Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio-Engineering

Bioethanol Production from Sequential Acidic-Alkaline Pretreated Sorghum Straw Hydrolysate

Fseha Gebremichael

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Advisor	Signature	Date
Professor Eduardo Ojito Cespedes	_____	_____
Internal Examiner	Signature	Date
Mr. Teshome Worku	_____	_____
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Name Signature

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Advisor Signature

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List of acronyms and abbreviations

AAiT	Addis Ababa institute of Technology
ANOVA	Analysis of variance
CI	Confidence interval
Df	Degree of freedom
FTIR	Fourier transform infrared spectroscopy
GHG	Greenhouse gas
HMF	Hydroxy methyl furfural
ODW	Oven dried weight
UNIDO	United Nations Industrial Development Organization
R^2	Regression coefficient
V	Volume
W	Weight

Abstract

The depletion and environmental problems associated with fossil fuels as main energy source motivates to look for alternative energy sources that do not compromise both food security and the environment. Sorghum is a fast-growing crop that can be harvested twice a year and produce both food (grain) and straw that can be utilized for ethanol production. The study explores the production of bioethanol from sequential acidic-alkaline pretreated sorghum straw hydrolysate. Sequential acid-alkaline pretreatment method was employed to obtain most intact monosaccharides from cellulose and hemicellulose. Dilute sulfuric acid (1%) pretreatment at 125 ℃ for 10 minutes was used at the first stage mainly for hemicellulose removal. In the second stage pretreatment, dilute sodium hydroxide (1.25 %) at 90 ℃ for 10 minutes was employed for delignification. The residues after the second pretreatment were hydrolyzed using dilute sulfuric acid of concentrations (2 %, 3%, and 4%), at temperatures (110 ℃, 125 ℃, and 140 ℃) and times (20, 40, and 30 minutes). The sugar concentration of the hydrolysates was determined using phenol sulfuric acid method. Three hydrolysates having sugar levels 30.42 g/L, 31.79 g/L, and 32.9875 g/L were selected for fermentation. Response surface methodology, Box-Behnken experimental design with design expert version 11.1.0.1 was applied to randomize and analyze the effects of the variables encountered in fermentation experiments. The experiments were done using the selected hydrolysates, varying size of inoculum (5 %, 10 %, and 15 %), and pH (4.5, 5, and 5.5) at 30 ℃ for 72 hours. The fermented samples were finally centrifuged and evaporated for purifying the ethanol. The yield of ethanol was determined after determining the percentage ethanol using digital density meter. FTIR analysis was done to determine the functional group of the product obtained and the result showed O-H, C-O, and C-H peaks similar to the standard ethanol. The results of the statistical analysis showed that all the three independent factors and the interaction between sugar level and pH affected the ethanol yield and a maximum of 0.617 mL/g yield was obtained at sugar level of 3.29875 g, pH of 5, and 15 % inoculum size. Generally, controlling fermentation parameters during the production of ethanol from sequential acidic-alkaline pretreated sorghum straw is a good choice in view of increasing yields of sugar and ethanol, decreasing dose and cost of chemicals, and minimizing waste generation.

***Key words**: Yield, Fermentation, Sequential Pretreatment, Sorghum Straw*

1 Introduction

1.1 Background

Relying on conventional fossil fuels for the entire energy supply is problematic for the world due to the severe consequences they cause, such as potential energy shortage that jeopardize national energy security (Guo, 2012). A variety of analysts including economists, policy makers, environmentalists, and engineers are engaged in solving the energy and climate challenges. As a result, some countries have managed to lessen their reliance on traditional energy systems through novel innovations and improved infrastructure, with socio-economic and environmental benefits. Yet most countries still depend mainly, in some cases almost completely, on fossil fuels. Ethiopia, for example, is a country where transport energy demand is entirely dependent on petroleum imports. Thus, security of petroleum supply or other sources of energy which can replace petroleum is critical in addition to the socio- economic and environmental reasons for Ethiopia to diversify the energy mix as the future of petroleum products reserve is uncertain with increase in price that makes the foreign currency expenditure intolerably high and affect transport tariff and price of other commodities negatively (Hiben, 2013).

Biofuel production is being considered as an alternative source of energy due to the prediction that there will be exhaustion of fossil fuel energy supply. Bioethanol is one such fuel which exhibits several advantages over conventional fossil fuels. This includes its renewable nature, ease of storage, the fact that it is free of sulfur, and contributes less to global warming and air pollution. In recent times, the application of bioethanol as a fuel replacement has become more appealing. (Rorke, Bosco, & Kana, 2017).

On the other hand, huge volumes of cellulosic materials, such as straws, stalks, peels etc. are being generated as waste from various agricultural activities. These materials can be exploited as sustainable resource for production of many organic fuels and bioenergy. They can reduce greenhouse gas emissions, enhance energy security, improve the economy, dispose problematic solid wastes, and improve air quality (Adelabu, Kareem, Adeogun, & Ademolu, 2018).

Every year, Ethiopia produces tons of Sorghum and huge amount of sorghum straw is produced as byproduct. An average 22,161,808 quintals of sorghum was produced between the years 2004-2008 (Alemayehu, Paul, & Sinafikeh, 2012). Producing bioethanol from this straw is important to reduce the impact caused by fossil fuels as it contains high amount of cellulose and hemicellulose which can be broken down to simple sugars which in turn can be fermented to produce ethanol (Adelabu et al., 2018)

1.2 Statement of the problem

The cost of fossil fuels is increasing at an alarming rate and the environment is being polluted by greenhouse gases like carbon dioxide, methane, nitrous oxide and sulfur containing compounds released from the use of fossil fuels which are in verge of depletion.

On the other hand, ethanol, a potential renewable form of energy and beverage, is commercially produced from edible feedstocks such as corn, maize, sorghum, barley etc. Use of these feedstocks competes with food security and results in increasing prices of fuel and food. So, using non-edible agricultural products can be a better choice to solve both energy and food security problems. As sorghum is one of the most cultivated crops in Ethiopia, huge amount of its straw is produced. This straw can be a potential source of ethanol as it has high amounts of cellulose and hemicellulose which can be broken down to simple sugars and ultimately fermented to ethanol.

Production of ethanol from lignocellulosic biomass involves four steps namely (pretreatment, hydrolysis, fermentation, and purification). Pretreatment is the rate-limiting step for bioethanol production from lignocellulosic biomass, and subsequently intensive studies have been undertaken to improve the pretreatment efficiency. However, so far, most pretreatment methods failed to achieve desirable sugar recovery from both cellulose and hemicellulose in the biomass, which is essential to improve process economics and competitiveness of bioethanol.

Previous researches on bioethanol production employed either single stage acidic or single stage alkaline pretreatment. Acid catalysts such as sulfuric acid and hydrochloric acid are less efficient in lignin removal while alkaline catalysts like lime are less effective in hemicellulose degradation. To address the issue, several researches were focused on sequential acidic-alkaline pretreatment. Sequential sulfuric acid-lime pretreatment was used for pretreatment of miscanthus (Guo, 2012). But lime was obtained to be less effective in lignin removal and it was recommended to use sodium hydroxide.

Fermentation has also a significant effect on yield of ethanol. The effects of fermentation parameters on ethanol production is not well studied. Yield of ethanol during fermentation is affected by temperature, pH, inoculum size, substrate concentration, time, etc. Thus, this research aims at studying the effects of fermentation parameters on ethanol production from the sequential pretreated (sequential sulfuric acid - sodium hydroxide) sorghum straw hydrolysate.

1.3 Objectives

1.3.1 General objective

The general objective of this research is bioethanol production from sequential acidic-alkaline pretreated sorghum straw hydrolysate.

1.3.2 Specific objectives

- Characterization of sorghum straw for its organic, ash, moisture content, cellulose, hemicellulose, and lignin contents.
- Comparing the yields of sequential and single pretreatment methods in terms of sugar yield.
- Investigating the effects of inoculum, pH, and hydrolysate concentration during fermentation.
- Determination of yield and functional group of the product (ethanol).

1.4 Significance

This research is crucial as it uses the available, cheap, and renewable waste for the production of valuable product. Moreover, it serves as a starting material for further researches, encourages farmers to improve sorghum production, and creates job opportunity for citizens if implemented.

2 Literature review

2.1 Biofuels use in the world

Biofuels, mainly produced from biomass, are a broad range of fuels in the form of solid biomass (bio-char), liquid fuels (bioethanol, biodiesel, and vegetable oil), and various biogases (biogas, bio-syngas, and bio-hydrogen). Biofuels have paid attention globally due to concerns on climate change, energy security, and dependency on import encumber of petroleum products. They are increasingly premeditated by many countries as much as practicable to substitute the fossil fuel source in the transport sector. At present bioethanol and biodiesel are the main biofuels for transport as both can be used in blended or neat form although neat usage requires engine modification. Bioethanol, derived from starch crops like sugarcane, sugar beets, corn, wheat, and sorghum is utilized blended with petroleum-based gasoline, and biodiesel, derived from oil crops like rapeseed, palm oil, jatropha, sunflower, and soy is utilized blended with petroleum based diesel (Hiben, 2013).

While bioethanol and biodiesel are the most typical biofuel types, the two top producers of ethanol in the world by far are the United States and Brazil, collectively producing almost 87% of the 21.8 billion gallons (82.5 billion liters) world ethanol production in 2011. United States was the major producer among the two representing 13.3 billion gallons (50.3 billion liters) of the world's ethanol production in 2011 using corn as the main feedstock accounting for the vast majority of the input whereas Brazil the second major producer uses sugarcane for its 5.6 billion-gallon (21.2 billion liters) ethanol production in 2011 (Hiben, 2013).

2.1.1 Bioethanol

Bioethanol is a liquid produced by distillation of fermented sugar obtained from various resources such as agriculture and forestry residue, dedicated starchy crops, woody and herbaceous crops, and organic portion of municipal solid waste. It has become favored due to its potential similarity on the convenient characteristics of petroleum products at competitive price (Hiben, 2013).

2.1.2 Bioethanol production in Ethiopia

Ethanol production in Ethiopia is linked with sugar factories and aimed for import substitute of petroleum products, enhance agricultural development and agro-processing, job creation, and export earnings. However, only a small fraction of the potentials is utilized yet and an alternate 5% and 10% ethanol blend has accessed in the capital city of the country (Hiben, 2013).

Ethiopia started modern sugar industry in 1951 at Wonji sugar factory as a share company founded by Ethiopian government and foreign investors followed by Shoa and Metehara sugar factories in 1962 and 1969 respectively. Five years later, in 1974, all sugar factories became in charge of the government ownership following the change of government and started operating under the Ethiopian Sugar Corporation which has embarked upon alcohol production program from molasses for use as blend with gasoline in 1979. The promoters of this project were Ministry of Industry and UNIDO which has paid for the feasibility study conducted by the State Alcohol Monopoly of Finland. Likewise, in 1984, a French company named SOFRECO has also made a feasibility study for the production of baker's yeast and bioethanol from molasses (Fessehaie, 2009).

When the sugar corporation was dissolved by law in 1992, all the factories were re-established as public enterprises and thus Ethiopian Sugar Industry Support Center came into existence in 1998 to provide common support to all factories as a share company of Ethiopian Insurance Corporation, the Development Bank of Ethiopia, and the three sugar factories. In 1999, Fincha sugar factory was established as a third sugar development project in the country. The same year, a technical committee was established consisting of the Ethiopian Sugar Industry Support Center, Finchaa Sugar Factory, Ethiopian Petroleum Enterprise, and Oil distributing companies in Ethiopia to address issues intended for successful commercialization of ethanol fuel. Later in April 2000 directives on production, distribution, and control of ethanol blended gasoline fuel were approved by the council of ministers of the Federal Democratic Republic of Ethiopia and then SOFRECO made another feasibility study in 2004 to expand Finchaa sugar factory, and concluded that the ethanol blended gasoline fuel was economical (Fessehaie, 2009).

Replacing the support center, in 2006, the house of peoples' representatives of the Federal Democratic Republic of Ethiopia established the Ethiopian Sugar Development Agency under proclamation No. 504/2006 with the purpose of implementing the government sugar development

policies and to assist public sugar enterprises in project development, research, training, and marketing in order to make them modern, efficient, and competitive with the vision of producing considerable amount of good quality sugar and ethanol at the lowest possible cost as compared to the world producers and gain significant market share in the world market. The same year, as a fourth sugar development project in the country, Tendaho sugar development project was also established (Fessehaie, 2009; "Sugar Corporation," 2013).

The current Sugar Corporation with a vision of increasing sugar development activities in a massive scale and structure came into existence on October, 2010 by replacing the former Ethiopian Sugar Development Agency under the council of ministers' regulation No.192/2010. The purposes of its establishment are growing sugarcane and other sugar yielding crops to process and produce sugar, sugar products, sugar byproducts, and products of sugar byproducts for sell in the domestic and export markets. Therefore, to complete the aforementioned purposes, the corporation is implementing expansion projects on the existing sugar factories while accordingly started constructing ten new sugar factories ("Sugar Corporation," 2013).

2.1.3 Feedstocks for bioethanol production

Bio-ethanol feedstocks can be divided into three major groups, namely:

- Sucrose-containing feedstocks such as sugarcane, sugar beets and sweet sorghum
- Starchy materials such as corn, sweet potatoes, cassava, wheat and barley
- Woody and grassy materials from agricultural and forest residues.

In the short term the production of bio-ethanol is dependent on sugary and starchy crops. The potential drawback of using sugar and starch crops is that they may be expensive to produce and may be needed for other uses. Lignocellulosic biomass has been envisaged to be the ultimate feedstock to provide large volumes of ethanol in the medium to longer term due to its low cost and high availability (Gaia, 2014).

The technology to produce bio-ethanol from sugary and starchy crops is known as first generation while the procedure of producing from lignocellulose is referred to as "second generation. With first generation technology, crops such as sugarcane, sweet sorghum and sugar beet are directly converted to bio-ethanol by yeasts. This is because these crops contain either glucose or sucrose.

In contrast, bio-ethanol from starchy crops such as corn and cassava requires the conversion of starch to simple fermentable sugars and then to bio-ethanol (Gaia, 2014).

2.1.3.1 Nature of Lignocellulosic Materials

Lignocellulosic materials consist of three major components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are structural carbohydrates that can be depolymerized through hydrolysis for ethanol production, while lignin is a complex aromatic polymer which forms a crust surrounding the carbohydrate fraction and performs as a barrier limiting the accessibility of carbohydrates to hydrolytic enzymes or chemicals. There are extensive interactions among these three components, which further improves the stiffness of biomass structure. Cellulose is the major structural component of any lignocellulosic biomass. It is a linear chain of several hundred to over ten thousand β-1, 4-linked D- glucopyranose units. Due to the existence of many equatorially oriented hydroxyl groups, cellulose molecules have a strong tendency to form intra-molecular hydrogen bonds which are responsible for the stiff and rigid nature of the cellulose molecule. Also, hydroxyl groups from one chain form inter-molecular hydrogen bonds with oxygens on another chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength(Xu, 2009).

Hemicellulose is a non-structural polysaccharide with low molecular weight. It has amorphous, heterogeneous, and branched structure, thus having low crystallinity. Monosaccharide residues constituting hemicelluloses include pentose (xylose and arabinose) and hexose (glucose, galactose, and mannose). Lignin is a three-dimensional complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose, stiffening and holding together the fibers of polysaccharides. It strengthens the cell wall structure, aids in conducting water in the plant stem, and provides protection against microbial attack and decay(Xu, 2009).

