Bioethanol Production from Brewer's Spent Grain through simultaneous saccharification and Fermentation process

Melese Nigusu Bogale

A Thesis Submitted to the Department of Applied Chemistry

College of Applied Natural Science

Presented in Partial Fulfilment of the Requirement for the Master of Science in Applied Chemistry (Industrial Chemistry)

Office of Graduate Studies

Adama Science and Technology University

March , 2026
Adama, Ethiopia

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DECLARATION

I hereby declare this MSc research thesis entitled **"Bioethanol Production from Brewer's Spent Grain through simultaneous saccharification and Fermentation process"** is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

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RECOMMENDATION OF ADVISOR

I, the advisor(s) of this thesis, hereby certify that I have read the revised version of the thesis entitled **"Bioethanol Production from Brewer's Spent Grain through simultaneous saccharification and Fermentation process"** prepared under my guidance by Melese Nigusu submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Chemistry (Industrial Chemistry). Therefore, I recommend the submission of a revised version of the thesis to the department following the applicable procedures.

Eshetu Bekele (PhD) ______________________

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I, the advisor of the thesis entitled **"Bioethanol Production from Brewer's Spent Grain through simultaneous saccharification and Fermentation process"** and developed by Melese Nigusu, hereby certifies that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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ESHETU BEKELE (PhD)		____________

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We, the undersigned, members of the Board of Examiners of the thesis by Melese Nigusu Bogale have read and evaluated the thesis entitled "I, the advisor of the thesis entitled **"Bioethanol Production from Brewer's Spent Grain through simultaneous saccharification and Fermentation process"** and developed by Melese Nigusu, hereby certifies that the recommendation and suggestions made by the Board of Examiners are appropriately incorporated into the final version of the thesis." and examined the candidate during open defence This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of master of science in Applied Chemistry (Industrial Chemistry).

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and College Graduate Committee (CGC).

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LIST OF ABBREVIATION

BSG ………………………………………Brewer's Spent Grains

GHG ……………………………………Greenhouse gases

SSF ………………………………………Simultaneous Saccharification and Fermentation process

XRD………………………………………X-ray Diffraction

DSC………………………………………..Differential Scanning Calorimetry

SEM………………………………………Scanning Electron Microscopy

UV-Vis ……………………………………Ultravaulate visible spectroscopy

H_2SO_4 ……………………………………Sulpheric Acid

NaOH……………………………………..Sodium Hydroxide

HCl ………………………………………Hydrochloric Acid

ANOVA …………………………………Analysis of Variance

FTIR ……………………………………..Fourier Transforms Infrared

SHF………………………………………Hydrolysis and Fermentation

RPM..Revolution Per Minute

EFB……………………………………… Empty Fruit Bunch

ABSTRACT

Increasing bioethanol production from biomass fermentation has gained worldwide attention to reduce fossil fuel depletion, rising oil prices, and environmental impacts. This study investigates production of bioethanol from Brewery Spent Grain using simultaneous Saccharification and fermentation process (SSF)".for the Produce of bioethanol from brewery spent grain using different methods for this research the BSG sample was pretreatment using torrifaction process at different temperature and time. After the torrifaction process the three components (cellulose, hemicellulose and lignin) was determine. Than the treated BSG sample was first stage NaOH followed by acid (H_2SO_4) hydrolysis pre-treatment was done. The selected torified sample was at temperatures of 120^0c and 220^0c for 30 minute and 60 minute for comparisons purpose. The 4% NaOH used to for lignin and hemicellulose removal, structural disruption and enhanced fermentable sugars and the acid was used to for hemicellulose hydrolysis, enhanced cellulose access and increased glucose yield. The second stage hydrolysis process using SSF. In this step both saccharification and fermentation process was done simultaneous using shaker incubator for three days the sample to yeast ratio was 5:1 and the sample to acid ratio was 1:10. Yeast was used to facilitate for the fermentation process and the acid was used to for hydrolysis conversion of cellulose in to glucose. The amount of glucose was calculated in to 24, 48 and 72h using glucose standard. Finally the distillation process was done and volume of the bioethanol was measured. The volume of the sample BSG approximately 0.9 mL/g or Percentage yield 90% .And there was a lot of characterization technics were take place. Those are FTIR, oxidation test, combustion of bioethanol and density determination. This work illustrated that BSG could be converted into bioethanol efficiently by pretreatment of torrefaction, chemical hydrolysis and SSF. Of the conditions tested, 120 °C torrefaction gave greater ethanol yields than 220 °C and achieved an ultimate yield of ~0.9 mL/g or 90%. These findings demonstrate that the bioethanol production could be significantly improved by optimizing the pretreatment and fermentation conditions, and it is a prospective way to convert of industrial wastes into value-added products such as bioenergy.

***Keywords:* Barley spent grains, Bioethanol, Diluted-acid hydrolysis, and SSF and** *Saccharomycescerevisiae,*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the study

In the 21th century, the world economy has been dominated by technologies that depend on fossil energy, such as coal, petroleum, or natural gas to produce fuels, chemicals, materials and power. The increasing concentration of CO_2 in the atmosphere and worries about global warming pose a threat to the ongoing reliance on fossil fuels to satisfy most of the world's energy (Friedlingstein, P., et al. (2025). The combustion (burning) of fossil fuel is responsible for 74 % of the CO_2 emission (European Commission, Joint Research Centre. (2025)). The increasing concern over the reliability of oil supply and its looming peak, along with the urgent need to reduce greenhouse gas (GHG) emissions linked to fossil fuel consumption to prevent harmful global climate changes, is fueling interest in alternative transportation fuels (International Energy Agency. (2024). Hence the development of alternative and eco-friendly energy source is critically important to address the aforementioned issue?

To reduce GHG emissions, we must shift from fossil fuel resources to renewable energies (such as biofuels) in order to create opportunities for lowering GHG emissions (IEA, 2006). Energy security and climate change imperatives require large-scale substitution of petroleum-based fuels as well as improved vehicle efficiency. Bioethanol as fuel has been known over hundred years. In 1860 Nicholas August Otto from Germany employed ethanol as fuel in his internal combustion engine. From the beginning of last century up to 1960, mixed ethanol with gasoline was used widely for transportation in many European countries such as Germany, France, Italy, Sweden, England as well as Brazil and USA. In 1960's the interest to use of ethanol decreased due to the low price of oil (in comparison with the price of ethanol). The new interest in bioethanol once more started in many countries (Brazil in 1979, USA in 1980 and Europe in 1990) owing to technological developments, market factors and some other factors such as national energy security concern and governmental motivations. a lot of country apply this bioethanol production for different consideration purpose. And also a huge amount of bioethanol was produce by those countries. Nowadays a large amount of bioethanol is made around the world. For instance, in 2008, USA was the first fuel ethanol

producer with 9000 million gallons. In that year Brazil with 6,472.2 million gallons and the European Union with 733.6 million gallons was the second and third producer of fuel ethanol in the world respectively (Renewable Fuels Association, 2025). The main raw materials in those countries (USA, Brazil and European Union) were sugar or starch (corn, wheat, sugar cane and sugar beets).the raw materials for the production of bioethanol was classified as in three those are first generation, second generation and third generations. Ethanol obtained from sugars and starch is referred to as the first generation bioethanol. Ethanol production from lignocelluloses biomass such as brewery spent grain is considered as second generation bioethanol. These types of biomass have shown potential as bioethanol feedstock on commercial scale. While bioethanol production from algae is still in an immature stage and confined to the laboratory research.

While Brewery spent grain (BSG) is a by-product of the brewing process, consisting of the solid residue remaining after mashing and/or lautering process. . For lignocellulosic biomass, pre-treatment is essential before hydrolysis to modify the structure of cellulose, enhancing enzyme accessibility (Singh and Bishnoi 2012; Avci et al. 2013)

1.2. Statement of the Problem

The challenge of energy security, arising from the dwindling global petroleum reserves, rising petroleum prices, and environmental issues, has prompted governments and researchers to seek alternative renewable energy sources that are technically viable, economically viable, and environmentally sound. In Ethiopia, bioethanol is primarily produced from sugarcane molasses, a by-product of the sugar industry. (Melaku, T., & Tekeste, F. (2016)). Ethiopia typically spends about 3 billion USD annually on fuel imports, which accounts for 77% of its total export revenue (PM Abiy Ahmed, as cited in Government of Ethiopia, 2021).. Additionally, fuel demand rises alongside the nation's economic growth. Conversely, there has been a rise in the number of breweries in recent years, which produce a significant amount of spent grain. This study suggests potential solutions for the previously mentioned issue and outlines methods for producing this biofuel, including detailed pretreatment techniques. Furthermore, it presents an innovative approach to bioethanol production through the SSF process.

1.3. Objectives of the study

1.3.1. General objective

The general objective of the study was to produce bioethanol from brewer's spent grain (BSGs) following simultaneous Saccharification and fermentation process (SSF).

1.3.2. Specific objective

The specific objectives of the study were: -

- Characterization of the BSG sample using different technics
- Employ torrefaction as a pretreatment techniques to enhance the quality of BSG for bioethanol production
- Develop a combined dilute base and acid pre-treatment on torified BSGs sample to remove lignin and hemicellulose, respectively.
- Production of bioethanol from BSG's sample using simultaneous Saccharification and fermentation process (SSF) at optimized physicochemical and microbiological Condition using extracted saccharomyces from yeast.

1.4. Significance of the Study

- This research conducted a preliminary analysis of brewery spent grain as a source for bioethanol production.
- It highlighted to researchers the potential of this inexpensive and widely available by-product for further studies.
- Additionally, this research significantly contributes to the replacement of fossil fuels with biofuels.
- Producing bioethanol from barley spent grain is classified as a second-generation biofuel process, as it does not create direct competition with human food, unlike first-generation biofuels derived from crops like corn, sugarcane, and soybean oil.
- Ethanol recovery from the water-ethanol mixture was carried out using distillation, along with an analysis of the ethanol produced.
- To compare the bioethanol fuel and petroleum fuel from fossil fuel.
- To produce this bioethanol in mass scale like developed country.

1.5 Scope of the Study

More importantly, the combustion or burning of fossil fuels contributes to the Couse of the following problem listed below. The release of CO_2 into the air is causing global warming, rising sea levels, pollution in cities, and the loss of biodiversity. Couse of a lots of disease by the emissions of different gases to the atmosphere. Thus, in order to combat climate change, the energy transition to low-carbon intensity fuels becomes imperative (Abas, N., Kalair, A., & Khan, N. (2015))The contemporary world's negative environmental, social, political, and energy security issues have raised interest in biofuels and other alternative energy sources. Nevertheless, the solution to the three major global issues of energy consumption, security, and climate change lies in alternative energy sources.

The creation and optimisation of bioethanol from lignocellulose biomass, or brewer's spent grain, a by-product of the brewing process, is the main topic of this study. Pre-treatment, fermentation, distillation, diluted-acid hydrolysis, and proximate analysis of BSG were the techniques employed in this study. Laboratory experiments were used to generate the statistical data and analysis of variance (ANOVA) was used to determine the generalising conclusion for each parameter on the optimal yield of total reduced sugar and to examine the impact of process parameters on the yield of total reduced sugar from the second stage hydrolysis process. By determining the concentration using absorbance, the standard glucose method is used to analyse the yield of total reduced sugar in hydrolyses. Ethanol was characterized using a pycenometer to determine the density by measuring the specific gravity, an oxidation test to determine the presence of alcohol in the sample using $K_2Cr_2O_7$ in acidic media, testing combustion of the distilled bioethanol, and FTIR to determine the functional groups found in the bioethanol. The process of separating the water-ethanol mixture is carried out using a distillation unit.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Bioethanol production and its Importance as an alternative fuel

In the literature that is currently available People have been using ethanol, also known as ethyl alcohol, since the beginning of time. About 70% of the world's bioethanol output was anticipated to come from the United States and Brazil in 2006. The Kyoto Protocol's rules, for example, called for a constant increase in demand. There are two methods for producing bioethanol: biological and synthetic. Ethylene is often catalytically hydrated in the vapour phase to produce synthetic ethanol. According to (Gencheva et al. (2012), the ethanol generated by this procedure is utilised as a solvent in 60% of cases and as a chemical intermediate in 40% of cases. Ninety-three percent of the world's bioethanol is produced by fermentation. Among microorganisms, Saccharomyces cerevisiae is the most common. Ninety-three percent of the world's bioethanol is produced by fermentation. Because of its high ethanol yield and great tolerance to relatively high ethanol concentrations, Saccharomyces cerevisiae is the most often utilised microbe for ethanol production. According to Vince et al. (2010), the majority of the ethanol generated is used as fuel (92%), industrial solvents and chemicals (4%), and beverages (4%). Comparing bioethanol to traditional fossil fuels, the former produces less smog due to its low toxicity, volatility, and photochemical reactivity (Idrees et al., 2013). Therefore, in order to maximise or enhance the yield while lowering or decreasing expenses, procedures to hydrolyse the lignocellulose materials must be implemented. Due to its limited domestic fuel supplies and the fuel crisis of the 1970s, Brazil is currently the world's second-largest producer of bioethanol, after the United States (Akanksha et al., 2014). Other countries have existing bioethanol production facilities. Although the most common forms of biofuel are bioethanol and biodiesel, the United States and Brazil are by far the world's two largest producers of ethanol. Brazil is the second-largest producer of ethanol, using sugarcane as the primary feedstock, while the United States is the largest producer, using maize as the primary feedstock, accounting for the great majority of the input (Novy et al., 2015).

Cellulose from biomass is used in the second generation method to manufacture ethanol (A. A. K. Chandel et al., 2011). 50–80% (dry basis) of lignocellulose biomass is usually composed of carbohydrates, which are polymers of sugar units with five and six carbons. The last several decades have seen a significant amount of study on renewable sources of liquid fuels to replace fossil fuels due to long-term economic and environmental concerns (P. Kumar et al., 2009).A sustainable and eco-friendly fuel, bioethanol is mostly made from biomass, such as corn and sugarcane. It boosts rural economies, lessens dependency on oil imports, and lowers greenhouse gas emissions as a fossil fuel substitute. To increase combustion efficiency and lower air pollutants like carbon monoxide and particulate matter, bioethanol can be mixed with petrol. The International Energy Agency (IEA) asserts that, when generated responsibly, bioethanol may greatly aid in reaching climate targets and is essential to the decarbonisation of the transportation sector (IEA, 2023)

2.2. World Bioethanol Production source

In available literature production of Bioethanol is produced in many countries around the world for a variety of strategic, environmental, and economic reasons. In addition to replacing conventional fossil fuels, bioethanol plays an important role in lowering greenhouse gas emissions and supporting national commitments to climate change mitigation. Its use improves energy security by decreasing dependence on imported petroleum while promoting the diversification of energy sources. The utilization of agricultural residues and biomass waste for bioethanol production further strengthens its role in sustainable waste management and industrial applications, making it a key component of renewable energy systems worldwide (Abas et al., 2015; Demirbas, 2009). Depending on the kind of feedstock utilised, the manufacturing of this bioethanol could be divided into three categories. (Pejin et al., 2016).

2.2.1. First-generation biofuels

The biofuels of the first generation are the primary feedstock for first-generation biofuels is food crops that comprise animal fats, sugar, starch, and vegetable oil. According to Kuila and Sharma (2016), biodiesel, which was primarily made from canola, soybean, and barley, and bioethanol, which is primarily made from corn, wheat, and sugarcane, are the most well-

known or widely used first-generation biofuels. Other forms of vegetable oil and biogas are next in line. The growing demand for first-generation biofuels and biomaterials raises a number of sustainability concerns.

2.2.2. Second-generation biofuels

The term "second-generation" biofuels refers to biofuels that have developed independently from biomass sources of lignocellulose and are a by-product of production, such as ethanol obtained from lignocellulose. The second-generation biofuels should have considerable potential for cost reduction and higher levels of production efficiency as more experience is obtained (IEA, 2008a). The conversion of cellulosic biomass into biofuels and chemicals offers a number of benefits, such as reduced reliance on fossil fuels, near carbon neutrality, greenhouse gas emissions, and increased energy security for the country.

