ADDIS ABABA INSTITUE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

BIOCHEMICAL ENGINEERING STREAM

BIO-BUTANOL PRODUCTION FROM NAPIER GRASS HYDROLYSIS USING CLOSTRIDIUM ACETOBUTYLICUM

BY

BIRUK YOHANNES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A MASTER'S DEGREE IN School of CHEMICAL BIOENGINEERING, Biochemical Engineering Stream

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ADDIS ABABA

ETHIOPIA

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A thesis submitted to the Research and Graduate School of Addis Ababa University, Addis Ababa Institute of Technology, School of Chemical and Bio-Engineering in partial fulfillment of the requirements for the degree of Masters of Science in Chemical Engineering (Biochemical Engineering Stream)

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Approved by the Examining Board

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Declaration

I Biruk Yohannes hereby Declared that this Master Degree thesis work titled Bio-Butanol Production from Hydrolysates of Napier Grass Using *Clostridium Acetobutylicum* is my novel work and this study has not been submitted for the award of in any program Addis Ababa institute of Technology or any other institutions I have carried out the presented study independently with the guidance and Support of the research advisor Dr. Solomon Kiros. Any other research or academic sources used in the study have been duly acknowledged.

Biruk Yohannes

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Contents

Abstract

Napier grass is a potential biomass for biofuel production due to its high adaptability and widespread availability in tropical areas such as Ethiopia. A compositional analysis of Napier grass collected from the Menagesha Mushroom farm in an Addis Ababa city suburb was performed, and microbes and enzymes were purchased from France. An alkaline pretreatment (2.5wt% NaOH, 1.5wt% urea, and 8wt% sample biomass) was mixed for 40 minutes at 121°C, followed by enzymatic hydrolysis (2ml Novozymes Cellic glucadase enzyme and 5gram pretreated biomass) shaken at 50rpm for 40 minutes at 50°C, and separated by centrifuge at 8500rpm to hydrolyze Napier grass. Carbohydrates make up 52.1 % of the Napier grass composition, making it an excellent carbon source. Acetone-Butanol-Ethanol fermentation (ABE) was carried out in a continuous fermentation process using co-cultured C. acetobutylicum bacteria. After optimizing the operating parameters pH, Temperature, substrate concentration, and fermentation time, the productivity of butanol was found to be 0.077 g lt^{-1} hr^{-1} with a 29.5 % efficiency. The yield of ABE was 0.0833 g lt^{-1} hr^{-1} when compared to the semi–Simultaneous Saccharification fermentation (sSSF) process, which combined alkaline pretreated biomass with enzymatic hydrolysis and co-cultured bacteria after 24 hours of hydrolysis followed by 96hrs of fermentation. Butanol efficiency at the s-SSF process is 33.6 %. When compared to previous studies, ABE fermentation of Napier grass yields a competitive yield.

Key words

Napier grass, Alkali-pretreatment, Enzymatic hydrolysis, Acetone-Butanol-Ethanol (ABE) fermentation, semi-Simultaneous Saccharification fermentation (sSSF), Bio-Butanol

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Acronyms

ABE ..... Acetone- Butanol- Ethanol

AFEX .....Ammonia Fiber Explosion

ANOVA ..... Analysis of Variance

ASTM .....American Society for Testing and Materials

AOAC……..……...Association of official Analytical Chemists

s-SSF..... semi–Simultaneous Saccharification Fermentation

BBD ..... Box-Behnken Design

CV ..... Coefficient of Variance

DRS..... Defuse Reflectance System

HMF ..... Hydroxy Methyl Furfural

FTIR..... Fourier Transform Infrared Spectroscopy

HPLC ..... High Pressure Liquid Chromatography

RSM ..... Response Surface Methodology

TRS ..... Total Reducing Sugar

DNS..... dinitro salicylic

HHV ..... Higher Heating Value

1. Introduction

1.1. Background

Given the depletion of fossil fuel reserves and the environmental impact of unwise use of fossil-based energy, as well as the emission of greenhouse gases (GHG), there is an urgent need to develop renewable and environmentally friendly alternatives to fossil fuels. Furthermore, global crude oil prices are rising, as is the number of natural disasters caused by climate change, presenting new opportunities and challenges for the biofuel industry. Many countries and organizations have recently focused on and become involved in new biofuel activities, and much research has been conducted to produce biofuel. The development of biofuels is stifled. One such option, in addition to nuclear, solar, and wind power, is the production of biofuels from biomass, cyanobacteria, or eukaryotic microalgae. Biofuels hold particular promise as liquid car fuels that can be blended with or even replaced by gasoline and diesel. (Biofuel, 2020).

Due to the depletion of these organic resources, the use of organic renewable energy resources such as biofuel production using microorganisms such as ethanol, butanol, and glycerol, among others, is required. In anticipation of fossil fuel depletion, rising transportation oil prices, and climatic changes, there has been an increase in research interest in the production of biofuel from renewable resources using microbial cells. This will prevent both resource depletion and pollution of the environment.

Butanol production was first described by Louis Pasteur, a French microbiologist, in 1862. Butanol is a next-generation biofuel that is produced through Clostridium-based fermentations. *Clostridium acetobutylicum* is a solvent producing strain isolated from agricultural soil that has been used to produce butanol and hydrogen. ABE fermentation and semi-simultaneous saccharification fermentation (sSSF) are used. Using clostridial strain, the sSSF process is a preferred method for converting lignocellulose biomass to solvents / butanol. It improves rates, yields, and solvent concentrations while requiring less capital investment than competing processes by eliminating the separate hydrolysis process. Butanol has significant advantages over bio-ethanol as a biofuel. Butanol is less corrosive and can be distributed through the gasoline pipeline system; it is less hygroscopic and can withstand water contamination better; it is less

evaporative and explosive due to its lower vapor pressure; it has a 30% higher energy density than ethanol and is more similar to gasoline; and, most importantly, it can be mixed in higher ratios with gasoline in existing cars without the engine being retrofitted (S. Kumar & Sani Editors, 2018).

Sugar sources from various sources, such as sugar beet, corn, sugar cane, molasses, and so on, are used to produce butanol. In addition to the substrates, di-ammonium phosphate is used as a nutrient for the microorganism Clostridium bacteria (Rathour et al., 2018).

Butanol is a biofuel alternative to ethanol and can be produced using Acetone-Butanol-Ethanol (ABE) fermentation with *Clostridium acetobutylicum*. Due to its high toxicity and separation cost, various fermentation/separation processes have been proposed. (Wang & Castelao, 2016). Moreover, various lignocellulosic biomasses have been investigated to obtain sugars for biofuels, including barley straw (M. Yang et al., 2014), sugarcane bagasse (Pang et al., 2016),(Travaini et al., 2016), corn stalk leaf (P. Li et al., 2016), blended softwood (Yamamoto et al., 2014), and rice straw (Rahnama et al., 2014). whereas the potential of Napier grass found in Ethiopia has yet to be investigated. Because of the abundance of these non-edible raw materials, there has been interest in shifting from sugar-based production to second-generation, lignocellulosic-based production. This research looked into the use of Napier/ Elephant grass (Pennisetum purpureum Schumach), a common fodder in tropical areas that is also considered an energy crop, for butanol production.

1.2. Statement of the Problem

There are two major concerns about continuing to rely on petroleum fuels. The first is the availability of fossil fuel resources; output is expected to reach its peak in the next 15-30 years due to the exponential increase in demand. On the other hand, global crude oil prices have risen dramatically in recent decades, and carbon emissions from the industry are a major contributor to climate change. Consuming fossil fuels has a negative impact on the global environment, accounting for 25% of global GHG (Green House Gas) emissions. (biofuel , 2019).

Ethiopia is a non-oil producing country that relies entirely on imports. Fuel imports are putting the country's hard-earned currency at risk, particularly at a time when the country is facing a severe shortage of hard currency due to a variety of internal and external factors, including the purchase

of pharmaceutical products. Ethiopia's 2019 fossil fuel expenditure was $4.5 billion, and it is expected to rise.

To alleviate these problems in Ethiopia, ethanol is mixed with gasoline to save hard currency and reduce environmental impact. However, ethanol has some limitations: it reduces fuel economy by 2-5 percent for a 10% blend, gasoline volatility increases when blended below 10% with ethanol because high vapor decreases fuel capability to reach the engine, high heat of vaporization decreases cold start performance, and the methane emissions increase.

With the limitations of ethanol mentioned above, butanol is chosen as a better bio fuel due to its superior performance. Butanol is a metabolic product of solventogenic clostridia that has been identified as a potential alternative transportation fuel as well as a fuel extender. (Paper et al., 2009) (Rathour et al., 2018) .

Butanol is produced by ABE fermentation, and previous research has shown that most fermentation technologies are batch fermenters, but theoretically continuous fermentation has a higher yield of production, so in these studies, butanol production in batch and continuous with bacteria clostridia strains using Napier grass was examined. Napier grass cultivation improves soil fertility and reduces soil erosion, especially in Ethiopia, where soil erosion is a major issue.

In addition to limitations and GHG emissions, producing biofuel from various sources has its own set of issues and shortfalls due to differences in microorganism properties. Using sugar-based raw materials such as corn, sweet potato, and sugar cane to produce bio-butanol or any other type of biofuel interferes with human and animal food supply, raising the fuel food debate. Producing bio-butanol from second generation biomass such as lignocellulose material such as Napier grass provides a versatile solution for environmental degradation by preventing soil erosion, and the leaves of the Napier grass can be used as animal feed.

1.3. Objectives of the Research

1.3.1. General Objective

The general objective of this research was to produce and characterize bio-butanol from Napier grass through semi continuous fermentation using clostridia strain bacteria

1.3.2. Specific Objectives

The specific objectives were as follows:

- Investigate the yield of monosaccharide sugar by hydrolyzing Napier grass using pretreatment and cellulase (endoglucanase) enzyme hydrolysis.
- Determining optimal operating parameters (temperature, pH, substrate concentration, and fermentation time) to improve butanol yield.
- Determine and perform the efficiency of Semi-simultaneous scarification fermentation (sSSF).
- Identify the characteristics of the optimized product.

1.4. Significance of the Study

The average price of Fossil fuel reaches it maximum value historically since 2008 economic recession, simultaneously the clement change caused by (GHG) gives a nightmare for the habitat of this world. All these problems forced the world to look for other sources for energy. Many nations and organizations have recently joined in new biofuel activities, and a lot of research works have been carried out to produce biofuel such as butanol from [acetone–butanol–ethanol (ABE) fermentation] worldwide. In order to address the aforementioned problems, it is highly important to search and study alternative sources of fuel such as biofuel butanol. Such studies are vital for the development of energy security and transition to bio-economy it also has contribution in minimizing global warming, job opportunities and significant impact on hard currency problem solution for countries like Ethiopia.

Ethiopia may have a chance to produce bio-butanol from lignocellulosic materials, primarily Napier grass. Napier grass is a tropical grass that has a high adaptability, a rapid growth rate, high carbon content makes it very good biomass. Bio-butanol is a renewable energy source. It can play a significant role in the energy sector when used as part of a strategic plan. There is currently no published research paper on the production of Bio-butanol from Napier Grass in specially in

Ethiopia this paper will help to open basic understanding for further research and production of bio-butanol from Napier grass.

Napier grass is second generation energy crop this makes cheap energy source. In addition to the cheap energy source butanol has many superior properties as alternative fuel when compared to ethanol. These includes

- Higher energy content (30541.21kJ per a litter on butanol vs 23322.8 kJ per a litter of ethanol gasoline contains about 31929.45kJ per litter
- Butanol is six time less evaporative than ethanol
- Butanol can be shipped through existing fuel pipelines
- Butanol can be replacement for gasoline liter for liter 50% to 50%
- Napier Grass has high cellulose and hemicellulos that can be hydrolysis to monosaccharide sugar.
- Napier grass is abundant in Ethiopia

1.5. Scope of the Study

The main purpose of this study is to analysis and study if Napier grass (Elephant grass) is a good source to produce bio-butanol. Generally, the study covers proximate analysis of raw material, alkaline pretreatment, and enzyme hydrolysis, identifying the content of total reducing sugar in hydrolysate. The individual and interaction effect of four operational parameters (Temperature, pH, sugar concentration and fermentation time) on the yield of bio-butanol. Furthermore, product will be investigated. In addition, the conventional fermentation product will be compared with the s-SSF yield of bio-butanol product.

2. Literature Review

2.1. Biofuel

Access to energy sources is critical for socioeconomic progress and sustainable human living. As a result, energy demand has increased by 200 % in recent years. The consumption of fossil fuels accounts for 80% of total consumption. (Pfenninger & Keirstead, 2015). The world is currently dealing with three major issues: hunger, a lack of energy, and environmental degradation. Finding a cheap and environmentally friendly alternative energy source is a pressing issue in today's world.

A biofuel is a fuel derived from living matter or manufactured from agricultural products such as starch, cereal crops such as sugarcane, corn, beets, wheat, and sorghum lignocellulose material such as sweet grass, biodegradable portion of industrial and municipal waste, Napier grass, or Elephant grass. A biofuel is a fuel made today from biomass, as opposed to a fuel made by the very slow geological processes that lead to the formation of fossil fuels such as oil. (Biofuel, 2020). Because biomass can technically be used directly as a fuel (e.g., wood logs), it is sometimes difficult to distinguish between biomass and biofuel. However, the term biomass is frequently used to refer to the biological raw material from which the fuel is made, or to some type of thermally/chemically altered solid end product, such as torrefied pellets or briquettes. The term "biofuel" typically refers to liquid or gaseous fuels used in transportation or heating. (Biofuel, 2020).

Brazil's PROALCOOL program, which began in 1975, was a successful experiment in using biofuel in automobiles. Because of the aforementioned issues, biofuels have emerged as a viable alternative to oil in the last five years. The main reasons for discovering and developing biofuels these days are their lower carbon emissions when compared to conventional fuels, the positive impact on rural development, and the never-ending rise in oil prices.

The type of biofuel used will be determined by a number of factors, the most important of which are the available feedstock and the energy that can be used locally. (Paper et al., 2009) (Biofuel, 2019). The first generation of biofuel is derived from food, while the second generation is derived from lignocellulosic biomass such as forest and grass. Algae and other microorganisms are used in the third generation. The fourth generation is made up of biodegradable components of industrial and municipal waste.

Biofuel has several advantages, including being a gasoline substitute in transportation fuel, a diesel substitute in transportation fuel, a flexible fuel, a combustion fuel such as household cooking, alcohol beverage, pharmaceuticals, and a feed stock for other chemical and biological products.

2.2. Bio-butanol

Butanol (C_4H_9OH) is a colorless, flammable alcohol with four carbon atoms that can form a straight-chain or a branched structure, resulting in different properties and thus isomers: n-Butanol (butan-1-ol, 1-butanol, n-butyl alcohol), iso-butanol (2-methylpropan-1-ol, isobutyl alcohol), sec-Butanol (butan-2-ol, 2-butanol, sec-butyl alcohol), tert-Butanol (2-methylpropan-2-ol, tert-butyl alcohol). Bio-butanol is one of the most promising renewable biofuels because of its high calorific value in addition to its high hydrophobicity, butanol is closer to gasoline that ethanol. ABE fermentation is the main process to produce bio-butanol by certain species of anaerobic spore forming grain positive microorganisms one of them are clostridium acetobutylicum (Tigunova et al., 2020).

2.2.1. Historical Background of Bio-butanol

Many countries have launched bioconversion programs to produce biochemical and bioenergy as a result of rising oil prices. Acetone butanol ethanol (ABE) fermentation by solventogenic clostridia is one of the oldest known industrial fermentations. In terms of scale production, it is one of the largest biotechnological processes ever known, ranking second only to ethanol fermentation by yeast. The actual fermentation, on the other hand, has been quite complicated and difficult to control.

During World War I, *clostridia acetobutylicum* was used to ferment industrial butanol and acetone. In 1911, Chime Wizemann, a Louis Pasture student, isolated the microbe that produced acetone.(Timung et al., 2021). England approached the young microbiologist and asked for permission to manufacture acetone for cordite. Until the 1920s, acetone was the desired product, but for every pound of acetone fermented, two pounds of butanol were produced. A thriving automotive paint industry reshaped the market, and by 1927, butanol was the primary product, with acetone as a byproduct.

People were familiar with the production of ethanol for the purpose of blending it with fuel in the 1970s, but they were unaware that dehydration was required to blend it with fossil fuels. They also

failed to recognize the distribution challenge, as ethanol cannot be transferred via the existing pipeline infrastructure. As a result of the ethanol selection, a lower grade, corrosive, difficult to purify, explosively dangerous, and highly evaporative alcohol is produced. (Lynd, 1996).

