L-PIPECOLIC ACID PRODUCTION IN *thrA* KNOCKOUT *Escherichia coli*

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การผลิตกรดแอล-พิพีโคลิกใน *Escherichia coli* ที่มีการน็อกเอาท์ยีน *thrA*

นายสุทธิลักษณ์ ควนวิไล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุทธิลักษณ์ ควนวิไล : การผลิตกรดแอล-พิพีโคลิกใน *Escherichia coli* ที่มีการน็อกเอาท์ยีน *thrA*. (L-PIPECOLIC ACID PRODUCTION IN *thrA* KNOCKOUT *Escherichia coli*) อ.ที่ปรึกษาหลัก : ผศ. ดร.กนกทิพย์ ภักดีบำรุง

กรดแอล–พิพีโคลิก (L-pipecolic acid, L-PA) เป็นกรดอะมิโนที่ไม่พบในโครงสร้างของโปรตีน แต่มีความสำคัญในแง่ของการเป็นสารตั้งต้นหลักสำหรับการสังเคราะห์สารประกอบที่มีฤทธิ์ทางเภสัชกรรม เช่น ยากดภูมิคุ้มกัน (immunosuppressant) และ ยาชา (anesthetic) การผลิตกรดอะมิโนด้วยกระบวนการหมักของจุลชีพกำลังได้รับความสนใจ ซึ่งหนึ่งในนั้น คือ *Escherichia coli* ที่ผ่านการทำพันธุวิศวกรรม สำหรับการผลิต L-PA จาก *E. coli* ที่ผ่านการทำพันธุวิศวกรรมนั้น L-lysine ในเซลล์จะถูกใช้เป็นสารตั้งต้น ดังนั้นงานวิจัยนี้ได้ศึกษาการเพิ่มปริมาณการผลิต L-PA โดยการน็อกเอาท์ยีน *thrA* ซึ่ง เข้ารหัสให้ homoserine dehydrogenase I ซึ่งเป็นเอนไซม์ที่นำเอาสารตัวกลางในวิถีการสังเคราะห์ไลซีนไปใช้ในการสังเคราะห์แอล-ทรีโอนีน ในการทำลายยีน *thrA* ได้ใช้วิธีการแทรกของอินทรอนกลุ่ม 2 ซึ่งมีความจำเพาะในการแทรกเข้าในยีนเป้าหมาย พบว่าประสิทธิภาพของอินทรอนกลุ่ม 2 ในการแทรกเข้ายีน *thrA* ใน *E. coli* นั้นคิดเป็น 65% โดยกำหนดชื่อ *E. coli* BL21(DE3) ที่ถูกน็อกเอาท์ยีน *thrA* นี้ว่า *E. coli* BL21(DE3) Δ*thrA* นอกจากนี้ได้ทำการสร้างรีคอมบิแนนท์พลาสมิด pE22-LPC*D* แล้ว ทรานส์ฟอร์มเข้าสู่ *E. coli* BL21(DE3) (ให้ชื่อว่า W-LPCD) และ *E. coli* BL21(DE3) Δ*thrA* (ให้ชื่อว่า KO-LPCD) โดย pE22-LPC*D* ประกอบด้วย *lysdh* (เข้ารหัสให้ lysine 6-dehydrogenase) จาก *Acromobacter denitrificans*, *proC* (เข้ารหัสให้ pyrroline-5-carboxylate reductase) จาก *Bacillus cereus* ATCC 11778 และ *lysC** และ *dapA** ซึ่งเข้ารหัสให้ aspartokinase และ dihydrodipicolinate synthase ที่ต้านการยับยั้งแบบย้อนกลับด้วยไลซีน ตามลำดับ การผลิต L-PA สูงสุดที่ประมาณ 0.57 กรัมต่อลิตร เมื่อเลี้ยงในอาหาร Ying ที่ใช้กลีเซอรอลเป็นแหล่งคาร์บอน หลังการเหนี่ยวนำด้วย IPTG 0.1 มิลลิโมลาร์เป็นเวลา 168 ชั่วโมง โดยมีค่า specific L-PA production เท่ากับ 0.049 กรัมต่อกรัมของเซลล์เปียก ซึ่งคิดเป็น 1.8 เท่า เมื่อเทียบกับค่าของ W-LPCD

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต .....
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Suttilak Khuanwilai : L-PIPECOLIC ACID PRODUCTION IN *thrA* KNOCKOUT *Escherichia coli*. Advisor: Asst. Prof. KANOKTIP PACKDIBAMRUNG, Ph.D.

L-pipecolic acid (L-PA) is a non-proteinogenic amino acid, however, it is an important precursor and a key ingredient of many pharmaceutically compound syntheses such as immunosuppressants and anesthetics. The production of amino acids via fermentation of microorganisms are getting attention, one of the widely used is an engineered *E. coli*. L-PA production in the engineered *E. coli* uses L-lysine in its cell as a substrate. This research aimed to increase L-PA production by *thrA* knockout. The *thrA* encodes homoserine dehydrogenase I, the enzyme which draws the intermediate in L-lysine biosynthesis pathway to synthesize L-threonine. The group II intron insertion was used for disruption of *thrA* by specific insertion at the target gene. The capability of group II intron insertion for *thrA* in *E. coli* (*E. coli* BL21(DE3) Δ*thrA*) was 65%. Moreover, pE22-LPC*D* was constructed and transformed into *E. coli* BL21(DE3) (namely W-LPCD) and *E. coli* BL21(DE3) Δ*thrA* (namely KO-LPCD). pE22-LPC*D* consisted of *lysdh* (encoding for lysine 6-dehydrogenase) from *Acromobacter denitrificans*, *proC* (encoding for pyrroline-5-carboxylate reductase) from *Bacillus cereus* ATCC 11778, and homologous *lysC** and *dapA** which encode for lysine feedback resistant aspartokinase and dihydrodipicolinate synthase, respectively. The highest L-PA production by KO-LPCD was 0.57 g/L when it was cultured in Ying medium containing glycerol as a carbon source at 198 hours after induction with 0.1 mM IPTG. The specific L-PA production was 0.049 g/g WCW, which was 1.8-fold of that obtained from W-LPCD.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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Suttilak Khuanwilai

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LIST OF ABBREVIATIONS

Ala	Alanine
bp	base pair
BLAST	basic local alignment search tool
BSA	bovine serum albumin
°C	degree Celsius
dNTP	2’-deoxynucleoside 5’-triphosphate
EDTA	ethylene diamine tetraacetic acid
g	gram
Glu	Glutamate
HPLC	high-performance liquid chromatography
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
L	liter
LB	Luria-Bertani
L-PA	L-pipecolic acid
Lys 6-DH	L-lysine-6-dehydrogenase
µg	microgram
µL	microliter

µM	micro molar
mA	milliampere
mg	milligram
mL	milliliter
mM	millimolar
M	mole per liter (molar)
ng	nanogram
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomole
SDS	sodium dodecyl sulfate
Thr	Threonine
Val	Valine
UV	ultraviolet

CHAPTER I

INTRODUCTION

Biomolecules are organic substances which take place naturally in living organisms. Most of them consist of carbon, hydrogen, oxygen, nitrogen in mainly and sometimes have sulfur and phosphorus. Biomolecules comprise macromolecules such as carbohydrate, protein, lipid and nucleic acid and small molecules such as monomer of macromolecules, secondary metabolites, and natural products. Nowadays, biomolecules can be applied for many features to increase their cost. For example, protein can be applied to generate biosensor, and sugar can be applied to synthesize nanomaterials.

One of the interesting biomolecules is an amino acid. Amino acids are organic compounds which have three main groups linked to α-carbon: carboxyl group (-COOH), amino group ($-NH_2$), and a side chain (R group). The main function of amino acids is the building block of protein in all organisms. However, the other group, non-proteinogenic amino acids also play a key role in living organisms. They are intermediates in biosynthesis and involve in protein post-translation. Moreover, they act as components of bacterial cell walls, neurotransmitters, and toxins. Interestingly, non-proteinogenic amino acids can be used as natural or man-made pharmacological compounds which commonly present in meteorites and in prebiotic. Since amino acids have many functions and involve in many biological processes, so they can be applied to many industries such as animal feed industry (increase essential amino acid), food industry (flavor enhancer by glutamic acid, nutrition improvement), agricultural industry (chelating ability for health of plants) and pharmaceutical and cosmetic industry (some derivatives of amino acids have pharmaceutical activity). Therefore, this study would focus on one amino acid, L-pipecolic acid.

1.1 L-pipecolic acid

L-pipecolic acid or L-PA is one of non-proteinogenic α-amino acids. The chemical structure of L-PA is homologous to proline: differing solely in the ring size. It is a carboxylic acid of piperidine. L-PA was first discovered as a plant constituent by Morrison and his coworker [1]. It is probably a product from the degradation of L-lysine and is broadly propagated in plants. In plants, L-PA acts as a critical regulator of plant systemic acquired resistance (plant SAR) [2]. The inadequate L-PA in plant cells is completely defective in SAR, and also present the reduction of basal resistance to bacterial infection [2] [3]. Moreover, L-PA could find in the other organisms, whether it be microorganism, animal, including human and could be a precursor of the natural bioactive molecule that involves communication between organisms [4]. In microorganism, it is a critical precursor of many useful microbial secondary metabolites, and it also acts as a compatible solute for several microorganisms [5] [6]. L-PA is detected in the hyphae of fungi and yeasts [7]. In mammals, there is a report from the experiment of Rothstein & Miller that L-PA plays a role in lysine metabolism [8]. In human, L-PA is synthesized and has been found in the form of physiological fluids (i.e., plasma, urine, and cerebrospinal fluid). The levels of L-PA in plasma of chronic liver diseases patients are higher than in normal one [9]. Patients with peroxisomal disorders, including Zellweger syndrome, infantile Refsum disease, or adrenoleukodystrophy also present high levels of L-PA in plasma, urine, and cerebrospinal fluid [10]. L-PA may play an important role in brain development, metabolism, and electrophysiology/ neurotransmitter regulation; however, its neurological roles are unclear [11].

L-PA also plays an important role in chemical, agricultural, and pharmaceutical industries [12] [13]. Especially, in the pharmaceutical industry, the chiral intermediates are more than 50% of all current drugs [14]. The high purity L-PA was used as a

precursor for the synthesis of many drug types such as immunosuppressor (macrolide-picolinate) rapamycin, FK-520, and FK-506 which are vital in the clinic [15] and the anesthetics bupivacaine, chloroprocaine and ropivacaine which vastly used in local anesthesia [16]. The chemical structure of drugs which use L-PA as a precursor are shown in Figure 1. Moreover, L-PA is one of the high-value compounds, and it has price around 968 USD per gram.

L-PA was first prepared by Ladenberg in 1891. This chemical method employs a hydrogenation reaction of α-picolinic acid hydrochloride using platinum oxide as a catalyst [17]. However, the chemical synthesis has many disadvantages such as unfriendly environment, high production cost, or unspecific product. Therefore, many research groups have attempted to synthesize L-PA via a biological process.

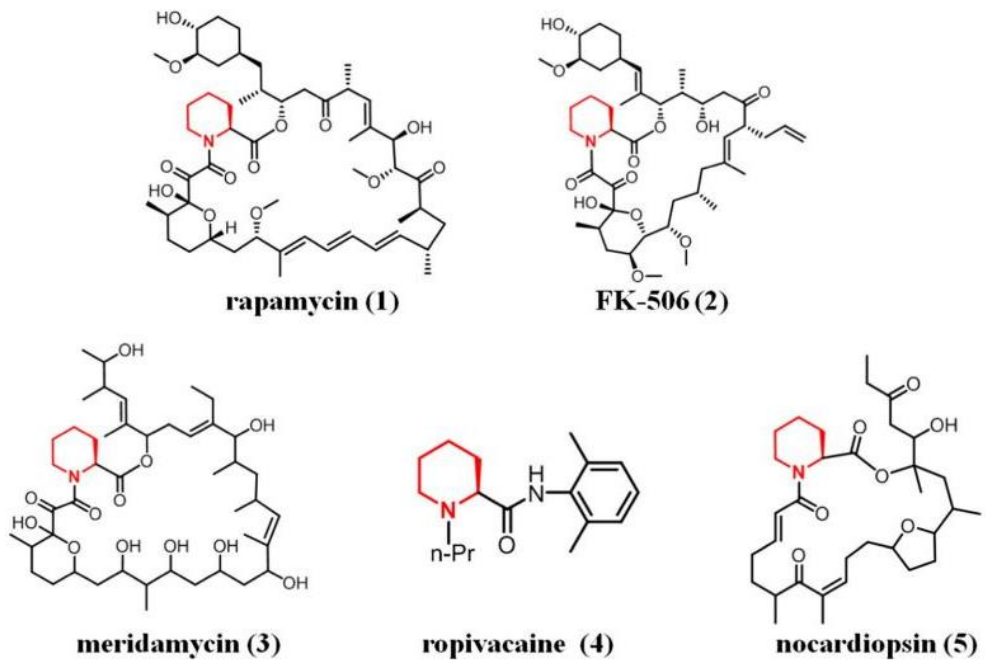


Figure 1 The chemical structure of pipecolate derivatives

The pipecolate groups are shown in red.

Source : Ying H. et al., 2017 [18]

1.2 L-pipecolic acid biosynthesis

Biosynthesis of L-PA has been vastly explored in many organisms because it closely relates to lysine metabolism [19]. In microorganisms, there are three main pathways which can convert L-lysine into L-PA. Firstly, the α-amino group is eliminated from lysine and the ε-nitrogen consolidates with the α-carboxyl group to generate L-PA. One important intermediate in this pathway is Δ^1-piperideine-2-carboxylate acid (P2C). Secondly, L-lysine is directly converted to L-PA using the activity of lysine cyclodeaminase [20]. Finally, the ε-nitrogen of L-lysine is eliminated and then α-nitrogen consolidates into L-PA via the important intermediate, Δ^1-piperideine-6- carboxylate acid (P6C) [21]. All of these pathways are shown in Figure 2.

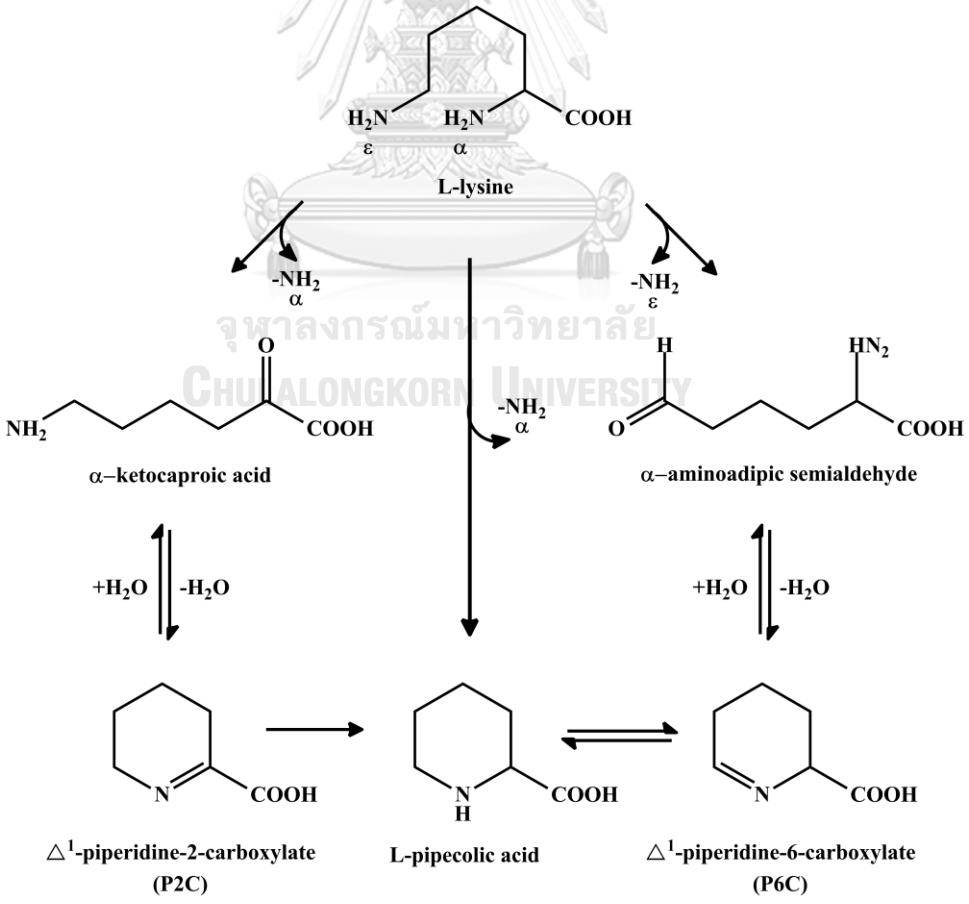


Figure 2 The pathways for converting L-lysine into L-pipecolic acid

In this study, we focused on the pathway which using P6C as an intermediate. Even though *E. coli* cannot synthesize L-PA in its cell, the transformation of some heterologous genes involving in L-PA synthesis could make *E. coli* to produce L-PA [22] [23]. So, our laboratory brought these pieces of knowledge to create *E. coli* strain, which could produce L-PA. This *E. coli* strain harbors *lysdh* encoding lysine-6-dehydrogenase (Lys 6-DH, ADK) from *Acromobacter denitrificans* K-1 [24] and *proC* encoding pyrroline-5-carboxylate reductase (P5CR) [25]. The reactions which converting L-lysine into L-PA by the activities of Lys 6-DH and P5CR are shown in Figure 3. These two genes were cloned into pET-17b to form pET-ADK-P5CR by Srimuang in 2010 [25] (Figure 4). After that, pET-ADK-P5CR was transformed into *E.coli* BL21 (DE3). After the cell was incubated in the reaction mixture containing 200 mM L-lysine in 200 mM Tris-HCl buffer, pH 9.0 for 24 hours, it was found that L-PA was produced approximately 1.74 g/L [25].

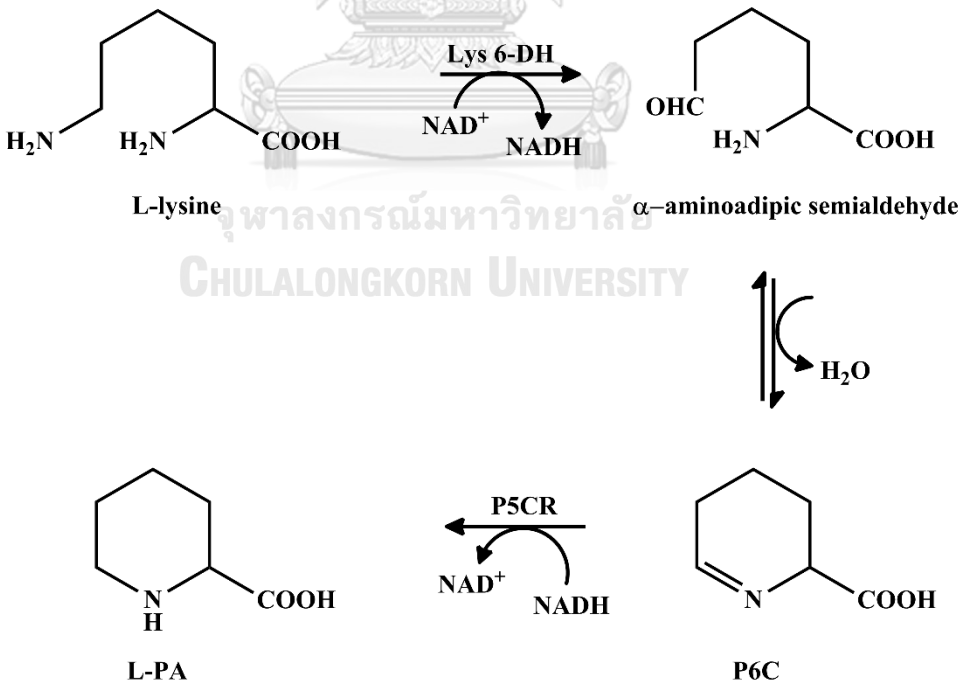


Figure 3 Reaction scheme of L-PA biosynthesis by Lys 6-DH and P5CR

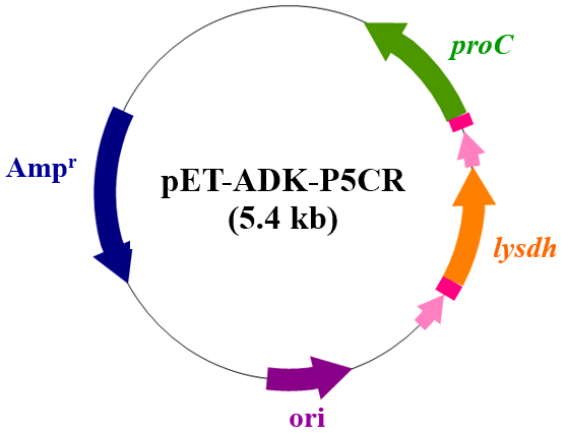


Figure 4 Map of recombinant plasmid pET-ADK-P5CR

However, production of L-PA by incubation in a reaction mixture is hard to use in large scale production or apply for industrial production. There are some drawbacks, such as complicated multistage or easily contaminated. So, fermentation is an interesting alternative process.

L-lysine is an important factor that we need to consider because it is used as a substrate for L-PA production. So, the incensement of L-PA production depends on the amount of L-lysine as well.

1.3 L-lysine biosynthesis

In *E. coli*, L-lysine is synthesized via diaminopimelate pathway (Figure 5). In this pathway, there are two key enzymes, aspartokinase (AK) and dihydrodipicolinate synthase (DHDPS), which are feedback inhibited by L-lysine. In *E. coli*, three isozymes of AK are found [26]; however, one of the isozymes, which is direct to L-lysine biosynthesis and involves in feedback inhibition by L-lysine is AK III. The AK III is encoded by *lysC*. The function of AK III is to convert L-aspartate into L-aspartyl-4-phosphate, which is the first reaction in the diaminopimelate pathway and

one of the rate-limiting steps in this pathway [27] [28]. In 2011, Chen and his coworkers reported that the mutation of Val at 339 to Ala (V339A) of AK III could remain the activity up to 95% when the concentration of L-lysine was increased to 20 mM [28].

DHDPS, which is encoded by *dapA* catalyzes the reaction for converting L-aspartate-semialdehyde to L-2,3-dihydrodipicolinate. This reaction is the first specific reaction for L-lysine biosynthesis and is another rate-limiting step of diaminopilmelate pathway. Geng and his colleagues studied feedback resistant inhibition of DHDPS in 2013 [29]. They mutated *E. coli* DHDPS at Glu 84 to Thr (E84T), it remained the highest activity up to 90% when the concentration of L-lysine was increased to 10 mM.

Therefore, in our previous study, Norasetsingh mutated *lysC* and *dapA* of *E. coli* BL21 (DE3) to *lysC** (V339A) and *dapA**(E84T), respectively. Then, the recombinant plasmid containing *lysC** and *dapA** was constructed and named pD-C*D* (Figure 6). The concept of his work is that the mutation of L-lysine feedback inhibition enzyme to L-lysine feedback resistant enzyme should give more production of L-lysine in *E. coli* cell when L-lysine in the cell is increased [30].

