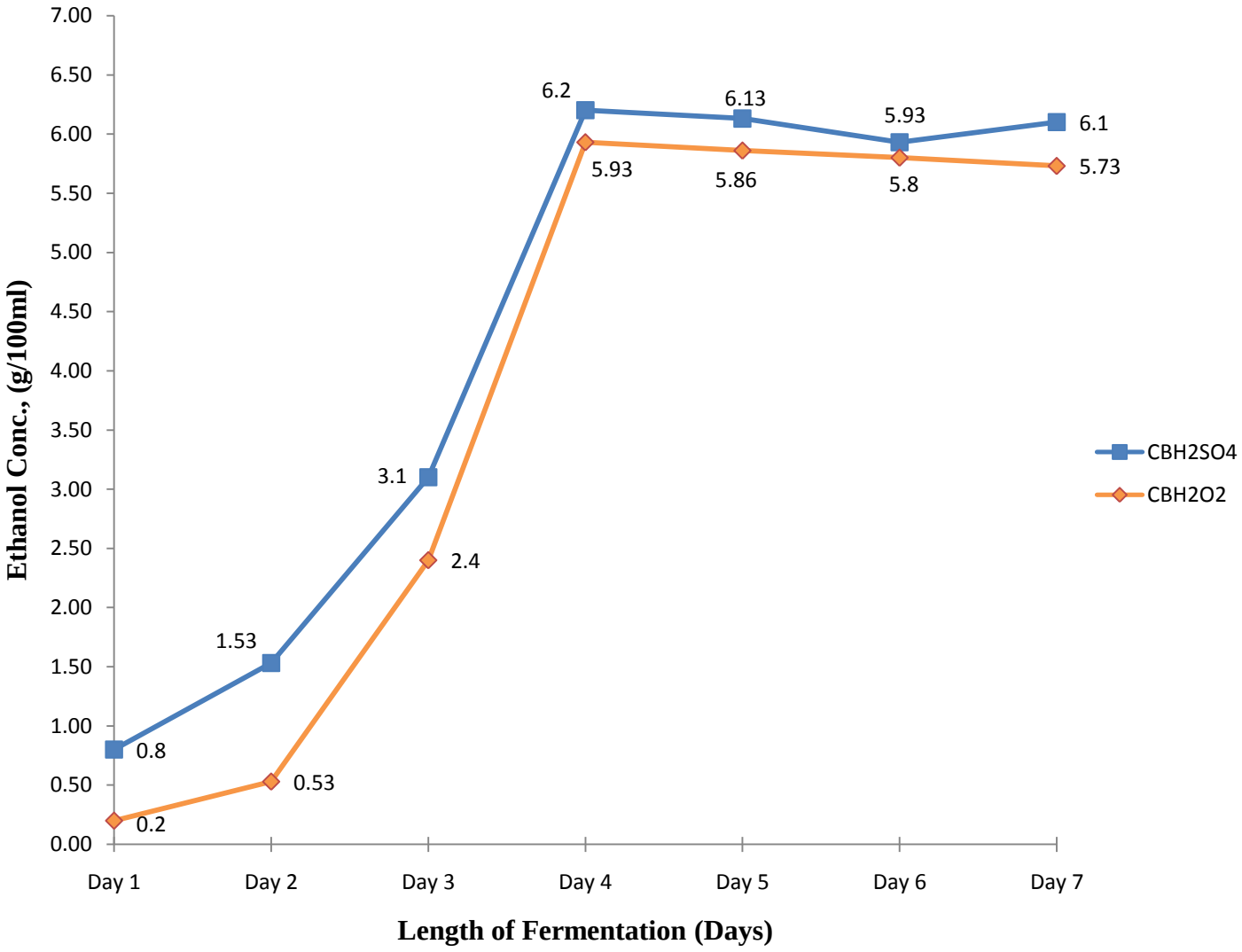
Figure 4.3: Ethanol tolerance property of the *S. cerevisiae* isolates

4.8Ethanol Yield by co-cultures of *A. niger*and *S. cerevisiae* from Sugarcane bagasse Treated with both Sulphuric acid and Hydrogen Peroxide

The result of ethanol yield from cane bagasse treated with sulphuric acid and hydrogen peroxide is presented in Figure 4.4. The highest and the optimum ethanol yield of this research was obtained from sulphuric acid-treated sugarcane bagasse (6.20%) whereas the highest yield of ethanol obtained from hydrogen peroxide-treated sugarcane bagasse was 5.93%.

4.9Ethanol Yield by co-cultures of *A. niger*and *S. cerevisiae* from Rice Stalk Treated withboth Sulphuric acid and Hydrogen Peroxide

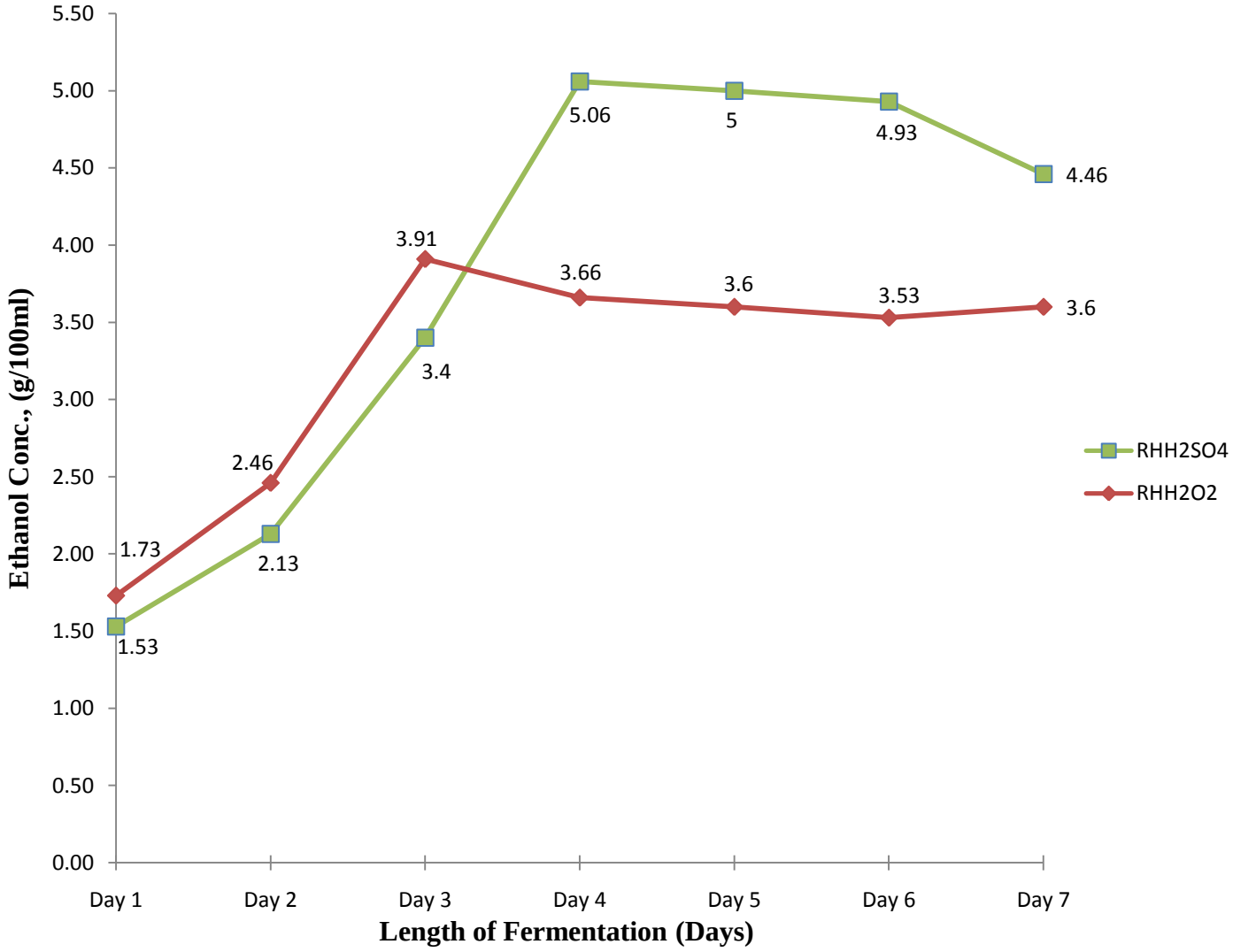
Figure 4.5shows the yield of ethanol obtained from the sulphuric acid and hydrogen peroxide treated rice stalk. The highest yield obtained from acid-treated rice stalk was 5.06% ethanol. Finally, the least quantity of ethanol produced was from alkaline treatment of rice stalk that gave an ethanol yield of 3.91%.