Cellulose, hemicellulose and lignin are not uniformly distributed within the cell walls. The structure and the quantity of these plant cell wall components vary according to species, tissues and maturity of the plant cell wall. Generally, lignocellulosic biomass consists of 35–50 % cellulose, 20–35 % hemicellulose, and 10–25 % lignin. Proteins, oils, and ash make up the remaining fraction. Below is the composition of some lignocellulosic materials.

Table 2-1 Types of lignocellulosic materials and their chemical composition

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Poplar	50.8 – 53.3	26.2 – 28.7	15.5 – 16.3
Oak	40.4	35.9	24.1
Eucalyptus	54.1	18.4	21.5
Pine	42 – 50	24-27	20
Spruce	45.5	22.9	27.9
Wheat straw	35 – 39	23 – 30	12 – 16
Barely straw	36 – 43	24 – 33	6.3 - 9.8
Rice straw	29.2 – 34.7	23 – 25.9	17 -19
Rice husk	28.7 – 35.6	12 – 29.3	15.4 -20
Corn cob	33.7-41.2	31.9 – 36	6.1 – 15.9
Sugar can bagasse	25 – 45	28 – 32	15-25
Grass	25 – 40	25 – 50	10 – 30
Switch grass	35 – 40	25 – 30	15 – 20
Sorghum straw	32 – 35	24 – 27	15 – 21

Source : (Isikgor & Becer, 2015)

2.1.3.1.1 Sorghum production in Ethiopia

Sorghum is the second most cultivated cereal crop in Ethiopia next to maize. Huge amount of sorghum is produced in the country annually. During the period of 2004-2008, an average of 22,161,808 quintals of sorghum was produced and its annual growth rate was 18.3 which is the second fastest growth rate next to maize (Alemayehu et al., 2012). A huge amount of sorghum straw is produced during the processing of the sorghum cereal. This straw has various components which can be utilized to produce second generation ethanol.

2.1.4 Uses of ethanol

Ethanol is used in alcoholic beverages. Alcoholic beverages vary considerably in their ethanol content and in the foodstuffs from which they are produced. Most alcoholic beverages can be broadly classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. Fermented beverages can be broadly classified by the food stuff from which they are fermented. Beers are made from cereal grains or other starchy materials, wines and ciders from fruit juices, and meads from honey. Fermented beverages may contain up to 15–20 % ethanol by volume, the upper limit being set by the yeast's tolerance for ethanol, or by the amount of sugar in the starting material. Absolute ethanol and 95 % ethanol are themselves good solvents, somewhat less polar than water and used in perfumes, paints and tinctures. Ethanol is used in medical wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62 % (Ayele, 2011).

The other and main use of ethanol is as a motor fuel and fuel additive. Ethanol and other alcohols can be used to power motor vehicles instead of gasoline. In almost all cases the ethanol is mixed with gasoline (Robati R. and Vazirzadeh1 M., 2013). Efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no GHG.

A number of market segments are available in the ethanol industry serving a wide range of uses in the medical sectors, pharmaceuticals, beverages, industrial, household, and transport uses. The market potential for bioethanol is therefore not just limited to transport fuel or energy production but has potential to supply the existing chemicals industry and house hold uses. However, the most prevalent use of bioethanol in Ethiopia is as a transport fuel in spark ignition engine vehicles and the current amount of ethanol fuel blended with gasoline is 10 % and the government is working to increase the share. The government is also working to start export in two years of time and to substitute household cooking fuel in the future (Yacob, 2013).

2.1.5 Bioethanol production process

Generally, the process of converting lignocellulose to bio-ethanol consists of four major steps: pretreatment of biomass to break down the main components, hydrolysis to depolymerize the broken components into monosaccharides, fermentation of hexoses and pentoses to ethanol, and final ethanol purification. The over-all production block diagram is shown in the Figure 2.1.

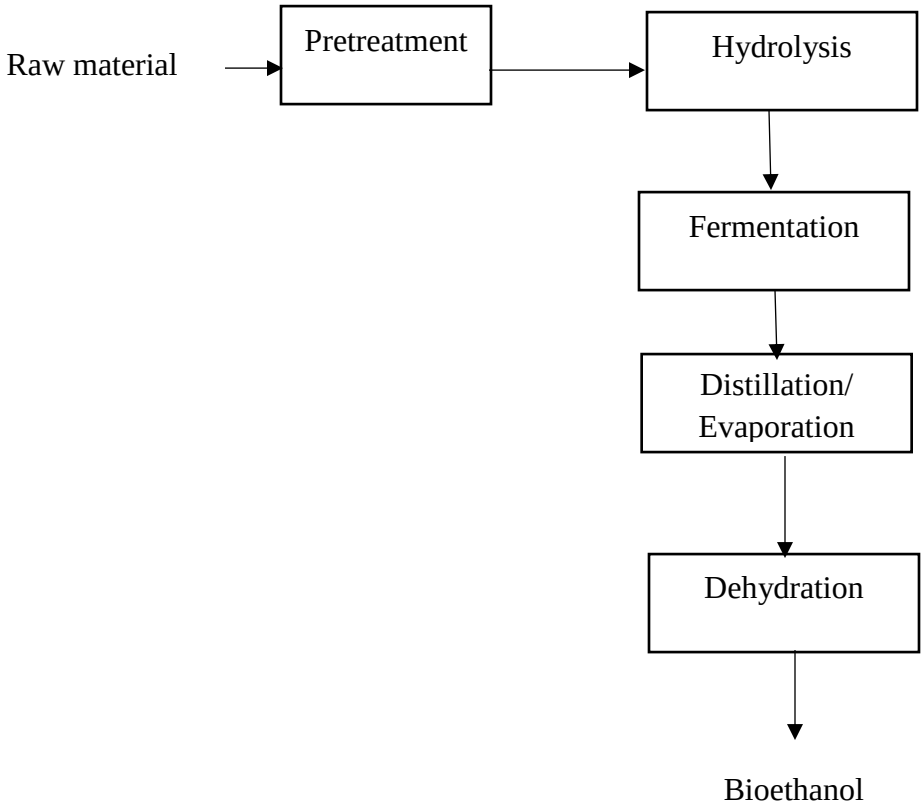

Figure 2-1 Flowsheet of Bioethanol Production

Source: (Guo, 2012)

2.1.5.1 Pretreatment

Pretreatment is among the most difficult steps due to its technical and economic challenges. First, lignocellulose is composed of three polymeric compounds: cellulose, hemicellulose, and lignin. The sugars to produce ethanol through fermentation are hexose (mainly glucose) and pentose (mainly xylose) derived from cellulose and hemicellulose individually. However, cellulose and hemicellulose are initially tightly bounded to lignin by hydrogen bonds and some covalent bonds, which form the backbone of lignocellulose. Secondly, pretreatment is costly step throughout the

conversion processes. Thirdly, since pretreatment is the first step of the conversion process, it strongly affects energy demand and costs of the downstream steps. (Guo, 2012).

The digestibility of cellulose present in lignocellulosic biomass is hindered by many physicochemical, structural, and compositional factors. In the conversion of lignocellulosic biomass to fuel, the biomass needs to be treated so that the cellulose in the plant fibers is exposed. Pretreatment uses various techniques, including ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion, to alter the structure of cellulosic biomass to make cellulose more accessible. The goal of pretreatment in biomass-to-biofuels conversion is depicted in Figure 2-2 (Kumar et al., 2009).

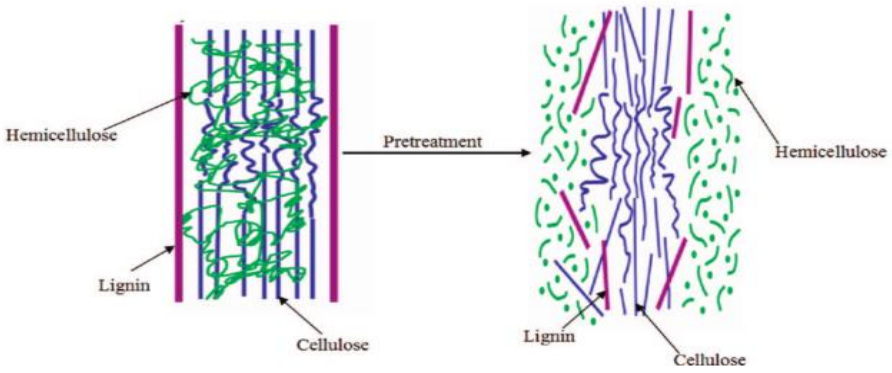

Figure 2-2 The role of pretreatment in production of biofuel from biomass

2.1.5.1.1 Single stage pretreatment

Since the pretreatment process is the rate-limiting step and the most challenging task, over the past 40 years, a number of technologies have been developed for lignocelluloses pretreatment. They can be roughly divided into four categories: physical (e.g. mechanical comminution, irradiation, electrical pretreatment), chemical, physicochemical, and biological pretreatments. The large power consumption of mechanical comminution, high cost of irradiation and insufficient research of electrical methods put physical pretreatment alone in an unpractical position. Although being considered environmentally friendly and saving energy, biological pretreatment sustains a fairly low processing rate which is intolerable for industrial application. Pretreatments with addition of chemicals were proved to be effective, however, a mixture of physical and chemical methods are more favored as physicochemical methods. Based on pH value of the applied catalyst,

physicochemical pretreatments can be further grouped into acid- based pretreatments, neutral pretreatments, alkaline pretreatments, and solvent based (Guo, 2012).

Currently, the strategy to choose a pretreatment technology for a particular biomass depends on the biomass composition and target products. However, for most biomass types, all three components (cellulose, hemicellulose, and lignin) account for the major part, and it would not be economically feasible to recover only one or part of the components. Therefore, it is highly desirable to develop a pretreatment technology for all major components which can reduce the production of inhibitory by-products (HU, G, Heitmann, 2008).

2.1.5.1.2 Multi-stage pretreatment

In view of the above discussion, different technologies and severities should be applied for multiple purpose optimizations. Different temperatures were applied in two-stage dilute-acid pretreatment of hybrid poplar. A low temperature (140 ℃) followed by a higher temperature (170 °C) was employed to deal with the easy-to-hydrolyze and hard-to- hydrolyze portion of xylan, respectively, achieving a 92 % xylose recovery with only 2 % of xylose degraded to furfural. However, the whole process focused only on maximizing xylose yield, leaving glucan, accounting for 42 % of the raw material, in a low conversion rate (Torget, R. and Hsu, 1994).

Guo (2012) applied sequential sulfuric acid-lime pretreatment of miscanthus at high temperatures for optimal production of ethanol. But lime pretreatment had its limitations in the two-stage pretreatment. Lime was less soluble in water at high temperatures and lead to inefficient delignification (Guo, 2012).

Sequential acid alkaline pretreatment of rice straw using sulfuric acid and sodium hydroxide was conducted in 2014. The enzyme Accellerase®1500 was employed for hydrolysis of the pretreated residue. The sequential acid alkaline pretreatment gave a good sugar yield compared to the single acid and base pretreatments, reduces chemical usage, and enzyme dose (Weerasai et al., 2014).

Besides the pretreatment technique, there are factors that affect the pretreatment efficiency and effectiveness which eventually affect sugar yield. Pretreatment is affected by solid to liquid ratio, temperature, time, and concentration of catalyst. High concentration of catalyst destructs sugars and it is costly. Besides, a study observed that monomer sugar yield decreased at high acid concentration, due to degradation of the product monomers by the acid (Palmarola-Adrados B,

Choteborska P, Galbe M, 2005). It was reported there was formation of spherical droplets on the surface of residual corn Stover following dilute-acid pretreatment at high temperature. It was suggested that the droplets formed were composed of lignin and possible lignin-carbohydrate complexes. It was demonstrated that these droplets were produced from corn Stover during pretreatment under neutral and acidic pHs at and above 130 °C and that they can deposit onto the surface of residual biomass. The deposition of droplets produced under certain pretreatment conditions (acidic pH, T>150 ℃) and captured on pure cellulose was shown to have a negative effect on the subsequent hydrolysis. Therefore, it is extremely important to carefully examine the appropriate dilute-acid pretreatment of lignocellulose biomass species (Kumar et al., 2009).

It was reported that dilute sulfuric acid solution and sodium hydroxide solution pretreatments gave good sugar yield when the solid to liquid ratio was 100 grams to 1000 mL. If the ratio is high the acids do not get the chance to strike the surface of the biomass while low ratio results in destruction of sugars (Tutt, 2015).

In an effort to save chemical, time, and energy cost and get better sugar yield, weerasai etal (2014) used optimized factors for sequential acid alkaline pretreatment of rice straw. The combined sequential process involved an initial hemicellulose solubilization by dilute acid using 1% (w/v) H_2SO_4 at 125 ℃ for 10 min, followed by alkaline delignification using 1.25 % NaOH at 90 ℃ for 10 min. Under these conditions, a glucose recovery yield of 70.9 % from saccharification of the cellulose enriched fraction was obtained with 2- to 4-fold savings in chemical usage as compared with single-step processes (Weerasai et al., 2014).

2.1.5.2 Hydrolysis

In the hydrolysis reaction, the complex chains of sugars that make up the hemicellulose are broken, releasing simple sugars. The complex hemi-cellulose sugars are converted to a mix of soluble five-carbon sugars, xylose and arabinose, and soluble six-carbon sugars, mannose and galactose. The rest of hemicelluloses are degraded to weak acids, furan derivates, and phenolics (Gupta, 2009). These compounds, however, are potential fermentation inhibitors.

Various methods for the hydrolysis of lignocellulosic materials have recently been described. The most commonly applied methods can be classified in two groups: chemical hydrolysis and enzymatic hydrolysis. Enzymatic hydrolysis is environmentally friendly but expensive. Besides,

the hydrolysis products (glucose and cellulose chains) inhibit the ability for enzymes to convert cellulose to glucose. As more product is formed, the enzymes become more inhibited by the excess glucose present. This ultimately slows down the hydrolysis process yielding low levels of usable hydrolysis product (Maurice, 2011). Among the chemical hydrolysis methods, acid hydrolysis is an easy and productive process. The rate of reaction is so fast which facilitates continuous processing (Zelelew, Gebrehiwot, & Fikre, 2018).

Hydrolysis by concentrated sulfuric or hydrochloric acids is a relatively old process. Concentrated acid processes are generally reported to give higher sugar yield e.g. 90 % of theoretical glucose yield and consequently higher ethanol yield, compared to dilute acid processes. Furthermore, the concentrated acid process can operate at low temperature (e.g. 40 ℃), which is a clear advantage compared to dilute acid processes. However, the concentration of acid is very high in this method e.g. 30-70 %, and dilution and heating of the concentrated acid during the hydrolysis process make it extremely corrosive. Therefore, the process requires either expensive alloys or specialized nonmetallic constructions, such as ceramic or carbon-brick lining. The acid recovery is an energy demanding process. In addition, when sulfuric acid is used, the neutralization process produces large amount of gypsum. Furthermore, the environmental impact strongly limits the application of hydrochloric acid. The high investment and maintenance costs have greatly reduced the potential commercial interest of this process (Taherzadeh, M. J., and Karimi, 2007).

2.1.5.3 Determination of sugar concentration of hydrolysates

Carbohydrates are quite heterogeneous, differing in primary structure (ring size and shape), degree of polymerization (mono vs. oligo vs. polysaccharides), macromolecular characteristics (linear structure vs. branched structure), linkage (i.e., α or β glyosidic linkage, linkage position), and charge. The physical and chemical differences give rise to disparate properties, including solubilities, reactivities, and susceptibility to digestive enzymes and chemicals. From an analytical perspective, the simplest situation is where there is only one type of carbohydrate present in a sample with minimal interfering compounds-measuring glucose oligomers in corn syrup, for example. In this case the disparate reactivities of sugars based on structure (e.g., ketose or hexose forms), charge, and type (e.g., glucose vs. arabinose) do not present a problem. It is the case most often though, especially with raw materials (e.g., seeds, cereal grains) and food products (ice cream, baked goods), that various types of carbohydrates are present in a sample with other

compounds including lipids, proteins, and minerals. The heterogeneity of this group of compounds can make analyzing the total carbohydrate content of a sample quite complex (Cui & Brummer, 2005).