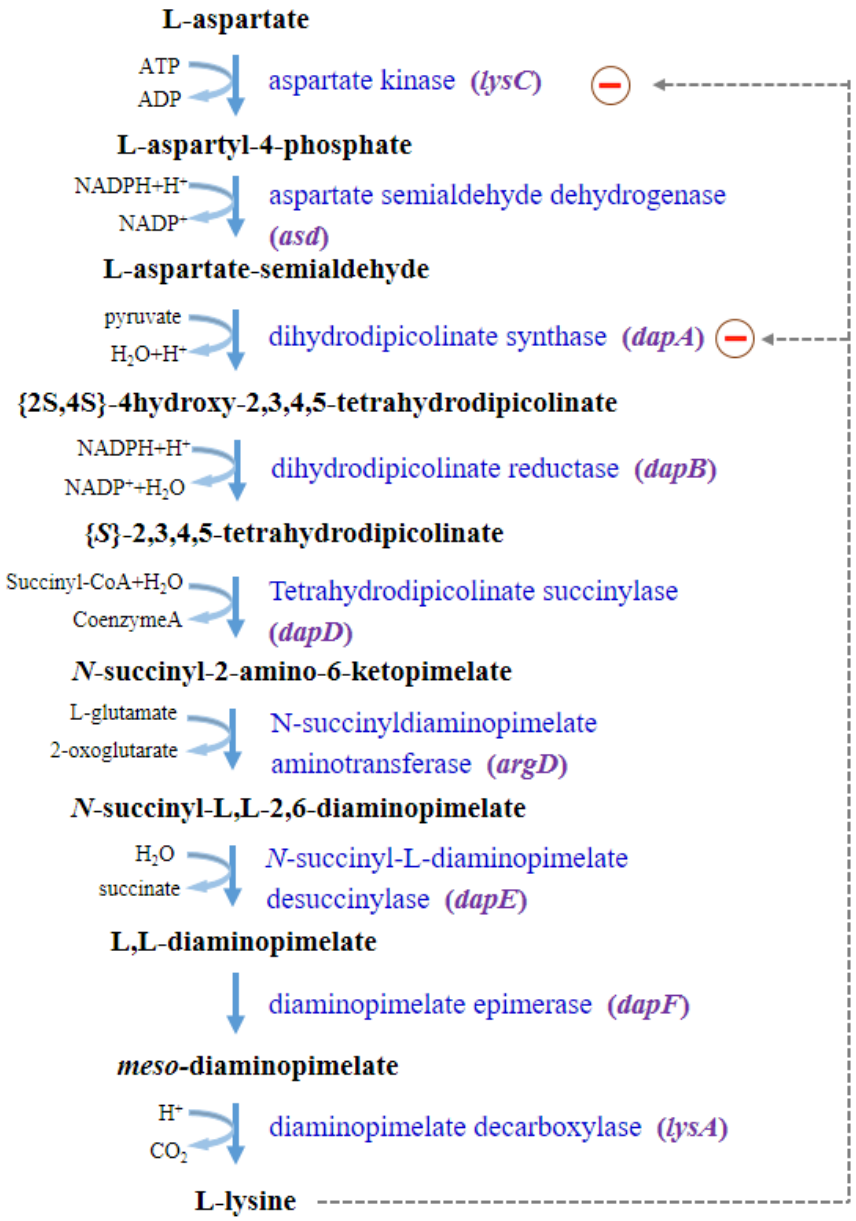


Figure 5 Schematic of diaminopimelate pathway

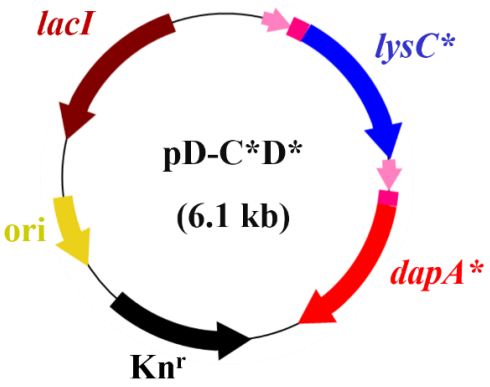


Figure 6 Map of recombinant pD-C*D*

Not only AK and DHDPS but also homoserine dehydrogenase affects on L-lysine production.

1.4 Homoserine dehydrogenase

Homoserine dehydrogenase (HSDH) can be found in many organisms such as plants, yeasts, and bacteria. The function of HSDH depends on each organism and also each type of an organism. For *E. coli*, HSDH consists of two domains that are N-terminal aspartokinase domain and C-terminal homoserine dehydrogenase domain. Thus, it is a bifunctional enzyme [31]. HSDH involves in aspartate metabolic pathway, the upstream pathway for L-lysine biosynthesis. It catalyzes at the third step of the aspartate metabolic pathway, which converts L-aspartate 4-semialdehyde into L-homoserine. L-homoserine is an intermediate in threonine biosynthesis. So, HSDH catalyzes the reaction at the branch of the L-lysine biosynthesis pathway that L-aspartate 4-semialdehyde is drawn to L-homoserine [32] (Figure 7).

There are two isozymes of HSDH, homoserine dehydrogenase I (HSDH I) and homoserine dehydrogenase II (HSDH II). They certainly possess remarkable similarities activity [33]. HSDH I is encoded from *thrA*. The nucleotide size of *thrA* is 2463 bp which locates at 336 to 2798 of *E. coli* BL21(DE3) genome. It is the key enzyme in the regulation of threonine biosynthesis [34] [35]. HSDH II is encoded from *metL*. The nucleotide size of *metL* is 2433 bp which locates at 4038382 to 4040814 of *E. coli* BL21(DE3) genome. This enzyme involved in methionine biosynthesis. It is an enzyme whose synthesis is controlled by the concentration of methionine in the intracellular pool [33]. However, HSDH I plays a significant role in enhancing quantitative expression more than HSDH II around 6-fold [36].

Therefore, in our hypothesis, if the *thrA* is knocked out, *E. coli* cell cannot produce HSDH I, the enzyme which draws L-aspartate semialdehyde from L-lysine biosynthesis pathway. So, the L-lysine production should be increased. For knockout method, the activity of group II intron was interesting.

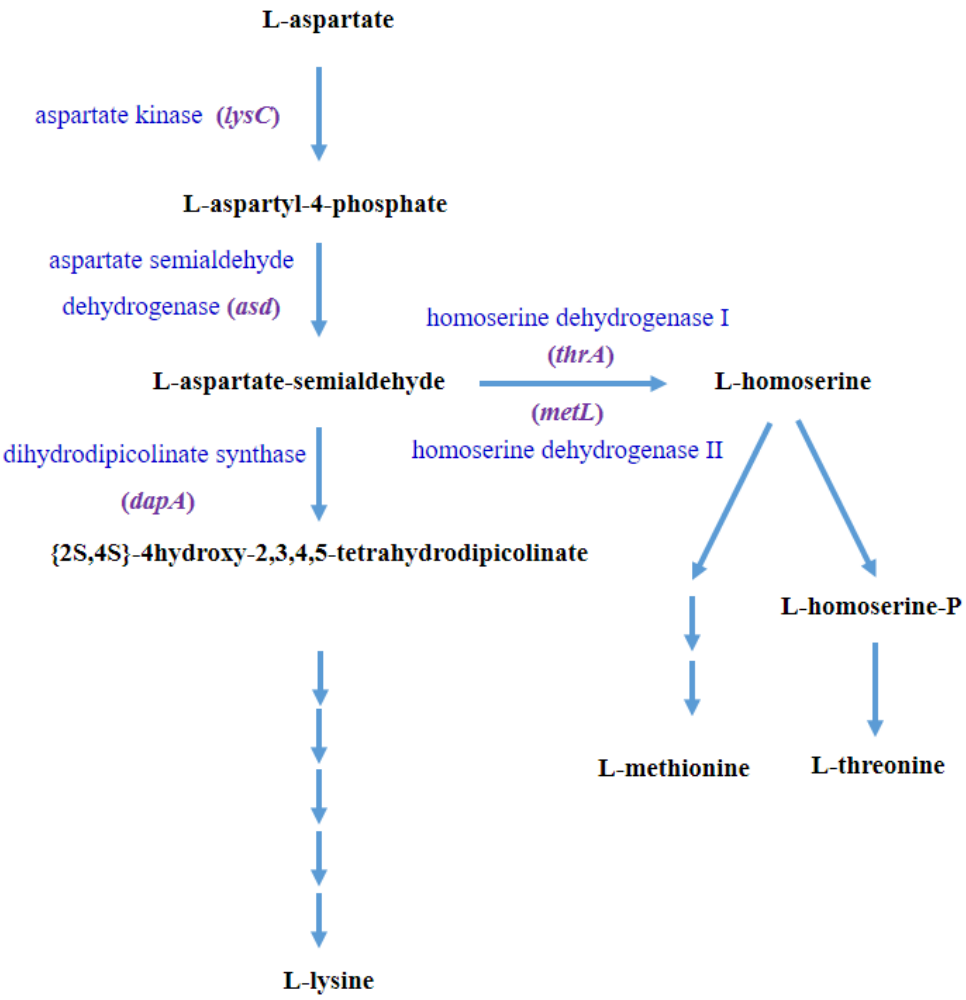


Figure 7 Schematic representation of brach in L-lysine biosynthesis pathway Homoserine dehydrogenase I and II can draw L-aspartate-semialdehyde, the intermediate in L-lysine biosynthesis pathway for L-threonine and L-methionine biosynthesis.

1.5 Group II intron

An intron is nucleotide fragments which are removed during maturation by RNA splicing [37]. The intron is classified into at least four classes: introns in nuclear protein-coding genes that are removed by spliceosomes (spliceosomal introns), introns in nuclear and archaeal transfer RNA genes that are removed by proteins (tRNA introns), Third self-splicing group I introns that are removed by RNA catalysis, and self-splicing group II introns that are removed by RNA catalysis. The types of introns are identified through the examination of the intron structure by DNA sequence analysis, genetic and biochemical analysis of RNA splicing reactions [37]. However, in this study, we focused on group II intron because of its application.

Group II introns are mobile genetic elements which are found in bacterial and organelle chromosomes. It possesses 1. an intron-encoded protein (IEP), LtrA which has reverse transcriptase activity, 2. a catalytically active intron RNA (ribozyme) which has RNA splicing or maturase, and 3. DNA endonuclease activities. Group II introns can mobilize autonomously with a high frequency to allelic sites, homing process [38]. The mobility can occur after the IEP assists the intron RNA fold into active form to promote splicing resulting in ligated to exon and an intron lariat-IEP ribonucleoprotein (RNP) complex. The RNP complex can recognize specific DNA target sites and promotes the integration of the intron RNA directly into one strand of DNA target by reverse splicing. The specific recognition site depends on the pairings of the exon binding sites (EBS1, EBS2, and δ) on the RNA together with the intron binding sites (IBS1, IBS2, and δ') on the DNA (shown in Figure 8). Then, the IEP cleavages the opposite strand, and it is used as a primer for reverse transcription of intron RNA insertion [39] [40] [41] [42]. After that cDNA copy of intron has completed the integration with genomic DNA by repair mechanism or cellular recombination [43] [44]. The intron mechanism is shown in Figure 9.

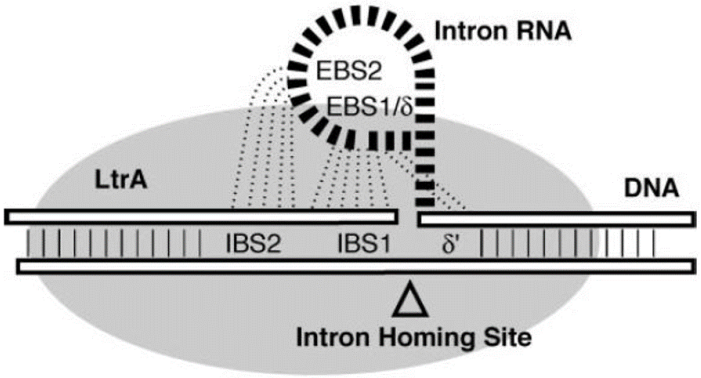


Figure 8 Schematic representation of target recognition by the RNP during retrohoming

Source: Ichiyanagi K. et al., 2003 [45]

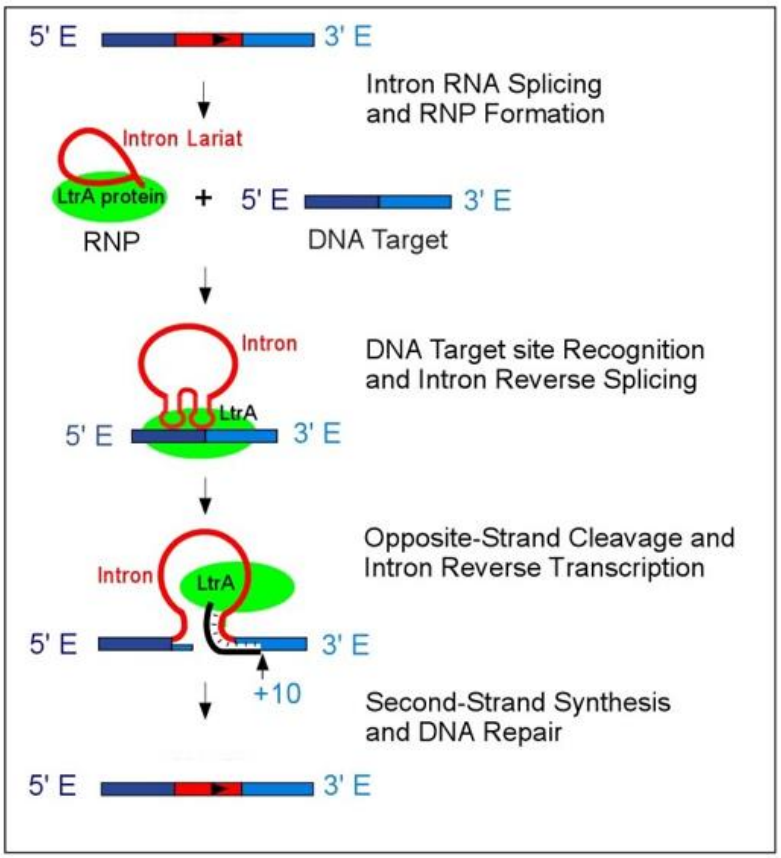


Figure 9 Mechanism of group II intron mobility

Source: Perutka J., 2012 [46]

1.6 L-pipecolic acid production by fermentation

Fermentation is the most popular method for the production of organic substance through the action of enzymes in microorganisms. So, it also was interesting for L-PA production because of easy preparation, high yield production, and high specific product. There are many reports on L-PA fermentation by *Corynebacterium glutamicum* [47] and *E. coli* [48].

In our previous work, Khuanwilai constructed recombinant plasmid, named pD-LPC*D* (consists of *lysdh*, *proC*, *lysC** and *dapA** on pRSFDuet-1) (Figure 10). Then, pD-LPC*D* was transformed into *E. coli* BL21(DE3). This *E. coli* strain was used for L-PA fermentation in glycerol minimal medium [49]. They could produce L-PA approximate 0.16 g/L [50]. The overview of L-PA production in this strain is shown in Figure 11.

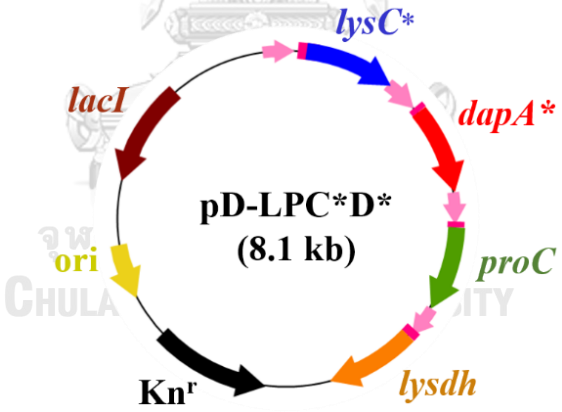


Figure 10 Map of pD-LPC*D*

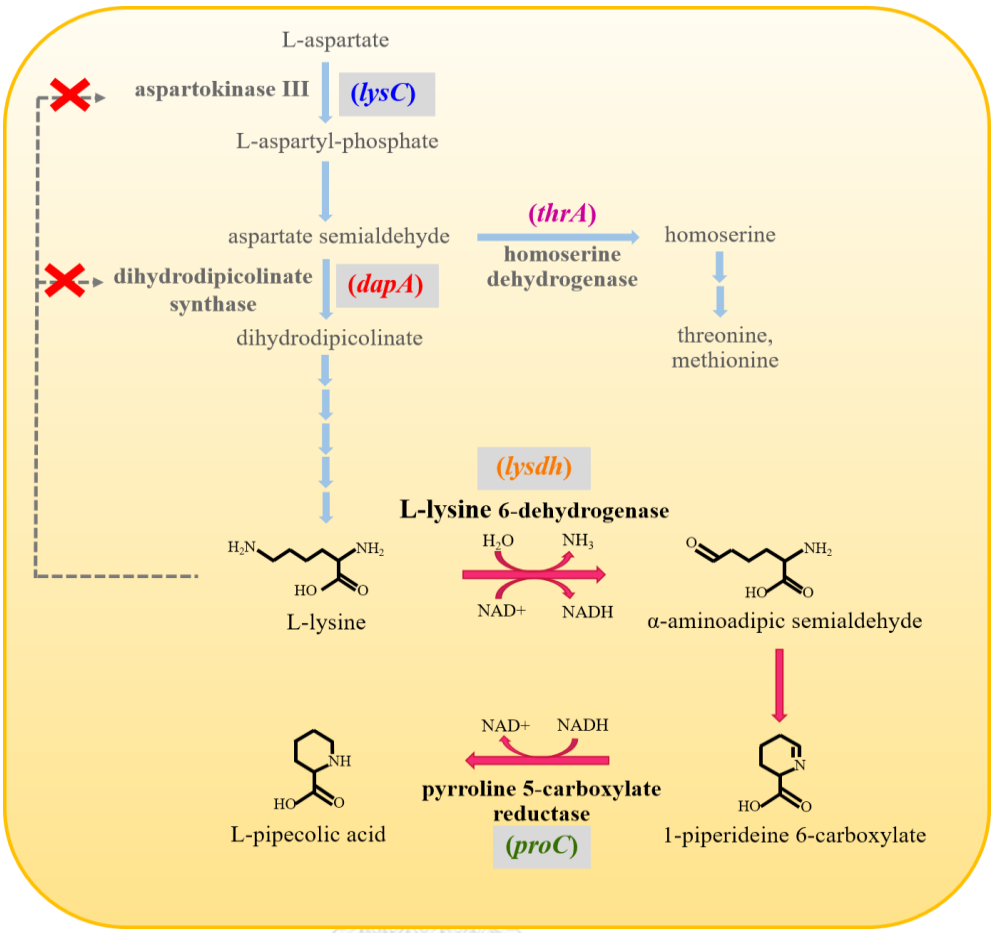


Figure 11 Overview of L-PA production in the engineered *E. coli*

1.7 Objective of this research

This research aimed to improve L-PA production in *E. coli* BL21(DE3) Δ*thrA* by feedback resistance *lysC** and *dapA** to increase L-lysine production in *E. coli* cell as well as the heterologous expression of *lysdh* and *proC* to transform L-lysine to L-PA. Therefore, the outline of this research composed of

1. To knock out *thrA* of *E. coli* BL21(DE3) by group II intron insertion
2. To construct pE22-LPC*D*, pET-22b(+) carrying *lysdh, proC, lysC** and *dapA** and transform into *E. coli* BL21(DE3) and *E. coli* BL21(DE3) Δ*thrA*
3. To select fermentation medium and compare L-PA production between wild-type and knockout strains

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model MLS-2420, Sanyo Electric Co.,Ltd, Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Sorvall Legend XTR, Thermo Scientific, USA

Centrifuge, microcentrifuge: Microfuge 22R, Beckman Instrument Inc., USA

Electrophoresis unit: Minis-150, Major Science, USA

Electroporator: MicroPulser™, Bio-Rad, USA

Electroporation cuvette: Gene Pulser®/E. coli Pulser® Cuvettes, Bio-Rad, USA

Gel Documentation: BioDoc-ItTm Imaging system, UVP, USA

Heating box: Model MD-01N Dry bath incubator, MS Major Science, USA

High Performance Liquid Chromatography (HPLC): UFLC, SHIMADZU, Japan

HPLC column: Reversed phase HPLC Inertsil ODS-3, 250 mm x 4.6 mm x 5 µm column, GL Sciences Inc., Japan

Incubator shaker: Innova™ 4080, New Brunwick Scientific, USA

Incubator shaker: Model E24R, New Brunswick Scientific, USA

Incubator oven: Series04067, Contherm Scientific., Ltd., New Zewland

Lamina flow: HT123, ISSCO, USA

Magnetic stirrer: Model Cerastir CH-1 series, Nickel-electro., Ltd., UK

Membrane filter: 0.45 µm Nylon Membrane Disc, Gs-Tek, USA

Microcentrifuge tubes: 1.5 mL, Nest biotechnology, China

Microwave oven: Model TRX1500, Turbora International Co., Ltd., Korea

PCR tubes: Thin-wall 0.2 mL, Axygen Hayward, USA

pH meter: Model S200, Mettler ToledoCo.,Ltd., Switzerland

Pipette tip: Axygen Inc., USA

Sonicator: Vibra cellTm, SONICS & MATERIALS, Inc., USA

Spectrophotometer: BioSpectrometer® kinetic, Eppendorf, Germany

Spin microtubes: Model microONE, Tomy Digital Biology Co., Ltd., Japan

Syringe: 3 mL, 5 mL latex free disposable syringe, Nipro Co., Ltd., Thailand

Syringe membrane filter: 0.2 µm Supor® Membrane Acrodisc® , PALL, USA

Thermo cycler: T100™, Bio-Rad, USA

UV transluminator: Model 2011 Macrovue, San Gabriel California, USA

Vacuum pump: Millipore Inc., USA

Vortex:Top Mix FB15024, Fisher Scientific, Inc., USA

2.2 Chemicals

Acetonitrile (HPLC grade): Duksan Pure Chemicals, Korea

Acrylamide: Sigma, USA

Agar: Becteriologocal agar powder, Himedia, India

Agarose: Serva, Germany

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo ErbaReagenti, Italy

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromphenol blue: Merck, Germany

Calcium chloride: Scharlau, Spain

Citric acid: Carlo Erba, Italy

Copper sulfate: Carlo Erba, Italy

Coomassie brilliant blue R-250: Sigma, USA

DNA marker: 100 base pair DNA ladder, Fermentas Inc., USA

DNA marker: GeneRuler 1 kb DNA Ladder, ThermoFisher Scienctific, Inc., USA

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., USA

Ethyl alcohol absolute: RCI Labscan, Thailand

Ethylene diaminetetraacetic acid di-sodium salt (EDTA-di-sodium salt): Scharlau Chemie S.A., Spain

Ferrous sulfate: Carlo Erba, Italy

Ferrous chloride: Ajax Finechem Pty Ltd., New Zeland

Glacial acetic acid: Carlo ErbaReagenti, Italy

Glycerol: Analytical Univar Reagent, Ajax finechem, Australia

Glycine: BDH, England

Hydrochloric acid: Carlo ErbaReagenti, Italy

Isopropylthio-β-D-galactosidase (IPTG): Serva, Germany

β- mercaptoethanol: Acros Organics, USA

Magnesium sulphate 7-hydrate: Carlo Erba, Italy

Maganese(II) sulphate: Carlo Erba, Italy

Methanol (HPLC grade): RCI Labscan, Thailand

N,N'-methylene-bis-acrylamide: Sigma, USA

N,N,N',N'-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagent, Italy

Ninhydrin: VWR Prolabo Range, France

dNTP: Biotechrabbit, Germany

Pancreatic digest of casein: Criterion, USA

Phenol reagent: sigma-aldrich, USA

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

L-pipecolic acid: Wako, Japan

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo ErbaReagenti, Italy

di-Potassium hydrogen phosphate: Carlo ErbaReagenti, Italy

Protein molecular weight marker: Tricolor protein ladder (10-180kDa), Biotech rabbit, Germany

RedSafe^{TM}: Nucleic acid staining solution 20,000X, Intron Biotechnology, Hongkong

Sodium chloride: BDH, England

tri-Sodium citrate dehydrate: Carlo Erba, Italy

Sodium dodecyl sulfate: Sigma, USA

di-Sodium hydrogen phosphate: Carlo Erba, Italy

Sodium hydroxide: Carlo Erba, Italy

Thiamine-HCl: Sigma, USA

Tris(hydroxymethyl)-aminomethane: Carlo Erba, Italy

Yeast extract: Scharlau microbiology, European Union

Zinc sulfate: BDH, England

2.3 Antibiotic

Ampicillin: USBiological, USA

Chloramphenical: Nacalai Tesque Inc., USA

Kanamycin: Sigma, USA

2.4 Kits

GenepHlow™ Gel Extraction Kit: Geneaid, Taiwan

Presto™ Mini Plasmid Kit: Geneaid, Taiwan

TargeTron® Gene Knockout System, Sigma-aldrich, USA

2.5 Enzymes and Restriction enzymes

Phusion High-Fidelity DNA Polymerase: Thermo Scientific, USA

Restriction enzymes: New England BioLabs, Inc., USA

T4 DNA ligase: Biotechrabbit, Germany

Taq DNA polymerase: Apsalagen, Thailand

2.6 Primers

The oligonucleotides were synthesized by Integrated DNA Technologies, Singapore. The primers in this work are shown in Table 1.

Table 1 The sequence of primers used in this work

Primer	Sequence	T_m (°C)	Experiment
thrA-IBS	5’-AAAAAAGCTTATAATTATCCTTAACCAC CGGCGTGGTGCGCCCAGATAGGGTG-3’	69.2	*thrA* knockout (*thrA*^{re*} amplification)
thrA-EBS1d	5’-CAGATTGTACAAATGTGGTGATAACAGA TAAGTCGGCGTGCGTAACTTACCTTTCTTTGT-3’	67.2	
thrA-EBS2	5’-TGAACGCAAGTTTCTAATTTCGATTGTGGT TCGATAGAGGAAAGTGTCT-3’	65.2	
EBS universal	5’-CGAAATTAGAAACTTGCGTTCAGTAAAC-3’	54	
thrA-F	5’-ATGCGAGTGTTGAAGTTCGGC-3’	58.3	*thrA* knockout confirmation
thrA-R	5’-TCAGACTCCTAACTTCCATGAGAGG -3’	57.3	

Note: The underlined sequences are restriction sites.

