Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio Engineering

Bio butanol Production from Brewery's Spent Grain Hydrolysate by *Clostridium/Eubacterium* and *Bacillus subtilis*

By

Meron Asteraye

July, 2018

Addis Ababa, Ethiopia

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of a Master's Degree in Chemical Engineering under Biochemical Engineering Stream

By

Meron Asteraye

Thesis Advisor

Dr. Solomon Kiros

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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)

By: Meron Asteraye

Approved by the Examining Board

Chairperson	Signature	Date
Dr. Solomon Kiros		
Advisor	Signature	Date
Prof. Eduardo Ojito		
Internal Examiner	Signature	Date
Dr. Hundessa Desalegn		
External Examiner	Signature	Date

DECLARATION

I declare that this thesis entitled "Bio butanol production from Brewery's Spent Grain Hydrolysate by *Clostridium/Eubacterium and Bacillus subtilis* "for the M.Sc. degree at the University of Addis Ababa, hereby submitted by me, is my original work and has not previously been submitted for degree at this or any other university, and that all resources of materials used for this thesis have been duly acknowledged.

Name: Meron Asteraye

Signature: ----------

Date of Submission: ----------

ABSTRACT

Butanol (butyl alcohol or n-butanol) is a four carbon straight chained alcohol which can be used as a liquid transportation biofuel. The objective of this study was production of bio butanol from brewery spent grain (BSG) which in effects to minimize energy cost and substituting nonrenewable energy by using renewable resources. The chemical and proximate compositions analysis was investigated. The moisture, ash, extractives, hemicellulose, lignin and cellulose contents were, 64.65, 4.2, 33.28, 17.79 and 17.87 wt. % respectively. The conversion of BSG to bio butanol can be achieved mainly by four process steps,pre-treatment, hydrolysis (dilute acid hydrolysis), fermentation and separation. Dilute acid hydrolysis was employed, because it is easy and productive process. Fermentation of the hydrolyzate was performed using 10 % v/v Clostridium/Eubacterium at 37^0C temperature, pH 6.5 and 96 hr fermentation time for all samples. The experiment was designed by Box-Behnken Design (BBD) using Design expert 7.0.0 software with three factors to investigate the effect of (temperature, reaction time and acid concentration). Hydrolysis temperature was varied from 115°C to 135℃, reaction time was varied from 30 to 40 minutes and the acid concentration was varied from 1.5% to 2%. Significance of the process variables were analyzed using analysis of variance (ANOVA) and the quadratic model was fitted to the experimental results. Thus, the influence of all experimental variables, factors and interaction effects on the response was investigated. Among the investigated components hydrolysis temperature, reaction time, acid concentration, interaction between temperature and reaction time, interaction between temperature and acid concentration contributed a significant effect on the yield of bio butanol. As the result of RSM optimization, the best yield of total reducing sugar (TRS) and bio butanol were found at 127.42^0C hydrolysis temperature, 34.32min reaction time and 1.65% w/w acid concentration. Under these conditions, 59.0 g/L total reducing sugar (TRS), 10.93 g/L biobutanol yield and 0.11 g/L.h of productivity were obtained. The optimized process variables were farther enhanced by co culturing Clostridium/Eubacterium and Bacillus subtilis. Different inoculum concentration ratios (10:1%, 10:3%, and 10:5% v/v) of Clostridium/Eubacterium to Bacillus subtilis) were used to ferment BSG hydrolysate. The results exhibited that the inoculum size of 10% Clostridium/Eubacterium with 1% Bacillus subtilis maximised the production of biobutanol to 13.76g/L and 0.14 g/L.h productivity.

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ACRONYMS

ABE	Acetone- Butanol- Ethanol
AFEX	Ammonia Fibre Explosion
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
BBD	Box-Behnken Design
BSG	Brewery Spent Grain
CV	Coefficient of Variance
DP	Degree of Polymerization
DRS	Defuse Reflectance System
EBI	Ethiopian Biodiversity Institute
HMF	Hydroxy Methyl Furfural
FT-IR	Fourier Transform Infrared Spectroscopy
GTP	Growth and Transformation Plan
MW	Molecular Weight
RSM	Response Surface Methodology
TRS	Total Reducing Sugar
VOC	Volatile Organic Compound

1. INTRODUCTION

1.1 Background

The rise in price of world fuel, the growing demand for energy, and concerns about emission of greenhouse gases as a result of combustion of fossil fuels are the key factors driving renewed interest in renewable energy sources particularly biofuels. Biofuels which are utilized for transportation and heating purpose are derived from biological sources such as grains, sugar crops, oil crops, starch, cellulosic materials (grasses and trees) and organic waste (Fraiture et al., 2008). Among the various biofuels including; bioalcohols, biodiesel, biogases, and bioelectricity (microbial fuel cells), bioalcohols have a great potential to reduce the dependency on fossil fuel, decrease greenhouse gas emissions, and serve as a transportation fuel and versatile chemical feedstock (Kharkwal et al., 2009). Among the renewable biofuels considered as a suitable substitute to petroleum-based gasoline, bio butanol has attracted a great deal of attention due to its unique properties such as high energy content, low volatility and less hygroscopicity (Karimi et al., 2015). Pfromm et al., (2010) has indicated that bio butanol has particular characteristics that can be used directly or blended with gasoline without the need to change any current vehicles and supplied with existing gasoline pipes.

Butanol (butyl alcohol or *n*-butanol) is a four carbon straight chained alcohol with a molecular formula of C_4H_9OH (MW 74.12). It can be produced by chemical and biological methods (Karimi, et al., 2015). Butanol is produced chemically using either the oxo process starting from propylene (with H_2 and CO over a rhodium catalyst) or the aldol process starting from acetaldehyde (Ezeji et al., 2007a). It can also be produced biologically through the Acetone-Butanol-Ethanol (ABE) fermentation using solventogenic *clostridium* strains (Wang et al., 2016). Bio butanol produced by fermentation has the potential to provide a new generation biofuel that is seen to offer a number of attractive features as a liquid transportation fuel. The reason that butanol has attracted renewed interest is that it offers certain advantages compared with ethanol. It contains a high energy density, less explosive and corrosive than ethanol and also less susceptible to separation in the presence of water. More importantly it can be used as a pure fuel in current combustion engines without any modifications (Kharkwal et al., 2009; Wang et al., 2016).

Fermentation substrate is an important factor influencing the cost of bio butanol production (Qureshi and Blaschek, 2000). Lignocellulosic biomass, such as agricultural and forest residues, are the most abundant renewable resource and have great potential as a substrate for fermentation. On the basis of utilization of feedstock, biofuels including bio butanol were classified into first generation and second-generation biofuels. In the first-generation biofuels, raw materials for bio butanol production were sugarcane and cereal grains. Taking into account the global including Ethiopian population and increased demand for food supply, this feedstock is not sustainable and cost-effective and thus expensive for production (Buyondo and Liu, 2012). Therefore, the current study was aimed in determining the development of alternative feedstocks that are widely available at low cost and abundant supply which is the key issue. In the second generation bio butanol production, lignocellulosic biomass is used as a substrate for fermentation (e.g. agricultural waste, paper waste, wood chips), which are abundant and non-edible (Visioli et al., 2014). One of them is Brewery spent grain (BSG) which is lignocellulosic residue. It is a by-product of the brewing process, consisting of the solid residue remaining after mashing and lautering. It represents around 85% of the total by-product generated. It consists primarily of grain husks and other residual compounds not converted to fermentable sugars by the mashing process. The chemical composition of BSG varies according to grain variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process (Ogunjobi et al., 2011). BSG contains mainly hemicellulose in the form of arabinoxylans from the barley grain and cellulose.

Currently, Ethiopia has developed Second Growth and Transformation Plan (GTP II) in 2010/11 to reach a medium income country by 2025 and its industrialization goals are set out in the industrial development strategy to give due attention for the expansion of eco-friendly industries. Subsequently, a number of tangible institutional changes have been implemented including the strengthening of existing and establishment of new industries with specialized capacity building and expansion of technology institutes. Consequently, the number and type of brewery industries are drastically increased to 18 (10 beers, 3 wine and around 5 alcoholic breweries). Unfortunately, most of the biomass wastes are not utilized and disposed in inappropriate ways. The accumulation of huge amounts of brewery wastes including spent grain, spent hop and spent yeast every year leads to environmental degradation and especially to significant loss of valuable material that could otherwise be exploited as great variety for biofuel.

Therefore, the conversion of brewery wastes to high quality bioenergy by advanced processes is a promising way to substitute fossil-based fuels and decrease greenhouse gas emissions. Bio butanol is an attractive liquid transportation biofuel produced biologically from renewable biomass. At present, bio butanol is considered a promising product of biomass fermentations because of its high demand and incur attractive price and potential industrial use as a solvent, chemical feedstock, and potential liquid fuel (Bellows et al., 1984). However, there are challenges in producing butanol from lignocellulosic biomass due to their recalcitrance to degradation as well as their unique chemical compositions (García et al., 2011). It has been acknowledged that the use of lignocellulosic biomass involves pretreatment and hydrolysis of raw material followed by fermentation of sugars to bio butanol. Unfortunately, during pretreatment using acid hydrolysis, complex mixtures of microbial inhibitors are generated and remain at inhibitory levels in the hydrolysates (Ezeji et al., 2007b). In addition, there were few reports on bio butanol fermentation by *Clostridium* and aerobic bacteria (Wu et al., 2016). Though some reports confirmed that aerobic bacteria, such as *B. subtilis* and *B. cereus*, had a positive interaction with anaerobic *Clostridium*, the fermentative parameters, stability of symbiotic system, inhibitory resistance dosage and role of aerobic bacteria was not clearly established. This indicates that although several studies have demonstrated the potential bioconversion of lignocellulosic biomass to bio butanol after pretreatment, to the researcher's knowledge, there is still lack of systematic study on bioconversion of lignocellulosic biomass to bio butanol. Thus, the present research was aimed to address the optimization gaps of the aforementioned parameters for the exploitation of brewery's spent grain as a renewable feedstock for bio butanol production by *Clostridium/Eubacterium* alone and mixed with *Bacillus subtilis.*

1.2 Statement of the problem

Currently, the rapidly growing industrialization and urbanization with increasing demand for fuel energy are the challenging factors leading the worldwide including Ethiopia to search for an alternative economical and eco-friendly energy sources. The non-renewable and depletion of fossil fuels, concerns for energy security and the need to respond to climate change have led to growing worldwide interests in biofuels (Koh and Ghazoul, 2008). Therefore, for the crises of fossil fuel depletion and environmental degradation, energy planning and technology improvement has become an important government agenda of the world including Ethiopia.

The energy system in Ethiopia is characterized by the predominance of biomass fuels which account nearly 85% of the total national energy consumption. The demand for petroleum fuels is increasing with increase in population and economic growth. Even though the share of petroleum fuels is about 7.4% of the total consumption, the increasing demand for it and the associated price hike have hit the Ethiopian national economy very hard. As a net importer of petroleum, Ethiopia is highly vulnerable to price shocks and supply problems of oil in the world market, and sometimes, the import price of petroleum fuel exceeded annual export earnings, resulting in a negative balance of trade. It is therefore the government's priority agenda for alternative fuels to partially substitute imported petroleum such as introduction of biofuel including biobutanol that will be produced from biomass waste. In fact, biomass provides a clean, renewable energy source that could dramatically improve our environment, economy, and energy security (Demirbas, 2009). Among the biomass waste fermentation product is Bio butanol which is much less evaporative than gasoline or ethanol, making it safer to use and generating fewer volatile organic compound (VOC) emissions (Singh and Singh, 2011).

Despite the presence of huge shortage of energy in the country, still the numbers of petroleum user transport vehicles, water pumps and industries are flourishing in Ethiopia which required high energy demand, such that Ethiopia is engage on searching and construction of alternative, renewable and sustainable energy technologies to satisfy the high demand for energy. In response, the government of Ethiopia has planned and developed wide range of renewable energy development programs in GTP II to ensure supply of modern energy services, in which biofuel development is one of the priorities given to be implemented.

However, Ethiopia lacks research, technology and technical expertise as well as monetary resources to explore bio butanol production from organic waste fermentation significantly. Thus, the present work was aimed to contribute in developing the techniques for bio butanol production and yield improvement.

Since, the feedstock of the bio butanol fermentation is a key issue for the economic success of the biotechnological route to produce bio butanol (Raganati et al., 2016) concerning the food chain and inflation of food prices caused by biofuel production from agricultural feed (sugar or starchy crops). Residues from agro-industries are particularly interesting as renewable due to their abundance and uncompetitive with food sources (Raganati et al., 2016). The use of such wastes besides providing alternative substrates helps to solve environmental problems, which are otherwise caused by their disposal (Aliyu and Bala, 2011). Moreover, to produce bio butanol at commercial scale, one of the biggest challenges is low butanol yield due to the formation of byproducts acetone and ethanol and low butanol tolerance capability of the microorganism. It should be noted that the hydrolysates of lignocellulosic biomass contain pentose sugars such as xylose and arabinose and hexose sugars of glucose, mannose and galactose. Unfortunately, current butanol-producing microorganism like clostridium strains cannot ferment the pentose sugars into bio butanol efficiently. To overcome this drawback, it is crucial to develop effective symbiotic system with improved bio butanol yield and tolerance which is the prerequisite for bio butanol fermentation.

In general, the present thesis was aimed to address those problems of the economic, environmental and energy aspects of reliance on fossil fuel, by studying the utilization of brewery's spent grain waste for the production of alternative biofuel, the so called bio butanol.

1.3 Objective of the study

1.3.1 General objective

The main objective of this thesis was bio butanol production from brewery's spent grain hydrolysate by *Clostridium/Eubacterium/* and *Bacillus subtilis.*

1.3.2 Specific objective

The specific objectives of this thesis were;

- ✓ To determine the proximate and chemical composition analysis of brewery's spent grain
- ✓ To quantify the total reducing sugar of brewery's spent grain hydrolysate
- ✓ To investigate the effects of process variables in hydrolysis (temperature, reaction time and H_2SO_4 concentration) using Box Behnken design of Response surface methodology (RSM)
- ✓ To determine the optimum operating conditions for high yield and quality of bio butanol and
- ✓ To study the effect of symbiotic type of microbe's, inoculum ratio of *Clostridium* to *Bacillus species)* by using the optimum operating condition on bio butanol yield

1.4 Significance of the study

The results of this study were aimed to provide essential baseline information about the potential of brewery's spent grain for the production of bio butanol. Investigating the conversion capacity of this low valuable biomass in to more valuable bio product, particularly bio butanol, was aimed to provide the researchers an insight towards;

- ✓ Insuring food security
- ✓ Reducing the economy of reliance on fossil fuel, thereof saving foreign exchange.
- ✓ Reducing the emission of greenhouse gases to the environment as a result of fossil fuel combustion by replacing with bio butanol

Generally, this study contributes to show the direction of an alternative fuel to fossil fuel which is technically feasible, economically competitive, environmentally acceptable and easily available raw material.

2. LITERATURE REVIEW

2.1 Butanol

Butanol (C_4H_9OH) is a colourless, flammable alcohol with 4 carbons, where the carbon atoms can form either a straight-chain or a branched structure, resulting in different properties and therefore in different isomers: n-Butanol (butan-1-ol, 1-butanol, n-butyl alcohol), isobutanol (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) (Jin et al., 2011).