Table 1: Global Fuel Ethanol Production by Country (2020–2024)

Country	2020	2021	2022	2023	2024
United States	13941	15016	15361	15580	16219
Brazil	8100	7320	7400	8470	8780
India	530	950	1220	1510	1630
EU	1310	1380	1420	1390	1440
China	940	900	960	1070	1900
Canada	429	434	447	454	464
Thailand	390	350	380	340	360
Argentina	210	270	310	310	310
Rest of World	630	690	722	806	807
Total World	26480	27310	28220	29930	31210

Data in millions of U.S. liquid gallons per year, sourced from the Renewable Fuels Association (RFA)

2.3. Bioethanol production in Ethiopia

Ethiopian ethanol manufacturing began in 1998–1999 at the Fincha sugar factory, which has a 1,907 m^3 annual capacity. With a 6,373 m^3 annual capacity, the Metahra sugar mill began producing ethanol in 2010–11. 19,805 m^3 of ethanol are being produced annually by both factories (source: Ethiopia Sugar Corporation). Molasses, a by-product of sugar manufacturing from the crystallisation process of sugarcane, serves as the primary raw material for this product. Many ethanol plants and cogeneration facilities should have been established as a result of the recent global awareness of the use of ethanol to replace petroleum and generate electricity in addition to sugar mill plants. There is a lot of sugar real estate in Ethiopia; for instance, the Sugar Development Agency manages the industries of Fincha, Metahra, and Wonji Showa. The majority of molasses-derived products are ethanol, but in order to be used as bioethanol, its use needs to catch the interest of government policymakers. Products made from ethanol that can be converted into liquid fuels for transportation are known as bioethanol or biofuel (ESDA, 2015). The ethanol produced by Ethiopia's sugar industries is used for a variety of purposes, including sanitizer, greasing, and perfume. There was no any ethanol production for biofuel purpose.

Table 2: Ethanol Productions in Ethiopia — Historical and Recent Data litter per year

Source	1998/9	1999	2000/1	2001/2	2002/3	2003	2004-2010	2011-2015	2025
Fincha sugar	1,907,000	720,000	1,790,571	209,444	894,624	911,431	32876161		
Fincha and Metehara	-	-	-	-	-	-	-	81,887,170	32.5 million L/year

Main source: Mebrhit Gebremariam Thesis Ethanol Production and Its Environmental and Socio-Economic Impacts in Ethiopia.

2.4. Feedstock for bioethanol Production

Ethanol can be produced synthetically from petroleum or by fermenting sugars microbiologically using second-generation biofuel. The three main types of raw materials utilised in the fermentation process to create ethanol are sugar, starch, and lignocellulose (Lin and Tanaka, 2006). "Any fermentable sugar can be converted to ethanol by yeast under the correct conditions." (Dussán and others, 2014). three primary classes comprise the feed stocks:

i) Saccharine products, such as fruit juices and sugarcane and sugar beet molasses.

ii) The starchy foods, like potatoes and cereals like sorghum, corn and barely.

iii) Cellulose products, such as wood and leftover lignocellulose

2.5. Lignocelluloses feedstock for bioethanol production

A liquid produced by distilling fermented sugar or glucose from a range of locally available resources, including forestry and agricultural residues, certain starchy crop material, woody and herbaceous crops, and the organic component of municipal wastes, is known as bioethanol (Chan-u-tit et al., 2013). Ethanol produced from lignocellulose and agro-industrial wastes is thought to be the most promising alternative to conventional methods since it offers a number of advantages, such as the fact that bioenergy does not compete with food supply and employs a greater variety of feedstock (Raposo et al., 2009).Dry lignocellulose content and common agricultural waste residue (Harmsen & Huijgen, 2010).

Table 3: Composition of Lignocellulose Feedstock

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Corn Stover	35.1–39.5	20.7–24.6	11.0–19.1
sugarcane bagasse	25–45	28–32	15–25
Rice straw	29.2–34.7	23–25.9	17–19
Switch grass	35–40	25–30	15-20
Hardwood stems	40–55	24-40	18–25
Barley straw	36–43	24-33	6.3-9.8
Sorghum straw	~26.9	~32.6	~5.3–5.9
Sugarcane straw	~29	~28.8	~32
BSG	12–25 %	20–35 %	12–28 %
Waste paper	35-40	10-20	5-10
Wheat straw	33-39	22-30	12-16

Source of the table Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. (2008).

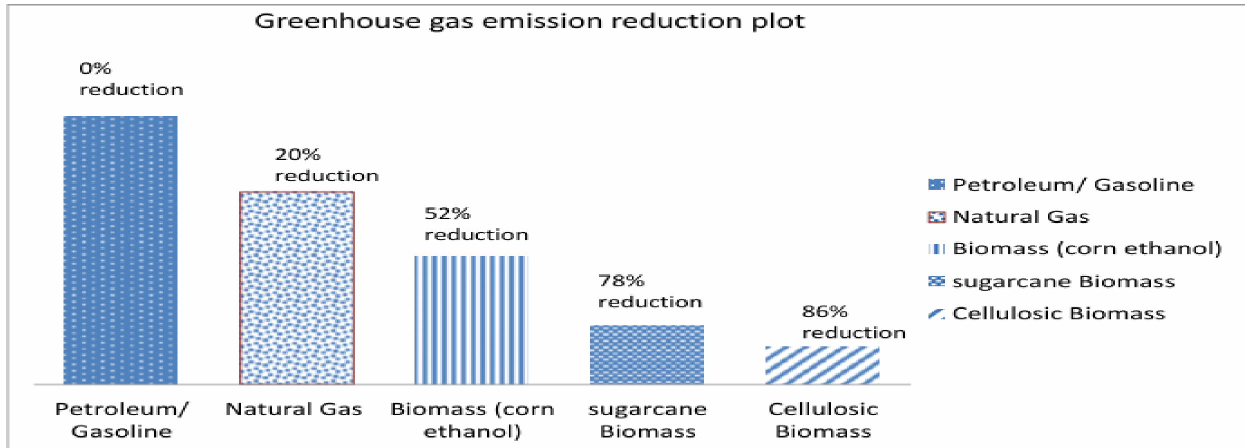

Figure 1: Energy and GHG emissions impact Source: (Chan-u-tit et al., 2013)

2.5.1. Cellulose

According to P. Kumar et al. (2009), cellulose is the primary structural component of plant cell walls and is present in an ordered fibrous structure. Since cellulose is made up of two glucose molecules and has the chemical formula $C_6H_{10}O_5$, it is more frequently thought of as a polymer of glucose. The image below illustrates the structure of one chain of the polymer (Orji et al., 2016).

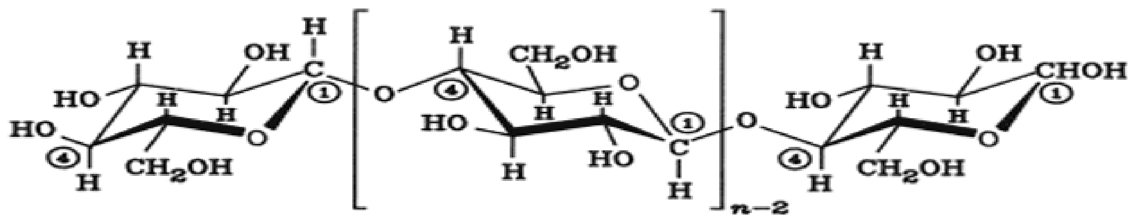

Figure 2: The Structure of single cellulose molecule

The linear polymer structure of cellulose was made up of D-glucose subunits connected by a (1, 4)-glycosidic bond. According to Limayem and Ricke (2012), cellulose is the repeat unit created by this linkage. It is part of the cellulose chain, and the long chain cellulose polymers are joined by hydrogen bonds and Van der Waals bonds. This results in the cellulose being

packed into micro fibrils, which are building blocks of fibrils and ultimately form the cellulose fibre.

2.5.2. Hemicellulose

The Hemicellulose was a polymer that is composed of several different sugars like, D-glucose, D galactose, D-mannose, D-xylose, L-arabinose, L-rhamnose and is sometimes referred to by the sugars they contain, for example, galactoglucomannan, arabinoglucoronoxylan, arabinogalactan, etc (Novy et al., 2015). The polymerization degree of hemicellulose is lower than cellulose, achieving an average of about 100-200 and due to the combination of several and for presenting majority part of amorphous structure, the hemicellulose is more soluble and easily degradable in water than cellulose (Dussán et al., 2014).the degradability of hemicellulose in water was higher than cellulose.

2.5.3. Lignin

The Lignin is a very complex molecule comprised of phenyl propane linked in a three-dimensional structure (Lenihan et al., 2012). Among the lignocellulose biomass (residues), the great interest has been focused on brewer`s spent grain, BSG due to its lower composition in lignin (L. Liguori et al., 2015). Because of the amount of lignin present in the BSG sample increase it affect the conversion of glucose in to bioethanol is difficult. Means it affects the hydrolysis process by inhabit the chemical reaction process.

2.5.4. Extractives and Ash

The term "extractives" or "ash" refers to any of the various substances (such as resins, phenolic, and other chemicals) found in biomass that are not essential components of the cellular structure. Using polar or non-polar solvents, such as hot or cold water, benzene, ether, methanol, or other solvents that do not break down the structure of the biomass, the extractives and ash compounds can be separated from the biomass. When biomass is burned, minerals such as calcium, magnesium, potassium, and other substances will be released as ash (Ktenioudaki et al., 2012).

2.6. Brewer`s spent grain

Lignocellulosic materials, the primary by-product of the brewing industry and accounting for around 85% of all by-products, were found in brewery discarded grains. The residue of malt and grain left over after mashing and lautering is this by-product of beer production; it is mainly a mixture of grain and grain husks from which the majority of its sugar or glucose contents were removed. They contain a lot of lignocellulose, which can be fermented to produce sugars. Additionally, research has been done on the enzymatic hydrolysis of BSG using an esterase and a xylanase to liberate the ferulic and p-coumaric acids. Few studies have been conducted to investigate this by product as a low-cost raw material for energy generation, despite the fact that it is produced in enormous quantities annually (Mussatto et al., 2006; Santos et al., 2003). The type of barley, the beginning material composition, the harvesting period, and the malting and mashing conditions all affect the chemical makeup of BSG. The quality and kind of adjuncts added during the brewing process, as well as the mashing techniques employed for sugar extraction, can all affect the composition and sugar concentrations of BSG (Santos et al., 2003). According to the research, BSG's composition varied, primarily consisting of cellulose and hemicellulose from barley grain arabinoxylans (Mussatto et al., 2006; Santos et al., 2003). It may also be used as an inexpensive feedstock for the ethanol manufacturing process. The chemical makeup of the brewer's spent grain changes according to the type of barley, when it is harvested, and the conditions of the brewing process (Mata et al., 2015).

Table 4: Chemical composition of Brewer`s spent grain (Muthusamy et al., 2014).

Components	% of dry material
Hemicellulose	19
Cellulose	34
Lignin	11.5
Protein fibre	20
Ash and extractives	2 - 4
Water	12.5

2.7. Sustainable utilization of BSG

Due to their affordability and accessibility, lignocellulose substrates have attracted a lot of attention lately due to their potential application in secondary fermentation procedures. However, BSG is not widely used, particularly in poor nations, and the process economy might benefit from new applications for this residue. According to reports, the following fields have been used (Aliyu and Bala, 2013).

2.7.1. Use of Brewery Spent Grain (BSG)

The potential use of lignocellulose substrates in secondary fermentation processes has recently attracted a lot of attention due to their affordability and accessibility. However, there is a lack of use for brewery leftover grain, particularly in developing nations, and the process economy would benefit from new applications for this residue. BSG is typically utilised. Formulations for animal nutrition and feed; manufacturing of building bricks; xylitol production; metal adsorption and immobilisation; lactic acid production; growth medium for microorganisms and enzymes; production of bioethanol; and extraction of Hydroxycinnamic acids (ferulic and p-coumaric).

2.8. Processing technologies for converting BSG to ethanol

There are multiple processing steps to convert lignocellulose biomass (BSG) into a value-added product, bioethanol. There are four basic steps to convert the lignocellulose biomass in to bioethanol those are: pre-treatment, hydrolysis, fermentation and distillation (Vince, 2010). The nature of the feedstock puts certain constraints on the technology required for the manufacture of ethanol. For example, molasses or sugar solutions can be fermented directly by yeast, using traditional and well-established technology. However, cellulose feedstock such as wood or bagasse or any lignocellulose biomass must be hydrolyzed in to component molecules and sugars before fermentation by one or more specifically selected microorganisms (Roberto, 2005).

2.8.1. Pre-treatment

Removing lignin and hemicellulose, decreasing cellulose's crystallinity, and increasing the lignocellulose materials' surface area and porosity were the primary objectives of the pre-

treatment procedure. The following conditions must be fulfilled by pre-treatment:
1. Enhance the production of sugars or the capacity to hydrolyse sugar later on;

2. Prevent or reduce the loss or degradation of carbohydrates;

3. Prevent or eliminate the production of by products that hinder the subsequent fermentation and hydrolysis processes; and be economical.

Pre-treatment techniques can be broadly categorised into several groups:

A. physical (grinding and milling),

B. chemical (alkali, diluted acid, and organic solvents),

C. Biological or a mix of these.

D. Physicochemical (auto-hydrolysis, steam pre-treatment, microwave assisted pre-treatment). For the economical pre-treatment of lignocellulose biomass for biological conversion to chemicals and fuels, the aforementioned pre-treatment technologies have shown promise.

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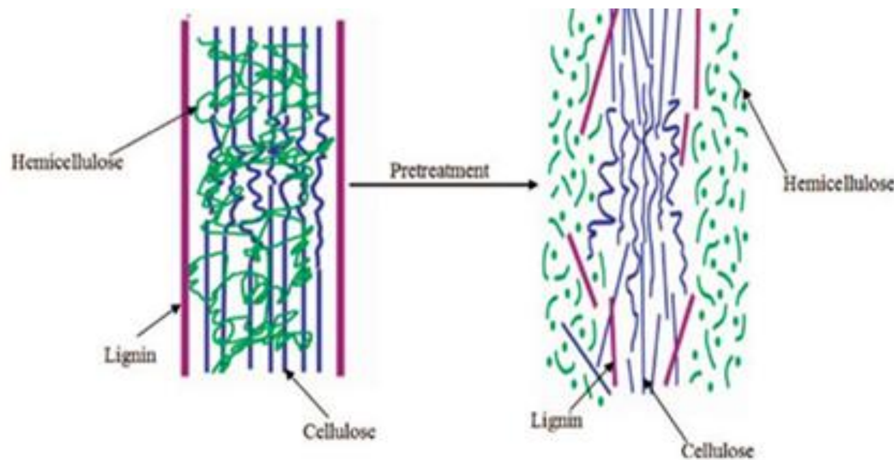


Figure 3: The role of pre-treatment in the conversion of biomass to fuel.

A. Physical pre-treatment

Mechanical commination

To lessen the crystallinity of cellulose, waste materials can be processed using a mixture of chipping, grinding, and milling. After chipping, the material size is typically 10–8 mm, and after milling, it is 0.2-2 mm. The cellulose crystallinity of brewer's spent grain can be broken down more effectively by a vibratory ball mill, which also improves the digestibility of

biomass. The power needed to combine agricultural materials mechanically depends on the biomass's final particle size and properties (Trajano and Wyman, 2013)

Mild Pyrolysis

Moreover, lignocellulose materials have been pre-treated via pyrolysis. Cellulose breaks down quickly to release gaseous compounds and leftover char when materials are heated over 300^0C (Da Silva and Chandel, 2012). Zheng and Rehmann (2014) state that at lower temperatures, less volatile compounds are generated and the breakdown was more slower. Although the process is aided by oxygen, mild acid hydrolysis (1N H2SO4, 970C, 2.5h) of the pyrolysis pre-treatment residues has led to a lower conversion of cellulose to reduced sugar, more specifically glucose. However, the process is energy-intensive and may produce harmful fumes.

B. Chemical pre-treatment

Alkaline Pre-treatment of BSGs

The pre-treatment in this investigation will be sodium hydroxide (NaOH), which degrades and ferments more readily than other alkalis such sodium carbonate, ammonium hydroxide, calcium hydroxide, and hydrogen peroxide (Rezende et al., 2011). The effects of diluted NaOH on the extraction of cellulose, hemicellulose, lignin, ash, and extractive from BSGs will be assessed at varying reaction pressures and times. As a result, various NaOH concentrations typically range from 0.5% to 5% at varying reaction pressures of 10, 15, and 20 Psi and times of 5, 20, and 35 minutes. A vertical autoclave (model VSL-52, India) with a digital temperature and time controller was used to pre-treat BSGs with diluted NaOH. The solid-to-liquid (NaOH) ratio was maintained at 1:10 (g/ml). Three-factor factorial treatment configurations (NaOH, pressure, and time) were used in the fully randomised experiment. Every therapy was carried out twice.

C. Physic-chemical pre-treatment

Steam explosion (Auto hydrolysis)

The most popular technique for pre-treating lignocellulose biomass is steam explosion. This process uses high-pressure saturated steam to treat chipped biomass, followed by a rapid drop in pressure, causing the materials to decompress explosively (P. Kumar et al., 2009). Before the material is subjected to air pressure, a steam explosion is usually started at a temperature

between 150 and 2600C (corresponding pressures of 0.69 and 4.83 MPa) for a few seconds to a few minutes. The technique increases the possibility of cellulose hydrolysis by causing hemicellulose degradation and lignin transformation as a result of the high temperature. Poplar chips that had been pre-treated by steam explosion had an enzymatic hydrolysis efficiency of 90% in 24 hours, but untreated chips only had 15% hydrolysis efficiency (Wang and Liu, 2014). Temperature, moisture content, chip size, and residence duration all influence steam explosion. High temperatures and short residence times (270^0C and 1min) or low temperatures and prolonged residence times (190^0C and 10min) can both produce the best hemicellulose solubilisation and hydrolysis (Olaoye and Kolawole, 2013).

