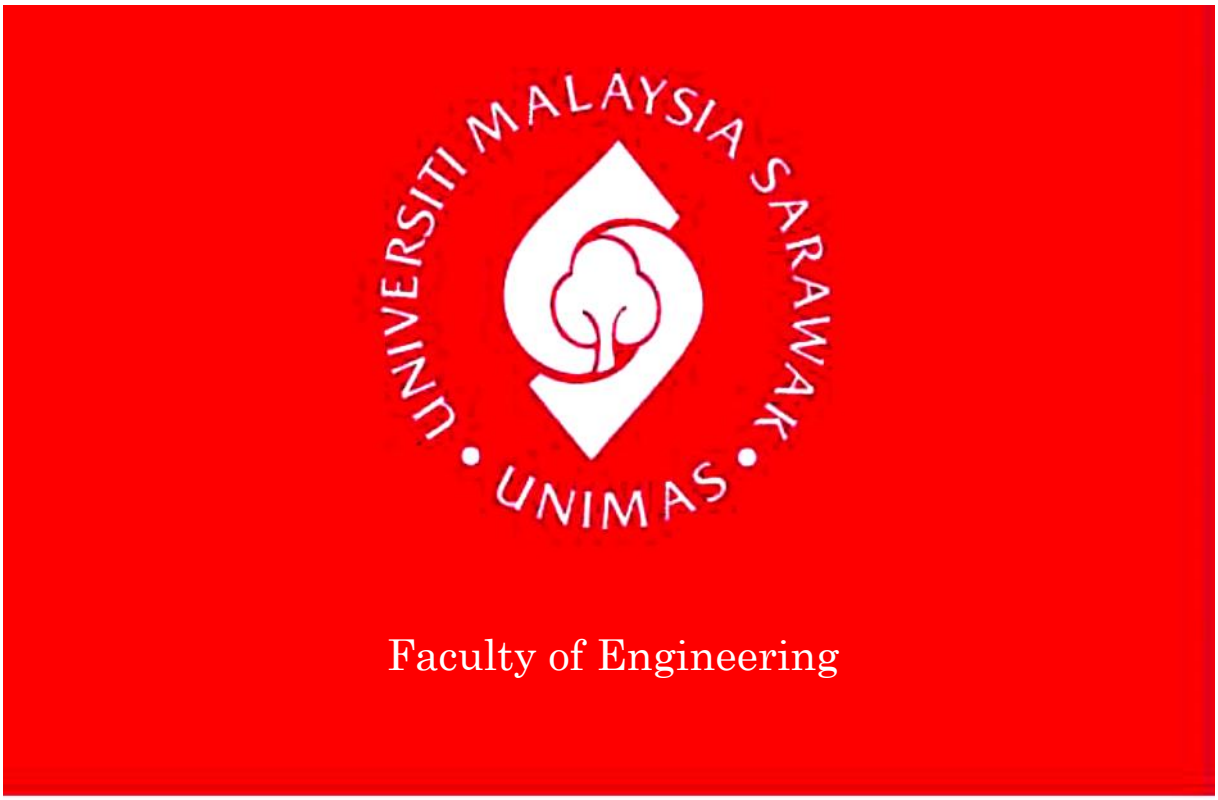

SCALING UP OF LACTIC ACID FERMENTATION USING *Enterococcus faecalis*

Eivo anak Kelvin

Bachelor of Engineering with Honours

(Chemical Engineering)

2018

UNIVERSITI MALAYSIA SARAWAK

Grade:

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Final Year Project Report	
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PhD	

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This declaration is made on the 25th of May 2018.

Student's Declaration:

I, EIVO ANAK KELVIN (45533), DEPARTMENT OF CHEMICAL ENGINEERING AND ENERGY SUSTAINABILITY, FACULTY OF ENGINEERING hereby declare that the work entitled, SCALING UP OF LACTIC ACID FERMENTATION USING *ENTEROCOCCUS FAECALIS* is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

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Name of the student (Matric No.)

