การปรับปรุงทางพันธุกรรมของ *Escherichia coli* เพื่อผลิตกรดแอล-แลกติก

นายวรรธนัย กาญจนพัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

GENETIC MODIFICATION OF *Escherichia coli* FOR L-LACTIC ACID PRODUCTION

Mr. Wattanai Kanjanapattanakul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2016

Thesis Title	GENETIC MODIFICATION OF *Escherichia coli* FOR L-LACTIC ACID PRODUCTION
By	Mr. Wattanai Kanjanapattanakul
Field of Study	Biotechnology
Thesis Advisor	Ruethairat Boonsombat

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

.....Dean of the Faculty of Science

(Associate Professor Polkit Sangvanich)

THESIS COMMITTEE

.....Chairman

(Associate Professor Nattaya Ngamrojanavanich)

.....Thesis Advisor

(Ruethairat Boonsombat)

.....Examiner

(Assistant Professor Sanit Piyapattanakorn)

.....External Examiner

(Wanilada Rungrassamee)

วรรธนัย กาญจนพัฒนกุล : การปรับปรุงทางพันธุกรรมของ *Escherichia coli* เพื่อผลิตกรดแอล-แลกติก (GENETIC MODIFICATION OF *Escherichia coli* FOR L-LACTIC ACID PRODUCTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ฤทัยรัตน์ บุญสมบัติ, 80 หน้า.

กรดแลกติกเป็นสารประกอบอินทรีย์ที่พบได้ในธรรมชาติซึ่งถูกนำมาใช้อย่างกว้างขวางในอุตสาหกรรมต่างๆ ทำให้ความต้องการของกรดแลกติกมีแนวโน้มที่จะเพิ่มสูงขึ้นอีกในอนาคต จึงได้เกิดการพัฒนาสายพันธุ์ของจุลินทรีย์ที่มีประสิทธิภาพในการผลิตกรดแลกติก และคุ้มค่าในการผลิต กรดแล็กติก สามารถผลิตได้จากการหมักของจุลินทรีย์เช่น แบคทีเรียผลิตกรดแลกติกและ เชื้อรา ในปัจจุบันเชื้อรา *Rhizopus oryzae* กำลังเป็นที่น่าสนใจเนื่องจากมันสามารถผลิตกรดแอล-แลกติกไอโซเมอร์แอลบริสุทธิ์ได้ แต่อย่างไรก็ตาม ในระหว่างการหมักโดยไม่มีอุปกรณ์เสริมการควบคุมลักษณะสัณฐานวิทยาของเชื้อราพันธุ์นี้จะสามารถทำได้ยาก เนื่องจากไมซีเลียมของราสามารถก่อให้เกิดปัญหากับถังปฏิกรณ์ชีวภาพแบบกวนได้ ในการที่จะแก้ปัญหาเหล่านี้ เทคนิคในการตัดต่อพันธุกรรมจึงถูกนำมาใช้เพื่อปรับปรุงสายพันธุ์ *Escherichia coli* ในโครงการวิจัยนี้จะใช้เทคนิกการแทนที่ยีน และการใช้พาหะที่แตกต่างกัน ตามขีดจำกัดของเทคนิกการแทนที่ยีนแล้ว การออกแบบชิ้นส่วนดีเอ็นเอสำหรับการผลิตกรดแอล-แลกติกจะประกอบด้วยส่วน open reading frame (ORF) ของยีน *ldhA* จาก *R. oryzae* ขนาบข้างโดยส่วน upstream และ downstream ของยีน *ldhA* จาก *E. coli* และวางแผนที่จะแทนที่ยีน *ldhA* บนโครโมโซมของ *E. coli* โดยการส่งถ่ายชิ้นส่วนยีนเข้าสู่ผู้รับด้วยพลาสมิด PKD46 อย่างไรก็ตามในการสร้างชิ้นส่วนดีเอ็นเอเพื่อการแทนที่ยีนนั้นไม่ประสบผลสำเร็จ สำหรับการใช้พาหะที่แตกต่างกันพลาสมิด pUC19 และ pBluescript II KS(+) จะถูกนำมาใช้ให้รับยีน *ldhA* จาก *R. oryzae* ทำการหมักเชื้อ *E. coli* ที่ดัดแปลงจากวิธีการทั้งสองด้วยอาหารเลี้ยงเชื้อที่มีกลูโคส 2% ที่ 37 ^{0}C ภายใต้สภาวะที่ไม่มีออกซิเจน เป็นเวลา 48 ชั่วโมง เชื้อ *E. coli* สายพันธุ์ TW2 ซึ่งมีพาหะ pUC19 ซึ่งมียีน *ldhA* จากเชื้อรา *R. oryzae* ประสบผลสำเร็จในการผลิตกรดแอลแลกติก และยังมีกิจกรรมของยีน *ldh* ที่มากกว่า แต่มีน้ำตาลไพรูเวทที่เหลือภายในเซลล์น้อยกว่า เชื้อ *E. coli* สายพันธุ์ RB24 (พาหะ pBluescript II KS(+) ซึ่งมียีน *ldhA* จากเชื้อรา *R. oryzae*) ประสิทธิภาพที่ดีกว่า pBluescript II KS(+) ในด้านการแสดงออกของยีน *ldhA* อาจจะเป็นผลจากความแข็งแกร่งของตัวควบคุมการแสดงออกที่น้อยกว่า อย่างไรก็ตามการผลิตกรดแอล-แลกติกของสายพันธุ์ TW2 สายพันธุ์ดั้งเดิม ซึ่งอาจเกิดจากการทำงานของ lactate dehydrogenase (LLDH) ตัวอื่นในสายพันธุ์ดั้งเดิม และการเปลี่ยนไปเส้นทางการหมักอื่นของสายพันธุ์ TW2

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5672210223 : MAJOR BIOTECHNOLOGY
KEYWORDS: LACTIC ACID / GENETIC ENGINEERING / GENE REPLACEMENT / ESCHERICHIA COLI / RHIZOPUS ORYZAE / DIFFERENT VECTOR SYSTEM

WATTANAI KANJANAPATTANAKUL: GENETIC MODIFICATION OF *Escherichia coli* FOR L-LACTIC ACID PRODUCTION. ADVISOR: RUETHAIRAT BOONSOMBAT, 80 pp.

Lactic acid is a chemical compound that has been used in many industrial applications. Therefore, effective process of lactic acid production is required to meet the higher demand of PLA plastic. The lactic acid can be produced by microbial fermentation including lactic acid bacteria (LAB) and fungi. Currently, *Rhizopus oryzae* has been interested due to its pure L(+)-lactic acid production. However, without additional applications, its morphology is difficult to control during fermentation process because its mycelium can cause a problem in stirred-tank bioreactor. To overcome such problem, genetic engineering technique for generating genetically modified *Escherichia coli* will be used. In this research, the gene replacement method and applied different plasmid system were used. According to a limitation of gene replacement technique, the DNA fragment for L(+)-lactic acid production was designed by containing ORF of *R. oryzae ldhA* gene flanked by upstream and downstream region of *E. coli ldhA* gene and planned to replace *E. coli* chromosomal *ldh* gene by linear transformation technique with plasmid pKD46. However, the construction of DNA fragment for gene replacement was unsuccessful. For applied different plasmid system, the pUC19 and pBluescript II KS(+) were used to harbor *R. oryzae ldhA* gene. The recombinant *E. coli* strains were fermented at 37 ^{o}C for 48 hours under anaerobic condition. The recombinant *E. coli* strain name TW2 which applied pUC19 vector harboring *R. oryzae ldhA* gene was successfully produced L(+)-lactic acid with higher yield and LDH activity, but lower intracellular pyruvate concentration than RB24 (pBluescript II KS(+) harboring *R. oryzae ldhA* gene). The better performances than pBluescript II KS(+) vector on *ldhA* expression may resulted from lower promoter strength. However, L(+) lactic acid production from TW2 strain was lower than wild type strain. This may come from the function of the other lactate dehydrogenase (LLDH) in wild type strain, and the shift to other fermentative pathways in TW2 strain.

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

Academic Year: 2016 Advisor's Signature .....

ACKNOWLEDGEMENTS

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

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

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

CONTENTS

Page

Page

Page

Page

LIST OF FIGURE

LIST OF TABLE

CHAPTER I
INTRODUCTION

Lactic acid is an organic compound with the formula $CH_3CH(OH)CO_2H$. In solid state, it white color and water-soluble. However, in liquid state, it is colorless. Lactic acid consists of two optical isomers: D (-) and L (+)-lactic acid. The acid was first discovered from sour milk by the Swedish chemist, Carl Wilhelm Scheele, in 1780. In 1857, Louis Pasteur proved that lactic acid also could be produced by fermentation of bacterial contamination, causing sour flavor in wines. Then, in 1881, the first industrial production of lactic acid by microbial process was established in the United States. The demand for lactic acid has been increase every year. The annual world market for lactic acid production was expected to reach 259,000 metric tons by the year 2012, and is forecasted to reach 367,300 metric tons by the year 2017 [1].

Lactic acid can be used in many applications. In food industry, lactic acid usually serves as a PH regulator or a preservative. It is sometime used as curing agent and flavoring agent. In pharmaceutical industry, lactic acid can be applied as a chiral intermediate to create other substances with desired stereo chemistry. Lactic acid is served as a good de-scaler, soap-scum remover, and an anti-bacterial agent in detergent. Because it is concerned as an environmental friendly agent, in polymer industry, lactic acid is uses as a monomer for polylactic acid (PLA) production. PLA is an interesting poly that can be produced from renewable resource and degraded by microorganisms in nature. Many PLA products are already available in the market to replace the petroleum-based products [1].

Lactic acid can be produced from both chemical synthesis and microbial fermentation. The former is usually involved in using lactonitrile, which is a byproduct from acrylonitrile synthesis [2]. Moreover, this chemical method has some limitations such as racemic mixtures between D (-) and L (+)-lactic acid. However, the other method, microbial fermentation by particular organisms can give pure optical isomer of lactic acid which benefits for industrial applications. Lactic acid can be produce by

many microorganisms such as bacteria, fungi, yeast, cyanobacteria, and algae. Lactic acid bacteria (LAB) also known as *Lactobacillus* strains are commercially preferable due to their high yield of lactic acid production and ability to tolerance in strong acidic conditions. However, LAB strains require complex nutrients such as vitamins, nucleotides, amino acids and peptides for their growth, resulting in high production cost. [3]. A filamentous fungus species named as *Rhizopus oryzae* recently has become interested for lactic acid fermentation due to its ability to produce pure L (+)-lactic acid. However, due to its morphology, it can cause some problems during process in stirred-tank bioreactor. Therefore, genetic engineering is applied to overcome such problems.

Genetic engineering or genetic modification is the direct manipulation of an organism's genome by receiving genetic materials from the other organisms to improve that organism or create a novel organism. First, the interested gene is isolated and amplified by using molecular techniques. Then, this DNA fragment was inserted into a selected host organism by direct transformation or by molecular vector to express this interested gene. In this research, *Escherichia coli* was selected as host. *E. coli* can naturally produce racemic forms of lactic acid mostly via anaerobic fermentation. Since *E. coli* 's genome is well studied, many researchers have been used it to harbor and express exogenous *ldh* genes to create pure lactic acid such as *E. coli* harboring plasmid with *ldh* gene from *Lactobacillus helveticus* [4], *Clostridium acetobutylicum* [5], *Streptococcus bovis* [6]. Moreover, due to its requirement of simple nutrients, some researchers have attempted to use other carbon sources such as xylose [7], sucrose [8], hexose and pentose [9] for fermentation by *E. coli*.

In previous research, *ldhA* gene from *R. oryzae* was inserted to pBluescriptII KS(+)plasmid, generating a plasmid named pRB85, and then, transformed into *E.coli* of which chromosomal *ldhA* and *pta* gene was knocked out, to allow the sole expression of L-lactate dehydrogenase from *R. oryzae* *ldhA* gene on pRB85 plasmid and reduce acetic acid production. The recombinant *E. coli* strain has achieved researcher goal by produce L (+)-lactic acid. However, the production yield was still low and there is a problem with genetic instability which is a problem of gene expression on plasmid [10].

In this research, gene replacement technique was applied to solve genetic instability problem. With this gene replacement method, some homologous regions between DNA fragment and chromosomal DNA are required [11]. However, *ldhA* genes from *R. oryzae* and *E. coli* are slightly homologous. Therefore, DNA fragment for gene replacement was constructed. This fragment was constructed by combining 3 fragments: open reading frame of *R. oryzae ldhA,* upstream and down region of *E. coli ldhA*. With this constructed fragment, *R. oryzae ldhA* gene was flanked by each homologous region to chromosomal *E. coli* DNA. The constructed fragment was planned to inserted into *E. coli* chromosome via λ Red system using plasmid PKD46 [12]. Although the original purpose of pKD46 was to inactivate chromosomal genes, many researchers have been applied this technique to inserted gene into host chromosomal genes such as *E. coli* producing 1-propanol [13] and *E. coli* producing L (+)-lactic acid from xylose [7].

This research also used different plasmid system to improve lactic acid production in *E. coli*. Since different copy number and promoter strength of the plasmids affect the protein expression level, it will further affect the enzymatic activity and flux of pathway [14]. There are some reports about expression of genes from different plasmid systems, for example, the expression of Biphenyl Dioxygenase-Encoding genes from *Rhodococcus globerulus* in pKS, pSK and pBBR1MCS [15], and *gshF* gene from *Streptococcus thermophiles* in pUC18, pUC19 and pET28a [16].

Objectives

- To create *E. coli* strain which *ldhA* gene on chromosomal DNA replaced by *ldhA* gene from *R. oryzae.*
- To study the expression of *ldh* gene of *R. oryzae* on pUC19 and pBluescript II KS(+) plasmid system.
- To study the efficiency of L (+)-lactic acid production using recombinant *E. coli* strain in shaking flask scale.

CHAPTER II
LITERATURE REVIEWS

2.1 Lactic acid

Lactic acid is an organic acid that can be found in nature. It has chemical formula as $C_3H_6O_3$ with and IUPAC name as 2-Hydroxypropanoic acid and its physical and chemical properties were describe in Table 2.1[17]. Lactic acid is an important product for many industries and biochemical processes in many organisms. It can be found as a white solid substance or colorless liquid. It has white color in solid state, whereas colorless in liquid state. However, it also has no odor and but miscible in water and ethanol. Lactic acid has 2 optical isomers known as L (+)-lactic acid and D (-)-lactic acid [18]. (Fig 2.1)

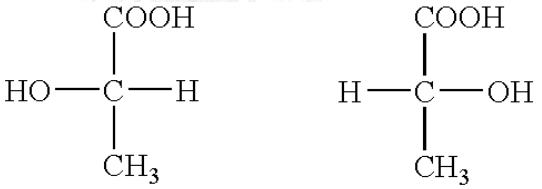


Figure 2.1 The two stereo isomers of lactic acid [18]

2.1.1 The physical and chemical properties of lactic acid

Table 2.1 Physical and chemical properties of lactic acid [17]

IUPAC Name	2-hydroxypropanoic acid
Molecular Formula	$C_3H_6O_3$ or $CH_3CHOHCOOH$
Molecular weight	90.078 g/mol
Color	Viscous, colorless to yellow liquid or colorless to yellow crystals

Odor	Weak unpleasant odor
Taste	Mild acid taste
Boiling point	122 °C at 15 mm Hg
Melting point	16.8 °C
Flash point	113 °C (235 °F) - closed cup
Solubility	Miscible with water
Density	1.2060 g/cu cm at 21 °C
Vapor pressure	0.0813 mm Hg at 25 °C
pH	The pH of a 10 wt% aqueous solution of lactic acid is 1.75
pKa	3.86 (at 20 °C)

2.1.2 Discovery of lactic acid

Lactic acid was first discovered in 1780 by a Swedish chemist, name Carl Wilhelm Scheele, who refined lactic acid from sour milk as impure brown syrup and named 'Mjölksyra', based on its origins. In 1857, Louis Pasteur proved that fermentation could also produce lactic acid (due to bacterial contamination). Pasteur's' experiment supported a hypothesis from Fre´my, a French scientist, that lactic acid could also be produced by fermentation. The first industrial production of lactic acid was established in the United States by microbial process in 1881.

2.1.3 Importance of lactic acid in industries

The demand for lactic acid has been increased every year. The annual world market for lactic acid production was expected to reach 259,000 metric tons by the year 2012, and is forecasted to reach 367,300 metric tons by the year 2017. Lactic acid has been classified by the US FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe) for use as a food additive, and it has been utilized in many applications including food, pharmaceutical and many other industries.

Food industry

Since lactic acid is classified as GRAS for use as a food additive by the US FDA [19], it has been used in many segment of the food industry for many severally applied applications such as flavoring, pH regulation, improved microbial quality, and mineral fortification. It is also used to increase shelf life, enhance flavor, and better control of food-borne pathogens in processed meat and poultry products. Lactic acid can be used in confectionery for flavoring and bringing the pH of the cooked mixture to the correct point for setting. The advantages of adding lactic acid in confectionery include its low inversion rate, ease of handling, and ability to produce clear candies. Another potential application of lactic acid in the food industry is the mineral fortification of food products [20].

Cosmetic industry

In cosmetic industry, lactic acid is usually used as moisturizers and pH regulators. It also applied as antimicrobial activity, skin lightening, and skin hydration. The moisturizing effect is performed through lactate's water retaining capacity, and the skin-lightening action of lactic acid is a result from the suppression of tyrosinase formation. The lactic acid and its salt are natural ingredients of the human body, which is considered as natural and safe formulation [21].

Chemical industry

Due to the composition of two functional groups; carboxylic and hydroxyl groups, lactic acid is mostly used as feedstock monomer for chemical conversion, such as propylene oxide, acetaldehyde, acrylic acid, propanoic acid, 2,3-pentanedione, and dilactide. It is also used for dyeing silk and other textile, printing woolens as a mordant, bating and plumping leathers, deliming hides, tanning vegetable, and fluxing soft solders[20].

