Characterization and Optimization of Lactic Acid Produced from Sugar Cane Molasses by Using Lactobacillus Plantarium Bacteria Isolated from "Kocho"

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A Thesis Submitted to
The School of Chemical and Bio-Engineering

Presented in partial Fulfillment of the Requirements for the Degree of Master of Science in process Engineering

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Addis Ababa University
Addis Ababa, Ethiopia
June, 2018

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This is to certify that the thesis prepared by Nesriya Jemal entitled: *Optimazation and Caharacterazation of Lactic Acid Produced from Sugar Cane Molasses by Using Lactobacillus Plantarium Bacteria Isolated from "Kocho"* and submitted in partial fulfillment of the requirements for the Dgree of Master of Science in process Engineering, complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.

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ABSTRACT

Production of lactic acid through fermentation of molasses using *Lactobacillus Plantarium* was investigated in this research. Fermentation was carried at different pH, temperature and incubation time. The fermentation were performed in 250 ml Erlenmeyer flasks containing 100 ml of MRS medium. Temperatures of 30^{o}C, 40^{o}C, and 50^{o}C; pH of 4, 6, and 8; with incubation time of 10-Hr, 20-Hr, and 30-Hr were used .Three level with three factor full factorial was applied for experimental design and statistical analysis using Design-expert 7.0.0.Agitation speed and carbon source were set as constant factors. A total of 54 experiments were conducted at conditions of previously specified incubation time, pH and temperature. Glucose concentration and the total amount of lactic acid produced were analysed by using Spectrophotometer method .The analysis of experimental results and the interaction effect were studied and the optimum yield of lactic acid production was found to be 17.407g/L at temperature, 40^{o}C, pH 6 and incubation time 30-Hr.Generally, the experimental results revealed that the incubation time, pH and temperature were the most important factors influencing production of lactic acid.

Keyword: Fermentation, Lactic Acid, Molasses, *Lactobacillus Plantarium*.

ACKNOWLEDGMENTS

This project wouldn't have been successfully come to an end without the kind support and guidance of many great people around. I would like to express my gratitude towards my adviser Dr. Zebene Kifile and the member of AAiT community for their kind cooperation and continuous assistance as well as for providing me necessary information regarding the project.

My special thanks and appreciation also goes to all AAiT Lab assistance for being so kind and helpful throughout my project preparation. I am very grateful for my parents, the most important people in my life who are the reason why I worked so hard for this project. I would like to thank my family for making me a strong and a responsible person.

They say "A friend in need is a friend in deed". It was such a blessing and an honor for being surrounded by amazing friends who were there for me always. I would like to take this opportunity to thank all of my friends specially Ato Edris Ali and Ato Fantahun Tarekegni for encouraging me, helping me and giving me an honest opinion whenever I needed.

DECLARATION

I the undersigned, declare that this thesis is my original work and has not been presented for the award of a degree in any university and all the sources of material used for this thesis have been duly acknowledged.

Nesriya Jemal Sherif .....

Signature

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June, 2018

Table of Contents

LIST OF TABLES

LIST OF FIGURES

List of abbreviations and acronyms

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AHA	Alpha hydroxy acid
Hr	Hour
HDPE	High density poly ethylene
HPLC	High performance liquid chromatography
LA	Lactic Acid
LAB .	Lactic acid bacteria
LDPE	Low density poly ethylene
MRS	De Man Rogosa and Shapes
PLA	Poly lactic acid
RRS	residual reducing sugar
VOC	Volatile organic compound

CHAPTER ONE

1. Introduction

1.1. Background

Lactic acid (IUPAC systematic name: 2-hydroxypropanoic acid), also known as milk acid, is a chemical compound that plays a role in several biochemical processes (Ionescu *et al*., 2008). Historically, it was first discovered in sour milk by Scheele in 1780, who initially considered it a milk component and the first commercial production of lactic acid started in the United States by a microbial process in 1881 (Vijayakumar and Aravindan, 2008).

Currently, it is the most widely utilized organic acid in the food, pharmaceutical, cosmetics and chemical industries (Champomier-Verg *et al*., 2002). More specifically, it can be used as neutralizers, solvents, cleaning agents, slow acid release agents and metal complexing agents. It has also been used in cosmetic industry as pH buffer, antimicrobial, skin rejuvenating and skin lightening (Vickroy, 1985).

Lactic acid can be manufactured by either chemical synthesis or fermentation. Since, it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources, the biotechnological production of lactic acid has received a significant amount of interest recently (Rojan *et al*., 2009).

Biotechnologically, it can be produced using microorganisms. Microorganisms with a capability to produce lactic acid can be divided into two groups namely bacteria and fungi. Most investigations of lactic acid production were carried out with lactic acid bacteria, and filamentous fungi (Zhang *et al*., 2007). There are two types of fermentation for the 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 (Rashid, 2008). However, only the homo-fermentative lactic acid bacteria are of industrial importance for lactic acid manufacture.

Eventhough the biotechnological production of lactic acid is common now a days, production of it from cheap raw materials is necessary. This is because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for its production should have the following characteristics: cheap, low levels of

contaminants, rapid production rate, high yield, little amount of by-product formation, ability to be fermented with some pre-treatment, and year-round availability (Vickroy, 1985).

Based on the above criteria, 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 corn, wheat, potato and cassava (Tonukari, 2004). But, there is also potetially high carbon content inside molasses that will use for lactic acid fermentation.

1.2. Statement of Problem

In the recent decades biotechnological production of lactic acid has gained a prime position in the industries as it is cost effective and eco-friendly. However, the main problem of conventional biotechnological lactic acid production and potentially, one of the most serious obstacles for the future fermentative production of lactic acid to compete with chemical synthesis are its higher production cost due to their utilization of high cost medium and its potential impact on the human food chain due to their utilization of food crops. Therefore, for economical lactic acid production by fermentation, low cost medium for it must be develop as soon as possible. In this case, sugar cane molasses represent a potentially inexpensive for the large-scale production by fermentation due to their huge abundance in many sugar industries as by-product (low price). So that, it is expected that cane molasses which can be available in larg amount at lower costs and riches in simple sugars could be used as a suitable and potential resource in substitution of other starchy agricultural products.

So, such assesments or searching of cheap raw materials are essential for the feasibility of the biotechnological production of lactic acid. Polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. The use of low-cost, non-food materials for lactic acid production appears to be more attractive because they do not have any impact on the human food chain. In addition to that biotechnological production of lactic acid can keep it's prime position over chemical synthesis of lactic acid which is the main cause to enviromental pollution and eco-unfriendly. Because, the manufacturing cost of lactic acid can be significantly reduced if low cost products such as molasses containing fermentable sugars could be used for the production of lactic acid.

The demand for lactic acid is dependent on the development of the manufacturing sector, particularly the pharmaceuticals, food, and soft drinks sub sectors. Ethiopia is costing huge amount money to import lactic acid. The country's requirement of lactic acid is entirely met through import. The quantity and value of lactic acid imported is presented in Table 1.1.

Table1.1 : Import data of lactic acid from 2011-2017.

Year	Qty (Tons)	Value (Birr)
2011	8.20	1,243,590
2012	9	1,500,123
2013	10	2,243,490
2014	11.09	3,432,211
2015	13.66	5,321,123
2017	15.67	8,214,984
2017	16.83	9,321,432

Source :- Ethiopian Revenues and Customs Authority.

1.3. Significance of the Study

The main significant of this study was to find cheap and non-food raw materials for the biotechnological production of lactic acid and to achive sustainable & feasible fermentation process of lactic acid production. Starts from the whole community until many polymer producers and other industrial users of lactic acid are all beneficiary either directly or indirectly due to low production cost of it. On the other hand, Ethiopia is producing more than 300,000 tons of sugarcane molasses. During this process, a large amount of molasses is generated as the by-product, which contains 40 to 60% sucrose, it can be converted to lactic acid by the use of microorganisms, and it will be achieve simultaneous benefits.

1.4. Objectives

1.4.1. General Objective

The main objective of this study was optimizaiton and characterization of lactic acid produced from sugar cane molasses by using lactobacillus plantarium bacteria isolated from "kocho".

1.4.2. Specific Objectives

- To determine simple sugar of cane molasses
- To formulate modify culture media for lactic acid production
- To produced and characterize lactic acid
- To determine optimum parameters of the fermentation processes such as temperature, pH, and incubation time for the maximum lactic acid yields from cane molasses.
- To determine the Total amount of Residual Reduced Sugar After Completion of Fermentation

1.5. Scope of the Study

This work involves investigation of lactic acid production starting from pretreatmetnt of molasses, media formulation, fermentation processes, and partial purification of lactic acid. The ultimate objective of the whole thesis is to improve value of sugar cane molasses for the efficient production of lactic acid by fermentation processes.

CHAPTER TWO

2. Literature Review

2.1. Historical Background

Lactic acid has a long history of uses for fermentation and preservation of human food stuffs. It was first discovered in sour milk by Scheele in 1780, who initially considered it a milk component. In 1857, however, Pasteur discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms. The first commercial production of lactic acid started in the United States by a microbial process in 1881 (Vijayakumar, 2008). Generally, there are a number of discovery related with lactic acid development such as Blondeau in 1847; Liebig in 1947; and Welceneus in 1873 (Holten, 1972). In the USA until 1963, Sterling Chemicals, Inc., was producing lactic acid by a chemical process using petroleum by products by the industrial level. In 1996, Sterling abandoned the lactic acid business, leaving lactic acid production again exclusively to fermentation companies (Severson, 1998).

2.2. Physical and Chemical Properties

Lactic acid (IUPAC systematic name: 2-hydroxypropanoic acid), also known as milk acid, is a chemical compound that plays a role in several biochemical processes. It is a carboxylic acid with a chemical formula of $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (AHA). In solution, it can lose a proton from the acidic group, producing the lactate ion (CH_3CH (OH) COO^-) (Ionescu *et al*., 2008).

Pure anhydrous lactic acid is a white crystalline solid with a low melting point of 53 ^{o}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.

Lactic acid is colorless, sour in taste, odorless and soluble in all proportions in water, alcohol and ether but insoluble in chloroform (Ameen, 2017).

Lactic acid is very corrosive; therefore corrosion resistance material such as high moly date stainless steel, ceramic, porcelain or glass lined vessel must be used for its production (Paturau, 1982). The presence of hydroxyl and carboxyl two functional groups permits a wide variety of chemical reactions for lactic acid. The primary classes of these reactions are oxidation, reduction, condensation and substitutions (Sridhar *et al*., 2012).

Table 2.1: Characteristics of Lactic Acid

Property	Characteristics
Optical activity	Exists as L(+), D(-) and racemic 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

Source: (Martin, 1996)

Lactic acid is a chiral compound with a carbon chain composed of a central (chiral) atom and two terminal carbon atoms (Fig.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.

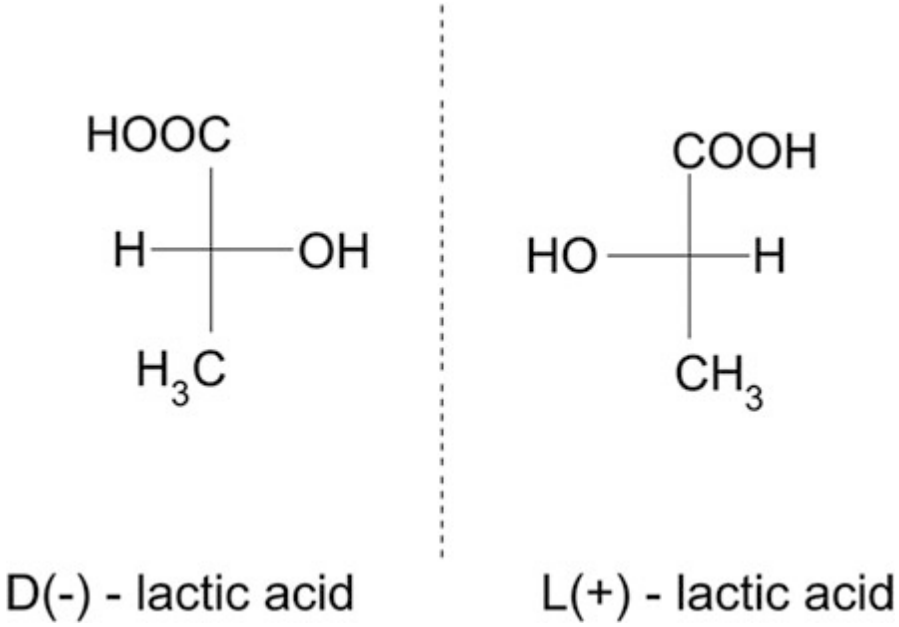


Figure 1.1: Chemical structure of L-(+)-lactic acid and D-(-)-lactic acid

2.2.1. 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 (Benthin and Villadsen, 1995). Some of the important applications of lactic acid are detailed below.

2.2.1.1. Food Industry

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

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

I. Confectionery

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

II. Beer and wine

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

III. Beverages

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

IV. Dairy Products

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

V. Bakery Products

For direct acidification of certain breads, lactic acid is the natural sour dough acid. The general appearance of a loaf of bread is greatly improved by the use of bacterial lactic acid, a larger loaf results per weight of bread with improved bloom, and colour of crust. Lactic acid is directly added to certain types of fermented dough crispy biscuits. Lactic acid added to dough increases the shelf life due to its retarding action on moulds and rope (Ameen, 2017). The sodium and calcium stearoyl acetylates find use as emulsifiers in the baking industry as they provide substantial quality improvement of baked products besides reducing shortening levels. In bakery products it is used for direct acidification of rye or rye-wheat breads. It increases butter stability and volume. Part of the egg albumen can be replaced by less

expensive calcium lactate. A large fraction (w > 50 %) of the lactic acid for food-related uses goes to produce emulsifying agents used in foods, particularly for bakery goods. These emulsifying agents are esters of lactate salts with longer chain fatty acids, and the four important products are calcium and sodium, stearoyl-2-1actylate, glyceryllactostearate and glyceryllactopalmirate. Among, the stearoyllactylates, the calcium salt is a very good dough conditioner, and the sodium salt is both a conditioner and an emulsifier for yeast leavened bakery products. The glycerates and palmitates are used in prepared cake mixes and other bakery products and in liquid shortenings (Goncalves *et al*., 1997).

VI. Meat and Meat Products

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

2.2.1.2. Pharmaceutical

Lactic acid is used in pharmaceutical industry as a very important ingredient. Pharmaceutical and food industries show presence for the L (+) lactic acid because the D (-) isomer is not metabolized by the human body. Lactic acid and its salts have been mentioned for various medical uses. They provide the energy and volume for blood besides regulation of pH. Calcium, sodium, ferrous and other salt of lactic acid are used in pharmaceutical industry in various formulations find use for their anti-tumor activity. Lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations and in tropical wart medications (Vickroy, 1991).

Biodegradable plastic made of poly (lactic acid) is used for suture that does not need to be removed surgically and has been evaluated for use as a biodegradable implant for the repair

of fractures and other injuries. The calcium salts of lactic acid are produced in a granular and powdered form.

Calcium lactate trihydrate is used in pharmaceuticals primarily as a dietary calcium source and also as a blood coagulant for use in the treatment of hemorrhages and to inhibit bleeding during dental operations. Sodium lactate is used in the production of some antibiotics and to buffer pharmaceutical preparations (Boontawan, 2010).

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 (Vickroy, 1991).

