Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio Engineering

Process Engineering Stream

Production of Lactic Acid from Taro Waste Starch:Comparative Analysis of Different Lactic Acid Bacteria Strains and Exploring their Potential as Bio preservative

Thesis Submitted to the School of Chemical and Bioengineering of Addis Ababa Institute of Technology, in Partial Fulfillment of the Requirements for the attainment of the Degree of Master of Science in Chemical Engineering

(Process Engineering Stream).

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This is to certify that the thesis prepared by Tensae Abera, entitled "Production of Lactic Acid from Taro Waste Starch:Comparative Analysis of Different Lactic Acid Bacteria Strains and Exploring their Potential as Bio preservative"and submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemical Engineering (process Engineering stream) complies with the regulation of the university and meets the accepted standards with respect to originality and quality.

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Declaration

I hereby declare that the thesis titled "Production of Lactic Acid from Taro Waste Starch: Comparative Analysis of Different Lactic Acid Bacteria Strains and Exploring their Potential as Bio Preservative," submitted for the degree of Masters of Science at Addis Ababa Institute of Technology, is an authentic piece of work created by me. This work has not been previously submitted or presented elsewhere for assessment. Whenever reference material from published or unpublished sources has been used, proper acknowledgment and citation have been provided.

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ABSTRACT

This study aimed to optimize fermentation parameters for maximizing lactic acid production from taro waste starch. Three bacterial strains, namely Lactococcus lactis, Enterococcus faecalis, and Lactobacillus paracasei, were selected for evaluation. The growth performance of these strains was assessed using an initial one variable at a time experimental design (OVAT). Each.variable, including temperature, incubation time, and pH, was varied individually while keeping the others constant to evaluate their effects on lactic acid yield. After the initial assessment, the fermentation parameters were optimized using response surface methodology with a central composite design. The optimized conditions were determined based on two replication experiments and the results from the analysis. The aim was to achieve maximum lactic acid yield from taro waste. Among the three bacterial strains evaluated, Lactococcus lactis demonstrated the highest potential for lactic acid production. Comparative analysis revealed that Lactococcus lactis outperformed Enterococcus faecalis and Lactobacillus paracasei in terms of lactic acid production. The optimization process confirmed the suitability of Lactococcus lactis for achieving higher yields of lactic acid from taro waste. Under the optimized conditions of a fermentation temperature of 35 °C, a fermentation time of 48 hours, and a pH of 6.5, Lactococcus lactis achieved the highest lactic acid yield of 33.74 g/L. The study suggests exploring alternative bacterial strains and considering factors beyond growth potential when evaluating strains for lactic acid production. Additionally, the potential use of lactic acid as a preservative for fruits and other food items can be further explored based on the study's findings.

Key words: Bio preservation,lactic acid, OVAT, taro waste

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ACRONYM

ASTM	American Society for Testing and Material
OVAT	One parameter at a time
OD	optical density
CFU	Colony Forming Unit
CCD	Central composite design
FTIR	Fourier-transform infrared spectroscopy
ANOVA	Analysis of variance
LAB	Lactic acid bacteria
FAO	Food and Agriculture Organization of the United Nations
LA	Lactic acid
RSM	Response surface methodology
TAC	tri carboxylic acid cycle
PLA	poly lactic acid
HPLC	High performance liquid chromatography
PLS	partial least square
DNS	Dinitrosalicylic acid
TA	titratable acidity
TSS	Total soluble liquid

1.INTRODUCTION

1.1 Back ground

Lactic acid, also known as 2-hydroxypropionic acid ($CH_3CHOH\ COOH$), is a naturally occurring organic acid that was initially identified in sour milk by the Swedish chemist Scheele in 1780(Ferguson et al., 2018).Its industrial production began with Fremi's discovery of fermentation-based lactic acid production in 1881(Choudhary & Kumari, 2021). Lactic acid can be generated through two primary methods: chemical synthesis or microbial fermentation using sugars derived from renewable sources (Castillo Martinez et al., 2013) However, biological production offers several advantages, such as the ability to obtain pure lactic acid, whereas chemical synthesis always results in a racemic mixture (Farooq et al., 2012).

In order to make the biotechnological production of lactic acid economically viable, it is crucial to have access to inexpensive raw materials.Various affordable raw materials, including starchy and cellulosic materials, whey, and molasses, have been utilized for lactic acid production (Hofvendahl & Hahn-Hägerdal, 2000). Among these options, there is currently a strong focus on starchy and cellulosic materials due to their abundance, cost-effectiveness, and renewable nature.(Åkerberg & Zacchi, 2000)(Richter & Berthold, 1998).

Starch, a naturally occurring biopolymer, possesses desirable characteristics such as biodegradability, affordability, renewability, and widespread availability in various plant species (Awol et al., 2020) The primary sources of starch worldwide include corn, potato, wheat, cassava, and sweet potato. However, these conventional sources are currently facing issues of market demand and diversification, the increasing global demand for starch puts pressure on convensional source. Therefore, conducting research and characterizing alternative starches can help alleviate the strain on limited sources of starch (Assefa et al., 2016).Given the extensive application of starch, it is crucial to focus on discovering new crops and plant components with high starch content and yield (Aprianita et al., 2014).

The economic viability of lactic acid production in the industry heavily relies on the cost and quality of the chosen raw materials. Therefore, it is crucial to select a raw material for industrial

lactic acid production that possesses specific characteristics, including low cost, minimal contaminants, fast fermentation rate, high lactic acid yield, minimal by-product formation, and year-round availability (Y. J. Wee, Kim, et al., 2006). The selection of a specific waste material for lactic acid production depends on factors such as waste availability, carbohydrate content, suitability for fermentation, and potential economic and environmental benefits. Taro waste is a viable option considering these factors.In this context, utilizing taro waste as a carbon source can meet the aforementioned criteria for lactic acid production. Taro, a tuber widely distributed and easily accessible in the South, West, and Southwestern regions of Ethiopia, has the potential to serve as an ideal substrate for lactic acid production(Yimer & Babege, 2018).

Microorganisms that possess the ability to produce lactic acid can be categorized into two main groups: bacteria and fungi. Lactic acid bacteria (LAB) and filamentous fungi have been extensively studied for lactic acid production(Z. Y. Zhang et al., 2007). LAB a group of gram-positive microorganisms are recognized as the primary and safe industrial-scale producers of lactic acid. Various LAB species, such as *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus,* and *Pediococcus*, are commonly used as starter cultures in industrial food fermentations. Among these LAB strains, Lactobacillus strains hold significant commercial importance due to their high tolerance to acidic conditions, high yield and productivity, and the potential for engineering to selectively produce L- or D-lactic acid(Abedi & Hashemi, 2020).

Lactic acid is currently widely utilized across various industries due to its diverse industrial applications. It is employed as probiotics, bio control agents, and bio preservatives (Lübeck & Lübeck, 2019). Its uses span across several sectors, including the food industry (confectionery, dairy products, bakery products), cosmetic industry, chemical industry, pharmaceutical industry, polymer industry, and leather tanning industries. The versatility of lactic acid allows for its incorporation in numerous products and processes within these industries.

1.2 Statement of problem

Root crops like taro have a limited shelf life due to their high moisture content, resulting in spoilage within 2-4 weeks after harvest. To minimize loss, taro is processed into nonperishable forms like flour and starch for shelf life extension,food security, convenience and versatility However, taro processing generates waste, including valuable taro waste produced during the production of taro flour, which is commonly used for various purposes such as making enjera by mixing with teff flour (a practice that is becoming more widespread in many regions). Waste is also generated at local markets where taro is commonly sold, as vendors discard peels and unused parts of the taro. Additionally, if taro roots are not sold promptly, they can rot, contributing further to waste. Farmers may also have surplus taro waste from harvesting or processing stages, and some roots may rot during growth due to viruses or other infections.

As taro production in Ethiopia has been steadily increasing over the years, there is a growing necessity to find alternative uses for taro beyond its traditional application as food. The rise in taro supply, as indicated by FAO production data, directly correlates with the amount of waste generated. Therefore, it is imperative to explore additional avenues for the productive utilization of taro waste in order to address this escalating issue effectively

Over the span of ten years, taro production has consistently risen, with a gradual increase observed each year. Notable spikes in production were seen in 2013, 2014, and 2017, where quantities reached 1.23 million, 1.4 million, and 1.5 million metric tons, respectively. The most substantial surge occurred in 2020, with taro production reaching 2.3 million metric tons. This demonstrates the sustained growth in taro production from year to year throughout the ten-year period.

By utilizing this waste as a carbon source we can produce lactic acid using lactic acid bacteria. This approach holds promise for a sustainable production process. However, different strains of bacteria exhibit varying capabilities in terms of sugar consumption and lactic acid production, with certain strains demonstrating superior performance. Therefore, it is crucial to identify the bacterial strain that yields the highest lactic acid output. The primary focus of this study is to determine the specific strain of lactic acid bacteria that can achieve the highest lactic acid yield

from waste *C. esculenta* tubers. Moreover, the fermentation process is influenced by several parameters, such as temperature, pH, substrate concentration, mixing rate, and fermentation time. To examine the production of lactic acid from waste *C. esculenta* tubers, the effects of these key parameters will be examined, and the fermentation conditions will be adjusted accordingly. And also this study promote the use of lactic acid as a natural preservative and improve food preservation.

1.3. Objectives

1.3.1. General Objective

The main objective of this study was to produce lactic acid from taro waste starch and compare different lactic acid bacteria strains and explore the potential as biopreservative.

1.3.2. Specific Objectives

- To extract and characterize taro waste starch.
- To determine the effect of hydrolysis processing variables such as temperature,pH and time on glucose yield.
- To assess the growth performance of different bacterial strains on the extracted glucose and determine their potential suitability for the production of lactic acid
- To investigate the effect of temperature, pH and time on the production of lactic acid.
- To find optimal operating conditions of lactic acid production
- To evaluate the bio preservative effect of lactic acid.

1.4. Research question

- Do taro waste could be utilized as a potential source of starch/fermentable sugars for the production of lactic acid through fermentation by lactic acid bacteria?
- Do pH, temperature and time affect the enzymatic hydrolysis of taro waste starch?
- Do bacteria strains have the potential to be used as candidates for the production of lactic acid?
- Do temperature, pH and time affect the production of lactic acid?

1.5. Significance of the Study

This study investigates the utilization of waste taro as a raw material for lactic acid production and its application in fruit bio-preservation. Its aim is to develop a more sustainable lactic acid production process by diversifying raw materials and reducing reliance on limited starch sources. It focuses on different lactic acid bacteria and their potential for production, aiming to identify those with higher yields through preliminary analysis. By doing so, the research provides valuable insights into efficient bacteria selection and factors influencing lactic acid yield, leading to improved industrial processes and potential cost savings. Additionally, the study highlights the versatility of lactic acid, demonstrating its various applications, such as bio-preservation of food and fruits for extended shelf life. By exploring lactic acid's potential as a natural preservative, the study emphasizes its importance in ensuring food safety and maintaining the freshness and quality of perishable products.

1.6. Scope of the Study

This study entirely focused on several key aspects. Firstly, it involved the extraction of starches from taro waste. Secondly, the study focused on characterizing the properties of the extracted starch. Additionally, the hydrolysis of starch was analyzed. The formulation of media and the inoculation of potential lactic acid bacteria onto the MRS agar medium (De Man Rogosa and Shapes) were also part of the study. Furthermore, the fermentation process was carried out, followed by the characterization of the resulting product. Lastly, the study explored the potential application areas of the product.

2. LITERATURE REVIEW

2.1. Background

Lactic acid's discovery and understanding have evolved through the investigations of notable figures. Ancient fermentation practices utilized microorganisms to convert sugars into alcoholic beverages. However, the specific compounds involved were not initially known(Vijayakumar et al., 2008)In 1780, the Swedish chemist Carl Wilhelm Scheele made a significant breakthrough. Scheele isolated lactic acid from sour milk, noting its sour taste and characteristic properties. He named the compound "lactic acid" due to its presence in milk (lactis is Latin for milk). Scheele's discovery marked the first identification and isolation of lactic acid, laying the foundation for further exploration(Kim et al., 2022).

The French chemist Louis Pasteur played a crucial role in advancing our understanding of lactic acid and its connection to fermentation. In the mid-19th century, Pasteur conducted numerous experiments to investigate the process of fermentation. He demonstrated that microorganisms, particularly lactic acid bacteria, were responsible for the conversion of sugars into lactic acid. Pasteur's work not only deepened our knowledge of the fermentation process but also established the importance of microorganisms in various industries, such as winemaking and dairy production.(Mohanty et al., 2015).Building upon Pasteur's findings, Johannes Wislicenus discovered the two forms of lactic acid: L-lactic acid and D-lactic acid, which are mirror images of each other. This breakthrough was crucial for understanding lactic acid's stereochemistry and properties(Ghaffar et al., 2014).

In the 20th century, research on lactic acid advanced in biochemistry and microbiology. Scientists discovered that it is a byproduct of anaerobic metabolism, commonly produced during intense exercise when muscles lack oxygen. Today, Lactic acid remains vital with diverse applications. Ongoing research explores its potential in biotechnology, environmental science, and sustainable materials(John et al., 2007).

2.2 Over view of lactic acid

Lactic acid, an organic compound classified as a carboxylic acid, is a clear, soluble liquid with a sour taste. It is commonly present in a variety of foods, beverages, and dairy products. Lactic acid occurs in two forms, L-lactic acid and D-lactic acid, with L-lactic acid being the more prevalent isomer in nature.

The production of lactic acid involves the fermentation of carbohydrates by specific microorganisms, particularly lactic acid bacteria. This fermentation process is extensively employed in the production of fermented food items such as yogurt, cheese, sauerkraut, and pickles. The conversion of sugars into lactic acid by lactic acid bacteria contributes to the tangy flavor and preservation of these foods(John et al., 2007).

Beyond its significance in food production, lactic acid exhibits diverse applications across various industries. In the pharmaceutical sector, lactic acid and its derivatives are utilized in the manufacturing of drugs, cosmetics, and personal care products. The textile industry also employs lactic acid for dyeing and printing processes. Moreover, lactic acid has gained attention as a valuable precursor for the synthesis of biodegradable polymers like polylactic acid (PLA), which find wide-ranging uses in packaging materials, medical devices, and 3D printing(Abdel-Rahman et al., 2011).

2.2.1physical and chemical properties

Lactic acid is a three-carbon organic acid with one terminal carbon atom in an acid or carboxyl group, the other in a methyl or hydrocarbon group, and a middle carbon atom with an alcohol carbon group. (Krishna et al., 2018). It is yellow to colorless (at 15℃ and 1 atm) and is the simplest hydroxycarboxylic acid (Komesu et al., 2017) and (Groot et al., 2022).

Table 2 1 Physical properties of lactic acid

Properties	Values
Molecular weight	90. 08
Density at 20℃(g/L)	1.249
Melting point	16.8ºC
Boiling point	82ºC at 0.5 mm Hg 122ºC at 14 mm Hg
Dissociation constant, Ka at 25ºC	$1.37x\ 10^{-4}$
Heat of combustion, ΔHc	1361 KJ/mole
Specific Heat, Cp at 20ºC	190 J/mole/ºC
pKa	3.86
Crystal structure	(S)-Lactic acid: orthorhombic,
Solid density (g/mL)	1.33 (solid, 20°C)
Solubility in water (wt%)	86 (20°C, monomeric (S)-lactic acid)

source- (Martin, 1996)

2.2.2 Recent work on lactic acid production

Recent studies have been focused on the production of lactic acid from glucose to make use of agro-industrial wastes as affordable and renewable resources, while also reducing environmental pollution. H.S. El-Sheshtawy conducted research where sixteen bacterial strains were isolated from these wastes. Chemical hydrolysis of the wastes using hydrochloric acid, sulfuric acid, and sodium hydroxide resulted in the highest yield of lactic acid. Optimal conditions for lactic acid production were determined, and calcium lactate obtained from the fermentation culture was treated with sulfuric acid to precipitate calcium sulfate. The filtrate containing the free organic acid was then evaporated to obtain pure lactic acid. The study showed that 5% HCl hydrolysis released the highest concentration of total reducing sugar, with cotton and coffee wastes yielding 8015.90 mg/L and 7761.55 mg/L, respectively. The bacterial strain Kosakonia cowanii (B2) exhibited the highest lactic acid concentration, reaching 24.97 g/L and 27.91 g/L after 24 and 48

hours, respectively, as analyzed by HPLC. On a larger scale, a maximum lactic acid production of 28.14 g/L was achieved in a fermenter after 72 hours(El-Sheshtawy et al., 2022).

In another study conducted by Sirisansaneeyakul, the focus was on producing lactic acid from glucose using immobilized cells of Lactococcus lactis IO-1. The study compared two methods of immobilization: entrapping the cells in alginate beads and encapsulating them in alginate membrane microcapsules. The fermentation process was optimized using the Taguchi method in shake flasks and further assessed in a production bioreactor with a packed bed of immobilized cells. The bioreactor operated by recycling the broth through the bed. The study revealed that microencapsulation was a more effective method of immobilization. Under optimized conditions, including an initial pH of 6.85 and specific nutrient concentrations, the volumetric productivity of lactic acid reached 1.8 g/L/h in shake flasks. By using a packed bed of encapsulated cells with broth recycling, the volumetric productivity increased to 4.5 g/L/h. Repeated batch runs using the packed bed system demonstrated its potential for lactic acid production(Sirisansaneeyakul et al., 2007).

Additionally, de Lima and colleagues utilized response surface methodologies to examine the effects of various factors on lactic acid fermentation by Lactobacillus sp. LMI8, which was isolated from cassava flour wastewater. They employed two central composite designs to evaluate the influence of cheese whey, corn steep liquor, ammonium sulfate, temperature, and pH control. The first design aimed to replace yeast extract with low-cost nitrogen sources to enhance cost-effective production. The optimal conditions determined were 55 g/L lactose, 15 g/L corn steep liquor, and 5.625 g/L ammonium sulfate, resulting in a maximum lactic acid concentration of 18.68 g/L. The second design investigated the impact of temperature and pH on lactic acid production and found that the highest lactic acid production of 52.37 g/L could be achieved at a temperature of 39.6 °C and a pH of 5.9. In shake flask fermentation, there was a significant 180% increase in lactic acid production after 30 hours, with an 86.12% conversion efficiency of the initial lactose. Furthermore, the lactic acid produced from whey lactose by Lactobacillus sp. LMI8 was mainly composed of optically pure D-lactic acid, accounting for more than 98% of the total lactic acid produced(de Lima et al., 2010).

These study findings demonstrate progress in utilizing glucose for lactic acid fermentation, which includes employing agro-industrial wastes as substrates, enhancing immobilization methods, and optimizing fermentation parameters to improve productivity

2.3 Lactic acid production technology

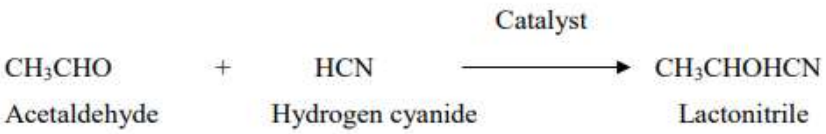
Lactic acid production technology encompasses the techniques and procedures utilized for large-scale manufacturing of lactic acid. Lactic acid, a versatile organic acid with wide-ranging industrial applications, is produced through various methods, including both chemical and biological approaches**.**

2.3.1Chemical synthesis

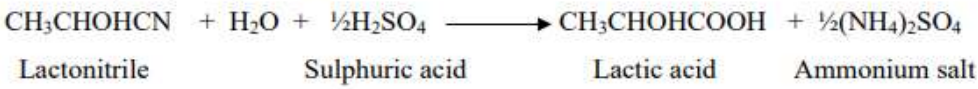
The commercial chemical synthesis process employs lactonitrile. Lactonitrile is formed by mixing hydrogen cyanide with acetaldehyde in the presence of a base. This reaction occurs in the liquid phase at high atmospheric pressure(Ghaffar et al., 2014) The crude lactonitrile is recovered and purified by distillation. It is subsequently hydrolyzed to lactic acid using strong HCl or H2SO4, yielding the desired ammonium salt and lactic acid. (Boontawan, 2010). Lactic acid is subsequently esterified with methanol to produce methyl lactate, which is extracted and purified via water under an acid catalyst to distillation before being hydrolyzed by produce

Both lactic acid and methanol are subjected to recycling. The responses shown below explain this process. (Narayanan et al., 2004)

(a) Addition of Hydrogen Cyanide



(b) Hydrolysis by H_2SO_4



(c) Esterification

(d) Hydrolysis by H_2O

Lactic acid manufacturing via a chemical approach is costly and reliant on byproducts from other industries that are based on fossil fuels. Biotechnological methods based on fermentation can alleviate the difficulties of expensive raw materials, product impurity, and reliance on other industries for raw materials(Pal et al., 2009).

2.3.2Biological Synthesis

Biological synthesis of LA is frequently a two-step process, with the first stage being LA fermentation and the second being product recovery and/or purity. LA fermentation requires the use of certain microorganisms, substrates, and purification methods. Several researchers have worked to develop economically viable methodologies for the biological production of LA (Kim et al., 2022).

Production of lactic acid by fermentation processes

Microbial fermentation accounts for over 90% of global lactic acid production (Hofvendahl & Hahn-Hägerdal, 2000) and it has grown in popularity due to its advantages over chemical synthesis. Lactic acid can be made by fermenting sugars or sugar-containing hydrolyzes,

converting starchy or cellulosic wastes in a single step using amyl lytic lactic acid generating microbes, or combining hydrolysis and fermentation with scarifying enzymes and an inoculum. There are various methods for biotechnological manufacture of lactic acid(Rojan et al., 2005).

2.3.2.1Fermentation via Lactic Acid Bacteria

One of the main characteristics of lactic acid bacteria, which are a kind of Gram-positive bacteria, is their capacity to ferment carbohydrates and generate lactic acid as the byproduct. These bacteria are anaerobic, non-respiring cocci or rods that belong to genera including Lactobacillus, Leuconostoc, Bifidobacteria, Pediococcus, and Streptococcus (Genera & Bacteria, n.d.).

Lactic acid bacteria are primarily distinguished by their capacity to ferment carbohydrates into lactic acid. The conventional method of identifying these microorganisms has been this procedure. Even though more species have been found, their significance in lactic fermentations is little(Roslina, 2008).

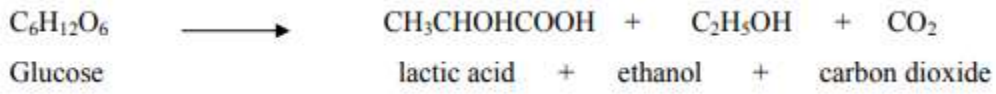
Lactic acid bacteria exhibit two primary forms of fermentation: hetero- and homo-fermentation. The only byproduct of fermentation produced by homo fermentative lactic acid bacteria is lactic acid. On the other hand, lactic acid is not the sole byproduct produced by hetero fermentative lactic acid bacteria; they also make acetic acid and ethanol.

Homo lactic fermentation

The fermentation of 1 mole of glucose yields two moles of lactic acid; (Romero-Garcia et al., 2009)

Hetero lactic fermentation

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



2.4 Nutrient demands for lactic acid fermentation

2.4.1 Carbon sources

Starch

Starch is the second most common substrate for LA fermentation, behind glucose. Numerous starchy materials, including as corn, maize, rice, rye, wheat, potato, barley, and cassava, have been investigated for their potential to produce LA. (Komesu et al., 2017)The production of LA requires the conversion of starchy sources to glucose, unless the LAB possesses amylolytic activity, which permits the direct production of LA from starchy materials. (Kim et al., 2022) Two fermentation processes are used to create glucose from starchy sources: liquefaction using thermostable α-amylase and saccharification using α-amylase and amyloglucosidase. One option for using the glucose generated by enzymatic treatment as a carbon source for LA fermentation is to use it directly (Massoud & M. Abd El-Razek, 2011).

Starchy materials such as wheat, corn, maize, cassava, potato, rice, rye, and barley can all be utilized to make lactic acid. One benefit is that starchy material can stop glucose repression, which is the process by which high glucose concentrations in the medium inhibit the growth of lactic acid bacteria. (Nakano et al., 2012) .Roughly 90% of the lactic acid sold commercially is produced via submerged fermentation of maize(Abdel-Rahman et al., 2013a).

Lignocellulose

The use of lignocellulose materials does not compete with the human food industry because they are naturally occurring, reasonably priced, and widely accessible as forestry residues, municipal solid waste, pulp, paper industry, and agricultural waste (such as wheat straw, rice straw, corn stalks, soybean residues, and sugarcane bagasse(Kim et al., 2022).

The inability of many bacteria to directly use lignocellulose for metabolism and product synthesis is the main obstacle to its use. It is crucial to use the right pretreatments to release lignin and hemicellulose for improved enzyme accessibility and enzyme hydrolysis, which releases glucose and xylose molecules, in order to transform polymeric lignocellulosic biomass into an easy-to-digest monomeric state. Efficient pretreatment and hydrolysis can address the primary technical and economic obstacles associated with lignocellulosic materials(Y. Wang et al., 2016).

