EVALUATION OF ELEPHANT GRASS (*PENNISETUM PURPUREUM*) AS SUBSTRATE FOR BIOETHANOL PRODUCTION USING CO-CULTURES OF *ASPERGILLUS NIGER* AND *SACCHAROMYCES CEREVISIAE*

BY

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DEPARTMENT OF MICROBIOLOGY,

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NIGERIA.

DECEMBER, 2014.

DECLARATION

I declare that the work in this thesis entitled **Evaluation of elephant grass (*Pennisetum purpureum*) as substrate for bioethanol production using co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*** has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Tochukwu Christian AGBODIKE

Name of student	Signature	Date

CERTIFICATION

This thesis entitledEVALUATION OF ELEPHANT GRASS (*PENNISETUM PURPUREUM*) AS SUBSTRATE FOR BIOETHANOL PRODUCTION USING CO-CULTURES OF *ASPERGILLUS NIGER* AND *SACCHAROMYCES CEREVISIAE*by Tochukwu Christian AGBODIKE meets the regulations governing the award of the degree of Masters of Science of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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.

ABSTRACT

Elephant grass (*Pennisetum purpureum*) was evaluated for its ethanol production potential using co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae*isolated from local sources. Proximate and lignocellulose analysis carried out on the plant sample showed that it had crude fibre, lignin, hemicellulose and cellulose contents of 31.5%, 26.78%, 18.76% and 34.16% respectively. *Aspergillus niger* strains were isolated from soil and bread and were further screened for both qualitative and quantitative cellulase production. Qualitative cellulase assay revealed clear zones around colonies indicative of enzyme activity on solid agar medium containing 0.1% carboxymethyl cellulose (CMC) for all the isolates. Quantitative cellulase assay showed that *A. niger* isolate AN-15 from soil gave highest cellulase yield of (0.1792 IU/ml/min) and was therefore selected as a co-culture with *S. cerevisiae*. *Saccharomyces cerevisiae* strains were isolated from palm wine and burukutu. Isolate PW-4 was selected for fermentation based on ethanol tolerance tests and assimilation of more sugars compared to other isolates. Fermentation of grass substrate was carried out at different concentrations ranging from 2-10% and highest ethanol yield of 1.68g/100ml was observed at an optimum substrate concentration of 6% though the yield was much less than that obtained from equal concentration of glucose (8.38g/100ml). Optimization of culture parameters for ethanol production showed maximum ethanol yield at pH 5, 35°C and agitation rate of 300 rpm. The results of the research also revealed that ethanol production by *S. cerevisiae* beyond the fourth day of fermentation is significantly reduced.

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CHAPTER ONE

1.0 INTRODUCTION

Ethanol fuel, ethyl alcohol (CH_3CH_2OH), is the same type of alcohol found in alcoholic beverages. It is an oxygenated fuel with a high octane value like that of petroleum fuels known to run combustion engines at higher compression ratios and thus provides superior performance (Wheals *et al*., 1999). The blending of ethanol into petroleum-based automobile fuels can significantly decrease petroleum use and decrease the release of greenhouse gas emissions. Furthermore, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. Methyl tertiary butyl ether is toxic and is a known contaminant in ground water. Thus, ethanol can be a substitute to mitigate the problems associated with the rising energy demands across the world as well as a way to reduce greenhouse gas emission to as high as 85% (Perlack *et al*., 2005).

Ethanol may be produced either from petroleum products or from biomass substrate. Today, most of the ethanol produced comes from renewable resources (Bothast and Saha, 1997). Although, most of the ethanol currently produced from renewable resources come from sugarcane and starchy grains, significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50% of all biomass in the biosphere such as agricultural residues are lignocellulosic biomass). The technological advances in recent years are promising to produce ethanol at low cost from lignocellulosic biomass (Bothast and Saha, 1997).

Bioethanol production from sugarcane and starch-rich feed stocks such as corn, potato, is considered a first generation process because it has already been developed (Joshi *et al*., 2011). The long-term viability of this process is in question because it requires

significantly increased amounts of cultivatable land and this may lead to significant hike in food prices that will ultimately lead to food insecurity (Mitchell, 2008). Estimates clearly point to the fact that first generation ethanol production process cannot sufficiently meet the global energy needs, therefore, second generation processes to produce bioethanol are gaining momentum(Joshi *et al*., 2011).

The second generation processes use lignocellulosic materials for this purpose and the biosphere clearly has sufficient supplies of lignocellulosic materials. The production of ethanol from lignocellulosic biomass (corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn cob, oat hull, corn fibre, woodchips and cotton stalk), energy crops such as switch grass and Alfa-Alfa, and various weeds such as *Saccharum spontaneum*, *Lantana camara*, *Eichhornia crassipes* (water hyacinth), has become one of the best alternatives because these sources have widespread abundance and the cost of their procurement is relatively cheap (Joshi *et al*., 2001). Even though the lignocellulosic biomass is abundant, the commercialization of the process to produce bioethanol from it is limited due to insufficient research, especially the research related to minimization of production cost. Bioethanol production from lignocellulosic materials relies on technologies that will efficiently hydrolyse cellulosic biomass to fermentable sugars. The hydrolysis process produces by-products that are toxic to yeast cells, thus interfering with the fermentation process (Palmqvist and Hahn-Hagerdal, 2000; Kádár and Réczey, 2004). Although several detoxification methods such as activated charcoal adsorption and lime treatment processes have been devised, an appropriate strategy for efficient hydrolysis of cellulose to fermentable sugars is still lacking (Kaya *et al*., 2000; Aden *et al*., 2002).

Recently, Brazil introduced a new kind of biofuel derived from Elephant grass (*Pennisetum purpureum*). Elephant grass or Napier grass or Uganda grass is a species of grass native to the tropical grasslands of Africa. It is a tall perennial plant, growing to 2–4.5 metres (6.6–14.8 ft.) tall, rarely up to 7.5 metres (25 ft.), with leaves 30–120 centimetres (12–47 in) long and 1–5 centimetres (0.39–2.0 in) broad. The plant was termed 'elephant grass' as a result of it being a favourite food of elephants. In the savannahs of Africa, it grows along lake beds and rivers where the soil is rich and local farmers cut the grass as forage for their animals. It looks like sugarcane but grows a lot faster and produces a much higher quantity of biomass. Elephant grass does reproduce sexually, but the seeds are very small and don't germinate well. The grass reproduces mainly through its rhizomes (root-like underground stems that produce roots below and send up shoots to the surface).Elephant grass can be very invasive and clogs the natural waterways which have to be cleared periodically. It likes tropical weather and can be killed by a light frost but the underground parts will stay alive if the soil doesn't freeze. Elephant grass produces 40 tons of biomass per hectare of crop, while sugarcane produces roughly between 15-20 tons in the same area(D.P.I & F, 2007).

Although this kind of crop is known in Europe and also in the United States, it was a Brazilian company that discovered the promising niche. Sykué Bioenergya based in Sao Paulo, Brazil, dedicated to producing energy from biological sources is investing about USD 80 million in a power plant powered by combusted dry biomass of elephant grass (http://www.energyfuturecoalition.org). Also Elephant grass as a source of biofuel is promising but the use of its cane as a biofuel is still under development. Research is needed to determine other ways of producing energy next to the process of combustion, for

example the use of its fibrous residue that remains after the juice is extracted, to produce ethanol. Until now, Elephant grass is proven effective only in small-scale applications, but it is obvious that this cannot be overlooked anymore(Joshi *et al*., 2011).

1.1 STATEMENT OF RESEARCH PROBLEM

The use of food crops such as corn, cassava tubers and sugarcane to produce biofuels is increasingly being discouraged due to the current worldwide rise in food prices. In order to minimize food-feed-fuel conflicts, it is necessary to integrate all kinds of bio waste into a biomass economy (Mahro and Timm, 2007)**.**

The demand for ethanol has been on the increase due to its various uses such as, chemical feedstock and more importantly as an alternative source of liquid fuel for automobiles. One of the ways of producing ethanol is through fermentation of crops which are rich in sugar or starch such as sugarcane, sugar beet, sweet sorghum, corn and cassava (Abouzeid and Steinkraus, 1983; Okolo *et al*., 1995). However, the major disadvantage of this process is that most of these crops are food crops and tend to increase the cost of production. In order to make the fermentation method cost effective and to meet the great demand for ethanol, research studies are now being directed in two areas namely; the production of ethanol from cheaper raw materials and the study of new microorganisms efficient in ethanol production (Pandey *et al*., 2000a; Akin-Osanaiye *et al*., 2008).

There has also been considerable debate about how useful bioethanol will be in replacing gasoline. Concerns about its production and use relate to increased food prices due to the large amount of arable land required for crops, as well as the energy and pollution balance of the whole cycle of ethanol production, especially from corn (Pimentel,

1998; Youngquist, 1997). The world population is estimated to increase from 6.7 billion to 8 billion by 2030 (USCB, 2008), on the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050, thus, the energy demands of the future is likely to play a key role in geo-political economics. Given this reality, nations around the world are investing in alternative sources of energy, including bioethanol (Campbell and Laherree, 1998). The leading nations in bioethanol production are Brazil and the USA, the latter being the world's largest producer of bioethanol. Asian countries altogether account for about 14% of world's bioethanol production (Carere *et al*., 2008). However, Nigeria ranks 1st in the global feedstock production for bioethanol production most of which is from cassava (Kura, 2014).

1.2 JUSTIFICATION OF RESEARCH

Bioethanol is a form of renewable energy because the energy is generated by using a resource, sunlight, which cannot be depleted and can be produced from agricultural feedstocks. It can be made from very common crops such as sugar cane, potato, manioc and corn. Creation of ethanol starts with photosynthesis causing a feedstock to grow. These feedstocks are then processed into ethanol (Pimentel, 1991).

Cellulosic ethanol offers great promise because cellulose fibres, a major and universal component in plant cell walls, can be used to produce ethanol. According to the International Energy Agency, cellulosic ethanol could allow ethanol fuels to play a much bigger role in the future than previously thought (IEA, 2006). In this respect, inexpensive raw materials such as agricultural wastes, lignocellulosic wastes, fruit wastes, vegetable wastes, municipal and industrial wastes can be used to produce ethanol cheaply (Abouzeid and Reddy, 1986; Park and Baratti, 1991; Schugerl, 1994; Akin-Osanaiye *et al*., 2008).

Increased biofuel production in most developed countries has contributed to the rise in food prices in the world. The concerns over fluctuating crude oil prices, concerns for energy security and climate change have prompted aggressive research and funding to encourage production and use of biofuels especially from lignocellulosic wastes. Hence, recent developments with cellulosic ethanol production and commercialization may allay some of these concerns (Azih, 2007).

The policy to blend ethanol with petroleum for domestic use is conceived to reduce the cost of fuel importation and to respond to strategies to reduce climate change. Ohimain (2010) stated that an investment of over $3.86 billion is already committed into construction of 19 ethanol bio-refineries, 10,000 units of mini-refineries in the 36 states of Nigeria including the Federal Capital Territory (FCT) and feedstock plantations for the production of over 2.66 billion litres of fuel grade ethanol per annum for 4 million families (Ohimain, 2010).

The high oil prices and current environmental concerns motivated Nigeria Global Bio-fuels Limited to invest about US$ 21 million in the sorghum to ethanol fuel production project. The fund will cover the purchase of about 10,000 hectares of virgin land (forests and grasslands) covering seven states (Osun, Oyo, Kwara, Ondo, Ekiti, Niger and Kogi) in Nigeria and seven ethanol plants in each of the seven states, to produce about 1 million litres of ethanol per plant on a daily basis. There is no doubt that the sorghum feedstock for the biofuels plants will compete with production of food crops in the proposed states, thereby exacerbating hunger in the nation. The bio-ethanol production in Nigeria targets staple food crops for its derivation, cultivated land to be used are high-value lands and cleared forest that will have a negative impact on the environment (Agboola *et al*., 2011).

1.3 AIM

The aim of this research work was to evaluate the potential of Elephant grass as substrate for bioethanol production by simultaneous saccharification and fermentation process using co-cultures of selected *Saccharomyces cerevisiae* and *Aspergillus niger.*

1.4 SPECIFIC OBJECTIVES

1. To isolate, characterize and screen *Saccharomyces cerevisiae* and *Aspergillus niger* from various sources.
2. To carry out proximate analysis of Elephant grass sample.
3. To produce bioethanol using Elephant grass as substrate and co-cultures of selected *S. cerevisiae* and *A. niger* strains.
4. To determine the physico-chemical quality of the bioethanol produced.
5. To optimize the environmental factors for bioethanol production by the selected organisms in co-cultures.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Ethanol

Ethanol, also called ethyl alcohol, pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colourless liquid. It is a psychoactive drug and one of the oldest recreational drugs. Best known as the type of alcohol found in alcoholic beverages, it is also used in thermometers, as a solvent, and as a fuel. In common usage, it is often referred to simply as alcohol or spirits. Ethanol is a straight-chain alcohol, and its molecular formula is C_2H_5OH. Its empirical formula is C_2H_6O(NCBI, 2012). Ethanol is the systematic name defined by the IUPAC nomenclature of organic chemistry for a molecule with two carbon atoms (prefix "eth-"), having a single bond between them (suffix "-ane"), and an attached -OH group (suffix "-ol") (Myers and Myers, 2007).

The fermentation of sugar into ethanol is one of the earliest organic reactions employed by humanity and the intoxicating effects of ethanol consumption have been known since ancient times. In modern times, ethanol intended for industrial use is also produced from ethylene (Ballinger and Long, 1960). Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including scents, flavourings, colourings, and medicines. In chemistry, it is both an essential solvent and a feedstock for the synthesis of other products. It has a long history as a fuel for heat and light, and more recently as a fuel for internal combustion engines.

2.1.1 History

Humans have used ethanol since prehistory as the intoxicating ingredient of alcoholic beverages. Dried residues on 9,000-year-old pottery found in China imply that

Neolithic people consumed alcoholic beverages (Roach, 2005). Although distillation was well known by the early Greeks and Arabs, the first recorded production of alcohol from distilled wine was by the School of Salerno alchemists in the 12th century. The first to mention absolute alcohol, in contrast with alcohol-water mixtures, was Raymond Lull (Forbes, 1948). In 1796, Johann Tobias Lowitz obtained pure ethanol by filtering distilled ethanol through activated charcoal. Antoine Lavoisier described ethanol as a compound of carbon, hydrogen, and oxygen, and in 1808, Nicolas-Théodore de Saussure determined ethanol's chemical formula. Fifty years later, Archibald Scott Couper published the structural formula of ethanol. It is one of the first structural formulas determined (AEOL, 2008).

Ethanol was first prepared synthetically in 1826 through the independent efforts of Henry Hennel in Great Britain and S.G. Sérullas in France. In 1828, Michael Faraday prepared ethanol by acid-catalyzed hydration of ethylene, a process similar to current industrial ethanol synthesis (*Lide*, *2000*). Ethanol was used as lamp fuel in the United States as early as 1840, but a tax levied on industrial alcohol during the civil war made its use uneconomical. The tax was however repealed in 1906 (Windholz, 1976). Original Ford Model T automobiles ran on ethanol until 1908. With the advent of Prohibition in 1920, ethanol fuel sellers were accused of being allied with moonshiners and ethanol fuel fell into disuse until late in the 20th century (Morrison and Boyd, 1972; Windholz, 1976).

2.1.2 Physical Properties

Ethanol is a volatile, colourless liquid that has a slight odour. It burns with a smokeless blue flame that is not always visible in normal light. The physical properties of ethanol stem primarily from the presence of its hydroxyl group and the shortness of its

carbon chain. Ethanol's hydroxyl group is able to participate in hydrogen bonding, rendering it more viscous and less volatile than less polar organic compounds of similar molecular weight (Dahlmann and Schneider, 1989).

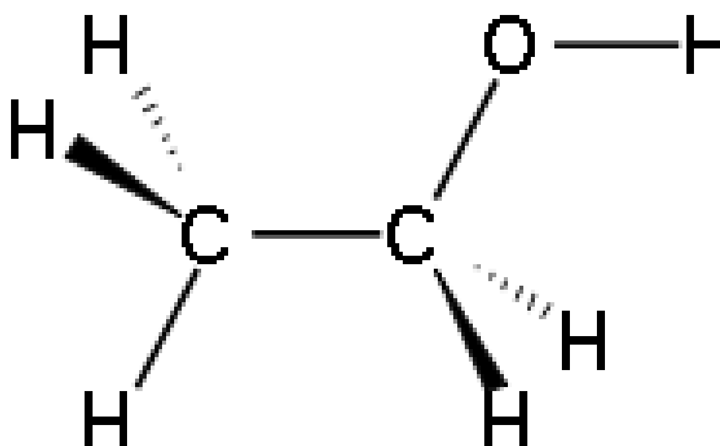


Figure 2.1. Structure of ethanol molecule.

Ethanol is a versatile solvent, miscible with water and with many organic solvents, including acetic acid, acetone, benzene, carbon tetrachloride, chloroform, diethyl ether, ethylene glycol, glycerol, nitromethane, pyridine, and toluene. It is also miscible with light aliphatic hydrocarbons, such as pentane and hexane, and with aliphatic chlorides such as trichloroethane and tetrachloroethylene (Costigan *et al.*, 1980). Ethanol's miscibility with water contrasts with that of longer-chain alcohols (five or more carbon atoms), whose water miscibility decreases sharply as the number of carbon increases.

The addition of even a few percent of ethanol to water sharply reduces the surface tension of water. This property partially explains the "tears of wine" phenomenon. When wine is swirled in a glass, ethanol evaporates quickly from the thin film of wine on the wall

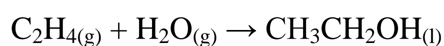
of the glass. As the wine's ethanol content decreases, its surface tension increases and the thin film "beads up" and runs down the glass in channels rather than as a smooth sheet. Mixtures of ethanol and water that contain more than about 50% ethanol are flammable and easily ignited. Alcoholic proof is a widely used measure of how much ethanol (i.e., alcohol) such a mixture contains.

2.1.3 Production of Ethanol

Ethanol is produced both as a petrochemical, through the hydration of ethylene and, via biological processes, by fermenting sugars with yeast. Which process is more economical depends on prevailing prices of petroleum and grain feed stocks (Streitweiser and Heathcock, 1976).

2.1.3.1 Ethylene hydration

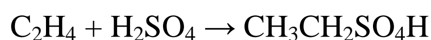
Ethanol for use as an industrial feedstock or solvent (synthetic ethanol) is often made from petrochemical feedstocks, primarily by the acid-catalysed hydration of ethylene, represented by the chemical equation



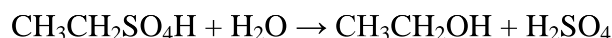
$$C_2H_{4(g)} + H_2O_{(g)} \rightarrow CH_3CH_2OH_{(l)}$$

The catalyst is most commonly phosphoric acid,adsorbed onto a porous support such as silica gel or diatomaceous earth. The Shell Oil Companywas the first company to use this catalyst for large-scale ethanol production in 1947. The reaction is carried out with an excess of high-pressure steam at 300 °C (Morais *et al.*, 1996; Badger, 2002). In the U.S., this process was used on an industrial scale by Union Carbide Corporation but now, only Lyondell-Basell uses it commercially. In an older process, first practiced on the industrial scale in 1930 by Union Carbide, but now almost entirely obsolete, ethylene was hydrated

indirectly by reacting it with concentrated sulphuric acid to produce ethyl sulphate, which was hydrolysed to yield ethanol and regenerate the sulphuric acid (Taherzadeh and Karimi, 2007a ; Taherzadeh and Karimi, 2007b).



$$C_2H_4 + H_2SO_4 \rightarrow CH_3CH_2SO_4H$$

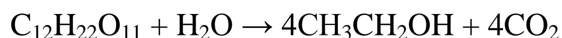


$$CH_3CH_2SO_4H + H_2O \rightarrow CH_3CH_2OH + H_2SO_4$$

2.1.3.2 Fermentation

Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as fuel, is produced by fermentation. When certain species of yeast (e.g. *Saccharomyces cerevisiae*) metabolizesugar they produce ethanol and carbon dioxide. The chemical equations below summarize the conversion:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$



$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CH_3CH_2OH + 4CO_2$$

The process of culturing yeast to produce alcohol is called fermentation. This process is carried out at around 35–40 °C. Toxicity of ethanol to yeast limits the ethanol concentration obtainable by brewing; higher concentrations, therefore, are usually obtained by fortification or distillation. The most ethanol-tolerant strains of yeast can survive up to approximately 15% ethanol by volume (Ritter, 2004). To produce ethanol from starchy materials such as cereal grains, the starch must first be converted into sugars. In brewing beer, this has traditionally been accomplished by allowing the grain to germinate, or malt, which produces the enzymeamylase. When the malted grain is mashed, the amylase converts the remaining starches into sugars. For fuel ethanol, the hydrolysis of starch into

glucose can be accomplished more rapidly by treatment with dilute sulphuric acid, fungal produced amylase, or some combination of the two (Badger, 2002).

2.1.3.3 Cellulosic ethanol

Sugars for ethanol fermentation can be obtained from cellulose. Until recently, however, the cost of the cellulase enzymes capable of hydrolysing cellulose has been prohibitive (Kompala, 2001). Cellulose-bearing materials typically also contain other polysaccharides, including hemicellulose. When undergoing hydrolysis, hemicellulose decomposes into mostly five-carbon sugars such as xylose. *S. cerevisiae*, the yeast most commonly used for ethanol production, cannot metabolize xylose. Other yeasts and bacteria are under investigation to ferment xylose and other pentoses into ethanol (Mick, 2008).

