ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAIT)

SCHOOL OF CHEMICAL AND BIO ENGINEERING

BIOCHEMICAL ENGINEERING STREAM

PRODUCTION OF BIOETHANOL FROM BREWERY SPEINT GRAIN USING CELLULASE ENZYME HYDROLYSIS

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A thesis submitted to the Research and Graduate School of Addis Ababa University, Addis Ababa Institute of Technology 5 kilo, School of Chemical and Bio engineering in partial fulfillment of the requirements for the attainment of the Degree of Master of Science in Chemical Engineering under biochemical Engineering stream.

Done by: Emru yidnekew

Advisor: Professor Eduardo Ojito

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DECLARATION

I declare that this thesis for the M.Sc. Degree at Addis Ababa University, hereby submitted by me, is my original work and has not previously been submitted for the degree at this or any other university, and that all resources of materials used in this thesis have been duly acknowledged.

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This thesis has been submitted for examination with my approval as University Advisor.

Name: Professor Eduardo Ojito ___________ ___________

Signature: _________

Date: _________

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List of Acronyms

AFEX	Ammonia fiber expansion
ANOVA	Analysis of variance
BSG	Barley spent grain
CCD	Central composite design
CI	Confidence interval
DAP	Dilute acid pretreatment
DNS	Dinitrosalicylic acid
FPA	Filter paper activity
GHG	Green-house gas emissions
HPLC	High-performance liquid chromatography
ICE	Internal combustion engines
LCC	Lignin-carbohydrate complex
LHW	Liquid hot water
MTBE	Methyl tertiary butyl ether
pH	Potential of hydrogen
RPM	Revolution per minute
RSM	Response surface methodology
SAA	Soaking aqueous ammonia
SEP	Steam explosion pretreatment
SHF	Simultaneous hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TRS	Total reducing sugar

Abstract

Bioethanol is a clear, colorless and mobile flammable liquid chemical compound with acceptable odour, and produced from biomass feed stocks through fermentation. The objective of this study was production of bioethanol from Barley spent grain by using cellulase enzyme hydrolysis. Decreasing of world petroleum reserves, fluctuation of petroleum price and environmental concerns, low yield cellulose acid hydrolysis and formation of inhibitory were the problem statement. Enzymatic hydrolysis method was used for cellulose hydrolysis, producing monomeric C6 sugars (glucose); enzymatic hydrolysis is more specific and high conversion rates can be achieved in long reaction times obtaining very high yields. Raw material characterization of BSG were, hemicelluloses content 28.4 %, cellulose content 27 %, and dry matter content 98.2 %. The aim of pretreatment is to disrupt recalcitrant structures of cellulosic biomass to make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars. The pretreated feedstock was mixed with 1.25 % (v/v) sulfuric acid solutions with a liquid-to-solid ratio of 20 %(w/w) and it was occurred on the temperature 121 ºC and time of 17 min, the severity factor coefficient was 0.198. Cellulase enzyme was produced from BSG using bacillus subtilus, under liquid state fermentation for 5 days at temperature of 37 ºC with pH of 5.0, in controlled incubator. For the enzyme hydrolysis, 26 full factorial central composite design (CCD) was applied to investigate the effect of temperature (40-50 ºC), pH (4.0-5.0), reaction time (24-72 hr.) and enzyme loading (1-2 %) using Design expert® 6 software. RSM was applied to investigate the interaction effect of hydrolysis process variables on the yield of ethanol from BSG. After hydrolysis process sugar content of the hydrolysate was quantified using spectrophotometer measuring its absorbance. Fermentation of the hydrolysate were performed 2.5 % Saccharomyces cerevisiae and 2.5 % fusarium oxysporum at 30 ºC temperature, pH 5.0 and 72 hr. fermentation time for all samples. Significance of the process variables were analyzed using analysis of variance. As the result of RSM optimization, the best yield of TRS was found at 40 ºC hydrolysis temperature, 4.5 PH, 48 hr. reaction time and 1.5 % v/v enzyme loading. Under these condition 96.55 %w/w and 53.68 % per 6.58 g dry barley spent grain of TRS and bio-ethanol respectively obtained.

Keywords: Bioethanol, Barley spent grains, *bacillus subtilus*, cellulase, enzyme hydrolysis, fermentation, *fusarium oxysporum* and *Saccharomyces cerevisiae*

1 INTRODUCTION

1.1 Background of the study

The use of biofuels for transport is becoming of increasing importance for a number of reasons, such as environmental concerns relating to climate change, depleting fossil fuel reserves, and reducing reliance on imports. This is leading to international, national and regional focus on alternative energy sources. Biofuels, processed from biomass, a renewable resource, are suggested as a direct substitute for fossil fuels in transport. Thus, current research and development drivers are the identification of potential renewable energy sources or biomass feedstock and their processing in order to produce alternatives to fossil fuels in transport, such as bioethanol, biodiesel, bio methanol and hydrogen. The relatively young biofuel industry is showing the first signs of consolidation, despite unproven business models. First-generation biofuels derived from corn, sugarcane and oilseed are currently available and are seen as an intermediate step to reduce GHG emissions and to diversify transport energy sources among other alternatives. However, they are available in limited volumes that do not make them serious replacements for petroleum.

Currently, bioethanol research is still continuously expanding. Not only serve as a renewable energy, bioethanol that is believed as most prospective bioenergy, can also be a solution for air pollution problem. While the production of first generation ethanol from starch materials has developed well, production process of second generation ethanol from lignocellulosic materials is still looking for its economical feasibility. Furthermore, first generation ethanol materials are also generally very important food sources that will impair food security issue and their availability are also not sufficient to cover the total required bioethanol production. Therefore, this research aims to elaborate lignocellulosic ethanol production prospective from the secondary generation of biomass as an effort to overcome bottleneck of lignocellulosic ethanol.

The improvement of living standard urges the hunt for sustainable energy in order to meet energy consumption across the world (Demirbas, 2010). On the other hand, the use of fossil fuels as the main energy resources caused the arising of worldwide problems such as environmental pollution and global warming (Hoekman, 2009). These led to the finding of environmentally friendly, renewable and sustainable energy by government, industrial and energy sector (Shafiee, 2009). Among renewable energies, priority was given to liquid biofuels as it represents about 40 % of the total energy consumption in the world (Tan, 2008). The use of liquid biofuels contributes to the reduction of greenhouse gas emissions, creation of job opportunities, regional development and supply security (Balat and Demirbas, 2006). Bioethanol is known as the most widely used biofuel in transportation sector and have a long history as alternative fuels. In 1984, Germany and France started to use bioethanol as a fuel in internal combustion engines (ICEs) (Demirbas and Karslioglu, 2007).

The depletion of fossil feedstock and climate changes, like global warming, due to green-house gas emissions (GHGs), have caused increasing attention in alternative renewable sources of energy, pushing the worldwide trend to produce and use bio-based products and biofuels in substitution of the fossil-based ones(Amore, 2014). The production of advanced biofuels (second generation biofuels) from lignocellulosic biomass has a number of technical difficulties associated with it due to the recalcitrant nature of the material (Chundawat, 2011). Bioethanol production from BSG is considered a 2nd generation biofuel process since it has no direct conflict with human food, as it happens with the 1st generation biofuels produced from agricultural crops, such as corn and sugarcane for bioethanol production, or soybean oil (Morais et al., 2010) and corn oil (Mata et al., 2012b) for biodiesel production. The most abundant renewable resource produced all around the world is symbolized by lignocellulosic biomasses (Prasad and Singh, 2007), which include food farming wastes, tree pruning residues agricultural residues, “green-grocer’s wastes” and the organic and paper fraction of urban solid wastes.

A global interest in using the lignocellulosic residues and waste as source of added value bio-products is rising due to their renewability, low cost, abundance, and non-competitiveness with food (Amore and Liguori, 2013), adapting the development of the bio-refinery concept and advancing sustainable waste management (Dusselier, 2014). Among the lignocellulosic residues, a higher interest is focusing on brewers’ spent grain (BSG). BSG is a solid residue of breweries consisting of exhaust grain husks, obtaining after mashing and lautering. Its composition changes based on the operative conditions adopt during harvest, malting and mashing time (Santos et al., 2003). BSG is a lignocellulosic material consisting of the husk of barley grain. It is the major by -product generated by the brewing industry, representing about 85 % of the total by-products (Mussatto et al., 2006).

Brewery spent grain (BSG) is a by-product of the brewing process, consisting of the solid residue remaining after mashing and lautering. It consists primarily of grain husks and other residual compounds not converted to fermentable sugars by the mashing process. BSG is the most abundant brewing by-product, corresponding to around 85% of total by-products (consisting of 85% malt and 15% wheat) generated and is mainly used as low-value cattle food or discarded as waste. The chemical composition of BSG varies according to barley variety, harvesting time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Huige, 1994; Santos et al., 2003). BSG contains mainly hemicellulose in the form of arabinoxylans from the barley grain and cellulose. BSG has the potential to serve as a low-cost feedstock for the production of ethanol since hemicellulose and cellulose content corresponds to 60% w/w of dry BSG (Dehnavi, 2009). On a dry weight basis, BSG contains about 40 - 50 % polysaccharides (consisting of 15 - 18 % cellulose, 24 - 31 % hemicellulose and 2 - 3 % starch) and 30 % or more proteins (Macheiner et al., 2003).

1.2 Statement of the problem

The goal of energy security resulting from the decreasing of world petroleum reserves, fluctuation of petroleum price and environmental concerns has motivated governments and researchers to look forward to others renewable energy sources that are technically feasible, economically competitive and environmentally acceptable. Among alternative energy sources, biofuel processed from lignocellulose, have been gaining increasing attention recently as a replacement for fossil fuels, hence bioethanol is an important biofuel. The industrial production of fuel ethanol in the world relies mainly on the fermentation of sugar and starch, but production of ethanol from such "first generation" feedstock is occasionally seen as contending with food production and increasing prices of food. So that productions of bioethanol from industrial wastes such as BSG confer the debate of "food versus energy‟ controversy.

Critical to the overall environmental impact of bioethanol is the type of feedstock used and its method of production. Using the acid hydrolysis cause corrosive condition and it is difficult to achieve high yield cellulose hydrolysis and formation of inhibitory by products. Therefore this study is concerning to solve such type of problem and to convert this low-value solid residue by product into highly valuable product by using enzyme hydrolysis.

Currently, numbers of brewery industries are shooting up in Ethiopia and they produce a high amount of barley spent grain around 309,002 kg is generated daily. At this time the by-product is mainly used as a low value cattle feed or simply deposited as a waste into land fill. However, BSG is a raw material of interest for application in different areas because of its low price, large availability throughout the year and valuable chemical composition. Beside this creates bad odour on the environment, so that Conversion of such waste into Bioethanol helps to reduce environmental pollution, reduce the country's hard currency and energy problem of developing countries like Ethiopia.

1.3 Objectives of the study

1.3.1 General objective

The general objective of the study was Production of Bio-Ethanol from Brewers' Spent Grain using cellulase Enzyme Hydrolysis

1.3.2 Specific objective

- To evaluate the effect of pretreatment parameters on the raw material brewery spent grain using combined severity factor.
- To quantify the sugar content of barley spent grain after the enzyme hydrolysis.
- Evaluate the effect of hydrolysis.

1.4 Significance of the study

Bioethanol is the most utilized non-fossil fuel for transportation globally. It can serve as an additive or substitute to gasoline and it is well-suited for automobile engines (Balat, 2011; Celik, 2008). Additionally, bioethanol has other varied uses which make it a highly valuable commodity. It can be used in domestic cooking as ethanol gels (Lloyd and Visagie, 2007), in fuel cells (Wang et al., 1995), for hydrogen production and as a precursor for other chemical commodities (Rass et al., 2007).

Bioethanol production from BSG is considered a 2nd generation biofuel process since it has no direct conflict with human food, as the case of 1st generation biofuels produced from agricultural crops, such as corn, sugar beet, sugarcane and soybean oil. BSG is rich in sugars and proteins. Ethanol is used for production of alcoholic beverages, for industrial purposes like a solvent, disinfectant, or chemical feedstock and, in recent years, as a blending agent with gasoline to increase octane and reduce carbon monoxide and other smog-causing emissions.

Ethanol is mainly used as an oxygenated fuel additive to reduce emissions of carbon monoxide, nitrous oxides and hydrocarbons. Numerous common ethanolic motor-fuel formulations are being used with increasing frequency. Ethanol has a higher octane rating than petroleum fuels enabling combustion engines to run at higher compression ratios and thus give superior net performance. In addition, ethanol exhibits higher vapour pressure and heat of vaporization than gasoline and therefore increased power outputs are observed while using ethanol Low-level ethanol blends such as E10 (10 % ethanol/90 % gasoline) can be used in conventional vehicles; while high level blends, such as E85 (85 % ethanol/15 % gasoline) can only be used in specially designed vehicles, such as flexible fuel vehicles (FFVs) (Kanauchi et al., 2001).

2 LITERATURE REVIEW

2.1 Bioethanol

The French chemist, Henri Braconnot, was the first to discover that cellulose could be hydrolyzed into sugars by treatment with sulfuric acid in 1819. The hydrolyzed sugar could then be processed to form ethanol through fermentation. The first commercialized ethanol production began in Germany in 1898, where acid was used to hydrolyze cellulose. In the United States, the Standard Alcohol Company opened the first cellulosic ethanol production plant in South Carolina in 1910. Later, a second plant was opened in Louisiana. However, both plants were closed after World War I due to economic reasons. The invention of ethanol as an automobile fuel is not a new. Already in 1908, Ford’s model T could be designed to run on either gasoline or alcohol (DiPardo, 2000). However, after World War II the need in using ethanol as a fuel declined because cheap gasoline made from petroleum was available.

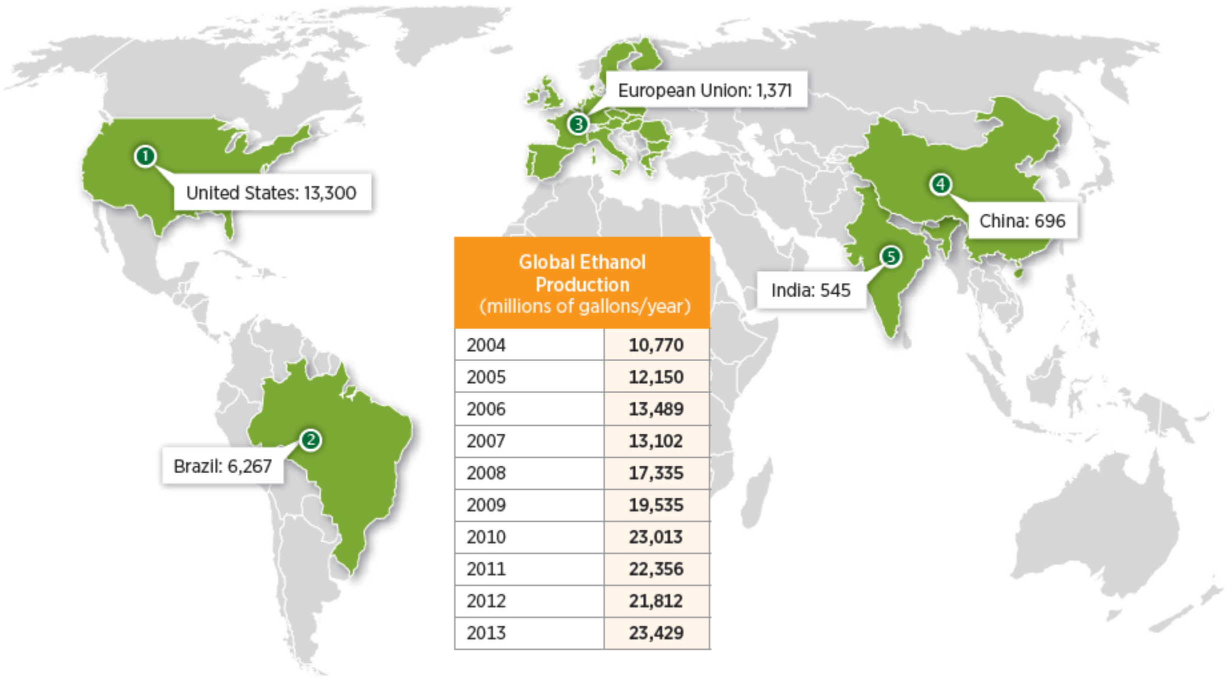
2.1.1 World fuel ethanol production

After World War II, the use of bioethanol was neglected due to its expensive production cost compared to petroleum fuel until the oil crisis in the 1970s (Demirbas, 2009). The interest in using bioethanol has been increasing since the 1980s and it has been considered as an alternative fuel in many countries. Global ethanol production increased from 13.12 billions of gallons in 2007 to 25.68 billions of gallons in 2015 with a slight decreased in 2012 and 2013 (Staniszewski et al., 2007).

United States is the largest ethanol producer with the production of nearly 15 billion gallons in 2015(Renewable Fuels Association US, 2015). The production of ethanol by United States and Brazil contribute to 85 % world's ethanol production. Bioethanol is also known as ethyl alcohol or chemically C_2H_5OH or EtOH. It can be used directly as pure ethanol or blended with gasoline to produce “gasohol” (Pejin et al., 2009). It can be used as a gasoline improver or octane enhancer and in bioethanol-diesel blends to reduce the emission of exhaust gasses (Balat, 2009). Bioethanol offers several advantages over gasoline such as higher octane number (108), broader flammability limits, higher flame speeds and increased heats of vaporization. In contrast to petroleum fuel, bioethanol is less toxic, readily biodegradable and produces lesser air-borne pollutants (John et al., 2011).

In 1970‟s, the interest in fuel ethanol was renewed due to the oil crisis (DiPardo, 2000). Today, there are many flexible fuel automobile models (vehicles that can run on mixtures of ethanol and gasoline containing up to 85 % ethanol) available from various manufacturers (BAFF, 2006).

About 99 % of the fuel ethanol is produced from cultivated crops (BAFF, 2006). Brazil has been the leading ethanol producer of the world. However, for the last few years USA has increased its production and in 2012 both countries have an annual production of about 16 million m^3 (F.O. Licht‟s World ethanol and biofuels report, 2006). The Brazilian ethanol is mainly produced from sugarcane. Brazil is the world leader in the use of ethanol as an automobile fuel. In Brazil, the ordinary gasoline, which is used in about 7, 000, 000 cars, contains about 24 % ethanol. In addition, 4 million automobiles drive on a blend of 95 % ethanol and 5% water (BAFF, 2006). In USA, ethanol is mainly produced from corn.



Top Five Regions (2013) **Ethanol Production** (millions of gallons/year)

Global Ethanol Production (millions of gallons/year)	
2004	10,770
2005	12,150
2006	13,489
2007	13,102
2008	17,335
2009	19,535
2010	23,013
2011	22,356
2012	21,812
2013	23,429

Figure 2.1 Top five ethanol production countries

(Data Source: F.O. Licht, cited in Renewable Fuels Association, Ethanol Industry Outlook 2008-2014 reports. Available at www.ethanolrfa.org/pages/annual-industry-outlook)

2.1.2 Ethanol production in Ethiopia

Ethanol production in Ethiopia has been started since 1998/99 in Fincha sugar factory with the capacity of 1,907 m^3 per year. In 2010/11 Ethanol production started in Metahra sugar factory with the capacity of 6,373 m^3 per year. Currently, both factories produce 19,805 m^3 ethanol per year (Ethiopia Sugar Corporation). The main raw material for this product is molasses which is a by-product of sugar production from the crystallization process. The worldwide recent awareness for the use of ethanol to replace petroleum and generation of power along with sugar mill plants should have led to setting up of number of ethanol plants and co-generations. Ethiopia has several sugar real estate's such as Fincha, Metehara and Wonji Shoa are among the industries which are run and administered by Sugar Development Agency. Among molasses derived products ethanol takes the largest part, but its utilization must attract the attention of the government policy makers in order to utilize as a bioethanol. Bioethanol or biofuel is ethanol based products that can process into liquid fuels for transport purposes (ESDA, 2015).

Table 2.1 Ethanol produced sugar factory in Ethiopia since 1998/99

Year	Ethanol produced (liters)		
	Fincha	Metahra	Total
1998/99	1,907,000	_	1,907,000
1999/00	720,000	_	720,000
2000/01	1,790,571	_	1,790,571
2001/02	209,444	_	209,444
2002/03	894,624	_	894,624
2003/04	911,431	_	911,431
2004/05	1,636,047	_	1,636,047
2005/06	6,847,816	_	6,847,816
2006/07	6,066,860	_	6,066,860
2007/08	5,330,337	_	5,330,337
2008/09	5,878,516	_	5,878,516
2009/10	7,116,585	_	7,116,585

2010/11	7,127,895	6,373,775	13,501,670
2011/12	6,794,000	7,658,000	14,452,000
2012/13	7,620,500	7,063,000	14,683,500
2013/14	11,678,000	7,767,000	19,445,000
2014/15	10,999,000	8,806,000	19,805,000
2015/16	2,744,432	6,789,456	9,533,888

Data source: Ethiopia Sugar Corporation

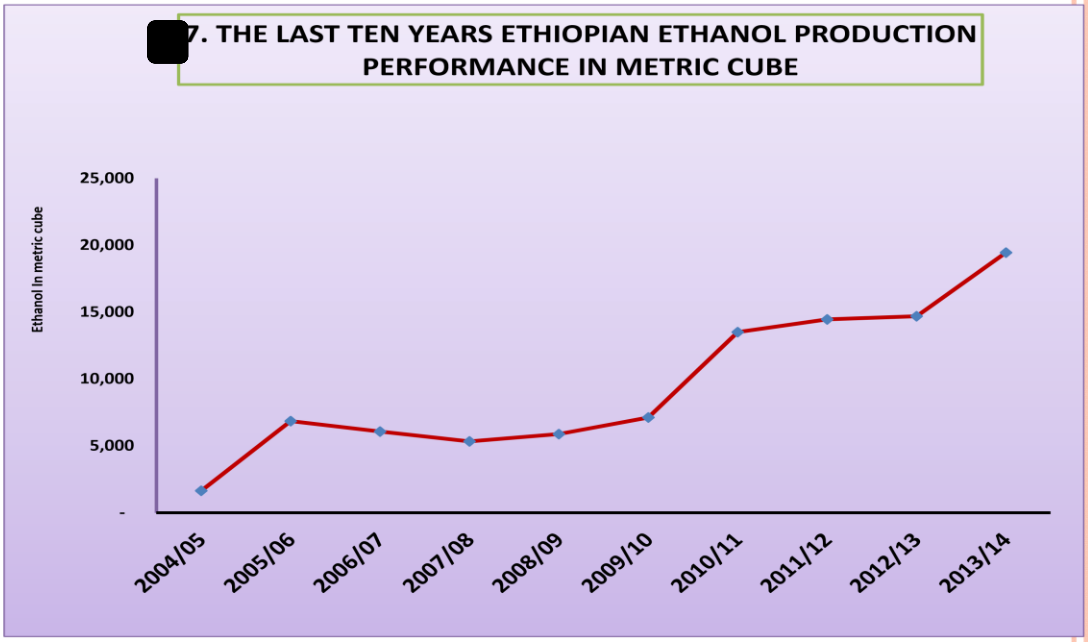


Figure 2.2 Ten years Ethiopia ethanol production performance

2.2 Generation of bioethanol

A variety of feedstocks from the first, second and third generation has been used in bioethanol production. The first-generation bioethanol involves feedstocks rich in sucrose (sugar cane, sugar beet, sweet sorghum and fruits) and starch (corn, wheat, rice, potato, cassava, sweet potato and barley).

Second-generation bioethanol comes from lignocellulosic biomass such as wood, straw and grasses. Third-generation bioethanol has been derived from algal biomass including microalgae and macro-algae (Nigam and Singh, 2011).

2.2 .1 First generation

Current commercial production of bioethanol is primarily based on sugar and starch crops (first-generation feedstock) (Hayes, 2009). The use of such feedstock is considered unsustainable due to competition with land and water, which are meant for food production; and unethical due to the controversial role in causing increase in food prices. First-generation biofuels, mainly from corn and other food-based crops are being used as a direct substitute for fossil fuels in transport. However, they are available in limited volumes that do not make them serious replacements for petroleum.

