การเพิ่มการผลิตกรดแอล-อะมิโนอะดิพิกใน *Escherichia coli* โดยวิศวกรรมเมแทบอลิซึมของกระบวนการชีวสังเคราะห์ไลซีนและกรดอะดิพิก

นายธีรภัทร์ นรเศรษฐ์สิงห์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ENHANCED PRODUCTION OF L-AMINOADIPIC ACID IN *Escherichia coli* BY METABOLIC ENGINEERING OF LYSINE AND ADIPIC ACID BIOSYNTHESIS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ธีรภัทร์ นรเศรษฐ์สิงห์ : การเพิ่มการผลิตกรดแอล-อะมิโนอะดิพิกใน *Escherichia coli* โดยวิศวกรรมเมแทบอลิซึมของกระบวนการชีวสังเคราะห์ไลซีนและกรดอะดิพิก (ENHANCED PRODUCTION OF L-AMINOADIPIC ACID IN *Escherichia coli* BY METABOLIC ENGINEERING OF LYSINE AND ADIPIC ACID BIOSYNTHESIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.กนกทิพย์ ภักดีบำรุง, 80 หน้า.

กรดแอล-อะมิโนอะดิพิก (L- AAA) เป็นกรดอะมิโนที่ไม่พบในโครงสร้างของโปรตีนแต่เป็นสารตั้งต้นที่สำคัญในการผลิตสารปฏิชีวนะในกลุ่มบีตาแลกแทม รวมทั้งเป็นสารตัวกลางในการผลิตยารักษาโรคสะเก็ดเงินและรูมาติซัม เนื่องจากการสังเคราะห์ L- AAA ในจุลชีพสามารถใช้แอล-ไลซีนเป็นสารตั้งต้น ดังนั้นการกลายยีนที่เข้ารหัสให้เอนไซม์ในวิถีการผลิตแอล-ไลซีนใน *Escherichia coli* เพื่อให้ไม่เกิดการยับยั้งการทำงานของเอนไซม์แบบย้อนกลับเมื่อปริมาณแอล-ไลซีนในเซลล์เพิ่มขึ้นน่าจะส่งผลให้มีการผลิต L-AAA เพิ่มมากขึ้นด้วย งานวิจัยนี้ได้ทำการสร้าง pD-Y*D*LP ซึ่งประกอบด้วยยีนกลาย *lysC* ที่ตำแหน่ง V339A ของแอสพาร์โทไคเนส III และยีนกลาย *dapA* ที่ตำแหน่ง E84T ของไดไฮโดรไดพิโคลิเนตซินเทสซึ่งต้านทานการยับยั้งการทำงานโดยแอล-ไลซีนและยีนที่เข้ารหัสให้เอนไซม์ที่เร่งปฏิกิริยาการสังเคราะห์ L-AAA จากแอล-ไลซีน ได้แก่ ไลซีนดีไฮโดรจีเนส (*lysdh*) จาก *Acromobacter denitrifican* และไพเพอริ-เดอีน-6-คาร์บอกซิเลตดีไฮโดรจีเนส (*pcd*) จาก *Pseudomonas putida* จากการทดลองยังไม่สามารถตรวจวัดการแสดงออกและกิจกรรมของแอสพาร์โทไคเนส III และการแสดงออกของไดไฮโดรไดพิโคลิเนตซินเทสได้อย่างชัดเจน อย่างไรก็ตาม ผลจากการวิเคราะห์ L-AAA ในอาหารเลี้ยงเชื้อโดยเทคนิค HPLC และ TLC บ่งชี้ว่า ยีนกลาย *lysC* และยีนกลาย *dapA* ภายใต้โปรโมเตอร์ T7 promoter ของ pRSF-Duet1 มีผลให้มีการผลิต L-AAA เพิ่มขึ้น

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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TEERAPAT NORASETSINGH: ENHANCED PRODUCTION OF L-AMINOADIPIC ACID IN *Escherichia coli* BY METABOLIC ENGINEERING OF LYSINE AND ADIPIC ACID BIOSYNTHESIS. ADVISOR: ASST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D., 80 pp.

L- Aminoadipic acid (L- AAA), a non- protein structure amino acid, is an important intermediate for many medicinal compounds such as antirheumatic drug, psoriasis and carcinostatic drug as well as a precursor in the production of β-lactam antibiotics. L-Lysine is known as one of precursors for L-AAA synthesis in microorganisms. To increase L-AAA production in *Escherichia coli*, releasing of allosteric inhibition of the enzymes in L-lysine biosynthesis pathway should be performed. pD-Y*D*LP containing V339A mutated *lysC* and E84T mutated *dapA* genes encoding for L-lysine feedback resistant aspartokinase III and dihydrodipicolinate synthase from *E. coli*, respectively, along with L–AAA synthesis genes, *lysdh* which encodes lysine dehydrogenase from *Acromobacter denitrifican* and *pcd* which encodes piperideine-6-carboxylate dehydrogenase from *Pseudomonas putida* was constructed. Proteins expression of aspartokinase III and dihydrodipicolinate synthase as well as the activity of aspartokinase III from the recombinant clone were not clearly detected. However, HPLC and TLC analysis indicated that the presence of V399A *lysC* and E84T *dapA* under T7 promoter of pRSF-Duet1 could elevate L-AAA titer when the recombinant clone was cultured in minimal medium.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
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CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

µg	microgram
µL	microliter
µM	micromolar
A	absorbance
AK I	aspartokinase I
AK II	aspartokinase II
AK III	aspartokinase III
Ala	L-alanine
ASA	L-aspartate-semialdehyde
bp	base pairs
dapA	dihydrodipicolinate synthase gene
DAPDC	diaminopimelate decarboxylase
DAPE	diaminopimelate epimerase
DHDP	dihydrodipicolinate
DHDPR	dihydrodipicolinate reductase
DHDPS	dihydrodipicolinate synthase
DNA	deoxyribonucleic acid
HPLC	high-performance liquid chromatography
HTPA	4-hydroxy-2,3,4,5-tetrahydro-L,L-dipicolinic acid
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-stranded nucleic acid

L-AAA	L-aminoadipic acid
LAT	lysine 6-aminotransferase
LL-DAP	L,L-2,6-diaminopimelate
lysC	aspartokinase III gene
LysDH	L-lysine 6-dehydrogenase
lysdh	L-lysine 6-dehydrogenase gene
M	mole per liter (molar)
MetL	aspartokinase II gene
mg	milligram
min	minute
mL	milliliter
mM	millimolar
NAD^+	nicotinamide adenine dinucleotide
nm	nanometer
NSDAP	*N*-succinyl-L,L-2,6,-diaminopimelate
^{o} C	degree Celsius
OD	optical density
pcd	1-piperideine-6-carboxylate dehydrogenase gene
P6CDH	1-piperideine-6-carboxylate dehydrogenase
PCR	polymerase chain reaction
PYR	pyruvate
THDP	L-2,3,4,5,-tetrahydrodipicolinate
thrA	aspartokinase I gene
v/v	volume by volume

Val	L-valine
α-AASA	α-aminoadipate-6-semialdehyde

CHAPTER I

Introduction

1.1 L-Aminoadipic acid

L-Aminoadipic acid (L-AAA) is a rare amino acid which is absent from protein structures. L-AAA is used as an important intermediate for medicines such as methotrexate derivative, antirheumatic drug, psoriasis and carcinostatic agents. Moreover, peptide antibiotics and peptide hormones are terminal-modified for physiologically active by L-AAA (Martin, 1998).

