Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio Engineering

(Environmental Engineering stream)

# Production of lactic acid from sugarcane bagasse

A Thesis Submitted to School of Chemical and Bio Engineering, Addis Ababa Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemical Engineering (Environmental Engineering)

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This is to certify that the thesis prepared by Mahamedberhan Edris, entitled "Production of lactic acid from sugarcane bagasse" and submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering (Environmental Engineering stream) complies with the regulation of the university and meets the accepted standards with respect to originality and quality.

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# Declaration

This thesis is prepared by Mahamedberhan Edris, entitled "Production of lactic acid from sugarcane bagasse*"* and submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering (Environmental Engineering stream) I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this or any other university, but it includes other work as a reference sources such as articles and which have been fully acknowledged and they are included in the references as well. I worked the thesis under the supervision of Dr.Ing.Zebene Kiflie Lecturer of AAiT, AAU School of chemical and bio engineering.

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# Abstract

Fossil fuel based plastic raw materials are not renewable which in questioned their sustainability as well as the plastic products of these raw material is sources of an overwhelming environmental problem due recalcitrant to degradation. Hence, there is an urgent need toward bio-based plastics research area particularly poly lactic acid (PLA). This study aimed to produce Lactic acid which is used as a raw material for bio-based plastic (PLA) by a bacteria Lacto bacillus plantarum using sugarcane bagasse as a carbon source. The effects of glucose concentration, time and pH on the lactic acid yield were studied. sugarcane bagasse was pretreated using dilute sulfuric acid as catalyst and a maximum glucose concentration of 3.755g/L was found at a temperature of 105°C, acid concentration of 2.75% and time of 3hrs.The fermentation experiments were performed in 250 mL Erlenmeyer flasks as small scale laboratory fermenter containing 100 mL of hydrolysate supplemented with 5g/L of yeast extract and 10mL of lacto-bacillus plantarum inoculum. The yield of lactic acid from the fermented broth was determined using HPLC. Response surface methodology (RSM) approach of Design expert software of version 11 was used to analyze the effects of glucose concentration (3.755g/L, 3.58.g/L and 3.42g/L), pH (4, 6 and 8) and incubation time (10hrs, 20hrs and 30hrs) on lactic acid production with their respective ranges keeping the temperature and agitation speed constant. A three-variable, three-level Box-Behnken design was used to develop a statistical model to describe the relationship between lactic acid concentration and the chosen independent variables and to observe their effects on the yield using RSM. The model was statistically significant ($p < 0.0001$), did not show lack of fit (p-value = 0.1141) and The Pred R-Squared of 0.9764 is in reasonable agreement with the Adj R-Squared of 0.9956.From the model regression equation developed, the linear terms of glucose concentration, pH and time and pure quadratic term of time as well as interaction of glucose concentration and pH, interaction of glucose concentration and time, and interaction of pH and time had positive effect on the response yield. On the other hand pure quadratic terms of glucose concentration and pH had negative effect on the response yield. The maximum yield of lactic acid of 15.79g/L was found at a higher glucose concentration of 3.755g/L, at a moderate pH of 6 and at a maximum time of 30hrs.



***Key words**: bagasse, lactic acid, hydrolaysate, bio-plastic, Poly lactic acid*

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# Acronyms and Abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ASL | Acid soluble lignin |
| CCIIDI | Chemical Construction Inputs Industry Development Institute |
| DOE | Design of expert |
| FDA | Food and Drug Administration |
| GRAS | Generally Recognized as Safe |
| HDPE | High density poly ethylene |
| HPLC | High performance liquid chromatography |
| LA | Lactic Acid |
| LAB | Lactic acid bacteria |
| LDPE | Low density poly ethylene |
| MRS | De Man Rogosa and Shapes |
| PLA | Poly lactic acid |
| PVC | polyvinyl chloride |
| PET | polyethylene terephthalate |
| PU | Polyutherene |
| PS | poly styrene |
| PP | poly propylene |
| RSM | Response Surface Method |

# CHAPTER ONE

# 1. Introduction

## 1.1. Background

Since 19 century human life was depending on petroleum based plastic materials. Since then these materials have been fabricated and used for different applications. A trial has been made to produce the first polymer, polyvinyl chloride (PVC) in 1838 but was not fabricated to be applied as plastic polymer at that time (Wade, 2006). The first successful production of polymer named Bakelite was accomplished in 1910 by Leo Baekeland. Due to the wide range of properties of these plastics almost every household and construction materials contain commodities that are fully or partly made up of these materials. The world population has dramatically increased as a result the demand for these materials frequently increasing due to their various usages. When these materials used it is an obvious to see their waste and severe environmental challenges around. This is happened because of their enormous quantity and disposal problem as they do not biodegrade for a very long time (Panesar, 2013).

Especially, in the developing countries like Ethiopia non-biodegradable plastic materials discarded after it has been used and end up with the most common handling system which is landfill which generates pollution itself (Liew and Khor, 2015).

Around the world, countries are taking actions to minimize these impacts by banning single use plastics and improving waste collection systems. However, these solutions are not far fully eradicating the different problems looming on the environment because of the petroleum based products. The environmental crises arising from the use of these materials led the world to find alternatives (Song, 2009).

In recent years, bio plastics became increased due to different reasons such as scarcity of oil, increase in the cost of petroleum based commodities, and growing environmental concerns with the dumping system being used. As a result an interest on production of plastics from renewable resources became an issue. There are different raw materials used for the productions of these materials one of the most commonly used raw material is lactic acid. Lactic acid ($CH_3CHOHCOOH$) is the most widely occurring hydroxyl carboxylic acid(Bayitse& Lg, 2015**)**.

The Food and Drug Administration (FDA) have made a decision on an approval of lactic acid to be GRAS (Generally Recognized as Safe)(Harvey,2009). After it has been considered as GRAS it became applicable in the areas of food, agricultural, textile, chemical, pharmaceutical and cosmetic industries.(Udachan& Sahoo, 2014). As far as the bio-plastics (PLA) produced from lactic acid are environmentally friendly; there is a growing demand for it to make a replace on the conventional plastics.

Lactic acid can be produced from different raw materials such as food sources and wastes or byproducts. Its production from different food sources such as sugarcane, potato, wheat is common on the developed countries but on the developing countries like Ethiopia it is not feasible to use food sources as raw material. In the developing countries mostly lactic acid is produced from different wastes or byproducts such as sugarcane bagasse. Sugarcane bagasse is an abundant source of lignocellulose that can be hydrolyzed to yield fermentable sugars for the production of lactic acid(Leelavatcharamas& Laopaiboon, 2009).

This study mainly dealt with development of a solution for the problems faced by the developing countries like Ethiopia because of the petroleum based plastics by producing an environmental friendly bio plastics raw material lactic acid. Lactic acid is a promising solution to be used for the production of bio plastics (PLA).

## 1.2. Statement of the problem

Nowadays, the world is under stress because of the environmental pollution coming from different hazardous pollutants. Especially, petroleum based plastics are the headache of the whole world because there is no technology which could degrade these materials. The production or manufacturing of plastic and plastic products from petroleum-based raw materials such as low and high density poly ethylene (LDPE & HDPE), poly propylene (PP), poly vinyl chloride (PVC), polyethylene terephthalate (PET), polyutherene (PU), poly styrene (PS) etc. are the causes for the un-degradable plastic wastes around the world. Particularly, Ethiopia is facing environmental impacts of these materials as well as losing a huge amount of money in foreign currency for importing. According to the Chemical Construction Inputs Industry Development Institute report these material costs the country 11.5 billion birr in average per year. It is the highest cost that takes our foreign currency next to petroleum (CCIIDI ,2018)

In addition, petroleum-based plastic products take more than 100 years to degrade. This has negative impact on the environment especially soil fertility and methane emission. Having seen these problems recently the world is shifting from the petroleum based plastics toward the bio-based plastics. In most developed countries the raw material used for the production of bio plastics (PLA) are mostly from different food source which is not a feasible on the case of developing countries. This shifting process also has to be applied on the developing countries like Ethiopia by utilizing wastes or byproducts such as bagasse for the production of bio-plastic (PLA) raw materials lactic acid. This thesis goes in line with our Climate-Resilient Green Economy (CRGE) strategy by producing environment friendly bio-plastic raw material lactic acid from bagasse. In doing so the foreign currency of the country invested on importing petroleum based raw materials could be reduced as well as it offsets the environmental impacts of their products and the impacts of bagasse itself by utilizing it as a carbon source for the production of lactic acid.

## 1.3. Scope of the study

This work starts with characterization of sugarcane bagasse, pretreatment and hydrolysis, media formulation, inoculation of potential lactic acid bacteria (LAB, Lactobacillus Plantarum) to the (De Man Rogosa and Shapes) MRS agar medium, finally fermentation process and characterization of the product were accomplished.

## 1.4. Objectives

### 1.4.1. General objective

The main objective of this research was to investigate the production of lactic acid from sugarcane bagasse by employing a bacterium called Lactobacillus Plantarum.

### 1.4.2. Specific objectives

The specific objectives of this work were to:

- ✓ characterize the physico-chemical analysis of sugar cane bagasse
- ✓ Determine the effect of fermentation process variables such as Glucose concentration, incubation time, and pH on lactic acid yield.
- ✓ find optimal operating conditions using response surface methodology
- ✓ characterize the product using HPLC

## 1.5. Significance of the study

Lactic acid produced from sugarcane bagasse a raw material for the bio-plastic PLA is considered to be a best option to replace the petroleum based conventional plastic raw materials. The significance of this study includes: Economically, if lactic acid is produced in Ethiopia, then the expenditure incurred for importing PP, LDPE, HDPE, PVC, PU, PS could be reduced. Environmentally, in doing so it makes our nation free of non-degradable petroleum based plastic products that meet our green economy strategy by reducing or if possible eliminating pollution. If this study becomes feasible and then scaled up there could be an implementation of new technology and also creating job opportunity in the country. This study could also be role model for others to work on converting such by products in to useful products like lactic acid.

# CHAPTER TWO

## 2. Literature review

Conventional plastics are putting the world under stress because of their economic and environmental impacts. As a result a shifting towards bio plastics is the best option on overcoming the overwhelming problems associated with the conventional one. The global production capacity of bio plastics has increased from 1.5 to 1.9 million tons in the period 2012-2015, and was forecasted to reach 6.7 million tons in 2018(Rivero, 2016). The share of plastics that are both bio-based and biodegradable, such as poly lactic acid (PLA) will increase to 2.5% of the total plastic production by 2020 (European Bioplastics, 2016 and Van ,2017). China-Korea, the United States (US), the European Union (EU) and Brazil are the top most bio-plastic producers, with capacity increases also expected in other countries of the Asian-Pacific region(European Bioplastics, 2016).

Currently the whole world shifted towards production of bio based plastics. Those materials are produced from different raw materials derived from renewable resources. Lactic acid is the most common raw material used for the production of bio plastics called poly lactic acid (PLA).Lactic acid has recently received an attention for the production of biodegradable plastics (PLA). Poly lactic acid (PLA) is biodegradable aliphatic polyester produced from renewable sources, which, due to its excellent physical and chemical properties and environment compatibility, nowadays it is considered as the best 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 (Xavier,2010).

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. It can be obtained by petrochemical synthesis or fermentation process. Nowadays, due to the improvements in bacterial fermentation of glucose, optically preferentially pure lactic acid (L +) is produced through fermentation process using different raw materials such as corn, potato, wheat and others. In the United States, LA now day is highly applicable in poly lactic acid formation which is in a very high demand of making biodegradable plastic (Kim and Ryu ,2006).

## 2.1. The Environmental and Economic Impacts of Conventional Plastics

Due to the changing consumption patterns, growing populations and increased urbanization, developing countries Like Ethiopia are facing significant challenges with regards to conventional plastic wastes. As a result Biodegradable plastic production becomes critical to our country Ethiopia because it goes with the green economy strategies. This thesis focused on production of lactic acid from sugarcane bagasse, a raw material for bio plastic poly lactic acid. The environmental and economic impacts of conventional plastics are summarized below(UNEP,2016).

Table 2-1: Comparison between conventional and bio based plastics

| Advantages of bio based plastics | disadvantages of petroleum based plastics |
|---|---|
| ▪ Biodegradable<br>▪ Potentially a much lower carbon footprint.<br>▪ Lower energy costs in manufacturing.<br>▪ Do not use scarce crude oil.<br>▪ can feel softer and more tactile<br>▪ Less likelihood of imparting a different taste to the product contained in a plastic container. | ▪ They are not biodegradable<br>▪ Toxic chemicals can leach out of drink and food Containers<br>▪ Wildlife threatened<br>▪ Catastrophic ocean spill<br>▪ High energy costs in manufacturing. |

Source:[UNEP,2016]

Table 2-2**:** Import data of petroleum based plastic raw material and their products cost

| S/No | Year of import | Net Wt in tones | Value in Birr |
|---|---|---|---|
| 1. | 2010 | 3005(PE),3124(PET), 1,816,302.88(PVC) | 4,541,726,448 |
| 2. | 2011 | 4608(PE),5226(PET), 647,045.00(PVC) | 5,868,587,970 |
| 3. | 2012 | 9750(PE),7271(PET), 1,072,166.00(PVC) | 7,465,715,460 |
| 4. | 2013 | 8063(PE),5283(PET), 648,015.90(PVC) | 8,362,574,619 |
| 5. | 2014 | 9050(PE),7340(PET), 751,277(PVC) | 9,750,812,205 |
| 6. | 2015 | 9600(PE),8463(PET), 921,458(PVC) | 11,456,512,610 |

Source :(CCIIDI, 2010-2015)

## 2.2. Different reviewed studies

Scientific literature regarding lactic acid production from lignocellulose materials mainly reports utilization of the cellulosic fraction. The investigation of lignocellulosic material fermentation to lactic acid began with the screening of strain (bacteria, fungus and yeast). According to Moldes et.al pentose ATCC 8041was the engineered strain that exhibited the best results for lactic acid production from acid hydrolyzed sugar cane bagasse with 3.9±1.7 g/l of concentration of glucose (16.2 g/L), yield. (Moldes et al,2006).

Improvement of fermentation was also reported with a mixed culture(Cui et al,2011).using mixed strains of Lb. rhamnosus and Lactobacillus brevis for the consumption of both cellulose and hemicellulose derived sugars from corn stover. During SSF using an NaOH-treated

corn stover by mixed fermentation, an improved lactic acid yield of 0.70 g/g was obtained, which was approximately 18.6% and 29.6% higher than that obtained from single strains of Lb. rhamnosus and Lb. brevis, respectively (Zui et al,2011). Similarly, 81 g/L of lactic acid was produced from cassava bagasse via the SSF method by mixed fermentation of Lb. casei and Lb. delbrueckii(John et al,2006).

## 2.3. Raw materials for the production of lactic acid by LAB

Raw material cost is one of the major factors in the economic production of lactic acid. In most developed countries pure sugars or edible crops has been a traditional substrate for lactic acid production that is advantageous in obtaining a pure lactic acid product and lowering costs of pretreatment and recovery. Since substrate cost cannot be reduced by process scale-up, extensive studies are currently underway to search for novel substrates for lactic acid production. Various materials have been considered as attractive alternative substrates and renewable resources, including byproducts of agricultural industries, food industries, and natural unutilized biomasses such as starchy biomass, lignocellulosic biomass (bagasse), whey, yogurt, glycerol, and algal biomass. Large amount of wastes is generated every year from the industrial processing of agricultural raw materials. Most of these wastes are used as animal feed or burned as alternative for elimination. However, such wastes usually have a composition rich in sugars, minerals and proteins, and therefore, they should not be considered wastes but raw materials for other industrial processes such as Lactic acid production (Taherzadeh & Karimi,2008).

Therefore, developments of processes that utilize cheap raw materials at minimal costs have been under extensive studies. These substrates can be roughly classified as starch-based non-processed biomasses, lignocellulosic non-processed biomasses, and waste or side stream feed stocks. The former are nowadays generally considered as non-ideal feed stocks due to ethical reasons. Extensive reviews including starch-based feed stocks are available elsewhere(John ,2007).

With respect to future applications, the most likely raw materials for the lactic acid production are industrial side streams and lignocellulosic biomasses such as sugarcane bagasse.

As in other bioconversion processes, also in lactic acid production the focus of research has turned towards the use of lignocellulosic feed stocks. The major driving forces are fossil fuel deprivation and general paradigm change to bio economy, and the abundance of lignocellulose

materials. Generally, the effective utilization of lignocellulosic biomass for biochemical processes is limited due to seasonal availability, scattered distributions and high logistics cost (Lin, 2006).

Other quite often referred raw materials include brewery residues, especially spent grain(Aliyu S, 2011). Additionally, there are various other proposed food industry residues that could fit to the lactic acid fermentation. The recently proposed include e.g. applepomace(Gullon, 2008), canned pineapple syrup(Nakanishi, 2010), cashew apple juice(Honorato, 2007), Jerusalem artichoke tubers(Ge, 2010),rice residues(Lu, 2009), sap from palmyra and oil palms(Chooklin, 2011), and spent coffee grounds (Mussatto, 2011).

Agricultural residues, such as sugarcane bagasse is rich in lignocellulosic biomass, which is mainly composed of cellulose, hemicellulose and lignin. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw (Pandey et al., 2000). Low ash content enhances the susceptibleness of hydrolysis process. (Cordeiroetal, 2009). It is a very promising raw material for the production of lactic acid. Especially in Ethiopia due to its availability and sustainability it is best raw material.