Whey

Whey, a significant by-product of the dairy industry, contains 5% lactose, 1% protein, 0.4% fat, and a few minerals, making it a powerful and appropriate source for the manufacture of LA. Worldwide dairy businesses produce a sufficient amount of whey for each liter of processed milk, contingent on the procedures followed, goods produced, and hygiene practices followed. Approximately half of the world's cheese-whey production is processed and made into different food items; the remaining half is used as cheese-whey-protein concentrates, 30% as powdered cheese whey, 15% as lactose and its byproducts, and the remaining 45% as liquid cheese whey (Marwaha & Kennedy, 1988).

Due to the fact that lactose makes up the majority of whey solids, along with water-soluble vitamins, minerals, and proteins, a number of biotechnological techniques have been developed to utilize whey to produce beneficial compounds with significant commercial value, such lactic acid. The emphasis of this review is on LAB and how well they utilize whey to produce lactic acid(Panesar et al., 2007).

Food Waste

Any substance produced during the food production process, as well as waste products created by the end user, might be considered food waste. Food waste is a good substrate for the fermentation of lactic acid because it contains a significant amount of carbohydrates. According to a number of studies, food wastes like kitchen residues and refuse, as well as municipal solid wastes, can produce lactic acid (López-Gómez et al., 2019),Model kitchen refuse mediums

include cereals, water, vegetables, meat, and fish, as well as mixtures of cooked rice, vegetables, meat, and bean curd (Tashiro et al., 2013), (Tashiro et al., 2013),(B. Zhang et al., 2008)Meat, veggies, rice, and noodles (Kwan et al., 2015).Fruit (banana, apple, and orange peels), vegetables (carrot, cabbage, and potato peels), baked fish, rice, and and vegetables (Kwan et al., 2015).vegetables such as carrot peel, cabbage, and potato peel, fruit such as banana peel, apple peel, and Fruit (banana, apple, and orange peels), vegetables (carrot, cabbage, and potato peels), baked fish, rice, and used tea leaves (Kitpreechavanich et al., 2016). rice, noodles, meat, and vegetables; rice, vegetables, and meat; coffee mucilage; and coffee pulp; and unsold bakery goods, such as cakes, breads, and pastries (Neu et al., 2016);and (Pleissner et al., 2016).

2.4.2 Nitrogen source

In order to produce lactic acid biotechnologically on a glucose or lactose-based media, supplements are needed, such as yeast extract(Linko, 1996) Volumetric productivity, substrate conversion, and lactic acid concentration were all significantly impacted by yeast extract supplementation. Thus, 10 g/l of yeast extract appears to be the most popular and efficient supplement (Helveticus et al., 1990).

Yeast extract is utilized as an element in medium for the development of microorganisms because it is high in nitrogen, vitamins, and other nitrogenous growth factor boosting chemicals. Furthermore, every published paper that has been published has demonstrated that the formation of lactic acid rises with the concentration of the supplement, particularly yeast extract(Lund et al., 1992).

Yeast extract typically contains 8-12% total nitrogen (organic and inorganic compounds), 50-75% protein, 3-5.2% fermentable nitrogen, 4-13% total carbohydrate, and very low lipid content. In microbial fermentations, the fermentation medium can cost about 30% of the entire cost (Domı et al., 2004)According to the literature, yeast extract is the most effective supply of growth factors for LAB among the nitrogen sources investigated. Various quantities of B (Barrette et al., 2001).

vitamins have been tested to replace yeast extract. Yeast extract definitely had the most significant influence on lactic acid generation of LAB, particularly in the early stages of

development, with lactic acid concentration increasing linearly with the rise of yeast extract level(Linko, 1996).

2.5 Lactic acid fermentation mode

The types of fermentation modes utilized vary depending on the substrate, the viscosity of the fermentation broth, and the microorganisms used and their development. (Krishna et al., 2018)Lactic acid production uses several fermentation modes, including batch, fed-batch, repeated, and continuous (Hofvendahl & Hahn-Hägerdal, 2000).

2.5.1 Batch Fermentation Mode

In batch fermentation, all of the necessary nourishment is provided before the fermentation process begins. To maintain a steady pH, most systems use acid or alkaline regulation (Abdel-Rahman et al., 2011)Batch fermentation is a closed system, thus the possibility of contamination is limited, and high lactic acid concentrations have been seenvalue (Abdel-Rahman et al., 2011). (Abdel-Rahman et al., 2011) ,(Hofvendahl & Hahn-Hägerdal, 2000).

The benefits of batch fermentation are simplicity of operation, high lactic acid concentration and production, and little contamination risk. However, batch fermentation has low productivity and inhibits substrate or product growth (Y. Wang et al., 2015).

2.5.2Fed-Batch Fermentation Mode

The fed-batch fermentation mode is a variation of batch fermentation. It comprises the same components as batch fermentation, including raw materials (carbon source), nitrogen supply, and other critical nutrients. (Ding & Tan, 2006) Nonetheless, throughout the fed-batch fermentation process, one or more of the necessary nutrients are provided aseptically at regular intervals without removing any fermentation broth. (Paulova et al., 2020) In fed-batch fermentation, response rates are determined by the amount of limiting nutrients delivered sequentially at regular intervals, such as carbon and nitrogen. In addition, restricting nutrients promotes microbial development, resulting in increased production. Fed-batch fermentation is a better option than batch fermentation (liu2005).

In fed-batch, by-product buildup is low, product concentration is high, and substrate inhibition is not present. Fed batch disadvantages include appropriate fermentation design and final product inhibition(Abdel-Rahman et al., 2013a) .Aside from increased yield and productivity in fed-batch, there is long-term product synthesis, low substrate concentration, and less substrate inhibition owing to the fermentation culture's constant feed input.(Abdel-Rahman et al., 2011).

2.5.3 Repeated Batch Fermentation Mode

Similar to the batch mode, in which all necessary nutrients are added at the beginning, is the repeated batch fermentation mode. The depleted nutrients are gradually replaced with an equal volume of fresh medium containing the same nutrients and culture broth as the initial fermentation broth (microbial cells recycling) for the subsequent fermentation process in repeated batch fermentation, according to (Y. J. Wee, Yun, et al., 2006), (Moni et al., 2018) .Although repeated batch fermentation requires special devices (e.g., special connection lines for cell concentration), this fermentation mode saves time and labor, and the mode gives a high growth rate (Abdel-Rahman et al., 2013b).

2.5.4 Continuous Fermentation Mode

The concentrations of substrates and products are continually maintained in the continuous fermentation mode because new medium is continuously introduced to the fermenter while the broth that already contains the utilized medium and cells is collected at the same rate(Abdel-Rahman et al., 2013b) Toxic metabolites are eliminated from the culture and replaced with nutrients that have been consumed. (Ying & Ma, 2021). The benefits of continuous fermentation include high lactic acid production and microorganism growth control rate(Abdel-Rahman et al., 2013b) Continuous fermentation has several disadvantages, such as significant equipment costs, insufficient use of carbon sources, and severe contamination concerns(Y. Wang et al., 2015).

2.6. Factor affecting lactic acid production

2.6.1 Effects of PH

Microorganisms' ability to assimilate nutrients and the activity of their enzymes are significantly impacted by pH. The fermentation of certain lactic acid producers is thought to be inhibited by increased un dissociated lactic acid in response to decreased pH brought on by lactic acid

generation (Abdel-Rahman et al., 2013b)The fermentation process' gradual pH drop may be related to the carbohydrates' conversion to lactic acid (Jawad et al., 2013).

The pH of the fermentation process is either adjusted initially or allowed to drop as a result of acid generation. Base titration, lactic acid extraction, adsorption, or electrodialysis are some methods used to regulate the pH (Hofvendahl & Hahn-Hägerdal, 2000).The pH of fermentation can be adjusted either by base titration, lactic acid extraction, adsorption, or electrodialysis, or it can be set first and then allowed to drop as a result of acid generation. The range of pH values that are ideal for the formation of lactic acid is 5.0 to 7.0. For Lactobacillus bacteria, which are known to tolerate lower pH than lactococci, a pH below 5.7 was ideal(Y. Wee et al., 2004).

The pH range for lactic acid generation is typically between 5.0 and 7.0.The cell began to use glucose earlier and more quickly at pH 6.5 than it did at other initial pH values. At the starting pH of 6.5, the maximum lactic acid concentration was reached (Boontawan, 2010) .Early in the fermentation process, a quick pH decrease is crucial to producing a high-quality end product (Alimentaria et al., 2005).

Table 2 2 Previous Studies on the Effect of pH on Lactic Acid Production

pH	Concentration of Lactic Acid	References
7.0	Highest yield (4 g/l)	(Y. J. Wee, Kim, et al., 2006)
4.0-7.0	Increase with the increasing of pH.	(Huang et al., 2005)
6.0-6.5	Highest yield of lactic acid obtained.	(Senthuran et al., 1999)
5.0-6.0	Highest yield (41.0 g/l)	(Tango & Ghaly, 1999)

2.6.2. Effect of temperature

One of the key elements influencing the development of microorganisms is temperature. The maximum temperature at which the growth rate reaches its maximum was found to be influenced by the properties of the microbe that was employed. In the meanwhile, certain microorganisms will eventually die or have reduced microbial activity if the ideal temperature is higher than or above the temperature required by them (Tango & Ghaly, 1999).In certain situations, the

temperature that produced the most productivity was lower than the temperature that produced the highest lactic acid mass concentration and yield (Siebold et al., 1995) yet, in other situations, the same temperature produced the best outcomes across the board (Linko, 1996) The majority of species can develop within a certain range of temperatures, although their rates of growth vary over this range. The pace at which enzymes in the cell catalyze chemical reactions controls the proliferation of microorganisms. The temperature range at which lactic acid bacteria may grow is 5 to 45 °C. With each rise in fermentation temperature between 30℃ and 40 °C, the lactic acid output rose. Lactic acid production decreases at 45 °C because growth is not at its optimal point at this temperature, resulting in a reduced output. The maximum lactic acid yield of 79.8% was reached at 40 °C (Busairi, 2010).

Table 2-3 Previous Studies on the Effect of temperature on L.A Production

Temperature (°C)	**Yield of Lactic Acid (g/L**	**References**
20-40	Optimum	(Jawad et al., 2013)
42	10.00	(Tango & Ghaly, 1999)
37	28.73	(Idris & Suzana, 2006)
30	60	(Åkerberg & Zacchi, 2000)
30	80	(Linko, 1996)

2.6.3 Effects of Fermentation Time

One of the crucial environmental factors influencing content, molecular mass, and sugar composition is fermentation duration. A higher initial substrate concentration results in a slower development phase. The growth phase, according to (Palaniraj & Nagarajan, 2012)is seen from 0 to 40 hours with varying starting substrate concentrations. The final stage occurs after more than 40 hours. The exponential phase lasted for twelve to forty hours. In addition, a research conducted by (Bhatt & Srivastava, 2008),) indicated that the rate of lactic acid generation remains nearly constant after 48 hours. The 48-hour period showed the maximum lactic acid concentration. Furthermore, as per (Vishnu et al., 2002the maximum production of lactic acid was obtained with low substrate obtained in 48 hours.

Table 2-4 Previous Studies on the Effect of time on Lactic Acid Production

Fermentation Time	Concentration of Lactic Acid	Reference
40 hour	52 g/l	(Palaniraj & Nagarajan, 2012)
After 40 hour	52 g/l	
After 48 hour	52 g/l	(Bhatt & Srivastava, 2008)
48 hour	78±1.2 (g/l)	
48 hour	8.7-9.6 g/l	(Vishnu et al., 2002)

2.7 Industrial application area of lactic acid

According to (John et al., 2007), lactic acid is a multipurpose compound with several uses in the food, pharmaceutical, leather, and textile industries.

2.7.1. food industries

Among its many uses in the food business are as a flavoring ingredient, pH regulator, preservative, shelf life extender, and infection control. Because lactic acid has a somewhat acidic nature and prevents germs from growing on food, it is utilized in food preservation (Scarnato et al., 2017) It works as a food preservative by inhibiting putrefying microorganisms, so preventing food from spoiling. Additionally, it is employed in the production of rye bread. To increase the shelf life of meat, poultry, and fish, lactic acid is added in the form of sodium or potassium lactate. Lactic acid is used as an acidulant in baked products, salad dressings, pickled vegetables, and drinks because of its mildly acidic flavor. (Y. J. Wee, Kim, et al., 2006). Lactic acid or its salts are finding new use in the packing and disinfection of carcasses, especially fish and poultry (Delgado et al., 2016) When lactic acid and its salts were added to aqueous solutions during processing, the shelf life was extended and the growth of anaerobic spoilage organisms like Clostridium botulinum was inhibited (Datta et al., 1995) The food industry uses lactic acid as an emulsifier; however, heat-stable emulsifiers that can be synthesized are needed for baking purposes (Castillo Martinez et al., 2013).

Lactic acid's inherent presence in dairy products, together with its strong antibacterial properties and dairy flavor, make it a great acidification agent for the dairy sector. It also improves the flavor of savory foods. Chocolates and other sweets include lactic acid to give them flavor and maintain the proper pH. The ability to produce transparent candies, handle chocolate and candy with ease, and have a low inversion rate are other benefits of including lactic acid into the process (Krishna et al., 2018) Lactic acid has a low inversion rate, is easy to handle, and may be used to make transparent candies, which are some of its benefits. Fortifying food items with minerals is another possible use for lactic acid in the food business.

2.7.2. Chemical industries

According to (Kim et al., 2022)lactic acid finds use as a pH regulator, descaling agent, neutralizer, cleaning agent, slow acid release agent, green solvent, and antibacterial agent. Because of its strong solvency power, lactic acid is a great polymer and resin remover (Wee et al. 2006). Lactic acid has two reactive functional groups—a carboxylic group and a hydroxyl group—which allow it to react chemically in a number of ways to produce useful compounds.

Large-volume oxygenated compounds including propylene glycol, propylene oxide, acrylic acid, and acrylate esters, as well as other chemical intermediates such lactate ester plasticizers, may be produced using lactic acid. It is possible to expand and combine the technological advancements in hydrogenolysis to produce propylene glycol from lactic acid in the future (Datta and Henry, 2006).

2.7.3. Pharmaceutical industries

Application in the pharmaceutical In the pharmaceutical sector, lactic acid is a crucial component. Since the human body cannot metabolize D-lactic acid, L+ lactic acid is crucial to the pharmaceutical industry. Numerous uses for lactic acids with various salts exist in the medical domain. They have the capacity to produce energy and aid in ph regulation. Anti-tumor action is demonstrated by lactic acid in conjunction with calcium, sodium, or iron (Alsaheb et al., 2015).

One of the most significant ingredients in pharmaceutical manufacturing is lactic acid. Pharmaceuticals indicate the existence of L(+) lactic acid since the human body does not

metabolize the D(-) isomer. Numerous academic publications have reported on the medicinal uses of lactic acid and its salts. In addition to controlling pH, they can provide blood volume and energy. The pharmaceutical industry uses calcium, iron, sodium, and other lactic acid salts in various formulations because they have anti-tumor potential. In addition, lactic acid and its salts are used in tropical verruca treatments and as an intermediary in pharmaceutical manufacturing to modify the pH of preparations (Martínez-Miranda et al., 2022).

Consequently, the following categories describe the uses of lactic acid that are now being used in the pharmaceutical industry: Parenteral,(Patil et al., 2007) (Manuscript et al., n.d.) Dialysis solution medication distribution system under control (Athanasiou et al., 1996)Ammonium lactate (Ademola et al., 2002),for conditions involving dry skin; mineral lactate formulations for illnesses including osteoporosis, anemia, and hypertension; and chiral synthesis.

The use of polylactic acid as a drug-releasing matrix is possible. Because PLA is quickly hydrolyzed to its monomers (hydroxyl acid monomers), which are easily metabolized by the human body through the tricarboxylic acid cycle (TAC) when applied to or added to the body, PLA is thought to be the most well-suited biopolymer (Athanasiou et al., 1996).

2.7.4. Cosmetic industries

A significant component of many cosmetic products is lactic acid. Because lactic acid has moisturizing, antibacterial, and renewing properties for the skin, it is utilized in the production of oral hygiene and hygiene products. Lactic acid derivatives, including lactate esters, are frequently employed due to their emulsifying and hygroscopic qualities(Gao et al., 2011) Creams, shampoos, lotions, and other items for the skin and hair are made with lactic acid. Many cosmetics have negative effects on the skin, such as roughness, inflammation, or dryness. Nevertheless, lactic acid cosmetics serve to address these issues and also enhance skin quality (Castillo Martinez et al., 2013).

When lactic acid is combined with other ingredients, it intensifies their beneficial characteristics. Lactic acid improves the lubricity, smoothing, and softening properties of hair and skin care products. Additionally, it offers defense and resistance against the drying impact of makeup. Because lactic acid cosmetics include vegetable oil, they feel greasy to the touch. (Heilig,

1994).Lactic acid treatment leaves skin smooth, thick, and wrinkle-free. Treatment with lactic acid has an anti-aging impact on the skin (Smith & Canaan, 1996).

Moisturizers:- Achieving flawless skin devoid of imperfections or wrinkles is a challenging task. Certain moisturizers contain lactic acid with the purpose of treating these and other skin care issues. One of the AHAs that has significant advantages for the skin is lactic acid. Because it minimizes the visibility of wrinkles and fine lines, it functions as an anti-aging tool. There may be obvious indications of skin sagging in the neck and cheeks as we age. The creation of collagen is encouraged by lactic acid, which firms the skin. Another important factor in lightening age spots is lactic acid(Bouwstra & Ponec, 2006).

Skin treatment: - Alpha hydroxyl acid lactic acid is utilized as a chemical exfoliator in skin care products. By removing dead skin cells that build up on the surface of healthy skin, lactic acid lotions help skin. Because they can't be absorbed, these dead cells produce dry, dull skin and can interfere with the effectiveness of your skin treatments. One of the most crucial aspects of a skin care routine is getting rid of this buildup (Evans & Johnson, 2010).

2.7.5. Polymer industries

Lactic acid polymers, often known as poly lactides, are aliphatic polyeaters that are extensively studied and employed in thermoplastic polyesters and bioplastics. 2-hydroxy propionic acid serves as the foundation for PLA and is the main component of PLA. PLA is a thermoplastic that can be composted and biodegraded; it comes from renewable plant sources (Lim et al., 2008).Petrochemical polymers can be substituted by these lactic acid polymers. The goal of PLA technologies is to produce polymers with better mechanical, chemical, and biological qualities than petrochemical polymers(Rasal et al., 2010).

A biodegradable polymer called biodegradable poly(L-lactic acid) (PLLA or PLA) has been given approval for use in food packaging in a number of nations, most notably the United States, the European Union, and Japan(Narayanan et al., 2004). Environmental worries about the disposal of plastics have led to a rise in interest in biodegradable polymers. Conventional plastic materials are not easily degraded in the environment because of their high molecular weight and hydrophobic character (Kharas et al., 1994).Because of their large molecular weight and

hydrophobic nature, conventional plastic materials are difficult to decompose in the environment (Kharas et al., 1994).

PLLA is a kind of biodegradable polymer that has the potential to replace non-biodegradable plastics made from petrochemicals in an ecologically beneficial way (D. R. Lu et al., 2009) .According to Kharas et al. (1994), PLLA is an aliphatic polyester that falls within the α-hydroxy group, making it the most easily biodegradable thermoplastic substance. There are 22 functional groups in L-lactic acid, including hydroxyl and carboxylic acid groups. Thus, this material may self-esterify to produce poly(L-lactide), a linear polymer, and L-lactide, a cyclic dimer (Nair & Laurencin, 2007).

High-molecular weight polylactic acid (PLLA) can be produced from L-lactic acid via ring-opening polymerization, polycondensation, or other techniques (chain extension, grafting). L-lactic acid is dehydrated to produce relatively low molecular weight polyester, which is then depolymerized to produce L-lactide, therefore preparing the ring-opening polymerization. Next, catalytic ring-opening polymerization is used to transform pure L-lactide monomer into poly(L-lactide) (Kharas et al., 1994). L-lactic acid is dehydrated by condensation and then extended in a chain to create polycondensation (Stolt, 2002)(Narayanan et al., 2004).The molecular weight and polymer processing factors determine the degree of crystallinity in PLLA, a semi-crystalline polymer with around 37% crystallinity. Its melting temperature is around 175°C, and its glass transition temperature is between 60 and 65°C (Middleton & Tipton, 2000).

It is heat-sensitive, particularly when the temperature rises beyond 190°C (Kharas et al., 1994). According to (Nair & Laurencin, 2007).PLLA has a high modulus (around 4.8 Giga Pascal, GPa), low extension, and strong tensile strength. However, in some applications, PLLA's thermal stability is insufficient to serve as a viable substitute for commercial polymers. The thermal breakdown of polylactic acid (PLLA) is caused by thermal operations including spin and melt molding (Tsuji & Fukui, 2003).

2.7.6. Other application

Other industries also make use of lactic acid. For instance, technical-grade lactic acid is widely employed in the vegetable and leather tanning industries as an acidulant to delim hides. In

several technological procedures, lactic acid is employed as a humectant, solvent, cleaning agent, descaling agent, and slow acid-releasing agent (John et al., 2007). It is utilized as solder flux, lithographic and textile printing developers, electroplating and electropolishing baths, detergent builders, alkyl resin modifiers, pH adjusting in hardening baths for cellophanes used in food packaging, and terminating agents for phenol formaldehyde resins. Furthermore, according to (Giménez et al., 2005). it is employed as an adhesive in the lamination and extraction of gelatin from fish waste.

LA is utilized as a decalcifier in restrooms and toilets because of its descaling characteristics. For degreasing, lactate esters like methyl or ethyl lactate are utilized. Due to its distinct complexing constant for Ni, LA is utilized in the Ni plating process. When some kinds of surfactants are produced for usage in specialty detergents and personal care products, LA is utilized as a neutralizer (Viljanmaa et al., 2003). PLA has gained popularity recently as a simple-to-print substance. PLA's remarkable printability and adaptability are the main reasons for its appeal in 3D printing. PLA has been used more and more in the biomedical area, particularly in recent years, for applications such scaffolds in tissue engineering. (Van den Eynde & Van Puyvelde, 2018).

According to (Giménez et al., 2005).it is also utilized as an adhesive for the lamination and extraction of gelatin from fish waste. In the process of making certain surfactants for use in specialized detergents and personal hygiene products, LA is utilized as a neutralizer. Additionally, it is used in the industrial glue lamination process for hot melt formulation. (Viljanmaa et al., 2003).

The recent reports on applications of LA and lactic acid bacteria in various fields are summarized below

- **Poly lactic acid (PLA**) is a highly processable, biocompatible plastic that is also environmentally beneficial. According to (Rasal et al., 2010).it has been applied in biomedical applications such as medical implants in the forms of rods, plates, fibers, and beads for bone and tissue engineering.

- **Poly lactic-glycolic acid polymer (PLGA)** is regarded as one of the top biomaterials for medication delivery in terms of functionality and appearance. Tailor-made PLGA is available for both short-term (one month) and long-term (up to six months) release, depending on the amount of time needed for the medicine to release. PLGA may be shaped into nano/micro spheres, hollow fibers, and millimeter-sized implants that can be injected into the human body through a variety of channels, depending on the requirements and kinds of materials to be encapsulated (drugs, peptides, and proteins) (Makadia & Siegel, 2011).
- **Lactic acid bacteria secrete exopoly saccharides** (EPS), which are considered to play a vital role in protection against desiccation, toxic compounds, bacteriophages and osmotic stress, and to permit adhesion to solid surfaces and biofilm formation. Apart from these, they have antitumor, antiulcer, immunomodulation and cholesterol lowering activities which have resulted in their applications in food industry. In addition, bacterial EPSs are resistant to human gastrointestinal juices selectively enhancing the colonization of human beneficiary bacteria in the colon whose function resembles probiotics that help in digesting polysaccharides present in the human diet such as fructooligosaccharides, galactooligo saccharides and inulin, which are not digested by the native human digestive system(Hongpattarakere et al., 2012).
- **Sporulated Lactobacilli**, Lactococcus, Pediococcus, For use in infant diapers, sanitary napkins, and panty liners, Bacillus coagulant (7.5*107 cfu/g) was combined with hard fat (hydrophobic carrier), polyacrylic acid (superabsorbent), and zeolite (odor absorbent). These bacteria are part of the typical microbial flora of the vagina, and using them in sanitary napkins can help women prevent vaginal infections (States, 2003).

Lactic acid bacteria are generally regarded as safe since they have GRAS and QPS (Qualified Presumption of Safety-EU) status. They are also crucial for the preservation of food. The primary way that lactic acid bacteria shield food is by the production of antifungal substances such fatty acids, carboxylic acids, ethanol, carbon dioxide, hydrogen peroxide, and bacteriocins. The flavor, texture, and nutritional content of food items can all be enhanced by these lactic acid

bacteria. Bread was preserved using L. plantarum FST 1.7, dairy products, fresh fruits, and vegetables were preserved using L. fermentum Te007, L. pentosus G004, L. paracasei D5, and P. pentosaceus Te010.(Pawlowska et al., 2012).

2.8. Method of starch hydrolysis

The second polysaccharide in nature is starch, which is produced by plants as a reserve substance. It is a biopolymer made up of two different types of molecules, amylose and amylopectin, formed by α-D-glucose bound α-(1→4) and α-(1→6). Hydrolysis, which can be accomplished with the aid of acids or enzymes, is the act of severing these boundaries. Acids or enzymes can be used to achieve hydrolysis.