t = 4.1472 *df = 6* *p = 0.0060*

Figure 4.4: Ethanol Yield by co-cultures of *A. niger*and *S. cerevisiae* from sugarcane bagasse treated with sulphuric acid and hydrogen peroxide

KEY: CB-H_2SO_4: Sulphuric acid-treated cane bagasse, CB-H_2O_2: Hydrogen peroxide treated cane bagasse.



t = 1.6967 *df = 6* *p = 0.1407*

Figure 4.5: Ethanol Yield by co-cultures of *A. niger*and *S. cerevisiae* from Rice Stalk Treated with Sulphuric acid and Hydrogen Peroxide

KEY: RS-H_2SO_4: Sulphuric acid-treated rice stalk, RS-H_2O_2: Hydrogen peroxide-treated rice stalk.

4.10FTIR spectrophotometry of the ethanol golden standard

The result of the Fourier transform infrared spectrophotometric analysis of the gold standard is given in Figure 4.6. It explains the specific wavelengths or their ranges within which specific functional groups of the test samples can be detected.

4.11FTIR spectrophotometry of the sulphuric acid-treated cane bagasse ethanol sample

Figure 4.7 shows the result of FTIR of the sulphuric acid treated cane bagasse ethanol sample designated (CB-H_2SO_4). The hydroxyl (OH) group of this sample had its absorbance at a wavelength of 3302.4cm^{-1} while it's (H-C-H) absorbance was at 2981.9cm^{-1}.

4.12FTIR spectrophotometry of the hydrogen peroxide-treated cane bagasse ethanolsample

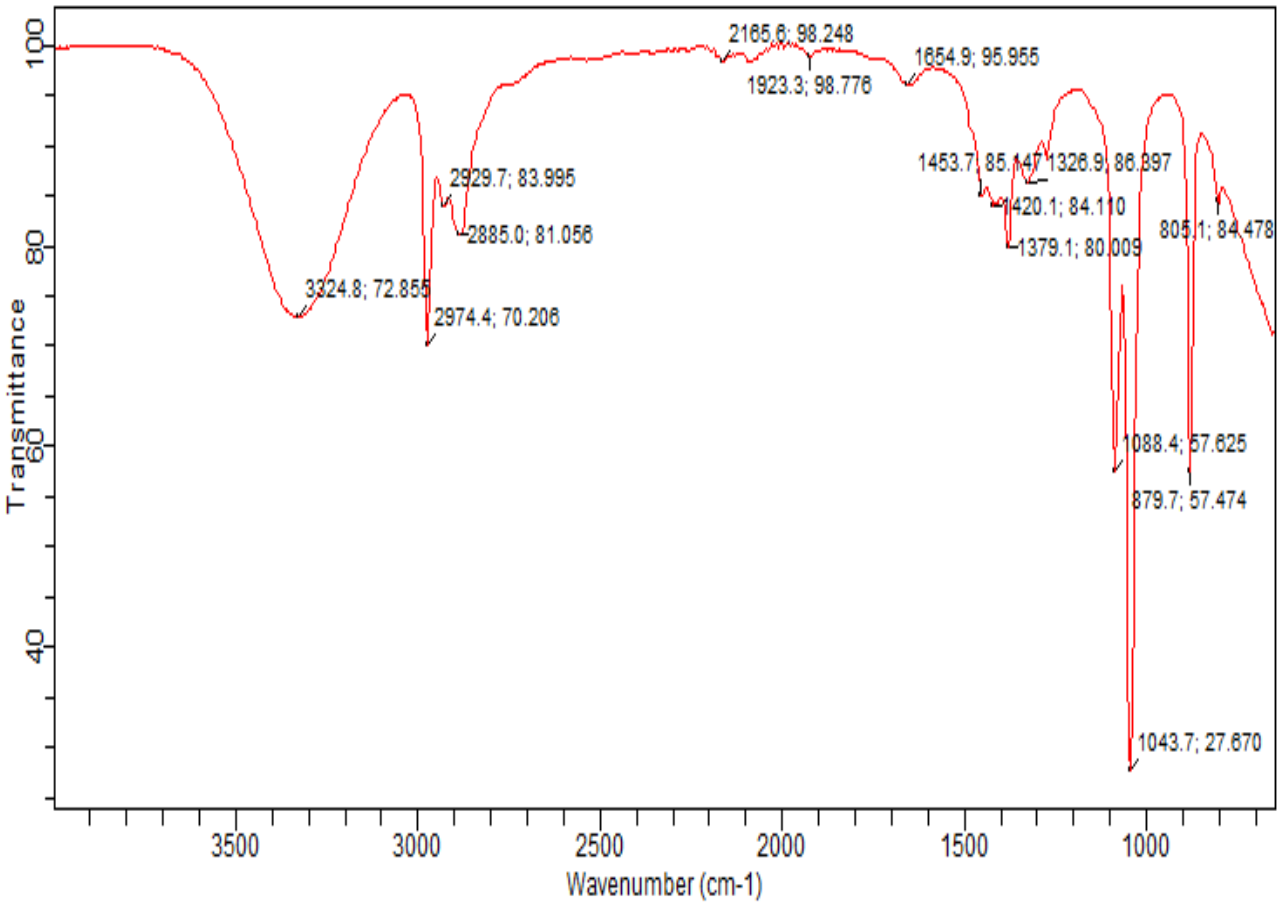
The result of the FTIR of the hydrogen peroxide-treated cane bagasse ethanol sample (CB-H_2O_2) is shown in Figure 4.8. The hydroxyl (OH) group of this sample had its absorbance at a wavelength of 3336.0 cm^{-1} while it's (H-C-H) absorbance was at 2981.9cm^{-1}.

4.13 FTIR spectrophotometry of the sulphuric acid-treated rice stalk ethanol sample

Figure 4.9 shows the result of FTIR of the sulphuric acid treated-rice stalk ethanol sample (RS-H_2SO_4). The absorbance of the hydroxyl (OH) group of this sample was at 3306.1cm^{-1} whereas it's (H-C-H) absorbance was at a wavelength of 2981.9cm^{-1}.