2.1.1 History of butanol fermentation

Acetone–butanol–ethanol (ABE) fermentation has an interesting history that goes all the way back to Louis Pasteur. Pasteur made the first observation in 1861 that bacteria produce butanol. In 1912, Chaim Weizmann (later to be recognized as the father of ABE fermentation) discovered a microorganism called *Clostridium acetobutylicum*, which was able to ferment starch to acetone, butanol, and ethanol (Ezeji et al., 2004).

In 1917 large-scale industrial plants were also erected in the USA and Canada (Jones and Woods, 1986). Because of the greater availability of potential agricultural substrates, the process was transferred to Canada in 1916. The first commercial ABE fermentation plant was built in Terra Haute, Indiana in 1918 by Commercial Solvents Corporation. This venture supplied butanol for conversion to butyl acetate as a primary component of paint lacquers. With the expiration of the Weizmann patent for the ABE process in 1936, ABE plants were built throughout the United States and this process went on to become a major industrial fermentation process recognized as second only to yeast-based ethanol fermentation (Ezeji et al., 2004).

Since the 1950s ABE fermentation declined continuously, and almost all butanol was produced via petrochemical routes, so it is called petro-butanol. The production of butanol by fermentation declined mainly because the price of petrochemicals dropped below that of starch and sugar substrates such as corn and molasses. Therefore, butanol production from fossil fuel became more popular and sealed the fate of ABE fermentation during this stage (Jin et al., 2011). Later in 1970s, the oil crisis gave a rise to the development of biofuels again (Jin et al., 2011). The rising cost of petrochemicals combined with the energy crisis of the early 1970s resulted in a renewed interest in ABE fermentation.

The decades of the 1980s and 1990s saw tremendous progress in the development of genetic systems for the solventogenic clostridia, which would allow for the development of strains with improved fermentation characteristics. Sentrachem in South Africa did employ ABE fermentation for production of butanol through the early 1980s, but it subsequently discontinued using the process. The plant in South Africa was closed in 1982 (Jones and Woods, 1986), and as the USSR disintegrated during the 1990s, their biobutanol production stopped (Antoni et al., 2007). In China, solvent fermentation was stepped down to complete closure only in 2004 (Chiao and Sun, 2007). Today most n-butanol are produced chemically from petroleum sources by either the oxo process starting from propylene (with H_2 and CO over a rhodium catalyst), or the aldol process starting from acetaldehyde (Brekke, 2007).

2.2 Biobutanol application and its utilization

Rising demand for alternative fuels and renewable energy owing to the harmful effects of synthetic fuels to the environment including global warming is expected to boost bio-butanol growth over the next seven years. Butanol is an important bulk chemical with a wide range of industrial uses. Most of the worldwide production is converted into methacrylate esters and acrylate. Other main derivatives include glycol ethers and butyl acetate, while derivatives with minor uses are amino resins and n-butylamines. Figure 2.1 shows the world bio-butanol market size by application, 2012-2022, (Kilo Tons) Forecasts to 2022.

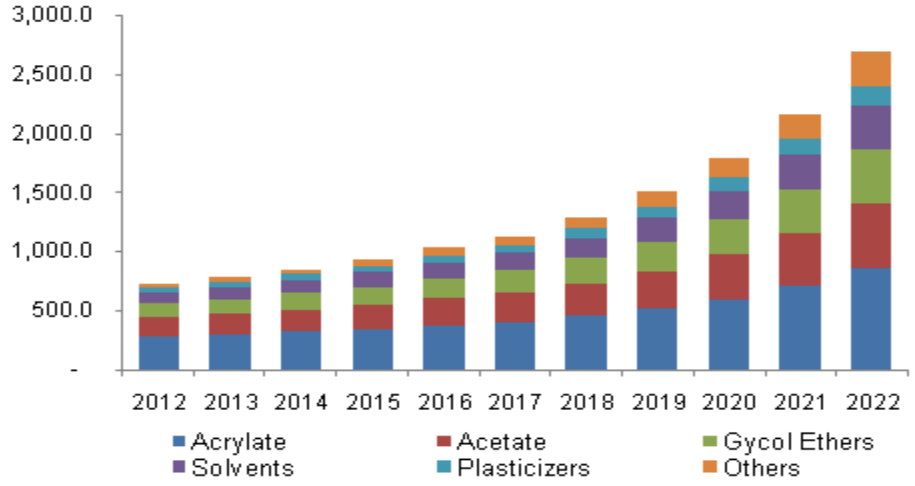

Figure 2.1: World bio-butanol market size by application, (Industry Analysis Report, 2014).

Bio-butanol is used as a key raw material in the production of paints, coatings, plasticizers and adhesives. It is used as a drop-in replacement of petroleum-based butanol in almost all applications. Applications, chemicals and products that use butanol include solvents (for paints, coatings, varnishes, resins, gums, dyes, camphor, vegetable oils, fats, waxes, shellac, rubbers and alkaloids), plasticizers (to improve how plastic material processes), coatings (as a solvent for a variety of applications, such as curable lacquers and cross-linked baking finishes), chemical intermediate or raw material (for producing many other chemicals and plastics, including safety glass, hydraulic fluids and detergent formulations), textiles (as a swelling agent and manufacturing garments from coated fabric), flotation agents, cleaners, floor polishes, cosmetics (including eye makeup, foundations, lipsticks, nail care products, personal hygiene products and shaving products), drugs and antibiotics, hormones, and vitamins (Dow, 2006.). A relatively new, but very important application is butanol as a biofuel.

It can be blended into gasoline and acts as bio-fuel which is expected to be one of its most popular commercial applications in the near future. Its ability to provide high energy content without compromising on fuel economy is a key feature which is responsible for encouraging various automobile manufacturers to explore this option. Depleting petroleum resources coupled with volatile oil prices has contributed towards its increasing need.

Table 2.1Properties of butanol compared with those of other fuels.

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, ^{0}C	79	13	35	<−40

Source: (Buyondo and Liu, 2012).

2.3 Feedstocks for biobutanol production

Feedstocks flexibility is seen to be an advantage. Biobutanol can be produced from the same biomass as used for ethanol (Green, 2011). Biomass is a sustainable feedstock for chemicals and energy products that could potentially enhance the energy independence of the world.

Biomass includes wood and logging residues, agricultural crops and their waste by-products, the organic portion of municipal solid waste, animal wastes, municipal biosolids (sewage), waste from food processing, and aquatic plants and algae (Demirbas, 2009).

The ability to utilize mixed sugars is of particular relevance for the use of inexpensive agricultural by-products and wastes as fermentation substrates, because fermentation substrate is an important factor influencing the cost of biobutanol production (Ezeji et al., 2007a). Although sucrose and starch are good substrates for butanol fermentation, increasing demand for these substrates for biofuel production has increased the prices of these feedstocks as well. Importantly, these crops potentially compete with food supply causing food versus fuel debate. The possible answer for obtaining enough fermentable carbon substrates without getting into the competition with food supplies resides in the efficient utilization of plentiful lignocellulosic biomass available on the earth. (Jang et al., 2012).

Lignocellulosic biomass comprising forestry, agricultural and agro-industrial wastes are abundant, renewable and inexpensive for the production of next generation biofuels. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer’s spent grains, switchgrass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn, sorghum and barley, among others. Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems. With their vast availability, they are considered to be a suitable alternative to the diminishing fossil fuels (Mussatto and Teixeira, 2010). Since, much of these biomasses are thrown away, turning these surplus discarded plant materials into biofuels is of great appeal and importance. The global energy consumption in 2008 being 533 EJ is projected for increase to 653 EJ by 2020 and 812 EJ by 2030. It is acceptable that a major proportion of this future energy supply (250–500 EJ per year by 2050) will be contributed by lignocellulosic biomass (Nanda et al., 2014).

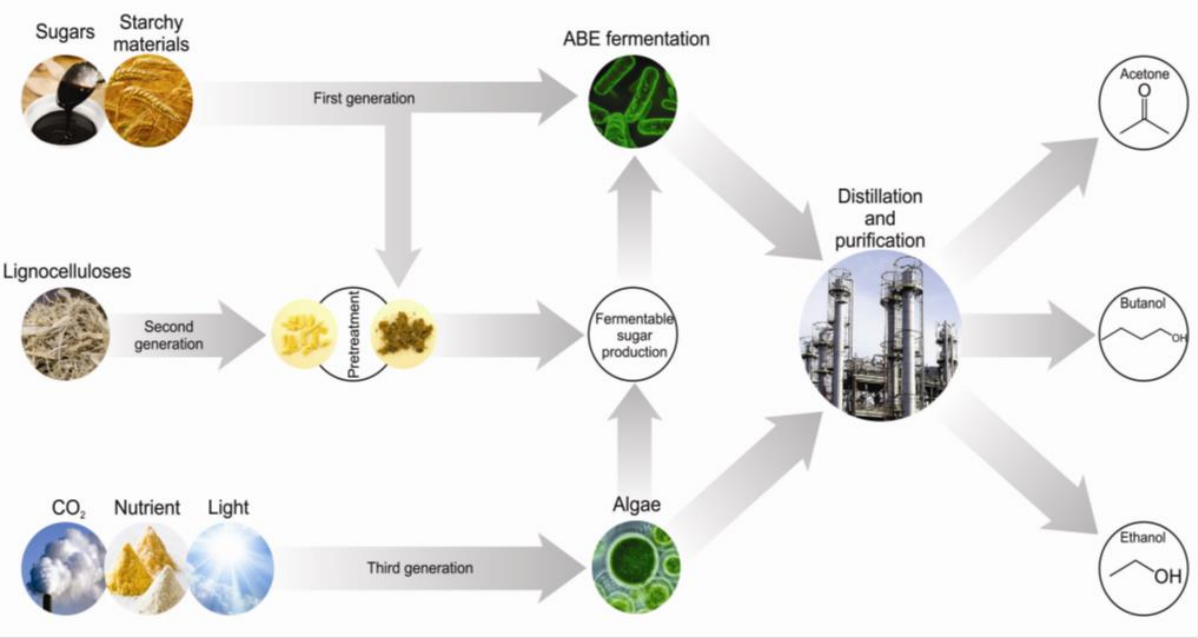

Figure 2.2: Overall process scheme for acetone, butanol, and ethanol production from biomass(Karimi et al., 2015).

2.3.1 Lignocellulosic biomass as a feedstock for biobutanol production

Lignocellulose is the largest of the world's carbon based renewable natural resources which is a combination of cellulose, hemicellulose and lignin. It exists in plants and trees. Cellulose is a polysaccharide consisting of multiple glucoses on a chain, hemicellulose is similar but consists of several different sugars, not only glucose. Lignin is a large macromolecule which acts as a glue binding together all the polysaccharides. These three substances are the main components of trees and the lignocellulose to be fermented (Green, 2011). Hemicelluloses are the second most abundant polysaccharides in nature, and represent about 20 to 35% of lignocellulosic biomass (Ezeji et al., 2007b). Hemicellulose has great potential as a fermentation substrate because it is least expensive and it is often underutilized or even an undesirable byproduct in certain industries and agricultural processes (Sun and Liu, 2012). Hemicellosic and cellulosic sugars offer the best alternative as a substrate for biobutanol production. On the other hand, lignin is a phenyl propane polymer which forms a complex network cross-linking the cellulose and hemicellulose together which needs to be removed.

2.4 Composition of lignocellulosic material

Lignocellulose is the primary building block of plant cell walls. Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Nanda, 2014; Kumar et al., 2009). The composition of these constituents can vary from one plant species to another. In addition, the ratios between various constituents within a single plant vary with age, stage of growth, and other conditions (Kumar et al., 2009). Biomass in general consists of 40-50% cellulose, 25-30% hemicellulose and 15-20% lignin and other extractable components (Menon and Rao, 2012).

2.4.1 Cellulose

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. The structure of cellulose is shown in Figure 2.3. Cellulose is composed of glucose polymers which are largely insoluble and exist in crystalline microfibrils making the sugars difficult to extract (Nanda et al., 2014). The glucose molecules ($C_6H_{12}O_6$) of the cellulose are produced by the tree through photosynthesis. Firstly, this monomer units are 20, transformed into glucose anhydrides ($C_6H_{10}O_5$) and then linked through oxygen atoms end-to-end to form linear polymers ($C_6H_{10}O_5$) *n.* The number of monomers per macromolecules (*n*) indicates the degree of polymerization (DP) in cellulose (Testova, 2006). This linear polymer consists of D-glucose subunits linked to each other by *β*-(1, 4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the *β*-(1, 4) -glycosidic linkages. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form (Kumar et al., 2009).

2.4.2 Hemicellulose

The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars.

These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-*o*methylglucuronic, D-glucuronic, and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1, 4)-glycosidic bonds and occasionally β-(1, 3)-glycosidic bonds. Also, hemicelluloses can have some degree of acetylation, for example, in heteroxylan. In contrast to cellulose, the polymers present in hemicelluloses are easily hydrolysable (Kumar et al., 2009). These polymers do not aggregate, even when they cocrystallize with cellulose chains. During pretreatment, firstly the side groups of hemicellulose react and then the linear backbone. Hemicellulose dissolves into water from around 150ºC or 180ºC under neutral conditions. But the extent of solubilization also depends on water content and pH. solubilisation begins at 120 ºC under dilute acid catalyst (Tai et al., 2012).

2.4.3 Lignin

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. It is present in the primary cell wall, imparting structural support, impermeability, and resistance against microbial attack. Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl-aryl, alkyl-alkyl, and aryl-aryl ether bonds link these phenolic monomers together. In general, herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents (Kumar et al., 2009). It binds cellulose and hemicellulose tightly, makes the cell wall compact and rigid. That is one of the reason that biomass must be pretreated to open its complex structure before enzymatic hydrolysis. Lignin normally starts to dissolve into water around 180ºC under neutral conditions. The solubility of the lignin in different pH depends on the compositions of precursor (Tai et al., 2012).

2.4.4 Extractives and ash

Any numbers of different compounds (resins, phenolics, and other chemicals) in biomass that are not an integral part of the cellular structure are called extractives. These compounds can be extracted from biomass by means of polar and non-polar solvents including hot or cold water, ether, benzene, methanol, acetone or other solvents that do not degrade the biomass structure (Dehnavi, 2009).

Minerals include: calcium, magnesium, potassium, and other materials, that will leave as ash when biomass is burned(Dehnavi, 2009).

2.5 Brewer's spent grains as a lignocellulosic feedstock

Brewers spent grain (BSG) is the most abundant brewing by-product, comprising of 85% of by-products generated, 31% of original malt weight and 20 kilograms per 100 liters of beer produced. It is the solid residue that remains from the barley after separation and filtration of the wort.

BSG as a lignocellulosic biomass contains hemicellulose, lignin, and high protein content, monosaccharides of xylose, glucose and arabinose, and minerals and amino acids. Although its composition of barley grain husk, pericarp and fragments of endosperm is contingent upon the barley type, harvest time and mashing conditions, carbohydrates comprise of around half of the dry mass of BSG, with the balance mostly being proteins and lignin (Buffington, 2014). Therefore, the carbohydrate content makes BSG a potential substrate for various applications; the sugars that are released after chemical or enzymatic processing, can be microbiologically converted into various bio products, such as organic acids, ethanol, glycerol, food additives and butanol, etc. (Nigam, 2017).

The chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process. (Mussatto et al., 2006) reported that the chemical composition of BSG as 16.8–25.4% cellulose, 21.8–28.4% hemicellulose, 11.9–27.8% of lignin, and 2.4-4.6% ash. BSG and other lignocellulosic wastes as a bio-feedstock were calculated through averages of four research studies by (Buffington, 2014) as shown in the Table 2.2.

Table 2.2: Composition of representative lignocellulosic feedstocks.

Material	Cellulose (wt. %)	Hemicellulose (wt. %)	Lignin (wt. %)
Barley Straw	33.8	21.9	13.8
Corn Cobs	33.7	31.9	6.1
Cotton Stalks	58.5	14.4	21.5
Oat Straw	39.4	27.1	17.5
Rice Straw	36.2	19.0	9.9
Rye Straw	37.6	30.5	19.0
Soya Stalks	34.5	24.8	19.8
Sugarcane Bagasse	40.0	27.0	10.0
Sunflower Stalks	42.1	29.7	13.4
Wheat Straw	32.9	24.0	8.9
BSG	22.2	26.8	14.1

Source; (Buffington, 2014)

Challenges associated with using BSG as a feedstock includes the existence of a complex outer layer, making it difficult to separate and convert, and a high moisture content (80% - 85%), making it susceptible to microbial growth and spoilage within a 7-10-day period (Buffington, 2014). Due to its high moisture and fermentable sugar contents, BSG is a very unstable material and is liable to deteriorate rapidly due to microbial activity (Mussatto et al., 2006). The moisture issue can be stabilized post production in the brewing process, deterioration of the feedstock through microbial activity can further reduce yields in the conversion process (Buffington, 2014).

2.5.1 Generation of brewery spent grain in Ethiopia

Currently, there is a rapid growth of brewery industries in Ethiopia. There are about eleven breweries in full function and production. There are also some breweries being expanded and expected to be completed for the next few years. Unfortunately, most of the biomass wastes are not utilized and disposed in inappropriate ways.

The accumulation of huge amounts of brewery wastes including spent grain, spent hop and spent yeast every year leads to environmental degradation and especially to significant loss of valuable material that could otherwise be exploited as great variety for biofuel. The production capacity of BSG in Ethiopia are presented in Table 2.3.

Table 2.3: Capacity of spent grain generated from Ethiopian brewery industries

No	Name of Brewery in Ethiopia	Capacity of spent grain generated (Kg per day)
1	Dashen(Gonder)	31,000
2	Dashen (debrebrhane)	21,830
3	Saint George Hawassa	32,980
4	Saint George Addis Ababa	33,592
5	Saint George Kombelcha	32,640
6	Meta	21,930
7	Bedele	21,930
8	Harar	22,950
9	Waliya	31,980
10	Habesha	21,780
11	Raya	20,500
	Total	263,736

Source: (Fissha, 2016).

2.6 Bio butanol production process

There are basically two big lines of production of butanol: from biomass (as “bio butanol”) and from fossil fuels (as “petro-butanol”), but both, bio butanol and petro-butanol, have the same chemical properties. Most of butanol produced today is synthetic and derived from a petrochemical reaction (Petro-butanol).

Petro-butanol is obtained by the propylene hydro formylation, well known as oxo route. Although common, this scenario is not the most interesting since synthetic butanol production costs are linked to the propylene market that is strongly linked to the price of crude oil (Trindade and Santos, 2017).

Bio-chemical production route of bio butanol

Renewable n-butanol can be produced from the fermentation of carbohydrates in a process better known as ABE fermentation since its major chemical products are acetone, butanol and ethanol. The fermentation is based on microorganism activity and bacteria from genus Clostridium are normally used. *Clostridium acetobutilicum* is widely used in butanol synthesis based on sugars, having as side products acetone and ethanol (Trindade and Santos, 2017). The production process of bio butanol from lignocellulosic biomass basically involves four steps: pre-treatment, hydrolysis, fermentation and separation.

- Biomass containing lignocellulosic is pretreated, allowing it to be used as substrate for the fermentation. Pretreatment can be based on sulfuric acid, peroxide, steam, hydrothermal or others, depending on the used material.
- The following process is detoxification to remove the inhibitors. The method can be done by activated charcoal, over-liming, electrodialysis and others. Selected process depends on the different feedstock and pre-treatment method used.
- Next process is the fermentation. This is a well-known process, also used for the sugar and starch-based feedstock. Again, different process can be used depending on the raw material and pre-treatment.
- After the fermentation, the product is recovered and purified, separating acetone, ethanol and butanol based on the boiling points of acetone, ethanol and butanol which are different from each other (56 °C, 78 °C and 118 °C, respectively).

2.6.1 Pre-treatment

In order to break the lignocellulosic framework and extract the fermentable sugars for bioconversion to higher fuel alcohols, a pretreatment method prior to conversion is required (Nanda et al., 2014).

The goal of pre-treatment is to alter the structure of cellulosic biomass to improve the ability to form sugars by hydrolysis. This is achieved by breaking the lignin seal, removing lignin and hemicellulose, or increasing the porosity of the biomass. Pre-treatment should improve the yield of the formation of fermentable sugars while avoiding the degradation or loss of carbohydrates and the formation of inhibitors for subsequent hydrolysis and fermentation processes (García et al., 2011). An ideal pretreatment should have the following properties: (1) disintegrate the lignin and hemicellulose complex with cellulose, (2) improve the sugar yield as a result of hydrolysis of cellulose and hemicellulose, and (3) prevent excessive degradation or loss of carbohydrate. Last but not the least, it should be cost-effective (Nanda et al., 2014). Physical, chemical, physico-chemical and biological treatments are the four fundamental types of pretreatment techniques known (Sarkar et al., 2012).

2.6.1.1 Physical pretreatment

Mechanical commination: The objective of the mechanical pretreatment is a reduction of particle size and crystallinity of lignocellulosic in order to increase the specific surface and reduce the degree of polymerization(Alvira et al., 2010). This can be achieved by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling. The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Sun and Cheng, 2002).

2.6.1.2 Physico-chemical pretreatment

Pretreatments that combine both chemical and physical processes are referred to as physico-chemical processes. The most important processes of this group includes: - steam explosion, catalyzed (SO_2 or CO_2) steam explosion, ammonia fiber explosion (AFEX), liquid hot water, microwave-chemical pretreatment (Menon and Rao, 2012).

Steam explosion (autohydrolysis): In steam explosion the biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160-260 ^{0}C (corresponding pressure, 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The biomass/steam mixture is held for a period of time to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. A steam explosion process using sugarcane bagasse as substrate was developed where the individual constituents were separated as pure cellulose, hemicellulose and lignin. The steam-explosion pretreatment process has been a proven technique for the pretreatment of different biomass feedstocks. It is able to generate complete sugar recovery while utilizing a low capital investment and low environmental impacts concerning the chemicals and conditions being implemented and has a higher potential for optimization and efficiency (Menon and Rao, 2012).

Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes. Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose. The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose. Typically, 20–25% of the initial dry matter is removed by water wash (Sun and Cheng, 2002).

Ammonia fiber explosion (AFEX): AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg ammonia/kg dry biomass, temperature 90 ^{0}C, and residence time 30 min. The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment (Sun and Cheng, 2002).

An advantage of AFEX is that the ammonia used during the process can be recovered and reused. Also, the downstream processing is less complex compared to other pretreatment processes. Thus, AFEX is not a very efficient technology for lignocellulosic biomass with relatively high lignin content such as woods and nut-shells. Furthermore, ammonia must be recycled after the pretreatment to reduce the cost and protect the environment. However, both the ammonia cost and the cost of recovery processes drive up the cost of the AFEX pretreatment (Menon and Rao, 2012).

Liquid-hot water pretreatment: In liquid hot water pretreatment, pressure is utilized to maintain water in the liquid state at elevated temperatures. It is a hydrothermal pretreatment method which releases high fraction of hemicellulosic sugars in the form of oligomers. The treatment generally occurs at temperatures of 170-230 ºC and pressures above 5 MPa for 20 min. It, however, also contributes to the production of small amounts of undesired degrading compounds such as furfural, carboxylic acid, that are very toxic to microbial growth (Sarkar et al. 2012).

2.6.1.1 Biological pretreatment

In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials. Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Sun and Cheng, 2002). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low (Sun and Cheng, 2002).

2.6.1.4 Chemical pretreatment

Chemical pretreatments that have been studied to date have had the primary goal of improving the biodegradability of cellulose by removing lignin and/or hemicellulose, and to a lesser degree decreasing the degree of polymerization (DP) and crystallinity of the cellulose component. Chemical pretreatment is the most studied pretreatment technique among pretreatment categories. The various commonly used chemical pretreatments includes: acid, alkali, organic acids, pH-controlled liquid hot water and ionic liquids (Menon and Rao, 2012). These methods are easy in operation and are have good conversion yields in short span of time (Sarkar et al. 2012).

Ozonolysis: Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw, bagasse, green hay, peanut, pine, cotton straw, and poplar sawdust. The degradation was essentially limited to lignin, and hemicellulose was slightly attacked, but cellulose was hardly affected. The rate of enzymatic hydrolysis increased by a factor of 5 following 60% removal of the lignin from wheat straw in ozone pretreatment. Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust. Ozonolysis pretreatment has the following advantages: (1) it effectively removes lignin; (2) it does not produce toxic residues for the downstream processes; and (3) the reactions are carried out at room temperature and pressure. However, a large amount of ozone is required, making the process expensive (Sun and Cheng, 2002).

Alkaline Pretreatment: Alkali pretreatment refers to the application of alkaline solutions to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of enzyme to the hemicellulose and cellulose. Sodium, potassium, calcium and ammonium hydroxide are appropriate chemicals for pretreatment (Balat, 2011). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks. Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Sun and Cheng, 2002). These processes utilize lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried out at ambient conditions, but pretreatment time is measured in terms of hours or days rather than minutes or seconds. Regardless the advantages, these methods present difficulties from the point of view of the process economy for obtaining fuels (Balat, 2011).

Acid pretreatment: Acid pretreatment is considered as one of the most important techniques and aims for high yields of sugars from lignocellulosics. It is usually carried out by concentrated or diluted acids (usually between 0.2% and 2.5% w/w) at temperatures between 130 ^{0}C and 210 ^{0}C (Sarkar et al. 2012).

Sulfuric acid is widely used for acid pretreatment among various types of acid such as hydrochloric acid, nitric acid and phosphoric acid. The acid medium attacks the polysaccharides, especially hemicelluloses which are easier to hydrolyze than cellulose. However, acid pretreatment results in the production of various inhibitors like acetic acid, furfural and 5 hydroxymethylfurfural. These products are growth inhibitors of microorganisms. Hydrolysates to be used for fermentation therefore need to be detoxified (Sarkar et al. 2012). Recently it has been demonstrated that the dilute acid prehydrolysis can achieve high reactions rates in short time and significantly improve cellulose hydrolysis. However, pretreatment operating conditions must be tailored to the specific chemical and structural composition of the various sources of biomass (Menon and Rao, 2012).

Dilute acid pretreatment

One of the well-studied and established methods of biomass pretreatment is the treatment of hemicellulosic material with dilute acid at an elevated temperature to obtain fermentable sugars (Ezeji et al., 2007b; Sun and Liu, 2012). Dilute sulfuric acid pretreatment solubilizes the hemicelluloses and thereby disrupts the lignocellulosic composite linked by covalent bonds, hydrogen bonds and van der Waals forces. Hemicellulose is removed when sulfuric acid is added and this enhances digestibility of cellulose in the residual solids (Mosier et al. 2005). The pretreatment process is used to overcome the recalcitrance of lignocellulose, increase enzyme efficiency, and improve the yields of monomeric sugars (Nanda et al., 2014).

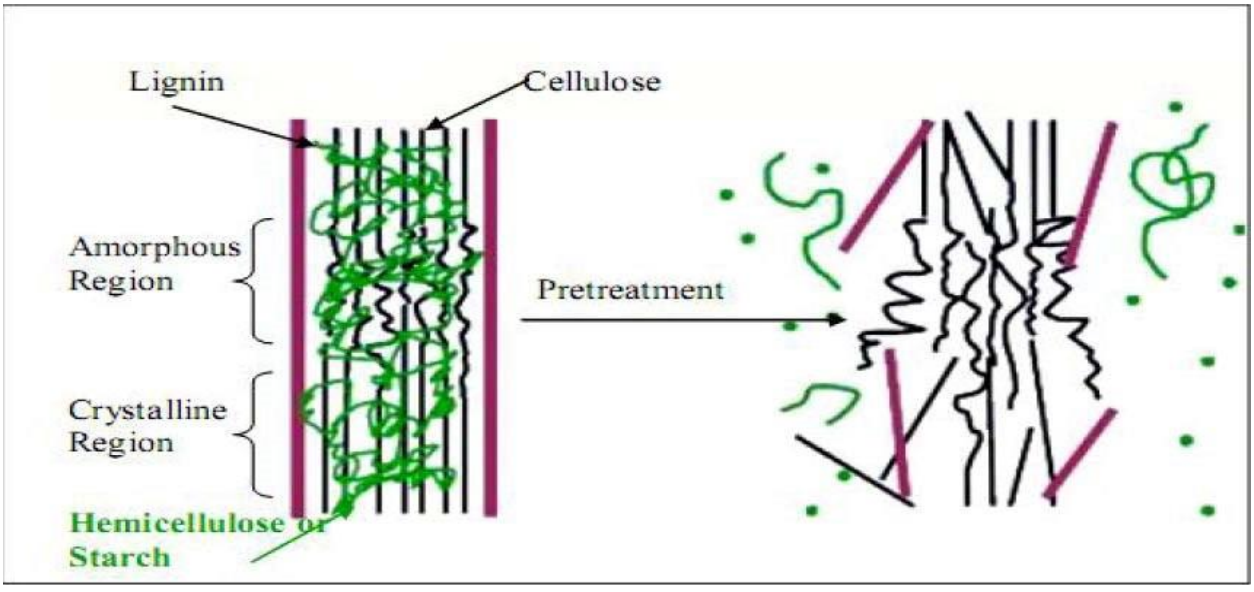

Figure 2.3:Schematic representation of biomass pretreatment (Mosier et al., 2005)

2.6.2 Hydrolysis

The hydrolysis process breaks down the cellulosic molecules exposed during pretreatment into glucose molecules and short chains. Hydrolysis can be carried out chemically via acid washes or biologically via enzymatic reactions. The most commonly applied methods can be classified in two groups: chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis (Balat, 2011).

Chemical hydrolysis

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical for a period of time at a specific temperature, and results in sugar monomers from cellulose and hemicellulose polymers. In the chemical hydrolysis, the pretreatment and the hydrolysis may be carried out in a single step. Acids are predominantly applied in chemical hydrolysis. Acid hydrolysis occurs by exposing the cellulosic material to either a dilute or concentrated acid. The acid reacts with the cellulosic material to produce glucose molecules and short chains. There are two basic types of acid hydrolysis processes: dilute acid and concentrated acid, each with variations (Balat, 2011).

Concentrated acid hydrolysis

Concentrated acid hydrolysis occurs at low temperatures and atmospheric pressure. The process is more efficient than its counter-part and has a high glucose yield. However, the process is time consuming, taking up to 120 hours to complete (Moe, 2006). In comparison to dilute acid hydrolysis, concentrated acid hydrolysis leads to little sugar degradation and gives sugar yields approaching 100%. The concentrated acid process offers more potential for cost reductions than the dilute acid process. However, environment and corrosion problems and the high cost of acid consumption and recovery present major barriers to economic success (Balat, 2011). Advantages of concentrated acid hydrolysis are the flexibility in terms of feedstock choice, high monomeric sugar yield as well as mild temperature conditions that are needed. Drawbacks of using concentrated acids are corrosive nature of the reaction and the need to recycle acids in order to lower cost.