2.2.1.3. Polymer Industry

In 1932, Carothers first produced aliphatic polyester of low molecular weight from lactic acid, but it had poor mechanical properties (Holten, 1972). In 1954, Dupont patented the production of a high molecular weight poly lactic acid. However, the development was terminated because of the hydrolytic degradability of the polymer (Lowe, 1954).In 1972; Ethicon produced high-strength co-polymers of lactic and glycolic acids. These polymers are now used as biocompatible fibres in resorb able sutures. They are slowly hydrolysed within the body to the constituent acids. For many years, growth in poly lactic acid production has been inhibited by the high cost of the lactic acid monomer. In late 1980s, new materials for lactic acid production by fermentation introduced lower-cost lactic acid than the petro chemically-derived product. Cargill, Inc. Minneapolis, MN now operates the world's largest poly (lactic acid) facility (Lunt, 1998).

In the United State, Europe and Japan, several companies are actively pursuing development and commercialization of poly (lactic acid) products. PLA polymers can be synthesized from various monomers. Low molecular weight polymers are obtained by step-growth

polymerization of lactic acid. Whereas high molecular weight polymers are synthesized by ring-opening polymerization of lactide.

Lactide is composed of two lactic acid units linked to form a diester cyclic monomer. Step growth polymerization of optically pure L-lactic acid (or pure D-lactic acid) and ring opining polymerization of optically pure L-lactide (or pure D-lactide) should lead to the same chain growth (Ionescu *et al*., 2008).

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

PLA polymers offer a broad balance of functional performance that makes them suitable for a wide variety of market applications. They are expected to compete with hydrocarbon-based thermoplastics on a cost or performance basis. It also exhibits a tensile strength and modulus comparable to some thermoplastics. Like PET (polyethylene terephthalate), these polymers resist grease and oil and offer good flavor and odor barrier. PLA polymers also provide for heat stability at lower temperature than polyolefin sealant resin (Datta and Tsai, 1995). The polymer can be processed by most melt fabrication techniques including thermoforming, sheet and film extrusion, blown film processing, fiber spinning and injection molding. This material biodegrades completely to carbon dioxide and water when composted in municipal or industrial facilities, unlike traditional degradable plastics that need ultraviolet radiation to degrade. PLA needs only water and thus will degrade in the landfills. Biodegradation of PLA proceeds by a two-step process. Initially hydrolysis produces progressive chain length reduction by what is essentially an ester interchange process. This reaction is catalyzed by

heat and pH. There are no bacteria involved in this phase of the process. When the chain length is reduced, producing very low molecular weight fragments, naturally occurring bacteria digest residues and liberate carbon dioxide and water (Lunt, 1998).

Lactic acid has recently received a great deal of attention as a feedstock monomer for the production of poly (lactic acid), which serves as a biodegradable commodity plastic. The optically pure lactic acid can be polymerized into a high molecular mass PLA through the serial reactions of poly condensation, de polymerization, and ring opening polymerization (Vijayakumar, 2008). The resultant polymer, PLA, has numerous uses in a wide range of applications, such as protective clothing, food packaging, mulch film, trash bags, rigid containers, shrink wrap, and short shelf-life trays (Benthin and Villadsen, 1995). Table shows the uses of various potential polymer products of lactic acid.

Table 1.2. Potential product from lactic acid

Product	Uses
Degradable plastics	Packaging ,films
Oxychemicals:	
Propylene glycol	Polymers, food deicers .humectants
acrylates	Polymer, plastics
‘‘green ‘chemicals/solvents:	
easters	Plasticizers, food processing
easter derivative	Packaging
Plant growth regulation	Same as above
Poly-L-lactates	Mulch film for vegetable and fruit crops

2.3. Lactic Acid 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 (Boontawan, 2010).

2.3.1. Lactic Acid Production by Chemical Reaction

Lactic acid can be manufactured by either chemical synthesis or fermentation. The synthetic manufacture of lactic acid on a commercial scale began around 1963 in Japan and United States (Ameen, 2017). Chemical synthesis of lactic acid produces a racemic lactic acid mixture. The commercial process for chemical synthesis is based on lacto nitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lacto nitrile. This reaction occurs in liquid phase at high atmospheric pressures. The crude lacto nitrile is recovered, and purified by distillation. It is then hydrolysed to lactic acid, either by concentrated HCl or by H_2SO4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate before being purified by the means of distillation, and is hydrolysed by water under acid catalyst to produce lactic acid and methanol. The chemical synthesis method produces a racemic mixture DL-lactic acid. This process is represented by the following reactions (Boontawan, 2010).

i. Addition of Hydrogen Cyanide

$$\underset{\text{Acetaldehyde}}{CH_3CHO} + \underset{\text{Hydrogen cyanide}}{HCN} \xrightarrow{\text{Catalyst}} \underset{\text{Lactonitrile}}{CH_3CHOHCN}$$

ii. Hydrolysis by H_2SO_4

$$\underset{\text{Lactonitrile}}{CH3CHOHCN} + H_2O + \tfrac{1}{2}H2SO4 \longrightarrow \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \tfrac{1}{2}\underset{\text{Ammonium salt}}{(NH_4)_2SO_4}$$

iii. Esterification

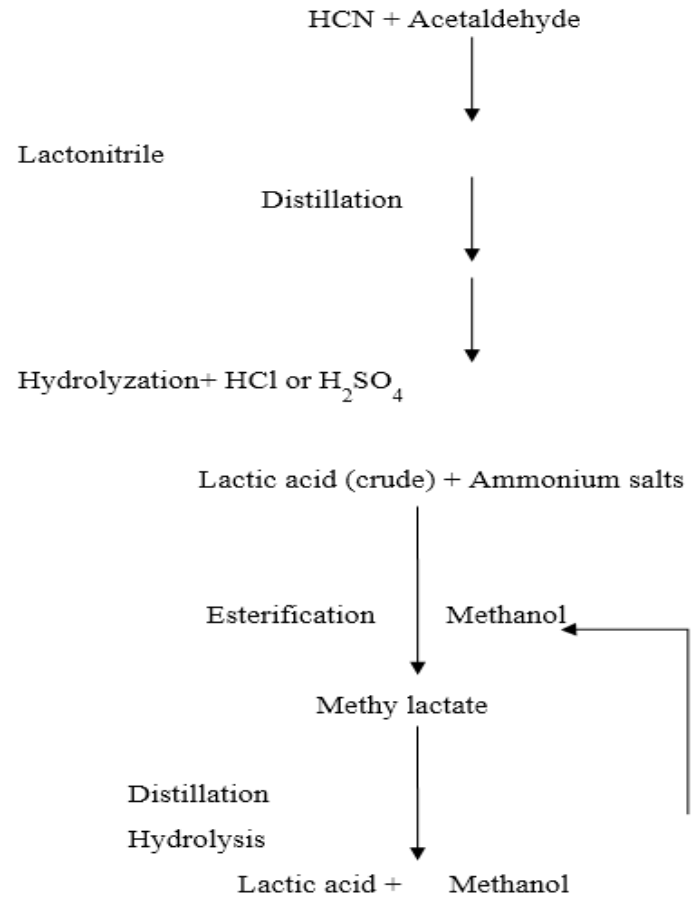
$$\underset{\text{Lactic acid}}{CH_3CHOHCOOH} + \underset{\text{Methanol}}{CH_3OH} \longrightarrow \underset{\text{Methyl lactate}}{CH_3CHOHCOOCH_3} + H_2O$$

iv. Hydrolysis by H_2O

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$

Methyl lactate — Lactic acid — Methanol

Generally lactic acid production by chemical method is summarized as follow.



2.3.2. Fermentation Processes

The mass production of lactic acid by using fermentation became widely used after the discovery of Lactobacillus sp. by the French chemist Loius Pasteur in 1856.Fermentation is a biochemical process in which carbohydrate molecules, e.g. glucose, are converted into energy, lactate, and other by-products depending on the type of microorganism involved in the fermentation process (Ameen, 2017).

Lactobacillus bacteria are able to produce lactic acid from carbohydrates such as glucose and lactose, and they are even living in our gastrointestinal system. Fermentation is a biochemical process in which carbohydrate molecules, e.g. glucose, are converted into energy, lactate, and

other by-products depending on the type of microorganism involved in the fermentation process (Rashid, 2008).

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 (Martin, 1996).

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

$$C_6H_{12}O_6 \longrightarrow 2CH_3CHOHCOOH$$

Glucose Lactic acid

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

$$C_6H_{12}O_6 \longrightarrow CH_3CHOHCOOH + C_2H_5OH + CO_2$$

Glucose Lactic acid + Ethanol + Carbon dioxide

Other fermentation types can occur depending on the fermentation raw materials and conditions (Rashid, 2008).

2.3.2.1. Fermentation through Lactic Acid Bacteria

Microorganisms with a capability to produce lactic acid can be divided into two groups namely bacteria and fungi. There are large number of species of bacteria and some species of moulds that possess the ability to form relatively significant quantities of lactic acid from carbohydrates. 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 (Holzapfel, 1995). 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 (Harvey, 1984)

Only the homo-fermentative lactic acid bacteria are of industrial importance for lactic acid manufacture. Homo-fermentative L (+) lactic acid producers are required if the lactic acid produced will be used as a feedstock for manufacture of 100% biodegradable plastics 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 (Benthin and Villadsen, 1995).

Next to the *Pediococcus*and lastly the homo-fermenters of the *Lactobacillus* species, which produce the most acid, follow the hetero-fermentative species of *Lactobacillus*, which produce intermediate amounts of acid. Homo-fermenters, convert sugars primarily to lactic acid, while hetero-fermenters produce about 50% lactic acid plus 25 % acetic acid and ethyl alcohol and 25% carbon dioxide. These other compounds are important as they impart particular tastes and aromas to the final product. (Vickroy, 1991).

Table 2.3. The fermentation types and products of lactic acid bacteria

Genus	**Fermentation type**	**Main product**	**Isomer**
Leuconostoc	hetrofermentative	Lactic acid(1) Acetic acid (1) CO_2 (1)	D(-)
Bifidobacteria	heterofermentative	Lactic acid (1) Acetic acid (1.5)	L(+)
Lactobacillus	hetrofermentative (pentose substrate)	Lactic acid (1) Acetic acid (1)	L(+), D(-), and DL
Lactobacillus	hemofermentative	Lactic acid(2)	L(+), D(-), and DL
Pedicoccus	homofermentative	Lactic acid(2)	DL and L(+)
Streptococcus	homofermentative	Lactic acid	(L+)

1) The number of moles the product when one mole of dextrose (glucose) is fermented.

{Source: (Kandler, 1983)}

2.3.2.2. Fermentation via Lactobacillus Bacteria

The lactic acid bacteria are a group of Gram positive bacteria. Other main characteristics include non-respiring, non-spore forming, cocci or rods, and produce lactic acid as the major end product from the fermentation of carbohydrates. They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, all fermented milks, cassava (to produce *gari*and*fufu*), and most fermented vegetables (Boontawan, 2010). There are numerous species of bacteria and fungi that are capable to producing relatively large amount of lactic acid from carbohydrates (Atkinson, 1991). However in industrial fermentation the use of various species of *Lactobacillus* is preferred because of their higher conversion, yield and rate of metabolism (Mercier, 1991).

Lactobacillus is more suited to grow in plant extracts .They are often found in carbohydrate containing substrates such as plants and materials of plant origin (Hammes and Whiley, 1993). It is believed that homo-fermentative *Lactobacillus* cultures are the most important commercial species for lactic acid production by fermentation (Vickroy, 1985)*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.

Table 2.4. Major and secondary products of Lactobacillus (L.) species

Species	Substrate	Major product	Secondary product
L. bulgaricus	Lactose	D(-)lactic acid	Acetaldehayed, aceton Diacetyle, ethanol
L. helveticus	Lactose	DL-Lactic Acid	Acetaldehyde, Acetic acid, Acetone,Diacetyle,ethanol
L. lactis	Lactose	D(-) Lactic acid	Acetaldehyde, Acetone, Diacetyle, Ethanol
L. acidophilus	Glucose	DL-Lactic	Acetaldehyde, , Ethanol

		Acid	
L. casei	Lactose	L(+) lactic acid	Acetic acid, Ethanol

The selection of an organism depends primarily on the carbohydrate to be fermented.

{Source: (Martin, 1996)}

2.4. Fermentation Mode

Fermentation is defined as an energy yielding process whereby organic molecules serve as both electron donors and electron acceptors. The molecule being metabolized does not have all its potential energy extracted from it. Hence, lactic acid bacteria are widely used as a low cost method for food preservation by fermentation and generally no or little heat is required during the fermentation.Lactic acid is most commonly produced in the batch mode but numerous examples of continuous culture exist as well as some fed batch and semi continuous/ repeated batch fermentations. When comparing batch and continuous fermentation modes, the former gave higher lactic acid concentration and yield in most of the studies. This is mainly due to that all substrate is used in the batch mode, whereas a residual concentration remains in the continuous one (Goksungu and Guvenc, 1997).

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 (Hofvendahl and Hahn-Hägerdal, 2000).

2.4.1. Batch Fermentation

Batch fermentation has been used to produce lactic add since production first tiegan in the 1890's (Thongwai, 1999). The major disadvantage of batch fermentation is that lactic acid concentration and productivity decrease due to inhibition of high substrate concentration. On the other hand, as the time goes by the concentration of lactic acid are increased but it can inhibit cell growth and product formation.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 (Thongwai, 1999). 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 (Neysens and Vuyst, 1991).

However, the total amount of lactic acid produced from batch fermentation can be depend on the type of raw material used.Literature reviews of lactic acid production from various materials shown in Table 2.5.

Table 2.5. Lactic acid fermentation from agricultural resources by batch fermetation

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

{Source: (Wee *et al.*, 2004)}

2.4.2. 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 (Holten, 1972). 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 (Ricica, 1996). 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 (Mehaia and Cheryan, 1987).

Batch, fed-batch, and continuous fermentations are the most frequently used methods for lactic acid production. Higher lactic acid concentrations may be obtained in batch and fed-batch fermentation than in continuous fermentation.The lactic acid production was found to depend on the choice of carbon substrate (Goksungu and Guvenc, 1997). 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. Several modifications have been done on the basic continuous process to increase the volumetric productivity such as the coupling of the fermentation unit with electro dialysis unit, ion-exchange unit, extraction unit or adsorption unit (John *et al*., (2007)).

2.5. Nutrient Requirement for Lactic Acid Fermentation

All lactic acid bacteria require a source of nutrients for metabolism. The fermentative bacteria require carbohydrates, either simple sugar such as glucose and fructose or complex carbohydrates such as starch or cellulose. The energy requirements of lactic acid bacteria are very high. Limiting amount of available substrate can stop their growth. It is necessary to supplement the fermentation media with sufficient nutrients for rapid lactic acid production. If small amounts of other nutrients were supplemented to the process, then the efficiencies of lactic acid fermentation would be improved significantly (Boontawan, (2010)).

2.5.1. Carbon Sources

The biotechnological production of lactic acid from cheap raw materials is necessary. This is because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. Raw materials for lactic acid production should have the

following characteristics: cheap, low levels of contaminants, rapid production rate, high yield, little amount of by-product formation, ability to be fermented with some pre-treatment, and year-round availability (Vickroy, 1985).

However, this is still economically unfavourable because the refined carbohydrates are too expensive that they eventually result in higher production costs. Therefore, there are attempts to select cheap raw materials for the economical lactic acid production. Cheap raw materials, such as wheat starch, cellulosic materials, whey, and molasses, have been used for lactic acid production. Among these, starch and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable. Consequently, inexpensive starch, starch derivatives or starch containing waste materials as substrate would offer great advantages when combine with minimal pre-processing and supplementation (Wee et al., 2004).

2.5.1.1. Alternative Carbon Sources

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 hydrolysed starch, has been the most commonly used substrates since the 50's (Vickroy, 1985). 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 hydrolysed 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 (Ricica, 1996).