2.2 .2 Second generation

Second-generation biofuels from forest and crop residues, energy crops and municipal and construction waste, will arguably reduce net carbon emission, increase energy efficiency and reduce energy dependency, potentially overcoming the limitations of first-generation biofuels. Over 90 % of the world’s bioethanol derives from crops (60 % from cane sugar and beet sugar and the remainder from grains, mainly corn starch using the (“first generation of ethanol plants”. The US ethanol industry uses corn as its main feedstock (Licht, 2008).

The goal of second-generation biofuels is to extend the amount of biofuel that can be produced sustainably by using biomass comprised of the residual non-food parts of current crops, as well as other crops that are not used for food purposes and also municipal, industrial and construction waste.

Second-generation biofuels are expected to reduce net carbon emission, increase energy efficiency and reduce energy dependency, potentially overcoming the limitations of first generation biofuels. Additionally, researches on third-generation biofuels (e.g. algae and cyanobacteria) and fourth-generation biofuels (e.g. bio-hydrogen and bioelectricity using photosynthetic mechanisms) is also being explored. (Gressel, 2008).

Table 2.2 selected second-generation biomass feedstocks

Energy crops	Agricultural and wood residues	Organic waste	Traditional breeding and genetically modified crops	Vegetable oils
Amaranth	Barn	Animal fats	Miscanthus	Calophyllum inophyllum
Bamboo	Citrus waste	Food waste	Switchgrass	Corn oil
Energy maize	Corn Stover	Municipal solid waste	Willow	Caster bean
Eucalyptus	Green waste	Olive pulp		Cottonseed
Grass	Industrial waste	Recycled cooking oil		Jatropha
Miscanthus	Sugarcane bagasse	Wastewater from pulp and paper industry		Palm
Oilseed rape	Sawdust	Wastewater from sugar factory		
Winter tritricade	Wheat straw	Organic waste		
Salix	Waste rice straw	Animal fats		
Sugar-beet	Wood	Food waste		
Sweet sorghum	Wood chips	Municipal solid waste		

Second-generation feedstocks in the form of lignocellulosic biomass are sustainable and fair alternatives because they are cheap, abundant and they do not exert undue pressure on land and food (Sims et al., 2010). Lignocellulose is composed of cellulose and hemicellulose carbohydrate polymers which are held together by lignin. The sugar monomers present in the carbohydrate polymers can be converted into ethanol via fermentation after hydrolysis using chemicals or cellulolytic and hemi cellulolytic enzymes (Balat, 2011). Lignocellulosic biomass is widely available in the form of agricultural residues, wood and forest residues, dedicated energy crops and grasses, industrial wastes and organic component of municipal solid wastes (Banerjee et al., 2010). Since most of these materials are regarded as waste, utilizing them for ethanol production is a means of relieving the environment of potential hazards. Moreover, their use in ethanol production has a lower net energy balance and lower greenhouse gases emissions and they are adaptable for use on marginal lands which are not suitable for food crops (Hayes, 2009; Sims et al., 2010). Additionally, only lignocellulosic ethanol has the potential to be produced and consumed sustainably under socio-economic considerations (Solomon, 2010).

2.2 .3 Third generation

The processes being developed to improve algal oil yields are broadly referred to as third generation biofuels technologies (Gressel, 2008). Microalgae have high lipid content and grow in aquatic environments, and hence would not compete for farmland and the global food supply chain. Among the biofuels that can be produced from microalgae, biodiesel is nearest to commercial production. Biodiesel has been widely studied for many years, as it is derived from other feedstocks, such as soybean oil, palm oil, and rapeseed (canola) oil (Argonne National Laboratory, 2008).

An example of the “third-generation biofuel plants” is to produce biomass without extensive use of land using macro-algae as another potential source of biofuel feedstock. Aquatic unicellular green algae, such as Chlorella Species, are typically considered for biodiesel production owing to their high growth rate, population density, and oil content. In addition to their high yields, macro-algae cultures are not land-intensive and may provide further benefits of wastewater remediation or nutrient reduction, but in terms of economical analysis it not visible (Campbell, 2008).

2.3 Feedstocks for bioethanol production

Almost any plant- based material can be an ethanol feedstock. All plants contain sugars, and these sugars can be fermented to make ethanol in a process called biochemical conversion. Plant material also can be converted to ethanol using heat and chemicals in a process called thermo-chemical conversion. According to scientists, approximately one tons of wastes are produced for every ten tons of bananas, made up of skins, leaves, and stems. Ethanol can be produced synthetically from petroleum or by the microbial fermentation of sugars. The three main groups of raw materials for the production of ethanol by fermentation are sugar, starch, and lignocellulose (Lin and Tanaka, 2006).

2.3.1 Substrates

Ethanol is produced from various kinds of substrates. The substrate used for ethanol production is chosen based on the regional availability and economical efficiency.

2.3.1.1 Sucrose containing materials

Ethanol is produced by fermentation. Fermentation process is a process to convert sugar to ethanol. Sucrose containing materials could simplify the ethanol production process. Fermentation involves microorganisms that use the fermentable sugars as 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 glucoses 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. Although fungi, bacteria, and yeast microorganisms can be used for fermentation, specific yeast (*Saccharomyces cerevisiae* also known as Bakers' yeast, since it is commonly used in the banking industry) is frequently used to ferment glucose to ethanol. Theoretically, 100 g of glucose will produce 51.4 g of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100 %. Other biomass feed stocks rich in. 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.

Sugar cane

Brazil is the world second biggest ethanol producer. In Brazil, sugarcane is the major substrate for ethanol (Goldemberg et al., 2008). Countries in Central America and Caribbean are suitable for sugarcane cultivation, and their ethanol production is increasing recently.

Sugar beet

Sugar beet is mainly cultivated in European countries (Power et al., 2008) since it grows under cold climate.

Sugar sorghum

Sugar sorghum is also a sucrose containing crop. It yields large amount of biomass and sugar due to its high photosynthetic efficiency (Giorgis et al., 1997).

2.3.1.2 Starchy materials

Starch is converted to sugar by saccharification followed by fermentation. Today, saccharification and fermentation are done simultaneously (SSF: simultaneous saccharification and fermentation). Starch-based materials that are commonly used for ethanol production include corn, potatoes, cassava, and various cereal grains. Starch is a biopolymer and defined as a homo-polymer consisting only one monomer, D-glucose (Pongsawatmanit et al., 2007). During bioethanol production from starch, it is necessary to break down the chains of this carbohydrate for obtaining glucose syrup, which can be converted into bioethanol by yeasts. This type of feedstock is the most utilized for bioethanol production in North America and Europe (Sanchez, 2007). Starch can be converted to fermentable sugar by a method called the hydrolysis technique. Hydrolysis is a reaction of starch with water, typically performed by cooking the starch at high and low temperatures which is normally used to break down the starch into fermentable sugar. Dextrin oligosaccharides are generated by adding α-amylase and gluco-amylase to obtain glucose (Wheals et al., 1999). A disadvantage of using starch based materials for ethanol production is high hydrolysis cost due to high energy costs of the cooking step and the high costs of the amylolytic enzymes (Lin and Tanaka, 2006).

Corn

It is relatively easy to obtain high purity starch from corn. As the world biggest corn producer, the United States mainly produces ethanol from corn, and this also makes the United States the world biggest ethanol producer.

Other starchy materials

Any kind of starch containing crop can be used to produce ethanol. Many researches on ethanol production from various starchy materials, such as potato (Quintero et al., 2008), sweet potato (Sree et al., 1999), cassava (Leng et al., 2008), and wheat (Murphy and Power, 2008), have been investigated.

a. Lignocellulosic Biomass as Source of Ethanol

Lignocellulose-based feedstocks that can be considered for ethanol production are wood residues, agricultural residues, and the spent sulfite liquor from pulp and paper mills. The advantage of using lignocellulose as raw material for ethanol production is due to its abundance and relatively cheap (Wheals et al., 1999).

In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production is difficult or relatively recalcitrant to hydrolysis (Gray et al., 2006). Because of its complex structure, this resists degradation. The basic structure of all lignocellulosic biomass consists of cellulose $(C_6H_{10}O_5)_x$, hemicelluloses $(C_5H_8O_4)_m$, and lignin $[C_9H_{10}O_3.(OCH_3)_{0.9-1.7}]_n$. Production of ethanol from lignocellulosic waste materials, such as sawdust from the forestry industry and BSG from Beer industries have a benefits from the fact that the energy input for the overall process can be kept low. Energy-related expenses for planting, fertilization and harvesting can be avoided if waste materials are used. If lignocellulosic waste materials are used, there will not be any competition for the limited agricultural land available, which instead might be needed for food production (Sun and Cheng, 2002).

Like sugar materials, starchy materials are also in the human food chain and are thus expensive. Fortunately, another alternative exists that is cellulosic material. Examples of cellulosic 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 from the human food chain makes cellulosic materials relatively inexpensive feedstocks for ethanol production (Badger, 2002). The global production of plant biomass, with over 90 % lignocelluloses content, is estimated to be about 2×10^{11} tons/year, where about $8\text{-}20\times10^{9}$ tons of primary biomass remain potentially accessible annually. Over the last few decades, extensive attention has been devoted to research on the conversion of lignocellulosic materials to ethanol. Lignocelluloses are complex mixtures of carbohydrate polymers, namely cellulose, hemicelluloses, lignin, and a small amount of compounds known as extractives.

The second generation ethanol feedstock primarily comprises feedstock called cellulosic feedstock. In the case of these feedstocks, ethanol is derived not from the starch component, but from the lignocellulosic component of the feedstock. A large number of non-food wild plants that grow in non-cultivated and non-arable lands, and even plant waste, contain lignocellulose; as a result, the second generation ethanol feedstock overcome the two main bottlenecks for the first generation feedstock: adversely effects on food prices, and inability to scale.

Many studies are going on for ethanol production from lignocellulosic biomass. Lignocellulosic materials include maize silage (Oleskowicz et al., 2008), barely hull (Kim et al., 2008), and paper sludge (Marques et al., 2008). The difficulties of using lignocellulosic materials are there poor porosity, high crystallinity, and lignin contents. Various kinds of pretreatment techniques have been investigated, such as steam (Linde et al., 2008), acid (Nichols et al., 2008), and alkali (Hu and Wen, 2008) treatments.

b. Lignocellulosic molecular components

The main components of the lignocellulosic biomass are cellulose, hemicellulose, lignin, and a remaining smaller part (extractives and ash). The composition of lignocellulose highly depends on its source. There is a significant variation of the lignin and hemi cellulose content of lignocellulose depending on whether it is derived from hardwood, softwood, or herbaceous/agricultural residue biomass.

The main components of lignocellulosic biomass are cellulose (30–35 %), hemicellulose (25–30 %) and lignin (10–20 %). In addition, lignocellulose contains protein, lipids, water and other items (Yang, 2001; Badger, 2000; Mielenz, 2001; Girio et al., 2010). Cellulosic and hemi-cellulosic polymers constitute approximately 70 % of the entire biomass and are connected to the lignin component through a variety of covalent bonds that give the lignocellulosic biomass significant robustness and resistance to bio- chemical or physical treatment (Edye 2008; Balat, 2008).

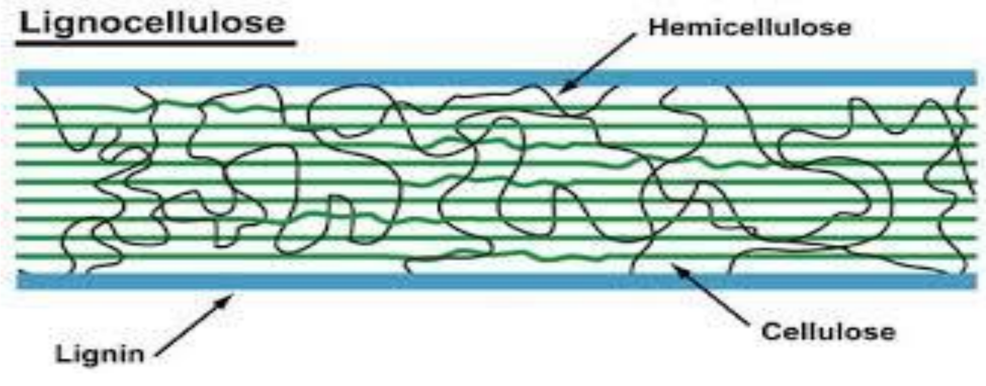


Figure 2.3 components of lignocellulose structure

c. Hemicellulose

Hemicellulose has a vague, soft and changeable structure of hetero-polymers including hexoses (glucose, galactose, mannose), pentoses (xylose, arabinose) and sugar (uronic-acids) (glucuronic, galacturonic, methyl-galacturonic) (Saha, 2003).

The hemi cellulosic chain consists of xylose (90 %) and arabinose (10 %). Xylan is the primary component of hemicellulose and its composition varies in each feedstock. For this reason, hemicellulose stands in need of wide variety of enzymes to be completely hydrolyzed into free monomers (Girio et al., 2010; Sun et al., 2004; Ebringerova et al., 2005).

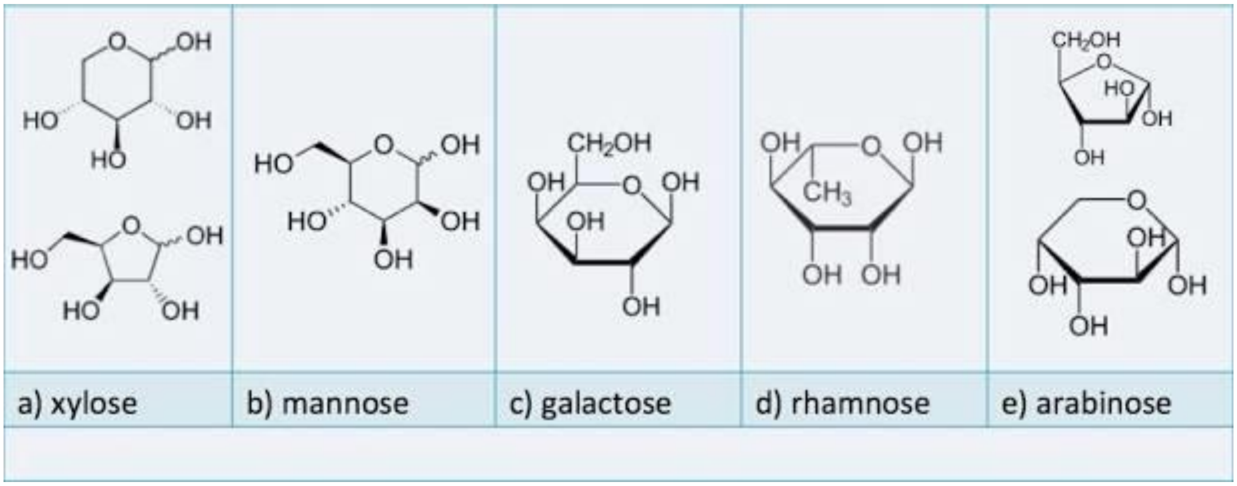


Figure 2.4 components of hemicellulose structure

d. Cellulose

Cellulose is a linear polymer which contains several thousand of 1, 4-b-glucosidic bonds connecting thousands of glucose units. The structure is crystallic because of the hydrogen bridges between the polymers. This large amount of hydrogen linkages provides toughness and compactness to the cellulose molecule. (Deguchi et al., 2006) refer that for the conversion of cellulosic crystalline to an amorphous structure, a temperature of 320 °C and a pressure of 25 MPa is required. Cellulose is the rifest organic polymer on earth and make up 30 % of plant biomass. However, cotton consists of almost 100% cellulose (Klemm et al., 2005).

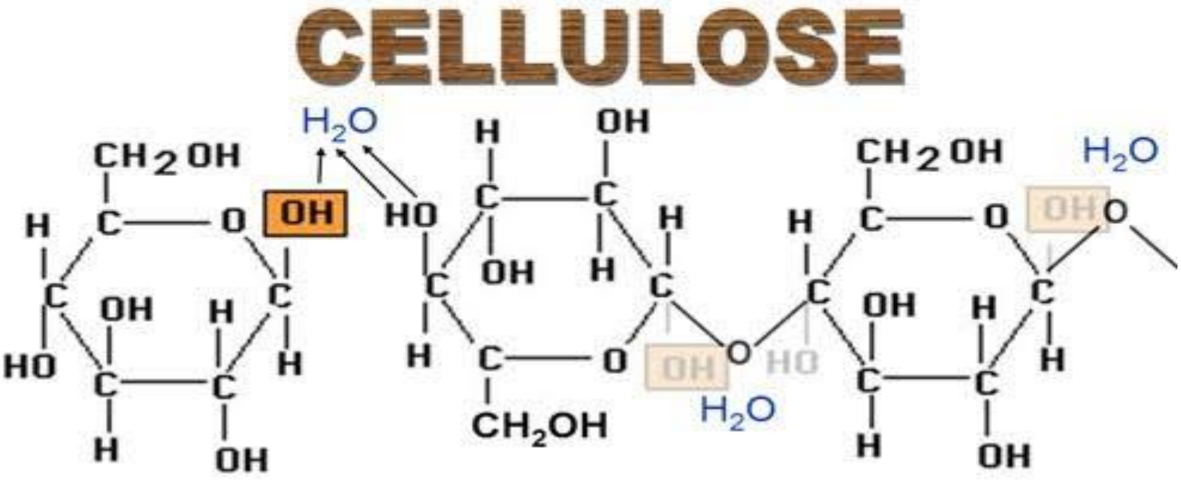


Figure 2.5 components of cellulose structure

e. Lignin

Lignin is a complex polymer coupled via covalent bonds to xylans rendering massiveness and stability to the plant cell wall. It contains three main monomers, coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Mielenz, 2001). Lignin is a copious natural polymer and a dominant constituent of wood (30–60 % for softwoods and 30–55 % for hardwoods), while agricultural residues and grasses contain 3–15 % and 10–30 % respectively (Demirbas, 2005). Contrarily, crop residues like corn Stover, rice and wheat straws contain particularly hemicellulose. (Chen et al., 2006) pointed out that lignin modification via genetic engineering techniques could increase the bioethanol yield and furthermore to be a potential source to give bio-refineries financial solvency.

Extractives and ash

Any numbers of different compounds (resins, phenolic, and other chemicals) in biomass that are not an integral part of the cellular structure are called extractives. These compounds can be extracted from biomass by means of polar and non-polar solvents including hot or cold water, ether, benzene, methanol, or other solvents that do not degrade the biomass structure.

f. Barley spent grain

Barley spent grains are Lignocellulosic materials, which are the main by-product of the brewing industry corresponding to around 85% of the total by-products. This byproduct of beer brewing consist of the residue of malt and grain which remains after the mashing and lautering; it is primarily a mixture of grain and grain husks from which a majority of its sugars were extracted. They are a rich source of lignocellulose, which may be converted to fermentable sugars. In addition, some investigation on the enzymatic hydrolysis of BSG has been carried out in order to release the ferulic and p-coumaric acids with an esterase and a xylanase. Although a huge amount of this byproduct is produced every year, only a few investigations have been carried out to consider it as a low-cost raw material for energy production (Mussatto et al., 2006; Santos et al., 2003).

The chemical composition of BSG varies according to the barley variety, harvesting time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Santos et al., 2003). Thus the composition of BSG as described in the literature is variable containing mainly hemicellulose in the form of arabinoxylans from the barley grain and cellulose (Mussatto et al., 2006; Santos et al., 2003) and has the potential to serve as a low-cost feedstock for the production of ethanol.

2.2.2.2 Availability of barley spent grain in Ethiopia

Currently, there is a rapid growth of brewery industries in Ethiopia. There are about twelve breweries in full function and production. There are also some breweries being expanded, and expected to be completed for the next few years. In Ethiopia around 309,002 kg barley spent grain is generated daily. The spent grain from Ethiopian breweries, now a days is only used for animal feed. The individual production capacity of the Brewery industries is presented in Table 2.3 below.

Table 2.3 Capacity of spent grain generated from Ethiopian brewery industries

No	Name of Brewery in Ethiopia	Capacity of spent grain generated Kg per day
1	Dashen (Gonder)	31,000
2	Dashen (debrebrhan)	21,830
3	Saint George Hawassa	32,980
4	Saint George Addis Ababa	33,592
5	Saint George Kombelcha	32,640
6	Meta	21,930
7	Bedele	21,930
8	Harar	22,950
9	Waliya	31,980
10	Habesha	21,780
11	Raya	20,500
Data source from Addis Ababa university 5 kilo campus department of chemical and bioengineering research paper June 2016		
12	Zebider	15,890

Data sources: Direct phone interview with the industry production manager

2.4 Bioethanol production process

There are two different methods (i.e. thermochemical and biochemical conversion) for bioethanol production from lignocellulose (Demirbas, 2007). Both methods conclude into fragments of lignin, hemicellulose and cellulose via degradation of lignocellulose. Polysaccharides are hydrolyzed into sugars and immediately are converted into bioethanol (Chandel et al., 2007; Gamage et al., 2010). However, these conversion technologies are not similar techniques. (Mu et al., 2010) State that the thermochemical route includes feedstock gasification at 800 °C with a catalytic reaction to ensue. This technology requires high level of heat and results into a synthesis gas (syngas) such as CO, H_2 and CO_2. Syngas can be chemically converted into a mixture of alcohols at 300 °C using MoS_2

as the catalyst. Alternatively, syngas can also be further processed into ethanol using the microorganism *Clostridium ljungdahlii*, *Saccharomyces cerevisiae* or *Zymomonas mobilis* (Younesi et al., 2005; Liu et al., 2014). In contrast to the thermochemical process towards syngas, the biochemical route includes mild physical and/or thermochemical pre-treatment, and biological pretreatment using hydrolytic enzymes to degrade cellulose and hemicellulose. The physical and/or thermochemical pretreatment is mainly used to submerge (crush) insubordinate substances and push cellulose availability/accessibility to cellulases and hemi-cellulases in the biological pretreatment to produce the monomeric sugars (Yang and Wyman, 2008); National Renewable Energy Laboratory, 2011).

The upstream process includes hydrolysis of cellulose and breakdown of hemicellulose into soluble sugars. Afterwards the sugars are converted into bioethanol via fermentation and pure ethanol is produced via distillation (Chandel et al., 2007; Zhu et al., 2009). Contemporaneously, the recalcitrant by-product, lignin, can be combusted and converted into power and heat (Gamage et al., 2010).

In general, biochemical conversion consists of four unit operations i.e. pretreatment, enzyme hydrolysis, fermentation and distillation (Sanchez and Cardona, 2008; Spatari et al., 2010). Nowadays, the biochemical approach is the most commonly used process (Fehrenbacher et al., 2009).

2.4.1 Pretreatment

During the whole process, pretreatment is the most crucial step since it has a large impact on the efficiency of the overall bioconversion. In lignocellulosic biomass, cellulose and hemicellulose are densely packed together with lignin, which serves several plant functions including protection against enzymatic hydrolysis (Leonowicz et al., 1999). The aim of pretreatment is to disrupt recalcitrant structures of cellulosic biomass to make cellulose more accessible to the enzymes that convert carbohydrate polymers into fermentable sugars. During the pretreatment, the removal of lignin and hemicellulose depends on the pretreatment technology, process conditions, and severity. For example, acidic chemical pretreatment removes most of hemicellulose, and lignin is condensed when pretreatment temperature reaches above 160 °C (Sannigrahi et al., 2010). On the contrary, the ammonia fiber expansion (AFEX) pretreatment does not significantly remove hemicellulose.

Numerous pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of fermentable sugars. Typical goals of pretreatment include (1) producing highly digestible solids to enhance sugar yields during enzyme hydrolysis, (2) avoiding the degradation of sugars including those derived from hemicellulose, and (3) minimizing the formation of inhibitors for subsequent fermentation steps (Brodeur et al., 2011).

The pretreatment methods can be classified into different categories according to various criteria (Alvira et al., 2010; Hendriks et al., 2009). It is common to differentiate pretreatment methods that are efficient at high, low, or neutral pH. Basically, low pH methods require addition of acids to increase the hydrolytic capacity while higher pH methods need pH-adjusting agents such as sodium hydroxide. The neutral pH methods, mainly liquid hot water (LHW) pretreatment, simply apply water in the process. However, the substrate medium in the neutral methods is weakly acidic due to the release of organic acids from the biomass during the pretreatments.