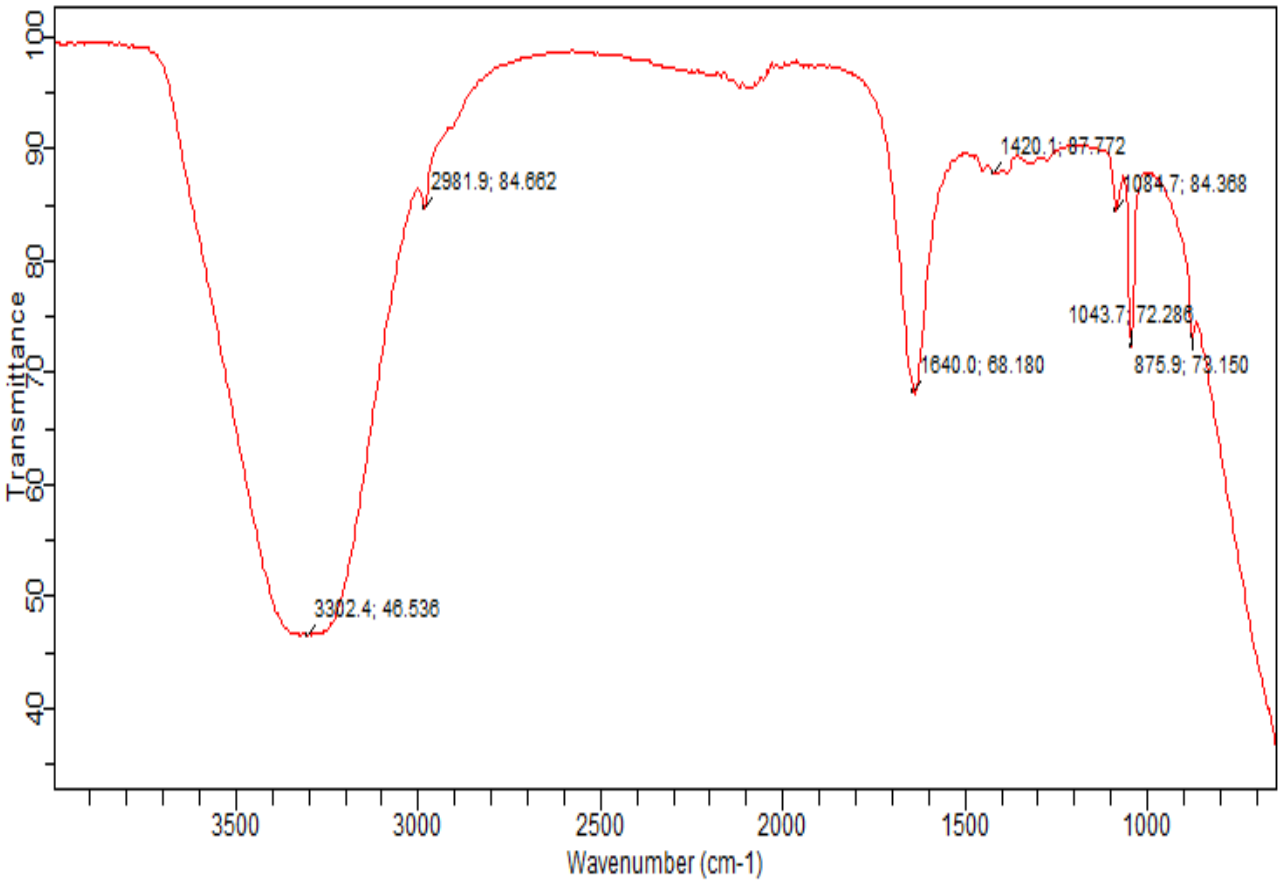
4.14 FTIR spectrophotometry of the hydrogen peroxide-treated rice stalk ethanol sample

The FTIR result of the hydrogen peroxide-treated rice stalk ethanol sample (RS-H_2O_2) is shown in Figure 4.10. The hydroxyl (OH) group of this sample had its absorbance at a wavelength of 3306.1 cm^{-1} meanwhile it's (-C=C) absorbance was at 1640.0 cm^{-1}.



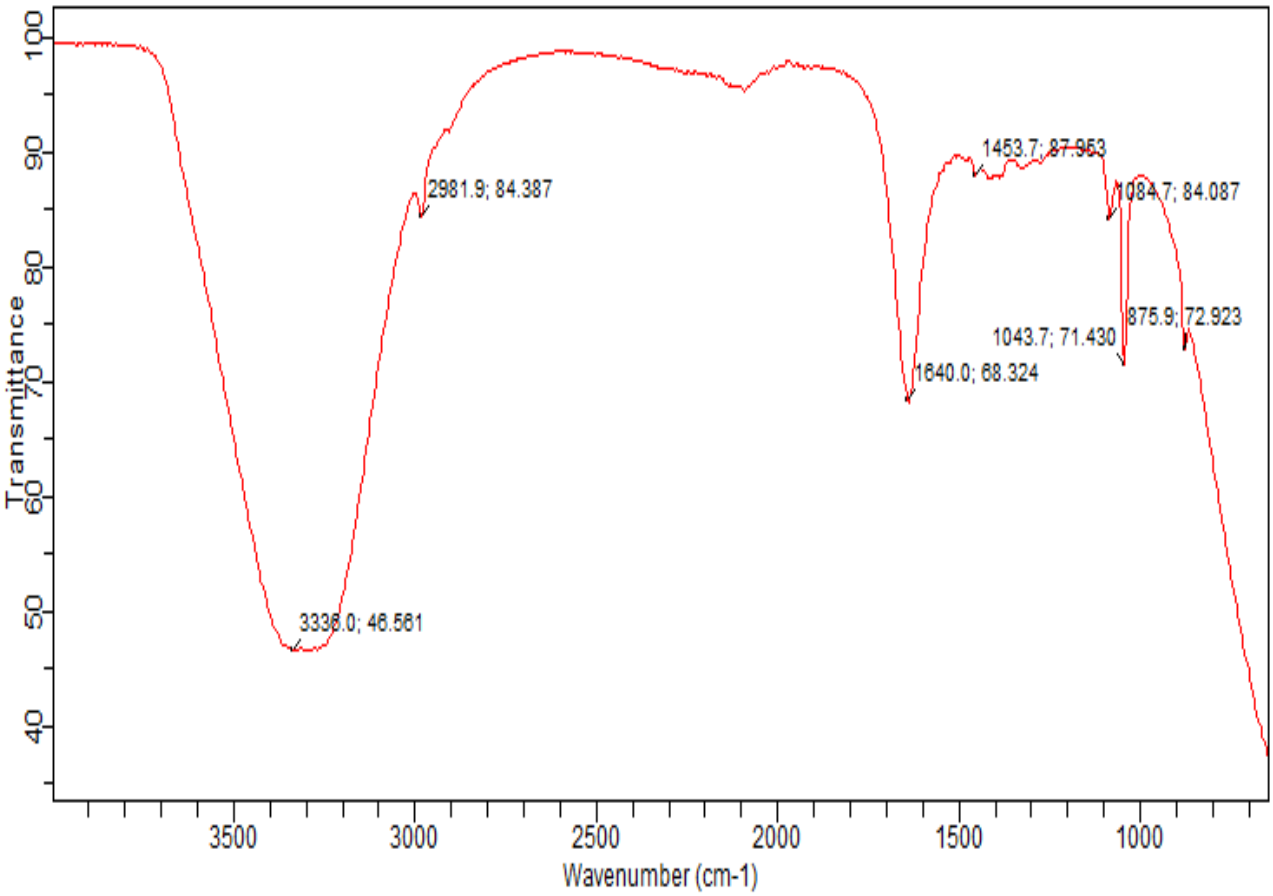
: ~~Ethanol~~ gold standard.