Table 2.6. Summarizes the substrates for lactic acid fermentation

Prinicipal substrate	Source
Lactose	Casein whey Cheese whey Sweet whey
Glucose	corn
Sucrose	Molasses Cane sugar Beet sugar
Other	Potatoes Cellulose Sorghum extract

{ Source: ((Martin, 1996)}

2.5.1.2. Nitrogen Sources

Biotechnological production of lactic acid on either glucose or a lactose based medium requires supplementation, for example, yeast extracts (Hujanen and Linko, 1994). Supplementation with yeast extract had a significant effect on lactic acid concentration, volumetric productivity, and substrate conversion. The most common and effective supplement therefore seems to be 10 g/l of yeast extract (Aeschimann and Stockar, 1990). When a yeast cell is inactivated, a natural digestion process called "autolysis" starts. During this process the yeast's own enzymes breakdown proteins and other parts of the cell. This causes the release of peptides, amino acids, vitamins and other yeast cell components which, once the insoluble components have been removed, is called "Yeast Extract". Yeast Extract is rich in nitrogen, vitamins and other nitrogenous growth factor stimulating compounds, and therefore is used as an ingredient in media for the cultivation of microorganisms. Moreover, all published reports have shown that lactic acid production increases with the concentration of the supplement (especially yeast extract) (Lund et al., 1992). The typical composition of yeast extract is 8 -12% of total nitrogen content (organic and inorganic compound), 50-75% of protein content, 3-5.2% of fermentable nitrogen content, 4-13% of total carbohydrate content, and very little of lipid content, respectively (Boontawan, 2010).

In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost (Rivas, 2004). Most studies reported on lactic acid production by lactic acid bacteria were performed in media containing expensive nutrients such as yeast extract and peptone (Mercier, 1992). In this context, the search for alternative, low cost media for lactic acid fermentation has an obvious economic interest. However, the development of an alternative nitrogen source is a prerequisite for the economical production of lactic acid, because yeast extract is a relatively expensive nitrogen source for industrials use. Therefore, it is essential to develop a more economical method for lactic acid fermentation, using materials as a cheaper nitrogen source (Goksungu and Guvenc, 1997).

2.6. 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 (Porro et al., 1999).

2.6.1. 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 (Tango and Ghaly, 1999).

2.6.2. 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 (Wee et al., 2004). It eventually influences the trans-membrane pH gradient and decreases the amount of energy available for cell growth (Goncalves *et al*., 1997).

The inhibitions of growth in Lactobacillus acidophilus were investigated, by acidification of cytoplasm via the acid produced, below an organism-specific threshold pH level of 4.4 (Kashket, 1987) .However, the mechanism for lactic acid inhibition of growth isn't 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 (Gatje and Gottschalk, 1991). This causes acidification of the cytoplasm and the collapse of the motive force, resulting in inhibition of nutrient transport (Bender and Marquis, 1987).

Therefore, to alleviate the inhibitory effect of lactic acid during the fermentation, it must be removed selectively in sit 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 electro dialysis 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 (Hano et al., 1993).

2.7. Factors Affecting on Lactic Acid Fermentation

However, there are still several researches that need to be addressed in order to produce lactic acid within the targeted cost, development of high performance lactic acid producing microorganisms and lowering the cost of the raw material. Many factors affected in lactic acid fermentation have been investigated. The optimization of fermentation processes requires profound knowledge of the factors determining microbial metabolism, and the influence of process parameters (Calvel *et al*., 2001). Lactic acid fermentation has been studied since 1935 using different types of microorganism and fermentation operation conditions such as pH, carbon source, temperature, inoculums size, initial substrate conditions and nitrogen source (Hofvendahl and Hahn-Hägerdal, 2000)).

2.7.1. Effect of Temperature

Temperature is one of the most important environment factors that affect lactic acid production. Various researchers have studied the effect of temperature on the lactic acid

production and they found the optimal temperature between 41-45 ^{o}C (Hofvendahl and Hahn-Hägerdal, 2000). Temperature and pH are the key environmental parameters that affect the fermentation process (Yuwono and Kokugan, 2008). Low temperature has been reported to positively influence the outgrowth of contaminating microorganism, thereby influencing the Performance of the lactic acid production were investigated (Neysens and Vuyst, 1991). The temperature giving the highest productivity lowers than the temperature resulting in highest lactic acid mass concentration and yield (Hujanen and Linko, 1994).

2.7.2. 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 optimal pH for lactic acid production varies between 5.0 and 7.0. The previous studies investigated the influence of culture pH on lactic acid fermentation from molasses where lactic acid fermentations were performed on a jar fermentor at 38 °C and pH 5.0-9.0 (Wee et al., 2004).

Maximum lactic acid concentration was attained at initial pH6.5 and further increase in initial pH beyond 6.5 does not improve the lactic acid production (Rashid, 2008). It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities (Vijayakumar and Aravindan, 2008). (Busairi, 2002). Also reported that lower production rate 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.

2.7.3. 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, glucose, sucrose, fructose and lactose. If these substrates contain high level of metal ions they must be removed prior to production (Wee et al., 2004). The purest product is obtained when a pure sugar is fermented, resulting in lower purification costs. However, this is economically unfavorable, because pure sugars are expensive and lactic acid is a cheap product (Vickroy, 1991).

2.7.4. Effect of Incubation Period

Previous reported represented that an increase in lactose utilization and subsequent lactic acid production was found incubation time up to 36 h and thereafter no improvement in both the functions was observed (Panesar et al., 2010). This could be attributed to the growth of the culture reached to the stationary phase and as a consequence of metabolism, microorganisms continuously change the characteristics of the medium and the environment. The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures (Gandhi *et al*., 2000).

In addition, the different optimal conditions reported by various 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 (Holzapfel, 1995); (Atkinson, 1991); and (Bender and Marquis, 1987).

2.7.5. 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 (Lund et al., 1992). Lactic acid increases with the increasing concentration of N_2 (Goncalves *et al*., 1997).

The addition of nutrients and higher nutrient concentrations generally had a positive effect on the lactic acid production. MRS medium, which contains yeast extract, peptone and meat extract was superior to yeast extract, which in turn was better than malt extract (Gandhi *et al*., 2000). This reflects the complex nutrient demands of lactic acid bacteria, being fastidious because of limited biosynthesis capacity. Yeast extract alone at high concentration gave higher lactic acid production than yeast extract and peptone in low amounts, but the opposite resulted when the concentration of yeast extract was kept constant and peptone was added (Gao *et al*., 2011).

2.7.6. 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^{o}C and agitation speed of 150 rpm, which was in accordance with a previous report (Hofvendahl and Hahn-Hägerdal, 2000), the optimal condition for lactic acid is pH 5.0-6.8, temperature 30-45^{0}c with continuously agitating at 100-200 rpm (Timbuntam, 2008).

2.8. Molasses

2.8.1. Types of Molasses

The term sugar cane molasses referred specifically to the final effluent obtained in the preparation of sucrose by repeated evaporation, crystallization and centrifugation of juices from sugar cane and from sugar beets (Hubert, 1963). Molasses is highly valued as an animal feed owing to its many beneficial properties. It is used to enhance and regularize the taste of mixed feeds and roughage, resulting in increased feed intake. The use of molasses requires little in the way of investment in technical infrastructure, particularly in the silage sector, where effective use can be achieved with very simple means. Molasses is also widely used in the fermentation industry, as it provides a good substrate for a range of fermentation technologies (www.liquid-energy.ch, 2018).

Generally, the Association of American Feed Control official describes the following types of molasses

i. **Cane Molasses** is a by-product of the manufacture or refining of sucrose from sugar cane. It must not contain less than 46% total sugars expressed as invert. If its moisture content exceeds 27%, its density determined by double dilution must not be less than 79.5^{0} Brix.
ii. **Beet Molasses** is a by-product of the manufacture of sucrose from sugar beets. It must contain not less than 48% total sugars expressed as invert and its density determined by double dilution must not be less than 79.5^{0}Brix.

iii. **Citrus Molasses** is the partially dehydrated juices obtained from the manufacture of dried citrus pulp. It must contain not less than 45% total sugars expressed as invert and its density determined by double dilution must not be less than 71.0^0Brix.

iv. **Hemicelluloses Extract** is a by-product of the manufacture of pressed wood. It is the concentrated soluble material obtained from the treatment of wood at elevated temperature and pressure without use of acids, alkalis, or salts. It contains pentose and hexose sugars, and has a total carbohydrate content of not less than 55%.

v. **Starch Molasses** is a by-product of dextrose manufacture from starch derived from corn or grain sorghums where the starch is hydrolysed by enzymes and/or acid. It must contain not less than43% reducing sugars expressed as dextrose and not less than 50% total sugars expressed as dextrose. It shall contain not less than 73% total solids.

2.8.2. Fermentation of Molasses

Many industrial plants use molasses as a substrate for microbial fermentations.

i. Yeast production

Yeast production: Baker's yeast (active dry yeast) is sold as pressed or dry yeast. It is used as a leavening agent and in bakeries. *Saccharomyces cerevisiaa*is the organism of choice for Baker's yeast. Before yeast fermentation, molasses is clarified by applying heat and subsequently removing sediments. The clear solution is then fed to a fermenter. The molasses is supplemented with certain salts, such as ammonium phosphate, ammonium sulphate, ammonia or magnesium sulphate to meet the requirement of the organisms (Hubert, 1963).

ii. Alcohol production

Ethyl alcohol fermentation is one of the oldest industrial uses for molasses. *Saccharomyces cerevisiaa* is the organism used. Inverted sugar (normally glucose) is converted into ethyl alcohol and carbon dioxide. Some fuel oil a mixture of higher boiling alcohols mainly amyl, isoamyl, isobutyl and normal propyl alcohol is formed during fermentation, approximately 0.1-0.5% by volume to the ethanol volume (Thongwai, 1999). Vinasse is the fermentation residue from molasses after alcohol distillation. It can be used as fertilizer and glycerol production (Olbrich, 1970).

iii. Organic acid production

There are many organic acids that are or can be produced by fermentation from molasses. Acetic acid, citric acid, glutamic acid, it aconic acid, aconitic acid, fumaric acid, malic acid and lactic acid are some examples (Thongwai, 1999).

CHAPTER THREE

3. Materials and Methods

3.1. Materials

Cane molasses, which is a by-product of the sugar industry, was collected from Wenji sugar factory located 98.3 km away from Addis Ababa and Lact. Plantarium bacteria was collected from Addis Ababa University; college of Natural and Computational Sciences , Microbiology Departmetnt which was isolated from enset plant for PhD dissertation.

MRS agar, Sodium acetate and magnesium sulphate were obtained from Neway P.L.C. On the other hand beef extract, yeast extract, dextrose, ammonium citrate and dipotassium sulphate were obtained from Atomic Educational Material Supply P.L.C. and the others (peptone, polysorbate and manganese sulphate) were obtained from Micron International Trading House P.L.C. All the chemicals and reagents used for analysis were analytical grade.

The instruments and various equipments needed included, UV-Spectrophotometer, incubator shaker, oven, digital balance, water bath, pH meter, autoclave, etc. was done at the experimental work .Characterization of molasses (such as moisture content, ash content, amount of reducing sugar and total amount of sugar), hydrolysis of molasses, fermentation process and characterization of lactic acid were conducted in the Food engineering, Environmental engineering, Analytical, Chemistry, Reaction, and Biochemical engineering laboratories in the School of Chemical and Bio Engineering of Addis Ababa Institute of Technology (AAiT), Addis Ababa University.

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3.2. Methods

3.2.1. Pretreatment of Cane Molasses by Acid Hydrolysis

Cane molasses, which was collected from Wenji sugar factory, was mixed with an equal quantity of double-glass distilled water (1:1) and hydrolyzed by adding 1 ml of 20% H_2SO4 in 100 ml of molasses solution. The acidified molasses solution was heated in a boiling water bath for 20 min. The clear supernatant molasses was diluted to 48% sugar level. Then, pH of the medium was adjusted to 6.5 with 4.0 M NaOH. Prior to sterilization and was left to stand overnight for clarification. The clear supernatant liquid was treated with activated charcoal (1:1) for 2 h in order to reduce its opacity and other interfering compounds.Then centrifuged at 3000 rpm for 15 min. the supernatant was used for fermentation compounds.

Figure 3.1. Treated molasses

3.2.2. Proximate Chemical Composition of Molasses

3.2.2.1. Determination of Total Reducing Sugar of Molasses

Benedict's solution is designed to detect the presence of reducing sugars. In hot alkaline solutions, reducing sugars reduce the blue copper (II) ions to brick red copper (I) oxide precipitate. As the reaction proceeds, the colour of the reaction mixture changes progressively from blue to green, yellow, orange and red. When the conditions are carefully controlled, the colouration developed and the amount of precipitate formed depends upon the amount of reducing sugars present. Hence, in most conditions, a sufficiently good estimation of the concentration of glucose-equivalent reducing sugars present in a sample can be obtained. The concentration of total reduced sugar (TRS) content of hydrolysates which obtained from hydrolysis is determined using digital spectrophotometer by using measuring absorbance v_s

sugar concentration at 540nm wavelength.To do that standard glucose dilution series solution is prepared at different concentration of 0,1,1.23,1.25,1.38 and 1.5g.Then, pipette 0.5ml from each of the dilution series in to labelled test tube, each containing 5ml of Benedict's solution then mix the solution by shaking. The labelled test tubes are heated at 90^{o}c water bath for 5 minutes. Each of the test tube are removed from the water bath and filter using filter paper to remove red precipitate formed when the reduced sugar in the sample reacted with Benedict reagent. After filtered precipitate % absorbance is measured using spectrophotometer at 540nm.Calibration curve is plotted to show the % of absorbance blue light by the standard glucose solution. (Frais.F, 2009)

Figure 3.2. Benedict solution

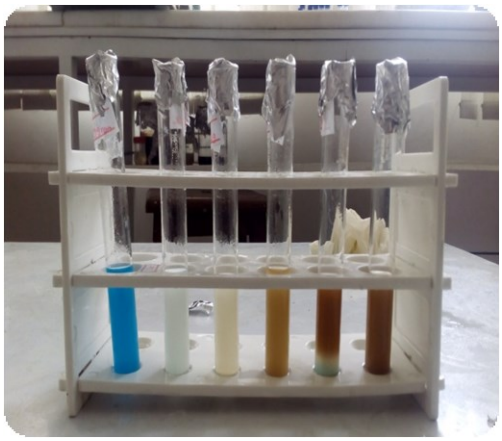

Figure 3.3. Sample tubes after water bath

Total reducing sugar was determined by pipette 0.5ml of each of the sample into labelled test tube each contain 5ml of Benedict's solution. Then solution was mixed by shaking and all the labelled test tubes were heated to 90^{o}c in the water bath. The test tube was removed from the water bath and filtered using filter paper to remove red precipitate formed when the reduced sugar in the sample reacted with Benedict reagent. After filtered precipitate % absorbance is

measured using spectrophotometer at 540nm, the concentration of sugar in each samples is measured are read from the calibration curve of the standard glucose solution.

$$CSRSUS = (absorbance\ of\ unkounsample) - (y - intercept)/slope$$

Where $CSRSUS$=concentration of total reducing sugar of unknown sample

3.2.2.2. Total Sugar Content of Molasses

The total sugar content of molasses which was collected from Wenji Sugar Factory, was read direct from density meter apparatus. Initially, molasses was diluted for 1:1 ratio with distilled water in order to make it readable by the apparatus.