When exact sugar concentration is needed, specific enzymatic assay methods are present. But these methods need sophisticated and costly equipment. So, simplified assay methods are available to determine approximate sugar concentration. The most applicable ones are phenol sulfuric acid and anthrone sulfuric acid method although phenol sulfuric acid is the most popular of these two. Because, the anthrone method is only applicable to solutions containing one hexose sugar (Cui & Brummer, 2005).

2.1.5.3.1 Phenol sulfuric acid assay

In the presence of strong acids and heat, carbohydrates undergo a series of reactions that leads to the formation of furan derivatives such as furfural and hydroxymethyl furfural. The initial reaction, a dehydration reaction is followed by the formation of furan derivatives, which then condense with themselves or phenolic compounds to produce dark colored complexes. The developed complex absorbs visible ultra-violate light, and the absorbance is proportional to the sugar concentration in a linear fashion. An absorbance maximum is observed at 490 nm for hexoses and 480 nm for pentoses as measured by a visible ultra-violate spectrophotometer (Basri, 2016; Cui & Brummer, 2005).

During the phenol sulfuric acid assay, phenol, in a 5 or 80% solution is added to a glass test tube containing a clear sample solution. Concentrated sulfuric acid is then added in a rapid stream directly to the surface of the liquid in the test tube. The mixture is thoroughly combined using a vortex mixer and then permitted to stand a sufficient time to allow for color development. The solution absorbance is read at 490 or 480 nm using a spectrophotometer, depending on the type of sugar present. Mixing and standing time should be kept the same for all samples to assure reproducible results. A calibration curve is constructed using the sugar being assayed. A stock aqueous sugar standard solution is used to prepare 5 or 6 standards. Each standard is subjected to the reaction procedure outlined above, transferred to a cuvette, and its absorbance is read at 480 or 490 nm. The absorbance of the blank should be subtracted from the absorbance of the standards manually, or the blank should be used to zero the spectrophotometer. A graph of absorbance vs.

concentration is constructed and the amount of analyte is derived from the calibration curve (Basri, 2016; Cui & Brummer, 2005).

2.1.5.4 Fermentation

The hydrolysate obtained from acid or enzymatic hydrolysis of lignocelluloses is then used for bioethanol fermentation by microorganisms such as yeast. Because such lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of bioethanol. The fermentation of ethanol is the biological process that converts fermentable sugars such as glucose and xylose to cellular energy with microorganisms which produce waste by-products ethanol and carbon dioxide anaerobically by the metabolic pathways of sugars (Udhayaraja & Narayanan, 2012). Below is the equation of alcoholic fermentation using yeasts.

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2 \qquad \Delta H = -74.986 \text{ kJ/mol} \qquad (2.1)$$

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \qquad \Delta H = -84.394 \text{ kJ/mol} \qquad (2.2)$$

2.1.5.4.1 Factors affecting fermentation of sugars.

The ethanol yield in fermentation process is affected by yeast variety, inoculum, sugar (glucose), temperature, pH, and inhibitory compounds present in the fermentation broth and time (Chacha, Dyrset, & Mtui, 2016). Yeasts as a group of micro-organisms has been quantitatively and commercially exploited as a fermentative species needed to carryout alcoholic fermentation and this has urged many scientists to study the factors governing its growth, survival and biological activities in different ecosystems. Among several yeasts Saccharomyces cerevisiae and S bayanus var. uvarum are the most important species present during fermentation process. Saccharomyces cerevisiae is most frequently and traditionally used microorganism for fermenting ethanol from starch-based residues at industrial scales. It has advantages such as its wide public acceptance, high fermentation rate and high ethanol tolerance that make it a good candidate for fermentation processes (Phutela & Kaur, 2014).

Temperature is also one of the parameters that is considered for the development of alcoholic fermentation because it affects both the kinetics of the process in terms of duration, rate of fermentation and the final quality of ethanol. The temperature determines how Saccharomyces

strains develop and how effectively they ferment. Some strains perform better at high temperatures and others at low temperatures. The temperature of fermentation can affect the development of different Saccharomyces strains. S.cerevisiae ferments best at temperatures around 30 ℃ (Maurice, 2011) . Any extreme of temperature during fermentation, either high or low, produces minimal concentrations of ethanol. This is partly because yeast does not grow well in temperatures much lower than 20 ℃ or much higher than 40 ℃ (Maurice, 2011). It was reported that temperature has a lesser effect on ethanol yield relative to pH and inoculum size (Chacha et al., 2016).

The pH of the growth medium is another important parameter for the successful progress of fermentation because it influences yeast growth as well as ethanol formation. It is reported the pH range of 4.8 - 5 (SumiGK, 2017) provides the conditions for Saccharomyces cerevisiae to be dominant species of the fermentation, and also for inhibition of wild yeasts (Maurice, 2011).

Yeast cells sense the amount and quality of external nutrients through multiple interconnected signaling networks, which allow them to adjust their metabolism, transcriptional profile and development program to adapt readily and appropriately to changing nutritional states. The highly interconnected signaling networks provide the cell with an environment that the cell can interpret the information to any nutritional condition. In a fermentative process, the variable used to establish the process kinetic has been the variation of biomass, variation of substrate content, product, oxygen consumed, heat evolved etc. Among these variables, biomass synthesis during the process and substrate consumption determines the fermentation It was reported that when the substrate concentration is increased beyond 25 % the effect of osmotic pressure becomes pronounced which seriously affects fermentation efficiency and leads to decreased ethanol production. High substrate concentrations have been shown to inhibit yeast growth and fermentation performance as a result of high osmotic pressure and low water activity (Panchal , G, 1980).

2.1.5.5 Separation

2.1.5.5.1 Evaporation

Evaporation is an operation used to remove a liquid from a solution, suspension, or emulsion by boiling off some of the liquid. It is thus a thermal separation, or thermal concentration, process. We define the evaporation process as one that starts with a liquid product and ends up with a more

concentrated, but still liquid and still pumpable concentrate as the main product from the process. There are actually a few instances where the evaporated, volatile component is the main product. In most cases it is essential that the product be subject to minimal thermal degradation during the evaporation process, requiring that temperature and time exposure must be minimized. Ethanol can be separated from a fermented product using an evaporator where the sample is subjected to heat up to the boiling point of ethanol (Mark, 2018).

2.1.5.5.2 Distillation

Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. This is because all materials have intermolecular interactions with each other, and two materials will co-distill during distillation. This means that proportion between two materials, in this case ethanol and water can be changed, and still, there are two materials in layers, the liquid and the vapor layer the liquid and the vapor layers (Onuki, 2005).

Whatever method of preparation is used, the ethanol is initially obtained in a mixture with water. The ethanol is then extracted from this solution by fractional distillation. Although the boiling point of ethanol, 78.3 ℃, is significantly lower than the boiling point of water, 100 ℃, these materials cannot be separated completely by distillation. Instead, an azeotrope mixture (i.e. a mixture of 95 % ethanol and 5 % water) is obtained, and the boiling point of the azeotrope is 78.15 ℃. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill from the distillation flask, and this material is the azeotrope of 95 % ethanol which has the lowest boiling point. If an efficient fractionating column is used, 95 % alcohol could be obtained first and then a small intermediate fraction of lower concentration, and then water. But no matter how efficient the fractionating column used, 95 % alcohol cannot be further concentrated by distillation because the vapor has exactly the same composition as the liquid; towards distillation, then, 95% alcohol behaves exactly like a pure compound.

2.1.5.5.3 Dehydration

After distillation, about 5 % of water remains in ethanol. Especially, this water is a big problem for fuel ethanol because the presence of this amount of water enhances the molecular polarity of ethanol when it is mixed with gasoline. Consequently, they separate into two phases, ethanol phase and gasoline phase. It is easy to imagine that this inhomogeneous fuel is not acceptable. Thus,

dehydration can be another issue (Onuki, 2005). For the ethanol to be usable as a fuel, water must be removed. Most of the water is removed by distillation, but the purity is limited to 95-96 % due to the formation of a low boiling water ethanol azeotrope. For blending with gasoline, purity of 99.5 to 99.9 % is required, depending on temperature, to avoid separation. Currently, the most widely used purification method is a physical absorption process using molecular sieves and another method is azeotropic distillation.

3 Materials and Methods

3.1 Materials

The following equipment and chemicals were used to conduct the research

Table 3-1 Major equipment used

Equipment used	Remark
Autoclaves LAMCS204 and LX-B50L	Sterilization, pretreatment, and hydrolysis
Electrical chamber furnace model VF2	Heating (ash content determination)
UV-VIS DOUBLE BEAM (UVD-3200)	Determination of sugar concentration
Oven	Drying
Plastic bag	Sample collection and packaging
Grinder type WRB80 C/2 q	Size reduction
Sieve type AS 200	Sieving/screening the sample
Analytical balance type EP214C	Weighing
pH meter (JENWAY 3505)	pH measurement and adjustment
Glove, eye glass and gown	Safety
Pyrex flasks of various volumes	To hold solutions
Graduated cylinders of different volumes	Volume measurement
Filter paper (quantitative)	Separation
Filtering crucible	Determination of insoluble lignin and hemicellulose
Vortex	Homogenization
Shaker incubator	Fermentation and inoculum preparation
Rotary evaporator	Purification of ethanol
DMA 4100 M (Density meter)	Determination of percentage volume of ethanol
Aluminum foils	Closing/covering flasks
Fourier transform infrared spectroscopy	Determination of functional groups of ethanol
Soxhlet extractor	Raw material characterization

Table 3-2 Chemicals used

Chemical used	Remark
Acetone	Extraction
95 %, and 98 % sulfuric acid (H_2SO_4)	Pretreatment, hydrolysis, determination of sugar concentration, and pH adjustment
Sodium hydroxide (NaOH)	pH adjustment, pretreatment, and hydrolysis
Phenol	Determination of sugar concentration
Distilled water	Washing and preparation of solutions
Yeast extract	Media preparation
Urea	
Potato dextrose broth	
Hydrous magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$)	

Sorghum straw was brought from a small village around Debirebrhan town, Ethiopia due to proximity of the area to the lab. Chemicals (sulfuric acid, sodium hydroxide, phenol, and acetone) were bought from Mollarie trading P.L.C., Cherkos sub city Addis Ababa, Ethiopia. While the chemicals used for media preparation (Yeast extract, Urea, Potato dextrose broth, and hydrous magnesium sulfate) were obtained in Biochemical Engineering lab, AAiT. The yeast Saccharomyces cerevisiae was used for the fermentation and it was brought from Ethiopian Biodiversity Institute.

3.2 Methods

3.2.1 Experimental frame work

The overall experimental tasks conducted are summarized in the figure below.

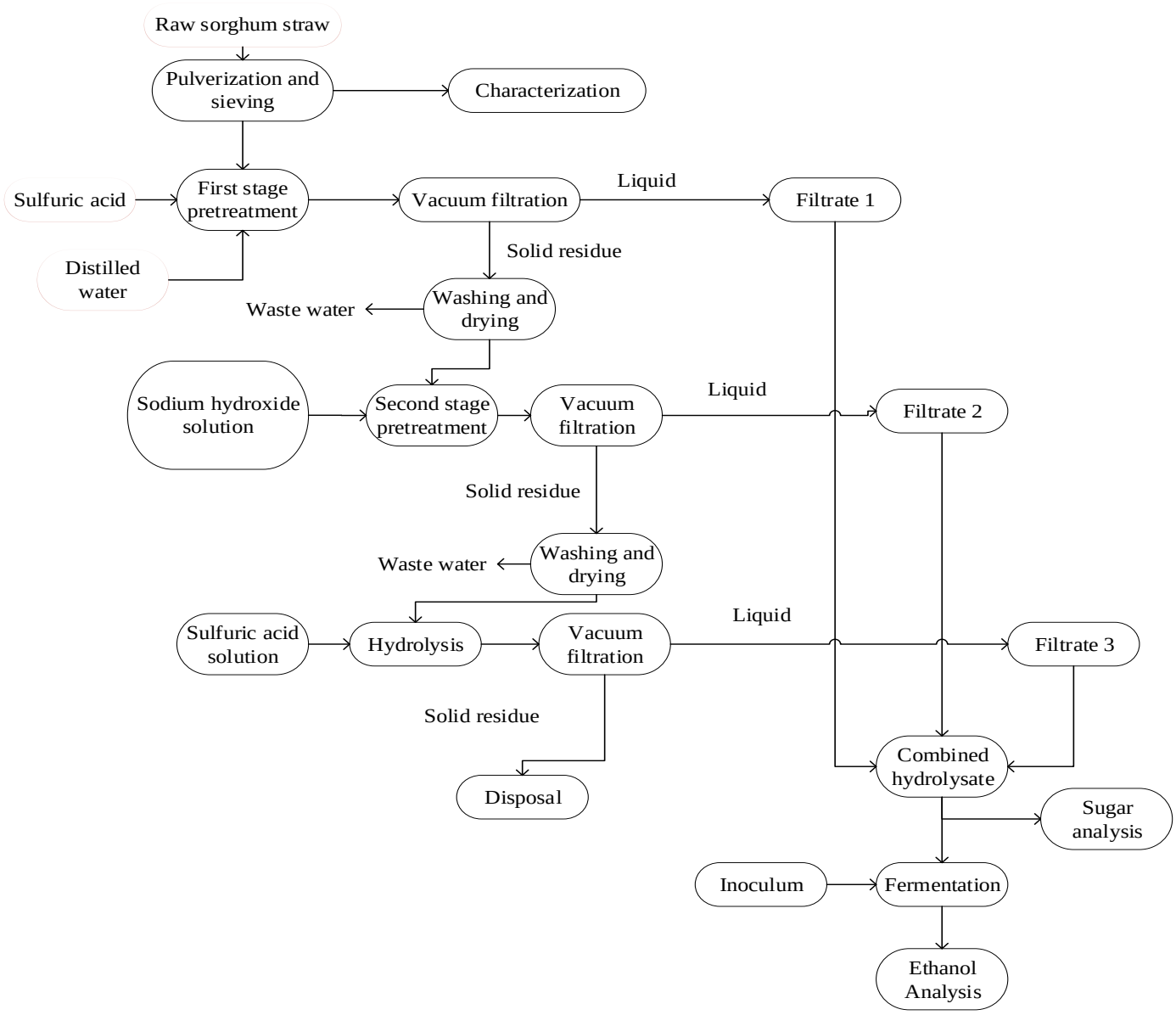

Figure 3-1 Overall experimental frame work

3.2.2 Sample collection and preparation

Three kilograms of sorghum straw was cut in to pieces using knife. The cut pieces were dried in sun for two days, ground with a grinder, sieved to pass 1 mm mesh according to NREL laboratory analytical procedure (Hames et al., 2008), and packed in plastic bag for further processing.

Figure 3-2 a) Cut sorghum straw b) Powder sorghum straw

3.2.3 Physio-chemical characterization of sorghum straw

Several experiments were conducted to assess the composition of the raw material. Moisture content, extractives content, and lignin were analyzed according to NREL laboratory analytical procedures (Sluiter, Hames, et al., 2008; Sluiter et al., 2012; Sluiter, Ruiz, et al., 2008).

