ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)

SCHOOL OF CHEMICAL AND BIO-ENGINEERING

(MSc. Program in Environmental Engineering)

Bioethanol production from Water Hyacinth by chemical Hydrolysis (Preliminary Study)

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A Thesis Submitted in Partial Fulfillment of the requirements for the Award of a Master's Degree in Chemical Engineering under Environmental Engineering

June 2016, ADDIS ABABA

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(Preliminary Study)

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ABSTRACT

Ethiopia, in order to reduce its dependency on imported petroleum fuels which consumes more than70% of the foreign currency earning, climate change issues and to tackle invasive weed problem such as Water hyacinth through integrated management approach of the concept of waste to Energy, a lot of research needs to be conducted.

Bearing this in mind, this study was conducted to conduct preliminary assessment on bioethanol production potential WH by chemical hydrolysis. Water hyacinth was collected from river Awash, the root was cut out and the leaf and stem part were properly washed, dried and chopped for further analyses.

Sample characterization result showed that Water hyacinth has high moisture content and the extractive free WH has 30%, 48% and 5% cellulose hemicellulose and lignin respectively. In this study Water hyacinth was hydrolysed with sulfuric acid (2-5 %v/v), temp (116-130 ^{o}C) and hydrolysis time (16-60min) and optimal reducing Sugar obtained was 31.152 g/L at acid concentration 3.97% v/v, Temperature at 129.91 ^{o}C and 57.82 minutes reaction time.

In this study, Ethanol yield obtained is extremely low. Ethanol yield ranges from 0.05% to 0.532% from obtained RS of WH hydrolysate with fermentation parameters of temperature 30^{o}C for 72hr and 150 RPM. The highest ethanol yield was not at the optimal value of factors for maximum reducing sugar but at increased acid concentration but at the lower temperature. This may be due to high content of hemicelluloses and at optimal values of factors pentose sugars are high and at increased acid concentration and temperature partial hydrolysis of the cellulose component has occurred to increase the hexoses sugars that are easily fermentable with the given condition.

These results suggest that refinement of the hydrolysis and fermentation process and application of the selected yeast which hydrolyze both hexose and pentose sugars could improve the efficiency of obtaining bioethanol from the water hyacinth.

Key words: Water hyacinth, Bioethanol, hydrolysis, reducing sugar, fermentation

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Table of Contents

List of Figures

List of Tables

Appendixes

Acronyms

AFEX - Ammonia Fiber Explosion

C2H5OH – Ethanol

ETOH- Ethanol

EM- Extractive free sample

FT - Fischer-Tropsch

Fig. -Figure

GHG- Greenhouse gas

GTP – Growth and Transformation Plan

g/l - gram per litter

g- gram

HMF - Hydroxymethyl furfural

LHW - Liquid hot water

MON - Motor octane number

MT - Metric ton

NREL- National Renewable Energy Laboratory

PH - Power of Hydrogen

RS- Reducing Sugar

RPM- Revolution Per minute

S. cerevisiea -Saccharomyces cerevisiae

w/v -Weight per volume

WH- Water Hyacinth

CO_2 - Carbon dioxide

°C- Degree Celsius

% -Percent

Wt%- weight percent

1. Introduction

1.1. Background

Every society requires energy services to meet basic human needs (e.g., lighting, cooking, space comfort, mobility, and communication) and to serve productive processes. The demand for energy and its associated services, to meet social and economic development and improve human welfare and health, is on the increase. Since approximately 1850, the global use of fossil fuels (coal, oil, and gas) has increased to dominate the energy supply, leading to a rapid growth in carbon dioxide (CO2) emission. Additionally, this has resulted in depletion of fossil fuel reserves which are the conventional energy resources so far and use of these conventional energy sources has become a cause for climate change. (IPCC, 2012)

Therefore, one of the challenges for the society is to meet the growing demand for energy for transportation, heating and industrial processes; also to provide raw materials for the industry in a sustainable way and to reduce greenhouse gas emissions. In order to cope with the challenges, our energy systems need to be renewable and sustainable, efficient and cost-effective, convenient and safe.

In order to provide dignified and quality of life of their people, developing countries like Ethiopia need to focus on improving and developing the energy sector service. Most of developing countries rely on imported fossil fuel for their energy provision. This makes them spend earned foreign currency from different activities, largely to import fossil fuel. Additionally, developing countries as part of a global system, they are victim of climate change and with limited adaptation capacity; the effect of climate change is worsen. Hence, developing countries need to be active participant in the alternative energy research activities which contribute to shift from use of fossil fuel to modern energy technologies.

Modern and renewable energy technologies include modern biomass energies, solar, wind, geothermal, hydropower etc. Deployment of Non- fossil fuel based modern and renewable

energy technologies also create enabling environment to use local resources and develop the local technical skill in general and employment creation.

Increase on world's energy demand and the progressive depletion of oil reserves motivate the search for alternative energy resources, especially for those derived from renewable materials such as biomass. (Saxena, R.C., Adhikari, D.K. and Goyal, H.B.,, 2009)

.Bioethanol produced from renewable biomass, such as sugar, starch, or lignocellulosic materials, is one of the alternative energy resources, which is both renewable and environmentally friendly. Although, the priority in global future ethanol production is put on lignocellulosic processing, which is considered as one of the most promising second generation biofuel technologies, the utilization of lignocellulosic material for fuel ethanol is still under improvement. (Mojolovic L., Pejin D.,Grujic o., Markov S.,Pejin J., Rakin M., Vukasinovic M.,Nikolic S.,Savic D.,, 2009)

In order to expand bioethanol production and use benefit, intensive and extensive research on potential bioethanol feedstocks, processing and utilization technologies needs to be in place for technical, economic and environmental viability.

Lignocellulose-rich biomass such as agricultural residues (e.g. straw), cellulosic crops on surplus land and forest biomass have been identified as a biomass resource to modern energy conversion such as for use as liquid biofuel with potential to fulfill various sustainability criteria while also reducing the risk of increased agricultural land-use competition (Berndes G., 2010)

The high growth rate of the water hyacinth with a growth rate of 17.5 tons/hectare/day under favorable condition (Shoeb, 2002) and that the daily average productivity of water hyacinth being 0.26 ton of dry biomass per hectare (Singh, 1984) in all seasons makes it as a potential lignocellulosic feedstock for bioethanol production. Other researches on its productivity revealed that depending on the time of the year and location production reaches upto 100-140 tons/year per hectare. (Nigam, 2002).

Water hyacinth is low in lignin content (10%) and contains high amounts of cellulose (20%) and hemicellulose (33%) (Bolenz S., 1990) imparting the fact that it can be potential for bioethanol production. The low lignin content suggests that the cellulose and the hemicelluloses are relatively easily acceptable for conversion to fermentable sugars to provide better yield biofuel i.e ethanol.

On the other hand water hyacinth is an adventive aquatic plant that threatens lakes and rivers ecosystem. The negative impacts of water hyacinth are due to its dense, impenetrable mats which restrict oxygen access to water. These mats affect fisheries and related commercial activities, functioning of irrigation canals, navigation/transport, hydroelectric programmes and tourism (Navarro L. &., 2000). Therefore, controlling and managing this weed is very important.

According to survery conducted between 2009 and 2011 on prevalence and severity of water hyacinth on water bodies within the rift valley in Ethiopia, it shows that it has become the major invasive alien weed in most of the water bodies. (Firehun Y. S., 2014)

Water bodies are complex ecosystems and include a variety of water plants that contribute to water quality and environmental health. In a healthy ecosystem there is usually a complex mix of plant species but sometimes the natural balances are disturbed. For example, increased nutrient loads entering water bodies, low water flows and increased temperatures often cause an excessive growth of some plants to the point where they become a problem.

The fact of water bodies supporting variety of plant growth and can be seen as a potential production sites for biofuel feed stocks in case of algae production and large lignocellulosic biomass such as water hyacinth. But this needs a lot of research on applicability without compromising the ecosystem balance and in a controlled manner.

The present research aims to conduct preliminary assessment in order to produce ethanol from water hyacinth by chemical hydrolysis and fermentation process.

Putting this weed into productive use have dual advantage in arresting under control the invasion and in reducing cost through integrated management of applying waste to energy concept.

1.2. Problem Statement

As a result of the prevailing economic development the transport sector is also developing resulting in demand increase for fuel. As dependant on use of imported fuel, Ethiopia needs to focus on developing local natural resources to be deployed for production of biofuels. Moreover, it has been declared that increasing use of fossil fuels has become cause of climate change endangering our earth. Therefore search on alternative and renewable source of energy is important theme of the time. While developing biofuels, it has to be noted that, Biofuels need not compromise food security and competition of land and other resources. Hence, bioethanol production requires alternative sources, such as wood and agricultural wastes and other alternative lignocellulosic feedstock.

On the other hand, Weeds like P. Julfira and Water hyacinth has become threat by invading important natural resources such as land and water in Ethiopia. It is an obvious fact that control management systems require incurring high amount of budget. Therefore converting these to useful products can contribute to minimizing cost through integrated management of applying waste to energy concept.

The current practice of controlling Water hyacinth is manual clearing of water bodies and, finally opens burning of the collected waste. Instead of burning these wastes, converting them to some useful product is recommended option.

Therefore the current study focus on studying effect of chemical hydrolysis to the yield of bioethanol production from water hyacinth as the possibility of using this option as environment management to contribute to mitigate water bodies deterioration, water ecosystem disturbance due to the plant's invasion of water bodies in addition to its being as a lignocelluloses biomass which can add up to being a feedstock for renewable fuel.

1.3. Scope and Objective

1.3.1. General Objective

The main objective of this study is to conduct preliminary assessment to produce ethanol from water hyacinth (Eichhornia Crassipes) using chemical hydrolysis and fermentation.

1.3.2. Specific Objective

- To characterize water hyacinth cellulose, hemicelluloses, lignin, moisture ash and content of from river Awash
- To evaluate the influence of Acid concentration, temperature, and treatment time on chemical hydrolysis of water hyacinth on yield of reducing sugar
- To conduct batch fermentation and evaluate ethanol yield using the hydolysate obtained from chemical hydrolysis of WH

1.3.3. Significance of the study

This research contributes to the effort of utilization of the abundantly available and renewable lignocellulosic feedstock option to the extraction and conversion of these materials to Bioethanol.

The current practice of Water hyacinth control is focused on trying to clear the weed from the water bodies through harvesting by manual and mechanical means and burning it. This system is not a recommended practice as it has impact on air pollution. Better solution other than burning the waste needs to be in place. Nowadays, a lot of effort is under way to mitigate the problem being caused by water and land invading weeds such as Water hyacinth and P.Julfira. Therefore, this study adds up to the research activities to include bioethanol production in applying "waste to Energy" environmental management concept as part of integrated management strategies in Controlling and halting the invasive water hyacinth and its disturbing effect on water bodies to a non- problematic level. Putting this weed to useful product can also reduce cost of the weed invasion management and control.

2. Literature review

2.1. Introduction

The world population is estimated to increases from 6.7 billion to 8 billion by 2030 (United States Census Bureau, 2008). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell CJ, 1998). Thus the energy demand of the future is likely to play a key role in geo-political economics. Given this reality, nations around the world are investing in alternative sources of energy, including bioenergy.

A considerable amount of research is currently being conducted on the production of bioenergy due to the increasing demand for fossil fuel and its limited quantities in reserve. Recently, more research has focused on using non-edible biomass as raw materials including lignocelluloses, celluloses, and marine algae rather than the first generation biomass such as starch and sugar biomass. Lignocellulosic biomass comes from agricultural products, forests, bush lands and water body plants.

On the other hand Land and water bodies invasion by different types of weeds has become a threat to the land and water resources. Weeds such as J. Prosopis and water hyacinth are some to name. Therefore currently researches are being conducted in view of managing and controlling these invasive species and as well as implementing integrated management system which includes converting these into useful products such as fuels and fertilizers. Therefore, currently researches are being conducted focusing on dual purpose to fulfill on demand of environmental control and looking for alternative energy demand. (Bergier, Salis, Miranda, Ortega, & Luengo, 2012)

The production of ethanol from lignocellulosic biomass such as corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn cob, oat hull, corn fiber, woodchips and cotton stalk and from energy crops such as switch grass and Alfa Alfa, and various weeds such as Prosopis Saccharum spontaneum, Lantana camara, Eichhornia crassipes (water hyacinth), etc. has become one of the best alternatives, because these sources have widespread abundance and probability of their use for biofuel production can be effective in lowering control cost of the weeds. (Hadar, 2013)

Lignocellulosic biomass is comprised of lignocellulosic materials consisting of lignin, cellulose and hemicellulose and bioethanol production relies on technologies that will efficiently hydrolyze cellulosic biomass to fermentable sugars. Efficient process technologies are still under search for large scale production realization with acceptable production cost range.

The natural and healthy ecosystem supports a complex mix of different plant species but sometimes the natural balances are disturbed causing excessive growth of some plants to the point where they become a problem. For example, increased nutrient loads entering water bodies, low water flows and increased temperatures often cause an excessive growth of some plants to the point where they become a problem. Such a point in case for instance is invasion of water bodies with water hyacinth. Or in other situation some plant species out compete for the scares resources and invade certain area and become the dominant species making others unable to survive like in the case of j.prosopis in both cases, some interventions are required to control them and as well as search for options to convert them to useful products so that the threat due to them can be minimized.

2.2. Overview on Water hyacinth and the Environment

Water hyacinth (Eichhornia crassipes) is a fresh water aquatic plant belonging to the family Pontederiaceae and is a native of Brazil and Equador region. But now, Water hyacinth, Eichhornia crassipes, is considered as one of the world's worst weeds (Center TD, 1999), invading lakes, ponds, canals, and rivers. Due to its extremely fast growth, the weed has become the major floating water weed of tropical and subtropical regions. It grows from few inches to a meter in height, floating on the surface of water (Bhattacharya, 2010). It tolerates extremes in water level fluctuations, seasonal variations, nutrient availability, pH, temperature, and toxic substances (Gopal, 1987) as cited by (Tham, 2012). It can even grow at salinity level upto 0.24% as shown in Indonesia (Kikuchi, 1997) . In the absence of natural enemies, the weed quickly becomes invasive, colonizing slow moving waters resulting in thick and extensive mats (Edwards, 1975) degrading aquatic ecosystems and limit their utilization (Hill, 2008).

The negative impacts of water hyacinth are due to its dense, impenetrable mats which restrict access to water. These mats affect fisheries and related commercial activities, functioning of irrigation canals, navigation/transport, hydroelectric programmes and tourism (Navarro L. &.,

2000). Ecologically, benthic and littoral diversity is reduced (Masifwa, 2001) (Midgley, 2006), while population of vectors of human and animal diseases such as bilharzias and malaria are increased with water hyacinth infestation as these plants interfere with pesticide application (Harley, 1996).

