การผลิตกรดแล็กติกโดย *Escherichia coli* ที่ได้รับยีน *ldhA* จาก *Rhizopus oryzae*

นางสาวธนวรรณ วัฒนพร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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LACTIC ACID PRODUCTION BY *Escherichia coli* HARBORING *ldhA* GENE FROM *Rhizopus oryzae*

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ธนวรรณ วัฒนพร : การผลิตกรดแล็กติกโดย *Escherichia coli* ที่ได้รับยีน *ldhA* จาก *Rhizopus oryzae* (LACTIC ACID PRODUCTION BY *Escherichia coli* HARBORING *ldhA* GENE FROM *Rhizopus oryzae*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ฤทัยรัตน์ บุญสมบัติ, 106 หน้า.

ในปัจจุบันได้มีการนำกรดแล็กติกมาประยุกต์ใช้ในอุตสาหกรรมอย่างกว้างขวาง เช่น อุตสาหกรรมอาหาร ยา เครื่องสำอาง อีกทั้งยังนำมาใช้เป็นสารตั้งต้นในการผลิตพลาสติกที่ย่อยสลายได้โดยธรรมชาติ กรดแล็กติกสามารถผลิตได้จากกระบวนการหมักโดยใช้จุลินทรีย์ เช่น รา *Rhizopus oryzae* ข้อดีของการผลิตกรดแล็กติกโดย *R. oryzae* คือ กรดแล็กติกที่ผลิตได้เป็นไอโซเมอร์แอลบริสุทธิ์ ใช้อาหารเลี้ยงเชื้อมีองค์ประกอบไม่ซับซ้อน แต่ปัญหาอย่างหนึ่งที่สำคัญคือลักษณะทางสัณฐานวิทยาของ *R. oryzae* มีผลต่อความหนืดของอาหารเลี้ยงเชื้อ ส่งผลให้ออกซิเจนไหลเวียนได้ไม่ดี เทคนิคทางพันธุวิศวกรรมจึงถูกนำมาประยุกต์ใช้ในการแก้ปัญหาเกี่ยวกับลักษณะทางสัณฐานวิทยาของรา *R. oryzae* โดยการใช้แบคทีเรีย *Escherichia coli* เป็นเซลล์เจ้าบ้าน งานวิจัยนี้มีจุดมุ่งหมายในการพัฒนาการผลิตกรดแล็กติกไอโซเมอร์แอลจาก *E. coli* สายพันธุ์ RB24 ซึ่งเป็นสายพันธุ์ที่ผ่านขั้นตอนการดัดแปลงทางพันธุกรรมโดยการนำพลาสมิดที่มียีน *ldhA* จาก *R. oryzae* ใส่เข้าไปในแบคทีเรีย *E. coli* สายพันธุ์ที่มีการทำลายยีน *ldhA* และ *pta* จากการทดลองเปลี่ยนแปลงตัวแปรในการหมักในระดับขวดเขย่า เมื่อใช้อาหารสูตร 56/2 พบว่า *E. coli* สายพันธุ์ RB24 มีการผลิตกรดแล็กติกได้เพียงเล็กน้อยในทุกสภาวะ คาดว่ากรดแล็กติกที่เกิดขึ้นนั้นมาจาก Lactate dehydrogenases (LldD และ Dld) เมื่อเปลี่ยนสูตรอาหารเป็น fermentation broth ที่อุดมไปด้วยสารอาหารในการหมักพบว่า *E. coli* สายพันธุ์ RB24 ผลิตกรดแล็กติกได้มากที่สุดที่ความเข้มข้น 7.45 กรัมต่อลิตร เมื่อใช้อาหารเลี้ยงเชื้อสำหรับการหมักที่น้ำตาลกลูโคสเริ่มต้น 30 กรัมต่อลิตร เป็นเวลา 48 ชั่วโมง ภายใต้ภาวะที่ไม่ให้อากาศ จากนั้นทำการเหนี่ยวนำพลาสมิดโดย IPTG เพื่อให้ยีน *ldhA* จาก *R. oryzae* มีการแสดงออกซึ่งผลิตกรดแล็กติกได้ปริมาณน้อยกว่าสายพันธุ์ RB24 ซึ่งคาดว่าอาจเกิดจากการกดการแสดงออกโดยน้ำตาลกลูโคสในอาหาร จากงานวิจัยนี้มีความเป็นไปได้ว่า LdhA จาก *R. oryzae* ไม่สามารถทำงานได้เต็มที่อยู่ใน *E. coli* ที่เป็นเซลล์เจ้าบ้าน ส่งผลให้มีการผลิตกรดแล็กติกได้ในปริมาณต่ำและยับยั้งการนำน้ำตาลกลูโคสเข้าสู่เซลล์ด้วยกระบวนการ phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) ทำให้พบน้ำตาลกลูโคสเหลือในอาหารปริมาณมาก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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THANAWAN WATTHANAPHORN: LACTIC ACID PRODUCTION BY *Escherichia coli* HARBORING *ldhA* GENE FROM *Rhizopus oryzae*. ADVISOR: RUETHAIRAT BOONSOMBAT, Ph.D., 106 pp.

Lactic acid is widely used for many industries such as food, pharmaceutical, cosmetic and use as monomer for producing biodegradable plastic. Lactic acid can be produced by microbial fermentation such as the fungus *Rhizopus oryzae*. Although this fungus has many advantages such as optically pure L(+)-lactic acid production and simple nutrient requirement, one of the obstacles for lactic production is its morphology that can increase in broth viscosity, but decrease in oxygen transfer. Therefore, genetic engineering was used to overcome the problem of *R. oryzae* morphology by using *Escherichia coli* as the host cell. The aim of this research is to study lactic acid production from the genetically modified *E. coli* strain RB24, *E. coli* with deactivated chromosomal *ldhA* and *pta* and harboring *R. oryzae ldhA* gene on the plasmid. After varying some fermentation parameters in shake flask level, It was suggested that a very small amount of lactic acid was detected when 56/2 minimal medium was used for 48 hours fermentation in all conditions. This lactic acid may come from the other Lactate dehydrogenases (LldD and Dld). When rich fermentation broth was used, the highest concentration of lactic acid at 7.45 g/L was obtained after being fermented with 30 g/L of initial glucose concentration under anaerobic condition for 48 hours. When IPTG inducible plasmid was used to express *R. oryzae ldhA* gene, smaller amount of lactic acid than that of RB24 strain may be resulted from glucose repression. From this study, it is possible that exogenous *R. oryzae* LdhA may not function properly in *E. coli* host. This was not only generation of low amount of lactic acid, but also inhibition of phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for glucose consumption that was investigated by high concentration of residual glucose.

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

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

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LIST OF ABBREVIATIONS

ackA	acetate kinase A gene
adhE	alcohol dehydrogenase E gene
bp	base pair
cat	chloramphenicol acetyltransferase gene
˚C	degree celsius
g	gram
kan	kanamycin resistance gene
ldhA	lactate dehydrogenase A gene
LDH	lactate dehydrogenase enzyme
LB	Luria-Bertani
µl	microliter
ml	milliliter
mM	millimolar
min	minute
M	molar
nm	nanometer
OD	optical density
plfB	pyruvate formate lyase B gene
pta	phosphotransacetylase gene
PCR	polymerase chain reaction
rpm	revolutions per minute

CHAPTER I

INTRODUCTION

Lactic acid, an organic acid discovered in 1780 by Scheele, is widely used in many industrial applications such as food, pharmaceutical industry, cosmetic, chemical and textile (Wee *et al*., 2006). Recently, the consumption of lactic acid trends to be increased rapidly. Due to the ability to be used as a monomer for poly(lactic acid), lactic acid can be applied in the production of biodegradable plastic which is a worldwide concern from the global warming problem (Datta *et al*., 1995; Litchfield, 1996).

Lactic acid has two optical isomers: L(+)- and D(-)-lactic acid. It can be produced by fermentation of many microorganisms or chemical synthesis (John *et al*., 2007). The production from chemical synthesis causes racemic DL-lactic acid. The advantages of microbial fermentation includes the potential to use low cost substrate and energy consumption and production of optically pure L(+)- or D(-)-lactic acid (Abdel-Rahman *et al*., 2013). The purity of lactic acid is important for industry application (Gao *et al*., 2011). The isomers of lactic acid produced from microorganisms are dependent on the enzyme lactate dehydrogenase. Lactate dehydrogenase catalyzes pyruvate to lactate in glycolysis (Ferain *et al*., 1994). The efficiency of poly(lactic acid) production also depends on purity of the isomers. It has been many studies about biological fermentation for lactic acid production which lactic acid bacteria are commonly used in various industries due to the high productivity. However, there still are some problems such as complex nutrient requirement (Chopin, 1993) and production of racemic L(+)- and D(-)-lactic acid from L-lactate dehydrongenase and D-lactate dehydrogenase, respectively (Abdel-Rahman *et al*., 2013). Other microorganisms such as a fungus, *Rhizopus oryzae*, can produce pure L(+)-lactic acid when starch or glucose is used as a carbon source (Oda *et al*., 2002; Yin *et al*., 1997). However, lactic acid production from *R. oryzae* has some limitations, for example, a high concentration of oxygen requirement, low yield lactic acid production, and the contaminations of fumaric and ethanol produced during lactic acid fermentation (Tay and Yang, 2002). Moreover, its filamentous morphology is not suitable for fermentation in bioreactors (Zhang *et al*., 2007). Therefore, we are interested in the expression of *ldhA* gene from *R. oryzae* in other microorganisms that requires simple nutrients for growth and produces pure and high L(+)-lactic acid.

The aim of this research is to apply genetic engineering techniques to overcome the problem of *R. oryzae* morphology. The genetically modified *Escherichia coli* strain RB24 used in this study is generated by transforming the plasmid harboring *R. oryzae ldhA* gene into *E. coli* background with knocked out chromosomal *ldhA* and *pta* to allow the expression of *R. oryzae ldhA* on the plasmid and reduce the contamination of other substances produced during fermentation, respectively (Chang *et al*., 1999). This *E. coli* strain RB24 is expected to combined the advantages of *E. coli*, which are rapid growth, simple nutritional requirements and well-studied genetic information, and *R. oryzae*, which are simple medium requirement and production of optically pure L(+)-lactic acid, in one particular organism.

The previous study suggests that L(+)-lactic acid production from *E. coli* RB24 in shake flask level under anaerobic condition gives a low yield of L(+)-lactic acid when fermented with 100 g/L glucose (5.03±4.149 g/L) (Boonsombat, 2013). Furthermore, the high residual glucose concentration may inhibit lactic acid production. Therefore, it is necessary to study lactic acid production from this *E. coli* harboring *R. oryzae ldhA* gene in various conditions, such as culture media, fermentation conditions and types of plasmid. This data will be useful for further improvement of lactic acid production from genetically modified *E. coli*.

Objective:

To study lactic acid production from *Escherichia coli* harboring *Rhizopus oryzae ldhA* gene on the plasmids in various conditions.

CHAPTER II

LITERATURE REVIEWS

2.1 Lactic acid

Lactic acid has been used for preservation of human food for a long time ago. It was discovered by Carl Wilhelm Scheele, a Swedish Chemist, in 1780. In 1881, the acid was commercially produced by Charles E. Avery at Littleton, Massachusetts, USA. (Narayanan *et al*., 2004).

Lactic acid is an organic acid consisting of carboxyl group (-COOH), hydroxyl group (-OH) and methyl group (-CH_3) on the molecule. Lactic acid has two optical isomers: L(+)- and D(+)-lactic acid represent in Figure 2.1. (Narayanan *et al*., 2004).

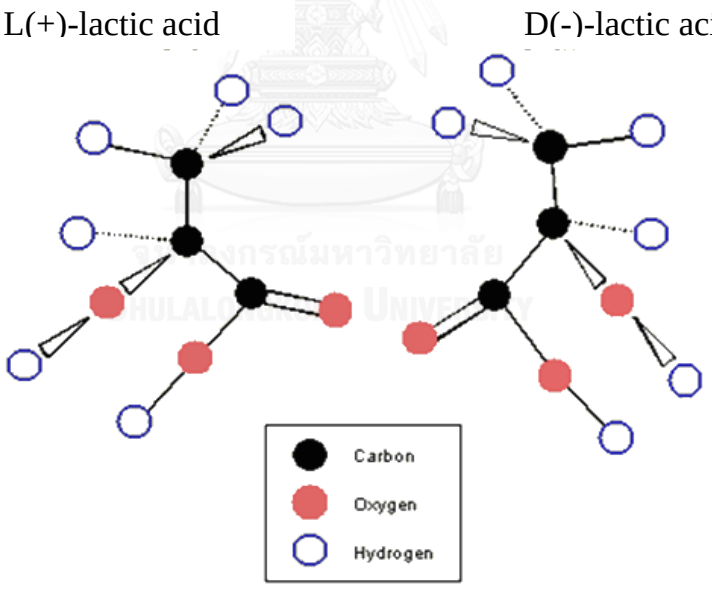


Figure 2.1 L(+)-lactic acid and D(-)-lactic acid structures.

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

Lactic acid is soluble in water but insoluble in organic solvents (Ren, 2010). Other properties of lactic acid are summarized in Table 2.1.

Table 2.1 Identification, physical-chemical properties and thermodynamic characteristics of lactic acid

Identification	**Physical-chemical properties**		
CAS number	D/L: [50-21-5]	Melting point	L: 53^{o}C
	L: [79-33-4]		D: 53^{o}C
	D: [10326-41-7]		D/L: 16.8^{o}C
Einecs No.	200-018-0	Boiling point	122^{o}C (12 mmHg)
H.S. Code	2918.11	Specific gravity	1.2 g/mL
Formula	$CH_3CH(OH)COOH$	Molar mass	90.08 g/mol

Thermodynamic characteristics	
Items	**Characteristics**
Dissociation constant *(K_a)*	0.000137 (at 25^{o}C)
Heat of dissociation *(ΔH)*	-63 cal/mol (at 25^{o}C)
Free energy of dissociation *(ΔF)*	5000 cal/mol
Heat of solution (ΔH)	1868 cal/mol(for L(+)-lactic acid at 25^{o}C)
Heat of dilution (ΔH)	-1000 cal/mol
Heat of fusion (ΔH)	2710 cal/mol (for racemic lactic acid) 4030 cal/mol (for L(+)-lactic acid)
Entropy of solution (ΔS)	6.2 cal/mol/^{o}C
Entropy of dilution (ΔS)	-3.6 cal/mol/^{o}C
Entropy of fusion (ΔS)	9.4 cal/mol/^{o}C (for racemic lactic acid) 12.2 cal/mol/^{o}C (for L(+)-lactic acid)
Heat of combustion (ΔH_c)	1361 KJ/mole
Specific Heat (C_p) at 20^{o}C	190 J/mole/^{o}C

(Reference: (Ren, 2010))

2.2 Lactic acid application

Lactic acid has been applied in many industry including food, pharmaceutical, cosmetic, agriculture industry and biodegradable plastic. The several applications of lactic acid are shown in Figure 2.2.

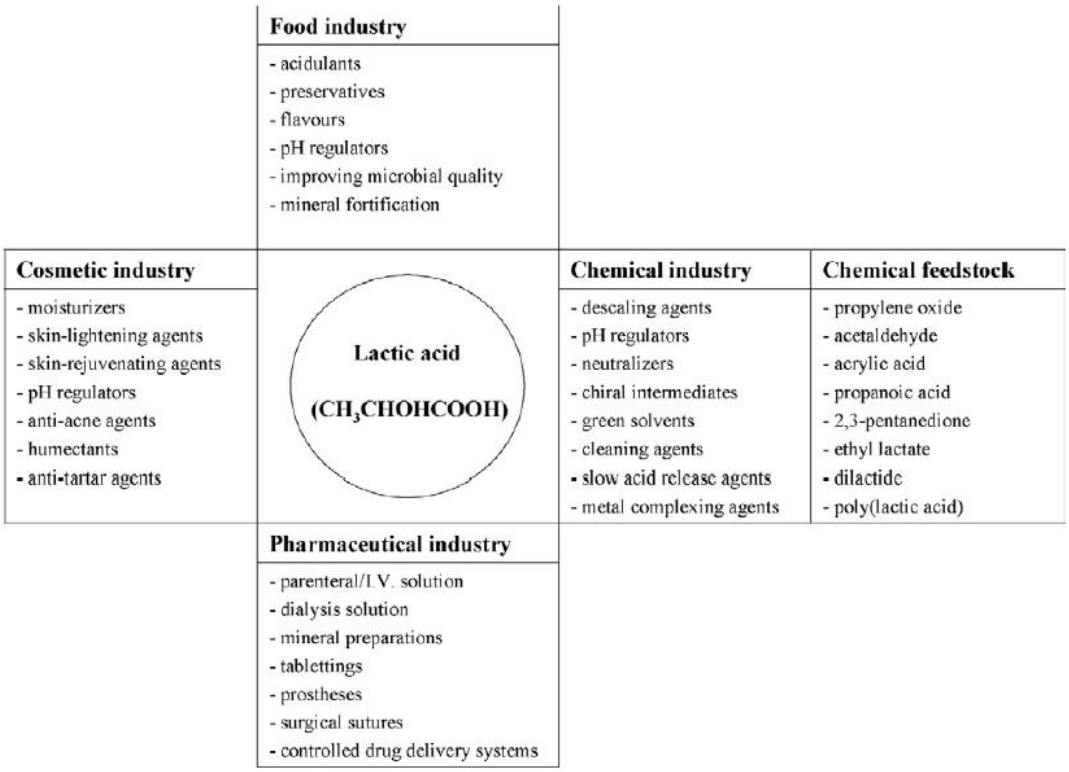


Figure 2.2 Diagram of the commercial uses and applications of lactic acid

(Reference: (Wee *et al*., 2006))

2.2.1 Food and beverage industry

Currently, most of lactic acid production is served for the demand in the food industry such as a food ingredient for pH control, using with citric acid and propanoic acid to achieve sour in food, dough conditioner, agitation withstand or other conditions in baking process, providing an increase shelf life, pathogen inhibition of food and use in cheese components. In addition, lactic acid can be used in beverage instead of citric acid, phosphoric acid and other mild acids.

2.2.2 Pharmaceutical industry

Biodegradable plastic, made from lactic acid, can be used for producing medical materials because it is more biocompatible for the implants and bioresorbable by biological system in human body. Biopolymers from lactic acid have been used for a long time with high potential such as sutures, staples, wound dressing, surgical implants, and orthopedic fixation devices.

2.2.3 Cosmetic industry

Lactic acid has been used as an emulsifier in cosmetic applications. There are many properties, for example, skin lightening which affects to tyrosinase inhibition, moisturizer to provide skin hydration and skin rejuvenation with accelerated peeling.

2.2.4 Polylactic acid

Nowadays, most packaging is made from petrochemical process. However, the major problem is the destruction process which leads to environmental pollution such as greenhouse effect. Recently, renewable plastics from renewable resources have come into the interest.

Polylactic acid (PLA) can be used for producing bioplastic that is degraded to carbon dioxide and water by nature microbial. PLA can be polymerized from lactic acid monomers which can be produced by microorganism fermentation from renewable resources such as cassava, corns and sugarcanes.

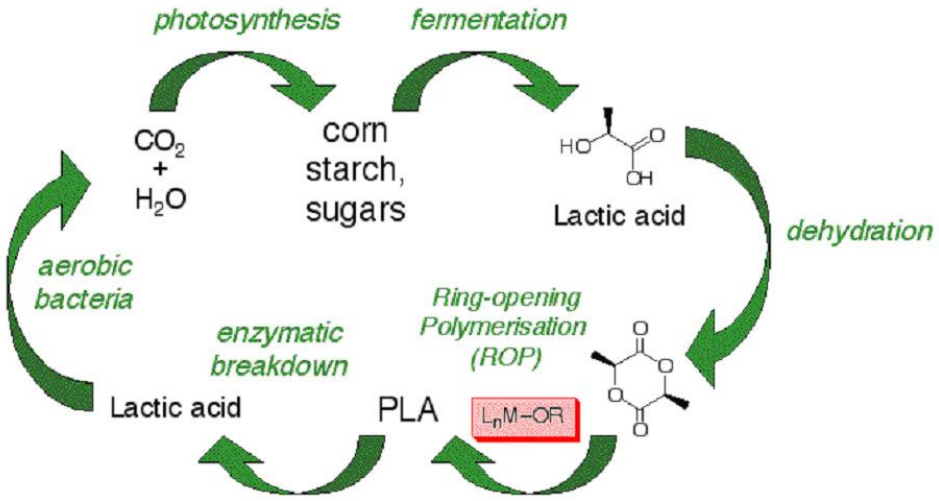


Figure 2.3 Environmental circulation of polylactic acid

(Reference: http://www.ch.ic.ac.uk)

The melting temperature range of PLA is 173 to 178 ^{o}C. However, the heat resistance can be up to 110 ^{o}C. The crystallinity of PLA is about 37% with glass transition temperature between 60 to 65 ^{o}C and it is suitable to biocompatibility. Although PLA cannot be degraded in the general environment, it is decomposed by plastic forming to landfill such as extrusion and injection molding.

PLA is a clear plastic that can be used for food packaging, but it is unsuitable at high temperature which limits to some applications, for example, food warp film, food containers, bag and textile. None of eruption mortality was reported from PLA but it may cause skin irritation if directly exposed. In addition, carcinogens have not been found in PLA, so it is safe for creatures and environment.

2.2.5 Agriculture industry

Lactic acid can be used as insecticide, herbicide and fertilizer components.

2.2.6 Other industries

Various applications of lactic acid are used in chemical and biological processes of other organic acid production such as propanoic acid, acetic acid and acylic acid. Lactic acid derivatives can be used in several applications, for example, package, fibers and dyeing.

2.3 Production of lactic acid

Lactic acid can be produced by chemical synthesis and microbial fermentation (Figure 2.4).

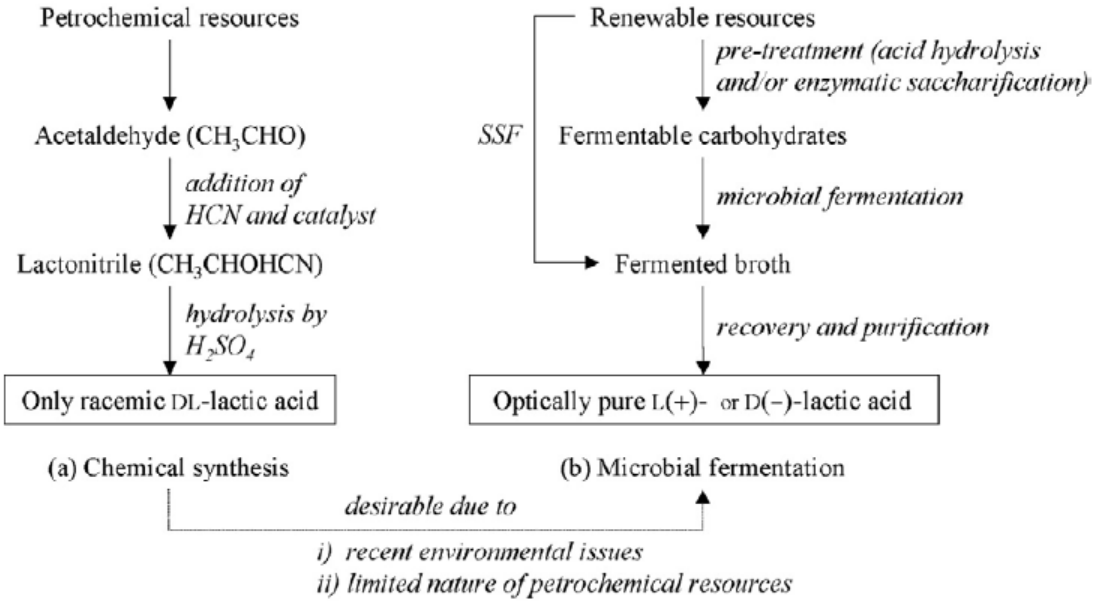


Figure 2.4 The two methods of lactic acid production

(Reference: (Wee *et al*., 2006)).

2.3.1 Chemical synthesis

The chemical synthesis can produce racemic mixture of D(-)-and L(+)-lactic acid. In the first step, hydrogen cyanide reacts with acetaldehyde in liquid phase and high pressures to produce lactonitrile. After that, lactonitrile is hydrolyzed by concentrated acid, such as hydrochlolic acid or sulfuric acid, to obtain ammonium salt and lactic acid. Then, lactic acid is esterified by methanol to produce methyl lactate that can be eliminated and purified by distillation and hydrolysis with acidic catalyst to produce lactic acid and methanol as the end products. The production of lactic acid by chemical synthesis can be summarized as the following reactions (Narayanan *et al*., 2004).

Step 1 Addition of hydrogen cyanide

CH_3CHO + HCN ⟶ $CH_3CHOHCN$
Acetaldehyde Hydrogen cyanide Lactonitrile

Step 2 Hydrolysis by sulphuric acid

$CH_3CHOHCN + H_2O + ½H_2SO_4 \longrightarrow CH_3CHOHCOOH + ½(NH_4)_2SO_4$
Lactonitrile Sulphuric acid Lactic acid Ammonium salt

Step 3 Esterification

$CH_3CHOHCOOH + CH_3OH \longrightarrow CH_3CHOHCOOCH_3 + H_2O$
Lactic acid Methanol Methyl lactate

Step 4 Hydrolysis by water

$CH_3CHOHCOOCH_3 + H_2O \longrightarrow CH_3CHOHCOOH + CH_3OH$
Methyl lactate Lactic acid Methanol

Due to the purification of lactic acid isomer being significant for the polylactic acid (PLA) efficiency, the chemical synthesis that produces racemic mixture of D(-) and L(+)-lactic acid isomers may be complicated for further application. Moreover, substrates for chemical synthesis process are from petrochemical resources that are high production cost and caused environmental pollution.

2.3.2 Microbial fermentation

Lactic acid can be produced by various microorganisms including bacteria, fungi, yeast, algae and cyanobacteria. The microbial fermentation gives some advantages compared to the chemical synthesis including low substrate cost and energy consumption (Datta and Henry, 2006). Lactic acid production from microbial fermentation is dependent of two types of enzymes, NAD-dependent L-lactate dehydrogenase and NAD-dependent D-lactate dehydrogenase, for converting pyruvic acid to lactic acid (Garvie, 1980). The efficient production of lactic acid has been attempted to be improved such as using various substrates from renewable resources, simple nutrient requirements, improving strains by genetic engineering for optically pure lactic acid, increases of yield and productivity with decrease of by-products. Currently, the genetic engineering has been more favorable to improve microbial strains for increasing yield and optical purity of lactic acid fermentation (Abdel-Rahman *et al*., 2013).

The microbial fermentation for lactic acid production generally used lactic acid bacteria (LAB), *Bacillus* strains, *Escherichia coli* and *Corynebacterium glutamicum*. The mainly limitations of these strains including (a.) they produce racemic mixture L-lactic acid and D-lactic acid by L-lactate dehydrogenase and D-lactate dehydrogenases; (b.) low production yield because of by-products; (c.) complex nutrient requirements and (d.) high risk of cell lysis from bacteriophage infection that inhibits lactic acid production. However, various studies reported that these problems could be solved by genetic engineering including (a.) knockout of genes for optically pure L- or D-lactic acid production; (b.) deletion of various genes that produce by-products to increased lactic acid yield; (c.) use of bacteria strains for lactic acid production in simple nutrient media and (d.) using mixed strains or developed strains for bacteriophage resistance (Abdel-Rahman *et al*., 2013).

Lactic acid bacteria (LAB)

LAB is a group of bacteria that produces lactic acid as a main product by using carbohydrate as a carbon source. LAB consists of bacteria in phylum Firmicutes including *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus* (Reddy *et al*., 2008). The optimal conditions for LAB growth include pH 5.5 to 6.5 and temperature of 5 to 45 ^{o}C. The major pathways of hexose and pentose metabolism in LAB are represented in Figure 2.5.

LAB can be classified as homofermentative and heterofermentative, dependent on production of end product, as shown in Table 2.2. Homofermentative LAB contains aldolase enzymes that produce lactic acid as major product. Therefore, this group is interested for scaling up in industrial lactic acid production. However, heterofermentative LAB produces other products, such as acetic acid, as by-products by phosphoketolase pathways causing of low lactic acid yield. *Lactobacilli* are preferable for lactic acid production in industrial scale because they have been studied for a long time with high acidic resistance.

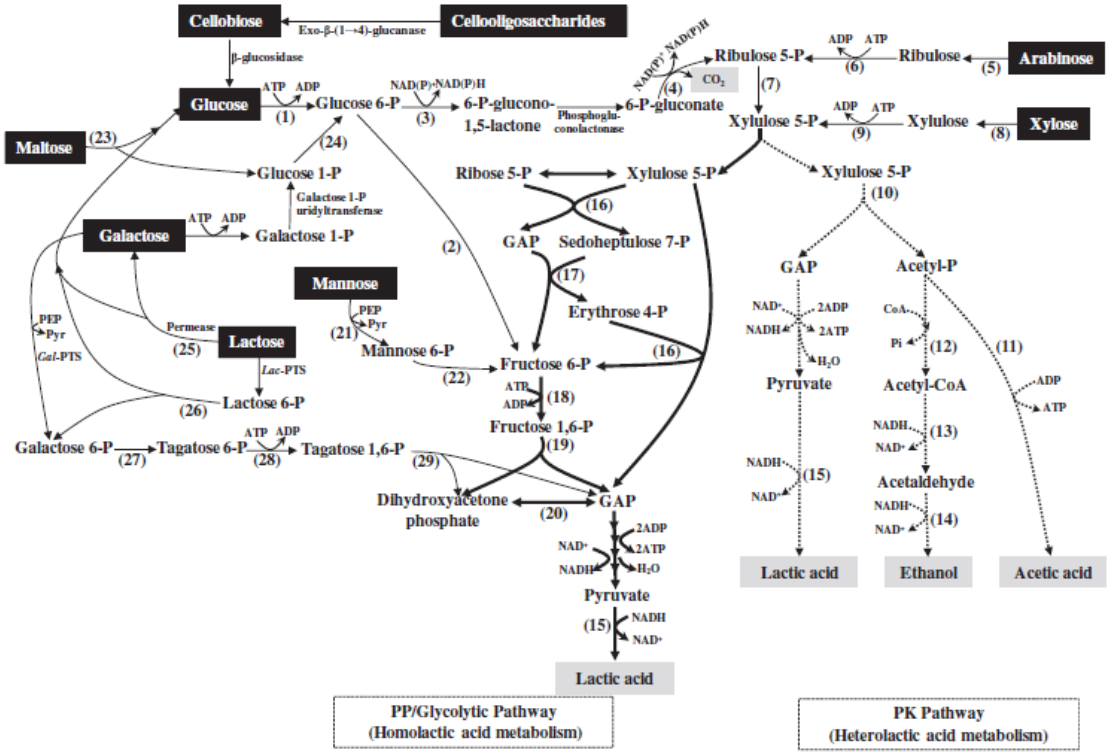


Figure 2.5 Metabolic pathways for lactic acid production from various sugars in lactic acid bacteria.