Pharmaceutical industry

In pharmaceutical industry, lactic acid can be used as electrolyte in many parenteral/I.V. (intravenous) solutions such as Lactated Ringer's or Hartmann's solutions, CAPD (continuous ambulatory peritoneal dialysis) solution, and dialysis solution for conventional artificial kidney machines. It is also applied for mineral preparation, including tablets, prostheses, surgical sutures, and controlled drug delivery systems. Moreover, lactic acid can be found in many pharmaceutical formulations such as topical ointments, lotions, anti-acne solutions, humectants, parental solutions and dialysis applications, and anti caries agent [22].

Polymer industry

In polymer industry, lactic acid can be used as monomer to create the biodegradable polymer, called Polylactic acid (PLA). The monomer can be converted to PLA by many polymerizations such as polycondensation, ring opening polymerization and azeotopic dehydration condensation. Although polycondensation (PC) such as solution polycondensation and melt polycondensation are the least expensive method, it is difficult to obtain a solvent-free high molecular weight PLA. On the other hand, ring-opening polymerization (ROP) is the most commonly used method to obtain high molecular weight PLA because this route can control PLA product's molecular weight. The ROP is carried out by ring opening of the lactide (cyclic dimmer of lactic acid) in the presence of a catalyst. The azeotropic condensation polymerization is a method to obtain high-molecular-weight polymer without the use of chain extenders or adjuvants. By this method., the azeotropically dehydrated of lactic acid and catalyst are in a refluxing, high-boiling, aprotic solvent under reduced pressures [23].

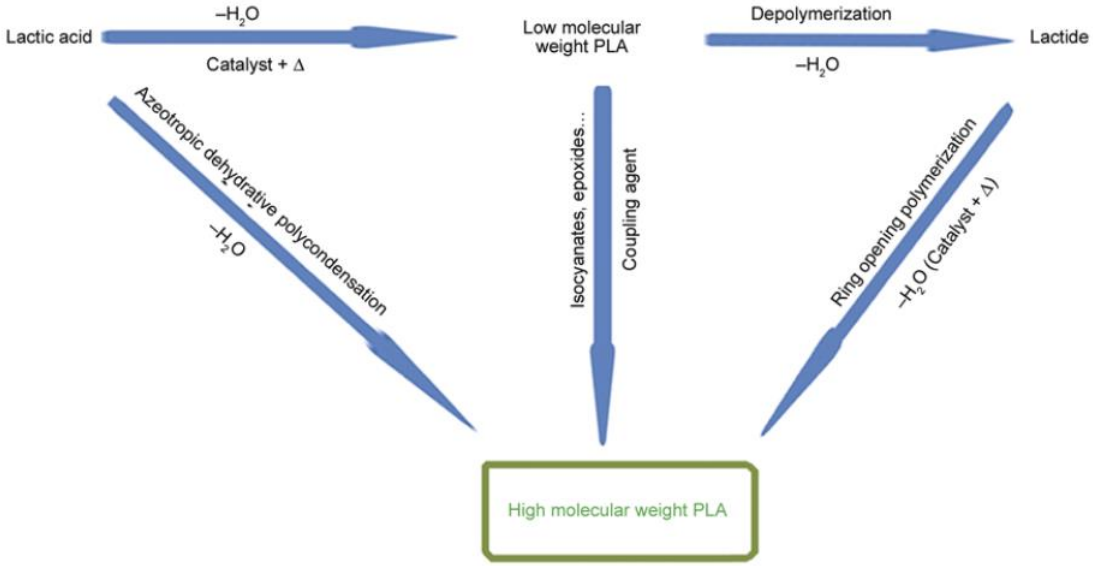


Figure 2.2 Polylactic acid (PLA) polymerization process [23]

2.1.4 Lactic acid production

Lactic acid can be produced either by chemical procedure using the lactonitrile route, which is a byproduct of acrylonitrile technology, or by microbial fermentation of carbohydrates such as molasses, corn syrup, whey, dextrose, cane and beet sugar.

2.1.4.1 Chemical synthesis for lactic acid production

Lactic acid is chemically synthesized from lactronitrile. In commercial procedure, acetaldehyde in liquid phase reacts with hydrogen cyanide under high pressure to produce lactronitrile. After the recovery and purification by distillation, lactronitrile is hydrolyzed by hydrochloric acid or sulfuric acid to produce lactic acid. Then, it is esterified with methanol to produce methyl lactate which is recovered and purified by distillation. The purified methyl lactate is hydrolyzed in acidic aqueous solution to produce lactic acid and methanol. Methanol is then recycled in the process.

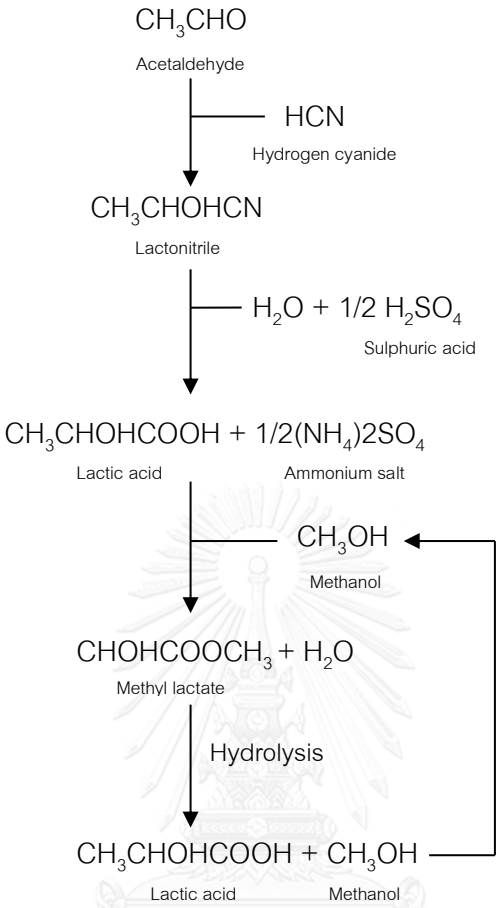


Figure 2.3 Chemical synthesis of lactic acid

There are other chemical methods for lactic acid synthesis such as base-catalyzed degradation of sugars, oxidation of propylene glycol, carbon monoxide and water at high temperature and pressure, hydrolysis of chloropropionic acid, and nitric acid oxidation of propylene. However, the lactic acid from chemical synthesis process is a racemic mixture of DL- lactic acid [24].

2.1.4.2 Microbial fermentation for lactic acid production

Microbial fermentation is a process that relatively fast with high yields of product. Moreover, either pure D (-) or L (+)-lactic acid is produced [25] depending on the strains being used [26]. With these benefits, this fermentation process is used in many industries. There are 2 main groups of microorganisms that can produce lactic acid; fungi and bacteria.

- Lactic acid production in Bacteria

Bacteria, including lactic acid bacteria (LAB), *Bacillus strains*, *Escherichia coli*, and *Corynebacterium glutamicum,* are preferable to be used for lactic acid production. Recently, LAB, considered as homofermentative lactic acid bacteria, are commercially used because they produce only lactic acid. However, LAB have some limitations, for example, they require appropriate environmental factors including temperature, pH, water activity, and the presence of inhibitory compounds [27]. With these reasons, LAB require a complex nutrition for growth [28]. Therefore, researchers have attempted to improve lactic acid production by using other microorganisms, which use simple nutritions to grow such as fungi.

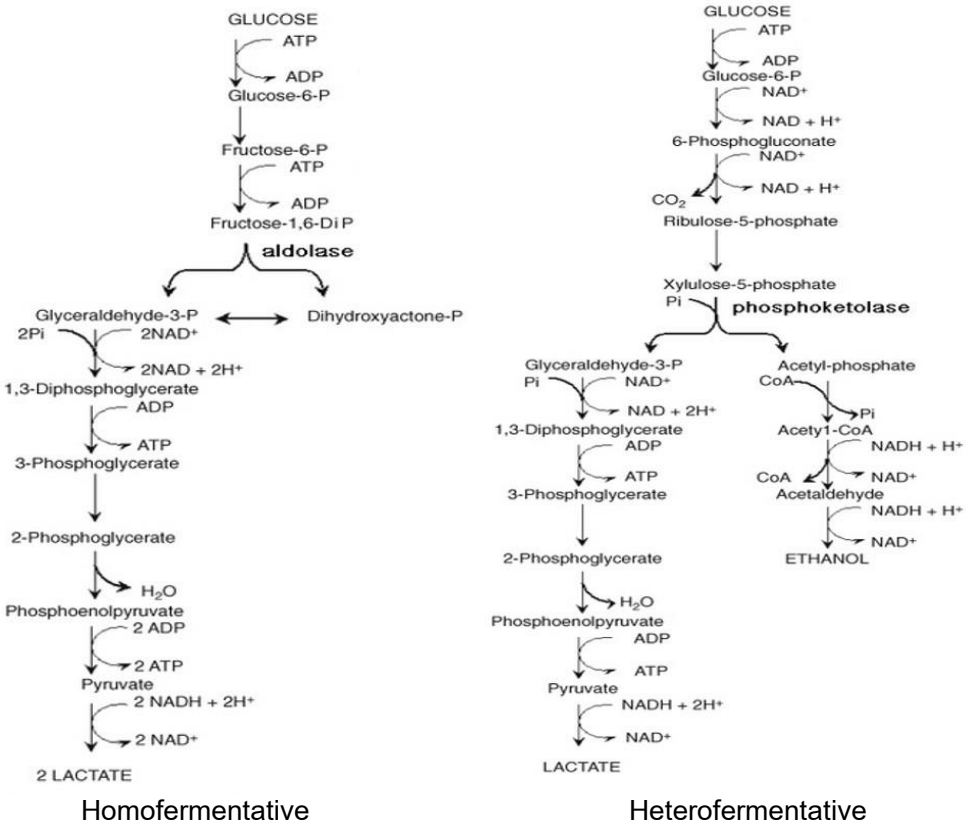


Figure 2.4 Metabolic pathways of homofermentative and heterofermentative lactic acid bacteria [29]

- Lactic production in fungi

There are many studies focused on the production of pure L (+)-lactic acid with the filamentous fungi in *Rhizopus* genus, especially *R. oryzae* [2, 30-32]. *Rhizopus* species can produce pure L (+)-lactic acid with simple nutritions [32-35]. It can also use renewable materials such as molasses [30] and lignocellulose [36]. However, there are some problems in lactic acid production by *Rhizopus* species such as undesirable byproducts including ethanol and fumaric acid [37]. Furthermore, due to its filamentous morphology, it can cause problem in stirred-tank bioreactor.

2.2 Characteristics of *Rhizopus oryzae*

R. oryzae is a filamentous fungus species classified in *Mucorales* order of *Zygomycota* phylum. Consisting of 4 main parts; sporangium, apophysis, rhizoid and sporangiophore, it is naturally found on decaying organic matter. Its hypae, or filaments, are for growing and expanding along the surface of substrate, and its root-like part, called rhizoid, penetrates through the substrate. *R. oryzae* digests food outside its body by rhizoid, and then digested food is transported inside its body. For growth, it can use many carbon sources such as glycerol, ethanol, lactic acid, glucose, mannose, fructose, sucrose, xylose, cellobiose, fatty acids, and oils [38]. The fungus species is a commercial filamentous fungus that can produce pure L (+)-lactic acid and ethanol as a by-product. However, some strains can also produce fumaric acid.

2.2.1 L (+)-lactic acid pathway

R. oryzae, can produce 4 major end products; ethanol, L (+)-lactic acid, L-malic acid and fumaric acid, from pyruvate during glucose metabolism. The formation of these products occurs due to the presence of dissolved oxygen in the medium. However, under anaerobic condition, carbon flow is directed toward ethanol pathway, catalyzed by enzymes called pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). However, this flow is shifted to organic acid pathway under aerobic condition. To

produce lactic acid, lactate dehydrogenase (LDH) with NADH catalyzes the conversion of pyruvate is to L (+)-lactic acid Furthermore, malic acid and fumaric acid are produced by malate dehydrogenase (MDH) and fumarase (FUM) in TCA cycle, respectively[38, 39].

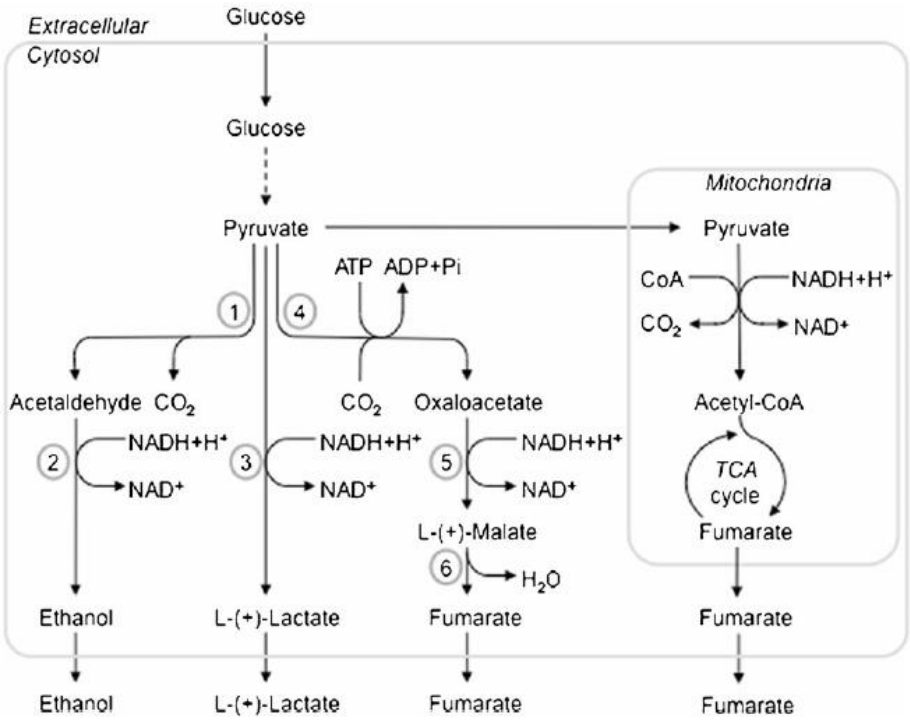


Figure 2.5 Glucose metabolism of *R. oryzae*. The numbers indicate key enzymes in each pathway: 1, pyruvate decarboxylase (PDC); 2, alcohol dehydrogenase (ADH); 3, lactate dehydrogenase (LDH); 4, pyruvate carboxylase (PYC); 5, malate dehydrogenase (MDH); 6, fumarase (FUM); 7, pyruvate dehydrogenase (PDH) [38]

2.2.2 Limitation of *R. oryzae* for lactic acid fermentation

Although *R. oryzae* fermentation can overcome many disadvantages from bacterial fermentation, it usually has lower lactic acid productivity. This may be resulted from the formation of other by-products, including ethanol and fumaric acid, and lower reaction rate caused by mass transfer limitation. The highly-interwoven hyphae are generally form sphere shape pallet that produce solute gradient through the spheres. As

the pallets increase in size, mycelium at the center of pallets become nutrient limited. Moreover, the filamentous morphology and the growth of mycelium on the impeller or on the electrode hamper optimal control of the process [40-43].

2.3 Characteristics of *Escherichia coli*

E. coli is a gram-negative, facultative anaerobic bacterium species with rod shape. It is commonly found in animal feces and lower intestines of mammals. *E. coli* was first discovered in1885 by a German bacteriologist, Theodor Escherich. Since then, *E. coli* has been commonly used for a variety of biological lab experiments and researches. *E. coli* has played an important role in modern biological engineering and industrial microbiology since the work of Stanley Norman Cohen and Herbert Boyer that created recombinant DNA by using plasmids and restriction enzymes. Genes can be introduced into *E. coli* using plasmids which allow high level of gene expression, and also used in industrial fermentation processes. One of the first applications of recombinant DNA technology was the manipulation of *E. coli* to produce human insulin [44, 45].

2.3.1 *E. coli* fermentative pathways.

E. coli usually conducts a mixed acid, including ethanol, acetic acid, lactic acid, formic acid and succinic acid from fermentation with glucose when oxygen is absent Ethanol production in wild type *E. coli* is catalyzed in a 2-step reaction by alcohol dehydrogenase (*adhE*) which converts acetyl – CoA into ethanol and generate 2 NAD^+ molecules. For acetic acid production phosphate acetyltransferase and acetate kinase are used and only 1 ATP molecule is produced. In this mixed- acid fermentation of *E. coli*. D (-)-lactic acid production is catalyzed by lactate dehydrogenase (*ldhA*) which converts pyruvate into D (-)-lactate and generate 1 NAD^+ molecule. For succinic acid production, wild type *E. coli* produce minor quantity of succinate under fermentative conditions due to the electron and ATP balance of the mixed-acid fermentation pathway.

Succinic acid is catalyzed by fumarate reductase which converts fumarate into succinate and generates 1 NAD^+ molecule[46-48].