WH has the high growth rate with a growth rate of 17.5 tons/hectare/day-on fresh weight basis under favorable condition (Shoeb, 2002) and that the daily average productivity of water hyacinth being 0.26 ton of dry biomass per hectare (Singh, 1984) in all seasons. Other researches on its productivity revealed that depending on the time of the year and location production reaches upto 100-140 tons/year per hectare on dry mass basis. (Nigam, 2002)

The biological attributes of water hyacinth as an ideal bioenergy crop includes: it is naturally grown vegetation- preferably perennials, high cellulose with low lignin content per unit volume of dry matter, easily degradable, fast growing, no completion for arable land, resists pests, diseases, no cross pollution with food crops. (Bhattacharya, 2010)

Water hyacinth is low in lignin content (10%) and contains high amounts of cellulose (20%) and hemicellulose (33%) (Bolenz S, 1990) (Poddar K, 1991) (Gressel.J., 2008). A typical biomass from land plants can have 30-50% cellulose, 20-40% hemicellulose and 15-30% lignins.

In plants, lignin (composed of phenylpropanoid groups) acts as a polymer around the hemicellulose microfibrils, binding the cellulose molecules together and protecting them against chemical degradation. Presence of Lignin complicates accessibility for cellulosic materials decomposition. The large amount makes biofuel production more complex. Their degradation is a high-energy process. Water hyacinth has low lignin, which means the cellulose and hemicellulose are more easily converted to fermentable sugar thus resulting in enormous amount of utilizable biomass for the biofuel industry. This makes it as a potential lignocellulosic feedstock for bioethanol production indiverting away the current bioethenol production from food sources and food security issues arising due to direct and indirect land use changes as a result of biofuel production.

In order to mitigate and contribute to the control the negative environmental effect of water hyacinth invasion and as well as looking into the potential biological attributes as a bioenergy potential a lot of researches are underway to use it as a biofuel feedstock.

2.3. Water hyacinth in Ethiopia

Water hyacinth was introduced into Africa from South America in the early 1900s (Mitchell, 1985) (Gopal, 1987) , but since the 1950s it has become a problematical weed in Southern Africa, the Congo basin and the Upper Nile (J. Rzoska, 1974) (Denny, 1984). In the East African region, the weed was first noticed almost simultaneously in Uganda, Tanzania and Kenya in 1987 (Ogwang, 2001). According to the same survey assessment conducted, the result shows that the weed infestation in Ethiopia was small and no subsequent action was taken by that time. (Firehun Y. S., 2014)

An exploratory survey and assessment of water hyacinth on the different water bodies of Ethiopia revealed that Water hyacinth was introduced in the water bodies of the Rift Valley in the 1950s as an ornamental plant and sporadic visit and some clean up attempt have been done in 1959, 1968, 1979, and 1988. (Stroud, 1994).

According to (Firehun, Abera, & Tariku, 2007) the weed began to proliferate on reservoirs, irrigation and drainage structures at Wonji-Shoa Sugar Estate, since 1996 when the plantation was flooded by overflow of Awash River that crosses the Koka Dam.

As of 2005, the gravity of the situation was quickly realized and it was decided to embark a management strategy of the weed nationally. An action-oriented control program involving manual, mechanical, biological and chemical measures (Firehun, Abera, & Tariku, 2007) and (GEF, 2009) was launched; but only the manual, chemical and mechanical control programs were effectively implemented. Although in some of the infested areas these control programs were implemented (Dula, 2008),

Recent assessment conducted between the year 2009 and 2011, result indicated that water hyacinth has become a major invasive alien weed in the Rift Valley of Ethiopia having successfully established and invaded the different water bodies. (Firehun Y. S., 2014). Lake Ellen and Lake Tana are also invaded with this weed. (Fessehaie, 2012)

Therefore, the prevailing situations according to the literature review made demands further studies on applying effective control methods that are environmentally sound and viable and

which are cost effective. These types of measures include use of this weed after being removed from the water bodies to be converted to a useful product such as Bioethanol.

2.4. Bioethanol as a fuel

Bioethanol and Ethanol are identical chemicals; Ethanol is named Bioethanol in order to designate the source. Therefore, Bioethanol refers to ethanol that is produced from Biomass sources. Otherwise, with respect to explaining about the ethanol properties, both names can be used interchangeably. Bioethanol (ethyl alcohol, grain alcohol, CH3–CH2–OH or ETOH) is a liquid biofuel which can be produced from different biomass feedstocks and conversion technologies. Ethanol is a volatile, flammable, and colorless chemical compound. It is a monohydric primary alcohol and it boils at 78.5 °C. It is miscible with water in all proportions. Ethanol that is completely free of water is called absolute ethanol. Ethanol forms a constant-boiling mixture, or azeotrope, with water that contains 95 % ethanol and 5 % water and that boils at 78.15 °C.

Bioethanol is an attractive alternative fuel because it is a renewable bio-based resource and it is oxygenated thereby provides the potential to reduce particulate emissions in compression ignition engines (Hansen AC, 2005;). The toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Hinman, 1990). An oxygenated fuel such as bioethanol provides a reasonable antiknock value and fuel combustion is more efficient, reducing hydrocarbons and particulates in exhaust gases.

Bioethanol has a higher octane number (108), Octane number is a measure of the gasoline quality and can be used for prevention of early ignition which leads to cylinder knocks. It also has broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline. These properties allow for a higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine(ICE) (Balat.M, 2007). Ethanol has a much higher latent heat of vaporization (855 MJ/kg) than petrol (293 MJ/kg) as well as a higher octane number (99) than petrol (80–100) and , as a result, pre-ignition does not occur when ethanol is used (Kumari, 2014) .

Bioethanol is appropriate for the mixed fuel in the gasoline engine because of its high octane number, and its low cetane number and high heat of vaporization impede self-ignition in the

diesel engine. So, ignition improver, glow-plug, surface ignition, and pilot injection are applied to promote self-ignition by using diesel–bioethanol blended fuel (Kim H, 2005.).

Disadvantages of bioethanol include its lower energy density than gasoline (bioethanol has 66% of the energy that gasoline has), its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water, and toxicity to ecosystems (MacLean HL, 2003). Ethanol is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emissions from combustion.

The other disadvantage in using ethanol as fuel is that aldehyde predominantly acetaldehydes emissions are higher than those of gasoline. However, acetaldehydes emissions generate less adverse health effects in comparison to formaldehydes emitted from gasoline engines (Gonsalves, 2006).

Developing and promoting use of bioethanol as fuel is an important measure for a country like Ethiopia because as dependent on imported fossil fuel. It is no wonder that the country has great interest on research activities that focus on advancing biomass conversion energy technologies like bioethanol production.

2.5. Bioethanol for fuel production and use in Ethiopia

Bioethanol production is not a new technology in Ethiopia, many people produce ethanol from different grains at household level using indigenous knowledge as part of beverage and food industries activities that are to be used as drinkable alcohol. There are also many refineries which process bioethanol for various purposes such as for pharmacetical purposes, as drinkable alcohol and for fuel that is used in beauty salons under different brand names. The alcohol concentration of these products varies.

However, ethanol with 99.5% concentration, called an absolute alcohol is currently produced in Metehara and Fincha sugar factories. This alcohol is used to be blended with gasoline and as a household cooking fuel.

Blending Ethanol with gasoline has commenced since 2008 with E5 (5% ethanol and gasoline blend) and in 2011 the blend increased to E10 (10% ethanol to 90 % gasoline). The blending status is shown in Table 2.5-2. Since Ethiopia is importing fuel for the transport and industrial

sectors whereby more than 75% of the foreign currency earning through export is taken back for importing fuel, looking for alternative fuel and maximizing locally available renewable source of energy such as biomass like bioethanol is a very important measure as emphasized by the Biofuel Development Coordination Directorate. (Status of Biofuel Development in Ethiopia, 2008). According to data from EPE, the quantity of imported fuel is increasing from time to time as is shown in (Table 2.5-1).

Table 2.5-1 Import data of fuels (source Ethiopian petroleum Enterprise, EPE, 2015)

Import	**2011/12**	**2012/13**	**2013/14**	**2014/15**
	Qty, M.ton	Qty, M.ton	Qty, M.ton	Qty, M.ton
Gasoline	161,470	193,032	211,598	233,100
Diesel	1,402,848	1,322,548	1,558,342	1,668,493
Kerosene	553,780	613,735	700744	712,981
Total	2,118,098	2,129,315	2,470,684	2,614,574

Table 2.5-2 Ethanol blend data (Source: BDCD, MoWIE, 2014)

Year	**Ethanol production, Liters**	**Ethanol blend, Liters**	**Rmk**
2008/09	5,878,578	6,790,000	for year 2013, it does not mean there was no production, data was not available at time of compilation
2009/10	7,116,585	3,400,000	
2010/11	7 ,131,509	9,800,000	
2011/12	13, 811,953	10,650,000	
2012/13	6 ,535, 396	8,630,000	
2013/14	-	7,650,683	
Total sum	**40,474,021**	**46,920,683**	

Ethanol for fuel is not limited to transport sector, however the household sector which currently is dependent on wood, charcoal, and kerosene is in need of cleaner fuels such as Ethanol. Using Ethanol fuel for the household sector has started by distributing clean cook stoves but progress is not still attractive due to lack of sufficient ethanol supply availability within the country. so far about 6000 ethanol cook stoves within Addis Ababa city and Refugee camps. Some private cook

stove producers also emerged looking at the potential. Ethanol blend use is not being implemented on a continuous basis due to intermittent supply of ethanol from the sugar factors.

2.6. Bioethanol feedstock types

Bioethanol feedstocks can be conveniently classified into three types: (i) sucrose-containing feedstocks (e.g. sugar beet, sweet sorghum and sugar cane), (ii) starchy materials (e.g. wheat, corn, and barley), and (iii) lignocellulosic biomass (e.g. wood, straw, and grasses).

Sugar feedstocks -Fermentation involve microorganisms that use the fermentable sugars for food and in the process produces ethanol and other byproducts. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to ethanol. However, since sugar materials are in the human food chain, these materials are usually too expensive to use for ethanol production. One example of sugar feedstock is sugar cane. Other biomass feedstocks rich in sugars (materials known as saccharrides) include sugar beet, sweet sorghum, and various fruits. However, these materials are all in the human food chain and, except for some processing residues are generally too expensive to use for fuel ethanol production.

Starchy feedstocks - another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials can also be fermented after breaking starch molecules into simple glucose molecules. Examples of starchy materials commonly used around the world for ethanol production include cereal grains, potato, sweet potato, and cassava. Cereal grains commonly used in the US for ethanol production include maize and wheat. A bushel of maize grain (from 25.3 kg with 15% moisture can produce from 9.4 to 10.9 L of pure ethanol, depending on the technology used).

Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Typically, hydrolysis is performed by mixing the starch with water to form slurry which is then stirred and heated to rupture the cell walls. Specific enzymes that will break the chemical bonds are added at various times during the heating cycle.

Lignocellulosic feedstock- these are inexpensive and abundantly available feedstocks for bioethanol production, unlike sugar and starchy materials these are not in the human food chain. Examples of lignocellulosic materials are paper, cardboard, wood, and other fibrous plant material. Cellulosic resources are in general very widespread and abundant. For example, forests comprise about 80% of the world's biomass. Being abundant and outside the human food chain makes cellulosic materials relatively inexpensive feedstocks for ethanol production.

In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production is difficult because of its complex structure, which resists degradation. It consists of cellulose, hemicelluloses and lignin component. The secondary and tertiary conformation of Cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose resistant to hydrolysis.

2.7. Composition and Structure of Lignocellulosic biomass

Lignocelluosic materials predominantly contain a mixture of carbohydrate polymers (cellulose and hemicelluloses) which is known as holocellulose, lignin, extractives and ashes. The basic structure of all lignocellulosic biomass consists of three basic polymers: cellulose $(C_6H_{10}O_5)_x$, hemicelluloses such as xylan $(C_5H_8O_4)_m$, and lignin $[C_9H_{10}O_3$ _ $(OCH_3)0.9\_1.7]_n$ in trunk, foliage, and bark. (Wiselogel, 1996)

Cellulose is a linear polysaccharide of glucose residues connected by ß-1, 4 linkages. Cellulose molecules are completely linear and have a strong tendency to form intra and intermolecular hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of micro-fibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions. Many properties of cellulose depend on its degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. The DP of cellulose can extend to a value of 17000, even though more commonly a number of 800-10000 units is encountered (Kirk-Othmer, 2001). For in-stance, cellulose from wood pulp has a DP between 300 and 1700. The cellulose degree of polymerization (DP) is about (10,000), although chemical pulping reduces this greatly. Cellulose molecules form intra-and inter-molecular hydrogen bonds that result in highly ordered crystalline as shown Fig 2.7-1structure of Cellulose

a consequence of its fibrous structure and strong hydrogen bonds cellulose has a high tensile strength and is insoluble in most solvents. Orientation of the linkages and additional hydrogen bonding makes the polymer rigid and difficult to break (Hamelinck, 2005,) (Sjostrom, 1981).

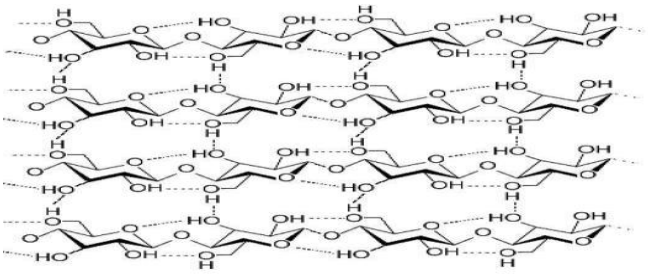

Fig 2.7-1structure of Cellulose

Hemicellulose is a short, highly branched chain of heteropolysaccharides (DP 100-200) built from hexoses (D-glucose, D-mannose, and D-galactose), pentoses (D-xylose, L-arabionose, and D-arabionse), and deoxyhexoses (L-rhamnose or 6-deoxy-L-mannose and rare L-fucose or 6-deoxy-L-galactose) as shown in **Error! Reference source not found.** Small amounts of uronic cids (4-O-methyl-D-glucoronic acid, D-galacturonic acid and D-glucuronic acid) are also present. (Kirk-Othmer, 2001). The composition of hemicellulose depends on the source of the raw material (Wiselogel et al., 1996). Hemicelluloses in hardwood contain mainly xylans (15-30 %) while in softwood galactoglucomannans (15-20%) and xylans (7-10%) predominant. The monosaccharides released upon hemicellulose hydrolysis include a large fraction of pentoses. The chemical and thermal stability of hemicellulose is lower than cellulose due to its lack of crystallinity and lower DP.

Hemicellulose(20-40 % of lignocellulose) extracted from plants possesses a high degree of polydispersity, polydiversity and polymolecularity (a broad range of size, shape and mass characteristics). However, the degree of polymerization does not exceed the 200 units whereas the minimum limit can be around 150 monomers.

Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders it soluble at elevated temperatures (Helsinki, 2003). The presence of acid highly improves the solubility of hemicellulose in water.