2.2.2. Challenges of ABE Fermentation

ABE fermentation was one of the most important biotechnological processes for the production of solvents during the first half of the twentieth century. ABE fermentation declined due to advances in the chemical synthesis of these solvents, combined with high substrate costs, but scientific interest in this process was reignited whenever the price of oil fluctuated. Despite the fact that most butanol fermentation plants had vanished by the 1980s, enormous progress had been made in the genetics and physiology of clostridia solvent production as a result of advances in biological science in the 1980s. This improves our understanding of the mechanisms involved in the formation of acids and solvents. The most important economic factor in ABE production, as it is in other bioprocesses, is the cost of substrate. Many developing countries prohibit the use of grain and certain lands for biofuel production due to the "food versus fuel" debate.

2.2.3. Current Utilization of Butanol

Rising demand for alternative fuels and renewable energy as a result of the negative environmental effects of fossil fuels, such as global warming, will drive bio-butanol growth in the coming years. Butanol is a significant bulk chemical that has a wide range of industrial applications. The vast majority of global output is converted into methacrylate esters and acrylate esters. Glycol ethers and butyl acetate are also important derivatives, while amino resins and n-butylamines have minor applications. Bio-butanol is an important raw material used in the production of paints, coatings, plasticizers, and adhesives. It is used as a direct replacement for petroleum-based butanol in almost all applications.

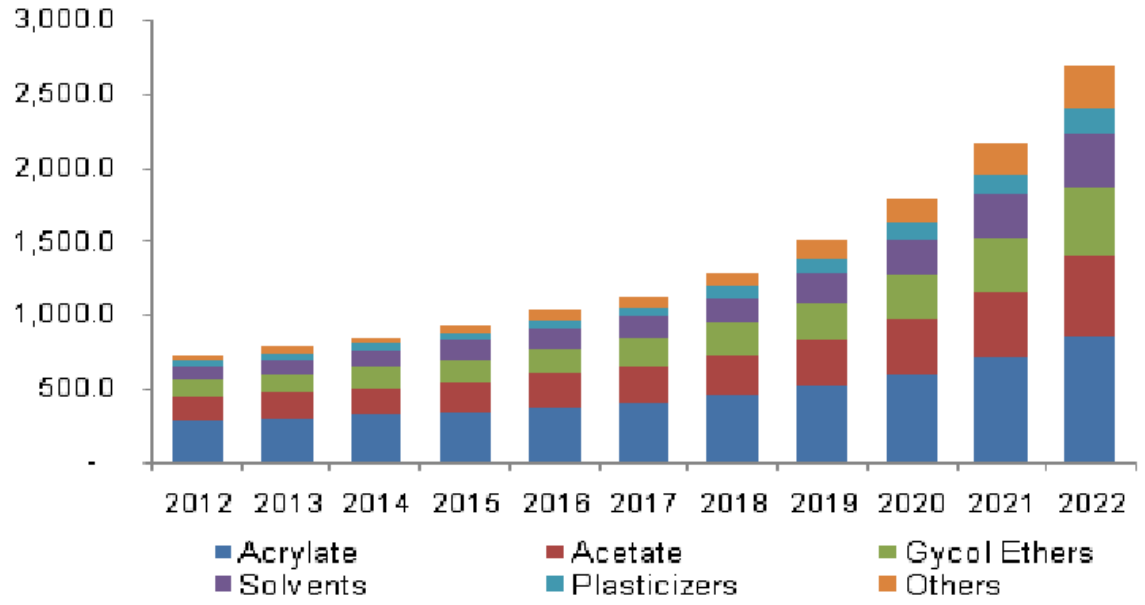

Figure 1 Butanol consumption as bio fuel (Zhen et al., 2020)

Table 2:1 Properties of Butanol Compared to other Energy (Buyondo and Liu 2012)

Properties	Methanol	Ethanol	Butanol	Gasoline
Energy density, MJ/L	16	21.2	29.2	32.5
Air-fuel ratio	6.5	9	11.2	14.6
Research octane number	136	129	96	91-99
Heat of Vaporization, MJ/kg	1.2	0.92	0.43	0.36
Flash point ℃	79	13	35	<-40

2.3. Feedstocks for Bio-butanol Production

Many studies are being conducted to investigate the use of agricultural wastes in the biofuel industry in order to achieve effective and efficient waste management. Biomass includes wood and logging residues, agricultural crops and waste byproducts, the organic portion of municipal solid waste, animal wastes, municipal bio solids (sewage), food processing waste, and aquatic plants and algae. Because fermentation substrate influences the cost of bio-butanol production, the ability to use mixed sugars is especially important for the use of low-cost agricultural byproducts and wastes as fermentation substrates.

Meanwhile, because fossil fuel is a nonrenewable source of energy, concerns about its sustainability, economic impact, and environmental impact have led to the use of renewable fuel sources. Locally, biomass energy is frequently available, and processing is feasible without a large capital investment. Furthermore, using biomass energy can help reduce GHG emissions, which can be harmful to public health. The various raw materials used in the fermentation process to produce butanol are classified into three types. Biomass from sugar, starches, and lignocellulose (Saliu & Sani, 2012).

2.3.1. Sugars

Sugars are a type of carbohydrate that contains molecules of carbon, hydrogen, and oxygen. It is a preferred energy source for cells and microorganisms. Fermentation is a process in which microorganisms consume fermentable sugars and produce butanol and other solvents. Glucose is the most common 6-carbon sugar used by Clostridium Strain microorganisms. As a result, biomass materials containing a high concentration of glucose or glucose precursors are the easiest to convert to butanol. However, because sugar materials are present in the human food chain, their use in the production of butanol is prohibitively expensive. High-sugar biomass feedstocks include sugarcane, sugar beet, sweet sorghum, and various fruits. These biomass materials, on the other hand, are all part of the human food chain and, with the exception of some processing residues, are generally too expensive to be used in the production of fuel butanol. (Badger, 2002).

2.3.2. Starch

Another potential butanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials that have been broken down to monomer molecules can be fermented. The most common starch materials used in the production of bio-butanol are cereal grains, potato, sweet potato, and cassava. Cereal grain is a type of grain that is commonly used in the production of ethanol in the United States. Starchy materials require hydrolysis to break down the macromolecule of starch to the monomer molecule glucose. Hydrolysis is carried out by combining starch and water to form a slurry, which is then stirred and heated to rupture the cell wall. During the heating and mixing process, a specific enzyme will be added to break the macromolecule bond. Because enzyme is temperature sensitive, the heating temperature must be in accordance with the requirements of the enzyme. (Badger, 2002).

2.3.3. Lignocellulose Biomass

Although sucrose and starch are good substrates for butanol fermentation, rising demand for these feedstocks for biofuel production has driven up their prices. Importantly, these crops may compete with food supplies, resulting in a food versus fuel debate. The efficient utilization of abundant lignocellulosic biomass available on Earth may hold the key to obtaining enough fermentable carbon substrates without competing with food supplies. Cellulose materials are the most abundant global source of biomass, but they are largely underutilized. The global production of plant biomass of which over 90% is lignocellulose, amount to about $200*10^9$ tons annually, where about 8 to $200*10^9$tons of the primary biomass remains potentially accessible (Saliu & Sani, 2012).

Cellulose is not consumed, and the lignocellulosic biofuel industries do not compete for raw materials. Cellulosic biomass, such as Napier grass and agricultural waste, is less expensive to produce and uses less energy.

Lignocelluloses are a complex substance made up of a mixture of carbohydrates polymers, cellulose and hemicellulose, which are tightly bound to lignin, a complex aromatic polymer, primarily by hydrogen bonds but also by some covalent bonds. Lignin prevents cellulose hydrolysis by acting as a physical barrier that prevents enzymes from accessing cellulose and hemicellulose materials. (Ladeira Ázar et al., 2020). Thus, delignification is the first step in the production of lignocellulose biofuels, releasing cellulose and hemicellulose from their lignin complex. It is a challenging, time-consuming, and critical task. (Saliu & Sani, 2012).

Cellulose from Napier grass, wood, agricultural residues, and other sources can be converted to monomer sugar via acid or alkali hydrolysis or enzyme action, and then fermented to produce butanol.

2.4. Composition of Lignocellulose Material

Lignocellulose materials contain polysaccharides such as cellulose and hemicellulose, lignin extractives, and ashes rather than readily bio-convertible monosaccharides. Polysaccharides must be hydrolyzed with chemicals and enzymes to become fermentable sugar.

Napier grass, also known as elephant grass, is a perennial bunch grass native to eastern and central Africa. (Nyambati et al., 2010). Napier grass cultivation improves soil fertility and reduces soil erosion; these characteristics, combined with its high cellulose and hemicellulose content, make

Napier grass an ideal competitive source for biofuel. According to UN Food and Agriculture Organization data from 2015, the production of Napier grass in most tropical areas, such as Nigeria, averaged 20.8 tons per acre.

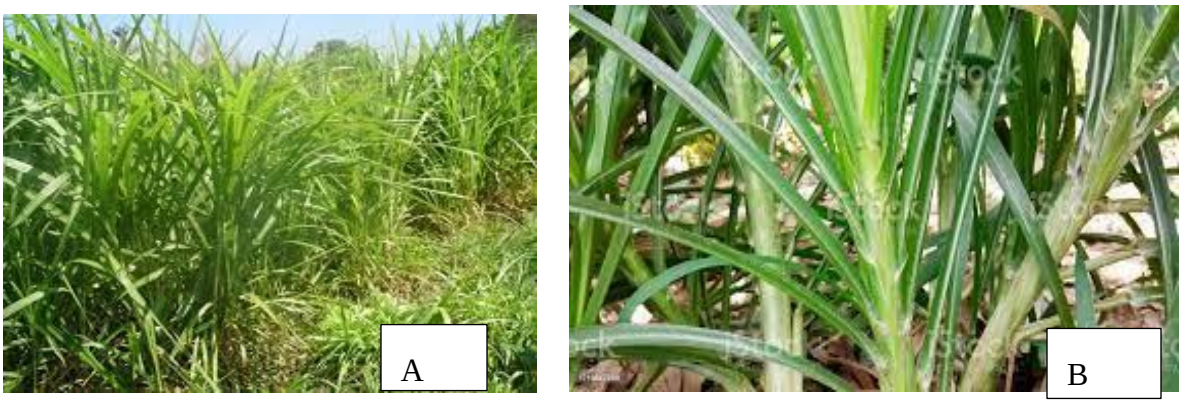

Figure 2 Napier Grass from Menagesha Mushroom Farm

Napier grass is typically made up of lignocellulose, which is found in plant cell walls. The main components of Napier grass biomass are cellulose (22.6-42.4wt %), hemicellulose (16.9-22.5wt %), and lignin (23-28wt %), with minor amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash. (Mohammed et al., 2015). The ratio of these constituents' composition can change depending on age, stage of development, and other factors (Mohammed et al., 2015). In general, biomass is composed of 40-50 % cellulose, 25-30% hemicellulose, and 15-20% lignin and other extractable components. (Crop et al., 2014). The table 2.2 shows composition of lignocellulose from different biomass.

Table 2:2: Content of Cellulose, Hemicellulose and Lignin in Common Biomass (Sun,Y. Cheng, J 2002)

Lignocellulose biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hard wood steam	40-55	24-40	18-25
Soft wood steam	45-50	5-35	25-35
Nut shells	25-30	25-30	30-40
Grass	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Cotton seed hair	80-95	5-20	0
Switch grass	45	31.5	12
Napier Grass	22.6-42.4	16.9-22.5	23-28

2.4.1. Cellulose

Cellulose is the primary structural constituent of plant cell walls and is found in an organized fibrous structure. Cellulose is composed of glucose polymers that are largely insoluble and exist in crystalline micro fibrils, making sugar extraction difficult (Mohammed et al., 2015). Through photosynthesis, the tree generates the glucose molecules ($C_6H_{12}O_6$) of cellulose. This monomer unit is first converted into glucose anhydrides ($C_6H_{12}O_5$), which are then end-to-end linked via oxygen atoms to form linear polymers ($C_6H_{12}O_5$). The number of monomers per macromolecule indicates the degree of polymerization (DP) in cellulose (n)(Xiao et al., 2017) (Rodríguez-Gutiérrez et al., 2014). This linear polymer is composed of D-glucose subunits connected by -(1, 4)-glycosidic bonds.

Cellobiose, the repeat unit formed by this linkage, is what makes up cellulose chains. Because of the hydrogen and van der Waals bonds that connect the long-chain cellulose polymers, the cellulose packs into microfibrils. Hemicelluloses and lignin protect the microfibrils. By breaking the -(1, 4) - glycosidic linkages with acid or enzymes, fermentable D-glucose can be produced from cellulose. In biomass, cellulose can be found in both crystalline and amorphous forms. The vast majority of cellulose is crystalline, with only a small percentage of unorganized cellulose

chains forming amorphous cellulose. Cellulose is more susceptible to enzymatic degradation when it is amorphous (Mohammed et al., 2015).

2.4.2. Hemicellulose

The main distinction between hemicellulose and cellulose is that hemicellulose has branches with short lateral chains made up of different sugars, whereas cellulose does not. Monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uranic acids (e.g., 4-omethylglucuronic, D-glucuronic, and D-galacturonic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches connected by -(1, 4)-glycosidic bonds and, on rare occasions, -(1, 3)-glycosidic bonds.

Hemicelluloses, such as heteroxylan, can also be acetylated to a degree. In contrast to cellulose, the polymers found in hemicelluloses are easily hydrolyzed (Mohammed et al., 2015). Even when co-crystallized with cellulose chains, these polymers do not aggregate. The hemicellulose side groups are the first to react during pretreatment, followed by the linear backbone. Hemicellulose dissolves into water at around 150^{o}C or 180^{o}C under neutral conditions. However, the extent of solubilization is also affected by water content and pH. Solubilization begins at 120^{o}C in the presence of a dilute acid catalyst (Rodríguez-Gutiérrez et al., 2014).

2.4.3. Lignin

Lignin is a large, complex molecular structure made up of cross-linked phenolic monomer polymers. It can be found in the primary cell wall, where it provides structural support, impermeability, and microbial resistance. Three phenyl propionic alcohols exist as lignin monomers: coniferyl alcohol (guaiacol propanol), coumaroyl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds connect these phenolic monomers. Herbaceous plants, such as grasses, contain the least lignin, whereas softwoods contain the most (Rodríguez-Gutiérrez et al., 2014). It tightly binds cellulose and hemicellulose, resulting in a compact and rigid cell wall. This is one of the reasons why biomass must be pretreated prior to enzymatic hydrolysis in order for its complex structure to be revealed. Under neutral conditions, lignin normally begins to dissolve in water around 180^{o}C. The solubility of lignin at different pH levels is determined by the composition of the precursor (Mohammed et al., 2015).

2.4.4. Ash and Extractive proteins

Extractives are any of a variety of compounds found in biomass that are not part of the cellular structure (resins, phenolics, and other chemicals). These compounds can be extracted from biomass using polar and non-polar solvents such as hot or cold water, ether, benzene, methanol, acetone, or other solvents that do not degrade the biomass structure (Rodríguez-Gutiérrez et al., 2014). Furthermore, when biomass is burned, minerals such as calcium, magnesium, potassium, and other materials are released as ash (Rodríguez-Gutiérrez et al., 2014).

2.5. Microorganisms

Following the discovery of the fermentation of sugar to butyric acid by Pasteur microorganisms responsible for the formation of butyrate in 1861, Clostridia species are responsible for the desired product. Butyrate is only used as a primary fermentation product by obligate anaerobes, which are divided into four genera. *Clostridia, butyrivibrio, Eubacterium,* and *fusobacterium* are all bacteria.*Clostridium acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum, C. saccharoacetobutylicum, C. aurantibutyricum, C. pasteurianum, C. sporogenes, Clostridium cadaveris, and Clostridium tetanomorphum* are the sole producers of biobutanol. Isolates of Clostridia can be obtained from soil, corn meal, or purifying materials. It is commercially extracted from potatoes, the roots of nitrogen-fixing legumes, and other crops. In the past, these organisms have been given a variety of specific names. The strains selected for industrial use are determined by the raw material used, which includes the substrate, the ratio of end products required, the need for additional nutrients, and phage resistance.