1.2 Role of L-aminoadipic acid in β-lactam antibiotics production

L-AAA is one of the three precursor amino acids along with L-cysteine and L-valine that condense to form the penicillins penam nucleus and the cephamycins and cephalosporins cephem nucleus (Aharonowitz et al. 1992). The mechanism given as "non-ribosomal peptides synthesis" comprises of the activation and condensation of all three amino acid components and epimerization of the L- to D-valine to create the tripeptide δ(L-α-aminoadipyl)-L-cysteinyl-D-valine (Aad-Cys-Val) (Banko et al. 1987; Jensen et al. 1988; Schwecke et al. 1992; Van Liempt et al. 1989). Then the cyclization of Aad-Cys-Val is catalyzed by the isopenicillin N synthase (IPN synthase or cyclase) to form isopenicillin N (IPN), a penam nucleus intermediate with an L-α-aminoadipyl side-chain, as shown in Figure 1.1. In penicillin-producing fungi, an isopenicillin-N acyltransferase catalyzed the exchange of α-aminoadipyl side-chain for phenylacetic acid. In cephalosporin- and cephamycin-producing microorganisms, isopenicillin N is converted to penicillin N by an isopenicillin N epimerase that

epimerizes the side chain of L-α-aminoadipyl to the D configuration (Jayatilake et al. 1981). Deacetoxycephalosporin C is created from Penicillin N by the deacetoxycephalosporin-C synthetase. Deacetoxycephalosporin C is hydroxylated to deacetylcephalosporin C by another 2-oxoglutarate-dependent dioxygenase. Deacetylcephalosporin C is acetylated to form cephalosporin C in the last reaction in cephalosporin-producing fungi. The addition of the C-3′ carbamoyl group and the C-7 methoxyl group during biosynthesis of cephamycin from actinomycetes (Martin, 1998).

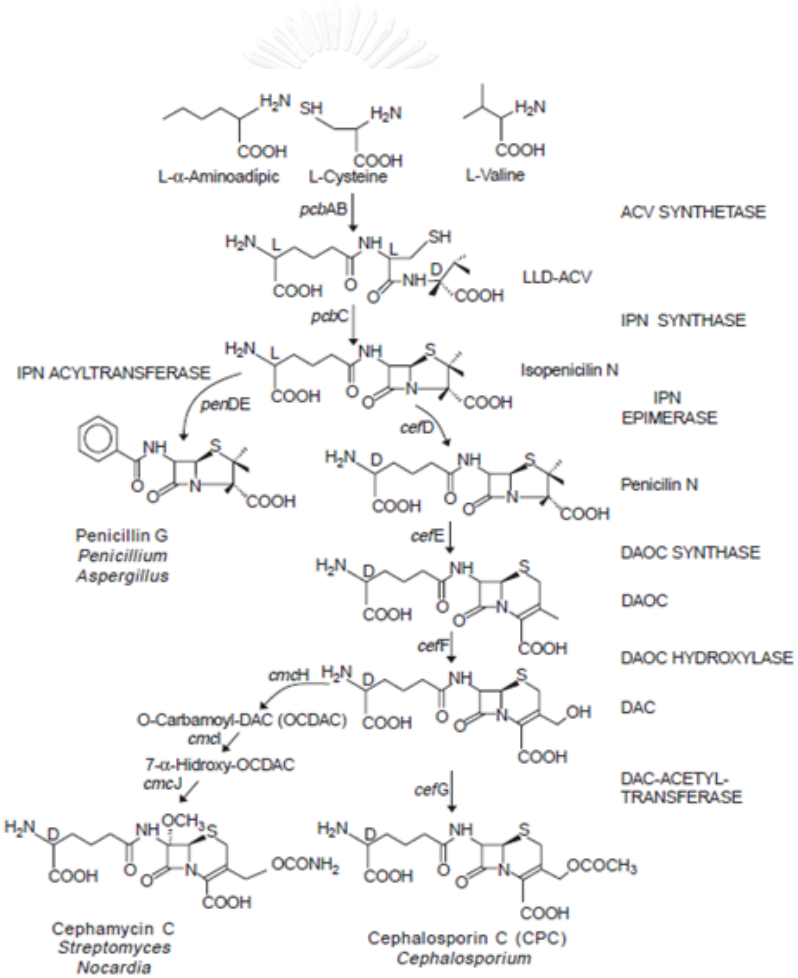

Figure 1.1 Biosynthetic pathways of β-lactam antibiotics and gene clusters encoding these pathways in different producing organisms (Martin, 1998)

1.3 L-Aminoadipic acid synthesis

L-AAA can be synthesized from both chemical and microbial method. However, the microbial method is now more suitable due to the lower cost. For the L-AAA production from microorganism, L-AAA is produced from L-pipecolic acid in *Alcaligenes*, *Pseudomonas* or *Kurthia* (Nakata *et al*, 1996). L-Lysine is also used for L-AAA production in *Agrobacterium*, *Klebsiella*, *Alcaligenes*, *Brevibacterium* or *Bacillus* (Nakata *et al*, 1996). However, these two methods have problems in mass production because L-pipecolic acid is expensive for the former methods and the reaction efficiency is usually low for the latter methods. Therefore, *Flavobacterium* is used for L-AAA production which comprises the step in converting an aminomethyl group of L-lysine to a carboxyl group.

The genes encoding lysine 6-aminotransferase (LAT) and 1-piperideine-6-carboxylate dehydrogenase (P6CDH) from *Flavobacterium lutescens* were cloned and sequenced by Fujii and coworkers (2000) to produce L-AAA from L- lysine by a two-step reaction. In the first reaction, L-lysine is converted to α-aminoadipate-6-semialdehyde by LAT (Soda *et al*., 1968) or Lysine-6-dehydrogenase (Misono and Nagasaki, 1982) which can be cyclized non-enzymatically to 1-piperideine-6-carboxylate. In the second step, α-aminoadipate-6-semialdehyde is converted to L-AAA by P6CDH (Fuente *et al.*, 1997). P6CDH is also called α-aminoadipate-6-semialdehyde dehydrogenase (α-AASDHA). It is notable that the conversion of L-lysine to L-AAA from microorganism preserves chirality unlike chemical synthesis. Therefore, it could improve the L-AAA production efficiency.

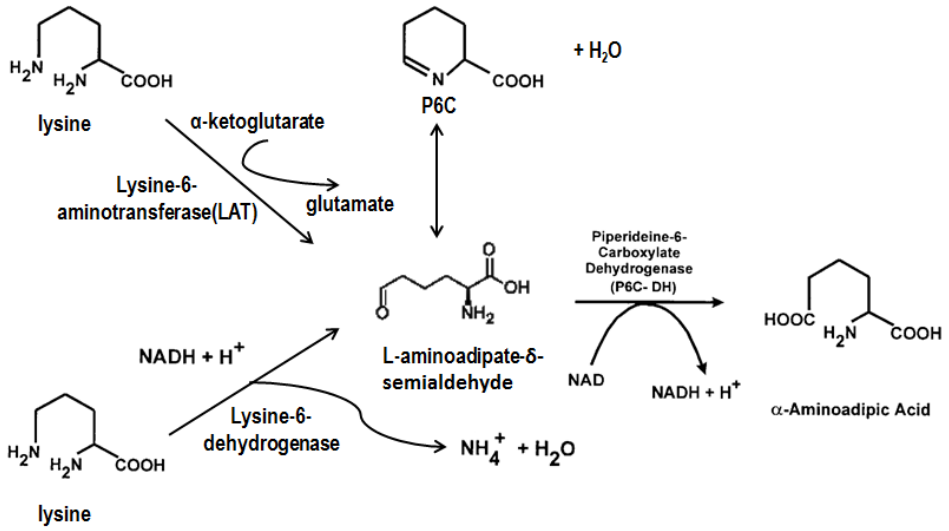

Figure 1.2 Conversion of L-lysine into α-aminoadipic acid by LAT or LysDH and P6CDH (Source: modified from Fuente *et al.*, 1997 and Fujii *et al.*, 2000)

1.4 L-Lysine-6-dehydrogenase

L-Lysine 6-dehydrogenase (LysDH, EC 1.4.1.18) catalyzes the conversion of L-lysine to form α-aminoadipate-6-semialdehyde (α-AASA) by oxidative deamination. Then α-AASA is cyclized non-enzymatically to 1-piperideine-6-carboxylate (P6C).

