การผลิตกรดแล็กติกในจุลินทรีย์ดัดแปลงพันธุกรรม *Escherichia coli*

นายฐากูร แย้มโอษฐ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

LACTIC ACID PRODUCTION IN GENETICALLY MODIFIED *Escherichia coli*

Mr. Thagoon Yamoat

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2014

Thesis Title	LACTIC ACID PRODUCTION IN GENETICALLY MODIFIED *Escherichia coli*
By	Mr. Thagoon Yamoat
Field of Study	Biotechnology
Thesis Advisor	Ruethairat Boonsombat, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Polkit Sangvanich, Ph.D.)

.....Thesis Advisor

(Ruethairat Boonsombat, Ph.D.)

.....Examiner

(Associate Professor Chanpen Chanchao, Ph.D.)

.....External Examiner

(Wanilada Rungrassamee, Ph.D.)

ฐากูร แย้มโอษฐ์ : การผลิตกรดแล็กติกในจุลินทรีย์ดัดแปลงพันธุกรรม *Escherichia coli* (LACTIC ACID PRODUCTION IN GENETICALLY MODIFIED *Escherichia coli*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ฤทัยรัตน์ บุญสมบัติ, 97 หน้า.

กรดแล็กติกเป็นกรดอินทรีย์สามารถแบ่งออกได้เป็น 2 ไอโซเมอร์ คือ กรดแอล-แล็กติก และดี-แล็กติก ซึ่งกรดแล็กติกถูกนำมาใช้ในอุตสาหกรรมการผลิตต่าง ๆ มากมาย รวมไปถึงการนำแล็กติกที่บริสุทธิ์มาใช้เป็นสารตั้งต้นในการผลิตพอลิแล็กไตด์ (Polylactide: PLA) เพื่อใช้ในการผลิตพลาสติกที่ย่อยสลายเองได้ (Biodegradable plastic) ถึงแม้ว่า *Rhizopus oryzae* จะมีศักยภาพในการผลิตกรดแอล-แล็กติกที่บริสุทธิ์แต่ยังมีข้อจำกัดบางอย่าง เช่น ลักษณะทางสัณฐานวิทยาไม่เหมาะสมต่อการหมักในถังปฏิกรณ์ชีวภาพ เป้าหมายในงานวิจัยนี้จึงเป็นการศึกษาการผลิตกรดแล็กติกใน *Escherichia coli* ที่มีการดัดแปลงพันธุกรรมของยีนที่เกี่ยวข้องในวิถีการหมัก และใน *E. coli* ที่มีการแสดงออกของยีน *ldhA* จาก *R. oryzae* บนพลาสมิด จากการทดลองนี้ *E. coli* สายพันธุ์ที่มีการทำลายยีน *pta* และ *ldhA* หากทำลายยีน *pflB* หรือ *adhE* เพิ่ม จะไม่สามารถเจริญเติบโตได้ในสภาวะที่ไม่มีออกซิเจน นอกเหนือไปจากนั้นการแสดงออกของยีน *ldhA* จาก *R. oryzae* บนพลาสมิด ไม่สามารถทำให้เจริญเติบโตได้ในสภาวะดังกล่าว ส่วนสายพันธุ์ที่มีการทำลายยีน *pta ldhA frdC* สามารถเจริญเติบโตได้ในสภาวะที่ไม่มีออกซิเจนแต่พบว่ามีการเจริญเติบโตน้อย แต่เมื่อมียีน *ldhA* จาก *R. oryzae* บนพลาสมิดมาแสดงออกพบว่าสามารถทำให้การเจริญเติบโตในสภาวะดังกล่าวดีขึ้น *E. coli* AP20 สายพันธุ์ที่มีการทำลายยีน *pta ldhA frdC* และมีการแสดงออกของยีน *ldhA* จาก *R. oryzae* บนพลาสมิดได้ถูกนำไปทดสอบการแสดงออกของยีนด้วยวิธี qRT-PCR แต่อย่างไรก็ตามพบว่ากิจกรรมของเอนไซม์ Lactate dehydrogenase และอัตราการเจริญเติบโตของสายพันธุ์ *E. coli* AP20 มีค่าน้อยกว่า *E. coli* สายพันธุ์ดั้งเดิม หลังจากทำการหมักในอาหารที่มีน้ำตาลกลูโคส 2 เปอร์เซ็นต์ พบการผลิตกรดแอล-แล็กติกในปริมาณที่น้อย และพบผลิตภัณฑ์อื่น ๆ มีการผลิตลดน้อยลงยกเว้นเอทานอล ทั้งนี้อาจเป็นเพราะว่าการหมุนเวียนของ NADH ไม่เพียงพอในวิถีการหมัก การใช้น้ำตาลกลูโคสถูกยับยั้งในกระบวนการ phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) หรือเป็นเพราะการแสดงออกของโปรตีนจากยูแคริโอตในโพรแคริโอต

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5571961623 : MAJOR BIOTECHNOLOGY

KEYWORDS: LACTIC ACID / ESCHERICHIA COLI / RHIZOPUS ORYZAE / LACTATE DEHYDROGENASE / P1 TRANSDUCTION

THAGOON YAMOAT: LACTIC ACID PRODUCTION IN GENETICALLY MODIFIED *Escherichia coli*. ADVISOR: RUETHAIRAT BOONSOMBAT, Ph.D., 97 pp.

Lactic acid is an organic acid, which can be classified into 2 isomers: L (+)-lactic acid and D (-)-lactic acid. It is widely used in various industrial applications, including as a potential precursor to synthesize polylactides (PLA) for biodegradable plastic which the production of optically pure monomers is essential. Although *Rhizopus oryzae* is a potential candidate for L-lactic acid production due to the production of an optically pure L-isomer of lactic acid, it has some limitations such as its unsuitable morphology for fermentation in bioreactors. The aim of this research is to study lactic acid production from genetically engineered *Escherichia coli* with inactivated genes involved in fermentative pathways and such mutants harboring plasmid with *R. oryzae ldhA* gene. The strains of deactivated *pta ldhA* with additional knocked out *pflB* or *adhE* could not grow anaerobically. Moreover, the exogenous *ldhA* from *R. oryzae* on the plasmid could not complement the growth under this condition. The deactivated *pta ldhA frdC* was able to grow, but poorly, under anaerobic condition, while *R. oryzae ldhA* gene could restore the fermentative growth. The *E. coli* strain of knocked out *pta ldhA frdC* with plasmid containing *ldhA* gene from *R. oryzae* (AP20) verified the expression of *R. oryzae ldhA* gene by qRT-PCR. Nevertheless, LDH activity and growth rate were lower than those of the wild type strain. After fermented in media with 2% initial glucose, a small amount of L-lactic acid was detected, but other fermentation byproducts seemed to be reduced, except for ethanol. This could be resulted from inadequate NADH recycle, inhibition of phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for glucose consumption, or expression of eukaryotic protein in prokaryotic host.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere thanks to my thesis advisor, Dr. Ruethairat Boonsombat, for all valuable helps, excellent suggestions, supports and constant encouragement throughout the course of this thesis.

I also would like to extend my profound appreciation to Associate Professor Dr. Polkit Sangvanich, Associate Professor Dr. Chanpen Chanchao and Dr. Wanilada Rungrassamee for serving as the committee and for their editorial assistance and comments.

I would like to thank The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and National Budget 2013 for financial support, staffs and students in Program in Biotechnology, the Faculty of Science and Institute of Biotechnology and Genetic Engineering, Chulalongkorn University for their friendship, supports and helps.

Finally, I would like to thank my family for the encouragement, kindly support and help to complete this entire thesis.

CONTENTS

Page

Page

Page

LIST OF FIGURE

Page

LIST OF TABLE

Page

LIST OF ABBREVIATIONS

$(NH_4)_2SO_4$	ammonium sulfate
ackA	acetate kinase A gene
adhE	alcohol dehydrogenase E gene
Amp^R	ampicillin resistance
bp	base pair
BSA	bovine serum albumin
$CaCO_3$	calcium carbonate
$CaCl_2$	calcium chloride
$Ca(NO_3)_2$	calcium nitrate
cat	chloramphenicol acetyltransferase gene
Cm^R	chloramphenicol resistance
$^{\circ}C$	degree Celsius
dNTP	deoxynucleoside triphosphates
DNA	deoxyribonucleic acid
K_2HPO_4	dipotassium hydrogen phosphate
Na_2HPO_4	disodium hydrogen phosphate

$FeSO_4$	ferrous sulfate
FRT	fiippase recognition target
FLP	flippase
frdC	fumarate reductase C gene
del	gene deletion
lacZ	gene encoding beta-galactosidase
::	gene insertion
g	gram
HCl	hydrochloric acid
Kan^r	kanamycin resistance gene
kb	kilo base pair
ldhA	lactate dehydrogenase A gene
LDH	lactate dehydrogenase ennzyme
l	liter
LB	Luria-Bertani
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
$MnCl_2$	manganese chloride

$MnSO_4$	manganese sulfate
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
µmole	micromole
mg	milligram
ml	milliliter
mM	millimolar
min	minute
M	molar
nm	nanometer
OD	optical density
pta	phosphotransacetylase gene
PCR	polymerase chain reaction
KOAc	potassium acetate
KH_2PO_4	potassium dihydrogen phosphate
$Y_{p/s}$	product yield coefficient (g product / g substrate)

plfB	pyruvate formate lyase B gene
qRT-PCR	quantitative reverse transcription PCR
rpm	revolutions per minute
RNA	ribonucleic acid
RbCl	rubidium chloride
NaOAc	sodium acetate
NaCl	sodium chloride

CHAPTER I
INTRODUCTION

Lactic acid, an organic acid, was first isolated from sour milk in 1780 by Swedish chemist, Carl Wilhelm Scheele. By natural fermentation, lactic acid is naturally composed in various foodstuff such as cheese, yogurt, soy sauce, sourdough, meat products and pickled vegetables. In 1875, Louis Pasteur proved that the acid was produced by certain microorganisms. Its empirical formula is $CH_3CH(OH)COOH$ or $C_3H_6O_3$. This carboxylic acid also plays a role in various biochemical processes. Moreover, other properties include no odor, transparent compound and ability to be miscible in water and ethanol. Lactic acid can be classified into two isomers: L (+)-lactic acid and D (-)-lactic acid. The L-isomer can be found in various organisms such as human, animals and microorganisms while the latter can be only produced by some microorganisms. The L-isomer is widely used in food industrial applications as a preservative and flavor additive. Unlike D-isomer, L-isomer causes neither any harms on human methabolism or any unhealthy conditions such as acidosis and decalcification [1]. In other industrial applications such as cosmetics, textile, phamaceutical and especially the use as a potential precursor for biodegradable plastic, when pure lactic acid (L or D-isomer) is used to synthesize for the polylactides (PLA), it gives a higher yield production than racemic LD-isomer [2, 3]. Thus, it is important to produce lactic acid with purity.

The demand of lactic acid has been estimated to grow as 5-8 % annually [4]. The world market of lactic acid production was expected to reach 259,000 metric tons in 2012 and 367,300 metric tons in the year 2017 [5]. NatureWorks LLC, the world's largest fermentative lactic acid manufacturers for polymer plant, started the world's first full-scale PLA plant in Blair, Nebraska, USA with the capability of 140,000 metric tons per year in 2003 [6]. NatureWorks LLC is also located in China and Thailand (2011).

Nowadays lactic acid can be produced by two ways: chemical synthesis and microbial fermentation. The former process is relied on petrochemical resources which

subsequently encounters many problems such as limited supply, global warming and racemic LD-isomer production. Therefore, microbial fermentation from renewable resource is a potential alternative to efficiently produce the pure isomer of lactic acid. Almost worldwide lactic acid production presently comes from the fermentative production route [7]. Most microorganisms capable of producing lactic acid include bacteria, fungi, yeast, cyanobacteria, and algae. Lactic acid-producing bacteria, so called lactic acid bacteria (LAB), are known as *Lactobacillus* strains. LAB strains are commercially used due to their high acid tolerance and high yield of lactic acid production. However, due to the lack of many biosynthetic capabilities under anaerobic condition, LAB strains usually require complex nutrients such as vitamins, nucleotides, amino acids and peptides for their growth resulting in hampering the lactic acid recovery and increasing in production costs [8-11]. One of the lactic acid-producing microorganisms is *Rhizopus oryzae*, a filamentous fungus species. Several researches indicate that *Rhizopus oryzae* is a potential candidate for L-lactic acid production due to the production of optically pure L-isomer of lactic acid with various raw materials including starch, cellulose, hemicellulose and different renewable resources [12]. However, L-lactic acid production from *R. oryzae* encounters many problems such as low productivity, ethanol contamination and its inappropriate morphology for fermentation without additional applications in any bioreactors [13]. To overcome these obstacles, recombinant DNA Technology has been used.

Recombinant DNA technology is a technique to join DNA molecules from two different species together, and then the recombinant DNA is put into a host organism to produce the interested products. Host organisms can be divided in two systems: prokaryotic system such as bacteria and eukaryotic system such as yeasts and plants. Many researchers prefer to use recombinant DNA technology to produce and improve lactic acid production due to the ability to produce a high yield of lactic acid in relatively short period of time. In this research, *Escherichia coli* was used as microorganism host to produce lactic acid. *E. coli* itself produces racemic DL-lactic acid during fermentative growth, therefore, genetically engineered strains expressing exogenous *ldh* genes,

mostly for L-lactic acid production, from various species have been attempted to be generated such as *E. coli* harboring plasmid with *L-ldh* gene from *Enterococcus faecalis* KK1 [14], *E. coli* harboring plasmid with *ldh* gene from *Streptococus bovis* [15] *E. coli* overexpressing L-lactate dehydrogenase gene from *Lactobacillus casei* [16]. *E. coli* has many advntages such as ability to utilize many substrate including glucose, xylose, sucrose and glycerol, relatively simple nutrient requirement, rapid growth and easy genetic manipulation [17].

Previous research was attempted to put *ldhA* gene from *R. oryzae* on the plasmid pBluescriptII KS(+), called pRB85 plasmid, and transformed into *E. coli* with knocked out chromosomal *ldhA* and *pta* background to allow the expression of L-lactate dehydrogenase only from *R. oryzae ldhA* gene on the pRB85 plasmid and reduce acetic acid contamination [18], respectively. This *E. coli* strain was expected to combine the advantages of *E. coli* and *R. oryzae* which should produce pure L-lactic acid with rapid growth. Nevertheless, when fermentation was performed for 48 hour by using 100 g/l glucose without ampicillin addition, L-lactic acid concentration of 5.03 ± 4.149 g/l was obtained from this strain [19]. Due to the productivity from this recombinant strain was still low and residual glucose was found in high concentration, in this research, lactic acid production from *E. coli* strains harboring plasmid with *R. oryzae ldhA* gene in the backgrounds with various inactivated chromosomal genes involving in fermentative pathways was focused on. Inactivation of genes on chromosomal DNA was manipulated by P1 transduction, a genetic technique for transferring target gene with selectable marker from *E. coli* donor strain to the recipient strain by P1 bacteriophage [20]. DNA pieces from the donor would be subsequently homologously recombined onto chromosomal DNA of the recipient. In this research, P1 transduction was applied to delete chromosomal *pta*, *ldha*, *adhE*, *plfB*, and *frdC* genes. Currently, several studies reported the use of metabolic engineered *E. coli* strains for lactic acid production such as a metabolically engineered *E. coli* for homofermentative production of D-lactic acid from sucrose [21].

The output from this project will be applied not only for the further process of L-lactic acid and D-lactic product, but also for the production of the higher scale and even the production of other products. Moreover, it will be useful for the study of optimization further.

Objective:

To study lactic acid production of genetically engineered *E. coli* harboring plasmid *R. oryzae ldhA* gene in backgrounds with various inactivated chromosomal fermentative genes in shake flask scale.

CHAPTER II
LITERATURE REVIEWS

2.1 Lactic acid

2.1.1 History of lactic acid

Lactic acid was the first discovered in 1780 by Carl Wilhelm Scheele, a Swedish chemist. It was isolated from sour milk as impure brown syrup of which the name based on its origins: 'Mjölksyra'. Then, in 1875, Louis Pasteur found that lactic acid was not a milk component, but a fermentation metabolite produced by certain microorganisms [22]. Later, in 1881, Frémy, a French scientist, produced lactic acid by fermentation which could be applied in industrial production scale in Massachusetts, USA.

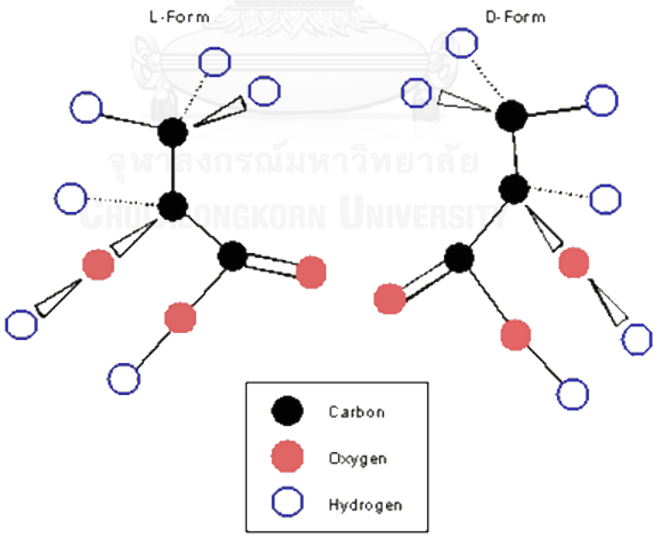


Figure 2.1 The two stereo isomers of lactic acid

(Reference: http://www.lactic-acid.com/history.html)

2.1.2 Characteristic of lactic acid

Lactic acid is a carboxylic acid with the chemical formula $C_3H_6O_3$ that plays a important role in various biochemical processes in organisms. Lactic acid is transparent compound with no odor and miscible in water and ethanol. Lactic acid can be classified into two isomer forms: L (+)-lactic acid and D (-)-lactic acid (Figure 2.1). L (+)-lactic is the biological isomer as its natural presence in the human and animal body and can be found in various microorganisms, while D (-)-lactic acid can be only produced by some microorganisms. The physical and chemical properties of lactic acid are summarized in Table 2.1 [23]. and the thermodynamic characteristics of lactic acid are represented in Table 2.2 [24].

Table 2.1 Physical and chemical properties of lactic acid.

Identification	CAS number	D/L: [50-21-5] L: [79-33-4] D: [10326-41-7]
	Einecs No.	200-018-0
	H.S. Code	2918.11
	Chemical formula	$CH_3CH(OH)COOH$
Physical & chemical properties	Melting point	L: 53 °C D: 53 °C D/L: 16.8 °C
	pKa at 25 °C	3.86
	pH (0.1% solution, 25 °C)	2.90
	pH (0.1N solution, 25 °C)	2.40
	Boiling point	122 °C (12 mmHg)
	Specific gravity	1.2 g/ml
	Molar mass	90.08 g/mol
	Solubility in water (g/100 g H2O)	Miscible

	Physical appearance	Aqueous solution, colorless to slightly yellow

Table 2.2 Thermodynamic characteristics of lactic acid.

Items	Thermodynamic characteristics
Dissociation constant	0.000137 (at 25 °C)
Heat of dissociation	-63 cal/mol (at 25 °C)
Free energy of dissociation	5000 cal/mol
Heat of solution	1868 cal/mol (for crystalline L(+) lactic acid at 25 °C)
Heat of dilution	-1000 cal/mol (for dilution with a large volume of water)
Heat of fusion	2710 cal/mol (for racemic lactic acid) 4030 cal/mol (for L(+) lactic acid)
Entropy of solution	6.2 cal/mol °C
Entropy of dilution	-3.6 cal/mol °C
Entropy of fusion	9.4 cal/mol °C (for racemic lactic acid) 12.2 cal/mol °C (for L(+) lactic acid)
Heat of combustion	-321220 cal/mol (for crystalline L(+) lactic acid at 25 °C) -325600 cal/mol (for liquid racemic lactic acid at 25 °C)
Heat of formation	-165890 cal/mol (for crystalline L(+) lactic acid at 25 °C) -163000 cal/mol (for liquid lactic acid) -164020 cal/mol (for lactic acid in dilute solution) -164080 cal/mol (for dissociated and diluted lactic acid)
Heat capacity	0.338 cal/g/°C (for crystalline lactic acid at 25 °C) 0.559 cal/g/°C (for liquid lactic acid at 25 °C)
Absolute entropy	34.0 cal/mol/°C (for crystalline L(+) lactic acid at 25 °C) 45.9 cal/mol/°C (for liquid racemic lactic acid at 25 °C)
Entropy off formation	-137.2 cal/mol/°C (for crystalline lactic acid at 25 °C)

	-125.3 cal/mol/°C (for liquid lactic acid at 25 °C)
Free energy off formation	-124980 cal/mol (for crystalline L(+) lactic acid at 25 °C) -126500 cal/mol (for liquid racemic lactic acid at 25 °C)

2.1.3 Application of lactic acid

Lactic acid is usually commercialized as a liquid. Pure and anhydrous racemic lactic acid is a white crystalline solid with a low melting point. Lactic acid is classified as generally recognized as safe (GRAS), was approved by the US Food and Drug Administration (FDA) and European regulatory authorities for being used as food and drug composition. The annual world market for lactic acid production was expected to reach 259,000 metric tons in 2012 and was expected to reach 367,300 metric tons by the year 2017 [5]. Approximately 85% of lactic acid demand is for food and food-related industries because it is a major component of dairy products while the other approximately 15% for the non-food application (Figure 2.2) [5, 6]. Nowadays, Lactic acid can be also used as a monomer to synthesize polylactic acid known as PLA, a potential precursor for biodegradable plastic. Therefore, the current market of lactic acid production is further expanding.

2.1.3.1 Food industry

In food and food related industries, lactic acid is usually served as a pH regulator, a preservative, or a flavoring agent in a variety of food and beverage products because it has low pH which can be used as a food preservative for inhibiting many species of bacteria and bacterial spoilage. Additionally, for bakery products, it was applied in the esterification reaction of calcium and sodium salts of lactate served as a dough conditioner and emulsifier. It has been reported that D-lactic acid is harmful to human metabolism due to being a cause of acidosis and decalcification. Therefore, only L-lactic acid is used in food and food related industries [1].

