ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL & BIO-ENGINEERING

SYNTHESIS AND PRODUCTION OF LACTIC ACID (LA) FROM FALSE BANANA/BULA USING LACTOBACILLUS PLANTARIUM

BY
YONAS ABATE

JUNE, 2016
ADDIS ABABA, ETHIOPIA

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A Thesis Submitted to the School of Graduate studies of Addis Ababa University, Addis Ababa Institute of Technology, in Partial Fulfilment of the Requirements for the Degree of Masters of Science in Chemical Engineering (Process Engineering).

ADVISOR: DR. BETELEY TEKOLA

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Approved by the Examining Board

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Abstract

The production of lactic acid from was optimized by central composite design, using bulla starch as carbon sources and MRS as yeast extract, meat extract, peptone and glucose source using *Lactobacillus Plantarium*. The fermentation were performed in 250 mL Erlenmeyer flasks containing 100 mL of MRS medium, maintained at 200 rpm, 40 g/l of starch (carbon source) , at temperature range from 30 °C to 40° C , pH 5 to 7 and time between 48 hours to 72 hours. The first two (agitation speed 200 rpm and carbon source 40 g/l) were set as constant. After data was analyzed by response surface method, it was revealed that Lactic acid production was significantly affected by temperature, time and pH of the samples. At central point of all factors: temperature 35 °C, time 60 hours and pH 6.25 maximal production of lactic acid was 17.09 g/L.

List of abbreviations and acronyms

AAIT	Addis Ababa Institute Technology.
AHA	Alpha Hydroxy Acid.
ATP	Adenosine Triphosphate.
CCD	Central Composite Design.
CCIIDI	Chemical and Construction Input Industry Development Institute.
EMP	Embden Meyerhof parnas.
GAP	Glyceraldehyde Phosphate.
GRAS	Generally Recognized As Safe.
HDPE	High Density Poly Ethylene.
LA	Lactic Acid.
LAB	Lactic Acid Bacteria.
LDPE	Low Density Poly Ethylene.
MRS	deMan Rogasa and Sharp.
PC	Poly Carbon.
PET	Poly Ethylene Terephthalate.
PLA	Poly Lactic Acid.
PP	Polypropylene.
PS	Polystyrene.
PU	Polyutherene.
PVC	Poly Vinyl Chloride.
RPM	Rotation per Minute.
VOC	Volatile Organic Compound.
^{0}C	Degree Centigrade.
µm	Micromere.

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CHAPTER ONE

I. Introduction

Enset (Ensete Ventricosum, Family Musaceae) is a plant indigenous to Ethiopia, it is often called false banana for its close resemblance to banana plant. The plant is the most important staple food for millions of people in the south and southwestern parts of Ethiopia. Enset plant contains starch as its major contents. The starch has been investigated for its physico-chemical properties including granule size, X-ray diffraction pattern, amylose content, gelatinization behavior, stability and various rhelogical properties of the gel. Based on its physico-chemical properties the starch was evaluated for various pharmaceutical applications such as in tablet binder and disintegrant. Several modifications were also attempted on the native enset starch so as to improve and modulate its physiochemical properties [1] .

Starch is the most abundant storage reserve carbohydrate in plants. It is found in many different plant organs, including seeds, fruits, tubers and roots, where it is used as a source of energy during periods of dormancy and re- growth. A range of native starches from different sources with highly different functionalities are already on the market.

Starch is made up of two polymers of D-glucose: amylose, an essentially unbranched α [1→ 4] linked glucan, and amylopectin, which has chains of α [1→ 4] linked glucoses arranged in a highly branched structure with α [1→ 6] branching links. Amylose and amylopectin make up 98–99 % of the dry weight of native granules, with the remainder comprising small amounts of lipids, minerals, and phosphorus in the form of phosphates esterified to glucose hydroxyls.

Lactic acid, commonly used in food, chemical and pharmaceutical industries, has recently received much attention for the production of biodegradable plastics. Poly lactic acid (PLA) is biodegradable aliphatic polyester produced from renewable sources, which, due to its excellent physical and chemical properties and environment compatibility, considered the best candidate to replace on the market petroleum-based plastics. It has a wide range of applications ranging from medical devices, such as suture treads and scaffolds, to commodity products like bottles and films for food packaging [2].

The monomer, lactic acid (LA), is the smallest optical active organic compound present in nature. Due to the presence of a chiral carbon, LA exists in the two optical isomers, L (+) and D (-) (Figure 1.1). It was first isolated in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. From sour milk and first commercially produced around 1880. It can be obtained by petrochemical synthesis or fermentation process. Nowadays, due to the improvements in bacterial fermentation of D-glucose, optically preferentially pure lactic acid is produced through the batch fermentation process of corn and other carbohydrates, which involve key steps as cell mass and protein removal and, LA recovery, purification and concentration. In the United States, LA is largely used in food applications such as acidic flavoring agent or buffering agent. Beside the above application now a day it is highly applicable in poly lactic acid formation which is in a very high demand of making biodegradable plastic [3].

The monomer**, lactic acid (LA),** is the smallest optical active organic compound present in nature. Due to the presence of a chiral carbon, LA exists in the two optical isomers, L (+) and D (-) (Figure 1.1). It was first isolated in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. From sour milk and first commercially produced around 1880. It can be obtained by petrochemical synthesis or fermentation process. Nowadays, due to the improvements in bacterial fermentation of D-glucose, optically preferentially pure lactic acid is produced through the batch fermentation process of corn and other carbohydrates, which involve key steps as cell mass and protein removal and, LA recovery, purification and concentration. In the United States, LA is largely used in food applications such as acidic flavoring agent or buffering agent. Beside the above application now a day it is highly applicable in poly lactic acid formation which is in a very high demand of making biodegradable plastic. (Wee *et al.*, 2006)

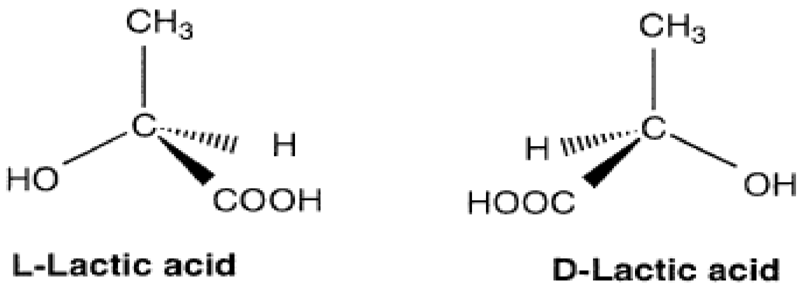


Fig 1.1 Lactic acid isomers

Thus biodegradable plastic production become critical to our country since Ethiopia is following and will follow green economy strategies. This thesis focused on production of lactic acid for manufacturing of poly lactic acid which is biodegradable bio plastic for packaging and other applications.

1.1 Statement of the problem

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), poly utherene (PU), poly styrene (PS) etc are costing Ethiopia huge amount money in foreign currency. These raw materials and their products cost the country 8 to 9 billion birr in average per year to import them. It is the highest cost that takes our foreign currency next to petroleum.(CCIIDI 2008 E.C report)

Table 1.1 Import data of petroleum based plastic raw material and their products cost from 2010-2015. (CCIIDI, plastic department 5 years strategic plan)

NO	Year of Import	Value in Eth Birr	Remark
1.	2010	4,541,726,448	
2.	2011	5,868,587,970	
3.	2012	7,465,715,460	
4.	2013	8,362,574,619	
5.	2014	9,750,812,205	
6.	2015	11,456,512,610	

In addition, petroleum based plastic products will take more than 100 yrs to degrade. This has negative impact on the environment specially soil fertility and methane emission. Therefore the above two problems should be addressed.

This thesis proposal wants to address the above two problems by saving foreign currency invested on importing petroleum based raw materials and produce environment friendly plastic raw materials for our green economy strategy.

1.2 Objective of the study

1.2.1 General objective

- Production and synthesis of lactic acid in laboratory scale from starch (obtained from false banana) via fermentation employing locally produced Lactobacillus Plantarum, which is raw material for biopolymer/bio-plastic.

1.2.2 Specific objectives

- To characterize the proximate and nutritional composition of locally sources Bulla starch
- To synthesize Lactic acid from Bulla starch via fermentation using Lactobacillus Plantarum microorganism
- To determine the effect of fermentation process variable; incubation period, temperature and pH on lactic acid yield.
- To find optimal operating conditions using central composite design

1.3 Scope of the study

This work involves investigation of lactic acid production starting from isolation of potential lactic acid bacteria (LAB), media formulation and fermentation processes.

1.4 Expected results

- High lactic acid yield from fermentation processes under the optimum condition will be obtained.
- Microorganism (*Lactobacillus Plantarum*) can utilize Bulla starch as carbon source with different controlled factors for lactic acid production.

1.5 Significance of the study

The significance of this study can be seen from five different angles.

- The first one is accompanying with economy. If poly lactic acid (PLA) will be produced in Ethiopia, then the cost will be incurred for importing PP, LDPE, HDPE, PVC, PU, PS and other will be greatly reduced. That means production of lactic acid from available renewable resource material in Ethiopia can be seen as huge import substitution.

- The second significance of this study is comprised on implementation of new technology in the country. As we know technology is transferring from developed countries to developing countries in transition of globalization**.** Technology transfer
- The third basic significance of this study is making our nation free of non-degradable petroleum based plastic products to meet our green economy strategy. Reduce or eliminate pollution. Because, carbon dioxide is absorbed from air when plant is grown, use of PLA has the potential to emit fewer greenhouse gases compared to competitive hydrocarbon-based polymers.
- The forth one is Significant energy savings; the energy used to process the raw material of PLA compared to that of petroleum product is much less.
- The last significance is that the ability to recycle back to lactic acid by hydrolysis or alcoholysis.

1.6 Scope and limitation of the study

- This work involves investigation of lactic acid production starting from isolation of potential lactic acid bacteria (LAB, Lactobacillus Plantarum), media formulation and fermentation processes. The ultimate objective of the whole thesis is to improve value of the agricultural product for the efficient production of lactic acid by fermentation processes. Lactic acid fermentation was performed using bulla starch as the main substrate.

CHAPTER TWO

II. LITERATURE REVIEW

Starch is the most abundant storage reserve carbohydrate in plants. It is found in many different plant organs, including seeds, fruits, tubers and roots, where it is used as a source of energy during periods of dormancy and re-growth [4]. A range of native starches from different sources with highly different functionalities are already on the market. Each starch is named according to its plant source, e.g. potato starch, maize starch, cassava starch, rice starch. These groups are distinctly different from each other with respect to chemical composition and physical properties [5].

Starch granules range in size from 1 to 100 μm diameters and shape of polygonal, spherical, lenticular, and can vary greatly in content, starch and organization of the amylase and amylopectine molecule the branching architecture of amylo-pectin, and the degree of crystallinity.

Amylose has a molecular weight ranging 10^5 – 10^6, corresponding to a degree of polymerization of 1000 –10,000 glucose units. Less than 0.5% of the glucoses in amylose are in α [1→ 6] linkage, resulting in a low degree of branching. Amylopectin is a much larger polymer, with a molecular weight about 10^8 and a degree of polymerization that may exceed one million. Most starches contain 60 – 90% amylopectin, although high- amylose starches, with as little as 30% amylopectin, and waxy starches with essentially 100% amylopectin are well known. Amylopectin has about 5% of its glucoses in α [1→ 6] linkage, giving it a highly branched, treelike structure and a complex molecular architecture that can vary substantially between different starches with regard to placement and length of branches [6].

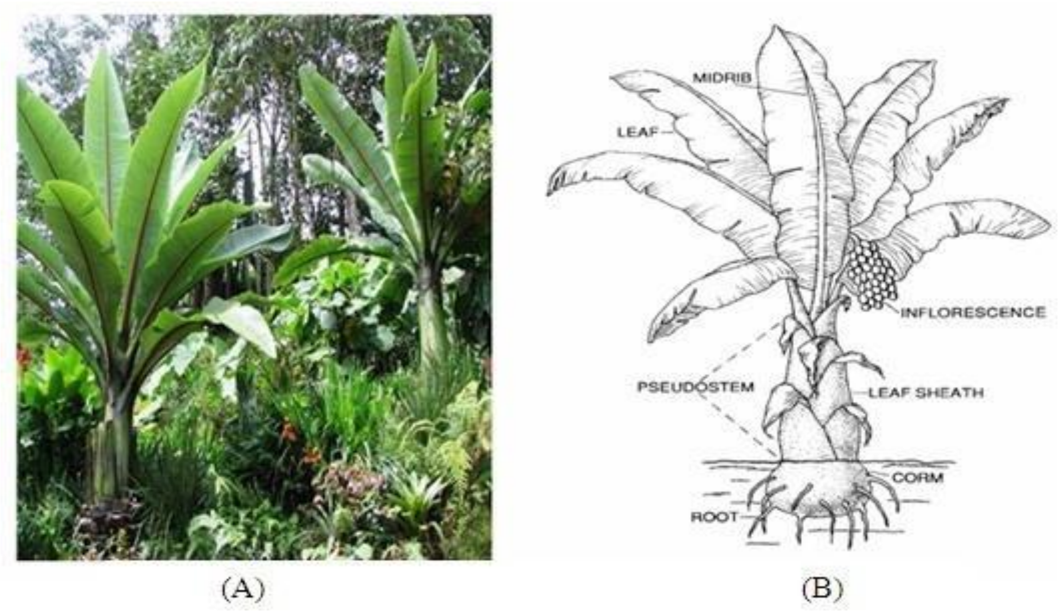
A great deal of attention has been devoted to starch and its derivatives mainly in the context of the food, plastics and pharmaceutical industries. This is not only because starch is readily available, inexpensive and inert material, but also because of the ease with which its physicochemical properties can be altered through chemical or enzymatic modification and/or physical treatment [7].

Commercial starches are obtained from cereals (corn and wheat) and from tubers and roots (particularly potato and cassava) and they dominate the world markets for starches in the food and pharmaceutical industries. Recently more attention has been focused on the development of some of the starch from different botanical sources as excipients in pharmaceutical formulation and bio degradable polymers for food packaging.

One of the potential alternative sources of starch in Ethiopia is *Ensete ventricosum.* Due to its high yielding potential, excellent caloric and nutrient source [8], wide adaptability, drought resistance, and multifunctional usage enset is considered an economic crop in Ethiopia. Starches from different botanical sources may not have identical properties with respect to their intended use. Indeed the chemical composition and physical characteristics of a starch are typical of its biological origin [9]. Hence the starch from each plant source will vary somewhat in appearance, composition and properties. The objective of the resent study was, therefore, to characterize this starch and provide information on its composition, morphology, and selected physico-chemical characteristics that may prove useful when considering various applications of the starch such as in the pharmaceutical, lactic acid, polymer, and the food industries.

