ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL AND BIO ENGINEERING

# LACTIC ACID PRODUCTION FROM BREWER'S SPENT GRAIN USING *LACTOBACILLUS PLANTARUM*

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**Yohannes Assefa**

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**School of Chemical and Bio Engineering**

This is to certify that the thesis prepared by Yohannes Assefa, entitled: *Lactic Acid Production from Brewer's Spent Grain Using Lactobacillus plantarum* and submitted in partial fulfilment of the requirement for the degree of Master of Science (Chemical and Bio Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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| Dr.S.Anuradha Jabasingh (Assoc. Professor) | ________ | ________ |
| Advisor | Signature | Date |
| **Dr.Solomon K.** | ________ | ________ |
| Internal Examiner | Signature | Date |
| **Eng. Gizachew** | ________ | ________ |
| External Examiner | Signature | Date |
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# DECLARATION

I declare that this thesis entitled "*Lactic Acid Production from Brewer's Spent Grain Using Lactobacillus plantarum* " has not been submitted in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. The work was under the guidance of Dr.S.Anuradha Jabasingh (Associate Professor) instructor in Addis Ababa University, School of Chemical and Bio Engineering.

Name: Yohannes Assefa

Signature: ___________________

Date of Submission: June, 2018

# ABSTRACT

Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries and has received increased attention for use as a monomer for the production of biodegradable poly (lactic acid). It can be produced by either biotechnological fermentation or chemical synthesis, but the former route has received considerable interest recently, due to environmental concerns and the limited nature of petrochemical feedstocks. The objective of this study was to produce lactic acid from Brewery Spent Grain by using *lactobacillus plantarum*. The production process was carried out in four main stages, such as pretreatment, hydrolysis, fermentation and recovery of lactic acid. Brewery spent grain was dried using oven at 80ºC temperature for 24 hr. Then for the hydrolysis, Box Behnken Design (BBD) was applied to investigate the effect of temperature (115-130ºC), reaction time (25-35min) and acid concentration (1.5-2M) using Design expert® 7 software. RSM was applied to investigate the interaction effect of the hydrolysis process variables and to find the optimum yield of lactic acid from BSG. After hydrolysis process, reducing sugar content of the hydrolyzate was quantified using quantitative benedict reagent solution. Fermentation of the hydrolyzate was performed using 150mL, *Lactobacillus plantarum* at 35ºC temperature, pH 5.0 – 5.5 and 200 rpm for 72hrs fermentation time for all samples. After fermentation recovery of lactic acid and purification process was carried out by centrifuging all samples at 5000 rpm for 5 min followed by filtration through 0.2µm paper filter. The concentration of lactic acid was determined by titration of the sample using 4M of sodium hydroxide. Significance of the process variables were analyzed using analysis of variance (ANOVA) and second order polynomial function was fitted to the experimental results. Thus, the influence of all experimental variables, factors and interaction effects on the response was investigated. Hydrolysis temperature, time, sulfuric acid concentration and interaction between reaction temperature and sulfuric acid concentration have significant effect on the yield of lactic acid. RSM optimization yielded the best yield of total reducing sugar and the maximum yield of lactic acid were obtained at 129.75ºC, 32.39 min and 1.89M. Under these condition 53.07% and 26.71% per 150ml of hydrolysate of total reducing sugar and lactic acid was obtained respectively.

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# ACRONYMS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| AOAC | Association of Official Analytical Chemists |
| ASTM | American Society for Testing and Material |
| ATP | Adenosine triphosphate |
| BBD | Box -Bhenken Design |
| BSG | Brewery spent grain |
| EBI | Ethiopian Biodiversity Institute |
| FTIR | Fourier Transform Infrared Spectroscopy |
| LA | Lactic Acid |
| LAB | Lactic Acid Bacteria |
| M | Molar |
| NADH | Nicotinamide adenine dinucleotide |
| PLA | Poly Lactic Acid |
| RSM | Response Surface Methodology |
| TSG | Treated Spent Grain |

# 1. INTRODUCTION

## 1.1 Background

Brewer`s spent grains (BSG) is the solid residue remaining after mashing and lautering, consisting primarily of grain husks and other residual compounds not converted to fermentable sugars by the mashing process. Brewer's spent grain(BSG) is the most abundant brewing by-product, corresponding to about 85% of total by-products generated(Aliyu and Bala, 2011). Traditionally this material has been landfilled or sold as animal feed (mostly for ruminants that can cope with the high fibre content). However, BSG is difficult to store because of the rapid deterioration owing to microbial activity and, therefore, it is not always suitable for food applications. In addition, the environmental implications of increased methane emission owing to this hard to digest material are gaining attention(Vanreppelen, et al, 2016).

Lactic acid, commonly used in food, chemical and pharmaceutical industries, has recently received much attention for the production of biodegradable plastics. Poly lactic acid (PLA) is biodegradable aliphatic polyester produced from renewable sources, which, due to its excellent physical and chemical properties and environment compatibility, considered the best candidate to replace on the market petroleum-based plastics. It has a wide range of applications ranging from medical devices, such as suture treads and scaffolds, to commodity products like bottles and films for food packaging(Aliyu & Bala, 2011).

The monomer, lactic acid (LA), is the smallest optical active organic compound present in nature. Due to the presence of a chiral carbon, LA exists in the two optical isomers, L (+) and D (-) (Figure 1.1). It was first isolated in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. From sour milk and first commercially produced around 1880. It can be obtained by petrochemical synthesis or fermentation process. Nowadays, due to the improvements in bacterial fermentation of D- glucose, optically preferentially pure lactic acid is produced through the batch fermentation process of corn and other carbohydrates, which involve key steps as cell mass and protein removal and, LA recovery, purification and concentration ( Wee, et al, 2006). In the United States, LA is largely used in food applications such as acidic flavoring agent or buffering agent(Andres et al., 2013). Beside the above application now a day it is highly applicable in poly lactic acid formation which is in a very high demand of making biodegradable plastic(Length, 2013). Lactic acid can be manufactured by either chemical synthesis or fermentation. The commercial process for chemical synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures(Vidra et al., 2017). The crude lactonitrile is recovered, and purified by distillation. It is then hydrolyzed to lactic acid, either by concentrated HCl or by $H_2SO_4$ to produce the corresponding ammonium salt and lactic acid. On the other hand, an optically pure L(+)- or D(–)-lactic acid can be obtained by microbial fermentation when the appropriate microorganism is selected.

Biotechnological processes for the production of lactic acid usually include lactic acid fermentation and product recovery and/or purification. There have been numerous investigations on the development of biotechnological processes for lactic acid production, with the ultimate objectives to enable the process to be more efficient and economical(Abdel-rahman et al., 2013). Biological production of lactic acid has attracted a great deal of interest due to its potential uses in many industries, and the production of polylactide polymer. Although the biological production of lactic acid has some advantages over chemical synthesis, it still requires the cheaper substrates for industrial feasibility(Bayitse and Lg, 2015).

Thus lactic acid production become critical to our country since Ethiopia is following and will follow green economy strategies. This thesis will focused on production of lactic acid for different applications.

## 1.2 Problem Statement

“Wastes are… resources out of place” This memorable quote rings especially true in the brewing industry. Many other industries have realized that their waste streams can be converted into profitable sources of revenue. For instance, the poultry industry currently sells poultry litter, the animal excreta mixed with a bedding material, to farmers as a nutrient-rich fertilizer. Similarly, dairy farmers use cattle excreta as a fertilizer or as a feedstock for anaerobic digestion. Converting a waste into a resource requires creativity as well as a comprehensive understanding of the by-product streams that the industry is generating. Now days, the numbers of brewery industries are increasing in Ethiopia and they generate a high amount of barley spent grain. This by-product is mainly used as a low value cattle feed or simply deposited as a waste into land fill. Beside this, Barley spent grain remains from animal feed also creates bad odor on the environment which in turn affects health of people who live around the area, so that Conversion of such waste into lactic acid helps to reduce environmental pollution and energy problem of developing country like Ethiopia. It is also used as a starting material for manufacture of biodegradable poly-lactate polymers. Due to its numerous applications, lactic acid production has a global market in excess of 100,000 to 150,000 tons per year, and an annual 15% growth rate of this market is predicted due to the possibility of making biodegradable plastics. With this increased demand comes a greater interest in finding alternative methods to produce lactic acid at the lowest cost possible.

## 1.3 Study Objectives

### 1.3.1General objective

The general objective of this study is lactic acid production from brewery spent grain using *lactobacillus plantarum*.

### 1.3.2 Specific objectives

The specific objectives are:

- Characterization of brewery spent gain, including proximate analysis (moisture content, volatile matter content, fixed carbon content and ash content), and chemical composition analysis (extractives, lignin, cellulose and hemicellulose).
- To synthesize Lactic acid from brewer's spent grain via fermentation using *Lactobacillus Plantarum*.
- Evaluate the effect of process variables (reaction time, reaction temperature and sulfuric acid concentration) on the yield of lactic acid using response surface methodology.
- Determine the amount of lactic acid produced and determine the model that better fits the process.

## 1.4 Significance of the study

Low cost medium for lactic acid fermentation have been developed for economic lactic acid fermentation. Agricultural products such as corn, potato, barley and wheat containing substantial amount of starch have been preferred as raw materials for lactic acid production. However, it is expected that brewery spent grain which can be supplied constantly at lower costs and riches in carbohydrate could be used as a suitable renewable resource in substitution of other agricultural products. Therefore, it is very attractive to develop a more economical method for lactic acid production using a cheaper carbon source. This research work focuses on the development of an efficient and economical process for the lactic acid production.

- Nowadays, there is great political and social pressure to reduce the pollution arising from industrial activities. Almost all developed and underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Consequently, most large companies no longer consider residues as waste, but as a raw material for other processes.

- The major motive for the production of lactic acid from brewer's spent grain is to ensure the demand of lactic acid for industries. The outcome of this study benefits industrial sectors as the research provides production method to treat BSG waste as a valuable product before discharging in to the environment.

## 1.5 Scope of the Study

The thesis work generally covers brewery spent grain preparation, characterization, acid hydrolysis of BSG, sugar content determination, media preparation for lactic acid bacteria, microorganism and inoculum cultivation, fermentation of the prepared hydrolysate and microorganism and recovery and purification of lactic acid and all of the characterization procedure are done by standard procedures and test methods.

# 2. LITERATURE REVIEW

Beer is the fifth most consumed beverage in the world next to tea, carbonates, milk and coffee and it continues to be a popular drink with an average consumption of 23 liters/person per year by population aged above 15. The brewing industry has an ancient tradition and is still a dynamic sector open to new developments in technology and scientific progress(Roberto, 2005).The brewing process uses malted barley and/or cereals, unmalted grains and/or sugar/corn syrups (adjuncts), hops, water, and yeast to produce beer. Most brewers use malted barley as their principal raw material. From this process, various byproducts are generated. The three most common byproducts are spent grains, spent hops and surplus yeast, which are generated from the main raw materials. These three brewery byproducts are available in large quantities throughout the year, but their use has still been limited being basically sold to local dairy farmers to be used as cattle feed, or simply as a land fill(Mussatto et al., 2006).

Spent grains are resulted from mashing process and obtained after rendering the malt and cereal grain content soluble in water. After extraction, the spent grains and wort (water with extracted matter) are called mash and need to be separated. The separated, brewers' spent grain (BSG) amount consists about 85% of the total byproducts(Oladeji, 2016). Brewers' spent grain (BSG) is a readily available, high volume low cost byproduct of brewing and is a potentially valuable resource for industrial exploitation. Although BSG is the main byproduct of the brewing process, it has received little attention as a marketable commodity, and its disposal is often an environmental problem(Anamaria et al., 2017).

However, for several years it has been utilized and sold as livestock feed and, being basically sold to local dairy farmers, or simply as a land fill(Géczi et al., 2015). Several attempts have been made in utilizing BSG in animal feeds, production of value added compounds (xylitol, lactic acid, among others), micro-organisms cultivation, or simply as raw material for extraction of compounds such as sugars, proteins, acids and antioxidants(Aliyu & Bala, 2011).

## 2.1 Composition of brewery spent grains (BSG)

Brewery spent grain is considered as a lignocellulosic material rich in protein and fiber, which account for around 20% and 70% of its composition, respectively. Microscopic examination of brewery spent grain shows the presence of numerous fibrous tissues from the surface layers of the original barley grain. The main components of these fibrous tissues are arabinoxylan, lignin (a polyphenolic macromolecule), and cellulose (a linear homo polymer of glucose units) (1Madubuike, P.C. and 2 Okolo, 2016).

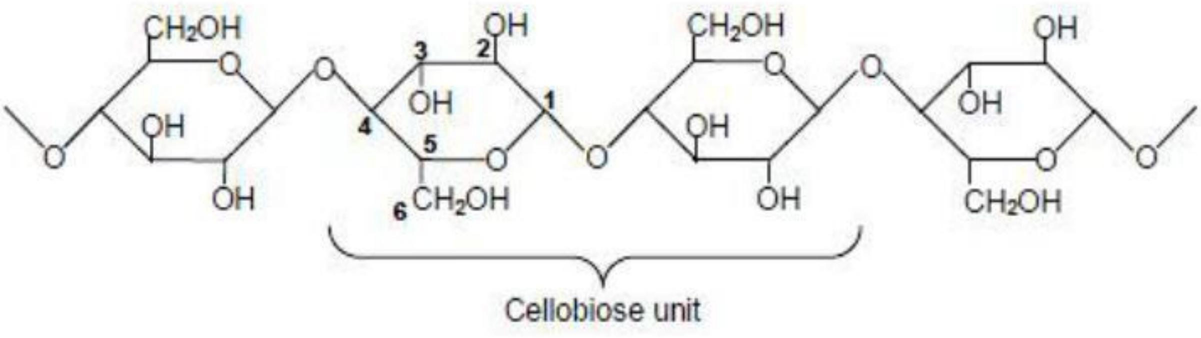


Figure 2.1: structure of cellulose

Chemical composition of brewery spent grain varies with types of barley used, harvesting time, characteristics of hops and adjuncts added, and brewery technology(Xi et al., 2014). According to Minerals, vitamins, and amino acids are also found in brewery spent grain. The mineral elements include calcium, cobalt, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium, and sulfur, all in concentrations lower than 0.5% each (Ogunjobi et al., 2011). The vitamins include (in ppm): biotin (0.1), choline (1800), folic acid (0.2), niacin (44), pantothenic acid (8.5), riboflavin (1.5), thiamine (0.7), and pyridoxine (0.7); protein bound amino acids include leucine, valine, alanine, serine, glycine, glutamic acid, and aspartic acid in the largest amounts, and tyrosine, proline, threonine, arginine, and lysine in smaller amounts. Cysteine, histidine, isoleucine, methionine, phenylalanine, and tryptophan also present in minor quantity(A. Fărcaş et al., 2014).

Brewery spent grain basically consists of the husk–pericarp–seed coat layers that covered the original barley grain. Depending on the evenness of malting more or less starchy endosperm and walls of empty aleurone cells may also remain. The starch content will be negligible, and some residues of hops introduced during mashing will be present depending on the brewing regime used. Thus, the major components of brewery spent grain will be the walls of the husk–pericarp– seed coat, which are rich in cellulose and non-cellulosic polysaccharides and lignin, and may contain some protein and lipid(Mussatto et al., 2006).

Figure 2-1: Fresh (left) and dried (right) BSG

Chemically, BSG is rich in polysaccharides, protein and lignin. Residual starch can contribute up to 13% of the dry weight and brewery spent grain from lager malts has higher protein content than that from ale. In general, at the point of production, brewery spent grain is a relatively uniform chemical feedstock available for industrial upgrading (Pires et al., 2012). Different components of brewer spent grain (BSG) is shown in Table 2.1 cited from different researchers.

Figure 2-2: Scanning electron microscopy of BSG particles. (a) Magnification 100 fold; (b) magnification 300 fold(Mussatto et al., 2006)

Table 2-1: Chemical composition of brewers' spent grain (BSG)

| Components | Kanauchi et al. 2001 | Mussatto and Roberto,2005 | Santos et al., 2006 | Huige, 1995 | Itodo et al., 2014 |
|---|---|---|---|---|---|
| Cellulose | 25.4 | 16.8 | - | - | 28.4 |
| Arabinoxyl | 21.8 | 28.4 | - | - | - |
| Lignin | 11.9 | 27.8 | - | - | 27.8 |
| Protein | 24 | 15.2 | 24.2 | 14 – 15 | 15.2 |
| Fat | - | - | 3.9 | - | - |
| Phenol | - | - | 18.7 | - | - |
| Lipid | 10.6 | - | - | - | - |
| Ash | 2.4 | 4.6 | 3.4 | - | 4.6 |

## 2.2 General uses of spent grain

The main current application of BSG is as cattle feed, where it can be utilized directly in wet form (as separated from the mashtun) or as dry material(Fărcaş et al., 2012). Animal growths in terms of body weight gain were reported by due to supplementation of brewery spent grain in their diet. The addition brewery spent grain in the cow's diet also increases milk yield, and milk TS concentration.

Since BSG is derived from materials utilized for humans, it can be incorporated into so many human diets, such as breads and snacks; especially where there is need to boost the fiber contents(Aliyu & Bala, 2011). According to the study of Pires et a.l., 2012 it revealed that wheat flour incorporating BSG had increased water absorption and improves its texture and nutritional value. Also, incorporation of BSG into monogastric diets is beneficial for intestinal digestion, alleviating both constipation and Diarrhoea. Such effects were attributed to the content of glutamine-rich protein, and to the high content of non-cellulosic polysaccharides and smaller a mounts of β-glucans(Madubuike and Okolo, 2016).

In energy perspective several alternatives have been investigated for BSG use in energy production, such as thermochemical conversion (pyrolysis, combustion)(Velebil, et al, 2016), biogas production and ethanol production(Liguori et al., 2015). Due to the energy crisis today, BSG has preferred to produce alternative energy sources because of its large availability, chemical composition and low cost (Mussatto, 2014). Generally, the following benefits of brewery spent grain are summarized in the areas of food, energy production and in chemical and biotechnological processes(Aliyu & Bala, 2011; Mussatto et al., 2006).

- Food ingredient
    - Animal nutrition
    - Human nutrition
- Energy production (bioethanol, biogas, thermochemical conversion)
- Charcoal production
- As a brick component
- Economic benefits
- Environmental benefits
- BSG use in chemical processes
    - Extraction of valuable compounds by chemical processes
    - Raw material for pulp and paper production
    - Adsorbent material
- Biotechnological processes
    - Extraction of valuable compounds by enzymatic processes
    - Substrate for cultivation of microorganisms
    - Substrate for enzyme production
    - Additive or carrier in brewing
    - Source of added-value products

### 2.2.1 Availability of Barley 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. In Ethiopia around 263,736 kg barley spent grain is generated daily. The spent grain from Ethiopian breweries, now a days is only used for animal feed.

Table 2.2: Capacity of spent grain generated from Ethiopian brewery industries,Source (Amare Fisha, 2016)

| 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** |

## 2.3 Lactic acid

It was the first organic acid to be commercially produced by fermentation, with production beginning in 1881. It is present in many foods both naturally or as a product of microbial fermentation. It is also a principal metabolic intermediate in most living organisms from anaerobic prokaryotes to humans. In 1839, Fremy performed lactic acid fermentation of several carbohydrates, such as sugar, milk sugar, mannite, starch and dextrin. A discovery that was then confirmed by Gay- Lussac, in 1840, Louradour prepared lactic acid by fermentation of whey and converted it into iron lactate by dissolution of metallic iron in it. Other fermentation experiments were performed by many different scientists to produce lactic acid from cane sugar beyond 1847(Pagana, et al, 2014). Blondeau discovered lactic acid as a fermentation product in 1847. Originally, the lactic acid of fermentation and that found in muscle tissue were regarded as identical. Liebig, who in 1947 re-examined meat extract, suspected that the two acids might not be identical. He asked Engelhardt to carry out an examination of the salts of the two acids. Engelhardt confirmed Liebig's thought that the contents of water of crystallization and the solubility of the salts of the two lactic acids differed and thus the acids were different(Kotzamanidis, et al, 2002). Welceneus, in 1873, proved they have the same structure, but different physical properties. It was also investigated by Pasteur as one of this first microbiological yeast cultures of distilleries, it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures when Lister isolated *Streptococcus lactis*. During this same period, Delbruck was endeavoring to find out the most favorable temperature for lactic acid fermentation in distilleries. He concluded that relatively high temperature favored high yields of lactic acid(Santos et al, 2013).