Raw materials for any product to produce 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(IRENA, 2017).

Based on the above criteria, sugar cane bagasse is best fitted for the production of lactic acid. The production of sugarcane bagasse globally was approximately 1900 million metric in the past 5 years. Brazil is by far the world's largest sugarcane producer with around 740 million ton cane crushed in the 2010/2011 harvest season, which is about 43% of the global production. The Table below summarizes the sugarcane crop production in selective countries between years 2009 and 2013 (Mokhena et al., 2008).

Compositions of Bagasse

Cellulose and hemicelluloses: They are present in the form of hollow cellulose in bagasse, which contributes to about 70 % of the total chemical constituents present in bagasse.

Lignin: It acts as a binder for the cellulose fibers and also behaves as an energy storage System (Centre & Industrial, 2016). Bagasse consists of water, fiber and small quantities of solids in solution in the following proportions. Water 46 - 57 % ( mean50%), Fiber 43% - 53 % ( mean 47%), Solids in solution (sugar) 2% - 6 % (mean 3%).It is a composition within certain limits as variable and depends in the varieties, their maturity. By definition the fiber of the bagasse is the component which is insoluble in water. It consists of mainly cellulose pentose and lignin. Cellulose is a polysaccharide having the general formula $(C_6H_{10}O_6)$ n and the main constituent of vegetable tissue.

The amount of organic waste obtained from the agriculture industry is abundant in the world but the utilization is still limited. In view of the shortage of conventional raw material for pulping and the increasing demand for paper products worldwide, non-wood plants and agricultural residues attracted renewed interest. Non-wood plants offer several advantages including short growth cycles (Ververis, et.all 2004).

Sugarcane (Saccarhum officinarum) bagasse is a residue produced in large quantities by sugar industries. In general, 1 ton of sugarcane bagasse generates 280 kg of bagasse, the fibrous by-product remaining after sugar extraction from sugarcane (Sun, J.X., et.all, 2004). However, the utilization of sugarcane bagasse is still limited and is mainly used as a fuel to power the sugar mill (Antaresti, et.all, 2002 and Charles, M, et.all, 2003). These polymers are important for the production of lactic acid.

Annual world sugarcane bagasse (Oryzae sativa) production was about 577 million tons for 1997 – 98. More than 50 countries contributed to this sum with the production of at least 100, 000 tons of bagasse annually. One of the countries is Malaysia. Among these large quantities of agricultural residues only a minor portion of the residues is reserved as animal feed. However a huge quantity of the remaining bagasse is not used and burnt in the fields.

Table2-3: Global sugarcane generation capacity

| Country | Year | Average production(million metric ton $yr^{-1}$) | Average annual yield of sugarcane(metric ton $ha^{-1}$) |
|---|---|---|---|
| **Brazil** | 2013 | 743 | 120 |
| **Mexico** | 2012 | 42.5-44.6 | 65 |
| **Colombia** | 2013 | 21.5 | 108 |
| **Argentina** | 2010 | 19 | 56 |
| **Cuba** | 2009 | 11.6 | 22.4 |
| **India** | 2012-2013 | 350 | 70 |
| **Thailand** | 2013 | 100.1 | 62.6 |
| **China** | 2013 | 125.5 | - |
| **South Africa** | 2013 | 20.3 | - |

Source: [Mokhena et al., 2008]

Ethiopia also has a well-established cane sugar industry, which is owned and operated by the Ethiopian Sugar Corporation (ESC). The country has nine sugar factories named Wenjishoa, Fincha, Tendaho, Welkayit, Arjodedissa, Metahara, Kassem, Omo-kuraz and TanaBeles. It is a common sense to expect a huge amount of by product bagasse from these factories.

Table2-4: Ethiopian factories sugarcane crushing capacity

| S.No | Name of factory | Sugar cane crushing capacity(TCD) | bagasse generation (TPD) |
|---|---|---|---|
| **1** | Wenjishoa | 6,250 | 2000 |
| **2** | Metehara | 5,000 | 1600 |
| **3** | Fincha | 12,000 | 3840 |
| **4** | Tendaho | 13,000 | 4160 |
| **5** | Wolkayit | 24,000 | 7680 |
| **6** | Arjodedissa | 8,000 | 2560 |
| **7** | Kassem | 6,000-10,000 | 2560 |
| **8** | Omo-kuraz | 6,000-6,500 | 2000 |
| **9** | Tanna Beles | 12,000 | 3840 |

Source: [Semret, 2014]

## 2.4. Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram positive, catalase negative, acid tolerant, non-respiring, non sporulating rod or cocci that synthesize lactic acid as the major metabolic end product during the fermentation of carbohydrates(Axelsson, 2004). They are heterogeneous group of bacteria which plays a significant role in a variety of fermentation processes. They ferment food carbohydrates and produce lactic acid as the main product of fermentation. The bacteria performing the conversion of carbohydrates to carboxylic acids, mainly lactic acid in traditional fermented foods, are called lactic acid bacteria (LAB). Food microbiologists used the term early, and 1919 the Danish bacteriologist Orla Jensen tried to define key features of LAB, unaware of the fact that LAB is not forming a systematically defined group based on evolutionary relationships; instead it can be regarded as a functional group used by food microbiologists, aiming at those bacteria that occur and multiply spontaneously in traditional lactic acid fermented foods. Furthermore, it is understood that LAB are harmless to human health. LAB includes species from around 20 different genera such as Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, and Vagococcus and Weisella. Lactobacillus is the largest of these genera, comprising around 80 recognized species(Makarova, 2006).

### 2.4.1. Lactobacilli

Lactobacilli are the largest genera in LAB. They are a very heterogeneous group, widespread in nature and containing the most acid tolerant species (Klaenhammer, 2002)(Makarova, 2006). Species such as Lactobacillus plantarum and L. casei can be found in a number of different environments whereas other species such as L. sanfransiscensis and L. delbrueckii are found only in certain habitats (Axelsson, 2004)

Lactobacillus plantarum is homofermentative LAB, metabolically very flexible and versatile, encountered in many environmental niches, and with broad applications, e.g. as a starter culture in vegetable(Salovaara, 2004)and meat (Mayo, 2002)fermentations; as probiotic for humans (Goossens, 2005)and animals (Demecková, 2002); and lately as a delivery vehicle for therapeutic compounds(Pavan, 2000).The complete genome of one L. plantarum strain has recently been sequenced(Kleerebezem, 2003). This strain has been shown to possess the largest chromosome

size (3.3Mb) within LAB. It has a circular chromosome with 3052 potential protein-encoding genes and more than 2500 predicted proteins with assigned biological function.(Axelsson, 2004).

## 2.5. Properties of lactic acid

Lactic acid is an organic acid with the official name2-hydroxypropanoic acid given by the International Union of Pure and Applied Chemistry (IUPAC). Pure lactic acid is a colourless and hydroscopic liquid; it can be defined a weak acid because of its partial dissociation in water (Eq. 2.1) and the correlated acid dissociation constant ($Ka = 1.38*10^{-4}$).

$$H_3C\text{-}CH\ (OH)\text{-}COOH \rightarrow H^+ + H_3C\text{-}CH\ (OH)\text{-}COO^- \qquad (2.1)$$

### 2.5.1. Isomers

Lactic acid is a chiral compound with a carbon chain composed of a central (chiral) atom and two terminal carbon atoms. A hydroxyl group is attached to the chiral carbon atom while one of the terminal carbon atoms is part of the carboxylic group and the other atom is part of the methyl group (Narayanan, 2004). As a result, two optically active isomeric forms of lactic acid exist: L (+) form, also named (S) lactic acid, and D (−) form, or (R) lactic acid. Pure and anhydrous racemic mixture of lactic acid is a white crystalline solid with a low melting point. L (+)-lactic acid is the biological isomer as it is naturally present in the human body; consequently, the importance of this form of lactic acid depends on the known biochemical synthesis(Narayanan, 2004)(p. OU et al.2011).

Table2-5: Physical and chemical properties of lactic acid

| Identification parameters | Description |
|---|---|
| **Compound name** | Lactic acid |
| **IUPAC name** | 2-Hydroxypropanoic acid |
| **Chemical formula** | $C_3H_6O_3$ |
| **Molecular mass** | 90.08 mol/g |
| **Appearance** | Color less and syrup liquid; alternatively, a white to yellow solid compound |
| **Taste** | Mild acid taste |
| **Odour** | Odour less |
| **Boiling point** | $122^oC$ |
| **Melting point** | $17^oC$ |
| **Specific gravity/density** | 1.2 |
| **Ka** | $1.38*10^{-4}$ |
| **PKa** | 3.86 |

## 2.6. Production technology

Lactic acid is a naturally occurring organic acid that can be produced by fermentation and chemical synthesis. However, it is more commonly produced from renewable resources via fermentation process. Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation, whereas the remainder is produced synthetically by the hydrolysis of lactonitrile. The chemical synthesis of lactic acid always results in a racemic mixture of lactic acid. Fermentative production of lactic acid offers the advantages in both utilization of renewable carbohydrates and production of optically pure L- or D-lactic acid, depending on the strain selected(Adsul et al,2011). In fermentation processes, bacteria or other microorganism produce lactic acid as they metabolize carbon containing raw material(Boontawan, 2010).

### 2.6.1. Synthetic manufacture

Lactic acid can be produced from the most part of its derivatives by means of suitable treatments (Ghaffar, 2014)(Vaidya, 2005). Lactonitrile (2-hydroxypropanenitrile, $CH_3CHOHCN$) is the most preferable of these compounds used in the chemical synthesis of lactic acid rather than other raw materials. Lactonitrile can be produced by the nucleophile addition of hydrogen cyanide (HCN) to the liquid phase of acetaldehyde ($CH_3CHO$) in alkaline media under high pressure (Eq. 2.2).

$$HCN + H_3C\text{-}CHO \rightarrow H_3C\text{-}CHOH\text{-}CN \qquad (2.2)$$

After recovery and distillation of the obtained impure lactonitrile (Narayanan et al. 2004), the purified compound can be treated (acid hydrolysis) by using concentrated hydrochloric acid (HCl) or concentrated sulfuric acid ($H_2SO_4$), with the resulting production of ammonium sulphate salt ($(NH_4)_2SO4$)and crude lactic acid (Eq. 2.3).

$$H_3C\text{-}CHON\text{-}CN + H_2O + H_2SO_4 \xrightarrow{\text{Hydrolysis}} H_3C\text{-}CHOH\text{-}COOH + \tfrac{1}{2}(NH4)_2SO_4 \qquad (2.3)$$

The produced (crude) lactic acid needs to be concentrated and purified. Methanol (CH3OH) can be used with the aim of producing methyl lactate ester, $CH_3CHOHCOOCH_3$ (Eq. 2.4).

$$H_3C\text{-}CHOH\text{-}COOH + H_3\text{-}C\text{-}OH \xrightarrow{\text{esterification}} H_3C\text{-}CHOH\text{-}COOCH_3 + H_2O \qquad (2.4)$$

Methyl lactate ester is subsequently collected, purified by distillation, and hydrolyzed in acidic aqueous solution to lactic acid, while methanol can be recycled in the same process. The resulting product is a racemic mixture of lactic acid(Narayanan, 2004).

## 2.7. Fermentation Mode

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. Lactic acid is most commonly produced in the batch mode but numerous examples of continuous culture exist as well as some fed batch and semi continuous/ repeated batch fermentations. When comparing batch and continuous fermentation modes, the former gave higher lactic acid concentration and yield in most of the studies. This is mainly due to that all substrate is used in the batch mode, whereas a residual concentration remains in the continuous one(Goksungu, 1997).

The most common fermentation processes are two types, continuous and batch process. The batch process is the preferable one because of the following advantages

- ✓ It is more safer than continuous because it has proper sterilization
- ✓ it has lower risk of infection by the foreign bacteria
- ✓ Lower risk of occurrence of strain mutation.
- ✓ It produces high yield of lactic acid industrially.

### 2.7.1. Microbial Fermentation

**I). Pretreatment and hydrolysis**

Pretreatment is a crucial process step in the biochemical conversion of lignocellulose biomass to fermentable sugars and finally to products like lactic acid. It is required to alter the structure of cellulosic biomass to make cellulose more susceptible to hydrolysis process that converts the carbohydrate polymers into fermentable sugars(Mosier,2005).

A number of studies on pretreatment of lignocellulose materials have been developed over the years. The productions of lactic acid involved enzymatic hydrolysis or by chemical hydrolysis have been reported in different journals. Chemical hydrolysis, usually acid hydrolysis, is one of the viable methods currently being developed as a promising means of producing sugar from lignocellulosic materials such as bagasse. The hydrolysis of cellulosic materials in mineral acids is strongly affected by the acid concentration, time and temperature (Sun et al,2009).Enzymatic hydrolysis of different raw materials was reported by. E.g. the hydrolysis of the corn stalks with the cellulose enzyme can produce glucose concentration of 4.8 g/L.

In most dilute acid treatments sulfuric acid is chosen as catalyst, in some cases hydrochloric acid or phosphoric acid is used. $H_2SO_4$concentrations ranged from 0.06 to 10% (w/v or w/w) and liquid/solid ratios (L/S) were mostly 10 or higher. Some studies used steam explosion combined with $SO_2$treatment and treatment temperatures ranged from 90-200$^{\circ}C$. in Acid Hydrolysis(sulfuric acid, Hydrochloric acid, carbonic acid and phosphoric acid) is added to the raw material and the mixture is held at elevated temperature for short period of time. Hydrolysis of hemicellulose then occurs, releasing monomeric sugars and soluble oligomers from the cell wall matrix into the hydrolysate. Hemicellulose removal increases porosity and improves enzymatic digestibility, with maximum enzymatic digestibility usually coinciding with complete hemicellulose removal(Chen, 2007). As an alternative to inorganic acids, organic acids such as maleic acid, fumaric acid can be used for dilute acid pretreatment(Kootstra, 2009).

Several companies use dilute acid as pretreatment method for fractionation of lignocellulosic biomass. Blue Sugars in the US has a demonstration plant for the production of ethanol from sugarcane bagasse. They combine dilute acid with mechanical action and co-ferment the C5 andC6-sugars. Cobalt Technologies, in cooperation with Rhodia and Andritz, are building a demonstration plant in Brazil for the production of butanol from sugarcane bagasse. They combine dilute acid hydrolysis with fermentation and claim that enzymatic hydrolysis is not necessary in their process(Sun and Cheng, 2002).

**II).Fermentation process**

Batch fermentation has been the method used industrially for lactic acid production. The inoculum size is usually 5-10 % of the liquid volume in the Fermenter. The inoculum can be propagated in seed tanks or taken from completed large scale fermentation. As the fermentation proceeds, the rate begins to slow because of the depletion of non-essential but stimulatory growth substances and the accumulation of the lactic acid. (Murray)(Moo)(Young)(1985).

There are two types of fermentation for these lactic acid bacteria, homofermentative and heterofermentative. Homofermentative lactic acid bacteria produce lactic acid as a sole end product; heterofermentative lactic acid bacteria produce other product such as acetic acid, ethanol as well as lactic acid the end product.

Only the homofermentative lactic acid bacteria are of industrial importance for lactic acid manufacture. Homo fermentative L (+) lactic acid producers are required if the lactic acid produced will be used as a feedstock for manufacture of 100% biodegradable plastics. The homo-fermentative of the Lactobacillus species, which produce the most acid, follow the heterofermentative species of Lactobacillus, which produce intermediate amounts of acid. Homofermentative, convert sugars primarily to lactic acid, while heterofermentative produce about 50% lactic acid plus 25 % acetic acid and ethyl alcohol and 25% carbon dioxide. These other compounds are important as they impart particular tastes and aromas to the final product (Vickroy, 1991).

(a) Fermentation and neutralization

$C_6H_{12}O_6$ + Ca $(OH)_2$ $\xrightarrow{\text{fermentation}}$ $(2CH_3CHOHCOO^-)\ Ca^{2+} + 2H_2O$ (2.5)

Carbohydrate Calcium hydroxide Calcium lactate

(b) Hydrolysis by H2SO4

$2(CH_3CHOHCOO^-)\ Ca^{2+} + H_2SO_4 \longrightarrow 2\ CH_3CHOHCOOH + Ca\ SO_4$ (2.6)

Calcium lactate Sulfuric acid lactic acid Calcium sulphate

(c) Esterification

$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$ (2.7)

Lactic acid Methanol Methyl lactate

(d) Hydrolysis by H2O

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$ (2.8)

Methyl lactate Lactic acid Methanol

The broth containing calcium lactate is filtered to remove cells, carbon treated, evaporated and acidified with Sulphuric acid to get lactic acid and calcium sulphate. The insoluble calcium sulphate is removed by filtration; lactic acid is obtained by centrifugation.

## 2.8. Factors affecting lactic acid production

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 that affect 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(Calvel, 2001). Lactic acid fermentation has been studied since 1935 using different types of microorganism and fermentation operation conditions such as pH, carbon source, sugar concentration, incubation time, temperature, inoculums size, initial substrate conditions and nitrogen source ((pp. Hofvendahl and Hahn-Hägerdal,2000).

