Utilization of Duckweed as a Carbon Source for the Production of Lactic Acid

Endriss Ali Umer

A Thesis Submitted to

The School of Chemical and Bio-Engineering

Presented in Fulfillment of the Requirements for the Degree of Master of Science (Chemical and Bio-Engineering)

Addis Ababa University

Addis Ababa, Ethiopia

June, 2017

Addis Ababa University

Addis Ababa Institutes of Technology

School of Chemical and Bio Engineering

This is to certify that the thesis prepared by Endriss Ali entitled: ***Utilization of Duckweed as a Carbon Source for Production of Lactic Acid*** **and submitted in partial fulfillment of the requirements for the Dgree of Master of Science in Biochemical Engineering, complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.**

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Advisor	**Signature**	**Date**

ABSTRACT

Despite a great deal of research work on lactic acid fermentation in the past, the production of lactic acid from duckweed has yet to be investigated. In this study lactic acid was produced from duckweed thourgh existing technologies of saccharification/acid hydrolysis and fermentation of starch would been used for duckweed-to-lactic acid conversion. For this purpose, small fronds of duckweed plants was harvested, crushed and prepared into a solution. The duckweed slurry was subjected to acid hydrolysis. Then, fermentation of duckweed hydro-lysate was done in shake flasks using Lactobacillus plantarum species which was isolated from enset. The immediate solutions were anlysed by using UV-Spectrophotometer (concentration of lactic acid) and serial dilution methocd (viable cell count). Finally, the optimum operation conditions of fermentation (i.e. incubation time, pH and temperature) were determined by using design expert software (version 10.0.1). and the optimum incubation time, pH and temprature were 21 hours, 6.4 and 34 ^{0}C respectively. The amount of lactic acid produced and number of viable cells at optimum operating conditions of fermentations were 15.6574g/L and 4.34004E+008.

Keyword: *Duckweed, Lactic acid, Fermentation, Culture medium and Yield*

ACKNOWLEDGMENTS

I am highly grateful to the Almighty God for the strength that he gave me and helped me stay focused on my vision in spite of all the challenges. I would like to thank the following people and organization for their help throughout the completion of this thesis.

First and foremost we would like to thank our advisor Professor Eduardo Ojito, Dr. Solomon Benor, and Dr. Mesfin Tafesse for their advice, guidance, support and encouragement throughout my thesis work. My gratitude also extends to Dr. Eng. Abubeker Yimam, Ato Brook Tesfamichael, Ato Dagem Zekaryas, Ato Fantahun Abeje, Miss Hanan Endris, and Miss Hana Bisrat for their valuable advices and suggestions during laboratory work.

I also take this opportunity to express my overwhelming gratitude to my parents for their blessing and unstinted co-operation in the present venture. Generally I would like to thank all the people who contributed in some way to the work described in this thesis.

DECLARATION

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

Endriss Ali Umer **..**

Signature

Date and Place of Submission:

School of Chemical and Bio-engineering, Addis Ababa Institute of Technology

June, 2017

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LIST OF ABBREVIATIONS

Abbreviations	Abbreviated word	Abbreviations	Abbreviated word
ABS	Absorbance	**L**	Liter
CFU	Colony forming unit	**mm**	Millimeter
DM	Dry matter	**M**	Molarity
et al.	el alia (and others)	**ppm**	Part per million
MRS	De Man Rogosa and Shapes	**g**	Gram
°C	Degree Celsius	**h**	Hour
HPLC	High Performance Liquid Chromatography	**mL**	Milliliter
%(w/v)	Percentage weight by volume	**min**	Minute
rpm	Revolution per minute	**OD**	Optical density.
g/s	Gram per second	**cm^2**	Centimeter square
g/mol	Gram per molar	**h^{-1}**	Per hour
%	Percentage		

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WELCOME!!!

1. Introduction

1.1. Background

Lactic acid (IUPAC systematic name: 2-hydroxypropanoic acid), also known as milk acid, is a chemical compound that plays a role in several biochemical processes. it was firstly discovered in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component (Benninga, (1990)). In 1950, the first commercial production of synthetic lactic acid started in Japan (Auras et al., (2011)). Currently, it is the most widely utilized organic acid in the food, pharmaceutical, cosmetics and chemical industries. According to (Champomier-Verg et al, (2002))a great interest in biodegradable lactide polymers has accelerated recently.

Due to it's diverse application and sharp increases in global market, lactic acid production is currently attracting a great deal of research and development (Vijayakumar et al., (2008)). More specifically, the sharp increases in manufacturing of biodegradable polylactic acid (PLA) materials have significantly increased the global interest in lactic acid production (Nampoothiri et al, (2010)). According to the Ethiopian Investment Agency, the demand for lactic acid in 2012 was nine tons, and the demand for this product is projected to reach 16.83 tons and 28.36 tons by the year 2018 and 2023, respectively. Due to this fact and its critical importance and increased demand by various industries, the Ethiopian Investment Bureau has put lactic acid production among the hundred priority investment areas (Ethiopian Revenues and Customs Authority, (2005-2015)). Therefore, reduction of lactic acid importation cost could be achieved through the application of biotechnology and utilization of inexpensive local materials.

Lactic acid can be manufactured by either chemical synthesis or fermentation. Since, it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources, the biotechnological production of lactic acid has received a significant amount of interest recently (John et al., (2007)).

Biotechnologically, it can be produced using microorganisms. Microorganisms with a capability to produce lactic acid can be divided into two groups namely bacteria and fungi. Most investigations of lactic acid production were carried out with lactic acid bacteria, and filamentous fungi (Zhang et al., (2007)). Fungal fermentation has some advantages, including it requires a

simple medium and produces L (+)-lactic acid, and they have amylolytic enzyme activity, which enables them to convert starch directly to L (+)-lactic acid. But, lactic acid producing bacteria (LAB) have received wide interest because of their high growth rate and product yield (Litchfield, (1996)). In this case, nutrients are the essential substances required in it's fermentation process. Supplement of sufficient carbon, nitrogen, phosphate, sulfur and other salts is vital to maintain the growth of microorganisms and formation of LA products (Axelsson and Ahrné, (2000)). In addition the productivity and yeild of lactic acid production is influenced by numerous factors, such as fermentation time, temperature, pH and etc (Hofvendahl and Hahn-Hägerdal, (2000)).

The commercial production of lactic acid using fermentation technology mainly depends on the cost of raw material used (Gao et al., (2011)). Therefore, it is compulsory to select a raw material for its industrial production with a number of characteristics. Cheap raw materials are necessary for the feasible economic production of it because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low-cost. Raw materials for its production should not only be of low cost, but with low levels of contaminants and low toxic materials capable of being fermented with little or no pre-treatment resulting in rapid production rate and high yield with little or no by-product formation and be available throughout the year (Rojan et al., (2009)). Eventhough corn starch is commonly used carbon source, lignocellulose, another carbohydrate source, is also an interesting raw material for biotechnological processes, owing to its renewable character, widespread distribution, abundance and low price (Zhang et al., (2007)). According to (Xin-Zhao, (2014)) 3-75 % w/w DM of duckweeds were starch and this was comparable with 24.0%, 26.2%, 19.9% and 19.0% starch compositions of cassava root, wheat, potato, and maize respectively (Tonukari, (2004)) and (Parigi-Bini, (1976)).

More and more, the botanic and structural characteristics of lignocellulostic aquatic plants as distinct from terrestrial plants, can be potentially advantageous for biotechenological lactic acid production, such as, Duckweed do not compete with food crops for arable land; They commonly have higher productivity because of their fast proliferation; Duckweed could be cultured in waste water system for decontamination; In addition, abundant nutrients such as protein, vitamin and minerals generated from lactic acid production are potentially useful as feed for animals.

1.2. Statment of the Problem

In the recent decades biotechnological production of lactic acid has gained a prime position in the industries as it is cost effective and eco-friendly (Rojan et al., (2009)). However, the main problem of conventional biotechnological lactic acid production and potentially, one of the most serious obstacles for the future fermentative production of lactic acid to compete with chemical synthesis are its higher production cost due to their utilization of high cost medium and its potential impact on the human food chain due to their utilization of food crops. Becuase, agricultural products such as corn, potato, and wheat containing substantial amount of starch have been preferred as raw materials for lactic acid production.

Therefore, for economical lactic acid production by fermentation, low cost medium for it must be develop as soon as possible. In this case, lignocellulosic materials from agricultural, agro-industrial, and forestry sources represent a potentially inexpensive and renewable carbohydrate feedstock for the large-scale production by fermentation of due to their abundance, low price, high polysaccharide content, and renewability. For example, it is expected that duckweed which can be available in larg amount at lower costs (perhaps at zero cost) and riches in carbohydrate could be used as a suitable renewable resource in substitution of other agricultural products.

So, such assesments or searching of cheap raw materials are essential for the feasibility of the biotechnological production of lactic acid. Because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively low cost. The use of low-cost non-food materials for lactic acid production appears to be more attractive because they do not have any impact on the human food chain. In addition to that biotechnological production of lactic acid can keep it's prime position over chemical synthesis of lactic acid which is the main cause to enviromental pollution and eco-unfriendly. In this case, with out any doubt lignocellulosic materials from agricultural, agro-industrial, and forestry sources or others such as duckweed from waterpond represent a potentially inexpensive and renewable carbohydrate feedstock for the large-scale fermentation of lactic acid due to their abundance, low price, high polysaccharide content, and renewability.

1.3. Significant of the Study

The main significant of this study was to find cheap and non-food raw materials for the biotechnological production of lactic acid and to achive sustainable & feasible fermentation process of lactic acid production. If this null hypotesis is true, starts from the whole community until many polymer producers and other industrial users of lactic acid are all beneficiary. Since duckweed can be easily grew and harvested from the given water pond they have, the majority of the community can supply to the producer. Thus it is expected that duckweed which can be harvested constantly at lower costs and riches in carbohydrate could be used as a suitable renewable resource in substitution of other agricultural products in production of lactic acid. Then finally all respective industries can fullfil their lactic acid demand by a lower cost relatively.

1.4. Objectives

1.4.1. General objective:

- The overall goal of this study is to utilize duckweed as a carbon source for production of lactic acid.

1.4.2. Specific objectives

- To characterize chemical composition of duckweed
- To conduct necessary pretreatement on duckweed
- To formulate Modified MRS broth medium for lactic acid production
- To analyse the main and interaction effect of selected fermentation parameters on total amount of lactic acid, cell growth and productivity of the process
- To determine the optimum temperature, pH, and incubation time for the maximum lactic acid yield, cell growth and productivity from duckweed for different objectives of optimization

1.5. Research hypothesis

Starchs of duckweed can be used for lactic acid production with acceptable yield and productivity by lactobacillus plantarum at the optimum production conditions.

2. Litrature Review

2.1. Short History of Lactic Acid

Lactic acid was firstly discovered in sour milk in 1780 by a Swedish chemist, Carl Wilhelm Scheele, who initially considered it as a milk component. In 1789, Lavoisier named this milk component (acide lactique), which became the possible origin of the current terminology for lactic acid. However, it was in 1857 when Louis Pasteur discovered that it was not a milk component, but a fermentation metabolite generated by certain microorganisms (Wee et al., (2006)).

A further description of the history of lactic acid by Holten and Benninga shows that industrial production of lactic acid started in the United States in the 1880s. A very patented and applied a process of fermentation of vegetable sugars. The actual application was the use of a mixture of calcium lactate and lactic acid as baking powder. Unfortunately, this application was not a big success, but other applications in food and textile dyeing were developed (Auras et al., (2011)).

In 1950, the first commercial production of synthetic lactic acid started in Japan. Lactonitrile was produced from acetaldehyde and hydrogen cyanide and hydrolyzed in the second stage to lactic acid. For some decades, synthetic lactic acid completed with lactic acid obtained by fermentation, but currently almost all lactic acid is produced by fermentation (Auras et al., (2011)).

2.2. Properities of Lactic Acid

Lactic acid (IUPAC systematic name: 2-hydroxypropanoic acid), also known as milk acid, is a chemical compound that plays a role in several biochemical processes. It is a carboxylic acid with a chemical formula of $C_3H_6O_3$. It has a hydroxyl group adjacent to the carboxyl group, making it an alpha hydroxy acid (AHA). In solution, it can lose a proton from the acidic group, producing the lactate ion ($CH_3CH\ (OH)\ COO^-$). Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents (Narayanan et al., (2004)). Lactic acid exists as two optical isomers. One is known as L-(+)- lactic acid or (S)-lactic acid, and its mirror image is D-(-)-lactic acid or (R)-lactic acid. L-(+)-lactic acid is the biologically important isomer.

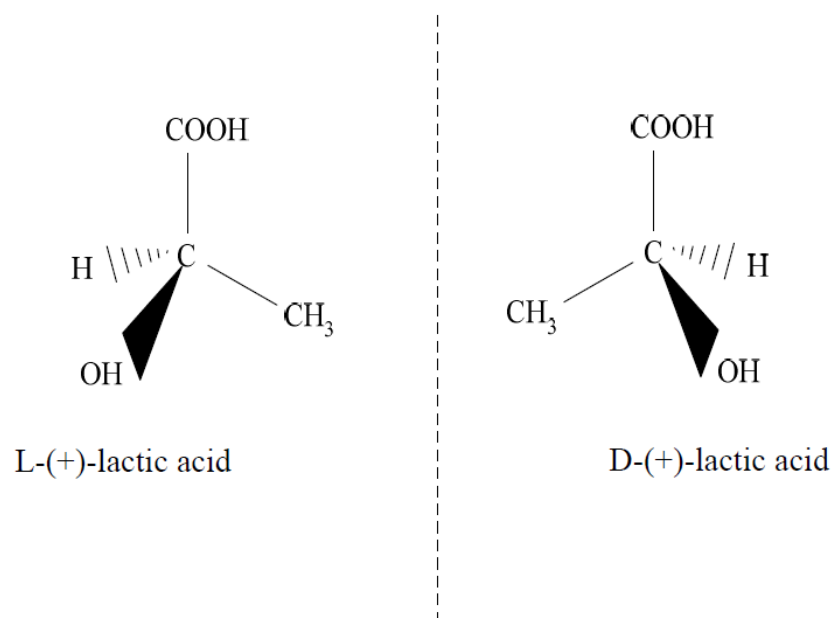


Fig. 1: Chemical structure of L-(+)-lactic acid and D-(-)-lactic acid.

Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents. It exhibits low volatility. Other properties of lactic acid are summarized in Table-1.

Table-1: Physiochemical properties of lactic acid.

Properties	**Value**
Empirical formula	$C_3H_6O_3$
Chemical name	2-hydroxypropanoic acid
Dissolution constant (k_a)	1.37×10^{-4}
Molecular weight	90.08 g/mol
Normal boiling point, °C	122 °C at 14 mmHg
Melting temperature, °C	L: 53 °C
	D: 53 °C
	D/L: 16.8 °C
Density, g/ml	1.1

[Source: (Datta et al, (1995))]

2.3. Application of Lactic Acid

Although lactic acid is most well-known for its alleged role in muscle fatigue, it has far more diverse applications in the commercial sector and is produced on a mass scale and used in several different sectors (American Research Council, (1992)). For example, lactic acid is used as a humectant, or moisturizer, in some cosmetics and as a mordant, a chemical that helps fabrics accept dyes, in textiles. It is also used in making pickles and sauerkraut, foods for which a sour taste is desired. Lactic acid is used in the dairy industry not only in making yogurt but in making cheese as well. It is also used in tanning leather. Lactic acid is important in the pharmaceutical industry as a starting material for other substances and is involved in the manufacturing of lacquers and inks. A related compound that is made from lactic acid is calcium stearoyl-2-lactylate, which is used as a food preservative (Roberts, (1990)).

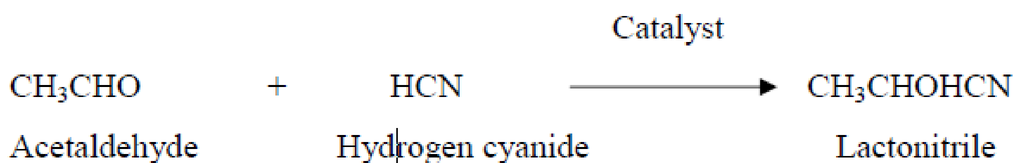
Recently, a great interest in biodegradable lactide polymers has accelerated researches on the production of pure L (+) - or D (-)-lactic acid as bulk raw material, and efforts have been made to enhance the productivity and economy of the L (+) - or D (-)-lactic acid production processes (Champomier-Verg et al, (2002)). The optically pure lactic acid can be polymerized into a high molecular mass poly-lactic acid (PLA) through the serial reactions of polymerization, de-polymerization, and ring opening polymerization (Vijayakumar et al., (2008)). Two molecules of lactic acid can be dehydrated to yield lactide, a cyclic lactone. A variety of catalysts can polymerize lactide to produce either heterotactic or syndiotactic polylactide, biodegradable polyesters with valuable (inter alia) medical properties, which are currently attracting much attention. These polymers are transparent and their degradation can be controlled by adjusting the composition, and molecular weight (Datta, (1995)).

2.4. Lactic Acid Production

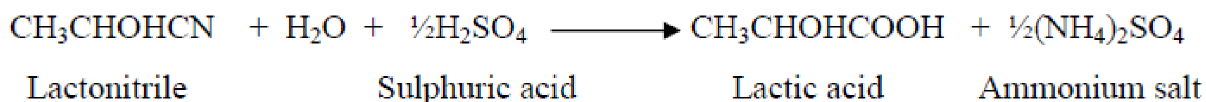
2.4.1. Lactic Acid Production by Chemical Reaction

Lactic acid can be manufactured by either chemical synthesis or fermentation. The commercial process for chemical synthesis is based on lactonitrile. Hydrogen cyanide is added to acetaldehyde in the presence of a base to produce lactonitrile. This reaction occurs in liquid phase at high atmospheric pressures (Boontawan, (2010)). The crude lactonitrile is recovered, and purified by distillation. It is then hydrolyzed to lactic acid, either by concentrated HCl or by H_2SO_4 to produce the corresponding ammonium salt and lactic acid. Lactic acid is then esterified with methanol to produce methyl lactate before being purified by the means of distillation, and is hydrolyzed by water under acid catalyst to produce lactic acid and methanol. The chemical synthesis method produces a racemic mixture DL-lactic acid. This process is represented by the following reactions (Narayanan et al., (2004)).

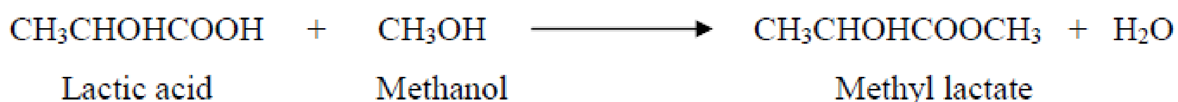
(a) Addition of Hydrogen Cyanide



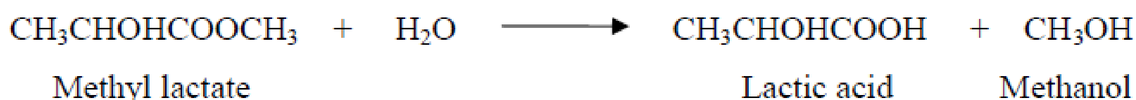
(b) Hydrolysis by H_2SO_4



(c) Esterification



(d) Hydrolysis by H_2O



2.4.2. Lactic Acid Production by Fermentation Processes

Fermentation is an energy yielding process in which organic molecules play role as both electron donors and electron acceptors. The molecule which is metabolized does not possess its whole potential energy extracted from it. Therefore, lactic acid bacteria are widely used as a cheap method for food maintenance by fermentation and usually no or little heat is required in fermentation. In batch fermentation process the culture is first grown in a series of inoculums vessels and after that transferred to the fermentor. The size of inoculum is usually 5-10% of the liquid volume in this fermentor. The fermentation is usually kept at 35-45 ^{0}C and at pH 5-6.5 by adding a suitable base, such as ammonium hydroxide. Other fermentations for lactic acid production are, fed-batch, repeated batch, and continuous batch. But the higher concentration of lactic acid has achieved in batch and fed-batch cultures than in others, whereas higher productivity has obtained by continuous cultures. Another advantage of the continuous batch over batch culture is that the process can be run for a long period of time. L (+) lactic acid is produced commercially in fermentation processes by lactic acid bacteria or fungi such as Rhizopus oryzae in submerged culture. Rhizopus sp. Can manufacture L (+) lactic acid from starch but the yield is very low as compared to lactic acid bacteria. 85% yield of L (+) lactic acid can be achieved using an airlift bioreactor under optimal conditions. The mycelia are not suitable enough for lactic acid as their morphology does not suit for fermentation because they increase the viscosity of the medium. They wrap up around the impellers and cause obstruction during sampling and in overflow lines. Small pellets of mycelia of R. oryzae are produced regulating the concentration of inoculated spores in pre culture. But there is a problem with pellets that they have insufficient mass transfer. Cotton like flock morphology can be obtained by mineral supports (Ghaffar et al., (2014)).