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.

(Reference: (Abdel-Rahman *et al.*, 2013))

However, LAB requires complex nutrition because of the inability to produce amino acids, peptides, nucleotides and vitamins for growth. This is a problem for recovery process of lactic acid and also increases production cost. Furthermore, LAB grows well at low temperature resulting in an increase in contamination risks and an obstacle for the simultaneous saccharification and fermentation (SSF) which requires higher temperature.

Table 2.2 Homofermentative and heterofermentative lactic acid bacteria.

Characterization	Homofermentative LAB	Heterofermentative LAB
Product	Lactic acid	Lactic acid, ethanol, diacetyl, formate, acetoin or acetic acid and carbon dioxide
Metabolic pathways	Hexose: Embden-Meyerhof pathways Pentose: pentose phosphate pathway	Hexose: phosphogluconate and phosphoketolase pathway Pentose: phosphoketolase pathway
Theoretical yield of lactic acid to sugars	Hexose: 1.0 g/g (2.0 mol/mol) Pentose: 1.0 g/g (1.67 mol/mol)	Hexose: 0.5 g/g (1.0 mol/mol) Pentose: 0.6 g/g (1.0 mol/mol)
Genera	*Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, some *Lactobacillus*	*Leuconostoc*, *Oenococcus*, some *Lactobacillus* species
Availability for commercial lactic acid production	Available due to high selectivity	Not available due to high by-product formation

(Reference: (Abdel-Rahman *et al*., 2013))

2.4 *Rhizopus oryzae*

Rhizopus oryzae, a species in the genus *Rhizopus*, is usually optically pure L(+)-lactic acid producer. The advantages of *R. oryzae* for lactic acid production compared to other microorganisms including use of various starchy biomasses without saccharification before utilize, simple nutritional requirement, easier process of cell separation than other microorganisms (Zhang *et al*., 2007). However, the filamentous form of *R. oryzae* effects on the fermentation broth circulation, the oxygen consumption and lactic acid yield.

R. oryzae consists of two genes of lactate dehydrogenase, *ldhA* and *ldhB*. L(+)-lactic acid production by *ldhA* to produced enzyme LdhA for conversion of pyruvate to lactate while grown on glucose or xylose as carbon source in culture media. In contrast, *ldhB* is transcribed when the fungus is grown on glycerol, ethanol or lactate, LdhB converts lactate to pyruvate (Skory, 2000).

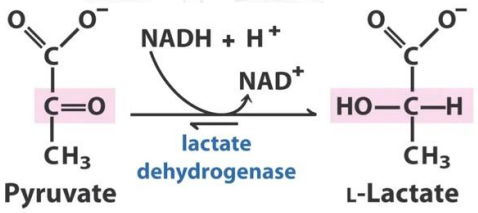


Figure 2.6 Lactate dehydrogenase (LDH) is an enzyme that catalyzes the stereospecific interconversion of lactate and pyruvate.

(Reference: http://www.proteopedia.org/wiki/index.php/User:Kelly_Roark/Sandbox1)

Immobilization techniques was applied in many studies for L(+)-lactic acid fermentation of *R. oryzae*. In 2013, Yamane and Tanaka studied L(+)-lactic acid production by *R. oryzae* using sponge-like cubic particles made of polyurethane foam for immobilization. The high L(+)-lactic acid concentration of 145 g/L produced by batch culture and 231 g/L produced by fed-batch culture were revealed (Yamane and Tanaka, 2013). However, the immobilization took a long time for entrapment between fungal cells and matrixes and limitation of surface area of the matrix (Z. Wang *et al*., 2010).

R. oryzae can be used several renewable resources and lignocellulosic biomasses. It was reported that the highest lactic acid concentration of 38.5 g/L was obtained when *R. oryzae* TS-61 was fermented by using molasses as carbon source and chicken feather protein hydrolysate (CEP) as nitrogen source (Taskin *et al*., 2012).

However, there are some limitations from lactic production by *R. oryzae* such as by-products (e.g., ethanol and fumaric acid), a high concentration of oxygen

requirement (Wee *et al*., 2006) and filamentous form of *R. oryzae* that result in an obstacle to mass transfer and bulk mixing (Bai *et al*., 2003).

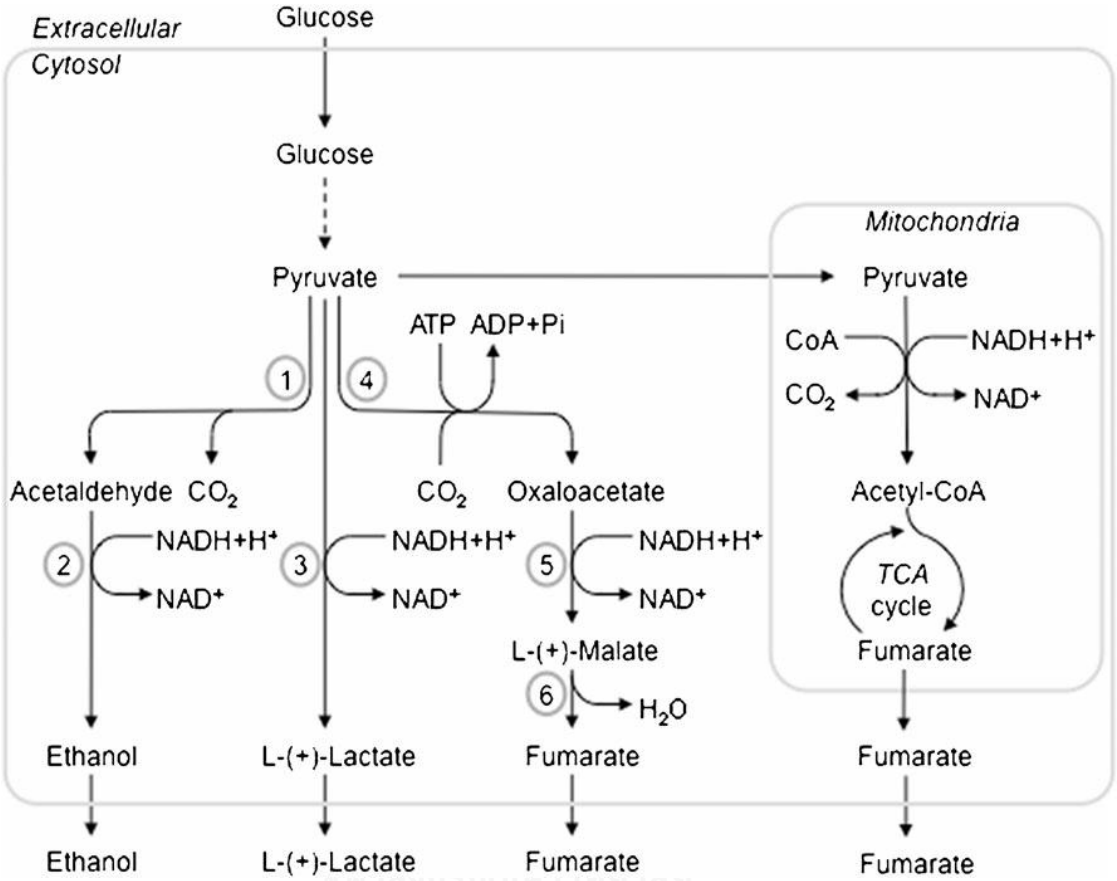


Figure 2.7 Glucose metabolism in *Rhizopus oryzae*.

The numbers indicate key enzymes in each pathway: 1, pyruvate decarboxylase (PDC); 2, alcohol dehydrogenase (ADH); 3, lactate dehydrogenase (LDH); 4, pyruvate carboxylase (PYC); 5, malate dehydrogenase (MDH); 6, fumarase (FUM).

(Reference: (Zhang *et al*., 2007))

2.5 *Escherichia coli*

E. coli was usually applied for lactic acid fermentation by using sugars as carbon and electron sources under anaerobic conditions. The glycolysis pathway converts sugar into two molecules of pyruvate, which is involved in releasing of two ATP and two NADH molecules. Under anaerobic conditions, pyruvate is converted to acetyl-CoA and carbon dioxide by the pyruvate dehydrogenase. This enzyme is activated under anaerobic conditions, controlled by the NADH/NAD^+ ratio. (Förster and Gescher, 2014).

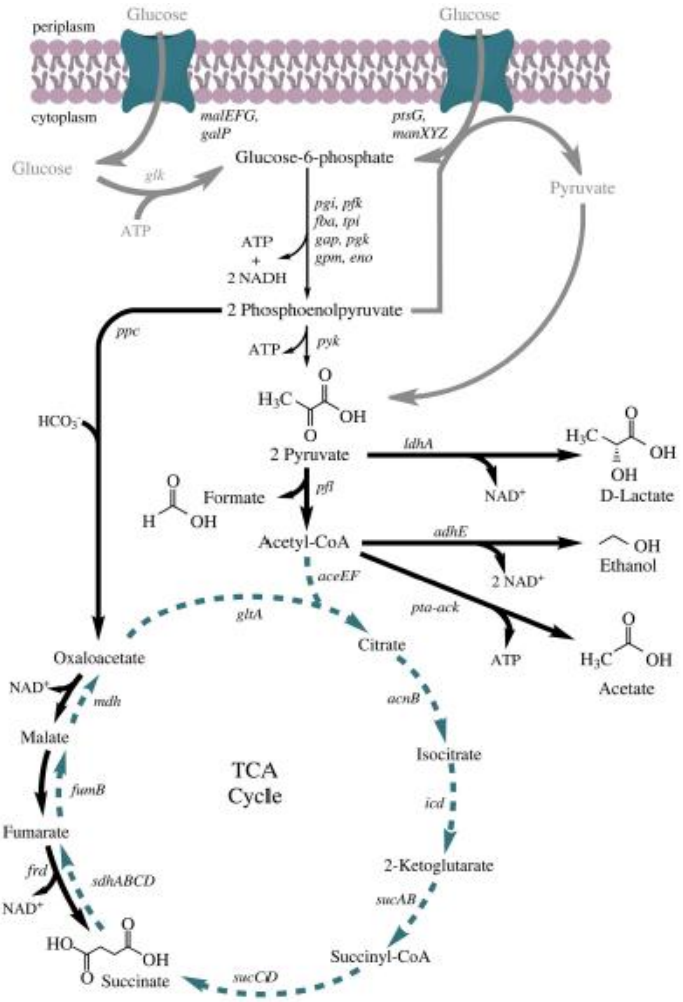


Figure 2.8 Anaerobic fermentative metabolism in *Escherichia coli*

Chemical structures are shown for all mixed-acid fermentation products and pyruvic acid. Bold gray arrows: glucose transport systems; thin black arrows: glycolysis; bold black arrows: fermentative reactions; dashed, green arrows: TCA cycle, only anabolic functions, completely active under oxic conditions. Genes: *malEFG* (maltose ABC transporter), *galP* (galactose: HC symporter), *ptsG* (fused glucose-specific PTS enzyme: IIB and IIC component), *manXYZ* (mannose PTS permease), *glk* (glucokinase), *pgi* (glucose-6-phosphate isomerase), *pfk* (6-phosphofructokinase), *fba* (fructose-bisphosphate aldolase), *tpi* (triosephosphate isomerase), *gap* (glyceraldehyde 3-phosphate dehydrogenase), *pgk* (phosphoglycerate kinase), *gpm* (phosphoglycerate mutase), *eno* (enolase), *pyk* (pyruvate kinase), *ppc* (phosphoenolpyruvate carboxylase), *ldhA* (lactate dehydrogenase), *pfl* (pyruvate formate lyase), *aceEF* (pyruvate dehydrogenase complex), *adhE* (alcohol dehydrogenase), *pta* (phosphate acetyltransferase), *ack* (acetate kinase), *gltA* (citrate synthase), *acnB* (aconitase), *icd* (isocitrate dehydrogenase), *sucA* (2-oxoglutarate decarboxylase), *sucB* (2-oxoglutarate dehydrogenase), *sucCD* (succinyl-CoA synthetase), *sdhABCD* (succinate dehydrogenase), *fumB* (fumarate hydratase), *frd* (fumarate reductase),and *mdh* (malate dehydrogenase). (Reference: (Förster and Gescher, 2014))

2.5.1 Metabolic engineering of *Escherichia coli*

Recently, the genetic engineering technique is preferable to efficiently improve lactic acid production in *E. coli* strains. This microorganism is advantageous due to its rapid growth (rapid hexose and pentose metabolisms), simple nutritional requirement and well-studied genetic information. The wild- type *E. coli* strain produces ethanol and several organic acids including lactic acid, acetic acid, succinic acid and formic acid. There have been many studies to improve lactic acid production in *E. coli* by using metabolic engineering with glucose, xylose, sucrose, glycerol as carbon sources. The genetically modified *E. coli* BAD-ldh strain, which was generated by transforming the pBAD vector containing *Enterococcus facelis* KK1 L-*ldh* gene into *E. coli* SZ85, gave the maximum L(+)-lactic acid concentration of 0.62 g/L from 1 g/L of fructose in 24 hours (Mulok *et al*., 2009). *E. coli* HBUT-D strain, which the chromosomal D(-)-lactate dehydrogenase gene (*ldhA*) was replaced by L(+)-lactate dehydrogenase (*ldhL*) from *Pedicoccus acidilactici* resulting in the *E. coli* strain called WYZ- L with improved expression of the sucrose operon (*cscA* and *cscKB*), gave the maximum L(+)-lactic acid concentration of 97 g/L from 100 g/L of sucrose with a greater than 99% of optical purity (Y. Wang *et al*., 2013). The *E. coli* K-12 with inactivated *pflB* and *ldhA* background produces ethanol as a major product (Kim *et al*., 2007). Furthermore, deletion of the *frd*, *ackA*, *focA* and *plfB* genes, called SZ420 strain, converted glucose and xylose to ethanol with a yield of 90% under an anaerobic condition (Zhou *et al*., 2008). Causey *et al*. (2003) constructed a homoacetate pathway in *E. coli* by deleting *focA-pflB, frdBC, ldhA* genes to decrease the other fermentation end products and minimize the production of biomass. Moreover, the citric acid cycle in the strains with *adhE* deletion was blocked by deletion of α-ketoglutarate dehydrogenase, and then acetate was produced with a yield of 86% (Causey *et al*., 2003).

Table 2.3 Improvement of lactic acid production in *E. coli* using genetic modification

Strain	Modification	Outcome	Reference
E. coli RR1	Deletion of phosphotransacetylase (*pta*) and PEP-carboxylase (*ppc*)	Lactate yield of 90% with glucose substrate	Chang *et al*., 1999
E. coli SZ85	Replacement of *E. coli* chromosomal *ldhA* by *ldhL* from *Pediococcus acidilactici*	Lactate yield of 94% with glucose and 82% with xylose substrate	Zhou *et al*., 2003
E. coli MG1655	Deletion of *pta*, alcohol dehydrogenase (*adhE*) and fumarate reductase (*frd*), inhibition of D(-)-lactic acid production by the methylglyoxal bypass and L-lactate dehydrogenase from *Streptococcus bovis* was added into the genome	L-Lactate yieid of 90% with glycerol	Mazumdar *et al*., 2013
E. coli VK-23	Encoding the enzymes of acrylate pathway (lactoyl-CoA dehydratase, acryloyl- CoA reductase and propionyl-CoA transferase)	Lactate yield of 96% with glucose substrate	Choudhary *et al*., 2014
E. coli BAD-ldh	*E. coli* harboring the pBAD vector containing *Enterococcus facelis* KK1 L-ldh gene into *E. coli* SZ85	The maximum lactic acid concentration of 0.62 g/L from 1.0 g/L of fructose	Mulok *et al*., 2009

CHAPTER III

METHODOLOGY

3.1 Material

3.1.1 Strain list

Strain	Partial genotype		Plasmid	Reference
	pta	***ldhA***		
JC13509[a]	+	+	-	Dr. Steven J Sandler
RB7	*kan*	*cat*	-	(Boonsombat, 2013)
RB24	*kan*	*cat*	pRB85	(Boonsombat, 2013)
RB29	*kan*	*cat*	pBluescript II KS(+)	RB7 harboring pBluescript II KS(+)
RB30	+	+	pRB85	JC13509 harboring pRB85
RB31	+	+	pKS(+)	JC13509 harboring pBluescript II KS(+)
THW1	*kan*	*cat*	pTN1	JC13059 harboring pTN1

[a] JC13509 used as wild type in this experiment contains partial genotype as F^- *lacMS286 ϕ80dIIlacBK1 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 (Boonsombat, 2013)

pTN1 is pQE30-Xa containing *ldhA* gene (ORF and promoter region) from *R. oryzae* NRRL395

3.2 Primer list

Primer	Sequence	Restriction site
prRB33	5’-CTCAGTTTATAGGATCCAAGCAGTC-3’	*BamH*I
prRB34	5’-TGTGTAAGCTTTACAATTCGATTGT-3’	*Hind*III
prRB42	5’-GCGTCGGGATCCAGTAGTGGAG-3’	*Hind*III
prRB43	5’-CTCAGTTTATAAAGCTTAAGCAGTC-3’	*BamH*I

3.3 Chemical and reagents

Chemical and reagents	Company and country
Acetic acid (CH_3COOH)	Merck, USA
Ammonium sulphate ($(NH_4)_2SO_4$)	Sigma Aldrich, USA
Ampicillin	Bio Basic, Canada
Arginine	Sigma Aldrich, USA
Bactro-agar	Himedia, India
Calcium carbonate ($CaCO_3$)	Sigma Aldrich, USA
Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$)	Carlo Erba Reagent, Italy
Copper(II) sulfate	Sigma Aldrich, USA
di-Sodium hydrogen phosphate (Na_2HPO_4)	Bio Basic, Canada
D-lactate	Sigma Aldrich, USA
Ethanol	Merck, USA
Formic acid	Carlo Erba Reagent, Italy
Glucose	Bio Basic, Canada
Glycerol	Sigma Aldrich, USA
Histidine	Sigma Aldrich, USA
Iron(II) sulphate heptahydrate ($FeSO_4.7H_2O$)	Merck, USA
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Bio Basic, Canada
L-lactate	Sigma Aldrich, USA

Chemical and reagents	Company and country
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	Bio Basic, Canada
Manganese(II) sulphate pentahydrate ($MnSO_4.5H_2O$)	Merck, USA
Peptone	Bio Basic, Canada
Potassium di-hydrogen phosphate (KH_2PO_4)	Merck, USA
Potassium hydrogen phosphate (K_2HPO_4)	Bio Basic, Canada
Proline	Sigma Aldrich, USA
Sodium chloride (NaCl)	Merck, USA
Succinate	Sigma Aldrich, USA
Sulfuric acid (H_2SO_4)	Merck, USA
Thiamine	Sigma Aldrich, USA
Yeast extract	Bio Basic, Canada

3.4 Equipments and supplies

Equipments and supplies	Company and country
Aminex HPX-87H column	Bio-Rad, USA
Anaero Anaerobic Gas Generator: model AnaeroPack	Misubishi Gas Chemical, Japan
Autoclave: model HICLAVE HV-50	Hirayama, Japan
Cellulose acetate filter, pore size 0.45 μm	Sartorius, Germany
Electronic balance : model FX-180	A&D Co., Ltd., Japan
Electronic balance : model FX-3000	A&D Co., Ltd., Japan
Erlenmeyer flask 125 ml	Pyrex, Germany
Erlenmeyer flask 250 ml	Pyrex, Germany
Freezer -20 ^{o}C	Sanyo, Japan
Freezer -70 ^{o}C	Thermo Fisher Scientific, USA
Growth Cabinet	Sanyo, Japan
High speed micro refrigerated centrifuge: model MTX-150	Tomy Seiko Co. Ltd., Japan
Laminar flow: model HF safe-12006, Heal Force	Heal Force, China
Micro auto pipette: model Discovery Comfort	High Tech Lab, Poland
Oven : model UNB-400	Memmert Co.,Ltd., Germany
pH meter	Mattler Toledo, USA
Pipette tips	Biologix Research, USA
Sumi chiral 0A-5000L column	SCAS, Japan
UV-visible recording spectrophotometer: model UV-160	Shimadzu, Japan

Equipments and supplies	Company and country
Vacuum pump: model DOA-V130-BN	Gast, USA
Vortex mixer: model Vortex-Genie2	Scientific Industries, USA
Water bath	Yamato, Japan
YSI Select Biochemistry Analyzer: model YSI 2700 selector	YSI Inc., USA
Incubator shaker: model innova 4300	New Brunswick Scientific, USA
High performance liquid chromatography	Shimadzu, Japan

3.5 Methods

3.5.1 Preparation of inoculum

To prepare seed culture, the *E. coli* strains (except for THW1 strain) were grown in 56/2 minimal or LB agar media at 37 ^{o}C for 24 hours. For strains harboring plasmids, the 100 µg/ml Ampicillin was added in the medium. The cells were transferred into 10 ml of 56/2 minimal or LB broth and incubated at 37 °C, 200 rpm for 8 hours in a rotary shaker (The final OD_{600} is approximately 1.0). The lactic acid fermentation was then performed as described in the next topic.

3.5.2 Lactic acid fermentation in shake flask level

The 5 ml of inoculum was transferred into 50.0 ml 56/2 minimal or fermentation broth containing $CaCO_3$ as pH controller. For the strains harboring plasmids, 100 µg/ml Ampicillin was added into all media. Fermentation conditions as in Table 3.1 were used in this experiment. The 2.0 ml of each sample was harvested at different points of time. Each set of experiment was performed for 3 repeats.

Table 3.1 Fermentation conditions for lactic acid production in this study.

Condition	
Aerobic	37 °C and 200 rpm for 48 hours
Limited oxygen	37 °C for 48 hours without shaking
Anaerobic	37 °C for 48 hours with Anaero Anaerobic Gas Generator

3.5.3 Lactic acid production in minimal medium

The 56/2 minimal medium was used to investigate lactic acid production from the following *E. coli* strains; JC13509, RB7, RB24, RB29, RB30 and RB31. The inoculum preparation and lactic acid fermentation in shake flask level was carried out as mentioned previously. For strains harboring plasmids, 100 µg/ml Ampicillin was added to all media.

3.5.3.1 The effect of initial concentration of glucose

To test the effect of initial concentration of glucose on lactic acid production, the concentration of initial glucose in 56/2 minimal medium was varied as 2.0, 10, 20, 30 and 50 g/L.

3.5.3.2 The effect of medium component on lactic acid production

The effect of medium components on lactic acid production, including carbon sources and calcium carbonate amount, were investigated. To test the effect of different carbon sources, lactic acid production in 56/2 minimal medium with 20 g/L, or 2.0% (w/v), of initial glucose was compared with that of 1.0% (v/v) initial glycerol. Then, the effect of $CaCO_3$ amount was tested by fermenting in 56/2 minimal medium with 10 g/L and 30 g/L (w/v) of $CaCO_3$.

3.5.4 Lactic acid production from different fermentation media

The lactic acid production when using 56/2 buffer was compared with fermentation broth. The steps of inoculum and fermentation were performed as described previously (Topic 3.4.1 and 3.4.2). For strains harboring plasmids, 100 µg/ml Ampicillin was added to all media. Then, the lactic acid production from variation of initial glucose concentrations (as 10, 20, 30, and 50 g/L) from each medium was observed.

3.5.5 Lactic acid production from different plasmid type

3.5.5.1 Construction of *E. coli* strain harboring *R. oryzae ldhA* gene on IPTG-induced plasmid

The *ldhA* gene from *R. oryzae* was amplified by PCR with the prRB42 and prRB43 primers. The 1800 base pair PCR product was cut with restriction enzymes *Hind*III and *Bam*HI, then cloned into these sites of the plasmid pQE-30 Xa (Figure 3.1). Plasmid, verified by restriction enzyme digestion and DNA sequencing, was named as pTN1. After that, pTN1 was transformed into *E. coli* strain RB7 competent cells to generate the strain named THW1 by chemical transformation.

To prepare the competent cells, a colony of RB7 strain was inoculated in 2 ml of LB broth and incubated at 37 ˚C, 200 rpm overnight, Then, the 100 µl of culture was diluted into 10 ml LB broth, and allowed to grow to early log phase, or the OD_{600} value was approximately 0.3. After being incubated on ice for 20 minutes, cells were harvested by centrifuging at 4 ˚C, 4,000 rpm for 10 minutes. The pellet was then resuspended in 3.3 ml of TBI with gently swirling on ice water and incubated on ice for 2 hours. Cells were harvested again by centrifuging at 4 ˚C, 4,000 rpm for 10 minutes. After being 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 carried out by adding 3 µl of pTN1 into 50 µl of RB7 competent cells. The mixture was incubated on ice for 20 minutes, then being incubated at 42 ˚C for 2 minutes. After that, the mixture was immediately returned to ice for 2 minutes. Then, 1 ml of LB broth was added to the reaction and incubated at 37 ˚C for 2 hours. Finally, the mixture was spread on LB agar plates with Ampicillin (100 µg/ml), and plates were incubated at 37 ˚C overnight.

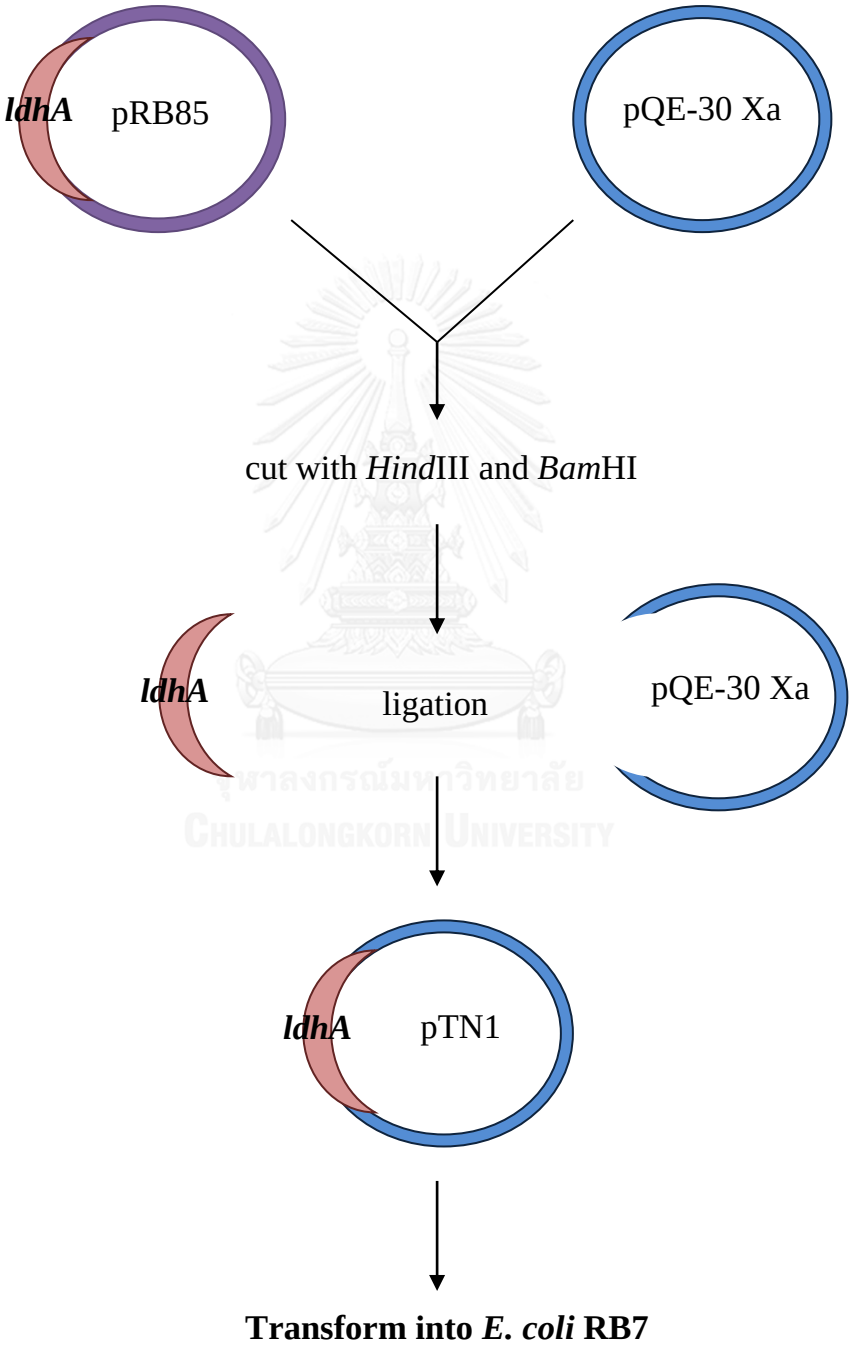


Figure 3.1 The diagram of the strain THW1 construction.

3.5.6 Comparison of lactic acid production from different plasmid types

The lactic acid production from the strain RB24 harboring plasmid pRB85 (pBluescript II KS(+)) derivative was compared with the strain THW1 harboring plasmid pTN1 (pQE-30 Xa derivative which could be induced by Isopropyl β-D-1-thiogalactopyranoside, or IPTG). Inoculum preparation in LB medium and fermentation in the fermentation broth was performed as mention above. Fermentation was performed in 3 conditions as described in Table 3.1 with 10 g/L, 20 g/L, 30 g/L, 50 g/L and 70 g/L of initial glucose. However, for the strain THW1, IPTG with the final concentration of 0.1 M was added in the step of inoculum preparation.

3.5.7 Measurement of strain growth

The growth curve was determined from the OD_{600} values at different time points. The 1.0 ml of each sample from fermentation culture was harvested every 3 hours for 12 hours. To eliminate the excess $CaCO_3$ that could cause light scattering, 0.37 M HCl was added prior to measure the absorbance by spectrophotometer at 600 nm (Skory, 2004).

3.5.8 Measurement of lactic acid, residual glucose and other fermentation products

The samples harvested at different points of time were centrifuged at 10,000 rpm for 7 minutes. Then, the supernatant was filtrated by cellulose membrane and diluted 10 times. The concentrations of lactic acid and residual glucose were prior observed by YSI select biochemistry analyzer. Then, total lactic acid, acetic acid, ethanol, formic acid, succinic acid and residual glucose concentrations were measured by high performance liquid chromatography (HPLC) with IR detector and an Aminex HPX-87H column chromatography using 5.0 mM H_2SO_4 as the mobile phase (injection volume 10 µl, 0.6 ml/min mobile phase, 50 ^oC column temperature). The concentrations of optical of D(-)-lactic acid and L(+)-lactic acid isomers from each sample were determined by UV detector at 254 nm and a Sumi chiral 0A-5000L column using 2.0 mM $CuSO_4$ as the mobile phase (injection volume 10 µl, 1.0 ml/min mobile phase, 40 ^oC column temperature). The concentrations fermentation products and residual glucose were calculated from standard calibration curve.