Thus, the aim of this research is to study the potential of enset starch as lactic acid production raw material.

Ensete ventricosum, Musaceae is a plant indigenous to Ethiopia, closely related to the banana tree [10]. Because it resembles the banana tree, but does not produce banana, enset is often referred as "False Banana". Instead, enset is a rich source of starch. The plant is cultivated in many parts of the country, particularly in the highlands of the south and the south-west and is widely used as a food source[11] . Domesticated enset is planted at altitudes ranging from 1,200 to 3,100 meters. However, it grows best at elevations between 2,000 and 2,750 meters. Most enset-growing areas receive annual rainfall of about 1,100 to 1,500 millimeters, the majority of which falls between March and September. The average temperature of enset growing areas is between 10 and 21 degrees centigrade, and the relative humidity is 63 to 80 percent.



(A) (B)

Figure 2.1: Enset plant in the garden (A) and its parts (B).

The majr foods obtained from enset are *kocho, bulla* and *amicho*. *Kocho* is the bulk of the fermented starch obtained from the mixture of the decorticated (scraped) leaf sheaths and grated corm (underground stem base). The two main products utilized as food are locally known as *kocho* and *bulla*. In the preparation of these two products, the pseudo- stem and corms are cut, crushed and the exuding liquid containing starch is collected. Most of the water is allowed to drain away and the wet starch, *bulla*, is collected leaving the fibrous material, *kocho* behind.

Kocho can be stored for long periods of time without spoiling. The quality of *kocho* depends on the age of the harvested enset plant, the type of clone (variety), and the harvesting season. Moreover, within one plant, the quality is influenced by the part of leaf sheath and corm processed. The preferred type is white in color and is obtained from the innermost leaf sheaths and inner part of the corm, while the lowest grade is blackish and is obtained from the outer leaf sheath and corm.

2.1 Physico-chemical properties of enset starch

2.1.1 Amylose content

The linear component of starch, viz., amylose, imparts defining characteristics to starch. Amylose content varies considerably among different starches have been carried out to obtain the starch of amylose contents varying from 0 to greater than 75 %. According to a study by Gebre-Mariam and Shimidt the amylose content of enset starch was estimated to be 29.0 %.

2.1.2 Other components in enset starch

The extracted starch is invariably accompanied by various other components ash, fiber, lipids, proteins and minerals, depending on a number of factors such as method of extraction, age of the crop, and environmental conditions. Some of these impart desirable qualities to the starch, while others affect the quality.

A study also revealed that fat and protein content of enset starch was significantly higher than potato starch but lower than maize starch. Enset starch contained higher content of ash than maize starch, but lower than potato starch.

2.2 Chemical composition and morphological characteristics

Starch is the major constituent of bulla. It accounts for more than 90% of bulla (on dry weight basis). The chemical composition of enset starch obtained by the isolation method described above is shown in Result and discussion part Table 4.1. Corresponding values for potato and maize starches determined simultaneously are given for comparison. Fat and protein content of enset starch was significantly higher than potato starch but lower than maize starch. Enset starch contained higher content of ash than maize starch but lower than potato starch. The true density of enset starch was 1.476.

2.3 Lactic acid

2.3.1 Historical background

It was the first organic acid to be commercially produced by fermentation, with production beginning in 1881[13]. It is present in many foods both naturally or as a product of microbial fermentation. It is also a principal metabolic intermediate in most living organisms from anaerobic prokaryotes to humans.

In 1839, Fremy performed lactic acid fermentation of several carbohydrates, such as sugar, milk sugar, mannite, starch and dextrin. A discovery that was then confirmed by Gay-Lussac, in 1840, Louradour prepared lactic acid by fermentation of whey and converted it into iron lactate by dissolution of metallic iron in it. Other fermentation experiments were performed by many different scientists to produce lactic acid from cane sugar beyond 1847 [14].

Blondeau discovered lactic acid as a fermentation product in 1847. Originally, the lactic acid of fermentation and that found in muscle tissue were regarded as identical. Liebig, who in 1947 re-examined meat extract, suspected that the two acids might not be identical. He asked Engelhardt to carry out an examination of the salts of the two acids. Engelhardt confirmed Liebig's thought that the contents of water of crystallization and the solubility of the salts of the two lactic acids differed and thus the acids were different[15].

Welceneus, in 1873, proved they have the same structure, but different physical properties. It was also investigated by Pasteur as one of this first microbiological yeast cultures of distilleries, it was not until the year 1877 that lactic acid bacteria were isolated in pure cultures when Lister isolated *Streptococcus lactis*. During this same period, Delbruck was endeavoring to find out the most favorable temperature for lactic acid fermentation in distilleries. He concluded that relatively high temperature favored high yields of lactic acid [16].

In the USA until 1963, lactic acid was produced solely by fermentation, when Sterling Chemicals, Inc., started producing lactic acid by a chemical process using petroleum by products, supplying nearly half the American demand for lactic acid. In 1996, Sterling abandoned the lactic acid business, leaving lactic acid production again exclusively to fermentation companies [17]. In the early 1990s, Ecological Chemical Products (EcoChem), a joint venture of E.I du Pont Nemours & Co., and Con Agra produced only 1 to 2 million pounds of lactic acid by fermentation of whey permeate. In 1993, the current leader in basic chemical fermentation, Archer Daniels Midland (ADM), entered the lactic acid business and produced, in a facility designed for 40 million pound per year, 10 million pounds of lactic acid from corn sugar. With a potential market for lactic acid in polymer production, the demand for lactic acid may reach as high as 2000 million and above per year [18] .

2.3.2 Physical and Chemical Properties

Pure anhydrous lactic acid is a white crystalline solid with a low melting point of 53 ^{0} C and appears generally in form of more or less concentrated aqueous solution, as syrupy liquid. It also can be a colorless to yellow liquid after melting or it dissolved in water. Lactic acid is considered as a stable substance and it is a combustible substance as well. Lactic acid is compatible with strong oxidizing agents. Normally lactic acid is observed as a clear to slightly yellowish liquid, typically supplied to formulators in an 88 to 92% concentration. Lactic acid normally appears in diluted or concentrated aqueous solution.

Table 2.2: Characteristics of Lactic Acid property

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

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

Table 2.3: Physical and thermodynamic properties of lactic acid [20].

Property	**Value**	**Isomer**
Molecular weight	90.08	D, L, DL
Melting Point, ^{o}C	52.8 53.0 16.8	D L DL
Boiling point (at 0.5mmHg), ^{o}C (at 14mmHg), ^{o}C	82.0 122.0	DL DL
Dissociation constant (K_a at $25^{o}C$)	3.83 3.79	D L
Heat of combustion (ΔH_c), cal/kg	3615	DL
Specific heat (C_p at $20^{o}C$), J/mol.^{o}C	190	DL
Specific rotation ($22^{o}C$, D line)	+2.6	L

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

Densities of aqueous solution of various lactic acid concentrations has shown that the density increased almost linearly with concentration and decreased almost linearly with temperature. The viscosity of lactic acid solution increased rapidly with the concentration and decreased rapidly with increasing temperature.

2.3.3 Application 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. Some of the important applications of lactic acid are detailed below.

2.3.3.1 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 [21].

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. These applications can be divided into:

- Medical/ pharmaceutical
    - Bone implants
    - Sutures
    - Ca-lactate in calcium tablets
    - Co-polymers in controlled drug release
    - Sodium lactate in dialysis solutions
- Skin and hair care (cosmetics industry)
    - Lactic acid (skin renewal process)
    - Sodium and ammonium-lactate (skin moisturizer)
    - Hair conditioner

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.

Natural L (+) lactic acid is used in many applications in cosmetics. Lactic acid is an alpha hydroxy 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 [21].

2.3.3.2 Food industry

Lactic acid occurs naturally in many food products. It has been in use as an acidulant, preservative and pH regulator for quite some time. Some of the important applications of lactic acid in the food industry are detailed below. 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 microflora. 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 [21].

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 an 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.

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 saved. Lactic acid is also used as an acidulant in dairy products like cheese and yogurt powder. The production of processed cheese can be greatly simplified if a sufficient amount of lactic acid is added to the freshly drained cheese curd to lower the pH to 4.8-5.2, then the curd can be processed without further curing, to adjust acidity and improved flavor, texture and stability.

2.3.3.3 Application of lactic acid in polymer industry

Recently, a great interest in biodegradable lactide polymers has accelerated researches on the production of pure L(+)- or D(-)-lactic acid as bulk raw material, and efforts have been made to enhance the productivity and economy of the L(+)- or D(-)-lactic acid production processes [22]. The optically pure lactic acid can be polymerized into a high molecular mass poly-lactic acid (PLA) through the serial reactions of polymerization, depolymerization, and ring opening polymerization [23] .Two molecules of lactic acid can be dehydrated to yield lactide, a cyclic lactone. A variety of catalysts can polymerize lactide to produce either heterotactic or syndiotactic polylactide, biodegradable polyesters with valuable (*inter alia*) medical properties, which are currently attracting much attention.

These polymers are transparent and their degradation can be controlled by adjusting the composition, and molecular weight. Their physical properties approach those of petroleum derived plastics. Lactic acid esters like ethyl/butyl lactate can be used as green solvents. They are high boiling, non-toxic, and biodegradable components. Poly L-lactic acid with low degree of polymerization can help in controlled release or degradable mulch films for large-scale agricultural applications [24]. Moreover, polymers of lactic acids are biodegradable thermoplastics. The resultant polymer, poly-lactic acid (PLA), has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, and short-life trays, respectively.

2.3.3.4 Polylactic acid

Polylactic acid (PLA) has been known since 1845 but not commercialized until early 1990. PLA belongs to the family of aliphatic polyesters with the basic constitutional unit lactic acid. The monomer lactic acid is the hydroxyl carboxylic acid which can be obtained via bacterial fermentation from corn (starch) or sugars obtained from renewable resources. Although other renewable resources can be used, corn has the advantage of providing a high-quality feedstock for fermentation which results in a high-purity lactic acid, which is required for an efficient synthetic process. L-lactic acid or D-lactic acid is obtained depending on the microbial strain used during the fermentation process.

PLA is a biocompatible and biodegradable material that has numerous applications in sustainable plastic products. Along with ease of disposal, lactic acid polymers also possess high tensile strength and can be used in the packaging industry and for medical and biological applications.

PLA can be synthesized from lactic acid by ***direct poly condensation reaction*** or ***ring-opening polymerization*** of lactide monomer. However, it is difficult to obtain high molecular weight PLA via poly condensation reaction because of water formation during the reaction. Nature Works LLC (previously Cargill Dow LLC) has developed a low-cost continuous process for the production of PLA [25]. In this process, low molecular weight pre-polymer lactide dimers are formed during a condensation process. In the second step, the pre-polymers are converted into high molecular weight PLA via ring opening polymerization with selected catalysts. Depending on the ratio and stereo-chemical nature of the monomer (L or D), various types of PLA and PLA copolymers can be obtained. The final properties of PLA produced are highly dependent on the ratio of the D and L forms of the lactic acid.

PLA is a commercially interesting polymer as it shares some similarities with hydrocarbon polymers such as polyethylene terephthalate (PET). It has many unique characteristics, including good transparency, glossy appearance, high rigidity, and ability to tolerate various types of processing conditions.

PLA is a thermoplastic polymer which has the potential to replace traditional polymers such as PET (polyethylene terephthalate), PS (polystyrene) and PC for packaging to electronic and automotive applications. While PLA has similar mechanical properties to traditional polymers, the thermal properties are not attractive due to low Tg of 60°C. This problem can be overcome by changing the stereochemistry of the polymer and blending with other polymers and processing aids to improve the mechanical properties, e.g., varying the ratio of L and D isomer ratio strongly influences the crystallinity of the final polymer. However, much more work is required to improve the properties of PLA to suit various applications.

PLA is widely used in many day-to-day applications. It has been mainly used in food packing (including food trays, tableware such as plates and cutlery, water bottles, candy wraps, cups, etc.). Although PLA has one of the highest heat resistances and mechanical strengths of all bio-based polymers, it is still not suitable for use in electronic devices and other engineering applications. PLA production requires less energy per kg as compared to PET and PP (42 MJ/kg for PLA as compared to 73 MJ/kg for PP and 80 MJ/kg for PET).

2.4 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. In fermentation processes, bacteria or other microorganism produce lactic acid as they metabolize carbon-containing (e.g. carbohydrate) raw material.

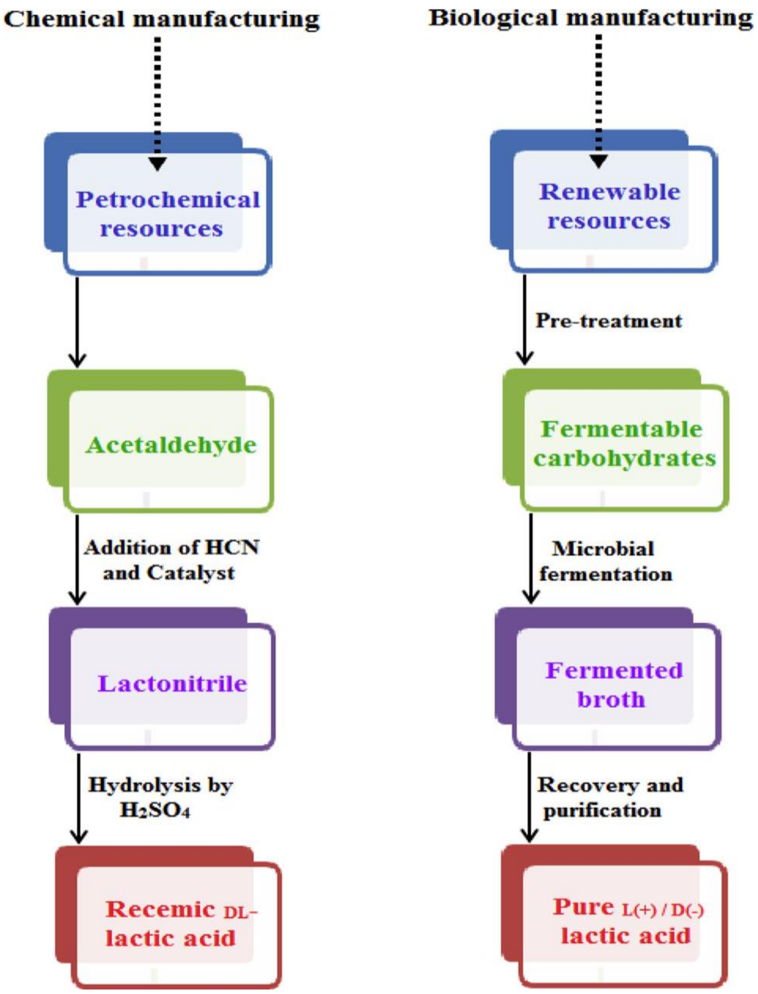


Figure: 2.2. Schematic representations of the two manufacturing processes of lactic acid for comparison