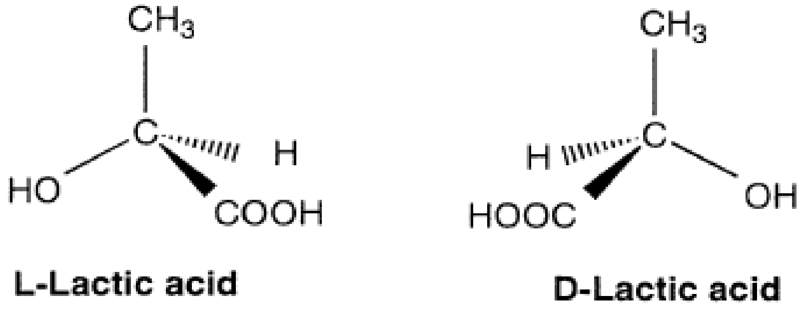


Figure 2.3: lactic acid isomers

In the USA until 1963, lactic acid was produced solely by fermentation, when Sterling Chemicals, Inc., started producing lactic acid by a chemical process using petroleum by products, supplying nearly half the American demand for lactic acid. In 1996, Sterling abandoned the lactic acid business, leaving lactic acid production again exclusively to fermentation companies. In the early 1990s, Ecological Chemical Products (EcoChem), a joint venture of E.I du Pont Nemours & Co., and Con Agra produced only 1 to 2 million pounds of lactic acid by fermentation of whey permeate. In 1993, the current leader in basic chemical fermentation, Archer Daniels Midland (ADM), entered the lactic acid business and produced, in a facility designed for 40 million pound per year, 10 million pounds of lactic acid from corn sugar. With a potential market for lactic acid in polymer production, the demand for lactic acid may reach as high as 2000 million and above per year.

### 2.3.1 Physical and Chemical Properties

Pure anhydrous lactic acid is a white crystalline solid with a low melting point of 53°Cand appears generally in form of more or less concentrated aqueous solution, as syrupy liquid. It also can be a colorless to yellow liquid after melting or it dissolved in water. Lactic acid is considered as a stable substance and it is a combustible substance as well. Lactic acid is compatible with strong oxidizing agents. Normally lactic acid is observed as a clear to slightly yellowish liquid, typically supplied to formulators in an 88 to 92% concentration. Lactic acid normally appears in diluted or concentrated aqueous solution(Onofre et al, 2017).

Lactic acid is the simplest hydroxy acid having an asymmetric carbon atom and it therefore exists in a racemic form and in two optically active form with opposite rotations of polarized light L(+) and D(-)lactic acid as shown in Figure 1.1. The optically active form of lactic acid is simply an equimolecular mixture of both and may be denoted as DL-lactic acid or racemic mixture. The optical composition does not affect many of the physical properties with important exception of the melting point of the crystalline acid. Table 2.3 shows a summary of lactic acid physical and thermodynamic properties(Pagana et al., 2014).

Table 2-3: Characteristics of Lactic Acid property

| **Properties** | **Characteristics** |
|---|---|
| Optical activity | Exists as L(+), D(-) and recemic mixture |
| Crystallization | Forms crystals when highly pure None or yellowish |
| Color | White crystalline solid |
| Odor | None |
| Solubility | Soluble in all proportions with water Insoluble in n chloroform, carbon disulphide |
| Miscibility | Miscible with water, alcohol, glycerol and furfural |
| Hygroscopicity | Hygroscopic |
| Volatility | Low |
| Self-esterification | In solutions of > 20% |
| Reactivity | Versatile; e.g. as organic acid or alcohol |

Table 2-4: Physiochemical properties of lactic acid

| **Property** | **Value** | **Isomer** |
|---|---|---|
| Empirical formula | $C_3H_6O_3$ | D, L, DL |
| Chemical name | 2-hydroxypropanoic acid | D, L, DL |

| | | |
|---|---|---|
| Dissociation constant ($K_a$ at 25ºC) | $1.37x10^{-4}$ | D, L, DL |
| Molecular weight | 90.08 g/mol | D, L, DL |
| Boiling point (at 0.5mmHg),ºC (at 14mmHg),ºC | 82.0 ºC<br>122.0 ºC | DL<br>DL |
| Melting Point | 52.8ºC<br>53.0 ºC<br>16.8 ºC | D<br>L<br>DL |

Table 2-5: Thermodynamic characteristics of lactic acid

| Items | Characteristics |
|---|---|
| Dissociation constant (Ka) | 0.000 137 (at 25ºC) |
| Heat of dissociation(ΔH) | -63 cal/mol (at 25 ºC) |
| Free energy of dissociation (ΔF) | 5000 cal/mol |
| Heat of solution (ΔH) | 1868 cal/mol<br>(for crystalline L( +) lactic acid at 25 ºC) |
| Heat of dilution (ΔH) | -1000 cal/mol<br>(for dilution with a large volume of water) |
| Heat of fusion (ΔH) | 2710 cal/mol (for racemic lactic acid)<br>4030 cal/mol (for L(+) lactic acid) |
| Entropy of solution (ΔS) | 6.2 cal/mol/ ºC |
| Entropy of dilution (ΔS) | -3.6 cal/mol/ ºC |
| Entropy of fusion (ΔS) | 9.4 cal/mol/ ºC (for racemic lactic acid)<br>12.2 cal/mol/ ºC (for L(+) lactic acid) |
| Heat of combustion ($\Delta H_{co}$) | -321220 cal/mol (for crystallineL(+) lactic acid at 25 ºC)<br>-325600 cal/mol (for liquid racemic lactic acid at 25 ºC) |
| Heat of formation ($\Delta H_{fo}$) | -165 890 cal/mol (for crystalline L( +) lactic acid at 25 ºC)<br>-163000 cal/mol (for liquid lactic acid)<br>-164020 cal/mol l (for lactic acid in dilute solution)<br>-164 080 cal/mol (for dissociated and diluted lactic acid) |
| Heat capacity($C_p$) | 0.338 cal/g/ ºC (for crystalline lactic acid at 25 ºC)<br>0.559 cal/g/ ºC (for liquid lactic acid at 25 ºC) |

| | |
|---|---|
| Absolute entropy ($S_o$) | 34.0 cal/mol/ ºC (for crystalline L(+) lactic acid at 25 ºC)<br>45.9 cal/mol/ ºC (for liquid racemic lactic acid at 25 ºC) |
| Entropy of formation ($\Delta S_{fo}$) | -137.2 cal/mol/ ºC (for crystalline lactic acid at 25 ºC)<br>-125.3 cal/mol ºC (for liquid lactic acid at 25 ºC) |
| Free energy of formation ($\Delta F_{fo}$) | -124980 cal/mol (for crystalline L(+) lactic acid at 25 ºC)<br>-126500 cal/mol (for liquid racemic lactic acid at 25 ºC) |

Holten (1971) reported that the solubility properties of the isomers are also different. The D (-) isomer is soluble in water, alcohol and acetone, ethyl ether and glycerol and is practically insoluble in chloroform. The racemic mixture is soluble in water, alcohol and furfural. It is practically insoluble in chloroform and acetic acid.

Densities of aqueous solution of various lactic acid concentrations has shown that the density increased almost linearly with concentration and decreased almost linearly with temperature. The viscosity of lactic acid solution increased rapidly with the concentration and decreased rapidly with increasing temperature(Onofre et al., 2017).

## 2.4 Microorganisms for the production of lactic acid

Microorganisms that can produce lactic acid can be divided into two groups: bacteria and fungi. The microorganisms selected for recent investigations of the biotechnological production of lactic acid are listed in Table 2.5. Although most investigations of lactic acid production were carried out with lactic acid bacteria (LAB), filamentous fungi, such as *Rhizopus*, utilize glucose aerobically to produce lactic acid. *Rhizopus* species such as *R. oryzae* and *R. arrhizus* have amylolytic enzyme activity, which enables them to convert starch directly to L (+)-lactic acid. Fungal fermentation has some advantages in that *R. oryzae* requires only a simple medium and produces L (+)-lactic acid, but it also requires vigorous aeration because *R. oryzae* is an obligate aerobe. In fungal fermentation, the low production rate, below 3 g/(L·h), is probably due to the low reaction rate caused by mass transfer limitation. The lower product yield from fungal fermentation is attributed partially to the formation of by-products, such as fumaric acid and ethanol.

Table 2-6: Microorganisms used for recent investigations of the biotechnological production of lactic acid

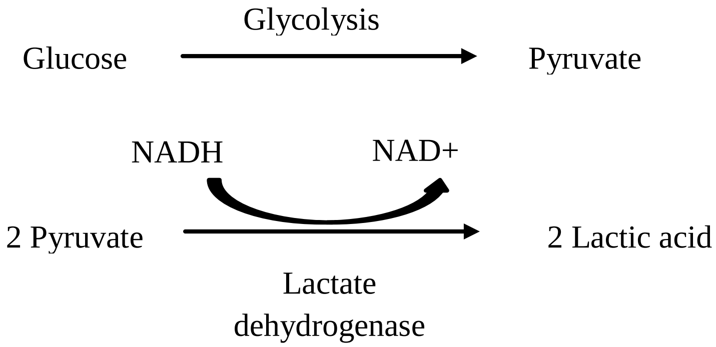
| *organism* | *Lactic acid g/L* | *Yield g/g* | *Productivity g/(L.h)* |
|---|---|---|---|
| *Rhizopus oryzae* ATCC 52311 | 83.0 | 0.88 | 2.6 |
| *Rhizopus oryzae* NRRL 395 | 104.6 | 0.87 | 1.8 |
| *Enterococcus faecalis* RKY1 | 144.0 | 0.96 | 5.1 |
| *Lactobacillus rhamnosus* ATCC 10863 | 67.0 | 0.84 | 2.5 |
| *Lactobacillus helveticus* ATCC 15009 | 65.5 | 0.66 | 2.7 |
| *Lactobacillus bulgaricus* NRRL B-548 | 38.7 | 0.90 | 3.5 |
| *Lactobacillus casei* NRRL B-441 | 82.0 | 0.91 | 5.6 |
| *Lactobacillus plantarum* ATCC 21028 | 41.0 | 0.97 | 1.0 |
| *Lactobacillus pentosus* ATCC 8041 | 21.8 | 0.77 | 0.8 |
| *Lactobacillus amylophilus* GV6 | 76.2 | 0.70 | 0.8 |
| *Lactobacillus delbrueckii* NCIMB 8130 | 90.0 | 0.97 | 3.8 |
| *Lactococcus lactis* ssp. *lactis* IFO 12007 | 90.0 | 0.76 | 1.6 |

### 2.4.1 Characterization of lactic acid bacteria

The lactic acid bacteria belong to two main groups, the homofermentors and the heterofermentors. The pathways of lactic acid production differ for both types(Maślanka & Adam 2015). Homofermentative produces mainly lactic acid whilst heterofermentative produces lactic acid plus appreciable amount of ethanol, acetate, and carbon dioxide, respectively.

The lactic acid bacteria are also divided into three groups based on fermentation patterns:

Homofermentative produces more than 85% lactic acid from glucose (Salminen et al., 2004). One mole of glucose can be converted to two moles of lactic acid, generating a net of 2 ATPs per mole of glucose metabolized.



Heterofermentative produces only 50% lactic acid and considerable amounts of ethanol, acetic acid and carbon dioxide, one mole of glucose converted to one mole of lactic acid, one mole of ethanol, and one mole of $CO_2$(Salminen et al., 2004). One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized. Because of the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration, and produce smaller colonies of 2-3 mm.

$$\text{Acetaldehyde} + \text{NADH} + \text{H}^+ \longrightarrow \text{Ethanol} + \text{NAD+}$$

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Ethanol}}{C_2H_5OH} + \underset{\text{Carbon dioxide}}{CO_2}$$

Less known heterofermentative species which produce DL-lactic acid, acetic acid and carbon dioxide. The ability of *lactobacilli* to convert lactose to lactic acid is employed in the successful treatment of lactose intolerance. People suffering from this condition cannot metabolize lactose due to lack or dysfunction of the essential enzyme systems. Lactic acid, by lowering the pH of the intestinal environment to 4 to 5, inhibits the growth of putrefactive organisms and E. coli, which require a higher optimum pH of 6 to 7. They are differentiated from other organisms by their ability to ferment hexoses to lactic acid(Sheeladevi & Ramanathan, 2011). The lactic acid bacteria have limited biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines, and typically a sugar as energy source. A rich medium is usually employed when cultivating LAB. These multiple requirements restrict their habitats to areas where the required compounds are abundant (animals, plants, and other multicellular organisms). LAB can grow at temperatures from 5-45 °C, and not surprisingly are tolerant to acidic conditions. Most strains are able to grow at pH 6(Kongo., 2013).

## 2.5 Nutrient requirement for lactic acid fermentation

All lactic acid bacteria require a source of nutrients for metabolism. The fermentative bacteria require carbohydrates, either simple sugar such as glucose and fructose or complex carbohydrates such as starch or cellulose. The energy requirements of lactic acid bacteria are very high. Limiting amount of available substrate can stop their growth. It is necessary to supplement the fermentation media with sufficient nutrients for rapid lactic acid production. If small amounts of other nutrients were supplemented to the process, then the efficiencies of lactic acid fermentation would be improved significantly(Farooq et al., 2012).

### 2.5.1 Carbon sources

The biotechnological production of lactic acid from cheap raw materials is necessary. This is because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should have the following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little amount of by-product formation, ability to be fermented with some pre-treatment, and year-round availability(Laopaiboon et al., 2009). However, this is still economically unfavorable because the refined carbohydrates are too expensive that they eventually result in higher production costs. Therefore, there are attempts to select cheap raw materials for the economical lactic acid production. Cheap raw materials, such as wheat starch, cellulosic materials, whey, and molasses, have been used for lactic acid production. Among these, starch and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable. Consequently, inexpensive starch, starch derivatives or starch containing waste materials as substrate would offer great advantages when combine with minimal preprocessing and supplementation( Wee et al., 2006).

### 2.5.2 Nitrogen sources

Biotechnological production of lactic acid on either a glucose or a lactose based medium requires supplementation, for example, yeast extract(Ooi., 2012). Supplementation with yeast extract had a significant effect on lactic acid concentration, volumetric productivity, and substrate conversion. The most common and effective supplement therefore seems to be 10 g/l of yeast extract(Siezen & Abee, 2002). When a yeast cell is inactivated, a natural digestion process called “autolysis” starts. During this process the yeast's own enzymes breakdown proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called “Yeast Extract”.

Yeast Extract is rich in nitrogen, vitamins and other nitrogenous growth factor stimulating compounds, and therefore is used as an ingredient in media for the cultivation of microorganisms. Moreover, all published reports have shown that lactic acid production increases with the concentration of the supplement (especially yeast extract)(Koirala, 2013). The typical composition of yeast extract is 8 -12% of total nitrogen content (organic and inorganic compound), 50-75% of protein content, 3-5.2% of fermentable nitrogen content, 4-13% of total carbohydrate content, and very little of lipid content, respectively. In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost. Most studies reported on lactic acid production by lactic acid bacteria were performed in media containing expensive nutrients such as yeast extract and peptone. In this context, the search for alternative, low cost media for lactic acid fermentation has an obvious economic interest. However, the development of an alternative nitrogen source is a prerequisite for the economical production of lactic acid, because yeast extract is a relatively expensive nitrogen source for industrials use. Therefore, it is essential to develop a more economical method for lactic acid fermentation, using materials as a cheaper nitrogen source(Orji et al., 2016).

## 2.6 Lactic acid fermentation processes

Fermentation is defined as an energy yielding process whereby organic molecules serve as both electron donors and electron acceptors. The molecule being metabolized does not have all its potential energy extracted from it. Hence, lactic acid bacteria are widely used as a low cost method for food preservation by fermentation and generally no or little heat is required during the fermentation. In fermentation, pyruvic acid molecules are turned into waste product and a little bit of energy (only two ATP molecules per molecule of glucose is produced by the homo fermentative pathway through the Embden-Meyerhof pathway(Hofvendahl, 2000). Yeast extract clearly exhibits the most significant effect on lactic acid production, especially at the beginning of growth, with the lactic acid concentration increasing nearly linearly with an increase in yeast extract level. The importance of yeast extract at the early stages of fermentation was probably caused by the amino acids and vitamins that are crucial for growth. However, if the medium contained a sufficient quantity of yeast extract from brewing as a source of nitrogen and growth factors, the amount of commercial yeast extract could be reduced or even replaced without a decrease in lactic acid production(Maache-rezzoug et al., 2009).

### 2.6.1 Batch fermentation

Lactic acid production in batch fermentation is widely studies in batch fermentation by lactic acid bacteria (Yun et al., 2006). The species of Lactobacillus used to ferment sugar depends mainly upon the type of carbohydrate being employed. Strains of *Lactobacillus delbruecki* find favor when production is based on maize sugar; the inoculum is grown up in batches and these are added directly to the fermenter containing several thousand gallons of medium(Wee et al., 2006). For whey fermentation, strains of Lactobacillus *bulgaricus* are necessary because this species is able to utilize the milk sugar (lactose). The fermentation proceeded for up to six days, and the concentration of sugar in the medium dropped to around 0.2 per cent (Dussán et al., 2014). An amylolytic lactic acid producing *Lactobacillus amylovorus* produced 36 g/L of lactic acid in mixed cultures with *Lactobacillus casei* at 37 °C in 48 h, when initial barley flour concentration was 180 g/L worth a yield of 20% (Javanainen and Linko, 1995). Lactic acid production was investigated for batch of *Enterococcus* faecalis RKY1, using wood hydrolyzate and corn steep liquor. When wood hydrolyzate (equivalent to 50 g/L glucose) supplemented with 15-60 g/L corn steep liquor was used as a raw material for fermentation, up to 48.6 g/L of lactic acid was produced with volumetric productivities ranging between 0.8 and 1.4 g/L/h with a yield of 97.2%(Wee et al., 2006). Literature reviews of lactic acid production from various materials shown in Table 2.6.

Table 2-7: Lactic acid fermentation from agricultural resources by batch fermentation. (Wee et al., 2006)

| Raw materials | Organisms | Lactic acid (g/L) | Productivity (g/L/h) |
|---|---|---|---|
| Waste paper | *Rhizopus oryzae* NRRL 395 | 49.1 | 1.8 |
| Molasses L. | *L. delbrueckii* NCIMB 8130 | 90.0 | 3.8 |
| Corn starch | *L. amylophilus* GV6 | 76.2 | 0.8 |
| Wheat hydrolyzate | *L. lactis* ATCC 19435 | 106.0 | 3.3 |
| A corn starch hydrolyzate | *L. rhamnosus* HG 09 | 57.6 | 1.6 |

Moreover, an amylolytic lactic acid bacterium was capable to produce 18.3 g/L of lactic acid from starch with a product yield of 61% (based on total carbohydrates consumed)(Zhang & Vadlani, 2013). However, the major disadvantage of batch fermentation is that lactic acid concentration and productivity decrease due to inhibition of high substrate concentration. This phenomenon is the typical property of batch fermentation. On the other hand, as the time goes by the concentration of lactic acid are increased but it can inhibit cell growth and product formation.

### 2.6.2 Fed-batch fermentation

Lactic acid production by *Lactobacillus casei* fermentation using different fed-batch feeding strategies was studied. According to the experimental results, exponential fed-batch culture is an effective method for the fermentation of L-lactic acid. In exponential feeding glucose solution (850 g/L), the maximum lactic acid concentration was obtained at 210 g/L. L-lactic acid yield, the maximal dry cell weight and productivity were up to 90.3%, 4.30 g/L, and 2.14 g/L/h, respectively. Comparing with the traditional batch culture, the exponential feeding glucose and yeast extract culture showed 56.5% improvement in L-lactic acid production, 68.6% improvement in dry cell weight and 59.7% improvement in productivity, respectively (Liguori et al., 2015). Batch, fed-batch, and continuous fermentations are the most frequently used methods for lactic acid production. Higher lactic acid concentrations may be obtained in batch and fed-batch fermentation than in continuous fermentation.

## 2.7 Factors affecting on lactic acid fermentation

However, there are still several researches that need to be addressed in order to produce lactic acid within the targeted cost, development of high performance lactic acid producing microorganisms and lowering the cost of the raw material. Many factors affected in lactic acid fermentation have been investigated. The optimization of fermentation processes requires profound knowledge of the factors determining microbial metabolism, and the influence of process parameters.