(continued)

Table 1 The sequence of primers used in this work

Primer	Sequence	T_m (°C)	Experiment
*Hin*dIIIT7lysCmu	5’-CCCAAGCTTGGCTCTCCCTTATGC-3’	62	*lysC*-dapA** fragment amplification
*Not*IdapAR	5’-ATAAGAATGCGGCCGCGGCGCGACTTTT -3’	69.7	
UpT7promoter	5’-ATCGAGATCTCGATCCCGCG-3’	60.7	DNA sequencing of *lysC** and *dapA**
dapAsequp-R	5’-TCGCCAGTGGTGCCAACAGAAACGATCG-3’	69.9	
DownlysC	5’-TACTGTATGGCCTGGAAGC-3’	55.5	
T7terminator (universal primer)	5’-GCTAGTTATTGCTCAGCGG-3’	54	

Note: The underlined sequences are restriction sites.

2.7 Bacterial strains

Escherichia coli TOP10 was used as a cloning host.

Escherichia coli BL21(DE3), genotype: F- *omp*T *hsdS*$_B$ (r^-_B m^-_B) *gal dcm* (DE3), was used as a host for *thrA* knockout and gene expression.

2.8 Plasmids

pACD4K-C was used for construction of *thrA* knockout *E. coli* BL21(DE3) (Appendix A).

pET-22b(+) was used for cloning and expression of *lysC**, *dapA**, *lysdh* and *proC* (Appendix B).

2.9 Media

2.9.1 Luria-Bertani broth (LB medium)

LB medium containing 1% pancreatic digest of casein (peptone C), 0.5% NaCl, and 0.5% yeast extract, was prepared (Sambrook et al. 1989). For agar plate, the medium was supplemented with 1.5% (w/v) biological agar. The medium was sterilized by autoclaving for 20 minutes at 121 °C and 15 psi. If needed, a selective antibiotic drug was then supplied.

2.9.2 Fermentation media

The fermentation media were used for the production of L-PA. In all of these media, glycerol was used as a carbon source. The pH of the media was adjusted to 7.0 by NaOH.

2.9.2.1 Yplus medium

Yplus medium was modified from Thongchuang et al., 2012 [49]. This medium contained 30 g/L glycerol, 50 g/L $(NH_4)_2SO_4$, 0.81 g/L $MgCl_2.6H_2O$, 2.43 g/L KH_2PO_4, 2.43 g/L K_2HPO_4, 2 g/L yeast extract, 0.0085 g/L thiamine-HCl, 2 mg/L $FeSO_47H_2O$, 2 mg/L $MnSO_4.H_2O$, 0.05 g/L $CaCl_2.2H_2O$ and 0.01 g/L $ZnSO_4.7H_2O$, pH 7.0.

2.9.2.2 Tplus medium

Tplus medium was modified from Thongchuang et al., 2012 [49]. This medium contained 30 g/L glycerol, 50 g/L $(NH_4)_2SO_4$, 0.81 g/L $MgCl_2.6H_2O$, 2.43 g/L KH_2PO_4, 2.43 g/L K_2HPO_4, 0.085 g/L yeast extract, 0.0085 g/L thiamine-HCl, 2 mg/L $FeSO_47H_2O$, 2 mg/L $MnSO_4.H_2O$, 0.05 g/L $CaCl_2.2H_2O$, 0.01 g/L $ZnSO_4.7H_2O$ and 0.476 g/L threonine, pH 7.0.

2.9.2.3 Gplus medium

Gplus medium was modified from Thongchuang et al., 2012 [49]. This medium contained 30 g/L glycerol, 50 g/L $(NH_4)_2SO_4$, 0.81 g/L $MgCl_2.6H_2O$, 2.43 g/L KH_2PO_4, 2.43 g/L K_2HPO_4, 0.085 g/L yeast extract, 0.0085 g/L thiamine-HCl, 2 mg/L $FeSO_47H_2O$, 2 mg/L $MnSO_4.H_2O$, 0.05 g/L $CaCl_2.2H_2O$, 0.01 g/L $ZnSO_4.7H_2O$ and 0.75 g/L glycine, pH 7.0.

2.9.2.4 Ning medium

Ning medium was modified from Ning et al., 2016 [51]. This medium contained 30 g/L glycerol, 2 g/L yeast extract, 4g/L peptone C, 1 g/L sodium citrate tribasic dihydrate, 2g/L KH_2PO_4, 0.7 g/L $MgSO_4$, 100 mg/L $FeSO_4$, 100 mg/L $MnSO_4$, 0.8 mg/L thiamine-HCl and 0.2 mg/L biotin, pH 7.0.

2.9.2.5 Ying medium

Ying medium was modified from Ying et al., 2017 [18]. This medium contained 30 g/L glycerol, 12 g/L peptone C, 8 g/L yeast extract, 2.1 g/L citric acid.H_2O, 2.5 g/L $(NH_4)_2SO_4$, 0.1 g/L $FeCl_3$, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L $K_2HPO_4.3H_2O$, 3 g/L KH_2PO_4 and 15.13 g/L $Na_2HPO_4.12H_2O$, pH 7.0.

2.10 Competent cell preparation

The competent cell in this research was prepared for electroporation. A single colony of *E. coli* BL21 (DE3) was grown in 5 mL of LB medium at 37 ºC for 16 hours with shaking at 250 rpm. The 1 mL of the fresh overnight culture of *E. coli* BL21(DE3) was inoculated into 100 mL of LB broth to use it as a starter, and then the cell culture was grown at 37 ºC for 18 h with shaking at 250 rpm. The 2 mL of starter was inoculated into 200 mL of LB medium and continued to grow at 37 ºC, 250 rpm until OD_{600} reached around 0.3-0.4. The culture was chilled and centrifuged at 3,000 xg for 10 minutes at 4 ºC. The supernatant was discarded and the cell pellet was washed for two times with cold sterile water. The washed cell was obtained by centrifugation at 3,000 xg for 10 minutes. Then, the cell was washed again with 25 mL of 10% cold sterile glycerol in distilled water and centrifuged at 3,000 xg for 15 minutes. Finally, the cell pellet was resuspended with 10% cold sterile glycerol to the final volume around 2 mL. The cell suspension was divided to each 50 µL and stored at -80 ºC.

2.11 Agarose gel electrophoresis

The agarose gel electrophoresis was used for analysis, separation, identification, or purification of DNA. In this research, TBE buffer (89 mM Tris-base, 89 mM boric acid and 20 mM EDTA) was used as a buffer for agarose gel preparing and running. Agarose powder was balanced and mixed with 1X TBE buffer, the percentage of agarose gel was depended on the size of DNA in each experiment. Then the mixture was heated in a microwave oven until agarose powder was completely dissolved. When the temperature of the agarose solution was decreased to 50-60 ^{o}C, RadSafe^{TM} was added to 5% (v/v) of final concentration before pouring into the tray. After the agarose gel was absolutely set, it was soaked in 1X TBE buffer in electrophoresis chamber. The samples were mixed with DNA loading dye (NEB, England) before loading into the well of agarose gel. The DNA samples were moved from cathode to anode. After the run, The DNA was visualized by UV light of a gel document machine. The size and intensity of DNA samples were compared with the bands of a DNA ladder.

2.12 *thrA* knockout in *E. coli* BL21(DE3)

thrA in *E. coli* BL21(DE3) was knocked out using Targetron gene knockout system kit**.**

2.12.1 Construction of pACD4K-C-*thrA*^{re*}

For *thrA* target site selection in *E. coli* BL21(DE3), Technical Services Scientist Team of Millporesigma predicted the potential intron insertion sites and designed primers for construction of intron re-targeting fragment which specific for the target site. Then the thrA-IBS, thrA-EBS1d, and thrA-EBS2 primers (Table 1)

were used to perform the PCR reaction for construction of intron re-targeting fragment (*thrA*^{re*}).

2.12.1.1 *thrA*^{re*} amplification

The four-primer master mix consisting of 10 µM thrA-IBS, 10 µM thrA-EBS1d, 2 µM thrA-EBS2 and 2 µM EBS universal was prepared. The intron PCR template, obtained from Targetron gene knockout system kit (Sigma-aldrich, USA) was used. The 50 µL of PCR reaction contained 1 µL of the four-primer master mix, 1 µL of intron PCR template, 25 µL of JumpStart REDTaq ReadyMix and 23 µL ultra-pure water. The PCR condition consisted of initial denaturation at 94 ^{o}C for 30 seconds, 30 cycles of denaturation at 94 ^{o}C for 15 seconds, annealing at 55 ^{o}C for 30 seconds, extension at 72 ^{o}C for 30 seconds, following by final extension at 72 ^{o}C for 2 minutes. The PCR product (*thrA*^{re*}) was made visible by 4% agarose gel electrophoresis.

2.12.1.2 Cloning of *thrA*^{re*} into pACD4K-C

The *thrA*^{re*} PCR product from section 2.12.1.1 was double digested with *Hin*dIII and *Bsr*GI to generate cohesive ends. The 20 µL of digestion reaction consisted of 200 ng of *thrA*^{re*}, 1X restriction enzyme buffer, 20 units of *Hin*dIII, 10 units of *Bsr*GI. Then, the ligation between digested *thrA*^{re*} and pACD4K-C linearized vector, which had the overhang site of *Hin*dIII and *Bsr*GI was performed to construct pACD4K-C-*thrA*^{re*} (Figure 12). The 1 µL of the ligation reaction was transformed into 50 µL of competent *E. coli* BL21(DE3) by electroporation. The transformant was added with 450 µL of LB medium and grown at 37 ^{o}C with shaking for 1 hour.

2.12.2 Induction of *thrA* knockout

The 100 µL of transformation reaction from section 2.12.1.2 was transferred into 3 mL LB medium containing 25 µg/mL chloramphenicol and 1% (w/v) glucose. The culture was incubated at 37ºC overnight with shaking. The 40 µL of the overnight culture was added into 2 mL of LB medium containing 25 µg/mL chloramphenicol and 1% glucose. The culture was grown at 37 ºC with shaking until OD_{600} reached around 0.2. The incubator was cooled to 30 ºC. Then, 0. 5 mM final concentration of IPTG was supplied, and the incubation was continued for 30 minutes with shaking. After the 30-minute induction step, the cells were centrifuged at 9,000 xg for 2 minutes. The cells were resuspended in 1 mL of LB medium containing 1% glucose and incubated at 30 ºC for 1 hour with shaking. The culture was plated on LB agar plate containing 30 µg/mL kanamycin.

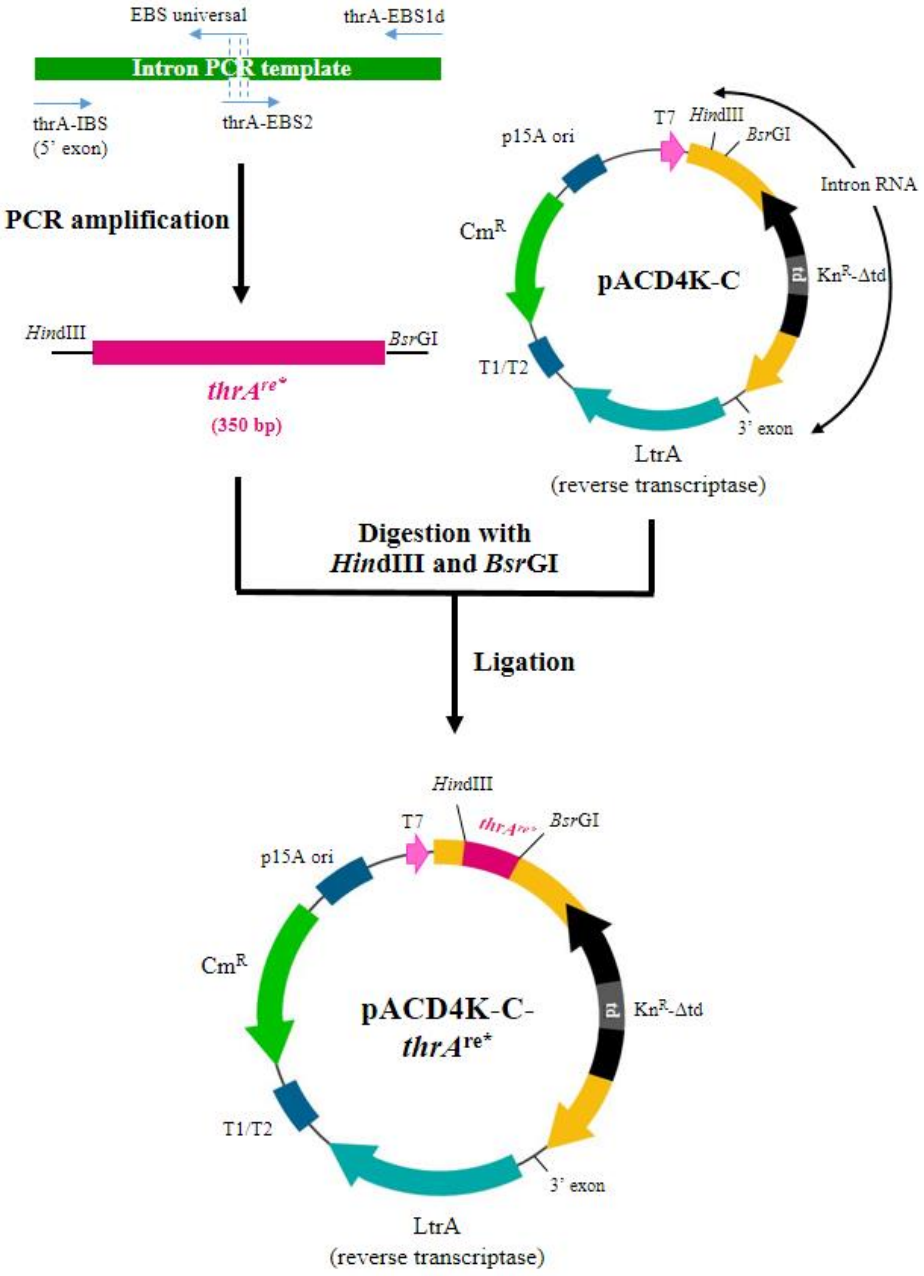


Figure 12 Construction of pACD4K-C-*thrA*^{re*}

2.12.3 Confirmation of *thrA* knockout

The *thrA* knockout colonies from section 2.12.2 were confirmed by colony PCR. The single growing colonies were picked and resuspended in 10 µL of ultra-pure water. The colony PCR reaction contained 10 µL of cell suspension, 0.2 µM of each primer, 2.5 units of *Taq* DNA polymerase, 1X reaction buffer, 200 µM of dNTP mix, 2 mM $MgCl_2$. The cycling instruction consisted of initial denaturation at 95 ^oC for 5 minutes, and 30 cycles of denaturation at 95 ^oC for 30 seconds, annealing at 58 ^oC for 30 seconds, extension at 72 ^oC for 3 minutes following by final extension at 72 ^oC for 10 minutes. The PCR product was visualized by agarose gel electrophoresis for verification of PCR product size.

In the confirmation process, 2 sets of PCR were performed. Firstly, gene specific primers (thrA-F and thrA-R, Table 1) were used for amplification. These primers were specific at the start and stop site of *thrA* to check the insertion of intron group II on *thrA* (Figure 13A). The other one used one gene specific primer and one intron specific primer (thrA-F and EBS universal, Table 1). In this set, one of the primers bound on *thrA* and the other one bound on the intron insertion to amplify across gene-intron junctions (Figure 13B).

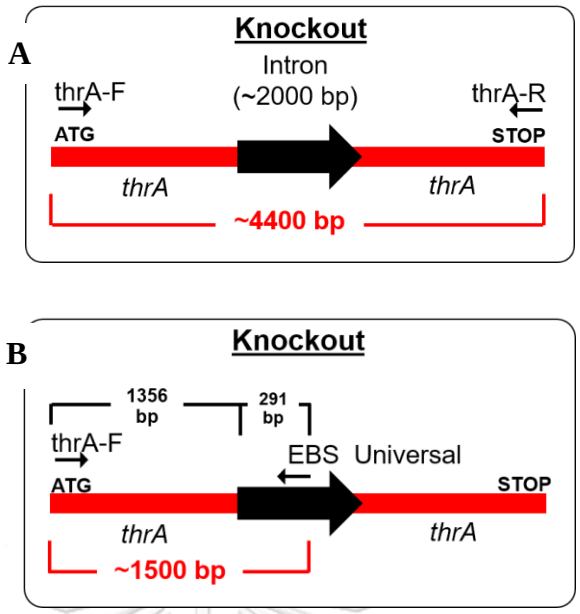


Figure 13 The diagram of PCR detection of intron insertions

2.13 Construction of pE22-LPC*D*

2.13.1 Cloning of pE22-C*D*

2.13.1.1 Recombinant DNA preparation

2.13.1.1.1 pET22-b(+) vector extraction

The *E. coli* BL21(DE3) which carrying pET-22b(+) vector was grown in 5 mL LB medium containing 100 µg/mL ampicillin at 37 ºC for 16 hours with shaking at 250 rpm. The vector was extracted as described in the protocol of Presto™ Mini Plasmid Kit in Appendix C.

2.13.1.1.2 pET-22b(+) vector preparation

pET-22b(+), the expression vector, was digested to linear form by *Hin*dIII and *Not*I. The 50 μL reaction mixture contained 1.5 μg pET-22b(+), 20 units of *Hin*dIII, and 20 units of *Not*I, 1X 2.1-digestion buffer (NEB, England). The reaction was incubated at 37 ^{o}C for 16 hours. The linear form of pET-22b(+) was collected from agarose gel using GenepHlow™ Gel/PCR Kit, as described in Appendix D.

2.13.1.2 DNA fragment (*lysC*-dapA) amplification**

2.13.1.2.1 pD-LPC*D* recombinant plasmid extraction

The *E. coli* BL21(DE3) which carrying pD-LPC*D* was grown in 5 mL LB medium containing 30 μg/mL kanamycin at 37 ^{o}C for 16 hours with shaking at 250 rpm. The recombinant plasmid was extracted by Presto™ Mini Plasmid Kit.

2.13.1.2.2 PCR amplification of gene fragment

2.13.1.2.2.1 Primer

The pair of primers which used for PCR amplification of *lysC**-*dapA** fragment were designed.

2.13.1.2.2.2 PCR condition

The pD-LPC*D* from 2.13.1.2.1 was used as a template. The PCR reaction in 50 μL contained 50 ng of pD-LPC*D*, 10 pmol of each primer, 1 unit of Phusion DNA polymerase, 1X Phusion HF buffer, 0.2 mM of each dNTPs, 3% DMSO. The cycling instruction consisted of initial denaturation at 98 ^{o}C for 30 seconds, and 30 cycles of denaturation at 98 ^{o}C for 10 seconds, annealing at 58 ^{o}C for 10 seconds, extension at 72 ^{o}C for 1 minute following

by final extension at 72 ºC for 10 minutes. The PCR product was visualized by agarose gel electrophoresis for verification of PCR product size. The PCR product was continued to purify by GenepHlow™ Gel/PCR Kit.

2.13.1.3 DNA fragment preparation

The PCR product from 2.13.1.2.2.2 was digested by *Hin*dIII and *Not*I. The 50 μL reaction mixture contained 1 μg gene fragment, 20 units of *Hin*dIII and 20 units of *Not*I, 1X NEBuffer 2.1 (NEB, England). The reaction was incubated at 37 ºC for 16 hours. The digested gene fragment was purified by GenepHlow™ Gel/PCR Kit.

2.13.1.4 Ligation of vector DNA and the gene fragment

The gene fragment (2.13.1.3) was ligated to the pET-22b(+) (2.13.1.1.2) with molar ration 6:1. The 20 μl of ligation mixture contained 100 ng of vector DNA, 260 ng of the gene fragment, 5x Rapid ligation buffer and 30 units of T4 DNA ligase. The ligation mixture was incubated at 25 ºC for 30 minutes. The recombinant plasmid from the ligation reaction was purified by GenepHlow™ Gel/PCR Kit. This recombinant plasmid was named pE22-C*D* (Figure 14).

2.13.1.5 Transformation

pE22-C*D* from 2.13.1.4 was transformed into competent cells of *E. coli* BL21(DE3) by electroporation. In the electroporation step, 5 μL of the recombinant plasmid was gently mixed with 50 μL of competent cell and placed on ice. The mixture was transferred into a cold electroporation cuvette. Then, the cuvette was applied one pulse by electroporator. The 500 μL of LB medium was added into the cuvette to mix with the transformant cell and transferred into 1.5 mL microcentrifuge tube. The cell suspension was incubated at 37 ºC with shaking for

1 hour. Finally, 300 µL of the transformant was plated on LB agar plate containing 100 µg/mL of ampicillin and incubated at 37 ºC overnight. The colonies which grew on the selective plate were selected to further experiment.

2.13.1.6 Confirmation of pE22-C*D* construction

The growing colonies from 2.13.1.5 were picked up to culture in 5 mL LB broth at 37 ºC with shaking at 250 rpm for plasmid extraction using Presto™ Mini Plasmid Kit. The recombinant plasmid was digested with *Hin*dIII and *Not*I by the method in section 2.13.1.1.2. The DNA fragments were identified by agarose gel electrophoresis. The recombinant plasmids with correct size were sent to perform DNA sequencing by Bioneer Inc. (Korea).

2.13.2 Cloning of pE22-LPC*D* and W-LPCD construction

2.13.2.1 Recombinant plasmid preparation

pE22-C*D* from 2.13.1 was digested to linear form by *Not*I and *Xho*I. The 50 µL of reaction mixture contained 2 µg pE22-C*D*, 20 units of *Not*I and 20 units of *Xho*I, 1X CutSmart® buffer (NEB, England) and adjusted the volume to 50 µl. The reaction was incubated at 37 ºC for 16 hours. The linear form pE22-C*D* of was collected from agarose gel using GenepHlow™ Gel/PCR Kit.

2.13.2.2 Insertion DNA preparation

pD-LPC*D* from 2.13.1.2.1 was digested to collect the *lysdh* and *proC* fragment (*proC-lysdh*) by *Not*I and *Xho*I. The 50 µL digestion mixture contained 1.6 µg pD-LPC*D*, 20 units of *Not*I and 20 units of *Xho*I, 1X CutSmart® buffer (NEB, England). The reaction was incubated at 37 ºC for 16 hours. The *proC-lysdh* fragment was separated and collected from agarose gel using GenepHlow™ Gel/PCR Kit.

2.13.2.3 Ligation pE22-C*D* with *proC-lysdh* fragment

The *proC-lysdh* fragment (2.13.2.2) was ligated to the pE22-C*D* (2.13.1) with molar ratio 1: 10 of vector: insert. The 20 µl of ligation mixture containing 150 ng of vector DNA, 450 ng of the gene fragment, 5x Rapid ligation buffer and 30 unit of T4 DNA ligase was incubated at 25 ºC for 30 minutes. The recombinant plasmid from the ligation reaction was purified by GenepHlow™ Gel/PCR Kit for further transformation. The recombinant plasmid was named pE22-LPC*D* (Figure 14).

2.13.2.4 Transformation of pE22-LPC*D*

pE22-LPC*D* from 2.13.1.4 was transformed into competent cells of *E. coli* BL21(DE3) by electroporation. In the electroporation step, 7 µl of the ligation reaction from 2.13.1.4 was gently mixed with 50 µl of competent cell and placed on ice. The mixture was transferred into a cold electroporation cuvette. Then, the cuvette was applied one pulse by electroporator. The 500 µl of LB medium was added into the cuvette to mix with the transformant cell and transferred into 1.5 mL microcentrifuge tube. The cell suspension was incubated at 37 ºC with shaking for 1 hour. Finally, 300 µL of transformant was plated on LB agar plate containing 100 µg/mL of ampicillin and incubated at 37 ºC overnight. The colonies which grew on the selective plate were selected to further experiment.