Dilute acid hydrolysis

The principle of this technique is to apply temperature and pressure in order to soften lignocellulosic providing better penetration of the acid, and then degrade carbohydrate part of wood into monosaccharides (Lee, 1999). During treatment various products are formed: monosaccharides (xylose, arabinose, mannose etc.), some sugar-dehydration products (furfural, hydroxyl methyl furfural), while lignin and part of cellulose remain as solid residue (Gladyshko, 2011). Dilute acid hydrolysis occurs in two-stages to take advantage of the differences between hemicellulose and cellulose. The first stage is performed at low temperature to maximize the yield from the hemicellulose, and the second, higher temperature stage is optimized for hydrolysis of the cellulose portion of the feedstock. The first-stage is conducted under mild process conditions (e.g. 0.7% H_2SO_4, 463 K) to recover the five-carbon sugars, while in the second stage only the remaining solids with the more resistant cellulose undergo harsher conditions (488 K, but a milder 0.4% H_2SO_4) to recover the six-carbon sugars (Balat, 2011).

Research works on the dilute acid hydrolysis of different lignocellulosic materials have defined optimal process conditions: temperature 80-200 ºC, sulfuric acid concentration 0.25-8 wt.%, and reaction time 10-2000 min (Gladyshko, 2011). Sulfuric acid is a commonly used acid due to low cost, non-volatileness and affordable corrosion strength (Mosier et al., 2005). Despite all of the benefits of sulfuric acid hydrolysis, some limitations take place including high corrosion rates and expensive construction materials. Also, liquors have to be neutralized before the sugars proceed to fermentation. Gypsum has problematic reverse solubility characteristics when neutralized with inexpensive calcium hydroxide (Mosier et al., 2005).

Enzymatic hydrolysis

Enzymatic hydrolysis occurs when enzymes are exposed to the pretreated biomass to decompose the biomass into simple sugars. The enzymes typically used are endocellulase, exocellulase, and Beta-glucosidase. The enzymes digest the lignin surface yielding cellulose. The endocellulase and exocellulase digest the cellulose into polysaccharide molecules. The polysaccharide molecules are then digested by the Beta-glucosidase yielding the final glucose product (Klass, 2008). Cellulase enzymes are produced by several microorganisms, commonly by bacteria and fungi. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic.

Bacteria belonging to Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora, and Streptomyces can produce cellulases effectively. Fungi such as Sclerotium rolfsii, P. chrysosporium and species of Trichoderma, Aspergillus, Schizophyllum and Penicilium are used to produce cellulases. Mutant strains of Trichoderma sp. (T. viride, T. reesei, T. longibrachiatum) have long been considered to be the most productive and powerful destroyers of crystalline cellulose (Balat, 2011). However, enzymatic hydrolysis can be problematic. The hydrolysis products (glucose and cellulose chains) inhibit the ability for enzymes to convert cellulose to glucose. As more product is formed, the enzymes become more inhibited by the excess glucose present. This ultimately slows down the hydrolysis process yielding low levels of usable hydrolysis product (D'amore, 1991).

Table 2. 4: Characteristics of hydrolysis processes used for lignocellulosic materials.

Hydrolysis process	Advantages	Disadvantages
Dilute acid (2–5 wt. %)	Low acid consumption Short processing time Acid recovery may not be required	High pressure and temperature needed Not so effective for cellulose hydrolysis Formation of undesirable by-products Corrosion of equipments
Concentrated acid (10–30 wt. %)	Low temperature High yield of sugar production	High acid consumption Longer reaction time (2–6 h) compared to dilute acid hydrolysis Acid recovery process needed, extra energy input Corrosion of equipments
Enzymatic	Mild conditions (40–50 ◦C, pH 4.5–5.0) High yield of sugar production No formation of inhibitors	Cost of enzymes Long hydrolysis time (several days) Feedstock pretreatment required

Sourse: (García et al., 2011).

2.6.3 Fermentation

The ABE fermentation is a proven industrial process that uses solventogenic clostridia to convert sugars or starches into solvents. During the fermentation of *Clostridia*, two separate growth phases occur. The first phase is the acidogenic phase that occurs during the exponential growth phase in which the acid forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. The second phase is the solventogenic phase during which acids are reassimilated and used in the production of butanol and other solvents like acetone and ethanol. During this stage, growth slows, the cells accumulate granulose and form endospores. The fermentation also produces carbon dioxide and hydrogen (Green, 2011: Jin et al., 2011). There are three major classes of products during fermentation: (i) solvents (acetone, ethanol and butanol); (ii) organic acids (acetic acid, lactic acid and butyric acid); (iii) gases (carbon dioxide, and hydrogen). The biosyntheses of acetone, butanol and ethanol share the same metabolic pathway from glucose to acetyl coenzyme A (acetyl-CoA) but branches into different pathways thereafter. The ABE process uses bacteria to produce a 6:3:1 ratio of butanol, acetone and ethanol (Jin et al., 2011).

2.6.4 Separation

Distillation is separation of mixtures based on the volatilities (boiling points) of the individual components that make up the mixture. The boiling point of acetone, ethanol and butanol is, 56 °C, 78 °C and 118 °C respectively.

2.7 Factors affecting fermentation

Fermentative biobutanol production is a biphasic fermentation process that is significantly affected by many factors, including initial pH, temperature, inoculum size and substrate type and concentration, product inhibition, oxygen concentration, etc. (Al-shorgani et al., 2014; Buyondo and Liu, 2012).

Effect of pH

The pH is a crucial factor that influences the yield of biobutanol. Proper pH can urge the metabolic shift from acidogenesis to solventogenesis. At the final stage of acidogenesis phase, acid production slows down due to effect of low pH.

To compensate for the unfavourable effect of low pH, organism shifts its metabolic activity from acidogenesis phase to solventogenesis phase (Buyondo and Liu, 2012). Cells in the exponential growth phase at higher pH produce acetate and butyrate during 'acidogenesis. In this phase, acetate and butyrate are consumed as substrates for biosynthesis of acetone and butanol while no growth is observed (Buyondo and Liu, 2012).

Effect of sugar concentration

Sugar concentration in the fermentation substrate has an important influence on bio butanol yield. A low sugar concentration can reduce cell growth, interrupt the acidogenesis phase, and further affect solvent formation; a high sugar concentration can directly inhibit cell growth and cause failure of the whole ABE fermentation (Wang et al., 2016).

Temperature

Temperature affects the activity of microorganisms and the conversion rate of fermentation products and is closely related to economic benefit (Jung et al., 2011). The temperature of the fermentation can affect overall yield, solvent ratios, and rate of solvent production. For instance, in the molasses fermentation at 30˚C yield of solvents was 31% with acetone contributing 23%, at 33˚C the yield was 30% with acetone contributing 26%, and at 37˚C yield was around 24% with acetone contributing 38% of total solvents (McNeil and Kristiansen, 1986). Most ABE fermentation processes are performed at optimum temperatures between 35˚C and 37˚C.

Anaerobic conditions

Clostridia are obligate anaerobes and thus their growth is significantly affected by oxygen. Four main hypotheses for oxygen toxicity were proposed: (1) Oxygen is itself a toxic agent. (2) Anaerobes require low redox potentials (Eh), (3) Organisms lacking catalase, e.g., the *Clostridia* are killed by water, formed by reducing some of the oxygen. (4) Oxygen is a more avid electron acceptor than the normal terminal oxidants so that anaerobes cannot maintain intracellular concentrations of electron donors such as NAD(P)H (Buyondo and Liu 2012). Compared with aerobes, the strict anaerobes need special equipment and complicated operation to eliminate oxygen in the culture medium, for example, adding reducing agents or flushing with N2 gas, which increased the total cost of ABE fermentation (Wu et al., 2016).

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 and Liu, 2012).

Product inhibition

Toxicity to solvents produced in the fermentation process is a major limitation preventing high productivity. These solvents interfere with the membrane permeability leading to leakage of cellular constituents, disrupt function of membrane proteins and alter fluidity of membrane. Solvent tolerant bacteria evade these reactions by cellular adaptations including altered membrane composition, efflux system that actively decreases the solvent concentration inside the cell, degradation of solvents or over-expressing stress proteins such as heat shock proteins.

2.7.1 Diversity of inhibitors in lignocellulose hydrolysates

The type of lignocellulosic material, the cell wall composition, and the thermochemical conditions employed for hydrolysis mostly determine the nature of the inhibitors. These inhibitory components must be removed from the lignocellulose hydrolysates prior to fermentation in order to attain suitable yields and productivities from the microorganisms. A number of inhibitors toxic to fermentative microorganisms are released during thermochemical degradation of the plant cell wall. Among these substances are furans, such as furfurals and 5-HMF; phenolics; weak acids (acetic acid, levulinic acid, formic acid, etc.); raw material extractives (acidic resins, tannic acids, and terpene acids); and heavy metal ions (iron, chromium, nickel, and copper), all of which have been recognized as potent inhibitory substances (Chandel et al., 2013). These toxic compounds inhibit the growth of fermenting microorganisms by affecting their sugar uptake rate and simultaneously decrease their rate of product formation.

Detoxification strategies for fermentation inhibitors

Several types of detoxification strategies, such as physical, chemical (Neutralization, Ca $(OH)_2$ overliming, Alkali (NaOH, Na_2SO_3, NH_3), Activated charcoal, Ion-exchangers (anionic and cationic) and extraction with ethyl acetate), biological (microbial and enzymatic), have been applied to remediate fermentation inhibitors (Chandel et al., 2013).

2.8 Biobutanol producing organisms

Biobutanol production is carried out exclusively by members of the genus Clostridia, including *Clostridium acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum, C. saccharoacetobutylicum, C. aurantibutyricum, C. pasteurianum, C. sporogenes, Clostridium cadaveris, and Clostridium tetanomorphum* (Buyondo and Liu, 2012). Several species of this bacteria (rod-shaped, spore forming, Gram positive and strict anaerobe) are capable of metabolizing different sugars, amino and organic acids, polyalcohols and other compounds to butanol, acetone and isopropanol; but butanol, being of relatively high value, is usually the most desired product (Ezeji et al., 2007b). Clostridia are largely saprotrophic organisms and live in virtually all anaerobic habitats containing organic matter, including soils, aquatic sediments, and anaerobic tissues (such as intestinal tracts of animals) (Tracy et al., 2012).

As an obligate anaerobe, *Clostridiums* were not able to survive in aerobic environments, but were often detected with aerobic bacteria simultaneously in habitats which were exposed to oxygen environments such as rice paddy soils and water retting pond. In these habitats, aerobic bacteria were considered to be essential for *Clostridiums* by supplying an anaerobic environment and consuming harmful metabolites. The cooperation between *Clostridiums* and aerobic bacteria were observed not only in natural habitats but also in many artificial systems (Wu et al., 2016). As such, they have developed an exceptionally broad capability to ferment organic compounds, including many simple and complex carbohydrates, also proteins, amino acids, and several other simple and complex organic molecules (Jin et al., 2011).

2.8.1 Co culture bacterial system

In nature, microorganisms often exist in highly diverse and complex communities rather than live in isolation. In these natural communities, microorganisms coexist stably by interacting with each other and exert various functions more effectively than single culture. As an obligate anaerobe, Clostridiums were not able to survive in aerobic environments, but were often detected with aerobic bacteria simultaneously in habitats which were exposed to oxygen environments such as rice paddy soils and water retting pond. In these habitats, aerobic bacteria were considered to be essential for Clostridiums by supplying an anaerobic environment and consuming harmful metabolites. The cooperation between Clostridiums and aerobic bacteria were observed not only in natural habitats but also in many artificial systems.

Co-culture of *Clostridium* and other bacteria which have enzymes capable of hydrolyzing cellulose and hemicellulose were induced in utilization of cellulosic biomass. In these co-culture systems, cellulose and hemicellulose were first hydrolyzed to glucose or butyric acid by cellulolytic strains, and then butanol was obtained subsequently by adding solvent-producing *Clostridium* species (Wu et al., 2016).

In an artificial syntrophic co-culture system, *Bacillus* was founded to be a good partner for creating anaerobic environment and pre-saccharification of substrate for co-cultured *Clostridium* strain. Some reports confirmed that aerobic bacteria, such as *B. subtilis* and *B. cereus*, had a positive interaction with anaerobic *Clostridium*, the role of aerobic bacteria was not established clearly (Wu et al., 2016). Their rationale was that strains of Bacillus generally have a high growth rate and secrete many saccharification-related extracellular enzymes into the medium, such as amylase, pectinase, protease, cellulose, and hemicellulase (Dwidar et al., 2013). Aerobic bacteria in the co-culture system are considered to sustain anaerobic environment for *Clostridiums* and different micro-organisms in the co-culture system may compete for substrates, therefore, the product yield and productivity would be influenced compared with single cultures (Wu et al., 2016).

(Abd-Alla and Abdel-Wahab, 2012) reported a maximum ABE productivity of 0.30 g/L.h and ABE yield of 0.42 from 75 g/L spoilage date fruits homogenate by mixed culture without addition of a reducing agent and N_2 flushing. (Thi et al., 2010) founded that when B. subtilis was co-cultured with *C. butylicum* without anaerobic pretreatment by reducing agent and N_2 flushing increased amylase activity 10 folds and enhanced ABE production 5.4 and 6.5 folds from soluble starch and cassava starch, respectively, compared to those of the pure culture of Clostridium itself.

3. MATERIALS AND METHODS

The experimental work was done in the laboratory of Addis Ababa institute of technology, school of chemical and bio Engineering.

3.1 Materials and reagents

The raw material used for the study was Brewery's Spent Grain (BSG). It was kindly supplied by BGI Ethiopia PLC (Industry) which is located in Addis Ababa.

The major equipment that were used during the experiments for, (raw material preparation, characterization of BSG, hydrolysis, quantifying total reducing sugar content, fermentation and product analysis) were: - Digital Weighing Balance, Sieves, Silica Crucible, Desiccators, Drying oven, Erlenmeyer Flasks (100ml, 250ml and 500ml) Electrical Muffle Furnace, Soxhlet Extractor, Cellulose Extraction Thimble, Watt-man Filter paper, Vacuum Filter, Measuring Cylinders, Water Bath, Stopwatch, test tubes, spectrophotometer, Autoclave, pH meter, Anaerobic jar, Candle, Shaker, incubator, Rotary evaporator, Density meter, Fourier Transform Infrared spectroscopy (FTIR) etc.

The major chemicals and analytical reagents used during series of experiments were: - Distilled Water, acetone, sulfuric acid, sodium hydroxide, quantitative Benedict reagent solution, glucose, Nutrient broth (HIMEDIA), Yeast extract, Potassium hydrogen phosphate (KH_2PO_4), *di* Potassium hydrogen ortho phosphate (K_2HPO_4), Ammonium chloride (NH_4Cl), Sodium chloride (NaCl), Magnesium Sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$), Manganese sulphate 1-hydrate, ($MnSO_4 \cdot H_2O$) and Iron Sulphate 7-hydrate ($FeSO_4 \cdot 7H_2O$) etc.