### 2.7.1 Effect of temperature

Temperature and pH are the key environmental parameters that affect the fermentation process (Kotzamanidis et al., 2002). Low temperature has been reported to positively influence the outgrowth of contaminating microorganism, thereby influencing the performance of the lactic acid production were investigated(Wasewar, 2005). The temperature giving the highest productivity lower than the temperature resulting in highest lactic acid mass concentration and yield. For *Lactobacillus amylophilus*, which is known to grow at 15 °C but not at 45 °C, the optimal temperatures were 25 °C and 35 °C for maximum productivity and yield, respectively(Roukas et al., 2002) Some previous reported investigated the cultivation temperature on the solid-state fermentation of lactic acid production by controlling the growth temperatures at 22, 30, 35, and 40 °C. The results from measuring the residual starch and reducing sugar in 4 h and 8 h indicated that there was increased in starch hydrolysis and reducing sugar accumulation as the temperature increased from 22-30 °C, and a further increase from 30-40 °C resulted in a slight improvement for the saccharification in both *Rhizopus oryzae* 2062 and *Rhizopus oryzae* 36017 cultures. Therefore, the lactic acid production and biomass growth were affected by the temperature.

### 2.7.2 Effect of pH

The fermentation pH is either set at the beginning and then left to decrease due to acid production or it is controlled by an addition of alkaline solutions. The optimal pH for lactic acid production varies between 5.0 and 7.0. A pH below 5.7 was optimal for *Lactobacillus* strains, which are known to tolerate lower pH than *lactococci*. The previous studies investigated the influence of culture pH on lactic acid fermentation from molasses where lactic acid fermentations were performed on a jar fermentor at 38 °C and pH 5.0-9.0. Although the optimum pH for cell growth of *Enterococcus faecalis* RKY1 was seen to be 8.0, the lactic acid fermentation at pH 7.0 was completed faster than that at pH 8.0. The cell growth at pH 5.0 almost ceased after 10 h of fermentation, the highest lactic acid mass concentration was obtained at pH 7.0 with a comparable yield with pH 6.0(Y. Wee et al., 2006). Moreover, some reported showed the effect of various initial pH on the lactic acid production of the immobilized *Lactobacillus delbrueckii* during the batch fermentation of liquid pineapple waste. At initial pH 6.5, cell started to utilize glucose earlier and at a faster rate than at other initial pH. Maximum lactic acid concentration was attained at initial pH 6.5. Further increase in initial pH beyond 6.5 does not improve the lactic acid production. It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities (Zhang & Vadlani, 2013).

### 2.7.3 Effect of incubation period

Previous reported represented that an increase in lactose utilization and subsequent lactic acid production was found incubation time up to 36 h and thereafter no improvement in both the functions was observed (Ine & Roberto, 2007). This could be attributed to the growth of the culture reached to the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures. In addition, the different optimal conditions reported by various workers for maximum lactic acid production could be explained by the differences in the nature of the strains and medium composition used in their studies(Zhang & Vadlani, 2013).

### 2.7.4 Effect of agitation

Different lactic acid bacterial strains differed in their requirement for growth conditions. The consequence of agitation speed on lactic acid fermentation efficiency was carried out. For the strain *Lactobacillus rhamnosus*, the maximum lactic acid concentrations could be achieved when fermentation was carried out at pH 6, temperature of 40°C and agitation speed of 150 rpm, which was in accordance with a previous report. The optimal condition for lactic acid is pH 5.0-6.8, temperature 30- 45°C with continuously agitating at 100-200 rpm(Roberto, 2005).

## 2.8 Purification techniques of lactic acid

A number of methods can be used for separation of lactate salt from fermented medium including extraction by organic solvents, ion-exchange separation, adsorption, vacuum distillation, and membrane separations. Each of these exhibits some advantages and disadvantages that are also reported with fermentation processes(Komesu et al., 2015). The choice of the separation process should be based on the efficient and economically usage of these extractants. Technological advancements in the major process components-fermentation, primary and secondary purification, polymerization, chemical conversion of lactic acid and its derivatives would enable low cost large volume and environment friendly production of lactic acid. Recent advancements in membrane based separation and purification would enable lactic acid production without producing salt or gypsum by products. In recently issued patents, an osmotolerant strain of lactic acid bacteria and a configuration of desalting electrodialysis, water splitting electrodialysis and ion exchange purification, a concentrated lactic acid product containing less than 0.1% proteinaceous components can be produced by a carbohydrate fermentation(Evangelista, 1994).

### 2.8.1 Adsorption

Recovery of lactic acids from fermentation broths presents a challenging separation problem, because of the dilute, complex nature of fermentation broths. Important characteristics of extractants and solid sorbents are a high capacity for the acid, a high selectivity for the acid as opposed to water and substrate (e.g., glucose), regenerability, and, depending upon the process configuration, the biocompatibility with microorganisms. Many fermentation processess, such as that for lactic acid production, are subject to end-product inhibition. If a solid sorbent can be used in situ or in an external recycle loop, higher overall yields can be achieved (Wasewar, 2005).

However, elution steps are the most difficult and problematic part as a large volume of eluent is required with adjusting of the pH.

### 2.8.2 Solvent extraction

Solvent extraction has been used for the purification of carboxylic acid such as lactic acid and succinic acid. But these solvents in-situ are toxic as they rupture the cell membrane causing the metabolite to leak out. Long chain alcohols such as 1-octanol and 1- decanol were found to be less toxic than other diluents. In addition, they reduce the toxicity of solvents on the cells. Bio-compatibility and price of the solvent are the major concern when using solvent extraction processes(Mussatto et al., 2013).

### 2.8.3 Membrane separation processes

As mentioned earlier, lactic acid production processes traditionally suffer from end-product inhibition. An undissociated lactic acid permits through the bacterial membrane and dissociates inside the cell. It eventually influences the transmembrane pH gradient and decreases the amount of energy available for cell growth (Komesu et al., 2017). In parallel with fermentation kinetic studies, the use of membranes to extract fermentation products from the aqueous phase was introduced many years ago in order to improve the traditional production processes. In many particular systems, membrane separations are the most effective techniques. Membrane separation has been widely used for purification concentration of fluid mixtures. Theory of membrane transport is concerned with chemical nature of the membrane, physical structure and physio-chemical properties of the mixture to be separation. The main interest will be focused on the absorption of organic compounds into the membrane surface and the diffusion across the membrane matrix(Laopaiboon et al., 2009). However, the fermentation step is needed for an economically favorable production.

### 2.8.4 Electrodialysis (ED) technique

Electrodialysis (ED) is usually considered as a desalination process, but there is much wider scope for this technique because ED is, in many cases, a powerful separation method when charged compounds have to be separated from a solution. For instance, ED can be used to recover free lactic acid from fermentation broth. Electrodialysis of the permeate allows good lactic acid separation and concentration, up to 13% w/v of lactic acid. The acid can further be concentrated by evaporation. The fermentation step is needed for an economically favorable production (Lund et al., 1992). Electrodialysis fermentation (EDF) is promising because it can remove produced lactic acid continuously from the fermentation, and maintain the pH of the broth. In this study, an EDF method with the conventional fermentation method was compared with EDF with continuous medium feed in order to increase the productivity of lactic acid. However, the acid can further be concentrated by evaporation which increase the cost of the process(Melzoch, 2003).

### 2.8.5 Electrodeionization (EDI) technique

Using current technology, the volumetric productivity of lactic acid fermentation is recognized to be rather small as accumulation of lactic acid inhibits the process by product inhibition. Unfortunately, owing to its physicochemical properties, lactic acid cannot be continuously extracted from the fermentation medium by simple operation, which means that a large proportion of the cost of lactic acid production is associated with recovery and purification operations(Y. Wee et al., 2006). The pH of the fermentation broth decrease and the fermentation is inhibited when lactic acid is produced. Therefore, the recovery of lactic acid from the fermentation broth is very important for increasing its productivity. Electrodeionization (EDI) utilizes chemical-free regeneration. EDI is a continuous process where ion exchange resins are being continuously regenerated by the DC electric field. Compared with deionization process of water, there is no breakthrough of ions as happens in conventional ion exchange operations, therefore the quality of the water remains at a constant high level of purity. The electric field also provides a bacteriostatic environment inside of the EDI cell, inhibiting the growth of bacterial and other organisms. EDI has significantly lower operating costs than conventional ion exchange processes. Only a relatively small amount of electric power is needed to provide high purity water(Melzoch, 2003).

### 2.8.6 In situ removal system product

Biocatalytic processes for the manufacture of small, highly functionalized molecules frequently have limited productivity. A common reason for this is the presence of the reaction products that can cause inhibitory or toxic effects (making poor use of the enzyme), or promote unfavorable equilibria (giving low conversions). In each case, the product needs to be removed as soon as it is formed in order to overcome these constraints and hence increase the productivity of the bio catalytic process. Therefore, in situ product removal and the process research are required for its implementation (Evangelista, 1994).

# 3. MATERIALS AND METHODS

## 3.1 Chemicals and equipment

The experimental work was carried out in the laboratory of Addis Ababa Institute of Technology, at the school of chemical and bio Engineering.

**Chemicals:** Sodium Hydroxide (NaOH, min. assay 98% BDH Chemicals Ltd pool England), Sulfuric acid ($H_2SO_4$, (98%, England)), beef extract, Yeast extract, tween 80, MgSO4.7$H_2O$, manganese sulphate, acetone, MRS broth, LAB (*Lactobacillus plantarum* ATCC 8014 from Ethiopian biodiversity institute).

**Equipment:** Digital balances (model-Sartorius with 0.01mg sensitivity, and model EP214C), Vacuum Filter (model-BN 3 STAATLICH, Berlin), Sieves (mesh size of 2.0 mm, Sortmks-3332, PFEUFFR, Germany), Shaking Incubator , Vertical Autoclave , pH- Meter , Ovens- Loading model 100 -800, spectrophotometer (Perkin Elmer), heating mantle, Soxhlet extractor set up, desiccator, Fourier Transform Infrared spectroscopy (FTIR).

## 3.2 Characterization of brewer's spent grain

Experiments were conducted to determine the moisture content, fixed carbon content, ash content and volatile matter content of air-dried biomass samples ground to particle size below 500 µm.

### 3.2.1 Proximate analysis

The proximate analysis gives moisture content (MC), volatile matter content, the fixed carbon content, the ash content (the inorganic residue remaining after combustion of the sample).

### 3.2.1.1 Moisture Content

Samples were weighed in clean preheated moisture crucible of known weight by using sensitive balance. The sample and crucible were kept in an oven 105℃ for an hour. The crucible was covered and transferred to desiccators, and weighed after reaching room temperature. The crucible was heated in the oven for another two hours and was re-weighed.

This was repeated until constant weight was obtained. The loss of weight was calculated as percent of weight and expressed as moisture content.

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{W_1} \times 100 \qquad \text{3.1}$$

Where:

W1= Initial weight

W2= weight after drying

#### 3.2.1.2 Volatile Matter Content

A crucible was weighed empty, and then samples were put in it. The sample and the crucible were placed in a muffle furnace for 30min at 600ºC. The crucible was removed from furnace and placed in a desiccators to cool, then reweighed. The process was repeated until constant weight was obtained.

Volatile content (%) = $\frac{W1- W2}{W1}$ x 100……………………………………………………………3.2

Where:

W1= Original weight of the sample

W2 = Weight of sample after cooling

#### 3.2.1.3 Ash Content

A crucible was weighed empty, and then samples were put in it. The sample and the crucible were placed in a muffle furnace for 2 hours at 550℃. The crucible was removed from furnace and placed in a desiccators to cool, then was reweighed.

Ash content (%) =$\frac{W1- W2}{W1}$ x 100………………..……………………………………………3.3

Where:

W1= Original weight of the sample

W2 = Weight of sample after cooling

#### 3.2.1.4 Fixed Carbon Content

This is the residue left after the moisture, volatile and ash is given up. It is deduced by subtracting from 100, the percentage of moisture, volatile matter and ash content. The fixed carbon content (FC) is given as

FC = 100 – (%moisture + %volatile matter + % ash)………………..………………………3.4

### 3.2.2 Compositional analysis of brewer's spent grain

#### 3.2.2.1 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. Residence times for the boiling and rising stages was carefully adjusted to 70 ºC and 25 min respectively on the heating mantle for a 4 h run period. After extraction, the sample was air dried at room temperature for few minutes. Constant weight

of the extracted material was achieved in a convection oven at 105 ºC. The %( w/w) of the extractives content was evaluated as the difference in weight between the raw extractive-laden biomass and extractive-free biomass

Extractive (%) = $\frac{W1 - W2}{W1}$ x 100…………………………………………………………….3.5

Where:

W1 = oven dry sample

W2 = extracted residue

### 3.2.2.2 Hemicellulose

1 g of extracted dried BSG was transferred into a 250 mL Erlenmeyer flask. 150 mL of 500 mol/m$^3$ NaOH was added. The mixture was boiled for 3.5 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.

Hemicellulose (%) = $\frac{W1 - W2}{W1}$ x 100.........................................................................…...3.6

Where:

W1 = oven dry sample

W2 = extracted residue

### 3.2.2.3 Lignin

1 g of dried extracted raw BSG was weighed in glass test tubes and 10 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, 280 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. Hydrolyzates were filtered through vacuum using a filtering crucible.

Lignin (%) = $\frac{W1 - W2}{W1}$ x 100.........................................................................…...............3.7

Where:

W1 = oven dry sample

W2 = extracted residue

### 3.2.2.4 Cellulose

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

$100 = WC + WH + WE + WL$……………………………………..…………………………3.8

$WC = 100 - WH + WE + WL$...............................................................................…...................3.9

Where:

WC = cellulose content

WH = hemicellulose content

WE = extractive

WL = lignin content

### 3.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectrum was recorded by passing a beam of infrared light through the sample. The functional group analysis of the raw brewery spent grain was carried using Fourier-Transformed Infrared (FTIR) spectroscopy. 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 and NaCl plate for liquid samples by thin film deposition technique. A regular scanning range of 400-4000 $cm^{-1}$ was used for 20 repeated scans at a spectral resolution of 4 $cm^{-1}$. All the spectra were recorded and processed using essential FTIR software.

## 3.3 Methods

### 3.3.1 Preparation of Spent Brewery Grains (BSG)

The main aim of the research was lactic acid production from brewery spent grain using *lactobacillus plantarum*. First the raw material was obtained from BGI brewery Ethiopia located in Addis Ababa. It was successively wash with distilled water to remove impurities, and then dried at 75°C. Afterward it was treated with sodium hydroxide solution of 0.5M for 4 hours at room temperature to enhance its metal sorption capacity(Mussatto et al., 2006). The excess of alkaline solution was removed by washing spent grains with distilled water until it was completely free from the base in successive washings, according to the procedure described by(Wee et al., 2006)This material was designated as TSG (Treated Spent Grains). Then TSG was dry in an oven at 75ºC for 24 h. The dried biomass was grind and sieve and the particles having sizes of 250-500 μm was used in the tests and stored at less than 4°C refrigerator.

### 3.3.2 Acid Hydrolysis

Diluted acids transform starch into glucose. Samples based on the BSG were put in Erlenmeyer flask. The ratio between BSG and acid solution was 1:10 W/V and The mixture of BSG and acid solution made 500 ml in Erlenmeyer flask.

#### 3.3.2.1 Procedures for Acid Hydrolysis

- Different concentration of sulfuric acid was prepared and added to the non-soluble component from pretreatment steps in the order of experimental design for all experiment.
- The BSG was then hydrolyzed in the autoclave at a temperature of 115℃, 122.5℃and 130℃, acid concentration 1.5M, 1.75M and 2.0M and time of 25min, 30min and 35min.
- After hydrolysis, the solid part was separated from the liquid by vacuum filtration unit to remove the non-fermentable component.
- The filtered hydrolyzate was neutralized with 4M NaOH until the pH became in a range of 5-5.5.

### 3.3.3 pH Adjustment

Before addition of any micro-organism to the above prepared samples, pH of these samples has to be adjusted. Otherwise the micro-organism was dying in high acidic or basic state. A pH of around 5.0-5.5 is maintained.

#### 3.3.3.1 Procedures in pH adjustment

- First the pH meter was calibrated by using buffer solution.
- The hydrolyze solution is acidic, so it needs highly basic solution to bring the pH in the range of 5.0-5.5.
- Sodium hydroxide solution was added drop wise to the other flask with constant stirring until the pH reaches to a range of 5-5.5.
- If suppose the pH goes beyond 5-5.5 concentrated sulfuric or hydrochloric acid was added drop wise to maintain the pH in the range.

### 3.3.4 Sugar content determination

Total reducing sugar of the hydrolyze sample was determined using Spectrometer, which measures the intensity of red light. Standard curve was plotted from known concentration of standard glucose and benedict solution reagent in digital spectrophotometer at 540nm.so that concentration sugar yield content of hydrolyzate which obtained from hydrolysis was determined using digital spectrophotometer (spectro uv-vis double beam pc 8 scanning auto cell UVD -3200) 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 color 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 concentration sugar present in a sample can be obtained.

#### 3.3.4.1 Calibration plot for glucose standard

The content reducing sugar concentration was determined by using UV-visible spectrophotometer at 540 nm wavelength of glucose absorbance and the quantification was made from calibration curve using glucose as standard and calculation was performed by equation of the linear regression obtained from calibration curve.

Stock glucose solution was made by dissolving a standard glucose with water at different concentration of 0.30, 0.25, 0.20, 0.15, 0.10, 0.05 and 0 g/ml; Pipette 0.5 ml from each of the dilution series was pipetted out into labeled test tubes, each containing 5 ml of Benedict's solution and then mix by shaking. Then all the labeled test tubes were heated at 90ºC water bath for 5 minutes. The test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent. After filtered the precipitate, % absorbance was measured using spectrophotometer at 540nm. Calibration curve was plotted to show the % of absorbance of blue light by the standard glucose solution.

#### 3.3.4.2 Determination of the total reducing sugar in the hydrolyze

In order determine the sugar content of the hydrolyze sample from the autoclave, 0.5 ml of samples is pipetted into labeled test tubes, containing 5 ml of Benedict's solution. All the labeled test tubes were heated to 90ºC in water bath for 5 minutes. Then test tubes were removed from the water bath and filtered using filter paper to remove red precipitate formed when reducing sugar in the samples reacted with Benedict reagent. After filtration the precipitate; % absorbance was measured using spectrophotometer at 540nm. The concentration of sugar in each samples were read from the calibration curve of the standard glucose solution.

$$\text{CTRSUS} = \frac{absorbance\ of\ unknown\ sample - y - intercept}{slope} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.10$$

Where

CTRSUS = Concentration of total reducing sugar of unknown sample.

### 3.3.4.3 Spectrophotometric Technique

The spectrophotometer is plugged in and turned on and allowed for 5 min to warm up. The wavelength is set at 540 nm and no light is transmitted by closing the light path. Then after blank sample was inserted in to the sample chamber using cuvette and setting the absorbance zero. Finally the cuvette was filled with the prepared samples and recorded the absorbance.

### 3.3.5 Culture media preparation

The aim of this step of the experiment is to culture the microorganism that is used for fermentation. *Lactobacillus plantarum ATCC 8014* was the microorganism used for conversion of glucose to lactic acid. Before conducting the fermentation, the media for the preparation of *Lactobacillus plantarum ATCC 8014* was prepared. For preparing one liter of media, the following chemicals were add to 1000mL distilled water as shown in Table 3.1

Table 3-1: Composition of MRS medium (1L)

| **Chemicals** | **gms/liter** |
|---|---|
| Protease peptone | 10.00 |
| Beef extract | 10.00 |
| Yeast extract | 5.00 |
| Dextrose | 20.00 |
| Tween 80 | 1.00 |
| Ammonium citrate | 2.00 |
| Sodium acetate | 5.00 |
| Magnesium sulphate | 0.10 |
| Manganese sulphate | 0.05 |
| Dipotasium phosphate | 2.00 |

From the above prepared media 250mL media prepared as above, *Lactobacillus plantarum ATCC 8014* was added using a 500mL conical flask.

#### 3.3.5.1 Sterilization

The purpose of this step is to remove the microorganisms which could share the glucose concentration prepared for lactic acid production from, *Lactobacillus plantarum ATCC 8014*. Accordingly, all the equipment that were used for fermentation purposes were sterilized. The sterilization was carried out at a temperature of 121ºC for 15 min in the autoclave.

### 3.3.6 Microorganism and inoculum cultivation

*Lactobacillus plantarum ATCC 8014* was grown in 25 mL test tubes containing 10 mL DeMan, Rogosa and Sharpe (MRS) broth. After 48 h static incubation at 37°C, 1 mL of this culture was then transfer to a new test tube with 10 mL MRS broth, and incubated at the same conditions. Then, the cells were harvested by centrifugation (1,100 rpm, 15 min) and resuspended in distilled sterilized water. Specific quality of this suspension was added to the fermentation process.