3.2.3.1 Total solids and Moisture content determination

Three aluminum dishes were dried by placing them in a 105 ^{o}C drying oven for four hours. The dishes were cooled in a desiccator. The cooled dishes were then weighed to the nearest 0.1 mg. Two grams of sample was weighed in to each dish. The dishes along with the added samples were put into a convection oven at 105 ^{o}C for five hours, removed, and cooled to room temperature in a desiccator. The dishes containing the oven-dried samples were weighed to the nearest 0. 1mg.The samples were then placed back in to an oven and dried until constant weight (± 0.1% change in the weight percent solids upon one hour of re-heating the sample.

The total solids and moisture contents of the powdered sorghum straw were calculated using the following formulas.

$$\text{Total solids (\%)} = \frac{(\text{Weight}_{\text{dry dish plus sample}} - \text{Weight}_{\text{dry dish}}) \times 100\ \%}{\text{Weight}_{\text{wet sample}}} \quad (3.1)$$

$$\text{Moisture content (\%)} = 100 - \text{total solids (\%)} \quad (3.2)$$

3.2.3.2 Determination of ash and organic matter

Three crucibles were dried by placing them in a 550 ℃ furnace for four hours. The crucibles were then cooled in a desiccator. The cooled crucibles were then weighed to the nearest 0.1 mg. The previously oven dried samples were placed into each crucible and the combined weight of the crucible and sample was weighed carefully and placed into furnace at 550 ℃ for 20 hours. The heated sample containing crucibles were then removed from the furnace, cooled in a desiccator, and weighed. The samples were placed back to the furnace and pyrolyzed until constant weight.

The percentage ash and organic matter were calculated as follows,

$$\text{Ash content (\%)} = \frac{(\text{Weight}_{\text{ash plus crucible}} - \text{Weight}_{\text{dry crucible}}) \times 100\ \%}{\text{Weight}_{\text{oven dried}}} \quad (3.3)$$

$$\text{Organic matter (\%)} = 100 - \text{ash (\%)} \quad (3.4)$$

3.2.3.3 Determination of extractives

5 gram of dried sorghum straw powder was loaded into a cellulose thimble. 300 mL of acetone was used for extraction using a Soxhlet extractor with a residence time of 4 hours and temperature of 70 ^{o} C. After extraction, the sample was air dried at room temperature for few minutes. Constant weight of the extracted material was achieved in a convection oven at 70 ^{o} C. The % (w/w) of the extractives content based on sample total weight basis was evaluated as the difference in weight between the raw extractive-laden sorghum straw and extractive-free sorghum straw.

The extractives content was also determined on dry weight bases as follow,

$$\text{Extractives (\%)} = (\text{Weight of thimble plus extractives-weight of thimble}) * 100 / \text{ODW} \quad (3.5)$$

3.2.3.4 Determination of hemicellulose

2 grams of extracted dried sorghum straw was transferred into a 500 mL conical flask. 150 mL of 0.5 molarity NaOH was added. The mixture was boiled at 100 ℃ for 3.5 h in an aut^{oc}lave. It was filtered after cooling through vacuum filtration and washed until neutral pH. The residue was dried to a constant weight at 105 ℃ in a convection oven. The difference between the sample weight before and after this treatment is the hemicellulose content (% w/w) of dry sorghum straw (Ayeni, Adeeyo, Oresegun, & Oladimeji, 2015).

3.2.3.5 Determination of lignin

0.3 g of dried extracted sorghum straw was weighed in 250 conical flask and 6 mL of 72 % H_2SO4 was added. The sample was kept at room temperature for 2 hours with carefully shaking at 10 minutes interval to allow for complete hydrolysis. The sample was removed from the bath and the acid was diluted by adding 84 mL distilled water. Then, the volume of the 4 % solution was adjusted to 86.73 mL, as demonstrated in Appendix H.

Then. the flask was placed in to autoclave and heated for 1 hour at 121 ℃. The hydrolyzed solution was vacuum filtered through filtering crucibles. The residue was then dried in a convection oven until constant weight was achieved (for 5 hours). Finally, the crucible containing the residue was put in to furnace, removed, cooled in a desiccator, and weighed. The difference in weight between the residue and the ash remained is the acid insoluble lignin.

3.2.3.6 Determination of cellulose

The cellulose content (% w/w) was determined by difference, assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components of the entire biomass

3.2.4 Pretreatment of sorghum straw powder

3.2.4.1 Single stage pretreatment

In the present study, acid only and base only pretreatment were conducted to compare the sugar yield with the sequential one. During the acid only pretreatment 70 grams of sample was mixed with 700 mL distilled water and 1.25 % H_2SO_4. The solution was then heated in an autoclave at 125 ℃ for 20 minutes, and filtered using vacuum filtration.

During the alkaline only pretreatment, 70 grams of sample was mixed with 1.5 % NaOH solution in a ratio of 1 to 10. The prepared solution was put in to autoclave for pretreatment at 90 ℃ for 20

minutes, cooled, and filtered. The residues in both treatments were washed twice to remove trace of acid and base, dried, and packed for hydrolysis.

3.2.4.2 Multi-stage pretreatment

Two subsequent pretreatment methods namely dilute Sulfuric acid and dilute sodium hydroxide treatment were performed aiming to get a better sugar yield. The treatment conditions used by Weerasai et al.(2014) to produce ethanol from rice straw were adopted for the present study's raw material. Several identical samples were prepared for the purpose of handling the experiment with the available equipment. Sample was mixed with distilled water in a ratio of 1:10 (g/mL), then pretreatment was carried out sequentially with both methods. During the dilute acid pretreatment, 100 mL dilute sulfuric acid solution of 1 % concentration was added to a solution prepared by mixing 70 grams of powder sorghum straw with 700 mL of distilled water. The solution was treated inside an autoclave and heated at temperature of 125 ℃ for 10 minutes.

Samples that had been pretreated as stated above were cooled and filtered. Each of the filtrate liquids were held in separate flasks and placed in a refrigerator for preservation while the residues were washed twice to remove the acid, dried in an oven, weighed, and packed in polyethylene bag for alkaline treatment.

During the alkaline pretreatment, 1.25 % sodium hydroxide was mixed with the dried solid residue in a ratio of 10: 1 (mL/g) and pretreated in an autoclave for 10 minutes at 90 ℃. The pretreated sample was filtered using vacuum filtration and the filtrates were mixed with previous filtrates and placed in a refrigerator for fermentation and other analysis while the residues were washed twice to remove the base, dried in an oven, weighed, and packed in polyethylene bag for hydrolysis treatment.

3.2.5 Hydrolysis

Dilute sulfuric acid was used for the purpose of hydrolysis. For the purpose of obtaining different sugar concentrations and compare single and sequential treatment, temperature, time, and acid concentration were varied in the hydrolysis experiment. The variables were varied standing from the results of Mebrhit (2016) who obtained an optimum sugar yield from corn cob with 3 % acid concentration, 124.3 ℃ and 30 minutes (Gebremariam, 2016).

The combination of the parameters was done using Box-Behnken design as follows. Acid concentration, temperature, and time were coded as A, B, and C respectively. Diluted H_2SO_4 concentration of 2%, 3% and 4% (v/v), temperature (110 ℃, 125 ℃ and 140 ℃) and time (20, 40 and 60) minutes were used. 17 experiments were done by varying the three hydrolysis parameters.

Table 3-3 Randomization of hydrolysis parameters

No	Acid concentration A (%)	Temperature B (℃)	Time C (min)	Sugar level
1	3	125	40	
2	2	125	60	
3	3	125	40	
4	3	140	60	
5	3	125	40	
6	2	140	60	
7	3	125	40	
8	3	110	60	
9	3	125	40	
10	4	125	20	
11	3	110	20	
12	4	125	60	
13	4	140	40	
14	2	125	20	
15	3	140	20	
16	4	110	40	
17	2	110	40	

3.2.6 Determination of total carbohydrates

The total carbohydrates of all the hydrolysates was determined using phenol sulfuric acid method. The method here was adopted from Cui (2005) and Basri and Brummer (2016) with little modification.

3.2.6.1 Preparation of reagents

5 % phenol solution was prepared by weighing 5 grams of phenol and diluting it with 100 mL distilled water while the 96 % sulfuric acid was prepared by mixing 97.96 mL of 98 % sulfuric acid and 2.04 mL distilled water.

3.2.6.2 Standard curve preparation

The glucose stock solution was prepared in such a way that a 3 gram of glucose (dextrose) was added in to 500 mL volumetric flask containing distilled water. The mix was stirred and its volume was adjusted to 300 mL by adding distilled water. The final concentration of the stock solution was then adjusted to 10 g/L and it was diluted to make 6 standard solutions. Table 3-3 shows how the standard solutions were prepared.

Table 3-4 Preparation of standard solutions

Dilutions	volume of solution (mL)	volume of distilled water	volume of stock that was taken (mL)	Concentration (g/L)
1	10	10	0	0
2	10	9	1	1
3	10	7	3	3
4	10	5	5	5
5	10	3	7	7
6	10	1	9	9

4 mL aliquot of each dilutions was taken and pipetted in to separate test tubes. 1 mL of 5 % phenol was added to the test tube containing the sample. After that, 5 mL of 96 % sulfuric acid was added to the mix and vortexed to keep uniformity. The mixture was kept at room temperature for 10 minutes and vortexed again. The mix was then placed in a water bath at 90 ℃ for 15 minutes. The test tubes were removed from water bath, cooled, and vortexed. Some amount of the mix from

each test tube was taken to clear cuvettes and the absorbance was read at 490 nm. The standard curve was then produced by plotting the absorbance versus concentration data.

3.2.6.3 Samples

4 mL aliquot of each of the 17 hydrolysates was taken and pipetted in to separate test tubes. 1 mL of 5 % phenol was added to the test tube containing the sample. After that, 5 mL of 96 % sulfuric acid was added to the mix and vortexed to keep uniformity. The mixture was kept at room temperature for 10 minutes and vortexed again. The mix was then placed in a water bath at 90 ℃ for 15 minutes. The test tubes were removed from water bath, cooled, and vortexed. Some amount of the mix from each test tube was taken to clear cuvettes and the absorbance was read at 490 nm.

3.2.7 Fermentation

The ethanol yield in fermentation process is affected by inoculum, sugar (glucose), temperature, nutrients, pH, and inhibitory compounds present in the fermentation broth and time. It was observed that pH and inoculum have a significant influence on bioethanol yield than temperature range. It was also shown that the yield was highly sensitive to a small change in pH (Chacha et al., 2016). It was obtained that ethanol production was increased with increasing sugar concentration and declined thereafter at high concentration (Adelabu et al., 2018). Standing from the results, three hydrolysates based on their sugar content (high level, medium level, and minimum level) were selected after determining the sugar concentration of all 17 hydrolysates for fermentation. The present study investigated the effects of levels of sugar, pH, and inoculum concentration. In addition, the pH range was made close standing from the previous research results.

Figure 3-3 Selected hydrolysates for fermentation

3.2.7.1 Media preparation

Before the actual fermentation process media was prepared. At the glance, a culture of saccharomyces cerevisiae was brought from Ethiopian Biodiversity institute with a 1.5 mL preservative.

The method by Alula and Mebrahtom (2014) was adopted to prepare a media for saccharomyces cerevisiae (Alula Gebregergs and Mebrahtom Gebresemati, 2014). The media was prepared by mixing 400 mL distilled water, 40-gram potato dextrose broth, 1-gram yeast extract, 4-gram urea, and 4-gram hydrous Magnesium sulphate in 500 mL conical flask. Then, the pH of the media was adjusted using 4 molar of sodium hydroxide to 5, closed tightly with aluminum foil, and sterilized in an autoclave at 121 ℃ for 15 minutes. Finally, the media was taken out of the autoclave and cooled.

3.2.7.2 Inoculation

The brought culture was added to the cooled medium in a laminar air hood (safety cabinet). Then it was closed using cotton material to allow entry of small amount of air which is used for the propagation of the yeast. Finally, the inoculated medium was incubated in a shaker incubator for 24 hours at 30 ℃.

3.2.7.3 Experimental design for fermentation

As stated above, 100 mL of sugar solution was taken from each of the three chosen hydrolysates for fermentation. Sugar level, inoculum size, and pH were varied according to the following the design.

Table 3-5 Randomization of fermentation parameters

No	Sugar level(g)	Inoculum size (% v/v)	pH	Yield (mL of ethanol per g of sugar)
1	3.042	5	5	
2	3.29875	5	5	
3	3.042	15	5	
4	3.29875	15	5	
5	3.042	10	4.5	
6	3.29875	10	4.5	
7	3.042	10	5.5	
8	3.29875	10	5.5	
9	3.179	5	4.5	
10	3.179	15	4.5	
11	3.179	5	5.5	
12	3.1792	15	5.5	
13	3.1792	10	5	
14	3.1792	10	5	
15	3.179	10	5	
16	3.1792	10	5	
17	3.1792	10	5	

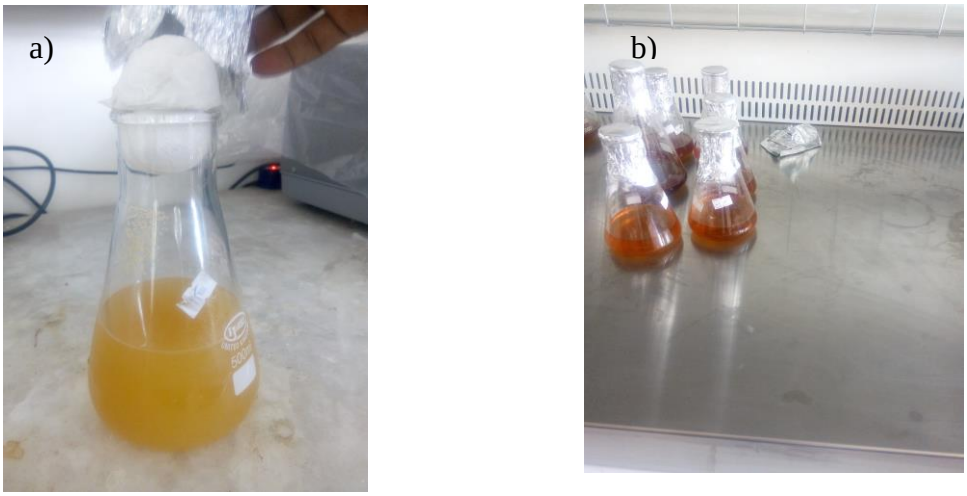

Figure 3-4 a) Inoculated medium b) Samples ready for fermentation

3.2.8 Centrifugation and evaporation

Fermented samples were centrifuged for 20 minutes with 6000 rpm. Then the centrifuged samples were evaporated in a rotary evaporator at 83 °C for two and half hours.

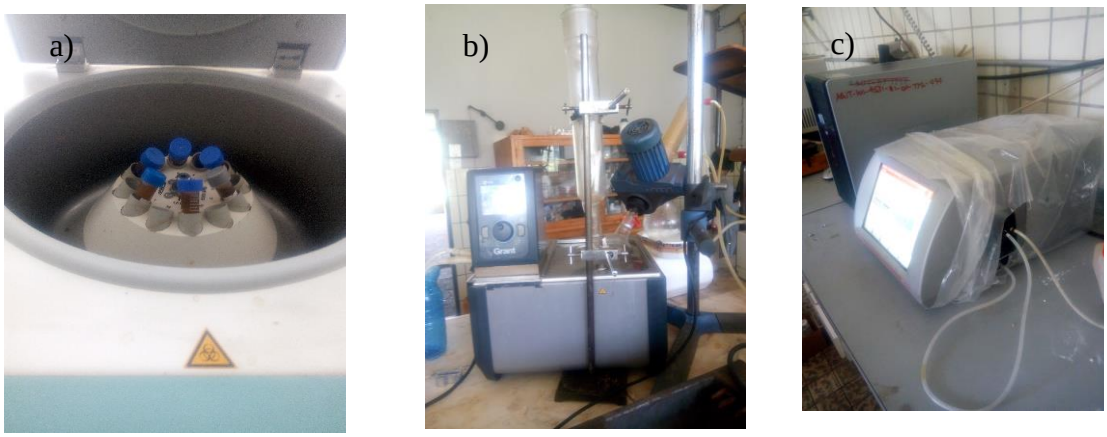

Figure 3-5 a) Centrifugation b) Rotary evaporator c) DMA 4100 M (digital density meter)

3.2.9 Product characterization

3.2.9.1 Determination of yield of ethanol

The volume percentage of ethanol was determined using digital density meter. A sample was injected to the equipment and the volume percentage of each sample was recorded. The yield (mL of ethanol per gram of sugar) was then determined as follows.