2.5.1. Culture Bacteria

Rather than living in isolation, microorganisms in nature frequently exist in highly diverse and complex communities. Microorganisms coexist in these natural communities for long periods of time by interacting with one another and performing various functions more effectively than a single culture. Clostridiums, as an obligate anaerobe, were unable to survive in aerobic environments, but were frequently found in oxygen-rich environments such as rice paddy soils and water retting ponds alongside aerobic bacteria. Aerobic bacteria were thought to be necessary for Clostridiums in these environments because they provided an anaerobic environment and

consumed harmful metabolites. Cooperation between Clostridiums and aerobic bacteria has been observed in a variety of artificial systems as well as in natural environments.

To induce cellulosic biomass utilization, co-cultures of Clostridium and other bacteria with enzymes capable of hydrolyzing cellulose and hemicellulose were used. In these co-culture systems, cellulolytic strains hydrolyzed cellulose and hemicellulose to glucose or butyric acid, and butanol was obtained later by adding solvent-producing Clostridium species (B. Wu et al., 2016).

Bacillus was discovered to be an excellent partner in an artificial syntrophic co-culture system for creating an anaerobic environment and pre-saccharification of substrate for co-cultured Clostridium strains. Although some reports confirmed that aerobic bacteria, such as B. subtilis and B. cereus, interacted positively with anaerobic clostridium, the role of aerobic bacteria was unclear (B. Wu et al., 2016). Bacillus strains grow quickly and secrete a variety of saccharification-related extracellular enzymes into the medium, including amylase, pectinase, protease, cellulose, and hemicellulose. Aerobic bacteria in the co-culture system are thought to maintain an anaerobic environment for Clostridiums, and different microorganisms in the co-culture system may compete for substrates, influencing product yield and productivity when compared to single cultures (B. Wu et al., 2016).

(Abd-Alla and Abdel-Wahab) obtained a maximum ABE productivity of 0.30 $gL^{-1}hr^{-1}$ and an ABE yield of 0.42 from 75 gL^{-1} spoilage date fruits homogenate by mixed culture without the addition of a reducing agent or N_2 flushing (P. Wu et al., 2016). Discovered that when B. subtilis was co-cultured with *C. acetobutylicum* without anaerobic pretreatment by reducing agent and N_2 flushing, amylase activity increased 10 folds and ABE production increased 5.4 and 6.5 folds from soluble starch and cassava starch, respectively, when compared to those of the pure Clostridium culture.

2.6. Sterilization

Many industrial food fermentation processes rely on microbial contamination prevention. Fermentation is heavily reliant on sterilizing fermentation media. Economic loss can occur if the fermentation media is not properly sterilized or if the media becomes contaminated. Media contamination can reduce product yield, alter the product, or inhibit the growth of fermentation microorganisms. As a result, proper fermentation media sterilization is critical. Various techniques

are used to sterilize various types of media and nutrient supplies used in fermentation. Various sterilization techniques are used depending on the situation. Fermentation techniques are used both in the laboratory and on a large scale.

Sterilization of fermentation media is accomplished by autoclaving, boiling, or passing steam through the media. Synthetic media can be sterilized quickly, whereas crude media takes longer. The type and characteristics of the media components determine the sterilization method used.

Media sterilization is the process of destroying or removing all forms of microbial life from aqueous feedstock. In industrial fermentations, components such as vessels, pipework, media, inlet air, and exhaust gases are frequently sterilized using a combination of wet-heat and filtration methods. Wet-heat methods are commonly used in the fermentation industry to destroy unwanted microorganisms because they are less expensive and more effective than dry-heat methods. Wet-heat sterilization is a popular method for killing all microorganisms, including bacterial spores. In an autoclave with a saturated steam atmosphere, these conditions can be met.

Sterilization takes longer because crude media is more viscous and may contain spores or highly resistant bacteria. Although crude media has a high sugar content, it should be sterilized properly because if a large amount of heat passes through the media for an extended period of time, it may caramelize the media's sugar content, resulting in media spoilage.

Filtration is used to sterilize fermentation media that contains substances that can change chemically and degrade due to heat. Some fermentation media include vitamins, enzymes, and volatile components that are easily destroyed by heat. As a result, to sterilize such media components, bacteriological filters should be used.

2.7. Bio-butanol Production from Napier Grass

Butanol can be made from two sources: fossil fuels or biomass. Currently, the majority of butanol production is derived from fossil fuels and their petrochemical derivatives. As a result of today's pressing environmental issues, researchers are looking for alternative methods to produce butanol. Napier grass is one of Ethiopia's most affordable and widely available bio energy sources.

The biological conversion process entails exposing biomass to specific microorganisms. Secondary fuels are produced as a result of the metabolic activity of microorganisms. The most common biological conversation process is the fermentation of butanol in an anaerobic digestion.

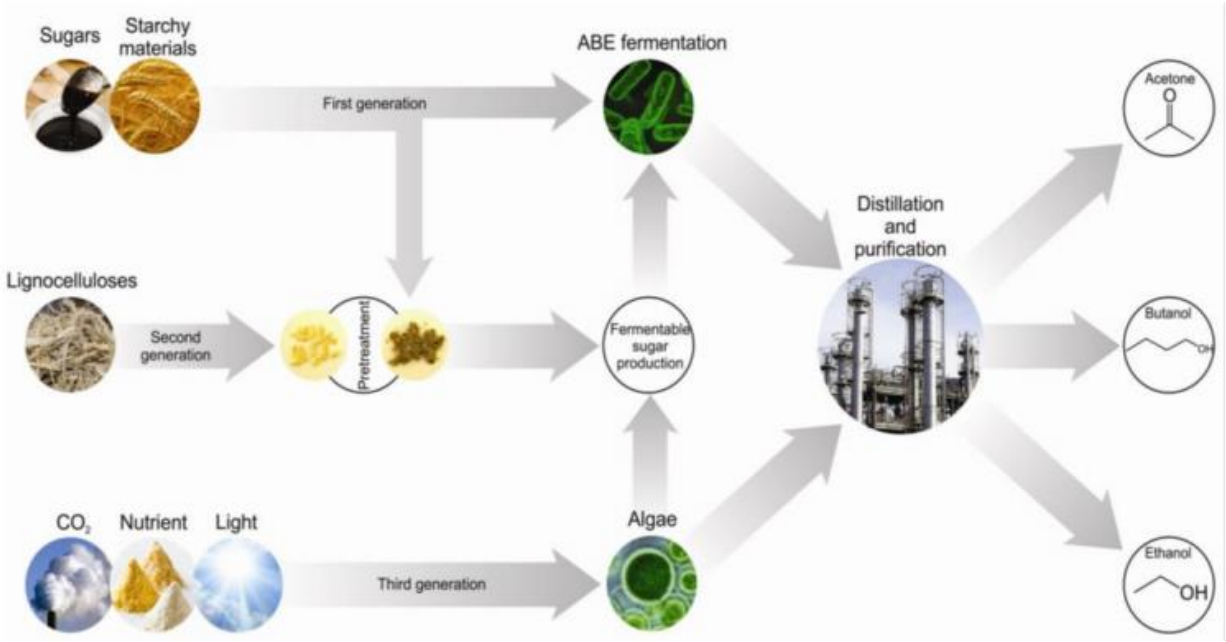

Figure 3 Schematic Illustration for Solvent Production (He et al., 2017)

Bio-Butanol Production

Fermentation is required for bio-butanol production. ABE fermentation is a microorganism-based fermentation process that converts monosaccharides into bio-butanol. It is referred to as solvent production because its primary chemical products are acetone, butanol, and ethanol. Fermentation is based on microorganism activity, and bacteria from the genus Clostridium strain are commonly used. *Clostridium acetobutylicum* is widely used in the synthesis of sugar-based butanol, producing acetone and ethanol as byproducts (Birgen et al., 2019). The process steps involved in the production of bio-butanol from lignocellulosic biomass are pretreatment, hydrolysis, fermentation, and separation.

2.7.1. Pretreatment

The goal of pretreatment is to modify the structure of lignocellulose biomass in order to improve its ability to form sugars via hydrolysis. This is achieved by breaking the lignin seal, removing lignin and hemicellulose, or increasing the porosity of the biomass. Pretreatment should increase

fermentable sugar yield while minimizing carbohydrate degradation or loss, as well as the formation of inhibitors for subsequent hydrolysis and fermentation processes.(Maurya et al., 2015). An ideal pretreatment results in the disintegration of the lignin and hemicellulose complex with cellulose, followed by an increase in sugar yield due to cellulose and hemicellulose hydrolysis. Finally, excessive carbohydrate degradation or loss is avoided. The four basic types of pretreatment techniques are physical, chemical, physicochemical, and biological treatments (Sarkar et al., 2012)

Before proceeding with chemical pretreatment, the size of Napier grass must be pulverized with a cutting mill to a particle size of 0.84mm. This will aid in increasing the surface area to volume ratio for improved extraction.

Hydrolysis is the process by which cellulose and hemicellulose polysaccharide chains are depolymerized into fermentable oligosaccharide or monosaccharide forms (Modenbach, 2013). Following the pretreatment process, the feedstocks for fermentation to biofuel can be hydrolyzed using one of two methods, the most common of which is chemical or biological hydrolysis. Furthermore, there are some other hydrolysis methods that do not rely on either of the two methods mentioned above. Gamma-ray, electron-beam, and microwave irradiation are among these methods. These processes, however, are not commercially viable.

2.7.1.1. Acidic Pretreatment

Acid pretreatment was invented in Germany in 1898. Mineral acids, such as sulfuric acid, are used in this method to break down hemicelluloses into monomeric sugars while also removing some lignin. Because optimum temperature necessitates a small amount of energy, this method necessitates a small amount of water (Z. Li et al., 2014). Diluted acid hydrolysis is the most commonly used technique for hemicellulose breakdown in this process; the use of diluted acid (1-4 percent) at moderate temperatures ranging from 120°C to 160°C has proven to be adequate for hemicellulose hydrolysis while promoting little sugar decomposition (Mussatto- Vencent et al., 2010)

Bensah et al., discovered that high temperature (120°C-210°C) and 5-8 bar pressure are used in a dilute acid (0.2-2.5 w/w %) process to achieve reaction times in seconds or minutes, making it suitable for continuous operations. Low acid consumption is a significant benefit in terms of cost

and process severity. Furthermore, low acid concentrations of less than 1% sulfuric or phosphoric acid release essential nutrients such as sulfur or phosphorus, which improves downstream fermentation (Zhang et al., 2015). It was also discovered that the most commonly used diluted acid hydrolysis pretreatment for biofuel production uses 0.05 - 2% sulfuric acid (w/v) at 120 to 220°C for 2 - 90 minutes.

Higher levels of hemicellulose sugar recovery result from pretreatment at high temperatures and longer reaction times. Higher pretreatment temperatures and longer pretreatment times, on the other hand, cause xylose and glucose degradation, resulting in the production of inhibiter products such as furfural and 5-hydroxmethyl furfural (HMF). These toxic byproducts suppress microbial growth and interfere with ABE fermentation. It was also discovered that Napier grass, which is used to produce xylose, is treated with 1.2 - 1.5 % sulfuric acid at 125°C to reduce the production of inhibitor compounds.

2.7.1.2. Alkaline pretreatment

Alkali pretreatment is more effective at dissolving lignin and preventing inhibitor production. Another important chemical pretreatment is alkaline pretreatment, which uses sodium or calcium hydroxide to improve cellulose digestibility. It is usually accompanied by a delignification process that affects the solubilization of a large number of hemicelluloses. The saponification reaction of intermolecular ester bonds crosslinking between hemicellulose and lignin underpins the reaction mechanism. Saponification breaks down lignin bonds, making cellulose more accessible to hydrolysis enzymes. The primary advantage is that residual alkali can be recycled and reused, and reaction temperature and pressure are reduced for effective lignin removal. The primary disadvantage is the incorporation of irreversible salts in the biomass during pretreatment (Wyman et al., 2005).

Sharma et al. removed lignin from SCT using an alkali pretreatment. Optimal conditions of 3% sodium hydroxide, 15% biomass loading, and 60 minutes incubation time at 121°C in a laboratory autoclave resulted in 89.9% lignin removal.

When compared to DAP in SCT, alkaline pretreatment removed about 89.9 % of the lignin (Sharma et al., 2018). The acid pretreatment, which used sulfuric acid in the presence of Triton X-100 as a surfactant, removed 50% of the lignin (Wyman et al., 2005). However, the biomass loaded

in the acid pretreatment was higher than in the alkaline pretreatment, and a decrease in solid concentration can increase lignin removal in the acid pretreatment. Furthermore, alkaline pretreatment has several advantages, including a high yield of hemicelluloses sugar, removal of lignin and hemicelluloses, increased cellulose exposure to enzyme, and removal of heavy metals in raw materials. (Guo, 2013) and also the production of inhibitors is very low.

2.7.2. Enzyme Hydrolysis

Biological pretreatment with lignin-degrading enzymes is typically performed under milder conditions and is effective in lignin removal. Peroxidases and oxidases are the most common lignocellulosic enzymes, with laccases being the most commonly used. Laccases are copper-containing oxidase enzymes that participate in the degradation of lignin for efficient biomass conversion. The main advantage is that they do not require a lot of energy and do not emit any harmful byproducts.

(Nikhita & Sachindra, 2021) employed laccase enzyme derived from Pleurotus Djamor for SCT pretreatment, and the studies revealed a 71.6 percent removal of lignin from SCT. Another study reported by (B. Yang et al., 2011), using laccase enzyme for SCT pretreatment resulted in 79.1 percent maximum delignification with optimum conditions of 21 (w/v) % substrate concentration at pH 7 incubated for 6hrs with an enzyme concentration of 500 IU/mL at 40°C.

Hydrolysis of cellulose material refers to the steps involved in converting carbohydrates polymers such as cellulose and hemicellulose into monomeric form. Endoglucanases enzyme can catalyze the cleavage of these polymers (Mosier et al., 2005) Porosity or accessible surface area, cellulose fiber crystallinity, and lignin and hemicellulose content have all been identified as factors influencing cellulosic biomass hydrolysis. Several methods for extracting monosaccharides from Napier grass have been investigated (Wen et al., 2015). On Napier grass, combined alkaline pretreatment and enzymatic hydrolysis yielded the highest glucose yield (g-glucose/g-cellulose) of 79.4 %.

2.7.3. Fermentation

Lignocellulose is frequently pretreated chemically or biologically before being hydrolyzed. The obtained hydrolysate is then used for bio-butanol fermentation by clostridia strain bacteria microorganisms. Because such lignocellulose hydrolysate contains not only glucose, but also a

variety of monosaccharides such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars in order to successfully industrially produce bio-butanol. Butanol fermentation is a biological process that converts fermentable sugars such as glucose and xylose to cellular energy using microorganisms that produce waste byproducts such as butanol, acetone, ethanol, and carbon dioxide anaerobically via sugar metabolic pathways.

2.7.3.1. Factors Affecting Fermentation

Fermentative bio-butanol production is a biphasic fermentation process influenced by numerous factors such as initial pH, temperature, inoculum size, substrate type and concentration, product inhibition, and oxygen concentration (Al-Shorgani et al., 2018).

A. Effect of pH

The pH of the solution has a significant impact on the yield of bio-butanol. Proper pH can stimulate the metabolic transition from acidogenesis to solventogenesis. Acid production slows down at the end of the acidogenesis phase due to the effect of low pH.

To compensate for the unfavorable effect of low pH, an organism's metabolic activity shifts from acidogenesis to solventogenesis (Buyondo & Liu, 2012). During acidogenesis, cells in the exponential growth phase at higher pH produce acetate and butyrate. Acetate and butyrate are consumed as substrates for the biosynthesis of acetone and butanol during this phase, but no growth is observed.

B. Effect of Sugar Concentration

The concentration of sugar in the fermentation substrate has a significant impact on bio-butanol yield. A low sugar concentration can inhibit cell growth, disrupt the acidogenesis phase, and further affect solvent formation; a high sugar concentration can directly inhibit cell growth and cause the entire ABE fermentation to fail (Lleixà et al., 2016).

C. Temperature

Temperature influences microorganism activity and the conversion rate of fermentation products, and it is closely related to economic benefit. The temperature of the fermentation can affect the overall yield, solvent ratios, and rate of solvent production. For example, in lignocellulose biomass fermentation at 30°C, the yield of solvents was 31%, with acetone contributing 23%, while at

33°C, the yield was 30%, with acetone contributing 26%, and at 37°C, the yield was around 24%, with acetone contributing 38% of total solvents. The majority of ABE fermentation processes are carried out at temperatures ranging between 35 and 37 degrees Celsius.