Final Year Project Supervisor's Declaration:

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APPROVAL SHEET

This final year projects report which entitled "Scaling up of Lactic Acid Fermentation Using *Enterococcus faecalis*" was prepared by Eivo Anak Kelvin (45533) as a partial KNC 4344 Final Year Project course fulfilment for the Bachelor of Engineering with Honours (Chemical Engineering) is hereby read and approved by:

……

CIRILO NOLASCO HIPOLITO
(Supervisor)

25th May 2018
Date

SCALING UP OF LACTIC ACID FERMENTATION USING *Enterococcus faecalis*

EIVO ANAK KELVIN (45533)

This project is submitted in partial fulfilment

of the requirement for the Degree of

Bachelor of Engineering with Honours

(Chemical Engineering)

Faculty of Engineering

University Malaysia Sarawak

2018

Dedicated to my beloved parents and siblings who always bestow us unconditional love, motivations and encouragements. All glory to God in the highest!

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ABSTRACT

Enterococcus faecalis is a Lactic Acid bacterium and was used to scaling up a lactic acid fermentation (LAF) from laboratory scale to pilot scale. Scaling up of LAF as the preferred embodiment of industry is a major concern in this study. Develop a large scale of LAF always became a huge challenge because of scarce data is available in large scale. LAF is studied in order to scale up the fermentation process from 3-litre bioreactor fermenter to 100-litre open stirred tank reactor (STR). Fermentations in batch mode for Lab and Pilot scales results were compared in terms of productivity, and yield by following the kinetics of the growth, lactic acid production, and glucose consumption. The fermentations were performed at pH 6.86, 45 C, and 100 rpm. Lactic acid (LA) produced by the strain was titrated in situ using 10M NaOH. Simulation of the fermentation was conducted by using SuperPro Software and data from pilot scale. The results of the fermentation for both scales were analysed and compared using t-test and ANOVA. The results showed the production rate for laboratory scale of laboratory 1, 2 and 3 is 5.06 g/L·h, 4.97 g/L·h, and 4.84 g/L·h respectively. While for pilot scale, the production rate of Pilot 1 and Pilot 2 is 1.53 g/L·h and 3.91 g/L·h respectively. The yield for laboratory 1, laboratory 2, laboratory 3, pilot 1 and pilot 2 is 0.93, 0.94, 0.93, 0.92 and 0.91 respectively. After comparing the production rate between laboratory and pilot scale using ANOVA, it was found that, there is no significant difference between both processes. Therefore, the LA production rate of pilot scale was the same as laboratory scale using *Enterococcus faecalis* bacteria.

Key words: *Enterococcus faecalis*, lactic acid, lactic acid fermentation, scaling up

ABSTRAK

Enterococcus faecalis adalah bakteria asid Laktik dan digunakan untuk meningkatkan fermentasi asid laktik (LAF) dari skala makmal ke skala pilot. Peningkatan LAF sebagai perwujudan industri adalah kebimbangan utama dalam kajian ini. Mengembangkan skala besar LAF sentiasa menjadi cabaran besar kerana data yang terhad terdapat dalam skala besar. LAF dipelajari untuk meningkatkan proses fermentasi dari fermenter bioreaktor 3-liter kepada reaktor tangki terbuka 100-liter (STR). Fermentasi dalam mod batch untuk keputusan Makmal dan Pilot telah dibandingkan dari segi produktiviti, dan hasil dengan mengikuti pertumbuhan kinetik, pengeluaran asid laktik, dan penggunaan glukosa. Fermentasi dilakukan pada pH 6.86, 45 C, dan 100 rpm. Asid laktik (LA) yang dihasilkan oleh bakteria dinaturalisasi menggunakan 10M NaOH. Simulasi fermentasi dilakukan dengan menggunakan Perisian SuperPro dan data dari skala pilot. Hasil fermentasi untuk kedua-dua skala dianalisis dan dibandingkan menggunakan T-test dan ANOVA. Keputusan menunjukkan kadar produktiviti untuk skala makmal 1, 2 dan 3 adalah 5.06 g/L·h, 4.97 g/L·h, dan 4.84 g/L·h masing-masing. Sementara untuk skala pilot, kadar pengeluaran Pilot 1 dan Pilot 2 masing-masing 1.53 g/L·h dan 3.91 g/L·h. Hasil untuk makmal 1, makmal 2, makmal 3, pilot 1 dan pilot 2 masing-masing adalah 0.93, 0.94, 0.93, 0.92 dan 0.91. Selepas membandingkan kadar produktiviti antara skala makmal dengan skala pilot menggunakan ANOVA, didapati bahawa tidak terdapat perbezaan yang signifikan antara kedua-dua proses. Oleh itu, kadar produktiviti skala pilot LA adalah sama dengan skala makmal yang menggunakan bakteria *Enterococcus faecalis*.