LysDH plays an important role in L-lysine metabolism. LysDH activity was screened among microorganisms from soil samples (Misono and Nagasaki, 1982). It was found that the LysDH activity was distributed narrowly only in *Agrobacterium tumefaciens, Alcaligenes faecalis, Bacillus spharicus, Klebsiella pneumonia* and *Pseudomonas fragi.* In 1991, LysDH was discovered in *Candida albicans* which can use L-lysine as the nitrogen and carbon source with accumulation of α-AASA. This enzyme in *Candida albicans* is strongly induced when cells were grown in L-lysine as

the nitrogen source (Hammer *et al*., 1991). Thermophilic and hyperthermophilic microorganisms were screened from a Japanese hot spring for more stable form of LysDH. The activity was found in a moderately thermophilic bacterium, *Geobacillus stearothermophilus* (Heydari *et al*, 2004).

In 2007, our research group found the activity of LysDH in *Achromobacter denitrificans* (Ruldeekulthamrong *et al*, 2007). However, the wild-type strain had the low yield of the enzyme, so the recombinant DNA technology was used to obtain an adequate amount of the LysDH. The *lysdh* gene was cloned and expressed in *Escherichia coli* BL21(DE3) using pET-17b as expression vector for producing LysDH. The LysDH activity from 600 ml cell culture was 1,664 unit with 1.89 unit/mg of specific activity. The enzyme was 2.8 fold purified with 47.43% recovery after purification by DEAE-Toyopearl 650M column chromatography and DEAE-Sephadex A50 column chromatography. The yield of recombinant LysDH from *A. denitrificans* and the enzyme properties showed a high potentiality for L-AAA production by coupling with PCDH.

1.5 1-Piperideine-6-carboxylate dehydrogenase

Fuente and coworkers in 1997 reported the finding of 1-piperideine-6-carboxylate dehydrogenase (P6CDH; EC 1.2.1.31) which is an enzyme in semialdehyde dehydrogenase family that catalyzes P6C conversion to L-AAA in the cephamycin C producer *Streptomyces clauligerus*. The enzyme was then purified and characterized. The native enzyme is a monomer with molecular weight of 56.2 KDa. The enzyme used P6C and NAD^+ efficiently with K_m of 14 μM and 115 μM,

respectively. Afterwards, the *pcd* gene encoding for P6CDH of *Streptomyces clavuligerus* was verified to be in the cephamycin C cluster (Perez-Llarena, 1998).

Our research group by Lertmongkolthum (2004) screened NAD^+ dependent P6CDH producing bacteria from soil in Thailand using minimal medium containing L-lysine as carbon and nitrogen sources. Fifty eight isolates from 20 soil samples were obtained. Their crude extracts were incubated with purified LysDH from *Achromobacter denitrificans* in the present of L-lysine as a starting material. TLC analysis showed that crude enzyme from 5 isolates could produce L-AAA. The activities of P6CDH in these isolates were confirmed by Sri-in (2007). The isolate that had the highest activity was selected and identified as *Pseudomonas putida* ADH3. However, the level of the enzyme in *P. putida* ADH3 was not enough for further purification and characterization. Therefore, *pcd* gene encoding for P6CDH was sequenced and cloned into *E. coli* BL21(DE3) using pET-17b expression vector (Pamorn, 2010). The molecular mass of the enzyme subunit determined by SDS-PAGE was 54 KDa. Optimum pH and temperature for enzyme reaction were 8.07 and 40 ^{o}C, respectively. The enzyme was stable in pH ranging from 7.0 to 8.5. The K_m values for acetaldehyde and NAD^+ were 1.18 and 0.24 mM, respectively. To produce L-AAA, the *pcd* was co-expressed with *lysdh* from *A. denitrificans* K-1 in *E. coli* BL21(DE3) under T7 promoter of pET-17b vector. The production of L-AAA, about 25 mM, was obtained by induction the recombinant clone with 0.2 mM IPTG for 4 h and then 0.05 g of cell wet weight was incubated in the reaction mixture containing 200 mM L-lysine in 200 mM Tris-HCl buffer, pH 8.5 for 24 h. Since L-lysine acts as the direct and only precursor for L-AAA, improvement of L-lysine production should be performed.

1.6 L-Lysine production

L-Lysine is an amino acid that is essential in mammalian diet, but can be synthesized *de novo* in bacteria, plants and some fungi. The diaminopimelate (DAP) pathway is the lysine biosynthesis pathway in bacteria, produces the important metabolites *meso*-2,6-diaminopimetate (*meso*-DAP) and lysine. The pathway in *E. coli* begins with aspartokinase (AK) that catalyzes the conversion of L-aspartate into L-aspartyl phosphate. Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) converts L-Aspartyl phosphate to L-aspartate-semialdehyde (ASA). ASA with pyruvate (PYR) are converted by dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) to form an unstable heterocyclic, (2*S*, 4*S*)-4-hydroxy-2,3,4,5-tetrahydrodipicolinate (HTPA). HTPA is then dehydrated to dihydrodipicolinate (DHDP) non-enzymatically. Then DHDP is reduced to form (*S*)-2,3,4,5,-tetrahydrodipicolinate (THDP) by NAD(P)H-dependent dihydrodipicolinate reductase (DHDPR, EC 1.3.1.26). THDP is then converted to *N*-succinyl-2-amino-6-ketopimelate (NSAKP) by tetrahydrodipicolinate succinylase (THPC-NST, EC 2.3.1.117). NSAKP is converted by *N*-succinyldiaminopimelate aminotransferase (NSDAP-AT, EC 2.6.1.17) to form *N*-succinyl-L,L-2,6,-diaminopimelate (NSDAP). NSDAP is then desuccinylated by *N*-succinyl-L-diaminopimelate desuccinylase (SDAP-DS, EC 3.5.1.18) to form L,L-diaminopimelate (LL-DAP). LL-DAP is converted by diaminopimelate epimerase (DAPE, EC 5.1.1.7) to meso-diaminopimelate (*meso*-DAP) (Wiseman, & Nichols, 1984). Finally the decarboxylation of *meso*-DAP is catalyzed by diaminopimelate decarboxylase (DAPDC, EC 4.1.1.20) to produce L-lysine and carbon dioxide (Ray *et al*., 2002).

There are two enzymes that L-lysine has been shown to allosterically inhibit which are AK and DHDPS. These enzymes catalyze the rate-limiting step in L-lysine biosynthesis. (Chen *et al.*, 2011; Geng *et al*, 2013).