D. Anaerobic Conditions

Clostridia are obligate anaerobes, so oxygen has a significant impact on their growth. Buyondo and Liu proposed four major hypotheses for the toxicity of oxygen. The first hypothesis was that oxygen is toxic on its own. The second hypothesis was that anaerobes require low redox potentials (Eh), and the third was those organisms' lacking catalase, such as Clostridia, are killed by water, which is formed by reducing some of the oxygen. Finally, because oxygen is a more avid electron acceptor than normal terminal oxidants, anaerobes are unable to maintain intracellular concentrations of electron donors such as NAD(P)H (Buyondo & Liu, 2012)

In comparison to aerobes, strict anaerobes require special equipment and complicated operations to eliminate oxygen from the culture medium, such as adding reducing agents or flushing with N_2 gas, which raises the total cost of ABE fermentation.

E. Agitation

In anaerobic fermentation the aim of agitation is limited in maintaining homogeneity of nutrient and microbes. Homogeneity is necessary for high mass transfer between nutrients and microbes to enhance the rate of fermentation by increased interfacial area (Buyondo & Liu, 2012).

F. Product Inhibition

Toxicity to solvents produced during the fermentation process is a significant barrier to high productivity. These solvents interfere with membrane permeability, causing leakage of cellular constituents, disrupting membrane protein function, and altering membrane fluidity. Solvent-tolerant bacteria avoid these reactions through cellular adaptations such as altered membrane composition, an efflux system that actively decreases the solvent concentration inside the cell, solvent degradation, or over-expression of stress proteins such as heat shock proteins.

2.7.3.2. Diversity of inhibitors in lignocellulose hydrolysates

The nature of the inhibitors is largely determined by the type of lignocellulose material used, the composition of the cell wall, and the thermochemical conditions used for hydrolysis. To achieve

suitable yields and productivities from the microorganisms, these inhibitory components must be removed from the lignocellulose hydrolysates prior to fermentation. Several inhibitors toxic to fermentative microorganisms is released during the thermochemical breakdown of the plant cell wall. Furans (furfurals and 5-HMF), phenolics, weak acids (acetic acid, clavulanic acid, formic acid, etc.), raw material extractives (acidic resins, tannic acids, and terpene acids), and heavy metal ions (iron, chromium, nickel, and copper) have all been identified as potent inhibitory substances (Chandel et al., 2013). These toxic compounds slow the growth of fermenting microorganisms by affecting their rate of sugar uptake while also slowing their rate of product formation.

2.7.3.3. Detoxification Strategies for Fermentation inhibitors

To remediate fermentation inhibitors, various detoxification strategies have been used, including physical, chemical (Neutralization, $Ca(OH)_2$ over liming, Alkali (NaOH, Na_2SO_3, NH_3), activated charcoal, ion-exchangers (anionic and cationic), and extraction with ethyl acetate), and biological (microbial and enzymatic) (Chandel et al., 2013).

2.7.4. Separation

The fermentation products are a mixture of acetone, butanol, and ethanol. There is a significant difference in volatility in the mixture, which can be easily separated using distillation.

Distillation is one of the purification steps for mixtures with significant volatility differences. Distillation is the process of separating two or more liquid mixtures based on their boiling points. However, several distillations are required to achieve high purification. This is due to the fact that all materials have intermolecular interactions, and two materials will co-distill during distillation (Onuki, 2005) (Mihret et al., 2016).

The three solvents acetone, butanol, and ethanol are formed in solution during the ABE fermentation process. As a result, fractional distillation is the simplest and most appropriate separation technique. Because the boiling points of acetone, ethanol, and butanol are 56^{o}C, 78^{o}C, and 117^{o}C, respectively, and there is a significant boiling point difference, (Mihret et al., 2016).

2.7.4.1. Dehydration

Some water remains in butanol after distillation. This water, in particular, is a major issue for butanol fuel because the presence of this amount of water increases the molecular polarity of butanol when it is mixed with gasoline. As a result, they separate into two phases: butanol and

gasoline. It's easy to see why this inhomogeneous fuel would be unacceptable. As a result, dehydration may be an issue (Onuki, 2005).

Water must be removed from butanol before it can be used as a fuel. Distillation removes the majority of the water, but the purity is limited to 97-98 % due to the formation of a low boiling water-butanol azeotrope. To avoid separation when blending with gasoline, a purity of 99.5 to 99.9 percent is required, depending on temperature. Physical absorption using molecular sieves is currently the most widely used purification method. Azeotropic distillation is another method. (Onuki, 2005) (Mihret et al., 2016).

3. Materials and Methods

3.1. Raw Materials Collection

Napier grass (Pennisetum Purpureum) was collected from Menagesha Mushroom Farm located in Menagesha town FenFene Special Zone Oromiya Region. The biomass material was then sun dried to 10% moisture content under the conditions defined by (Mussatto & Roberto, 2006). Napier grass was first pulverized, and then the powder was sieved through 0.84 mm, to collect particles smaller than 0.84 mm. The sieved Napier grass powder was dried in a 45-60 °C oven until the weight remained constant. The sieved Napier grass powder was then stored at room temperature in zipper bags for further analysis and experiments. Microorganisms' clostridium acetobutylicum ATCC 824 was purchased from Louis Pasteur institute Paris France stored in LB broth in serum bottle which was filled with nitrogen to keep anaerobic environment. Enzyme of Novozymes Cellic c Tec 2 was purchased from Switzerland.

3.2. Materials

Most of the experimental work was done in the laboratories of Addis Ababa institute of Technology at School of Chemical and Bioengineering and School of Civil and Environmental Engineering waste water laboratory.

For the production and characterization of bio-butanol the following equipment's and chemical were utilized.

3.3. Equipment and Chemicals

Table 3:1 List of Equipment's and Chemicals

No	Equipment's	Model	Chemicals
1	Polyethene bag		Distilled Water
2	Cooler		Acetone
3	Digital Weighing Balance	OHAUS Explorer Pro	Sulfuric acid
4	pH meter,	Denver Instrument Model 250	Sodium hydroxide
5	Density Meter		
6	Test tubes, volumetric flask, bakers, measuring cylinders		Quantitative Benedict reagent solution
7	Batch and continues fermenter		Nutrient broth
8	Water bath	Grant TXF200	Yeast extract
9	Incubator, oven, autoclave		Potassium hydrogen phosphate
10	Desiccators		Ammonium chloride
11	Laminar flow		Potato Dextrose Agar
12	Micropipette		Diammonium phosphate
13	Microscope		
14	Anaerobic jar		
15	Spectrophotometer	Lambda 950 PerkinElmer UV-Vis spectrometer	
16	Fourier Transform Infrared Spectrometer		
17	High Performance Liquid Chromatogram (HPLC)	Waters 600	
18	Centrifuge	Universal 320R	
19	Bomb Calorimeter	Cussons	
20	Colony counter		

3.4. Characteristics of Napier Grass

3.4.1. Proximate Analysis

The proximate analysis was performed using the procedure of AOAC (Association of official Analytical Chemists 1996) official methods of Analysis, and Forage Fiber Analysis Agricultural Handbook (Thiex, 2009).

A. Moisture Content

To obtain the moisture content of the Napier grass in percent, 2g sample was weighed and then dried at 105 ± 3 °C for 24hrs until a constant weight was obtained. After 24hrs, the Napier grass was withdrawn, cooled and then weighed to the nearest 0.0001g. The moisture content was found on a percentage basis. The moisture content was determined by using equation 3.1.

$$\%\ of\ moisture\ content = \frac{m_1 - m_2}{m_1} * 100 \quad \text{.....3.1}$$

Where:

m_1: $Intal\ Mass\ of\ Sample\ brefore\ drying$

m_2: $Fanial\ Mass\ of\ Sample\ after\ Dring$

B. Ash Content

Ash content of Napier grass was determined following ASTM 2014 methods. The crucible was oven dry, cooled and weighted until constant weight. 3g of Napier grass sample was measured and placed in the crucible. Then crucible and sample were weighed to the nearest 0.0001g. The Napier grass sample was ignited at 525 ± 25 ºC for about 4hrs (ASTM D1102-84). After 4hrs, the crucible was carefully withdrawn and cooled in a desiccator then weighed. The ash content was calculated on percentage basis according to equation 3.2.

$$Ash\ content\ \% = \frac{WAA - TWOC}{OSW} * 100 \quad \text{.....3.2}$$

Where

WAA : $Weight\ after\ ignition$

$TOWC$: $Tare\ Weight\ of\ crucible$

$OSW : Original\ sample\ Weight$

3.4.2. Chemical Composition Analysis

Compositional analysis of the lignocellulose material, extractives, cellulose, hemicellulose and lignin content was analyzed using gravimetric method under the conditions defined by (Ayeni et al., 2015).

A. Extractive

3 g of dried Napier grass was loaded into the cellulose thimble. With the Soxhlet extractor set up, 200 mL of acetone was used as solvent for extraction. After about 8 hours' extraction, the sample was air dried at room temperature for few minutes. Constant weight of the extracted material was achieved in a convection oven at 105°C. The % (w/w) of the extractives content was evaluated as the difference in weight between the raw extractive-laden Napier grass and extractive-free Napier grass. Equation 3.3. was used to obtain the % extractive content of Napier grass sample:

$$Extractive\ \ \% = \frac{m_1 - m_2}{m_1} * 100 \quad \text{.....3.3}$$

Where:

$m_1 : Mass\ of\ sample\ before\ Soxhelet$

$m_2 : Mass\ of\ sample\ After\ Soxhelet\ and\ oven\ Drying$

B. Hemicellulose

10 g of extracted dried Napier grass was transferred into a 250 mL Erlenmeyer flask. 150 mL of 500 mol m^{-3} NaOH was added. The mixture was boiled for 3.5 hrs. with distilled water. It was filtered after cooling through vacuum filtration and washed until neutral pH. The residue was dried to a constant weight at 105 ºC in a convection oven. The difference between the sample's weights before and after this treatment is the hemicellulose content (%w/w) of dry Napier grass.

$$concentration\ of\ Hemicellulose\ \ \% = \frac{m_2 - m_1}{m_3} * 100 \quad \text{.....3.4}$$

Where:

$m_1 : Weight\ of\ over\ dryed\ filter\ paper\ (g)$

$m_2 : Weight\ of\ filter\ paper\ after\ Soxhelet\ and\ dryed(g)$

$m_3 : weight\ of\ Sample\ after\ oven\ dryed\ (g)$

C. Lignin

1.8 g of dried extracted raw Napier grass was weighed in glass test tubes and 18 mL of 72% H_2SO_4 was added. The sample was kept at room temperature for 2hrs with carefully shaking at 30 min intervals to allow for complete hydrolysis. After the initial hydrolysis, 50 mL of distilled water was added. The second step of hydrolysis was made to occur in an autoclave for 1hrs at 121 °C. The slurry was then cooled at room temperature. The hydrolysate was filtered through vacuum using a filtering crucible. The acid insoluble lignin was determined by drying the residue at 105 ºC and accounting for ash by incinerating the hydrolyzed sample at 575 ºC in a muffle furnace (Conesa & Götz, 2008).

$$concentration\ of\ Lignin\ \ \% = \frac{m_2 - m_1}{m_3} * 100 \quad \text{.....3.5}$$

Where

$m_1 : Weight\ of\ oven\ dryed\ filter\ paper\ (g)$

$m_2 : Weight\ of filterd\ lignin\ and\ filter\ paper\ after\ dryed\ (g)$

$m_3 : Weight\ of\ oven\ dryed\ sample\ (g)$

D. Cellulose

The cellulose content (%w/w) was calculated by difference, assuming that extractives, hemicellulose, lignin, ash, and cellulose are the only components of the entire Napier Grass biomass (Ayeni et al., 2015).

$$Cellulose\ conc.\% = 100 - Ash\ content - Extractive - lignin - Hemicellilose \quad \text{.....3.6}$$

3.5. Pretreatment of Napier grass

To obtain the highest glucose yield from the hydrolysis of Napier grass, an alkaline pretreatment followed by enzyme hydrolysis was used. (He et al., 2017). 2.5wt% sodium hydroxide, 1.5wt% urea, and 8% of the sample were loaded and mixed for 40 minutes at 121°C. This eliminates the need for any additional separation costs to extract sugar from the pretreatment and enzymatic

processes for the subsequent fermentation process. The use of alkali-pretreatment avoids the formation of furfural fermentation inhibitors, which are produced during the acid-pretreatment process and necessitate an additional separation process (He et al., 2017). The sample is alkaline treated, which dissolves the lignin and exposes the cellulose and hemicellulose. Following enzyme addition, the exposed macromolecules are hydrolyzed, and cellulose and hemicellulose begin to form their monomers, which is glucose.

The solid residues in the centrifuge tube were washed with deionized water to remove the majority of the residual chemicals. The pretreated solid was weighed after drying the solid residues to determine the weight loss percentage.

Enzymatic Hydrolysis

Following alkali pretreatment, enzymatic hydrolysis was used. Cellulase enzyme was used, which was purchased in Switzerland from Novozymes cellic. 5 g of pretreated substrate was mixed with 100 mL of 0.05 M citrate buffer (pH 5.0) containing 0.2 wt.% sodium azide and 2 mL of cellulase enzyme. For 40 minutes at 50°C, the reaction mixture was gently shaken at 50 rpm. The mixture was then centrifuged at 8500rpm for 10 minutes to separate the insoluble solid from the supernatant. Using dinitro salicylic acid (DNS) methods and a spectrometer, the concentration of monosaccharides in the supernatant was determined (Marsden et al., 1982a)

3.5.1. Determination of Total Reducing Sugar using DNS method

DNS method provides a quick and simple estimate of the extent of scarification, by measuring the total amount of reducing sugars in the hydrolysate. It is, however, susceptible to interference from citrate buffer and other substances, as well as the varying reactivity of the various reducing sugars. When complex substrates, such as sugar cane bagasse, are used, these interferences become more visible (Marsden et al., 1982b)

When using the manual method 1ml of sugar solution and 5 ml of DNS reagent were mixed in test tube and placed in boiling water bath, in Addis Ababa case 92 °C for about 5 minutes and transferred to an ice bath for rapid cooling and then transfer to 25 °C water bath to measure the absorbance. The absorbance was then measured using a spectrometer at 540 nm (Saqib & Whitney, 2011). To identify the concentration of sugar sample using spectrometer calibration curve was prepared using different concentrations (Marsden et al., 1982b). Standard glucose

solution was prepared at different concentration of, 25, 50, 75, 100, 125 and 150 µg L^{-1}. Then, 1 ml of each standard glucose solution was added to test tubes containing 5 ml of DNS reagent and shaken together. Each test tube was immersed in a 92^{o}C boiling water bath for 5 minutes before being cooled in a 25^{o}C water bath for 10 minutes. Using a Lambda 950 spectrophotometer, the absorbance of standard samples was measured at 540 nm. Finally, a calibration curve was obtained by plotting standard concentration versus absorbance, and a linear equation was developed.

The reducing sugar from the hydrolysis process was determined using the same procedure, and the absorbance obtained from this was calculated using the calibration curve equation (Hu et al., 2008).

3.6. Microorganisms Incubation

All media and equipment were sterilized at 121^{o}C for 15 minutes prior to cell inoculation. The pre-culture was prepared in anaerobic conditions in order to propagate the microbe, adapt the substrate, and create a favorable environment during fermentation. The pH of the pre-culture was initially adjusted to 6.8. The pre-culture solution was then heat-shocked for 5 minutes at 42^{o}C; upon immediate temperature upshift, the cellular level of sigma 32, which is responsible for heat-shock gene transcription, increases rapidly and transiently. The increase in sigma 32 is due to increased synthesis as well as stabilization of sigma 32, which is normally very unstable (Yura et al., 1993) following which; the cultures were transferred into a shaking incubator controlled at 37^{o}C and 150 rpm for 18–24 hrs. The medium for main culture of C. acetobutylicum was LB-s medium (He et al., 2017):10 gL^{-1} sodium chloride, 5 g L^{-1} yeast extract, 0.11 g L^{-1} $FeSO_4 \cdot 7H_2O$, 0.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 0.008 g L^{-1} $CaCl_2$.

Colony counting

A 0.1ml pre-cultured Clostridium acetobutylicum was dropped onto a plate and spread with a spreader over the entire plate. After 24hrs, the plate was removed from the incubator and colonies were counted using a colony counter. (Laramore, 2017).