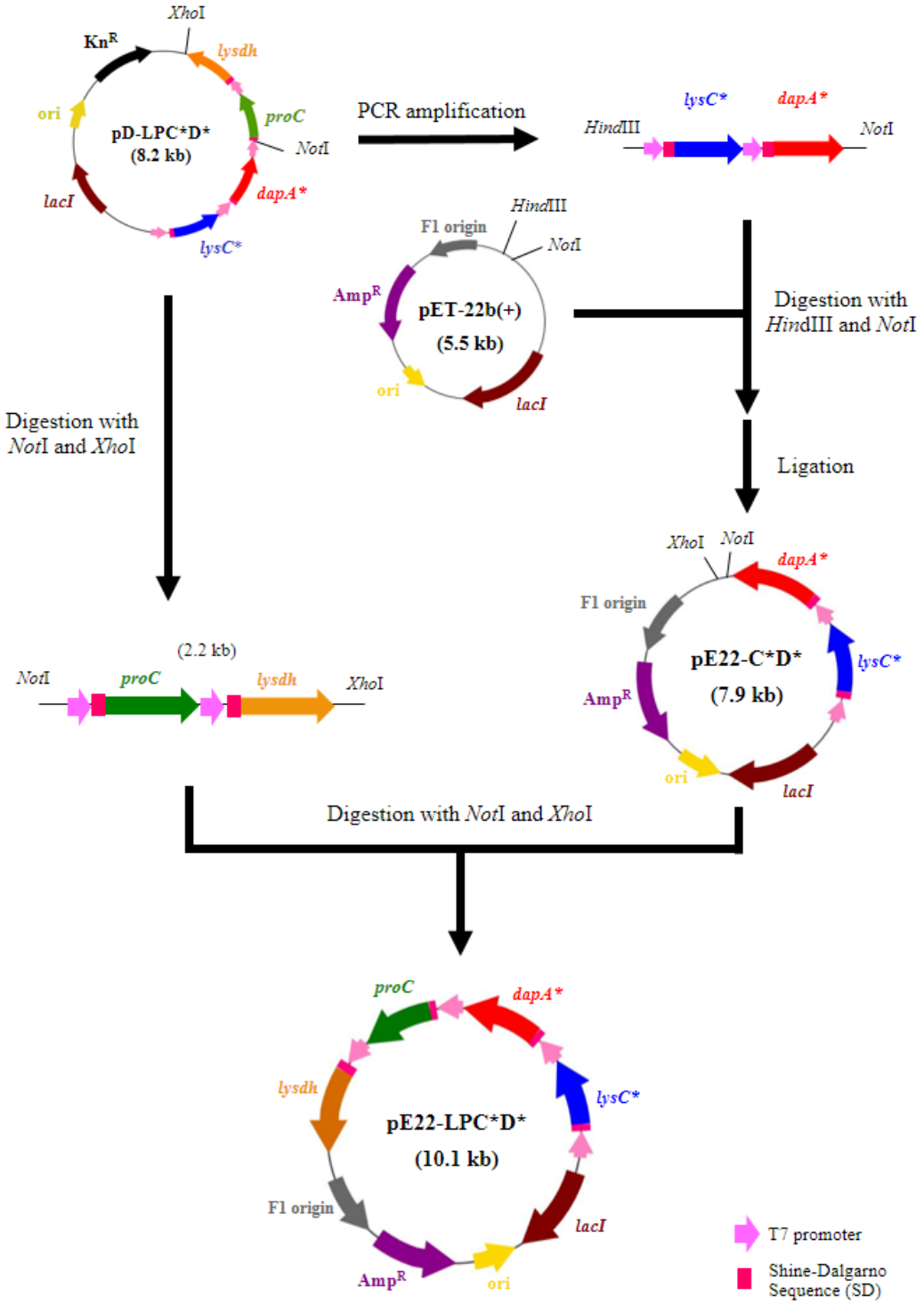


Figure 14 pE22-LPC*D* construction map

2.13.2.5 Confirmation of pE22- LPC*D* construction

The growing colonies from 2.13.2.4 were picked up to culture in 5 mL LB broth at 37 ºC with shaking for plasmid extraction using Presto™ Mini Plasmid Kit. The recombinant plasmids were digested with *Not*I and *Xho*I as described in section 2.13.2.1. The DNA fragments were identified by agarose gel electrophoresis and PCR amplification of involving genes. The successful pE22-LPC*D* construction, which carried by *E. coli* BL21(DE3) was collected to use in the L-PA production part. The recombinant clone was named W-LPCD.

2.14 Construction of KO-LPCD

The extracted pE22-LPC*D* from 2.13.2.5 was transformed into *E. coli* BL21(DE3) Δ*thrA* by electroporation. The 2 ng of pE22-LPC*D* was transformed into 50 µl of the competent cell, *E. coli* BL21(DE3) Δ*thrA*. The successful transformant was selected on LB agar plate containing 100 µg/mL of ampicillin and 30 µg/mL of kanamycin. This recombinant clone was named KO-LPCD (*E. coli* BL21(DE3) Δ*thrA* carrying pE22-LPC*D*).

2.15 Expression of cloned genes

Each *E. coli* BL21 (DE3) strain shown in Table 5 was cultured overnight in 5 mL LB broth at 37 ºC with shaking for using as the starter. The starters were inoculated into 100 mL of LB medium. When OD_{600} reached 0.6, IPTG at the final concentration of 0.4 mM, was added. The cultures were continuously incubated for 4 hours after that the cells were collected by centrifugation at 5,000 xg for 10 minutes,

washed with 0.85% NaCl and extraction buffer, respectively. The cell pellets were dissolved again in extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% β-mercaptoethanol and 1.0 mM EDTA) and were broken by ultrasonic cell disruption. The crude extracts were centrifuged at 10,000 xg 5 minutes to collect the supernatants for SDS-PAGE analysis.

Table 2 The *E. coli* strains used in expression experiment

Strain	**Description**	**Reference**
pDuet	*E. coli* BL(DE3) carrying pRSFDuet-1 vector	khuanwilai, 2016
pET22b	*E. coli* BL(DE3) carrying pET22b(+) vector	This work
pD-ADK	*E. coli* BL(DE3) carrying pD-ADK recombinant plasmid, pRSFDuet-1 carrying *lysdh*	khuanwilai, 2016
pD-P5CR	*E. coli* BL(DE3) carrying pD-P5CR recombinant plasmid, pRSFDuet-1 carrying *proC*	khuanwilai, 2016
pE22-C*D*	*E. coli* BL(DE3) carrying pE22-C*D* recombinant plasmid, pET22b(+) carrying *lysC** and *dapA**	This work
W-LPCD	*E. coli* BL21(DE3) carrying pE22-LPC*D*, pET22b(+) carrying *lysC**, *dapA**, *proC* and *lysdh*	This work
KO-LPCD	*E. coli* BL21(DE3) Δ*thrA* carrying pE22-LPC*D*, pET22b(+) carrying *lysC**, *dapA**, *proC* and *lysdh*	This work

2.15.1 Protein measurement

The protein concentration of crude extract of each *E. coli* strain was measured by the modified Bradford method [52]. The 50 µL of crude extract was mixed with 200 µL of Bradford's reagent (0.5% (w/v) Coomassie Brilliant Blue G-250, 25% (v/v) absolute ethanol, and 50% (v/v) of 85% phosphoric acid). The reaction mixture was incubated at room temperature for 15 minutes. The protein concentration was determined by measuring the absorbance at 595 nm and calculated from the standard curve of standard BSA.

2.15.2 SDS-PAGE analysis

The SDS-PAGE was used to evaluate the molecular weight and intensity of protein. The SDS-PAGE system was performed using the method of Bollag et al., 1996 [53] (Appendix E). The slab gel consisted of 0.1% SDS (w/v) in 10% separating gel and 5% stacking gel. The buffer which used for running the protein in the slab gel was Tris-glycine buffer (25 mM Tris, 192 mM glycine and 0.1% SDS), pH 8.3. For the protein loading preparation, the crude extracts were mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol), then the reaction mixtures were boiled for 10 minutes. The electrophoresis was performed at a constant of 20 mA per slab gel. The gel was stained with a staining solution (1% (w/v) Coomassie Blue R-250, appendix F) for 30 minutes with shaking. Then, the gel was washed with the destaining solution (Appendix F). The size of protein and the protein band intensity were analyzed by comparing with Tricolor protein ladder.

2.16 L-pipecolic acid production

2.16.1 Shake flask fermentation

Each W-LPCD and KO-LPCD strain was cultured in 5 mL LB broth, which contained only 100 µg/mL ampicillin for W-LPCD and 100 µg/mL ampicillin and 30 µg/mL kanamycin for KO-LPCD for 16 hours with shaking. After that 50 µL of each culture was inoculated into 100 µL of LB medium, which containing the same antibiotic. The cells were cultured for 18 hours with shaking for using as the starter. The 1% (v/v) starters were transferred into each fermentation medium as described in section 2.9.2. After OD_{600} reached 0.6, the cultures were induced by IPTG at the final concentration of 0.1 mM. The samples from each fermentation flask were collected every 24 hours until 8 days for monitoring their growth and L-pipecolic acid production.

2.16.2 HPLC determination of L -pipecolic acid titer

2.16.2.1 Sample preparation

The samples from 2.16.1 were centrifuged at 10,000 xg for 10 minutes. The supernatants were collected and filtered through 0.22 µm syringe filter. After that, the amino acid was derivatized by the method modified from Moulin et al., 2002 [54]. Firstly, 100 µL of the sample was mixed with 20 µL of 1.8 M HCl and 20 µL of 250 mM $NaNO_2$. The reaction mixture was stood for 30 minutes at room temperature. After that, 20 µL of 250 mM NH_4Cl was added. After shaking, 100 µL of 12 M HCl was added and the reaction was incubated at 95 ^oC in an oven. After 20 minutes of incubation, the reaction was stopped by adding 100 µL of 10 M NaOH. The reaction mixture was freeze-dried by lyophilizer. The dried sample was resuspended with 100 µL of ultra-pure water. The supernatant was collected by centrifugation at

10,000 xg for 5 minutes. The 2 µL of supernatant was added into 500 µL of 4% (w/v) ninhydrin in glacial acetic acid. After mixed vigorous, the reaction was incubated at 95 ºC for 40 minutes in an oven. Finally, the reaction was filtered through a 0.22 µM nylon syringe filter and kept in HPLC vials.

2.16.2.2 HPLC analysis

The production of L-PA by each *E. coli* clone was determined by HPLC method using Inertsil ODS-3 (C-18) column. The mobile phase was 80% acetonitrile: 20% ultra-pure water, and the flow rate was 1 mL/minute. The standards and samples obtained from 2.16.2.1 were injected in 20 µL. Photodiode array detector was used. L-PA derivative was detected at the wavelength of 570 nm. The L-PA concentrations were calculated from the standard curve of L-PA.

CHAPTER III

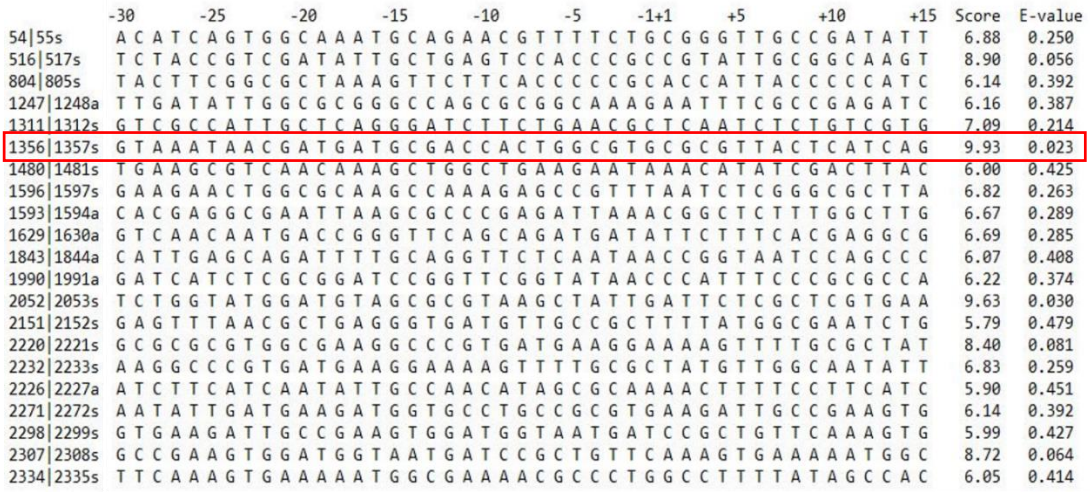
RESULTS

3.1 *thrA* knockout

3.1.1 Target site selection and primer design for *thrA* knockout

The sequence of *thrA* from *E. coli* BL21(DE3) (accession No. CP001509.3) was sent to Technical Services Scientist Team of Millporesigma for prediction of potential insertion sites. We got 21 predicted sites, as shown in Figure 15. As recommended by Targetron® Gene Knockout System user guide, the predicted insertion site, which gives the highest score and lowest E-value should be the best position for gene knockout. Therefore, in this research, the insertion between nucleotide 1356 and 1357 of the sense strand of *thrA*, which gave the highest score at 9.93 and lowest E-value at 0.023 was selected.

After getting the knockout site, three intron re-target primers, thrA-IBS, thrA-EBS1d, and thrA-EBS2, were designed by Technical Services Scientist Team of Millporesigma while the sequence of ESB universal was fixed and this primer was included in Targetron® kit. The sequences of all primers are shown in Table 3.



	-30	-25	-20	-15	-10	-5	-1+1	+5	+10	+15	Score	E-value
54|55s	A C A T C A G T G G C A A A T G C A G A A C G T T T T C T G C G G G T T G C C G A T A T T										6.88	0.250
516|517s	T C T A C C G T C G A T A T T G C T G A G T C C A C C C G C C G T A T T G C G G C A A G T										8.90	0.056
804|805s	T A C T T C G G C G C T A A A G T T C T T C A C C C C G C A C C A T T A C C C C C A T C										6.14	0.392
1247|1248a	T T G A T A T T G G C G C G G G C C A G C G C G G C A A A G A A T T T C G C C G A G A T C										6.16	0.387
1311|1312s	G T C G C C A T T G C T C A G G G A T C T T C T G A A C G C T C A A T C T C T G T C G T G										7.09	0.214
1356|1357s	G T A A A T A A C G A T G A T G C G A C C A C T G G C G T G C G C G T T A C T C A T C A G										9.93	0.023
1480|1481s	T G A A G C G T C A A C A A A G C T G G C T G A A G A A T A A A C A T A T C G A C T T A C										6.00	0.425
1596|1597s	G A A G A A C T G G C G C A A G C C A A A G A G C C G T T T A A T C T C G G G C G C T T A										6.82	0.263
1593|1594a	C A C G A G G C G A A T T A A G C G C C C G A G A T T A A A C G G C T C T T T G G C T T G										6.67	0.289
1629|1630a	G T C A A C A A T G A C C G G G T T C A G C A G A T G A T A T T C T T T C A C G A G G C G										6.69	0.285
1843|1844a	C A T T G A G C A G A T T T T G C A G G T T C T C A A T A A C C G G T A A T C C A G C C C										6.07	0.408
1990|1991a	G A T C A T C T C G C G G A T C C G G T T C G G T A T A A C C C A T T T C C C G C G C C A										6.22	0.374
2052|2053s	T C T G G T A T G G A T G T A G C G C G T A A G C T A T T G A T T C T C G C T C G T G A A										9.63	0.030
2151|2152s	G A G T T T A A C G C T G A G G G T G A T G T T G C C G C T T T T A T G G C G A A T C T G										5.79	0.479
2220|2221s	G C G C G C G T G G C G A A G G C C C G T G A T G A A G G A A A A G T T T T G C G C T A T										8.40	0.081
2232|2233s	A A G G C C C G T G A T G A A G G A A A A G T T T T G C G C T A T G T T G G C A A T A T T										6.83	0.259
2226|2227a	A T C T T C A T C A A T A T T G C C A A C A T A G C G C A A A A C T T T T C C T T C A T C										5.90	0.451
2271|2272s	A A T A T T G A T G A A G A T G G T G C C T G C C G C G T G A A G A T T G C C G A A G T G										6.14	0.392
2298|2299s	G T G A A G A T T G C C G A A G T G G A T G G T A A T G A T C C G C T G T T C A A A G T G										5.99	0.427
2307|2308s	G C C G A A G T G G A T G G T A A T G A T C C G C T G T T C A A A G T G A A A A A T G G C										8.72	0.064
2334|2335s	T T C A A A G T G A A A A A T G G C G A A A A C G C C C T G G C C T T T T A T A G C C A C										6.05	0.414

Figure 15 Target site prediction for *thrA* knockout

The red box represented the selected site for the *thrA* knockout. The number, -30,-25…+10,+15 represented the counting nucleotide from knockout insertion site (+ = forward direction, - = reverse direction). The "s" represented sense strand and "a" represented anti-sense strand.

Table 3 Sequence of primers for *thrA* knockout at the selected site

Insertion site	**Primer name**	**Primer sequence (5' to 3')**
1356/1357	thrA-IBS	AAAAAAGCTTATAATTATCCTTAACCACC GGCGTGGTGCGCCCAGATAGGGTG
	thrA-EBS1d	CAGATTGTACAAATGTGGTGATAACAGAT AAGTCGGCGTGCGTAACTTACCTTTCTTTGT
	thrA-EBS2	TGAACGCAAGTTTCTAATTTCGATTGTGGT TCGATAGAGGAAAGTGTCT
	EBS universal	CGAAATTAGAAACTTGCGTTCAGTAAAC

3.1.2 *thrA*^{re*} PCR amplification

For knockout *thrA* in *E. coli* BL21(DE3), *thrA*^{re*} fragment, which consisted of the specific site with the target gene, was needed to generate. Two pairs of primer were prepared as described in 2.12.1. These primers were used for assembly PCR. The 5'-primer, thrA-IBS, had *Hin*dIII restriction site and the 3'-primer, thrA-EBS1d, had *Bsr*GI restriction site. In addition, the other two primers, thrA-EBS2 and EBS universal, acted as capture primers to ligate the PCR products from each primer together, as shown in Figure 16. *thrA*^{re*} PCR product was visualized by 1% agarose gel electrophoresis, as shown in Figure 17 with the size around 350 bp.

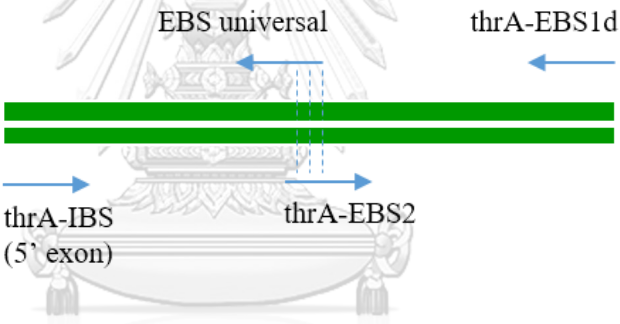


Figure 16 Diagram of primer binding for *thrA*^{re*} PCR amplification

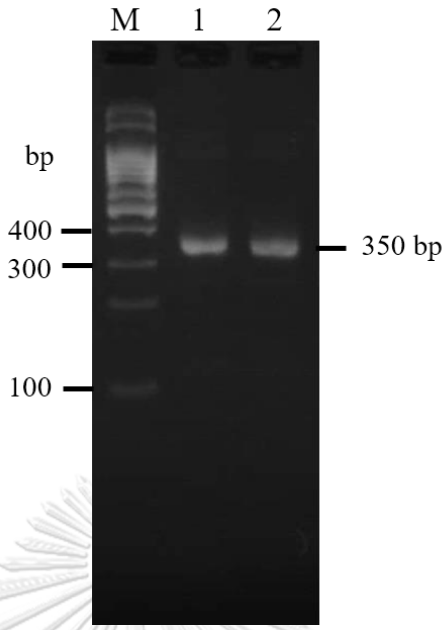


Figure 17 *thrA*^{re*} PCR product

Lane M : 100 bp DNA ladder

Lane 1, 2 : *thrA*^{re*} PCR product

3.1.2 Construction of pACD4K-C-*thrA*^{re*}

The first step of pACD4K-C-*thrA*^{re*} construction was digestion of *thrA*^{re*} from section 3.1.1 with *Hin*dIII and *Bsr*GI. The digestions were prepared by the process described in section 2.1212.2. The result was visualized by 4% agarose gel electrophoresis, as shown in Figure 18.

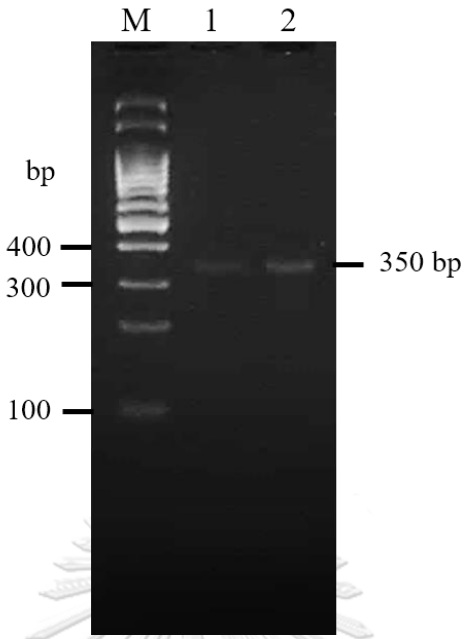


Figure 18 Digestion pattern of *thrA*^{re*} with *Hin*dIII and *Bsr*GI

Lane M : 100 bp DNA ladder

Lane 1, 2 : *Hin*dIII and *Bsr*GI-digested *thrA*^{re*} fragment

The digested *thrA*^{re*} fragment gave a size around 350 bp. Then, this digested fragment was further ligated with pACD4K-C linear vector (Sigma, USA). After that, pACD4K-C-*thrA*^{re*} was transformed into *E. coli* BL21(DE3) by the method described in 2.12.1.2.

3.1.3 *thrA* knockout induction

After transformation as described in 2.12.1.2, the transformant was screened in LB medium containing 25 µg/mL chloramphenicol. The transformant that contained pACD4K-C-*thrA*^{re*} was induced by IPTG as described in 2.12.2. After the induction, we found 80 colonies which could grow on LB agar plate containing 30 µg/mL kanamycin.

3.1.4 *thrA* knockout confirmation

The twenty single colonies from section 3.1.3 were randomly picked for confirmation by colony PCR as described in 2.12.3.

3.1.4.1 PCR using gene specific primers

The gene specific primers, *thrA*-F and *thrA*-R, were used to perform colony PCR. The colony PCR products were analyzed through 0.8% agarose gel electrophoresis, as shown in Figure 19.

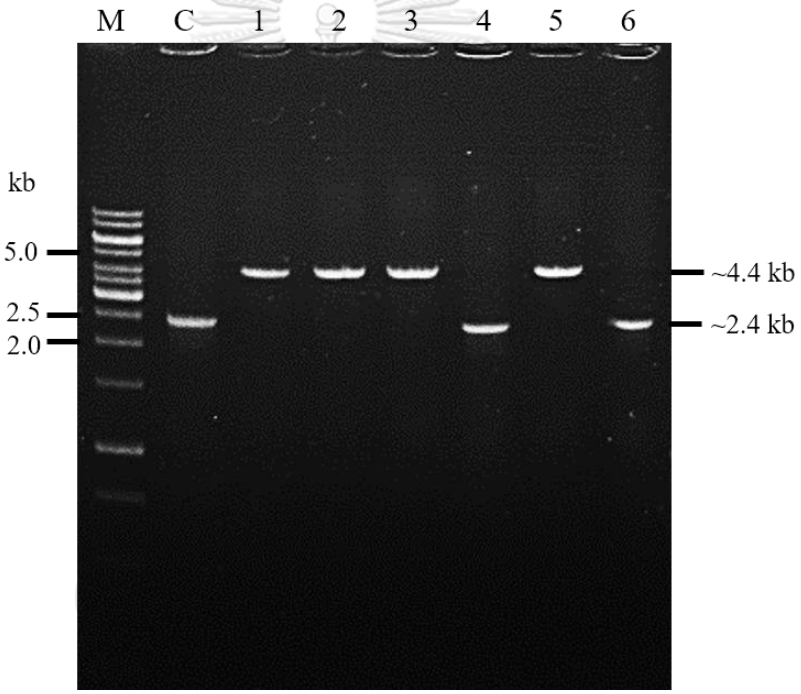


Figure 19 Colony PCR analysis of *thrA* knockout *E. coli* using gene specific primers

Lane M : GeneRuler 1 kb DNA ladder

Lane C : Negative control, colony PCR amplification of wild-type *E. coli* BL21(DE3)

Lane 1-6 : Colony PCR amplification of colony 1-6

The site of colony PCR product of negative control, was around 2.4 kb, in wild-type *E. coli* BL21 (DE3). For colony PCR of colony 1, 2, 3 and 5 gave DNA band around 4.4 kb that was close to the size of *thr*A including the size of intron insertion (~ 2 kb). On the other hand, the colony PCR of colony 4 and 6 gave 2.4 kb in size that was only the size of *thrA*. So, the colonies which gave the PCR product around 4.4 kb were selected for confirmation in the next step.

3.1.4.2 PCR using gene specific primer and intron specific primer

Gene specific primer (*thrA*-F) and intron specific primer (EBS universal) were used to perform colony PCR. The PCR products were visualized through 0.8% agarose gel electrophoresis, as shown in Figure 20.

As expected, the colonies which gave the positive results from section 3.1.4.1 gave a DNA band around 1.5 kb as shown in lane 1, 2, 3, 4 and 5 of Figure 20. The 1.5 kb came from 1,356 bp of *thrA* fragment from the start codon to intron insertion site and 219 bp from the intron insertion fragment. Contrarily, wild-type *E.coli*, a negative control, and the colonies which gave the negative result from section 3.1.4.1 did not give the band. So, the colonies which gave a DNA fragment around 1.5 kb were collected to use in the further experiment.