CHAPTER IV

RESULT AND DISCUSSION

The previous study revealed the *E. coli* harboring *R. oryzae ldhA* gene on the plasmid (strain RB24) could produce lactic acid, however, a small and inconsistent amount of the lactic acid was obtained (Boonsombat, 2013). Therefore, to improve the production of lactic acid, it is important to study lactic from *E. coli* harboring *R. oryzae ldhA* gene, including the strain RB24, in various conditions. In this study, $CaCO_3$ was served as neutralizing agent and Ampicillin was added in the media to maintain the plasmid.

4.1 Lactic acid production in minimal medium

In this experiment, minimal medium was prepared from 56/2 phosphate buffer. The 56/2 minimal medium was composed of components that met the minimal requirement for JC13509 derivative strains. Studying the lactic acid fermentation in minimal medium should be easy to manipulate factors in the medium that may be affected on lactic acid production. The steps of inoculum and fermentation were performed as described previously (Topic 3.4.1 and 3.4.2). Fermentation conditions in this experiment were divided as aerobic, limited oxygen and anaerobic.

The growth curve of *E. coli* JC13509, RB7, RB24, RB29, RB30 and RB31 in 56/2 minimal medium at 37 ^{o}C in 3 conditions for 12 hours is represented in Figure 4.1. The wild type strain, JC13509, seemed to grow better than other strains in all conditions. However, the slightly lower growth from the other strains may be resulted from many factors. The strain RB7 contains deactivated chromosomal *ldhA* and *pta* that may be affected on growth. The others, including RB24, RB29, RB30 and RB31, harbors plasmid, therefore, it is possible that the limited nutrients in minimal medium was shared for plasmid replication, other that cell biomass and growth.

For lactic acid production and residual glucose concentration by using 56/2 minimal medium with 20 g/L of initial glucose concentration, it was found that, after being fermented for 48 hours, the strain RB24 could produce lactic acid in all 3 conditions (Figure 4.2). However, the highest lactic acid concentration was measured in limited condition. Although the system of *ldhA* expression in *E.coli* is functioned during fermentative growth, the RB24 strain harboring the *ldhA* from *R. oryzae* on the plasmid may still require some oxygen for LdhA expression and function as the native enzyme. Furthermore, residual glucose concentrations of all strains in all conditions

were more than 10 g/L (Figure 4.2). This excess glucose could inhibit lactic acid production. Therefore, it was important to study suitable initial glucose concentration for lactic acid production in 56/2 minimal medium.

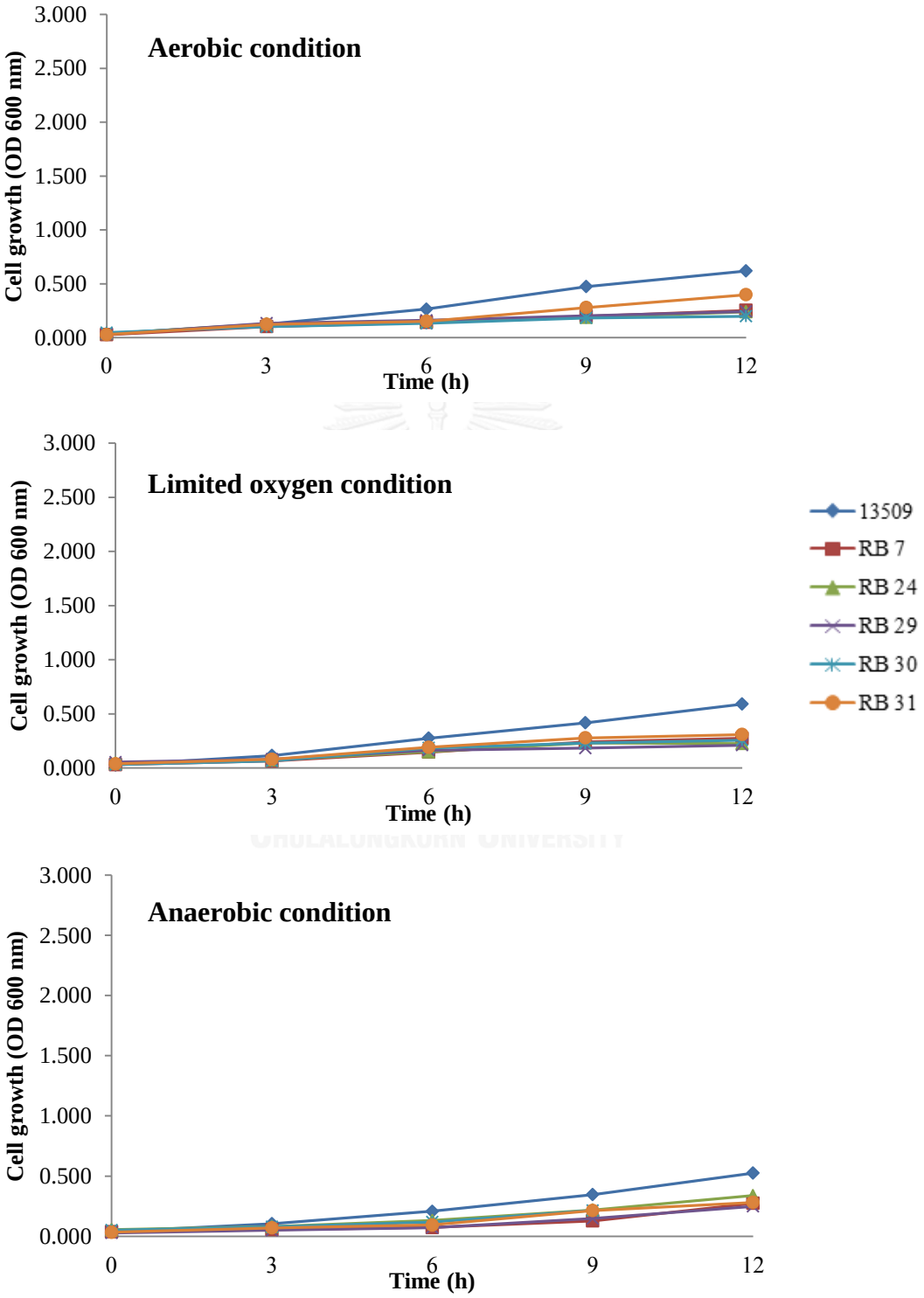
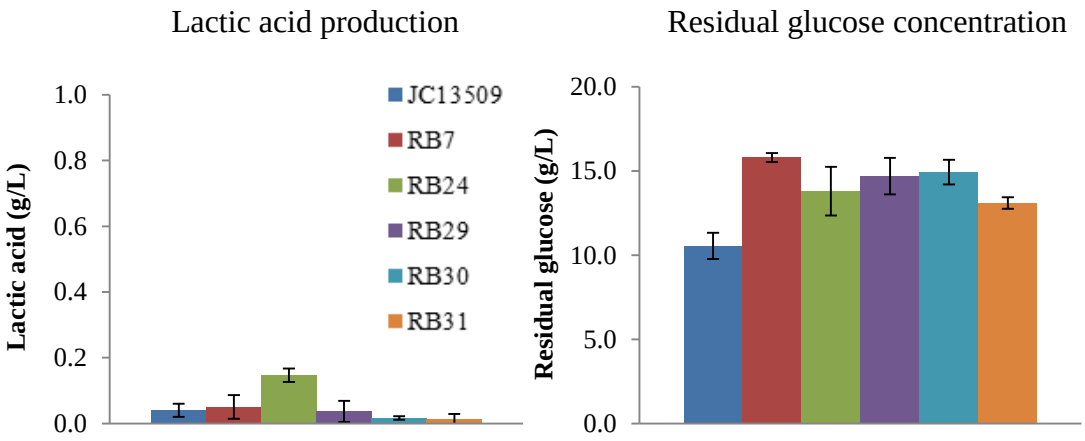
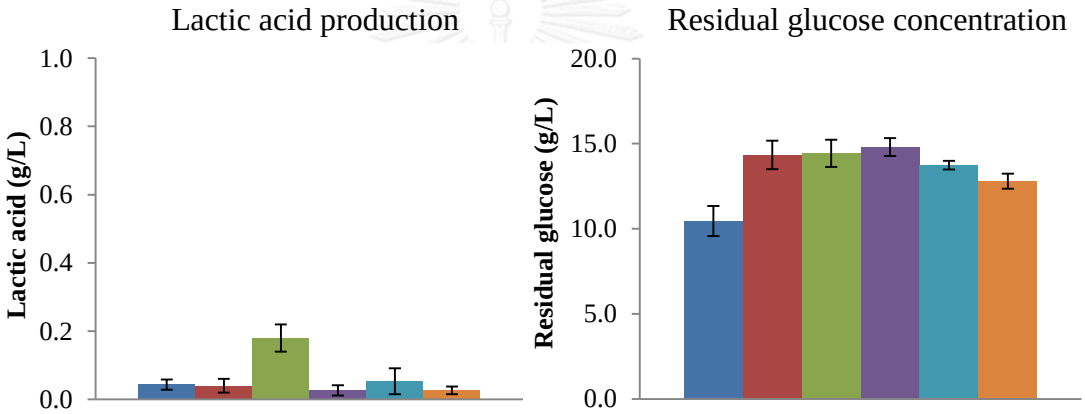


Figure 4.1 Cell growth during lactic acid fermentation in 56/2 minimal medium under aerobic, limited oxygen and anaerobic conditions ($p < 0.05$).

A. Aerobic condition



B. Limited oxygen condition



C. Anaerobic condition

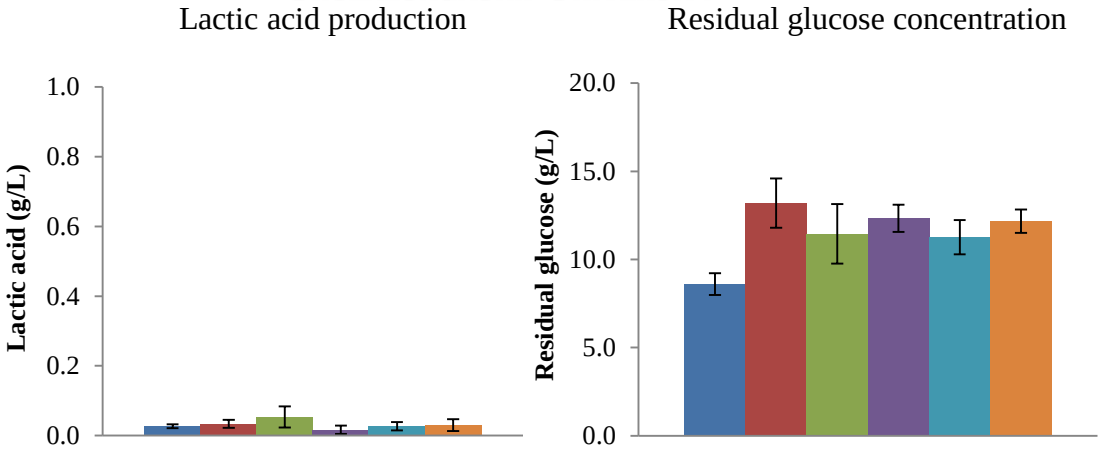


Figure 4.2 Lactic acid and residual glucose concentrations after being fermented of strains in 56/2 minimal medium with initial glucose concentration of 20 g/L under A. aerobic, B. limited oxygen, C. anaerobic conditions at 48 h ($p < 0.05$).

4.1.1 The effect of initial concentration of glucose

In this study, fermentation was also carried out by using 56/2 minimal medium with 3 conditions, but 5 initial glucose concentrations were varied. Amino acids (arginine, thiamine, histidine, proline) were served as nitrogen source. Growth curves of different initial glucose concentrations during fermentation under aerobic, limited oxygen and anaerobic conditions were represented in Figure 4.3-4.5, respectively. From Figure 4.3, under aerobic condition, growth of wild type strain, JC13509, tended to be better than others, however, at some glucose concentrations, the strain RB31 (wild type background with pBluescript II KS(+)) has similar growth to that of wild type. It is possible that, some initial glucose concentrations, it has enough nutrients and energy for the wild type background strain to both produce biomass and replicate plasmid. From Figure 4.4 and 4.5, under limited and aerobic conditions, growth of wild type strain seemed to be slightly better than other strains for all initial glucose concentrations. With these conditions, chromosomal *ldhA* and *pta* is somehow required for growth, moreover, nutrients and energy for growth were shared for plasmid replication.

The lactic acid production and residual glucose concentration at 48 hours of all fermentation conditions were represented in Figure 4.6. In this experiment, the result suggested the similarity of lactic acid production in all fermentation conditions. As expected, the strain RB24 could produce the highest amount of lactic acid due to the *R. oryzae ldhA* gene on the plasmid. Even though the strain RB30 also contains pRB85, pBluescript II KS(+) with *R. oryzae ldhA*, it contains *E. coli* chromosomal *ldhA*. The previous study from Chang *et al.* in 1999 suggested that exogenous *ldhA* could not express if chromosomal *ldhA* was still exist (Chang *et al*., 1999). However, lactic acid production could be detected from the strains without *R. oryzae ldhA*, including JC13509, RB7, RB29 and RB30, which may resulted from the other L-lactate dehydrogenase, LldD, of *E. coli* (Keseler *et al*., 2013).

Although the *E. coli* strain RB24 was expected to produce lactic acid, it seemed not to produce lactic acid in all conditions nor all initial glucose concentration. Considering of lactic acid production and residual glucose in fermentation media, the optimal initial glucose concentration when using 56/2 minimal medium in this study was 10 g/L. Less than 10 g/L of glucose concentration was used when fermented with 10 g/L to 50 g/L initial concentration of glucose. This may be caused from components in 56/2 minimal medium could not be utilized inappropriately for producing lactic acid. Therefore, one of the important factors for lactic acid fermentation by microorganism is nutrient requirement that may be varied upon the strain of each organism (Lawford and Rousseau, 1996). Lactic acid can be produced by many microorganisms with the use of different media, nitrogen sources and carbon sources depended on microbial metabolites (Bhalla *et al*., 2007). Therefore, studying

of effect of different components in media on lactic acid production is important to further improve lactic acid production.

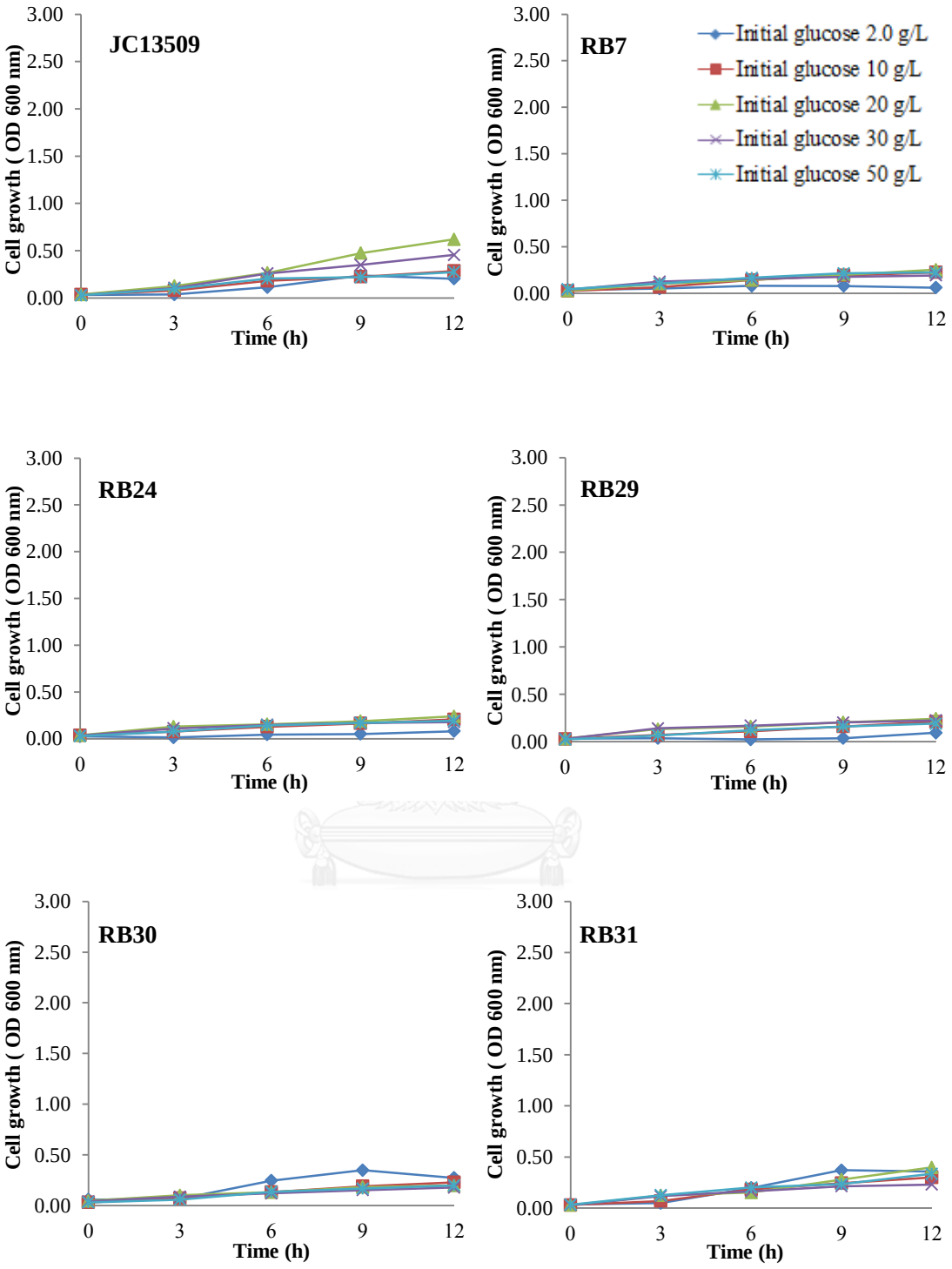


Figure 4.3 Cell growth during lactic acid fermentation in 56/2 minimal medium with 2 g/L, 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose under aerobic condition (37 °C with 200 rpm for 12 h).

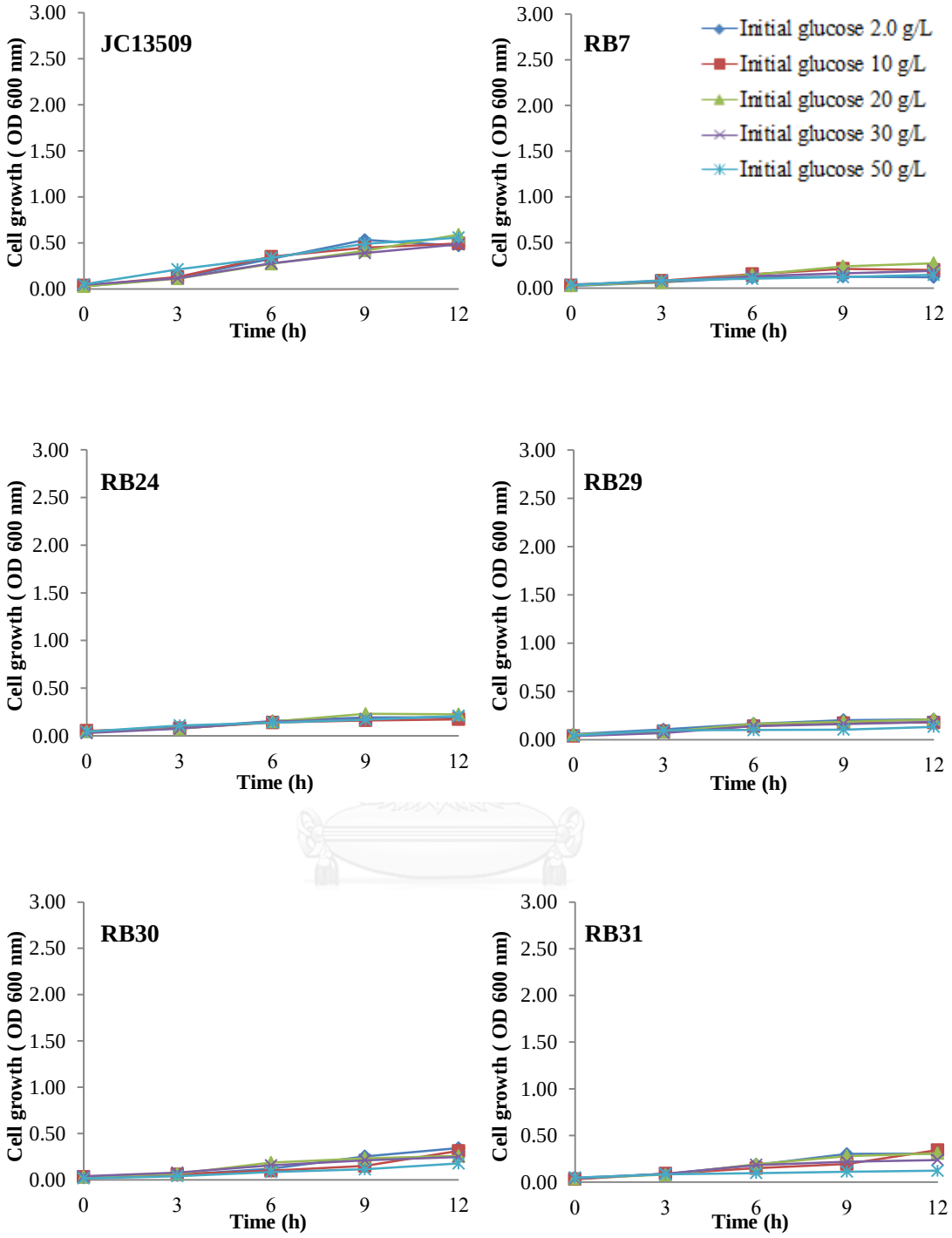


Figure 4.4 Cell growth during lactic acid fermentation in 56/2 minimal medium with 2 g/L, 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose under limited oxygen condition (37 °C without shaking for 12 h).

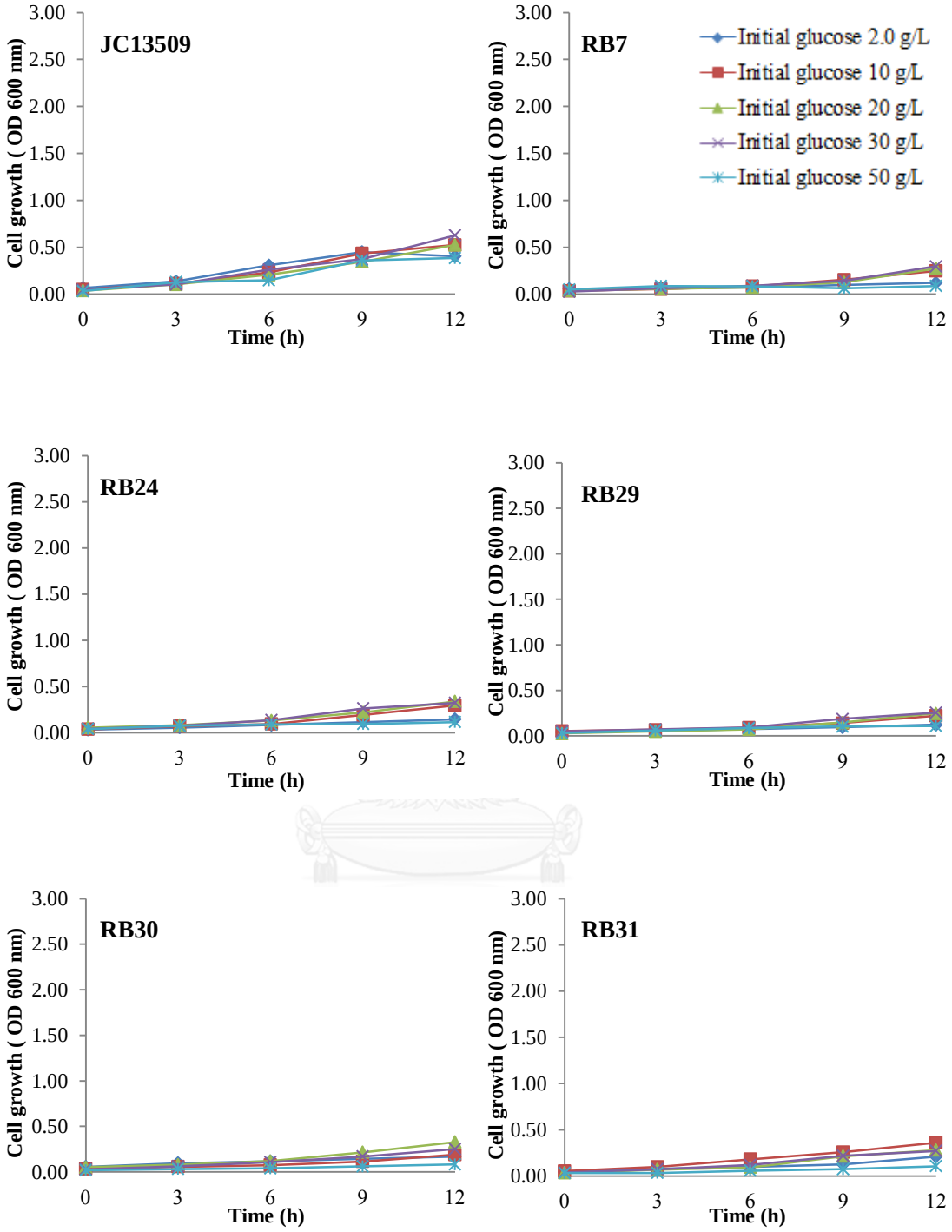
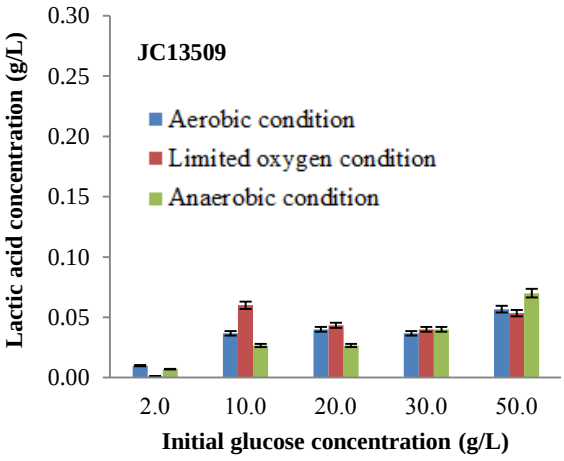
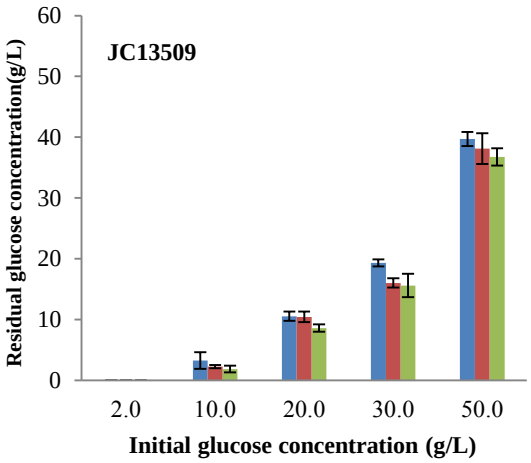
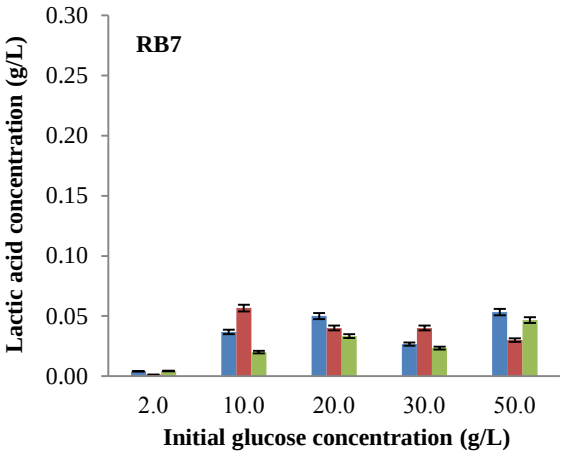
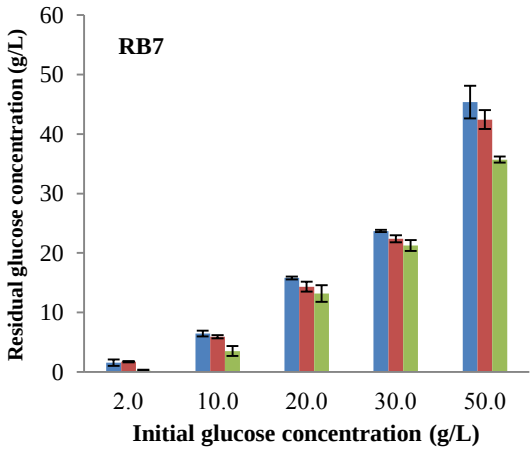


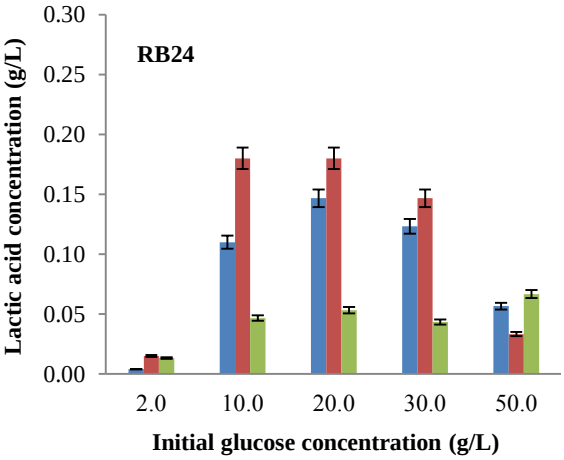
Figure 4.5 Cell growth during lactic acid fermentation in 56/2 minimal medium with 2 g/L, 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose under anaerobic condition (37 °C without shaking for 12 h, anaerobically).

