JIMMA UNIVERSITY

JIMMA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL ENGINEERING

Optimization of Fermentation Condition and Characterization for Lactic Acid Production from Khat ("*Catha edulis*") Waste by Using Immobilized Lactobacillus Plantarum

---

By

SISAY FANTA

A Thesis Submitted to Jimma University, Jimma Institute of Technology, School of Chemical Engineering in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Process Engineering

ADVISOR   **CO-ADVISOR**

EDO BEGNA (Ph.D.)   **Mr. ABRHA G/MESKEL**

November 2021

Jimma, Ethiopia

JIMMA UNIVERSITY

JIMMA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL ENGINEERING

Optimization of Fermentation Condition and Characterization for Lactic Acid Production from Khat ("*Catha edulis*") Waste by Using Immobilized Lactobacillus Plantarum

A Thesis Submitted to Jimma University, Jimma Institute of Technology, School of Chemical Engineering in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Process Engineering

By

SISAY FANTA

<u>ADVISOR</u>   <u>CO-ADVISOR</u>

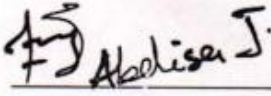EDO BEGNA (Ph.D.)   Mr. ABRHA G/MESKEL
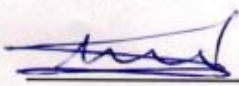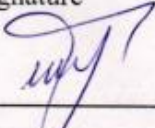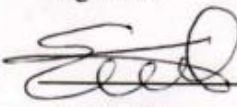
November 2021

Jimma, Ethiopia

JIMMA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL ENGINEERING

This is to certify that the thesis prepared by **Sisay Fanta** entitled **"Optimization of Fermentation Condition and Characterization for Lactic Acid Production from Khat ("*Cathus edulis*") Waste by using Immobilized Lactobacillus Plantarum "** and submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science in Chemical Engineering (Process Engineering) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Approved By the Examining Board

Abdisa Jabesa (PhD)	*Abdisa J.*	03/11/2021
External Examiner	Signature	Date
Lema Deme (MSc)	*[signature]*	04/01/2022
Internal Examiner	Signature	Date
Edo Begna (PhD)	*[signature]*	04/01/2022
Advisor	Signature	Date
Abrha G/Meskel (MSc)		
Co-Advisor	Signature	Date
Samuel Gesessse (MSc)		
Chairperson	Signature	Date
Ermias Girma (MSc)	*[signature]*	04/01/2022
School dean	Signature	Date

DECLARATION

This thesis is prepared by Sisay Fanta, entitled "**Optimization of fermentation condition and characterization for lactic acid production from khat ("*Catha edulis''*) waste by using immobilized lactobacillus plantarum**" and submitted in partial fulfillment of the requirements for the degree of master of science in chemical engineering (process engineering stream). I confidently declare that this thesis is my original work, has not been presented for a degree in this or any other university, but it includes other work as reference sources, which have been fully acknowledged. I worked the thesis under the supervision of Dr. Edo Begna and Mr. Abrha G/meskel Lecturers of JU, JiT, School of Chemical Engineering.

Researcher Name	**Signature**
SISAY FANTA	_____
Date of submission	_____
Main Advisor	**Signature**
EDO BEGNA (Ph.D.)	_____
Co-Advisor	**Signature**
Mr. ABRHA G/MESKEL	_____

ABSTRACT

Huge amounts of petroleum-based plastic wastes are generated annually possessing serious environmental problems that must be addressed. Poly lactic acid, which is an eco-friendly bioplastic product that can be used as an alternative to fossil-based plastics has gotten the attention of many researchers. Accordingly, owing to its great potential as a building block for the synthesis of poly lactic acid, the demand for lactic acid has grown substantially in recent years. Lactic acid production from lignocellulosic biomass is becoming more popular as a way to solve the problem associated with first-generation biomass. In the present study, the optimum fermentation conditions for maximum lactic acid production from khat waste biomass by using immobilized lactobacillus plantarum was investigated. Characterization of the khat waste like proximate analysis, chemical composition analysis, Fourier Transform Infrared Spectroscopy, and some physio-chemical properties of the product was done. The chemical composition analysis result revealed that khat waste has cellulose (43.7 $\pm$ 0.71%), hemicellulose (17.42 $\pm$ 0.85%), and lignin (30.63 $\pm$ 0.93%). The investigated physio-chemical properties (density, kinematic viscosity, odor, reactivity, and miscibility) and functional groups of the product were in good agreement with the standard one. The effect of cell immobilization on the yield of lactic acid has been investigated and the yield obtained from immobilized lactobacillus plantarum cell is 24.7% higher than the free cells'. The effects of three process parameters (incubation temperature, incubation time, and pH) on the yield of lactic acid were investigated. Response surface methodology with a central composite design was used for the optimization of lactic acid yield and process parameters. Based on the analysis, the optimum fermentation conditions were found to be incubation temperature of 42.3 ᵒC, incubation time of 40.0 hrs, and pH of 6.18. Under these conditions, the maximum lactic acid yield obtained was 23.05 g/L. The experimental yield of lactic acid (22.98 $\pm$ 0.10 g/L) is in smooth agreement with the predicted one (23.05 g/L), showing the suitability of the quadratic model used. The exponential growth of lactobacillus plantarum cell was observed from the kinetic model of microbial growth study under optimum conditions. The applicability of the produced product for poly lactic acid synthesis was investigated. Generally, the findings of the study suggest that utilization of khat waste as a potential carbohydrate source and immobilization of lactobacillus plantarum cells for lactic acid production is worthful.

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACRONYMS

CADR	Compound Annual Growth Rate
CCD	Central Composite Design
DM	Dry Matter
FC	Fixed Carbon
HDPE	High-density polyethylene
LA	Lactic Acid
LAB	Lactic Acid Bacteria
LCB	Lignocellulosic Biomass
LDPE	Low-density polyethylene
MRS	De Man Rogassa and Sharpe
PET	Polyethylene terephthalate
PE	Polyethylene
PLA	Poly Lactic Acid
PP	Polypropylene
RSM	Response Surface Methodology
SD	Standard Deviation
VM	Volatile Matter

1. INTRODUCTION

1.1. Background of the study

Nowadays, researchers have been focusing on the sustainable management of plastic wastes on earth by finding eco-friendly alternative resources for the production of plastics. As an alternative to fossil-based plastics, the idea of producing bioplastics is gaining popularity in front of many researchers. According to the studies conducted so far, products with similar performance can be achieved by using biological feedstocks instead of fossil-based resources (Cinar et al., 2020; Jing *et al.*, 2019). Therefore, the production of bioplastic has been attracting a great deal of research and development.

Recently, several bioplastic products have been researched, developed, and grown rapidly. The sustainability and biodegradability of these products increase their attractiveness. They would easily degrade through the enzymatic actions of microorganisms if disposed to the environment (Qian *et al.*, 2018; Saha *et al.*, 2019). The main bioplastic used so far to prepare these innovative materials are some aliphatic polyesters (e.g. polylactic acid and polyhydroxyalkanoates), various polypeptides (i.e. soy proteins, collagen, and gelatin), and numerous polysaccharides (i.e. starch, chitin, pectin) obtained from plant or animal feedstocks. Among these, Poly Lactic Acid (PLA) which is aliphatic polyester and a biodegradable product is one of the most commercially successful bioplastics (Garlotta, 2019; Li *et al.*, 2020). As compared with most other biodegradable plastics, it has better mechanical strength, biocompatibility, and transparency. It is commonly produced from a lactic acid monomer via ring polymerization reaction (Estivéz Rivadulla, 2019).

Lactic Acid (LA) is a potential feedstock for the production of PLA. Furthermore, it has been widely used in various cosmetic, pharmaceutical, food, polymer, and chemical industries (Wang *et al*., 2020). The first commercial production of LA was started in Japan in 1950 (Auras *et al.*, 2011). Nowadays, it is produced commercially either by chemical synthesis or microbial fermentation (Chinenye *et al.*, 2021). The chemical synthesis method involves the derivation of LA from petrochemical products, which relies on fossil fuel depletion, and generates the racemic LA mixture (D-L-LA) via Lacto nitrile hydrolysis. On the contrary, the microbial fermentation method explores the possibility of using renewable resources for the production of environmentally friendly LA products. It generates optically pure (L (+) or D (-) LA isomer) via

microbial fermentation depending on the strain selected (Alves *et al*., 2020; Chinenye *et al*., 2021). Since it compromises an alternative technique to avert environmental pollution caused by the petrochemical industry and the inadequate supply of fossil-based resources, the biotechnological production of LA has received significant attention recently (Ali, 2017).

The LA production is strongly dependent on the cost of raw material used (Ali, 2017). Therefore, it is essential to select a raw material for its industrial production with a number of factors. These include availability, food confliction, cost, production rate, and yield with little or no by-product formation (Rojan *et al*., 2020). By compromising the above criteria, lignocellulose biomass is the preferred substrate to meet the huge demand for LA production (Abdel-rahman and Sonomoto, 2016). Khat (*Catha edulis*) is a flowering evergreen plant. It belongs to the Celastrus family (Yusuf, 2008). The most favored part of the leaves is the young shoot near the top of the plant. However, the other major part ''khat garaba'' is simply disposed to the environment and considered as waste. However, khat waste is lignocellulose biomass, which has enough amount of cellulose and hemicellulose (Abdulhafiz, 2014; Gabriel *et al*., 2021). It is abundantly available in different cities of Ethiopia like Jimma, Aweday, Wolkite, Harar, Dire Dawa, Addis Ababa, etc. (Rameshwar & Argaw, 2016).

Moreover, the production of LA through microbial fermentation method mainly depends on microbes (bacteria, fungi, and yeast) used for fermentation. The yield and productivities of fungal and yeast strains are very low as compared with bacterias' (Subramanian et al., 2015). Among these bacteria, lactobacillus is the main species that can be utilized for LA production as free or immobilized cells. However, factors such as incubation temperature, incubation time, and pH are substantially affecting the metabolic process of the microbes. Therefore, optimization of fermentation is mandatory for finding the major factor influencing the metabolic process. Rather than single parameter optimization, the optimization by response surface methodology has benefits including saving time, space, and raw material (Thakur *et al*., 2018).

Keeping in view the above ideas, the current study has been carried out to optimize the fermentation process parameters (incubation temperature, incubation time, and pH) for maximizing the LA production by using immobilized *lactobacillus plantarum* cells via Response Surface Methodology – Central Composite Design (RSM-CCD). Sodium alginate was used for

immobilization. Khat waste was hydrolyzed and then fermented to LA. Hence, this study obsoletes the idea of khat waste and makes it a potential resource for LA production.

1.2. Statement of the problem

Nowadays, due to its multi functionalities, the demand for PLA is dramatically increased in different sectors. Consequently, the requirement of its monomer LA is incredibly increased throughout the world. However, most of the commercialized LA productions use carbohydrate sources such as sugar beet, sugarcane, wheat, potato, whey, and corn (Ghaffar *et al.*, 2014). These resources are highly conflicting with human food. Therefore, to date, the researchers are finding an alternative feedstock like lignocellulosic biomass to overcome this problem. Microbial fermentation is the common method used for LA production from lignocellulosic biomass. Nevertheless, during the fermentation process, most of the researchers have not addressed some crucial concepts. These include lack of cell immobilization along with homofermentative pathway. Therefore, owing to the lack of cell immobilization, numerous factors such as the substrate and product inhibitors, organic solvents, and salt are substantially affecting the fermentation process. Besides, in the heterofermentative pathway, several byproducts, which cause an increment of purification cost are formed. Not only this but also, process parameters such as incubation temperature, incubation time, and pH are significantly affecting the metabolic process of the microbes. Therefore, optimization of fermentation conditions for finding the major factor influencing the LA yield by overcoming the aforementioned problem is indispensable.

Moreover, solid waste management is the main challenge of municipal governments. Especially, in urban areas, a huge amount of waste is generated in daily human activities. Khat waste is one of the solid waste, which is daily disposed to the environment. Though the daily consumption of khat by millions of people in Ethiopia is increasing, very little concern was given about the waste related to khat. Averagely, an individual consumes about 0.4 kg per day and around 70% of residue was generated per kg of chewed khat (Maria, 2019; Fikadu, 2018). Mostly in the rainy seasons, the town's khat waste together with other solid wastes serve as the breeding ground for vectors (mosquito and flies) that spread diseases (Yusuf, 2008). These aforementioned problems reveal that there is a gap of awareness in the public on proper management of the wastes.

Having this all in mind, using khat waste as a potential carbohydrate feedstock for the production of LA is striking. Thus, rather than disposed as a waste, it was utilized for LA production.

Generally, this study addresses the aforementioned problems and endeavor to overcome such problems by reusing the khat waste for producing environmentally friendly LA product.

1.3. Objective of the study

1.3.1. General objective

The general objective of this study is to characterize and optimize lactic acid produced from khat waste biomass by using immobilized *lactobacillus plantarum*. The produced product can be used as a potential feedstock for poly lactic acid synthesis.

1.3.2. Specific objectives

- To determine the physio-chemical composition of khat waste. This includes cellulose, hemicellulose, lignin, extractives, moisture, ash, DM, VM, and FC contents;

- To investigate the effect of immobilization of *lactobacillus plantarum* cell on the yield of lactic acid obtained from the fermentation process;

- To determine and optimize the effects of major fermentation parameters (incubation temperature, incubation time, and pH) on the yield of lactic acid by using RSM-CCD;

- To develop the kinetic model for *lactobacillus plantarum* growth during the fermentation process (i.e. under optimum fermentation conditions);

- To determine the physio-chemical properties of the obtained lactic acid product. This includes kinematic viscosity**,** density, odor**,** specific gravity, functional group, miscibility, and reactivity;

- To investigate the applicability of the produced product for poly lactic acid synthesis.

1.4. Hypothesis of the study

The immobilized *lactobacillus plantarum* cell can utilize khat waste carbohydrate as a carbon source with different controlled factors for LA production. The effects of substrate and product inhibitors are substantially reduced. Therefore, the yield of LA obtained from this biomass is good.

1.5. Significance of the study

This study has a lot of significance for various sectors including government, public, individual, and industries. Since the feedstock for LA production is abundantly available, the capacity of the LA manufacturing industry will be increased. Moreover, the yield of LA obtained was optimized. Consequently, the PLA manufacturing polymer industry will be increased.

Therefore, if this study is implemented, then the expenditure incurred for importing PP, LDPE, HDPE, PVC, PU, PS will be reduced. Thus, the environment will not be suffered more from plastic pollution derive from petroleum. Consequently, the demand for PLA products will be satisfied. Furthermore, once the reusability of the khat waste for LA production is fulfilled, the awareness of people on collecting this waste rather than disposing it into the environment will be increased. Hence, the cities of our country will be kept clean and the green economy strategy will be ensured.

1.6. Scope of the study

This study includes the task starting from khat waste collection to production of LA along with the raw material and product characterization. Moreover, the effects of some major fermentation process variables (incubation temperature, pH, and incubation time) were determined. Furthermore, the optimum values of these parameters were also determined. Lastly, the obtained product is compared with the literature and discussed.

2. LITERATURE REVIEW

2.1. Lactic acid

LA is an organic acid, which has both a carboxyl group (COOH) and hydroxyl group (-OH). First, it was discovered in sour milk in 1780 by Swedish chemist Carl Wilhelm Scheele. Primarily, it was considered as a milk component. Nine years later, Lavoisier termed this milk component as "acide lactique", which developed to the current terminology for lactic acid. However, in 1857 Louis Pasteur discovered that LA was not a milk component, rather a fermentation metabolite generated by different microbes (Wee *et al.*, 2006). In 1881, in support of Pasteur's discovery, a French scientist Frémy produced LA by fermentation and this gave rise to the first microbial fermentation industrial production of LA in the United States (Ghaffar *et al.*, 2014; Auras *et al.*, 2011).

In recent years, due to its great potential as a building block for the production of PLA materials, which is an alternative to petroleum-based plastics the demand for LA has grown substantially (Abdel-rahman and Sonomoto, 2016; Wang *et al.*, 2020; Saha *et al.*, 2019). The global demands for LA were estimated to be 714,200 tons in 2013 and annually grow with the rate of 18.7% in the international market (Global Lactic Acid Market Report, 2019). In support of this, the Ethiopian investment agency data reveals that the demand for LA in 2012 was nine tons, and the demand for this product is projected to reach 28.36 tons by the year 2023.

2.2. Current trends of production of lactic acid

In this study, to date, scientific literature regarding LA production at a commercial scale and research and development level has been reviewed. Generally, about 90% of total LA produced throughout the world is via microbial fermentation (Koekemoer, 2018; Zhang and Vadlani, 2015). Nevertheless, most of the commercialized LA productions are extremely uses in the following carbohydrate sources such as sugarcane, wheat, potato, whey, corn starch, and barley malt (Battula *et al.*, 2019). Owing to these resources are highly conflicting with human food, numerous researchers have highly striving to overwhelm these problems. Nowadays, they are finding an alternative feedstock like lignocellulosic biomass, which does not conflict with human food and enables to produce a sustainable product. Some articles regarding the production of LA from lignocellulosic biomass are summarized in Table 2.1.

Table 2.1 Summary of some articles on lignocellulosic biomass for production of LA

Feedstock	Pretreatment and Hydrolysis	Fermenting Microorganism	LA Yield $(g \times g^{-1})$	Reference
Rice Straw	Dilute H_2SO_4	*lactobacillus plantarum*	0.69	Tu *et al*., 2019
Corn Stover	NaOH pretreatment	*Lactobacillus brevis* and *lactobacillus plantarum*	0.78	Zhang and Vadlani, 2015
Wheat straw	Ionic liquid	Immobilized *Lactobacillus brevis*	0.70	Grewal and Khare, 2017
Beechwood	Organosolv pretreatment	*Lactobacillus delbrueckii*	0.69	Karnaouri *et al*., 2020
Sugarcane bagasse	Ionic liquid	*Lactobacillus brevis*	0.52	Grewal and Khare, 2018
Corn stover	NaOH pretreatment	*Lactobacillus pentose*	0.66	Hu *et al*., 2016
Wheat straw	Ionic liquid	*Lactobacillus brevis*	0.49	Grewal and Khare, 2018

> **Note**: $(g \times g^{-1})$ indicates that gram of LA produced from gram of biomass (cellulose + hemicellulose).

Generally, the above-summarized articles are endeavored to overcome the problem associated with a first-generation feedstock that conflicts with human food. Besides, they add various raw materials for the production of sustainable LA products. However, they have their own gaps, which need to be filled to meet the current demand for LA products, especially on biodegradable PLA productions. These gaps are discussed later in section 2.11.

2.3. Properties of lactic acid

Lactic acid (IUPAC systematic name: 2- hydroxypropanoic acid), is an essential organic acid that is being widely used around the globe in a range of industrial and biotechnological applications (Ghaffar *et al.*, 2014). It is colorless, sour in taste, odorless, and soluble in all proportions in water, alcohol, and ether but insoluble in chloroform (Ameen and Caruso, 2017). Because of the presence of hydroxyl and carboxyl functional groups, LA can possess a wide variety of chemical reactions for LA. These include reactions that include oxidation, reduction, condensation, and substitutions (Sridhar *et al.*, 2012). The Physiochemical properties of LA are summarized in Table 2.2.

Table 2. 2 Physiochemical properties of LA

Identification Parameters		Description	Reference
Compound name		Lactic Acid	Komesu *et al.*, 2017;
IUPAC Name		2-Hydroxypropanoic acid	Mohanty *et al.*, 2015
Chemical formula		$C_3H_6O_3$	and
Molecular mass (g/mol)		90.08	Sridhar *et al.*, 2012
Taste		Mild acid taste	
Odor		Odorless	
Crystallization		Forms crystal when highly pure	
Normal Boiling point (ºc) at 14 mmHg		122	
Melting point (ºc)	L(+)	53	
	D(-)	52.8	
Density (g/ml)		1.2	

Dissolution constant (Ka)	1.37×10^{-4}

2.3.1 Isomers of lactic acid

LA is a chiral compound, which has a carbon chain composed of a central atom and two terminal carbon atoms. A hydroxyl group is attached to the chiral carbon atom while one of the terminal carbon atoms is part of the carboxylic group and the other atom is part of the methyl group (Narayanan *et al.*, 2004). Thus, the two optically active isomeric forms of LA exist: L (+) - form, and its mirror image is D (−) – form is depicted on Fig 2.1. However, the optical composition does not affect most of the physical properties with the important exception of the melting point of the crystalline acid (Ou *et al.,* 2011).

The optical purity of the obtained isomers of LA highly relies on the species used in the fermentation broth. Optically pure L (+) - LA is widely produced by using all species of rhizopus via aerobic fermentation, and some species of lactobacillus (*Lactobacillus brevis* and *Lactobacillus casei*) via anaerobic fermentation. On the contrary, D (-) - LA is produced by using some species of Lacto bacillus (*Lactobacillus bulgaricus* and *Lactobacillus delbrukii*) via anaerobic fermentation (Garlotta, 2019; Abdel-rahman and Sonomoto, 2016; Mirdamadi *et al.*, 2002).
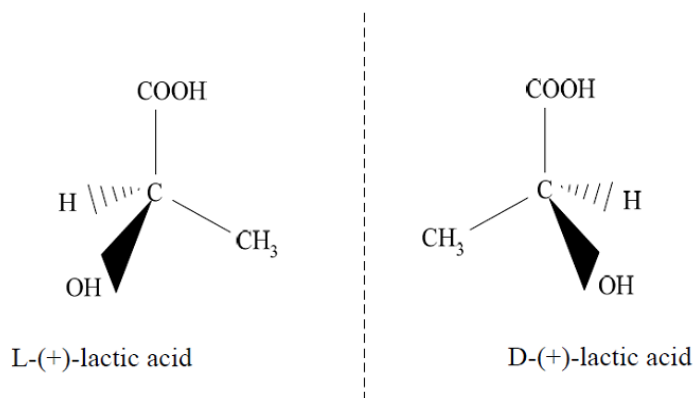
Figure 2. 1 Chemical structure of L (+) and D (−) lactic acid isomers

Source:- (Mirdamadi *et al. (2002))*

2.4. Application of lactic acid

Even though LA has the most eminent application for the production of biodegradable PLA materials, it has far more diverse applications in the commercial and different sectors. These

include food, pharmaceutical, chemical, and cosmetics industries (Chinenye *et al.*, 2021; Tarraran and Mazzoli, 2018). These were summarized in Table 2.3.

Table 2. 3 Application of LA in different sectors

Field	Application
Polymers Industries (PLA)	❖ Food containers
	❖ Protective clothing
	❖ Trash bags
	❖ Rigid containers
Food Industries	❖ Preservatives
	❖ Acidulants
	❖ pH regulators
	❖ Bacterial inhibitions
Pharmaceutical Industries	❖ Dialysis solution
	❖ Mineral preparation
	❖ Surgical sutures
	❖ Controlled drug delivery system
Chemical Industries	❖ Neutralizers
	❖ Chiral intermediates
	❖ Green solvents
	❖ pH regulators
	❖ Cleaning agents
Cosmetics Industries	❖ Moisturizers
	❖ Anti-acne agents
	❖ Humectants
	❖ Anti-tartar agents
	❖ Skin lightening agents

Source:- (Ali, 2017; Wee *et al.*, 2006)

2.5. Market of lactic acid product

2.5.1. World market of lactic acid

Globally, the market for LA has witnessed significant growth as it is used as a substitute for toxic chemicals and plastics. Especially, the production of PLA increases the demand for LA tremendously. Besides, its demand was increased in sectors such as the food, pharmaceutical, chemical, and cosmetics industries (Grand View Research, 2019). Diagrammatically, it was depicted as Fig 2.2.
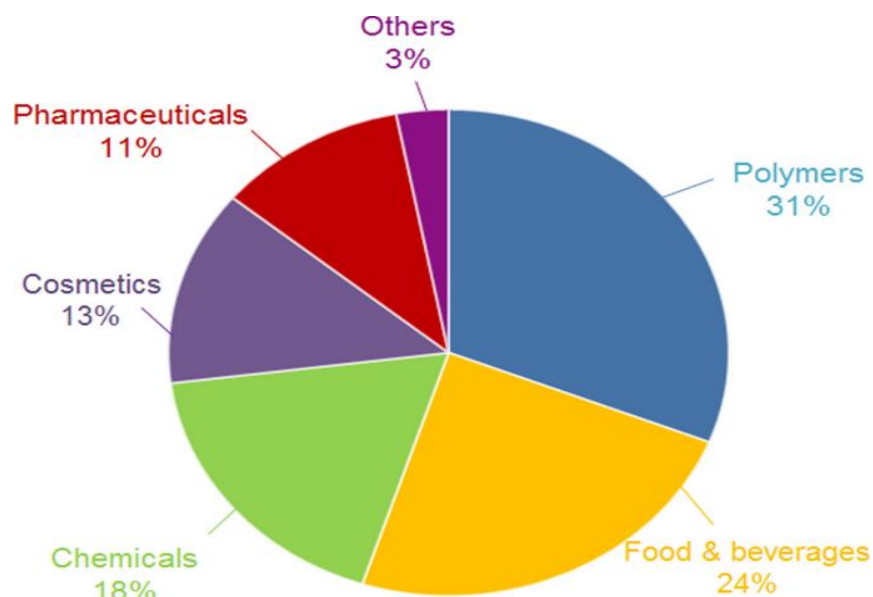
Figure 2. 2 Pie chart representation of LA demand

Source: (Grand View Research, 2019)

2.5.1. Ethiopia's market of lactic acid

Similarly, in Ethiopia, the demand for LA in different sectors is increasing annually. However, owing to the lack of commercialized LA production, Ethiopia is costing a huge amount of money to import LA. The country's demand for LA is entirely met through imports (Nesryia, 2018). The quantity and value of LA imported are presented in Table 2.4.

Table 2. 4 Import data of LA in Ethiopia from 2011-2017

Year	Qty (Ton)	Value (Birr)
2011	8.2	1,243,590
2012	9	1,500,123
2013	10	2,243,490
2014	11.09	3,432,211
2015	13.66	5,321,123
2016	15.67	8,214,984
2017	16.83	9,321,432

Source:- (Nesryia, 2018)

2.5.3 Market of poly lactic acid product

Due to the utilization of petroleum-based plastic products such as PP, PET, PE, LDPE, and HDPE adversely affect the environment, utilization of an alternative environmentally friend bio-based (i.e. PLA) product is substantially increased (Sungyeap Hong, 2014). The global PLA market size is valued at USD 525.47 million in 2020 and is expected to grow at a compound CAGR of 18.1% from 2021 to 2028. The demand for the product is mostly driven by the end-use industries, such as agriculture, transport, textile, and packaging (Grand View Research, 2019). Fig 2.3 shows the demands of PLA in different sectors.
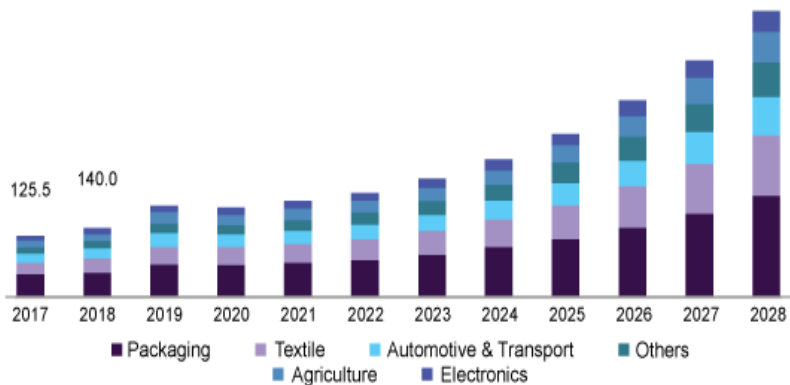
Figure 2. 3 PLA market size
Source:-(Grand View Research, 2019)

2.6. Raw materials to produce lactic acid

Raw material cost is one of the major factors in the economic production of LA. There are three main groups of raw materials available for the production of LA by fermentation. These include sugar, starch, and lignocellulose. Prematurely, the first generation for microbial fermentation of LA is highly reliant on the utilization of sugar feedstocks such as sugarcane, sugar beets, sweet potatoes, fruits, and sweet sorghum. Hence, they are highly conflicting with human food especially in developing countries. Therefore, researchers are striving to shift the current LA productions from the first to second generation (starch and lignocellulose) biomass (Chinenye *et al.*, 2021).