3.1.1 Raw material preparation

The BSG sample was transported to AAIT biochemical laboratory in a plastic bag and it was then washed with water in order to remove the wort residues and other unwanted matter. The feedstock material was then sun dried to 10% moisture content under the conditions defined by (Mussatto and Roberto, 2005). The dried BSG was milled to a powder with maximum particle size of 2 mm and sieved in to appropriate particle size and stored until required for processing or analysis. Then its compositional analysis was done to estimate cellulose, hemicellulose, lignin and other components.

3.2 Characterization of Brewery's Spent Grain

3.2.1 Proximate analysis

Moisture Content: - To obtain the percent moisture content of the BSG, sample was weighed and then dried at 105 ± 3 °C for 24 hours until a constant weight was obtained. After 24 hours, the BSG was withdrawn, cooled and then weighed to the nearest 0.001g. The moisture content was found on a percentage basis. The moisture content was determined by the following formula:

$$\%\ Moisture\ content\ = \frac{W1-W2}{W1} \times 100 \quad \text{.....[3.1]}$$

Where;

w_1 = weight of BSG sample before drying

w_2 = weight of BSG sample after drying.

Ash content: Ash content of BSG was determined following ASTM methods. The crucible was oven dry, cooled and weighted until constant weight. 3g BSG sample was measured and placed in the crucible. Then crucible and content were weighed to the nearest 0.001g. The BSG sample was ignited at 525 ± 25 ºC for about 4 hours (ASTM D1102-84). After 4 hours, the crucible was carefully withdrawn and cooled in a desiccator then weighed. The ash content was calculated on percentage basis:

$$\%\ Ash\ content\ = \frac{W2}{W1} \times 100 \quad \text{.....[3.2]}$$

Where;

w_1 = weight of BSG sample before ignited

w_2 = weight of BSG sample after ignition

3.2.2 Chemical compositions analysis

The chemical compositional analysis of the BSG sample i.e., extractives, cellulose, hemicellulose and lignin was analyzed using gravimetric method under the conditions defined by (Ayeni et al., 2015).

Extractives: 3 g of dried BSG 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 BSG and extractive-free BSG. The following formula was used to obtain the % extractive content of BSG sample:

$$\%\, Extractives = \frac{W1-W2}{W1} \times 100 \quad \text{.....[3.3]}$$

Where;

W_1=weight of oven-dry test specimen

W_2=weight of oven-dry extraction residue

Hemicellulose: 1 g of extracted dried BSG was transferred into a 250 mL Erlenmeyer flask. 150 mL of 500 $\mathrm{mol/m^3}$ NaOH was added. The mixture was boiled for 3.5 h 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 weight before and after this treatment is the hemicellulose content (%w/w) of dry BSG.

$$\%\, Hemicellulose = \frac{W2-W1}{Ws}\, x\, 100 \quad \text{.....[3.4]}$$

Where;

W_1 – Oven dry weight of filter paper, g

W_2 – Oven dry weight of filtrated hemicellulose + weight of filter paper, g

W_S – Oven dry weight of sample, g

Lignin: 1.8 g of dried extracted raw BSG was weighed in glass test tubes and 18 mL of 72% H_2SO_4 was added. The sample was kept at room temperature for 2 h with carefully shaking at 30 min intervals to allow for complete hydrolysis. After the initial hydrolysis, 504 mL of distilled water was added. The second step of hydrolysis was made to occur in an autoclave for 1 h 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.

The acid soluble lignin fraction was determined by measuring the absorbance of the acid hydrolyzed samples at 320 nm. The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin.

$$\% \, Lignin \; content \; = \frac{W2 - W1}{Ws} x100 \ldots\ldots\ldots [3.5]$$

Where;

W_1 – Oven dry weight of filter paper, g

W_2 – Oven dry weight of filtrated lignin + weight of filter paper, g

W_S – Oven dry weight of sample, g

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 biomass

$$\% \, Cellulose \; content = 100 - extractive \; + hemicellulose \; + lignin \; + ash \ldots\ldots [3.6]$$

3.3 Methods

3.3.1 Dilute-acid pre-treatment

Among all the available pre-treatment methods, dilute sulphuric acid pre-treatment has been widely considered to open the crystalline complex of cellulose. Moreover, this method is also reported to be inexpensive, convenient and effective chemical treatment for hydrolysing variety of lignocellulosic biomass (Qureshi et al., 2008). Hemicellulose is removed when sulfuric acid is added and this enhances digestibility of cellulose in the residual solids (Mosier et al. 2005).

The brewery spent grain was pretreated by using 1.25% (*v*/*v*) sulphuric acid in a solid-to-liquid ratio of 1:8 (*w*/*w*) at 120 °C for 17 min in an autoclave under the conditions defined by (Mussatto and Roberto, 2005). When the treatment time had elapsed, temperature dropped by delaying at room temperature. After treatment the resulting solid material was separated by vacuum filtration and the filtrate (hemicellulosic sugar) was preserved in another conical flask and kept for fermentation. The resulting solid material was then washed by distilled water to remove sulphuric acid from it and prepared to a second hydrolysis step.

3.3.2 Dilute acid hydrolysis

However, glucose from cellulose is not usually hydrolyzed under the range of operational conditions commonly used for diluted acid treatment. The main purpose of this process is to degrade cellulose in to its monomer in the optimal condition of temperature, acid concentration and reaction time (Mussatto and Roberto, 2005). Research works on the dilute acid hydrolysis of different lignocellulosic materials have defined optimal process conditions at: temperature 80-200°C, sulfuric acid concentration 0.25–8 wt.%, and reaction time 10-2000min (Gladyshko, 2011).

The process conditions that were used for hydrolysis were temperature (115°C, 125°C and 135°C), reaction time (20min, 25min and 30min) and sulfuric acid concentration (1.25%, 1.5% and 1.75%).

Filtration

After hydrolysis the lignin and degraded cellulose which is called monomeric sugar was separated by vacuum filtration to remove the non-fermentable lignin portion. After the solid part was separated, it was washed by distilled water twice. The washing was performed in order to extract all residual soluble sugars from the solid. Then the filtrate (cellulosic sugar) obtained in the hydrolysis process was mixed with the previously recovered hemicellulosic sugar during the dilute acid pretreatment process.

pH Adjustment

Initial pH of the medium for biobutanol production is an important factor which significantly affects fermentation process (Al-shorgani et al., 2014). The pH of the hydrolysate was adjusted by addition of 6 M NaOH until the pH became in a range of 6.0 - 6.5.

3.3.3 Fermentation

Microorganisms

Vials of *Clostridium/Eubacterium* and *Bacillus subtillus* were generously obtained from Ethiopian Biodiversity Institute (EBI), Addis Ababa.

Inoculum preparation

Clostridium/Eubacterium used for the fermentation was anaerobically cultured in a nutrient broth (NB) under static condition at 37 ºC for 24–48 h and *Bacillus subtillis* was aerobically cultured in a nutrient broth (NB) (HIMEDIA) under shaking condition at 200 rpm and 37 ºC for 12–24 h under the conditions defined by (Ndaba et al., 2015).

Fermentation condition

The fermentation media consisted of the following components: 5 g/L yeast extract, 2 g/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 0.01 g/L NaCl and supplemented by 5 g/L $CaCO_3$ under the conditions defined by (Thi et al., 2010) with some modification.

NH_4Cl and Yeast extract were used as inorganic and organic nitrogen sources. Yeast extract provides various amino acid and it is a growth factor for the microbes. (KH_2PO_4 and K_2HPO_4 K_2HPO_4) are a kind of phosphate commonly used for a buffer, especially in a bacteriological culture medium, which has a buffering effect that can maintain the pH during fermentation. ($MgSO_4.7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4.7H_2O$ and NaCl) were used as mineral salts. $CaCO_3$ supplementation to the fermentation media allows to both improve the exploitation of the feedstock and to enhance solvent production (Raganati et al., 2012).

The prepared media was autoclaved at 121°C for 15min. The fermentation process was carried out anaerobically inside of anaerobic jar incubated at 37°C for 96 hrs.

In addition to the single species fermentation, co-culture fermentation of BSG hydrolysate was also conducted on the optimized hydrolysate. Different inoculum concentration ratios (10:1, 10:3, and 10:5% v/v of *Clostridium/Eubacterium* to *Bacillus subtilis*) were used to ferment BSG hydrolysate to investigate the influence of co-culture bacterial system variable on the yield of biobutanol under the conditions defined by (Wu et al., 2016) with some modification.

3.3.4 Separation

Rotary evaporator was used for the separation biobutanol from the broth based on the volatilities (boiling points) of the individual components that make up the mixture. The boiling point of acetone, ethanol and butanol is, 56°C, 78°C and 118°C respectively.

3.3.5 Determination of total reducing sugar using Benedict's test

The concentration of total reducing sugar (TRS) content of hydrolysate was determined using digital spectrophotometer by measuring absorbance vs. sugar concentration at 540nm wave length. Quantitative benedict solution and standard glucose solution was used for assays to plot the calibration curve. Benedict's solution is designed to detect the presence of reducing sugars. In hot alkaline solutions, reducing sugars reduce the blue copper (II) ions to brick red copper (I) oxide precipitate. As the reaction proceeds, the color of the reaction mixture changes progressively from blue to green, yellow, orange and red. When the conditions are carefully controlled, the coloration developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of glucose-equivalent reducing sugars present in a sample can be obtained.

Calibration curve Plot for standard glucose

- ☞ Standard glucose solution was prepared at different concentration of, 25, 20, 15, 10, 5, 1 and 0%.
- ☞ 1ml of each of the standard glucose solution was added into labelled test tubes, each containing 5 ml of benedict's solution and mixed by shaking.
- ☞ The labelled test tubes were heated in 90^{o}C water bath for 5 minutes.
- ☞ As soon as the test tubes removed from the water bath the samples were filtered using filter paper to remove any red precipitate formed when reducing sugar in the samples reacted with Benedict's reagent.
- ☞ The absorbance of the samples was read using spectrophotometer at 540nm.
- ☞ Finally, a calibration curve was plotted to show the % of absorbance of red light by the standard glucose solutions. Concentration of standard glucose with its absorbance

Determination of the reducing sugar amount on the hydrolysates were carried out by using the same procedure made for the standard glucose concentrations and the absorbance of red light by the unknown sample (hydrolysate) was read. Then the concentration of glucose-equivalent reducing sugars present in each hydrolysate were read from the calibration curve of the standard glucose solution.

$$\text{CTRSUS} = \frac{(\text{absorbance of unknown sample}) - (\text{y} - \text{intercept})}{\text{slope}} \quad \text{.....[3.7]}$$

CTRSUS = Concentration of total reducing sugar of unknown sample

$$\% \text{ TRS} = \frac{\text{glucose concentration of sample} \times \text{volume of hydrolysate}}{\text{gram of sample used}} \times 100 \ldots\ldots\ldots\ldots\ldots [3.8]$$

TRS = Total reducing sugar

FTIR analysis of the produced BSG bio butanol

In order to determine the functional groups of the BSG powder and the bio butanol produced was analysed using FTIR (Fourier Transform Infrared spectroscopy) method by passing a beam of infrared light through the sample.

The FTIR spectra were recorded on spectrum 65 FT-IR (perkinElmer) equipped with KBr beam splitter. Diffuse reflectance system (DRS) was used for powder samples (BSG powder) and NaCl plate for liquid samples (bio butanol) by thin film deposition technique. A regular scanning range of 400-4000 cm^{-1} was used. All the spectra were recorded and processed using essential FTIR software and the result was compared with the standard.

3.4 Experimental design

Experimental data analysis was done using Design-Expert 7.0.0 software. The experimental design selected for this study was response surface methodology, three-factor quadratic model, Box-Behnken Design (BBD) and the response variable measured was the bio butanol yield. The three independent variables studied for the hydrolysis process were temperature, reaction time and acid concentration as summarized in Table 3.1 with their respective levels and the proposed BBD in Table 3.2.

Table 3.1: Independent variables and levels for Box Behnken Design

Independent Variables	Unit	Levels		
		-1	0	1
Temperature	ºC	115	125	135
Reaction time	min	30	35	40
Acid concentration	%	1.5	1.75	2

Table 3.2: The proposed Box Behnken Design (BBD) with the response of bio butanol yield

Std	Run	Factor 1: Temperature (^{0}C)	Factor 2: Reaction time (min)	Factor 3: Acid concentration (%)	Response 1: Biobutanol yield (ml/25g)
1	8	115.00	30.00	1.75	
2	16	135.00	30.00	1.75	
3	15	115.00	40.00	1.75	
4	6	135.00	40.00	1.75	
5	1	115.00	35.00	1.50	
6	7	135.00	35.00	1.50	
7	11	115.00	35.00	2.00	
8	13	135.00	35.00	2.00	
9	5	125.00	30.00	1.50	
10	4	125.00	40.00	1.50	
11	2	125.00	30.00	2.00	
12	14	125.00	40.00	2.00	
13	10	125.00	35.00	1.75	
14	17	125.00	35.00	1.75	
15	12	125.00	35.00	1.75	
16	9	125.00	35.00	1.75	
17	3	125.00	35.00	1.75	

4. RESULTS AND DISCUSSION

4.1 Proximate and chemical compositions analysis of brewery's spent grain

Table 4.1 presents the proximate analysis of brewery's spent grain. The values 64.65 wt. % and 4.2 wt. % were obtained for moisture and ash content respectively. Moisture content analysis is used for the determination of proportionality of solid to liquid ratio in the pretreatment and hydrolysis method, whereas; in the preservation and handling of the biomass. The high amount of water in the wet residue may result in other limiting factors, such as difficulties in long distance transport and storage. Ash is defined as the inorganic and the mineral matter of a biomass. Ash content of a biomass is required to be small for the downstream processing of bio butanol.

(Muthusamy, 2014) reported that the moisture and ash content of the brewer's spent grain ranges from 75-80% and 3-5% respectively. According to reports by (Thavasiappan et al., 2016; Senthilkumar et al., 2010), brewers' spent grain has a moisture of around 69.98% and a total ash content of 4.65% (Thavasiappan et al., 2016) and 5.76% (Senthilkumar et al. 2010). In other literature reviews high a moisture content of (80% - 85%) was reported, making it susceptible to microbial growth and subsequent spoilage within a short period of time (7 - 10 days) (Buffington, 2014; Aliyu and Bala, 2011). (Mussatto et al., 2006; Mussatto et al., 2008) determined the ash content of brewer's spent grain in ranges from 2.4-4.6 wt.%.

The present result on ash content was consistent with the work described by (Muthusamy, 2014; Thavasiappan et al., 2016; Mussatto et al., 2006; Mussatto et al., 2008) and it is suitable for decreasing sludge formation in the downstream processing of bio butanol. In the contrary the present result found for the moisture content of brewer's spent grain under analysis Table 4.1 was different with the work described by (Aliyu and Bala, 2011; Muthusamy, 2014; Senthilkumar et al., 2010; Thavasiappan et al., 2016; Buffington, 2014). The reason for such differences might be due to barley variety, growth environment, harvesting time, malting and mashing conditions in the process.