2.1.4 Ethanol Purification

Ethylene hydration or brewing produces an ethanol–water mixture. For most industrial and fuel uses, the ethanol must be purified. Fractional distillation can concentrate ethanol to 95.6% by volume (89.5 mole %). This mixture is an azeotrope with a boiling point of 78.1 °C, and cannot be further purified by distillation. Common methods for obtaining absolute ethanol include desiccation, using adsorbents such as starch, corn grits, or zeolites, which adsorb water preferentially, as well as azeotropic distillation and extractive distillation. Most ethanol-fuel refineries use an adsorbent or zeolite to desiccate the ethanol stream (Lei *et al.*, 2002).

2.1.5 Grades of Ethanol

2.1.5.1 Denatured alcohol

Pure ethanol and alcoholic beverages are heavily taxed, but ethanol has many uses that do not involve consumption by humans. To relieve the tax burden on these uses, most

jurisdictions waive the tax when an agent has been added to the ethanol to render it unfit to drink. These include bittering agents such as denatonium benzoate and toxins such as methanol, naphtha, and pyridine. Product of this kind is called *denatured alcohol* (Andrews, 2008)

2.1.5.2 Absolute ethanol

Absolute or anhydrous alcohol refers to ethanol with low water content. There are various grades with maximum water contents ranging from 1% to Parts per million (ppm) levels. Absolute alcohol is not intended for human consumption. If azeotropic distillation is used to remove water, it will contain trace amounts of the material separation agent (e.g. benzene). Absolute ethanol is used as a solvent for laboratory and industrial applications, where water will react with other chemicals, and as fuel alcohol. Spectroscopic ethanol is an absolute ethanol with a low absorbance in ultraviolet and visible light, fit for use as a solvent in ultraviolet-visible spectroscopy (Chakrabartty, 1978).

2.1.5.3 Rectified spirits

Rectified spirit, an azeotropic composition containing 4% water, is used instead of anhydrous ethanol for various purposes. Wine spirits are about 188 proof and the impurities are different from those in the 190 proof laboratory ethanol (Eyidogan *et al.*, 2010).

2.1.6 Uses of Ethanol

2.1.6.1 Ethanol fuel

The largest single use of ethanol is as a motor fuel and fuel additive. Brazil has the largest national fuel ethanol industry. Gasoline sold in Brazil contains at least 25% anhydrous ethanol. Hydrous ethanol (about 95% ethanol and 5% water) can be used as fuel

in more than 90% of new cars sold in the country. Brazilian ethanol is produced from sugar cane and noted for high carbon sequestration . The US uses Gasohol (max 10% ethanol) and E85 (85% ethanol) ethanol/gasoline mixtures in most modern and light duty vehicles with internal combustion. Ethanol may also be utilized as a rocket fuel, and is currently in lightweightrocket-powered racing aircraft (Reel, 2006).

Ethanol as a fuel reduces harmful tailpipe emissions of carbon monoxide, particulate matter, oxides of nitrogen, and other ozone-depleting pollutants. Argonne National Laboratory analysed the greenhouse gas emissions of many different engine and fuel combinations. Comparing ethanol blends with gasoline alone, they showed reductions of 8% with the biodiesel/petrodiesel blend known as B20, 17% with the conventional E85 ethanol blend, and that using cellulosic ethanol lowers emissions by 64% (http://www.energyfuturecoalition.org/biofuels/benefits_env_public_health.html).

World production of ethanol in 2006 was 51 gigalitres (1.3×10^{10} US gal), with 69% of the world supply coming from Brazil and the United States. More than 20% of Brazilian cars are able to use 100% ethanol as fuel, which includes ethanol-only engines and flex-fuel engines (Jones, 2008).

2.1.6.2 Alcoholic beverages

Ethanol is the principal psychoactive constituent in alcoholic beverages, with depressant effects on the central nervous system. It has a complex mode of action and affects multiple systems in the brain, the most notable one being its agonistic action on the gamma-aminobutyric acid (GABA) receptors. Similar psychoactives include those that also interact with GABA receptors, such as gamma-hydroxybutyric acid (GHB) (Chastain,

2006). Ethanol is metabolized by the body as an energy-providing nutrient, as it metabolizes into acetyl CoA, an intermediate common with glucose and fatty acid metabolism that can be used for energy in the citric acid cycle or for biosynthesis. Alcoholic beverages vary considerably in ethanol content and in foodstuffs they are produced from. Most alcoholic beverages can be broadly classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. The ethanol content of a beverage is usually measured in terms of the volume fraction of ethanol in the beverage, expressed either as a percentage or in alcoholic proof units.

2.1.6.3 Feedstock

Ethanol is an important industrial ingredient and has widespread use as a base chemical for other organic compounds. These include ethyl halides, ethyl esters, diethyl ether, acetic acid, ethyl amines, and to a lesser extent butadiene(Eyidogan *et al.*, 2010).

2.1.6.4 Antiseptic

Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62% v/v as an antiseptic. Ethanol kills organisms by denaturing their proteins and dissolving their lipids and is effective against most bacteria and fungi, and many viruses, but is ineffective against bacterial spores (McDonnell and Russell, 1999).

2.1.6.5 Treatment for poisoning by other alcohols

Ethanol is sometimes used to treat poisoning by other, more toxic alcohols, in particular methanol and ethylene glycol. Ethanol competes with other alcohols for the

alcohol dehydrogenase enzyme, lessening metabolism into toxic aldehyde and carboxylic acid derivatives, and reducing one of the more serious toxic effect of the glycols to crystallize in the kidneys (Barceloux *et al.,* 2002).

2.1.6.6 Solvent

Ethanol is miscible with water and is a good general purpose solvent. It is found in paints, tinctures, markers, and personal care products such as perfumes and deodorants. It may also be used as a solvent in cooking, such as in vodka sauce (Streitweiser and Heathcock, 1976).

2.2 Lignocellulosic Ethanol Production

Long-term economic and environmental concerns have resulted in a great amount of research in the past couple of decades on renewable sources of liquid fuels to replace fossil fuels. Burning fossil fuels such as coal and oil releases CO_2, which is a greenhouse gas and a major cause of global warming (Yat *et al*., 2008). With only 4.5% of the world's population, the United States is responsible for about 25% of global energy consumption and 25% of global CO_2 emissions (Yat *et al*., 2008). The average price of gasoline in 2005 was $2.56 per gallon, which was $0.67 higher than the average price of gasoline in the previous year (Yat *et al*., 2008). Yet, in June 2008, the average price of gasoline in the United States reached $4.10 per gallon (D.O.E., 2008).

Conversion of abundant lignocellulosic biomass to biofuels as transportation fuels presents a viable option for improving energy security and reducing greenhouse emissions. Unlike fossil fuels, which come from plants that grew millions of years ago, biofuels are produced from plants grown today. They are cleaner burning than fossil fuels, and the short

cycle of growing plants and burning fuel made from them does not add CO_2 to the atmosphere. It has been reported that cellulosic ethanol and ethanol produced from other biomass resources have the potential to cut greenhouse gas emissions by 86% (Wang *et al.*, 2007).

Lignocellulosic materials such as agricultural residues (e.g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), and dedicated crops (switchgrass, salix) are renewable sources of energy. These raw materials are sufficiently abundant and generate very low net greenhouse emissions. Approximately 90% of the dry weight of most plant materials is stored in the form of cellulose, hemicellulose, lignin, and pectin (Yat *et al.*, 2008). The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for conversion to fuel. For the conversion of biomass to fuel, the cellulose and hemicellulose must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them.

2.2.1 Structure of Lignocellulosic Biomass

Lignocellulose is the primary building block of plant cell walls. Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble non-structural materials such as non-structural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Jorgensen *et al.*, 2007). The composition of these constituents can vary from one plant species to another and the chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003). For example, hardwood has greater amounts of cellulose, whereas wheat straw and leaves have more hemicellulose (Table 2.1) (Sun and Cheng, 2002). In addition, the ratios between various constituents

Table 2.1. Lignocellulose contents of common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
S32 rye grass (early leaf)	21.3	15.8	2.7
S32 rye grass (seed setting)	26.7	25.7	7.3
Orchard grass (medium maturity)	32	40	4.7
Grasses (average values for grasses	25-40	25-50	10-30

(Source: Howard *et al*., 2003)

within a single plant vary with age, stage of growth, and other conditions (Perez *et al*., 2002).

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure (Figure 2.2). This linear polymer consists of D-glucose subunits linked to each other by β-(1,4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils.

Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the β-(1, 4)- glycosidic linkages. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form. The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-*o*-methylglucuronic, D-glucuronic, and D-galactouronic acids).

The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1, 4)-glycosidic bonds and occasionally β-(1, 3)-glycosidic bonds (Kuhad *et al*., 1997). In addition, hemicelluloses can have some degree of acetylation, for example, in heteroxylan. In contrast to cellulose, the polymers present in

hemicelluloses are easily hydrolysable. These polymers do not aggregate, even when they co-crystallize with cellulose chains.

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack (Perez *et al*., 2002). Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds link these phenolic monomers together. In general, herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents (Table 2.1) (Howard *et al*., 2003; Kumar *et al*., 2009).

Large amount of lignocellulosic wastes are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. Sadly, much of the lignocellulose waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon (Levine, 1996). However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals and cheap energy sources for fermentation, improved animal feeds and human nutrients. Lignocellulytic enzymes also have significant potential applications in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture.

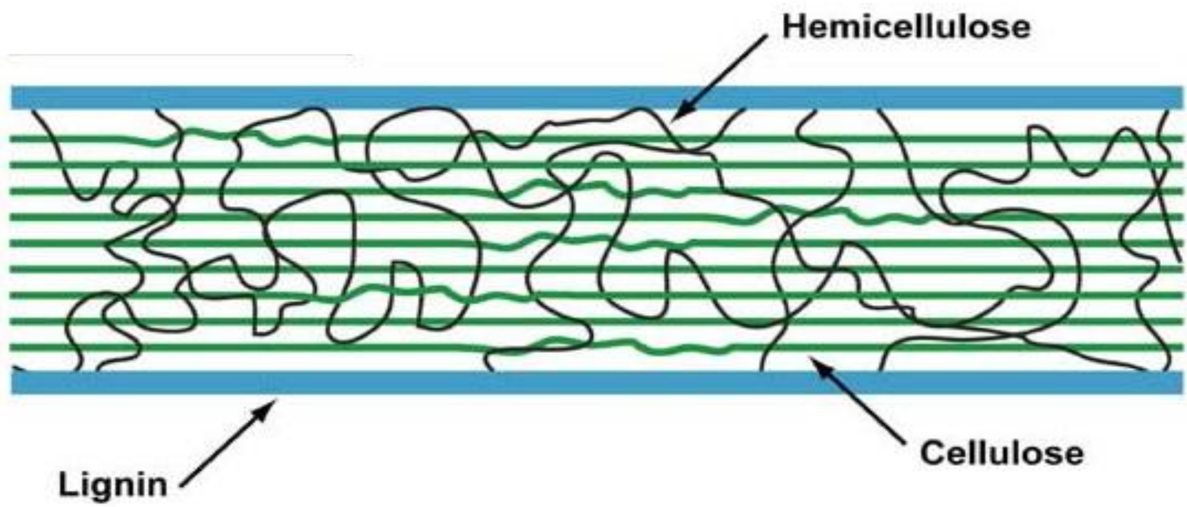


Figure 2.2. Structure of Lignocellulosic Biomass. (Source**:** Kumar *et al.* 2009)

2.2.2 Value-Added Products from Lignocellulosic Wastes (LCW)

Biomass can be considered as the mass of organic material from any biological material, and by extension, any large mass of biological matter and a wide variety of biomass resources are available on our planet for conversion into bioproducts. These may include whole plants, plant parts (e.g. seeds, stalks), plant constituents (e.g. starch, lipids, protein and fibre), processing by-products (distiller's grains, corn solubles), materials of marine origin and animal by-products, municipal and industrial wastes (Smith *et al*., 1987). These resources can be used to create new biomaterials and this will require an intimate understanding of the composition of the raw material whether it is whole plant or constituents, so that the desired functional elements can be obtained for bioproduct production.

Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues. Biodevelopment of biowastes provide a wide range of affordable renewable value-added products from LCW (Pandey *et al*., 2000b; van Wyk, 2001; Howard *et al*., 2003).

2.2.2.1 Reducing sugars

Fermentable sugars, come first in the value chain of processed LCW with glucose, xylose, xylitol, cellobiose, arabinose, pentose and galactose being the main reduced sugars produced (Akmar and Kennedy, 2001; Saha, 2003; Rodríguez-Chonga *et al*., 2004; Yáñez *et al*., 2004; Sepúlveda-Huerta *et al*., 2006; Tabka *et al*., 2006; Hanchar *et al*., 2007; Singh *et al*., 2008; Li *et al*., 2008; Kim *et al*., 2008). In these sugar-producing processes, hydrolysable sugars yield of up to 83.3% has been achieved at the reaction temperatures of 37° - 50°C for 6 – 179 h at pH 5 - 6. The size of substrate added determines the amount of

the saccharification products (Baig *et al*., 2004). In the enzymatic hydrolysis step using celluclast® supplemented with novozym®, a degree of saccharification of 100% has been achieved (Marques *et al*., 2008). Some transgenic plant residues have been reported to yield nearly twice as much sugar from cell walls compared to wild types (Chen and Dixon, 2007).

Glucose seems to be the major monosaccharide product from LCW. The challenge facing depolymerisation of hemicellulose into fermentable sugars is the requirement for a consortium of enzymes to complete the hemicellulose hydrolysis, leading to high enzyme costs. Efforts to overcome the problem include process improvement and the use of modified microorganisms that produce the required hemicellulose enzymes (Lu and Mosier, 2007; Haan *et al*., 2007).

2.2.2.2 Bio-fuel

Worldwide, there is a growing concern over the fossil oil price increase, the security of the oil supply and the negative impact of fossil fuels on the environment, particularly greenhouse gas emissions (Hahn-Hägerdal *et al*., 2006). Conversion of LCW to biofuels provides the best economically feasible and conflict-free second-generation renewable alternatives (Rubin, 2008). Significant advances have been made towards bioconversion of plant biomass wastes into bioethanol, biodiesel, biohydrogen, biogas (methane).

Production of ethanol from sugars or starch from sugarcane and cereals, respectively, impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Hence, the technology development focus for the production of ethanol has shifted towards the utilization of residual lignocellulosic

materials to lower production costs (Howard *et al*., 2003). Currently, research and development of saccharification and fermentation technologies that convert LCW to reducing sugars and ethanol, respectively, in eco-friendly and profitable manner have picked tempo with breakthrough results being reported (Lin and Tanaka, 2006; Prasad *et al*., 2007; Patel *et al*., 2007; Pasha *et al*., 2007; Taherzadeh and Karimi, 2007b; Sánchez and Cardona, 2008).

Ethanol yield of 6 - 21% has been obtained through fermentation of agricultural and municipal residues (Mtui and Nakamura, 2005; Sjöde *et al*., 2007; Li *et al*., 2007; Akin-Osanaiye *et al*., 2008; Cara *et al*., 2008; Sørensen *et al*., 2008). While microaeration enhances productivity of bioethanol from LCW using ethanologenic *E. coli* (Okuda *et al*., 2007), simultaneous saccharification and fermentation (SSF) using recombinant *Saccharomyces cerevisiae* result to as high as 62% of the theoretical value (Itoh *et al*., 2003).

The principal benefits of performing the enzymatic hydrolysis together with the fermentation, instead of in a separate step after the hydrolysis, are the co-fermentation of both hexoses and pentoses during SSF, reduced end-product inhibition of the enzymatic hydrolysis and the reduced investment costs (Kádár and Réczey, 2004; Olofsson *et al*., 2008). Life cycle assessment (LCA) shows that bio-ethanol from LCW results to reductions in resource use and global warming (von Blottnitz and Curran, 2007). The long-term benefits of using waste residues as lignocellulosic feedstocks will be to introduce a sustainable solid waste management strategy for a number of lignocellulosic waste materials; contribute to the mitigation in greenhouse gases through sustained carbon and nutrient recycling; reduce the potential for water, air, and soil contamination associated

with the land application of organic waste materials; and to broaden the feedstock source of raw materials for the bio-ethanol production industry (Champagne, 2007).

2.2.2.3 Enzymes

Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992a,b; Beauchemin *et al*., 2001, 2003). It is estimated that approximately 20% of the >1 billion US dollars of the world's sale of industrial enzymes consists of cellulases, hemicellulases and pectinases and that the world market for industrial enzymes will increase in the range of 1.7-2.0 billion US dollars by the year 2005 (Bhat, 2000).

In the baking industry, xylanases are used for improving desirable texture, loaf volume and shelf life of bread. A xylanase, Novozyme 867, has shown excellent performance in the wheat separation process (Christopherson *et al*., 1997). Hemicellulases are used for pulping and bleaching in the pulp and paper industry where they are used to modify the structure of xylan and glucomannan in pulp fibres to enhance chemical delignification (Suurnäkki *et al*., 1997).

A patented Lignozyme® process is effective in delignifying wood in a pilot pulp- and paper process (Call and Mücke, 1997). In bio-pulping where lignocellulolytic enzymes were used the following was achieved: tensile, tear and burst indexes of the resultant paper improved, brightness of the pulp was increased and an improved energy saving of 30-38% was realised (Scott *et al*., 1998). Laccases can degrade a wide variety of synthetic dyes

making them suitable for the treatment of wastewater from the textile industry (Rosales *et al*., 2002). Organisms such as the white rot fungi producing lignases could be used for the degradation of persistent aromatic pollutants such as dichlorophenol, dinitrotoluene and anthracene (Gold and Alic, 1993).

2.2.2.4 Food and feed

Bioconversion of lignocellulosic agro-residues through mushroom cultivation and single cell protein (SCP) production offer the potential for converting these residues into protein-rich palatable food and reduction of the environmental impact of the wastes. Mushroom cultivation provides an economically acceptable alternative for the production of food of superior taste and quality which does not need isolation and purification (Israilides and Philippoussis, 2003; Philippoussis *et al*., 2007).

Mushrooms with increased number of fruit bodies and high contents of protein and total carbohydrates are obtained when LCW substrates are used in combination. On the other hand, SCP production from LCW offers a potential substrate for conversion of low-quality biomass into an improved animal feed and human food. SCP is the protein extracted from cultivated microbial biomass. It can be used for protein supplementation of a staple diet by replacing costly conventional sources like soymeal and fishmeal to alleviate the problem of protein scarcity. Moreover, bioconversion of agricultural and industrial wastes to protein-rich food and fodder stocks has an additional benefit of making the final product cheaper (Anupama and Ravindra, 2000).

2.2.3 Bioprocessing of Lignocellulosic Materials

Technologies are currently available for all steps in the bioconversion of lignocelluloses to ethanol and other chemical products. However, these technologies must be improved and new technologies developed to produce renewable biofuel and other bioproducts at prices that can compete with current production costs. The feedstock costs can be minimized by initially focusing on agricultural residues and waste materials.

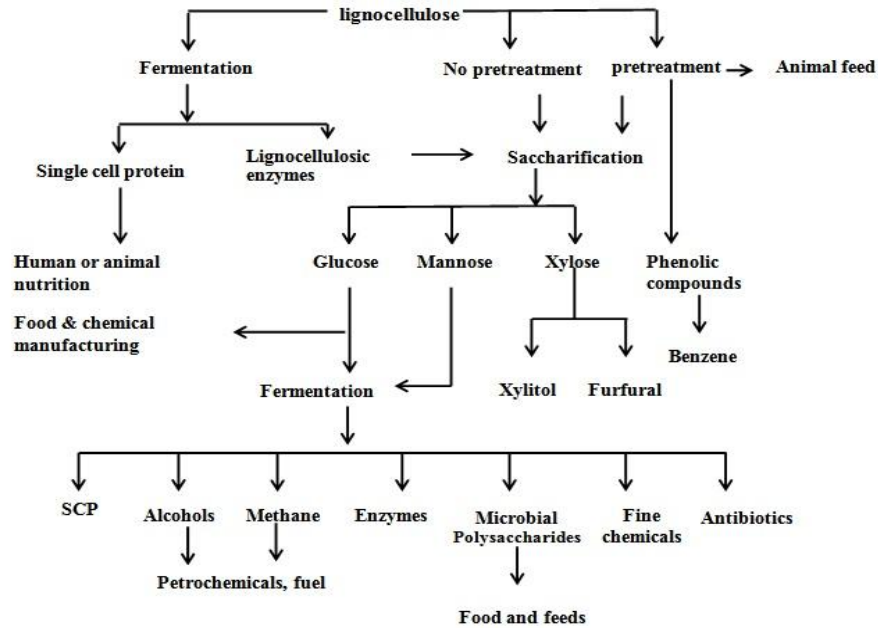
Processing involves the use of biocatalysts, whole microorganisms or their enzymes or enzymes from other organisms to synthesize or bioconvert raw materials into new products; recover/purify such bioproducts and subsequently any needed downstream modifications. Bioconversions of lignocellulosic materials to useful, higher value products normally require multi-step processes (Figure 2.3) which include: (i) pretreatment (mechanical, chemical or biological) (Grethlein, 1984; Grethlein and Converse, 1991), (ii) hydrolysis of the polymers to produce readily metabolizable molecules (e.g. hexose or pentose sugars), (iii) bio-utilization of these molecules to support microbial growth or to produce chemical products and (iv) the separation and purification (Smith *et al*., 1987).