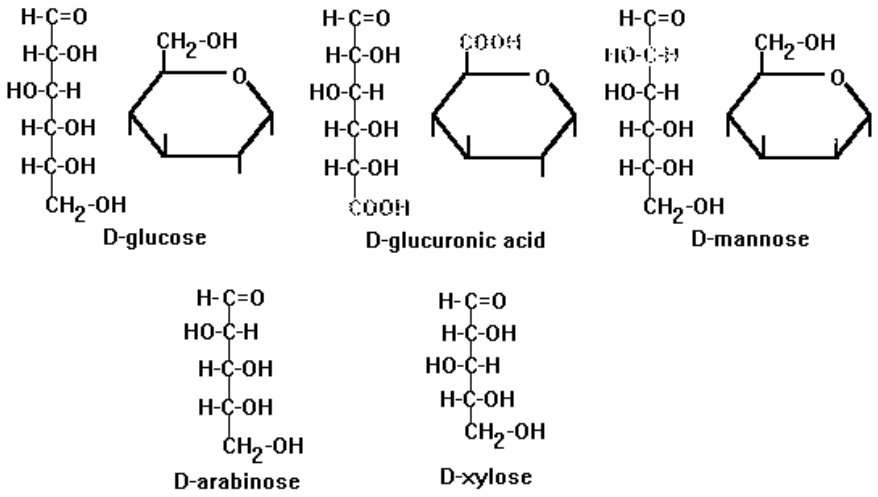

Fig 2.7-2 sturcture of Hemicellulose

Lignin is a phenylpropane-based polymer and is the largest non-carbohydrate fraction of lignocellulose. It is constructed of three monomers: coniferyl alcohol, sinapyl alco-hol, and coumaryl alcohol **.** Each has an aromatic ring with dierent sub-stituents. Unlike cellulose, lignin cann't be depolymerized to its original monomers.

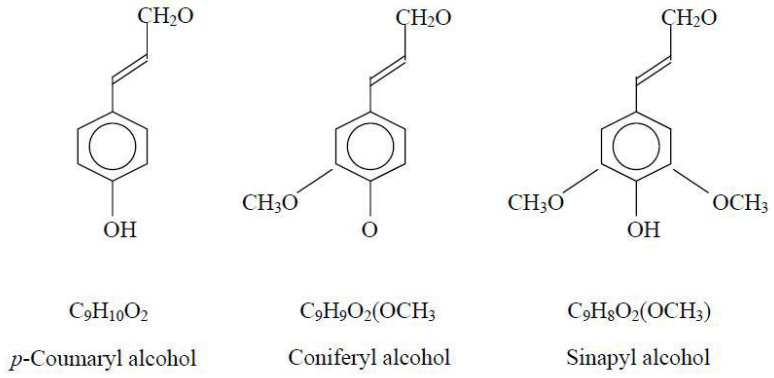

Fig 2.7-3 building blocks of Lignin

Lignin and hemicellulose form a sheath that surrounds the cellulosic portion of the biomass. Generally, structural features can be categorized into two groups: physical and chemical. Physical structural features include cellulose crystallinity, degree of cellulose polymerization, pore volume, accessible surface area and particle size. Chemical structural features include the contents of lignin, hemicellulose and acetyl groups.

Lignin in wood behaves as an insoluble three-dimensional network. It plays an important role in the cell's endurance and development, as it affects the transport of water, nutrients and metabolites in the plant cell. It acts as binder between cells creating a composite material that has a remarkable resistance to impact, compression and bending.

Extractives are wood compounds that are soluble in neutral organic solvents or water. The extractives usually represent a minor fraction between 1-5 % of lignocellulosic materials. They contain a large number of both lipophilic and hydrophilic constituents. Extractive includes terpenoids and steroids, fats and waxes, phenolic constituents and inorganic compounds. The amount of carbohydrate polymers and lignin depend on the type of materials. (Garrote, 1999)

2.8. Ethanol production technologies

Ethanol can be produced Either chemically, by hydration of ethylene, which is derived from crude oil or natural gas, or by fermentation of sugar containing feeds, starchy feed materials or lignocellulosic materials. Hydration of ethylene is represented by the following reaction:

$$C_2H_4 + H_20 - CH_3CHOH \text{ .....Eq. 2.8-1}$$

Petroleum ethanol product is made by the catalytic hydration of ethylene with sulfuric acid as the catalyst. It can also be obtained via ethylene or acetylene, from calcium carbide, coal, oil gas, and other sources.

Ethanol produced from biomass source is normally termed as Bioethanol to indicate that it is produced from renewable biomass source.

The two primary ways of producing fuel ethanol from cellulosic feedstock are: Biochemical conversion process and Thermo chemical conversion process (Jhonson, 2010). Ethanol for beverages, and fuel, is mainly produced thermo chemical conversion i.e fermentation.

$$C_6H_{12}O_6 - 2C_3HCH_2OH + 2CO_2 \text{.....Eq. 2.8-2}$$

2.9. Lignocellulosic biomass to Ethanol conversion technologies

Complex carbohydrates like cellulose and hemicelluloses are first converted into their component sugars through a hydrolysis process and then the sugars are anerobically fermented

into biofuel such as bioethanol. At present only the cellulose and hemicelluloses components of plant biomass can be converted into biofuels by the action of anaerobic microbes. The major obstacle in increasing biofuel production is to find an efficient way to break down complex plant polymers into simpler derivatives. Biomass first is ground into smaller particles or chips, these are then pretreated to breakdown the hemicelluloses and as well to unlock the cellulose.

Lignocellulosic biomass can be converted to ethanol using either a biochemical or thermochemical platform.

2.9.1. Thermochemical Technologies

Thermochemical transforms the lignocellulosic feedstock into carbon monoxide and hydrogen (syngas) by partial combustion .These gases can be converted to liquid transportation fuels or commodity chemicals by catalytic or biological pathways. The biological process converts carbon monoxide to ethanol using a non-yeast fermentation microorganism (eg Clostridium ljungdahlii). Alternatively, the syngas can be fed to a catalytic reactor where the carbon monoxide and water are combined via a metalcatalysed process to produce ethanol, other higher alcohols and liquid fuels (Fischer-Tropsch liquids). Gasification is important because lignin, which constitutes about 25 – 30% of cellulosic biomass, is also converted to syngas.

2.9.2. Ethanol production (Biochemical method)

In biochemical conversion the plant fibre is separated into its component parts; cellulose, hemicelluloses, and lignin hence the term lignocellulosic or cellulosic ethanol. The cellulose is then further broken down to simple sugars that are fermented to produce ethanol. Typically the process is carried out in four stages

1. Physical and chemical pretreatment of the plant fibres to expose the holocellulose (cellulose and hemicelluloses) and reduce cellulose crystallinity,
2. Hydrolysis of the cellulosic polymer, with enzymes or acids, to monomer sugars such as glucose, galactose, xylose etc.
3. Microbial fermentation of these simple sugars to ethanol, and
4. Distillation to produce 99.5% pure alcohol.

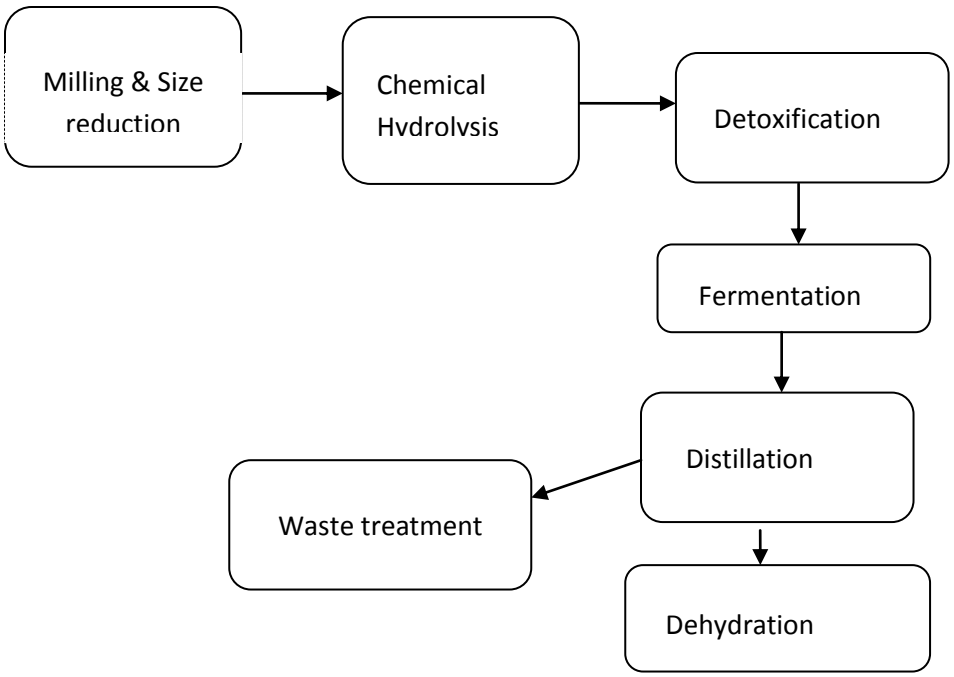

Fig 2.9-1 Lignocellulosic to ethanol processing scheme via chemical hydrolysis

2.9.2.1. Pretreatment

Pretreatment of lignocellulose has received considerable research globally due to its influence on the technical, economic and environmental sustainability of cellulosic ethanol production. Some of the most promising pretreatment methods require the application of chemicals such as acids, alkali, salts, oxidants, and solvents. Thus, advances in research have enabled the development and integration of chemical-based pretreatment into proprietary ethanol production technologies in several pilot and demonstration plants globally, with potential to scale-up to commercial levels.

Without any pretreatment, the conversion of cellulose to sugar is extremely slow, since cellulose is well protected by the matrix of lignin and hemicelluloses in macrofibrils. Therefore pretreatment of these materials is necessary upstream process to enhance porosity of the lignocelluotic material for enzyme accessibility and to remove lignin in order to increase the rate of hydrolysis of cellulose to fermentable sugars prior to the enzymatic process (Galbe, 2002) (Kumar, 2009) .

2.9.2.2. Hydrolysis

The process converting the biomass biopolymers to fermentable sugars is called **hydrolysis**. There are two major hydrolysis methods: Chemical hydrolysis and enzymatic hydrolysis. The hydrolysis of cellulose and hemicelluloses can be carried out chemically by dilute acids such as sulfuric acid or with use of enzymes. The first and older method uses acids as catalysts, second method uses enzymes called cellulases. The chemical hydrolysis is described in detail in this thesis as it is the hydrolysis method involved in this project.

In addition, there are also some hydrolysis processes which don't use chemicals or enzymes such as hydrolysis by gamma ray, electronic beam irradiation or microwave irradiation, however, these processes are still under research and far from commercial application. (Taherzadeh M. , 1999).

Complete hydrolysis of cellulose results in glucose, whereas the hemicellulose gives rise to several pentose and hexoses. While softwood hemicelluloses are mainly composed of mannose, the dominant sugar in hemicelluloses derived from hardwood and crop residues is usually xylose (Karimi., 2006b) (Taherdadeh, 1997a)

There are several advantages and disadvantages of dilute acid and enzymatic hydrolysis. See Table 2.9-1) Enzymatic hydrolysis is much more efficient than acid hydrolysis with a high possibility of close to hundred percent conversion of cellulose to monomeric sugars under mild condition where as acid hydrolysis require either highly concentrated acid or high temp situation and even less yield when compared with enzymatic hydrolysis. Furthermore, inhibitory products by enzymatic hydrolysis are low. However, the cost of enzyme is very high as compared to cost of acid. (Taherzadeh & Karimmi, 2007)

2.9.2.2.1. Enzymatic hydrolysis

Enzymatic hydrolysis is carried out by cellulase enzymes which are highly specific, and the products of the hydrolysis are usually reducing sugars including glucose. Unlike chemical hydrolysis, enzymatic hydrolysis is conducted at mild conditions at a pH of 4.8 and temperature of 45-50 _C, which is optimum for the cellulase enzyme.

Enzymatic hydrolysis methods have shown distinct advantages over acid based hydrolysis. The very mild process conditions give potentially higher yields, the utility cost is low (no corrosion problems). Therefore, this is the method of choice for future cellulosic-to-ethanol processes

(Duff, 1996). But the process takes several days whereas it is only a few minutes in the case of chemical hydrolysis. Moreover, the final product of enzymatic hydrolysis inhibits the enzyme and ultimately affects the process unless they are removed immediately after they are formed. Apart from this, a major bottleneck in lignocellulosic ethanol production, at present, is the cost of the enzymes.

2.9.2.2.2. Chemical hydrolysis

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicelluose polymers. Acids are predominantly applied in chemical hydrolysis.

Acid hydrolysis can be divided into two groups: concentrated acid and dilute acid hydrolysis. Concentrated hydrolysis is comparatively an old process and generally are reported to give higher monomer sugar yield even 90 % of the theoretical yield and it can be processed under low temp such as 40 0c. However the high concentration of acid make it corrosive and neutrazation step produces large amount of gypsum.

Dilute acid hydrolysis can be used either as a pretreatment preceding enzymatic hydrolysis or as the actual method of hydrolysis of lignocelluose to its monomers (MANDERSON, 1995). Dilute acid processes are conducted under high temperature and pressure, and have reaction times in the range of seconds or minutes, which facilitates continuous processing. A main drawback of dilute acid process, particularly in single stage is degradation of the monomer sugars and formation of products which lower yield of sugar and as well are inhibitory to fermentation process to ethanol. These inhibitors are HMF (hydroxymethylfurfural), Furfural,Levulinic acid,acetic acid, formic acid, uronic acid, 4-hydroxybenzoic acid, vanillic acid,vanillion,cinnamhaldehyde, formaldehyde, phenol etc (Taherzadeh M. , 1999). Some inhibitors exist in the feedstock but most are formed during hydrolysis process.

Two stage dilute acid process minimizes formation of these degradation products. In the first stage much of hemicelluloses sugars are released which comprises both pentose and hexose sugars where as the second stage releases more glucose of the cellulose part. But there is still a potential to release glucose (40-60 %) from cellulose in the first stage.

Most dilute acid processes are limited to a sugar recovery efficiency of around 50%. The reason for this is that at least two reactions are part of this process. The first reaction converts the cellulosic materials to sugar and the second reaction converts the sugars to other chemicals. Unfortunately, the conditions that cause the first reaction to occur also are the right conditions for the second to occur. Thus, once the cellulosic molecules are broken apart, the reaction proceeds rapidly to break down the sugars into other products—most notably furfural, a chemical used in the plastics industry. Not only does sugar degradation reduce sugar yield, but the furfural and other degradation products can be poisonous to the fermentation microorganisms.

Table 2.9-1 Comparison of Acid hydrolysis vs enzymatic hydrolysis

Variables	Dilute acid	Enzymatic
Hydrolysis condition	high temp, pressure required	Mild
Hydrolysis yield	Comparably low	Close to 100%
Product inhibition to hydrolysis process	Yes	No
Formation of inhibitory by products	Yes such as	No
Time of hydrolysis	Relatively short	Long period required- several days may be required
Cost of catalyst	Cheaper	Expensive

2.9.2.3. Chemical hydrolysis processes and reactors

Batch reactors are used for hydrolysis in lab scale and pilot plants. However, plug flow, percolation, concurrent and shrinking bed reactors are being investigated for dilute acid hydrolysis process. Much improvement was obtained with higher yield of conversion (50-60%) and shorter retention time even less than 30 second with use of plug flow reactors. However, much improvement was not possible due to difficulty in control of retention time and heat transfer limitation within the biomass particles (Lee, Iyer, & Torget, 1999). Percolation reactor is also attractive as high solid/ liquid ration can be used and there is no need to separate the liquid and the solid. Upto 95% xylose was obtained using this reactor. Countercurrent reactors were also found to minimize sugar degradation and product dilution by removing sugars from the reaction zone before substantial degradation occurs and consequently raising the yield and

concentration of sugar. Use of shrinking bed reactor have sugar yield higher than 95% from hemicelluloses and 85% from cellulose in a multiple percolation reactor system.