In general, an optically pure L (+) - or D (–)-lactic acid can be obtained by microbial fermentation when the appropriate microorganism is selected. The optical purity of lactic acid is crucial to the physical properties of poly (lactic acid), and an optically pure L (+) - or D (–)-lactic acid, rather than racemic DL-lactic acid. Lactic acid can be polymerized to produce a high crystalline PLA that is suitable for commercial uses. As a result, the biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of

petrochemical resources (Han and Pan, (2009)). An Overview of both production methods is given in the **Figure-2.**

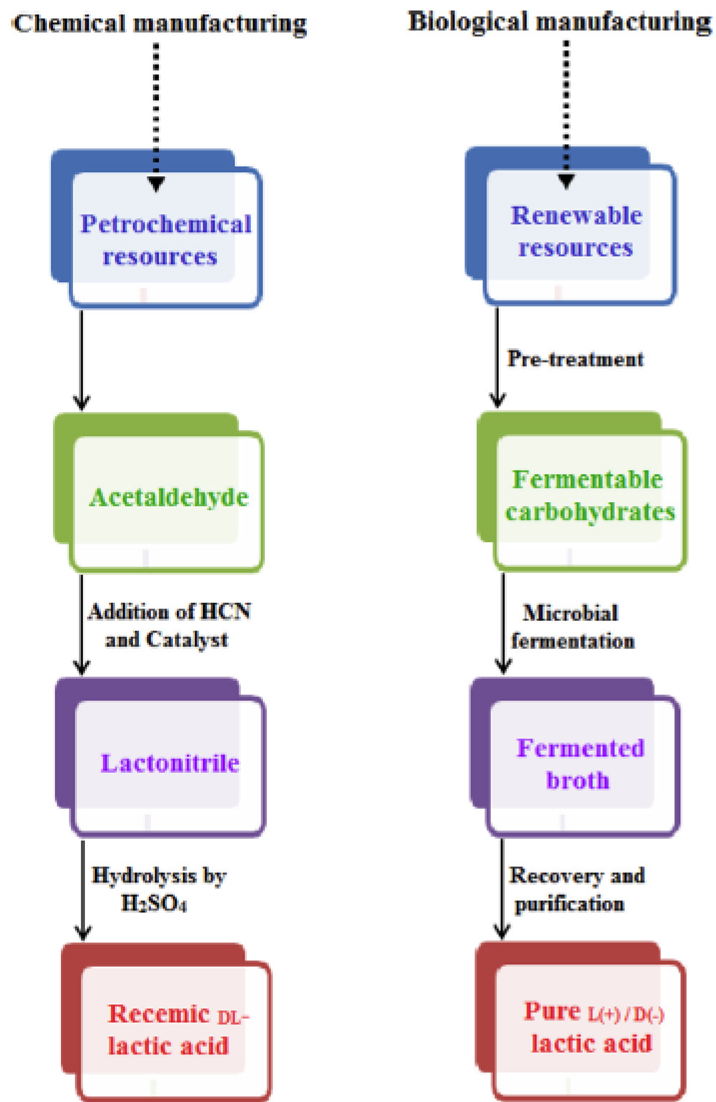


Fig. 2: A schematic representations of the two manufacturing processes of lactic acid.

2.5. Microbial Sources for Lactic Acid Fermentation

Microorganisms with a capability to produce lactic acid can be divided into two groups namely bacteria and fungi. Most investigations of lactic acid production were carried out with lactic acid bacteria, and filamentous fungi. Fungal fermentation has some advantages, including it requires a simple medium and produces L (+)-lactic acid, and they have amylolytic enzyme activity, which enables them to convert starch directly to L (+)-lactic acid. However, in many cases fungal fermentation showed low production rate, below 3.0 g/L/h, is probably due to the low reaction rate caused by mass transfer limitation of substrate. The lower product yield from fungal fermentation is partially attributed to the formation of by-products, such as fumaric acid and ethanol (Wee et al., (2006)).

Although there have been persistent attempts to produce lactic acid through fungal fermentation, LAB have been commonly used for the production of lactic acid due to the aforementioned disadvantages of fungal fermentation. Moreover, lactic acid bacteria were performed the desirable activities which they promote (Vijayakumar et al., (2008)).

Lactic acid bacteria are generally gram-positive bacteria. They are non-motile, have non-spore-forming rods and cocci. They do not synthesize porphyrins and cytochromes there for why they cannot generate ATP (Champomier-Verg, (2002)). Lactic acid bacteria grow under anaerobic conditions as they do not use oxygen for their energy manufacture, but they are also capable of growing in the presence of oxygen. They remain protected from byproducts of oxygen (e.g. H_2O_2) due to peroxidases they have and are aero tolerant anaerobes. They are distinguished from other organisms because of their capability to ferment hexoses to lactic acid. They are divided generally in two categories according to their fermentation patterns, homo fermentative and hetero fermentative bacteria (Ghaffar et al., (2014)).

The choice of an organism primarily depends on the carbohydrate to be fermented. Lactobacillus delbreuckii subspecies delbreuckii are able to ferment sucrose. Lactobacillus delbreuckii subspecies bulgaricus is able to use lactose. Lactobacillus helveticus is able to use both lactose and galactose. Lactobacillus amylophylus and Lactobacillus amylovirus are able to ferment starch. Lactobacillus lactis can ferment glucose, sucrose and galactose. Lactobacillus pentosus have been used to ferment sulfite waste liquor (Narayanan et al., (2004)).

2.6. Nutrientional Requirement for Lactic Acid Fermentation

All lactic acid bacteria require a source of nutrients for metabolism. Generally, lactobacillus has complex nutritional requirements (Ghaffar et al., (2014)). The fermentative bacteria require carbohydrates, either simple sugar such as glucose and fructose or complex carbohydrates such as starch or cellulose. The energy requirements of lactic acid bacteria are very high (Antonio, (2000)). Limiting amount of available substrate can stop their growth. It is necessary to supplement the fermentation media with sufficient nutrients for rapid lactic acid production. If small amounts of other nutrients were supplemented to the process, then the efficiencies of lactic acid fermentation would be improved significantly (Boontawan, (2010)).

Table-2: Nutritional requirement of lactic acid bacteria

Composition*		
No.	**Ingredients**	**Gms / Litre**
1	Proteose peptone	10.000
2	Beef extract	10.000
3	Yeast extract	5.000
4	**Dextrose**	**20.000**
5	Polysorbate 80	1.000
6	Ammonium citrate	2.000
7	Sodium acetate	5.000
8	Magnesium sulphate	0.100
9	Manganese sulphate	0.050
10	Dipotassium phosphate	2.000

[Source: (HiMedia Laboratories Pvt. Ltd. Technical Data, (2015))**]**

2.6.1. Commonly Kown Material as a Carbon sources

The commercial production of lactic acid using fermentation technology mainly depends on the cost of raw material used. Therefore, it is compulsory to select a raw material for industrial production of lactic acid with a number of characteristics. Cheap raw materials are necessary for the feasible economic production of lactic acid because polymer producers and other industrial users usually require large quantities of lactic acid at a relatively lowcost. Raw materials for lactic acid production should not only be of low cost, but with low levels of contaminants and low toxic materials capable of being fermented with little or no pre-treatment resulting in rapid production rate and high yield with little or no by-product formation and be available through out the year (Rojan et al., (2009)).

Renewable raw materials, including starch and lignocellulose) can be used as a substrate for fermentation of lactic acid. Starch is a type of polysaccharide that mainly exists in tubers including potatoes and cassava, and seeds of grains such as wheat, corn and rice. However, corn starch is usually subjected for enzymatic hydrolysis prior to fermentation. Lignocellulose, another carbohydrate source, is also an interesting raw material for biotechnological processes, owing to its renewable character, widespread distribution, abundance and low price (Zhang et al., (2007)).

Lignocellulose mainly consists of cellulose, hemicellulose and lignin. Cellulose is a linear **β**-D-glucan, whereas hemicellulose is a heteropolysaccharide and the hydrolysate includes xylose, glucose, mannose, arabinose, galactose and traces of other sugars, depending on the source of lignocellulose. Lignin is a polymer of three closely-related phenylpropane moieties. Due to its low bio accessibility, a number of chemical and physical methods, such as acid or base hydrolysis and steam explosion, have been developed and used to hydrolyze lignocellulytic materials to oligosaccharide, prior to fermentation by lactic acid producing bacteria. Biological and biochemical pretreatment methods for conversion of cellulose materials into sugars appear to be attractive alternatives from both economical and environmental viewpoints (Zhang et al., (2007)).

The following table indicated that different materials have different amount of starch to utilize as a carbon source.

Table-3: Chemical composition of different starch sources

Chemical Composition (% dry basis)	Starch sources			
	Cassava root	Wheat (whole grains)	Potato (whole grains)	Maize (whole grains)
Moisture	70.0	68.3	75.8	75.0
Starch	24.0	26.2	19.9	19.0
Fiber	2.0	2.0	1.1	2.7
Protein	1.0	1.2	1.8	3.2
Other substances	3.0	2.3	1.4	0.1

Source: (Tonukari, (2004)) & (Parigi-Bini, (1976)).

2.6.2. Duckweed as an Alternative Carbon Source

Duckweed is tiny aquatic flowering plant that floats in large quantities on still water, often forming an apparently continuous green layer on the surface. It's species are the smallest of all flowering plants (Gandhi et al., (2000)). They are monocotyledons belonging to the botanical family Lemnaceae and are classified as higher plants or macrophytes, although they are often mistaken for algae. The family consists of four genera, Lemna, Spirodela, Wolffia, and Wolffiella among which about 40 species have been identified so far (Skillicorn et al., (1993)). Duckweed is one of the world's most abundant plants and contains the smallest higher plants, having tiny leaf-like fronds, flat growth habit, and vegetative reproduction (Cummingsand Englyst, (1988)).

Fig. 3: Sizes of three species of duckweed (Lemna minuta < Lemna minor < Spirodela)

Some research has investigated the chemical compounds present in duckweed (See **Table-4**). In many cases, duckweed has high proportions of protein and carbohydrate (29.1 – 45% by DM of protein and 14.1 - 43.6% by DM of carbohdrate). The relatively high proportion of protein suggests use as animal feed, and duckweed is used for this purpose (Xin-Zhao, (2014)). In addition, starch can be a very significant component of duckweed; the level is variable, 3 - 75 % by DM, and it has been used as a carbon source for biotechenological production of lactic acid.

2.6.2.1. Chemical Composition of Duckweed

Duckweed has high carbon content and high productivity but the crude protein content of duckweeds depend mainly on the N content of the water upon which they grow, and there are also some variations in amino acid content of duckweed proteins. The starch amount of duckweed also can be altered by control by provide limited amount of nutrient in to the pond in which they are growing. Moreover, duckweed has less cell wall materials than other aquatic plants. The crude protein content of duckweed can be as high as 39.3 % in dry basis and it is rich in carbohydrates. (Emmanuel-Nuamah, (2016)).

Table-4: The chemical compounds of duckweed

Parameters	**Chemical Composition**
Dry Matter (% of wet mass)	4.5 - 4.7
Crude fiber (% of DM)	11.0 - 18.1
Crude protein (% of DM)	29.1 - 45
Ash (% of DM)	15.9 - 19
Carbohydrate (% of DM)	14.1 - 43.6
Starch (% of DM)	3 - 75
Cellulose (% of DM)	NA
Hemicellulose (% of DM)	NA
Lignin (% of DM)	NA

[Source: (Xin-Zhao, (2014))**]** {NA means not reported.}

2.6.2.2. Advantages of Utilization of Duckweed as Alternative Carbon Source

The botanic and structural characteristics of aquatic plants as distinct from terrestrial plants, can be potentially advantageous for biotechenological lactic acid production, such as,

i. Duckweed do not compete with food crops for arable land;
ii. They commonly have higher productivity because of their fast proliferation;
iii. Duckweed could be cultured in waste water system for decontamination;
iv. In addition, abundant nutrients such as protein, vitamin and minerals generated from lactic acid production are potentially useful as feed for animals

2.7. Lactic Acid Fermentation Processes

Lactic acid fermentations are generally inexpensive, and often little or no heat is required in their preparation, making them fuel efficient as well. Lactic acid bacteria are generally fastidious on artificial media, but they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow. Leuconostocs and lactic streptococci generally lower the pH to about 4.0 to 4.5, and some of the lactobacilli and pediococci to about pH 3.5, before inhibiting their own growth (American Research Council, (1992)).

Lactic acid fermentation is known to be end product inhibited fermentation by an un-dissociated form of lactic acid. Several studies have been carried out to overcome this problem. It has found that using extractive lactic acid fermentation technique could give a lactic acid yield of 0.99g/l and lactic acid productivity of 1.67 g/l/h over a conventional batch reactor which gave a yield of 0.83 g/l and lactic acid productivity of 0.31 g/l/h (Srivastava et al., (1992)). Using an air-lift bioreactor under optimum conditions could produce L (+) lactic acid with a yield of 85%. A high productivity can be obtained using membrane recycle reactor, but it has a potential drawback of fouling. At high cell densities the cells are put under stress and start producing the D-isomer of the product. Ingeneral, the two reactor systems results in high yields and productivities of lactic acid: - a continuous cell recycle fermentation process (**Figure- 4**) and a fed batch fermentation (**Figure- 5**). (Narayanan et al., (2004)). However, lactic acid production in batch fermentation is widely studies in batch fermentation by lactic acid bacteria (Boontawan, (2010)).

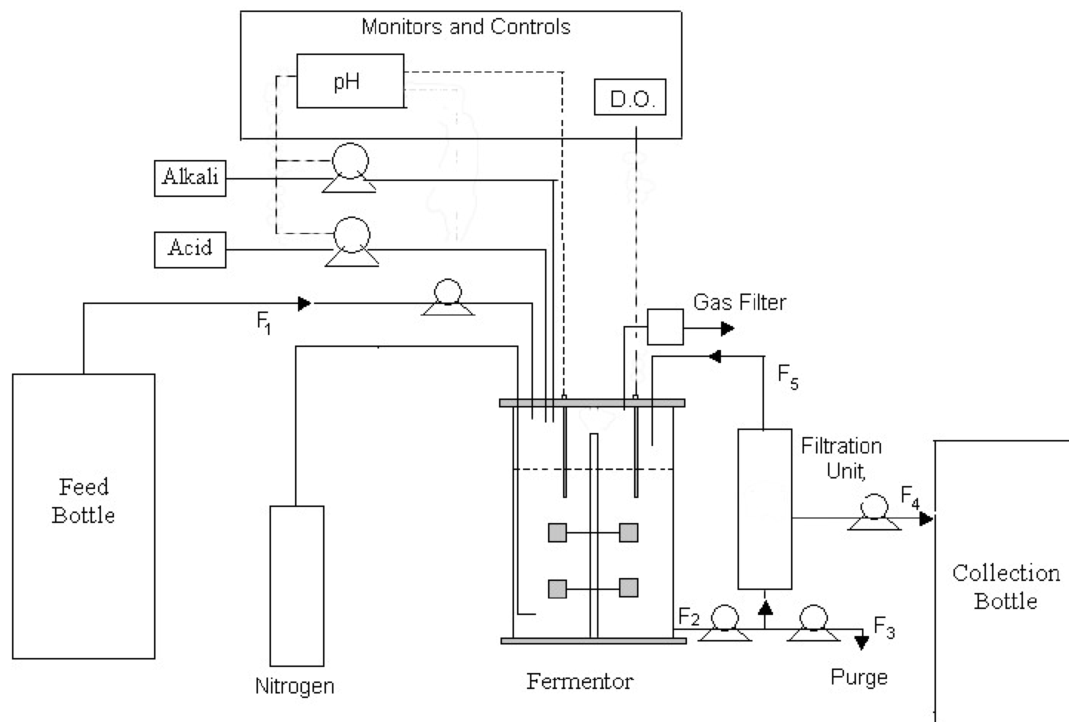


Fig. 4: Sketch of the fermentor is shown with all the connection for monitoring and control of the continuous lactic acid fermentation process with continuous cell recycling.

- F_1: feed rate (the dilution rate was maintained by maintaining the feed rate);
- F_2: rate at which broth was taken out of the fermentor;
- F_3: rate of purging (the sample from the purge were regularly analyzed for biomass and metabolites concentrations inside the fermentor);
- F_4: rate at which permeate was taken out from the filtration unit;
- F_5: rate at which biomass was recycled back to the fermentor.

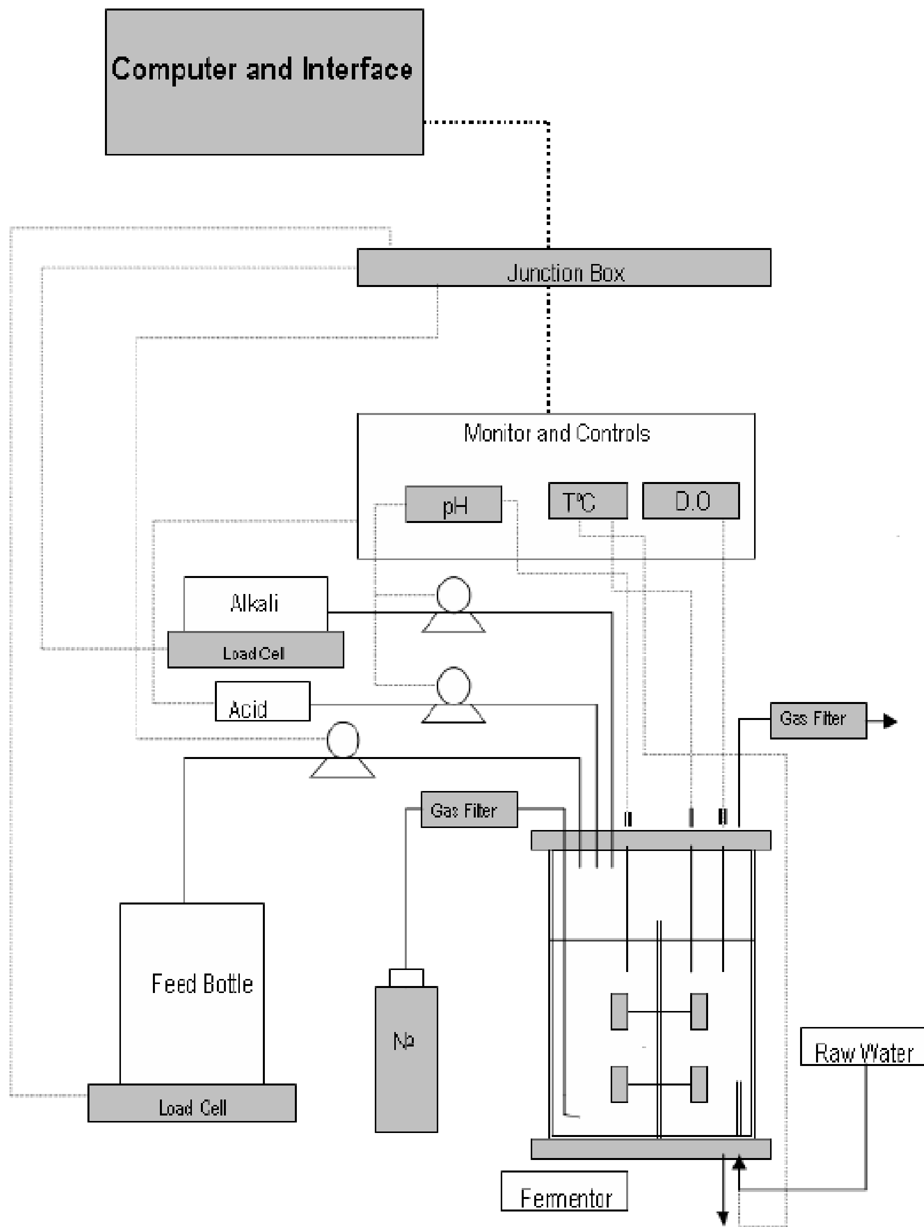


Fig. 5: Schematic of lactic acid fermentation in fed-batch fermenter

2.8. Factors Affecting on Lactic Acid Fermentation

The productivity and yeild of lactic acid production is influenced by numerous factors of which grape productivity is one of the most important factors. The production of lactic acid with desired objectives, however, is not possible without good fermentatin parameter knowledge and effective quality control. Critical control points (CCP) during the lactic acid production process must be identified to ensure optimum lactic acid production. Eventhough, many factors are responsible for good or bad lactic acid fermentation process, the effect of temprature, pH, and incubation time are the most dominant one (Hofvendahl and Hahn-Hägerdal, (2000)).

2.8.1. Effect of Temperature

Temperature and pH are the key environmental parameters that affect the fermentation process. Low temperature has been reported to positively influence the outgrowth of contaminating microorganism. The temperature giving the highest productivity lower than the temperature resulting in highest lactic acid mass concentration and yield. For Lactobacillus amylophilus, which is known to grow at 15 °C but not at 45 °C, the optimal temperatures were 25 °C and 35 °C for maximum productivity and yield, respectively (Boontawan, (2010)).

2.8.2. Effect of pH

The fermentation pH is either set at the beginning and then left to decrease due to acid production or it is controlled by an addition of alkaline solutions. The optimal pH for lactic acid production varies between 5.0 and 7.0. A pH below 5.7 was optimal for Lactobacillus strains, which are known to tolerate lower pH than lactococci. The previous studies investigated the influence of culture pH on lactic acid fermentation from molasses where lactic acid fermentations were performed on a jar fermentor at 38 °C and pH 5.0-9.0. Although the optimum pH for cell growth of Enterococcus faecalis RKY1 was seen to be 8.0, the lactic acid fermentation at pH 7.0 was completed faster than that at pH 8.0. The cell growth at pH 5.0 almost ceased after 10 h of fermentation, the highest lactic acid mass concentration was obtained at pH 7.0 with a comparable yield with pH 6.0 (Wee et al., (2006)).

Moreover, some reported showed the effect of various initial pH on the lactic acid production of the immobilized Lactobacillus delbrueckii during the batch fermentation of liquid pineapple waste. At initial pH 6.5, cell started to utilize glucose earlier and at a faster rate than at other initial pH. Maximum lactic acid concentration was attained at initial pH 6.5. Further increase in initial pH beyond 6.5 does not improve the lactic acid production. It is possible that the higher initial pH brought too much stress on the microorganism metabolic abilities (Vijayakumar et al., (2008)).

2.8.3. Effect of Incubation Period

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

2.9. Purification of Lactic Acid

A number of approaches can be used for separation of lactate salt from fermented medium including extraction by organic solvents, ion-exchange separation, adsorption, vacuum distillation, and membrane separations. Each of these exhibits some advantages and disadvantages. The choice of the separation process should be based on the efficient and economically usage of these extractants. Technological advancements in the major process components-fermentation, primary and secondary purification, polymerization, chemical conversion of lactic acid and its derivatives would enable low cost large volume and environment friendly production of lactic acid (Boontawan, (2010)). Recent advancements in membrane based separation and purification would enable lactic acid production without producing salt or gypsum by products (Narayanan et al., (2004)).

The recovery of lactic acid must be improved in order to reduce lactic acid losses and to increase purity. Purification or product recovery is an important step in production of lactic acid that is associated with separation and purification of lactic acid form fermentation broth. Fermentation broth contains a number of impurities such as residual sugars, color, nutrients and other organic acids, as part of cell mass. These impurities must be removed from the broth in order to achieve more pure lactic acid. To recover and purify the L (+) - lactic acid produced from the microbial fermentation media economically and efficiently, ion exchange chromatography is used among the variety of downstream operations. It is extremely selective and gives product recovery at very low cost within a short period of time. The other purposes were to analyze the end product purity, to check adsorption or desorption behaviors of lactic acid and to examine the applicability of this method for industrial usage (Narayanan et al., (2004)).

Process strengthening and monomer grade lactic acid has been achieved in high purity by advancement of a new membrane-integrated technology. It has lesser the processing steps, chemical requirement and energy expenditure. The fastidious modular design provides a great elasticity in action of the system which the modern industrialized sector is looking for dreadfully in this era of shrunken profit edge. Most of the time maximum removal of impurities and lactic acid recovery has been achieved at maximum pressures of trans-membrane (Ghaffar et al., (2014)).

