PRODUCTION OF BIOETHANOL FROM ELEPHANT GRASS (*Pennisetum purpureum)* STEM

By

MOSUNMADE OLUKEMI AIYEJAGBARA

DEPARTMENT OF CHEMICAL ENGINEERING

AHMADU BELLO UNIVERSITY, ZARIA.

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Mosunmade Olukemi AIYEJAGBARA, B Sc (OAU) 1998
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DEPARTMENT OF CHEMICAL ENGINEERING
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DECLARATION

I declare that the work in this Project Thesis entitled "Production of Bio–Ethanol from Elephant Grass (*Pennisetum purpureum)* Stem" has been carried out by me in the Department of Chemical Engineering under the supervision of Dr. B. O. Aderemi, Prof. Sani Ibrahim (Department of Biochemistry) and Dr. A. O. Ameh. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project thesis was previously presented for another degree or diploma at this or any other institution

Mosunmade Olukemi Aiyejagbara	**Date**

CERTIFICATION

This project thesis entitled "PRODUCTION OF BIO–ETHANOL FROM elephant GRASS (*Pennisetum purpureum)* STEM by MOSUNMADE OLUKEMI AIYEJAGBARA meets the regulations governing the award of the degree of Masters of Science in Chemical Engineering of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dr. B.O. Aderemi Chairman, Supervisory Committee	Date
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Prof. S. Ibrahim Member, Supervisory Committee	Date
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Dr. A.O. Ameh Member, Supervisory Committee	Date
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Dr. I.A. Mohammed- Dabo Head of Department	Date
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Prof. A.Z. Hassan Dean, Postgraduate School Ahmadu Bello University, Zaria	Date

DEDICATION

This work is dedicated to the almighty God for His love, mercy and preservation of my life.

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ABSTRACT

The production of bio-ethanol from Elephant grass (*Pennisetun purpureum)* stem was carried out using elephant grass stem as a feedstock and a combination of *Aspergillus niger* at 0.2%(w/v) 0.4%(w/v), 0.6%(w/v), 0.8%(w/v) and 1%(w/v) concentrations and *Saccharomyces cerevisiae* (brewer's yeast) at 0.5% (w/v), 1%(w/v), 1.5%(w/v), 2%(w/v) and 2.5%(w/v) concentrations as cells by simultaneous saccharification and fermentation (SSF). The study determined the most suitable pre-treatment method from the following pretreatment methods; 1M H_2SO_4, 0.1M H_2SO_4, 1M NaOH, 0.2M NaOH, Boiling, and 3M NH_4OH. IM NaOH pre-treatment gave the highest cellulose and lowest lignin content. The effects of temperature at 25^oC, 30^oC, 35^oC, 40^oC and 45^oC; pH values of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5; substrate concentration values of 1%(w/v), 2%(w/v), 3%(w/v), 4%(w/v) and 5%(w/v); particle size range of 53-106µm, 106-150µm, 150-250µm, 250-300µm and 300-425µm; and cell loading combination of *Aspergillus niger* at 0.2%(w/v) 0.4%(w/v), 0.6%(w/v), 0.8%(w/v), 1%(w/v) concentrations and *Saccharomyces cerevisiae* (brewer's yeast) at 0.5% (w/v), 1%(w/v), 1.5%(w/v), 2%(w/v), 2.5%(w/v) on the hydrolysis and fermentation process were investigated to obtain optimum conditions of fermentation. The optimum conditions of fermentation were obtained at temperature of 35^{0}C, pH value of 5.5, substrate concentration of 30g/l, particle size range of 250-300µm and *Aspergillus niger* to yeast ratio of 0.6/1.5 after 72 hours of fermentation time. Also substrate concentration of 30g/l, gave highest ethanol concentration of 23.4g/l and a yield of 78%. From the research, the kinetic Parameters which are reaction constant k and order of reaction n were evaluated to be 8.172×10^{-8} l/g.s and 2 respectively.

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C_s	Initial substrate concentration -
r_p	Production rate (g/l.s)
r	Initial rate of reaction (g/l.s)
K_m	Michealis Menten's constant (g/l)
r_{max}	Maximum rate of reaction (g/l.s)
k	Reaction constant for 2^{nd} order reaction (l/g.s)
n	Order of reaction
A. niger	*Aspergillus niger*
SEM	Scanning electron microscope

CHAPTER ONE

INTRODUCTION

1.1 Preamble

Energy availability, supply and use play a central role in the way societies organize themselves, from individual welfare to social and industrial development. By extension, energy accessibility and cost is a determining factor for the economical, political and social interrelations among nations. Considering energy sources, human society has dramatically increased the use of fossil fuels in the past 50 years in a way that the most successful economies are large consumers of oil. However, geopolitical factors related to security of oil supply, high oil prices and serious environmental concerns, prompted by global warming, the use of petrol for transportation which accounts for one-third of greenhouse gas emissions (Wyman, 1996), have led to a push towards decrease in its consumption. Indeed, the world's strongest economies are deeply committed to the development of technologies aiming at the use of renewable sources of energy. Within this agenda, the substitution of liquid fuel gasoline by renewable ethanol is of foremost importance. Biomass – derived ethanol represents one of the more promising commodities for long term sustainability of transportation fuels (Chum and Overend, 2001).

Brazil has been a front-runner in the use of renewable fuels. The partial substitution of gasoline by ethanol started in 1975. At the time of the first oil crisis, in the 1970s, the country imported 85% of its oil needs, hence the potential for ethanol production from sugarcane as a transportation fuel was a strategy in line with the Government policy regarding energy supply independence. Although in the mid-1970s, environmental concern was not a major driving force for finding alternative to gasoline, it is worth pointing out the global environmental benefits that have resulted from this policy since then. Presently the Brazilian fleet of 20 million cars runs on either a gasoline blend containing 22-24% ethanol or on 100% ethanol. Ethanol consumption is forecast to increase as the number of “flex-fuel" cars, with engines able to run on either gasoline blend or ethanol, from the present 4 million to 15 million in 2013 (Elba and Maria, 1996). Currently, established technologies in fuel-ethanol industry are primarily based on the fermentation of sugars derived from starch and sugar crops. However, conversion of starch and sugars to ethanol also has concerns, since it draws its feed stock from food streams (Elba and Maria, 1996).

Happily, large amounts of lignocellulosic wastes are generated through agricultural practices, paper-pulp industries, yard wastes, animal and human waste and many other agro-industries. Lignocellulose waste is often disposed-off by biomass burning, causing environmental pollution problem and consequently affects the public health, which is not restricted to developing countries, but is considered a global phenomenon (Cheng and Anderson, 1997).

Furthermore, the chemical properties of lignocelluloses components make them of enormous biotechnological values for the production of affordable fuel ethanol.

Also, it is less expensive than starch and sugar crops and is also renewable and available in large quantities.

Studies on simultaneous saccharification and fermentation of corn stover using a thermostable cellulase complex and 1.8g/l compressed baker's yeast have been reported to produce highest ethanol concentration of 33.8g/l in the simultaneous saccharification and fermentation with 11.5% corn stover (Öhgren *et al,* 2006). Shengdong *et al* (2005) studied the simultaneous saccharification and fermentation (SSF) of pre-treated rice straw by *Saccharomyces cerevisiae* with 10% w/v substrate, 40°C, pH 5.3, 72 hours, and 15 mg cellulase/g rice straw from Trichoderma reesei with an ethanol yield of 61.3% of the theoretical yield. The fermentation of olive tree pruning hydrolysate by *Pachysolen tannophilus* was found to give an ethanol yield of 0.38g/g sugar at 30°C and pH 3.5 (Romero *et al*., 2007). Separate hydrolysis of elephant grass carried out for 72 hrs using novozymes and fermentation of the hydrolysate obtained by *saccharomyces cerevisiae* for 10hrs at 30^{0}C gave 1.8g/L ethanol, stoichiometric yield being 94.44% (Isaias *et al*., 2011). Agbodike et al (2013) also carried out simultaneous saccharification and fermentation of elephant grass using co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae.* A combination of alkaline and steam pre-treatment was used. Optimum fermentation parameters were reported to be temperature of 35^{0}C, pH of 5, and substrate concentration of 6% at 72hrs fermentation time.

Nonetheless lignocellulosic conversion is not without its challenge. The crystalline nature of cellulose and the presence of lignin as cement in its structure are known to constitute a strong barrier to enzymatic activities. The keen interest of researchers in this field has resulted into several novel ways of mitigating against this challenge.

Hence, this work focuses on using elephant grass to produce ethanol, being one of the commonest lignocellulosic materials in the tropic. It is inedible, morphorlogically similar to sugar cane and has very little patronage presently for any known commercial end use.

Furthermore, this investigation is based on the single pot fermentation technology i.e. simultaneous saccharification and fermentation due to its widely reported advantages over the traditional two pots technology

1.2 Research Problems

1. While much attention has been devoted to starch and sugar conversion to fermentable sugar, little attention has been given to naturally occurring agricultural menace called grass in our environment as a source of wealth.

2. The practice of indiscriminate bush burning causes environmental pollution. Therefore the development of alternative agricultural waste disposal which is environmentally friendly is of utmost importance.

3. Effective exploitation of these new comers requires systematic studies to establish most favourable conditions for their processing, and development of relevant kinetic parameters for reactor design and analysis.

1.3 Research Justification

This study is necessary because the production of bio-ethanol from lignocellulosic biomass like elephant grass is a better choice than starch and sugar crops because of the comparative accessibility and abundance of forest, agricultural, and other cellulosic resources which do not compete with existing food chain.

In addition, converting elephant grass into valuable product like glucose and ethanol provides a potential alternative for treatment and disposal of such material.

The traditional method of producing ethanol from grains such as corn and wheat uses some type of fossil fuel to heat the boilers in the distillation columns and power the process.

New lignocellulosic biomass conversion processes can be mostly run on the otherwise waste lignin by-product, saving cost, energy and the environment.

1.4 Research Aim and objectives

The aim of this work is to produce bio-ethanol from the stem of elephant grass.

The specific objectives are:

1. To determine the best pre-treatment method and the optimum process conditions for pre-treatment at varying temperature, residence time, and liquid/solid ratio.
2. Production of bio-ethanol from the elephant grass stem using a combination of *Aspergillus niger* and *Saccharomyces cerevisae* as cells to produce enzymes for the hydrolysis and fermentation process.
3. To determine the optimum cultural conditions that favor production of bio-ethanol from elephant grass employing the method of Simultaneous Saccharification and Fermentation, SSF.

4. Investigation into the effects of particle size, substrate concentration, cell loading, temperature and pH on yield of the bio-ethanol produced.

1.5 Scope of the study

The scope of this work will be, to determine the best pre-treatment method of elephant grass for the production of bio-ethanol using the method of Simultaneous Saccharification and Fermentation, SSF. The process parameters that maximize bio-ethanol production will also be investigated and kinetic parameters evaluated.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Cellulose

Cellulose is the major polymeric component of plant material and is the most abundant polysaccharide on Earth (Liming and Xueling, 2004). It is one of the three major components of all plant cell walls with two other components; hemicelluloses and lignin. The digestive systems of man and most other animals (except ruminants) do not contain the necessary enzymes (cellulase) for hydrolyzing β – glucosidic linkages. However, cellulases are found in ruminants, various insect, fungi, bacteria and algae (Lee, 1992). Tremendous quantities of waste cellulose materials exist which are classified as municipal wastes, industrial wastes and agricultural wastes. The agricultural wastes represent the largest class of cellulosic waste while a few of these agro–waste are fed to some farm animals, most are being burnt, buried or otherwise discarded indiscriminately, thus causing environmental pollution. Proper treatments could change them from liabilities to assets (Vlesenko *et al*., 1997).

Table 2.1: Cellulose, Hemi-cellulose, And Lignin Contents in Common Agricultural Residues And Wastes.

LIGNOCELLULOSIC MATERIAL	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
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
wheat straw	30	50	15
sorted refuse	60	20	20
Leaves	15-20	80-85	0
cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
waste papers from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	-	-
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
swine waste	6.0	28	Na

Adopted from Jorgensen *et al.* (2007)

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. The structure of cellulose is shown in Figure 2.1. 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. 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.

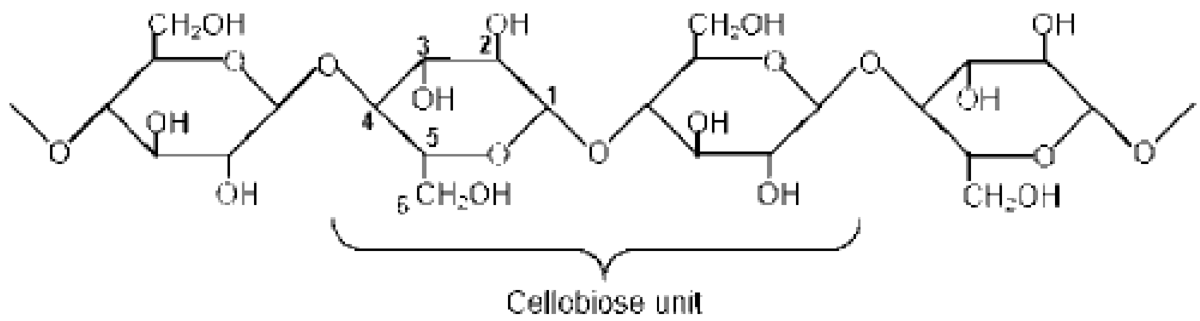


Figure 2.1**:** Illustration of a Cellulose Chain.

The main feature that differentiates hemi-cellulose from cellulose is that hemi-cellulose 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.Also, hemicelluloses can have some degree of acetylation, for example,in heteroxylan. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolyzable. 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. Lignocellulosic biomass typically contains 50% - 80% (dry basis) carbohydrates that are polymers of five carbon and six carbon sugar units. Most carbohydrates can be processed either chemically or biologically to yield biofuels such as ethanol. Employment of enzymes for the hydrolysis of lignocellulose is considered the prospectively most viable strategy to offer advantages such as higher yields, minimal by-product formation, low energy requirements, mild operating conditions, and environmentally friendly processing. The physiochemical and structural compositions of lignocelluloses are however resistant to direct enzymatic hydrolysis of cellulose (Beguin and Aubert, 1994). Therefore, pre-treatment step is invariably required to render the cellulose amenable to enzymatic attack prior to the enzymatic hydrolysis, by breaking down the shield formed by lignin and hemicelluloses, disruption of the crystalline structure and reducing the degree of polymerisation of cellulose. Although pre-treatment has been viewed as one of the expensive processing steps within the conversion of biomass to fermentable sugar, the pre-treatment is also believed to have great potential for the improvement of efficiency and reduction of cost (Himmel *et al.*, 1994). The goal of pretreatment in biomass-to-biofuels conversion is depicted in Figure 2.2

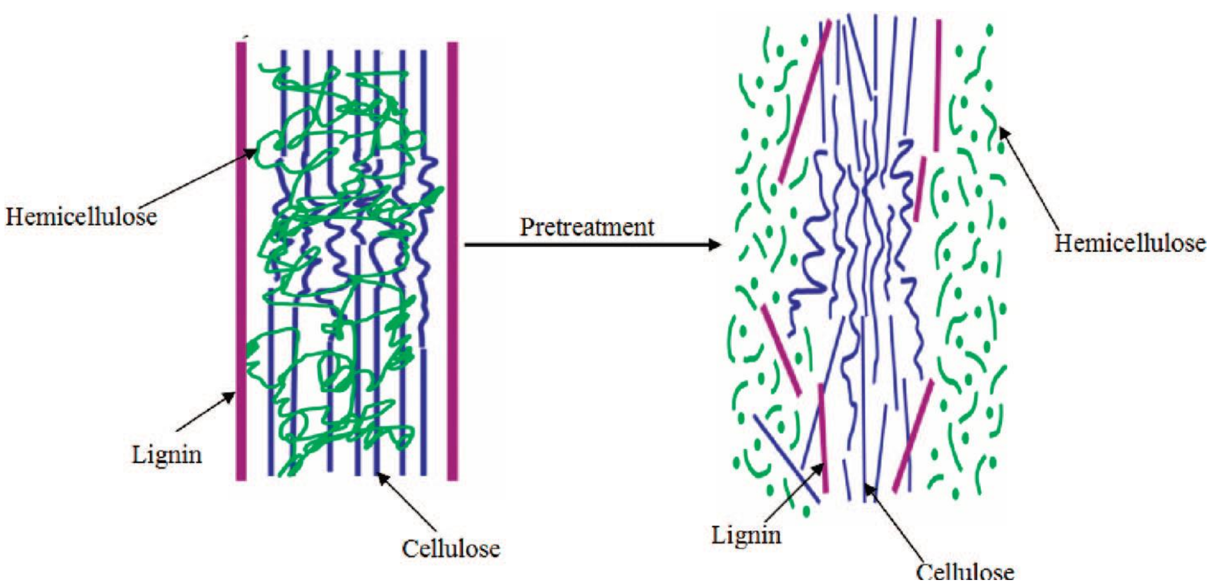


Figure 2.2: Schematic of the Role of Pretreatment in the Conversion of Biomass to Fuel.