Figure 4.6: FTIR spectrophotometry of the ethanol gold standard



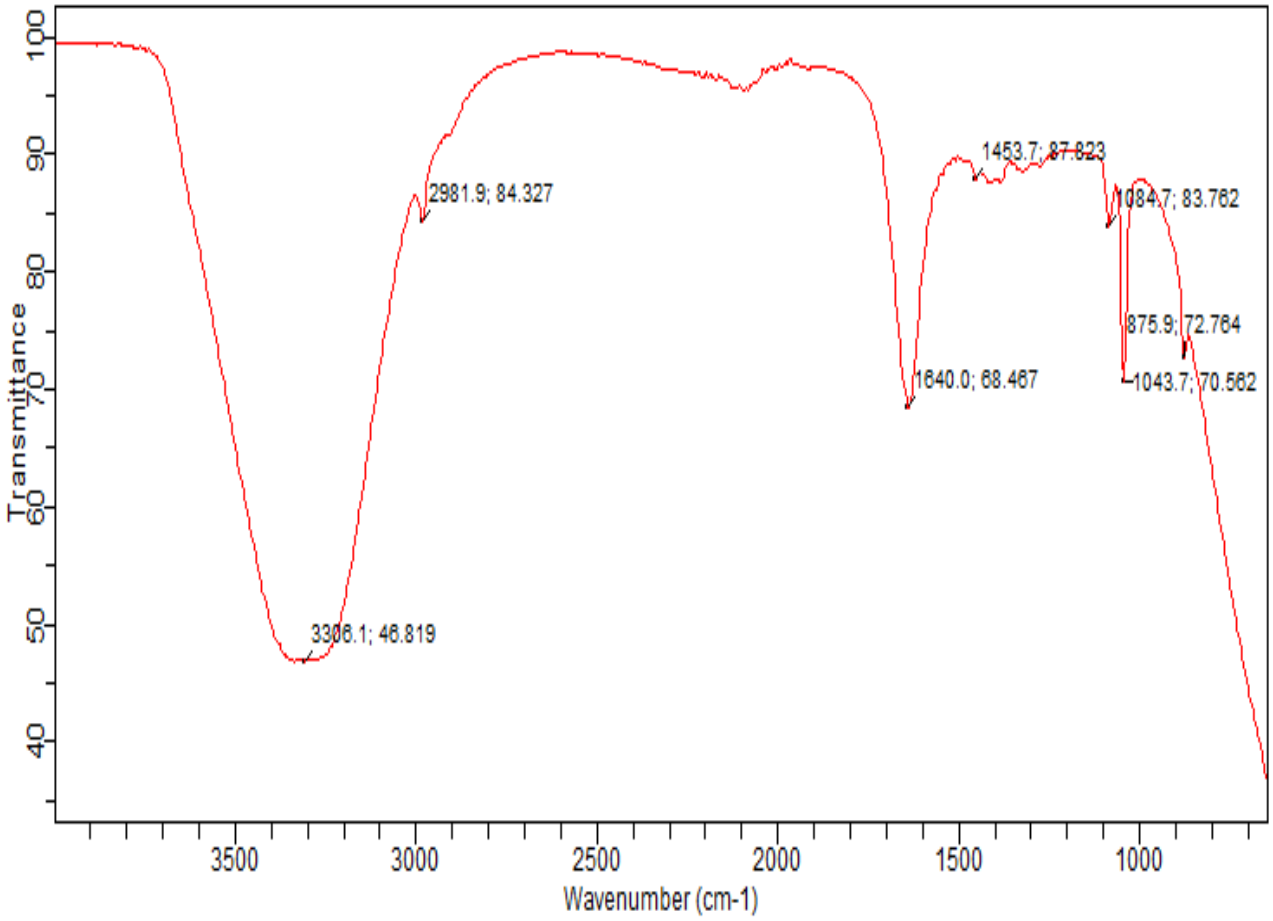
: ~~CB-H_2SO_4~~ Ethanol sample.

Figure 4.7: FTIR spectrophotometry of the sulphuric acid-treated cane bagasse (CB-H_2SO_4) ethanol sample



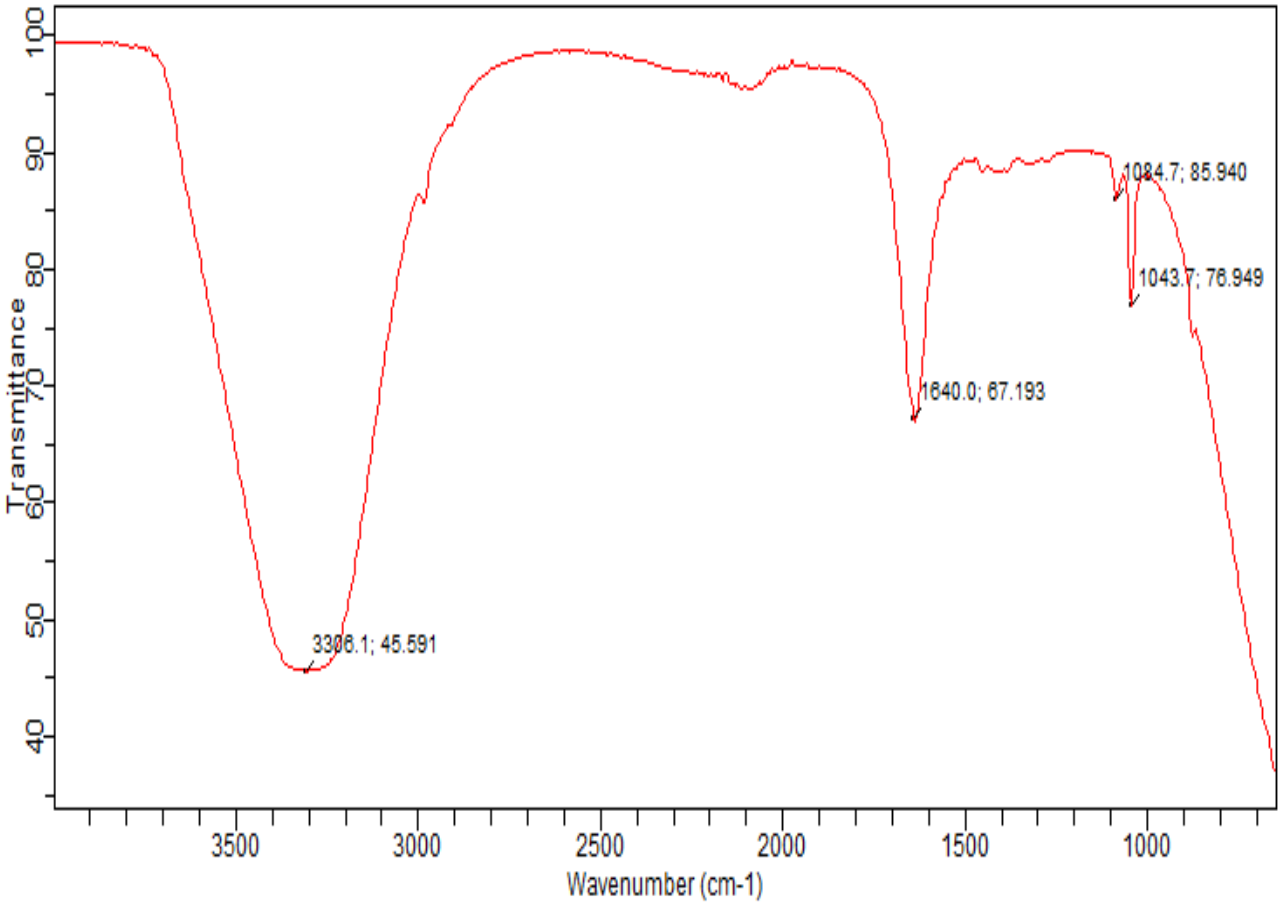
: ~~CB-H_2O_2~~ Ethanol sample.

Figure 4.8: FTIR spectrophotometry of the hydrogen peroxide-treated cane bagasse (CB H_2O_2)ethanol sample



: ~~RS-H_2SO_4~~ Ethanol sample.

Figure 4.9: FTIR spectrophotometry of the sulphuric acid-treated rice stalk (RS-H_2SO_4) ethanolsample



: ~~RS H_2O_2~~ Ethanol sample.