2.4.1 Synthetic methods

The synthetic manufacture of lactic acid on a commercial scale began around 1963 in Japan and United States [26] .Chemical synthesis of lactic acid produces a racemic lactic acid mixture. Lactonitrile produced by combining of hydrogen cynide and acetaldehyde in liquid phase reaction at atmospheric pressure as shown in the reaction (1). The crude lactonitrile is recovered and purified by distillation and is then hydrolyzed into lactic acid using either concentrated sulfuric or hydrochloric acid, producing an ammonium salts as a by-product (2). This crude preparation is esterified with methanol to produce methyl lactate(3). Methyl lactate is recovered, purified by distillation and then hydrolyzed under acidic conditions to produce a purified lactic acid (4) , which is further concentrated and packaged. The sequence of the reactions is demonstrated as the follows:

1) Addition of hydrogen cyanide

$$\mathrm{HCN + CH3CHO \longrightarrow CH3CH(OH)CN}$$

2) Hydrolysis by H_2SO_4 or HCl

$$\mathrm{CH_3CHOHCN + H_2O + \tfrac{1}{2}H_2SO_4 \longrightarrow CH_3CHOHCOOH + \tfrac{1}{2}(NH_4)_2SO_4}$$

Lactonitrile Sulphuric acid Lactic acid Ammonium salt

$$\mathrm{CH3CH(OH)CN + 2H2O + HCl \longrightarrow CH3CH(OH)CO2H + NH4Cl}$$

3) Esterification

$$\mathrm{CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O}$$

Lactic acid Methanol Methyl lactate

4) Hydrolysis by H_2O

$$\mathrm{CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH}$$

Methyl lactate Lactic acid Methanol

There are other routes for chemically synthesizing of lactic acid, for example: oxidation of propylene glycol; reaction of acetaldehyde with carbon monoxide and water at elevated temperatures and pressure; hydrolysis of chloropropionic acid and nitric acid oxidation of propylene. However, none of these processes are commercialized [27]. Due to the growing demand for lactic acid for biodegradable thermoplastics, there is a need for pure chiral forms, D- or L- lactic acid. Chemical synthesis produces a racemic mixture of lactic acid, D and L isomeric forms.

2.4.2 Fermentation processes

Fermentation processes are characterized by biological degradation of substrate (starch) by a population of microorganism (biomass) into metabolites such as ethanol, citric acid and lactic acid [28] .Lactic acid is produced from mono or disaccharide via the Embden Mayerhof glycolysis. Under anaerobic condition, the pyruvic acid produced is reduced to lactic acid by the enzyme lactic dehydrogenase.

2.4.2.1 Fermentation through lactic acid bacteria

Lactic acid bacteria are a group of Gram-positive bacteria, non-respiring, non-spore forming, cocci or rods, anaerobic bacteria that excrete lactic acid as the main fermentation product into the medium if supplied with suitable carbohydrate. Lactic acid bacteria have been traditionally defined by the formation of lactic acid as a sole or main end product from carbohydrate metabolism [29].

Historically, bacteria from the genera *Lactobacillus*, *Leuconostoc, Bifidobacteria, Pediococcus* and *Streptococcus* are the main species involved. Several more have been identified but play minor role in lactic fermentations [30].

There are two types of fermentation for these lactic acid bacteria, homo-fermentative and hetero-fermentative. Homo-fermentative lactic acid bacteria produce lactic acid as a sole end product; hetero-fermentative lactic acid bacteria produce other product such as acetic acid, ethanol as well as lactic acid the end product. The fermentation type and products of lactic acid as the end products of lactic acid bacteria have been summarized in Table 2.4.

2.4.2.1.1 Homolactic fermentation

The fermentation of 1 mole of glucose yields two moles of lactic acid;

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{Lactic acid}}{2CH_3CHOHCOOH}$$

2.4.2.1.2 Heterolactic fermentation

The fermentation of 1 mole of glucose yields 1 mole each of lactic acid, ethanol and carbon dioxide;

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{lactic acid}}{CH_3CHOHCOOH} + \underset{\text{ethanol}}{C_2H_5OH} + \underset{\text{carbon dioxide}}{CO_2}$$

Only the homofermentative lactic acid bacteria are of industrial importance for lactic acid manufacture. Homofermentative L(+)lactic acid producers are required if the lactic acid produced will be used as a feedstock for manufacture of 100% biodegradable plastics and or as a physiological active food additive. All species of *Streptococcus* produce L(+)lactic acid as the main end product when growing rapidly under conditions of carbohydrate excess, however in most cases, *Streptococcus* requires complex culture media, which often contain expensive meat extracts, peptone and blood or serum. Also under glucose limiting conditions and at low dilution rates in continuous culture, other end products including formate, acetic acid and ethanol are produced by Streptococcus.

Next to the *Pediococcus* and lastly the homo-fermenters of the *Lactobacillus* species, which produce the most acid, follow the heterofermentative species of *Lactobacillus*, which produce intermediate amounts of acid. Homo-fermenters convert sugars primarily to lactic acid, while hetero-fermenters produce 50% lactic acid, 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 [31].

Table 2.3: The fermentation types and products of lactic acid bacteria [32].

Genus	Fermentation type	Main product	Isomer
Leuconostoc	heterofermentative	lactic acid (1) acetic acid (1) CO_2 (1)	D(-)
Bifidobacteria	heterofermentative	lactic acid (1)	L(+)
		acetic acid (1.5)	
Lactobacillus	heterofermentative	lactic acid (1)	L(+), D(-
	(pentose substrate)	acetic acid (1)	and DL
Lactobacillus	homofermentative	lactic acid (2)	L(+), D(-
			And DL
Pediococcus	homofermentative	lactic acid (2)	DL, L(+)
Streptococcus	homofermentative	lactic acid (2)	L(+)

2.4.2.2 Fermentation via Lactobacillus Bacteria

There are numerous species of bacteria and fungi that are capable to producing relatively large amount of lactic acid from carbohydrates[33].However in industrial fermentation the use of various species of *Lactobacillus* is preferred because of their higher conversion, yield and rate of metabolism[34].

Lactobacillus is more suited to grow in plant extracts (Crueger, 1984). They are often found in carbohydrate containing substrates such as plants and materials of plant origin [35].It is believed that homofermentative *Lactobacillus* cultures are the most important commercial species for lactic acid production by fermentation [36].*Lactobacillus* cultures produce either L(+) or D(-)lactic acid or DL mixture. The species producing L(+)-lactic acid from cellulosic substrate have the most potential for future uses. In general, the desirable characteristics of potential industrial Lactobacillus cultures are the ability to rapidly and completely convert cheap substrate to L(+)-lactic acid with a minimum amount of nitrogenous substance supplement. Several bacterial strains (*Lactobacillus rhamnosus*, *L. casei and L. delbrueckii*) can be used in fermentation. *Lactobacillus delbrueckii* as in Figure 2.4 are used more commonly than the fungus by virtue of the bacteria's high rates of production and high conversion efficiency. The major and secondary products for this bacteria strain are shown in Table 2.5

Table 2.5: Major and secondary products of *Lactobacillus* (L.) species (Martin, 1996)

Species	Substrate	Major product	Secondary product
L. bulgaricus	Lactose	D(-)Lactic	Acetaldehyde, Acetone,
	Lactose	acid	
	Lactose	DL-Lactic	
	Glucose	acid D(-)	Diacetyle, Ethanol
L. helveticus		Lactic acid	
	Lactose	DL-Lactic	Acetaldehyde, Acetic
		acid	acid, Acetone, Diacetyle,
			Ethanol
L. lactis			
	Glucose		Acetaldehyde, Acetone,
L. acidophilus			Diacetyle, Ethanol
L. casei			Acetaldehyde, Ethanol
L. delbrueckii			
		L(+) lactic	Acetic acid, Ethanol

Additional by-products may include glycerol, formate, pyruvate, succinate and minnitol. Only the homofermentative organisms are of industrial importance for the lactic acid manufacture, which grow optimally at temperatures around 37^{O}C and at a pH of 5-6.5. As shown in Table 2.5 and 2.6, several species have been identified that produce predominantly one isomer.

Table 2.6: Lactic acid isomer produced by *Lactobacillus* species

L(+) lactic acid producer	D(-)lactic acid producer	DL-lactic acid
L. rhamnosus	L. coryniformis	L. acidophilus
L. amylophilus	L.bulgaricus	L. helveticus
L. bavaricus	L. jensenii	
L.casei L.maltaromicus	L. lactis	
L. delbrueckii		

The selection of an organism depends primarily on the carbohydrate to be fermented. Lactose is fermented by *L. bulgaricus*, *L. casei* or *S. lactis* while glucose is fermented by *L. delbrueckii* and *L. leichmannii*. Xylose is fermented by *L. pentoaceticus*.

2.4.3 Fermentation operating condition and parameters

Lactic acid fermentation has been studied since 1935 using different types of microorganism and fermentation operation conditions such as pH, carbon source, temperature, inoculum size, initial substrate conditions and nitrogen source [37].A batch process in which the conditions undergo a continuous change as a result of consumption of nutrients, multiplication of cells and accumulation of products, etc normally carries out the lactic acid fermentation. The culture condition vary from the strain which grow efficiently with good acid production on one carbon source will frequently not do so on another [38].Several parameters and operating condition effect the optimal production of lactic acid which includes:

2.4.3.1 Microbial strain

Selection of the production strains is one of the most important parameters of successful production. First, strain development in the lactic acid industry does not only aim at high yields and productivities but also at the ability to transform cheap raw materials and to utilize substrates with constituents that maybe harmful to the production strain. Strain selection for these complex properties has generally been accomplished empirically.

A large number of bacteria have the ability to produce lactic acid. Strains of Lactobacillus were compared with regard to the fermentation of various sugars. Strain giving the highest lactic acid concentration and yield usually also showed the highest productivity. On lactose, including whey and milk, Str. thermophilus was in most studies superior to Lactobacillus delbrueckii spp. bulgaricus and L. lactis. In wheat flour hydrolysate L. lactis showed the highest productivity, whereas Lb. delbrueckii spp. delbrueckii resulted in the highest lactic acid concentration and yield. Generally the temperature used was adjusted to the optimum for each organism [39]. But for this specific study Lactobacillus Plantarum was used.

2.4.3.2 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 source is capable of producing lactic acid, including molasses, fruits waste, starch, glucose, sucrose, fructose and lactose. If these substrates contain high level of metal ions they must be removed prior to production. 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.

2.4.3.3 Nitrogen sources

The medium composition has been investigated from many aspects, including the addition of various concentrations of nutrient. 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 [40]. Lactic acid increases with the increasing concentration of N_2.

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. 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.

2.4.3.4 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 41-45 ^{O}C [41]. Lactic acid bacteria can be classified as thermophilic or mesophilic. *Lactobacillus delbrueckii* is mesophilic bacteria, which grow at 17-50 ^{O}C and have optimum growth between 20-40 ^{O}C (Buchta, 1983). The yield increased with each increase at temperature level of fermentation (30 to 40 ^{O}C). The lactic acid production begins to decrease when the temperature is above 45^{O}C. The highest yield at 79.8% was achieved at temperature of 40 ^{O}C [42].

2.4.3.5 Effect of pH

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. The fermentation pH is set either at the beginning or then left to decrease due to acid production, or it is controlled by base titration, or by extraction, adsorption or electrodialysis of lactic acid. Various researchers studied the effect of pH on lactic acid production and found that the optimum pH for lactic acid production is between 5-7 [43].and found that the effect of pH on lactic acid production is important and the optimal pH was 6.0 with lactic acid production found to be 54.97 g/l and the yield value 79%.

When the controlled pH was increased to 6.5, lactic acid production and yield value was reduced to 21.88 g/l and 31.25% respectively [44]. Busairi (2002) also reported that lower production rate of 11.59 g/l or 16.55% yield was obtained with lower pH of 5.5. In all cases, titration to a constant pH resulted in higher or equal lactic acid concentration, yield and productivity in comparison with no pH control.

Previous reported represented that an increase in lactose utilization and subsequent lactic acid production was found incubation time up to 36 h and thereafter no improvement in both the functions was observed [45]. 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 48h has been generally used for lactic acid production using different lactobacilli cultures [46].In addition, the different optimal conditions reported by various workers for maximum lactic acid production could be explained by the differences in the nature of the strains and medium composition used in their studies.

2.4.3.7 Effect of agitation

Different lactic acid bacterial strains differed in their requirement for growth conditions. The consequence of agitation speed on lactic acid fermentation efficiency was carried out. For the strain Lactobacillus rhamnosus, the maximum lactic acid concentrations could be achieved when fermentation was carried out at pH 6, temperature of 40 ^{0}C and agitation speed of 150 rpm, which was in accordance with a previous report [47] the optimal condition for lactic acid is pH 5.0-6.8, temperature 30-45 ^{0}C with continuously agitating at 100-200 rpm [48].

2.5 Inhibition of lactic acid fermentation

The major problems associated with lactic acid production are substrate inhibition, end-product (lactic acid) inhibition, and by-product formation, respectively. There are different strategies to check the end-product inhibition for example neutralization of lactic acid with suitable alkali, but there are fewer attempts to account for the substrate inhibition [49].End product inhibition occurs due to rise in pH of extracellular medium during lactic acid production that results in disturbance of $NADH/NAD^+$ ratio. It has been evidenced that conversion of pyruvate to lactic acid requires high cytosolic NADH and a high $NADH/NAD^+$ ratio increases *ldh* activity [50].

2.5.1 Substrate inhibition

At high concentrations, some substrates also inhibit the enzyme activity. Uncompetitive inhibition is substrate inhibition which occurs at high substrate concentrations. It happens when two molecules of substrate can bind to the enzyme, and thus block activity. Some paper showed that high initial lactose concentration of 100 g/L in cheese whey reduced both the specific growth rate and substrate utilization rate due to the substrate inhibition phenomenon [51].