Table 4.1: Proximate compositions analysis of brewer's spent grain

Component	Run 1	Run 2	Average
Moisture (wt. %)	62.53	66.77	64.65
Ash (wt. %)	3.8	4.6	4.2

The present result for chemical compositional analysis of brewery's spent grain shown in Table 4.2 were cellulose (17.87wt%), hemicellulose (28.86wt.%), lignin (15.79wt.%) and extractives (33.28wt.%). (Mussatto et al., 2006) reported that the chemical composition of BSG as 16.8–25.4% cellulose, 21.8–28.4% hemicellulose and 11.9–27.8% of lignin. The present result found for the cellulose, hemicellulose and lignin content of the brewer's spent grain under analysis Table 4.2 are consistent with the work described by (Mussatto et al., 2006). The raw material analysis revealed that the spent grains contained a high amount of hemicellulose and low lignin. Hemicellosic sugars offer the best alternative as a substrate for bio butanol production (Buyondo and Liu, 2012). Several species of *clostridia* are capable of converting hemicellulose sugars to bio butanol. The low lignin content makes its bioconversion to bio butanol particularly attractive.

The high value of total extractives in the sample indicated higher amount of acetone soluble components such as terpenes, terpenoids, tannins, resins, fats, waxes, lipids, proteins, and organic acids.

Table 4.2: Chemical compositions analysis of brewery's spent grain

Component	Composition (wt. %)
Cellulose	17.87
Hemicellulose	28.86
Lignin	15.79
Extractives	33.28

4.1.1 Functional group analysis of brewery's spent grain by FTIR

Lignocellulosic biomass from different lignocellulosic materials appear quite different, but the chemical composition is fairly similar although with different magnitudes of constituents.

Compositional variation and physical organization at the microscopic level determine the ability to perform a desired function for most materials. FTIR has been mostly successful in accurate analysis of both major and minor constituents of lignocellulosic biomass. The major compositions of lignocellulosic biomass are cellulose, hemicellulose and lignin. While the minor constituents include minerals, pectin, waxes and water-soluble components.

The functional group analysis of Brewery's Spent Grain (BSG) was obtained by Fourier Transform Infrared (FTIR) transmission spectra. The spectrum in (Figure 4.1) revealed the presence of a number of characteristic bands in the range of (400–4,000 cm^{-1}).

A broad band noticed from (3610 - 3100 cm^{-1}) corresponds to polymeric involvement of hydroxyl groups (OH stretching vibration), and bonded O–H stretching vibration present in carbohydrates (hemicellulose, cellulose and lignin). The C–H stretching bands (1500–1300 cm−1) and a C–O stretching band at 1040 cm^{-1} are attributed to the presence of cellulosic structure. The absorption at 1640 cm−1 implies the presence of C=O of carboxylic acid and their derivatives. The absorption (1700–1560 cm^{-1}) corresponds to the aromatic skeletal vibrations due to the presence of lignin. The absorptions (1200–900 cm^{-1}) are predominantly dominated by a sequence of bands owing to C–O, C–C, C–O–C, and C–O–P stretching vibrations of polysaccharides as well as CH_3, CH_2 (Coates, 2000).

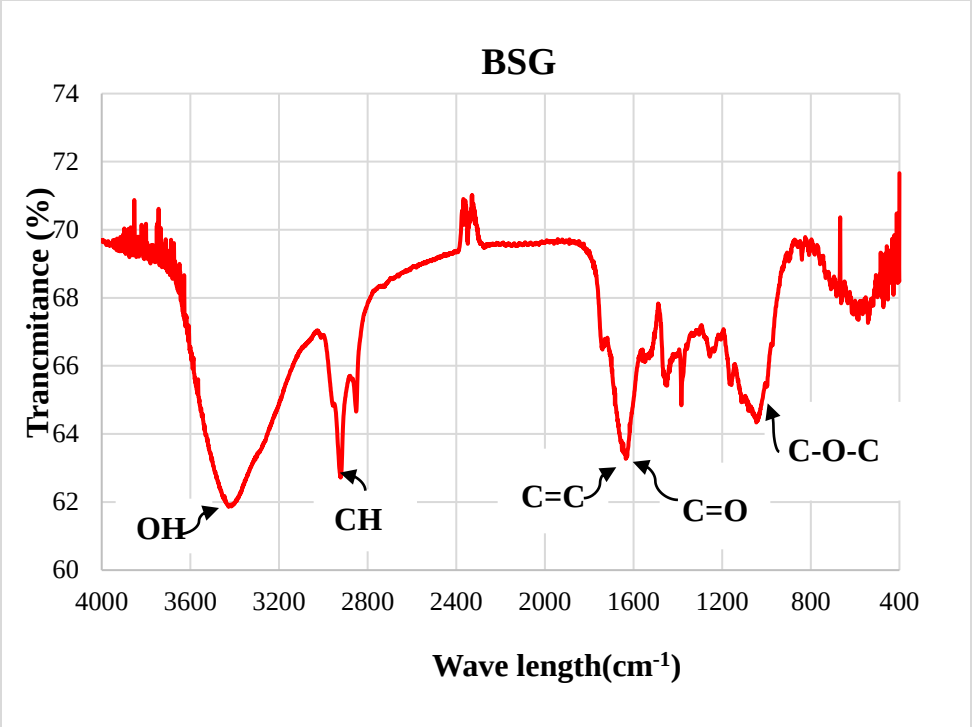

Figure 4.1: FTIR spectra of Brewery Spent Grain (BSG powder)

4.2 Determination of total reducing sugar

The concentration of total reducing sugar of unknown sample was determined using equation 3.7 which was obtained from the calibration curve of standard glucose concentration. (Figure 4.2). From Table 4.3 it is clear that the absorbance and concentration of sugar has inverse relationship.

The CU (II) ions in the Benedict's solution are reduced to CU (I) ions which causes the color change. As the concentration of reducing sugar increases, the nearer the final color is to brick-red and the greater the precipitate formed i.e. redder CU (I) oxide is formed. The sample which have less sugar concentration will remain with high amount of CU (II) oxide, blue color which is unreacted with the Benedict's reagent due to this, absorbance of the sample increases.

But in the sample, which have high reducing sugar concentration, CU (II) oxide is completely react with the Benedict's reagent and form CU (I) oxide red color precipitate and absorbance of the sample decreases.

Table 4. 3: Concentration of standard glucose and its absorbance

Concentration of standard glucose (g/ml)	0	0.01	0.05	0.10	0.15	0.20	0.25
Absorbance	1.012	0.981	0.912	0.842	0.781	0.658	0.573

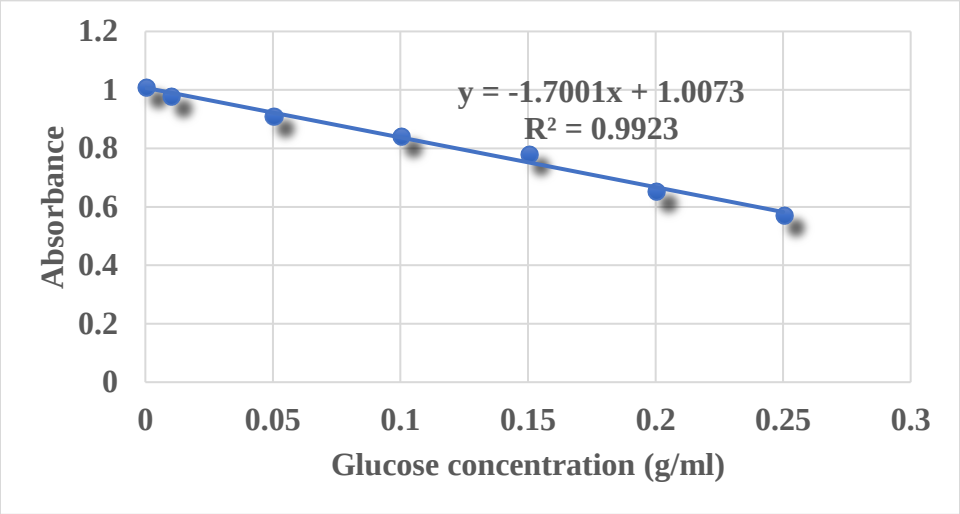

Figure 4. 2: Calibration curve of standard glucose concentration

4.3 Statistical analysis of the experimental results

4.3.1 Effect of hydrolysis process variables on the yield of bio butanol

In this study, RSM based on a Box-Behnken design was implemented which can reflect the nature of the response surface in the experimental region and investigate the three independent process variables including acid concentrations, temperature and reaction time to evaluate their effect on the production of bio butanol. Design-Expert Software 7.0.0 was used in the least squares regression analysis of variance (ANOVA). The statistical software program is used to generate the model equation, interaction effects of the independent variables and surface plots using the fitted equation obtained from the regression analysis holding one of the independent variables constant. The BBD conditions and their respective responses are shown in Table 4.4.

Table 4. 4: Box-Behnken design with actual and predicted values of bio butanol yield

Order		Independent process variables				Yield of Bio butanol (ml/25g of BSG)	
Std	Run	Temperature (ºC)	Reaction time (min)	Acid concentration (%)	Yield of TRS (g/L)	Actual value	Predicted value
1	8	115.00	30.00	1.75	35.5	1.90	1.95
2	16	135.00	30.00	1.75	47.2	2.30	2.32
3	15	115.00	40.00	1.75	37.8	2.00	1.97
4	6	135.00	40.00	1.75	41.1	2.10	2.05
5	1	115.00	35.00	1.50	37.2	2.00	1.99
6	7	135.00	35.00	1.50	56.1	2.50	2.51
7	11	115.00	35.00	2.00	46.6	2.20	2.19
8	13	135.00	35.00	2.00	44.9	2.10	2.11
9	5	125.00	30.00	1.50	51.9	2.40	2.36
10	4	125.00	40.00	1.50	49.6	2.30	2.34
11	2	125.00	30.00	2.00	53.7	2.40	2.36
12	14	125.00	40.00	2.00	43.7	2.10	2.14
13	10	125.00	35.00	1.75	58.4	2.60	2.64
14	17	125.00	35.00	1.75	58.4	2.60	2.64
15	12	125.00	35.00	1.75	59.0	2.70	2.64
16	9	125.00	35.00	1.75	58.4	2.60	2.64
17	3	125.00	35.00	1.75	59.0	2.70	2.64

The statistical significance of the model was analyzed using ANOVA as shown in Table 4.5. The Model F-value of 34.48 and Model P value (Prob>F) of less than [<0.0001] imply the model was significant.

The P values are used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The "Lack of Fit F-value" of 1.39 implies the Lack of Fit is not significant relative to the pure error. There is a 36.78% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 4. 5: Analysis of variance for response surface quadratic model of bio butanol

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	1.09	9	0.12	34.48	< 0.0001	significant
A-Temperature	1.09	1	0.10	28.93	0.0010	
B-Reaction time	0.10	1	0.031	8.93	0.0203	
C-Acid concentration	0.031	1	0.020	5.71	0.0481	
AB	0.020	1	0.022	6.43	0.0389	
AC	0.022	1	0.090	25.71	0.0014	
BC	0.090	1	0.010	2.86	0.1348	
A^2	0.010	1	0.47	133.00	< 0.0001	
B^2	0.47	1	0.23	65.03	< 0.0001	
C^2	0.23	1	0.049	13.90	0.0074	
Residual	0.049	7	3.500E-003			
Lack of Fit	0.025	3	4.167E-003	1.39	0.3678	not significant
Pure Error	0.013	4	3.000E-003			
Cor Total	0.012	16				

Note: A, B and C are temperature, reaction time and acid concentration respectively.

Values of P value "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2, C^2, AB, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. In term of the determination of interactions between the variables, the *P* values can provide an understanding of the pattern of the interactions as well as the effect of each variable on the investigated response. This shows that the hydrolysis temperature, reaction time, acid concentration, interaction between temperature and reaction time, interaction between temperature and acid concentration, square of temperature, square of the reaction time and square of the amount of acid concentration affects the yield of bio butanol significantly.

Model adequacy check

Another useful statistic here was the one labeled "Adeq Precision". This measures the signal to noise ratio that is, the ratio of the range of variation in the predicted response to an estimate of the standard error of the predictions. The adequacy of a model is determined through R-Squared (multiple correlation coefficient), CV (coefficient of variation) and *P* values. R-Squared value closer to 1 denotes better correlation between the experimental and predicted values.

The "Pred R-Squared" of 0.8030 is in reasonable agreement with the "Adj R-Squared" of 0.9496 as shown in the table 4.6. A ratio greater than 4 is desirable. The ratio of 15.207 indicates an adequate signal. This model can be used to navigate the design space.

The fit of the model was also expressed by the coefficient of regression R-Squared, which was found to be 0.9779 indicating that 97.79% the variability in the response could be explained by the model. The closer the value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R-Squared (0.9779) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. This implies that the prediction of experimental data is quite satisfactory. The Coefficient of Variation (CV) value as the ratio of the standard error of the estimate to the mean value of the observed response indicates the degree of precision with which the experiments are compared. Generally, the higher the value of the CV is, the lower the reliability of the experiment. Here a lower value of CV (2.55) indicates greater reliability of the experiments performed. PRESS is the predicted residual sum of squares for the model which is a measure of how well a particular model fits each point in the design.

Table 4. 6: Model adequacy measures

Std. Dev.	0.059	R-Squared	0.9779
Mean	2.32	Adj R-Squared	0.9496
C.V.	2.55	Pred R-Squared	0.8030
PRESS	0.22	Adeq Precision	15.207

The actual and predictive model in terms of coded factors excluding the insignificant term is presented below. These terms can be used to re-create the results of this experiment, but they cannot be used for modeling future responses.

Final equation in terms of coded factors:

$$\text{Biobutanol yield} = +2.64 + 0.11 \times A - 0.062 \times B - 0.050 \times C - 0.075 \times A \times B - 0.15 \times A \times C - 0.33 \times A^2 - 0.23 \times B^2 - 0.11 \times C^2 \quad \text{.....4.1}$$

Final equation in terms of actual factors:

$$\text{Biobutanol yield} =$$

$$-86.27937 + 1.00000 \times \text{Temperature} + 0.826 \times \text{Reaction time} + 13.32000 \times \text{Acid concentration} - 1.50000E-003 \times \text{Temperature} \times \text{Reaction time} - 0.060000 \times \text{Temperature} \times \text{Acid concentration} - 3.32500E-003 \times \text{Temperature}^2 - 9.30000E-003 \times \text{Reaction time}^2 - 1.72000 \times \text{Acid concentration}^2 \quad \text{.....4.2}$$

4.3.2 Individual effect of hydrolysis process variables on bio butanol yield

The individual effects of the operating conditions on the bio butanol yield were investigated using the Analysis of Variance (ANOVA) and the hydrolysis process variables were found to affect the bio butanol yield significantly by observing the p-value less than 0.05. This result demonstrated that the advantage of using BHD surface response for experimental data analysis in capturing the individual process variables that affect the bio butanol yield. The significant individual effects were temperature, A, reaction time, B and acid concentration, C.

Effect of acid concentration

The resulting plot of acid concentration versus bio butanol yield (Figure 4.3) depicts the yield of bio butanol is significantly affected by acid concentration. At higher level of acid concentration, the amount of bio butanol yield was low compared to lower acid concentration. It can be seen from the figure that with increasing acid concentration until it reaches 1.75% indicated an increase in the yield of bio butanol, the yield of bio butanol then starts to drop as the acid concentration tend to increase above 1.75%. Such behavior may be attributed to the following reasons. The degradation of hemicellulosic sugars of pentoses, hexoses, and the lignin present. Among these degradation substances are furans, such as furfurals and 5-hydroxymethyl-2-furaldehyde (HMF); phenolics; weak acids (acetic acid, levulinic acid, formic acid, etc.) which have been recognized as potent inhibitory substances to the fermentative clostridia (Ji et al., 2012). And the generation of these inhibitory compounds might cause a lower yield of total reducing sugar concentration which has direct relation with the yield of bio butanol.