Another way to do the grouping is based on the principal mechanism acting during pretreatment. As such, the methods can be classified as physical, physicochemical, and chemical pretreatments. In this review, mainly the first classification is used. The acidic pretreatments include dilute acid pretreatment (DAP), steam explosion pretreatment (SEP), and organosolv pretreatment. The neutral pretreatment is LHW, and the sodium hydroxide/lime pretreatment and AFEX are classified into the alkaline pretreatments. Besides the above methods, pretreatment of biomass with high pressure ozone (ozonolysis) was recently developed in bioethanol production (Sannigrahi et al., 2012). Pretreatment methods can be roughly divided into different categories: physical (milling and grinding), physicochemical (steam pretreatment/auto-hydrolysis), chemical (alkali, dilute acid, and organic solvents), biological or a combination of these. The above pretreatment technologies have been promising for cost-effective pretreatment of lignocellulosic biomass for biological conversion to fuels and chemicals.

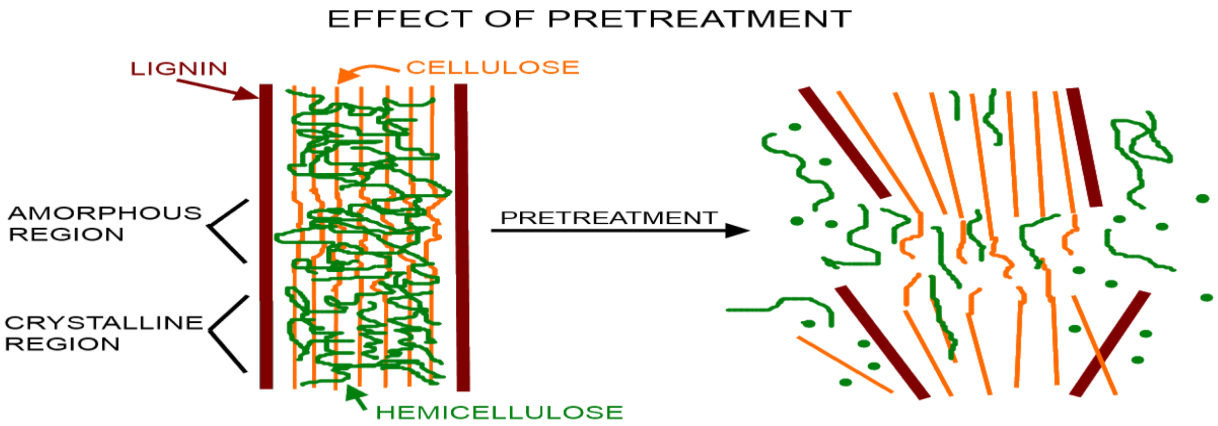


Figure 2.6 the role of pretreatment in the conversion of biomass to fuel (Hsu et al., 1980)

2.4.1.1 Physical pretreatment

a. Mechanical pulverization

Lignocellulosic biomass can be pulverized by chipping, grinding, shearing, or milling. The goal of mechanical pulverization is to reduce the particle sizes of the biomass and to increased surface area to volume ratio leads to improved cellulose hydrolysis. Some of the crystalline structure of cellulose is also destroyed using these methods, though the amount varies according to the type of biomass and power applied in milling or grinding. A vibration ball mill is the most effective mechanical tool for breaking the crystalline structure of cellulose (Millet et al., 1976). Mechanical pulverization methods generally are high cost and do not remove the lignin or hemicellulose.

2.4.1.2 Physicochemical pretreatment

a. Steam explosion pretreatment

b. Process description

SEP is one of the most common pretreatments applied to fractionate biomass components and increase its chemical and biological reactivity. In this method, biomass is treated with high-pressure saturated steam, and then, the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160–260 °C (corresponding pressure, 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure (Kumar et al., 2009). Addition of an acid catalyst such as H_2SO_4 because it is inexpensive to steam explosion can significantly increase its hemicellulose sugar yields (Wyman et al., 2005). SEP applies physicochemical pretreatment for lignocellulosic biomass.

The mechanical effects are caused by a sudden decompression to separate the fibers in the biomass (Alvira et al., 2010). The most important factors affecting the effectiveness of steam explosion are temperature, residence time, and the combined effect of both temperature (T)and time (t), which is described by the severity factor (R0) as follows (Alfani et al., 2000):

$$R0 = t \times \exp\left[\frac{T-100}{14.75}\right] \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.1)$$

Higher temperatures result in an increased removal of hemicelluloses from the solid fraction and an enhanced cellulose digestibility; they also promote higher sugar degradation. Previous studies indicated the optimal conditions for maximum sugar yield with a severity factor (R_0) between 3.0 and 4.5 (Alfani et al., 2000).

2.4.1.2.1.2 Mode of action

During the SEP pretreatment, the steam penetrates the plant cell wall, causing hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis (Taherzedeh and Karimi, 2007). The external acid (H_2SO_4) or the acetic acid released from acetylated hemicellulose serves to catalyze and further hydrolyze hemicellulose in the biomass. In addition, water itself also possesses certain acid properties at high temperature, thereby contributes to the hydrolysis hemicellulose during the SEP (Weil et al., 1997). In general, the hemicelluloses are dissolved as oligosaccharides after acid hydrolysis, which are partially further hydrolyzed to monosaccharides. Under high temperature and acid conditions, these monosaccharides can be degraded into inhibitor compounds (i.e. furfural and HMF) for the subsequent enzymatic hydrolysis (Oliva et al., 2014). In addition, the acids also lead to the acidolysis of lignin and the cleavages of β-O-4 linkage, which are similar to the LHW and DAP pretreatments (Li et al., 2007). As steam explosion pretreatment conditions get more severe, the condensation and re-polymerization reactions will take place between the decomposition products of hemicelluloses, leading to the formation of pseudo-lignin, as observed in DAP pretreatment (Negro, 2003; Li and Gellerstedt, 2008).

During the SEP pretreatment, the structure changes of cellulose are mainly on the degree of polymerization and degree of crystallinity. (Asada et al., 2012) Observed that the DP of cellulose decreased significantly as the steaming time increased until reaching a minimum value of about 700 when examining the bagasse SEP pretreatment, which was due to the de-polymerization of cellulose at a relatively short steaming time. The steam explosion pretreatment also affects cellulose crystallinity. After the SEP pretreatment, the increase of cellulose crystallinity was reported by several researchers in the literature (Negro, 2003; Corredor et al., 2009; Cherian et al., 2010). Generally, this increase was attributed to the selective hydrolysis of amorphous portions (Corredor et al., 2009) and transformation of some amorphous portions into crystalline portions in the cellulose structure (Deguchi et al., 2008). Furthermore, cellulose can be also degraded and form HMF during SEP; the mechanism of HMF formation in steam explosion pretreatment is similar to that in DAP (Hu et al., 2012).

In addition, after the SEP pretreatment, the accessible pore volume and accessible surface area of the pretreated fibers are progressively increased, which are probably the result of hemicellulose extraction and lignin redistribution (Wong et al., 2004).

c. Advantage and disadvantage

SEP pretreatment is an attractive process because it makes limited use of chemicals and it does not result in excessive dilution of the resulting sugars. Moreover, it requires low energy input. The disadvantage of SEP is its incomplete destruction of lignin-carbohydrate matrix resulting in the risk of condensation and precipitation of soluble lignin components making the biomass less digestible. Other limitation of this process is the formation of fermentation inhibitors at higher temperatures (Agbor et al., 2011).

2.4.1.3 Ammonia fiber expansion pretreatment

a. Process description

The AFEX pretreatment, also known as ammonia fiber explosion pretreatment, is another physicochemical process, much like SEP, in which the biomass material is subjected to liquid anhydrous ammonia under high pressures and moderate temperatures and is then rapidly depressurized. This swift pressure release leads to a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers (Chundawat et al., 2007). The AFEX is usually performed to treat moist biomass (0.1–2 g H_2O/g dry biomass) with liquid ammonia (0.3–2 g NH_3/g dry biomass) while heating (60–100 ºC) the biomass-water-ammonia mixture for a period of time (5–60 min) before rapidly releasing the pressure (Balan et al., 2009). AFEX can be carried out in either a batch or a flow-through reactor. The major parameters influencing the AFEX process are ammonia loading, temperature, pressure, moisture content of biomass, and residence time (Teymouri et al., 2014). During the AFEX pretreatment, about 95 % of the ammonia can be recovered in the gas phase and recycled, with a small amount of ammonia that remains in the lingo-cellulosics which might serve as a nitrogen source for the microbes in the fermentation process (Chundawat et al., 2010).

b. Mode of action

During the AFEX, the physicochemical treatment induces the disruption in the lignin carbohydrate linkage, hemicellulose hydrolysis, and ammonolysis of glucuronic cross-linked bonds, and partial de-crystallization of the cellulose structure, all leading to a higher accessible surface area for enzymatic attack (Teymouri et al., 2014). In AFEX pretreatment, the removal of acetyl groups from hemicellulose results in the formation of acetamide and acetic acid, but it is reported that AFEX removes the least amount of acetyl groups from lignocellulosic material compared to other leading pretreatment technologies (Kumar et al., 2009). In addition, the ammonia also causes a series of ammonolysis (amide formation) and hydrolysis reactions (acid formation) in the presence of water, which cleave LCC ester linkages (Chundawat et al., 2011). The major effect of AFEX on cellulose is the de-crystallization, which is mainly due to the generation of more amorphous portions in cellulose during this process (Kumar et al., 2009). These chemical structural changes lead to the formation of highly porous structures on the fiber cell wall, which greatly enhance enzyme accessibility to the cellulosic micro-fibrils (Chundawat et al., 2011).

c. Advantage and disadvantage

The important advantages of AFEX pretreatment include lower moisture content, lower formation of sugar degradation products due to moderate conditions, almost complete recovery of solid material and high cellulose digestibility in the subsequent enzymatic hydrolysis. One concern for this process is the cost of ammonia and the need for recycling technologies (Zheng et al., 2009).

2.4.1.4 Chemical pretreatment

Chemical pretreatment involves the de-polymerization of lignocellulosic materials with chemical agents such as acid, inorganic solvents, alkali and peroxidases.

a. Dilute-acid pretreatment

Acid pretreatment firstly developed in Germany in 1898. In this method concentrated or dilute mineral acids like sulfuric acid are used in order to break down hemicelluloses into monomeric sugars and simultaneously removing part of the lignin. Dilute acid hydrolysis is widely used due to the high reaction time that can be achieved with hemicellulose which significantly improve the availability of the cellulose fraction for hydrolysis (Sanchez et al., 2008). The reaction is carried out between 121-220 ºC under pressure; however temperature (~121 ºC) are optimal to reduce the formation of inhibitors .The reaction time dependent on the temperature used. This method needs a small amount of water since a small amount of energy is required to get an optimum temperature. Some advantages of this method are: (1) High yield of hemicelluloses sugar, (2) Remove of lignin and hemicelluloses in this method increases exposing of cellulose to enzyme, (3) Remove of heavy metals in the raw materials. Some disadvantages of this method are: (1) Neutralization of acids is necessary, (2) Degradation of hemicelluloses sugar, (3) production of inhibitors like acetic acid and furfural, (4) High cost of reactor due to high pressure and temperature and resistance to low pH (Sanchez, 2004; Pilcher et al., 2004).

b. Ozonolysis

Ozonolysis of biomass is usually carried out at low temperature (20–30 ºC), and the ozone flow rate ranged from 0.5 to 0.8 L/min. The ozone consumption (% dry wt. of biomass) is 2–7 % (Taherzadeh and Karimi, 2008; Mtui, 2009). Ozone treatment is one way of reducing the lignin content of lignocellulosic wastes. This results in an increase of the in vitro-digestibility of the treated material, and unlike other chemical treatments, it does not produce toxic residues. Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben and Miron, 1981), bagasse, and green hay, peanut, pine (Neely 1984), cotton straw (Ben and Shefet, 1983), and poplar sawdust (Vidal and Molinier, 1988). Research indicated (Garcia-Cubero et al., 2009) that ozone is highly reactive toward compounds incorporating conjugated double bonds and functional groups with high electron densities. Therefore, the moiety, most likely to be oxidized in ozonization of lignocellulosic materials, is lignin due to its high content of C=C bonds. Thus, during the ozonolysis, the degradation is mainly limited to lignin. Ozone attacks lignin releasing soluble compounds of less molecular weight, mainly organic acids such as formic and acetic acids (Garcia et al., 2009). The main advantages linked to this process are the lack of any degradation products that might interfere with subsequent hydrolysis or fermentation and the reactions occurring at ambient temperature and normal pressure. Furthermore, the fact that ozone can be easily decomposed by using a catalytic bed or increasing the temperature means that processes can be designed to minimize environmental pollution. A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive and less applicable (Contreras et al., 2002). However, recently, (Hu et al., 2012) demonstrated that a lower charge of ozone could be used to enhance the enzymatic digestibility of cellulose, if the ozone-treated biomass was not washed and thein situ-generated acids were employed in a subsequent DAP.

c. Alkaline pretreatment

Alkaline pretreatments have received numerous studies as another major chemical pretreatment technology besides acidic pretreatments. Alkaline pretreatments can be divided into two groups based on the chemical used: (1) pretreatments that use sodium or calcium hydroxide and (2) pretreatments that use ammonia (Carvalheiro et al., 2008). The process description and alternations of major chemical components for these two types of alkaline pretreatments are discussed in the following sections.

d. Sodium hydroxide and lime pretreatment

e. Process description

Both sodium hydroxide and lime pretreatments have been shown to be effectively enhancing cellulose digestibility (Wu et al., 2011; Kaar and Holtzapple, 2000). However, lime has received much more attentions than sodium hydroxide since it is inexpensive (about 6 % cost of sodium hydroxide) (Peters and Timmerhaus, 1991), has improved handling, and can be recovered easily by using carbonated wash water (Mosier et al., 2005). In comparison with other pretreatment technologies, the sodium hydroxide and lime pretreatments usually use lower temperatures and pressures, even at ambient conditions. Pretreatment time, however, is recorded in terms of hours or days which are much longer than other pretreatment processes. In the alkaline pretreatment, the residual alkali could be reused through the chemical recycle/recovery process, which may make the system more complex due to the need for chemical recovery (Zheng et al., 2009; Pavlostathis and Gossett, 1985). In order to increase the pretreatment efficiency usually, the particle size needs to be reduced to 10 mm or less (Mosier et al., 2005).

f. Mode of action

The sodium hydroxide and lime pretreatments are basically a delignification process, in which a significant amount of hemicellulose is solubilized as well. The major effect is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, the alkaline pretreatment could swell cell wall and improve cell wall accessibility for the subsequent enzymatic hydrolysis. The proposed reaction mechanism is believed to be saponification of intermolecular ester bonds cross-linking hemicellulose and lignin (Zheng et al., 2009). The saponification leads to the cleavage of lignin-carbohydrate complex (LCC) linkages, and the expose of cellulose micro-fibrils can increase enzymatic digestibility of cellulose. Acetyl groups and various uronic acid substitutes are also removed by alkali, thereby reducing steric hindrance of hydrolytic enzymes and increasing the accessibility of carbohydrates to enzymes (Wan et al., 2011). Furthermore, the degraded hemicellulose could also form furfural and HMF in the hydrolysates, but the amount is typically lower than that with DAP (Taherzadeh and Karimi, 2008). In addition, alkaline pretreatment decreases the DP of cellulose and causes swelling of cellulose, leading to an increase in its internal surface area (Fan et al., 1987). This makes cellulose more accessible for enzymes in the subsequent hydrolysis stage. In terms of cellulose crystallinity change during the pretreatment, research indicated that the amorphous regions suffered greater peeling reactions than the crystalline regions, and the occurrence of the peeling actions of the amorphous regions leads to an increase of cellulose crystallinity (Wu et al., 2011). During the alkaline pretreatment, lignin suffered delignification, which has similarities to soda chemical pulping technologies (Mosier et al., 2005; Chakar and Ragauskas, 2004). In addition, recent studies indicated that the alkali pretreatment could also increase the fiber porosity due to the disruption of biomass structures (Keshwani and Cheng, 2009; Xu et al., 2010).

g. Advantage and disadvantage

Compared with DAP, alkaline pretreatments have some practical operational advantages including lower reaction temperatures and pressures. A significant disadvantage of alkaline pretreatment is the conversion of alkali into irrecoverable salts and the incorporation of salts into the biomass during the pretreatment reactions so that the treatment of a large amount of salts becomes a challenging issue for alkaline pretreatment (Zheng et al., 2009). In addition, during the lime pretreatment, the precipitation of calcium oxalate on the heat exchanger and process streams is another issue to be considered since it may cause severe problems in the plants (Galbe and Zacchi, 2012).

h. Soaking in aqueous ammonia pretreatment

Another type of process utilizing ammonia is soaking aqueous ammonia (SAA), which appears as an interesting alternative since it is performed at lower temperature (30–75 °C) (Millet et al., 1976). The main purpose of SAA pretreatment is the removal of lignin, which is the physical and chemical barrier that inhibits accessibility of enzyme to the cellulose substrate (Kang et al., 2012). It is regarded as a valuable pretreatment methodology due to the retention of the hemicellulose fraction and removal of lignin after pretreatment (Kim and Lee, 2006). SAA has been shown to be able to remove 74 % of the lignin from corn Stover while retaining >85 % of the Xylan and nearly 100 % of the glucan (Kim and Lee, 2005). This allows easy downstream utilization of sugars in a single co-fermentation process in which net sugar yield was increased due to the presence of hemicelluloses (Gao et al., 2012). SAA utilizing lower temperatures and less extreme pH reduces the associated chemical and energy costs and may also reduce the formation of carbohydrate degradation products. However, the SAA pretreated biomass pretreated shows relatively lower enzymatic digestibility due to its mild pretreatment conditions (McIntosh and Vancov, 2011), (Pryor et al., 2012).

i. Organosolv pretreatment

j. Process description

In the organosolv pretreatment, numerous organic or aqueous solvent mixtures can be utilized, including methanol, ethanol, acetone, ethylene glycol, and tetra-hydro-furfuryl alcohol, in order to solubilize lignin and hemicellulose, providing treated cellulose suitable for enzymatic hydrolysis (Mielenz, 2001). Although several organic solvents can be applied in the organosolv pretreatments, the low molecular weight alcohols such as ethanol and methanol are favored solvents mainly due to their lower boiling points. The preferred conditions of organosolv process is generally in the following ranges: a cooking temperature of 180–195 ºC, a cooking time of 30–90 min, an ethanol concentration of 35–70 % (w/v), and a liquor to solid ratio ranging from 4:1 to 10:1. The pH of the liquor ranges from 2.0 to 4.0. In some studies, these mixtures are combined with acid catalysts (HCl, H_2SO_4, oxalic, or salicylic) to break hemicellulose bonds and lignin linkages (Hallac et al., 2010). A high yield of xylose can usually be obtained with the addition of acid. However, this acid addition can be avoided for a satisfactory delignification by increasing process temperature (above 185 ºC) (Duff et al., 1996). Usually, in the organosolv pretreatment, high lignin removal (>70 %) and minimum cellulose loss (less than 2 %) could be achieved (Papatheofanous et al., 1995).

k. Mode of action

During the organosolv pretreatment, the largest component, cellulose, is partially hydrolyzed into smaller fragments that largely remain insoluble. (Sannigrahi et al. 2012) Revealed the degree of cellulose crystallinity increases and the relative proportion of para-crystalline and amorphous cellulose decreases after the organosolv pretreatment of Loblolly pine.

The second largest component, hemicellulose is hydrolyzed mostly into soluble components, such as oligosaccharides, monosaccharides, and acetic acid. Acetic acid can act as catalyst for the rupture of lignin-carbohydrate complex (Zhao et al., 2009). Some of the pentose sugars are subsequently dehydrated under the operating conditions to form furfural (Kumar et al., 2009). The third major polymer component, lignin, is hydrolyzed into lower molecular weight fragments that dissolve in the aqueous ethanol liquor.

In addition, the de-polymerization of lignin occurs primarily through cleavage of β-O-4 linkages (Meshgini and Sarkanen, 1989). Moreover, lignin condensation was also observed in organosolv pretreatment (Evtiguin et al., 1997). In addition, the hydrolysis of hemicellulose and degradation of lignin in organosolv pretreatment also lead to the increase of cellulose surface accessibility (Chandra et al., 2011).

m. Advantage and disadvantage

Compared with other pretreatments, organosolv pretreatment has some advantages as follows:(1) organic solvents are always easy to recover by distillation and recycled for pretreatment; (2) the chemical recovery in organosolv pulping processes can isolate lignin as a solid material and carbohydrates as a syrup, both of which show promise as chemical feedstocks (Lora and Aziz, 1985), (Aziz and Sarkanen, 1989). It seems that organosolv pretreatment is feasible for bio-refinery of lignocellulosic biomass, which considers the utilization of all the biomass components. However, there are inherent drawbacks to the organosolv pretreatment. Organic solvents are always expensive, so it should be recovered as much as possible, but this causes increase of energy consumption. In addition, organosolv pretreatment must be performed under extremely tight and efficient control due to the volatility (flammability) of organic solvents which increase capital cost. Moreover, removal of solvents from the system is necessary since the solvents might be inhibitory to enzymatic hydrolysis and fermentative microorganisms (Gray et al., 2006).

Table 2.4 Effect of different chemical pretreatment technologies on the structure of lignocellulose

Pretreatment Method	Increase of accessible surface area	Cellulose DE crystallization	Hemicellulose Solubilization	Lignin Removal	Generation of inhibitor compound	Lignin structure alteration
DAP	H	L	H	L	H	H
SEP	H	L	M	L	H	M
Organosolv	H	L	H	H	H	H
LHW	H	L	M	L	L	M
NaOH	H	L	M	M	L	H
AFEX	H	H	L	L	L	H
SAA	H	L	L	H	L	L
Ozonolysis	H	L	M	H	L	H
High effect, Moderate effect, Low effect						

Data source: Alvira et al., 2010; Brodeur et al., 2011 and Li et al., 2007

2.4.1.5 Biological treatment

Biological pretreatment employs wood degrading microorganisms, including white, brown, and soft rot fungi, and bacteria to modify the chemical composition and/or structure of the lignocellulosic biomass so that the modified biomass is more amenable to enzyme digestion. Most biological pretreatment so far has focused on the degradation of lignin in lingo-cellulosic biomass. However, degradation of lignin usually accompanies the loss of cellulose and hemi-cellulose. In order to reduce and eliminate the sugar loss during biological pretreatment, the microbial strains should have low cellulase activity. White rot fungi are the most widely studied for biological pretreatment since they can degrade lignin more effectively and more specifically.

Biological pretreatment appears to be a promising technique and has very clear advantages, including no chemical requirement, low energy input, mild environmental conditions, and an environmentally friendly working manner. However, biological pretreatment is very slow (taking from weeks to a year) and requires careful control of growth conditions and a large amount of space to carry out. In addition, most lignolytic microorganisms solubilize or consume not only lignin but also hemicellulose and cellulose (Mosier et al., 2005).

2.4.2 Hydrolysis

Hydrolysis is a process where carbohydrate polymers are converted to simple fermentable sugars. This is facilitated through the pretreatment process, which changes the structure of the biomass (larger pores and higher surface area), thus allow the enzymes or chemical to enter the fiber (Alfani et al., 2000). Current technology for conversion of lignocellulose to bioethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by microorganisms. Hydrolysis is essential before fermentation to release the fermentable sugars. In the process, cellulose is cleaved to glucose, while hemicellulose results in several pentoses and hexoses (Taherzadeh and Karimi, 2007).

$$cellulose \xrightarrow{hydrolysis} glucose \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.2)$$

$$hemicellulose \xrightarrow{hydrolysis} pentose\ and\ hexoses \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.3)$$

The hydrolysis step can be performed in different ways, either acidic (dilute and concentrated) or enzymatic hydrolysis (Galbe and Zacchi, 2002).

2.4.2.1 Diluted acid hydrolysis

Chemical hydrolysis means primarily the use of acids; diluted or concentrated. Due to environmental and corrosion problems, dilute-acid hydrolysis has been prioritized instead of concentrated acid (Balat, 2011). In chemical hydrolysis, the pretreatment and the hydrolysis can be combined. Hydrolysis by dilute-acid occurs under high temperature and pressure with a short residence time, resulting in degradation of hemicellulose and cellulose. However, the glucose yield is low, glucose decomposition occurs and there will be a formation of high amount of undesirable by-products. The harsh conditions (acid together with high temperature and pressure) lead to high utility costs and the process will also require downstream neutralization (Su et al., 2006).