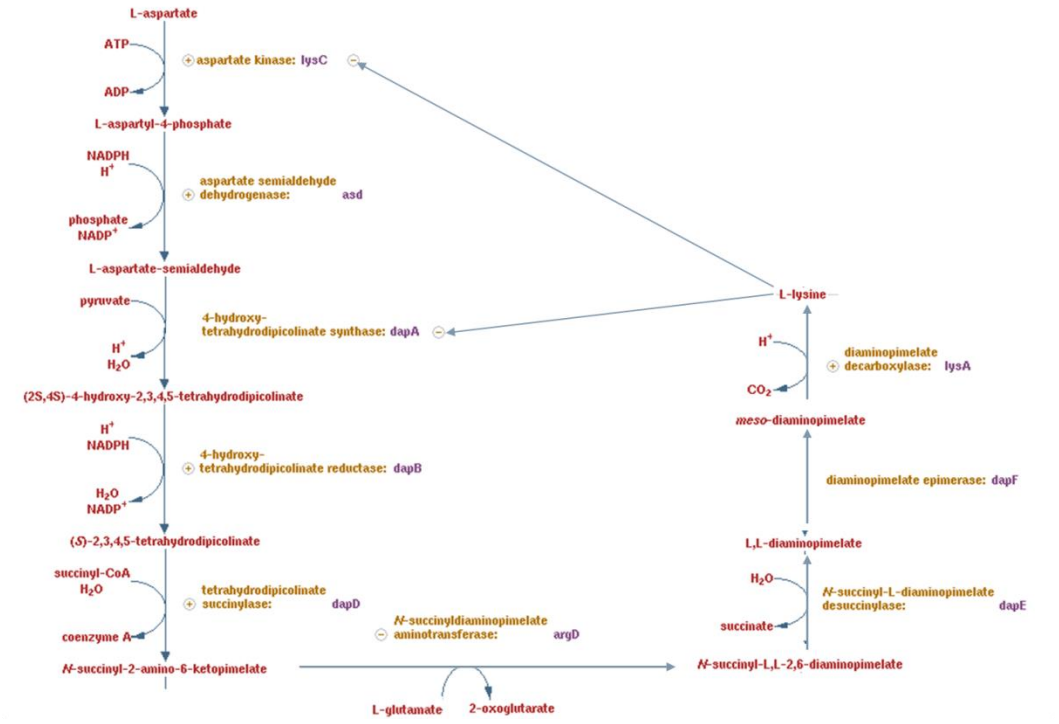

Figure 1.3 DAP pathway for L-lysine biosynthesis in *E. coli* (Source: Riley, 1993)

1.7 Aspartokinase

Aspartokinase or aspartate kinase (AK) catalyzes the aspartate phosphorylation and regulates many industrially important amino acids biosynthesis such as L-lysine, L-threonine and L-methionine (Yoshida *et al.,* 2007). All the AK identified so far is oligomeric enzymes. Both homo-oligomers and hetero-oligomer were found such as *Arabidopsis thaliana* AKI is homo-oligomer of an identical subunit while *Corynebacterium glutamicum* AK is a heterotetramer. In *E. coli.*, there are three isozymes of aspartate kinase. AK I (encoded by *thrA*) and AK II (encoded by *MetL*) are bifunctional enzymes that catalyze a phosphorylation and then after an intervening reduction by a different enzyme, a second reduction to produce the intermediate homoserine. However, AK III (encoded by *lysC*) is a monofunctional enzyme.

AK I and AK III of *E. coli* are allosteric enzymes. AKI is allosterically inhibited by threonine and it synthesis is repressed by threonine and leucine while AK III is repressed and inhibited by lysine (Kataoka *et al*., 2006) Kikuchi and coworkers (1999) identified residues and regions of the polypeptide essential for feedback inhibition by L-lysine of AK III using chemical mutagenesis. They found that the L-lysine-insensitive mutants were mutated in amino acid residues 323-352, and at position 250.

The crystal structure of AK III complex with substrates (R state) or lysine (T state) were solved by Kataoka and his colleges (2006). AK III consists of an N-terminal where catalysis domain is located and a C-terminal where regulatory domain is located. The regulatory domain has two motifs called as ACT domains which are responsible for the L-lysine binding as shown on Figure 1.4 (Chipman and Shaanan, 2001). In 2011, Chen and coworkers used molecular dynamic simulation and

statistical coupling analysis of sequences from enzyme aspartokinase family to identify important sequences for allosteric regulation of AK III. Mutation of Val 339 to Ala (V339A) that destroyed the hydrogen bond with L-lysine showed the highest remaining activity (> 95%) when L-lysine concentration was increased to 20 mM.

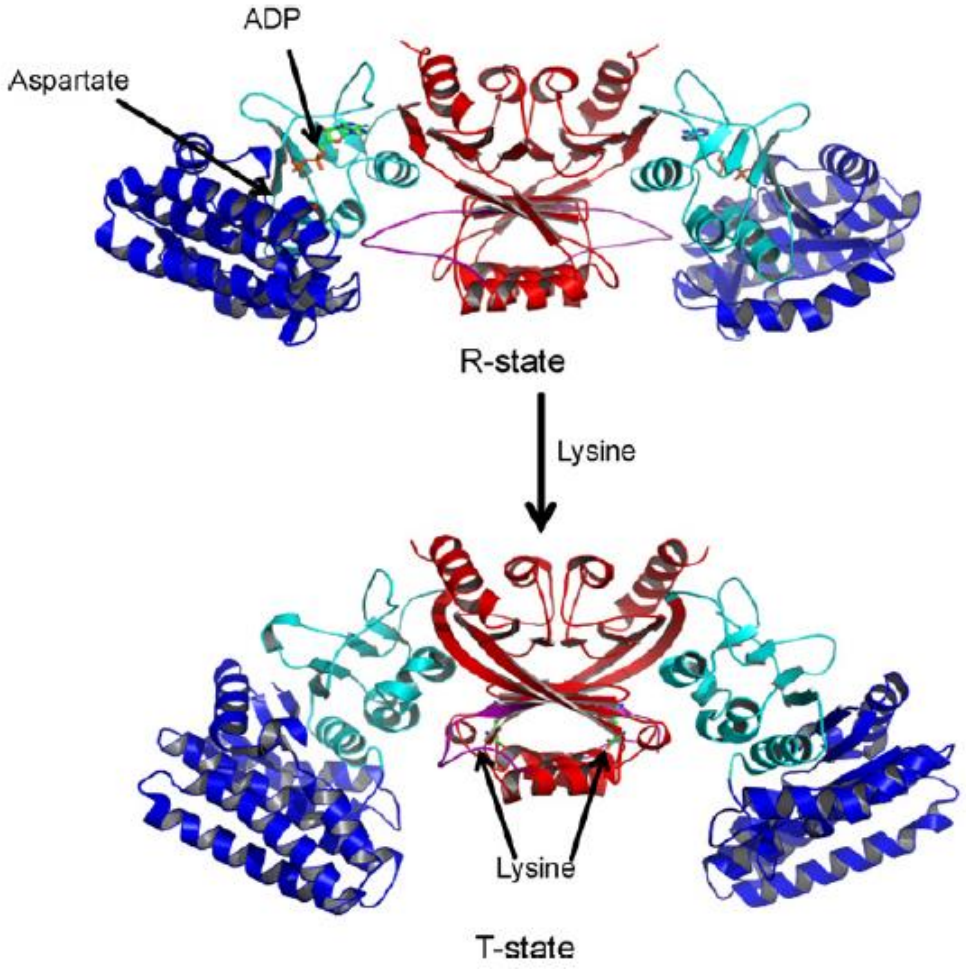

Figure 1.4 Conformational transition of AK III by allosteric regulation of L-lysine The allosteric region that showed the largest conformational change is colored in purple (Source: Chen *et al.*, 2011)