Figure 4.10: FTIR spectrophotometry of the hydrogen peroxide-treated rice stalk (RS H_2O_2)ethanol sample

CHAPTER FIVE

5.0 DISCUSSION

The overall obstinately uncooperative nature of the lignocellulosic substrates in fact is an obstruction to its industrial exploitation, thus, a pretreatment step is necessary to increase the surface area of cellulose by removing the lignin seal, solubilizing the hemicelluloses, disrupting crystallinity and increasing the number of the pores and then making the substrate easily accessible or influenced for enzymatic hydrolysis or microbial biodegradation for optimal bioethanol production and recovery. In this research, pre-treatments of sugar cane bagasse and rice stalk substrates were carried out using dilute sulphuric acid and alkaline hydrogen peroxide. Similar results on the proximate composition analysis of sugarcane bagasse hydrolysate were reported by Omoniyi and Olorunnisola (2014) to have a higher 86% carbohydrate content as compared to that shown by the findings of this research (84.15%), lower ash content 3.3% as compared by that of this research work (6.30%), lower protein 2.3% as compared to that of this research work (3.05%) and higher moisture 7.5% as compared to that this work (3.30%).

This corroborates with the findings of Dussán*et al.* (2014) who showed final ash content of acid-treated sugarcane bagasse to be 6.89% as compared to 6.30% of this research work. Contrary to that, the findings of Damisa*et al*. (2008) reported high ash content of 7.02% from acid-treated corn straw as the highest ash content recorded in this research (7.95%) is from alkaline hydrogen peroxide-treated cane bagasse probably because of the high delignification ability of the alkaline treatment. The renowned higher delignification potential of alkali is been justified by its mechanism of action which is ought to be the saponification of the bonds cross-linking lignin and hemicelluloses (intermolecular ester bonds) causing the opening of the obstinately

uncooperative, crystalline configuration of cellulose so that an increased and overall, optimal enzyme activity can be achieved.

The mechanism of action of acid treatment of lignocellulosic material is believed to be the heterogeneous breakage of hydrogen bonds by both hydrolytic chemical reaction (acid) and physical factors as established by Xiang *et al*. (2003) who also used 210^{o}C and 0.07% H_2SO_4 to demonstrate that once the crystalline formation of cellulose is disrupted and glucose units are freed, they interact with acid soluble lignin forming a lignin-carbohydrate complex. This also is in agreement with the work of Houghton *et al*. (2006) who stated that acidic treatment of substrate is a promising technique that provides a good sugar yield but if re-annealing of cellulose that lead to horrification of cellulose in to microfibrils did not occur. By virtue of its highest carbohydrate (CHO) content of 84.15%, high ash (mineral salts) content of 6.30% and significantly good protein (nitrogen source) content of 3.05%, sulphuric acid-treated hydrolysate of cane bagasse stands out to be the best and thus recommended substrate for bioethanol production. The rest of the treatment options of the substrates provide conditions with higher complement influence on biomass production. This is because for instance, hydrogen peroxide-treated rice stalk is comparably having the best and highest amalgamative and cumulative effect of ash and protein contents (5.10 and 7.00) representing high mineral salt and protein source respectively that are required by *S. cerevisiae* for enhanced cellular growth and bioethanol production, probably this may affect the speed of production and maximum yield to be obtained within the shortest period of time as observed in RS-H_2O_2 substrate.

In this research work, *A. niger* was isolated from garden soiland identified following cultural and microscopic characteristics. Slide culture technique was employed for the microscopic identification of *A.niger*. A heavy dark brown and black irregular & roughened conidial head and

dull yellow hyphae were observed. This agrees with the description of the species by Klitch, (2002),an identification atlas titled identification of common *Aspergillus* spp. The findings of the current research work is in agreement with that of Wijedasa and Liyanapathirana (2012). *Aspergillus niger*isolate GS02 had 6.3mm and 17.1mm zones of amylase and cellulase hydrolysis respectively thus, the selected isolate for the ethanol production. The higher zone of clearance or hydrolysis produced by an isolate after cellulose hydrolysis was the function of its ability to produce higher amount of the enzyme cellulase that was being targeted for hydrolysis and saccharification of the biomass in this research work. This ought to be because of availability of the substrate and how well they adaptand established as only strains with pre-adaptive ability to starch and cellulose hydrolysis are likely to dominating in such environments. This is similar to the work of Ado *et al.* (2010) although 7.2mm was reported to be their best zone of clearance.

Saccharomyces cerevisiae was also isolated from palm wine using dilution and plating method as above. Three different isolates were obtained from different palm wine samples and subjected to different test for the purpose of identification. As mentioned earlier on, only three yeast isolates were identified as *S. cerevisiae* based on their colony characteristics as shape, smoothness, colour, elevations & margin, microscopic examination & budding formation and biological & physiological characteristics as illustrated inLarone(2002).Considering the differences in their colony characteristics, microscopic examination and physiology, these isolates were designated PW01, PW02 and PW03. The findings of this research work are in agreement with that of Monek*et al*., (2008) and that of Brooks (2008) who were able to isolate and identify six morphologically different yeast strains from orchard soil of Nsukka Nigeria and eight yeast isolates from a ripe banana peels out of which only three of the eight were identified as *S. cerevisiae* respectively and likewise, the work of Ali *et al*., (2014) who were able to

successively isolate and identify 7 yeast strains by adopting the same identification scheme above also argues in favour of the findings of this work.

Viability of a cell is its ability to live, reproduce and form a colony (Tong and Herbert 2004). The assessment of the viability of yeast is of utmost importance for fermentation and for bioengineering systems such as fermentation reactors. This viability can be checked in several ways among which the promising one is by examining the micrographic images of the cells after staining them with specific dyes that are specific to the cells metabolic activities or membrane properties. In the case of methylene blue dye, there is a reaction with oxidoreductases of the viable cells and thus, the dye of choice. The viable cells appear relatively colourless on the blue background as compared to the nonviable once that appear dark blue. This findings agrees with that of Tong and Herbert (2004). Just like the Gram positive bacteria, the microscopic examination of the isolates after Gram's staining uncovers Gram positive vegetative cells appearing bluish black, oval in shape and with a clear bud formation.

As a part of the scheme for yeast identification, the physiologic behaviour of various sugar fermentation of the test isolates were examined using Arabinose, Fructose, Galactose, Glucose, Lactose, Maltose and Sucrose where all the isolates were able to assimilate and ferment all the sugars but Arabinose and Maltose with varying gas production potentials. This was ascertained as a result of acid production after the assimilation that turns the colour of the medium from colourless to pink or red due to the presence of Andrade indicator in the Andrade peptone water used. Similar results were reported by Kumar *et al*. (2011) and Ali *et al*. (2014) who showed that the above mentioned sugars were fermented by the *S. cerevisiae* isolates but the exceptions above.

The findings of thermotolerance test of this research work shows that there is a weak growth of PW02 and a good growth of PW01 and PW03 at 40^0C but at temperatures above that, the growth was inhibited. This might be as a result of the mesophilic characteristic or preferences of the yeast *S. cerevisiae*. While at higher temperatures no growth was recorded. This might be attributed to denaturation of enzymes and thus growth inhibition, absence of growth at lower temperatures might as a result of enzymes inactivation that leads to stunted growth. Patil and Patil(2006) reported good growth of yeast at 37^0C. Ali *et al*. (2014) reported a frail growth at 40^0C and a very feeble growth of only 2 of their isolates out of 7 at 42^0C. The findings of this research work lies between the findings of these two Authors.