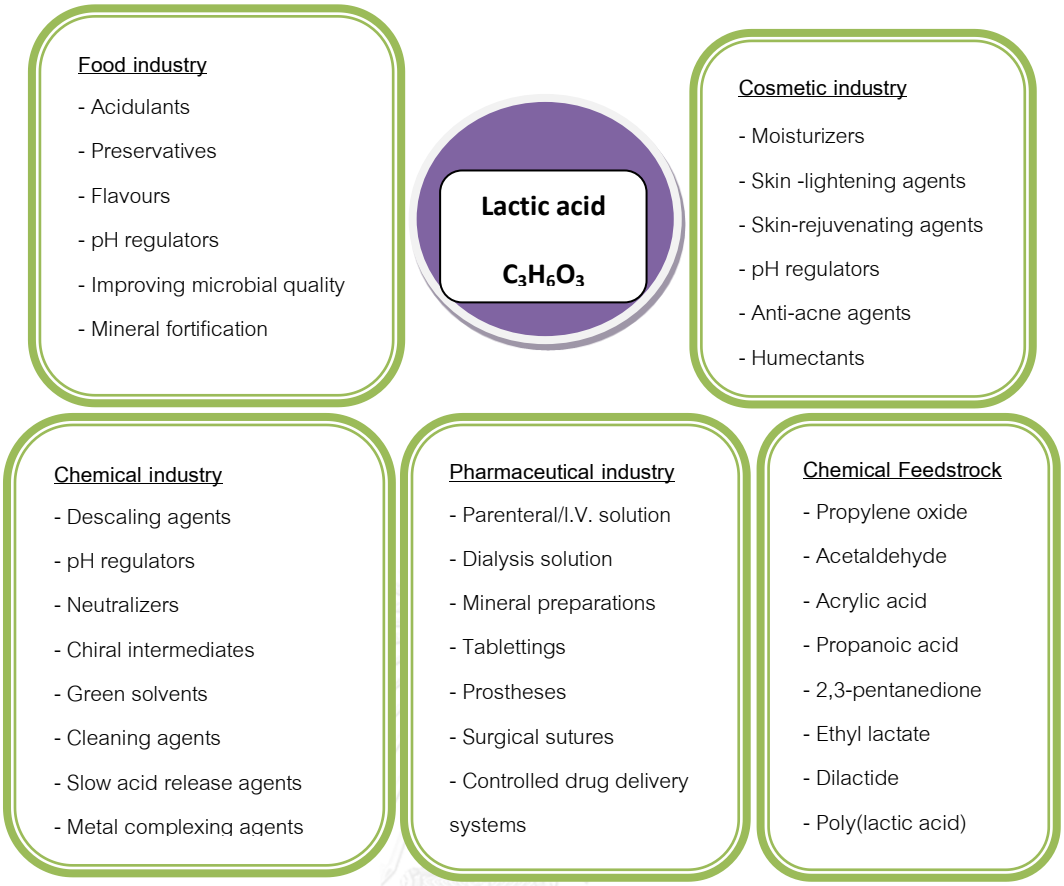


Figure 2.2 The diagram of commercial uses and applications of lactic acid its salt [25].

2.1.3.2 Cosmetic industry

Lactic acid, used as an active compound for reducing acne breakouts, the appearance of wrinkles and the other signs of aging, is a popular ingredient in skincare products. It can also improve the skin texture and hydration level by being used as humectant and moisturizer for retaining the moisture which. Moreover, lactic acid has an ability to mildly exfoliate the outer layer of skin resulting in a softer, younger looking complexion and speeding up the rate of cell turnover. Lactic acid may reduce skin tones when being used for cleansing. It also functions as a pH regulator of *cosmetic* product to make a suitable pH in cosmetic formulation. Furthermore, ethyl lactate, a lactic acid derivative, is used in many anti- acne preparations because of its excellent solvency power against oils and polymeric stains without toxicological effects and environmental impacts [26].

2.1.3.3 Pharmaceutical and medical industry

The sodium salt of lactic acid has been mainly applied in pharmaceutical industry as an electrolyte in many intravenous therapy solutions such as Hartmann's solutions, CAPD (continuous ambulatory peritoneal dialysis) solution used to replace body fluid and mineral salts, and dialysis solution for conventional artificial kidney machines. In the form of calcium salt, it can be used for calcium deficiency therapy and being an effective anti-carrying agent. Due to its ability to be polymerized as polylactic acid (PLA), it can be used for producing a sustainable plastic with biocompatible property that can be further applied as tablets, prostheses, surgical sutures, and controlled drug delivery systems. Moreover, lactic acid is used in a wide variety of mineral preparations [27].

2.1.3.4 Chemical industry

Lactic acid are used increasingly in various chemical products and chemical processes, such as a descaling agent, pH regulator, neutralizer, chiral intermediate, solvent, cleaning agent, slow acid-release agent and metal complex agent. The ester form is especially used as a feedstock monomer for the chemical conversion, including acetic acid, acrylic acid, and propionic acid, in household cleaning products. Natural lactic acid is an alternative solvent in many fine mechanical cleaning applications. Due to its high solvency power and solubility, lactic acid is an excellent remover of polymer and resins. Since lactic acid offers better descaling properties than conventional organic descalers, it is often used in many decalcification products, such as bathroom cleaners, coffee machines, and toilets [1, 28].

2.1.3.5 Polymer industry

Lactic acid can be used to synthesize polylactic acid, so called PLA, a potential precursor for biodegradable plastic. It has been reported that pure lactic acid (D or L-isomer) gave a higher yield of PLA productions than racemic LD-isomer [2, 3]. Therefore, it is important to produce lactic acid with purity for synthesize PLA. In the

recent years, the lactic acid polymer technology is widely commercially produced. Due to a biodegradable and biocompatible polymer, PLA is concerned as an environmental friendly choice to replace petrochemical plastics. Because of the high strength and thermo plasticity properties of PLA, it can be applied in the productions of packaging, textiles, and medicals products. PLA has been considered as a renewable plastic since lactic acid can be produced by fermentation of feedstock biomass including sucrose, corn starch and tapioca starch. PLA from fermentative processes is expected to replace many conventional petrochemical plastics [29, 30]. Furthermore, a variety of PLA applications should result in a great potential for expanding the market of lactic acid production. Polylactide can be divided into poly-L-lactide(PLLA), poly-D-lactide(PDLA) and poly-DL-lactide(PDLLA). These are synthesized from the polymerization of L-lactide, D-lactide and Meso lactide (50:50 mixture of D and L isomers), respectively (Figure 2.3) [31]. These polymers can be produced by using several techniques, including direct polycondensation, ring-opening polymerization of lactide and azeotropic dehydrative polycondensation (Fig. 2.4) [32]. The different properties of any PLA, such as the melting temperature and degree of crystallinity, are resulted from the ratio of L : D isomer [33].

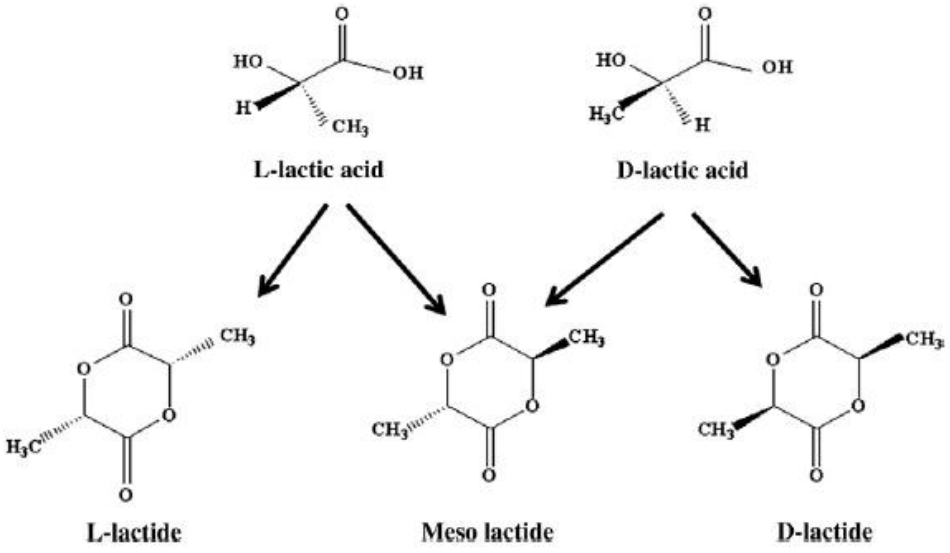


Figure 2.3 Stereoforms of lactides [6].

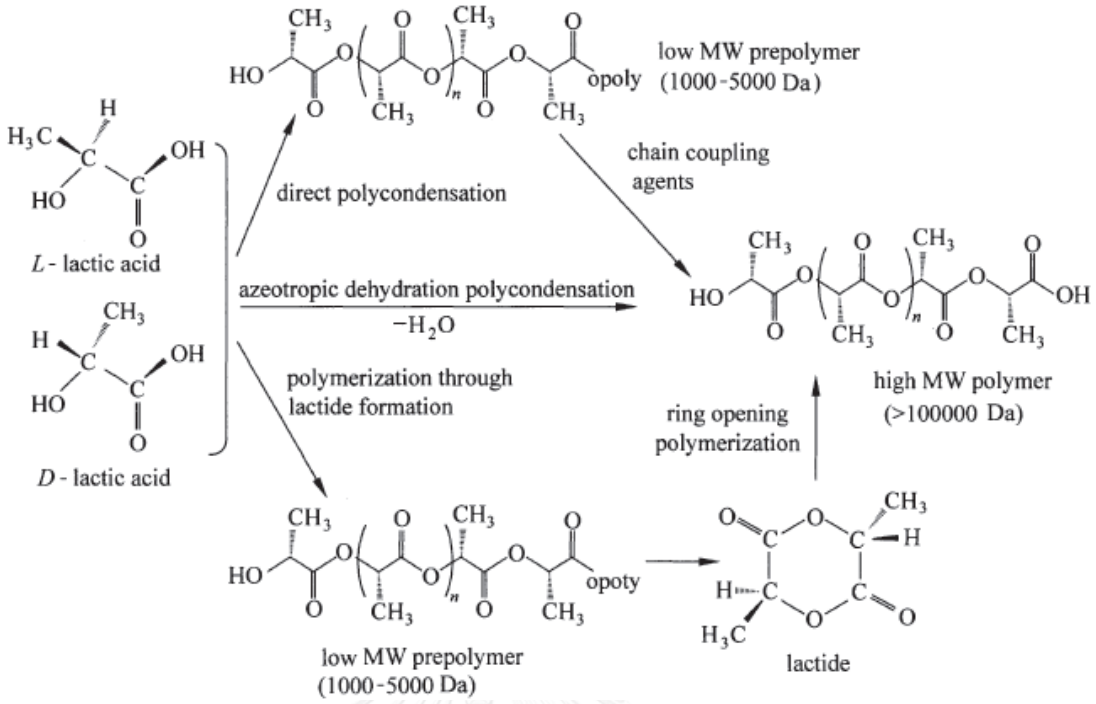


Figure 2.4 Chemical synthesis of Polylactide [32].

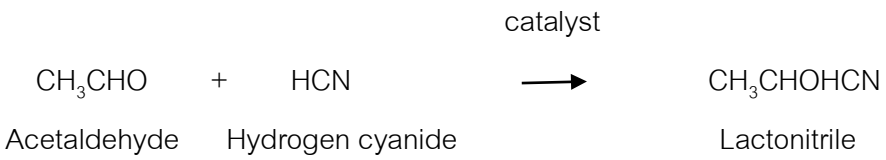
2.1.4 Lactic acid production

Lactic acid is a natural organic acid which can be produced either by chemical procedure involving formation of lactonitrile from acetaldehyde and hydrogen cyanide, or by microbial fermentation of carbohydrates such as glucose, sucrose, or lactose or by chemical synthesis.

2.1.4.1 Chemical synthesis

For this process, the chemical reaction is mainly based on petrochemical resources. As the following reactions, the hydrolysis of lactonitrile by strong acids provides only the racemic mixture of D- and L-lactic acid [34].

1) Addition of HCN: The reaction begins with mixing hydrogen cyanide with acetaldehyde under high atmospheric pressure to produce lactonitrile.

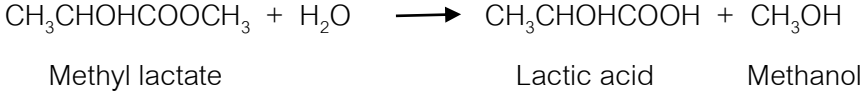


2) Hydrolysis by H_2SO_4: The crude lactonitrile is recovered and purified by using a distillation technique. Then, the purified lactronitrile is hydrolyzed to lactic acid, either by hydrochloric acid or sulphuric acid to produce ammonium salt and lactic acid.

.

3) Esterification: Lactic acid is subsequently esterified by methanol to produce methyl lactate which is further removed and purified by distillation

4) Hydrolysis by H_2O: Methyl lactate is hydrolyzed by mixing with water and acid catalyst resulting in lactic acid and methanol. Methanol is usually recycled for using in previous esterification step.



However, a racemic mixture of D(-)-lactic acid and L(+)-lactic acid is obtained from this chemical synthesis. This limitation causes a problem for further industrial applications in which an optical purity of L isomer form is preferable due to its properties [25, 35].

2.1.4.2 Microbial fermentation

Besides racemic LD-isomer production, the drastic conditions of chemical synthesis contribute to uneconomical and environmentally harmful effects from the petrochemical industry [23]. The limited supply of petrochemical resources, crude oil or natural gas is also a worldwide concern. Therefore, the microbial fermentation from renewable resource is a potential alternative method for commercially producing lactic acid with isomer purity. The natural lactic acid producing microorganisms can be divided into two main groups: bacteria and fungi. Depending on strains of microorganisms, substrates, and cultivation conditions, mixture of D(-) and L(+)-isomer or optically pure isomer can be obtained from the fermentation [36]. Moreover, genetically modified microorganisms have become interested for lactic acid production.

1) Bacteria

Lactic acid producing bacteria can be classified into four main groups, which are lactic acid bacteria (LAB), *Bacillus strains*, *Escherichia coli*, and *Corynebacterium glutamicum*. Most of commercial lactic acid production is carried out with LAB. On the basis of the nature fermentation, LAB which can be grouped as (1.) homofermentative lactic acid bacteria, producing lactic acid without other metabolic products and (2.) hetrofermentative lactic acid bacteria, producing lactic acid along with other products such as ethanol, diacetyl, formate, acetoin or acetic acid, and carbon dioxide. (Figure 2.5, 2.6). The homofermentative LAB, such as bacteria in genera *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Pediococcus*, are available for the commercial lactic acid production. In general, bacterial lactic acid fermentation encounters several

limitations, including racemic lactic acid from the expression of both L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), low yield production, byproduct formation, specific nutrient supplementation such as yeast extract and vitamin-B, high risk of bacteriophage infection that can result in cell lysis and subsequent cessation of lactic acid production [11, 36], and pH control during the fermentation process for bacterium growth, lactic acid recovery and purification in the downstream process [37]. Therefore, the attempts have been made to improve lactic acid production with microorganisms that are less nutritionally demanding but more acid-tolerant, such as fungi from the genus *Rhizopus* [38].

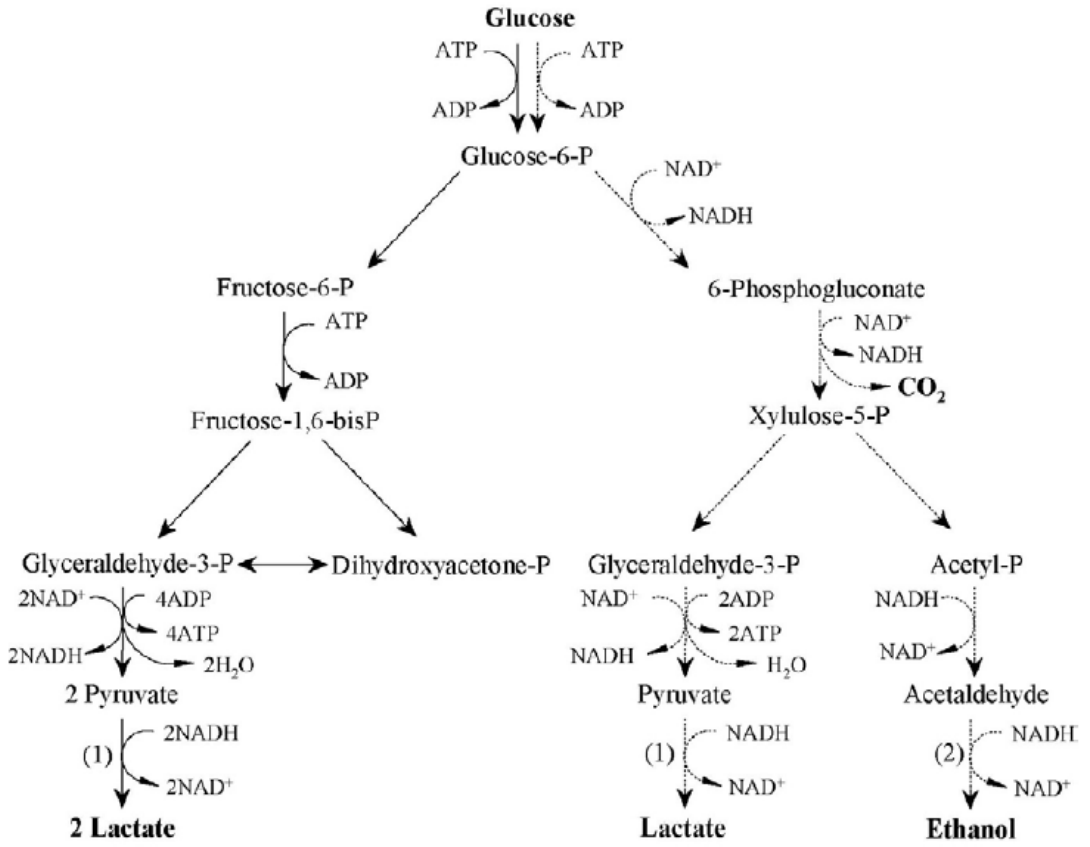


Figure 2.5 Metabolic pathways of lactic acid bacteria, the homofermentative pathways (solid lines) and the heterofermentative pathways (dotted line) [25].

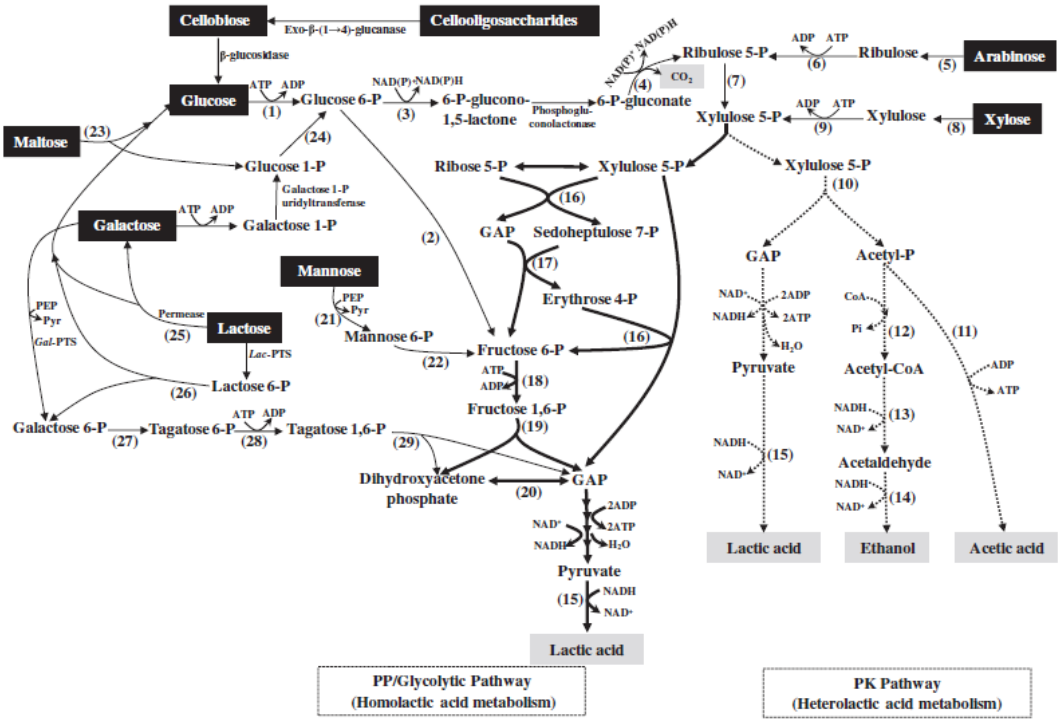


Figure 2.6 Metabolic pathways of lactic acid bacteria for lactic acid production from various sugars substrate. Enzymes: (1) hexokinase; (2) glucose 6-phosphate isomerase; (3) glucose 6-phosphate dehydrogenase; (4) 6-phosphogluconate dehydrogenase; (5) arabinose isomerase; (6) ribulokinase; (7) ribulose 5-phosphate 3-epimerase; (8) xylose isomerase; (9) xylulokinase; (10) phosphoketolase; (11) acetate kinase; (12) phosphotransacetylase; (13) aldehyde dehydrogenase; (14) alcohol dehydrogenase; (15) lactate dehydrogenase; (16) transketolase; (17) transaldolase; (18) 6-phosphofructokinase; (19) fructose bisphosphate aldolase; (20) triosephosphate isomerase; (21) mannose phosphotransferase system; (22) phosphomannose isomerase (23) maltose phosphorylase; (24) phosphoglucomutase ; (25) β-galactosidase; (26) phospho-β-galactosidase; (27) galactose 6-phosphate isomerase; (28) tagatose 6-phosphate kinase; and (29) tagatose 1,6-diphosphate aldolase. Solid lines indicate the homofermentative pathway. Thick-solid lines and dashed lines indicate PP/glycolytic pathway and PK pathway, respectively. Lac-PTS: phosphoenolpyruvate-lactose phosphotransferase system [39].

2) Fungi

Nowadays, the studies of optically pure L-lactic acid production from several species of the filamentous fungi *Rhizopus* genus, especially *R. oryzae*, have been focused on [40-42]. The *Rhizopus* strains can be a candidate for producing lactic acid with simple nutrient requirement for growth, pure L-isomer production [43-45], lower cost of downstream process [46], and valuable fungal biomass. Furthermore, *Rhizopus* strains can use agricultural residues such as sugarcane, cassava pulp, corn corps, and paper pulp sulfite liquor as carbon sources [39, 46]. In addition, many studies discovered that *R. oryzae* has protease, urease, ribonuclease, pectate lyase, and polygalacturonase [47]. However, there are some limitations to produce lactic acid by *Rhizopus* strains, such as production of undesirable byproducts such as ethanol and fumaric acid [48-50], aeration requirement for more than 0.3 gO_2/l/h of the oxygen transfer rate [51, 52], and the filamentous nature of the *Rhizopus* strains that causes potential issues in mass transfer, bulk mixing, and lactic acid recovery [53].

3) Genetically engineered microorganisms

Genetic engineering plays a role to overcome limitations of bacterial and fungal fermentation problems, for example, the engineered *Lactobacillus helveticus* CNRZ32 for production of pure L-(+)-lactic acid by deletion of either *ldhD* or *ldhL* gene [54], and the engineered of *R. oryzae* for the production of platform chemicals [55]. Moreover, for expressing the L-(+)-Lactate dehydrogenase gene (*ldhL*) from *Pediococcus acidilactici*, the optimization of lactic acid yields was done by the deletion of genes involing in byproduction generation such as genes encoding for pyruvateformate lyase (formic acid production), alcohol dehydrogenase (ethanol production) and acetate kinase (acetic acid production) [56]. Various strains of *E. coli* producing lactic acid on chemically defined media was constructed by genetic engineering [57]. The LAB strains were also improved for blocking steps in phage life cycle [58, 59]. Using of mixed strains for

fermentation or development of phage resistant mechanism strains are necessary to prevent bacteriophage infected to LAB strains [60].