2.9.2.4. Factors influencing hydrolysis yield

The following are factors which influence hydrolysis yield:-

- Feedstock type or substrate(composition of material), degree of polymerization of cellulose, configuration of cellulose chain and association with other prote tive polymeric structures witin such as lignin, hemicellulose pectin, mineral, proteins etc
- reaction Time
- Temperature
- Hydrolysis Chemical concentration and diffusivity
- Solid to liquid ratio/ dispersion in the reactor
- Neutralizing capacity of the lignocelluloses
- Movement of solution during heating
- Surface area to volume ratio
- Released sugar concentration(higher concentration in excess of 10% may result in reversion phenomena whereby much of the glucose present may not free but as a dimer, oligomers and anhydrosugars which are not usable in the fermentation process (Harris, 1984)
- Presence of metals and metallic ions which can catalyzes glucose decomposition (Xiang, 2004)

2.9.2.5. Hydrolysis products and by products

The anticipated products of hemicellulose hydrolysis are glucose, galactose , xylose and arabinose (ARA,) and these were indeed detected in the reaction mixtures. The C6-sugars from the hemicellulose fraction (glucose and galactose) are expected to be converted to HMF and subsequently to LA. Both C5-sugars (xylose and arabinose) are known to be decomposed to furfural (FUR,). The furfural concentration also shows an optimum with respect to reaction time, indicating subsequent reactions under these conditions. Acetic acid is most likely formed from the hydrolysis of the acetyl groups present in the hemicelluloses.

The cellulose in the water hyacinth is broken down into low molecular weight fragments and ultimately to glucose by the action of the acid catalyst. Subsequently, the glucose is decomposed to 5-hydroxymethylfurfural (HMF), which is further converted in a serial mode to LA and formic acid .

The rate of sugar decomposition under dilute acid hydrolysis condition for instance (0.8% acid at 180 oc is ordered as stated by (Xiang, 2004) and cited by Bioresource.

Xylose> arabinos > mannose>Galactose> Glucose

Therefore, xylose is more sensitive to high acidity and high temperature condition decomposing to furfural. On the other hand glucose is more resistant to harsh conditions.

2.9.3. Fermentation

Fermentation of lignocellulosic hydrolyzates is more difficult than traditional fermentation systems from grains or sugar cane due to the fact that hydrolyzates contain different inhibitor compounds depending on the type of lignocelluosic materials and on the chemistry and nature of pretreatment and hydrolysis processes. Moreover. Hydrolyzates contain different types of sugars coming from the hemicellulose (mixture of C5 and C6 Sugars)which require different types of ferementing micro organisms. For instance the most common yeast type which is used in the conventional ethanol production technology which is known as Saccharomyces Cerevisiae can't ferment xylose which is one of the monomers obtained in hemicelluloses hydrolysis.

the conversion of lignocellulosic material to sugar and then to ethanol is governed by the following equation

$$(C_6H_{10}O_5)_n + nH_2O - nC_6H_{12}O_6 + yeast - 2nC_2H_5OH + 2nCO_2 \quad \text{Eq. 2.9-1}$$

According to the reactions, the theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide CO2 per kg of xylose and glucose (Hamelinck, 2005,).

2.9.3.1. Fermenting micro organisms

A large number of yeasts, bacteria and filamentous fungi are reported to produce ethanol as the main ferementation product. None of these microorganisms naturally meets all the requirements for the lignocellulosic ethanol production. Microorganisms for bioethanol fermentation can best

be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment.

Photosynthetic processes in plants produce simple and complex sugars, which can be decomposed by fermentation in the presence of micoorganisms such as Escherichia coli, Klebsiella oxytoca, Saccharomyces cerevisiae, and Zymomonas mobilis (Dien, MA, & TW, 2003) to produce bioethanol.

One of the major challenges in producing ethanol from the fermentation of lignocellulosic materials is difficulty of fermenting xylose with yeast, S. Cervisiae which is widely used microorganism in ethanol production. This yeast does not have genes encoded for xylose reductase(XR) and xylitol dehdrogenase(XDH) and can't utilize xylose (Jeffries T.W and Jin Y.S, 2004). There have therefore been intensive efforts to introduce other wild type organisms that can utilize xylose and produce ethanol or genetically modify the organism for this purpose. There are severally naturally occurring ethanol producing bacteria,yeast and fungi that utilize xylose such as yeast species, candida, pichia, schizosaccharomyces, kluveromyces and pachysolen, fungi of species Fusarium, Mucor, Ryzopus, Monilia and paecilomyces and bacteria of species Clostridium, Bacillus, Bacteroides, Thermoanaarobacter and Ervinia (Abbi, 1996) (Flores, 2000).The majority of organisms cannot tolerate bioethanol concentrations above 10–15 % (w/v) (Hettenhaus, 1998).

2.9.3.2. Effect of influencing factors in Fermentation

The performance parameters of fermentation are temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas, 2004).

High sugar concentration by default is expected to give high ethanol yield however, high sugar concentration inhibit microbial metabolishm by increased osmotic pressure. According to some research a sugar concentration of 10-18% is usually satisfactory, although other concentrations are used (Dunn, 1959).

Presence of Inhibitory compounds, different fermentation techniques and presence of different types of monomer sugars have effects on the fermentation yield. Different inhibitors may dominate in terms of concentration but interaction and combined action is key for the inhibitory actions (Clark & Mackie, 1984) Acetic acid is not the by product of hydrolysis process but it is

also a byproduct of ferementation process. Acetic acid production is not dependant on the severity of hydrolysis process as it is formed from acetylated hemicellulosic sugars which require mild processing conditions. Acetic acid can be formed even higher than 10 g/l (Taherzadeh.M, 1997b). Undissociated acetic acid is the one to have effect on ferementation. The dissociation occurs after the undissociated acetic acid diffuse throught the cell member and this lowers the pH. Saccharomyces Services tolerates a maximum 5g/l concentration of acetic acid (Taherzadeh.M, 1997b). Cell growth is more sensitive to the presence of furfural than is the ethanol production from glucose. Furfural can be converted by the yeast to less inhibitory compounds which are furfural alcohol and furoic acid (Taherzadh, Niclasson, & Liden, 2000c) HMF is not as severely toxic to S.cerevisiae as furfural (Taherzadeh, 2000b).

As the conversion of furfural is four times higher than HMF which means furfural stays longer in the medium. HMF is major degradation product of hexoses but subsequently levulinic and formic acids predominates (Harris, 1984). A large number of phenolic/aromatic compounds have been detected in dilute acid hydrolyzates (Larsson C, 2000) which are believed to be degradation products of lignin and or which are present as extractives. Transformation of aromatic compounds occurs during fermentation, phenolic compounds are also important inhibitors, however, they can be assimilated by S. cerevisiae in the fermentation process (Delgene's, 1996). Concentration of the phenolic/aromatic compounds are few milligrams per liter probably due to low water solubility of many of the phenolic compounds or to a limited degradation of lignin during the hydrolysis process.

Several of the inhibitory compounds found in hydrolyzates can be biotransformed or in a few cases can fully be metabolized by yeast. This implies possibility of in-situ detoxification of the hydrolyzate during the fermentation. Therefore, ferementation technique also influence ethanol yield. For instance, too high a feed rate (with higher concentration of inhibiting compounds limits cell growth and production of ethanol. Therefore an optimum feed rate must be identified for a particular hydrolyzate in order to minimize inhibition effect for batch fermentation.

Adaptation of microorganisms to the fermentation media is also a factor which influence yield of ethanol production by fermentation. This can be taken as an alternative approach to detoxification for increasing ethanol production.

2.9.4. Product recovery by Distillation

A distillation system separates the bioethanol from the fermentation broth. Large quantities of energy are required to concentrate the ethanol to 95.6% (azeotrope mixture of ethanol with water).

The first step is to recover the bioethanol in a distillation or beer column, where most of the water remains with the solids part. The product (37% bioethanol) is then concentrated in a rectifying column to a concentration just below the azeotrope (95%) (Hamelinck, 2005,). Therefore in simple distillation maximum concentration achievable is 37% bioethanol concentration.

Bioethanol from cellulosic biomass has likely lower product concentrations (5 wt %) than in bioethanol from corn. The maximum concentration of bioethanol tolerated by the microorganisms is about 10 wt% at 303 K but decreases with increasing temperature.

2.10. Lignocellulosic to Ethanol Technologies Technical challenges

2.10.1. Thermochemical

Contamination – various components of the biomass feedstock can cause problems in the gasification and catalytic synthesis stages. Contaminants such as tars and inorganic components (Halides, alkalis, ash) present in the syngas can deactivate the catalysts and must be removed prior to catalytic conversion.

The formation of tars, and measures to deal with their removal, are significant challenges in biomass gasification. Advances in catalyst preparations are also needed in order to make large scale biomass to liquid facilities practical.

Ethanol yields up to 50% have been obtained using synthesis gas-to-ethanol processes. Some processes that first produce methanol and then use catalytic shifts to produce ethanol have obtained ethanol yields in the range of 80%. Unfortunately, like the other processes, finding a cost-effective all-thermochemical process has been difficult.

2.10.2. Biochemical

Pretreatment - the usefulness of cellulose as a feedstock has been limited by its rigid structure and difficulty to breakdown into simple sugars. Cost-effective pretreatments are needed to liberate the cellulose from the lignin/hemicellulose matrix and reduce its crystallinity. Pretreatments of increasing severity are needed as feedstock recalcitrance increases from nonwoods (agricultural residues) to hardwoods to softwoods.

Many pretreatments are currently being explored, ranging in chemistries from very acidic to mildly alkaline, such as dilute acid, ammonia fibre expansion (AFEX), wet oxidation, organosolv, steam explosion. The ideal pretreatment must also minimize the formation of degradation products that can inhibit the subsequent hydrolysis and fermentation processes.

Lignin – As lignin is mainly responsible for lignocellulosic recalcitrance, particularly in softwoods, studies have shown its separation during pretreatment greatly enhances cellulose accessibility and enzyme effectiveness. Pretreatments that minimise lignin redeposition and condensation on the fibre surfaces are favoured. Separation of lignin and production of specialty lignin co-products also has the potential to improve the overall economics.

Hydrolysis - Cellulose is broken down into individual glucose units by chemical and enzyamatic hydrolysis. Enzymatic hydrolysis uses cellulase enzymes, under mild conditions. Research is ongoing to find cost-effective enzyme systems that produce high sugar yields at accelerated rates and without the formation of inhibitory byproducts. Currently, per unit cost of enzymes is considered to be a deterrent to the commercial success of the biochemical pathway. Alternative strategies to reduce enzyme cost include the recycling of enzymes and the use of polymers to reduce the binding of enzymes to the substrate (MABEE, 2006).

Fermentation - The hydrolysate contains both 5-carbon (pentose) and 6-carbon (hexose) sugars. The conversion of pentose sugars into ethanol is less efficient than conversion of hexose sugars. A system of mixed-sugar fermenting microorganisms is required to utilise the full range of sugars present and thus maximise the production of ethanol. Metabolic engineering is on-going to find low-cost, microorganisms capable of C5 and C6 sugar co-fermentation that are also resistant to inhibitors (acetic acid, furfural) that may be present. (BECA.ORG)

3. Materials and Methods

3.1. Materials and chemicals

3.1.1. Chemicals and reagents

Sulfuric Acid, Sodium hydroxide, DW

Yeast Sacharomacies cervisiea, Yeast extract, Peptone and dextrose (YEPD), urea, Magnesium Sulphate

For cellulose and lignin determination- Dintrosalsilic acid(DNS), DW, D glucose

For Ethanol Determination- Potassium Dichromate, DW, anhydrous ethanol

3.1.2. Equipments and instruments

Cutting mill

Sieves

Vertical Autoclave – for hydrolysis and sterilization of equipments

K2Vaccum filter

Simple distillation set up

PH-Meter -to adjust the pH of the hydrolyzates before fermentation Shaking incubator

Digital balances

Shaker (adjustable temperature and speed) for fermentation

Magnetic stirrer- for neutralization

Digital spectrophotometer- for determination of glucose and ethanol

3.2. Methods

3.2.1. Feedstock collection and experimental design

Whole, fresh Water Hyacinth plants were collected from river Awash was washed with tap water and leaves and stalks were dried in sunlight after cutting out the roots and used as feedstock. River awash is one of the big and fresh water rivers in Ethiopia and which is mainly used for irrigation Agriculture. It is found in the rift valley. Its course is entirely contained within the

boundaries of Ethiopia and empties into a chain of interconnected lakes that begin with Lake Gargori and end with Lake Abbe.

Response Surface Methodology (RSM) with Central Composite Design (CCD) was used to optimize the hydrolysis condition of WH. The design matrix of the variables in the coded and real units was depicted in Table 3.2-1 with the experimental values of total sugar and ethanol concentrations as responses.

Central composite design is a general series of experiments that have been developed to efficiently serve as a base for deriving the mathematical model to estimate the coefficients of a quadratic model. It contains an embedded factorial or fractional factorial design with center points that is augmented with a group of (star points) that allow estimation of curvature.

Table 3.2-1 Factors and Values in the Experimental Run

Variable	Unit	Min	Maximum	Coded, min= -1,Cpt = 0, max = +1		
Acid Conc	% V/V;	2	5	-1=2	0= 3.5	+1=5
Temp	oC	116	130	-1= 116	0=123	+1=130
Time	Min	16	60	-1= 16	0= 38	+1= 60

3.2.2. Substrate preparation and pretreatment

Only the shoots and leafy part of the water hyacinth plant were used. Leaves and petioles were washed manually with tap water to remove the waste particles and were chopped into small pieces (1-2cm) and is dried on open air. Then the cleaned and dried WH was ground into powdered form. The dried powder material was reserved at room temperature for further work.

3.2.3. Hydrolysis

Hydrolysis experiments were carried out in 500 ml Erlenmeyer flasks with a constant solids concentration of 12%, acid concentration (2-5%) and reaction time (16 – 60 minutes) and temperature ranging from 116 – 130 ^{O}C according to central composite experimental design and using Design Expert 7 Soft ware. Response surface optimization was used as it is more advantageous than the traditional single parameter optimization due to its time saving. In each

case total of 31 run was conducted with 3 replicates at center point and 2 replicates each for axial and factorial points. Optimal yield of reducing sugar was evaluated for ethanol production.

In this experiment 25 gm open air and sun dried Water hyacinth was used and to achieve 12% solid concentration 300 ml of distilled water was used. In order to have uniform mixing first about half of the water was poured out on to the measured biomass and then an acid amount to bring about 2%-5% acid concentration was poured into the remaining half of the hydrolyzing water was added then for each run. Since water hyacinth is a bulk biomass, it takes quite some time in order to have uniform soaking and therefore was it was allowed for 1hr soaking before putting into the autoclave for each respective run.