Lignocellulose obtained from agricultural, forestry, and industrial manufacturing waste has enough amounts of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are the major parts, which provide the fermentable sugar by passing through pretreatment followed by hydrolysis. Consequently, this sugar is fermented to the desired product based on the selected microorganism. Due to this complex production process, many factors can affect the yield obtained from these feedstocks (Tu *et al.*, 2019). Hence, the selection of appropriate materials with fermenting microorganisms is indispensable. Some factors that need to be taken into account while selecting Lignocellulosic biomass include the ability to produce a high yield of fermentable sugar, negligible or no formation of byproduct, high productivity, and less contamination so that not much pretreatment is required (Edris, 2019).

Khat waste (''khat garaba'') is one of lignocellulosic biomass which contains cellulose (39 - 55%), hemicellulose (12.8 – 13.5%), lignin (25.6 %-31%) (Gabriel *et al.,* 2021; Abdulhafiz,

2014; Tessfaw *et al.*, 2020). Additionally, Tessfaw *et al.* (2020) study revealed that khat waste biomass has some amount of nitrogen (0.97–1.68%) and phosphorous (0.58–0.76%). These elements are very essential in the fermenter, which is used as the nutrition for fermenting microorganisms. Moreover, khat waste is the largest quantity of solid waste generated in the cities and towns, which conflicts with the clean policy of the country. Furthermore, this waste together with other solid wastes serves as the breeding ground for rodents and vectors (mosquito and flies) that spread diseases (Yusuf, 2008). Therefore, utilizing this biomass for LA production is worthy.

2.7. Cultivation and consumption of khat

The production, sale, and consumption of khat are legal in the countries where its use is traditional of those cultures. These include Djibouti, Kenya, Uganda, Ethiopia, Somalia, and Yemen. In recent times, a greater number of farmers are engaged in growing and producing stimulant crops such as khat and coffee than those growing fruits (Abdulhafiz, 2014). Ethiopia is one of the world's largest khat producers, which has recently become the fastest-growing export commodity. Currently, over two million farmers produce khat on more than 250,000 hectares of land (Gabriel *et al.*, 2021; Gessesse Dessie, 2013; Wuletaw, 2018).

The harvestable part of khat has consumable and non-consumable parts. The chewable portion is succulent and tender while the other part is unfit for consumption (Abdulhafiz, 2014). Culturally, all stages of generations including children, younger, elder, pregnant, and breastfeeding women have utilized khat (Gabriel *et al.*, 2021).

2.7.1. Availability of khat waste

In Ethiopia, annually different organic wastes are being produced by different activities like agro-industries, industries, and municipal solid waste (Rameshwar and Argaw, 2016). Khat waste is one of the solid waste, which was generated daily throughout 24 hrs especially in cities of export centers like Dire Dawa, jigjiga, and mojo. Besides, it was daily generated in each city of our country (Beneberu, 2017; Filaba, 2008).

Chewing of khat ("*Catha edulis*") is a common habit in some countries of East Africa and the Arabian Peninsula. Generally, it is estimated that around 20 million population have been

chewing khat per day in the Arabian Peninsula and Eastern Africa region including Ethiopia. Ethiopia is believed to be the country of origin of khat where millions chew khat per day (Teklie et al., 2017). Therefore, as mentioned above, a large portion of this khat has been disposed to the environment, while a little amount of part was chewed. Hence, it can be concluded that this biomass is abundantly available.

2.8. Lactic acid production technology

Lactic acid is a naturally occurring organic acid that can be manufactured industrially via microbial fermentation and chemical synthesis (Juodeikiene et al., 2019). The former method, microbial fermentation, has superior advantage because an optically pure LA can be obtained by choosing a suitable strain of microorganism, whereas chemical synthesis always results in a racemic mixture of LA isomers (Abdel-rahman and Sonomoto, 2016; Wee et al., 2006). The block flow diagrams for LA production via the two above mentioned methods are depicted in Fig 2.6.
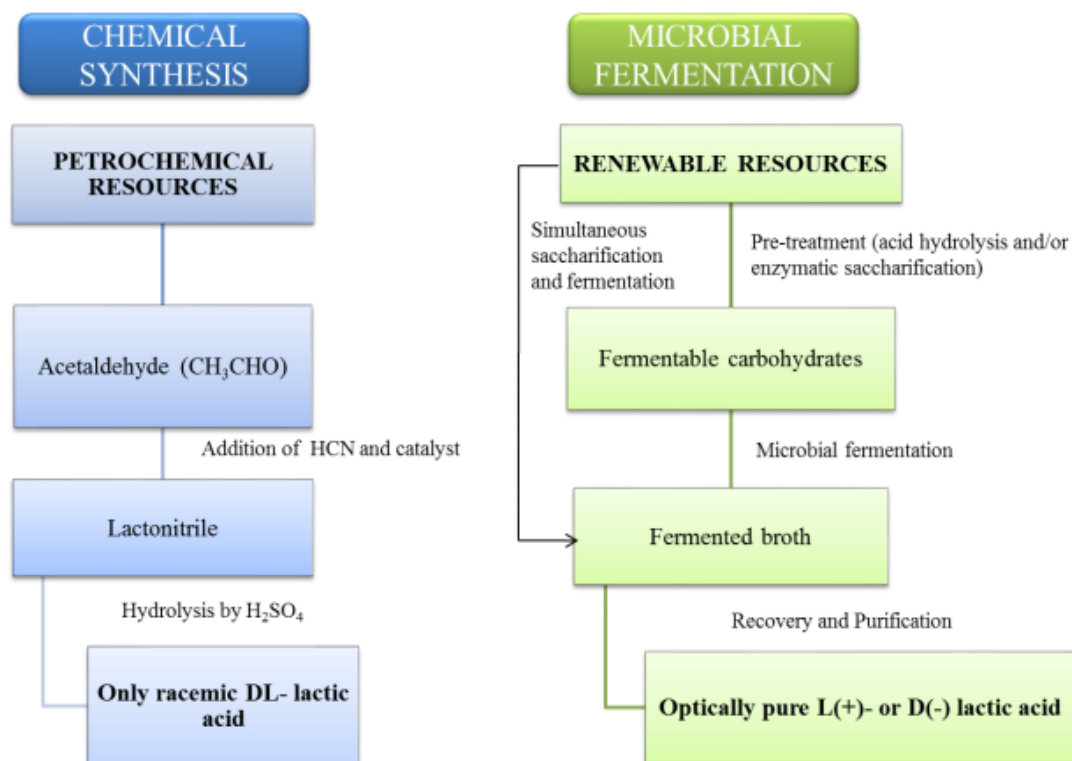
Figure 2. 4 Block flow diagram of the two manufacturing processes of LA

Sources :- (Wee et al., 2006)

2.8.1. Chemical synthesis method

This method of LA production is highly dependent on petrochemical feedstocks. The commercial LA production process for this method is based on lactonitrile. Initially, Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction takes place in the liquid phase at high atmospheric pressures. Then, the crude lactonitrile is recovered and purified by distillation. It is then hydrolyzed to LA, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and LA. Finally, LA is esterified with methanol to produce methyl lactate before being purified by the means of distillation and hydrolyzed by water under an acid catalyst to produce a racemic mixture of DL-lactic acid and methanol (Narayanan *et al*., 2004).

2.8.2. Microbial fermentation method

This is the most dominant production method for LA. It is estimated that about 90% of total LA produced throughout the world is via microbial fermentation (Koekemoer, 2018). In fermentation processes, bacteria or other microorganisms produce LA as they metabolize feedstocks, which contains carbon (Mora-Villalobos et al., 2020). The technology of LA production from renewable biomass feedstock consists of several steps and varies depending on the type of raw materials used. It becomes more sophisticated as the raw materials turn from sugars to starches and cellulosic materials. For the production of LA from lignocellulose feedstock, it should go through four major steps. These are - pretreatment, hydrolysis, fermentation, and purification.

A. Pretreatment

Pretreatment of biomass is a technically challenging process, which takes a large part of the process cost. As revealed in Fig 2.7, the main purpose of the pretreatment is to remove lignin, reduce cellulose crystalline, and increase the porosity of the materials. This can be done via acid-based, mild alkaline-based, hydrothermal processing, steam explosion, oxidative, chemical pulping processes method, etc. The selection of appropriate method takes into account the following requirements:- improve the formation of reducing sugar, avoid the degradation/loss of carbohydrate; no / little formation of byproducts inhibitory to the subsequent processes, easily recovering lignin for modification into valuable co-products, and minimum requirement of heating and power (Jönsson and Martín, 2016; Robak and Balcerek, 2018).
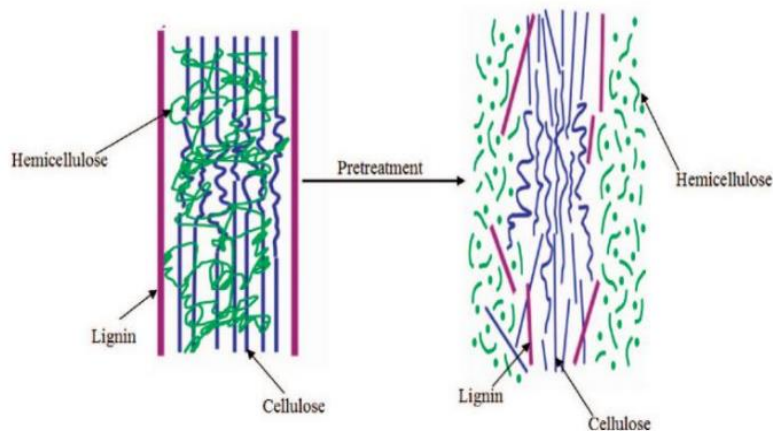
Figure 2. 5 Lignocellulosic biomass pretreatment representation   source:- (Harun *et al.*, 2016)

Among the above-listed methods, the acid-based method is one of the most favorable pretreatment methods, which is implemented in commercial production. Especially, dilute sulfuric acid pretreatment has been studied for a wide range of lignocellulosic biomass. It yields a high recovery of the hemicellulosic sugars and solid cellulose fraction in pretreatment with enhanced enzymatic convertibility (Jönsson and Martín, 2016).

B. Hydrolysis

Hydrolysis is a process where complex carbohydrate polymers are converted to fermentable sugars. As shown in Fig 2.8, in the hydrolysis reaction, the complex chains of sugars in lignocellulose are broken and yield simple monomers (C_6-sugars). The complex cellulose sugars are converted glucose, whereas the complex hemicellulose sugars are converted to a mix of soluble five-carbon sugars, xylose and arabinose, and soluble six-carbon sugars, mannose, and galactose. This process is accomplished either with the help of chemical or enzymatic hydrolysis (Fisiaha, 2016; Robak and Balcerek, 2018). Chemical hydrolysis means primarily the use of acids; diluted or concentrated. Both of them are carried out to break down the bond between the cellulose's monomer molecules. Due to some factors such as environment, cost, and corrosion problems, dilute-acid hydrolysis has been widely used instead of concentrated acid. On the other hand, enzymatic hydrolysis can occur under milder conditions (typically 40-50^{0}C and pH 4.5-5). It has the succeeding advantages as compared to chemical hydrolysis. These are - process low utility cost, low corrosion problems, environmentally friendly, and low toxicity of the hydrolyzates (Fisiaha, 2016; D. Kim, 2018). However, it has some draw back as compared to

dilute-acid hydrolysis; longer retention time, enzymes are more expensive than acid, and end-product inhibition can occur (Kang *et al.*, 2014; Keikhosro and Karimi, 2007).
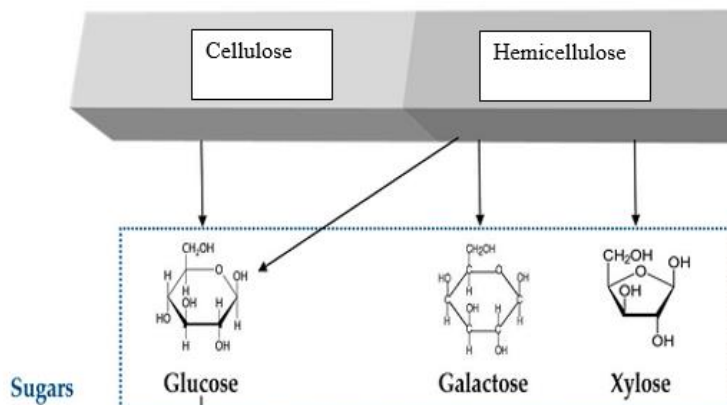
Figure 2. 8 A schematic representation of the major hydrolysis reaction yield

Source:- (D. Kim, 2018)

C. Fermentation

Fermentation is a metabolic process that makes a chemical change in the organic substrate by the action of microbes. Fermenting microorganisms are used for the conversion of fermentable sugar into LA. Many microorganisms such as bacteria, yeast, and fungi can be used for conversion. However, the yield and productivities of fungal and yeast strains are very low compared with bacteria. These LA-producing bacteria are grouped into four main categories including Lactic Acid Bacteria (LAB), Corynebacterium glutamicum, Escherichia coli, and Bacillus spp. (Grewal et al., 2020). Among these all, LAB is the most commonly used microbes; because it is safe, has high capacity tolerance to acidic pH, and capacity to generate LA at very high yields. Hence, the most frequently used organism in industrial processes is different species of LAB (Subramanian et al., 2015).

Usually, LAB ferments glucose efficiently by oxidizing NADH generated during glycolysis, with pyruvate serving as the electron acceptor to form LA product. There are two types of fermentation for these LAB, homofermentative and heterofermentative. Homofermentative lactic acid bacteria produce LA as the only end product. These include *lactobacillus delburuki*, *lactobacillus plantarum*, and *lactobacillus casei* (Behera et al., 2018; Doran-Peterson et al., 2008). On the contrary, heterofermentative lactic acid bacteria including actococcus lactis, *lactobacillus brevis*, *lactobacillus pentosus*, and *lactobacillus xylosus* produce other by-products

such as acetic acid, ethanol, and CO_2 in addition to the LA product (Abedi and Hashemi, 2020; Bintsis, 2018). Generally, Homofermentative LAB is the most promising organism for the industrial production of LA (Bintsis, 2018).

Homofermentative reaction

The fermentation of 1 mole of glucose yields two moles of LA

$$C_6H_{12}O_6 \longrightarrow 2\ C_3H_6O_3 \tag{2.1}$$

Heterofermentative reaction

The fermentation of 1 mole of glucose yields 1 mole each of LA, acetic acid, ethanol, and carbon dioxide

$$C_6H_{12}O_6 \longrightarrow C_3H_6O_3 + CH_3COOH + C_2H_5OH + CO_2 \tag{2.2}$$

D. Purification

Purification or product recovery is an important step in the production of LA that is associated with the separation/purification of LA from fermentation broth. The fermented product contains a different number of impurities such as residual sugars, color, and other organic acids. These impurities must be removed from the broth in order to achieve more pure LA. To recover and purify the LA produced from the microbial fermentation media there are different techniques that the researcher has been using. These include ion-exchange chromatography, membrane-integrated technology, reactive distillation, etc. Among these, reactive distillation is the simplest and easiest way of separation which can provide 95% pure LA (Rao *et al.*, 2014).

2.9. Factors affecting lactic acid fermentation

There are many factors, which affect the production of LA from numerous raw materials. However, fermentation parameters are the major factors, which affect the yield of LA produced (Abate, 2016; Edris, 2019; Hofvendahl, 2000; Olszewska-widdrat *et al.*, 2020). These include pH, incubation time, incubation temperature, inoculums size, carbon source, initial substrate conditions, and nitrogen source. Among these, incubation temperature, pH, and incubation time are the major factors (Chinenye *et al.*, 2021; Ali, 2017; Alrefaey *et al.*, 2020; Edris, 2019; Nesryia, 2018; Thakur *et al.*, 2018). Henceforth, researchers are studying these parameters to optimize the yield of LA products (Nesryia, 2018; Wang *et al.,* 2020)

Table 2. 5 Major factors that affect LA fermentation

S.No	Factors	Effects	Reference
1.	Incubation Temperature	❖ Numerous researchers have studied the effect of temperature on LA production by different microorganisms and they found the optimal temperature range between 30-45 ºc. ❖ Too low or high of temperature may cause denaturation of the cells, and results in poor yield of production; especially for mesophilic microorganisms	Edris, 2019; Hofvendahl, 2000
2.	pH	❖ The optimal pH for LA production varies from 5.0 - 7.0. ❖ It is promising that the higher initial pH brought too much stress on the microorganism's metabolic abilities.	Abate, 2016; Chimenye *et al*., 2021
3.	Incubation Time	❖ Generally, the incubation period of 10-48 hr has been used for LA production using different microorganism cultures ❖ Besides, the different optimal conditions reported by various researchers for maximum LA production could be explained by the differences in the nature of the strains and medium composition used in their studies.	Ali, 2017; Edris, 2019

2.10. Cell immobilization

Cell immobilization is the mechanism of localizing the cells in distinct support or matrix. This improves the stability and performance of the cell during the operation of fermentation processes (Zhu, 2011). It has attracted great attention in the fields of scientific research and industry. There

are several immobilization techniques developed to date. Among this Cross-linking method is the most widely used method by researchers. Because as compared to the others (disulfide bonding, covalent bonding, adsorption bonding, and metal bonding), it does not require support that significantly decreases the cost (Górecka and Jastrzębska, 2011). Sodium alginate (SA) is the most widely used material to prepare immobilized cell beads based on a cross-linking reaction with Ca^{2+} (Wang e*t al*., 2020). As it was shown in Table 2.6, immobilized cells have many advantages over free cells.

Table 2. 6 Comparisons of immobilized cell with free cell

S.N<u>o</u>	Free Cell	Immobilized Cell
1.	Could not Survive inhibitor effects	Capable to Survive inhibitor effects
2.	Less stable	Long term operation stable
3.	Difficult for product recovery	Convenient for product recovery
4.	Less yield as compared to immobilized cell	Higher yield
5.	Less cell density in the fermenter	Higher cell density in the fermenter
6.	Death of cell due to contamination over long fermentation period	High biological activity maintained for a long fermentation period
7.	Cells cannot be reused	Reuse of cells for a prolonged period due to cell regeneration

Source:- (Zhu, 2011; Zur *et al*., 2016)

Generally, immobilization provides special stability for the microorganism against environmental stresses (organic solvents, salts, inhibiting substrates and products, poisons, self-destruction, etc.) (Thakur *et al*., 2018; Zhu, 2011).

2.10.1. Model for substrate utilization, product formation, and cell growth

During the fermentation process, the model can predict how the rates of substrate consumption, product formation, and cell growth within the beads, and bulk liquid. As shown in Fig 2.10**,** the fermentation with the immobilized cell is depicted (Nakasaki *et al.*, 1989).

| (a) . Overall section of the reactor | (b) the close-up view of the bead |
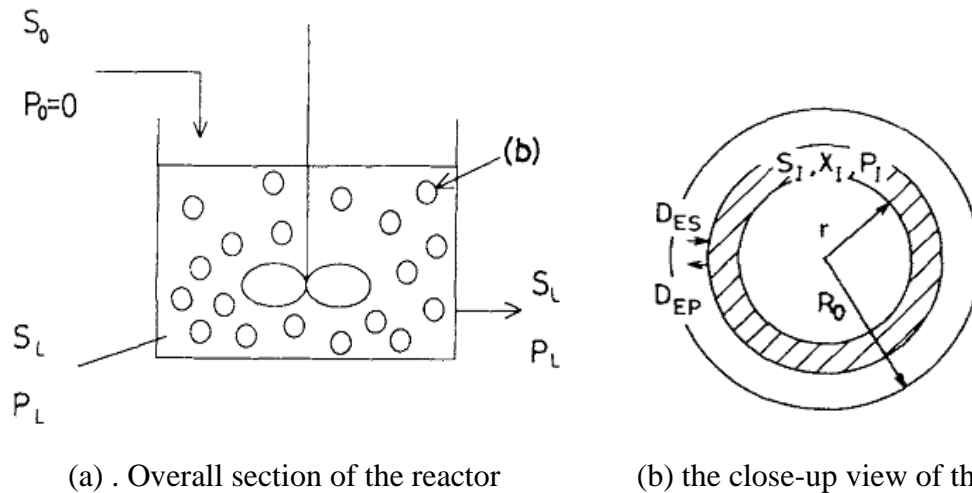
Figure 2. 9 Image of immobilized cell fermentation   **Source:-** (Nakasaki *et al.*, 1989)**.**

Some Assumptions need to be considered for developing the model. These include, the cells are immobilized inside spherical gel beads, and no leakage will occur, there is a uniform distribution of cells throughout the gel beads at the beginning of the reaction, the substrate diffuses into the gel beads where reactions take place, and the diffusion occurs symmetrically from all directions. Hence, based on these premises, the unsteady-state balance equations are as follows.

In the bead, the models are developed as the below

- ❖ For substrate utilization

$$\frac{\partial S_I}{\partial t} = D_{ES}\left(\frac{\partial^2 S_I}{\partial r^2} + \frac{2}{r} \times \frac{\partial S_I}{\partial r}\right) - (m + \frac{\mu}{Y_{\frac{x}{s}}})X_I$$

(2.3)

- ❖ For product formation

$$\frac{\partial P_I}{\partial t} = D_{EP}\left(\frac{\partial^2 P_I}{\partial r^2} + \frac{2}{r} \times \frac{\partial P_I}{\partial r}\right) + Y_{\frac{P}{s}}(m + \frac{\mu}{Y_{\frac{x}{s}}})X_I \qquad (2.4)$$

- ❖ For cell growth

$$\frac{\partial X_I}{\partial t} = \mu X_I \tag{2.5}$$

$$\mu = \frac{\mu_{max} S_I}{K_S + S_I} \tag{2.6}$$

where, S, P, and X are the concentrations of substrate, product, and cell, respectively, the subscripts I refers to the immobilized cell in the beads, D_{ES} and D_{EP} are the effective diffusivities of substrate and product, respectively, m is the specific maintenance rate, r:- radius, and μ_{max} is the maximum specific growth rate.

As it was shown in the model above, in the bead, there is both spatial and temporal variation of substrate utilization, product formation, and cell growth. However, in the bulk external liquid as shown in equations (B-1) and (B-2), only temporal variation of substrate utilization and product formations have happened.

2.11. Summary of research gaps

Thanks to the researchers, they have strived to overcome the problem associated with first-generation feedstocks and the sustainability of the product by finding alternative lignocellulosic biomass feedstocks. However, there is a crucial concept, which has not been addressed yet. These gaps were summarized in Table 2.7.

Table 2. 7 Summary of gaps in some articles on lignocellulosic biomass for production of LA

Articles	Fermenting microorganism	LA yield	Gap
Tu *et al.* (2019)	*Lactobacillus plantarum*	0.69	♠ Inhibitors (substrate and product) affect this microbe
Zhang and Vadlani (2015)	*Lactobacillus brevis* and *Lactobacillus plantarum*	0.78	♠ Inhibitors (substrate and product) affect this microbe. ♠ *Lactobacillus brevis* follows the heterofermentative pathway and increases purification cost
Grewal and Khare (2017)	Immobilized *Lactobacillus brevis*	0.70	♠ It follows the heterofermentative pathway and results in an increment of purification cost
Hu *et al.* (2016)	*Lactobacillus pentose*	0.66	♠ Inhibitors (substrate and product) affect this microbe ♠ It follows the heterofermentative pathway
Karnaouri *et al.* (2020)	*Lactobacillus delbruecki*	0.69	♠ Inhibitors (substrate and product) affect this microbe
Grewal and Khare (2018)	*Lactobacillus brevis*	0.49	♠ Inhibitors (substrate and product) affect this microbe ♠ it follows the heterofermentative pathway

As it is revealed in Table 2.7, owing to lack of cell immobilization, a major of microbes used for fermentation are highly affected by inhibitors (substrate and product). Furthermore, most of the microorganisms follow the heterofermentative pathway, which yields another by-product that increases the purification cost substantially. Therefore, the yield of LA obtained is not adequate. Hence, the lack of cell immobilization for fermenting microbes of hydrolyzed biomass (i.e. glucose) via homofermentative pathway is a major concept, which did not addressed in the above-summarized articles. Furthermore, some resources used in the above articles are still conflicting with other products. Bagasse is the most powerful raw material for boilers in many sugar industries for steam generation, which was already installed in many sugar factory including Ethiopian sugar factories. Therefore, these aforementioned gaps enthusiastic the researcher to fill these gaps. In general, this study is motivated to produce sustainable LA product through microbial fermentation by using immobilized *Lactobacillus plantarum* from khat waste biomass. By doing so, all the above problems associated with LA production from lignocellulosic biomass would be overwhelmed.

3. METHODOLOGY

3.1. Materials and chemicals

3.1.2. Materials

The equipments used in this study are listed below. DNP-9052 Electric thermostatic incubator, made in china. 85-2A Temperature magnetic stirrer, CLB-201-04 Vertical Laminar Flow Cabinet, Funce gerber colony counter, Citizon® digital mass balance, HH-S6 Water bath six holes digital, made in china, NDJS viscometer, pH meter, plastic bag, mortar, vacuum filter, Autoclave reactor, petri dish, ceramic crucible, centrifuge, FTIR spectroscopy, UV-visible spectrophotometry.

3.1.2. Chemicals

The chemicals used in this study are listed below.