In general, lactic acid can be separated and substantially purified from fermentation broths by several membrane-based unit operations as shown in the **Figure-6.**

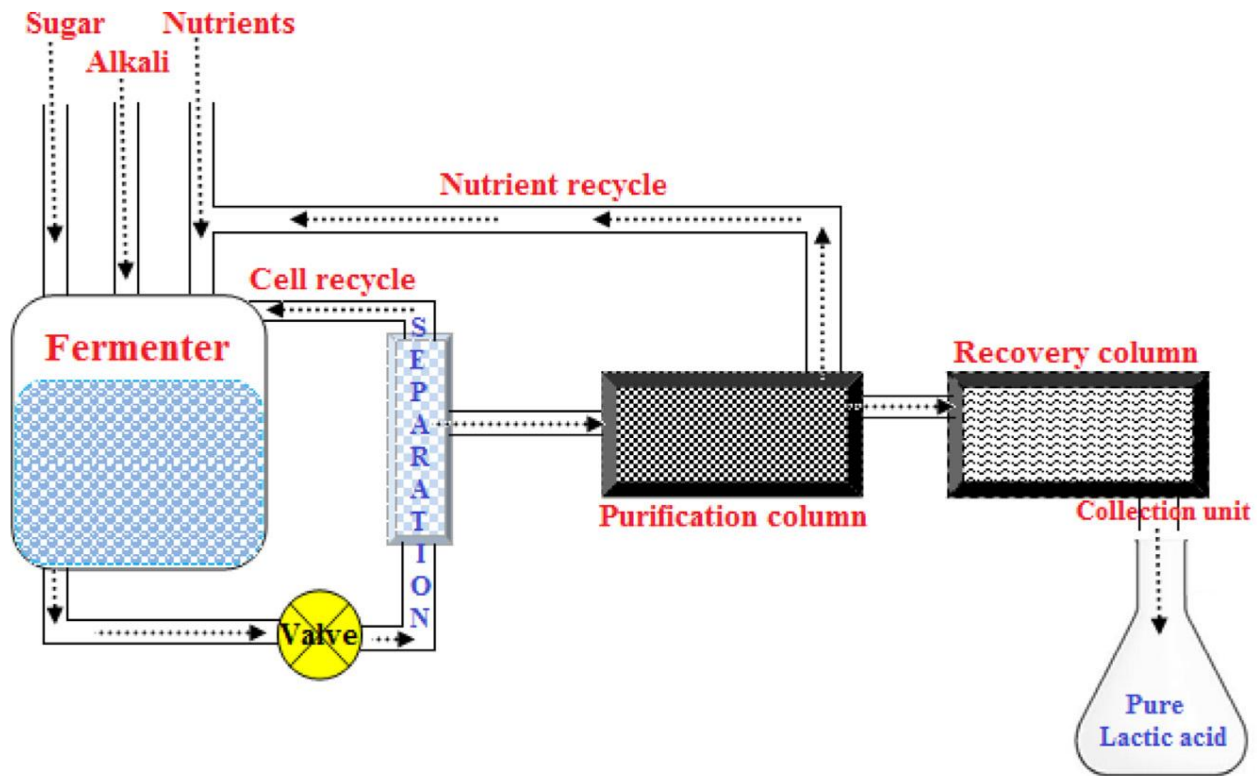


Fig. 6: Substantial purification of lactic acid from fermentation broths by several membrane-based unit operations (columns).

3. Materials and Methods

3.1. Experiment Location, Materials, Chemicals and Reagents

3.1.1. Experiment Location

The preparation of duckweeds were partially conducted in Adiss Ababa Science and Technology University (AASTU). Experiment on the characterization of duckweeds (such as moisture content, total carbon content and starch content), hydrolysis of duckweed, Fermentation process and characterization of lactic acid were conducted in the Food engineering, Enviromental engineering, Analytical, Chemistry, Reaction, Size reduction, and Biochemical engineering laboratories in the School of Chemical and Bio Engineering of Addis Ababa Institute of Technology (AAiT), Addis Ababa University.

3.1.2. Sample Collection and Transportation

3.1.2.1. Collection of Duckweeds

Duckweed plants were collected from a pond located at AASTU from january to february, 2017. I was collected by gravitational filtration. And then, dried duckweed was transported to AAIT for further pretreatment and experimental work.

Fig. 7: Collection of Duckweed

3.1.2.2. Collection of Lactic Acid Bacteria

One hundred [and] eleven different strains and five different types of lactic acid bacteria (4-lactobacillus brevis, 10-lac. Paracasei, 77-lac.plantarum, 11- Leuconostoc mesenteroides and 9-undefined LAB strains) were collected from College of Natural and Computational Sciences (Arat-Kilo Campus of AAU). Those bacteria were isolated from kocho by (Alemu-Gonfa, (2016)) and their DNA sequences were determined & characterized a year before in Germany for a PHD dissertation.

3.1.2.3. Collection of Nutrients and Chemical Agents

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

3.1.2.4. Collection of Other Chemicals

Both sulpheric acid and calcium hydroxide were purchased from Atomic Educational Material Supply P.L.C.

3.1.3. Chemicals and Equipments

There was a number of equipment such as, UV-Spectrophotometr, incubator shaker, microscope, oven, digital balance, water bath, pH meter, autoclave, pestle and mortar etc. used during the experimental work. All the chemicals and reagents used for analysis (such as H_2SO_4 and DNS reagent) were analytical grade.

3.2. Methodology

The production of lactic acid from lignocellulosic biomass involves four main steps: pretreatment, acid hydrolysis/enzymatic saccharification, fermentation and purification.

3.2.1. Pretreatment of Duckweed

Two types of duckweed in which their varieties are differnent were collected from two different ponds which is located in AASTU. Both types of duckweed were cleaned with tap water and then renised with disttled water. Then, cleaned duckweeds were subjected to drying with the help of sun over the net just to avoid its moisture content and dried duckweeds were grounded in to micro-scale powder by using pestle and mortar. Finally, differnent polysacharide of duckweeds were decomposed in to monosacharides through hydrolysis by using 1% sulfuric acid solution at 121 °C for 1 h.

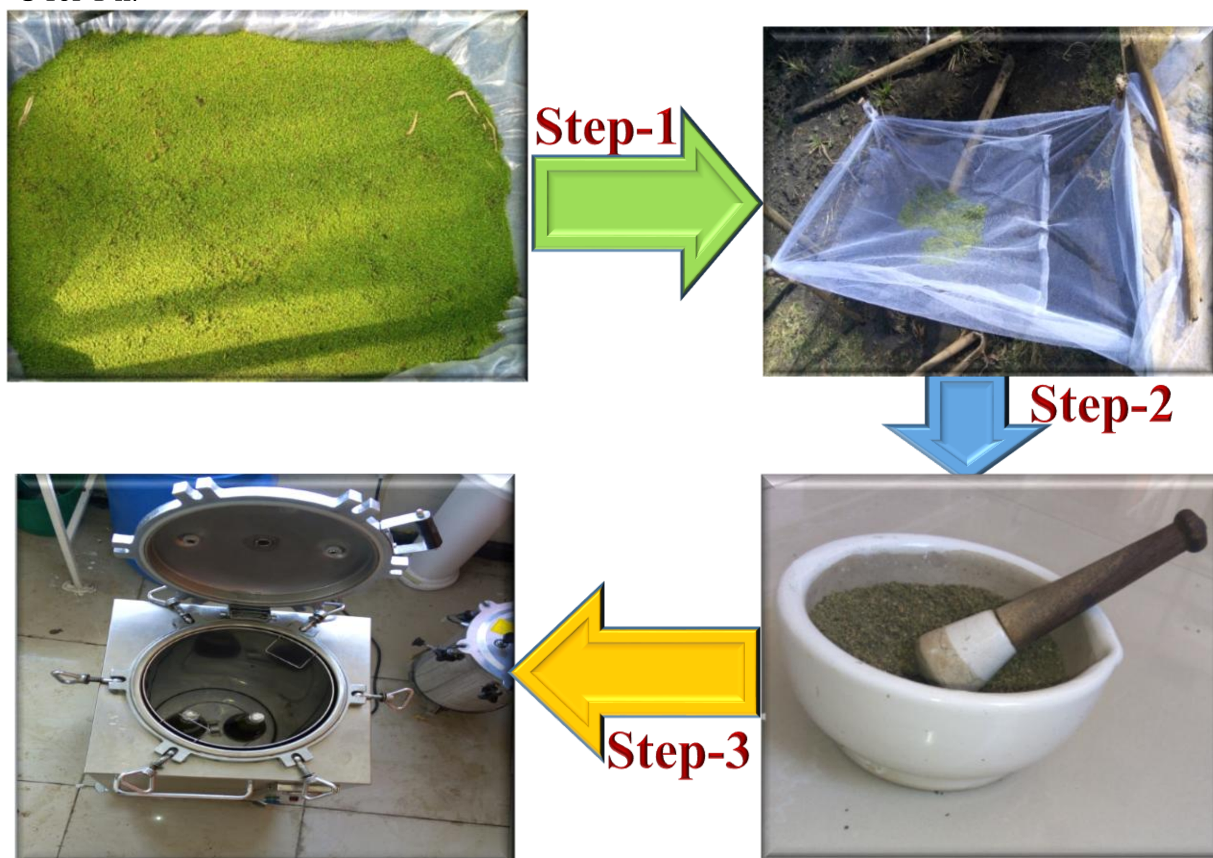


Fig. 8: Steeps of duckweed pretreatment

3.2.2. Characterization of Duckweeds

3.2.2.1. Determination of Moisture Content

Six plates was dried in an oven at 105°C for 5h and placed in a desiccator to cool. The weight of the crucible (W_1) for each was determined. 5-grams of each samples from both duckweed samples were weighted in this dry plates (W_2), dried at 105°C for 12 hour and reweighed again (W_3). All weights are measured in grams. The moisture content was determined by the following relationship: Then finally take the average.

$$\text{Moisture content(MC)} = \frac{W_2 - W_3}{W_2 - W_1} \text{x100}$$

Moisture is reported as grams of water per hundred grams of wet sample

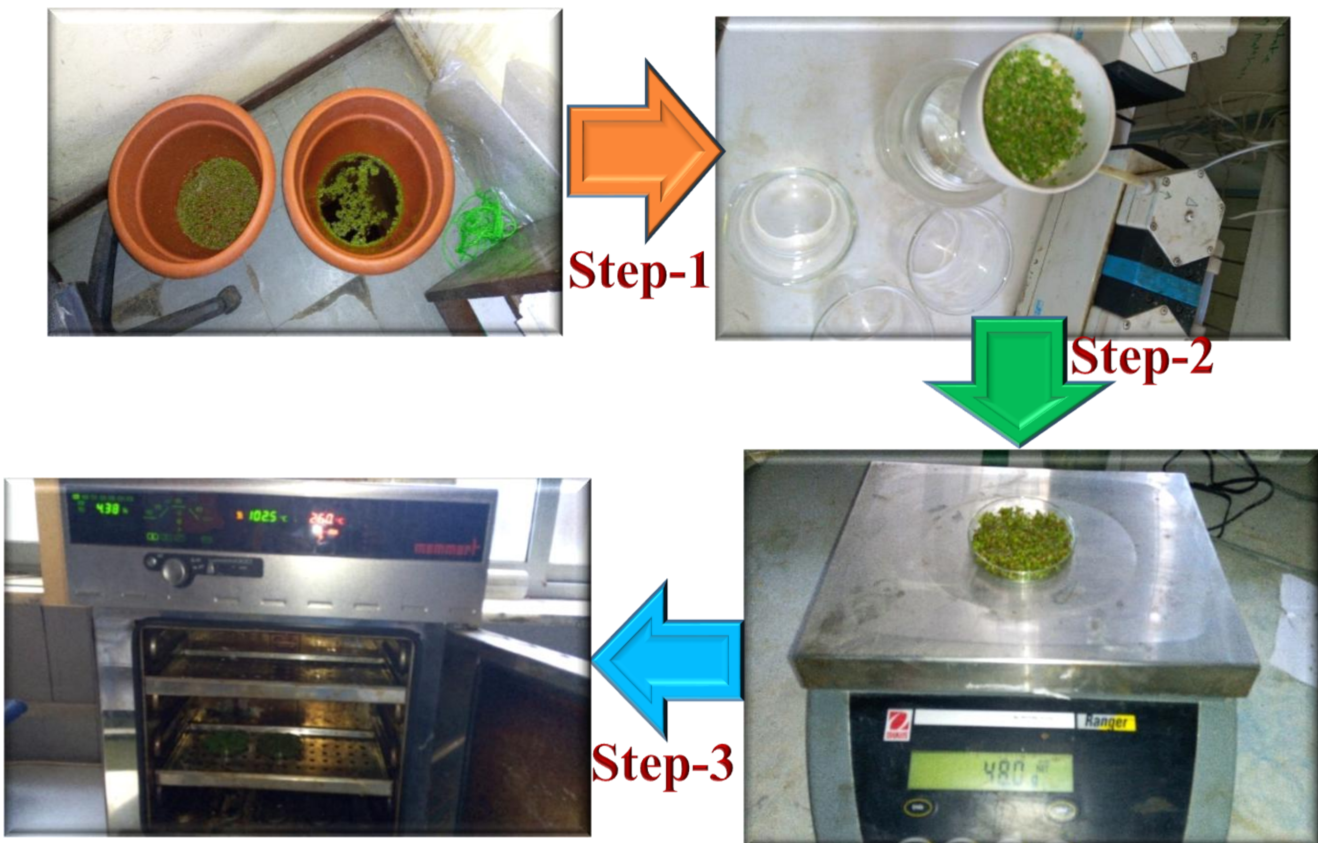


Fig. 9: Steeps to determine moisture content

Finally, Step-5 is re-weighing of dried dukweed.

3.2.2.2. Determination of Ash Content

The apparatus used for total ash analysis were three crucible and muffle furnace. Six empty crucible of capacity 45 ml was weighed and 5 g samples added in it. This was put in to a muffle furnace where the temperature was raised to about 550ºC for 12 h. The dish was placed in a desiccators, cooled and weighed. The ash content was calculated on dry mass basis by the following relationship:

$$\%ash = \frac{W_2 - W}{W_1 - W} x100$$

Where: W: Weight in grams of the empty dish.

Fig. 10: Furnace apparatus to determine ash content

3.2.2.3. Amount of Disolved Material with 1% H_2SO_4 Hydrolysis

Hydrolysate products were islolated in to two (filter cake and filtrate which contain disolved sugars) by using vaccum filtration. Then, Filter cake of both types of duckweed were dried inside an oven at 105^0C for the whole night and dried cake (W_2) were measured by using digital balance. Amount of disolved material was calculated from intial weight of duckweeds(W_1). The result is reported as grams of disolved material per hundred grams of sample.

$$\text{Desolved Material} = \frac{W_1 - W_2}{W_1} x100$$

.

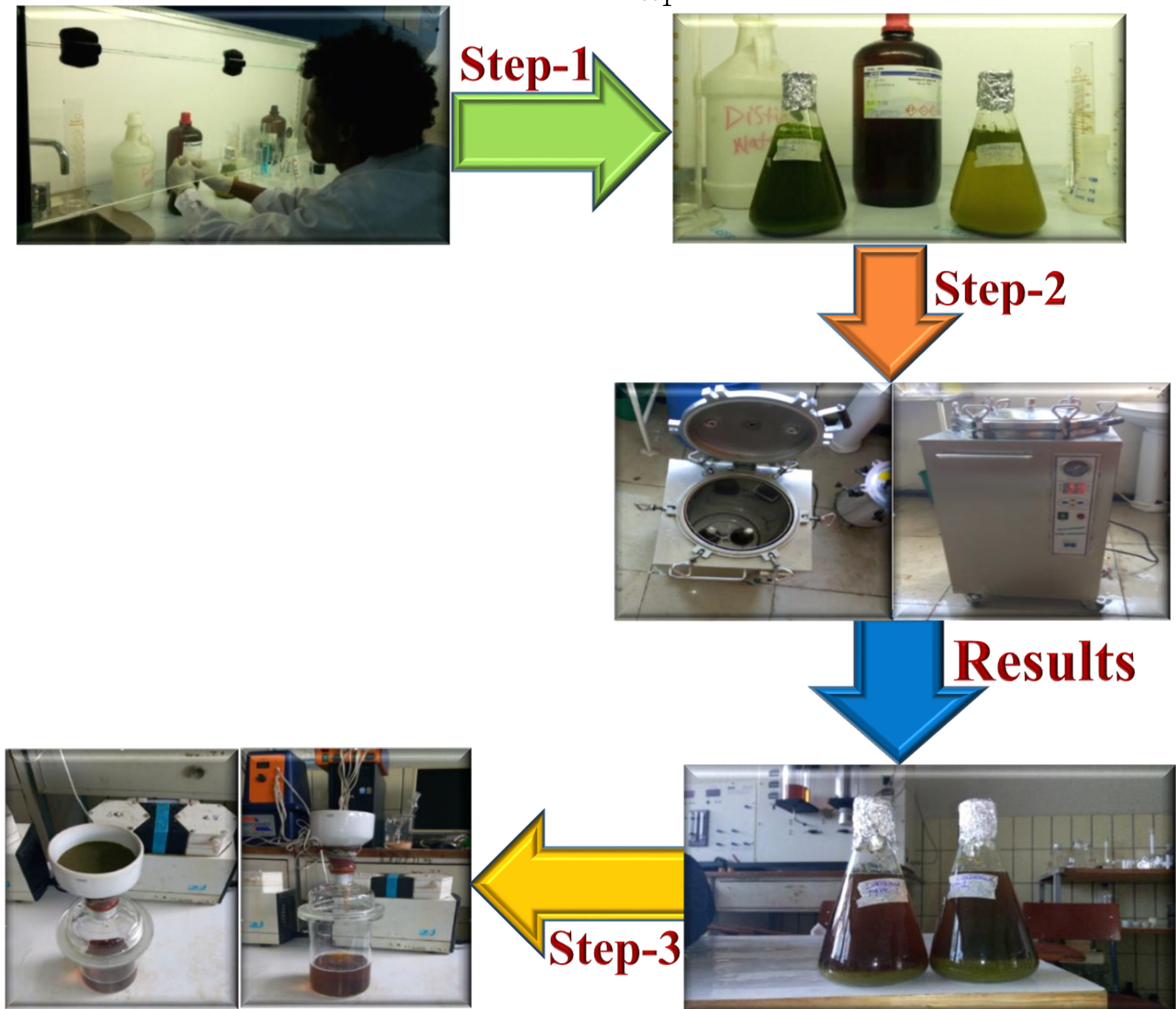


Fig. 11: Steeps to determine total amount of dissolved materials

3.2.2.4. Determination of Starch Content

3.2.2.4.1. Standard Curve of Reducing Sugar Concentration by DNS Method Analysis

Step-1:- Standard stock solution of glucose: Standard stock solution was prepared by dissolving 100 mg of glucose in 100 mL of distilled water and working standard was prepared by diluting 10 mL of stock solution to 100 mL with distilled water.

Step-2:- Preparation of di-nitro-salicylic acid (DNS) reagent: One gram of dinitrosalicylic acid, 200 mg of crystalline phenol, and 50 mg of sodium sulphite were simultaneously dissolved in 100 mL of 1 per cent NaOH solution by stirring. Forty per cent of Rochelle salt solution (sodium-potassium tartrate solution) was prepared.

Step-3:- Preparation of standard curve: Standard curve was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard glucose solution and the volume was made up to 3 mL by adding distilled water. 3mL of DNS reagent was added and the mixture was heated for five 45 minutes in a boiling water bath. After the development of the color, 1 mL of 40 per cent Rochelle salt solution was added (in warm contents) and mixed thoroughly. After cooling the tubes, the absorbance was recorded at a wavelength of 540 nm using UV-Spectrophotometer.

Fig. 12: UV-Spectrophotometer to determine starch content of duckweeds

3.2.2.4.2. Estimation of Reducing Sugars

Reducing sugars were estimated by the method of Miller (1950). Test tubes containing 3 mL sample and 3 mL DNS reagent were heated for 15 min. in a boiling water bath. One mL Rochelle salt solution was added to each tube and the tubes were allowed to cool to room temperature and O.D. was measured at 540 nm by using UV-Spectrophotometer. The concentration of reducing sugars (as glucose) was calculated from the pevious standard curve.

Then, amount of starch was calculated according to the following equation:

$$\frac{\text{A x (ABS sample} - \text{ABS blank)} - \text{B}}{\text{total mass}} \text{x100\%}$$

Where 'A' is stands for the gradient and 'B' represents intercept which were calculated from glucose standard curve.

3.2.3. Medium Formulation of Lactic Acid Fermentation by Lactic Acid Bacteria

The pH of hydro-lysates were adjusted to desired pH by using 0.1% $Ca(OH)_2$. The liquefied hydro-lysates of duckweed then used as the substrate for further fermentation as a main carbon source. Then, hydrolysates of duckweed was mixed with other nutrients and chemical agent. For that 10g/L of proteose peptone, 10g/L of beef extract, 5g/L of yeast extract, 1g/L of polysorbate 80, 2g/L of ammonium citrate, 5g/L of sodium acetate, 0.100g/L of magnesium sulphate, 0.050g/L of manganese sulphate, and 2g/L of dipotassium phosphate) were weighed and mixed with liquefied hydrolysate.