### 2.8.1. Effect of Temperature

Temperature is one of the most important environment factors that affect lactic acid production. Various researchers have studied the effect of temperature on the lactic acid production and they found the optimal temperature between 30-45$^{\circ}$C(pp. Hofvendahl and Hahn-Hägerdal,2000). Temperature and pH are the key environmental parameters that affect the fermentation process(pp. Yuwono and Kokugan,2008) Low temperature has been reported that positively influence the outgrowth of contaminating microorganism, thereby influencing the Performance of the lactic acid production were investigated (pp. Neysens and Vuyst,1991). The temperature giving the highest productivity lowers than the temperature resulting in highest lactic acid mass concentration and yield (Hujanen and Linko, pp. ,1994).

### 2.8.2. Effect of pH

pH is also affects the fermentation process as a result it is a must to adjust it based on the characteristics of the strain going to be used. There are various ways to control pH of the fermentation process. It can be set at the beginning and then left to decrease due to the acid production. In cases, when the pH is controlled, base titration can be carried out(., pp. Wee et al,2004). It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities(Vijayakumar) and(Aravindan)(2008).(Busairi, 2002). titration to a constant pH resulted in higher or equal lactic acid concentration, yield and

productivity in comparison with no pH control. The optimal pH for lactic acid production lies between the ranges 6.0- 8.

### 2.8.3. Effect of Carbon Sources

A number of different substrates have been used to fermentative production of lactic acid by lactic acid bacteria. A wide variety of carbon sources capable of producing lactic acid, including molasses, sugarcane bagasse, fruits waste, glucose, sucrose, fructose and lactose. If these substrates contain high level of metal ions they must be removed prior to production (Wee, 2004). The purest product is obtained when a pure sugar is fermented, resulting in lower purification costs. However, this is economically unfavorable, because pure sugars are expensive and lactic acid is a cheap product (Vickroy, 1991).

### 2.8.4. Effect of Incubation Time

Previous study represented that an increase in lactose utilization and subsequent lactic acid production was found incubation time up to 30 h and thereafter no improvement in both the functions was observed(Panesare et al, 2010). 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 10-48hr has been generally used for lactic acid production using different lactobacilli cultures(Gandhi et al, 2000).In addition, the different optimal conditions reported by various researchers for maximum lactic acid production could be explained by the differences in the nature of the strains and medium composition used in their studies(Holzapfel, 1995);(Atkinson, 1991); and (Bendand Marquis, 1987).

### 2.8.5. Effect of Nutrient Sources

The medium composition has been investigated from many aspects, including the addition of various concentrations of nutrients. The lactic acid bacteria require substrates with high nitrogen content and have a particular demand for B vitamins. The nutrients are added in the form of malt sprout, corn steep liquor, and yeast extract. Lactic acid production increases with the concentration of the supplement especially yeast extract. The highest production rate was found with addition of 5-15 g/l yeast extract(Lund et al, 1992). Lactic acid increases with the increasing concentration of Nitrogen (Goncalves et al, 1997).The addition of nutrients and higher nutrient concentrations generally had a positive effect on the lactic acid production. MRS medium, which contains yeast extract, peptone and meat extract was superior to yeast extract, which in turn was better than malt extract (Gandhi et al, 2000). This reflects the complex nutrient demands of lactic acid bacteria, being fastidious because of limited biosynthesis capacity. Yeast extract alone at high concentration gave higher lactic acid production than yeast extract and peptone in low amounts, but the opposite resulted when the concentration of yeast extract was kept constant and peptone was added(Gao et al, 2011).

### 2.8.6. Effect of Agitation speed

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 train Lactobacillus rhamnosus, the maximum lactic acid concentrations could be achieved when fermentation was carried out at pH 6, temperature of 40$^{\circ}$C and agitation speed of 150 rpm, which was in accordance with a previous report (Hofvendahl and Hahn, 2000), the optimal condition for lactic acid is pH 6.0- 8, temperature 30-45$^{\circ}$C with continuously agitating at 100-200 rpm(Timbuntam, 2008).

## 2.9. Applications of Lactic Acid

Lactic acid is sold in food, pharmaceutical and technical grades. Since the lactic acid has gained increasing importance and has been used in a great variety of applications, its salt, ester and many derivatives have been developed. The uses of lactic acid can be broken down by grade and by lactic acid derivatives (Benthin)(Villadsen)(1995). Some of the important applications of lactic acid are detailed below.

### 2.9.1. Food and beverage Industries

Approximately 85% of lactic acid produced is used in food and food-related industries and the rest (~ 15 %) of the uses are for non-food industrial applications (Datta)(Tsai)(1995). Lactic acid is used in the food industry for several aspects. It has a long history of uses for fermentation and preservation of human food stuffs. It occurs naturally in many food products. It has been in used as an acidulant, preservative and pH regulator for quite some time. There are many properties of lactic acid, which make it a very versatile ingredient in the food industry. It has a pronounced preservative action, and it regulates the micro flora. It has been found to very effective against certain type of microorganisms. Sometimes a combination of lactic acid and acetic acid is used as it has a greater bactericidal activity. Because it occurs naturally in many food stuffs, it does not introduce a foreign element into the food. The salts are very soluble, and this gives the possibility of partial replacing the acid in buffering the acid in buffering systems (Vickroy, 1991).

Lactic acid is non-toxic and is deemed “Generally Recognized as Safe” (GRAS) as a general purpose food additive in the USA. The same status is accorded in many other countries too. The calcium salt of lactic acid, calcium lactate, has greater solubility than the corresponding salt of citric acid. In such products, where turbidity caused by calcium salts is a problem, the use of lactic acid gives products, which are clear. L (+) Lactic acid is the natural lactic acid found in biological systems and hence its use as acidulant does not introduce a foreign element into the body. Lactic acid are widely used in food industry such as confectionery as acidulant, beverages industries as natural flavoring, a preservatives for fermented vegetable and meat, and also an vital element for producing dairy’s product(Vijayakumar)(Aravindan)(2008).Some of the important applications of lactic acid in the food industry are detailed below.

**I. Confectionery**

Lactic acid finds use as an acidulant in the confectionery industry. It is a better acidulant than citric acid since the sugar inversion is less when used for hardboiled candies. It does not have the initial burst of flavor and tanginess of citric acid. Lactic Acid imparts a mellower and lasting sourness and enhances the flavor much more. The use of buffered lactic acid in continuous production lines for high boiled sweets is a more recent application. Liquid buffered lactic acid may be converted easily to the molten syrups, even at the high temperatures used in depositing lines. In sugar confectionery it is used in continuous production lines for high boiled sweets (like bonbons) to make perfectly clear sweets, with minimum sugar inversion and with no air trapped. Lactic acid is used in confectionery, not only for flavor, but also to bring the pH of the cooked mix to the correct point for setting (Hujanen)(Linko)(1994).

**II. Beer and wine**

Lactic acid is a natural beer acid and hence it is used for pH adjustments during the mashing process and in wort cooking. Lactic acid improves the microbial stability and also enhances the flavor of beer during the manufacturing process( Buchta, 1983).

**III. Beverages**

Lactic acid is used as an acidulant in delicately flavored soft drinks and fruit juices. It does not mask or over power the natural flavor. Its flavor enhancing property makes the beverage more palatable and leaves a lingering taste. Lactic acid is preferred over citric acid for these reasons. Use of buffered lactic acid improves the taste and flavor of man beverages, such as soft drinks, mineral water and carbonated fruit juicesetc(Atkivnson, 1991).

**IV. Dairy Products**

Direct acidification with lactic acid, in dairy products such as cottage cheese, is preferred to fermentation as the risks of failure and contamination can be avoided. The processing time also can be reduced. Lactic acid and calcium lactate are used extensively in the production of Channa and Panneer by direct acidification. Lactic acid is also used as an acidulant in dairy products like cheese, margarine and yogurt powder. In dairy products such as cottage cheese, addition of lactic acid is preferred to fermentation(Gandhi et al, 2000).

## V. Bakery Products

For direct acidification of certain breads, lactic acid is the natural sour dough acid. The general appearance of a loaf of bread is greatly improved by the use of bacterial lactic acid, a larger loaf results per weight of bread with improved bloom, and colour of crust. Lactic acid is directly added to certain types of fermented dough crispy biscuits. Lactic acid added to dough increases the shelf life due to its retarding action on moulds and rope(Ameen, 2017).

The sodium and calcium stearoyl acetylates find use as emulsifiers in the baking industry as they provide substantial quality improvement of baked products besides reducing shortening levels. In bakery products it is used for direct acidification of rye or rye-wheat breads. It increases butter stability and volume. Part of the egg albumen can be replaced by less expensive calcium lactate. A large fraction (w > 50 %) of the lactic acid for food-related uses goes to produce emulsifying agents used in foods, particularly for bakery goods. These emulsifying agents are esters of lactate salts with longer chain fatty acids, and the four important products are calcium and sodium, stearoyl-2-1actylate, glyceryllactostearate and glyceryllactopalmirate. Among, the stearoyllactylates, the calcium salt is a very good dough conditioner, and the sodium salt is both a conditioner and an emulsifier for yeast leavened bakery products. The glycerates and palmitates are used in prepared cake mixes and other bakery products and in liquid shortenings(Gonclaves et al, 1997).

## VI. Meat and Meat Products

Lactic acid is widely used in meat products as an antimicrobial agent. Decontamination of beef, poultry and pork carcasses in slaughterhouse operations is practiced to reduce salmonella infection. In sausages, sodium lactate is used to reduce water activity and achieve higher shelf life. Recent research publication indicates the use of hot lactic acid spray on carcasses where reduction of over 99 % of E. coli has been observed. Lactic acid is also used in the improvement of shelf-life of buffalo meat(Naveena, 2006). An emerging new use for lactic acid or its salts is in the disinfection and packaging of carcasses, particularly those of poultry and fish, where the addition of aqueous solutions of lactic acid and its salts during the processing increased shelf life and reduced the growth of anaerobic spoilage organisms such as clostridium botulinum(Hovlten, 1972).

### 2.9.2. Pharmaceutical

Lactic acid is used in pharmaceutical industry as a very important ingredient. Pharmaceutical and food industries show presence for the L (+) lactic acid because the D (-) isomer is not metabolized by the human body. Lactic acid and its salts have been mentioned for various medical uses. They provide the energy and volume for blood besides regulation of pH, Calcium, sodium, ferrous and other salt of lactic acid are used in pharmaceutical industry in various formulations find use for their anti-tumor activity. Lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications (Vickroy,1991).Biodegradable plastic made of poly (lactic acid) is used for suture that does not need to be removed surgically and has been evaluated for use as a biodegradable implant for the repair of fractures and other injuries. The calcium salts of lactic acid are produced in a granular and powdered form. Calcium lactate trihydrate is used in pharmaceuticals primarily as a dietary calcium source and also as a blood coagulant for use in the treatment of hemorrhages and to inhibit bleeding during dental operations. Sodium lactate is used in the production of some antibiotics and to buffer pharmaceutical preparations (Boontawan, 2010;Boontawan, 2010).

Natural L (+) lactic acid is used in many applications in cosmetics. Lactic acid is an alpha hydroxyl acid (AHA) and is found in the skin. It is used as a skin-rejuvenating agent, pH regulator. It is a common ingredient in moisturizers, skin whiteners and anti-acne preparation. Since L (+) Lactic acid is naturally present in the skin, lactic acid and sodium lactate are extensively used as moisturizing agents in many skin care products. Lactic acid is also used as a pH-regulator. It is one of the most effective AHAs and has the lowest irritation potential. Lactates are regarded as skin whitening agents that have been shown to produce a synergistic effect when combined with other skin whitening agents (Vickroy,1991).

### 2.9.3. Chemical industry

Currently, lactic acid is considered the most potential feedstock monomer for chemical conversions, because it contains two reactive functional groups, a carboxylic group and a hydroxyl group. Lactic acid can undergo a variety of chemical con- versions into potentially useful chemicals,suchas propylene oxide(via hydrogenation), acetaldehyde(via decarboxylation), acrylic acid (via dehydration), propionic acid (via reduction),2,3-pentane-dione (via condensation), and dilated (via self-esterification). In the chemical industries, lactic acid is used in the dyeing of silks and other textile goods, as a mordant in the printing of woolens, in the bating and plumping of leathers, in the deliming of hides, in vegetable tanning, and as a flux for soft solders. The water-white grade is used in plastic industry. Lactic acid functions as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-re- lease agent, metal complexing agent, antimicrobial agent, and humectant. Natural lactic acid has an emerging use as an excellent and safe solvent, which is alternative in many fine mechanical cleaning applications. Due to the high solvency power and solubility of lactic acid, it is an excellent re- mover of polymer and resin

### 2.9.4. Cosmetic industry

Lactic acid offers natural ingredients for cosmetic applications. Although primarily used as moisturizers and pH regulators, they possess multiple other properties such as antimicrobial activity, skin lightening, and skin hydration. The moisturizing effect is related directly to lactate’s water retaining capacity, and the skin-lightening action of lactic acid is produced by the suppression of the formation of tyrosinase. Since they are natural ingredients of the human body, lactic acid and its salt fit perfectly into the modern trend towards natural and safer formulations, and they produce such effects as skin lightening and rejuvenation which makes them very useful as active ingredients in cosmetics. Lactic acid is popularly known as an alpha hydroxy acid (AHA) in the cosmetics industry. It is widely used as a milder alternative to glycolic acid. It is primarily used as an anti-aging chemical claimed to soften lines, reduce photo damage from the sun, improve skin texture and tone and improve overall appearance. Precautions should be taken when using lactic acid as a cosmetic agent because it can increase sensitivity to the sun's UV radiation.

### 2.9.5. Polymer Industry

In 1932, Carothers first produced aliphatic polyester of low molecular weight from lactic acid, but it had poor mechanical properties(Holten,1972). In 1954, DuPont patented the production of a high molecular weight poly lactic acid. However, the development was terminated because of the hydrolytic degradability of the polymer(Lowe,1954).In 1972;Ethicon produced high-strength co-polymers of lactic and glycolic acids. These polymers are now used as biocompatible fibres in restorable sutures. They are slowly hydrolyzed within the body to the constituent acids. For many years, growth in poly lactic acid production has been inhibited by the high cost of the lactic acid monomer. In late 1980s, new materials for lactic acid production by fermentation introduced lower cost lactic acid than the petro chemically-derived product. Cargill, Inc. Minneapolis, MN now operates the world's largest poly (lactic acid) facility (Lunt, 1998).In the United State, Europe and Japan; several companies are actively pursuing development and commercialization of poly (lactic acid) products. PLA polymers can be synthesized from various monomers. Low molecular weight polymers are obtained by step-growth polymerization of lactic acid where as high molecular weight polymers are synthesized by ring opening polymerization of lactide. Lactide is composed of two lactic acid units linked to form a diester cyclic monomer. Step growth polymerization of optically pure L + lactic acid (or pure D-lactic acid) and ring opining polymerization of optically pure L-lactide (or pure D-lactide) should lead to the same chain growth(Ionescu et al,2008).

Actually dramatic differences in main chain structures are observed as soon as one deals with stereo copolymers of L-and D-lactic acid repeating units. The step growth polymerization of mixtures of L- and D-lactic acid leads to poly (D, L-lactic acid) with a random distribution of the L- and D-lactyl units, whereas ring opening polymerization of the lactide dimmers lead to non-random distribution because chains grow through a pair addition mechanism (Cassanas et al., 1998). The difference in the crystallinity of poly (D, L-lactic acid) and poly (L-lactic acid) has important practical ramifications(Auras et al,2011). Since poly (D, L-lactic acid) is an amorphous polymer; it is usually considered for applications such as drug delivery where it is important to have homogenous dispersion of the active species within a monophasic matrix. On the other hand, the semi crystalline poly (L-lactic acid) is preferred in applications where high mechanical strength and toughness is required (i.e. Sutures and orthopedic

devices).PLA polymers offer a broad balance of functional performance that makes them suitable for a wide variety of market applications. They are expected to compete with hydrocarbon-based thermoplastics on a cost or performance basis. It also exhibits a tensile strength and modulus comparable to some thermoplastics. Like PET (polyethylene terephthalate), these polymers resist grease and oil and offer good flavor and odor barrier. PLA polymers also provide for heat stability at lower temperature than polyolefin sealant resin(Datta and Tsai,1995). The polymer can be processed by most melt fabrication techniques including thermoforming, sheet and film extrusion, blown film processing, fiber spinning and injection molding. These materials biodegrade completely to carbon dioxide and water when composted in municipal or industrial facilities, unlike traditional degradable plastics that need ultraviolet radiation to degrade. PLA needs only water and thus will degrade in the landfills. Biodegradation of PLA proceeds by a two-step process. Initially hydrolysis produces progressive chain length reduction by what is essentially an ester interchange process. This reaction is catalyzed by heat and pH. There are no bacteria involved in this phase of the process. When the chain length is reduced, producing very low molecular weight fragments, naturally occurring bacteria digest residues and liberate carbon dioxide and water(Lunt,1998).

Lactic acid has recently received a great deal of attention as a feedstock monomer for the production of poly (lactic acid), which serves as a biodegradable commodity plastic. The optically pure lactic acid can be polymerized into high molecular mass PLA through the serial reactions of poly condensation, de polymerization, and ring opening polymerization (Vijaya,2008).

The resultant polymer, PLA, has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays(Benthin and Villadsen,1993).

### 2.9.6. Other applications

Technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Lactic acid is being used in many small scale applications like pH adjustment hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electro-polishing baths, detergent builders. It is also used for the extraction of fish skin gelatin. In recent days it is used in the field of soft tissue augmentation and also used as adhesive in lamination industries.