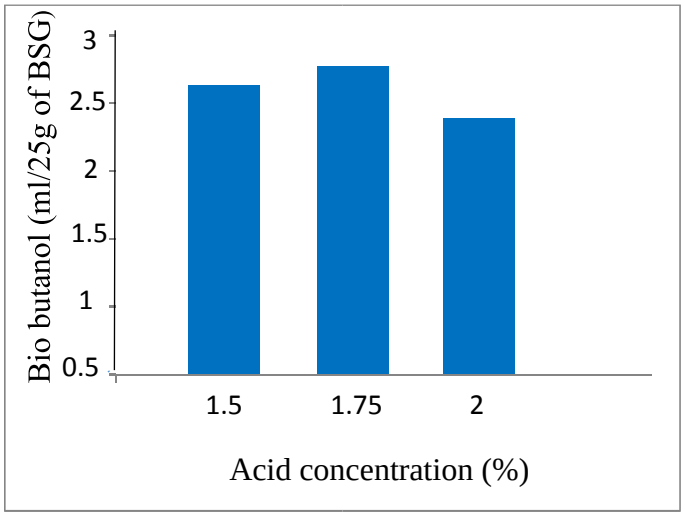

Figure 4.3: Effect of hydrolysis acid concentration on bio butanol yield at a fixed temperature (125 ºC) and reaction time (35min)

Effect of temperature

The resulting plot of temperature versus bio butanol yield (Figure 4.4) indicated when hydrolysis temperature varied from 115°C to 135 ºC. The effect of hydrolysis temperature was found to affect the bio butanol yield positively. As the temperature varied from 115°C to 125 ºC yield of bio butanol observed to increase.

Therefore, the optimum hydrolysis temperature was found to be 125°C. When the temperature was increased more than the optimal level (125°C), a decrease in the yield of bio butanol was observed and this may be due to the formation of degradation products which are inhibitory substances. In other way bio butanol yield was low at low level of temperature this might be due the reason that the temperature was not sufficient enough to convert cellulosic sugars to monomeric sugars on the hydrolysis stage or possibly due to low reducing sugar levels in the hydrolysate.

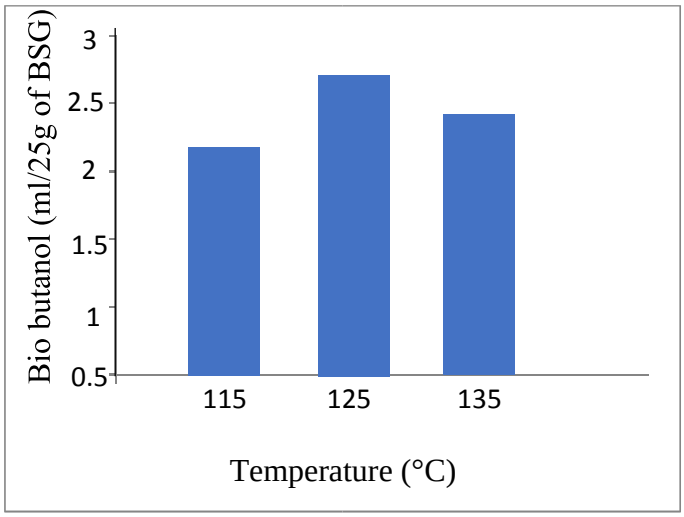

Figure 4. 4: Effect of hydrolysis temperature on bio butanol yield at a fixed reaction time (35 min) and acid concentration (1.75%)

Effect of reaction time

As indicated in Figure 4.5, when reaction time was varied from 30min to 35min at a fixed temperature (125ºC) and acid concentration (1.75%) increased bio butanol yield was observed. From the analysis of variance, reaction time was found to have a significant effect on bio butanol yield by using F-test ($p<0.05$). The optimal hydrolysis reaction time was found to be 35 min and further increase beyond 35min observed to show a decrease in yield of bio butanol due to the formation of inhibitory substances to the fermentative clostridia since the presence of inhibitory substances cause inhibition of cell division and commencement of solvent production and cell differentiation (Ji et al., 2012).

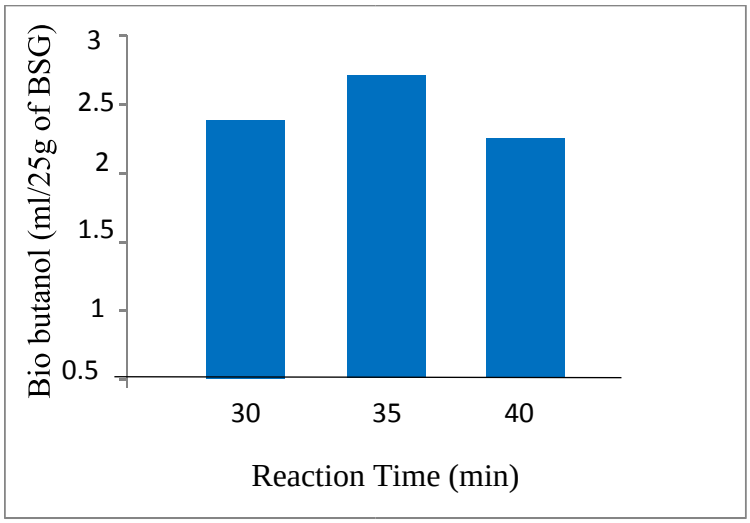

Figure 4.5: Effect of hydrolysis reaction time on bio butanol yield at a fixed temperature (125 ^{0}C) and acid concentration (1.75%)

4.3.3 Interaction effect between hydrolysis process variables

The interaction effects and optimal levels of temperature, reaction time and acid concentrations were determined by plotting the response surface curves. To study the interactive effect of factors on the biobutanol production, the response surface methodology of quadratic model was used and 3D surface was drawn. Response surface plots as a function of two factors at a time, maintaining other factor at fixed levels are more helpful in understanding both the main and the interaction effects of these factors. The interaction effects of the variables and optimal levels of each variable by maintaining the other factor constant were determined by plotting the response surface 3D graphs as shown in Figures: 4.6, 4.7 and 4.8.

Interaction effect of temperature and reaction time at constant acid concentration

When, the acid concentration was fixed at 1.75% and the response surface curves representing the interaction effects of two variables, i.e. reaction time and temperature, on the production of bio butanol was plotted in Figure 4.6. The shapes of the response surface curves showed a moderately positive interaction between these two variables on the production of bio butanol. Temperature obviously affect the response more than does reaction time. The increase in temperature from 115°C to 125 ^{0}C approximately increased bio butanol production at a fixed acid concentration.

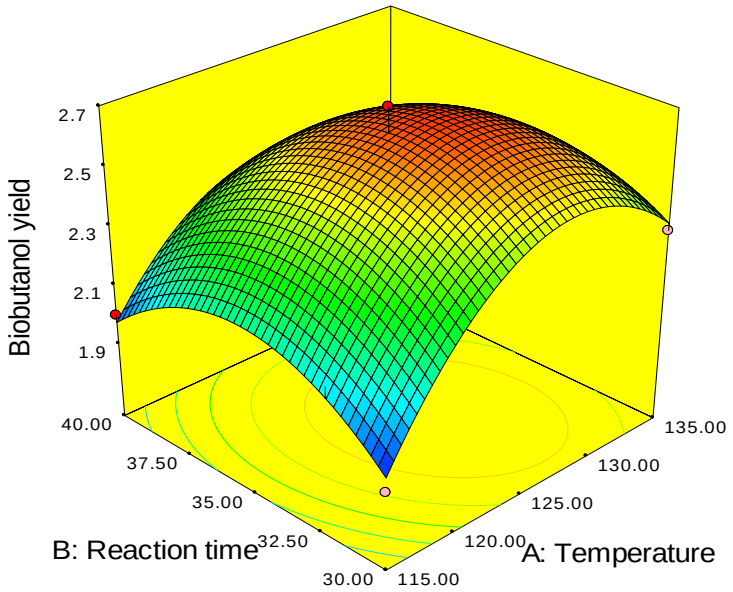

Figure 4. 6: Interaction effect of temperature and reaction time at a fixed acid concentration (1.75%)

Interaction effect of temperature and acid concentration at constant reaction time

Figure 4.7 depicted that a maximum bio butanol production was obtainable at a medium concentration of both temperature (125 ºC) and concentration of acid (1.75%). Temperature obviously affect the yield considerably when compared with acid concentration. But the shape of the response surface indicates a large effect of both variables on the yield of bio butanol production. While at higher levels of both temperature and acid concentration, the yield of bio butanol was observed declined. At lower temperature and acid concentration the cellulose might not hydrolysed to simple sugars and at higher acid concentration and temperature the cellulose might convert to other product which is not fermentable. Temperature and acid concentration were observed strong interaction effect in hydrolysis process.

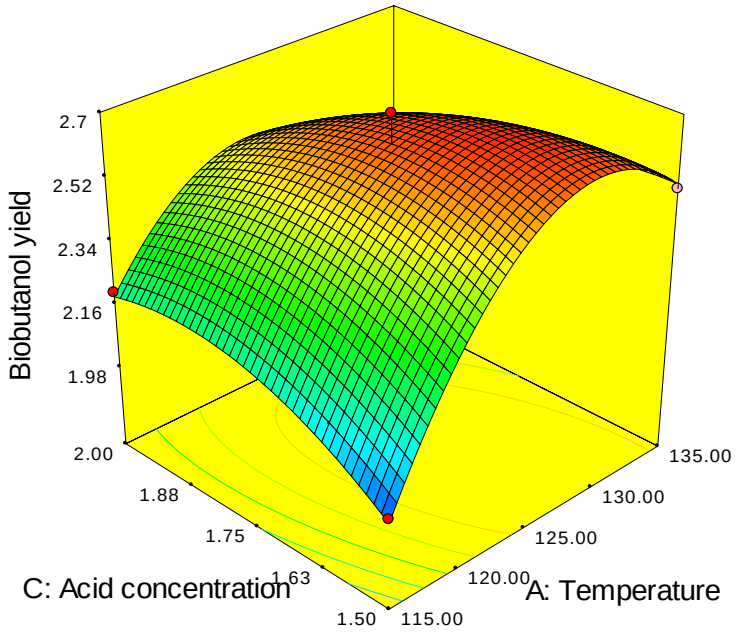

Figure 4.7: Interaction effect of temperature and acid concentration at a fixed reaction time (35 min)

Interaction effect of reaction time and acid concentration at constant temperature

Figure 4.8 depicts the 3D response surface plot of the interaction of reaction time and acid concentration on bio butanol production. Even if both variables of the process affect the yield independently the shape of the response surface plot on Figure 4.8 showed that they have no interaction on bio butanol yield. Reaction time affects the response more than does acid concentration the yield of bio butanol was observed high when both are at the center i.e. 35min and 1.75% and the yield was 2.7 ml/25g of BSG. The increase in reaction time from 30 to 35 min increases bio butanol production and further increase beyond 35min declined the yield this may probably due to the decomposition of sugar and the formation of degradation products toxic to the fermentative microorganism. This is due to the fact that lignocellulosic material is decomposed in to unwanted product or not fermentable molecules if it exposed into high temperature for long time.

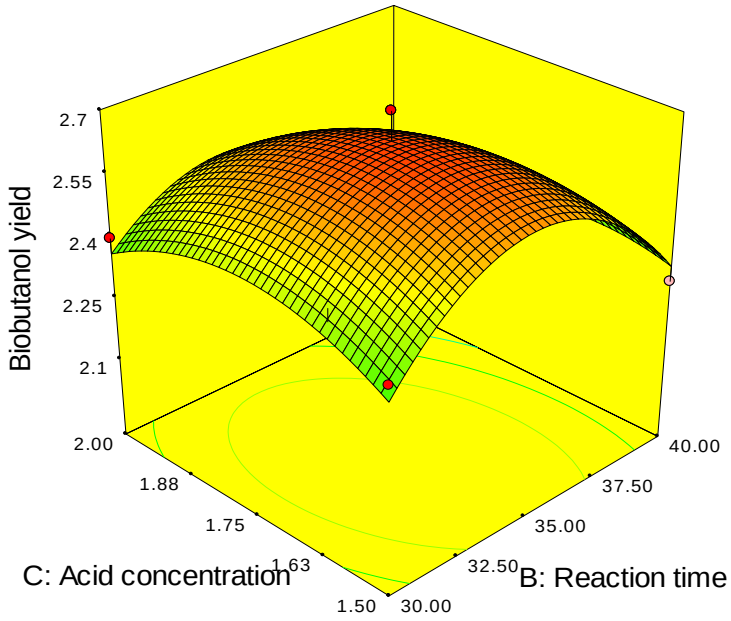

Figure 4. 8: Interaction effect of reaction time and acid concentration at a fixed temperature (125ºC)

4.4 Optimization of process factors and response variable

Optimization of hydrolysis process factors and responses was carried out by a multiple response method called desirability (D) function to optimize different combinations of process parameters. The process factors (temperature, reaction time and acid concentration) and the process responses were optimized based on the principles of optimization as shown in Table 4.7. The principles of optimization tell us maximize economic benefit by minimizing process cost, the process variables need to set as much as possible at their minimum value and the response variable bio butanol yield was set to maximum. Numerical optimization was used to optimize the hydrolysis process variables. Once the optimum hydrolysis process variables were obtained, fermentation was carried out at this optimum condition by using *Clostridium/Eubacterium*.

Table 4. 7: Summary of factors, response and goal of optimization

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	Is in range	115	135	1	1	3
Reaction time	Is in range	30	40	1	1	3
Acid concentration	Is in range	1.5	2	1	1	3
Biobutanol yield	Maximize	1.9	2.7	1	1	3

Based on desirability analysis one optimum point was selected based on the operating cost and the product quantity as well as quality. Desirability function was used to identify the optimum levels of factors. To get maximum desirable response the optimized combinations of process variables was selected i.e. 0.962 from Table 4.8.

Table 4. 8: Optimum possible solution obtained from design expert software

Number	Temperature ^{0}C	Reaction time (min)	Acid concentration (%)	Biobutanol yield (ml/25g of BSG)	Desirability	
1	127.72	34.32	1.65	2.66938	0.962	Selected

4.5 Model validation

The optimum process variables value obtained from the design expert software shown in the Table 4.8 were temperature of 127.72^{0}C, reaction time of 34.33 min, and acid concentration of 1.65 %. Under these conditions, the predicted bio butanol yield was 2.669 ml/25g of BSG. In order to confirm the validity of the RSM model results, experiment was conducted at the above-specified optimum process conditions predicted by the model and the result showed that 59.0 g/L total reducing sugar (TRS) and 2.7 ml/25g of BSG bio butanol yield was obtained.

This might be due to the supplementation of $CaCO_3$ in the fermentation. ($CaCO_3$) promotes bio butanol production by increasing the stability of membrane proteins and increase the clostridium's tolerance against the accumulation of bio butanol thereby improving the exploitation of the feedstock, provided the presence of bivalent ions (Ca^{2+}) (Raganati et al., 2012).