2.2 *Rhizopus oryzae*

The filamentous fungus species *Rhizopus oryzae*, also known as *R. arrhizus, R. javanicus, R. japonicas, R. thermosus,* or *R. schizans*, is commonly isolated from soil, plant materials and dead organic matter. *R. oryzae* is an obligate aerobe, which is able to assimilate various carbon sources. It is rhizoid formation with white cottony colonies at first, and then become brownish grey to blackish-grey depending on the amount of sporulation which can be found as bread mold (Figure 2.7). Its hyphae are tube-like, smooth walled, non septate, simple or branched, and the cell wall contains chitosan and chitin instead of glucans, mannans, and chitin which are different from ascomycetes and basidiomycetes. This fungus belongs to the zygomyces group, which reproduced by forming asexual and sexual spores. In asexual reproduction, sporangiospores (non-motile spores) are produced inside a spherical structure, the sporangium. Sporangia are supported by a large apophysate columella atop a long stalk, the sporangiophore. Sporangiophores arise among distinctive, root-like rhizoids. In sexual reproduction, a dark zygospore is produced at the point where two compatible mycelia fuse. Upon germination, a zygospore produces colonies that are genetically different from either parent [61]. In addition, *R. oryzae* potentially produces a wide range of metabolites, including enzymes, ester, organic acids, volatile materials, polymers, and bioalcohols. This fungus species is particularly recognized as the commercially perspective of L(+)-lactic acid producer and also used for making fermented foods and alcohol beverages in Asia [62].

Figure 2.7 The morphological characteristic of *R. oryzae*. A: a colony on potato dextrose agar after 7 day incubation. B: sporangiums and sporangiophores. C: a columella. D: sporangiospores. E: Rhizoids [63].

2.2.1 *Rhizopus oryzae* metabolism

For *R. oryzae*, all fermentable carbon sources are metabolized to pyruvate which can be used to produce several fermentation metabolite products in glucose metabolism; depending on the cultivation condition (Fig. 2.8).The pyruvate is used in both cytosol and mitochondria [64]. In the mitochondrial part which is responsible for growth and energy production, pyruvate is oxidized to acetyl-CoA, NADH and CO_2 by pyruvate dehydrogenase (PDH) complex. PDH is a complex enzyme consisting of 3 enzyme domains: pyruvate dehydrogenase, dihydrolipoyl transacetylase, dihydrolipoyl dehydrogenase. The complex enzyme requires many cofactors for fuctioning such as TPP, lipoate coenzyme A, and FAD. After that, acetyl-CoA is further entered into TCA cycle for generating ATP, NADH and fumaric acid which secreted extracellulaly. For the cytosolic part, many metabolites such as lactic acid, ethanol, and fumaric acid are generated. For glucose metabolism of *R. oryzae*, 1 mol of glucose can be converted to 1.5 mol of lactic acid by the reaction of lactate dehydrogenase (LDH)(EC 1.1.1.27). LDH

is an enzyme that requires NADH as a cofactor for converting pyruvate to L(+)-lactic acid and NAD^+. LDH has isozymes which are encoded by 2 genes, *ldhA* and *ldhB*. At the initial cell growth and L(+)-lactic acid synthesis, NAD^+-dependent LDH encoded by *ldhA* gene is responsible for converting pyruvate to L(+)-lactic acid until the glucose level is exhausted. The other NAD^+-independent LDH, encoded by *ldhB*, is involved in the reverse reaction, a conversion of L(+)-lactic acid to pyruvate, which can be activated in the culture containing non-fermentable substrates such as glycerol and ethanol. In addition, when glucose is depleted, the decreasing level of lactic acid is perhaps caused by *ldhB* activation [65, 66].

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDHA}} \text{L(+)-lactate} + \text{NAD}^+$$

$$\text{L(+)-lactate} + \text{NAD}^+ \xrightarrow{\text{LDHB}} \text{Pyruvate} + \text{NADH}$$

For metabolic route of ethanol, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are involved. In an O_2 limiting condition, pyruvate is converted to acetaldehyde by the reaction of PDC (EC 4.1.1.1). PDC is an isoenzyme which requires thiamine pyrophosphate (TPP) as a co-factor. It is encoded by two genes: *pdcA* and *pdcB* which could be induced by glucose addition under anaerobic condition. Moreover, PDC is a key enzyme for ethanol production and respiration by contributing acetyl CoA to the TCA cycle [67]. Finally, acetaldehyde is converted to ethanol by the reaction of alcohol dehydrogenase (ADHs) (EC 1.1.1.1). ADHs are oxidoreductase enzymes which consist of two ADH isoenzymes, including ADH I and ADH II. The ADH I is a fermentative enzyme related to the ethanol formation while the ADH II is less expressed [68, 69]. In addition, *Rhizopus* ADH is similarity to yeast ADH [69].

The fumaric acid production of *R. oryzae* is in the cytosol by using pyruvate carboxylase, malate dehydrogenase, and fumarase for catalyzing [70]. According to the lactic acid and fumaric acid production, R. oryzae strains can be divided into type I and type II. The former type contains both *ldhA* and *ldhB* genes and primarily produces

lactic acid as the main product. However, the latter type only has *ldhB* gene and produces fumaric acid as the major product [71]. It was suggested that fumaric acid production from type II strains was resulting from the lack of capability to produce lactic acid from pyruvate, contributing to excess pyruvate available for producing fumaric acid or ethanol [70].

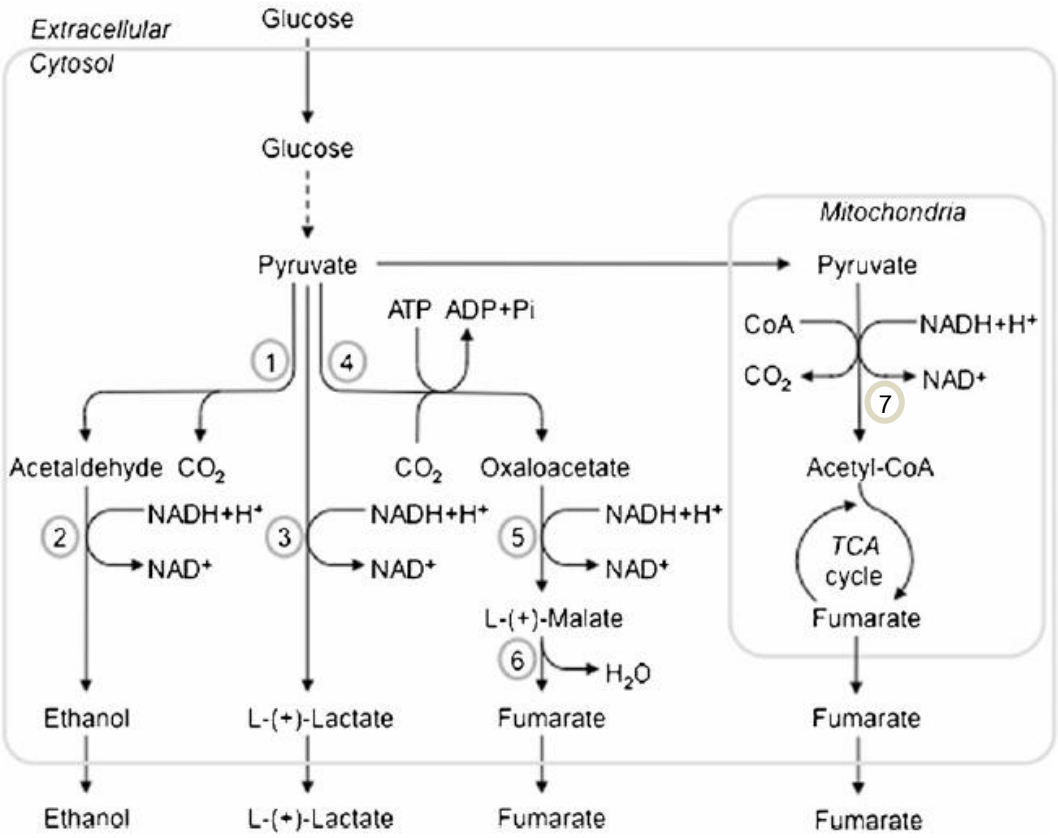


Figure 2.8 Glucose metabolism of *R. oryzae*. The numbers indicate key enzymes in eachpathway: 1, pyruvate decarboxylase (PDC); 2, alcohol dehydrogenase (ADH); 3, lactate dehydrogenase (LDH); 4, pyruvate carboxylase (PYC); 5, malate dehydrogenase (MDH); 6, fumarase (FUM); 7, pyruvate dehydrogenase (PDH) [55, 70].

2.2.2 Limitation of using *R. oryzae*

Even though *R. oryzae* has many advantages for lactic acid production, it has some limitations for fermentation. Many previous studies reported less lactate yield production and lower productivity from R. oryzae than those of LAB. Moreover, without additional applications, *R. oryzae* has unsuitable morphology for fermentation in any bioreactors and it can produce undesirable byproducts such as ethanol and fumaric acid. Although byproduct formation can be controlled by several fermentation parameters such as pH, medium composition, neutralizing agent, fungal morphology, and oxygen transfer [46], all parameters are related and required to be optimized. In addition, all of these parameters are able to affect enzyme activities of all metabolites. Therefore, there was an attempt to introduce *ldhA* gene of *R. oryzae* to express LDHA in *E. coli* for improving L-lactic acid production [19].

2.3 *Escherichia coli*

E. coli is a gram-negative bacteria, was identified in 1885 by the German pediatrician named Theodor Escherich. It is *a facultative* anaerobe that can grow in both anaerobic and aerobic conditions. *E. coli* is rod-shaped bacterium (Figure 2.9) that is commonly distributed in the intestine of many other animals and human. Most *E. coli* strains are harmless in animal or human health, but some serotypes can cause serious food poisoning in their hosts [72]. The harmless strains are normal flora, mostly stayed in the gut of animal and human, which are useful for their hosts by producing vitamin K2 [73] and also prevent pathogen colonization in the intestine [74]. *E. coli* and the other facultative anaerobes are found in the gut as about 0.1% gut flora [75]. Fecal–oral transmission is the main route for transferring the pathogenic strains of the bacterium. Cells can survive outside the host body for a limited amount of time, which are the indicator organisms to use for testing environmental samples for fecal contamination

[76]. *E. coli* can be grown easily and inexpensively in a laboratory, and has been intensively examined as prokaryotic model organism for over 60 years.

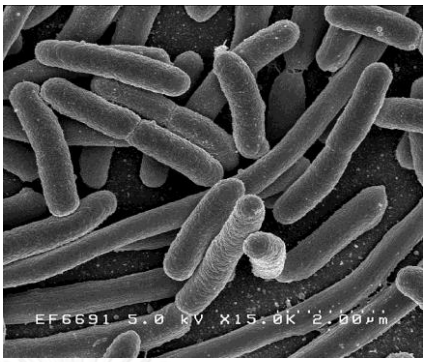


Figure 2.9 The scanning electron micrograph of *Escherichia coli*.
(http://en.wikipedia.org/wiki/Escherichia_coli)

E. coli is the most widely studied prokaryotic model organism and important in the fields of biotechnology and microbiology. It is a favorite organism for genetic engineering, which has served as the host organism for the majority of work with recombinant DNA. In desirable conditions, it can be constructed to produce unlimited quantities of an introduced gene product with a short period of time to reproduce due to its easy and rapid *growth* in the laboratory. Nowadays, many studies have applied genetic engineering methods for improving the fermentation efficiency by using *E. coli* strains due to their ability to use hexose and pentose sugars for metabolism, rapid growth rate, simple nutritional requirements and easy genetical manipulation. The wild-type *E. coli* can generally produce a mixture of several organic acids (lactic acid, acetic acid, formic acid and succinic acid) and ethanol during anaerobic growth [17, 57, 77]. To enhance lactic acid production in *E. coli* strains, it has been genetically engineered with modified genes involving in metabolic pathways affecting to lactic acid production pathway [18, 57, 78, 79]. Several researches reported lactic acid production from metabolic engineered *E. coli* strains when being fermented with xylose [14], glucose

[14, 18, 56, 57, 80, 81], sucrose [21, 80] and glycerol [82]. Although, the productivity and lactic acid production from engineered *E. coli* strains in the terms of concentrations and tolerance of lactic acid were still lower than many LAB and *Bacillus* spp. [18, 57, 83] but it still benefited in the other terms such as lower cost from using inexpensive resources for fermentation.

2.3.1 *E. coli* fermentative pathway

E. coli can produce a mixture of products such as lactic acid, acetic acid, succinic acid, formic acid and ethanol from sugars by fermentation under anaerobic conditions and in the absence of alternative electron acceptors. The main product is acetic acid or lactic acid depended on *E. coli* strain while ethanol and formic acid with smaller amounts of succinic acid [17, 57, 84] and the gaseous products, such as hydrogen and carbon dioxide, are also found to be produced in substantial amounts. The fermentative pathways for generating products are varied in response both to the pH of the culture medium and the type of the fermentation substrate. Normally, the ratio of the various fermentation products is manipulated in order to balance the number of reducing equivalents generated during glycolytic breakdown of the substrate. The enzymes and corresponding genes involved in these fermentation pathways are described in Figure 2.10. When *E. coli* is grown in absence of oxygen, the energy must be supplied either by anaerobic respiration coupled to electron acceptors such as nitrate, trimethylamine oxide or fumarate, or by fermentation [85]. Glucose is transported into the cell by the phosphotransferase system and catabolized to pyruvate in both aerobic and anaerobic conditions. However, the metabolisms of pyruvate between aerobic and anaerobic conditions are different. Under an aerobic condition, NADH is generated during both of the glycolysis and the Krebs cycle, and then associated reactions are reoxidized by the operation of the respiratory chain [86]. During fermentation or anaerobic conditions, neither the respiratory chains linked to oxygen nor those linked to alternative electron acceptors are functional. Even though the Krebs cycle and pyruvate dehydrogenase reactions generated a high amount of NADH, it is

inoperative under the anaerobic condition. However, NADH produced by glycolysis must be reoxidized to NAD^+ from the reaction on fermentative pathway, so the glycolytic sequence can be continued. Therefore, the key issue in fermentation is the recycling of NADH by conversion of pyruvate to fermentation products [17].

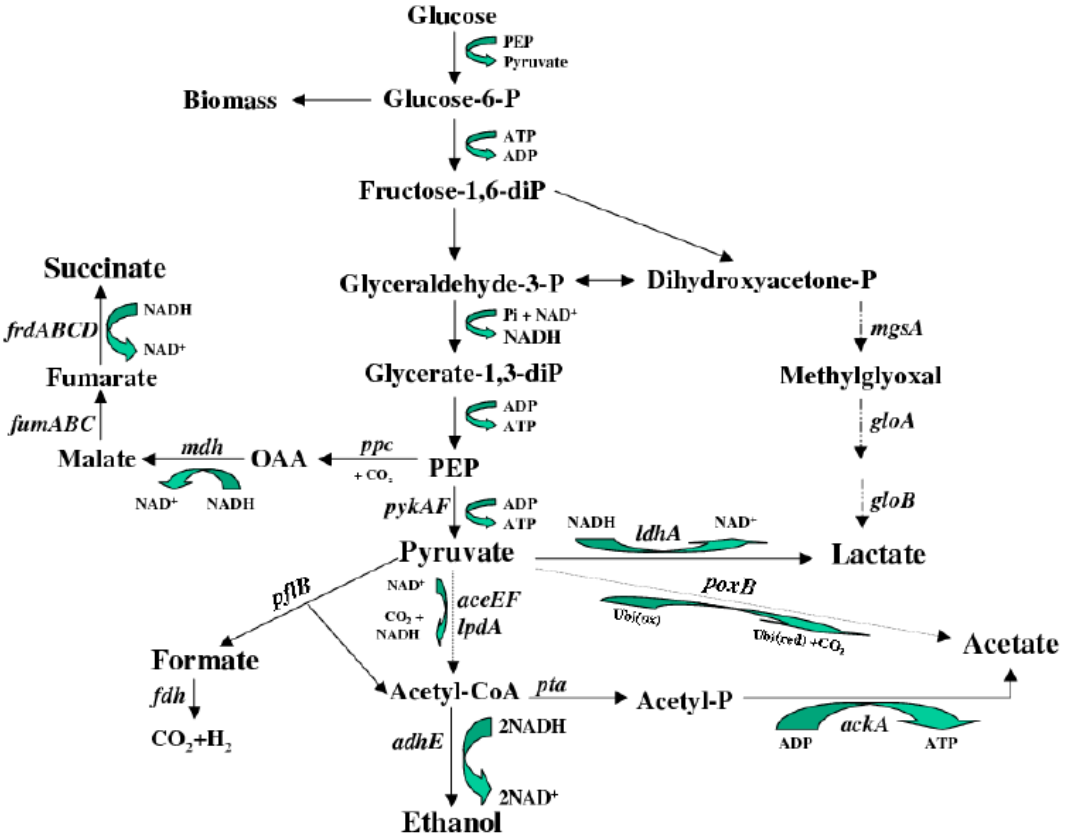
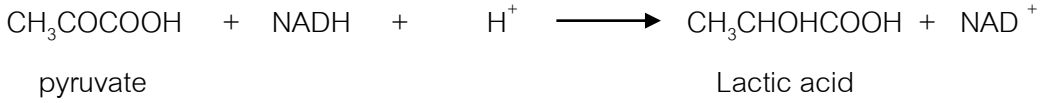


Figure 2.10 Carbon central metabolic pathway in wild type *E. coli*. Solid arrows represent central fermentative pathways, dotted arrows represent microaerobic pathway (*poxB*), and dash arrow represents minor lactate producing pathway (*mgsA*, *gloAB*). Genes: *ldhA*: lactate dehydrogenase, *pflB*: pyruvate formate-lyase, *pta*: phosphate acetyltransferase, *ackA*: acetate kinase, *adhE*: alcohol dehydrogenase, *fumABC*: fumarase, *frdABCD*: fumarate reductase, *fdh*: formate dehydrogenase, *pykAF*: pyruvate kinase, *ppc*: PEP carboxylase, *aceEF*/*lpdA*: acetyltransferase/dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex, *mdh*: malate

dehydrogenase, *mgsA*: methyglyoxal synthase, *gloAB*: glyoxylase, and *poxB*: pyruvate oxidase [87].

2.3.2 Lactic acid production in *E. coli*

In wild type *E. coli* strain, there are three lactate dehydrogenase (LDH) enzymes which interconvert to lactic and pyruvic acids. Two of them are membrane-bound flavoproteins, one for the D-isomer and the other for the L-isomer, which can convert lactate to pyruvate during oxidative growth on lactate [88], but they are not participated in fermentation *in vivo* [88, 89]. The wild type *E. coli* has only one fermentative lactate dehydrogenase (LDH), which is soluble and NAD-linke, to convert pyruvate to lactate during anaerobic growth [90]. This enzyme was first suggested to be used for producing the L-isomer [90] , but it was revealed later that D-lactate is also synthesized by this enzyme [91]. Therefore, in *E. coli*, the LDH enzymes are present in both aerobic and anaerobic conditions. The LHD level increases by 5 to 10-fold during anaerobic growth, especially growth with low pH medium, while such acid induction does not occur in aerobic condition [92]. Moreover, the fermentative LDH activity is increased when pyruvate concentration is accumulated [93]. The lower pyruvate concentration, the lower activation of the enzyme. Despite converting a significant fraction of the pyruvate to lactic acid when substantial pyruvate levels accumulated under fermentation [93], during normal aerobic growth, pyruvate never rises to a sufficient level to activate the LDH and is therefore not wasted. During high pyruvate accumulation or low pH condition, pyruvate may be converted to lactate by the fermentative lactate dehydrogenase (LDH) as the following reaction:



2.4 P1 transduction for genetic engineering in *E. coli*

P1 transduction is a genetic technique for transferring selectable mutations of interesting gene from one *E. coli* strain to the other strain. P1 is a bacteriophage that is

used to infect a recipient host, then DNA pieces from the donor is transferred into the new stain and the fragment of DNA is homologously recombined onto chromosomal DNA of target strain [20]. Generally, the target DNA from the donor contain selective marker, such as an antibiotic resistant gene, in the appropriated region. After being infected by P1, *E. coli* donor cells are lysed, and then P1 phages carrying donor gene including target gene with marker would get off and infect the other *E. coli* cells (recipient strain). DNA fragments from P1 phages are released into the recipient cells of which target gene with marker can homologously recombined onto chromosomal DNA, then further selected on agar plate with appropriated method, such as selected antibiotic plates. The principle of P1 transduction is represented in Figure 2.11.

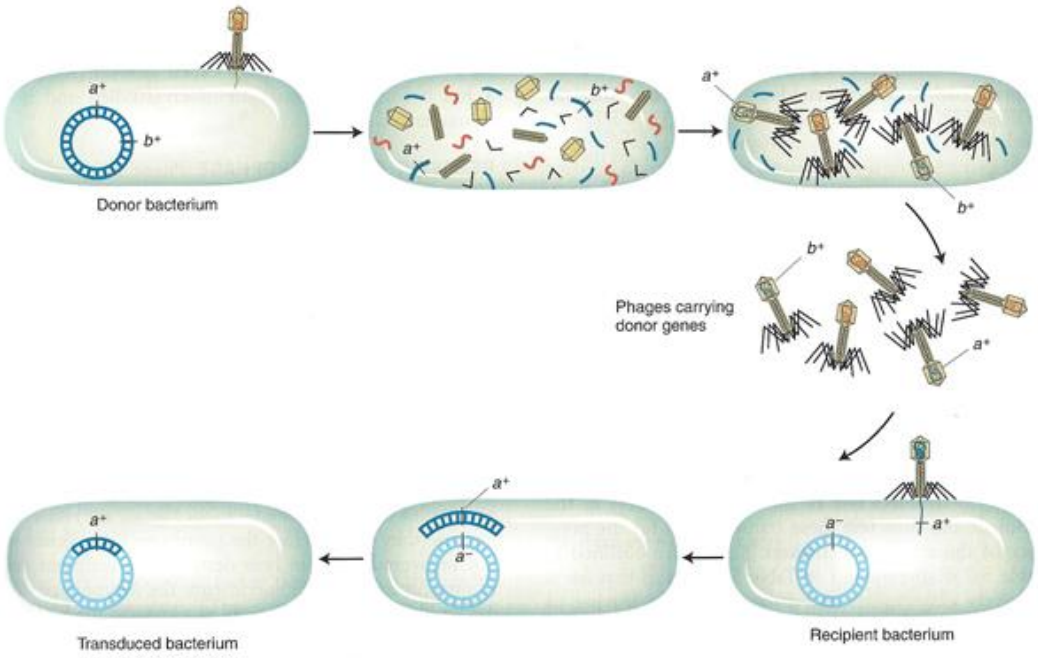


Figure 2.11 The principle of P1 transduction.