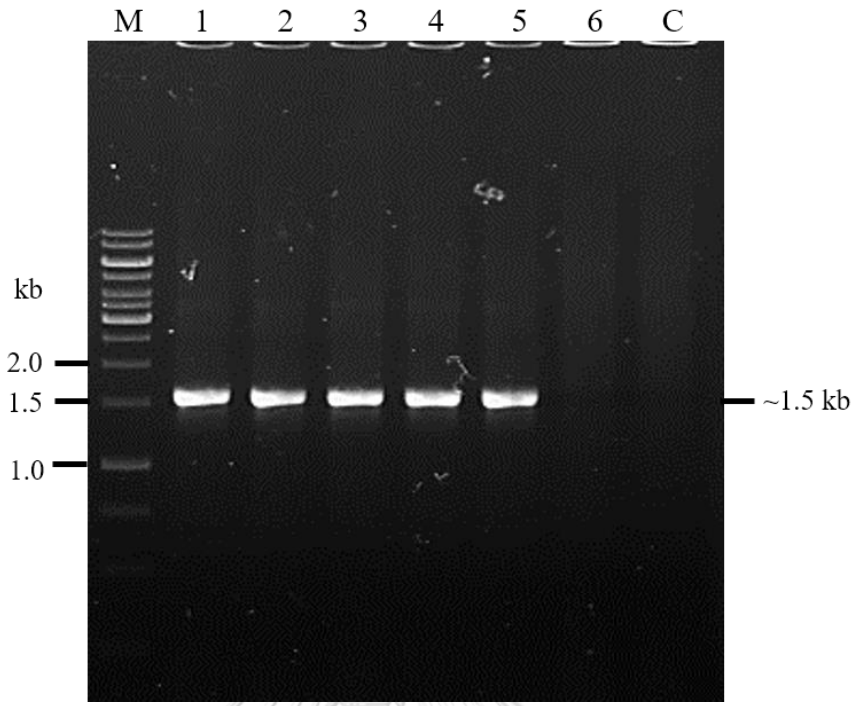


Figure 20 Colony PCR analysis of *thrA* knockout *E. coli* using gene specific primer and intron specific primer

Lane M : GeneRuler 1 kb DNA ladder

Lane 1-5 : Colony PCR of *thrA* knockout tranformants

Lane 6 : Colony PCR of colony 6 which gave the negative result in Figure 19

Lane C : Negative control, colony PCR of wild-type *E. coli* BL21(DE3)

3.2 pE22-LPC*D* construction

3.2.1 Cloning of pE22-C*D*

3.2.1.1 PCR amplification of *lysC*-dapA** fragment

The *lysC*-dapA** was amplified using pD-LPC*D* as a template. The primers for the amplification were *Hin*dIII-T7-*lysC**, the 5'-primer consisting of *Hin*dIII restriction site, and *Not*I-*dapA**, the 3'-primer consisting of *Not*I restriction site. The PCR amplification was performed according to the condition as described in 2.13.1.2.2.2. The PCR product was analyzed through 0.8% agarose gel electrophoresis. The *lysC*-dapA** PCR fragments gave a size around 2.5 kb as shown in lane 1 and 2 in Figure 21. The 2.5 kb was from *lysC** (1,399 bp), *dapA** (947 bp) and their upstream and downstream regions. The *lysC*-dapA** fragment was purified from agarose gel by GenepHlow™ Gel/PCR Kit for further experiment.

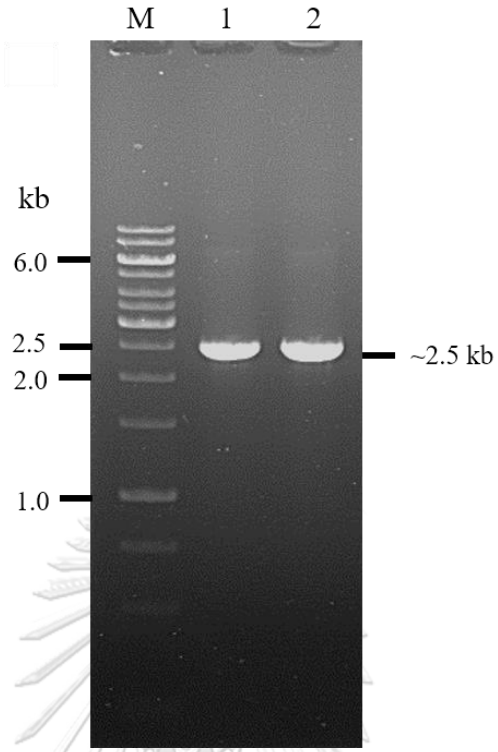


Figure 21 *lysC*-dapA** fragment from PCR amplification using pD-LPC*D* as a template

Lane M : GeneRuler 1 kb DNA ladder

Lane 1 and 2 : *lysC*-dapA** fragment

3.2.1.2 Digestion of *lysC*-dapA fragment and pET-22b(+)**

The *lysC*-dapA** fragment and pET-22b(+) were digested with *Hin*dIII and *Not*I described in section 2.13.1.3 and 2.13.1.1.3, respectively. The digestion patterns of them were analyzed through 0.8% agarose gel electrophoresis, as shown in Figure 22.

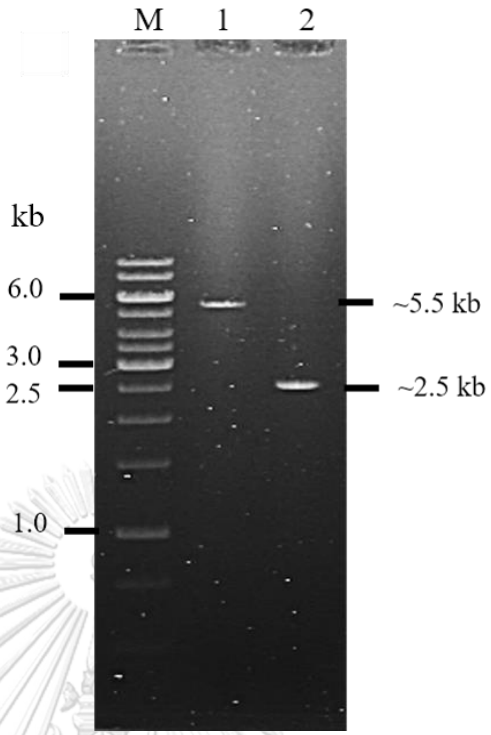


Figure 22 Digestion pattern of *lysC*-dapA** fragment and pET-22b(+)

Lane M : GeneRuler 1 kb DNA ladder

Lane 1 : *Hin*dIII/*Not*I-digested pET-22b(+)

Lane 2 : *Hin*dIII/*Not*I-digested *lysC*-dapA** fragment

The pET-22b(+) was completely digested to linear form with a size around 5.5 kb. For *lysC*-dapA** fragment, DNA band with size around 2.5 kb was obtained after the digestion. Then, these two linear DNA fragments were ligated together and transformed into *E. coli* BL21 (DE3).

3.2.1.3 Verification of pE22-C*D* construction

The colonies which could grow on LB agar plate containing 100 µg/ml of ampicillin were randomly picked for plasmid extraction. In this experiment, six single colonies were extracted for their plasmid. The obtained plasmids were visualized through 0.8% agarose gel electrophoresis, as shown in Figure 23.

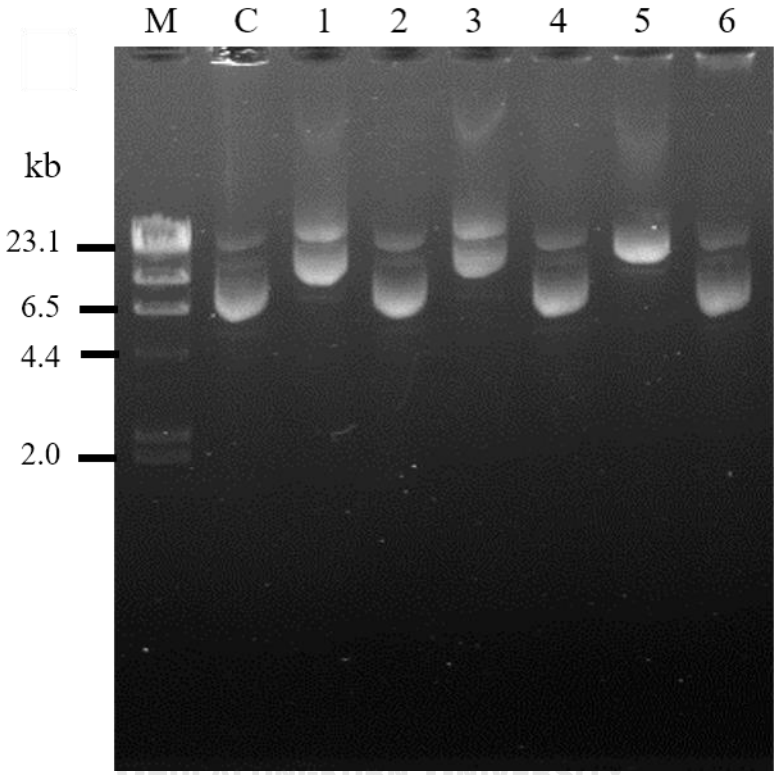


Figure 23 Plasmid extraction of picked colonies

Lane M : Lambda DNA/*Hin*dIII marker

Lane C : pET-22b(+) vector

Lane 1-6 : Recombinant plasmid of picked colony No.1 to 6, respectively

From the agarose gel electrophoresis, colony No. 1, 3, 5 (in lane 1, 3, 5, respectively) gave larger size when compared with that of pET-22b(+) vector in lane C. So, it might have the insertion of *lysC*-dapA** fragment into pET-22b(+) vector. Therefore, in this experiment, transformant No. 1 and 5 were selected to verify the insertion by restriction digestion, as shown in Figure 24.

The restriction pattern of pE22-C*D* from transformant No. 1 and 5 gave a band at the size around 8 kb when the plasmids were digested with *Hin*dIII. When they were digested with *Hin*dIII and *Not*I, they gave DNA bands at 5.5 kb and 2.5 kb which were the same size as pET-22b(+) vector and *lysC*-dapA** fragment, respectively. These results confirmed that *lysC*-dapA** fragment was successfully inserted into pET-22b(+) vector to construct pE22-C*D*. After that DNA sequencing was performed by Bioneer Inc. (Korea) to verify *lysC*-dapA** fragment.

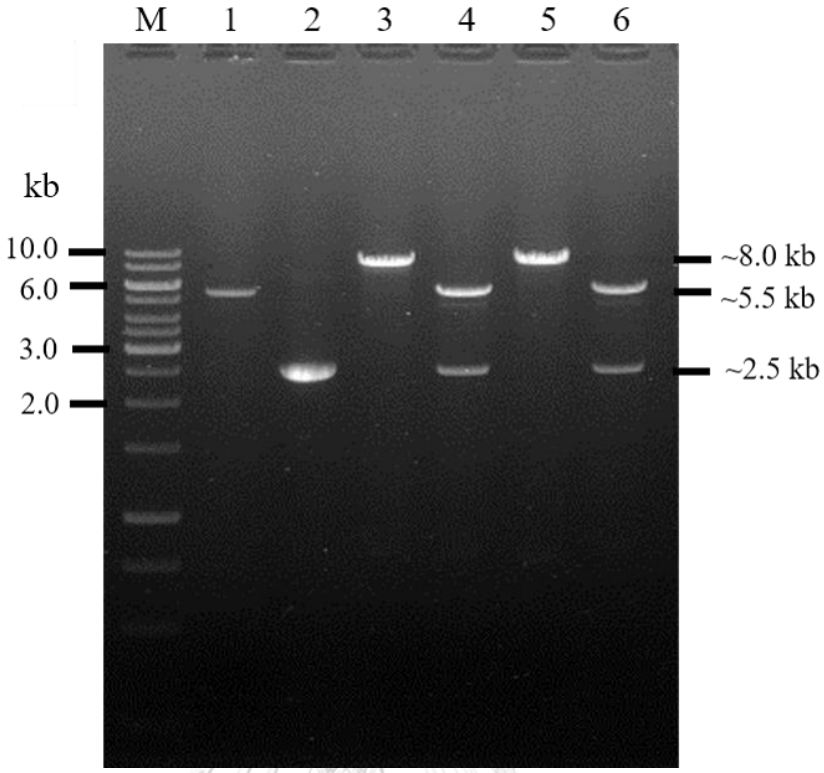


Figure 24 Restriction pattern of pE22-C*D*

Lane M : GeneRuler 1 kb DNA ladder

Lane 1 : *Hin*dIII/*Not*I-digested pET-22b(+)

Lane 2 : *Hin*dIII/*Not*I-digested *lysC*-dapA** fragment

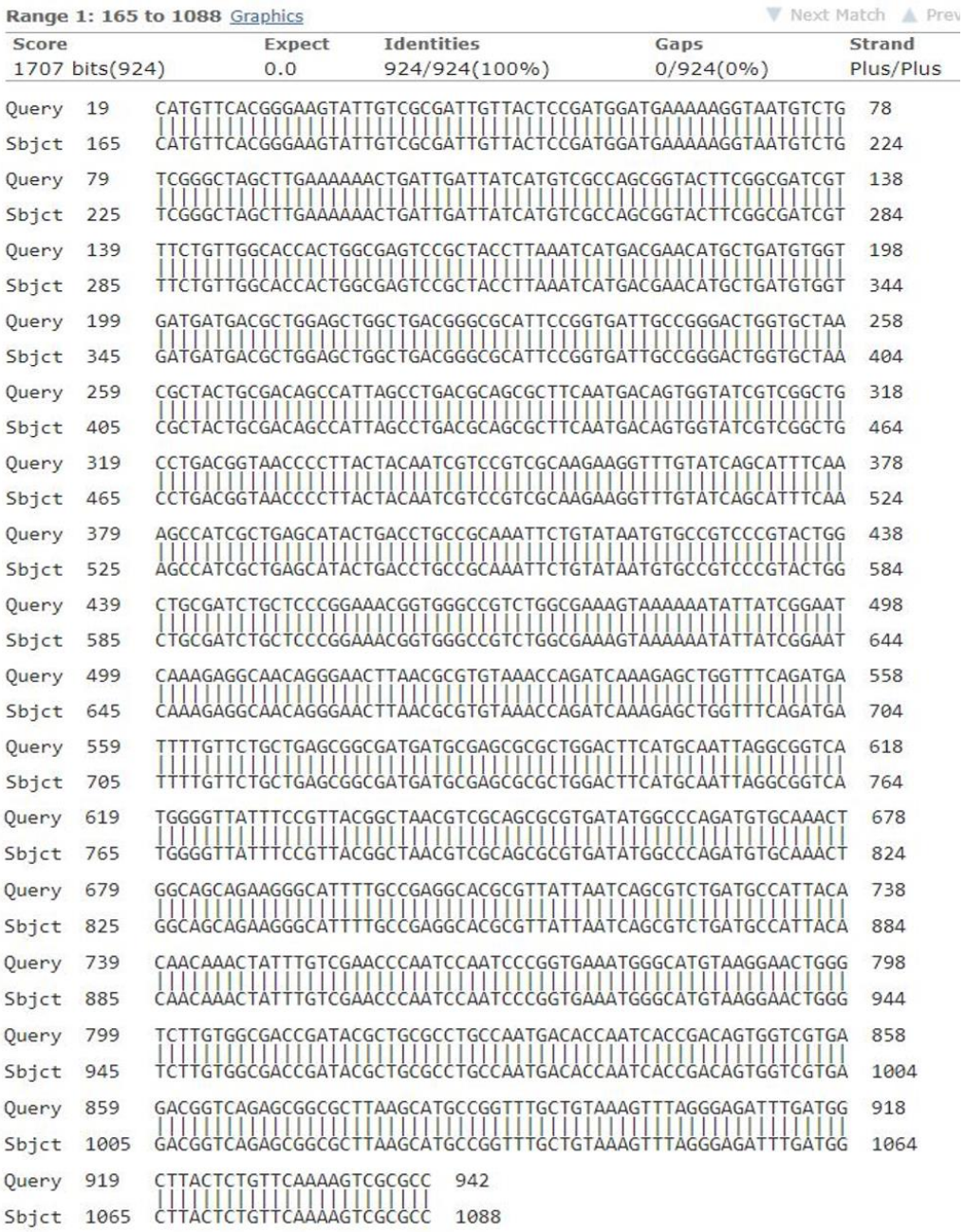
Lane 3 : *Hin*dIII-digested pE22-C*D* from transformant No.1

Lane 4 : *Hin*dIII/*Not*I-digested pE22-C*D* from transformant No.1

Lane 5 : *Hin*dIII-digested pE22-C*D* from transformant No.5

Lane 6 : *Hin*dIII/*Not*I-digested pE22-C*D* from transformant No.5

For DNA sequencing of *lysC**-*dapA** fragment, two pairs of primers were used. The first pair was UpT7promotor (forward) and dapAsequp (reverse) primers. These primers were used for DNA sequence analysis of *lysC** and its upstream region. The other pair was DownlysC (forward) and T7terminator (reverse) primer, both primers were used for the analysis of the DNA sequence of *dapA** and its upstream region. After getting the sequencing data, they were compared with *dapA**, *lysC** and their upstream region sequence reported by Norasetsingh in 2016 [30] using blastn of NCBI. The nucleotide sequence comparison of *dapA** on pE22-C*D* with reference *dapA** nucleotide sequence is shown in Figure 25. The result showed 100% of identities between the query and reference nucleotides. In parallel, the sequence of *lysC** on pE22-C*D* was compared with that of reference *lysC** using blastn as shown in Figure 26. The result also showed 100% identities between the query and reference nucleotides of *lysC**. So, the pE22-C*D* which carrying the corrected nucleotides sequence of *dapA** and *lysC** could be used in the further experiment.



Range 1: 165 to 1088 Graphics ▼ Next Match ▲ Prev

Score	Expect	Identities	Gaps	Strand
1707 bits(924)	0.0	924/924(100%)	0/924(0%)	Plus/Plus

```
Query  19    CATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCGATGGATGAAAAAGGTAATGTCTG  78
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165   CATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCGATGGATGAAAAAGGTAATGTCTG  224

Query  79    TCGGGCTAGCTTGAAAAAACTGATTGATTATCATGTCGCCAGCGGTACTTCGGCGATCGT  138
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225   TCGGGCTAGCTTGAAAAAACTGATTGATTATCATGTCGCCAGCGGTACTTCGGCGATCGT  284

Query  139   TTCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTGATGTGGT  198
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285   TTCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTGATGTGGT  344

Query  199   GATGATGACGCTGGAGCTGGCTGACGGGCGCATTCCGGTGATTGCCGGGACTGGTGCTAA  258
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345   GATGATGACGCTGGAGCTGGCTGACGGGCGCATTCCGGTGATTGCCGGGACTGGTGCTAA  404

Query  259   CGCTACTGCGACAGCCATTAGCCTGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCTG  318
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405   CGCTACTGCGACAGCCATTAGCCTGACGCAGCGCTTCAATGACAGTGGTATCGTCGGCTG  464

Query  319   CCTGACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTTCAA  378
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465   CCTGACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTTCAA  524

Query  379   AGCCATCGCTGAGCATACTGACCTGCCGCAAATTCTGTATAATGTGCCGTCCCGTACTGG  438
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  525   AGCCATCGCTGAGCATACTGACCTGCCGCAAATTCTGTATAATGTGCCGTCCCGTACTGG  584

Query  439   CTGCGATCTGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGAAT  498
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  585   CTGCGATCTGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGAAT  644

Query  499   CAAAGAGGCAACAGGGAACTTAACGCGTGTAAACCAGATCAAAGAGCTGGTTTCAGATGA  558
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  645   CAAAGAGGCAACAGGGAACTTAACGCGTGTAAACCAGATCAAAGAGCTGGTTTCAGATGA  704

Query  559   TTTTGTTCTGCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCATGCAATTAGGCGGTCA  618
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  705   TTTTGTTCTGCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCATGCAATTAGGCGGTCA  764

Query  619   TGGGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGGCCCAGATGTGCAAACT  678
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  765   TGGGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGGCCCAGATGTGCAAACT  824

Query  679   GGCAGCAGAAGGGCATTTTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGCCATTACA  738
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Sbjct  825   GGCAGCAGAAGGGCATTTTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGCCATTACA  884

Query  739   CAACAAACTATTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAGGAACTGGG  798
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  885   CAACAAACTATTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAGGAACTGGG  944

Query  799   TCTTGTGGCGACCGATACGCTGCGCCTGCCAATGACACCAATCACCGACAGTGGTCGTGA  858
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  945   TCTTGTGGCGACCGATACGCTGCGCCTGCCAATGACACCAATCACCGACAGTGGTCGTGA  1004

Query  859   GACGGTCAGAGCGGCGCTTAAGCATGCCGGTTTGCTGTAAAGTTTAGGGAGATTTGATGG  918
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Sbjct  1005  GACGGTCAGAGCGGCGCTTAAGCATGCCGGTTTGCTGTAAAGTTTAGGGAGATTTGATGG  1064

Query  919   CTTACTCTGTTCAAAAGTCGCGCC  942
             ||||||||||||||||||||||||
Sbjct  1065  CTTACTCTGTTCAAAAGTCGCGCC  1088
```

Figure 25 The nucleotide sequence comparison of *dapA**

Query represented nucleotide sequence of *dapA** on pE22-C*D*.

Sbjct represented nucleotide sequence of *dapA** reference from Norasetsingh [30].

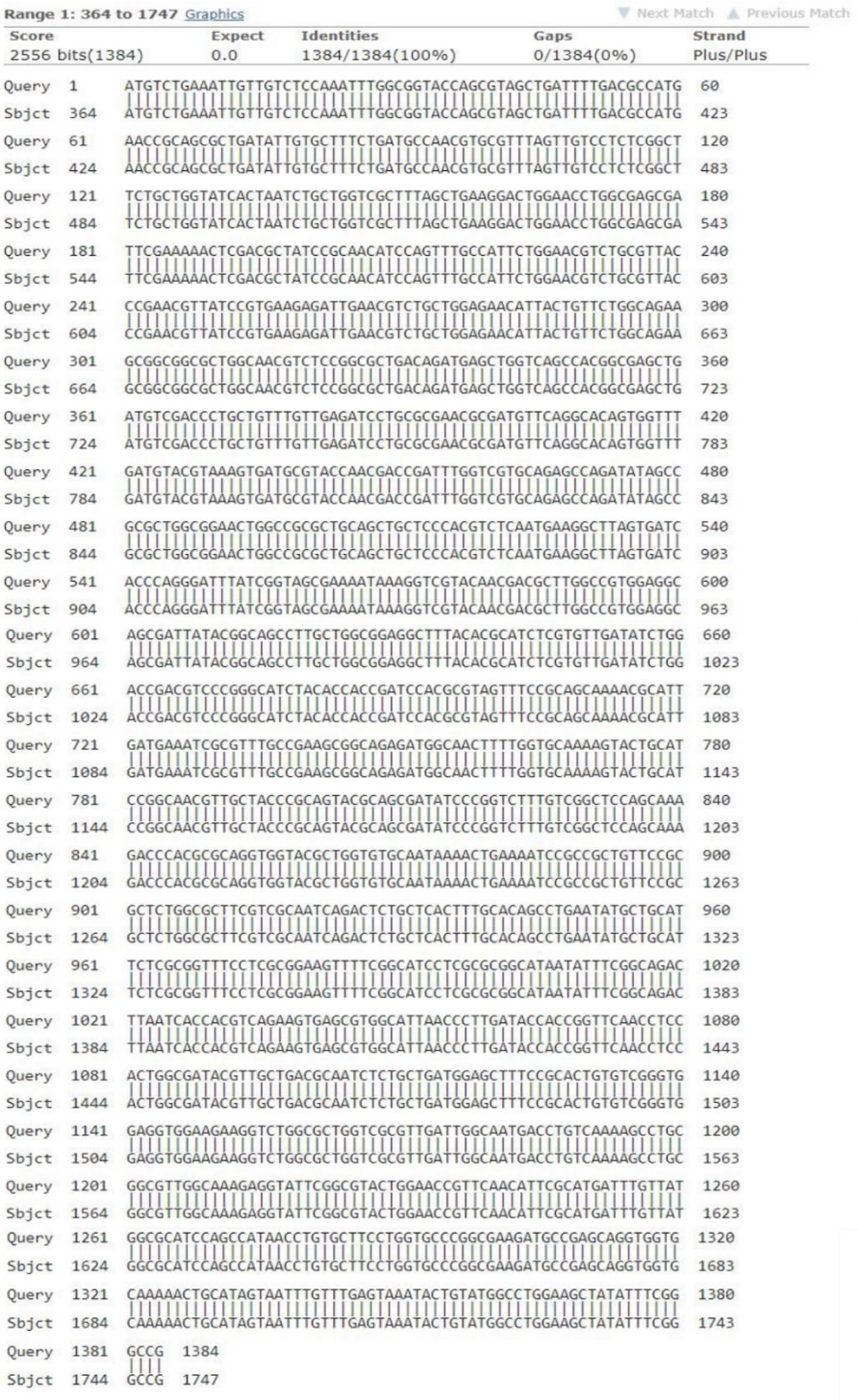

Figure 26 The nucleotide sequence comparison of *lysC**

Query represented nucleotide sequence of *lysC** on pE22-C*D*.