1.8 Dihydrodipicolinate synthase

Dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) catalyzes the L-aspartate-semialdehyde (ASA) and pyruvate condensation to form 4-hydroxy-2,3,4,5-tetrahydro-L,L-dipicolinic acid (HTPA), the first specific reaction for the L-lysine biosynthesis (Shedlars and Gilvarg 1970; Yugari and Gilvarg 1965). In some organisms, the activity of DHDPS is regulated by L-lysine from a process of allosteric inhibition. DHDPS allosteric inhibition from L-Lysine has been identified in several plants, Gram-negative and Gram-positive bacterial species to date. DHDPS from plant are frequently strongly inhibited by L-lysine (IC50 = 0.01-0.05 mM). However, DHDPS sensitivity to L-lysine of Gram-negative bacteria such as *E. coli* are significantly less compare with their plant DHDPS with an IC50 value of 0.25-1.0 mM. Moreover, the enzyme from Gram-positive bacteria such as *Corynebacterium glutamicum* (Cremer et al., 1988) show little or no sensitivity to L- lysine. Since allosteric inhibition of DHDPS is directly correlated to L-lysine production, many efforts have been made for enhanced L-lysine production by relieve the feedback inhibition.

In *E. coli*, the enzyme is homotetramer. The monomer contains 292 amino acids with two domains. A structure of a $(\beta/\alpha)_8$ TIM-barrel (residues 1-224) of the N-terminal domain contains active site located inside the center of the barrel. The C-terminal domain (residues 225-292) consists of three α-helices and contains several key residues that important for tetramerisation (Dobson *et al.*, 2005). The tetramer can be called as a dimer of dimers, monomers A & B and C & D have the strong interactions which known as tight dimer interface, and weaker interactions of the dimers A-B and C-D which known as weak dimer interface (Dobson *et al*., 2005) as

shown in Figure 1.5. Muscroft-Taylor and coworkers (2010) found that introducing point mutations to *E. coli* DHDPS at locus L197D and Y107W. The mutant could produce L-lysine resistance isolable monomeric DHDPS. However, the enzyme activity is lower than the wild-type enzyme.

In 2013, Geng and coworkers studied sequence and structure comparisons of DHDPS between two industrially important DHDPSs, the L-lysine-sensitive DHDPS from *E. coli* and L-lysine-insensitive DHDPS from *C. glutamicum* to identify important residue related to allosteric regulation. Site-directed mutagenesis at Glu 84 to Thr (E84T) of *E. coli* DHDPS (encoded by *dapA* gene) showed the highest remaining activity (>90%) when L-lysine concentration was increased to 10 mM.

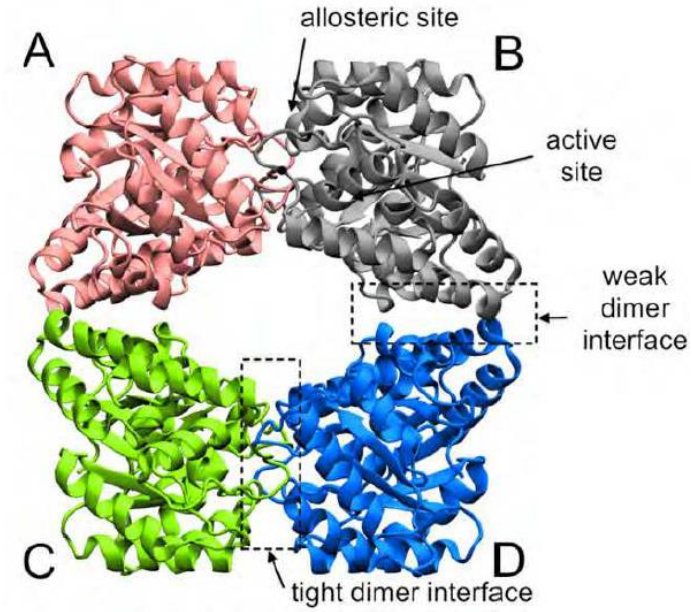

Figure 1.5. *E. coli* DHDPS structure. The active sites, allosteric sites, dimerisation interface (tight dimer interface) and tetramerisation interface (weak dimer interface) are shown (PDB: 1YXC). (Source: Dogovski *et al*., 2012)

1.9 Objective of this research

In our previous works, pET-LP which contains *A. denitrificans lysdh* gene encoding LysDH and *P. putida* ADH 3 *pcd* gene encoding P6CDH was constructed by Pamorn (2011). The resting cell of the recombinant clone was proved to produce L-AAA when L-lysine was used as a starting substance. Since L-lysine acts as the direct and only precursor for L-AAA biosynthesis, improvement of the biosynthetic pool of the precursor L-lysine should increase the yield of L-AAA in minimal medium fermentation. Therefore, the objectives of this research are:

1. To clone *lysC* and *dapA* from *E. coli*
2. To construct V339A mutated *lysC* (*lysC**) and E84T mutated *dapA* (*dapA**) to produce AK III and DHDPS that can resist to the allosteric inhibition by L-lysine using site directed mutagenesis
3. To co-express *lysC** and *dapA** with *lysdh* and *pcd* in *E. coli* BL21(DE3)
4. To determine the L-AAA production of the mutant

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave (MLS-3020, SANYO electric Co., Ltd., Japan)

Autopipette (Pipetman, Gilson, France)

Benchtop centrifuge (SorvallBiofuge Primo, Kendro Laboratory Products L.P., USA)

Chirex 3126 (D)-penicillamine size 150 mm dimension 4.6 mm (Phenomenex, USA)

Dry bath incubator (MD-01N, Major Science, USA)

Electroporator (MicroPulserTMelectroporator, Bio-Rad Laboratories, Inc., USA)

Gel Doc (BioDoc-It® Imaging System with M-20 UV Transilluminator, UVP®, Inc., USA)

High performance liquid chromatography (Shimadzu, Japan)

Magnetic hotplate stirrer (CH-1E, Nickel Electro-Clifton, UK)

Microfuge Centrifuge (22R, Beckman coulter, USA)

pH meter (S20-K SevenEasyTM, Mettler-Toledo, Switzerland)

Refrigerated centrifuge (Avanti J-30I High-Performance Centrifuge, Beckman Coulter, Inc., USA)

Shaking incubator (Excella E24R, New Brunswick Scientific, USA)

Spectrophotometer (Beckman DU 530, Beckman Coulter, Inc., USA)

Thermo cycler (Mastercycler® Family, Eppendorf, Germany)

UV Transilluminator (MacroVueTM UV-25, Hoefer Inc., USA)

Vacuum/pressure pump (Model number. WP6111560, Millipore Inc., USA)

Vortex shaker (Topmix FB15024, Thermo Fisher Scientific Inc., USA)

2.2 Disposable materials

Membrane filter (NYLON membrane filters, 0.45 μm, 47 mm, Vertical Chromatography Co., Ltd., Thailand)

Microcentrifuge tube (1.5 mL microcentrifuge tube, MCT-150, Axygen Inc., USA)

PCR tube (0.2 mL thin-wall domed-cap PCR tube, PCR-02D-C, Axygen Inc., USA)

Pipette tip (10 μL, 200 μL and 1000 μL pipette tip, Axygen Inc., USA)

Syringe (3 mL and 10 mL disposable syringe, Nissho Nipro Co., Ltd., Japan)

Syringe filter (0.2 μm, 13 mm, VertiPure^{TM} PTFE, Vertical Chromatography Co., Ltd., Thailand)