Actual volume of ethanol = % v/v of ethanol * total volume of ethanol after evaporation

Ethanol yield = Actual volume of ethanol/sugar level

% yield of ethanol = ((Actual volume of ethanol* density of pure ethanol) /sugar level)) *100

3.2.9.2 Characterization of ethanol using FTIR

The sample that contained high ethanol percentage during the volume percentage measurement was tasted for its functional group after further purification. The analysis was done in Addis pharmaceutical factory S.C. A drop of the sample was put in to the sample holder, potassium bromide, and the percentage transmittance versus wave number were recorded and the spectrum was plotted.

4 Results and discussion

4.1 Characterization of sorghum straw

The moisture, ash, organic matter, and total solids contents of the sorghum straw were obtained and the results are in Table 4-1.

Table 4-1 Proximate analysis of sorghum straw

Physical composition	Weight percentage (dry basis)
Total solids	92.428
Moisture	7.572
Organic matter	98.75
Ash	1.25

These results are relatively similar to the finding of Mutepe in 2012. A moisture content of 91.8 % and 2.9 % of ash were obtained (Mutepe, 2012).

Similarly, the extractives, lignin, Hemicellulose, and cellulose contents of the sorghum straw on dry basis were obtained as given in Table 4-2.

Table 4-2 Chemical composition of sorghum straw

Component	% on dry basis
Extractives	13.4
Hemicellulose	30.3
Lignin	16.41
Cellulose	38.6

The high extractives content is attributed to the presence of sucrose in the inner part of the straw (Phutela & Kaur, 2014). The above results are relatively different from earlier studies by sikgor & Becer (2015) who obtained the compositions of sorghum straw as 32 -35 % cellulose, 24-27 % hemicellulose, and 15-21 % lignin and (Mutepe, 2012) who found the cellulose, hemicellulose, and lignin contents of sweet sorghum bagasse to be 28.53, 19.07, and 3.93 respectivelly. The small

difference comes from the type of cultivars of sorghum (Batista, Pimentel, Dias, Teófilo, & Barros, 2018).

4.2 Total carbohydrates

The total carbohydrate content of the hydrolysates produced through sequential acid-base pretreatment and subsequent hydrolysis at different acid concentration, time, and temperature was determined using phenol sulfuric acid method. In addition, the sugar concentration of hydrolysates produced using single acid and single base pretreatment and subsequent hydrolysis was also determined. The standard solutions and corresponding absorbances are tabulated below in Table 4-2. The standard curve prepared by fitting the concentration vs absorbance linearly is also shown in Figure 4-2.

Table 4-3 Standard solutions and corresponding absorbances

Concentration of standard solution (g/L)	Absorbance
1	0.096
3	0.274
5	0.414
7	0.718
9	0.836

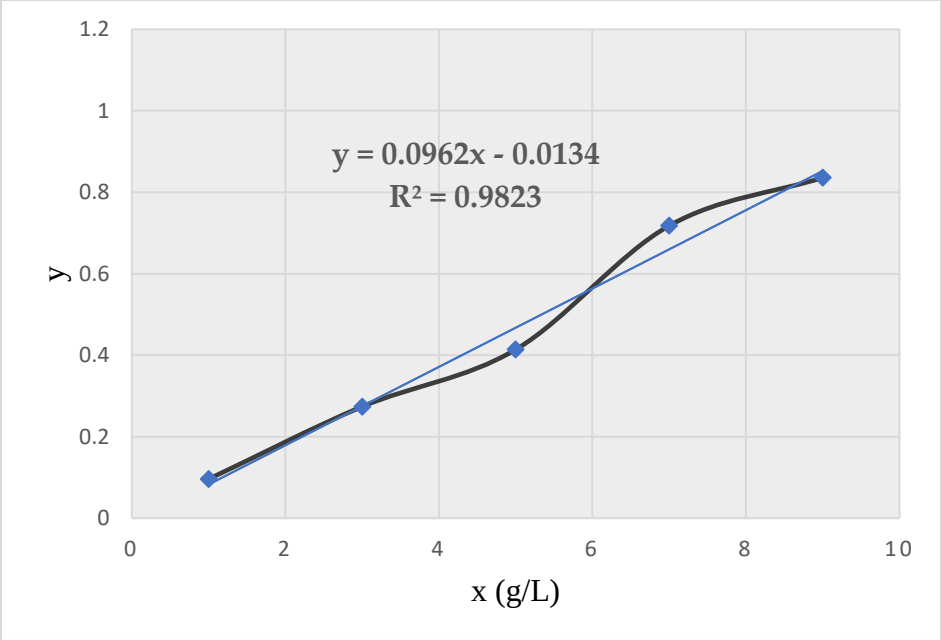

Figure 4-1 Standard glucose curve

The concentrations of the samples were calculated from the equation of the standard curve. x = (y + 0.0134) / 0.00962. Where, x and y represent concentration and absorbance respectively.

Table 4-4 Sugar concentration of hydrolysates from sequential acid-base pretreatment

Code	acid concentration %	Temperature ℃	Time (min.)	absorbance	concentration (g/L)
A_H	3	125	40	3.069	30.98129
B_H	3	125	40	3.063	31.97921
C_H	3	125	40	3.114	32.50936
D_H	3	125	40	3.103	32.39501
E_H	3	125	40	3.12	32.57173
F_H	2	110	40	3.154	32.92516
G_H	4	110	40	3.16	32.98753
H_H	2	140	40	2.967	31.07484
I_H	4	140	40	2.913	30.41996
J_H	2	125	20	3.041	31.75052
K_H	4	125	20	2.935	30.64865
L_H	2	125	60	3.145	31.72973
M_H	4	125	60	3.045	31.7921
N_H	3	110	20	3.105	32.4158
O_H	3	140	20	2.932	30.61746
P_H	3	110	60	3.146	32.842
Q_H	3	140	60	3.069	32.04158

As shown in the above table the maximum, minimum, and middle sugar levels of 32.98753, 30.41996, and 31.7921 g/L respectively were selected for fermentation. It is shown that the maximum sugar level was obtained at the conditions of acid concentration, temperature, and time of 4%, 110 ℃, and 40 minutes, respectively. The sugar concentrations of the hydrolysates are cloth i.e. there is no big difference between them. This is because the identical treatment conditions employed during the sequential acidic-alkaline pretreatment stage.

Previous researches on sweet sorghum bagasse showed a glucose concentration of 0.68 g/g (Mutepe, 2012) while the present study obtained 0.424 g/g . The difference could be due to the difference in sorghum variety and the efficiency and sophistication of the equipment employed.

From Table 4.4 and the statistical analysis of the hydrolysis experiment, sugar concentration was increased with increasing time, and decreased with increasing acid concentration and temperature. The decrease in sugar concentration with increasing acid concentration and temperature is due to degradation of monomeric sugars (xylose, glucose) to furfural and HMF or may be derived from dehydrating or oxidizing by sulfuric acid on glucose or it could be attributed from the conversion of glucose to levulinic and formic acid which leads to decrease in glucose yield (Zelelew et al., 2018). These substances are toxic substances for yeast and can inhibit the yeast growth. The observed results are in agreement with previous result by Demis et al (2018) who reported that a higher sugar yield (55.8 %) after hydrolysis with distilled water only, and Mutepe (2012) who reported that sugar yield from sorghum bagasse was increased with increasing time.

Table 4-5 Concentration of single stage pretreated sorghum straw hydrolysates

Hydrolysate type	Absorbance	Concentration (g/L)
Alkali pretreated	2.536	26.50104
Acid pretreated	2.536	27.61331

After determining the sugar concentration of hydrolysates obtained with hydrolysis parameters (temperature = 110 ℃, Acid concentration = 4 % and time= 40 minutes), sequential pretreatment gave a better sugar yield than the single stage acidic and alkaline This is because the sequential pretreatment provides efficient removal of lignin and efficient degradation of hemicellulose to monomeric sugars (Guo, 2012).

4.3 Characterization of ethanol

4.3.1 Ethanol yield

The yield of all 17 runs was expressed in terms of mL ethanol per g glucose and the results are tabulated in table 4-6.

Table 4-6 Ethanol yield

Glucose equivalent (g)	Inoculum (v/v %)	pH	Yield mL of ethanol per g of glucose equivalent	Ethanol purity (%)	Percentage yield
3.042	5	5	0.4041	49.17	31.883
3.29875	5	5	0.512	48.23	40.375
3.042	15	5	0.482	41.93	38.023
3.29875	15	5	0.617	63.66	48.724
3.042	10	4.5	0.394	36.29	31.061
3.29875	10	4.5	0.5913	33.63	46.653
3.042	10	5.5	0.376	45.77	29.678
3.29875	10	5.5	0.415	48.99	32.809
3.179	5	4.5	0.424	42.13	33.458
3.179	15	4.5	0.527	54.03	33.874
3.179	5	5.5	0.358	42.15	28.243
3.179	15	5.5	0.449	46.08	35.451
3.179	10	5	0.5449	48.12	42.992
3.179	10	5	0.562	47.01	44.333
3.179	10	5	0.576	42.35	45.194
3.179	10	5	0.564	41.73	45.532
3.179	10	5	0.582	44.05	45.915

From Table 4-6, a maximum of 0.48742 g of ethanol per g of glucose was obtained. The result is in agreement with the yield obtained in previous researches. Mutepe (2012) obtained an ethanol

yield of 0.48 g. g^{-1} of sugar from sweet sorghum juice fermentation. and 0.45 g. g^{-1} of sugar from sweet sorghum bagasse fermentation with Saccharomyces Cerevisiae.

4.3.2 FTIR spectrum

Alcohols have a characteristic infrared spectrum associated with O-H, C-O and C-H stretching vibrations. When run as a liquid film, a region with a very intense and broad band at 3500 - 3200 cm^{-1} indicate the O-H stretch of alcohols, while the region 1260 - 1050 cm^{-1} confirms the C-O stretch of alcohols (William R et al., 2019). 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 (Radu, Ă, Penu, & Litescu, 2012). Figure 4-2 shows FTIR Spectrum analysis of ethanol from sorghum straw. The O-H, C-H, and C-O stretch looks like the same as standard ethanol (see Appendix D).

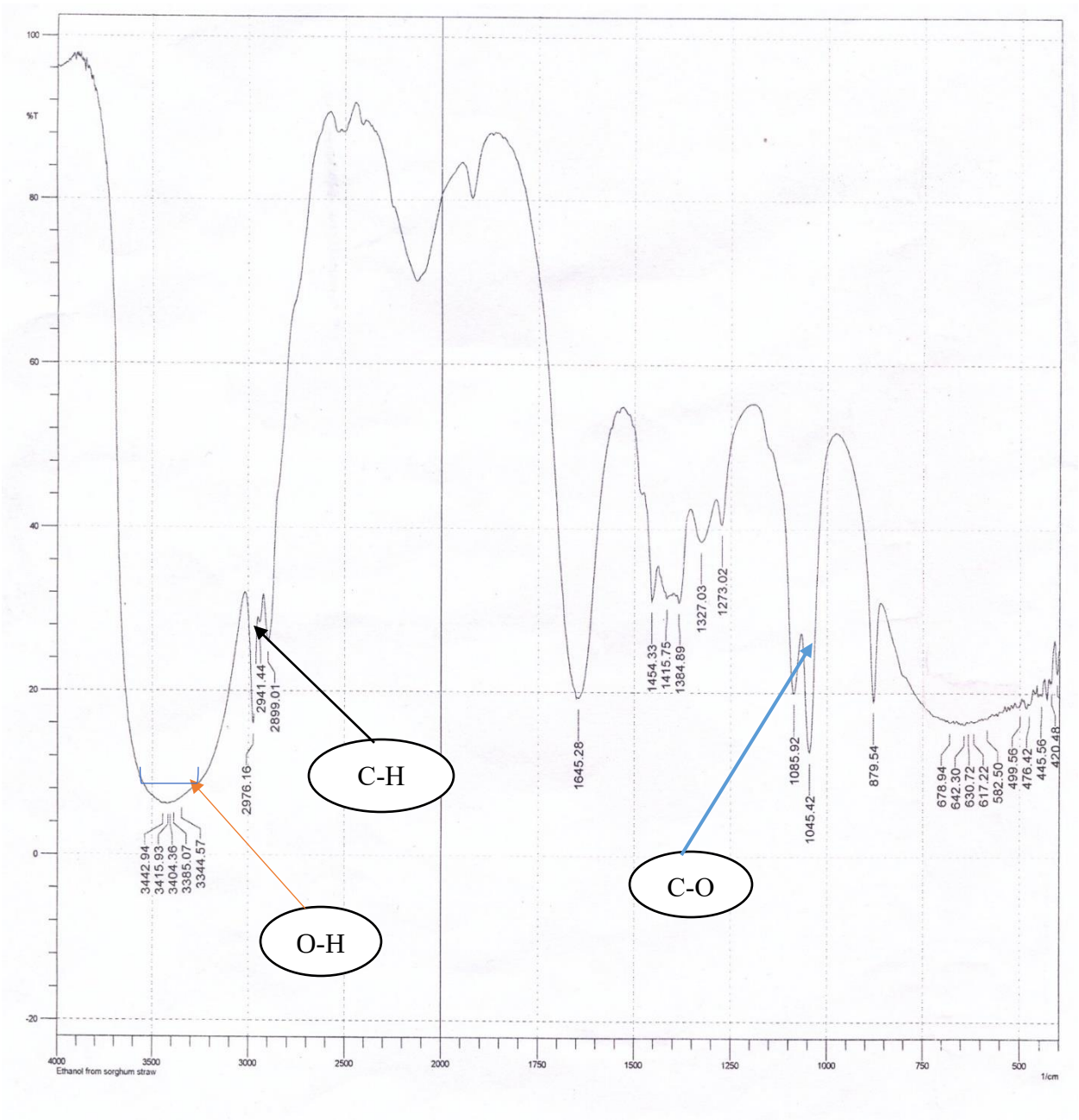

Figure 4-2 FTIR spectrum of the produced ethanol

4.4 Statistical Analysis of Experimental results

4.4.1 Design summary

Design expert software version 11.1.0.1 using Box-Behnken design was employed for analysis of variance in which the ethanol yield was taken as a response while pH, sugar level, and inoculum size were the independent variables. The design summary is shown in the table 4-7. The analysis of variance in which sugar level was taken as a response while acid concentration, temperature, and time were the independent variables during the hydrolysis experiment was also done (see appendix F).

Table 4-7 Design summary

File Version	11.1.0.1		
Study Type	Response Surface	Subtype	Randomized
Design Type	Box-Behnken	Runs	17
Design Model	Quadratic	Build time (minutes)	1

4.4.2 Analysis of variance (ANOVA)

The results of analysis of variance are shown in table 4-8.