Sbjct represented nucleotide sequence of *lysC** reference [30].

3.2.2 Cloning of pE22-LPC*D*

3.2.2.1 Digestion of *proC-lysdh* fragment and pE22-C*D*

The pD-LPC*D* was digested with *Not*I and *Xho*I by the method as described in 2.13.2.2. Then, *proC-lysdh* fragment was collected and purified via agarose gel electrophoresis. Moreover, pE22-C*D* was also digested with *Not*I and *Xho*I as described in 2.13.2.1. The digested DNA fragments were analyzed by 0.8% agarose gel electrophoresis, as shown in Figure 27.

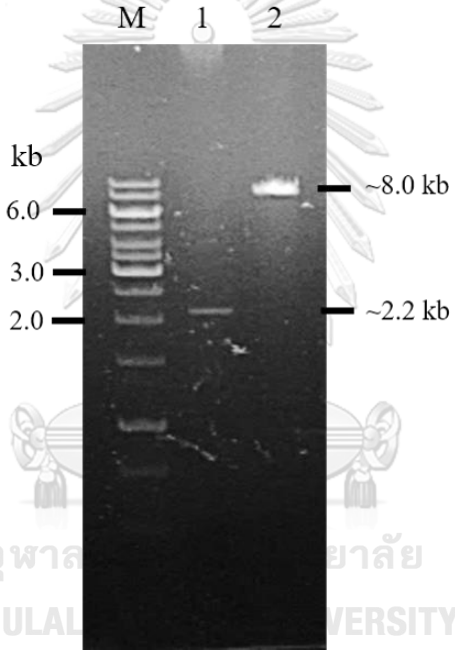

Figure 27 Digestion pattern of *proC-lysdh* fragment and pE22-C*D* with *Not*I and *Xho*I

Lane M : GeneRuler 1 kb DNA ladder

Lane 1 : *Not*I/*Xho*I-digested *proC-lysdh* fragment

Lane 2 : *Not*I/*Xho*I-digested pE22-C*D*

The electrophoresis showed digested band of *proC-lysdh* fragment around 2.2 kb and that of pE22-C*D* around 8.0 kb. Then, these fragments were ligated together by the method described in section 2.13.2.3 to construct the recombinant plasmid, named pE22-LPC*D*. After that, the plasmid was transformed into *E. coli* BL21(DE3) cell to construct W-LPCD strain. After transformation, the transformants carrying pE22-LPC*D* were selected on LB agar plate containing 100 μg/mL of ampicillin. The growing colonies were randomly picked for plasmid extraction and confirmation. The extracted pE22-LPC*D* was analyzed through 0.8% agarose gel electrophoresis, as shown in Figure 28.

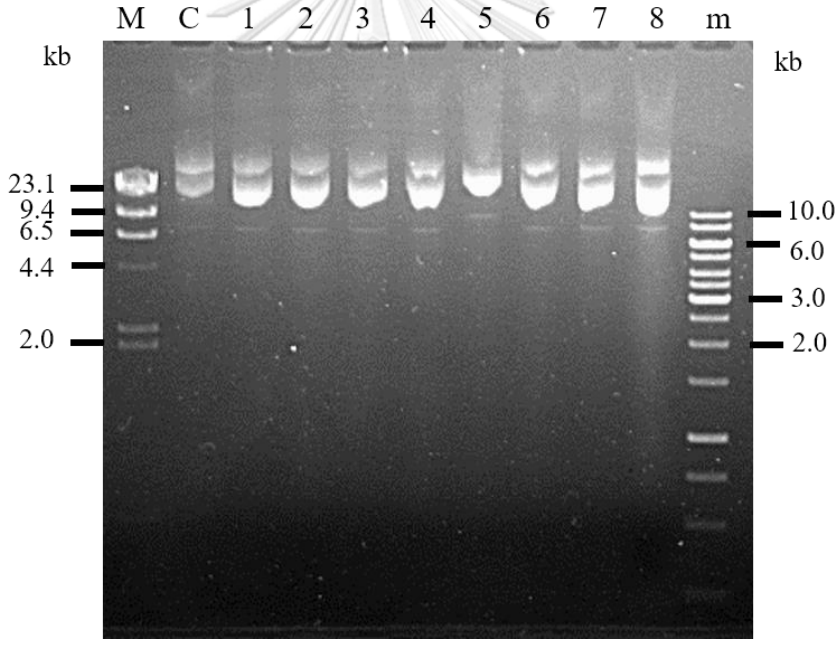

Figure 28 Plasmid extraction of picked transformants

Lane M : Lambda DNA/*Hin*dIII marker

Lane C : pE22-C*D*

Lane 1-8 : Recombinant plasmid of transformant No.1 to 8, respectively

Lane m : GeneRuler 1 kb DNA ladder

From the agarose gel electrophoresis, only transformant No. 5 (in lane 5) gave the larger size of plasmid when compared with pE22-C*D* in lane C. So, it might have the insertion of *proC-lysdh* fragment into pE22-C*D*. Therefore, the recombinant plasmid from transformant No. 5 was selected to verify the insertion by restriction digestion, as shown in Figure 29.

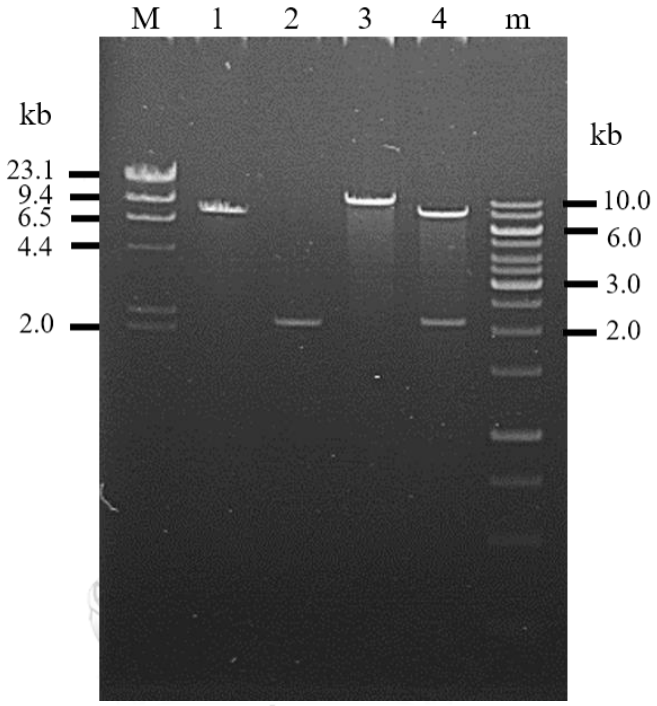

Figure 29 Restriction pattern of pE22-LPC*D*

Lane M : Lambda DNA/*Hin*dIII marker

Lane 1 : *Not*I/*Xho*I-digested pE22-C*D*

Lane 2 : *Not*I/*Xho*I -digested *proC-lysdh* fragment

Lane 3 : *Xho*I -digested pE22-LPC*D* from transformant No.5

Lane 4 : *Not*I/*Xho*I -digested pE22-C*D* from transformant No.5

Lane m : GeneRuler 1 kb DNA ladder

The restriction pattern showed that when pE22-LPC*D* was digested by *Xho*I, transformant gave one band which slightly higher than digested pE22-C*D*. And when it was double digested with *Not*I and *Xho*I, it gave two bands at 8.0 kb and 2.2 kb which were the same size as pE22-C*D* and *proC-lysdh* fragment, respectively. This result confirmed that *proC-lysdh* fragment was successfully inserted into pE22-C*D* to construct pE22-LPC*D*. The *E. coli* BL21(DE3) carrying pE22-LPC*D* was named W-LPCD strain.

After we got recombinant pE22-LPC*D*, it was transformed into *E. coli* BL21(DE3) Δ*thrA* as described in section 2.14. The transformants which could grow on LB agar plate containing 100 μg/mL of ampicillin and 30 μg/mL of kanamycin were picked, and they were confirmed. This new recombinant clone was named KO-LPCD.

3.3 Expression of the recombinant proteins

The expression of *proC, lysdh, lysC** and *dapA** under T7 promoter in each recombinant strain was determined. The recombinant strains were *E. coli* BL21(DE3) harboring pRSFDuet-1, pET-22b(+), pD-ADK [50], pD-P5CR [50] and pE22-C*D*, pE22-LPC*D* (W-LPCD) as well as *E. coli* BL21(DE3) Δ*thrA* harboring pE22-LPC*D* (KO-LPCD). Each strain was induced by 0.4 mM IPTG for 4 hours in LB medium. After that, the recombinant cells were disrupted by sonication, and the crude extracts were collected as described in section 2.15. The proteins expression was observed via SDS-PAGE, as shown in Figure 30. From the SDS-PAGE, Lane 1 and 2 were used as controls. Lane 3, 4 and 5 were used as reference proteins. The results showed that Lys 6-DH, P5CR and AK III, which had the size around 39, 29 and 48 kDa, respectively, gave strong protein band both in W-LPCD and KO-LPCD strains.

On the other hand, the protein band of the mutated DHDPS which had the size around 31.2 kDa was not clearly seen on the SDS-PAGE. In addition, the protein expression pattern between knockout (KO-LPCD) and unknockout (W-LPCD) strains was not significantly different.

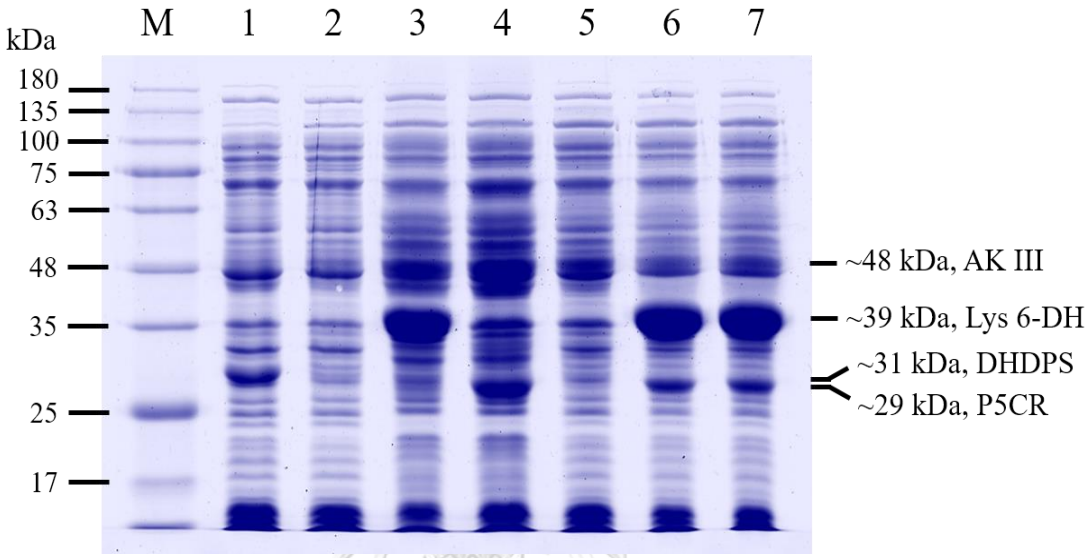

Figure 30 SDS-PAGE of crude extract of each *E. coli* clones after induction by 0.4 mM IPTG for 4 hours

*note: The name of recombinant plasmids which began with pD was derived from pRSFDuet-1, whereas pE22 was derived from pET-22b(+).

Lane M : TriColor Protein Ladder, ready to use (10-180 kDa)

Lane 1 : Crude extract of *E. coli* BL21(DE3) harboring pRSFDuet-1

Lane 2 : Crude extract of *E. coli* BL21(DE3) harboring pET-22b(+)

Lane 3 : Crude extract of *E. coli* BL21(DE3) harboring pD-ADK

Lane 4 : Crude extract of *E. coli* BL21(DE3) harboring pD-P5CR

Lane 5 : Crude extract of *E. coli* BL21(DE3) harboring pE22-C*D*

Lane 6 : Crude extract of *E. coli* BL21(DE3) harboring pE22-LPC*D* (W-LPCD)

Lane 7 : Crude extract of *E. coli* BL21(DE3) Δ*thrA* harboring pE22-LPC*D* (KO-LPCD)

3.4 L-pipecolic acid production

W-LPCD and KO-LPCD strains were cultured in each fermentation medium; Yplus, Tplus, Gplus, Ning, and Ying as described in section 2.16.1. The cultures were induced by 0.1 mM IPTG. The samples from each fermentation flask were collected every 24 hours for growth analysis by optical density monitoring (OD_{600}) and L-PA determination by HPLC. The represented data is average from triplicate experiments. The growth and L-PA production profiles are shown in Figure 31 and 32, respectively. KO-LPCD and W-LPCD strain showed the highest growth when they were cultured in Ying medium. W-LPCD gave higher growth than KO-LPCD until 96 hours after that growth of W-LPCD was not significantly different from that of KO-LPCD. However, the error bars of W-LPCD were rather broad. In the other media, the growth of both strains differed more than 2-3 times. For Ning medium, W-LPCD gave higher growth than KO-LPCD. Their growth curves were nearly constant after 72 hours. The growth patterns of these two strains in Yplus medium were similar to those of Ning medium, but their growths were lower around 0.7-0.8 times. The growth in Tplus medium of W-LPCD was similar within Ning medium while the growth in Gplus medium was similar to Yplus medium. On the other hand, the growth of KO-LPCD was ostensibly low in both of Tplus and Yplus media. So, we set criteria that to compare the L-PA production between KO-LPCD and W-LPCD, the OD_{600} of both strains in the same media had to higher than 2.0. Therefore, we brought the samples from Yplus, Ning, and Ying media to detect L-PA titer.

For L-PA production profiles as shown in Figure 32, the highest L-PA production came from KO-LPCD in Ying medium at 168 hours after induction, with 0.57 g/L of L-PA titer. At this point, W-LPCD gave the lower L-PA titer at 0.40 g/L. The L-PA production was increased with increasing the time although the L-PA production rate after 120 hours was slightly decreased. In Ning medium, KO-LPCD

gave higher L-PA production (0.30 g/L) than W-LPCD (0.21 g/L) at 168 hours however their L-PA production rates were lower than in Ying medium. For Yplus medium, the L-PA production from KO-LPCD and W-LPCD were not different before 72 hours. After 72 hours, W-LPCD gave higher L-PA production (0.17 g/L) than KO-LPCD (0.10 g/L).

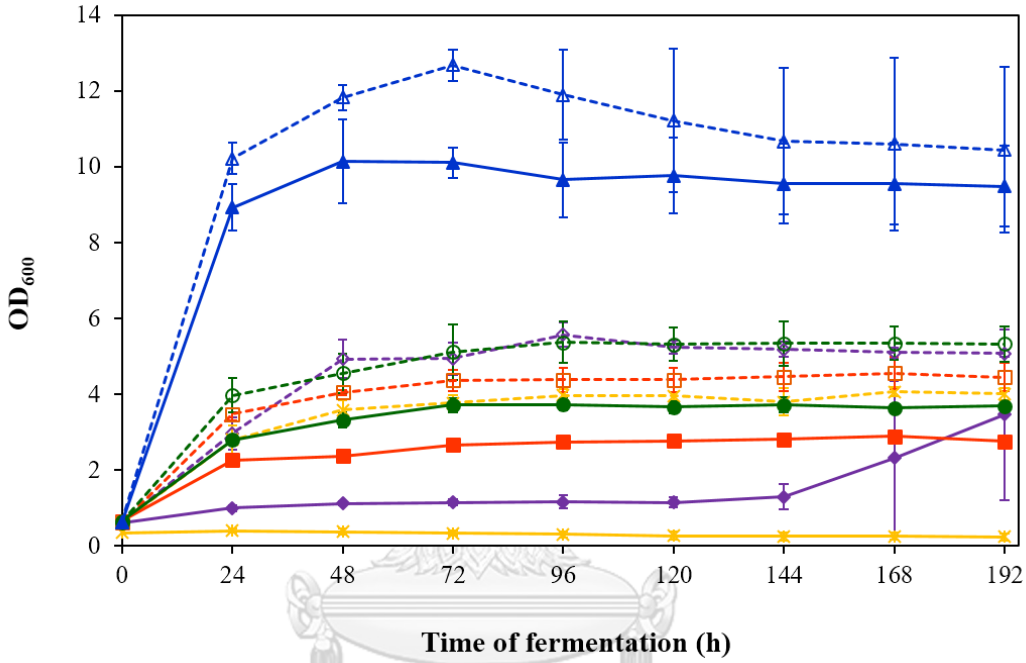

Figure 31 Growth curve of recombinant clones in various fermentation media. The data came from three independent experiments.

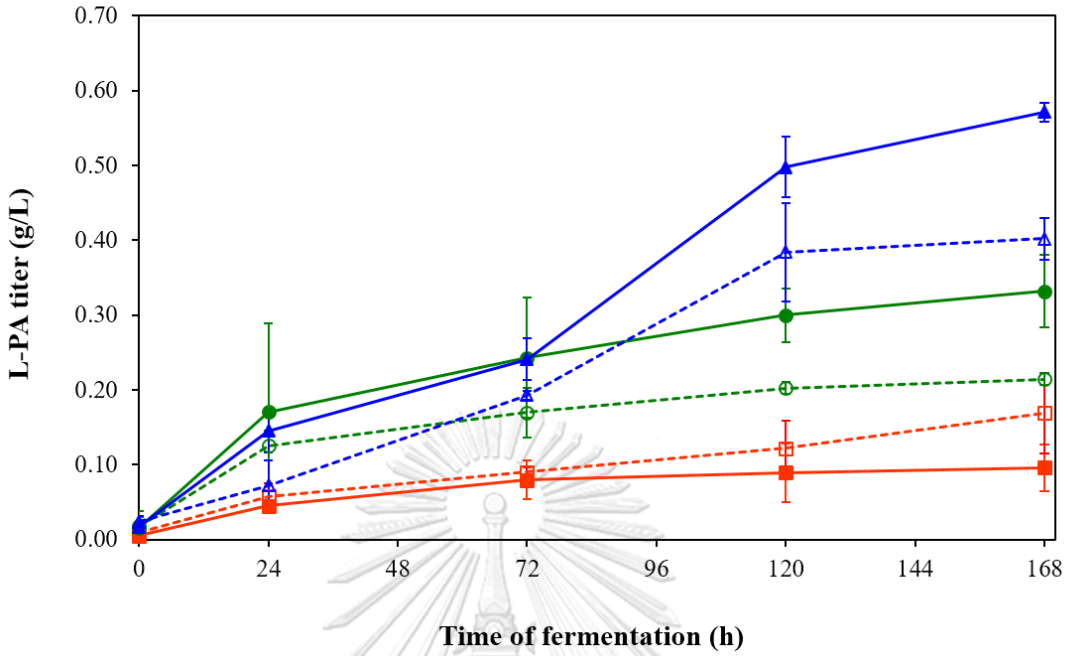

Figure 32 L-PA production profile of recombinant clones in various fermentation media

The data came from three independent experiments.

Since Ying medium gave the highest growth and L-PA production, the data from the fermentation in Ying medium at the highest production time were selected to analyze the effect of *thrA* knockout to L-PA production. The comparison of L-PA production between KO-LPCD and W-LPCD was performed, and the results are shown in Table 7 and Figure 33. L-PA titer which produced by KO-LPCD was higher than that of W-LPCD around 1.4-fold. Moreover, the specific production from KO-LPCD was 1.8-fold of that obtained from W-LPCD.

Table 4 Comparison of L-PA production

Strain	**L-PA titer (g/L)**	**Wet cell weight (g/L)**	**Specific production (g/g WCW)**
KO-LPCD	0.571 ± 0.01	11.7 ± 1.3	0.049±0.006
W-LPCD	0.408 ± 0.02	15.1 ± 2.2	0.027±0.003

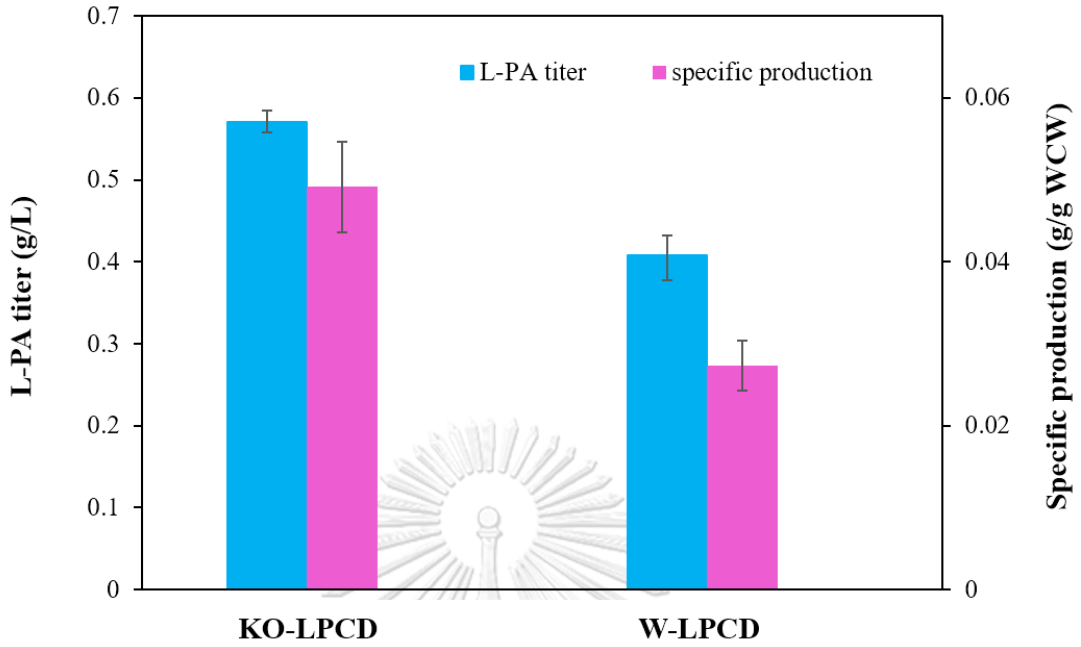

Figure 33 The effect of *thrA* knockout on L-PA production

CHAPTER IV

DISCUSSIONS

L-pipecolic acid (L-PA) is produced by *E. coli* only if it is engineered. The heterologous genes, *lysdh* and *proC*, was transformed into *E. coli* cell to make them produced L-PA [25]. Furthermore, L-PA production in *E. coli* involves in L-lysine metabolism because L-lysine is used as a substrate. So, the essential factor in enhancing L-PA production is the amount of L-lysine in the cell. For enhancement of L-lysine, the amino acid residues at the regulatory site of L-lysine feedback inhibition enzymes (AK III and DHDPS) in L-lysine biosynthesis were changed to deprive the feedback effect. Site-directed mutageneses were performed on the nucleotide sequence of *lysC* (encoding AK III) and *dapA* (encoding DHDPS). Moreover, homoserine dehydrogenase encoded by *thrA* can draw the intermediate in L-lysine biosynthesis pathway to synthesize L-threonine. Thus, the inactivation of homoserine dehydrogenase I by *thrA* knockout should give more L-lysine production and lead to more production of L-PA.

4.1 *thrA* knockout in *E. coli* BL21(DE3)

For *thrA* knockout, TargeTron® Gene Knockout System was chosen. TargeTron® Gene Knockout System is based on the principle of disruption of bacterial genes by insertion of group II introns. TargeTron system is site specific; thus, it is enabled re-targeting of introns to insert efficiently into virtually any desired DNA target. Moreover, it is an easy method to disrupt the gene and save time to perform the experiment. The processes started with target site prediction on *thrA* for group II intron insertion. The site of insertion, which gave the highest knockout efficiency score was between 1356 and 1357 of *thrA*. This site located around the middle of the

gene sequence. So, after transcription of *thrA* with intron insertion, the inserted homoserine dehydrogenase I should not fold to an active form. To confirm the *thrA* knockout, we performed colony PCR in two steps using 1. gene specific primers and 2. one gene specific primer and one intron specific primer. Colony PCR from gene specific primers can indicate that the gene is inserted by group II intron. In addition, the colony PCR from one gene specific primer and one intron specific primer in the second step can roughly indicate the location of intron insertion on the gene. From this research, the 20 growing colonies on the selective plate was brought to analyze *thrA* knockout. The result indicated that 13 colonies showed *thrA* knockout. So, the capability of TargeTron® Gene Knockout System in this research was 65%. However, the capability of group II intron insertion might differ for each gene and each organism. For example, the intron insertion capability to disrupt *Seb* and *Has* in *Staphylococcus aureus* were 37 and 100%, respectively [55] as well as the intron insertion capability to disrupt *plc* (alpha toxin gene) in *Clostridium perfringens* was 5.3% [56]. For the stability of group II intron insertion, the clone in this study was subcultured more than seven times and kept more than one year for storage. The clone still grew on medium containing kanamycin antibiotic and the colony PCR of it was similar to the original.