2.2.4 Pretreatment Technologies for Lignocellulosic Wastes

2.2.4.1 Mechanical pretreatment

Mechanically based pretreatment technologies are aimed at reducing the size of LCW to facilitate subsequent treatments. Reduction of biomass size below #20 sieves shows the best mechanical performance (de Sousa *et al*., 2004). Mechanical pretreatment technologies increase the digestibility of cellulose and hemicellulose in the lignocellulosic biomass. The use of mechanical chopping (de Sousa *et al*., 2004); hammer milling (Mani *et*

al., 2004); grind milling (Mtui and Nakamura, 2005); roll milling (Qi *et al*., 2005); vibratory milling (Guerra *et al*., 2006) and ball milling (Inoue *et al*., 2008) have proved



successful as a low cost pretreatment strategy.

Figure 2.3. Generalised process stages in lignocellulose bioconversion into value-added bioproducts. (Source: Howard *et al*. 2003)

2.2.4.2 Physical pretreatment

Elevated temperatures and irradiation are the most successful physical treatments in the processing of LCW. Thermogravimetric treatment of wood waste under both inert and oxidant atmospheres from room temperature up to 1100 K leads to moisture loss; hemicellulose, cellulose and lignin decomposition (Lapuerta *et al*., 2004). On the other hand, pyrolysis of nutshells, straws, sawdust and municipal solid wastes at temperatures of 600 - 1200 K result to yields of char, liquid and gaseous products of up to 55% of the

original LCW (Puértolas *et al*., 2001; Demirbas, 2002; Bonelli, 2003; Chen and Leung, 2003; Álvarez *et al*., 2005; Phan *et al*., 2008; Zabaniotou *et al*., 2008).

Irradiation can cause significant breakdown of the structure of LCW. Microwave irradiation at a power of up to 700 W at various exposure times resulted to weight loss due to degradation of cellulose, hemicellulose and lignin, and the degradation rates are significantly enhanced by the presence of alkali (Zhu *et al*., 2005a, 2005b, 2006). In addition, gamma radiation has been shown by Yang *et al*. (2008) to cause significant breakdown of the structure of powder of 140 mesh wheat straw, leading to weight loss and glucose yield of 13.40% at 500 kGy.

2.2.4.3 Physicochemical pretreatment

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

Addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion of LCW can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete liquefaction of hemicellulose, glucan, xylan, mannan, galactan, and arabinan (Jeoh and Agblevor, 2001; Sun and Cheng, 2002). Such pretreatments also lead to higher digestion efficiencies during production of monosaccharides, oligosaccharides, lactic acid, antibacterial violet pigments and methane gas (Liu *et al*., 2002; Kim *et al*., 2003; Asada *et al*., 2005; Wang and Chen, 2007; Öhgren *et al*., 2007).

2.2.4.4 Chemical pretreatment

Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicellulose and cellulose from LCW. Powerful oxidizing agents such as ozone and H_2O_2 effectively remove lignin; does not produce toxic residues for the downstream processes; and the reactions are carried out at room temperature and pressure (Sun and Cheng, 2002).

When these pretreatments are performed by using 0.5-2 M alkali at 120-200°C, they substantially facilitate saccharification and improve enzymatic hydrolysis of LCW. Dilute and concentrated acids at high temperature are suited for hydrolysis of LCW. Studies by del Campo *et al*. (2006) and Karimi *et al*. (2006) have established that 0.5% H_2SO_4 is optimal for treatment of wastes from vegetables and rice straw, respectively. More concentrated H_2SO_4 (up to 2.5 M) has been shown to be able not only to hydrolyse cellulose and hemicellulose, but also in separating lignin and other organic components from LCW (Iranmahboo *et al*., 2002; Alma and Acemioglu, 2004; Okafoagu and Nzelibe, 2006; Rahman *et al*., 2007). Sulphur (IV) Oxide (SO_2) and fly ash in flare gas; HNO_3, HCl and polyhydric alcohol in the presence of sulphuric acid are also useful in LCW pretreatment

(Fan, 2003; Herrera *et al*., 2004; Kobayashi *et al*., 2004; Rodríguez-Chonga *et al*., 2004; Hassan and Shukry, 2008).

2.2.4.5 Biological pretreatment

Biological treatment involves the use of whole organisms or enzymes in pretreatment of LCW. Both fungi and bacteria are used for biotreatment of LCW. Commercial preparations of fungal and bacterial hydrolytic and oxidative enzymes are also widely used instead of these microorganisms. Fungal pretreatment of agricultural residues is a new method for improvement of digestibility (Sinegani *et al*., 2005). White-, brown- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials whereby brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Sun and Cheng, 2002).

Bacterial pretreatment of LCW involves both anaerobic and aerobic systems. Anaerobic degradation utilizes mainly mesophilic, rumen derived bacteria (Han and Shin, 2002; Hu and Yu, 2005, 2006; Neves *et al*., 2006; Hu *et al*., 2008; Yue *et al*., 2008). Aerobic-anaerobic systems have an upper hand when it comes to degradation of LCW richer in lignin content (Ammary, 2004; Mshandete *et al*., 2005, 2008) while in aerobic system alone the actinomycete,*Streptomyces griseus,*is able to produce high levels of extracellular hydrolytic enzyme that degrade lignocellulose (Arora *et al*., 2005). *Escherichia coli* and *Klebsiella oxytoca* strains have been genetically engineered to produce microbial biocatalysts that produce bioethanol from lignocellulosic materials (Jarboe *et al*., 2007; Peterson and Ingram, 2008).

Enzymatic pretreatment of LCW utilize hydrolytic and oxidative enzymes that are mainly derived from fungi and bacteria. Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (endo-1,4-glucanohydrolase) which attacks regions of low crystallinity in the cellulose fibre, creating free chainends; (2) exoglucanase or cellobiohydrolase (CBH) (1,4-β-glucan cellobiohydrolase) which degrades the molecule further by removing cellobiose units from the free chain ends and (3) β-glucosidase which hydrolyses cellobiose to produce glucose (Sun and Cheng, 2002). In addition, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, feruloylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Nikolov *et al*., 2000; Draude *et al*., 2001; Aranda *et al*., 2004; Mtui and Nakamura, 2005, Roman *et al*., 2006; Georgieva *et al*., 2008).

During the enzymatic hydrolysis, cellulose is degraded by cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol. Ligninolytic enzymes are primarily involved in lignin degradation in oxidative reactions that are mainly free radical driven in the presence (or sometimes absence) of mediators. The main enzymes involved are lignin peroxidase, manganese peroxidase and laccase (Hao *et al*., 2006; Mtui and Nakamura, 2007, 2008; Mtui and Masalu, 2008). The hydrolytic and oxidative enzymatic reactions are mainly carried out at 30° - 45°C with low enzyme loading rate at reaction time of 6 - 26 h.

2.3 The Genus *Aspergillus*

Members of the genus *Aspergillus* are septated moulds that belong to;

Kingdom: Fungi

Phylum: Ascomycota

Class: Eurotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as moulds on the surface of a substrate, as a result of the high oxygen tension. They grow on carbon-rich substrates like monosaccharides (such as glucose) and polysaccharides (such as amylose). *Aspergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *A. niger* is a prime example of this; it can be found growing on damp walls, as a major component of mildew(Bennett, 2010).

These moulds produce upright conidiophores that are simple and terminate in globose or clavate swelling. Members of this genus possess erect stalk (hypha) and thick spore heads. The mycelium of the *Aspergillus* is like that of many fungi, the hyphae are well developed, profusely branched septated hyaline; their cells are multinucleate (Alexopoulous and Mims, 1979). The conidiophores are either septate or non-septate and arise from one-foot cell in the mycelium as a branch perpendicular to the long axis of the cell and usually about mid-way its length. The conidiophores swell into a vesicle at the end bearing a sterigmata from which the conidiophore cut-off, the foot cells may twist and their

connection with hyphae may be composed of a continuous series of foot cells each bearing short conidiophores (Raper and Fennell, 1965).

The mycelium is usually non-coloured, the submerged part is vegetative and the aerial part is fertile, colonies are often zonate, sterigmata are simple or compound and may be coloured. The conidia are in chains with colours ranging from green, black or brown. The mould grows well and the rate of colony growth and diameter attained within a specified time on a specified medium differs from group to group and from species to species (Raper and Fennel, 1965; Frazier and Westhoff, 1988).

2.3.1 *Aspergillus niger* (Black mould)

This is the most common of all the *Aspergillus* species, characterized by its very dark brown to black colonies, biseriate conidial heads with large vesicles, faily long metulae and irregularly roughened conidia (Raper and Fennell, 1965; Klich and Pitt, 1988). On Malt Extract Agar (MEA), conidial areas are black; mycelia white and inconspicuous; reverse uncoloured and colonies granular to floccose. This ubiquitous species is commonly isolated from soils, plant litter, plant rhizospheres, seeds, dried fruits and nuts. It is one of the most commonly reported fungi from foods and indoor environments (Klich, 2002).

Aspergillus niger has been documented to sustain growth in freezing temperatures, which indicates it as a thermotolerant species that can also survive at very high temperatures. Its thermotolerant abilities enable growth in a wide temperature range from 6 to 47°C with a preferred optimum temperature at 35°-37°C. The fungus is capable of growing over a very wide pH range, from 1.4 to 9.8 pH. The growth ability in various temperature ranges, pH ranges as well as the abundant amount of conidiospores allow the

species to be continuously widespread. Conidiospores are distributed by air (Schuster *et al*., 2002).

2.3.1.1 A. niger in Biotechnology

In the field of biotechnology, *A. niger* is a valuable asset to microbiology for its ability to produce a variety of useful substances (Oliveira *et al.,* 2008). The use of microorganisms in biotechnology is common, however *A. niger* is considered to be one of the most essential of those microorganisms (Schuster *et al*., 2002). The significance of *A. niger* is the industrial role that it plays in the production of proteins, enzymes and fermentation. It is capable of producing heterologous proteins, such as human cytokine interleukin-6 (Semova *et al.,* 2006). This very useful microbe is even referred to as an "industrial workhorse" because of the frequent use in many applications. Many enzymes including amylases, lipases, cellulases, xylanases, glucoamylase and proteases are produced by this fungus. *A. niger* is usually used in fermentation for the production of these various enzymes (Pel *et al*., 2007; Andersen *et al*., 2008).

Using a solid-state fermentation (SSF) method, glucoamylase was produced by *A. niger* where the fungus was inoculated with nutrient supplies and the study showed continuous production of the enzyme (Varzakas *et al.,* 2007). *A. niger* strain ATCC 1015 is most well-known as the strain that produces citric acid. Citric acid serves the purpose of improving taste, nutrition and shelf life of food products in the food industry (Pel *et al.*, 2007).

A. niger can produce alpha-galacatosidase, which is an enzyme that is capable of breaking down certain non-digestible oligosaccharides in the digestive tract. Those non-

absorbable oligosaccharides that are not broken down usually cause flatulence (gas). *A. niger* is used in dietary supplement, Beano (Schuster *et al*., 2002) which can lower frequency of flatulence or other abdominal issues caused by those oligosaccharides that have not been fully digested (Rajoka *et al.,* 2008).

2.4 The Genus *Saccharomyces*

Saccharomyces is a genus in the kingdom of fungi that includes many species of yeast. It belongs to:

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Saccharomycotina

Class: Saccharomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Saccharomyces is from Greek "saccharo" (sugar) and "myco" (fungus) and means *sugar fungus*. Many members of this genus are considered very important in food production. It is known as the brewer's yeast or baker's yeast. They are unicellular and saprophytic fungi. One example is *Saccharomyces cerevisiae*, which is used in making wine, bread, and beer. Other members of this genus include *Saccharomyces bayanus*, used in making wine, and *Saccharomyces boulardii*, used in medicine.

Colonies of *Saccharomyces* grow rapidly and mature in three days. They are flat, smooth, moist, glistening or dull, and cream to tannish cream in colour. The inability to use nitrate and ability to ferment various carbohydrates are typical characteristics of *Saccharomyces*.Generally, they have a diameter of 2-8µm and length of 3-25µm.

Blastoconidia (cell buds) are observed. They are unicellular, globose, and ellipsoid to elongate in shape. Multilateral (multipolar) budding is typical. Pseudohyphae, if present, are rudimentary and hyphae are absent (Walker, 1998).

Saccharomyces produces ascospores, especially when grown on V-8 medium, acetate ascospor agar, or Gorodkowa medium. These ascospores are globose and located in asci. Each ascus contains 1-4 ascospores. Asci do not rupture at maturity. Ascospores are stained with Kinyoun stain and ascospore stain(Walker, 1998).

2.4.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a eukaryotic microbe. More specifically, it is globular-shaped, creamy to white coloured yeast belonging to the Fungi kingdom, which includes multicellular organisms such as mushrooms and moulds. Natural strains of the yeast have been found on the surfaces of plants, the gastrointestinal tracts and body surfaces of insects and warm-blooded animals, soils from all regions of the world and even in aquatic environments (Martini, 1993). Most often it is found in areas where fermentation can occur, such as the on the surface of fruit, storage cellars and on the equipment used during the fermentation process (Mortimer, 2000)

S. cerevisiae is also considered to be a "model organism" by scientists. Its big advantage is that it is both a unicellular and eukaryotic organism. Another advantage is its fast growth grate. On a normal yeast medium, it takes 90 minutes for the yeast population to double and colonies are usually visible 2-3 days after placing them on fresh medium (Sherman, 2002).

2.4.1.1 Application to Biotechnology

One of the oldest applications of *Saccharomyces cerevisiae* in biotechnology is its role in the fermentation of alcoholic beverages and food production. It is the critical component in the fermentation process that converts sugar into alcohol; an ingredient shared in beer, wine and distilled beverages. It is also used in the baking process as a leavening agent; yeast-releasing gas into their environment results in the spongy-like texture of breads and cakes (Landry *et al.,* 2006).

In brewing beer, two different types of yeasts are produced in the fermentation process, depending on the type of beer created. Top-fermenting yeasts, also known as ale yeasts, form foam on top of the wort, the liquid containing the sugars used to be converted into ethanol. The yeast stays at the top of the tank, and begins to ferment at warm temperatures. This process is used in the creation of ales, porters, stouts and wheat beer (Bekatorou, 2006). Bottom-fermenting yeasts, also known as lagers yeast, ferment at cooler temperatures, and the yeast settle at the bottom of the tank (Talaro and Talaro, 2002).

In food production, *S. cerevisiae* also acts as a leavening agent. During preparation, dried yeast cells are added with the rest of the ingredients. While baking, yeast reacts with its environment and releases gas. This gas is trapped, forming holes as it bakes. This contributes to the spongy-like texture of breads and cakes seen after baking. While dried yeast cells include a leavening agent, unleavened yeast could also be used to add flavour to the bread (Bekatorou, 2006).

2.5 Elephant Grass (*Pennisetum purpureum*)

Elephant grass, also called Napier grass, gigante (Costa Rica) or mfufu (East Africa), is a robust perennial plant with a vigorous root system, sometimes stoloniferous with a creeping rhizome. Its culms are usually 1.8-3.6m high and branching upwards. Leaf-sheaths are glabrous or with tubercle-based hairs, leaf-blades are 20-40mm wide with margins thickened and shiny. Inflorescence exists as a bristly false spike up to 30cm long, dense, usually yellow-brown in colour, more rarely purplish (Chippendall, 1955).

It is native to subtropical Africa (Zimbabwe), now introduced into most tropical and subtropical countries and usually grows during the summer in damp grassland and forest edges. It is similar in appearance to sugarcane, but has narrower leaves and does not grow tothe same height (cane will grow to 6m). Elephant grass grows best in high-rainfall areas (in excess of 1,500mm per year), but its deep root system allows it to survive in dry times. It survives drought quite well when established because of its deep root system and grows best in deep, fertile soils through which its roots can forage. Friable loams are preferable but it is susceptible to frost (Russell and Webb, 1976). Elephant grass will stand heavy grazing and provides a great bulk of feed (Harrison and Snook, 1971), especially if fertilized and irrigated. It is suited to rapid rotational grazing, which must not be severe enough to hinder regrowth and only the leaves are eaten when the grass is near maturity (Ware-Austin, 1963).

Elephant grass is one of the most valuable forage, soilage and silage crops in the wet tropics and will give very effective control of erosion in its own ecological niche. As an environmental weed, elephant grass can form bamboo-like, densely tufted clumps that become invasive in bushland vegetation. Garden plantings and the dumping of garden

waste in bushland are the main sources of infestation of this weed. Unmanaged forage plantings that are not grazed also contribute to infestations. Elephant grass is an opportunistic weed with the ability to persist in disturbed areas, out-competing other native vegetation (D.P.I & F, 2007).

Figure 2.4. Elephant Grass

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of samples

3.1.1 Sample collection for *Aspergillus niger*

A hundred grams of soil sample was gotten from the lawn in the Department of Microbiology, Ahmadu Bello University, Zaria. Bread sample purchased from a local retail shop was moistened and kept in a cupboard for 7 days to allow the growth of moulds.

3.1.2 Sample Collection for *Saccharomyces cerevisiae*

Fermented palm wine sample was purchased and collected into a pre-washed and sterile bottle from a palm wine dealer along Nupe Street, Sabon-Gari Zaria. "Burukutu" (local beverage) was also purchased and collected into a pre-washed and sterile bottle from a local beer parlour in Palladan, Zaria.

3.1.3 Sample Collection and Identification of Elephant Grass

Elephant grass sample was harvested along the bank of Galma River, Jos road, Zaria, Kaduna state. The plant sample was taken to the herbarium section in the Department of Biological Sciences, Ahmadu Bello University, Zaria for identification and was given the voucher number 475. A voucher specimen was also deposited.

3.2 Media for Isolation

Malt extract agar (MEA) (Biotech)was used for the isolation of both *A. niger* and *S. cerevisiae*. The medium was prepared according to manufacturer's instruction and sterilized at 121°C for 15 minutes.

3.3 Isolation of Test Organisms

3.3.1 Isolation of *A. niger*

Twenty-five grams of the soil sample was dissolved in 250ml of sterile water in a conical flask. The flask was vigorously shaken and allowed to stand for an hour. Ten millilitres of the supernatant was taken and dispensed into a test tube from which 1ml was taken and a 10-fold dilution was carried out into tubes containing 9ml of distilled water each to obtain dilutions up to 10^{-5}. Aliquots of 0.1ml of dilutions 10^{-3}, 10^{-4} and 10^{-5} were aseptically transferred onto sterile petri dishes containing solidified MEA and spread with a sterile bent glass rod in order to prevent excessive growth of mould the plates. The plates were incubated at room temperature for 5 days. The plates were observed for mould growth with black sporulation which was then subcultured onto fresh MEA plates to obtain pure cultures. The pure isolates were then transferred onto fresh MEA slants and stored for further use in the refrigerator at 4°C. For the bread samples, mould growth on the bread surface with black spores were aseptically transferred onto fresh MEA plates and subsequently incubated, purified by continuous subculture until pure culture was gotten and stored as above.

3.3.2 Isolation of *S. cerevisiae*

A loopful of the fermented palm wine sample was aseptically streaked solidified MEA plates. The streaked plates were incubated at room temperature for 3 days. Moist, raised and creamy colonies observed were aseptically picked and subcultured onto fresh MEA plates to obtain pure cultures. The pure isolates were then transferred onto fresh MEA slants and stored for further use in the refrigerator at 4°C. Same procedure as above

was carried out using the Burukutu sample and also an industrial strain was obtained from one of the breweries in Kaduna.

3.4 Identification of Test Organisms

3.4.1 Identification of *A. niger* Isolates

The *A. niger* isolates were identified according to Klich (2002) as follows:

3.4.1.1 Colonial morphology

The colonial appearance was observed on the MEA plates after 5 days. These include the colour of the spores and the colour on the reverse of the plates.

3.4.1.2 Microscopic morphology

Wet mounts of the mould isolates were made using a flamed inoculating needle to aseptically pick a portion of the mycelial mat. This was placed on a clean glass slide, a drop of methylene blue dye was added and the slide was covered with a clean cover slip. The preparation was observed under the x40 objective of the light microscope for the characteristics of the conidia and the arrangement of the spores on the conidia.

3.4.2 Identification of *S. cerevisiae* Isolates

The yeast isolates were identified as follows:

3.4.2.1 Colonial morphology

The colonial appearance was observed on the MEA plates after 48 hours. These include the size, shape and pigment formation.

3.4.2.2 Microscopic and vegetative morphology

Wet mounts of yeast isolates were made by using a flamed wire loop to aseptically pick a colony onto a clean glass slide. A drop of methylene blue dye was added onto the slide and then covered with a clean cover slip. The preparation was observed under the x40 objective of the light microscope.

3.4.2.3 Pseudomycelium or true mycelium observation

Presence of pseudomycelium or true mycelium was observed using corn meal agar. The medium was prepared according to the manufacturer's instruction. After sterilization and cooling, a streak of the yeast strain was made across the agar surface toward one side of the petri dish with a small inoculum. Two point inoculations were made on either side of the streak. Furthermore, sterile coverslip was placed over the centre of the streak. The plates were incubated for 5 days at room temperature before examination. The areas of inocula under the slips were observed for pseudomycelium or true mycelium formation.

3.4.2.4 Assimilation of sugars

The yeast isolates were confirmed further using the API 20 C AUX (Biomereuix) test kit. The kit comprises of seventeen different sugars which include; glucose, sucrose, maltose, raffinose, galactose, lactose, arabinose, inositol, sorbitol, methyl-glucopyranoside, cellobiose, trehalose, melezitose, N-acetyl glucosamine, glycerol and adonitol. Freshly cultured (24h) yeast cells were introduced into API wells according to the manufacturer's instruction and observed for turbidity after 48 hours.

3.4.2.5 Pellicle formation

Pellicle formation was observed using malt extract broth. The broth was prepared according to the manufacturer's instruction and dispensed into universal bottles. The broth was inoculated with the selected yeast strains after cooling and incubated for 7 days. Formation of film on the broth surface indicates pellicle formation.