Kata kunci: *Enterococcus faecalis*, asid laktik, fermentasi asid laktik, skala besar

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ABBREVIATIONS

μ	Microorganisms specific growth rate
μ_{max}	Maximum specific growth rate
AHA	Alpha hydroxy acid
$Ca(OH)_2$	Calcium hydroxide
cdw	Cell dry weight
DCW	Dry Cell Weight
DNS	Dinitrosalicylic acid
DO	Dissolve oxygen
g_c	Centrifugal force
GRAS	Generally recognized as safe
HPLC	High Performance Liquid Chromatography
Ks	Half-velocity constant
LA	Lactic acid
LAB	Lactic acid bacteria
NaOH	Sodium hydroxide
pH	Potential hydrogen
PLA	Poly-lactic acid
$q_{p\ max}$	Maximum specific production rate
$q_{s\ max}$	Maximum specific sugar utilization rate
$r_{A\ Lab}$	Laboratory production rate
$r_{A\ STR}$	Stirred tank reactor production rate
ROP	Ring opening polymerization
r_p	Production rate
rpm	Revolution per minute
r_s	Substrate consumption rate
r_x	Cell growth rate
STR	Stirred tank reactor
$Y_{P/S}$	Yield of product per substrate
$Y_{X/S}$	Yield of biomass per substrate
α	growth-associated constant

NOMENCULTURE

%	Percentage
˚Brix	Degree Brix
˚C	Degree Celsius
µm	Micrometre
g	Gram
g g^{-1}	Gram per gram
g g^{-1} h^{-1}	Gram per gram per hour
g/L	Gram per litre
g/L·h	Gram per litre per hour
g/ml	Gram per milli-litre
g/mol	Gram per mole
h	Hour
J/g·K	Joule per gram per Kelvin
J/mol·˚C	Joule per mole per Degree Celsius
KJ/mol	Kilo-Joule per mole
L	Litre
M	Molar
ml	Milli-litre
mmol L^{-1}	Milli-mole per litre
mPa·s	Millipascal-second
N	Normality
wt %	Weightage percentage
$\boldsymbol{\rho}$	Density

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In modern chemical terminology, lactic acid known as 2-hydroxypropionic acid and it belongs to carboxyl functional group. In nature, it is widely occurring as carboxylic acid and known as milk acid. Swedish chemist, Carl Wilhelm Scheele in 1790, first isolated it but Charles E. Avery has first produced it commercially in Littleton, Massachusetts, the USA in 1881 (Ren, 2010).

Lactic Acid (LA) contains two reactive functional groups, which are carboxylic group and hydroxyl group. Due to the functional groups, it was considered the most potential feedstock monomer for chemical conversions (Wee, et al., 2006). LA can be produced by two different routes, which are by chemical synthesis or by microbial fermentation. Chemical synthesis process usually from petrochemical resources and produces a racemic mixture of DL-lactic acid, which is the main disadvantage of the process. On the other hand, microbial fermentation has an advantage in term of utilization of biomass, low production temperature, low energy consumption and high purity of LA by selecting an appropriate strain of Lactic Acid Bacteria (LAB). Due to its wide range of application, the demand of LA increased considerably. (Abdel-Rahman, et al., 2011). The four main applications of LA are food, chemicals, cosmetic and pharmaceutical industries. Approximately 70% of LA produced is used in the food industry because of its role in the production of cheese and yogurt (Martinez, et al., 2013). In manufacturing industry of diary product from LAB, it is convenient to divide them into two groups, which are mesophiles and thermophiles (Robinson, 1993). The worldwide demand for LA is increasing every year where it is estimated roughly to be 130 000 to 150 000 tons per year (Ghaffar, et al., 2014).