2.8.1. Acid hydrolysis

Using an acid as a catalyst, glucose molecules are broken down into smaller parts in a process known as acid hydrolysis. The glucose molecule's glycosidic bonds are broken during this process of hydrolyzing glucose. Individual glucose units in polysaccharides like cellulose and starch are joined by glycosidic linkages. By providing a proton (H+) to the glycosidic link, the acid catalyzes the hydrolysis step, causing a hemiacetal intermediate to develop. Subsequent hydrolysis of this intermediate can produce individual glucose molecules.(S. Wang & Copeland, 2013) Starch was frequently hydrolyzed with the use of catalytic acids like sulfuric and hydrochloric acids(Mironescu & Mironescu, 2017).

Starch slurry serves as the starting point for the production of glucose syrup. It is subsequently purified using ion-exchange resins or powdered or granular activated carbon methods, and neutralized with sodium carbonate. However, because starch hydrolysate contains glucose, maltose, and other sugars, there may be a danger of microbial contamination. (S. Wang & Copeland, 2013)**.** Many industries employ acid conversion to produce glucose syrup because of its benefits, which include low material costs, fast hydrolysis times, and ease of installation, particularly for the sugar confectionery business.

A starch slurry that is typically generated at a temperature of 20 to 30 °C and has a dry material content of between 30 and 40 weight percent is acidified to a pH of 2 or less and then undergoes

a series of temperature, pressure, and time changes to facilitate hydrolysis Higher temperatures, made possible by operating under pressure, significantly shorten reaction times.

2.8.2. Enzyme Hydrolysis

Because enzymatic hydrolysis is simple to regulate, efficient even under moderate ambient temperatures, and produces no byproducts, it is currently replacing acid hydrolysis in most cases. Plant and microbial amylase enzymes are used in the enzymatic hydrolysis of (Mironescu & Mironescu, 2017) Gelatinization, hydrolysis (liquefaction and saccharification), carbon treatment, ion exchange treatment, and evaporation to final particles are all steps in the commercial hydrolysis of starch by enzyme hydrolysis (Peter Hull, 2010).Below is a description of the enzymatic manufacturing procedure.

The enzyme amylase, which is produced from, breaks down starch. An endoacting enzyme called α-amylase rapidly breaks down the whole starch structure by hydrolyzing α-(1, 4)-glycoside bonds inside the starch molecule at random(Singh et al., 2007).

2.8.2.1 Gelatinization

Starch has to be formed into slurry and boiled to produce a gel or paste in order for an enzyme to be efficient in converting it. Next, the temperature and pH must be changed to within the enzyme's operating range (Peter Hull, 2010) Simply put, gelatinization is the process of disrupting hydrophobic interactions and inter-and intramolecular hydrogen bonds inside the starch granule. This leads to granular swelling, solubilization, loss of birefringence, crystallite melting, and viscosity development(Megavitry & Nurhijrah, 2019).

For each kind of starch, a particular temperature is required for gelatinization. It is determined by the ratio of amylose to amylopectin, granule size, moisture content, and degree of crystallinity inside the granule (Rahman et al., 2022).

About 30–35% of the starch slurry should be made up of solids. The initial high viscosity of the starch paste is the reason why the starch slurry has less solids than acid hydrolysis; by utilizing less starch solids, the paste's viscosity is brought down to more manageable proportions. It is

possible that the enzyme will be killed if shearing reduces the viscosity of the starch paste (Peter Hull, 2010).

2.8.2.2 Liquefaction

Traditionally, liquefaction refers to the melting of starch gel to produce a reduced viscosity by hydrolyzing starch into smaller oligosaccharide molecules or dextrin by employing the α-amylase enzyme (Bednarska, n.d.).

The parameters that are appropriate for the enzyme to be used in such an enzymatic process dictate the pH and temperature of the process (Rahman et al., 2022).

In this procedure, water soluble maltose and higher maltodextrin oligomers are produced by the hydrolysis of the α-1,4-glycosidic linkages in pregelatinized starch by thermostable α-amylase (Tochtrop et al., 2011).Enzymatic liquefaction necessitates meticulous regulation of reaction parameters, including calcium level, pH, temperature, duration, and percentage of solids, in order to guarantee effective hydrolysis, limit enzyme expenses, and lower the likelihood of issues during downstream process.

2.8.2.3 Saccharification

Saccharification, or the liquefaction of starch into a sugar syrup, is the second enzymatic step. A single enzyme (glucoamylase) or a combination of enzymes (glucoamylase and pullulanase) hydrolyzes liquid dextrin further to produce glucose during saccharification (Megavitry & Nurhijrah, 2019).According to the functioning state of the saccharifying enzyme(s), the liquefied hydrolysate containing 30–35% (w/w) solids is now adjusted to pH 4-4.5 and 55–65°) (Rahman et al., 2022).

During saccharification, glucose is the breakdown product that results from the addition of glucoamylase. Glucose is separated from the non-reducing end of the substrate molecule. The hydrolysis rate of maltotriose, and especially maltose, is lower than that of higher saccharides, and the breakdown of α-l,6-linkages is slower than that of α-1,4-linkages. Eventually, starch is almost entirely converted to glucose is obtained.

Recently, pullulanase has seen a rise in uses in the starch-based sectors, particularly in those that aim to produce glucose. During the saccharification process, pullulanase, an essential de branching enzyme, is widely used to hydrolyze the α-1,6 glucosidic linkages in starch, amylopectin, pullulan, and related oligosaccharides. This completes and effectively converts the branched polysaccharides into small fermentable sugars. Two sequential enzymatic processes are involved in the commercial production of glucose: saccharification, which further converts maltodextrins into glucose, and liquefaction, which occurs after α-amylase has gelatinized the material. Pullulanase has been utilized in the saccharification process to raise the final glucose concentration while using less glucoamylase. Consequently, the process of reversion, which entails the synthesis of saccharides from glucose molecules again, is stopped(Hii et al., 2012).

2.9. Taro in Ethiopia

With a history spanning more than 9,000 years, taro is among the world's oldest food crops. Originally cultivated in Southeast Asia, this ancient crop has expanded globally and is currently a significant crop in Asia, the Pacific, Africa, and the Caribbean (Rao et al., 2010).The main reason this tropical plant is produced is for its tasty corms. This hardy and very varied plant can have leaves up to two meters tall. Its peltate leaves have a robust, more than one-meter-long petiole. It grows or naturally occurs next to 500–2000 m streams or waterfalls. It is grown in South-East Asia and the Hawaiian Islands in moist environments for its starch-rich tubers(Hedberg, 1996).

It has been widely grown as a food crop and a means of solving economic issues in Ethiopia's heavily inhabited, highly precipitation regions in the country's southwest, west, and south. Since the famine of 1984, Ethiopians have recognized the potential of this crop (Tewodros et al., 2013).

Figure 2.1 taro and its growing area in Ethiopia

Among the primary root crops (Irish potato, cassava, enset, and others) planted in the country's main producing areas, taro ranks third in terms of area coverage and output during the previous five years, with sweet potatoes coming in second. As stated by (Dagne et al., 2014). Some colloquial terms for taro are "godare" in Amharic and "Boyna" in Wolaitta, which are comparable to "taro" in English.

According to The federal democratic republic of Ethiopia central statistical agency agricultural sample survey for production of major crops (source-Minister of agriculture)

Table 2 5 Production of Crops for Private Peasant Holdings for Meher Season (Production In Quintals)

	2008	2009	2010	2011	2012	2013
Potato	9,432,334.43	9,214,031.85	9,689,696.44	10,444,363.59	9,245,283.61	11,418,717.25
Sweet potato	13,723,268.22	19,397,611.90	18,484,137.40	14,911,089.33	17,558,547.48	15,988,384.91
Taro	12,112,217.60	12,179,164.45	11,797,769.33	14,633,644.48	14,527,644.92	23,279,722.57

2.9.1. Taro starch

Colocasia esculenta, a starchy tuber that is esteemed for its culinary and therapeutic properties across the world, is the scientific name for taro root. The starch derived from processing taro roots has certain qualities that make it a desirable option for this use(T. J. Lu et al., 2008).

Taro root starch's high starch concentration makes it a plentiful supply for starch extraction, which is one of its main benefits as a feedstock. Moreover, taro root starch's aptitude for lactic acid synthesis is greatly influenced by its composition, particularly the ratio of amylose to amylopectin. This ratio has an impact on the starch's physicochemical characteristics and how it behaves during fermentation.

Taro, a plant belonging to the Araceae family, is well known for having a high starch content as well as a rich protein, mineral, and vitamin composition. Taro corm quality, which refers to the swelling subterranean stem from which taro root starch is extracted, is determined by several parameters including composition, starch concentration, cellulose and lignin levels, and taste. Current investigations center on the growth of the amyloplast, the organelle in charge of starch production in plant cells, as well as the physicochemical characteristics and processes of taro starch extraction(Warkoyo, 2019).

2.9.2. Taro root starch as a feedstock for lactic acid

The white, odorless powder known as taro root starch is made from the corms of the taro plant (Colocasia esculenta) and is used in cooking for its thickening qualities. It has a high amylopectin content, which is readily hydrolyzed into sugars that may be fermented. Taro root starch is a desirable option for the manufacture of lactic acid since it also has a reasonably high starch content. Taro root starch's high starch content and advantageous composition make it an excellent feedstock for the synthesis of lactic acid. Taro root's appeal as a sustainable feedstock alternative is further enhanced by its availability in different places(Petrova et al., 2013).

There are numerous crucial processes in the conversion of taro root starch into lactic acid. Firstly, the starch is degraded by acid or enzyme hydrolysis to produce glucose. Fermentable sugars that are released during this hydrolysis act as substrates for the fermentation of lactic acid. The glucose is subsequently fermented under controlled circumstances by lactic acid bacteria,

such as Lactobacillus species, to form lactic acid. The productivity and yield from taro root starch may be increased by optimizing the fermentation process(Moorthy et al., 2021).

Numerous techniques for removing starch from taro roots and turning it into lactic acid have been investigated in research investigations. Research has concentrated on employing enzymes or acids to hydrolyze taro root starch into fermentable sugars. Additionally, research has been done to evaluate the effectiveness of lactic acid bacteria's fermentation of taro root starch hydrolysates in lactic acid production. Researchers have also explored optimizing fermentation conditions, such as pH, temperature, and nutrient supplementation, to maximize lactic acid yield from taro root starch.

2.10 Characterization method of lactic acid

2.10.1 Determination of Lactic Acid by Ferric Chloride

Ferric chloride can be used to do spectroscopic analysis on lactic acid. The lactic acid and ferric chloride reaction, which results in a colorful complex that can be detected with spectroscopic methods, is the basis for this procedure. Ferric chloride measurement of lactic acid is extensively employed in a number of sectors, including clinical diagnostics, pharmaceuticals, and food and beverage. The development of a colorful complex between ferric chloride and lactic acid is the basis for the spectroscopic measurement of lactic acid using this method. When lactic acid and ferric chloride combine, a complex with a distinct visual absorption spectrum is created. The quantitative measurement of lactic acid in solution is made possible by this complex's ability to absorb light at particular wavelengths (Borshchevskaya et al., 2016).

2.10.2. Determination of Lactic Acid content by titration

One often used technique in analytical chemistry is the titration method for determining concentration of lactic acid. Using an acid-base titration, this technique depends on the neutralization reaction between lactic acid and a strong base, such as sodium hydroxide (NaOH).During the titration procedure; slowly add the burette's standardized NaOH solution to the titration flask's lactic acid sample solution while continually spinning the flask. The

phenolphthalein indicator's pink hue vanishes as the lactic acid and NaOH react. This signifies that the lactic acid has been neutralized(Niro & No, 2006).

2.10.3 Determination of lactic acid content using FTIR analysis

A Thermo Nicolet 380 FTIR spectrometer with Attenuated Transmission and an internal reflection accessory consisting of composite zinc selenide and diamond crystals were used to perform FTIR spectroscopy studies. Each spectrum had an average of 32 scans at a resolution of 4 cm-1. The lactic acid spectra were gathered throughout a range of 4000 to 400 cm-1. Pre-processing methods like polynomial substrate and Gaussian smoothing were used to eliminate high-frequency noise and baseline shifts from the spectra in order to improve the quality of the data. The program Delight Version 3.2.1 was used to analyze the data, making it possible to identify distinctive peaks linked to the functional groups of lactic acid.

A partial least squares (PLS) model was utilized for quantitative analysis in order to forecast the lactic acid contents in the examined samples. PLS latent variable count was maximized to reduce overfitting and improve prediction precision. For analyzing the content and structure of lactic acid samples, FTIR spectroscopy is a flexible method that is often used in the food and beverage, materials science, and pharmaceutical industries(Huisuo et al., 2018).

2.10.4. Determination of lactic acid content using HPLC analysis

.The lactic acid samples were analyzed using the same HPLC process that was previously developed using the calibration curve. The autosampler was used to inject the samples into the column, and the chromatograms were examined using the established HPLC parameters and the calibration curve. This made it possible to precisely measure the amounts of lactic acid present in the samples.

3. Materials and Methods

3.1. Chemicals and Equipment's

Sample collection:-In this study, taro waste was collected from Arbaminch in southern Ethiopia and Shiromeda in Addis Ababa.

Chemicals:- Ondea Pro's Enzymes, which include α-amylase and pullulanase, were obtained from Dashin Brewery Share Company in Debre Birhan, Ethiopia. Various reagents, such as 3,5-dinitrosalicylic acid (DNS)(98%), sodium metabisulphite,(96.5%) sodium hydroxide(98.8%), sulfuric acid(98%), standard lactic acid(99.5%), MRS agar, MRS broth, and alcohol(70%), were purchased from suppliers in Addis Ababa. Additional chemicals and reagents, including distilled water and various compounds, were obtained from the Faculty of Chemical and Bio Engineering Laboratory at Addis Ababa Institute of Technology. All the chemicals and reagents used in this analysis were of analytical grade, ensuring their suitability for precise measurements.

Equipments:-The main equipment utilized in this study were Test tubes, crucibles, measuring cylinders, aluminum foil, cotton roll, label paper, extraction thimbles, Vacuum filter, pH meter, Erlenmeyer flasks, mechanical stirrer, digital balance, Soxhlet apparatus, water bath, vacuum evaporator, Analytical balance Oven, Filter paper, Buchner funnel membrane Centrifuge, muffle Furnace, Autoclave, Incubator shaker, UV-spectrophotometer, refractometer, colony counter, vortex mixer, biological safety cabinet, refrigerator.

Bacteria source: The bacteria (*Lactococcus lactis, Enterococcus faecalis,* and *Lactobacillus paracasei*) were obtained from the 'Ethiopian Biodiversity Institute' in Addis Ababa.

3.2. Experimental methods

The production of lactic acid typically require a sequence of steps, as depicted in Figure 3.1.This diagram illustrates the processes involved in the production of lactic acid.

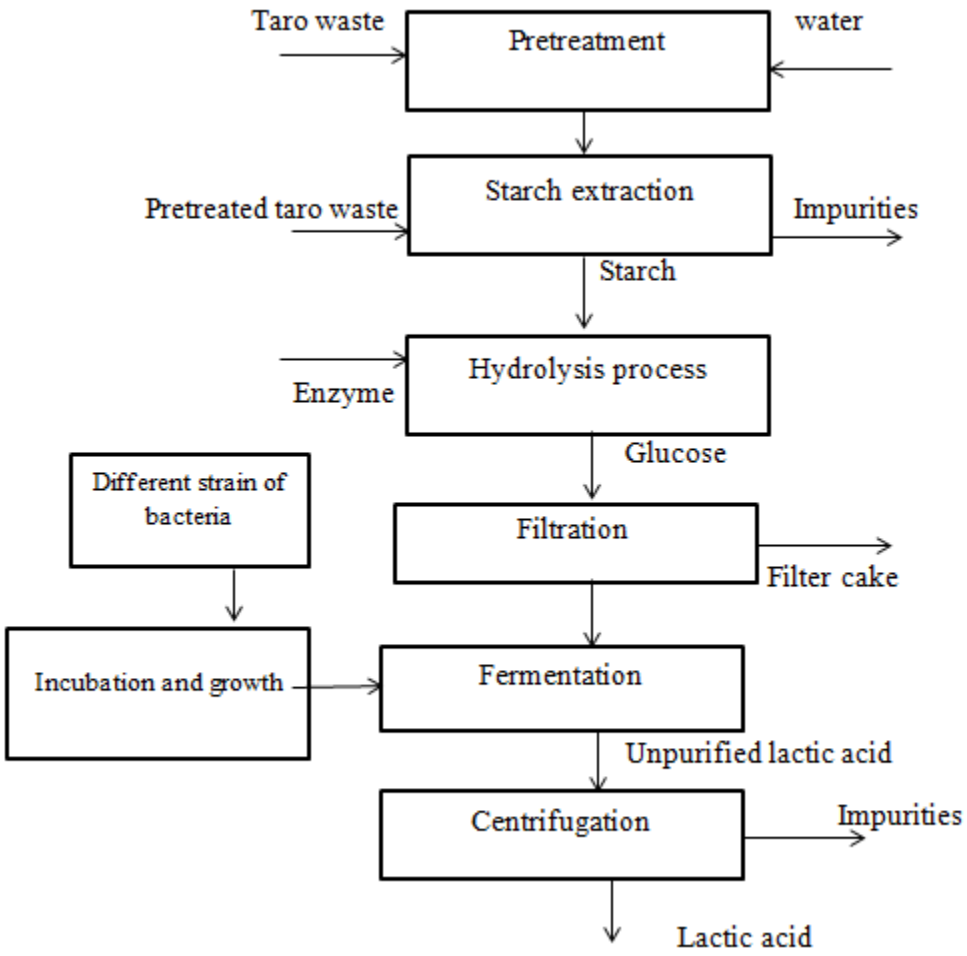


Figure 3-1 Process flow diagram for lactic acid production from taro waste

3.2.1. Raw material collection and starch extraction process

For this research, taro waste samples were obtained from two locations: Arbaminch in southern Ethiopia and Shiromeda in Addis Ababa.In the described method by Gebre-Mariam and Schmidt, the isolation of starch from the waste taro involved several steps. Initially, the taro

waste was washed and cleansed to remove any impurities. The cleaned tubers were then peeled and blended to form slurry. This slurry was mixed with water and 0.075% (w/v) sodium metabisulphite, (Chemistry et al., n.d.) Agitated well to create a suspension, and passed through a fine muslin cloth to remove cell debris, resulting in a translucent suspension. The suspension was allowed to settle for a period of two to three days, during which heavier starch particles settled at the bottom of the container while other contaminants remained suspended or floated. Once the suspension became translucent after multiple washings of the sediment starch, the supernatant liquid was carefully drained off, leaving behind the settled starch mixture. The starch was then spread out on a tray to dry, followed by grinding it into a fine powder using a grinder after it had completely dried (Gebre-Mariam & Schmidt, 1998).

The amount of isolated starch was calculated using the equation below:

$$\% \text{ yield of starch} = \frac{\text{mass of starch obtained}}{\text{mass of tuber used}} \times 100\% \quad \text{.....Eq1}$$

3.3 Proximate Analysis of starch

3.3.1 Determination of Moisture Content

The moisture content determination method, as described in (Getachew et al., 2016), involved the following steps: Porcelain dishes and lids were dried in an air drying oven at 100°C for one hour to remove any moisture, cooled in a desiccator, and weighed (W_1). The samples were mixed, and dried dishes (W_2) were supplemented with approximately 2g of starch. The dishes and their contents were then dried in an oven at 105°C for three hours, allowed to cool in a desiccator, and weighed again (W_3).

Finally, the moisture content was calculated using the following formula.

$$\text{Moisture content (\%)} = \frac{W3-W1}{W2-W1} * 100\% \quad \text{.....Eq2}$$

Where, W_1 denotes the weight of the dish and lid when they are initially empty,

W_2 the weight of the dish and starch following the addition of the sample, and

W_3: the weight of the dish, starch, and dried sample.

3.3.2Determination of Ash content

The ash value of starch was determined following the method outlined by (Puwastien P, 2011) the procedure involved several steps to accurately calculate the ash content. Firstly, a nickel crucible was carefully weighed and heated to a consistent weight at 105°C. Once the crucible had cooled down, it was filled with 2g sample of starch. The choice of nickel crucible was important due to its resistance to high temperatures. To ensure the majority of carbon content was evaporated, the crucible containing the starch sample was subjected to combustion in a muffle furnace at a temperature of 550°C for duration of two hours. This high temperature and extended duration ensured the complete combustion of organic matter, leaving behind the inorganic ash content. After the combustion process, the crucible was allowed to cool to room temperature. The remaining residue, referred to as the ash, was carefully weighed. To ensure complete combustion, the heating and cooling process was repeated until a constant weight of ash was obtained, indicating that all organic material had been completely burned off. Finally, the weight of the ash was determined by subtracting the initial weight of the crucible from the total weight, including the ash residue.

$$\text{Ash Content} = \frac{Wa - Wc}{Wb - Wc} \times 100\% \quad \text{.....Eq3}$$

Where, $W_{a,}$ is the weight after complete ashing + crucible

W_b is the weight before ashing + crucible

W_c is the weight of the empty crucible

3.3.3 Determination of Protein content

The crude protein content in the sample was determined using the Kjeldahl technique, as described in(Digestion & Weigh, n.d.) This method involved acid digestion of the sample, which converted the organic nitrogen into ammonia. The liberated ammonia was then distilled off and collected. The collected ammonia was subsequently titrated to determine its nitrogen content. By applying a conversion factor specific to the sample, the nitrogen concentration was multiplied to calculate the crude protein content. The Kjeldahl technique provided an effective means of

quantifying the protein content in the sample through the measurement of nitrogen, which is an essential component of proteins.

Digestion: To facilitate the digestion of the 0.5 g starch sample in the Kjeldahl tube, a combination of orthophosphoric acid and concentrated sulfuric acid was added. Following thorough mixing, a gradual addition of 35 milliliters of 30% hydrogen peroxide caused a vigorous reaction. After the initial reaction subsided, the tubes were shaken for a few minutes before being returned to the rack. Each tube was then treated with a catalyst solution consisting of 3 g of potassium sulfate, 100 g of potassium sulfate, and 0.5 g of copper sulfate. This mixture was allowed to stand for approximately 15 minutes before undergoing digestion. The digestion process took place in a digester set at 420°C for an additional hour. To prevent sulfate precipitation within the solution, 15 ml of deionized water was added. Finally, the tubes were transferred to a fume hood for cooling, ensuring proper handling of the potentially hazardous fumes produced during the digestion process.

Distillation To prepare the boric acid indicator solution, a flask was filled with 25 ml of the solution, and the tips of the flask were immersed in the sample. Positioned beneath the distiller's condenser, the flask collected the distilled solution after digestion and dilution. The tubes used in the digestion process were rinsed twice with approximately 5 ml of deionized water, and the rinses were combined with the solution. A small amount of water was added to aid in washing, followed by the introduction of 25 ml of a 40% sodium hydroxide solution. Once the flask was securely sealed, the steam was activated. As a result, ammonium in the form of ammonia was released. The steam distiller was then configured, and the digest was steam-distilled into the receiving solution containing boric acid for approximately five minutes. This process allowed for the determination of the instrument blank and facilitated further analysis.

Titration Following the completion of distillation, the sample underwent titration using standardized 0.1N H_2SO_4 solution. The purpose was to determine the amount of standardized acid needed to neutralize the boric acid present in the sample. The volume of standardized acid required for neutralization was carefully recorded. It is important to note that blank testing was conducted for all reagents used in the process, and their respective volumes were calculated and

accounted for in the analysis. This ensured accurate measurements and precise determination of the neutralization point during the titration process (Angell et al., 2015).

Nitrogen %= $\frac{[(V1-V0)\times N\mathrm{HCL}\times 14]}{W} \times 100\%$.....Eq 4

$$P = F \times N$$

Where;

- V_1: The volume in milliliters of the standard hydrochloric acid solution used in the titration for the test.
- V_0 represents the volume in ml of the standard hydrochloric acid solution used in the titration for the blank.
- N_{HCl}: indicates the normality of the HCl used, which is typically 0.1N.
- F:- is the conversion factor, with a value of 6.25, used to convert nitrogen content to crude protein content.
- N: - corresponds to the percentage of nitrogen in the sample.
- W: - denotes the sample weight on a dry matter basis.
- P: - represents the crude protein percentage.
- 14.00 is the molecular weight of nitrogen, in grams per mole.