4.2 Construction of pE22-LPC*D*

This part was performed to solve the problem about the selectable recombinant plasmid in *E. coli* host cell. As it was mention, the *thrA* knockout in *E. coli* using TargeTron® Gene Knockout System could be selected with kanamycin antibiotic. Unfortunately, the recombinant plasmid which containing the desired genes, pD-LPC*D* was also selected with kanamycin antibiotic. To screen the recombinant plasmid which, was transformed into *E. coli* BL21(DE3) Δ*thrA*, the selective antibiotic was needed to change. The best choice in that time was to change harboring vector from

pRSFDuet-1 to pET-22b(+) by subcloning of these desired genes. pET-22b(+) was chosen because it was T7 system for expression of the inserted gene, which is similar to pRSFDuet-1.

4.3 Expression of the involving genes

From the SDS-PAGE, the expression of lysine 6-dehydrogenase; Lys 6-DH and pyrroline-5-carboxylate reductase; P5CR were clearly seen when *lysdh* and *proC* were induced by 0.4 mM IPTG for 4 hours. Especially, Lys 6-DH, it gave very strong protein band. The result was similar to the report of Ruldeekulthamrong in 2007 [57]. *lysdh* could overexpress even if it was not induced. For P5CR, its size and band intensity were similar to the result of Srimaung in 2010 [25]. The mutated AKIII showed the band at around 48 kDa although its expression was not strong like Lys 6-DH and P5CR. On the other hand, the expression of DHDPS could not be detected. One of the reason was that its size was close to the other proteins. Norasetsingh in 2016 also reported that it very hard to display the clear band of DHDPS [30]. However, the existence of mutated AK III and DHDPS could give more production of L-PA [50] and more production of L-aminoadipic acid that use L-lysine as a substrate [30].

4.4 Growth of the recombinant clones

Before the fermentation experiment was performed, the media for fermentation had been searched. At first, the minimal media from Thongchuang [49] and Rathchaneeladdajit [58] which used for L-phenylalanine production by engineered *E. coli*, was used to culture wild-type and *thrA* knockout *E. coli*. These media containing glycerol and $(NH_4)_2SO_4$ as sole carbon and nitrogen sources, respectively. The results showed that they gave meager growth of *thrA* knockout *E. coli*, especially

in medium from Rathchaneeladdajit, the OD_{600} of this strain was lower than 0.2 while OD_{600} from Thongchuang medium was around 0.3. So, Thongchuang medium was chosen to further use in this study. This minimal medium was modified for growing the *thrA* knockout *E. coli*. L-threonine, L-glycine, and yeast extract were added into Thongchuang medium to generate Tplus, Gplus, and Yplus media, respectively. Since *thrA* knockout effects directly on L-threonine biosynthesis, the *thrA* knockout should make the cell lacking L-threonine. So, L-threonine was supplied into the minimal medium to prove the presumption. Moreover, in KEGG PATHWAY Database shows that there is another way to synthesize L-threonine, via reversible reaction from glycine and acetaldehyde catalyzed by threonine aldolase (Appendix J). So, L-glycine was supplied to occur the condensation with acetaldehyde in *E. coli* cell. Meanwhile, yeast extract was also supplied because it composes of the highly short peptide chain, high concentration of B vitamins, sugar, and mineral. So, it might improve the growth of *thrA* knockout *E.coli*. Moreover, Ning medium which used to grow *thrA* knockout *E. coli* [51], and Ying medium which used for L-PA production by engineer *E. coli* [18], were also modified to this work. Glycerol was substituted to glucose in Ning and Ying media, respectively.

The result showed that Tplus medium gave higher growth than in the Thongchuang medium about three times, but the growth was still lower than that of wild-type *E.coli*. Although L-threonine concentration was also varied, the growth was limited. It indicated that the cell required L-threonine for its growth; however, other substances were also required for its normal growth. For Gplus medium, it could not improve the growth. So, the supplementation of L-glycine could not lead to the recovery of L-threonine. For Yplus medium, it could elevate the growth of *E. coli* BL21(DE3) Δ*thrA* better than the other two media mentioned before. It indicated that the supplement of yeast extract components was effective for the growth of

thrA knockout *E. coli*. In Ning medium, it elevated higher growth than in Yplus medium. Since Ning medium contained high protein supplement, yeast extract, and casein. For Ying medium, *E. coli* BL21(DE3) Δ*thrA* could not grow equivalent to wild-type *E. coli* BL21(DE3), however, the difference of them was lower when compared to the other media. Therefore, it can assume that the high protein or small peptide components could improve the growth of *E. coli* BL21(DE3) Δ*thrA*. However, growth of the knockout strain could be improved by optimization of culture medium. Ning et al. in 2016 [51], also disrupted *thrA* in *E. coli*. The growth of *thrA* knockout strain was lower than wild-type strain around 23%. We also found that the growth of *thrA* knockout strain in Ying medium was lower than wild-type about 22%. However, the experiment gave a work perk that homoserine dehydrogenase I was the essential enzyme in *E. coli*.

4.5 Production of L-pipecolic acid

At present, the researches about the production of L-PA by biological process, fermentation, and enzymatic reaction are dramatically increased. For example, in 2015 Tani et al. reported the production of L-PA using one-pot synthesis by incubation the engineered *E. coli* JM109 (overexpressing gene encoding L-lysine racemase, L-lysine α-oxidase, Δ^1-piperideine-2-carboxylate reductase and glucose dehydrogenase) in the reaction mixture containing 0.4 M DL-lysine, 0.5 M glucose, 0.2 mM $NADP^+$, 10,000 units/L catalase from bovine, and 4 g/L deforming agent. They earned 45.1 g/L of L-PA [48]; however, the method was a risk to contamination, and L-lysine has a high price.

In 2017, Ying and coworker overexpressed genes encoding AK III, DHDPS, diaminopimelate carboxylase and lysine cyclodeaminase in *E. coli* BL21(DE3). Since lysine cyclodeaminase has poor catalytic efficiency, L-PA production of the engineered

E. coli using fed-batch fermentation was only 5.33 g/L [18]. In addition, Pérez-García and coworker reported that the L-PA production using fed-batch fermentation by an engineered *Cyanobacterium glutamicum*, overexpressing the gene encoded for L-lysine 6-dehydrogenase and pyrroline 5-carboxylate reductase, obtained 14.4 g/L of L-PA [47]. In this study, we earned just 0.57 g/L of L-PA titer that was lower more than nine fold when compared to all mention reports. However, in this study, we reached the objectives. It can be ascribed that *thrA* knockout in *E. coli* can increase the flux of L-lysine biosynthesis that resulted in more L-PA production. When it was compared with wild-type L-PA production *E. coli*, it was increased L-PA production around 29% although its growth is lower. However, our study was just a preliminary experiment. The culture components and also culture conditions should be optimized for more L-PA production.

CHAPTER V

CONCLUSIONS

1. The *thrA* in *E. coli* BL21(DE3) was successfully knocked out by insertion of group II intron between nucleotide 1356 and 1357 of the sense strand of *thrA*. The knockout strain was named *E. coli* BL21(DE3) Δ*thrA*.

2. The pE22-LPC*D* containing *lysC**, *dapA**, *lysdh*, and *proC* was successfully constructed and transformed into *E. coli* BL21(DE3) wild-type and *E. coli* BL21(DE3) Δ*thrA*.

3. Protein expression of KO-LPCD and W-LPCD strains did not significantly different on SDS-PAGE analysis. The expression of Lys 6-DH, P5CR, and AK III, which have the size around 39, 29 and 48 kDa, respectively, was clearly seen while that of DHDPS could not be detected.

4. The *thrA* knockout led to the reduction of growth in *E. coli*, whereas the L-PA production was increased.

5. Ying medium containing contained 30 g/L glycerol, 12 g/L peptone C, 8 g/L yeast extract, 2.1 g/L citric acid.H_2O, 2.5 g/L $(NH_4)_2SO_4$, 0.1 g/L $FeCl_3$, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L $K_2HPO_4.3H2O$, 3 g/L KH_2PO_4 and 15.13 g/L $Na_2HPO_4.12H_2O$, gave the highest growth and L-PA production in both knockout and wild-type strains.

6. KO-LPCD strain could produce 0.571 g/L of L-PA when it was cultured in Ying medium for 168 after induction by 0.1 mM IPTG, with specific production of 1.8-fold higher than the wild-type strain.

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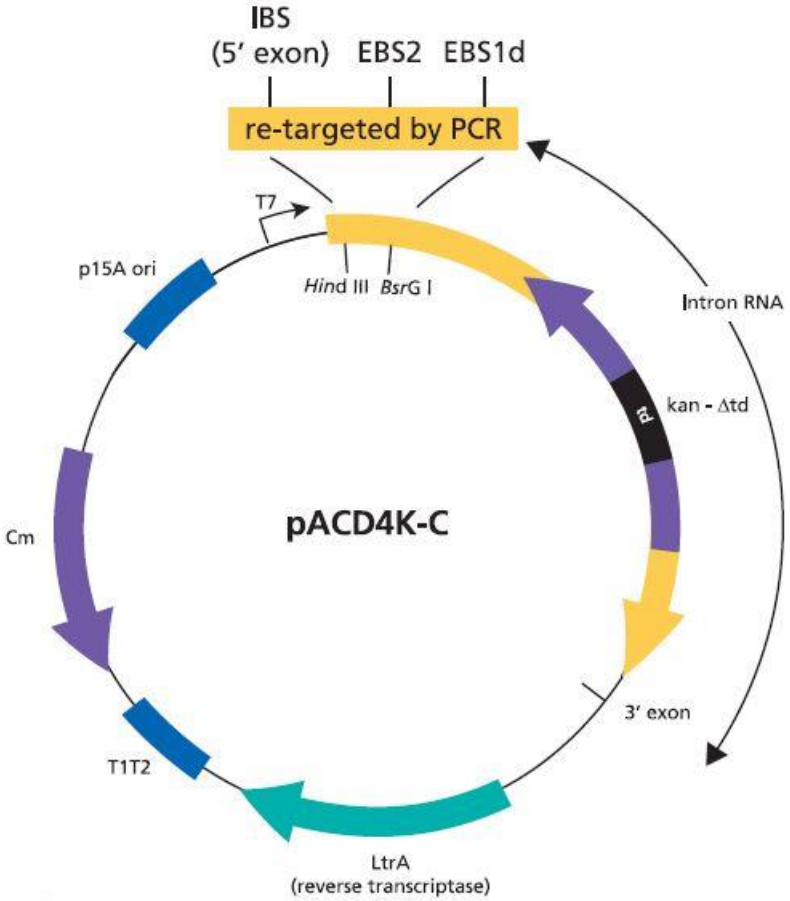
APPENDICES

APPENDIX A

Map of pACD4K-C

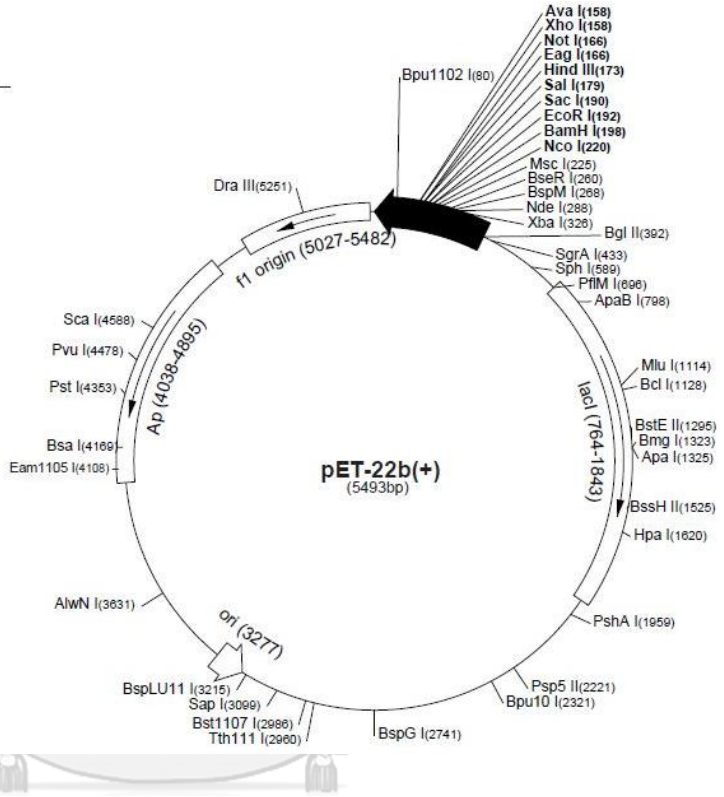


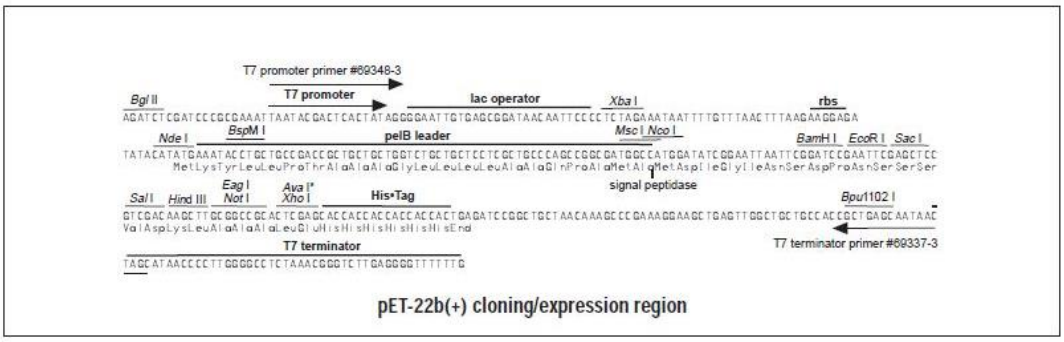
Note: pACD4K-C plasmid had a size of 7675 bp in total. The plasmid is propagated in medium containing chloramphenicol. Splicing of the group II intron results in excision of the *td* intron. Removal of *td* restores the kan ORF prior to chromosomal insertion.

APPENDIX B

Restriction Map of pET-22b(+)

pET-22b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
bla coding sequence	4038-4895
f1 origin	5027-5482





pET-22b(+) cloning/expression region

APPENDIX C

Modified protocol of Presto™ mini plasmid kit

Presto™ mini plasmid kit was designed for quick isolation of plasmid DNA.

1. Harvesting

   The bacterial cell culture 3 mL was centrifuged at 10,000 xg for 2 minutes and collected the cell pellet.

2. Resuspension

   The cell pellet was added 200 µL of PD1 buffer (contained RNase A). The cell was completely resuspended by vortex.

3. Cell lysis

   To lyse the cell, 200 µL of PD2 buffer was added to resuspend sample. Then, it was gently mixed by inverting the tube 10 times and let stand at room temperature for 2 minutes.

4. Neutralization

   The suspension was neutralized by addition of 300 µL PD3 buffer. Then, it was mixed immediately by inverting the tube 10 times and centrifuged at 10,000 xg for 15 minutes.

5. DNA binding

   All of the supernatant was transferred to PDH column and centrifuged at 10,000 xg for 1 minutes.

6. Wash

   The 400 µL of W1 buffer was added into the PDH column. Then, it was centrifuged at 10,000 xg for 1 minutes. The flow-through in the collection tube was discarded. After that, the 600 µL of Wash buffer was added into the PDH column then was centrifuged at 10,000 xg for 1 minutes. The flow-through was discarded and the column was centrifuged again to dry column.

7. Elution

   The dried column was transferred into new 1.5 microcentrifuge tube. The 50 µL of elution buffer was added and let stand for 10 minutes. After that, the tube was centrifuged at 10,000 xg for 5 minutes to elute the purified DNA.

APPENDIX D

Modified protocol of GenepHlow™ Gel/PCR kit

1. Sample preparation

   1.1 Gel dissociation

   The agarose gel slice containing relevant DNA fragment 0.3 g was added 500 µL of Gel/PCR buffer. After mixed, it was incubated at 58 ºC for 15 minutes and inverts the tube every 3 minutes, ensures the gel slice completely dissolved.

   1.2 PCR reaction

   The 50 µL of PCR reaction was added 5 volumes of Gel/PCR buffer and mixed by vortex.

2. DNA binding

   All of the sample from section 1 (maximum of 800 µL) was transferred into DFH column and centrifuged at 10,000 xg for 1 minute. The flow-through was discarded.

3. Wash

   The 400 µL of W1 buffer was added into DFH column. The column was centrifuged at 10,000 xg for 1 minute. After that, it was added by 600 µL of Wash buffer and let stand for 1 minute. Then the flow-through was discarded by centrifugation at 10,000 xg for 1 minute. And the column was dried by centrifuged again at 10,000 xg for 5 minutes.

4. DNA Elution

   The dried column was transferred into new 1.5 microcentrifuge tube. The elution buffer, the volume depend on each experiment, was added and let stand for 10 minutes. After that, the tube was centrifuged at 10,000 xg for 5 minutes to elute the purified DNA

APPENDIX E

Preparation for SDS-PAGE analysis

1. Stock solution

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane	24.2	g

Adjusted pH to 8.8 with 1 N HCl and adjusted volume to 100 mL with distilled water.

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane	12.1	g

Adjusted pH to 6.8 with 1 N HCl and adjusted volume to 100 mL with distilled water.

10% (w/v) SDS

Sodium dodecyl sulfate (SDS)	10	g

Added distilled water to a total volume of 100 mL.

50% (w/v) Glycerol

100% Glycerol	50	mL

Added distilled water to a total volume of 100 mL.

1% (w/v) Bromophenol blue

Bromophenol blue	100	mg

Brought to 10 mL with distilled water and stirred until dissolved.

The aggregated dye was removed by filtration.

APPENDIX E (continued)

2. Working solutions

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2	g
N, N′-methylene-bis-acrylamide	0.8	g

Adjusted volume to 100 mL with distilled water.

Filtered and stored in dark (brown bottle) at 4^{oC}

Solution B (1.5 M Tris-HCl, pH 8.8 and 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75	mL
10% (w/v) SDS	4	mL
Distilled water	21	mL

Solution C (0.5 M Tris-HCl, pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50	mL
10% (w/v) SDS	4	mL
Distilled water	46	mL

10% (w/v) Ammonium persulfate

Ammonium persulfate	0.5	g
Distilled water	5.0	mL

APPENDIX E (continued)

Electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS)

Tris (hydroxymethyl)-aminomethane	3.0	g
Glycine	14.4	mL
SDS	1	g

Dissolved and adjusted to total volume to 1 liter with distilled water

(final pH should be approximately 8.3)

5x Sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (w/v) bromophenol blue)

1 M Tris-HCl (pH 6.8)	0.6	mL
50% (v/v) Glycerol	5.0	mL
10% (w/v) SDS	2	mL
1% (w/v) Bromophenol blue	1	mL
β-Mercaptoethanol	0.5	mL
Distilled water	1.4	mL

APPENDIX E (continued)

3. SDS-PAGE

12.5% Separating gel

Solution A	4.2	mL
Solution B	2.5	mL
Distilled water	3.3	mL
10% (w/v) Ammonium persulfate	50	µL
TEMED	5	µL

5.0% Stacking gel

Solution A	0.67	mL
Solution C	1.0	mL
Distilled water	2.3	mL
10% (w/v) Ammonium persulfate	30	µL
TEMED	5	µL

APPENDIX F

Protein staining solution

Staining solution, 1 liter

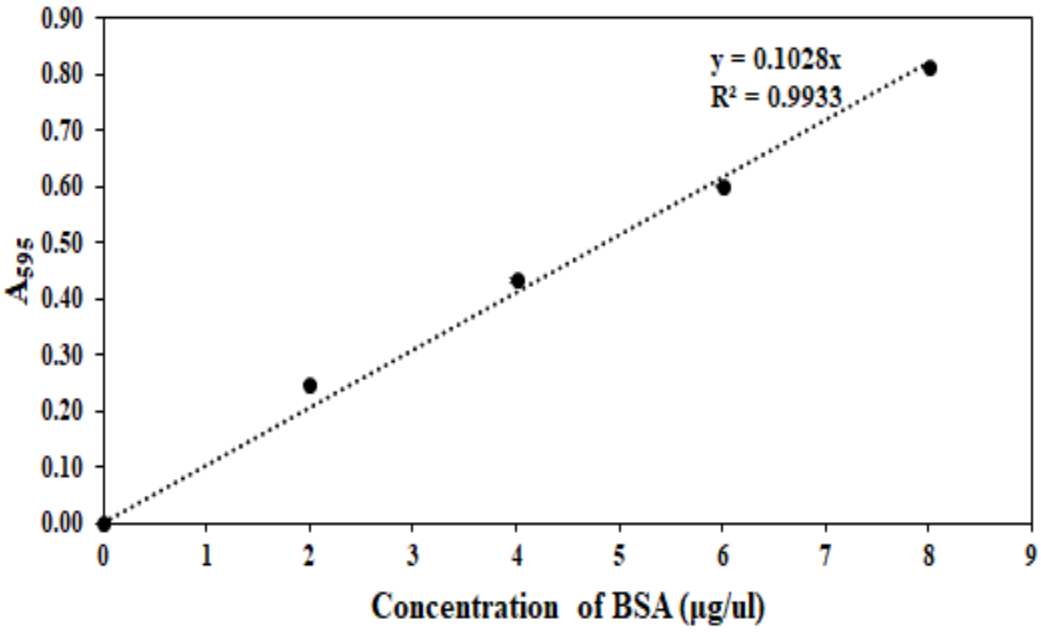
Coomassie brilliant blue R-250	1.0	mL
Methanol	450	mL
Distilled water	450	mL

Destaining solution, 1 liter

Methanol	100	mL
Glacial acetic acid	100	mL
Distilled water	800	mL

APPENDIX G

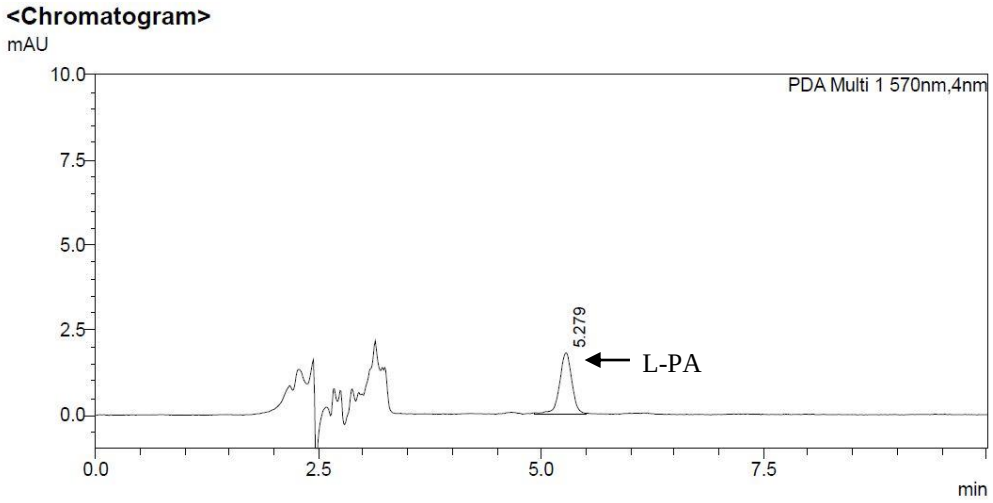
Standard curve for protein determination by Bradford's method



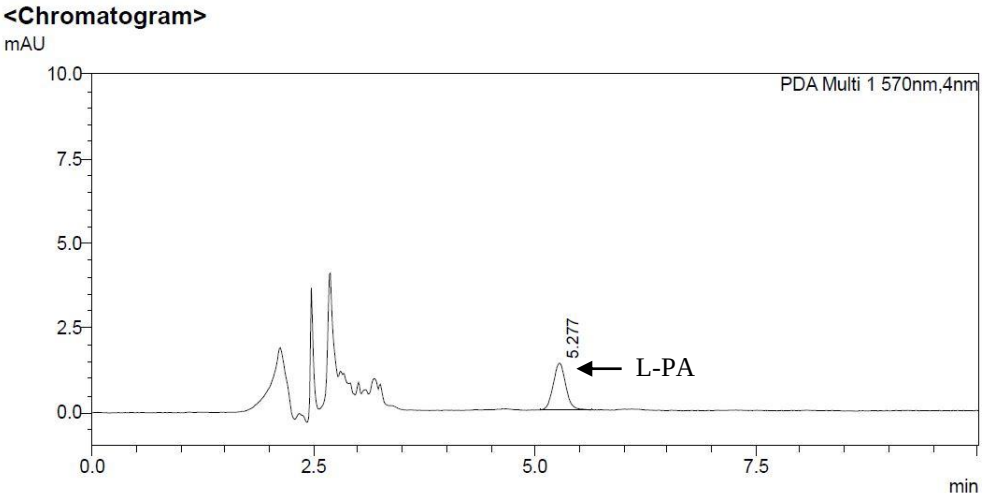
APPENDIX H

HPLC profile of L-pipecolic acid

A.) L-PA standard at 0.1 g/L



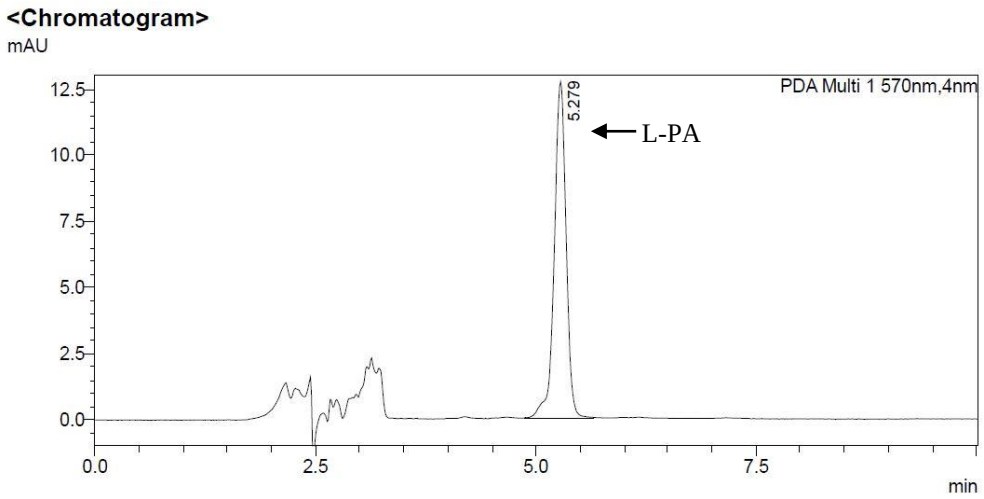
B.) Sample from Ying medium at 24 hours after induction (0.114 g/L)



APPENDIX H (continue)

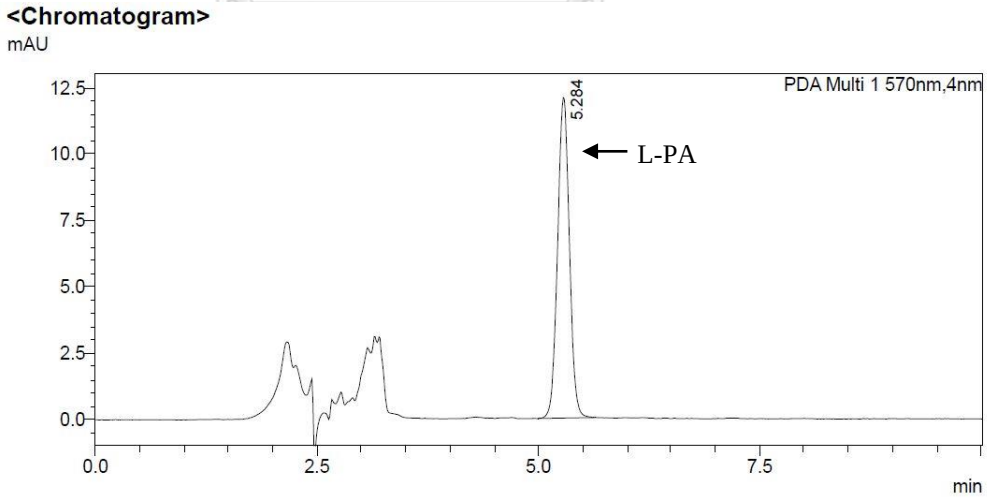
HPLC profile of L-pipecolic acid

C.) L-PA standard at 0.5 g/L



D.)