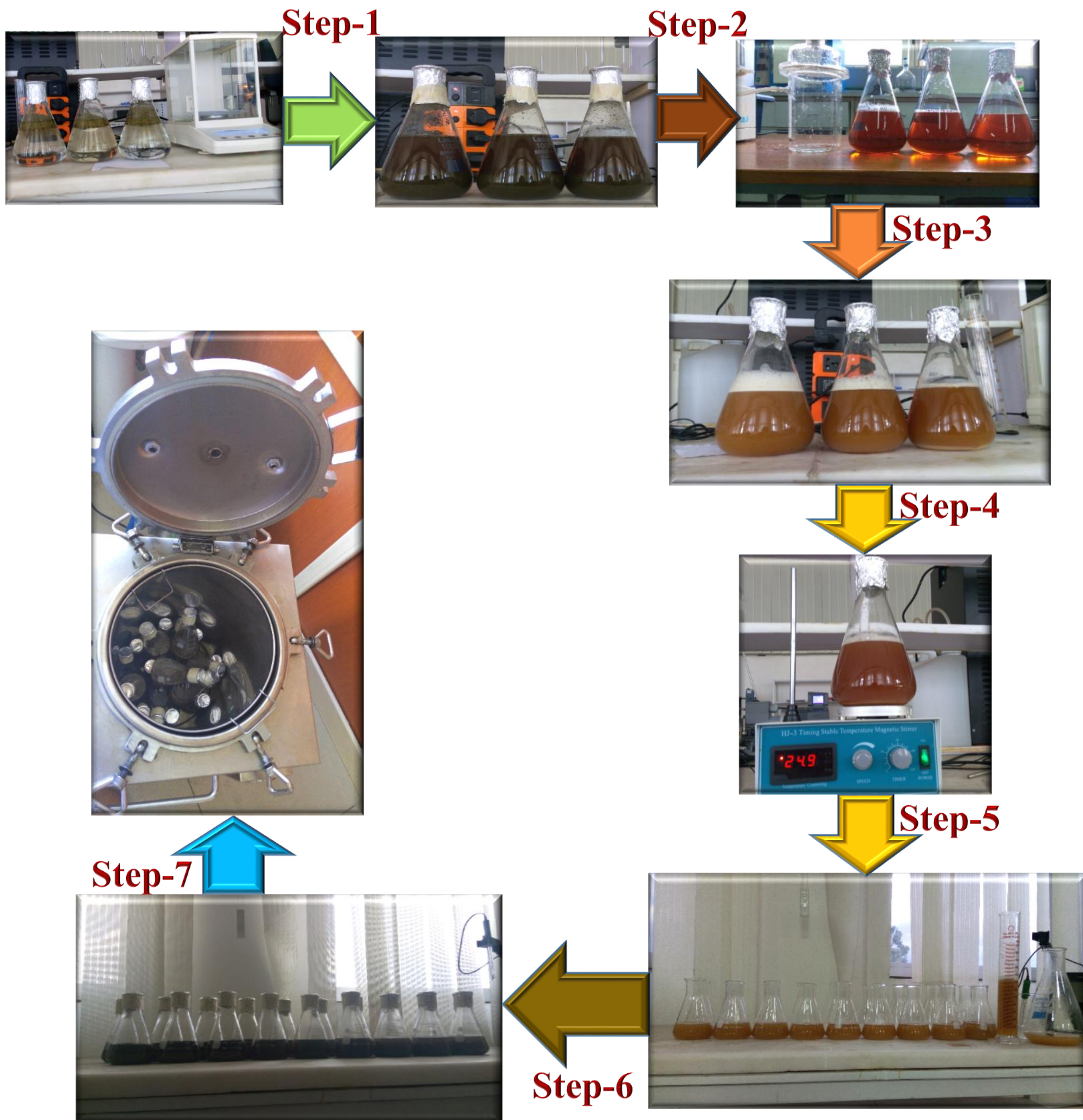


Fig. 13: Steeps of culture medium formulation

3.2.4. Design of Fermentation Experiment

Three factors with three level and two replication was selected for this particular experiment. Temprature, pH, and incubation time are factors. 30 °C, 40 °C and 50 °C are three levels selected for temprature factor; 4, 6 and 8 are three levels selectered for pH factor and 10-hr, 20-hr and 30-hr are three levels selected for incubation time factor. Then all of these experiments were performed twice for the sake of certainity and the experimentswere conducted based on their randomized run order. Finally, the interaction and main effects of this parameter were analysed and the optimum condtion of fermentation was determined by using Design-Expert Soft-ware (version 10.0.1).

Table-5: Design of fermentation experiment

Factors	Replication=2		
	Levels of Factors		
Temprature	30 °C	40 °C	50 °C
pH	4	6	8
Fermentation time	10-hr	20-hr	30-hr

3.2.5. Lactic Acid Fermentation Processes

All fermentations were performed in an incubator shaker with 250 mL flask each. The inoculum size of 10% (v/v) was prepared in MRS medium at 30 °C for 12 hs using an incubator at 200 rpms agitation. Then, this 10%(v/v) inoculum size of lactobacillus lactic were added in to sterilized culture medium that was prepared before. After that, all experiments were performed at corresponding growth temperature and pH of each randomized experimental run order without aeration. The pH was monitored manually by the addition of 5M NH_4OH and measuring with a pH meter. Then, fermentation were end with their respective incubation time.

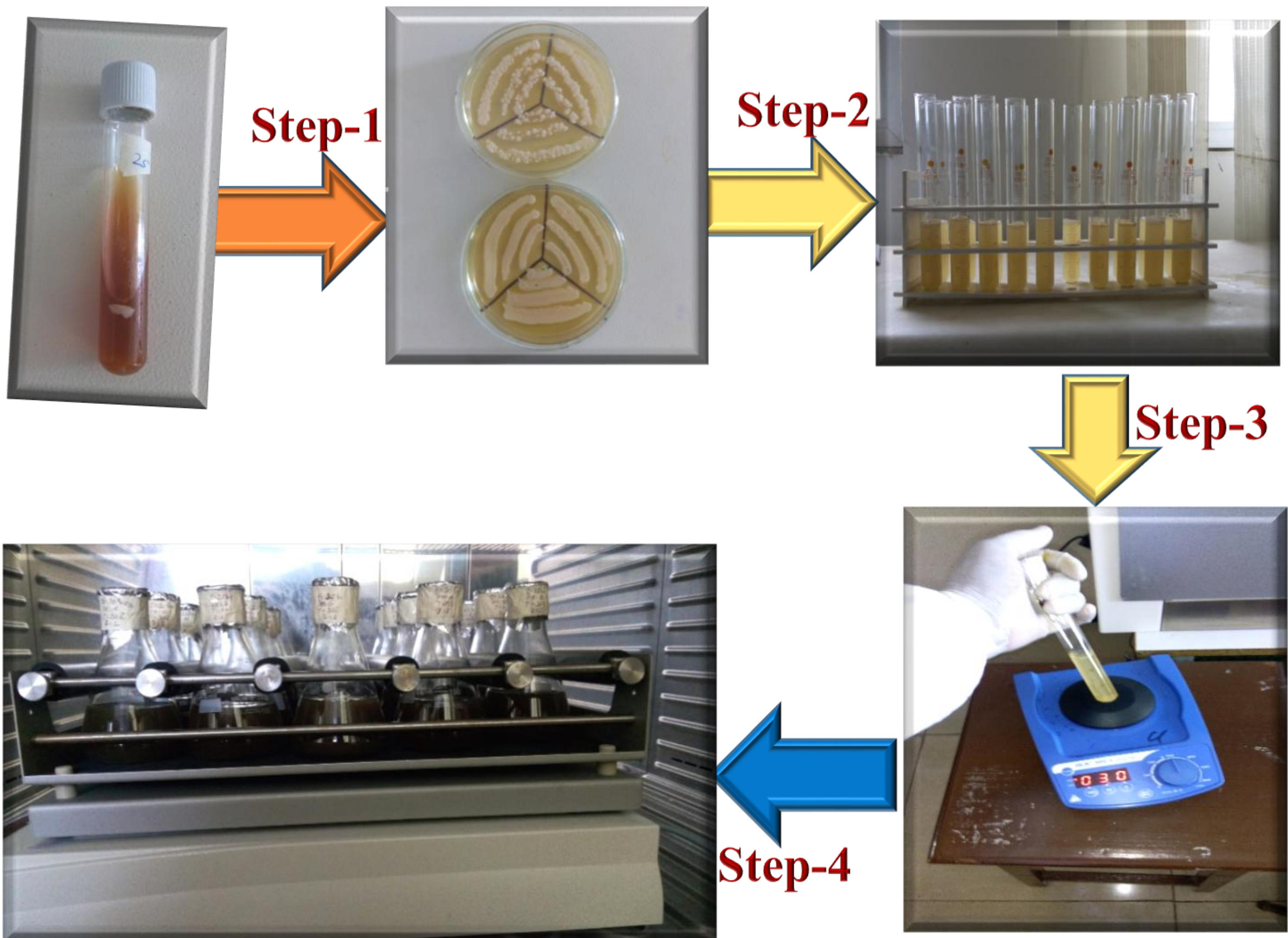


Fig. 14: Scale up processes for lactic acid fermentation

3.2.6. Analysis of Fermentation Broth

During the end of every incubation time of fermentation process, a 10 mL samples were withdraw under aseptic conditions. After centrifugation at 12,000 rpms for 10 mins, the supernatant was collected for further analysis.

3.2.6.1. Growth and Biomass Measurement

Growth and biomass (number of bacterias) were measured with serial dilutions. 6 small, sterilized test tubes and labelled from 1 to 6 were prepared. Then, the orginal samples were diluted independently by the following ways.

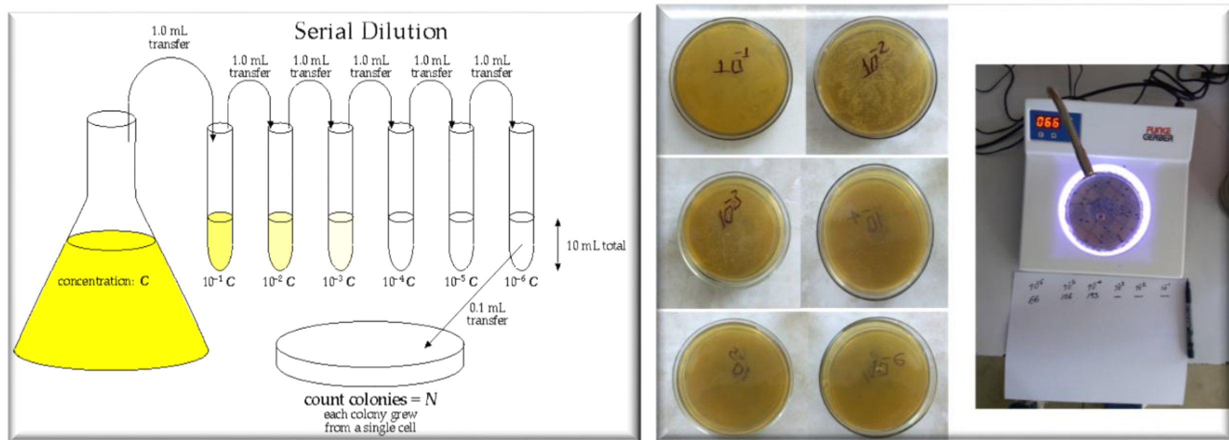


Fig. 15: Serial dilution steeps to count viable cells

Then, the number of bacteria per mL of diluted sample was calculated by the following equation:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

For example, if for the 1x 10^{-6} dilution plate you plated **0.1 mL** of the diluted cell suspension and counted 200 bacteria, then the calculation would be: **200/0.1 mL x 10^{-6} or 200/10^{-9} or 2.0 x 10^{9} bacteria per mL.**

3.2.6.2. Lactic Acid Analysis

At end of every incubation time of fermentation process, a 10-ml sample were collected under aseptic condition. The result could have been expressed as lactic acid and the yield (%) could have been calculated as the grams of acid that would produced from 100 gs of reducing sugars consumed. At specific intervals of time, the vials was removed and the fermented broth was centrifuged at 12,000 rpms for 10 mins and the supernatant was analyzed by UV-Spectrophotometer. The cultured medium was filter through 0.45µm membrane filter. The culibration curve was constructed by the following way. Lactic acid with known concentration (from 89% cp grade lactic acid) was placed 10 mL volumetric flask and diluted with water. A series of lactic acid solutions were prepared from the stock solution using two-fold dilution. Then, a test solution (50 µL) containing lactic acid was added to 2 mL of 0.2% solution of iron(III) cloride and stirred and absorbance was measured at 390 nm against the reference solution (2 mL of 0.2% $FeCl_3$ solution). The color of the solution was stable for 15 min (Borshchervskaya et al., (2016)).

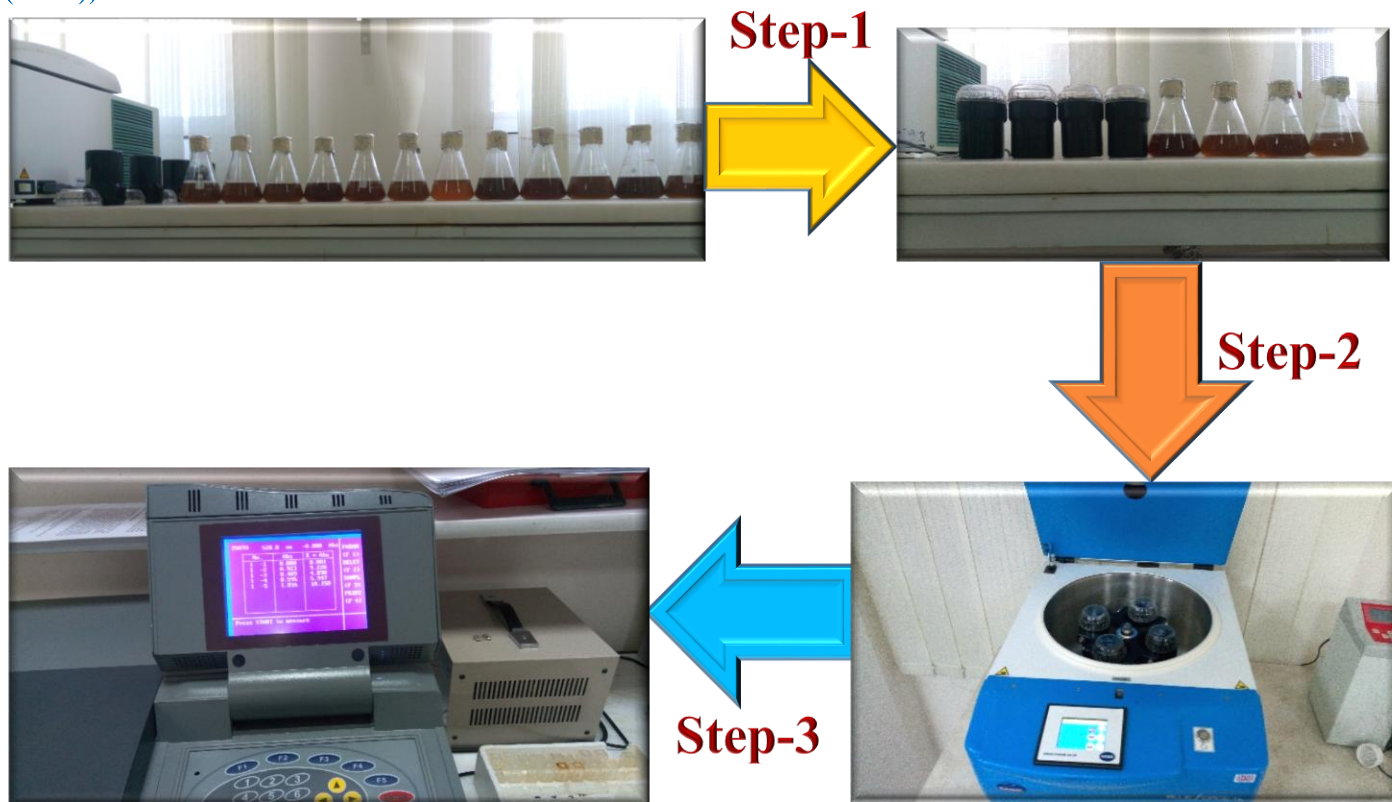


Fig. 16: Steeps to determine total amount of lactic acid

4. Results and Discussions

4.1. Proximate Chemical Composition of Duckweeds

Four important components of both duckweeds were studied for multiple reasons. (1)inorder to identify a better type of duckweed to utilize as alternative carbon source for the production of lactic acid, (2)to prepere modified MRS medium from selected duckweed species and to prevent substrate inhibition on fermentation due to excess suply of glucose. Specially, the amount of starch present inside duckweeds were the major screening criteria to select better duckweed as a carbon source and it also gaves an exellent information to prepere modified MRS medium for lactic acid fermentation.

Table-6 shows moisture contents, ash contents, total disolved materials and starch contents of both duckweeds. The amount of ash content in percent were 21.3% & 19.7%; the moisture content were 94.2 % & 94.01%; the amount of disolved materials were 44.4% & 52.7% and the amount of starch were 17.52 % w/w DM & 19.2% w/w DM of duckweed type-1 and duckweed type-2 respectively. Even though, the best amount of starch present in local duckweed plants were 19.2 % w/w DM, the amount of strarch present in both duckweeds fail the range of 3 - 75 % w/w DM as stated by (Xin-Zhao, (2014)). Acording to Xin Zhao's explaniation starch accumulation of duckweeds vary based on their type and the nutrient condition of waste water that they are growing. But in this case, both duckweeds were grow with similar nuttrient condition of waste water which was collected in differnt ponds. So; its' difference of starch composition came from their varieties. On the hand, the percentage ash contents of both duckweeds were not fail in the range 15.9-19% which was stated by (Xin-Zhao, (2014)). This will make both duckweed unique and different as it compare with previously studied duckweeds. In priniciple, the ash content varies and depends on many factors, such as the variety of duckweed, the terrain, the fertilization, and the climate (Calvel et al., (2001)). So, the previous deviation of ash content may come from the unique climatic nature of Ethiopia.

Table-6: Chemical composition of different duckweeds

Type of duckweed	Moisture content	Ash content	Starch content	Total disolved material after 1% H_2SO_4 hydrolysis
Type-1 (it looks Lemina minor)	94.2%	21.3%	17.52 % w/w DM	44.4%
Type-2 (it looks Lemna minuta)	94.01%	19.7%	19.2% w/w DM	52.7%

Therefore, based on different criateria; such as high starch accomulation capacity, fast growth and easily to prepare; duckweed type two was selected to substitute the traditional carbon sources of lactic acid fermentation process.

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

4.2.1. The Effect of Intial pH on Cell Growth

Figure-17 presents the effect of intial pH on cell growth during lactic acid fermentation process with modified MRS broth. The maximum number of cells were $4.23x10^8$ cells , $4.81x10^8$ cells and $4.41x10^8$ cells at pH 4, 6 and 8 respectively. That means the optimum amount of pH for this particulat bacteria is around 6. This result is similar to those of (Giraud et al., (1991)), who found an optimum pH of Lactobacillus plantarum of 5.5 to 6.5. In addition, it was observed in the present work that the pH had a considerable effect on the growth rate but had little influence on total lactic acid yield.

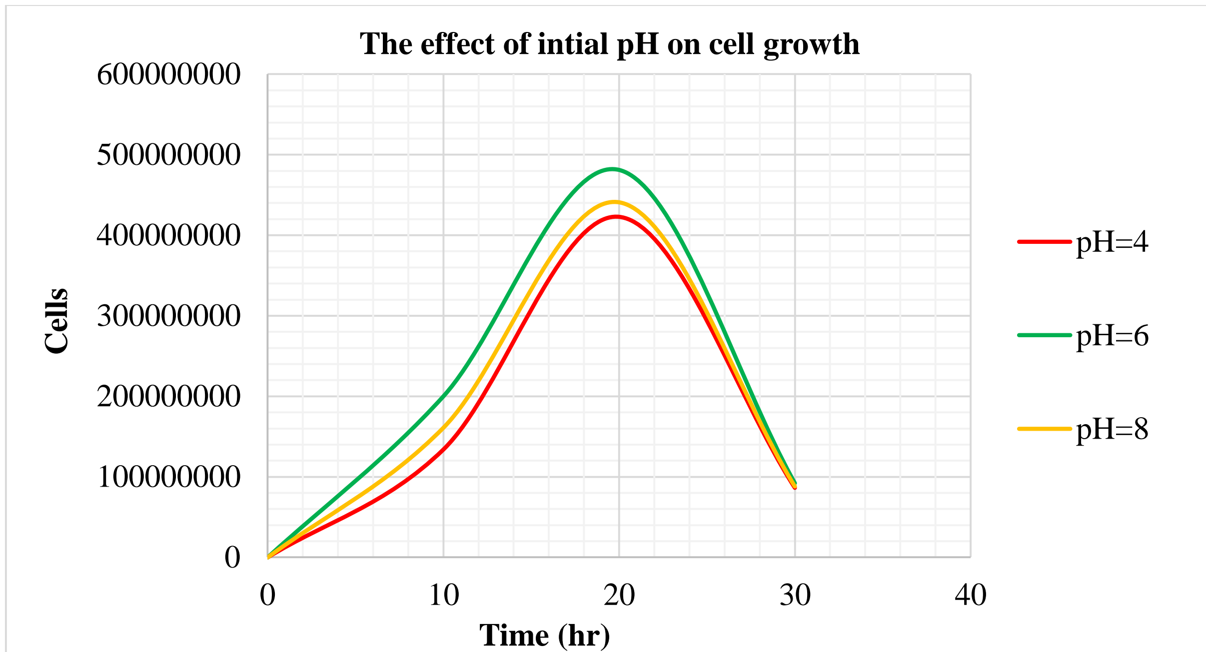


Fig. 17: The effect of initial pH on cell growth

4.2.2. The Effect of Temperature on Cell Growth

Figure-18 presents the effect of temperature on cell growth during lactic acid fermentation with modified MRS broth. The maximum number of cells were 4.81×10^8 cells, 4.54×10^8 cells and 3.9×10^8 cells at 30 °C, 40 °C and 50 °C temperature respectively. From this the optimum temperature can be estimated around 30 °C. According to (Adamberg et al, (2003)) explanarion ATP production capacity was an important factor in determining the maximum growth rate of lactic acid bacteria (LAB). The decrease of Y_{ATP} with increase of temperature above its optimum value resulted in the decrease of specific growth rate. In general, the effect of temperature on specific growth rate and on lactate production rate Q_{LAC} can be described by Arrhenius equation

$$ln\mu = lnA - \frac{E_a}{R}\frac{1}{T}$$

$$lnQ_{LAC} = lnA - \frac{E_a}{R}\frac{1}{T}$$

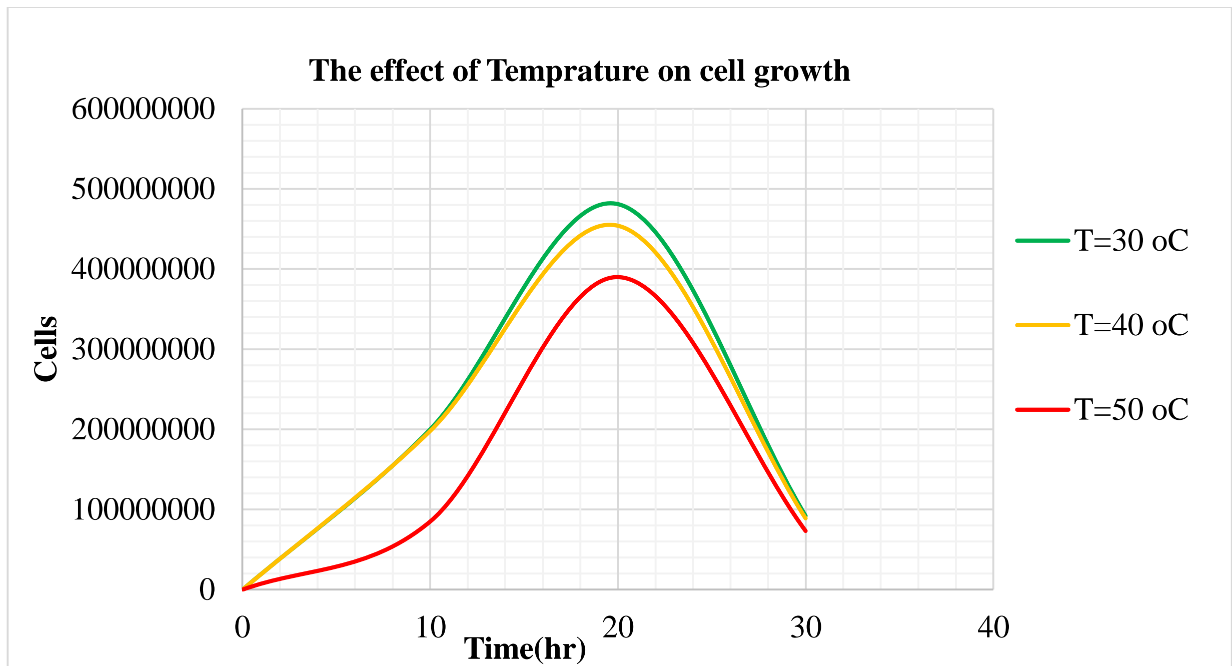


Fig. 18: The effect of temperature on cell growth

4.2.3. The Effect of Intial pH on Total Amount of Lactic Acid

Figure-19 presents the effect of intial pH on total amount of lactic acid produced from modified MRS broth at 30 ^{o}C operating temperature. The maximum amount of lactic acid produced from 104.12 gram duckweed were 16.71g/L, 16.94g/L and 16.86g/L at 4, 6 and 8 intial pH respectively. That means, reletively large amount of lactic acid was produced at pH 6. Figure-17 and figure-18 show that there is direct relationship between number of cells and total amount of lactic acid produced (for any increased in number of cells, the amount of lactic acid also increased). So, relatively large amount of lactic acid was produced at pH due to relatively large amount of cells were produced in the respective pH.