2.5.2 End-product inhibition

For microorganisms, limitation of growth and acid production by the end- product is well known. Lactic acid production processes traditionally suffer from end-product inhibition. The inhibition mechanism of lactic acid is probably related to the solubility of the un-dissociated lactic acid within the cytoplasmic membrane and the insolubility of dissociated lactate, which causes acidification of cytoplasm and failure of proton motive forces [52].It eventually influences the trans-membrane pH gradient and decreases the amount of energy available for cell growth [53]. The inhibition of growth in *Lactobacillus acidophilus* was investigated, by acidification of cytoplasm via the acid produced, below an organism-specific threshold pH level of 4.4 [54].However, the mechanism for lactic acid inhibition of growth is not fully understood. The accepted mechanism of inhibition by weak organic acids is related to the solubility of the non-dissociated form within the cytoplasm membrane and the insolubility of the ionised acid form [55]. This causes acidification of the cytoplasm and the collapse of the motive force, resulting in inhibition of nutrient transport [56]. In addition, effect of inhibition by acetic acid on anaerobic bacteria is related to the accumulation of metabolic intermediates of terminal reactions [57]. It suggested that the capacity of lactobacilli to tolerate lower pH values than other lactic acid bacteria, such as *Streptococcus* and *Leuconostocos*, originates from their more basic cytoplasmic pH values, allowing higher concentrations of non- dissociated acids to be tolerated. In addition, it could also be related to the higher activity of the ATPase responsible for the extrusion of H^+, or related to the more efficient excretion of lactic acid by lactobacilli [58]. The lactic acid is excreted in *Lactobacillus helveticus* as transported lactate ions and by simple diffusion (of the non-dissociated form of lactic acid). This could be due to the decrease of intracellular pH, which reduces ATPase activity, since it has been shown the ATPase isolated from different lactic acid bacteria have different pH optima, ranging from 5.5 to 7.5 depending on their origin [59].

Therefore, to alleviate the inhibitory effect of lactic acid during the fermentation, it must be removed selectively *in situ* from the fermentation broth. Recently, various attempts have been carried out to remove the lactic acid simultaneously as it is formed. There are studied the reactive extraction of lactic acid from the fermented broth. They indicated that *in situ* extraction was possible with the use of di-*n*-octylamine and with adjustment of the fermentation broth to a pH 5.0 by ammonia. In their study, the system of electrodialysis fermentation with a level meter was the most efficient system and a higher yield could be obtained if the glucose concentration in the broth could be controlled to remain at a lower level [59].

2.6 Fermentation mode

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.

On the other hand, the continuous mode generally resulted in higher productivities. The major reason is probably that the continuous cultures were run at a high dilution rate, where the advantages over the batch mode are most pronounced. Varying the dilution rates in continuous culture affects both the substrate and nutrient concentrations. However the effects on the yield and productivities were inconclusive. Fed batch, semi continuous and repeated batch mode gave higher yields than the batch mode [61].

In this section, the types of microorganism and the range of operation conditions used will be described briefly in order to provide the background for the present study which will be helpful in selecting the appropriate microorganism and operational conditions for lactic acid fermentation of Bulla starch.

2.6.1 Batch fermentation

The basic fermentation process is batch. The culture is grown in a series of inoculums vessels and then transferred to the production fermentor. The inoculum size is usually 5-

10% of the liquid volume in the fermentor. The fermentation is typically controlled at 35-45^{o}C and at pH 5-6.5 by the addition of the suitable base, such as ammonium hydroxide.

At a pH of 5.0, Venkatesh (1997) attained a lactic acid concentration of 62 g/L in 6 days of simultaneous fermentation using *T.reesei* and *L. bulgaricus*. However, at a pH of 4.2, the lactic acid concentration dropped down to 18 g/l at the end of 6 days. Product concentrations of lactic acid have been reported as high as 115 g/L in 11 hours on whey permeate and yeast extract medium with *Lactobacilli bulgaricus* (Mehaia and Cheryan, 1987). At pH 5-6.5, for enzyme thinning corn starch, concentrations greater than 150 g/L in 30 hours have been reported with *Lactobacillus amylovorus* (Cheng *et al*., 1991). The molar conversion of carbohydrates was 94-95% for the two examples. Benthin and Villadsen (1995) produced optically pure D (-) lactic acid by fermentation of lactose with *L. bulgaricus*. The product was purified by crystallization as magnesium d-lactate followed by extraction with butanol. The overall yield of D (-) lactic acid was 72% and the purity was more than 99%.

The major limitation of the batch fermentation process is that both the presence of the lactic acid in the fermentation and the associated drop in pH, reduce the cells ability to secrete lactic acid. Adding a basic solution such as $CaCO_3$ will precipitate the Ca-lactate and prevent the pH drop, however, this precipitate has to be dissolved using another acid such as sulfuric acid. While this process is not technically difficult, it is expensive on a large scale and consumes large quantities of other chemicals. Instead, removing the produced lactic acid during the fermentation process can eliminate both of these events.

2.6.2 Continuous fermentation

Continuous fermentation may be conducted to obtain fermentation products as a laboratory tool in the study of the physiology, metabolism or genetics of microorganisms or to produce microorganisms efficiently. It is characterized by the inflow of fresh nutrient medium into the culture vessel and the outflow at the same rate of the medium modified by the metabolic activity of the organisms together with part of the grown organisms. The concentration of all components, cells, substrates and products is identical in the whole cultivation volume and therefore in the out flowing fluid as well.

This type of fermentation can also be in a multi-stage process. The application of the multi-stage continuous system becomes necessary when we are concerned with the formation of certain products, with the chemical transformation of complex molecules by cells that are in a certain physiological state or with the stabilization of a certain enzymatic system [61]. The efficiencies and advantages of continuous process over the batch processes; stability, ease of control and increase in the productivity, make the continuous process more attractive for the industry than a simple batch process. Nevertheless, continuous charge of the nutrients and substrate may lead to substantial losses that will add to the cost of the final product.

The lactic acid production was found to depend on the choice of carbon substrate. At steady state, yeast extract requirements for lactic acid production were lower when glucose was used as a substrate than with the lactose fermentation. Consequently, more growth factors were needed for lactose fermentation than for the glucose.

2.7 Substrate of lactic acid production via fermentation

Several carbohydrate materials have been used for the commercial production of lactic acid by fermentation. Refined sucrose from cane and beet sugar, followed by dextrose and maltose from hydrolyzed starch, has been the most commonly used substrates since the 50's [62]. However, sugar and starch also have food and feed value and their sources are limited. Several raw materials or by-products have been evaluated as potential inexpensive substrates for lactic acid production.

The raw materials for the fermentation process consist of carbohydrates and nutrients for growth of the cells. For large-scale fermentation, the carbohydrates have primarily been lactose from whey or hydrolyzed corn syrup. The latter is predominantly glucose with some higher saccharides. A large number of carbohydrates materials have been used, tested or proposed for the manufacture of lactic acid by fermentation. Table 2.7 summarizes the substrates for lactic acid fermentation.

Table 2.7: Summary of the substrates for lactic acid fermentation [63]

Principal substrate	Source
Lactose	Casein whey Cheese whey Sweet whey
Glucose	Corn
Sucrose	Molasses Cane sugar Beet sugar
Other	Potatoes Cellulose Sorghum extract Bulla

It is useful to compare feedstock based on the following desirable qualities:

1. Low cost
2. Low levels of contaminants
3. Fast fermentation rate
4. High lactic acid yield
5. Little or no by-product formation
6. Ability to be fermented with little or no pretreatment
7. Year- round availability

Crude feedstock has been avoided because high levels of extraneous materials can cause separation problems in the recovery stages. Use of pentose sugars results in the production of acetic acid that will incur extra process equipment for separation. Sucrose from cane and beet sugar, whey containing lactose and maltose and dextrose from hydrolyzed starch are presently used commercially. Since the 50’s, potato, molasses and cheese whey have been studied as substrate for lactic acid production[64]. The results showed that cheese whey is a good inexpensive substrate for lactic acid production. However, the amount of whey supply is limited.

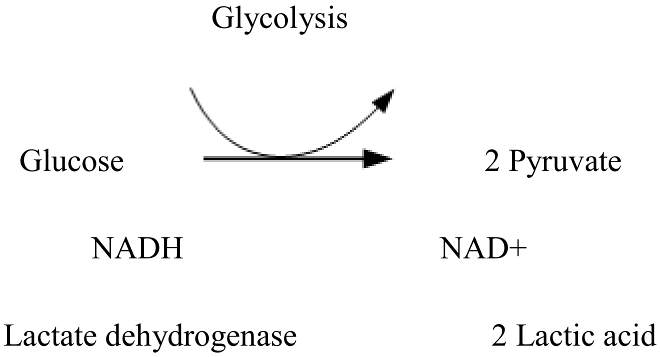
Strain

The microorganism used in this study was Lactobacillus planterum. subsp. planterum A145, and A155strain, a mesophilic homo-fermentative lactic acid bacterium. It was obtained from southern nation nationality and people Sheka zone Masha woreda from Enset plant and isolated in AAU (Addis Ababa University), department of nutrition Addis Ababa, Ethiopia.

The lactic acid bacteria belong to two main groups, the homo-fermentors and the hetero-fermentors. The pathways of lactic acid production differ for both types. Homo-fermentative produces mainly lactic acid whilst hetero-fermentative produces lactic acid, appreciable amount of ethanol, acetate, and carbon dioxide, respectively.

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

1. Homo-fermentative:- produces more than 85% lactic acid from glucose (Salminen et al., 1998). One mole of glucose can be converted to two moles of lactic acid, generating a net of 2 ATPs per mole of glucose metabolized.



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

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

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

3. Less known hetero-fermentative species which produce DL-lactic acid, acetic acid and carbon dioxide.

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

There are two main hexose fermentation pathways that are used to classify LAB genera. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolize one mole of glucose in the Embden-Meyerhof-Parnas (EMP) pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles ATP per glucose consumed. Representative homolactic LAB genera include Lactococcus, Enterococcus, Streptococcus, Pediococcus and group I lactobacilli.

Hetero-fermentative LAB utilizes the pentose phosphoketolase pathway. One mole Glucose-6-phosphate is initially dehydrogenated into 6-phosphogluconate, and subsequently decarboxylated to yield one mole of CO_2. The resulting pentose-5-phosphate is cleaved into one mole glyceraldehyde phosphate (GAP) and one mole acetyl phosphate. Theoretically, the end products (including ATP) are produced in equimolar quantities from the catabolism of one mole glucose. Obligate hetero-fermentative LAB includes Leuconostoc, Oenococcus, Weissella, and group III lactobacilli.

2.8 Lactic acid fermentation models

The kinetic models play an important role in monitoring and predicting fermentation process. In batch fermentation the kinetic model provides information to predict the rate of cell mass of product generation, while continuous fermentation predicts the rate of product formation under given conditions [65].

The models contain kinetic of growth, substrate utilization and product formation. According to this view, the cell, growth models can be divided into unstructured and structured types. Most of the available mathematical models for lactic acid fermentation process are unstructured. Unstructured model are the simplest, they take the cell mass as a uniform quantity without internal dynamics whose reaction rate depends only upon the conditions in the liquid phase of the reactor. This model contains a small number of parameters which can easily be estimated on the basis of steady state experiments and open ended and can rather easily be extended to describe more complex systems[66].

2.8.1 Kinetics of microbial growth

Batch growth of a microorganism consists of the following phases: lag phase, transition phase, exponential or logarithmic phase, a second transition phase, stationary phase and death phase [67].The rate of microbial growth is given by equation 2.1

$$\frac{dx}{dt} = \mu X \tag{2.1}$$

Where: X = the concentration of microbial biomass in gram/liter
μ = the specific growth rate in hours^{-1}
t = fermentation time in hours

During the exponential growth phase, the specific growth rate of the cells, μ, is constant and reaches its maximum, μ_{max} as seen in equation 2.2.

$$\frac{dx}{dt} = \mu_{max} X \tag{2.2}$$

The kinetic of microbial growth in lactic acid fermentation has been studied by Mercier and Yerushalmi (1991) and Norton and Vullemard (1994). They used the logistic model that express the relationship of the growth rate and two kinetic parameters such as the maximum specific growth (μ_{max}). The two parameters were estimated by non-linear regression using the least square methods.

$$\frac{dx}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}} \right) \qquad 2.3$$

Integration of equation (2.3), gives;

$$X_t = \frac{X_o X_m \exp(\mu_{max} t)}{X_m - X_o + X_o \exp(\mu_{max} t)} \qquad 2.4$$

An unstructured model, which is frequently used in the kinetics description of microbial growth, is the Monod equation. This model expresses that the specific growth rate of microorganism increase if the substrate concentration in the medium is increased, however the increase in specific growth rate becomes progressively less if the substrate concentration level is higher. The relationship between μ and the residual growth-limiting substrate is represented in the equation below:

$$\mu = \mu_{max}\left(\frac{S}{Ks + S}\right) S \qquad 2.5$$

K_S is the substrate utilization constant numerically equal to substrate concentration when μ is half μ_{max} and is a measure of the affinity of the organism for its substrate. The kinetics of microbial growth by combining equation (2.1) with (2.5) was proposed by Hanson. This model is represented in the equation below:

$$\frac{dx}{dt} = \mu_{max}\left(\frac{S}{K_s + S}\right) X \qquad 2.6$$

Similar model has been proposed by Suscovic *et al.* (1992) and they assumed that the death rate cannot be neglected. The equation is as follows:

$$\frac{dx}{dt} = \mu_{max}\left(\frac{S}{Ks+S}\right) X - K d\ X \qquad (2.7)$$

2.8.2 Kinetic model of substrate utilization

The substrate utilization kinetics for lactic acid fermentation using *Lactobacillus delbrueckii* may be expressed by the equation proposed by Monteagudo *et al.* (1997) which considers both substrate consumption for maintenance and substrate conversion to biomass and product. The rate of substrate utilization is related stochiometrically to the rates of biomass and lactic acid formation. The substrate requirement to provide energy for maintenance is usually assumed to be first order with respect to biomass concentration, mX. The equation is express as the follows:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dx}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + mX \qquad (2.8)$$

The parameters of the biomass yield on the utilized substrate $Y_{X/S}$, the product yield on the utilized substrate ($Y_{P/S}$) and maintenance coefficient (m) were estimates by non-linear regression analysis. A similar model was used for the kinetics of substrate utilization in lactic acid fermentation using *Lactobacillus amylophilus* by [68] and *Streptococcus cremoris,*[69]have also proposed simpler models. They assumed that since the maintenance coefficient is much smaller than the specific growth rate, it can be omitted, thus only the substrate utilization for conversion of biomass and product is considered. The equation has the following form:

$$-\frac{dS}{dt}=\frac{1}{Y_{X/S}}\frac{dx}{dt}+\frac{1}{Y_{P/S}}\frac{d}{dt} \qquad (2.9)$$

The simplest model has been proposed by [70] Suscovic *et al.* (1992). they assumed that the substrate utilization only for conversion of biomass. By the combining of Monod equation to this model can be obtained the following equation:

$$-\frac{dS}{dt}=\frac{1}{Y_{X/S}}\mu_{max}\left(\frac{S}{K_s+S}\right)X \qquad (2.10)$$

The parameters of biomass yield on the utilized substrate ($Y_{X/S}$) and saturation constant (K_S) can be estimated using linear regression analysis.