As there is a constant decrease in cellular growth because of the increase in ethanol concentration in the media, ethanol tolerance of the yeast strains was taken as a concentration of ethanol after which there is a sharp decrease in growth. The findings of this work revealed that PW01 and PW03 isolates tolerated 10% ethanol concentration while isolate PW02 tolerated up to 15% ethanol. Ethanol tolerance in yeast cells might be the result of constitutive expression of MPR1 gene coding for N-acetyl transferase that reduces the production or level of reactive oxygen species (ROS) in the cell thus conferring ethanol stress tolerance to the cells concerned. Thus, as the ethanol concentration increases, production of ROS decreases hence, ethanol tolerance of the cells increases as suggested by Du and Takagi (2007).It could also be a function of oleic acid component of lipid membrane (an unsaturated fatty acid). That cells under ethanol stress alter their fatty acid composition to achieve a compensatory increase in their membrane fluidity caused by ethanol stress i.e., ethanol stress tolerance is achieved by counteracting the membrane fluidizing effect of ethanol, a function of fatty acid composition of the lipid membrane. Here, the predominant unsaturated fatty acids of *S. cerevisiae* that are responsible for

the tolerance are palmitoleic and oleic acids produced by the formation of a Z(cis) double bond between carbon atoms 9 and 10 of saturated 16 and 18-carbon fatty acids through action of acyl coenzyme A (CoA) as suggested by (You *et al*., 2003 and Appiah, 2013). The results obtained in this research work is slightly superior to that of Ali *et al*, (2014) who showed that 2 yeast strains (TA) and (C2) tolerates up to 14% ethanol because the yeast isolate PW02 tolerates up to 15% ethanol. However, Tikka *et al*. (2013) reported that only 1 yeast strain (YDE) was able to tolerate a maximum of 12% ethanol unlike the PW01 and PW03 of this work that tolerates only 10% ethanol. Following the complete identification scheme and having shown to be able to tolerate up to 15% of ethanol, grow at 40^0C, assimilate and ferment all the test sugars known to be fermented by its likes and have the macroscopic and microscopic features of its kind, the isolate PW02 is thus the qualified candidate for various biotransformations and surely for the purpose it is been isolated for hence, it is been used for the production. This might be the function of how well it was adapted in its previous environment from which it is been isolated from i.e. palm wine as suggested by Ado *et al*. (2010).

The ethanol standard specific gravity curve was prepared by plotting the specific gravities of different known concentration of ethanol against their concentrations. The plot reveals that the specific gravities of the different samples decrease with increase in concentration. The coefficient of determination (R^2) of 0.9888 was gotten for the relationship. The equation of the relation between specific gravities and the concentration is also given on the graph and it indicates that a negative relationship exist between the two variables that is to say, the ethanol specific gravities are inversely proportional to the their respective concentrations. This standard curve was used to compare with the values of specific gravities obtained in this research work in order to ascertain the concentration (volumetric analysis) of the ethanol produced.

The highest bioethanol yield of this research work was obtained from sulphuric acid-treated sugarcane bagasse substrate (6.20g/100ml) than with the hydrogen peroxide-treated cane bagasse substrate(5.93g/100ml) followed by sulphuric acid -treated rice stalk(5.06g/100ml) and the least ethanol concentration produced comes from alkaline hydrogen peroxide-treated rice stalk (3.91g/100ml). This might be the result of the influence of a complement of high percentages of ash and protein (6.30% and 3.05% respectively) contents as well as the relatively higher carbohydrate content of 84.15% observed with the sulphuric acid-treated cane bagasse after proximate composition analysis. It is clearly notable that the quantity of ethanol produced is dependent to a greater extent upon the monomeric sugar units available for microbial assimilation as the higher the sugar units available, the higher the ethanol yield to be produced thou, not all the sugars available can beused up as it is the combination of both pentoses and hexoses this is because *S. cerevisiae* cannot ferment both pentose and hexoses unless if it is been genetically engineered.Because of the fact thatthe maximum ethanol yield was obtained from sulphuric acid treatment of cane bagasse in this research work, it is thus recommended to be the best option for ethanol production by virtue of its relatively higher sugar 84.15%, ash 6.30% and protein 3.05% contents. The result of the current research is in contrast to that ofLakkana*et al*. (2012) who obtained 9.62g/100ml as the optimum ethanol produced from sweet sorghum stalks, whereas the work of Arifa*et al*. (2010) and Ado *et al*. (2010) that demonstrated 7.68g/100ml and 4.30g/100ml respectively are in partial agreement with the current workmeanwhile,the result of this work did not correlate with that of Ester *et al*., (2014) who demonstrate 2.80g/100ml from fermentation of sugarcane bagasse too.

Fourier Transform Infrared Analysis (FTIR) meantfor qualitative determination of ethanol was carried out on the samples produced using the FTIR spectrophotometer. This works by exciting

the chemical bonds with infrared light and the different chemical bonds (of the concerned sample) in this excited state absorbs light energy at specific frequencies unique to their various bonds. The wave number of the peak tells what types of bonds are present. This shows the peaks at which the hydroxyl (O-H) bond (that appears much broader than the rest of the peaks) and the carbon hydrogen (H-C-H) bonds are formed and the range of wavelength for the absorption of ethanol is 3100-3600cm^{-1} stretching, 1440-1500cm^{-1} bending and 2800-3000cm^{-1} stretching & bending for OH and H-C-H groups respectively. For functional group analysis, attention is meant to be paid only to the functional group 1400cm^{-1}-4000cm^{-1} region as the rest i.e., <1400cm^{-1} is what is been called the finger print region cheaply because different compounds have different finger prints peculiar to them as it can be seen on the FTIR sketch for the standard as compared to the various test samples. Different other peaks can also be seen on the standard and not the rest of the test samples for example the 2165.6cm^{-1} and 1923.3cm^{-1}. This is ought to be the absorption peaks for either different impurities as a result of handling or the additives in general including stabilizers that have been used after production. The sharpness and number of peaks present on the standard and not the rest of the samples are undoubtedly because of formulation and polishing that the standard ethanol have been subjected to and as well, the number and different types of distillations for example azeotropic distillation it has passed through before it is been dispatched.

For sulphuric acid treated cane bagasse ethanol sample, the O-H stretch and the H-C-H symmetric and asymmetric stretches are located at a wavelength of 3302.4cm^{-1} and 2981.9cm^{-1} respectively meanwhile the O-H stretches and the H-C-H symmetric and asymmetric stretches for alkaline hydrogen peroxide treated cane bagasse ethanol sample were located at 3336.0 cm^{-1} and 2981.9 cm^{-1} respectively. However, the O-H stretch and the H-C-H symmetric and

asymmetric stretches of sulphuric acid treated rice stalk ethanol sample were located at 3306.1 cm^{-1} and 2981.9 cm^{-1} respectively whereas, the O-H stretch and the H-C-H symmetric and asymmetric stretches of alkaline hydrogen peroxide treated rice stalk ethanol sample were located at 3306.1 cm^{-1} but interestingly unlike the rest, there is no H-C-H stretches absorbance (the absorbance of saturated part of the compound for this sample). This may perhaps be largely due to either excess dilution or oxidation of the sample that is ought to have taken place. This is supported by the fact that the chemical agents (sulphuric acid and hydrogen peroxide) used for pretreatment are all oxidizing agents. This leads to formation of unsaturation because of the removal of hydrogen atom leading to realization of alkene with –C=C symmetric stretch and this is what is been observed at a wavelength of 1640.0 cm^{-1}. Further oxidation will lead to formation of alkynes with –C≡C stretching that will be found at wavelength range of 2100-2200 cm^{-1}.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The proximate compositions of sugarcane bagasse and rice stalk treated with sulphuric acid and hydrogen peroxide were determined to be (6.30% ash), (3.05% protein), (84.15% carbohydrates) and (4.75 ash), (5.25 protein), (80.55 carbohydrates) respectively while that of sugarcane bagasse and rice stalk treated with alkaline peroxide were (7.95% ash), (1.75% protein), (83.20% carbohydrates) and (5.10% ash), (7.00% protein), (77.65% carbohydrates) respectively.

Aspergillus niger (GS02) isolated from soil had6.3mm and 17.1mm zones of starch and cellulose hydrolysis respectively and *Saccharomyces cerevisiae* (PW02) isolated from palm wine withstood up to 15% ethanol and grew even at 40ºC temperature.

The maximum ethanol yield obtained via simultaneous saccharification and fermentation was from sulphuric acid-treated sugarcane bagasse (6.20g/100ml).While, 5.06g/100ml was obtained from sulphuric acid-treated rice stalk.