Syringe filter (0.2 μm, 25 mm, Acrodisc®, Non-pyrogenic, Pall Corporation, USA)

2.3 Markers

GeneRuler™ 1 kb DNA Ladder (#SM0311, Fermentas Inc., USA)

λ/*Hin*dIII marker (#SM0101,Thermo Scientific™, USA)

2.4 Kits

Gel/PCR DNA fragment extraction kit (DF300, Geneaid, Biotech Ltd, Taiwan)

High-speed plasmid mini kit (PD300, Geneaid, Biotech Ltd, Taiwan)

2.5 Chemicals

Adenosine 5′-triphosphate disodium salt hydrate (Sigma, USA)

Agar, Bacteriological grade (Criterion, USA)

Agarose (FMC Bioproducts, USA)

L-Aminoadipic acid (Wako, Japan)

Ammonium sulphate (Carlo Erba, Italy)

L-Aspartic acid (Sigma, USA)

Bovine serum albumin (Sigma, USA)

Bromphenol blue (Merck, Germany)

Calcium chloride (Scharlau, Spain)

Copper sulfate (Carlo Erba, Italy)

Ethyl alcohol absolute (Carlo Erba, Italy)

Ethylenediaminetetraacetic acid disodium salt, EDTA (Merck, Germany)

Ferrous sulfate (Fluka, Switzerland)

Ferric chloride (Ajax Finechem, Australia)

Glacial acetic acid (Carlo Erba, Italy)

Glycerol (Ajax Finechem, Australia)

Hydroxylamine hydrochloride (Fluka, Switzerland)

Isopropyl-β-D-thiogalactopyranoside (IPTG), Dioxane Free (US Biological, UK)

Kanamycin (Sigma, Switzerland)

Magnesium chloride (Carlo Erba, Italy)

Manganese (II) sulphate monohydrate (Carlo Erba, Italy)

Methanol, HPLC grade (Merck, Germany and LAB SCAN, Thailand)

Pancreatic digest of casein (Criterion, USA)

Potassium dihydrogen phosphate (Carlo Erba, Italy)

Potassium hydroxide (Ajax Finechem, Australia)

Sodium chloride (Carlo Erba, Italy)

Sodium citrate (Carlo Erba, Italy)

Sodium hydroxide (Carlo Erba, Italy)

Thiamine-HCl (Sigma, USA)

Yeast extract (Scharlau, Spain)

Zinc sulfate (BDH, England)

Other common chemicals were products obtained from Sigma, USA; BDH, UK; Fluka, Switzerland; Merck, Germany; Ajax Finechem, Australia; Carlo Erba, Italy; and Lab Scan, Thailand.

2.6 Enzymes and restriction enzymes

Pfu DNA polymerase (Promega, USA)

Restriction enzymes (New England BioLabs, Inc., USA)

RNase A (Sigma, USA)

T4 DNA ligase (Biotechrabbit, Germany)

2.7 Bacterial strains and plasmids

E. coli TOP10 was used for gene cloning and *E. coli* BL21 (DE3) was used to overexpress all genes in this study. All plasmids used are shown in Table 2.1.

2.8 Media

2.8.1 Luria-Bertani broth

Luria-Bertani (LB) medium consisted of 1% casein peptone, 0.5% yeast extract and 0.5% NaCl. For agar plate, 1.5% agar was added. An antibiotic was added as depending on plasmid.

2.8.2 Minimal medium

In this work, carbon and nitrogen sources were glycerol and $(NH_4)_2SO_4$ respectively. This medium contained (g/L): glycerol 30; $(NH_4)_2SO_4$ 60; KH_2PO_4 3.00; K_2HPO_4 12.00; $MgSO_4.7H_2O$ 0.3; thiamine-HCl 7.5×10^{-3}; $FeSO_4.7H_2O$ 7.5×10^{-2}; $MnSO_4.H_2O$ 3.6×10^{-5}; Na-Citrate 1.0; NaCl 1.0; $Al_2(SO_4)_3.18H_2O$ 3.0×10^{-6}; $CoSO_4.7H_2O$ 1.5×10^{-6}; $CuSO_4.5H_2O$ 3.8×10^{-6}; H_3BO_3 7.5×10^{-7}; $Na_2MoO_4.2H_2O$ 4.5×10^{-6}; $NiSO_4.6H_2O$ 3.8×10^{-6}; $CaCl_2.2H_2O$ 1.5×10^{-2}; $ZnSO_4.7H_2O$ 2.3×10^{-5}, pH 7.0.

Table 2.1 Plasmids used in this work

Plasmid	Characteristic	Reference
pET-LP	pET-17b, carrying *lysdh* and *pcd* genes	Pamorn, 2011
pD-Y	pRSFDuet-1, carrying *lysC* gene	This work
pD-D	pRSFDuet-1, carrying *dapA* gene	This work
pD-Y*	pRSFDuet-1, carrying V339A mutated *lysC* gene	This work
pD-D*	pRSFDuet-1, carrying E84T mutated *dapA* gene	This work
pD-Y*D*	pRSFDuet-1, carrying V339A mutated *lysc* and E84T mutated *dapA* genes	This work
pD-LP	pRSFDuet-1, carrying *lysdh* and *pcd* genes	This work
pD-Y*D* LP	pRSFDuet-1, carrying V339A mutated *lysc*, E84T mutated *dapA* genes, *lysdh* and *pcd* genes	This work

2.9 Chromosomal DNA extraction

Chromosomal DNA of *E. coli* BL21(DE3) was extracted using method of Frederick et al., (1995). A single colony of *E. coli* BL21(DE3) was inoculated into 5 mL of LB medium and incubated at 37 °C for 18 h with 250 rpm shaking. Each 1.5 mL of cell culture was centrifuged at 10,000xg for 2 min. The pellet was resuspended in 550 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The cell suspension was then treated with 3 µL of 5 mg/mL lysozyme, 2 µL of 10 mg/mL RNase A, 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K and incubated at 37 ◦C for 1 h. Then the DNA was extracted with a phenol-chloroform (1:1 v/v) and centrifuged at 10,000xg for 10 min. The upper phase was transferred to a new microcentrifuge tube. DNA was precipitated by the addition of 5 M NaCl to the final concentration of 1 M and 2 volumes of cold absolute ethanol and kept at -20 °C for at least 30 min. the DNA pellet was collected by centrifugation at 10,000xg for 10 min at 4 ◦C and washed well with cold 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of sterile ultrapure water. DNA concentration was estimated by agarose gel electrophoresis.

2.10 Amplification of *lysC* and *dapA* genes

The *lysC* and *dapA* genes were amplified by PCR technique using chromosomal DNA of *E. coli* BL21(DE3) as a template. The sequences of both genes were retrieved from NCBI database for primer design. The primers are shown in Table 2.1. PCR reaction mixture (50 µl) contained 50 ng of the chromosomal DNA, 0.2 mM dNTPs, 10 pmole of each primer, 5 µl 10xbuffer with $MgSO_4$ and 2.5 Unit of

Pfu DNA polymerase. Condition for gene amplification was pre-denaturation (95 °C, 2 min), 30 cycles of denaturation (95 °C, 1 min), annealing (60 °C, 35 sec), and extension (72 °C, 2 min), and the final extention (72 °C, 5 min). The sizes of the PCR products were verified by agarose gel electrophoresis. Then *lysC* and *dapA* fragments were used for furture construction of pD-Y and pD-D, respectively.