3.2.4. Hydrolysate separation and preparation for fermentation

After each hydrolysis, the sample was filtered using vacuum filter and the hydrolysate (the liquid part) was used for fermentation. Hydrogen ions (H+) in a fermentation broth affect yeast growth, ethanol production rate, byproduct formation, and bacterial contamination control. If the pH value is less than five during fermentation, bacterial growth is severely repressed. The pH value range for growth of most strains of *sacharomyces cerevisiae* is 2.4-8.6, with an optimum of 4.5. Yeast sugar fermentation rates are relatively insensitive to pH values between 3.5 and 6. Before the fermentation experiment was conducted, the PH was adjusted using 10N NaOH until the PH became 5 as it is considered suitable for the fermenting micro. Yeast and fungi tolerate a range of pH 3.5–5.0 (Aminifarshidmehr, 1996)

3.2.5. Fermentation and Distillation

Saccharomyces cerevisiae was purchased from commercial Baker's yeast seller found in Addis Ababa was used for the ethanol fermentation. Inoculum was prepared by transferring some cells into 250 ml flask containing 50 ml of culture medium containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose and was subsequently incubated at 30 oC for 24 h. This was used to inoculate the fermentation medium. The inoculums to solution ratio of 1:10 were used for fermentation purposes. Fermentation was allowed for 3 days (72 h) at 30ºC with 150 rpm.

After the fermentation, product was distilled using oil bath to separate the alcohol. Simple distillation technique was used as shown in the picture below (fig 3.2.1).

Fig 3.2-1 Distillation set up

3.3. Analytical Methods

3.3.1. Compositional analysis

3.3.1.1. Moisture content and Total Solids determination

Moisture content was determined after drying of 1000 gm fresh water hyacinth (roots cut out) the WH sample in open air to constant Weight. The initial weight minus the final weight gives the moisture content. The final weight is taken as the total solids. (Sluiter, Version 07-08-2011)

$$\boldsymbol{Moisture\ Content}\ \% = \frac{Wi - Wf}{Wi}\ \boldsymbol{X}\ \mathbf{100}\% \quad \text{.....Eq. 3.3-1}$$

Where, Wi, weight of sample before drying (g),

Wf, weight of sample after drying

3.3.1.2. Determination of extractives

A 10 g of the dried Water Hyacinth sample was placed into the timble which was plugged with small amount of cotton and placed in a soxhlet extraction tube (fig 3.3-1). The boiling flask contain 2:1 solvent mixture containing 95% ethanol alcohol and 5% distillted toluene was used and the extraction was conducted for 8hrs. The whole procedure was done according to ASTM E:1690

$$\boldsymbol{Ethanol\ toluene\ extracted}\ \% = \frac{M1 - M2}{M1}\mathbf{100} \quad \text{.....Eq. 3.3-2}$$

Where M1 = total dried water hyacinth with the solvent and

M2= is the final weight after extraction for 8hrs.

Fig 3.3-1 Extraction processes of WH Extractives

3.3.1.3. Ash content

Ash content as expressed as the percentage of residue remaining after dry oxidation (oxidation at 550 to 600 ^{o}C). (Sluiter, Version 07-08-2011). Crucibles were initially dried and weighed according to the procedure and 2gm of the WH sample was put into the dried and tared crucibles and this was put into a furnace set at 575 ^{o}c. Three replicates were taken and the average ash content was determined after 4hrs. The ash content is determined by taking the final weight over the initial.

$$\boldsymbol{Ash\ content}\% = \frac{\boldsymbol{wt.crucible\ and\ ash - wt.crucible}}{\boldsymbol{Oven\ Dried\ wt.(ODW)}}\ \boldsymbol{X\ 100}\ldots\ldots\ldots\ldots\ldots\text{Eq. 3.3-3}$$

Where, Oven Dried Wt is calculated as follows as per summative closure lab analytical procedure (LAP) in NERL Procedure.

$$\boldsymbol{ODW} = \frac{\boldsymbol{wt.air\ dried\ sample\ X\ \ Total\ solid}\%}{\boldsymbol{100}}\ldots\ldots\ldots\ldots\ldots\text{Eq. 3.3-4}$$

In which case, Total solid% needs to be determined again by the following equation,

$$\boldsymbol{Total\ Solid}\ \% = \frac{\boldsymbol{wt.dry\ pan\ plus\ dry\ solids - wt\ dry\ pan}}{\boldsymbol{wt.sample\ as\ recieved}}\ \boldsymbol{X\ 100}\ldots\ldots\ldots\ldots\ldots\text{Eq. 3.3-5}$$

3.3.1.4. Cellulose Determination

Extractive free water Hyacinth sample was used in order to determine the cellulose content according to Kurschner-Hoffner approach. Accordingly, 2g extractive free water hyacinth sample was treated with 50ml of alcoholic nitric acid solution under reflux during four cylces of 1hr and after each cycle, the solution was removed for a fresh volume. The alcoholic nitric acid solution consisted of mixing one volume of solution of nitric acid (68%(w/w) with four volume of 97% ethyl alcohol. Then the precipitate obtained was washed and dried and weighed in order to determine the Cellulose according to the following equation;

$$\boldsymbol{Cellulose}\ \% = \frac{\boldsymbol{C(g)}}{\boldsymbol{EM(g)}}\ \boldsymbol{X}\ \mathbf{100}\% \quad \text{.....Eq. 3.3-6}$$

Where, C= Washed and Dried cellulose residue

EM= Extractive free sample

3.3.1.5. Determination of Kalson lignin

The Lignin content of WH was determined according to NERL procedure and accordingly about 900mg of the sample was measured into a beaker and 9ml of 72% sulfuric acid was added and was mixed with a Teflon stir rod to mix thoroughly. This was placed into a water bath set at 30 ^{o}C and incubated for 1hr with frequent stirring of every 5 minutes without removing from the bath. After completion, the acid was diluted to 4% concentration by adding 252 ml deionized water and was put into autoclave set at 121 ^{o}C for 1hr. then the material was removed and filtered in order to determine the Kalson lignin.

The precipitates were collected with sintered glasses (4G) by suction filtration and washed with water. The sinters with the acid insoluble lignin (Klason lignin) were dried at 103°C, cooled in desiccator and weighed and klason lignin was determined as follows;

$$\boldsymbol{Kalson\ lignin}\ \% = \frac{\boldsymbol{P(g)}}{\boldsymbol{M(g)}} \quad \text{.....Eq. 3.3-7}$$

Where, P=precipitate [g], and M= dry weight of extracted sample [g].

3.3.1.6. Determination of Hemicelluloses

Indirect means of quantifying technique was used to determine the quantity of Hemicelluloses. Even though direct measurement of Hemicelluloses is not conducted and that the Holocellulose (Cellulose and Hemicelluloses) content was estimated as total reducing sugar by the DNS solution from the hydrolysate obtained during lignin determination whereby it is expected that both hemicelluloses and cellulose are hydrolysed to RS. Therefore, measuring these RS is expected to give total content of both hemicelluloses and hemicellulose and that percent of Cellulose content was already determined , the rest fraction was expected to be hemicellulose.

3.3.2. Reducing sugar determination after acid hydrolysis

3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959) was used to determine the Reducing sugars released after acid hydrolysis. 3, 5-dinitrosalicylic acid 10.0 g, Na2SO3- 0.5 g ,Na-K tratrate - 182.0 g, NaOH- 10.0 g, Phenol - 2.0 g, Deionized water 1000 ml.

10 g of NaOH were added into 700 ml of deionized water and mixed in order to add the 300 g Na-K tratrate. When the solution dissolved, 10 g 3, 5-dinitrosalicylic acid was then added and continuously stirred. After that, the 0.5 g of Na2SO3and 2.0 g of phenol was dissolved, respectively. Finally, the volume was adjusted to 1,000 ml by deionized water and kept away from light.

Reducing sugar determination procedure:

0.5 ml from each sample from each run was mixed with 0.5 ml of DNS solution. The mixture was boiled for 10 min.

The sample was cooled down by immersing the sample tube into cold water immediately. Five ml of water was added into the prepared samples. The mixture was mixed well, and measured at absorbance 540 nm.

Absorbance at 540 nm was converted to glucose or galactorunic acid concentration with the standard curve prepared.

Standard curve to be used in the determination of RS was developed as follows; stock solution of 0.9 g/100ml using D- glucose was prepared, then 200µL, 300 µL, 400µL were taken in a

separate tube and were diluted with 5ml distilled water. These gave concentration of 0.36mg/ml, 0.54mg/ml and 0.72mg/ml which where the absorbance of each concentration was reading using digital spectrophotometer. The dilution factor taken was 25.

3.3.3. Evaluating performance of Acid hydrolysis

The performance of diluted acid hydrolysis was calculated as follows:

$$Hydrolysis\ yield = \frac{Rs\ \ g\ *0.9}{Cellulose\ \ g\ + Hemicellulose(g)} * 100 \quad \text{.....Eq. 3.3-8}$$

The 0.9 value corrects the contribution of a mole mass of water for each hydrolyzate glycosidic bond.

3.3.4. Determination of Ethanol

Ethanol content was determined by Dichromate assay that is, 0.25 M potassium dichromate solution and 6M sulfuric acid (H2 SO4) were prepared for subsequent use in ethanol content determination. Reaction of potassium dichromate with the presence of dilute sulfuric acid produces chromium (III) sulfate which is a blue green compound which has maximum absorbance at 560nm. The reaction is expected to be as follows:-

2K2Cr2O7 + 8H2SO4 + 3CH3CH2OH → 2Cr2 (SO4)3 + 2K2SO4 + 3CH3COOH + 11 H2O

Orange yellow **Blue Green**

Potassium Dichromate **Chromium(III)**

This method was used to determine ethanol content of each run using a calibration curve prepared as follows: alcohol concentration of 2.5%,5%, and 10% were prepared and 1ml is drawn from each solution and was mixed to react with a 5ml potassium dichromate and 5ml sulfuric acid which were prepared as described above and after 5 minutes of reaction 39ml of distilled water was added and reading was taken with a digital spectrophotometer set at 560nm which was the maximum absorbance for the blue green color generated by the reaction and with calibration curve the concentration of the ethanol was determined for each run.

Fig 3.3-2 Ethanol content determination

3.3.5. Evaluating Ethanol production efficiency

Ethanol yield was calculated based on the available glucose. It was expressed on either molar or weight basis. The relative ethanol yield (efficiency) was then calculated as a ratio of the ethanol yield and the theoretical maximal yield (0.51 g/g) (Hettenhaus, 1998).

$$\boldsymbol{FE}\ \% = \frac{\boldsymbol{Ethanol\ yield\ obtained}}{\boldsymbol{theoretical\ maximum\ ethanol\ yield\ from\ cellulosic\ material}} \times \mathbf{100}\% \ \textbf{……..3.3-9}$$

Where, FE is Fermentation Efficiency

4. Result and Discussion

4.1. Characterization of Water Hyacinth

The result of the compositional analysis of the Water Hyacinth are presented Table 4.1-1

Table 4.1-1 summary of compositional analysis of WH

	Constituents	% weight
	Total Solids(TS)	7
	Moisture	93
	Ash (% of TS)	10.2
	Extractives(% of TS)	13
	Cellulose(% EM)	30
	Hemicellulose(% of EM)	48
	Kalson lignin(% EM)	5

Note that percent of cellulose, hemicelluloses and lignin are obtained and calculated from Extractive free sample basis denoted by (EM) throughout the document. Therefore, these quantities may show lower value if taken from the total solid.

The Average ash content is 10.2 %, Compared to ash content from literatures for instance, the Work of Hector and his team has reported ash content of 19.1% and 22.9% percent from two WH samples taken from two river and discussed that these results are higher than determined by (Nigam, 2002) where he reported 15.1 and reasoned out that higher result might be due to contamination of the Water resources. This work shows relatively lower yield but the Ash content is still higher when compared to other biomass such as corn stover. Therefore, probably this shows that contamination of River Awash is lower than those rivers where the researchers have conducted their compositional analysis of Water Hyacinth.

Cellulose content is higher than reported by Nigaam (2002) but near to (Abraham & Kurup, 1996) result which is 35% and that of Alfaro's and his teams' work, (Alfaro, 2013) which is 31.67 %

The present result has shown that WH collected from Awash River has 5% lignin content. In line to the present result several research report has recorded lignin content 3.5 %- 4.4%. However Gunnarsson & Petersen, 2007 has recorded lignin content of 9.3% which is different from the present finding. This may be due to the fact that Acid Soluble Lignin (ASL) determination is not

included in the present work. If this has been worked out it can be seen that even higher Lignin content may be reported.

Even though direct measurement of Hemicelluloses is not conducted and that the Holocellulose (Cellulose and Hemicelluloses) content was estimated as total reducing sugar by the DNS solution from the hydrolysate obtained during lignin determination whereby it is expected that both hemicelluloses and cellulose are hydrolysed to RS. Therefore, measuring these RS is expected to give total content of both hemicelluloses and hemicellulose and that percent of Cellulose content was already determined , the rest fraction was expected to be hemicellulose.

This result confirms that WH has high hemicellulose which is reported by many researchers such as (Nigam, 2002) and as reported by Hector and the team (Perez, 2013).Much of the research shows that the water hyacinth hemicellulose substantially predominates. Similarly, in this study the hemicellulose fraction is still higher. The low content of lignin present in the water hyacinth allow better utilization of cellulose and hemicelluloses for release of much of the reducing sugars such as the glucose and xylose for fermentation to ethanol and as well to conversion to other useful products such as xylitols.

4.2. Maximum theoretical yield of RS and Ethanol and statstical analysis of results

Table 4.2-1 Maximum RS and Ethanol yield (own analysis)

	Qty (g/g)	Expected RS g/g (from the components)	Expected Ethanol g/g potential convertible RS content	Remark
Total (cellulose and Hemicellulose content content g/g of WH(g)	0.68	0.611	0.550	this is calculated based on extractive free sample and taking 0.9 conversion respective components to RS and again 0.9 conversion of RS to Ethanol
only cellulose content g/g of WH	0.204	0.183	0.165	
Only Hemicellulose contet g/g	0.326	0.293	0.264	

The maximum theoretical yield for RS and Ethanol as shown in Table 4.2-1 was used to analyse the hydrolysis and fermentation results which are presented in Table 4.3-1 and Table 4.3-2.

4.3. Reducing Sugar yield and statstical analysis of experiment

The experimental results under the various conditions according to the experiment design and the data has shown that reducing sugar (RS) obtained vary from 19.46g/L to 30.64g/L of WH hydrolysate. The result shows yield of hydrolysis on basis of RS obtained per holocellulose content with 0.9 conversion factor shows that 25.8% to 40.6%.