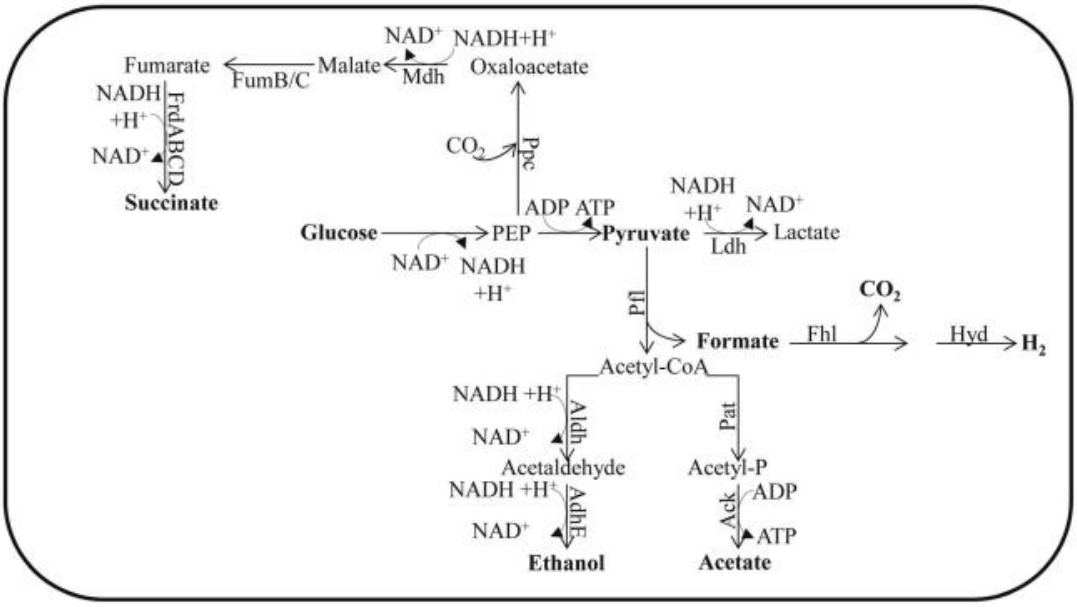


Figure 2.6 Fermentative pathways of *E. coli*. The enzyme designations are: *Ack* for acetate kinase; AdhE for alcohol dehydrogenase; Aldh for aldehyde dehydrogenase; Fhl for formate hydrogen lyse; FrdABCD for fumarate reductase; FumB for fumarase B (anaerobic); FumC for fumarase C; Ldh for lactate dehydrogenase; Mdh for malate dehydrogenase; Pfl for pyruvate formate lyse; Ppc for phosphoenolpyruvate carboxylase and Pat for phosphotransacetylase [46].

2.3.2 Lactic acid production in *E. coli*.

Many researchers have improved lactic acid production by using *E. coli* as a host because of its well-studied genome that makes it easy to be genetically manipulated. *E. coli* also has a rapid growth rate and simple nutrition requirement. There are at least two ways to enhance lactic acid production in *E. coli*, one is selection of homofermentative strains, and the other is using *ldh* gene from other microorganisms. The early homofermentative experiment was conducted by Chang et al. (1999) by deleting phosphotransacetylase (*pta*) and PEP-carboxylase (*ppc*) genes in competing pathways of the *E. coli* RR1 strain. This mutant was reported that it could produce

lactate with a yield of almost 90% of the theoretical maximum in a 2-phase fermentation process, with an aerobic growth phase and an anaerobic production phase at pH 7. However, there were many attempts to insert *ldh* genes from different microorganism such as *Plasmodium falciparum* [49], *Lactococcus lactis* [50], *Pediococcus acidilactici* [51] into *E. coli*. In addition, foreign genes were used to create recombinant in homofermentative *E. coli* strains with different carbon sources from glucose such as xylose [7], glycerol [52], sucrose [8], hexose and pentose [9].

2.4 Genetically engineered microorganisms for lactic acid production

Genetic engineering has been used to overcome bacteria and fungi's problem in fermentation, for example, the engineered *E. coli* SZ85 for pure L (+)-lactic acid production by replacing *E. coli ldhA* gene with *Pediococcus acidilactici ldhL* gene [51], the engineered *Saccharomyces cerevisiae* pLdhA68X for lactic acid production by inserting plasmid harboring *R. oryzae ldhA* gene into *S. cerevisiae* [53], and the engineered *E. coli* RR1 for optical D (-)-lactic acid production and for optical L (+)-lactic acid production [54].

2.5 Gene replacement technique

Gene replacement technique is carried out by using the ability of many bacteriophages that they can encode their own homologous recombination systems. The current technique, λ Red (*γ*, *β*, *exo*) function, gives a greatly enhanced rate of recombination over that exhibits by *recBC, sbcB* or *recD* mutants when using linear DNA [12]. Many researchers have used this technique to create recombinant *E. coli* strain in their studies [8, 51, 55]. However, this technique has its limitation that target gene was required at least 40 bp homologous with target site [56]. However, in this study, when *ldhA* gene from *R. oryzae* was aligned with *ldhA* gene from *E. coli* (Figure 2.7), lack of homology was investigated (less than 40% similarity). Moreover, amino acid sequences of these LDHs from these two species also revealed lack of similarity (less than 15%, Figure 2.8).

Figure 2.7 DNA sequence comparision between *ldhA* gene from *R. oryzae* (sequence line 1) and *ldhA* gene from *E. coli* (sequence line 2) using Emboss Needle program (EMBL-EBI, UK)

```
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 522
# Identity:      37/522 ( 7.1%)
# Similarity:    66/522 (12.6%)
# Gaps:         393/522 (75.3%)
# Score: 22.0
#
#
#=======================================

EMBOSS_001         1 MKLAVYSTKQYDKKYLQQVNESFGFELEFFDFLLTEKTAKTANGCEAVCI     50

EMBOSS_001         1 --------------------------------------------------       0

EMBOSS_001        51 FVNDDGSRPVLEELKKHGVKYIALRCAGFNNVDLDAAKELGLKVVRVPAY    100

EMBOSS_001         1 --------------------------------------------------       0

EMBOSS_001       101 DPEAVAEHAIGNMMTLNRRIHRAYQRTRDANFSLEGLTGFTMYGKTAGVI    150
                                                     ..::.|.| ::
EMBOSS_001         1 ----------------------------------------MVLHSKVA-IV     10

EMBOSS_001       151 GTGKIG--VAMLRILKGFGMRLLAFDPYPSAAALELGVEYVDLPTLFSES    198
                     |.|.::|  .|...:.|.....::..|..|.....::    :||      :
EMBOSS_001        11 GAGAVGASTAYALMFKNICTEIIIVDVNPDIVQAQV----LDL------A     50

EMBOSS_001       199 DVISL-HCPLTPENYHLLNEAAFEQMKNGVMIVNTSRGALIDSQAAIEAL    247
                     |..|: |.|        :...:.|:.....::|.|: ||
EMBOSS_001        51 DAASISHTP--------IRAGSAEEAGQADIVVITA-GA-----------     80

EMBOSS_001       248 KNQKIGSLGMDVYENERDLFFEDKSNDVIQDDVFRRLSACHNVLFTGHQA    297
                      .|:.|.....:.|..                 ||.|.:    :..|.|.
EMBOSS_001        81 -KQREGEPRTKLIERN----------------FRVLQS----IIGGMQP    108

EMBOSS_001       298 FL----------TAEALTSISQTTLQNLSNLEKGETCPNELV*-------    330
                     ..          ..:.||.|::|    ||.|.     ||:::.
EMBOSS_001       109 IRPDAVILVVANPVDILTHIAKT----LSGLP-----PNQVIGSGTYLDT    149

EMBOSS_001       331 --------------------------------------------------    330

EMBOSS_001       150 TRLRVHLGDVFDVNPQSVHAFVLGEHGDSQMIAWEAASIGGQPLTSFPEF    199

EMBOSS_001       331 --------------------------------------------------    330

EMBOSS_001       200 AKLDKTAISKAISGKAMEIIRLKGATFYGIGACAADLVHTIMLNRKSVHP    249

EMBOSS_001       331 --------------------------------------------------    330

EMBOSS_001       250 VSVYVEKYGATFSMPAKLGNRGVEQIYEVPLTEEEEALLVKSVEALKSVE    299

EMBOSS_001       331 ----------------------    330

EMBOSS_001       300 YSSTKVPEKKVHATSFSKSSC*    321
```

Figure 2.8 Protein sequence comparision between *ldhA* gene from *R. oryzae* (sequence line 1) and *ldhA* gene from *E. coli* (sequence line 2) using Emboss Needle program (EMBL-EBI, UK)

2.6 The pKD46 plasmid for gene replacement

The pKD46 plasmid carries λ red genes from bacteriophage. The plasmid was created by Datsenko and Wanner [12] with original purpose to delete chromosomal gene by FRT recognizing site-specific recombinase. However, it has been applied to replace host chromosomal gene with other interested genes such as replacement of *E. coli ldhA* gene by *Pediococcus acidilactici ldhL* gene [51]. The map of plasmid pKD46 [57] is represented in figure 2.9.

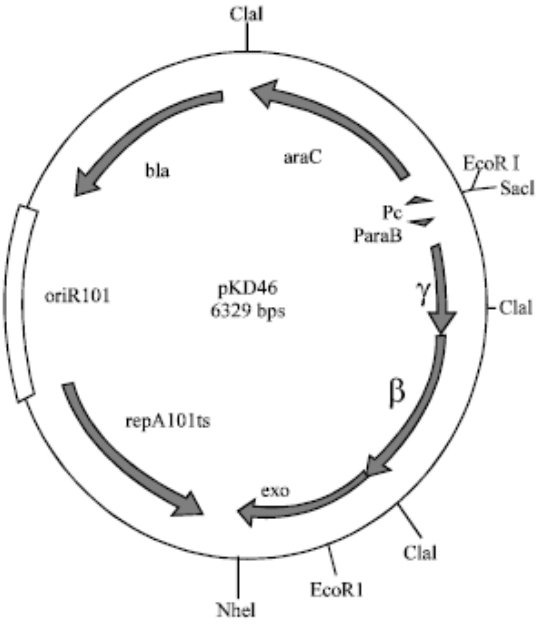


Figure 2.9 Plasmid PKD46 map [57].

2.7 Gene expression under different plasmid systems

Some researchers have applied different plasmid systems to improve the expression of their interested genes with different gene expression activities. In 2015, Chen wang and co-workers enhanced glutathione production by expressing *gshF* gene in various vector systems in *E. coli*. They found that different plasmid systems gave different performance in *gshF* expression [16]. In 2012, Xiangping Wu and co-workers also used different plasmid systems to express *lipA* and *lipB* genes from a screened

strain named AB which belongs to *Pseudomonas aeruginosa*. They founded the similar expression level of *lipA* in the three expression plasmid systems, but different active LipA enzyme level. However, the expression and active enzyme level from *lipB* were different [58].

2.8 pUC19 plasmid vector

The pUC19 plasmid is one of a series of plasmid cloning vectors created by Joachim Messing and co-workers. The plasmid is a circular double stranded DNA with 2686 base pairs. It is most widely used for recombinant DNA technology. With the use of this plasmid, the non-recombinants can be visually distinguished from recombinants by differentiation of colony color on particular growth media. The plasmid contains amp^R gene (ampicillin resistance gene), and an N-terminal fragment of β-galactosidase (*lacZ*) gene of *E. coli*. The map of plasmid pUC19 [59] is represented in figure 2.10.

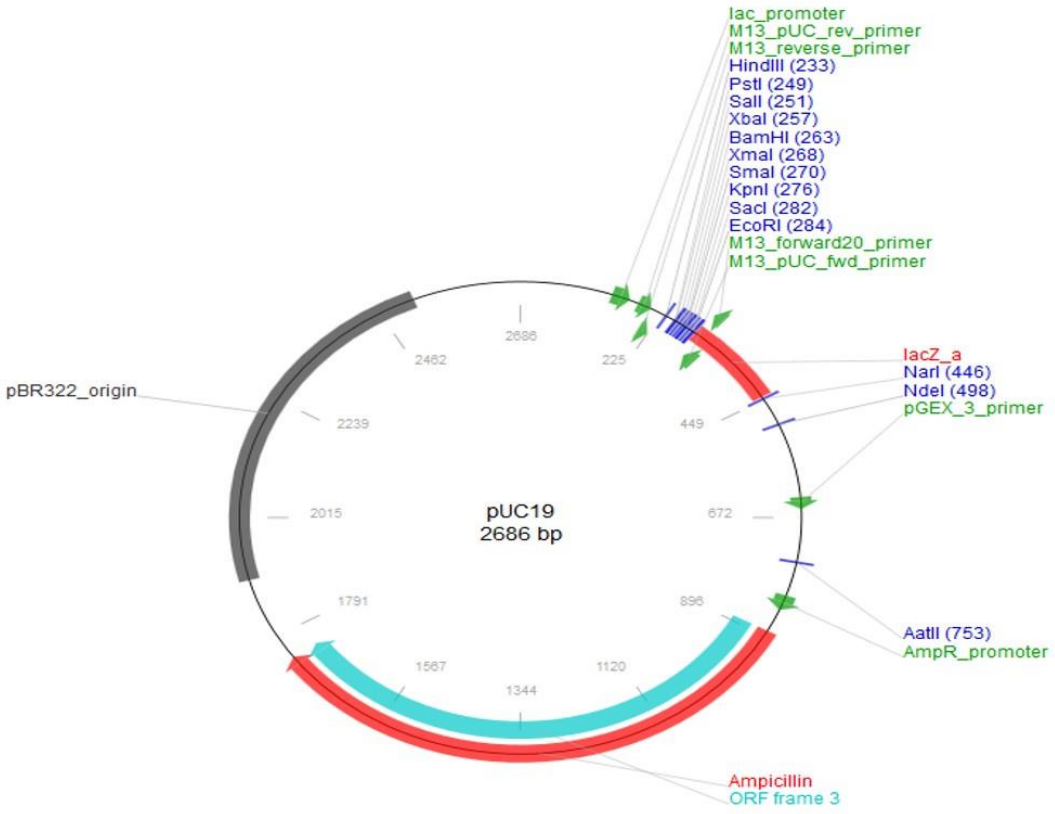


Figure 2.10 Vector pUC19 map [59].

2.9 pBluescript II KS(+) plasmid vector

The pBluescript (pBS) or pBluescript II is a commercial phagemid (plasmids with a phage origin) designed to simplify used for cloning and sequencing procedures, including the construction of nested deletions for DNA sequencing, generation of RNA transcripts *in vitro* and site-specific mutagenesis and gene mapping. The pBluescript phagemid contains multiple cloning site with 21 unique restriction enzyme recognition sites flanked by T7 and T3 RNA polymerase promoters, amplicillin antibiotic resistance and f1 helper phage origin of replication. The multiple cloning site sequence is located within *lacZ* gene. If the gene is disrupted by successful insertion of a DNA sequence, the white coloration is revealed in blue-white screening which can distinguish a successful recombination from those phagemids which were not altered. This is usually achieved via solid growth media with X-gal. The map of plasmid pBluescript II KS(+) [60] is represented in figure 2.11.

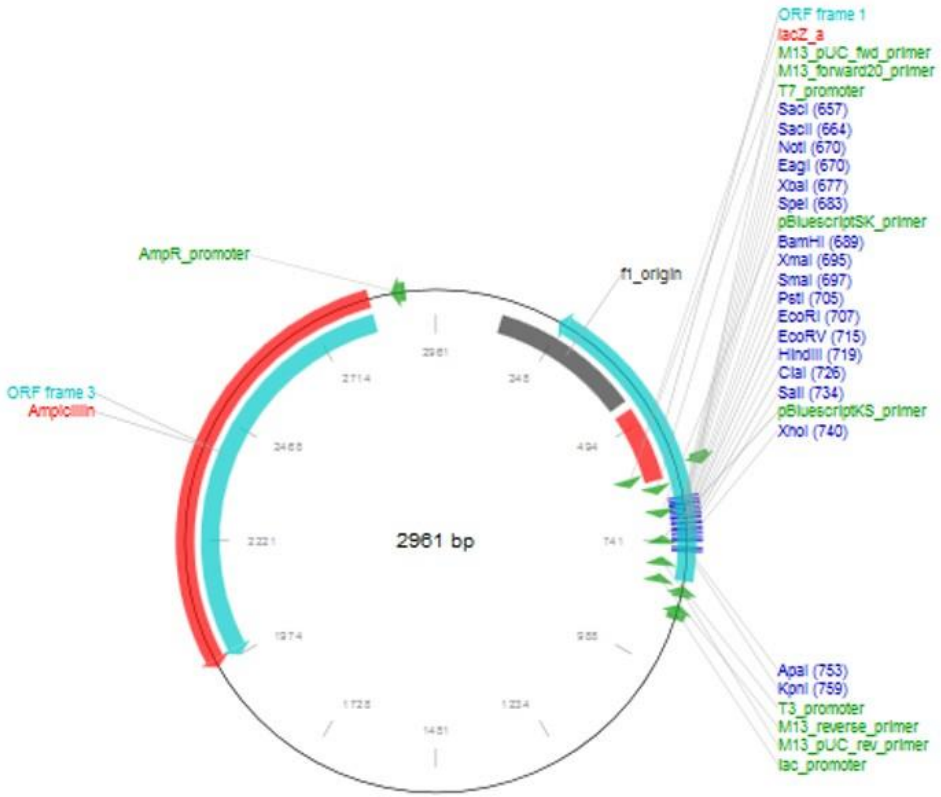


Figure 2.11 vector pBluescript II KS(+) map [60].

CHAPTER III
METHODOLOGY

3.1 List of strains used in this study

Strain	Partial genotype	Plasmid	Reference
JC13509[a]	-	-	Dr. Steven J Sandler
RB7	*ldhA::cat pta::kan*	-	Ruethairat (2013)
RB24	*ldhA::cat pta::kan*	pRB85	Ruethairat (2013)
TW2	*ldhA::cat pta::kan*	pTW	This study

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

pRB85 is pBluescriptII KS(+) containing *ldhA* gene (ORF and promoter region, Figure B1) from *R. oryzae* NRRL395.