### 3.3.7 Fermentation Medium

150mL of hydrolysate was prepared according to the requirements of *Lactobacillus plantarum ATCC 8014*, containing yeast extract, beef extract, tween 80, ammonium citrate, peptone, MgSO4.7 $H_2O$ and sodium acetate, based on the amount of total sugar present in the hydrolysate. Then autoclaved at 121°C for 15 min.

### 3.3.8 Procedure for Fermentation

Prior to performing the fermentation, the samples were conditioned to a temperature of 37ºC. All the fermentation experiments were carried out at this temperature. The pH was checked before adding the culture media and it was maintained by using 4M NaOH. The pH (5.0-5.5) was optimum for the *Lactobacillus plantarum ATCC 8014* culture. The cultured media and the hydrolyzate sample were mixed with the proportion of 1:10 in Erlenmeyer flask. Then the mixture was placed in the shaker incubator at a temperature of 35ºC and 200 rpm for 72 hours.

### 3.3.9 Method of lactic acid recovery

The fermented liquor obtained after the above fermentation contains lactic acid together with various impurities such as the un-reacted raw materials, cells and culture media-derived saccharides. The samples were centrifuged at 5000 rpm for 5 minutes and filtered through 0.2µm paper filter.

### 3.3.9.1 Determination of Lactic Acid Concentration in Fermented Broth

**Materials**

- 100mL Conical flask with a wide neck and a stopper (12 per group or use 1 flask per group and ensure they rinse it well between samples).
- 1 x Burette per
- Measuring cylinder (50 – 100 ml)
- Phenolpthalein in dropper bottle
- Filter paper (for white background) 1 sheet per group
- 0.1M Sodium hydroxide (NaOH ) approximately 70 ml per group
- Distilled water
- Balance

**Method**

25mL of the fermented broth was added to the conical flask and 5 drops of Phenolpthalein indicator is added. 4M sodium hydroxide is filled the burette. Start the titration until the solution retains a very slight pink tinge and record the volume of sodium hydroxide used. The percentage of lactic acid in the sample is:

% lactic acid =

$$\frac{(\text{ml of NaOH used})\ (\text{conc NaOH})\ (0.090\ \text{ milli equivalent weight of lactic acid})}{\text{Weight of Sample}}(100)\ldots\ldots\ldots\ldots3.11$$

## 3.4 Experimental Design for hydrolysis of BSG

The reducing sugar was produced using treated spent grain, sulfuric acid, sodium hydroxide and distilled water. Experimental data analysis was carried out using Design- Expert 7.0.0 software.

The experimental design selected for this study was response surface methodology, three-level-three-factor Box-Behnken Design (BHD) and the response variable measured was the percentage of sugar present.

The three independent variables studied for the hydrolysis of BSG were the reaction temperature, sulfuric acid concentration and reaction time. The effect of the independent variables and interaction effect were analyzed to obtain maximum percentage of reducing sugar. In addition to achieve maximum conversion of reducing sugar, 2mL cell suspension and 5.5 pH was used as inoculum.

Three-level-three-factor Box Behenken Design was used in the optimization study which requires 17 experiments to be conducted. The seventeen experiments were done and the data was statistically analyzed using Design-Expert Software 7.0.0 to obtain a suitable model equation for the hydrolyste preparation for the production lactic acid.

Table 3-2 lists the range and levels of the three independent variables studied. The lower and higher levels are chosen by considering the operating limits of hydrolysis reaction.

Table 3-2: Independent variables and levels used in the BHD for the hydrolysis of BSG

| Variable (Factors) | Factor Coding | Unit | Levels | | |
|---|---|---|---|---|---|
| | | | -1 | 0 | 1 |
| Reaction time | $X_1$ | min | 25 | 30 | 35 |
| Reaction temperature | $X_2$ | ºC | 115 | 122.5 | 130 |
| Sulfuric acid concentration | $X_3$ | Molar | 1.5 | 1.75 | 2.0 |

Below in Table 3-3 the complete experimental design matrix of BBD for the factorial design was shown. The order in which the runs were made was randomized to minimize systematic errors.

Table 3-3: Design matrix for Box Behnken Design

| Run | Temprature( ºC) | Time (min) | Sulfuric acid concentration (Molar) |
|---|---|---|---|
| 16 | 25.00 | 115.00 | 1.75 |
| 6 | 35.00 | 115.00 | 1.75 |
| 1 | 25.00 | 130.00 | 1.75 |
| 12 | 35.00 | 130.00 | 1.75 |
| 17 | 25.00 | 122.50 | 1.50 |
| 7 | 35.00 | 122.50 | 1.50 |
| 13 | 25.00 | 122.50 | 2.00 |

| | | | |
|---|---|---|---|
| 11 | 35.00 | 122.50 | 2.00 |
| 9 | 30.00 | 115.00 | 1.50 |
| 5 | 30.00 | 130.00 | 1.50 |
| 14 | 30.00 | 115.00 | 2.00 |
| 3 | 30.00 | 130.00 | 2.00 |
| 2 | 30.00 | 122.50 | 1.75 |
| 15 | 30.00 | 122.50 | 1.75 |
| 10 | 30.00 | 122.50 | 1.75 |
| 4 | 30.00 | 122.50 | 1.75 |
| 8 | 30.00 | 122.50 | 1.75 |

# 4. RESULTS AND DISCUSSION

## 4.1 Proximate analysis of the raw BSG

Figure 4-1 shows that the proximate analysis of BSG. Moisture content, volatile matter, ash content and fixed carbon content of BSG was 10.8, 73.0, 3.9 and 12.3% respectively. The raw BSG sample was rich in volatiles (73.0%) and fixed carbon but low in ash content (3.9%). The BSG supplied had a moisture content of about 10.8%, which is a well-tolerated value for production of lactic acid. Bio- mass with higher fixed carbon content has higher lactic acid yield.

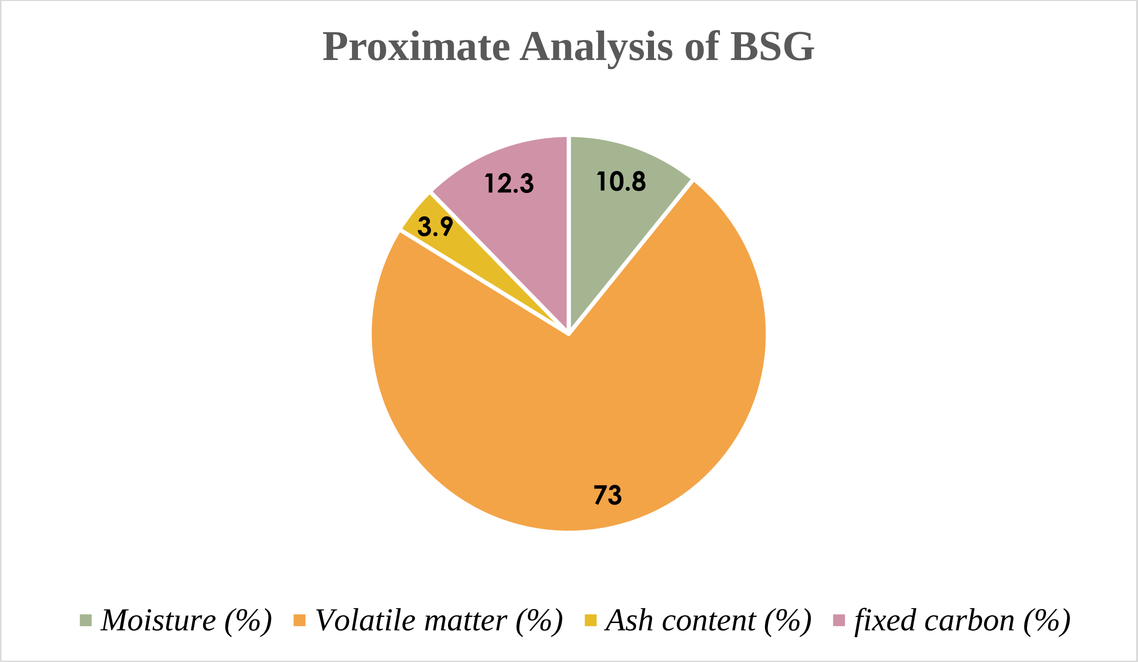


Figure 4-1: proximate analysis of BSG

## 4.2 Chemical composition analysis

Literature data for BSG of chemical composition analysis range from 32.5 to 45.6% of cellulose, 39.8% hemicellulose, and 3% extractive(Pahor et al., 2008). Samuel, 2011 also reported, 59.4 % cellulose, 6.5% hemicellulose, and 22.2% lignin. The results from this study are in a comparable range with literature values as reported by the abovementioned researchers. Therefore, the determination of cellulose and hemicellulose can be applied to quantify the theoretical production of lactic acid. However, *lactobacillus plantarum* converts BSG in to lactic acid using acid hydrolysis. In this study, BSG contained high contents of the total hemicellulose of approximately

40% and 30% cellulose. The lower the lignin content the easer hydrolysis condition, and decrease formation of toxic chemicals such as, aromatic, polyaromatic, phenolic and aldehydic.

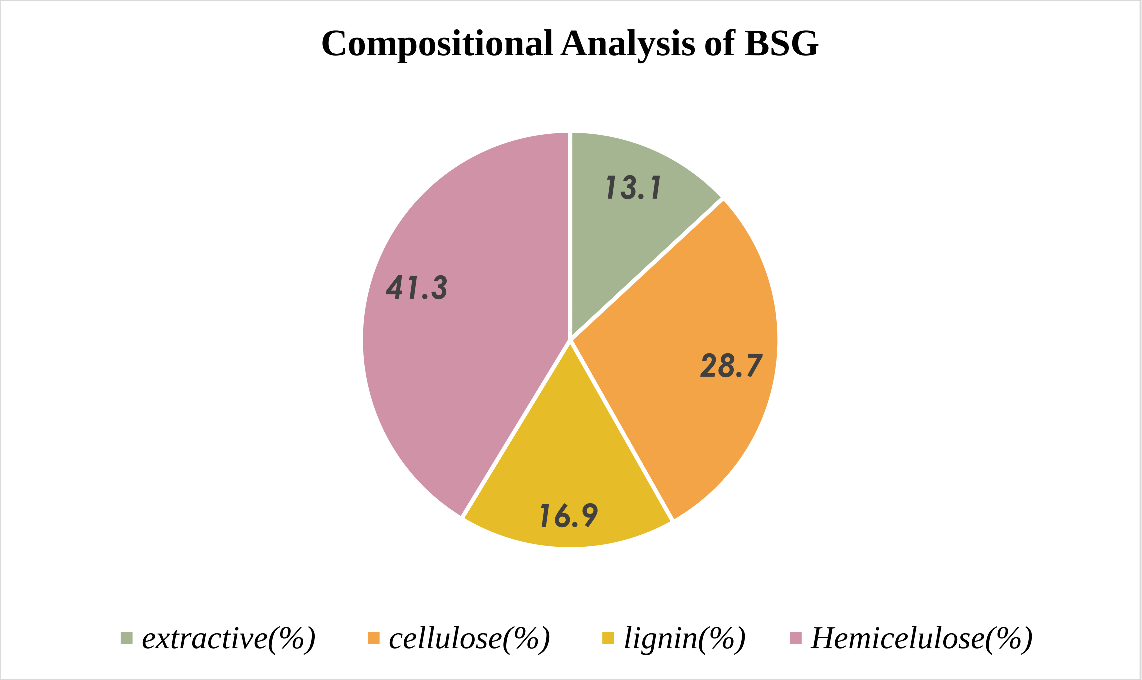


Figure 4-2: compositional analysis of BSG

## 4.3 Effect of acid hydrolysis on the lactic acid production

### 4.3.1 Total reducing sugar content after hydrolysis

Total reducing sugar of the hydrolyzate sample was determined using calibration curve which was plotted from the known concentration of standard glucose reacted with Benedict solution reagent and absorbance of standard glucose-benedict solution after reaction was measured using digital spectrophotometer at 540nm wavelength.

In this study, the total reduced sugar content obtained after the hydrolysis process was investigated. The powdered BSG through hydrolysis at different temperature, time and acid concentration on the amount of sugar produced was investigated; glucose concentration and its absorbance and there results are shown in Table 4.1 and the results of glucose calibration curve are shown in figure 4.3

Table 4-1: Concentration of standard glucose and its absorbance

| Std.Glucose concentration (g/ml) | Absorbance |
|---|---|
| 0 | 0 |
| 0.05 | 0.503 |
| 0.10 | 0.545 |
| 0.15 | 0.584 |
| 0.20 | 0.626 |
| 0.25 | 0.665 |
| 0.30 | 0.710 |

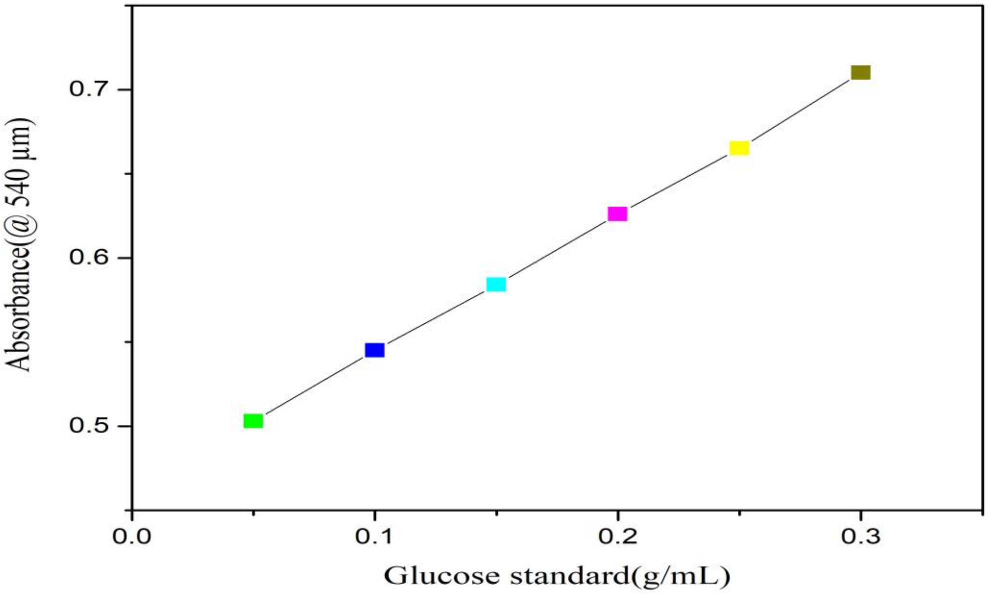


Figure 4-3: Calibration curve of glucose standard for determination of glucose content

The concentrations of unknown sugar samples were determined from a standard curve of glucose ($y = 0.821X + 0.462$; $R^2 = 0.999$)

| Item | Value | Equation |
|---|---|---|
| Intercept | 0.462 | $Y = 0.821X + 0.462$ |
| Slope | 0.821 | |
| Adj. R-Square | 0.999 | |

### 4.3.2 FT-IR analysis of BSG before and after hydrolysis

FTIR analysis was performed to detect information on the nature of the bonds and to identify different functional groups available on the BSG. As it can be seen from Figure 4.4 brewery spent grains contains functional groups of O-H and C-O stretching vibration of primary alcohol, N-H stretching vibration of amine), C-H stretching and bending vibration of alkane groups, C=O stretching vibration carbonyl, C=C stretching vibrations of alkene, C–F stretching vibrations of alkyl halide. All functional groups, their types of vibrations, wave number and intensity found in brewery spent grain are given in Table 4.2.

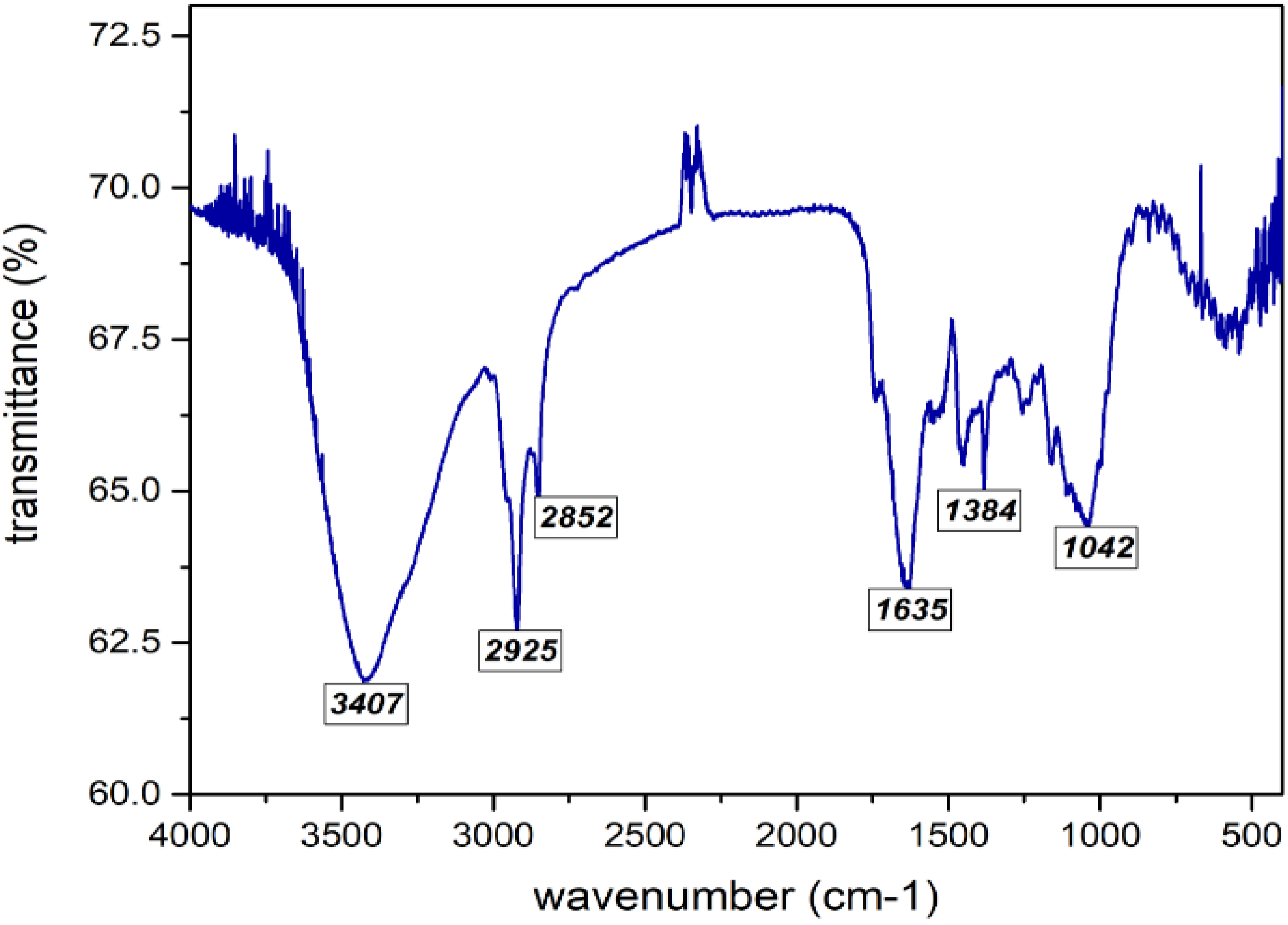


Figure 4-4: FTIR spectra for spent brewery grain before hydrolysis

Table 4-2: Characteristic IR absorption frequencies of BSG functional groups

| **Functional groups** | **Types of vibration** | **Wave number (cm-1)** | **Intensity** |
|---|---|---|---|
| O-H (Alcohol) | Stretch, H-bound | 3407 | Strong, Broad |
| N-H (amine) | Stretch | 3407 | Medium |
| C-H (Alkane) | Stretch | 2925 | Strong |
| C=C (alkene) | Stretch | 1635 | Variable |
| -C-H (Alkane) | Bending | 1384 | Variable |
| C-F (alkyl halide) | Stretch | 1042 | Strong |
| C-O (Alcohol) | Stretch | 1042 | Strong |

As seen in Figure 4.4 and 4.5 broad peak representing hydroxyl and amine groups shifts from 3407 to 3420 $cm^{-1}$ after hydrolysis of BSG. The increase in wave number of this peak is attributed to the removal of double bond on hydroxyl and amine groups. The peaks at 2925, 1635, 1042 $cm^{-1}$ corresponds C-H stretching vibration of alkane, -C-H bending vibration of alkane, C-O stretching vibration in primary alcohol showed a reduction in wave number after hydrolysis which are attributed to the removal of hydroxyl group. The other constant peak values before and after hydrolysis are worth nothing that the bond with functional groups were not observed after hydrolysis.