2.2 Cellulose Chemistry

Despite its simple chemical composition, cellulose exists in a number of crystalline and amorphous topologies. Its insolubility and heterogeneity make native cellulose a recalcitrant substrate for enzymatic hydrolysis. Microorganisms meet this challenge with the aid of a multi-enzymatic system. Specific enzymes act in synergy to elicit effective hydrolysis (Lee and Fan, 1982).

It is now accepted for as a general concept that cellulolysis is performed by the synergistive action of three types of enzymatic activities namely: Endoglucanases, Exoglucanases and Glucosidases as typified in figure 2.3.

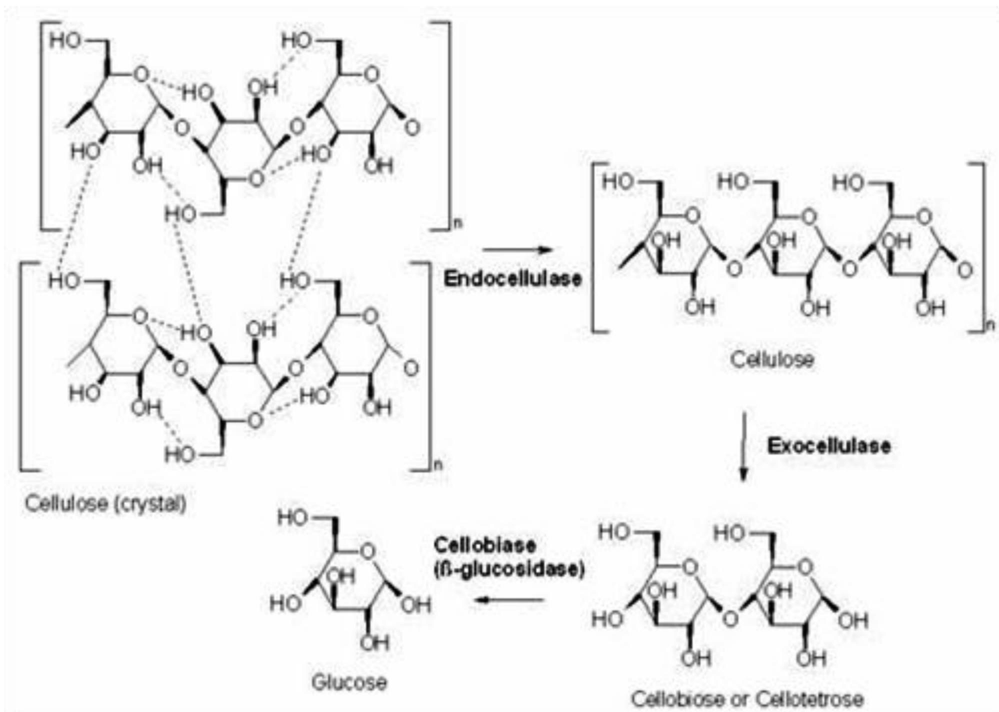


Figure 2.3: Reaction Pathway from Cellulose to Glucose.

2.3 Cellulases

Many fungi are capable of producing extra cellular enzymes that can degrade cellulose. They are *Trichoderma viride, Aspergillus niger, Aspergillus flavus* etc. Bacteria such as cellulomonas along with *Clostridium thermacellum* can also produce cellulases.

Typical proportion of the endoglucanase and exoglucanase in the extra cellular protein from *Trochoderma reseei* grown on cellulose are 15% - 25% and 35% - 55% respectively. The endoglucanase hydrolyses cellulose molecule randomly within the molecule and are more effective in combination with cellobiohydrolases. Cellobiohydrolases cleave cellobiose and glucose from the non-reducing end of cellulose molecule. β-Glucosidase converts cellobiose to glucose, with some indication of the ability to degrade Oligosaccharides. Endoglucocanases and Cellobiohydrolases must adsorb to the cellulose before a reaction can occur. β-glucosidase, however, remains in solution and hydrolyses the cellobiose in solution (Lee, 1992).

2.4 Cellulose Conversion

Interest in cellulose degradation has centered on production of products, usually via glucose and as alternative method of disposal of cellulosic waste, e.g domestic refuse.

Also, it has been found that cellulosic waste have a great potential as a feedstock for producing fuels and chemicals (Wen *et al*., 2004).

Kaur *et al*. (1998) made some efforts to produce glucose from rice straw and sawdust employing cellulase from *Trichoderma viride.* The enzyme has high thermal stability and is not easily inhibited by impurities in the substrate material. However, the conversion of cellulosic biomass to useful substance such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugars, still suffers severe problem, that are economic and technical in nature. The economic problems consist exclusively of the cost of the production of cellulase and of the pretreatment of native cellulose substance to enhance their susceptibility to enzymatic attack. Of all, enzyme production is the most expensive factor, which has been estimated to be 50% of the total cost of the glucose production from cellulosic materials. Any means that is capable reducing this cost significantly would have a dramatic effect on the cost of production of useful substances from renewable resources (Taniguchi *et al*., 1983).

2.5 Pre-Treatment of Lignocellulosic Material

The beneficial effects of pre-treatment of lignocellulosic materials have been recognized for a long time (Himmel *et al.*, 1994). The goal of the pretreatment process is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic material. Pretreatment must meet the following requirements:

1. Improve the formation of sugars or the ability to subsequently form sugars by hydrolysis.
2. Avoid the degradation or loss of carbohydrate.
3. Avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes.
4. Must be cost-effective.

Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pre-treatment/autohydrolysis, Ammonia fiber explosion, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), biological, electrical, or a combination of these. The following pretreatment technologies are potentially cost-effective for lignocellulosic biomass conversion to fuels and chemicals.

2.5.1 Physical pre-treatment.

Mechanical comminution.

Comminution of lignocellulosic materials through a combination of chipping, grinding, and/or milling can be applied to reduce cellulose crystallinity. The size of the materials is usually 10-30 mm after chipping and 0.2-2 mm after milling or grinding (Sun and Cheng 2002). Vibratory ball milling was found to be more effective than ordinary ball milling in reducing cellulose.

2.5.2 Physicochemical pre-treatment.

***Steam explosion*.**

Steam explosion is the most commonly used method for the pre-treatment of lignocellulosic materials. In this method, biomass is treated with high pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160-260^{0}C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicelluloses degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Removal of hemicelluloses from the microfibrils is believed to expose the cellulose surface and increase enzyme accessibility to the cellulose microfibrils (Kabel *et al.*, 2007). Lignin is removed only to a limited extent during the pretreatment but is redistributed on the fiber surfaces as a result of melting and depolymerization/repolymerization reactions. The removal and redistribution of hemicellulose and lignin increase the volume of the pre-treated sample. Rapid flashing to atmospheric pressure and turbulent flow of the material causes fragmentation of the material, thereby increasing the accessible surface area. Depending on the severity of the pre-treatment, some degradation of the cellulose to glucose can take place. Water acts as an acid at high temperatures. Addition of SO_2 (or H_2SO_4) or CO_2 [typically 0.3-3% (w/w)] in steam explosion can decrease time and temperature, effectively improve hydrolysis, decrease the production of inhibitory compounds, and lead to complete removal of hemicellulose. The factors that affect steam-explosion pretreatment are residence time, temperature, chip size, and moisture content. Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short residence time (270^0C, 1 min) or lower temperature and longer residence time (190^0C, 10 min) (Parveen *et al.*, 2009).

The advantages of steam-explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same particle size reduction (Hotzapple *et al.*, 1991). Steam explosion is recognized as one of the most cost-effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods. Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix, and generation of compounds that might be inhibitory to microorganisms used in downstream processes (Parveen *et al.*, 2009).

Ammonia fiber explosion (AFEX)

Ammonia fiber explosion is a physicochemical pre-treatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The AFEX process is very similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1-2kg of ammonia/kg of dry biomass, the temperature is 90^0C, and the residence time is 30 min. AFEX pretreatment can significantly improve the fermentation rate of various herbaceous crops and grasses. During pretreatment only a small amount of the solid material is solubilized; that is, almost no hemicellulose or lignin is removed. The hemicelluloses is degraded to oligomeric sugars and deacetylated, which is most likely the reason that the hemicellulose is not soluble. The structure of the material is changed, resulting in increased water holding capacity and higher digestibility. Over 90% hydrolysis of cellulose and hemicellulose was obtained after AFEX pre-treatment of bermuda grass (approximately 5% lignin) and bagasse (15% lignin) (Holtzapple *et al.*, 1991). However, the AFEX process was not very effective for biomass with higher lignin content. The ammonia fiber explosion pretreatment simultaneously reduces lignin content and removes some hemicelluloses while decrystallizing cellulose. It can have a profound effect on the rate of cellulose hydrolysis. The cost of ammonia, and especially of ammonia recovery, drives the cost of the AFEX pretreatment. (Holtzapple *et al.*, 1991).

2.5.3 Chemical pre-treatment

Acid hydrolysis.

Concentrated acids such as H_2SO_4, and HCl have also been used to treat lignocellulosic materials Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible.

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. Sulfuric acid at concentrations usually below 4% wt, has been of the most interest in such studies as it is inexpensive and effective. High temperature in the dilute-acid treatment is favorable for cellulose hydrolysis. Two types of dilute-acid pretreatment processes are typically used: a high-temperature ($T>160^0C$), continuous-flow process for low solids loadings (weight of substrate/weight of reaction mixture) 5-10%, and a low-temperature ($T<160^0C$), batch process for high solids loadings (10-40%). Although dilute-acid pretreatment can significantly improve cellulose hydrolysis, its cost is usually higher than those of physicochemical pretreatment processes such as steam explosion or AFEX. Neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. Dilute-acid pretreatment is also known to have a negative influence on the enzymatic hydrolysis of biomass. In a recent article, Selig *et al.* (2007) reported the formation of spherical droplets on the surface of residual corn stover following dilute-acid pretreatment at high temperature. They suggested that the droplets formed were composed of lignin and possible lignin-carbohydrate complexes. It was demonstrated that these droplets were produced from corn stover during pretreatment under neutral and acidic pHs at and above 130^0C and that they can deposit onto the surface of residual biomass. The deposition of droplets produced under certain pretreatment conditions (acidic pH, $T>150^0$C) and captured on pure cellulose was shown to have a negative effect on the enzymatic saccharification of the substrate. Therefore, it is extremely important to carefully examine the appropriate dilute-acid pretreatment of lignocellulose biomass species. It has been shown that materials that have been subjected to acid hydrolysis can be harder to ferment because of the presence of toxic substances (Galbe and Zacchi 2007).

Furthermore, acid pretreatment results in costly materials of construction, high pressures, neutralization and conditioning of hydrolysate prior to biological steps, slow cellulose digestion by enzymes, and nonproductive binding of enzymes to lignin.

Alkaline hydrolysis

Some bases can be used for the pretreatment of lignocellulosic materials, and the effect of alkaline pretreatment depends on the lignin content of the material. Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies. Alkali pretreatment can be carried out at ambient conditions, but pretreatment times are in the order of hours or days rather than minutes or seconds. Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents. Of these four, sodium hydroxide has been studied the most. However, calcium hydroxide (slake lime) has been shown to be an effective pretreatment agent and is the least expensive per kilogram of hydroxide. It is possible to recover calcium from an aqueous reaction system as insoluble calcium carbonate by neutralizing it with inexpensive carbon dioxide; the calcium hydroxide can subsequently be regenerated using established lime kiln technology.

2.5.4 Biological pretreatment.

Most pretreatment technologies require expensive instruments or equipment that has high energy requirements, depending on the process. In particular, physical and thermo chemical processes require abundant energy for biomass conversion. Biological treatment using various types of rot fungi, a safe and environmentally friendly method, is increasingly being advocated as a process that does not require high energy for lignin removal from a lignocellulosic biomass, despite extensive lignin degradation. In biological pretreatment processes, microorganisms such as brown-, white-, and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccase. These enzymes are regulated by carbon and nitrogen sources. White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials. Hatakka (1983), studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in 5 weeks. Akin *et al*. (1995), also reported the delignification of Bermuda grass by white-rot fungi. The biodegradation of Bermuda grass stems was improved by 29-32%, after 6 weeks, using *Ceriporiopsis subvermispora* and by 63-77% using *Cyathus stercoreus*. Advantages of biological pre-treatments are low energy requirement and mild operation conditions. Nevertheless, the rate of biological hydrolysis is usually very low, so this pretreatment requires long residence times (Sun and Cheng, 2002). Table 2.2 shows the summary of the various pre-treatment methods of linocellulosic materials.

Table 2.2: Summary of Various Process Used For the Pre-treatment of Lignocellulose Biomass

pretreatment process	**Advantages**	**Limitation and disadvantages**
mechanical comminution	reduces cellulose crystallinity	power consumption usually higher than inherent biomass energy
steam explosion	causes hemicelluloses degradation and lignin transformation; cost-effective	destruction of a portion of the xylan fraction; incomplete disruption of the lignin – carbohydrate matrix; generation of compounds inhibitory to microorganisms
AFEX	increase accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream process	not efficient for biomass with high lignin content
CO_2 explosion	increase accessible surface area; cost-effective; does not cause formation of inhibitory compounds	does not modify lignin or hemicelluloses
Ozonolysis	reduces lignin content; does not produce toxic residues	large amount of ozone required; expensive
acid hydrolysis	hydrolyzes hemicelluloses to xylose and other sugars; alters lignin structure removes hemicelluloses and lignin; increases accessible surface	high cost; equipment corrosion; formation of toxic substances
alkaline hydrolysis	removes hemicelluloses and lignin; increases accessible surface area	long residence times required; irrecoverable salts formed and incorporated into biomass
Organosolv	hydrolyzes lignin and hemicelluloses	solvents need to be drained from the reactor, evaporated, condensed, and recycled; high cost
Pyrolysis	produces gas and liquid products	high temperature; ash production
pulsed electrical field	ambient conditions; disrupts plant cells; simple equipment	process need more research
biological	degrades lignin and hemicelluloses; low energy requirements	rate of hydrolysis is very low

Adapted from Parveen *et al* (2009)

2.6 Factors Affecting Enzymatic Hydrolysis of Cellulose

Past techno-economic modeling work have identified the relatively large contribution that enzymatic hydrolysis adds to the cost of producing ethanol from lignocellulosic substrates. This cost was primarily due to high concentration of enzyme and long incubation time that was required to obtain a complete hydrolysis. Hydrolysis of cellulose in a batch fashion is generally characterized by a limited logarithmic phase, associated with the rapid release of soluble sugars, followed by a declining rate of sugar production as the reaction proceeds. Several studies have shown that the specific hydrolysis rates decline rapidly with increased conversion of the substrate. There are several proposed explanations for the diminishing rate of hydrolysis. One proposal is that as hydrolysis proceeds, the substrates become enriched in the more recalcitrant cellulose as the less recalcitrant amorphous cellulose is hydrolyzed. Other possible contributing factors to the declining hydrolysis rate include enzyme denaturation through thermal, mechanical and chemical actions (Sharma et al., 2001, Lee and Fan, 1982). However, the complete mechanism of cellulose hydrolysis has not been determined, primarily due to the complexity of both the substrate and the enzymatic system required to hydrolyze crystalline cellulose. Consequently, the characteristics of a typical batch hydrolysis reaction, such as denaturation or inhibition, the intrinsic structural features of the substrate as the reaction progress are yet to be properly understood. Only some of these factors are readily available for manipulation within the design or operation of the enzymatic hydrolysis step (James and David 1992).