(http://www.discoveryandinnovation.com/BIOL202/notes/lecture15.html)

CHAPTER III
METHODOLOGY

3.1 Strain list

Strain	Partial genotype					Plasmid	Reference
	adhE	*frdC*	*ldhA*	*pflB*	*pta*		
JC13509[a]	+	+	+	+	+	-	Dr. Steven J Sandler
JW0886-1	+	+	+	*kan*	+	-	Coli genetic stock center
JW1228-1	*kan*	+	+	+	+	-	Coli genetic stock center
JW4113-1	+	*kan*	+	+	+	-	Coli genetic stock center
RB5	+	+	*cat*	+	+	-	[19]
RB6	+	+	+	+	*kan*	-	[19]
RB7	+	+	*cat*	+	*kan*	-	[19]
RB24	+	+	*cat*	+	*kan*	pRB85	[19]
AP1	+	+	+	*kan*	*del*	-	JW0886-1→RB6 that *kan* gene was removed
AP2	+	*kan*	+	*del*	*del*	-	JW4113-1→AP1 that *kan* gene was removed
AP3	*kan*	*del*	+	*del*	*del*	-	JW1228-1→AP2 that *kan* gene was removed
AP4	*kan*	*del*	*cat*	*del*	*del*	-	RB5→AP3

Strain	Partial genotype					Plasmid	Reference
	adhE	*frdC*	*ldhA*	*pflB*	*pta*		
AP5	*kan*	*del*	*cat*	*del*	*del*	pRB85	AP4 harboring pRB85
AP7	*kan*	+	+	+	*del*	-	JW1228-1→RB6 that *kan* gene was removed
AP8	+	*kan*	+	+	*del*	-	JW4113-1→RB6 that *kan* gene was removed
AP9	+	+	*cat*	*kan*	*del*	-	RB5→AP1
AP10	+	*kan*	*cat*	*del*	*del*	-	RB5→AP2
AP11	*kan*	+	+	*del*	*del*	-	JW1228-1→AP1 that *kan* gene was removed
AP12	*kan*	+	*cat*	+	*del*	-	RB5→AP7
AP13	+	*kan*	*cat*	+	*del*	-	RB5→AP8
AP14	*kan*	+	*cat*	*del*	*del*	-	RB5→AP11
AP15	*kan*	*del*	+	+	*del*	-	JW1228-1→AP8 that *kan* gene was removed
AP16	*kan*	*del*	*cat*	+	*del*	-	RB5→AP15
AP17	+	+	*cat*	*kan*	*del*	pRB85	AP9 harboring pRB85
AP18	+	*kan*	*cat*	*del*	*del*	pRB85	AP10 harboring pRB85
AP19	*kan*	+	*cat*	+	*del*	pRB85	AP12 harboring pRB85

Strain	Partial genotype					Plasmid	Reference
	adhE	*frdC*	*ldhA*	*pflB*	*pta*		
AP20	+	*kan*	*cat*	+	*del*	pRB85	AP13 harboring pRB85
AP21	*kan*	+	*cat*	*del*	*del*	pRB85	AP14 harboring pRB85
AP22	*kan*	*del*	*cat*	+	*del*	pRB85	AP16 harboring pRB85

[a] JC13509 used as wild type in this experiment contains partial genotype as F^- *lacMS286* ***ϕ****80dIIlacBK1 sulB103 argE4 his-4 thi-1 xyl-5 mtl-1* Sm^R $T6^R$, Derivative of *E. coli* SK362. It was obtained from Dr. Steven J. Sandler, University of Massachusetts, Amherst.

+ means wild type gene.

– means not harboring any plasmid.

pRB85 is pBluescriptII KS(+) containing *ldhA* gene (ORF and promoter region) from *R. oryzae* NRRL395 [19]

3.2 Primer list

Primer	Sequence	Target gene
prRB1	ACAGGTGGATCCGTCCTTTG	*ldhA* of *E. coli*
prRB4	GGAATACGGAATTCTGGATCACG	*ldhA* of *E. coli*
prRB13	GATGGGATCCATCGTCGCAAA	*pta* of *E. coli*
prRB16	CGTCGAATTCCGGCAGTACG	*pta* of *E. coli*
prRB47	CAAGCTCAAGTCCTTGACCTTGCAGATGCTCG	*ldhA* of *R. oryzae*
prRB48	GGTCGTGTCAAGGTAGGTACCGGA	*ldhA* of *R. oryzae*
frdC1	ATGACGACTAAACGTAAACCGTATG	*frdC* of *E. coli*
frdC2	TTACCAGTACAGGGCAACAAACAGG	*frdC* of *E. coli*
adhE1	GCTGCTGCAGATGCTCGAATCCC	*adhE* of *E. coli*
adhE2	GCAGCAGTACGGTCGCCCGGTGCG	*adhE* of *E. coli*
pflB1	ATGTCCGAGCTTAATGAAAAGTTAGCC	*pflB* of *E. coli*
pflB2	TTACATAGATTGAGTGAAGGTAC	*pflB* of *E. coli*

3.3 Chemical and reagents

Chemical and reagents	Company and country
Acetic acid	Merck, USA
Agarose	Research Organic, USA
Ammonium sulfate	Sigma Aldrich, USA
Ampicillin	Bio Basic, Canada
Bacto-agar	Himedia, India
Bovine serum albumin	Sigma Aldrich, USA
Calcium carbonate	Sigma Aldrich, USA
Calcium chloride	Bio Basic, Canada

Chemical and reagents	Company and country
Calcium nitrate	Carlo Erba Reagent, Italy
Chloramphenicol	Sigma Aldrich, USA
Chloroform	Merck, USA
Coomassie brilliant blue R 250	Bio Basic, USA
Copper sulfate	Sigma Aldrich, USA
Dipotassium hydrogenphosphate	Bio Basic, USA
Disodium hydrogen phosphate	Bio Basic, USA
Dithiothreitol	Bio Basic, Canada
D-lactate	Sigma Aldrich, USA
Ethanol	Merck, USA
Ethidium Bromide	Bioexcellence, India
Ethylenediaminetetraacetic acid disodium salt dihydrate	Bio Basic, USA
Ferrous sulfate	Merck, USA
Formic acid	Carlo Erba Reagent, Italy
Glucose	Bio Basic, USA
Glycerol	Sigma Aldrich, USA
IPTG (Isopropyl-β-D-Thiogalactopyranoside)	Bio Basic, Canada
Kanamycin	Bio Basic, USA
L-lactate	Sigma Aldrich, USA
Lysozyme	Bio Basic, USA
Magnesium Sulfate	Bio Basic, Canada
Manganese chloride	Sigma Aldrich, USA
Manganese sulfate	Merck, USA
MOPS (Morpholinepropanesulfonic acid)	Sigma Aldrich, USA
NADH (Nicotinamide adenine dinucleotide)	Merck, USA

Chemical and reagents	Company and country
Peptone	Bio Basic, USA
Phosphoric acid	Merck, USA
Potassium acetate	Thermo Fisher Scientific, USA
Potassium dihydrogen phosphate	Merck, USA
Rubidium chloride	Sigma Aldrich, USA
Sodium acetate	Carlo Erba Reagent, Italy
Sodium chloride	Merck, USA
Sodium pyruvate	Sigma Aldrich, USA
Succinate	Sigma Aldrich, USA
Sulfuric acid	Merck, USA
Thymidine	Sigma Aldrich, USA
Tris-hydrochloride	Bioexcellence, India
Tris-maleate	Sigma Aldrich, USA
Trizma Base	Sigma Aldrich, USA
Yeast extract	Bio Basic, USA

3.4 Equipments and supplies

Equipments and supplies	Company and country
Agarose gel electrophoresis equipments: model Mupid-Exu	Mupid, Japan
Aminex HPX-87H column	Bio-Rad, USA
Anaero Anaerobic Gas Generator: model AnaeroPack	Mitsubishi Gas Chemical, Japan
Autoclave: model HICLAVE HV-50	Hirayama, Japan
Biological safety cabinet class II : model Hfsafe-1200	Shanghai Lishen Scientific Equipment, China
Cellulose acetate filter, pore size 0.45 µm	Sartorius, Germany
Centrifuge tubes 50 ml	Labcon, USA
Cryogenic vials	Biologix Research, USA
Erlenmeyer flask 125 ml	Pyrex, Germany
Erlenmeyer flask 250 ml	Pyrex, Germany
Favorprep plant total RNA purification mini kit	Favorgen Biotech, Taiwan
Freezer -20 °C: model SF-C997	Sanyo, Japan
Freezer -70 °C: model Forma 8600	Thermo Fisher Scientific, USA
Geneon PCR kit	Intron, Korea
High speed refrigerated centrifuge: model 6500	Kubota, Japan
High speed refrigerated micro centrifuge: model MTX-150	Tomy Seiko, Japan
High-pressure liquid chromatography	Shimadzu, Japan
Hot plate stirrer: model C-MAG HS7	Ika, Germany

Equipments and supplies	Company and country
Incubator shaker: model innova 4300	New Brunswick Scientific, USA
Laboratory bottle	Duran, Germany
Micro auto pipette: model Discovery comfort	High Tech Lab, Poland
Microcentrifuge tube	Labcon, USA
Microplate spectrophotometer: model Multiskan GO	Thermo Fisher Scientific, USA
Microtiter microplates, UV plate, 96 Well	Costar, USA
PCR thermocycler: model T100 thermal cycler	Bio-Rad, USA
PCR tube	Labcon, USA
Petridish sterilized	Kappa Disposable Plastic
pH meter	Mettler Toledo, Switzerland
Pipette tips	Biologix Research, USA
Real-Time PCR thermocycler: model Light Cycler 480	Roche, France
SpinClean plasmid miniprep kit	Mbiotech, Korea
Sumi chiral 0A-5000L column	SCAS, Japan
SYBR Lo-ROX One-step kit	Mbiotech, Korea
Test tube	Pyrex, Germany
UV-visible recording spectrophotometer: model UV-160	Shimadzu, Japan
Vacuum pump: model DOA-V130-BN	Gast, USA
Vortex mixer: model Vortex-Genie2	Scientific Industries, USA
Water bath shaker: model R-86	New Brunswick Scientific, USA
WaterPro PS/UF Polishing Stations: model 9000701	Labconco, USA

3.5 Methods

3.5.1 Construction of *E. coli* strains containing inactivated genes involved in fermentative pathway

In this study all *E. coli* mutant were developed from *E. coli* RB6 (3.1). Strains with inactivated genes were constructed by P1 transduction technique. Preparation of transducing lysate started with inoculating a colony of each donor strain (RB6, JW0886, JW1228, or JW4113) in 5 ml LB broth with selective antibiotic and incubated at 37 °C, 200 rpm overnight. Then, 0.2 ml of the overnight culture was diluted into 10 ml LB broth antibiotic and allowed to grow until reaching log phase (3-4 hours). After that, 0.5 ml of log phase donor culture was mixed with 0.5 ml of P1 lysate stock with 1:10 dilution and 3 ml of LCTG top agar. The mixture was poured onto LCTG plate and incubated upright at 37 °C overnight. Next, top agar layer was scraped off and transferred to a screw cap centrifuge tube containing 1 ml of chloroform and 5 ml LB broth, and then mixed by vortexing. After centrifugation at 4,000 rpm for 5 min, the supernatant, containing P1 phages carrying fragment DNA of donor strain, was transferred to another tube containing 0.5 ml chloroform and stored at 4 °C until used. The P1 transduction was begun by inoculating a colony of recipient strain in 5 ml LB broth with selective antibiotic at 37 °C, 200 rpm overnight. The 0.2 ml of overnight culture was then diluted into 10 ml LB broth with selective antibiotic and allowed to grow to log phase (3-4 hours). Next, 5 ml of log phase recipient culture was centrifuged at 4,000 rpm for 5 min. The pellet was then resuspended in 0.5 ml of LB broth, 0.25 ml of 30 mM $CaCl_2$, 0.25 ml of 60 mM $MgSO_4$ and 0.5 ml of P1 transducing lysate (1:10 dilution). The mixture was incubated at room temperature for 30 min. After being centrifuged at 4,000 rpm for 5 min, cells were washed once with 5 ml of 56/2 buffer. The transduced cells were harvested by centrifugation at 4,000 rpm for 5 min and resuspended in 1 ml of 56/2 buffer. 0.3 ml of transduced cell solution was spread onto LB agar with selective antibiotic. Plates were incubated at 37 °C for 2 days [94].

3.5.2 Removal of *kan* insert by FLP-FRT recombination system

Insertion of *kan* gene in *pta*, *pflB*, *adhE* and *frdC* are located between two *frt* sites. To remove this *kan* insert, the pLH29 plasmid [95] was transformed to the target strain by chemical transformation and then, selected for chloramphenicol resistance on LB agar plate. Then, one colony of transformants was inoculated in LB broth with 25 μg/ml chloramphenicol at 37 °C, 200 rpm until reaching early log phase. The expression Flp was induced by adding 3mM IPTG for 1 hour at 37 °C, 200 rpm. Then, the culture was diluted back cells LB broth without chloramphenicol and allowed cells to grow until log phase (3-4 hours) at 37 °C 200 rpm to remove pLH29 plasmid from cells. This step was repeated in several times to increase a chance of getting rid of pLH29 plasmid. After that, cells were serially diluted and spread on LB agar. Plates were incubated at 37 °C overnight, and then 50-100 colonies were selected and streaked onto LB agar with kanamycin, LB agar with chloramphenicol and LB agar. After being incubated at 37 °C overnight, kanamycin and chloramphenicol sensitive colonies were selected. Finally, the loss of *kan* insert was verified by polymerase chain reaction [20]. Specific primers for genes in this study are listed in 3.2.

3.5.3 Chemical Transformation

The plasmids were transformed into competent cells by chemical transformation [95]. To prepare the competent cells, a colony of target strain was inoculated in 2 ml of LB broth with appropriated antibiotic. After being incubated at 37 °C overnight, 200 rpm overnight, the 100 μl of culture was diluted into 10 ml LB broth with selective antibiotic, and then allowed to grow to early log phase (OD_{600} = 0.3). Then, the culture was incubated on ice for 20 min. Cells were harvested by centrifuging at 4 °C, 4,000 rpm for 10 min. The cell pellet was resuspended in 3.3 ml of TBI with gently swirling on ice water. The mixture was incubated on ice for 2 hours. Next, cells were harvested again

by centrifuging at 4 °C, 4,000 rpm for 10 min. Then, cell pellet was resuspended in 1.1 ml TBII. The 100 µl of aliquot competent cells was kept into each tube at -80 °C until used. The chemical transformation was performed by mixing 3 µl of plasmid with 50 µl competent cells. The mixture was incubated on ice for 20 min, then heat-shocking by being incubated at 42 °C for 2 min. After that, the mixture was returned to ice immediately for 2 min. Next, 1 ml of LB broth was added to the transformation reaction and incubated at 37 °C for 1-2 hours. Finally, the mixture was spread on LB agar plates with appropriate antibiotic, and plates were incubated at 37 °C overnight.

3.5.4 Lactic acid fermentation in shake flask level

Each *E. coli* strain was streaked on cultivation slant and incubated at 37 °C overnight. Next, cells on the overnight cultivation slants were transferred to 50 ml of pre-culture broth. After being incubated at 37 °C, 200 rpm for 6 hours, 5 ml of culture was transferred to 45 ml fermentation broth of which final OD_{600} is approximately 1.0. (0.37 M HCl was added to solubilize the excess $CaCO_3$ prior to measure the absorbance by spectrophotometer at 600 nm [13]). The fermentation was performed at 37 °C for 48 hours under anaerobic condition. For the cultures of strains harboring plasmids, 100 µg/ml Ampicillin was added in all media. After being fermented for 48 hours, 1 ml of each sample was harvested and centrifuged at 10,000 rpm for 7 min. The supernatant was kept for analyzing the concentrations of residual glucose, ethanol and fermentation acids, such as acetic acid, formic acid, lactic acid and succinic acid by a high-pressure liquid chromatography (HPLC).

3.5.5 Measurement of fermentation products by high-performance liquid chromatography *(*HPLC)

The concentrations of residual glucose, ethanol, acetic acid, formic acid, lactic acid and succinic acid were determined by using a high-pressure liquid chromatography (HPLC) equipped with IR detector and an Aminex HPX-87H column chromatography. The analysis was performed at 50 °C of which compounds were eluted by 5 mM sulfuric acid with elution rate 0.60 ml/min. The optical purity of lactic acid produced was determined by HPLC equipped with UV detector at 254 nm using a Sumi chiral 0A-5000L column to separate the L (+)-lactic acid and D (-)-lactic acid. The chromatography was performed at 40 °C and the compounds were eluted by 2 mM copper sulfate with elution rate 1 ml/min. Glucose, ethanol, acetic acid, formic acid, succinic acid, L (+)-lactic acid D (-)-lactic acid were used as calibration standard.

3.5.6 Measurement of *R. oryzae ldhA* expression by quantitative reverse transcription PCR (qRT-PCR)

The gene expression of *ldhA* of *R. oryzae* was measured by qRT-PCR using LightCycler 480 Real-Time PCR with SYBR Lo-ROX One-step kit. The mRNA was extracted from cells by Favorprep plant total RNA purification mini kit. The purified total mRNA was used as the template to amplify 326 base pairs of partial *R. oryzae ldhA* gene with prRB47 and prRB48 primers during qRT-PCR process. The 20 µl of each qRT-PCR reaction consisted of 1 µg of purified total mRNA (measured by Nanodrop 2000), 0.5 µM of each primers, 10 µl of 2x Mbiotech SYBR Lo-ROX one-step mix, 0.2 µl of reverse transcriptase, 0.4 µl of RNase inhibitor and DEPC-H_2O. The qRT-PCR reaction was carried out as the following: 45 °C for 10 min (reverse transcription), 95 °C for 2 min (pre-denature and polymerase activation), 40 cycles of 95 °C for 15 second (denature), 60 °C for 15 second (annealing), 72 °C for 20 second (extension). The fluorescent

absorbance was detected in each cycle and calculated into CP value (Crossing point) after the qPCR processing was finished.

3.5.7 Lactate dehydrogenase (LDH) activity assay

LDH activity was assayed by measuring NADH oxidation at 340 nm UV absorbance using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., USA). Cells, harvested from 5 ml of 48 hour anaerobic culture without the $CaCO_3$ by centrifugation, were washed once in Tris-maleate buffer (100 mM Tris-maleate, 1 mM dithiothreitol with pH 6.5) and resuspended in Tris-maleate buffer to reach approximately final OD600 as 1.0. Then, cells were permeablized by treating 0.1 ml of this suspension with 10 µl of chloroform and vigorously mixing for 15 second with a vortex. After chloroform was allowed to be settled, the upper layer containing permeabilized cells was used to assay LDH activity. The assay mixture contained 30 µl of 1 M sodium pyruvate, 30 µl of 6.4 mM NADH, 400 µl of 50 mM morpholinepropane-sulfonic acid buffer with pH 7.0, 530 µl of distilled H2O, and 10 µl of crude enzyme. LDH activity (initial rate) was measured at room temperature for 5 min and reported as micromole of NAD produced per min per milligram of total cell protein [56] which was measured by Bradford protein assay [96].

CHAPTER IV
RESULT AND DISCUSSION

4.1 Results

4.1.1 Construction of *E. coli* strains containing inactivated genes involved in fermentative pathway

The mutants with inactivated genes on chromosomal DNA were constructed by P1 transduction and verified by polymerase chain reaction. The expected sizes of PCR products of partial *E. coli ldhA*, *pta*, *frdC, adhE* and *pflB* were 794, 980, 396, 2,258 2,283 base pairs, respectively. When these genes were inactivated, the expected PCR products could not be obtained comparing to the control, JC13509 (Figure 4.1).

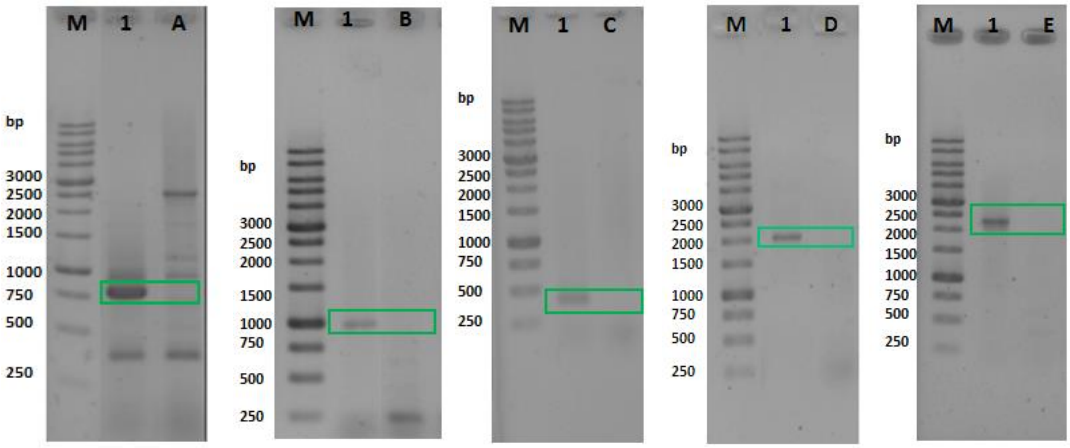


Figure 4.1 Verification of inactivated genes by on 1% Agarose gel. (A.) *ldhA* by using prRB1,4 primers (B.) *pta* when using prRB13,16 primers (C.) *frd* when using frdC1,2 primers (D.) *adhE* when using adhE1,2 primers (E.) *pflB* when using pflB1,2 primers Lane M: 1 kb DNA ladder marker. Lane 1: PCR product of wild type *E. coli* JC13509. Lane A: PCR product of inactivated gene.

4.1.2 The growth of mutant strains

The growth results of mutant strains were also tested by incubating at 37 °C for 48 hours under aerobic and anaerobic condition on cultivation plate with appropriate antibiotic, as shown in Table 4.1. All of mutant strains could grow under aerobic condition. The single mutants of *ldhA, pta, pflB, frdC* and *adhE* could grow under both aerobic and anaerobic conditions. However, mutants of deactivated *pta ldhA* with additional knocked out *pflB* or *adhE* were synthetically lethal under anaerobic condition and *R. oryzae ldhA* could not complement the loss of chromosomal *ldhA* in these *E. coli* strains. The result from Table 4.1 reveals that the deactivated *pta ldhA* mutants and *pta ldhA frdC* (strain RB7 and AP13, respectively) grow poorly under anaerobic condition. The *R. oryzae ldhA* from pRB85 plasmid could restore the growth of both mutants (strain RB24 and AP20).

Table 4.1 Growth *E. coli* strains under aerobic and anaerobic condition after incubated on cultivation plate at 37 °C for 48 hours.