Table 3. 1 Summary of chemicals used in this study

S.No	Chemical Name	Formula	Grade
1	Sulfuric acid	H_2SO_4	98%
2	Sodium hydroxide	NaOH	Analytical
3	Lactobacillus MRS broth	-	Analytical
4	Phenol	C_6H_6O	Analytical
5	Acetone	C_3H_6O	Analytical
8	Distilled water	D ionized water	Laboratory Grade
9	Phenolphthalein	$C_{20}H_{14}O_4$	Laboratory reagent
10	Lactic Acid	$C_3H_6O_3$	Standard
11	Glucose	$C_6H_{12}O_6$	Analytical
12	Calcium chloride	$CaCl_2$	Analytical

| 13 | Sodium Alginate | $C_6H_9NaO_7$ | Laboratory reagent |

3.2. Experimental setup

The experimental procedure to produce LA from khat waste biomass via microbial fermentation by using immobilized *lactobacillus plantarum* is shown in Fig 3.1.
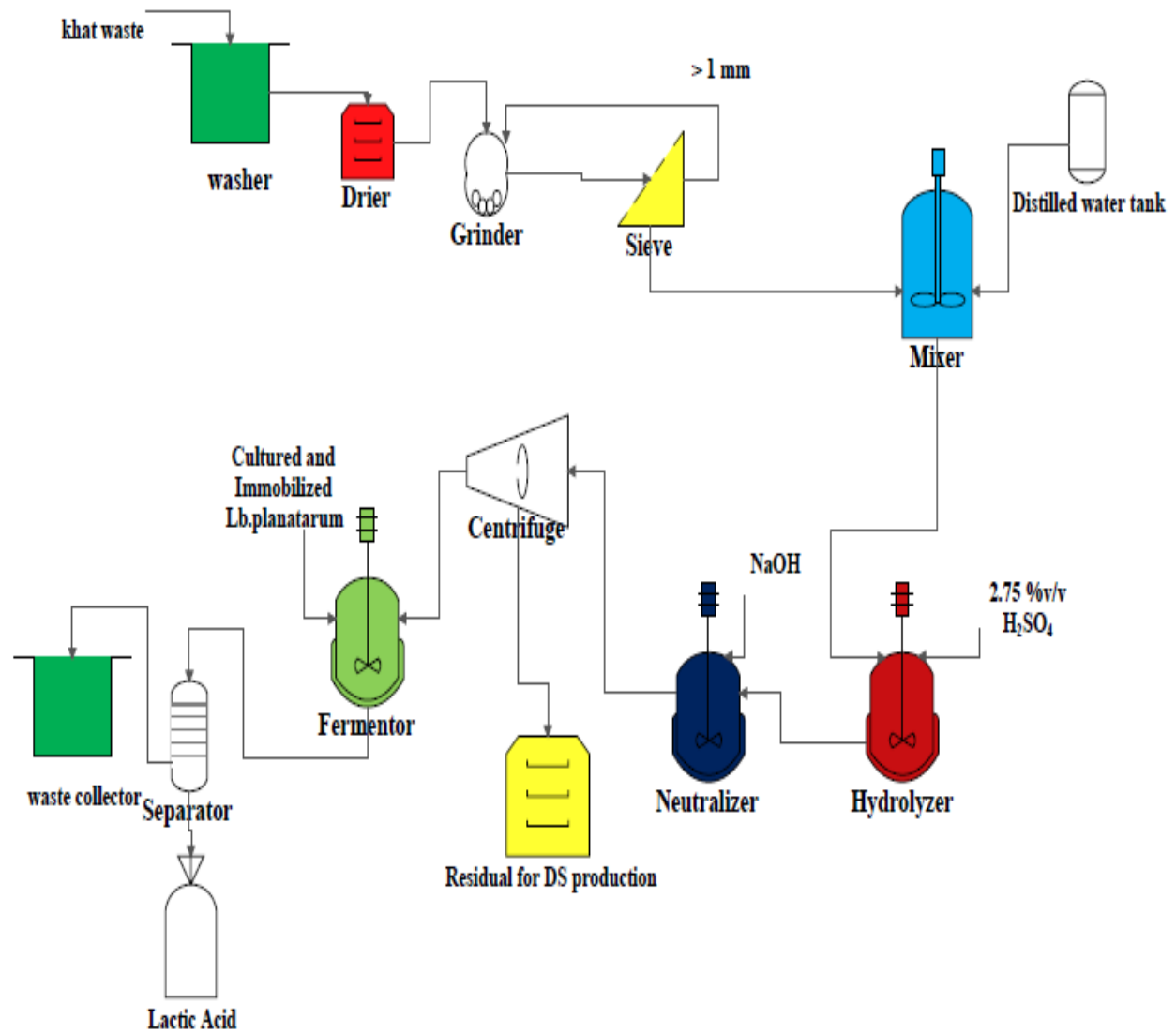
Figure 3. 1 Process flow diagram for production lactic acid

3.3. Experimental methods

The procedure followed in this study was mentioned as follows.

3.3.1. Sample collection and pretreatment

The sample was collected from Jimma city in different areas of chewing places (Sato samaro and markato). The collected waste was washed with water to remove any unnecessary materials and dried in the sunlight for seven days. The dried sample was grounded into fine particles to facilitate the rate of reaction in the subsequent process. The particles were separated based on their size by using a sieve, which has a mesh size of 1mm. The maximum particle size of the grounded sample was 1mm. The samples of larger particle size greater than 1mm was ground again until all particle sizes become (≤1mm).

3.3.2. Proximate analysis of khat waste

A. Moisture content determination

The moisture content was determined by using ASTM standard E1756 – 08 methods (Cai *et al.*, 2017). Three different empty petri-dish (45.40 g, 45.80 g, 42.0 g) were measured by using digital mass balance. Five grams of the sample was measured on this balance by putting on a separated petri dish. The prepared samples were taken to the oven and dried for three hours at 105°c. The samples were cooled and their mass was measured. Again, they were returned to the oven at the same temperature as in the previous step and waited for 30 min. The process was continued until the consecutive mass difference becomes less than 1mg. Finally, these samples were taken out and measured.

Then, the moisture content of the sample is determined by the following formula,

$$ \textbf{❖} \quad \% Moisture = \frac{W_l}{W_b} \times 100\% $$

(3.1)

where, W_b is the weight of the sample before dried (g), and W_l is the loss of weight after dried (g)

B. Ash content determination

The ash content was determined by using ASTM standard E1755 −01 method (Cai *et al.*, 2017). Three different empty crucibles (33.96 g, 34.12 g, 38.45 g) were measured by using digital mass balance. Five grams of the sample was measured on this balance by putting on a separated crucible. The prepared samples were taken to the oven and dried for three hours at 105 °c and then taken to a furnace and burned at 550°c for 4 hours. The burned samples were cooled and their mass was measured. Again, they were returned to the furnace at the same temperature as in the previous step and waited for 30 min. The process was continued until the consecutive mass difference becomes less than 1mg. Finally, these samples were taken out and measured. The ash content of the sample is determined by the following formula.

$$ \text{❖} \quad \% Ash = \frac{W_{ash}}{W_b} \times 100\% $$

(3.2)

where, W_b is the initial weight of the sample before burned (g) and W_{ash} is the weight of the sample after burned (g)

C. Dry matter content determination

The dry matter content was determined by using ISO 638:2008 standard method (Abate, 2016). Total dry matter (TDM) was calculated from moisture content.

Total dry matter or total solid is calculated by the following formula,

$$ \text{❖} \quad \% DM = (100\% - \% Moisture) $$

(3.3)

D. Volatile matter content determination

The Volatile Matter (VM) content was determined by using the European Standard EN15148-2009 method (Edris, 2016). Three different empty crucibles (28.53 g, 31.17 g, 35.47 g) were measured by using digital mass balance. Five grams of the sample was measured on this balance by putting on a separated crucible. The samples were covered with aluminum foil and kept in a furnace at a temperature of 920°C for 10 minutes and then cooled for 30 minutes. Finally, these samples were taken out and weighed.

The percentage of volatile matter is computed as below:

$$\text{\%}VM = \frac{W_i - W_f}{W_i} \times 100\%$$

(3.4)

where, W_i is the initial weight of the sample (g) and W_f is the weight of the sample after ignition (g)

E. Fixed carbon content determination

The Fixed Carbon (FC) content was determined by subtracting the sum of percentage compositions of moisture content, volatile matter content, and ash content from 100 %.

$$\text{\%}FC = (100\% - \%Moisture - \%Ash - \%VM)$$

(3.5)

3.3.3. Chemical composition analysis of khat waste

A. Qualitative determination of khat waste chemical compositions

The qualitative cellulose, hemicellulose, and lignin content were determined by using FTIR spectroscopy (Gonultas and Candan, 2018). The functional groups present in khat waste are known from data of wavenumber along with their absorbance peaks.

B. Quantitative determination of khat waste chemical compositions

(i). Hemicellulose content

The hemicellulose content was determined by using ASTM D5896-96 method (Lin *et al.,* 2010). Two grams of the sample was transferred into a 250 mL Erlenmeyer flask and 10 ml of 0.5M of NaOH was added to it. The mixture was boiled for 3 h in the water bath at 80°c. The boiled sample was cooled and filtered by using vacuum filtration and washed with distilled water seven times until neutral pH is achieved. Finally, the residue was dried to a constant weight at 105 ºC for 24 hrs in a convection oven.

The hemicellulose content (%w/w) is computed as below:

$$\text{\%}Hemicellulose = (W_i - W_f) \times 100\%$$

(3.6)

where, W_b is the initial weight of the sample (g) and W_l is the final weight of the sample (g)

(ii). Lignin content

The lignin content was estimated according to the method of the institute of paper chemistry, Appleton, Wisconsin as described in (Lin *et al.*, 2010). Two grams (mesh size 2 mm) of the sample was weighed and taken into glass test tubes and 25 mL of 72% H_2SO_4 was added to it and well mixed for 2 hrs at 25°c. The mixture was transferred to a circular bottom flask and diluted with distilled water to make a 3% acidic solution. The solution was boiled for 2 hrs by using a hot plate at 95°c. The hydrolyzed sample was filtered with a vacuum filtration unit and washed until free from acid. Finally, it was dried with crucible in the oven at 105°c and weighed to constant weight.

The lignin content of the sample is determined by the following formula.

❖ $$\% Lignin = \frac{W_{ad}}{W_{bd}} \times 100\%$$

(3.7)

where, W_{bd} is the initial weight of the sample (g) and W_{ad} is the weight of sample after dried (g)

(iii). Extractive content

The extractive contents were determined according to the method used by (Lin *et al.*, 2010). Twenty-five grams of sample was taken into the flask and 300 mL of acetone was used for extraction. The sample was placed on a soxhlet extractor and heat is supplied to the solvent and the extraction process was started after the acetone is boiled (i.e. 56°c). The residence time for the extraction process is 4 hrs. Finally, the left residual solid was dried in the oven at 105 °C for 24 hrs.

Then % (w/w) of the extractives was calculated as below.

❖ $\% Extractives = (W_i - W_f) \times 100\%$ (3.8)

where, W_i is the initial weight of the sample (g) and W_f is the weight of extractive free khat waste powder (g)

(iv). Cellulose content

The cellulose content of khat waste (%w/w) was calculated by subtracting extractives, hemicellulose, and total lignin from 100 %.

❖ $\% Cellulose = (100\% - \% Lignin - \% Hemicellulose - \% Extractives)$ (3.9)

3.3.4. Sample preparation for hydrolysis process

First, 1000 ml of the conical flask was washed and dried. Then, a hundred grams of ground sample was added to the flask and mixed with 1000 ml distilled water. This was the sample that was ready for the subsequent process (i.e. hydrolysis process).

3.3.5. Acid hydrolysis process

The prepared sample was transferred to another 1000 ml conical flask. Then, 50 ml of 2.75% (v/v) of H_2SO_4 was added to the sample and soak for 24hr to mix it well. The mixed sample was placed in the autoclave reactor at 105 ℃ for 1 hrs. This ready for fermentation process.

3.3.6. Neutralization process

Since the hydrolyzation process was carried out by sulfuric acid, the pH of the hydrolyzed sample is too low. Not only this but also, since the residual solid part of the hydrolyzed sample after centrifuged is going to be used as cattle feed (i.e. dried solid) the hydrolyzed sample should be neutralized before centrifuge separation. 10 M of NaOH was prepared for neutralization process. The sample was neutralized by adding the prepared solution of NaOH in dropwise until it was neutralized

3.3.7. Centrifuge separation

The sample taken from hydrolysis had some insoluble solid parts, which should be separated before it was taken to the fermentation process. Thus, the mixture of the sample was feed to the tube of a centrifuge. After that, the samples were separated according to their phase. Finally, the liquid part was sent to the fermenter, whereas the residue part is taken to another flask to produce Dried Solid (DS).

3.3.8. Sugar content determination

The concentration of glucose in the hydrolyzed sample was determined by using a digital UV spectrophotometer method. A quantitative standard glucose solution was used to plot the calibration curve (Edris, 2019; Nielsen, 2010).

A. **Calibration plot for glucose standard (known concentration of glucose)**

A standard glucose solution was prepared (by dissolving one gram of glucose in 100 ml of distilled water) and serially diluted by two fold dilutions. 1 mL of the phenol solution (prepared by dissolving 5 g of phenol in 100 mL of distilled water) was added to each test tube, and carefully mixed. 5 mL of concentrated sulfuric acid (96%) was added with the help of a burette to each test tube. The prepared solution was carefully mixed using a vortex, and it was left at room temperature for 10 minutes. The mixture was heated at 90 $^\circ$c in the water bath for 15 minutes and cooled. The intensity of absorbance for the solution was measured using a spectrophotometer at 490nm against distilled water. Finally, the calibration curve was plotted (absorbance vs concentration).

B. Determining the amount of glucose in the hydrolyzed sample

The hydrolyzed sample with dilute sulphuric acid was taken from hydrolysis. 1 mL of the phenol solution (prepared by dissolving 5 g of phenol in 100 mL of distilled water) was added to the test tube, and carefully mixed. 5 mL of concentrated sulfuric acid (96%) was added with the help of a burette to the test tube. The solution was carefully mixed using vortex and it was left at room temperature for 10 minutes. The mixture was heated at 90 $^\circ$c in the water bath for 15 minutes and cooled. The intensity of absorbance for the sample was measured using a UV spectrophotometer at 490nm against distilled water. Finally, the concentration of sugar in the samples was calculated from the calibration curve by using the following formula.

$$Cocentration\ of\ glucose\ \left(\frac{g}{L}\right) = \frac{Absorbance\ of\ unknown\ sample - Y_{intercept}}{slope\ of\ the\ graph}$$

(3.10)

3.3.9. Lactobacillus plantarum collection, inoculum preparation, and growth

A. Collection of lactobacillus plantarum

The vial of *lactobacillus plantarum* was collected from the Ethiopian bio-diversity institute. The existence of this cell was checked by using phase-contrast microscopy and the existence of the rod-shaped living cell, which shows the presence of this *lactobacillus plantarum* cell was observed. After that, it was placed in a deep freeze (-4 °C) until the MRS medium was prepared.

<div align="center">(a)   (b)</div>

Figure 3. 2 Images of "(a) *Lactobacillus plantarum* collected   (b) Microscopy and scanned result of microscopy on the Personal Computer (PC)".

B. Media preparation and inoculation

Thirteen grams of MRS broth was dissolved in 150 ml of distilled water in a 250 ml conical flask at 25°c. The prepared MRS broth medium was sterilized for 15 min at 121°c. The vial of *lactobacillus plantarum* was inoculated in this prepared medium and incubated for 36 hrs at 37°c. Cell growth was monitored analytically by measuring the optical density (OD) of the medium in a glass cuvette with UV-spectrophotometer at 520 nm (Sutton, 2011). The microbes were grown as a result the optical density increased from the absorbance of 1.54 to 1.72. Next, the multiplied *lactobacillus plantarum* were ready for the fermentation process.

3.3.10. Immobilization of lactobacillus plantarum

The *lactobacillus plantarum* immobilization was accomplished by using procedure adopted by ((Thakur *et al*., 2018; Wang et al., 2020). The inoculated *lactobacillus plantarum* was taken to the petri- dish. Then, 3% of sodium alginate and 1.5% calcium chloride was prepared and added to it respectively. Finally, the immobilized *lactobacillus plantarum* was collected and transferred to the prepared MRS broth. This is ready for fermentation.
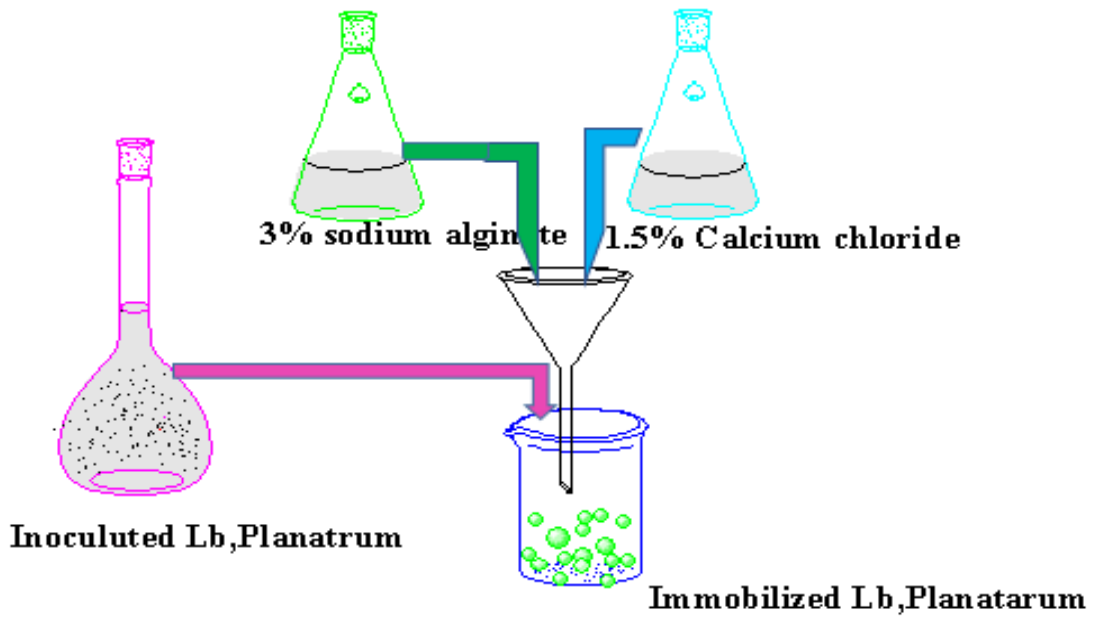
Figure 3. 3 Experimental setup for immobilization of *lactobacillus plantarum* cell
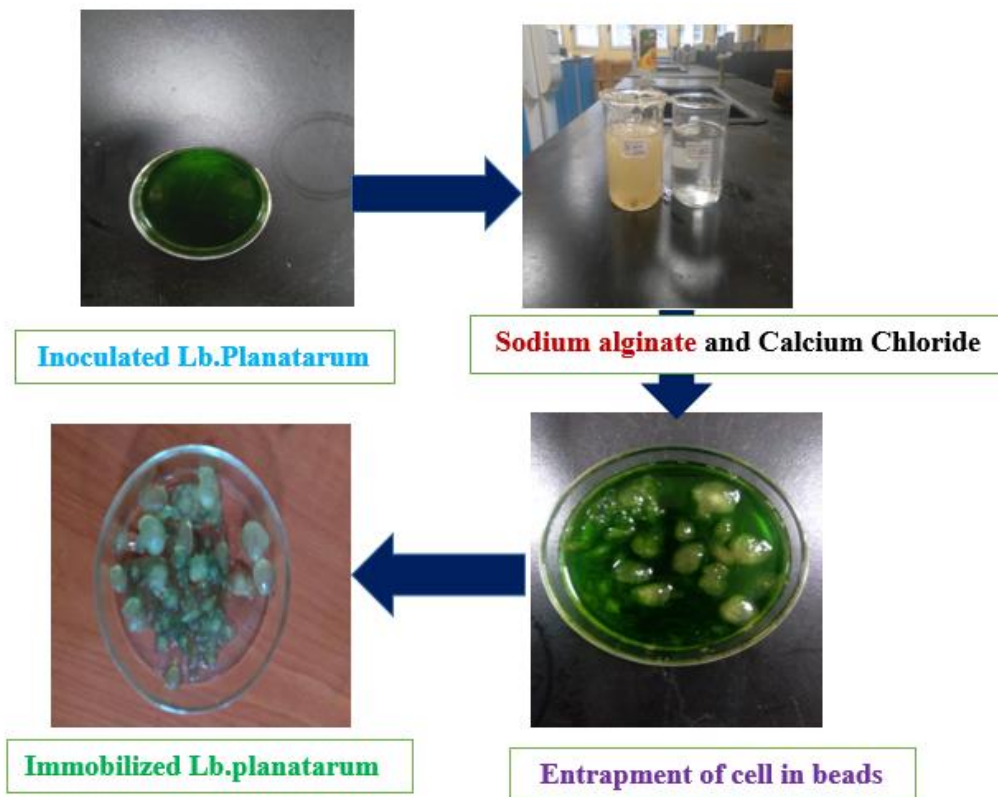
Figure 3. 4 Immobilization of *lactobacillus plantarum* cell

3.3.11. Effect of immobilization of Lb. plantarum on the yield of lactic acid

In the beginning, the 500ml conical flask was sterilized by heat. Then, the sample with 10% (free and immobilized) inoculum with the cell was placed into an incubator separately and incubated with their respective temperature of 37.5 ^{o}c, and pH of 5.5. Finally, the samples were taken out with the time interval of 12 hrs (0, 12 hrs, 24 hrs, 36 hrs, and 48 hrs), and then their LA content was determined.

3.3.12. Design of experiment for fermentation

Rather than using single parameter optimization, the optimization by response surface methodology offers more advantages like saving time, space, and raw material (Thakur *et al.*, 2018). The design expert (v11) software was used to design the experiment and carry out optimization. From Response Surface Methodology (RSM), Central Composite Design (CCD) was used in this study to design the experiment with three independent process variables (incubation temperature, pH, and incubation time) at three levels with 6 replication at the center point of each factor. As it was stated in section 2.9, these major fermentation parameters are selected based on the previous studies with some modification on their levels by taking into account the immobilized *lactobacillus plantarum* cell are utilized in this study. Other factors including substrate concentration, nutrients, and others were kept constant. Finally, the main and interaction effects of this parameter were analyzed by using this design-expert software**.**

Table 3. 2 Design factors with their lower, center, and upper value

Factor	Parameter	Unit	Code	Level (alpha value = 1.38)		
				Low	Center	High
1.	Incubation temperature	^{o}c	A	30	37.5	45
2.	Incubation time	hr.	B	10	29	48
3.	pH		C	5	6	7

The RSM-CCD contains 2^K factorial runs, n_o center runs, and 2K axial runs in which the total experimental runs (N) could be computed as:

$$N = 2^k + 2k + n_o$$

(3.11)

Hence, in this work, the number of experimental runs required is

$$N = 2^3 + 2*3 + 6 = 20$$

Generally, twenty experimental runs having six center points were designed by RSM-CCD. The RSM gave the empirical relationship between the response function and the independent variables. The quadratic response model was based on all linear terms, quadratic terms, and linear interaction terms according to the following equation (Thakur *et al.*, 2018) given by equation (3.12):

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

(3.12)

where: Y= predicted response (i.e. LA content), β_0 = constant-coefficient, β_i = linear coefficients, β_{ii} = quadratic coefficients, β_{ij}= interaction coefficients, X_i and X_j were studied independent fermentation parameters.

3.3.13. Lactic acid content determination

Determination of LA produced was done using UV-Visible spectrophotometry method (Borshchevskaya *et al.*, 2016; Mahato *et al.*, 2021).

A. Plotting of calibration curve

Ten grams of LA with the known purity (88%) was placed in a 100-mL volumetric flask and diluted with distilled water. A stock solution with the concentration of LA 88 g/L was obtained. The series of LA solutions were prepared from the stock solution using two-fold dilutions. Then, a solution of iron(III) chloride (0.2%) was prepared. iron(III) chloride (0.2 g) was placed in a 100-mL volumetric flask, diluted to the mark with water, and stirred to the complete dissolution of the salt. The solution was kept at room temperature 25 °C. Next, a solution of LA (20 ml) of a corresponding concentration was added to 10 mL of a 0.2% solution of iron(III) chloride and stirred. The absorbance of the obtained colored solutions was measured at 390 nm. The reference solution contained 2 mL of a 0.2% solution of iron (III) chloride.

B. Determination of lactic acid content

The fermented liquid was separated from the immobilized cells by using vacuum filtration. Then, the supernatant was diluted with distilled water. The solution of iron (III) chloride (0.2%) was prepared. Iron(III) chloride (0.3 g) was placed in a 100-mL volumetric flask, diluted to the mark with water, and stirred to the complete dissolution of the salt. The solution was kept at room temperature 25 °C. Next, A prepared solution (20 ml) containing LA with a corresponding concentration was added to 10 mL of a 0.2% solution of iron (III) chloride and stirred. The absorbance of the obtained colored solutions was measured at 390 nm. The reference solution contained 2 mL of a 0.2% solution of iron (III) chloride. Finally, the LA content was be calculated from the developed calibration curve.

3.3.14. Residual sugar determination

To determine the fermenting efficiency of immobilized *lactobacillus plantarum* in the fermentation process, the residual sugar was determined at the end of the fermentation process (i.e. optimum incubation temperature and pH of 42.2 °c, and 6.3 respectively). Hence, the samples were taken out with a time interval of 12 hrs (0, 12 hrs, 24 hrs, 36 hrs, and 48 hrs), and the content of LA and residual sugar were determined. Therefore, it was determined by a method stated in sections 3.3.12, and 3.3.8 respectively.

3.3.15. Kinetic model for lactobacillus plantarum growth

This study used the serial dilution counting method (Ben-David and Davidson, 2014). First, the incubator was sterilized by heat. Then, five different samples with 10% inoculum were placed into an incubator and fermented at the optimum conditions above for 48 hrs. Finally, the samples were taken out with the time interval of 12 hrs (0, 12 hrs, 24 hrs, 36 hrs, and 48 hrs), and the number of the living *lactobacillus plantarum* was counted by using a colony counter. The kinetic model for microbial growth is given by a first-order linear ordinary differential equation.

$$\frac{dX_I}{dt} = \mu X_I - k_d X_I$$

(3.13)

where, X_I is an immobilized cell, μ is specific growth rate , K_d is the death rate constant. However, the growth of microbial cells has occurred in the exponential phase. Likewise, since the conditions are suitable for cell growth, we can reasonably assume that there is insignificant

death of microbial cells occurred in this phase. Therefore, $K_d = 0$, by using the assumption made in section 2.10.1. The model was developed as given below.

$$\frac{dX}{dt} = \mu X_I$$

(3.14)

Integrating the above equation yields $\int_{X_0}^{X} \frac{dX}{X} = \mu \int_{0}^{t} dt$

$$\ln(\frac{X}{X_o}) = \mu t$$

(3.15)

It   can   be   rewritten   as $\frac{X}{X_o} = e^{\mu t}$

(3.16)

$$X(t) = X_o e^{\mu \times t}$$

(3.17)

Then, the graph for $\boldsymbol{ln\left(\frac{X}{X_o}\right)}$ vs **t** was plotted and the slope (i.e. μ) was computed from it.

$$No\ of\ living\ cell\ (\frac{CFU}{ml}) = \frac{No\ of\ colonies}{0.1 \times Dilution\ factor}$$

(3.18)

(a)   (b)

<p align="center">(c)   (d)</p>

Figure 3. 5 Images of "(a) serial dilution of each sample (b, c) inoculating on the plate (d) counting by colony counter"

3.3.16. Lactic acid separation

Lactic acid produced from the fermentation process is recovered from any other by-products by using the reactive distillation method (Kim *et al*., 2000; Komesu et al., 2017; Rao *et al*., 2014). In the beginning, 400 ml of fermented product is taken to 500 ml round bottom flask. Then, 100 ml of methanol was added to it. This is an esterification reaction. The reaction was continued at 65 °c for 1 hrs. Next, the produced mixture of methyl lactate and water was transferred to the next distillation. After that 100 ml of distilled water was used to hydrolysis the mixtures into LA, methanol, and water. The mixture was distilled at 100 °c for 4 hrs. Finally, LA remained in the round bottom flask was collected.