2.6.1 Enzyme-related factors affecting hydrolysis

Improvement in the hydrolysis rates of lignocellulosic materials can be by increasing, to a certain extent, the amount of cellulose used in the conversion process. Cellulase dosage of 10FPU/g cellulose is often used in the laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48 – 72h) at a minimal enzyme cost (Gregg and Saddler, 1996). However, it would be desirable if considerably higher hydrolysis rates and glucose yield could be obtained using even lower enzyme concentrations (Sun and Cheng, 2002).

Attempts to enhance the rate of cellulose hydrolysis have met with some success, end-product inhibitions of cellulases are well known for most cellulose systems. Cellobiose (basic repeating of cellulose) is thought to be a stronger inhibitor of the cellulases than glucose. Several methods have been proposed to overcome the end-product inhibition resulting from the rapid accumulation of sugars during hydrolysis. These include the use high concentration of enzymes, the supplementation of cellulase with exogenous β-glucosidase activity; the elimination of sugars from hydrolyses by ultra filtration or the simultaneous saccharification and fermentation of the substrate (Cao *et al*., 1996). It has been shown that the endoglucanases and cellobiohydrolases are inhibited by increased concentrations of cellobiose, whereas β-glucosidases are more sensitive to glucose accumulation. Although the glucanases are also inhibited by glucose, drop in the overall enzyme activity of the system due to glucose accumulation is relatively small compared to that associated with cellobiose accumulation. Also, it was found that periodic removal of product sugars was effective at initially reducing end-product inhibition. However, it was found that extended incubation (≥48hrs) was still required before complete hydrolysis could be obtained. End product inhibitions appeared to greatly affect the initial rate of hydrolysis while other factors, such as increasing substrate recalcitrance, reduced the overall rate yield of hydrolysis (James and David, 1992, Duff and Murray, 1996)

2.6.2 Effects of substrate concentration

Substrate concentration is one of the main factors that affect the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheng and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, and consequently lowers the rate of the hydrolysis. Kademi and Baratti, (1996) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate (Avicel) to the cellulase from *Trichoderma reeseei,* (grams of cellulose per filter paper unit) was greater than 5. Their reports show that the optimum substrate to enzyme ratio was 1:25 for the microcrystalline substrate. Two independent experiments were conducted in the investigation of the effects of substrate concentration on enzymatic hydrolysis; one at fixed enzymes concentration, the other at fixed enzyme to substrate ratio. For each experimental run, glucose concentration and yield showed an opposite trend, with glucose concentration increasing, and yield decreasing with increasing substrate concentration (Wen *et al.,* 2004, Tengborg *et al*., 2001).

When enzymes concentration was fixed, increasing substrate concentration resulted in more cellulose available for hydrolysis. However, the amount of enzyme was not proportionally increased and as a result, more intact cellulose was present in the system. When the ratio of enzyme to substrate was fixed, sufficient enzyme could be supplied with increased substrate concentrations. As a result, glucose yield remained almost constant within the substrate concentration ranging from 10 to 50g/l. Further increase in substrate concentration (50-100g/l) resulted in a lower glucose yield. This may be due to the end-product inhibition caused by high concentration of glucose (Wen *et al.,* 2004). However, in simultaneous saccharification and fermentation system of ethanol production, this inhibition is eliminated as product of hydrolysis are spontaneously converted by yeast to ethanol and carbon (iv) oxide (Abouzied and Reddy,1986). Banerjee *et al*.,(2004) reported that by increasing starch concentration, there was an increase in ethanol yield and maximum ethanol production at 12.5g/100ml.

2.6.3 Effects of substrate particle size

The enzymatic hydrolysis of cellulosic materials is a heterogeneous reaction, with soluble enzyme attaching to solid cellulose and converting it into soluble sugars. The particle size of the substrate is an important parameter for the hydrolysis because it influences the contact between enzyme and substrate (Gan *et al.,* 2003). Wen *et al.,* (2004) reported that hydrolysis of animal manure for reducing sugar production using particles ranging from 590 - 350µm enhanced the glucose yield by 29%. However, further decrease in the particle size resulted in no significant change in glucose yield.

Recent works reported by Highina *et al,* (2006) shows that hydrolysis of rice straw for reducing sugar production and reducing the particle size from 425 - 75µm increases the glucose yield from 59% to 82% probably due to increase in surface area made available by rice straw for enzyme attack. However, decreasing the particle size further below 75µm resulted in no significant change in glucose yield.

2.6.4 Effects of pH

pH is the measurement of the gram of hydrogen ions per liter of a solution. Its scale varies from 0 for extreme acidity (1 gram – ion of hydrogen ions per liter) to 14 for extreme alkalinity (1 gram – ion of hydroxyl ion per liter). It is expressed as:

$$pH = \log \frac{1}{(H^{+})} \qquad 2.1$$

Where (H^{+}) is the hydrogen ion concentration in the solution.

Medium pH is an important parameter affecting growth and product formation. Most organisms will function over a pH range 3 – 4 pH units. Their maximum growth rate falls over 1 – $1^{1}/_{2}$ pH unit range. Because pH is so important, it is controlled in most fermentation either by means of a buffer or pH control system.

Various factors have been attributed to decrease ethanol fermentation. This includes pH of the fermentation medium and loss of sugar transport ability at high initial sugar concentration. (Salmon and Maurico, 1994).

Ekumankama *et al*., (1997) recorded an optimum pH of 4.5 – 5.0 for ethanol production by *S. cerevisiae*. This is in agreement with those of Adams and Flynn, (1982), and Morris and Sarad, (1990). In a region outside the optimum pH, the cells of S. cerevisiae were observed to be less tolerant to the environment and hence less active and less efficient in substrate utilization (Ekumankama *et al*., 1997).

2.6.5 Effects of temperature

Microbial growth and product formation are the result of a series of complex chemical reactions. Like all chemical reactions, they are influenced by temperature. Growth may be described by;

$$\frac{dx}{dt} = \mu x - \alpha x \text{ or } \frac{1}{x}\frac{dx}{dt} = \mu - \alpha \qquad 2.2$$

Where μ is the specific growth rate of the microorganism, α is dilution rate while $\frac{dx}{dt}$ is growth rate-death rate and x is the concentration of biomass.

Most microorganisms fall within three growth temperatures range. Those with a temperature for maximum growth rate of 20^0C are psychrophilic, those around 30 - 35^0C are mesophilic and those above 50^0C are thermophilic. Special enrichment and isolation programmes are often used to produce thermo tolerant strains that can grow at temperature above 40^0C (Kiran *et al*., 2000). In typical ethanol fermentation process, where exothermic metabolic reaction increases to above 40^0C, had been reported to have a consequential reduction in ethanol productivities (Kiran *et al.*, 2000). Where cooling is affordable, it adds to overhead cost of the ethanol production (Gera *et al.*, 1997)

The temperature of fermentation medium is one of the critical factors that have direct effect on many industrial processes. Low temperature was observed to reduce activities of enzymes, giving no significant impact on yield. In the same study when the temperature of the medium was increased above 30^0C, biosynthesis decreased. This was attributed to the fact that high temperature can cause denaturation of enzymes such as cellulase or accumulation of other by-products and enzyme catabolism repressed (Pandey *et al.,* 2000). In another study, optimum temperature for maximum ethanol production using cellulase in co-cultures was observed to be 30^0C (Verma *et al*., 2000).

2.7 Ethanol

Ethanol is a clear, colourless chemical compound made from the sugars found in crops such as maize, sugar beets, and sugarcane. In the 1850s, nearly 90 million gallons of ethanol were produced every year in the united states. At that time it was used as a fuel for lamps. It was also consumed as an alcoholic beverage. In 1862 the union congress put $2 per gallon excise tax on alcoholic beverages to help finance the civil war. The tax made ethanol too expensive to be used for lighting, so people started using kerosene and methanol instead (Keith, 2009).

In 1896, Henry Ford built his first automobile, the quadricycle, to run on pure ethanol. Ford designed his model T to run on a mixture of gasoline and ethanol. During World War I, ethanol use increased rapidly, not only as a fuel but in manufacturing of war materials also. The year 1919 brought prohibition and a denaturing process was developed which made ethanol poisonous and undrinkable. In the 1920s ethanol was replaced as a booster to gasoline by other products (Keith, 2009).

The prohibition ended in 1933, and ethanol production rose to 600 million gallons a year to meet the need of World War II. After the war, production once again decline because there were no more government contracts. Farmers began exporting grains formerly used to make ethanol to help feed countries whose agriculture had been destroyed by the war. Large supplies of cheap foreign oil made gasoline less expensive than ethanol.

In the 1970s, the US placed embargoes on gasoline suppliers from foreign sources, and interest in ethanol as an alternative fuel rose again. Concerns about global warming and dependence on foreign oil had caused interest in ethanol as an alternative fuel source to grow in recent years. In 2006, 112 ethanol plants, mostly in the Midwest, produced about 5 billion gallons of ethanol.

2.7.1 Uses of ethanol

Ethanol has found wide use both in industry and in the laboratory as a solvent and an extracting agent. It is useful as a fuel and in the preparation of pharmaceuticals and perfumes. It is used for the production of various liquors, acid, vanishes, and dyes, cosmetics, printing inks and plastics. The largest market of ethanol is as a beverage and partly in form of liquors (Cheremisinorff, 1979).

2.7.2 Ethanol as disinfectants

Like many chemical disinfectants, alcohols are generally considered to be non specific antimicrobials because of a multiplicity of toxic effect mechanism (Hugo and Russell, 1996). This has important implication for the spectrum, speed, and ultimately, overall effectiveness of ethanol as a disinfectant. The predominant mode of action appears to stem from protein coagulation/denaturation, with associated interference with cellular metabolism, disruption of cytoplasmic integrity and cellolysis (Mc Donnel and Russell, 1999).

2.7.3 Ethanol as domestic lighting agent

There is also extensive use of kerosene for lighting and cooking in less developed countries. Ethanol can have a role in reducing petroleum dependency in this area too. There is a potential for bio-ethanol from feed stocks grown locally replacing kerosene use in domestic lighting. A 50% (w/w) ethanol – water mixture has been reported in specially designed stoves and lantern for rural areas (Anil 2009).

2.7.4 Ethanol as transportation fuel

Ethanol is a very high octane fuel, replacing lead as an octane enhancer in gasoline and increases the antiknock capacity of the fuel, the higher the octane rating, the slower the fuel burns and the less likely the engines will knock (Chereminisinoff, 1979). Unlike gasoline, ethanol is biodegradable. It is used as alternative fuel when blended with gasoline. There are numerous advantages of blending ethanol with gasoline that emits lower quantities of carbon mono oxide (CO), nitrogen oxides (NOx), and hydrocarbon after combustion compared to that of gasoline alone. As a fuel, ethanol is the most commonly used additive to increase the octane rating and improve the emissions quality of gasoline (Addison and Hiraga, 2004). Because ethanol is made from crops that absorb carbon (IV) oxide and give off oxygen, it helps reduce the total volume of greenhouse gas emission (Farabee, 2009). Ethanol fuel is the same type of alcohol found in alcoholic beverages. It can be used as fuel, mainly as a bio-fuel alternative to gasoline and is widely used in cars in Brazil. It is easy to manufacture and process and can be made from very common crops such as sugar cane, maize grain and cellulosic materials, it is therefore an increasingly common alternative to gasoline in some parts of the world.

Ethanol can be mass produced by fermentation of sugar or by hydration of ethylene ($CH_2 = CH_2$) from petroleum and other sources. Currently, interest in ethanol mainly lies in bio-ethanol, produced from starch, cellulose or sugar in a wide variety of crops, but there has been considerable debate about how useful bio-ethanol will be in replacing fossil fuels in vehicles. Recent developments with cellulosic ethanol production commercialization may allay some of these concerns.

According to the International Energy Agency, cellulosic ethanol could allow ethanol fuels to play a much bigger role in the future than previously thought. Cellulosic ethanol offers promises as resistant cellulose in plant cell walls can be used to generate ethanol. Crops such as switch grass are also promising cellulose sources that can be produced in many regions of the United State (Mick, 2008).

2.7.5 Other uses of ethanol

Ethanol is used in manufacturing of surface coating, yeast growth medium, solvent for resins, fats fatty acids, oils, hydrocarbons. It is also used in direct food additives like preparation of acetic acids, acetaldehyde, and cleaning preparations, and in dyes and explosives (Robert, 1990). Mono–, di –, and tri – ethyl amines, produced by catalytic reaction of ethanol with ammonia, are a significant outlet for ethanol. In the synthesis of ethyl acrylate, the esterification of acrylic acid is a major use for ethanol.

2.7.6 Chemistry of bio-ethanol

Simple sugar (Glucose) is synthesized in plant by photosynthesis (Robert, 1990)

$$6CO_2 + 6H_2O + light \rightarrow C_6H_{12}O_6 + 6O_2 \qquad 2.3$$

During ethanol fermentation, glucose is decomposed into ethanol and carbon dioxide.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + heat \qquad 2.4$$

During combustion, ethanol reacts with oxygen to produce carbon dioxide water and heat.

$$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O + heat \qquad 2.5$$

Glucose itself is not the only substrate in the plant that is fermentable. The simple sugar fructose also undergoes fermentation. Three other compounds in the plant can be fermented often breaking them up by hydrolysis into the glucose or fructose molecules that compose them. Starches and cellulose are molecules that are strings of glucose molecules, and sucrose is a molecule of glucose bonded to a molecule of fructose.

Ethanol may also be produced industrially from ethene (ethylene). Addition of water to the bond converts ethene to ethanol.

$$CH_2 = CH_2 + H_2O \rightarrow CH_3CH_2OH \qquad 2.6$$

This is done in the presence of an acid which catalyzes the reaction, but is not consumed (Robert, 1990).

2.7.7 Properties of ethanol

The physiochemical properties of alcohols are associated with their chemical structures. Factors such as water solubility or miscibility, solvency, surface tension, vapour pressure and protein denaturing vary with chemical structures and help explain corresponding variation in biological activity (Morrison, and Boyd *et al*., 1972). The properties are shown in Table 2.3.

Table 2.3: Physiochemical Properties of Ethanol

Molecular formula	C_2H_5OH
Molar mass	46.07gmol^{-1}
Appearance	Colourless liquid
Density	0.789/cm^3
Melting point	-114.3^{0}C
Boiling point	78.4^{0}C
Solubility in water	Miscible
Acidity (pka)	15.9
Viscosity	1.200cp (20^{0}C)
Dipode moment	1.69D (gas)

Adapted from Morrison and Boyd (1972)

2.7.8 Bio-ethanol production process

Bio-ethanol from entirely microbial source is the most economical and widely used way of producing ethanol (Kiran *et al*., 2000). The biological way of producing ethanol from renewable biomass had received considerable attention in recent years (Seeman *et al.*, 2007).

The basic steps for large scale production of ethanol are microbial (yeast) fermentation of sugar, distillation, and dehydration. Prior to fermentation, some crops require saccharification of cellulose which is called cellulolysis. (Verma *et al*., 2000).