2.8.3 Kinetics of Lactic Acid Production

Lactic acid fermentation that was described by Luedeking and Piret [71], Norton *et al.* (1994) reported that lactic acid production was strongly linked to biomass production. Basically three types of fermentation can be distinguished such as growth associated product formation, mixed growth associated product formation and non-growth associated product formation [72].

Many researchers used the mixed growth associated product formation for lactic acid production kinetics. This model was described by Luedeking and Piret [73] and is represented below:

$$dP/dt=\alpha\, dx/dt+\beta X \qquad (2.11)$$

Where dP / dt is the volumetric product formation rate, α is the growth associated product formation and β is the non-growth associated product formation.

Mathematical modeling and estimation of kinetics parameters for lactic acid production using high-glucose, high fructose and high sucrose syrup by *L. delbrueckii* have been studied by Suscovic [74]. The growth associated lactic acid production constant (α) and non-growth associated product formation constant (β) were estimated by linear regression and obtained values of α always higher than β.

The kinetics model for lactic acid production on beet molasses using *L. delbrueckii* was proposed by Monteagudo [75]. Using model given by Luedeking and Pilet [76], it improved by the addition of a term indicating dependence of the rate of lactic acid production on inhibitor concentration the lactic acid. The model has the following form:

$$dP/dt=[dx/dt + X][1- p/p_{max}] \qquad (2.12)$$

The parameters were estimated by non-linear regression analysis and similar results were also obtained as reported by previous researcher Suscovic [77]. *et al*. (1992).

CHAPTER THREE

III.MATERIALS AND METHODS

3.1 Chemicals and equipment

Basically the chemicals that are required for the experiments in this study were divided into three categories: chemicals for immobilization, Bulla starch characterization, fermentation of Bulla and media LAB growth (MRS broth and agar) .All the chemicals used are analytical grade and purchased from various suppliers. The Lactic acid standard used in this study was obtained from SIGMA (Code No.L-6402 and L-0625) and lactobacillus MRS broth from TITAN BIOTECH LTD.

Figure 3.1: MRS broth

3.1.1. Collection and pretreatment of bulla starch (carbon source)

The bulla starch used for this study was bought from Sheka Zone Masha woreda in Southern Nations, Nationalities and Peoples Region (SNNPR).

Steps followed to get bulla starch

a) Scraping the leaf sheath, peduncle, and grated corm into a pulp;
b) Squeezing liquid containing a starch from the pulp
c) Allowing the resultant starch to concentrate into a white powder; and
d) Rehydrating with water. It is considered the best quality enset food and is obtained

mainly from fully matured enset plants.

3.1.2. Proximate composition of bulla starch

Characterization and proximate composition analysis were done at the laboratory of food and nutrition in Ethiopian Public Health Institute.

3.1.2.1 Determination of moisture content

Accurately weighed 5 g of sample of bulla was taken in a tared aluminium dish. Dried in an air oven at 100 ^{0}C for 5 hours and cooled in a desiccator and weighed. It was dried again for 30 minutes, cooled in a dessicator and weighed. The process was repeated 3 times until the difference in two successive weighing is less than 1 mg. the lowest weight was recorded and amount of moisture calculated.

$$\% \text{ Moisture} = \frac{\text{Wt of sample} - \text{Wt sample after dried in oven}}{\text{Wt of sample}} \quad (3.1)$$

3.1.2.2 Determination dry matter content

Total dry matter was just calculated from moisture content. Total dry matter or total, %Total solids = (100 - %Moisture) (3.2)

3.1.2.3 Determination of total ash content analysis

Weigh accurately about 5 g of sample in a tared silica / platinum dish Char the material carefully on a burner and transfer the dish to a muffle furnace and ash at a temperature of 550± 10 ^{0} C until the ash is free of Carbon . Heat the dish again at 550 ± 10 ^{0}C for 30 minutes Cool in a desiccator and weigh. Repeat this process of heating for 30 minutes, cooling in a dessicator and weighing until the difference between two successive weighing's less than 1 mg. The lowest weight was recorded and amount of total ash calculated.

Total ash content = Wt of sample before put in muffle furnace – Wt of sample after dried and burned in the furnace (3.3)

3.1.2.4 Fat content analysis

Fat was extracted, continuously, with an organic solvent (toluene). Solvent was heated and volatilized, then was condensed above the sample. Solvent was continuously dripped through the sample to extract the fat. Fat content was measured by weight loss of sample or weight of fat removed.

350 ml petroleum ether was put in the flask, 6 glass boiling beads were added, and extracted for 6h. 250ml beaker was labeled and put on the Soxhlet extraction unit. A sample in thimbles was placed in the beaker after extraction and before drying.

Thimbles from the Soxhlet extractor were removed using tongs, air dried overnight in a hood, then dried in a vacuum oven at 70ºC, for 24h. Samples were cooled and dried in a desiccator then reweighed.

3.1.2.5 Protein content analysis

5 g of the sample was weighed and transfered to a 500 ml Kjeldahl flask. No portion of the sample clings to the neck of the flask. 0.7 g. of copper sulphate, 15 g. of Potassium Sulphate and 40 ml of concentrated sulphric acid was added to the flask.

Two glass beads were added to the flask. The flask was placed in an inclined position on the stand in the digestion chamber and digested. The flask was gently heated at low flame until the initial frothing ceases and the mixture boils steadily at a moderate rate. During heating the flask was rotated several times.

Heating was continued for one hour. The color of the digest was changed to pale blue. The digest was cooled and slowly 200 ml of water was added. A piece of granulated Zinc and sodium hydroxide solution (450g/l) were added and carefully pour down the side of the flask to make the contents strongly alkaline (about 110 ml) before mixing the acid and alkaline layer.

The flask was connected to a distillation apparatus incorporating an efficient flash head and condenser. A delivery tube was fitted to condenser which dips just below the surface of the pipetted volume of standard acid contained in a conical flask receiver. (Precaution: The receiving solution must remain below 45 degrees Celsius to prevent loss of ammonia). The contents of the digestion flask was mixed and boiled until 150 ml have distilled into the receiver. 5 drops of methyl red indicator was added and titrated with 0.1N sodium hydroxide solutions. A blank titration was simultaneously carried out. 1 ml of 0 .1 N H_2SO

= 0.0014 g N.

3.1.2.6 Total carbohydrate analysis

The carbohydrate content of a food can be determined by calculating the percent remaining after all the other components have been measured:

% Total carbohydrates = 100 - %moisture - %protein - %fat - % ash. (3.4)

3.1.3 Culture media

The culture media used was MRS (deMan Rigosa Sharpe) medium, which suggested by DSMZ catalogue (1993). The compositions for 1 liter MRS medium are shown in Table 3.1

Table 3.1: Composition of MRS medium (1L)

Material	Composition(g)
$MgSO4.7H2O$	0.58
$MnSO4$	0.25
Sodium acetate	2
$K2HPO4$	2
Diammonium citrate	5
Yeast extract	5
Meat extract	5
Peptone	10
Glucose	20
Tween-80	1ml

3.1.4 LAB isolation

A 10g bulla was taken aseptically and dissolved in 90ml of distilled and autoclaved water serially diluted 6 times (dilution factor 10^{-6}) and 0.1ml was spread evenly on MRS agar plates, supplemented with sodium azide (0.2 g/l), bromocresol purple (0.12 g/l) and 2% (w/v) sucrose. Appropriate dilution was spread placed on MRS agar and incubated at 30 0C for 24 hours. The plate was counted. The colony were isolated and grown in MRS broth at 30 ^{0}C for 24 hours. The colony was purified by streaked plate method many times. Pure colonies were kept at 4 ^{0}C for fermentation process.

3.1.5 Fermentation of bulla starch into lactic acid

3.1.5.1 Fermentation setup

The fermentation process was done at AAIT,school of Chemical and Bio-Engineering laboratory of food, on apparatus called New Brunswick Scintific, Excella E24 Incubator shaker series. The incubator has temperature controlling unit in degree centigrade, time controlling unit in hours and agitation controlling unit in RPM.

3.1.5.2 Fermentation Process variable study using experimental Design

The fermenter was set at different time in hour, constant agitation speed, pH and temperature in degree centigrade.

The effect of experimental values of lactic acid concentration obtained under different conditions of selected parameters at a temperature range of (30-45)^{0}C, time (24-72) hours and pH (4-7) keeping substrate concentration and agitator speed constant. The substrate concentration was set to 200 RPM, substrate concentration 40g/l.

The process variables for this specific study were pH, temperature and time. Other parameters except the above mentioned such as agitation speed, carbon source and other factors constant.

In this work, different parameters were tested in order to get the lactic acid from kocho(Bulla starch), and its product. Central composite design (CCD) was used for analysis.

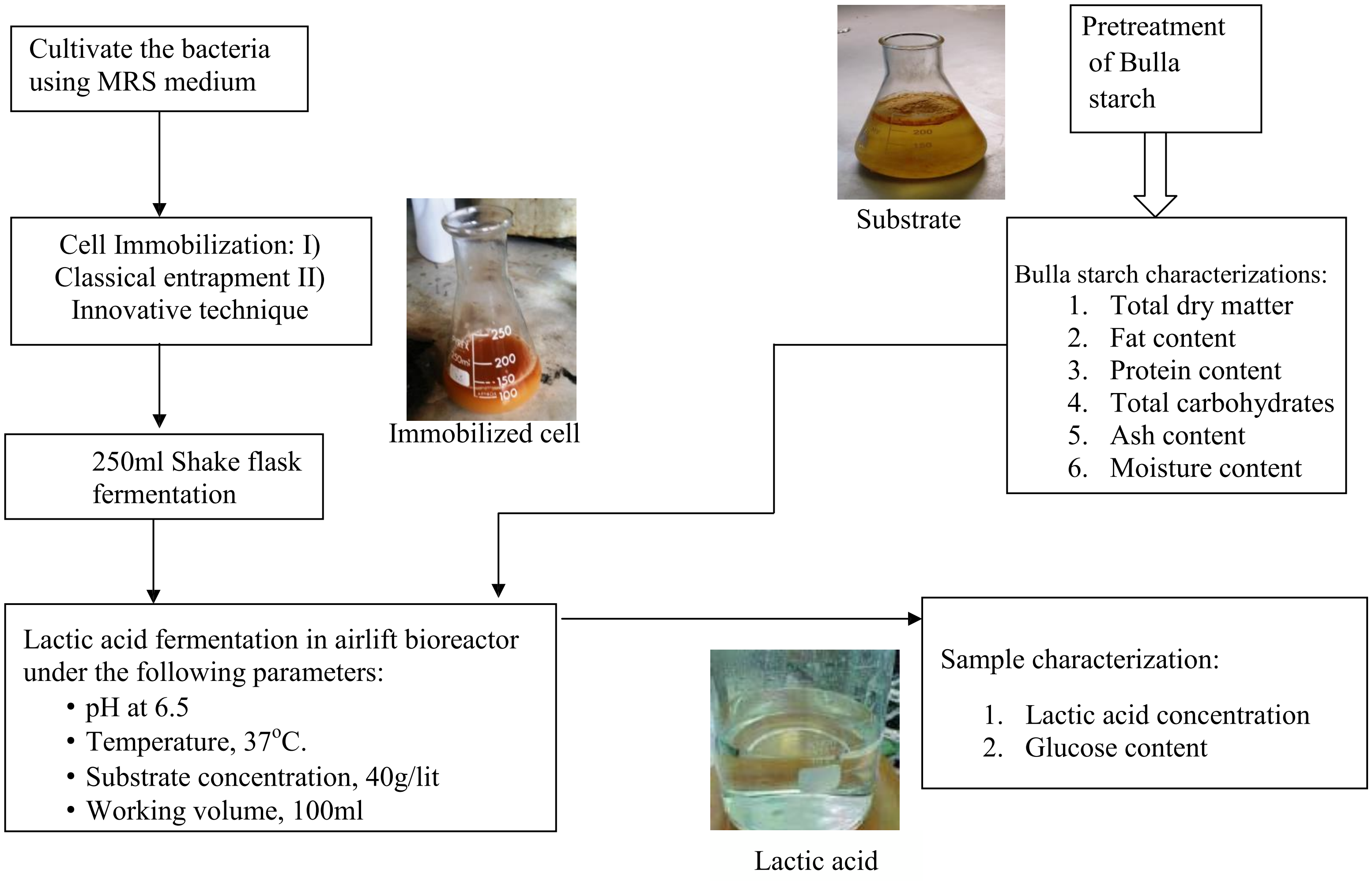
The influence of pH, Temperature, Time and yeast autolysate on the fermentation of bulla starch was studied using CCD with three replicates at the center, total of 21 experiments

Three 250ml glass flasks with working volume of 100ml sample were placed in incubator. The samples were of different pH (The first one with 5.5, the second one with 6.5 and the last with 7). The incubator was set to 200 rpm temperature 30 ^{0}C and time 24 for the fermentation. This was done for the remaining temperatures and time.

3.1.5.3 Method of lactic acid recovery and analysis

Lactic acid and lactose concentrations were measured at Addis Ababa University, department of analytical chemistry laboratory, by HPLC equipped with a Shim-pack CLC-ODS column. The HPLC was calibrated with standard Lactic acid. The column, maintained at 75°C, was eluted with 4 M H_2SO_4 at a flow rate of 0.4 ml/min for 20 min. The retention time of lactic acid under these conditions was 18 min. The samples were centrifuged at 5000 rpm for 5 minutes and then filtered through 0.2µm paper filter. To obtain desired peak height, sample size of 10µl of the clear solution were injected to HPLC.

Figure3.1 Process flow diagram for lactic acid production.



CHAPTER FOUR

IV. RESULTS AND DISCUSSIONS

4.1 Influence of carbon sources (Bulla starch) on lactic acid production

For this specific study Proximate composition analysis was done at Ethiopian health and nutrition research institute. The enset starch showed 18.25 % (w/w) moisture, 0.4639 % (w/w) protein, 0.1127 % (w/w) fats, 80.5143% total carbohydrate, 81.75 % dry matter and 0.1596% (w/w) ash.

Table 4.1 composition of bulla starch

No.	Starch component	enset
1.	Moisture	18.25
2.	Ash	0.1596
3.	Fat	0.1127
4.	Protein	0.4639
5.	Total carbohydrate	80.5143
6.	Dry matter	81.75

Result for lactic acid fermentation by using Bulla starch as the main carbon source , modified MRS broth containing 40 g/L of carbon sources(lactose, sucrose, maltose, and fructose) as a main substrate, different parameters were tested in order to get the lactic acid.

Central composite design (CCD) was used for analysis. The results are displayed in Table 4.2. Analyzing Table 4.3, the best results of all the responses evaluated were obtained in the experiments located at the central point.