Lactic acid has better descaling properties than conventional organic descales due to which reason it is used in many decalcification applications such as cleaners for toilets, bathrooms etc. Lactate esters like ethyl, methyl lactate etc. are used for degreasing since they have excellent action for oils, oligomeric and polymeric stains. Lactic acid is used in Ni plating process because of its unique complexing constant for Ni. Lactic acid is used as a pH regulator and complexing agent in various binder systems for water–based coatings such as electro-deposition coatings. Lactates find use as neutralizers in the production of certain types of surfactants, used in special detergents and personal care products.

# CHAPTER THREE

## 3. MATERIALS AND METHODS

The experimental procedure for the production of Lactic acid from sugarcane bagasse is depicted in Figure 3-1.

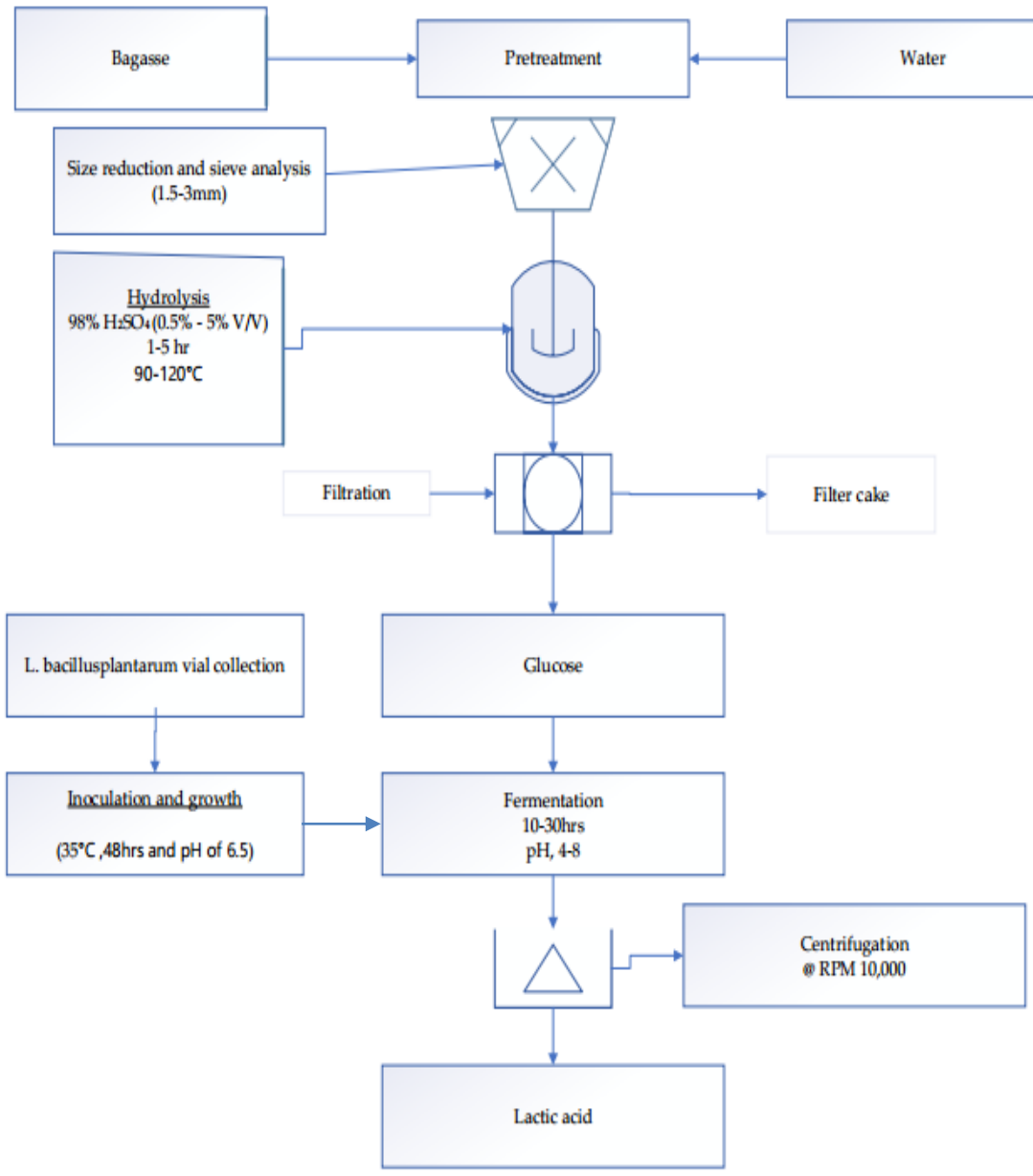



Figure 3-1**:** Block diagram of experimental set up

Experimental work such as pretreatment of the raw material (bagasse), size reduction and sieve analysis, hydrolysis, determination of the glucose content of the hydrolysate, inoculation and growth of lacto bacillus plantarum were conducted in the laboratory of the School of Chemical and Bio Engineering, Addis Ababa Institute of Technology-Addis Ababa University, Addis Ababa. The lactic acid content of the fermented broth was analytically determined using HPLC in Addis pharmaceutical factory, Adigrat.

All chemicals and reagents used in this study were of analytical grade. The Raw material sugarcane bagasse was collected from Wonjisugar factory. Reagents such as sulfuric acid, phenol, sodium hydroxides, dextrose, yeast extract, beef extract, sodium acetate, ammonium citrate, polysorbate 80, die-potassium phosphate, magnesium sulfate was collected from NwayP.L.C, Addis Ababa. The rest reagents used such as standard lactic acid, potassium dihyrdogen phosphate, Acetonitrile, manganese sulfate, Acetone and Gelatin peptone were collected from Elay Trading P.L.C, Addis Ababa. Distilled water from school of chemical and bioengineering laboratory of environmental engineering has been used in the entire work of the experiment.

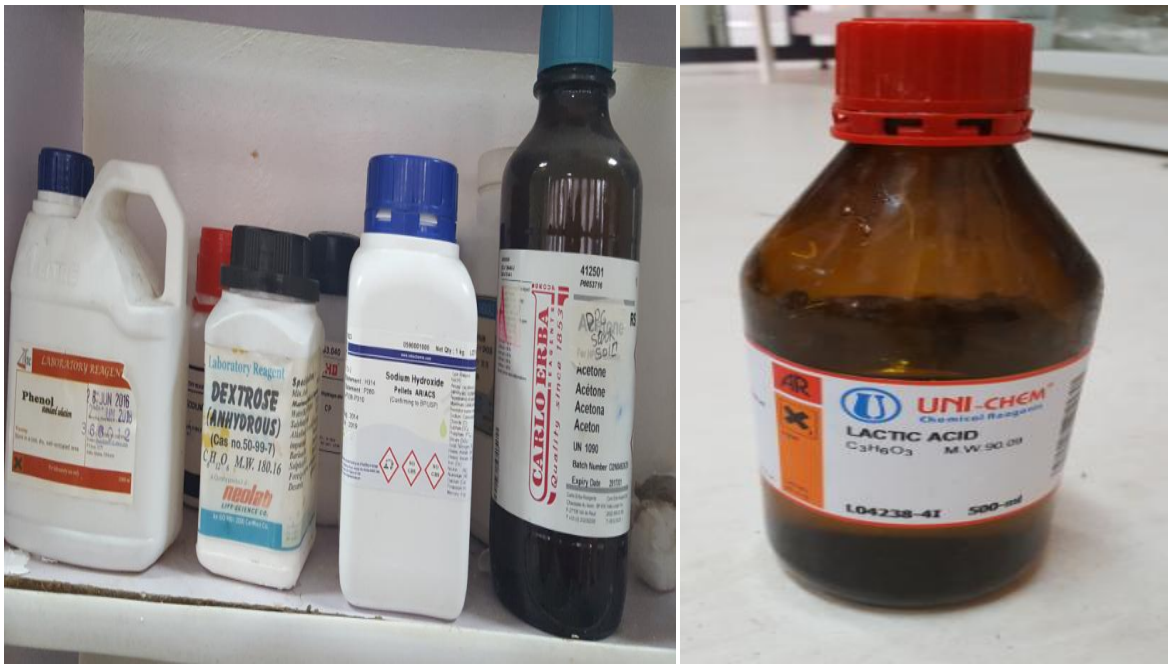



Figure 3-2**:** Different analytical chemicals

## 3.1. Materials and Methods

### 3.1.1. Materials used

The chemicals used on this study are depicted here in the table below:

Table 3-1: chemicals used

| S/No | Name of chemical | Chemical formula | Grade | Remark |
|---|---|---|---|---|
| **1** | Sulfuric acid | $H_2SO_4$ | 96% and 98% | Reagent for hydrolysis and pH adjustment |
| **2** | Sodium hydroxide | NaOH | Analytical | For pH adjustment |
| **3** | Phenol | $C_6H_6O$ | Analytical | Reagent for standard graph |
| **4** | acetone | $C_3H_6O$ | Analytical | Reagent for extraction |
| **5** | MRS | Not defined | Analytical | Medium |
| **6** | Potassium Die hydrogen phosphate | $KH_2PO_4$ | HPLC grade | Reagent as mobile phase |
| **7** | Acetonitrile | $CH_3CN$ | HPLC grade | Reagent as mobile phase |
| **8** | Distilled water | D ionized water | Laboratory grade | Used throughout the lab work |
| **9** | Lactic acid | $C_3H_6O_3$ | Analytical | Reagent for standard |

### 3.1.2. Equipment used

The different pieces of equipment used in this study were: Crusher with a model of Elektromotor fabric 7311, West Germany, UV-Spectrophotometer UV-VIS DOUBLE BEAM PC 8 SCANNING AUTO CELL UVD-3200 USA, Autoclave ADOLF WOLF sanoclve Robert-Bosch-str 13,Flow fast V cabinet model of flow fast V 15P,Furnace Model number of VF2, UK, Centrifuge (UNIVERSAL 320R), HPLC (high performance liquid chromatography): An Elite La Chrom (Hitachi, USA),pH meter with model of model 3505,S/NO 38490,Analyticalbalance with Model of FA2004, maximum weighing200g,Number201803173, readability, 0.0001g , Filter paper membrane filters paper type with diameter 45µm,Sox late extractor NS 60/40 Witeg Germany, Erlenmeyer flasks with size of 250 mL, shaker memmer model 100-800, Sieves:

retsch as 200 Gmbh 4278 Haan, Germany, Water bath with Model HH-S4,Test tubes:50 mL,Buchner funnel, Measuring cylinders Oven: intercontinental equipment, Refrigerator, Thermometer, Aluminum foil, Extraction thimbles and Vacuum filter.

## 3.2. Experimental methods

### 3.2.1. Raw material preparation and pretreatment

The carbon source sugarcane bagasse a by-product of sugar industry was collected from Wenji sugar factory located in Oromiya Regional State near Nazareth City about 120 km away from Addis Ababa. The sample was first washed with tap water several times to remove the impurities and it was washed again with distilled water and dried in the sun for one week and then dried at 105˚C for 24 hrs. Finally, it was ground in a crusher and screened into size range of 1.5-3.0 mm. (Sanchez et al., 2011).

Figure 3-3: Sugarcane bagasse

### 3.2.2. Proximate analysis of sugarcane bagasse

**Moisture content determination:** The moisture content was determined by loss on drying method. Subsequent incubation was accomplished at the interval of 30 min until constant weight achieved. A crucible amount weighed, and 5g of sugarcane bagasse was added and then the crucible was placed in an electric hot air oven maintained at 105˚C for 3 hours. After that it was cooled in dissector and then measured its mass, again it is returned to the oven at the same temperature as in the previous step and waited for 30 min. the process is continued until the consecutive mass difference becomes less than 1m.g.Finally, the moisture content was calculated as follows.

$$\%Mc = \frac{(W_1+W_2)-W_3}{W_2} * 100 \quad (3.1)$$

Where $M_c$ = Moisture content (%)

$W_1$= weight of container, g

W2 = weight of sample, g

W3 = weight of sample after draying, g

**Ash content determination:** 5g of powdered sugarcane bagasse was accurately weighed and transferred into a crucible. The crucible and its contents were gently heated over Bunsen burner flame until it became free from moisture and completely charred and then placed into a preheated muffle furnace at a temperature of 550°C for 4 hours. The crucible was cooled in a desiccator and weighed. The heating, cooling and weighing cycle was repeated until constant weight was achieved, and then the weight lost was recorded as the ash content of the activated carbon sample. The % ash content (dry basis) was calculated as follows:

$$\%\text{Ash content} = \frac{(M_1-M_2)}{M_3} * 100 \quad (3.2)$$

Where, $M_1$ = mass of sample after ignition, g

$M_2$=mass of empty dish, g

$M_3$ = mass of the sample, g

Figure 3-4**:** Ash content determination process

**Volatile Matter content Determination:** 1g of air-dried, powdered sugarcane bagasse sample was put in crucible covered with aluminum foil and was kept in a furnace at a temperature of 920°C for 10 minutes and then it was cooled in desiccators for 30 minutes. It was removed and weighed then the percentage of weight loss gave the volatile matter content(Hamid et al. , 2016). The percentage of volatile matter is computed as:

$$(\%)\ \text{Volatile matter} = \frac{(W_1 - W_2)}{W_1} * 100 \quad (3.3)$$

Where, $W_1$=Initial mass of sample as delivered

$W_2$ =Mass of the sample after ignition

**Fixed carbon content determination:** It was determined by subtracting the sum of percentage compositions of moisture content, volatile matter content, and ash content from 100.

$$\%FC = 100 - \%\text{moisture content} - \%\text{volatile matter content} - \%\text{ash content} \quad (3.4)$$

The sugar cane bagasse was subjected to compositional analysis using the gravimetric method. Materials used in this study were all unscreened.

**Extractives Determination:** 5g of dried sugarcane bagasse was loaded into cellulose thimble and then subjected to an extraction with the Soxlet extractor set up.150 mL of acetone was used as solvent for extraction. Residence times for the boiling and rising stages was carefully adjusted to 70°C and 25 min respectively on the heating mantle for a 4 hrs run period. After extraction, the sample was air dried at room temperature for few minutes. Constant weight of the extracted bagasse was achieved in a convection oven at 105°C for 24hrs. Then the %(w/w) of the extractives was calculated as the difference in weight between the raw bagasse and extractive free bagasse (Signorelli, 1999, Xu, 2004 and Yan, 2010).

$$\%\text{extractives} = (\text{raw bagasse} - \text{extractive free bagasse}) * 100 \quad (3.5)$$

Figure 3-5**:** Soxlet extraction process

**Hemicellulose Determination:** 1 g of extracted dried bagasse 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 using vacuum filtration and washed with distilled water seven times and neutral pH is achieved. The residue was dried to a constant weight at 105$^o$C for 24hrs in a convection oven. The difference between the sample weight before and after this treatment is the hemicellulose content (%w/w) of dry biomass (Blasi, 1999, Xu, 2004 and Lin, 2010).

% Hemicellulose (%w/w) = (weight before treatment – weight after treatment)*100 (3.6)

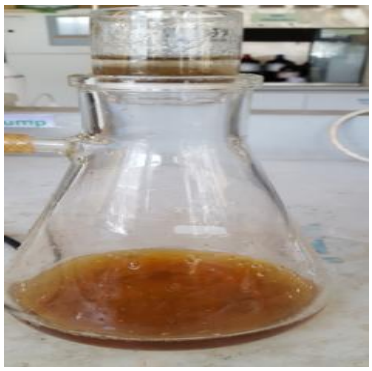


Figure 3-6**:** Hemicellulose filtration process

**Lignin Determination:** 0.3 g of extracted and dried bagasse was weighed in glass test tubes and 3 mL of 72% $H_2SO4$ was added. The sample was kept in water bath at room temperature for 2 h with carefully shaking at 30 min intervals to allow for hydrolysis. After the initial hydrolysis, 84 mL of distilled water was added which means the percent concentration of sulfuric acid was decreased to 2.57%. The second step of hydrolysis was made to occur in an autoclave for 1 h at 121 °C and then cooled at room temperature. Hydrolyzates were filtered through vacuum using a filtering crucible. The acid insoluble lignin was determined by drying the residues at 105 $^{o}$C for 24hrs and accounting for ash by incinerating the hydrolyzed samples for 4 hrs at 575$^{o}$C in a muffle furnace. The acid soluble lignin fraction was determined by measuring the absorbance of the filtrate sample at 320 nm using(equa.3.7) . The lignin content was calculated as the summation of acid insoluble lignin and acid soluble lignin (Hames, 2008).