3.5 Preparation of Standard Reagents

3.5.1 Preparation of 3, 5- Dinitro Salicylic Acid (DNS) Reagent

Sixty grams of Rochelle's salt (Potassium Sodium Tartrate) was weighed into a 100ml beaker and dissolved with about 70ml of distilled water. Sixteen grams of sodium hydroxide (NaOH) pellets was weighed separately in another beaker and also dissolved in 30ml of distilled water. The NaOH solution was transferred into the beaker containing the Rochelle's salt solution and was properly mixed. Two grams of DNS reagent was weighed into a 250ml beaker and was dissolved with 20ml of distilled water. The beaker containing the DNS reagent was placed in a water bath set at 90°C and the mixture of the Rochelle's salt and NaOH solutions were added to it. This was continuously stirred while heating until there was complete dissolution. The solution was then transferred into a 200ml volumetric flask and was made up the 200ml mark with distilled water. The volumetric flask was properly shaken to ensure proper mixing, absorbance of the DNS was taken using a Sherwood 254 colorimeter at 540nm to ensure that the absorbance was within the range of 0.300 – 0.350. The reagent was finally dispensed into an amber bottle to avoid photooxidation and then stored in a refrigerator for further use.

3.5.2 Preparation of 0.05M Sodium Citrate Buffer pH 4.8

The buffer solution was prepared by first dissolving 9.605g of citric acid in 1000ml of distilled water and also dissolving 14.7g of sodium citrate in 1000ml of distilled water. A total of 100ml of the buffer pH 4.8 was then prepared by mixing 46ml of the citric acid solution and 54ml of the sodium citrate solution.

3.6 Preparation of Standard Curves

3.6.1 Preparation of Glucose Curve for Total Saccharifying Cellulase (FPase) Assay

A stock solution of 10mg/ml of anhydrous glucose was prepared by dissolving 1g of glucose in 100ml of distilled water. To a set of 4 test tubes, 1ml each of the glucose stock solution was added followed by 0.5, 1.0, 2.0 and 4.0ml of sodium citrate buffer (pH 4.8) respectively in the tubes to obtain glucose concentrations of 6.7, 5.0, 3.3 and 2.0 mg/ml respectively. Aliquots of 0.5ml of the various glucose standards were transferred into another set of tubes to obtain 3.35, 2.5, 1.65 and 1.0 mg/0.5ml concentrations respectively. These were used to construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5ml) plotted against Absorbance at 540nm (A_{540}) (Appendix 1).

3.6.2 Preparation of Glucose Curve for Reducing Sugars Measurement

A glucose stock solution of 1% ($^{w}/_{v}$) (10 mg/ml) was prepared by dissolving one gram of anhydrous glucose in 100ml of distilled water. The stock solution was diluted with distilled water to prepare various concentrations ranging from 0.5mg/ml to 4.0mg/ml. One millilitre of each concentration was added to a separate 20ml test tube one and thereafter of DNS reagent was added to each test tube, cotton plugged and boiled in a water bath for 5 minutes. After cooling, 10ml quantity of distilled water was added and the absorbance determined at 540nm using a colorimeter (Sherwood 254). The absorbance values obtained

were plotted against the various glucose concentrations to obtain a glucose standard curve (Appendix 2) and the curve was used to determine the reducing sugar concentrations of the fermentation broth in accordance to the method described by Amadi *et al*. (2004).

3.6.3 Preparation of Standard Ethanol Density Curve

Different concentrations of ethanol were prepared from absolute ethanol using the formula; $C_1V_1 = C_2V_2$

Where

C_1 = Concentration of the absolute ethanol

V_1= Volume of the absolute ethanol

C_2= Concentration of the standard ethanol solution to be prepared

V_2= Volume of the standard ethanol to be prepared

A series of seven dilutions of ethanol in distilled water were prepared by varying concentrations from 2% to 14% ($^v/_v$) ethanol. The density bottles were pre-weighed with their stoppers before preparing the solution and reweighed after the preparation. Thus, the densities of these solutions were determined as follows;

$$\text{Density} = \frac{\text{Weight of the density bottle+ solution} - \text{Weight of empty density bottle}}{\text{Volume of solution.}}$$

The densities of the solutions were plotted against ethanol concentrations to obtain a standard density curve (Appendix 3) for ethanol as described by Amadi *et al*. (2004).

3.6.4 Preparation of Standard Ethanol Specific Gravity Curve

The specific gravity values of the standard ethanol solution were determined using the formula;

$$\text{Specific gravity} = \frac{\text{Density of ethanol solution}}{\text{Density of equal volume of distilled water}}$$

The specific gravity values were plotted against the ethanol concentration to obtain standard specific gravity curve (Appendix 4) for ethanol (Amadi *et al.*, 2004).

3.7 Screening of *A. niger* Isolates

Mould isolates were screened for cellulase activities to determine the isolate for fermentation of elephant grass as follows:

3.7.1 Qualitative Cellulase Assay

A.niger isolates were inoculated onto sterile MEA plates containing 0.1% Carboxymethyl cellulose (CMC), ampicillin (50µg/ml) and tetracycline (50µg/ml). The plates were incubated at room tremperature for 5 days after which they were flooded with one millilitre of 0.1% Congo red dye solution for 30 minutes. This was followed by destaining with 1M NaCl for 15 minutes after which the plates were observed for clear zones around the colonies indicating cellulase activity.

3.7.2 Quantitative Enzyme Assay

3.7.2.1 Preparation of spore suspension

The buffer solution for spore suspension comprised of 2.5% Tween 80 in normal saline solution which was prepared by dissolving 5ml of Tween 80 in 195ml of normal saline. The solution was dispensed into universal bottles and sterilised at $121^{o}C$ for 15 minutes after which it was allowed to cool and stored at $4^{o}C$ in a refrigerator pending use. The spore suspension was prepared by wetting 5 day old slant cultures of *A. niger* with 10ml of the buffer solution. The spores were harvested using a sterile wire loop to break the

clumps to obtain a homogenous spore suspension and a haemocytometer was used for spore count.

3.7.2.2 Enzyme production

A. niger isolates were grown on a slightly modified medium described by Mandels and Webber (1965). The medium contained (per litre of distilled water): Carboxymethyl Cellulose (CMC) (2.5g), Urea (0.3g), $FeNH_4(SO_4)_2$ (1.4 g), Na_2HPO_4 (2.0 g), $CaCl_2$ (0.3 g), $MgSO_4.7H_2O$ (0.3 g), protease peptone (1.0 g), $MnSO_4.7H_2O$ (1.6 mg), $ZnSO_4.7H_2O$ (1.4 mg) and $CoCl_2$ (2.0 mg). The pH of the medium was adjusted to 5.0 and then 25 ml each of the liquid medium were placed in 100 ml Erlenmeyer flasks and sterilized at 121°C for 15 minutes. After sterilization, the flasks were inoculated with 10^6 spores/ml and incubated at 30°C for 4 days with the agitation speed of 120rpm. At the end of fermentation period the culture filtrate was centrifuged at 8000 rpm for 10 min at 4°C using a Jouan GR-2022 refrigerated centrifuge and supernatants obtained after centrifugation were used as the crude extracellular enzyme sources.

3.7.2.3 Measurement of Total saccharifying cellulase (FPase) activities

This was carried out as described by Ghose (1987). One millilitre of 0.05M sodium citrate buffer (pH 4.8) was added to a test tube. Aliquots of 0.5ml of enzyme were then added and the solution was heated to 50°C after which Whatman No. 1 filter paper strip, 1.0× 6.0cm ($\simeq$ 50mg) was added as source of cellulose. The solution was mixed and the tube was incubated at 50°C for 1hour. To another set of test tubes, 1.5ml of the buffer solution was added (control) and to the other 1ml of the buffer solution and 0.5ml of the enzyme was added (to serve as enzyme blank). Three ml of DNS was added to all the test tubes including the spec zero, enzyme blanks and the glucose standards, boiled for

5minutes in a vigorously boiling water bath and there after transferred to a cold water bath. Twenty ml distilled water was added and the solution was mixed by completely inverting the tubes several times. Colour formation was measured against spec zero at 540nm using a colorimeter (Sherwood 254) and the absorbance of the sample was subtracted from that of the enzyme blank. Using the standard curve prepared, the absorbance values of the samples were converted into glucose concentrations and then multiplied with the enzyme unit constant (0.185) to obtain the concentration of the enzyme. One unit of FPase activity is expressed as 1µmol of glucose released per ml per min.

3.8 Screening of Yeast Isolates

The yeast isolates were screened to influence the choice of strain for fermentation as follows:

3.8.1 Growth at 50% ($^{W}/_{V}$) Glucose Concentration

A basal medium composed of 50% ($^{w}/_{v}$) glucose, 1% yeast extract, 1.5% ($^{w}/_{v}$) peptone and 2.5g (^{w}/v) agar was prepared and sterilized for 15minutes at 121^{0}C. After cooling, the medium was aseptically dispensed into sterile petri dishes. The plates were inoculated with yeast isolates, incubated at room temperature (28-30^{0}C) for 5 days and observed for growth.

3.8.2 Fermentation of 50% ($^{W}/_{V}$) Glucose

The ability of selected yeast isolates to ferment 50%($^{w}/_{v}$) glucose was determined by using basal medium containing 50% ($^{w}/_{v}$) glucose, 0.75% ($^{w}/_{v}$) peptone, 1.5% ($^{w}/_{v}$) yeast extract. Ten millilitres aliquots of the medium were dispensed into test tubes with inverted Durham tubes and sterilized for 15minutes at 121^{0}C. After cooling, the tubes were

inoculated with 0.5ml aliquots of yeast isolate suspension from yeast extract medium, incubated at room temperature and examined for sugar fermentation and gas production within 5 days.

3.8.3 Ethanol Tolerance Test

A basal medium comprised of 1% ($^{w}/_{v}$) yeast extract, 2% ($^{w}/_{v}$) peptone, and 2% ($^{w}/_{v}$) glucose was prepared and used for this test. Ten millilitres aliquots of the Yeast extract-Peptone-Glucose medium was dispensed into test tubes and sterilized at 121^{0}C for 15minutes. After cooling to about 45^{0}C, different volumes of absolute ethanol were added aseptically to the tubes to bring the final ethanol concentrations to 5%, 10% and 15% respectively. The medium was inoculated with 0.1ml aliquot of 2 McFarland suspension of 48-hour culture of yeast strains and incubated for 72 hours at room temperature. Presence of sediment or turbidity indicated growth.

3.9 Proximate Analysis of Elephant Grass Sample

The proximate analysis of the Elephant grass sample was carried out according to AOAC methods (1990) to determine the moisture, ash, crude lipid, crude protein, crude fibre and soluble carbohydrate contents as follows:

3.9.1 Determination of Moisture Content

A clean crucible was dried to a constant weight in an air oven at 105°C, cooled in a desiccator and weighed (W_1). Two grams of finely ground sample was weighed into the previously labelled crucible and reweighed (W_2). The crucible containing the sample was dried in an oven to a constant weight (W_3).

The percentage moisture content was calculated thus:

$$\% \text{ Moisture content} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

3.9.2 Determination of Ash Content

A porcelain crucible was dried in an oven at 100^oC for 10 minutes, cooled in a desiccator and weighed (W_1). Two grams of the finely ground sample was placed into a previously weighed porcelain crucible and reweighed (W_2). It was first ignited and then transferred into a furnace, which was then set at 550^oC. The sample was left in the furnace for 8 hours to ensure proper ashing. The crucible containing the ash was then removed and cooled in the desiccator and weighed (W_3).

The percentage ash content was calculated as:

$$\% \text{ Ash content} = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

3.9.3 Determination of Crude Lipid Content

A clean, dried 500ml round bottom flask containing few anti-bumping granules was weighed (W_1) and 300ml of petroleum ether at 40^oC for the extraction was poured into the flask fitted with soxhlet extraction unit. The round bottom flask and a condenser were connected to the soxhlet extractor and cold water circulation was turned on. The heating mantle was switched on and the heating rate was adjusted until the solvent was refluxing at a steady rate. Extraction was carried out for 6 hours. The solvent was recovered and the oil was dried in the oven at 70^oC for 1 hour. The flask and oil were cooled and then reweighed (W_2).

The lipid content was calculated thus:

$$\% \text{ crude lipid content} = \frac{W_2 - W_1}{\text{Weight of sample}} \times 100$$

3.9.4 Determination of Crude Fibre Content

Two grams of the finely ground sample was weighed out into a 500ml round bottom flask. A 100ml of 0.25M sulphuric acid solution was added and the mixture boiled under reflux for 30 minutes. The hot solution was quickly filtered under suction. The insoluble matter was washed several times with hot water until it was acid free. It was quantitatively transferred into the flask and 100ml of hot 0.31M NaOH solution added and the mixture was boiled again under reflux for 30 minutes and quickly filtered under suction. The soluble residue was washed with boiling water until it was base free. It was dried to constant weight in the oven at 100°C, cooled in a desiccator and weighed (C_1). The weighed sample (C_1) was then incinerated in a muttle furnace at 550°C for 2 hours, cooled in a desiccator and reweighed (C_2).

The loss in weight on incineration = $C_1 - C_2$. Therefore the calculation was carried out thus:

$$\% \text{ Crude fibre} = \frac{C_1 - C_2}{\text{Weight of original sample}} \times 100$$

3.9.5 Determination of Nitrogen and Crude Protein Content

Exactly 1.5g of the ground defatted sample in an ashless filter paper was dropped into a 300ml Kjeldahl flask. Twenty-five millilitres concentrated sulphuric acid and 3g of digesting mixed catalyst (weighed separately into an ashless filter) was also dropped into the flask. The flask was then transferred to the Kjeldahl digestion apparatus. The sample was digested until a clear green colour was obtained. The digest was cooled and diluted to

100ml with distilled water. Twenty millilitres of the diluted digest was measured into a 500ml Kjeldahl flask containing anti-bumping chips and 40ml of 40% NaOH was slowly added by the side of the flask. A 250ml conical flask containing a mixture of 50ml 2% boric acid and 4 drops of mixed indicator was used to trap the ammonia being liberated. The conical flask and the Kjeldahl flasks were then placed on the Kjeldahl apparatus with the tubes inserted into both flasks. The Kjeldahl flask was heated to distil the NH_3 (distillate) evolved which was then collected in the flask containing the boric acid solution. From the point when the boric acid turned green, the system was allowed for about 10 minutes for complete distillation of ammonia present in the digest. The distillate was subsequently titrated with 0.1M HCl.

The %Nitrogen was calculated thus:

$$\%\text{Nitrogen } (N_2) = \frac{14 \times M \times V_1 \times T_v \times 10}{\text{Weight of sample (mg)} \times V_a}$$

$$\%\text{Crude protein} = \%\text{Nitrogen } (N_2) \times 6.25$$

Where M = actual molarity of acid

T_v = titre volume of HCl used

V_1 = total volume of diluted digest

V_a = aliquot volume distilled

3.9.6 Determination of Soluble Carbohydrates

Soluble carbohydrates content was determined using the formula;

Soluble carbohydrates = 100 – (moisture + crude lipid + ash + crude protein + crude fibre).

3.10 Determination of Lignocellulose Content

The lignocellulose assay of the Elephant grass was carried out according to the AOAC methods (1990) to determine the lignin, cellulose and hemicellulose contents as follows:

3.10.1 Determination of Neutral Detergent Fibre (NDF)

3.10.1.1 Principle

A neutral detergent solution is used to dissolve the easily digested pectin and plant cell contents (proteins, sugars and lipids), leaving a fibrous residue (NDF) that is primarily cell wall components of plants (cellulose, hemicellulose and lignin). Detergent is used to solubilize the proteins and sodium sulphite also helps remove some nitrogenous matter. EDTA is used to chelate calcium and remove pectin at boiling temperatures; triethylene glycol helps to remove some non-fibrous matter from concentrate feeds.

3.10.1.2 Preparation of Neutral detergent solution

The neutral detergent solution was prepared by mixing two solutions; one consisting of 93g of disodium Ethylenediamine Tetra acetate Dihydrate (EDTA) and 34g of sodium borate in distilled water to which 15g of sodium lauryl sulphate and 10ml of ethylene glycol were added and the other consisting of 22.8g of anhydrous disodium hydrogen phosphate in distilled water.

3.10.1.3 Procedure

Sample was oven dried at 55^oC then ground to pass a 1mm screen. A fritted glass crucible was dried overnight at 100^oC and hot weighed (W_1). About 0.5g of sample was weighed (W_2) into a 600ml Berzelius beaker. Extraction heating (reflux) unit was pre-heated to a temperature that permits boiling of neutral detergent solution within 5 minutes

and 0.5g of sodium sulphite was added using previously calibrated scoop. Fifty millilitres of neutral detergent solution was then added and the beaker was swirled until the sample and sodium sulphite were completely suspended. The beaker was placed on the heating unit under a cool water condenser and allowed to boil and then refluxed for 60 minutes. Sample was removed from heating unit and allowed to settle for 1 minute before it was filtered. Fritted glass crucible for filtering was preheated by adding 40 ml of boiling water and the water was removed with a vacuum. The residue in fritted glass crucible was filtered and washed twice by adding 30 to 40 ml of boiling water and allowing soaking for 2 minutes each time after which the sample was rinsed twice with 30 ml of acetone also allowing 2 minutes soaking time between rinses. Crucible was dried at 100°C for overnight and hot weighed (W_3).

The calculation of the NDF was given thus:

$$\%NDF = \frac{W_3 - W_1}{W_2} \times 100$$

3.10.2 Determination of Acid Detergent Fibre (ADF)

3.10.2.1 Principle

An acidified quaternary detergent solution is used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose, lignin and heat damaged protein and a portion of cell wall protein and minerals (ash). ADF is determined gravimetrically as the residue remaining after extraction.

3.10.2.2 Preparation of Acid detergent solution

The acid detergent solution was prepared by dissolving 20g of Cetyltrimethyl ammonium bromide (CTAB) in 1L of sulphuric acid.

3.10.2.3 Procedure

Sample was oven dried at 55°C and ground to pass through a 1mm sieve. A fritted glass crucible was dried overnight at 100°C and hot weighed (W_1). About 1g of sample was weighed (W_2) into Berzelius beaker, 100ml of acid-detergent solution was added at room temperature and the beaker was placed on a heater under the cold water condenser. The beaker containing the sample was heated to boiling; reducing heat to avoid foaming as boiling began, and then reflux for 1hour from onset of boil. After about 30 minutes, the beaker was removed swirled and content was filtered through tare fritted glass crucible with gentle suction. The Berzelius beaker was rinsed with boiling water while inverted over the crucible to ensure quantitative transfer of all fibre particles into the crucible. The residue was washed with boiling water until no more foam was formed and then with acetone until no more colour was removed. The crucible with its content was dried in an oven at 100°C overnight and was allowed to cool in a desiccator and then weighed (W_3).

The calculation of the ADF was given thus:

$$\%ADF = \frac{W_3 - W_1}{W_2} \times 100$$

Hence,

$$\%Hemicellulose = NDF - ADF$$

3.10.3 Determination of Acid Detergent Lignin (ADL)

3.10.3.1 Principle

An acidified quaternary detergent solution is used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose, lignin and heat damaged protein and a portion of cell wall protein and minerals (ash). ADF is determined

gravimetrically as the residue remaining after extraction. ADL is determined gravimetrically as the residue remaining upon ignition after 72% H_2SO_4 treatment.

3.10.3.2 Procedure

The 50ml crucible containing the residue from the ADF analysis was placed in a 50 ml beaker for support, the contents of the crucible were covered with cooled (15^oC) 72% $H_2SO_4$and stirred with a glass rod to a smooth paste, breaking all lumps. The crucible was then filled about halfway with acid, was stirred and kept at about 20-30^oC. After 3 hours, a vacuum was used to filter off excess acid and the content was washed with hot water until free from acid. The crucible was dried at 105^oC overnight, cooled in a desiccator for at least 1 hour and weighed (W_1). The crucible was then ignited in a muffle furnace at 500^oC for 2 hours after which while still hot, was placed in desiccator, cooled to constant temperature and reweighed (W_3).

The calculation of the ADL was given thus:

$$\%ADF = \frac{W_1 - W_3}{W_2} \times 100$$

Hence,

$$\%Cellulose = ADF - ADL$$

3.11 Fermentation of Elephant Grass

3.11.1 Sample Preparation and Pretreatment

The plant sample was prepared and pretreated as described by Roy *et al.* (1993). Plant sample was sun-dried and pulverized to pass through a 0.5mm sieve. Combined alkaline and steam pretreatment was carried out by soaking the powder in 2M NaOH, ensuring the powder was totally submerged and autoclaving at 121^oC for 2hours after

which it was neutralised by continuous washing with warm distilled water. The pretreated sample was dried in an oven at 65°C for 3 days.

3.11.2 Inocula Preparation

A. niger and *S. cerevisiae* inocula were prepared by using slant cultures to inoculate 100ml of sterile growth medium contained in 250ml Erlenmeyer flasks. The flasks were incubated with shaking (200rpm) at 30°C for 5 days (Ado *et al*., 2009).