3.2.2.3. Moisture Content

Moisture content measurement was carried out as follow. A sample of 5g is accurately weighed into a dish and dried in an air oven at 105±2^{o}C for about 4 hours. The sample was then cooled in a desiccator and weighted. The process of drying, cooling and weighing was repeated after an hour until two consecutive weighs did not deviate by more than 1 milligram. The moisture content was calculated according to equation below:

$$Moisturecontent = \frac{w_1 - w_2}{w_2 - w} * [100]$$

Where:

w = weight empty dish (g)

w_1 = weight dish and sample before drying (g)

w_2 = weight dish and sample after drying (g)

3.2.2.4. Determination of Total Ash Content of Molasses

About 5 g of sample Weighed accurately in a tared silica / platinum dish Char material carefully on a burner and transfer the dish to a muffle furnace and ash at a temperature of550± 10^0C until the ash is free of Carbon. Heat the dish again at 550 ± 10 0C for 30minutes Cool in a desiccator and weigh. Repeat this process of heating for 30 minutes, cooling in a desiccator 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)

3.2.2.5. Determination of Dry Matter Content of Molasses

Total dry matter was just calculated from moisture content which is already determined before. Based on law of conservation of mass total dry matter can be calculated as follows,

%Total solids = (100 - %Moisture)

3.2.2.6. Determination of Sugar in the Total Solid Content

$$Q = 100 * S/T$$

(Q denotes purity quotient of molasses; S is sugar content; T represents dry substance.)

3.2.3. Measuring pH

An accurate and practical method for measuring pH involves the use of a pH meter. The pH meter is a potentiometer which measures the potential developed between a glass electrode and a reference electrode. To obtain accurate results the pH meter need to be calibrated before using. The calibration is normally performed using a standard pH meter with standard pH 4.00, 7.00 and 10.00 buffers. When using the pH meter, care must be taken to rinse the electrode carefully with the test solution and immersed in the solution to sufficient depth. The pH reading was taken after a minimum five minute.

3.2.4. Medium Formulation for Lactic Acid Fermentation by Lactic Acid Bacteria

The new culture medium was prepared by substituting dextrose (the main ingredient of comertially availabellactobacillus MRS agar) with molasses hydrolysates to got modified medium. so, the treated cane molasses wasmixed with other ingredients of comertially available lactobacillus MRS agar (10g/L of proteose peptone, 10g/Lof beef extract, 5g/L of yeast extract, 1g/L of polysorbate 80, 2g/L of ammonium citrate, 5g/L of sodium acetate, 0.100g/L of magnesium sulphate, 0.050g/L of manganese sulphate, and 2g/L ofdipotassium phosphate (HiMedia Laboratories Pvt. Ltd.Technical, (2015).

3.2.5. Inoculums Preparation

The culture in the petri dish was aseptically inoculated into a 25ml test tube which contains 10ml MRS medium. The biological safety cabinet must be swabbed with disinfectant (alcohol) to reduce the risk of contamination and the work must be accomplished in minimum time to prevent exposure. The loop was flamed and allowed to cool before transferring the bacteria. The mouth of the fermentation mediums was flamed before and after adding the culture. The inoculating loop was re-flamed after completing. The test tube was then incubated in the incubator shaker at 37 ^{o}C, 150 rpm for 24 hours (Sakamoto, M. and Komagata, K, (1996)).

Figure 3.3. Lactobacillus planetarium bacteria colonies grown on MRS agar

3.2.6. Lactic Acid Fermentation Processes

All batch mode fermentations were performed in an incubator shaker with 250 mL flask each . The inoculum size of 10% (v/v) was prepared in MRS medium at 30 °C for 12 h using an incubator at 200 rpms agitation. Then, this 10 % (v/v) inoculum size of lactobacillus lactic were added in to sterilized culture medium that was prepared before. After that, all experiments were performed at corresponding growth temperature and pH of each randomized experimental run order without aeration. The pH was monitored manually by the addition of 2M NaOH and measuring with a pH meter. Then, fermentation were end with their respective incubation time.

Figure 3.4. Fermentation setup (Incubator shaker series)

3.2.7. Design of Fermentation Experiment

Three factors with three level and two replication is selected for this particular experiment. Temprature, pH, and incubation time are factors. 30 °C, 40 °C and 50 °C are three levels for temprature factor. 4, 6 and 8 are three levels of pH factor and 10-hr, 20-hr and 30-hr are three levels for incubation time factor) keeping substrate concentration and agitator speed constant. The substrate concentration was set to 200 RPM Then all of these experiment will performed twice for the sake of certainity. Finally the interaction and main effects of this parameter can be analyse by using Design-Expert.

Table 3.1. *Experimental factors and level*

Factors	Levels		
Temperature (^{0}C)	30	40	50
Ph	4	6	8
incubation time	10	20	30

3.2.8. Fermentation Product Analysis

3.2.8.1. Total Amout of Lactic Acid

An efficient and inexpensive spectrophotometric method had been selected in this research for the determination of lactic acid. The method is based on the spectrophotometric determination of the coloured product of the reaction of lactate ions with iron (III) chloride at 390 nm. At the end of each fermentation, a 15 mL samples were withdrawn under aseptic conditions and centrifuge at 6,000 rpm for 10 min, the supernatant was collected for further Analysis.Then, a test solution (50 µL) containing lactic acid was added to 2 mL of 0.2% solution of iron(III) chloride and stirred and absorbance was measured at 390 nm against the reference solution (2 mL of 0.2% $FeCl_3$ solution). The color of the solution was stable for 15 min (Borshchervskaya *et al.,* (2016)).

Intially, callibration curve from stndard lactic acid was constructed. About 5 g lactic acid with the know concentration (89%, ρ = 1.2 g/mL) was placed in a 10-mL volumetric flask and diluted with water. A stock solution with the x concentration of lactic acid 89 g/L was obtained. A series of lactic acid solutions was prepared from the stock solution using two-fold dilutions. The dependence of the absorbance of coloured solutions on the concentration of lactic acid taken for the reaction was used for the calculation of the parameters of linear equation corresponding to the linearity range of the calibration curve.

Therefore, iron (III) chloride (0.2 g) was placed in a 100-mL volumetric flask and a test solution (50 µL) containing lactic acid was added to 2 mL of a 0.2% solution of iron (III) chloride and stirred. Absorbance was measured at 390 nm against the reference solution (2 mL of a 0.2% $FeCl_3$ solution). The reaction and measurements were performed at 25 ± 5°C and the colour of the solution was stable for 15 min. The concentration of lactic acid in each samples was measured by read from the calibration curve of lactic acid.

3.2.8.2. Residual Reduced Sugar

Residual reduced sugar was determined at the end of each fermentation process.Becuase; it can be good indicator of fermentation efficiency. Sometimes, total amount of reducing sugar will be consumed. However, it doesn't mean necessary converted in to lactic acid; it might be converted to other organic compound by an other microorganisms.

In these case, determination of residual reducing sugar is very importatnt in addtion to determination of total amount of lactic acid produced from fermentation. And it was determine by a method stated by section 3.2.2.1.

CHAPTER FOUR

4. Results and Discussions

4.1. Proximate Compostion of Molasses

As shown by table 4.1 the percentage moisture content, dry matter content, ash content, total amount of reducig sugar, total amount of non-reducing sugar and amount of total sugar were 15.2%, 84.8%, 14.1%, 14.46%, 34% and 48% respectively. According to (Dividich *et al*., 1978), research the percentage moisture content of molasses was determined around 18.5%. There are also some other litrature which have nearly similar result of molasses compostion with these researh work. For example, Letco liquid energy trading company quality control data show that the percentage of total sugar, moisture and ash were 45-58%, 18-25% and 7-15% (www.liquid-energy.ch, 2018). The purity of molases used for this research and pH are 56.6% and 6.7. According to (Hubert, 1963), the purity of molasses and pH are 60% and 6.5.

The percentage composition of reduced sugar in molasses was determined by

Absorbance 1	Absorbance 2	Mean	Concentration (mg/ml)
1.049	1.064	1.0565	2.8625
1.038	1.202	1.12	2.7826

$$\%TRS = \frac{Concentration\ of\ unknown\ sample}{Gram\ of\ sample\ used} * V_{\text{molass}} * 100\%$$

Concentration of molasses=2.8452 g/L

Volume of molases = 0.5 L

Gram of sample used=10g

$\%TRS = \frac{2.8452\ \text{g/l}}{10\text{g}} * 0.5\ \text{l} * 100\% =$ **14.46%**

Table 4.1. Experimentally determined compostion of molasses

Compostions	Percentage
Moisture content	15.2%
Dry matter content	84.8%
Ash content	14.1%
Amount of reducing sugar	14.46%
Amount of non reducing sugar	34%
Amount of total sugar	48%

4.2. The Effect of Different Operation Condition on Lactic Acid Production

4.2.1. The Effect of temperature on Total Amount of Lactic Acid Produced from Molasses by Lactobacillus Plantarum Bacteria

Figure 4.2 presents the effect of temperature on total amount of lactic acid produced from molasses by lac. Plantarum bacteria fermentation. The maximum amount of total amount lactic acid produced during molasses fermentation, at the center point of the other experimental level (pH=6), were 16.062g/L, 17.397g/L, and 13.473 g/L at 30^{o}C, 40 ^{o}C, and 50 ^{o}C respectively. On the other hand, the minimum amount of lactic acid produced were 14.260g/L, 15.403g/L, and 11.591g/L with the respective fermentation temperatures from lower level to higher level. In both case, the results clearly show that the total amount of lactic acid increase with temperature from 30 ^{o}C to 40^{o}C. However,it starts to decrease with temperature from 40 ^{o}C to 50 ^{o}C.These result clearly indicates, the optimum fermentation temperature might be around 40 ^{o}C.According to (Adamberg *et al,* (2003))explanation ATP production capacity was an important factor in determining the maximum growth rate of lactic acid bacteria (LAB). The decrease of YATP with increase of temperature above its optimum value resulted in the decrease of specific growth rate and subsequently results for decrease on total amount of lactic acid.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.

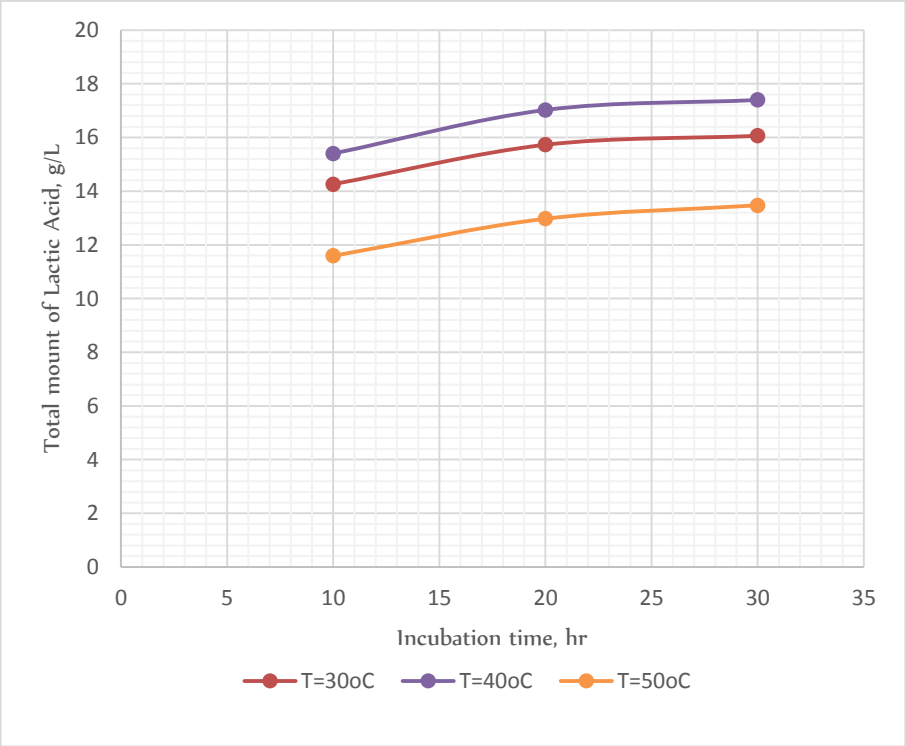

Figure 4.2. The effect of temperature on total amount of lactic acid at pH 6

4.2.2. The Effect ofpH on Total Amount of Lactic Acid Produced from Molasses by Lactobacillus Plantarum Bacteria

Figure 4.3 shows the effect of pH on total amount of lactic acid produced from molasses fermentation by lac. Plantarum bacteria. In these case, the total amount of lactic acid produced, at 40^{o}C of fermentation temperature, were 16.88g/L, 17.401 g/L, and 15.906g/L at pH 4, 6, and 8 respectively. Looking back, the minimum total amount of lactic acid produced were 15.703 g/L, 16.403g/L, and 14.755g/L at the pH 4, 6, and 8 respectively. Which means the optimal pH of fermentation condition during molasses to lactic acid conversion by lac. Plantarum bacteria might be in the range of 6 and 8.The optimum pH for lactic acid fermentation using Lactobacillus Planetarium is 6. Increasing pH beyond these values does not result in any increase of lactic acid yield. An environment, which is too acidic and alkaline, is not conducive for lactic acid production.

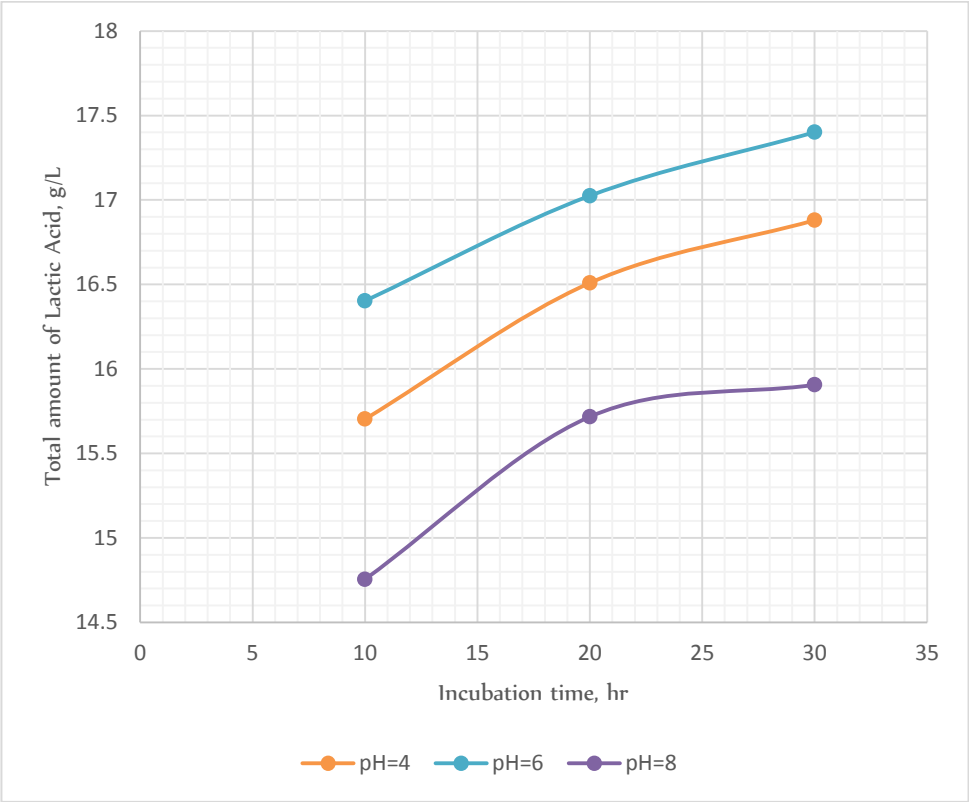

Figure 4.3. The effect of pH on total amount of lactic acid at 40^{o}C

4.2.3. The Effect of Temperature, pH and Incubation Time on the Total Amount of Lactic Acid

In general, figure 4.4 presents the effect of temperature, pH and incubation time on the total amount of lactic acid produced from molasses. This is the overall summery of the result for all 27 samples. The total amount of lactic acid produced from molasses fermentation were 12.472 g/L, 15.603g/L, and 11.074g/L at 30^{o}C, 40, and 50 operating temperature with 4 intial pH and 10-hr incubation time; 14.227g/L, 16.382g/L, and 11.584g/L at 30 ^{o}C, 40 ^{o}C, and 50^{o}C with 6 intial pH and 10-hr incubation time; 11.937g/L, 15.725g/L, and 9.73g/L at 30 ^{o}C, 40^{o}C, and 50 ^{o}C with intial pH of 8 and the same incubation time with before; 14.102g/L, 16.453g/L, and 12.103 at 30 ^{o}C, 40 ^{o}C, and 50 ^{o}C with 20-hr incubation time and 4 intial pH; 15.642g/L, 17.025g/L, and 12.759g/L at usual sequace iof operating temperature with 6 intial pH and 20 hr incuabation time; 13.553g/L, 16.678g/L and 11.755g/L at 30 ^{o}C, 40 ^{o}C, and 50 ^{o}C with intial pH 8 and 20 hr incubation time; 14.372g/L, 16.842g/L, and 12.721 g/L at 30 ^{o}C, 40 ^{o}C, and 50 ^{o}C with pH 4 and 30 hr incubation time; 16.064g/L, 17.384g/L, and 13.473 g/L at 30 ^{o}C, 40 ^{o}C and 50 ^{o}C with intial pH 8 and 30 hr incubation time; and 14.097g/L,

16.905g/L, and 12.063g/L at 30 ^{o}C, 40 ^{o}C, and 50 ^{o}C with intial pH 8 and incubation hour of 30 hr. For each run, all temperature, ph and incubation time significantlly affect the total amount of lactic acid produced from molasses fermentation and it is very important to determine their statistical value of how it affect the response.