Strain	Growth under aerobic condition	Growth under anaerobic condition
JC13509	+++	+++
RB5	+++	+++
RB6	+++	+++
RB7	+++	+
RB24	+++	+++
AP1	+++	+++
AP2	+++	+++
AP3	+++	+
AP4	+++	-
AP5	+++	-
AP7	+++	+++

Strain	Growth under aerobic condition	Growth under anaerobic condition
AP8	+++	+++
AP9	+++	-
AP10	+++	-
AP11	+++	+++
AP12	+++	-
AP13	+++	+
AP14	+++	-
AP15	+++	+++
AP16	+++	-
AP17	+++	-
AP18	+++	-
AP19	+++	-
AP20	+++	+++
AP21	+++	-
AP22	+++	-

+++ means normal growth

+ means poor growth

– means no growth

4.1.3 Lactic acid production in shake flask level

The JC13509, RB7, RB24, AP3 and AP20 strains were selected for fermentation with 2% glucose in shake flask. After being incubated at 37 ^{0}C under anaerobic condition for 48 hours, the residual glucose concentrations, ethanol, formic acid, lactic acid and succinic acid concentrations of each culture were measured by High-performance liquid chromatography (HPLC) as summarized in Figure 4.2 and Table B9. All selected strains produced insignificant amount of formic acid. The reduction of acetate was found in

strains with inactivated *pta* (RB7, RB24, AP3 and AP 20). The highest concentration of L-lactic acid was come from *E. coli* AP20, but only 0.411 g/l was detected. The strain AP3 was highest D-lactic acid producer with 6.315 g/l. However, as expected, succinic acid concentration was reduced in AP3 and AP20 strains due to the inactivation of *frdC* and ethanol concentration was reduced in AP3 strain due to the inactivation of *adhE* (Figure 2.10). The growth curve of each selected strain under anaerobic conditions was represented in Figure 4.3. The growth of each mutant was lower than that of wild type (*E. coli* JC13509), especially in AP3, which poorly grew under anaerobic condition, corresponding to the experiment indicated in Table 4.1. This result is also consistent with the research of Zhou and coworker in which *E. coli* SZ63, with knocked out *focA-pflB frdBC adhE ackA*, grew poorly under an anaerobic condition [56, 57].

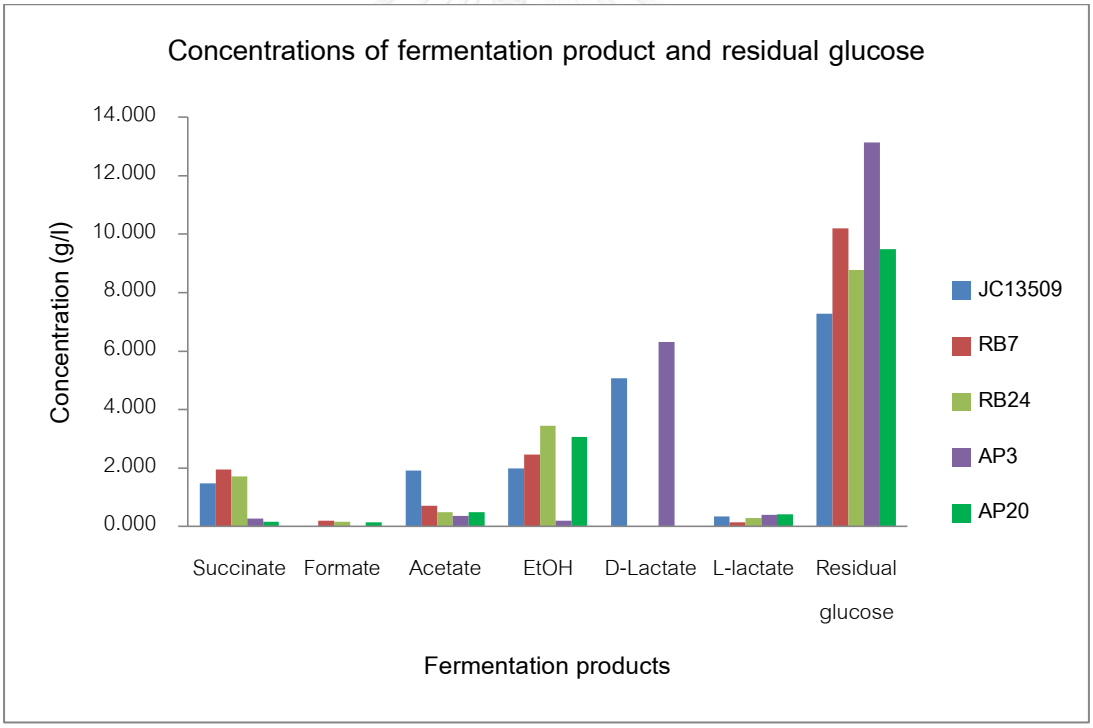


Figure 4.2 Concentrations of fermentation products after 48 hour of fermentation under anaerobic condition.

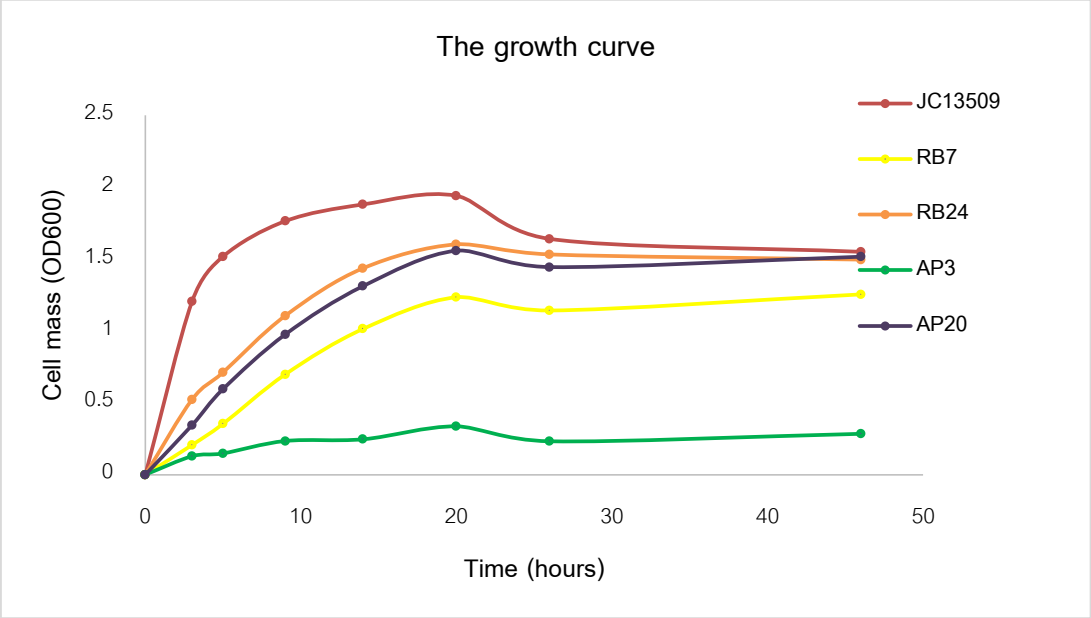


Figure 4.3 The growth curves of *E. coli* strains when being fermented in shake flask under anaerobic condition with 2% glucose for 48 hours.

4.1.4 LDH activity

The selected strains were fermented with 2% glucose without $CaCO_3$ addition under anaerobic condition for 48 hours. Then, LDH activity was assayed by measuring NADH oxidation at UV absorbance 340 nm due to the catalyzing of lactate dehydrogenase in the conversion of pyruvate to lactate and NADH to NAD^+. In this study, LDH activity was reported as micromole of NAD produced per min per milligram of total cell protein as shown in Table 4.2. *E. coli* AP3 strain revealed the highest LDH activity of 1.087 µmole/mg of protein.min, but not significantly from that of the wild type (JC13509). The LDH activities of the others were reduced comparing to that of the wild type strain.

Table 4.2 LDH activity of each selected strain after being fermented at 37 °C for 48 hours under anaerobic condition.

Strains	LDH activity (μmole[1]/ mg of protein[2] per min)
JC13509	0.842[a,c] ±0.145
RB7	0.219[b] ±0.152
RB24	0.402[a,b] ±0.182
AP3	1.087[a,c] ±0.175
AP20	0.647[a] ±0.096

[a, b, c] Statistics analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) using Duncan's Multiple Range Test (DMRT). The same superscripts are not significantly different from each other ($P<0.05$).

[1] Micromole of NAD produced

[2] Milligram of total cell protein

4.1.5 Measurement of *R. oryzae ldhA* expression by quantitative reverse transcription PCR (qRT-PCR)

To test the expression of *R. oryzae ldhA* on pRB85 plasmid in *E. coli* strains, qRT-PCR was performed (Figure 4.4). The selected strains were fermented in 50 ml fermentation media with 2% glucose and 100 μg/ml ampicillin, under anaerobic

condition for 48 hours. The CP value (Crossing point) was calculated after the qPCR processing was finished and summerized in Table 4.3. The expression of *ldhA* gene from *R. oryzae* on pRB85 plasmid in RB24 and AP20 was detected by qRT-PCR with CP values of 18.69 and 18.59, respectively. The strain JC13509, RB7 and AP3 were used as the negative controls which indicated that *R. oryzae ldhA* expression was not found in these strains. The qPCR products were also verified by agarose gel electrophoresis as shown in Figure 4.5 which revealed only 326 base pair of specific product.

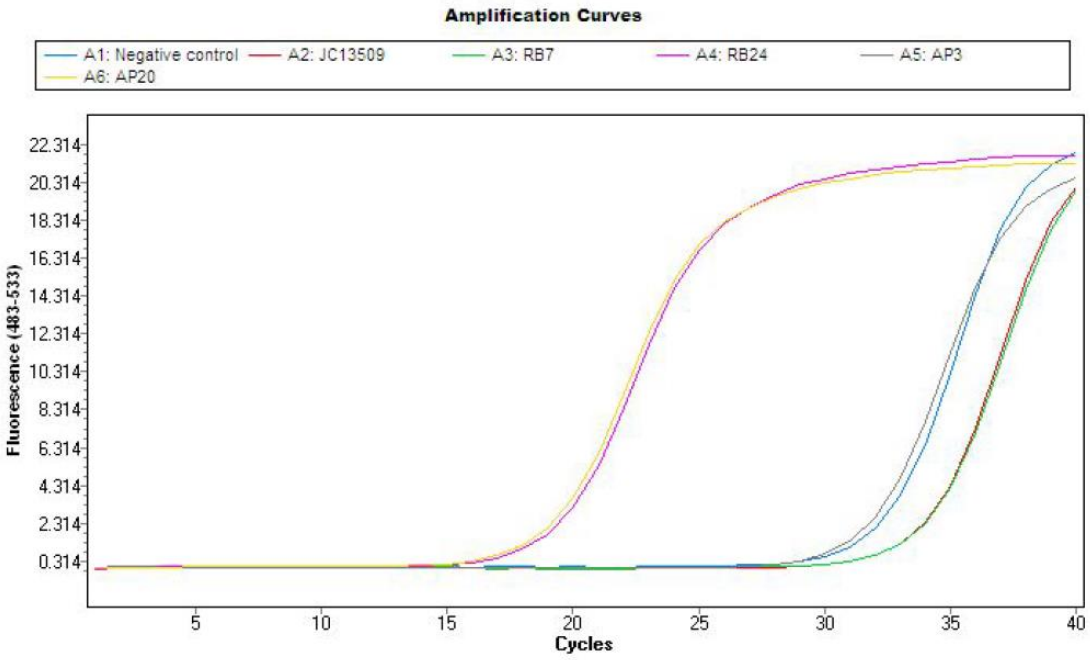


Figure 4.4 The expression of *ldhA* gene from *R. oryzae* on the pRB85 plasmid in *E. coli* strains after being fermented 48 hours under anaerobic condition with 2% glucose. The gene expression was measured by qRT-PCR with prRB47 and prRB48 primers.

Table 4.3 CP value (Crossing point) of each selected strain after the qPCR processing was finished, calculated by 480 light cycler software.

Strains	CP
Negative control	31.92±ND
JC13509	33.97±ND
RB7	34.16±ND
RB24	18.69[a]±0.54
AP3	31.5±ND
AP20	18.59[a]±0.56

[a] Statistics analysis by SPSS with t-test using paired samples test, The same superscripts are not significantly different from each other ($P<0.05$).

ND = Not detected

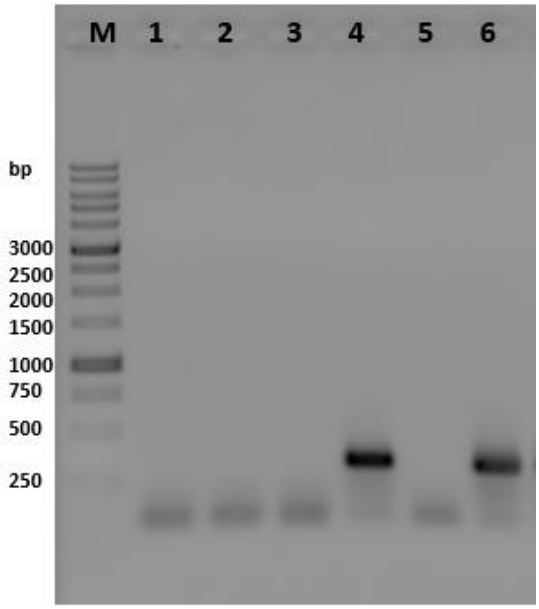


Figure 4.5 The qPCR products on 1% Agarose gel. The expected size of qPCR product of partial *R. oryzae ldhA* gene with prRB47 and prRB48 primers was 326 base pairs. Lane M: 1 kb DNA ladder marker. Lane 1: PCR buffer. Lane 2: qPCR product of

JC13509. Lane 3: qPCR product of RB7. Lane 4: qPCR product of RB24. Lane 5: qPCR product of AP3. Lane 6: qPCR product of AP20.

4.2 Discussion

The purpose of this research was to investigate the lactic acid and other byproduct production from genetically engineered *E. coli* with inactivated genes involved in fermentative growth and such mutants harboring plasmid *R. oryzae ldhA* gene. A previous study revealed that *E. coli* with *pta::kan* and *ldhA::cat* background grew poorly under anaerobic condition; however, growth could be restored when this strain obtained plasmid with *R. oryzae ldhA* gene [19]. From the same research, this strain RB24 could produce small amount of L-lactic acid under an anaerobic condition. This study hypothesized if genes involved in other pathways of fermentative metabolism were knocked out (Figure 2.10), lactic acid production should be altered and it should be useful for further improvement of L-lactic acid production from *E. coli*. Therefore, P1 transduction was used for inactivating chromosomal *pta, frdC, pflB, adhE* and *ldhA* genes. Then, the aerobic and anaerobic growth of each mutant *E. coli* strain was tested on cultivation plate incubated at 37 $^{\circ}$C for 48 hours. Although all strains could grow aerobically, mutants of deactivated *pta ldhA* with additional knocked out *pflB* or *adhE* were synthetically lethal under anaerobic condition. The former corresponded to the previous studies in which *ldh* and *pfl* could not grow fermentatively due to the inability to regenerate NAD^{+} under this condition [66, 92]. On the other hand, this study was opposed to the previous study [66] in which the exogenous *ldhA* from *R. oryzae* could not complement the loss of chromosomal *ldhA* under anaerobic condition. Moreover, in contrast to previous study [97], the deactivated *ldhA adhE* mutants were unable to anaerobically grow. This disagreement may come from many factors such as difference in growth media and conditions. For example, the strains in this study contained additional deactivated *pta*. Furthermore, this study used 20 g/l of initial glucose concentration while the study of Skory in 2000 used 0.4%. The l*dhA* from *R. oryzae* from the plasmid pRB85 could not restore the growth of *pta ldhA adhE* mutants, either.

Because *ldhA* mutants showed an induction of stress response [98], it is possibly that, from the condition in this research, the exogenous *ldhA* from *R. oryzae* expressed from its own promoter could not function efficiently, while some products from *pflB* and *adhE* pathways would be required to cope with this situation.

The deactivated *pta ldhA* and *pta ldhA frdC* mutants (RB7 and AP13, respectively) could grow poorly under anaerobic condition (Table 4.1). However, the *R. oryzae ldhA* from pRB85 plasmid could restore the growth of both mutants (RB24 and AP20, Table 4.1). The expression of *R. oryzae ldhA* from the strain RB24 and AP20 was confirmed by qRT-PCR with the primer prRB47 and prRB48 targeting *ldhA* of *R. oryzae* and showed the expression of this gene at a similar level in both strains (Figure 4.4, Table 4.3). However, a small amount of the L-lactic acid was detected from strains harboring plasmid with *R. oryzae ldhA* gene (RB24 and AP20). Corresponded to the previous study, it was reported low concentration L-lactic acid (0.901 – 2.252 g/l) was detected when *ldhA* of *R. oryzae* was expressed in *E. coli* with knocked out *ldhA* and *pfl* background, was detected [66]. It was suggested that exogenous *R. oryzae* LdhA may not function completely efficiently in this condition. When considered with lower LDH activities of the RB24 and AP20 strains than that of the wild type strain (Table 4.2), it is also possible that the recycling of NADH by conversion of pyruvate to L-lactic acid and NAD^+, NAD^+ is not enough to use in glycolysis for ATP production under anaerobic condition [99]. The investigation of slower growth rate of the strain RB24 and AP20 than that of wild type (Figure 4.3) may also be resulted from this reason. During anaerobic growth of *E. coli*, ATP was derived from substrate level phosphorylation and acetate forming reaction [17, 100]. However, strains in this study were inactivated *pta* mutants, so ATP was only derived from substrate level phosphorylation [101] (Figure 2.10). Furthermore, considering of residual glucose concentration, the strains may encounter with a problem in phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for glucose consumption. It is possible that this fermentative pathway inactivation causes an accumulation of pyruvate. This accumulation inhibits Cya, resulting in lower cAMP, and then subsequently inhibits PTS for glucose transport [102]. Although the

concentration of pyruvate could not be detected in the fermentation broth, it possibly remained in the *E. coli* cells. Furthermore, this may be indicated that *ldhA* gene from eukaryotic *R. oryzae* may not function efficiently in prokaryotic *E. coli* host.

The result of fermentation in shake flask of *E. coli* JC13509 supported Zhou and his colleague experiment [57, 84] in which lactate was a main product when fermented under anaerobic condition. However, the strain AP20 shows a reduction of byproducts, except for ethanol which may be resulted from the the redirection of anaerobic pathway when the others were knocked out. Therefore, to improve L-lactic acid production from the strain AP20, *ldhA* expression, other inhibitors in fermentative growth and factors in the fermentation process should be focused on further. Moreover, an improvement in glucose transport is important for further study.

CHAPTER V
CONCLUSION

Conclusion

In this study, many lactic acid producing strains were engineered from *E. coli* to efficiently produce lactic acid. The genetic modification of *E. coli* opens the possibility for this host organism exploitation to produce useful biochemical products. However, the mutants of deactivated *pta ldhA* with *pflB* or *adhE* were synthetically lethal under anaerobic condition. The exogenous *R. oryzae ldhA* gene on the plasmid could not restore the fermentative growth of those mutants. It is possible that in this study's condition, exogenous *R. oryzae ldhA* still relied on some products in *pflB* and *adhE* pathways to cope with an induction in stress response. Nevertheless, the exogenous *ldhA* from *R. oryzae* could complement the slow growth of *pta ldhA frdC* mutants. Although *ldhA* from *R. oryzae* on the plasmid could be expressed in *pta ldhA frdC* background (AP20). The resulting strain, AP20, efficiently fermented glucose into L-lactic acid with an optical purity of 96.7 %, a small amount of the L-lactic acid was detected. The low concentration of L-lactic acid along with lower LDH activity and growth rate may result from inadequate NADH recycle, inhibition of phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for glucose consumption, or expression of eukaryotic protein in prokaryotic host. However, for the strain AP20, the fewer amounts of byproducts, except for ethanol, compared to that of the wild type were measured.

Suggestion

To improve of L-lactic production, optimization of LDH expression, fermentation process and upscale production of L-lactic acid should give more yields because the parameters such as the pH values, oxygen limitation and revolution per minute (rpm) can be controlled in bioreactor. Furthermore, other factors in L-lactic acid production

from the strain AP20 should be further further optimized. Due to the relatively high concentration of residual glucose, parameters in glucose phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) should be focused on further. The inactivation of genes in one fermetative pathway could effect the others and also metabolic flux, so specific inhibitors in such target pathway should be studied.

REFERENCES

1. Datta R, Tsai SP, Bonsignore P, Moon SH, Frank JR: **Technological and economic potential of poly(lactic acid) and lactic acid derivatives**. *FEMS Microbiology Reviews* 1995, **16**(2-3):221-231.
2. Lunt J: **Large-scale production, properties and commercial applications of polylactic acid polymers**. *Polymer Degradation and Stability* 1998, **59**(1-3):145-152.
3. Sodergard A, Stolt M: **Properties of lactic acid based polymers and their correlation with composition**. *Progress in Polymer Science* 2002, **27**(6):1123-1163.
4. Yadav AK, Chaudhari AB, Kothari RM: **Bioconversion of renewable resources into lactic acid: an industrial view**. *Critical Reviews in Biotechnology* 2011, **31**(1):1-19.
5. Martinez FAC, Balciunas EM, Salgado JM, Domínguez JM, Converti GA, Oliveira RPDS: **Lactic acid properties, applications and production: a review**. *Trends in Food Science & Technology* 2013, **30**(1):70-83.
6. John RP, Nampoothiri KM, Pandey A: **Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives**. *Applied Microbiology and Biotechnology* 2007, **74**(3):524-534.
7. Abdel-Rahman MA, Tashiro Y, Sonomoto K: **Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits**. *Journal of Biotechnology* 2011, **156**(4):286-301.
8. Hofvendahl K, Hahn-Hagerdal B: **Factors affecting the fermentative lactic acid production from renewable resources(1)**. *Enzyme and Microbial Technology* 2000, **26**(2-4):87-107.
9. Lapierre L, Germond JE, Ott A, Delley M, Mollet B: **D-Lactate dehydrogenase gene (*ldhD*) inactivation and resulting metabolic effects in the *Lactobacillus***

johnsonii strains La1 and N312. *Applied and Environmental Microbiology* 1999, **65**(9):4002-4007.