3.11.3 Media Preparation for Fermentation

A synthetic medium containing yeast extract base glucose broth and a complex medium containing the pretreated elephant grass were used for ethanol production. The synthetic medium containing (per 100ml); Glucose, 1g; Peptone, 0.1g and Yeast extract, 0.2g with the glucose concentrations ranging from 2 to 10% (2 to 10g) and the complex medium containing (per 100ml); $FeNH_4(SO_4)_2$, 0.1g; $(NH_4)_2HPO_4$, 0.25g; Urea, 0.3g and Peptone, 0.5g with the Elephant grass concentrations also ranging from 2 to 10% (2 to 10g) were prepared and dispensed into 2000ml Erlenmeyer flasks each containing 1000ml of the medium. The pH of the media were adjusted to 5.0 and then sterilized in an autoclave. The flasks containing synthetic medium were inoculated with *S. cerevisiae* while those containing complex medium were inoculated with both *S. cerevisiae* (3×10^8 cells/ml) and *A. niger* (2.5×10^6 spores/ml). The flasks were incubated at ambient temperatures on an orbital shaker set at 200rpm for 5 days (Abouzied and Reddy, 1986).

3.12 Analytical Procedure

Two hundred millilitres of the sample was collected from each flask at 24-hour intervals and 10ml was centrifuged at 4000rpm for 15 minutes to remove the cells, the

supernatant fluid was filtered through Whatman filter paper No. 1 and the filtrate used to determine the total cellulase (FPase) activity, reducing sugar concentrations and cell dry weight. The remaining 190ml was distilled and used for quantitative and qualitative ethanol determination.

3.12.1 Measurement of Cell Dry Weight

Cell biomass was determined by centrifuging ten millilitres sample, drying the cells to obtain a constant weight at $60^{o}C$, and expressing the dry weight as grams per 100 ml growth medium.

3.12.2 Determination of Reducing Sugar Concentration

One millilitre of Benedict's reagent was added to one ml of each sample. The reagent reacted with the reducing sugar and the copper (ii) ions were reduced to give a reddish brown precipitate which confirmed the presence of reducing sugar (Amadi *et al*., 2004). The concentration of the reducing sugar present in the samples were determined by adding one ml of DNS reagent to one ml of the sample and boiling for five minutes after which 10ml distilled water was added. The absorbance of each sample was determined at 540nm using SHERWOOD 254 colorimeter. Thus, the corresponding concentration values were extrapolated from the glucose standard curve.

3.12.3 Quantitative and Qualitative Determination of Ethanol Concentration

The volume and the weight of the distillate were measured and recorded after which the density of the distillate was calculated using the formula;

$$\text{Density} = \frac{\text{Mass}}{\text{Volume}}$$

The concentration of the distillate was obtained by extrapolation using the density value from the standard ethanol density curve.

3.13 Statistical Analyses

Statistical Packages for Social Sciences (SPSS v.19) software was used to perform independent *t*-test to compare ethanol yields of individual substrate concentrations of both glucose and pretreated elephant grass sample. One-way analysis of variance (ANOVA) to compare the ethanol yields from all the substrate concentrations of elephant grass and also the ethanol yields after optimization of culture parameters.

CHAPTER FOUR

4.0 RESULTS

4.1 Characterisation of *Aspergillus niger*Isolates

Aspergillus niger presented a woolly growth at first which progressed to dense black sporulation with a pale yellow reverse on MEA plates (Plate I). The microscopic structures were long smooth-walled conidiophores terminating in globose swellings (Plate II) as presented on Table 4.1.

4.2 Characterisation of *Saccharomyces cerevesiae* Isolates

Different strains of *Saccharomyces cerevesiae* were isolated from burukutu and palm wine while an industrial strain (Is) was gotten from Kaduna brewery, Kaduna state. Isolates BK-1, BK-2, BK-3, BK-4, BK-5 and BK-6 were gotten from burukutu and isolates PW-1, PW-2, PW-3 and PW-4 were gotten from palm wine (Table 4.2). The isolates were characterised based on morphological characteristics (Plates III and IV) and also based on their ability to assimilate certain sugars (Table 4.3).

4.3 Screening of *A. niger* Isolates

4.3.1 Qualitative Enzyme Assay

Mould isolates were grown on medium containing carboxymethyl cellulose (CMC) as carbon source. After staining, clear zones indicative of cellulase activity was observed (Plate V).

Plate I. Colonial morphology of *Aspergillus niger* on Malt Extract Agar (MEA) plate

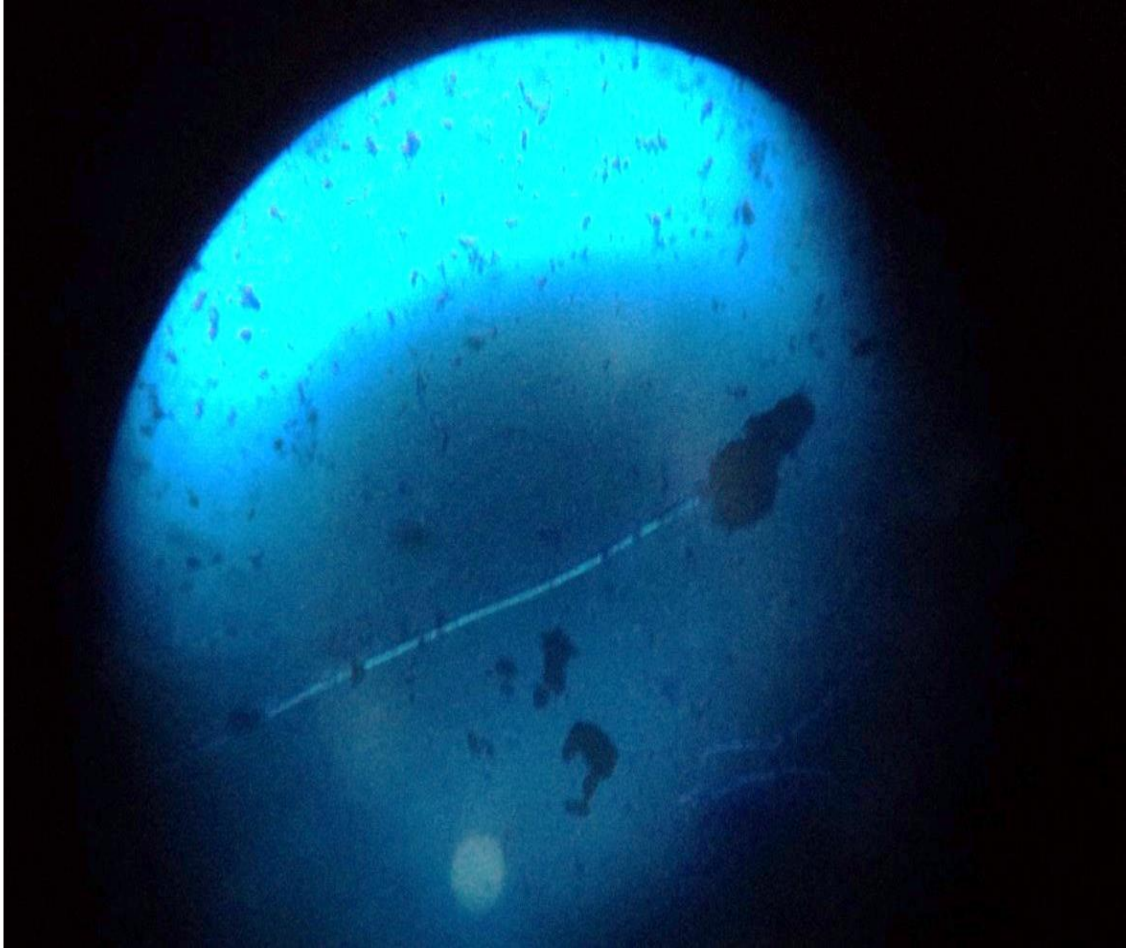


Plate II. Microscopic structure of *A. niger* conidiophore (x40).

Table 4.1. Characterisation of *A. niger* isolates.

Isolate	Macroscopic		Microscopic		Tentative identity	Source
	Colonial morphology	Reverse of plate	Structure of conidiophore	Arrangement of spores on conidia		
AN-1	Woolly growth at first, then dense black sporulation	Pale yellow	Long and smooth-walled terminating in globose swelling	Biseriate phialides forming radiate conidial heads	*A. niger*	Soil
AN-2	"	"	"	"	"	"
AN-4	"	"	"	"	"	Soil
AN-7	"	"	"	"	"	Bread
AN-8	"	"	"	"	"	Soil
AN-11	"	"	"	"	"	"
AN-12	"	"	"	"	"	Soil
AN-13	"	"	"	"	"	Bread
AN-14	"	"	"	"	"	Bread
AN-15	Woolly growth at first, then dense black sporulation	Pale yellow	Long and smooth-walled terminating in globose swelling	Biseriate phialides forming radiate conidial heads	*A. niger*	Soil

Key:
AN = *A. niger*

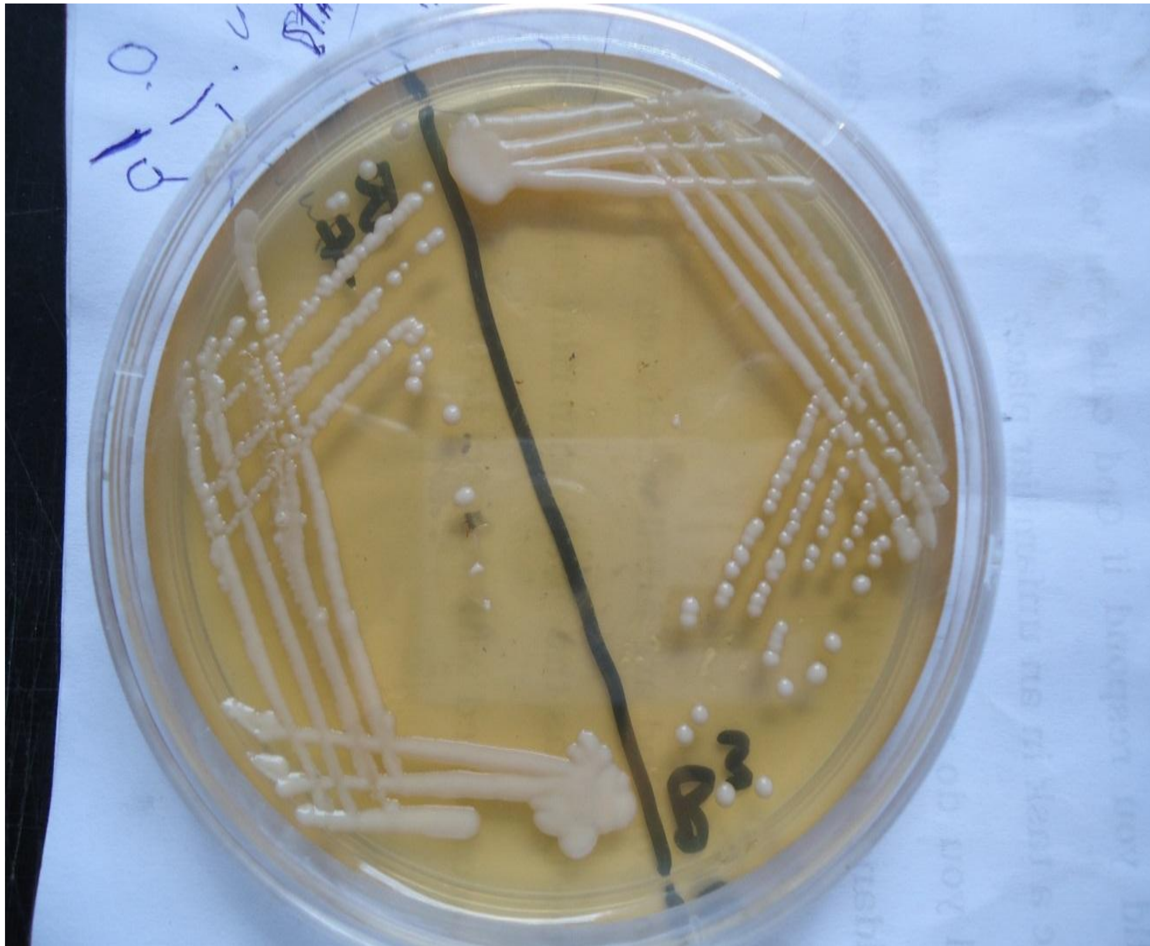


Plate III. Colonial morphology of *Saccharomyces cerevisiae* on Malt Extract Agar (MEA) plate.

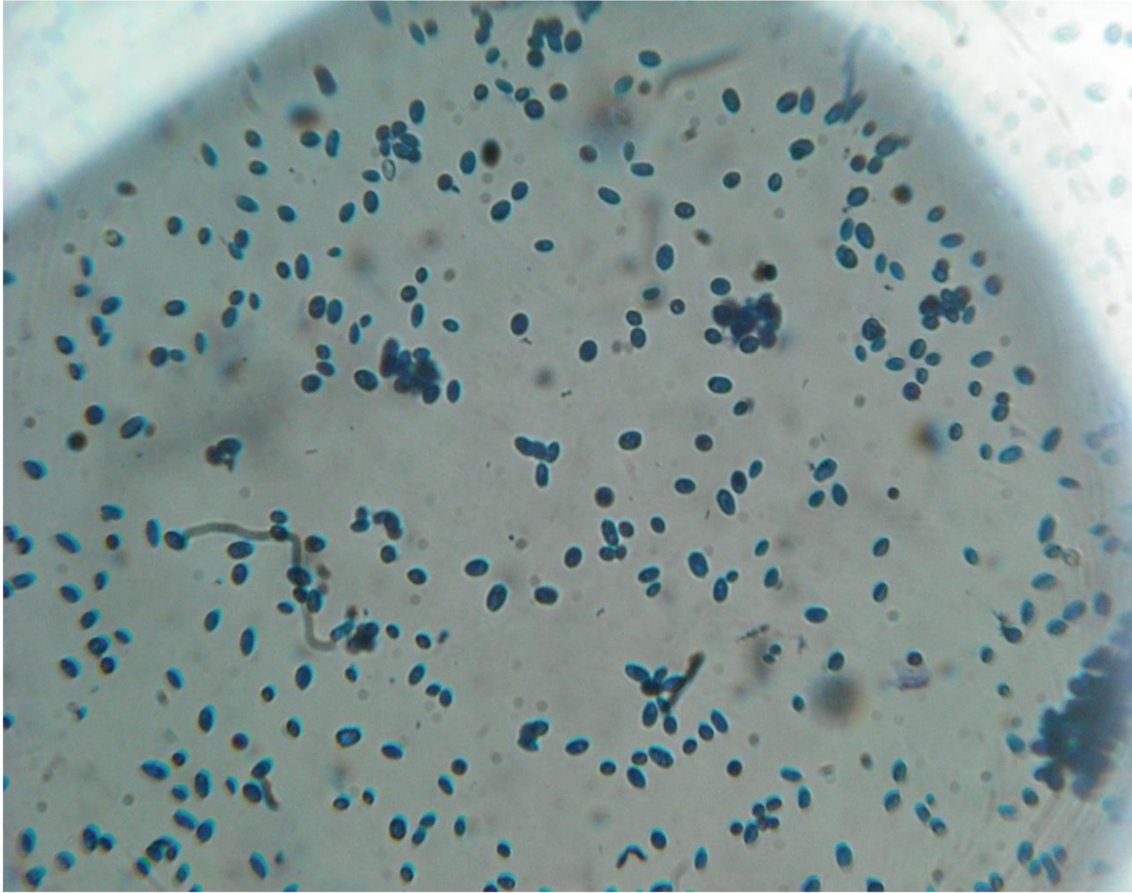


Plate IV. Microscopic morphology of *S.* cerevisiae (x10).

Table 4.2. Characterisation of *S. cerevisiae* isolates.

Isolate	Colonial morphology	Vegetative morphology	Presence of pseudomycelium or true mycelium	Pellicle formation	Tentative identity	Source
BK-1	Moist, creamy and raised colonies	Oval shaped and budding	-	-	*S. cerevisiae*	Burukutu
BK-2	"	"	-	-	"	"
BK-3	"	"	-	-	"	"
BK-4	"	"	-	-	"	"
BK-5	"	"	-	-	"	"
BK-6	"	"	-	-	"	Burukutu
PW-1	"	"	-	-	"	Palm wine
PW-2	"	"	-	-	"	"
PW-3	"	"	-	-	"	"
PW-4	"	"	-	-	"	Palm wine
Is	Moist, creamy and raised colonies	Oval shaped and budding	-	-	*S. cerevisiae*	Brewery

Key:

BK = burukutu - = no growth

PW = palm wine Is = industrial strain

Table 4.3. Identification of yeast isolates based on assimilation of various sugars using API 20 C AUX kit.

Isolate	Fermentable sugars																			Tentative identity
	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	
BK-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	*S. cerevisiae* 1
BK-2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	“
BK-3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	“
BK-4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	“
BK-5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	“
BK-6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	*S. cerevisiae* 1
PW-1	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	*S. cerevisiae* 2
PW-2	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	“
PW-3	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	“
PW-4	+	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+	+	+	+	“
Is	+	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+	+	-	+	*S. cerevisiae* 2

Key:

BK = burukutu PW = palm wine Is = industrial strain + = growth - = no growth
GAL= D-galactose INO= inositol SOR= D-sorbitol GLY= glycerol GLU= D-glucose
CEL= D-cellobiose MAL= D-maltose ARA= L-arabinose TRE= D-trehalose SAC= D-saccharose
XYL= D-xylose XLT= xylitol RAF= D-raffinose ADO = adonitol MLZ= D-melezitose
LAC= D-lactose (bovine origin) 2KG= calcium 2-céto-gluconate
MDG= methyl-α-D-glucopyranoside NAG= N-acetyl-glucosamine

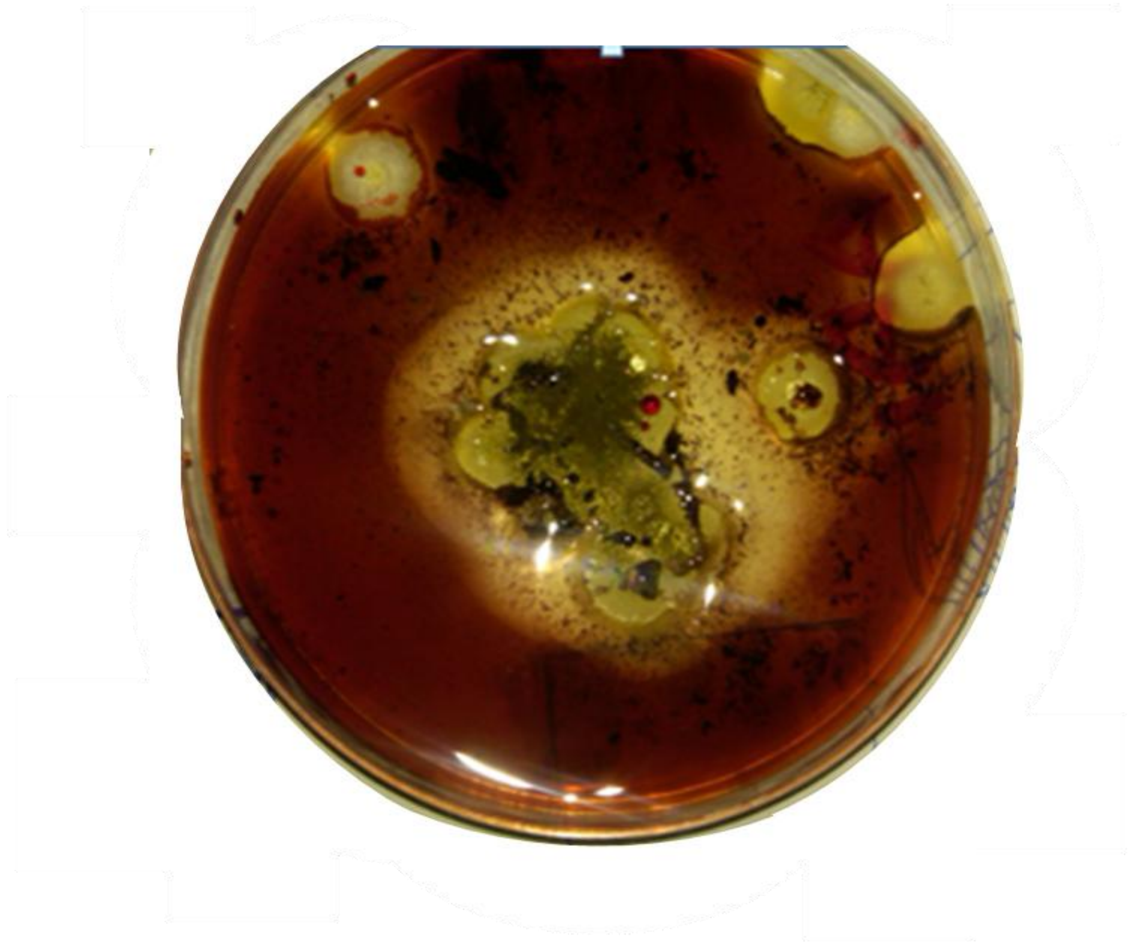


Plate V. ***Aspergillus niger*** **showing clear zone around the growth indicating enzyme activity on solid agar medium.**

4.3.2 Quantitative Enzyme Assay

Further enzyme assay to quantify enzyme load was carried out and the result ispresented in Table 4.4. Based on the results of the assay, isolate AN-15 was chosen for the fermentation process.

4.4 Screening of *S. cerevisiae* Isolates

Table 4.5 shows the results of the screening of yeast isolates. From the results of the screening and assimilation of sugars, isolate PW-4 was selected for the fermentation for its ability to assimilated more sugars than the other isolates in combination with its ability to grow at 15% ethanol concentration.

4.5 Proximate Analyses and Lignocellulose Content of Elephant Grass Sample

Table 4.6 shows the results of proximate analysis and the lignocellulose contents analysis carried out on the grass sample.