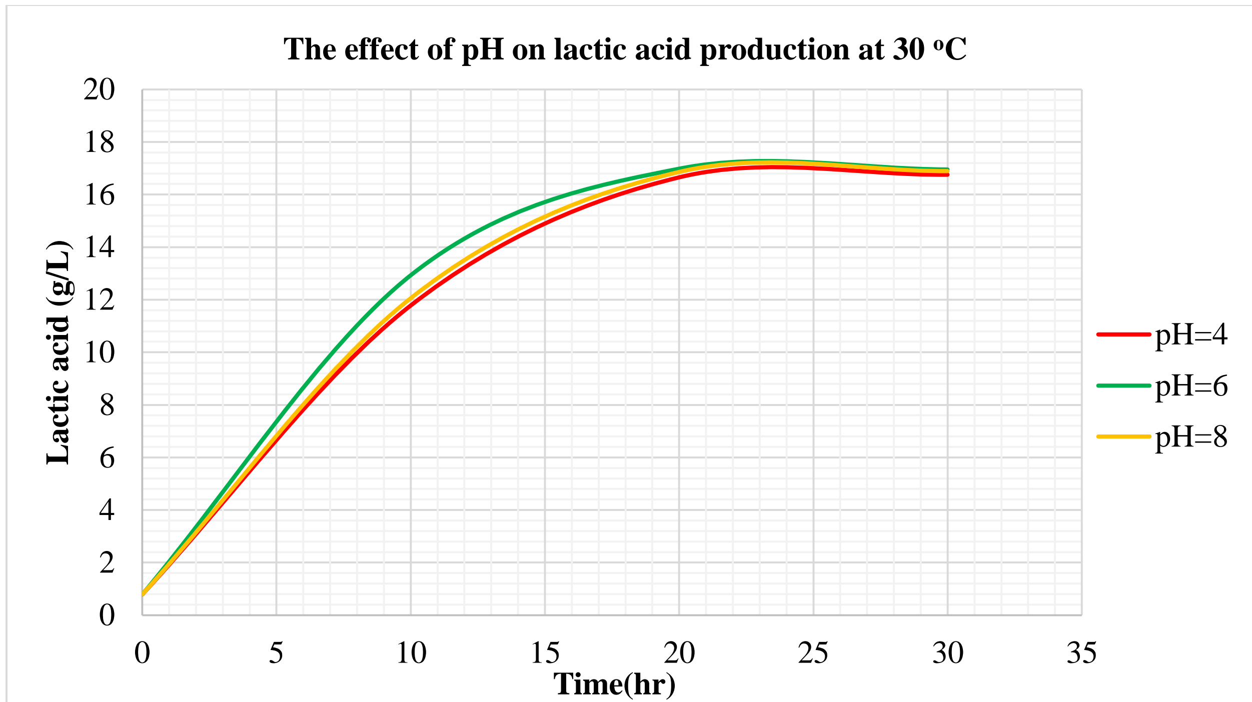


Fig. 19: The effect of initial pH on the amount of lactic acid produced

4.2.4. The Effect of Temperature on Total Amount of Lactic Acid

Figure-20 presents the effect of temperature on total amount of lactic acid produced from modified MRS broth medium at around optimum pH. The maximum amount of lactic acid produced from 104.12 gram duckweed were 17.01 g/L, 16.27 g/L and 13.12 g/L at 30 ºC, 40 ºC and 50 ºC operating temperature. Relatively maximum amount of lactic acid were produced at 30 ºC operating temperature due to similar reasons of the previous case (relatively large amount of cells were grew at 30 ºC).

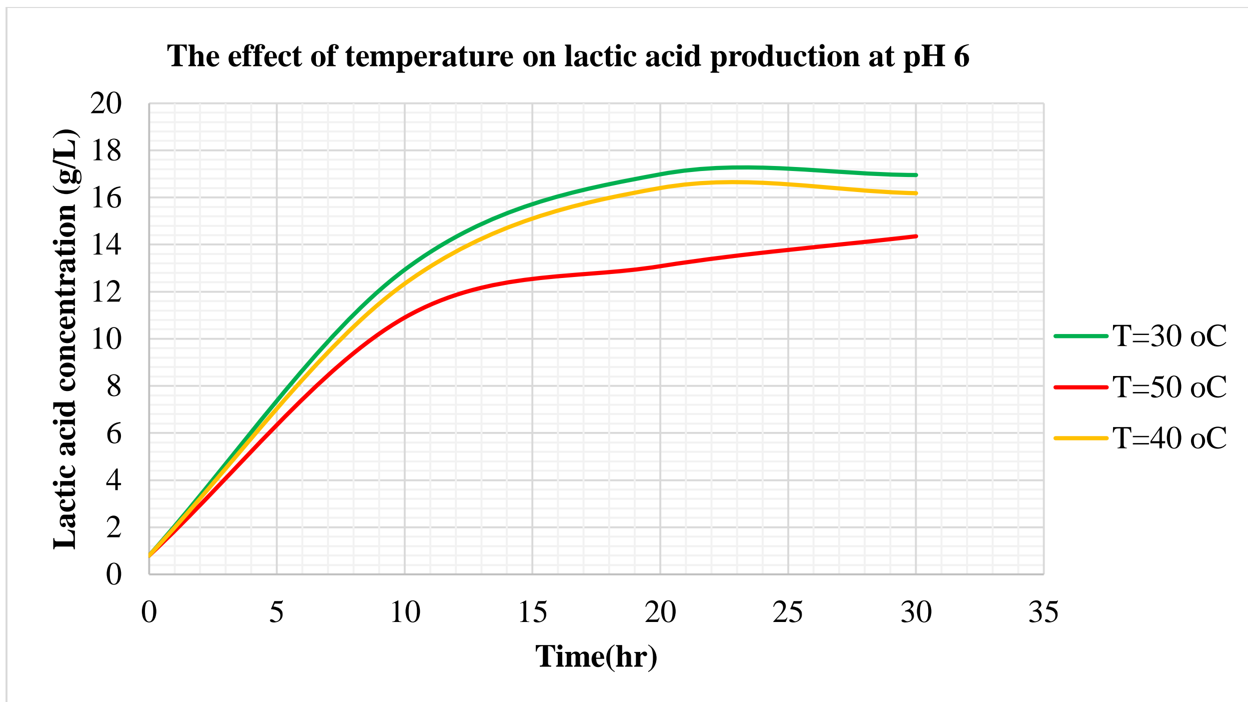


Fig. 20: The effect of initial pH on the amount of lactic acid produced

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

In general, figure-21 presents the effect of temperature, pH and incubation time on the total amount of lactic acid produced from duckweed. This is the overall summery of the result for all 27 samples. The average amount of lactic acid produced from 104.12 gram duckweed were 11.78 g/L, 11.3 g/L and 9.71 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 4 intial pH and 10 hr incubation time; 12.93 g/L, 12.34 g/L and 10.9 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 6 intial pH and 10 hr incubation time; 12.06 g/L, 11.54 g/L and 10.53 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 8 intial pH and 10 hr incubation time; 16.65 g/L, 15.98 g/L and 12.82 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 4 intial pH and 20 hr incubation time; 16.98 g/L, 16.4 g/L and 13.08 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 6 intial pH and 20 hr incubation time; 16.86 g/L, 16.21 g/L and 12.91 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 8 intial pH and 20 hr incubation time; 16.75 g/L, 16.4 g/L and 14.01 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 4 intial pH and 30 hr incubation time; 16.95 g/L, 16.18 g/L and 14.35 g/L at 30 ºC , 40 ºC and 50 ºC operating temperature with 6 intial pH and 30 hr incubation time; 16.89 g/L, 16.16 g/L and 14.2 g/L at 30 ºC , 40 ºC and 50 ºC

operating temperature with 8 intial pH and 30 hr incubation time respectively. These show that the total amount of lactic acid produced from duckweed was negetively affected by temperature in the range between 30 and 50 ^{o}C. However, variations on total amount of duckweed were showed that direct relation with both pH and incubation time till certain optimum condition was developed. After that, inverse relation was developed beteewn pH and the total amount of lactic acid produced. Where as the total amount of lactic acid produced was became constant once the optimum incubation time developed.

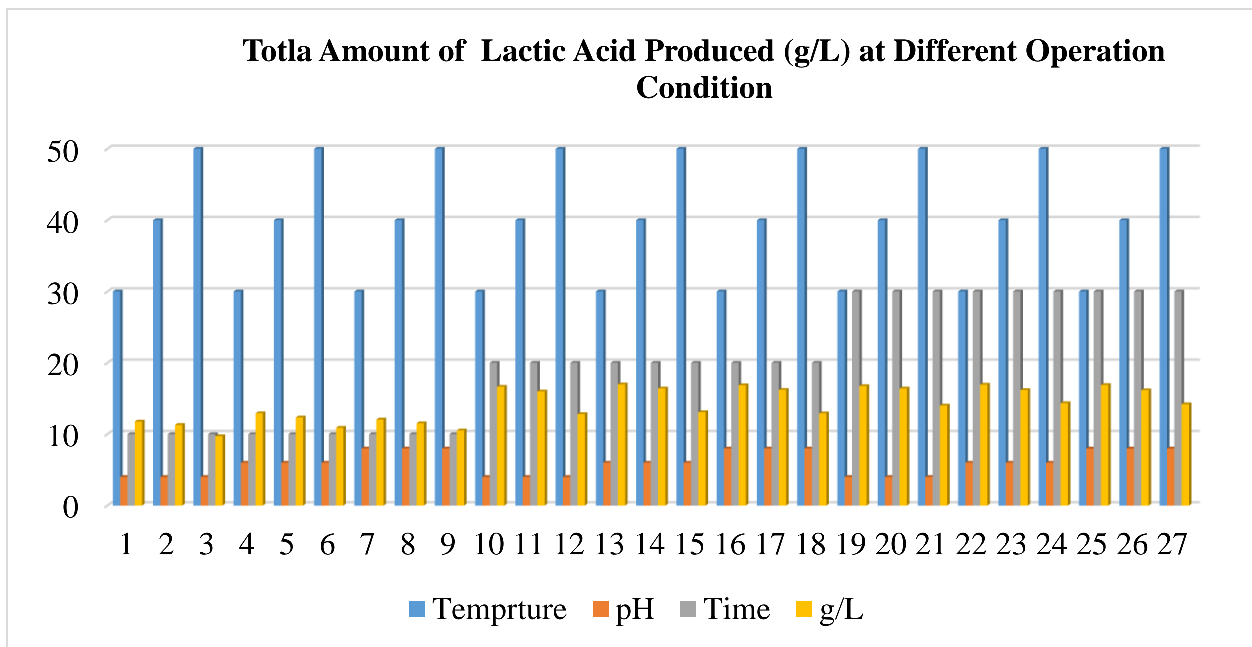


Fig. 21: Total amount of lactic acid produced at different operating condition

4.3. Statstical Analysis of the Exerimental Results

Appendix A presents experimental results of three factors with three levels and two repilicants and the following tables show statsitical analysis of the experimental results by using Design-Expert Soft-ware (version 10.0.6).

Table-7: Design summary for three variables and three level factorial design

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

Design Summary...						
Factor	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	
A	Temperature	^oC	Numerical	30	50	
B	pH		Numerical	4	8	
C	Incubation time	hr.	Numerical	10	30	
Response	**Name**	**Units**	**Minimum**	**Maximum**	**Trans**	**Model**
Y_1	Lactic acid con.	g/L	9.64	17.01	None	Quadratic Polynomial
Y_2	Cell growth	Numbers	$4.01x10^7$	$4.81x10^8$	None	Quadratic Polynomial
Y_3	Productivity	g/hr.	0.13	1.29	None	RMain effects

The ANOVA results from three factial model equation described in table-7. The analysis of variance (ANOVA) was carried out to assess the significance of quadratic polynomial equation fittness for the each response variables and independent variables. The model was significant due to high F-Value of 483.1, 6.366E+007 & 57.16 and low Probability value of 0.0001 for lactic aicd concentration, cell growth and productivity respectively.

Then, a measure of the statistical accuracy of an estimate was done by using Design Expert software at optimum incubation time. Figure-22 shows that the standard error of design or dispersion of sample means around the population mean on 3D-surface.

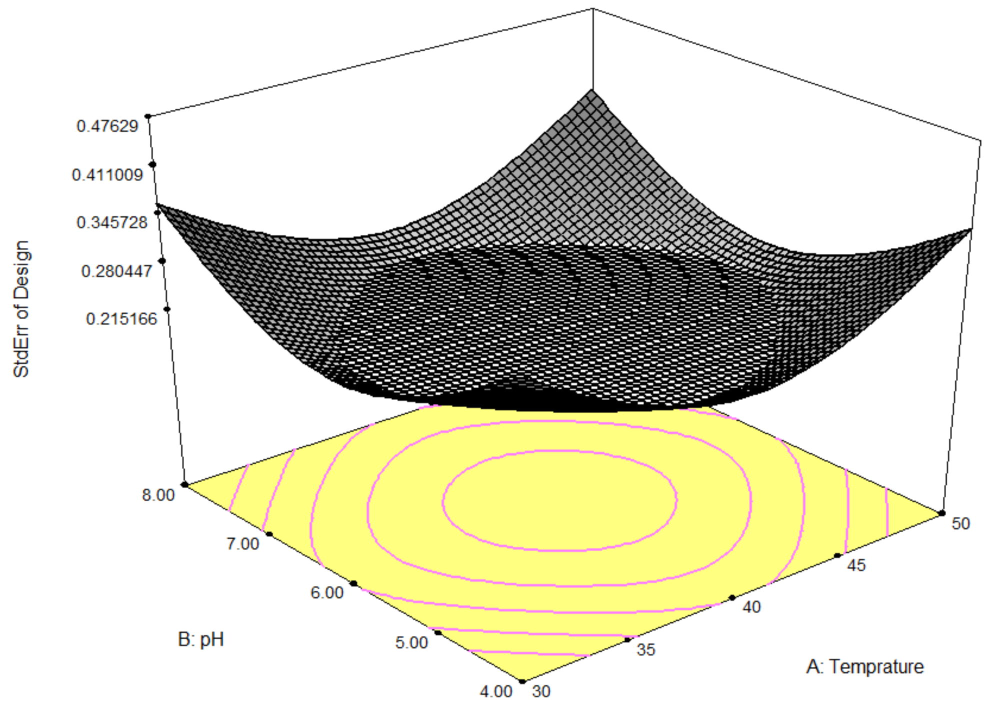


Fig. 22: Standard error of design

4.3.1. Analysis of Variance (ANOVA) for Quadratic Models of Lactic Acid Concentration

Table-8 shows that both temperature and incubation time were highlly influential process variable for the generation of lactic acid. Whereas, intial pH was less significant process variable. Ingeneral, the significance of the main effects and interaction effects are shown by Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, A^2, B^2, C^2, AC are significant model terms. That means only these variables had more impact on the total amount of lactic acid produced. However, values greater than 0.1000 indicate the model terms are not significant.

Table-8: Analysis of Variance (ANOVA) for quadratic models of lactic acid concentration

Response: Lactic acid yield					
Source	Sum of Squares	DF	Mean Square	F Value	P-value, Prob > F
Model	282.57	10	28.26	131.58	< 0.0001 significant
A-Temperature	71.26	1	71.26	331.84	< 0.0001
B-pH	0.60	1	0.60	2.80	0.1017
C-incubation time	164.14	1	164.14	764.32	< 0.0001
A^2	7.18	1	7.18	33.44	< 0.0001
B^2	2.27	1	2.27	10.59	0.0022
C^2	35.93	1	35.93	167.29	< 0.0001
AB	0.035	1	0.035	0.16	0.6873
AC	0.96	1	0.96	4.45	0.0407
BC	0.13	1	0.13	0.62	0.4348
ABC	0.060	1	0.060	0.28	0.5997
Residual	9.23	43	0.21		
Lack of Fit	8.61	16	0.54	23.21	< 0.037 not significant
Pure Error	0.63	27	0.023		
Cor Total	291.80	53			

The Model F-value of 131.58 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Finally, by applying multiple regression analysis to the experimental data, the following second degree polynomials was found to represent the relationnship between the total amount of lactic acid produced and the most significant process variables.

Final Equation in Terms of Actual Factors:

Lactic acid yield= -6.23769 + 0.46986*Temperature + 1.12333*pH + 0.9343*incubation time -7.73611E-003*Temperature^2 - 0.10882*pH^2 - 0.017303*Incubation time^2 + 8.04167E003*Temperature * pH -1.58333E-004*Temperature*Incubation time + 8.52083E-003*pH*Incubation time - 3.06250E-004*Temperature*pH*Incubation time

Table-9: Adequateness of the model for lactic acid yield

Std. Dev.	0.15	R-Squared	0.9979
Mean	14.16	Adj R-Squared	0.9958
C.V.	1.07	Pred R-Squared	0.9914
PRESS	2.50	Adeq Precision	67.481

The "Pred R-Squared" of 0.9914 is in reasonable agreement with the "Adj R-Squared" of 0.9958. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case a ratio of 67.481 indicates an adequate signal. This model can be used to navigate the design space.

4.3.2. Analysis of Variance (ANOVA) for Quadratic Models of Cell Growth

Table-11 shows that all temperature, pH and incubation time were highlly influential process variable for cell growth. Ingeneral, the significance of the main effects and interaction effects are shown by values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, A^2, B^2, C^2, AC are significant model terms and values greater than 0.1000 indicate the model terms are not significant.

Table-11: Analysis of Variance (ANOVA) for quadratic models of cell growth

Response: Cell growth					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	1.243E+018	10	1.243E+017	799.38	< 0.0001 significant
A-Temperature	3.489E+016	1	3.489E+016	224.40	< 0.0001
B-pH	2.181E+015	1	2.181E+015	14.02	0.0005
C-Incubation time	2.949E+016	1	2.949E+016	189.66	< 0.0001
A^2	7.288E+015	1	7.288E+015	46.87	< 0.0001
B^2	9.835E+015	1	9.835E+015	63.24	< 0.0001

C^2	1.154E+018	1	1.154E+018	7421.38	< 0.0001
AB	1.320E+013	1	1.320E+013	0.085	0.7722
AC	4.670E+015	1	4.670E+015	30.04	< 0.0001
BC	1.591E+014	1	1.591E+014	1.02	0.3174
ABC	4.818E+014	1	4.818E+014	3.10	0.0855
Residual	6.687E+015	43	1.555E+014		
Lack of Fit	6.687E+015	16	4.179E+014		<0.143 not significant
Pure Error	0.000	27	0.000		
Cor Total	1.250E+018	53			

The Model F-value of 799.38 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Then, by applying multiple regression analysis to the experimental data, the following second degree polynomials was found to represent the relationnship between the total amount of lactic acid produced and the most significant process variables.

Final Equation in Terms of Actual Factors:

Cell growth= -1.30722E+009 + 1.68822E+007*Temprature + 1.12817E+008*pH + 1.22960E+008*Incubation time -2.46444E+005*Temprature^2 - 7.15694E+006*pH^2 - 3.10111E+006*Incubation time^2 - 5.11667E+005*Temprature * pH -25125.00000*Temprature*Incubation time -1.22625E+006*pH*Incubation time +27437.50000*Temprature*pH*Incubation time

Table 12: Adequateness of the model for cell growth

Std. Dev.	0.000	R-Squared	1.0000
Mean	2.086E+008	Adj R-Squared	1.0000
C.V.	0.000	Pred R-Squared	1.0000
PRESS	0.000	Adeq Precision	

The "Pred R-Squared" of 1.0000 is in reasonable agreement with the "Adj R-Squared" of 1.0000.

4.3.3. Analysis of Variance (ANOVA) for Quadratic Models of Productivity

Table 13: Analysis of Variance (ANOVA) for quadratic models of productivity

Response: Productivity					
Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	2.87	3	0.96	40.30	< 0.0001 significant
A-Temperature	0.077	1	0.077	3.24	0.0777
B-pH	3.414E-003	1	3.414E-003	0.14	0.7060
C-Incubation time	2.79	1	2.79	117.51	< 0.0001
Residual	1.19	50	0.024		
Lack of Fit	0.50	23	0.022	0.87	0.6298 not significant
Pure Error	0.68	27	0.025		
Cor Total	4.05	53			

The Model F-value of 40.30 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. In this case only incubation time was the significant model term.