Ammonia fiber explosion (AFEX)

Another kind of physic-chemical pre-treatment is called AFEX, in which lignocellulose materials are exposed to liquid ammonia for a while at high temperatures and pressures before the pressure is quickly lowered. Similar in principle to steam explosion, AFEX typically involves a dosage of 1-2 kg of liquid ammonia per kilogramme of dry biomass, a temperature of 900C, and a 30-minute retention period (Li, 2010). Many herbaceous crops and grasses can have their scarification rates considerably increased by the AFEX pre-treatment. Lucerne, wheat straw, barely straw, maize Stover, rice straw, municipal solid waste, softwood newsprint, switch grass, aspen chips and bagasse are just a few of the lignocellulose materials that can be pre-treated with it. In contrast to acid pre-treatment and acid-catalyzed steam explosion, the AFEX pre-treatment does not considerably solubilise hemicellulose (Than, 2017).

D. Biological pre-treatment

Using wood-degrading microorganisms, such as bacteria and fungi that cause white, brown, and soft rot, this treatment approach alters the lignocellulose biomass's chemical makeup and/or structure to make it more digestible by enzymes. Up to now, the majority of biological pre-treatment has been on lignin breakdown in the cellulosic biomass. However, the loss of cellulose and hemi-cellulose is typically accompanied by lignin degradation. The microbial strains should have low cellulose activity in order to reduce and eliminate the sugar loss during biological pre-treatment. Since they can more efficiently and precisely break down

lignin, white rot fungi are the most researched fungi or biological pre-treatment. With its many obvious benefits, such as low energy input, mild ambient conditions, no chemical demand, and an eco-friendly operating method, biological pre-treatment seems like a promising solution. Biological pre-treatment, on the other hand, is extremely sluggish (lasting weeks to a year) and necessitates more room and precise control over growth conditions. Furthermore, the majority of lignolytic microbes eat or solubilise cellulose and hemicellulose in addition to lignin (Mosier et al., 2005).

2.8.2. Hydrolysis

Under the presence of suitable catalyst (acid or enzyme) the reaction between biomass and water to produce simple sugar or glucose this process is called hydrolysis. (Bokulich et al., 2013). At the time of this reaction, the released polymer sugars, cellulose and hemicellulose are hydrolyzed in to free monomer molecules readily available for fermentation conversion to bioethanol (Novy and Longus, 2015). Hydrolysis was categorized as in to two those are one stage or first stage and two stages or second stage hydrolysis. There are different types of hydrolysis; chemical, biological and enzymatic, steam explosion, ionic liquids and hot water hydrolysis of these methods, chemical and enzymatic are dominant (Tsoutsos and Bethanis, 2011). Chemical hydrolysis is classified as either alkaline (base) or acid hydrolysis (Parsapour, 2012). Alkaline solutions like sodium hydroxide, lime and usually also ammonia can remove lignin and some part of hemicellulose (Dehnavi, 2009). The Acid (H_2SO_4, HCl, H_3PO_4, HNO_3) pre-treatments was first established in Germany in 1898 (Vigo-ourense and Lagoas, 2004). Acid hydrolysis can be categorized in to two those are concentrated or dilute acid hydrolysis (Axelsson et al., 2012). Concentrated acid hydrolysis is potent agents to the conversion of hemicellulose and cellulose to monosaccharide sugars, however it is adverse to environment, corrosive and dangerous and needs of reactors which are resistant to corrosion (P. Kumar et al., 2009). The hydrolysis is done by enzymatic method, which uses enzymes to hydrolyze hemicellulose and cellulose into simple sugars or glucose. Biological hydrolysis using microorganisms are known as brown-, white- and soft-rot fungi attack lignin and hemicellulose within waste materials, brown-rot fungi attack mainly hemicellulose, whilst white- and soft-rots fungi attack both cellulose and soft-rots fungi attack both cellulose and lignin (Chandel, A. K., Singh, O. V., Rao, L. V., & Narasu, M. L. (2011)) the enzymatic

Consequently, for the improvement of the reaction productivity, it is essential the pre-treatment of the raw material. Generally, three hydrolysis alternatives exist after pre-treatment processes (Verardi et al., 2014):to get a good result those three hydrolysis methods was recommended those are

A. Acid hydrolysis (Concentrated and Dilute acid hydrolysis)
B. Enzymatic hydrolysis
C. Alkaline hydrolysis

Among these methods, of hydrolysis dilute acid mediated hydrolysis has been found more effective and less costly in complete hemicellulose and cellulose hydrolysis in short reaction time (Dussán et al., 2014).

A. Acid Hydrolysis

Cellulose and hemicellulose polymers in lignocellulose biomass are broken down by acid-catalysed hydrolysis, which produces individual sugar molecules that can then ferment to produce ethanol. While other mineral acids like HCl, H_3PO4, HNO_3, and others are utilised, sulphuric acid is the acid that is most studied during hydrolysis. Two types of acid hydrolysis can be applied Dilute-acid and concentrated-acid hydrolysis. (Chandel, A. K., Singh, O. V., Rao, L. V., & Narasu, M. L. (2011).

The use of concentrated acids for the hydrolysis of lignocellulosic biomass is a long-established approach, recognized for its ability to produce high sugar yields—reaching up to 90% of the theoretical glucose output. This efficiency translates into greater ethanol production compared to dilute-acid hydrolysis methods (Jose and Reinaldo, 2011). One of the benefits of concentrated-acid hydrolysis is that it operates effectively at relatively lower temperatures, around 400°C. Despite this advantage, the method requires acid concentrations between 30% and 70%, making the system extremely corrosive due to the subsequent dilution and heating steps (Akanksha et al., 2014). To withstand such harsh conditions, the equipment must be built from expensive corrosion-resistant materials like specialty alloys, ceramics, or carbon-brick linings. Furthermore, acid recovery is energy-intensive, and neutralization with sulfuric acid results in large volumes of gypsum waste. Environmental concerns also limit the use of alternatives like hydrochloric acid. These technical and environmental challenges, along with high costs for setup and maintenance, have greatly hindered the commercial feasibility of the process (Ktenioudaki, A., Chaurin,(2012)

Dilute-Acid Hydrolysis

Dilute acid hydrolysis of lignocellulosic biomass offers several benefits that enhance its appeal as a processing method. One key advantage is that it typically eliminates the need for a separate pre-treatment step, as the acid can sufficiently penetrate the lignin structure (Tsoutsos and Bethanis, 2011). Additionally, this method generally avoids the need for acid recovery and poses a lower risk of equipment corrosion compared to concentrated acid techniques. Despite these advantages, one-stage dilute acid hydrolysis has a major drawback: the sugars produced can degrade during the reaction process, resulting in the formation of undesirable by-products. To reduce sugar decomposition and inhibitor formation at high temperatures, a two-stage hydrolysis strategy is often implemented (Than, 2017).When applying dilute acid hydrolysis to brewer's spent grain (BSG), several operational factors must be considered—such as temperature, acid concentration, reaction duration, particle size of the biomass, and the liquid-to-solid ratio. Among these, temperature, acid concentration, and reaction time are particularly influential in maximizing sugar yield while limiting the generation of fermentation inhibitors (Roberto, 2005). The acid acts as a catalyst in the hydrolysis reaction, which is illustrated in the work of Arredondo et al. (2009).

Cellulose → hydrolysis → Glucose

Hemicellulose → hydrolysis → pentose and hexoses

B. Enzymatic Hydrolysis

Enzymatic hydrolysis of cellulose is driven by highly specific enzymes that bind to lignocellulosic material to facilitate its breakdown. A well-known microorganism used in the commercial production of cellulolytic enzymes is *Trichoderma reesei*, which plays a central role in the enzyme industry for generating a wide variety of enzyme products (Maache-Rezzoug et al., 2009). When producing ethanol using enzymatic hydrolysis, two main process configurations are available: Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF). In SHF, the hydrolysis and fermentation steps are carried out independently, allowing for temperature optimization tailored to each stage. However, a drawback of SHF is the accumulation of cellobiose, which inhibits cellulase activity, and the requirement for two distinct processing steps (Akanksha et al., 2014). To address these issues, the SSF method integrates hydrolysis and fermentation into a single process. This configuration enables fermenting microorganisms to consume glucose as it is

released, thus minimizes enzyme inhibition. Despite its advantages, SSF also faces limitations, particularly the challenge of aligning the optimal operating temperatures for both cellulose activity and microbial fermentation.

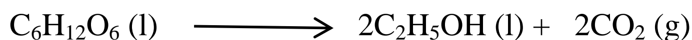
C. Alkaline Hydrolysis

For the pre-treatment of lignocellulosic materials, alkaline hydrolysis can be carried out using a variety of bases, such as sodium hydroxide NaOH, calcium hydroxide $Ca(OH)_2$, potassium hydroxide KOH, and ammonium hydroxide NH_4OH. Alkaline hydrolysis mainly done for the removing of lignin and also the effectiveness of this process was influenced by the lignin content present in the material. The underlying mechanism of alkaline hydrolysis is thought to involve the saponification of intermolecular ester bonds that link xylan hemicelluloses and other constituents. As crosslinks are removed, the porosity of the lignocellulosic materials increases (Than, 2017). Treatment with dilute NaOH results in the swelling of lignocellulosic materials, which enhances the surface area, decrease the degree of polymerization, lowers crystallinity, separates structural linkage between lignin and carbohydrates, and disrupts the lignin structure (Chandel et al., 2012).for this research work done using sodium hydroxide 4% at 60 minute hydrolysis process.

2.8.3. Fermentation

After the pre-treatment and hydrolysis of BSG, the subsequent phase in bioethanol production is the fermentation of sugars into ethanol (Mata et al., 2015). The production of bioethanol primarily occurs through three fermentation methods: batch, fed-batch, and continuous (Rojas-Chamorro et al., 2017). In batch fermentation, the feedstock, along with microorganisms, nutrients, and other components, is introduced into the fermentation vessel at the outset, culminating in the recovery of ethanol. Conversely, in the fed-batch approach, one or more components are added to the vessel during the fermentation process (Zabed et al., 2014). Continuous fermentation entails a steady supply of ingredients and the simultaneous removal of the output from the fermentation vessel (Soupioni and Koutinas, 2013). The role of microorganisms in the fermentation of sugars is essential for the production of bioethanol (Zabed et al., 2014). In the fuel ethanol production sector, fermentation is a biochemical process where simple sugars, such as glucose, fructose, and sucrose, are converted into ethanol by the yeast Saccharomyces cerevisiae (Ccopa et al., 2013For this research work was

use simple distillation method. And also the second stage hydrolysis and the fermentation process was take place once using SSF methods.



$$C_6H_{12}O_6\,(l) \longrightarrow 2C_2H_5OH\,(l) + 2CO_2\,(g)$$

Factors Influencing Fermentation process

The effectiveness of the fermentation process is influenced by various factors, including: Selection of microorganism, Pre-treatment method, Hydrolysis technique, Type of raw material and additionally, environmental conditions such as pH, temperature, substrate concentration, and ethanol levels play a significant role. For instance, typical fermentation conditions for Saccharomyces cerevisiae are generally a pH of 5.0 and a maximum temperature of 37°C (Alfani et al., 2000).

2.8.4. Distillation

At the time of fermentation process the amount of glucose remain at different time 24h, 48h and 72h was calculated. The fermentation broth, commonly referred to as mash or beer, consists of a combination of ethanol, water, cellular material, fuel oil, and various other constituents present in the fermentation medium, including residual sugars, non-fermentable sugars, and by-products of hydrolysis (Erdelji et al., 2007). After the fermentation process is completed, distillation technique is followed. Distillation is a technique employed to separate two or more liquid substances based on their differing boiling points. The boiling point of a mixture is determined by the vapour pressures of its individual components (Thiago et al., 2014).for this research work was done using simple distillation technique.

2.8.5 Alternative approaches for generating ethanol from cellulosic materials.

Ethanol is produced from lignocellulosic feedstock using a variety of bioconversion techniques. These consist of:

1. SHF: Independent Fermentation and Hydrolysis
2. SHFR: In-Situ Recovery via Separate Hydrolysis and Fermentation
3. SSF. Simultaneous fermentation and saccharification
4. In-situ recovery combined with simultaneous saccharification and fermentation (SSFR)
5. Consolidated Bioprocessing, or CBP

SHF and SSF are the most widely used of these technologies in the synthesis of bioethanol today. The two distinct steps of the SHF method—hydrolysis and fermentation—are commonly used for starch-based raw materials. Enzymes such as glucoamylase and amylase catalyse the conversion of starch into fermentable sugars in this process.

Usually, different reactors are used for these reactions. The build-up of sugars during hydrolysis, which can prevent enzymatic activity, is a significant drawback of SHF. Furthermore, this approach is typically more expensive and time-consuming. However, SSF—a procedure created in collaboration between the University of Arkansas and the Gulf Oil Company—addresses a number of SHF's drawbacks. In SSF, fermentation and enzymatic hydrolysis occur concurrently in a single bioreactor. By preventing sugar accumulation, this integration preserves enzyme efficiency. Because ethanol is continuously produced in a single system, co-culturing two compatible microorganisms in the same vessel further improves process efficiency, shortens the production timeline, and lowers contamination concerns. (Gauss, W. F., Smith, J. R., & Brown, L. M. (1976).According to research, SSF typically produces better productivity and greater ethanol concentrations than SHF.

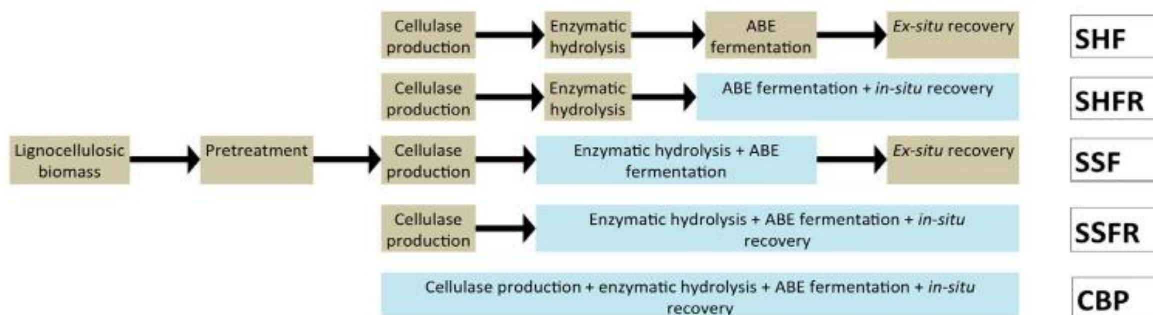


Figure 4: Source M.F. Ibrahim et al. Renewable and Sustainable Energy Reviews 79 (2017) 1241–1254

2.9. Advantages and Effects of Converting Cellulosic Biomass to Ethanol

Reduction of Greenhouse Gas Emissions

One of the key environmental advantages of converting cellulosic biomass into fuels and chemicals is its considerably lower net greenhouse gas emissions compared to conventional fossil fuels. In ethanol production, for example, the residual solids and non-fermentable by-products left over from processing can be repurposed to generate heat and electricity for the facility itself. This reduces or even eliminates the dependence on fossil fuels in fully developed bioconversion plants (Ruiz & Teixeira, 2012). A similar approach can be applied to

the production of other bio-based chemicals, provided that their heat and power demands are not excessively high. Additionally, many lignocellulosic crops grow with minimal agricultural input, such as fertilizers, which further reduces the energy needed for biomass cultivation (Saadavate et al., 2013).

International Fuel Market

Energy demand is rapidly increasing in developing countries, with a growing emphasis on improved mobility driving up the need for transportation fuels. Since transportation systems heavily depend on petroleum, meeting this rising demand poses a significant challenge without the development of alternative fuel sources. Additionally, this approach has the potential to stimulate economic development (Acacio et al., 2014).

Sustainable Production of Organic Fuels and Chemicals

When considering sustainable resources to meet human needs, biomass stands out as the only practical option for the renewable production of organic fuels and chemicals. Liquid fuels, in particular, are especially well-suited for transportation due to their ease of storage and handling, along with their relatively high energy content (Yohannan & Walker, 2008). As a result, converting sugars—produced through the hydrolysis of cellulose and hemicellulose—into ethanol or other liquid biofuels via fermentation offers a promising pathway for sustainable fuel production. This method also helps reduce the overwhelming dependence of the transportation sector on petroleum, which currently supplies over 96% of its energy needs. Although sugar and starch are currently the dominant sources for fermentation processes, cellulosic biomass has the potential to provide a large, low-cost supply of sugars suitable for expanded industrial use, provided that processing technologies become more cost-effective (Li, 2010).