3.7. Fermentation

In this fermentation process the independent parameters used are temperature, pH, concentration of sugar substrate, fermentation time, while agitation speed, and other nutrients values like nitrogen were kept constant. Fermentation was conducted from 34^{o}C to 40^{o}C, for fermentation

time with residence time of 48 - 96 hrs. In addition to temperature and residence time, pH was also optimized from 5 - 7 since the microorganisms operate at this pH range. Hydrolysates of Napier grass sugar concentration were also optimized from 2 % to 10 % w/w. and the optimum agitation speed for this process was set as 150 rpm (Glassner et al., 1991).

3.7.1. ABE Fermentation

The fermentation medium and conditions were varied to achieve a high level of total solvents and butanol using hydrolyzed Napier grass as fermentation substrate. The initial fermentation medium contains 2% (w/w) substrates, and the pH adjusted to 5 with saturated NaOH before autoclaving for 20 min at 121 °C. Fermentation parameters including sugar content of hydrolysate, nitrogen source, pH, temperature, buffering agent, trace element, and inoculum size is optimizing. The experiments perform in a 250ml Erlenmeyer flask with 150 mL working volume for 72 hrs.

Under optimized fermentation conditions, batch culture is performed in a 500ml stirred bioreactor with a 250ml fermentation medium. Because sodium chloride has been reported to be a strong inhibitor of cell growth in ABE fermentation, the pH of the medium is carefully adjusted with freshly prepared saturated NaOH to avoid the introduction of HCl. (Qureshi et al., 2001). To ensure an anaerobic environment, external CO_2 gas is aerated into a bioreactor through microbial filter before inoculation.

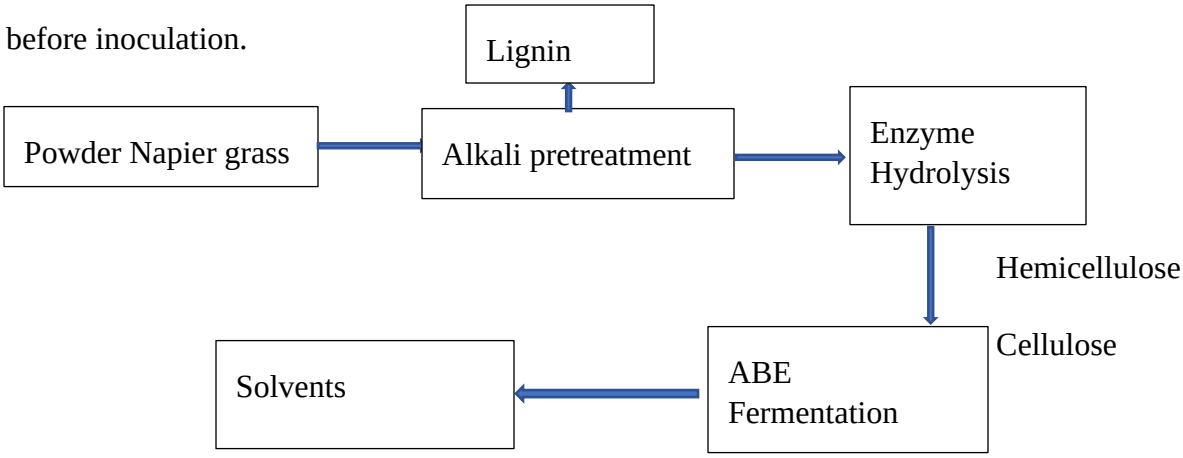

Figure 4 ABE Fermentation

3.7.2. Semi-simultaneous Scarification Fermentation

sSSF was (Shen & Agblevor, 2010) started with enzymatic hydrolysis reaction where 12g of alkaline pretreated sample mixed with 100ml of distilled water and 3ml of endoglucanases and beta-glucanase enzymes. This hydrolysis lasted 24 hours at 50°C and 200rpm, after which the ABE fermentation was initiated by adding 12ml (360CFU ml^{-1}) of pre-cultured Clostridium

acetobutylicum to the solution. This count corresponds with research found at (Mann et al., 2012) where the pH was adjusted to 6 and the fermentation continued for about 96 hours, with samples taken every 24 hours to analyze the butanol yield.

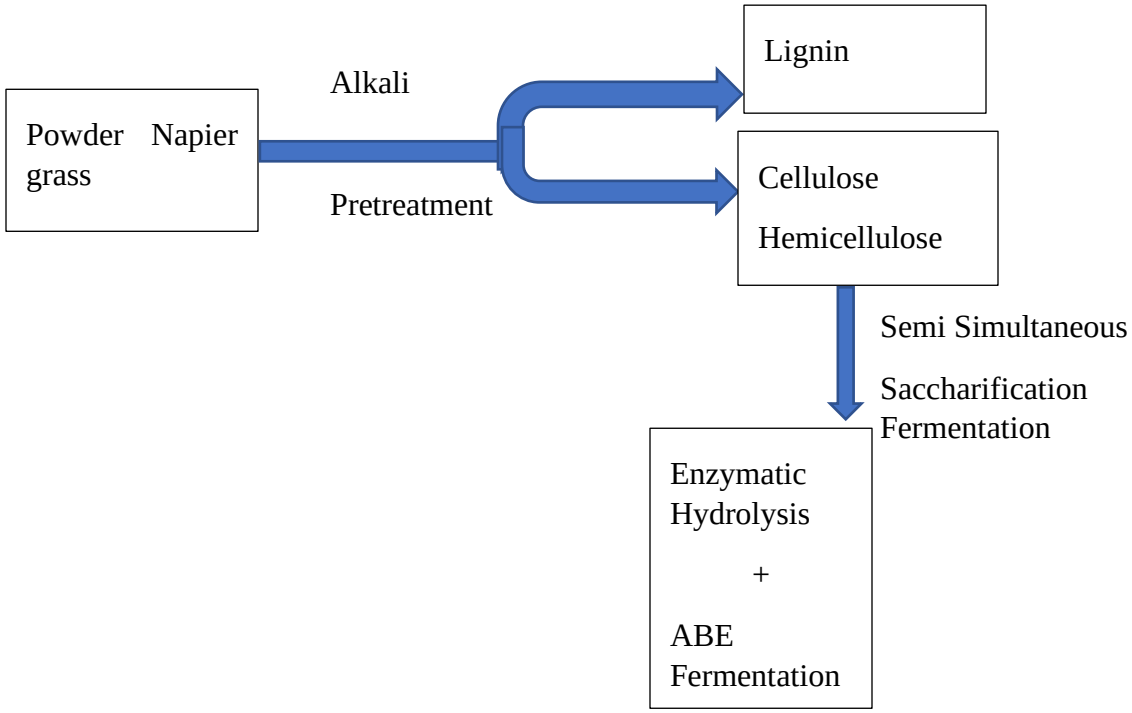

Figure 5 s-SSF Process Diagram (He et al., 2017)

3.8. Distillation

The downstream process is primarily concerned with product separation and purification. Different types of downstream operations are chosen based on the characteristics of the product. Having said that, because the output of ABE fermentation is a mixture of acetone, butanol, and ethanol, these products must be separated by distillation. Distillation is a thermal separation process that is based on the difference in solution volatility. The volatility of acetone, butanol, and ethanol solutions differs significantly. The distillation process is well used to separate each component's volatility. The operation was carried out at temperatures ranging from 57^{o}C to 80^{o}C because the boiling temperature of acetone is 57^{o}C and that of ethanol is 78^{o}C, whereas butanol has a higher boiling point of 117^{o}C, resulting in Butanol as the bottom product. The separation was carried out in a rotary evaporator connected to a vacuum pump.

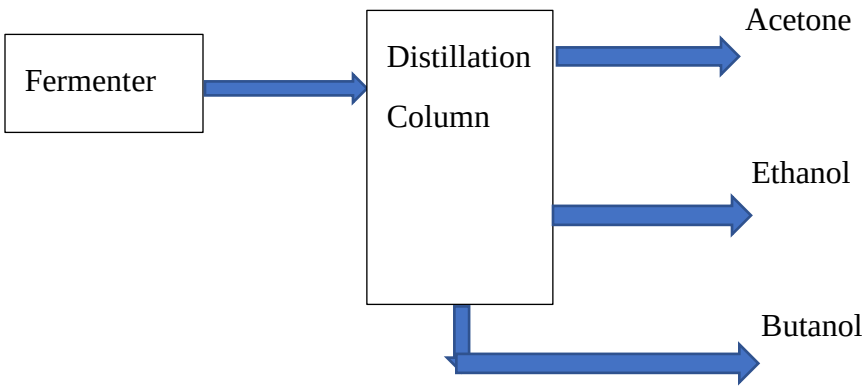

Figure 6 Distillation

3.9. Characterization of the Product

Using the DNS method and a spectrometer, a sample of broth was taken to test the unconverted total reducing sugar content. The fermented product was then fractionally distilled to separate ethanol and acetone from the main target product, butanol. A 250 ml sample was taken from a fermenter and filtered to determine the amount of alcohol in the broth after distillation. The sample was then distilled in order to separate the alcohol from the water. Distilled water was used to dilute the collected distillate back to exactly 250ml. A density meter was used to determine the concentration of alcohol in the solution (Nordon et al., 2005). Whereas the calorific value of the product was determined using Bomb calorimeter.

In order to determine the bio-functional butanol's groups. A beam of infrared light was passed through the sample to perform Fourier Transform Infrared Spectroscopy (FTIR). The FTIR spectra were captured using a Spectrum 65 FT-IR equipped with a KBr beam splitter. Thin film deposition techniques were used to create a NaCl plate for liquid samples (bio-butanol). A scanning range of 400-4000 cm^{-1} was used on a regular basis.

High Liquid Performance Chromatography was used for qualitative analysis (HPLC). HPLC (waters 600, with components external column and UV-Vis detector at wave length of 250) was used to analyze the broth sample. Because the column does not have an oven, the broth was examined at room temperature with a flow rate of 0.5ml min^{-1} using HPLC grade mobile phase containing 0.005M sulfuric and a column type of column (is reverse phase C18) (M. Kumar et al., 2014).

3.10. Experimental Design

Response surface methodology (RSM) with Box Behnken Design (BBD) was used to carry out the experiment and optimize the identified variables. RSM is a set of mathematical and statistical techniques that can be used to model and analyze problems in which a response of interest is influenced by several variables and the goal is to optimize this response (Montgomery, 2017). The RSM, which is based on statistical principles, was used as an interesting strategy to implement process conditions that lead to optimal bio-butanol production while requiring the least number of experiments. As a result, the analysis was carried out using BBD experiments. BBD helps to minimize the run and also does not deviate from the set value, but its limitation is that it repeats the same run more than three times and by using the design expert 12.3.0 software package, as shown in table 3.2.

Table 3:2 Box Behnken Design with the response of bio-butanol yield

	Factor 1	Factor 2	Factor 3	Factor 4
Run	A: Substrate	B: Fermentation time	C: pH	D: Temperature
	w/w%	Hrs.		^{o}C
1	2	48	6	37
2	2	72	5	37
3	6	48	7	37
4	10	96	6	37
5	6	72	6	37
6	10	72	5	37
7	10	48	6	37
8	2	72	6	34
9	10	72	6	34
10	6	72	6	37
11	6	96	6	40
12	6	48	6	40
13	10	72	7	37

14	2	96	6	37
15	6	72	6	37
16	6	72	5	34
17	6	48	6	34
18	2	72	7	37
19	6	72	6	37
20	6	96	7	37
21	6	48	5	37
22	6	72	7	34
23	6	96	6	34
24	6	72	5	40
25	6	72	6	37
26	6	96	5	37
27	6	72	7	40
28	10	72	6	40
29	2	72	6	40

4. Results and Discussion

4.1. Characterization of Napier Grass

4.1.1. Proximate Analysis

Moisture content measures the amount of water in Napier grass. This analysis was used to determine the proportionality of solid to liquid ratio in the pretreatment and hydrolysis quality. Ash content measures amount of inorganic impurities in Napier grass that have negative impact on ABE fermentation. The composition of dried Napier grass collected from Menagesha Mushroom farm was characterized and the proximate analysis is shown in table 4.1.

Table 4:1 Proximate Analysis of Napier Grass

Composition	**Weight percentage (dry Basis)**
Moisture	9
Ash content	7.8

The water and ash content of Napier grass from proximate analysis were 9 % and 7.8 % respectively, as it is shown in table 4.1. These values are higher than those in the previous study where 7.3 % ash and 7.5 % water content were reported (He et al., 2017). The main reason for difference might be due to species variation, harvesting time, age and the difference climate in Ethiopia and Thailand.

4.1.2. Chemical Analysis

Determination of cellulose and hemicellulose can be applied to quantify the theoretical production of butanol. Even though, microorganisms cannot utilize cellulose or hemicellulose complex, it is necessary to know the Napier grass chemical composition since the complexes will be converted to their respective monomer after hydrolysis.

Eliana et al. found that the chemical composition of Napier grass is in the range 22.6-42.4 % wt. for cellulose, 16.9-22.5 % wt. for hemicellulose, 19-30.6 % wt. for lignin, and 3.5-11.68 % wt. for extractives (Eliana et al., 2014). He et al. reported 36.8 % wt. of cellulose, 18 % wt. of hemicellulose, 23.4 % wt. of lignin and 7 % wt. of extractives (He et al., 2017). The Napier grass in this study exhibited comparable compositional range with the aforementioned studies as shown in Table 4.2.

Napier grass has 35.2 wt. % of cellulose and 16.9 wt. % of hemicellulose implying Napier grass has high potential for bio-butanol production. However, the concentration of lignin in the sample is also high 22.4 wt. %, suggesting the needs to select efficient hydrolysis method in order to prevent formation of inhibitors and toxic chemicals that have negative effect on ABE fermentation process.

Table 4:2: Chemical Composition of Napier Grass

Composition	**Weight percentage (dry Basis)**	**He.et.al wt. %**
Extract	8.7	7
Hemicellulose	16.9	18
Lignin	22.4	23.8
Cellulose	35.2	36.8

4.2. Determination of Total Reducing Sugar

Determining the total sugar before fermentation aids in understanding and designing the fermentation process. Following the DNS method, a standard curve of total reducing sugar after alkaline pretreatment and enzymatic hydrolysis was plotted. Table 4.3 and Figure 7 show the calibration data and curve, respectively.

Table 4:3 Standard Data and Absorbance

Absorbance	Reducing Sugar ($\mu g\ L^{-1}$)
0.092568	25
0.24144	50
0.3895	75
0.53944	100
0.70014	125
0.985623	150

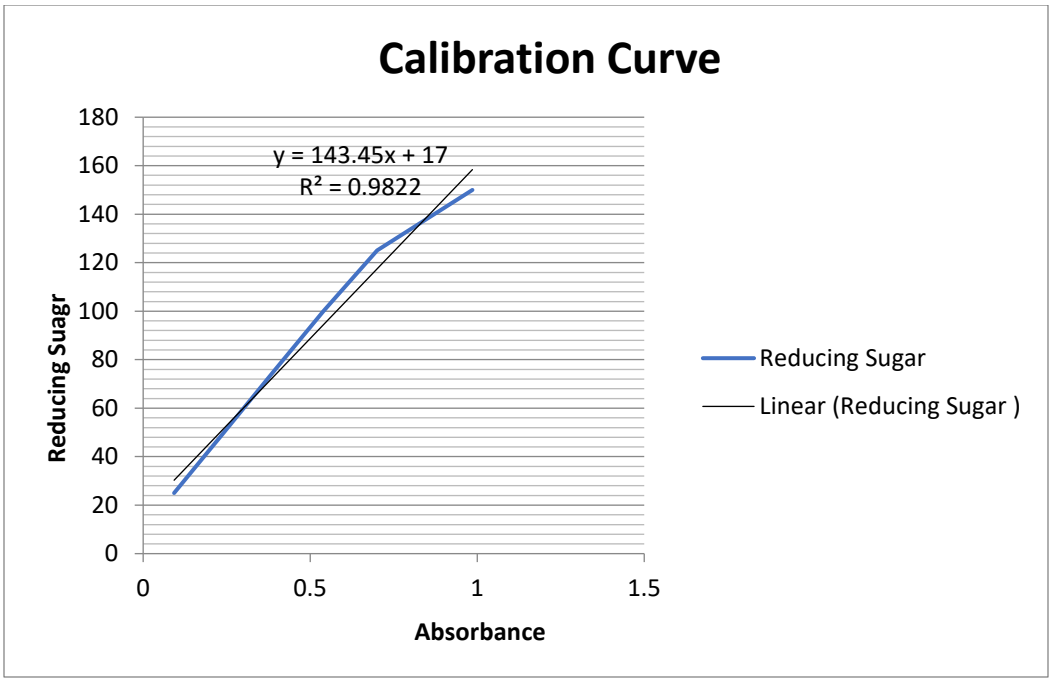

Figure 7 Reducing Sugar Calibration Graph

The calibration of standard sample and the main sample absorbance was performed using Lambda 950 UV-Vis's spectrometer and equation 4.1 was developed from the calibration curve.