L-lactic acid (L-LA) has received much attention because it has many industrial application and mainly because of its use as a starting material for the synthesis of poly-lactic acid (PLA), a biodegradable plastic. PLA is one of the most promising polymers that could play an important role in solving worldwide environmental problems (Rydz, et al., 2014). For the past 15 years, PLA has been study widely (Mehta, et al., 2005). PLA belongs to the group of aliphatic polyesters and it usually made of a-hydroxy acids (Garlotta, 2002). In last decade, world LA production has expanded 10-fold due to increased demand for green products derived from LA, including ethyl lactate and PLA (Miller, et al., 2011). The company Cargill has produced PLA commercially, since 2002 (Waldron, 2009). However, it seems difficult that PLA can compete with conventional plastics derived from petrochemicals because it is exclusively dependent on its cost in commercial production (Avérous & Pollet, 2012). The industrial production of LA is an old-fashioned industry since 1881 (Ren, 2010). It has been a long process to develop this industry since it is difficult to produce LA commercially as an intermediate for biodegradable plastics, because it is well reported that LAB require expensive nitrogen sources such as yeast extract and the substrate is an issue in this fermentative process (Abbasiliasi, et al., 2017). Furthermore, it is also very difficult to separate cells from the culture broth in the process of recovery and purification of LA (Yang, et al., 2007). Thus, the commercial-scale production of L-LA by bacteria as an intermediate for biodegradable plastics is controversial from the viewpoint of economics and engineering (Miura, et al., 2003). Some researchers claim the use of fungi such as *Rhizopus* for the commercial production of L-LA because they do not require organic nitrogen sources and are easily separated from the culture broth in the process of recovery and purification of the LA produced (Maas, et al., 2006). However, this fermentative process is also controversial since there are many problems associated with the growing of the fungus and low productivity (Magnuson & Lasure, 2004). The low production rate in fungal fermentation, below 3 g/L·h, is most likely due to the low reaction rate caused by mass transfer limitations. Lower product yields in fungal fermentation are also partially attributed to the formation of by-products, such as fumaric acid and ethanol (Wee, et al., 2006). Therefore, the seek for microorganism that can overcome these kind of problems are focus of many research studies. Upon the success of isolation, purification of novel microorganism, the next step is to test the fermentative capacities of the isolated strain and then the scaling up to pilot plant level to assess the parameters optimized in laboratory scale can be reproduced in pilot level with the aim to further go to commercial scale. The

scaling up of lactic acid fermentation (LAF) as the preferred embodiment of industry is a major concern in this study. Develop a large scale of LAF always became a huge challenge because of scarce data available in large scale.

Enterococcus faecalis is a new strain isolated in the laboratory of Biochemistry of the faculty of Resource Science and Technology at UNIMAS. The strain is a thermophilic microorganism and is able to produce more than 100 g/L of LA. Moreover, the strain has a production rate of approximately 5 g/L·h. This production rate is among the maximum productivity reported for a LAB such as a genus Lactobacillus (Hofvendahl & Hahn-Hägerdal, 2000). However, there is no report on this new isolate in the literature and less on the scaling up to at least 100-litre Stirred Tank Reactor (STR).