Table 4.3-1 yield of Reducing sugar from WH

Run	Acid Conc, %	Temp, 0C	Time, min	Glucose Absorbance	RS mg/ml or (gm/liter) hydrolysate from the absorbance curve reading	Actual RS mg/ml or (gm/liter) in the hydrolysate	RS generated in 250ml hydrolysate(calculated) (g)	RS obtained g/g of WH calculated	RS obtained from 25 gm feedstock (g)	Yield of hydrolysis on basis of RS obtained per holocellulose content with 90% converion (%)
A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	K
1	5.0	130	60	0.260	1.17	29.25	7.31	0.292	7.3	38.8%
2	3.5	123	38	0.255	1.14	28.55	7.14	0.285	7.1	37.9%
3	5.0	130	16	0.230	1.00	25.05	6.26	0.251	6.3	33.2%
4	5.0	116	16	0.209	0.88	22.12	5.53	0.221	5.5	29.3%
5	3.5	130	38	0.264	1.19	29.81	7.45	0.298	7.5	39.5%
6	3.5	123	16	0.245	1.09	27.2	6.79	0.271	6.8	36.0%
7	3.5	116	38	0.239	1.05	26.31	6.58	0.263	6.6	34.9%
8	5.0	116	60	0.245	1.09	27.15	6.79	0.271	6.8	36.0%
9	5.0	123	38	0.250	1.11	27.85	6.96	0.278	7.0	36.9%
10	5.0	116	60	0.243	1.07	26.87	6.72	0.269	6.7	35.6%
11	5.0	130	16	0.228	0.99	24.77	6.19	0.248	6.2	32.9%
12	3.5	130	38	0.269	1.22	30.50	7.63	0.305	7.6	40.5%
13	2.0	123	38	0.212	0.90	22.53	5.63	0.225	5.6	29.9%
14	2.0	130	60	0.223	0.96	24.07	6.02	0.241	6.0	31.9%

15	3.5	123	38	0.257	1.15	28.83	7.21	0.288	7.2	38.2%
16	2.0	116	60	0.218	0.93	23.37	5.84	0.234	5.8	31.0%
17	3.5	123	60	0.270	1.23	30.64	7.66	0.306	7.7	40.6%
18	3.5	123	60	0.265	1.20	29.95	7.49	0.299	7.5	39.7%
19	2.0	116	60	0.216	0.92	23.09	5.77	0.231	5.8	30.6%
20	3.5	123	38	0.253	1.13	28.27	7.07	0.283	7.1	37.5%
21	2.0	116	16	0.190	0.78	19.46	4.86	0.195	4.9	25.8%
22	2.0	130	60	0.223	0.96	24.07	6.02	0.241	6.0	31.9%
23	3.5	123	16	0.222	0.96	23.93	5.98	0.239	6.0	31.7%
24	5.0	130	60	0.263	1.19	29.67	7.42	0.297	7.4	39.3%
25	2.0	130	16	0.209	0.88	22.12	5.53	0.221	5.5	29.3%
26	2.0	123	38	0.210	0.89	22.26	5.56	0.223	5.6	29.5%
27	2.0	116	16	0.206	0.87	21.70	5.42	0.217	5.4	28.8%
28	5.0	123	38	0.249	1.11	27.71	6.93	0.277	6.9	36.7%
29	5.0	116	16	0.211	0.90	22.40	5.60	0.224	5.6	29.7%
30	3.5	116	38	0.252	1.13	28.13	7.03	0.281	7.0	37.3%
31	2.0	130	16	0.207	0.87	21.84	5.46	0.218	5.5	29.0%

**** Note: RS concentration(refer column F) used to be read by the RS vs Absorbance curve is diluted and hence (column G) is the result column which is the actual concentration of RS obtained per each experiment run***

Effect of acid concentration and Temperature

The results show that maximum RS was obtained at 3.5% acid concentration which is is a concentration in between the min 2% and max 5%. However yield began to decline as the concentration of acid increased as shown in fig 4.3-1 below.

It has been reported by many researchers that acid hydrolysis can be applied to convert available hemicelluloses and cellulose in order to obtain RS which can be potential basis for production of many chemicals including production of Ethanol. It has been reported by many researchers that Sulphuric acid at higher temperature degrades the xylose and glucose into furfural and HMF respectively. Hence, similar reason can apply for the reason for decline of yield in this experiment. In literature written by (Ogawa, 2008) Approximately 36 + 1 % reducing sugars are available for fermentation during pretreatment step at 110-130 °C.

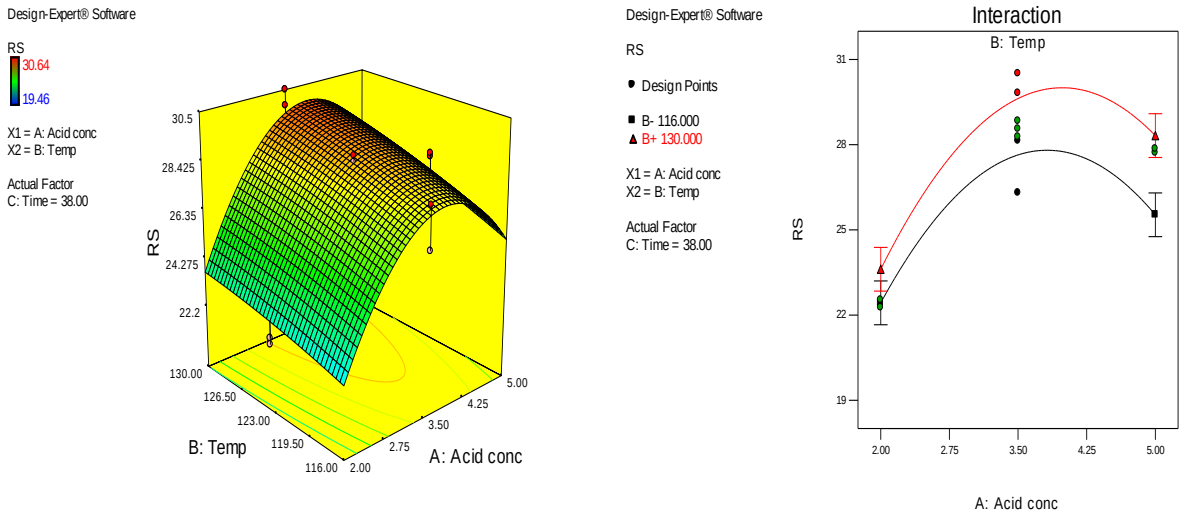

Fig. 4.3-1 Effect of Acid Conc. with temp at Constant time

Effect of time on hydrolysis

As RS production was shown in figure 4.3-2, the present study found that efficient WH hydrolysis occurred when the sample was incubated at 123 ^{o}C for 1hr.

The quantity of reducing sugars at higher temperature and long time was expected to be less due to degradation of the xylose and arabinose into furfural at higher temperature and long time but from this experiment run with acid conc 3.5% max reducing sugar was obtained at higher

temperature and time. This may indicate that acid concentration upto 3.5 % can be tolerable to bring deterioration and instead higher hydrolysis yield was obtained.

The cellulosic residue and the byproducts formed during treatment steps were different for varying time at different temperature. In conventional practice high temperature and long time for treatment hydrolyzed more hemicellulose (Ogawa, 2008). Therefore, probably in this experiment more hemicellulose hydrolyis has occurred which can contributed to the total RS.

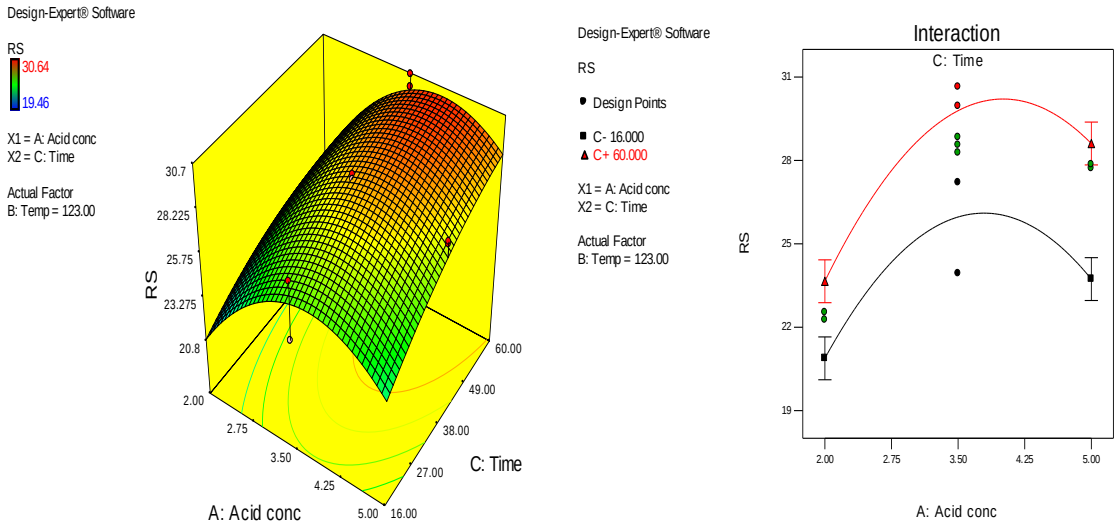

Fig. 4.3-2 Effect of temp and time keeping Acid constant

Optimization acid hydrolysis for optimal value of RS release

Upon estimating the optimal value, it was found that with acid concentration 3.91% v/v, Temperature at 129.91 ^{o}C and 57.82 minutes reaction time, $\pm$31.152 g/L of reducing sugar was obtained. This value is comparable with other researchers' value. For instance research by Takagi Toshiyuki shows that water hyacinth saccharified with 3 % (v/v) sulfuric acid gave glucose at 21.5 ± 2.9 g/l and reducing sugars at 33.3 ± 2.1 g/l (Takagi, Uchida, & Matsushima, 2012). And Similar results were reported by (Guillermo, 2013) which is 33.3 g/L. The detail analysis is annexed.

4.4. Ethanol produced and Statstical Analysis of experiment

Table 4.3.2-1 shows the experimental results under the various conditions according to the experimental design and the data analysis that Ethanol yield from generated RS of WH hydrolysate ranging from 0.049 % to 0.521% Whereas the generated ethanol from the fermentation broth is found to range from 0.56% to 6.79%.

In this study Ethanol yield obtained is extremely low. This may be due to the fact that the WH has high hemicelluloses content and the hydrolysis process mainly converts the components of hemicelluloses sugar such as xylose where by conversion of xyloses components of RS by the fermentation yeast, Saccaromyces Cerviciea is not satisfactory as already indicated by researches.

Table 4.4-1 Ethanol yield table

Run	Ethanol abs	Ethanol concentration % obtained using E. standard curve	Ethanol water solution after each expriment al measured run (ml)	pure ethanol obtained (calculated) (ml)	Pure Ethanol obtained , (mg)	Produced ethanol (g/L)	)Ethanol content % (V/V) in 250 ml hydrolysate**	total producible ethanol from obtained RS (g)	total producible ethanol from obtained RS (g/ L)	Ethanol yield obtained from obtained RS (%)	ethanol yield(%) = Ethanol obtained / ethanol expected from WH produced
A	**I**	**M**	**N**	**O**	**P**	Q	R	S	T	U	**V**
1	0.105	44%	29.0	12.73	10.04	0.040	5.09%	3.36	13.42	0.299%	0.14%
2	0.083	33%	27.0	9.03	7.13	0.029	3.61%	3.28	13.10	0.218%	0.10%
3	0.120	51%	28.0	14.28	11.27	0.045	5.71%	2.87	11.50	0.392%	0.16%
4	0.142	61%	26.0	15.98	12.61	0.050	6.39%	2.54	10.15	0.497%	0.18%
5	0.067	26%	29.0	7.50	5.92	0.024	3.00%	3.42	13.68	0.173%	0.08%
6	0.078	31%	27.0	8.39	6.62	0.026	3.36%	3.12	12.46	0.213%	0.09%
7	0.080	32%	28.0	8.97	7.08	0.028	3.59%	3.02	12.08	0.234%	0.10%
8	0.110	46%	29.0	13.42	10.59	0.042	5.37%	3.12	12.46	0.340%	0.15%
9	0.130	56%	26.0	14.50	11.44	0.046	5.80%	3.20	12.78	0.358%	0.16%
10	0.112	47%	27.0	12.75	10.06	0.040	5.10%	3.08	12.33	0.326%	0.14%
11	0.124	53%	26.0	13.76	10.86	0.043	5.50%	2.84	11.37	0.382%	0.15%
12	0.067	26%	28.0	7.24	5.71	0.023	2.90%	3.50	14.00	0.163%	0.08%
13	0.036	11%	30.0	3.35	2.64	0.011	1.34%	2.59	10.34	0.102%	0.04%
14	0.075	30%	27.0	8.01	6.32	0.025	3.20%	2.76	11.05	0.229%	0.09%
15	0.085	34%	27.0	9.29	7.33	0.029	3.72%	3.31	13.23	0.222%	0.10%
16	0.038	12%	30.0	3.63	2.86	0.011	1.45%	2.68	10.73	0.107%	0.04%
17	0.070	27%	29.0	7.91	6.24	0.025	3.17%	3.52	14.07	0.178%	0.09%
18	0.069	27%	30.0	8.04	6.35	0.025	3.22%	3.44	13.74	0.185%	0.09%
19	0.036	11%	26.0	2.90	2.29	0.009	1.16%	2.65	10.60	0.086%	0.03%

20	0.084	34%	27.0	9.16	7.23	0.029	3.66%	3.24	12.97	0.223%	0.10%
21	0.023	5%	28.0	1.40	1.10	0.004	0.56%	2.23	8.93	0.049%	0.02%
22	0.035	11%	28.0	2.99	2.36	0.009	1.20%	2.76	11.05	0.085%	0.03%
23	0.075	30%	27.0	8.01	6.32	0.025	3.20%	2.75	10.99	0.230%	0.09%
24	0.103	43%	27.0	11.60	9.15	0.037	4.64%	3.40	13.62	0.269%	0.13%
25	0.030	8%	26.0	2.16	1.70	0.007	0.86%	2.54	10.15	0.067%	0.02%
26	0.038	12%	25.0	3.03	2.39	0.010	1.21%	2.55	10.22	0.093%	0.03%
27	0.027	7%	27.0	1.86	1.47	0.006	0.74%	2.49	9.96	0.059%	0.02%
28	0.132	57%	26.0	14.75	11.63	0.047	5.90%	3.18	12.72	0.366%	0.17%
29	0.145	63%	27.0	16.98	13.40	0.054	6.79%	2.57	10.28	0.521%	0.19%
30	0.068	26%	27.0	7.11	5.61	0.022	2.84%	3.23	12.91	0.174%	0.08%
31	0.033	10%	27.0	2.63	2.07	0.008	1.05%	2.43	9.70	0.085%	0.03%

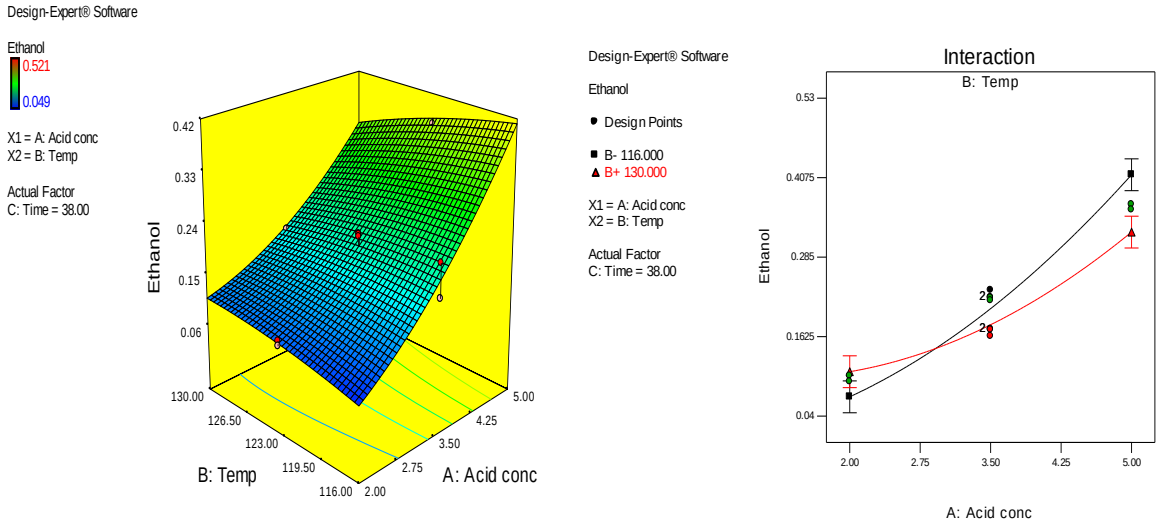

Fig 4.4-1 Effect of temperature and Acid concentration keeping time constant on Ethanol yield

From the analysis as indicated in **Error! Reference source not found.** increased ethanol oncentration was obtained for those fermentation broths with hydrolysis condition of increasing acid concentration but with lowering of temperature. However, ethanol production slightly declined as temperature increased to maximum with slightly lowered acid concentration.