3.3.17. Physio-chemical characterization of lactic acid

The physical and chemical properties of LA produced were characterized as follow.

A. Specific gravity and density determination

The specific gravity of the LA was determined by using a pycnometer (STP-4.3, 2006). First, the 100 ml pycnometer was cleaned and weighed. Then, the bottle was filled with LA and

reweighed by inserting a stopper. Next, after washing and drying the bottle, it was filled with water and weighed. Finally, the specific gravity of LA was determined as below.

$$SG = \frac{W_1 - W_0}{W_2 - W_0}$$

(3.19)

where, W_0 is the mass of empty bottle (g), W_1 :- is the mass of empty bottle and sample (g), and W_2 :- is the mass of empty bottle and water (g)

Then, density was computed as below.

$$\rho_{LA} = SG \times \rho_{water}$$

(3.20)

where, ρ_{LA} is the density of LA product ($\frac{kg}{m^3}$) and ρ_{Water} is the density of water @ 4 °c ($\frac{kg}{m^3}$)

B. Dynamic and kinematic viscosity determination

First, 80 ml of LA product was added to the 100 ml beaker. Then, NDJS Viscometer was set on and ready for reading the dynamic viscosity of the sample. After that, the spindle L_2 was connected to the viscometer and inserted in the sample preparation. Finally, the result of the sample dynamic viscosity was displayed on the screen of the viscometer. Subsequently, the kinematic viscosity of the sample was computed by dividing the obtained dynamic viscosity by the density of the sample.

$$\eta_s = \frac{\eta_d}{\rho_s}$$

(3.21)

where, η_s is the kinematic viscosity of the sample ($\frac{m^2}{s}$), η_d is the dynamic viscosity of the sample ($\frac{kg}{m.s}$), and ρ_s is the density of the sample at a given temperature ($\frac{kg}{m^3}$).

C. Functional group determination

FTIR Spectroscopy was used to determine the functional group of LA. The functional groups present in LA were known from data of wavenumber along with their absorbance peaks.

D. Reactivity determination

Reactivity of LA with sodium hydroxide is determined according to the method adopted by (Tam *et al.*, 1997). First, 25 ml of sample was added to the beaker**.** Then, 25ml of distilled NaOH was added to the sample prepared. This yields sodium lactate**.** Finally, the formed product was compared with the standard sodium lactate.

E. Miscibility determination

First, 10ml of the sample was added to the test tube**.** Then, 10ml of distilled was added to it**.** Finally, the phase separation occurrence was checked.

3.3.19. Applicability of produced LA for PLA synthesis

The suitability of this LA product formed, for the synthesis of PLA was justified via a direct polycondensation reaction. In the beginning, the LA product is feed to the evaporator for removal of free water at 95°c for 1 hr. Then, it was transferred to stirred reactor and the polymerization reaction was started by using sulfuric acid as a catalyst. The reaction proceeded for six hrs at 95 °c. Finally, the polymerized lactic acid (PLA) was collected.

3.3.20. Summary of methods

```
                        ┌─────────────────────────────┐
                        │    Khat waste collection     │
                        └─────────────────────────────┘
                                      │
                                      ▼
┌──────────────────┐    ┌─────────────────────────────────────────┐
│                  │    │             Pretreatment                │
│ Distilled water  │───▶│   ❖ Washing                             │
│                  │    │   ❖ Drying, grinding, and               │
└──────────────────┘    │   ❖ Sample preparation and treatment    │
                        └─────────────────────────────────────────┘
                                      │
                                      ▼
┌──────────────────┐    ┌─────────────────────────────────────────┐
│                  │    │              Hydrolysis                 │
│ 2.75% (v/v) of   │───▶│                                         │
│ H₂SO₄            │    │   ❖ Temp @105°c for 1 hrs               │
└──────────────────┘    └─────────────────────────────────────────┘
                                      │
                                      ▼
            ┌─────────────────────────────┐      ┌──────────────────┐
            │    Centrifuge Separation    │─────▶│  For Dried Solid │
            └─────────────────────────────┘      └──────────────────┘
                                      │
                                      ▼
┌──────────────────┐    ┌─────────────────────────────┐
│  Lb. plantarum   │    │         Fermentation        │
│  ❖ Inoculating   │───▶│   ❖ @ Optimum conditions    │
│    and           │    │                             │
│    immobilization│    └─────────────────────────────┘
└──────────────────┘                  │
                                      ▼
                        ┌─────────────────────────────┐
                        │ Separation (Reactive        │
                        │            distillation)    │
                        └─────────────────────────────┘
                                      │
                                      ▼
                        ┌─────────────────────────────┐
                        │ Lactic Acid (Physio-chemical│
                        │ characteristics determination)│
                        └─────────────────────────────┘
```

Figure 3. 6 Production process of LA

4. RESULT AND DISCUSSION

4.1. Physio-chemical characterization of the khat waste

The result obtained from the experiment conducted was discussed as the following.

4.1.1. Proximate analysis of khat waste

The moisture, ash, dry matter, volatile matter, and fixed carbon contents of khat waste were calculated by using equations (3.1), (3.2), (3.3), (3.4), and (3.5) respectively. Details calculations are available in Appendix A. As it is revealed in Table 4.1, the result obtained from this study is close to the works of literature. The little deviation from the literature might be due to some factors such as geographical locations, equipment, and measurement errors. Moisture content analysis is used to determine the proportionality of the solid to liquid ratio in the given sample. The properties of materials such as flowability, product quality, and shelf time were highly affected by the moisture content. For bulk powder materials, an increment of moisture content results in an increment of cohesive and adhesive force of the particles because of the formation of interparticle bonds. As the result, the flowability, shelf time, and product quality of the substances were decreased. Accordingly, since the investigated moisture content of the khat waste is benign, the powder of khat waste was stayed safe for a long period. Similarly, Probst *et al.* (2013) and Fitzpatrick *et al.* (2004) investigated different effects of biomass moisture contents'.

Table 4. 1 Proximate analysis of khat waste comparisons with the literature value

No	Moisture (%)	Ash (%)	DM (%)	VM (%)	FC (%)	Reference
1	10.85	4.35	-	70	14.80	Fikadu, 2018
2	-	5.31	-	-	-	Abdulhafiz, 2014
3	-	3.40	-	-	-	Gabriel *et al.* 2021
4	13.33 ± 0.31	7.1 ± 0.42	86.67 ± 0.31	54.89 ± 0.86	24.68 ± 0.24	**This study**

Moreover, ash content analysis is used to determine the contents of inorganic impurities present in the sample. As the ash content increased, the sludge formed through the production process is also increased, which results in the increment of purification cost. Thus, since the investigated ash content of the khat waste is less, utilization of khat waste for LA production results in a low amount of sludge. Accordingly, Teshome (2020) stated the analogous effects of ash content on biochemical product production from corncobs biomass. Moreover, the Fixed Carbon (FC) content analysis is used to determine the solid combustible carbon residue that remains after the solid biomass is heated at high temperature, excluding the VM, moisture, and ash contents. This is important to determine some thermodynamic properties of materials like Higher Heating Value (HHV). The higher the carbon content the higher HHV. In the same manner, Cavalaglio *et al.* (2020) reported the similar effects of FC and VC contents of biomass on the thermodynamic properties of materials.

4.1.2. Chemical composition of khat waste

A. Qualitative determination

Functional groups present in khat waste were characterized by using FTIR Spectroscopy. As revealed in Fig 4.1, the result from the FTIR spectrum showed that there were different functional groups present in khat waste biomass. The FTIR spectra of the sample indicated two main absorbance regions in a range of 700 - 1,745 cm^{-1} and 2,800 - 3,500 cm^{-1}. In graph analysis of khat waste biomass, 800 cm^{-1} is attributed to bending vibration of arenes C-H bond in lignin. The peak observed at 897 cm^{-1} shows the presence of β-(1, 4) glycosidic linkage between glucose unit in cellulose and hemicellulose. Similarly, Gonultas and Candan (2018) and Rashid *et al.* (2016) reported the same result with these outcomes.

Moreover, the peak observed at 1052 cm^{-1} is attributed to the C-O antisymmetric stretching vibration of the glycosidic ring in cellulose and hemicellulose. The peak observed at 1160 cm^{-1} is related to the pyranose ring C-O-C asymmetric stretching of cellulose and hemicellulose. The peak at 1365 cm^{-1} is attributed to C-H bending or polysaccharide aromatic C-O vibration and aliphatic C-H stretching mode of cellulose, hemicellulose, and lignin. The peak observed at 1598 cm^{-1} is due to aromatic ring stretching in lignin. As well, the peak located at 1745 cm^{-1} is related to the C=O stretching vibration of the carboxylic group of lignin and hemicellulose. The peak observed at 2860 cm^{-1} is attributed to the C-H stretching vibration of methylene in cellulose and

hemicellulose. The peak around 3345 cm^{-1} is attributed to the O-H stretching of the intermolecular hydrogen bond of hydroxyl groups. These investigation is in smooth agreement with the previous studies (Hospodarova *et al.*, 2018; Raspolli Galletti *et al.*, 2015). Therefore, this study qualitatively confirmed that the khat waste has cellulose, hemicellulose, and lignin.
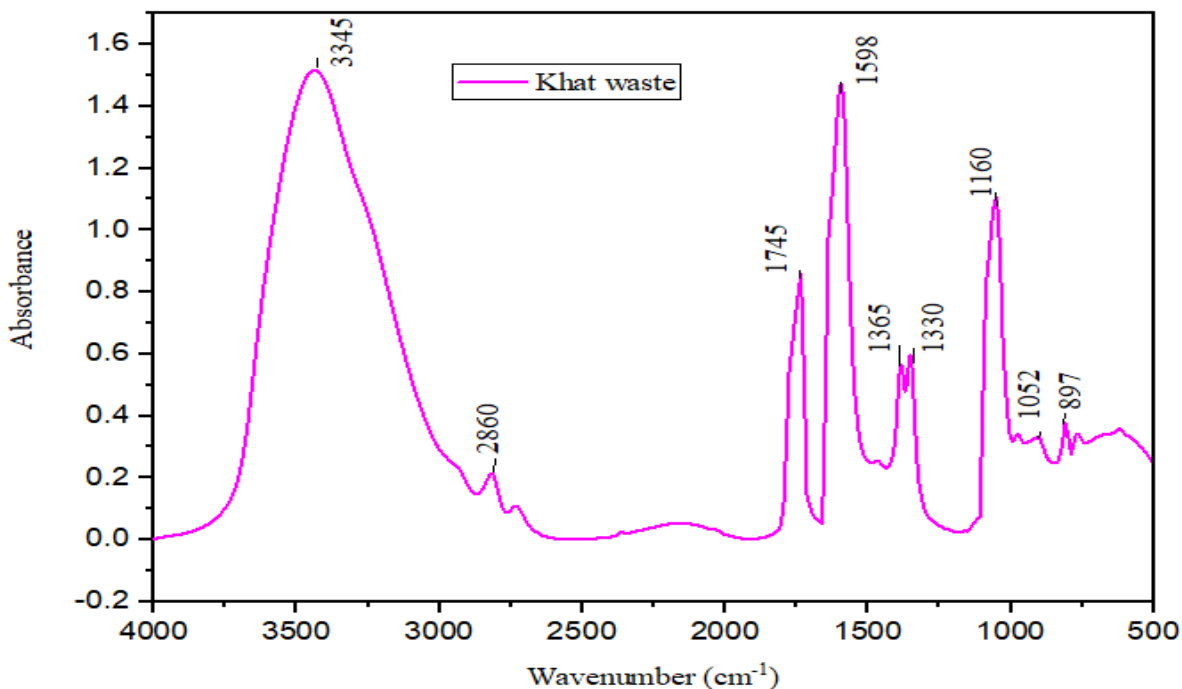
Figure 4. 1 Chemical composition of khat waste by using FTIR Spectroscopy

Table 4. 2 Summary on functional group analysis of khat waste chemical compositions

Wavenumber (cm^{-1})	Functional group
3345	O-H stretching of the intermolecular hydrogen bond of hydroxyl groups
2860	C-H stretching vibration of methyl and methylene in cellulose
1745	C = O stretching vibration of the carboxylic group of lignin and hemicellulose
1598	Aromatic ring stretching in lignin
1365	C-H bending or C-O vibration and aliphatic C-H stretching mode of cellulose, hemicellulose, and lignin.
1330	CH$_2$ wagging frequency of the cellulose

1052	C-O antisymmetric stretching vibration of the glucosidic ring in cellulose
897	β-(1, 4) glucosidic linkage between glucose unit in cellulose and hemicellulose

B. Quantitative determination

The hemicellulose, lignin, extractives, and cellulose contents of khat waste were calculated by using equations (3.6), (3.7), (3.8), and (3.9) respectively as shown in Appendix A. As it is shown in Table 4.3, the investigation of this study is in good agreement with the works of literature. The deviation from the literature might be due to factors such as geographical locations and measurement errors. In the same way, Xu (2020) and Ahorsu *et al.* (2018) stated the effects of geographical locations on the chemical compositions (cellulose, hemicellulose, and lignin) of different biomasses. The cellulose content of this khat waste biomass is relatively higher than the other lignocellulosic biomass feedstocks used for biochemical and biofuel productions. Essentially, cellulose is a carbohydrate polymer, which is built up from glucose molecules. Therefore, the researcher can reasonably deduce that khat wasste might provide enough amount of fermentable sugar by passing through pretreatment followed by hydrolysis.

Table 4. 3 Chemical compositions of khat waste comparisons with the literature value

N<u>o</u>	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Reference
1	59.0	-	31.7	-	Abdulhafiz, 2014
2	39.4	12.75	28.67	-	Gabriel et al., 2021
4	43.70 ± 0.71	17.42 ± 0.85	30.63 ± 0.93	8.25 ± 0.41	**This study**

4.2. Determination of reducing sugar (glucose) yield from the hydrolysis process

The concentration of glucose in the hydrolyzed sample was determined by using a digital UV spectrophotometer by measuring absorbance versus sugar concentration at 490 nm wavelength.

A. The calibration plot for glucose standard (known concentration of glucose)

The collected data from the experiment was depicted as Fig 4.2.

Figure 4. 2 The plot for standard glucose solution

$$Y = 0.1096x + 0.0408 \tag{4.1}$$

B. The amount of glucose in the hydrolyzed sample was determined as below.

$$Concentration\ of\ glucose\ \left(\frac{g}{L}\right) = \frac{Absorbance\ of\ unknown\ sample - 0.0408}{0.1096}$$

(4.2)

Table 4. 4 The experimental results of absorbance for the unknown sample

S.No	Absorbance	Concn (g/L)
1	1.532	13.61
2	1.583	14.07
3	1.558	13.85
Average value		13.84

Standard Deviation (SD)	0.23
	13.84 ± 0.23

The average of the above three-run results was 13.84 ± 0.23 g/L. Thus, this finding discovered that khat waste has enough amount of cellulose and hemicellulose which can be converted to a fermentable sugar (i.e. glucose) at 105 ºc, 2.75%, and 1 hrs for temperature, acid conc<u>n</u>, and retention time respectively. This result is significantly higher than Edris (2019), who obtained 3.76g/L under the same operating conditions. This might be due to the cellulose contents of sugarcane bagasse (18.7%) observed by Edris (2019) is substantially lower than this work (43.7%). Hence, the utilization of khat waste as a potential feedstock for LA production is relatively worthy.

4.3. Lactic acid fermentation and its content determination

The fermentation process for each run was carried out and their LA content was determined. The calibration curve developed with the known concentration of LA was depicted first and then the equation used to determine the LA contents of each sample with their respective conditions was attained.

A. The calibration plot for LA standard (known concentration of LA)

The collected data from the experiment was depicted as Fig 4.3.

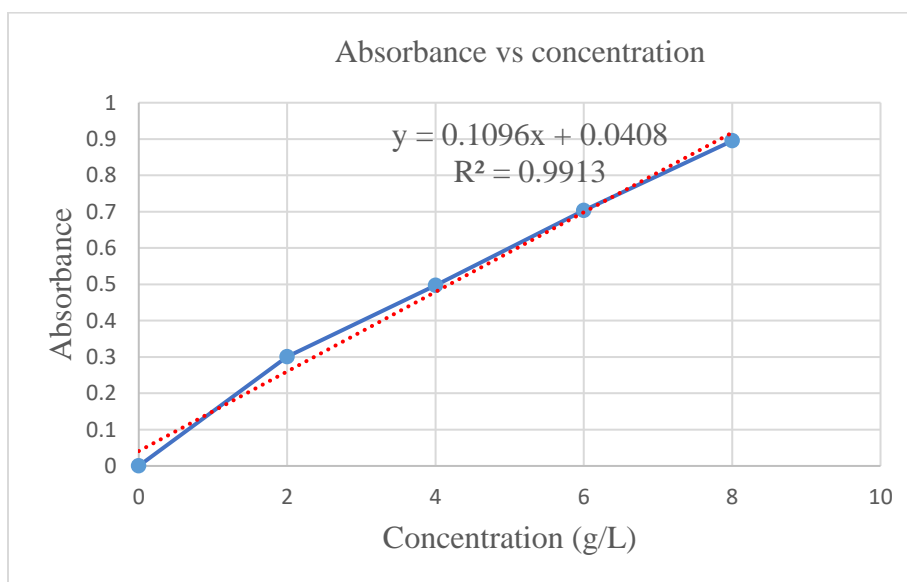Absorbance Vs Concentration

$y = 0.1336x + 0.022$
$R^2 = 0.9972$
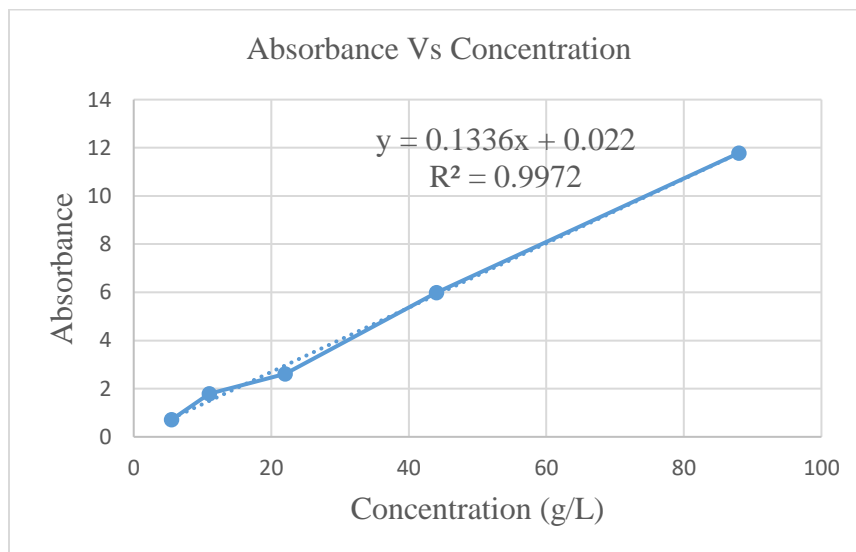
Figure 4. 3 The plot for standard LA solution

$$Y = 0.1336x + 0.022 \tag{4.3}$$

Then, the content of LA in the fermented sample was determined by using equation (4.4).

$$\text{Concentration of LA} \left(\frac{\text{g}}{\text{L}}\right) = \frac{Absorbance\ of\ unknown\ sample - 0.022}{0.1336}$$

(4.4)

4.4. The effect of Lactobacillus plantarum immobilization on the yield of LA

The result obtained from the experiment conducted to determine the effect of using immobilized and free *lactobacillus plantarum* on the LA yield is shown in Fig 4.4. As evident from Fig 4.4, during the first 22 hrs, the LA yield obtained from immobilized *lactobacillus plantarum* (9.65 g/L) is less as compared with the free cells' (10.74 g/L). The reason behind this result is at the outset there is the limitation of mass transfer (i.e. product and substrate) through the bead in immobilized cells. The immobilized cell needs time for diffusion of substrate and product into the beads and from the bead respectively. However, as the incubation time increased to 36 hrs the LA yield obtained from immobilized *lactobacillus plantarum* (17.86 g/L) is substantially higher than the free cells' (13.45 g/L), in percent about 24.7% higher. This indicates that, an immobilized *lactobacillus plantarum* has high fermenting efficiency and excellent stability over a long period of the fermentation process. Owing to its capacity to overcome the substrate and product inhibitors, the cells could utilize the hydrolyzates of khat waste efficiently and produce a huge amount of LA yield. On the contrary, as incubation time increased, the free cells fermenting efficiency were highly affected by different inhibitors, which consequently decreased the metabolic activities of cells, even up to death. Subsequently, the yield of LA obtained was low as compared to the immobilized cells'. Correspondingly, Thakur *et al.* (2018) and Wang *et al.* (2020) investigated the same effect of cell immobilization on fermenting efficiency of different lactobacillus species.
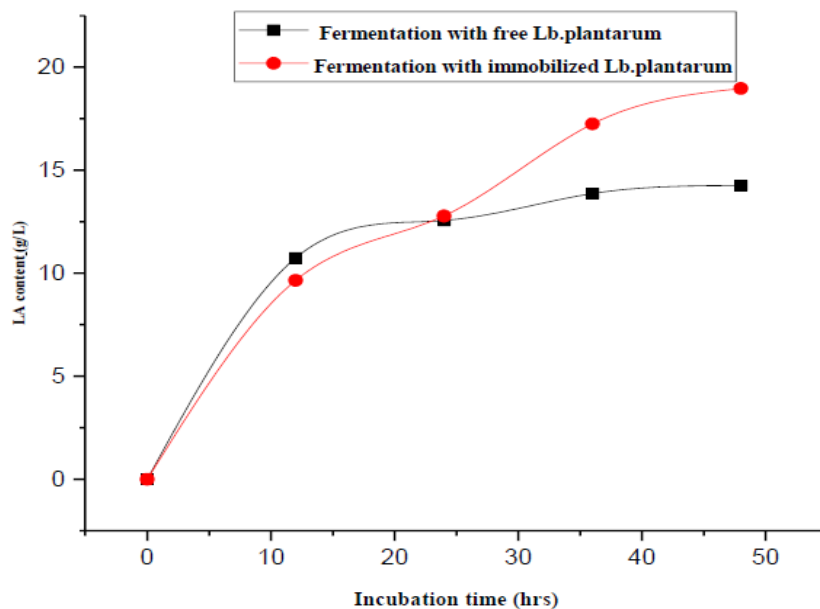
Figure 4. 4 Time profiles for LA fermentation by using *lactobacillus plantarum* cell

4.5. Statistical analysis of the experimental (fermentation) results

4. 5.1. Experimental data reports

The correlation between LA yield and process variable was analyzed by the RSM-CCD modeling technique. Table 4.8 shows the experimental data reports obtained from design expert software.

Table 4. 5 The value of experimental and predicted data reports from RSM-CCD

	Factor 1	Factor 2	Factor 3	Response	
R. No	A: Incubation Temperature (°c)	B: Incubation Time (hrs)	C: pH	LA content (g/L)	
				Actual	Predicted
1	30	10	7	13.12	13.56
2	37.5	29	6	20.98	21.73
3	47.37	29	6	22.96	22.97
4	30	10	5	9.23	9.71
5	37.5	29	4.68	20.61	19.99
6	45	48	5	22.56	22.35
7	37.5	29	7.32	21.49	21.57

8	37.5	29	6	22.12	21.73
9	30	48	7	19.89	19.64
10	30	48	5	19.98	20.15
11	37.5	29	6	22.07	21.73
12	45	10	5	17.54	18.03
13	37.5	3.99	6	16.01	14.98
14	45	48	7	21.14	20.90
15	37.5	29	6	21.21	21.73
16	37.5	54.00	6	21.34	21.83
17	45	10	7	20.87	20.93
18	37.5	29	6	21.97	21.73
19	37.5	29	6	21.31	21.73
20	27.63	29	6	17.23	16.67

4.5.2. Fit summary for the suggested model

Generally, there are four main models used to fit the experimental results obtained from batch fermentation experiments. As presented in Table 4.9, the quadratic model with a correlation regression coefficient (R^2) of 0.9817 is suggested to denote the empirical relationship between the response function and the independent variables. As it is shown in Table B-1 under Appendix B, the linear and 2FI models have a significant lack of fit. Besides, the linear model could not incorporate the curvature effects of the independent parameters, whereas the cubic model is aliased.

Table 4. 6 Fit summary for the suggested model

Source	Std. Dev.	R²	Adjusted R²	Predicted R²	PRESS	
Linear	2.27	0.6420	0.5748	0.3654	145.65	
2FI	2.03	0.7669	0.6593	-0.1546	265.00	
Quadratic	**0.6475**	**0.9817**	**0.9653**	**0.8964**	**23.77**	**Suggested**

	Cubic	0.5610	0.9918	0.9739	-0.1715	268.87	Aliased

4.5. 3. Analysis of Variance

Analysis of variance (ANOVA) was used to confirm the statistical significance of every factor and their respective interactions in the developed quadratic model as already shown in Table 4.10. The model F-value in regression tests the null hypothesis (i.e. all coefficients of independent parameters in the model were zero). In the present study, the model F-value (59.72) is greater than critical $F_{0.001\ (9,\ 5)}$ is (27.24) which implies we have to reject the null hypothesis. Therefore, the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Besides, P-values less than 0.050 indicate the model terms are statistically significant. In this case, A, B, C, AB, BC, A², B², C² are significant model terms. Furthermore, the Lack of Fit F-value of 2.36 implies the Lack of Fit is not significant relative to the pure error. There is an 18.40% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good.

Table 4. 7 Analysis of variance (ANOVA) for the suggested quadratic model

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	
Model	225.32	9	25.04	59.72	< 0.0001	Significant
A- Incubation Temperature	65.64	1	65.64	156.57	< 0.0001	
B- Incubation Time	77.59	1	77.59	185.08	< 0.0001	
C- pH	4.11	1	4.11	9.82	0.0106	
AB	18.70	1	18.70	44.60	< 0.0001	
AC	0.4465	1	0.4465	1.07	0.3264	
BC	9.53	1	9.53	22.72	0.0008	
A²	8.42	1	8.42	20.08	0.0012	
B²	25.66	1	25.66	61.21	< 0.0001	
C²	2.09	1	2.09	4.99	0.0496	

Residual	4.19	10	0.4192			
Lack of Fit	2.94	5	0.5888	2.36	0.1840	not significant
Pure Error	1.25	5	0.2496			
Cor Total	229.52	19				

4.5.4. Model adequacy analysis

The adequacy of the model can be deduced considering the regression coefficients of R^2. Hence, R^2 was used to determine the relationship between the experimental and the predicted responses. As represented in Table 4.11, the response of R^2 was 0.9817, which recommends that 98.17% of the response variability in LA yield can be described by the analyzed process parameters, and it could not describe nearly about 1.83% of the variation of the response. It was obtained that the value of R^2 is very close to 1, showing the very correlation between the experimental and predicted value. The value of R^2 greater than 0.80 is acceptable (Bayuo *et al.*, 2020). Therefore, the model is enough adequate. Moreover, the coefficients of R^2 (0.9817) and adjusted R^2 (0.9653) shown in Table 4.8 indicate the close agreement of experimental and predicted values.