3.2 List of oligonucleotide sequence used in this study

Primer	Sequence	Target gene
prRB5 prRB6	(+) GTAGCGCGTACGATGATTCCGGGGATC (-) CCATGCCGTACGTGTAGGCTGGAGCTG	*cat* gene introducing *BsiW*I site (underlined)
prRB40 prRB51	(+) CGCCCGGATCCCGCAGTTGCTGGAT (-) CCAACGATGGCGACCTTTGAGTGTAATAC CATAAGACTTTCTCCAGTGATGTTGAATCAC	Upstream region of *E. coli ldhA* gene (Figure B2) introducing *BamH*I site (underlined) and homologous region to *R. oryzae ldhA* (red)

prRB49 prRB50	(+) ATGGTATTACACTCAAAGGTCGCC (-) TCAACAGCTACTTTTAGAAAAGGAAGT	ORFs of *R. oryza ldhA* gene (Figure B1)
prRB41 prRB52	(-) GCGTCGGATCCAGTAGTGGAG (+) ACTTCCTTTTCTAAAAGTAGCTGTTGATA ATCTTGCCGTACGCCTGCATTCCAGGGGACG	Downstream region of *E. coli ldhA* gene (Figure B2) introducing *BamH*I site from prRB41 and *BsiW*I in prRB 52 (underlined) and homologous region to *R. oryzae ldhA* (red)
prRB47 prRB48	CAAGCTCAAGTCCTTGACCTTGCAGATGCT CGGGTCGTGTCAAGGTAGGTACCGGA	*R. oryzae ldhA* gene
16sF 16FR	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	*E. coli ldhA* gene

3.3 Chemical and reagents

Chemical and reagents	Company and country
Acetic acid	Merck, USA
Agarose	Research Organic, USA
Ampicillin	Bio Basic, Canada
Bovine serum albumin	Sigma Aldrich, USA
Calcium carbonate	Sigma Aldrich, USA
Chloroform	Merck, USA
Copper sulfate	Sigma Aldrich, USA
Dipotassium hydrogenphosphate	Bio Basic, USA
Dithiothreitol	Bio Basic, Canada
D-lactate	Sigma Aldrich, USA
Ethanol	Merck, USA
Ethidium Bromide	Bioexcellence, India
Ethylenediaminetetraacetic acid disodium salt dihydrate	Bio Basic, USA
Ferrous sulfate	Merck, USA
Glucose	Bio Basic, USA
Glycerol	Sigma Aldrich, USA
L-lactate	Sigma Aldrich, USA
Lysozyme	Bio Basic, USA
Magnesium Sulfate	Bio Basic, Canada
Manganese sulfate	Merck, USA
MOPS (Morpholinepropanesulfonic acid)	Sigma Aldrich, USA
NADH (Nicotinamide adenine dinucleotide)	Merck, USA
Peptone	Bio Basic, USA

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

3.4 Equipments and supplies

Equipments and supplies	Company and country
Agarose gel electrophoresis equipments: model Mupid-Exu	Mupid, Japan
Aminex HPX-87H column	Bio-Rad, USA
Anaero Anaerobic Gas Generator: model AnaeroPack	Mitsubishi Gas Chemical, Japan
Autoclave: model HICLAVE HV-50	Hirayama, Japan
Biological safety cabinet class II : model Hfsafe-1200	Shanghai Lishen Scientific Equipment, China
Cellulose acetate filter, pore size 0.45 µm	Sartorius, Germany
Centrifuge tubes 50 ml	Labcon, USA
Cryogenic vials	Biologix Research, USA
Erlenmeyer flask 125 ml	Pyrex, Germany
Erlenmeyer flask 250 ml	Pyrex, Germany

Equipments and supplies	Company and country
Freezer -20 °C: model SF-C997	Sanyo, Japan
Freezer -70 °C: model Forma 8600	Thermo Fisher Scientific, USA
Green PCR Master Mix 2x	Biotechrabbit, Germany
High speed refrigerated centrifuge: model 6500	Kubota, Japan
High speed refrigerated micro centrifuge: model MTX-150	Tomy Seiko, Japan
High-pressure liquid chromatography	Shimadzu, Japan
Hot plate stirrer: model C-MAG HS7	Ika, Germany
Incubator shaker: model innova 4300	New Brunswick Scientific, USA
Laboratory bottle	Duran, Germany
Micro auto pipette: model Discovery comfort	High Tech Lab, Poland
Microcentrifuge tube	Labcon, USA
Microplate spectrophotometer: model Multiskan GO	Thermo Fisher Scientific, USA
Microtiter microplates, UV plate, 96 Well	Costar, USA
MyGo Pro	IT-IS Life Science, Ireland
NucleoSpin® Gel and PCR Clean-up	MACHEREY-NAGEL, Germany
NucleoSpin® RNA	MACHEREY-NAGEL, Germany
PCR thermocycler: model T100 thermal cycler	Bio-Rad, USA
PCR tube	Labcon, USA
Petridish sterilized	Kappa Disposable Plastic
pH meter	Mettler Toledo, Switzerland
Pipette tips	Biologix Research, USA

Equipments and supplies	Company and country
Precision nanoScript 2 Reverse Transcription kits	Primerdesign, United Kingdom
Pyruvate Assay Kit	Biovision, USA
qPCR BIO SyGreen Mix LO-ROX	PCR Biosystem, United Kingdom
SpinClean plasmid miniprep kit	Mbiotech, Korea
Sumi chiral 0A-5000L column	SCAS, Japan
Test tube	Pyrex, Germany
UV-visible recording spectrophotometer: model UV-160	Shimadzu, Japan
Vacuum pump: model DOA-V130-BN	Gast, USA
Vortex mixer: model Vortex-Genie2	Scientific Industries, USA
Water bath shaker: model R-86	New Brunswick Scientific, USA
WaterPro PS/UF Polishing Stations: model 9000701	Labconco, USA

3.5 Methods

3.5.1. Construction of DNA fragment for gene replacement

According to a limitation of gene replacement technique, as mentioned previously in Topic 2.5, that the replacing fragment require at least 40bp homologous region (same 40 bp contigous nucleotides) to target site, DNA fragment for L (+)-lactic acid production was required to be constructed due to the lack of homology (Figure 2.7). The fragment was designed by combining 3 fragments

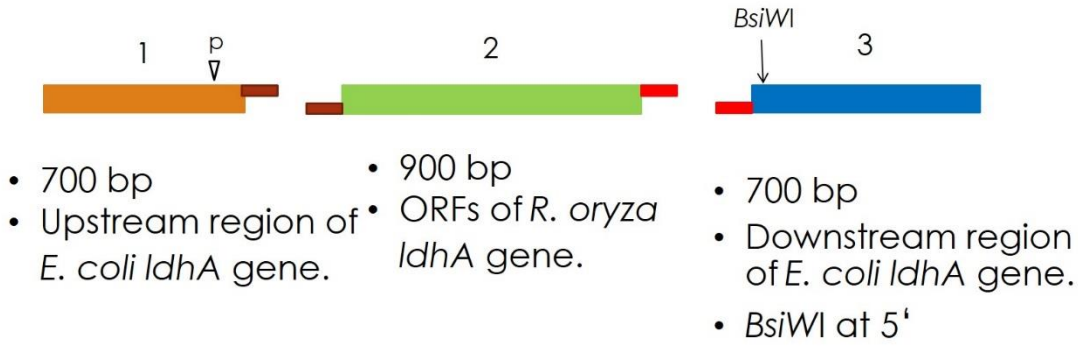


Figure 3.1 Schematic diagram of DNA fragment construction for replacing chromosomal *E. coli ldhA* with *R. oryzae ldhA*

The first fragment, named as Fragment 1 containing the upstream region of *E. coli ldhA* gene (Figure B2), was constructed using *E. coli* JC13509 genome as DNA template. The construction was started by growing *E. coli* JC13509 on LB agar plate and incubated at 37 °C overnight. Then, two colonies of overnight growth bacteria were used for DNA extraction. The colonies were put in a test tube containing 1 ml of distilled water and boiled for 10 minutes in a water bath, and then centrifuged for 5 minutes at 1000 rpm [61]. The 3 µl of the supernatant were used as template for amplifying DNA fragment by PCR with primers prRB40 and prRB51. The PCR product was purified by agarose gel electrophoresis technique and NucleoSpin® Gel and PCR Clean-up (Macherey- nagel, Germany).

The second fragment, named as Fragment 2 containing the ORF of *R. oryzae ldhA* gene (Figure B1) and restriction site of *BsiWI*, was constructed using plasmid pRB85, extracted from *E. coli* RB24 strain, as DNA template. The construction was started by growing RB24 strain in 5 ml of LB broth with 100 µg/ml of Amplicilin and incubated at 37 °C, 200 rpm for overnight. Then, 1 ml of overnight culture was used to extract plasmid by SpinClean plasmid miniprep kit (Macherey- nagel, Germany). The 3 µl of the extract was used as template for amplifying DNA fragment by PCR with primers prRB49 and prRB50. The PCR product was also purified by agarose gel electrophoresis technique and NucleoSpin® Gel and PCR Clean-up (Macherey- nagel, Germany).

The third fragment, named as Fragment 3 containing downstream region from *E. coli ldhA* stop codon (Figure B2), was constructed with the same method as Fragment 1 except for the use of prRB 41 and prRB52 as primers for DNA fragment amplification by PCR. The Fragment 1 was connected to the Fragment 2 by crossover PCR from the homologous region designed in prRB49 and prRB50 primers, generating a new fragment named Fragment 1+2. The Fragment 1+2 was then fused to Fragment 3 by crossover PCR from the homologous region designed in prRB41 and prRB51 primers, generating a new fragment named Fragment 1+2+3. Each fragment was purified by agarose gel electrophoresis technique and NucleoSpin® Gel and PCR Clean-up (Macherey- nagel, Germany). The purified Fragment 1+2+3 was clone into pCR®2.1-TOPO® (Invitrogen, USA) by added 4µl purified fragment, 1µl salt solution and 1 µl pCR2.1 vector to a 0.5ml centrifuge tube, and then incubated at room temperature for 15 minutes. The 4µl of this mixture was then added to 50µl *E. coli* DH5α, and mixed by gentle flicking. After being incubated on ice water for 20 minutes, the reaction was heat shocked at 42°C for 30 seconds, and then incubated on ice water for 2 minutes. The 1 ml LB broth was added to the reaction cloned and incubated at 37°C 250 rpm for 6 hours before spreading on LB solid plate with 100µg/ml of Ampicillin and 40 µg/ml of X-gal, and then incubated at 37°C overnight. The DNA sequence was verified by DNA sequencing (Macrogen, South Korea). The transformed *E. coli* DH5α and its plasmid were named WK1 and pWK1, respectively.

3.5.2. Introducing *cat* gene

The pWK1 and pRB74 plasmids were extracted by SpinClean plasmid miniprep kit. The plasmid prRB74 was used as DNA template for amplification of *cat* gene by PCR with primer pRB5 and pRB6. The PCR product was purified by agarose gel electrophoresis technique and NucleoSpin® Gel and PCR Clean-up. Then, plasmid pWK1 and PCR product of amplified *cat* gene were cut by restriction enzyme *BsiW*I for *cat* gene insertion. The 15 µl of purified plasmid pWK1 or PCR product of amplified *cat* gene, 2 µl of NEBuffer 3.1, 2 µl of nuclease-free water and 1µl of restriction enzyme

*BsiW*I were mixed in a 1.5 ml centrifuge tube and incubated at 55 °C for 1 hour. The digested pWK1 plasmid and *cat* gene were purified from 1% of agarose gel by NucleoSpin® Gel and PCR Clean-up. The 5 µl of digested plasmid pWK1 and 15 µl of *cat* gene were ligated with 1 µl of ligase in 3 µl of 10x ligation buffer and 6 µl of ddH_2O at 16 ° C overnight. The ligation reaction was transformed into *E. coli* DH5α and selected on LB plate with 25 µg/ml of Chloramphicol.

3.5.3 Linear transformation

Plasmid pKD46 was first transformed into *E. coli* RB7 and selected on LB plate with 100 µg/ml of Amplicillin at 30 °C. The selected colony was cultured at 30 °C in LB broth with 100 µg/ml of Amplicillin and 0.2% arabinose to induce the expression of the lambda *red* gene. After a couple of hours of incubation, cells were harvested to subsequently generating chemically competent cells [62]. The 2 µL of linear DNA was added to 40 µL of competent cells before being transferred to an ice-cold electroporation cuvette. The standard electroporation process was then performed [62]. Cells were incubated at 37 °C for 2-4 hours, then plated out on LB chloramphenicol plate and incubated at 37 °C overnight.

3.5.4 Construction of *E. coli* strain harboring plasmid pUC19 with *R. oryzae* ldhA gene.

The *E. coli* RB24 lab stock was inoculated in 5 ml LB broth with 100 µg/ml of Amplicilin and incubated at 37 °C, 200 rpm for overnight. Then, 1 ml of overnight was harvested for pRB85 plasmid extraction by SpinClean plasmid miniprep kit. The plasmid was digested by *Hind*III and *BamH*I restriction enzymes. The 1800 bp of target DNA band was extracted from 1% agarose gel and further purified. The plasmid pUC19 was also cut with *Hind*III and *BamH*I restriction enzymes. Then, the digested DNA fragment and pUC19 was ligated by T4 ligase, and named this recombinant plasmid as pTW. The plasmid pTW was transformed into *E. coli* RB7 by chemical transformation technique and selected on LB solid media with Amplicillin and X- gal at 37 ° C for

overnight. Several colonies were selected for plasmid extraction and subjected for DNA sequencing. A verified clone was named as TW2 strain.

3.5.5. Determination of *R. oryzae ldhA* gene expression by quantitative reverse transcription PCR (qRT-PCR)

The expression of *R. oryzae ldhA* gene was measured by qRT-PCR using MyGo Pro Real-Time PCR with qPCR BIO SyGreen Mix LO-ROX. The mRNA was extracted from cells by NucleoSpin® RNA kit (Macherey- nagel, Germany). The purified total mRNA was used as template for cDNA synthesis by Precision nanoScript 2 Reverse Transcription kits (Primerdesign, UK). Then, this cDNA was used for qRT-PCR. The 20 µl of each qRT-PCR reaction was consisted of 1 µg of cDNA, 0.8 µl of primer (prRB47 with prRB48 and 16sF with 16FR), 10 µl of 2x qPCR BIO SyGreen Mix LO-ROX, 7.4 µl of RNase/DNase free water. The qRT-PCR reaction was carried out as the following: 95 °C for 2 min (pre-denature), 40 cycles of 95 °C for 15 second (denature), 60 °C for 15 second (annealing), 72 °C for 20 second (extension). The fluorescent absorbance was detected in each cycle and calculated into CP value (Crossing point), which turn in to relative quantification after the qRT-PCR processing was finished. The reaction to amplify 16s rRNA housekeeping gene with 16sF and 16FR primers was used as a reference.

3.5.6 Lactic acid fermentation in shaking flask level

Each *E. coli* strain was streaked on cultivation slant and incubated at 37 °C overnight and then, cells were transferred from cultivation slant to 50 m of pre-culture broth and incubated at 37 °C, 200 rpm for 6 hours. The 5 ml of pre-culture was then added to 45 ml fermentation broth of which the final OD_{600} was approximately 1.0. (0.37 M HCl was added to solubilize the excess $CaCO_3$ prior to measure the absorbance by spectrophotometer at 600 nm). The fermentation was performed at 37 °C for 48 hours under anaerobic condition since, after 48 hour L (+)-lactic production rate was steady [63]. The 1 ml of each fermentation experiment was harvested and centrifuged at 10,000

rpm for 7 min. The supernatant was kept for analyzing the concentrations of residual glucose and lactic acid by a high-pressure liquid chromatography (HPLC) and the pellet was kept for qRT-PCR, LDH activity assay and pyruvate concentration assay.

3.5.7 Measurement of lactic acid during the fermentation by high-performance liquid chromatography (HPLC)

The concentrations of residual glucose and lactic acid were determined by using a high-pressure liquid chromatography (HPLC) equipped with IR detector and an Aminex HPX-87H column chromatography. The analysis was performed at 50 ˚C of which compounds were eluted by 5 mM sulfuric acid with elution rate 0.60 ml/min. The optical purity of lactic acid was determined by HPLC equipped with UV detector at 254 nm using a Sumi chiral 0A-5000L column to separate the L (+)-lactic acid and D (-)-lactic acid. The chromatography was performed at 40 ˚C and the compounds were eluted by 2 mM copper sulfate with elution rate 1 ml/min. Glucose, L (+)-lactic acid D (-)-lactic acid $\geq$ 99.0% from Sigma Aldrich were used as calibration standard.

3.5.8 Determination of Lactate dehydrogenase (LDH) activity during the fermentation

The purpose of this experiment was to measure the efficiency of the LDH enzyme in each strain. The selected strains were fermented with 2% glucose and $CaCO_3$ under anaerobic condition. After 48 hours, LDH activity was assayed by measuring UV absorbance of NADH oxidation at 340 nm (Figure 3.2) using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., USA). After solubilize the excess $CaCO_3$by adding 0.37 M HCl, cells were harvested from 5 ml of 48 hours' anaerobic culture by centrifugation. Then, cells were washed once in Tris-maleate buffer (100 mM Tris-maleate, 1 mM dithiothreitol with pH 6.5) and resuspended in Tris-maleate buffer to reach approximately final OD_{600} as 1.0. Then, cells were permeabilized by treating 0.1 ml of this suspension with 0.1 ml of chloroform and vigorously mixing for 15 second with a vortex. After chloroform being settled, the upper layer containing

permeabilized cells was used to assay LDH activity. The assay mixture contained 30 µl of 1 M sodium pyruvate, 30 µl of 6.4 mM NADH, 400 µl of 50 mM morpholinepropane-sulfonic acid buffer with pH 7.0, 530 µl of distilled H_2O, and 10 µl of crude enzyme. LDH activity (initial rate) was measured at room temperature for 5 min and reported as micromole of NAD produced per min per milligram of total cell protein [64] which was measured by Bradford protein assay [65].

Figure 3.2 Lactate dehydrogenase activity [66]

3.5.9 Determination of intracellular pyruvate concentration after fermentation for 48 hours

The purpose of this experiment was to measure pyruvate concentration that remained in the cell after 48 hours of fermentation with 2% glucose and $CaCO_3$ under anaerobic condition. Cells of *E. coli* strain were lysed by using lysozyme freeze/thaw procedure. The cell pellet was resuspended in TAE buffer then 1 mg/ml of lysozyme was added frozen at -80 °C for 10 minutes, and thawed at 37 °C for 30 minutes. After the steps of freeze- and-thaw were repeated for 3 times, the sample was centrifuged at 12000 rpm for 1 minute. The supernatant was harvested for pyruvate concentration assay according to the manual of Pyruvate Assay Kit (Biovision, USA).