2.7.9 Process description of simultaneous saccharification and fermentation (SSF)

New bioprocess technologies had been developed that have the potential to lower the cost of ethanol production and improve the competitiveness of ethanol as a fuel additive (Bothas and Saha, 1997) one of these technologies is the simultaneous saccharification and fermentation (SSF) technique.

This process has been found to be superior to saccharification and subsequent fermentation (Krishna *et al*., 2001). Due to the rapid assimilation of sugar by the yeast, several methods had been proposed to overcome the end-product inhibition resulting from the rapid accumulation of sugar during hydrolysis. These include the use of SSF by co-culturing two organisms with synergistic relationship (Abouzied and Reddy, 1986; Cao *et al.*, 1996).

In simultaneous saccharification and fermentation (SSF), rapid fermentation of sugar to ethanol by *S. cerevisiae* had been found to keep the sugar concentration low enough to prevent feedback inhibition of cellulase activity observed in monoculture of *Aspergillus niger* (Abouzied and Reddy, 1986). In their studies, they observed that monoculture of *A. niger* tended to favour higher cell mass production, whereas in the co-culture, most of the substrate carbon is utilized for ethanol production. The process involved the enzymatic hydrolysis of cellulose to fermentable sugar and their conversion to ethanol in the same fermenter, thereby preventing the inhibiting effects of sugar on cellulase activity (Abouzied and Reddy, 1986). The conversion of cellulose biomass to useful substances such as liquid fuels through the enzymatic hydrolysis of polysaccharides, into fermentable sugar, still suffers severe problems that are economical and technical in nature. The economic problems consist exclusively of cost of production and purification of hydrolytic enzymes. It has been suggested therefore that any means that is capable of reducing this cost significantly would have a dramatic effect on the cost of production of useful substance, such as ethanol from renewable resources (Taniguchi *et al.*, 1983).

The process was found to increase yield of ethanol not only by eliminating inhibition effects, but also by eliminating the need for separate reactors for saccharification and fermentation (Deshpande *et al.*, 1983).

2.7.10 Fermentation

Fermentation is a series of chemical reactions that converts sugar to ethanol. The fermentation reaction is caused by yeast or bacteria, which utilize or breakdown certain sugar to ethanol and carbon (IV) oxide. The simplified fermentation reaction equation for the 6 – carbon sugar, glucose is

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad 2.7$$

Ethanol toxicity to yeast limits the ethanol concentration obtainable by brewing. The most ethanol tolerant strains of yeast can survive up to approximately 15% ethanol by volume (Mills and Eckluand, 1987).

Ethanol is produced by microbial fermentation of the sugar. Two major components of plants, starch and cellulose are both made up of sugars, and can in principle be converted to sugar during saccharrification. Currently, only the sugar and sugar portions can be economically converted. However, there is much activity in the area of cellulosic ethanol where the cellulose part of a plant is broken down to sugar and subsequently converted to ethanol (Krishna et al., 2001).

2.7.11 Distillation

Water must be removed for ethanol to be usable as a fuel, active disinfectant, solvent and extract. Most of the water is removed by distillation, but the purity is limited to 95 -96% due to formation of low boiling water/ ethanol azeotrope (Synder and Kalf, 1994).

In another method to obtain absolute alcohol, a small quantity of benzene is added and the mixture is then distilled. Absolute alcohol is obtained in the third fraction, which distils over at 78.3^0C (Morrison and Boyd, 1972). This is because a small amount of the benzene used remains in the solution, absolute alcohol produced by this method is not suitable for consumption as benzene is carcinogenic (Synder and Kalf, 1994).

2.7.12 Dehydration

There are basically five dehydration processes to remove the water from an azeotropic ethanol/water mixture. The first process used in many early fuels ethanol plants, is called azeotropic distillation and consists of adding benzene or cyclohexane to the mixture. When these components are added to the mixture, they form a heterogeneous azeotropic mixture which when distilled produces anhydrous ethanol in the column bottom, and vapour mixture of water and cyclohexane/benzene (Synder and Kalf, 1994). When condensed, this becomes a two phase liquid mixture. Another early method called extractive distillation consists of adding a ternary component which will increase ethanol relative volatility, when the ternary mixture is distilled, it will produce anhydrous ethanol on the top stream of the column (Synder and Kalf, 1994). New process uses molecular sieves to remove water from fuel ethanol. In this process, ethanol vapour under pressure passes through a bed of molecular sieve beads. The beads pores are sized to allow absorption of water excluding ethanol. After a period of time, the bed is regenerated under vacuum to remove the absorbed water. Two beds are used so that one is available to absorb water while the other is being regenerated. (Synder and Kalf, 1994).

CHAPTER THREE

MATERIALS AND METHOD

3.1 List of Materials and Equipment

The materials used include:

1. Sodium hydroxide pellets (Analar grade) M&B Ltd Dagenham England.

2. Sodium acetate (Analar grade) BDH Laboratory reagent Poole England.

3. Acetic Acid (Analar grade) BDH Laboratory reagent Poole England.

4. Tetraoxosulphate (vi) acid (Analar grade) M&B Ltd Dagenham England.

5. Aqueous Ammonia Solution (Analar grade) BDH Laboratory reagent Poole England.

6. Distilled water.

7. Magnesium Sulphate (Analar grade) BDH, England.

8. Ammonium phosphate (Analar grade) BDH, England.

9. Potato destrose ager (PDA) (Analar grade).

10. Yeast (*Saccharomyces cerevisiae*) obtained from Nigeria Breweries Kaduna

11. Potassium dichromate (Analar grade).

List of Equipment

The equipment used includes:

1. Weighing balance (ae Adam pw 184).
2. Cooled incubator (Gallenkamp model).
3. Digital pH meter (Jenway 3150).
4. 20ml Sample bottles.
5. Immersion Thermometer.

6. Oven (Gallenkamp model).
7. Beer analyzer (Alcohol meter) Anton par model from Nigerian Breweries Kaduna.
8. Magnetic hot pate stirrer regulator (Gallen kamp model).
9. Laboratory mill (Thomas wite model 4).

3.2 Culturing of *Aspergillus Niger*

3.2.1 Preparation of culture medium (potato dextrose agar, PDA)

PDA medium was used in culturing the micro-organism. It was composed of 200mg-peeled Irish potato, 20g of dextrose (analytical grade) and 20g of Agar-agar powder (Analytical grade). The Irish Potato was cut into small pieces and boiled in 500ml of distilled water for about 20-25minutes. The extract was filtered through a muslin cloth into a 100ml flask, after which dextrose and agar were added and reboiled for 10 minutes to obtain complete dissolution. Distill water was added to the potato extract-dextrose – agar solution and made up to 1 litre. The medium was corked properly and secured with aluminium foil paper after which it is sterilized in an autoclave (Griffing and George 31-417 model) for 20 minutes at a pressure of 15psi and temperature of 121^{O}C to prevent contamination. The content of the flask was cooled to 45^{O}C and 5ml of Streptomycin Sulphate solution was added to prevent the growth of bacteria (Alexoponlus and Benke, 1984).

3.2.2 Pour plating

Petri dishes were washed with detergent and rinsed with tap water. Thereafter they were arranged in a canister and dried in an oven (Gallenkamp model) for 2 hours. The mouth of the conical flask containing the medium was flame sterilized to destroy surface micro organism and then the medium was poured into petri dishes, covered and left to solidify (Alexoponlus and Benke, 1984).

3.2.3 Isolation of organisms

Maize grains were surface sterilized with sodium hypochlorite and then rinsed with distilled water. This was done to destroy secondary pathogens. The maize grains were then inoculated into the petri dishes containing the PDA medium using flame sterilized forceps. The petri dishes were incubated at 28-30^{O}C for 3 days and observed for growth.

3.2.4 Identification of fungus

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

3.2.5 Sub-culturing

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

3.3 Sample Collection/Preparation

The elephant grass (*Pennisetum purpureum*) stem was obtained from National Animal Production Research Institute (NAPRI) ABU Zaria, shown in figure 3.1. It was dried to a constant weight in an oven (Gallenkamp model) at a temperature of $105^{O}C$ for 48hours, it was then milled using a Laboratory mill (Thomas wite model 4). A screen analysis was carried out with various sieve sizes to obtain desired particle sizes.

(a) (b) (c)

Figure 3.1: (a) Elephant grass (b) Stem (c) Leaf

3.4 Pre-Treatment of Substrate

Various pretreatment methods like acid, alkaline pretreatment and boiling were carried out to determine the best pretreatment method. The various pretreatment methods and operating conditions are shown in Table 3.1. For each pre-treatment method, 10 grams of the substrates was added to 100mls of solution.

After pretreatment the samples were filtered and washed repeatedly with distilled water until pH of 7 was attained. The elephant grass was then dried in an oven at $50^{O}C$ to a constant dry weight for 48hours.

Table 3.1: Operating Conditions for the Pre-treatment Methods

PRETREATMENT METHODS	TIME (minutes)	TEMPERATURE (^{o}C)	SOLID/LIQUID RATIO
1M H_2SO_4	90	100	1:10
0.1M H_2SO_4	90	100	1:10
1M NaOH	90	55	1:10
0.2M NaOH	90	55	1:10
Boiling	25	50	1:10
3M NH_4OH	30	90	1:10

Adopted from Parveen *et al* (2009

3.5 Substrate Analysis

The substrate analysis of the various pretreated and the untreated sample were determined by Van Soest System of feed analysis (Galyean, 2010).

3.6 Simultaneous Saccharification and Fermentation (SSF) of Elephant Grass

In a typical run 100ml of 0.1M sodium acetate buffer solution was poured into 250ml Erlenmeyer flask, 0.2g of *Aspergillus niger*(crude enzyme), 0.5g of S*accharomyces cerevisiae* (brewer's yeast),2g of untreated or treated elephant grass were added and 0.1g of $MgS0_4$, 0.2g of $(NH_4)H_2PO_4$ were added as nutrient.

The flask was corked properly, sealed with aluminum foil paper and incubated at $30^{O}C$ for 5days in an incubator. All the experiments were performed in triplicate. Samples were withdrawn everyday for analysis. The ethanol concentration was determined using an Anton par beer analyzer (Alcohol meter). The residual glucose concentration was also determined using Trinder's method (clinical chemistry lab manual).

In separate runs, the effect of pretreatment, substrates concentration, particle size, cell loading, reaction temperature reaction time and medium pH on the extent of fermentation were studied as follows:

3.6.1 Effect of substrate concentration

This was investigated by carrying out the simultaneous saccharification and fermentation at 10g/l, 20g/l, 30g/l, 40g/l and 50g/l substrate concentration, while other reaction conditions were kept constant (Highina et al.,2006).

3.6.2 Effect of substrate particle size

The particle sizes of the untreated elephant grass were selected by passing it through different sizes of standard sieves. Fractions of particle sizes of 53-106µm, 106-150µm, 150-250μm, 250-300μm and 300-425μm were then fermented and analyzed for ethanol concentration.

3.6.3 Effect of *Aspergillus niger* concentration

Fermentation was carried out at different *A.niger* concentration of 0.2%(w/v), 0.4%(w/v), 0.6%(w/v), 0.8%(w/v) and 1.0%(w/v) at constant substrates concentration and constant yeast concentration (Highina et al., 2006). Other conditions were identical to the normal fermentation conditions. Ethanol concentrations were measured with time.

3.6.4 Effect of yeast concentration

Fermentation was carried out at different yeast concentration of 0.5%(w/v), 1.0%(w/v), 1.5%(w/v), 2.0%(w/v) and 2.5%(w/v) at constant *Aspergillus niger* concentration. Other conditions were identical to the normal fermentation conditions. Ethanol concentration was measured with time.

3.6.5 Effect of temperature

The experiment was carried out at different temperature of 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C using the incubator for 5days at different temperatures. Other fermentation parameters were held constant (Omemu *et al*,2005).

3.6.6 Effect of pH

This was investigated by carrying out the fermentation at initial pH adjusted to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 using Sodium acetate buffer (Omemu *et al*,2005)

The methodology for the production of ethanol from elephant grass stem is shown in Figure 3.2

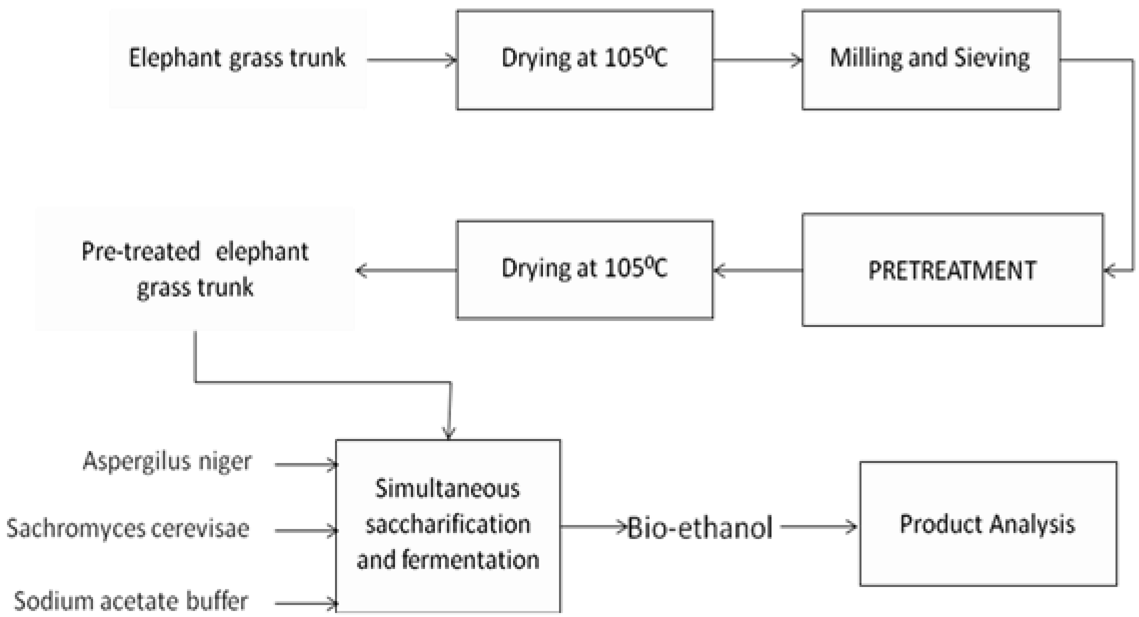


Figure3.2: Methodology for Bio-Ethanol production

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents the results of this work, which includes: determination of suitable pre-treatment method, simultaneous saccharification and fermentation and optimizing cultural conditions under varying process parameters (i.e. substrate concentration, cell concentration, temperature, particle size and pH).

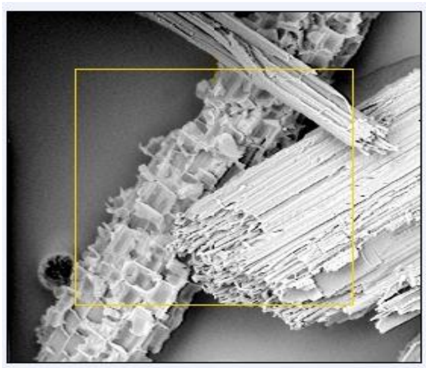
4.2 Determination of suitable Pre-treatment Method

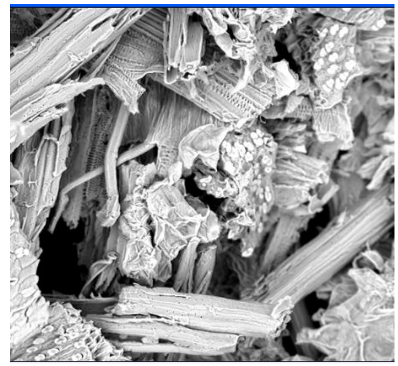
Table 4.1 shows the substrate analysis for the pretreated samples. The result showed that 1M sodium hydroxide pretreatment gave the lowest lignin content of 7.93% and the highest cellulose content of 64.69%. This implies that the substrate has sufficient cellulose to be hydrolyzed to glucose and subsequently to ethanol. This was corroborated by the SEM analysis, plate 4.1 which compared the untreated elephant grass with the sodium hydroxide pretreated elephant grass. It shows the fact that sodium hydroxide pretreatment of the lignocellulosic materials caused swelling, leading to an increase in internal surface area, decrease in crystalinity and disruption of the lignin structure. The result also showed low hemicellulose content of the 1M tetraoxosulphate (vi) acid pretreated sample. This implies that the hemicellulose was hydrolysed by the acid to simple sugar.