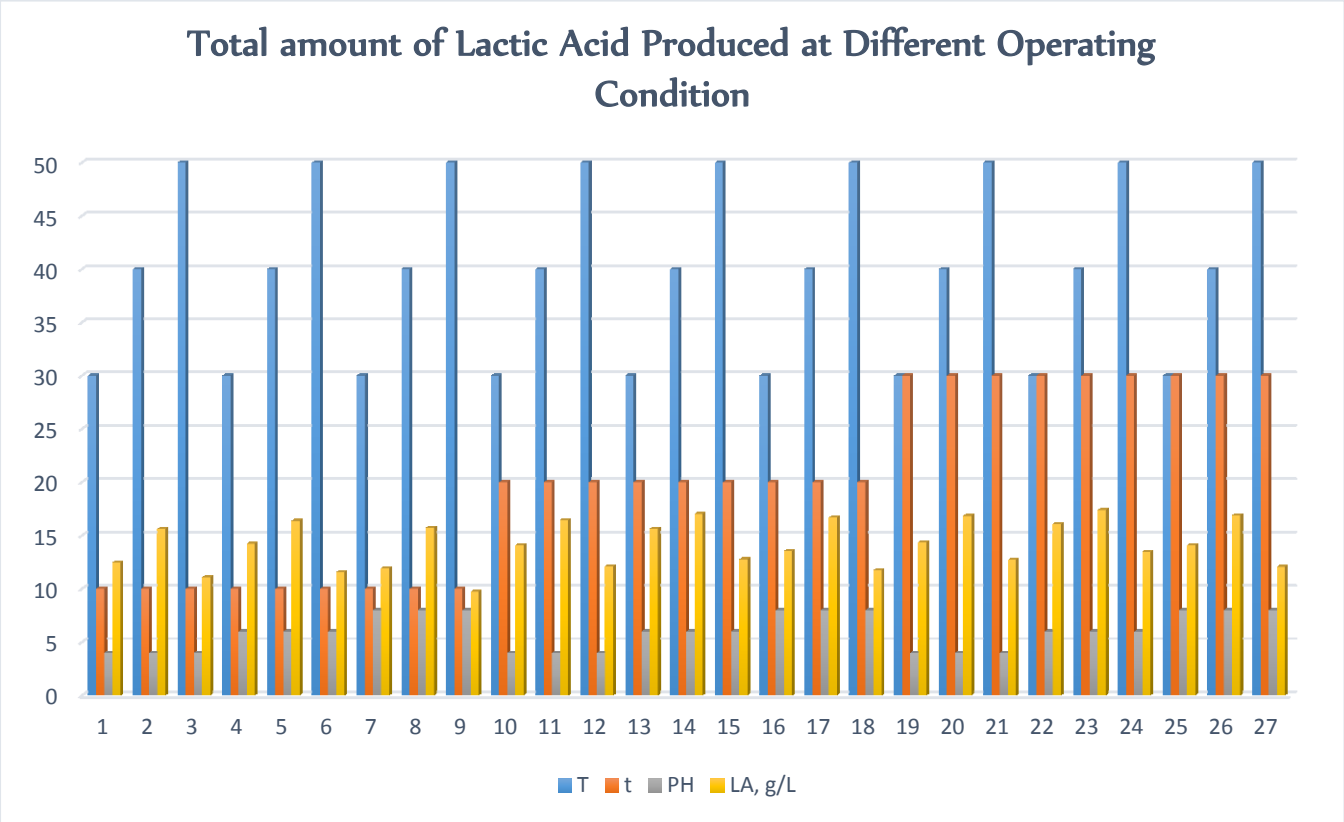

Figure 4.4. Total amount of lactic acid produced at different operating condition

4.2.4. Total amount of Residual Reduced Sugar after Completion of Fermentation

The amount of recedual sugare content at the end of each fermentation condition detrmined by spectrophotometer which was used to read the absorbance at a wavelength of 540 nm and then use the calibration curve of standard glucose is used. The equation $RRS = A - 0.001/0.3151$was used to calculate the residual sugare content. The graphical illustration of RRS variation with time is as shown in Figure 4.5 the plot shows that the RRS -time graph is non-linear. The concentration of RRS decreased between 10 and 30 hr.when the temprature raise by 10^{0}C theconcentration of RRS was 4.826mg/ml at atemprature of 40^{0}C as compared to 5.24mg/ml at atemparature of 30^{0}C for the same fermantation time. at atemprature of 50^{0}C

the concentration of RRS was 5.512 mg/mL in order to get low RRS at the some fermantation time approch the fermentation temprature to 40^0C.

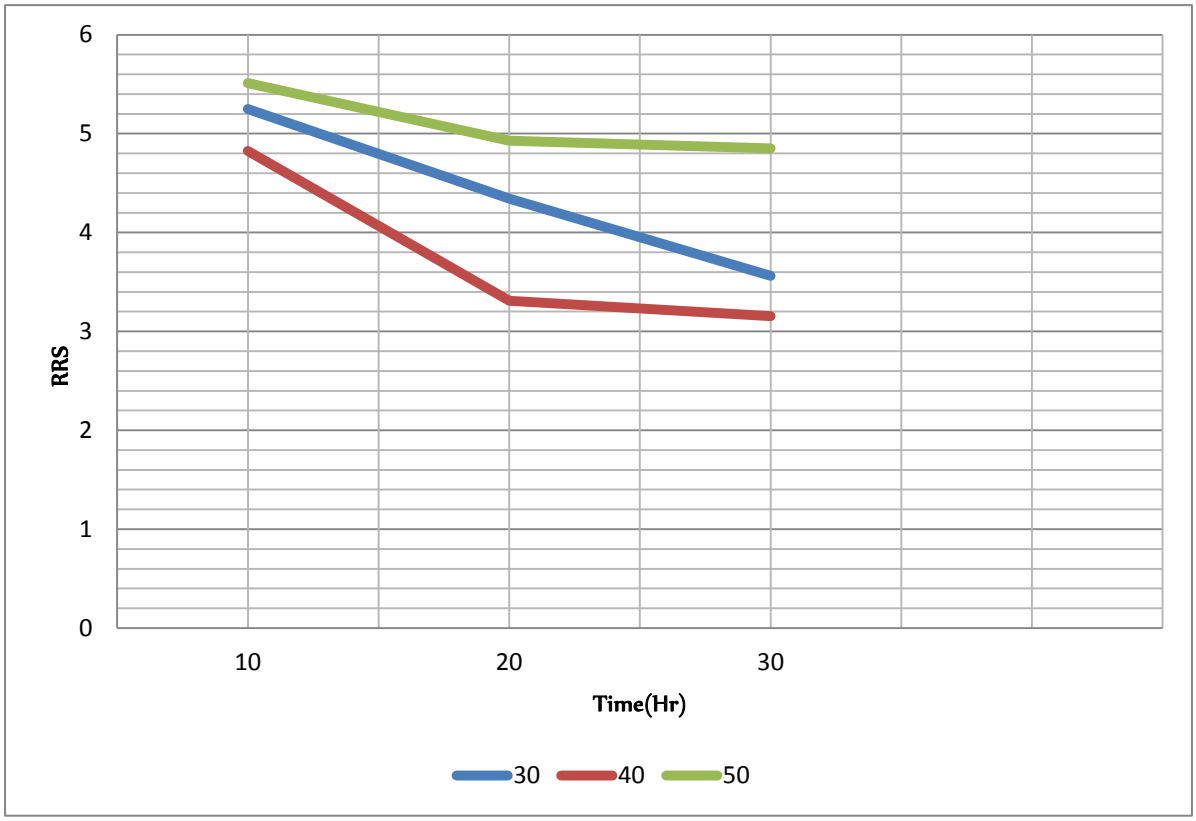

Figure 4.5. Effect of temperature on residual reduced sugar

4.3. Statistical Analysis of the Experimental Results

Appendix A presents experimental results of three factors with three levels and two replicates and the following tables show statistical analysis of the experimental results by using Design-Expert Soft-ware (version 7.0.0).

Table 4.2. Design summary for three variables and three level factorial design

Design Summary	
Study type	Factorial
Initial Design	Full Factorial
Center Points	0
Design Model	Quadratic Polynomial
Experiments	54

Design Summary.....						
Factor	Name	Units	Type	Low Actual	High Actual	
A	Temperature	^{o}C	Numeric	30.00	50.00	
B	pH		Numeric	4.00	8.00	
C	time	Hr.	Numeric	10.00	30.00	
Response	Name	Units	Analysis	Minimum	Maximum	Mean
Y1	Total amount of lactic acid produced	g/L	Polynomial	9.73	17.41	14.20

The ANOVA results from three factorail model equation described in table 4.2. The analysis of variance (ANOVA) was carried out to assess the significance of quadratic polynomial equation fitness for the response variable and independent variables. The model was significant due to high F-Value of 154.18 and low Probability value of 0.0001 for total amount of lactic acid.

Then, a measure of the statistical accuracy of an estimate was done by using Design Expert soft-ware at optimum incubation time. Figure-4.6 shows that the standard error of design or dispersion of sample means around the population mean on 3D-surface.

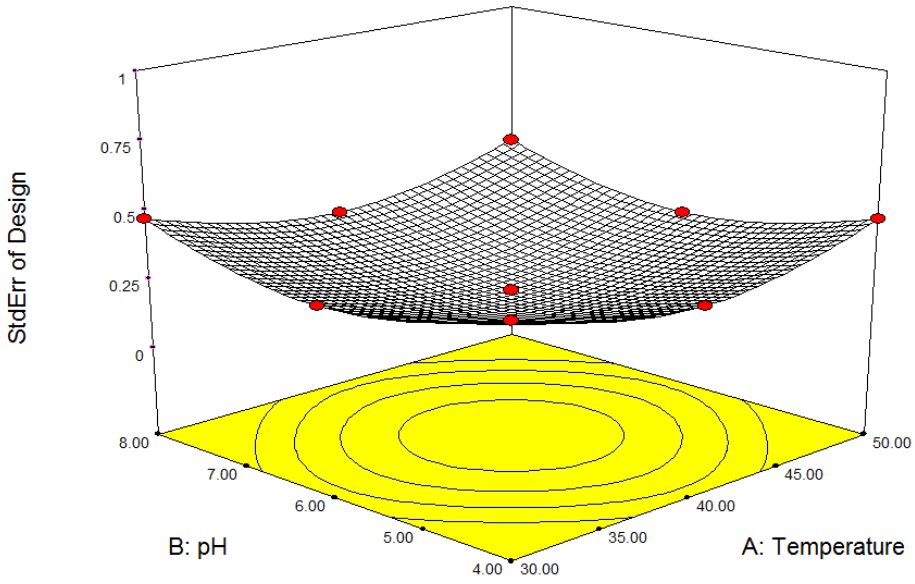

Figure 4.6. Standard error of design

4.3.1. Analysis of Variance (ANOVA) for Quadratic Models of Total Amount of Lactic Acid Prouduced

Table 4.3 shows that all temperature, pH and incubation time were highlly influential process variable for the generation of lactic acid. Whereas, only the interaction effect of intial pH and operating temperature alone was significant interaction. Ingeneral, the significance of the main effects and interaction effects are shown by Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, A^2, B^2, C^2, AC are significant model terms. That means all these variables had more impact on the total amount of lactic acid produced. However, values greater than 0.1000 indicate the model terms are not significant

Table *4*.3. Analysis of Variance (ANOVA) for quadratic models of total amount of lactic acid

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F	
Model	241.21	9	26.80	154.18	< 0.0001	significant
A-Temperature	42.12	1	42.12	242.33	< 0.0001	
B-pH	1.46	1	1.46	8.40	0.0058	
C-time	25.59	1	25.59	147.24	< 0.0001	
AB	0.12	1	0.12	0.72	*0.0094*	
AC	2.183E-003	1	2.183E-003	0.013	0.42	
BC	0.11	1	0.11	0.62	0.4355	
$A^{\wedge 2}$	153.84	1	153.84	885.02	< 0.0001	
$B^{\wedge 2}$	15.88	1	15.88	91.36	< 0.0001	
$C^{\wedge 2}$	2.08	1	2.08	11.97	0.0012	
Residual	7.65	44	0.17			
Lack of Fit	7.17	17	0.42	23.71	< 0.1	not significant
Pure Error	0.48	27	0.018			
Cor Total	248.86	53				

The Model F-value of 154.18 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Finally, by applying multiple regression analysis to the experimental data, the following second degree polynomials was found to represent the relationship between the total amount of lactic acid produced and the most significant process variables.

Final Equation in Terms of Actual Factors:

Total Amount of Lactic Acid Produced =

- 48.96710 + 2.77972* Temperature
+ 3.42753* pH + 0.23459* time
- 3.60146E-003 * Temperature * pH
- 9.53750E-005* Temperature * time
+ 3.34854E-003* pH * time
- 0.035805*Temperature^2-0.28760* pH^2
- 4.16386E-003* $time^{\wedge 2}$

Table 4.4. Adequateness of the model for total amount of lactic acid

Std. Dev.	0.42	R-Squared	0.9693
Mean	14.20	Adj R-Squared	0.9630
C.V. %	2.94	Pred R-Squared	0.9537
PRESS	11.52	Adeq Precision	43.641

The "Pred R-Squared" of 0.9537 is in reasonable agreement with the "Adj R-Squared" of 0.9630. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case a ratio of 43.641 indicates an adequate signal. This model can be used to navigate the design space.

4.3.2. Diagonostics of The Model

The plots as shown in figure 4.7, the residuals follow a normal distribution, the points in the plots follow linearity, this shows that the quadratic polynomial model satisfies the assumptions analysis of variance (ANOVA).