3.3.4 Determination of Crude Fat content

To prepare for extraction, the extraction flasks underwent a thorough cleaning process, followed by drying at 100 degrees Celsius for one hour. After cooling in desiccators for 30 minutes, the flasks were weighed. The bottom of the extraction thimbles was covered with a layer of cotton that was approximately 2 cm thick and free from fat. Fresh samples, weighing around 10 grams, were carefully added to thimble and another layer of cotton, also about 2 cm thick and free from fat was placed on top to cover the samples. These prepared thimbles, containing the sample, were then inserted into a Soxhlet extraction chamber. The cooling water was activated, and 90 ml of diethyl ether with a boiling point of 55°C was introduced into the extraction flask through the condenser. Over the course of a 3-hour extraction period, the diethyl ether gradually evaporated

from the extraction flask. After completing each extraction process, the extraction cups were removed from the extractor, dried for 30 minutes at 102°C in an oven to eliminate any moisture, cooled for 30 minutes in a desiccator, and then weighed to obtain the final weight of the extracted substances. This meticulous procedure ensured accurate measurements and consistent extraction results(November, 2006).

$$\text{Crude fat (\%)} = \frac{W2-W1}{W0} \times 100\% \quad \text{.....Eq 5}$$

Where

W_1- indicates the initial weight of the extraction flask prior to the extraction weight of extraction

W_2= refers to the final weight of the extraction flask after the extraction process has been completed.

W_0 = weight of fresh Sample

3.3.5 Determination of Crude fiber content

A weighted sample of 2 grams was placed in a 500-ml beaker, and approximately 200 ml of a 1.5% sulfuric acid solution was added. To observe the procedure, a watch glass was positioned over the beaker's rim, and the liquid was boiled for 30 minutes. Subsequently, 20 ml of a 28% KOH solution was introduced, and the solution was gently heated for an additional 30 minutes. Throughout this heating period, the solution level was carefully maintained by adding distilled water. The sample was then subjected to washing using a solution composed of sulfuric acid and NaOH. (Nutrition, n.d.) Afterward, the washed sample was dried by baking it for two hours at 120°C in an oven. Once cooled in a desiccator for 30 minutes at room temperature, the sample was weighed and transferred to a muffle furnace. The sample underwent further treatment in the muffle furnace at 550°C for 30 minutes. After cooling once again in a desiccator, the sample was reweighed. Finally, the provided equation was utilized to calculate the crude fiber content based on the obtained measurements and observations(International, n.d.)**.**

$$\text{Total crude fiber} = \frac{W2-W1}{WS} \times 100\% \quad \text{.....Eq6}$$

Where:

W1 = Weight of crucible with sample before drying

W2 = Weight of crucible with sample after drying

W3 = Weight of sample

DeterminationCarbohydrate content

The total carbohydrate content is derived by subtracting the sum of the percentages of ash, moisture, protein, fat, and fiber from 100.The calculation can be summarized as (Saed & El-waseif, 2018).

Total Carbohydrate% = 100 - % (ash+ moisture + protein+ fat + fiber) ……..……Eq7

3.3.6 Determination of Amylose content

0.10g sample was weighed and placed in a 100 cm^3 volumetric flask. To the flask, 9 cm^3 of 1 M sodium hydroxide solution and 1 cm^3 of 99% ethanol were added carefully. The components were thoroughly mixed, and the resulting sample solution was boiled in water for 10 minutes to achieve starch gelatinization. After cooling, the solution was agitated and diluted with distilled water to reach the mark on the flask. In a separate 100 cm^3 volumetric flask, a 5 cm^3 portion of the starch solution was combined with 1 cm^3 of 1 M acetic acid and 2 cm^3 of iodine solution. The solution was then diluted to the desired concentration using distilled water. The absorbance of the solution at 620 nm was measured using a spectrophotometer. The absorbance of the sample solution was subtracted from the absorbance of a blank solution prepared in a similar manner. The amylose and amylopectin contents were calculated using specific equations(Hassan et al., 2013).

Amylose content (%) = 3.06×absorbance ×20 ……..Eq 8

Amylopectin (%) = 100 - % amylose content…….…….Eq 9

3.4Enzymatic hydrolysis

To initiate the enzymatic hydrolysis process, the starch solution underwent gelatinization, which involves breaking the intermolecular bonds within starch molecules using heat and water. In this case, a 10 (w/v) isolated starch suspension was prepared by mixing it thoroughly with distilled water in an Erlenmeyer flask. The resulting slurry was homogenized to ensure uniformity, and the pH was adjusted using either 1M HCl or NaOH. The suspension was then heated to 70°C and maintained at this temperature for 40 minutes using a water bath, promoting starch gelatinization(Terms, 2014). This gelatinization process allowed for subsequent enzymatic hydrolysis to occur.

3.4.1 Liquefaction and saccharification

After gelatinization, the starch solution was cooled gradually to reach room temperature. Precise pH adjustments were made using either H_2SO_4 or NaOH solutions, resulting in pH values of 5.5, 5.3, and 5.5. The next step involved adding the 'Ondea Pro' enzyme at a concentration of 0.05% (v/v) to the solution. The mixture was then gently stirred for 2, 3, or 4 hours at 200 rpm in a water bath, while carefully maintaining temperatures at 65°C, 70°C, and 75°C. During this process, the α-amylase enzyme effectively liquefied the starch solution by breaking down the α-1,4-glycosidic bonds, while the pullulanase enzyme facilitated saccharification by cleaving the α-1,6-glycosidic linkages in the starch. These enzymatic actions resulted in the conversion of starch into smaller sugar molecules(Marchal & Tramper, 1999).

3.4.2. Clarification:

Following the hydrolysis process, the crude glucose was subjected to filtration using filter paper. This filtration step served multiple purposes, including the removal of impurities that originated from starch particles that were not fully broken down during hydrolysis. Additionally, the filtration helped improve the color and stability of the glucose solution. By passing the solution through the filter paper, unwanted substances and particles were effectively separated, resulting in a purer and more desirable glucose product (Lambri et al., 2014).

3.4.3 Preliminary experiments for hydrolysis

One-variable-at-a-time (OVAT) experimentation is a method where process factors are tested individually, one at a time, while keeping other factors constant. The objective is to assess the

influence of each parameter in isolation and identify the optimal ranges that result in improved outcomes or higher yields.. By systematically varying one factor at a time while holding others constant, OVAT allows for a focused analysis of the impact of each parameter, aiding in the identification of key variables and their optimal settings for achieving desired results (Vanaja & Rani, 2016). The basis for these values relies on the manufacturer's recommendations.

Table 3-1factor range for hydrolysis

Factor	Levels	
	High	Low
Temperature(℃)	75	65
pH	5.5	5
Time(h)	2	4

Table3-2 OVAT experimental design for enzyme of 0.05% (v/v)

Run	**Temperature(**℃)	**pH**	**Time(h)**
1	70	5	3
2	70	5.3	3
3	70	5.5	3
4	65	5	3
5	70	5.3	3
6	75	5.5	3
7	70	5.3	2
8	70	5.3	3
9	70	5.3	4

3.4.4 Determination of glucose

3.4.4. 1Spectrophotometeric analysis

Total Reducing sugar determination was analyzed using the method described by (Djalal et al., 2019).To prepare 100 ml of DNS,the solution, 30g of Potassium sodium tartrate tetrahydrate was dissolved in 20mL of distilled water. A 2N sodium hydroxide solution was created by dissolving 20mL of sodium hydroxide in water, ensuring complete dissolution through stirring with a glass rod. Additionally, 50mg of sodium sulfite and 200mg of phenol were dissolved in 10mL of water. Meanwhile, 3,5-dinitrosalicylic acid was dissolved in 40mL of distilled water using a magnetic stirrer on a hot plate set at a temperature range of 90-95°C. The solutions were gradually added to the 3,5-dinitrosalicylic acid solution while stirring with the magnetic stirrer. The resulting mixture was then filtered using filter paper to remove any impurities. Finally, the filtered solution was transferred to dark glass bottles and stored at room temperature(Miller Gail Lorenz, 1959).

To prepare the standard solution, 100 mg of anhydrous dextrose (glucose) was dissolved in a 100 mL volumetric flask using distilled water. Various aliquots of the standard glucose solution, ranging from 0.1 mL to 1 mL, were transferred into separate dry test tubes. A blank tube containing only distilled water was also prepared. Each tube's volume was adjusted to 1 mL using distilled water. Then, 1 mL of DNS (3,5-dinitrosalicylic acid) reagent was added to all the tubes. The tubes were incubated in a boiling water bath for 5 minutes and subsequently allowed to cool to room temperature. Finally, 8 mL of distilled water was added to each tube.

The spectrophotometer was adjusted to the specific wavelength of 540 nm, known as the maximum absorbance wavelength for glucose. A cuvette containing a solution without glucose, called the blank solution, was placed in the sample compartment of the spectrophotometer and used as a reference to zero the absorbance. Subsequently, the absorbance of each standard solution was measured at 540 nm using the spectrophotometer, and the corresponding absorbance values were recorded. These absorbance values were then plotted against the concentration of each standard solution on a graph, with concentration on the x-axis and absorbance on the y-axis. A straight line was drawn through the points on the graph, and the

slope of this line represented the molar absorptivity of glucose. The molar absorptivity signifies how strongly glucose absorbs light at 540 nm, providing a quantitative measure of glucose concentration in unknown samples based on their absorbance readings.

The standard graph was used to determine how much reducing sugar was in the sample solution.

$$Y = mx + b$$

Where: y = absorbance

x = concentration

m =slope

b = intercept

$$\text{Conc. unknown sample} = \frac{\text{Absorbance of unknown substance} - (\text{y-intercept})}{\text{Slope}} \quad \ldots\ldots\ldots\ldots\ldots\text{eq 10}$$

To determine the concentration of glucose in an unknown sample, the absorbance of the unknown sample at 540 nm was measured using the spectrophotometer and cuvettes. The obtained absorbance value was then compared to the calibration curve, which was created using the known concentrations of the standard solutions and their corresponding absorbance values. By locating the absorbance value of the unknown sample on the y-axis of the calibration curve, the corresponding concentration value on the x-axis was identified. This allowed for the determination of the concentration of glucose in the unknown sample. It is crucial to store the standard solutions in a refrigerator prior to use to ensure their stability and prevent any degradation that may affect their accuracy in calibration.

3.4.4.2. FTIR analysis of glucose

The functional group analysis of glucose was conducted using Fourier-Transformed Infrared (FTIR) spectroscopy. The infrared spectrum was obtained by passing an infrared light beam through the sample. A spectrum 65 FT-IR instrument (PerkinElmer) equipped with a KBr beam splitter was used for recording the FTIR spectra (Louise Nybacka, 2016)**.**For powder samples, a diffuse reflectance system (DRS) were employed, while a NaCl plate was used for liquid

samples through thin film deposition technique. The spectra were recorded within a regular scanning range of 400-4000 cm^{-1}, with 20 repeated scans at a spectral resolution of 4 cm^{-1}. All the spectra were recorded and processed using essential FTIR software(Shen et al., 2003).

3.5.Growth of bacteria strain

3.5.1. Preliminary experiments for bacterial growth

One-variable-at-a-time (OVAT) experimentation based on growth of bacteria .For the purpose of conducting One-variable-at-a-time (OVAT) experimentation on lactic acid production, three bacterial strains, namely *Lactococcus lactis, Enterococcus faecalis,* and *Lactobacillus paracasei,* were selected. The goal of this experiment is to evaluate and compare these bacterial strains based on their pH levels, number of bacteria growth, and amount of reducing sugars.

Table3-3 optimum parameter range for bacteria strain growth

	Types of bacteria	Temperature(℃)	Ph	Time (h)	Reference
1	*L.b paracasei* (strain A)	28-33	6.3-8	24-48	(Abbasiliasi et al., 2014),(Adu et al., 2020)
2,	*Enterococcus faecalis* (strain B)	33-40	6-8	24-48	(Abe & Honda, 2023), (Gardini et al., 2001),
3,	*Lactococcus lactis* (strain C)	30-39	6.3-6.9	24-48	(Chen et al., 2015),(Sánchez et al., 2008)

Growth of bacterial cell number

To evaluate bacterial growth and quantify the number of bacteria, serial dilutions were performed. A set of six small sterilized test tubes, labeled from 1 to 6 in sequential order, was prepared. The samples were then diluted individually using the following techniques

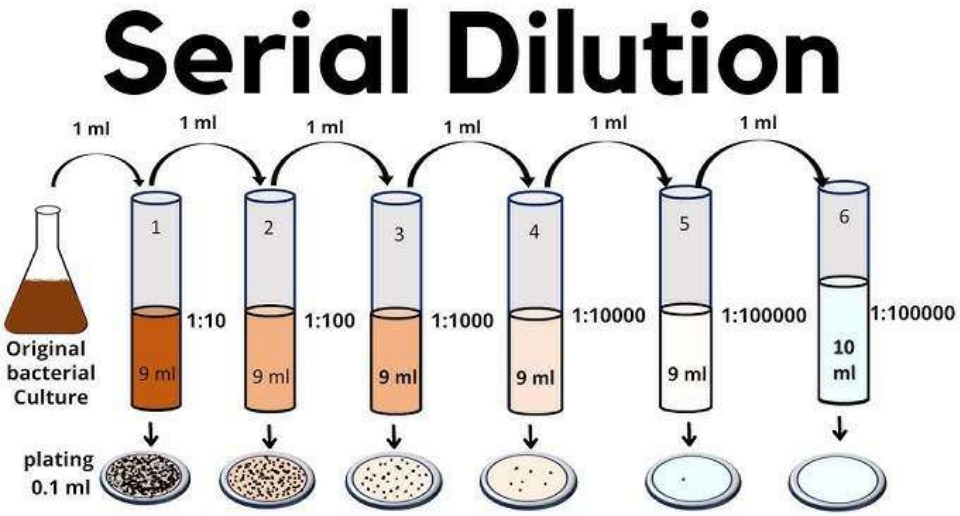


Figure3-2 Serial dilution for bacteria

Individual culture tubes were prepared for each bacterium, and the respective bacterial strains were inoculated into them. Incubation was conducted until the bacteria reached the desired growth stage. Simultaneously, a series of dilution tubes labeled with different dilution factors (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) was prepared, with each tube containing 9 mL of sterilized water. Then, 1 mL of bacterial culture from each culture tube was transferred into the corresponding dilution tube, starting with the initial dilution. Thorough mixing was ensured using a vortex mixer for proper dilution. And The MRS Agar culture medium was prepared, and MRS agar plates were labeled with the specific bacteria species. Subsequently, 0.1 mL of the contents from each dilution tube was taken and plated onto separate MRS agar plates for further analysis(Davis et al., 2005).

The sample was evenly spread on the agar surface using a sterile spreader. Following this, the agar plates with the inoculated samples were placed in a controlled incubator set at temperatures varying from 30 to 40℃ to facilitate the growth of the respective bacteria. Incubation periods ranging from 24 to 60 hours were implemented to allow adequate time for colony development. After completing the specified incubation duration, the agar plates were removed from the incubator. Each plate underwent examination, and the visible colonies for each bacterial strain were enumerated. A colony counter was used to mark and prevent the duplication of counted

colonies. The colony count for each bacterial strain was individually recorded as Colony Forming Units per milliliter (CFU/ml). Using a colony counter, the colonies were marked, and subsequently, the average colony count was calculated for each dilution and bacterial strain(Brugger et al., 2012).

Colony Count = (Number of Colonies Counted) / (Dilution Factor x Volume Plated)…Eq 12

And also the growth of each strain of bacteria was facilitated by the hydrolysis of glucose, with a glucose concentration of 55g/L. The initial optical density (OD) of 0.03, pH control at 6.5, and the specified temperature and time range were maintained for preliminary analysis in an incubator under anaerobic conditions

Table 3-4 preliminary analysis of bacteria growth conditions(OVAT)

Strain	Temperature (°C)			pH control	Time(h)		
A	28	30	33	6.5	24	42	60
B	33	38	40	6.5	24	42	60
C	30	34	39	6.5	24	42	60

3.6. Fermentation process

3.6.1.Fermentation medium preparation

A fermentation medium was prepared in 40 Erlenmeyer flasks, each with a volume of 250mL. Lactococcus lactis bacteria, chosen for their high potential based on initial analysis, were used for the fermentation process. The medium was prepared by taking 100 mL of a fermented broth containing glucose at a concentration of 84g/L, obtained from the hydrolysis process. It was supplemented with 5g/L of yeast extract, 10g/L of proteose peptone, 10g/L of 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 of dipotassium phosphate. To ensure sterility, the prepared medium underwent sterilization at 121°C for 15

minutes, followed by cooling for approximately 2 hours in a sanitized and disinfected hood with proper air circulation. The necessary precautions were taken to minimize exposure during the process. The inoculation loop was sterilized and allowed to cool before transferring the bacteria. A 10mL inoculum of Lactococcus lactis was added from the prepared culture to each of the 40 flasks. With the added inoculum, the prepared medium was now ready for fermentation.

3.6.2 Microbial Collection, Inoculum Preparation, and Cultivation

The vial containing Lactococcus lactis bacteria was kept in a deep freeze at -4°C until the MRS medium was prepared. To prepare the inoculum, a mixture of different reagents such as proteose peptone, beef extract, yeast extract, dextrose, polysorbate 80, ammonium citrate, sodium acetate, magnesium sulfate, manganese sulfate, and dipotassium phosphate was used.following the protocol established by Rogosa and Sharpe in 1960.

A 250 mL MRS broth medium was prepared and sterilized at 121°C for 15 minutes. The vial containing Lactococcus lactis was used to inoculate 10 mL of the MRS medium, which was then incubated at 37°C for 48 hours. The growth of the cells was monitored by measuring the optical density (OD) of the medium at 620nm using a 1.5 mL glass in a UV-spectrophotometer. As the cells multiplied, the optical density increased. The resulting Lactococcus lactis bacteria were transferred to the remaining 240 mL of the previously prepared and sterilized MRS broth and incubated for another 48 hours at 37°C. Finally, the medium was ready for fermentation.

3.6.3Purification of lactic acid

To prepare the lactic acid solution for filtration, solid impurities are first removed by employing a centrifuge. Following this, the setup for filtration is prepared by placing a Buchner funnel on top of a filtration flask, ensuring its cleanliness. A suitable solvent or a small amount of lactic acid solution is used to dampen the filter paper, which is then carefully positioned inside the Buchner funnel, ensuring full coverage. A vacuum source is connected to the filtration flask, creating a passive suction effect. The lactic acid solution is poured slowly into the Buchner funnel, allowing it to pass through the filter paper aided by gravity. Throughout the filtration process, the procedure is observed, and adjustments to the vacuum level are made if necessary.

Finally, the filtered lactic acid solution is collected in a clean container, ready for further analysis or use(Komesu et al., 2017).

3.6.4 Characterization of lactic acid

3.6.4.1 Spectrophotometric determination of lactic acid

To create a stock solution, 1.2 g of lactic acid with a concentration of 89% is diluted in a 10-mL volumetric flask. The resulting concentration of the stock solution is denoted as "x." A solution of iron (III) chloride is prepared by dissolving 0.3 g of the salt in a 100 mL volumetric flask filled with water. For the measurement process, 50 μL of the lactic acid solution is mixed with 2 mL of a 0.2% iron (III) chloride solution. The resulting solution exhibits a distinct color, and its absorbance is measured at a wavelength of 390 nm. A reference solution of 2 mL of 0.2% iron (III) chloride is also prepared and used for comparison during the absorbance measurement. The same process is repeated for the test solution, where 50 μL of the lactic acid solution is added to 2 mL of the 0.2% iron (III) chloride solution. The mixture is stirred, and the absorbance is measured against the reference solution at 390 nm. Throughout the reaction and absorbance measurements, the temperature is maintained at 25 ± 5°C, and the color of the solution remains stable for a duration of 15 minutes(Borshchevskaya et al., 2016).

3.6.4.2. Determination of lactic acid by titration

The fermentation medium was titrated with 1M NaOH using phenolphthalein as an indicator through the treatable acidity method to measure lactic acid production. In order to determine the lactic acid content in the fermentation broth, the centrifuged and filtered supernatant of fermented SGB and BNP hydrolate was transferred to a 500 ml flask. The flask was then treated by adding three drops of phenolphthalein indicator, followed by titration with 1M NaOH until the appearance of a pink color, indicating the endpoint for recording the volume of NaOH used. Titratable acidity was calculated as weight/volume (w/v)(Niro & No, 2006). as shown below

LA (g/lt) =

$$\frac{\text{volume of NaOH consumed for titration} * \text{gram Eq. wt of LA} * \text{N NaOH}}{\text{volume of fermentation broth used for titration}}$$

3.6.4.3. FTIR analysis

FTIR spectroscopy, as an alternative to HPLC for monitoring lactic acid in fermentation processes, involved acquiring absorbance spectra within the 650-4000 cm^{-1} range. An IR-Prestige FTIR spectrometer with an ATR accessory captured the spectra, each comprising 128 scans at a spectral resolution of 4 cm^{-1}.

Instrument calibration was conducted using appropriate standards; with background spectra measured using a blank sample. Samples were positioned on a holder, and instrument parameters were adjusted accordingly before recording the infrared spectrum. Subsequently, spectral analysis tools or software were utilized for processing the obtained spectrum data. By creating a calibration curve based on known lactic acid standards, absorption intensities at specific peaks could be correlated to lactic acid concentrations. The results were analyzed and the lactic acid concentrations were quantified and reported (Păucean et al., 2017).

3.7.Experimental design and statistical analysis for fermentation

In this specific experiment, three factors with three levels were chosen: temperature, pH, and incubation time. The temperature levels selected were 25°C, 30°C, and 35°C, while the pH levels chosen were 5.5, 6, and 6.5. For incubation time, the levels selected were 12 hours, 30 hours, and 48 hours. The experiments were conducted in a randomized order, and the Design-Expert software was used to analyze the main effects and interactions of these parameters and determine the optimal fermentation conditions.

The fermentation process took place in an incubator shaker located in the biochemical laboratory of AAU School of Chemical and Bioengineering. The samples were prepared and incubated within a temperature range of 25-35°C, with incubation times ranging from 12 to 48 hours. The pH during fermentation was maintained between 5.5 and 6.5. The incubator shaker operated at a constant speed of 180rpm, and the fermentation was conducted under anaerobic conditions to prevent the entry of oxygen. Once the specified incubation time was reached, the fermentation process was stopped. The resulting fermented broth was separated using a centrifuge, which ran

at 6,000rpm for 20 minutes. This process allowed for the collection of the clear liquid containing lactic acid.

Design Expert software version 6.0.8 was utilized in this study to implement a central composite design (CCD). The purpose of this design was to investigate the effects of process factors on lactic acid production during fermentation. The chosen process variables were temperature, pH, and time, and their respective ranges were determined based on the outcomes of the One-variable-at-a-time (OVAT) experiments. The glucose concentration and agitation rate were considered constant variables throughout the study. The below factor range is based on the previous study (Sirisansaneeyakul et al., 2007)(Abbasiliasi et al., 2014).

Table 3-5 Factors and levels for fermentation

Factors	Replication=2	
	Levels of factor	
	High	Low
Temperature(℃)	25	35
Ph	5.5	6.5
Time(h)	12	48

Table 3-6 experimental design for fermentation of lactic acid in CCD

Std order	**Run Order**	**Temperature (℃)**	**pH**	**Time (hr.)**
1	14	25.00	5.50	12.00
2	15	35.00	5.50	12.00
3	10	25.00	6.50	12.00
4	16	35.00	6.50	12.00
5	6	25.00	5.50	48.00
6	13	35.00	5.50	48.00

7	20	25.00	6.50	48.00
8	11	35.00	6.50	48.00
9	18	21.59	6.50	30.00
10	9	38.41	6.50	30.00
11	2	30.00	5.16	30.00
12	12	30.00	6.84	30.00
13	8	30.00	6.00	-0.27
14	1	30.00	6.00	60.27
15	5	30.00	6.00	30.00
16	3	30.00	6.00	30.00
17	7	30.00	6.00	30.00
18	19	30.00	6.00	30.00
19	4	30.00	6.00	30.00
20	17	30.00	6.00	30.00

3.8.Application of lactic acid as bio preservative

Comparatively, avocado is more sensitive than other fruitsdue to its susceptibility to physical damage, ethylene gas, oxidative reactions, and microbial spoilage and necessitates bio preservation for various reasons. Its higher fat content makes it prone to spoilage and oxidation, resulting in a shorter shelf life.due to its delicate flesh texture and high moisture content, avocado is susceptible to enzymatic browning and microbial growth, which can compromise its quality and safety. The selection of avocado as a bio preservation ingredient is based on its nutrient richness, high fat content, moisture retention ability, neutral flavor, and extended shelf stability. These combined attributes make it an ideal choice for preserving the nutritional value, quality, and safety of bio preserved products.(soares& ITO,2000).

3.8.1. Preparation and treatment of bio preserved avocado samples

Directly applying lactic acid to fruit for biopreservation is often recommended over using fermented fruit with LAB for several reasons. It allows for precise control over the concentration

of lactic acid, ensuring consistent preservation outcomes. This method utilizes standardized solutions, enhancing quality control. Moreover, direct application enables immediate use of the fruit without the need for fermentation, which is especially useful for packaged fruits like olives and jam.(M.zelman,2024) However, it's important to note that in some cases, such as with unpackaged fruits like avocados in certain countries, direct application may require analysis of the results to assess its effectiveness.

Large fruits like avocados, direct application of lactic acid for biopreservation can be challenging through immersion due to the fruit's size and density. However, there are alternative methods that can be used to achieve effective preservation:' surface spraying .

The recommended concentration for acid solutions used in fruit preservation typically ranges from 0.5% to 2%. However, concentrations higher than 2% can have a detrimental impact on the quality and taste of the fruit. In this case, an average concentration of 1% lactic acid was used, following the recommended dilution guidelines to ensure the appropriate concentration for spraying on avocados.(filannion 2020).