Competition with Food

One of the major challenges to the long-term economic sustainability of biofuels—particularly ethanol—is their potential competition with food resources. According to the Food and Agriculture Organization (FAO), food security exists when "all people, at all times, have physical, social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life" (Zabed et al., 2014). Diverting crops or agricultural inputs meant for food production toward bioethanol

manufacturing can drive up food prices and reduce availability, as both sectors compete for essential resources like land, water, and fertilizers.

2.10. Factors affecting conversion of biomass into ethanol

The choice of microorganism, source material, pre-treatment technique, hydrolysis technique, and environmental elements like pH, temperature, substrate, and ethanol concentration all affect how well a fermentation process works. Type and Composition of Biomass

Cellulose and hemicellulose content: Higher levels of fermentable sugars (like glucose and xylose) make conversion more efficient. Lignin content: High lignin acts as a barrier, making it harder to access the sugars during hydrolysis. Moisture content: Affects pretreatment efficiency and microbial activity Common conditions for fermentation with S.cerevisiae are normally pH 5.0 and a temperature of maximum 37ºC (Alfani et al., 2000). The mixture of inhibitors inhibits the growth and ethanol production of the 24 microorganism. Different bacteria have varying tolerance against these inhibitors, thus S. cerevisiae has proven to be the most robust one (Almeida et al., 2007). In addition, ethanol, the product itself, has an inhibiting effect on the fermenting microorganism, thus limits the conversion of glucose to ethanol (Olsson and Hahn-Hägerdal, 1996).

Important Research on BSG and Related Waste

Numerous research papers have been published regarding the use of BSG as a feedstock for the auto hydrolysis pretreatment process, which contains a high solid content of 20-25%. The process yields high glucose concentrations of approximately 43.7 and 57.7 g/L, along with ethanol levels around 42.27 g/L, achieving a yield of approximately 94% when utilizing whole slurry. The pre-treatment of BSG feedstock with phosphoric acid, when compared to the SSF and SHF processes, shows that at low to medium solids (5-10% w/v), both SSF and SHF yield similar results. However, at higher solid concentrations (15%), SHF proves to be more advantageous, resulting in ethanol production of approximately 22.5 g/L. In the case of alkaline-acid pretreatment and enzymatic hydrolysis, a process involving multiple yeasts yielded ethanol levels of approximately 12-13 g/L, with a yield of about 0.28 g ethanol per gram of glucose, which is roughly 50-55% of the theoretical maximum. Furthermore, nutrient supplementation has been shown to enhance fermentation. The combination of BSG feedstock

and the co-cultures of fungi and yeast (A. oryzae and S. cerevisiae) resulted in ethanol production of approximately 37 g/L within a span of 10 days through consolidated bioprocessing (CBP). This method, while exhibiting lower productivity, also leads to reduced energy and water consumption, with a sequential degradation process that follows the order of hemicellulose to cellulose.

2.11 Physical and Chemical characteristics of Ethanol

Table 5: The physical and chemical properties of ethanol

Property	Value / Description
Chemical Formula	C_2H_5OH or CH_3CH_2OH
Molecular Weight	46.07 g/mol
Physical State	Liquid
Color	Colorless
Odour	Characteristic, pleasant, alcoholic
Taste	Burning, slightly sweet (not recommended for tasting)
Boiling Point	78.37 °C (173.07 °F)
Melting Point	–114.1 °C (–173.4 °F)
Density	0.789 g/cm³ at 20°C
Solubility in Water	miscible in all proportions
Vapour Pressure	5.95 kPa at 20°C
Viscosity	1.2 cP at 20°C
Flash Point	13°C (closed cup)
Auto ignition	365°C (689°F)
Flammability	Highly flammable
pH (in water)	~7 (neutral, but can be slightly acidic)
Reactivity	Reacts with strong oxidizers, acids, and alkali metals
Functional Group	Hydroxyl (-OH)
Chemical Class	Alcohol
Toxicity	Toxic in large amounts; depresses central nervous system
Uses	Solvent, fuel, disinfectant, beverage (alcoholic drinks)

Source: The source the National Institute for Occupational Safety and Health pocket

2.12. Use of ethanol

Ethanol has a lot of importance in the production of different materials as a raw material some of the use of ethanol is listed below in table 6.this research was explained the bioethanol from BSG sample as a fuel with blending gasoline.

Table 6: The importance of ethanol

1. Cleaning Glass	16. Food Colouring and Flavour
2. Removing Grease and Stains	17. Fuel
3. Disinfectant	18. Antidote
4. Household Heating	19. Medicinal Solvent
5. Sanding	20. Manufacture of Fuel Cells
6. Pest Control	21. Industrial Use
7. Removing Mold and Mildew	22. Cannabis Oil Extraction
8. Cleaning and Removing Paint	23. Biotechnology
9. Removing Sticky Residues	24. Cooling
10. Removing Permanent Marker	25. Alcohol Thermometer Fluid
11. Facial Products	26. Manufacture of Alcoholic Beverages
12. Manufacturing Hand Sanitizers	27. Mouthwash
13. Food Preservative	28. Medical Wipes
14. Manufacture of Paints	29. Manufacturing Perfumes
15. Hair Products	30. Manufacturing of Iron Supplements

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and reagents

The chemicals and reagents used in this study include Sulfuric acid (H_2SO_4) (98%), used for hydrolysis process and determination of cellulose and lignin by diluting. NaOH (LOBA CHEME PVT.LTD, 98%) used for second stage hydrolysis process and determination of amount of hemicellulose content present in BSG. distilled water. Ethanol (SAMIRTECH-CHEM, 96%) and detergents were used for equipment cleaning and distilled water was used to prepare aqueous solutions and to rinse equipment. All the experimental works is done in the laboratories of School of natural science laboratory. All chemicals and reagents used in this study were analytical grades.

3.1.2. Apparatus and equipment

The apparatus and equipment used for production and optimization of bioethanol from BSG during this research work were appropriate for laboratory-scale pretreatment, fermentation, and ethanol recovery processes those were as follow. (Whatman No. 42 filter paper (GE Healthcare Life Sciences, United Kingdom), plate dryer, beaker, conical flasks, volumetric flasks, Erlenmeyer flask, burette, analytic balance and digital electronic balance (FA2104, German), rotary hammer mill, oven, furnace (MF2015, India), autoclave, desiccator, crucible, vacuum pump, vacuum filtration, fermenter, agitator incubator, water bath, simple distillation unit, pH meter pH meter (model Mettler-Toledo GmbH), Fourier transform infrared spectrometer (FT-IR) model FT/IR6600, JAPAN), UV-Vis-2000 AUG, USA).

3.2. Experimental procedures

3.2.1. Collection and preparation of raw brewery spent grain (BSG)

The BSG used for this study were collected from united beverage or Anbesa brewery in Mojo located in east Shewa Zone of Oromia Ethiopia. It is located approximately 63.8 Km from Addis Ababa city and 16.81 Km from Adama town. The industries produce beer from barley malt and the lautering technology used in the process of mashing that led to the production of BSG is Lautertun. A total of 10 kg of fresh sample was collected and transported to the

Adama Science and Technology University laboratory for preparation and analysis. The sample was dried under sunlight for five days, ground using a mechanical grinder, and sieved to obtain particles smaller than 2 mm.

3.2.2. Physicochemical analysis of BSG

3.2.2.1 Moisture content

The moisture content of brewery spent grain samples was determined according to the method described by Ktenioudaki and Gallagher (2012) and ASTM E1209-67. Approximately 2 g of each sample was dried in a WTB Binder oven at 105 °**C**. The dried samples were then cooled in a desiccator for 2 hours and weighed. The drying, cooling, and weighing process was repeated until a constant weight was achieved.

Moisture Content% $= \frac{M1-M2}{M1} \times 100$……………………………………………1

Where, M_1 is the amount (g) sample collected

M_2 is the amount (g) after drying

3.2.2.2 Ash content

The ash content of the sample was evaluated in accordance with ASTM A365-407. A dried sample weighing 0.8 g was placed in a Nabertherm B150 muffle furnace and heated at 550 °**C** for 3 hours to ensure complete combustion of organic matter. After heating, the residue was cooled and weighed. The ash content was calculated using Equation (2).

Ash Content (%) = $(M_2 / M_1) \times 100$ ………………………………………….. (2)

where M_1 represents the mass of the sample prior to incineration (g), and M_2 denotes the mass of the remaining ash after incineration (g).

3.2.2.3 Pre-treatment of BSG using torrifaction

Torrifaction of brewery spent grain (BSG) was carried out using a muffle furnace at different temperatures and residence times. For each run, 40 g of dried BSG was placed in the furnace once the target temperature of 120, 150, 180, or 220 °C was reached and torrefied for 10, 30, 60, or 90 minutes**.** After completion of the residence time, the samples were removed from the furnace and cooled under ambient air conditions. The torrefied material was then ground and

sieved to a particle size of 2 mm using a 3½ mesh sieve**.** A total of 16 treatment combinations were conducted in triplicate, and the average values were reported.

3.2.3. Determination of biomass composition: cellulose, hemicellulose, and lignin contents

3.2.3.1 Cellulose content determination

Cellulose content was determined following the National Renewable Energy Laboratory (NREL) procedure described by Sluiter et al. (2008). The method is based on acid hydrolysis of biomass**,** followed by gravimetric determination of the cellulose fraction.Approximately 0.3 g of torrefied sample (denoted as M_1) was treated with 3 mL of 72% (w/w) sulfuric acid (H_2SO_4) and stirred at 30 °C for 1 hour in a temperature-controlled environment. The hydrolysate was then diluted with distilled water to obtain a final sulfuric acid concentration of 4%, after which the mixture was autoclaved at 120 °C for 1 hour. After autoclaving, the mixture was allowed to cool to room temperature and subsequently filtered using Whatman filter paper. The residue was thoroughly washed with hot distilled water until the filtrate reached neutral pH (≈7). The retained solid fraction was then dried at 105 °C to constant weight**,** cooled in a desiccator, and weighed (denoted as M_2).A portion of the dried solid residue was further placed in a furnace and heated at 575 ± 25 °C for 24 hours to determine the ash content. The cellulose content was calculated by correcting the dried residue mass for ash content using Equation (3).

$$\text{Cellulose (\%)} = [(M_2 - \text{Ash}) / M_1] \times 100 \quad (3)$$

Where M_1 is the mass of the dried sample (g)

M_2 is the mass of the dried solid residue after acid hydrolysis (g).

3.2.3.2 Hemicellulose content determination

The hemicellulose content was determined using a modified ASTM procedure (Templeton & Crocker, 2008). A 0.5 g representative sample of ground biomass was treated with 100 mL of 4% (w/w) NaOH and maintained at 60 °C for 2 hours. The resulting mixture was then filtered under vacuum**,** and the solid residue was thoroughly washed with distilled water until the filtrate reached neutral pH (~7)**.**The solid fraction obtained after filtration was dried at 105 °C

for 24 hours until a constant weight was achieved. The weight loss of the dry residue after alkali treatment was used to calculate the hemicellulose content using Equation (4):

Hemicellulose (%) = $[(M_1 - M_2) / M_1] \times 100$ ……………………………….. (4)

Where M_1 is the initial biomass weight (g)

M_2 is the weight of the residue after alkali treatment (g).

3.2.3.3 Lignin content determination

The amount of lignin was determined by taking 0.3g of from the terrified sample using laboratory analytical procedure. (ASTM E1758-01 International. (2001)).3ml of 72% H_2SO_4 was added in to a pressure tube and stirred using stir rode for one minute until the sample is thoroughly mixed. The mixed sample was placed in a water bath at 30°C for 60 minute to incubate the sample. Than the sample was stirred every 5 to 10 minutes without removing the sample from the bath to increase acid to particle contact and uniform hydrolyses process. After completed of 60 minute hydrolysis the tube was removed from the water bath.84 ml of deionized water was added using an automatic burette to dilute the acid in to 4% and mixed the sample several time to stop the phase separation of acid layers with high and low concentrations.The tube was placed in an autoclave safe rack and the rack was placed in the autoclave at 121 °C sugar recovery standard. Before removing the covers, let the hydrolyzate cool gradually at room temperature once the autoclave cycle is finished. For four hours, the crucible was dried in an oven set to 105 °C. The sample was then allowed to cool in a desiccator until its weight remained consistent. The dry residue and crucible weights were noted. The purpose of this stage was to identify lignin that was acid insoluble.The amount of lignin present the given samples were determines using the following formula.

$$\%\text{lignin} = \frac{\text{mass of filter paper plus residu} - \text{mass of filter paper}}{\text{Initial biomass}} \times 100 \quad \ldots\ldots 5$$

And also the acid soluble lignin was determined using the hydrolysis liquor. a liquor obtained from lignin determination was to determine the ASL some samples were selected (220^{0}C for 60 minute in triplicate). The absorbances of the liquors were determined using UV- visible spectrophotometer at 280 wavelengths. The amount of acid soluble lignin present in the liquid was calculated using the following formula.

$$\text{ASL\%} = \frac{\text{Uvabs} \times \text{Volume filtrate} \times \text{Dilution}}{\varepsilon ODW \text{sample} \times \text{pathlength}} \times 100 \quad \ldots\ldots 6$$

Where UV-abs = average UV-Vis absorbance for the sample at appropriate wavelength

Volume hydrolysis liquor = volume of filtrate,

ε = Absorptivity of biomass at specific wavelength

ODWsample = weight of sample in milligrams

Path length = path length of UV-Vis cell in cm

3.2.4. Chemical pretreatment (first stage hydrolysis) of torified BSG using dilute NaOH followed by dilute acid (H_2SO_4)

During the first stage of hydrolysis, 3 g of selected torrefied BSG samples, denoted as BSG120 and BSG220, torrefied for 30 and 60 minutes respectively, were subjected to a two-step pre-treatment. First, the samples underwent mild alkaline pre-treatment using 0.1 M NaOH at a 1:10 solid-to-liquid ratio under warm conditions in a water bath. This was followed by mild acid hydrolysis with sulfuric acid (H_2SO_4) at varying concentrations of 0.5%, 1.75%, and 3% (1:10 solid-to-liquid ratio) and temperatures of 90, 120, and 150 °C, as summarized in Table 6. These treatments were performed to remove lignin and hemicellulose**,** respectively (Fan et al., 1987).

Table 6 first stage NaOH followed by acid hydrolysis process

treatments	pre-treatment conditions	pre-treatment conditions
BSG120, 30	4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4, 90 ^{O}C, 30,60 and 90 minute
BSG120, 30	4% NaOH at 30^0C for 60 minute	1.75% H_2SO_4, 120^0C, 30,60 and 90 minute
BSG120, 30	4% NaOH at 30^0C for 60 minute	3% H_2SO_4, 150^0C, 30,60 and 90 minute
BSG120, 60	4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4, 90^0C, 30,60 and 90 minute
BSG120,60	4% NaOH at 30^0C for 60 minute	1.75% H_2SO_4, 120^0C, 30,60 and 90 minute
BSG120,60	4% NaOH at 30^0C for 60 minute	3% H_2SO_4, 150^0C, 30,60 and 90 minute
BSG 220,30	4% NaOH at 30^0C for 60	0.5% H_2SO_4, 90^0C, 30,60 and 90

	minute	minute
BSG 220,30	4% NaOH at 30^0C for 60 minute	1.75 % H_2SO_4 120^OC 30,60 and 90 minute
BSG 220,30	4% NaOH at 30^0C for 60 minute	3% $H2SO_4$ 150^OC 30,60 and 90 minute
BSG 220,60	4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4, 90^0C, 30,60 and 90 minute
BSG 220,60	4% NaOH at 30^0C for 60 minute	1.75 % H_2SO_4 120^OC 30,60 and 90 minute
BSG 220,60	4% NaOH at 30^0C for 60 minute	3% $H2SO_4$ 150^OC 30,60 and 90 minute

3.2.5. Second Stages Hydrolysis process using simultaneous saccharification and fermentation (SSF)

The second stage hydrolysis process was done using the SSF method (Olofsson, K., Wiman, M., & Lidén, G. (2008). 5g of BSG sample was mixed with 50ml of 3% sulphuric acid (1.10) ratio and heated at 220^0c for 60 minute. The pH of the solution was adjusts 5 using 2M of NaOH. After heated 1g of yeast was added in (1:5 ratio) in to 250 ml flask and placed in a shaker at optimized temperature of 35^0c at 150 RPM. The amount of sugar was measured at different time of fermentation at 24hr, 48hr and 72hr using UV spectroscopy.