The main drawbacks of dilute acid hydrolysis, especially those performed in batch processes, are sugar degradation during hydrolysis, low overall sugar yield and the formation of several byproducts which inhibit the fermenting microorganism (Balat, 2011). The high hydrolysis temperature also causes corrosion problems even at low acid concentration and accelerates the sugar degradation. One way to reduce the sugar degradation is to perform a two -stage dilute acid hydrolysis. Here, mainly hemicellulose is hydrolyzed at relatively mild conditions to recover the 5-carbon sugars while the second stage is conducted under harsh conditions to recover the 6-carbon sugars (Balat, 2011). A two-stage dilute acid hydrolysis is generally preferred over a one stage hydrolysis, since e.g. Sugar degradation is reduced and less inhibitor is produced.

2.4.2.2 Concentrated Acid Hydrolysis

Hydrolysis of cellulosic materials by concentrated sulfuric or hydrochloric acids is a relatively old process. The concentrated acid process uses relatively mild temperatures, and the only pressures involved are those created by pumping materials from vessel to vessel. Reaction times are typically much longer than for dilute acid (Demirbas, 2005). This method generally uses concentrated sulfuric acid followed by a dilution with water to dissolve and hydrolyze or convert the substrate into sugar. This process provides a complete and rapid conversion of cellulose to glucose and hemicelluloses to 5-carbon sugars with little degradation. The critical factors needed to make this process economically viable are to optimize sugar recovery and cost effectively recovers the acid for recycling. The solid residue from the first stage is dewatered and soaked in a 30 to 40 % concentration of sulfuric acid for one to four hours as a pre-cellulose hydrolysis step. The solution is again dewatered and dried, increasing the acid concentration to about 70 %. After reacting in another vessel for 1 to 4 hr. at low temperatures, the contents are separated to recover the sugar and acid. The acid solution from the second stage is recycled to the first stage to provide the acid for the first stage hydrolysis.

2.4.2.3 Enzymatic hydrolysis

Hydrolysis process takes place after pretreatment to break down the feedstocks into fermentable sugar for bioethanol production as well as chemical and enzymatic methods are the most common techniques for hydrolyzing cellulose. In the chemical method, the hydrolysis is catalyzed by an acid; whereas in the enzymatic method, cellulolytic enzymes act as catalysts (Martin et al., 2002). Currently, enzymatic hydrolysis has attracted increasing attention as an alternative to acid hydrolysis because the process is highly specific, can be performed under milder reaction conditions (pH around 5.0 and temperature less than 50 ºC) with lower energy consumption and lower environmental impact. In addition, it does not present corrosion problems, and gives high yield of pure glucose with low formation of by-products that is favorable for the hydrolysate use in bioconversion processes (Liao et al., 2005; Martin et al., 2002). Due to these advantages, the enzymatic hydrolysis has been extensively studied in several lignocellulosic substrates, including rice straw (Vlasenko et al., 1997), rice hull (Sharma et al., 2001), corn Stover (Varga et al., 2002), corncob (Chen et al., 2007), dairy manure (Liao et al., 2005), softwoods (Pan et al., 2005), and sugarcane bagasse (Martin et al., 2002). Enzymatic hydrolysis requires enzymes to hydrolyze the feedstocks into fermentable sugars. Three types of enzymes that are commonly used for cellulose breakdown such as endo-β—1,4-glucanases, cellobio-hydrolases and β-glucosidases. The activity of cellulase enzyme is influenced by the concentration and source of the enzyme. Cellulose will be degraded into reducing sugars under mild reaction conditions (pH: 4.5–5.0, temperature: 45–50 ºC). Moreover, it does not cause corrosion problem in the reactors which can result in high sugar yields. The efficiency of enzymatic hydrolysis is influenced by optimized conditions such temperature, time, pH, enzyme loading and substrate concentration. The amount of fermentable sugar obtained increases as the enzyme load increases while cellulose load decreases.

Enzymatic hydrolysis can occur under milder conditions (40-50 ºC and pH 4.5-5), which give rise to two advantages of the process; low utility cost since there is low corrosion problems and low toxicity of the hydrolyzates (Taherzadeh and Karimi, 2007). In addition, it is also an environmental friendly process (Balat, 2011). However, enzymatic hydrolysis has longer hydrolysis time, and enzymes are more expensive than acid (Taherzadeh and Karimi, 2007). However, many experts consider enzymatic hydrolysis as the most cost-effective process in the long run (Hamelinck et al., 2005) and it is thought to be the key process to achieve an economically viable ethanol production (Horn and Eijsink, 2010).

Enzymatic hydrolysis of cellulose is a reaction carried out by cellulases enzymes, which are highly specific (Beguin, 1994). Cellulases are a mixture of three enzymes involved in the hydrolysis process of cellulose.

Reaction path way

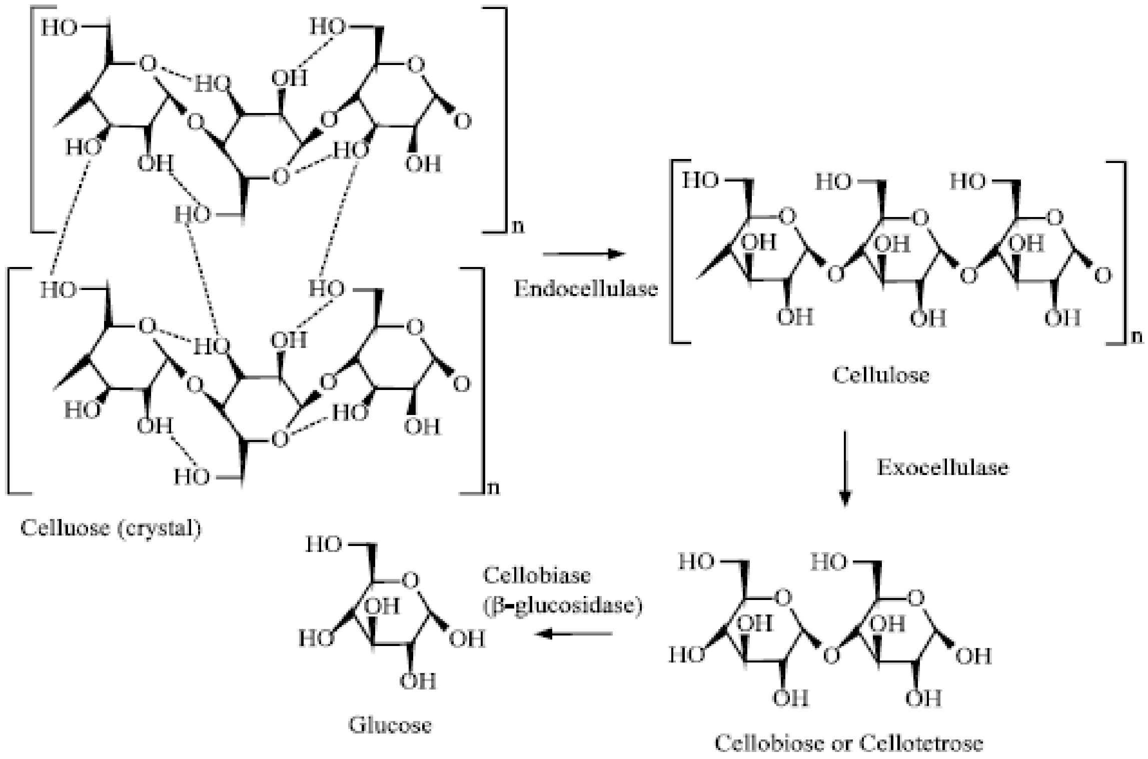


Figure 2.7 Reaction pathway from cellulose to glucose (Enzyme india, 2008)

The above reaction pathway shows the progression from the cellulose substrate to the glucose product through the activity of three enzymes: endocellulase, exocellulase and β-glucosidase. A consortium of enzymes- endoglucanase, exoglucanase and β-glucosidase (cellobiase), collectively known as cellulase, are needed to break down cellulose into its constituent glucose monomers. The endocellulase and exocellulase enzymes incrementally cleave cellobiose molecules off of the long chain polysaccharide molecules. This cellobiose intermediate then becomes the substrate in the hydrolysis reaction with β-glucosidase, producing the final glucose product. Cellulases are industrially important enzymes that can catalyze on cellulose material to produce fermentable sugars like mannose, glucose, xylose and arabinose.

a. Source of cellulases enzymes

Cellulases are industrially important enzymes that can catalyze on cellulose material to produce fermentable sugars like mannose, glucose, xylose and arabinose (Bajpai, 1999). Cellulases are obtained from various microorganisms: bacteria such as *Bacillus subtilus, Bacillus circulans, Bacillus sphaericus, Bacillus pumilus, Cellulomonas flavigena* (Long et al., 2012) and fungi like *Aspergillus species*. (Verma et at., 2012). Mainly, bacteria are more efficient for producing cellulases enzyme than fungi because of their high cell cultivation rate(Xia and Cen, 1999) Cellulases are widely used in processing industries such as fuel, textile, food, detergent and cosmetics, pulp and paper (Liu and Yang, 2007). Besides this, cellulases act as an additive in cattle and pig feeds (Maurya et al., 2012).

Cellulases enzyme production was investigated using various Solid substrates such as cellulose bagasse (Singhania. Et al., 2006), egg shell waste (Alam et al., 2008), ligno cellulosic waste from xylose industry (Zhang et al., 2004), lignocellulosic waste from the vinegar industry (Alam et al., 2009) wheat bran (Guruchandran and Sasikumar., 2011), sugar cane bagasse (Latifian et at., 2007), domestic wastewater sludge (Hideno et.al., 2011), sweet potato (Dos Santos et al., 2012), Oil palm lignocellulosic biomass in the form of empty fruit bunches (Taherzadeh and Karimi, 2007), Saw dust (Vyas, 2005), rice bran (Durand et at., 1997), rice straw (Agarwal et al., 2009), potato peel (Kumar et al., 2009), banana waste (Martín et al., 2002), BSG and ground nut shell waste (Cardona et al., 2007). In recent years, solid state fermentation has been preferred for enzyme production than submerged fermentation due to low water consumption, low energy requirement, low chances of contamination for producing high quality of product and easy separation of product, but bacteria’s need a liquid state fermentation for production of cellulase enzyme (Stephen et al., 2012).

2.4.4. Filter paper activity (FPase) assay for cellulase

The FPA is the key method for analysis of total cellulase activity.

The DNS reagent is used as a colorimetric method for the determination of reducing sugars, such as glucose. It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color-developing reaction. Sodium bisulfite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars. The cellulase activity was 1.5u/ml determined using equation 3.8.

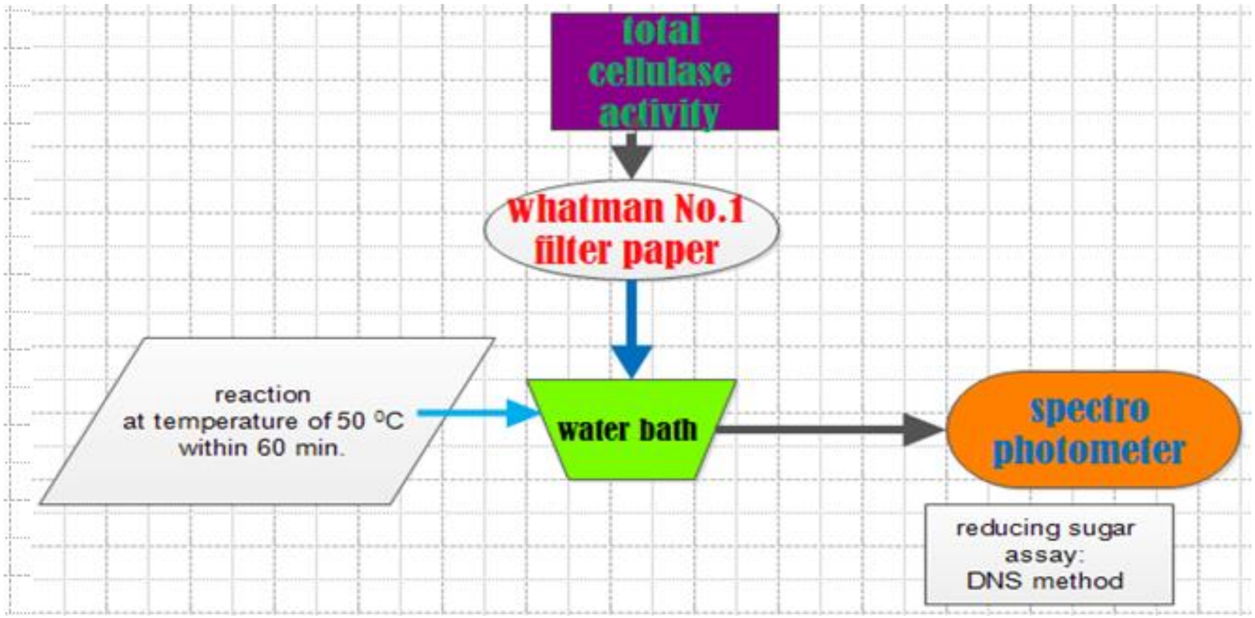


Figure 2.8 total cellulase activity

2.4.3 Fermentation

Fermenting microorganisms are used for the conversion of monomeric sugars to ethanol and other end products. Topically *Saccharomyces cerevisiae* converts the sugars into ethanol under anaerobic conditions at a temperature of 30 ºC. In this other by-products are generated in the form of CO_2 and N-based compounds. *Saccharomyces cerevisiae* is a prevalent microorganism and provides a high yield of ethanol (12.0–17.0% w/v; 90% of the theoretical yield) from sugars (Claassen et al., 1999). Ethanol production is influenced by pH of the broth as it affects bacterial contamination, yeast growth, fermentation rate and byproduct formation. The permeability of some essential nutrients into the cells is influenced by the concentration of H^+ in the fermentation broth. Moreover, the survival and growth of yeasts is influenced by the pH in the range of 2.75–4.25 (Fleet et al., 1993). In fermentation for ethanol production, the optimum pH range of *Saccharomyces cerevisiae* is 4.0–5.0 (Martin et al., 2002). When the pH was below than 4.0, a longer incubation period is required but the ethanol concentration was not reduced significantly. However, when then pH was above 5.0, the concentration of ethanol reduced substantially (Claassen et al., 1999).

The SHF is the traditional method for bioethanol production. Several studies have reported the weakness of *Saccharomyces cerevisiae* to ferment only hexose sugars and the interest for versatileacting microorganisms increased, so pentose sugars are fermented by fungus species (Martin et al., 2002).

2.4.4 Distillation

The end-product from fermentation process is a mixture of ethanol–water and requires further separation through a distillation process. Fractional distillation is a very common process to separate ethanol from water based on their different volatilities. The distillation column is heated and on the top of the column the distillate (bioethanol) is collected as it has lower boiling point (78.3 ºC) whereas water's boiling point is (100 ºC). However, the concentration of the ethanol distillate is about 92 % and further dehydration is required to obtain 99 % ethanol (Hamelinck et al., 2005).

2.5. Parameters affecting enzyme hydrolysis of lignocellulosic materials

The efficient use of enzymes depends on process variables such as temperature, pH, reaction time, enzyme concentration, pulp consistency (substrate concentration), intensity of agitation, and presence of other chemical species that may inhibit or accelerate their rates of reaction (Kaya et al., 2000).

2.5.1 Effect of the temperature

Enzymatic hydrolysis can occur under milder conditions (40-50 ºC and pH 4.5-5), which give rise to two advantages of the process; low utility cost since there is low corrosion problems and low toxicity of the hydrolyzates (Taherzadeh and Karimi, 2007).

2.5.2 Effect of enzyme loading

The type of enzyme or cellulase used would have an effect on the amount of reducing sugars released due to the variation of enzyme components in the cellulase or the activity of the enzymes. In a typical system, higher enzyme loadings would generate higher cellulose conversion rates due to the increase in the catalytic conversion of the cellulose material to sugars. However, from an economic standpoint it is necessary to produce sugars at low enzyme loadings because the cellulase enzyme is an expensive mixture that may end up being the main cost of the entire process.

2.5.3 Properties of the substrate

The properties of the substrate can affect the hydrolysis. These properties are: neutralizing capacity, proportion of easily hydrolysable hemicellulose and cellulose, amount and rate of hydrolysis of the difficult to hydrolyze materials, the length of the macromolecules, degree of polymerization of cellulose, configuration of the cellulose chain, and association of cellulose with other protective polymeric structures with the plant cell wall such as lignin, pectin, hemicellulose, protein, and mineral elements. Particle size is also one of the effective parameters.

2.5.4 The acidity of the system

Another parameter affecting the hydrolysis is the acidity of system. The acidity is dependent on the type and concentration of the acid used, amount of acid solution, amount of acid released from the biomass during hydrolysis, liquid to solid ratio, the neutralizing capacity of the lignocellulose, and movement of the solution during heating. When dilute acid hydrolysis is applied in a continuous process such as a screw fed co-current reactor, it requires a relatively short residence time. Therefore, the penetration of acid catalyst in the biomass, as well as dispersion in the reactor, can significantly affect the overall reaction, and consequently the reactor performance. Diffusivity of sulfuric acid is dependent on the nature of the lignocellulosic materials (Demibras, 2005).

2.5.5 Time of enzyme hydrolysis

Enzymatic hydrolysis is more specific and high conversion rates can be achieved in long reaction times (48 – 72 hr.). Research in this area usually focuses on obtaining very high yields, mostly at the expense of long reaction times, using high enzymes and low solids loading (Dias et al., 2013a). Taking into consideration that most of the cellulose is converted under 24 hr. (Stephen et al., 2012).

2.6 Factors Affecting Fermentation

2.6.1 Effect of sugar concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. The amount of ethanol produced is proportional to the amount of sugar added; thus, high sugar concentrations are desired. However, too high sugar concentrations can inhibit metabolism due to increased osmotic pressure. Very low levels of sugar may limit the rate of ethanol production

(Jones et al., 1981).Hence, each fermentation process will have an optimal glucose or equivalent sugar concentration (Sofer and Zaborsky, 1981).

2.6.2 Effect of temperature

Temperature has an important factor on the growth rate of the microorganisms and the rate of ethanol production. Wine and beer fermentations are generally conducted below 20 °C, whereas higher temperatures (30-38 °C) are being examined for industrial alcohol production by yeast cultures (Sofer and Zaborsky, 1981). Too high temperature kills yeast, and low temperature slows down yeast activity and growth. Thus, specific range of temperature is required (Onuki, 2005).

Most of fermentation process using *Saccharomyces cerevisiae* was carried out at 30 ºC whereas fermentation using *Kluyveromyces marxianus* was performed at 42 ºC. The ideal temperature for bioethanol production depends on the ideal temperature of the yeasts.

2.6.3 Effect of pH

A very important factor for cellular growth is external pH. Most alcoholic yeast fermentations are conducted below pH 4.5, although this may not be the optimal pH for growth or ethanol production. Yeast cultures can grow over a wide range from 3.0 to 8.0 with an optimum for growth generally in the slight acidic range. Shifts in pH can also affect the final ratio of organic waste products produced by yeast cultures. Thus, the optimal pH for a fermentation process must support a balance among ethanol production, cellular growth, and physicochemical effect on waste product pathways. Low pH values in yeast fermentation help to inhibit growth of contaminating bacterial cultures. Bacterial cultures generally have a pH optimum around 7.0-7.5, with less tolerance than yeast to acid conditions (Sofer and Zaborsky, 1981).

2.6.4 Effect of agitation rate

Agitation rate controls the permeability of nutrients from the fermentation broth to inside the cells and removal of ethanol from the cell to the fermentation broth. The greater the agitation rate, the higher the amount of ethanol produced. Besides, it increases the amount of sugar consumption and reduces the inhibition of ethanol on cells. The common agitation rate for fermentation by yeast cells is 110–200 rpm. Excess agitation rate is not suitable for smooth ethanol production as it causes limitation to the metabolic activities of the cells. (Zabed et al., 2014).

2.6. 5 Effect of inoculum size

Inoculum concentration does not give significant effects on the final ethanol concentration but it affects the consumption rate of sugar and ethanol productivity. The common inoculum size employed in bioethanol production are 2.5 % and 10 % (Zhang et al., 2009).

2.6.6 Time of fermentation

Fermentation time affect the growth of microorganisms. Shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms. On the other hand, longer fermentation time gives toxic effect on microbial growth especially in batch mode due to the high concentration of ethanol in the fermented broth. Complete fermentation can be achieved at lower temperature by using longer fermentation time which results in lowest ethanol yield (H. Zabed et al., 2014).

2.6.7 Ethanol concentration

Concentration of ethanol in the fermentation broth can directly affect the growth rate of the culture and its ability to convert sugar to ethanol. Inhibitory and toxicity level of ethanol vary from culture to culture. Higher temperature lowers the tolerance of the organism. At temperatures above 35 ºC, current strains lose viability at ethanol concentrations of 10 % (w/v) (Hettenhaus,1998).

2.6.8 Osmotic tolerance

The semi-permeable membrane surrounding the cell must be able to withstand wide osmotic pressure changes in extracellular fluids that impact the relative osmotic pressure difference. If not, the cells may be severely damaged or even killed. The cells may burst in a hypotonic solution, when the solution becomes more dilute than the intracellular fluid. If hypertonic, the cells will shrink from the osmotic pressure difference. Osmotic pressure limits can be one of the factors that restrict maximum substrate concentration (Sofer and Zaborsky, 1981).

2.6.9 Inhibitor tolerance

McMillan, (1994) grouped the fermentation inhibitors into three classes:

(1) Compounds originating in the biomass by hydrolysis. These include organic acids such as acetic, glucuronic and galacturonic acids from the hemicellulose, and phenolic compounds from the lignin. The inhibitoriest of these for both yeast and bacteria is acetic acid and solubilized

lignin.(2) Compounds formed by degradation of the products resulting from pretreatment and hydrolysis of the biomass. Furfural from xylose and hydroxyl-methyl-furfural from glucose lead this group. It is completed by an assortment of aldehydes, acids and alcohols from lignin, sugar and protein degradation.(3) Compounds from other sources. Metal ions resulting from equipment corrosion, sulfites, sulfur dioxide and lactic acid introduced with other streams containing nutrients, cleaning solutions and backset.

2.6.10 Microorganisms

The industrial fermentation of lignocellulose hydrolysate to ethanol requires microorganisms, which have a broad substrate range, and which produce ethanol with high yield and productivity. Such microorganisms must also tolerate ethanol and inhibitors formed in the pretreatment process. Most research efforts have been devoted to the development of efficient xylose-fermenting microorganisms (Jeffries, 2006; Kotter and Ciriacy, 1993). Two groups of microorganisms- enteric bacteria and some yeasts and fungus - are able to ferment pentoses, but with low ethanol yields. Furthermore, in the case of xylose fermenting yeasts (*Pachysolen tannophilus*, *Candida shehatae*, *and Pichia stipitis*), large-scale utilization is hampered by their sensitivity to high concentrations of ethanol (≥40 g/l), the requirement for carefully monitored microaerophilic conditions, high sensitivity to inhibitors, and the inability to ferment xylose at low pH.

2.7 FT-IR Characterization of the Produced Bioethanol

2.7.1 Infrared spectroscopy

Infrared spectroscopy (IR) is an analytical technique utilizing infrared adsorption. Infrared with different wavelengths are passed through a liquid sample. Infrared is adsorbed by a compound, and the absorbability of infrared varies among different compounds and different infrared wavelengths. Samples are identified by comparing absorbability of infrared. IR does not have as high resolution as GC or HPLC. However, the equipment is relatively cheap and analysis is simple and quick. Thus, it utilizes more for quality assurance (Lachenmeier, 2007) and classification purposes (Pontes et al., 2006).

3 MATERIAL AND METHODS

The experimental work was done in laboratory of Addis Ababa institute of Technology ,School of Chemical and Bio-Engineering Addis Ababa Ethiopia.