FTIR analysis revealed the presence of the ethanol functional groups from all the ethanol samples produced and all the functional groups had their absorption peaks within their normal ranges of 3100-3600cm^{-1}, 2800-3000cm^{-1} and 1600-1675cm^{-1} for hydroxyl (OH), methyl (CH_3) and methylene (CH_2) functional groups respectively.

6.2 Recommendations

1. There is need for species improvement of the yeast in order for it to be able to ferment pentose and hexose at the same time for this will improving the overall production efficiency of ethanol even at commercial level.
2. Further research should be conducted on the exploration of other lignocellulosic biomass with bioethanol production potentials, determine a suitable pretreatment for their maximum sugar recovery and even attempt their genetic improvement such that lignocellulosic plants with increased yields of fermentable sugars, requiring less costly preprocessing will be available for bioethanol production.
3. As it is essential to start projecting into available solutions against the years of depleted oil reserves that lies ahead, governmental and non-governmental organizations at all levels should encourage and sponsor large scale production of bio-ethanol from cellulosic materials to create employment.

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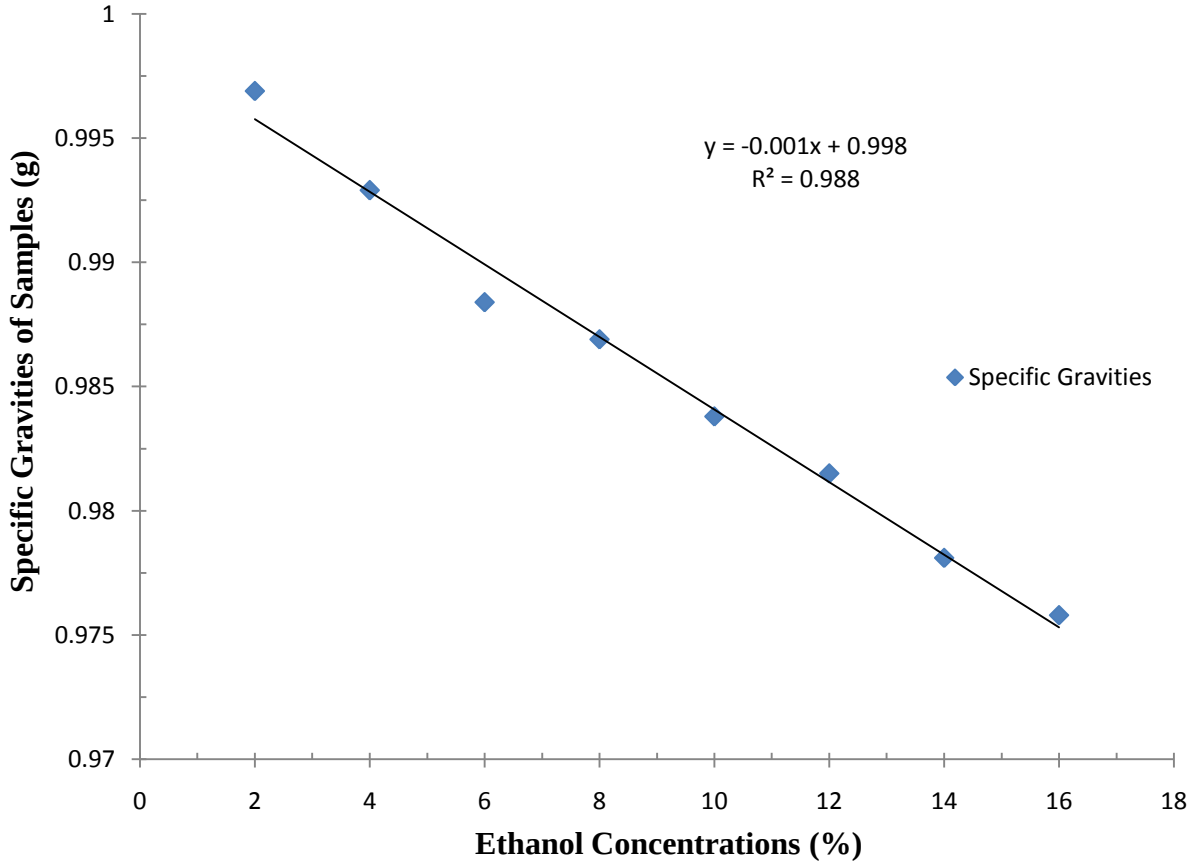
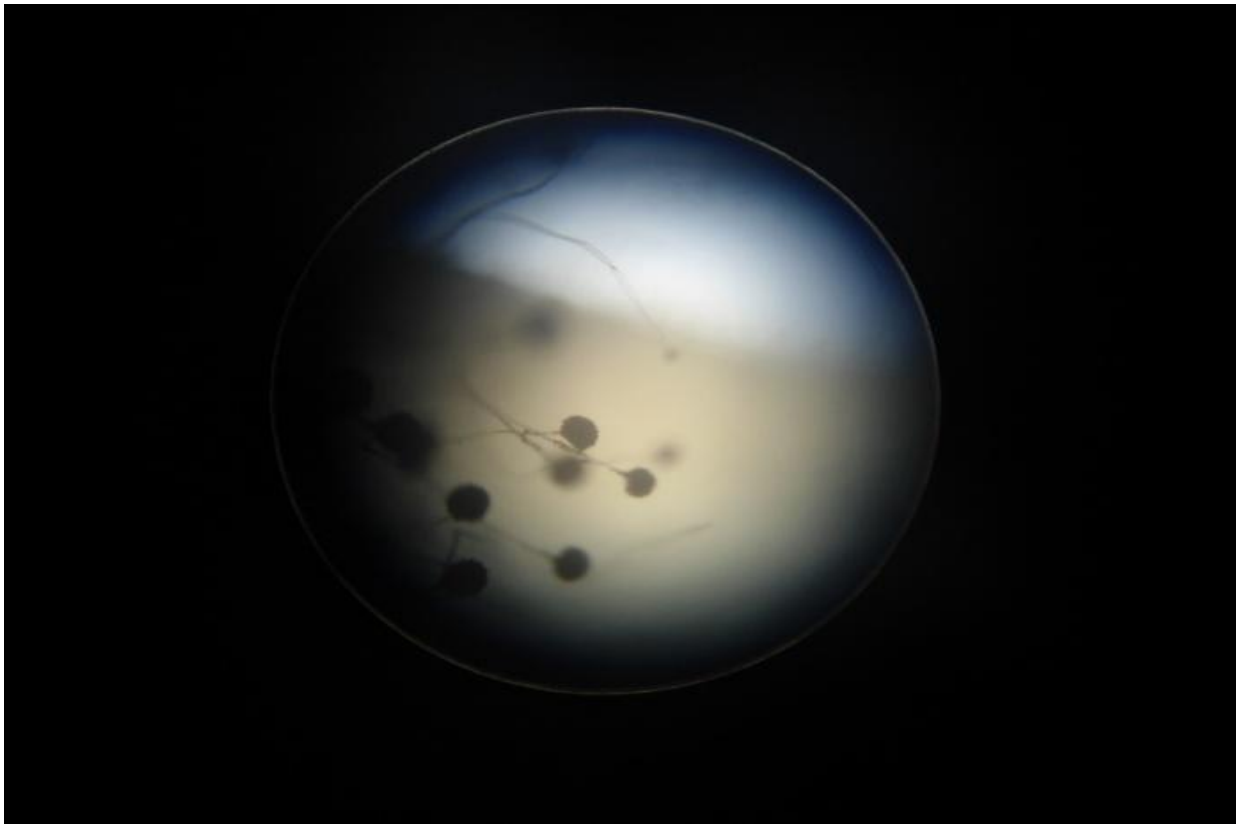
APPENDICES