%Lignin content = %( acid insoluble lignin (ash)) + %( acid soluble lignin (ASL)) = ash + equa.3.7

$$\%ASL = (UV\ abs * Volume\ of\ filtrate) * \frac{dilution}{\epsilon * ODW\ sample} * 100n \quad (3.7)$$

Where: UVabs = average UV is absorbance for the acid soluble sample at a wavelength of 320

$\epsilon$ = Absorbitivity of bagasse at 240nm wavelengths

Volume hydrolysis liquor = volume of filtrate, 86.73 mL

$$\text{Dilution} = \frac{volume\ sample + volume\ diluting}{volume\ sample} \quad (3.8)$$

$$\text{ODW} = \frac{Weightairdrysample * \%\ total\ solids}{100} \quad (3.9)$$

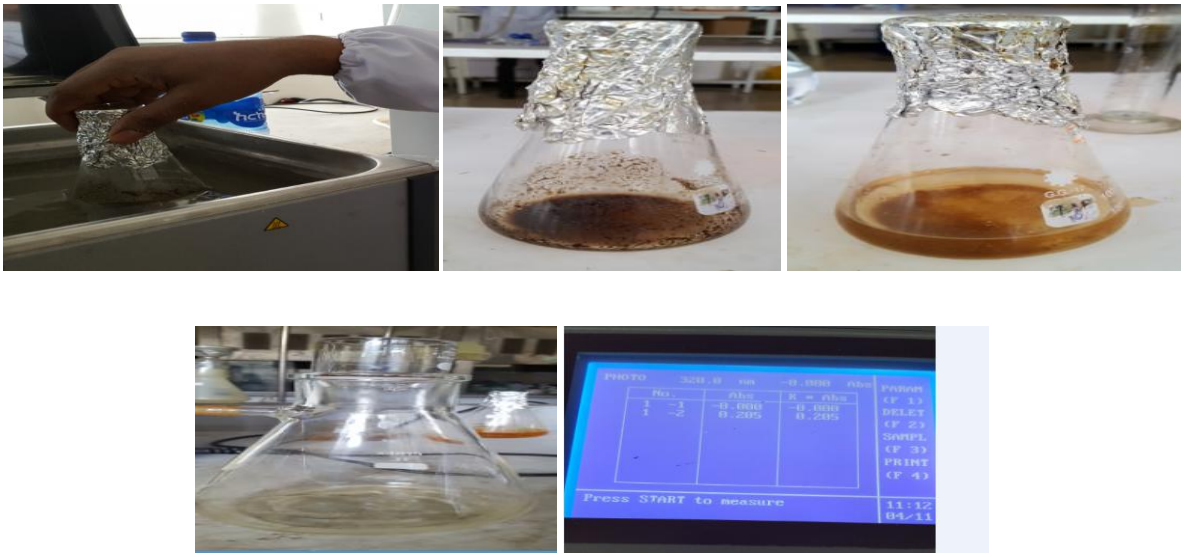



Figure 3-7**:** Lignin determination process

**Cellulose Determination:** The cellulose content of bagasse (%w/w) was calculated by subtracting extractives, hemicellulose, and total lignin from 100.Assuming that extractives, hemicellulose, total lignin, and cellulose are the only components of the entire bagasse (Blasi, 1999; Li, 2004 and Lin, 2010).

%Cellulose = (100 –%extractives – %hemicellulose − %total lignin) (3.10)

### 3.2.3. Acid Hydrolysis of sugar cane bagasse

The hydrolysis process was accomplished with three detrimental factors temperature, acid concentration and time in an autoclave. This is done to investigate the effects of these factors on the yield of glucose from the local bagasse. A Pretreated 480gof bagasse was hydrolyzed by 0.5%, 2.75%, and 5 % (v/v) of $H_2SO_4$. The temperature of the hydrolysis was controlled at 90, 105 and 120 $^oC$, and the reaction time was varied at 1, 3 and 5 hrs. All conditions were carried out using a LSR of 15 mL liquor/g dry weight of sugarcane bagasse (Aguilar, 2002, Herrera, 2004 and Chong, 2004).

After hydrolysis the hydrolaysate was filtered with the help of vacuum filter and the supernatants was determined for glucose content using UV spectrophotometer by the phenol sulfuric acid method.

Table 3-2: Experimental Range and Level of the Independent Variables

| **Variables** | **Symbols** | **Coded levels** | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| **Time(hr)** | X1 | 1 | 3 | 5 |
| **Acid concentration()** | X2 | 0.5 | 2.75 | 5 |
| **Temperature ($^0C$)** | X3 | 90 | 105 | 120 |

### 3.2.4. Calibration for glucose concentration measurement using UV-spectrophotometer

A stock solution was prepared by measuring accurately 0.05 g of glucose and dissolving in 500 mL distilled water. Five test tubes were used for preparation of the standard solution the four test tubes each was filled with 4, 6, 8, and 10 mL of standard glucose solution respectively the one test tube was placed with 10 mL of distilled water served as blank. 1 mL of the phenol solution (prepared by dissolving 5 g of phenol in 100 mL of distilled water) was added to each test tube and carefully mixed. 5 mL of concentrated sulfuric acid (96%) was also added with the help of burette to each test tube. The solution was carefully mixed using vortex and it was left at room temperature for 10 minutes and then it was placed in a water bath for 15 min at a temperature of 90 $^{\circ}C$ and then it is cooled in running water. Finally, the absorbance of the solution was detected by the help of UV-spectrophotometer at 490 nm against distilled water as a reference.

These absorbance (AU) values were plotted against the sugar concentration (g/L) of the standard solution then from these data the standard graph was developed (see Figure 3-8). The slope was calculated (absorbance over concentration) using linear regression and used to determine the amount of glucose in the samples (SASTA, 1985).

Table 3-3: Solutions used for calibration

| **Concentration of the stock solution (g/L)** | **Water that was taken (mL)** | **Stock solution that was taken (mL)** | **Phenol that was taken (mL)** | **Sulfuric acid that was taken (96%) (mL)** | **Concentration of glucose (g/L)** | **Absorbance of the standard solution** |
|---|---|---|---|---|---|---|
| **0** | 10 | 0 | 1 | 5 | 0 | 0 |
| **1.6** | 6 | 4 | 1 | 5 | 40 | 0.48 |
| **3.6** | 4 | 6 | 1 | 5 | 60 | 0.608 |
| **6.4** | 2 | 8 | 1 | 5 | 80 | 0.641 |
| **10** | 0 | 10 | 1 | 5 | 100 | 0.9345 |

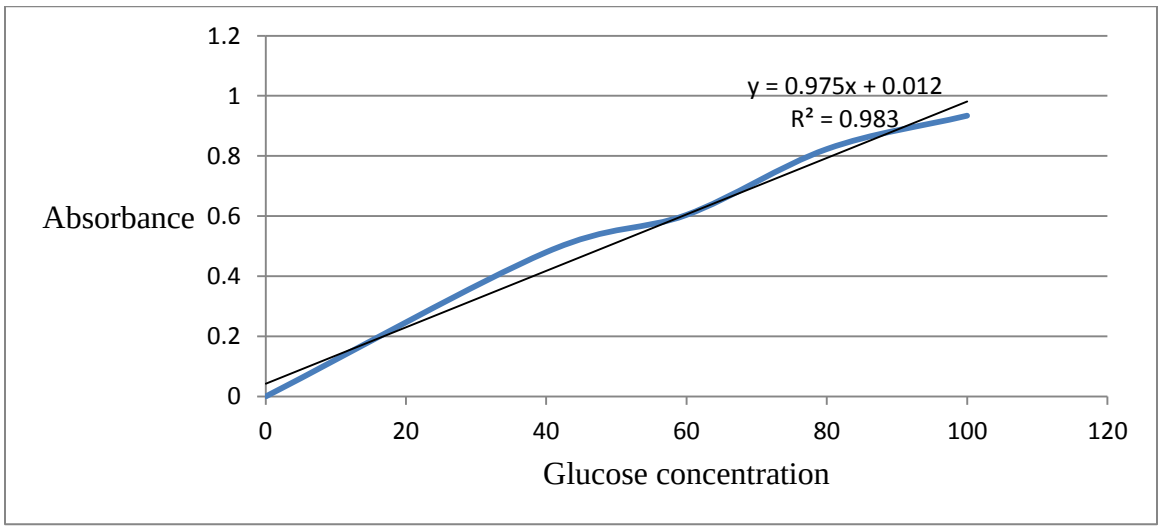



Figure 3-8**:** Calibration curve

Slope = 0.975 AU g

Intercept = 0.012

### 3.2.5. Determination of samples equivalent glucose

The same procedure was applied what is done in the above except here the glucose was taken from the one produced in this study. The glucose concentration of the hydrolaysate samples were determined by using the developed formula from the standard graph.

Then glucose content of samples was calculated using the slope of the graph as follows.

$$\text{Glucose concentration } \left(\frac{g}{l}\right) = \frac{(absorbance\ of\ sample - intercept)}{slop\left(AU\frac{g}{l}\right)} \quad (3.11)$$

Figure 3-9**:** Glucose content determination process

### 3.2.6. Microbe collection, inoculum preparation and growth

The vial of lacto-bacillus Plantarum bacteria was collected from Ethiopian bio-diversity institute and placed on a deep freeze (-4°C) until the MRS medium was prepared. The inoculum preparation of this experiment was done by mixing the following reagents g/L(Proteose peptone 10.000,Beef extract10.000,Yeast extract 5.000,Dextrose 20.000,Polysorbate 80, 1.000,Ammonium citrate2.000,Sodium acetate 5.000,Magnesium sulphate 0.100,Manganese sulphate0.050,Dipotassium phosphate 2.000 and Final pH 6.5±0.2) (at 25°C))(Rogosa and Sharpe, 1960,).250 mL of MRS broth medium was prepared and sterilized for 15 min at 121°C then half of the vial was inoculated in to 10 mL MRS medium and incubated for 48 hrsat37°C.Cell growth was monitored analytically by measuring the optical density (OD) of the medium in a 1.5 mL glass cuvette with UV-spectrophotometer at 620nm (Pharmacia LKB Biotechnology, 2011). Then the microbes were grown as a result the optical density increased from the absorbance of 1.74 to 1.98. The multiplied lacto bacillus plantarum was transferred to the remaining previously prepared and sterilized 240 mL of MRS broth and incubated for 48 hrs at 37°C. Finally, the denser and jelly like material became ready for fermentation. (Hi Media Laboratories Pvt, 2015).

Figure 3-10**:** Lacto bacillus Plantarum growth process

### 3.2.7. Fermentation medium preparation

The fermentation medium was prepared in 250mL of 17 Erlenmeyer flask by taking 100 mL of fermented broth with 3.755g/L,3.59g/L, and 3.42 g/L high to low glucose concentration produced in the hydrolysis process with a supplement of 5g/L of yeast extract. The pH was adjusted to the chosen one (4, 6, 8) with the help of $H_2SO_4$ and NaOH (for decreasing and increasing respectively). The prepared medium was sterilized for 15 min at121$^{\circ}$C and cooled for about 2 hrs on swabbed and disinfected hood flow fast V cabinet and the work was accomplished in minimum time to prevent exposure. The loop was flamed and allowed to cool before transferring the bacteria. Then the inoculum was added in such a way: The mouth of the fermentation medium flasks and the inoculating loop were flamed before adding the culture. After completing the sterilization process a 10mL of inoculum of the L.bacillus plantarum was added from the prepared to a series of flasks and then it became ready for fermentation. (Sakamoto and Komagata, 1996).

Figure 3-11: Fermentation media preparation process

### 3.2.8. Design of Fermentation Experiment

Three factors with three levels were selected for this particular experiment. Glucose concentration, pH, and incubation time were the factors. 3.755 g/L, 3.59 g/L and 3.42 g/L were three levels selected for glucose concentration; 4, 6 and 8 were three levels selected for pH and 10hrs, 20hrsand 30hrs were three levels selected for incubation time. Then all of these experiments were conducted based on their randomized run order. Finally, the interaction and main effects of these parameter were analyzed and the optimum condition of fermentation parameters were determined by using Design-Expert software version 11.

### 3.2.9. Fermentation process

The fermentation was conducted in the incubator shaker found on AAU school of chemical and bioengineering biochemical laboratory. The prepared samples were incubated at a constant temperature of 37°C with their respective incubation time of 10-30hrs, pH range of 4-8 as well as glucose concentration range of 3.755g/L -3.42g/L. The speed of the incubator shaker was kept constant at180rpm and the fermentation was takes placed in anaerobic condition. Then the fermentation ceased at its respective incubation time and the fermented broth was separated by the help of centrifuge running at10000rpm for 10 min. Finally the clear liquid collected as lactic acid (Sakamoto and Komagata, 1996).

Figure 3-12**:** Fermentation and centrifugation process

### 3.2.10. HPLC analysis

The process of determination of lactic acid from the fermented broth was conducted with the help of HPLC in Addis pharmaceutical S.C, Adigrat, Ethiopia. This is an efficient method selected for the determination of lactic acid concentration. The estimation of the concentration was accomplished using An Elite LaChrom (Hitachi, USA) liquid chromatography consisting of a system controller, binary pump (L-2130), auto injector (L-2200), column oven (L-2300) and photodiode array detector (DAD) (L2456).

The solvents used were of HPLC grade. The column used was Ace 5 C18 and the pH and temperature were kept at 2.5 and 20°C, respectively. The mobile phase used was a mixture of10 mM $KH_2PO_4$ (1.36 g/L)(potassium die-hydrogen phosphate) and Acetonitrile with 99:1 composition. This mixture was prepared in such a way that 1.36 g of potassium die-hydrogen phosphate was dissolved in 1L of distilled water and 990 mL of this solution was mixed with 10mL of Acetonitrile and delivered isocratic, at a flow rate of 0.2 mL/min.1.5 mL of standard lactic acid (87.5% purity) was injected in to the HPLC and its peak height was displayed as indicated in Figure 3-13 at a detection time of 3.26 min. Detection and identification were performed using a photodiode array detector at 220 nm. Likewise, the desired peak height of the samples was also obtained using a photodiode array detector at 220 nm with a sample size of1.5mLat a detection time of 3.26min (Jannicke, Remme and Astrid, 2006).The obtained peak height at which the maximum lactic acid was found is indicated in Figure3-14.

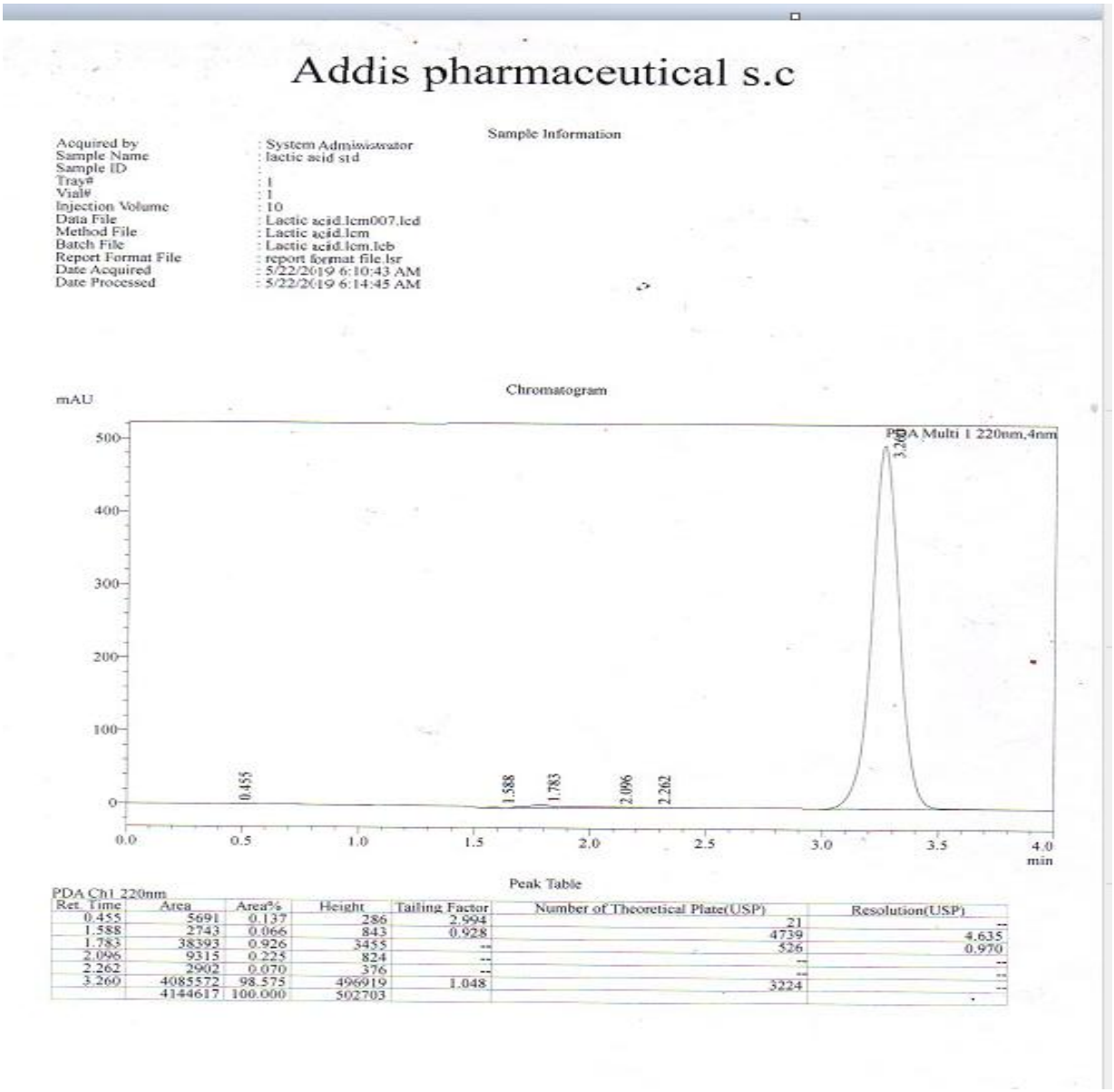


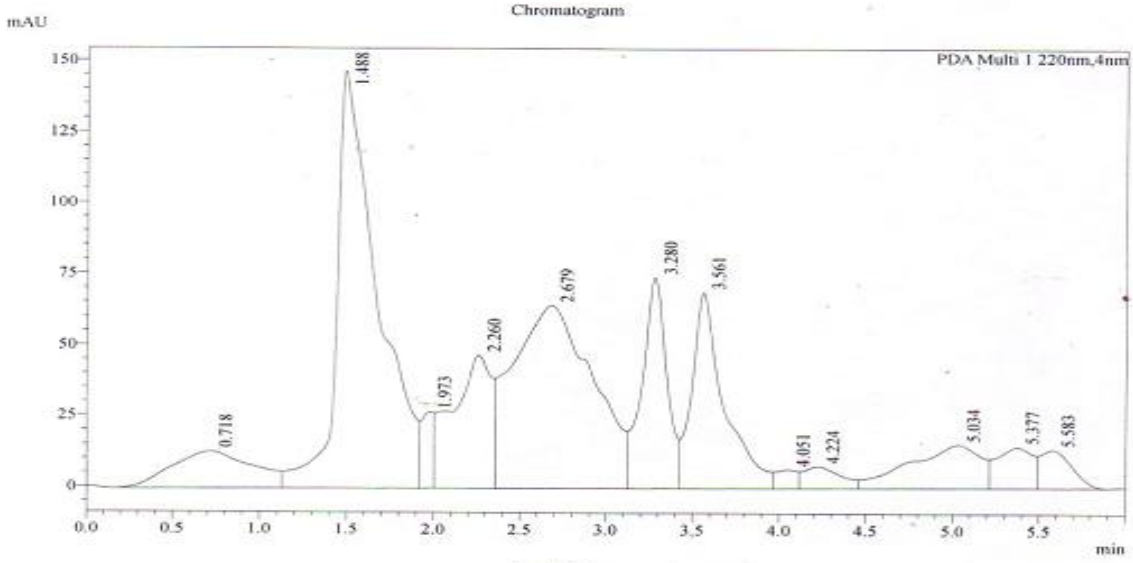

Figure 3-13**:** Graph of standard lactic acid

# Addis pharmaceutical s.c

Sample Information

| | |
|---|---|
| Acquired by | : System Administrator |
| Sample Name | : lactic acid 12 |
| Sample ID | : |
| Tray# | : 1 |
| Vial# | : 13 |
| Injection Volume | : 10 |
| Data File | : Lactic acid.lcm025.lcd |
| Method File | : Lactic acid.lcm |
| Batch File | : Lactic acid.lcm.lcb |
| Report Format File | : report format file.lsr |
| Date Acquired | : 5/22/2019 8:46:57 AM |
| Date Processed | : 5/22/2019 8:52:59 AM |