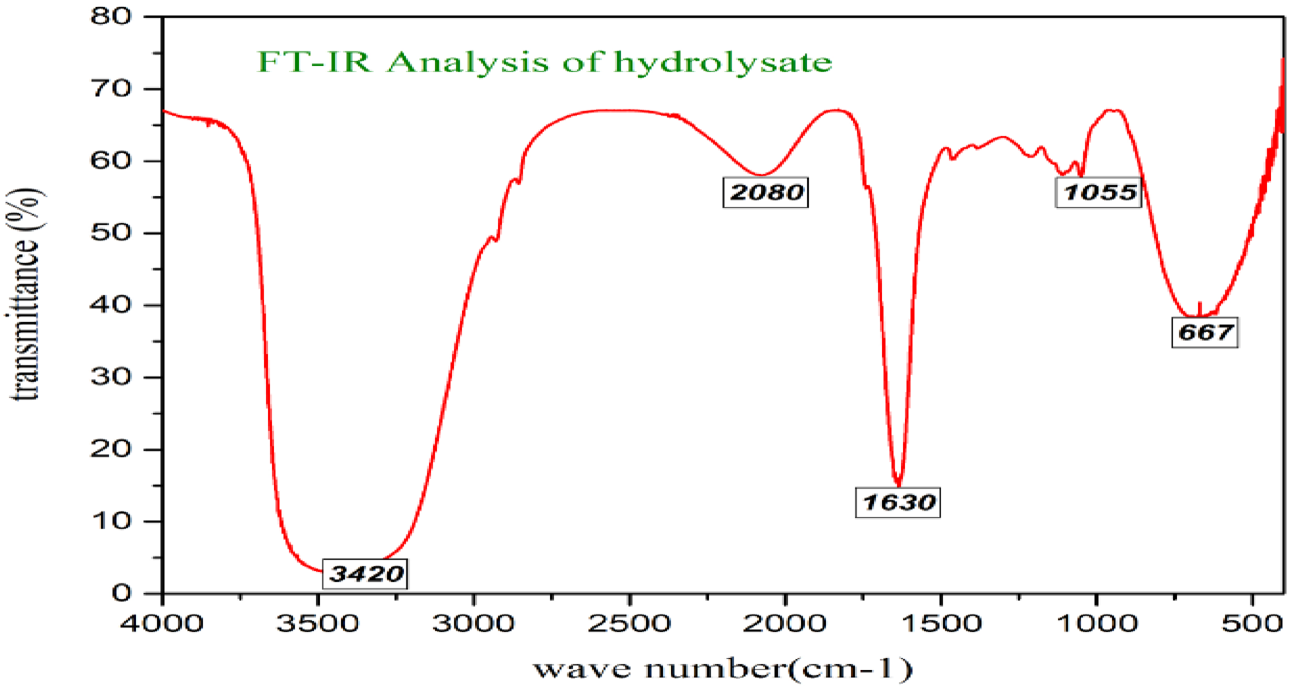


Figure 4-5: FTIR spectra for spent brewery grain after hydrolysis

Table 4-3: Glucose concentration (%) and lactic acid produced at various reaction times, temperature and acid concentration.

| **Run** | **A: Reaction time (min)** | **B:Reaction temperature (ºC)** | **C:Acid concentration (M)** | **Total reducing sugar(%)** | **Lactic acid yield (%)** |
|---|---|---|---|---|---|
| 1 | 35.00 | 130.00 | 1.75 | 52.42 | 23.9 |
| 2 | 30.00 | 130.00 | 2.00 | 51.26 | 27.56 |
| 3 | 25.00 | 122.50 | 2.00 | 43.21 | 24 |
| 4 | 25.00 | 122.50 | 1.50 | 33.75 | 27.19 |
| 5 | 30.00 | 122.50 | 1.75 | 42.63 | 23.9 |
| 6 | 30.00 | 130.00 | 1.50 | 39.85 | 27.78 |
| 7 | 30.00 | 115.00 | 1.50 | 37.65 | 25.63 |
| 8 | 25.00 | 130.00 | 1.75 | 58.65 | 26.51 |
| 9 | 35.00 | 122.50 | 2.00 | 35.22 | 23.89 |
| 10 | 30.00 | 122.50 | 1.75 | 45.11 | 23.56 |
| 11 | 25.00 | 115.00 | 1.75 | 55.98 | 27.61 |
| 12 | 30.00 | 122.50 | 1.75 | 45.23 | 24.29 |
| 13 | 30.00 | 122.50 | 1.75 | 43.46 | 24 |
| 14 | 30.00 | 122.50 | 1.75 | 43.26 | 25.08 |
| 15 | 35.00 | 115.00 | 1.75 | 48.5 | 20.19 |
| 16 | 35.00 | 122.50 | 1.50 | 24.6 | 17.03 |
| 17 | 30.00 | 115.00 | 2.00 | 48.85 | 26.63 |

Table 4.3 shows that the glucose concentration increases with increase in temperature. Based on this, the maximum yield of glucose for 1.75M of acid concentration, at 130°C and a hydrolysis time of 25 min was 58.65%. The glucose concentration was observed to increase at 1.75M acid concentration, (25 – 30) min and (115 – 122.5) ºC temperature. This may be due to the formation of other intermediates products. Table 4.3 shows the variation in the lactic acid yield at different reaction time, temperature and acid concentration. To analyze the experimental results, Design expert® 7.0.0 software was used.

From the Table 4.3, the maximum lactic acid yield of 27.78% was obtained at run 6, at 130℃, 1.5M acid concentration, and at 30 min. While the minimum yield of 17.03% was obtained at run 16, at a temperature of 122.5℃, 2M acid concentration, and 35 min of hydrolysis time. The decrease and increase of the yield depends on the level of factors. There resulting data, from Table 4.3, were analyzed using Design expert® 7.0.0 software to determine the effect of reaction time, temperature and acid concentration. The dependent variable used as a response parameter was the lactic acid yield. All experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to external factors.

Table 4-4: Design summary

| | |
|---|---|
| Study type | Response surface Methodology |
| Initial point | Box-Behnken Design |
| Center point | 5 |
| Design Model | quadratic polynomial |
| Run | 17 |
| Blocks | No |

To determine whether or not the quadratic model is significant, it was vital to perform analysis of variance (ANOVA), (Table 4.5). The probability values (p-values) were used to execute as a device to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the p- values, the bigger the significance of the corresponding coefficient.

## 4.4 Statistical analysis on factors affecting the yield of lactic acid

The experimental design selected for this study is the Box-Behnken Design (BBD) and the response variable measured yield of Box Behnken design are the commonly used experimental design models for three level three factor experiments. Box Behnken designs (BBD) always have three levels for each factor and are purpose built to fit a quadratic model. The Box Behnken design does not have runs at the extreme combinations of all the factors, but compensates by having better prediction precision in the center of the factor space. 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 variable constant. The BBD conditions and their respective responses and the ANOVA are given in Table 4-5 and Table 4-6 respectively.

Table 4-5: Experimental and predicted values

| Diagnostics Case Statistics | | | | |
|---|---|---|---|---|
| Standard Order | Actual Value | Predicted Value | Residual | Leverage |
| 1 | 27.61 | 27.58 | 0.029 | 0.75 |
| 2 | 20.19 | 20.1 | 0.089 | 0.75 |
| 3 | 26.51 | 26.6 | -0.089 | 0.75 |
| 4 | 23.9 | 23.93 | -0.029 | 0.75 |
| 5 | 27.19 | 27.52 | -0.33 | 0.75 |
| 6 | 17.03 | 17.42 | -0.39 | 0.75 |
| 7 | 24 | 23.61 | 0.39 | 0.75 |
| 8 | 23.89 | 23.56 | 0.33 | 0.75 |
| 9 | 25.63 | 25.33 | 0.3 | 0.75 |
| 10 | 27.78 | 27.36 | 0.42 | 0.75 |
| 11 | 26.63 | 27.05 | -0.42 | 0.75 |
| 12 | 27.56 | 27.86 | -0.3 | 0.75 |
| 13 | 23.9 | 24.17 | -0.27 | 0.2 |
| 14 | 24.29 | 24.17 | 0.12 | 0.2 |
| 15 | 25.08 | 24.17 | 0.91 | 0.2 |
| 16 | 23.56 | 24.17 | -0.61 | 0.2 |
| 17 | 24 | 24.17 | -0.17 | 0.2 |

### 4.4.1 Regression Model Equation

The model equation that correlates the response (lactic acid yield) to the hydrolysis process variables in terms of actual value after excluding the insignificant terms is given below. The predicted model for percentage of degree of conversion in terms of the coded factors is given in equation (4.1).

Lactic acid yield $= + 24.17 - 2.54A + 0.71B + 0.56C + 1.20AB + 2.51AC - 0.31BC - 1.74A^2 + 2.13B^2 + 0.60C^2$……………………………………………………...……………………..4.1

Where:

A is the reaction time in min

B is the reaction temperature in ºC

C is the sulfuric acid concentration in Molar

### 4.4.2 Model adequacy check

The model was tested for adequacy by analysis of variance. The regression model was found to be highly significant with the correlation coefficients of determination of R-Squared, adjusted R Squared and predicted R-Squared having a value of 0.9807, 0.9558 and 0.8440 respectively. The quality of the model developed could be evaluated from their coefficients of correlation. The value of R-squared for the developed correlation is 0.9807. It implies that 98.07% of the total variation in the percentage of yield is attributed to the experimental variables studied. The graph of the predicted values obtained using the developed correlation versus actual values is shown in Figure 4-7. The results in Figure 4-7 demonstrated that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in capturing the correlation between the three hydrolysis reaction process variables to the percentage of lactic acid yield. The adequacy of the model was further checked with analysis of variance (ANOVA) as shown in Table 4-6, based on a 95% confidence level, F – value is a test for comparing model variance with residual (error) variance. If the variances are close to the same, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response with the P – value less than 0.05. It is calculated by model mean square divided by residual mean square. Here The Model F-value of 39.43 implies the model is significant. There is only a 0.01% chance that a "Model F Value" this large could occur due to personal error or disturbance.

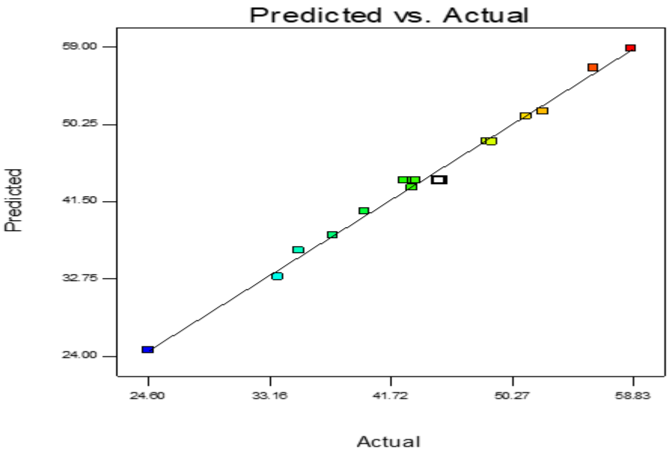


Figure 4.6: Predicted versus actual percentage yield of the lactic acid in hydrolysis of BSG

Table 4-6: ANOVA for the regression model equation and coefficients

| **ANOVA for Response Surface Quadratic Model** | | | | | | |
|---|---|---|---|---|---|---|
| **Analysis of variance table [Partial sum of squares - Type III]** | | | | | | |
| **Source** | **Sum of Squares** | **DF** | **Mean Square** | **F Value** | **p-value Prob > F** | **Remarks** |
| Model | 121.42 | 9 | 13.49 | 39.43 | < 0.0001 | significant |
| A-reaction time | 51.51 | 1 | 51.51 | 150.55 | < 0.0001 | |
| B-reaction temperature | 4.05 | 1 | 4.05 | 11.83 | 0.0108 | |
| C-sulfuric acid concentration | 2.48 | 1 | 2.48 | 7.23 | 0.0311 | |
| AB | 5.78 | 1 | 5.78 | 16.9 | 0.0045 | |
| AC | 25.25 | 1 | 25.25 | 73.8 | < 0.0001 | |
| BC | 0.37 | 1 | 0.37 | 1.09 | 0.3317 | |
| A^2 | 12.79 | 1 | 12.79 | 37.39 | 0.0005 | |
| B^2 | 19.09 | 1 | 19.09 | 55.8 | 0.0001 | |
| C^2 | 1.54 | 1 | 1.54 | 4.5 | 0.0717 | |
| Residual | 2.4 | 7 | 0.34 | | | |
| Lack of Fit | 1.08 | 3 | 0.36 | 1.09 | 0.4485 | not significant |
| Pure Error | 1.32 | 4 | 0.33 | | | |

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC,$A^2$, $B^2$ , $C^2$are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

This shows that the reaction time, reaction temprature and lactic acid concentration, interaction between reaction time and reaction temprature, interaction between reaction time to sulfuric acid concentration, interaction between reaction temprature to sulfuric acid concentration, square of the reaction time,square of reaction temprature and square of sulfuric acid concentration affects the percentage of lactic acid yield significantly.

The "Lack of Fit F-value" of 1.09 implies the Lack of Fit is not significant relative to the pure error.  There is a 44.85% 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.

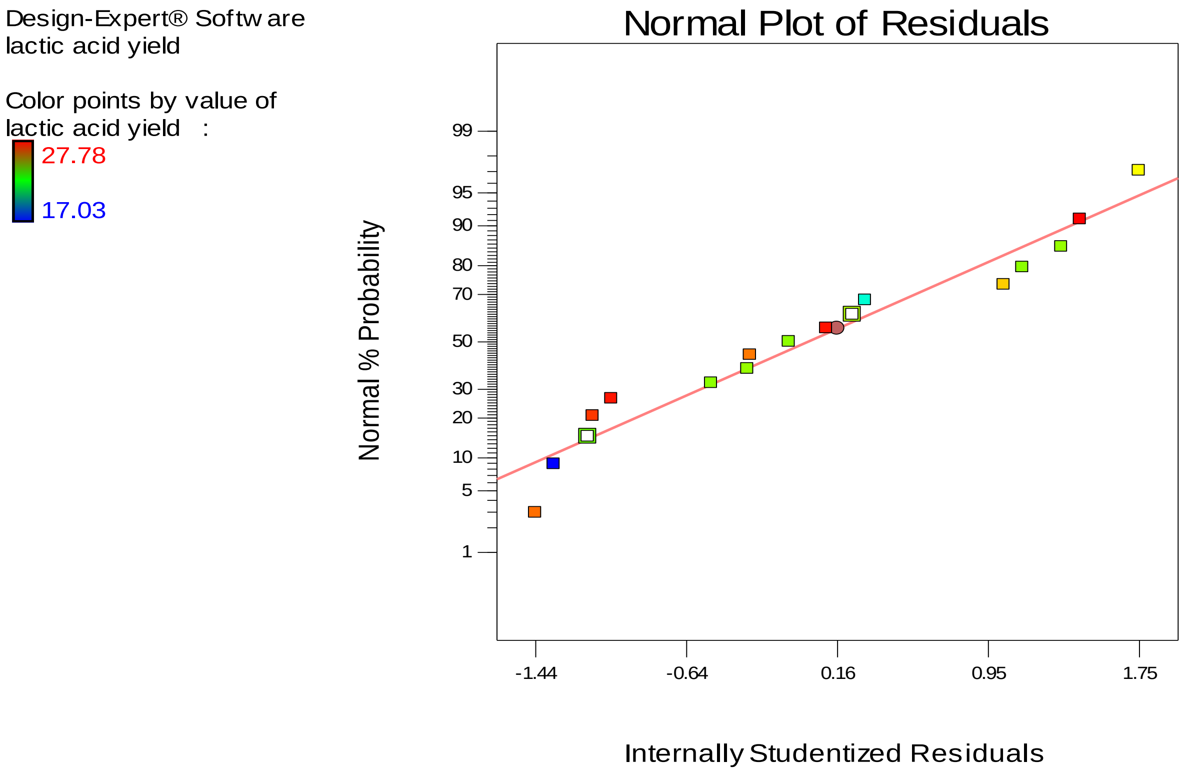


Figure 4-7: Normal plots of residuals

The normal probability plot indicates the residuals following a normal distribution, in which case the points follow a straight line. This shows that the quadratic polynomial model satisfies the assumption of ANOVA. The error distribution is approximately normal.

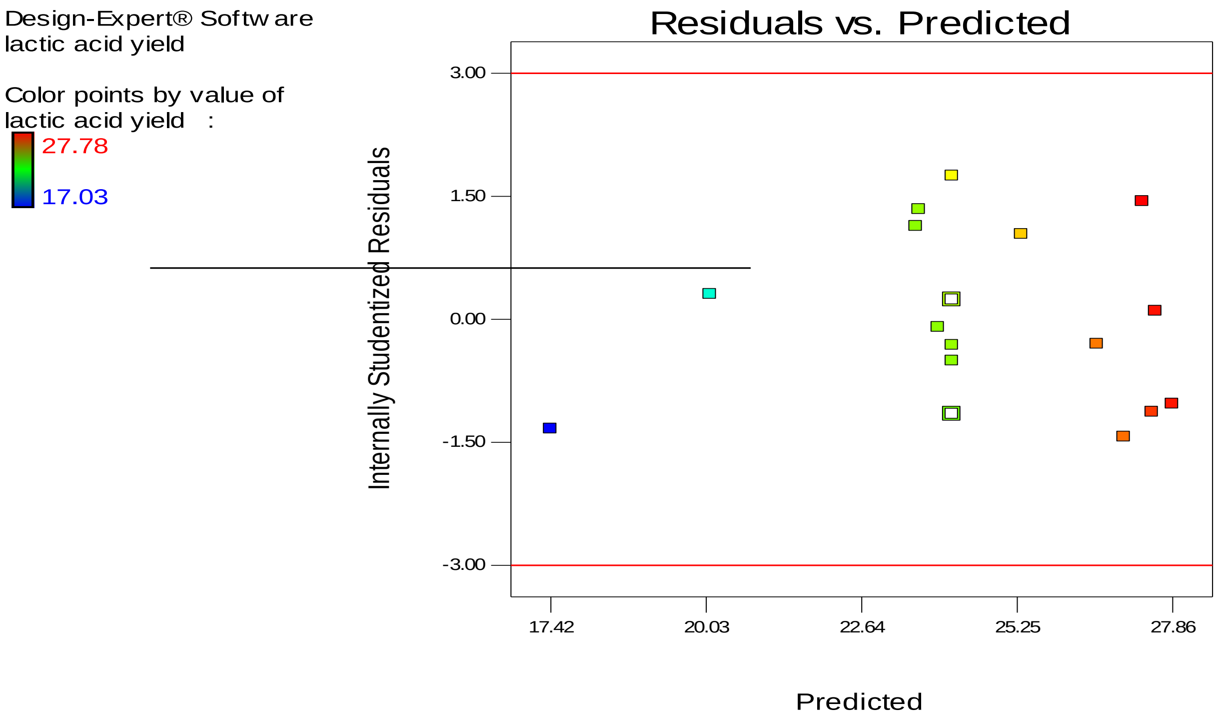


Figure 4-8: Plot of residuals versus model predicted values

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

## 4.5 Effect of Individual Process Variables

The effects of the operating conditions on the lactic acid yield were examined and the optimal values were determined in this study.

### 4.5.1 Effect of time on lactic acid yield

The resulting plot of time versus the lactic acid yield, when Acid concentration and hydrolysis temperature were actual factors, was depicted in Figure 4.10. As shown from the plot increasing reaction time from 25 to 35 minute, lactic acid yield decreased the reason was due to formation of degradation product.