However when simultaneous saccharification and fermentation was carried out on both the sodium hydroxide pretreated and untreated sample, the untreated gave a higher yield of 83% while the pretreated sample gave a yield of 42%. The sodium hydroxide pretreated and untreated sample was tested for glucose using the trinder's method. It was discovered that the untreated sample has about 35% glucose, while there was no presence of glucose in the sodium hydroxide pretreated sample. This accounted for the higher yield of ethanol from the untreated sample. Agbodike et al (2013), also reported lower yield of ethanol from pretreated sample of elephant grass stem, when the same concentration of the grass and glucose were fermented under the same reaction condition respectively. This could be due to loss of fermentable sugar during the pretreatment washing process. However when the residence time of pretreatment was increased from 1.5hours to 8hours, as shown in Table 4.2, the sodium hydroxide pretreated sample gave a yield of 86%. This could be as a result of the increase in cellulose content. However, considering the cost and time of pretreatment, the untreated sample was used in this work.

Table 4.1: Substrate analysis of each pretreatment method

PREATREMENT METHODS	% LIGNIN	% HEMICELLULOSE	% CELLULOSE
1M H_2SO_4	16.09	2.82	60.28
0.1M H_2SO_4	15.31	15.33	56.62
1M NaOH	7.93	15.43	64.69
0.2M NaOH	10.14	23.88	58.61
BOILING	13.53	19.28	52.14
3M NH_4OH	15.47	18.40	52.80
UNTREATED	16.47	17.92	49.12





(a) (b)

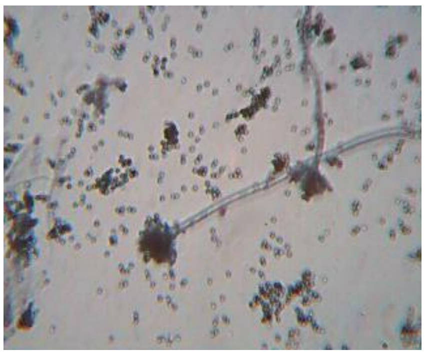
Plate 4.1: SEM analysis for (a) untreated and (b) 1M NaOH pre-treated elephant grass stem *500

Table 4.2: Substrate analysis for 1M NaOH pre-treatment at varying residence time.

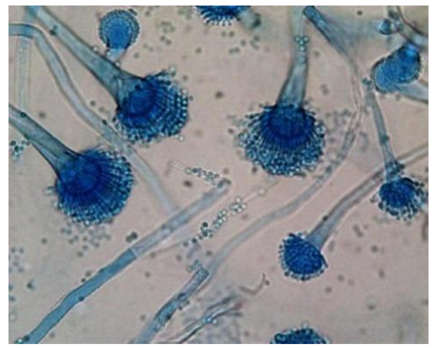
Residence time (hours)	% Lignin	% Hemi-cellulose	% cellulose
2	7.93	15.43	64.69
4	7.65	15.20	65.30
6	7.25	14.69	65.50
8	7.01	14.25	65.80
10	7.01	14.23	65.80

4.3 Isolation of *Aspergillus Niger*

The growth of *Aspergillus niger* was monitored on potato dextrose agar (PDA) at 30^{O}C. The spores initially observed as white turned to yellow which later become dense dark brown to carbon black mass. A sample of the black colony was sub-cultured ten times, which produced a fairly homogenous black colony. When viewed under a microscope (Olympus venon –T microscope model) its morphological structure presented in plate 4.2a compared with the standard structure of *Aspergillus niger* given by Robert and Ellen (1988) shown in plate 4.2b.



(a)



(b)

Plate 4.2 (a) Morphological structure of *Aspergillus niger *250* and (b) standard morphological structure of *Aspergillus niger *300 (Robert and Ellen, 1988)*

4.4 Simultaneous Sacharification and Fermentation of Elephant Grass Stem

4.4.1 Fourier Transform Infrared Analysis (FTIR)

The qualitative determination of ethanol was done using the FTIR spectrophotometer, which works by exciting chemical bonds with infrared light and is best for identification of organic materials. The different chemical bonds in this excited state absorb the light energy at frequencies unique to the various bonds. The wave number of the peak tells what types of bonds are present. The result is presented in Figure 4.1, which showed the peaks at which the hydroxyl (O-H) bond and the carbon hydrogen (C-H) bond is formed.

The O-H bond is present at a wavelength of 3433.41cm^{-1} while the C-H bond is present at a wavelength of 2090.91cm^{-1}

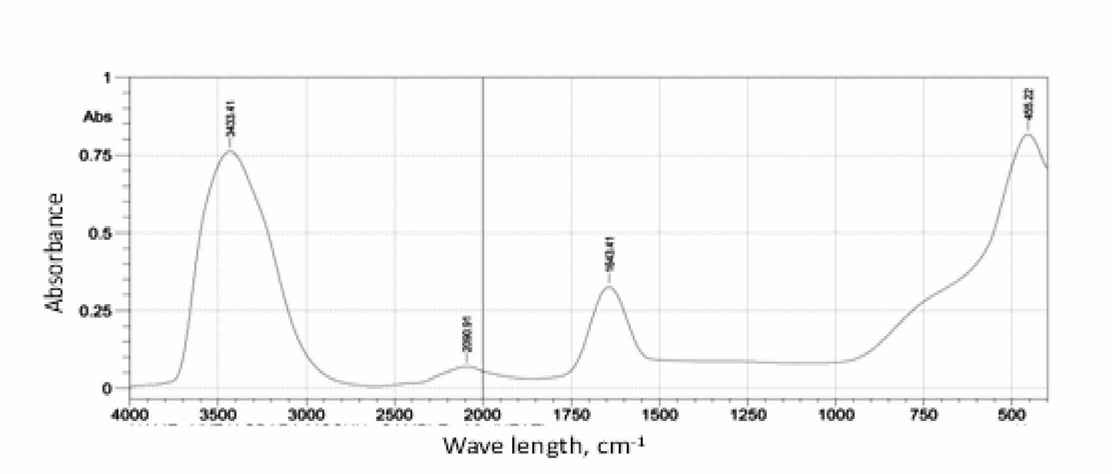


Figure 4.1: FTIR analysis result for ethanol

4.4.2 Effect of pre-treatment on ethanol yield

The experiment was done at temperature of 35^{0}C, pH of 5.0, substrate concentration of 30g/l, A.niger/yeast ratio 0.6/1.5%(w/v), and particle size 250- 300µm. Figure 4.2 shows the effect of pretreatment on ethanol yield. It can be seen from the figure that at 72 hours of fermentation time, 83% and 86% yield were attained when untreated and NaOH pretreated elephant grass were fermented respectively. This could be as a result of the presence of soluble sugar in the untreated sample, which was lost during pre-treatment.

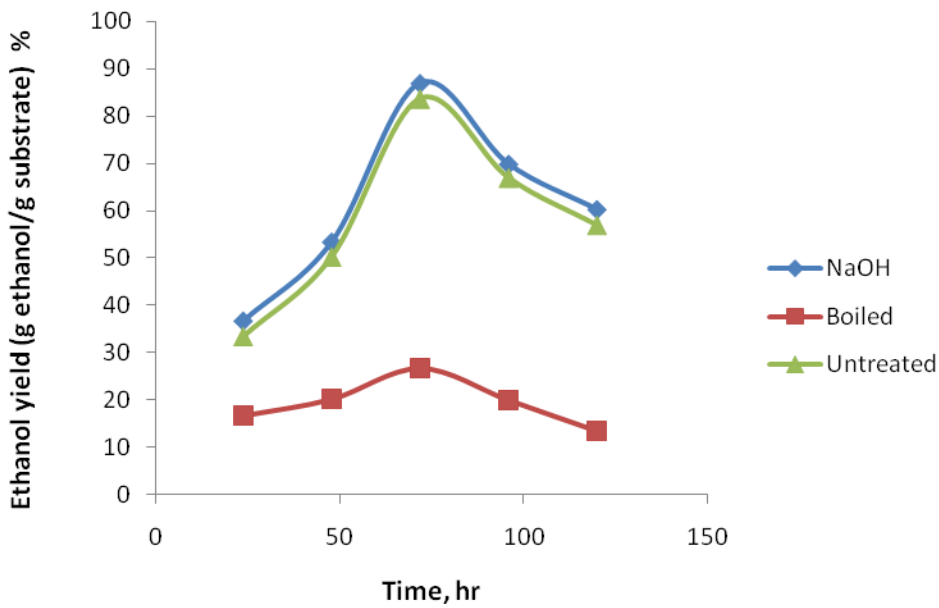


Figure 4.2: Effect of pre-treatment on ethanol yield

4.4.3 Effect of substrate concentration on ethanol yield

The effect of substrate concentration on ethanol yield was studied using elephant grass stem as substrate by carrying out the experiment at different initial substrate concentrate of 10g/l, 20g/l, 30g/l, 40g/l and 50g/l. Other reaction conditions kept constant are temperature 30^0C, pH 5.0, A.niger/yeast ratio 0.6/1.5%(w/v), and particle size 150-250µm. The result is shown in Figure 4.3. The result showed that as substrate concentration increases from 10g/l to 30g/l ethanol yield increased to a maximum of 78.1% after 72 hours of fermentation. Further increase in substrate concentration up to 50g/l resulted in low ethanol yield. The decrease in ethanol yield beyond the optimum concentration of 30g/l could be that at substrate concentration higher than the optimum, there is product inhibition. This implies that the ethanol produce inhibits the activity of the yeast, hence the drop in ethanol yield.

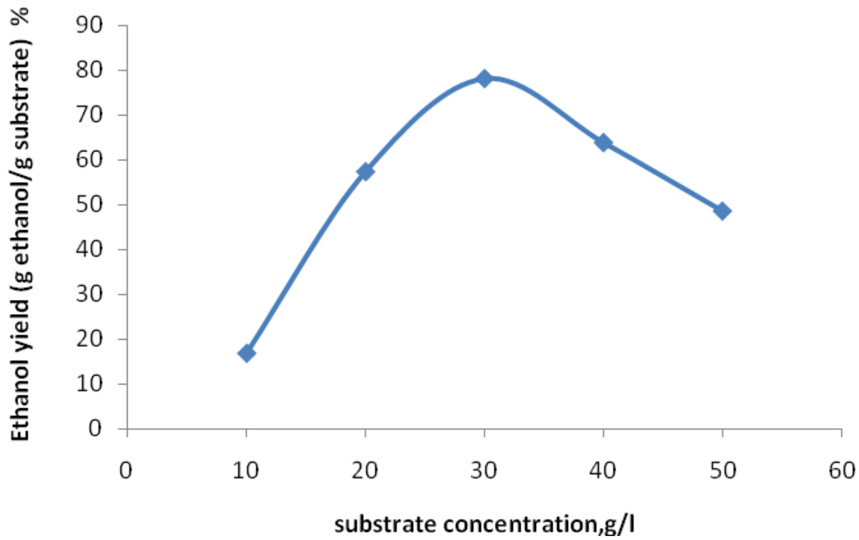


Figure 4.3: Effect of substrate concentration on ethanol yield

4.4.4 Effect of substrate concentration on residual sugar

At fixed *A.niger* and yeast concentration, the effect of substrate concentration on residual sugar was investigated. The result is presented in Figure 4.4, which showed that as the substrate concentration increases from 1-3 % low residual sugar was observed, which corresponds to increase in ethanol yield in figure 4.2. While substrate concentration greater than 3 % shows high residual sugar and decrease in ethanol yield. Similar results had also been observed for other materials such as animal manure, and sugarcane bagasse (Wen et al, 2004, Manonmani and Streekantiah, 1987).

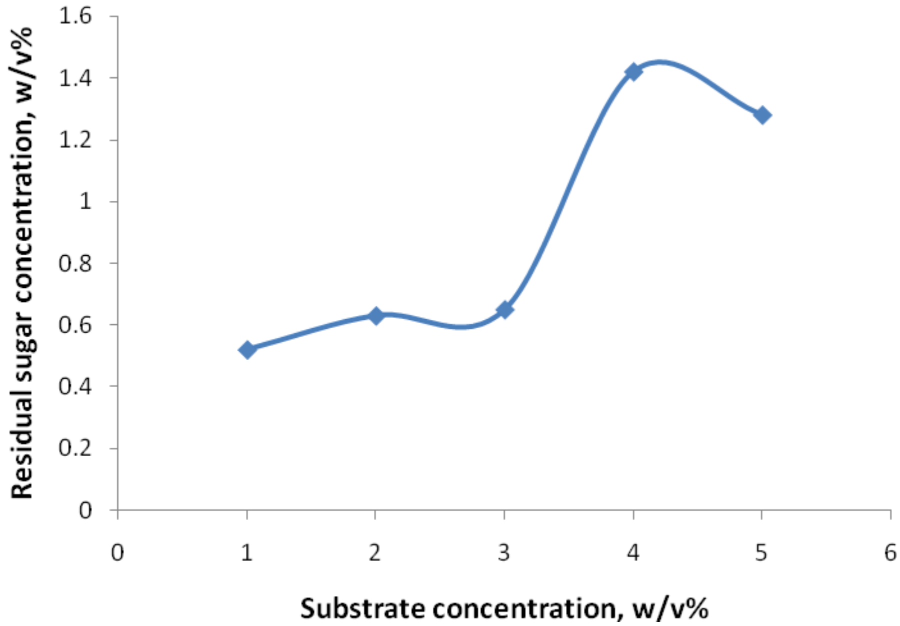


Figure 4.4: Effect of substrate concentration on residual sugar

4.4.5 Effect of temperature on ethanol yield

The following fermentation parameters were kept constant: pH 5.5, 3% w/v substrate concentration, 150-250µm particle size and A.niger/yeast ratio 0.6/1.5%w/v. The effect of temperature on ethanol yield was then studied by at different incubation temperature of 25^{O}C, 30^{O}C, 35^{O}C, 40^{O}C and 45^{O}C. The result is shown in Figure 4.5 which showed an optimum ethanol yield of 77.6% at 35^{O}C after 72hours of fermentation. Lowest ethanol yield of 28.1% was observed when the temperature increased to 45^{O}C. This shows that the optimum temperature is 35^{O}C and might be due to the fact that enzyme activity and other chemical reaction in the cells are favored at that temperature. Badal *et al* (2005) also reported maximum *E.coli* activity on wheat straw at 35^{O}C, while Jiayi and Lee (2010) reported optimum ethanol yield from paper sludge using *Z.mobilis* and *S. cerevisae* at 37^{O}C.