CHAPTER IV
RESULT AND DISCUSSION

4.1 Results

4.1.1 Construction of DNA fragment for gene replacement.

The Fragment 1, the 700 bp upstream region containing a promoter and ribosome binding site of *E. coli ldhA* gene of *E. coli ldhA* gene, was amplified by PCR with primers prRB40 and prRB51, and then visualized on agarose gel (Figure 4.1A). The Fragment 2, containing the ORF of *R. oryzae ldhA* gene, was amplified by PCR with primers prRB49 with prRB50. The expected 900 bp PCR product was revealed (Figure 4.1B). These 2 fragments were extracted from the gel and further used for cross-over PCR.

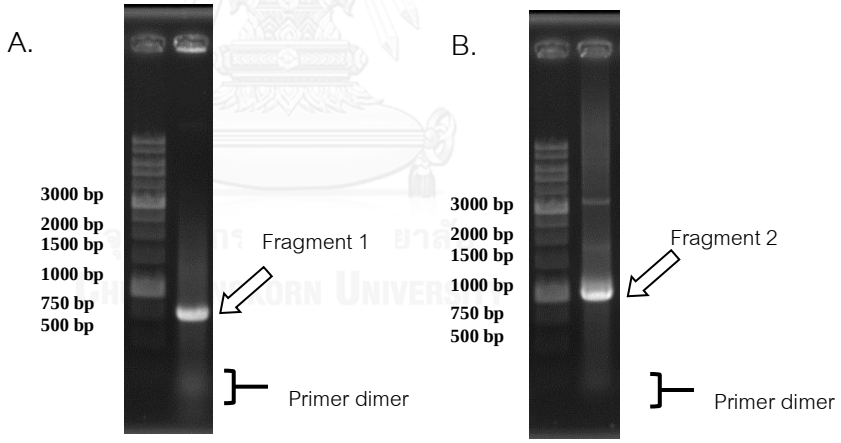


Figure 4.1 The PCR products of (A.) Fragment 1 (B.) Fragment 2

Then, the primerless cross over PCR technique was performed to anneal the Fragment 1 and 2 by their overlapping regions. Then, the cross-over fragment was amplified by PCR using prRB40 and prRB50 primers. The approximately 1600 bp PCR product was revealed on agarose gel (Figure 4.2). Therefore, this combined fragment, named as Fragment 1+2, consists of promoter, ribosome binding site of *E. coli ldhA*

gene and ORF of *R. oyzae ldhA* gene (Figure B1). The Fragment 1+2 was cloned into pCR®2.1-TOPO® (Invitrogen, USA), and transformed into *E. coli* DH5α for maintenance. Plasmid was extracted and digested with *EcoR*I (Figure B1) to verify the insertion of the Fragment1+2 (Figure 4.3). The nucleotide sequence of the Fragment 1+2 was also verified by DNA sequencing (Macrogen, Korea).

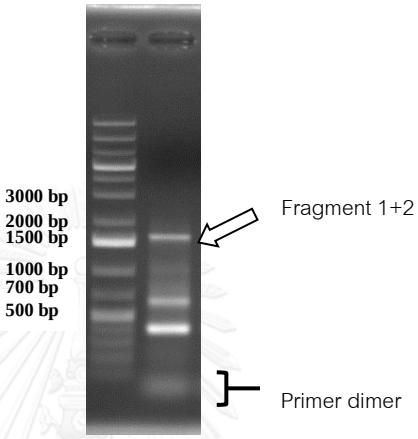


Figure 4.2 The cross-over PCR product of Fragment 1+2 with the expected 1600 bp DNA band.

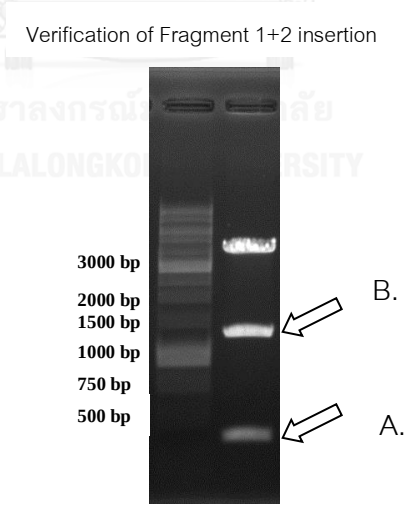


Figure 4.3 Verification of Fragment 1+2 insertion into pCR®2.1-TOPO® by *EcoR*I digestion. The expected (A.) 400 bp and (B.) 1300 bp DNA bands could be visualized from 1% agarose electrophoresis.

The Fragment 3, the 700 bp downstream region containing the terminator of *E. coli ldhA* gene of *E. coli ldhA* gene (Figure B2.), was amplified by PCR with primers prRB41 with prRB52. The expected size of this DNA fragment was extracted for further annealing with the Fragment 1+2 (Figure 4.4).

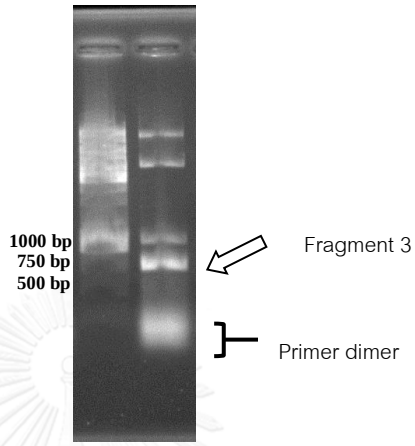


Figure 4.4 Verification of an approximately 700 bp PCR product which amplified by PCR with prRB41 and prRB52 primers

The primerless cross over PCR technique was performed to anneal the Fragment 1+2 and 3 by their overlapping regions. Then, the cross-over fragment was amplified by PCR using prRB40 and prRB41 primers. Unfortunately, the approximately 2300 bp PCR product was not revealed on agarose gel (Figure 4.5)

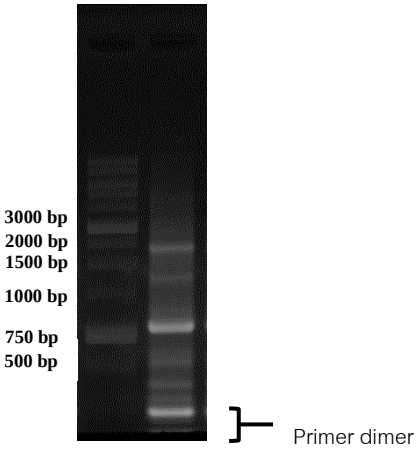


Figure 4.5 The cross-over PCR product of Fragment 1+2 with Fragment 3, however, the expected 2300 bp DNA band could not investigated.

Because the connection between Fragment 1+2 and Fragment 3 was unsuccessful, the new strategy was attempted by constructing Fragment 2+3 to increase the overlapping regions for further primerless crossover PCR of Fragment 1+2 and 2+3). The cross-over PCR of Fragment 2+3 was amplified by PCR using prRB41 and prRB49 primers. The approximately 1600 bp PCR product was revealed on agarose gel (Figure 4.5).

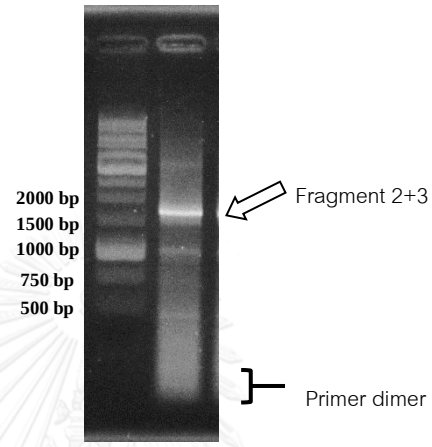


Figure 4.6 The cross-over PCR product of Fragment 2+3 with the expected 1600 bp DNA band.

However, the yield of Fragment 2+3 PCR product was too low after performed gel purification by using NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Germany). Therefore, the Fragment 2+3 was not observered on agarose gel.

The annealing of Fragment 1+2 and 3 was unsuccessful due to the limitation of PCR technique; possibly, target fragment was too large for this *Taq* DNA polymerase. Optimization of PCR parameters including times and temperatures adjustment was attempted for several times, however, it was still unsuccessful. Therefore, the other method to improve L (+)-lactic acid production by using different plasmids harboring *R. oryzae ldhA* gene was considered.

4.1.2 Construction of *E. coli* strain harbor plasmid pUC19 with *R. oryzae ldhA* gene

The pRB85 plasmid extracted from *E. coli* RB24 by SpinClean plasmid miniprep kit was digested by *Hind*III and *BamH*I restriction enzymes. The 1800 bp of target DNA fragment containing Ribosome binding site, promoter and ORF of *R. oryzae ldhA* gene (Figure B1) was revealed on agarose gel (Figure 4.7).

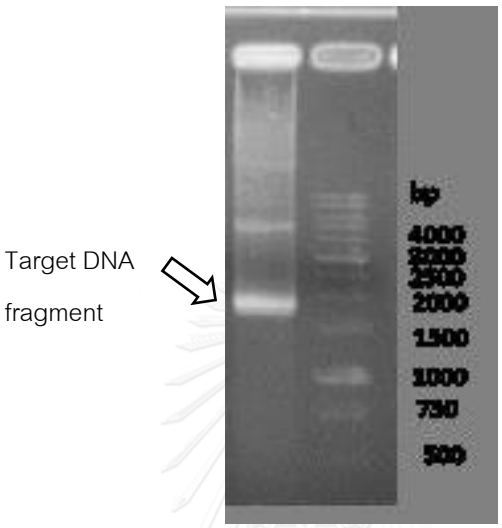


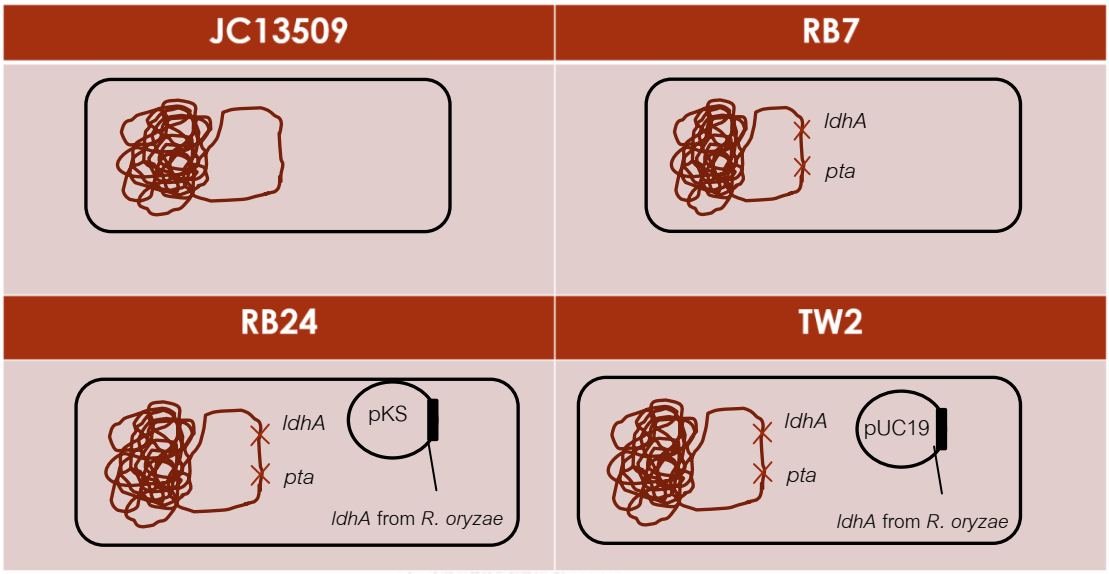
Figure 4.7 Target DNA fragment from pRB85 digested by *Hind*III and *BamH*I

Then, target DNA fragment was ligated with plasmid pUC19 digested *Hind*III and *BamH*I. The ligation reaction was transformed into *E. coli* RB7 and selected with blue-white screening technique. The selected clone was subsequently subjected for plasmid extraction and verified the inserted fragment by DNA sequencing. This verified recombinant *E. coli* was named as TW2.

4.1.3 Lactic acid production in shake flask level

The JC13509, RB7, RB24 and TW2 strains were selected for fermentation with 2% glucose medium at 37 ^{0}C under anaerobic condition.

Table 4.1 Strains used for experiment for lactic acid production



After being incubated for 48 hours, the residual glucose concentrations and lactic acid concentrations of each culture were measured by High-performance liquid chromatography (HPLC) as reported in Figure 4.5. The highest concentration of D (-)-lactic acid and L (+)-lactic acid was produced from the wild-type JC13509 at 2.613± 1.069 and 8.053 g/L± 0.847, respectively (Table 4.2, Figure 4.8). However, the aim of this research was to improve L (+)-lactic acid production. Although the wild type strain produced the highest yield of L (+)-lactic acid, the highest yield of D (-)-lactic was also obtained. However, when growth curve of each strain during 48 hour of fermentation was observed, the *E. coli* RB7, RB24 and TW2 have reached the stationary phase, but *E. coli* JC13509 has a slower growth rate than other strains (Figure 4.9). Nevertheless, when our two engineered strains harboring plasmids with *R. oryzae ldhA* gene were compared, the TW2 strain with pUC19 derivative seemed to produce more L (+)-lactic acid, but less D (-)-lactic acid, than the RB24 strain with pBluescript II plasmid (Figure 4.8).

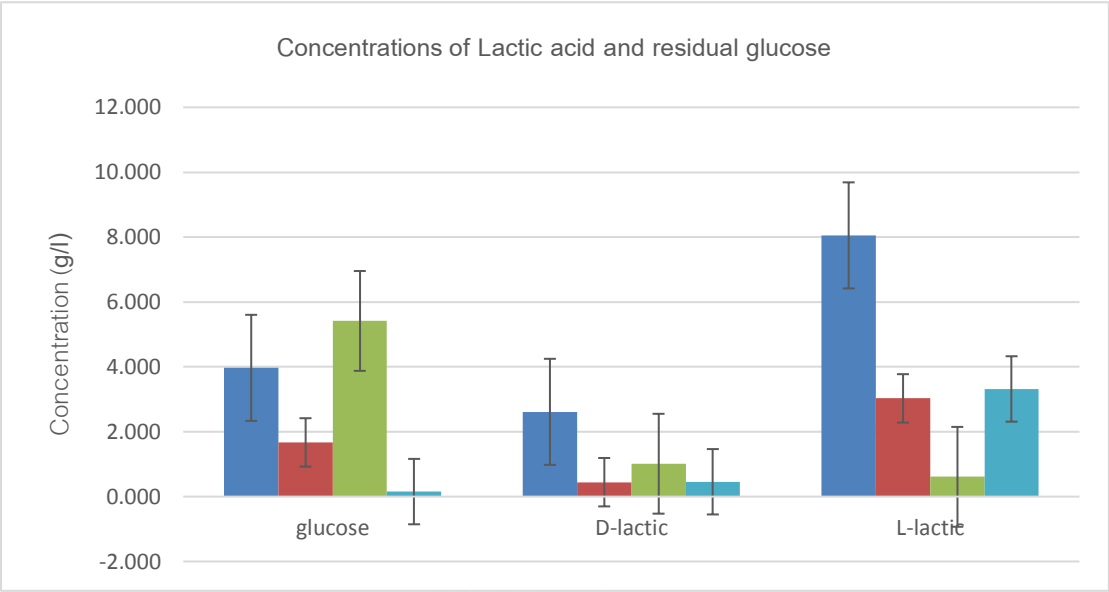


Figure 4.8 Concentrations of residual glucose and lactic acid after 48 hours' fermentation from each selected *E. coli* strain; ■ for JC13509; ■ for RB7; ■ for RB24; ■ for TW2.

Table 4.2 The concentrations from selected *E. coli* strain of residual glucose and lactate harvested after 48 hours fermentation under anaerobic condition.

Strains	Concentration (g/l) (Mean±S.D.)		
	Residual Glucose	D-lactate	L-lactate
JC13509	3.970 ± 0.434 ^{a}	2.613 ± 1.069 ^{A}	8.053 ± 0.847 ^{1}
RB7	1.67 ± 1.355 ^{a}	0.445 ± 0.162 ^{B}	3.028 ± 0.799 ^{2}
RB24	5.415 ± 0.317 ^{a}	1.014 ± 0.070 ^{B}	0.609 ± 0.049 ^{2}
TW2	0.155 ± 0.028 ^{b}	0.458 ± 0.303 ^{B}	3.319 ± 0.911 ^{2}

$^{a,b \text{ or } A,B \text{ or } 1,2,3}$ Statistics analysis by SPSS variance (ANOVA) with post hoc comparison (one-way) using Duncan's Multiple Range Test (DMRT). The same superscripts are not significantly different from each other (p<0.05).

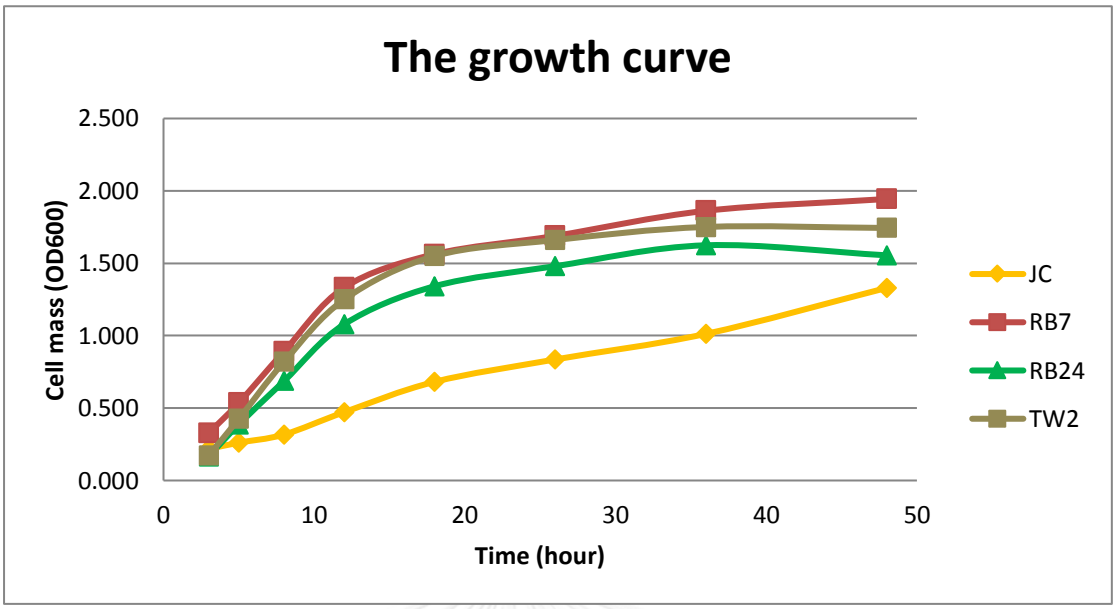


Figure 4.9 Growth curve of *E. coli* strains during fermentation in shake flask under anaerobic condition with 2% glucose for 48 hours.