4.6 Fermentation of Substrate for Ethanol Production

4.6.1 Effect of Glucose Concentrations on Biomass Yield

Figure 4.1 shows the effect of glucose concentration on the biomass yield. Highest biomass yield was observed at 4% glucose concentration with 1.043g/100ml on day 1, 1.319g/100ml on the second day then a sharp decline on the third day (0.541g/100ml) and finally a plateau on the fourth and fifth days with 0.303 and 0.297g/100ml respectively.

Table 4.4. Quantitative screening of *A. niger* isolates for cellulase activity.

Isolate	FPase assay	
	Absorbance	**Enzyme activity (IU/ml/min)**
AN-1	0.07	0.0784
AN-2	0.14	0.1568
AN-4	0.13	0.1456
AN-7	0.12	0.1344
AN-8	0.09	0.1008
AN-11	0.05	0.0560
AN-12	0.02	0.0224
AN-13	0.07	0.0784
AN-14	0.08	0.0896
AN-15	0.16	0.1792*

Key:

Fpase = Filter paperase (total cellulase)

AN = *A. niger*

* = Highest cellulase activity

Table 4.5. Screening of yeast isolates

Isolate	Growth at 50% Glucose concentration	Fermentation of 50% glucose with gas production	Ethanol tolerance		
			5%	10%	15%
BK-1	+++	+++	+++	+++	+++
BK-2	+++	+++	+++	+++	++
BK-3	+++	+++	+++	++	++
BK-4	+++	+++	+++	+++	++
BK-5	+++	+++	+++	+++	+
BK-6	+++	+++	+++	+++	+
PW-1	+++	+++	+++	+	-
PW-2	+++	+++	+++	+	+
PW-3	+++	+++	+++	+++	+
PW-4	+++	+++	+++	+++	++
Is	+++	+++	+++	+++	++

Key:

BK = Burukutu - = no growth +++ = dense growth

PW = palm wine + = slight growth

Is = Industrial strain ++ = moderate growth

Table 4.6. Proximate analysis and lignocellulose content of Elephant grass.

Parameter	Content (%)
Moisture	16.26
Ash	5
Crude lipid	22.9
Crude protein	7.47
Crude fibre	31.5
Soluble carbohydrate	16.87
Lignin	26.78
Cellulose	34.16
Hemicellulose	18.76

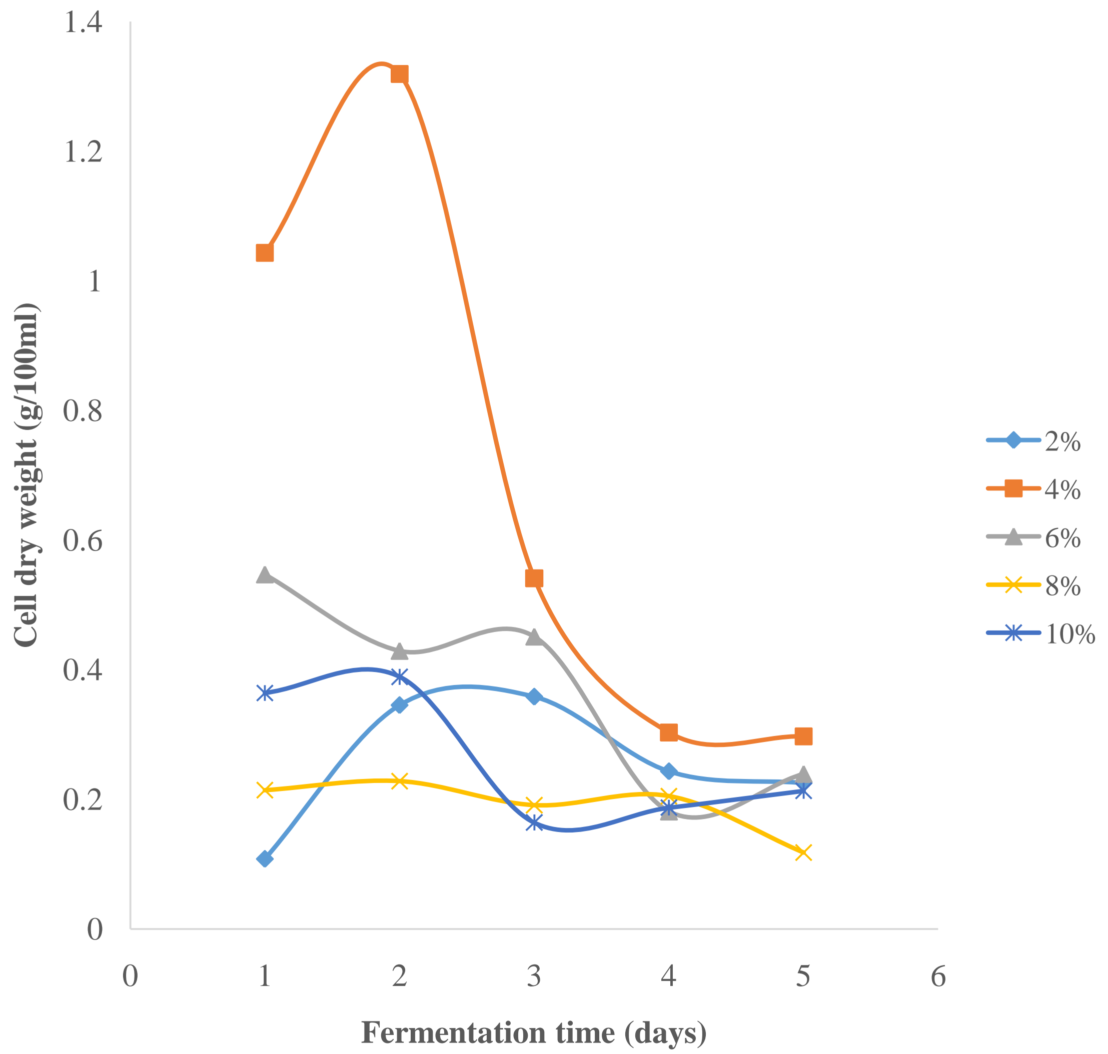


Figure 4.2. Effect of glucose concentrations on cell dry weight

4.6.2 Determination of Residual Sugar after Fermentation

Figure 4.2 shows the residualsugar concentration after fermentation. Results show a sharp decline in reducing sugar concentrations from day 1 to day 2 at 2% (1.24 to 0.1354mg/ml), 4% (2.73 to 0.1354mg/ml) and 6% (1.64 to 0.1741mg/ml) glucose concentrations and then flattened out from day 2 to day 5 respectively. There was a steady decline from day 1 to day 2 (3.212 to 2.979mg/ml), then a sharp drop from day 2 to day 3 (2.979 to 0.8899mg/ml) and finally a steady decline again from day 3 through day 5 (0.8899 to 0.8318 to 0.4837mg/ml respectively) at 8% glucose concentration. At 10% glucose concentration, the reducing sugar concentration gradually decreased from day 1 to 3 (3.212 to 2.786 to 2.545mg/ml respectively) followed by a sharp drop on day 4 (0.5997mg/ml) and slight decrease on the fifth day (0.4837mg/ml).

4.6.3 Effect of Glucose Concentrations on Ethanol Yield

Figure 4.3 shows the effect of glucose concentrations on ethanol yield. Results showed that highest yield was observed at 10% substrate concentration. Ethanol yield was highest on day 1 (10.09g/100ml) followed by a sharp decline on day 2 (6.47g/100ml) then a gradual increase through day 4 (7.26g/100ml and 9.17g/100ml) respectively and finally a sharp drop again on day 5 (3.46g/100ml). The specific gravity values from day 1 through day 5 were 0.98406, 0.98484, 0.98496, 0.9819 and 0.98483 respectively while the corresponding densities of the distillates ranged from 0.98218, 0.98290, 0.98302, 0.98016 and 0.98289 g/ml respectively.

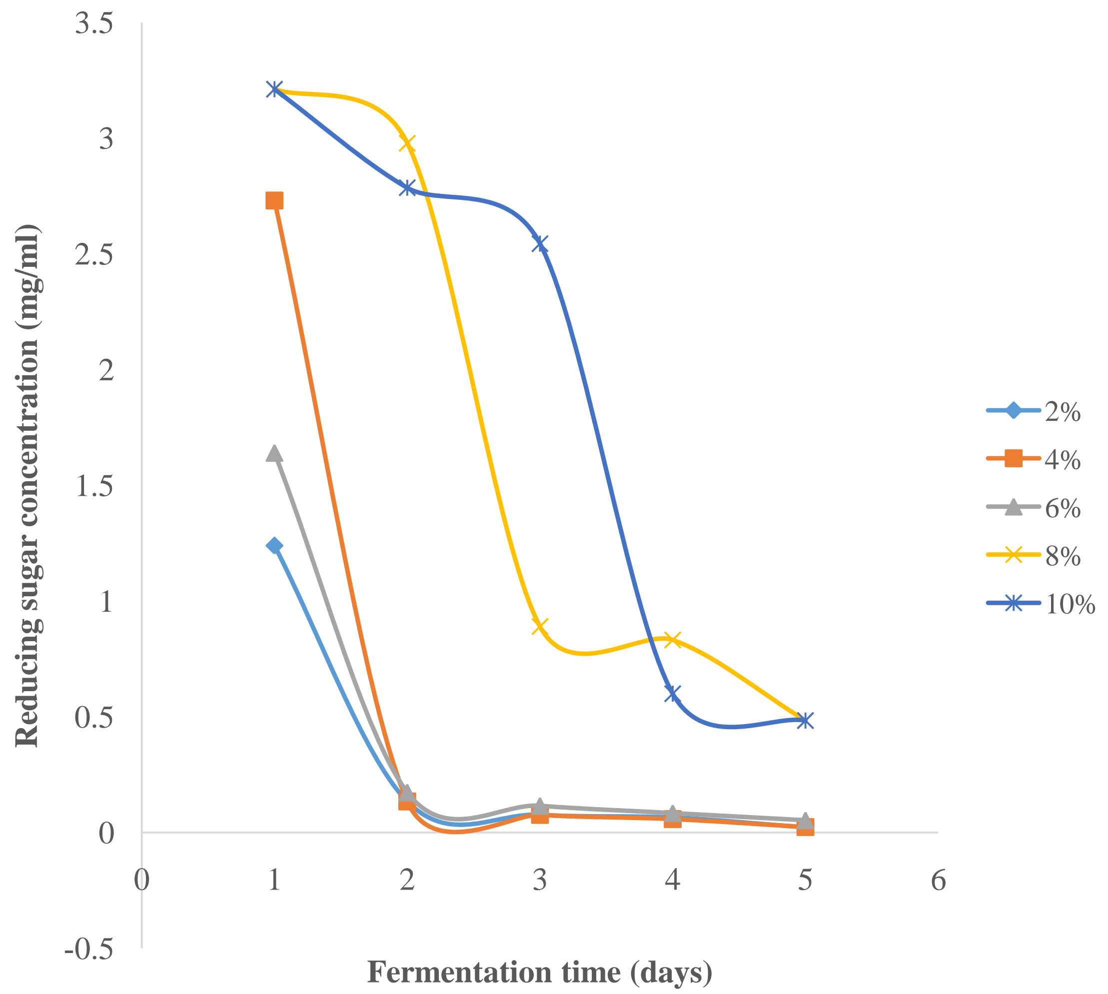


Figure 4.3. Residual sugar concentrations after fermentation

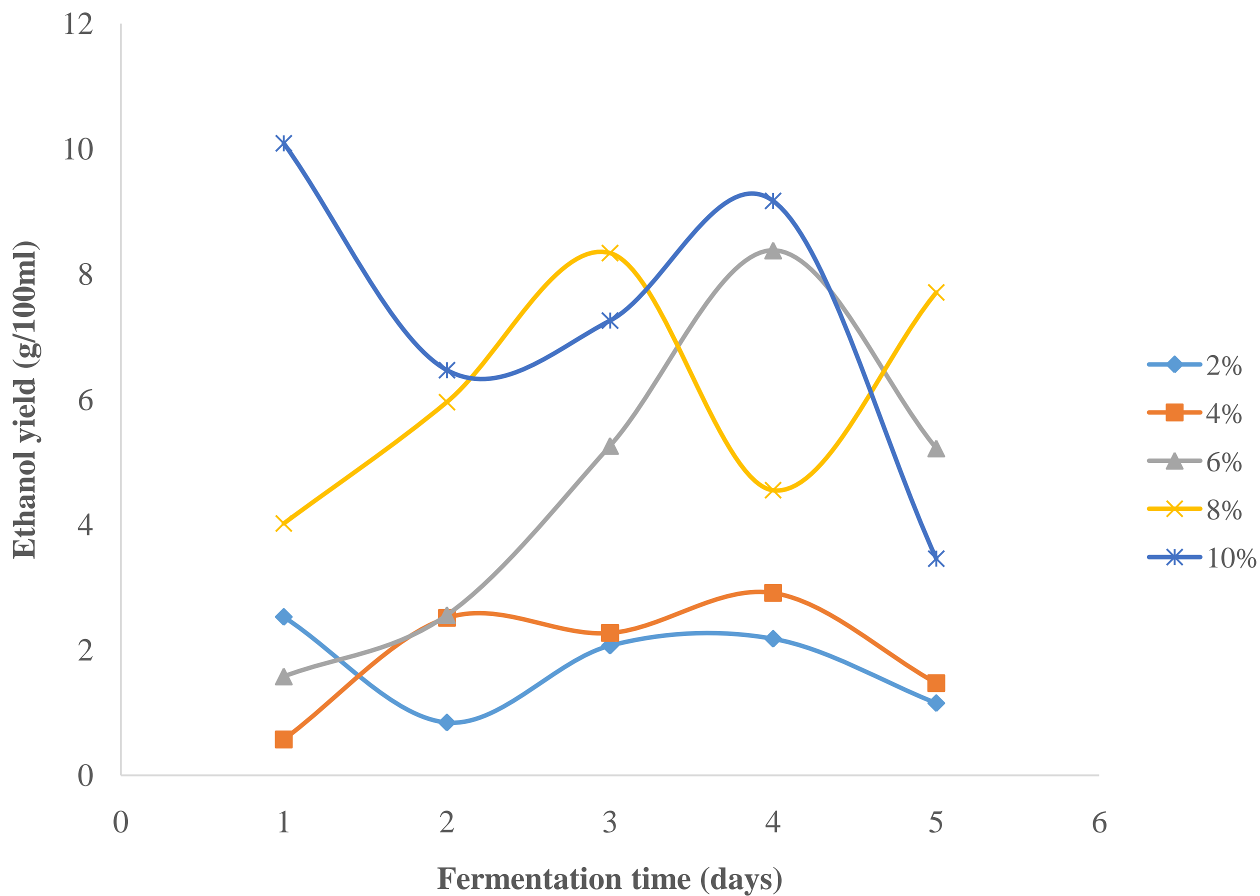


Figure 4.4. Effect of glucose concentrations on ethanol yield

4.6.4 Effect of Pretreated Elephant Grass Concentrations on Biomass Yield

Figure 4.4 shows the effect of pretreated elephant grass samples on the biomass yield. Results show that biomass yield peaked on the day 2 at the different substrate concentrations. Highest yield was observed at 6% substrate concentration on day 2 (0.428g/100ml). There was a gradual decline in biomass yield all through from day 1 through day 5 of fermentation with the exception of 2% substrate concentration where there was a sharp decline from day 2 to day 3 (0.231 to 0.118g/100ml).

4.6.5 Effect of Pretreated Elephant Grass Concentrations on Reducing Sugar Yield

The effect of various concentrations of pretreated sample on the reducing sugar concentration was studied and is presented in Figure 4.5. The highest reducing sugar concentration was observed on second day of fermentation at 6% substrate concentration (2.3796mg/ml) after which there was a sharp decline to 1.6831mg/ml on the third day then subsequent steady decline up to the 5thday.

4.6.6 Effect of Pretreated Elephant Grass Concentrations on Ethanol Yield

The effect of various concentrations of pretreated sample on the ethanol yield was studied and is shown in Figure 4.6. Results show that there was a gradual increase in yield from day 1 to day 2 at all substrate concentrations with exception of 8% where yield dropped on the second day (from 1.05 to 0.85g/100ml). Highest yield (1.68g/100ml) was observed at 6% substrate concentration on the third day of fermentation and this dropped on the fourth day (1.59g/100ml) and sharply again to 1.16g/100ml on the fifth day. The densities of the distillates from the 1st to 5th day of fermentation were 0.99284, 0.99259, 0.99254, 0.99286 and 0.99318 g/ml respectively with specific gravity values of 0.99549, 0.99522, 0.99516, 0.99551 and 0.99585 respectively.