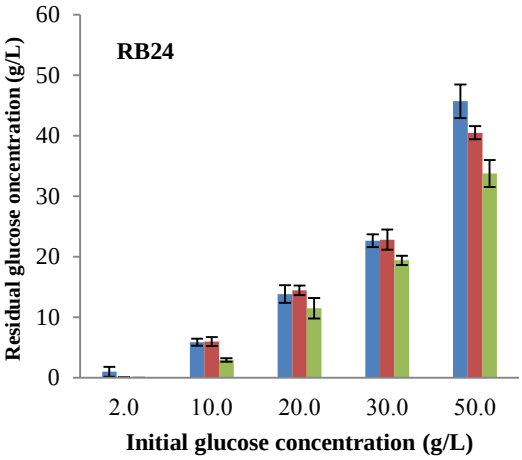












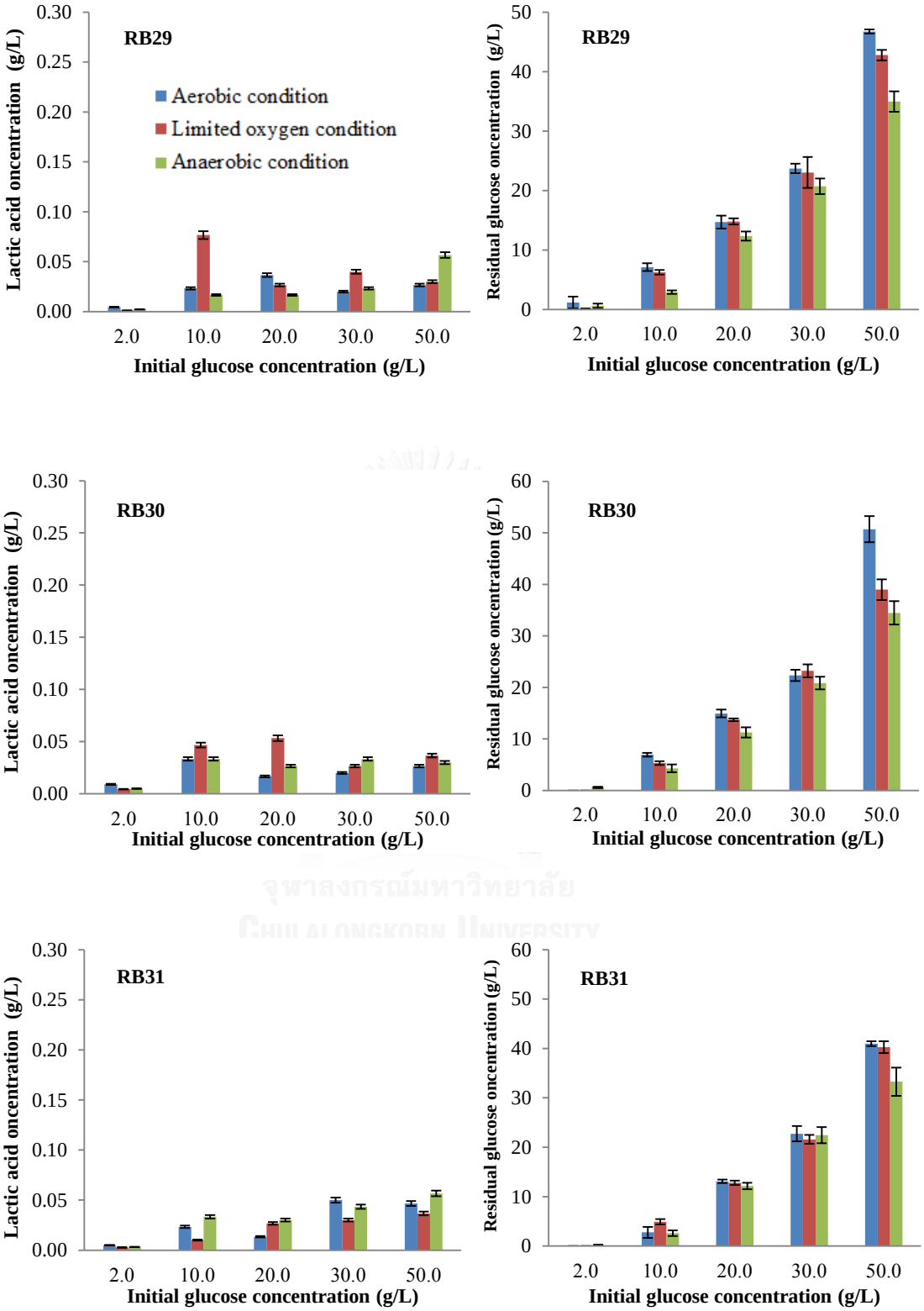


Figure 4.6 Lactic acid and residual glucose concentrations after being fermented in 56/2 minimal medium with 2 g/L, 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose under aerobic, limited oxygen and anaerobic conditions at 48 h ($p < 0.05$).

4.1.2 The effect of medium component on lactic acid production

In this experiment, carbon sources and amount of $CaCO_3$ neutralizer of 56/2 minimal medium were studied. From Figure 4.7, lactic acid production after being fermented under anaerobic condition at 48 hours from 20 g/L of initial glucose (2% (w/v) initial glucose) was compared with 1% (v/v) initial glycerol. Lactic acid concentration when using 20 g/L of initial glucose as carbon source was higher than 1% (v/v) glycerol. It is possible that C6 carbon source is more suitable for metabolism and fermentation than C3 carbon source. Moreover, during fermentation, decrease in pH due to insufficient amount of $CaCO_3$ that may affect glycerol dehydrogenase activities, glycerol metabolism enzyme (Gonzalez *et al*., 2008). Moreover, the excess amount of $CaCO_3$ neutralizer was disadvantageous for lactic production from this RB24 *E. coli* strain. From Figure 4.8, When 30 g/L of $CaCO_3$ in 56/2 minimal medium was used for fermentation, at 48 hours, lower amount of lactic acid could be detected than that of 10 g/L of $CaCO_3$. Possibly, too much $CaCO_3$ may inhibit to lactic acid production (Bhalla *et al*., 2007).

From all previous experiments, very small amount of lactic acid was detected. It is possible that 56/2 minimal medium is not suitable for lactic acid fermentation. This medium only meets the minimal requirement for *E. coli* JC13509 derivatives that cells may possibly choose to utilize provided nutrients for necessary functions, such as maintaining cellular biochemical activities for surviving, rather than lactic acid production. Furthermore, with the situation of limited nutrients, small amount of lactic acid that was measured from fermentation under aerobic and limited oxygen conditions may be resulted from the other L-lactate dehydrogenase, LldD that functions during aerobic metabolism (Keseler *et al*., 2013). Therefore, medium for this *E.coli* RB24 strain to ferment lactic acid was the next issues to study.

Figure 4.7 Comparison of lactic acid production after being fermented with 20 g/L of initial glucose and 1.0% (v/v) initial glycerol in 56/2 minimal medium under anaerobic condition (37 ^{o}C without shaking, anaerobically) at 48 h ($p < 0.05$).

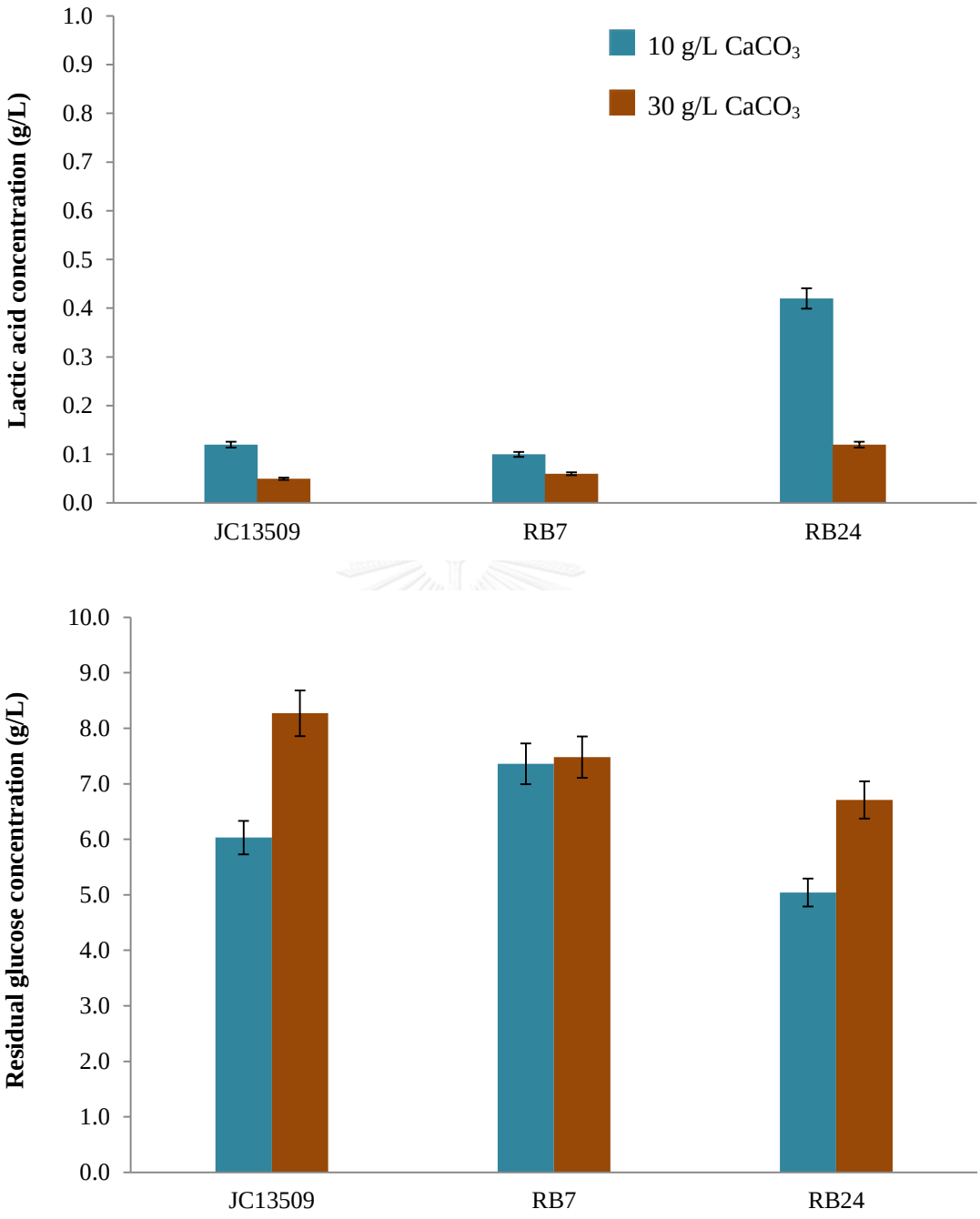


Figure 4.8 Comparison of lactic acid production and residual glucose concentrations after being fermented with 10 g/L and 30g/L of $CaCO_3$ neutralizer in 56/2 minimal medium under anaerobic condition (37 ^{o}C without shaking, anaerobically) at 48 h ($p < 0.05$).

4.2 Lactic acid production from different fermentation media

From the previous experiments, even the highest concentration of lactic acid was still low. It was hypothesized that amino acids (arginine, thiamine, histidine, proline) are served as nitrogen source in minimal medium was not enough for lactic acid production for the *E. coli* RB24. In this experiment, a kind of rich medium was used. Lactic acid production from 3 fermentation conditions and initial glucose concentrations of 10 g/L, 20 g/L, 30 g/L and 50 g/L were studied. Cell growth during fermentation in aerobic, limited oxygen and anaerobic conditions are represented in Figure 4.9-4.11, respectively. As expected, all strains grew better in fermentation broth better than 56/2 minimal medium in all conditions and all initial glucose concentrations. One of the major differences between 56/2 minimal medium and fermentation broth is nitrogen source. Four amino acids (arginine, thiamine, histidine and proline) are served as nitrogen sources in 56/2 minimal medium while yeast extract and peptone were served as nitrogen sources in fermentation broth. Therefore, fermentation broth should provide nutrients such as polypeptides, amino acid, vitamins and many growth factors suitable for metabolism of *E. coli* (Hofvendahl and Hahn-Hägerdal, 2000) that are sufficient for cell growth (Milić *et al*., 2007). Furthermore, yeast extract rich in vitamin B which increase lactic acid production (Aeschlimann and Stockar, 1990).

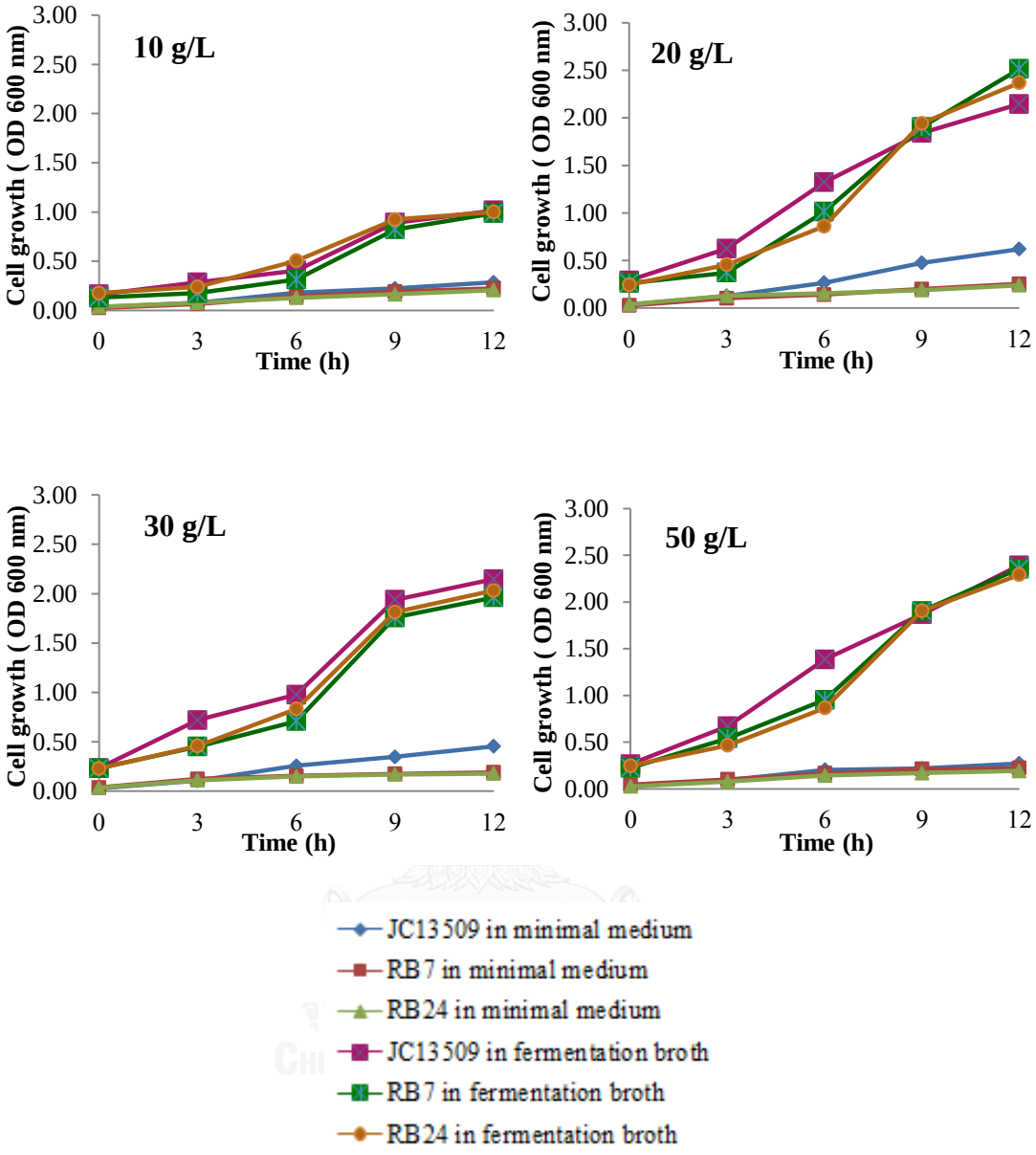


Figure 4.9 Cell growth during lactic acid fermentation in 56/2 minimal medium and fermentation broth during fermentation under aerobic condition (37 ^{o}C with 200 rpm for 12 h) ($p < 0.05$).

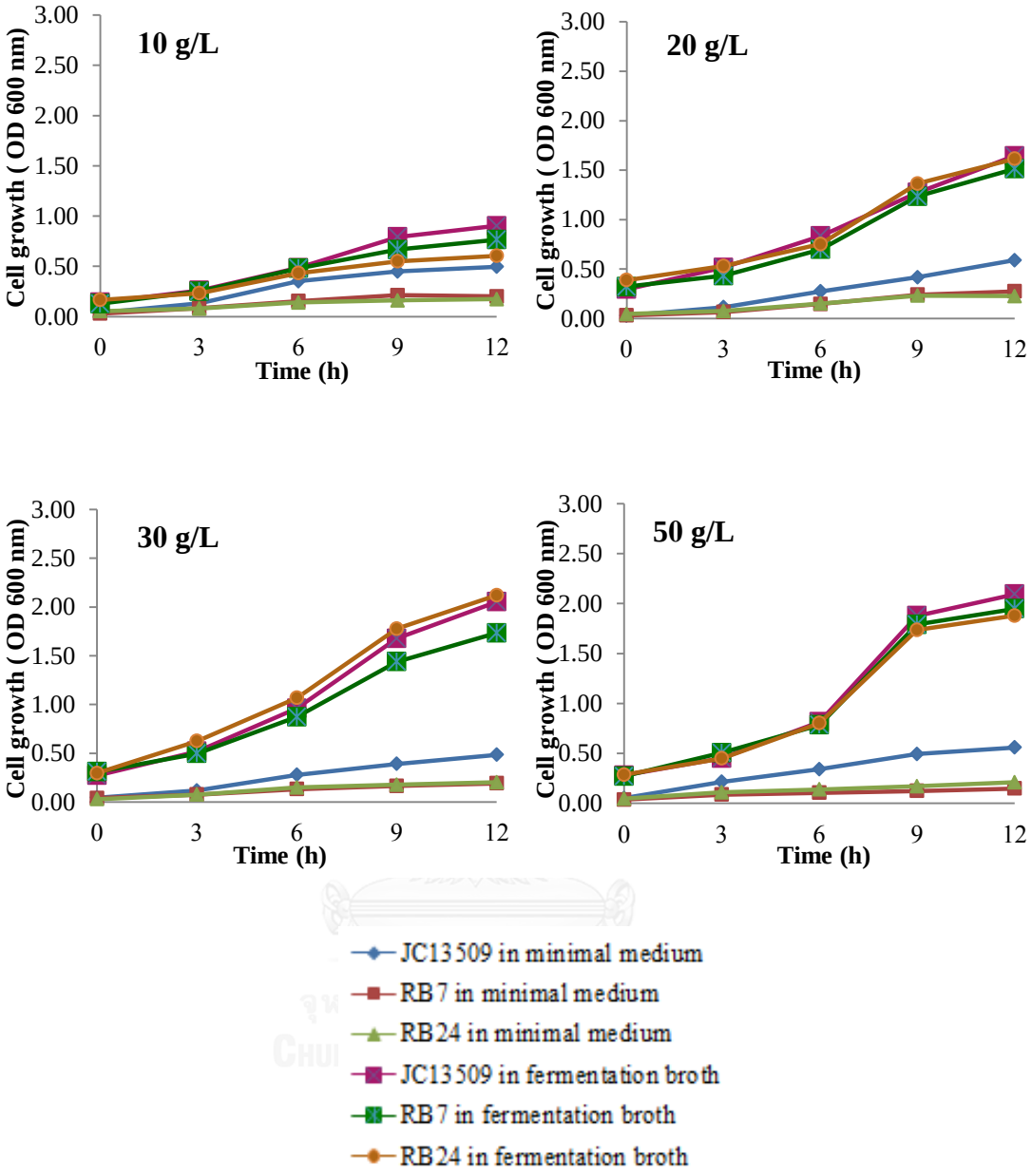


Figure 4.10 Cell growth during lactic acid fermentation 56/2 minimal medium and fermentation broth during fermentation under limited oxygen condition (37 ^{o}C without shaking for 12 h) ($p < 0.05$).

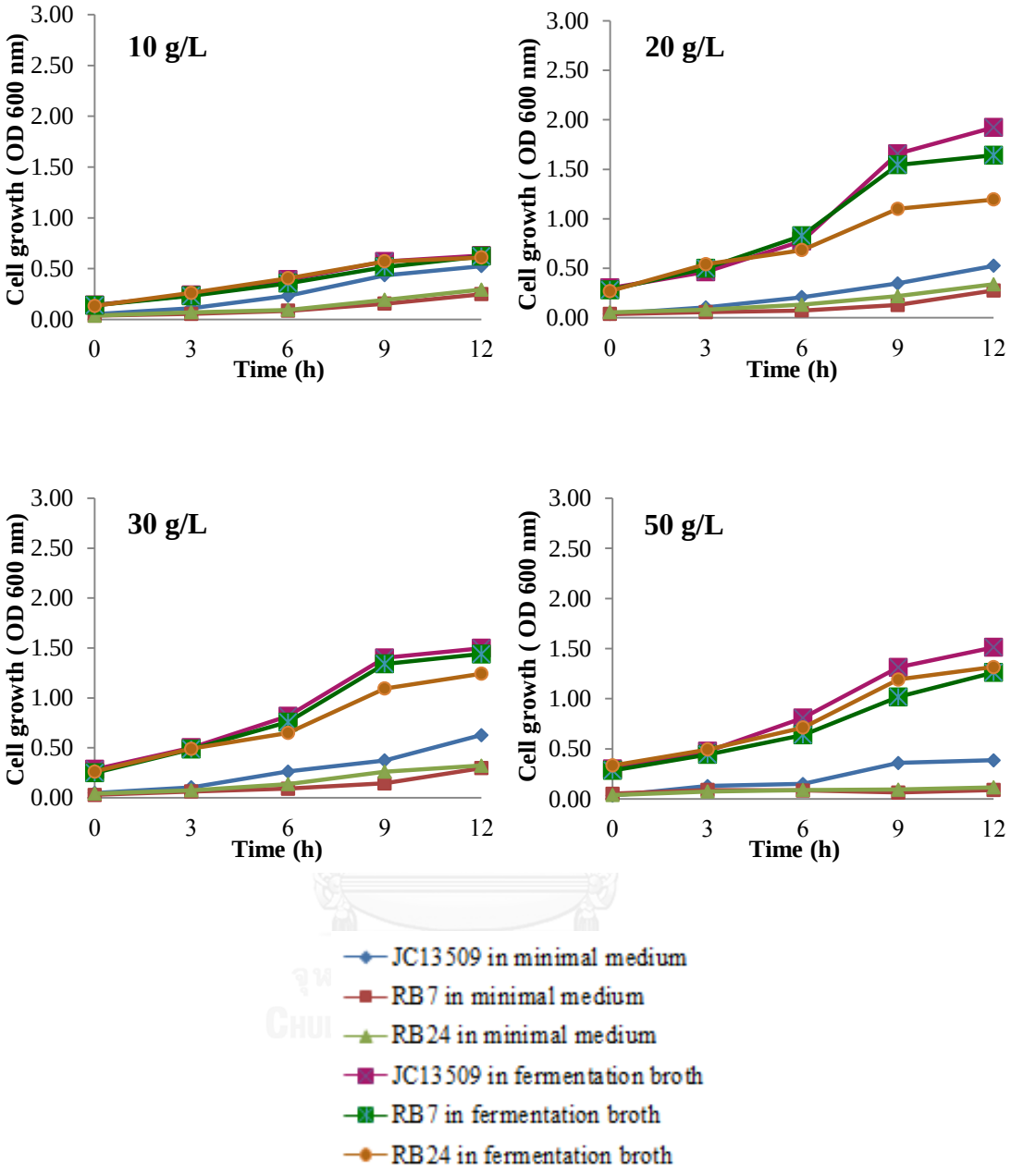


Figure 4.11 Cell growth during lactic acid fermentation in 56/2 minimal medium and fermentation broth during fermentation under anaerobic condition (37 ^{o}C without shaking for 12 h, anaerobically) ($p < 0.05$).

The lactic production and residual glucose concentrations from the *E. coli* RB24 when being fermented with 56/2 minimal medium and fermentation broth in 3 fermentation conditions and 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose concentrations at 48 hours were represented in Figure 4.12- 4.14. As expected, lactic acid production from the strain RB24 was better in fermentation broth than 56/2 minimal medium. This may be resulted from component in fermentation broth that were rich and adequate for both cell growth and lactic acid production. However, the optimal condition for fermentation by fermentation broth was found under anaerobic condition.

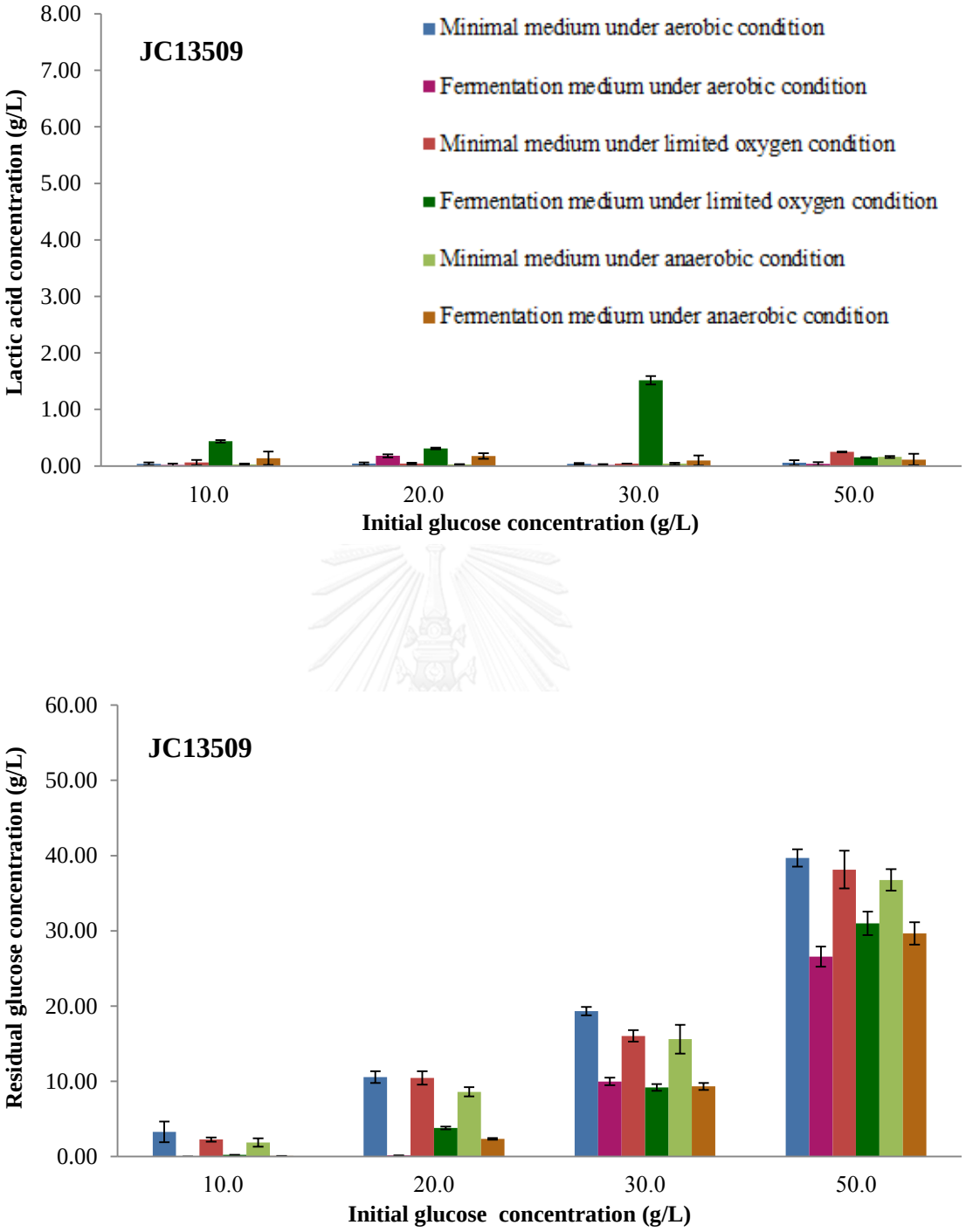


Figure 4.12 Comparison of lactic acid production by *E. coli* JC13509 after being fermented under aerobic, limited oxygen and anaerobic conditions at 48 h with 56/2 minimal medium and fermentation broth containing 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose concentration ($p < 0.05$).

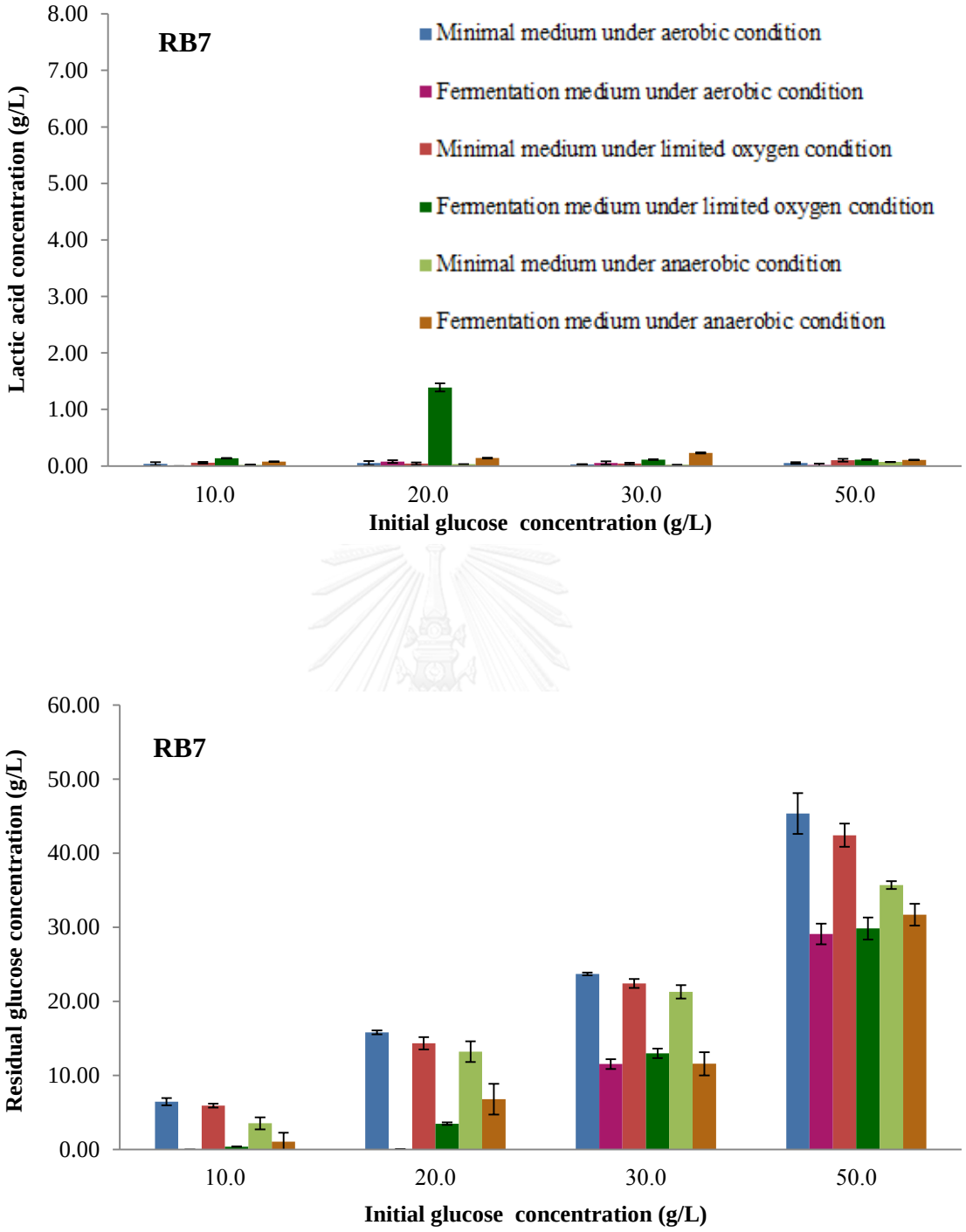


Figure 4.13 Comparison of lactic acid production by *E. coli* RB7 after being fermented under aerobic, limited oxygen and anaerobic conditions at 48 h with 56/2 minimal medium and fermentation broth containing 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose concentration ($p < 0.05$).