Figure 4.3 showed the fermentation performances of various sugars in terms of cell concentration, and lactic acid production, respectively. The maximum cell growth and the highest lactic acid concentration were obtained after 24 h of the fermentation time. These experiments were operated with non-controlled pH: therefore; pH of fermentation broth rapidly decreased due to accumulation of lactic acid. The fermentation was almost ceased after 24 h when pH reached a value of around 3.08 as shown in figure 4.1.

Figure 4.1 pH of fermentation after 24 hour

In fact, there were several reports demonstrating that lactic acid fermentations could be operated, or only work at all, under conditions where the pH exceeded the p*K*a of the lactic acid produced (p*K*a = 3.86). This was because lactic acid in an un-dissociated form severely inhibited cell growth (Wasewar, 2005).

Design Expert 6.0.8 was used with central composite design (CCD) method having 20 runs and 1 replicates.

Table 4.2 Experimental result of lactic acid fermentation

NO.	Std	Run	Block	Factor 1	Factor 2	Factor 3	Response 1
				A:Temprature, T degree centigrade	B:Time, t Hours	C:pH	lactic acid concentration g/l
1.	2	1	Block 1	40.00	48.00	5.50	16.13
2.	19	2	Block 1	35.00	60.00	6.25	17.03
3.	8	3	Block 1	40.00	72.00	7.00	17.12
4.	17	4	Block 1	35.00	60.00	6.25	17.3
5.	4	5	Block 1	40.00	72.00	5.50	15.12
6.	9	6	Block 1	30.00	60.00	6.25	16.33
7.	6	7	Block 1	40.00	48.00	7.00	16.01
8.	7	8	Block 1	30.00	72.00	7.00	16.66
9.	18	9	Block 1	35.00	60.00	6.25	17.25
10.	3	10	Block 1	30.00	72.00	5.50	16.23
11.	12	11	Block 1	35.00	72.00	6.25	17.33
12.	10	12	Block 1	40.00	60.00	6.25	16.22
13.	14	13	Block 1	35.00	60.00	7.00	16.68
14.	11	14	Block 1	35.00	48.00	6.25	16.56
15.	16	15	Block 1	35.00	60.00	6.25	17.09
16.	20	16	Block 1	35.00	60.00	6.25	17.12
17.	13	17	Block 1	35.00	60.00	5.50	16.55
18.	1	18	Block 1	30.00	48.00	5.50	16.1
19.	15	19	Block 1	35.00	60.00	6.25	17.17
20.	5	20	Block 1	30.00	48.00	7.00	15.89

Design Summary

Table 4.3 Design summary

Study Type	Response Surface			**Experiments**	20	
Initial Design	Central Composite			**Blocks**	No Blocks	
Design Model	Quadratic					
Response Model	**Name**	**Units**	**Obs**	**Minimum**	**Maximum**	**Trans**
Y1	lactic acid concentration		20	15.12	17.33	None
Quadratic						
Factor High Coded	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	**Low Coded**
A	Temprature, T	degree centigrade		Numeric	30.00	40.00-1.000
B	Time, t	hours	Numeric	48.00	72.00	-1.000
C	pH		Numeric	5.50	7.00	-1.000

3 Factors: A, B, C

Design Matrix Evaluation for Response Surface Quadratic Model

No aliases found for Quadratic Model

Degrees of Freedom for Evaluation

Model	9
Residuals	10
Lack 0f Fit	*5*
Pure Error	*5*
Corr Total	19

Table 4.4 Design Matrix evaluation response surface quadratic model

Power at 5 % alpha level for effect of

Term	**StdErr****	**VIF**	**Ri-Squared**	**1/2 Std. Dev.**	**1 Std. Dev.**	**2 Std. Dev.**
A	0.32	1.00	0.0000	11.1 %	29.9 %	81.3 %
B	0.32	1.00	0.0000	11.1 %	29.9 %	81.3 %
C	0.32	1.00	0.0000	11.1 %	29.9 %	81.3 %
A^2	0.60	1.82	0.4500	11.7 %	32.3 %	84.8 %
B^2	0.60	1.82	0.4500	11.7 %	32.3 %	84.8 %
C^2	0.60	1.82	0.4500	11.7 %	32.3 %	84.8 %
AB	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
AC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
BC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %

****Basis Std. Dev. = 1.0**

Measures Derived From the $(X'X)^{-1}$ Matrix

Std	**Leverage**	**Point Type**
1	0.7932	Fact
2	0.7932	Fact
3	0.7932	Fact
4	0.7932	Fact
5	0.7932	Fact
6	0.7932	Fact
7	0.7932	Fact
8	0.7932	Fact
9	0.4909	Axial
10	0.4909	Axial
11	0.4909	Axial
12	0.4909	Axial
13	0.4909	Axial
14	0.4909	Axial
15	0.1182	Center
16	0.1182	Center
17	0.1182	Center
18	0.1182	Center
19	0.1182	Center
20	0.1182	Center
Average =	**0.5000**	

Maximum Prediction Variance (at a design point) = 0.793
Average Prediction Variance = 0.500
Condition Number of Coefficient Matrix = 5.500
G Efficiency (calculated from the design points) = 63.0 %
Scaled D-optimality Criterion = 2.727
Determinant of $(X'X)^{-1}$ = 2.219E-9
Trace of $(X'X)^{-1}$ = 1.884

Table 4.5 Correlation Matrix of Regression Coefficients

Correlation Matrix of Regression Coefficients

	Intercept	A	B	C	A^2	B^2	C^2
Intercept	1.000						
A	-0.000	1.000					
B	-0.000	-0.000	1.000				
C	-0.000	-0.000	-0.000	1.000			
A^2	-0.219	0.000	0.000	0.000	1.000		
B^2	-0.219	0.000	0.000	0.000	-0.375	1.000	
C^2	-0.219	-0.000	-0.000	-0.000	-0.375	-0.375	1.000
AB	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000
AC	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000
BC	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000	-0.000

	AB	AC	BC
AB	1.000		
AC	-0.000	1.000	
BC	-0.000	-0.000	1.000

Table 4.6 Correlation matrix of factors

Correlation Matrix of Factors [Pearson's r]

	A	B	C	A^2	B^2	C^2	AB	AC	BC
A	1.000								
B	0.000	1.000							
C	0.000	0.000	1.000						
A^2	0.000	0.000	0.000	1.000					
B^2	0.000	0.000	0.000	0.600	1.000				
C^2	0.000	0.000	0.000	0.600	0.600	1.000			
AB	0.000	0.000	0.000	0.000	0.000	0.000	1.000		
AC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	
BC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.000	1.000

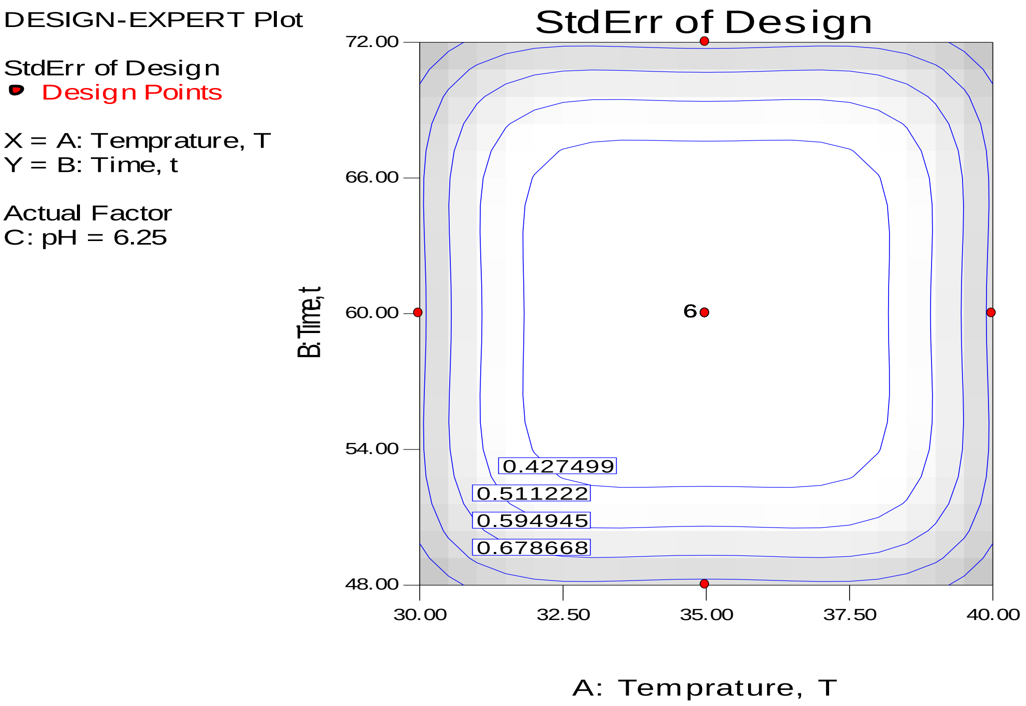


Figure 4.2: Standard Error of Design on contour graph

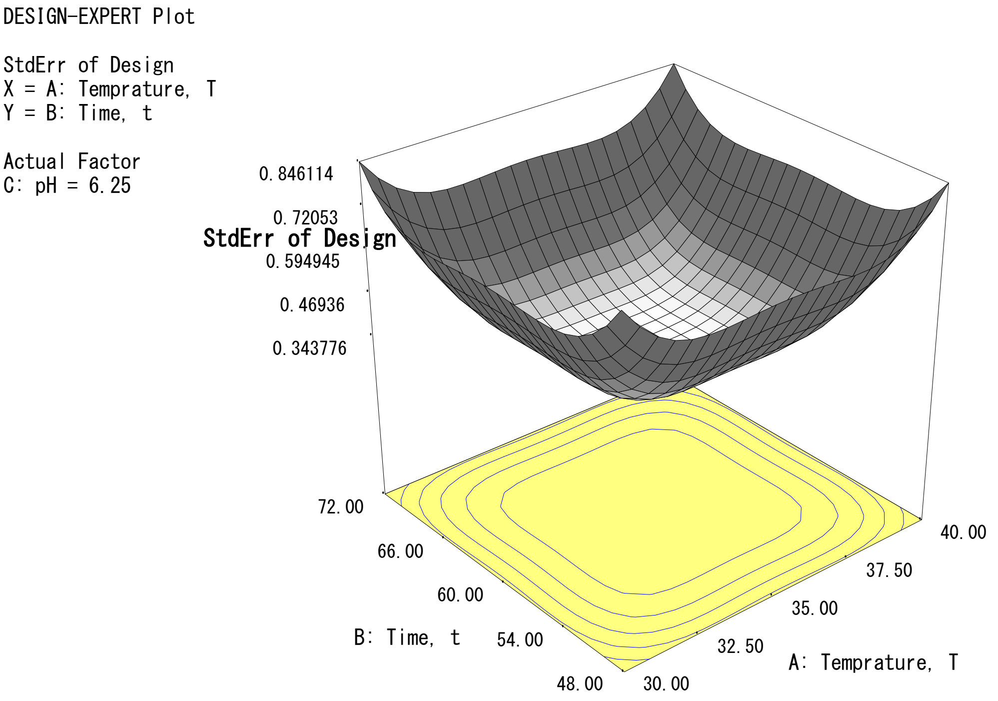


Figure 4.3: Standard Error of Design on 3D graph

Response: lactic acid concentration

***** WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	5507.55	1	5507.55			Suggested
Linear	0.85	3	0.28	0.78	0.5203	
2FI	1.38	3	0.46	1.36	0.2990	
Quadratic	3.72	3	1.24	18.33	0.0002	Suggested
Cubic	0.44	4	0.11	2.88	0.1193	Aliased
Residual	0.23	6	0.039			
Total	5514.17	20	275.71			

"Sequential Model Sum of Squares": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Linear	5.72	11	0.52	50.77	0.0002	
2FI	4.34	8	0.54	53.00	0.0002	
Quadratic	0.62	5	0.12	12.20	0.0079	Suggested
Cubic	0.18	1	0.18	17.59	0.0085	Aliased
Pure Error	0.051	5	0.010			

"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.60	0.1281	-0.0354	-0.6017	10.60	
2FI	0.58	0.3362	0.0298	-3.1209	27.27	
Quadratic	0.26	0.8979	0.8060	-0.2911	8.54	Suggested
Cubic	0.20	0.9650	0.8893	-32.4574	221.40	Aliased

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Response:lactic acid concentration

Table 4.6 ANOVA for Response Surface Quadratic Model Analysis of variance

ANOVA for Response Surface Quadratic Model Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	5.94	9	0.66	9.77	0.0007
A	*0.037*	*1*	*0.037*	*0.55*	*0.4751*
B	*0.31*	*1*	*0.31*	*4.64*	*0.0568*
C	*0.50*	*1*	*0.50*	*7.36*	*0.0218*
A^2	*1.17*	*1*	*1.17*	*17.26*	*0.0020*
B^2	*9.551E-004*	*1*	*9.551E-004*	*0.014*	*0.9077*
C^2	*0.27*	*1*	*0.27*	*3.95*	*0.0751*
AB	*0.080*	*1*	*0.080*	*1.18*	*0.3021*
AC	*0.34*	*1*	*0.34*	*5.10*	*0.0476*
BC	*0.95*	*1*	*0.95*	*14.09*	*0.0038*
Residual	0.68	10	0.068		
Lack of Fit	*0.62*	*5*	*0.12*	*12.20*	*0.0079*
Pure Error	*0.051*	*5*	*0.010*		
Cor Total	6.62	19			

Std. Dev.	0.26	R-Squared	0.8979
Mean	16.59	Adj R-Squared	0.8060
C.V.	1.57	Pred R-Squared	-0.2911
PRESS	8.54	Adeq Precision	10.332

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	17.07	1	0.089	16.87	17.27	
A-Temprature, T	-0.061	1	0.082	-0.24	0.12	1.00
B-Time, t	0.18	1	0.082	-6.168E-003	0.36	1.00
C-pH	0.22	1	0.082	0.040	0.41	1.00
A^2	-0.65	1	0.16	-1.00	-0.30	1.82
B^2	0.019	1	0.16	-0.33	0.37	1.82
C^2	-0.31	1	0.16	-0.66	0.038	1.82
AB	-0.10	1	0.092	-0.30	0.10	1.00
AC	0.21	1	0.092	2.712E-003	0.41	1.00
BC	0.34	1	0.092	0.14	0.55	1.00

Final Equation in Terms of Coded Factors:

lactic acid concentration = $+17.07 -0.06\,A+0.18\,B+0.22C -0.65A^2+0.019B^2-0.31C^2-0.10\,AB +0.21AC +0.34\,B\,C$