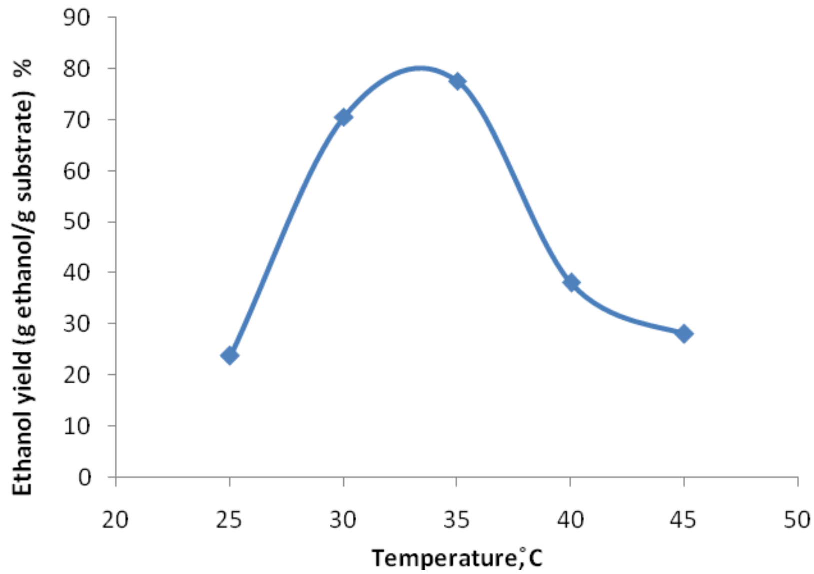


Figure 4.5: Effect of temperature on ethanol yield

4.4.6 Effect of temperature on residual sugar

Effect of temperature on the hydrolysis of elephant grass is shown in Figure 4.6. The result showed that low glucose concentration of 6.5g/L was observed at 35^{O}C which was the temperature at which high ethanol yield was recorded. This implies that more of the glucose was converted to ethanol at that temperature after 72hours of fermentation. High glucose concentration of 18.9g/L was recorded at 45^{O}C. This could be that glucose formation is favored at that temperature while yeast is less active at that temperature. Highina *et al* (2006), also reported optimum glucose yield at 45^{O}C, in hydrolysis of rice straw by *Aspergillus niger*.

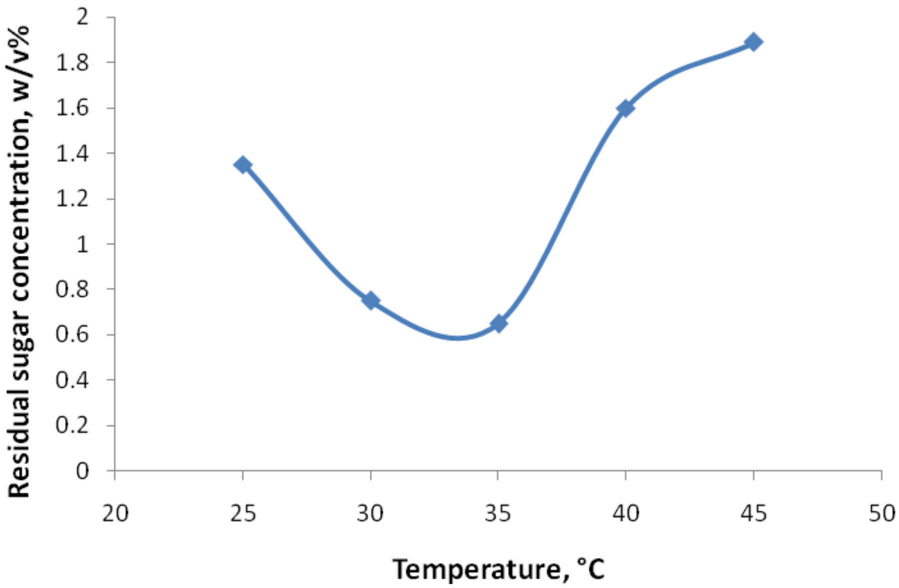


Figure 4.6: Effect of temperature on residual sugar

4.4.7 Effect of particle size on ethanol yield

The result of particle size effect on fermentation of elephant grass at pH of 5.0, temperature of 30^{0}C, substrate concentration of 3% w/v and A.niger/yeast ratio 0.6/1.5%w/v is presented in Figure 4.7. It can be seen from the result that as the particle size increases from 53µm to 300µm ethanol yield increases from 24.9% to 80.1%. Further increase in particle size to 425µm, results in decrease of ethanol yield. This implies that as the particle size increases beyond 300µm surface area available for enzymatic attack decreases. The low yield recorded between 53µm to 250µm is contrary to expectation, and could be that the cellulose content of the smaller particle size was lost to the larger particle size i.e.300µm-425µm during the screen analysis.

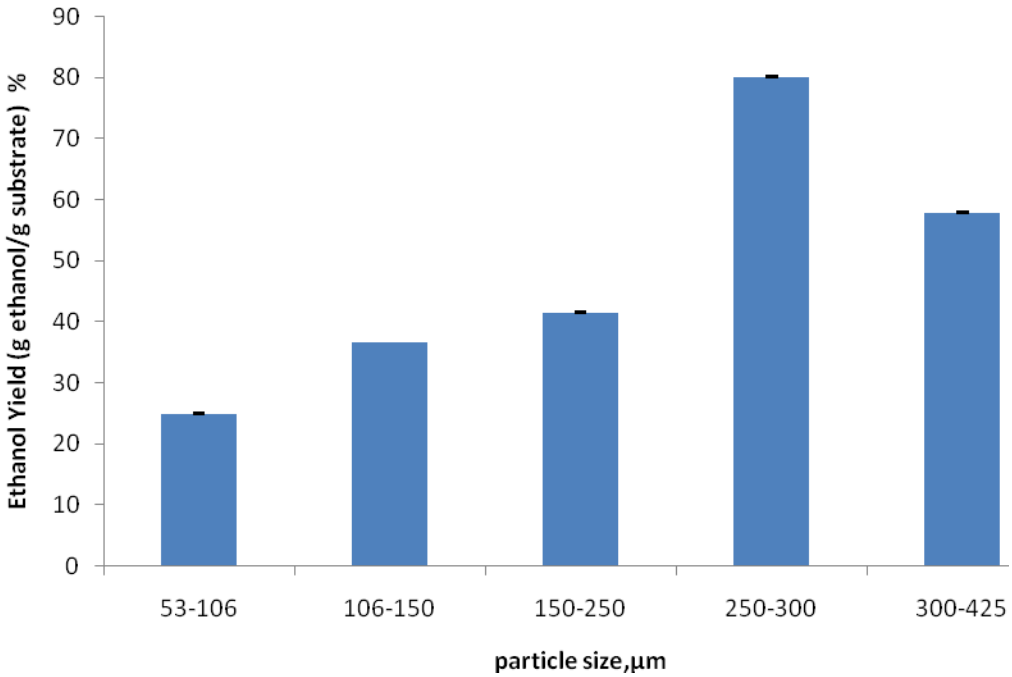


Figure 4.7: Effect of particle size on ethanol yield

4.4.8 Effect of particle size on residual sugar

The effect of particle size on residual sugar concentration is shown in Figure 4.8. The result showed that low glucose concentration of 5.6g/L was observed in the range of 250-300µm. This represented the range that optimum ethanol yield was recorded after 72hours of fermentation. This implies that more of the glucose was converted to ethanol, hence the low glucose concentration.

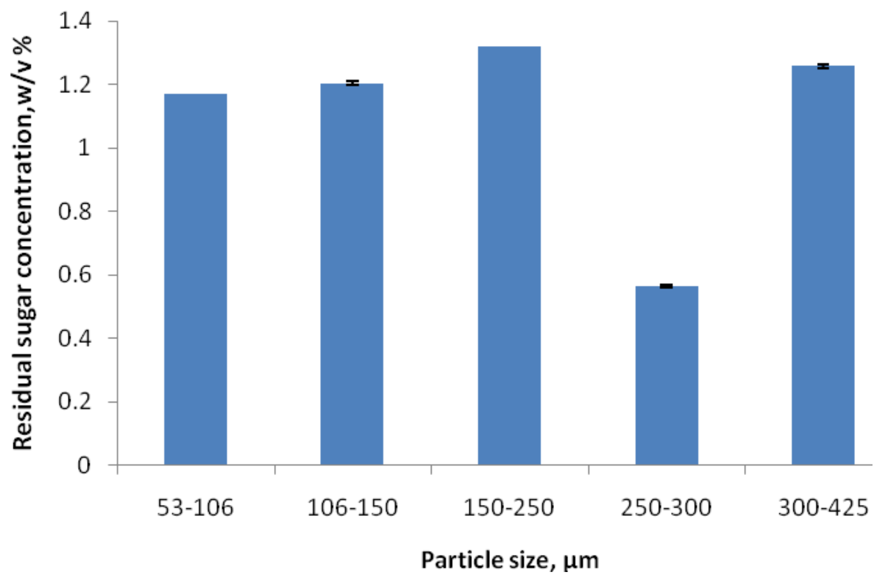


Figure 4.8: Effect of particle size on residual sugar

4.4.9 Effect of pH on ethanol yield

The effect of pH on ethanol yield was investigated by conducting the fermentation at different controlled pH of 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. All other fermentation conditions kept constant were temperature 30^{0}C, 3% w/v substrate concentration, 106-150µm particle size and A.niger/yeast ratio 0.6/1.5% w/v. The result is shown in Figure 4.9. As the pH increased from 3.5 to 5.5, the ethanol yield increased to a maximum of 79.8% at pH of 5.5. Further increase in pH to 6.5 resulted in decrease in the yield of ethanol. The decrease in ethanol yield may be due to the fact that enzymes are more active in mildly acidic medium (pH of 4.5-5.0 for *A. niger* cells and pH of 5.0-5.5 for yeast cells), hence the increase in pH of the medium away from the more acidic medium result in increase in the yield of ethanol from pH of 3.5 to 5.5. But there was decrease in the yield of ethanol as the pH tends towards neutral medium. The reported pH tolerance range for yeast is 3.5-6.5 while that of *A. niger* is 3.5-8.0 (Rezael et al 2009).

Also metabolic processes are generally temperature and pH dependent. Similar result was reported by Ohgren *et al*, (2007) in simultaneous sacharification and fermentation of corn stover in which the highest ethanol concentration was obtained at pH 5.5

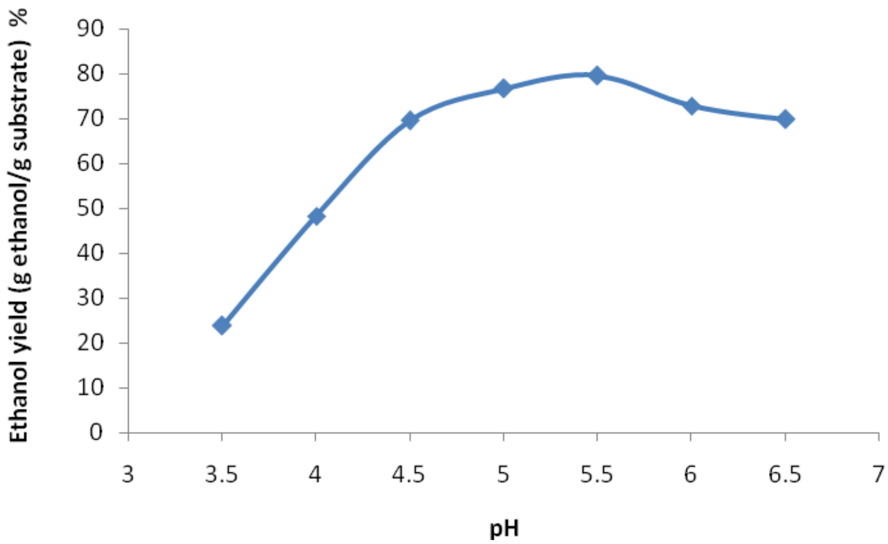


Figure 4.9: Effect of pH on ethanol yield

4.4.10 Effect of pH on residual sugar

The effect of pH on residual sugar concentration is shown in Figure 4.10; the pH of 4.5 gives the maximum glucose concentration of 9.03g/L after 72hours of fermentation. This is in agreement with the result obtained by Tengborg *et al.*, (2001) in which the maximum glucose yield was obtained at a pH of 4.8. Minimum glucose concentration of 6.1g/l was observed at pH 5.5. This is because yeast is more active at this pH, therefore, more of the glucose was converted to ethanol, hence the low glucose concentration. It was observed that glucose concentration increases at pH 6 - 6.5. This implies that A.niger activities also likely increase at this pH.

The rate of enzyme reaction is influenced by pH because enzyme is a protein which consists of amino acids residues. These residues possess basic, neutral or acidic groups which can be positively or negatively charged at any given pH. Also an enzyme is catalytically active when the amino acid residues at the active site each possess a particular charge. Therefore, the fraction of the catalytically active enzyme depends on the pH (Lee, 1992).

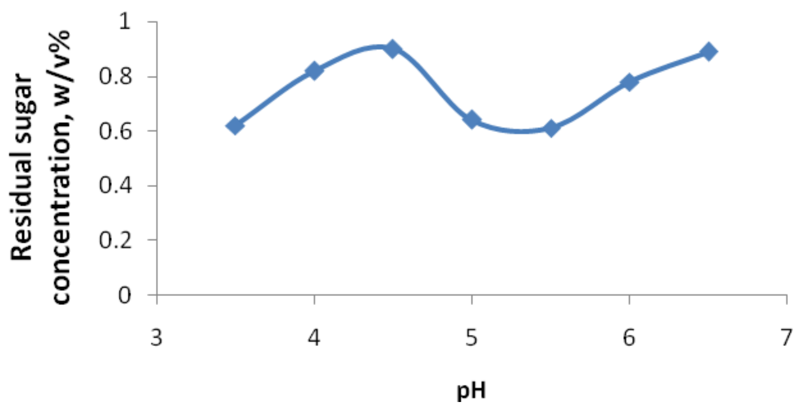


Figure 4.10: Effect of pH on residual sugar

4.4.11 Effect of cell loading

Two cells *Aspergillus niger and saccharomyces cerevisiae* (brewer's yeast) were used in the fermentation. To investigate the effect of cell loading, *Aspergillus niger* concentration was kept constant while the yeast concentration was varied. Similarly yeast concentration was kept constant while the *Aspergillus niger* concentration was varied, this was done to get the optimum *Aspergillus niger*/yeast ratio.

4.4.11.1 Effect of *Aspergillus niger* concentration on ethanol yield

At fixed yeast concentration and other fermentation conditions kept constant, the effect of *Aspergillus niger* concentration on ethanol yield was investigated. The result is presented in Figure 4.11; the result shows that as *A. niger* concentration increases from 0.2% w/v to 0.6% w/v ethanol yield increases from 66.9% to 82.4% after 72hours of fermentation. Subsequent increase in *A. niger* concentration beyond 0.6% w/v resulted in decrease of ethanol yield. This may be due to the fact that as more cells were introduced and substrate concentration was not increased proportionally, the glucose produced was being used up by the cells for survival, hence resulting in low ethanol yield.

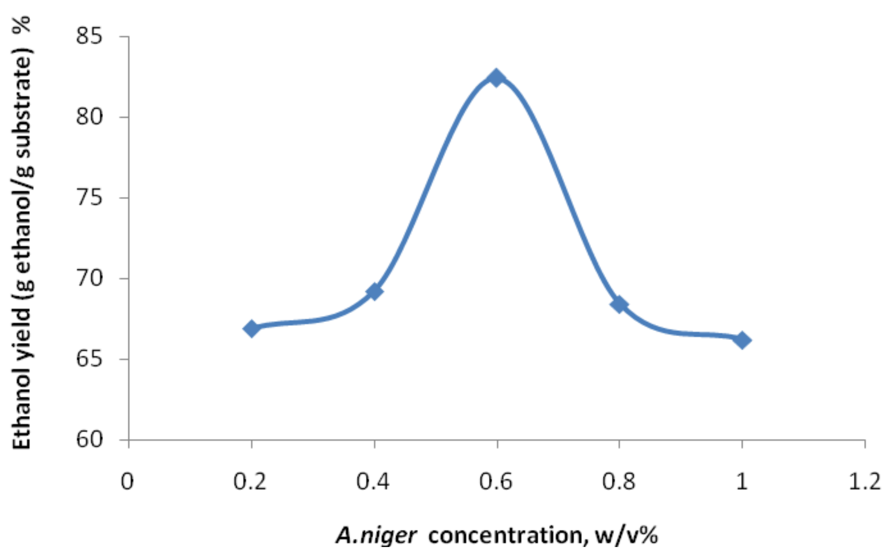


Figure 4.11: Effect of *A. niger* concentration on ethanol yield

4.4.11.2 Effect of yeast concentration on ethanol yield

A. niger at fixed concentration keeping other fermentation conditions constant was used. The effect of yeast concentration on ethanol yield was investigated. The result is shown in Figure 4.12 which showed that as yeast concentration increases from 0.5%w/v to

1.5%w/v ethanol yield increase from 36.6% to 83.6% after 72hours of fermentation. Further increase in yeast concentration beyond 1.5%w/v resulted in decrease of ethanol yield. This may be as a result of more yeast consuming the limited glucose for self sustenance, thereby resulting in low yield of ethanol.