4.1.4 LDH activity

The LDH activity assay was performed by measuring NADH oxidation at UV absorbance 340 nm due to the catalyzing of lactate dehydrogenase in the conversion of pyruvate to lactate and NADH to NAD^+(Figure 3.2). In this study, LDH activity was reported as micromole of NAD produced per min per milligram of total cell protein as represented in Table 4.3. The *E. coli* TW2 strain revealed the highest LDH activity of 0.66 µmole/mg of protein per min. However, it was not significantly different from the other strains.

Table 4.3 LDH activity of each selected *E. coli* strain after 48 hours of fermentation under anaerobic condition.

Strains	LDH activity (μmole[1]/ mg of protein[2] per min)
JC13509	21.519 ± 4.877 [a]
RB7	23.276 ± 6.559 [a]
RB24	21.422 ± 2.709 [a]
TW2	59.084 ± 46.137 [a]

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

[1] Micromole of NAD produced

[2] Milligram of total cell protein

4.1.5 Pyruvate assay

Pyruvate assay was performed by measuring pyruvate at 570 nm UV absorbance. Pyruvate concentration, represented in Table 4.4, was reported as milligram per milliliter as. As expected, the RB 7 strain RB7 revealed the highest intracellular pyruvate concentration of 4.479 ±4.701 mg/mL due to the deletion of *ldhA* and *pta* causing the accumulation of pyruvate that could not converted to lactate and acetate (Figure 2.6). Moreover, the lowest intracellular pyruvate concentration was observed in the TW2 strain, corresponding to the highest LDH activity as reported in Table 4.3

Table 4.4 The concentration of intracellular pyruvate from each selected *E. coli* strain after 48 hours of' fermentation under anaerobic condition.

Strains	Pyruvate concentrate (Mg/mL)
JC13509	1.701±0.392
RB7	4.479±4.701
RB24	0.889±0.267
TW2	0.526±0.091

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

As the results mentioned above, when our two strains harboring plasmid with *R. oryzae ldhA* gene were compared, the strain TW2 containing pUC19 derivatives seemed to give higher efficiency to produce L (+)-lactic acid than the strain RB24 containing pBluescript II derivative. One of the possibilities was that the strain TW2 expressed more *R. oryzae* LdhA enzyme. To prove this hypothesis, the qRT-PCR technique was performed to measure the transcriptional expression level of *R. oryzae ldhA* from the plasmid in both *E. coli* strains. The selected strains were fermented in 50 ml fermentation media with 2% glucose and 100 µg/ml of ampicillin, under anaerobic condition for 48 hours. The relative quantification graph was calculated after the qPCR processing was finished and summarized in Figure 4.10. As expected, the higher expression was observed in the strain TW2.

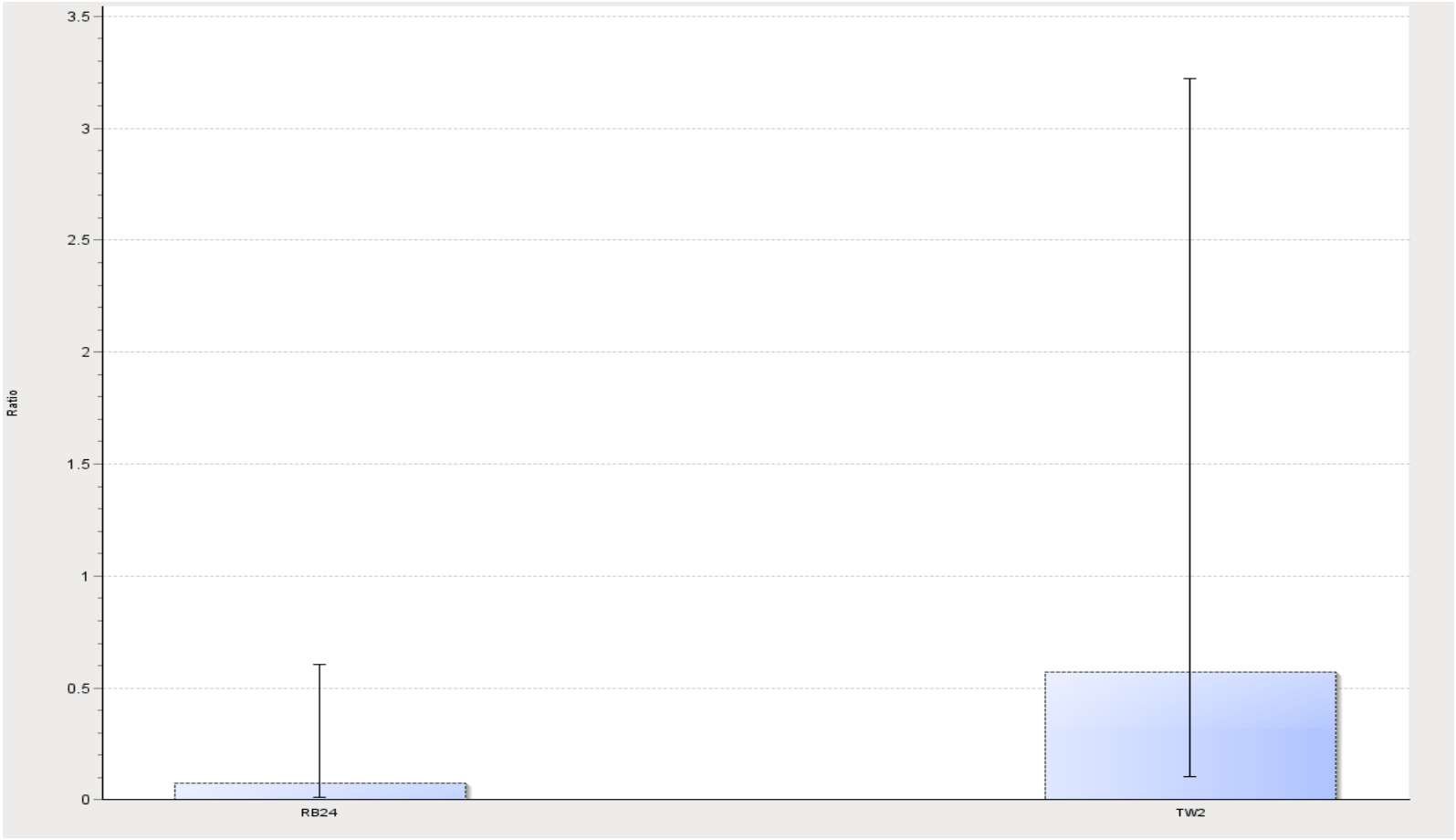


Figure 4.10 The relative quantification graph from expression of *ldhA* gene from *R. oryzae* on the pRB85 plasmid in *E. coli* RB24 strain and on the pTW plasmid on TW2 strain after being fermented 48 hours under anaerobic condition with 2% glucose. The gene expression was measured by qRT-PCR with prRB47 and prRB48 primers detecting R. oryzae ldhA gene (X-axis: strain, Y-axis: ratio).

4.2 Discussion

From a previous study, *E. coli* strain RB24 harboring pBluescript II KS(+) vector system with *ldhA* gene from *R. oryzae* was used for L (+)-lactic acid production. However, the strain RB24 could produce small amount of L (+)-lactic acid under an anaerobic condition [10]. To improve L (+)-lactic acid production from genetically engineered *E. coli*, the replacement of *R. oryzae ldhA* gene on *E. coli* chromosomal DNA was initially attempted. Since, the homologous between *ldhA* gene from *E. coli* and *R. oryzae* are 39.6%, which less than 40%, without any of same 40 nucleotide contigous region between these two *ldhA* gene (40 bp homologous region) (Figure 2.7), DNA fragment for gene replacement was constructed. The Fragment 1 and 2 was successfully amplified (Figure 4.1) and the connection between Fragment 1 and 2 by crossover PCR gave the expected band of 1,600 bp Fragment 1+2 (Figure 4.2). However, due to the limitation of PCR technique, the connection between Fragment 1+2 and Fragment 3 was unsuccessful (Figure 4.5). It is possibly that the designed fragment is too large for this kind of *Taq* DNA polymerase and this crossover PCR technique. According to general protocol of standard PCR [67], primer size is recommended as 20-30 oligonucleotides. However, prRB 51 and prRB 52 consist of 61 oligonucleotides because of the design of homologous regions for gene replacement method. With the large size of primers, it was possible that some primers had self-binding, called primer dimer, instead of annealing to DNA template. As seen in Figure 4.1, 4.2, 4.4, and 4.5, the primer dimers were revealed as bands lower than 100 bp size [68]. To avoid the primer dimer problem, a carefully designed primer with other PCR techniques, such as hot start PCR are suggested [68]. Moreover, according to the PCR product (~3200 bp), although the manual of Taq polymerase states that up to 5 kbp of product can be used, many protocols suggested that the PCR products should not be large. The large size of product can decrease the amplification efficiency [68].

The other alternatives to improve L (+)-lactic acid production from genetically engineered *E. coli* of this study was to apply different vector system. In this study, the strain TW2 with pUC19 vector system was used to compare with the strain RB24 with

pBluescript II KS(+) vector system. After being fermented at 37 ˚C for 48 hours under anaerobic condition, L (+)-lactic acid production from TW2 was lower than JC13509 (Figure 4.8). The high concentration of lactic acid in this wild type strain was possibly come from the function of other lactate dehydrogenases in *E. coli*. Generally, *E. coli* contains 3 types of Lactate dehydrogenase, encoding from *ldhA*, *dld* and *lldD* gene which the *ldhA* and *dld* were responsible for D (-)-lactic acid production. The presence of L (+)-lactic acid in JC13509 after 48 hour of fermentation may cause from *lldD* gene expression which normally functions as aerobic respiratory L(+)-lactate dehydrogenase. It is possible that there was a leak of O_2 during fermentation process, or an activation of *lldD* gene expression during anaerobic fermentation due to some unidentified regulators such as the presence of nitrate under anaerobic conditions [69]. This may also cause slower growth of *E. coli* JC13509 that still gradually grew and did not yet enter stationary phase, even though most of strains already reached this phase (Figure 4.9). The activation of lldD gene in JC13509 strain can be further proved by qRT-PCR of lldh gene which can detect the expression of this gene. Moreover, high L (+)-lactic acid production can also cause slower growth of *E. coli* JC13509, supported by the study of Dandan and co-workers in 2014 [70]. They found the competition between lactic acid production and cell growth in E. coli. The expression of lactate dehydrogenase can be resulted in a used of pyruvate converted to lactate, limiting metabolic flux for cell synthesis and eventually retarding cell growth. However, the ratio of L (+) and D (-)-lactic acid were higher in TW2 than JC13509 (Table C5), which is commercially preferable for industrial production, since the expense for L (+)and D (-)-lactic acid separation was high.

However, when two engineered strain with different plasmid system were compared, the TW2 strain was able to produced more L (+)-lactic acid than the RB24 strain, supported by Cheng and his colleague experiment [16]that different vector systems made the different performances of gene expression. This may resulted from promoter strength and copy number of each strain. *R. oryzae ldhA* gene on TW2, a pUC 19 derivative controlled by *lac* promoter, was preferable for *E. coli* to express

recombinant protein than RB24, a pBluescript II derivative controlled by T7 promoter because the *lac* promoter was naturally found in normal *E. coli*. The result from real time PCR (Figure 4.10) revealed the higher relative quantification graph from expression of *ldhA* gene from *R. oryzae* from the TW2 than RB24 strain, supported this hypothesis of preferable *lac* promoter. Moreover, the LDH activity from TW2 was also higher than RB24 (Table 4.3), which may come from the higher gene expression, the higher enzyme produced, leading to higher enzyme activity. Furthermore, the promoter can also cause different copy numbers in each strain. Craig W. Adams and *G.* Wesley Hatfiel [71] carried out the experiment to explain the effect of promoter strength on plasmid copy numbers and discovered that strong promoter could cause the decrease in plasmid copy numbers, possibly caused by tighter binding of RNA polymerase to the promoter leading to slower rate of transcription. In this study, T7 promoter on pRB85 in the RB24 strain was stronger than *lac* promoter on pUC19 in the TW2 strain and may cause the lower copy number of *ldhA* mRNA, leading to lower production of LDH enzyme, and finally lower production of L (+)-lactic acid from RB24. When pyruvate, a substrate for LDH activity in *E. coli* fermentative pathway (Figure 2.6), in the cell was determined, it was corresponded with LDH activity that pyruvate in TW2 was lower than that of RB24 (Table 4.3), suggesting that high amount of pyruvate was converted to other products, so small amount was remained in the cell.

Although the result of qRT-PCR, LDH activity and intracellular pyruvate concentration (Figure 4.11, Table 4.3, and, Table 4.4 respectively) suggested that the higher expression of *R. oryzae ldhA* gene and higher function of LDH from TW2 strain than those of RB24, the unexpected production L (+)-lactic acid production was still observed (Figure 4.8). Due to the deletion of chromosomal *ldhA* and *pta* pathways in TW2 strain (Figure 2.6), there was a possibility that pyruvate may converted into ethanol pathway, then low L (+)-lactic production was observed, even though glucose was used up (Figure 4.8) and low pyruvate concentration was detected (Table 4.4). Furthermore, the LDH activity was determined by the conversion of NAD^+ to NADH that can be catalyzed not only by LDH, but also the products of *aldh* and *adhE* in ethanol pathway

(Figure 2.6). Therefore, LDH activity reported in Table 4.3 was possibly from the NADH generated in ethanol pathway. To prove this possibility, the concentration of ethanol will be further measured. Moreover, it was suggested that, by deleting genes in the competing fermentation pathway in *E. coli*, including *pflB*, *pta*, *adhE*, *frdABCD*, *ald*) an increase in D (-)-lactic acid production was observed because other byproduct production pathways were blocked. Furthermore, it was also suggested that the inhibition of ethanol production pathway may increase L (+)-lactic acid production as well [8] since *pta* and *E. coli ldhA* in TW2 was deleted, metabolic flux should flow to *R. oryzae ldhA* which control L (+)-lactic acid production.

CHAPTER V
CONCLUSION

Conclusion

To improve L (+)-lactic acid production, the genetic modification of *E. coli* was applied by harboring *R. oryzae ldhA* responsible for optical pure L (+)-lactic acid production in its own fungus species. In this study, gene replacement technique was first selected due to the benefit of gene stability, therefore antibiotic was not required during the fermentation. This should lead to easier manipulation and save some costs in the production. However, according to the limitation of gene replacement that at least 40 bp homologous region is required, the new DNA fragment for doing this is needed to be constructed. The fragment was designed by flanking the ORF of *R. oryzae ldhA* (Fragment 2) with upstream (Fragment 1) and downstream (Fragment 3) regions of *E. coli ldhA*. Although, Fragment 1 and 2 was successfully fused by crossover PCR (Fig. 4.2), the connection between Fragment 1+2 and 3 was unsuccessful. It is possible that the designed fragment is too large for this kind of *Taq* DNA polymerase and this PCR technique. The other strategy was attempted by using different plasmid systems. Although using plasmid can cause lower gene stability and antibiotic is required to maintain the plasmid, one of the advantages is higher copy number of gene and gene

expression. Moreover, some studies suggested the different effect of gene expression on different plasmid systems. In this study, the *E. coli* TW2 strain with pUC19 harboring *R. oryzae ldhA* was compared with the RB24 strain with pBluescript II KS(+) harboring the same gene. The results revealed that TW2 strain gave higher amount of L (+)-lactic acid production than that of RB24 strain. Moreover, higher *R.oryzae ldhA* expression and higher LDH activity with lower concentration of intracellular pyruvate were observed in the TW2 strain. This may be resulted from lower promoter strength in pUC19 leading to produce higher copy number of target gene. Although the result pointed out the possibility of TW2 strain to produce L (+)-lactic acid with high efficiency, the low yield of target product was unexpected observed. It is possible that the main fermentative pathway in TW2 strain was shifted to ethanol pathway controlled by *aldh* and *adhE* genes. Furthermore, among the selected strains, the highest yield of lactic acid observed in wild type strain (JC13509) may resulted from the function of other lactate dehydrogenases in normal *E. coli*.

Suggestion

- For gene replacement technique, a new primer design is required to combine all DNA fragments together. Moreover, and PCR parameters are required to be optimized. The other DNA fusion techniques are also needed to be considered to generate this large DNA fragment.
- Since *R. oryzae* is a eukaryotic microorganism, *ldhA* gene from *R. oryzae* might not express properly in *E. coli* host which is a prokaryotic microorganism. A study about eukaryotic gene express in prokaryotic microorganism is needed.
- The results revealed that the TW2 strain could be use most of glucose for fermentation and the lowest amount of pyruvate was remained in the cells. However, the unexpected low yield of L (+)-lactic acid was observed. This suggested that the substrate was already converted to other products, besides L (+)-lactic acid. Because the LDH activity was determined by the oxidation of NADH which also occurred in ethanol pathway, it is possible that, in TW2 strain, substrates in fermentative pathway were converted to

ethanol. To prove this hypothesis, the concentration of ethanol is required to be measured.