Final Equation in Terms of Actual Factors:

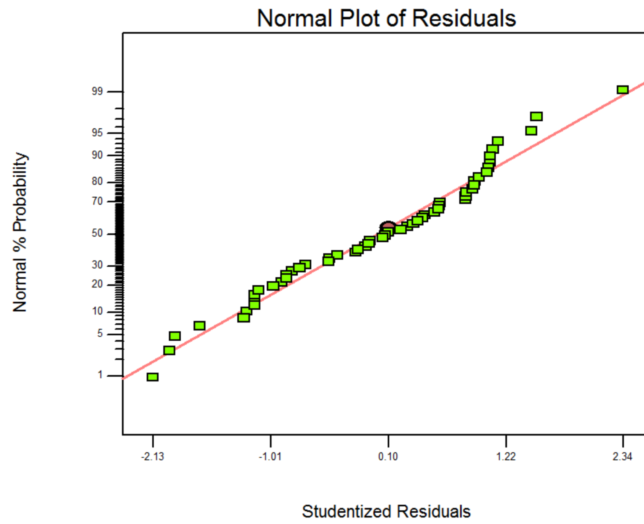
Productivity= 1.50226 - 4.62278E-003*Temprature + 4.86944E-003*pH -0.027822*Incubation time

Table 14: Adequateness of the model for productivity

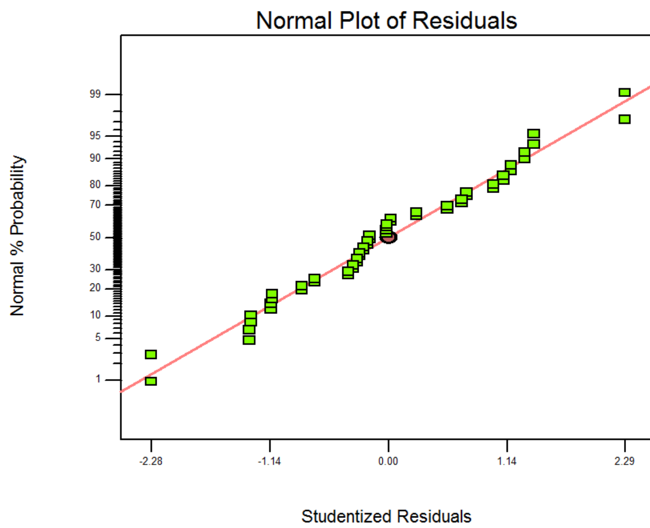
Std. Dev.	0.15	R-Squared	0.7074
Mean	0.79	Adj R-Squared	0.6899
C.V.	19.49	Pred R-Squared	0.6594
PRESS	1.38	Adeq Precision	15.947

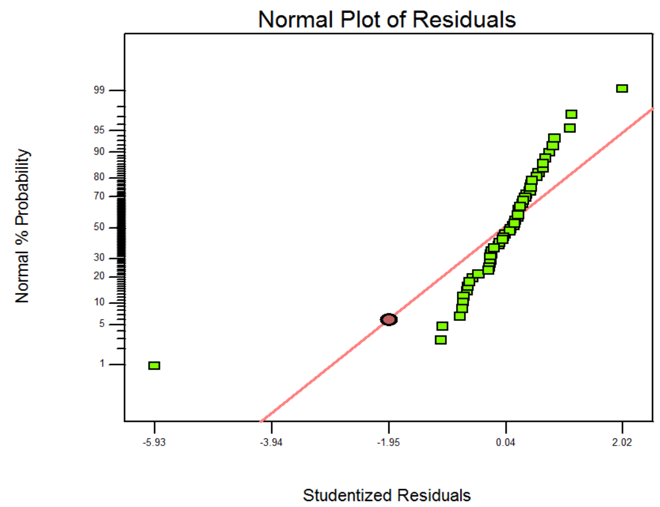
The "Pred R-Squared" of 0.6594 is in reasonable agreement with the "Adj R-Squared" of 0.6899. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case, ratio of 15.947 indicates an adequate signal. This model can be used to navigate the design space.

The plots as shown in figure-23, the residuals follow a normal distribution, the points in the plots follow linearity, this shows that the quadratic polynomial model satisties the assumptions analysis of variance (ANOVA).



(A)

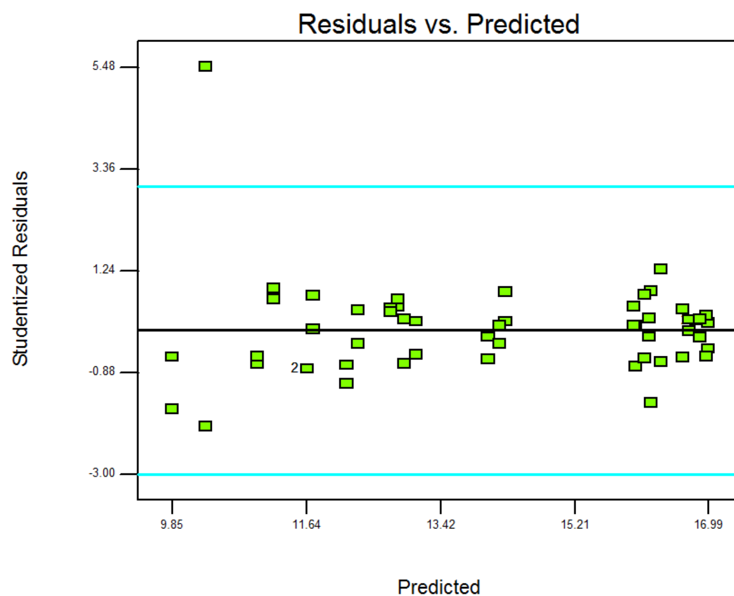




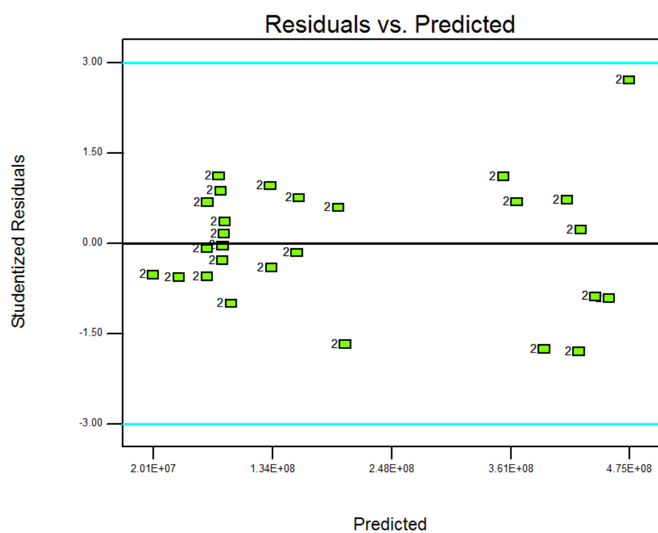
(B) **(C)**

Fig. 23: Normal plot of residuals (A) =Lactic acid yield, (B) = Cell growth and (C) =Productivity

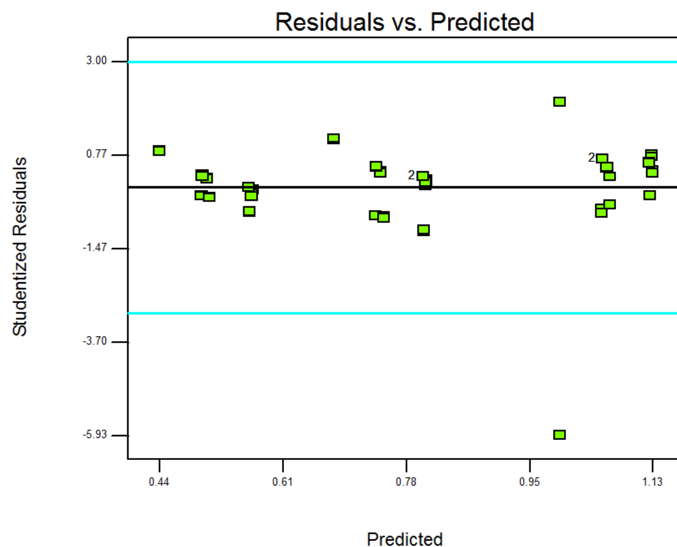
The plots in figure-24 show contstant range of residuals across the praph which is justifiable no need for a transformation to minimize personal error.



(A)



(B)



(C)

Fig. 24: Residual Vs. predicted values (A) =Lactic acid yield, (B) = Cell growth & (C) =Productivity

4.4. Interaction Effects of Experimental Variables on the Required Responses

4.4.1. Interaction Effect of Experimental Variables on Lactic Acid Yield

The effects of different operating variables on yield of total lactic acid were obtained by keeping one variable constant at the center point and varying the other variables within experimental range. The resulting response with interaction effect of operating variables were plotted with the following figurers. Figure-25, 26 & 27 show the interaction effect of temperature & pH; temperature & incubation time and incubation time & pH on generation of lactic acid at 20 hours incubation time respectively.

Figure-25 presents the interaction effect of temperature and pH on total mount of lactic acid with 3D graph. The graph shows that the effect of temperature was higher than the interaction effect of temperature and pH on total amount of lactic acid. Because, the reponse surface was almost parallel with the imaginary plane passing through (50,8,9.64)and (30,8,17.01). Slightly curved structure of the response surface shows their interaction effect was little and insignificant. The *P*-value (Table-8) also indicated that the interaction effect of pH and temperature had limited significance. The same results have been observed by (Gorret et al., (2001)).

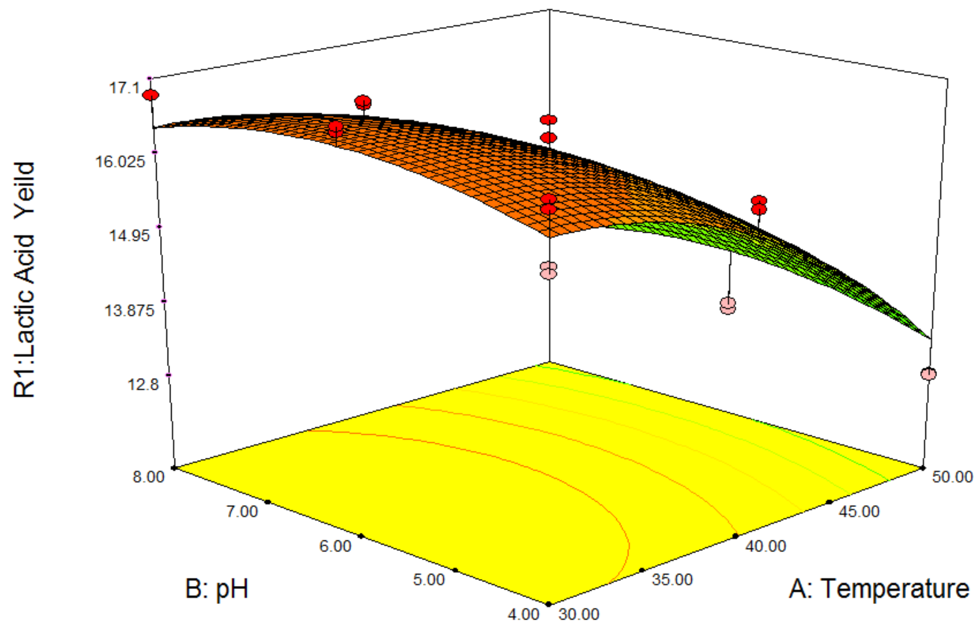


Fig. 25: The interaction effect of temperature and pH on total amount of lactic acid

On the other hand, figure-26 and 27 show the interaction effect of temperature & incubation time; and pH and incubation time on total amount of lactic acidat at pH 6 and 40 ^{o}C respectively. In this case, the interaction between temperature & incubation time had significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum for both factors. However, the interaction effect of pH & incubation time was limited significance. The same results have been observed by (Gorret et al., (2001)).

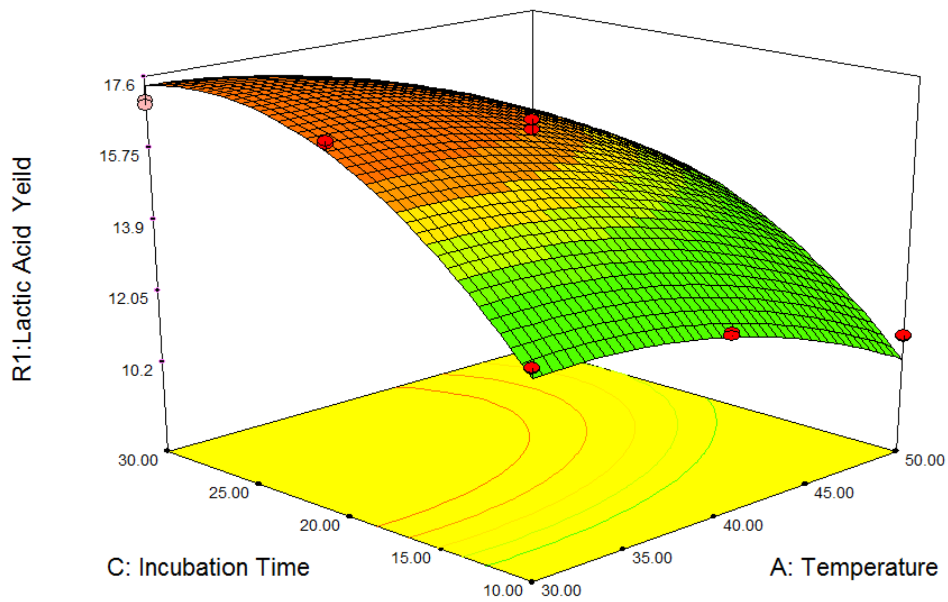


Fig. 26: The interaction effect of temperature and incubation time on total amount of lactic acid

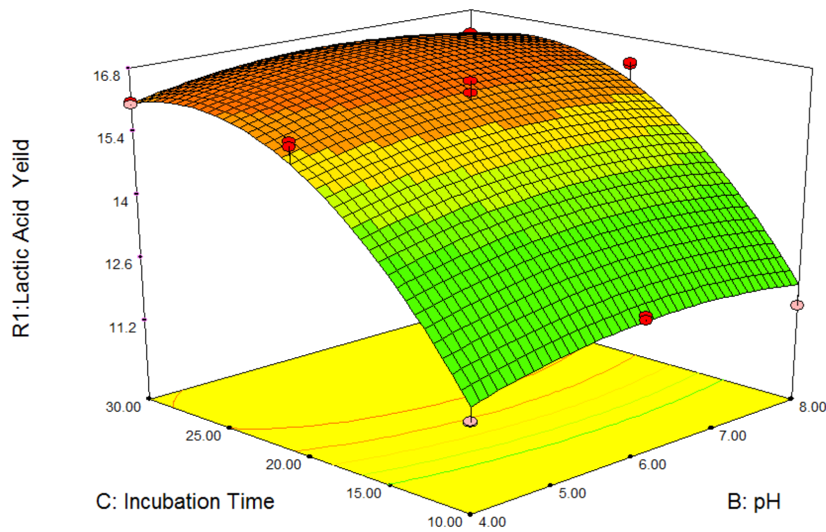


Fig. 27: The interaction effect of pH and incubation time on total amount of lactic acid

4.4.2. Interaction Effect of Experimental Variables on Cell Growth

Figure-28, 29 and 30 show an interaction effect of temprature & pH; temperature & incubation time and incubation time & pH on cell growth respectively. In this case, all temperature pH, temperature & incubation time, and pH & incubation time have significant quadratic effects ($P < 0{\cdot}05$), indicating the presence of an optimum for all factors. The same results have been observed by (Tripathi et al., (2015)).

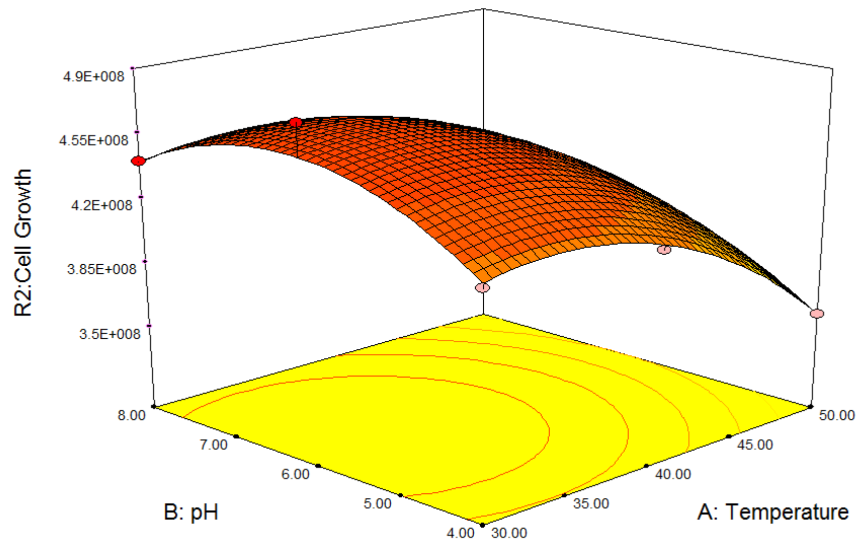


Fig. 28: The interaction effect of temperature and pH on cell growth

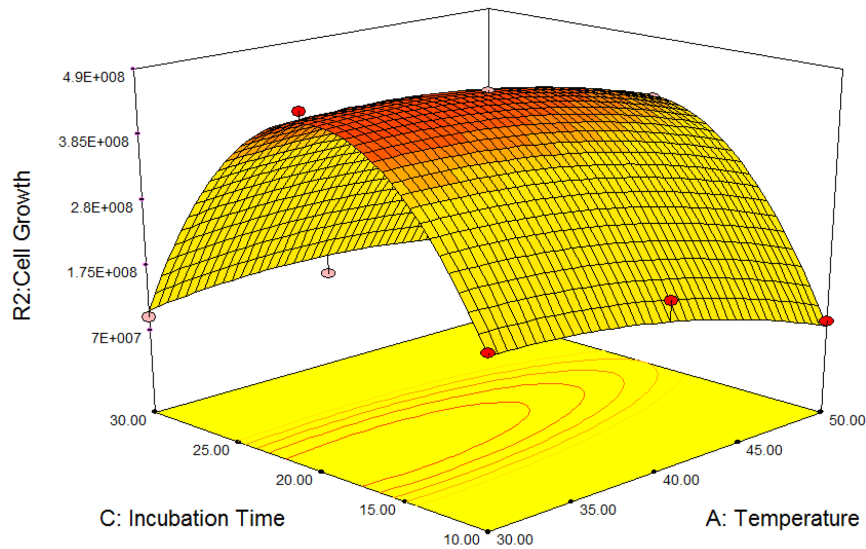


Fig. 29 : The interaction effect of temperature and incubation time on cell growth

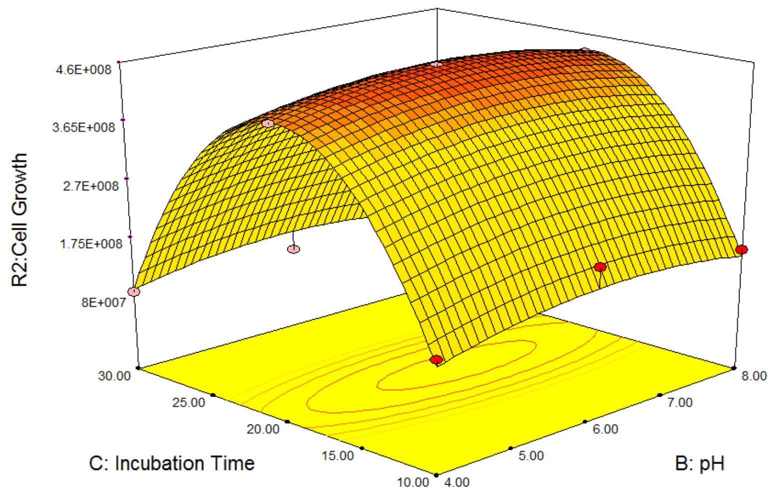


Fig. 30: The interaction effect of pH and incubation time on cell growth

4.4.3. Interaction Effect of Experimental Variables on Productivity

Figure-31, 32 and 33 show the interaction effect of temprature & pH; temperature & incubation time and incubation time & pH on productivity at 20 hours incubation time, pH 6 and 40°C temperature respectively. Similar with the previous case; all temperature & pH, temperature & incubation time, and pH & incubation time have significant quadratic and linear effects ($P < 0{\cdot}05$), indicating the presence of an optimum for all factors. The same results have been observed by (Roslina-Rashid, (2008)).