Final Equation in Terms of Actual Factors:

lactic acid concentration =
-15.34401
+1.56578 * Temprature, T
-0.18203 * Time, t
+2.97986 * pH
-0.026055 * Temprature, T^2
+1.29419E-004 * Time, t^2
-0.55354 * pH^2
-1.66667E-003 * Temprature, T * Time, t
+0.055333 * Temprature, T * pH
+0.038333 * Time, t * pH

Table 4.7: Diagnostics Case Statistics

Standard	**Actual**	**Predicted**			**Student**	**Cook's**	**Outlier Run**	**Order**
		Value	**Value**	**Residual**	**Leverage**	**Residual**	**Distance**	**t**
	Order							
1	16.10	16.24	-0.14	0.793	-1.150	0.507	-1.171	18
2	16.13	15.90	0.23	0.793	1.954	1.465	2.358	1
3	16.23	16.10	0.13	0.793	1.100	0.464	1.113	10
4	15.12	15.36	-0.24	0.793	-2.055	1.620	-2.565	5
5	15.89	15.58	0.31	0.793	2.648	2.689	4.595 *	20
6	16.01	16.07	-0.060	0.793	-0.507	0.099	-0.487	7
7	16.66	16.82	-0.16	0.793	-1.361	0.711	-1.431	8
8	17.12	16.91	0.21	0.793	1.743	1.165	1.982	3
9	16.33	16.48	-0.15	0.491	-0.788	0.060	-0.772	6
10	16.22	16.35	-0.13	0.491	-0.723	0.050	-0.705	12
11	16.56	16.91	-0.35	0.491	-1.877	0.340	-2.213	14
12	17.33	17.26	0.068	0.491	0.366	0.013	0.349	11
13	16.55	16.53	0.018	0.491	0.096	0.001	0.091	17
14	16.68	16.98	-0.30	0.491	-1.608	0.249	-1.771	13
15	17.17	17.07	0.10	0.118	0.424	0.002	0.406	19
16	17.09	17.07	0.023	0.118	0.096	0.000	0.091	15
17	17.30	17.07	0.23	0.118	0.956	0.012	0.952	4
18	17.25	17.07	0.18	0.118	0.752	0.008	0.734	9
19	17.03	17.07	-0.037	0.118	-0.150	0.000	-0.142	2
20	17.12	17.07	0.053	0.118	0.219	0.001	0.208	16

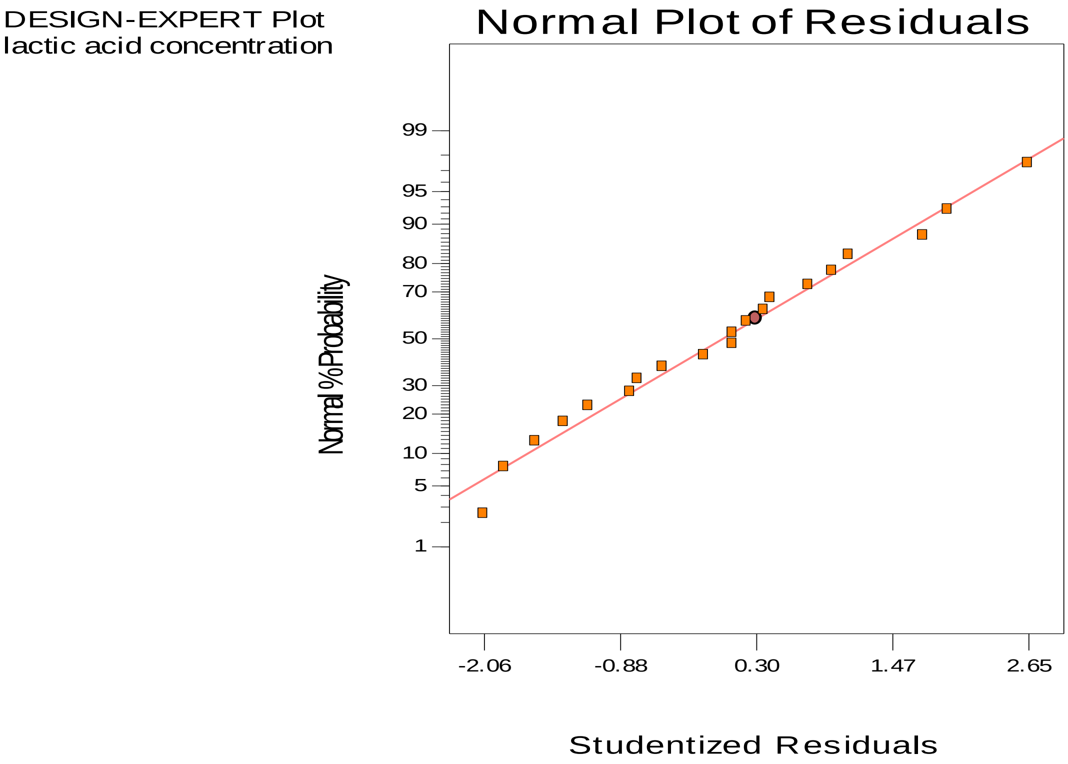


Figure 4.4 : normal plot of Residuals graph

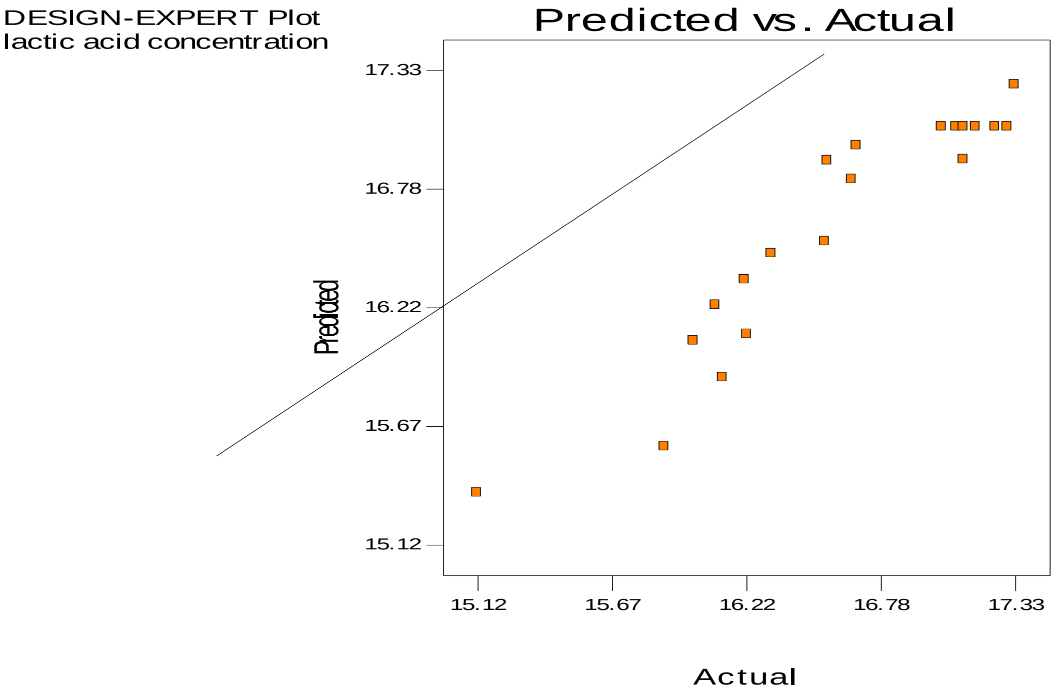


Figure 4.5: predicted vs Actual graph

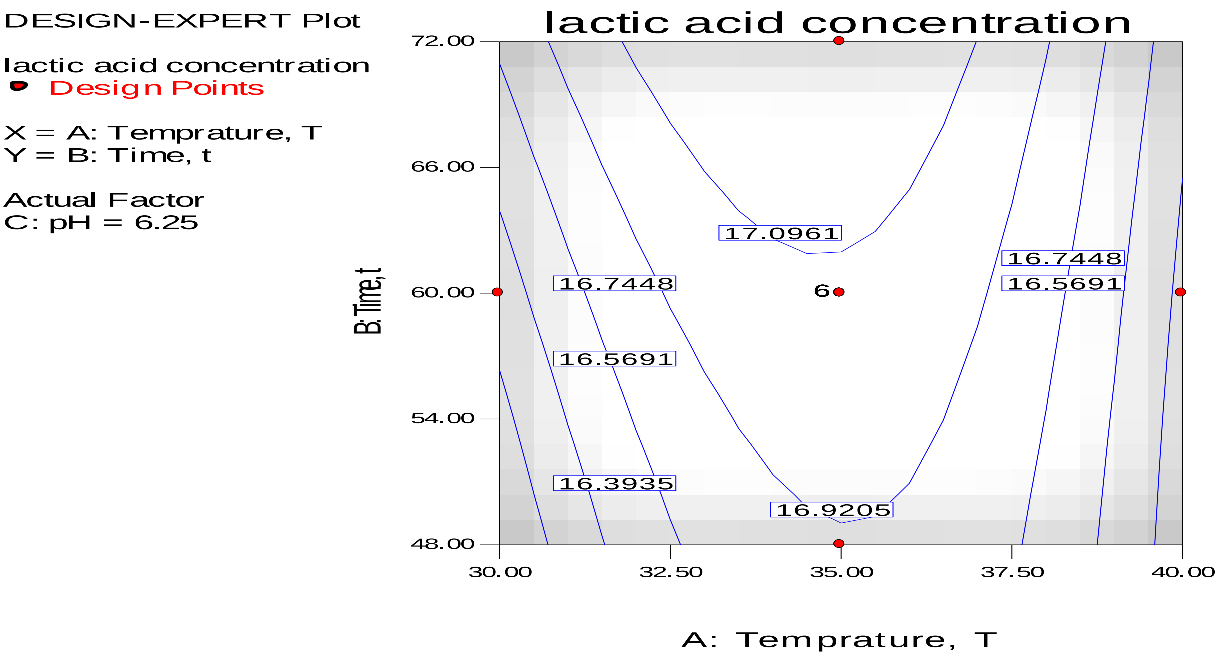


Figure 4.6: Temperature vs time contour graph for lactic acid concentration

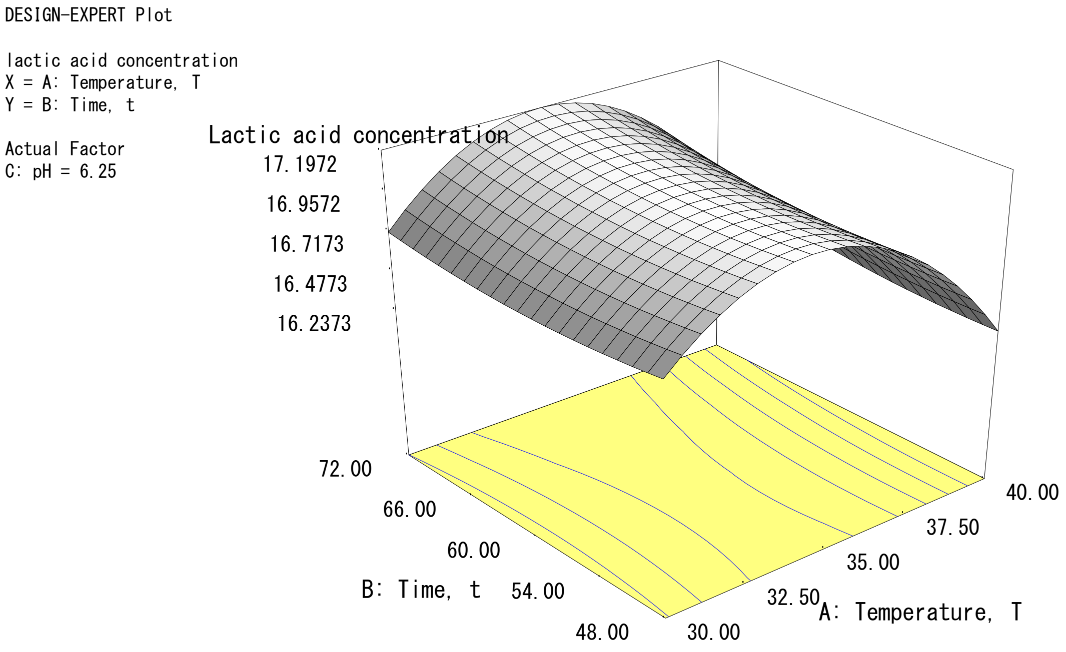


Figure 4.7: Temperature vs time 3D graph for lactic acid concentration

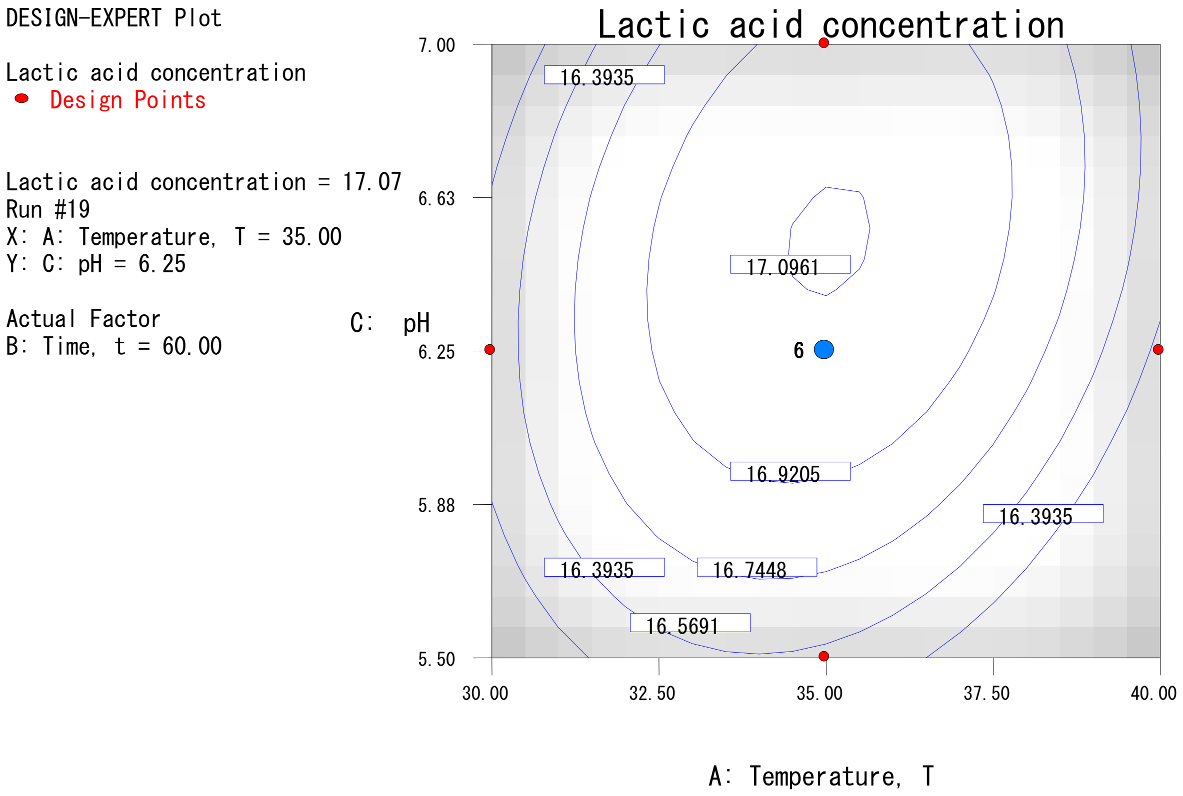


Figure 4.8: Temperature Vs pH contour graph for lactic acid concentration (g/l)