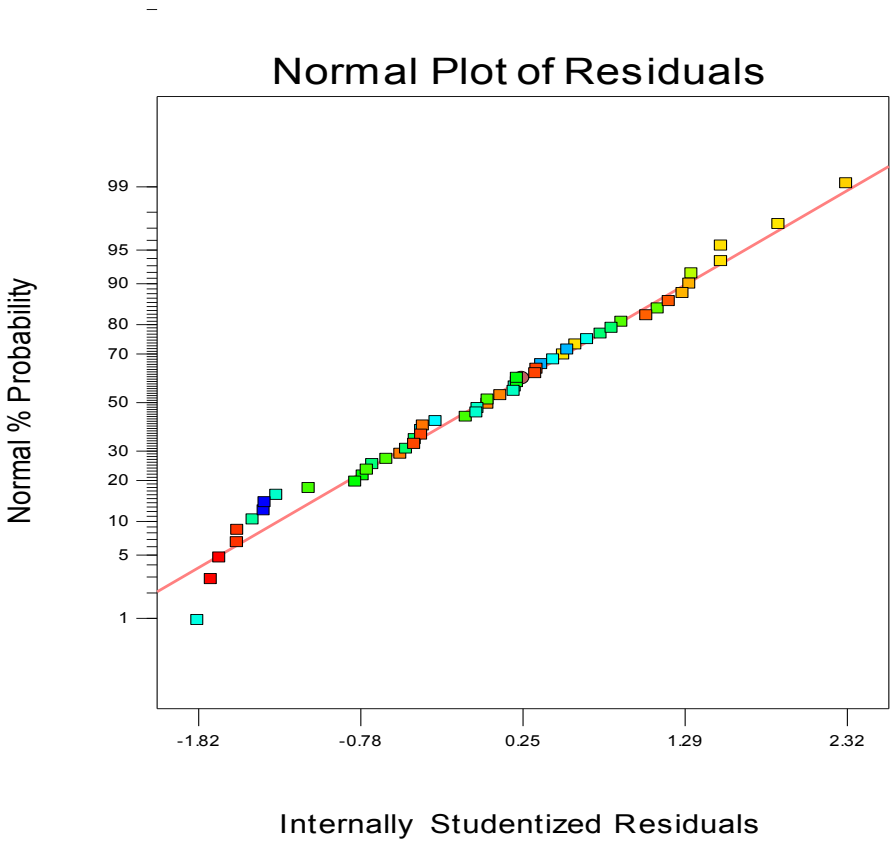

Figure 4.7. Normal plot of residuals

On the other hand, both the plots in figure 4.8 and 4.9 show constant range of residuals across the graph which is justifiable no need for a transformation to minimize personal error.

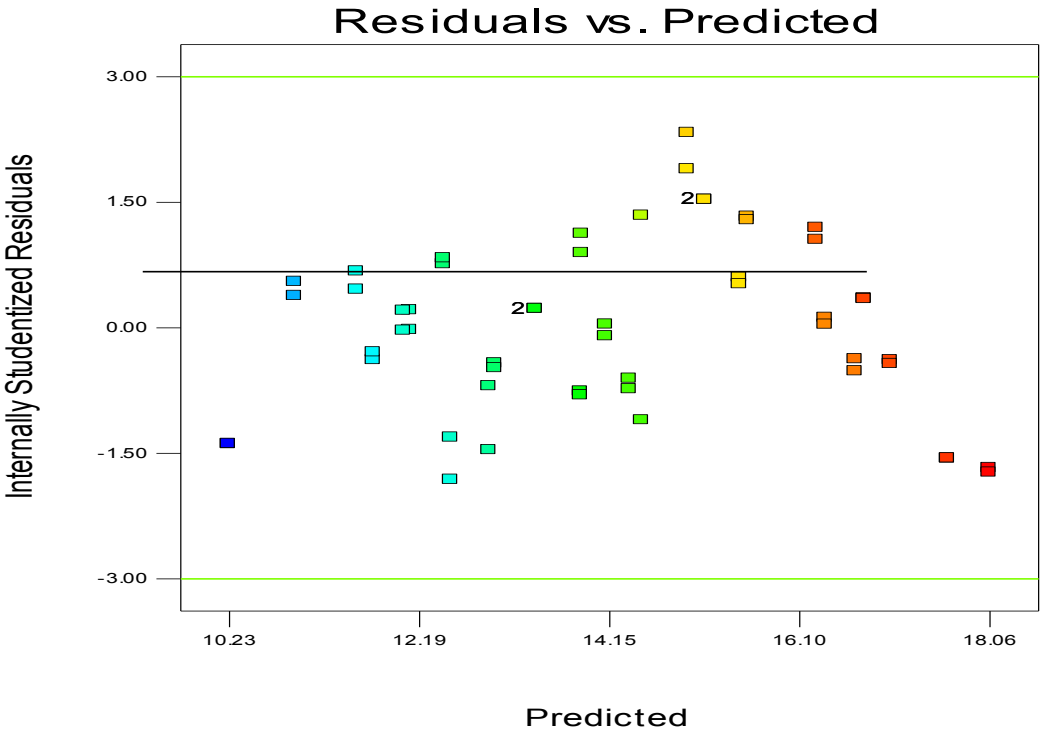

Figure 4.8. Residual vs. predicted plots

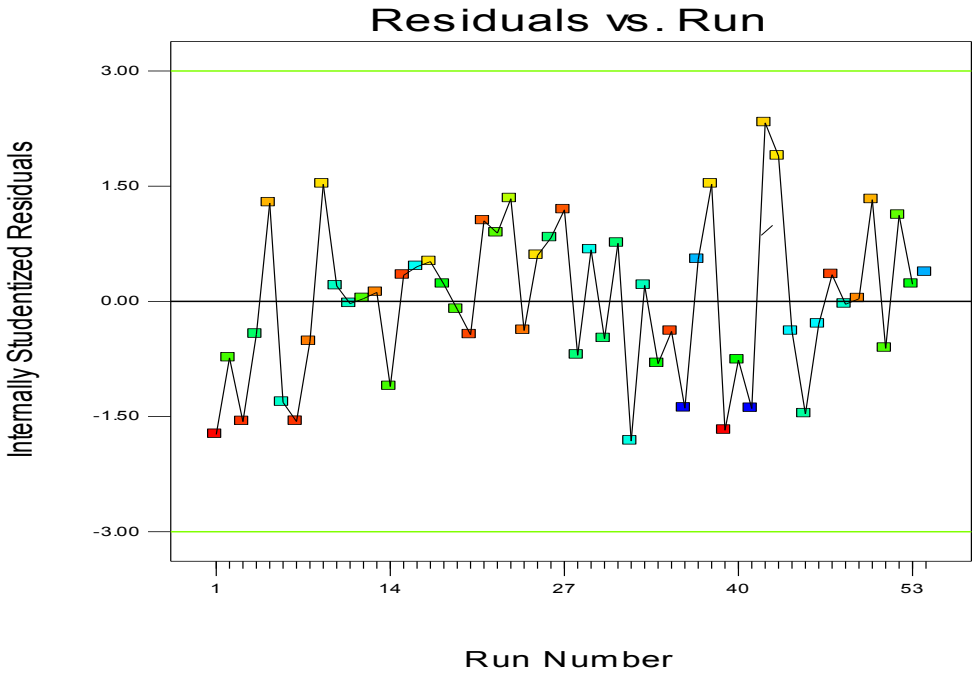

Figure 24.9. Residual vs. run plots

4.3.3. TheInteraction Effect of Experimental Variables on Total Amount of Lactic Acid

The effects of different operating variables on total amount of total lactic acid were obtained by keeping one variable constant at the center point and varying the other variables within experimental range. The resulting response with interaction effect of operating variables were plotted with the following figures. Figure 4.10, 4.11 & 4.12 show the interaction effect of temperature & pH; temperature & incubation time and incubation time & pH on generation of lactic acid at 20 hours incubation time respectively.

Figure 4.10 presents the interaction effect of temperature and pH on total amount of lactic acid with 3D graph. The graph shows that, boththe effect of main parameters and their interactions are highly significant. Because, the response surface is absolutly curved structure and it shows that, the interaction effect was also highly dominant on production of lactic acid. The *P*-value (Table-4.2) also indicated that the interaction effect of pH and temperature is significance. The same results have been observed by (Gorret et al., (2001)); (Hofvendahl and Hahn-Hägerdal, 2000); and (Mercier, 1991).

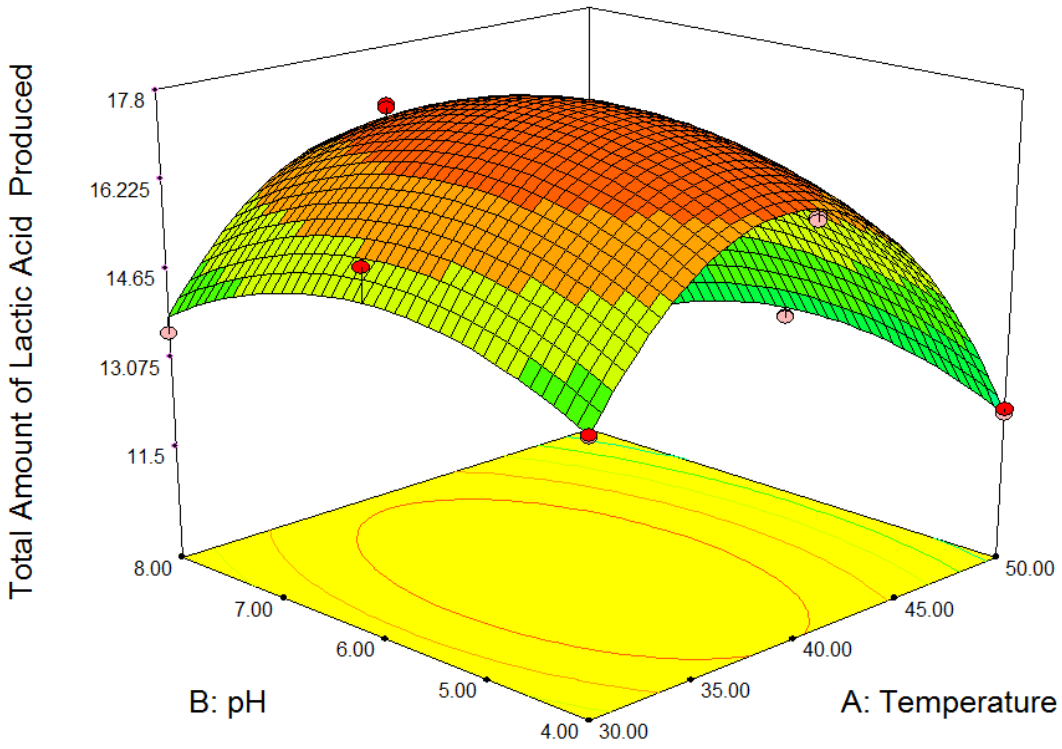

Figure 4.10. The interaction effect of temperature and pH on total amount of lactic acid

On the other hand, figure 4.11 and 4.12 show the interaction effect of temperature & incubation time; and pH and incubation time on total amount of lactic acidat at pH 6 and 40 ^{o}C respectively. In this case, both the interaction between temperature & incubation time; and the interaction effect between pH and incubation time had limited significance ($P > 0{\cdot}05$). This is indicated by slightly curved structured plot of those interaction. These results was supported by (Giraud et al., (1991)); (Gorret et al., (2001)); and (Rashid, 2008).

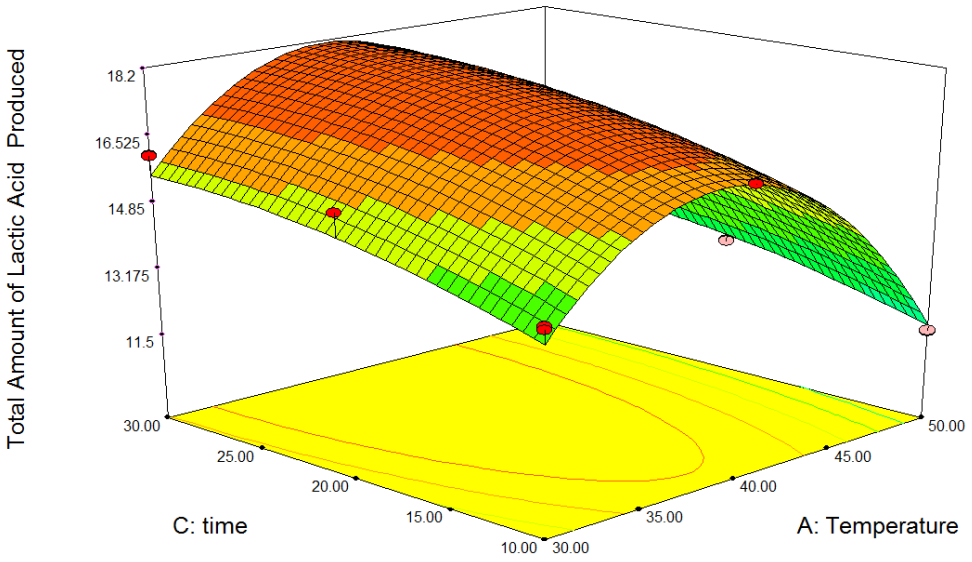

Figure 4.11. The interaction effect of temperature and time on total amount of lactic acid

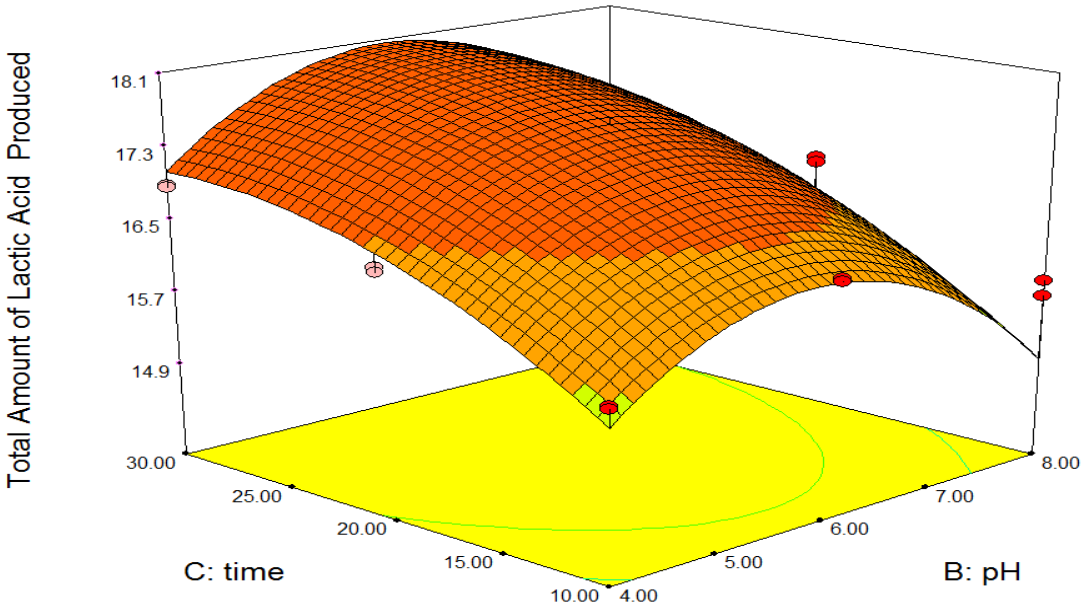

Figure 4.12. The interaction of ph and time on total amount of lactic acid

4.4. Optimization for Maximum Amount of Lactic Acid Production from Molasses by Lactobacillus Plantarum Bacteria

Table 4.5 pesents optimization for maximum lactic acid produciton with the given constrainnts and the maximum amount of lactic acid will be 15.5864g/L at 33.55^{o}C, 5.81 Ph and 10 hr incubation time with 0.856 desirability.i.e is selected optimum solution. However, there are a number of feasible solution that will fullfil the given constraints, such as 15.5779g/L at 33.53 ^{o}C, 5.78 initial pH and 10-hr incubation time with 0.856 desirability; 15.771g/L at 33.52 ^{o}C, 5.83 initial pH and 10 hr incubation time with 0.856 desirability; 15.5532g/L at 33.46 ^{o}C, 5.8 initial ph and 10 hours incubation time with usual desirability; 15.4602g/L at 33.39, 5.32 initial ph, and 10 hr incubation time with 0.853 desirability; and 15.7804g/L at 34.96 ^{o}C, 4.89 initial ph and 10 hr incubation time with 0.84 desirability. Solution fivelooks like the best option for the maximum lactic acid production, but it is with low desirability.