$Y = 143.45X + 17$ ($R^2 = 0.9822$) ……...4.1.

The sample was diluted 200 times after hydrolysis and DNS treatment methods because the apparatus detects at microlevel (ppm) and thus dilution was important to reduce noise in the spectra. The sample absorbance at 540 nm was calculated as 0.88322 using equation 4.1, and the total reducing sugar concentration was $28.74 gL^{-1}$. From a 5g sample load, He et al. recovered 3.9 g total reducing sugar. In this study, 3.2 gram of total reducing sugar could be recovered, which was less than 18.19 percent of the previous study (He et al., 2017). The main reason for such a disparity could be the age and type of Napier grass.

4.3. Characterization of Butanol

4.3.1. Calorific Value

Heating value is highly recommended and significant property of fuels. Since it gives the energy content of the bio-fuel. Heating value can be expressed as gross and net calorific value depend on the phase of water exist in the exhaust. When water phase is in liquid form the heating value is gross calorific value, or if water exists in gas or vapor phase, it called net calorific value. Having a simple clarification about heating value in this study gross calorific value or higher heating value was determined using bomb calorimeter and the value obtained was 6,757.86 kcal L^{-1}, this result is on the same range that was reported on previous study (Kuszewski, 2018) 6,928.15 kcal L^{-1} .The calculation and data are presented at Annex A.

Density measurement of the product is in Appendix B

4.3.2. Fourier Transform Infrared Spectroscopy (FTIR) of Bio-Butanol

Alcohols' IR absorption properties are associated with hydrogen bond O-H, C-H, and C-O stretching vibration. When running as a liquid, the region 3500 – 3200 cm^{-1} with highly intense and broadband indicated the alcohol's O-H stretch. While the region 1260-1050cm^{-1} confirms the C-O stretch bond, the region 2880 cm^{-1} represents – CH_2, and the region 2930 cm^{-1} represents – CH_3. (Bodirlau et al., 2007). Figure 8 illustrated the FTIR spectra of the bio-butanol. Due to the conformation of these regions on Figure 8 shown assures that the product obtained by using Napier grass as biomass and with ABE fermentation was bio-butanol.

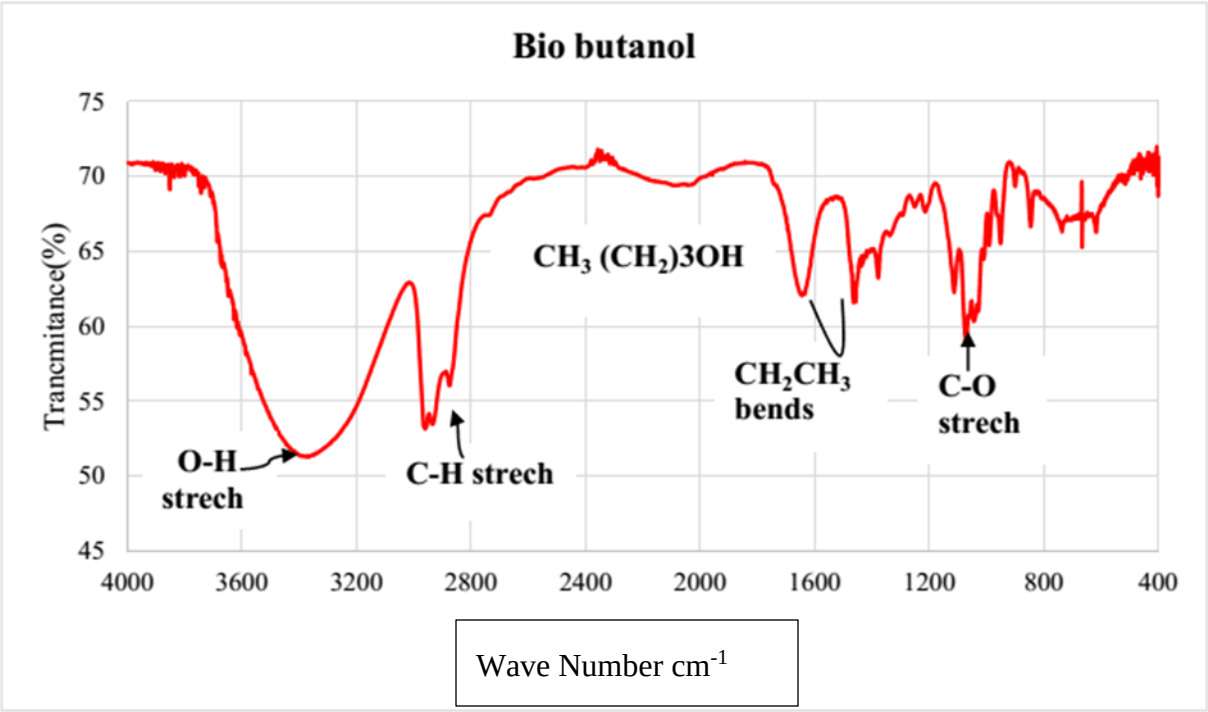

Figure 8 FTIR spectra of Bio-Butanol

4.3.3. High Performance Liquid Chromatography HPLC of Bio-butanol

Bio-Butanol Sample was analyzed by HPLC (waters 600, with components external column and UV-Vis detector at wave length of 250nm. The broth was examined at room temperature since the column do not have oven column temperature was at room temperature, with flow rate of 0.5ml min^{-1} using HPLC grade mobile phase consisted with 0.005M sulfuric and column type is column (is reverse phase C18) (M. Kumar et al., 2014). Figure 9 showed the chromatographs of broth sample for optimized parameters with flow rate of 0.5ml min^{-1} at room temperature.

All components in this sample were eluted in the increasing order of retention time with Glucose at 23 min, Acetic acid at 40 min, Ethanol at 24 min, Butyric acid at 58 min, Acetone at 59 min and butanol 95 min at 570 psi column pressure (M. Kumar et al., 2014). Figure 9 shows the existence of bio-butanol in the product.

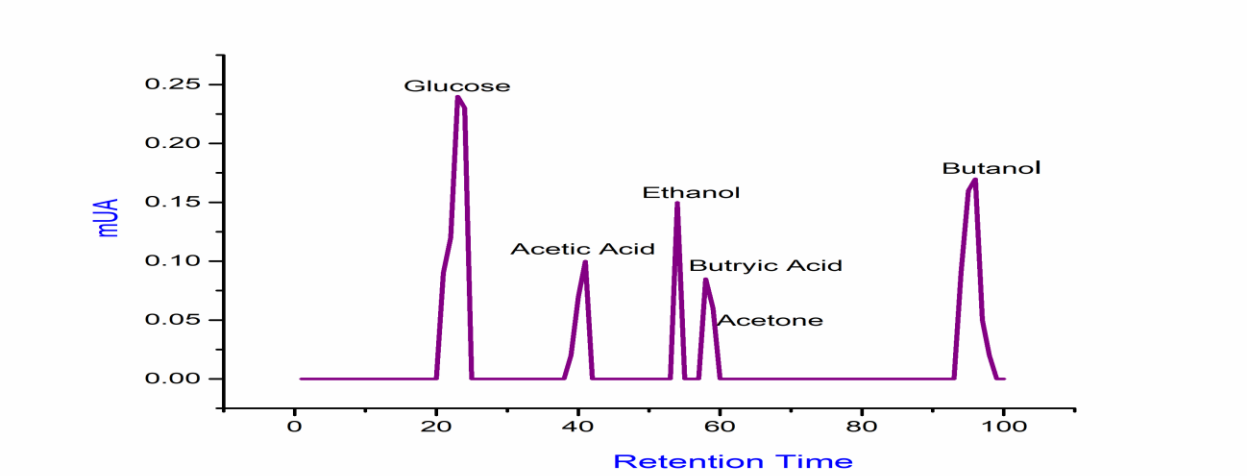

Figure 9 HPLC Chromatographs of Residual Sugar, Acetic Acid, Ethanol, Butyric Acid, Acetone and Butanol

4.4. Statistical Analysis of Experimental Results

BBD was used to investigate the four independent process variables, sugar substrate concentrations, temperature, fermentation time, and pH, and to reflect the nature of the response surface in the experimental region. To assess their impact on bio-butanol production, the least squares regression analysis of variance was performed using design expert software 12.0.3.0. (ANOVA). The statistical software program was used to generate the model equation, interaction effects of the independent variables, and surface plots based on the fitted equation obtained from the regression analysis while holding one of the independent variable constants.

The production of bio-butanol was investigated in 29 different runs under BBD conditions by varying the independent variable, and the results are shown in table 4.4. To produce bio-butanol, separate pretreatment and enzymatic hydrolysis were used, followed by ABE fermentation. The yield varies according to operational parameters, raw material properties, and concentration. This separated enzyme hydrolysis followed by ABE fermentation process yielded a productivity of 0.077g L^{-1} hr^{-1}. This result was in the same ballpark as previous studies, which reported productivity values of 0.08 g L^{-1} hr^{-1} and 0.05 g L^{-1} hr^{-1}. (He et al., 2017), (M. Yang et al., 2015).

Table 4:4 BBD Matrix and the Corresponding Response, Bio-butanol Yield

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Run	A:Substrate Con.	B:Fermentation Time	C:pH	D:Temprature	Butanol Yield
	% w/w	Hrs,		^{o}C	g/L
1	6	48	6	34	7.5
2	6	48	5	37	8.4
3	2	72	7	37	9
4	10	96	6	37	10.6
5	6	72	6	37	16.3
6	2	72	5	37	7.9
7	10	48	6	37	9.8
8	2	48	6	37	5.6
9	6	72	6	37	16.8
10	10	72	7	37	10.3
11	2	72	6	34	7.1
12	6	72	7	34	7.9
13	2	96	6	37	9.7
14	6	96	6	40	11.3
15	10	72	6	34	8.1
16	6	96	6	34	10.3
17	6	48	7	37	9.2
18	2	72	6	40	8.5
19	10	72	5	37	8.2
20	6	96	5	37	9.8
21	6	72	6	37	15.9
22	6	72	5	34	6.5
23	6	72	6	37	16.03
24	6	72	5	40	8.9
25	6	72	7	40	10.8
26	10	72	6	40	9.7
27	6	72	6	37	15.4
28	6	96	7	37	11.2
29	6	48	6	40	10.1

Depending on the operational parameters, hydrolysis of Napier grass followed by fermentation with *Clostridium Acetobutylicum* bacteria produced 5.6 g L^{-1} to 16.03 g L^{-1} of butanol. The findings suggest that Napier grass has the potential to be used in the production of bio-butanol. The maximum butanol product was obtained at a substrate concentration of 6 w/w, 72 hours of fermentation, a pH of 6, and a temperature of 37 °C. The minimum butanol product, on the other hand, was obtained at 2 w/w percent substrate, 48 hours of fermentation, pH of 6, and 37 °C.

Table 4:5 ANOVA for Response Surface Quadratic Model of Bio-butanol Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	253.39	14	18.10	46.57	< 0.0001	significant
A-Substrate Con.	6.60	1	6.60	16.99	0.0010	
B-Fermentation Time	12.61	1	12.61	32.44	< 0.0001	
C-pH	6.31	1	6.31	16.23	0.0012	
D-Temprature	11.80	1	11.80	30.37	< 0.0001	
AB	2.72	1	2.72	7.01	0.0191	
AC	0.2500	1	0.2500	0.6433	0.4359	
AD	0.0100	1	0.0100	0.0257	0.8748	
BC	0.0900	1	0.0900	0.2316	0.6378	
BD	0.6400	1	0.6400	1.65	0.2202	
CD	0.0625	1	0.0625	0.1608	0.6944	
A^2	103.64	1	103.64	266.68	< 0.0001	
B^2	53.51	1	53.51	137.69	< 0.0001	
C^2	81.62	1	81.62	210.02	< 0.0001	
D^2	89.87	1	89.87	231.25	< 0.0001	
Residual	5.44	14	0.3886			
Lack of Fit	4.38	10	0.4377	1.65	0.3337	not significant
Pure Error	1.06	4	0.2660			
Cor Total	258.83	28				

Factor coding is **Coded**.

Sum of squares is **Type III – Partial**

The F-value test compares model variance to residual (error) variance. If the variances are close to one another, the ratio will be close to one, and no factors are likely to have a significant effect on the response. It is calculated by dividing the model mean square by the residual mean square. In this model, the F-value of 46.57 indicates that the model is significant. An F-value this large could occur due to noise only 0.01 % of the time.

Prob >F values less than 0.050 indicate that model terms are significant. A, B, C, D, AB, A2, B2, C2, and D2 are all significant model terms in this case. Values greater than 0.1 indicate that the terms in the models are not significant. The lack of Fit F-value of 1.65 indicates that the lack of Fit is insignificant in comparison to the pure error. There is a 33.37 % chance that a large lack of Fit of F-value could occur due to noise.

Adeq. Precision is defined as the signal-to-noise ratio, which is the ratio of the predicted response's range of variation to an estimate of standard error of prediction. R-squared (multiple correlation coefficient), CV (coefficient of variation), and P-Value are used to assess model adequacy. A higher R-squared value indicates a stronger correlation between the experimental and predicted values.

Table 4:6 R^2 Value for the Quadratic Model

Std. Dev.	0.5257		**R^2**	0.9790
Mean	12.91		**Adjusted R^2**	0.9588
C.V. %	4.07		**Predicted R^2**	0.8962
			Adeq Precision	21.1024

The Predicated R^2 of 0.8962 is in a reasonable agreement with the Adjusted. R^2 of 0.9588 as shown in table 4.6, their difference is 0.0626 which is less than 0.2 showing very acceptable range. Another factor to see is the Adeq. Precision where a ratio greater than 4 is desirable 21.1024 signifies this model can be used to navigate the design space.

The fit of the model was also expressed by the coefficient of regression R^2 which was to be 0.9790 implying 97.9 % variability in the response could be explained by the model. The value of R (correlation coefficient) close to 1 correlation between the experimental and predicted values. In this study the value of R^2 is 0.9790 indicating a close agreement between the experimental results and theoretical values predicted by the model equation.

4.5. Determination of Optimum Operating Parameters

In this study, the effects of operating conditions on bio-butanol yield were investigated, and optimal values were determined. The results of experimental work are discussed in order to identify major factors. For optimum fermentation conditions in terms of improving yield and bringing out the required biochemical reaction, it was necessary to optimize parameters such as pH, temperature, and substrate concentration, which resulted in effective conversation of reducing sugar at the optimum fermentation time.

4.5.1. Effect of Substrate Concentration

Favorable substrate concentration for the microbe during ABE fermentation was investigated in the range of 2 % to 10 % w/w. The total production was increase when the substrate concentration was raised from 2 % to 6 % w/w and maximum bio-butanol of 16.03 g $L^{-1}$was recovered at 6%w/w. When the substrate concentration was increased further from 6 % to 10 % w/w the production of bio-butanol was decreased sharply.

As shown figure 10, when the sugar concentration is low, the microbe starts consuming it and makes butyric and acetic acid and, these acids immediately start to convert to butanol and acetone solvents. Hence, at lower sugar concentration the production of butanol increase and reaches its maximum until 6 % w/w. when the concentration was further increased the substrate acts as an inhibitor for C. acetobutylicum showing similar pattern with the previous reports (He et al., 2017) (Berezina et al., 2009).