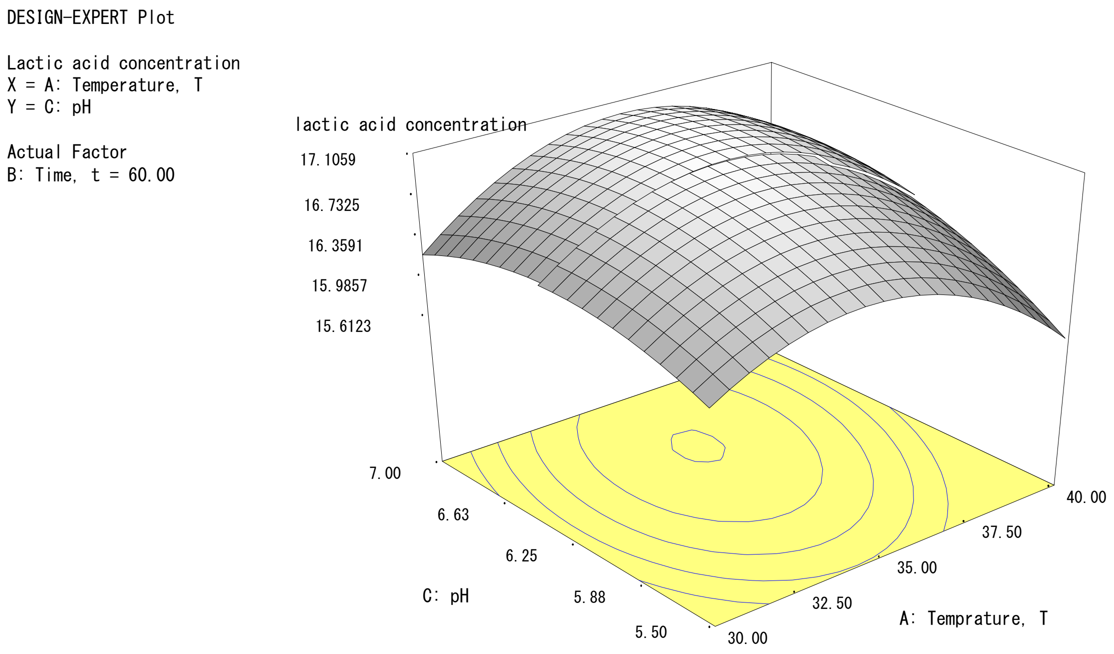


Figure 4.9: Temperature vs pH 3D graph for lactic acid concentration (g/l)

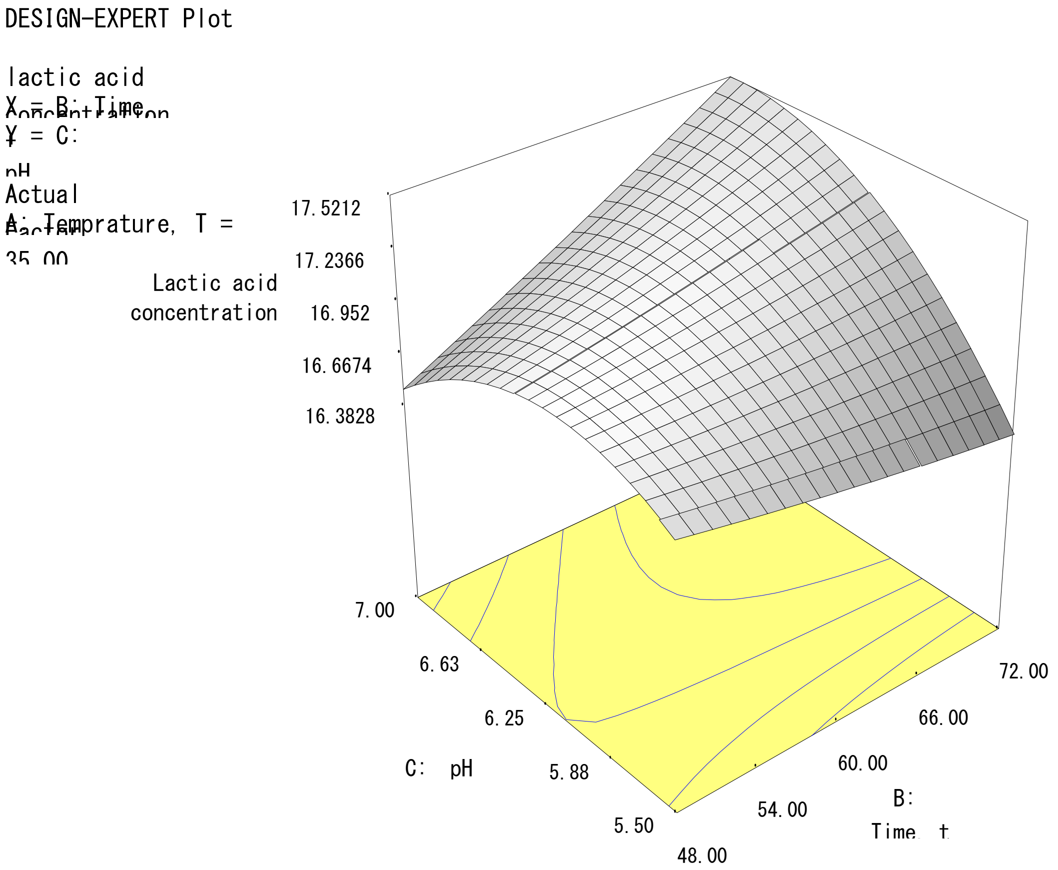


Figure 4.10: Time Vs pH 3D graph for lactic acid concentration (g/l)

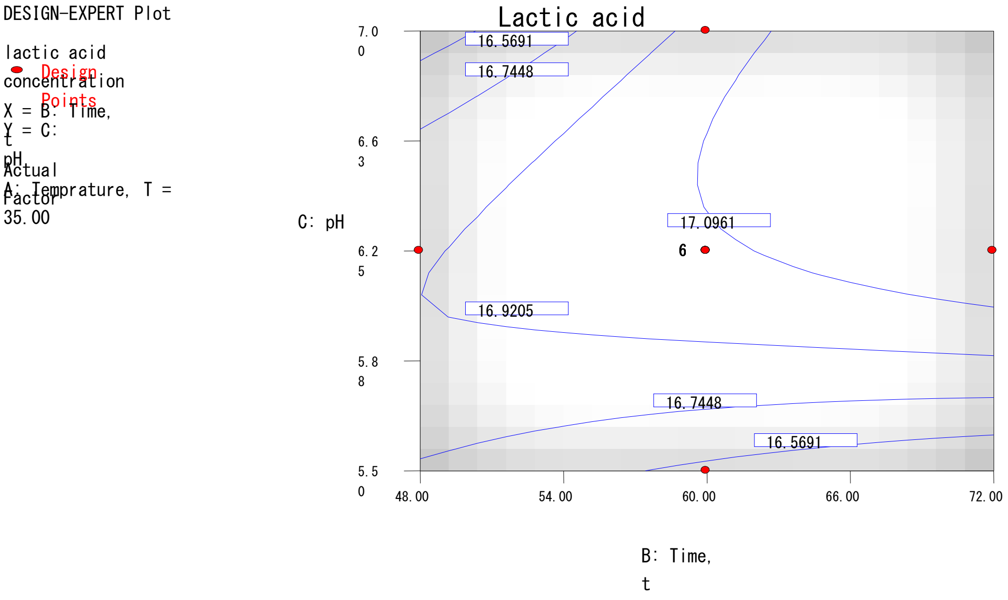


Figure 4:11 Time Vs pH contour graph for lactic acid concentration (g/l)

4.2 Effect of Bulla starch on fermentation performance

The bulla starch used from SNNPR, sheka zone, Massha woreda contains high starch content with low quantity of impurities.

The effect of initial concentration of bulla starch on lactic acid production was investigated by addition of Bulla starch into the fermentation medium at initial concentrations 40g/l. The results in Table 4.7 showed that *L. Plantarium* could utilize all types of carbon source. The maximum lactic acid concentration was found to be 17.33 g/L which resulted in the production yield of nearly 82%.

However, as the refined sugars are expensive and it is not economical attractive for industrial production. The experimental results for all initial bulla starch concentrations revealed that bulla starch possesses low specific growth rates, and product concentrations. The low specific growth rates indicated that there was low activity of amylolytic enzyme resulting in slow break down of bulla starch molecule.

Generally, lactic acid bacteria are deficient in amylolytic characters especially for the highly branched starch. Bulla starch contains percentage of amylo-pectin of approximately 80%, therefore, it is necessary to hydrolyze the bulla starch prior to beginning the fermentation for their utilization. Traditionally, the gelatinized Bulla starch can be treated with amylase (liquefaction) and gluco-amylase (saccharification) to get the hydrolyzed product. Hydrolyzed bulla starch showed its high potential to be used as the representative carbon source; although, the final acid concentration was lower than the others (refined sugars, especially glucose and fructose). This was probably due to the lower amount of available reducing sugar in the hydrolyzed bulla starch. However, these results showed that hydrolyzed bulla starch possessed comparable fermentation performance with other carbohydrates.

But for this specific study the starch used is not hydrolyzed and recommended it for further study. It is obvious that pre-treatment (hydrolyzed) of bulla starch generally increases the lactic acid yield, fermentation efficiency, and lactic acid productivities.

4.3 Influence of factors on lactic acid fermentation

4.3.1 Effect of various temperature on lactic acid production

This work examined the optimum environment condition for microbial growth, and lactic acid production. Temperature adaptations of bacterial growth under different temperature regimes were studied. Better understanding of the temperature effects on lactic acid fermentation will facilitate improvement of the production process. The temperature were monitored and controlled during the fermentation process at elevated temperatures of 30, 35, 37, 40 and 45 ^{0}C with fermentation time 72 h as shown in Figure 4.6 and 4.7. The results showed that highest yield obtained at temperature 35^{0}C and at 72hr of time for the lactic acid production 17.33. Temperature is the most important factor on nutrient utilization and cell viability. Higher temperature stimulated the rapid growth of lactic acid bacteria resulted in a rapid decline in pH, and consequently suppressed the growth of *L. Plantarum*. Although *L.Plantarum* could grow at 45 ^{0}C, the optimum temperature for its growth was observed between 30 and 37 ^{0}C.

In industrial fermentation processes, the operating temperature of the fermenter is often raised to optimum level to increase microbial activity which depends on the characteristics of the microorganism used as well as on the environmental conditions. The results indicated that temperature had a pronounced influence on the growth of these microorganisms during lactic acid fermentation.

Moreover, the temperature affects the rate of biochemical reactions, the activity of extracellular enzymes, the generation time, and the activity of the microorganisms involved. Some literatures reported that the rate of reaction for microorganisms increases with increasing temperature until a limiting maximum temperature is reached (45^0 C), after which the growth rate decreases very rapidly (Peleg, 1996). However, when the temperature of the medium is above or below that required for optimum growth, the microbial activity is substantially reduced and the organisms may eventually die.

The fermentation conditions of various temperature were studied, the pH value of the fermentation broth decreased from 6.0 to values less than 3.08 as fermentation proceeded reaching conditions unfavorable for cell growth.

Lactic acid production in the fermentation broth depended on the prevailing conditions of temperature. For this reason the control of temperature were necessary during lactic acid fermentation.

4.3.2 Effect of various pH on lactic acid production

The effect of pH on optimum Lactic acid production is clearly revealed in Figure 4.8 and 4.11. The optimum pH for lactic acid fermentation using Lactobacillus Planatrum A145 is 6.5. Increasing pH beyond these values does not result in any increase of lactic acid yield. The bacteria, Lactobacillus Planatrum seems to grow well in neutral environment with a pH in the region of 5.5 to 7.5, but best at pH 6.5. An environment, which is too acidic and alkaline, is not conducive for lactic acid production.

CHAPTER FIVE

V. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The central composite design was proved to be a useful and applicable tool for determining the behavior of the variables studied in the production of lactic acid. The Lactobacillus Plantarium strain using MRS agar was used to achieve significant results regarding lactic acid production. Under optimal conditions a lactic acid concentration of 17.33 g/l was obtained.

This study was carried out in order to use bulla starch as carbon source and Lactobacillus Plantarium bacterial strain for the production of lactic acid under different factors such as temperature, time and pH of initial fermentation samples. Fermentation was carried out in 250ml glass flask with in aerobically.

This was done because of the nature of Lactobacillus Plantarum. Lactobacillus Plantarum can act as both home fermentative and hetero fermentative bacteria. Since the objective of the study was the production of lactic acid from bulla starch and the bacterial strain in an aerobic condition will convert the carbon source to lactic acid only.

Lactic acid obtained by biotechnological process is preferred for industrial applications, especially, bioplastic industry. Lactic acid bacteria are good organisms for lactic acid fermentation. *L. Plantarium* is a homo-fermentative LAB used extensively in this study. It exhibited more than 90 % lactic acid production yield which is desirable for industrial application. The intention of the present investigation was to study growth and lactic acid production at different environments, temperatures, and pH values as well as media compositions, etc

It was observed from the laboratory works the three factors (temperature, time and pH) greatly affect the fermentation of the starch to the response (lactic acid) formation and concentration.

5.2. Recommendation

Further study should be conducted with different factors in combination or separately as they affect production and purification of lactic acid. The parameters which were taken in this study as constant such as carbon source, agitation speed, nitrogen source, volume of fermenter and others can affect the production of lactic acid. Optimization and scale up of the product should be studied.

For purification of lactic acid, different techniques had been introduced; such as, solvent extraction, adsorption, direct distillation, electro dialysis, electrode ionization and esterification. However, such purification procedures were difficult because of the low volatility of lactic acid (122 °C at 166.73 Pa), with its affinity to water, and its tendency to self-polymerize.

Once we obtained pure lactic acid, it can be then easier to polymerize in to PLA (poly lactic acid), which is now a days considered as backbone of packaging industry.

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