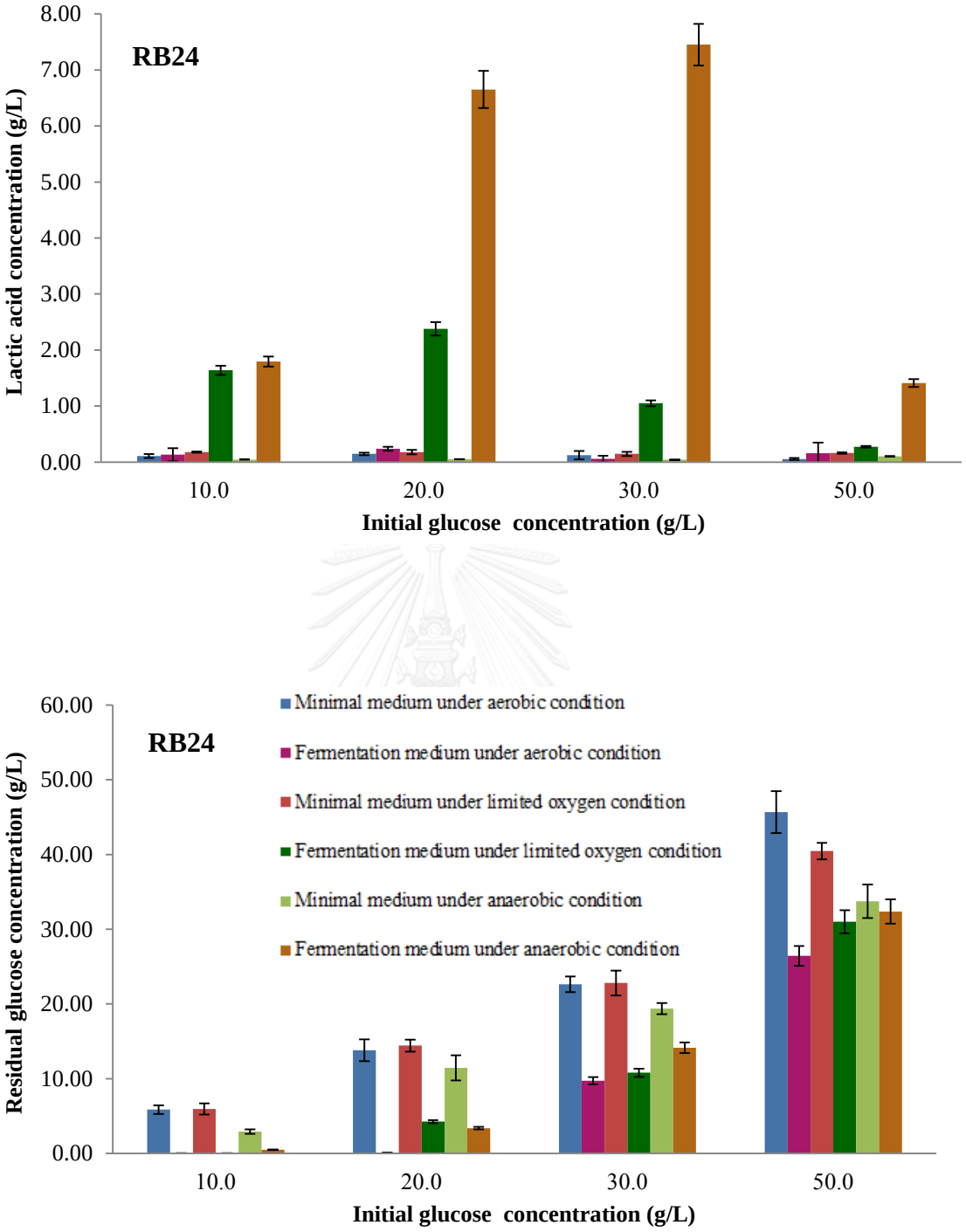


Figure 4.14 Comparison of lactic acid production by *E. coli* RB24 after being fermented under aerobic, limited oxygen and anaerobic conditions at 48 h with 56/2 minimal medium and fermentation broth containing 10 g/L, 20 g/L, 30 g/L and 50 g/L of initial glucose concentration ($p < 0.05$).

By using fermentation broth with rich nutrients, under aerobic condition, lactic acid fermentation was performed at 37 ^{o}C, 200 rpm for 48 hours. In this condition, with oxygen in the system, the cell could grow rapidly. From Figure 4.12-4.14, with the initial glucose concentration of 10 g/L and 20 g/L, at the end of the fermentation, it seemed that glucose was used up. However, with initial glucose concentration of 30 g/L and 50 g/L, it was suggested that only 20 g/L of glucose was used. However, under this condition, the strain RB24 could not produce lactic acid. It was probably caused by oxygen in fermentation system. Oxygen can serves as electron acceptor for aerobic metabolism, therefore glucose in cell chooses to enter glycolysis in aerobic metabolism, that resulted in higher energy obtained, rather fermentative growth. Moreover, in aerobic condition, half of substrate carbon has been converted to cell mass (Causey *et al*., 2003).

Under the limited oxygen condition (Figure 4.12-4.14), the residual glucose concentration tended to be similar to ones in aerobic condition. During fermentation with 10 g/L and 20 g/L of initial glucose concentration, the strain RB24 produced lactic acid with concentration of 1.64 g/L, or 0.18 g/g of yield and concentration of 2.02 g/L of lactic acid, or of 0.12 g/g of yield, respectively. However, the amount of lactic acid was still low. It was possible that glucose entered glycolysis until oxygen was used up, then, it entered to fermentative growth that can produce lactic acid.

Under anaerobic condition (Figure 4.12-4.14), lactic acid fermentation was performed in the closed system without oxygen in the system at 37 ^{o}C, for 48 hours. With this condition, the strain RB24 gave the highest lactic acid concentration of 7.45 g/L with a yield of 0.42 g/g when using 30 g/L of initial glucose. It was suggested that regulation of lactic acid expression required some components in *E. coli* anaerobic metabolism even though the *R. oryzae ldhA* produce lactic acid aerobically. Moreover, with the higher initial glucose concentration (50 g/L), the lower amount of lactic acid production. It is possibly caused by inhibition of lactic acid production by high glucose concentration (Gonçalves *et al*., 1991).

Although the highest concentration of lactic acid was detected as 7.45 g/L, considering with costs, it was not high enough. There were some factors were required to study for further improvement of lactic production. Due to *R. oryzae* gene was on the plasmid, gene product could be inconsistently produced and plasmid could be lost. Therefore, in the next topic, plasmid type will be focused on. It was hypothesized that if *R. oryzae ldhA* gene was on an inducible plasmid, recombinant LdhA should be produced in higher amount and resulting in higher amount of lactic acid.

4.3 Lactic acid production from different plasmid type

4.3.1 Construction of *E. coli* strain harboring *R. oryzae ldhA* gene on IPTG-induced plasmid

Although *E. coli* strain RB24 could produce the highest concentration of lactic acid when using fermentation medium with 30 g/L of initial glucose in anaerobic condition (7.45 g/L), the amount was still low. A hypothesis was that the expression of *R. oryzae ldhA* on the plasmid on pBluescript II KS(+) may be not enough and inconsistent. Therefore, in this experiment, the pQE-30 Xa plasmid was selected, so the target gene could be forced to be expressed. The *R. oryzae ldhA* was amplified from pRB85 with primer 42 and 43. The approximately 1800 base pairs DNA fragment of *R. oryzae ldhA* (Figure 4.15) was cloned into pQE-30 Xa plasmid under the control of *lac* promoter that could be induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG). This constructed plasmid was named as pTN1 and then, transformed into *E. coli* strain RB7 to generate the strain name THW1. Before transferring to fermentation broth, the strain THW1 was induced by 0.1 M IPTG, and then also fermented for 48 hours with fermentation broth in 3 conditions, with various initial glucose concentrations as 10 g/L, 20 g/L, 30 g/L, 50 g/L and 70 g/L. Cell growth under aerobic, limited oxygen and anaerobic conditions were represented in Figure 4.16-4.18, respectively. Growth of all strains tended to be similar in most conditions and initial glucose concentrations. However, in some conditions, such as 20 g/L of initial glucose under anaerobic condition, the RB24 strain seemed to grow slightly slower than the other strains. It is possible that some component on pRB85 may somehow cause lower growth in these conditions.

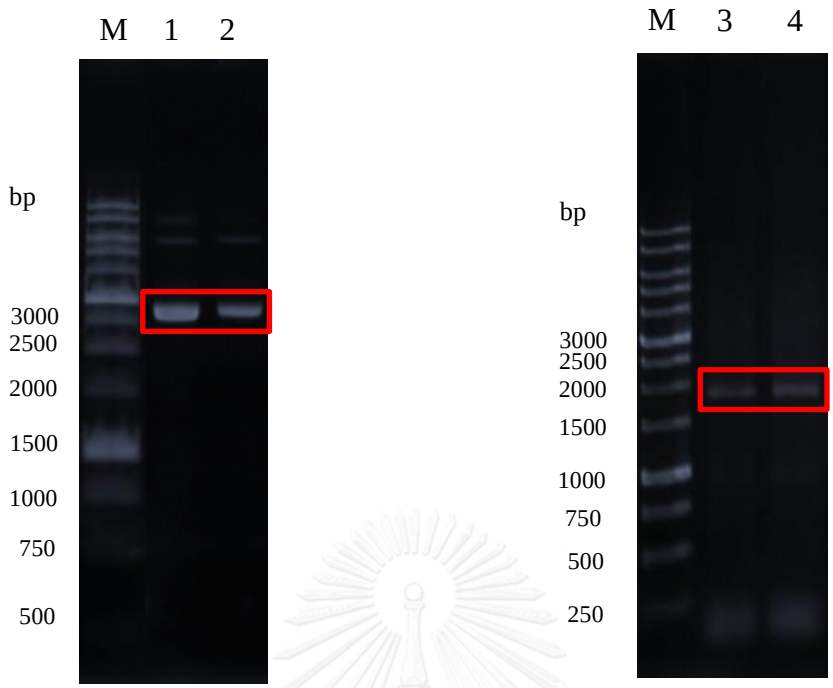


Figure 4.15 1% Agarose gel electrophoresis of the plasmid pQE-30 Xa and DNA fragment of *R. oryzae ldhA* cut with *Hind*III and *Bam*HI enzymes.

The size of plasmid pQE-30 Xa (lane 1-2) and DNA fragment of *R. oryzae ldhA* (lane 3-4) are approximately 3500 and 1800 base pairs, respectively. Lane M is 1 kbp DNA ladders marker.

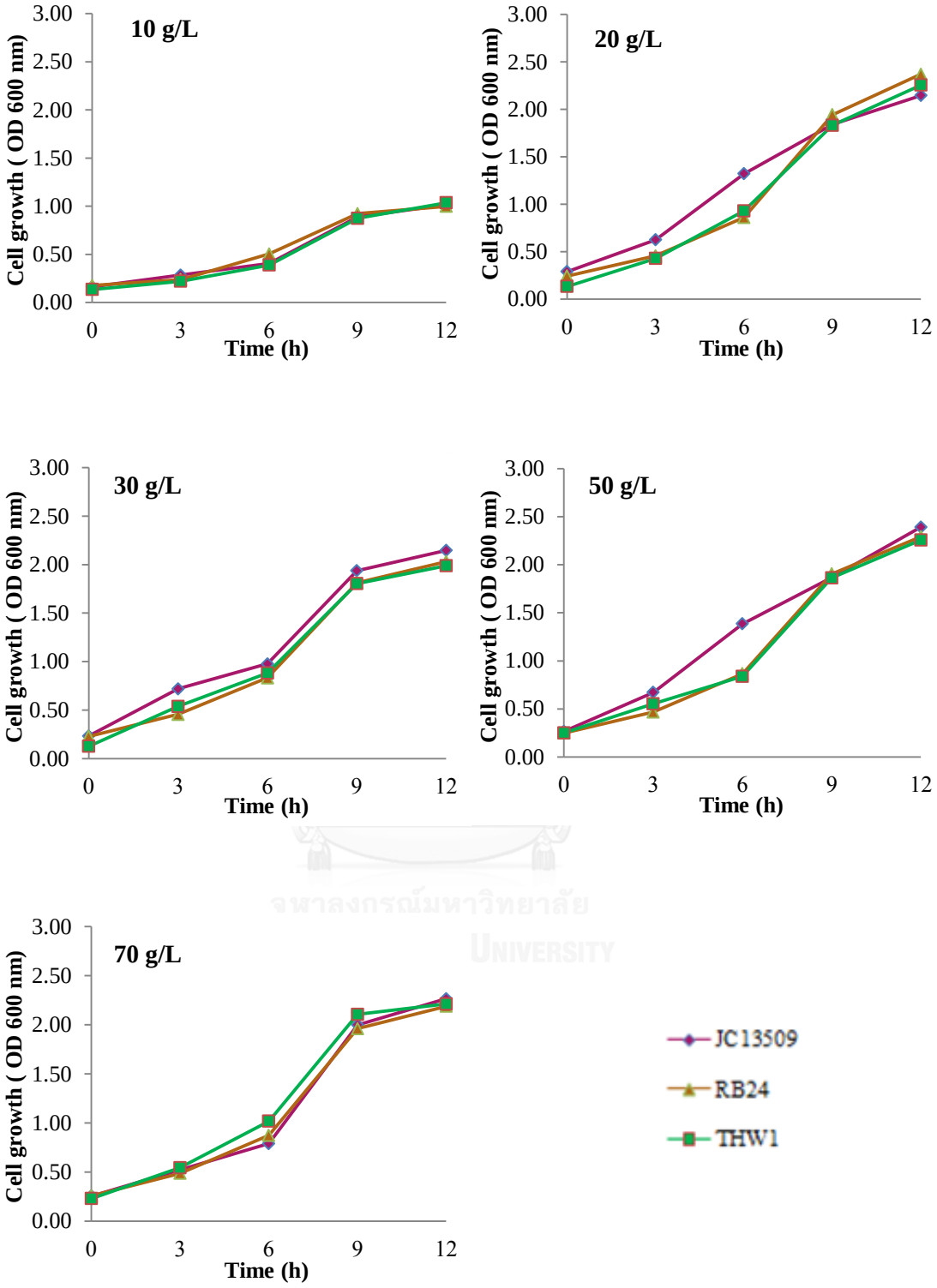


Figure 4.16 Cell growth during lactic acid fermentation of JC13509, RB24 and THW1 strains using fermentation broth with 10 g/L, 20 g/L, 30 g/L, 50 g/L and 70 g/L of initial glucose concentration under aerobic condition for 12 h ($p < 0.05$).

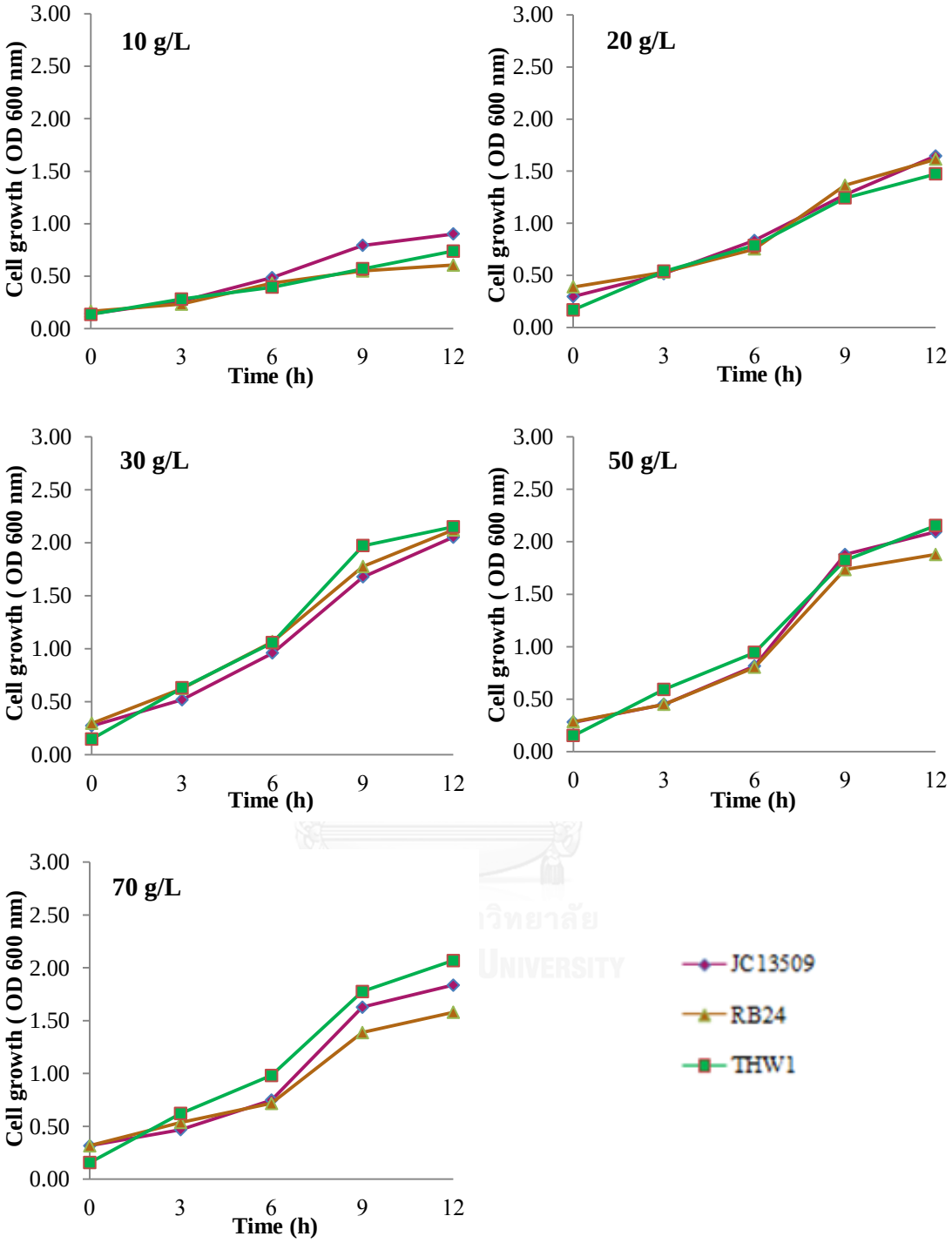


Figure 4.17 Cell growth during lactic acid fermentation of JC13509, RB24 and THW1 strains using fermentation broth with 10 g/L, 20 g/L, 30 g/L, 50 g/L and 70 g/L of initial glucose concentration under under limited oxygen condition for 12 h ($p < 0.05$).

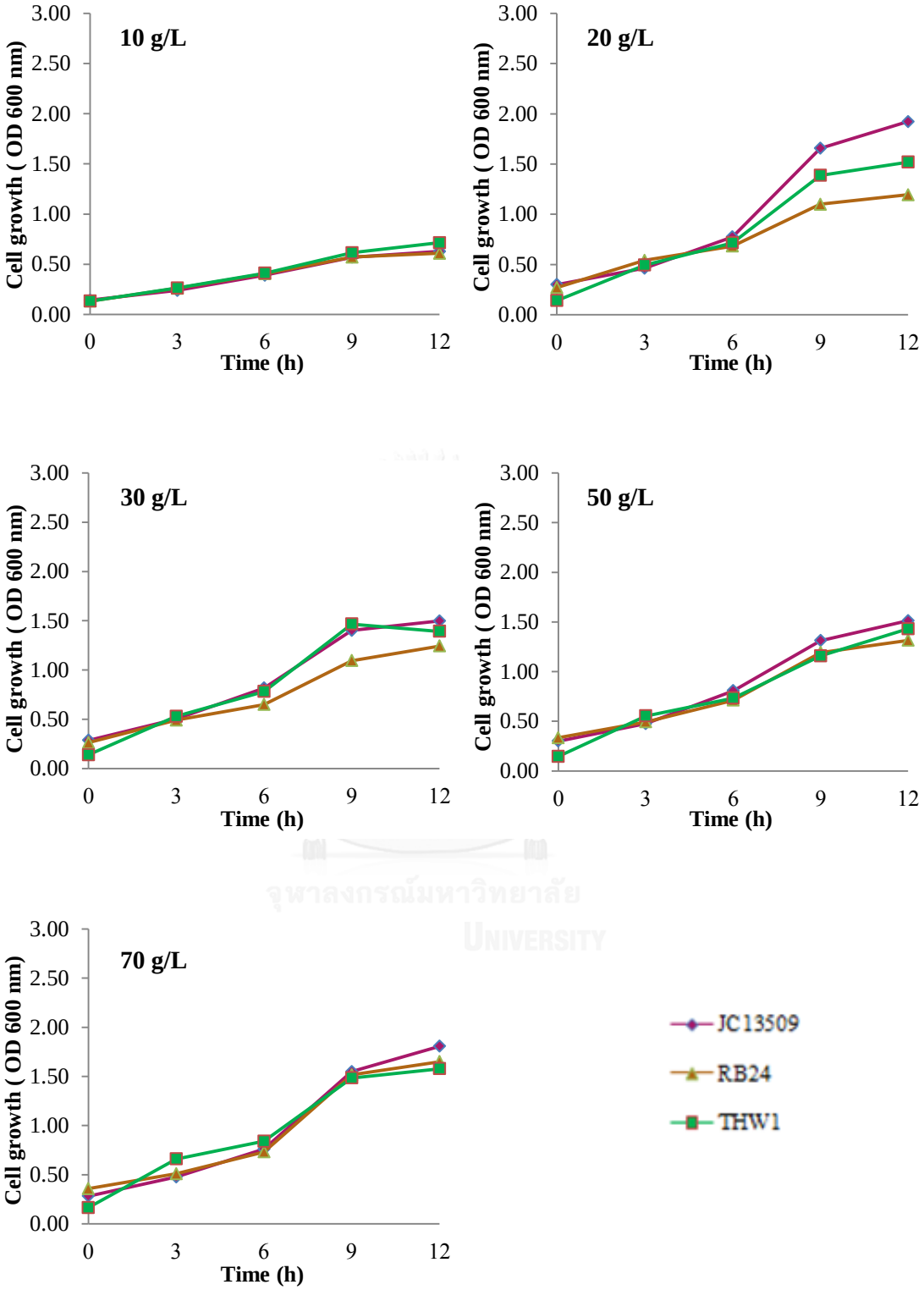


Figure 4.18 Cell growth during lactic acid fermentation of JC13509, RB24 and THW1 strains using fermentation broth with 10 g/L, 20 g/L, 30 g/L, 50 g/L and 70 g/L of initial glucose concentration under anaerobic condition for 12 h ($p < 0.05$).

4.3.2 Comparison of lactic acid production from different plasmid types

The lactic acid production by THW1 was compared with RB24 by using fermentation broth with various initial glucose concentrations. The strain THW1 was induced by IPTG before. Lactic acid production and residual glucose concentration under aerobic, limited oxygen and anaerobic conditions were represented in Figure 4.19-4.21, respectively.

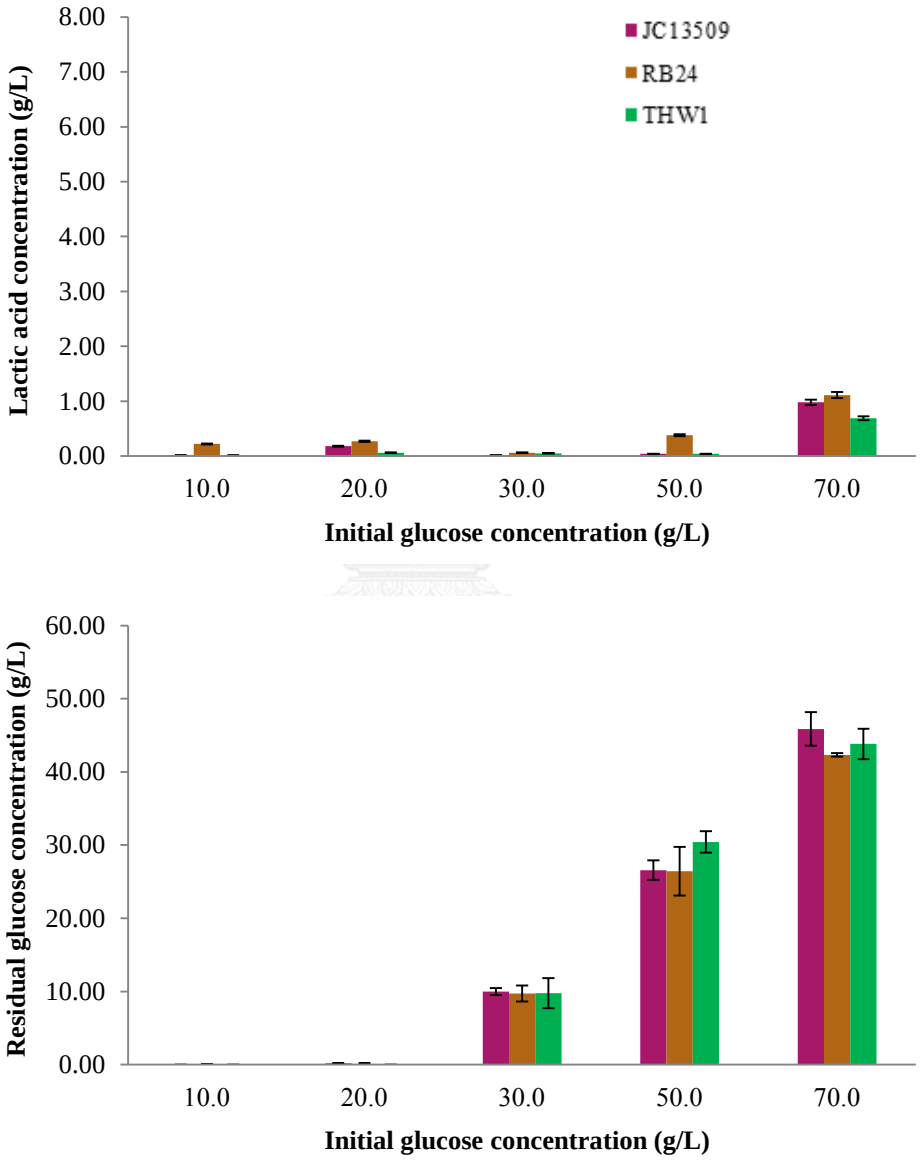


Figure 4.19 Lactic acid production and residual glucose concentrations from the strain THW1 with IPTG induction compared with RB24 under aerobic condition after being fermented with various initial glucose concentrations for 48 h ($p < 0.05$).

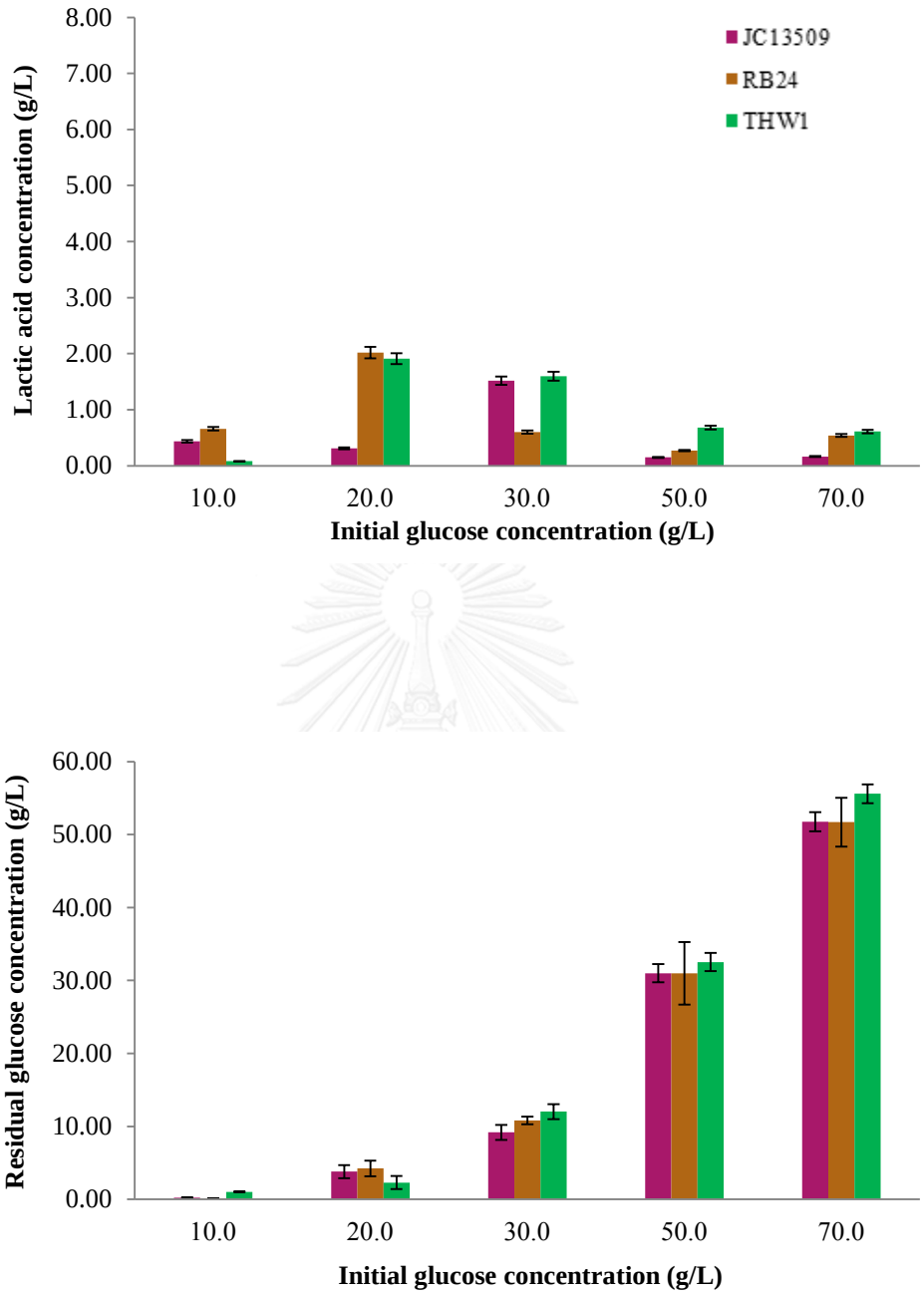


Figure 4.20 Lactic acid production and residual glucose concentrations from the strain THW1 with IPTG induction compared with RB24 under limited oxygen condition after being fermented with various initial glucose concentrations for 48 h ($p < 0.05$).