As mentioned before, there is not much research for the past 10 years for LA scaling up fermentation in pilot scale. However, there is one research conducted on the application of the biorefinery concept to produce L-LA from soybean vinasse at laboratory and pilot scale (Karp, et al., 2010). This research using soybean vinasse as a substrate and *Lactobacillus agilis* LPB 56 as a LAB. The soybean vinasse is a by-product of ethanol fermentation of soybean molasses. The soybean vinasse must undergo pretreatment before it can be used as a medium in LA production. The research found that the kinetic and yield parameters for laboratory scale were 0.864 and 0.0162 for $Y_{P/S}$ and $Y_{X/S}$, 0.0145 g/L h (r_x), 1.32 g/L h (r_s) and 1.13 g/L h (r_p). While for the pilot plant, kinetic and yield parameters were 0.849 and 0.0353 for $Y_{P/S}$ and $Y_{X/S}$, 0.0278 g/L h (r_x), 0.915 g/L h (r_s) and 0.863 g/L h (r_p). By comparing these data, product yield from sugar ($Y_{P/S}$) slightly decrease after the scaling up from 0.864 to 0.849. Nevertheless, both productivities are low.

From the research, it can be seen that different substrate used in the scaling up of LAF. Moreover, the substrate must undergo pretreatment before it can be used as a medium in LA production. The disadvantage of pretreatment process is it will cause an increase in capital cost in the production where the cost of pretreatment is considered including capital cost (Yang, et al., 2013). Thus, there is a need to conduct a study on the scaling up of lactic acid fermentation using an open stirred tank reactor (STR) as the preferred embodiment of the industry

The study was carried out in 3-litre jar fermenter batch system for lab scale experiment and 100-litre open stirred tank reactor (STR) for scale-up by using *Enterococcus faecalis* as a preferred microorganism. The data from the laboratory scale was used to conducting simulation using SuperPro software.

1.2 Scope of Study

The scope of the study focuses on scaling up of LAF in open stirred tank reactor (STR) using glucose. The study includes on the reactor modelling of the LAF and fermentation simulation (SuperPro Software) based on the fermentation of pilot scale data. The LAB used in the study is *Enterococcus* faecalis and the fermentation system is a batch system for both laboratory scale and pilot scale.

1.3 Aim and Objectives

The aim of the study is the scaling up of LAF using an open STR to study whether the parameter obtained in laboratory scale can be reproduced at higher scale. In order to prove the hypothesis some objectives are required as the following:

i. To develop a bioprocess to produce LA using *Enterococcus faecalis* in laboratory scale as well as in pilot scale.

ii. To compare the kinetic and yield parameters in bioprocess to produce LA for laboratory scale and pilot scale.

iii. To compare the performance of LAF in the pilot scale and laboratory scale in term of productivity.

iv. To perform process simulation using SuperPro software.

1.4 Hypothesis

The LA production rate of pilot scale is the same as laboratory scale using *Enterococcus faecalis* bacteria. Mathematically it can be expressed as Null Hypothesis as follow:

$$r_{A_{Lab}} = r_{A_{STR}} \quad \textbf{1.1}$$

CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid

Lactic acid (LA) is also known as milk acid and it is an acid ingredient of sour dairy products fermented fruits, vegetables, and sausages. Earlier it was discovered by Swedish chemist in 1780 by Carl Wilhelm Scheele where it was isolated from sour milk as impure brown syrup. Lactic acid is a carboxylic acid that contains a hydroxyl group adjacent to the carboxyl group thus, it is considered as an alpha hydroxy acid (AHA). It is non-volatile, odorless organic acid and is classified as GRAS (generally recognized as safe) for use in food by the Food and Drug Administration and other regulatory agencies (Yang, et al., 2013).

Nearly every form of organized life produces LA. Human, for example, LA is produced by every organ except red blood cell by cytoplasm in the cell through anaerobic metabolism of glucose. This process is called glycolysis where the anaerobic breakdown of glucose to LA (Herlihy, 2014). In microorganism, LA can be produced by certain bacteria and these bacteria known as "lactic acid bacteria" (LAB) which is a group of gram-positive bacteria. The LAB produces LA as the main fermentation product of carbohydrates and in taxonomic classification, they belong to the phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales*. Other than *Lactobacillaceae,* other families include *Enterococcaceae*, *Aerococcaceae*, *Carnobacteriacea*, *Leuconostocaceae*, and *Streptococcaceae* (Lahtinen S. , et al., 2012).