Nine Ripe avocados of uniform size were selected for the treatment.The avocados are meticulously cleaned and checked for any dirt or debris, ensuring their surface is free from contaminants. The area where the spraying will be conducted is sanitized to minimize the risk of contamination, and careful consideration is given to select a clean and well-ventilated space. The spraying process takes place within a sanitized food-grade container or on a clean surface, allowing any excess solution to be captured and reducing wastage. The lactic acid solution, prepared as recommended, is loaded into a spray bottle. Starting from the top and progressing downwards, the avocados are evenly sprayed, with attention given to rotating them to achieve uniform coverage on all sides. The stem area, as well as any crevices or blemishes on the fruit's surface, are also sprayed. This spraying procedure is repeated for each individual avocado. Following the spraying, the avocados are given 30 minute to air dry, facilitating the adherence of the lactic acid solution to their surfaces. Once the avocados have completely dried, they stored under suitable conditions (M.zelman,2024).

3.8.2. Physiochemical analysis of untreated and treated fruit

1, pH measurement;pH measurements were conducted using a pH meter to determine the acidity of the bio preserved avocado samples. The pH values obtained were recorded and analyzed. The pH of the control samples was also measured for comparison(B.A. Orhevba 2011).

2. TSS Measurement:

A weight of 150 grams of avocado flesh was measured using a digital scale, and the weight was recorded. The weighed avocado flesh was transferred to a clean blender. The avocado flesh was blended until a smooth puree was formed. A small portion of the puree was taken and filtered through cheesecloth to remove any solid particles. The filtered avocado puree was collected in a clean, dry container. The digital refractometer was calibrated, and a few drops of the filtered avocado puree were placed onto the prism or sample well of the refractometer. The TSS value displayed on the refractometer was read and recorded. The TSS value was reported in degrees Brix(M.salameh 2022).

3, **Titratable Acidity (TA):**

A standardized solution of sodium hydroxide with a 0.1N concentration is prepared. A 10 mL volume of the fruit juice is pipetted into a clean, dry flask. Next, 0.2 mL of 0.5% phenolphthalein is added as an indicator. The fruit juice is slowly titrated with the standardized NaOH solution while the flask is swirled. The titration is continued until the indicator undergoes a color change, typically from pink to colorless. The volume of NaOH solution used for the titration is then noted. The titratable acidity (TA) of the fruit juice is calculated using the following formula:(B.A. Orhevba 2011).

TA (g/L) = (Volume of NaOH solution used × N × equivalent factor) / Sample volume.

4, color Analysis:

The avocados should be cut into uniform slices for consistent presentation during color analysis, and care should be taken to avoid damaging the flesh while handling the samples. The colorimeter should be placed on the surface of each avocado sample, ensuring good contact between the device and the sample, The color values obtained from the colorimeter for each avocado sample should be recorded. Typically, colorimeters provide values for attributes such as L* (lightness), a* (red-green component), and b* (yellow-blue component) in the chosen color space.

3.8.3. Sensory evaluation of treated fruit

1, **Visual Inspection** the bio preserved avocado samples were compared with the control samples in terms of color, texture, and appearance.

2, Sensory evaluation a sensory evaluation was conducted to assess the taste, odor and overall quality of the bio preserved avocados in comparison to the control samples. Due to the insufficient purification of the lactic acid, tasting the solution was not feasible. Therefore, only the odor of the samples was evaluated.

4. RESULT AND DISCUSSION

4.1. Proximate analysis of starch

5kg of starch was extracted from 20 kg of taro waste . The achieved yield of 25% which is within the acceptable range of 10-30% reported by the FAO and (Adane et al., 2006).

Table 4.1 presents the physio-chemical characteristics of the extracted starch, including moisture content, ash content, crude fat, protein, crude fiber, amylose content, and amylopectin content. The values obtained were compared with the standard values provided by the ASTM)

Table 4.1 proximate analysis of taro starch

Properties	**ASTM standard value** (Warkoyo, 2019)	**Experimental values of this study**
Moisture content (%)	10-12%	11.09 ± 0.001
Ash content (%)	0.1-0.3%	0.25 ± 0.002
Crude fat (%)	<0.2%	0.19 ± 0.012
Protein (%)	<0.5%	0.293 ± 0.003
Crude fiber	0.5-1.5%	0.9 ± 0.46
Amylose content (%)	20-30%	19.94 ± 0.54
Amylopectin content (%)	70-80%	80.05±0.002
Carbohydrate	87-89%	87.277
pH	5-7	5.5

The moisture content of the taro starch sample was found to be 11.09%, which is within the recommended range of 10-12%. according to the ASTM standard (Wu et al., 2006).This indicates that the moisture level of the taro starch is suitable for storage and processing,

maintaining its quality. The ash content of the taro starch was analyzed and found to be 0.25%, which is within the acceptable range of 0.1-0.3% specified by the ASTM standard (Dereje, 2021) A lower ash content suggests that the taro starch is relatively pure, containing minimal inorganic impurities. This purity is important for efficient enzymatic hydrolysis.

The taro starch sample exhibited a crude fat content of 0.19%, slightly below the maximum limit of <0.2% set by the ASTM standard. This low fat content is advantageous for glucose production, as fats can hinder enzymatic reactions and reduce yields. The protein content of the taro starch was determined to be 0.293%, which is within the acceptable range of <0.5%. This relatively low protein content indicates that the taro starch is unlikely to cause viscosity issues or complications during enzymatic hydrolysis. Furthermore, the crude fiber content of the taro starch was measured at 0.9%, which is within the ASTM range of 0.5-1.5%.

The taro starch sample was found to have an amylose content of 19.94%, slightly below the lower limit of 20-30% indicated in the ASTM standard. Lower amylose content can enhance the digestibility and solubility of starch. On the other hand, starches with higher amylose content tend to exhibit higher gelatinization temperatures and form stronger gels (Cornejo-Ramírez et al., 2018).

The analysis of the taro starch revealed an amylopectin content of 80.05%, which is within the range of 70-80%. This relatively high amylopectin content suggests that the taro starch has the potential to form stable gels, as mentioned (Svihus et al., 2005)Furthermore, the carbohydrate content of the taro starch was determined to be 87.277%, which aligns with the expected range of 87-89% specified by the ASTM standard. This indicates that the taro starch is suitable for efficient glucose production.

4.2Enzymatic hydrolysis .

4.2.1 Determination of Reducing Sugar

Table 4-2 glucose standard curve determination

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Glucose conc(g/L)	0	0.1	0.3	0.5	0.7	0.9	1
Absorbance at 540 nm	0.0003	0.00477	0.01371	0.02265	0.03159	0.04053	0.045

The relationship between glucose concentration (x) and absorbance (Y) was established through the application of a linear regression model to the data points, as described in the studies conducted by Lee et al. (2017) and Asghar et al. (2014). The resulting calibration curve equation was determined as Y = 0.0447X+0.0003. this equation demonstrates a direct correlation between the absorbance measured at 540 nm and the concentration of glucose, which aligns with the principles of the Beer-Lambert Law. The absorbance values of standard glucose solutions were measured using UV detection, enabling the determination of glucose content in the hydrolysate of taro waste starch. By utilizing a linear equation with glucose as the reference compound, the concentration of reducing sugars in the hydrolysate was calculated. The concentrations of the hydrolysate samples were determined by applying the equation of the standard curve: X = (Y + 0.0003) / 0.0447.Where X represents the glucose concentration and Y represents the absorbance value.

The concentrations of unknown sugar samples were determined using a conventional glucose curve (Y = 0.0447X+0.0003, R^2 = 0.9944). By applying this established standard, the study successfully identified the highest glucose yield, as shown in Table 0.7

Using the specified standard equation, the study effectively calculated the highest glucose yield, as evidenced by the data presented in Table 0.7, The table indicates that the maximum glucose yield achieved was 84 g/L, while the minimum yield recorded was 72 g/L. These results demonstrate the range of variability in glucose production under different experimental conditions or factors.

4.2.2. Preliminary experiments analysis result for glucose yield

Before conducting enzymatic hydrolysis, preliminary experiments were conducted to identify the optimal parameters that could lead to improved production yields. These preliminary studies aimed to gain a better understanding of the factors influencing enzymatic hydrolysis and identify the critical parameters that require control or adjustment for optimal results. The OVAT (One Variable at a Time) experimental design, commonly used in scientific research, was employed for this purpose.

The OVAT approach involves systematically altering one parameter while keeping others constant, allowing for an examination of its impact on the final outcome. In this particular study, the OVAT design was utilized to analyze the effects of temperature, and time, with a control pH which were selected based on existing literature and their known relevance to enzymatic hydrolysis. The insights obtained from this investigation are valuable for developing optimization strategies aligned with the research objectives.

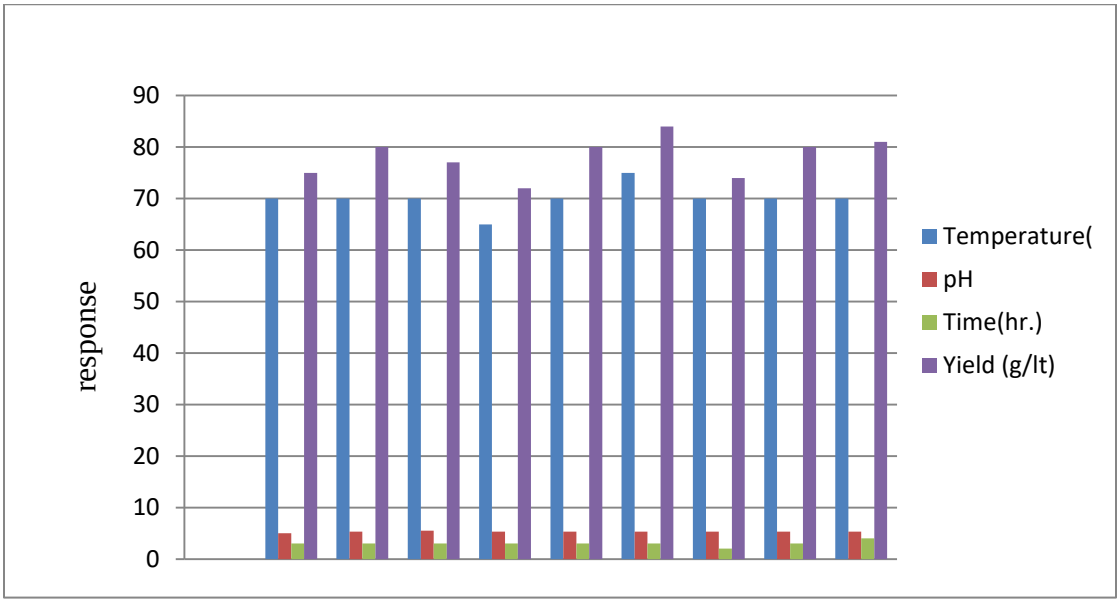


Figure 4-1 priliminary expement analysis for hydrolysis of reducing sugar

Based on the data provided in Table 0-7, the highest glucose yield achieved was 84 g/L. This optimal yield was obtained under specific conditions, including a reaction temperature of 75°C, a

pH of 5.3, and a reaction period of 3 hours. These particular settings resulted in the most favorable outcome in terms of glucose production. Conversely, the study observed the lowest yield of 72 g/L, which was associated with different conditions: a reaction temperature of 65℃, a pH of 5.3and a shorter reaction period of 3 hours. These altered settings led to a less favorable outcome in terms of glucose yield.

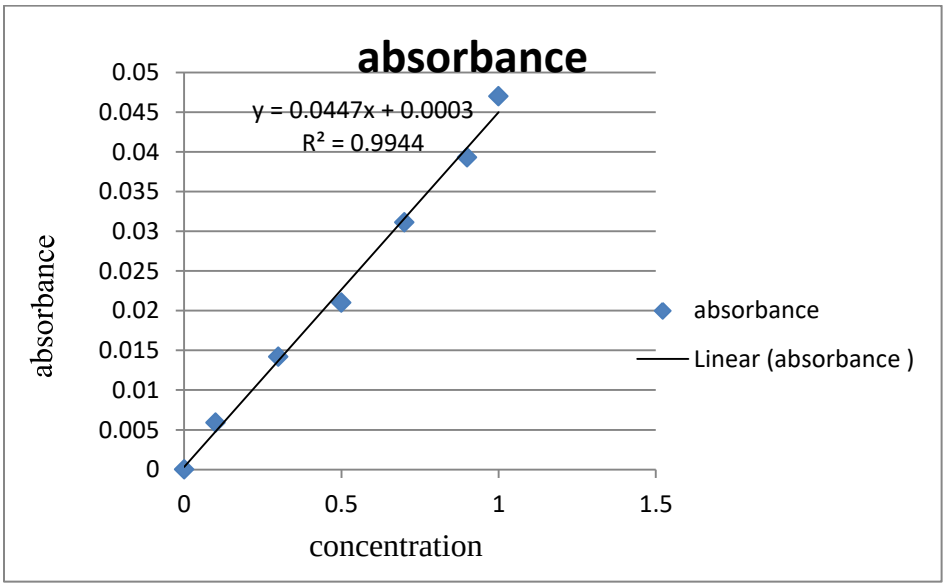


Figure 4.2 standard calibration curve for determining glucose content

4.2.3 FT-IR analysis of glucose

The FTIR analysis results for glucose reveal distinct peaks that provide insights into its structural features and functional groups. The observed indicate the presence of various functional groups in glucose.The peak at 879 cm^{-1} suggests the presence of pyranose ring vibrations, which are characteristic of glucose's ring structure. The peak at 1180 cm^{-1} corresponds to C–O stretching vibrations, indicating the presence of carbon-oxygen bonds within the molecule. The peak at 1290 cm^{-1} is associated with C–O–C or C–C stretching vibrations, indicating specific functional groups within glucose. The peak at 1505 cm^{-1} suggests the presence of C=C stretching vibrations in conjugated systems, potentially indicating double bonds or aromatic ring structures. The peak at 1387 cm^{-1} corresponds to -CH bending vibrations, indicating carbon-hydrogen bonds.

The peak at 1829 cm^{-1} signifies the presence of a carbonyl group (C=O), further characterizing the glucose molecule. The peak at 2384 cm^{-1} indicates -OH stretching vibrations, suggesting the presence of hydroxyl groups. The peak at 2981 cm^{-1} corresponds to aliphatic -CH_2 stretching vibrations, indicating the presence of methylene groups. Finally, the peak at 3832 cm^{-1} represents hydroxyl (-OH) stretching vibrations, potentially originating from multiple hydroxyl groups in glucose. these FTIR peaks provide valuable information about the molecular structure and functional groups present in glucose. Based on this analysis, the FTIR results for glucose is within the expected range of interpretations, confirming the molecule's structural characteristics and chemical composition in line with standard FTIR analysis of glucose.

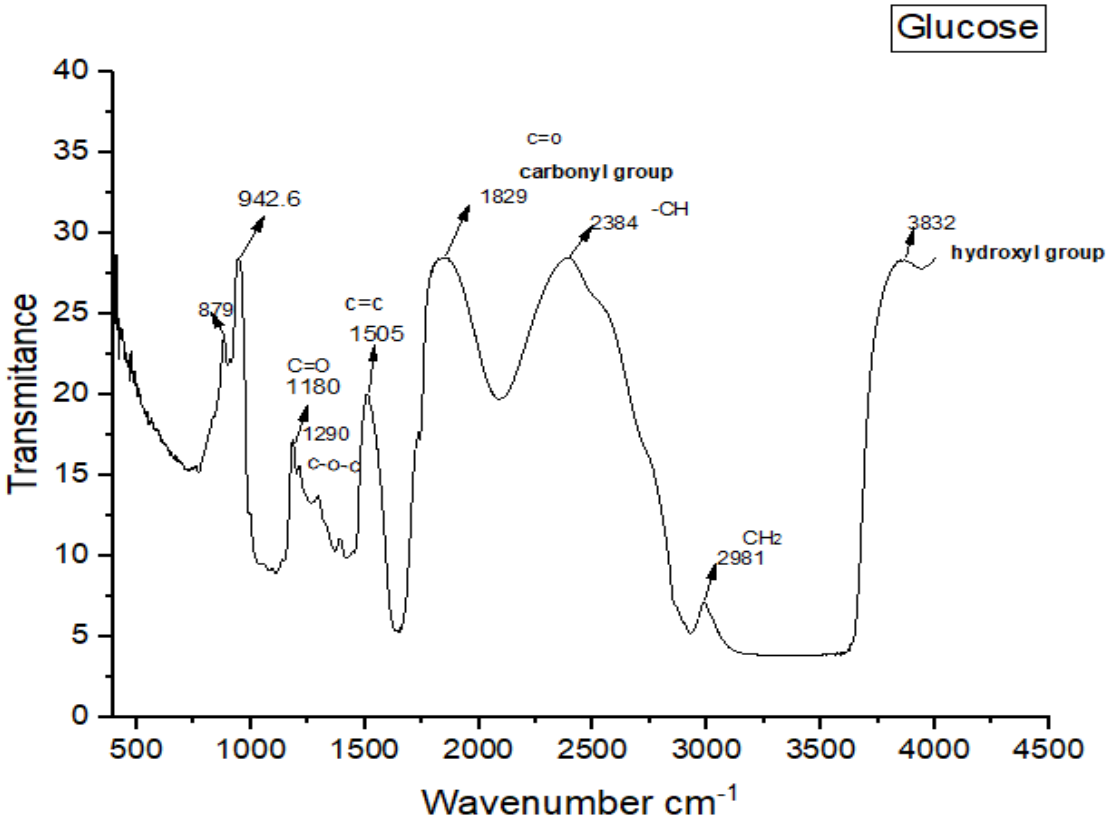


Figure4-3 FTIR analysis graph for hydro lysate glucose

4.3 Lactic acid production process

4.3.1 Bacteria strain selection Preli minary experiment

The optimal temperature ranges have been determined for three bacterial strains: *L.b paracasei*, *E. faecalis*, and *L. lactis*. According to the literature, the optimal temperature range for *L.b paracasei* is reported to be around 28-33℃, indicating that this temperature range is most favorable for the growth and activity of *L.b paracasei*. (Abbasiliasi et al., 2014),(Adu et al., 2020) Similarly, for *E. faecalis*, the optimal temperature range falls between 33-40℃.(Abe & Honda, 2023), (Gardini et al., 2001), while for *L. lactis*, the literature suggests an ideal temperature range of approximately 30-39℃. (Chen et al., 2015),(Sánchez et al., 2008)It is important to note that the mentioned time range is likely based on previous experiments conducted in the respective studies.

4.3.1.1Effects of temperature on cellular growth of bacteria

This study examined the impact of temperature on bacterial growth, focusing on three strains: Strain A, Strain B, and Strain C. Each strain exhibited an optimal temperature range for efficient growth. Strain A showed the highest effectiveness at approximately 33°C, Strain B preferred 38°C, and Strain C performed best at 34°C.The study also highlighted the presence of minimum and maximum growth temperatures for bacteria. The minimum temperature represented the lowest threshold for bacterial survival and reproduction, below which growth was significantly slowed. The maximum temperature indicated an upper threshold beyond which bacterial growth diminished, resulting in decreased growth efficiency.

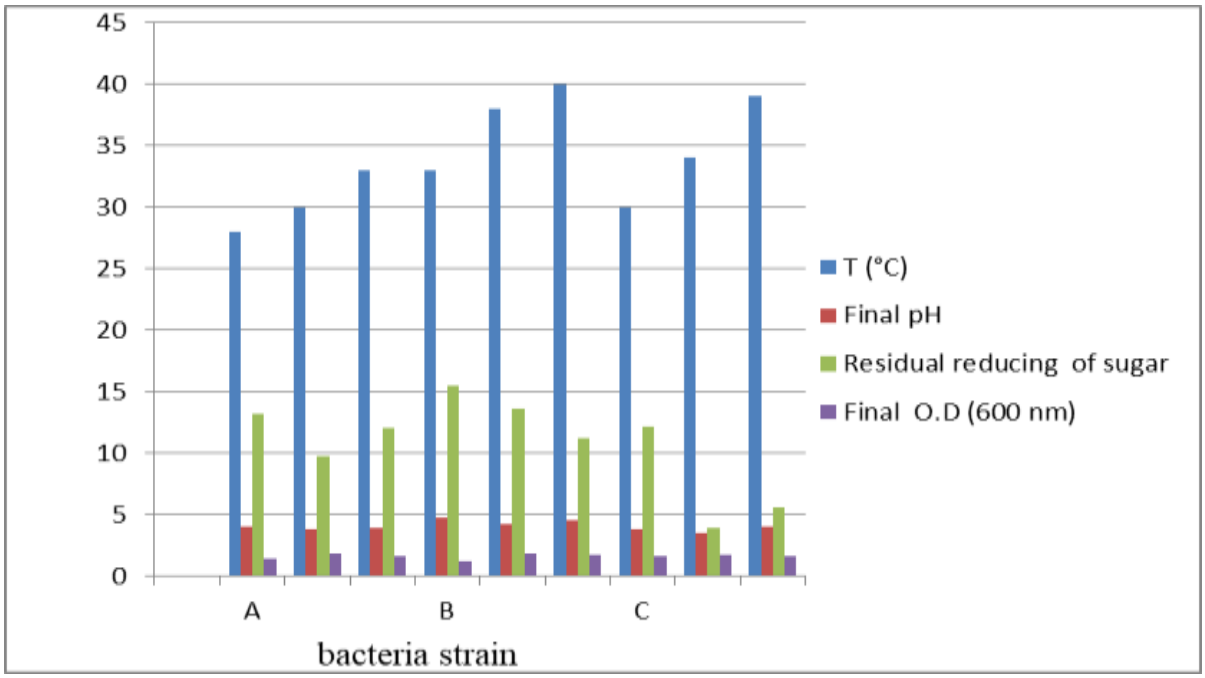


Figure 4-4Effects of temperature on bacteria growth

Figure 4-4 presented the results of the preliminary analysis, showing the relationship between temperature and various growth parameters for the three strains. For Strain A, at 30°C, there was increased acidity (decreased pH), enhanced sugar consumption (indicating metabolic activity), and higher optical density (OD) values (indicating cell growth and biomass production). Strain B exhibited high sugar consumption and increased OD values at 38°C. Strain C displayed significant sugar consumption, the lowest pH value among the tested temperatures, and the highest OD value at 34°C, indicating optimal growth and biomass production.

In conclusion, Strain C demonstrated the best performance among the three strains, with optimal growth parameters observed at 34°CStrain A performed best at 30°C in terms of sugar consumption, pH, and OD, while Strain B showed higher sugar consumption at 38°C but with reduced growth.

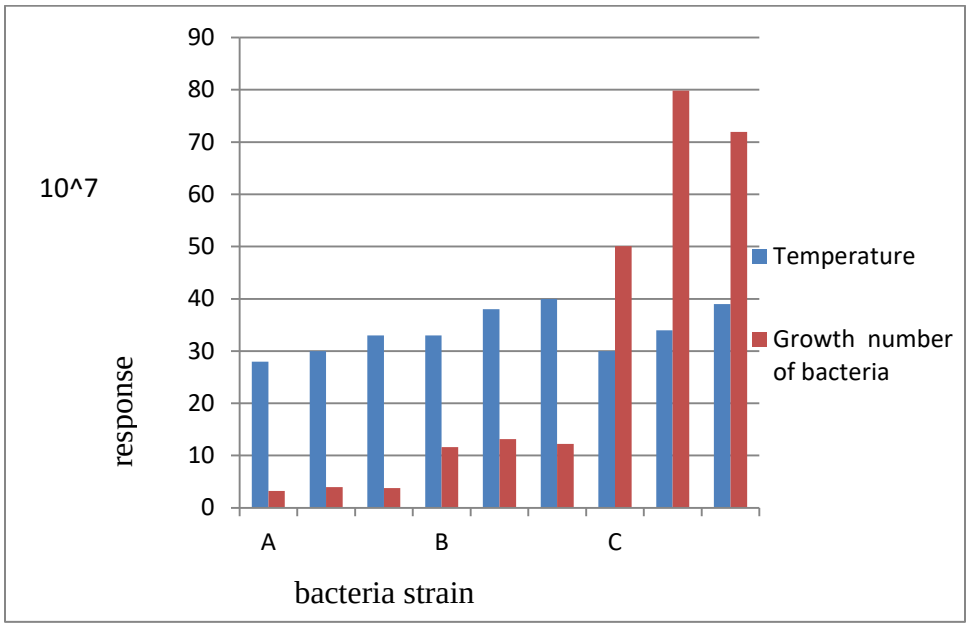


Figure4-5 impacts of temperature on cellular growth

In this study, the impact of temperature on bacterial growth was investigated. Temperature was found to significantly influence bacterial growth, with each strain exhibiting an optimal temperature range for efficient growth. The study focused on three bacterial strains: Strain A, Strain B, and Strain C., it was observed that Strain A was most effective at approximately 33°C. This temperature was identified as the optimal condition for promoting growth in Strain A. Strain B is 38°C, indicating that this temperature is the most favorable for its growth. Strain C performed best at 34°C, demonstrating its preference for this specific temperature to achieve optimal growth.