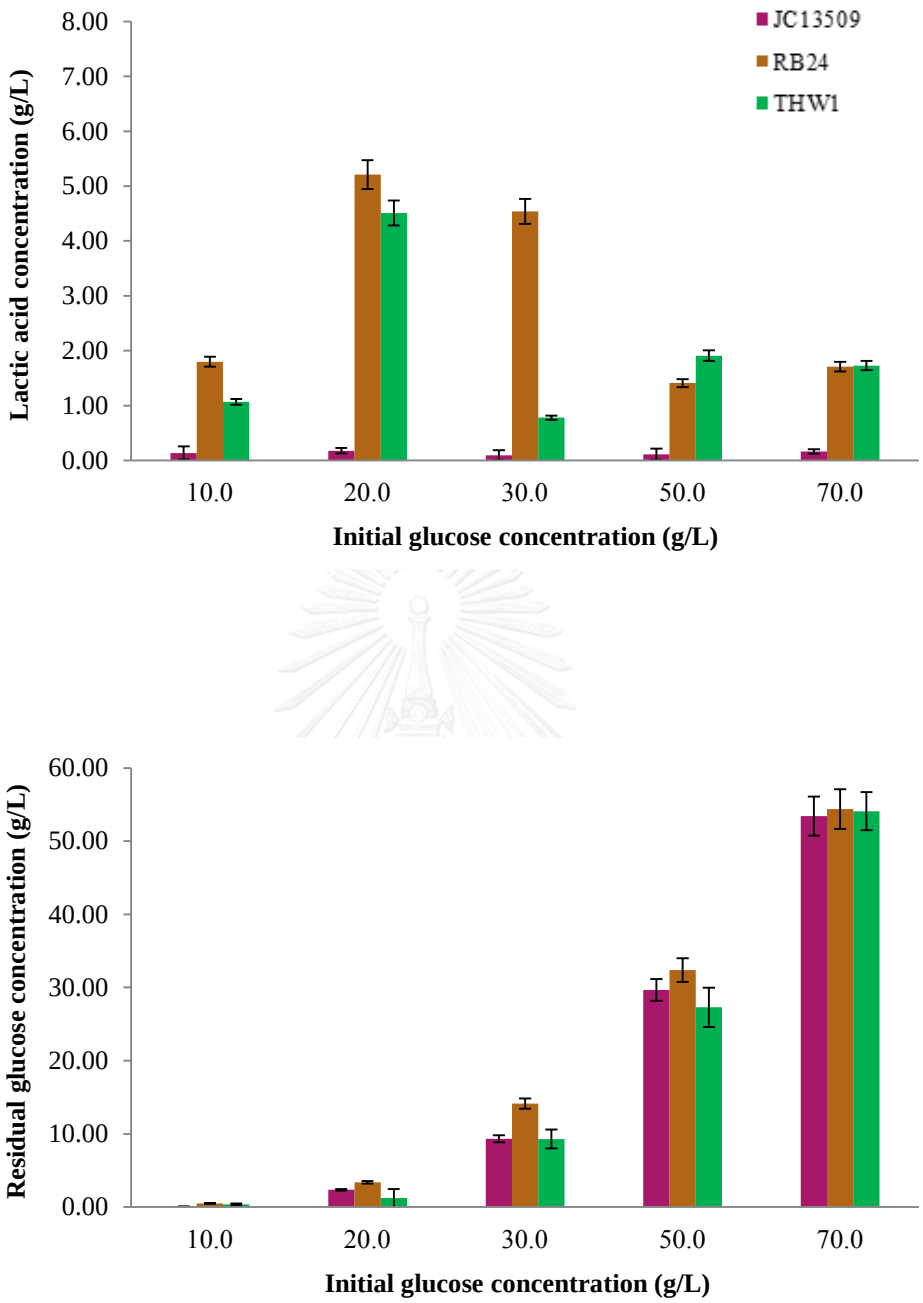


Figure 4.21 Lactic acid production and residual glucose concentrations from the strain THW1 with IPTG induction compared with RB24 under anaerobic condition after being fermented with various initial glucose concentrations for 48 h ($p < 0.05$).

The results from Figure 4.19, 4.20 and 4.21 suggested that even though the expression of *R. oryzae ldhA* was forced to be expressed in THW1 strain, unfortunately, the amount of lactic acid was not higher than that of RB24 strain as hypothesized. One possibility was that IPTG was added only once prior to fermentation and used up. Furthermore, it is possible that glucose in the medium affects to formation of CAP/cAMP complex that could repress *lac* promoter on the pQE-30 Xa plasmid (Donovan *et al*., 1996). Gosset in 2005 improved phosphoenolpyruvate:carbohydrate phosphotransferase system in *E. coli* that modifications can be improve glucose transport system (Gosset, 2005). To better understand in lactic acid production in these strains, other fermentation products after being fermented with 20 g/L and 30 g/L of initial glucose under anaerobic condition were measured and represented in Figure 4.22. As expected, D(-)-lactic acid was detected in relatively high amount from wild type JC13509 strain compared with RB24 and THW1 because of the exist of chromosomal *ldhA* gene that involved in D(-)-lactic acid production, a main product of *E. coli* fermentative growth in shake flask level (Zhou *et al*., 2003). However, D(-)-lactic acid amount was also higher than L(+)-lactic acid in the strain RB24 and THW1. It is possible that recombinant LdhA from eukaryotic *R. oryzae* could not function efficiently in prokaryotic *E. coli* host. Moreover, D(-)-lactic acid that detected in these RB24 and THW1 strains, even though the chromosomal *ldhA* gene was deactivated, may resulted from the other D-lactase dehydrogenase, the product of *dld* gene. Furthermore, during fermentation, accumulation of by-products such as succinate, acetate and ethanol, may cause a decrease in pH. The optimal pH can affect an increase in lactic acid and an decrease in by-products production (Mulok *et al*., 2009; Yoo *et al*., 1996).

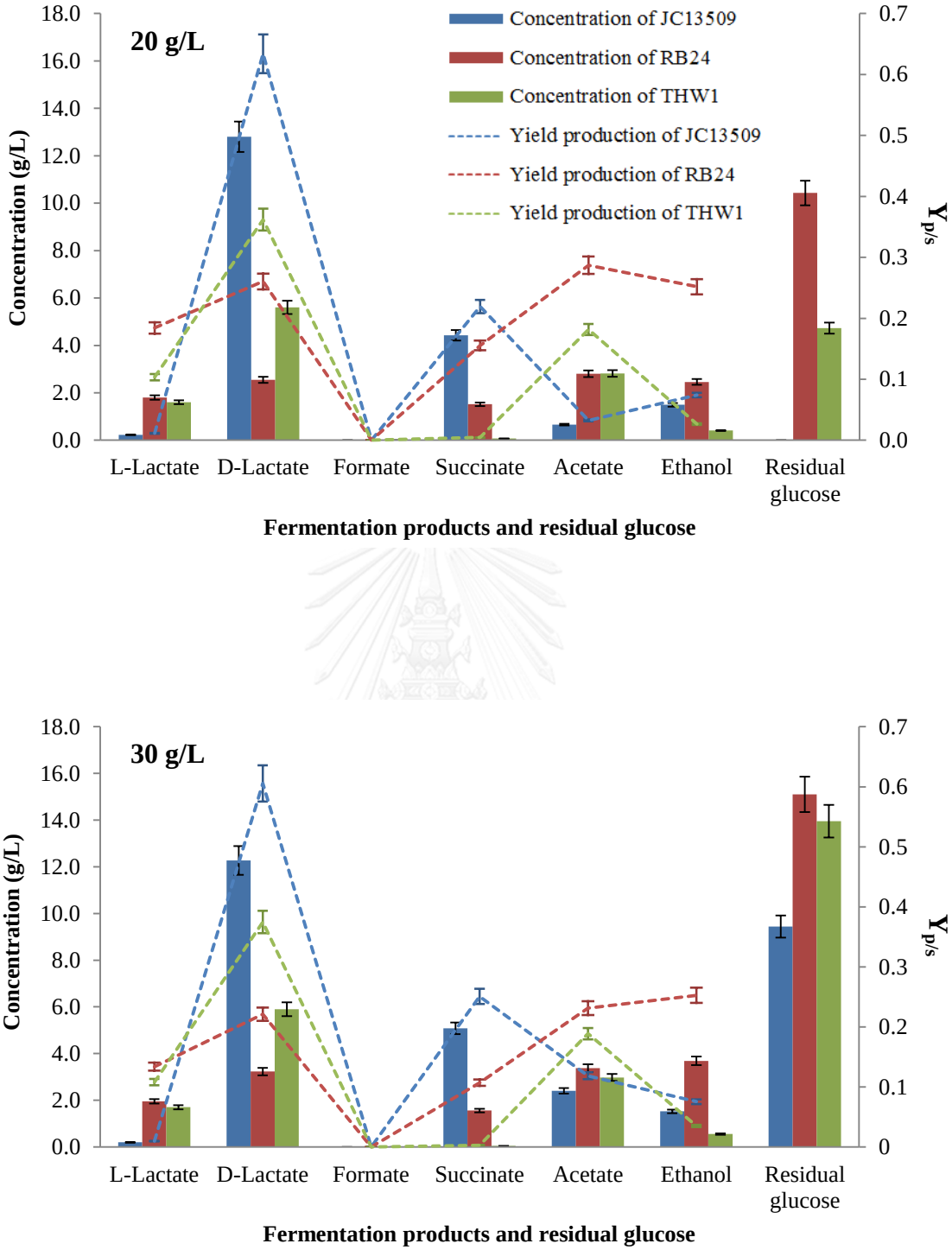


Figure 4.22 Fermentation products, residual glucose concentrations and yield production of each fermentation product after being fermented under anaerobic condition for 48 h with 20 g/L and 30 g/L of initial glucose ($p < 0.05$).

Table 4.1 Comparison of genetic modification in *E. coli* for lactic acid production from previous study compare with this study.

Strain	Modification	Outcome	Reference
E. coli RB24	*E. coli* JC13509 with deactivated chromosomal *ldhA* and *pta*, harboring *R. oryzae ldhA* gene on the plasmid	Lactate yield of 42% with glucose substrate	This study
E. coli RR1	Deletion of phosphotransacetylase (*pta*) and PEP-carboxylase (*ppc*)	Lactate yield of 90% with glucose substrate	Chang *et al*., 1999
E. coli SZ85	Replacement of *E. coli* chromosomal *ldhA* by *ldhL* from *Pediococcus acidilactici*	Lactate yield of 94% with glucose and 82% with xylose substrate	Zhou *et al*., 2003
E. coli MG1655	Deletion of *pta*, alcohol dehydrogenase (*adhE*) and fumarate reductase (*frd*), inhibition of D(-)-lactic acid production by the methylglyoxal bypass and L-lactate dehydrogenase from *Streptococcus bovis* was added into the genome	L-Lactate yieid of 90% with glycerol	Mazumdar *et al*., 2013
E. coli BAD-ldh	*E. coli* harboring the pBAD vector containing *Enterococcus facelis* KK1 L-ldh gene into *E. coli* SZ85	The maximum lactic acid concentration of 0.62 g/L from 1.0 g/L of fructose	Mulok *et al*., 2009
E. coli FBR11	*E. coli* harboring plasmid with *ldh* gene from *Streptococcus bovis*, the chromosomal *pfl* and *ldh* mutations .	Lactate yield of 93% with glucose substrate	Dien *et al*., 2001

From all experiments, it was found that a small amount of the lactic acid was detected from strains harboring plasmid with *R.oryzae ldhA* gene (RB24 and THW1). The previous study also suggested that low amount of lactic acid was obtained from *E. coli* with deactivated *ldhA* and *pflB* background that harbors *R. oryzae ldhA* gene (Skory, 2000). Then, compare with genetic modification of *E. coli* for lactic acid production from previous study (Table 4.1), *E. coli* RB24 lower amount of lactate yield than previous study. It is possible that eukaryortic *R. oryzae ldhA* may not function efficiently in prokaryotic *E. coli* host. Moreover, the lower enzyme activity may affect the recycling of NADH in the conversion of pyruvate to lactic acid and NAD^+, so NAD^+ is not enough to use in glycolysis for ATP production under anaerobic condition (Sánchez *et al*., 2005). In *E. coli* cell, under anaerobic condition, ATP was derived from substrate level phosphorylation and acetate forming reaction (Dien *et al*., 2001; Kabir *et al*., 2005). However, expected strains for L(+)-lactic acid production in this study, including RB24 and THW1, are *pta* inactivated. Therefore, for anaerobic growth of these strains, ATP was only derived from substrate level phosphorylation (Bunch *et al*., 1997). Furthermore, considering of residual glucose concentration, the strains may encounter with a problem in glucose transportation. An accumulation of pyruvate may occurs in the cells due to the less effective recombinant LdhA that could not convert pyruvate to lactate effectively (Figure 2.8). This pyruvate accumulation can inhibit *Cya*, resulting in lower cAMP, and then subsequently inhibits PTS (phosphoenolpyruvate:carbohydrate phosphotransferase system) for glucose transport (Steinsiek and Bettenbrock, 2012). Although the concentration of pyruvate could not be detected in the fermentation broth, it possibly remained intracellularly.

A benefit from our *E. coli* strains harboring *R.oryzae ldhA* gene, RB24 and THW1 is the reduction in some fermentation by-products (Figure 4.22). This may resulted from the redirection of anaerobic pathway. Therefore, to improve L(+)-lactic acid production these strains, *ldhA* expression, recombinant LdhA function efficiency, other inhibitors in anaerobic growth and other factors in the fermentation process should be further studied. Moreover, glucose transport is also required to study further.

CHAPTER V

CONCLUSION

Conclusion

There a lot of studies to improve the effectiveness for lactic acid production and expected to applied to an industrial scale. Genetic engineering was used to constructed *E. coli* strains harboring exogenous *ldhA* gene. However, to further improve lactic acid production, it is important to study lactic acid production from these *E. coli* strains in various conditions.

In this study, *E. coli* strain RB24, a genetically modified strain that harbors *ldhA* from *R. oryzae*, was used to study lactic acid production in shake flask level. The previous study suggested that this strain could produce lactic acid, but low yield was obtained. In this experiment, 56/2 minimal medium was first used because it was easy to vary some composition that may effect on lactic acid production from the *E. coli* RB24 strain. It was found that very small amount of lactic acid was detected from the strain RB24 after being fermented for 48 hours in all conditions. Furthermore, by varying medium components, lactic acid concentration using 56/2 minimal medium with 20 g/L of initial glucose as carbon source was higher than 1% (v/v) glycerol and fermentation medium with 10 g/L of $CaCO_3$ was higher than 30 g/L of $CaCO_3$. However, small amount of lactic acid that detected when being fermented with 56/2 minimal medium may be resulted from other oxygen induced L-lactate dehydrogenase, LldD, because of the detection of lactic acid from the strain without *R.oryzae ldhA* gene along with an amount of lactic acid from aerobic and limited oxygen conditions that seemed to be higher than that from anaerobic conditions. Moreover, in this minimal medium, slow cell growth was found and less than 10 g/L of glucose concentration was used when fermented with 10 g/L to 50 g/L of initial concentrations of glucose. Therefore, 56/2 minimal medium was not suitable for lactic acid fermentation due to the limited nutrients. In this situation, cells was possibly used this limited nutrients for maintaining necessary biochemical activities for survival rather that producing biomass, cell division, or producing lactic acid.

When using rich medium for fermentation, as expected, a higher cell growth in fermentation broth than 56/2 minimal medium could be detected. Moreover, the highest concentration of lactic acid (7.45 g/L) was obtained when fermentation medium with 30 g/L of initial glucose concentration under anaerobic condition. However, the amount of lactic acid produced was still low which could not use for

further application or upscaling. Moreover, the higher initial glucose concentrations tended to give the lower amount of lactic acid. This was possibly caused by the inhibition of lactic acid production by high glucose concentration. Moreover, lactic acid production from the strain RB24 is depended on *R. oryzae ldhA* gene on the plasmid. Even though one of the advantages of using plasmid for gene expression is more copy numbers, it is hard to control the stability. Then, *R. oryzae* was forced to be expressed by cloning under the *lac* promoter of pQE-30 Xa which is induced by IPTG, resulted in the strain named THW1. The lactic acid production from THW1 strain was still low and even lower than RB24. It is possible that, in this case, glucose in the medium may repress *lac* operon. When other fermentation products were measured, D(-)-lactic acid could be detected in the RB24 and THW1 strains, even though the chromosomal *ldhA* gene was deactivated. This may be resulted from the other D-lactase dehydrogenase, the product of *dld* gene.

From all experiments, it was suggested that *R. oryzae ldhA* gene could express in *E. coli* host, but the exogenous eukaryotic protein may not function efficiently in prokaryotic host which it may cause lower enzyme activity. Then, lactic acid could not be efficiently generated from pyruvate. Besides low amount of lactic acid production, pyruvate is accumulated which further inhibits PTS (phosphoenolpyruvate:carbohydrate phosphotransferase system) for glucose transport. This may be a reason why a relatively high residual glucose concentration was detected.

Suggestion

The yield of lactic acid in this study was low. To solve this problem, one of the solutions is to continue to find new conditions for fermentation. However, the result suggested that varying some parameters such as types of media, initial glucose concentrations and fermentation conditions were not enough to obtain high yield of lactic acid from the *E. coli* harboring plasmid with *R. oryzae ldhA* gene. Therefore, it is interesting to do more genetic modification of the strain RB24. The genetically engineered *E. coli* involving in other anaerobic metabolic pathways such as *adhE*, *plfB* and *frdABC* may improve the lactic acid production by redirection of lactic acid pathway.

The *R. oryzae ldhA* gene expression in the *E. coli* strain RB24 may be quite complicated to be regulated due to the presence of both *R. oryzae ldhA* promoter (on the plasmid) and *E. coli ldhA* promoter on the host chromosome. Furthermore, for the strain RB24, the *R. oryzae ldhA* expression is dependent on gene on the plasmid that is quite difficult to control the stability. Therefore, to solve these problems, the new genetically modified *E. coli* strain should be constructed by replacing chromosomal *E.*

coli ldhA with *R. oryzae ldhA* under the control of *E. coli ldhA* promoter. By integrating gene onto the chromosome, the gene stability can be increased and easier to maintain. Moreover, it should be advantageous because antibiotic is not required for maintaining plasmid. With the control of only host promoter, it should be easier to find the conditions suitable for *R. oryzae ldhA* expression and further improve lactic acid production.

As the results suggested the ineffective recombinant LdhA expressed from *R.oryzae ldhA* in *E. coli* host, it is important to study the activity of this LdhA. Moreover, parameters in glucose phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) should be focused on further to solve the problem of high residual glucose concentration that may inhibit lactic acid production.

In addition, during fermentation, because other factors such as pH can affect to lactic acid and by-products yields, pH in fermentation media should be controlled. Furthermore, time and temperature for IPTG induction of the *R. oryzae ldhA* gene on the IPTG-inducible plasmid should be optimized to increase the target gene expression efficiency.

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APPENDIX

APPENDIX A

MEDIA AND SOLUTIONS

1. Ampicillin (100mg/ml)

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

2. LB medium

Peptone	10	g
Yeast extract	5	g
NaCl	10	g

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

3. LB agar

Add 2% (w/v) agar in LB medium and sterilize at 121 °C for 15 minutes.

4. Fermentation broth

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

The concentration of glucose in fermentation broth was varied as 10, 20, 30, 50, 70 g/L in the study. Dissolve in dH_2O, adjust pH to 6.8 and sterilize at 121 °C for 15 minutes.

3.1 Salt solution

$MgSO_4.7H_2O$	400	mg
$MnSO_4.5H_2O$	20	mg
$FeSO_4.7H_2O$	20	mg

Dissolve in 10 ml of dH_2O.

5. 5X 56 phosphate buffer

Na_2HPO_4	48.4	g
KH_2PO_4	25.5	g
$MgSO_4.7H_2O$	1.0	g
$(NH_4)_2SO_4$	10.0	g
$Ca(NO_3)_2.4H_2O$	0.05	g
$FeSO_4$	0.0025	g

Dissolve in 5000 ml of dH_2O.

6. 56/2 phosphate buffer

5X 56 phosphate buffer	500	ml
dH_2O	500	ml
Arginine	0.2	g
Thiamine	0.001	g
Histidine	0.1	g
Proline	10.0	g

Sterilize by autoclaving at 121 ^oC for 15 minutes.

7. 56/2 minimal medium

Add glucose and $CaCO_3$ to the 56/2 phosphate buffer before use. 10 g/L of $CaCO_3$. In this study, the concentration of glucose in fermentation broth was varied as 2.0, 10, 20, 30, 50 g/L in the study. Use 1% (v/v) of glycerol instead of glucose in the experiment of varying carbon source. $CaCO_3$ was varied as 10 g/L and 30 g/L in this study.

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

9. TB-II (Transformation buffer II)

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

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

APPENDIX B

HPLC STANDARD CURVE

1. Standard curve of DL-lactic acid

For the standard curve, 2.0, 1.5, 1.0, 0.50, 0. 25 and 0.10 g/L of standard DL-lactic acid were applied in HPLC**.**

Table B1 DL-lactic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

DL-lactic acid concentration (g/L)	Peak area
0.10	2323097
0.25	5502485
0.50	11133362
1.00	21970144
1.50	32656214
2.00	45035488

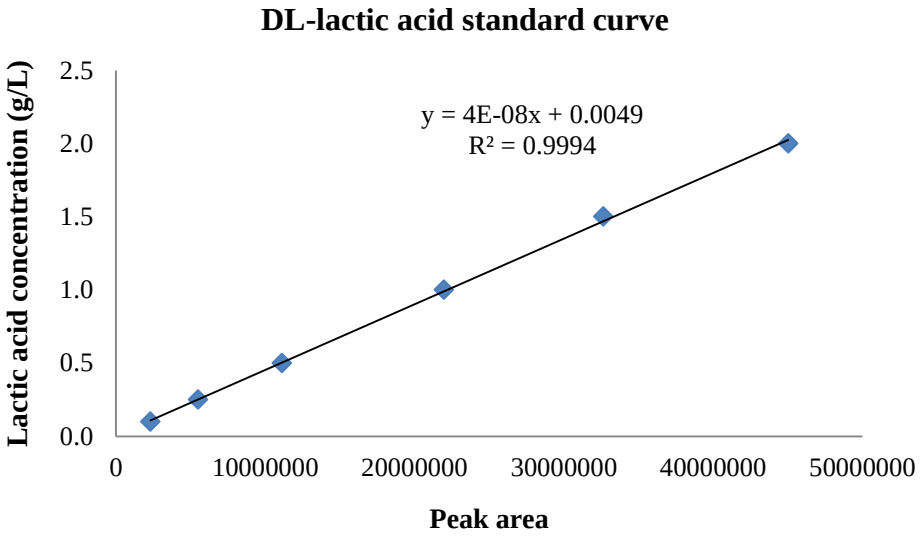

Figure B1 A Standard DL-lactic acid curve from HPLC by using Aminex HPX-87H column with RI detector for DL-lactic acid determination.

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

$$y = 4E\text{-}08x + 0.0049$$

which x was the peak area of each sample and y was the DL-lactic acid concentration in each sample. With this calculation, the average DL-lactic acid concentrations from JC13509, RB7, RB24, RB29, RB30 RB31 and THW1 strains (from 3 repeats of each set of experiment) are represented in Table C1-C10.

2. Standard curve of succinic acid

For the standard curve, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/L of standard succinic acid were applied in HPLC.

Table B2 Succinic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

Succinic acid concentration (g/L)	Peak area
0.03125	2016
0.0625	6419
0.125	12123
0.25	25759
0.5	55848
1.0	107602
2.0	209656

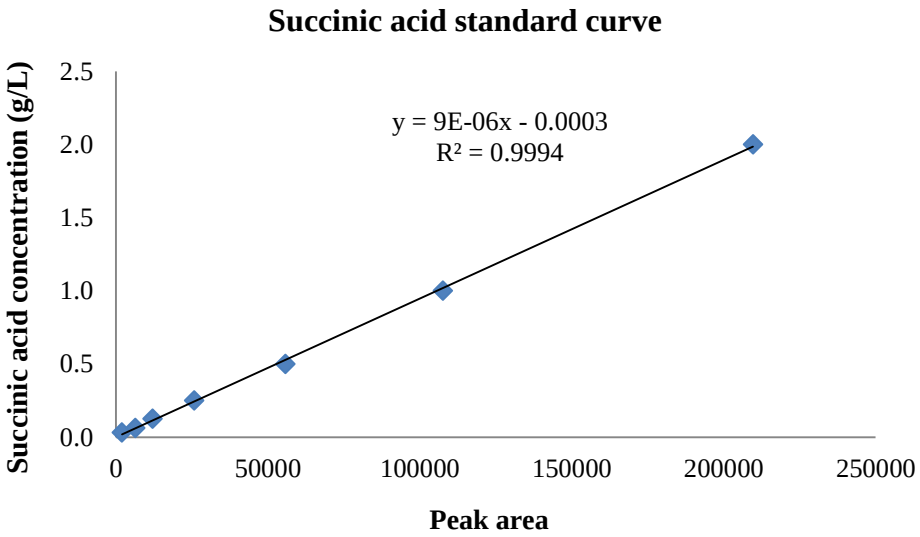

Figure B2 A standard succinic acid curve from HPLC by using Aminex HPX-87H column with RI detector for succinic acid determination.

The equation from the graph in Figure B2 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:

$$y = 9E\text{-}06x - 0.0003$$

which x was the peak area of each sample and y was the succinic acid concentration in each sample. With this calculation, the average succinic acid concentrations from JC13509, RB24 and THW1 strains are represented in Table C11.

3. Standard curve of acetic acid

For the standard curve, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/L of standard acetic acid were applied in HPLC.

Table B3 Acetic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

Acetic acid concentration (g/L)	Peak area
0.03125	849
0.0625	3811
0.125	13762
0.25	19742
0.5	41151
1.0	77699
2.0	147315

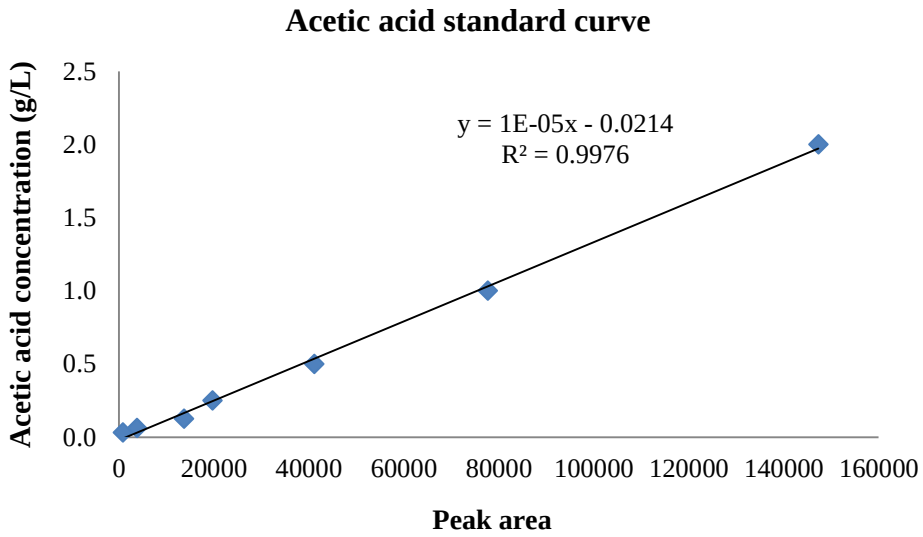

Figure B3 A standard acetic acid curve from HPLC by using Aminex HPX-87H column with RI detector for acetic acid determination.

The equation from the graph in Figure B3 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:

$$y = 1E\text{-}05x - 0.0214$$

which x was the peak area of each sample and y was the acetic acid concentration in each sample. With this calculation, the average acetic acid concentrations from JC13509, RB24 and THW1 strains are represented in Table C11.

4. Standard curve of ethanol

For the standard curve, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/L of standard ethanol were applied in HPLC.

Table B4 Ethanol concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

Ethanol concentration (g/L)	Peak area
0.03125	1011
0.0625	3422
0.125	5763
0.25	10156
0.5	27029
1.0	51759
2.0	102829

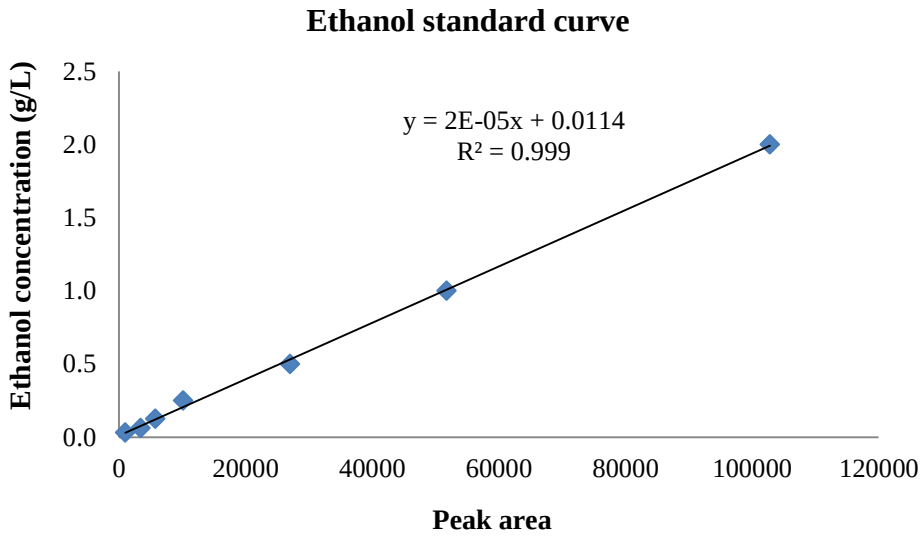

Figure B4 A standard ethanol curve from HPLC by using Aminex HPX-87H column with RI detector for ethanol determination.

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

$$y = 2E\text{-}05x + 0.0114$$

which x was the peak area of each sample and y was the ethanol concentration in each sample. With this calculation, the average ethanol concentrations from JC13509, RB24 and THW1 are represented in Table C11.

5. Standard curve of formic acid

For the standard curve, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/L of standard formic acid were applied in HPLC.