The LA molecule consists of three carbons and because of its chiral carbon atom, it exists in two enantiomeric forms, which are L-LA and D-LA. L-LA and D-LA also called S-LA and R-LA respectively. **Figure 2.1** shows the structure arrangement of L-LA and D-LA. These isomers can be further upgrade or synthesis into polymer known as

poly-lactic acid (PLA). The synthesis of PLA can undergo two major routes where are direct polycondensation of LA and ring opening polymerization (ROP) of lactide (Lorenzo & Androsch, 2017). **Table 2.1** shows the physical properties of the LA.

Table 2. 1: Physical properties of LA (Auras, et al., 2010*)*

Property	**Value**
CAS number	General: 50-21-5 L-lactic acid: 79-33-4 D-lactic acid: 10326-41-7
Molecular weight (g/mol)	90.08
Formula	$C_3H_6O_3$
Density (g/ml, 20 ˚C)	1.186 (80.8% in water)
Melting point (˚C)	18 (racemic) 53 (chiral pure)
Boiling point (˚C)	122 (at 14 mmHg)
Crystal structure	L-lactic acid: orthorhombic, space group $P2_12_12_1$
Solid density (g/ml)	1.33 (solid, 20 ˚C)
Solubility in water (wt%)	86 (20 ˚C, monomeric L-lactic acid)
Heat capacity (J/mol·˚C)	190 (DL-lactic acid, 20 ˚C)
Heat of solution (kJ/mol)	7.79 (at 20 ˚C)
Heat of fusion (kJ/mol)	L-lactic acid: 16.8
Viscosity (mPa s)	28.5 (85.3% solution in water, 25 ˚C)
pK_a	3.86
Specific heat (J/g K) at 25 ˚C	Crystalline L-lactic acid: 1.41 Liquid lactic acid: 2.34

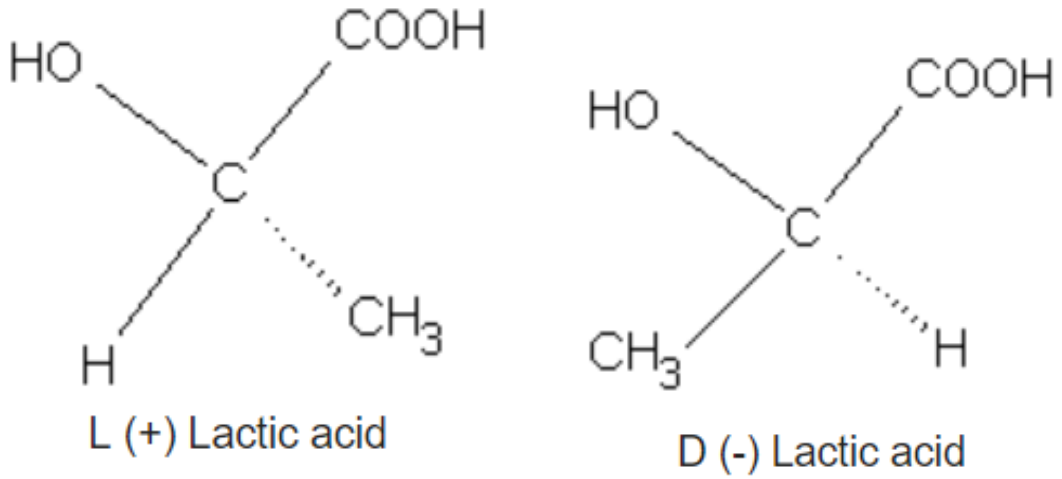

Figure 2. 1: LA structure arrangement (Narayanan, et al., 2004)

2.2 Application of Lactic Acid

Lactic acid is considered as well know organic acid as it has a wide range in any industrial application. The four main applications of LA are in food, chemicals, cosmetic and pharmaceutical industries. Besides LA plays a big role in the preparation of fermented dairy products, wine making, curing fish, vegetable baking, and preparation of pickling (Alsaheb, et al., 2015). **Figure 2.2** shows the wide application of LA in various industries.