The predicted R² of 0.8964 is in reasonable agreement with the Adjusted R² of 0.9653; i.e. the difference is less than 0.2. Adequate Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of this model is 28.965, which indicates an adequate signal. The low coefficient of variation (3.29%) was obtained, which is the standard deviation divided by the mean indicating the good precision of the experiments.

Table 4. 8 Fit statistics for the suggested quadratic response surface model

Std. Dev.	0.6475	R²	0.9817
Mean	19.68	Adjusted R²	0.9653
C.V. %	3.29	Predicted R²	0.8964
		Adequate Precision	28.9645

4.5. 5. Development of a model equation by using RSM-CCD

The model equation developed by using design expert software was used to determine the relationship between responses and process variables. As shown in Table 4.9, the quadratic response surface model was selected, because it efficiently fits the statistics. Thus, the quadratic response surface model that relates the yield of LA with independent parameters (coded variables) was developed as shown in equation (4.5) and used for regression analysis. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. Additionally, it is useful for identifying the relative impact of the factors by comparing the factor coefficients.

$$LA(\frac{g}{L}) = 21.73 + 2.39A + 2.60B + 0.599C - 1.53AB - 1.09BC - 1.10A^2 - 1.92B^2 - 0.548C^2 \quad (4.5)$$

where, A is incubation temperature ($^{\circ}$c), B is incubation time (hrs), and C is pH

In general, the negative coefficients describe that the factors negatively affect the yield of LA, which means an increment of the factors level results in a decrement of LA yield. Whereas the positive coefficients indicate that, the factors positively affect the yield of LA that means an increment of the factors level results in an increment of LA yield. As evident from equation 4.5, the effect of B on the yield of LA was dominant as compared to the effect of A and C.

4.5. 6. Diagnostic plot for response surface design assessment

A diagnostic plot indicates a graphical representation of the model that can be used to interpret the probability of variation of the values. The normal plot of residuals is shown in Fig 4.5. As it is observed from the normal probability plot, the residuals are following a normal distribution, thus the residuals were approximated along a straight line confirming that the normality assumption is satisfied. This indicates the selected quadratic polynomial model is satisfactory for the LA content analysis.

Normal Plot of Residuals

Figure 4. 5 The diagnostic plot showing the normal plot of residuals

In addition, as shown in Fig 4.6, there is a close agreement between actual and predicted values. The predicted values obtained by the model were very close to the experimental values and lies reasonably close to the straight line. Therefore, the result indicates that the actual value is in good agreement with the predicted values as shown in the plot.

Figure 4. 6 The diagnostic plot showing actual vs predictive value

Another very important diagnostic tool for confirming the adequacy of the fitted model for predicting the response is a residual versus predicted plot, which indicates the random scattering of the residuals. If the model is correct and the assumptions are satisfied, the residuals should be structureless, in particular, they should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. Fig 4.7 indicates that the residuals of the predicted values were randomly scattered on the plot, justifying no need for any alteration to minimize personal error.

Design-Expert® Software

LA content

Color points by value of
LA concn:

9.23 ▬▬▬ 22.96
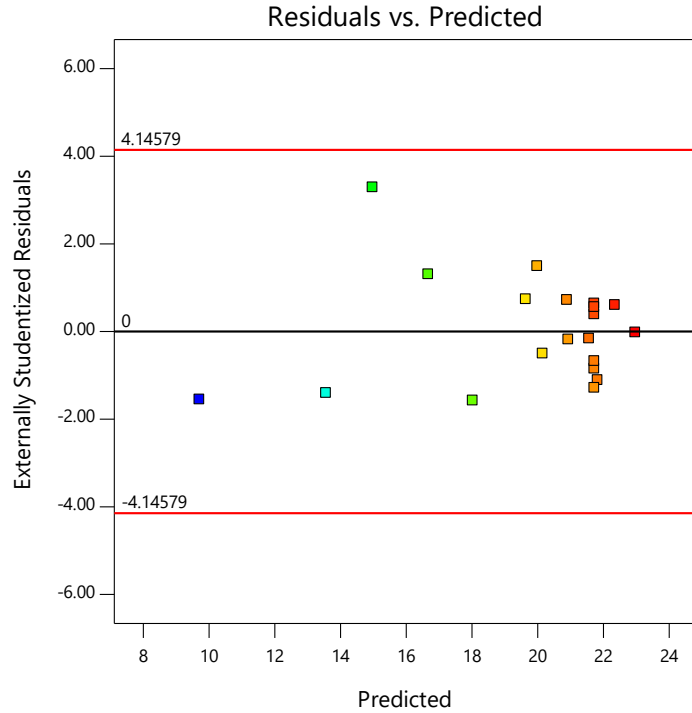
Figure 4. 7 The plot of predicted versus residual for the yield

4.6. The main effect of model parameters on the yield of lactic acid

4.6. 1. Effect of incubation temperature

The yield of LA obtained from the fermentation process was found to be substantially affected by incubation temperature. As shown in Fig 4.8, an increment of LA yield was observed with an increase in the incubation temperature. During the first 30 $^{\circ}$c to 35 $^{\circ}$c of incubation temperature, LA contents of the fermented product were very low; it is 18.86 g/L. However, as incubation temperature increased to a nearby 42 $^{\circ}$c, the LA contents of the fermented product were significantly increased to 22.89g/L, which is 21% higher. This result confirmed a positive effect of incubation temperature on the yield of LA shown in equation 4.5. The reason behind this result is that an increment of incubation temperature results in a substantial increment of the diffusion of substrate and product through the beads. As a result, the cells could obtain the substrate and the metabolic process was taking place in a good manner along with the diffusion of the generated LA product from the beads. Furthermore, since *lactobacillus plantarum* is categorized under mesophilic microorganisms (10 - 45 $^{\circ}$c) with high temperature, its metabolic activity is good as the incubation temperature gets increased to a higher level (Abate, 2016).

In the same manner, Thakur *et al.* (2018) observed the positive effect of incubation temperature in the fermentation process with other immobilized lactobacillus spp. on the substrate and product diffusion through the beads. However, further increment of the incubation temperature beyond this optimum point results in the gradual decrements of the LA contents. This indicates that a higher incubation temperature beyond the optimum decreases the number of viable cells and metabolic activities of the cells. Correspondingly, Mahato *et al.* (2021) reported a similar observation with the present study.
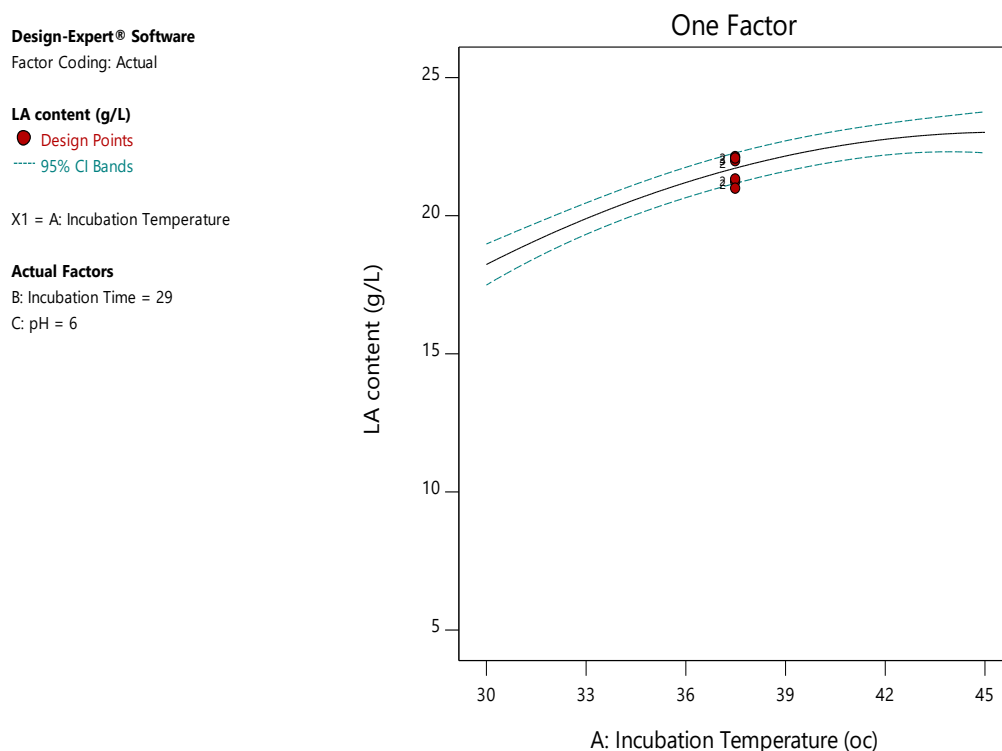
Figure 4. 8 Effects of incubation temperature on LA yield

4.6.2. Effect of incubation time

The yield of LA obtained from the fermentation process was also found to be dependent on incubation time. Incubation time is another important factor, which substantially affects the yield of LA obtained from the fermentation process. As depicted in Fig 4.9, during the first 12 hrs, LA content of the fermented product 16.65 g/L was obtained which is very low. Because of *lactobacillus plantarum* needs enough time to ferment the entire glucose as well to adapt to the new conditions, fermentation at this condition provides a low yield of LA product. Moreover, the diffusion of the substrate through the beads needs time. Accordingly, at the initial stage of

fermentation, the cells couldn't obtain enough amount of glucose for the metabolic process and result in a decrement in LA yield. Similarly, Edris (2019) stated the same phenomena with this investigation.

However, as incubation time gets increased to 40 hrs an increment of LA yield (22.67 g/L) was observed, which is 26.5% higher. This result validated a positive effect of incubation time on the yield of LA shown in equation 4.5. This shows that in this period the number and metabolic activity of the cells were very high since the cells adapted to the conditions and synthesized the molecules (i.e. DNA, RNA, and other molecules) which is necessary for their growth. Therefore, the cells are matured and fermented a huge amount of glucose efficiently. This performance of the cells is stable over the range of incubation time as compared with free cells. However, beyond this level of incubation time (40 hrs), there is a decrement of LA yield. This is due to a depletion in the substrate and the amount of available nitrogen fermentation medium for the metabolism process. The present finding is in smooth agreement with (Wang *et al*., 2020).
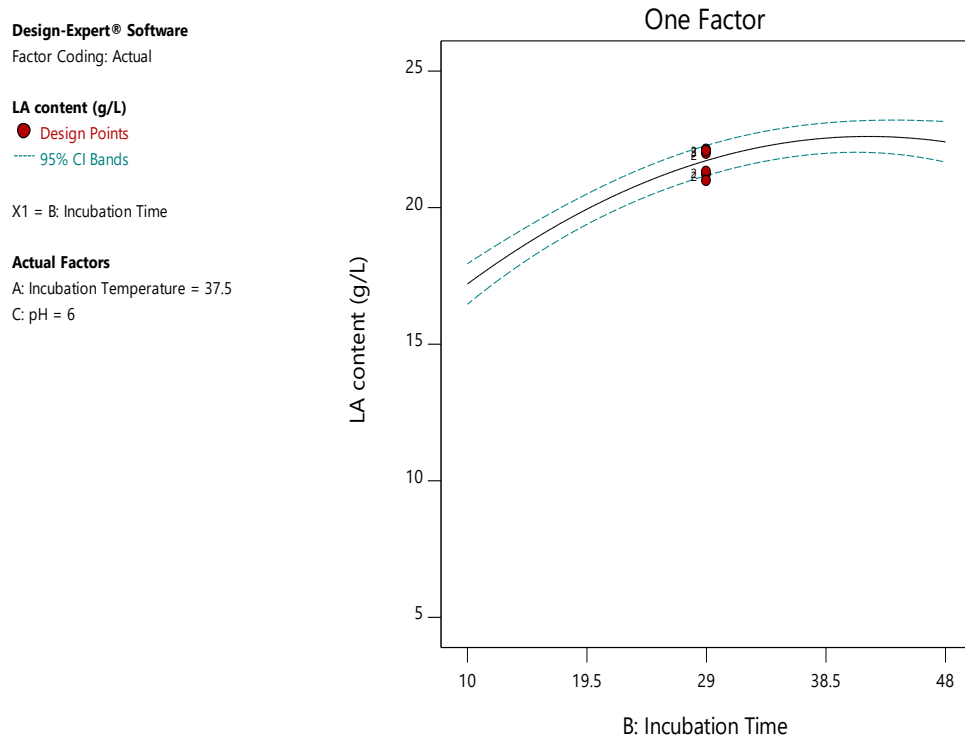
Figure 4. 9 Effects of incubation time on LA yield

4.6.3. Effect of pH

In addition to the above factors, pH was another major factor by which the yield of LA obtained from the fermentation process was affected. As shown in Fig 4.10, an increment of LA yield was observed with the increase in the pH. During the first 5 to 5.5 values of pH, LA yields of the fermented product obtained is 20.79 g/L. However, as pH increased to a nearby 6.18, the LA contents of the fermented product were increased to 21.83g/L, which is 5% higher. This result validated a positive effect of pH on the yield of LA shown in equation 4.5. This revealed that the lower the pH (i.e. higher H^+ concentration) of the media causes the diffusion of H^+ through the plasma membrane into the cytoplasm of the cells and leads to the decrement in the internal pH of the cells. Consequently, the drastic variation in cytoplasmic pH can harm the cells by inhibiting their activity and membrane transport of nutrients. Similarly, Panesar *et al.* (2010) investigated the effect of pH on the cellular metabolism process and microbial growth of microbial cells. Owing to *lactobacillus plantarum* is a prokaryotic cell, it cannot survive at lower internal pH and even die. This revealed that at lower pH of media, the metabolic activity of the *lactobacillus plantarum* cells are less and decreased LA yield. Then, as the pH of the fermentation medium increased to 6.18, the internal pH of the cells was also kept around its normal level (i.e. around 6.5) and the plasma membrane of the cells did not affected by the pH. Accordingly, the metabolic activity of the cells is increased and their fermenting efficiency is significantly increased. Subsequently, the yield of LA obtained was increased. Moreover, Bhushan *et al.* (2015) reported that a higher acidic pH caused changes in the intracellular ionic environment and damaged protein structure, which was harmful to cell growth and metabolism, results in a decrement of product yield. However, the further increment of the pH of fermented media beyond this optimal value of 6.18 leads to a less further increment of LA. Because as the pH of fermented broth gets larger, growth of *lactobacillus plantarum* cells are suppressed and their metabolic activities were inhibited. Therefore, fermenting efficiency of the *lactobacillus plantarum* cells were substantially decreased and the amount of additional LA produced was significantly reduced. Accordingly, Bahry *et al.* (2019) reported the same phenomena for the effect of pH on fermenting efficiency of an immobilized *lactobacillus rhamnosus*.
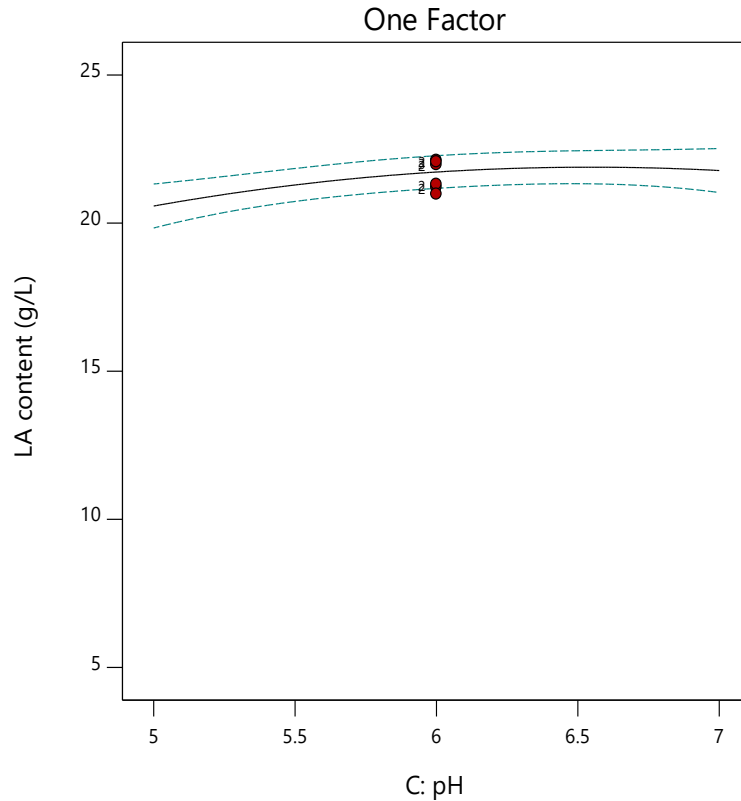
Figure 4. 10 Effects of pH on LA yield

4.7. The interaction effect of model parameters on the yield of lactic Acid

As it was revealed in section 4.5.3 above, the interaction effects of the independent parameters on the yield of LA were significant. A high significant interaction effect was observed between incubation time and pH; incubation time and temperature. These were elaborated as the below.

4.7.1. Effect of incubation temperature and time

The interactive effect of incubation temperature and incubation time on the yield of LA was depicted on the 3D surface plot in Fig 4.11. As discussed in the above sections, regardless of incubation time, the increment of incubation temperature results in an increment of LA yields. The same is true for incubation time. However, upon their interaction, as evident from Fig 4.11 the simultaneous increment of incubation time along with incubation temperature results in a decrement of LA yield. At a low level of incubation time, an increment of incubation temperature results in an increased LA yield. Conversely, at a high level of incubation time, an increment of incubation temperature results in a decreased in LA yield just after a slight initial increase in it. This outcome exposed that the interaction between incubation temperature and

incubation time has a negative effect on LA yield. Additionally, a negative sign coefficient of incubation temperature and incubation time in the developed model equation 4.5 indicated this effect. The reason behind this result is the fermentation carried out at high temperature (beyond optimum temperature, 42 °C) over a long period might cause the reduction in metabolic activities of the *lactobacillus plantarum* and depletion of the substrate. Consequently, the vial cells of *lactobacillus plantarum* substantially decreased which in turn leads to the decrement of LA yield. Because, if the cells were kept at high temperature for a long period, the cells would not survive to grow and possess their metabolism. Moreover, another researcher Edris (2017) and Thakur *et al.* (2018) investigated the negative effect of incubation temperature and incubation time on the yield of LA by using *lactobacillus plantarum.*
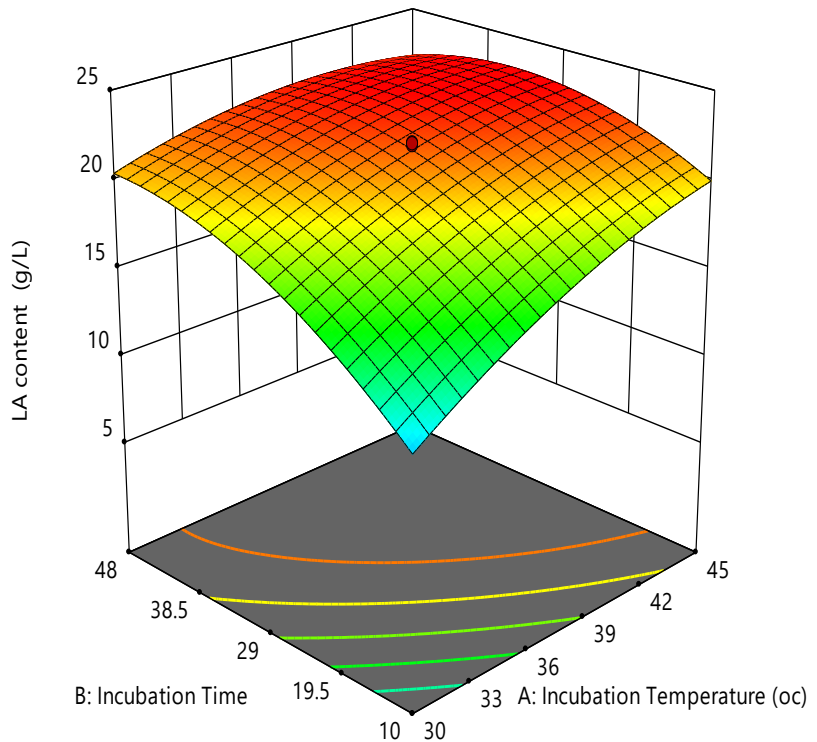
Figure 4. 11 3D surface plot for the interaction effects of incubation temperature and time on LA yield

4.7.2. Effect of pH and incubation time

The interactive effect of pH and incubation time on the yield of LA was depicted on the 3D surface plot as shown in Fig 4.12. It is observed that, at a low level of pH, an increment of incubation time leads to a sharper increment of LA yield. Nevertheless, at a high level of pH, an increment of incubation time results in a slight increment of LA for a period and then starts decreasing. This result showed that the interaction between pH and incubation time has a negative effect on LA yield. The negative sign coefficient of pH to incubation time in the developed model equation 4.5 showed this negative effect. The reason behind this effect is the increment of both pH and incubation might cause the reduction in metabolic activities of the *lactobacillus plantarum*. Because, if the fermentation process was carried out a high pH of media (low H^+ Concentration) for a long period, then a significant amount of H^+ would be diffused from the internal cytoplasm of the cells to the surrounding(media) which results in a decrement of the H^+ and increment of pH of the cells'. Therefore, if the lactobacillus species were kept at a high pH (low H^+ Concentration) for a long period, the plasma membrane of the cells would be disturbed (Panesar *et al.*, 2010). Consequently, the growth of *lactobacillus plantarum* cells was suppressed and their metabolic activities were inhibited. In turn, the production of LA was decreased. This result is in good agreement with works of Bahry *et al.* (2019).
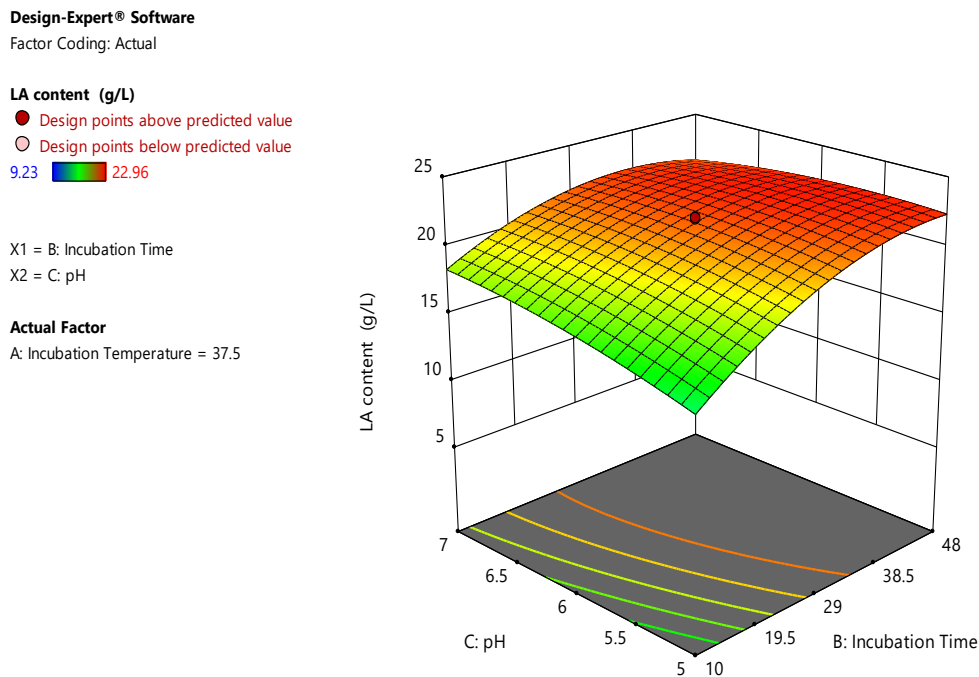
Figure 4. 12 3D surface plot for the interaction effects of incubation time and pH on LA yield

4.8. Numerical optimization of lactic acid yield

The main objective of this study is to optimize the yields of LA produced from khat waste biomass. It means to produce a higher yield of LA within the limited available resources. Therefore, the optimum condition for the selected major fermentation parameters was determined by using the numerical optimization feature of design-expert software. All factors and response with their constraints for optimizing criteria were listed in Table 4.12. The ultimate goal of the response was to maximize the LA yield obtained from the fermentation process within the given range of process variables. The obtained optimum conditions were evaluated by the composite desirability, which has a value from 0 to 1.

Table 4. 9 Ultimate goals of the response and constraints of process variables for optimization

Name	Goal	Lower Limit	Upper Limit
A: Incubation Temperature	is in range	30	45
B: Incubation Time	is in range	10	48
C: pH	is in range	5	7
LA content	maximize	9.23	22.96

Totally, around 100 possible optimal solutions had been generated. However, as shown in Table 4.13, the most appropriate solution was selected. These include incubation temperature (42.3 °c), incubation time (40.01 hrs), pH (6.18), and LA content (23.05 g/L). The highest composite desirability of 1.0 at optimum conditions was obtained, which indicates the degree of satisfaction of the optimum conditions for the ultimate goal of response was successfully attained.

Table 4. 10 The selected optimum condition by RSM

Number	Incubation Temperature (°c)	Incubation Time (hrs)	pH	LA Content (g/L)	Desirability	
1	42.30	40.01	6.18	23.05	1.00	**Selected**
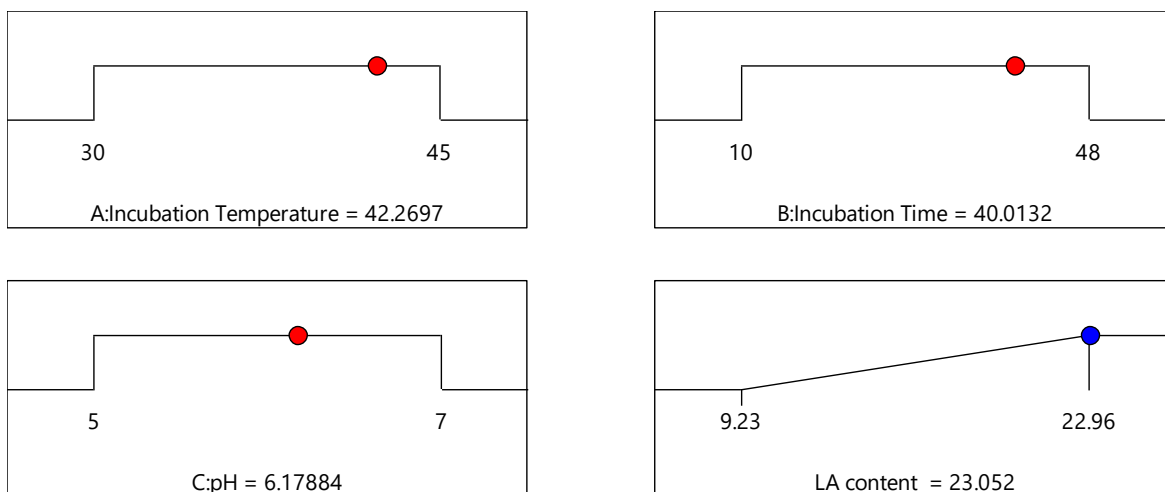
Desirability = 1.000
Solution 1 out of 100

Figure 4. 13 Ultimate goals for constraint and response in ramp style

4.8.1. Model validation

To validate the model developed, three replications of an experiment were done. As shown in Table 4.14, the model was experimentally validated. The result obtained from the experiment (22.98 ± 0.10 g/L) is very close to the result obtained from the model (23.05 g/L) used by design-expert software. Therefore, the high yield of LA obtained from this experiment revealed that the immobilized *lactobacillus plantarum* cells performing high fermenting efficiency and outstanding stability during the fermentation process under optimum conditions.