Table 7: For second-stage hydrolysis, factors and corresponding operating values

Factors	Unit			
Temperature	^0C	35	35	35
Time	hr	24	48	72
P^H	-	5	5	5

3.2.5.1. Glucose content determination

The total reducing sugar content of the hydrolysates obtained at 24, 48, and 72 hours was estimated using a digital spectrophotometer at a wavelength of 540 nm. A calibration curve was prepared using standard glucose solutions and quantitative Benedict's reagent to relate absorbance to sugar concentration. Benedict's reagent was used to identify reducing sugars, which reduce copper (II) ions to form red-brown cuprous oxide (Cu_2O) precipitate**.** For the calibration, a series of glucose standards (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M) was prepared. 0.5 mL of each glucose solution was pipetted into labelled test tubes containing 5 mL of Benedict's solution. The mixtures were heated in a water bath at 90 °C for 5 minutes to allow the reduction reaction to occur. After heating, the samples were cooled to room temperature and filtered to remove the Cu_2O precipitate. The absorbance of the filtrate was then measured at 540 nm using the spectrophotometer. The resulting absorbance values were plotted against the known glucose concentrations to generate the calibration curve, which was subsequently used to determine the reducing sugar concentration in the hydrolysate (Sluiter et al., 2006).

Determination of the total reducing sugar in the hydrolyzate

The concentrations of total reducing sugars were determined from the calibration curve of the standard glucose solution.at different time 24h, 48h and 72h.using the formula listed below.

$$\text{Concentration of sugar} = \frac{\text{Absorbance} - \text{y intersept}}{Slope} \quad \text{...} 7$$

3.2.6. Distillation (Ethanol Separation)

This study paper states that distillation is the final step in the ethanol production process. It's a purification phase. Distillation is the process of separating two liquids based on their different boiling points. However, many distillation techniques are needed to attain high purity. For this investigation, simple distillation was employed at 85 °C, and the amount of ethanol in each sample was quantified.

3.2.7. Characterization of bioethanol

Oxidation of alcohols

To know the presence of alcohols in the distilled ethanol, oxidation of alcohols was tested. A heated solution of potassium dichromate (VII) ($K_2Cr_2O_7$) combined with diluted H_2SO_4 can oxidize alcohols. The $C_2O_7^{-2}$ ions in solution are reduced to Cr^{3+} ions as the alcohol oxidizes. As a result, the orange color of the solution turns green.

FT-lR determination of RAW and Torified BSG

The chemical structure of Raw BSG and samples torified at two different temperatures (120, 220^{0}C) and residence times (30, 60min) were examined using FTIR spectrometer (Spectrum 65, PerkinElmer) in the wavenumber range of 400–4000 cm^{-1} at Addis Ababa University. The KBr method was used by mixing 1.5 mg of biomass thoroughly with 200 mg KBr powder and pressed into 13 mm discs using a press.

Specific gravity of ethanol

A 20^{0}C pycnometer was used to measure the ethanol's specific gravity or density. After washing and drying the 25 ml pycnometer, the mass (M_0) was measured. Then, the bottle was filled with ethanol, and the stopper was placed. The mass (M_1) was then measured. After the bottle was cleaned, dried, and measured, the ethanol was replaced with water to yield (M_2). The following is the formula for specific gravity (Sp.gr):

$$\text{Specific gravity} = \frac{M1-Mo}{M2-M0} = \frac{mass\ of\ ethanol}{mass\ of\ an\ equal\ volume\ of\ water} \ldots\ldots\ldots\ldots 8$$

Density of ethanol = Specific gravity $*$ $density\ of\ water$

The density of water at 20°C is approximately 1 g/mL.
Where: M_0-mass (g) of empty bottle

M_1- mass (g) of bottle + sample

M_2 - mass (g) of bottle + water

Combustion test of distilled ethanol

A qualitative combustion test was performed to confirm the flammability of bioethanol obtained from BSG after distillation and to observe its fuel characteristics. Approximately 2mL of distilled ethanol was placed in a clean glass dish. The ethanol was ignited using a spark or lighter, and the combustion was observed carefully under safe laboratory conditions. Observations focused on flame color, flame stability, soot formation, and any residue left in the dish. This test was repeated in triplicate to ensure consistency.

CHAPTER FOUR

4. RESULT AND DISCUSSION

4.1. Proximate analysis of BSG

4.1.1 Moisture content of BSG

As indicated in Table 8, the moisture content of the BSG samples in wet basis that was, before they lost water due to exposure to sunlight was 60%, 60%, and 59.5%, respectively. Thus the average moisture content of the three samples was 59.83%.Determination of moisture content was critical value for the production of bio fuel from BSG means the samples that contain higher content of moisture it require makes it highly perishable, promotes microbial growth (bacteria), leading to spoilage and loss of usable material. Drying or pre-treatment was often needed to extend shelf life. Moisture significantly lowers the calorific value (heating value) of the biomass so that the sample was needed efficient drying or use of low-moisture pre-treatment techniques is critical to maintain overall energy balance. (Barrozo, M. A. S. (2024).

Table 8: The Moisture Content of BSG sample at 105^0C a triplicate sample, in %

samples	Mass of crucible(g)	Mass of crucible + sample after dry	Moisture content %
Sample 1	24.86	25.66	60
Sample 2	25.58	26.38	60
Sample 3	26.89	27.7	59.5
average	-	-	59.83

4.1.2 Ash content determination

The ash content of the samples was 3% each and also the average of ash content was 3% as duplicated in Table 9.The ash content in Brewer’s Spent Grain (BSG) plays a significant role in biofuel production, influencing both the efficiency and quality of the fuel, as well as equipment maintenance and environmental impact the presence of higher ash content was

lower calorific value (energy per unit mass), reduces the overall efficiency of thermochemical conversion processes (e.g., combustion, gasification, pyrolysis).The ash content of the this sample was very low this indicate that the sample had good efficiency of thermochemical conversion and had good combustion state. 3% ash content in Brewer's Spent Grain (BSG) is generally considered good and acceptable for most biofuel production processes. A lower percentage means more organic material is available for conversion into energy (ASTM International (2013) *ASTM E1755-01(2013)*.

Table 9: The ash content of the sample at 550^0c in a triplicate sample in %

samples	Mass of crucible	Mass of crucible with ash	Ash %
1	24.86	24.884	3
2	25.58	25.604	3
3	26.87	26.894	3
Avg	-	-	3

4.1.3 Pre-treatment of breweries spent grain

Before determine the amount of three components (cellulose, hemicellulose and lignin) first the sample was homogenized by pre-treatments. In this research the sample was treated by torrifaction process. Torrifying BSG sample had a number of benefits such as; improved fuel properties, increased fixed carbon and reduced volatilize-torrefaction drives off moisture and volatile compounds, enriching the remaining material in carbon content, which boost fuel quality and decrease moisture and making the material more stable. simple to handle or easier to store, and resistant to microbial spoilage Mościcki, K., & Pawlak-Kruczek, H. (2021). After torified at 120°C, 150°C, 180°C and 220°C for 10 minute, 30 minute, 60 and 90 minute. the sample was stored in a good place to minimize contamination and then the three components were determined.

4.1.4 Cellulose content determination

The amount of cellulose was determined by taking 0.3g of all terrified sample in triplicate totally 48 samples (120^0c, 30 minute to 220^0c 90 minute) but for comparison purpose 120^0c 30 minute and 60 minute and 220^0c30 and 60 minute were selected for further analysis. The amount of cellulose was determined using equation 3 and the results shown in Table 10.the % of cellulose content was increase when the torrifayed temperature increase. ASTM E1758 and NREL protocols. (ASTM International. (2024). *ASTM E1758-24)*

$$\textbf{Cellulose\%} = \frac{\textit{mass of dried sample} - \textit{ash}}{\textit{initial biomass weight}} \times 100$$

Table 10: The amount of cellulose content present in BSG sample

Sample ID	Initial biomass	mass of oven dried after hydrolysis + paper	mass of filter paper	Ash	% cellulose
120^0c for 30 min	0.3	1.5828	1.4583	0.03	31.5
120^0c for 30 min	0.3	1.586	1.4606	0.03	31.8
120^0c for 30 min	0.3	1.5822	1.4571	0.03	31.7
120^0c for 60 min	0.3	1.5839	1.4582	0.03	31.9
120^0c for 60 min	0.3	1.5866	1.4606	0.03	32
120^0c for 60 min	0.3	1.5834	1.4571	0.03	32.1
220^0c for 30 min	0.3	1.5872	1.4573	0.03	33.3
220^0c for 30 min	0.3	1.5886	1.4581	0.03	33.5
220^0c for	0.3	1.5884	1.4582	0.03	33.4

30min					
220^0c for 60 min	0.3	1.5915	1.4601	0.03	33.7
220^0c for 60 min	0.3	1.5892	1.4584	0.03	33.6
220^0c for 60 min	0.3	1.5899	1.4582	0.03	33.9
Average					≈32.69

4.1.5 Hemicellulose content determination

The amount of hemicellulose was determined by taking 0.5g of all terrified sample in triplicate totally 48 samples (120^0c, 30 minute to 220^0c 90 minute) but for comparison purpose 120^0c 30 minute and 60 minute and 220^0c30 and 60 minute were selected for further analysis. The amount of hemicellulose was determined using equation 4 and the value was listed below in Table 11. The % of hemicellulose content was decrease when the torrifayed temperature increases. ASTM E1758 and NREL protocols.

Hemicellulose (%) = $\frac{M1 - M2}{M1} \times 100$

Table 11: The amount of hemicellulose content present in BSG sample

Sample ID	Initial biomass	mass of oven dried after hydrolysis with paper	mass of filter paper	residue after alkali treatment	% hemicellulose
120^0c for 30 min	0.5	1.8386	1.4581	0.3805	23.9
120^0c for30 min	0.5	1.8408	1.4583	0.3825	23.5
120^0c for 30 min	0.5	1.8424	1.4579	0.3845	23.1
120^0c for 60 min	0.5	1.8446	1.4601	0.3845	23.1
120^0c for 60 min	0.5	1.8462	1.4592	0.387	22.6
120^0c for 60 min	0.5	1.8444	1.4584	0.386	22.8

220^0c for 30 min	0.5	1.8579	1.4584	0.3995	20.1
220^0c for 30 min	0.5	1.8602	1.4592	0.401	19.8
220^0c for 30min	0.5	1.8603	1.4583	0.402	19.6
220^0c for 60 min	0.5	1.8626	1.4601	0.4025	19.5
220^0c for 60 min	0.5	1.8621	1.4586	0.4035	19.3
220^0c for 60 min	0.5	1.8627	1.4582	0.4045	19.1
Average					≈ 21.39

4.1.6 Lignin content determination

The amount of lignin was determined by taking 0.3g of all terrified sample in triplicate totally 48 samples (120^0c, 30 minute to 220^0c 90 minute) but for comparison purpose (120^0c 30 minute and 60 minute and 220^0c30 and 60 minute) were selected for further analysis. The amount of lignin was determined using equation 5 and the value was listed below in table 12.and also the acid soluble lignin was determined using absorbance value of the selected sample 220^oc for 60 minute sample in triplicate using equation 6.the % amount of lignin was decreases when the torrifayed temperature increase. (ASTM E1758 and NREL protocols) (2001).

$$\%\text{lignin} = \frac{\text{mass of filter paper plus residu} - \text{mass of filter paper}}{\text{Initial biomass}} \times 100$$

$$\text{ASL\%} = \frac{\text{Uvabs} \times \text{Volume filtrate} \times \text{Dilution}}{\varepsilon ODW\text{sample} \times \text{pathlength}} \times 100$$

Table 12: The amount of lignin content present in BSG sample

Sample ID	Initial biomass	mass of oven dried after hydrolysis with paper	mass of filter paper	% lignin
120^oc for 30 min	0.3	1.6405	1.4611	59.8
120^oc for 30 min	0.3	1.5971	1.4189	59.4

120^0c for 30 min	0.3	1.6401	1.4601	60
120^0c for 60 min	0.3	1.6356	1.4583	59.1
120^0c for 60 min	0.3	1.6388	1.4621	58.9
220^0c for 60 min	0.3	1.6383	1.458	58.6
220^0c for 30 min	0.3	1.6218	1.4601	53.9
220^0c for 30 min	0.3	1.6202	1.4579	54.1
220^0c for 30min	0.3	1.6221	1.4604	54
220^0c for 60 min	0.3	1.5771	1.4145	54.2
220^0c for 60 min	0.3	1.5759	1.4291	53.8
220^0c for 60 min	0.3	1.5997	1.4389	53.6
Average				≈ 56.57

The amount of cellulose, hemicellulose, lignin, ash content and moisture content of the BSG sample in % was approximately 32.69, 21.39, 56.57, 3 and 59.83 respectively. Generally this result good for bioethanol production because of the cellulose and hemicellulose content was very high and also the ash was very acceptable related to other literature review. Mussatto, S. I. (2014) but the amount of lignin content was too high and required further treatments. Lignin is not fermentable and forms a rigid barrier around cellulose and hemicellulose, making them harder to access. High lignin content reduces enzyme efficiency and can significantly lower ethanol yields unless effective pretreatment applied.

The amount of acid soluble lignin was also determined using absorbance value of selected sample (220^0c.60min) at 280 nm.to estimate how much of acid soluble lignin was passing though. Using the above formula equation 6.The amount of acid soluble lignin was listed below in Table 13.

Table 13: The amount of acid soluble lignin present in selected sample

Sample id	Abs	path length cm	ε	ODW	Volume of filtrate	ASL%
220^0c.60min	3.000	10	25	0.3	86ml	344
220^0c.60min	2.904	10	25	0.3	85ml	224.6
220^0c.60min	2.898	10	25	0.3	84ml	324.6

4.2 Hydrolysis

4.2.1 First stage NaOH followed by dilute acid (H_2SO_4) hydrolysis pre-treatments

This stage was the fundamental stage of pre-treatments. Because to enhance the glucose concentration and to decrease the inhibition and also to increase the amount of cellulose present in the BSG sample. The components of the BSG sample were very tightly bound especially by lignin. When we used NaOH treatments, breaks down lignin disrupts, lignin-carbohydrate bonds, and swells the cellulose fibres, making cellulose more accessible. After sodium hydroxide pre-treatment, the samples were followed to dilute sulphuric acid pre-treatment using different acid concentration (0.5%, 1.75% and 3%) and temperature (90^0c, 120^0c and 150^0c) respectively in triplicate. From dilute acid hydrolysis the cellulose obtained from alkali hydrolysis was converted in to simple sugar (glucose) the amount of glucose passing through the acid filtrate was calculated and the result was listed below in table 4.7 from standard value**.**(Agustina, T. E. (2017).

4.2.2 Calibration plot for glucose standard

The absorbance and concentration of glucose standards were measured using spectrophotometer. Derive the equation to determine the glucose concentration of the sample using the standard values. (Gómez Alegría, C.2020).

Table 14: The absorbance value of standard glucose

Concentration of glucose standard (molarity)	Absorbance value
0.1	0.106
0.2	0.110
0.3	0.114
0.4	0.117
0.5	0.119
0.6	0.121

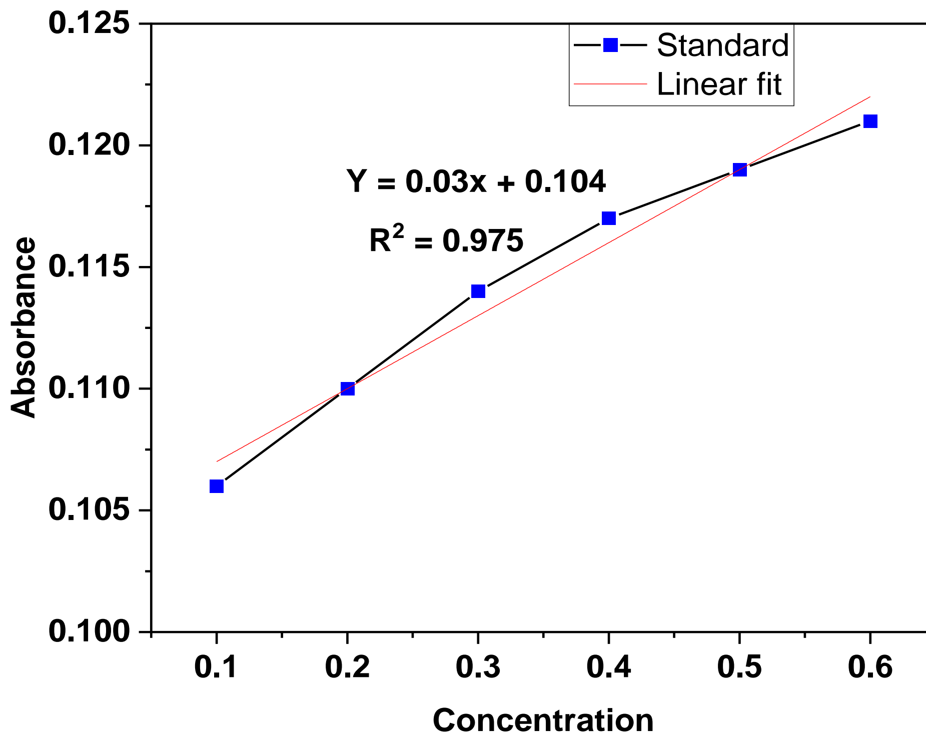


Figure 5: The calibration curve for standard glucose solution

The reduce content was determined using the standard glucose value. However, this value was not applied to all treated samples only to selected samples: 12:30, 120:60, 220:30, and 220:60 at a 3% concentration.

$$C = \frac{Abs - b}{M}$$

Where C: represents the total reduced sugar content.