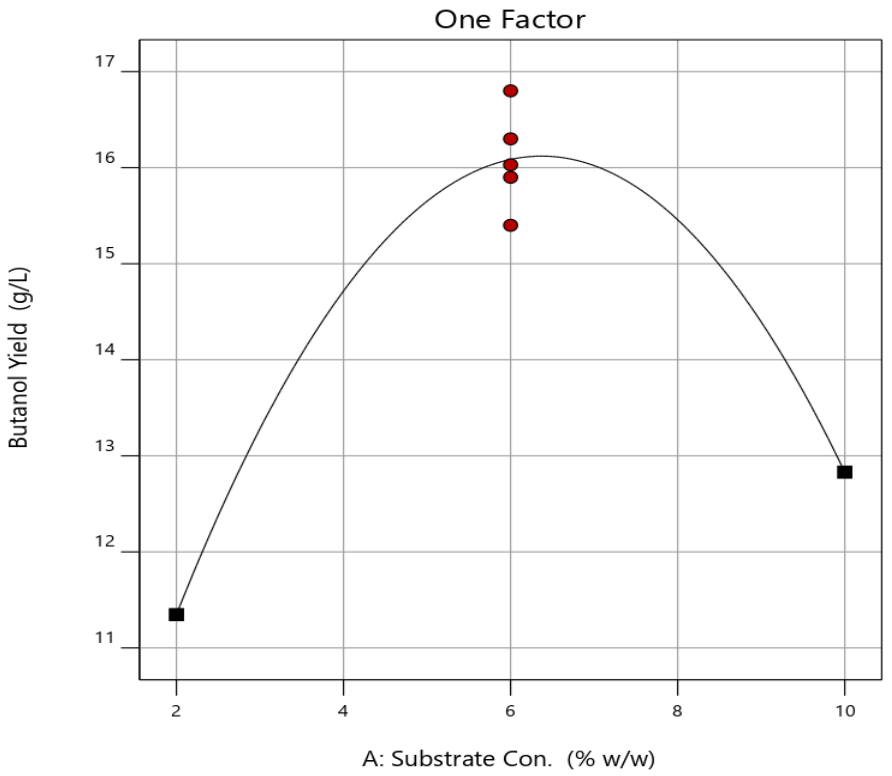

Figure 10: Effect of Substrate Concentration

4.5.2. Effect of Temperature

The main parameter in order to have proper fermentation process is optimizing fermentation temperature since most microorganisms are very sensitive to temperature. The effect and allowable optimum temperature to produce butanol and possible higher product is shown in figure 11. The effect of temperature was analyzed starting from 34^{o}C to 40^{o}C and an increment in butanol yield was observed starting from 34^{o}C and reaches its maximum at 38^{o}C. After that, the yield started to decline due to the inefficiency of C. acetobutylicum bacteria caused by high temperature effect (Shao & Chen, 2015) (Stein et al., 2017).

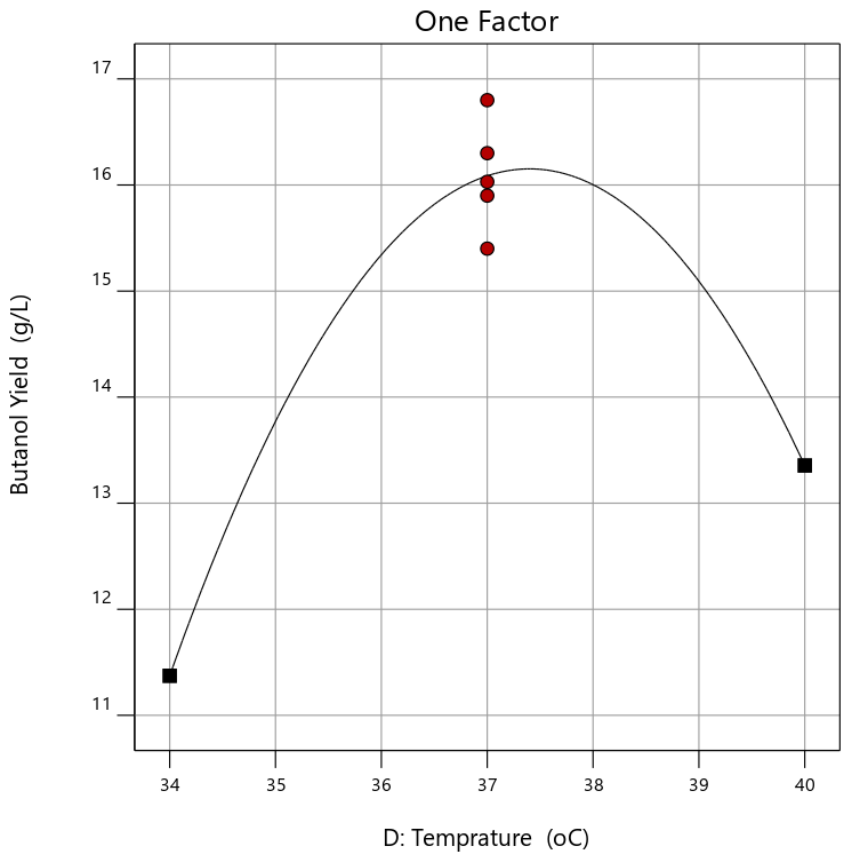

Figure 11 Effect of Temperature

4.5.3. Effect of Fermentation Time

Fermentation time is a crucial parameter in order to get considerable amount of the product. ABE fermentation process has two phase, acidogenic and solventogenic phase. Acidogenic phase is manifested by decreasing the pH from 5.6 to 4.9 in the first 12hrs while solventogenic phase starts immediately after 12hrs if the process is controlled and is accompanied by converting organic acids to ABE solvent. In this phase the pH shows increment up to 6.3. as depicted in figure 12 butanol yield increased up to 72hrs where it reaches its maximum and after that the yield enters to declining stage because of toxicity of butanol that kills the microorganisms (Ni et al., 2012).

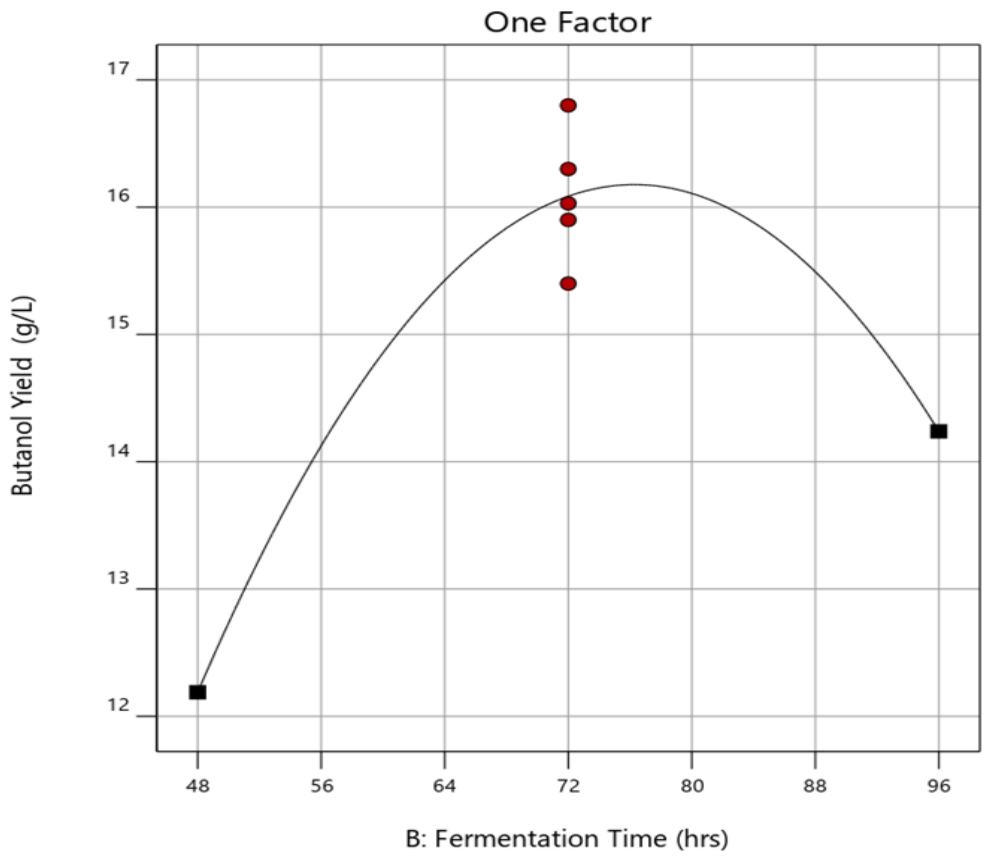

Figure 12: Effect of Fermentation Time

4.5.4. Effect of pH

The effect of pH on butanol production is shown in figure 13. To determine the effect of pH in ABE fermentation, it is necessary to understand the metabolic path way of *C. acetobutylicum.* In the first 12hrs. the fermentation process produces more acid due to acidogenic phase. After the first 12hrs, controlling the pH is crucial since fermentation will proceed at acidogenic phase and the pH decreases further until it reaches 3.6 for additional 12 hrs. hindering the production of solvents. Below 3.6 pH, the fermentation broth will be more acidic creating unfavorable condition for the microbe. When the pH was maintained between 5 and 6, the production of bio-butanol increased constantly. At pH 6, butanol production reaches its maximum and starts to decrease dramatically due to formation of NaCl inhibitor (Tsai et al., 2014). NaCl is formed by the addition of NaOH to adjust and keep the pH at the desirable condition. This study confirms that a suitable pH control method can increase butanol production in ABE fermentation.

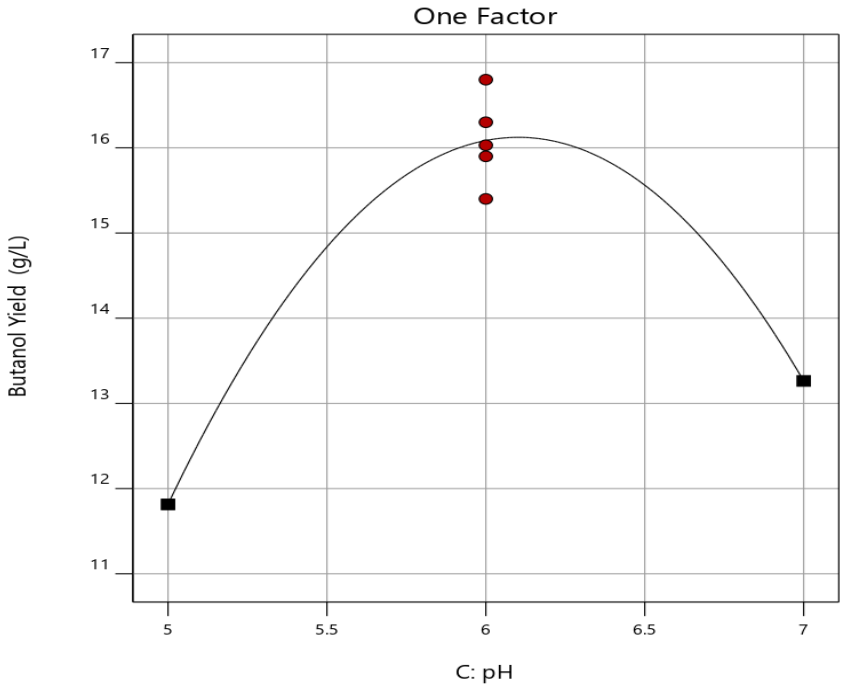

Figure 13 Effect of pH

4.5.5. Interaction effect of Sugar Concentration and Fermentation Time

Interaction effect of sugar concentration and fermentation time on butanol yield have been analyzed. For a constant temperature and pH, variation in the yield of bio-butanol was observed. When the sugar concentration is 2% w/w, the butanol yield is 5.6 g L^{-1} lower than the yield of 10 % w/w sugar concentration, 9.8 g L^{-1}, at 48hrs of fermentation time. This initial productivity difference due to sugar concentration was minimized when the fermentation time was further increased to 96 hr. When fermentation time was increased from 48hrs to 96hrs, the butanol yield with 2 % w/w sugar concentration was increased from 5.6 g L^{-1} to 9.7 g L^{-1} while for the 10 % w/w sugar concentration, butanol yield was increased only from 9.8 g L^{-1} to 10.6 g L^{-1}. It is clearly seen from figure 12 that lower sugar concentration needs more fermentation time to yield bio butanol compared to high sugar concentration which exhibited a very small change in the final product for higher fermentation time.

High sugar concentration act as inhibitor while low sugar concentration affects the growth and activity of *C. acetobutylicum* because of limitation of food source. In addition, longer fermentation time affects the metabolic activity of microbe, by producing organic acid and NaCl salt inhibits

the growth of microbe. Accumulation of butanol has toxicity property for microorganisms negatively affecting the yield.

Figure 14 shows that when the sugar concentration and fermentation time start increasing form 2 % w/w to 6 % w/w and 48 to 72hrs, respectively, the production of butanol increased to the highest level of 16.8 g L^{-1}. However, productivity couldn't exceed more than 16.8 g L^{-1} because the increment in concentration of butanol has the property of toxicity for the microbe. In addition to toxicity, formation of NaCl has inhibiting effect on fermentation process (Abulude, 2004). Hence, it is realized that both sugar concentration and fermentation time have strong relation in the production of bio butanol.

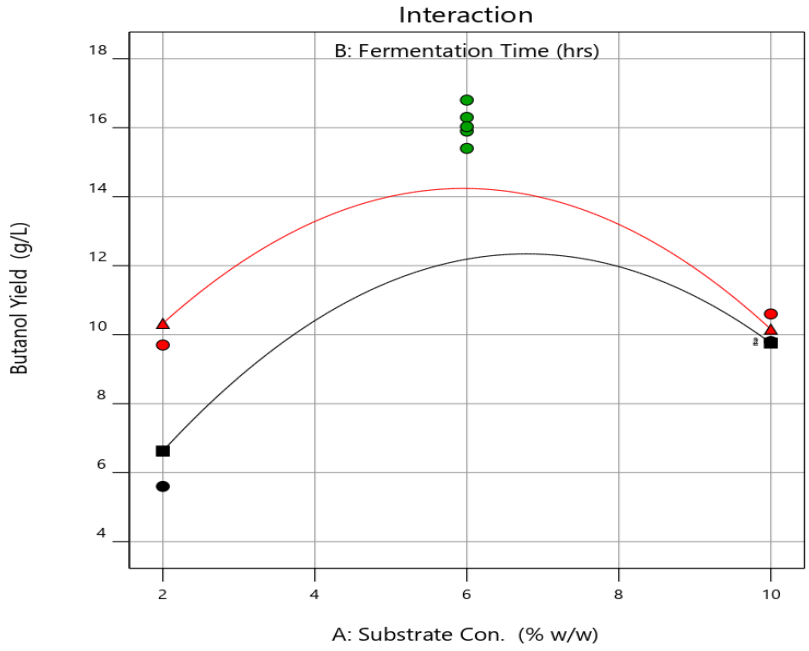

Figure 14 Interaction effects of Sugar concentration and Fermentation time

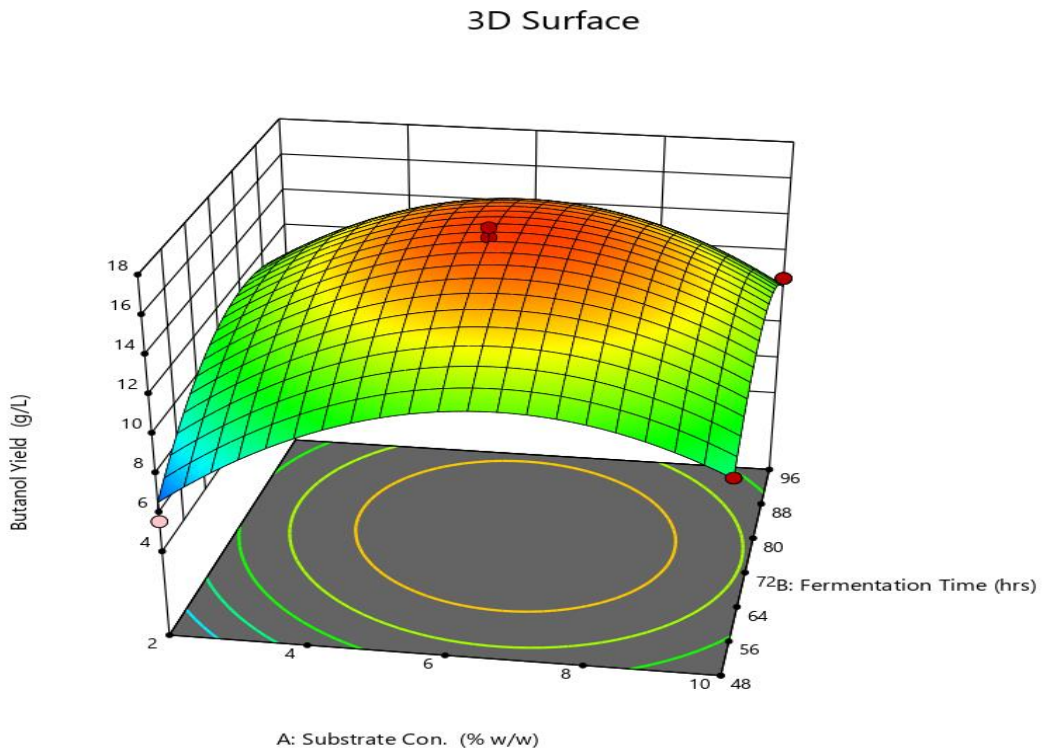

Figure 15: 3D surface interaction between Sugar concentration and Fermentation time

4.6. Optimization

Optimization of butanol yield was carried out by multiple response methods called Desirability (D) function to optimize the different combination of process parameters. The main aim was to maximize economic benefit or increase the yield of butanol by minimizing the process cost. To investigate the optimum values of butanol production in ABE fermentation from Napier grass using alkaline pretreatment and enzyme hydrolysis, the optimization constraints are summarized in table 4.7.