Table 4. 11 Model validation experimental data

Run No	LA (g/L)
1	22.99
2	23.07
3	22.89
Average	22.98
SD	0.10
	22.98 ± 0.10

4.9. Residual sugar determination

To determine the fermenting efficiency (i.e. glucose conversion to LA) of immobilized *lactobacillus plantarum* in the fermentation process, the residual sugar contents of the fermented sample at optimum conditions (incubation temperature of 42.2 °C and incubation time of 40 hrs) was determined with the time interval of 12 hrs. The result obtained from the experiment conducted for this particular objective is collected in Table 4.15 and plotted as shown in Fig 4.14. As it was depicted in Fig 4.14, during the first 12 hrs, about 43.13 % of glucose fed to the fermentor had been converted by *latobacillus plantarum* and yields 10.89 g/L of LA. This indicated that, because of factors such as lack of adaption to the new condition and diffusion limitation at the starting, the cells did not convert the entire available substrate into LA. Consequently, a huge amount of glucose was left unconverted. However, as incubation time increased to 36 hrs (i.e. almost near optimum incubation time), 89.40% of glucose was converted and yields 22.96 g/L of LA. Because these conditions are very suitable for the metabolism process of *lactobacillus plantarum*, as well as the substrate and product formed were diffused through the bead properly. Thus, this outcome mentioned the utilization of immobilized *lactobacillus plantarum* for LA fermentation process at optimum conditions can efficiently convert the given glucose into LA product. Accordingly, Wang *et al*. (2020) reported that fermentation with immobilized Lactobacillus species increases the fermenting efficiency and the stability of the microbes, which results in the high conversion of glucose to LA.
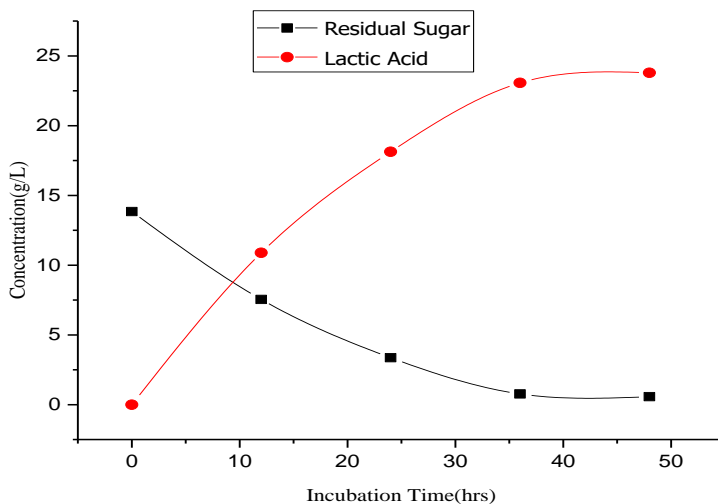
Figure 4. 14 Time profiles of LA and residual sugar during the fermentation process

4.10. Kinetic model for lactobacillus plantarum growth

The result obtained from an experiment conducted for the *lactobacillus plantarum* cell growth is shown in Fig 4.15. During the first 5 hrs, there is no significant growth of *lactobacillus plantarum* cells, which means they are in the lag phase. This indicated that, *lactobacillus plantarum* requires enough time to adapt to the new environment. In this phase, the cells are synthesizing some molecules, which are necessary for their growth in the next phase. The same phenomena were reported by Ali (2017) and Markov (2011).
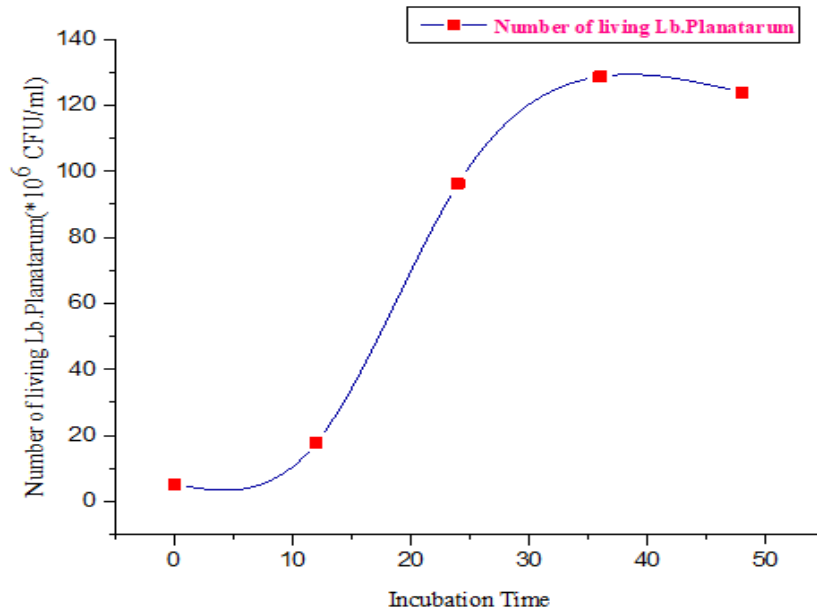
Figure 4. 15 Time profiles of *lactobacillus plantarum* growth during the fermentation process

Following the lag phase, there is a log phase in which the growth of microbial cells is started. In this cycle, owing to the molecules necessary for the growth (DNA, RNA, and other molecules) are generated, the metabolic activity of the cells is very high. The cells are well matured and utilized the nutrients efficiently. Hereafter, the single cell is divided into two (i.e. binary fission) and the number of new cells that appeared per unit time is proportional to the existing cells. Accordingly, Bailey (2018) reported the same phenomena for lactobacillus species in the log phase. As it was described in section 3.3.2, to develop the pseudo-first-order kinetic model for *lactobacillus plantarum* growth, the log (exponential) phase was used. Hence, as it was revealed in Fig 4.16, this growth cycle includes the incubation time from 12 - 36 hrs. Therefore, the specific growth rate ($i.e. \mu$) was computed from these data as the below.

Then, the graph for $ln\left(\frac{X}{X_o}\right)$ vs t was plotted as below and the slope (i.e. μ) was computed
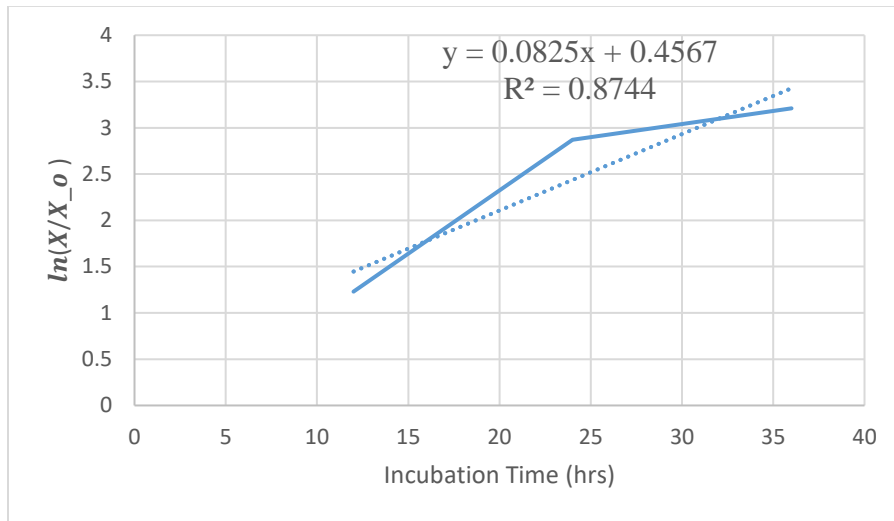
Figure 4. 16 Pseudo-first-order growth of *lactobacillus plantarum* during fermentation

Then, from the above equation, the value of $\mu =0.083$ is obtained. Therefore, the pseudo-first-order kinetic growth of *lactobacillus plantarum* is described as below.

$$X(t) = 5.2\times10^6 e^{0.083t}$$
$$12 \leq t \leq 36$$

4.6

where X is N$\underline{o}$ of living *lactobacillus plantarum* cell (CFU/ml), t is incubation time (hrs)

As shown in equation 4.6 above, the kinetic model developed is in smooth agreement with the experimental data. During the first 12 hrs, there is 17.8×10^6 CFU/ml of living cells. However, upon an increment of incubation time to 36 hrs, it is increased to 96.3×10^6 CFU/ml. This indicate that because the conditions are convenient for the growth of the cell, the cell is multiplied rapidly. Subsequently, as the incubation time goes beyond 40 hrs, the phase came to the stationary phase. At this stage, since the number of new cells formed and died were equal, the number of microbes present stayed the same throughout this cycle. The reason behind this result is, with an increment of incubation time there is a decrement of nutrients available for microbial cell growth. Therefore, the cells are competing for the nutrients and becoming less active metabolically. Lastly, the microbial cells come to be in the death phase. In this cycle, the quantities of nutrients available for microbial cell growth were substantially decreased and the

accumulation of inhibitory molecules was started. Therefore, the number of vial *lactobacillus plantarum* cells were considerably reduced. This result is in good agreement with reports of Bailey (2018) and Markov (2011).

4.11. Separation of lactic acid

LA obtained from fermentation was recovered from any other impurities via the reactive distillation method. The result obtained from this particular experiment was discussed as follows. The recovery of LA involves two reversible reactions, esterification and hydrolysis, catalyzed by a sulfuric acid catalyst.

1. Esterification

$$C_3H_6O_3 \quad + \quad CH_3OH \quad \longrightarrow \quad C_4H_8O_3 \quad + \quad H_2O \quad (4.6)$$

Lactic Acid Methanol Methayl Lactate

2. Hydrolysis

$$C_4H_8O_3 \quad + \quad H_2O \quad \longrightarrow \quad C_3H_6O_3 \quad + \quad CH_3OH$$

Methayl Lactate Lactic Acid Methanol
(4.7)

$$C_3H_6O_3 \quad + \quad CH_3OH \quad \underset{\textbf{Hydrolysis}}{\overset{\textbf{Esterification}}{\rightleftarrows}} \quad C_4H_8O_3 \quad + \quad H_2O$$

Lactic Acid Methanol Methyl Lactate
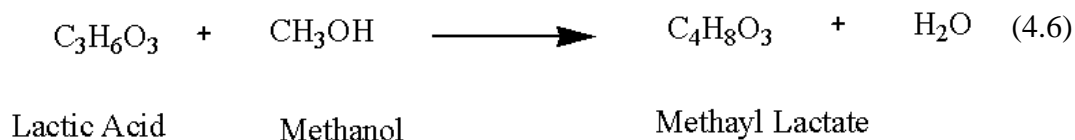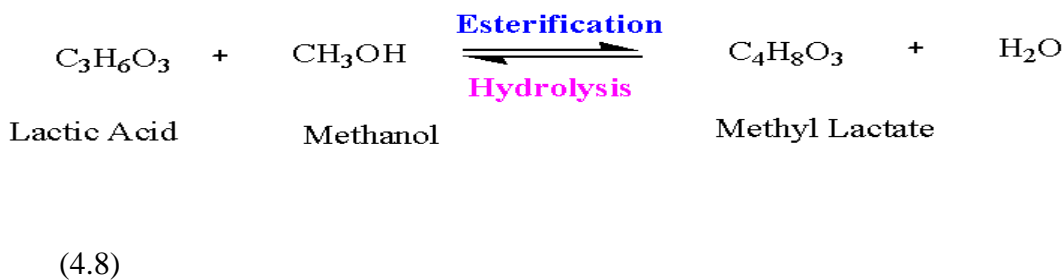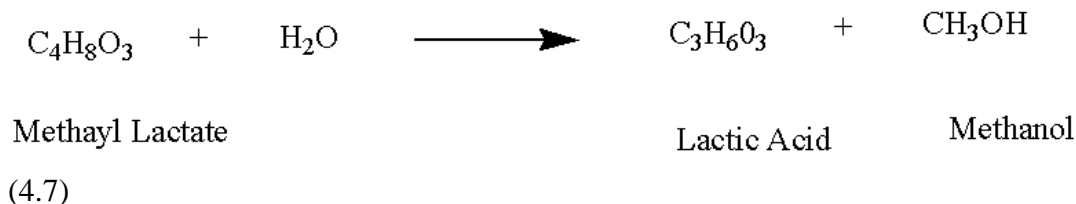
The two reactions can be written as below.

(4.8)

In the esterification reaction, the addition of methanol to LA yields methyl lactate and water. Whereas in the hydrolysis reaction, the additions of distilled water favor the reaction in the reverse direction. It means the reaction reversed in a backward direction to produce LA and methanol. In support of this, Le chatelier's principle shows that the addition of the product to an

equilibrium system will shift the equilibrium to the reactant side and the reverse is true. Therefore, in the hydrolysis reaction, the addition of distilled water decomposes the methyl lactate formed into LA and methanol. Consequently, this mixture was separated based on their boiling point (relative volatility) difference. This result was in corroborate with the work of Rao *et al*. (2014).

4.12 Characterization of the product

A. Functional group determination

FTIR spectroscopy was used to determine the functional group of LA. As depicted in Fig 4.17, the result from the FTIR spectrum showed that there were different functional groups present in LA product. The spectra of the sample showed absorbance peaks at 768 cm^{-1}, 936 cm^{-1}, 1055 cm^{-1}, 1372 cm^{-1}, 1612 cm^{-1}, 1738 cm^{-1}, 2855 cm^{-1}, and 3389 cm^{-1}. There are two absorbance peaks of the O–H group observed. The first peaks observed at 3389 cm^{-1} are attributed to the O–H stretching of the alcohol (hydroxyl) component whereas the 2855 cm^{-1} are attributed to the O–H stretching of the acid (carboxyl) component. The peak observed at 1738 cm^{-1} is related to the C=O stretching vibration of the carboxylic acid group of LA. Besides, the peak attained at 1372 cm^{-1} is attributed to the asymmetric deformation mode of CH_3. Moreover, the peak observed at 1055 cm^{-1} is contributed to the stretching modes of C–C functional groups in LA. Furthermore, the lower range of the region below 978 $cm{-1}$ generally represents different kinds of C-H, C-O, and CH_3 vibrations (rocking, deformation, stretching). These results were in a smooth agreement with previous findings reported by (Huisuo *et al*., 2018; Paucean *et al*., 2017; Tripathi *et al*., 2015).
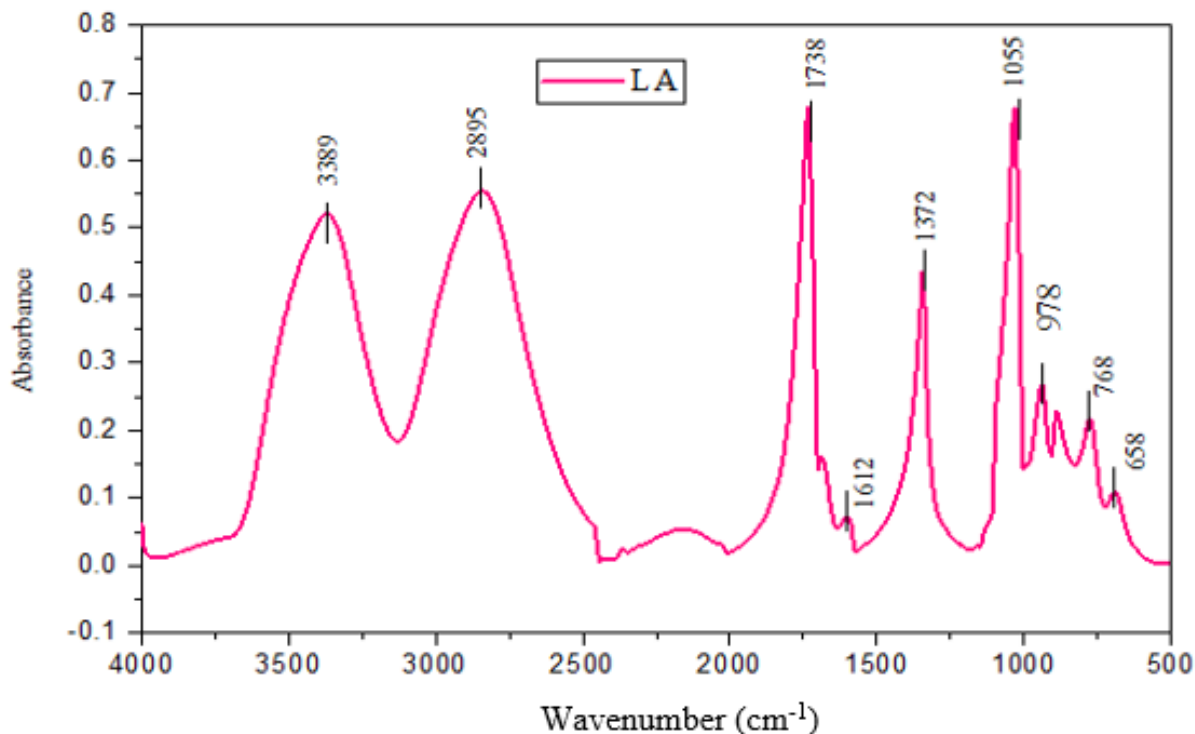
Figure 4. 17 Chemical composition of LA by using FTIR Spectroscopy

B. Physio-chemical characterization of the product

The physical and chemical properties of the product such as specific gravity, kinematic viscosity, odor, reactivity, and miscibility were investigated. As it is shown in Table 4.19, the physio-chemical properties of the produced product agree with the standard values. However, the little deviation from the standard (literature) values occurred due to some factors such as lack of equipment efficiency used for purification, lack of accuracy of the equipment used for measuring, and human error. Therefore, this result assured that the product obtained is the desired product (lactic acid).

Table 4. 12 Comparison of the obtained product with the standard

Properties	Standard (Ameen and Caruso, 2017; Mohanty *et al.,* 2015; Sridhar *et al.,* 2012)	The current study
Specific gravity	1.20	1.12

Kinematic viscosity@25ºc ($\frac{m^2}{s}$)	7.1×10^{-2}	6.07×10^{-2}
Odor	Odorless	Odorless
Reaction with NaOH	React and yields sodium lactate	React and yields sodium lactate
Miscibility in water	Completely miscible	Completely miscible

4.13. Applicability of LA produced for PLA synthesis

As it is demonstrated in section 3.3.19, the suitability of this LA product for the synthesis of PLA was confirmed. During the first one hours, a rotary evaporator removed the free water present in the LA product. The carboxylic acid group can form a hydrogen bond with each other or other groups to form a dimer (i.e. oligomer consisting of two monomers) and water molecules. Thus, in the stirred reactor the carboxyl (-COOH) and hydroxyl (–OH) groups of LA were directly reacted with each other by using a sulfuric acid catalyst and yields an oligomer of ester along with water molecules. Because the oxygen from the hydroxyl group broke the bond between carboxyl groups and formed an ester group and water. This product, which has an ester functional group, is called PLA. The reaction is shown below.
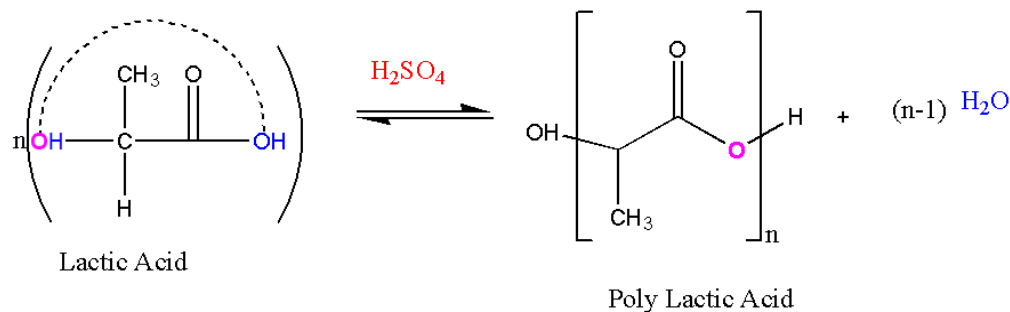
Fig 4. 18 Polymerization reaction for PLA

As the reaction time increased to six hrs, the oligomer formed was developed to low molecular weight PLA, approximately it is < 5000 Daltons. The same result was observed by Estivéz Rivadulla (2019). Therefore, the researcher can deduce that this LA produced can be used as the potential feedstock for PLA synthesis.

4.14. Summary of result and discussion

Table 4. 13 Summarization of the major part of the experimental result and discussions

Study	Result	Discussion
1. Chemical composition of khat waste	+ Cellulose = 43.7% + Hemicellulose = 17.42% + Lignin = 30.63%	Owing to its high contents of cellulose and hemicellulose, which can provide glucose via hydrolysis, the utilization of khat waste as a potential feedstock for LA production is worthy.

2.	Effects of cell immobilization on the yield of LA	Around optimum incubation time 🞣 LA $_{immobilized\ cell}$:(17.86 g/L) 🞣 LA $_{free\ cell}$:(13.45 g/L)	After 36 hrs of incubation time, the yield of LA obtained from the immobilized cell is 24.7% higher than free cells'. This revealed that an immobilized *cell* has high fermenting efficiency and excellent stability over a long period of the fermentation process
3.	The main effect of major fermentation parameters	🞣 Positively affect the LA yield obtained from the fermentation process	An increment of these parameters (Inc. temperature, Inc. time, and pH) results in an increment of LA yield up to optimum point. This is mainly because an increment of one factor at a time results in a substantial increment of the metabolic activities of the cell and the diffusion of substrate and product through the beads.
4.	The interaction effect of major fermentation parameters	🞣 Negatively affect the LA yield obtained from the fermentation process	Upon their interactive effects, an increment of their interaction (Inc. temperature and Inc. time; Inc. time and pH) results in a decrement of LA yield. This is mainly because an increment of two factors at a time results in a depletion of a substrate, substantial decrement of the metabolic process of the cell, and death of the cell.

5. Numerical optimization	+ Inc. Temperature = 42 °c + Inc. Time = 40 hrs + pH= 6.2 + LA yield = 23.05 g/L	The optimum conditions obtained from the model were validated with the experiment. At this condition, the LA yield obtained was maximum.
6. Residual sugar determination	+ Residual sugar =1.47 g/L	At optimum conditions, approximately only 10.6% of glucose is left unconverted. This showed that utilization of immobilized cell for LA fermentation process at optimum conditions can efficiently convert the given glucose into LA product.
7. kinetic model of microbial growth	+ $X(t) = 5.2 \times 10^6 e^{0.083t}$	As it was shown on the equation, during the fermentation period of 12 hrs to 36 hrs, *lactobacillus plantarum* was grown exponentially. Hence, in this cycle, the cells are multiplied rapidly.
8. Characterization of LA product obtained	+ Specific gravity: 1.12 + Kinematic viscosity(m^2/s): 6.1×10^{-2} + Odor: odorless + React with NaOH + Miscibility in water	The physio-chemical properties of the produced product were very close to the standard. Therefore, the researcher can reasonably deduce that the obtained product is LA.
9. Suitability of LA for PLA synthesis	+ Successfully synthesized low molecular weight PLA via a direct condensation reaction	The Carboxyl(-COOH) and hydroxyl groups(-OH) of LA were directly reacted with each other by using a sulfuric acid catalyst and yields PLA along with water molecules.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In the present study, optimization of fermentation condition and product characterization for lactic acid production from khat waste biomass by using immobilized *lactobacillus plantarum* was successfully attained. The chemical composition analysis result revealed that khat waste has good amount of cellulose (43.7 ± 0.71%) and hemicellulose (17.42 ± 0.85%), which could be utilized as a carbohydrate source for lactic acid production. The effect of immobilization on the yield of LA has been investigated and the yield obtained from immobilized *lactobacillus plantarum* cell is 24.7% higher than the free cells'. The effects of the major fermentation parameters (incubation temperature, incubation time, and pH) on the yield of lactic acid were investigated. Response surface methodology with a central composite design was used for the optimization of lactic acid yield and process parameters. Based on the analysis, the optimum fermentation conditions acquired by the quadratic model for maximum LA yield were, 42.3 ᵒC, 40.0 hrs, and 6.18 for incubation temperature, incubation time, and pH respectively. Under these optimum conditions, the LA yield obtained from fermentation was 23.05 g/L. The result from the model also confirmed that all the independent fermentation parameters significantly affected the yield of LA and especially, the incubation time was the dominant one. The outcome from residual sugar content determination at optimum conditions is about 1.47 g/L (i.e. in percent about 10.6% of glucose left unconverted) which shows the utilization of immobilized Lb. planatrum for fermentation at optimum conditions can efficiently convert the given glucose into LA product. The exponential growth $(X(t) = 5.2*10^6 e^{0.083t})$ of *lactobacillus plantarum* cell was observed from the kinetic model of microbial growth study under optimum conditions. The investigated physio-chemical properties (specific gravity, kinematic viscosity, odor, reactivity, and miscibility) and functional groups of the obtained product were very similar to the standard product. Finally, the applicability of the produced product for PLA synthesis was confirmed and polymerization of the obtained LA product is very suitable for PLA synthesis. Generally, this study demonstrated that the utilization of khat waste as a potential carbohydrate source and immobilization of *lactobacillus plantarum* cell for LA production is worthful.

5.2. Recommendations

To explore the possibility of commercial lactic acid production from khat waste biomass by using immobilized *lactobacillus plantarum*, further investigations are recommended. These are-

- Along with the above studied major fermentation parameters, it is better to investigate the effect of bead size and substrate concentration on the yield of lactic acid;
- Besides, it is recommended that using co-fermentative lactobacillus species, to utilize both glucose and xylose which is the second abundant carbohydrate source next to glucose;
- Moreover, it is better to separate non-reducing sugar developed during the hydrolysis process to minimize their effects on fermentation;
- Furthermore, it is better to recover lactic acid obtained from fermentation by using advanced purification methods such as membrane separation;
- Lastly, it is highly recommended that the polymerization techniques used for Poly Lactic Acid Synthesis should be via Ring-opening polymerization.

REFERENCE

Abate, Y. (**2016**). Synthesis and Production of Lactic Acid From False Banana / Bula by Using Lactobacillus plantarum, **MSc Thesis,** Addis Ababa University,Ethiopia.

Abdel-rahman, M. A., & Sonomoto, K. (**2016**). Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. **Journal of Biotechnology**, 236, 176–192. https://doi.org/10.1016/j.jbiotec.2016.08.008

Abdel-rahman, M. A., Tashiro, Y., Zendo, T., Sakai, K., & Sonomoto, K. (**2016**). Highly e ffi cient L -lactic acid production from xylose in cell recycle continuous fermentation using. **RSC Advances**, 6, 17659–17668. https://doi.org/10.1039/C5RA27579B

Abdulhafiz, Y. (**2014**). Liquid Fuel Production through Pyrolysis of Khat and Plastic waste mixture. **MSc Thesis**, Addis Ababa University,Ethiopia.

Abedi, E., & Hashemi, S. M. B. (**2020**). Lactic acid production – producing microorganisms and sources-state of art.**Heliyon**,6(10),e04974. https://doi.org/10.1016/j.heliyon.2020.e04974

Ali, E. (**2017**). Utilization of Duckweed as a carbon sources for production of Lactic Acid. **Msc Thesis**, Addis Ababa University,Ethiopia.