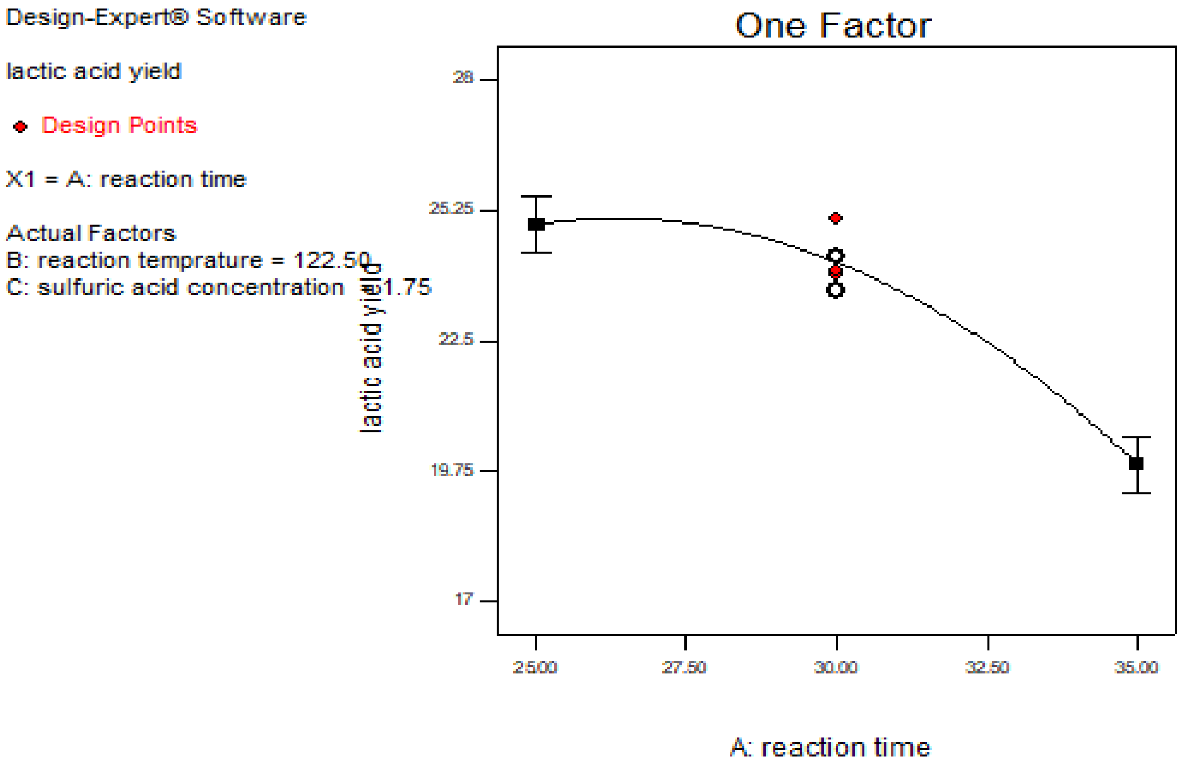


Figure 4-9: Effect of time on the lactic acid yield

### 4.5.2 Effect of temperature on lactic acid yield

The resulting plot of temperature versus the lactic acid yield, when Acid concentration and hydrolysis time were actual factors, was depicted in Figure 4.11. From the plot as temperature increases from 115°C to 122.5°C, lactic acid yield was decreased which is due to further transformation of other by product. From the plot as temperature increases from 122.5°C to 130°C, lactic acid increased to 27.78% by weight. Therefore, the optimum temperature was found to be 130°C and the yield at this temperature was 27.78%.

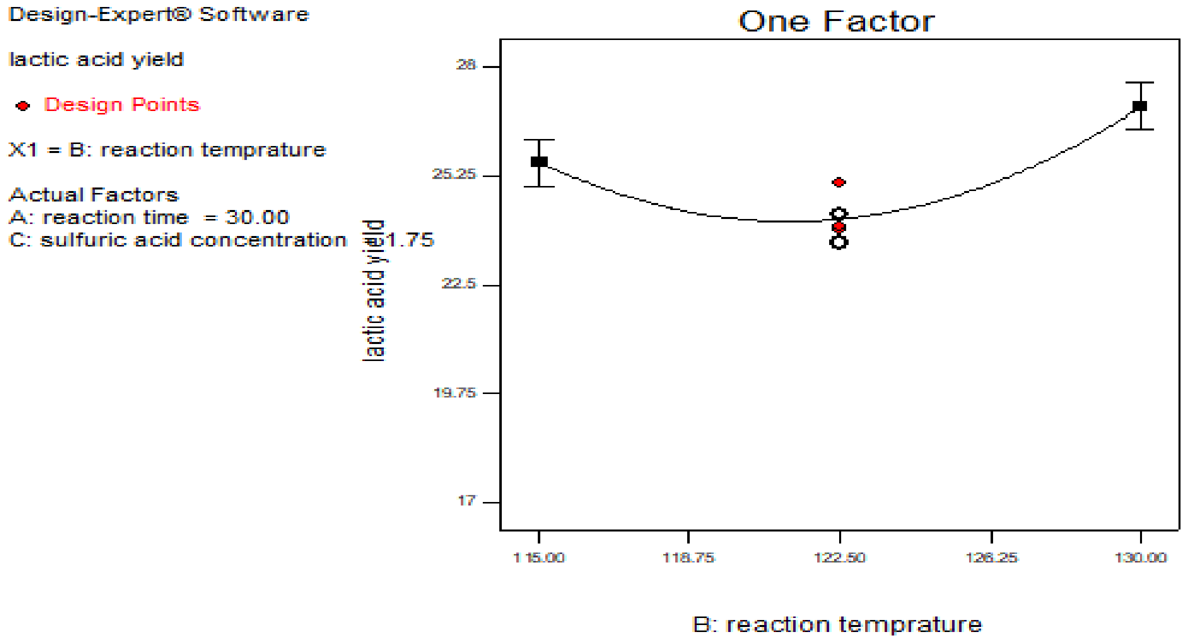


Figure 4-10: Effect of temperature on the lactic acid yield

### 4.5.3 Effect of sulfuric acid concentration on the lactic acid yield

The resulting plot of acid concentration versus the lactic acid yield, when time and hydrolysis temperature were actual factors, was depicted in Figure 4.11. As shown in figure the yield of lactic acid was affected slightly by the acid concentration, as the concentration of acid increase from 1.5M to 2M the yield slightly increases. Therefore, the optimum acid concentration was found to be 1.75M and the yield at this acid concentration was 25.25%.

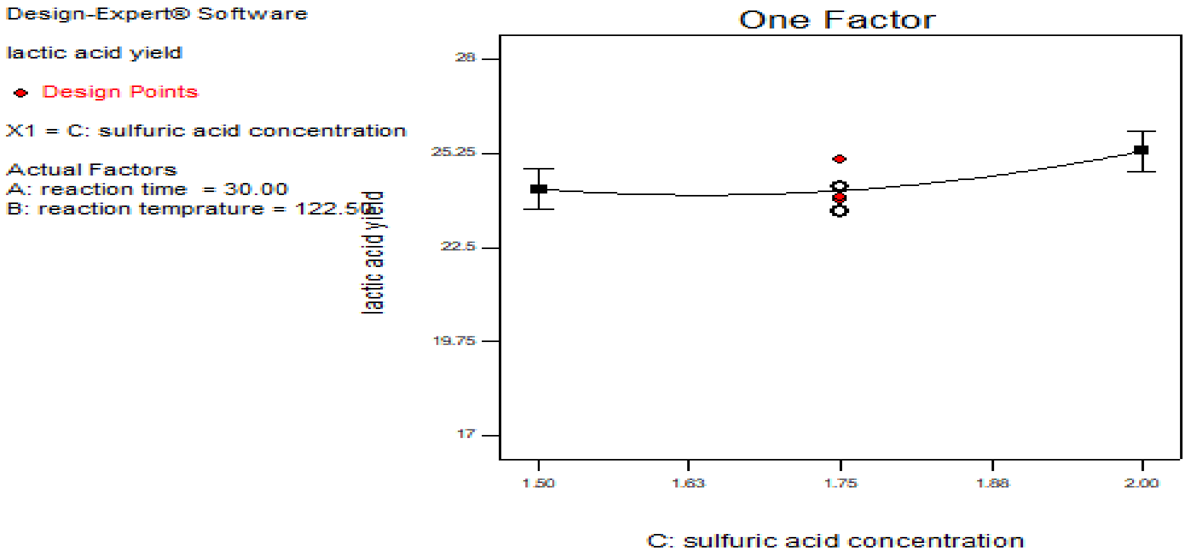


Figure 4-11: Effect of Acid concentration on the lactic acid yield

## 4.6 Response surface and contour plot on the experimental variables

Lactic acid production can be affected by many parameters starting from sample preparation to fermentation, the hydrolysis steps has a complex connection with independent variables. The best way of showing the effects of this parameter for the yield of lactic acid are to generate response surface plots of the equation. In order to analyze the regression equation of the model, three-dimensional surface and 2D contour plots were obtained by plotting the response on the Z axis against any two variables while keeping the other variable at center level. These plots are created to analyze the change in the response surface. Conical shape response surface plot indicates optimum operating conditions. The response optimized value for the production of lactic acid was based on the two process variables described on the response surface plot. The effect of the independent variables and their mutual interaction on the yield of lactic acid can be seen in Figures below.

Interaction effects are effects that independent variable enact on one another. All controllable factors are obvious variables which affect the output of the response variable. In this research, there are three controllable factors in the acid hydrolysis step, namely: hydrolysis time, hydrolysis temperature and sulfuric acid concentration. The main effects of sulfuric acid concentration and hydrolysis time depended on the level of hydrolysis temperature.

### 4.6.1 The Effects of Temperature and Time

As it observed from the temperature and time have an interaction on the lactic acid yield and it has maximum effect on the yield of lactic acid at a time of 27.5 min until the temperature reach 122.5ºC. Beyond this, temperature 122.5ºC the yield of lactic acid was gradually decreased. This might be due to the cause that the glucose was not converted to lactic acid. The maximum amount is found in between the high and low temperature. At fixed hydrolysis time at low level of temperature the amount of lactic acid yield is slightly higher. At high level of hydrolysis temperature, the amount of lactic acid yield is low compared to the middle maximum point. This might be due to the reason that reducing sugar was converted to other by products.

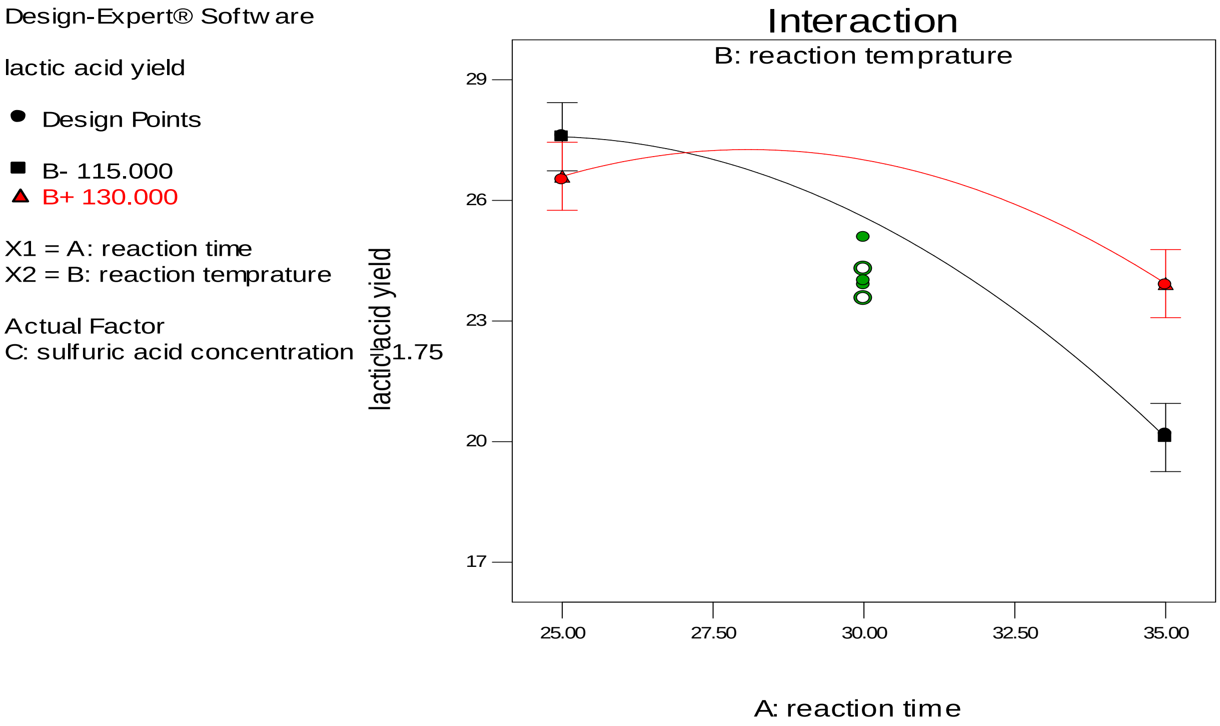


Figure 4-12: Effect and interaction of temperature and time on the yield of lactic acid at center of sulfuric acid concentration

Figure 4.13 and 4.14 shows the contour plots and response surface respectively developed as a function of temperature and time, while the acid concentration was kept constant at 1.75M. It was observed that the yield of lactic acid was changed, when the temperature change from 122.5 ºC to 130 ºC and time changes from 25min to 30min the yield of lactic acid reaches at the peak and beyond 122.5 ºC and time beyond 30min the yield of lactic acid was slightly decreased. The reason for this observation is due to the reason that when the glucose exposes to high temperature and longer time, the sugar which obtained from hydrolysed sugar degraded into not fermentable product and gives low yield of lactic acid.

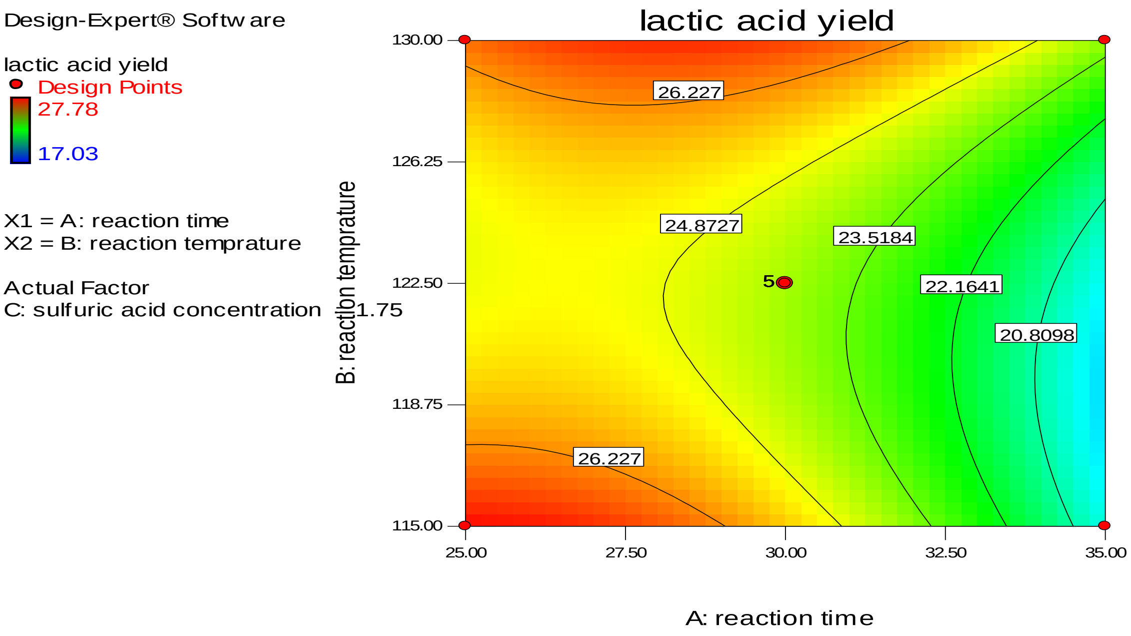


Figure 4-13: Contour plots of the effect of temperature and time on the lactic acid yield at constant sulfuric acid concentration

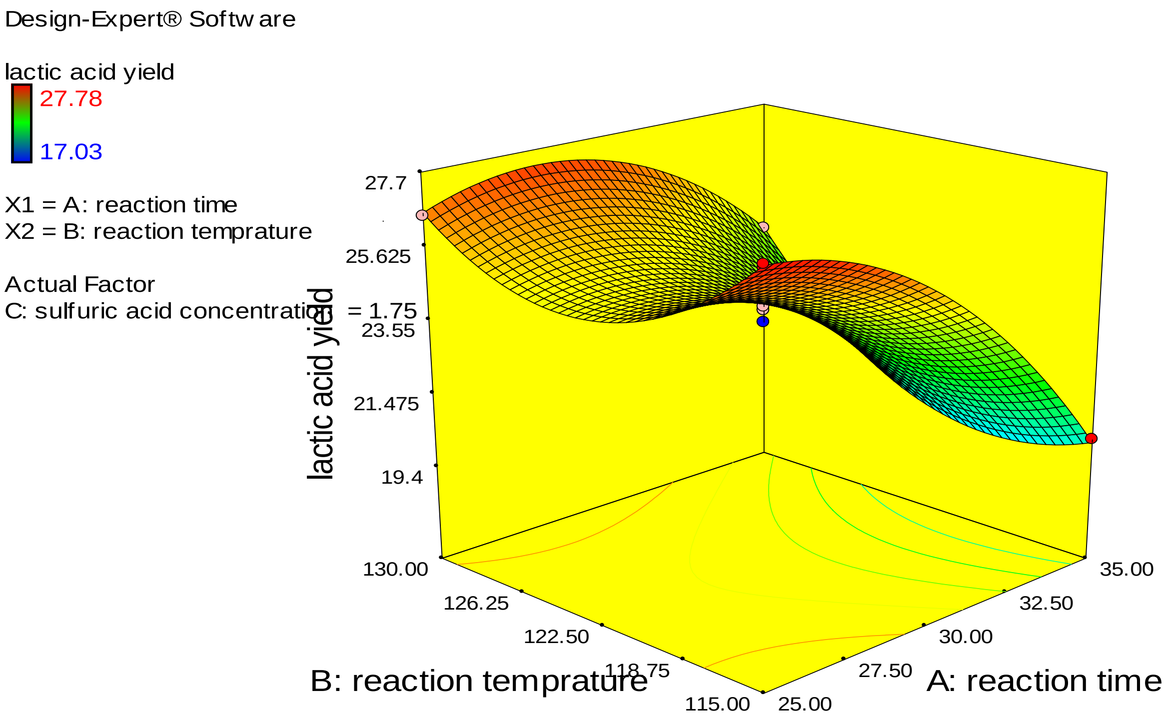


Figure 4-14: Response surface plots of the effect of temperature and time on the lactic acid yield at Constant sulfuric acid concentration.

### 4.6.2 The Effects of sulfuric acid concentration and time

The graph in Figure 4.15 shows the interaction effect of sulfuric acid concentration and reaction time on lactic acid yield by keeping the temperature at122.5 ºC. The graph shows the effect of sulfuric acid concentration on lactic acid yield at fixed time when the temperature held at center point. The red and black color shows the high and low level of time. When the acid concentration increases at a time of 30min the amount of lactic acid yield decreases. As it observed from figure 4.16, the acid concentration and time have an interaction effect on the lactic acid yield and it has maximum effect on the yield of lactic acid at a moderate time until the acid concentration reach 1.75M and the reaction time was 29 min. But, beyond 1.75 M acid concentration at fixed time the yield of lactic acid decreases due to the fact that the reducing sugar is decomposed in to unwanted product. This could be due to the reason that the amount of simple sugar is low at low level of the acid concentration and time. At high level of acid concentration and time, the amount of lactic acid yield is lower compared to the lower and middle point.