Table 4-8 Analysis of variance

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.1152	9	0.0128	77.39	< 0.0001	Significant
A-Sugar level	0.0287	1	0.0287	173.50	< 0.0001	
B-inoculum size	0.0178	1	0.0178	107.33	< 0.0001	
C-pH	0.0143	1	0.0143	86.47	< 0.0001	
AB	0.0002	1	0.0002	1.11	0.3271	
AC	0.0063	1	0.0063	37.87	0.0005	
BC	0.0000	1	0.0000	0.2176	0.6550	
A^2	0.0035	1	0.0035	20.99	0.0025	
B^2	0.0047	1	0.0047	28.20	0.0011	
C^2	0.0364	1	0.0364	220.07	< 0.0001	
Residual	0.0012	7	0.0002			
Lack of Fit	0.0003	3	0.0001	0.5475	0.6759	Not significant
Pure Error	0.0008	4	0.0002			
Cor Total	0.1164	16				

The Model F-value of 77.39 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicates that model terms are significant. In this case, A, B, C, AC, A^2, B^2, C^2 are significant model terms.

The Lack of Fit F-value of 0.55 implies the Lack of Fit is not significant relative to the pure error. There is a 67.59% chance that a Lack of Fit F-value of this large could occur due to noise.

4.4.3 Model adequacy measures

Table 4-9 Model adequacy measures

Std. Dev.	0.0129	R^2	0.99
Mean	0.4928	Adjusted R^2	0.9773
C.V. %	2.61	Predicted R^2	0.9426
		Adequacy Precision	26.8083

The Predicted R^2 of 0.9426 is in reasonable agreement with the Adjusted R^2 of 0.9773; i.e. the difference is less than 0.2 which shows that the model represents the actual data. Adequacy Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 26.808 indicates an adequate signal. This model can be used to navigate the design space. The value of coefficient of determination is also close to 1 which indicates the model represents the actual data.

4.4.4 The regression Model

The model equations, which relates the response yield of ethanol to the independent variables in terms of the coded and actual factors are given below. The yield (ml of ethanol per g of glucose) is represented by Y_{PS} for brevity.

Final equation in terms of the coded factors is shown below.

$$Y_{PS} = 0.0599\ A + 0.0471\ B - 0.0423\ C + 0.0068\ AB - 0.0396\ AC - 0.003\ BC - 0.0287\ A^2 - 0.0333\ B^2 - 0.093\ C^2 + 0.5658 \quad (4.1)$$

The final equation in terms of the actual factors was represented as follows,

$$Y_{PS} = 1.144913\ \text{sugar level} + 0.008592\ \text{inoculum size} + 5.60168\ \text{pH} + 0.001055\ \text{sugar level} * \text{inoculum size} - 0.061654\ \text{sugar level} * \text{pH} - 0.0012\ \text{inoculum size} * \text{pH} - 0.017423\ \text{sugar level}^2 - 0.001332 \quad (4.2)$$

4.4.5 Graphical analysis

The adequacy of model describing the variation of the ethanol yield as function of the independent variable was also be checked using graphical analysis of residuals. The model adequacy was checked using the following 3 plots.

4.4.5.1 Normal probability plot

The normal probability plot is a graphical method used to check whether the error occurred during the experimental analysis is normally distributed throughout the experiment. The normality assumption of analysis of variance is valid if all the data points lie along the straight line of normality pot. The normal probability plot of residuals versus internally studentized residuals is shown in Figure 4.3. The figure shows that the data points are approximately located along the straight line. This indicates that the quadratic polynomial model developed satisfies the normality assumption of analysis of variance. This means error distribution is throughput the experiment is approximately normal.

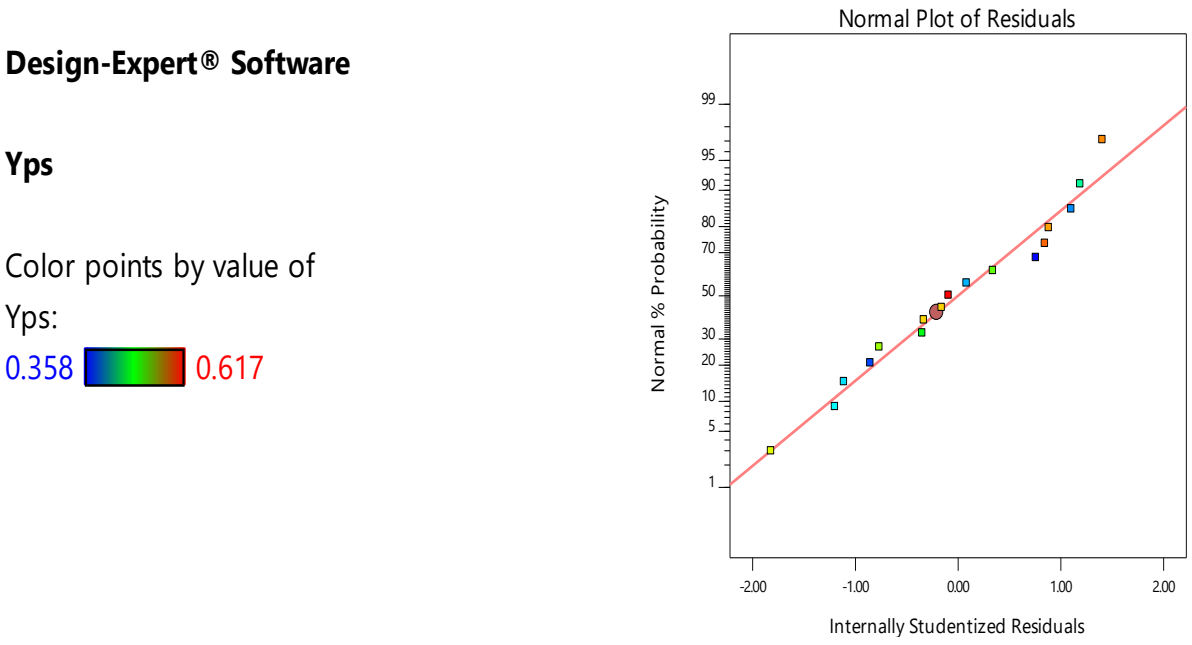

Figure 4-3 Normal plot of residuals

4.4.5.2 Actual versus Predicted plot

The plot of actual value versus predicted value is another graphical method that can be used to determine the adequacy of the model. A model is considered to be perfect if the slope of the line is unity (all data points are perfectly along the line) which corresponds zero error. The graph of the predicted value versus actual value of this study is shown in Figure 4-4. The graph tells that all data points are approximately close the line of perfect fit. This indicates that the actual data are closely related to the data predicted from the model.

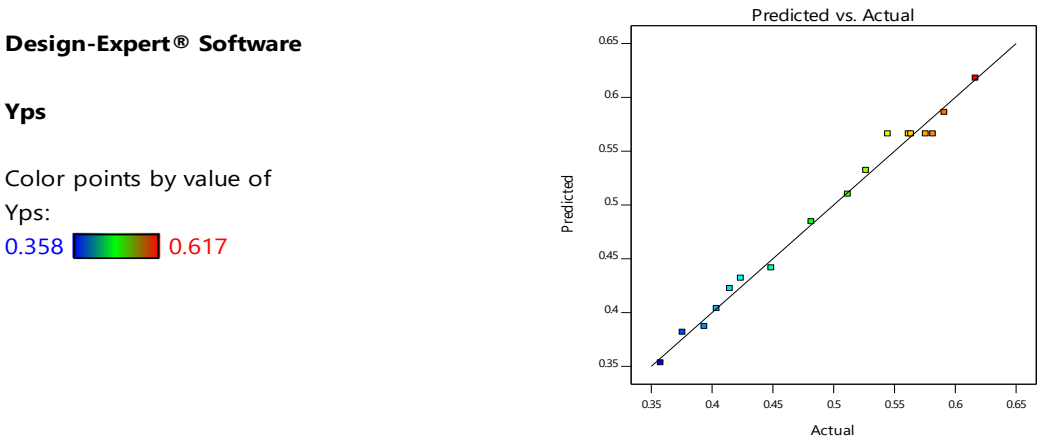

Figure 4-4 actual data vs predicted data value

4.4.5.3 Residual verse Predicted Plot

The plot of the residuals versus predicted values is another graphical technique used to assess the validity of the model. The model developed to describe the process is considered to be perfect if the residuals are structureless. This graph is used to assess non-linearity, unequal error variances, and outliers. The residual value versus predicted value plot of the current study shown in figure 4-5. From the graph, it was observed that the residual values are not uniquely structured. That means there is no need of an improvement for minimizing personal error.

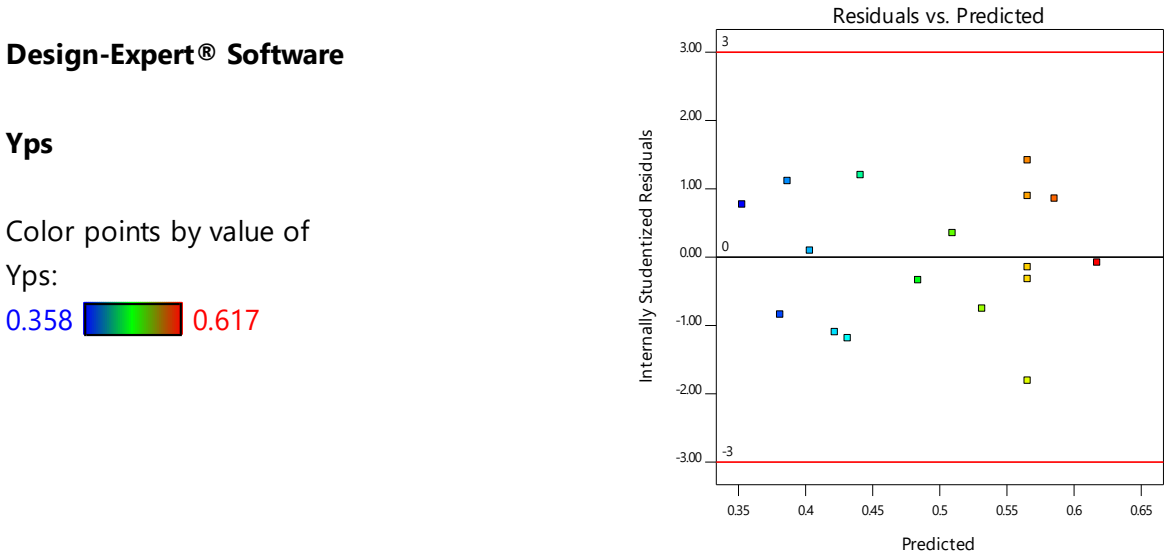

Figure 4-5 Residuals vs predicted value diagnostic plot

4.4.6 Factors affecting the yield of ethanol

Observing the analysis of variance, yield of ethanol was highly affected by all the three factors (sugar level, pH, and inoculum size). Besides, it was affected by the interaction between sugar level and pH although it is less significant than the individual factors.

4.4.6.1 Effect of single factors

4.4.6.1.1 Effect of sugar concentration

From the regression model equation, the coefficient of A and A^2 are positive and negative respectively. These numbers show that yield is increased at the glance with increasing sugar concentration and decreases eventually at very high sugar level. The effect of sugar level on ethanol yield when the inoculum size and pH are held 13.4 % and 5.25 is shown in figure 4.6.

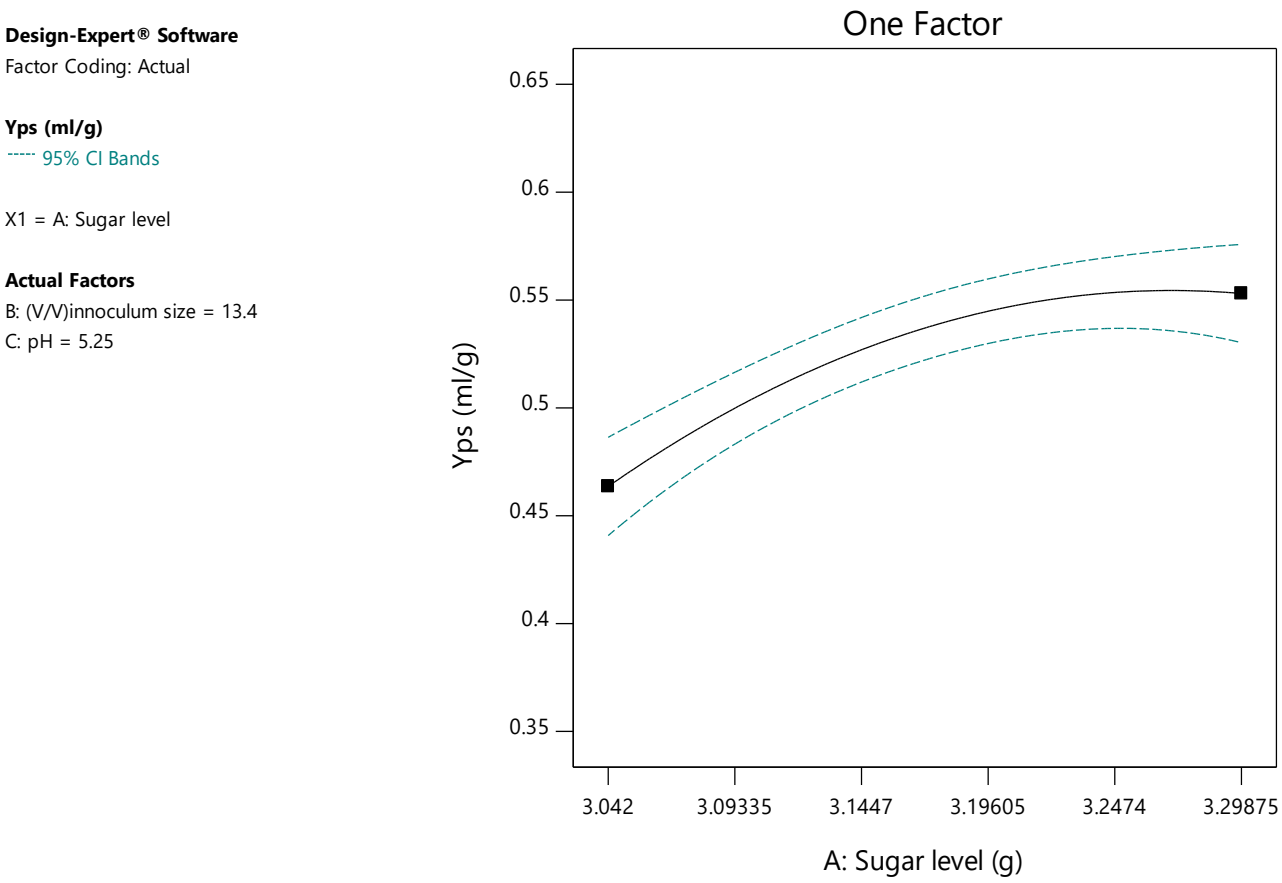

Figure 4-6 Effect of sugar level on ethanol yield

The ethanol yield at low sugar level is too low. This is because of the low concentration of fermentable sugars (Mutepe, 2012). It is observed that the ethanol yield at sugar level of 3.2613 is 0.554696 mL/g which is higher than the sugar yield at sugar level of 3.29875 (0.553016 mL/g). This is because of the negative effect caused by osmotic pressure at higher sugar concentration (Mary Anupama.P, 2010). These results are in agreement with that of Adelabu et al.(2018). Ethanol productions at different substrate concentration of sorghum straw by two yeasts were observed. The ethanol yield increased gradually with the use of 5 % to 7.5 % and thereafter declined (Adelabu et al., 2018).

4.4.6.1.2 Effect of inoculum

The probability value of less than 0.0001 in case of the inoculum size shows that yield is greatly affected by it. From the regression model, the coefficients of B and B^2 are 0.0471 and – 0.0333 respectively indicates that yield of ethanol is increased with increasing inoculum size until it

reaches maximum yield and eventually decreases at high values of inoculum size. Figure 4-7 shows the effect of inoculum size on ethanol yield when sugar yield and pH are held constant at 4.5 and 3.29875 g respectively.