Alrefaey, H. M. A., Abdel-Rahman, M. A., Hassan, S. E. D., El-Din, M. N., & Azab, M. S. (**2020**). Sequential optimization of the fermentation factors with integrating seed culture adaptation for increased biorefinery of beet molasses to lactic acid. **Biomass Conversion and Biorefinery**. https://doi.org/10.1007/s13399-020-00773-3

Alves, R., de Oliveiraa, C., Rossellb, C. E. V., Pereiraa, G. A. G., & Rubens Maciel Filhoc. (**2020**). Second-generation (2G) Lactic Acid Production and New Developments – **A Mini-review. Chemical engineering transactions,** 80, 223–228. https://doi.org/10.3303/CET2080038

Ameen, S., & Caruso, G. (**2017**). Lactic Acid in the Food Industry. **Springer**. https://doi.org/10.1007/978-3-319-58146-0

Auras, A., Rafael, Lim, L.-T., Selke, S. E. M., & Tsuji, H. (**2011**). Poly(lactic acid): Synthesis, Processing, and Applications. **Wiley Series on Polymer Engineering and Technology**.

Bahry, H., Abdalla, R., Pons, A., Taha, S., & Vial, C. (**2019**). Optimization of lactic acid production using immobilized Lactobacillus Rhamnosus and carob pod waste from the Lebanese food industry. *Journal of Biotechnology*, *306*(October), 81–88. https://doi.org/10.1016/j.jbiotec.2019.09.017

Bailey, R. (**2018**). *Phase of Bacterial Growth* (pp. 1–11).

Battula, S., Gantala, S., Besetty, T., Narayana, S., & Gopinadh1, R. (**2019**). Industrial production of lactic acid. *International Journal of Biotech Research*, *1*(1), 42–54. https://doi.org/10.1016/B978-0-12-809633-8.09142-1

Behera, S. S., Ray, R. C., & Zdolec, N. (**2018**). Lactobacillus plantarum with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. *BioMed Research International*, *2018*, 1–18. https://doi.org/10.1155/2018/9361614

Ben-David, A., & Davidson, C. E. (**2014**). Estimation method for serial dilution experiments. *of Microbiological Methods*, *107*, 214–221. https://doi.org/10.1016/j.mimet.2014.08.023

Beneberu, S. (**2017**). Generation, Composition and Characteristics of Urban Solid Waste in a Major Khat Producing and Marketing Area in Eastern Ethiopia. *International Journal of Environmental Protection*, *8*, 1–25. https://doi.org/10.5963/IJEP0105002

Bhushan, B., Pal, A., & Jain, V. (**2015**). Improved Enzyme Catalytic Characteristics upon Glutaraldehyde Cross-Linking of Alginate Entrapped Xylanase Isolated from Aspergillus flavus MTCC 9390. *Enzyme Research*, *2015*, 1–10. https://doi.org/10.1155/2015/210784

Bintsis, T. (**2018**). Lactic acid bacteria as starter cultures : An update in their metabolism and genetics. *Microbiology*, *4*(4), 665–684. https://doi.org/10.3934/microbiol.2018.4.665

Borshchevskaya, L. N., Gordeeva, T. L., Kalinina, A. N., & Sineokii, S. P. (**2016**). Spectrophotometric determination of lactic acid. *Journal of Analytical Chemistry*, *71*(8), 755–758. https://doi.org/10.1134/S1061934816080037

Cai, J., He, Y., Yu, X., Banks, S. W., Yang, Y., Zhang, X., Yu, Y., Liu, R., & Bridgwater, A. V. (**2017**). Review of physicochemical properties of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews*, *76*, 309–322. https://doi.org/10.1016/j.rser.2017.03.072

Chinenye, M., Sun, F., Rose, M., Song, G., Damascene, J., & Ajeje, S. (**2021**). Trends and hassles in the microbial production of lactic acid from lignocellulosic biomass. ***Environmental Technology & Innovation***, *21*, 1–24. https://doi.org/10.1016/j.eti.2020.101337

Cinar, S. O., Chong, Z. K., Kucuker, M. A., Wieczorek, N., Cengiz, U., & Kuchta, K. (**2020**). Bioplastic Production from Microalgae : A Review. ***Environmental Research and Public Health***, *17*, 1–21. https://doi.org/10.3390/ijerph17113842

Doran-Peterson, J., Cook, D. M., & Brandon, S. K. (**2008**). Microbial conversion of sugars from plant biomass to lactic acid or ethanol. ***Plant Journal***, *54*(4), 582–592, . https://doi.org/10.1111/j.1365-313X.2008.03480.x

Edris, M. (**2019**). Production of lactic acid from sugarcane bagasse, **MSc Thesis,** Addis Ababa University,Ethiopia.

Estivéz Rivadulla, S. (**2019**). Production facility of polylactic acid from lactic acid.

Fikadu, E. (**2018**). Production and Characterization of Fuel Briquette from Khat (Catha Edulis Forsk) Residue for Diversification of Household, **MSc Thesis**, Hawasa University ,Ethiopia

Filaba, M. (**2008**). Waste Management Problems in Jimma, Southern Ethiopia. ***Ethiopian Journal of Environmental Studies and Management***, *1*(2), 8–15. . https://doi.org/10.4314/ejesm.v1i2.41575

Fisiaha, A. (2016). *Bioethanol Production from Barley- Spent grain(Brewer Spent Grain),* **MSc Thesis,** Addis Ababa University,Ethiopia.

Gabriel, T., Wondu, K., & Dilebo, J. (**2021**). Valorization of khat (Catha edulis) waste for the production of cellulose fibers and nanocrystals. ***Plos One***, *16*(2), e0246794. https://doi.org/10.1371/journal.pone.0246794

Garlotta, D. (**2019**). A Literature Review of Poly (Lactic Acid) A Literature Review of Poly (Lactic Acid). ***Polymers and the Environment***, *9*(2), 63–84. . https://doi.org/ 10.1023/A:1020200822435

Gessesse Dessie. (**2013**). Favouring a Demonised Plant Khat and Ethiopian smallholder-

enterprises (Issue 51). http://www.diva-portal.org/smash/record.jsf?pid=diva2:586499

Ghaffar, T., Irshad, M., Anwar, Z., Aqil, T., Zulifqar, Z., Tariq, A., Kamran, M., & Ehsan, N. (**2014**). Recent trends in lactic acid biotechnology : A brief review on production to purification. *Journal of Radiation Research and Applied Sciences*, *7*(2), 222–229. https://doi.org/10.1016/j.jrras.2014.03.002

Global Lactic Acid Market Report. (**2019**). Data intelligence for market research LLP publisher

Gonultas, O., & Candan, Z. (**2018**). Chemical characterization and ftir spectroscopy of thermally compressed eucalyptus wood panels. *Maderas: Cienciay Tecnologia*, *20*(3), 431–442. https://doi.org/10.4067/S0718-221X2018005031301

Górecka, E., & Jastrzębska, M. (**2011**). Immobilization Techniques And Biopolymer Carriers – A Review. *Biotechnology and Food Sciences Research*, *75*(1), 27–34.

Grand View Research. (**2019**). Global Lactic Acid Market Size, Share & Trend Analysis Report 2019-2025. In *Marketsandmarkets.Com*. https://www.grandviewresearch.com/industry-analysis/lactic-acid-and-poly-lactic-acid-market

Grewal, J., & Khare, S. (**2017**). Lactic acid production by Lactobacillus brevis mediated bioprocesses utilizing lignocellulosic agro-wastes. *4460*, 1–3.

Grewal, J., & Khare, S. K. (**2018**). One-pot bioprocess for lactic acid production from lignocellulosic agro-wastes by using ionic liquid stable Lactobacillus brevis. *Bioresource Technology*, *251*, 268–273. https://doi.org/10.1016/j.biortech.2017.12.056

Guyot, J. P., & Calderon, M. (**2000**). Effect of pH control on lactic acid fermentation of starch by Lactobacillus manihotivorans LMG 18010 T. *Journal of Applied Microbiology*, *88*,176–182.

Harun, S., Sohrab, H., Azem, M., & Karim, A. (**2016**). A Review of the Thermal Pretreatment of Lignocellulosic Biomass towards Glucose Production: Autohydrolysis with DIC Technology. *Bioresources Technology*, *11*(4), 10625–10653. https://doi.org/10.15376/biores.11.4.

Hofvendahl, K. (**2000**). Factors affecting the fermentative lactic acid production from renewable resources. *Enzyme and Microbial Technology*, *26*, 87–107.

Hospodarova, V., Singovszka, E., & Stevulova, N. (**2018**). Characterization of Cellulosic Fibers by FTIR Spectroscopy for Their Further Implementation to Building Materials. *American Journal of Analytical Chemistry*, *09*(06), 303–310. https://doi.org/10.4236/ajac.2018.96023

Hu, J., Lin, Y., Zhang, Z., Xiang, T., Mei, Y., Zhao, S., Liang, Y., & Peng, N. (**2016**). High-titer lactic acid production by Lactobacillus pentosus FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. *Bioresource Technology*, *214*, 74–80. https://doi.org/10.1016/j.biortech.2016.04.034

Huisuo, H., Grün, I. U., Ellersieck, M., & Clarke, A. D. (**2018**). Use of HPLC and FTIR as a tool for analysis of lactic acid in restructured fish products. *Journal of Nutrition, Food Research and Technology*, *1*(2), 42–48. https://doi.org/10.30881/jnfrt.00010

Jing, M., Sui, G., Zhao, J., Zhang, Q., & Fu, Q. (**2019**). Enhancing crystallization and mechanical properties of poly (lactic acid)/ milled glass fiber composites via self-assembled nanoscale interfacial structures. *Composites Part A*, *117*, 219–229. . https://doi.org/10.1016/j.compositesa.2018.11.020

Jönsson, L. J., & Martín, C. (**2016**). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, *199*, 103–112. https://doi.org/10.1016/j.biortech.2015.10.009

Juodeikiene, G., Bartkiene, E., Zadeike, D., & Klupsaite, D. (**2019**). Biorefinery approach for the utilization of dairy by-products and lignocellulosic biomass to lactic acid. In *Biorefinery*. pp. 31–53. https://doi.org/10.1515/9783110538151-002

Kang, Q., Appels, L., Tan, T., & Dewil, R. (**2014**). Bioethanol from Lignocellulosic Biomass : Current Findings Determine Research Priorities. *The Scientific World Journal*, *2014*, 1–13. https://doi.org/org/10.1155/2014/298153

Karnaouri, A., Asimakopoulou, G., Kalogiannis, K. G., Lappas, A., & Topakas, E. (**2020**). Efficient D-lactic acid production by Lactobacillus delbrueckii subsp. bulgaricus through

conversion of organosolv pretreated lignocellulosic biomass. ***Biomass and Bioenergy***, *140*, 105672–110582. https://doi.org/10.1016/j.biombioe.2020.105672

Keikhosro, T. and, & Karimi, M. J. (**2007**). Acid Based Hydrolysis Process of Ethanol from Lignocellulosic Biomass : A Review. ***Bioresources***, *2*(3), 472–499.

Kim, D. (**2018**). Physico-chemical conversion of lignocellulose: Inhibitor effects and detoxification strategies: A mini review. ***Molecules***, *23*(2), 1–21. https://doi.org/10.3390/molecules23020309

Kim, J. Y., Kim, Y. J., Hong, W. H., & Wozny, G. (**2000**). Recovery process of lactic acid using two distillation columns. ***Biotechnology and Bioprocess Engineering***, *5*(3), 196–201. https://doi.org/10.1007/BF02936594

Koekemoer, T. (**2018**). Lactic acid production from sugarcane bagasse and harvesting residues, **MSc Thesis**, at Stellenbosch University.

Komesu, A., de Oliveira, J. A. R., Martins, L. H. da S., Maciel, M. R. W., & Filho, R. M. (**2017**). Lactic acid production to purification: A review. ***Bioresources Technology***, *12*(2), 4364–4383. https://doi.org/10.15376/biores.12.2.4364-4383

Lin, L., Yan, R., Liu, Y., & Jiang, W. (**2010**). In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: Cellulose, hemicellulose and lignin. ***Bioresource Technology***, *101*(21), 8217–8223.   . https://doi.org/10.1016/j.biortech.2010.05.084

Mahato, A. K., Kumari, L., Singh, R. S., & Alam, M. T. (**2021**). Fermentative study on optimization of lactic acid production from cane sugar by Lactobacillus spp. *European Journal of Molecular and Clinical Medicine*, *8*(2), 712–723. https://www.embase.com//results?subaction=viewrecord&id=L2011094351&from=export

Markov, S. M. (**2011**). On the mathematical modelling of microbial growth : some computational aspects. ***Computing***, *5*, 153–168.

Mirdamadi, S., Sadeghi, H., Sharafi, N., Fallahpour, M., Mohseni, F. A., & Bakhtiari, M. R. (**2002**). Comparison of lactic acid isomers produced by fungal and bacterial strains. ***Iranian Biomedical Journal***, *6*(3), 69–75.

Mohanty, J. N., Das, P. K., Nanda, S., Nayak, P., & Pradhan, P. (**2015**). Comparative analysis of crude and pure lactic acid produced by Lactobacillus fermentum and its inhibitory effects on spoilage bacteria. *Pharma Innovation Journal TPI*, *38*, 38–42.

Mora-Villalobos, J., Montero-Zamora, J., Barboza, N., Rojas-Garbanzo, C., Usaga, J., Redondo-Solano, M., Schroedter, L., Olszewska-Widdrat, A., & Lopez-Gomez, J. (**2020**). Multi-Product Lactic Acid Bacteria Fermentations. *Fermentation MDPI,* *6*(23), 1–21. https://doi.org/10.3390/fermentation6010023

Nakasaki, K., Murai, T., & Akiyama, T. (**1989**). Dynamic modeling of an immobilized cell reactor - Application to aerobic reactions. *Applied Biochemistry and Biotechnology*, *22*(3), 279–288. https://doi.org/10.1007/BF02921762

Narayanan, N., Roychoudhury, P. K., & Srivastava, A. (**2004**). L (+) lactic acid fermentation and its product polymerization. *Electronic Journal of Biotechnology*, *7*(2), 168–179. https://doi.org/10.2225/vol7-issue2-fulltext-7

Nesryia, J. (**2018**). Characterization and Optimization of Lactic Acid Produced from Sugar Cane Molasses by Using Lactobacillus plantarium Bacteria Isolated from "'kocho.'",**MSc Thesis,** Addis Ababa University, Ethiopia

Nielsen, S. S. (**2010**). Phenol-Sulfuric Acid Method for Total Carbohydrates. *Springer*, 39–44. https://doi.org/10.1007/978-1-4757-5250-2_6

Olszewska-widdrat, A., Alexandri, M., Pablo, L., Schneider, R., & Venus, J. (**2020**). Batch and Continuous Lactic Acid Fermentation Based on A Multi-Substrate Approach. *Microorganisms*, *8*(7), 1084, 1–14. https://doi.org/10.3390/microorganisms8071084

Ou, M. S., Ingram, L. O., & Shanmugam, K. (**2011**). L(+)-Lactic acid production from non-food carbohydrates by thermotolerant Bacillus coagulans. *Journal of Industrial Microbiology and Biotechnology*, *38*(5), 599–605. https://doi.org/10.1007/s10295-010-0796-4

Panesar, P. S., Kennedy, J. F., Knill, C. J., & Kosseva, M. (**2010**). Production of L(+) Lactic Acid using Lactobacillus casei from Whey. *Brazilian Archives of Biology and Technology*, *53*(1), 219–226. https://doi.org/10.1590/S1516-89132010000100027

Păucean, A., Vodnar, D. C., Mureşan*, V., Fetea, F., Ranga, F., Man, S. M., And, S. M., &

Socaciu, C. (**2017**). Monitoring lactic acid concentrations by infrared spectroscopy : a new developed method for lactobacillus fermenting media with potential food applications. *Acta Alimentaria*, *46*(4), 420–427. https://doi.org/10.1556/066.2017.0003

Probst, K. V, Ambrose, R. P. K., Pinto, R. L., Bali, R., Krishnakumar, P., & Ileleji, K. E. (**2013**). The Effect of Moisture Content on the Grinding Performance of Corn and Corncobs by Hammer milling. *American Society of Agricultural and Biological Engineers*, *56*(3), 1025–1033.

Qian, S., Zhang, H., Yao, W., & Sheng, K. (**2018**). Effects of bamboo cellulose nanowhisker content on the morphology , crystallization , mechanical , and thermal properties of PLA matrix biocomposites. *Composites Part B*, *133*, 203–209. . https://doi.org/10.1016/j.compositesb.2017.09.040

Rameshwar, H. Y., & Argaw, A. (**2016**). Manurial value of khat waste vermicompost from Awday , Harar town , Manurial value of khat waste vermicompost from Awday ,Ethiopia. *International Journal of Recycling of Organic Waste in Agriculture*, 1–8. https://doi.org/10.1007/s40093-016-0121-y

Rao, V. V. B., Kumar, P. S., Sailu, C., & Rao, S. R. M. (**2014**). Recovery of Lactic Acid by Reactive Distillation. *Journal of Applied Sciences*, *14*(12), 1289–1293. https://doi.org/10.3923/jas.2014.1289.1293

Raspolli Galletti, A. M., D'Alessio, A., Licursi, D., Antonetti, C., Valentini, G., Galia, A., & Nassi O Di Nasso, N. (**2015**). Midinfrared FT-IR as a tool for monitoring herbaceous biomass composition and its conversion to furfural. *Journal of Spectroscopy*, *2015*, 1–13. https://doi.org/10.1155/2015/719042

Robak, K., & Balcerek, M. (**2018**). Review of second generation bioethanol production from residual biomass. *Food Technology and Biotechnology*, *56*(2), 174–187. https://doi.org/10.17113/ftb.56.02.18.5428

Rojan, G., Anisha, K. ., A, N., & R., P. (**2020**). Direct Lactic Acid Fermentation: Focus on Simultaneous Saccharification and Lactic Acid Production. *Biotechnology Advances*, *27*, 145–152.

Saha, D., Samal, S., Biswal, M., Smita, M., & Nayak, S. K. (**2019**). Preparation and characterization of poly (lactic acid)/ poly (ethylene oxide) blend film : . *Wileyonlinelibrary*, *68*, 164–172. https://doi.org/10.1002/pi.5718

Sridhar, G., Poloju, S., & Kishore, C. (**2012**). Oxidation of Lactic Acid. https://doi.org/10.3923/ja.2015.37.42

STP-4.3. (**2006**). *Density – Pycnometer Calibration*. 1–6.

Subramanian, M. R., Talluri, S., & Christopher, L. P. (**2015**). Production of lactic acid using a new homofermentative Enterococcus faecalis isolate. *Microbial Biotechnology*, *8*(2), 221–229. https://doi.org/10.1111/1751-7915.12133

Sungyeap Hong, C. L. (**2014**). An Overview of the Synthesis and Synthetic Mechanism of Poly (Lactic acid). *Modern Chemistry & Applications*, *02*(04). https://doi.org/10.4172/2329-6798.1000144

Sutton, S. (**2011**). Measurement of microbial cells by optical density. *J. Validation Techn*, *17*(1), 46–49. http://www.microbiologynetwork.com/content/JVT_2011_v17n1_Measurement-of-Microbial-Cells-by-Optical-Density.pdf

Tam, M. S., Gunter, G. C., Craciun, R., Miller, D. J., & Jackson, J. E. (**1997**). Reaction and Spectroscopic Studies of Sodium Salt Catalysts for Lactic Acid Conversion. *American Chemical Society*, *1997*(36), 3505–3512.

Tarraran, L., & Mazzoli, R. (**2018**). Alternative strategies for lignocellulose fermentation through lactic acid bacteria : the state of the art and perspectives. *FEMS*, *365*(126), 1–14. https://doi.org/orcid.org/0000-0001-7674-8187

Teklie, H., Gonfa, G., Getachew, T., Defar, A., Bekele, A., Bekele, A., Gelibo, T., Amenu, K., Tadele, T., Taye, G., Getinet, M., Chala, F., Mudie, K., Guta, M., Feleke, Y., Shiferaw, F., Tadesse, Y., Yadeta, D., Michael, M. G., … Teferra, S. (**2017**). Prevalence of khat chewing and associated factors in Ethiopia: Findings from the 2015 national non-communicable diseases STEPS survey. *Ethiopian Journal of Health Development*, *31*, 320–330.

Tessfaw, Z. A., Beyene, A., Nebiyu, A., Pikoń, K., & Landrat, M. (**2020**). Co-composting of khat-derived biochar with municipal solid waste: A sustainable practice of waste

management. *Sustainability (Switzerland)*, *12*(24), 1–14. https://doi.org/10.3390/su122410668

Thakur, A., Panesar, P. S., & Saini, M. S. (**2018**). Parametric optimization of lactic acid production by immobilized Lactobacillus casei using box-Behnken design. *Periodica Polytechnica Chemical Engineering*, *62*(3), 274–285. https://doi.org/10.3311/PPch.11403

Tu, W.-L., Hsu, T.-C., Wang, C.-A., Guo, G.-L., & Chao, Y. (**2019**). Using Novel Lactobacillus plantarum to Produce Lactic Acid from Lignocellulosic Biomass . *Bioresources Technology*, *14*(2), 3873–3885.

Wang, J., Huang, J., Laffend, H., Jiang, S., Zhang, J., Ning, Y., & Fang, M. (**2020**). Optimization of immobilized Lactobacillus pentosus cell fermentation for lactic acid production. *Bioresources and Bioprocessing*, *7*, 1–14. https://doi.org/10.1186/s40643-020-00305-x

Wee, Y. J., Yun, J. S., Kim, D., & Ryu, H. W. (**2006**). Batch and repeated batch production of L(+)-lactic acid by Enterococcus faecalis RKY1 using wood hydrolyzate and corn steep liquor. *Journal of Industrial Microbiology and Biotechnology*, *33*(6), 431–435. https://doi.org/10.1007/s10295-006-0084-5

Wee, Y., Kim, J., & Ryu, H. (**2006**). Biotechnological Production of Lactic Acid and Its Recent Applications. *Biotechnology*, *44*(2), 163–172.

Wuletaw, M. (**2018**). Public discourse on Khat (Catha edulis) production in Ethiopia: Review. *Journal of Agricultural Extension and Rural Development*, *10*(10), 192–201. https://doi.org/10.5897/jaerd2018.0984

Yusuf, M. (**2008**). Composting of khat and Related Materials As Solid waste management option in Awaday Town, **MSc Thesis,** Addis Ababa University,Ethiopia

Zhang, Y., & Vadlani, P. V. (**2015**). Lactic acid production from biomass-derived sugars via co-fermentation of Lactobacillus brevis and Lactobacillus plantarum. *Journal of Bioscience and Bioengineering*, *119*(6), 694–699. https://doi.org/10.1016/j.jbiosc.2014.10.027

Zhu, Y. (**2011**). Immobilized Cell Fermentation for Production of Chemicals and Fuels.

Bioprocessing for Value-Added Products from Renewable Resources, 373–396. https://doi.org/10.1016/B978-044452114-9/50015-3

Zur, J., Wojcieszyńska, D., & Guzik, U. (**2016**). Metabolic responses of bacterial cells to immobilization. *Molecules*, *21*(7), 1–15. https://doi.org/10.3390/molecules21070958

APPENDICES

Appendix A: Calculations

1. Moisture content determination

Table A-1 Moisture content determination

S.N	1A	1B	1C	Standard Deviation (SD)
Weight of the sample(g)	5.0	5.0	5.0	
Weight of empty Petridish(g	45.4	45.8	42.0	
Weight of sample with petridish before dried(g)	49.4	50.8	47.0	
Weight of sample with petridish after dried(g)	48.72	50.15	46.33	
Loss of the weight(g)	0.68	0.65	0.67	
Moisture content (%) $= \frac{W_l}{W_b} *$ **100**%	13.6	13.0	13.4	0.31
Average value (%)	$(\frac{13.6+13.0+13.4}{3}) = \underline{13.33}$			13.33 ± 0.31

2. Ash content determination

Table A-2 Ash content determination

S.N	2A	2B	2C	SD
Weight of the sample(g)	5.0	5.0	5.0	
Weight of empty crucible(g	33.96	34.12	38.45	
Weight of sample with crucible before burned(g)	38.96	39.12	43.45	
=weight of ash formed (g)	0.36	0.33	0.37	
Ash content (%) $= \frac{W_{ash}}{W_b} *$ **100**%	7.2	6.6	7.4	0.42
Average value (%)	$(\frac{7.2+6.6+7.4}{3}) = \underline{7.1}$			7.1 ± 0.42

3. Volatile matter content determination

Table A-3 volatile matter content determination

S.N	3A	3B	3C	SD
Weight of the sample(g)	5.0	5.0	5.0	
Weight of empty crucible(g	28.53	31.17	35.47	
Weight of sample with crucible before burned(g)	33.53	36.17	40.47	
Weight of sample with crucible after ignition (g)	30.74	33.435	37.76	
$W_i - W_f$ (g)	2.79	2.735	2.705	
Volatile mater $(\%) = \dfrac{W_i - W_f}{W_b} * 100\%$	55.8	54.7	54.10	0.86
Average value (%)	$(\dfrac{55.8+54.7+54.1}{3}) = \underline{54.89}$			54.89 ± 0.86

4. Hemicellulose Content determination

Table A-4 Hemicellulose Content determination

S.N	4A	4B	4C	SD
Initial weight of the sample(g)	2.0	2.0	2.0	
Final weight of the sample(g)	1.831	1.835	1.819	
Hemicellulose content $= (W_i - W_f) * 100\%$	16.87	16.46	18.10	0.85
Average value (%)	$(\dfrac{16.87+16.46+18.10}{3}) = \underline{17.42}$		17.42 ± 0.85	

5. Lignin Content determination

Table A-5 Lignin Content determination

S.N	5A	5B	5C	SD
Initial weight of the sample(g)	2.0	2.0	2.0	
Final weight of the sample(g)	0.590	0.613	0.630	
Lignin content $= \frac{W_f}{W_i} * 100\%$	29.50	30.65	31.35	0.93
Average value (%)	$(\frac{29.50+30.65+31.35}{3})= \underline{30.63}$			30.63 ± 0.93

6. Extractive Content determination

Table A-6 Extractive Content determination

S.N	6A	6B	6C	SD
Initial weight of the sample(g)	2.0	2.0	2.0	
Final weight of the sample(g)	1.913	1.919	1.921	
Extractives content $= (W_i - W_f) * 100\%$	8.70	8.15	7.90	0.41
Average value (%)	$(\frac{8.70+8.15+7.9}{3}) = \underline{8.25}$			8.25 ± 0.41

7. Cellulose Content determination

Table A-7 Lignin Content determination

F. $Cellulose (\%) = 100\% - (Lignin + hemicellulose + extractives)\%$
$$= 100\% - (17.42 + 30.63 + 8.25)\% = 43.70\% \pm 0.71$$

8. Specific gravity and density determination

Table A-8 Specific gravity and density determination

S.N	
The empty weight of the pycnometer(W_o)((g)	30.645
The final weight of the sample with pycnometer(W_2)(g)	138.956
The final weight of the distilled water with a pycnometer (W_1)((g)	127.438
Specific gravity $= \dfrac{W_1-W_o}{W_2-W_o}$	$\dfrac{138.96 - 30.65}{127.44 - 30.65}$ 1.12
Density($\dfrac{kg}{m^3}$) $\rho_{LA} = SG * \rho_{Water}$	1.12*1000 $\dfrac{kg}{m^3}$ 1120

9. Viscosity determination

Table A-9 Viscosity determination

S.N	
Dynamic viscosity of the sample($\dfrac{kg}{m.s}$)	68.00
Kinematic viscosity($\dfrac{m^2}{s}$) $= \dfrac{\eta_d}{\rho_s}$	$\dfrac{68.00\ \frac{kg}{m.s}}{1120\ \frac{kg}{m^3}}$ $6.07*10^{-2}$

10. F- Critical determination

Table A-10 F- Critical determination

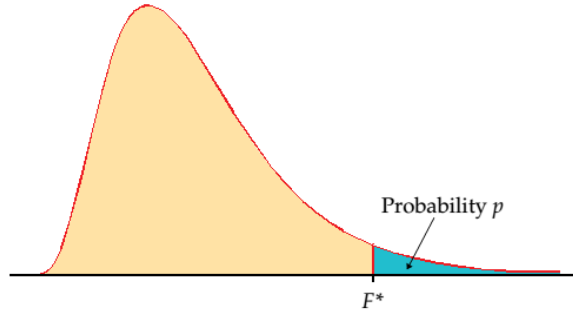Table entry for p is the critical value F* with probability p lying to its right.