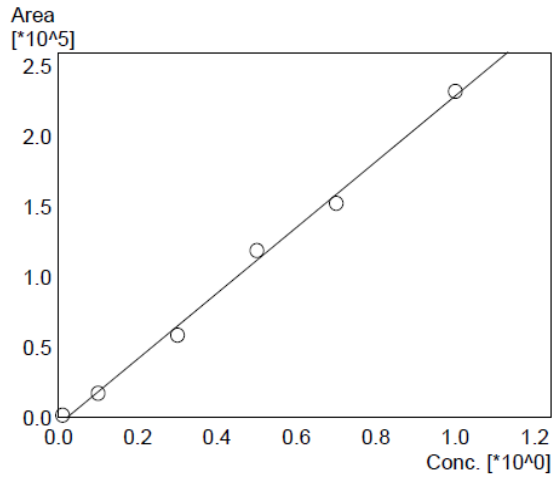
E.) Sample from Ying medium at 120 hours after induction (0.507 g/L)



APPENDIX I

Standard curve of L-pipecolic acid by HPLC

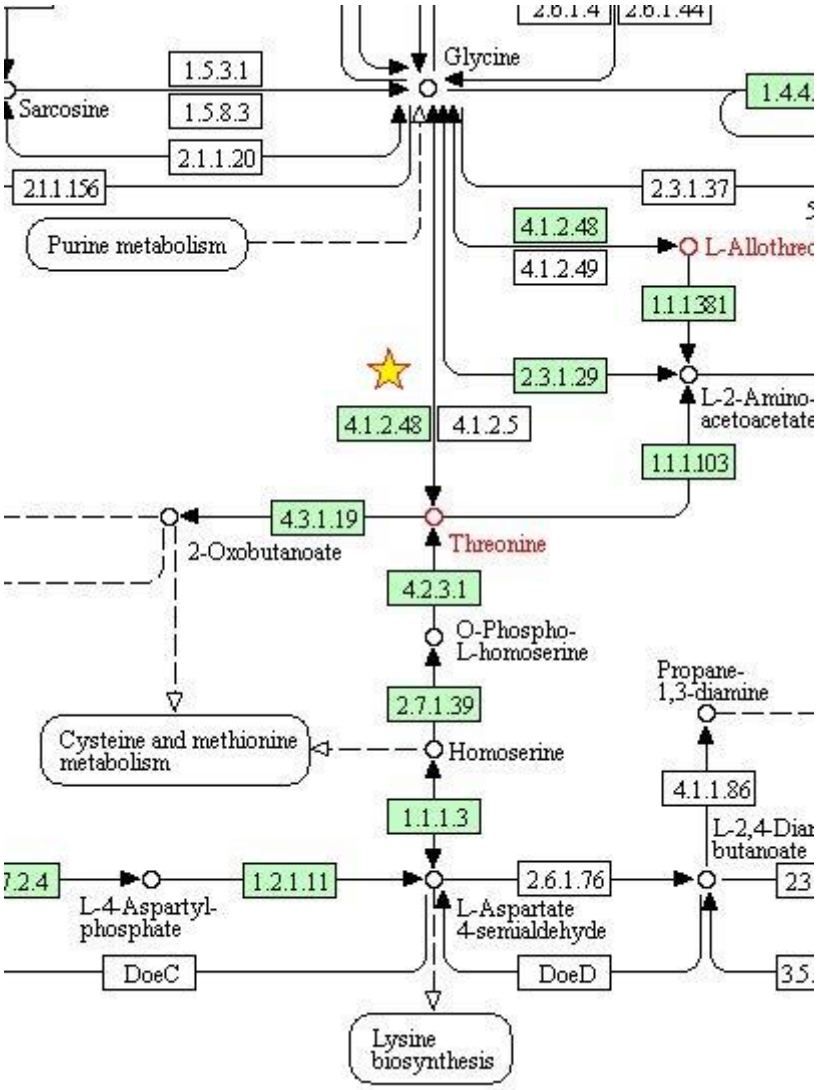
Name : L-PA
Quantitative Method : External Standard
Function : f(x)=233781*x-4739.53
Rr1=0.9979179 Rr2=0.9958402 RSS=1.608918e+008
MeanRF: 2.079068e+005 RFSD: 2.541258e+004 RFRSD: 12.223064
FitType : Linear
ZeroThrough : Not Through
Weighted Regression : None
Detector Name : PDA-M20A



#	Conc.(Ratio)	MeanArea	Area
1	0.01	1896	1896
2	0.1	17388	17388
3	0.3	58760	58760
4	0.5	118993	118993
5	0.7	152551	152551
6	1	232142	232142

APPENDIX J

L-threonine synthesis from condensation of L-glycine and acetaldehyde



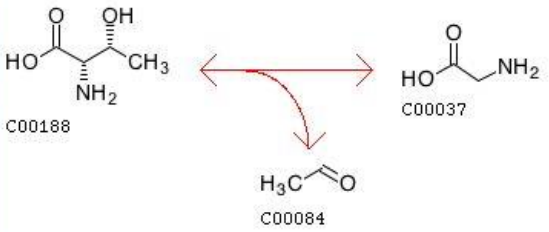
= L-threonine alldolase

APPENDIX J (continue)

KEGG

REACTION: R00751

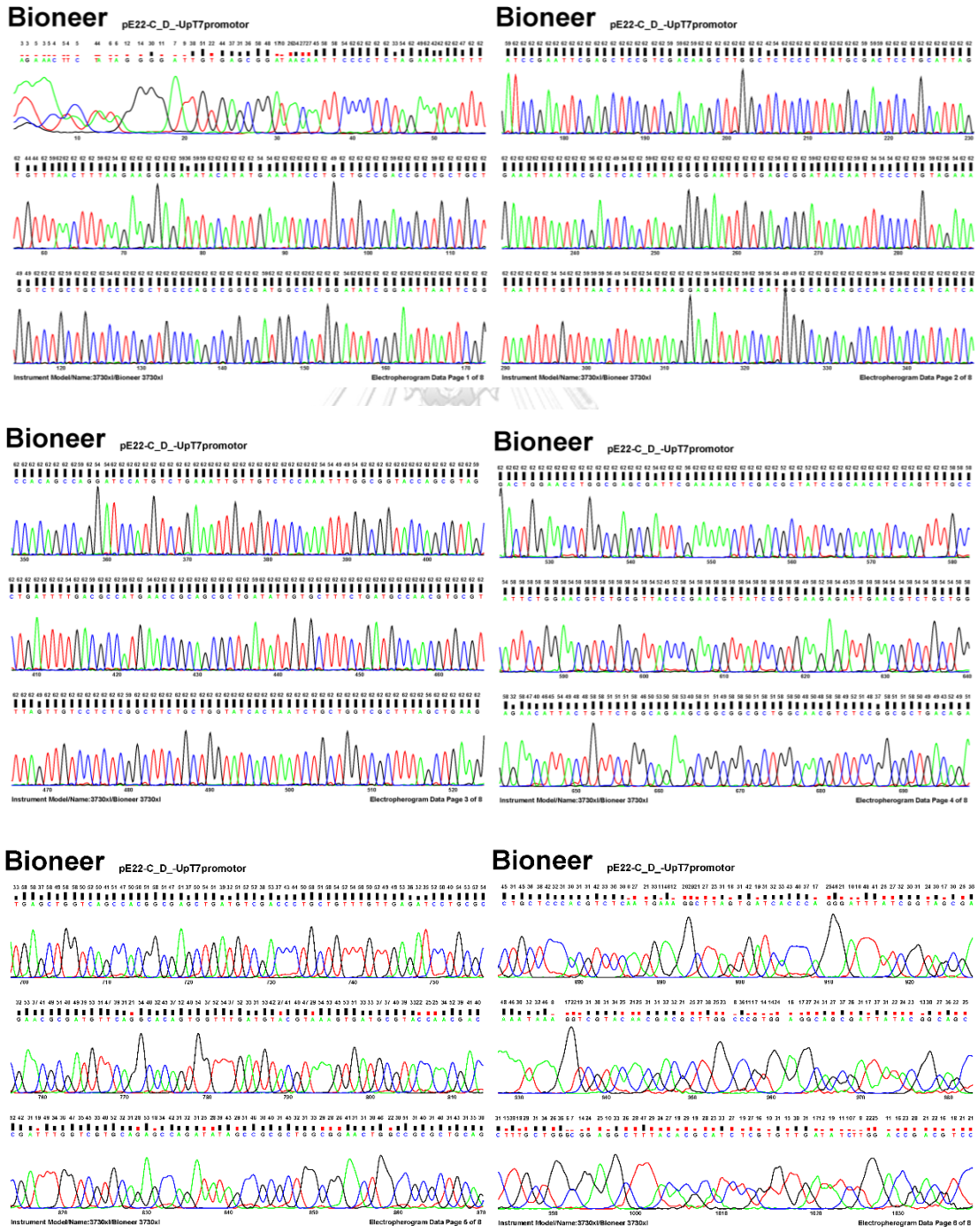
Help

Entry	R00751 Reaction
Name	L-threonine acetaldehyde-lyase (glycine-forming)
Definition	L-Threonine <=> Glycine + Acetaldehyde
Equation	C00188 <=> C00037 + C00084
	 C00188 C00037 C00084
Reaction class	RC00312 C00037_C00188 RC00372 C00084_C00188
Enzyme	4.1.2.5 4.1.2.48
Pathway	rn00260 Glycine, serine and threonine metabolism rn01100 Metabolic pathways rn01110 Biosynthesis of secondary metabolites rn01120 Microbial metabolism in diverse environments rn01130 Biosynthesis of antibiotics rn01230 Biosynthesis of amino acids
Orthology	K01620 threonine aldolase [EC:4.1.2.48]
Other DBs	RHEA: 19628
LinkDB	All DBs

APPENDIX K

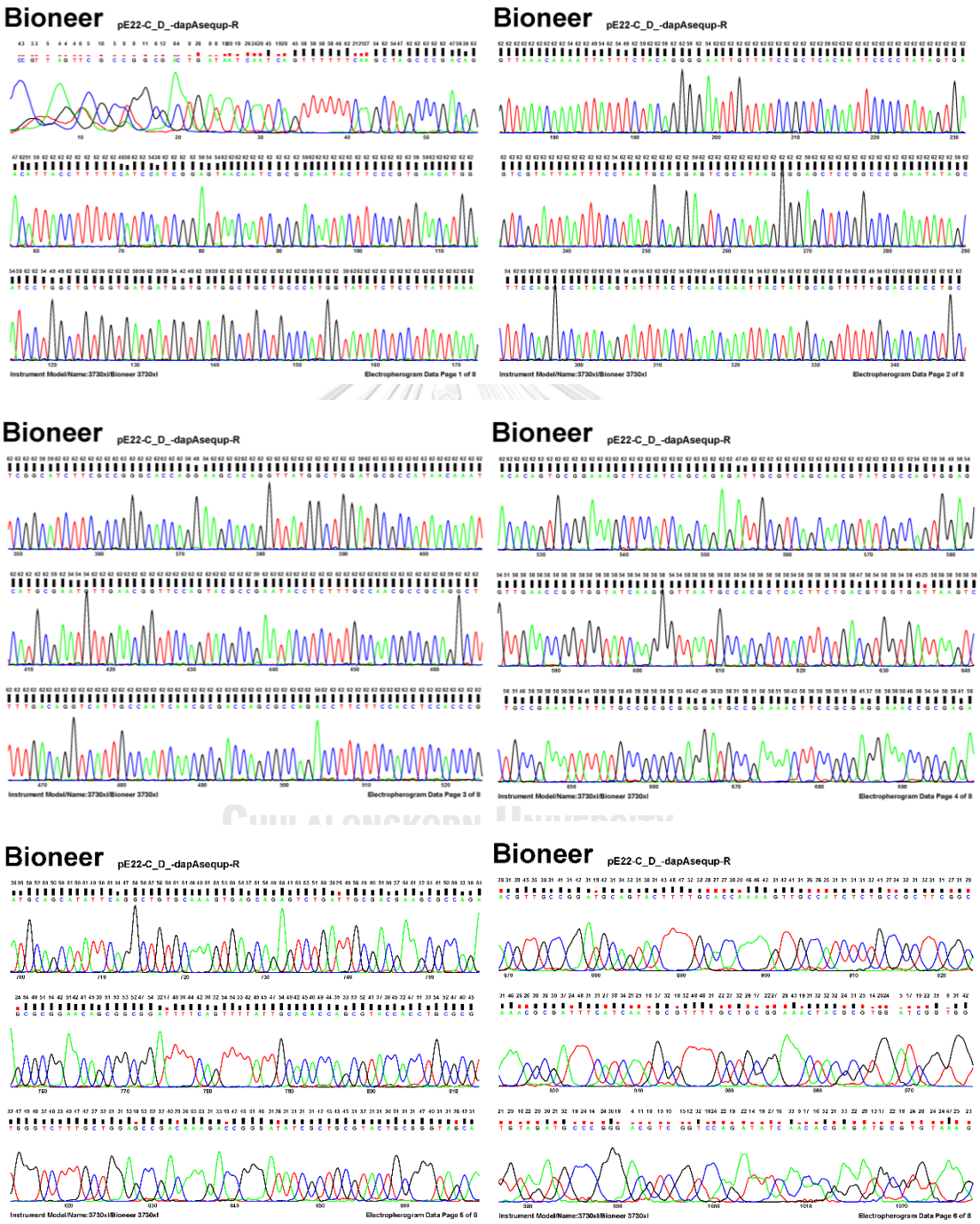
The sequencing chromatogram of *lysC** in pE22-C*D* using primers, UpT7promoter (A) and dapAsequp-R (B)

A)



APPENDIX K (continue)

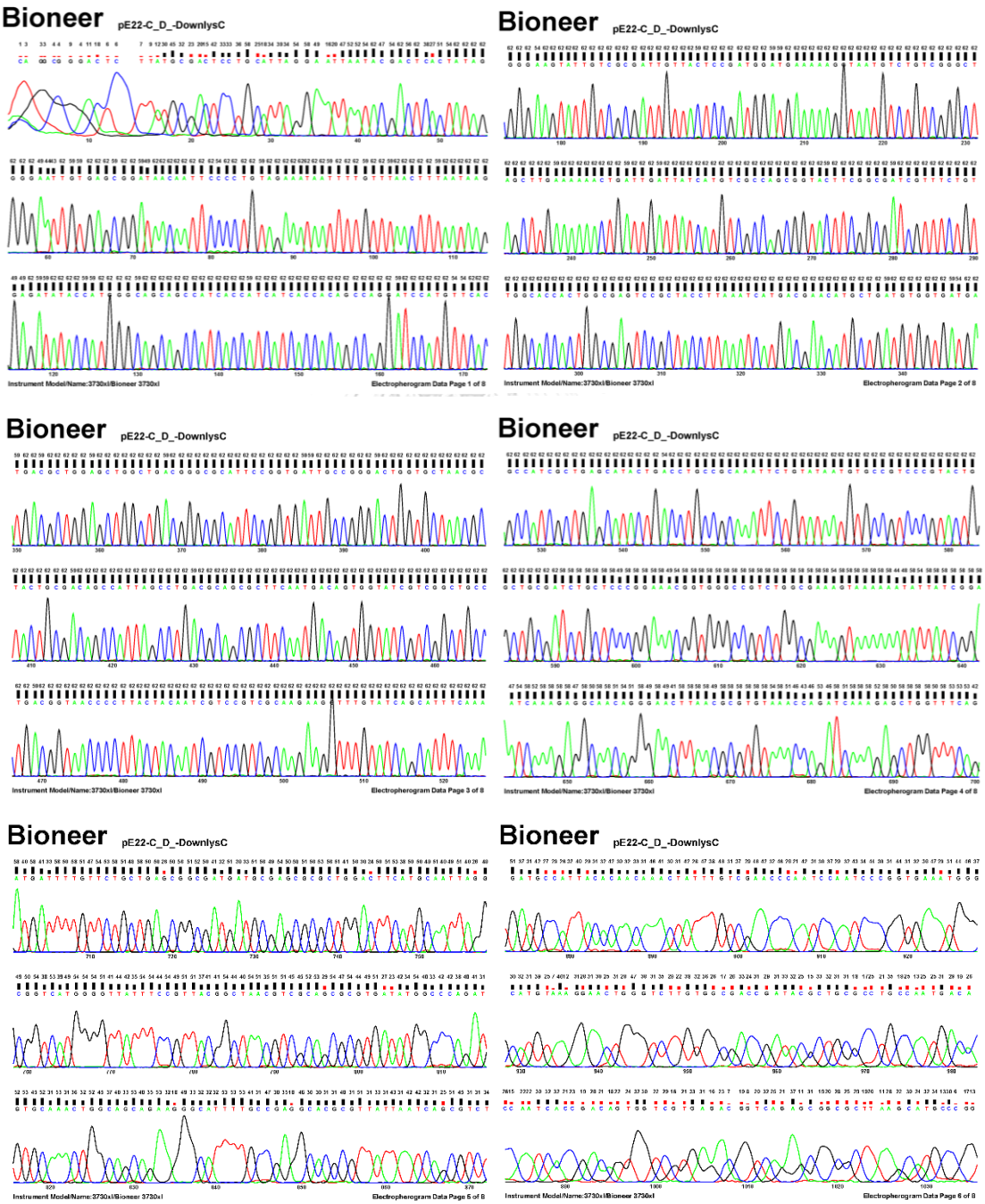
B)



APPENDIX K

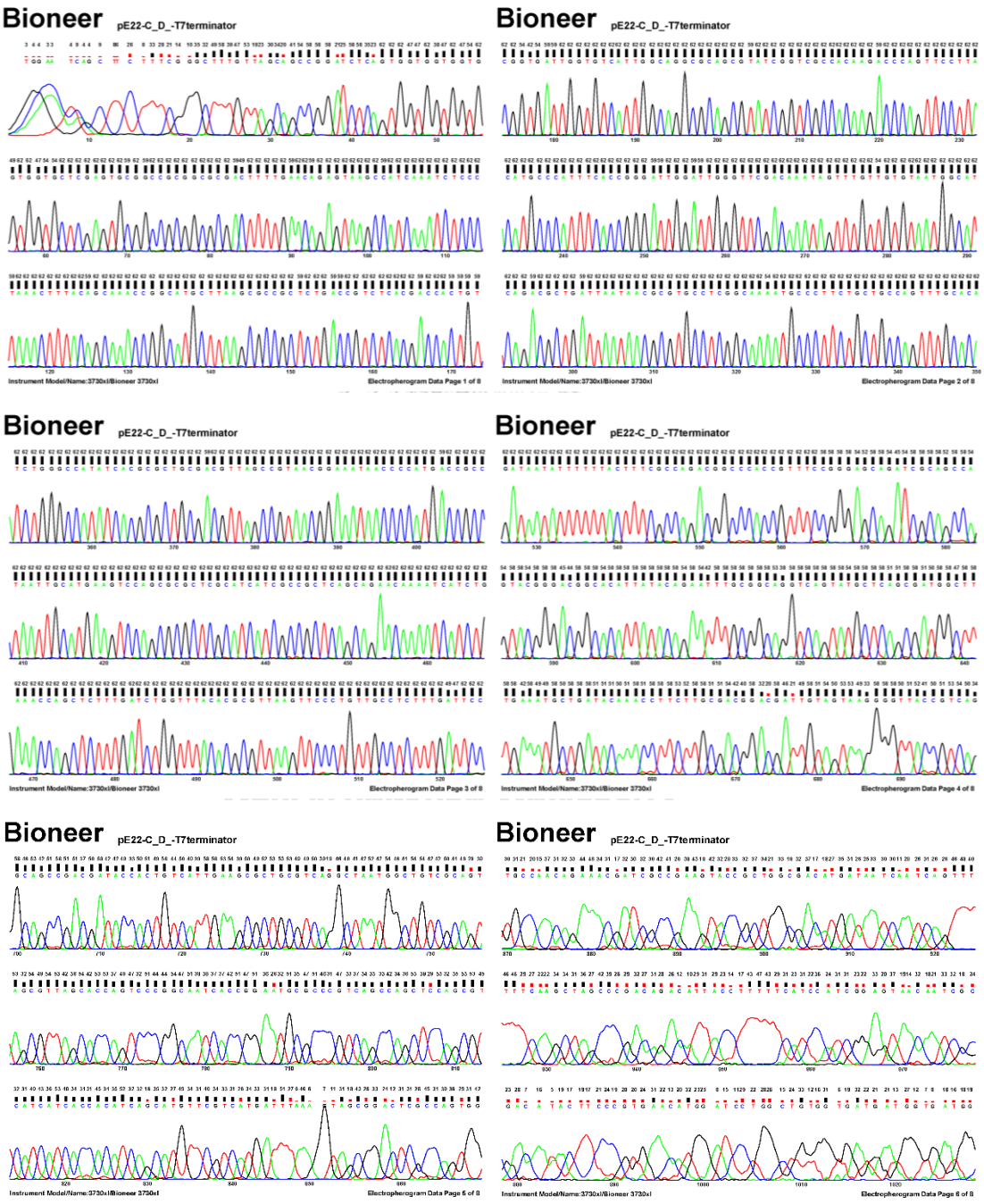
The sequencing chromatogram of *dapA** in pE22-C*D* using primers, DownlysC (A) and T7terminator (B)

A)



APPENDIX K (continue)

B)



VITA

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AWARD RECEIVED	-