10. Reddy G, Altaf M, Naveena BJ, Venkateshwar M, Kumar EV: **Amylolytic bacterial lactic acid fermentation - a review**. *Biotechnology Advances* 2008, **26**(1):22-34.
11. Singh SK, Ahmed SU, Pandey A: **Metabolic engineering approaches for lactic acid production**. *Process Biochemistry* 2006, **41**(5):991-1000.
12. Woiciechowski AL, Soccol CR, Ramos LP, Pandey A: **Experimental design to enhance the production of L-lactic acid from steam-exploded wood hydrolysate using *Rhizopus oryzae* in a mixed-acid fermentation**. *Process Biochemistry* 1999, **34**(9):949-955.
13. Skory CD: **Lactic acid production by *Rhizopus oryzae* transformants with modified lactate dehydrogenase activity**. *Applied Microbiology and Biotechnology* 2004, **64**(2):237-242.
14. Dien BS, Nichols NN, Bothast RJ: **Recombinant *Escherichia coli* engineered for production of L-lactic acid from hexose and pentose sugars**. *Journal of Industrial Microbiology and Biotechnology* 2001, **27**(4):259-264.
15. Tengku ET, Zainal M, Chong ML, Yoshihito S, Raha AR, Mohd AH: **Engineering of *E. coli* for increased production of L-lactic from *Enterococcus facelis* KK1**. *African Journal of Biotechnology* 2009, **8**(18):4597-4603.
16. Dien BS, Nichols NN, Bothast RJ: **Fermentation of sugar mixtures using *Escherichia coli* catabolite repression mutants engineered for production of L-lactic acid**. *Journal of Industrial Microbiology and Biotechnology* 2002, **29**(5):221-227.
17. Clark DP: **The fermentation pathways of *Escherichia coli***. *FEMS Microbiology Reviews* 1989, **5**(3):223-234.
18. Chang DE, Jung HC, Rhee JS, Pan JG: **Homofermentative production of D- or L-lactate in metabolically engineered *Escherichia coli* RR1**. *Applied and Environmental Microbiology* 1999, **65**(4):1384-1389.

19. Boonsombat R: **Production of L-lactic aid from *Escherichia coli* haboring recombinant plasmid with *Rhizopus oryzae***. *Life Science Journal* 2013, **10**(4):2217-2221.

20. Thomason LC, Costantino N, Court DL: ***E. coli* genome manipulation by P1 transduction**. USA: John Wiley & Sons, Inc.; 2001.

21. Wang Y, Tian T, Zhao J, Wang J, Yan T, Xu L *et al*: **Homofermentative production of D-lactic acid from sucrose by a metabolically engineered *Escherichia coli***. *Biotechnology Letters* 2012, **34**(11):2069-2075.

22. Benninga H: **A history of lactic acid making**. Dordrecht, Netherlands: Kluwer Academic Publishers; 1990.

23. Thongchul N: **Production of lactic acid and polylactic acid for industrial applications**. In: *Bioprocessing technologies in biofinery for sustainable production of fuels, chemicals and polymers.* Edited by Yang TS, Ensashy WE, Thongchul N. John Wiley & Sons, Inc.: New Jersey, USA; 2013: 293-316.

24. Ren J: **Biodegradable poly(lactic acid): synthesis, modification, processing and applications**. Berlin, Germany: Springer Berlin Heidelberg; 2010.

25. Wee YJ, KimJin JN, Ryu HW: **Biotechnological production of lactic acid and its recent applications**. *Food Technology and Biotechnology* 2006, **44**(2):163-172.

26. Martin AM: **Fermentation process for the production of lactic acid**. In: *Lactic acid bacteria: current advances in metabolism, genetics and applications.* Edited by Bozolu TF, Ray B, vol. 98. Berlin, Germany: Springer; 1996: 270-301.

27. Middleton JC, Tipton AJ: **Synthetic biodegradable polymers as orthopedic devices**. *Biomaterials* 2000, **21**(23):2335-2346.

28. Victory TB: **Lactic acid**. In: *Comprehensive biotechnology: the principles, applications and regulations of biotechnology in industry, agriculture and medicine.* Edited by Moo-Young M, vol. 3. New York, USA: Pregamon Press; 1985: 761-774.

29. Tokiwa Y, Jarerat A: **Biodegradation of poly (L-lactide)**. *Biotechnology Letters* 2004, **26**(10):771-777.

30. Tokiwa Y, Calabia BP: **Biodegradability and biodegradation of poly(lactide)**. *Applied Microbiology and Biotechnology* 2006, **72**:244–251.

31. Madhavan NK, Nair NR, John RP: **An overview of the recent developments in polylactide (PLA) research**. *Bioresource Technology* 2010, **101**(22):8493-8501.

32. Ma PX: **Tissue Engineering**. In: *Encyclopedia of polymer science and technology.* Edited by Kroschwitz JI, vol. 12, 3 edn. USA: John Wiley & Sons Inc.; 2004.

33. Martin O, Averous L: **Poly(lactic acid): plasticization and properties of biodegradable multiphase systems**. *Polymer* 2001, **42**(14):6209-6219.

34. Narayanan N, Roychoudhury PK, Srivastava AL: **L-lactic acid fermentation and its product polymerization**. *Electronic Journal of Biotechnology* 2004, **7**(2):167-179.

35. Datta R, Henry M: **Lactic acid: recent advances in products, processes and technologies - a review**. *Journal of Chemical Technology & Biotechnology* 2006, **81**(7):1119-1129.

36. Budhavaram NK, Fan Z: **Production of lactic acid from paper sludge using acid-tolerant, thermophilic Bacillus coagulan strains**. *Bioresource Technology* 2009, **100**(23):5966-5972.

37. Jin B, Yin P, Ma Y, Zhao L: **Production of lactic acid and fungal biomass by *Rhizopus* fungi from food processing waste streams**. *Journal of Industrial Microbiology and Biotechnology* 2005, **32**(11-12):678-686.

38. Maris AJ, Konings WN, Dijken JP, Pronk JT: **Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes**. *Metabolic Engineering* 2004, **6**(4):245-255.

39. Abdel-Rahman MA, Tashiro Y, Sonomoto K: **Recent advances in lactic acid production by microbial fermentation processes**. *Biotechnology Advances* 2013, **31**(6):877-902.

40. Bai DM, Li SZ, Liu ZL, Cui ZF: **Enhanced L-lactic acid production by an adapted strain of *Rhizopus oryzae* using corncob hydrolysate**. *Applied biochemistry and biotechnology* 2008, **144**(1):79-85.

41. Taskin M, Esim N, Ortucu S: **Efficient production of L-lactic acid from chicken feather protein hydrolysate and sugar beet molasses by the newly isolated *Rhizopus oryzae* TS-61**. *Food and Bioproducts Processing* 2012, **90**(4):773-779.

42. Wu X, Jiang S, Liu M, Pan L, Zheng Z, Luo S: **Production of L-lactic acid by *Rhizopus oryzae* using semicontinuous fermentation in bioreactor**. *Journal of Industrial Microbiology and Biotechnology* 2011, **38**(4):565-571.

43. Bulut S, Elibol M, Ozer D: **Effect of different carbon sources on L-lactic acid production by *Rhizopus oryzae***. *Biochemical Engineering Journal* 2004, **21**(1):33-37.

44. Martak J, Schlosser S, Sabolova E, Kristofı L, Rosenberg M: **Fermentation of lactic acid with *Rhizopus arrhizus* in a stirred tank reactor with a periodical bleed and feed operation**. *Process Biochemistry* 2003, **38**(11):1573-1583.

45. Oda Y, Yajima Y, Kinoshita M, Ohnishi M: **Differences of *Rhizopus oryzae* strains in organic acid synthesis and fatty acid composition**. *Food Microbiology* 2003, **20**(3):371-375.

46. Zhang ZY, Jin B, Kelly JM: **Production of lactic acid from renewable materials by *Rhizopus* fungi**. *Biochemical Engineering Journal* 2007, **35**(3):251-263.

47. Thompson DP, Eribo BE: **Extracellular enzyme production by *Rhizopus* and *Mucor* species on solid media**. *Canadian Journal of Microbiology* 1984, **30**(1):126-128.

48. Litchfield JH: **Lactic acid,microbially produced**. In: *Encyclopedia of microbiology.* Edited by Schaechter MO. Oxford, England: Academic Press; 2009: 362–372.

49. Magnuson J, Lasure L: **Organic acid production by filamentous fungi**. In: *Advances in fungal biotechnology for industry, agriculture, and medicine.* Edited by Tkacz JS, Lange L. New York, USA: Kluwer Academic/Plenum Publishers; 2004: 307–340.

50. Vink ETH, Davies S, Kolstad JJ: **ORIGINAL RESEARCH: The eco-profile for current Ingeo® polylactide production**. *Industrial Biotechnology* 2010, **6**(4):212-224.

51. Liu T, Miura S, Yaguchi M, Arimura T, Park EY, Okabe M: **Scale-up of L-lactic acid production by mutant strain *Rhizopus* sp. MK-96-1196 from 0.003 m3 to 5 m3 in airlift bioreactors**. *Journal of Bioscience and Bioengineering* 2006, **101**(1):9-12.

52. Liu Y, Liao W, Liu C, Chen S: **Optimization of L-lactic acid production using pelletized filamentous *Rhizopus oryzae* NRRL 395**. *Applied Biochemistry and Biotechnology* 2006, **131**(1-3):844-853.

53. Bai DM, Jia MZ, Zhao XM, Ban R, Shen F, Li XG *et al*: **L-lactic acid production by pellet-form *Rhizopus oryzae* R1021 in a stirred tank fermentor**. *Chemical Engineering Science* 2003, **58**(3–6):785-791.

54. Kyla-Nikkila K, Hujanen M, Leisola M, Palva A: **Metabolic engineering of *Lactobacillus helveticus* CNRZ32 for production of pure L-lactic acid**. *Applied and Environmental Microbiology* 2000, **66**(9):3835-3841.

55. Meussen BJ, Graaff LH, Sanders JP, Weusthuis RA: **Metabolic engineering of *Rhizopus oryzae* for the production of platform chemicals**. *Applied Microbiology and Biotechnology* 2012, **94**(4):875-886.

56. Zhou S, Shanmugam KT, Ingram LO: **Functional replacement of the *Escherichia coli* D-lactate dehydrogenase gene (*ldhA*) with the L-lactate dehydrogenase gene (*ldhL*) from *Pediococcus acidilactici***. *Applied and Environmental Microbiology* 2003, **69**(4):2237-2244.

57. Zhou S, Causey TB, Hasona A, Shanmugam KT, Ingram LO: **Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110**. *Applied and Environmental Microbiology* 2003, **69**(1):399-407.

58. Allison GE, Klaenhammer TR: **Phage resistance mechanisms in lactic acid bacteria**. *International Dairy Journal* 1998, **8**(3):207-226.

59. Forde A, Fitzgerald GF: **Bacteriophage defence systems in lactic acid bacteria**. *Antonie Van Leeuwenhoek* 1999, **76**(1-4):89-113.

60. Hassan A, Frank J: **Starter cultures and their use**. In: *Applied dairy microbiology.* Edited by Marth EH, Steele JL. New York, USA: Marcel Dekker, Inc; 2001: 151-206.

61. Gryganskyi AP, Lee SC, Litvintseva AP, Smith ME, Bonito G, Porter TM *et al*: **Structure, function, and phylogeny of the mating locus in the *Rhizopus oryzae* complex**. *Public Library of Science ONE* 2010, **5**(12):e15273.

62. Ghosh B, Ray RR: **Current commercial perspective of *Rhizopus oryzae* : a review**. *Journal of Applied Sciences* 2011, **11**(14):2470-2486.

63. Kwon JH, Kim J, Kim WI: **First report of *Rhizopus oryzae* as a postharvest pathogen of apple in Korea**. *Mycobiology* 2011, **39**(2):140-142.

64. Wright BE, Longacre A, Reimers J: **Models of metabolism in *Rhizopus oryzae***. *Journal of Theoretical Biology* 1996, **182**(3):453-457.

65. Pritchard GG: **An NAD-dependent L- lactate dehydrogenase from *Rhizopus oryzae***. *Biochimica et Biophysica Acta* 1971, **250**:25-34.

66. Skory CD: **Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae***. *Applied and Environmental Microbiology* 2000, **66**(6):2343-2348.

67. Acar S, Yucel M, Hamamcı H: **Purification and characterisation of two isozymes of pyruvate decarboxylase from *Rhizopus oryzae***. *Enzyme and Microbial Technology* 2007, **40**(4):675-682.

68. Skory CD: **Induction of *Rhizopus oryzae* pyruvate decarboxylase genes**. *Current Microbiology* 2003, **47**(1):59-64.

69. Yoneya T, Sato Y: **Alcohol dehydrogenase from *Rhizopus javanicus***. *Applied and Environmental Microbiology* 1979, **37**(6):1073-1078.

70. Skory CD, Ibrahim AS: **Native and modified lactate dehydrogenase expression in a fumaric acid producing isolate *Rhizopus oryzae* 99-880**. *Current Genetics* 2007, **52**(1):23-33.

71. Saito K, Saito A, Ohnishi M, Oda Y: **Genetic diversity in *Rhizopus oryzae* strains as revealed by the sequence of lactate dehydrogenase genes**. *Archives of Microbiology* 2004, **182**(1):30-36.

72. Vogt RL, Dippold L: ***Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June-July 2002**. *Public Health Reports* 2005, **120**(2):174-178.

73. Palaniappan C, Sharma V, Hudspeth ME, Meganathan R: **Menaquinone (vitamin K2) biosynthesis: evidence that the *Escherichia coli menD* gene encodes both 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase and alpha-ketoglutarate decarboxylase activities**. *Journal of Bacteriology* 1992, **174**(24):8111-8118.

74. Hudault S, Guignot J, Servin A: ***Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection**. *Gut* 2001, **49**(1):47-55.

75. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M *et al*: **Diversity of the human intestinal microbial flora**. *Science (New York, N.Y.)* 2005, **308**(5728):1635-1638.

76. Ishii S, Sadowsky MJ: ***Escherichia coli* in the environment: Implications for water quality and human health**. *Microbes and Environments* 2008, **23**(2):101-108.

77. Graef MR, Alexeeva S, Snoep JL, Teixeira MJ: **The steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli***. *Journal of Bacteriology* 1999, **181**(8):2351-2357.

78. Kochhar S, Hottinger H, Chuard N, Taylor PG, Atkinson T, Scawen MD *et al*: **Cloning and overexpression of *Lactobacillus* helveticus D-lactate dehydrogenase gene in *Escherichia coli***. *European Journal of Biochemistry* 1992, **208**(3):799-805.

79. Taguchi H, Ohta T: **D-lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxyacid dehydrogenase family. Cloning, sequencing, and expression in *Escherichia coli* of the D-lactate dehydrogenase gene of**

Lactobacillus plantarum. *The Journal of Biological Chemistry* 1991, **266**(19):12588-12594.

80. Zhou S, Yomano LP, Shanmugam KT, Ingram LO: **Fermentation of 10% (w/v) sugar to D-lactate by engineered *Escherichia coli* B**. *Biotechnology Letters* 2005, **27**(23-24):1891-1896.

81. Zhu Y, Eiteman MA, DeWitt K, Altman E: **Homolactate fermentation by metabolically engineered *Escherichia coli* strains**. *Applied and Environmental Microbiology* 2007, **73**(2):456-464.

82. Mazumdar S, Clomburg JM, Gonzalez R: ***Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol**. *Applied and Environmental Microbiology* 2010, **76**(13):4327-4336.

83. Portnoy VA, Herrgard MJ, Palsson BO: **Aerobic fermentation of D-glucose by an evolved cytochrome oxidase-deficient *Escherichia coli* strain**. *Applied and Environmental Microbiology* 2008, **74**(24):7561-7569.

84. Kessler D, Knappe J: **Anaerobic dissimilation of pyruvate**. In: *Escherichia coli and Salmonella: cellular and molecular biology*. vol. 2. USA: The American Society for Microbiology Press 1996: 199-205.

85. Gottschalk G: **Bacterial metabolism**. New York, USA: Springer-Verlag; 1985.

86. Ingledew WJ, Poole RK: **The respiratory pathways of *Escherichia coli***. *Microbiology Reviews* 1984, **48**:222-271.

87. Kaemwich J: **Metabolic engineering of *Escherichia coli* to efficiently produce succinate in mineral salts media**. University of Florida: University of Florida; 2008.

88. Haugaard N: **D- and L-lactic acid oxidases of *Escherichia coli***. *Biochimica et Biophysica Acta* 1959, **31**(1):66-72.

89. Kline ES, Mahler HR: **The lactic acid dehydrogenases of *Escherichia coli***. *Annals of the New York Academy of Sciences* 1965, **119**(3):905-919.

90. Tarmy EM, Kaplan NO: **Interacting binding sites of L-specific lactic dehydrogenase of *Escherichia coli***. *Biochemical and Biophysical Research Communications* 1965, **21**(4):379-383.

91. Tarmy EM, Kaplan NO: **Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B**. *Journal of Biological Chemistry* 1968, **243**(10):2579-2586.

92. Mat-Jan F, Alam KY, Clark DP: **Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase**. *Journal of Bacteriology* 1989, **171**(1):342-348.

93. Tarmy EM, Kaplan NO: **Kinetics of *Escherichia coli* B D-lactate dehydrogenase and evidence for pyruvate-controlled change in conformation**. *Journal of Biological Chemistry* 1968, **243**(10):2587-2596.

94. Malke H: **A short course in bacterial genetics - a laboratory manual and handbook for *Escherichia coli* and related bacteria**. *Journal of Basic Microbiology* 1993, **33**(4):278-278.

95. Sambrook J, Maniatis T, Fritsch EF: **Molecular cloning: a laboratory manual(2nd edition)**. USA: Cold Spring Harbor Laboratory Press; 1989.

96. Daniel MB, Michael DR, Stuart JE: **Protein Methods(2nd edition)**. USA: John Wiley & Sons, Inc.; 1996.

97. Sanchez AM, Bennett GN, San KY: **Efficient succinic acid production from glucose through overexpression of pyruvate carboxylase in an *Escherichia coli* alcohol dehydrogenase and lactate dehydrogenase mutant**. *Biotechnology Progress* 2005, **21**(2):358-365.

98. Kabir MM, Ho PY, Shimizu K: **Effect of *ldhA* gene deletion on the metabolism of *Escherichia coli* based on gene expression, enzyme activities, intracellular metabolite concentrations, and metabolic flux distribution**. *Biochemical Engineering Journal* 2005, **26**(1):1-11.

99. Bunch PK, Mat-Jan F, Lee N, Clark DP: **The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli***. *Microbiology* 1997, **143**:187-195.

100. Boeck A, Sawers G: **Fermentation**. In: *Escherichia coli and Salmonella: cellular and molecular biology*. vol. 2. USA: The American Society for Microbiology Press; 1996: 262-282.

101. Hasona A, Kim Y, Healy FG, Ingram LO, Shanmugam KT: **Pyruvate formate lyase and acetate kinase are essential for anaerobic growth of *Escherichia coli* on xylose**. *Journal of Bacteriology* 2004, **186**(22):7593-7600.

102. Steinsiek S, Bettenbrock K: **Glucose transport in *Escherichia coli* mutant strains with defects in sugar transport systems**. *Journal of Bacteriology* 2012, **194**(21):5897-5908.

APPENDIX

APPENDIX A

MEDIA AND SOLUTIONS

1. Antibiotics

Ampicillin (100mg/ml)

Dissolve 1000mg ampicillin in 10 ml dH_2O. Working solution is 100 µg/ml. Prepare in 1 ml aliquots and store at -20°C.

Chloramphenicol (25mg/ml)

Dissolve 100 mg chloramphenicol in 1 ml 100% ethanol. Add 9 ml dH_2O and store at 4°C. Working solution is 25 µg/ml.

Kanamycin (50mg/ml)

Dissolve 500 mg in 10 ml dH_2O. Working solution is 50 µg/ml. Prepare in 1 ml aliquots and store at -20°C.

2. LB broth

Peptone	10 g
Yeast extract	5 g
NaCl	10 g

Dissolve in 1000 ml of dH_2O and autoclave 121 °C for 15 min.

3. LB agar

Peptone	10 g
Yeast extract	5 g
NaCl	10 g
Bacto-agar	20 g

Dissolve in 1000 ml of dH_2O and autoclave 121 °C for 15 min.

4. Salt solution

$MgSO_4.7H_2O$	0.4 g
$MnSO_4.5H2O$	0.02 g

$FeSO_4.7H_2O$	0.02 g

Dissolve in 10 ml of dH_2O

5. **Cultivation plate and slant**

Glucose	10 g
Yeast extract	5 g
Peptone	5 g
KH_2PO_4	0.25 g
K_2HPO_4	0.25 g
$CaCO_3$	5 g
Bacto-agar	10 g
Salt solution	5 ml

Adjust final volume to 1000 ml with dH_2O and pH 6.8, autoclave 121 °C for 15 min.

6. **Pre-culture broth**

Glucose	10 g
Yeast extract	5 g
Peptone	5 g
KH_2PO_4	0.25 g
K_2HPO_4	0.25 g
$CaCO_3$	5 g
Salt solution	5 ml

Adjust final volume to 1000 ml with dH_2O and pH 6.8, autoclave 121 °C for 15 min.

7. **Fermentation broth**

Glucose	20 g
Yeast extract	5 g
Peptone	5 g
KH_2PO_4	0.25 g
K_2HPO_4	0.25 g
$CaCO_3$	20 g

Salt solution	5 ml

Adjust final volume to 1000 ml with dH_2O and pH 6.8, autoclave 121 °C for 15 min.

8. **5X 56 Phosphate buffer**

Na_2HPO_4	48.4 g
KH_2PO_4	25.5 g

Dissolve in dH_2O 800 ml and heat/stir until dissolved. Cool to RT before proceeding.

$MgSO_4$.7H2O	1 g
$(NH_4)_2SO_4$	10 g
$Ca(NO_3)_2.4H_2O$	0.051 g
$FeSO_4$	0.0025 g

Adjust final volume to 5000 ml with dH_2O and pH 7, solution may appear cloudy.

9. **56/2 Phosphate buffer**

dH_2O	500 ml
5X 56 Phosphate buffer	500 ml

Autoclave 121 °C for 15 min.

10. **LCTG plates**

1 M $CaCl_2$	4 ml
Glucose	2 g
Bacto-agar	20 g
1% thymidine (filter sterilized)	2 ml

Adjust final volume to 1000 ml with dH_2O and autoclave 121 °C for 15 min.

11. **LCTG top agar**

Bacto-agar	7 g
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Dissolve in 1000 ml of dH_2O and autoclave 121 °C for 15 min. Heat until melted before use.

12. TB-I (Transformation buffer I)

KOAc	1.47 g
$MnCl_2$	5.0 g
RbCl	6 g
$CaCl_2$	0.74 g
15% glycerol	75 ml

Adjust final volume to 500 ml with dH_2O. Sterilize by filtering through 0.22 µm filter and store at 4 °C.

13. TB-II (Transformation buffer II)

MOPS	1.04 g
$CaCl_2$	5.5 g
RbCl	0.6 g
15% glycerol	75 ml

Adjust final volume to 500 ml with dH_2O. Sterilize by filtering through 0.22 µm filter and store at 4 °C.

14. 50X TAE

Trizma base	485 g
Disodium dihydrate EDTA	36 g
$NaOAc.7H_2O$	41 g

Dissolve in 1 l of dH_2O and bring pH to approximately 8 using glacial acetic acid (approximately 180 ml). Adjust volume to 2 l with dH_2O. Do not autoclave.

15. 1X TAE

For use as gel electrophoresis buffer, add 200ml of 50X TAE to large 10 l jug with approximately 9.8 l dH_2O.

APPENDIX B

DETERMINATION OF EACH FERMENTATION PRODUCT CONCENTRATION BY HPLC

1. Standard curve of succinic acid

For the standard curve, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/l of standard succinic acid were applied in HPLC.

.