Chromatogram




Peak Table

PDA Ch1 220nm

| Ret. Time | Area | Area% | Height | Tailing Factor | Number of Theoretical Plate(USP) | Resolution(USP) |
|---|---|---|---|---|---|---|
| 0.718 | 416324 | 4.969 | 12831 | | 6 | |
| 1.488 | 2394476 | 28.580 | 146552 | | 238 | 0.970 |
| 1.973 | 131685 | 1.572 | 26653 | | 6 | 0.263 |
| 2.260 | 737078 | 8.798 | 46922 | | 173 | 0.143 |
| 2.679 | 2077649 | 24.799 | 64240 | | 90 | 0.462 |
| 3.280 | 734601 | 8.768 | 73896 | | 2238 | 0.856 |
| 3.561 | 898660 | 10.726 | 68695 | | 2193 | 0.965 |
| 4.051 | 55954 | 0.668 | 6521 | | 121 | 0.552 |
| 4.224 | 115411 | 1.378 | 7658 | | 606 | 0.160 |
| 5.034 | 439592 | 5.247 | 15197 | | 417 | 0.968 |
| 5.377 | 208413 | 2.488 | 14320 | | 599 | 0.368 |
| 5.583 | 168233 | 2.008 | 13415 | | 845 | 0.250 |
| | 8378075 | 100.000 | 496901 | | | |

Figure 3-14: Graph of the maximum lactic acid yield

$$Concentration of lactic acid \left(\frac{g}{l}\right) = \frac{peak area of sample}{peak area of standard} * purity\ of\ standard \quad (3.12)$$

Figure 3-15**:** HPLC lactic acid determination process

# CHAPTER FOUR

## 4. RESULTS AND DISCUSSION

### 4.1. Characterization of Sugarcane Bagasse

The results are presented in table 4-1.

Table 4-1: Experimentally determined compositions of sugarcane bagasse

| Characteristics | Value obtained (%) | Factory data |
|---|---|---|
| **Moisture content** | 46.23 | 49.85 |
| **Ash content** | 4.363 | N/A |
| **Volatile matter** | 34.82 | N/A |
| **Fixed carbon content** | 14.587 | N/A |
| **Extractives** | 6.6 | N/A |
| **Hemicellulose** | 47.43 | N/A |
| **Lignin** | 27.6 | N/A |
| **Cellulose** | 18.37 | N/A |

Table 4-2: Compositions of sugarcane bagasse from literature

| Characteristic | Value from literature (%) | |
|---|---|---|
| **Moisture content** | 28–31(Ayeni,2015 ,Omoniyi& Olorunnisola,2014) | 7 (Olorunnisola, 2014) |
| **Ash content** | 4.1±0.3 (Ayeni,2015,Omoniyi&Olorunnisola, 2014) | 2-15 (Pham et al., 2015) 7.21 (Olorunnisola, 2014) |
| **Volatile matter** | 69.4 - 81.7 (Ayeni, 2015 and Omoniyi & Olorunnisola, 2014) | 70.94 (Olorunnisola, 2014) |
| **Fixed carbon content** | 12 – 16 (Ayeni et al, 2015 and Omoniyi & Olorunnisola, 2014) | 16 (Olorunnisola, 2014) |
| **Extractives** | 2.14±0.6 (Ayeni et al., 2015) and Omoniyi & Olorunnisola, 2014) | |

| | |
|---|---|
| **Hemicellulose** | 33.28±0.8 (Ayeni et al., 2015 and Omoniyi & Olorunnisola, 2014) 31 (DOE US Department of Energy, 2006) 33.8 (Sunetal, 2004). |
| **Lignin** | 25.20±1.1 (Ayeni ,2015) and Omoniyi & Olorunnisola, 2014) 11 (DOE US Department of Energy, 2006) 18.1 (Sunetal, 2004). |
| **Cellulose** | 35.28±1.2 (Ayeni, 2015 and Omoniyi & Olorunnisola, 2014) 43 (DOE US Department of Energy, 2006) 43.6 (Sunetal, 2004). |

The experimental physico-chemical analyses of bagasse of this study result indicated in table 4-divates with the literature studied, the reason could be due to the local weather condition, laboratory handling system and variety of the sugarcane.

## 4.2. Glucose content of the acid hydrolyzed samples

The glucose content of the hydrolyzed samples was determined using the phenol sulfuric acid method. As shown in table 4-3, the maximum glucose concentration of 3.755 g/L was achived at a temprature of 105°C,at a time of 3hrs and at an acid concentration of 2.75%.

Table 4-3: All runs of glucose concentration of hydrolyzed samples

| STD | Run | Factor 1: Acid concentration (%) | Factor 2: time (hrs.) | Factor 3:temperature(°C) | Response 1: Glucose concentration(g/L) |
|---|---|---|---|---|---|
| **1** | 8 | 0.5 | 1 | 105 | 3.45 |
| **2** | 10 | 5 | 1 | 105 | 3.51 |
| **3** | 15 | 0.5 | 5 | 105 | 3.52 |
| **4** | 14 | 5 | 5 | 105 | 3.52 |
| **5** | 1 | 0.5 | 3 | 90 | 3.28 |
| **6** | 6 | 5 | 3 | 90 | 3.39 |
| **7** | 12 | 0.5 | 3 | 120 | 3.35 |
| **8** | 4 | 5 | 3 | 120 | 3.32 |
| **9** | 5 | 2.75 | 1 | 90 | 3.37 |
| **10** | 13 | 2.75 | 5 | 90 | 3.21 |
| **11** | 7 | 2.75 | 1 | 120 | 3.19 |
| **12** | 17 | 2.75 | 5 | 120 | 3.39 |
| **13** | 16 | 2.75 | 3 | 105 | 3.755 |
| **14** | 3 | 2.75 | 3 | 105 | 3.71 |
| **15** | 11 | 2.75 | 3 | 105 | 3.74 |
| **16** | 2 | 2.75 | 3 | 105 | 3.752 |
| **17** | 9 | 2.75 | 3 | 105 | 3.73 |

The glucose concentration found here in this study is below the literature the reason could be due to the utilization of single strain lactobacillus plantarum, variety of the sugarcane and the laboratory conditions.

## 4.3. Statistical Analysis

Table 4.4 shows the results of the 17 experimental runs. Table 4-5 also illustrates the observed values and predicted values carried out according to the BBD. BBD is the commonly used experimental design models for three level three factor experiments. BBD always has three levels for each factor and is purpose built to fit a quadratic model. The BBD 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. While a run or two can be botched in these designs the accuracy of the observations in the remaining runs is critical to the dependability of the model. Design-Expert Software 11was 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.

Table 4-4: Box-Behnken Design Matrix for Lactic Acid Production

| | | **Factor 1** | **Factor 2** | **Factor 3** | **Response 1** |
|---|---|---|---|---|---|
| **Std** | Run | A:glucose concent | B:pH | C:Time | Lactic acid |
| | | g/L | | hr | g/L |
| **1** | 16 | 3.19 | 4 | 20 | 12.37 |
| **2** | 5 | 3.755 | 4 | 20 | 12.68 |
| **3** | 3 | 3.19 | 8 | 20 | 12.01 |
| **4** | 4 | 3.755 | 8 | 20 | 14.03 |
| **5** | 9 | 3.19 | 6 | 10 | 14.21 |
| **6** | 11 | 3.755 | 6 | 10 | 12.61 |
| **7** | 8 | 3.19 | 6 | 30 | 11.45 |
| 8 | 7 | 3.755 | 6 | 30 | 15.79 |
| **9** | 15 | 3.47 | 4 | 10 | 14.27 |
| **10** | 13 | 3.47 | 8 | 10 | 12.33 |
| **11** | 10 | 3.47 | 4 | 30 | 11.87 |
| **12** | 12 | 3.47 | 8 | 30 | 14.87 |
| **13** | 17 | 3.47 | 6 | 20 | 13.849 |
| **14** | 1 | 3.47 | 6 | 20 | 13.845 |

| | | | | | |
|---|---|---|---|---|---|
| **15** | 14 | 3.47 | 6 | 20 | 13.73 |
| **16** | 6 | 3.47 | 6 | 20 | 13.849 |
| **17** | 2 | 3.47 | 6 | 20 | 13.845 |

The maximum lactic acid concentration of 15.79g/L was achievedat ata pH of 6, a maximum time of 30hrs and a maximum glucose concentration of 3.755g/L.this concentration is below the litrature the reason could be due to the application of single strain lacto bacillus plantarum,labratory handling system and variety of the sugarcane.

Table 4-5: Diagnostics Case Statistics

| Run Order | Actual Value | Predicted Value | Residual |
|---|---|---|---|
| **1** | 13.85 | 13.82 | 0.0214 |
| **2** | 13.85 | 13.82 | 0.0214 |
| **3** | 12.01 | 11.97 | 0.0425 |
| **4** | 14.03 | 14.09 | -0.0600 |
| **5** | 12.68 | 12.72 | -0.0425 |
| **6** | 13.85 | 13.82 | 0.0254 |
| **7** | 15.79 | 15.70 | 0.0862 |
| **8** | 11.45 | 11.47 | -0.0163 |
| **9** | 14.21 | 14.30 | -0.0863 |
| **10** | 11.87 | 11.91 | -0.0438 |
| **11** | 12.61 | 12.59 | 0.0162 |
| **12** | 14.87 | 14.90 | -0.0263 |
| **13** | 12.33 | 12.29 | 0.0437 |
| **14** | 13.73 | 13.82 | -0.0936 |
| **15** | 14.27 | 14.24 | 0.0262 |
| **16** | 12.37 | 12.31 | 0.0600 |
| **17** | 13.85 | 13.82 | 0.0254 |

## 4.4. Experimental Design Analysis

The experimental design selected for analysis of variance was response surface methodology (RSM) and under response surface methodology, BOX- Behnken was selected. The response of the analysis was Lactic acid yield. The response yield was arranged from11.45 – 15.79g/L.The ratio of maximum to minimum yield was 1.38. A ration greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformation has a little effect. The aim of the model fit summary was maximizing the adjusted R-Squared and predicted R-Squared. Model significances was checked for both model and model factor, linear model factors glucose concentration (A,) fermentation time (B) and pH(C) and, quadratic model factors; pure quadratic terms ($A^2$,$B^2$,$C^2$) and interaction quadratic terms (AB,AC,BC) depending on the F and P values.

Table 4-6: Analysis of variance (ANOVA) for the Response Of lactic Acid yield

| Source | Sum of square | df | Mean square | F value | p-value prob > F | |
|---|---|---|---|---|---|---|
| **Model** | 21.97 | 9 | 2.44 | 403.09 | < 0.0001 | significant |
| **A-glucose concentration** | 3.21 | 1 | 3.21 | 530.56 | < 0.0001 | |
| **B-pH** | 0.5253 | 1 | 0.5253 | 86.74 | < 0.0001 | |
| **C-Time** | 0.0392 | 1 | 0.0392 | 6.47 | < 0.0001 | |
| **AB** | 0.7310 | 1 | 0.7310 | 120.71 | < 0.0001 | |
| **AC** | 8.82 | 1 | 8.82 | 1456.55 | < 0.0001 | |
| **BC** | 6.10 | 1 | 6.10 | 1007.41 | < 0.0001 | |
| **$A^2$** | 0.7988 | 1 | 0.7988 | 131.89 | < 0.0001 | |
| **$B^2$** | 1.60 | 1 | 1.60 | 263.44 | < 0.0001 | |
| **$C^2$** | 0.0679 | 1 | 0.0679 | 11.21 | <0.0001 | |
| **Residual** | 0.0424 | 7 | 0.0061 | | | |
| **Lack of Fit** | 0.0314 | 3 | 0.0105 | 3.82 | 0.1141 | not significant |
| **Pure Error** | 0.0110 | 4 | 0.0027 | | | |
| **Cor Total** | 22.01 | 16 | | | | |

The model was statistically significant as seen from the very low p value (<0.0001) as shown in Table 4-6. The Model F-value of 403.09 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. 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 glucose concentration, pH, and incubation time as well as interaction between glucose concentration and pH, interaction between glucose concentration and incubation time, interaction between pH and incubation time, square of the glucose concentration, square of pH and square of incubation time affects the yield of lactic acid production significantly.

The Lack of Fit F-value of 3.82 implies the Lack of Fit is not significant relative to the pure error. There is an 11.41% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit. The model fit summary statistics are listed in Table 4-7.

Table 4-7:Analysis of variance (ANOVA) fit statistics for the Response of lactic Acid yield

| **Std.Dev** | **0.0778** | **R-Squared** | **0.9981** |
|---|---|---|---|
| **Mean** | 13.39 | Adj R-Squared | 0.9956 |
| **C.V. %** | 0.5812 | Pred R-Squared | 0.9764 |
| | | Adeq Precision | 70.9970 |

The Predicted $R^2$ of 0.9764 is in reasonable agreement with the Adjusted $R^2$ of 0.9956; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of this study which is 70.997 indicates an adequate signal. This model can be used to navigate the design space.

Table 4-8: Lactic Acid Model Coefficient Estimate and Confidence Interval

| Factor | Coefficient Estimate | Df | Standard Error | 95%CI Low | 95% CI High | VIF |
|---|---|---|---|---|---|---|
| **Intercept** | 13.82 | 1 | 0.0348 | 13.74 | 13.91 | |
| **A-glucose concentration** | 0.6338 | 1 | 0.0275 | 0.5687 | 0.6988 | 1.0000 |
| **B-pH** | 0.2563 | 1 | 0.0275 | 0.1912 | 0.3213 | 1.0000 |
| **C-Time** | 0.0700 | 1 | 0.0275 | 0.0049 | 0.1351 | 1.0000 |
| **AB** | 0.4275 | 1 | 0.0389 | 0.3355 | 0.5195 | 1.0000 |
| **AC** | 1.49 | 1 | 0.0389 | 1.39 | 1.58 | 1.0000 |
| **BC** | 1.23 | 1 | 0.0389 | 1.14 | 1.33 | 1.0000 |
| **$A^2$** | -0.4356 | 1 | 0.0379 | -0.5252 | -0.3459 | 1.01 |
| **$B^2$** | -0.6156 | 1 | 0.0379 | -0.7052 | -0.5259 | 1.01 |
| **$C^2$** | 0.1269 | 1 | 0.0379 | 0.0373 | 0.2166 | 1.01 |

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicates multi co-linearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

The application of RSM offers an empirical relationship between the response and the independent variables. The mathematical relationship between the reponse and the independent variables glucose concentration(A), pH(B) and time(C) in terms of coded and and actual factors can be determined by Design Expert Software. The model equation that correlats the response to the process variables is given in equation below.