3.1 Material and Equipment's

3.1.1 Task one

Materials used for raw material characterization of brewery spent grain were: Oven(V 230, W 160, A 7, fuse 10, code DAS4200 Made of Italy), muffle furnace, Sieves, Electronics weighting balance, Soxhlet extraction system, vacuum pump filter, UV-Vis spectrophotometer (SPECTRA UV-VIS BOUBLE BEAM PC 8 SCANNING AUTO CELL UVD-3200, serial No-8UVD 11200 lambomed, Inc, U.S.A country of origin U.S.A), and Electric Thermostatic Heated Dry Box (Model. 202-0A, Made in Sweden, Tem-Range.50-250 ºC,Voltage.220±22v,Rated Frequency. 50HZ± HZ, Powder. 1.2 kw Date of Manufacturing 04/22/2009).

3.1.2 Task two

Materials used during production of cellulase enzyme were: autoclave (Electrical Heated Vertical Steam Sterilizer Model LX-B50LCDigital , Made in China, working Ac 220 V,50 HZ, Power 2.5 kw, Des Temperature 129 ºC, Working Pressure 0.165 Mpa, No 16-006, Netwet.39 kg, Volume 0.05 m^3), thermostatic Incubator shaker (Model. DH-500A, Temperature Range +5-65, Power. 0.4kw, Bio base Biodustry (Shandong) Co, Ltd, N0.51.South Gengye, and Road. Jinan City, China), centrifuge (model REF 1406, SN 0005379-02.00,Max speed-15000 rpm made in Germany, andrcas Hettich GmbH and CO.KG 78532.TUttingey,Germany) and water-bath (Type: TXF200 model, serNo.T41611001, supply 200-240 v, Grant instrument (Cambridge) Ltd Shepreth SG8 6GB made in England).

3.1.3 Task three

Materials used during quantifying sugar content of Barley spent grain were: Electronics weighting balance, Electric Thermostatic Heated Dry Box (Model. 202-0A, Made in Sweden, Tem-Range.50-250 ºC,Voltage.220±22v,Rated Frequency. 50HZ± HZ, Powder. 1.2 kw Date of Manufacturing 04/22/2009), quantitative Benedict reagent solution, centrifuge (model REF 1406, SN 0005379-02.00,Max –speed-15000 rpm made in Germany, andrcas Hettich GmbH and CO.KG 78532.TUttingey,Germany),Water-Bath (Type:TXF200 model, serNo.T41611001,supply

200-240 v, Grant instrument (Cambridge) Ltd Shepreth SG8 6GB made in England), Double Beam Pc Scanning Spectrophotometer UV-VIS Dual (model-spectro-UVD-3000/UVD-3200, serial No-8UVD 11200 lambomed, Inc, U.S.A country of origin U.S.A), Autoclave (Electrical Heated Vertical Steam Sterilizer Model LX-B50 LC Digital , Made in China, working Ac 220 V,50 HZ, Power 2.5 kw, Des Temperature 129 ºC, Working Pressure 0.165 Mpa, No 16-006, Netwet.39 kg, Volume 0.05 m^3), Thermostatic Incubator shaker (Model. DH-500A, Temperature Range +5-65, Power. 0.4kw, Bio base Biodustry (Shandong) Co, Ltd, N0.51.South Gengye, Road. Jinan City, China).

3.1.4 Task four

Materials used during the separation and quantification of ethanol after fermentation were: simple distillation, Double Beam Pc Scanning Spectrophotometer UV-VIS Dual (Model-spectro-UVD-3000/UVD-3200, serial No-8UVD 11200 lambomed, Inc, U.S.A country of origin U.S.A), and Fourier transform infrared spectroscopy (prinks Elmer spectrum, 65 FTlR).

3.1.1 Quantify the sugar content of barley spent grain

3.1.2 Evaluate the effect of Enzyme Hydrolysis Process Variables (Temperature, PH, Enzyme loading, and time) in the Yield of reducing sugar

The effect of hydrolysis process variables were evaluated using analysis of variance (ANOVA) and the material and equipment were, 98% Sulfuric acid (H_2SO_4), Sodium hydroxide (NaOH), cellulase enzyme, Dextrose sugar, yeast extracts, Urea and $MgSO_4.7\,H_2O$, Yeast (*Saccharomyces cerevisiae*), bacteria(*bacillus subtilus*), digital pH meter (Janway, model 3510), Thermometer, Thermostatic Incubator shaker (Model. DH-500A, Temperature Range +5-65, Power. 0.4kw, Bio base Biodustry (Shandong) Co, Ltd, N0.51.South Gengye, and Road. Jinan City, China).

3.2 Experimental methods

3.2.1 Quantify the sugar content of barley spent grain

3.2.1.1 Raw material preparation

Barley Spent grain (BSG) which remains after the mashing and lautering process, was obtained from Heineken brewery industry, Addis Ababa, Ethiopia. This sample was packed in polyethylene bags and transported to Addis Ababa institute technology (AAiT), School of chemical and bio engineering laboratory. The Samples used for this study were prepared in biochemical engineering laboratory. 200 g of BSG was washed in order to remove unwanted matter and dried at 70 ºC for 24hr. until 10.8 % moisture content remain. Then the dried sample was sieved and milled the oversize in to appropriate particle size which is less than 0.5 mm. The milled sample was sterilized at 121 ºC for 15 min and stored at less than 4 ºC refrigerator.

3.2.1.2 Raw material characterization

3.2.1.2.1 Particle size classification

The particle size classification was done using a sieve system with meshes of different openings' sizes and respective vibrator. The masses of the fraction collected in each sieve were recorded. Until the particle size reached to 0.5 mm.

3.2.1.2.2 Hemicelluloses content determination

The hemicelluloses content was determined using a Soxhlet extraction system, by adding 200 mL NaOH solution 2 % (w/v) to a 2 g BSG sample and allowing it to boil for 4 hr. After this time the material was filtered and washed with deionized water, and placed in an oven at 105 ºC, until constant mass was obtained. The hemicellulose content was determined by the ratio of the final mass to the mass of BSG initially weighed.

$$hemicellulose\ content = \frac{final\ mass}{initial\ weight\ of\ the\ BSG} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots.. (3.1)$$

3.2.1.2.3 Cellulose content determination

Raw cellulose contents of brewery spent grain were determined by using weendize method, 2 g of BSG sample in 250 ml flask, added 1.25 % of 250 ml sulfuric acid to remove all glycosid. Besides boiled for 30 minutes, filtered and washed with hot water several times. Added 1.25 percent of sodium hydroxide to remove protein by hydrolysis and fats by saponification, then boiled for 30 minutes. Filtered and washed several times with hot water. Finally residue was dried at 105 ºC for 15 min, cooled and weighed.

$$cellulose\ content = \frac{final\ dried}{total\ initial\ weight\ of\ BSG} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots.. (3.2)$$

3.2.1.2.4 Determination of moisture content

Moisture content was determined using the standard official methods of analysis of the AOAC (1990). This involved drying to a constant weight at 105 ºC and calculating moisture as the loss in weight of the dried samples. The crucible was thoroughly washed and dried in an oven at 100 ^{0}C for 30 min and allowed to cool inside desiccators. After cooling, they were weighted using a weighing balance and their various weights were recorded as (W_1). Then, 2 g of the finely-ground samples were put into the crucible and weighed to determine (W_2). Thereafter, the sample and crucible were placed inside the oven and dried at 100 ºC for 4 hr., then cooled and weighed at the same temperature for 30 min until constant weights were obtained to get (W_3). Then, the moisture content of the samples were calculated as the following equation:

$$\frac{(initial\ weight\ of\ filled\ crucible) - (final\ weight\ of\ filled\ crucible)}{(initial\ weight\ of\ filled\ crucible) - (initial\ weight\ of\ empty\ crucible)} * 100\% \ldots (3.3)$$

3.2.1.2.5 Determination of ash content

Total ash content of the samples was determined using furnace incineration, as described by AOAC (1990), based on the vaporization of water and volatiles with burning organic substance in the presence of oxygen in the air to carbon dioide at a temprature of 550 ºC (drying). About 2 g of finely-ground dried sample was placed in a porcelain crucible and incinerated at 525 ºC for 6 hr. in an ashing muffle furnace (KW2.2,V220,A 10,NR72158,TYPE VMK3,CH21150; Germeny) until ash was obtained. The ash was cooled in a desiccator and weighed using the following equation.

$$\left(\frac{weight\ of\ ash}{weight\ of\ original}\right) * 100 = \%\ ash \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.4)$$

3.2.1.2.6 Determination of dry matter content

Dry matter represents everything contained in a sample except water; this includes protein, fiber, fat, and minerals. In practice, it is the total weight of the sample minus the weight of water, expressed as a percentage. In this study, it was determined by drying the sample in an oven (V 230, W 160, A 7, fuse 10, code DAS4200 Made of Italy) until the sample reached a stable weight. The sample was weighed and about 2 g placed in a dry crucible. The crucible and sample were oven-dried for 24 hr. after that, the crucible and sample were weighed. The percentage of dry matter was calculated and recorded (Nennich and chase, 2007). The formula was as follow

$$\left(\frac{Dry\ weight}{total\ Weight}\right) * 100 = \%\ dry\ matter \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots..\ldots\ldots\ldots\ldots.(3.5)$$

3.2.1.3 Pretreatment (diluted acid hydrolysis)

The process parameters were fixed at best process conditions e.g. (1.25 % H_2SO of the total solution, 17 min reaction time and 121 ºC) for extraction of hemi cellulosic sugars (Mussatto and Roberto, 2006).The pretreated feedstock was mixed with 1.25% (v/v) sulfuric acid solutions in 1000-mL closed universal flasks with a liquid-to-solid ratio of 20 % (w/w). The mixtures were allowed to stand for 10 min at room temperature in order to equilibrate the acid concentrations between the bulk phase and the biomass. The pretreatment was performed in an autoclave at 121 ºC from, 1.65 pH, and 11- 19 min time. The flasks were placed inside the autoclave at 100 °C, and the heating time to reached 121 ºC. Frist grinded the brewery sample to 0.5 mm using micro grinder, Measured 180 g using electronic balance then put in 2000 ml flasks. It could pretreatment using autoclave at temperature 121 ºC and time of 11-19 min.

Calculate the severity factor using equation (3.6) as mentioned below:

$$Log\ (CS) = \ log\ \left[t\ exp\ \left(\frac{T-100}{14.75}\right)\right] - pH \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(3.6)$$

3.2.1.4 Enzyme hydrolysis

The main purpose of this process was to degraded cellulose in to its monomer in the optimal condition of temperature, PH, Enzyme concentration and reaction time. The solution separated from the pretreatment was washed and dried before introduced in to the enzyme hydrolysis. In enzyme hydrolysis, the process conditions were optimized: Temperature between (40 ºC and 50 ºC), pH (4.0 and 5.0), and reaction time between (24 hr. and 72 hr.) and enzyme loading between (1 % and 2 % of enzyme) using full factorial central composite design (CCD).

a. Strain Specification

Bacillus subtilus isolated from soil and water was obtained from Ethiopian Biodiversity Institute Culture Collection, Addis Ababa, Ethiopia. The medium used for cultivating *Bacillus subtilus* consist of Nutrient agar.

b. Liquid State Fermentation

Solid substrate BSG is taken in Erlenmeyer flasks. Production medium containing (g/l); K_2HPO_4 (1); KH_2PO_4 (1); KCl (0.5); $MgSO_4$ (0.5); $FeSO_4.7H_2O$ (0.01); Yeast extract (2); and *bacillus subtilus* (1 ml) was introduced in to the liquid medium. Liquid medium containing mineral salt medium is stir well, sterilize in an autoclave at 121 ºC at 15 min and cool. Then 20 g BSG as source of cellulose with sterile medium under aseptic condition and incubate at room temperature, but the fermentation carry out with pH between 4.0 and 5.0 for both cellulase produced using BSG cellulose and refined cellulose powder, and the optimum pH was observed at 5.0 minimum and maximum activity of 45 ºC and 40 ºC respectively of cellulase produced by *Bacillus subtilis* (Russ, 2005).

c. Enzyme Extraction from liquid State Fermentation

Samples obtaining from the liquid fermentation are centrifuged at 10000 rpm at 4 ºC for 10min and the supernatant collect is taken as crude enzyme solution.

d. Filter paper activity (FPase) assay for cellulase

The FPA is the key method for analysis of total cellulase activity. In 1976, the FPA was developed by (Mandels et al., 1976). the FPA uses a 1×6-cm strip of Whatman no. 1 filter paper as the standard substrate because it is readily available and inexpensive (Coward-Kelly et al., 2003). This standard filter paper method has been reviewed by Ghose, (1987). The International Unit (IU) of filter paper activity (FPase) (FPU) is defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions, where assay conditions refer to the conditions such as pH and temperature at which the enzymes are held at during the assay and depend largely on the properties of the enzyme, varying widely between cellulases and microorganisms. Reducing sugar is estimated as glucose by the Miller method. This assay is performed so that 0.5 mL of diluted enzymes releases about 2 mg of glucose equivalents in 60 min, as determined by the 3, 5- dinitrosalicylic acid (DNS) assay (Miller, 1959; Wood and Bhat, 1988).

The DNS reagent is used as a colorimetric method for the determination of reducing sugars, such as glucose. It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color-developing reaction. Sodium bisulfite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars.

DNS will be added at the last step of the enzyme assay to stop the reaction. To promote full color development, samples have to be boiled vigorously and the absorbance of diluted samples will be read at 540 nm (Zhang et al., 2009).

Description: this protocol is a little modified but it is based on IUPAC procedure. Basically, this measures the ability of the cellulose to hydrolyze both crystalline and amorphous regions of cellulose. Therefore, it is generally believed that FPase assay is the best measure of the activity of both endo- and exo-type in a cellulase with a strip of filter paper (Whatman#1, 50mg, 1×6 cm) for 1 hr. at the given temperature.

The standard procedure for the filter paper assay is as follows;

1. Add 1 ml buffer to a test tube having a cup.
2. Add 0.5 ml enzyme, diluted in the buffer.at least two dilutions must be made of mg of glucose in the reaction conditions.
3. Temperature on 50 ºC or specific temperature and add one filter paper strip into the test tube.
4. Incubate 50 ºC for 1 hr.
5. Boil the mixture for 20 min.
6. Add 20 ml water. Mix it by vigorous shaking.
7. Filter the mixture with a glass filter paper.
8. The filtrate is measured against reference at 540 nm.

Reference; 1.5 ml buffer, 3 ml DNS, 20 min boil, 20 ml water

Blank; 1ml buffer, 0.5ml enzyme, 3 ml DNS, 20 min boil, 20 ml water

Unit calculation:

1. Construct a linear glucose standard using the absolute amounts of glucose (mg/0.5ml) plotted against 540 nm.
2. Using this standard, translate the absorbance values of the sample tubes (after subtraction of the enzyme blank) into glucose.
3. Translate the dilutions used into enzyme concentrations;

$$concentartion = \frac{1}{Dilution} = \frac{volume\ of\ enzyme\ indilution}{totalvolume\ of\ dilution} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots. (3.7)$$

4. Estimate the concentration of enzyme which would have released exactly 2 mg of glucose by plotting glucose produced against enzyme concentration on semi logarithmic paper.
5. Calculate FPase Unit (FPU):

$$FPU = \frac{0.37}{(\text{enzyme concentration to released 2mg glucose})} \frac{\text{unit}}{\text{ml}} \dots\dots\dots\dots (3.8)$$

The unit of FPU is based on the international unit (IU)

1 IU= 1 micromole min^{-1} of substrate converted

= 1 micromole min^{-1} of glucose formed during the hydrolysis reaction

= 0.18 mg min^{-1} when product is glucose

3.2.1.5 Sugar content determination

The concentration of total reducing sugar (TRS) content of hydrolysate which obtained from enzyme hydrolysis was determined using Double Beam Pc Scanning Spectrophotometer UV-VIS Dual or double beam labo med.inc. (Model-spectro-UVD-3000/UVD-3200, serial No-8UVD 11200 lambomed, Inc, U.S.A country of origin U.S.A) by measuring absorbance vs. Sugar concentration at 540 nm wave length. Quantitative benedict solution and standard glucose solution was used for assays to plot the calibration curve. Benedict’s solution is designed to detect the presence of reducing sugars. In hot alkaline solutions, reducing sugars reduce the blue copper (II) ions to brick red copper (I) oxide precipitate. As the reaction proceeds, the color of the reaction mixture changes progressively from blue to green, yellow, orange and red. According the color changed the amount of reducing sugar was changed, so the red color indicated maximum reducing sugar. When the conditions are carefully controlled, the colour developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of glucose-equivalent reducing sugars present in a sample can be obtained.

a. Calibration plot for glucose standard

1. Standard glucose dilution series solution were prepared at different concentration of 0%, 2%, 4%, 6%, 8%, and 10%.

2. Pipette 1 mL from each of the dilution series into labeled test tubes, each containing 4 mL of Benedict’s solution. Mixed by vortex.
3. All the labeled test tubes were heated at 90 ± 1 ºC water bath for 5 minutes.
4. The test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent.
5. After filtered the precipitate, % absorbance were measured using spectrophotometer at 540 nm.
6. Calibration curve was plotted to show the % of absorbance of blue light by the standard glucose solution.

b. Determination of the total reducing sugar in the hydrolysate

1. Pipetted 1 mL of each of the samples into labeled test tubes, each containing 4 mL of Benedict’s solution. Mix by vortex.
2. All the labeled test tubes were heated to 90 ± 1 ºC in water bath for 5 minutes.
3. The test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent.
4. After filtered the precipitate, % absorbance was measured using spectrophotometer at 450nm.
5. The concentration of sugar in each samples were read from the calibration curve of the standard glucose solution.

$$y(absorbance) = slope * (concentration) - absorbance \dots\dots\dots\dots\dots\dots\dots..\dots.(3.9)$$

$$CTRSUS = \frac{(absorbance\ of\ unkown\ sample) - (Y\ intercept)}{slope} \dots\dots\dots\dots.\dots\dots..(3.10)$$

Where

CTRSUS = Concentration of total reducing sugar of unknown sample

3.2.2 Evaluate the effect of hydrolysis process variables (Temperature, PH, enzyme loading and time) in the yield of reducing sugar

The sugar (glucose) content yield of enzyme hydrolysis is defined as

$$\text{Sugar yield } (\%) = \left(\frac{\text{M sugar}}{\text{M polysaccharides} * \text{f}}\right) * 100\% \dots\dots\dots\dots\dots\dots\dots.\dots\dots\dots\dots(3.11)$$

Where

M= sugar is amount of glucose released by enzyme hydrolysis

M= polysaccharide is the amount of cellulose in the raw material

f= is the conversion factor from polysaccharides to monomeric sugars (180/162) for glucose and (150/132) for Xylan to xylose (Zeng et al., 2007).

3.2.2.1 Fermentation

The fermentation process was carried out in shaker incubator, at 30 ºC, with stirring at 160 rpm, for a 72 hr. All assays were performed with 2.5% (v/v) of inoculum. The prepared hydrolyzates were adjusted to pH of 5.0 which is optimum for *Saccharomyces cerevisiae* using sodium hydroxide solution.

a. Media Preparation for *sachermaysis servesia*

The culture medium was prepared in 250 mL test tube by composed of (g/l): Yeast extract (10); Dextrose (20); Urea (5); Mg SO_4.7 H_2O (5); Peptone (20).

Procedures in Media Preparation

- The media was sterilized at 121 ºC for 15 min and 0.50 g of Saccharomyces cerevisiae were added into 100ml prepared media at 250 mL conical flask.
- The conical flasks were properly covered with aluminum foil and placed to a shaker incubator for 24 hr., at 30 ºC and 200 rpm.

b. Media preparation for growth of *Fusarium oxysporum*

The culture medium was prepared in 250 ml flask composed of (g/l):NH_4CL (10); K_2HpO_4 (3); KH_2PO_4 (0.5); $CaCL_2$ (0.2); $MgSO_4$ (0.1); $FeCL_2.7H_2O$ (0.2); distil water (500 ml) at temperature of 30 ºC anaerobic growth and1 ml of *fusarium oxysporum*.

The Procedure of Fermentation for both microbes

- The hydrolyzates sample was conditioned at temperature of 30 ºC. This temperature was favorable for both microbes (*Saccharomyces cerevisiae and fusarium oxysporum*) in the fermentation.
- The pH of the sample was adjusted using NaOH to make solution pH from (4.5-5.0) to establish a favorable condition for *Saccharomyces cerevisiae* and *fusarium oxysporum*.
- The hydrolysate sample with 2.5 % inoculum was placed into shaker incubator at 30 ºC and 160 rpm for 3 days.
- After 72 hr. fermentation, the samples were taken out and introduce into distillation to separate the hydrous ethanol.

- Finally characterized using Infrared spectroscopy.

3.2.2.2 Ethanol Separation

Distillation is the final step in the production of ethanol. It is a purifications step. Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. Separation was carried out by simple distillation at 78.3 ºC for 3 hrs.

3.2.2.3 Spectrophotometric method for the determination of bioethanol

Colorimetric methods for the determination of ethanol, being more sensitive, require a smaller quantity of sample (e.g. 1 ml or a lower alcohol content in solution (e.g. less than 1%). However, these methods are not selective and, requiring the distillation of the sample, are subject to the quantitative recovery of distillate. It is of common knowledge that potassium dichromate oxidizes ethanol to acetic acid. This reaction is used in the analysis of wines, ethanol solutions and beverages.

Requirements

1. Chromic acid reagent (5 g of potassium chromate, H_2O, 50 ml concentrated sulfuric acid)
2. Ethanol sample
3. Ethanol standard
4. Distilled water

Procedure

1. Collect 1 ml ethanol sample by distillation of the fermented sample.
2. To 1ml of sample/solution, added 25 ml of chromic acid reagent.
3. Placed the tube in a water bath at 70 ºC for 15 min.
4. Taken out the tubes and immediately 24 ml of distilled water to it to stop the reaction.
5. Measured the absorbance at 600 nm.

$$\text{Percentage of ethanol in sample (\%)} = \left(\frac{Cs}{Cu}\right) * \left(\frac{Au}{As}\right) * 100 \dots\dots\dots\dots\dots..(3.12)$$

Where

Cs = Concentration of standard, Cu = Concentration of sample, Au = Absorbance of standard, As = Absorbance of sample.

3.3 Design of the Experiment

Data analysis was carried out by DESIGN EXPERT version 6.0 software (central composite design) to evaluate the effects of the process variables; temperature (40 ºC, 45 ºC and 50 ºC), pH(4.0, 4.5, 5.0) reaction time (24 hr., 48 hr. and 72 hr.) and enzyme loading (1, 1.5 and 2%v/v). 45 ºC, 4.5 pH, 48 min and 1.5% enzyme concentration was used center point. A CCD experimental design with 26 experiments were employed, which includes 24 non center points and 2 trails for replication of the central points to estimate error based on the pattern generated through software. The response variable was yield of reducing sugar content after hydrolysis. This design of the experiment helps us to optimize of process parameters using Response Surface Methodology (RSM). Significance of the result was set from analysis of variance (ANOVA).

Table 3.1 Minimum, central and Maximum values of factors

Factor name	Unit	Low	Centre point	High
Temperature	ºC	40	45	50
pH		4.0	4.5	5.0
Enzyme loading	%	1	1.5	2
Time	hr.	24	48	72

4 RESULT AND DISCUSSION

4.1 Determination the sugar content of barley spent grain based on task one

4.1.1 Raw material characterization

Table 4.1 raw material characterization

Particle size	0.5 mm
Hemicelluloses content	28.4 %.........................equation (3.1)
Cellulose content	27 %.......................... equation (3.2)
Moisture content	10.8 %....................... equation (3.3)
Ash content	3.5 %........................ equation (3.4)
Dry matter content	98.2% ……………..equation (3.5)

4.1.2 Evaluation of combined severity factor on the pretreatment method

Frist grinded the brewery sample to 0.5 mm using micro grinder, Measured 30 gram using electronic balance then put in 1000 ml flasks. It could pretreatment with pH 1.65, sterilization using autoclave at temperature 122 ºC with time of 11-19 min, so let's evaluated the results using combined severity factor.