- To improve L (+)-lactic acid production from our engineered *E. coli*, inhibition of other fermentative pathways in *E. coli* should increase L (+)-lactic acid production efficiency by making lactate pathway as sole or main fermentative pathway, for example, inhibiting *aldh* and *adhE* genes in ethanol production pathway

REFERENCES

1. Abdel-Rahman, M.A., Y. Tashiro, and K. Sonomoto, *Recent advances in lactic acid production by microbial fermentation processes.* Biotechnology advances, 2013. **31**(6): p. 877-902.
2. Skory, C.D., *Isolation and expression of lactate dehydrogenase genes from Rhizopus oryzae.* Applied and environmental microbiology, 2000. **66**(6): p. 2343-2348.
3. Nancib, A., et al., *Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by Lactobacillus casei subsp. rhamnosus.* Bioresource Technology, 2005. **96**(1): p. 63-67.
4. KOCHHAR, S., et al., *Cloning and overexpression of Lactobacillus helveticusd ‐ lactate dehydrogenase gene in Escherichia coli.* European Journal of Biochemistry, 1992. **208**(3): p. 799-805.
5. Contag, P., M. Williams, and P. Rogers, *Cloning of a lactate dehydrogenase gene from Clostridium acetobutylicum B643 and expression in Escherichia coli.* Applied and environmental microbiology, 1990. **56**(12): p. 3760-3765.
6. Wyckoff, H., et al., *Cloning, sequence, and expression of the L-(+) lactate dehydrogenase of Streptococcus bovis.* Current microbiology, 1997. **34**(6): p. 367-373.
7. Zhao, J., et al., *Homofermentative production of optically pure L-lactic acid from xylose by genetically engineered Escherichia coli B.* Microbial cell factories, 2013. **12**(1): p. 57.
8. Wang, Y., et al., *Homofermentative production of D-lactic acid from sucrose by a metabolically engineered Escherichia coli.* Biotechnology letters, 2012. **34**(11): p. 2069-2075.
9. Dien, B., N. Nichols, and R. Bothast, *Recombinant Escherichia coli engineered for production of L-lactic acid from hexose and pentose sugars.* Journal of industrial microbiology & biotechnology, 2001. **27**(4): p. 259-264.

10. Boonsombat, R., *Production of L-lactic Acid from Escherichia coli Harboring Recombinant Plasmid with Rhizopus oryzae ldhA Gene.* Life Science Journal, 2013. **10**(4).
11. Chand, S., et al., *Biotechnology in India II*. Vol. 85. 2003: Springer.
12. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proceedings of the National Academy of Sciences, 2000. **97**(12): p. 6640-6645.
13. Choi, Y.J., et al., *Metabolic engineering of Escherichia coli for the production of 1-propanol.* Metabolic engineering, 2012. **14**(5): p. 477-486.
14. Peretti, S.W. and J.E. Bailey, *Simulations of host–plasmid interactions in Escherichia coli: Copy number, promoter strength, and ribosome binding site strength effects on metabolic activity and plasmid gene expression.* Biotechnology and bioengineering, 1987. **29**(3): p. 316-328.
15. McKay, D.B., et al., *Heterologous expression of biphenyl dioxygenase-encoding genes from a gram-positive broad-spectrum polychlorinated biphenyl degrader and characterization of chlorobiphenyl oxidation by the gene products.* Journal of bacteriology, 1997. **179**(6): p. 1924-1930.
16. Wang, C., et al., *Heterologous gshF gene expression in various vector systems in Escherichia coli for enhanced glutathione production.* Journal of biotechnology, 2015. **214**: p. 63-68.
17. https://pubchem.ncbi.nlm.nih.gov/compound/lactic_acid#section=Top.
18. https://biology.stackexchange.com/questions/20002/what-happens-to-lactic-acid-i-eat.
19. Datta, R., et al., *Technological and economic potential of poly (lactic acid) and lactic acid derivatives.* FEMS microbiology reviews, 1995. **16**(2-3): p. 221-231.
20. Vijayakumar, J., R. Aravindan, and T. Viruthagiri, *Recent trends in the production, purification and application of lactic acid.* Chemical and biochemical engineering quarterly, 2008. **22**(2): p. 245-264.

21. Wee, Y.-J., J.-N. Kim, and H.-W. Ryu, *Biotechnological production of lactic acid and its recent applications.* Food Technology and Biotechnology, 2006. **44**(2): p. 163-172.

22. Alsaheb, R.A.A., et al., *Recent applications of polylactic acid in pharmaceutical and medical industries.* J Chem Pharm Res, 2015. **7**: p. 51-63.

23. Garlotta, D., *A literature review of poly (lactic acid).* Journal of Polymers and the Environment, 2001. **9**(2): p. 63-84.

24. Ghaffar, T., et al., *Recent trends in lactic acid biotechnology: A brief review on production to purification.* Journal of Radiation Research and Applied Sciences, 2014. **7**(2): p. 222-229.

25. Martinez, F.A.C., et al., *Lactic acid properties, applications and production: a review.* Trends in food science & technology, 2013. **30**(1): p. 70-83.

26. Narayanan, N., P.K. Roychoudhury, and A. Srivastava, *L (+) lactic acid fermentation and its product polymerization.* Electronic journal of Biotechnology, 2004. **7**(2): p. 167-178.

27. Saeed A, H. and I. Salam A, *Current limitations and challenges with lactic acid bacteria: A review.* Food and Nutrition Sciences, 2013. **2013**.

28. Zhou, S., et al., *Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered Escherichia coli W3110.* Applied and Environmental Microbiology, 2003. **69**(1): p. 399-407.

29. http://textbookofbacteriology.net/lactics_2.html.

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

31. Oda, Y., et al., *Lactic acid fermentation of potato pulp by the fungus Rhizopus oryzae.* Current Microbiology, 2002. **45**(1): p. 1-4.

32. Zhou, Y., et al., *Optimization of L-lactic acid production from glucose by Rhizopus oryzae ATCC 52311.* Applied biochemistry and biotechnology, 1999. **78**(1-3): p. 401-407.

33. Soccol, C., et al., *Potential of solid state fermentation for production of L (+)-lactic acid by Rhizopus oryzae.* Applied Microbiology and Biotechnology, 1994. **41**(3): p. 286-290.

34. Hang, Y., H. Hamamci, and E. Woodams, *Production of L (+)-lactic acid byRhizopus oryzae immobilized in calcium alginate gels.* Biotechnology letters, 1989. **11**(2): p. 119-120.

35. Tay, A. and S.T. Yang, *Production of L (+) ‐ lactic acid from glucose and starch by immobilized cells of Rhizopus oryzae in a rotating fibrous bed bioreactor.* Biotechnology and bioengineering, 2002. **80**(1): p. 1-12.

36. Maas, R.H., et al., *Lactic acid production from xylose by the fungus Rhizopus oryzae.* Applied microbiology and biotechnology, 2006. **72**(5): p. 861-868.

37. Engel, C.A.R., et al., *Fumaric acid production by fermentation.* Applied microbiology and biotechnology, 2008. **78**(3): p. 379-389.

38. Meussen, B.J., et al., *Metabolic engineering of Rhizopus oryzae for the production of platform chemicals.* Applied microbiology and biotechnology, 2012. **94**(4): p. 875-886.

39. Vodnar, D.C., et al., *L (+)-lactic acid production by pellet-form Rhizopus oryzae NRRL 395 on biodiesel crude glycerol.* Microbial cell factories, 2013. **12**(1): p. 92.

40. Ghosh, B. and R.R. Ray, *Current commercial perspective of Rhizopus oryzae: a review.* J Appl Sci, 2011. **11**(14): p. 2470-2486.

41. Park, E.Y., Y. Kosakai, and M. Okabe, *Efficient Production of l ‐ (+) ‐ Lactic Acid Using Mycelial Cotton ‐ like Flocs of Rhizopusoryzae in an Air ‐ Lift Bioreactor.* Biotechnology progress, 1998. **14**(5): p. 699-704.

42. Coban, H.B. and A. Demirci, *Enhancement and modeling of microparticle-added Rhizopus oryzae lactic acid production.* Bioprocess and biosystems engineering, 2016. **39**(2): p. 323-330.

43. Hamamci, H. and D.D. Ryu, *Production of L (+)-lactic acid using immobilized Rhizopus oryzae reactor performance based on kinetic model and simulation.* Applied biochemistry and biotechnology, 1994. **44**(2): p. 125-133.

44. https://en.wikipedia.org/wiki/Escherichia_coli.

45. https://microbewiki.kenyon.edu/index.php/Escherichia_coli.

46. Catalanotti, C., et al., *Fermentation metabolism and its evolution in algae.* Frontiers in plant science, 2013. **4**.

47. Stokes, J., *Fermentation of glucose by suspensions of Escherichia coli.* Journal of bacteriology, 1949. **57**(2): p. 147.

48. Förster, A.H. and J. Gescher, *Metabolic engineering of Escherichia coli for production of mixed-acid fermentation end products.* Frontiers in bioengineering and biotechnology, 2014. **2**.

49. Bzik, D.J., B.A. Fox, and K. Gonyer, *Expression of Plasmodium falciparum lactate dehydrogenase in Escherichia coli.* Molecular and biochemical parasitology, 1993. **59**(1): p. 155-166.

50. Llanos, R.M., A.J. Hillier, and B.E. Davidson, *Cloning, nucleotide sequence, expression, and chromosomal location of ldh, the gene encoding L-(+)-lactate dehydrogenase, from Lactococcus lactis.* Journal of bacteriology, 1992. **174**(21): p. 6956-6964.

51. Zhou, S., K. Shanmugam, and L. Ingram, *Functional replacement of the Escherichia coli D-(−)-lactate dehydrogenase gene (ldhA) with the L-(+)-lactate dehydrogenase gene (ldhL) from Pediococcus acidilactici.* Applied and environmental microbiology, 2003. **69**(4): p. 2237-2244.

52. Mazumdar, S., J.M. Clomburg, and R. Gonzalez, *Escherichia coli strains engineered for homofermentative production of D-lactic acid from glycerol.* Applied and environmental microbiology, 2010. **76**(13): p. 4327-4336.

53. Skory, C.D., *Lactic acid production by Saccharomyces cerevisiae expressing a Rhizopus oryzae lactate dehydrogenase gene.* Journal of industrial microbiology & biotechnology, 2003. **30**(1): p. 22-27.

54. Chang, D.-E., et al., *Homofermentative production of d-orl-lactate in metabolically engineered Escherichia coli RR1.* Applied and Environmental Microbiology, 1999. **65**(4): p. 1384-1389.

55. Wang, Y., et al., *Engineering and adaptive evolution of Escherichia coli W for L-lactic acid fermentation from molasses and corn steep liquor without additional nutrients.* Bioresource technology, 2013. **148**: p. 394-400.

56. Yu, D., et al., *An efficient recombination system for chromosome engineering in Escherichia coli.* Proceedings of the National Academy of Sciences, 2000. **97**(11): p. 5978-5983.

57. http://www.medwelljournals.com/fulltext/?doi=javaa.2011.2090.2094.

58. Wu, X., et al., *In vivo functional expression of a screened P. aeruginosa chaperone-dependent lipase in E. coli.* BMC biotechnology, 2012. **12**(1): p. 58.

59. https://www.addgene.org/50005/.

60. https://www.addgene.org/vector-database/1944/.

61. Dashti, A.A., et al., *Heat treatment of bacteria: a simple method of DNA extraction for molecular techniques.* Kuwait Med J, 2009. **41**(2): p. 117-122.

62. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual*. 1989: Cold spring harbor laboratory press.

63. Martinez, A., et al., *Low salt medium for lactate and ethanol production by recombinant Escherichia coli B.* Biotechnology letters, 2007. **29**(3): p. 397-404.

64. Zhou, S., K.T. Shanmugam, and L.O. Ingram, *Functional replacement of the Escherichia coli D-lactate dehydrogenase gene (ldhA) with the L-lactate dehydrogenase gene (ldhL) from Pediococcus acidilactici.* Applied and Environmental Microbiology, 2003. **69**(4): p. 2237-44.

65. Kruger, N.J., *The Bradford method for protein quantitation.* Basic protein and peptide protocols, 1994: p. 9-15.

66. https://en.wikipedia.org/wiki/Lactate_dehydrogenase.

67. Lorenz, T.C., *Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies.* Journal of visualized experiments: JoVE, 2012(63).

68. http://www.sciencedirect.com/topics/neuroscience/primer-dimer.

69. Nishimura, T., et al., *Gene expression profiling of Corynebacterium glutamicum during anaerobic nitrate respiration: induction of the SOS response for cell survival.* Journal of bacteriology, 2011. **193**(6): p. 1327-1333.

70. Niu, D., et al., *Highly efficient L-lactate production using engineered Escherichia coli with dissimilar temperature optima for L-lactate formation and cell growth.* Microbial cell factories, 2014. **13**(1): p. 78.

71. Adams, C.W. and G.W. Hatfield, *Effects of promoter strengths and growth conditions on copy number of transcription-fusion vectors.* Journal of Biological Chemistry, 1984. **259**(12): p. 7399-7403.

APPENDIX

APPENDIX A

MEDIA AND SOLUTIONS

1. Antibiotics

Ampicillin (100mg/ml)

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

Chloramphenicol (25mg/ml)

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

Kanamycin (50mg/ml)

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

2. LB broth

Peptone	10 g
Yeast extract	5 g
NaCl	10 g

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

3. LB agar

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

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

4. Salt solution

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

$FeSO_4.7H_2O$	0.02 g

Dissolve in 10 ml of dH_2O

5. **Cultivation plate and slant**

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

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

6. **Pre-culture broth**

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

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

7. **Fermentation broth**

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

Salt solution	5 ml

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

8. 5X 56 Phosphate buffer

Na_2HPO_4	48.4 g
KH_2PO_4	25.5 g

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

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

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

9. 56/2 Phosphate buffer

dH_2O	500 ml
5X 56 Phosphate buffer	500 ml

Autoclave 121 °C for 15 min.

10. TB-I (Transformation buffer I)

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

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

11. TB-II (Transformation buffer II)

MOPS	1.04 g
$CaCl_2$	5.5 g
RbCl	0.6 g

15% glycerol	75 ml

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

12. 50X TAE

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

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

13. 1X TAE

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

APPENDIX B

ldhA GENE MAP FROM *Escherichia coli* AND *Rhizopus oryzae*

Figure B1 The approximately 1800 bp of nucleotide sequence from *R. oryzae ldhA* inserted in pRB24 and pTW plasmid. This gene does not contain any introns and the ORF is in red. The theoretical promoter (CAAT and TATA sites) is represented in pink boxes. The ATG start and TAA stop codon are represented in yellow and sky blue boxes, respectively. The polyadenation signal, part of mature mRNA production, is highlighted in grey. The *EcoR*I and primer binding sites are also indicated.

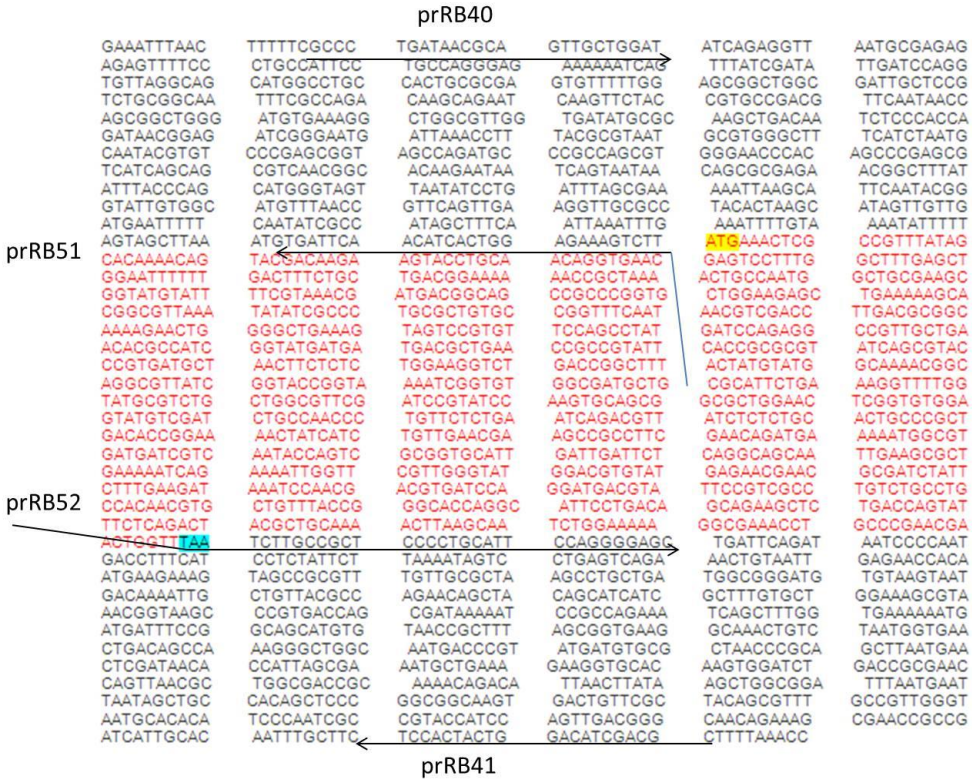


Figure B2 The nucleotide sequence from *E. coli ldhA* used for gene replacement method. The ORF is in red. The ATG start and TAA stop codon are represented in yellow and sky blue boxes, respectively. The primer binding sites are also indicated.

APPENDIX C

DETERMINATION OF LACTIC ACID AND GLUCOSE CONCENTRATION BY HPLC

1. Standard curve of glucose

For the standard curve, 6, 5, 4, 3, 2 and 1 g/l of standard glucose were applied in HPLC

Table C1 Concentrations of standard glucose and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector.