Table 4.5. Optimization for maximum lactic acid production with the given constraint

Constraints					
Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
time	minimize	10	30	1	1
Total amount of lactic acid produced	maximize	9.728	17.407	1	1
Solutions					
Number	Temperature	pH	time	Total amount of lactic acid produced	Desirability
1	33.55	5.81	10.00	15.5864	0.856Selected
2	33.53	5.78	10.00	15.5779	0.856
3	33.52	5.83	10.00	15.5771	0.856
4	33.46	5.81	10.00	15.5532	0.856
5	33.39	5.32	10.00	15.4602	0.853
6	34.96	4.89	10.00	15.7804	0.840

The desirability function is the most popular solution for the multy-response optimization problem. Desirability is an objective function that ranges from zero outside of the limit to one at the goal. The numerical optimization finds a point that maximizes the desirability function. The desirability value of 0.856 corresponded to the maximum lactic acid production in the given condition of constraints. The following histogram shows how well each variable satisfied the criteria: values near one are good.

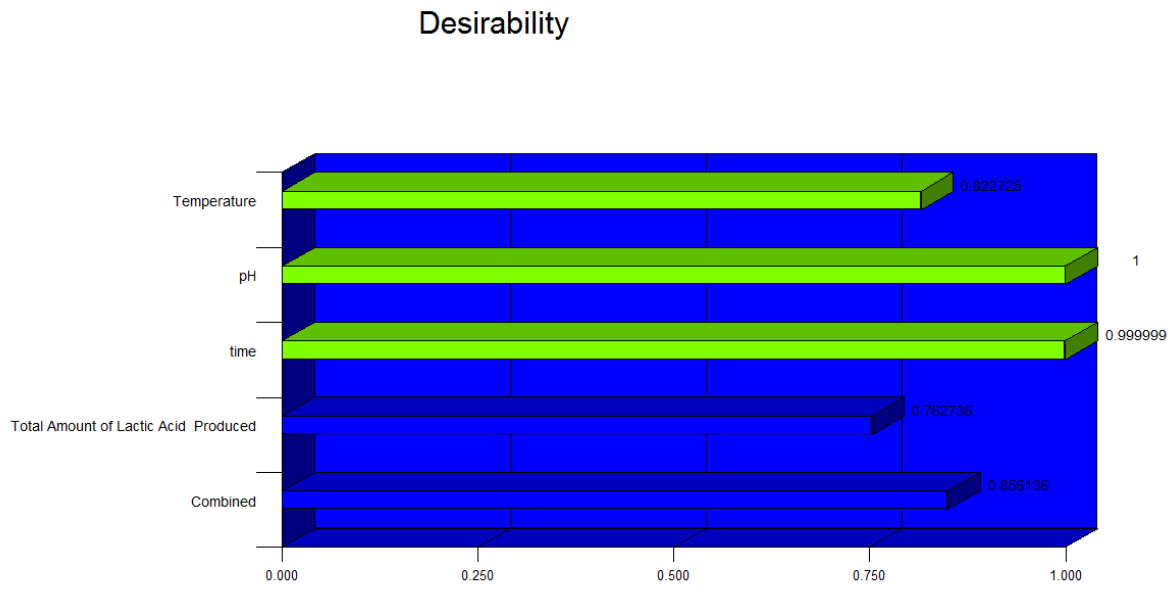

Figure 4.13. Desirability of optimization process

CHAPTER FIVE

5. Conclusions and Recommendations

5.1. Conclusions

This study was carried out in order to utilize sugar cane molasses for the production of lactic acid by using Lactobacillus Planetarium bacterial under different conditions such as temperature, incubation time and intial pH of fermentation processes and the maximum yield of lactic acid was found 17.407g/L which obtained at a temperature of 40^0C, pH of 6 and 30hr incubation time.

The new culture medium was prepared by substituting dextrose (the main ingredient of comertially availabel lactobacillus MRS agar) with molasses hydrolysates to got modified medium.The fermentations were performed in an incubator shaker with 250 mL flask each. The lowest amount of residual reducing sugare during the experiments was 2.471 g/L and it was obtained at a temperature of 50^0C, pH of 8 and 10 h incubation time.

A three -level full factorial design was used to determine the significant factors and the optimal condition of the process variable. These experiments have identified that pH, temperature, incubation time are the significant factors. The optimal values of tested variables were found to be 33.55^{o}C, 5.81, 10 h temperature, pH and incubation time. The optimum values of lactic acid with the given constraints was 15.5864g/L with 0.856 desirability.

The experiments has identified that, all temperature, initial pH and incubation time of fermentation process were significant factors and further analyses were carried out to study the detail correlation between lactic acid production and these factors. For Example, as the initial pH increased, the lactic acid production increase until the critical pH of 6 is reached. Beyond this pH, lactic acid production begins to decrease. A similar trend is observed for the temperature, the total amount of lactic acid produced increased with increase of temperature until a critical temperature of 40^{o}C. Beyond 40^{o}C, a reversal trend were occurred. Generally, sugar cane molasses with optimized fermentation condition can be used as best alternative carbon source for the production of lactic acid.

5.2 Recommendations

In this study, 250mL volume conical flasks were used during each fermentation processes. However, it may not have the same result during scale up work. Since, the experimental results of this research work show a great possibility of using sugar cane molasses for the effective production of lactic acid, additional study shoud be conducted in order to know the effect of scale up on the yield of lactic acid before it becomes Commercialized.

In addtion to that, the effect of major fermentation parameters such as temperature, intial pH and incuabation time alone were analysed on total amount of lactic acid. However, there are other minor parameters such as carbon source, agitation speed, nitrogen source, volume of fermenter, and others, which will affect the total amount of lactic acid. Eventhough the individual effect of each parameter on fermentation processes is limited, their sum up effect will be significant enough. So, it should be considered in future research in combination or separately in order to know how they affect the total amount of lactic acid.

In this study, the total amount of lactic acid was determined by using spectrophotometer with its specific wave length and there is no way to know the other compostions of fermented borths. So, it better to know the whole compostion of fermented broth with HPLC and also the kinetics of lactic acid production, which was not incorporated with this work should be studied in future researches.

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APPENDICES

Appendix A

Experimental Results for Optimization

No	Temperature, ^{o}C	pH	Incubation time,hr	Total amount of Lactic acid
1	30.00	4.00	10.00	12.6589
2	30.00	4.00	10.00	12.3838
3	40.00	4.00	10.00	15.718
4	40.00	4.00	10.00	15.688
5	50.00	4.00	10.00	11.047
6	50.00	4.00	10.00	11.107
7	30.00	6.00	10.00	14.291
8	30.00	6.00	10.00	14.203
9	40.00	6.00	10.00	16.419
10	40.00	6.00	10.00	16.387
11	50.00	6.00	10.00	11.573
12	50.00	6.00	10.00	11.608
13	30.00	8.00	10.00	12.0454
14	30.00	8.00	10.00	11.864
15	40.00	8.00	10.00	15.837
16	40.00	8.00	10.00	15.673
17	50.00	8.00	10.00	9.728
18	50.00	8.00	10.00	9.73
19	30.00	4.00	20.00	14.074
20	30.00	4.00	20.00	14.127
21	40.00	4.00	20.00	16.537
22	40.00	4.00	20.00	16.481
23	50.00	4.00	20.00	12.083
24	50.00	4.00	20.00	12.173
25	30.00	6.00	20.00	15.728
26	30.00	6.00	20.00	15.728
27	40.00	6.00	20.00	17.024
28	40.00	6.00	20.00	17.025
29	50.00	6.00	20.00	12.804
30	50.00	6.00	20.00	12.782
31	30.00	8.00	20.00	13.565
32	30.00	8.00	20.00	13.547
33	40.00	8.00	20.00	16.745
34	40.00	8.00	20.00	16.689
35	50.00	8.00	20.00	11.719
36	50.00	8.00	20.00	11.802
37	30.00	4.00	30.00	14.964
38	30.00	4.00	30.00	14.084

39	40.00	4.00	30.00	16.896
40	40.00	4.00	30.00	16.879
41	50.00	4.00	30.00	12.717
42	50.00	4.00	30.00	12.742
43	30.00	6.00	30.00	16.073
44	30.00	6.00	30.00	16.057
45	40.00	6.00	30.00	17.407
46	40.00	6.00	30.00	17.386
47	50.00	6.00	30.00	13.473
48	50.00	6.00	30.00	13.473
49	30.00	8.00	30.00	14.093
50	30.00	8.00	30.00	14.138
51	40.00	8.00	30.00	16.907
52	40.00	8.00	30.00	16.904
53	50.00	8.00	30.00	12.018
54	50.00	8.00	30.00	12.104

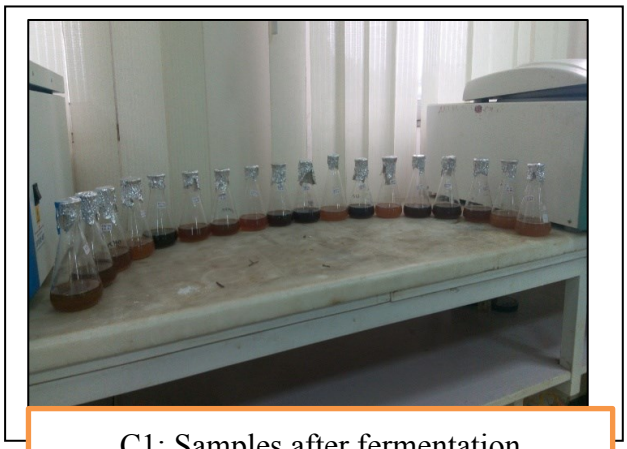
Appendix B

Residual reducing sugar content

No	Temperature, ^{o}C	pH	Incubation time,hr	Residual reducing sugar, g/L
1	30.00	4.00	10.00	5.249
2	30.00	4.00	10.00	5.307
3	40.00	4.00	10.00	4.826
4	40.00	4.00	10.00	4.922
5	50.00	4.00	10.00	5.512
6	50.00	4.00	10.00	5.504
7	30.00	6.00	10.00	4.472
8	30.00	6.00	10.00	4.472
9	40.00	6.00	10.00	4.381
10	40.00	6.00	10.00	4.296
11	50.00	6.00	10.00	4.815
12	50.00	6.00	10.00	4.82
13	30.00	8.00	10.00	6.417
14	30.00	8.00	10.00	5.618
15	40.00	8.00	10.00	4.947
16	40.00	8.00	10.00	5.061
17	50.00	8.00	10.00	7.362
18	50.00	8.00	10.00	7.362
19	30.00	4.00	20.00	4.341
20	30.00	4.00	20.00	4.286
21	40.00	4.00	20.00	3.311
22	40.00	4.00	20.00	3.468
23	50.00	4.00	20.00	4.753
24	50.00	4.00	20.00	4.753
25	30.00	6.00	20.00	3.913
26	30.00	6.00	20.00	3.762
27	40.00	6.00	20.00	2.358
28	40.00	6.00	20.00	2.754
29	50.00	6.00	20.00	4.928
30	50.00	6.00	20.00	4.898
31	30.00	8.00	20.00	4.294
32	30.00	8.00	20.00	4.294
33	40.00	8.00	20.00	3.485
34	40.00	8.00	20.00	3.691
35	50.00	8.00	20.00	6.054
36	50.00	8.00	20.00	6.023
37	30.00	4.00	30.00	4.58
38	30.00	4.00	30.00	3.155
39	40.00	4.00	30.00	3.755
40	40.00	4.00	30.00	3.31
41	50.00	4.00	30.00	4.851

42	50.00	4.00	30.00	4.905
43	30.00	6.00	30.00	3.564
44	30.00	6.00	30.00	3.231
45	40.00	6.00	30.00	2.707
46	40.00	6.00	30.00	2.421
47	50.00	6.00	30.00	3.341
48	50.00	6.00	30.00	3.167
49	30.00	8.00	30.00	3.647
50	30.00	8.00	30.00	4.634
51	40.00	8.00	30.00	3.196
52	40.00	8.00	30.00	3.781
53	50.00	8.00	30.00	5.676
54	50.00	8.00	30.00	5.958