2.11 Plasmid extraction

A single colony of *E. coli* TOP10 harboring pRSFDuet-1 was inoculated in 5 mL of LB medium containing selective antibiotic. Then the culture was incubated at 37 °C overnight with shaking at 250 rpm. After that, cell pellet was gathered by centrifugation at 5,000 x g for 2 min at room temperature. Then, plasmid was extracted using High-Speed Plasmid Mini kit (Geneaid Biotech).

2.12 Agarose gel electrophoresis

The PCR products were separated and analyzed by agarose gel electrophoresis. 0.8% (w/v) agarose in 1×TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) was melted by microwave oven. The solution was cooled to about 55-50 ^{o}C and then 1 µl of red safe dye per 20 mL of the solution was added to the solution before pouring into the casting tray with proper comb and sit about 10 – 15 minute for gel solidification. The DNA samples were mixed with 6x loading buffer (30% glycerol and 0.25% bromphenol blue), then loaded into the well. Electrophoresis was performed at 100 volt. The DNA bands were observed by exposure to UV light. The concentration and molecular weight of DNA sample was compared with the standard DNA marker (GeneRuler 1 kb DNA Ladder).

2.13 Double digestion by restriction enzymes

For double digestion, Cutsmart buffer was used. Twenty µl of reaction mixture containing 1 µg of DNA fragment or plasmid, 1x of Cutsmart buffer, 10 µl of each restriction enzyme was incubated at 37 °C for 3 h. After that the digested fragments were separated by agarose gel electrophoresis and purified by Gel/PCR DNA fragment extraction kit (DF300, Geneaid, Biotech Ltd, Taiwan)

2.14 Ligation

The reaction was performed in 20 µl of reaction mixture containing 120 ng of plasmid vector, 700 ng of insert, 1x of T4 DNA ligase buffer and 5 Unit of T4 DNA ligase. Ligation mixture was mixed and incubated at 16 °C for 16 h.

2.15 Transformation of plasmid

2.15.1 Preparation of competent cell

A single fresh colony of *E. coli* BL21(DE3) or *E. coli* Top10 from LB agar plate was grown in 100 mL of LB medium starter at 37 °C with 250 rpm shaking for 24 h then the 50 mL of starter was inoculated into 1 liter of LB medium and continued incubation when optical density at 600 nm (OD_{600}) of cell culture reached 0.3-0.4, the culture was chilled on ice for 30 min and centrifuged at 2,500xg at 4 °C for 15 min. The cell pellets were washed with cold autoclaved distilled water by 1.5-2 of medium volume. Finally, the pellets were washed with appropriate amount of 10% cold glycerol, centrifuged at 2,500xg at 4 °C for 15 min and resuspended with 10% cold glycerol to the final volume of 2-3 mL. Then 50 µL of cell suspension was aliquoted to 1.5 mL microcentrifuge tube and stored at -80 °C.

2.15.2 Electroporation

Both 0.1 cm electroporation cuvette and holder were chilled on ice. Competent cells were thawed on ice. After that, 4 µL of ligation products were mixed with 50 µL competent cells and placed on ice for 1 min. This mixture was transferred to a cold cuvette. After the cuvette was placed into electroporation chamber and one pulse for electroporation was applied, 0.5 mL of LB medium was added and then quickly resuspended with pipette. Then cell suspension was transferred to new tube and incubated at 37 °C for 1 h with shaking. Finally, 0.2 mL cell suspension was spread on LB agar plate containing an appropriate concentration of antibiotic and incubated at 37 °C for 18 h.

2.16 Nucleotide sequencing

The plasmids were sent to Bioneer, Korean for sequencing. The primers for DNA sequencing are shown in Table 2.3. The nucleotide sequences were compared by using ClustalW2 Multiple Sequence Alignment tools (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The protein sequences from nucleotide sequences were predicted using ExPASy translate tool (http://web.expasy.org/translate/).

Table 2.2 Primers for cloning of *lysC* and *dapA* genes

Primer	**Sequence (5' to 3')**	**Target gene**	**Direction**	**Tm (°C)**
BamHIlysCF	CGGGATCCATGTCTGAAATTGTTGTCTCCAAATTTGGCGG	*lysC*	forward	67.6
SacIlysCR	CGAGCTCCGGCCCGAAATATAGCTTCCAGGCC	*lysC*	reverse	69.5
BamHIdapAF	CGGGATCCATGTTCACGGGAAGTATTGTCGCG	*dapA*	forward	67
NotIdapAR	ATAAGAATGCGGCCGCGGCGCGACTTTTGAACAGAGTAAGC	*dapA*	reverse	70.5

Table 2.3 Oligonucleotide primers used for DNA sequencing

Primer	**Sequence (5' to 3')**	**Target**	**Direction**	**Tm (°C)**
ACYC Duet Up1	GGATCTCGACGCTC TCCCT	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Forward	55
Duet Down1	GATTATGCGGCCGT GTACAA	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Reverse	52
lysCsequp-R	GCAGATTAGTGAT ACCAGCAGAAGCCG	Upstream of pD-Y from nucleotide 142 of *lysC* gene in pD-Y	Reverse	61.3
dapAsequp-R	TCGCCAGTGGTGCC AACAGAAACGATCG	Upstream of pD-D from nucleotide 140 of *dapA* gene in pD-D	Reverse	64.3

2.17 Mutagenesis

The pD-Y*, a plasmid withV339A mutated *lysC*, and pD-D*, a plasmid with E84T mutated *dapA* genes, were constructed using QuikChange II Site-Directed Mutagenesis Kit. The pD-Y and pD-D were used as templates. The mutagenic primers are shown in Table 2.4. Reaction mixture were prepared by combining 5µl of 10× reaction buffer, 5–50 ng of dsDNA template, 125 ng of each primer, 1 µl of dNTP mix, then sterile water was added to a final volume of 50 µl and 1 µl of *Pfu* DNA polymerase (2.5 U/µl). The mutated plasmid were amplified using the cycling parameters which were pre-denaturation (95 °C, 2 min), 12 cycles of denaturation (95 °C, 1 min), annealing (60 °C, 1 min), and extension (72 °C, 6 min), and the final extention (72 °C, 10 min). One µl of the *Dpn* I restriction enzyme (10 U/µl) was added directly to each amplification reaction. Then reaction mixtures were mixed and incubated at 37 °C for 1 h. One µl of the reaction mixtures were transformed to *E. coli* TOP10 competent cells by electroporation and 0.2 ml of the competent cells were spreaded on LB agar plate with 30 µg/mL kanamycin and incubated at 37 °C for 18 h. The mutated plasmids were extracted and the mutation were confirmed by DNA sequencing.