Calculate the combined severity factor using equation (3.6):

$$\mathrm{Log}\ (CS) = \log\left[\mathrm{t}\ \exp\left(\frac{\mathrm{T}-100}{14.75}\right)\right] - \mathrm{pH} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots . (3.6)$$

Where t= time, T= temperature and CS= combined severity factor

Table 4.2 result of combined severity factor

Type of parameter	unit	Different pretreatment values							
temperature	C0	121	121	121	121	121	121	121	121
pH		1.65	1.65	1.65	1.65	1.65	1.65	1.65	1.65
Time	min	11	12	13	14	15	16	17	18
Log(CS)		0.009	0.0474	0.082	0.114	0.144	0.1724	0.198	0.22

From the above table the best pretreatment was occurred on the temperature 121 ºC and time of 17 min, the severity factor coefficient was 0.198, because less than 0.198 maximum lignin was produced as well as greater than 0.198 combined severity factor hemicellulose and cellulose were converted to other simple components' (Mussatto and Roberto, 2006).

4.1.4 Total reducing sugar content after enzyme hydrolysis based on task two

Total reducing sugar of the hydrolysate sample was determined using calibration curve which was plotted from the known concentration of standard glucose reacted with Benedict solution reagent and absorbance of standard glucose-benedict solution after reaction, was measured using digital spectrophotometer at 540 nm wavelength. As we seen from the calibration curve below the absorbance and concentration of sugar has direct relationship because in the sample which have less sugar concentration remain high amount of Cu (II) oxide (blue color) un react with the reducing sugar due to this absorbance of the sample increase but in the sample which have high sugar concentration Cu (II) oxide is completely react with the reducing sugar and form Cu (I) oxide (red color) precipitate and absorbance of the sample increases.

Table 4.3 standard concentration and absorbance

Std. Glucose concentration (g/ml)	Absorbance
0	0
2	0.024
4	0.10
6	0.15
8	0.27
10	0.31

The graph of fitted line plot was designed using Minitab.v17.1.0. Software.

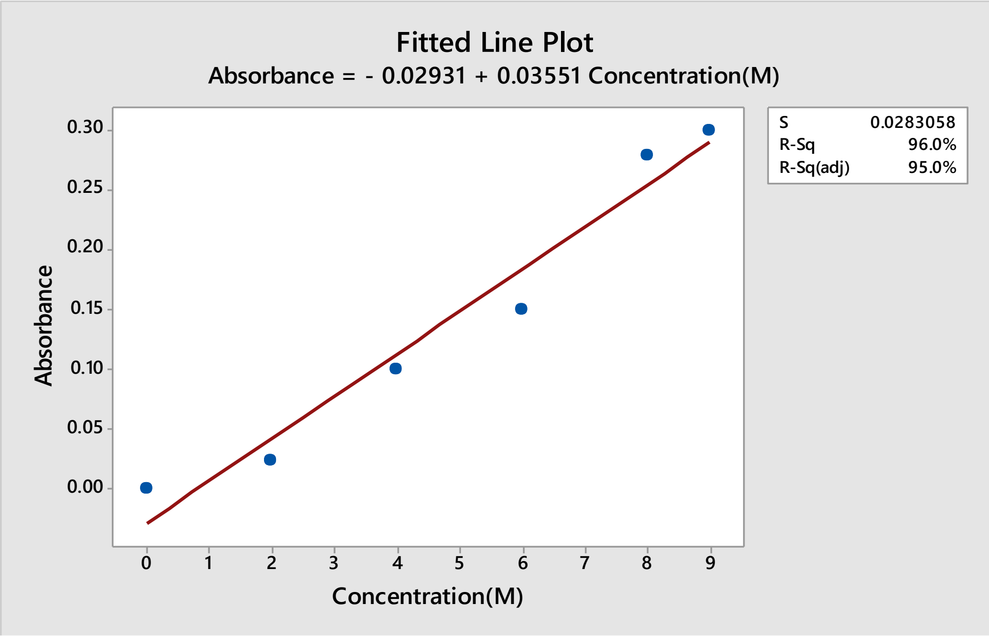


Figure 4.2 graph for concentration of standard glucose and its absorbance

4.2 Evaluate the effect of hydrolysis process variables in the yield of reducing sugar based on task three

Table 4.4 effect of hydrolysis process variables

Std.	Run	Temperature (°C)	pH	Time (hr.)	Enzyme con (%)	Concentration TRS(% m/v)	Yield of TRS after hydrolysis (% w/w)	Best
1	18	40.00	4.00	24.00	1.00	21.05	70.89	
2	17	50.00	4.00	24.00	1.00	7.67	25.82	
3	19	40.00	5.00	24.00	1.00	8.26	27.81	
4	10	50.00	5.00	24.00	1.00	7.38	24.87	
5	3	40.00	4.00	72.00	1.00	23.43	78.9	

6	16	50.00	4.00	72.00	1.00	7.30	24.58	
7	15	40.00	5.00	72.00	1.00	20.62	69.44	
8	22	50.00	5.00	72.00	1.00	20.89	70.34	
9	23	40.00	4.00	24.00	2.00	23.79	80.12	
10	11	50.00	4.00	24.00	2.00	19.17	64.55	
11	20	40.00	5.00	24.00	2.00	10.06	33.87	
12	7	50.00	5.00	24.00	2.00	10.00	33.67	
13	9	40.00	4.00	72.00	2.00	9.92	33.4	
14	14	50.00	4.00	72.00	2.00	9.98	33.6	
15	12	40.00	5.00	72.00	2.00	23.03	77.56	
16	21	50.00	5.00	72.00	2.00	9.68	32.6	
17	6	40.00	4.50	48.00	1.50	28.67	96.55	Selected
18	4	50.00	4.50	48.00	1.50	17.19	57.89	
19	13	45.00	4.00	48.00	1.50	10.00	33.67	
20	8	45.00	5.00	48.00	1.50	10.06	33.87	
21	26	45.00	4.50	24.00	1.50	6.14	20.69	

22	5	45.00	4.50	72.00	1.50	6.93	23.35	
23	24	45.00	4.50	48.00	1.00	8.15	27.43	
24	1	45.00	4.50	48.00	2.00	8.68	29.23	
25	25	45.00	4.50	48.00	1.50	16.86	56.78	
26	2	45.00	4.50	48.00	1.50	16.36	55.08	

4.2.1 Spectrophotometric method for the determination of bioethanol based on task four

Using equation 3.9 the result of percentage ethanol yield after fermentation was as fellow:

Table 4.5 yield of ethanol with respective microbes

Type of microbe in fermentation	Absorbance	% Yield of ethanol
Fermentation one with *sachermaysis servesia* only	0.148	52.59%
Fermentation two with *fusarium oxysporum* and *sachermaysis servesia*	0.145	53.68%
Fermentation three with *fusarium oxysporum* only	0.151	51.55%

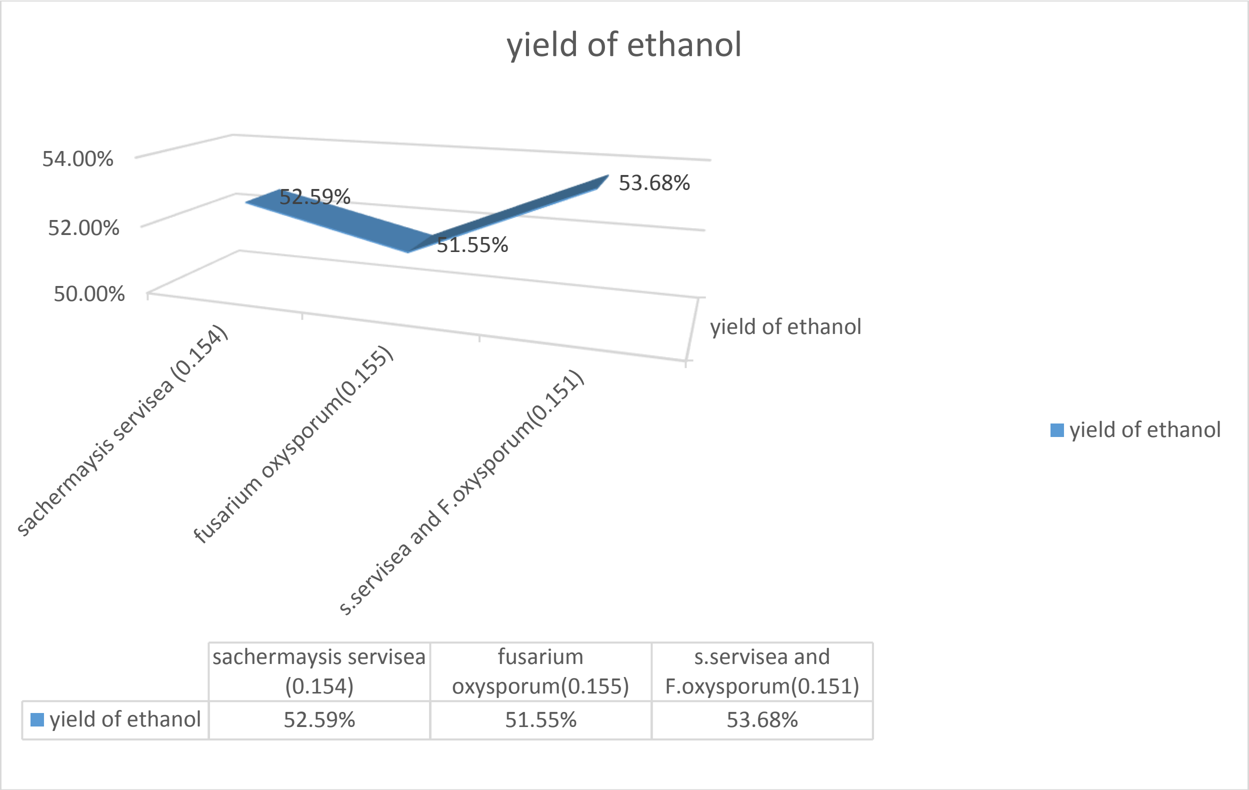


Figure 4.3 yield of ethanol with different microbes

The yield of ethanol from three experiments were as follow, which means 1ml of each three samples i.e. *sachermaysis servesia*, ethanol produced using *fusarium oxysporum* and ethanol produced using combined microbes (*sachermaysis servesia* and *fusarium oxysporum*) gave as 52.59 %, 51.55 % and 53.68 % of ethanol product respectively. The maximum product was produced from the combined co-culture of the *fusarium oxysporum* and *sachermaysis servesia* as compared to individual microbes.

4.2.2 Statistical Analysis of the experimental results

4.2.2.1 Analysis of variance (ANOVA)

The analysis of variance of the cubic regression model was a significant model, from evident of Fisher‟s F test with a very low probability value [(P-model > F) =0.0001]. From Table 4.6 it was observed that The Model F-value of 3.63 implies the model is significant ,and the Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, A^2, BC, and CD are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The coefficient for the linear effect of temperature was highly significant, and that of pH, time and enzyme loading were least significant. It were also observed that there are an interactions effect between pH with time and time with enayme loading.

Table 4.6 Analysis of variance (ANOVA) for Response Surface Quadratic Model

Source	Sum of squares	DF	Mean Square	F value	Prob > F	
Model	10472.87	14	748.06	3.63	<0.0189	Significant
A	2236.02	1	2236.02	10.85	<0.0072	
B	95.68	1	95.68	0.46	0.5098	
C	209.99	1	209.99	1.02	0.3345	
D	0.12	1	0.12	5.902E-004	0.9811	
A^2	3727.56	1	3727.56	18.08	<0.0014	
B^2	71.90	1	71.90	0.35	0.5668	
C^2	744.37	1	744.37	3.61	0.0839	
D^2	295.33	1	295.33	1.43	0.2565	
AB	285.27	1	285.27	1.38	0.2643	
AC	73.96	1	73.96	0.36	0.5613	
AD	104.55	1	104.55	0.51	0.4912	
BC	2515.52	1	2515.52	12.20	<0.0050	
BD	43.03	1	43.03	0.21	0.6567	
CD	1038.77	1	1038.77	5.04	<0.0463	
Residual	2267.82	11	206.17			
Lack of Fit	2266.37	10	226.64	156.84	0.0621	not significant
Pure Error	1.45	1	1.45			
Cor Total	12740.68	25				

The regression coefficients and the corresponding 95 % CI (Confidence Interval) High and Low were presented in table 4.7 below. If zero was in the range High and Low 95 % Confidence interval, the factors has no effect. From the 95 % CI High and Low values of each model term, it could be concluded that the regression coefficients of temperature and the interaction terms of temperature and time as well as time and enzyme loading have highly significant effect in the yield of reducing sugar production.

The "Lack of Fit F-value" of 156.84 implies there is a 6.21 % chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 4.7. Regression coefficients and the corresponding 95% CI High and Low

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	43.28	1	5.69	30.76	55.81	
A-temperature	-11.15	1	3.38	-18.59	-3.70	1.00
B-pH	-2.31	1	3.38	-9.75	5.14	1.00
C-time	3.42	1	3.38	-4.03	10.86	1.00
D-enzyme loading	-0.082	1	3.38	-7.53	7.37	1.00
A^2	38.15	1	8.97	18.40	57.90	2.16
B^2	-5.30	1	8.97	-25.05	14.45	2.16
C^2	-17.05	1	8.97	-36.80	2.70	2.16
D^2	-10.74	1	8.97	-30.49	9.01	2.16
AB	4.22	1	3.59	-3.68	12.12	1.00
AC	-2.15	1	3.59	-10.05	5.75	1.00
AD	2.56	1	3.59	-5.34	10.46	1.00
BC	12.54	1	3.59	4.64	20.44	1.00
BD	-1.64	1	3.59	-9.54	6.26	1.00
CD	-8.06	1	3.59	-15.96	-0.16	1.00

The following second order polynomial model was derived to explain the yield TRS after enzyme hydrolysis from brewery spent grain

Final Equation in Terms of Actual Factors:

$$\text{Yield of TRS after hydrolysis} = +3159.39664-147.84816 \times \text{temperature}+69.82089\times \text{pH} +0.095166\times \text{time}+144.43706\times \text{enzyme loading}+1.52605\times \text{temperature}^2-21.19467\times \text{pH}^2-0.029598\times \text{time}^2-42.95467\times \text{enzyme loading}^2+1.68900\times \text{temperature} \times \text{pH}-0.017917\times \text{temperature} \times \text{time}+1.02250\times \text{temperature} \times \text{enzyme loading}+1.04490\times \text{pH} \times \text{time}-6.56000 \times\text{pH}\times\text{enzymeloading}-0.67146\times\text{time}\times\text{enzymeloading}\cdots\cdots\cdots\cdots(4.1)$$

Table 4.8. Model adequacy measures

Std. Dev.	14.36
R-Squared	0.9220
Mean	46.79
Adj R-Squared	0.855
C.V.	30.69

Pred R-Squared	-0.2494
PRESS	15918.36
Adeq Precision	6.983

The regression coefficient (R^2) quantitatively evaluates the correlation between the experimental data and the predicted responses. Results of R^2= 0.9220 and Adj-R^2= 0.855 obtained explicates that the predicted values were found to be in good agreement with experimental values. Since the R^2 value is closer to 1.0 it indicates that the regression line perfectly fits the data. Similar to that in this investigation, R^2 obtained was 0.9220, which was close to 1. Results imply that the predicted values were found to be in good agreement with experimental values (R^2= 0.9220 and Adj-R^2= 0.855), indicating the achievement of the RSM. The model‟s goodness of fit was checked by regression coefficient (R^2). In this case, the value of the coefficient (R^2=0.9220) from Table 4.8 indicated that only 5.47 % of the total variance was not explained by the developed regression model. The obtained R^2 values suggest good adjustments to the experimental results. The adjusted determination coefficient (Adj-R^2= 0.855) was also satisfactory for confirming the significance of the model. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 6.983 indicates an adequate signal. This model can be used to navigate the design space.A negative "Pred R-Squared" implies that the overall mean is a better predictor of your response than model.

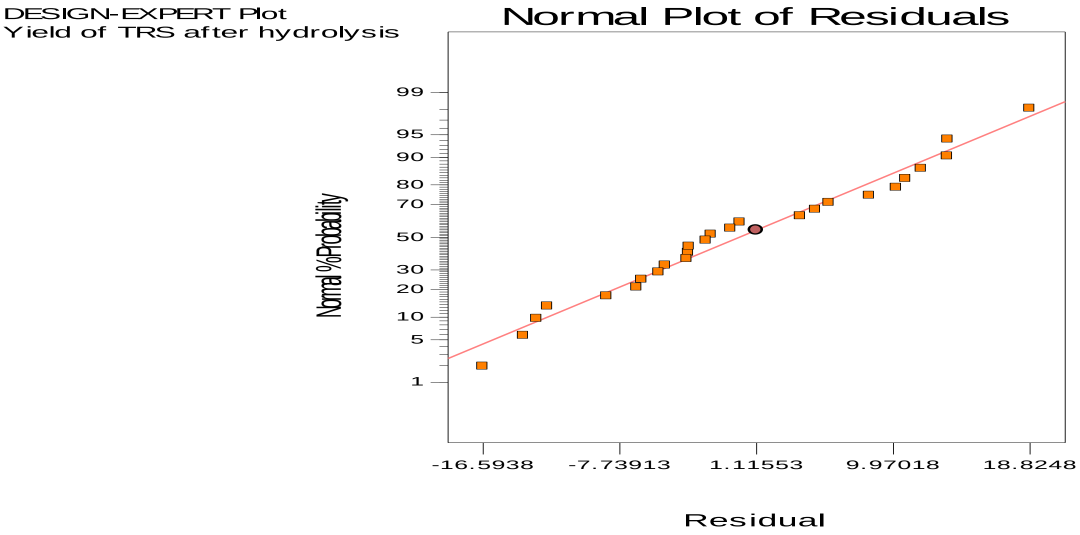


Figure 4.4. Normal plots of residuals

From the plot as shown above, the normal probability plot indicates the residuals following by the normal % probability distribution, in the case of this experimental data the points in the plots shows fitted to the straight line in the figure, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.

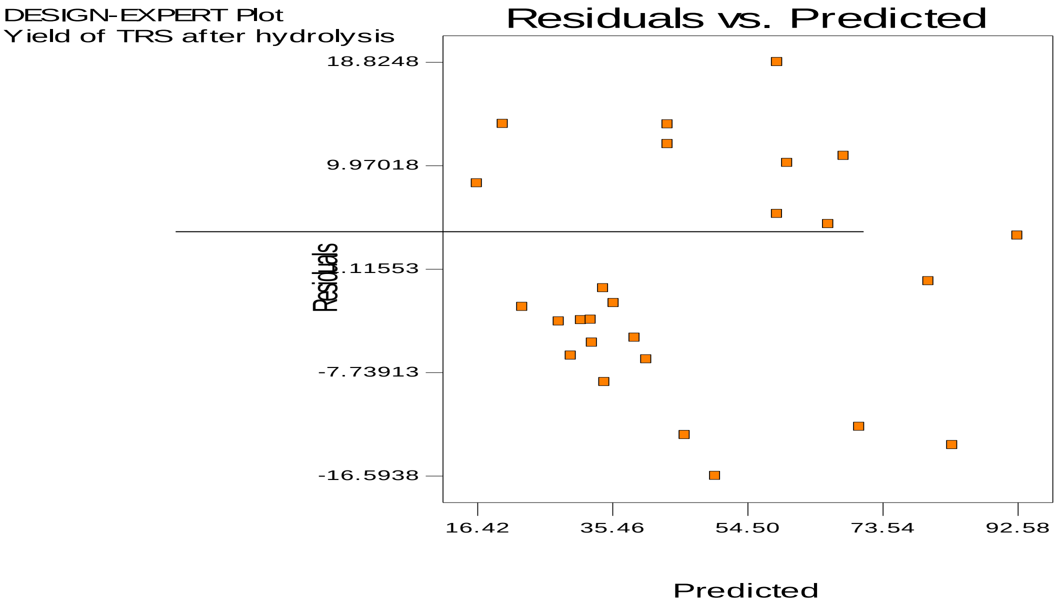


Figure 4.5. Residual versus predicted values

If the model is correct and the assumptions are satisfied, the residuals should be structure less; in particular, they should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. The plot shows random scatter which justifying no need for an alteration to minimize personal error.

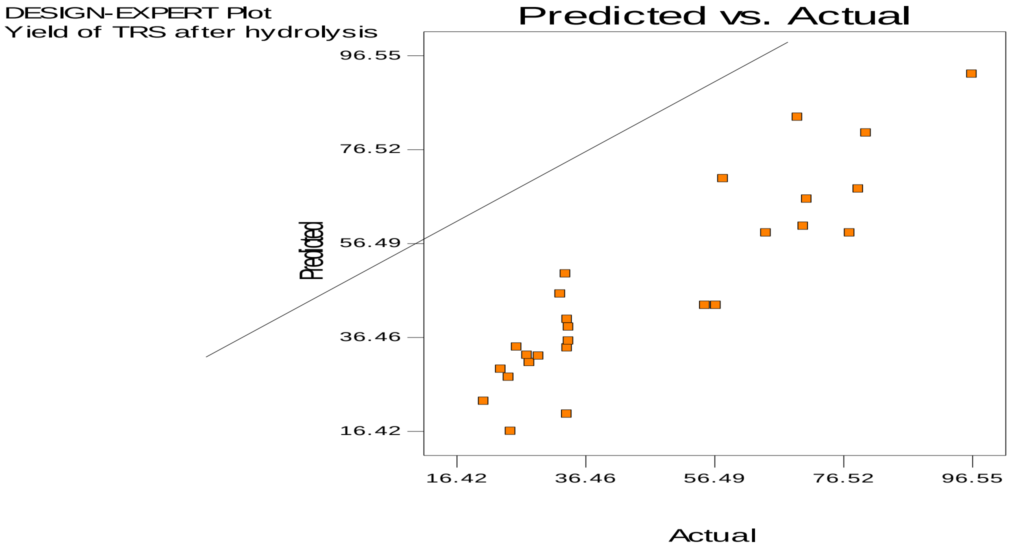


Figure 4.6 precidctor and acutal value

From graph 4.6 the precdictor and actual value of individual experment was not equal ,but the midum acutal value versus the predicted value was lined at temperature 40 ºC, pH 4.0, time 24 hr. and enyme loading 2 %, both had a value of 80.12 % w/w and the maximum was occurred 96.55 % and 92.51 % respectivly.

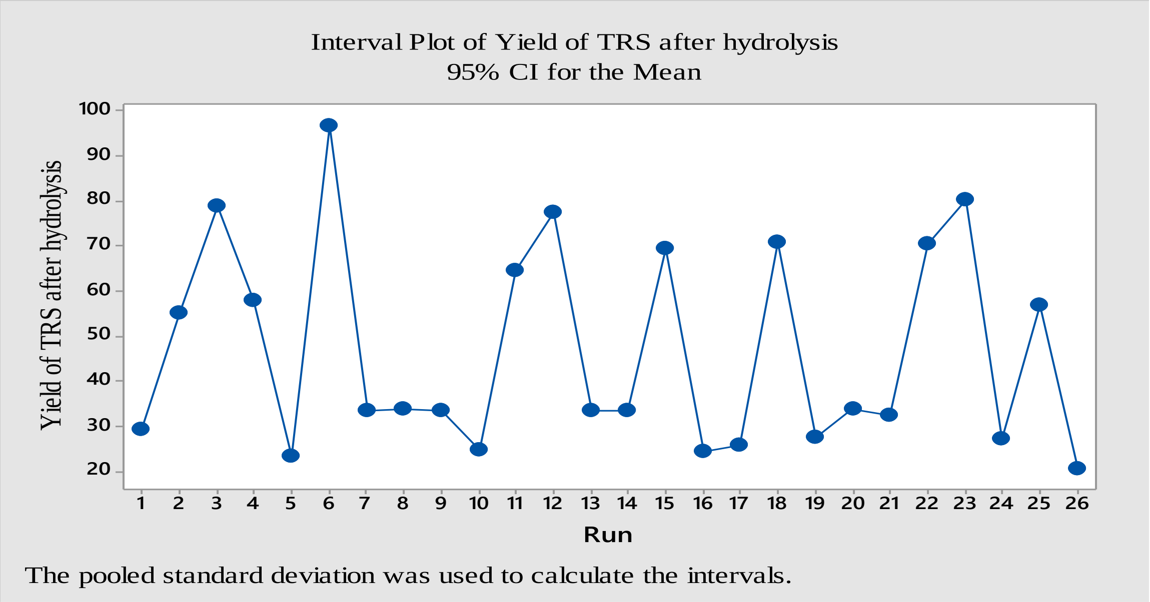


Figure 4.7 run versus yield of TRS after hydrolysis

The run versus yield of TRS after hydrolysis on figure 4.7 were different for individual experment, so that the mean of run 7, run 8 and run 9 aswel as run13, and run14 were nearly the same,but different for other run. The minmum and the maximum Yield of TRS after hydrolysis were occurred on the run 26 and run 6 , which were 20.69 w/w% and 96.55 w/w% repectivily.