Appendix C: Different Lab photo's



C1; Samples after fermentation

C2; Labeling Samples

C3; Final products

C4; planetarium bacteria

C5; shaker incubator

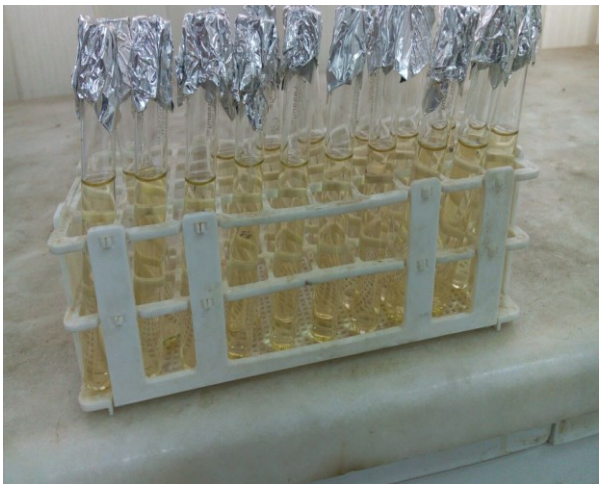
C6; fermented products on centrifuge

C7; PH adjustment

C8; samples sterilization

C9; lactic acid determination

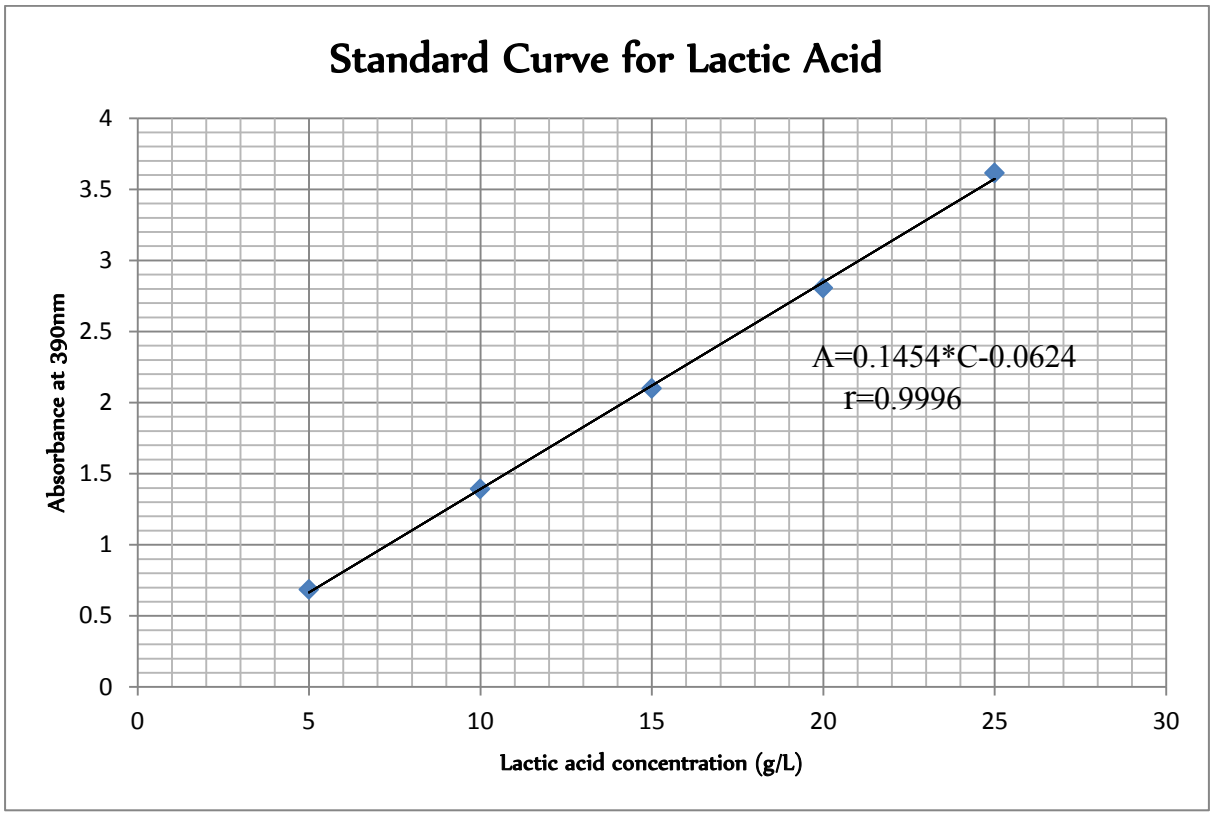
C10; $FeCl_3$ solutions



C11; MRS medium

Appendix D

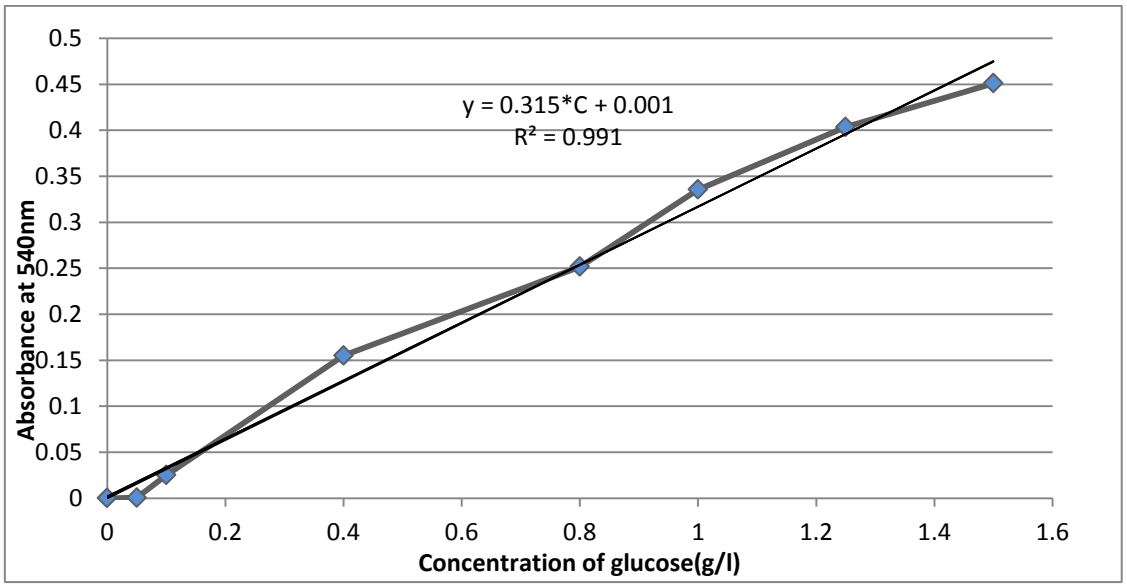
Standard curve of lactic acid



LA Concentration (g/L)	Absorbance at 390nm
5	0.6848
10	1.3918
15	2.098
20	2.8058
25	3.6128

Appendix E

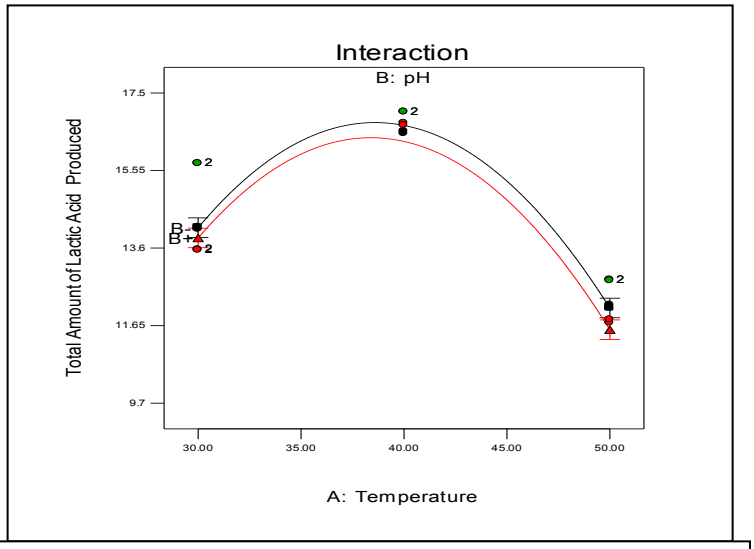
Standard curve of glucose

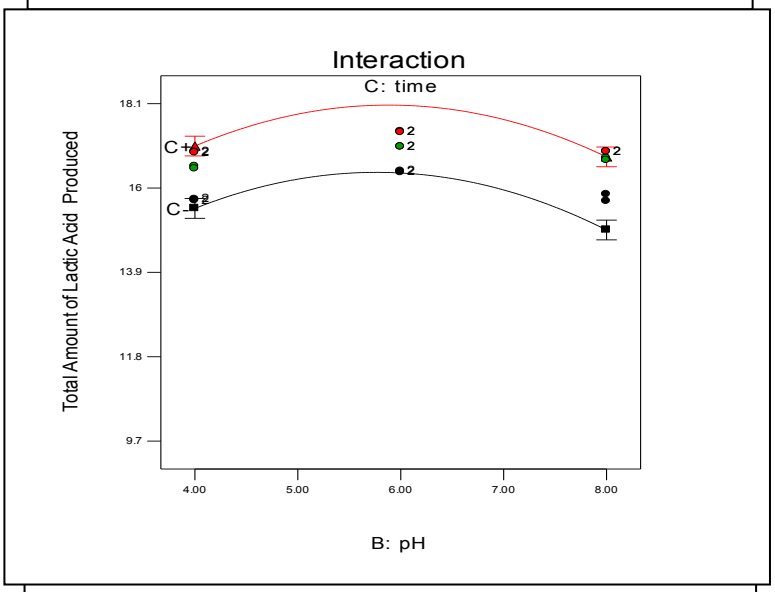


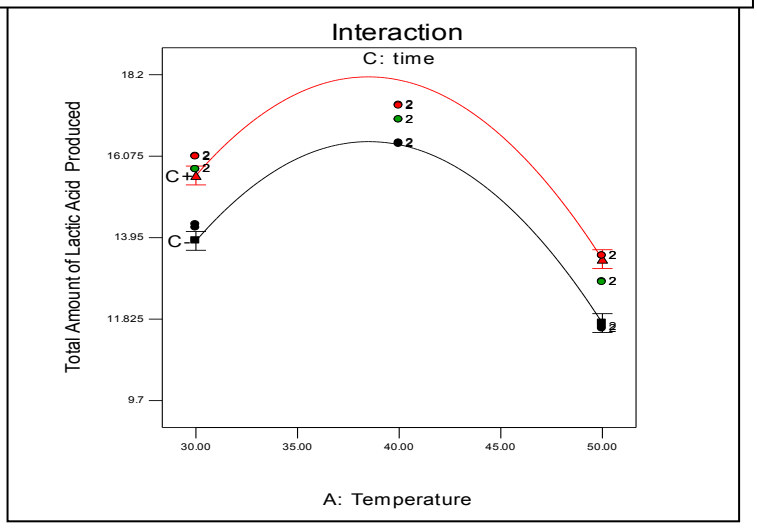
Glucose concentration (mg/mL)	Absorbance (nm)
0	0
0.05	0.0005
0.1	0.025
0.4	0.155
0.8	0.2515
1	0.3355
1.25	0.4035
1.5	0.451

Appendix F

The interaction effect of each factors on total amount of lactic acid produced

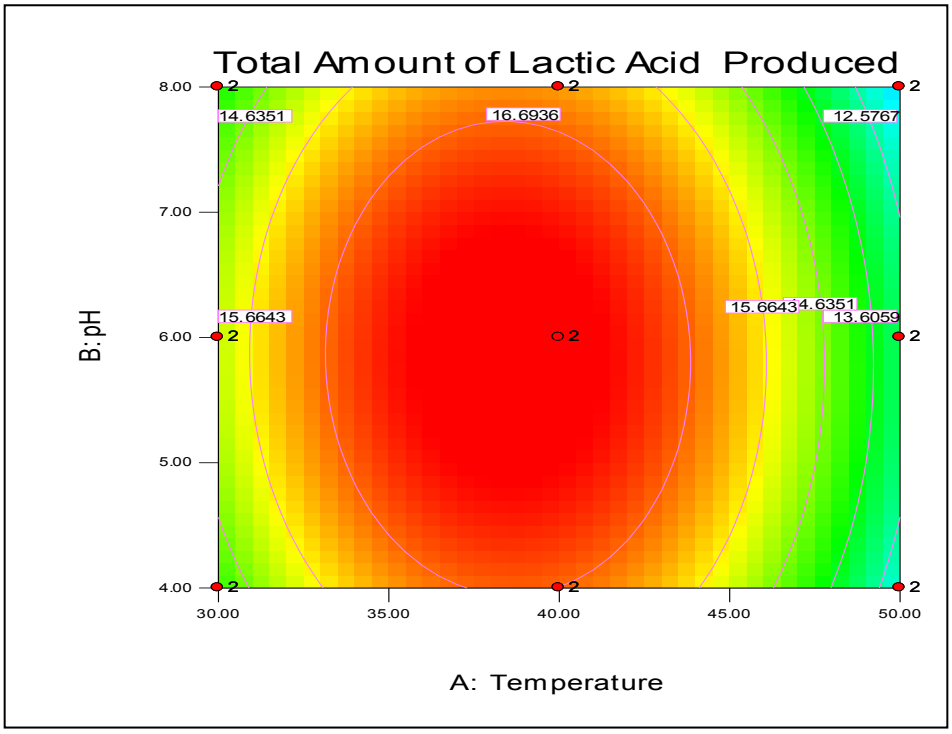


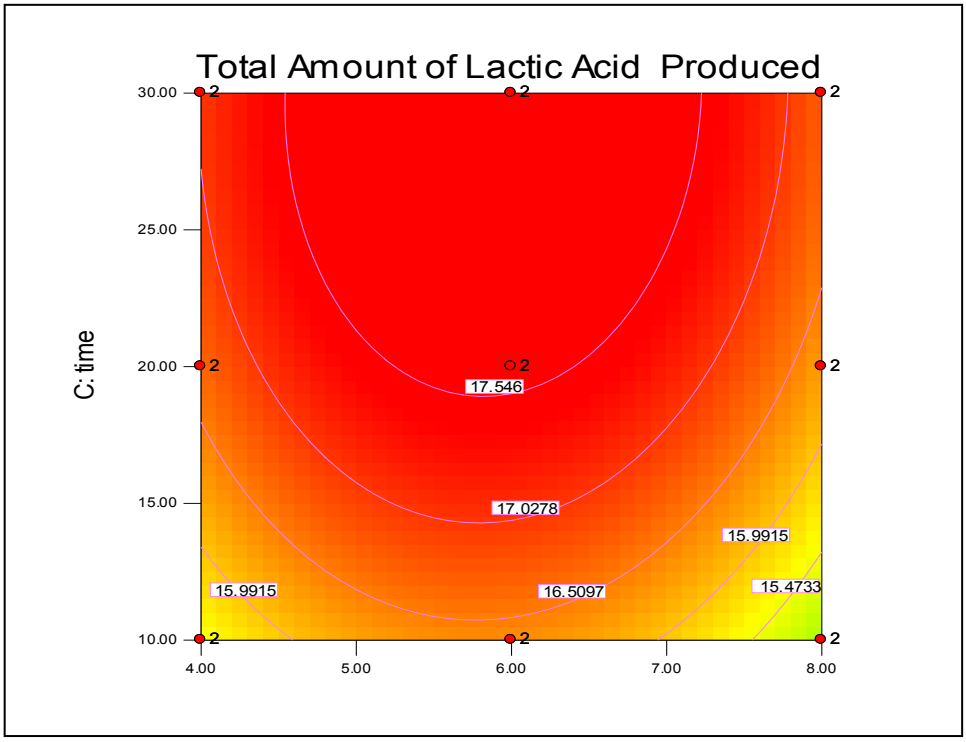


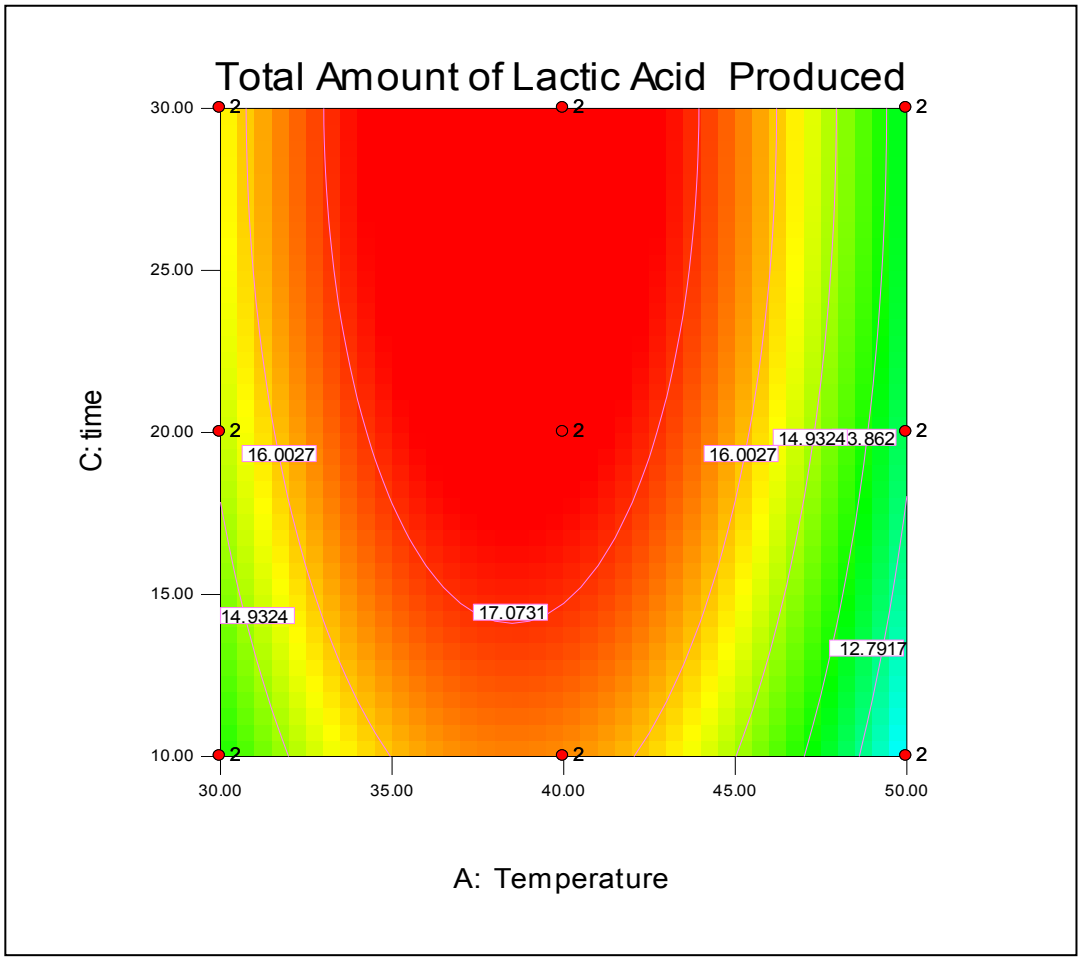


Appendix G

The contour plot of each interaction effect on total amount of lactic acid produced






Total Amount of Lactic Acid Produced
C: time
A: Temperature
30.00
25.00
20.00
15.00
10.00
30.00
35.00
40.00
45.00
50.00
16.0027
16.0027
14.93243.862
14.9324
17.0731
12.7917