Table B1 Concentrations of standard succinic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard succinic acid curve in Figure B1.

Standard succinic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	209546	208904	210518	209656±812.603
1	106983	108342	107481	107602±687.533
0.5	55987	55763	55794	55848±121.371
0.25	25745	25942	25590	25759±176.417
0.125	11987	12345	12037	12123±193.876
0.0625	6342	6445	6470	6419±67.845
0.03125	2001	1956	2091	2016±68.739

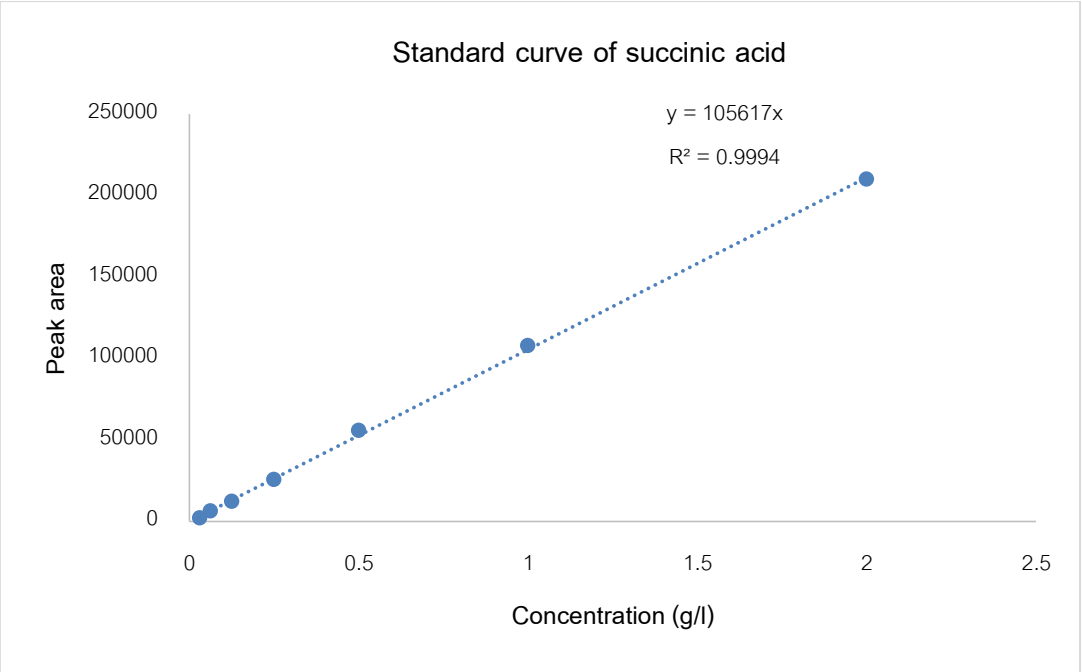


Figure B1 A standard succinic acid curve from HPLC by using Aminex HPX-87H column with RI detector for succinic acid determination

The equation from the graph in Figure B1 was used to calculate the succinic acid concentration in the each fermented sample. From this graph, the succinic acid concentration was calculated as the following:

$$y = 105671x$$

which y was the peak area of each sample and x was the succinic acid concentration in each sample. With this calculation, the average succinic acid concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

2. Standard curve of acetic acid

For the standard curve, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/l of standard acetic acid were applied in HPLC.

Table B2 Concentrations of standard acetic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard acetic acid curve in Figure B2.

Standard acetic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	146934	147456	147555	147315±333.648
1	77345	77742	78010	77699±334.579
0.5	41134	41024	41295	41151±136.297
0.25	19754	19703	19769	19742±34.598
0.125	13721	13793	13772	13762±37.027
0.0625	3829	3821	3783	3811±24.576
0.03125	850	864	833	849±15.524

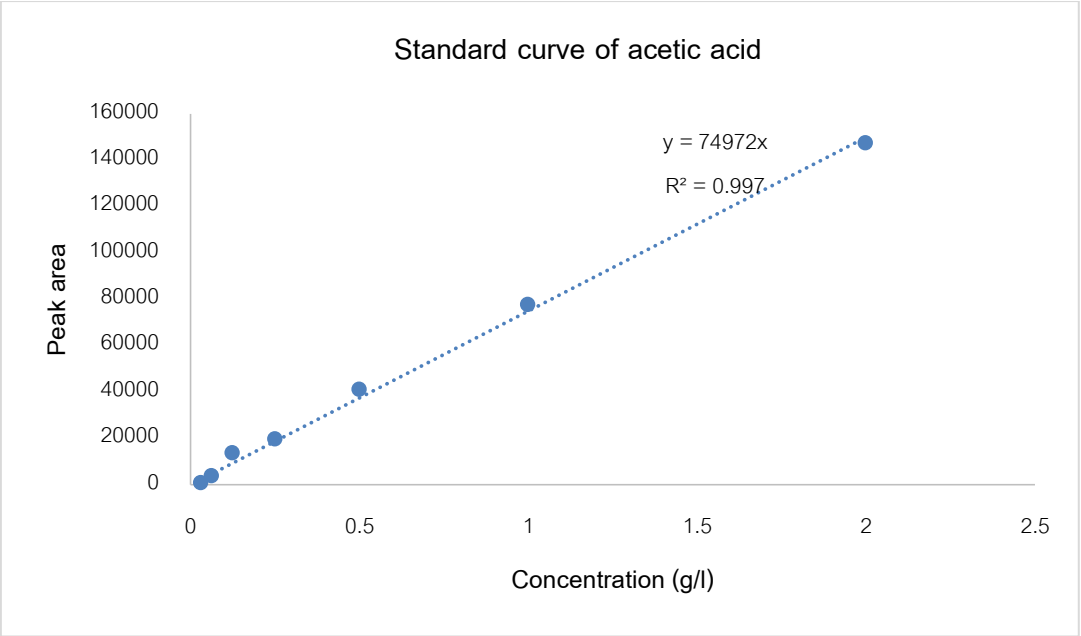


Figure B2 A standard acetic acid curve from HPLC by using Aminex HPX-87H column with RI detector for acetic acid determination

The equation from the graph in Figure B2 was used to calculate the acetic acid concentration in the each fermented sample. From this graph, the acetic acid concentration was calculated as the following:

$$y = 74972x$$

which y was the peak area of each sample and x was the acetic acid concentration in each sample. With this calculation, the average acetic acid concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

3. Standard curve of ethanol

For the standard curve, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/l of standard ethanol were applied in HPLC.

Table B3 Concentrations of standard ethanol and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard ethanol curve in Figure B3.

Standard ethanol concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	102756	102332	103399	102829±537.233
1	51734	51832	51711	51759±64.257
0.5	27059	26932	27096	27029±86.017
0.25	10231	10123	10114	10156±65.108
0.125	5758	5744	5787	5763±21.932
0.0625	3420	3447	3399	3422±24.062
0.03125	989	1012	1032	1011±21.517

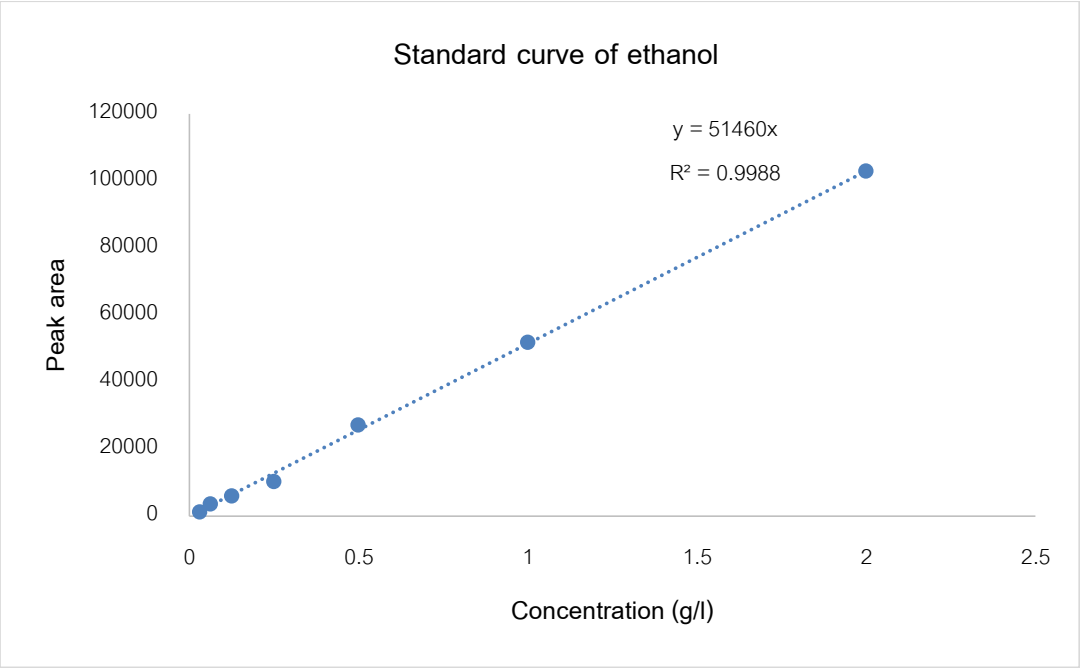


Figure B3 A standard ethanol curve from HPLC by using Aminex HPX-87H column with RI detector for ethanol determination

The equation from the graph in Figure B3 was used to calculate the ethanol concentration in the each fermented sample. From this graph, the ethanol concentration was calculated as the following:

$$y = 51460x$$

which y was the peak area of each sample and x was the ethanol concentration in each sample. With this calculation, the average ethanol concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

4. Standard curve of formic acid

For the standard curve, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/l of standard formic acid were applied in HPLC.

Table B4 Concentrations of standard formic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard formic acid curve in Figure B4.

Standard formic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	68321	69945	68002	68756±1041.984
1	24934	24353	25428	24905±538.086
0.5	13435	13422	13454	13437±16.093
0.25	6143	6123	6181	6149±29.462
0.125	2956	2993	3009	2986±27.185
0.0625	1442	1511	1526	1493±44.800
0.03125	762	796	770	776±17.776

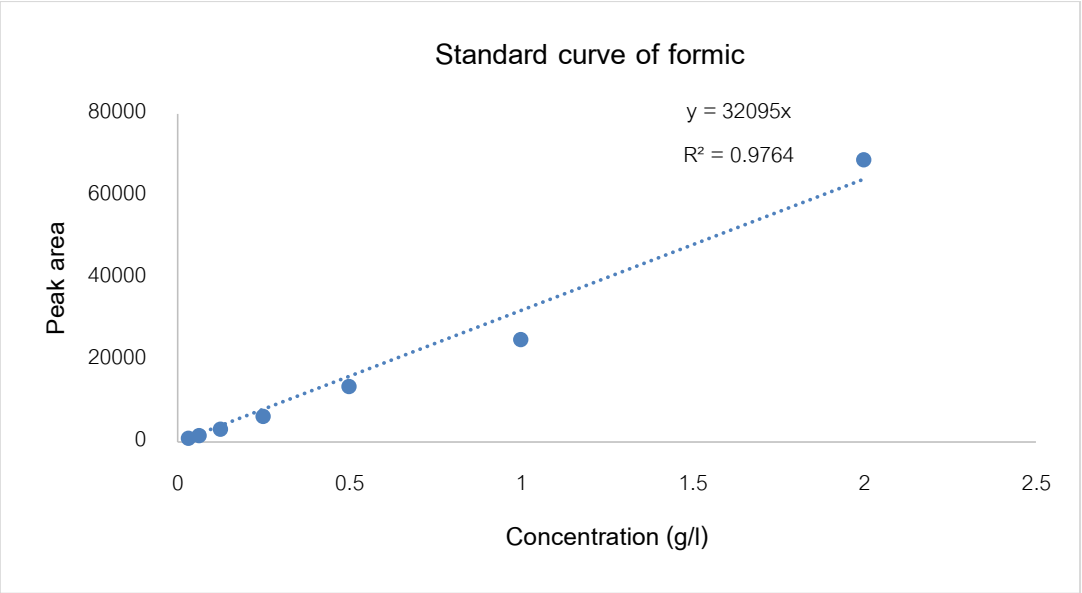


Figure B4 A standard formic acid curve from HPLC by using Aminex HPX-87H column with RI detector for formic acid determination

The equation from the graph in Figure B4 was used to calculate the formic acid concentration in the each fermented sample. From this graph, the formic acid concentration was calculated as the following:

$$y = 32095x$$

which y was the peak area of each sample and x was the formic acid concentration in each sample. With this calculation, the average formic acid concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

5. Standard curve of glucose

For the standard curve, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/l of standard glucose were applied in HPLC

Table B5 Concentrations of standard glucose and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard glucose curve in Figure B5.

Standard glucose concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
4	590332	591103	589525	590320±789.068
2	279436	278453	280707	279532±1130.062
1	146301	146335	146237	146291±49.759
0.5	75316	75219	75113	75216±101.533
0.25	34414	34121	34698	34411±288.512
0.125	17432	17335	17358	17375±50.685
0.0625	8634	8698	8717	8683±43.486
0.03125	4021	3998	3957	3992±32.419

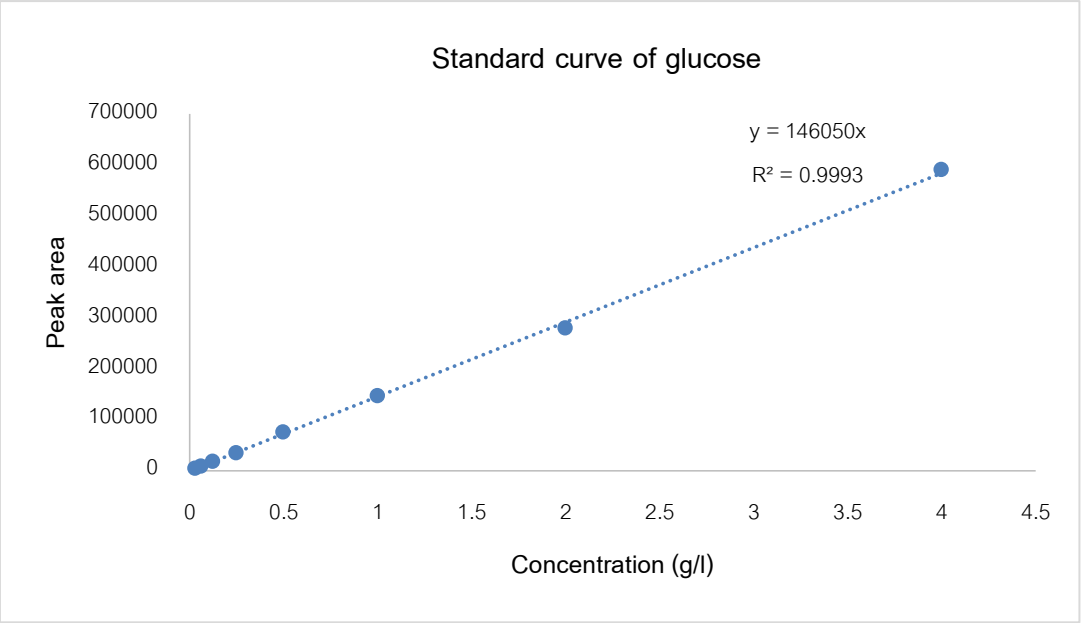


Figure B5 A standard glucose curve from HPLC by using Aminex HPX-87H column with RI detector for glucose determination

The equation from the graph in Figure B5 was used to calculate the glucose concentration in the each fermented sample. From this graph, the glucose concentration was calculated as the following:

$$y = 146050x$$

which y was the peak area of each sample and x was the glucose concentration in each sample. With this calculation, the average glucose concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

6. Standard curve of L-lactic acid

For the standard curve, 2, 1, 0.5, 0.125 and 0.03125 g/l of standard L-lactic acid were applied in HPLC.

Table B6 Concentrations of standard L-lactic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard L-lactic acid curve in Figure B6

Standard L-lactic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	9376564	9369743	9385956	9377421±8140.404
1	4835469	4835648	4833565	4834894±1154.422
0.5	2143654	2144032	2141686	2143124±1259.605
0.125	571453	572144	571665	571754±353.993
0.03125	137532	137631	137715	137626±91.602

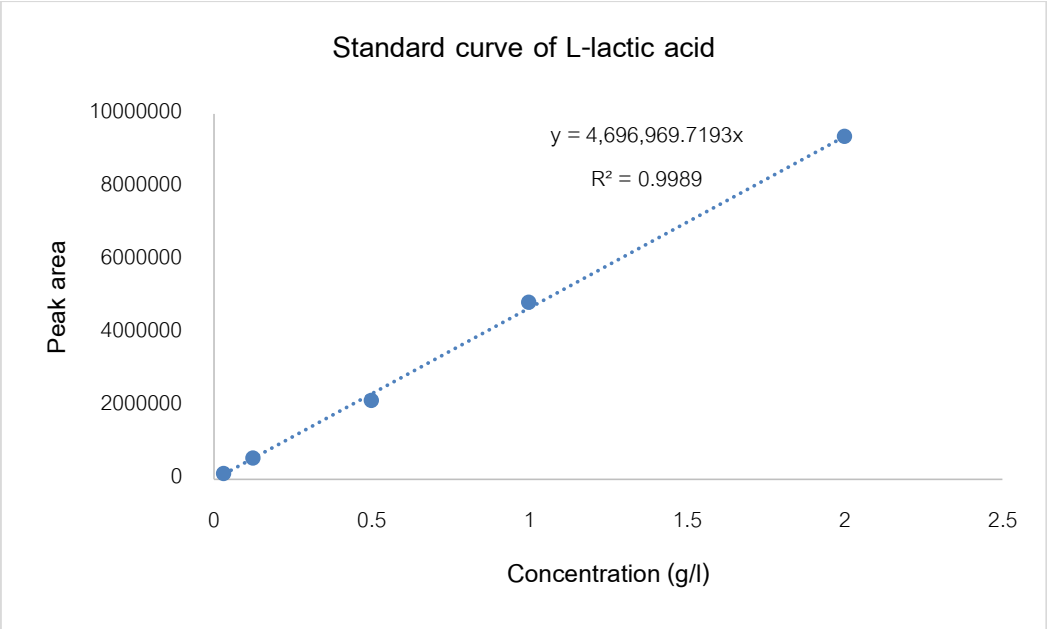


Figure B6 A standard L-lactic acid curve from HPLC by using chiral column with UV detector at 254 nm for L-lactic acid determination

The equation from the graph in Figure B6 was used to calculate the L-lactic acid concentration in the each fermented sample. From this graph, the L-lactic acid concentration was calculated as the following:

$$y = 4{,}696{,}969.7193x$$

which y was the peak area of each sample and x was the L-lactic acid concentration in each sample. With this calculation, the average L-lactic acid concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

7. Standard curve of D-lactic acid

For the standard curve, 2, 1, 0.5, 0.125 and 0.03125 g/l of standard D-lactic acid were applied in HPLC.

Table B7 Concentrations of standard D-lactic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector for creating the standard D-lactic acid curve in Figure B7.

Standard D-lactic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
2	10607839	10608264	10608431	10608178±305.226
1	5414675	5413974	5415076	5414575±557.764
0.5	2397523	2397904	2398012	2397813±256.887
0.125	636890	636912	637168	636990±154.544
0.03125	146283	146223	146376	146294±77.091

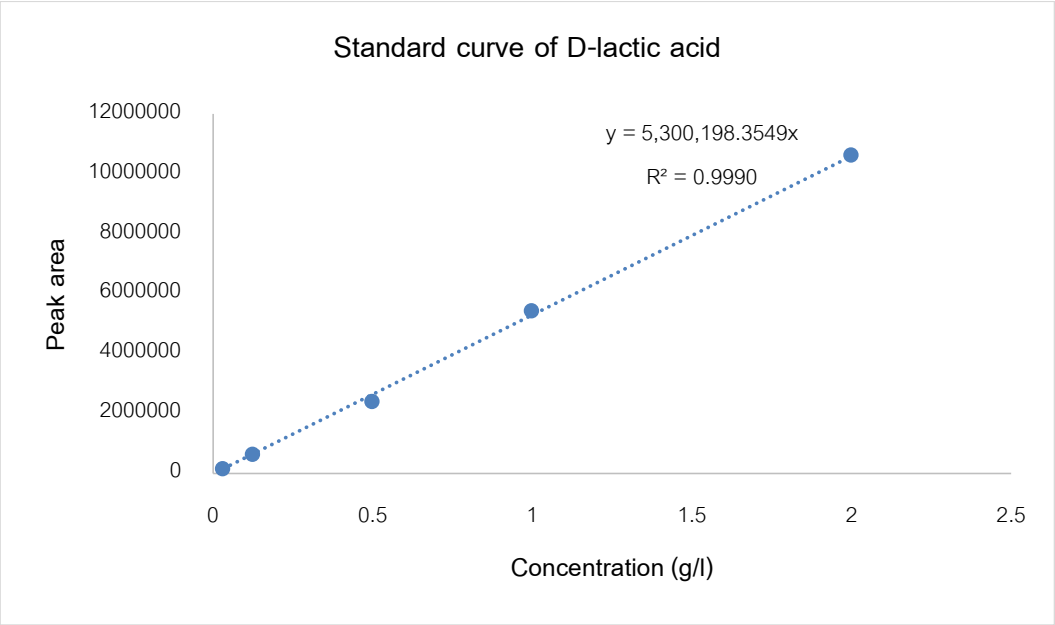


Figure B7 A standard D-lactic acid curve from HPLC by using chiral column with UV detector at 254 nm for D-lactic acid determination.

The equation from the graph in Figure B7 was used to calculate the D-lactic acid concentration in the each fermented sample. From this graph, the D-lactic acid concentration was calculated as the following:

$$y = 5{,}300{,}198.3549x$$

which y was the peak area of each sample and x was the D-lactic acid concentration in each sample. With this calculation, the average D-lactic acid concentrations from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table B9.

8. Retention time of fermentation products

.