4.2.2.3 Individual effect of experimental variables on the yield of reducing sugar

Effect of Temperature

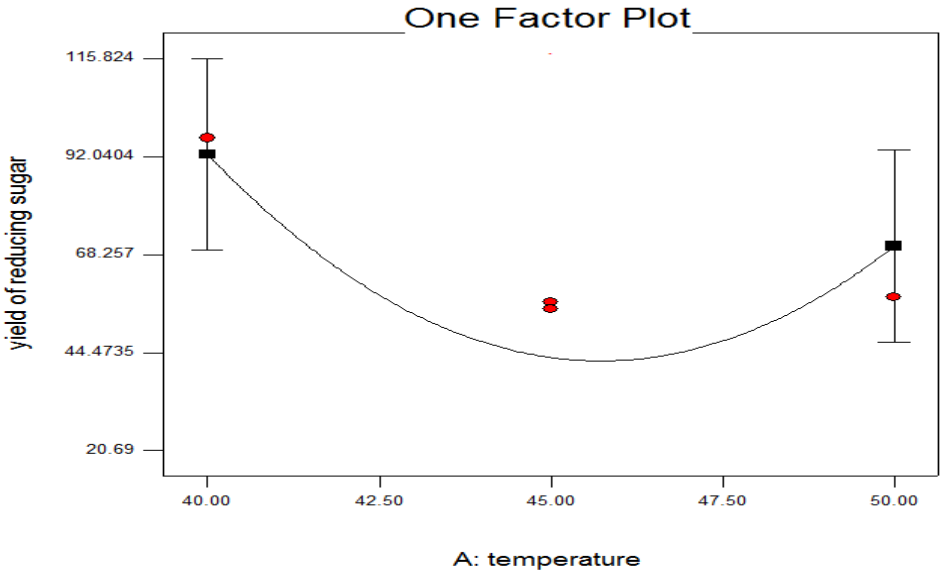


Figure 4.8 effect of temperature on yield of reducing sugar at constant of pH, time and enzyme loading in the center point

Figure 4.8 represents the effect of hydrolysis temperature on the yield of total reducing sugar after enzyme hydrolysis at constant time and acid concentration at the center point. As shown on the figure 4.9 the yield of TRS is not sensitive to the hydrolysis temperature. Yield of reducing sugar was highly decreased as temperature increase from 40 ºC to 45 ºC, but from 45 ºC to 50 ºC the yield of reducing sugar was slightly increased. Optimum yield of reducing sugar was obtained around 40 ºC hydrolysis temperature. Beyond 40 ºC hydrolysis temperature the yield of reducing sugar is highly decrease, because at high temperature enzyme activity is decreased swiftly and cellulose is not degraded well (Taherzadeh and Karimi, 2007).

Effect of pH

As shown in figure 4.10 below the yield of TRS after hydrolysis was affected slightly by pH, as the pH increase from 4.0 to 4.5 the yield highly increase, beyond 4.5 the yield of TRS after enzyme hydrolysis highly decreases, because high concentrations of acid may decompose the sugar and producing inhibitor molecules for the fermentation process (Demibras, 2005)

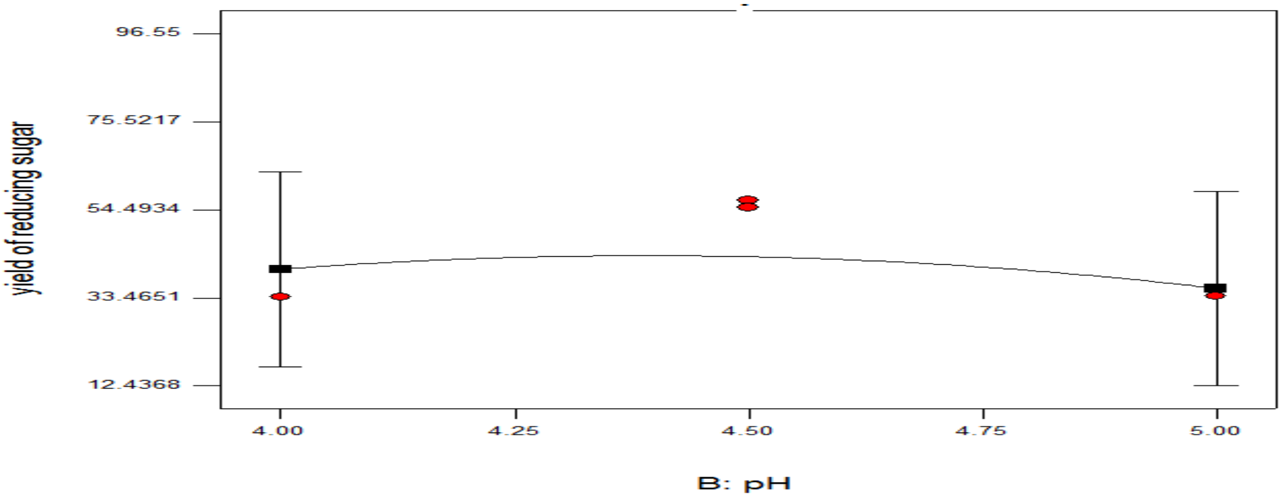


Figure 4.9 effect of pH on yield of reducing sugar after enzyme hydrolysis at constant temperature, time and enzyme in the center point

Effect of time

As it observed from figure 4.10 below the yield of reducing sugar was highly affect by hydrolysis time, as the hydrolysis time increased from 24hr. to 48 hr. the yield slightly increased (Stephen et al., 2012). Beyond 48 hr. up to 72 hr. hydrolysis time the yield of TRS highly decreased (Dias et al., 2013a). Therefore the minimum and maximum yield of TRS after enzyme hydrolysis were occurred at 72 hr. and 48 hr. of hydrolysis time respectively.

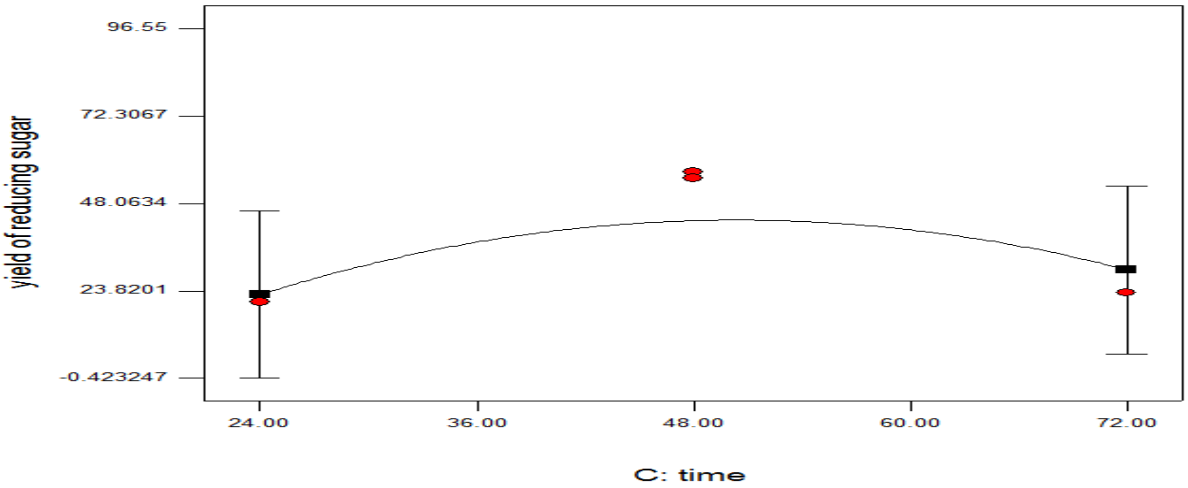


Figure 4.10 shows the effect of time on the enzyme hydrolysis for the yield of TRS at constant temperature, pH and enzyme loading in the center point.

Effect of Enzyme loading

Figure 4.11 effect of enzyme loading on yield of TRS after enzyme hydrolysis was evaluated as follow. The effect of enzyme loading was increased slightly from 1 % up to 1.5% as well as the yield of reducing sugar increased, but when the enzyme loading slightly increased the yield of TRS after hydrolysis was decreased (Dias et al., 2013a), so the minimum and maximum yield of reducing sugar occurred at 1% and 1.5 % of enzyme loading respectively.

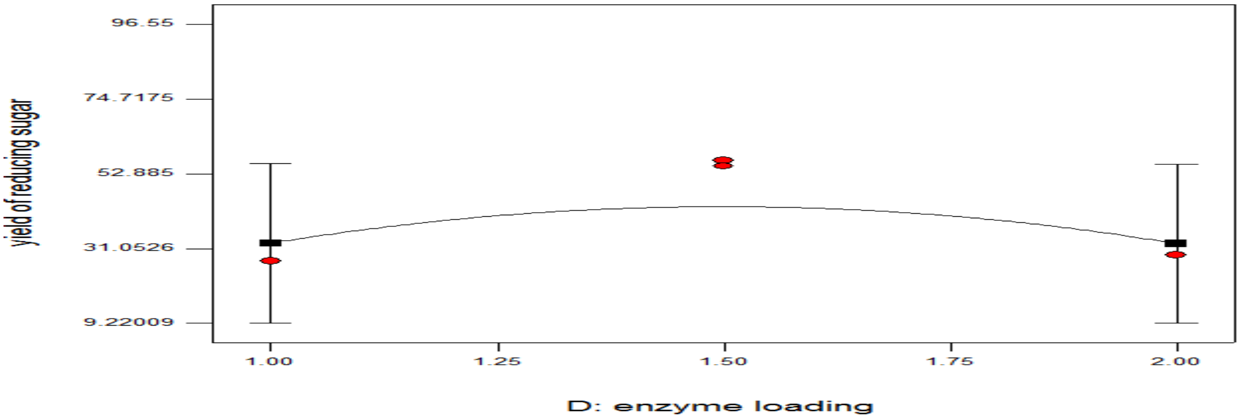
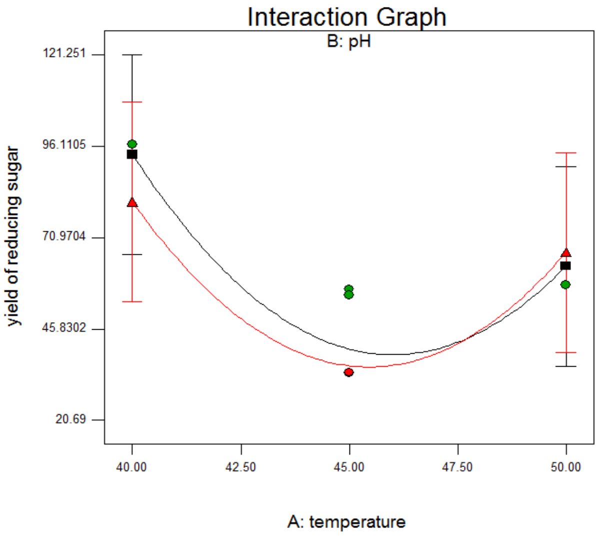


Figure 4.11 shows the effect of enzyme loading on the enzyme hydrolysis for the yield of TRS at constant temperature, pH and time in the center point

4.2.2.4 Interaction effect of experimental variables on the yield of reducing sugar



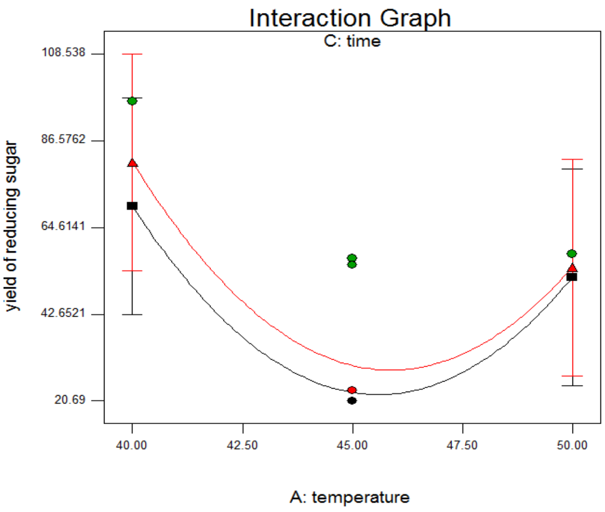
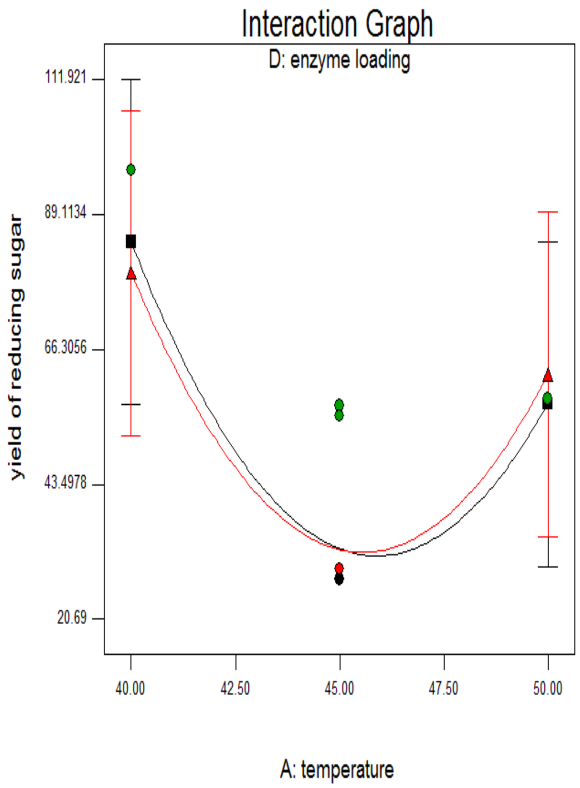


Figure 4.12 interaction effect of temperature and pH figure 4.13 interaction effect of temperature and time



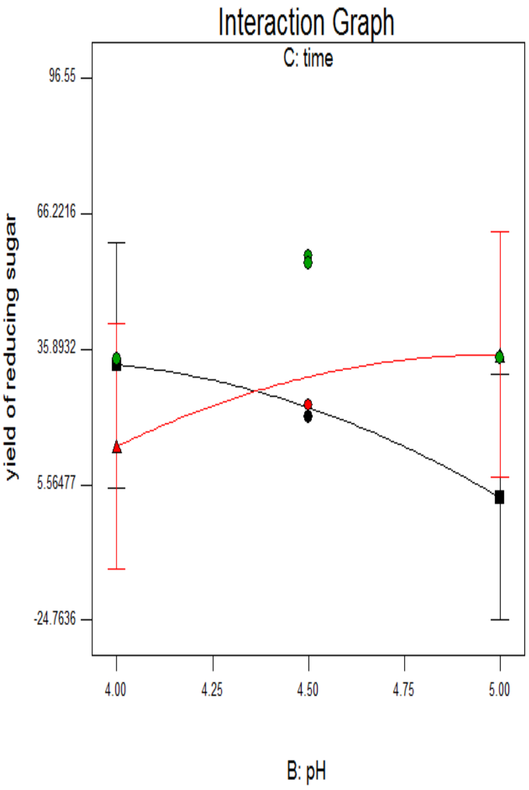
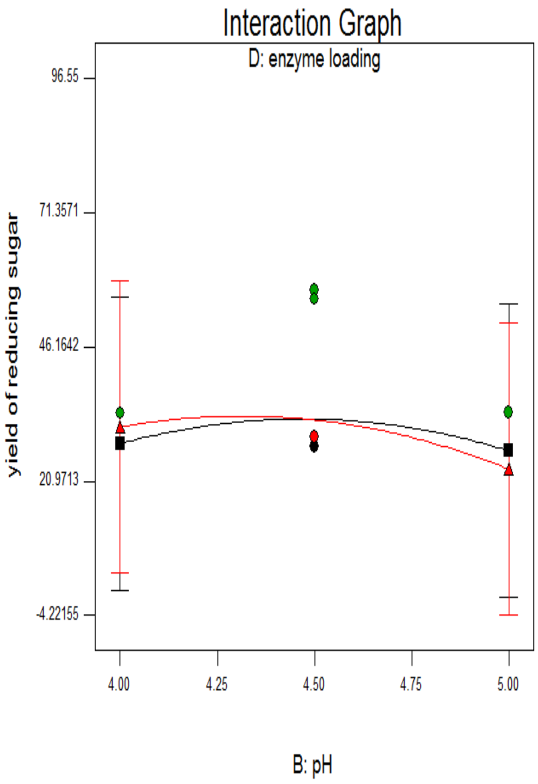


Figure 4.14 Interaction effect of temperature and enzyme loading figure 4.15 Interaction effect of pH and time



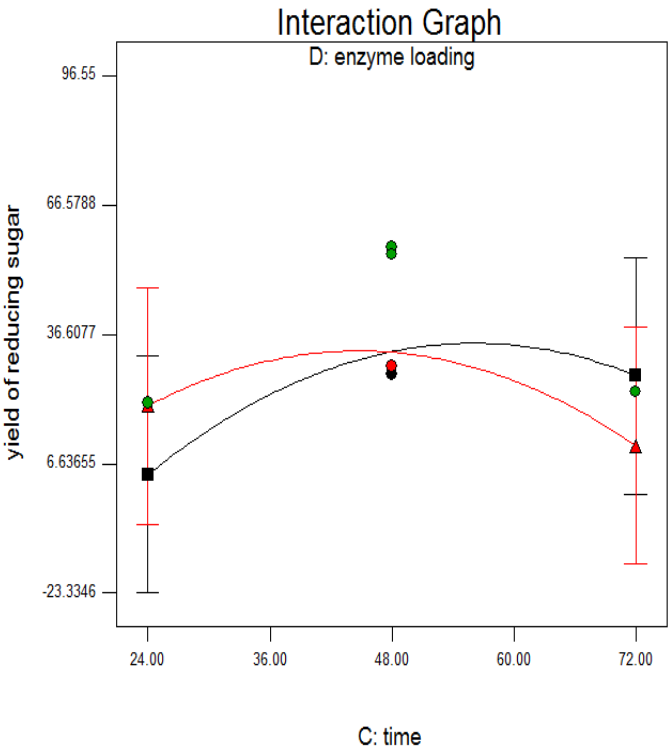


Figure 4.16 Interaction effect of pH and enzyme loading figure 4.17 Interaction effect of time and enzyme loading

From figure 4.15 ,which was effect of pH and time addtionally figure 4.17, which was effect of time and enzyme loading had high interaction on the yield of reducing sugar after enzyme hydolysis , but figure 4.12,figure4.13,figure 4.14 and figure4.16 were sginfcantly negligable effect on the on the yield of reducing sugar after enzyme.

4.2.2.4 Contour plot on the experimental variables

In order to analyze the regression equation of the model, 2D contour plots was obtained by plotting the response (yield of reducing sugar) on the Z axis against any two variables while keeping the other two variables at zero level. The effect of the independent variables on the yield of reducing sugar can be seen in Figures (4.18-4.23) below.

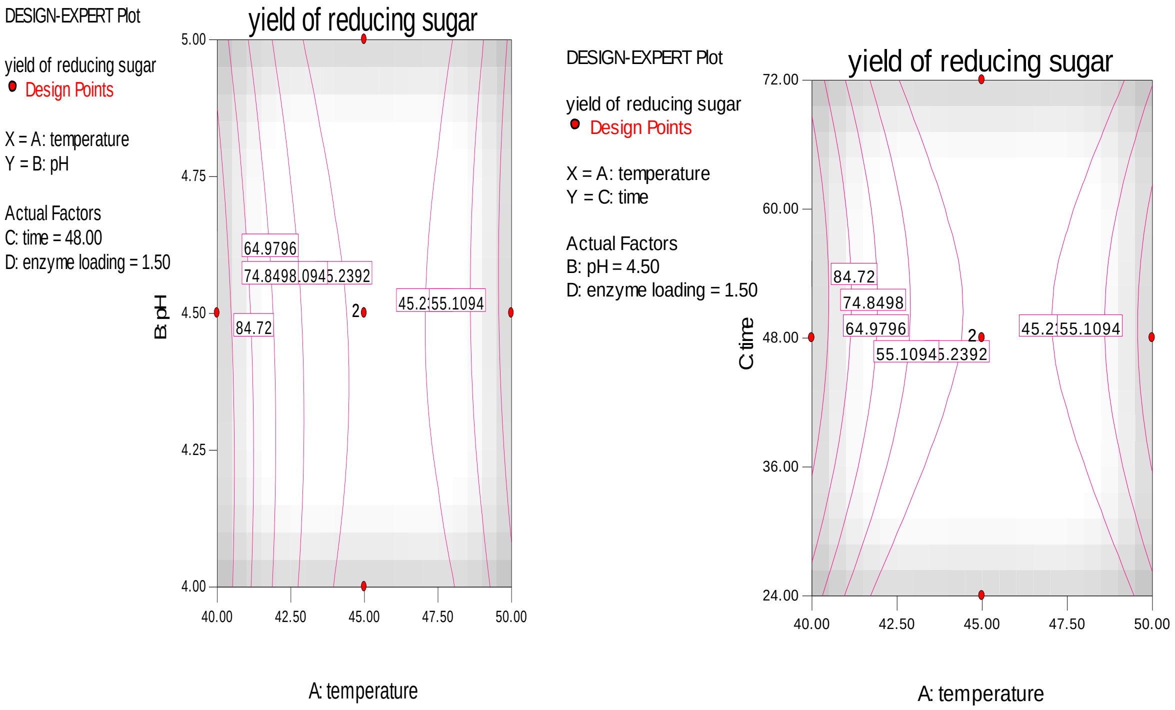


Figure (4.18 and 4.19) Contour plots of the effect of temperature with pH, and time at fixed time and enzyme loading as well as at fixed pH and enzyme loading respectively on the yield of reducing sugar

As shown from figure 4.18 represented contour plot the maximum yield of reducing sugar was occurred at temperature of 41 ºC and pH of 4.5, additionally from figure 4.20 represented counter plot the maximum yield of reducing sugar was occurred at temperature of 40 ºC and time of 50 hr.

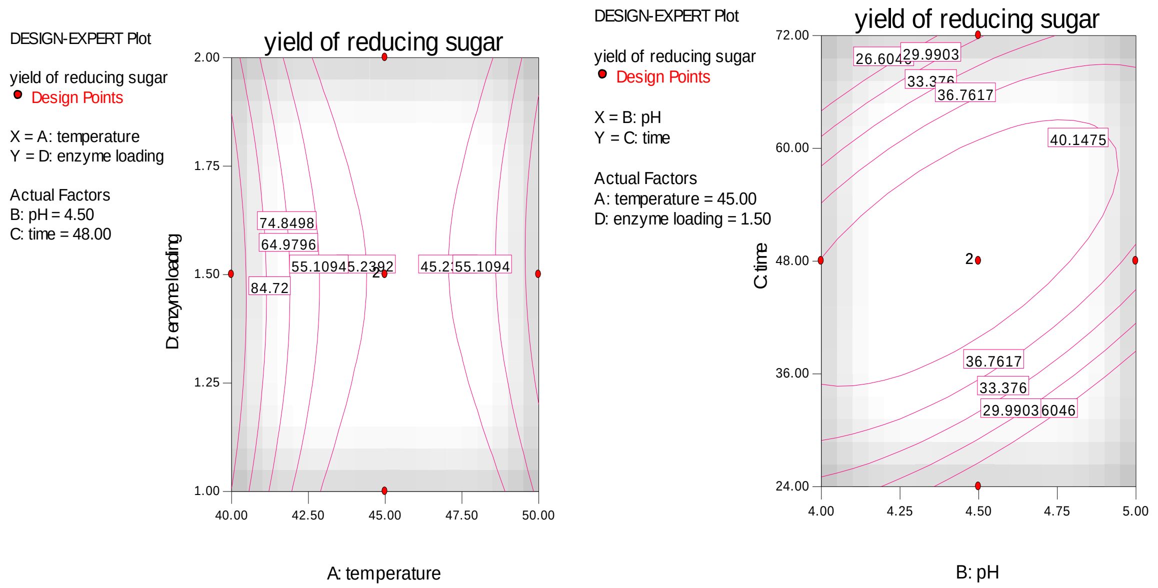


Figure (4.20 and 4.21) Contour plots of the effect of temperature with enzyme loading, effect of pH and time at fixed pH and time as well as at fixed temperature and enzyme loading respectively on the yield of reducing sugar

From figure 4.20 shown as represented contour plot the maximum yield of reducing sugar was carried out on temperature of 41 ºC and 2% enzyme loading, but from figure 4.21 maximum yield of reducing sugar was occurred at pH 4.75 and time of 60 hr.