Probability p

F*

TABLE E

F critical values

	p	Degrees of freedom in the numerator
		1	2	3	4	5	6	7	8	9
1	.100	39.86	49.50	53.59	55.83	57.24	58.20	58.91	59.44	59.86
	.050	161.45	199.50	215.71	224.58	230.16	233.99	236.77	238.88	240.54
	.025	647.79	799.50	864.16	899.58	921.85	937.11	948.22	956.66	963.28
	.010	4052.2	4999.5	5403.4	5624.6	5763.6	5859.0	5928.4	5981.1	6022.5
	.001	405284	500000	540379	562500	576405	585937	592873	598144	602284
2	.100	8.53	9.00	9.16	9.24	9.29	9.33	9.35	9.37	9.38
	.050	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38
	.025	38.51	39.00	39.17	39.25	39.30	39.33	39.36	39.37	39.39
	.010	98.50	99.00	99.17	99.25	99.30	99.33	99.36	99.37	99.39
	.001	998.50	999.00	999.17	999.25	999.30	999.33	999.36	999.37	999.39
3	.100	5.54	5.46	5.39	5.34	5.31	5.28	5.27	5.25	5.24
	.050	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81
	.025	17.44	16.04	15.44	15.10	14.88	14.73	14.62	14.54	14.47
	.010	34.12	30.82	29.46	28.71	28.24	27.91	27.67	27.49	27.35
	.001	167.03	148.50	141.11	137.10	134.58	132.85	131.58	130.62	129.86
4	.100	4.54	4.32	4.19	4.11	4.05	4.01	3.98	3.95	3.94
	.050	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00
	.025	12.22	10.65	9.98	9.60	9.36	9.20	9.07	8.98	8.90
	.010	21.20	18.00	16.69	15.98	15.52	15.21	14.98	14.80	14.66
	.001	74.14	61.25	56.18	53.44	51.71	50.53	49.66	49.00	48.47
5	.100	4.06	3.78	3.62	3.52	3.45	3.40	3.37	3.34	3.32
	.050	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77
	.025	10.01	8.43	7.76	7.39	7.15	6.98	6.85	6.76	6.68
	.010	16.26	13.27	12.06	11.39	10.97	10.67	10.46	10.29	10.16
	.001	47.18	37.12	33.20	31.09	29.75	28.83	28.16	27.65	27.24
6	.100	3.78	3.46	3.29	3.18	3.11	3.05	3.01	2.98	2.96
	.050	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10
	.025	8.81	7.26	6.60	6.23	5.99	5.82	5.70	5.60	5.52
	.010	13.75	10.92	9.78	9.15	8.75	8.47	8.26	8.10	7.98
	.001	35.51	27.00	23.70	21.92	20.80	20.03	19.46	19.03	18.69
7	.100	3.59	3.26	3.07	2.96	2.88	2.83	2.78	2.75	2.72
	.050	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68
	.025	8.07	6.54	5.89	5.52	5.29	5.12	4.99	4.90	4.82
	.010	12.25	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.72
	.001	29.25	21.69	18.77	17.20	16.21	15.52	15.02	14.63	14.33

Degrees of freedom in the denominator

Equations

In the bulk liquid

❖ **For substrate utilization**

$$\frac{dS_L}{dt} = \frac{F}{V_L}(S_O - S_L) - \frac{3V_L}{R_O^3 V_L} * \int_0^{Ro} r^2 \frac{\partial S_I}{\partial t} dr - \frac{3V_I}{R_O^3 V_L} * \int_0^{Ro} r^2 (m + \frac{\mu}{Y_{\frac{x}{s}}}) X_I dr \quad \text{(B-1)}$$

❖ **For product formation**

$$\frac{dP_L}{dt} = \frac{F}{V_L}(P_O - P_L) - \frac{3V_L}{R_O^3 V_L} * \int_0^{Ro} r^2 \frac{\partial P_I}{\partial t} dr - \frac{3V_I}{R_O^3 V_L} * Y_{\frac{P}{s}} \int_0^{Ro} r^2 (m + \frac{\mu}{Y_{\frac{x}{s}}}) X_I dr \quad \text{(B-2)}$$

Appendix B: Some important results generated from design expert software

Table B-1: Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	80.93	11	7.36	29.47	0.0008	
2FI	52.26	8	6.53	26.17	0.0011	
Quadratic	**2.94**	**5**	**0.5888**	**2.36**	**0.1840**	**Suggested**
Cubic	0.6402	1	0.6402	2.56	0.1702	Aliased
Pure Error	1.25	5	0.2496			

The selected model should have insignificant lack-of-fit
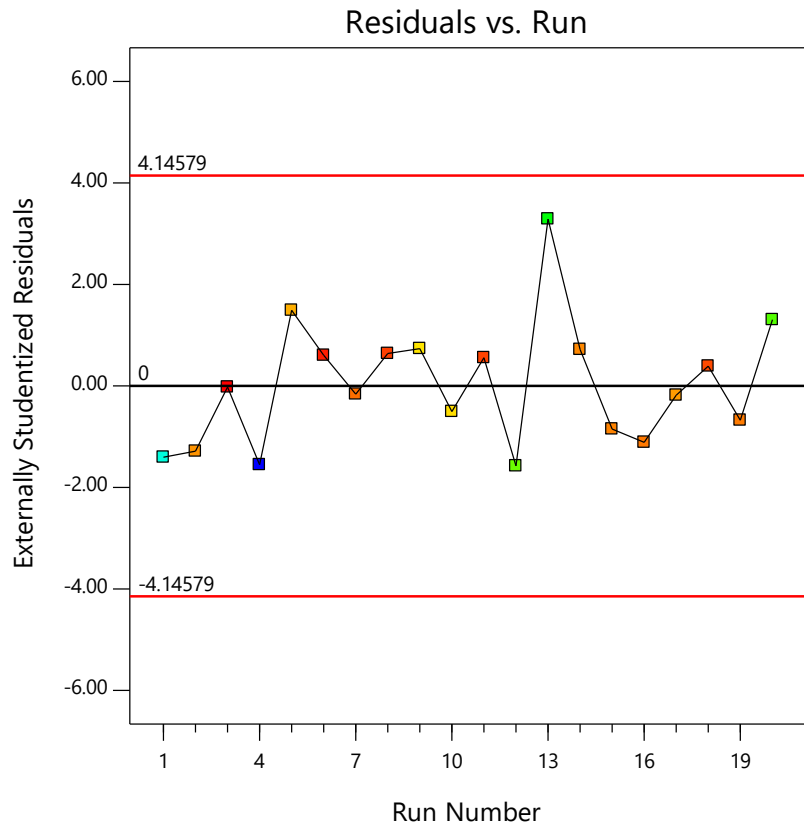
Figure B-1: Residual vs run
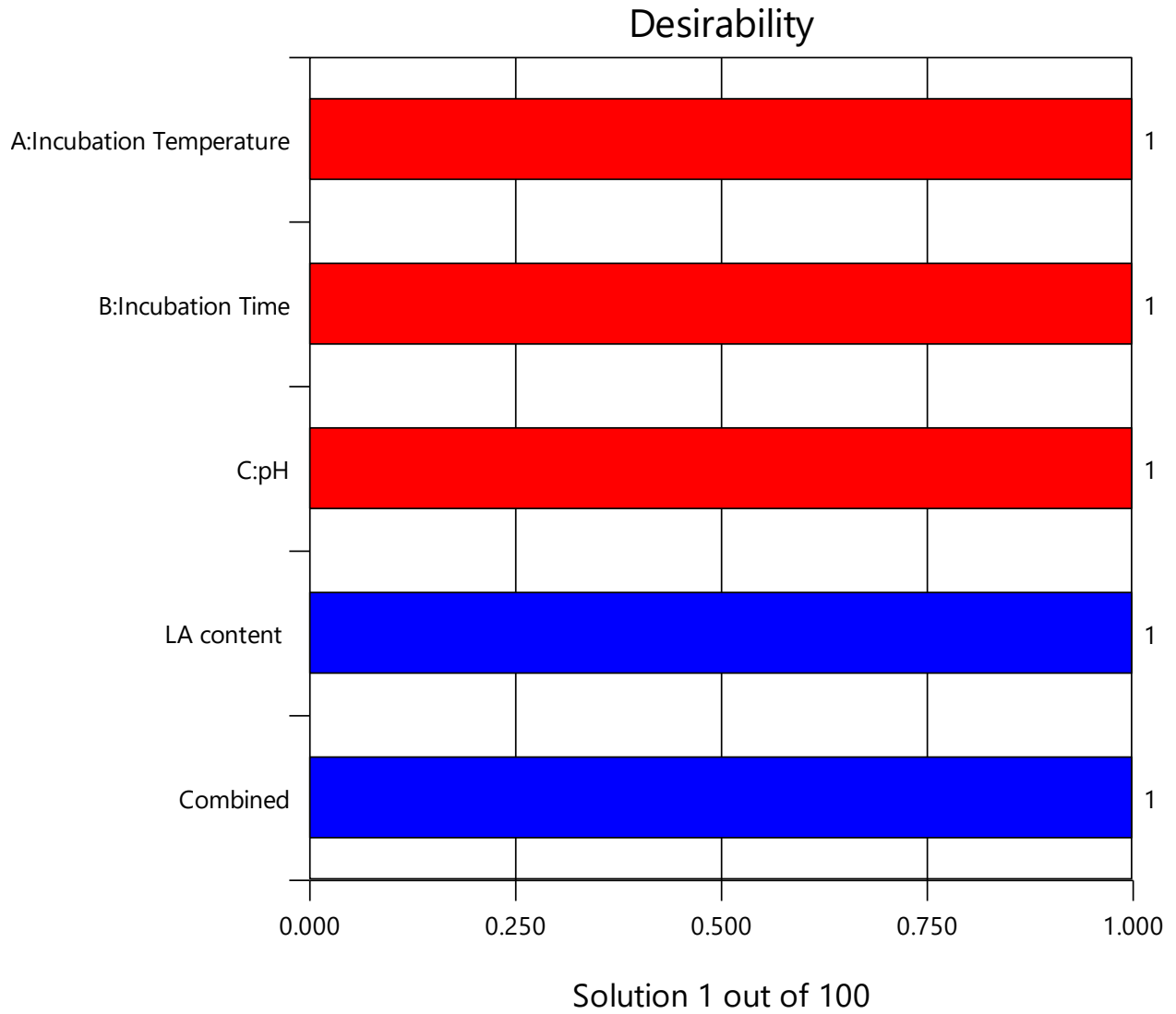
Figure B-2: Desirability

Figure B-3: Perturbation

Table B-2: Solutions for optimization

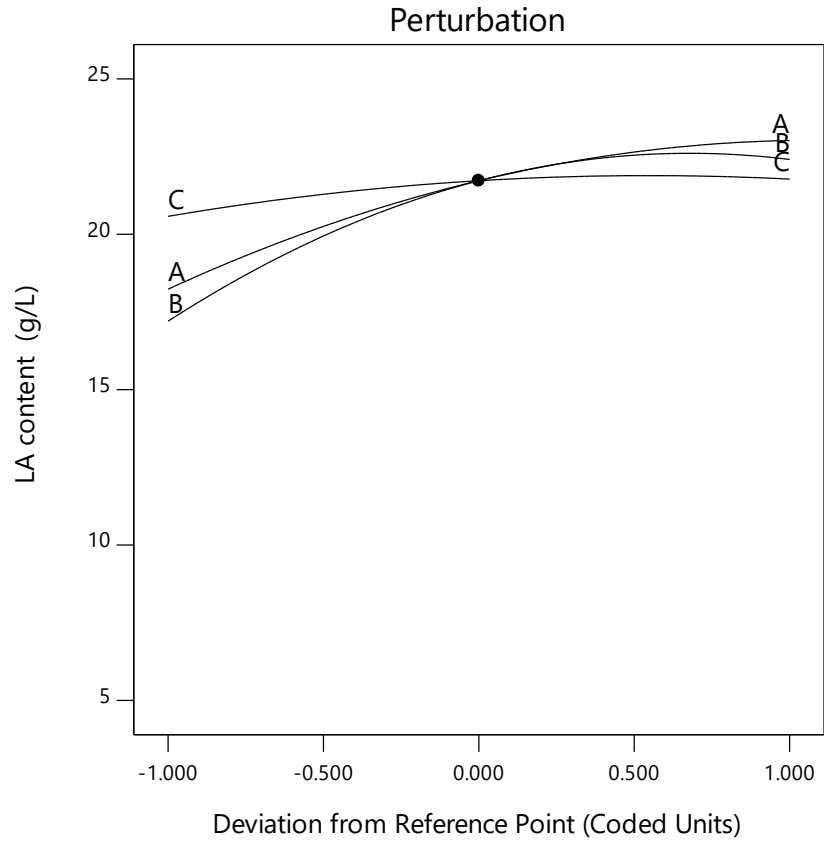| Number	Incubation Temperature	Incubation Time	pH	LA content	Desirability	
1	**42.270**	**40.013**	**6.179**	**23.052**	**1.000**	**Selected**
2	41.667	44.059	5.667	22.970	1.000	
3	44.952	30.279	6.734	23.000	1.000	
4	42.777	43.431	5.546	22.984	1.000	
5	41.394	37.252	6.494	22.962	1.000	
6	41.793	38.211	5.886	23.121	1.000	
7	43.018	33.331	6.625	23.024	1.000	
8	41.340	37.100	6.347	23.027	1.000	
9	41.608	43.139	5.418	22.970	1.000	
10	41.065	35.167	6.462	22.965	1.000	
11	43.004	38.228	5.325	22.976	1.000	
12	42.097	38.419	6.437	22.990	1.000	
13	42.266	32.075	6.543	23.002	1.000	
14	43.492	33.170	5.913	23.127	1.000	
15	41.664	40.916	5.504	23.032	1.000	
16	41.686	39.999	6.264	23.006	1.000	
17	44.089	34.281	6.313	23.155	1.000	
18	44.166	42.407	5.546	22.965	1.000	
19	42.146	35.893	6.088	23.134	1.000	
20	42.554	39.743	5.312	22.976	1.000	
21	44.186	40.779	6.135	22.967	1.000	
22	41.000	40.002	6.230	22.991	1.000	
23	41.610	43.313	5.451	22.975	1.000	
24	43.096	39.278	6.257	23.048	1.000	
25	41.228	34.810	6.086	23.036	1.000	
26	40.903	40.060	5.675	23.034	1.000	
27	42.563	40.137	6.057	23.087	1.000	
28	41.671	40.196	5.973	23.086	1.000	
29	42.376	37.949	5.681	23.114	1.000	
30	42.285	34.633	5.753	23.054	1.000	
31	44.906	38.027	5.450	23.020	1.000	
32	44.673	41.091	5.524	22.985	1.000	
33	41.413	40.155	6.118	23.045	1.000	
34	42.936	42.289	5.994	22.990	1.000	
35	41.796	39.229	6.091	23.093	1.000	
36	42.369	40.510	5.897	23.103	1.000	
37	44.757	34.748	5.630	23.086	1.000	
38	44.669	31.414	6.710	23.013	1.000	
39	41.937	39.239	6.069	23.102	1.000	
40	44.092	32.306	6.295	23.152	1.000	
41	42.952	35.517	5.674	23.094	1.000	
42	42.545	37.285	6.144	23.142	1.000	
43	44.809	28.985	6.763	22.976	1.000	
44	42.238	40.556	5.560	23.072	1.000	
45	42.174	42.672	5.994	22.985	1.000	
46	44.594	39.807	5.570	23.046	1.000	
47	40.538	38.450	6.290	22.968	1.000	

48	43.801	32.013	6.742	22.988	1.000
49	44.676	34.763	6.468	23.078	1.000
50	41.117	42.447	5.597	23.009	1.000
51	42.365	34.119	6.478	23.057	1.000
52	41.101	42.641	5.771	23.012	1.000
53	41.284	42.166	5.897	23.022	1.000
54	40.579	37.446	6.110	23.016	1.000
55	43.282	42.936	5.499	22.983	1.000
56	41.535	37.466	6.177	23.087	1.000
57	43.801	29.933	5.990	23.017	1.000
58	43.994	33.896	6.690	22.993	1.000
59	43.025	33.755	6.471	23.091	1.000
60	44.024	29.173	6.449	23.048	1.000
61	42.952	41.805	5.506	23.035	1.000
62	41.729	42.114	5.518	23.023	1.000
63	44.908	33.111	6.715	22.975	1.000
64	42.242	40.715	5.317	22.974	1.000
65	41.975	41.422	5.375	22.992	1.000
66	41.334	38.600	6.422	22.963	1.000
67	43.668	32.966	5.602	22.990	1.000
68	43.391	36.756	6.296	23.121	1.000
69	44.320	30.967	6.751	22.992	1.000
70	44.122	31.662	5.975	23.113	1.000
71	42.909	35.702	5.736	23.120	1.000
72	41.148	36.618	5.838	23.044	1.000
73	41.943	35.798	6.594	22.966	1.000
74	43.034	39.956	6.033	23.097	1.000
75	41.376	43.808	5.693	22.978	1.000
76	42.123	35.770	6.603	22.970	1.000
77	44.524	32.410	6.663	23.030	1.000
78	43.990	30.799	6.718	23.004	1.000
79	44.183	35.232	5.457	23.007	1.000
80	43.457	32.687	6.720	22.990	1.000
81	44.269	33.151	6.379	23.142	1.000
82	44.372	27.474	6.531	22.982	1.000
83	41.456	35.118	5.940	23.051	1.000
84	43.615	38.564	5.389	23.024	1.000
85	42.152	38.728	5.898	23.135	1.000
86	41.066	42.697	5.429	22.962	1.000
87	41.474	38.005	5.495	22.985	1.000
88	42.904	39.499	5.363	23.010	1.000
89	44.619	27.165	6.594	22.969	1.000
90	43.760	32.338	6.623	23.049	1.000
91	43.226	36.436	6.582	22.989	1.000
92	40.881	37.335	6.266	23.015	1.000
93	42.032	41.320	5.506	23.042	1.000
94	43.792	38.544	6.380	23.002	1.000
95	42.419	35.850	5.565	23.024	1.000
96	40.595	37.083	6.303	22.978	1.000
97	44.567	32.356	6.050	23.157	1.000
98	42.199	36.641	5.803	23.120	1.000

Appendix C: Some important experimental images

<center>(a)   (b)</center>
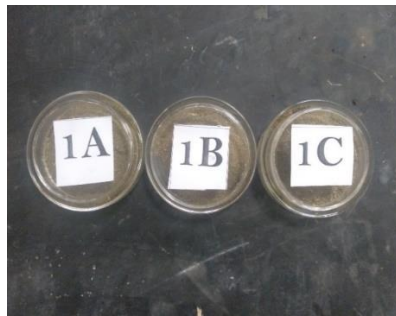
Figure C-1 Images of (a) khat waste collected and (b) grinding process

<center>(a)   (b)</center>

Figure C-2 images of "(a) sample taken for moisture content determination   (b) oven drying"

<center>(a)   (b)</center>

Figure C-3 Images of "(a) sample taken for ash content determination"   (b) furnace"

(a)
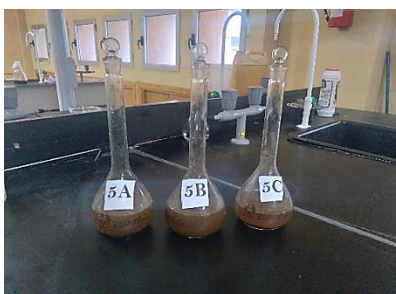

(b)   (c)

Figure C-4 Images of "(a) sample taken for hemicellulose content determination (b) Water bath (c) vacuum filtration"

(a)
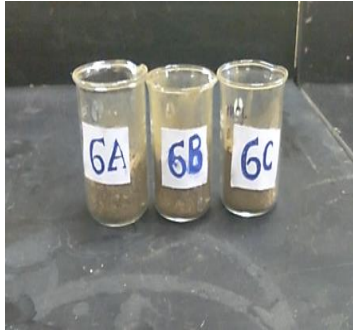

(b)   (c)

Figure C-5 Images of "(a) sample prepared for lignin content determination (b) hot plate (c) vacuum filtration"

(a)   (b)

Figure C-6 Images of (a) sample taken for extractive content determination (b) soxhlet extractor

(a)   (b)

Figure C-7 Images of "(a) the standard glucose solution prepared (b) UV spectrophotometer"

(a)   (b)

Figure C-8 Images of "(a) the sample taken from hydrolysis (b) UV spectrophotometer"

(a)   (b)

Figure C-9 Images of "(a) Lactobacillus plantarum cultured in MRS broth   (b) incubator"

(a)   (b)

(c)   (d)

Figure C-10 Images of **"**(a,b) samples prepared for fermentation (c,d) incubator"

(a)   (b)

Figure C-11 Images of **"**(a) samples prepared to be fermented (b) incubation process**"**

(a)   (b)

Figure C-12 Images of "(a) the standard LA solution prepared (b) UV spectrophotometer"

(a)   (b)

Figure C-13 Images of **"**(a) the sample prepared to be fermented at optimum condition   (b) Incubation process"

(a)

(b)

(c)

Figure C-14 Images of "(a) Esterification process (b) hydrolysis process, (c) LA product"

(a)

(b)

(c)

(d)

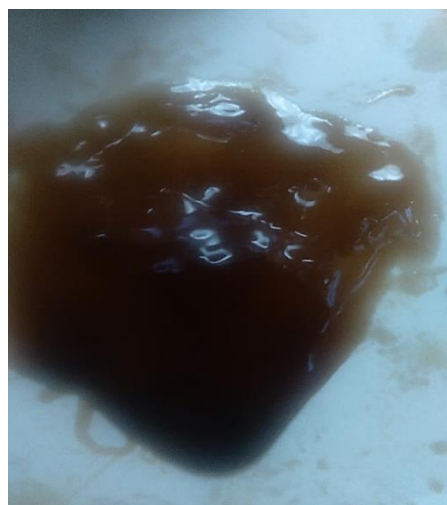**Figure C-15** Images of "(a) Evaporator (b) Hot plate with a stirrer (c, d) PLA product obtained"
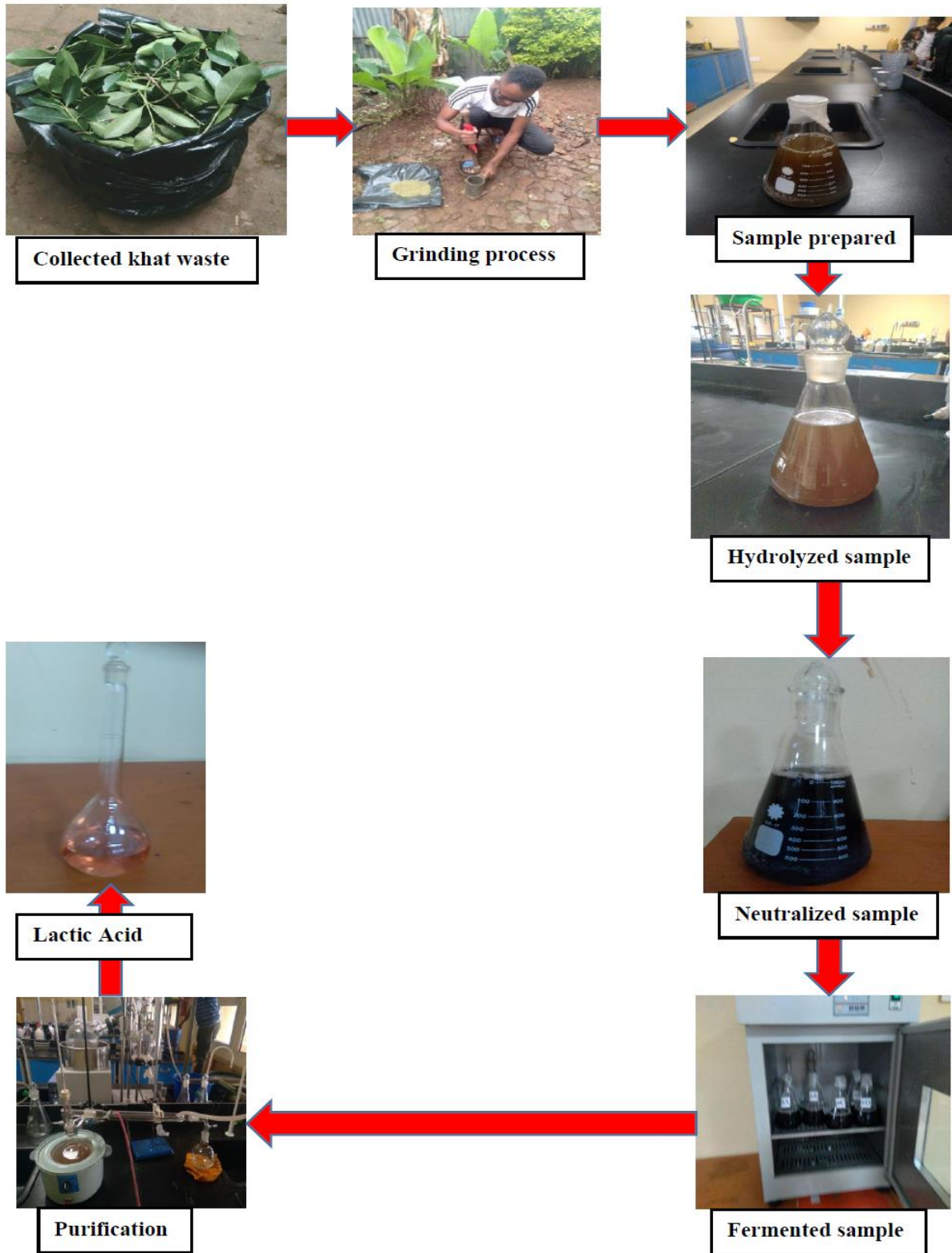
Figure C-16 Production process