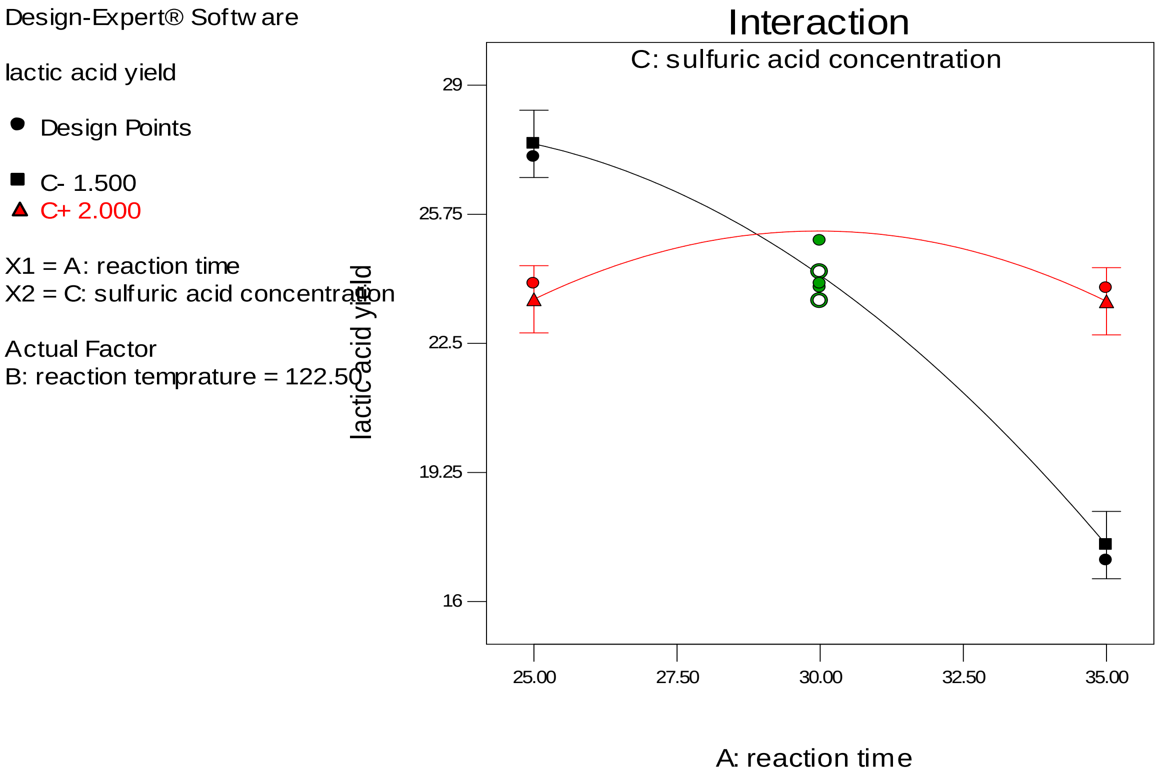


Figure 4-15: Interaction effects of acid concentration and time on the yield of lactic acid, when the temperature was constant

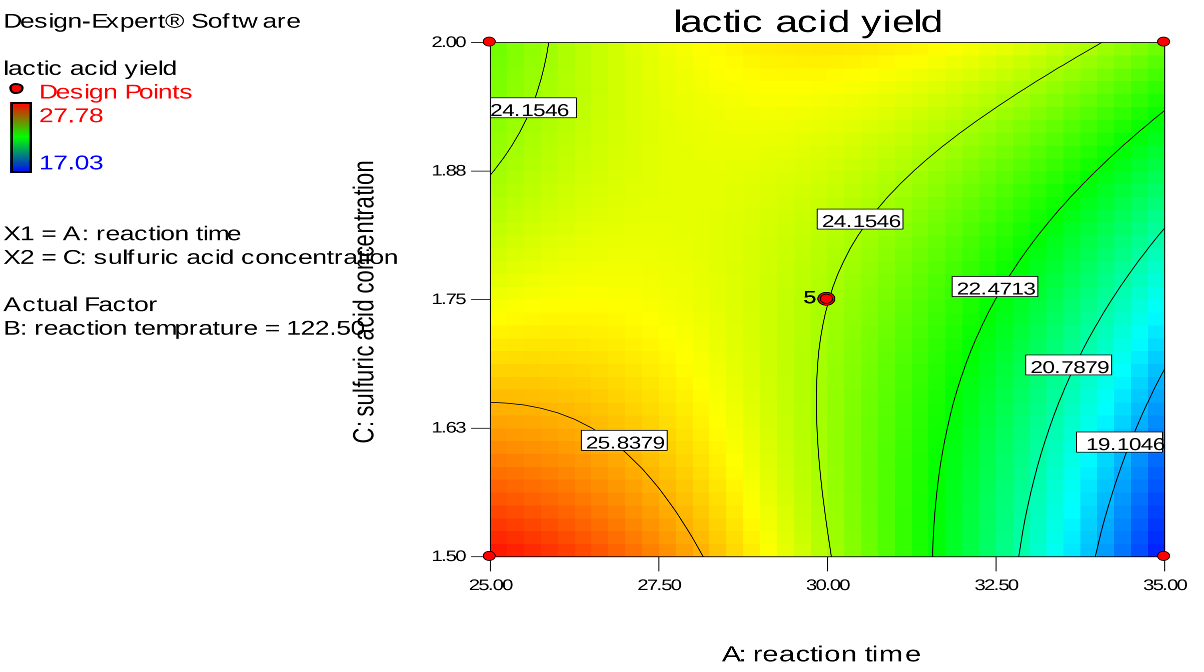


Figure 4-16: Contour plot of the effect of acid concentration and time on the yield of lactic acid at constant temperature

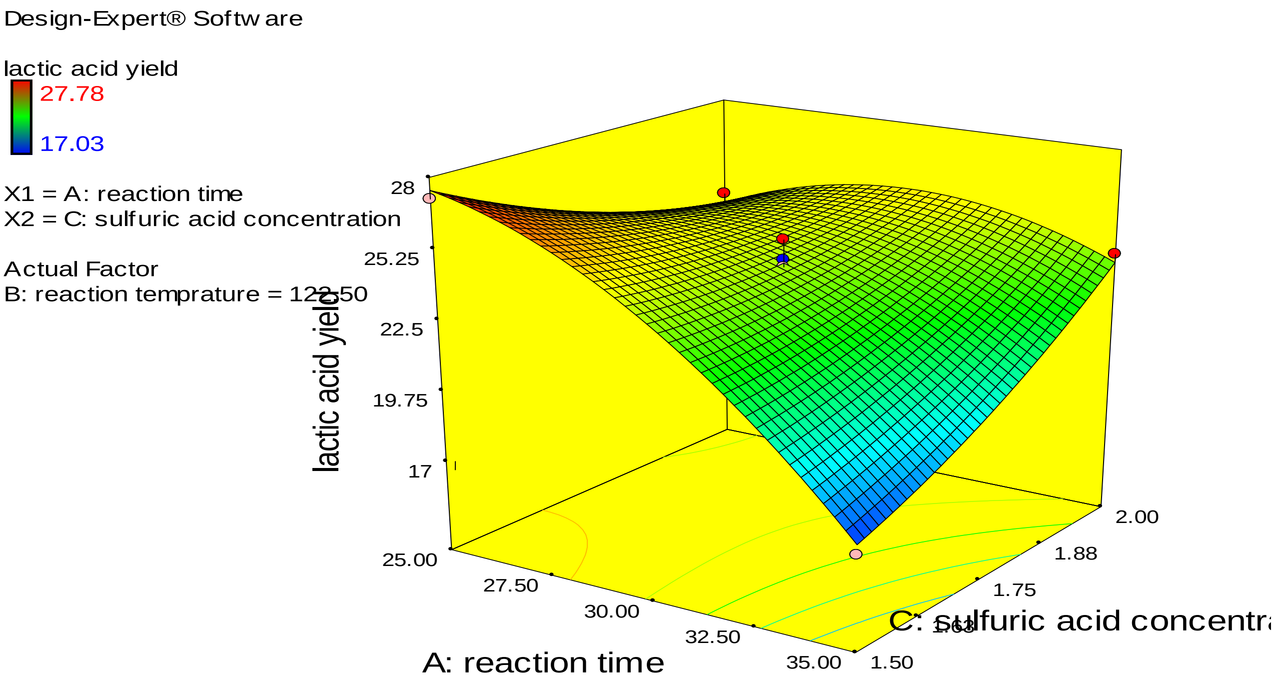


Figure 4-17: Response surface plot of the effect of acid concentration and time on the yield of lactic acid at constant temperature

Figure 4.16 and 4.17 shows the contour plots and response surface developed as a function of sulfuric acid concentration and time respectively, while the temperature was kept constant at 122.5ºC. Upon increasing the acid concentration 1.5M to 1.75M with a decrease of hydrolysis time from 35min to 25min, the yield of lactic acid increased. The highest yield was obtained at 30min of hydrolysis time and 1.75M sulfuric acid concentration.

### 4.6.3 Effect of Temperature and Acid concentration

The figure 4.18 shows of temperature and acid concentration on the lactic acid yield when time was at the center point. As it observed from figure the temperature and sulfuric acid concentration have no interaction on the lactic acid yield and it has maximum effect on the yield of lactic acid at higher and lower acid concentration when the temperature reach 115ºC and 130 ºC respectively. At the middle level of sulfuric acid concentration the amount of lactic acid yield is lower compared to lower and higher acid concentration.

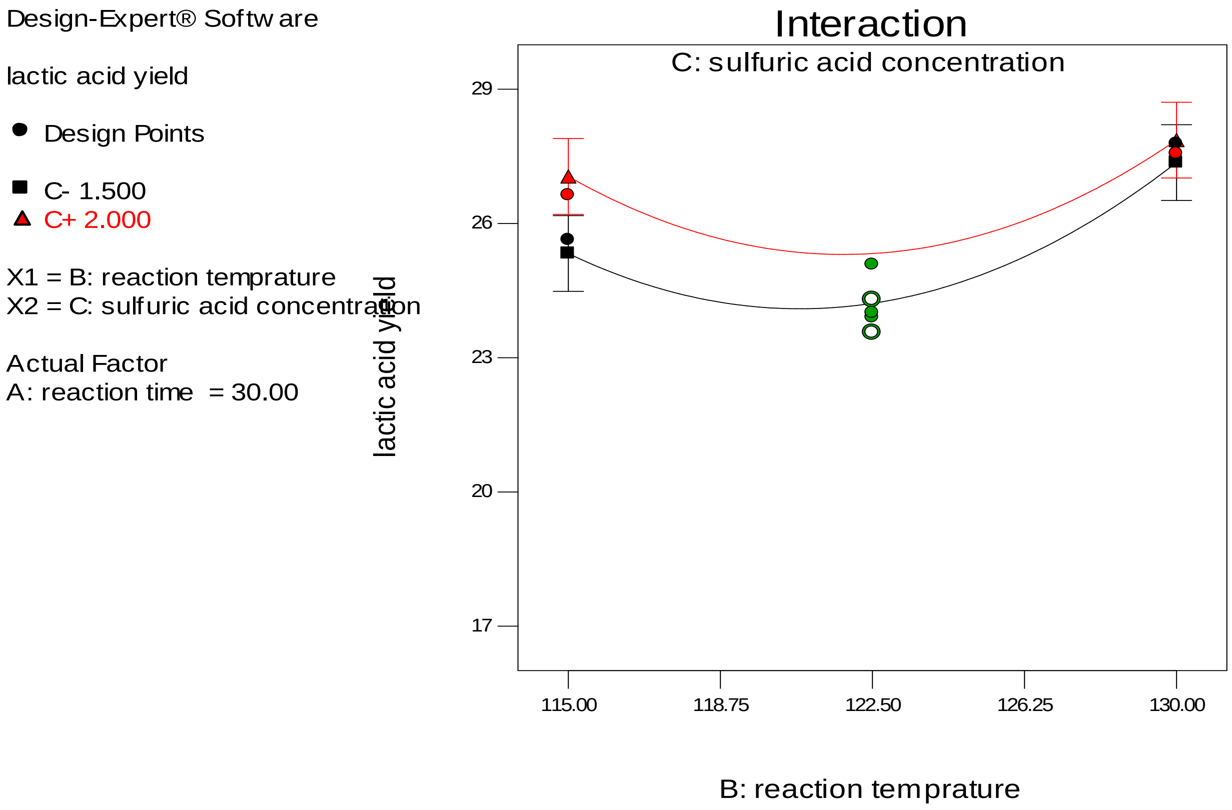


Figure 4-18: Effect and Interaction of temperature and sulfuric acid concentration on the yield of lactic acid at center of time

Figure 4.19 and 4.20 represents the contour plot and response surface developed as a function of temperature and sulfuric acid concentration respectively, while time was kept constant at 30min. the yield of lactic acid increased slightly with hydrolysis temperature from 115 to 122.5ºC and acid concentration from 1.5M to 1.75M. However, upon increasing the hydrolysis temperature beyond 122.5 ºC, and acid concentration beyond 1.75M there was a gradual decline in the yield, because reducing sugar degrade might be occurred.

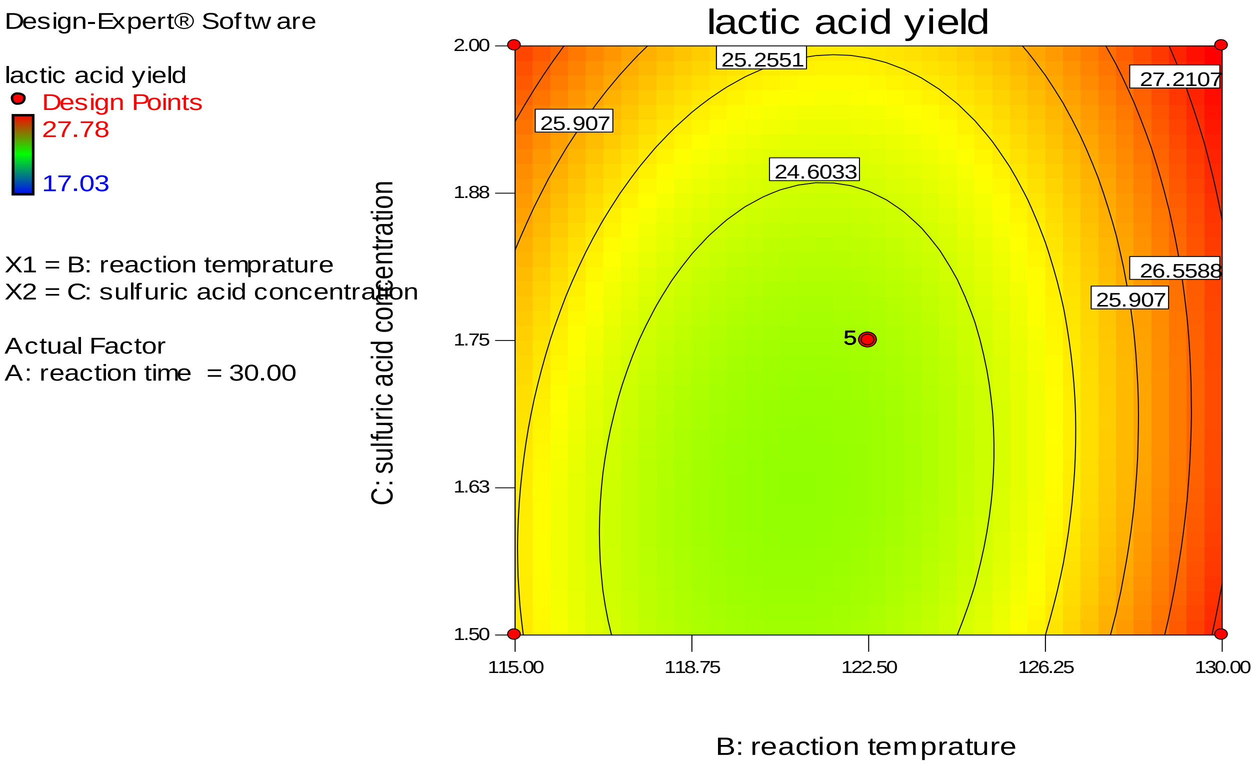


Figure 4-19: Contour plot of the effect of temperature and acid concentration at constant time

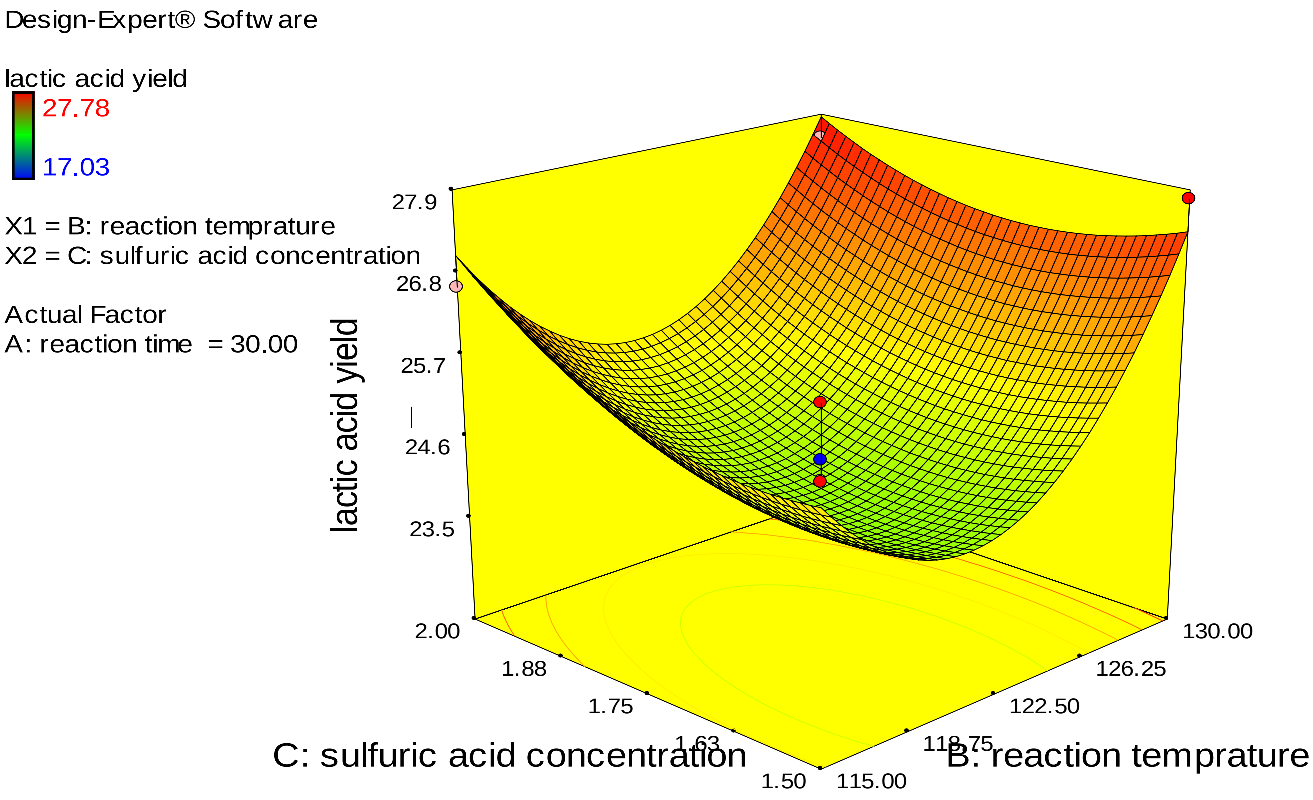


Figure 4-20: Response surface plot of the effect of temperature and sulfuric acid concentration at constant time

## 4.7 Optimization of operating process variables in hydrolysis process using RSM

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. It also has important applications in the design, development, and formulation of new products, as well as in the improvement of existing product designs. Optimization of lactic acid yield was carried out by a multiple response method called desirability (D) function to optimize different combinations of process parameters. The goal of optimization was to maximize economic benefit or increasing lactic acid yield by minimizing process cost. The optimization of hydrolysis criteria for lactic acid production from Brewery spent grain using *lactobacillus plantarum* are summarized as follows:

Table 4-7: Constraints applied for optimization

| Name | Goal | Lower Limit | Upper Limit | Lower Weight | Upper Weight | Importance |
|---|---|---|---|---|---|---|
| Reaction time (min) | is in range | 25 | 35 | 1 | 1 | 3 |
| Reaction temperature(ºC) | is in range | 115 | 130 | 1 | 1 | 3 |
| Sulfuric acid concentration (M) | is in range | 1.5 | 2 | 1 | 1 | 3 |

By using the numerical optimization criteria in Table 4.7, the design expert solution was obtained. The possible solution for this model with the given factors that change the amount of the produced lactic acid yield and reducing sugar concentration is shown in Table 4.8. The optimum possible solutions in hydrolysis of brewery spent grain for yield of maximum of concentration reducing sugar and lactic acid are presented in Table 4.8

Table 4-8: Optimum possible solutions

| No | Reaction time(min) | Reaction temperature(ºC) | Sulfuric acid concentration(M) | Desirability | Remark |
|---|---|---|---|---|---|
| 1 | 34.78 | 116.58 | 1.51 | 1 | |
| 2 | 34.04 | 120.31 | 1.62 | 1 | |
| 3 | 31.63 | 118.53 | 1.62 | 1 | |
| 4 | 34.16 | 127.18 | 1.94 | 1 | |
| 5 | 27.63 | 120.32 | 1.93 | 1 | |
| 6 | 33.96 | 129.45 | 1.62 | 1 | |
| 7 | 29.47 | 127.2 | 1.97 | 1 | |
| 8 | 32.85 | 120.14 | 1.8 | 1 | |
| 9 | 34.83 | 115.8 | 1.81 | 1 | |
| 10 | 32.39 | 129.75 | 1.89 | 1 | Selected |
| 11 | 28.21 | 128.38 | 1.56 | 1 | |
| 12 | 29.71 | 118.63 | 1.58 | 1 | |
| 13 | 26.71 | 116.8 | 2 | 1 | |
| 14 | 29.77 | 120.53 | 1.61 | 1 | |
| 15 | 25.25 | 119.27 | 1.86 | 1 | |

| | | | | | |
|---|---|---|---|---|---|
| 16 | 29.33 | 125.53 | 1.89 | 1 | |
| 17 | 26.96 | 125.76 | 1.59 | 1 | |
| 18 | 34.77 | 124.76 | 1.54 | 1 | |
| 19 | 26.81 | 116.6 | 1.98 | 1 | |
| 20 | 33.34 | 119.1 | 1.59 | 1 | |
| 21 | 27.5 | 125.14 | 1.99 | 1 | |
| 22 | 30.3 | 122.53 | 1.6 | 1 | |
| 23 | 26.39 | 116.57 | 1.86 | 1 | |
| 24 | 28.08 | 129.1 | 1.63 | 1 | |
| 25 | 32.36 | 118.04 | 1.5 | 1 | |
| 26 | 34.32 | 128 | 1.63 | 1 | |
| 27 | 34.06 | 126.36 | 1.77 | 1 | |
| 28 | 30.74 | 125.08 | 1.62 | 1 | |
| 29 | 25.35 | 121.73 | 1.71 | 1 | |
| 30 | 28.89 | 124.39 | 1.81 | 1 | |

Based on the above analysis best local maximum for reducing sugar and lactic acid yield 53.07 and 26.71% was predicted at acid concentration 1.89M, temperature 129.75°C and time 32.39 minutes and the value of desirability obtained was 100%. To validate the optimum conditions predicted by the model using desirability ramp, triplicate experiments were conducted using the optimized hydrolysis process parameters and mean percentage conversion value of 24.89% was obtained and the results are closely related with the data obtained from optimization analysis using desirability functions.