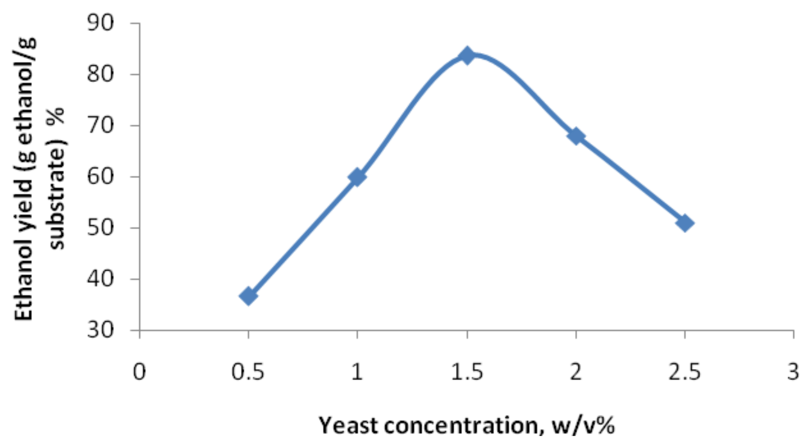


Figure 4.12: Effect of yeast concentration on ethanol yield

4.5 Kinetic Parameters

The development of the kinetic parameters is based on the following observations and assumptions

1. The cells were introduced at their exponential growth phase
2. Ethanol was the main product of interest
3. The reaction was viewed as enzyme been secreted by the cells into the solution, enabling the overall system to be treated as that of enzyme- substrate kinetics.
4. Volume of reactant was assumed to be constant during the fermentation period.

Table A7.1 in appendix VII shows the results obtained from the slopes taken from figures A8.1-A8.5 in appendix VIII. The slopes at 24hours represent the initial rate. Figure 4.13 shows the plot of initial rate versus initial substrate concentration

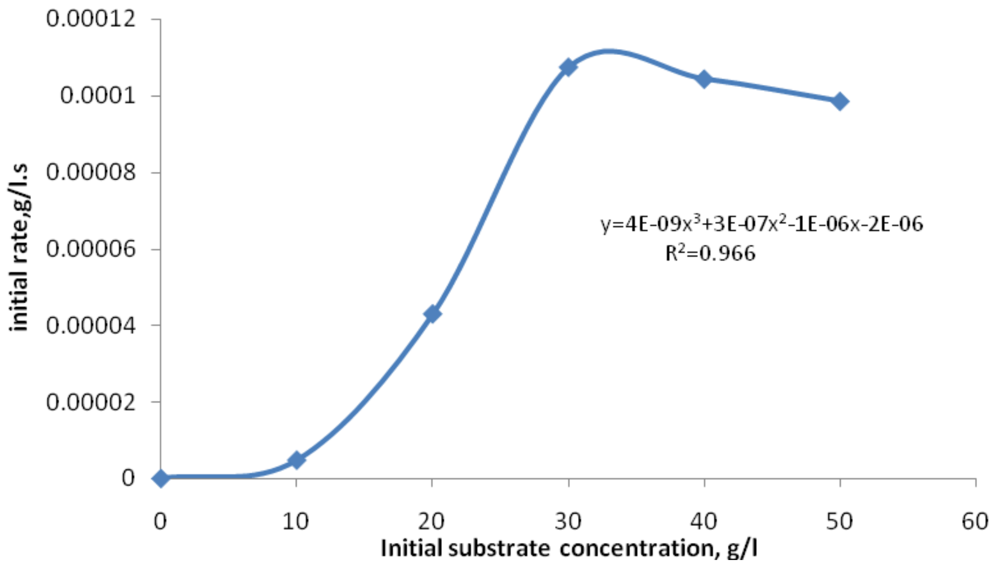


Figure 4.13: Initial rate versus initial substrate concentration

The Michaelis Mentens kinetics is given in equation 4.1.

$$r_p = \frac{r_{max}\, C_s}{K_M + C_s} \qquad 4.1$$

Where r_{max} is the maximum rate and k_M is the Michaelis Menten constant.

These are the kinetic parameters which need to be determined experimentally.

Applying the line weaver-burk method to linearise the rate expression by inverting equation 4.1 it yields

$$\frac{1}{r_p} = \frac{1}{r_{max}} + \frac{K_M}{r_{max} \cdot C_s} \qquad 4.2$$

A plot of the reciprocal of rate (1/r), versus the reciprocal of initial substrate concentration ($1/C_s$) is expected to yield a straight line with an intercept $1/r_{max}$ and slope $K_{M/}r_{max}$, if the data agrees with the Michaelis Menten model.

A plot of this using the generated experimental data given in Table A7.1 is shown in Figure 4.14, from which it can be seen that the intercept gives a negative value. This implies that the data does not agree with the Michaelis Menten model.

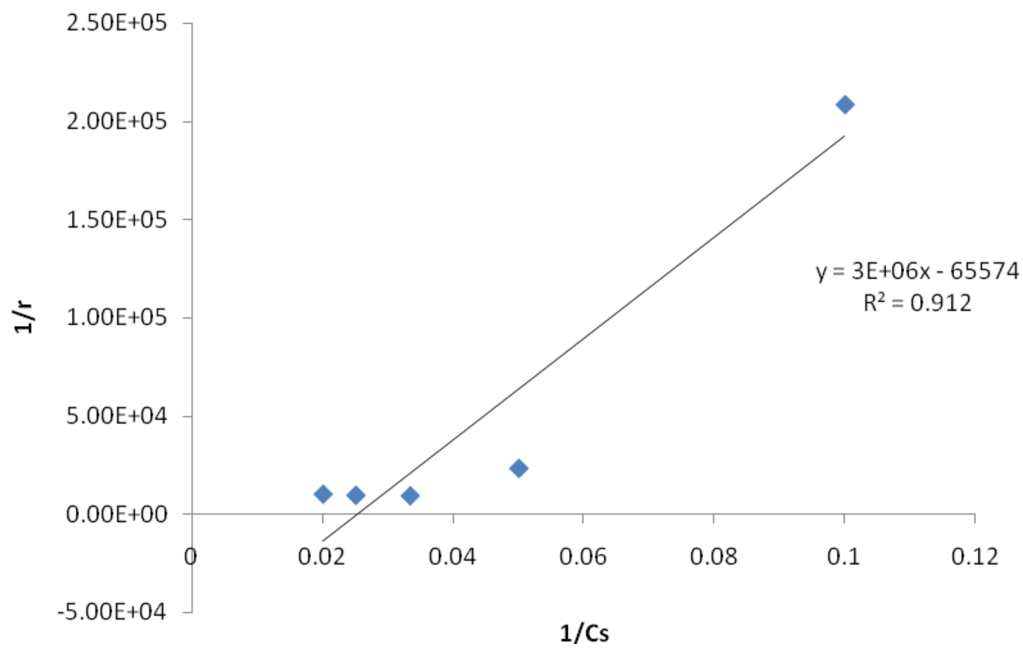


Figure 4.14: Line weaver-Burk plot

In view of this, the power law was used. The power law is given in equation 4.3, where r is the rate of reaction, k is the reaction constant, C_s is substrate concentration and n is the order of reaction. Therefore, k and n are the kinetic parameters to be determined.

$$r = kC_s^n \qquad 4.3$$

Taking the natural logarithm of both sides of equation 4.3 gives equation 4.4.

$$\ln r = \ln k + n \ln C_s \qquad 4.4$$

A plot of ln r against ln C_s will give a straight line with slope n and intercept ln k. This is presented in Figure 4.15 from which k and n were evaluated to be 8.172 x 10^{-8} l/g.s and 2 respectively. The order of reaction n is 2, this explains the reason why the Michaelis Menten model did not agree with the data used. The Michaelis Menten model is only suitable for zero and first order reactions.

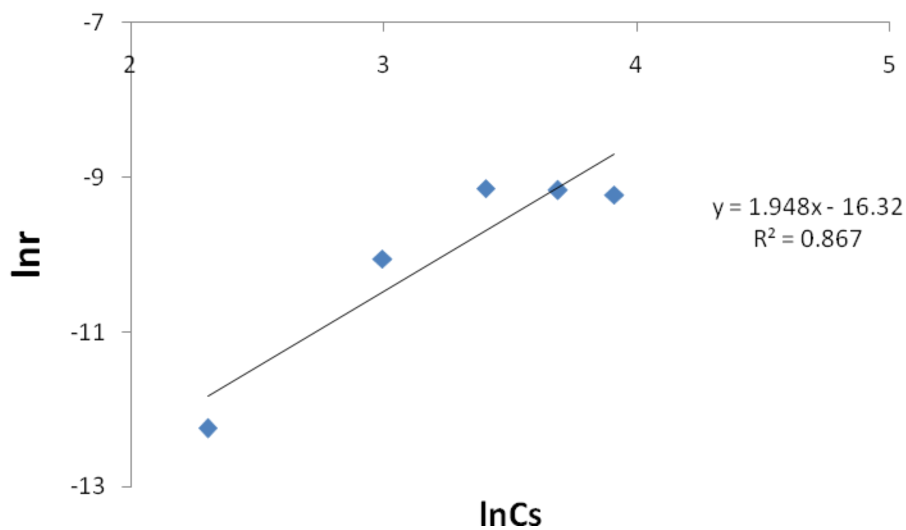


Figure 4.15: plot of ln r versus ln C_s

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

From the results obtained from this work the following conclusions can be drawn

1. The best pre-treatment condition for elephant grass stem was using 1M NaOH for 8hrs contact time, at 55°C and 1:10 solid/Liquid ratio. However because of presence of soluble sugar in the untreated sample which is lost during pretreatment, the untreated sample was used in the fermentation.
2. The optimum fermentation conditions obtained were temperature 35°C, substrate concentration 30g/l, particle size 250-300µm, pH 5.5, *A. niger/yeast* ratio 0.6/1.5% w/v and fermentation time of 72 hours.
3. At optimum substrate concentration of 30g/l, ethanol concentration of 23.4g/l was obtained.
4. The kinetic parameters of the reaction k and n were obtained to be 8.172×10^{-8} l/g.s and 2 respectively.

5.2 RECOMMENDATIONS

With regard to the results obtained and the problems encountered in this work, the following recommendations are made:

1. Further studies can be done on this work by removing the soluble sugar present in the substrate to ferment separately, after which the sugar free substrate can now be pre-treated and fermented separately.
2. Government at all levels should encourage and sponsor small scale production of bio-ethanol from cellulosic materials to create employment.

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APPENDICES

APPENDIX I

Table A1: Effect of pre-treatment on ethanol yield at pH 5.0, 35^0C, 250-300µm particle size *A.niger*/yeast ratio 0.6/1.5% w/v

PRETREATMENT METHOD	**yield at 24hr**	**yield at 48hr**	**yield at 72hr**	**yield at 96hr**	**yield at120hr**
NaOH	36.60	53.30	86.90	69.80	60.20
BOILED	16.70	20.20	26.70	20.00	13.50
UNTREATED	33.40	50.20	83.60	66.90	56.90

APPENDIX II

Effect of substrate concentration on ethanol yield and residual sugar concentration

Table A2.1: Effect of substrate concentration and fermentation time on ethanol yield at pH 5.0, 30^0C, 150-250μm particle size *A.niger*/yeast ratio 0.6/1.5% w/v.

Substrate concentration g/l	**Yield at 24hr**	**Yield at 48hr**	**Yield at 72hr**	**Yield at 96hr**	**Yield at 120hr**
10	5.00	8.00	16.70	12.00	9.60
20	17.30	37.50	57.20	52.50	47.80
30	30.90	64.00	78.10	72.60	68.10
40	22.70	45.50	63.80	59.8	58.7
50	16.86	41.20	48.50	46.90	42.30

Table A2.2: Effect of substrate concentration and fermentation time on residual sugar concentration

Substrate concentration w/v%	**Conc. at 24hr**	**Conc. at 48hr**	**Conc. at 72hr**	**Conc. at 96hr**	**Conc. at 120hr**
1	0.90	0.78	0.52	0.34	0.19
2	1.64	1.23	0.63	0.61	0.31
3	2.10	1.06	0.65	0.58	0.02
4	3.01	2.10	1.42	0.68	0.24
5	4.01	2.41	1.28	0.58	0.35

APPENDIX III

Effect of temperature on ethanol yield and residual sugar concentration

Table A3.1: Effect of temperature and fermentation time on ethanol yield at pH 5.5, 3% w/v substrate concentration, 150-250μm particle size *A.niger*/yeast ratio 0.6/1.5% w/v

Temperature, °C	Yield at 24hr	Yield at 48hr	Yield at 72hr	Yield at 96hr	Yield at 120hr
25	4.90	17.20	23.90	28.20	36.60
30	16.60	50.10	70.60	65.60	64.10
35	17.2	55.90	77.60	73.60	65.90
40	10.10	31.90	38.10	36.20	34.90
45	6.90	14.20	28.10	24.90	16.60

Table A3.2: Effect of temperature and fermentation time on residual sugar concentration

Temperature, °C	Conc. at 24hr	Conc. at 48hr	Conc. at 72hr	Conc. at 96hr	Conc. at 120hr
25	2.35	1.85	1.35	1.21	0.23
30	2.49	1.28	0.75	0.58	0.01
35	2.43	1.08	0.65	0.62	0.03
40	2.55	1.83	1.60	0.86	0.54
45	2.25	2.01	1.89	1.52	1.01

APPENDIX IV

Effect of particle size on ethanol yield and residual sugar concentration

Table A4.1: Effect of particle size and fermentation time on ethanol yield at pH 5.0, 30^0C, 3% w/v substrate concentration, *A.niger*/yeast ratio 0.6/1.5% w/v.

Particle size, µm	**Yield at 24hr**	**Yield at 48hr**	**Yield at 72hr**	**Yield at 96hr**	**Yield at 120hr**
53-106	8.10	16.60	24.90	23.20	18.20
106-150	25.20	31.70	36.70	28.10	21.60
150-250	29.20	37.20	41.60	36.10	30.90
250-300	29.30	60.40	80.10	73.40	70.44
300-425	18.60	40.80	57.90	59.60	49.10

Table A4.2**:** Effect of particle size and fermentation time on residual sugar concentration.