Abs: stands for the unknown sample's absorbance,

B: for the standard curve's intercept, and

M: for the standard curve's slope, expressed in millilitres per gramme.

Table 15: The amount of reduced sugar for selected sample of filtrate `

Sample at 3%	Absorbance	Amount of glucose
120:30	0.699	19.833
120:60	0.563	15.3
220:30	0.350	8.2
220:60	0.210	3.5

The above table showed that the amount of sugar that passed though filtrate was very small in 220: 60 torified samples than 120:30 and 120:60 torified samples. At 120°C for 30 minutes, torrefaction is mild, which means: cellulose are largely preserved, remaining available for acid hydrolysis, The biomass structure becomes slightly more open, making it easier for acid to penetrate and break down polysaccharides into soluble sugars (like glucose and xylose) and These sugars are water-soluble and easily pass through filtration into the hydrolysate. In contrast, the 220°C for 60-minute torrefied sample underwent severe thermal treatment, which degrades hemicellulose before acid hydrolysis can act on it. Causes sugar decomposition into compounds like furfural and HMF, which are not measured as sugars and may not be soluble. The amount of lignin Promotes condensation and cellulose crystallinity, making hydrolysis less efficient. Results in lower sugar content in the filtrate**.**

The amount of cellulose residue was measured after first stage sodium hydroxide followed by dilute acid hydrolysis. The result was listed below in table 4.8.from this result observed that the mass of residue was increase when the concentration of acid increase. And also 220.30 minute and 220.60 minute sample were some little increment was observed rather than 120(30 and 60)

Table 16: The mass of residue after sodium hydroxide followed by acid hydrolysis

pre-treatment conditions	pre-treatment conditions	Mass of residue (g)			
		120:30	120:60	220:30	220:60
4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4 90^0c:30min	2, 2.1, 2	2.1, 2, 2.2	2.3, 2.1, 2.1	2.2, 2.3,2
4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4 90^0c:60min	2.3, 2, 2.2	2.3, 2.1,2	2, 2.3,2.1	2.3, 2.1, 2.2
4% NaOH at 30^0C for 60 minute	0.5% H_2SO_4 90^0c:90min	2.4, 2.1, 2.3	2.1, 2.1,2.1	2, 2.2 , 2	2.1, 2.3 ,2
4% NaOH at 30^0C for 60 minute	1.75% H_2SO_4 120^0c:30min	2.1, 2 ,2.3	1.9, 2.2 , 2	1.9, 2 , 2.2	2, 1.9 ,2.1
4% NaOH at 30^0C for 60 minute	1.75% H_2SO_4 120^0c:60min	2.3, 2.1, 2.2	2.2, 2.1, 2	2.1, 1.9 , 2	2.1, 2 1, 2
4% NaOH at 30^0C for 60 minute	1.75% H_2SO_4 120^0c:90min	2, 2.3 ,2	2, 2.3, 2	2.1, 2.1 ,2	2, 2.1,2
4% NaOH at 30^0C for 60 minute	3% H_2SO_4,150^0c :30 min	1.9, 2 ,1.9	2.1, 2,2, 2	2, 2.1 ,2.1	2, 2 ,2.1
4% at 30^0C for 60 minute	3% H_2SO_4,150^0c :60min	2.1, 1.9 , 2	2.2, 1.9 , 2.1	2.1, 2, 2	2.2, 2 ,2.1
4% NaOH at 30^0C for 60 minute	3% H_2SO_4,150^0c: 90min	1.9, 2 ,2.1	2, 2.1, 2	1.9, 2 ,2.1	2, 2.1 ,2

4.2.3 Second Stages Hydrolysis process using simultaneous scarification and fermentation (SSF)

The samples of residue from first stage sodium hydroxide followed by acid hydrolysis were collected and combine the triplicate in to one. Than 5g of sample was taken for second stage hydrolysis process. By adjust the acid in to 3%. The P^H of the solution was adjusting by P^H meter in to 5. Yeast was used to for fermentation process. The ratio of sample to yeast was 1:5.The ratio of the sample to acid was 1:10 than placed in to 250ml flask. The fermentation and the saccharification process was take place in to shaker incubator for three days. By reducing product inhibition and doing away with the need for separate reactors for

fermentation and saccharification, the SSF method boosts ethanol yields.Because yeast assimilates sugars quickly during SSF, the procedure also proved to be superior to saccharification and subsequent fermentation. Ethanol was fermented concurrently with reducing sugars generated during saccharification or cellulose hydrolysis. In order to determine the remaining glucose concentration using the standard glucose concentration, the samples were taken out at 24, 48, and 72 hours. From 220^0c:60 minute torrifayed samples. the spectrophotometer method was used to measure the absorbance of glucose at a wavelength of 540 nm in order to calculate its concentration.The absorbance and the glucose value were listed below in table 4.9 Hari Krishna, S., & Chowdary, G. V. (2000). The absorbance data was done in ASTU pharmaceutical laboratory.

Table 17: **The amount of glucose in different fermentation time**

sample id220^0c:60min	Abs 24h	Glucose content	Abs 48h	Glucose content	Abs 72h	Glucose content
3% 90^0c 30 min	2.683	85.96	2.312	73.6	1.701	53.23
3% 90^0c 60 min	2.976	95.73	2.35	74.86	1.901	59.9
3% 90^0c 90min	2.587	82.76	2.34	74.53	1.831	57.56
3% 120^0c 30 min	2.345	74.7	2.341	74.56	1.761	55.23
3% 120^0c 60 min	2.419	77.16	2.351	74.9	1.791	56.23
3% 120^0c 90min	2.852	91.6	2.33	74.2	1.851	58.23
3% 150^0c 30 min	2.552	81.6	2.021	63.9	1.711	53.56
3% 150^0c 60 min	2.417	77.1	2.205	70.03	1.685	52.7
3% 150^0c 90 min	2.373	75.63	2.332	74.26	1.798	56.46

The amount of glucose content remain at different time of fermentation (24h,48h and 72h) was plotted using the glucose concentration and the absorbance value from the above table. Graph plotted below.

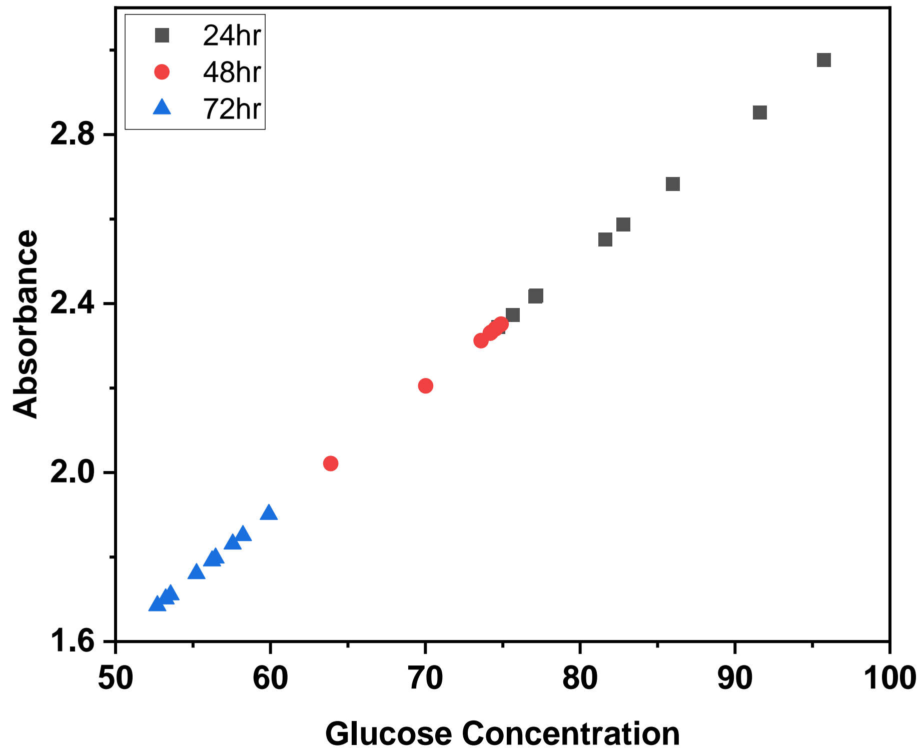


Figure 6: The graph of glucose concentration vs. absorbance

From the above table 4.10 and the above graph were explained the relationship between glucose concentration and the absorbance of the sample. From the above graph when the absorbance increases the concentration also increase and vies versa. When the fermentation day increases the amount of glucose was decrease. The glucose amount was decreases during fermentation over time because microorganisms (like yeast or bacteria) use the glucose as a source of energy and carbon to grow and carry out metabolic activities. As time goes on, microbes multiply and more glucose was used and Glucose was being converted to ethanol/lactic acid + CO_2, so its amount naturally drops. The concentration of glucose value for this research was expected and good change from 24h to 48h to 72h fermentation.

4.3 Distillation (Ethanol Separation)

Distillation was the final step to get the biofuel. The fermented BSG sample was after 72h duration of shaker incubator in to distillation set up after distillation process the amount of biofuel of each sample was measured. The volume of biofuel of each samples were listed in table 4.11 below. The biofuel volume of 120:30, 120:60, 220:30 and 220:60 torrefaied samples were some a little bet difference was observed in 120:30 and 120:60 samples was greater than 220:30 and 220:60 samples. Because The 220:30 and 220:60 samples produced higher biofuel efficiency because the torrefaction temperature of 220°C caused more thermal breakdown of biomass, especially cellulose, leading to the formation of more volatile compounds that contribute to biofuel production. In contrast, 120°C is too low for significant torrefaction, so less biofuel is generated. The volume was measured in graduated cylinder in ml.(D., Novak, S., & Šantek, B. (2018).

Table 18: The volume of the sample after distillation process

sample id120:30	Volume	sample id120:60	volume	sample id220:30	Volume	sample id220:60	Volume
3% 90^{0}c 30 min	5.5	3% 90^{0}c 30 min	5	3% 90^{0}c 30 min	4	3% 90^{0}c 30 min	4
3% 90^{0}c 60 min	5.5	3% 90^{0}c 60 min	5	3% 90^{0}c 60 min	4.5	3% 90^{0}c 60 min	4
3% 90^{0}c 90min	5.5	3% 90^{0}c 90min	5	3% 90^{0}c 90min	4	3% 90^{0}c 90min	4
3% 120^{0}c 30 min	5.5	3% 120^{0}c 30 min	5.5	3% 120^{0}c 30 min	4	3% 120^{0}c 30 min	4
3% 120^{0}c 60 min	5.5	3% 120^{0}c 60 min	5.5	3% 120^{0}c 60 min	4.5	3% 120^{0}c 60 min	4
3% 120^{0}c 90min	5.5	3% 120^{0}c 90min	5.5	3% 120^{0}c 90min	4.5	3% 120^{0}c 90min	4
3% 150^{0}c 30 min	5.5	3% 150^{0}c 30 min	5.5	3% 150^{0}c 30 min	4.5	3% 150^{0}c 30 min	4.5
3% 150^{0}c 60 min	5.5	3% 150^{0}c 60 min	5.5	3% 150^{0}c 60 min	4.5	3% 150^{0}c 60 min	4
3% 150^{0}c 60 min	5.5	3% 150^{0}c 60 min	5.5	3% 150^{0}c 60 min	4.5	3% 150^{0}c 60 min	4

4.4. Characterization of bioethanol

4.4.1. Oxidation of alcohols

The dichromate test indicated the presence of alcohol functional groups in all samples, as evidenced by the color change from orange to green. However, the extent of this change varied among the samples. The 120:30 min and 120:60 min samples exhibited a weaker or incomplete color transition compared to the others. Overall, samples torrefied at higher temperatures showed a more pronounced color change than those torrefied at lower temperatures, suggesting a greater relative abundance of oxidizable alcohol groups at elevated torrefaction conditions. (McMurry, J. (2023).

FT-lR determination of BSG Bioethanol

With the use of lR correlation charts, the functional group of the raw sample, 120:30, 12:60, 220:30, and 220:60 samples was identified using Prinks Elmer spectrum 65 FT-lR. From this FTIR results were four picks was observed. Those picks had own transmittance and wave number. Based on this value we predict the functional group of each picks. A. A. & Khluchina, N. A. (2023).According to some journals the functional group and their wave numbers were listed below. Based on this truth we predict the functional group present in BSG sample. S. K. Sharma (ed.) 2018.

Table 19: IR spectroscopy functional group vs wavenumber range

Functional group	Type of vibration	Wavenumber range	Note
O–H (alcohol)	Stretching (broad)	3200–3600	Broad, due to H-bonding
O–H (carboxylic acid)	Stretching (very broad)	2500–3300	Very broad, overlaps C–H
N–H (amine or amide)	Stretching	3300–3500	Sharp, may have one or two peaks
C–H (alkane)	Stretching	2850–2960	Common in most organic compounds
C–H (alkene)	Stretching	3010–3100	Just above alkane C–H
C–H (aromatic)	Stretching	3000–3100	Overlaps with alkene C–H
C≡C or C≡N	Triple bond stretching	2100–2260	Sharp, weak to medium intensity

C=O (carbonyl)	Stretching	1650–1750	Very strong, sharp
– Aldehyde	Stretching	1720–1740 + C–H at ~2720	2 peaks: C=O and aldehyde C–H
– Ketone	Stretching	1705–1725	Strong
– Carboxylic acid	Stretching	1700–1725	Very strong
– Ester	Stretching	1735–1750	Strong
– Amide	Stretching	1630–1690	Strong, also N–H bend around 1600
C=C (alkene)	Stretching	1600–1680	Medium intensity
C=C (aromatic)	Stretching	1450–1600	Multiple peaks
C–O (ether, ester, alcohol)	Stretching	1000–1300	Strong, often two peaks

Based on the above information in table 4.12 the BSG samples was contain four picks and obtained four functional group basically those are O-H alcohol (at 3300 cm^{-1}), C-H alkane ,alkene and alkyne (2973cm^{-1}), C=O carbonyl (1665 cm^{-1}) and C-O ether, ester (1025cm^{-1}).

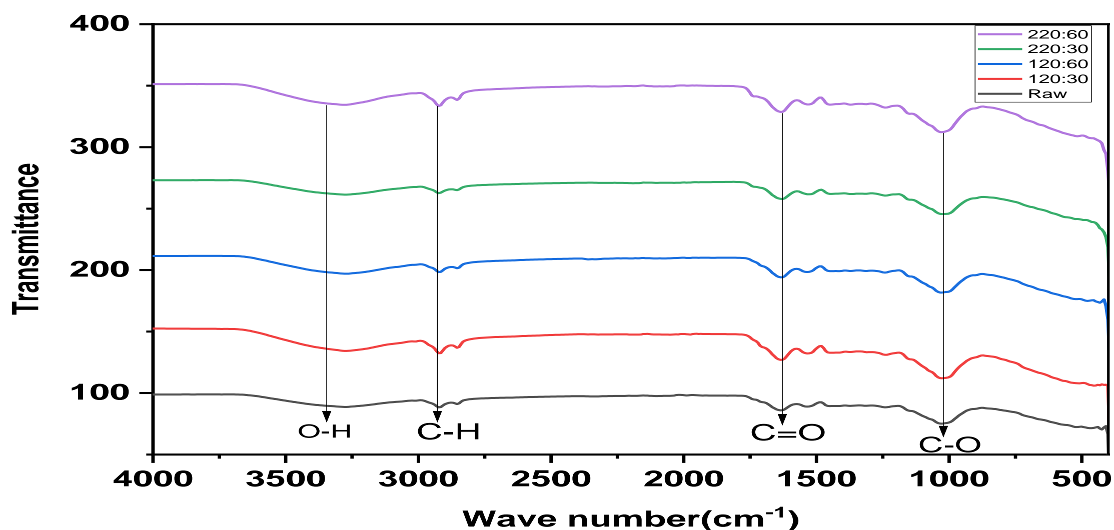


Figure 7: The FTIR result graph of the separated samples

Figure 18 shows the FTIR spectra of the raw BSG and torrefied samples produced at different temperatures and residence times (120:30, 120:60, 220:30, and 220:60). Although all samples retain the main spectral features of lignocellulosic biomass, clear differences in peak intensity and shape demonstrate progressive chemical modification with increasing torrefaction severity. The raw BSG spectrum was characterized by a strong and broad O–H stretching

band in the 3200–3500 cm^{-1} regions, reflecting the abundance of hydroxyl groups from cellulose, hemicellulose, and lignin. Pronounced C–H stretching bands around 2900–3000 cm^{-1} indicate aliphatic structures, while a distinct C=O band near 1650–1700 cm^{-1} suggests the presence of carbonyl-containing compounds. A strong C–O stretching band at approximately 1000–1100 cm^{-1} confirms the dominance of polysaccharide structures. This spectrum represents the chemically oxygen-rich nature of untreated BSG. Torrefaction at 120 °C for 30 minutes cause's only minor spectral changes relative to the raw sample. The O–H band shows a slight decrease in intensity, indicating limited dehydration. Other functional group bands remain largely unchanged, suggesting that this mild treatment was insufficient to significantly alter the chemical structure of BSG. Increasing the residence time to 60 minutes at 120 °C results in more noticeable, though still moderate, changes. The O–H stretching band further decreases and slight intensity changes appear in the C–O region. These trends indicate gradual degradation of hemicellulose and partial loss of oxygenated functional groups, while the overall biomass structure remains largely intact. At 220 °C, even with a shorter residence time, clear chemical transformations were evident. The O–H band was substantially reduced, reflecting enhanced dehydration and breakdown of hydroxyl-rich components. The relative intensity of aliphatic C–H bands increases, while the carbonyl band becomes more pronounced, suggesting structural rearrangement and formation of oxygen-containing groups during thermal decomposition.