Final Equation in Terms of Coded Factors:

$$\text{Lactic acid} = +13.82 + 0.6338*A + 0.2563*B + 0.0700*C + 0.4275*A*B + 1.49*A*C + 1.23*B*C - 0.4356*A^2 - 0.6156*B^2 + 0.1269*C^2 \quad (4.1)$$

Were, A = glucose concentration

B = pH

C = time

From the regression model equation developed in terms of coded factors, the reponse yield was affected by linear terms glucose concentration (A) ,pH(B) and time (C) and, pure quadratic terms ($A^2$,$B^2$,$C^2$) and interaction quadratic terms (AB,BC,AC). On the basis of the coefficint in equation, it was evident that the lactic acid yield increase with the increase in glucose concentration (A),pH (B) and incubation time(C), which have positive linear effect on yield but glucose concentration has a more weightly linear effect on lactic acid yield compare to incubation time and pH with a value of 0.6338. Pure quadratic terms ($A^2$,$B^2$) have negative effect on the lactic acid yield but pure qadratic $C^2$ has a posetive effecton the lactic acid yeild as a result it has substenial effect than the other quadratic terms.interaction of glucose concentrationb and pH (AB),interaction of glucose concentration and time (AC), and interaction of pH and time (BC) have positive effect on lactic acid yield.

The coded model equation is useful for identifying the relative impact of the comparing the factor coeeficients.

Final Equation in Terms of Actual Factors:

$$\text{Lactic acid} = -106.99973 + 89.78144 * \text{glucose concentration} - 3.83830 * \text{pH} - 3.59484 * \text{time} + 1.27612 \text{ glucose concentration} * \text{pH} + 0.886567 \text{ glucose concentration} * \text{time} + 0.061750 \text{ pH} * \text{time} - 15.52417 \text{ glucose concentration}^2 - 0.153888\text{pH}^2 + 0.001270 \text{ time}^2 \quad (4.2)$$

Model equation of both coded and actual factors can be used to make prediction about the response for given levels of each factors.

## 4.5. 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 with a value of 0.9981, 0.9956 and 0.9764 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 0.9981. It implies that 99.81% of the total variation in the percentage of conversion 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 4-3. The results in Figure 4-3 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 obtaining the correlation between the three variables to the production of Lactic acid. 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 each other, 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. The effectiveness of the model could also be measured for the sake of assurance its approximation to the true value. Thus, regression coefficient, $R^{2,}$ could be used for checking its adequacy. The regression value is between 0 and 1,and as it approach to 1 it fits well to the experimental data otherwise it indicates failure of approximation .in this study,$R^2$, 0.9981 was obtained ,which was close to one and the value Adj- $R^2$ was 0.9956.and it is in a reasonable agreement with $R^2$.

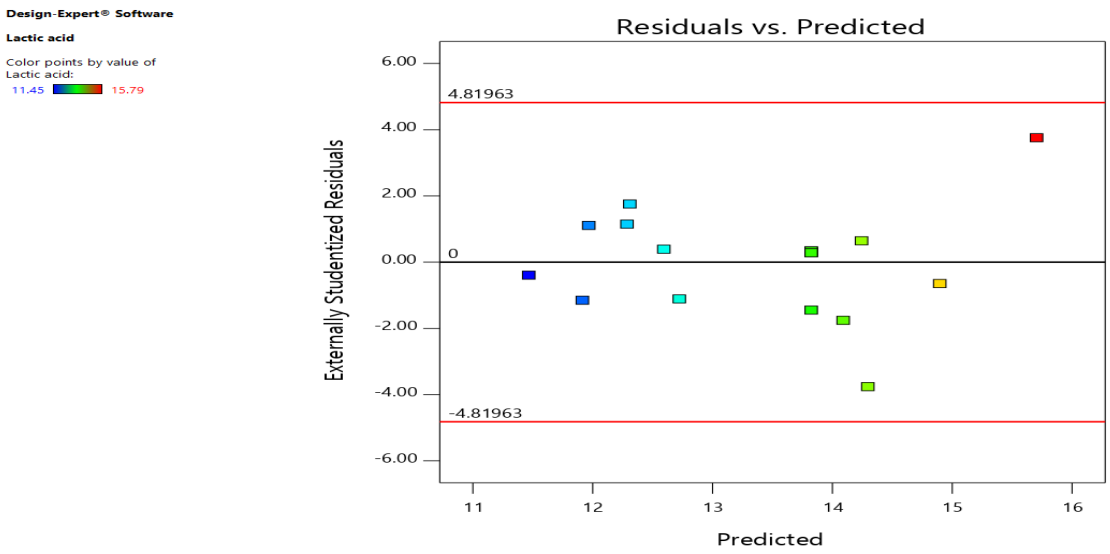



Figure 4-1: Predicted Vs Actual Experimental Value of lactic Acid Yield

## 4.6. Effect of single factors on Lactic Acid yield

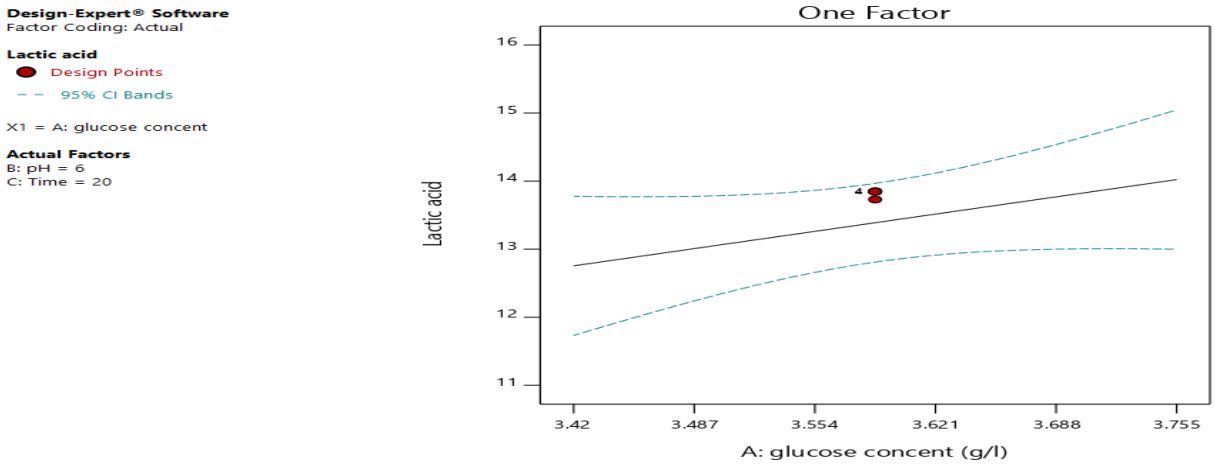
As it is observed in equation 4.1, all the three factors affects the yield of lactic acid positively which means the yield of lactic acid is directly proportional to all the three factors: glucose concentration, to some extent(up to pH 6) and time as these parameters increase the yield of lactic acid increases.

The effect of glucose concentration on lactic acid yield is shown in Figure 4-4a.The production of lactic acid by L.bacillus plantarum was strongly dependent on concentration of glucose. At the lower concentration of glucose the yield of lactic acid found to decline this is because of a low glucose concentration leads to low carbon source, which consequently results in increased competition for nutrients. But as glucose concentration increases the lactic acid yield was also increased (Wee et al., 2004).

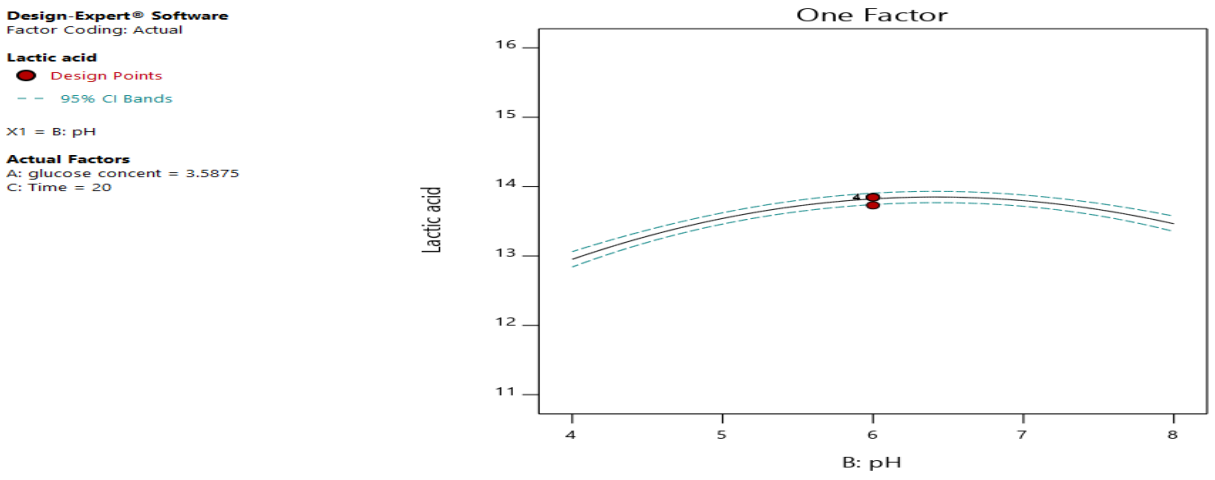
From the model equation developed on this study pH positively affects the yield of lactic acid. As indicated in Figure 4-4 (b), at the beginning of the fermentation, the yield of lactic acid was low due to the L.bacillus plantarum difficulty to adapt to the media. But at the middle at a pH of 6 the maximum lactic acid yield was achieved which is the optimum pH for L.bacillus plantarum to ferment sugar.(Wee et al., 2006). As the pH exceeds the optimum one, the yield of lactic acid

begins to decline due to the higher pH brought too much stress on the L.bacillus plantarum metabolic abilities (Vijayakumar et al., 2008).

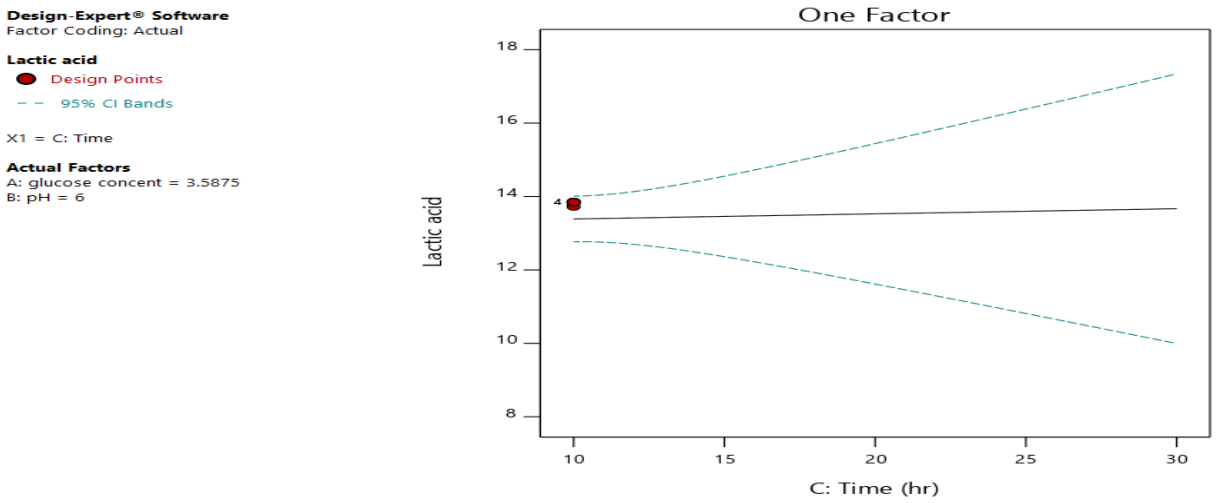
In this study as it is indicated in Figure 4-4 (c), time positively affects the yield of lactic acid. The maximum amount of lactic acid was obtained at the optimum fermentation time which is 30hrs. The fermentation time within the range of 10 – 20hrs the production of lactic acid was lees due to the growth phase of L.bacillus plantarum as a result of difficulty to adapt the environment (Panesar et al., 2010).




(a)




(b)




(c)

Figure 4-2: single effect of glucose concentration (a); pH (b); time (c) on lactic acid yield

## 4.7. Interactive Effects of Parameters between Process Variables

The interaction of the three factors also significantly affects the lactic acid yield. Three dimensional (3D) response surface plots (plotted in order to understand the interaction between the variables and the optimum level of each variable) and contours plots were generated to determine the optimum levels of the variables that were investigated in this study. The plots were generated by varying three of the variables within the experimental range. The resulting response surface shows the effect of glucose concentration, pH and time on lactic acid production. The significance of the interaction between the corresponding variables was indicated by saddle nature of the contour plots.

An interaction occurs when the response was different depending on the setting of the factors. The plot makes it easy to interpret the interaction. They appear with two non-parallel lines, indicates that the effect of one factor depends on the level of other.

If the plotted points fall outside the range, the differences are unlikely to be caused by error alone and can be attributed to the factor. As it is shown from equation 4.1 the effects of interaction factor on lactic acid yield can be understood easily by coefficient of interaction factors. There were three interaction factors analyzed by the model equation. These are:

- AB- glucose concentration and pH
- AC- glucose concentration and time
- BC- pH and time

Among these, three interaction factors interaction of glucose concentration and time (AC) was the most significance factor for lactic acid production as response, because it has the highest coefficient (1.49) of the rest. The sign of the coefficient of the interaction factor indicates the effect of interaction factor on lactic acid yield. Therefore, the interaction factors with positive sign have positive effect on lactic acid yield (as interaction factors increase lactic acid yield increase).whereas, interaction factors with negative signs have a negative effect on lactic acid production (as the interaction factors increase lactic acid yield decrease).unfortunately in this study all the three interaction factors have a positive sign as a result they affects the yield of lactic acid positively. The 3D response surface and contour plots of the effect of interaction of glucose concentration, pH and time with the response of lactic acid production was discussed below.

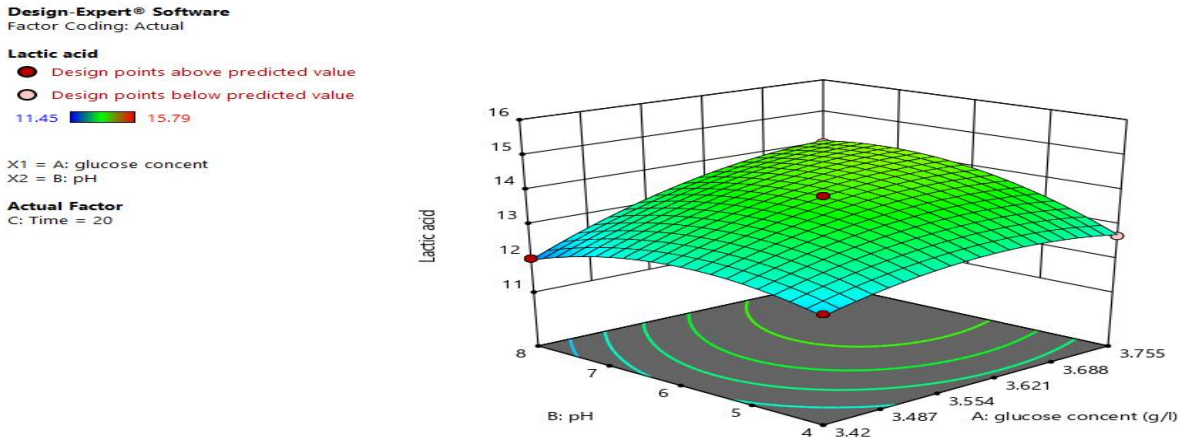
### 4.7.1. Effect of glucose concentration and pH on lactic Acid Yield

Figure 4-5 (a) and (b)shows the effect of glucose concentration and pH on lactic acid production. The trend observed indicates that lactic acid production was favored at high glucose concentration and optimum pH of 6 this is evident from the increase in lactic acid concentration when the glucose concentration was increased and pH was at its optimum which is 6.

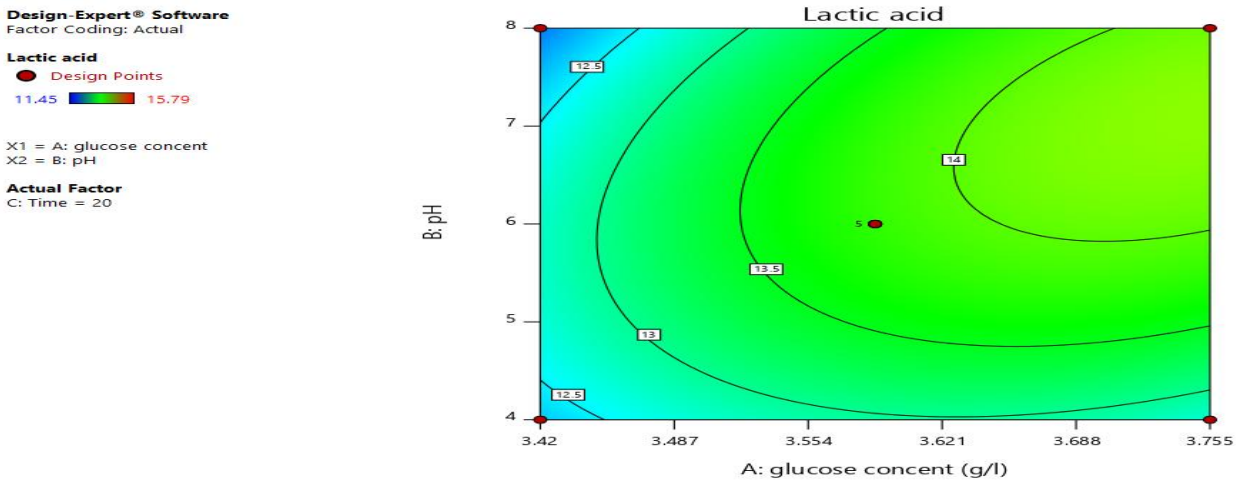
The concentration of glucose used for fermentation determines the amount of fermentable sugars that can be liberated for the purpose of producing lactic acid. Since lactic acid production is enhanced at increased sugar concentration, it will be expected that increasing the glucose concentration will also have a similar effect.