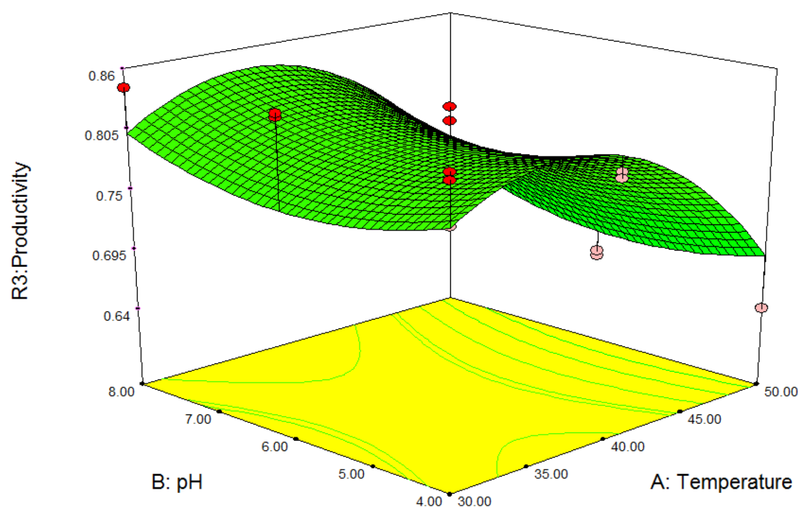


Fig. 31: The interaction effect of temperature and pH on productivity of Fermentation process

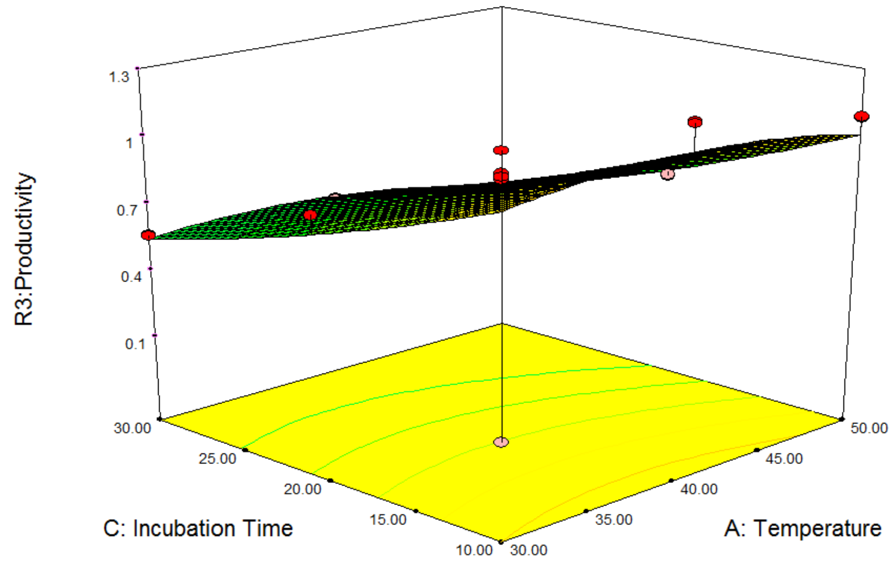


Fig. 32: The interaction effect of temperature and incubation time on productivity of fermentation process

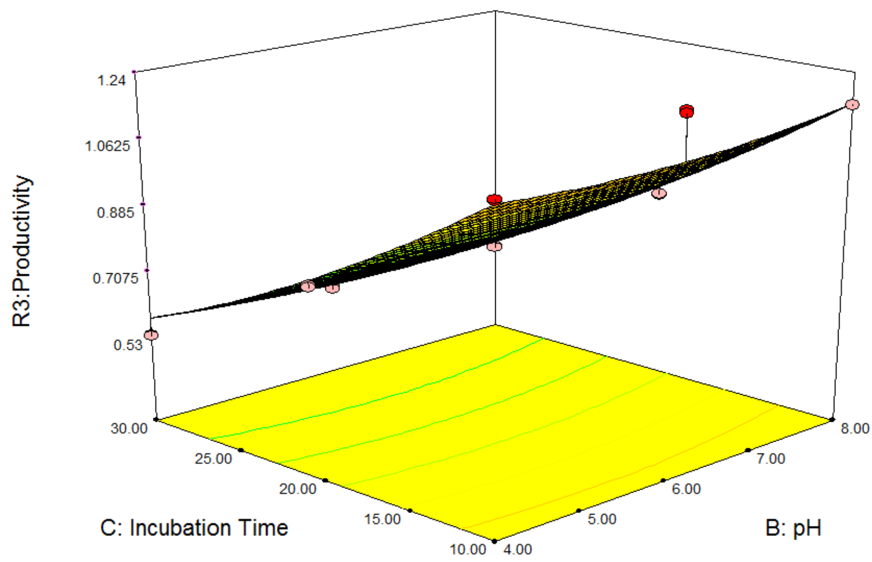


Fig. 33: The interaction effect of pH and incubation time on productivity of fermentation process

4.5. Optimizations

Now, optimization is very important in order to make the best or in order to select the most effective use of a situation or resource. So, the following tables show the process of optimizaton with the help of Design-Expert Soft-ware (version 10.0.6) for differenet objectives of optimazation.

4.5.1. Optimization for Maximum Yield of Lactic Acid

Table-15 presents optimization for maximum lactic acid yield alone i.e the maximum amounts of lactic acid that will be produced at optimum operating temprature, pH, and incubation time with out considering the other response variables. The maximum amount of lactic acid produced will be 15.3381g/L at 30ºC, 6.22, & 15.44-hr with 0.826 desirability; 15.3279 g/L at 30.00 ºC, 6.25 & 15.42-hr with 0.826 desirability; 15.3011 g/L at 30.00 ºC, 6.20, & 15.35-hr with 0.826 desirability; 15.1332 g/L at 30.00 ºC, 5.55, & 15.05-hr with 0.823 desirability; 15.8101 g/L at 30.00 ºC, 6.24, & 16.72-hr with 0.822 desirability; and 15.6702 g/L at 30.00 ºC, 5.56, & 16.46-hr (0.821 desirability) optimum temperature, pH, and incubation time respectively. But 30ºC, 6.22, & 15.44-hr with 0.826 desirability were selected as optimum temperature, pH, and incubation time respectively due to it's high desirability and lactic acid yield.

Table-15: Optimization for maximum lactic acid yield

Constraints					
Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	minimize	10	30	1	1
Lactic acid yield	maximize	9.64	17.01	1	1
Solutions					
Number	Temperature	pH	Incub. time	Lactic acid yield (g/L)	Desirability
1	**30.00**	**6.22**	**15.44**	**15.3381**	**0.826 Selected**
2	30.00	6.25	15.42	15.3279	0.826
3	30.00	6.20	15.35	15.3011	0.826
4	30.00	5.55	15.05	15.1332	0.823
5	30.00	6.24	16.72	15.8101	0.822
6	30.00	5.56	16.46	15.6702	0.821

4.5.2. Optimization for Maximum Cell Growth

Table-16 presents optimization for maximum cell growth alone i.e the maximum growth of bacteria that will be produced at optimum operating temprature, pH, and incubation time with out considering the other response variables. The maximum number of bacteria that will be produced were 4.10691E+008 cells at 30.00 ºC, 6.39, & 15.23-hr with 0.853 desirability; 4.11073E+008 cells at 30.00 ºC, 6.37, & 15.25-hr with 0.853 desirability; 4.09864E+008 cells at 30.00ºC, 6.29, & 15.20-hr with 0.853 desirability; 4.03129E+008 cells at 30.00 ºC, 5.99, & 14.98-hr with 0.852 desirability; 4.10865E+008 cells at 30.00ºC, 5.82. & 15.33-hr with 0.851 desirability; and 4.16641E+008 cells at 30.00ºC, 5.58, &15.67-hr (0.849 desirability) optimum temperature, pH, and incubation time respectively. But 30ºC, 6.39, 15.23-hr with 0.853 desirability were selected as optimum temperature, pH, and incubation time respectively due to it's high desirability and number of cells.

Table-16: Optimization for maximum cell growth

Constraints					
Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	minimize	10	30	1	1
Cell growth	maximize	4.01E+007	4.81E+008	1	1

Solutions					
Number	Temperature	pH	Incubation time	Cell growth (Number of bacteria)	Desirability
1	**30.00**	**6.39**	**15.23**	**4.10691E+008**	**0.853 Selected**
2	30.00	6.37	15.25	4.11073E+008	0.853
3	30.00	6.29	15.20	4.09864E+008	0.853
4	30.00	5.99	14.98	4.03129E+008	0.852
5	30.00	5.82	15.33	4.10865E+008	0.851
6	30.00	5.58	15.67	4.16641E+008	0.849

4.5.3. Optimization for Maximum Productivity

Table-17 presents optimization for maximum productivity alone i.e maximum productivity will be acheived at optimum operating temprature, pH, and incubation time with out considering the other response variables. The maximum productivity that will be acheived were 1.12412 g/L*hr at 30.00ºC, 8.00, & 10.01-hr with 0.949 desirability; 1.12358 g/L*hr at 30.00ºC, 7.85, & 10.00-hr with 0.949 desirability;1.12275 g/L*hr at 30.00ºC, 7.68, & 10.00-hr with 0.949 desirability; 1.116494 g/L*hr at 30.00 ºC, 6.40, &10.00-hr with 0.947 desirability; 1.11495 g/L*hr at 30.00ºC, 6.08, & 10.00-hr with 0.946 desirability; 1.11146g/L*hr at 30.00ºC, 5.36, & 10.00-hr with 0.945 desirability; 1.10506g/L*hr at 30.00 ºC, 4.05, & 10.00-hr with 0.943 desirability; 1.10477g/L*hr at 30.01 ºC, 4.00, & 10.00-hr with 0.943 desirability; and 1.09946 g/L*hr at 31.16 ºC, 4.00, & 10.00-hr (0.923 desirability) optimum temperature, pH, and incubation time respectively. But 30.00ºC, 8.00, & 10.01-hr with 0.949 desirability were selected as optimum temperature, pH, and incubation time respectively due to it’s high desirability and productivity.

Table-17: Optimization for maximum productivity

Constraints					
Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	minimize	10	30	1	1
Productivity	maximize	0.1294	1.292	1	1

Solutions					
Number	Temperature	pH	Incubation time	Productivity (g/L.hr)	Desirability
1	**30.00**	**8.00**	**10.01**	**1.12412**	**0.949 Selected**
2	30.00	7.85	10.00	1.12358	0.949
3	30.00	7.68	10.00	1.12275	0.949
4	30.00	6.40	10.00	1.11649	0.947
5	30.00	6.08	10.00	1.11495	0.946
6	30.00	5.36	10.00	1.11146	0.945
7	30.00	4.05	10.00	1.10506	0.943
8	30.01	4.00	10.00	1.10477	0.943
9	31.16	4.00	10.00	1.09946	0.923

4.5.4. Optimization for Maximum Yield and Productivity Simultaneously

Table-18 presents optimization for maximum yield and productivity together i.e the maximum amount of lactic acid that will be produced with maximum productivity at optimum operating temprature, pH, and incubation time with out considering the cell growth. Because, our interest is lactic acid production not cell mass. Then, the maximum amount of lactic acid that will produce and productivity that will be acheived were 14.5528g/L & 1.01643 g/L*hr at 30.00^{o}C, 6.36, & 13.59-hr with 0.804 desirability; 14.5547g/L & 1.0164g/L*hr at 30.00^{o}C, 6.39, & 13.60-hr with 0.804 desirability; 14.5602g/L & 1.01611g/L*hr at 30.00^{o}C, 6.41, & 13.61-hr with 0.804 desirability; 14.5021 & 1.019491g/L*hr at 30.00 ^{o}C, 6.35, & 13.48-hr with 0.804 desirability; 14.5591 & 1.01456g/L*hr at 30.00^{o}C, 6.07, & 13.61-hr with 0.803 desirability; and 13.1493g/L & 1.07058g/L*hr at 30.00^{o}C, 4.60, & 11.34-hr optimum temperature, pH, and incubation time respectively. But 30.00^{o}C, 6.36, & 13.59-hr with 0.804 desirability were selected as optimum temperature, pH, and incubation time respectively due to it's high desirability, yield and productivity.

Table-18: Optimization for maximum yield and productivity simultaneously

Constraints

Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	minimize	10	30	1	1
Lactic acid yield	maximize	9.64	17.01	1	1
Productivity	maximize	0.1294	1.292	1	1

Solutions

No.	Temperature	pH	Incub. time	Lactic acid (g/L)	Productivity (g/L.s)	Desirability
1	**30.00**	**6.36**	**13.59**	**14.5528**	**1.01643**	**0.804 Selected**
2	30.00	6.39	13.60	14.5547	1.0164	0.804
3	30.00	6.41	13.61	14.5602	1.01611	0.804
4	30.00	6.35	13.48	14.5021	1.01949	0.804
5	30.00	6.07	13.61	14.5591	1.01456	0.803
6	30.00	5.86	13.70	14.5922	1.01083	0.803
7	30.00	4.60	11.34	13.1493	1.07058	0.774

4.5.5. Optimization for Maximum Yield, Cell Growth and Productivity in the Given Ranges of Experimental Variables

Table-19 presents optimization for maximum yield, cell growth and productivity i.e the maximum amount of lactic acid that will be produced with maximum productivity and maximum cell growth at optimum operating temprature, pH, and incubation time in their ranges. since the process is type -I i.e yield of lactic acid and cell groth have direct relationship, it is important to take in to account during optimization of process condition. In this case only one solution was found. The maximum amount of lactic acid that will produce with maximum cell growth and productivity were 16.5643g/L, 4.60936E+008cells, & 0.860024g/L*hr at 30.02°C, 6.35, & 19.21-hr with 0.827 desirability.

Table-19: Optimization for maximum yield, cell growth and productivity

Constraints					
Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	is in range	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	is in range	10	30	1	1
Lactic acid concentration	maximize	9.64	17.01	1	1
Cell growth	maximize	1.9E+007	4.81E+008	1	1
Productivity	maximize	0.1294	1.292	1	1

Solutions							
Number	Temperature	pH	Incubation time	Lactic acid con.	Number cells	Productivity	Desirability
1	**30.02**	**6.35**	**19.21**	**16.5643**	**4.60936E+008**	**0.860024**	**0.827 Selected**

4.5.6. Optimization for Maximum Yield, Cell Growth and Productivity with the Objectives of Minimizing Temperature and Incubation Time

Table-20 presents optimization for maximum lactic acid yield, cell growth and productivity i.e the maximum amount of lactic acid that will be produced with maximum productivity and maximum cell growth at optimum operating temprature, pH, and incubation time approach to minimum operating temperature and incubation time. Then, the maximum amount of lactic acid that will produce with maximum cell growth and productivity were 15.6574 g/L, 4.34004E+008 cells, & 0.941168 g/L*hr at 30.00^{o}C, 6.37, & 16.30-hr with 0.810 desirability; 15.6725g/L, 4.34484E+008cells, & 0.939322g/L*hr at 30.00^{o}C, 6.19, & 16.33-hr with 0.810 desirability; and 15.5114g/L, 4.26497E+008cells, & 0.952333 g/L*hr at 30.00^{o}C, 6.61, & 15.94-hr (0.809 desirability) optimum temperature, pH, and incubation time respectively. But 30.00^{o}C, 6.37, & 16.30-hr with 0.810 desirability were selected as optimum temperature, pH, and incubation time respectively due to it's high desirability, yield, cell growth and productivity.

Table-20: Optimization for maximum yield, cell growth and productivity

Constraints					
Name	Goal	Lower Limit	Upper Limit	Weight	Weight
Temperature	minimize	30	50	1	1
pH	is in range	4	8	1	1
Incubation time	minimize	10	30	1	1
Lactic acid yield	maximize	9.64	17.01	1	1
Cell growth	maximize	4.01E+007	4.81E+008	1	1
Productivity	maximize	0.1294	1.292	1	1

Solutions							
Number	Temperature	pH	Incubation time	Lactic acid yield	Cell growth	Productivity	Desirability
1	**30.00**	**6.37**	**16.30**	**15.6574**	**4.34004E+008**	**0.941168**	**0.810 Selected**
2	30.00	6.19	16.33	15.6725	4.34484E+008	0.939322	0.810
3	30.00	6.61	15.94	15.5114	4.26497E+008	0.952333	0.809

The desirability function is the most popular solution for the multy-response optimization problem. Desirability is an objective function that ranges from zero outside of the limit to one at the goal. The numerical optimizaition finds a point that maximizes the desirability function. The desirability value of 0.810 corresponded to the maximum lactic acid yeild, cell numbers, and productivity in the given range og parameters. The following histogram shows how well each variable satisfied the criteria: values near one are good.

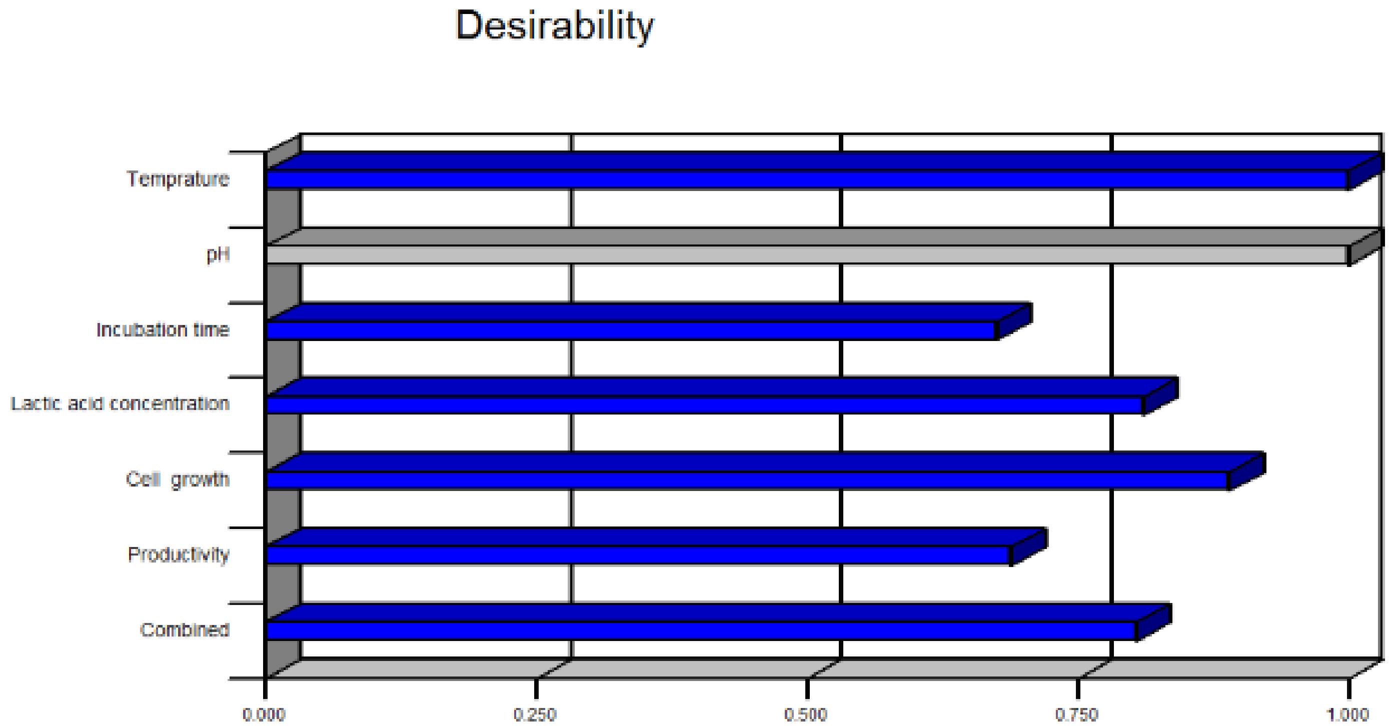


Fig. 34: Desirability of optimization process

4.6. Validation of the Model

The suitabiliy of the model equation for predicting the optimum response values was tested using the optimum conditions mentioned above. The results obtained from three replications demonstrated that the maximum amount of lactic acid, cell numbers, and productivity obtained were 16.05g/L, 4.70004E+008cells, and 0.941262g/L*hr respectively. Those results were close to the predicted values of 15.6574g/L lactic acid, 4.34004E+008 number of cells, and 0.941168g/L*hr productivity. Those results indicate that there is excellent correlation between experimental and predicted values and in turn proves the validity of the model.

Table 4-15: Maximum amount of lactic acid, number of cells and productivity at optimum points

Run	Temp. °C	pH	Time, hr	LA, g/L	Growth, No. of cells	Productivity, g/L*hr
1	30	6.37	16.30	16.07	4.73004E+008	0.951324
2	30	6.37	16.30	16.13	4.67004E+008	0.933216
3	30	6.37	16.30	15.97	4.71004E+008	0.939247

5. Conclussions and Recommendations

This final chapter is written to summarize all the results and discussion of the data presented in Chapter 3, 4 and 5. Recommendation for further study is also suggested for lactic acid fermentation by using duckweed.

5.1. Conclusions

This study was carried out in order to utilize of dense colonies of duckweed for the production of lactic acid as a carbon source. The first experimental steps were to evaluate the duckweeds to ensure the availability of nutrient needed to support the growth and consequently the production of lactic acid and screening of the best duckweed species for maximum lactic acid production. The best way to screen this duckweed was evaluation of each duckweeds in terms of their holding capacity of starch that can be converted in to simple sugar easily. The results indicated that 19.2% was presented in Lemna minuta species's of duckweed.

The second stage of the experiment was tailored to evaluate several parameters that were thought to influence the lactic acid production using duckweed as a carbon source. A three-level full factorial design was used to determine the significant factors and the optimal condition of the process variable. These screening experiments have identified that pH, temperature, and incubation time are the significant factors.

Since the screening experiments has identified the significant factors to be initial pH, temperature, incubation time and their interaction effects further activities were carried out to study in detail the correlation between lactic acid production, cell growth, productivity and these factors. The regression analysis carried out on the third stage revealed that there is a fairly strong correlation between initial pH, temperature, incubation time, and cell growth consequently on lactic acid production and productivity, whereby as the initial pH is increased, the cell growth increase until the critical initial pH of 6.37 is reached. Beyond this initial pH, the cell growth begins to decrease. A similar trend is observed for the temperature, where cell growth increased when the temperature is increased until a critical temperature of 37°C. Beyond 37°C, a reversal trend occurred.

Finally, the optimal values of tested variables with setted objectives were found to be: initial pH at 6.37, temperature, 30°C and incubation time, 16.30 hr. The maximum amount of lactic acid, cell growth and productivity predicted were determined and comparison was made between the mathematical model results and the experimental results. The results obtained from three replications demonstrated that the maximum amount of lactic acid, cell numbers, and productivity obtained were 16.05g/L, 4.70004E+008cells, and 0.941262g/L*hr respectively. Those results were close to the predicted values of 15.6574g/L lactic acid, 4.34004E+008 number of cells, and 0.941168g/L*hr productivity. In general, the preliminary results indicated that lactic acid produced using duckweed as a carbon source was comparable with those produced from other carbon source's material inculuding of starchy food crops.

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5.2. Recommendations

Based on the gaps identified in this research work, the following recommendations are made for the future researchers to bring integrated and valuable knowledge contributions to the science community.

- The screening process, and regression analysis carried out up to this extent are considered as at the preliminary stage for further optimization of the fermentation process.It is evident that this research has both national and international benefits may be attributed to solve both social and enviromental problems. Therefore, more attentions should be given and the effect of an other fermentation parameters (including minor fermentation parameters) should be analysise and incorporate in to procesess optimization.
- Since hydrolysis process has been employed as a first step in the production of lactic acid from duckweed, the effect of process parameters (time, temperature and H_2SO_4 concentration) should be analyse. Finally, the optimum point of each process parameters should be determine inorder to get the optimum amount of reducing sugar from hydrolysate of duckweed.
- The effect of non-reducing sugars and other components of duckweed hydrolysate on fermentation process should be analysed. Because, all components of hydrolysates of duckweed were subjected for fermentation with out any purification and it's effect on total amount of lactic acid produced during fermentation was not analysed.
- In both hydrolysis and fermentation optimization process, parameters sensitivity analysis can be conducted to obtain an insight into the influence of the parameters.