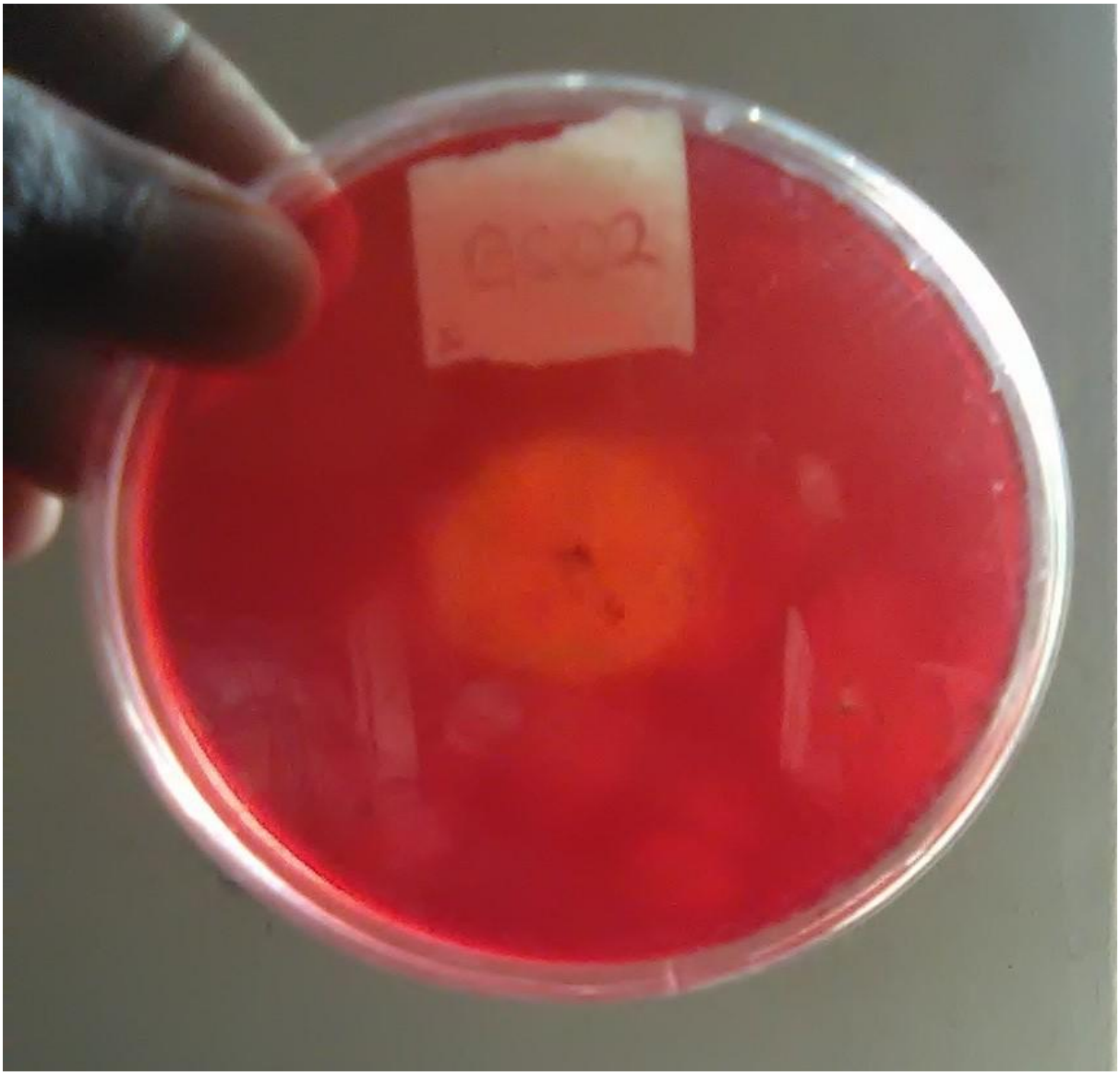
Appendix I: Ethanol standard specific gravity curve.



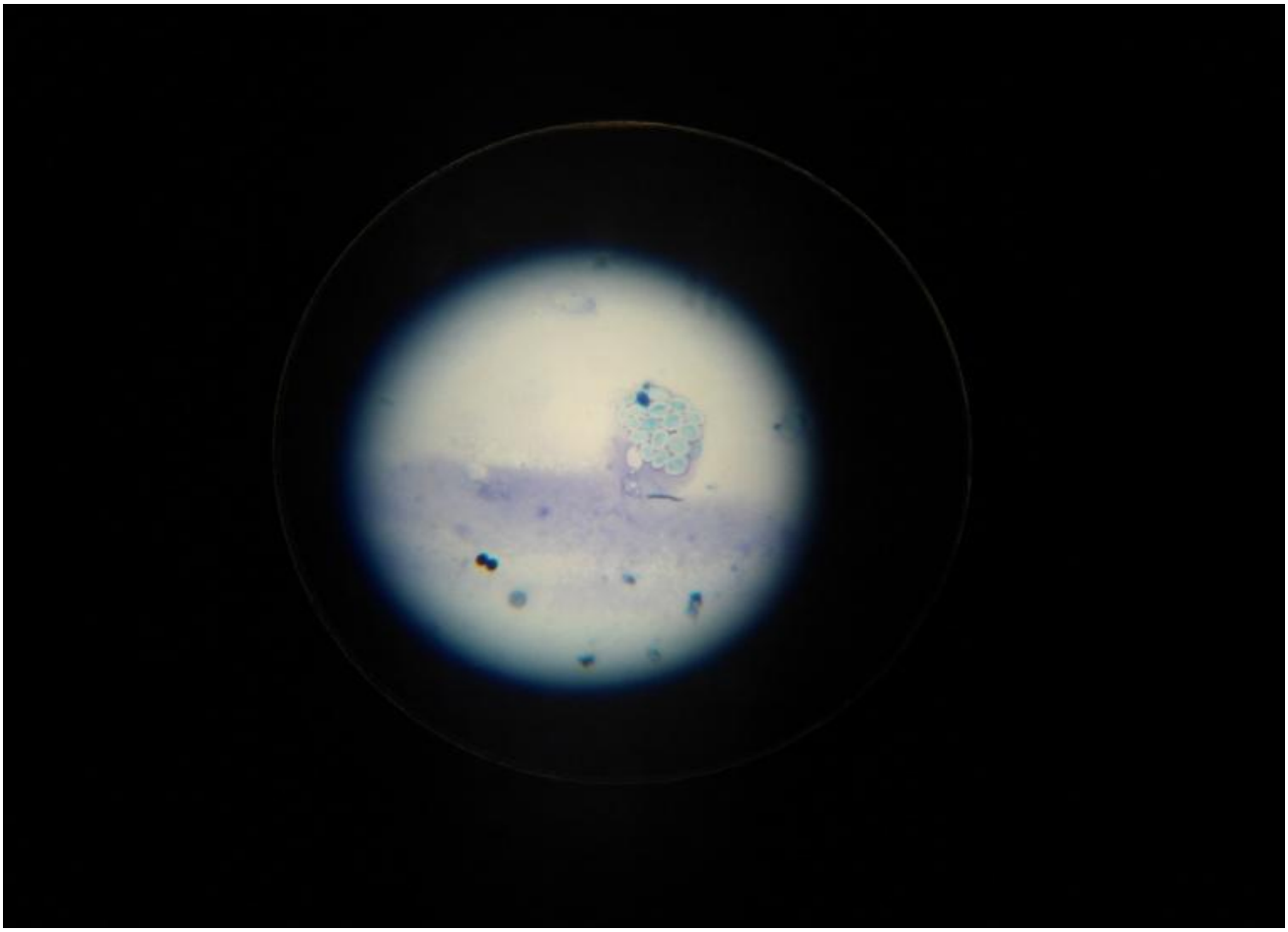
Appendix II: Micrographic image of *A.niger* x100.



Appendix III**:** Screening for cellulase production by *A. niger*isolates



Appendix IV: Micrograph of *S. cerevisiae* methylene blue staining x1000.



Appendix V: Micrograph of *S. cerevisiae* after Gram's staining x1000.