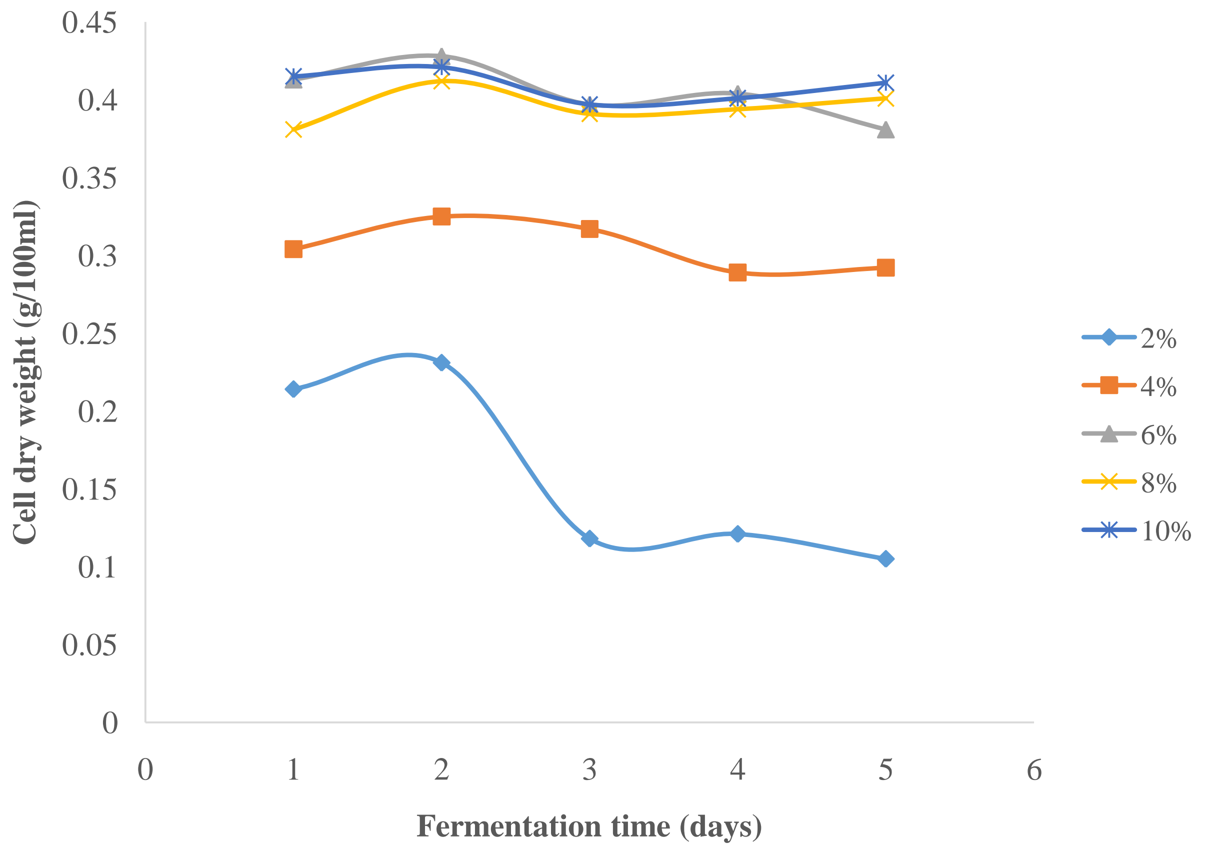


Figure 4.5. Effect of pretreated sample concentrations on cell dry weight

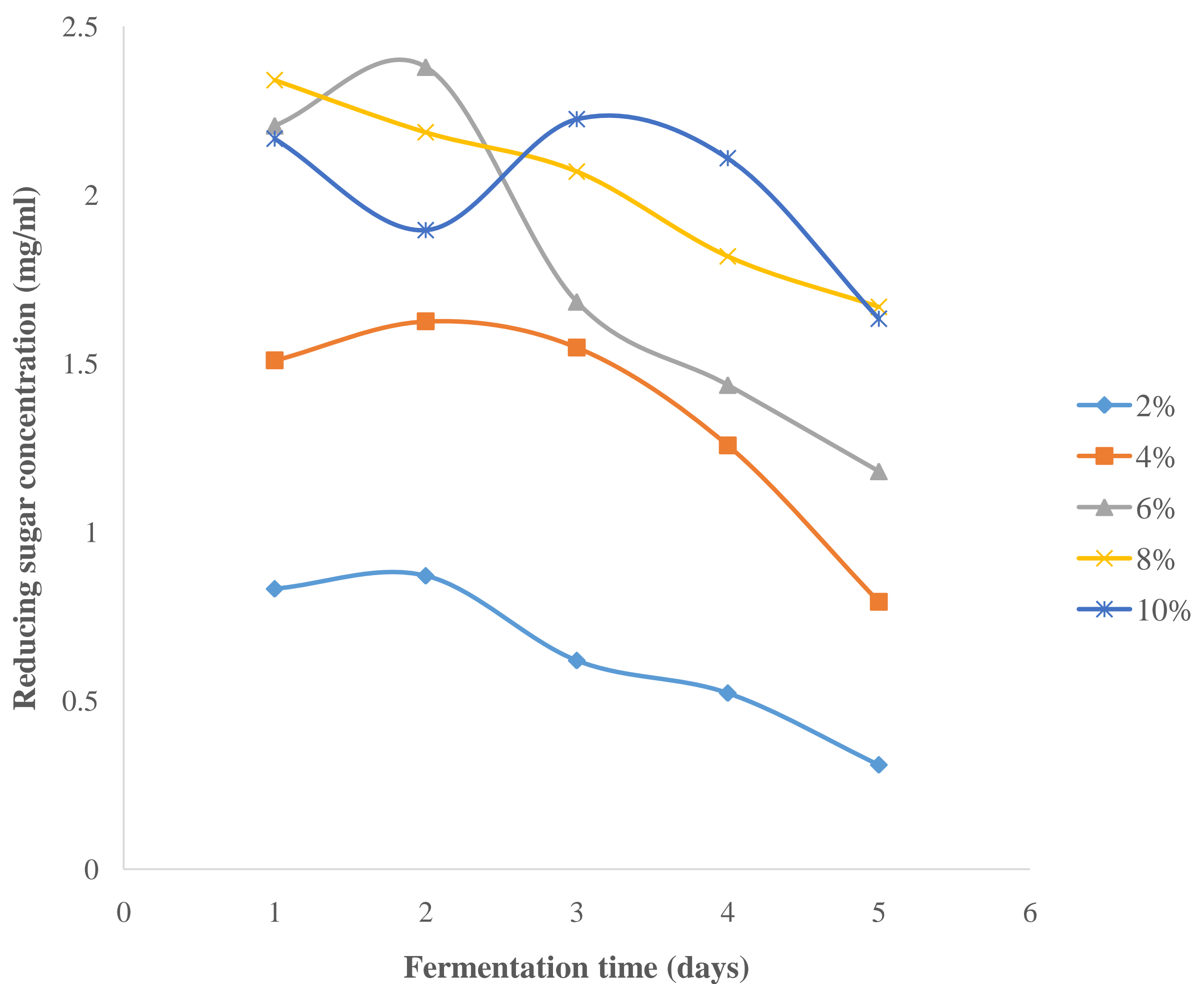


Figure 4.6. Effect of pretreated sample concentrations on reducing sugar yield

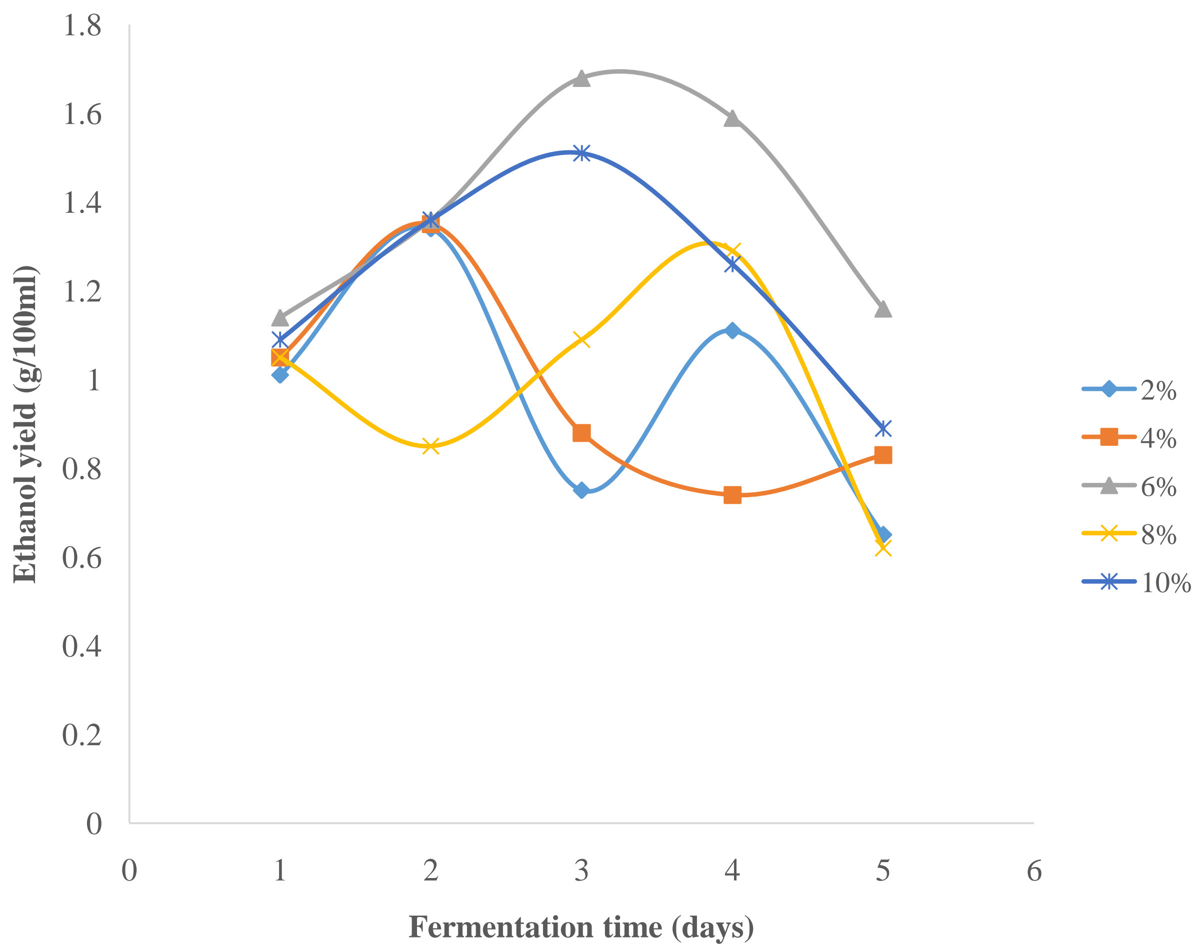


Figure 4.7. Effect of pretreated sample concentrations on ethanol yield

4.7 Optimization of Culture Parameters for Ethanol Production from 6% Pretreated Substrate.

4.7.1 Effect of pH on Ethanol Yield

The effect of pH on ethanol yield at 6% substrate concentration, 200 rpm and 30°C (Figure 4.7) shows that there was a gradual increase in ethanol yield from day 1 to day 3 after which there was a decline in the yield on the fourth day at all pH except pH 6 and pH 4 where the yield dropped from the first to the second day. There was also a drop in yields from day 4 to 5 at all pH conditions except at pH 3 and 7 which both showed increase. Maximum yield (1.68g/100ml) was observed at pH 5 and 6 on the third and fourth days respectively. The densities of the distillates from day 1 to 5 of fermentation at pH 5 were 0.99284, 0.99259, 0.99254, 0.99286 and 0.99318 g/ml respectively with specific gravity values of 0.99549, 0.99522, 0.99516, 0.99551 and 0.99585 respectively.

4.7.2 Effect of Temperature on Ethanol Yield

The effects of temperature on ethanol yield at 6% substrate concentration, pH 5 and 200 rpm is shown in Figure 4.8. It was observed that there was a gradual increase in the ethanol yield from day 1 which peaked on day 3 and then a steady decline from the fourth through the fifth day with the exception of fermentation at 35°C where ethanol yield peaked on the fourth day of fermentation before declining on the fifth day. Maximum ethanol yield (1.86 g/100ml) was also observed at 35°C on the fourth day. The density values of the distillate at 35°C from day 1 to day 5 were 0.99213, 0.99209, 0.99230, 0.99233 and 0.99242 g/ml respectively with specific gravity values of 0.99473, 0.99468, 0.99492, 0.99494 and 0.99504 respectively.

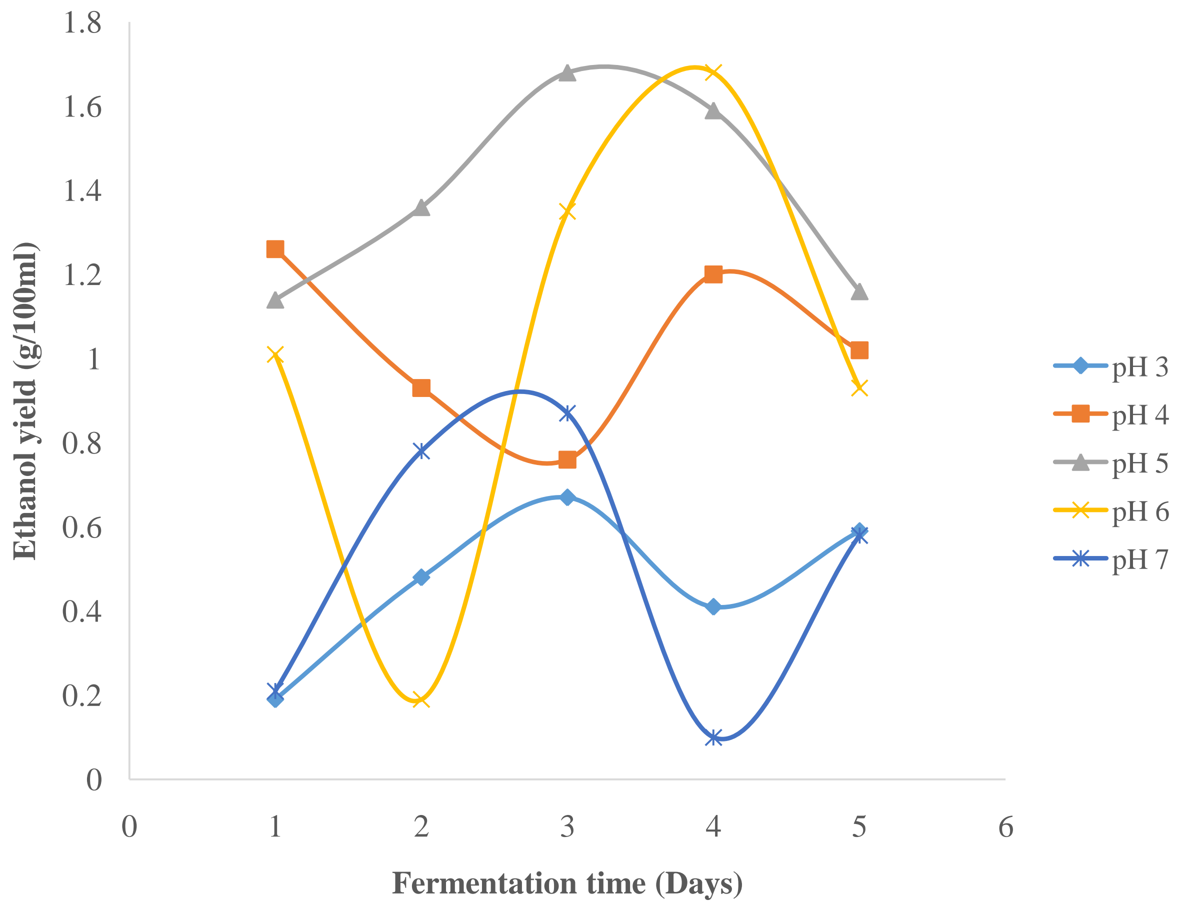


Figure 4.8. Effect of pH on ethanol yield.

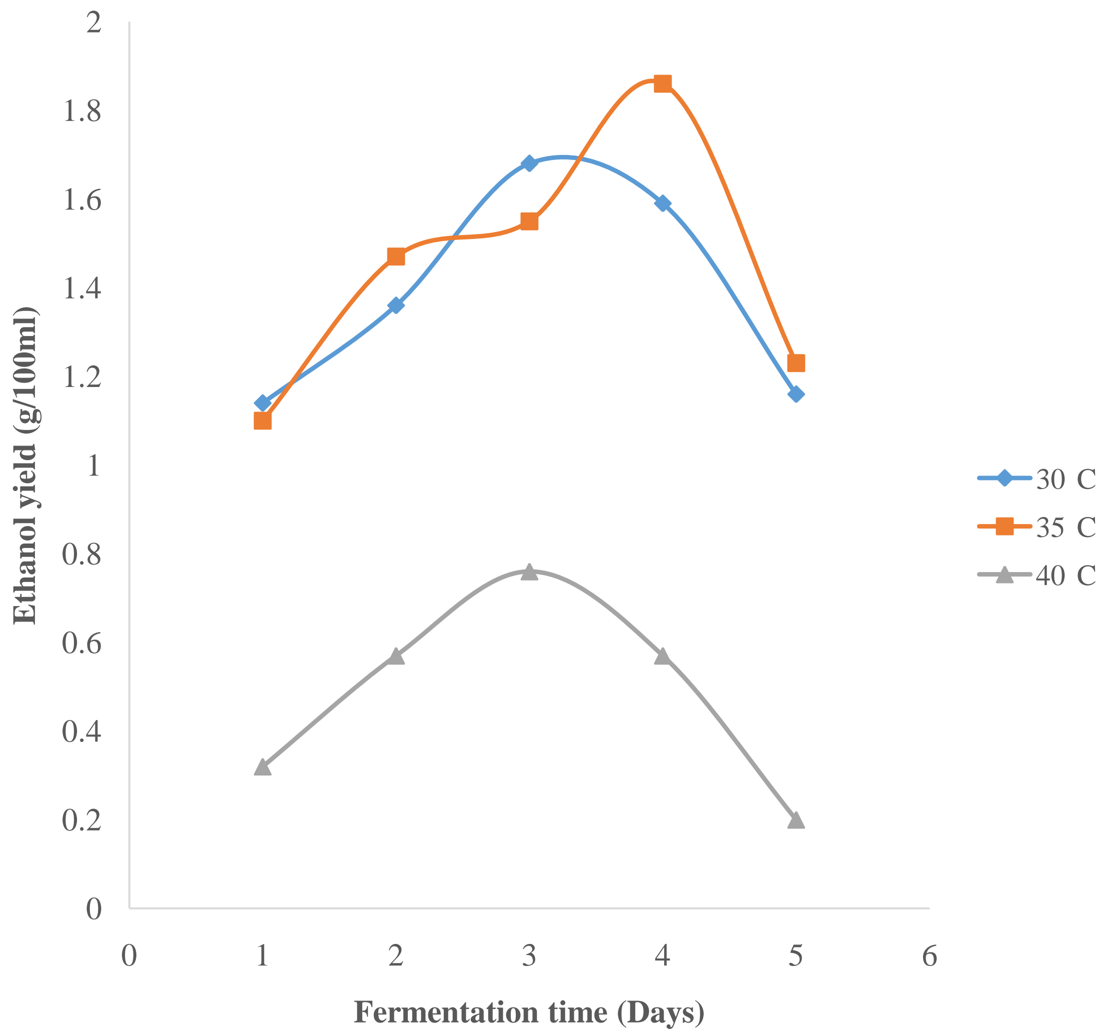


Figure 4.9. Effect of temperature on ethanol yield

4.7.3 Effect of Agitation Rate on Ethanol Yield

Fermentation was carried out at 6% substrate concentration, pH 5 and 35°C while varying the agitation rate and the result is shown on Figure 4.9. Ethanol yield peaked on the third day of fermentation except at 200 rpm which had its peak on the fourth day. Maximum ethanol yield of 1.99 g/100ml was observed at 300 rpm and the density of the distillates were 0.99245, 0.99209, 0.99213, 0.99254 and 0.99196 g/ml respectively from day 1 to 5 while the specific gravity values were 0.99509, 0.99468, 0.99473, 0.99516 and 0.99486 respectively.

4.7.4 Effect of Length of Time on Ethanol Yield

Ethanol yield was determined for 10 days by bringing optimum conditions to bear, i.e. 6% substrate concentration, 35^{0}C, 300 rpm and pH 5.0. The maximum yield was produced on the fourth day being 1.52 g/100ml (Figure 4.10).

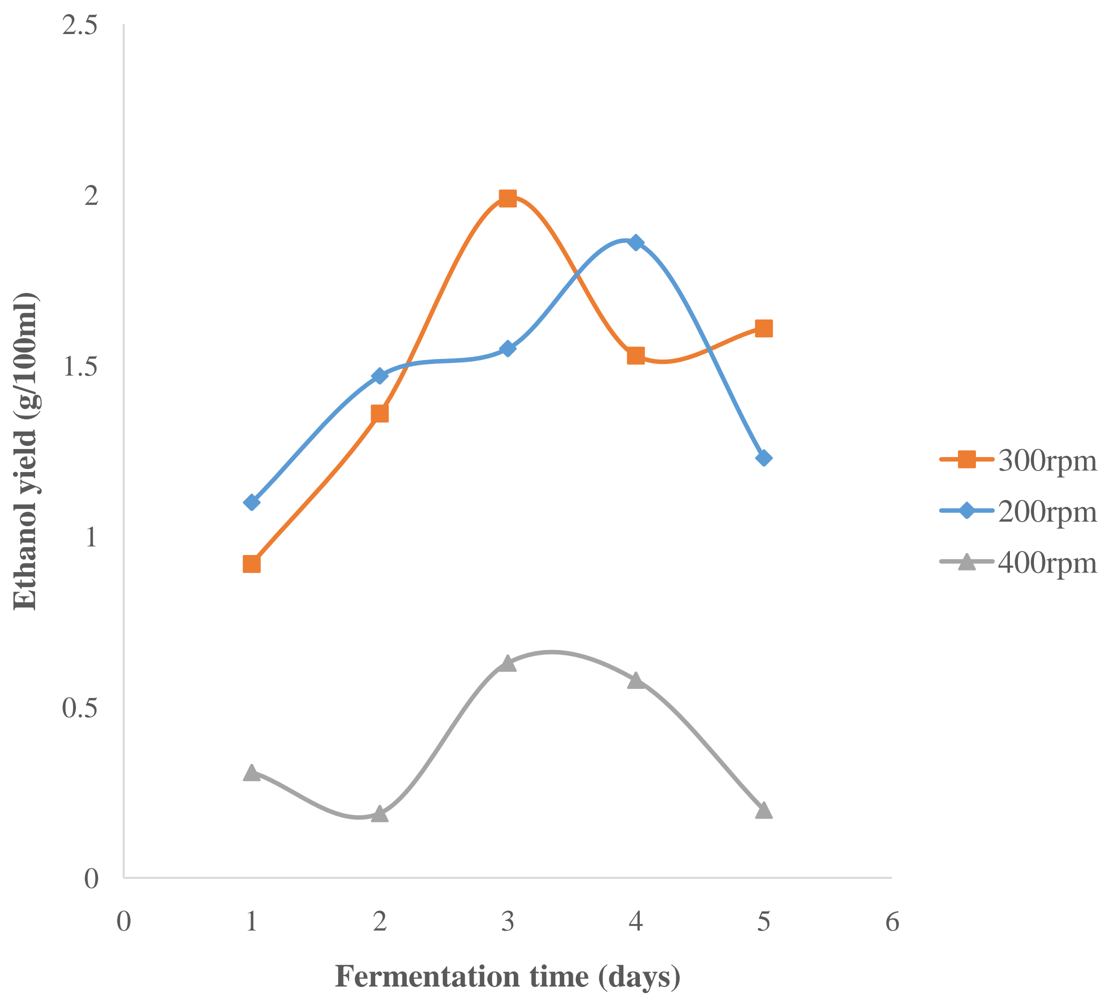


Figure 4.10. Effect of agitation rate on ethanol yield

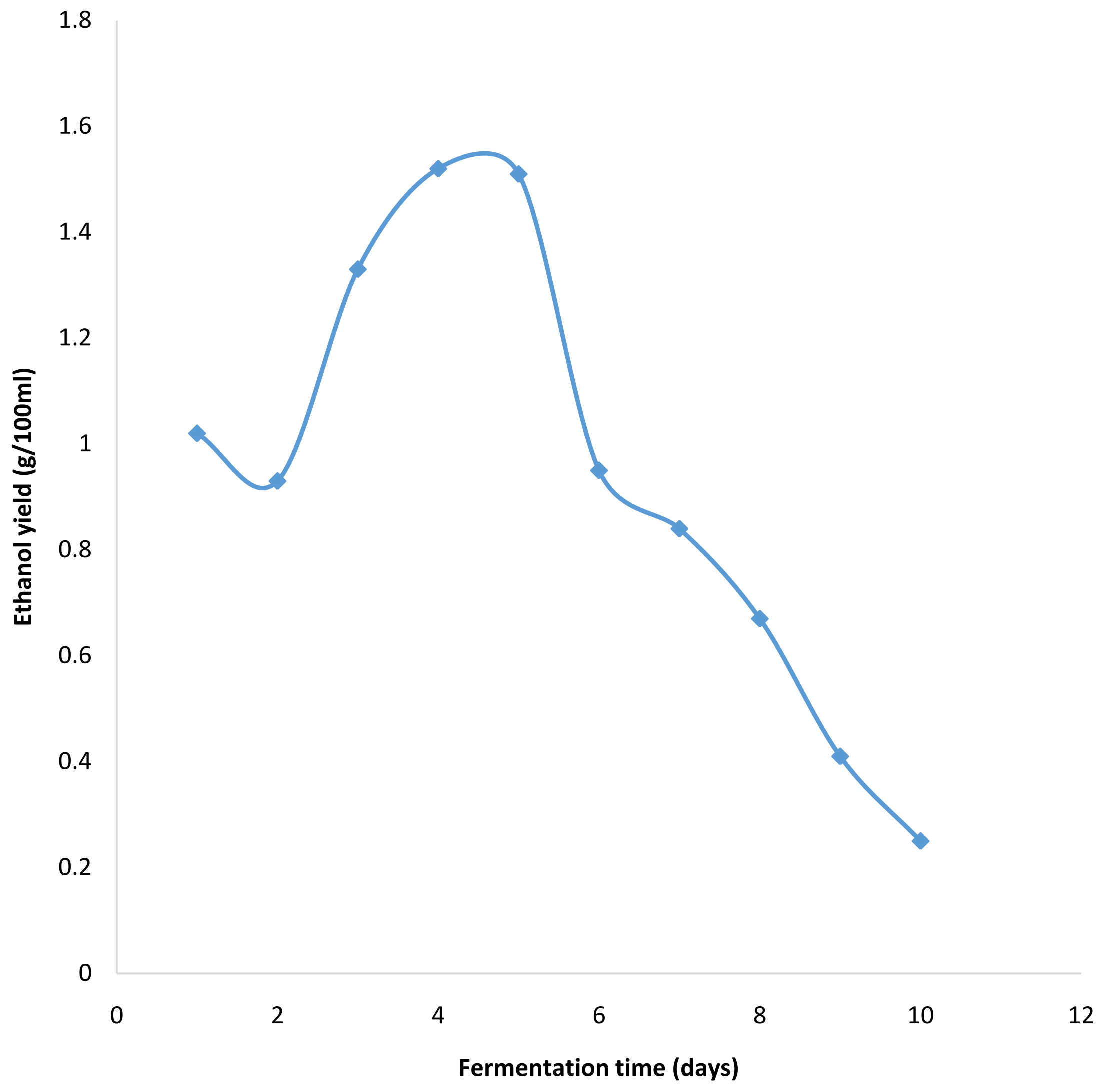


Figure 4.11. Effect of length of time on ethanol yield.