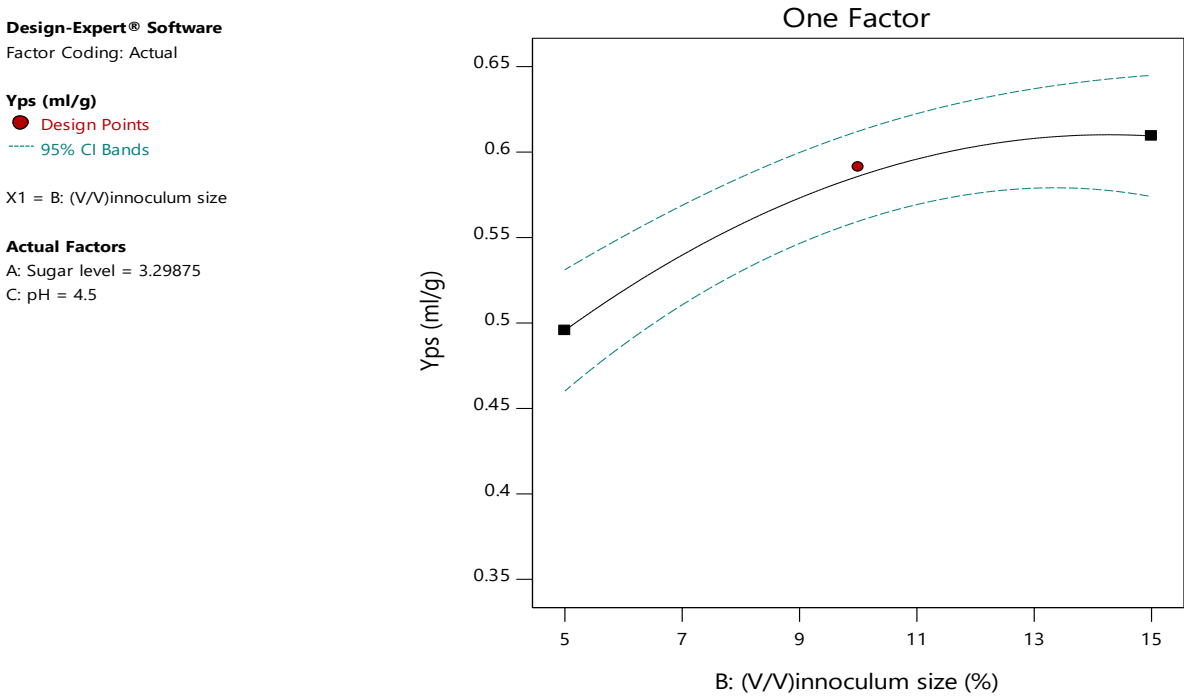

Figure 4-7 Effect of inoculum size on ethanol yield

The results show that the ethanol yield increased with increasing inoculum size and decline at very high inoculum size. These results coincide with previous studies by giving maximum ethanol yield at some point optimum which is neither too low nor too high. Elhussieny, 2014 showed that there was observed higher ethanol yield at 2.5 mL inoculum size than 0.5 and 3 mL (Elhussieny, 2014) while Adelabu et eal, 2018 showed that highest volume of ethanol was produced with 7.5 % inoculum concentration, followed by 5 % inoculum concentration while 15 % produced the least bioethano l(Adelabu et al., 2018). Lower ethanol production at lower inoculum size is probably due to the less cells which are insufficient to use the fermentation medium for yeast optimal activity, while the decreased yield at higher inoculum size might probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (Adelabu et al., 2018).

4.4.6.1.3 Effect of pH on ethanol yield

The ethanol yield was maximum at pH 5 followed by pH 4.5 and 5.5. Yield was sharply increased at the glance and reaches its maximum point at the middle. Then it goes decreasing with increasing pH. The plot where sugar level and inoculum size were held constant at 3.042 g and 15 % respectively is shown below in graph 4-8.

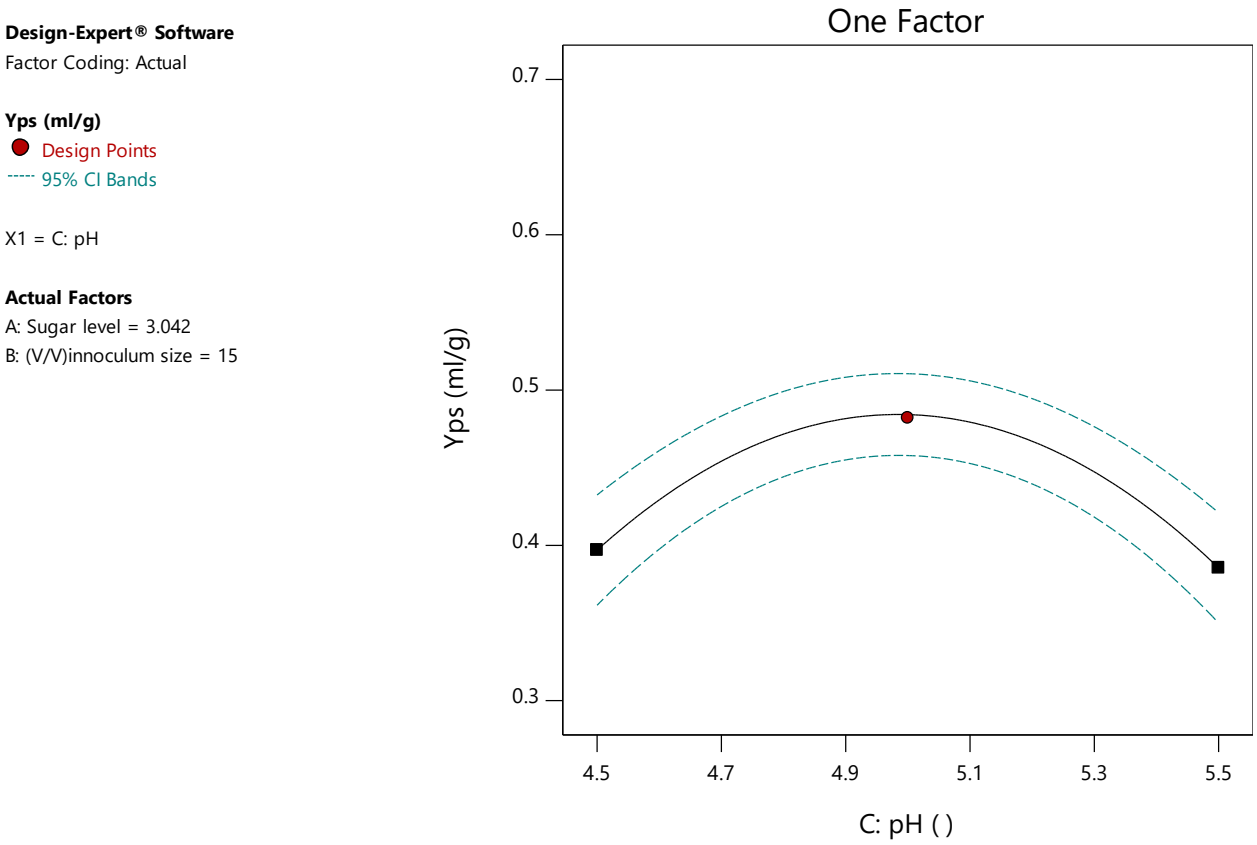

Figure 4-8 Effect of pH on ethanol yield

As observed from the graph, the ethanol yield is high at pH around 5 and relatively low yield was observed at pH 4.5 and 5.5. This is because the optimum pH of saccharomyces cerevisiae is in between 4.8 and 5 (SumiGK, 2017).

4.4.6.2 Interaction effects

From the analysis of variance, only the interaction between sugar level and pH significantly affected the yield of ethanol. The effect of the interaction between sugar level and pH on ethanol yield was analyzed and the results were plotted using both interaction and 3D.

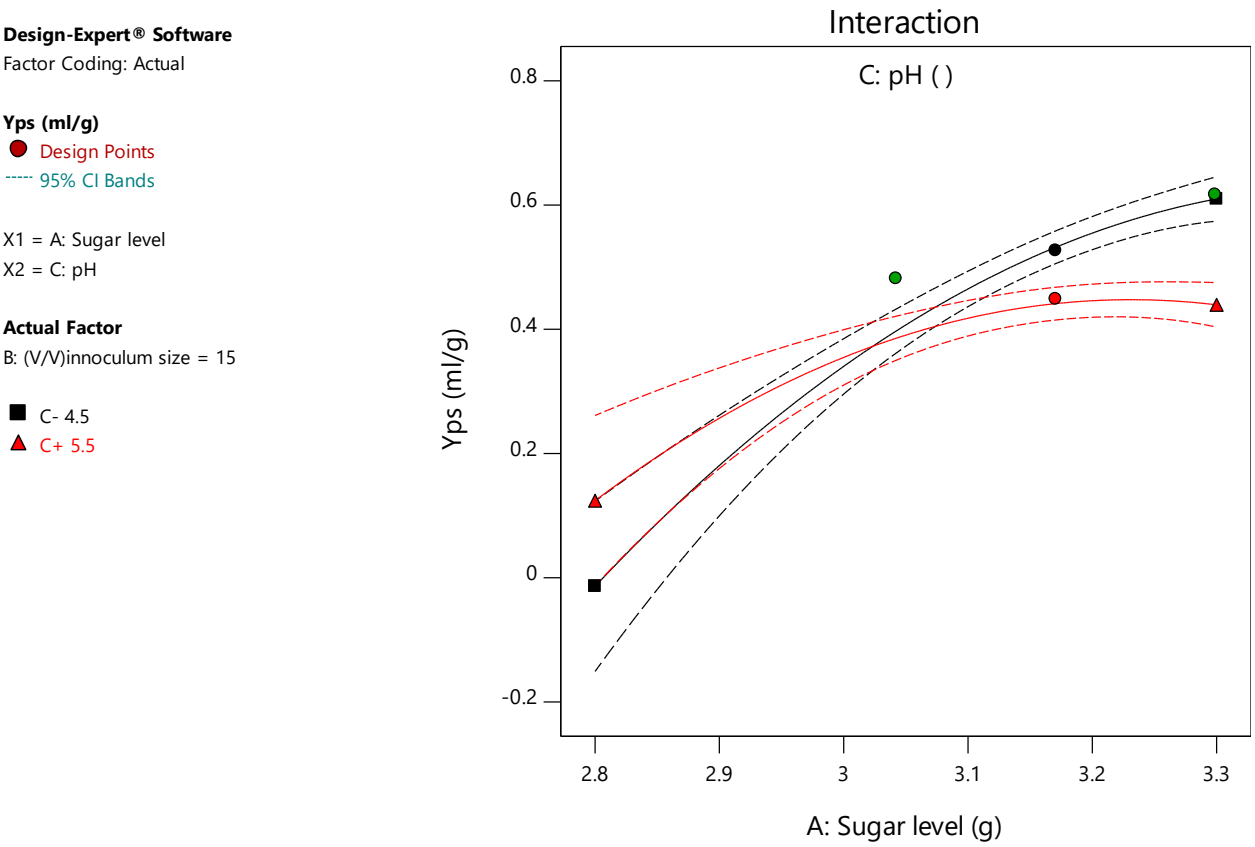

Figure 4-9 Interaction plot of pH and sugar level on ethanol yield

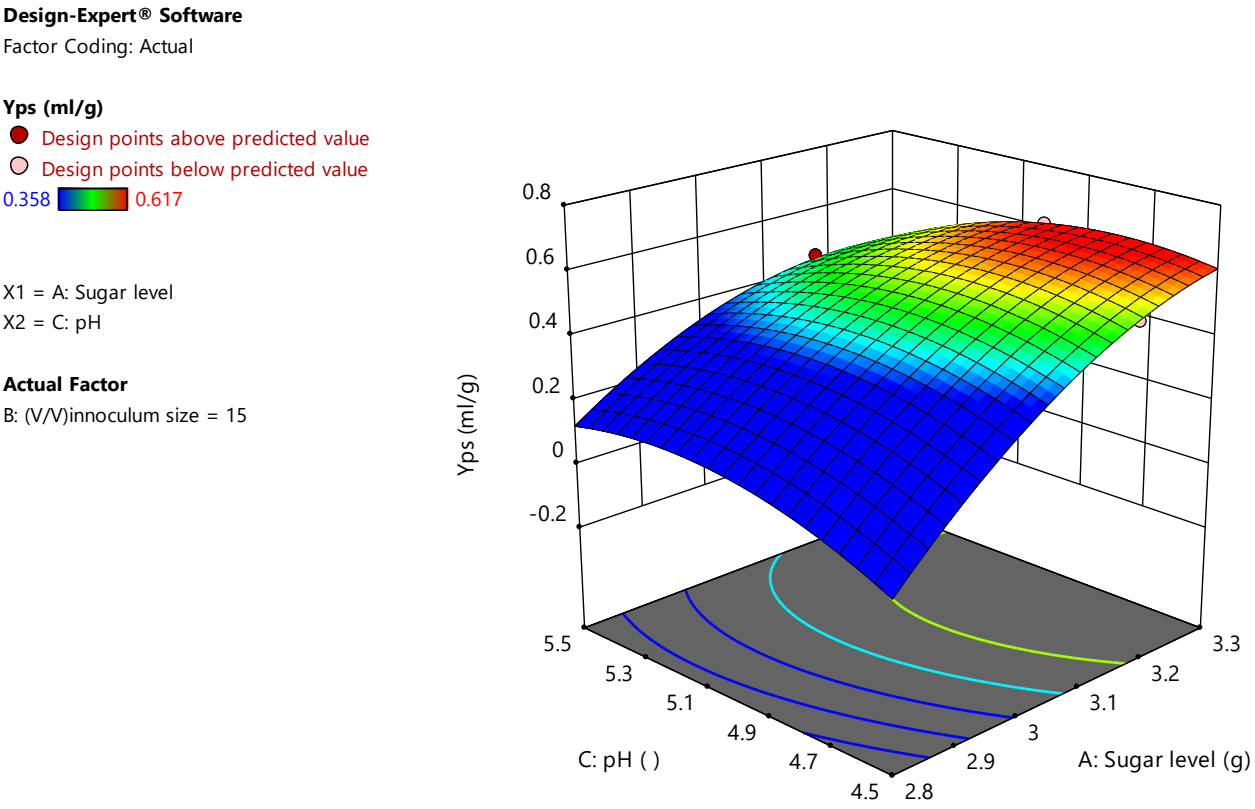

Figure 4-10 3D plot of the pH and sugar level interaction

As observed from the above plots, the ethanol yield increases gradually with increasing the concentration of sugar and pH at constant inoculum size of 15 until the yield reaches maximum at pH around 5.1. Further increase in pH leads to decrease in the yield of ethanol with increasing sugar level. So, the interaction between high sugar concentration and high pH leads to decrease in ethanol yield. This is because of inhibitory effect of the alkaline sugar on the activity of the saccharomyces cerevisiae which works favorably between pH 4 and 5.

5 Conclusions and recommendations

5.1 Conclusions

Sugar level, inoculum size, and pH affect the yield of ethanol during fermentation. In this study, these three variables were varied from 3.042 – 3.29875 g, 5 % - 15 %, and 4.5 - 5.5 respectively and the results showed that pH, inoculum size, and sugar concentration affect ethanol yield significantly. The maximum ethanol yield of 0.617 mL/g was obtained at pH 5, sugar concentration of 3.29875 g, and 15 % inoculum size.