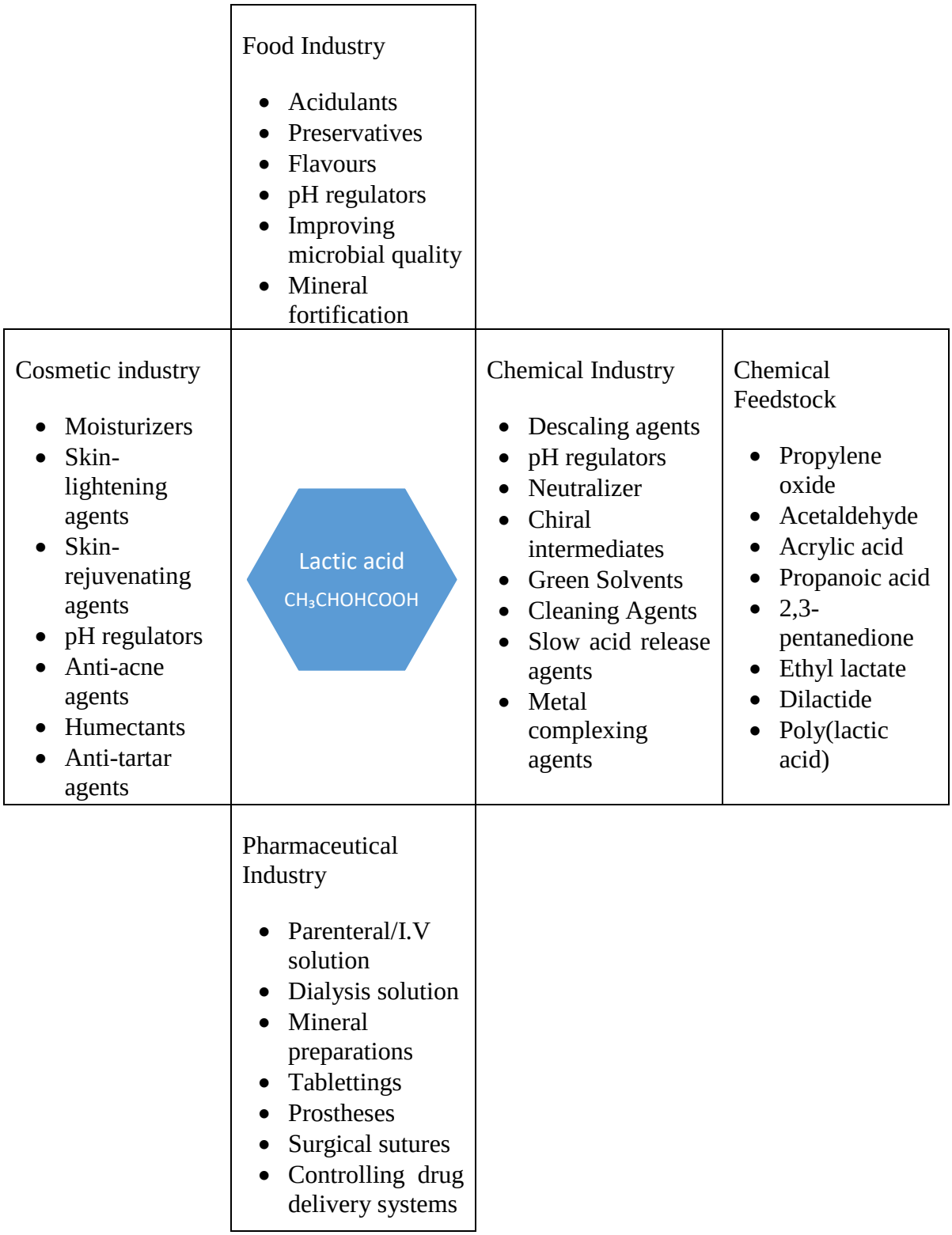

Figure 2. 2: Commercial uses and applications of LA (Wee, et al., 2006)

In the food industry, LA is used as a flavoring, pH regulator; improve microbial quality and mineral fortification. In commercial scale, it is used in processed meat and poultry industries where it provides with increase shelf life, enhanced flavor and better