The 220:60 sample exhibits the most significant spectral changes among all samples. The O–H band was weakest, indicating extensive removal of hydroxyl groups. The C–O stretching band shows a marked decrease, consistent with advanced degradation of cellulose and hemicellulose. Simultaneously, the increased prominence of C=O and C–H bands suggests enrichment of thermally stable, carbon-rich structures. These changes confirm that high temperature combined with longer residence time leads to substantial chemical transformation of BSG. Generally the Comparative analysis of all five samples demonstrates that torrefaction severity strongly governed the chemical evolution of BSG. Mild conditions (120 °C) result in limited dehydration and minor structural changes, whereas higher temperature and longer treatment duration (220:60) significantly reduce oxygenated functional groups and promote carbon enrichment. These transformations were consistent with the progressive

decomposition of hemicellulose followed by cellulose, while lignin becomes relatively more dominant. Such chemical changes were advantageous for applications requiring improved fuel quality and thermal stability.

4.4.2. Specific gravity of bioethanol

The density of ethanol was calculated using the above formula of equation 8. First the specific gravity of the selected sample was measured then multiplies with the density of water. The density of bioethanol was calculated for the selected sample only. Those are 120^0:30 minute and 220^0:30 minute distilled bioethanol (Tekounegnin, & Okale, A. N. (2011)

Table 20: The density of the selected samples

120^0:30	Volume	Density	220^0:30	volume	Density
3% 90^0c 30 min	5.5	0.891gmL^{-1}	3% 90^0c 30 min	4	0.785 gmL^{-1}
3% 90^0c 60 min	5.5	0.891gmL^{-1}	3% 90^0c 60 min	4.5	0.778 gmL^{-1}
3% 90^0c 90min	5.5	0.891gmL^{-1}	3% 90^0c 90min	4	0.785 gmL^{-1}

4.4.3. Combustion test of distilled ethanol

The combustion of ethanol was tested by putting small amount of ethanol in to a glass dish and turn on the light; little light was observed but not continually burn because of the ethanol quality and the fuel properties of the ethanol in 120^0c 30 and 60 minute very little. But in 220^0c 30 and 60 minute the flam was very high like fuel. Barraza-Botet, C. L. Wagnon, S. W. & Wooldridge, M. S**.** (2016).The combustion reaction of ethanol is illustrated below

$$C_2H_5OH\ (L) + 3O_2\ (g) \longrightarrow 2CO_2\ (g) + 3H_2O\ (L)$$

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The production of bioethanol is sensitive issue know a day because a lot of country suffering by the shortage of diesel for transportation purpose and also the fossil fuel is the non-renewable natural resource. Using of this non-renewable natural resource for different functions had also a problem for different disease. Because at the time combustion of the fossil fuels a lot of disease causing gasses are exerted in to the atmosphere. Those gasses are directly contact with human at the time of inhaling. Using of bioethanol instead of fossil fuels had potential advantages rather than disadvantages. Such as

A. Renewable Source of plants

The biomass for bioethanol production is corn, sugarcane, wheat BSG and cellulosic materials is not need millions years to form like fossil fuel can be produce annually.

B. Lower Greenhouse Gas Emissions

The amount of carbon dioxide emits in bioethanol combustion is less than fossil fuels.

C. Energy Security and Diversification

It reduces or decreases the oil import from other country. And also introduce domestic energy production and promote national energy independence.

D. Cleaner Combustion

The bioethanol burns cleaner than gasoline. And decrease the emission of CO, SOx and unburned hydrocarbons.

E. Agricultural and Rural Development

Promote a market for agricultural products and residues and boosting the economies by creating jobs for producers, transport and bioethanol processing.

F. High Octane Rating

This product has a high octane rating and increasing the engine performance and decreasing Knocking. Often used as a fuel additive such as E10,E85 to increase the gasoline quality.

G. Biodegradable and Safer to Handle the bioethanol

The bioethanol is less toxic and biodegradable than fossil fuels like gasoline. And has low risk of environmental damage.

H. Potential for Waste Utilization

This biofuel are produce from non-food biomass for example agricultural waste, forestry residues, algae and reduces waste and promotes circular economy or the principle changes of waste in to value added products.

I. Used in Existing Engines (Blended)

By blended with gasoline and used in combustion engines for example E10 = 10% ethanol, 90% petrol.s

5.2. Recommendations

However, for the coming study should consider the production and optimization of bioethanol from brewing spent grain (BSG) was applicability of the bioethanol and also how to blend with gasoline. i recommended that when hydrolysed the sample we must be careful using of the acid and base concentration. Handling of acid and base are basic consideration. I recommend that all steps in short like Characterize, Apply Effective Pre-treatment, Optimize Saccharification (Enzymatic Hydrolysis), Use Appropriate Microorganisms for Fermentation, Monitor and Minimize Inhibitors, Consider Simultaneous Saccharification and Fermentation (SSF), Maximize Ethanol Recovery and Analyze Economic and Environmental Viability.

5.3 Summary

At the production of bioethanol from brewery spent grain the sample were directly collected from Mojo Anbesa beer industry or united beverage directly from the machine and had a lot of moisture, the amount of moisture content was determined approximately 60%, the ash content also determined using furnace the ash content was approximately 3%. The sample was dried for five days by using sun light. After drying, Then the dried sample was sieved and milled the over size in to appropriate particle size which is less than 2mm. Finally the sample was ready for further analysis. The bioethanol production from brewery spent grain according to this research work basically four steps. The first pretrtement technique was torrefaction process. Torrefaction is a mild thermal treatment process used to improve the fuel properties of biomass. It involves heating biomass (like wood chips, agricultural residues, or brewers' spent grain—BSG) in the absence of oxygen, typically between 200°C and 300°C, for a set

period. The torrefaction process was take place 40g of sample at different temperature and time (120^0C, 150^0c, 180^0c and 220^0c for 10 minute, 30 minute, 60 minute and 90 minute) in triplicate totally 48 torrefied sample. The main purpose of torrefaction is reduce moisture, increase energy density, improve grind ability, enhance stability, Reduce smoke & Volatiles Removes tars and volatiles, resulting in cleaner combustion and for uniformity purpose. After this process the amount of cellulose, hemi cellulose and lignin was determined. Depend on the amount of those three particles the followed step was first stage hydrolysis and second stage hydrolysis of selected (120^0c 30 minute and 60 minute) and (220^0c 30 minute and 60 minute) sample was done. The first stage hydrolysis processes were done using 4% sodium hydroxide for 6o minute followed to 0.5%, 1.75% and 3% dilute sulphuric acid hydrolysis. The main importance of this first stage sodium hydroxide followed to dilute acid hydrolysis was to remove the lignin from the sample and to convert the cellulose material in to glucose conversion. Than the second stage was done using SSF method means scarification and fermentation was take place one using shaker incubator. The ratio of acid to sample was 10.1 and the sample to yeast ratio is sample was 5.1. Scarification is the enzymatic or chemical breakdown of complex carbohydrates (like cellulose and hemicellulose) into simple sugars (mainly glucose and xylose), which can then be fermented by microorganisms (like yeast) to produce ethanol**.** The amount of glucose at different time (24h, 48h and 72h) was determined. The final step was distillation it is the separation technics of ethanol from water at 78-80^0c. After distillations some characterization technics was done to know the bioethanol in detailed. Oxidation of alcohols, FTIR, density, and combustion of alcohols were characterized.

CHAPTER SIX

References

Abas, N., Kalair, A., & Khan, N. (2015). *Review of fossil fuels and future energy technologies*. **Energy Reports, 1**, 1–10. https://doi.org/10.1016/j.egyr.2014.11.001

Alfani, F., Gallifuoco, A., Saporosi, A., Spera, A., & Cantarella, M. (2000). Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw. *Journal of Industrial Microbiology and Biotechnolgy*, 184–192.

Arredondo, V., & Junior, R. C. A. (2009). Ethanol Production from Banana Fruit and its Lignocellulosic Residues. *Exergy and Renewability Analysis* *, *12*(3), 155–162.

ASTM International. (2001). *ASTM E1758-01: Standard method for the determination of carbohydrates by high performance liquid chromatography*. West Conshohocken, PA: ASTM International. https://doi.org/10.1520/E1758-01

ASTM International. (2024). *Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography (ASTM E1758-24)*. ASTM International.

ASTM International. (2025). *Standard Test Method for Determination of Cellulose/Hemicellulose-Derived Glucan and Galactan Content in Solid Corn and Corn-Sorghum Blended Biomass Samples (ASTM E3417-25)*. ASTM International.

Axelsson, L., Franzén, M., Ostwald, M., Berndes, G., Lakshmi, G., & Ravindranath, N. H. (2012). *Perspective: Jatropha cultivation in southern India: Assessing farmers' experiences*.

AGRIS - home. (n.d.). Retrieved 15 April 2025, from http://agris.fao.org/agris.

ASTM International. (2020). *Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography (ASTM E1758-01(2020))* [Standard]. ASTM International.

ASTM International. (2001). *ASTM E1758-01: Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography*. ASTM International. https://doi.org/10.1520/E1758-01

ASTM International. (2013). *ASTM E1755-01(2013): Standard Test Method for Ash in Biomass*. ASTM International. https://doi.org/10.1520/E1755-01R13

Barraza-Botet, C. L., Wagnon, S. W., & Wooldridge, M. S. (2016). Combustion chemistry of ethanol: Ignition and speciation studies in a rapid compression facility. *The Journal of Physical Chemistry A, 120*(38), 7408–7418. https://doi.org/10.1021/acs.jpca.6b06725

Bokulich, N. A., & Bamforth, C. W. (2013). The microbiology of malting and brewing. *Microbiology and Molecular Biology Reviews: MMBR*, *77*(2), 157–172. doi:10.1128/MMBR.00060-12

Bušić, A., Marđetko, N., Kundas, S., Morzak, G., Belskaya, H., Šantek, M. I., Komes, D., Novak, S., & Šantek, B. (2018). Bioethanol production from renewable raw materials and its separation and purification: A review. *Food Technology and Biotechnology, 56*(3), 289–311. https://doi.org/10.17113/ftb.56.03.18.5546

Bridgeman, T. G., Jones, J. M., Shield, I., & Williams, P. T. (2008). Torrefaction of reed canary grass, wheat straw and willow to enhance solid fuel qualities and combustion properties. *Fuel, 87*(6), 844–856. https://doi.org/10.1016/j.fuel.2007.05.041

Chan-u-tit, P., Laopaiboon, L., Jaisil, P., & Laopaiboon, P. (2013). High Level ethanol production by nitrogen and osmoprotectant supplementation under very high gravity fermentation conditions. *Energies*, *6*(2), 884–899. doi:10.3390/en6020884

Demirbas, A. (2005). Bioethanol from Cellulosic Materials: A Renewable Motor Fuel from Biomass. *Biomass. Energy Sources*, *27*, 327–337.

Dussán, K. J., Silva, D. D. V., Moraes, E. J. C., Arruda, P. V., & Felipe, M. G. A. (n.d.). Dilute-acid hydrolysis of cellulose to glucose from sugarcane bagasse. *Chemical Engineering Transactions*. doi:10.3303/CET1438073

Da Silva, S. S., & Chandel, A. K. (Eds.). (2012). *D-Xylitol* (2012th ed.) [PDF]. doi:10.1007/978-3-642-31887-0

Demirbas, A. (2005). Bioethanol from Cellulosic Materials: A Renewable Motor Fuel from Biomass. *Biomass. Energy Sources*, *27*, 327–337.

Dussán, K. J., Silva, D. D. V., Moraes, E. J. C., Arruda, P. V., & Felipe, M. G. A. (n.d.). Dilute-acid hydrolysis of cellulose to glucose from sugarcane bagasse. *Chemical Engineering Transactions*. doi:10.3303/CET1438073

Hari Krishna, S., & Chowdary, G. V. (2000). Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass**.** *Journal of Agricultural and Food Chemistry, 48*(5), 1971–1976. https://doi.org/10.1021/jf991296z

Jackowski, M., Niedzwiecki, Ł., Mościcki, K., & Pawlak-Kruczek, H. (2021). *Synergetic co-production of beer colouring agent and solid fuel from brewers' spent grain in the circular economy perspective*. Sustainability, 13(18), 10480. https://doi.org/10.3390/su131810480

Kostryukov, S. G., Matyakubov, H. B., Masterova, Y. Y., Kozlov, A. S., Pryanichnikova, M. K., Pynenkov, A. A., & Khluchina, N. A. (2023). *Determination of lignin, cellulose, and hemicellulose in plant materials by FTIR spectroscopy*. Journal of Analytical Chemistry, 78**,** 718–727. https://doi.org/10.1134/S1061934823040093

Kopylovich, M. N., Ribeiro, A., Alegria, E., & Martins, N. M. R**.** (2015). Catalytic oxidation of alcohols: Recent advances. In *Advances in Organometallic Chemistry* (Vol. 63, pp. 91–174). https://doi.org/10.1016/bs.adomc.2015.02.004

McMurry, J**.** (2023). *Organic Chemistry*. OpenStax. Section 17.7: Oxidation of Alcohols. Retrieved from https://openstax.org/books/organic-chemistry/pages/17-7-oxidation-of-alcohol

Novy, V., Longus, K., & Nidetzky, B. (2015). From wheat straw to bioethanol: integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production. *Biotechnology for Biofuels*, *8*(1), 46. doi:10.1186/s13068-015-0232-0

Nikolic, S., Pejin, J., & Mojovic, L. (2016). Challenges in bioethanol production: Utilization of cotton fabrics as a feedstock. *Chemical Industry and Chemical Engineering Quarterly*, *22*(4), 375–390. doi:10.2298/ciceq151030001n

National Renewable Energy Laboratory (NREL). (2011). *SSF Experimental Protocols: Laboratory Analytical Procedures for Hydrolysis and Fermentation of Biomass* (LAP-009). NREL. https://www.nrel.gov/docs/gen/fy11/42630.pdf

Orji, F. A., Dike, E. N., Lawal, A. K., Sadiq, A. O., Suberu, Y., Famotemi, A. C., & Elemo, G. N. (2016). *Properties of Bacillus species Cellulase Produced Using Cellulose from Brewers Spent Grain (BSG) as Substrate*. 142–148

Olofsson, K., Wiman, M., & Lidén, G. (2008). *Controlled feeding of cellulases improves conversion of xylose in simultaneous saccharification and co-fermentation of pretreated spruce*. Enzyme and Microbial Technology, 42(10), 624–630. https://doi.org/10.1016/j.enzmictec.2008.02.004

Parsapour, A. (2012). *Biogas Production System as an - Upcycler ‖ Biogas Production Systemas an - Upcycler*.

Rivera-Díaz, P., Gómez Camargo, D. E., Ondo-Méndez, A., & Gómez Alegría, C. (2020). A colorimetric bioassay for quantitation of both basal and insulin-induced glucose consumption in 3T3-L1 adipose cells. *Analytical Biochemistry*, *598*, Article 113720. https://doi.org/10.1016/j.ab.2020.113720

Rojas-Chamorro, J. A., Romero, I., Ruiz, E., Cara, C., & Castro, E. (n.d.). Comparison of fermentation strategies for ethanol production from pretreated brewers spent grain. *Chemical Engineering Transactions*. doi:10.3303/CET1761104

Santos, M., Jimenez, J. J., Bartolome, B., Gomez-Cordoves, C., & Nozal, D. (2003). Variability of brewers' spent grain within a brewery. *Food Chem*, *80*, 17–21.