The yield of sugar in hydrolysates is affected by the type of pretreatment and pretreatment parameters. Sugar yield of hydrolysates was determined using phenol sulfuric acid method and the sequential acidic- alkaline pretreatment method gave a good sugar yield compared to the single acidic and alkaline pretreatments.

The FTIR spectrum of the product indicated the presence of O-H, C-H, and C-O stretches similar to the standard ethanol's FTIR spectrum. This confirmed that product obtained is ethanol. On other hand, the compositional analysis of the sorghum straw gave high amount of cellulose and hemicellulose relative to lignin. This shows that sorghum straw is a good source of sugar for ethanol production.

In conclusion, controlling fermentation parameters during the production of ethanol from sequential acidic-alkaline pretreated sorghum straw is a good choice in view of increasing yields of sugar and ethanol, decreasing dose and cost of chemicals, and minimizing waste generation.

5.2 Recommendations

Based on the outcomes of this research work and overall understanding of the production process of ethanol, the following recommendations are suggested.

- Saccharomyces cerevisiae doesn't ferment pentoses. So, future researches on optimization of fermentation parameters on bioethanol production from sequentially pretreated sorghum straw using other microorganisms shall be studied. Candida species, genetically engineered E coli, and genetically engineered saccharomyces cerevisiae can solve this problem.
- The present study investigated one variety of sorghum straw from Debrebrhan area, Ethiopia. Investigating the effects of fermentation parameters on ethanol production from different sorghum straw varieties for ethanol production is recommended.
- Detoxification of the hydrolysates shall be done before proceeding to fermentation to minimize inhibitors which potentially affect the ethanol yield.

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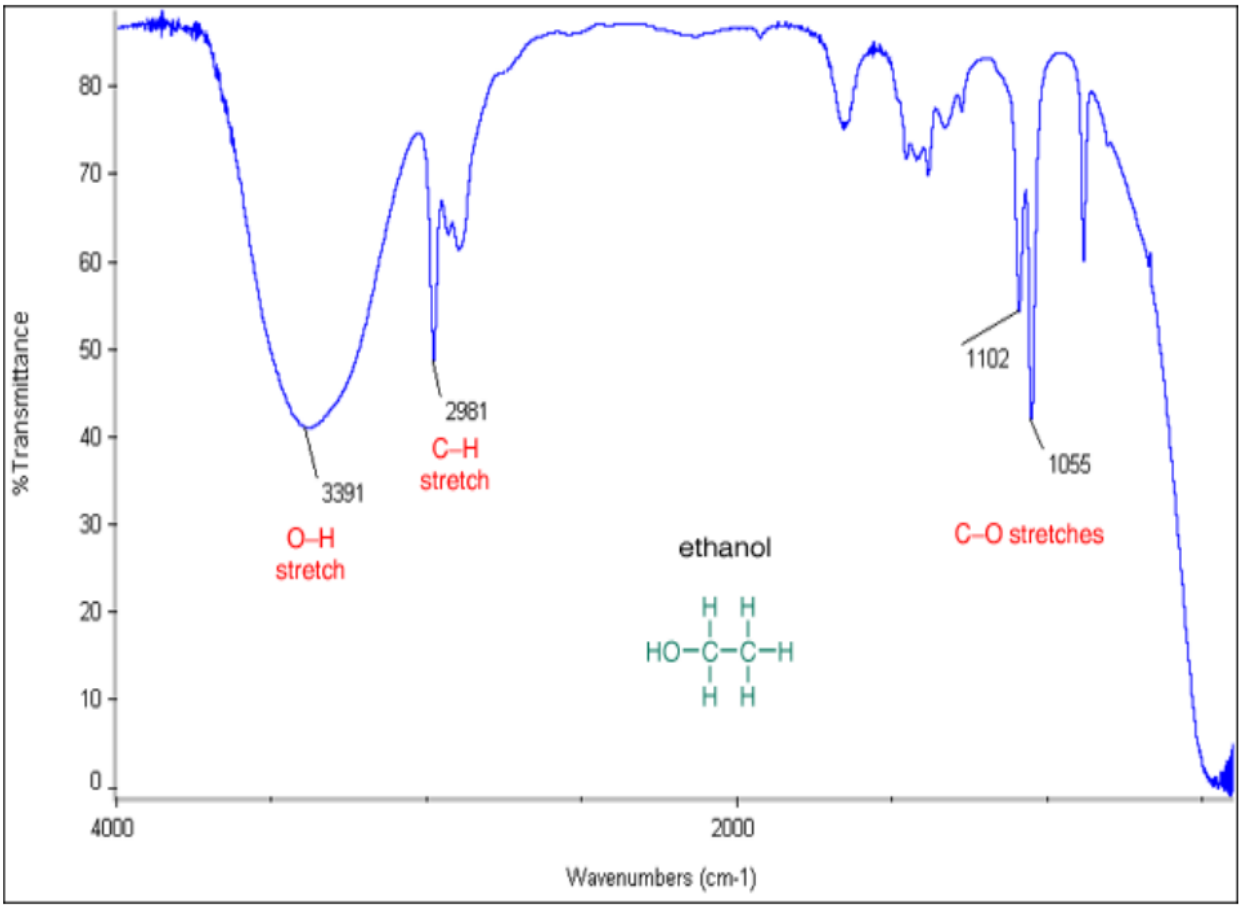
Appendices

Appendix A: Dilute acid pretreated samples

Appendix B: Dilute alkali pretreated samples

Appendix C: Hydrolyzed samples

Appendix D: Infrared spectrum of standard ethanol



Source: (William et al., 2019)

Appendix E: Properties of ethanol

Molecular formula	$CH_3CH_2O_5$
Appearance	Colorless
Boiling point	78.4 ^{O}C
Density and phase at room temperature	0.789g/cm^3, liquid respectively
Solubility	Fully soluble in water
Viscosity at 20 ^{O}C	1.2 cp

Appendix F: Statistical analysis of hydrolysis parameters

ANOVA for Linear model

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.0888	3	0.0296	20.53	< 0.0001	significant
A-A	0.0081	1	0.0081	5.59	0.0422	
B-B	0.0650	1	0.0650	45.07	< 0.0001	
C-C	0.0158	1	0.0158	10.93	0.0306	
Residual	0.0187	13	0.0014			
Lack of Fit	0.0160	9	0.0018	2.59	0.1584	not significant
Pure Error	0.0027	4	0.0007			
Cor Total	0.1075	16				

The Model F-value of 20.53 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 2.59 implies the Lack of Fit is not significant relative to the pure error. There is a 18.62% chance that a Lack of Fit F-value this large could occur due to noise.

Fit Statistics

Std. Dev.	0.0380	R^2	0.8257
Mean	3.06	Adjusted R^2	0.7855
C.V. %	1.24	Predicted R^2	0.7137
		Adequacy Precision	13.9074

The Predicted R^2 of 0.7137 is in reasonable agreement with the Adjusted R^2 of 0.7855; i.e. the difference is less than 0.2. Adequacy Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case the ratio of 13.9074 indicates an adequate signal. This model can be used to navigate the design space.

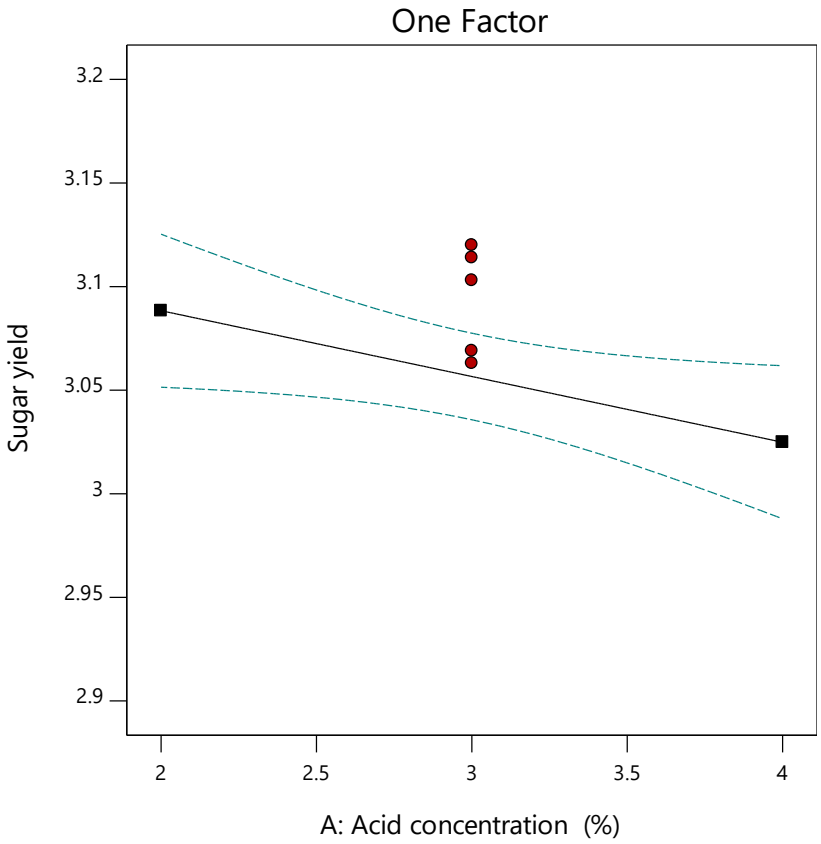
Effect of acid concentration

Design-Expert® Software
Factor Coding: Actual

Sugar yield
Design Points
95% CI Bands

X1 = A: Acid concentration

Actual Factors
B: Temperature = 125
C: Time = 40



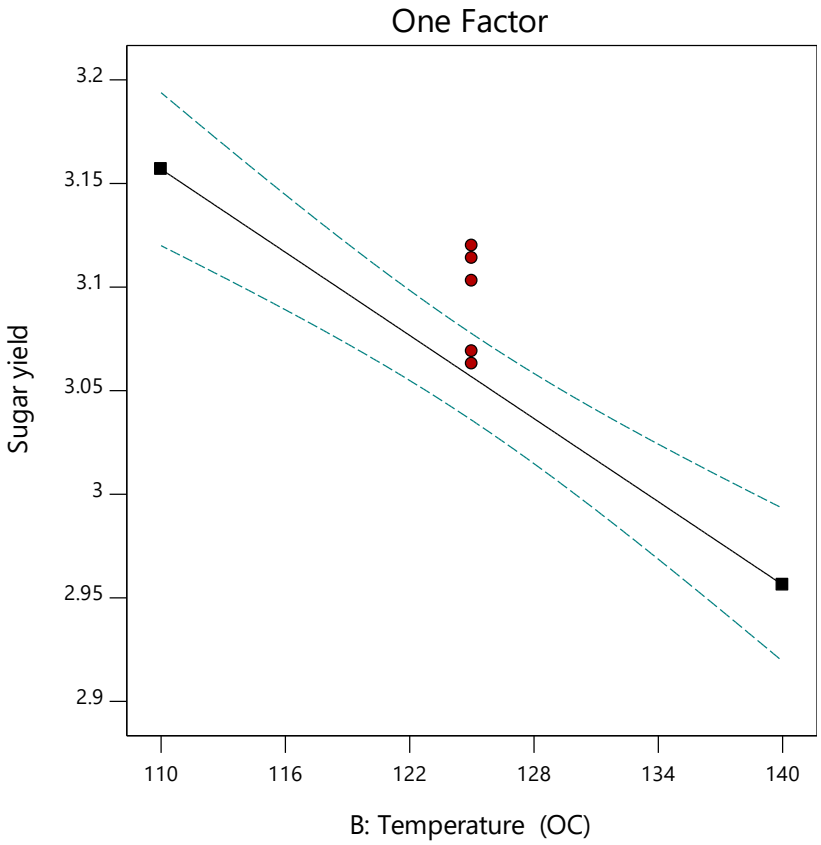
Effect of temperature

Design-Expert® Software
Factor Coding: Actual

Sugar yield
Design Points
95% CI Bands

X1 = B: Temperature

Actual Factors
A: Acid concentration = 3
C: Time = 40



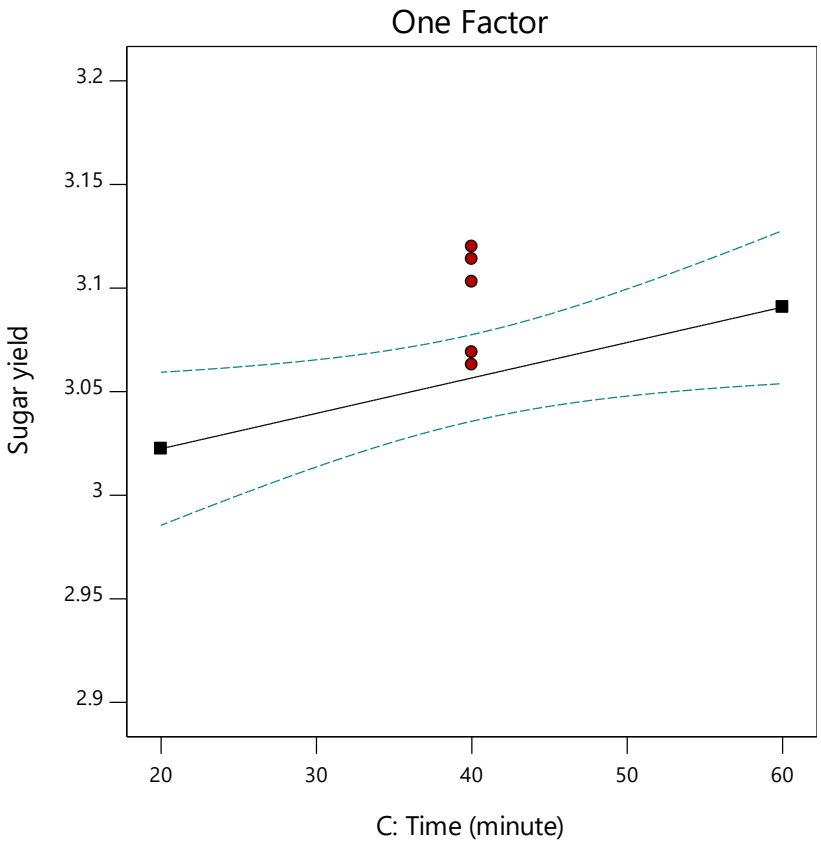
Effect of time

Design-Expert® Software
Factor Coding: Actual

Sugar yield
Design Points
95% CI Bands

X1 = C: Time

Actual Factors
A: Acid concentration = 3
B: Temperature = 125



Appendix G: Soxhlet extraction set up

Appendix H: Adjustment of the volume of acid used in lignin determination

The volume of 4 % sulfuric acid used during the determination of lignin was adjusted as demonstrated in the following calculations.

- ✓ Density 72 % H_2SO_4= d 72 % H_2SO_4 = 1.6338 g/mL
- ✓ Density H_2O= d H_2O= 1.00 g/mL
- ✓ Density 4 % H_2SO_4 = d 4 % H_2SO_4= 1.025 g/mL
- ✓ The weight of 3.00 mL 72 % H_2SO_4 = 3.00 mL 72 % H_2SO_4 x d72 % H_2SO4= 4.90 g 72% H_2SO_4
- ✓ The composition of 3 mL of 72 % H_2SO_4 = 4.90 g 72% H_2SO_4 x 72 % (acid weight) = 3.53 g acid
- ✓ 4.90 g 72% H_2SO_4 x 28 % (water weight) = 1.37 g water
- ✓ The concentration of H_2SO_4 after dilution = 3.53 g acid / (84.00 g H_2O + 4.90 g 72 % H_2SO_4) = 3.97 % H_2SO_4 (w/w)
- ✓ The total volume of solution present after dilution: (4.90 g H_2SO_4 + 84.00 g H_2O) / (d4 % H_2SO_4) = 86.73 mL