Furthermore, the study highlighted that bacteria have both a minimum and maximum growth temperature. The minimum temperature represents the lowest temperature at which bacterial survival and reproduction can occur. Below this threshold, bacterial growth is significantly slowed down. On the other hand, the maximum temperature signifies the upper threshold beyond which bacterial growth diminishes. At temperatures exceeding this threshold, the growth efficiency of bacteria decreases

4.3.1.2Effects of time on cellular growth of bacteria

Based on the data presented in Figure 4-5, the optimal time for growth was observed at 60 hours for both Strain A and Strain B. At this time point, these strains exhibited characteristics such as low pH, high sugar consumption, and increased optical density (OD), indicating optimal growth and biomass production. Strain C, on the other hand, demonstrated the best performance at 42 hours, with substantial sugar consumption, the lowest pH value, and the highest OD value at this time point.

Overall, it can be concluded that Strain C performed best at 42 hours, while Strain A and Strain B showed optimal growth characteristics at 60 hours. The findings suggest that each strain has a specific time frame in which they perform optimally in terms of sugar consumption, pH, and OD. Additionally, the bacterial counts for each strain increased with longer incubation times, indicating a growth-promoting effect of extended incubation.

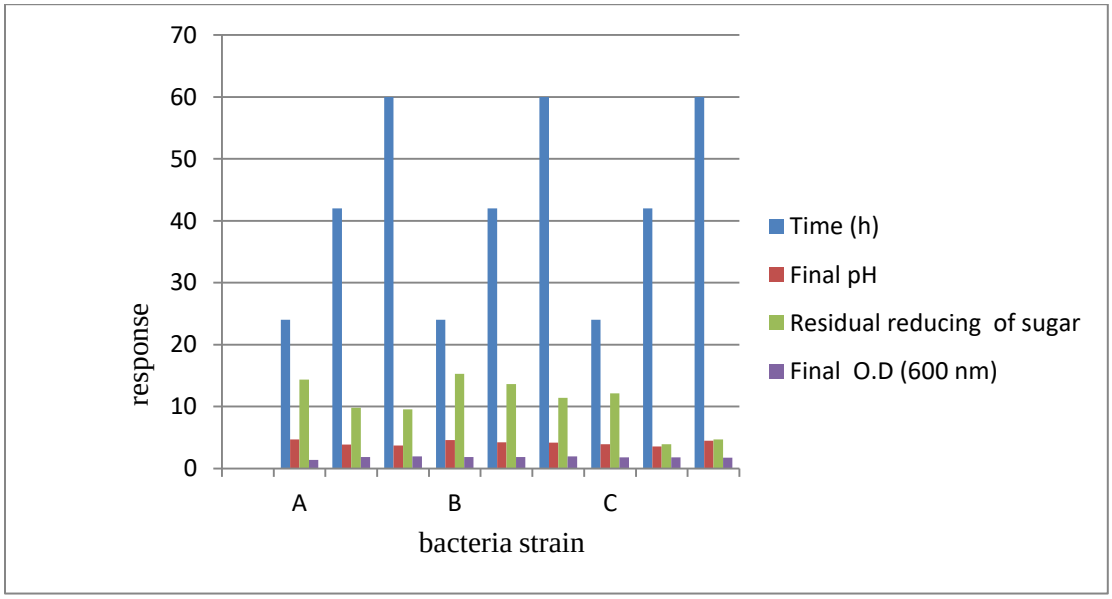


Figure4-6 impacts of temperature on cellular growth

For Strain A, the optimal incubation time for growth was observed at 60 hours, with a corresponding bacteria count of 5.416×10^7. When comparing this to the growth at 24 and 42 hours, it is evident that the bacterial population increased as the incubation time increased. This suggests that Strain A requires a longer incubation period to reach its maximum growth potential.

Moving on to Strain B, the optimal incubation time for growth was also observed at 60 hours, with a growth count of. 1.3209×10^8 Similar to Strain A, the population size increased with longer incubation times. However, it is noteworthy that Strain B exhibited a lower growth rate compared to Strain A..

For Strain C, the optimal incubation time for growth was observed at 42 hours, with a bacterial count of 7.9857×10^8. Unlike Strain A and Strain B, Strain C did not show a continuous increase in growth with longer incubation times. The bacterial count at 60 hours slightly decreased compared to the count at 42 hours. This suggests that Strain C may have reached its maximum growth potential or entered a stationary phase by the 60-hour mark.

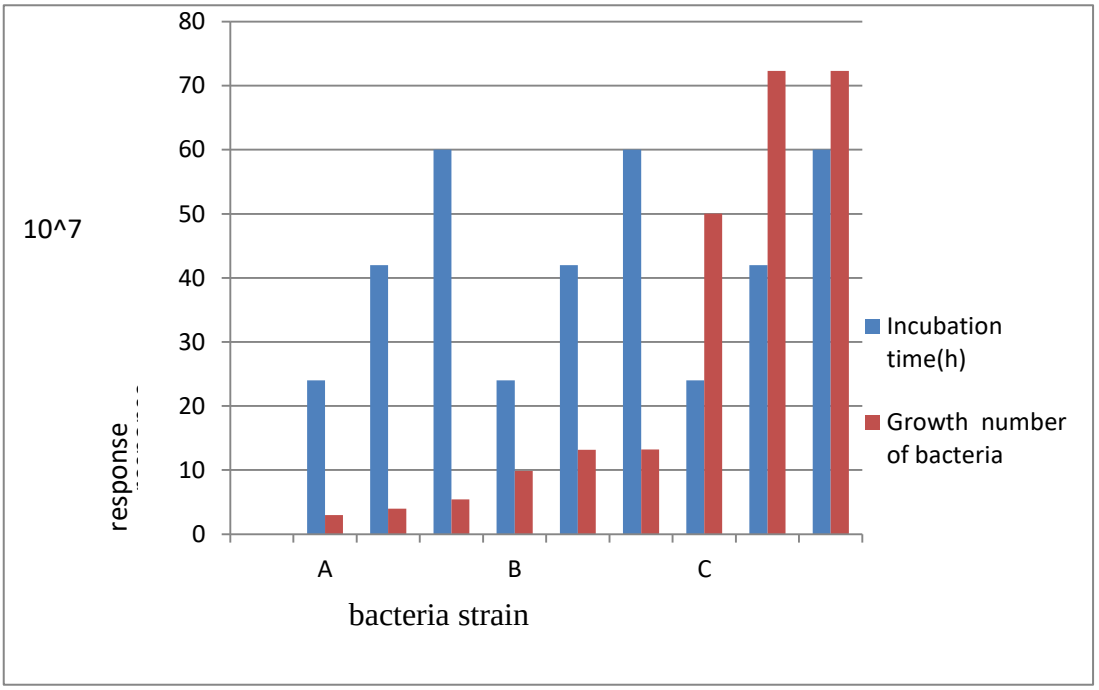


Figure4-7 impacts of temperature on cellular growth

4.3.2.Optimization of lactic acid production

A central composite design (CCD) was employed to enhance the production of lactic acid by optimizing various factors, namely temperature, pH, and time. These factors were examined at three different levels. To ensure accuracy, a total of twenty experiments were conducted in duplicate. The obtained data was then analyzed using Design-Expert 6.08 software through multiple regression analysis. The main objective of this approach was to identify the optimal reaction conditions that would result in maximum lactic acid production. Based on the preliminary analysis, it was determined that *Lactococcus*, among the three bacteria considered, exhibited superior potential. Therefore, *Lactococcus* was selected for further optimization in the production of glucose.

Optimization of lactic acid production using *Lactococcus lactis*

Table 4.3 Design factors with their lower, center and higher value

Factor	**Parameter**	**Unit**	**Code**	**Level**		
				Low	Center	High
1	Fermentation temperature	°C	A	25	30	35
2	pH		B	5.5	6	6.5
3	Fermentation time	H	C	12	30	48

The utilization of central composite design (CCD) allowed for the exploration of factors within and beyond the predefined range. One of the advantages of CCD is its capability to incorporate factorial points, center points, and star points, enabling the evaluation of factor levels that are below and above the range examined.

Table 4-4 CCD methodology for lactic acid production by L.lactis

Std order	Run Order	Temperature (℃)	pH	Time (hr.)	Actual yield (g/l)	Predicted yield (g/l)	Yield (%)
1	14	25.00	5.50	12.00	13.56	13.39	16.14
2	15	35.00	5.50	12.00	25.77	25.13	30.68
3	10	25.00	6.50	12.00	22.26	21.47	26.50
4	16	35.00	6.50	12.00	27.15	26.84	32.32
5	6	25.00	5.50	48.00	24.78	24.98	29.50
6	13	35.00	5.50	48.00	32.66	33.33	38.88
7	20	25.00	6.50	48.00	31.27	31.80	37.22
8	11	**35.00**	**6.50**	**48.00**	**33.74**	**33.79**	**40.17**
9	18	21.59	6.50	30.00	21.42	21.50	25.50
10	9	38.41	6.50	30.00	32.96	33.04	39.24
11	2	30.00	5.16	30.00	25.52	25.43	30.38
12	12	30.00	6.84	30.00	32.35	32.60	38.51
13	8	30.00	6.00	-0.27	14.78	15.86	17.60
14	1	30.00	6.00	60.27	32.37	31.45	38.54
15	5	30.00	6.00	30.00	31.48	31.70	37.48
16	3	30.00	6.00	30.00	31.48	31.70	37.48
17	7	30.00	6.00	30.00	31.48	31.70	37.48
18	19	30.00	6.00	30.00	31.48	31.70	37.48
19	4	30.00	6.00	30.00	32.72	31.70	38.95
20	17	30.00	6.00	30.00	31.56	31.70	37.57

4.3.3. Analysis of variance (ANOVA)

Analysis of variance (ANOVA) for the yield of lactic acid obtained using central composite design (CCD) is shown in table below.

Where A: Temperature

B: pH

C: time

Table 4-5 Analysis of variance (ANOVA)

Source	Sum of squares	Df	Mean Square	F Value	Prob > F	
Model	687.78	9	76.42	144.28	< 0.0001	significant
A	160.77	1	160.77	303.53	< 0.0001	
B	62.16	1	62.16	117.36	< 0.0001	
C	293.33	1	293.33	553.79	< 0.0001	
A^2	35.26	1	35.26	66.57	< 0.0001	
B^2	12.93	1	12.93	24.42	< 0.0001	
C_2	116.43	1	116.43	219.81	< 0.0001	
AB	20.26	1	20.26	38.24	0.0005	
AC	5.70	1	5.70	10.75	0.0188	
BC	0.79	1	0.79	1.49	0.8388	
Residual	5.30	10	0.53			
Lack of fit	4.04	5	0.81	3.23	0.3495	not significant
Pure error	1.25	5	0.25	144.28	< 0.0001	

The model's statistical significance is indicated by the F-value of 144.28, implying that the probability of obtaining such a large F-value by chance is only 0.01%. When the p-value associated with the F-test is below 0.05, it suggests that the model terms collectively have a significant impact. In this particular case, the following terms are deemed significant: Temperature, pH, Time, Square of temperature, Square of pH, Square of time, Interaction between temperature and pH, and Interaction between temperature and reaction time. If any p-value exceeds 0.1, it indicates that those terms are not significant. To potentially improve the

model's performance, it is recommended to simplify the model by excluding such terms (unless they are necessary for hierarchical purposes).

The Lack of Fit F-value of 3.23 implies that the Lack of Fit is not statistically significant when compared to the pure error. This suggests that there is an 11.23% probability of obtaining such a large Lack of Fit F-value due to random noise. A lack of fit that is not significant is desirable since it indicates a good fit of the model. The p-values associated with the model coefficients (Temperature, pH, Time, Square of temperature, Square of pH, Square of time, Interaction between temperature and pH, and Interaction between temperature and reaction time in linear form) are all less than 0.0001. This indicates that the yield of lactic acid is influenced by all the process variables and the interaction between temperature and pH, as well as the interaction between temperature and reaction time.

4.3.4. Model adequacy checking

To assess the model's effectiveness in explaining the variation in lactic acid yield based on process variables, various metrics are employed: R-Squared, Adjusted R-Squared, Predicted R-Squared, and Adequacy Precision. The corresponding values are provided below.

Table 4-6Model adequacy check

Std. Dev.	**0.73**	**R-Squared**	**0.992**
Mean	**28.04**	Adj R-Squared	0.9855
C.V.	**2.60**	Pred R-Squared	0.9520
PRESS	**33.23**	Adeq Precision	39.637

The model's accuracy and its ability to explain the data are demonstrated by the strong agreement between the "Pred R-Squared" value of 0.9520 and the "Adj R-Squared" value of 0.9855. The "Adeq Precision" metric, with a ratio of 39.637, exceeds the desired threshold of 4, indicating a strong signal relative to noise. This confirms that the model effectively captures the relationship between process variables and lactic acid yield.

Based on these results, it can be confidently stated that the model is reliable and well-suited for navigating the design landscape. With high values for "Pred R-Squared" and "Adj R-Squared," along with a robust signal-to-noise ratio, the model provides precise predictions and facilitates informed decision-making in optimizing lactic acid yield.

4.3.5 The regression model equation

The following is the model equation that describes how the response to independent variables is related to coded factors:

With regard to the coded factor, the final equation is:

yield = + 31.70 + 3.43 * A + 2.13 * B + 4.63 * C-1.56* A^2-0.95 * B^2- 2.84 * C^2 -1.59* A * B+0.84 * A * C -0.31* B * C

Where A; temperature

B; pH

C;time

4.3.6. Graphical analysis

The adequacy of the developed model was assessed through graphical analysis, which involved examining expected vs. actual yield, residual vs. predicted yield, and a residual normal plot. When all data points in a functional model align perfectly, it indicates the absence of error. In Figure 4-5, the graph depicting predicted vs. actual yield demonstrates a close alignment between the model's projections and the experimental data. This indicates a high level of agreement, highlighting the accuracy and reliability of the model in estimating yield.

Figure 4-6, the Residual vs. Expected Yield graph, illustrates the relationship between residual values (the differences between predicted and actual yields) and expected yield values. Notably, the residuals do not exhibit any discernible pattern or trend. This suggests that there is no significant need to reduce personal error or make adjustments to the model. The scatter of

residual values indicates that the model effectively accounts for variations in the data, without any apparent biases or systematic errors.

In Figure 4-7, the Normal Plot of Residuals graph demonstrates a close alignment between the experimental data points and a straight line. This indicates that the errors follow a normal distribution, satisfying an important assumption in statistical modeling. The model effectively captures data variability, ensuring its reliability.

Predicted vs actual yield

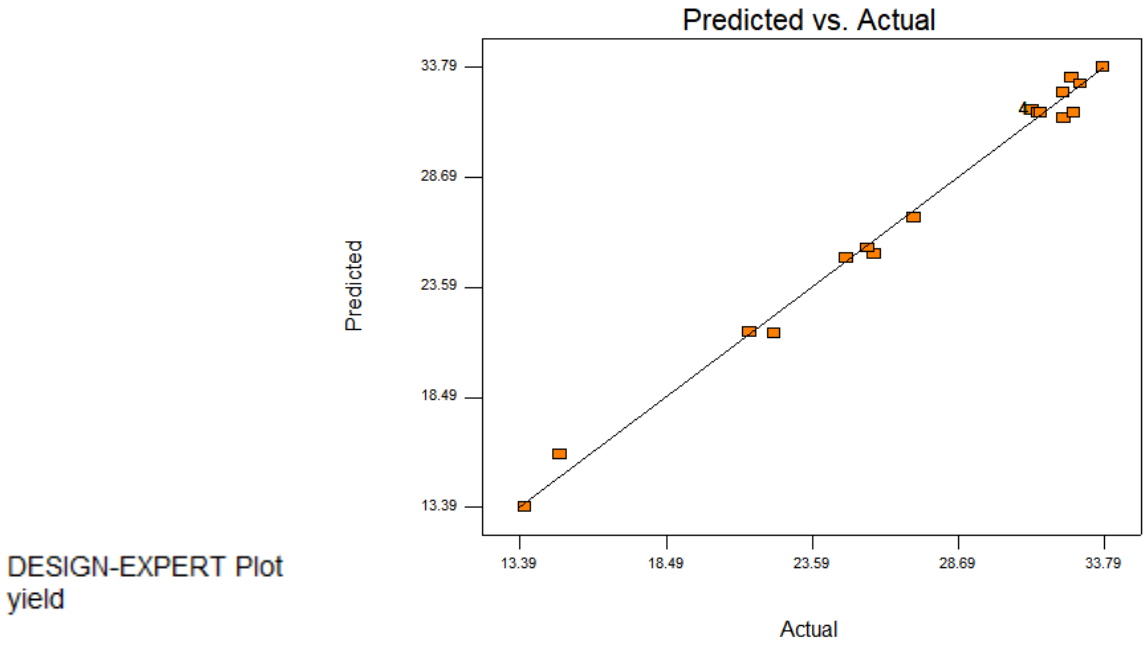


Figure 4.8Predicted versus actual yield graph

Residual versus predicted yield

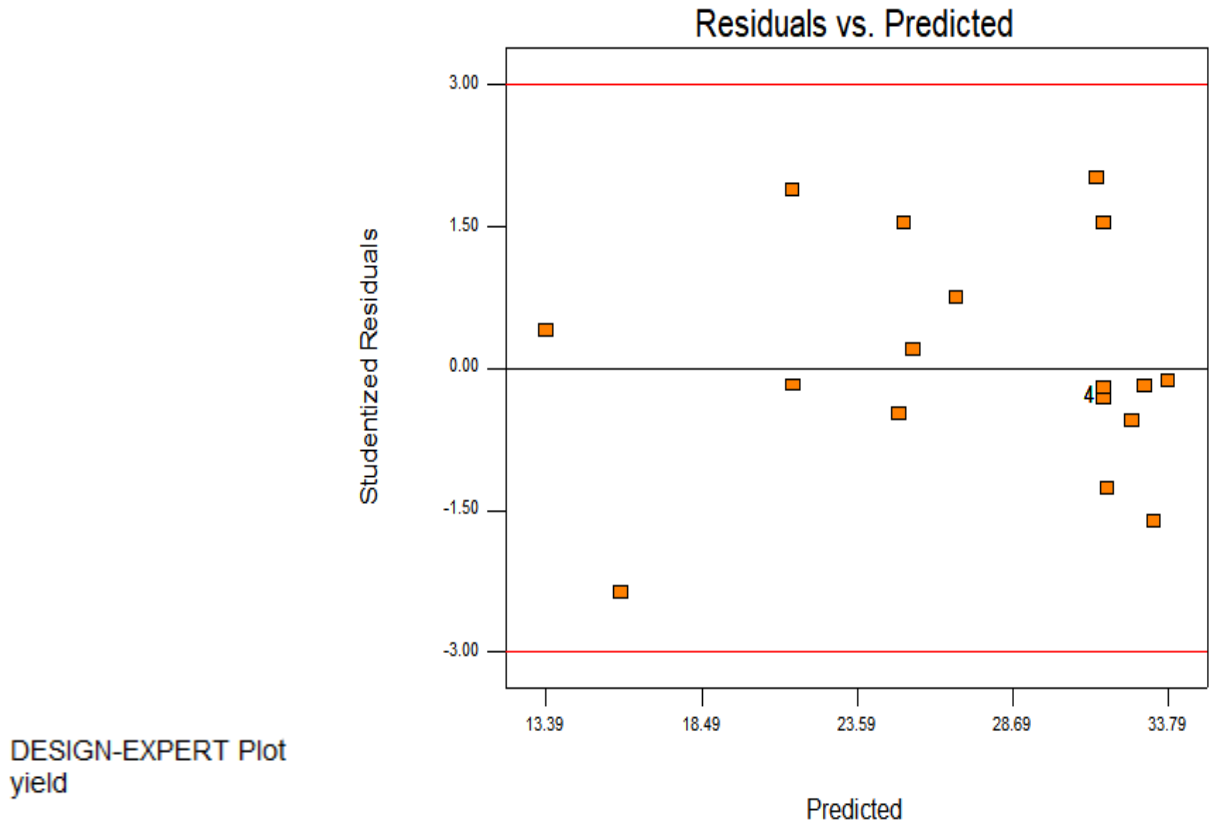


Figure 4-9Residual versus predicted yield graph

Normal plot of residuals

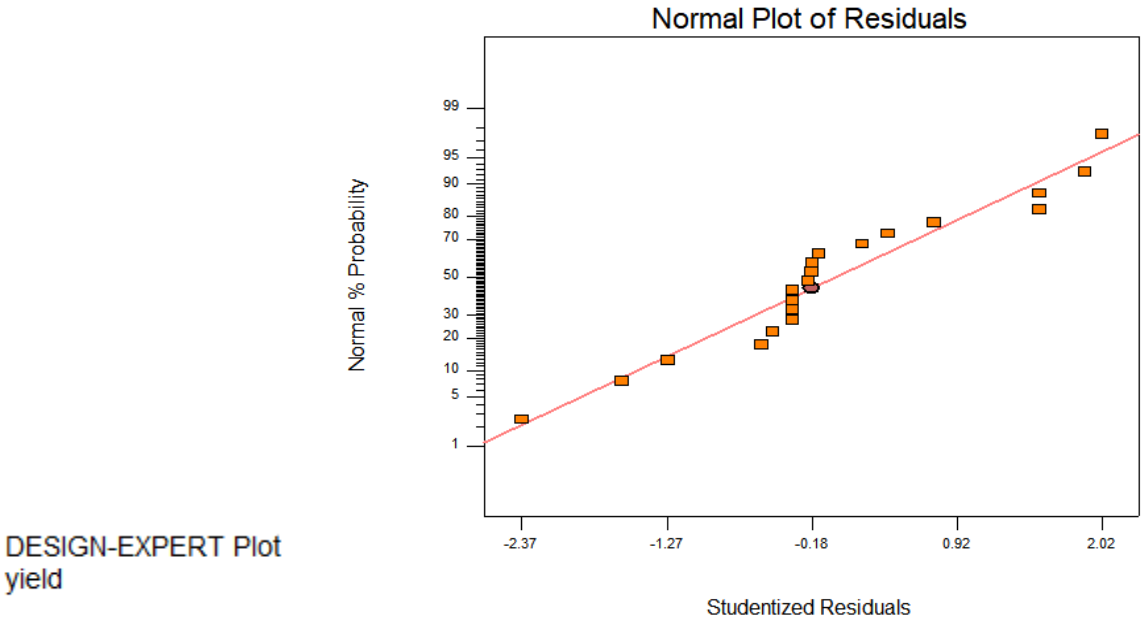


Figure 4-10 normal versus Residual yield graph

4.3.7 Effect of process parameters on yield of lactic acid

The ANOVA analysis indicated that the reaction process was significantly influenced by the process variables: temperature, pH, and time. Furthermore, significant interactions were observed between temperature and pH, as well as between temperature and reaction time in linear form. These results highlight the combined impact of temperature and pH, as well as temperature and reaction time, on the reaction process, underscoring the importance of considering these interactions when optimizing the process. Further discussion will delve into the individual effects of each process variable.

4.3.7.1 Effect of temperature

The temperature variable was identified as a crucial factor influencing the yield of lactic acid. Figure 4-6 clearly illustrates the relationship between temperature and lactic acid yield. The experimental data revealed a linear increase in lactic acid yield with higher temperatures within the range of 25℃ to 35℃.

The findings indicate that as the temperature increased within this range, the yield of lactic acid consistently increased. Furthermore, the highest yield of lactic acid was obtained at 35℃, indicating that this temperature level was optimal for achieving the highest yield. This observation underscores the importance of temperature control in lactic acid production. It suggests that increasing the temperature within the specified range can have a positive impact on lactic acid yield, potentially due to improved reaction kinetics or enhanced enzyme activity at higher temperatures.