### 4.7.1 Validation of the model

According to the Box Behenken design result using Design-Expert® 7 software, an experiment with hydrolysis time, temperature and sulfuric acid concentration were conducted in order to study the outcome or effect of the design. The experiment was carried out at the optimized conditions. Based on the second-order models, numerical optimization was carried out to maximize the yield of lactic acid, using the response optimizer in Design expert®7. The optimal values test factors were 1.89M Acid concentration, 129.75°C temperature and 32.39 min reaction time (obtained from Table 4.8). Lactic acid yield of 26.71% obtained and was in good agreement with the predicted one. Therefore the model is considered to be accurate and reliable for predicting the yield of lactic acid.

# 5. CONCLUSION AND RECOMMENDATION

## 5.1 Conclusion

Lactic acid obtained from BSG using *lactobacillus plantarum* is preferred for industrial applications, especially, bioplastic industry. Lactic acid bacteria are good organisms for lactic acid fermentation. *Lactobacillus plantarum* is a homo-fermentative LAB used extensively in this study. It exhibited more than 27.78% lactic acid production yield which is desirable for large scale lactic acid production. The intention of the present investigation was to study growth and lactic acid production at different environments, reaction time, reaction temperatures, and sulfuric acid concentration values.This study examines the possibility of brewery spent grain for lactic acid production. The conversion of brewery spent grain to lactic acid was carried out with pretreatment, dilute acid hydrolysis, microorganism and inoculum cultivation, fermentation and purification and recovery process steps.

Characterization of the BSG was performed using the ASTM, AOAC and FTIR. From the result, it was observed that BSG contains high amount of cellulose and hemicellulose and O-H (Alcohol), N-H (amine), C-H (Alkane), C=C (alkene), -C-H (Alkane), C-F (alkyl halide) and C-O (Alcohol) functional groups; which confirm the presence of high carbon content in the raw BSG.

The experimental design was conducted by Box Behnken design (BBD) to study the effects of three variables, time (25, 30, and 35minute), temperature (115, 122.5, and 130°C) and sulfuric acid concentration (1.5, 1.75, and 2M). The optimum operating condition was found to be at a temperature of 130°C, acid concentration 1.75M, and a hydrolysis time of 25minutes. At these optimum operating conditions the maximum yield of reducing sugar and lactic acid was found to be 39.85 % and 27.78% respectively. Quadratic model was employed to correlate the operating variables with the response. From the analysis of variance, the interaction between reaction temperature and sulfuric acid concentration have significance effect on the yield of lactic acid by using F-test ($p<0.05$). The maximum, observed, value of lactic acid productivity recorded was 27.78% and this is in a good agreement with the predicted value of 27.36%. Based on this study, it is evident that the chosen method of optimization was efficient, and reliable. From this result, it can be concluded that Barley spent grain (BSG) has the potential to serve as a low-cost feedstock for the production of lactic acid by using *lactobacillus plantarum* and sulfuric acid hydrolysis process is very effective.

## 5.2 Recommendation

The current major markets for lactic acid are food-related industries, but the emerging markets for PLA polymer would cause a significant increase in growth of lactic acid consumption. Currently, the worldwide consumption of lactic acid is estimated to be 130,000-150,000 (metric) tons per year, and the commercial prices of food grade lactic acid range between 1.38 US$/kg(for 50 % purity) and 1.54 US$/kg (for 88 % purity). Technical grade lactic acid with 88 % purity has been priced as much as 1.59 US$/kg.

Based on the current investigation the following recommendations are forwarded:

- ♣ Further researches have to be carried out to increase the yield of lactic acid from Brewery spent grain by using other microorganisms which are capable of converting 5- and 6-carbon sugar into lactic acid.
- ♣ Optimization of the Fermentation processes parameters are recommended to maximize the yield of lactic acid from BSG.
- ♣ Alternative purification and recovery methods of lactic acid such as adsorption, solvent extraction, electrodialysis(ED) and membrane separation need to be done in order to investigate the variation that could be arise on the quality and quantity of the lactic acid yield as a result of using different purification and recovery methods.
- ♣ BSG is rich in cellulose and hemicelluloses, due to this it is used as a low valuable raw material in different applications, such as for production of xylitol, poly lactic acid, biogas and bioethanol. But in developing country this valuable by-product is used as low-cost animal feed and the remaining are deposited into landfills. Further research is required to utilize this largely available by-product, Brewery Spent Grain.

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# APPENDICES

## Appendix A: IR-Spectroscopy: Functional Group Identification

Infrared spectroscopy (IR spectroscopy or Vibrational Spectroscopy) is the spectroscopy that deals with the infrared region of the electromagnetic spectrum that is light with a longer wavelength and lower frequency than visible light. It covers a range of techniques, mostly based on absorption spectroscopy.

The exact frequency at which a given vibration occurs is determined by the strengths of the bonds involved and the mass of the component atoms. For a more detailed discussion of these factors. In practice, infrared spectra do not normally display separate absorption signals for each of the 3n-6 fundamental vibrational modes of a molecule. The number of observed absorptions may be increased by additive and subtractive interactions leading to combination tones and overtones of the fundamental vibrations, in much the same way that sound vibrations from a musical instrument interact. Furthermore, the number of observed absorptions may be decreased by molecular symmetry, spectrometer limitations, and spectroscopic selection rules. One selection rule that influences the intensity of infrared absorptions, is that a change in dipole moment should occur for a vibration to absorb infrared energy. Absorption bands associated with C=O bond stretching are usually very strong because a large change in the dipole takes place in that mode.

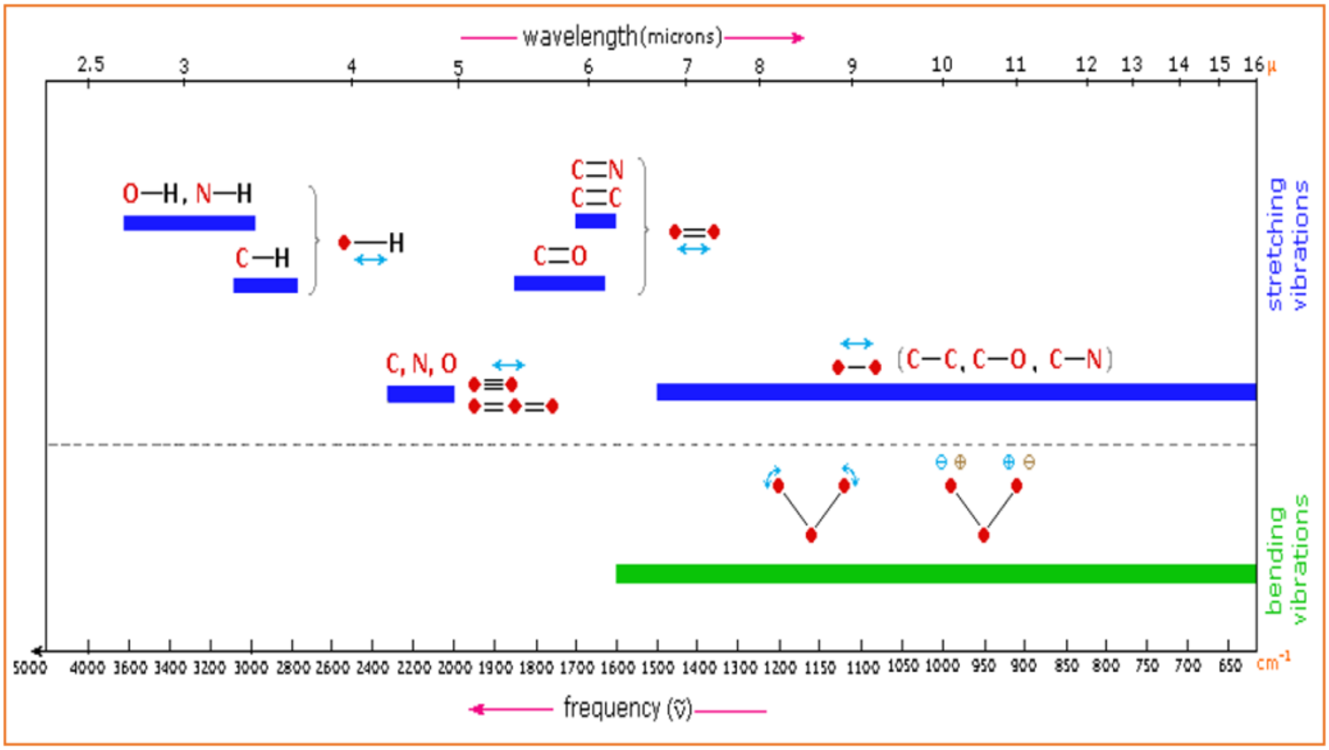

Table A1: Characteristic IR absorption frequencies of organic functional groups

| Functional Group Names | Absorption Ranges(cm-1) | Type of Vibration causing IR absorption |
|---|---|---|
| Alkanes | 3000-2800 | H-C-H asymmetric & symmetric stretch |
| | 1500-1440 | H-C-H bend |
| Alkenes | 3100-3000 | C=C-H asymmetric stretch |
| | 1675-1600 | C-C=C symmetric stretch |
| Alkynes | 3300-3200 | C-H stretch |
| | 2200-2100 | C-C stretch |
| Aromatic Rings | 3100-3000 | C=C-H asymmetric Stretch |
| | 1600-1580 | C-C=C symmetric stretch |
| | 1500-1450 | C-C=C asymmetric stretch |
| Phenols & alcohols | 3600-3100 | Hydrogen-bonded O-H stretch |
| Carboxylic Acids | 3400-2400 | Hydrogen-bonded O-H stretch |
| | 1730-1650 | C=O stretch |
| Ketones | 1750-1625 | C=O stretch |
| Aldehydes | 1750-1625 | C=O stretch |
| | 2850-2800 | C-H stretch off C=O |
| | 2750-2700 | C-H stretch off C=O |
| Esters | 1755-1650 | C=O stretch |
| | 1300-1000 | C-O stretch |
| Amines-Primary | 3500-3100 | N-H stretch |
| | 1640-1560 | N-H bend |
| Amines-Secondary | 3500-3100 | N-H stretch |
| Nitriles | 2300-2200 | C-N stretch |
| Nitro Groups | 1600-1500 | N=O stretch |
| | 1400-1300 | N=O bend |
| Amides | 3500-3100 | N-H stretch |
| | 1670-1600 | C=O stretch |
| | 1640-1550 | N-H bend |

## Appendix B: Data analysis by design expert 7.0.0 software

Table: B1 Box Behnken design for hydrolysis

| Std | run | factor 1 reaction time | factor 2 reaction temperature ºC | factor 3 acid concentration Molar | response 1 total reducing sugar % | response 2 lactic acid yield % |
|---|---|---|---|---|---|---|
| 1 | 11 | 25 | 115 | 1.75 | 55.98 | 27.61 |
| 2 | 15 | 35 | 115 | 1.75 | 48.5 | 20.19 |
| 3 | 8 | 25 | 130 | 1.75 | 58.65 | 26.51 |
| 4 | 1 | 35 | 130 | 1.75 | 52.42 | 23.90 |
| 5 | 4 | 25 | 122.5 | 1.5 | 33.75 | 27.19 |
| 6 | 16 | 35 | 122.5 | 1.5 | 24.6 | 17.03 |
| 7 | 3 | 25 | 122.5 | 2 | 43.21 | 24.00 |
| 8 | 9 | 35 | 122.5 | 2 | 35.22 | 23.89 |
| 9 | 7 | 30 | 115 | 1.5 | 37.65 | 25.63 |
| 10 | 6 | 30 | 130 | 1.5 | 39.85 | 27.78 |
| 11 | 17 | 30 | 115 | 2 | 48.85 | 26.63 |
| 12 | 2 | 30 | 130 | 2 | 51.26 | 27.56 |
| 13 | 5 | 30 | 122.5 | 1.75 | 42.63 | 23.90 |
| 14 | 12 | 30 | 122.5 | 1.75 | 45.23 | 24.29 |
| 15 | 14 | 30 | 122.5 | 1.75 | 43.26 | 25.08 |
| 16 | 10 | 30 | 122.5 | 1.75 | 45.11 | 23.56 |
| 17 | 13 | 30 | 122.5 | 1.75 | 43.46 | 24.00 |

Table: B2 Analysis of variance for total reducing sugar yield

| Response 1 reducing sugar | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA for Response Surface Quadratic Model | | | | | | |
| Analysis of variance table [Partial sum of squares - Type III] | | | | | | |
| Source | Sum of Squares | DF | Mean Square | F Value | p-value Prob > F | remark |
| Model | 1139.3 | 9 | 126.59 | 108.03 | < 0.0001 | significant |
| A-reaction time | 118.97 | 1 | 118.97 | 101.52 | < 0.0001 | |
| B-reaction temperature | 15.68 | 1 | 15.68 | 13.38 | 0.0081 | |
| C-sulfuric acid concentration | 227.8 | 1 | 227.8 | 194.4 | < 0.0001 | |
| AB | 0.39 | 1 | 0.39 | 0.33 | 0.5818 | |
| AC | 0.34 | 1 | 0.34 | 0.29 | 0.6087 | |
| BC | 0.011 | 1 | 0.011 | 9.41E-03 | 0.9254 | |
| A^2 | 0.07 | 1 | 0.07 | 0.06 | 0.8138 | |
| B^2 | 427.69 | 1 | 427.69 | 364.98 | < 0.0001 | |
| C^2 | 389.17 | 1 | 389.17 | 332.11 | < 0.0001 | |
| Residual | 8.2 | 7 | 1.17 | | | |
| Lack of Fit | 2.76 | 3 | 0.92 | 0.68 | 0.6104 | not significant |
| Pure Error | 5.44 | 4 | 1.36 | | | |
| Cor Total | 1147.5 | 16 | | | | |

Table: B3 Analysis of variance for lactic acid yield

| Response 2 lactic acid yield | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA for Response Surface Quadratic Model | | | | | | |
| Analysis of variance table [Partial sum of squares - Type III] | | | | | | |
| Source | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | remark |
| Model | 121.42 | 9 | 13.49 | 39.43 | < 0.0001 | significant |
| A-reaction time | 51.51 | 1 | 51.51 | 150.55 | < 0.0001 | |
| B-reaction temprature | 4.05 | 1 | 4.05 | 11.83 | 0.0108 | |
| C-sulfuric acid concentration | 2.48 | 1 | 2.48 | 7.23 | 0.0311 | |
| AB | 5.78 | 1 | 5.78 | 16.9 | 0.0045 | |
| AC | 25.25 | 1 | 25.25 | 73.8 | < 0.0001 | |
| BC | 0.37 | 1 | 0.37 | 1.09E+00 | 0.3317 | |
| A^2 | 12.79 | 1 | 12.79 | 37.39 | 0.0005 | |
| B^2 | 19.09 | 1 | 19.09 | 55.8 | 0.0001 | |
| C^2 | 1.54 | 1 | 1.54 | 4.5 | 0.0717 | |
| Residual | 2.4 | 7 | 0.34 | | | |
| Lack of Fit | 1.08 | 3 | 0.36 | 1.09 | 0.4485 | not significant |
| Pure Error | 1.32 | 4 | 0.33 | | | |
| Cor Total | 123.82 | 16 | | | | |

## Appendix C: Laboratory equipment and sample photos

Fig: 1 Raw BSG

Fig: 2 treated spent grain

Fig: 3 grind and sieved TSG

Fig 4: characterization of BSG

Fig: 5 hydrolysis of BSG

Fig: 6 chemicals for media preparation

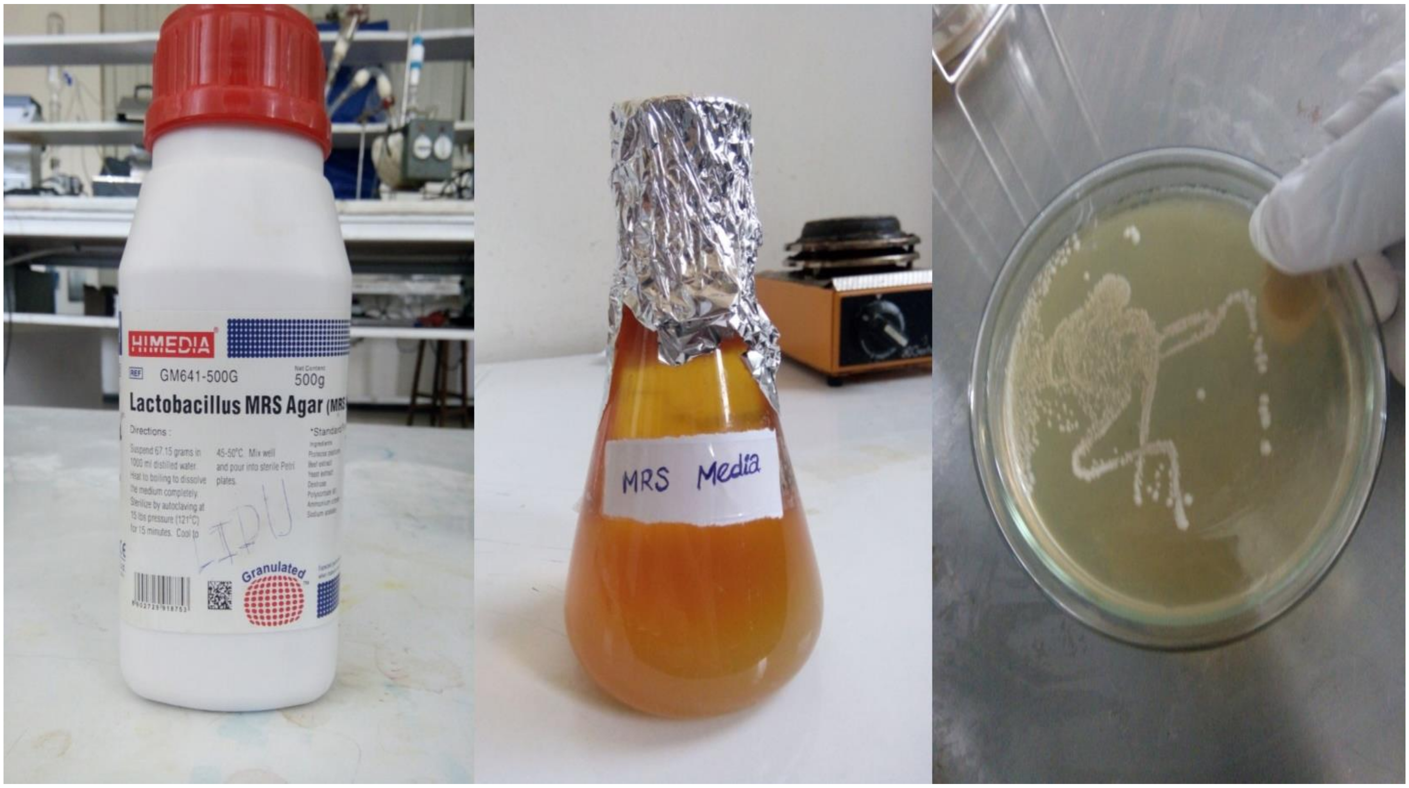

Fig: 7 isolation of lactobacillus plantaum in solid media

Fig: 8 mass growth of lactobacillus plantarum in liquid media

Fig: 9 fermentation medium preparation and sterilization

Fig: 10 fermentation and characterization of lactic acid