Table B5 Formic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

Formic acid concentration (g/L)	Peak area
0.03125	776
0.0625	1493
0.125	2986
0.25	6149
0.5	13437
1.0	24905

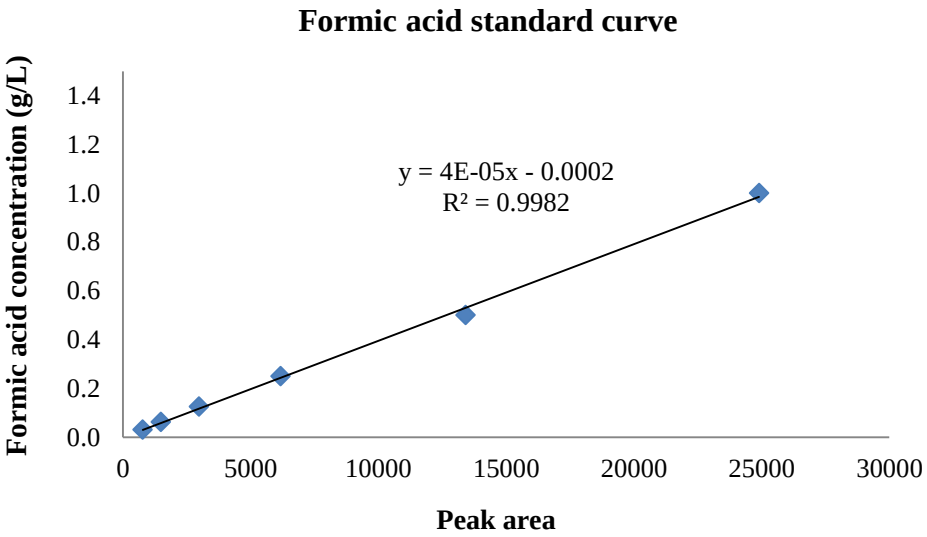

Figure B5 A standard formic acid curve from HPLC by using Aminex HPX-87H column with RI detector for formic acid determination**.**

The equation from the graph in Figure B5 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:

$$y = 4E\text{-}05x - 0.0002$$

which x was the peak area of each sample and y was the formic acid concentration in each sample. With this calculation, the average formic acid concentrations from JC13509, RB24 and THW1 strains are represented in Table C11.

6. Standard curve of residual glucose in medium culture

For the standard curve, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 g/L of standard glucose were applied in HPLC

Table B6 Glucose concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Aminex HPX-87H column with RI detector.

Glucose concentration (g/L)	Peak area
0.03125	3992
0.0625	8683
0.125	17375
0.25	34411
0.5	75216
1.0	146291
2.0	279532
4.0	590320

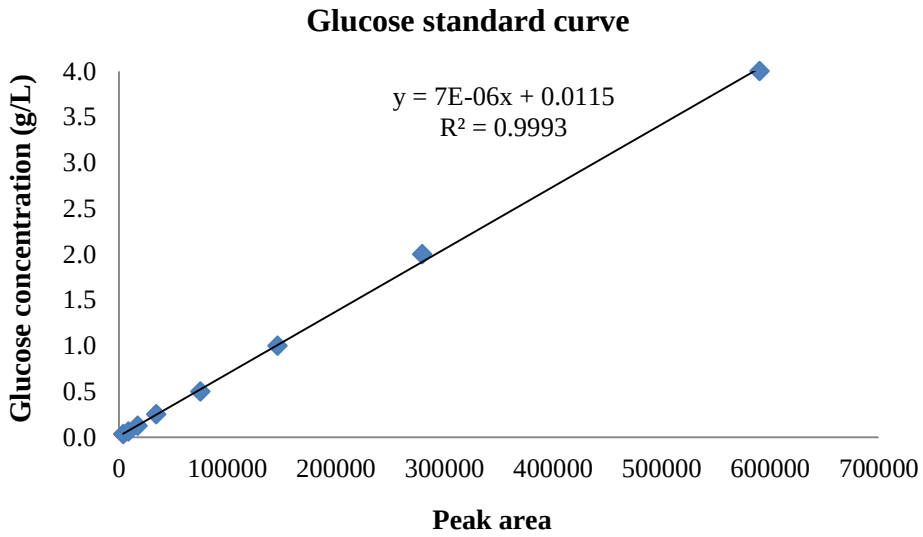

Figure B6 A standard glucose curve from HPLC by using Aminex HPX-87H column with RI detector for glucose determination.

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

$$y = 7E\text{-}06x + 0.0115$$

which x was the peak area of each sample and y was the residual glucose concentration in each sample. With this calculation, the average glucose concentrations from all strains are represented in Table C1-C11.

7. Standard curve of L(+)-lactic acid

For the standard curve, 2.0, 1.5, 1.0, 0.50, 0.25, 0.10 and 0.05 g/L of standard L(+)-lactic acid were applied in HPLC.

Table B7 L(+)-lactic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Sumi chiral 0A-5000L column with UV detector at 254 nm.

L(+)-lactic acid concentration (g/L)	Peak area
0.05	454907
0.10	670652
0.25	1798165
0.50	2617617
1.00	5472796
1.50	8076543
2.00	10814629

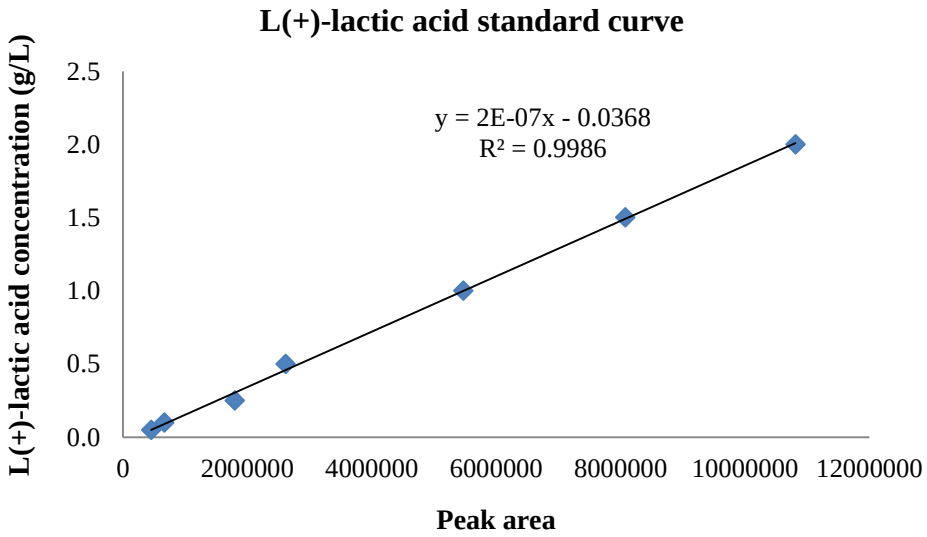

Figure B7 A standard L(+)-lactic acid curve from HPLC by using Sumi chiral 0A-5000L column with UV detector at 254 nm for L(+)-lactic acid determination

The equation from the graph in Figure B7 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:

$$y = 2E\text{-}07x - 0.0368$$

which x was the peak area of each sample and y was the L(+)-lactic acid concentration in each sample. With this calculation, the average L(+)-lactic acid concentrations from JC13509, RB24 and THW1 strains are represented in Table C11.

8. Standard curve of D(-)-lactic acid

For the standard curve, 2.0, 1.5, 1.0, 0.50, 0.25, 0.10 and 0.05 g/L of standard D(-)-lactic acid were applied in HPLC.

Table B8 D(-)-lactic acid concentration and peak area by high performance liquid chromatography (HPLC) measured by HPLC using Sumi chiral 0A-5000L column with UV detector at 254 nm.

D(-)-lactic acid concentration (g/L)	Peak area
0.05	515153
0.10	756693
0.25	2003667
0.50	2916220
1.00	6079535
1.50	8948894
2.00	11976627

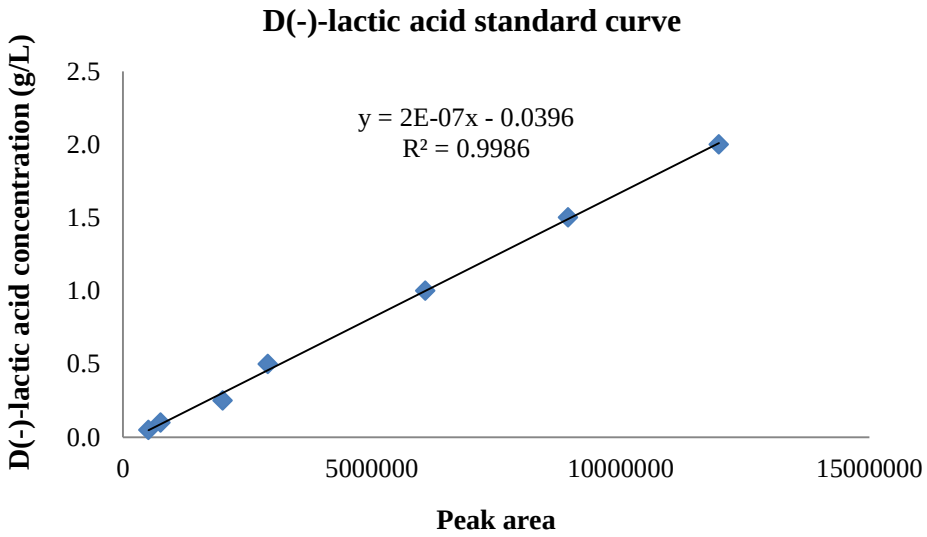

Figure B8 A standard D(-)-lactic acid curve from HPLC by using Sumi chiral 0A-5000L column with UV detector at 254 nm for D(-)-lactic acid determination

The equation from the graph in Figure B8 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:

$$y = 2E\text{-}07x - 0.0396$$

which x was the peak area of each sample and y was the D(-)-lactic acid concentration in each sample. With this calculation, the average D(-)-lactic acid concentrations from JC13509, RB24 and THW1 strains are represented in Table C11.

9. Retention time of fermentation products

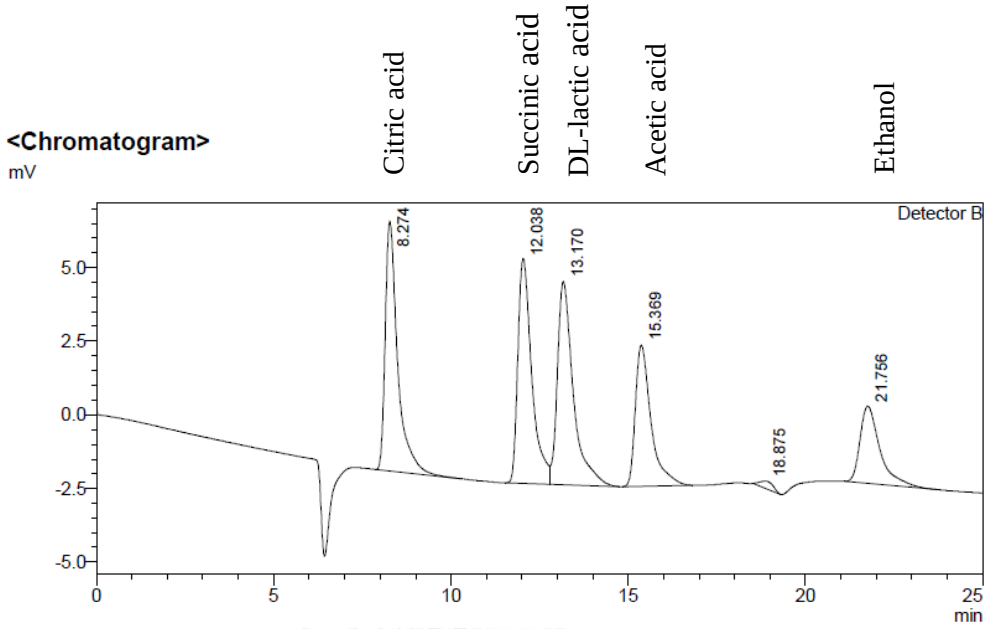

Figure B9 The chromatogram of acetic acid, citric acid, ethanol, DL-lactic acid and succinic acid by using a high performance liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

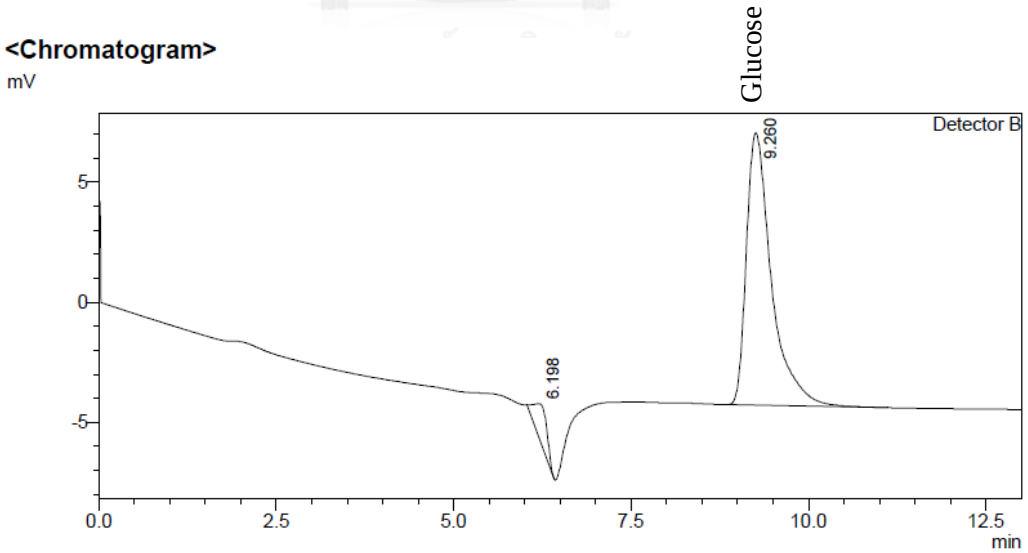

Figure B10 The chromatogram of glucose by using a high performance liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

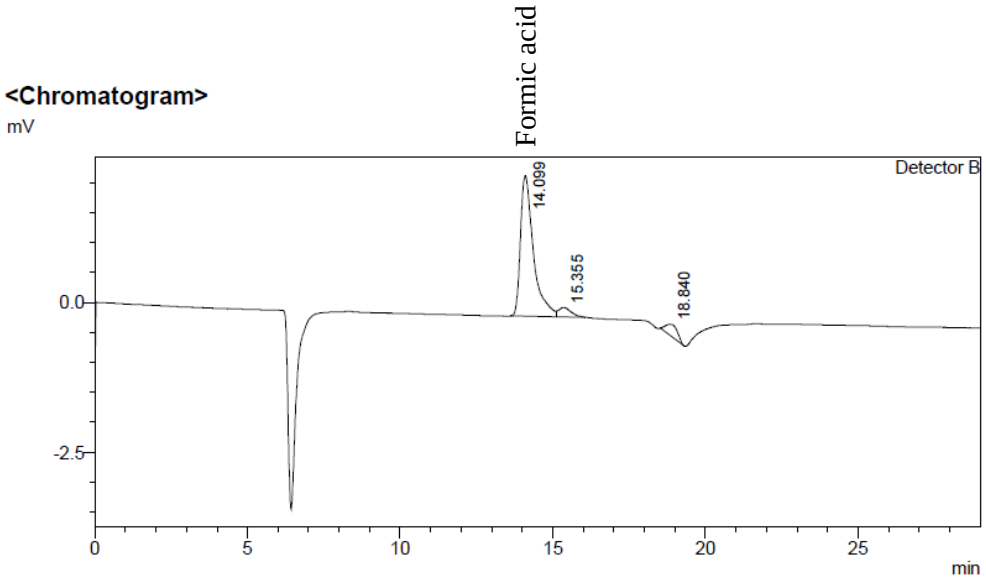

Figure B11 The chromatogram of formic acid by using a high performance liquid chromatography (HPLC) equipped with Animex HPX-87H column and IR detector.

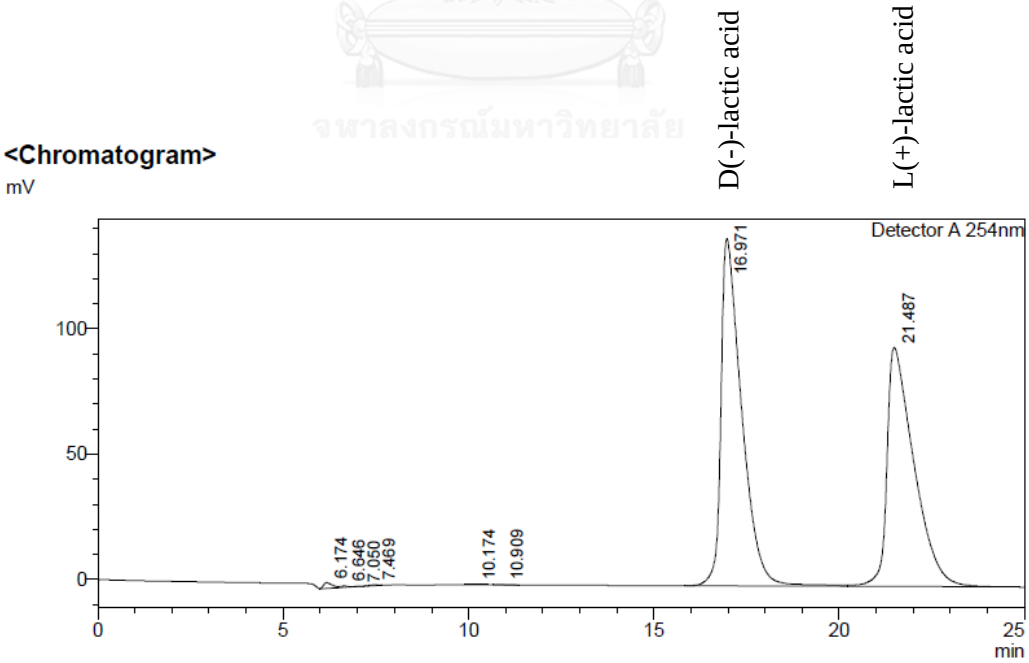

Figure B12 The chromatogram of D(-)-lactic acid and L(+)-lactic acid by using a high performance liquid chromatography (HPLC) equipped with Sumi chiral 0A-5000L column and UV detector at 254 nm.

Table B9 The retention time of each fermentation product by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and Sumi chiral 0A-5000L column.

Product	Retention time in Aminex column (min)	Retention time in Sumi chiral column (min)
Acetic acid	15.36-15.39	ND
Ethanol	21.75-21.77	ND
Formic acid	14.10-14.30	ND
DL-lactic acid	9.20-9.30	ND
D(-)-lactic acid	ND	16.90-17.20
L(+)-lactic acid	ND	21.40-21.80
Succinic acid	12.00-12.10	ND

APPENDIX C

LACTIC ACID AND RESIDUAL CONCENTRATIONS

Table C1 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using 56/2 minimal medium with various initial glucose concentrations under aerobic condition (37 ^{o}C, 200 rpm).

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.0	JC13509	0.00±0.00	0.01±0.01	0.01±0.01	0.01±0.01	1.58±0.64	0.46±0.40	0.18±0.16	0.00±0.00
	RB7	0.00±0.00	0.01±0.01	0.01±0.01	0.00±0.00	1.66±0.69	1.60±0.53	1.53±0.50	1.55±0.55
	RB24	0.00±0.00	0.01±0.01	0.01±0.01	0.00±0.00	1.76±0.24	1.40±0.20	0.99±0.86	0.96±0.84
	RB29	0.00±0.00	0.01±0.01	0.00±0.00	0.01±0.01	1.82±0.33	1.55±0.55	1.17±1.01	1.15±1.00
	RB30	0.00±0.00	0.01±0.01	0.01±0.01	0.01±0.01	1.79±0.23	0.00±0.00	0.00±0.00	0.00±0.00
	RB31	0.00±0.00	0.01±0.01	0.01±0.01	0.01±0.01	1.82±0.27	0.00±0.00	0.00±0.00	0.00±0.00
10.0	JC13509	0.03±0.01	0.02±0.02	0.03±0.03	0.04±0.03	9.76±0.50	7.41±0.21	5.16±0.77	3.28±1.37
	RB7	0.02±0.01	0.03±0.03	0.02±0.02	0.04±0.03	10.04±0.31	7.78±0.48	6.77±0.65	6.46±0.49
	RB24	0.02±0.01	0.07±0.02	0.06±0.05	0.11±0.04	10.20±0.41	7.41±0.99	6.32±0.92	5.85±0.57
	RB29	0.03±0.01	0.03±0.04	0.02±0.01	0.02±0.01	9.70±0.25	7.85±0.40	7.51±0.39	7.10±0.66
	RB30	0.02±0.01	0.04±0.04	0.03±0.02	0.03±0.03	9.97±0.40	7.83±0.50	7.31±0.51	6.93±0.63
	RB31	0.03±0.01	0.05±0.03	0.03±0.03	0.02±0.02	9.90±0.21	6.92±0.50	4.65±0.66	2.77±1.13
20.0	JC13509	0.03±0.01	0.04±0.02	0.03±0.02	0.04±0.02	20.17±0.32	13.00±0.44	11.50±0.89	10.55±0.78
	RB7	0.01±0.01	0.08±0.06	0.05±0.01	0.05±0.04	20.40±1.04	17.20±0.36	16.30±0.20	15.80±0.27
	RB24	0.01±0.01	0.13±0.09	0.10±0.11	0.15±0.02	19.27±0.35	15.47±1.07	15.10±1.67	13.80±1.45
	RB29	0.02±0.02	0.04±0.03	0.04±0.04	0.04±0.03	20.13±0.60	16.30±1.10	15.53±1.07	14.70±1.08
	RB30	0.03±0.01	0.07±0.01	0.02±0.01	0.02±0.01	20.90±1.04	16.63±0.21	16.03±0.31	14.93±0.72
	RB31	0.03±0.01	0.03±0.04	0.01±0.01	0.01±0.02	19.83±0.55	14.97±0.76	13.83±0.35	13.10±0.35
30.0	JC13509	0.01±0.01	0.06±0.04	0.06±0.02	0.04±0.02	29.53±1.07	22.00±0.66	20.40±0.36	19.33±0.57
	RB7	0.02±0.01	0.04±0.02	0.04±0.04	0.03±0.01	30.80±0.66	25.70±0.40	24.47±0.32	23.70±0.20
	RB24	0.03±0.01	0.14±0.09	0.13±0.05	0.12±0.08	28.93±1.24	24.87±1.19	23.73±0.86	22.63±1.05
	RB29	0.03±0.01	0.03±0.03	0.03±0.04	0.02±0.02	30.40±0.30	25.90±1.01	24.73±0.80	23.70±0.79
	RB30	0.02±0.01	0.06±0.03	0.04±0.03	0.02±0.02	29.93±0.51	25.00±0.27	23.77±0.74	22.33±1.19
	RB31	0.01±0.02	0.02±0.02	0.04±0.04	0.05±0.01	30.43±0.42	25.33±1.10	23.60±1.51	22.73±1.56
50.0	JC13509	0.03±0.02	0.04±0.01	0.06±0.01	0.06±0.05	49.45±0.88	46.83±0.90	44.60±0.70	39.70±1.15
	RB7	0.03±0.03	0.04±0.00	0.05±0.04	0.05±0.02	51.60±1.13	48.47±1.80	47.47±1.88	45.37±2.75
	RB24	0.04±0.01	0.03±0.03	0.06±0.02	0.06±0.02	50.03±2.06	47.40±2.70	46.40±2.59	45.67±2.80
	RB29	0.04±0.02	0.02±0.01	0.05±0.02	0.03±0.01	51.43±1.24	48.90±0.61	47.57±0.06	46.73±0.35
	RB30	0.03±0.02	0.02±0.02	0.03±0.03	0.03±0.01	51.77±2.38	49.40±1.74	48.43±2.05	50.70±5.15
	RB31	0.04±0.00	0.01±0.02	0.04±0.05	0.05±0.01	50.50±0.87	47.37±0.75	43.17±0.85	40.97±0.47

Table C2 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using 56/2 minimal medium with various initial glucose concentrations under limited oxygen condition (37 ^{o}C without shaking).

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.0	JC13509	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.91±0.03	0.00±0.00	0.00±0.00	0.00±0.00
	RB7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.96±0.03	1.78±0.14	1.75±0.11	1.72±0.09
	RB24	0.00±0.00	0.01±0.01	0.02±0.01	0.02±0.00	1.91±0.02	1.05±0.09	0.09±0.16	0.01±0.01
	RB29	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.87±0.05	1.13±0.25	0.25±0.15	0.10±0.10
	RB30	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.92±0.04	0.24±0.22	0.03±0.06	0.00±0.00
	RB31	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.93±0.04	0.29±0.14	0.00±0.00	0.00±0.00
10.0	JC13509	0.03±0.01	0.05±0.04	0.04±0.03	0.06±0.05	10.03±0.51	3.24±0.36	2.38±0.32	2.26±0.28
	RB7	0.01±0.00	0.04±0.04	0.04±0.03	0.06±0.02	10.80±0.40	6.78±0.57	6.12±0.27	5.91±0.26
	RB24	002±0.01	0.16±0.05	0.18±0.04	0.18±0.01	10.11±0.42	6.89±0.41	6.14±0.76	5.95±0.74
	RB29	0.02±0.01	0.03±0.01	0.05±0.02	0.08±0.02	10.00±0.54	6.84±0.45	6.43±0.36	6.25±0.38
	RB30	0.01±0.01	0.04±0.02	0.04±0.02	0.05±0.02	10.26±0.52	6.00±0.43	5.54±0.43	5.32±0.36
	RB31	0.02±0.01	0.04±0.04	0.02±0.01	0.01±0.02	10.04±0.42	5.63±0.72	5.19±0.48	4.92±0.55
20.0	JC13509	0.02±0.03	0.03±0.01	0.04±0.04	0.04±0.02	20.90±0.46	11.93±1.19	10.75±0.78	10.46±0.89
	RB7	0.02±0.00	0.05±0.04	0.02±0.01	0.04±0.02	20.17±0.55	15.97±0.67	14.77±0.90	14.33±0.84
	RB24	0.03±0.02	0.18±0.03	0.14±0.05	0.18±0.04	20.53±0.90	16.07±0.91	14.87±0.85	14.43±0.80
	RB29	0.01±0.01	0.03±0.03	0.02±0.01	0.03±0.2	20.20±0.46	16.60±0.46	15.47±0.86	14.80±0.53
	RB30	0.01±0.01	0.02±0.01	0.05±0.01	0.05±0.04	20.67±0.78	15.70±0.61	14.17±0.31	13.73±0.25
	RB31	0.02±0.02	0.02±0.02	0.03±0.02	0.03±0.01	19.87±0.60	15.03±0.71	13.30±0.56	12.80±0.44
30.0	JC13509	0.02±0.02	0.04±0.04	0.05±0.02	0.04±0.00	29.33±0.67	19.80±0.56	16.63±0.67	16.03±0.76
	RB7	0.01±0.01	0.02±0.02	0.02±0.02	0.04±0.02	29.53±0.80	24.67±0.55	22.90±0.66	22.40±0.60
	RB24	0.02±0.02	0.11±0.08	0.16±0.07	0.15±0.04	29.80±0.61	24.87±1.40	23.13±1.76	22.80±1.67
	RB29	0.02±0.02	0.02±0.01	0.02±0.01	0.04±0.02	30.40±0.53	25.23±1.36	23.77±2.22	23.03±2.60
	RB30	0.02±0.02	0.02±0.01	0.03±0.02	0.03±0.02	29.93±0.49	25.40±0.79	23.57±1.19	23.20±1.25
	RB31	0.03±0.02	0.02±0.00	0.02±0.02	0.03±0.03	29.50±0.82	24.87±1.10	22.40±0.66	21.60±0.90
50.0	JC13509	0.04±0.02	0.30±0.01	0.30±0.03	0.25±0.01	48.30±3.30	43.47±2.40	40.83±2.90	38.15±2.51
	RB7	0.03±0.02	0.08±0.02	0.09±0.01	0.10±0.03	47.93±1.50	46.07±2.78	44.40±1.47	42.43±1.58
	RB24	0.03±0.03	0.12±0.02	0.14±0.01	0.16±0.01	45.63±1.63	44.17±2.11	42.13±1.27	40.47±1.10
	RB29	0.01±0.00	0.08±0.02	0.12±0.01	0.15±0.03	45.93±2.17	44.87±2.55	43.13±2.06	42.77±0.90
	RB30	0.02±0.02	0.09±0.01	0.24±0.01	0.20±0.02	46.80±0.92	45.07±1.31	41.00±0.66	38.97±2.03
	RB31	0.06±0.01	0.07±0.01	0.19±0.01	0.20±0.01	46.73±1.24	45.50±0.36	41.80±1.21	40.27±1.19

Table C3 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using 56/2 minimal medium with various initial glucose concentrations under anaerobic condition (37 °C without shaking for 12 h, anaerobically).