CHAPTER FIVE

5.0 DISCUSSION

Ten isolates of *A. niger* isolated from spoilt bread (3) and soil samples (7) were identified. The isolates showed characteristic woolly growth between 1-3 days of inoculation after which there was dense black sporulation on the plate typical of *A. niger*. The high frequency of isolation from soil sample is because of their ubiquity in such environments where they play an important role in many essential processes such as organic matter decomposition and elemental release by mineralisation (Christensen, 1989). This agrees with the work of Rabah *et al.* (2010) who also reported a high frequency of *A. niger* among fungi isolated from soil sample.

Ten yeast isolates were also obtained from palm wine (4) and burukutu (6). The reason for isolation of yeasts from palmwine is because the palm sap of a palm tree is a rich medium capable of supporting the growth of several types of microorganisms including the yeasts (Santiago-Urbina and Ruiz-Teran, 2014). Also the isolation of the yeast from burukutu agrees with the findings of Eze *et al.*(2011) who reported that *S. cerevisiae* found in burukutu serves as an agent of fermentation of the beverage.The ability of the palmwine isolates to assimilate more sugars than those from burukutu could be attributed to the fact that palm wine contains a host of different sugars including glucose, raffinose, saccharose (sucrose) and galactose as reported by Okafor (1972).

Qualitative screening test showed clear zones around the mould growth which is indicative of cellulase production and it also showed that all the mould isolates were capable ofproducing the cellulase enzyme as observed generally for most *Aspergillus* species (Ong *et al.,* 2004). Quantitative enzyme assay for the total saccharifying cellulase

(FPase) was carried out on all the isolates and it was observed that isolates obtained from the soil gave a high yield of FPase compared to those from bread with the exception of AN-7, which was isolated from bread but still gave a high cellulase yield. This high cellulase yield by soil isolates could be attributed to their being isolated from a habitat rich in organic matter. Decay and decomposition of leaves and other plant parts adds rich organic matter to soil. *Aspergillus* species and other moulds play an important role in the soil because they are adept at recycling starches, hemicelluloses, celluloses, pectins and other sugar polymers (Carroll and Wicklow, 1992).

Yeast isolates screened showed positive results for growth at 50% glucose concentration and fermentation of 50% glucose with gas production. Isolates were further subjected to ethanol tolerance test and it was observed that all the isolates grew at 5% and 10% ethanol concentrations except PW-1 and PW-2. This test further showed that at 15%ethanol concentration just one isolate (BK-1) was able to well. The ethanol tolerance of some of the isolates agrees with the works of Khaing *et al.* (2008) and Kumar *et al.* (2011) who reported ethanol tolerance of some yeast cells as high as 20% and 15% respectively. This tolerance to such high ethanol concentrations has been linked with the ability of some yeast cells to increase the amount of monounsaturated fatty acids e.g. oleic acid, in cellular lipids (You *et al.*,2003).

Media were constituted for the production of bioethanol using various concentrations of elephant grass ranging from 2% to 10% with same concentrations of glucose serving as control. The results of the tests showed that the media containing glucose produced more ethanol than those containing the grass at equal concentrations as there was statistical significance ($P < 0.05$) between them except at 6% concentration where

there was no statistical significance ($P > 0.05$). The production of a lesser quantity of bioethanol from the grass than from glucose could be due to loss of fermentable sugar during the pretreatment and washing processes of the grass preparation before fermentation. Pretreatment with either acid (H_2SO_4) or alkaline (NaOH) generally reduce the availability of total carbohydrate and reducing sugar for fermentation as levels of reducing sugar in a sample determine to some extent the percentage of ethanol that will be produced from the fermenting medium (Kumar *et al*., 2009).

Results of the optimization studies carried out showed that maximum ethanol yield was observed at 6% substrate concentration. Decrease in ethanol yield with increase in substrate concentration could be attributed to the production of inhibitory compounds e.g. furfurals, which could adversely affect ethanol yield (Kumar *et al*., 2009). It could also be due to substrate inhibition that sometimes occurs when excessive amounts of substrates are present whereby there are so many substrate molecules competing for the active sites on the enzyme surfaces that they block the sites and prevent any other substrate molecules from occupying them (Worthington Biochemical Corporation, 1972). This study agrees with the findings of Zakpaa *et al.* (2009) who reported an optimum substrate concentration of 6% using corncobs as substrate with co-culture of *A. niger* and *S. cerevisiae* for ethanol production.

The pH of a solution has several effects on the structure and activity of enzymes and hence saccharification. Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups vary, according to their acid dissociation constants, with the pH of the solution. Thus, pH affects the reactivity of the catalytically active groups (Chaplin and Bucke, 1990). Ethanol yield

increased from pH 3 to a maximum at pH 5 after which it decreased down to pH 7. Kinetics of optimum pH 5 agrees with the report of Abouzied and Reddy (1986) and Ado *et al.* (2009) that the optimal pH for growth and ethanol production by *Saccharomyces cerevesiae* was between pH 5 and 6. There was a high significant difference in the ethanol yields across the various pH ($P < 0.05$) which was attributed to pH 4, 5 and 6.

Temperature has complex effect on enzyme activity and hence saccharification. It affects the speed of molecules; the activation energy of the catalytic reaction and thermal stability of the enzyme. Ethanol production increased from 30°C to 35°C after which it decreased at 40°C. The increase in ethanol yield with temperature could be due to corresponding increase in kinetic energy and the decline after the optimum due to enzyme denaturation (Chaplin and Bucke, 1990; Shuler and Kargi, 1997). Occurrence of higher ethanol at 35^{0}C than at 40^{0}C agrees with the report of Kádár and Réczey (2004) who demonstrated that ethanol yield by free cells of *Saccharomyces cerevesiae* decreased at temperature beyond 35^{0}C. There was also a high statistical difference ($P < 0.05$) at 30^{0} and 35^{0}C.

The effect of agitation rate on the amount of ethanol produced was evident as it was observed that maximum ethanol was produced at 300rpm. This agrees with the findings of Ado *et al.* (2010) who selected an agitation rate of 300rpm as the preferable rate at which the isolates performed best. Abouzeid and Reddy (1986) also confirmed that the presence of excessive oxygen through agitation will promote respiration and cell growth at the expense of ethanol production. There was a high statistical difference ($P < 0.05$).

When the optimum fermentation parameters were brought to bear i.e. 6% substrate concentration, 35°C, 300 rpm and pH 5.0; it was observed that ethanol yield did not increase beyond the 4th day of fermentation. This also agrees with the work of Ado *et al*. (2010) who reported that an increase in the length of fermentation beyond five days neither enhanced ethanol yield or cell dry weight. However, statistical analysis using one way ANOVA without replication showed that there was significant difference in the ethanol yield given the optimization variation ($P < 0.05$).

As a general rule, an increase in solute concentration in a solution, leads to an increase in the density and specific gravity of the solution. When the density and specific gravity values of the ethanol produced were juxtaposed with the concentrations, it was observed that an increase in the concentration led to a decrease in the density and specific gravity values. This could be attributed to the low molecular weight and high solubility of ethanol in water because of the -OH functional group which is also present in water (http://www.britannica.com/EBchecked/topic/13366/alcohol).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Recently, there is a growing interest in biofuel production in most countries because of the increasing concerns about hydrocarbon fuel shortage and global climate changes, also for enhancing agricultural economy and producing local needs for transportation fuel. Ethanol can be produced from biomass by the hydrolysis and sugar fermentation processes. In this study, ethanol was produced without using expensive commercial enzymes instead cellulase was produced by *Aspergillus niger*. The findings from this study shows maximum ethanol yield of 1.68 g/100ml from 6% pretreated elephant grass substrate. Though much less than that from 6% glucose (8.38 g/100ml), it proves that with good environmental conditions, microorganisms of desired characteristics and genetic stability, ethanol is attainable from this lignocellulose waste hence, converting waste to wealth. The result of this study further shows that simultaneous saccharification and fermentation of elephant grass substrate is feasible. It equally revealed the fact that optimization of culture condition could enhance ethanol production from elephant grass using co-culture technique, thereby increasing the economy, in terms of percentage of cellulose fermentation to ethanol. It also showed that increasing temperature, pH, agitation rate and length of fermentation beyond certain level will not increase ethanol yield.

6.2 Recommendations

From the findings of this study, it is hereby recommended that:

1. Other pretreatment processes such as steam explosion, should be employed to ascertain their efficiency in disrupting the lignocellulose structure of Elephant grass hence, the release of the cellulose fraction for fermentation into ethanol.
2. It is also of utmost importance that the hemicellulose fraction of the plant sample which also contains fermentable sugars be also harnessed for its ethanol production potential.
3. A system of simultaneous saccharification and co-fermentation should be employed in the fermentation of plant sample to enable the simultaneous conversion of both hexoses and pentoses into bioethanol.
4. Finally, Elephant grass should also be considered as substrate for bioconversion into other beneficial value added products such as enzymes which include phytases, cellulases and hemicellulases.

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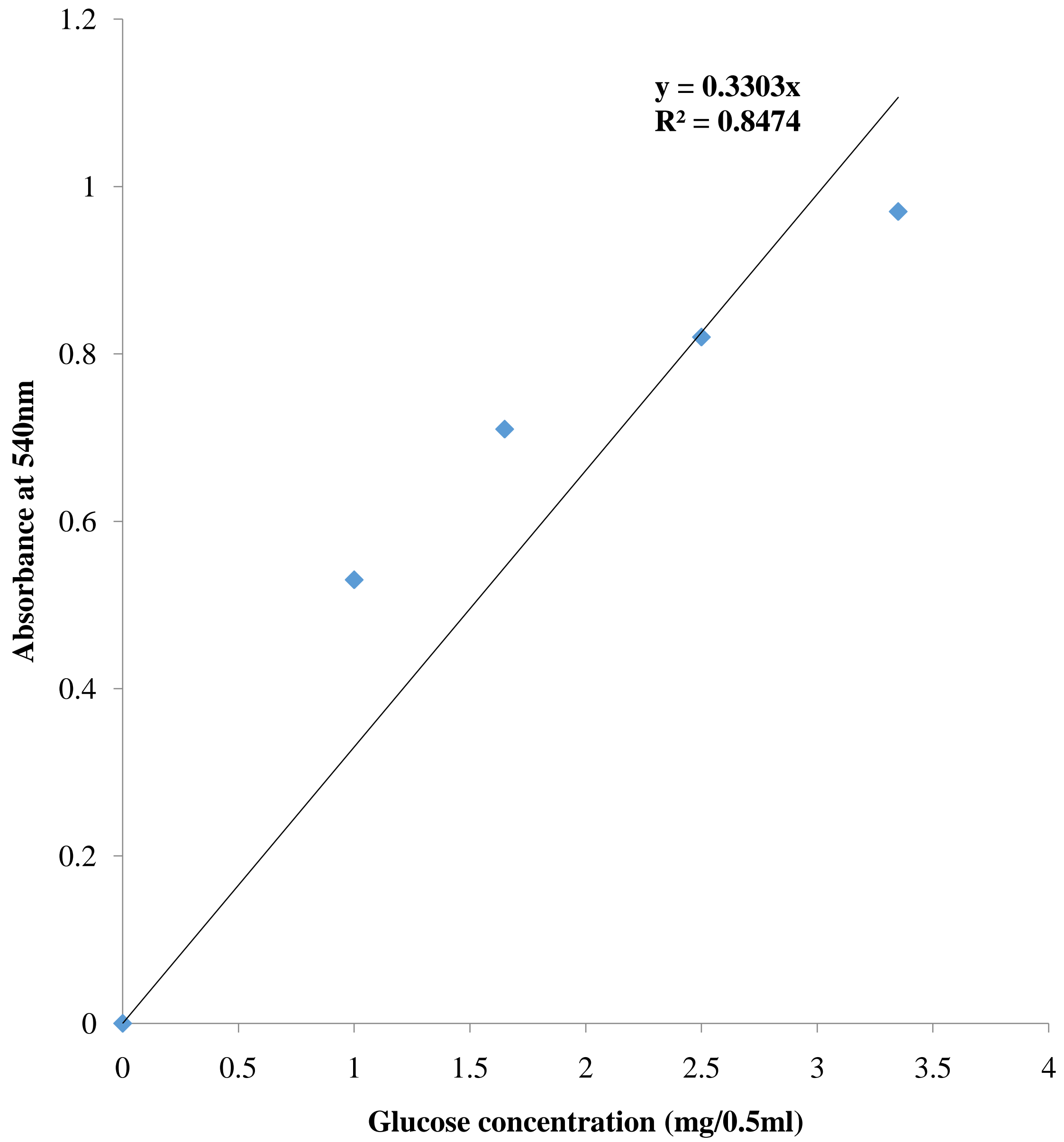
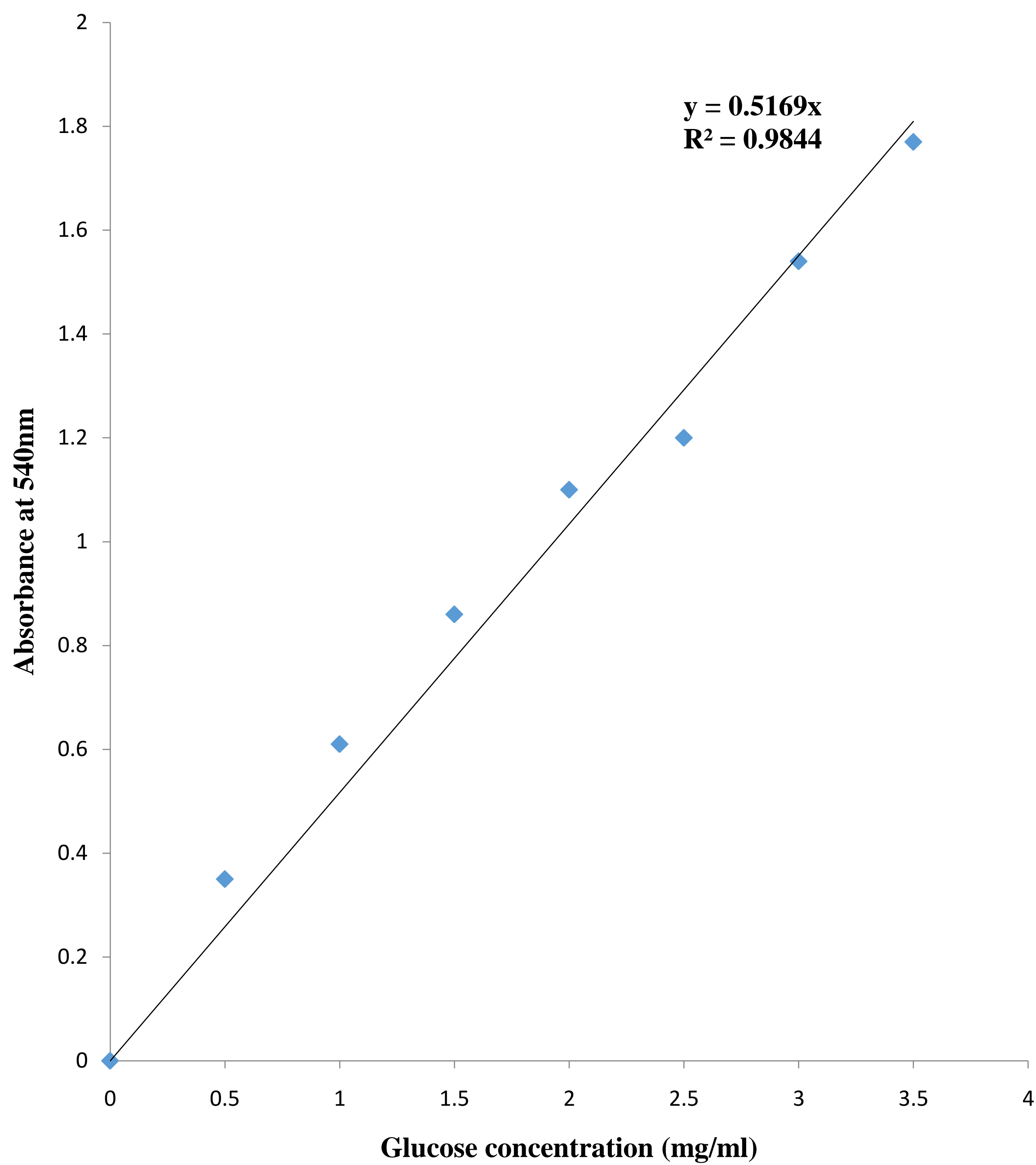
APPENDICES

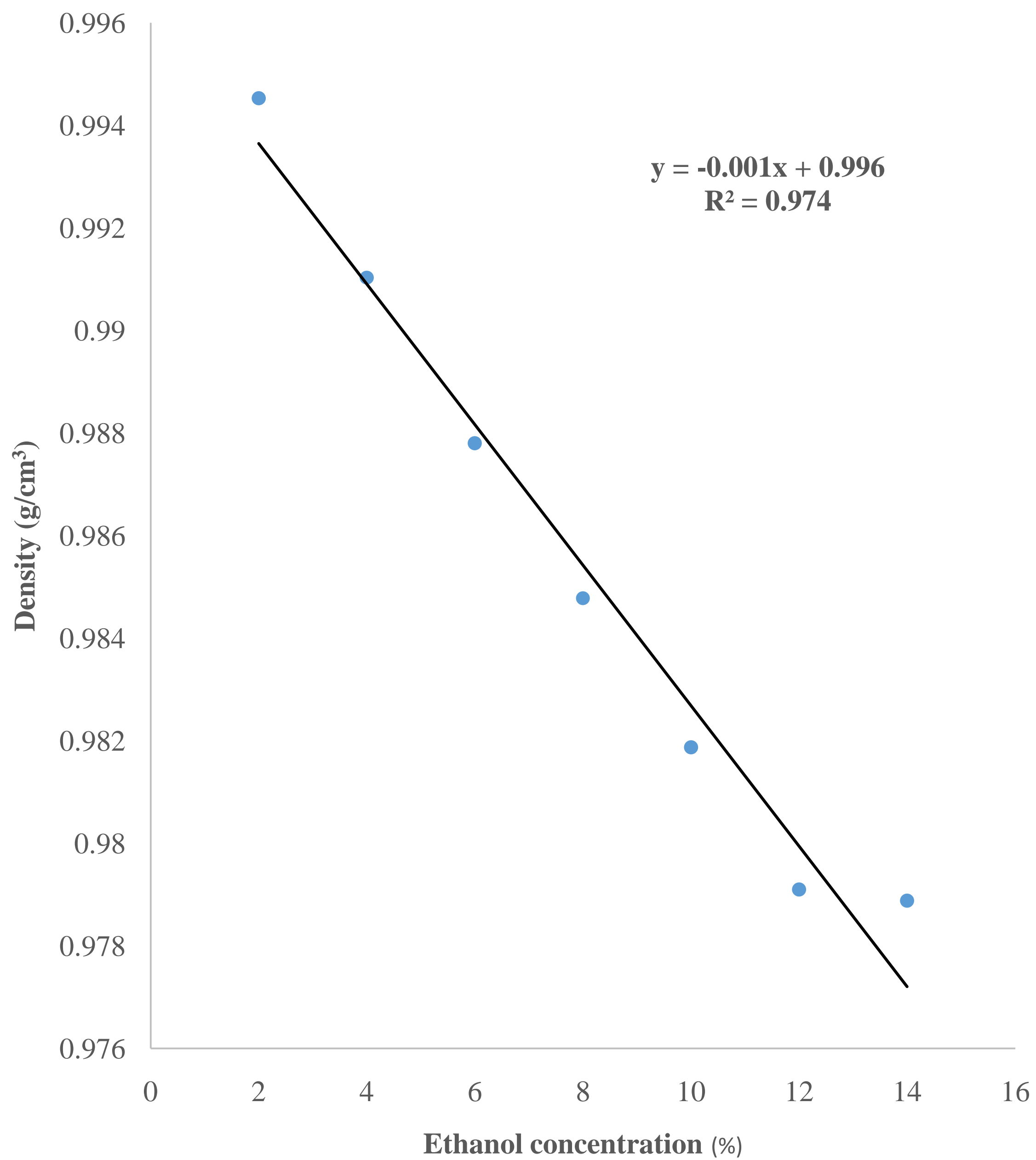
Appendix 1: Standard glucose curve for Total cellulase (Fpase) Assay



Appendix 2: Standard glucose concentration curve for reducing sugars.



Appendix 3: Standard density curve of ethanol



Appendix 4: Standard Specific gravity curve of ethanol