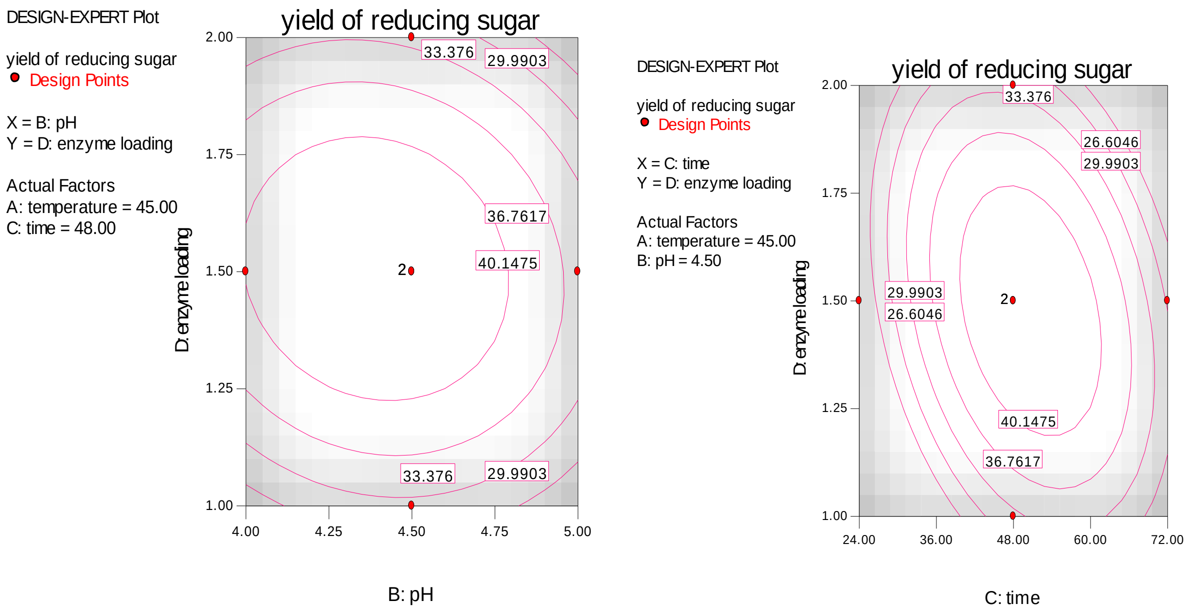


Figure (4.22 and 4.23) Contour plots of the effect of pH with enzyme loading, effect of time and enzyme loading at fixed temperature and time as well as at fixed temperature and pH respectively on the yield of reducing sugar

As shown from figure 4.22 the contour plot the maximum yield of reducing sugar was 40.1475 w/w% was occurred in the region of pH 4.75 and 1.75 % enzyme loading, but from figure 4.23 contour plot the maximum yield of reducing sugar was occurred at time of 50 hr. and 1.75 % enzyme loading.

4.2.2.4 Response surface on the experimental variables

In order to analyze the regression equation of the model, three-dimensional surface was obtained by plotting the response (yield of reducing sugar) on the Z axis against any two variables while keeping the others two variables at zero level. These plots are created to analyze the change in the response surface. Conical shape response surface plot indicates optimum operating conditions. The response optimized value for the production of bioethanol was based on the two process variables described on the response surface plot. The effect of the independent variables on the yield of reducing sugar can be seen in Figures (4.24-4.29) below.

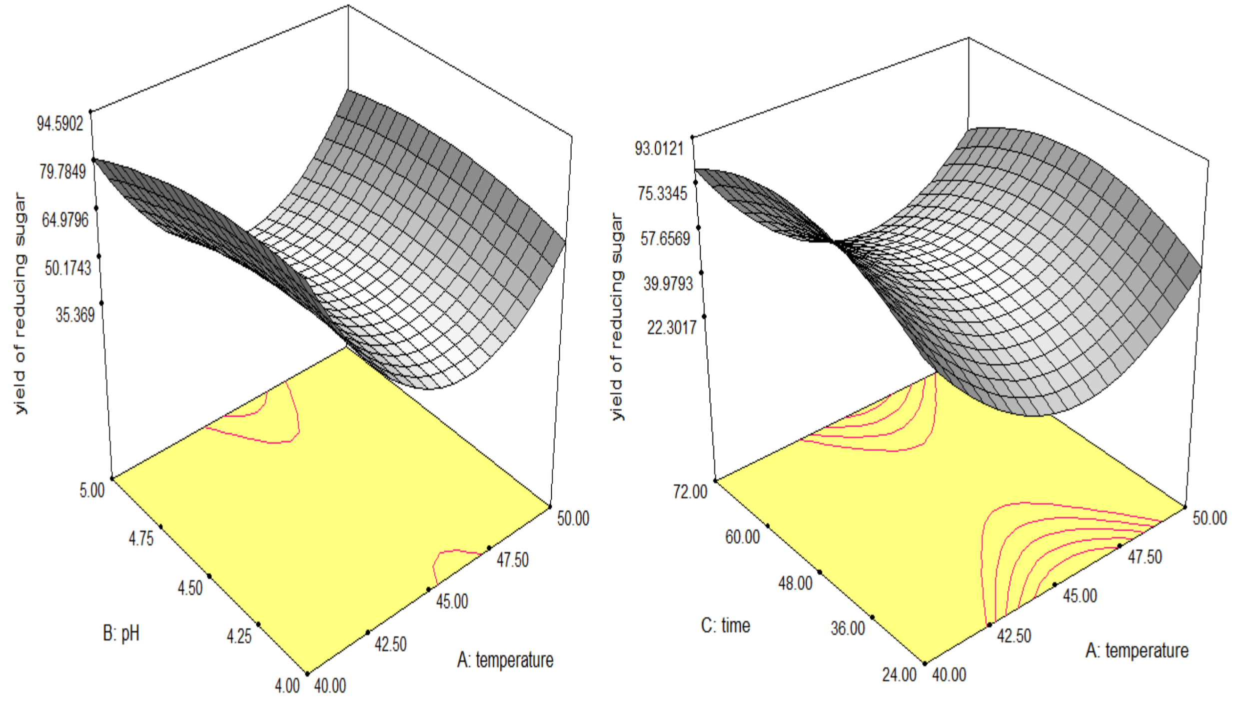


Figure (4.24 and 4.25). Response surface plots of the effect of temperature and pH, as well as the effect of temperature and time on the yield of reducing sugar at fixed time and enzyme loading, at fixed pH and enzyme loading respectively

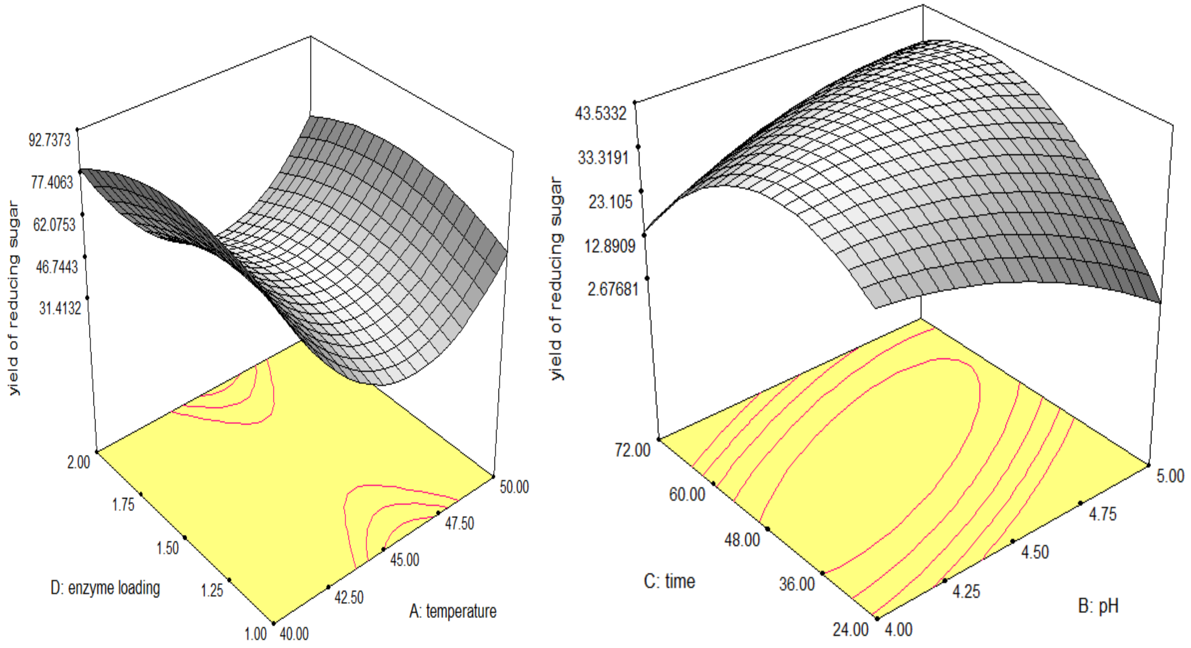


Figure (4.26 and 4.27). Response surface plots of the effect of temperature and enzyme loading, as well as the effect of pH and time on the yield of reducing sugar at fixed pH and time, at fixed temperature and enzyme loading respectively

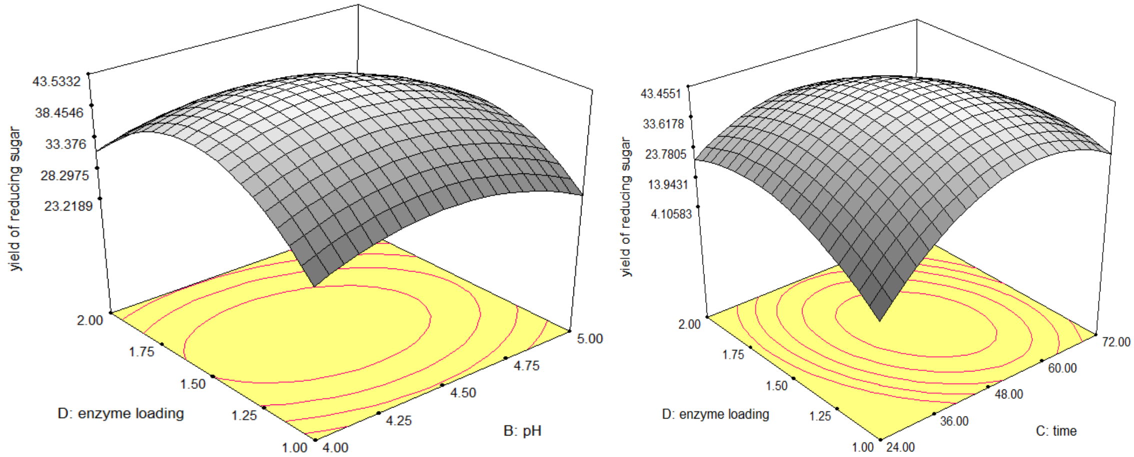


Figure (4.28 and 4.29). Response surface plots of the effect of pH and enzyme loading, as well as the effect of time and enzyme loading on the yield of reducing sugar at fixed temperature, and time at fixed temperature and pH respectively

From figure (4.24 and 4.25). Represents the response surface developed as a function of temperature and pH, as well as function of temperature and time on the yield of reducing sugar at fixed time and enzyme loading, at fixed pH and enzyme loading respectively, which hadn't a significant effect on the yield of reducing sugar after enzyme hydrolysis and the shape of the surface was not conical, explained there were no effect on yield of reducing sugar.

From figure (4.26).represent response surface plot developed as function of temperature and enzyme loading, on the yield of reducing sugar at fixed pH 4.5 and at fixed time 48 hr. weren't significant effect. But, from figure 4.29 represent response surface plot was developed as function of pH and time on the yield of reducing sugar, at fixed temperature and enzyme loading 45 ºC and 1.5% respectively were increased up to the optimum point of pH 4.5 and time 48 hr. with a value of 43.5332 w/w%, after that the yield of reducing sugar was dropped to 2.67681 w/w% at pH 5.0 and time 72 hr.

Figure 4.28 Shown that Response surface plots of the effect of pH and enzyme loading, temperature kept constant at 45 ºC, and time kept constant at 48 hr., the yield of reducing sugar increased slightly, but from the pH 4.5 and enzyme loading 1.5 % highly decreased. As well as Figure 4.29 the response surface plot explained of time and enzyme loading on the yield of reducing sugar at, temperature kept constant at 45 ºC and pH kept constant at 4.5 were slightly decreased. The highest yield of reducing sugar was observed at 4.5 hydrolysis pH and 1.5 % enzyme loading.

4.3 Model validation

4.4 FT-IR Characterization of the Produced Bioethanol

The functional groups of BSG Bioethanol were determined by using prinks Elmer spectrum 65 FT-lR with the help of lR correlation charts in Addis Ababa University, 4kilo campus. The lR spectrum was reported by % transmittance. The wave number region for the analysis was 4000-400 cm^{-1} (in the mid-infrared range).

Alcohols have characteristic IR absorptions associated with the O-H, C-O and the C-H stretching vibrations. When run as a liquid film the region 3500-3200 cm^{-1} with a very intense and broad band indicated the O-H stretch of alcohols, while the region 1260-1050 cm^{-1} confirms the C–O stretch. The bands at around 2880 and 2930 cm^{-1} were assigned as the symmetric stretching modes of the $–CH_2$ and$–CH_3$ groups, respectively (Coates and Meyers, 2000; Yu et al, 2007). This ascertains that the product obtained from Barley spent grain (BSG) is definitely ethanol due to the confirmation of these regions (Figure 4.6.1).

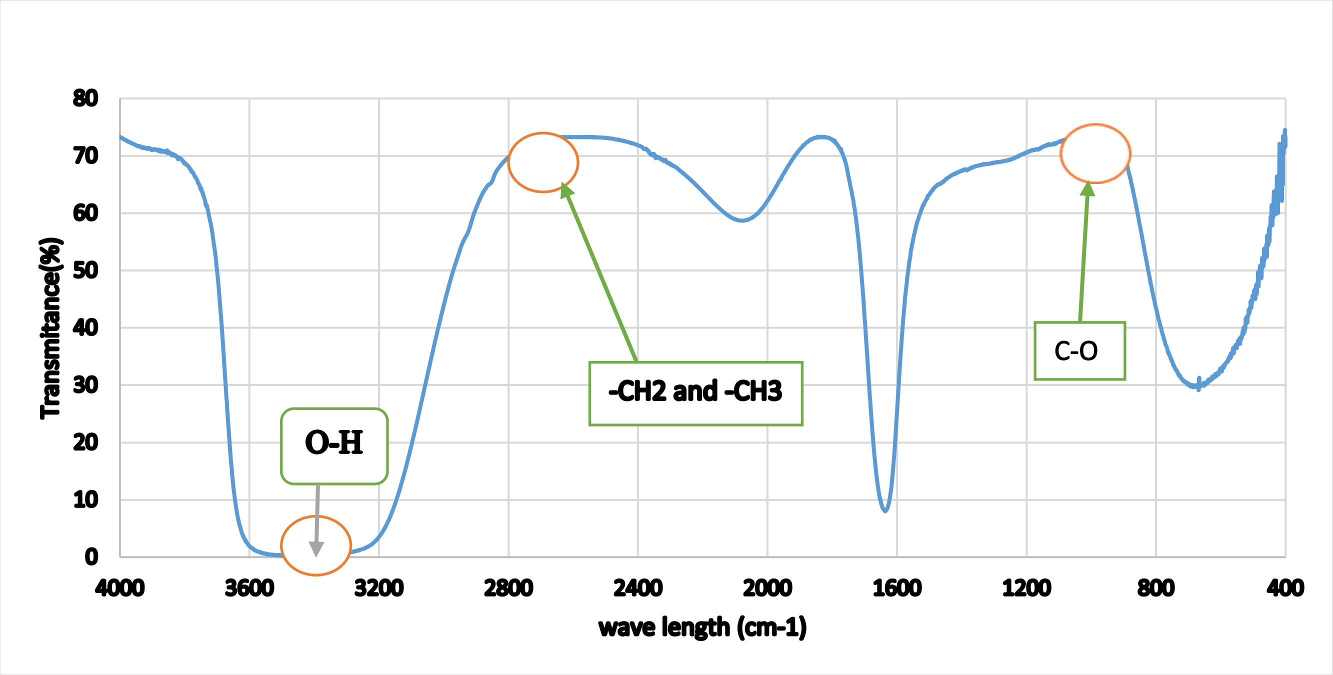


Figure 4.6.1 Fourier transform Infrared spectra of the produced bioethanol from Barley spent

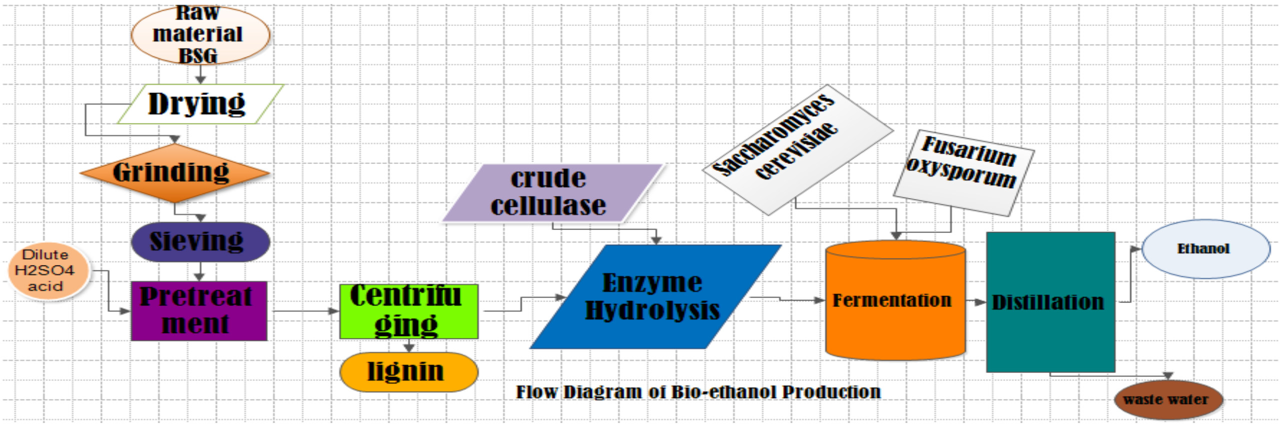


Figure 4.6.2 Flow diagram of bioethanol production

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The biomass pretreatment and the intrinsic structure of the biomass itself are primarily responsible for its subsequent hydrolysis.. Therefore, pretreatment of biomass is an extremely important step in the synthesis of biofuels from lignocellulosic biomasses.

In this study pretreatment was occurred at temperature 120 ºC, pH 1.65 and time of 17 min, which given 0.198 combined severity factor and the effect of the hydrolysis process variable (temperature, pH, time and enzyme leading) in the yield of reducing sugar were investigated and optimized using response surface methodology. Based on the analysis of variance (ANOVA) hydrolysis temperature, pH, time and enzyme loading interaction between temperature and acid concentration have significant effect on both the yield of reducing sugar and ethanol.

Maximum yield of reducing sugar was obtained when the temperature of the process was slightly small, because enzyme are sensitive for temperature. Enzyme hydrolysis gave maximum total reducing sugar than acid hydrolysis, which were 96.55 % reducing sugar using enzyme but, 54.67 % reducing sugar using acid hydrolysis (Amare, 2016: Liao et al. 2005; Martin et al., 2002) as well as the value of 96.55 % for cellulose conversion into glucose attained in the enzyme hydrolysis step was higher than the acid hydrolysis of 72 % obtained by (Mussatto et al., 2005).

Positive yield of ethanol was obtained at a medium pH and low temperature which was 40 ºC as well as at medium time and enzyme concentration. As the result of RSM optimization at 40 ºC,4.5 pH, 48 hr. and 1.5 % enzyme loading were the hydrolysis temperature, pH, time and acid concentration, respectively afforded 96.55 % w/w yield of reducing sugar and 53.68 % ethanol respectively. All points were located near to the central point of the design. Based on this study, it is evident that the chosen method of optimization was efficient, and reliable. From this result, it can be concluded that Barley spent grain (BSG) has the potential to serve as a low-cost feedstock for the second generation of ethanol production and enzyme hydrolysis process was very effective, furthermore the yield of ethanol was maximum with the respective yield of total reducing sugar. The reason for this result is fungus *fusarium oxysporum* which has the ability to ferment the 5-carbon sugars to produce ethanol (Martin et al., 2002).

5.2 Recommendation

Based on the current investigation the following recommendations are forwarded:

Optimization of hydrolysis variables are carried out in this study. But further optimization of the Pretreatment, and Fermentation are recommended to maximize the yield of ethanol from BSG.

Enzyme hydrolysis is an attractive, efficient and environmental friendly to hydrolysis cellulose materials, so it needs further optimization of liquid state fermentation for maximum productivity.

For quantification of hydrolysed reducing sugar further experiential equipment's are wonderful in terms of quality and quantity with no significant error, in terms of using spectrophotometer for quantification of reducing sugar HPLC will be a good exemplary for individual quantification of hydrolysed reducing sugar.

Further research have to be carried out on the optimization of using co-culture microbes for seek of increasing yield of ethanol on the fermentation.

Controlled and jacketed fermenter is good for maximum ethanol production than controlled incubator conical flasks, because the fermenter helps process parameters are easily controlled and adjusted.

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Appendixes

Appendix A: laboratory equipment's and sample photos

A1

A2

A3

A1 original spent grain, A2 molecular sieve up to 0.2mm, A3 fine spent grain with particle size of 0.2mm

A4

A5

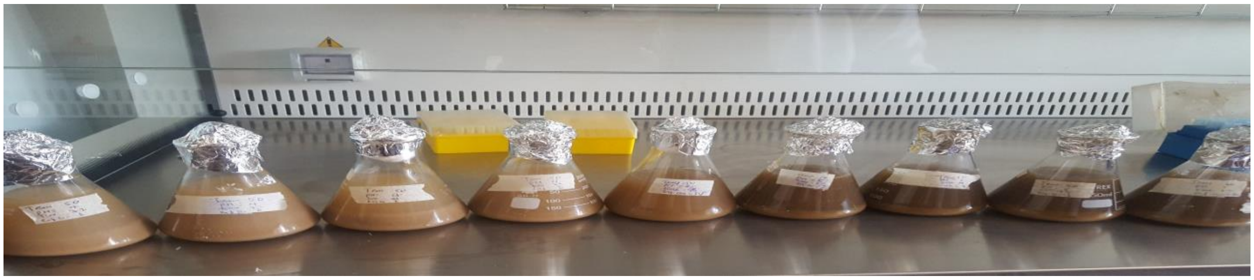
A4 muffle furnace for determination of ash content, A5 treated cellulose component

A6

A6 samples ready for enzyme hydrolysis with pH 4 and 4.5

A7

A7 samples ready for enzyme hydrolysis with pH 4.5 and 5



A8

A8 samples ready for enzyme hydrolysis at temperature of 50 C^0,pH 4,4.5 and 5

A10

A11

A10 fermentation of hydrolysed samples in incubator shaker, A11 simple distillation for separation of ethanol from solution

Appendix B: General Characteristics of bioethanol

Molecular formula	C_2H_5OH
Molecular mass	46.07g/mol
Appearance	colorless liquid
Water solubility	miscible
Density	0.789kg/lit
Boiling point	78.5 C^0
Freezing point	-117 C^0
Flash point	12.8 C^0 (lower temperature ignition)
Ignition point	425 C^0
Explosion limit	lower 3.5%V/V: upper 19%V/V
Vapor pressure	38 C^0 50mmHg
Lower heating value	@20 C^0 29.800kJ/Kg
Higher heating value	@20 C^0 21.09kJ/l
Specific heat	1 kcal/kg@60 C^0
Acidity	@pka 15.9
Viscosity	1200mpa.s (20 C^0)
Refractive index (nd)	1.36 (25 C^0)
Octane number	108