Soupioni, M., Golfinopoulos, A., Kanellaki, M., & Koutinas, A. A. (2013). Study of whey fermentation by kefir immobilized on low cost supports using 14C-labelled lactose. *Bio resource Technology*, *145*, 326–330. doi:10.1016/j.biortech.2012.12.131

Sharma, S. K. (Ed.). (2018). *Handbook of Materials Characterization* (Chapter 9). Springer International Publishing AG, part of Springer Nature. https://doi.org/10.1007/978-3-319-92955-2_9

Silva, N. C., Santos, A. O., Duarte, C. R., & Barrozo, M. A. S. (2024). *Refractance Window Drying as an Alternative Method for Brewer's Spent Grain Preservation*. *Applied Biosciences, 3*(1), 71-86. https://doi.org/10.3390/applbiosci3010005

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2012). Determination of Structural Carbohydrates and Lignin in Biomass (NREL/TP-510-42618). National Renewable Energy Laboratory. https://www.nrel.gov/docs/gen/fy13/42618.pdf

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2006). *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples (NREL/TP-510-42623).* National Renewable Energy Laboratory. https://www.nrel.gov/docs/gen/fy08/42623.pdf

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2008). *Determination of Ash in Biomass (NREL/TP-510-42622).* National Renewable Energy Laboratory. https://www.nrel.gov/docs/gen/fy08/42622.pdf

Tsoutsos, T., & Bethanis, D. (2011). Optimization of the dilute acid hydrolyzator for cellulose-to-bioethanol saccharification. *Energies*, *4*(10), 1601–1623. doi:10.3390/en4101601

Appendix

The following figures was showed that when I do this research work starting from the first work Abe to the final steps. The figures were listed below with explanations in alphabetical order.

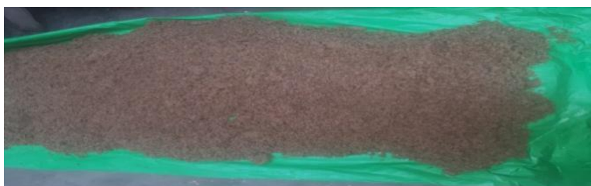


Figure 8: Appendix A the wet sample directly from the industry

Figure 9: Appendix B a dried sample after dry sun light

Figure 10: Appendix c the milled dry sample Figure 11: Appendix D at the time of sieving using appropriate sieve

Figure 12: Appendix E 48 torrified sample using furnace

After torrifaction process the amount of cellulose, hemi cellulose and lignin of 48 samples was determined and the values were listed below.

The cellulose content of all treated sample

Table 21: the cellulose content of all treated sample

Sample ID	Initial Dry Biomass (g)	mass of dried residue with paper	mass of paper	Dried Residue (g)	Ash Content (g)	Cellulose (%)
120^0c for 10 min	0.3	1.6149	1.4871	0.1278	0.03	32.6
120^0c for 10 min	0.3	1.607	1.487	0.12	0.03	30
120^0c for 10 min	0.3	1.613	1.4873	0.1257	0.03	31.9
120^0c for 30 min	0.3	1.61613	1.4901	0.12603	0.03	32.01
120^0c for 30 min	0.3	1.616	1.4873	0.1287	0.03	32.9
120^0c for 30 min	0.3	1.6164	1.4901	0.1263	0.03	32.1
120^0c for 60 min	0.3	1.6158	1.4874	0.1284	0.03	32.8
120^0c for 60 min	0.3	1.6145	1.4873	0.1272	0.03	32.4
120^0c for 60 min	0.3	1.6083	1.4796	0.1287	0.03	32.9
120^0c for 9o min	0.3	1.6158	1.4871	0.1287	0.03	32.9
120^0c for 9o min	0.3	1.6151	1.4876	0.1275	0.03	32.5
120^0c for 9o min	0.3	1.6139	1.487	0.1269	0.03	32.3
150^0c for 10 min	0.3	1.61623	1.4872	0.12903	0.03	33.01
150^0c for 10 min	0.3	1.6166	1.4873	0.1293	0.03	33.1
150^0c for 10 min	0.3	1.6089	1.4799	0.129	0.03	33
150^0c for 30 min	0.3	1.6169	1.4873	0.1296	0.03	33.2
150^0c for 30 min	0.3	1.6167	1.4874	0.1293	0.03	33.1
150^0c for 30 min	0.3	1.6171	1.4872	0.1299	0.03	33.3

150^{0}c for 60 min	0.3	1.6168	1.4875	0.1293	0.03	33.1
150^{0}c for 60 min	0.3	1.6176	1.4874	0.1302	0.03	33.4
150^{0}c for 60 min	0.3	1.6181	1.4876	0.1305	0.03	33.5
150^{0}c for 90 min	0.3	1.6175	1.4873	0.1302	0.03	33.4
150^{0}c for 90 min	0.3	1.6179	1.4871	0.1308	0.03	33.6
150^{0}c for 90 min	0.3	1.6189	1.4872	0.1317	0.03	33.9
180^{0}c for 10min	0.3	1.6182	1.4871	0.1311	0.03	33.7
180^{0}c for 10min	0.3	1.6184	1.487	0.1314	0.03	33.8
180^{0}c for 10min	0.3	1.61867	1.4873	0.13137	0.03	33.79
180^{0}c for 30 min	0.3	1.6188	1.4872	0.1316	0.03	33.89
180^{0}c for 30 min	0.3	1.619	1.4873	0.1317	0.03	33.9
180^{0}c for 30 min	0.3	1.6195	1.4872	0.1323	0.03	34.1
180^{0}c for 60 min	0.3	1.6191	1.4871	0.132	0.03	34
180^{0}c for 60 min	0.3	1.61933	1.4873	0.13203	0.03	34.01
180^{0}c for 60 min	0.3	1.6199	1.4873	0.1326	0.03	34.2
180^{0}c for 90 min	0.3	1.61917	1.4872	0.13197	0.03	33.99
180^{0}c for 90 min	0.3	1.62017	1.4873	0.13287	0.03	34.29
180^{0}c for 90 min	0.3	1.6201	1.4872	0.1329	0.03	34.3
220^{0}c for 10 min	0.3	1.6209	1.4874	0.1335	0.03	34.5
220^{0}c for 10 min	0.3	1.6208	1.4876	0.1332	0.03	34.4
220^{0}c for 10 min	0.3	1.6215	1.4874	0.1341	0.03	34.7
220^{0}c for 30 min	0.3	1.6212	1.4877	0.1335	0.03	34.5
220^{0}c for 30 min	0.3	1.622	1.4876	0.1344	0.03	34.8
220^{0}c for 30 min	0.3	1.6223	1.4873	0.135	0.03	35

220^0c for 60 min	0.3	1.6218	1.4871	0.1347	0.03	34.9
220^0c for 60 min	0.3	1.6235	1.4873	0.1362	0.03	35.4
220^0c for 60 min	0.3	1.6249	1.4872	0.1377	0.03	35.9
220^0c for 90 min	0.3	1.6371	1.4874	0.1374	0.03	35.8
220^0c for 90 min	0.3	1.6347	1.4874	0.1377	0.03	35.9
220^0c for 90 min	0.3	1.6368	1.4874	0.1386	0.03	36.2

The hemicellulose content result of all treated sample

Table 22: The hemicelluloses contents of all treated sample

Sample ID	Initial Dry Biomass (g)	Ash Content (g)	mass of paper	mass of residue with paper after alkali treatment	Post-Alkali Residue (g)	Hemicellulose (%)
120^0c for 10 min	0.5	0.03	1.4878	1.8633	0.3755	24.9
120^0c for 10 min	0.5	0.03	1.4871	1.8641	0.377	24.6
120^0c for 10 min	0.5	0.03	1.4872	1.8667	0.3795	24.1
120^0c for 30 min	0.5	0.03	1.487	1.8665	0.3795	24.1
120^0c for 30 min	0.5	0.03	1.4871	1.8671	0.38	24
120^0c for 30 min	0.5	0.03	1.4873	1.86755	0.38025	23.95
120^0c for 60 min	0.5	0.03	1.4874	1.8689	0.3815	23.7
120^0c for 60 min	0.5	0.03	1.4901	1.87265	0.38255	23.49
120^0c for 60 min	0.5	0.03	1.4873	1.8716	0.3843	23.14
120^0c for 9o min	0.5	0.03	1.487	1.87195	0.38495	23.01
120^0c for 9o min	0.5	0.03	1.4875	1.872	0.3845	23.1
120^0c for 9o min	0.5	0.03	1.4875	1.8725	0.385	23
150^0c for 10 min	0.5	0.03	1.4874	1.8724	0.385	23

150^0c for 10 min	0.5	0.03	1.4873	1.87235	0.38505	22.99
150^0c for 10 min	0.5	0.03	1.4872	1.87235	0.38515	22.97
150^0c for 30 min	0.5	0.03	1.4871	1.87255	0.38545	22.91
150^0c for 30 min	0.5	0.03	1.4873	1.8728	0.3855	22.9
150^0c for 30 min	0.5	0.03	1.4875	1.8735	0.386	22.8
150^0c for 60 min	0.5	0.03	1.4874	1.8754	0.388	22.4
150^0c for 60 min	0.5	0.03	1.4876	1.8771	0.3895	22.1
150^0c for 60 min	0.5	0.03	1.49	1.87995	0.38995	22.01
150^0c for 90 min	0.5	0.03	1.4873	1.8768	0.3895	22.1
150^0c for 90 min	0.5	0.03	1.487	1.877	0.39	22
150^0c for 90 min	0.5	0.03	1.4874	1.87765	0.39025	21.95
180^0c for 10min	0.5	0.03	1.4875	1.87795	0.39045	21.91
180^0c for 10min	0.5	0.03	1.4875	1.87805	0.39055	21.89
180^0c for 10min	0.5	0.03	1.4873	1.8783	0.391	21.8
180^0c for 30 min	0.5	0.03	1.4874	1.8824	0.393	21.4
180^0c for 30 min	0.5	0.03	1.4871	1.88165	0.39455	21.09
180^0c for 30 min	0.5	0.03	1.4876	1.88255	0.39495	21.01
180^0c for 60 min	0.5	0.03	1.4873	1.8823	0.395	21
180^0c for 60 min	0.5	0.03	1.4876	1.8827	0.3951	20.98
180^0c for 60 min	0.5	0.03	1.4873	1.8828	0.3955	20.9
180^0c for 90 min	0.5	0.03	1.4873	1.88385	0.39655	20.69
180^0c for 90 min	0.5	0.03	1.4874	1.8832	0.3958	20.84
180^0c for 90 min	0.5	0.03	1.4875	1.88535	0.39785	20.43

220^0c for 10 min	0.5	0.03	1.4877	1.8862	0.3985	20.3
220^0c for 10 min	0.5	0.03	1.4874	1.8869	0.3995	20.1
220^0c for 10 min	0.5	0.03	1.4872	1.88715	0.39995	20.01
220^0c for 30 min	0.5	0.03	1.4875	1.8876	0.4001	19.98
220^0c for 30 min	0.5	0.03	1.4901	1.8906	0.4005	19.9
220^0c for 30 min	0.5	0.03	1.4874	1.8887	0.4013	19.74
220^0c for 60 min	0.5	0.03	1.4873	18903	0.403	19.4
220^0c for 60 min	0.5	0.03	1.487	1.88935	0.40235	19.53
220^0c for 60 min	0.5	0.03	1.4877	1.89165	0.40395	19.21
220^0c for 90 min	0.5	0.03	1.4878	1.8918	0.404	19.2
220^0c for 90 min	0.5	0.03	1.487	1.892	0.405	19
220^0c for 90 min	0.5	0.03	1.4878	1.8929	0.4051	18.98

Lignin content result of all treated sample

Table 23: the lignin content result of all treated sample

Sample ID	Initial Dry Biomass (g)	mass of oven dried after hydrolysis with paper	mass of filter paper	% of lignin
120^0c for 10 min	0.3	1.5422	1.4873	18.3
120^0c for 10 min	0.3	1.5453	1.4901	18.4
120^0c for 10 min	0.3	1.5444	1.4901	18.1
120^0c for 30 min	0.3	1.5438	1.4901	17.9
120^0c for 30 min	0.3	1.5414	1.4874	18
120^0c for 30 min	0.3	1.5396	1.487	17.4
120^0c for 60 min	0.3	1.5393	1.4738	17.3
120^0c for 60 min	0.3	1.5387	1.4871	17.2

120^0c for 60 min	0.3	1.5385	1.4872	17.1
120^0c for 9o min	0.3	1.5385	1.4872	17.1
120^0c for 9o min	0.3	1.5332	1.4789	18.1
120^0c for 9o min	0.3	1.5337	1.4797	18
150^0c for 10 min	0.3	1.5277	1.4749	17.6
150^0c for 10 min	0.3	1.5409	1.4872	17.9
150^0c for 10 min	0.3	1.5405	1.4871	17.8
150^0c for 30 min	0.3	1.5432	1.4901	17.7
150^0c for 30 min	0.3	1.5395	1.487	17.5
150^0c for 30 min	0.3	1.5397	1.4872	17.5
150^0c for 60 min	0.3	1.5398	1.487	17.6
150^0c for 60 min	0.3	1.5393	1.4871	17.4
150^0c for 60 min	0.3	1.5395	1.4873	17.4
150^0c for 90 min	0.3	1.5419	1.49	17.3
150^0c for 90 min	0.3	1.542	1.4901	17.3
150^0c for 90 min	0.3	1.5387	1.4871	17.2
180^0c for 10min	0.3	1.538	1.487	17
180^0c for 10min	0.3	1.5377	1.487	16.9
180c for 10min	0.3	1.5383	1.487	17.1
180^0c for 30 min	0.3	1.5378	1.4871	16.9
180^0c for 30 min	0.3	1.5372	1.4874	16.6
180^0c for 30 min	0.3	1.5363	1.4871	16.4
180^0c for 60 min	0.3	1.5365	1.4873	16.4

180^0c for 60 min	0.3	1.5362	1.4873	16.3
180^0c for 60 min	0.3	1.5356	1.4873	16.1
180^0c for 90 min	0.3	1.5348	1.4871	15.9
180^0c for 90 min	0.3	1.5353	1.4873	16
180^0c for 90 min	0.3	1.518	1.4706	15.8
220^0c for 10 min	0.3	1.5372	1.4901	15.7
220^0c for 10 min	0.3	1.5343	1.4872	15.7
220^0c for 10 min	0.3	1.5343	1.4901	15.7
220^0c for 30 min	0.3	1.5337	1.4873	15.5
220^0c for 30 min	0.3	1.534	1.4872	15.6
220^0c for 30 min	0.3	1.5259	1.4797	15.4
220^0c for 60 min	0.3	1.5252	1.4796	15.2
220^0c for 60 min	0.3	1.5166	1.471	15.2
220^0c for 60 min	0.3	1.5323	1.4873	15
220^0c for 90 min	0.3	1.5322	1.4869	15.1
220^0c for 90 min	0.3	1.5316	1.4869	14.9
220^0c for 90 min	0.3	1.5322	1.4872	15

From the above three table were observed that the three components was not the same amount as the temperature and the treatment time increase because of Different Thermal Stabilities. Hemicellulose was least thermally stable, Starts to degrade as low as 150°C, and significantly decomposes by 180–200°C.It breaks down into soluble sugars, acids, and volatile compounds. It is branched and amorphous, so it's easily hydrolysed or thermally decomposed. Lignin More thermally stable than hemicellulose, but less stable than cellulose**.** Begins partial depolymerisation around 180°C, continues above 200°C. Complex, cross-linked structure resists full breakdown at moderate temperatures. Decomposes more slowly, but over time and with higher heat, it starts to solubilize or form char. cellulose Most

thermally stable. Crystalline structure resists breakdown. Only begins to degrade significantly at 250–300°C.During treatment up to 220°C, cellulose remains mostly intact. That why the amounts of cellulose increase but the hemicellulose and lignin content was decrease. Summary of the three components was listed below.

Table 24: The three Component vs Behaviour as Temperature

Component	Behaviour as Temperature Increase	reasons
Cellulose	Appears to increase	Thermally stable; others degrade
Hemicellulose	Decreases rapidly	Low thermal stability (starts at ~150°C)
Lignin	Decreases slowly	Cross-linked, degrades above ~180°C

Figure 13: First stage hydrolysis sodium hydroxide followed by dilute acid hydrolysis filtration process**.**

Figure 14: Second stage hydrolysis process using shaker incubator and SSF process Figure 15: Glucose content determination at 24h, 48h, and 72h

Figure 16: At distillation time to separate the fermented ethanol in to water at 78-80^{0}c in analytical laboratory

Figure 17: Standard glucose solution preparation Figure 18: The oxidation of alcohols test

Figure 19: The collected ethanol after distillation process