Standard glucose concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
6	992870	1004006	1081078	1025985±48036.02
5	892775	879351	847612	870246±22581.68
4	616953	677032	N/A	646993±42482.27
3	553366	537108	N/A	545237±11496.14
2	355174	369514	363464	362717±7199.099
1	196095	182813	186496	6857±074

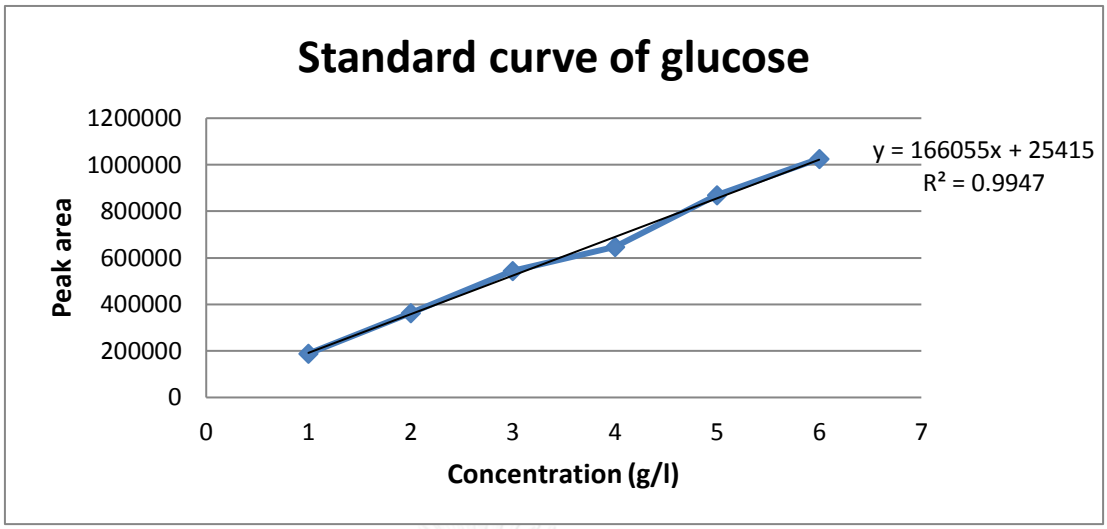


Figure C1 A glucose standard curve from HPLC by using Aminex HPX-87H column with RI detector for glucose determination.

The equation from the graph in Figure C1 was used to calculate the glucose concentration in each fermented sample. From this graph, the glucose concentration was calculated as the following:

$$y = 166055x + 25415$$

Which y was the peak area of each sample and x was the glucose concentration in each sample. With this calculation, the average glucose concentrations from JC13509, RB7, RB24 and TW2 strains (from 3 repeats of each set of experiment) are represented in Table C1

.

2. Standard curve of Lactic acid

For the standard curve, 6, 5, 4, 3, 2 and 1 g/l of standard L (+)-lactic acid were applied in HPLC.

Table C2 Concentrations of standard Lactic acid and the average peak areas measured by HPLC using Aminex HPX-87H column with RI detector.

Standard Lactic acid concentration (g/l)	Peak area			Average (Mean±S.D.)
	1	2	3	
6	758122	831619	787121	792287±37019.87
5	622344	638785	664810	641980±21412.49
4	543222	512169	508118	521170±19204.99
3	352997	454142	365162	390767±55220.37
2	237584	239534	205963	227694±18844.55
1	121187	121626	128786	123866±4266.207

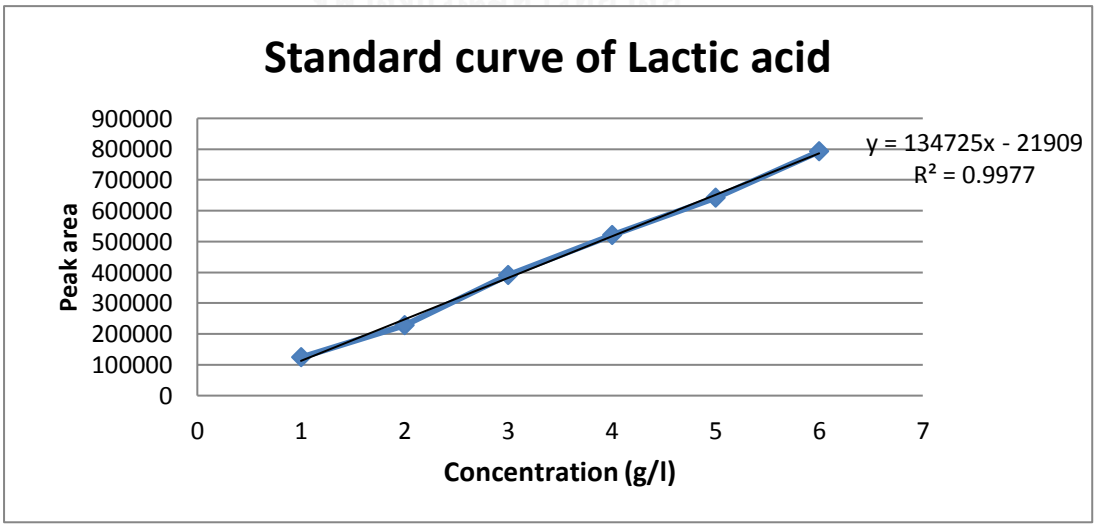


Figure C2 A lactic acid standard curve from HPLC by using Aminex HPX-87H column with RI detector for Lactic acid determination.

The equation from the graph in Figure C2 was used to calculate the Lactic acid concentration in each fermented sample. From this graph, the Lactic acid concentration was calculated as the following:

$$y = 134725x - 21909$$

Which y was the peak area of each sample and x was the L (+)-lactic acid concentration in each sample. With this calculation, the average L (+)-lactic acid concentrations from JC13509, RB7, RB24, TW1 and TW2 strains (from 3 repeats of each set of experiment) are represented in Table C2.

3. The concentrations of residual glucose and Lactic acid from all strains in this study

Table C3 The concentrations of residual glucose and Lactate measured by using a high-pressure liquid chromatography (HPLC) equipped with Animex HPX-87H column and Chiral column, from all strains constructed in this research.

Strains	Concentration (g/l) (Mean±S.D.)			
	Residual Glucose	Lactate	D-lactate	L-lactate
JC13509	3.970±0.434	10.769±1.593	2.613±1.069	8.053±0.847
RB7	1.67±1.355	2.795±0.577	0.445±0.162	3.028±0.799
RB24	5.415±0.317	0.658±0.071	1.014±0.070	0.609±0.049
TW2	0.155±0.028	3.344±0.361	0.458±0.303	3.319±0.911

4. The yield production of lactic acid

Table C4 The yield production of lactic acid calculated by gram of product production/gram of glucose consumption.

Strains	Yield (Mean±S.D.)		
	Lactate	D-lactate	L-lactate
JC13509	0.615±0.007	0.126±0.027	0.472±0.017
RB7	0.171±0.009	0.029±0.001	0.191±0.012
RB24	0.045±0.005	0.069±0.004	0.042±0.003
TW2	0.168±0.018	0.023±0.015	0.167±0.046

5. The ratio of L (+) and D (-)-lactic acid

Table C5 The ratio of L (+) and D(-)-lactic acid from each strain calculated by gram of L (+)-lactic acid/gram of D (-)-lactic acid.

Strains	Ratio of L (+) and D (-)-lactic acid
JC13509	3.082±1.084
RB7	6.81±0.961
RB24	0.601±0.089
TW2	7.255±3.614

APPENDIX D

DETERMINATION OF LDH ACTIVITY AND TOTAL CELL PROTEIN

1. Standard curve of NADH

For the standard curve, the UV absorbance from the reactions of 0.32, 0.16, 0.08, 0.04, 0.02, 0.01 and 0 µmol/ml of standard NADH with the volume of 200 µl/well were measured at 340 nm by microplate spectrophotometer. The absorbance values are represented in Table D1.

Table D1 The amount of standard NADH and its average 340 nm absorbance value for creating the standard NADH curve in Figure D1.

NADH (µmole)	Absorbance at 340 nm			Average (Mean±S.D.)
	1	2	3	
0.000	0.111	0.105	0.104	0.107±0.004
0.01	0.11	0.11	0.108	0.109±0.001
0.02	0.116	0.117	0.118	0.117±0.001
0.04	0.151	0.153	0.137	0.147±0.009
0.08	0.196	0.195	0.179	0.19±0.01
0.16	0.317	0.306	0.287	0.303±0.015
0.32	0.522	0.537	0.461	0.507±0.040

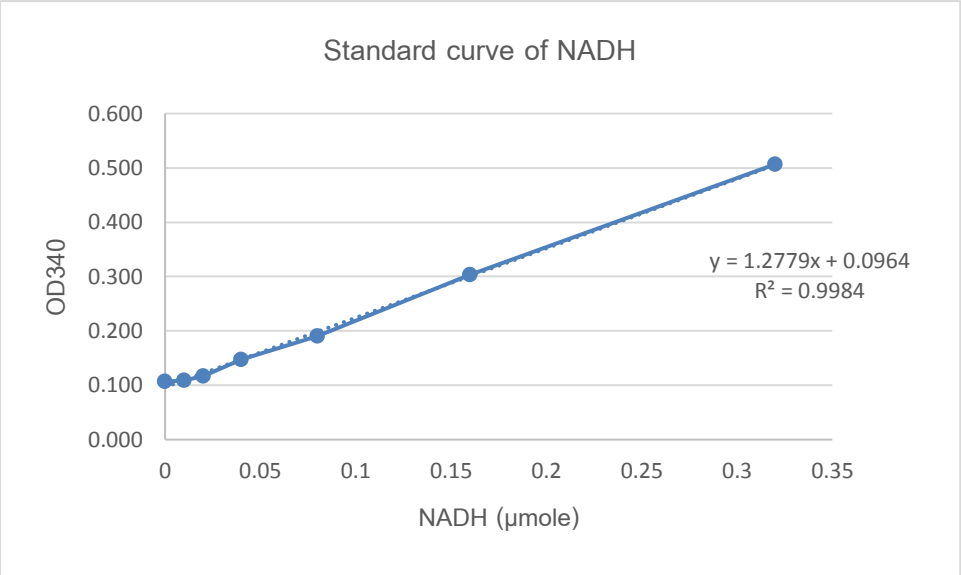


Figure D1 A standard NADH curve for calculating the amount of NADH in the reaction of LDH activity assay

The equation from the graph in Figure D1 was used to calculate the amount of NADH in the reaction of LDH activity assay. The amount of NADH was calculated as the following:

$$y = 1.2779x + 0.0964$$

which y was the absorbance value at 340 nm of each reaction and x is the amount of NADH in the reaction of each sample. With this calculation, the average amount of NADH from JC13509, RB7, RB24, TW1 and TW2 strains (from 3 repeats of each set of experiment) are represented in Table D1

Table D2 The absorbance value at 340 nm of each *E. coli* strain after fermented 48 hours by using LDH activity assay.

Sample	Absorbance at 340 nm			Average (Mean±S.D.)
	1	2	3	
Blank	0.111	0.105	0.104	0.107±0.004
JC13509	0.718	0.71	0.799	0.742±0.049
RB7	0.697	0.838	0.803	0.779±0.073
RB24	0.729	0.702	0.751	0.727±0.024
TW2	0.709	N/A	0.706	0.707±0.002

Table D3 The amount of NADH of each *E. coli* strain after fermented 48 hours by using LDH activity assay

Sample	NADH (µmole)			Average (Mean±S.D.)
	1	2	3	
JC13509	0.403	0.396	0.466	0.422±0.038
RB7	0.387	0.496	0.469	0.451±0.057
RB24	0.411	0.39	0.429	0.41±0.019
TW2	0.395	N/A	0.393	0.394±0.002

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

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

which a was the initial amount of NADH (0.192 µmol), b was the molecular weight of NADH (665.43 g/mol), and c was the molecular weight of NAD^+(664.43 g/mole). The

average NAD^+ productions (from 3 repeats of each set of experiment) from JC13509, RB7, RB24 and TW2 strains from this equation is represented in Table D4.

Table D4 The amount of NAD^+ produced of each selected *E. coli* strain after being fermented for 48 hours.

Sample	Produced NAD^+ (μmole)			Average
	1	2	3	(Mean±S.D.)
JC13509	0.21	0.204	0.273	0.229±0.038
RB7	0.194	0.304	0.277	0.258±0.057
RB24	0.219	0.198	0.236	0.218±0.019
TW2	0.203	N/A	0.201	0.202±0.002

2. Standard curve of BSA

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

Table D5 Concentrations of standard BSA and the average 595 nm absorbance values for creating the standard BSA curve in Figure D2.

BSA	Absorbance at 595 nm			Average
(μg/ml)	1	2	3	(Mean±S.D.)
0	0.686	0.68	0.689	0.685±0.005
5	0.669	0.735	0.702	0.702±0.033
10	0.693	0.742	0.706	0.714±0.025
20	0.736	0.781	0.762	0.76±0.023
50	0.796	0.841	0.833	0.823±0.024
100	0.965	0.973	0.97	0.969±0.004
250	1.26	1.324	1.3	1.295±0.032

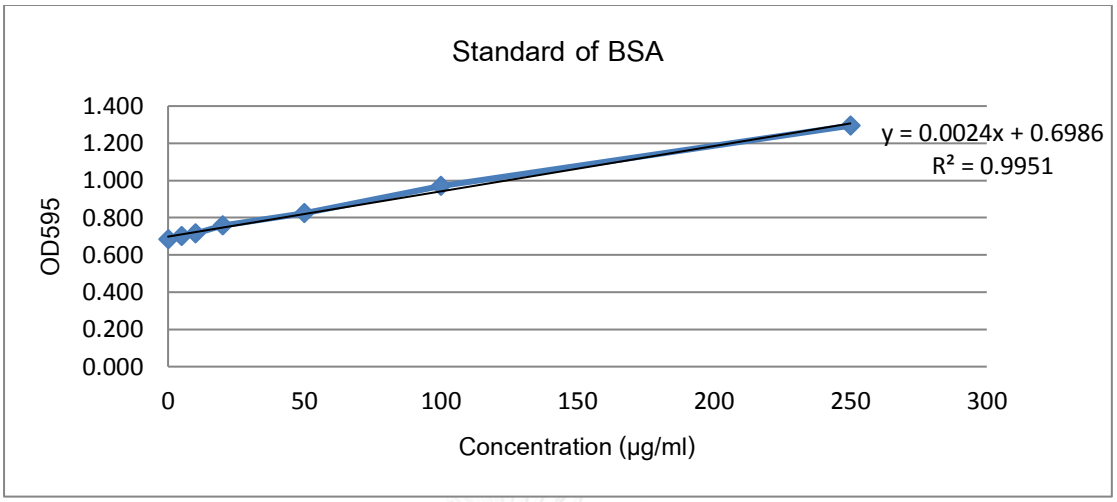


Figure D2 A standard BSA curve for total protein determination from Bradford assay measured at 595 nm by microplate spectrophotometer.

The equation from the graph in Figure D2 was used to calculate the total protein in Bradford assay. The total protein was calculated as the following:

$$y = 0.0024x + 0.6986$$

which y was the 595nm absorbance value of each sample and x is the total protein concentration of each sample. With this calculation, the average values of the total protein from JC13509, RB7, RB24, and TW2 (from 3 repeats of each set of experiment) are represented in Table D7.

Table D6 The 595 nm. absorbance value of each selected *E. coli* strain after being fermented for 48 hours

Sample	Absorbance at 595 nm			Average (Mean±S.D.)
	1	2	3	
Blank	0.686	0.68	0.689	0.685±0.005
JC13509	1.208	1.252	1.184	1.215±0.034
RB7	1.291	1.226	1.202	1.24±0.046
RB24	1.216	1.187	1.161	1.188±0.028
TW2	1.067	N/A	0.804	0.935±0.186

Table D7 The total protein concentration of each selected *E. coli* strain after being fermented 48 hours.

Sample	Protein concentration (µg/ml)			Average (µg/ml)
	1	2	3	
JC13509	212.25	230.444	202.389	215.028±14.233
RB7	246.833	219.611	209.75	225.398±19.207
RB24	215.722	203.5	192.677	203.963±11.535
TW2	153.5	N/A	43.778	98.639±77.585

3. LDH activity calculation

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

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

which a was the amount of produced NAD^+ (μmole), b is the total protein (mg) and c is the time of reaction (min). The LDH activities of JC13509, RB7, RB24, AP3 and AP20 are represented in Table D8. The average values from 3 repeats of each set of experiment were summarized in Table 4.2

Table D8 The LDH activity of each selected strain after incubated for 5 min at room temperature.

Sample	Produced NAD^+(μmole)	Total protein (mg)*	Time (min)	LDH activity (μmole/mg.min)
JC13509(1)	0.210	0.002	5	19.823
JC13509(2)	0.204	0.002	5	17.716
JC13509(3)	0.273	0.002	5	27.018
RB7(1)	0.194	0.002	5	15.738
RB7(2)	0.304	0.002	5	27.674
RB7(3)	0.277	0.002	5	26.417
RB24(1)	0.219	0.002	5	20.277
RB24(2)	0.198	0.002	5	19.473
RB24(3)	0.236	0.002	5	24.515
TW2(1)	0.203	0.002	5	15.580
TW2(2)	N/A	N/A	5	N/A
TW2(3)	0.201	0.000	5	22.886

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

VITA

Mr. Wattanai kanjanapattanakul was born on October, 1989 in Chiangmai, Thailand. He graduate with a bachelor degree of science in field of Food science and nutrition from Faculty of Science, Srinakharinwirot University in 2011. He has studied for a Master degree of Science in Biotechnology, the Faculty of science, Chulalongkorn University since 2013.

Academic Presentation

Kanjanapattanakul, W., and Boonsombat, R., GENETIC MODIFICATION OF Escherichia coli FOR L-LACTIC ACID

PRODUCTION. The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference, 17-20 November 2015, Mandarin Hotel Bangkok by Centre Point, Bangkok, Thailand.