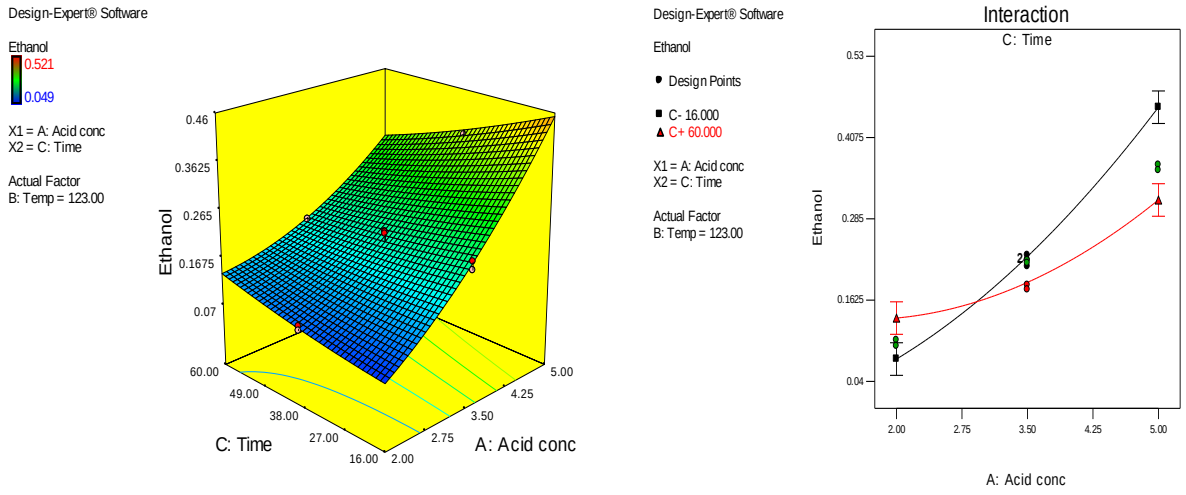

Fig 4.4-2 Effect of Acid concentration and time keeping temp constant on Ethanol yield

From Fig 4.4-2, with fermentation broth that was prepared with hydrolysis condition of t high acid concentration and with minimum hydrolysis time ethanol produced keeping temperature at 123^{o}C was maximum; this shows that hydrolysis product is rich with hexose sugars which can be easily fermented with yeast saccharomyces cerevisiae.

Higher yield of ethanol was achieved at higher temperature probably, this may indicate two important facts one is it proves that glucose is tolerant to sever condition and decline of RS might be due to xylose degradation. This situation can show also that xylose tolerance to harsh condition is less that of glucose confirming the result of Xiang's work (Xiang, 2004).

And the other fact for higher yield may be explained by enhancement of partial hydrolysis of cellulose component releasing glucose which is convertible by the yeast S.Cerevisiae. Details of the analysis are annexed.

1.1. Conclusion

This preliminary assessment mainly characterizes the Water hyacinth from the river Awash to determine the cellulose, hemicelluloses and lignin content and conduct acid hydrolysis to obtain Reducing Sugars which can be potential sources for bioethanol production. According to the characterization work, it was found out that the hemicellulose which is mainly composed of C5 sugars such as xylose is the highest and the lignin content is very minimal.

From this research it is also shown that the increase in H2SO4 concentration, reaction time and temp to the optimum level increased the total sugar concentration. Upon estimating the optimal value, it was found that with acid concentration 3.97% v/v, Temperature at 129.91 ^{o}C and 57.82 minutes reaction time, 31.152 g/L of reducing sugar was obtained.

However, with the fermentation set up used the Ethanol yield obtained is extremely low (that Ethanol yield ranging from 0.05% to 0.532% from obtained RS of WH hydrolysate. The acid hydrolysis result showed availability of RS upto 40% but satisfactory fermentation of these sugars to ethanol was not achieved. This could be due to conversion of hemicelluloses derived sugar monomers to inhibitory products.

The other observation is, higher ethanol production was not obtained at the optimal reducing sugar available within the studied parameter but for those which are hydrolized at higher acid concentration whereby the RS yield has shown to decline. This suggests that hydrolysis of cellulose components started but at the same time at higher side of the parameters degradation of hemicellulose products could be the reason for decrease of the total RS components due to increased temperature. In the meantime there is also a possibility of decomposition of cellulose into mainly hexoses C6 sugars hexoses sugars that can be easily fermented by the yeast strain used.

1.2. Recommendation

In this work, it was possible to measure total Reducing Sugar. But in order to understand the whole mechanism and appropriately define effect of the acid hydrolysis, identifying and quantifying the type reducing sugars i.e how much is the glucose, xylose, galactose, arabinose etc is important. This helps to deeply understand and quantify the pentose and hexose sugars. This helps to understand which component contributes to decline of RS production at higher acid and higher temperature.

Ethanol production in this work with the conducted fixed fermentation condition did not result in satisfactory yield; this indicates that further analysis is required to analyze effect of different parameters such as fermentation yeast strain and fermentation physico-chemical parameters. Therefore detail analysis of fermentation parameters is also relevant future work. Especially, since the majority component is hemicelluloses, xylose monomers are expected to be highest, therefore, it is also recommended that further research to be conducted with xylose fermenting yeast strains.

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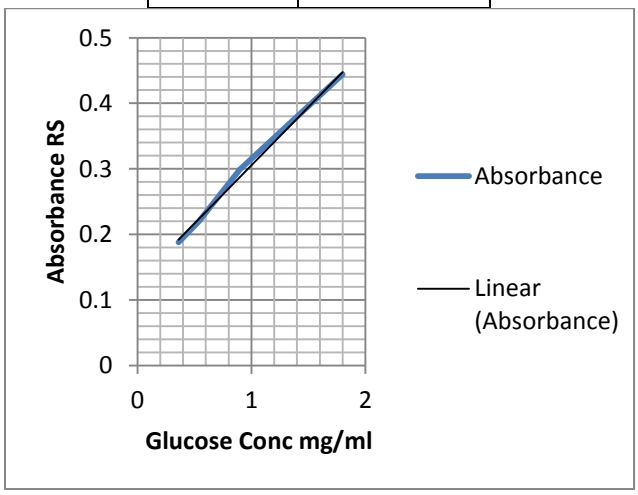
Appendix

Appendix 1 Glucose vs Absorbance standard curve

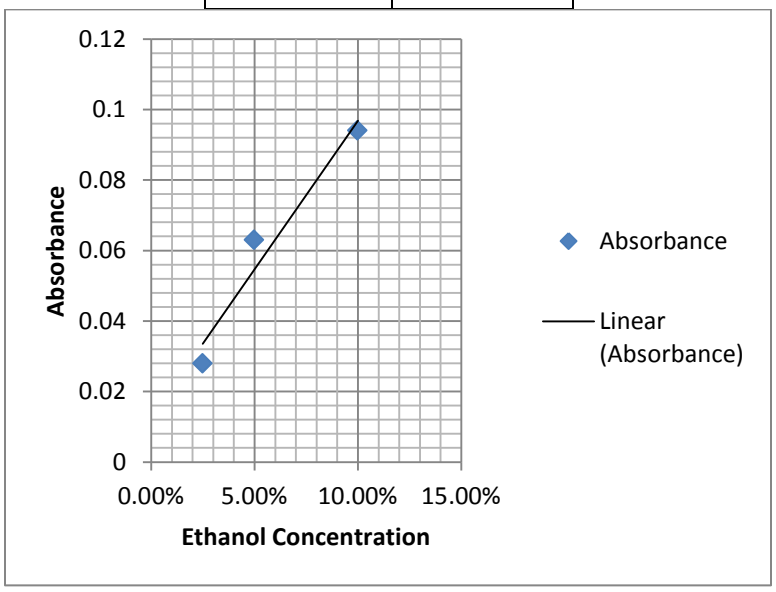
Glucose Conc mg/ml	Absorbance
0.36	0.098
0.54	0.154
0.9	0.23
1.8	0.365



This standard curves are used to develop results that are indicated in Table 4.3-1 yield of Reducing sugar from WHTable 4.3-1

Appendix 2 Appendix 3Ethanol conc vs Absorbance standard Curve

Ethanol conc	Absorbance
2.50%	0.028
5%	0.063
10%	0.094



This standard curves are used to develop results that are indicated in Table 4.3-1 yield of Reducing sugar from WHTable 4.4-1

Appendix 3 Design Summary for RS and Ethanol response

Study Type	Response Surface		Runs	31							
Initial Design	Central Composite		Blocks	No Blocks							
Design Model	Quadratic										
Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Std. Dev.		
A	Acid conc	%	Numeric	2	5	-1	1	3.5	1.205		
B	Temp	degcent	Numeric	116	130	-1	1	123	5.623		
C	Time	minutes	Numeric	16	60	-1	1	38	17.671		
Response	Name	Units	Obs	Analysis	Min	Max	Mean	Std. Dev.	Ratio	Trans	Model
Y1	RS	g/L	31	Polynomial	19.46	30.64	25.76645	3.172002	1.574512	None	Quadratic
Y2	Ethanol	%	31	Polynomial	5	63	32.93548	15.39153	12.6	None	Quadratic

Appendix 4 Rs design model evaluation

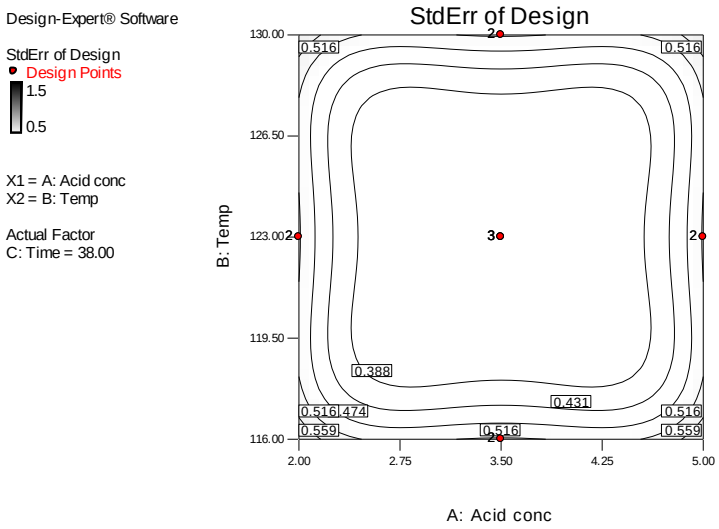
3 Factors: A, B, C						
No aliases found for Quadratic Model						
Aliases are calculated based on response selection,						
taking into account missing datapoints, if necessary.						
Watch for aliases among terms you need to estimate.						
Degrees of Freedom for Evaluation						
Model	9					
Residuals	21					
Lack 0f Fit	5					
Pure Error	16					
Corr Total	30					
A recommendation is a minimum of 3 lack of fit df and 4 df for pure error.						
This ensures a valid lack of fit test.						
Fewer df will lead to a test that may not detect lack of fit.						
				Power at 5 % alpha level for effect of		
Term	StdErr**	VIF	Ri-	0.5 Std.	1 Std.	2 Std.

			Squared	Dev.	Dev.	Dev.
A	0.2236068	1	0	18.7 %	56.9 %	98.9 %
B	0.2236068	1	0	18.7 %	56.9 %	98.9 %
C	0.2236068	1	0	18.7 %	56.9 %	98.9 %
AB	0.25	1	0	15.9 %	47.9 %	96.8 %
AC	0.25	1	0	15.9 %	47.9 %	96.8 %
BC	0.25	1	0	15.9 %	47.9 %	96.8 %
A^2	0.43788991	1.3607892	0.2651323	19.3 %	58.7 %	99.2 %
B^2	0.43788991	1.3607892	0.2651323	19.3 %	58.7 %	99.2 %
C^2	0.43788991	1.3607892	0.2651323	19.3 %	58.7 %	99.2 %
**Basis Std. Dev. = 1.0						
Standard errors should be similar within type of coefficient. Smaller is better.						
Ideal VIF is 1.0. VIF's above 10 are cause for alarm,						
indicating coefficients are poorly estimated due to multicollinearity.						
Ideal Ri-squared is 0.0. High Ri-squared means terms are correlated						

with each other,						
possibly leading to poor models.						
Power should be approximately 80% for the effect you want to detect.						
Be sure to set the Model (on previous screen) to be an estimate of the terms you expect to be significant.						
Measures Derived From the (X'X)^-1 Matrix						
Std	Leverage	Point Type				
1	0.398	Fact				
2	0.398	Fact				
3	0.398	Fact				
4	0.398	Fact				
5	0.398	Fact				
6	0.398	Fact				
7	0.398	Fact				
8	0.398	Fact				
9	0.398	Fact				
10	0.398	Fact				
11	0.398	Fact				
12	0.398	Fact				
13	0.398	Fact				
14	0.398	Fact				
15	0.398	Fact				
16	0.398	Fact				
17	0.271	Axial				

18	0.271	Axial				
19	0.271	Axial				
20	0.271	Axial				
21	0.271	Axial				
22	0.271	Axial				
23	0.271	Axial				
24	0.271	Axial				
25	0.271	Axial				
26	0.271	Axial				
27	0.271	Axial				
28	0.271	Axial				
29	0.126	Center				
30	0.126	Center				
31	0.126	Center				
Average =	0.323					
Leverages are far from being close to 1.0.						

Appendix 5 Model design standard error



Appendix 6 ANOVA Reducing sugar analysis using design expert

All the important data and information generated by the design expert are presented in the Appendix for reference and those data important to mention are presented here as follows.