Table 4:7 Optimization Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight
A: Substrate Con.	is in range	2	10	1	1
B: Fermentation Time	minimize	48	96	1	1
C: pH	is target = 6	5	7	1	1
D: Temperature	is in range	34	40	1	1
Butanol Yield	maximize	5.6	16.8	1	1

Desirability lies between 0 and 1; it represents the closeness of response to its ideal value. If a response falls with unacceptable interval, the desirability is zero (0) and if it falls with the ideal intervals or the response has ideal value the desirability is one. In this study based on the analysis, the best local maximum butanol production of 15.649 g L^{-1} exhibited higher desirability of 0.86 at 6.51 % w/w sugar substrate concentration, 64.65hrs fermentation time, pH of 6 and temperature 37.459^{o}C.

Table 4:8 Optimum Possible Solution Based on Optimization of Constraints

Number	Substrate Con.	Fermentation Time	pH	Tempratur e	Butanol Yield	Desirability	Desirability (w/o Intervals)	
1	6.510	64.650	6.000	37.459	15.649	0.839	0.860	Selected
2	6.507	64.492	6.000	37.457	15.631	0.839	0.860	
3	6.541	64.670	6.000	37.456	15.651	0.839	0.860	
4	6.515	64.857	6.000	37.468	15.671	0.839	0.860	
5	6.476	64.622	6.000	37.469	15.646	0.839	0.860	
6	6.524	64.548	6.000	37.488	15.637	0.839	0.860	
7	6.458	64.833	6.000	37.452	15.668	0.839	0.860	
8	6.476	65.004	6.000	37.440	15.687	0.839	0.860	
9	6.613	64.548	6.000	37.478	15.634	0.839	0.860	
10	6.606	65.159	6.000	37.492	15.699	0.839	0.860	
11	6.379	65.211	6.000	37.501	15.704	0.839	0.860	
12	6.518	64.920	6.000	37.591	15.670	0.839	0.860	

4.7. Model Validation

The optimum process variables obtained from the design is show in Table 4.8 and the optimum value was realized at a temperature of 37.459ºC, fermentation time 64.655hrs, pH 6 and total reducing sugar 6.51 % w/w. Under this condition the predicted amount of bio-butanol yield is 15.649 g L^{-1}. In order to confirm the validity of the model, experiments were conducted at the specified optimum condition and an average of 15.3 g L^{-1} of bio-butanol was obtained. Therefore, the model is considered to be valid and reliable for predicting the optimum bio-butanol yield

Two-sided Confidence = 95%

Table 4:9 Validation Confirmation

Solution 1 of 12 Response	Predicted Mean	Predicted Median	Observed	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
Butanol Yield	15.6488	15.6488		0.623389	3	0.449752	14.6842	15.3	16.6134

4.8. Determination of bio-butanol yield in sSSF

The production of butanol was analyzed and compared with a previous batch fermentation method, sSSF. In sSSF, alkali pretreated Napier grass was converted to bio-butanol while avoiding additional steps (Shen & Agblevor, 2010). To produce glucose, a concentration of 12 g L^{-1} alkaline pretreated Napier grass was mixed with 3 ml of beta glucanase and Endoglucanase enzyme. The viscosity of the suspension decreased as a result of this process. The *C. acetobutylicum* was inoculated after 24 hours, and the ABE fermentation was carried out concurrently. After the process is completed, the maximum amount of bio-butanol recovered at optimized parameters 64.65hrs, 6 pH, and 37.459 °C is 17.6 g L^{-1}. sSSF has a higher product yield when compared to conventional pretreatment and hydrolysis separately, as well as ABE fermentation. sSSf produced a high butanol productivity of 0.083 g $L^{-1}h^{-1}$ with a 33.6 % efficiency. It has a very high yield when compared with a previous report with butanol productivity of 0.08 g $L^{-1}h^{-1}$ and 31% efficiency (He et al., 2017). The result from sSSF revealed that Napier grass biomass has the potential to produce

butanol via ABE fermentation. Table 4.10 shows different biomass and processes to produce butanol yields compared to this study.

$$Efficiency = \frac{mass\ of\ butanol_{produced}}{Mass\ of\ butanol_{Therortical}} \quad \text{.....4.1.}$$

on the bases of mass of the substrate the calculation is at Appendix C

Table 4:10 Comparison of ABE Fermentation Products

Biomass	**Pretreatment, Enzyme loading and butanol producing Strain**	**Butanol Productivity g/lh**	**Butanol yield**	**Butanol Efficiency**	**Reference**
Napier Grass	2.5%w/w, 5mlNovozymatic Cellic CTec2 , C.acetobutylium	0.077	0.16	29.5%	This Study
Napier Grass	2.5%w/w, 5mlNovozymatic Cellic CTec2 , C.acetobutylium (sSSF)	0.083	0.18	33.6%	This Study
Barley Straw	2%w/w H_2SO_4 cellulast 1.5L and novozyme 188, C. acetobutylicum DSM1731	0.05	0.2	36%	(M. Yang et al., 2015)
Rice Straw	2%W/w NaOH Trichoderma Harzianum SNR33 C. acetobutylium ATCC824	0.01	0.14	13%	(Rahnama et al., 2014)
Pine Wood	Autohydrolysis nethod 25FPU cellulose and 40IU β glucosidase C. acetobutylium NRRL B-591	0.05	0.2	19%	(Amiri & Karimi, 2015)
Sugar Cane	1%NaOH, 50mMH_2SO_4 for hydrolysis of cellulose C. acetobutylium	0.15	0.23		(Kong et al., 2016)
Sugar Cane	Ozonolysis method ,30CBU Novozymes 188 ceelulast C. acetobutylium DSM792	0.01	0.07	12%	(Travaini et al., 2016)
Corn Stalk leaf	0.5w/w NaOH, 40FPU, cellulose C. acetobutylium ABE 1301	0.05	0.24	31%	(Cai et al., 2016)
Napier Grass	0.5%w/w NaOH, 40FPU Novozymes Cellic CTec C.acetobutylium ATCC824	0.08	0.22	31%	(He et al., 2017)

5. Conclusion and Recommendation

5.1. Conclusion

The possibility of producing bio-butanol from Napier grass was investigated in this study. Because Napier grass contains 52.1 % carbohydrates, it is a potential biomass for ABE fermentation to produce bio-butanol. Alkaline pretreatment combined with enzymatic hydrolysis yielded 78 % of the reducing sugar yield, demonstrating the ability of Napier grass to efficiently transfer from polysaccharides to its monomer structure. Furthermore, this study demonstrated the production of butanol using *C. acetobutylicum* bacteria and ABE fermentation.

Bio-butanol was chemically characterized, and its higher heating value of 6,757.86 kcal L^{-1} meets the desired range for use as fuel. Furthermore, the optimized bio-butanol product's FTIR spectroscopy revealed O-H, C-O, C-H, – CH_2, and –CH_3 functional groups at the specified wavenumbers, confirming the presence of butanol in the product. The HPLC results also confirmed.

BBD conducted an experimental design to investigate the effect of four operational parameters: pH, temperature, sugar substrate concentration, and fermentation time. The optimum operating conditions were found to be 37.459 °C, pH 6, 64.65 hours of fermentation time, and 6.51 wt. % reducing sugar concentration. Butanol yield was 15.649 g L^{-1} at these optimum operating conditions.

To correlate the operating variables with the response, a quadratic model was used. According to the ANOVA test, all four operating variables have a significant effect on bio-butanol productivity. The maximum observed value of butanol productivity recorded was 0.077 g $L^{-1}hr^{-1}$ and 29.5 % efficiency for separated enzyme hydrolysis and ABE fermentation process, while sSSF process obtained 0.083 g $L^{-1}hr^{-1}$ productivity and 33.6 % efficiency.

Based on this research, it is clear that the optimization method chosen was efficient, and the sSSF process has high productivity while also minimizing the process. As a result, the selected model adequately fit the data of the response variable, and Napier grass is an excellent potential biomass for the production of bio-butanol.

5.2. Recommendation

Based on the current investigation the following recommendations are forwarded.

- ❖ Further study on the effect of hydrolysis on alternative methods
- ❖ Since bacteria is very sensitive in case of fermentation, other enzymes or yeast that can produce butanol from Napier grass can be studied.
- ❖ For better yield of bio-butanol it will be great to use recombinant, genetically engineered microorganisms, bacteria or yeast.
- ❖ Napier grass have different chemical composition depending on its age and height. Thus, further study can be performed on effect of such properties on butanol production.
- ❖ Feasibility study on Ethiopia's Napier grass for butanol production can be performed in order to commercialize it.
- ❖ HPLC column with oven and standard solvents will help to understand the exact quantity

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Appendices

Appendix A: Higher Heating value of Butanol

The heating value of a fuel is the amount of thermal energy released per unit amount of fuel when the fuel is completely burned and the combustion products are cooled back to the initial temperature of the combustible mixtures. It determines the amount of energy in a fuel. The HHV of any fuel, including butanol, was determined using an ASTM D2015 bomb calorimeter and the ASTM D2015 standard method. With the help of an oxygen container, an oxygen – bomb was pressurized to 3 Mpa. After the jacket and bucket temperatures equilibrated to within acceptable accuracy of each other, the bomb was automatically detonated.

$$HHV = \frac{(M_w C_w + M_{capp})(t_m + c - t_o) - \sum b}{\dot{M}}$$

Where

M_w: $Mass\ of\ water$

C_w : $calorific\ value\ water$

M_{capp} : $Specific\ heat\ capacity\ of\ water$

t_m : $Final\ temprature\ c$: $correcting\ factor$

t_o: $initial\ Temprature$

$\sum b$: $calarofic\ value\ of\ ignited\ wire$

$\dot{M}$: $mass\ of\ the\ sample$

$$HHV = \frac{(2000 + 305.26)(5.6 + 0.011602 - 1.9) - (14 - 2.5)1.5}{0.98}$$

$$HHV = 5{,}473.87\ {}^{cal}/_{g}\ = 6{,}757.86\ {}^{kval}/_{L}$$

Appendix B Density Measurements Using Density Meter

The measurement was taken at room temperature 25^{o}C

Table Ap 1 Density Measurement

Measurement	w/w % of alcohol
0.9973	2.5
0.9687	10
0.9464	15
0.9225	20
0.9126	25
0.9094	30
0.8968	35
0.8801	40
0.8786	45
0.8735	50
0.8610	55
0.8562	60
0.8487	65
0.8423	70
0.8398	75
0.8301	80
0.8284	85
0.8215	90
0.8195	95
0.8102	100

Appendix C Design Summary

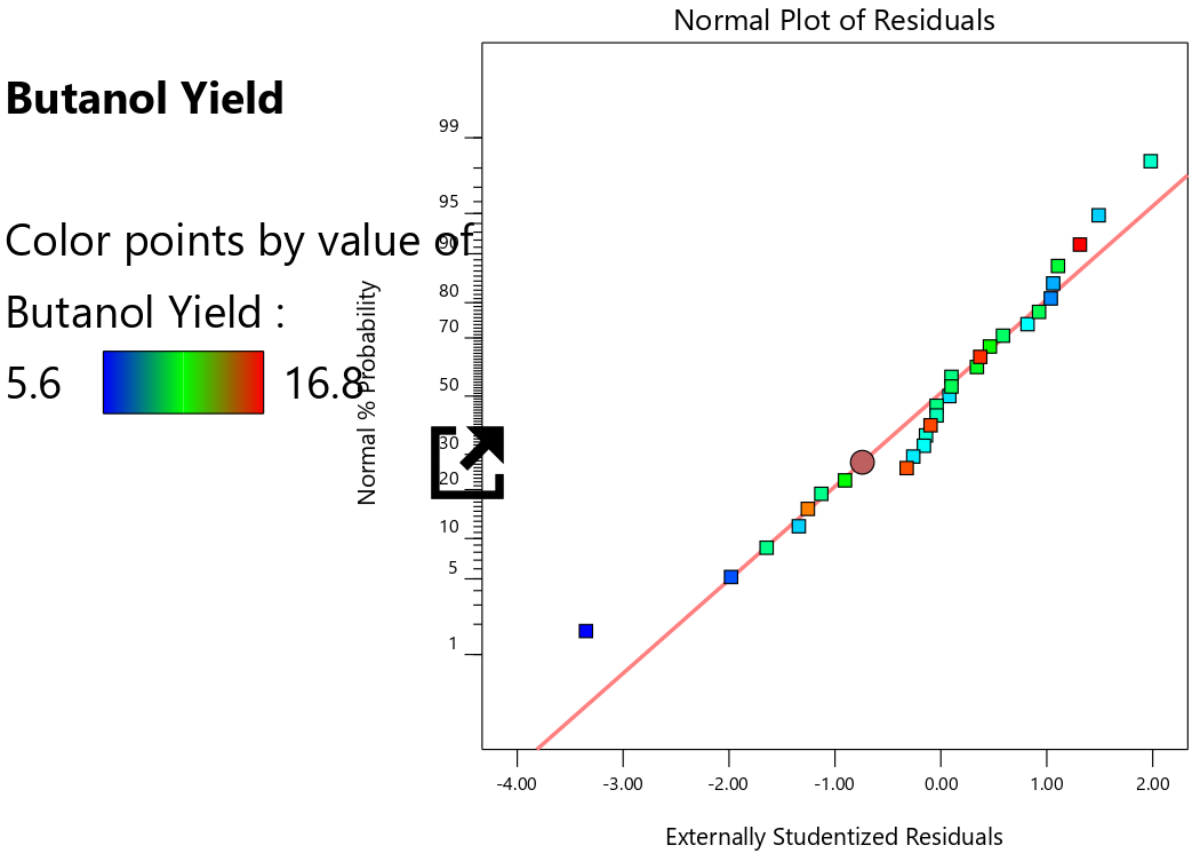

Figure Ap 1 Normal plot

Table Ap 2 Design Build Information

File Version	12.0.3.0		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	29
Design Model	Quadratic	**Blocks**	No Blocks
Build Time (ms)	122.00		

Appendix D Efficiency

$$efficiency\ on\ ABE\ fremenation\ is = \frac{0.145}{0.5} = 0.29 = 29\%$$

$$efficiency\ sSSF = \frac{0.168}{0.5} = 0.336 = 33.6\%$$

Theoretically from one gram of Sugar substate 0.5g of butanol is produced

Appendix E Laboratory work Pictures

Figure Ap 2Dried and copped Napier grass

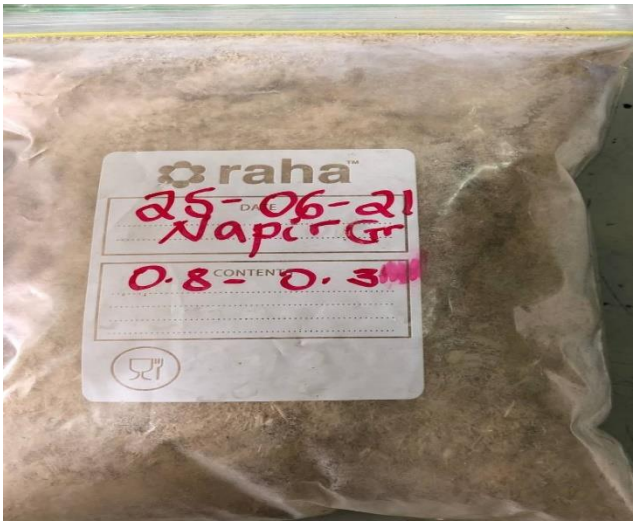

Figure Ap 3 0.8mm size Sample

Figure Ap 4 Alkaline pretreated and Enzyme hydrolyzed samples

Figure Ap 5 Microbe pre-culting

Figure Ap 6Fermentation in Incubator and Modified continues Fermenter

Figure Ap 7 Bio-Butanol Product