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
2.0	JC13509	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.91±0.02	0.00±0.01	0.00±0.00	0.00±0.00
	RB7	0.00±0.00	0.00±0.01	0.00±0.00	0.00±0.00	1.96±0.06	1.78±0.21	1.75±0.13	1.72±0.16
	RB24	0.00±0.00	0.01±0.01	0.02±0.00	0.02±0.01	1.91±0.06	1.05±0.13	0.09±0.20	0.01±0.00
	RB29	0.00±0.00	0.00±0.00	0.00±0.01	0.00±0.00	1.87±0.03	1.13±0.10	0.25±0.21	0.10±0.37
	RB30	0.00±0.00	0.00±0.01	0.00±0.00	0.00±0.01	1.92±0.02	0.24±0.06	0.03±0.13	0.00±0.14
	RB31	0.00±0.00	0.00±0.01	0.00±0.00	0.00±0.01	1.93±0.03	0.29±0.09	0.00±0.08	0.00±0.10
10.0	JC13509	0.03±0.02	0.05±0.04	0.04±0.02	0.06±0.02	10.03±0.18	3.24±0.47	2.38±0.69	2.26±0.54
	RB7	0.01±0.01	0.04±0.02	0.04±0.02	0.06±0.03	10.80±0.57	6.78±0.98	6.12±0.64	5.91±0.82
	RB24	0.02±0.02	0.16±0.09	0.18±0.03	0.18±0.01	10.11±0.23	6.89±0.81	6.14±0.49	5.95±0.31
	RB29	0.02±0.02	0.03±0.02	0.05±0.02	0.08±0.01	10.00±0.42	6.84±0.35	6.43±0.37	6.25±0.29
	RB30	0.01±0.02	0.04±0.01	0.04±0.02	0.05±0.03	10.26±0.27	6.00±0.80	5.54±0.82	5.32±0.78
	RB31	0.02±0.01	0.04±0.03	0.02±0.01	0.01±0.02	10.04±0.47	5.63±0.65	5.19±0.50	4.92±0.55
20.0	JC13509	0.02±01	0.03±0.02	0.04±0.01	0.04±0.01	20.90±0.53	11.93±1.01	10.75±0.51	10.46±0.62
	RB7	0.02±0.02	0.05±0.01	0.02±0.01	0.04±0.01	20.17±0.42	15.97±1.38	14.77±1.53	14.33±1.40
	RB24	0.03±0.02	0.18±0.04	0.14±0.02	0.18±0.03	20.53±0.32	16.07±1.23	14.87±1.30	14.43±1.69
	RB29	0.01±0.01	0.03±0.04	0.02±0.01	0.03±0.01	20.20±0.38	16.60±1.14	15.47±0.67	14.80±0.78
	RB30	0.01±0.02	0.02±0.01	0.05±0.03	0.05±0.01	20.67±0.49	15.70±1.36	14.17±1.19	13.73±0.97
	RB31	0.02±0.01	0.02±0.02	0.03±0.02	0.03±0.02	19.87±0.70	15.03±0.46	13.30±0.81	12.80±0.67
30.0	JC13509	0.02±0.01	0.04±0.03	0.05±0.03	0.04±0.02	29.33±0.76	19.80±2.25	16.63±1.85	16.03±1.92
	RB7	0.01±0.02	0.02±0.01	0.02±0.03	0.04±0.02	29.53±0.60	24.67±1.10	22.90±0.76	22.40±0.91
	RB24	0.02±0.02	0.11±0.05	0.16±0.02	0.15±0.04	29.80±0.40	24.87±0.76	23.13±1.01	22.80±0.76
	RB29	0.02±0.01	0.02±0.01	0.02±0.01	0.04±0.01	30.40±0.32	25.23±1.12	23.77±1.37	23.03±1.31
	RB30	0.02±0.01	0.02±0.03	0.03±0.01	0.03±0.02	29.93±0.67	25.40±0.92	23.57±1.33	23.20±1.22
	RB31	0.03±0.02	0.02±0.04	0.02±0.03	0.03±0.01	29.50±0.30	24.87±0.59	22.40±1.32	21.60±1.62
50.0	JC13509	0.04±0.03	0.30±0.03	0.30±0.02	0.25±0.02	48.30±0.83	43.47±0.87	40.83±0.91	38.15±1.42
	RB7	0.03±0.01	0.08±0.03	0.09±0.02	0.10±0.02	47.93±1.07	46.07±0.80	44.40±1.06	42.43±0.52
	RB24	0.03±0.03	0.12±0.03	0.14±0.03	0.16±0.02	45.63±1.99	44.17±2.40	42.13±2.43	40.47±2.24
	RB29	0.01±0.04	0.08±0.04	0.12±0.02	0.15±0.04	45.93±2.66	44.87±2.43	43.13±2.38	42.77±1.72
	RB30	0.02±0.04	0.09±0.04	0.24±0.04	0.20±0.03	46.80±1.96	45.07±2.11	41.00±2.48	38.97±2.27
	RB31	0.06±0.02	0.07±0.02	0.19±0.05	0.20±0.04	46.73±2.94	45.50±2.61	41.80±2.55	40.27±2.85

Table C4 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using 56/2 minimal medium with various components under anaerobic condition (37 °C without shaking for 12 h, anaerobically).

Growth medium	*E. coli* strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
56/2 phosphate buffer + amino acid (arginine, thiamine, histidine and proline) + 1.0 % (v/v) glycerol	JC13509	0.00±0.00	0.00±0.00	0.01±0.01	0.01±0.01	ND	ND	ND	ND
	RB7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	ND	ND	ND	ND
	RB24	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	ND	ND	ND	ND
56/2 phosphate buffer + amino acid (arginine, thiamine, histidine and proline) + 20 g/L glucose	JC13509	0.00±0.00	0.01±0.01	0.03±0.02	0.04±0.02	19.37±1.27	17.14±1.36	13.82±1.22	10.46±2.54
	RB7	0.00±0.00	0.00±0.00	0.02±0.01	0.04±0.01	20.15±0.86	18.06±2.18	16.56±1.75	14.33±1.37
	RB24	0.01±0.00	0.00±0.00	0.07±0.04	0.18±0.07	19.66±0.55	18.31±0.94	15.94±2.19	14.43±1.61
fermentation medium + yeast extract + peptone + KH_2PO_4 + K_2HPO_4 + salt solution + 20 g/L glucose + 30 g/L $CaCO_3$	JC13509	0.00±0.00	0.01±0.01	0.04±0.03	0.05±0.02	20.43±0.74	15.62±0.77	11.67±1.53	8.27±1.85
	RB7	0.00±0.00	0.02±0.01	0.03±0.02	0.06±0.03	19.97±0.69	14.73±1.19	9.44±2.16	7.48±2.52
	RB24	0.00±0.00	0.02±0.01	0.08±0.03	0.12±0.05	20.51±0.62	14.58±1.46	9.83±1.72	6.71±2.38
fermentation medium + yeast extract + peptone + KH_2PO_4 + K_2HPO_4 + salt solution + 20 g/L glucose +10 g/L $CaCO_3$	JC13509	0.02±0.01	0.03±0.02	0.05±0.02	0.12±0.03	20.32±0.49	14.66±1.29	10.11±0.78	6.03±1.46
	RB7	0.00±0.00	0.02±0.02	0.06±0.03	0.10±0.04	20.65±0.94	13.45±0.68	9.84±2.35	7.36±1.25
	RB24	0.04±0.01	0.11±0.05	0.32±0.18	0.42±0.22	20.73±0.58	14.51±1.38	10.62±1.30	5.04±0.89

ND = Not determined

Table C5 Lactic acid fermentation with *E. coli* strains were fermented under aerobic condition (37 ^{o}C, 200 rpm) using fermentation medium with yeast extract as nitrogen source.

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
10.0	JC13509	0.03±0.04	0.02±0.02	0.02±0.02	0.02±0.02	10.03±0.24	4.62±0.13	0.07±0.07	0.00±0.00
	RB7	0.00±0.01	0.00±0.01	0.00±0.00	0.00±0.00	10.37±0.15	5.29±0.50	0.26±0.10	0.00±0.01
	RB24	0.04±0.05	0.03±0.02	0.05±0.03	0.13±0.12	10.34±0.47	4.78±0.80	0.15±0.16	0.02±0.03
	RB29	0.03±0.04	0.06±0.03	0.02±0.02	0.07±0.05	10.01±0.36	5.22±0.21	0.10±0.13	0.00±0.01
	RB30	0.00±0.00	0.00±0.02	0.02±0.02	0.02±0.03	10.31±0.35	5.12±0.23	0.20±0.24	0.04±0.04
	RB31	0.03±0.06	0.03±0.01	0.00±0.00	0.01±0.01	9.93±0.16	5.87±0.34	0.21±0.12	0.02±0.01
20.0	JC13509	0.04±0.04	0.17±0.02	0.16±0.04	0.18±0.03	20.13±0.31	13.60±0.92	0.97±0.46	0.17±0.29
	RB7	0.04±0.03	0.09±0.03	0.09±0.05	0.08±0.03	20.23±0.31	12.07±0.55	0.23±0.40	0.01±0.02
	RB24	0.04±0.03	0.22±0.03	0.22±0.04	0.24±0.04	20.60±0.20	12.47±0.35	1.82±3.15	0.11±0.13
	RB29	0.05±0.02	0.03±0.02	0.02±0.01	0.03±0.03	20.40±0.27	12.67±0.55	0.48±0.50	0.09±0.12
	RB30	0.07±0.05	0.03±0.02	0.04±0.03	0.05±0.06	20.37±0.49	12.60±0.10	0.88±0.14	0.03±0.04
	RB31	0.03±0.01	0.03±0.03	0.05±0.04	0.04±0.04	20.33±0.06	12.27±0.61	0.25±0.35	0.01±0.01
30.0	JC13509	0.02±0.01	0.02±0.02	0.02±0.02	0.02±0.02	29.37±0.42	19.17±1.21	12.70±1.42	9.98±0.30
	RB7	0.00±0.01	0.03±0.04	0.05±0.02	0.05±0.03	30.10±0.70	21.17±1.46	15.03±0.71	11.53±0.67
	RB24	0.03±0.03	0.06±0.05	0.06±0.04	0.06±0.06	29.97±0.21	19.87±1.10	14.77±0.91	9.72±1.09
	RB29	0.02±0.02	0.02±0.02	0.02±0.02	0.01±0.01	30.40±0.20	19.37±0.95	15.10±0.79	11.07±0.49
	RB30	0.01±0.01	0.01±0.02	0.03±0.02	0.03±0.04	29.90±0.36	19.83±1.60	15.47±0.91	11.87±1.54
	RB31	0.01±0.01	0.01±0.01	0.01±0.01	0.00±0.00	30.33±0.47	19.53±1.69	15.53±1.35	11.07±0.96
50.0	JC13509	0.01±0.01	0.03±0.02	0.04±0.03	0.04±0.03	49.43±1.62	40.33±2.69	33.10±4.36	26.57±5.58
	RB7	0.01±0.02	0.02±0.02	0.03±0.03	0.02±0.02	50.37±0.76	41.10±1.61	33.57±6.31	29.10±1.39
	RB24	0.06±0.07	0.10±0.15	0.13±0.15	0.16±0.19	50.27±1.33	40.97±3.09	34.20±3.35	26.43±3.34
	RB29	0.00±0.00	0.02±0.02	0.02±0.01	0.05±0.01	50.43±0.32	40.87±1.68	32.93±2.20	25.93±3.40
	RB30	0.06±0.05	0.04±0.08	0.07±0.10	0.13±0.21	49.63±0.45	40.83±1.24	35.93±1.23	28.40±3.90
	RB31	0.01±0.01	0.02±0.01	0.01±0.02	0.02±0.02	51.00±0.62	41.07±0.64	34.37±2.95	27.50±2.59
70.0	JC13509	0.08±0.11	0.45±0.63	0.91±1.41	0.98±1.54	71.47±0.65	62.27±0.49	55.60±2.11	45.87±2.88
	RB7	0.09±0.10	0.32±0.39	0.49±0.63	0.56±0.74	71.57±2.50	62.10±0.53	56.63±2.30	44.80±1.05
	RB24	0.22±0.07	0.83±0.44	2.14±1.05	2.43±1.15	70.63±0.91	60.30±0.61	52.23±1.68	42.33±0.25
	RB29	0.02±0.01	0.01±0.01	0.02±0.02	0.05±0.05	70.90±0.89	61.93±0.90	54.03±2.35	43.83±3.27
	RB30	0.08±0.08	0.25±0.10	0.34±0.16	0.39±0.20	70.20±0.76	58.30±1.38	49.77±2.63	39.50±2.78
	RB31	0.01±0.02	0.05±0.05	0.08±0.07	0.09±0.07	70.63±1.38	60.70±0.95	53.27±1.82	43.77±0.81

Table C6 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using fermentation broth with various initial glucose concentrations under limited oxygen condition (37 ^{o}C without shaking).

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
10.0	JC13509	0.02±0.02	0.01±0.02	0.10±0.10	0.44±0.41	10.15±0.27	7.70±0.34	2.77±0.66	0.24±0.41
	RB7	0.02±0.03	0.05±0.03	0.11±0.10	0.14±0.12	9.76±0.30	7.71±0.44	3.13±0.92	0.38±0.61
	RB24	0.08±0.04	0.35±0.17	1.52±0.82	1.64±0.85	9.53±0.18	7.49±0.30	2.70±0.35	0.01±0.02
	RB29	0.00±0.01	0.05±0.03	0.12±0.07	0.32±0.21	10.20±0.37	7.85±0.60	2.37±0.53	0.21±0.17
	RB30	0.01±0.02	0.03±0.04	0.07±0.06	0.22±0.15	9.84±0.39	7.54±0.31	2.29±0.74	0.12±0.21
	RB31	0.03±0.04	0.01±0.02	0.05±0.05	0.14±0.13	9.88±0.22	7.74±0.57	2.26±0.68	0.01±0.02
20.0	JC13509	0.16±0.06	0.24±0.07	0.26±0.10	0.31±0.13	20.63±0.47	15.60±0.89	9.55±0.86	3.80±3.29
	RB7	0.17±0.06	0.69±0.35	1.12±0.58	1.39±0.62	19.90±0.52	14.87±1.36	10.81±1.94	3.48±0.54
	RB24	0.20±0.06	0.96±0.25	1.74±0.39	2.02±0.32	20.20±0.82	17.20±0.40	13.43±0.47	4.25±1.07
	RB29	0.03±0.03	0.13±0.13	0.14±0.10	0.14±0.13	20.33±0.25	15.37±1.56	10.69±1.37	4.09±1.22
	RB30	0.04±0.04	0.08±0.09	0.16±0.14	0.19±0.19	20.27±0.55	13.93±0.55	9.25±0.26	2.88±0.55
	RB31	0.02±0.02	0.09±0.08	0.11±0.12	0.13±0.11	20.47±0.21	16.87±0.31	11.28±1.76	4.02±0.57
30.0	JC13509	0.16±0.14	0.41±0.47	1.23±0.92	1.52±0.35	29.53±0.59	19.70±3.06	14.15±3.81	9.18±4.42
	RB7	0.14±0.06	0.11±0.10	0.15±0.13	0.11±0.10	29.60±0.80	21.00±0.70	17.50±0.90	3.10±3.10
	RB24	0.18±0.02	0.34±0.19	0.51±0.33	0.60±0.41	27.70±0.72	20.20±1.45	15.53±0.49	0.53±0.53
	RB29	0.04±0.03	0.08±0.04	0.12±0.11	0.11±0.13	28.73±0.76	21.60±0.92	18.20±0.79	0.95±0.95
	RB30	0.03±0.03	0.06±0.09	0.08±0.13	0.09±0.12	28.86±1.56	20.23±0.74	17.03±1.76	1.07±1.07
	RB31	0.02±0.03	0.03±0.02	0.01±0.01	0.01±0.02	28.30±0.79	20.60±0.90	17.40±0.78	0.47±0.47
50.0	JC13509	0.22±0.01	0.14±0.10	0.21±0.01	0.15±0.15	51.50±0.20	44.90±0.70	37.33±0.83	31.00±2.31
	RB7	0.11±0.03	0.11±0.08	0.12±0.01	0.11±0.11	51.27±0.67	44.07±1.10	40.27±0.76	29.83±4.02
	RB24	0.22±0.01	0.25±0.18	0.26±0.13	0.27±0.27	51.17±0.15	43.57±0.86	38.03±4.29	31.00±4.29
	RB29	0.08±0.05	0.09±0.12	0.12±0.13	0.07±0.07	51.37±0.55	43.87±0.35	37.17±1.75	28.77±2.45
	RB30	0.09±0.07	0.04±0.04	0.01±0.01	0.02±0.02	51.23±0.31	44.53±1.07	37.87±1.78	32.67±2.27
	RB31	0.10±0.05	0.06±0.09	0.08±0.09	0.11±0.11	51.03±0.98	43.90±1.38	36.17±3.23	30.73±3.08
70.0	JC13509	0.13±0.10	0.11±0.14	0.17±0.14	0.16±0.14	70.87±1.22	63.27±1.27	58.27±2.35	51.77±2.49
	RB7	0.09±0.03	0.17±0.13	0.20±0.18	0.20±0.20	70.63±1.00	64.20±0.66	58.30±1.23	50.87±0.65
	RB24	0.22±0.01	0.36±0.09	0.41±0.10	0.54±0.17	71.47±0.15	64.73±0.91	59.50±2.07	51.70±3.34
	RB29	0.10±0.06	0.12±0.09	0.15±0.11	0.16±0.15	70.90±0.40	63.90±0.82	57.67±2.22	51.00±1.77
	RB30	0.11±0.04	0.04±0.03	0.06±0.06	0.06±0.10	70.63±0.86	63.93±0.15	61.23±1.56	54.17±1.88
	RB31	0.06±0.04	0.04±0.02	0.07±0.07	0.10±0.03	70.63±0.55	63.27±1.56	58.27±3.45	52.13±1.99

Table C7 Lactic acid and residual glucose concentrations when *E. coli* strains were fermented by using fermentation broth with various initial glucose concentrations under anaerobic condition (37 °C without shaking for 12 h, anaerobically).

Initial glucose (g/L)	Strain	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
10.0	JC13509	0.02±0.02	0.06±0.04	0.10±0.10	0.14±0.12	9.57±0.12	6.61±0.20	3.15±0.10	0.09±0.11
	RB7	0.01±0.01	0.05±0.04	0.06±0.06	0.08±0.09	9.57±0.22	7.15±0.24	2.74±0.67	1.04±1.21
	RB24	0.16±0.02	0.87±0.06	1.61±0.16	1.80±0.19	9.39±0.24	7.51±0.46	2.08±0.74	0.49±0.38
	RB29	0.04±0.04	0.11±0.12	0.15±0.19	0.23±0.23	9.35±0.14	7.40±0.12	2.26±0.60	0.89±0.69
	RB30	0.01±0.01	0.05±0.07	0.07±0.09	0.12±0.12	9.46±0.19	7.27±0.52	2.43±0.57	0.38±0.49
	RB31	0.01±0.01	0.02±0.03	0.05±0.05	0.09±0.09	9.49±0.14	7.11±0.38	1.69±0.91	0.56±0.51
20.0	JC13509	0.20±0.01	0.18±0.07	0.21±0.02	0.14±0.05	20.10±0.61	14.77±0.55	9.29±0.01	2.35±1.04
	RB7	0.04±0.03	0.08±0.06	0.10±0.10	0.14±0.12	20.50±0.53	16.57±0.85	12.13±2.46	6.78±2.09
	RB24	0.20±0.01	0.65±0.73	5.84±1.84	6.65±2.00	21.03±0.60	12.26±4.47	8.45±5.03	3.37±3.38
	RB29	0.03±0.04	0.02±0.01	0.05±0.04	0.17±0.12	20.00±0.36	15.47±1.00	11.42±1.87	6.11±3.45
	RB30	0.02±0.01	0.05±0.05	0.07±0.08	0.12±0.11	20.63±0.67	13.90±0.70	10.17±0.64	4.04±1.12
	RB31	0.02±0.03	0.02±0.02	0.04±0.02	0.28±0.19	20.00±0.53	14.03±0.31	9.65±0.24	2.11±1.10
30.0	JC13509	0.02±0.02	0.09±0.11	0.08±0.11	0.10±0.09	30.03±0.40	22.03±1.22	15.43±2.83	9.32±4.27
	RB7	0.07±0.08	0.14±0.10	0.20±0.02	0.23±0.12	29.17±0.42	22.83±1.50	17.67±0.92	11.56±1.56
	RB24	0.23±0.03	0.79±0.69	5.88±2.77	7.45±2.78	29.43±0.31	21.27±2.16	17.73±2.64	14.13±3.53
	RB29	0.03±0.01	0.07±0.07	0.08±0.09	0.08±0.07	29.20±1.25	20.97±0.57	16.87±1.99	11.55±1.69
	RB30	0.03±0.05	0.10±0.11	0.12±0.16	0.20±0.13	29.77±0.71	22.87±0.60	17.37±0.96	13.40±1.38
	RB31	0.02±0.03	0.09±0.07	0.08±0.11	0.06±0.10	29.07±0.49	21.93±1.31	17.50±1.21	11.94±1.84
50.0	JC13509	0.10±0.07	0.10±0.06	0.11±0.03	0.11±0.10	50.30±0.46	44.33±0.72	35.90±0.66	29.67±2.12
	RB7	0.10±0.02	0.13±0.03	0.15±0.03	0.11±0.06	49.56±0.76	43.70±1.05	39.63±1.00	31.70±1.47
	RB24	0.18±0.04	0.68±0.11	1.14±0.26	1.41±0.44	51.63±1.01	43.33±0.81	40.30±1.90	32.37±2.07
	RB29	0.07±0.04	0.05±0.02	0.10±0.06	0.09±0.04	50.27±0.80	44.87±0.35	40.13±1.08	34.57±4.08
	RB30	0.01±0.01	0.06±0.07	0.06±0.04	0.07±0.09	50.87±0.55	44.83±0.68	39.87±3.16	34.63±2.39
	RB31	0.02±0.02	0.09±0.10	0.11±0.12	0.10±0.10	51.13±1.42	44.23±1.27	40.53±2.91	34.03±4.12
70.0	JC13509	0.05±0.03	0.12±0.06	0.18±0.04	0.16±0.04	70.47±0.55	64.47±0.95	57.73±1.33	53.43±1.37
	RB7	0.11±0.11	0.40±0.35	0.84±0.97	0.97±1.14	70.50±0.36	63.70±0.92	56.77±1.85	53.70±1.10
	RB24	0.20±0.70	0.75±0.07	1.46±0.26	1.71±0.40	70.10±0.70	63.57±0.93	59.93±1.55	55.40±0.90
	RB29	0.03±1.12	0.07±0.06	0.04±0.04	0.08±0.08	70.93±1.12	62.43±0.85	54.23±3.42	49.20±4.16
	RB30	0.03±0.45	0.07±0.10	0.12±0.12	0.15±0.15	69.83±0.45	63.13±1.27	57.70±0.87	51.87±1.44
	RB31	0.01±0.97	0.08±0.06	0.12±0.06	0.10±0.08	69.97±0.97	63.50±0.99	57.07±3.09	50.77±3.67

Table C8 Lactic acid and residual glucose concentration of *E. coli* strains harboring different plasmid types under aerobic condition (37 ^{o}C, 200 rpm).

Strain	Initial glucose (g/L)	Lactic acid (g/L)				Residual glucose (g/L)			
		0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
JC13509	10	0.03±0.04	0.02±0.02	0.02±0.02	0.02±0.02	10.03±0.24	4.62±0.13	0.07±0.07	0.00±0.00
	20	0.04±0.04	0.17±0.02	0.16±0.04	0.18±0.03	20.13±0.31	13.60±0.92	0.97±0.46	0.17±0.29
	30	0.02±0.01	0.02±0.02	0.02±0.02	0.02±0.02	29.37±0.42	19.17±1.21	12.70±1.42	9.98±0.30
	50	0.01±0.01	0.03±0.02	0.04±0.03	0.04±0.03	49.43±1.62	40.33±2.69	33.10±4.36	26.57±5.58
	70	0.08±0.11	0.45±0.63	0.91±1.41	0.98±1.54	71.47±0.65	62.27±0.49	55.60±2.11	45.87±2.88
RB24	10	0.04±0.05	0.03±0.02	0.05±0.03	0.13±0.12	10.34±0.47	4.78±0.80	0.15±0.16	0.02±0.03
	20	0.04±0.03	0.22±0.03	0.22±0.04	0.24±0.04	20.60±0.20	12.47±0.35	1.82±3.15	0.11±0.13
	30	0.03±0.03	0.06±0.05	0.06±0.04	0.06±0.06	29.97±0.21	19.87±1.10	14.77±0.91	9.72±1.09
	50	0.06±0.07	0.10±0.15	0.13±0.15	0.16±0.19	50.27±1.33	40.97±3.09	34.20±3.35	26.43±3.34
	70	0.22±0.07	0.83±0.44	2.14±1.05	2.43±1.15	70.63±0.91	60.30±0.61	52.23±1.68	42.33±0.25
THW1	10	0.00±0.00	0.02±0.02	0.02±0.02	0.01±0.00	10.80±0.79	5.27±0.49	0.08±0.07	0.00±0.00
	20	0.00±0.01	0.01±0.02	0.04±0.03	0.06±0.07	20.73±0.59	11.47±1.11	1.72±0.75	0.00±0.00
	30	0.00±0.00	0.02±0.02	0.04±0.03	0.05±0.03	30.80±0.82	20.13±0.57	14.77±0.55	9.76±2.06
	50	0.01±0.01	0.04±0.02	0.04±0.02	0.04±0.03	50.30±0.99	39.90±1.77	34.20±1.40	30.43±1.46
	70	0.02±0.01	0.03±0.02	0.50±0.31	0.69±0.34	71.27±0.42	60.87±1.55	54.10±1.41	43.83±2.07

Table C9 Lactic acid and residual glucose concentration of *E. coli* strains harboring different plasmid types under limited oxygen condition (37 ^{o}C without shaking).

Limited oxygen condition									
Strain	**Initial glucose (g/L)**	**Lactic acid (g/L)**				**Residual glucose (g/L)**			
		0 h	**12 h**	**24 h**	**48 h**	**0 h**	**12 h**	**24 h**	**48 h**
JC13509	**10**	0.02±0.02	0.01±0.02	0.10±0.10	0.44±0.41	10.15±0.27	7.70±0.34	2.77±0.66	0.24±0.41
	20	0.16±0.06	0.24±0.07	0.26±0.10	0.31±0.13	20.63±0.47	15.60±0.89	9.55±0.86	3.80±3.29
	30	0.16±0.14	0.41±0.47	1.52±0.35	1.52±0.35	29.53±0.59	19.70±3.06	14.15±3.81	9.18±4.42
	50	0.22±0.01	0.14±0.10	0.21±0.01	0.15±0.15	51.50±0.20	44.90±0.70	37.33±0.83	31.00±2.31
	70	0.13±0.10	0.11±0.14	0.17±0.14	0.16±0.14	70.87±1.22	63.27±1.27	58.27±2.35	51.77±2.49
RB24	**10**	0.08±0.04	0.35±0.17	1.52±0.82	1.64±0.85	9.53±0.18	7.49±0.30	2.70±0.35	0.01±0.02
	20	0.20±0.06	0.96±0.25	1.74±0.39	2.02±0.32	20.20±0.82	17.20±0.40	13.43±0.47	4.25±1.07
	30	0.18±0.02	0.34±0.19	0.51±0.33	0.60±0.41	27.70±0.72	20.20±1.45	15.53±0.49	0.53±0.53
	50	0.22±0.01	0.25±0.18	0.26±0.13	0.27±0.27	51.17±0.15	43.57±0.86	38.03±4.29	31.00±4.29
	70	0.22±0.01	0.36±0.09	0.41±0.10	0.54±0.17	71.47±0.15	64.73±0.91	59.50±2.07	51.70±3.34
THW1	**10**	0.01±0.01	0.04±0.04	0.05±0.02	0.08±0.09	10.14±0.58	7.69±0.16	3.21±0.54	1.03±0.06
	20	0.02±0.02	0.76±0.54	1.52±0.51	1.91±0.64	20.17±0.42	14.87±0.40	9.44±0.75	2.30±0.89
	30	0.12±0.04	0.37±0.23	1.29±0.47	1.60±0.40	29.73±0.35	20.47±0.86	16.63±0.83	12.03±1.02
	50	0.16±0.03	0.33±0.06	0.48±0.14	0.68±0.19	51.03±0.70	44.70±0.87	38.30±1.57	32.53±1.26
	70	0.18±0.04	0.28±0.10	0.39±0.12	0.61±0.23	71.00±0.53	64.80±1.23	59.43±0.91	55.60±1.30

Table C10 Lactic acid and residual glucose concentration of *E. coli* strains harboring different plasmid types under anaerobic condition (37 °C without shaking for 12 h, anaerobically).

Anaerobic condition									
Strain	**Initial glucose (g/L)**	**Lactic acid (g/L)**				**Residual glucose (g/L)**			
		0 h	**12 h**	**24 h**	**48 h**	**0 h**	**12 h**	**24 h**	**48 h**
JC13509	**10**	0.02±0.02	0.06±0.04	0.10±0.10	0.14±0.12	9.57±0.12	6.61±0.20	3.15±0.10	0.09±0.11
	20	0.20±0.01	0.18±0.07	0.21±0.02	0.14±0.05	20.10±0.61	9.29±0.01	2.35±1.04	1.06±0.30
	30	0.02±0.02	0.09±0.11	0.08±0.11	0.10±0.09	30.03±0.40	22.03±1.22	15.43±2.83	9.32±4.27
	50	0.10±0.07	0.10±0.06	0.11±0.03	0.11±0.10	50.30±0.46	44.33±0.72	35.90±0.66	29.67±2.12
	70	0.05±0.03	0.12±0.06	0.18±0.04	0.16±0.04	70.47±0.55	64.47±0.95	57.73±1.33	53.43±1.37
RB24	**10**	0.16±0.02	0.87±0.06	1.61±0.16	1.80±0.19	9.39±0.24	7.51±0.46	2.08±0.74	0.49±0.38
	20	0.20±0.01	0.65±0.73	5.84±1.84	6.65±2.00	21.03±0.60	12.26±4.47	8.45±5.03	3.37±3.38
	30	0.23±0.03	0.79±0.69	5.88±2.77	7.45±2.78	29.43±0.31	21.27±2.16	17.73±2.64	14.13±3.53
	50	0.18±0.04	0.68±0.11	1.14±0.26	1.41±0.44	51.63±1.01	43.33±0.81	40.30±1.90	32.37±2.07
	70	0.20±0.70	0.75±0.07	1.46±0.26	1.71±0.40	70.10±0.70	63.57±0.93	59.93±1.55	55.40±0.90
THW1	**10**	0.17±0.04	0.39±0.08	0.83±0.15	1.07±0.21	9.89±0.18	6.02±0.62	2.55±0.54	0.36±0.13
	20	0.25±0.04	1.34±0.24	3.66±0.82	4.51±0.77	20.27±0.45	14.10±1.41	8.66±1.44	1.23±1.22
	30	0.18±0.03	0.37±0.14	0.53±0.19	0.78±0.30	29.90±0.70	20.23±2.25	12.83±1.29	9.30±1.28
	50	0.22±0.04	0.86±0.34	1.38±0.55	1.91±0.72	51.93±0.42	42.50±2.60	33.43±2.40	27.30±2.69
	70	0.26±0.05	0.73±0.15	1.42±0.42	1.73±0.40	70.87±0.60	60.20±3.82	54.10±5.67	54.10±2.61

Table C11 Concentrations of fermentation products after 48 h fermentation under anaerobic condition with initial glucose concentrations of 20 g/L and 30 g/L.

Initial glucose (g/L)	Strain	L(+)-lactic acid (g/L)	D(-)-lactic acid (g/L)	Formic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Residual glucose (g/L)
20	**JC13509**	0.23±0.12	12.81±1.04	0.00±0.00	4.43±0.46	0.66±0.35	1.50±0.46	0.25±0.14
	RB24	1.80±0.58	2.55±0.78	0.00±0.00	1.52±0.73	2.81±0.73	2.46±0.52	10.43±1.28
	THW1	1.60±0.39	5.60±0.57	0.00±0.00	0.07±0.04	2.81±0.69	0.41±0.16	4.73±1.47
30	**JC13509**	0.20±0.09	12.27±1.35	0.00±0.00	5.08±0.79	2.40±0.88	1.53±0.63	9.44±1.63
	RB24	1.95±0.62	3.23±0.53	0.00±0.00	1.56±0.46	3.38±0.74	3.69±0.71	15.10±2.48
	THW1	1.70±0.41	5.90±0.77	0.00±0.00	0.05±0.04	2.97±0.83	0.55±0.32	13.95±1.35

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Academic Presentation;

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