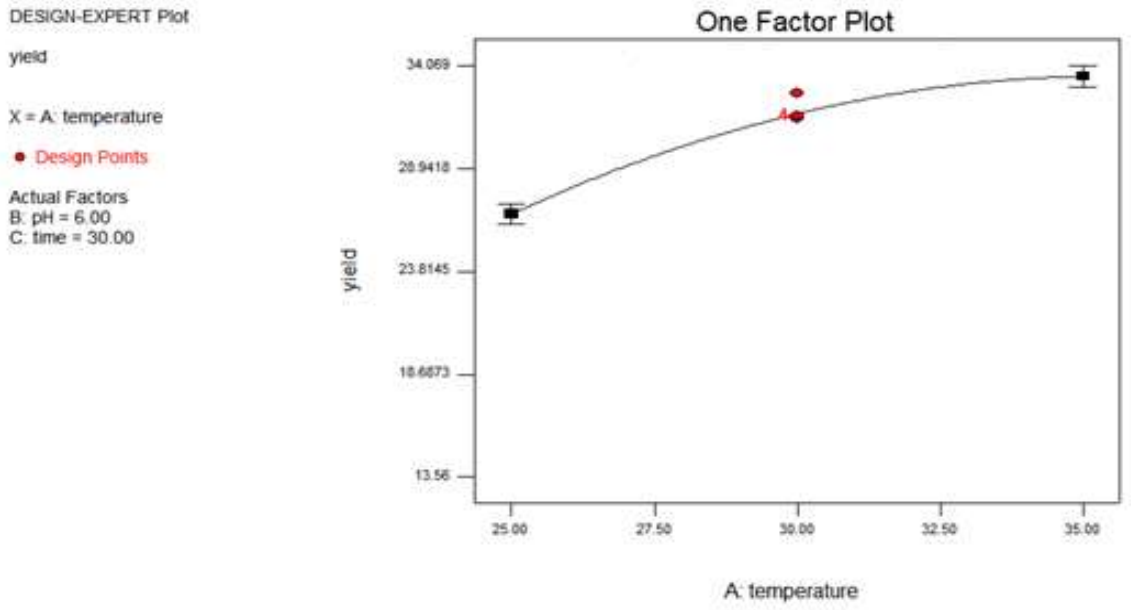


Figure 4.11 Effect of temperature graph

The analysis conducted as a design expert reveals valuable insights into the effect of temperature on lactic acid yield. It was observed that raising the temperature above 35°C resulted in a slightly decrease in lactic acid production. This finding suggests that temperatures exceeding 35°C have a detrimental impact on the overall efficiency of lactic acid fermentation. Furthermore, during the preliminary study on bacteria selection, it was discovered that the highest number of bacteria, reaching 7.9857×10^8, was obtained at a temperature of 34°C. However, when the temperature was increased to 39°C, there was a decline in the bacterial count. This observation strongly supports the notion that the optimal temperature for achieving maximum lactic acid production lies around 35°C but ont only for this .Interestingly, Jun Chen's research findings align with your own observations. Chen observed that *Lactococcus lactis*, the bacteria commonly involved in lactic acid fermentation, experiences hindered growth and an inability to thrive at temperatures exceeding 37°C. This emphasizes the critical role of maintaining suitable temperature conditions for the successful cultivation and growth of *Lactococcus lactis*, which directly impacts lactic acid production.. (Chen et al., 2013). (Adamberg et al., 2003)

4.3.7.2 Effects of pH

The pH variable is a critical factor that significantly influences the yield of lactic acid production. The relationship between pH and lactic acid yield was investigated, and the results were depicted in Figure 4-9. This figure clearly illustrates that as the pH increased from 5.5 to 6.5, there was a corresponding increase in the yield of lactic acid. Notably, the highest yield of lactic acid was achieved when the pH was carefully controlled at 6.5.

This finding aligns with prior research conducted by Cock and De Stouvenel in 2006, where they studied the fermentation of lactic acid by *Lactococcus lactis*. Their research also observed optimal yields of lactic acid within the pH range of 5.5 to 6.5. The consistency between these studies strengthens the validity of the current findings and emphasizes the significance of pH control in optimizing lactic acid production. (Cock & De Stouvenel, 2006)

The experimental data clearly demonstrates a notable relationship between pH and lactic acid yield. Within the pH range of 5.5 to 6.5, an increase in pH directly correlates with an increase in

lactic acid yield. This suggests that maintaining a slightly mild acidic environment favors higher lactic acid production. The underlying mechanisms behind this pH-dependent effect on yield could be attributed to the influence of pH on the enzymatic activity of the microorganisms involved in lactic acid fermentation.

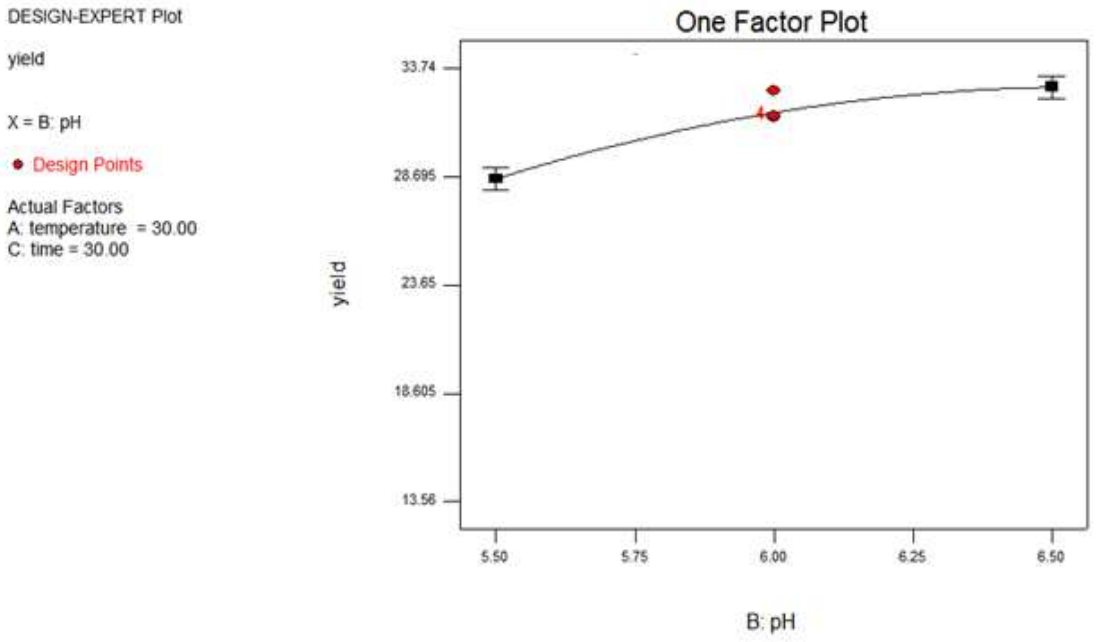


Figure 4.12 Effect of pH graph

4.3.7.3 Effects of time

The duration of fermentation is a critical factor that significantly influences the yield of lactic acid production. Extending the fermentation time initially enhances lactic acid production and improves the utilization of available substrates, leading to higher yields. This prolonged fermentation period also contributes to a lower final pH, which further enhances lactic acid production. The data presented in Figure 4-10 clearly demonstrates that increasing the reaction time from 12 to 48 hours results in an increased yield of lactic acid. Therefore, the optimal

duration for lactic acid production in this study was determined to be 48 hours.However, it is important to note that extending the fermentation time beyond 48 hours has a negative impact on lactic acid yield. This observation is supported by my preliminary analysis, where it was found that the bacterial count decreased from 7.9857×10^8 at 42 hours to 7.2312×10^8 at 60 hours. These findings indicate that the optimum fermentation time lies around 48 hours.

Several factors associated with prolonged fermentation durations can account for the decline in lactic acid yield. One such factor is nutrient depletion, where the available nutrients in the fermentation medium become exhausted over time. As a result, the growth and metabolic activity of lactic acid bacteria become limited, leading to reduced lactic acid production.

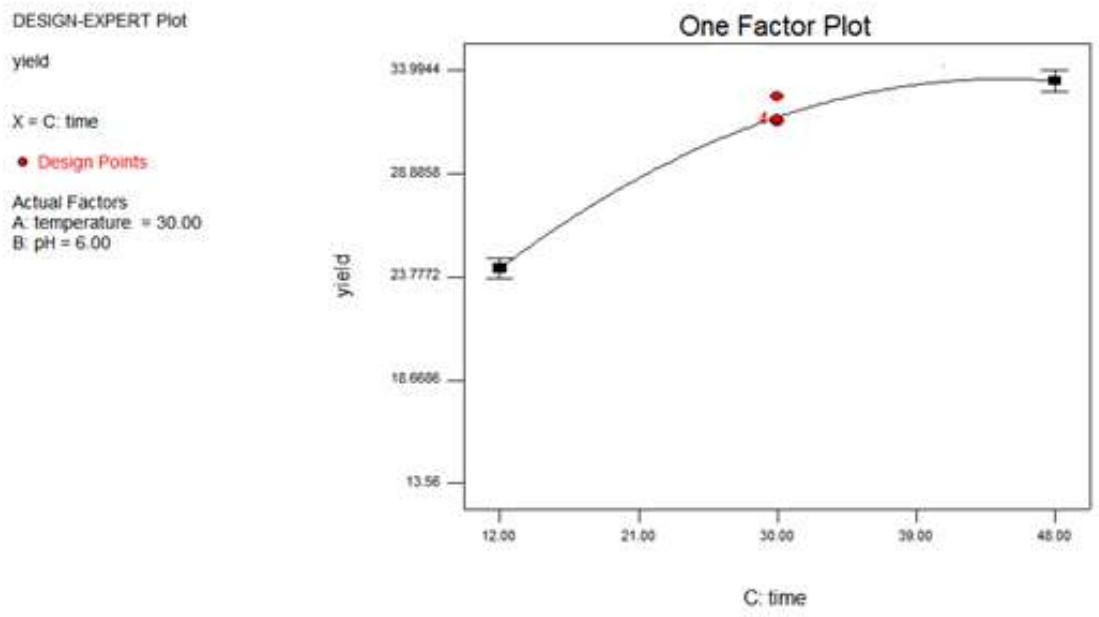


Figure 4.13Effect of time graph

4.3.8.Effect of interaction between process parameters

The analysis of variance (ANOVA) reveals that the interaction between temperature and pH, as well as temperature and fermentation time, has a significant impact on lactic acid yield. This

finding provides valuable insights into optimizing lactic acid fermentation processes. By utilizing response surface (3D) and contour plots, it becomes possible to visually examine how these process factors influence lactic acid yield. These plots offer a graphical representation of the effects and interactions of temperature, pH, and fermentation time, aiding in the optimization of lactic acid production.

4.3.8.1. Effect of interaction between temperature and pH

The response surface (3D) and contour plots presented in Figure 4-11 and Figure 4-12 provide a visual representation of the relationship between temperature, pH, and the yield of lactic acid. These plots demonstrate how variations in temperature and pH values influence lactic acid production and emphasize the significance of their interaction. Both figures illustrate that increasing temperature and pH lead to higher lactic acid yields. Within the observed range, the yield of lactic acid increased from 13.56% to 33.64% as the temperature rise from 25 to 35 ℃ and the pH was elevated from 5.5 to 6.5. The lowest lactic acid yield was observed at a temperature of 25 ℃ and a pH of 5.5, indicating that this combination was less favorable for lactic acid production. Conversely, the maximum yield of lactic acid was achieved at 35 ℃ and a pH of 6.5. This suggests that these specific conditions of higher temperature and slightly mild acid pH were optimal for lactic acid production.

The contour plot in Figure 4-12 further supports the observation of a significant interaction between temperature and pH. The contour lines on the plot represent points of equal lactic acid yield, allowing for a visual understanding of how changes in temperature and pH influence lactic acid production. The contour lines appear closer together around the optimal conditions of 35 ℃ and pH 6.5, indicating that small variations in temperature and pH within this range have a more pronounced impact on lactic acid yield.

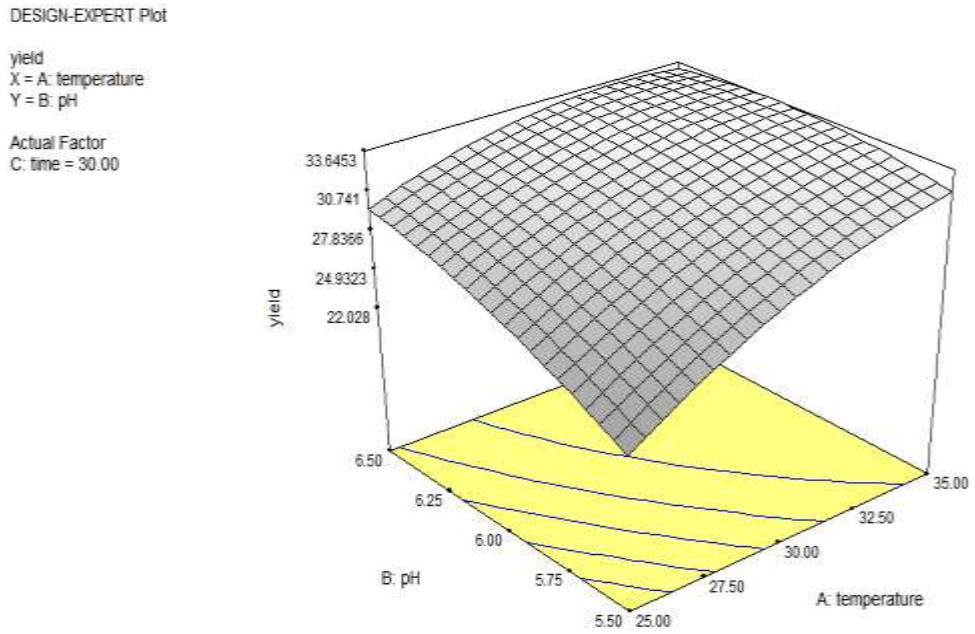


Figure 4-14 Interaction between temperature and pH 3D plot

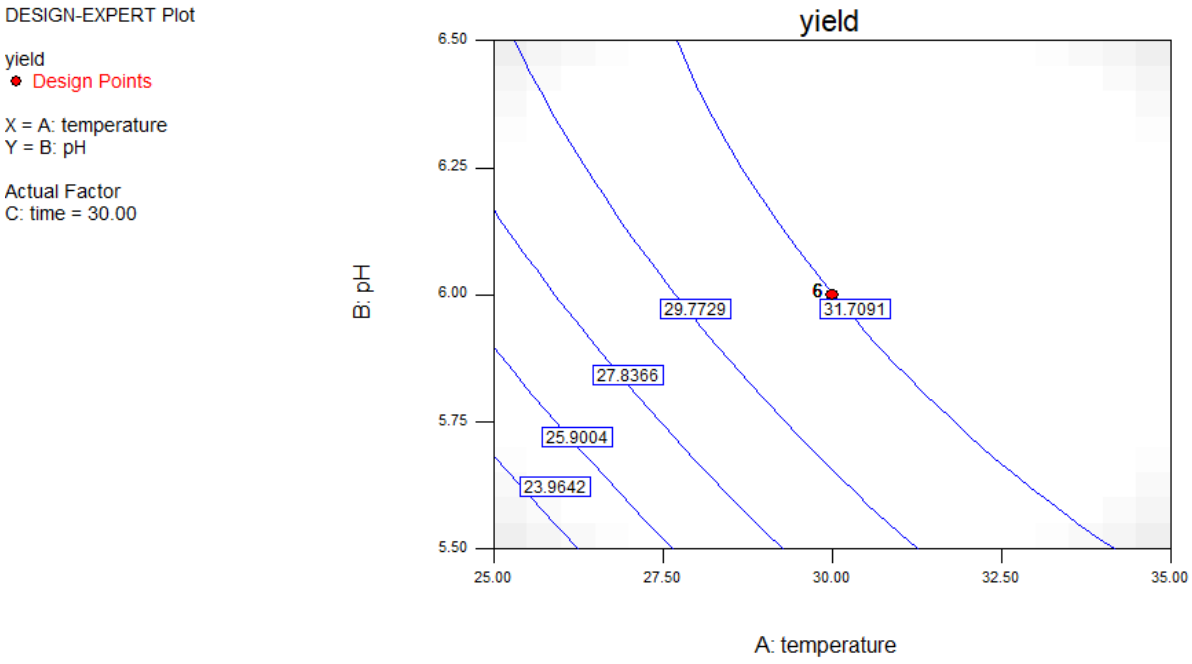


Figure 4.15 Interaction between temperature and pH counter plot

4.3.8.2Effect of interaction between temperature and time

The relationship between temperature, fermentation time, and the yield of lactic acid is depicted in the response surface (3D) and contour plots presented in Figure 4-13 and Figure 4-14. These plots visually illustrate the impact of varying temperature and fermentation time on lactic acid production, highlighting the significance of their interaction. Both figures clearly demonstrate that increasing temperature and fermentation time result in higher lactic acid yields. Within the range studied, the yield of lactic acid increased from 13.56% to 33.64% as the temperature was raised from 25 to 35 ℃ and the fermentation time was extended from 12 to 48 hours. These findings indicate that higher temperatures and longer fermentation times are favorable for lactic acid production. The lowest yield of lactic acid was observed at a temperature of 25 ℃ and a fermentation time of 12 hours, indicating that this combination was less optimal for lactic acid production. Conversely, the maximum yield was achieved at 35 ℃ and a fermentation time of 48 hours. These results suggest that these specific conditions of higher temperature and longer fermentation time were optimal for maximizing lactic acid production.

The contour plot in Figure 4-14 further supports the observation of a significant interaction between temperature and fermentation time. The contour lines on the plot connect points of equal lactic acid yield, providing a visual representation of how changes in temperature and fermentation time influence lactic acid production. The contour lines are closer together around the optimal conditions of 35 ℃ and 48 hours, indicating that small variations in temperature and fermentation time within this range have a more pronounced impact on lactic acid yield.

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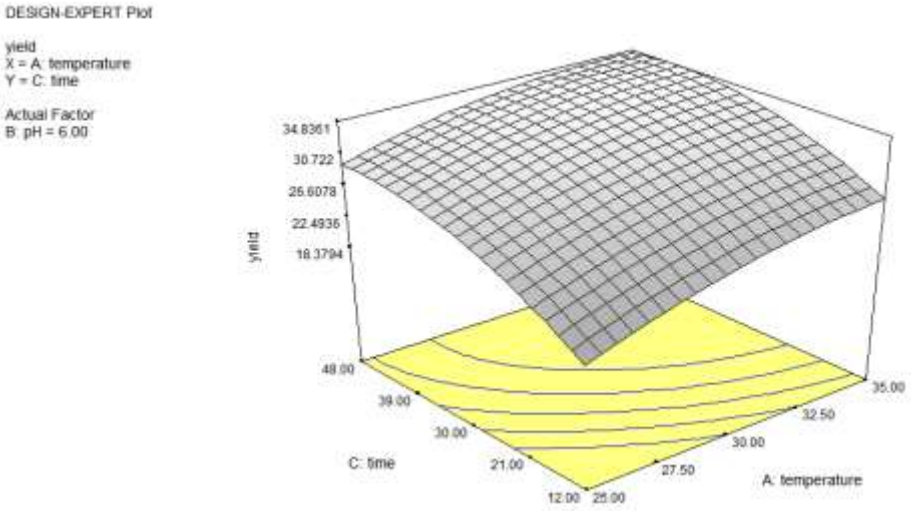

Figure 4.16 Interaction between temperature and time 3D plot

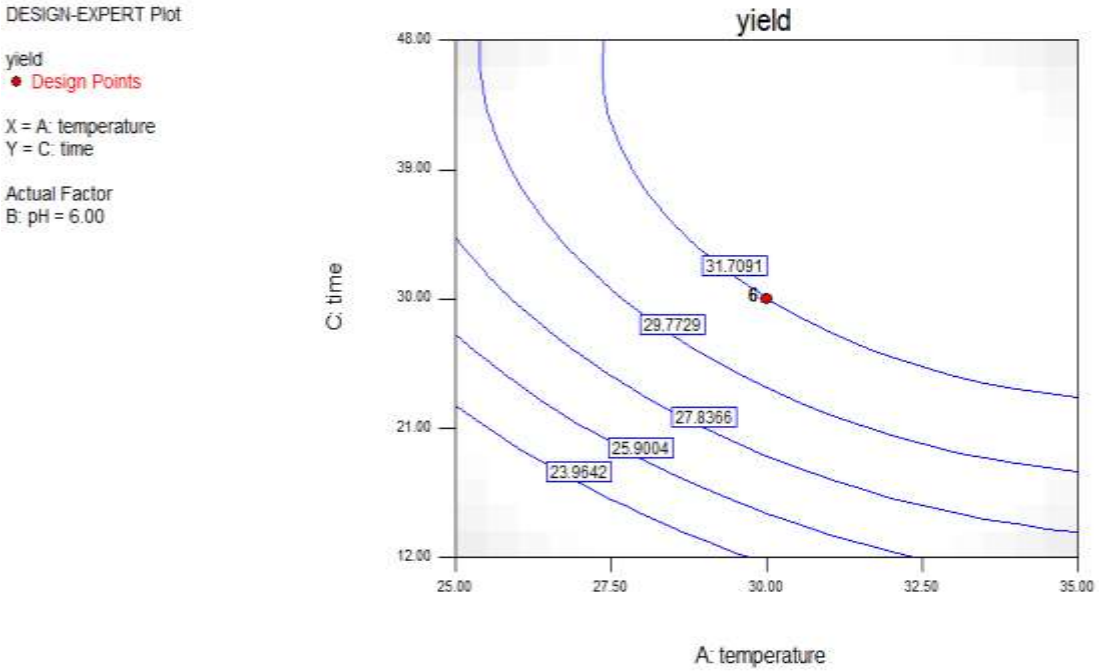

Figure 4.17 Interaction between temperature and time contour plot

4.3.9 Spectrophotometric Measurements of Lactic Acid content

Ferric chloride ($FeCl_3$) is widely utilized as a colorimetric reagent for spectrophotometric measurements of lactic acid. Its role involves detecting and quantifying lactic acid in a sample by forming a colored complex.

Table 4-7lactic acid standard curve determination

LA Concentration (g/L)	Absorbance at 390nm
3	0.1548
6	0.5418
9	0.8798
12	1.2258
15	1.576

The concentration of lactic acid (x) can be estimated using the equation Y = 0.1097x - 0.1338, where Y represents the absorbance value obtained at 390 nm. As shown in figure 4-13. This equation assumes a linear relationship between the concentration of lactic acid and the corresponding absorbance measured. The calibration curve equation, Y = 0.1097x - 0.1338, demonstrates a direct correlation between the absorbance values and the concentration of lactic acid. By applying this equation, the concentration of lactic acid in a sample can be determined by measuring its absorbance and utilizing the established linear relationship.

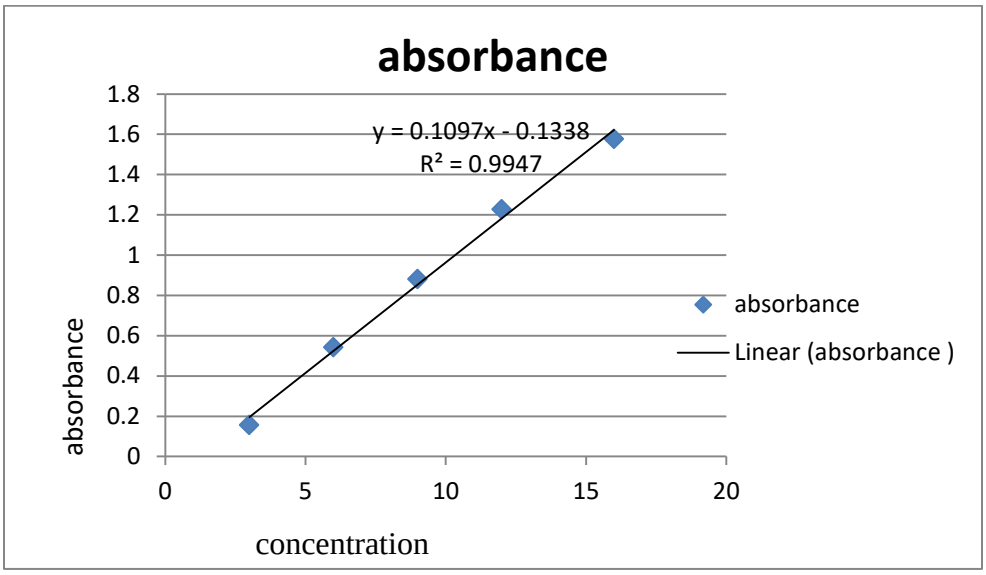

Figure 4-18 calibration curve for lactic acid

Table4-8 lactic acid yields using spectrophotometric method

Std order	Run Order	Temperature (℃)	pH	Time (hr.)	yield(g/l)
1	14	25.00	5.50	12.00	13.5
2	15	35.00	5.50	12.00	23
3	10	25.00	6.50	12.00	20.5
4	16	35.00	6.50	12.00	25.65
5	6	25.00	5.50	48.00	21.24
6	13	35.00	5.50	48.00	32.01
7	20	25.00	6.50	48.00	28.79
8	**11**	**35.00**	**6.50**	**48.00**	**33.48**
9	18	21.59	6.50	30.00	20.54
10	9	38.41	6.50	30.00	32.21
11	2	30.00	5.16	30.00	23.46
12	12	30.00	6.84	30.00	31.25

13	8	30.00	6.00	-0.27	12.25
14	1	30.00	6.00	60.27	31.67
15	5	30.00	6.00	30.00	33.22
16	3	30.00	6.00	30.00	31.48
17	7	30.00	6.00	30.00	31.48
18	19	30.00	6.00	30.00	30.5
19	4	30.00	6.00	30.00	31.5
20	17	30.00	6.00	30.00	30.95

4.3.10Titrimetric analysis for lactic acid content determination

The determination of lactic acid concentration can be accomplished through a titration method, which involves adding a measured volume of a strong base (NaOH) to the lactic acid solution until the reaction reaches the equivalence point. The concentration of lactic acid can be determined by applying the appropriate titration formula. This method serves as an alternative to the spectrophotometric method for accurately determining the concentration of lactic acid.