ANOVA for Response Surface Quadratic Model(RS)					
Analysis of variance table [Partial sum of squares - Type III]					
	Sum of		**Mean**	**F**	**p-value**
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**
Model	295.97	9	32.89	43.32	< 0.0001
A-Acid conc	76.17	1	76.17	100.34	< 0.0001
B-Temp	19.72	1	19.72	25.98	< 0.0001
C-Time	73.11	1	73.11	96.32	< 0.0001
AB	2.58	1	2.58	3.39	0.0796
AC	4.43	1	4.43	5.84	0.0249
BC	0.04	1	0.04	0.06	0.8163
A^2	69.80	1	69.80	91.95	< 0.0001
B^2	0.02	1	0.02	0.03	0.8746
C^2	3.47	1	3.47	4.57	0.0444
Residual	15.94	21	0.76		
Lack of Fit	5.01	5	1.00	1.47	0.2554
Pure Error	10.93	16	0.68		
Cor Total	311.91	30			

According to the Design expert Values of "Prob > F" less than 0.0500 indicate model terms are significant and Values greater than 0.1000 indicate the model terms are not significant. In this case A, B, C, AC, A^2, C^2 are significant model terms and BC and B^2 are not significant.

Crossed factors (AA, CC and BB) have no physical meaning but they are incorporated to improve the mathematical fit of statistical model based on experimental data. The Acid temperatue interaction did not exert a significant effect within the experimental design boundaries, it has great contribution to obtain fermentable sugars as it is shown in the P-value in the table above.

Std. Dev.	0.87	R-Squared	0.95
Mean	25.77	Adj R-Squared	0.93
C.V. %	3.38	Pred R-Squared	0.89
PRESS	33.57	Adeq Precision	19.63

The "Lack of Fit F-value" of 1.47 implies the Lack of Fit is not significant relative to the pure error. There is a 25.54% chance that a "Lack of Fit F-value" this large could occur due to noise which is good according to the design expert and. "Adeq Precision" measures the signal to noise ratio. The ratio of 19.628 shows adequate signal as a ratio greater than 4 is desirable. Therefore, This model can be used to navigate the design space. The statistical analysis yielded a mathematical model to predict outcomes within the range of each variable. The regression equation, with variables in encoded units and with the terms that have a significant and measurable effect on the release of RS, is as follows:

$$\boldsymbol{RS = 28.69 + 1.95A + 0.99B + 1.91C + 0.4AB + 0.53AC - 0.051BC - 3.66A^2 - 0.061B^2 - 0.82C^2} \quad \cdots\cdots\cdots \textbf{Eq 1}$$

Where RS is the concentration of RS (g/L), *A* is the concentration of solids (% w/v), *B* is the concentration of sulfuric acid (% v/v) and *C* is the reaction time. The regression coefficient value for this model ($R^2 = 0.95$) indicates that the quadratic model fitted explains 95% of the variability in the production of RS.

Name	**Goal**	**Limit**	**Limit**	**Weight**	**Weight**	**Importance**
Acid conc	is in range	2	5	1	1	3
Temp	is in range	116	130	1	1	3
Time	is in range	16	60	1	1	3
RS	maximize	19.46	30.64	1	1	5
Solutions						
Number	Acid conc	Temp	Time	RS	Desirability	
1	4.09	126.73	56	30.655	1	
2	4.08	127.08	59.94	30.818	1	
3	3.88	128.96	50.9	30.735	1	
4	4.09	126.36	59.44	30.699	1	
5	4.48	129.92	54.74	30.796	1	
6	4.08	127.73	54.85	30.763	1	
7	3.97	126.9	55.14	30.650	1	
8	4.46	129.77	59.51	30.971	1	
9	3.68	128.7	58.14	30.772	1	
10	4.08	126.86	56.33	30.686	1	
11	4.21	129.96	49.57	30.794	1	
12	3.72	128.21	54.61	30.661	1	
13	3.97	129.91	57.82	31.152	1	
14	4.13	129.26	55.31	30.993	1	
15	3.88	127.43	54.51	30.671	1	

ANOVA for Response Surface Quadratic Model(Ethanol)					
Analysis of variance table [Partial sum of squares - Type III]					
	Sum of		**Mean**	**F**	**p-value**
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**
Model	0.4885	9	0.0543	67.967	< 0.0001
A-Acid conc	0.4056	1	0.4056	507.861	< 0.0001
B-Temp	0.0033	1	0.0033	4.168	0.0540
C-Time	0.0079	1	0.0079	9.918	0.0048
AB	0.0166	1	0.0166	20.758	0.0002
AC	0.0425	1	0.0425	53.270	< 0.0001
BC	0.0033	1	0.0033	4.104	0.0557
A^2	0.0067	1	0.0067	8.427	0.0085
B^2	0.0004	1	0.0004	0.481	0.4958
C^2	0.0002	1	0.0002	0.313	0.5818
Residual	0.0168	21	0.0008		
Lack of Fit	0.0025	5	0.0005	0.570	0.7218
Pure Error	0.0142	16	0.0009		
Cor Total	0.5053	30			

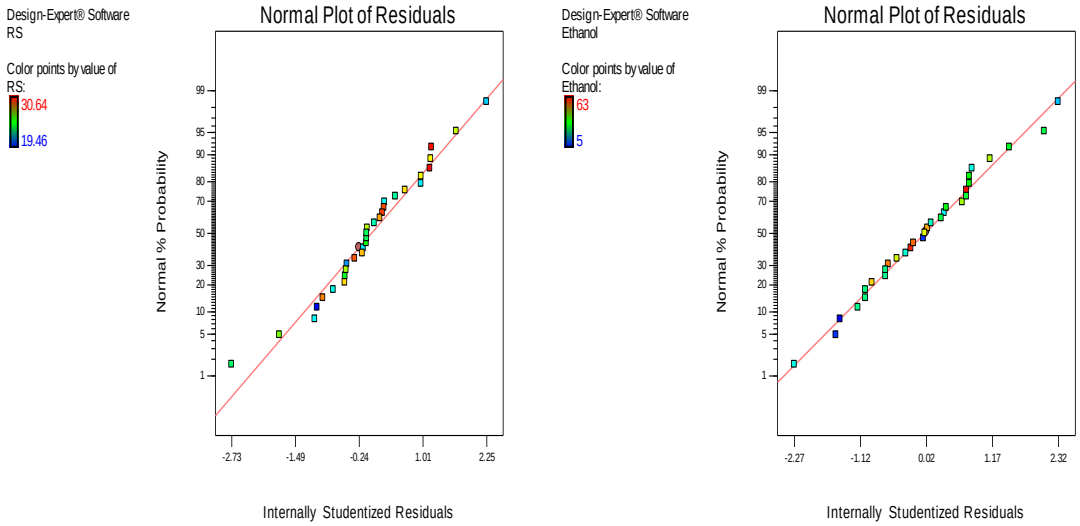
According to the Design expert Values of "Prob > F" less than 0.0500 indicate model terms are significant and Values greater than 0.1000 indicate the model terms are not significant. In this case A,AB, AC, A^2, are significant model terms and the rest are not significant.

Std. Dev.	0.0283	R-Squared	0.967
Mean	0.2277	Adj R-Squared	0.953
C.V. %	12.4100	Pred R-Squared	0.917
PRESS	0.0418	Adeq Precision	28.181

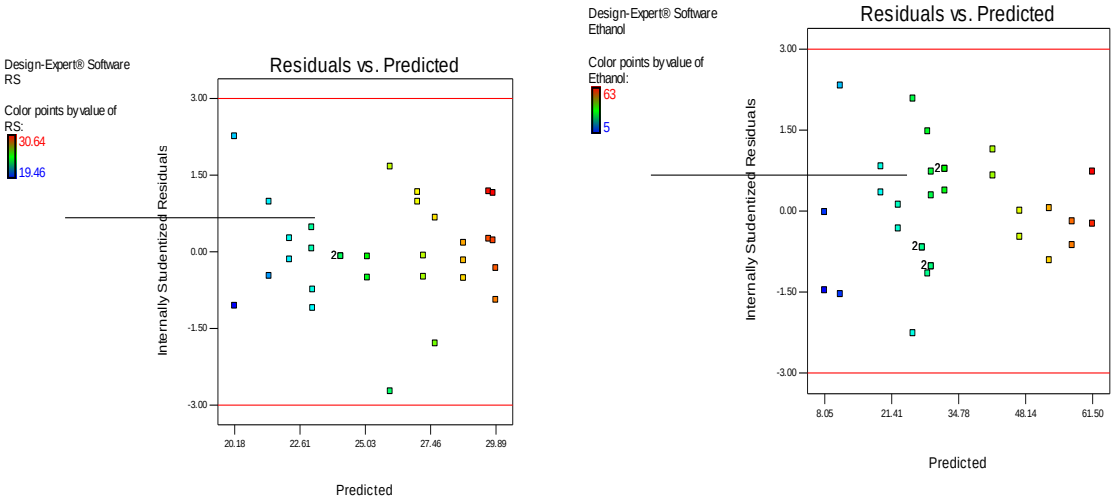
Optimization values for Ethanol yield

		Lower	Upper	Lower	Upper	
Name	Goal	Limit	Limit	Weight	Weight	Importance
Acid conc	is in range	2	5	1	1	3
Temp	is in range	116	130	1	1	3
Time	is in range	16	60	1	1	3
RS	is in range	19.46	30.64	1	1	3
Ethanol	maximize	0.05	0.532	1	1	3
Solutions						
Number	Acid conc	Temp	Time	RS	Ethanol	Desirability
1	5	116	16	22.22486	0.513	0.961
2	4.99	116	16.09	22.29013	0.510	0.955
3	5	116.63	16	22.36523	0.509	0.953
4	5	116	26.91	24.06736	0.465	0.862
5	5	116.06	27.48	24.165	0.463	0.856
6	5	116	33	24.92255	0.440	0.810
7	5	117.57	51.81	27.10128	0.365	0.653
8	2.3	130	60	26.02451	0.162	0.231
8 Solutions found						

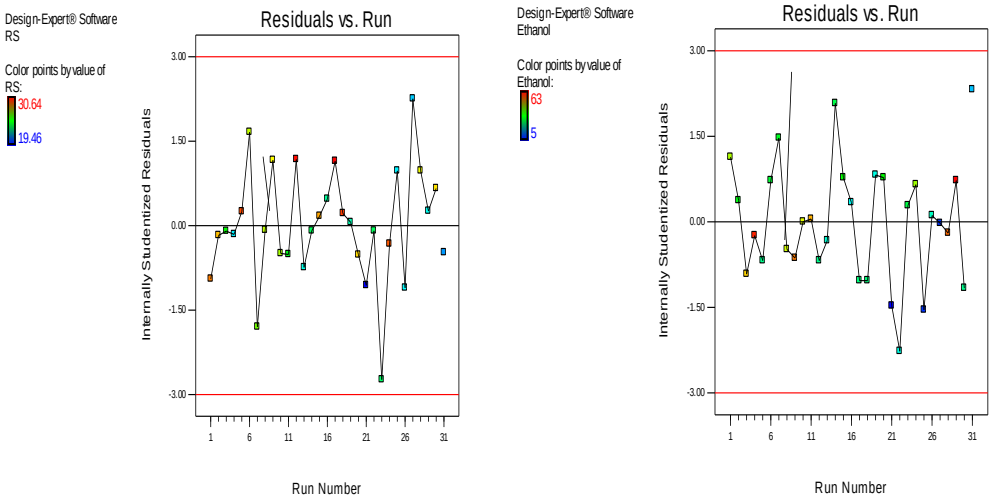
Appendix 7 fig RS analysis Normal plot of Residuals RS and Ethanol



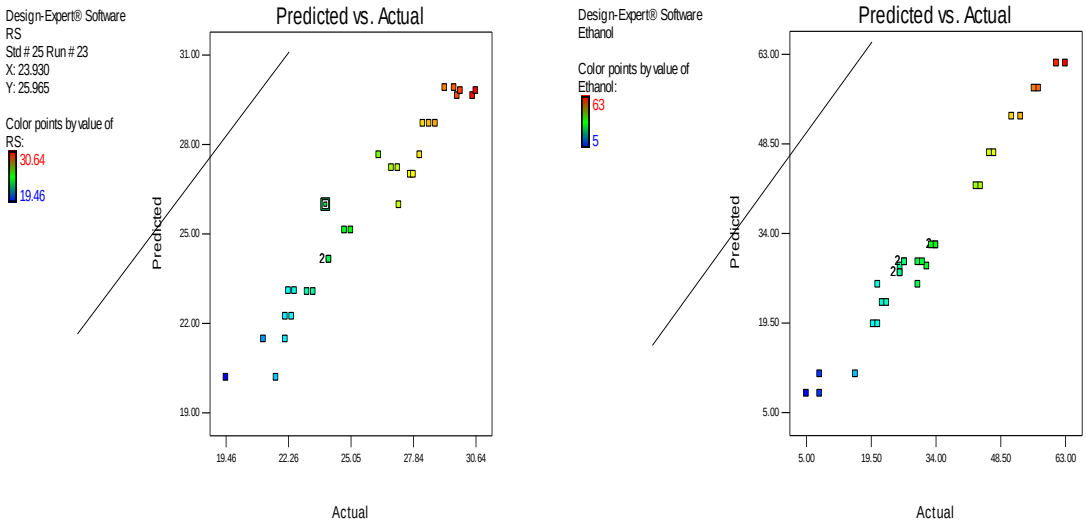
Appendix 8 RS and Ethanol analysis Residual vs predicted



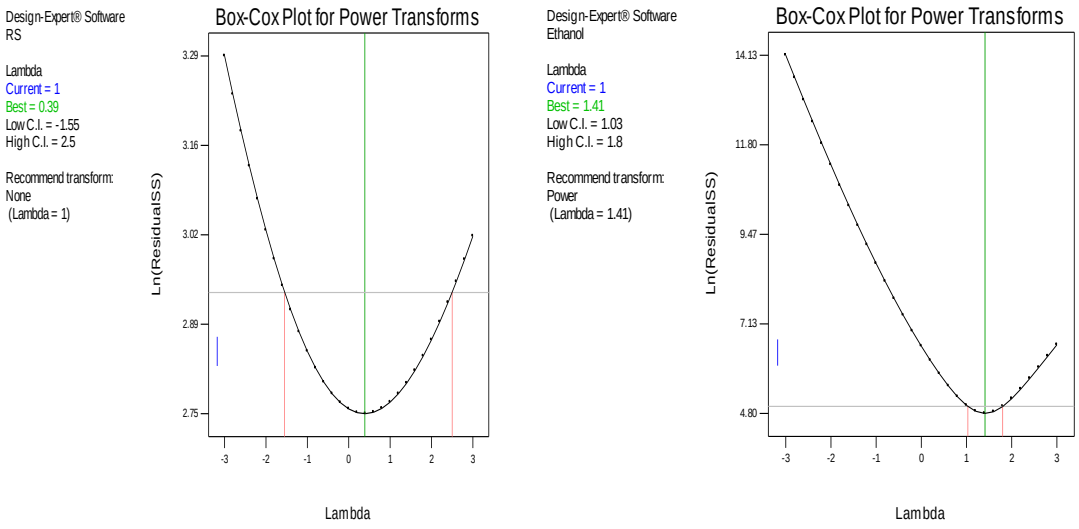
Appendix 9 RS analysis Residual vs Run plot



Appendix 10 RS analysis predicted vs actual plot



Appendix 11 Box-Cox plot for power transforms



Appendix 12 Residual vs Acid conc for RS analysis