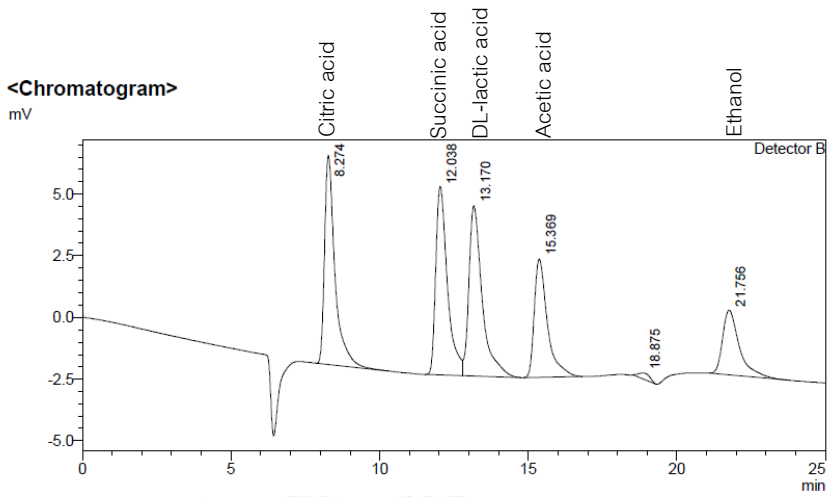


Figure B8 The chromatogram of acetic acid, citric acid, ethanol, DL-lactic acid and succinic acid by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

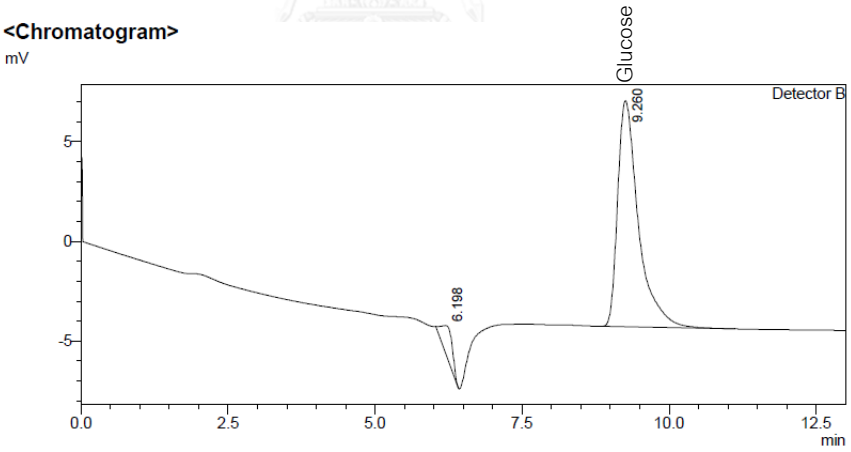


Figure B9 The chromatogram of glucose by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

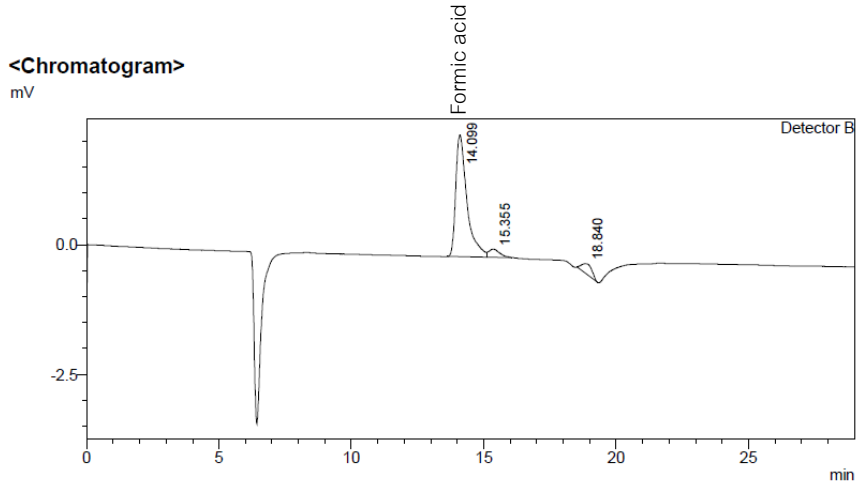


Figure B10 The chromatogram of formic acid by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

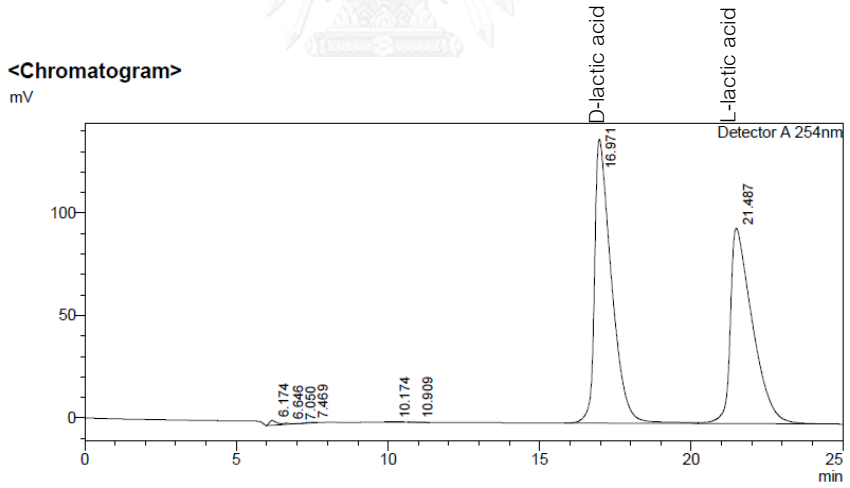


Figure B11 The chromatogram of D-lactic acid and L-lactic acid by using a high-pressure liquid chromatography (HPLC) equipped with Chiral column and UV detector at 254 nm.

Table B8 The retention time of each fermentation product by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and Chiral column.

Products	Retention time in Aminex column (min)	Retention time in Chiral column (min)
Acetic acid	15.36-15.39	ND
Ethanol	21.75-21.77	ND
Formic acid	14.10-14.30	ND
Glucose	9.20-9.30	ND
DL-lactic acid	13.10-13.20	ND
D-lactic acid	ND	16.90-17.20
L-lactic acid	ND	21.40-21.80
Succinic acid	12.00-12.10	ND

9. **The concentrations of residual glucose and fermentation products from all strains in this study**

Table B9 The concentrations of the fermentation products, measured by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and Chiral column, from all strains constructed in this research

Strains	Concentration (g/l) (Mean±S.D.)									
	Residual Glucose	Succinate	Lactate	Formate	Acetate	Ethanol	D-lactate	L-lactate	%purity D form	%purity L form
JC13509	7.287±3.216	1.485±0.196	5.487±0.375	0.000±0.000	1.910±0.513	1.982±0.748	5.064±0.397	0.348±0.101	93.656±1.291	6.344±1.291
RB5	8.255±0.338	1.639±0.179	0.302±0.027	1.929±0.029	2.692±0.186	3.545±0.258	0.033±0.011	0.193±0.009	14.511±4.828	85.489±4.828
RB6	10.340±2.129	1.162±0.122	7.996±0.741	0.000±0.000	0.082±0.116	0.418±0.177	7.815±0.742	0.320±0.018	96.040±0.576	3.960±0.576
RB7	10.200±3.894	1.960±1.300	0.058±0.032	0.201±0.308	0.702±0.190	2.458±0.477	0.011±0.018	0.148±0.020	5.994±10.381	94.006±10.381
RB24	8.767±4.284	1.720±0.261	0.097±0.005	0.170±0.140	0.499±0.024	3.453±1.075	0.006±0.005	0.292±0.055	2.052±1.793	97.948±1.793
AP1	11.499±0.213	0.834±0.130	7.774±0.010	0.000±0.000	0.120±0.048	0.365±0.011	7.275±0.184	0.385±0.024	94.978±0.182	5.022±0.182
AP2	13.463±0.753	0.075±0.004	5.519±0.584	0.000±0.000	0.095±0.036	0.319±0.070	5.403±0.449	0.313±0.031	94.520±0.083	5.480±0.083
AP3	13.133±2.228	0.282±0.281	6.375±0.503	0.000±0.000	0.366±0.382	0.197±0.341	6.315±0.865	0.395±0.018	94.049±0.745	5.951±0.745
AP7	11.353±0.633	1.155±0.049	8.372±0.280	0.000±0.000	0.138±0.050	0.000±0.000	7.699±0.145	0.448±0.040	94.504±0.361	5.496±0.361
AP8	10.580±1.051	0.184±0.025	7.886±0.537	0.000±0.000	0.152±0.013	0.663±0.006	7.623±0.225	0.366±0.064	95.409±0.896	4.591±0.896
AP11	10.152±0.295	0.805±0.346	6.398±3.409	0.000±0.000	0.115±0.004	0.139±0.014	8.385±0.237	0.326±0.003	96.255±0.064	3.745±0.064
AP13	13.000±1.921	0.214±0.072	0.134±0.040	1.491±0.189	0.386±0.059	3.601±0.483	0.207±0.033	0.188±0.019	52.347±5.162	47.653±5.162
AP15	13.348±2.254	0.073±0.008	6.243±2.263	0.000±0.000	0.110±0.013	0.156±0.036	7.324±0.721	0.372±0.033	95.125±0.861	4.875±0.861
AP20	9.479±2.342	0.171±0.046	0.105±0.015	0.145±0.110	0.492±0.029	3.064±0.64	0.014±0.014	0.411±0.146	3.261±2.398	96.739±2.398

10. The yield production of each fermentation product

Table B10 The yield production of each fermentation product calculated by gram of product production/gram of glucose consumption.

Strains	$Y_{p/s}$ (Mean±S.D.)						
	Succinate	Lactate	Formate	Acetate	Ethanol	D-Lactate	L-Lactate
JC13509	0.116±0.009	0.433±0.049	0.000±0.000	0.149±0.032	0.154±0.049	0.400±0.048	0.028±0.009
RB5	0.118±0.014	0.022±0.002	0.138±0.005	0.193±0.014	0.255±0.024	0.002±0.001	0.014±0.001
RB6	0.099±0.007	0.680±0.060	0.000±0.000	0.006±0.009	0.034±0.009	0.664±0.057	0.028±0.006
RB7	0.191±0.102	0.006±0.003	0.018±0.027	0.072±0.020	0.249±0.016	0.001±0.002	0.015±0.003
RB24	0.157±0.023	0.009±0.003	0.013±0.010	0.046±0.012	0.304±0.024	0.000±0.000	0.028±0.014
AP1	0.078±0.011	0.727±0.015	0.000±0.000	0.011±0.004	0.034±0.000	0.680±0.004	0.036±0.002
AP2	0.009±0.000	0.631±0.012	0.000±0.000	0.011±0.005	0.037±0.011	0.619±0.002	0.036±0.000
AP3	0.037±0.030	0.956±0.215	0.000±0.000	0.048±0.042	0.023±0.041	0.947±0.235	0.059±0.013
AP7	0.107±0.002	0.772±0.019	0.000±0.000	0.013±0.005	0.000±0.000	0.711±0.028	0.041±0.001
AP8	0.016±0.004	0.679±0.015	0.000±0.000	0.013±0.000	0.057±0.005	0.658±0.040	0.032±0.008
AP11	0.066±0.027	0.528±0.270	0.000±0.000	0.010±0.000	0.012±0.001	0.696±0.003	0.027±0.000
AP13	0.023±0.004	0.014±0.002	0.164±0.016	0.042±0.004	0.396±0.039	0.024±0.008	0.021±0.007
AP15	0.009±0.003	0.695±0.079	0.000±0.000	0.013±0.002	0.018±0.000	0.844±0.134	0.044±0.015
AP20	0.018±0.009	0.033±0.023	0.012±0.009	0.049±0.015	0.292±0.005	0.001±0.001	0.042±0.021

APPENDIX C

DETERMINATION OF LDH ACTIVITY AND TOTAL CELL PROTEIN

1. Standard curve of NADH

For the standard curve, the UV absorbance from the reactions of 0.192, 0.096, 0.048, 0.024, 0.012, 0.006 and 0 µmol/ml of standard NADH with the volume of 200 µl/well were measured at 340 nm by microplate spectrophotometer. The absorbance values are represented in Table C1.

Table C1 The amount of standard NADH and its average 340 nm absorbance value for creating the standard NADH curve in Figure C1.

NADH (µmole)	Absorbance at 340 nm			Average (Mean±S.D.)
	1	2	3	
0.000	0.000	0.000	0.000	0.000±0.000
0.006	0.019	0.024	0.020	0.021±0.003
0.012	0.039	0.039	0.038	0.039±0.001
0.024	0.073	0.076	0.075	0.075±0.002
0.048	0.139	0.148	0.148	0.145±0.005
0.096	0.285	0.295	0.289	0.290±0.005
0.192	0.593	0.593	0.581	0.589±0.007

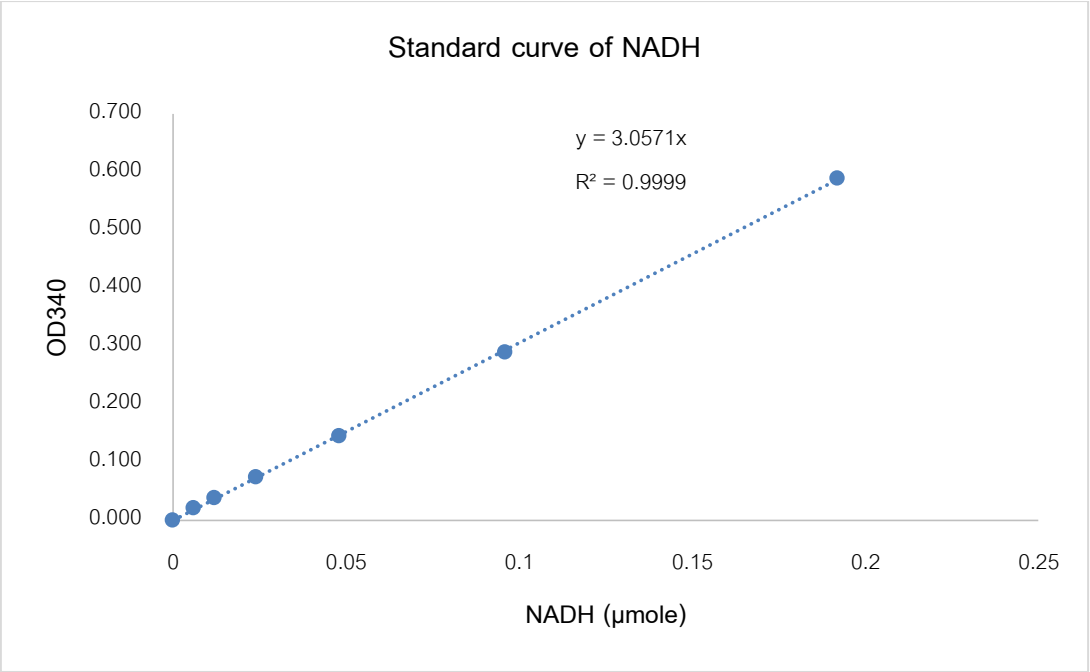


Figure C1 A standard NADH curve for calculating the amount of NADH in the reaction of LDH activity assay.

The equation from the graph in Figure C1 was used to calculate the amount of NADH in the reaction of LDH activity assay. The amount of NADH was calculated as the following:

$$y = 3.0571x$$

which y was the absorbance value at 340 nm of each reaction and x is the amount of NADH in the reaction of each sample. With this calculation, the average amount of NADH from JC13509, RB7, RB24, AP3 and AP20 strains (from 3 repeats of each set of experiment) are represented in Table C3.

Table C2 The absorbance value at 340 nm of each *E. coli* strain after fermented 48 hours by using LDH activity assay.

Sample	Absorbance at 340 nm			Average (Mean±S.D.)
	1	2	3	
Blank	0.335	0.332	0.335	0.334±0.002
JC13509	0.892	0.902	0.905	0.899±0.007
RB7	0.917	0.911	0.900	0.909±0.009
RB24	0.891	0.908	0.885	0.894±0.012
AP3	0.886	0.888	0.883	0.886±0.003
AP20	0.893	0.878	0.902	0.891±0.012

Table C3 The amount of NADH of each *E. coli* strain after fermented 48 hours by using LDH activity assay.

Sample	NADH (μmole)			Average (Mean±S.D.)
	1	2	3	
JC13509	0.182	0.186	0.186	0.185±0.002
RB7	0.190	0.189	0.185	0.188±0.003
RB24	0.182	0.189	0.180	0.183±0.005
AP3	0.180	0.182	0.179	0.181±0.002
AP20	0.183	0.179	0.185	0.182±0.003

The amount of NADH was then used to calculate into NAD^+ production as the following:

$$\frac{(a - NADH)}{b} \times c = NAD^+ \text{ μmole}$$

which a was the initial amount of NADH (0.192 μmol), b was the molecular weight of NADH (665.43 g/mol), and c was the molecular weight of NAD^+(664.43 g/mole). The average NAD^+ productions (from 3 repeats of each set of experiment) from JC13509, RB7, RB24, AP3 and AP20 strains from this equation is represented in Table C4.

Table C4 The amount of NAD^+ produced of each selected *E. coli* strain after being fermented for 48 hours.

Sample	Produced NAD^+ (μmole)			Average (Mean±S.D.)
	1	2	3	
JC13509	0.0100	0.0060	0.0060	0.0073±0.0023
RB7	0.0020	0.0030	0.0070	0.0040±0.0026
RB24	0.0100	0.0030	0.0120	0.0083±0.0047
AP3	0.0120	0.0100	0.0130	0.0116±0.0015
AP20	0.0090	0.0130	0.0070	0.0097±0.0031

2. Standard curve of BSA

The BSA was used as the standard protein in Bradford assay. For the standard curve, the 595 nm UV absorbance from 300, 200, 175, 150, 125, 100, 75, 50, 25 and 0 μg/ml of standard BSA with the volume of 200 μl/well were measured microplate spectrophotometer.

Table C5 Concentrations of standard BSA and the average 595 nm absorbance values for creating the standard BSA curve in Figure C2.

BSA (µg/ml)	Absorbance at 595 nm			Average (Mean±S.D.)
	1	2	3	
0	0	0	0	0.000±0.000
25	0.068	0.063	0.062	0.064±0.003
50	0.129	0.148	0.127	0.134±0.012
75	0.129	0.13	0.117	0.125±0.007
100	0.177	0.185	0.188	0.184±0.006
125	0.227	0.221	0.209	0.219±0.009
150	0.257	0.276	0.277	0.270±0.011
175	0.286	0.286	0.291	0.288±0.003
200	0.307	0.287	0.302	0.299±0.010
300	0.38	0.37	0.402	0.384±0.016

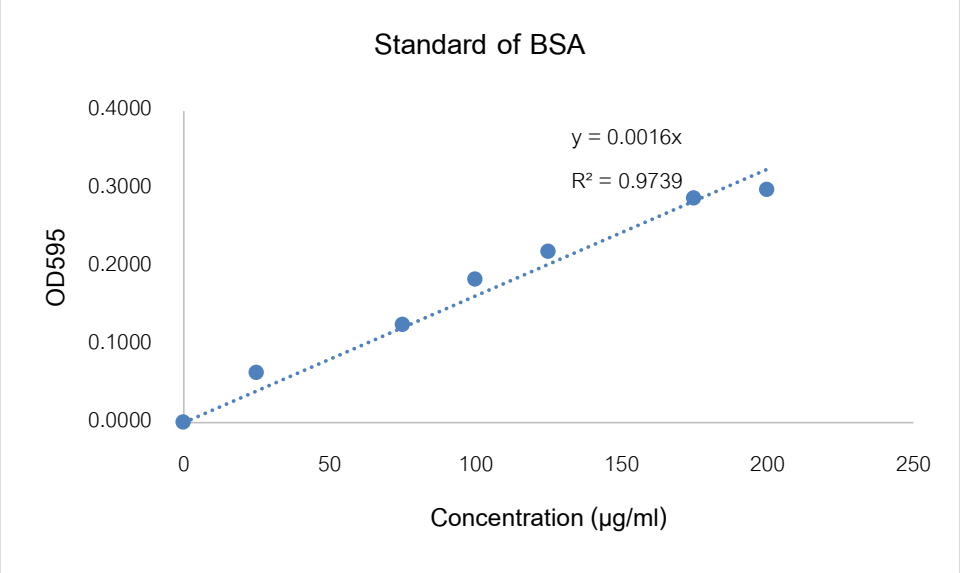


Figure C2 A standard BSA curve for total protein determination from Bradford assay measured at 595 nm by microplate spectrophotometer.

The equation from the graph in Figure C2 was used to calculate the total protein in Bradford assay. The total protein was calculated as the following:

$$y = 0.0016x$$

which y was the 595 nm absorbance value of each sample and x is the total protein concentration of each sample With this calculation, the average values of the total protein from JC13509, RB7, RB24, AP3 and AP20 (from 3 repeats of each set of experiment) are represented in Table C7.

Table C6 The 595 nm absorbance value of each selected *E. coli* strain after being fermented for 48 hours.

Sample	Absorbance at 595 nm			Average (Mean±S.D.)
	1	2	3	
Blank	0.181	0.191	0.185	0.186±0.005
JC13509	0.362	0.327	0.276	0.322±0.043
RB7	0.448	0.501	0.459	0.469±0.028
RB24	0.540	0.546	0.516	0.534±0.016
AP3	0.365	0.362	0.340	0.355±0.014
AP20	0.462	0.462	0.355	0.426±0.062

Table C7 The total protein concentration of each selected *E. coli* strain after being fermented 48 hours.

Sample	Protein concentration (μg/ml)			Average (μg/ml)
	1	2	3	
JC13509	113.042	84.646	57.125	84.938±27.959
RB7	166.771	193.396	171.138	177.101±14.279
RB24	224.542	221.729	206.958	217.743±9.445
AP3	114.583	106.479	96.771	105.944±8.918
AP20	175.396	169.104	106.438	150.313±38.127

3. LDH activity calculation

The LDH activity was calculated from the NAD^+, production, total protein and time of reaction as the following:

$$\frac{a}{b \times c} = \text{LDH activity μmole/mg of protein/min}$$

which a was the amount of produced NAD^+ (μmole), b is the total protein (mg) and c is the time of reaction (min). The LDH activities of JC13509, RB7, RB24, AP3 and AP20 are represented in Table C8. The average values from 3 repeats of each set of experiment were summerized in Table 4.2

Table C8 The LDH activity of each selected strain after incubated for 5 min at room temperature.

Sample	Produced NAD^+(μmole)	Total protein (mg)*	Time (min)	LDH activity (μmole/mg.min)
JC13509(1)	0.010	0.002	5	0.885
JC13509(2)	0.006	0.002	5	0.709
JC13509(3)	0.006	0.001	5	1.050
RB7(1)	0.002	0.003	5	0.120
RB7(2)	0.003	0.004	5	0.155
RB7(3)	0.007	0.003	5	0.409
RB24(1)	0.010	0.004	5	0.445
RB24(2)	0.003	0.004	5	0.135
RB24(3)	0.012	0.004	5	0.580
AP3(1)	0.012	0.002	5	1.047
AP3(2)	0.010	0.002	5	0.939
AP3(3)	0.013	0.002	5	1.343
AP20(1)	0.009	0.004	5	0.513
AP20(2)	0.013	0.003	5	0.769
AP20(3)	0.007	0.002	5	0.658

* Total protein in milligram was calculated from protein concentration (Figure C7), 20 μl of crude enzyme was used for LDH activity assay

VITA

Mr. Thagoon Yamoat was born on May 30, 1990 in Nakhonpathom, Thailand. He graduated with a Bachelor degree of Science in field of Biology from Faculty of Science, Chulalongkorn University in 2011. He has studied for a Master degree of Science in Biotechnology, the Faculty of Science, Chulalongkorn University since 2012.

Academic Presentation;

Yamoat, T., and Boonsombat, R. Improvement of lactic acid production in Escherichia coli harboring plasmid with Rhizopus oryzae ldhA gene. The Society for Biotechnology International Forum 2013, 28-30 August 2013, Bitec Bang Na Hall 101, Bangkok, Thailand.