REFERENCES*

By Endriss Ali Umer ((2017))*

Adamberg, Kask S, Laht TM, and Paalme T, K. ((2003)). The Effect of Temperature and pH on the Growth of Lactic Acid Bacteria. International Journal of Food Microbiology, Pg:171–183.

Alemu-Gonfa ((2016)). Synergetic Effects Mandillo During Enset Fermentation: Chemical Nature and Efficiency. Addis Ababa: Addis Ababa University.

American Research Council, O. ((1992)). Applications of Biotechnology in Traditional Fermented Foods. Washington, D.C.: National Academy Press.

Antonio, G. R. ((2000)). Enhanced Production of L-(+)-Lactic Acid in Chemostat by Lactobacillus Casei DSM 20011 Using Ion-Exchange Resins and Cross-Flow Filtration in a Fully Automated Pilot Plant Controlled via NIR. Biotechnology and Bioengineering No.67, Pg: 147-156.

Auras, A. Rafael, Loong-Tak Lim, Susan E. M. Selke, Hideto Tsuji ((2011)). Wiley Series on Polymer Engineering and Technology: Poly(lactic acid):Poly(lactic acid): Synthesis, Structures, Properties, Processing, and Applications. Tokyo: John Wiley & Sons.

Axelsson and Ahrné, L. ((2000)). Lactic acid bacteria. Applied microbial systematics.

Batey, I. ((1982)). Starch Analysis Using Thermostable Alpha-Amylase. J. Starch.No. 34, Pg:125-128.

Benninga, H. ((1990)). A History of Lactic Acid Making: A Chapter in the history of Biotechnology (Chemists and Chemistry). Springer.

Borshchervskaya, T. L. Gordeeva, A.N. Kalinina, and S.P. Sineokii, L.N. ((2016)). Spectrophotometer Determination of Lactic Acid. Jornal of Analytical Chemistry , Pg:775-758.

Calvel, Abdulayef D.A., and Wangermann E., R. J. ((2001)). Factor Affecting Ash Content. Aspen Publishers.

Champomier-Verg, J., Aravindan, R., and Viruthagiri, T. s. M. ((2002)). Lactic Acid Bacteria and Proteomics: Current Knowledge and Perspectives. Journal of Chromatography B. 771, Pg: 329-342.

Cummingsand Englyst. ((1988)). Improved Method for Measurement of Dietary Fibre as Non-Starch Polysaccharides in Plant foods. Jornal of Assoc. Anal Chem. 71, Pg:808-814.

Datta, R., Tsai, S. P., Bonsignore, P., Moon, S. H., and Frank, J., R. T. ((1995)). Technological and Economic Potential of Poly(Lactic Acid) and Lactic Acid Derivatives. FEMS Microbiology Reviews. 16, Pg: 221-231.

Davison, B. E., Llanos, R. L., Cancilla, M. R., Redman, N. C., and Hillier, A. J. ((1995)). Current Research on the Genetics of Lactic Acid Production in Lactic Acid Bacteria. International Dairy Journal No.5, Pg: 763-784.

Duff and Murray, W. S. ((1996)). Bioconversion of Forest Products Industry Waste Cellulosics to Fuel Ethanol: A Review. Bioresour. Technol. 55,, Pg: 1–33.

Emmanuel-Nuamah. ((2016)). Chemical and Nutritional Compostion of Duckweed. pg:5-12.

Ethiopian Revenues and Customs Authority. ((2005-2015)). Import /Export Information. Adiss Ababa.

Gandhi, D. N., Patel, R. S., Wadhwa, B. K., Bansal, N., Kaur, M., and Kumar, G. ((2000)). Effect of Agro-Based By-Products on Production of Lactic Acid in Whey Permeate Medium. Journal of Food Science and Technology, Pg: 292-295.

Gao, Cuiqing Ma and Ping Xu,Chao. ((2011)). Biotechnological Routes Based on Lactic Acid Production From Biomass. Biotechnology Advances, Pg: 930–939.

Ghaffar, M Irshad, Z Anwar, T Aqil, Z Zulifqar ((2014)). Recent Trends in Lactic Acid Biotechnology: A Brief Review on Production to Purification. Journal of Radiation Research and Applied Sciences, Pg:222-229.

Giraud, E., Brauman, A., Kekele, S., Lelong, B., and Raimbault, M. ((1991)). Influence of pH and Initial Lactate Concentration on the Growth of Lactobacillus Plantarum. Appl Microbio1 Biotechnology, Pg:96-99.

Gorret, JL Maubois, and JM Engasser, N. ((2001)). Study of the Effects of Temperature, Ph and Yeast Extract on Growth and Exopolysaccharides Production. Jornal of Applied Microbiology.

Gyunter, E. P. ((2008)). Production of Polysaccharides by Callus Cultures of Common Duckweed. Appl Biochem Microbiol No.. 44(1), Pg:104-109.

Han and Pan, X. ((2009)). A Model for Simultaneous Crystallisation and Biodegradation of Biodegradable Polymers. Biomaterials. No.30, Pg: 423-430.

Hendrickson, R. D. ((2007), April 29 – May 2, http://sim.confex.com 29th Symposium on Biotechnology for Fuels and Chemicals. Auxiliary Enzymes for Hydrolysis of Cellulose and Hemicellulose from Liquid Hot Water Pretreated Distillers Grains.

HiMedia Laboratories Pvt. Ltd. Technical Data. ((2015)). Lactobacillus MRS Agar M641. Mumbai: HIMEDIA Laboratories Pvt. Ltd.

Hofvendahl and Hahn-Hägerdal, K. B. ((2000)). Factors Affecting the Fermentative Lactic Acid Production from Renewable Resources. Enzyme Microb Technology, Pg:87-107.

Iyer, P. R. ((2002)). Biomass Thermochemical Characterization. Third Edition; pp.38.

John, Madhavan Nampoothiri, Ashok Pandey, Rojan P. ((2007)). Fermentative Production of Lactic Acid From Biomass: An Overview on Process Developments and Future Perspectives. Applied Microbiology and Biotechnology, Pg: 524-534.

Laloknam, S. S. ((2009)). Detection of Amylase Activity from Fruit and Vegetables in Anundergraduate Classroom. . Asian Journal of Food and Agro-Industry vol.2, Pg: 402-411.

Leng, J. S. ((1995)). Duckweed - A Potential High-Protein Feed Resource for Domestic Animals And Fish. Livestock Research for Rural Development Vol.7 (N0.1), Pg:12-15.

Lin and Tanaka, S. Y. ((2006)). Ethanol Fermentation From Biomass Resources: Current State and Prospects. Appl. Microbiol. Biotechnology No. 69, Pg:627–642.

Litchfield, J. ((1996)). Microbiological Production of Lactic Acid. Advances in Applied Microbiology.

Megazyme. ((2011)). www.Total Starch Assay Procedure.com. Retrieved from www.megazyme.com/downloads/en/data/K-TSTA.pdf.

Miller, G. L. ((1959)). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry vol.31, Pg: 426-428.

Mohamed, A. A.-R. ((2011)). Lactic Acid Production from Lignocellulose-Derived Sugars Using Lactic Acid Bacteria: Overview and Limits. Journal of Biotechnology No. 156, Pg:286–301.

Nampoothiri, Nair NR, John RP., Madhavan. ((2010)). An Overview of The Recent Developments in Polylactide (PLA) Research., Pg: 493-501.

Narayanan, N., Roychoudhury, P. K., and Srivastava, A. ((2004)). L (+) Lactic Acid Fermentation and Its Product Polymerization. Electronic Journal of Biotechnology vol. 7, Pg: 167-179.

Panesar, P. S., Kennedy, J. F., Knill, C. J., and Kosseva, M. ((2010)). Production of L(+) Lactic Acid Using Lactobacillus Casei from Whey. Brazilian Archives of Biology and Technology., Pg: 53: 219-226.

Parigi-Bini, R. ((1976)). Influence of Ensiling Maize Ears and Grain on Chemical Composition, Conservation Losses and Digestibility. Animal Feed Science and Technology vol.1, Pg: 301-311.

Roberts, M. ((1990) November 5). U.S. News and World Report 109. "Keeping Yogurt Honest-Its Reputation as a Health Food May Not Always be Deserved.", p. 76.

Rojan, G.S., Anisha, K.M,. Nampoothiri, and A., Pandey R.P. ((2009)). Direct Lactic Acid Fermentation: Focus on Simultaneous Saccharification and Lactic Acid Production. Biotechnology Advances No. 27, Pg:145–152.

Roslina-Rashid. ((2008)). Optimization and Modeling of Lactic Acid Production From Pineapple Waste. Faculty of Chemical and Natural Resources Engineering,Universiti Teknologi Malaysia.

Sigma-Aldrich. ((2011), 12 11). UK Datasheets. Retrieved from www.sigmaaldrich.com.

killicorn, W., Spira, W., Journey P.- The World Bank Group ((1993)). Duckweed Aquaculture. A New Aquatic Farming System for Developing Countries. . Washington D.C.: A world Bank Publication.

Srivastava, Pradip K.Roychoudhury and Vikram Sahai, Aradhana ((1992)). Extractive Lactic Acid Fermentation Using Ion Exchange Resins. Biotechnology and Bioengineering, Pg: 607-613. vol. 39,.

Thang V. H. and Novalin, S. ((2008)). Green Biorefinery: Separation of Lactic Acid from Grass Silage Juice by Chromatography using Neutral Polymeric Resin. Bioresource Technology vol.99:, Pg:4368-4379.

Tonukari, N. J. ((2004)). Cassava and the Future Of Starch. . Electronic Journal o fBiotechnology vol.7, Pg:5-8.

Tripathi, S.K., Srivastava, Prashant Singh, R.P Singh, S.P., Singh, Alok Jha, Poonam Yadav, A.D. ((2015)). Optimization of process variables for Enhanced Lactic Acid Production Utilizing Paneer Whey as Substrate in SMF. Applied Food Biotechnology, Pg: 46-55.

Vijayakumar, J., Aravindan, R., and Viruthagiri, T. ((2008)). Recent Trends in The Production, Purification And Application of Lactic Acid. Chemical and Biochemical Engineering Quarterly. 22, Pg: 245-264.

Wee, Y. J., Kim, J. N., and Ryu, H. W. ((2006)). Batch and Repeated Batch Production of L (+)-lactic acid by Enterococcus Faecalis RKY1 Using Woodhydrolyzate and Corn Steep Liquor. Journal Industrial Microbiology Biotechnolog No. 33, Pg: 431-435.

Xin-Zhao. ((2014)). Optimization of the Production of Bioethanol from Duckweed. University of East Anglia.

Zhang, B., Jin, J.M., and Kelly, Z.Y. ((2007)). Production of Lactic Acid from Renewable Materials. Biochemical Engineering Journal 35, Pg: 251–263.

APPENDICES

Appendix A

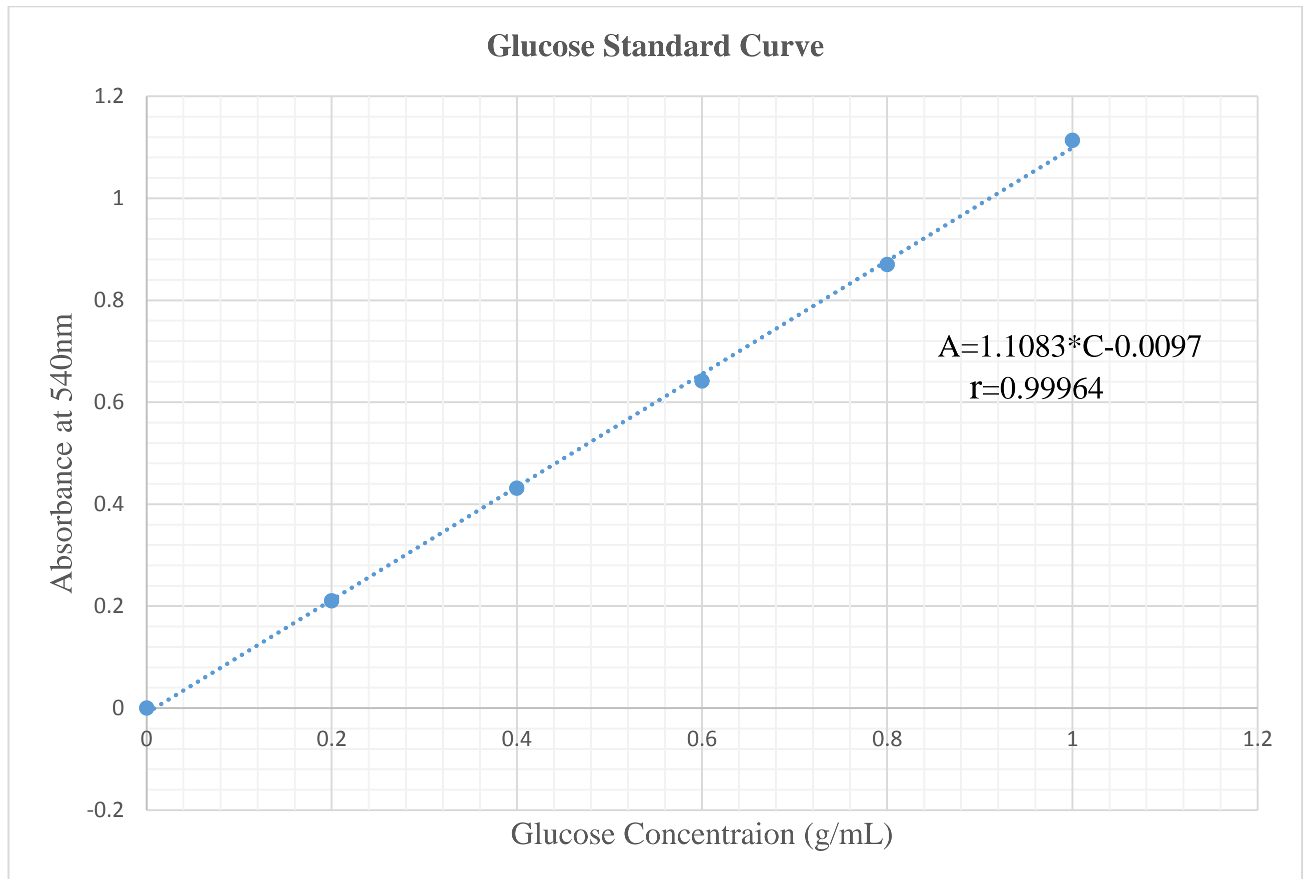
Experimental results for optimization

Std	Run	Block	Factor 1 A: Temprature; ºC	Factor 2 B: pH	Factor 3 C: incubation time; hr	Response 1 R-1: Lactic acid con.; g/L	Response 2 R-2: Cell growth; Numbers	Response 3 R-3: Productivity; g/hr
48	1	Block 3	50	6	30	14.39	7.33E+007	0.4797
47	2	Block 3	50	6	30	14.31	7.33E+007	0.477
26	3	Block 1	30	6	20	17.01	4.81E+008	0.8505
16	4	Block 2	40	8	10	11.54	1.57E+008	1.154
37	5	Block 1	30	4	30	16.73	8.64E+007	0.5577
29	6	Block 3	50	6	20	13.03	3.9E+008	0.6515
13	7	Block 1	30	8	10	12.08	1.61E+008	1.208
32	8	Block 1	30	8	20	16.86	4.41E+008	0.843
19	9	Block 1	30	4	20	16.71	4.23E+008	0.8355
52	10	Block 2	40	8	30	16.07	8.65E+007	0.5357
14	11	Block 1	30	8	10	12.03	1.61E+008	1.203
36	12	Block 3	50	8	20	12.85	3.69E+008	0.6425
5	13	Block 3	50	4	10	9.78	1.9E+007	0.978
15	14	Block 2	40	8	10	11.54	1.57E+008	1.154
39	15	Block 2	40	4	30	16.06	8.57E+007	0.5353
23	16	Block 3	50	4	20	12.82	3.57E+008	0.641
54	17	Block 3	50	8	30	14.17	7.12E+007	0.4713
43	18	Block 1	30	6	30	17	9.21E+007	0.5667
7	19	Block 1	30	6	10	12.92	2E+008	1.292
46	20	Block 2	40	6	30	16.03	8.9E+007	0.5343
40	21	Block 2	40	4	30	16.01	8.57E+007	0.5337
11	22	Block 3	50	6	10	10.89	8.5E+007	1.089
42	23	Block 3	50	4	30	13.98	7.01E+007	0.466
3	24	Block 2	40	4	10	11.28	1.32E+008	1.128
1	25	Block 1	30	4	10	11.82	1.34E+008	1.182
33	26	Block 2	40	8	20	16.18	4.29E+008	0.809
30	27	Block 3	50	6	20	13.12	3.9E+008	0.656
20	28	Block 1	30	4	20	16.58	4.23E+008	0.829
10	29	Block 2	40	6	10	12.29	1.98E+008	1.229
51	30	Block 2	40	8	30	16.24	8.65E+007	0.5413
35	31	Block 3	50	8	20	12.97	3.69E+008	0.6485
49	32	Block 1	30	8	30	16.91	8.81E+007	0.5637
12	33	Block 3	50	6	10	10.91	8.5E+007	1.091
17	34	Block 3	50	8	10	10.04	1.09E+008	1.004
50	35	Block 1	30	8	30	16.86	8.81E+007	0.562
18	36	Block 3	50	8	10	11.01	1.09E+008	1.101
9	37	Block 2	40	6	10	12.38	1.98E+008	1.238
22	38	Block 2	40	4	20	15.92	4.17E+008	0.796
38	39	Block 1	30	4	30	16.76	8.64E+007	0.5587

4	40	Block 2	40	4	10	11.31	1.32E+008	1.131
45	41	Block 2	40	6	30	16.33	8.9E+007	0.5443
24	42	Block 3	50	4	20	12.81	3.57E+008	0.6405
53	43	Block 3	50	8	30	14.22	7.12E+007	0.474
27	44	Block 2	40	6	20	16.27	4.54E+008	0.8135
21	45	Block 2	40	4	20	16.03	4.17E+008	0.8015
41	46	Block 3	50	4	30	14.04	7.01E+007	0.468
25	47	Block 1	30	6	20	16.94	4.81E+008	0.847
34	48	Block 2	40	8	20	16.23	4.29E+008	0.8115
44	49	Block 1	30	6	30	16.89	9.21E+007	0.563
8	50	Block 1	30	6	10	12.94	2E+008	0.1294
6	51	Block 3	50	4	10	9.64	1.9E+007	0.964
28	52	Block 2	40	6	20	16.52	4.54E+008	0.826
31	53	Block 1	30	8	20	16.86	4.41E+008	0.843
2	54	Block 1	30	4	10	11.73	1.34E+008	1.173

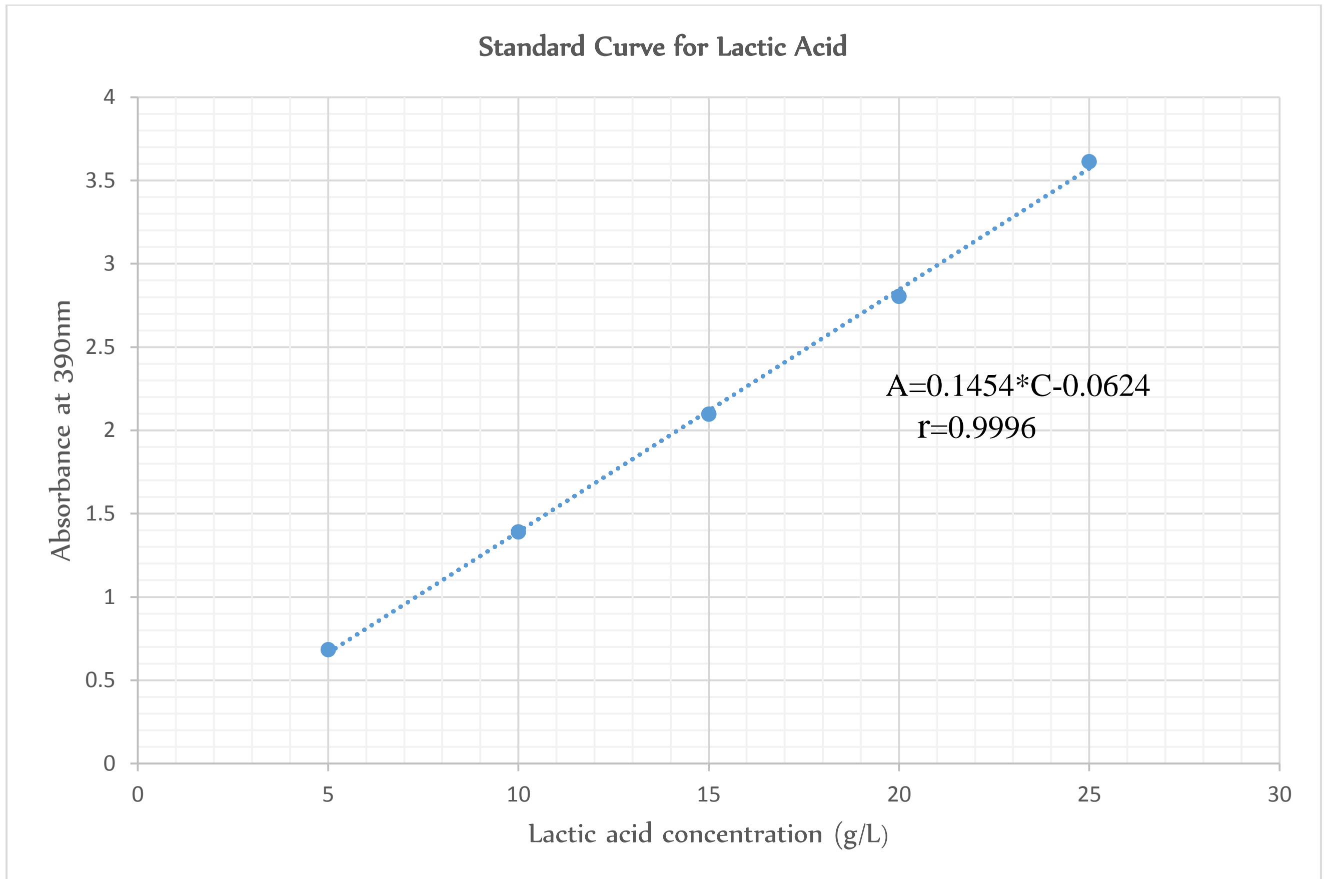
Appendix B

Standard curve of reducing sugar



Appendix C

Standard curve of lactic acid



Appendix D

Researcher performance inside the laboratory