Table 4. 9: Model validation

Number	Temperature	Reaction time	Acid concentration	TRS (g/L)	Biobutanol (ml/25g of BSG)
Predicted	127.72	34.32	1.65	_	2.66938
Experimental	127.7	34	1.65	59.0	2.7

Therefore, the model was valid and capable of predicting the maximum bio butanol yield i.e. numerical optimization can be taken as optimal value because the predicted value was close enough with the experimental value.

Table 4. 10: Comparison of BSG hydrolysate for bio butanol production with other biomasses

Different feedstocks	TRS (g/L)	Biobutanol concentration (g/L)
BSG hydrolysate (This study)	59.0	10.93
Wheat straw hydrolysate (Qureshi, et al., 2007)	60.2	12.5
Food waste (Huang, et al.,2015)	60.1	9.45
Fruit peels extract (Raganati et al., 2016)	70.0	14

Table 4.10 depicts that same feedstocks used for bio butanol production along with the maximum product obtained from each. The result of fermentation experiment carried out under this study indicated that the BSG hydrolysate can be a potential feedstock for bio butanol production.

In a similar fermentation considering the single bacterial system as a control experiment, experiments were carried out under the same operating conditions but by applying co-culture bacterial system i.e. *Clostridium/Eubacterium* and *Bacillus subtilis*. Co-culture (symbiotic) bacterial system was used to compare the bio butanol yield when fermented by single butanol fermenting bacteria *(Clostridium/Eubacterium)* alone and the mixed culture with *Bacillus subtilis*. The symbiosis was used by varying the amount of *Bacillus subtilis* inoculation size at three levels

i.e. (1%, 3% and 5%) while keeping the other constant 10% (v/v). The operating conditions were; pH adjusted in a range of (6-6.5), temperature was kept at 37°C and incubated for 96 hours. The effect of the inoculum size of *B. subtilis* on biobutanol production (1, 3 and 5%) (*v/v*) was investigated with 10% (v/v) *Clostridium/E.* as summarized in the Table 4.11. The results exhibited that the inoculum size of 10% *Clostridium/Eubacterium* with 1% *Bacillus subtilis* maximised the production of biobutanol to 13.76g/L and 0.14 g/L.h productivity.

The present result implied that increased bio butanol production when fermentations were carried out by using the co culture system. Similarly, (Abd-Alla and Abdel-Wahab, 2012) also reported a maximum bio butanol yield and productivity of 10.78 g/L and 0.15 g/L.h respectively from (54.3 g/L equivalent glucose concentration) of spoilage date fruits by co culture system. This may probably due to the reason that *Bacillus subtilis* played an important role in the fermentation likely functioning as an oxygen consumer in the co culture system to create an anaerobic environment for *Clostridium/Eubacterium*. And (Thi et al., 2010) founded that when *B. subtilis* was co-cultured with *C. butylicum* enhanced bio butanol production of 5.4 and 6.5 folds from soluble starch and cassava starch, respectively, compared to those of the pure culture of Clostridium itself.

Table 4.11: Biobutanol yield for the optimum BSG hydrolysate by co culture bacterial system

Inoculation ratio		Biobutanol concentration (g/L)	Fermentation time (hr.)	Biobutanol yield/ (59.0 g/L TRS)	Biobutanol productivity (g/L.h)
Clostridium/ Eubacterium (%)	*Bacillus subtilis (%)*				
10	0	10.93	96	0.185	0.11
	1	13.76	96	0.233	0.14
	3	11.34	96	0.192	0.12
	5	9.31	96	0.158	0.10

4.6 Characterization of bio butanol by FTIR

Alcohols produce characteristic infrared bands due to O–H stretching, C–O stretching and C-H stretching bands, which are both sensitive to hydrogen bonding. For alcohols, the broad O–H stretching band is centred at 3600 cm−1. C–O stretching in alcohols produce a strong band in the 1300–1000 cm−1 region. The bands at around 2880 and 2930 cm-1 were assigned as the symmetric stretching modes of the $–CH_2$ and$–CH_3$ groups, respectively (Coates, 2000). This ascertains that the product obtained from Brewery's spent grain hydrolysate was bio butanol.

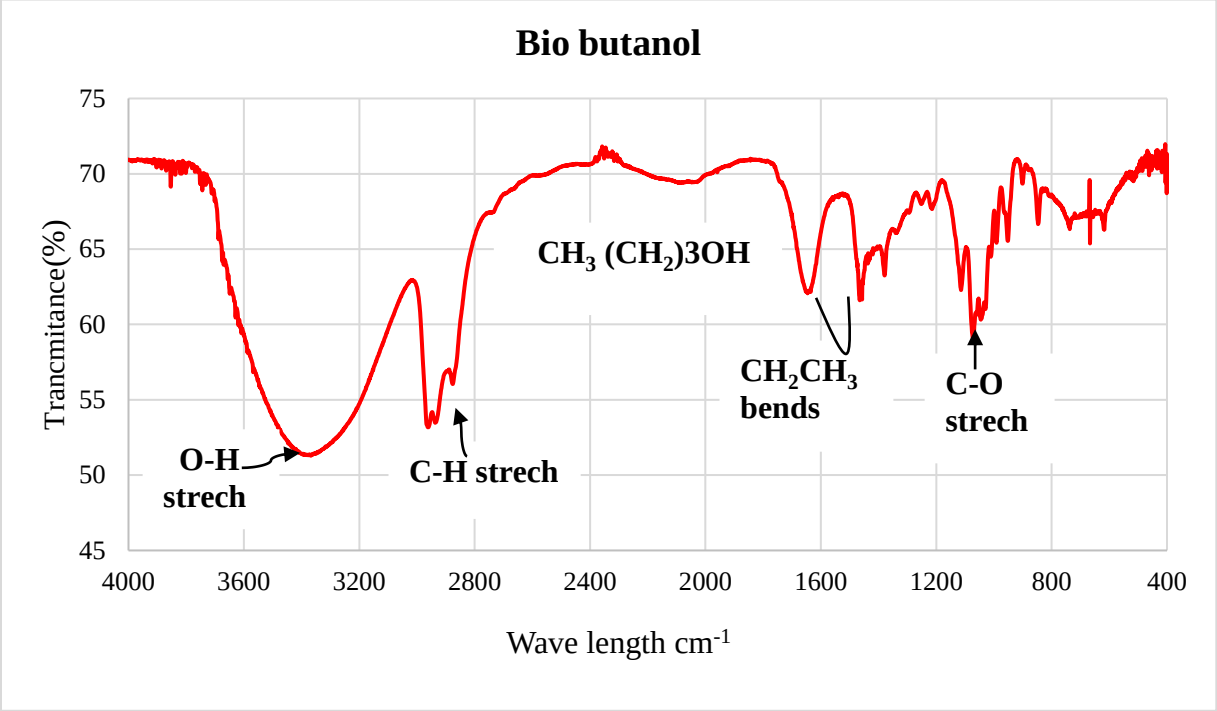

Figure 4. 9: Fourier transform Infrared spectra of bio butanol from brewery's spent grain

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The rise in price of world fuel, the growing demand for energy, and concerns about emission of greenhouse gases as a result of combustion of fossil fuels are the key factors driving renewed interest in renewable energy sources particularly biofuels. The reason that butanol has attracted renewed interest is that it contains a high energy density, less explosive and corrosive than ethanol and also less susceptible to separation in the presence of water. Since the feedstock of the bio butanol fermentation is a key issue for the economic success. Alterative feedstock search is the main problem for bio production of butanol. BSG is the most abundant by-product generated from the beer-brewing process, representing approximately 85% of the total by-products obtained.

This study examines the possibility of BSG for bio butanol production. The proximate and chemical compositions analysis was carried out using ASTM procedures. The proximate ash (4.2wt. %) and moisture (64.65wt. %) contents were obtained. The chemical compositions cellulose (17.87wt %), hemicellulose (28.86wt. %), lignin (15.79wt. %) and extractives (33.28wt. %) were obtained. The conversion of brewery's spent grain (BSG) to bio butanol was carried out with dilute acid pretreatment, dilute acid hydrolysis, fermentation and distillation process steps.

The effect of the hydrolysis process variable (temperature, reaction time and acid concentration) in the yield of bio butanol were investigated and optimized using response surface methodology. Based on analysis of variance (ANOVA) hydrolysis temperature, reaction time, acid concentration, interaction between temperature and reaction time, interaction between temperature and acid concentration contributed a significant effect on the yield of bio butanol. As the result of RSM optimization, the best yield of total reducing sugar (TRS) and bio butanol were found at Since, 127.42 ^{0}C hydrolysis temperature, 34.32 min reaction time and 1.65 % w/w acid concentration. Under these conditions, 10.93 g/L biobutanol yield and 0.11 g/L.h of productivity were observed. This is in a good agreement with the predicted value of 10.76 g/L and 0.11g/L.h respectively. In a similar fermentation considering the above as control experiment, experiments were carried out under the same operating condition but by applying co-culture bacterial system i.e. *Clostridium/Eubacterium* and *Bacillus subtilis*. The symbiosis was used by varying the amount of *Bacillus subtilis* inoculation size at three levels i.e. (1,3 and 5% v/v) while keeping Clostridium/E. constant ,10% (v/v).

The results exhibited that the inoculum size of 10% *Clostridium/Eubacterium* with 1% *Bacillus subtilis* maximized the production of bio butanol to 13.76g/L and 0.14 g/L.h productivity. Generally based on this study, it is evident that the chosen method of optimization was efficient, and reliable. From this result, it can be concluded that brewery's spent grain (BSG) has the potential to serve as a low-cost feedstock for the production of bio butanol.

5.2 Recommendations

This study would like to suggest the following recommendations:

It is recommend that in this study dilute acid hydrolysis variables are optimised; future studies should include optimisation of pretreatment process, optimisation of fermentation process and optimisation of methods for cost-effective recovery of fermentation products to obtain maximum yield of bio butanol from brewery's spent grain BSG.

In this study the co culture bacterial system for enhanced bio butanol yield was performed only by varying the inoculation size of *Bacillus subtilis* while keeping the other fermentation parameters constant (temperature, pH, substrate concentration etc.) but creating suitable condition for both bacteria is very important concept not covered in this research which needs further investigation.

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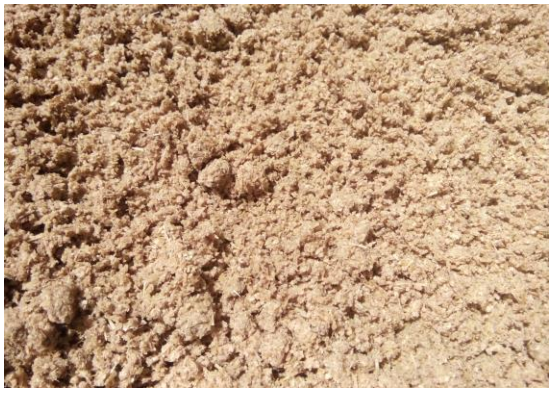
APPENDIX

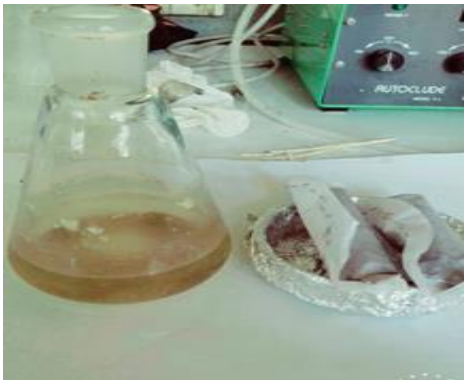
Appendix A: Laboratory equipments and sample photos

Supporting pictures during the study



A: Raw BSG sample

B: sun dried BSG sample

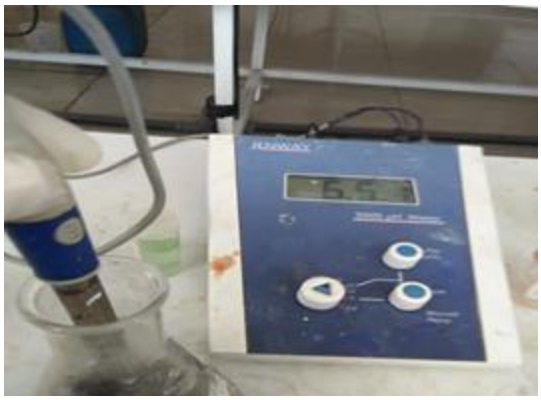


A: Determination of extractives

B: Determination of lignin

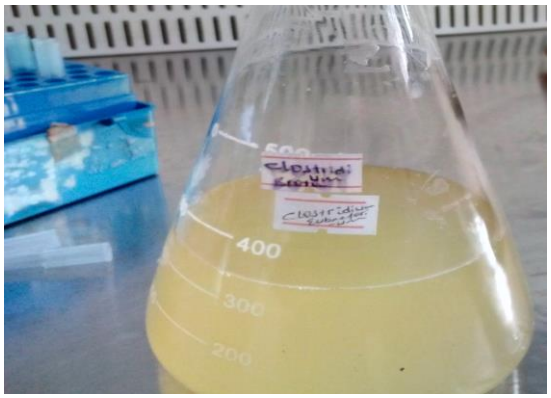
A: Autoclave

B: Vacuum filtration of the hydrolysate

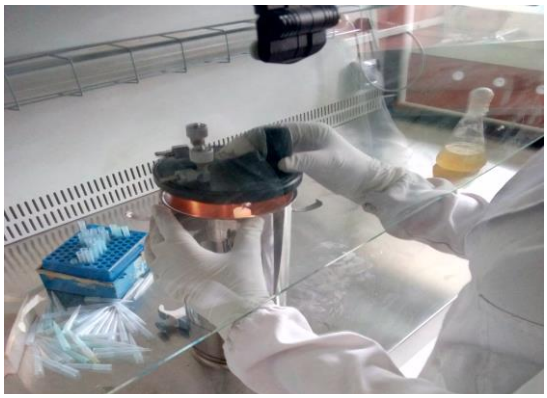


A: pH adjustment

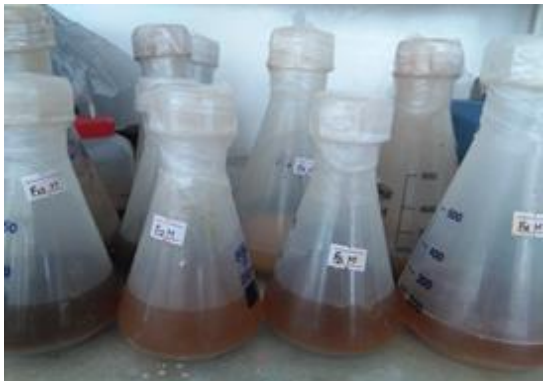
B: Nutrient broth prepared for growth



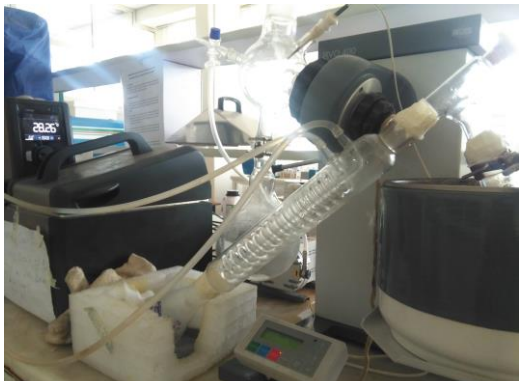
A: Actively growing *Clostridium/E.*



B: Anaerobic jar



A: Fermented broth



B: Separation of the product by distillation