Table 4-9 lactic acid yield using titration method

Std order	**Run Order**	**Temperature (℃)**	**pH**	**Time (hr.)**	**yield**
1	14	25.00	5.50	12.00	13.62
2	15	35.00	5.50	12.00	28.54
3	10	25.00	6.50	12.00	24.02
4	16	35.00	6.50	12.00	28.65
5	6	25.00	5.50	48.00	28.32
6	13	35.00	5.50	48.00	33.31
7	20	25.00	6.50	48.00	31.75

8	**11**	**35.00**	**6.50**	**48.00**	**34**
9	18	21.59	6.50	30.00	22.3
10	9	38.41	6.50	30.00	33.71
11	2	30.00	5.16	30.00	27.58
12	12	30.00	6.84	30.00	33.45
13	8	30.00	6.00	-0.27	17.31
14	1	30.00	6.00	60.27	33.07
15	5	30.00	6.00	30.00	29.74
16	3	30.00	6.00	30.00	32.21
17	7	30.00	6.00	30.00	32.21
18	19	30.00	6.00	30.00	32.46
19	4	30.00	6.00	30.00	33.94
20	17	30.00	6.00	30.00	32.17

The optimization result was obtained by calculating the average value derived from both titration and spectrophotometry methods.

4.3.11. FT-IR analysis for lactic acid content determination

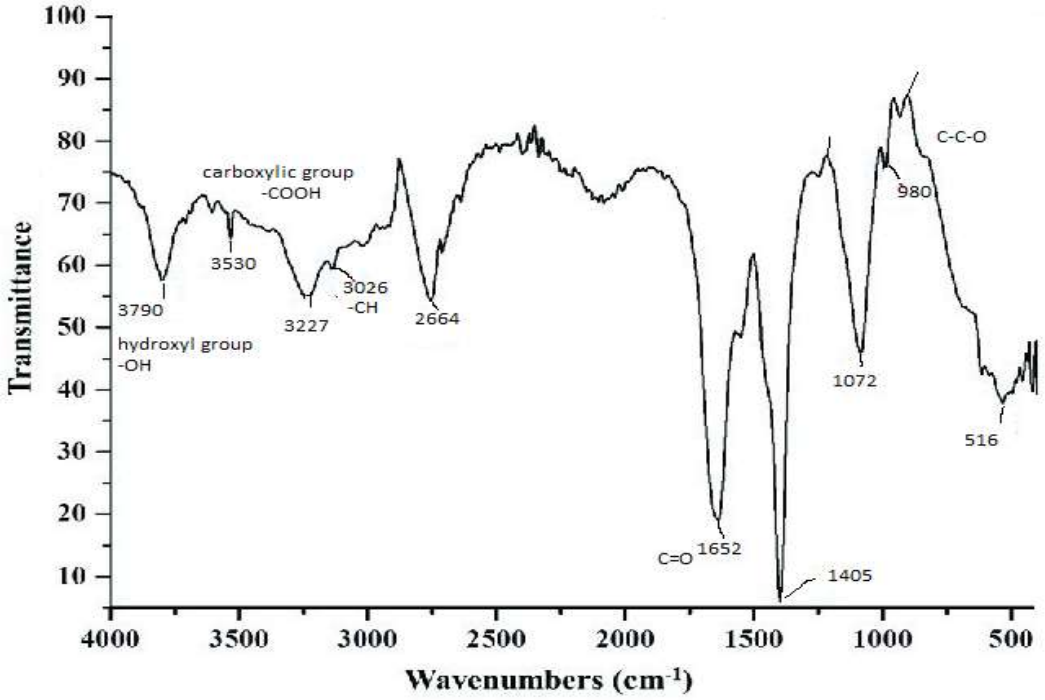

Figure 4-19 FTIR analysis of lactic acid

The peak at 3790 cm^{-1} indicates the stretching vibration of the hydroxyl group (-OH) in lactic acid. This group is commonly found in alcohols and organic acids. A prominent peak at 3530 cm^{-1} corresponds to the stretching vibration of the carboxylic acid group (-COOH), which is a defining characteristic of lactic acid. The wavenumber 2227 cm^{-1} is typically associated with the stretching vibration of the carbonyl group (C=O) present in lactic acid. This group is commonly found in organic acids and esters. At 3026 cm^{-1}, there is a peak that can be attributed to the stretching vibration of the sp^2 hybridized carbon-hydrogen bonds (C-H) present in lactic acid. The peak at 2664 cm^{-1} corresponds to the stretching vibration of the sp^3 hybridized C-H bonds in lactic acid, which are commonly found in alkanes and organic acids. Another distinct peak is observed at 1652 cm^{-1}, indicating the stretching vibration of the carbonyl group (C=O) in lactic acid. At 1405 cm^{-1}, a peak is observed, which can be attributed to the bending vibration of the O-H bond in lactic acid. The peak at 1072 cm^{-1} corresponds to the stretching vibration of the C-O bond present in lactic acid. This group is commonly found in carboxylic acids and esters. At 980 cm^{-1}, a peak is observed, which can be attributed to the bending vibration of the C-C-O bond in lactic acid.

4.4 Application of Lactic acid for fruit biopreservation

4.4.1 Physiochemical analysis of untreated avocado

Table 4-10 Physiochemical analysis of untreated avocado

Parameter	pH	Titratable acidity (%)	TSS (ºBrix)	Surface Color	L*	a*	b*
	5.46 ± 0.04	0.26 ± 0.05	8.87± 0.29		55.00 ± 0.54b	-13.33 ± 0.27	26.00 ± 1.94b

The assessment of acidity levels in avocados relies on two crucial parameters: pH and titratable acidity. These parameters significantly influence the storage and processing quality of the fruit. Avocado typically exhibits a pH value of approximately 5.46 ± 0.04, indicating a slightly acidic nature. When examining the titratable acidity of avocados, it has been observed that different parts of the fruit, particularly the pulp, exhibit relatively low levels of acidity. Several studies, including the research conducted by Athmaselvi et al. (2014), have associated avocados with low titratable acidity values with higher susceptibility to spoilage and reduced shelf life. These findings suggest that avocados with low titratable acidity are more prone to perishability.

Soluble solids, mainly in the form of sugars such as fructose and glucose, are significant components present in fruits and have a notable impact on sensory characteristics, particularly taste and sweetness. According to a study by Vinha et al. (2014), sugars like fructose and glucose are the major soluble solids found in fruits.

In the case of avocados, the total soluble solids content of the pulp was determined to be 8.87 ± 0.29 ºBrix, as indicated in Table 4-10. The ºBrix unit is commonly used to express the concentration of soluble solids in fruit juices, representing the percentage of total soluble solids by weight. In the context of avocados, it reflects the sugar content and other dissolved compounds present in the avocado pulp.The total soluble solids content is a crucial parameter for evaluating the quality and sensory attributes of fruits. In the case of avocados, the measured ºBrix value provides an indication of the expected sweetness and flavor intensity of the fruit.

Color:-Table4-10 provides a comparison of the L*, a*, and b* values for avocado fruit, offering interesting insights into its color characteristics. Specifically, it is observed that the pulp of the avocado has higher L* and b* values compared to other parts. These values indicate that the pulp has a lighter color and contains a higher concentration of yellow pigments, such as flavonoids and carotenoids, which contribute to its visual appearance. Additionally, the pulp exhibits a negative a* value (-13.33 ± 0.27), indicating the presence of green pigments, including chlorophylls. This negative value suggests that the pulp contains a significant amount of green color components, contributing to its overall color profile. Analyzing the L*, a*, and b* values allows us to conclude that the avocado pulp possesses an appealing color combination. It displays a light and yellowish hue, attributed to the presence of flavonoids and carotenoids, while also containing green pigments like chlorophylls.

4.4.2 Physicochemical analysis of treated result

Table 4-11 Physiochemical analysis of biopreservation

Parameter	**Before treated**	**After treated**			**control**
		Day 1	Day 3	Day 6	Day6
pH	5.46 ± 0.04	4.74± 0.05	4.75± 0.08	4.76± 0.01	6.41 ± 0.03
Titratable acidity (%)	0.26 ± 0.05	0.50± 0.09	0.49 ± 0.07	0.48 ± 0.07	0.19± 0.04
TSS (ºBrix)	8.87± 0.29	8.84± 0.29	8.81 ± 0.07	8.80 ± 0.07	9.23± 0.07
Surface Color					
L*	55.00 ± 0.54b	53.67	52.03 ± 0.16	51.99 ± 1.3 30-70	47.03± 0.02
a*	-13.33 ± 0.27	-13.45	-11.12 ± 2.6	- 11.30± 0.09 -10-20	-8.45± 0.09
b*	26.00 ± 1.94b	24.78	23.6 ± 1.2	22.87± 0.77 10-30	19.54± 0.05

The initial pH of the avocado before treatment was slightly acidic at 5.46 ± 0.04. After treatment, the pH significantly decreased to 4.74 ± 0.05 on Day 1, followed by slight increases to 4.75 ±

0.08 on Day 3 and 4.76 ± 0.08 on Day 6. The pH remained acidic throughout the follow-up period, with no significant pH increase observed. Various factors such as buffering capacity, enzymatic processes, can influence these pH changes. And also The titratable acidity before treatment was 0.26 ± 0.05. After treatment, it increased to 0.50 ± 0.09 on Day 3, further decreased to 0.49 ± 0.07 on Day 3, and slightly decreased to 0.48 ± 0.07 on Day 6.

These changes were minimal and within the range of measurement variability.The total soluble solids (TSS) content before treatment was 8.87 ± 0.29 ºBrix, indicating sweetness. After treatment, TSS remained relatively stable, with slight decreases on Day 1 (8.84 ± 0.29 ºBrix) and Day 3 (8.81 ± 0.07 ºBrix). These changes were minimal and within the range of measurement variability, suggesting no significant impact on the sweetness of the avocados.

In conclusion, the surface color of the treated samples remained within a relatively narrow range throughout the experiment, indicating that the avocado was well treated. Initially, the L*, a*, and b* values were recorded as 55.00 ± 0.54, -13.33 ± 0.27, and 26.00 ± 1.94, respectively. After treatment, minimal changes were observed in the color parameters. On Day 3, the L* value decreased slightly to 52.03 ± 0.16, indicating a subtle darkening effect. This trend continued, with the L* value further decreasing to 51.99 ± 1.3 on Day 6, still within an acceptable range. The a* and b* values also showed a minor decreasing trend, suggesting a slight decrease in both redness (a*) and yellowness (b*) of the treated samples over time. Overall, the minimal color changes within the specified range indicate that the avocado was effectively treated without significant alteration to its surface color.

Figure 4-20 biopreservation of avocado sample

4.4.3 Sensory evaluation treated fruit

Visual inspection

During the first day of the experiment, a visual inspection was carried out to compare the bio-preserved avocado samples with the control samples. The bio-preserved avocados were observed to have a slightly darker color and a softer texture compared to the control avocados. These differences can be attributed to the preservation process affecting pigmentation and texture. While there were no significant changes in the overall appearance of the avocados, these observations provided valuable insights for the subsequent days of the experiment.

On the third day, a follow-up visual inspection was conducted to compare the bio-preserved avocados with the controls. The bio-preserved avocados continued to exhibit a slightly darker color and a softer texture, consistent with the observations from the previous day. However, overall appearance differences between the two groups remained minimal, indicating a consistent trend in color and texture alterations throughout the experiment.

On the sixth day, another visual inspection was performed to compare the bio-preserved avocado samples with the controls. The bio-preserved avocados maintained their slightly darker color and

softer texture.These visual assessments indicate stability in the properties of the avocados, with no significant changes in appearance noted over the observation period.

Sensory analysis

A sensory evaluation was conducted on the first day to assess the bio-preserved avocado samples. Due to difficulties in purifying lactic acid, taste evaluation was not possible, and the focus shifted to evaluating the odor. The bio-preserved avocados emitted a slight tangy scent, presumably caused by the presence of lactic acid. In contrast, the control samples maintained a neutral avocado odor. Despite the challenges in taste assessment, the odor evaluation provided indications of the preservation method's impact on sensory properties. Subsequent evaluations in the following days will further enhance the sensory analysis.

The sensory evaluation of the bio-preserved avocado samples continued on the third day, with a specific focus on odor assessment. Due to issues with lactic acid purification, taste evaluation was not feasible. The tangy scent observed on the first day persisted in the bio-preserved avocados, likely due to the presence of lactic acid. This consistent odor suggested minimal changes in lactic acid concentration within a day. In comparison, the control samples retained their neutral avocado scent, highlighting the contrast in aroma between the bio-preserved and control avocados. Despite the limitations in taste assessment, the ongoing aromatic evaluation provided continuous evidence of lactic acid presence in the bio-preserved avocados. The sustained tangy odor in the bio-preserved samples, juxtaposed with the neutral scent of the controls, continued to emphasize the impact of the preserves condition method on the sensory attributes of the avocados.

On the sixth day of the experiment, the focus remained on evaluating the odor of the bio-preserved avocado samples, as taste assessment was still hindered by lactic acid purification issues. During this evaluation4, the bio-preserved avocados continued to exhibit a persistent tangy scent, likely due to the presence of lactic acid resulting from the preservation process. This consistent aroma indicated minimal changes in lactic acid concentration throughout the experiment.

Generally the characterization of avocado after treatment with lactic acid suggests that the product lactic acid has the potential to be used as a preservative for fruits and other food products. The findings indicate that lactic acid treatment can help maintain the acidity and stability of avocados, as observed through the pH and titratable acidity measurements.

5. CONCLUSIONS AND RECOMMENDATION

5.1. Conclusion

This study successfully optimized fermentation parameters for maximizing lactic acid production from taro waste. The initial assessment using the one variable at a time experimental design revealed that *Lactococcus lactis* exhibited the most promising performance among the tested bacterial strains. Subsequently, the effects of temperature, incubation time, and pH on lactic acid yield were evaluated individually while keeping other variables constant.To further enhance lactic acid production, response surface methodology with a central composite design was employed to optimize the fermentation parameters. The central composite design allowed for the exploration of a wide range of parameter combinations and captured the complex interaction effects. Through this optimization process, the optimal conditions for lactic acid production were determined to be an fermentation temperature of 35 °C, an fermentation time of 48 hours, and a pH of 6.5.Under these optimized conditions, *Lactococcus lactis* demonstrated the highest lactic acid yield of 33.74 g/L. This result highlights the effectiveness of the selected bacterial strain and the significant impact of the optimized fermentation parameters on lactic acid production from taro waste.The findings of this study have important implications for the utilization of taro waste as a sustainable resource for lactic acid production. By optimizing the fermentation parameters, the study has provided valuable insights into maximizing lactic acid yield and enhancing the efficiency of the production process.

5.2 Recommendation

Based on the findings of this thesis, the following recommendations are suggested for future research:

1) Evaluate alternative bacterial strains for lactic acid production to explore additional options for industrial-scale production. This could involve screening a broader range of bacterial strains, including both lactic acid bacteria (LAB) and other microbial species, to identify potential candidates with higher lactic acid production capabilities.
2) Consider factors beyond growth potential when evaluating bacterial strains for lactic acid production, including substrate utilization, byproduct formation, and process economics. While growth potential is important, it is essential to evaluate other factors that can impact the overall feasibility and efficiency of lactic acid production.
3) Further optimize fermentation parameters such as agitation rate, carbon source concentration, and nitrogen source supplementation to enhance lactic acid yield. The optimization of fermentation parameters is a continuous process that can lead to improved lactic acid production.
4) Conduct further sensory evaluations to understand the impact of lactic acid treatment on the sensory attributes and overall quality of preserved food. Sensory evaluations play a crucial role in determining the acceptability and consumer preference for preserved foods treated with lactic acid. Future research should include sensory tests involving trained sensory panels or consumer panels to assess the sensory attributes such as flavor, texture, aroma, and appearance of the preserved fruit
5) Conduct microbial tests to assess the microbial load on the treated fruit. Ongoing monitoring and testing of the microbial load on the preserved fruit are essential for ensuring food safety and quality. Future research should continue to conduct microbial tests to assess the presence of spoilage microorganisms, pathogens, and any other microbial contaminants in the treated fruit.
6) Explore the use of varying concentration of lactic acid to enhance the biopreservation of fruits and achieve improved out come

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APPENDIX

Appendix A Extraction and characterization of starch

Figure A-1 extraction of starch

Figure A-2 soxhlet extraction chamber

Figure A-3 furnace

Table A-1 Moisture content of starch

Trial	Starch weight Before drying(g)	Starch weight after drying(g)	Moisture content %
1	2.002	1.781	11.04
2	2.308	2.052	11.09
3	2.153	1.913	11.14
Average moisture content			11.09

Table A-2 ash content of starch

Trial	Starch weight Before ash(g)	Starch weight after ash(g)	ash content %
1	2.452	2.446	0.25
2	2.115	2.110	0.225
3	2.347	2.341	0.275
Average ash content			0.25

Table A-3 fat content of starch

Trial	Starch weight Before extract (g)	Starch weight after extract (g)	fat content %
1	12.117	12.093	0.2
2	10.453	10.443	0.19
3	12.343	12.321	0.18
Average fat content			0.19

Table A-4 protein content of starch

Trial	Titration volume of HCl(ml)	protein content %
1	1.465	0.2905
2	1.3185	0.293
3	1.4895	0.2955
Average protein content		0.293

Table A-5 crude fiber content of starch

Trial	Starch weight Before ash (g)	Starch weight after ash(g)	C.fiber content %
1	2.115	1.21	0.905
2	2.02	1.125	0.895

3	2.54	1.64	0.9
Average C. fiber content			0.9

Table A-6 amylose content of starch

Trial	Absorbance at 620nm	amylose content %
1	0.327	19.935
2	0.328	19.94
3	0.328	19.945
Average amylose content		19.94

Appendix B

hydrolysis and characterization of glucose

Figure A-4 Experimental setup for hydrolysis

Figure A-5 spectrophotometer for glucose analysis

Figure A-6 solution for glucose standard

Table A-7 hydro sate glucose yield

No	Absorbance at 540 nm	Glucose conc (g/L)
1	3.3528	75
2	3.5763	80
3	3.6657	82
4	3.4869	78
5	3.3915	76
6	3.5316	79
7	3.7551	**84**
8	3.4364	77
9	3.6194	81
10	3.2147	72
11	3.7094	83
12	3.5763	80

Table A-8 OVAT experimental design result for glucose after hydrolysis

Run	Temperature(℃)	Ph	Time(hr.)	Yield (g/lt)
1	70	5	3	75
2	70	5.3	3	80
3	70	5.5	3	77
4	65	5.3	3	72
5	70	5.3	3	80
6	75	5.3	3	**84**
7	70	5.3	2	74

8	70	5.3	3	80
9	70	5.3	4	81

Figure A-9 sterilization

Figures A- 10centrifugation

Figure A-11 preparation and LAB on MRS agar

Figure A-12 colony counter

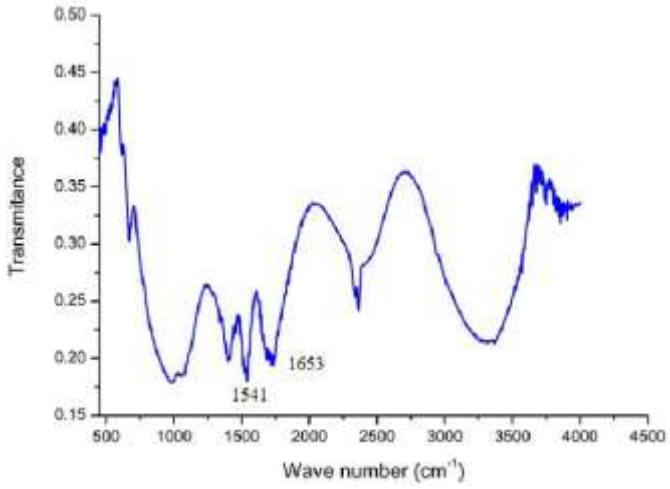

Figure A-14 FTIR for standard glucose

Figure A-13vortex mixer

Table A-9avarage yield of lactic acid

Std order	**Run Order**	**Temperature (℃)**	**pH**	**Time (hr.)**	**Spectro. yield**	**Titration yield**	**Average yield (g/lt)**
1	14	25.00	5.50	12.00	13.5	13.62	13.56
2	15	35.00	5.50	12.00	23	28.54	25.77
3	10	25.00	6.50	12.00	20.5	24.02	22.26
4	16	35.00	6.50	12.00	25.65	28.65	27.15
5	6	25.00	5.50	48.00	21.24	28.32	24.78

6	13	35.00	5.50	48.00	32.01	33.31	32.66
7	20	25.00	6.50	48.00	28.79	31.75	31.27
8	11	35.00	6.50	48.00	33.48	34	33.74
9	18	21.59	6.50	30.00	20.54	22.3	21.42
10	9	38.41	6.50	30.00	32.21	33.71	32.96
11	2	30.00	5.16	30.00	23.46	27.58	25.52
12	12	30.00	6.84	30.00	31.25	33.45	32.35
13	8	30.00	6.00	-0.27	12.25	17.31	14.78
14	1	30.00	6.00	60.27	31.67	33.07	32.37
15	5	30.00	6.00	30.00	33.22	29.74	31.48
16	3	30.00	6.00	30.00	31.48	32.21	31.48
17	7	30.00	6.00	30.00	31.48	32.21	31.48
18	19	30.00	6.00	30.00	30.5	32.46	31.48
19	4	30.00	6.00	30.00	31.5	33.94	32.72
20	17	30.00	6.00	30.00	30.95	32.17	31.56

Figure A-15preliminary fermentation

FigureA-16 titration of lactic acid

Table A-10 Regression coefficient and corresponding 95% CI

Factor	**Coefficient Estimate**	**DF**	**Standard Error**	**95% CI Low**	**95% CI High**	**VIF**
Intercept	31.70	1	0.30	31.03	32.36	
A-T	3.43	1	0.20	2.99	3.87	1.00
B-pH	2.13	1	0.20	1.69	2.57	1.00
C-time	4.63	1	0.20	4.20	5.07	1.00
A2	-1.56	1	0.19	-1.99	-1.14	1.02
B2	-0.95	1	0.19	-1.37	-0.52	1.02
C2	-2.84	1	0.19	-3.27	-2.42	1.02
AB	-1.59	1	0.26	-2.16	-1.02	1.00
AC	-0.84	1	0.26	-1.42	-0.27	1.00
BC	-0.31	1	0.26	-0.89	0.26	1.00

Table A-11 Growth of bacteria indifferent condition

Bacteria type	Temperature	pH	Incubation time(h)	Growth number of bacteria
A	28	6.5	42	3.241×10^7
	30	6.5	42	3.980×10^7
	33	6.5	42	3.753×10^7
B	33	6.5	42	1.1648×10^8
	38	6.5	42	1.3176×10^8
	40	6.5	42	1.2209×10^8
C	30	6.5	24	5.0051×10^8
	34	6.5	42	7.9857×10^8

	39	6.5	60	7.1910×10^8

Bacteria type	Temperature	pH	Incubation time(h)	Growth number of bacteria
A	30	6.5	24	2.9866×10^7
	30	6.5	42	3.980×10^7
	30	6.5	60	5.416×10^7
B	38	6.5	24	0.9878×10^8
	38	6.5	42	1.3176×10^8
	48	6.5	60	1.3209×10^8
C	34	6.5	24	5.0051×10^8
	34	6.5	42	7.9857×10^8
	34	6.5	60	7.2312×10^8

Table A-12 priliminary analysis of LAB in different parameter

Bacteria strain	**T (°C)**	**Time (hr.)**	**pH Control initial**	**Final pH**	**Initial reducing sugar (g/l)**	**Residual reducing of sugar (g/l)**	**Initial O.D (600 nm)**	**Final O.D (600 nm)**
A	30	24	6.5	4.71 ± 0.04	55	**14.342**	0.03	1.38 ± 0.07
	30	42	6.5	3.87 ± 0.05	55	9.806	0.03	1.85 ± 0.04
	30	60	6.5	3.73 ± 0.41	55	9.545	0.03	1.93 ± 0.02
B	38	24	6.5	4.59 ± 0.07	55	15.3	0.03	1.85 ± 0.07
	38	42	6.5	4.21 ± 0.09	55	13.62	0.03	1.87 ± 0.02
	38	60	6.5	4.17 ± 0.11	55	11.39	0.03	1.94 ± 0.02
C	34	24	6.5	3.94 ± 0.11	55	12.14	0.03	1.82 ± 0.02

	34	42	6.5	3.53 ± 0.16	55	**3.928**	0.03	1.79 ± 0.02

Bacteria strain	**T (°C)**	**Time (h.)**	**pH Control initial**	**Final pH**	**Initial reducing sugar (g/l)**	**Residual reducing of sugar (g/l)**	**Initial O.D (600 nm)**	**Final O.D (600 nm)**
A	28	42	6.5	4.02 ± 0.04	55	**13.192**	0.03	1.45 ± 0.07
	30	42	6.5	3.87 ± 0.05	55	9.806	0.03	1.85 ± 0.04
	33	42	6.5	3.94 ± 0.41	55	12.013	0.03	1.59 ± 0.02
B	33	42	6.5	4.79 ± 0.07	55	15.52	0.03	1.23± 0.07
	38	42	6.5	4.21 ± 0.09	55	13.62	0.03	1.87 ± 0.02
	40	42	6.5	4.55 ± 0.11	55	11.23	0.03	1.74 ± 0.02
C	30	42	6.5	3.87 ± 0.11	55	12.14	0.03	1.64 ± 0.02
	34	42	6.5	3.53 ± 0.16	55	**3.928**	0.03	1.79 ± 0.02
	39	42	6.5	3.99 ± 0.14	55	5.583	0.03	1.68 ± 0.02
	34	60	6.5	4.50 ± 0.14	55	4.712	0.03	1.75 ± 0.02