The increase in lactic acid concentration in the course of fermentation could be attributed to the consumption of sugar substrate by the L.bacillus plantarum cells to produce lactic acid. Similarly increase in glucose concentration for the production of lactic acid from sugarcane bagasse increases its yield was observed in this study.

As observed in the Figure 4-5(a) and (b) with rising glucose concentration and pH to some extent (6) lactic acid production was the maximum yield. It was obtained at the maximum glucose concentration of 3.755 g/L, pH of 6. The optimum fermentation pH and glucose concentration of the fermentation medium displayed a significant overall positive effect on lactic acid concentration as shown in Figure 4-5 (a) and (b). This observation could be attributed to the fact that these are the optimum ranges of the factors required to produce high amount of lactic acid.




(a)




(b)

Figure 4-3: Effects of glucose concentration and pH on lactic Acid yield(a): 3D plot and (b)Corresponding Contour Plot

### 4.7.2. Effect of glucose concentration and Incubation Time on lactic Acid Yield

As observed in the Figure 4-6(a) and (b), maximum glucose concentration and incubation time was the fermentation conditions that results in maximum lactic acid yield. When the incubation time and glucose concentration increase from low to high lactic acid yield also increases. The decrease in lactic acid production beyond the certain level of incubation time and glucose concentration is due to decrease in sugar content leads to the lag growth phase of L.bacillus plantarum, decrease in amount of available nitrogen fermentation medium. The low amount of lactic acid in the short incubation time is due to; L.bacillus plantarum needs enough time to ferment the entire carbon source as well as to adapt the environment (Panesar et al., 2010).

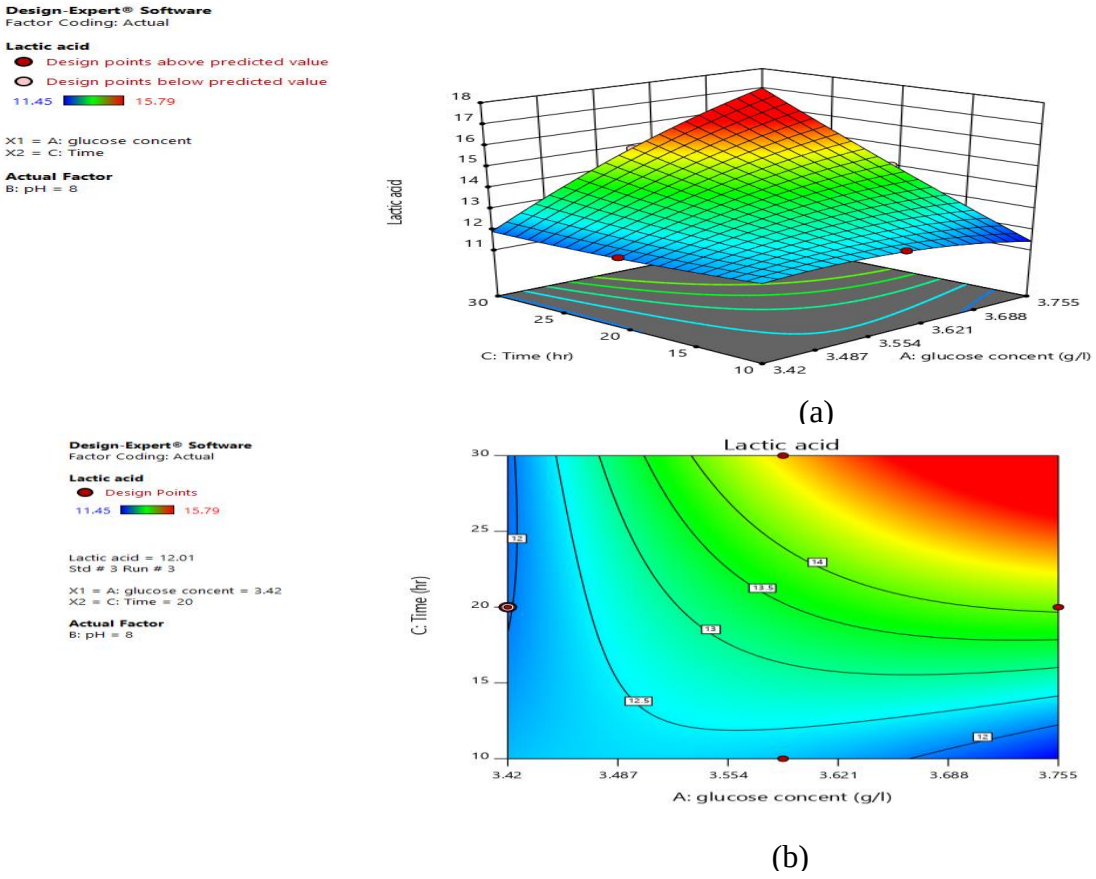
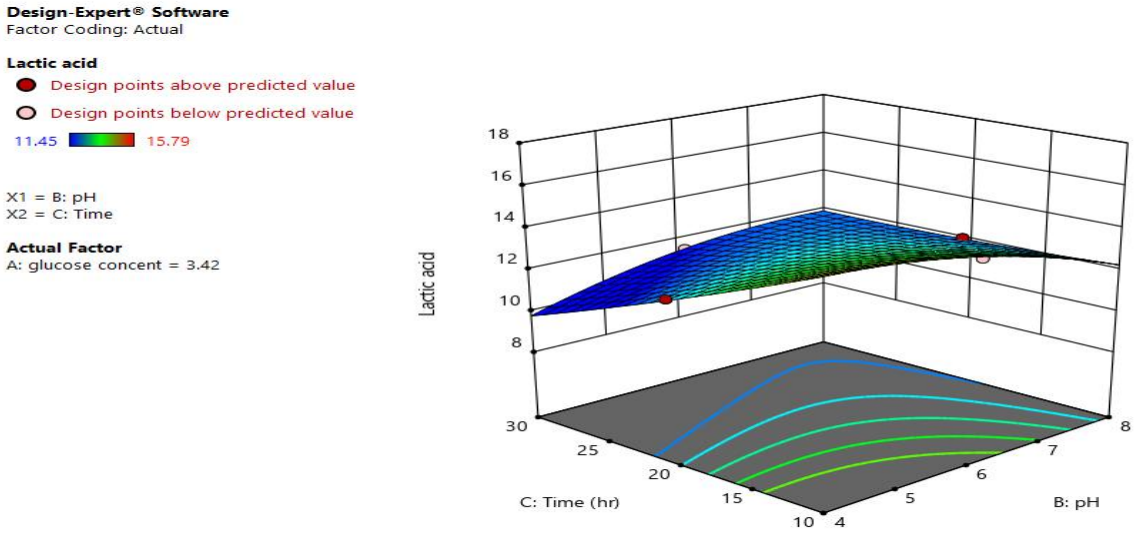


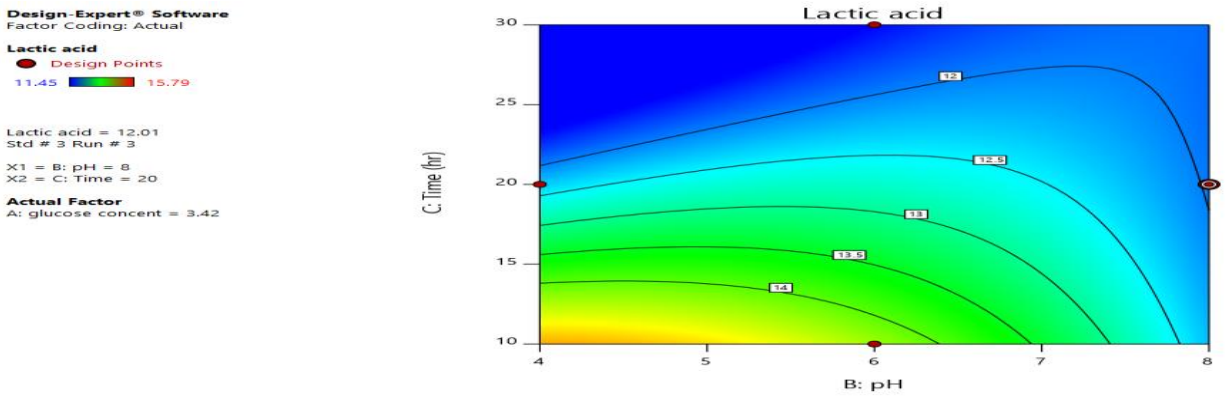

Figure 4-4: Effects of glucose concentration and time on lactic Acid yield (a): 3D plot and (b) Corresponding Contour Plot

### 4.7.3. Effect of pH and Incubation Time on lactic Acid Yield

The effect of fermentation time and pH on the response yield of lactic acid shown in the form of 3D and surface contours as illustrated in the Figure 4-7(a) and (b). Maximum lactic acid yield was produced at maximum time and moderate pH. As the fermentation time and pH rise to the center point the yield of lactic acid was increased but above and below the center point the yield was declined.




(a)




(b)

Figure 4-5: Effects of pH and time on lactic Acid yield (a): 3D plot and (b) Corresponding Contour Plot

# CHAPTER-FIVE

## 5. CONCLUSIONS AND RECOMMENDATIONS

### 5.1. Conclusions

In this study, sugarcane bagasse was used as a carbon source for the production of lactic acid. The experimental conditions for acid pretreatment of sugarcane bagasse were investigated to determine the optimum conditions that give the highest concentration of glucose, and the highest concentration of glucose of 3.755g/L was achieved at a temperature of 105$^{\circ}$C, at an acid concentration of 2.75% and time of 3hrs.

The response surface methodology was proved to be a useful and applicable tool for determining the behavior of the variables studied in the production of lactic acid. The intention of the present investigation was to use sugarcane bagasse as carbon source for Lactobacillus Plantarum bacterial strain for the production of lactic acid under the three factors namely glucose concentration,pH and time. It was observed from the design expert software that the three factors glucose concentration; time and pH positively affect the production of lactic acid. A statistically significant model ($p<0.0001$) was developed to describe the relationship between lactic acid yield and the chosen independent variables. The statistical model showed a good fit with the experimental data ($R^2=0.9981$) with a low standard deviation.

According to the model regression equation developed, lactic acid yield was positively affected by linear effects of all the three factors as well as their quadratic interactions and pure quadratic of time, and negatively affected by pure quadratic of glucose concentration and pH. In this study, based on the analysis of experimental results, it is demonstrated that the individual factors and their interaction effects are significant model terms on lactic acid yield. This shows that the capability of the design of the experimental analysis was successfully capturing these effects. Maximum concentration of glucose, incubation time and moderate level of pH was favorable for lactic acid production. Optimal values of fermentation which gave maximum lactic acid yield were selected using design expert.

After conducting the experiments, the maximum lactic acid yield of 15.79g/L was obtained at selected optimum conditions of 3.755g/L of glucose concentration, 30hrs of time and pH of 6. The observed quantitative difference in the quantity of the lactic acid produced was due to glucose concentration, incubation time and pH variability. Thus, determination of the appropriate amount of glucose concentration, optimum incubation time and pH needs to be considered to get the maximum amount of lactic acid.

Finally, it can be concluded that utilization of sugarcane bagasse for lactic acid production saves valuable foreign exchange by reducing petroleum-based plastic raw materials from abroad.

## 5.2. Recommendations

Based on the conclusion of this research work and overall understanding of the synthesis of sugarcane bagasse for the production of lactic acid for PLA production application, the following recommendations are stated:

- Further investigation on different pretreatment for the sugarcane bagasse such as steam explosion, Ozonolysis should be studied.
- In this study, $H_2SO_4$ was used for the hydrolysis of sugarcane bagasse and a further study should be investigated by comparing hydrolysis using different acids together such as HCl and $H_3PO_4$ as well as by varying their concentrations.
- The parameters which were taken in this study as constant such as agitation speed, nitrogen source, volume of Fermenter and others can affect the production of lactic acid and should further be studied as well as optimization and scale up of the product should be considered.
- Further studies should be accomplished in various topics related to utilization and best incorporation techniques of different wastes together in lactic acid production.

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# Appendices

## Appendix A: Experimental Result

Table A-1: Calculation of moisture content of bagasse

| Run | Sample weight(g) | | | Moisture Content (%) | Average Moisture Content (%) |
|---|---|---|---|---|---|
| | W1 | W2 | W3 | | |
| **1** | 35.7<br>38.6<br>35539.69 | 540.38<br>5 | 43.29 | 46.3<br>46.1<br>46.3 | 46.21 |
| **2** | 40.37<br>43.27<br>39.67 | | | 46.29<br>46.11<br>46.27 | 46.25 |
| **3** | 40.37<br>43.27<br>39.67 | | | 46.28<br>46.11<br>46.27 | Total average = 46.23 |

$$\%\mathrm{Mc} = \frac{((\mathrm{w1} + \mathrm{w2}) - \mathrm{w3}))}{\mathrm{w2}}$$

Table A-2**:** Glucose content of the hydrolaysate

| STD | Run | Factor 1 Acid concentration (%) | Factor 2 time (hr.) | Factor 3 temperature($^{o}$C) | Absorbance of sample @490nm | Glucose concentration(g/l) |
|---|---|---|---|---|---|---|
| **1** | 8 | 0.5 | 1 | 105 | 3.364 | 3.45 |
| **2** | 10 | 5 | 1 | 105 | 3.42 | 3.51 |
| **3** | 15 | 0.5 | 5 | 105 | 3.43 | 3.52 |
| **4** | 14 | 5 | 5 | 105 | 3.43 | 3.52 |
| **5** | 1 | 0.5 | 3 | 90 | 3.19 | 3.28 |
| **6** | 6 | 5 | 3 | 90 | 3.30 | 3.39 |
| **7** | 12 | 0.5 | 3 | 120 | 3.27 | 3.35 |
| **8** | 4 | 5 | 3 | 120 | 3.24 | 3.32 |
| **9** | 5 | 2.75 | 1 | 90 | 3.29 | 3.37 |
| **10** | 13 | 2.75 | 5 | 90 | 3.13 | 3.21 |
| **11** | 7 | 2.75 | 1 | 120 | 3.11 | 3.19 |
| **12** | 17 | 2.75 | 5 | 120 | 3.30 | 3.39 |
| **13** | 16 | 2.75 | 3 | 105 | 3.66 | 3.755 |
| **14** | 3 | 2.75 | 3 | 105 | 3.62 | 3.71 |
| **15** | 11 | 2.75 | 3 | 105 | 3.65 | 3.74 |
| **16** | 2 | 2.75 | 3 | 105 | 3.66 | 3.752 |
| **17** | 9 | 2.75 | 3 | 105 | 3.64 | 3.73 |

Slope = 0.975AU g /l

$$\text{Glucose}\left(\frac{\text{g}}{\text{l}}\right) = \text{Absorbance of the sample} - \text{intercept/ Slope(AU } \frac{\text{g}}{\text{L}}$$

Table A-3**:** Lactic acid yield of all runs

| **Std** | **Run** | **Factor 1 A:glucose concentration (g/l)** | **Factor 2 B:pH** | **Factor 3 C:Time(hr)** | **peak area of standard** | **peak area of sample** | **Response 1 Lactic acid(g/l)** |
|---|---|---|---|---|---|---|---|
| **1** | 16 | 3.42 | 4 | 20 | 4085572 | 566539 | 12.37 |
| **2** | 5 | 3.709 | 4 | 20 | 4085572 | 592057 | 12.68 |
| **3** | 3 | 3.42 | 8 | 20 | 4085572 | 565184 | 12.01 |
| **4** | 4 | 3.755 | 8 | 20 | 4085572 | 655092 | 14.03 |
| **5** | 9 | 3.42 | 6 | 10 | 4085572 | 663496 | 14.21 |
| **6** | 11 | 3.73 | 6 | 10 | 4085572 | 588789 | 12.61 |
| **7** | 8 | 3.42 | 6 | 30 | 4085572 | 524612 | 11.45 |
| **8** | 7 | 3.755 | 6 | 30 | 4085572 | 734601 | 15.79 |
| **9** | 15 | 3.5875 | 4 | 10 | 4085572 | 666298 | 14.27 |
| **10** | 13 | 3.5875 | 8 | 10 | 4085572 | 757507 | 12.33 |
| **11** | 10 | 3.5875 | 4 | 30 | 4085572 | 554237 | 11.87 |
| **12** | 12 | 3.5875 | 8 | 30 | 4085572 | 692175 | 14.87 |
| **13** | 17 | 3.5875 | 6 | 20 | 4085572 | 646652 | 13.849 |
| **14** | 1 | 3.5875 | 6 | 20 | 4085572 | 644072 | 13.845 |
| **15** | 14 | 3.5875 | 6 | 20 | 4085572 | 644054 | 13.73 |
| **16** | 6 | 3.5875 | 6 | 20 | 4085572 | 646652 | 13.849 |
| **17** | 2 | 3.5875 | 6 | 20 | 4085572 | 644084 | 13.845 |

$$\text{Concentration of lactic acid}\left(\frac{\text{g}}{\text{l}}\right) = \frac{\text{peak area of sample}}{\text{peak area of standard}} * \text{purity of standard}$$

# Appendix B: Images of experimental works


Fig B-1: Images of process of the experiment