Particle size, µm	**Conc. at 24hr**	**Conc. at 48hr**	**Conc. at 72hr**	**Conc. at 96hr**	**Conc. at 120hr**
53-106	2.35	1.45	1.17	0.64	0.04
106-150	2.21	1.35	1.20	0.42	0.03
150-250	2.10	1.46	1.32	0.58	0.02
250-300	2.10	1.13	0.56	0.67	0.32
300-425	2.32	1.72	1.26	0.52	0.17

APPENDIX V

Effect of pH on ethanol yield and residual sugar concentration

Table A5.1: Effect of pH and fermentation time on ethanol yield at 30^0C, 3% w/v substrate concentration 106-150µm particle size, *A.niger*/yeast ratio 0.6/1.5% w/v.

pH	**Yield at 24hr**	**Yield at 48hr**	**Yield at 72hr**	**Yield at 96hr**	**Yield at 120hr**
3.5	7.22	8.20	23.80	33.70	34.67
4	9.80	24.80	48.20	44.80	35.00
4.5	14.20	36.20	69.60	67.40	55.00
5	21.20	40.30	76.60	71.10	61.80
5.5	24.10	54.10	79.80	78.10	64.80
6.0	25.20	51.20	72.80	71.90	60.70
6.5	20.20	39.80	69.90	64.10	54.00

Table A5.2: Effect of pH and fermentation time on residual sugar concentration

pH	**Conc. at 24hr**	**Conc. at 48hr**	**Conc. at 72hr**	**Conc. at 96hr**	**Conc. at 120hr**
3.5	2.23	1.60	0.62	0.52	0.22
4.0	2.24	1.72	0.82	0.64	0.33
4.5	2.39	1.84	0.90	0.76	0.42
5	2.29	1.52	0.64	0.42	0.16
5.5	2.25	1.42	0.61	0.33	0.12
6.0	2.23	1.38	0.78	0.41	0.23
6.5	2.38	1.74	0.89	0.53	0.38

APPENDIX VI

Effect of cell loading

Table A6.1: Effect of *A. niger* concentration and fermentation time on ethanol yield at 30^0C, 3% w/v substrate concentration, 250-300µm particle size, 1.5%w/v yeast conc.

***A.niger* conc. (w/v) %**	**Yield at 24hr**	**Yield at 48hr**	**Yield at 72hr**	**Yield at 96hr**	**Yield at 120hr**
0.2	27.20	43.20	66.90	62.90	59.60
0.4	30.60	45.80	69.20	65.80	62.70
0.6	36.60	49.90	82.40	79.60	76.60
0.8	35.40	45.40	68.40	65.60	62.60
1.0	31.90	45.60	66.20	62.20	57.60

Table A6.2**:** Effect of yeast concentration and fermentation time on ethanol yield at 30^0C, 3% w/v substrate concentration, 250-300µm particle size, 0.6%w/v *A.niger* conc.

Yeast conc. (w/v)%	**Yield at 24hr**	**Yield at 48hr**	**Yield at 72hr**	**Yield at 96hr**	**Yield at 120hr**
0.5	13.30	26.10	36.60	33.00	26.70
1	20.10	28.40	59.90	53.40	47.10
1.5	33.40	50.20	83.60	66.90	56.90
2	30.10	47.10	68.40	56.80	50.20
2.5	21.60	39.20	51.00	45.90	40.00

APPENDIX VII

Table A7.1: Initial rates at various initial substrate concentrations

initial substrate concentration, Cs (g/l)	initial rates, r (g/l.s)	1/Cs (l/g)	1/r (l.s/g)
10	4.80E-06	0.1	2.08E+05
20	4.29E-05	0.05	2.33E+04
30	1.07E-04	0.0333	9.32E+03
40	1.04E-04	0.025	9.60E+03
50	9.84E-05	0.02	1.02E+04

Table A7.2: Natural logarithm of C_s and r

initial substrate concentration, Cs (g/l)	initial rates, r (g/l.s)	ln C_s	ln r
10	4.80E-06	2.30	-12.25
20	4.29E-05	2.99	-10.06
30	1.07E-04	3.40	-9.14
40	1.04E-04	3.68	-9.17
50	9.84E-05	3.91	-9.23

$$\text{Initial rate} = \text{slope at 24Hrs} = \frac{\text{change in glucose concentration}}{\textit{change in time}}$$

For substrate concentration of 10g/l

$$\text{slope at 24Hrs} = \frac{0.84-0.08}{44-0} = 0.0173_{g/lhr} = 4.8\text{x}10^{-6}\ _{g/l.s}$$

APPENDIX VIII

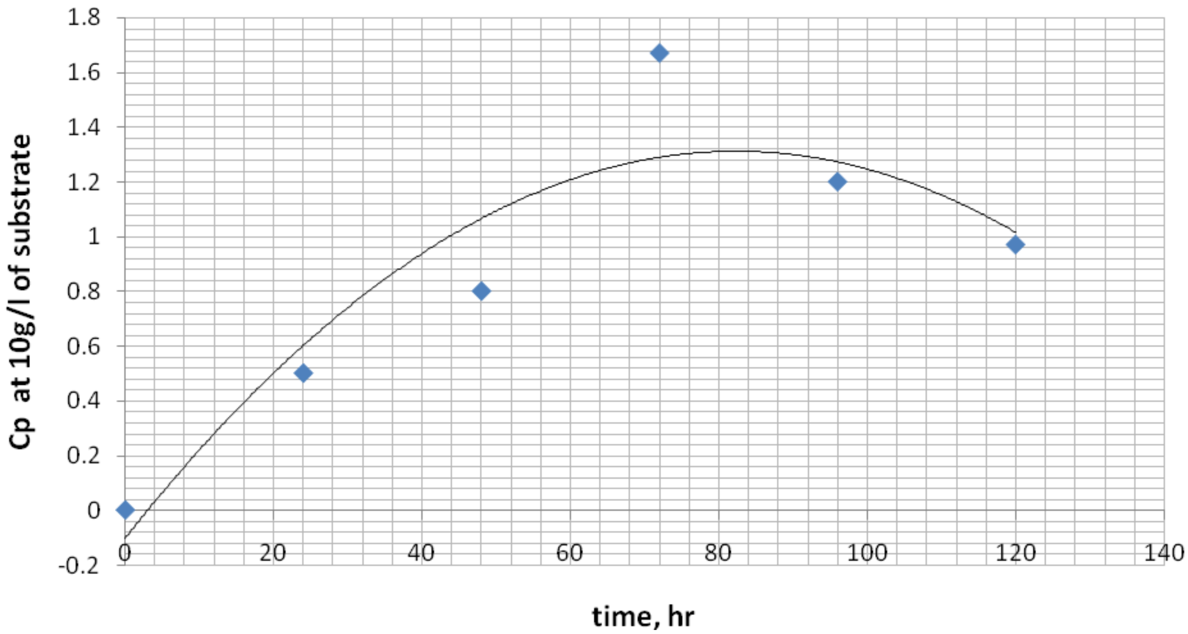
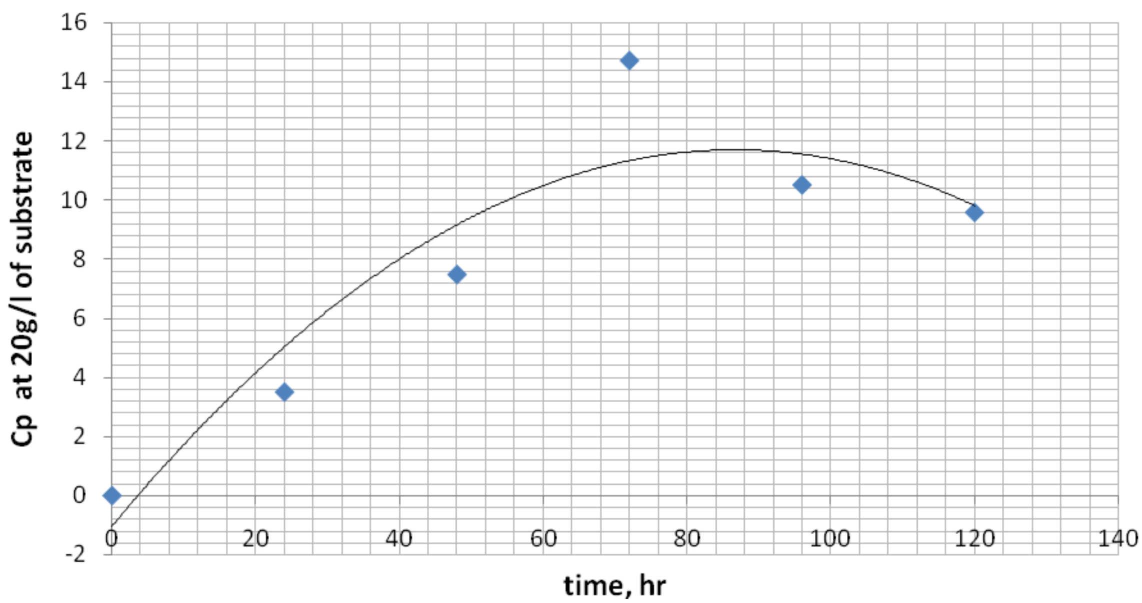


Figure A8.1: Concentration-time curve at 10g/l



FigureA8.2: Concentration-time curve at 20g/l

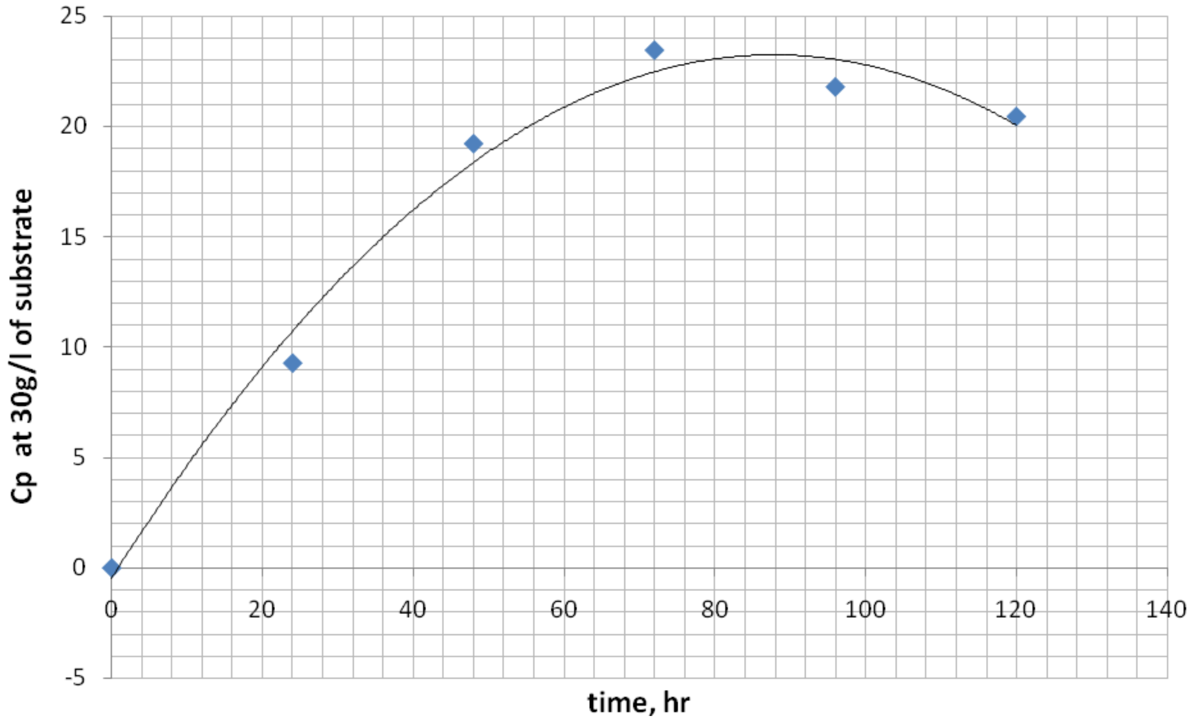


Figure A8.3: Concentration-time curve at 30g/l

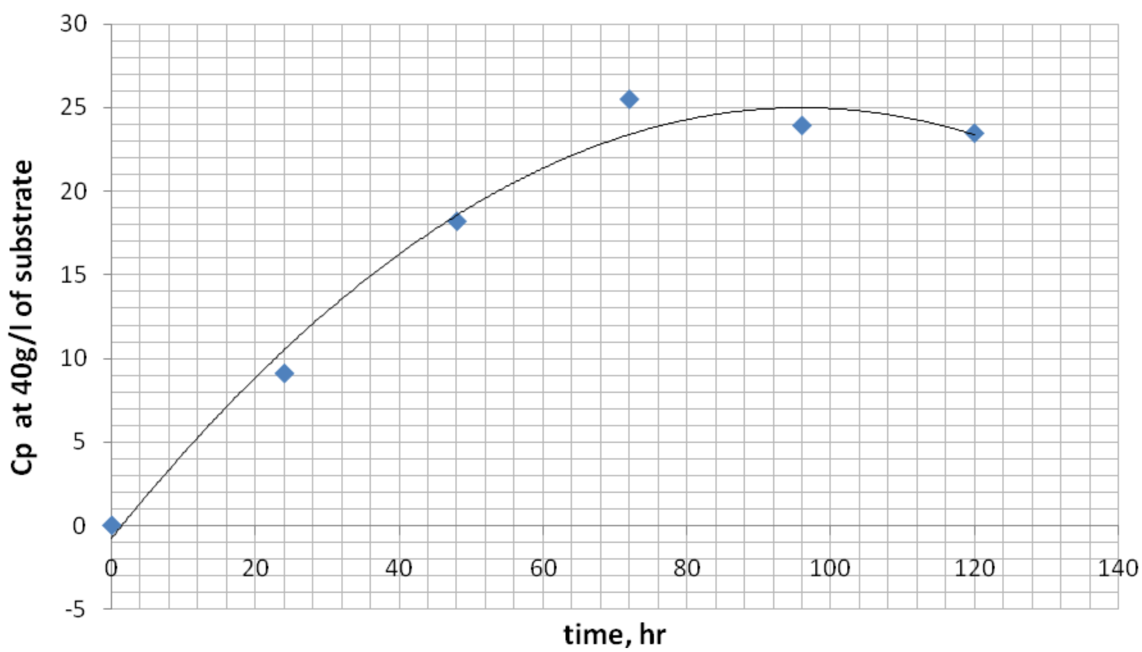


Figure A8.4: Concentration-time curve at 40g/l

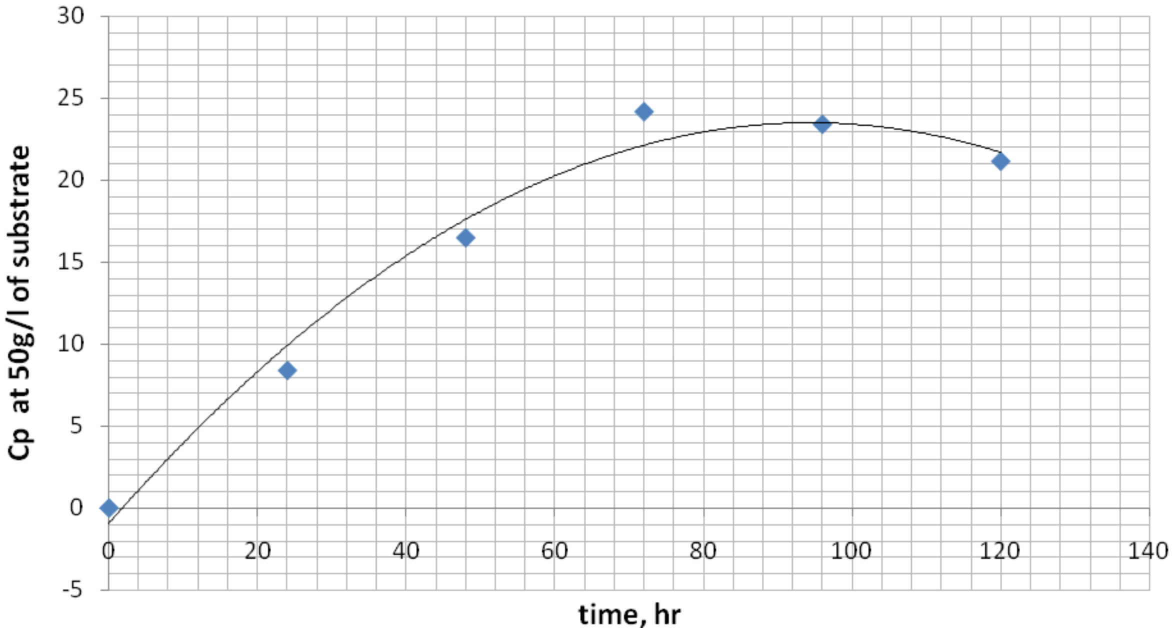


Figure A8.5: Concentration-time curve at 50g/l