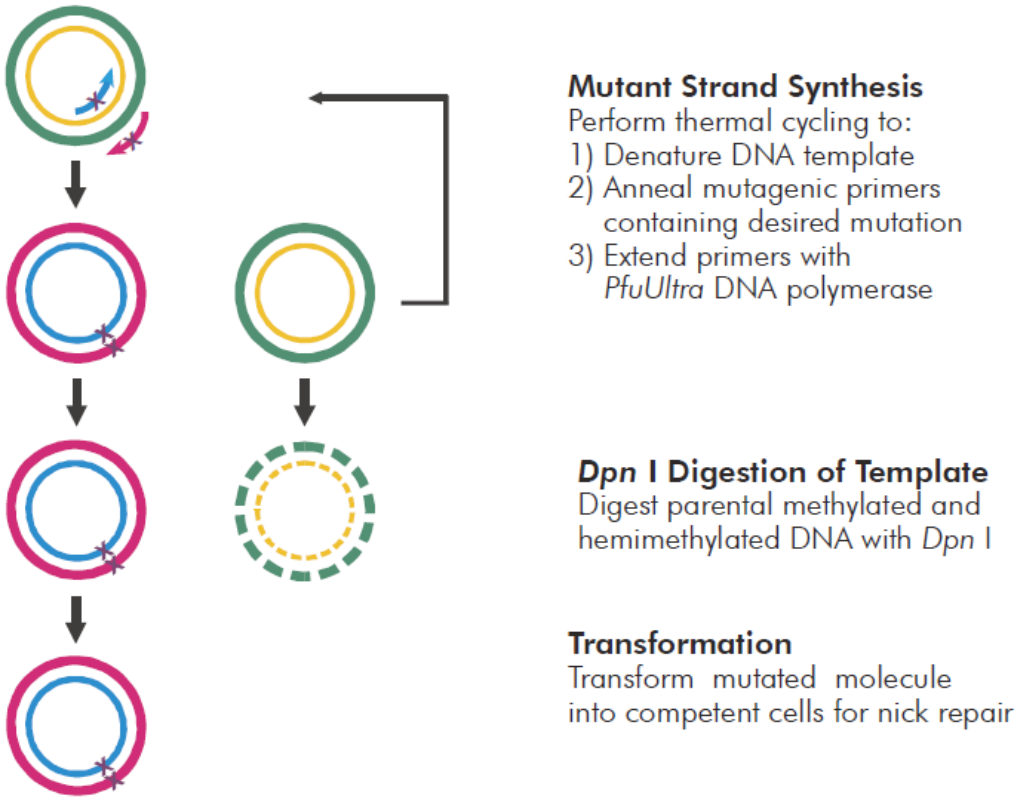

Figure 2.1 Overview of the QuikChange site-directed mutagenesis method (Source: Dhuffman, 2010)

2.18 Construction of pD-Y*D*LP

To construct pD-Y*D*LP, recombinant plasmid pD-Y*, pD-D* and pET-LP were used as sources of V339A mutated *lysC*, E84T mutated *dapA* as well as *lysdh* and *pcd*, respectively as shown in Figure 2. The mutated *dapA* gene was amplified by PCR using forward primer 5'-ATCGTCAGAGCTCCCCTTATGCGACTCCTGCAT TAGG-3' with restriction site of *Sac*I and reverse primer 5'- ATAAGAATGCGG CCGCGGCGCGACTTTTGAACAGAGTAAGC-3' with restriction site of *Not*I. PCR reaction mixture (50 µl) contained 50 ng of the pD-D* as template, 0.2 mM dNTPs, 10 pmole of each primer, 1xbuffer with $MgSO_4$ and 2.5 Unit of *Pfu* DNA polymerase. Condition for amplified gene was pre-denaturation (95 °C, 2 min), 30 cycles of denaturation (95 °C, 1 min), annealing (60 °C, 35 sec), and extension (72 °C, 2 min), and the final extention (72 °C, 5 min).The sizes of the PCR products were verified by agarose gel electrophoresis. The PCR product was digested with *Sac*I and *Not*I as same as pD-Y* then ligated together to gain pD-Y*D*. The *lysdh* and *pcd* genes fragment from pET-LP was obtained by digestion pET-LP with *Bgl*II and *Not*I and applied to agarose gel electrophoresis. Then *lysdh* and *pcd* genes fragment was extracted from agarose gel by Gel/PCR DNA fragment extraction kit. The *lysdh* and *pcd* fragment was ligated with the *Bgl*II and *Not*I digested pD-Y*D*to produce pD-Y*D*L

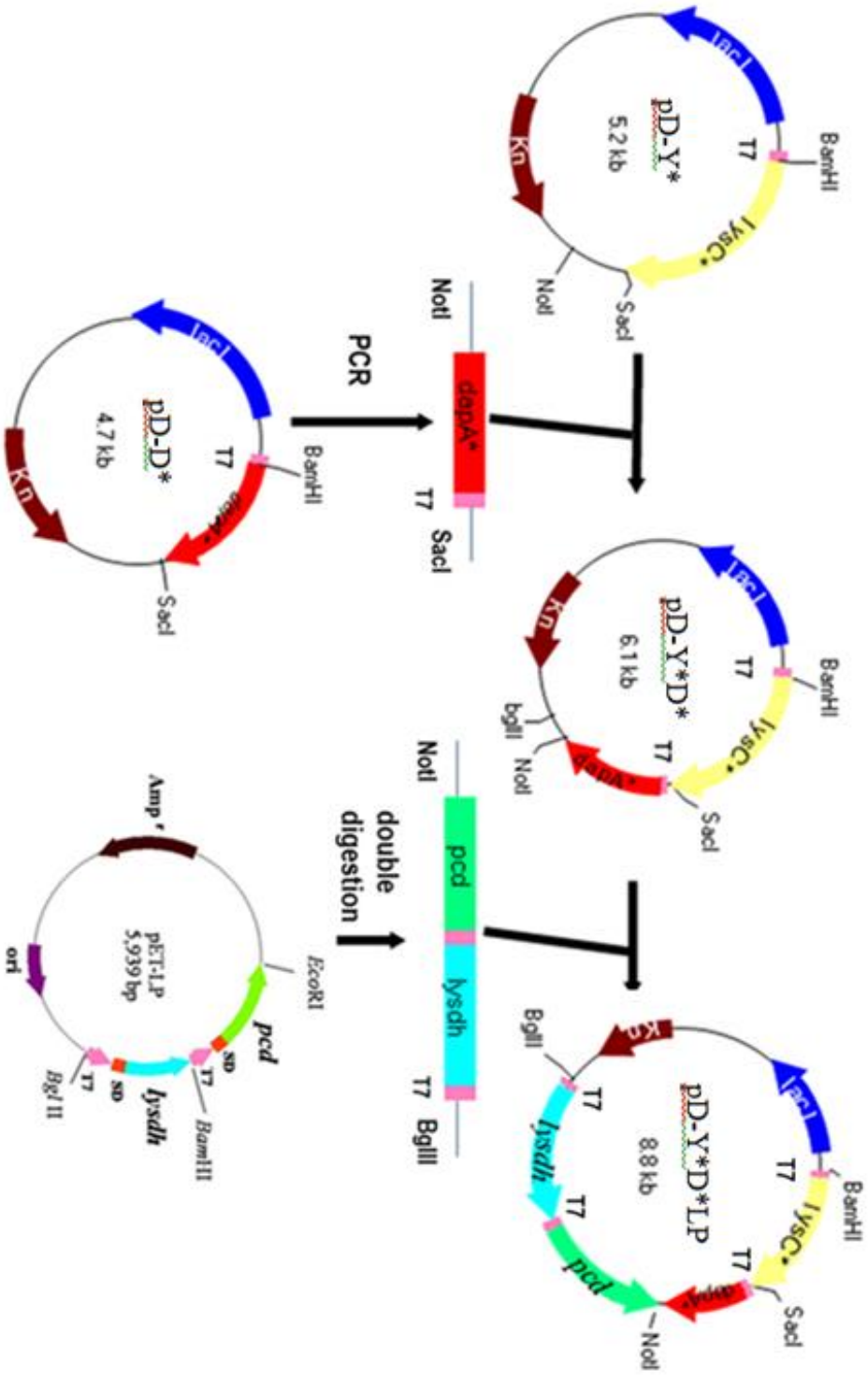

Figure 2.2 Construction of pD-Y*D*LP

Table 2.4 Mutagenic primers used for site-directed mutagenesis

Primer	**Sequence (5' to 3')**	**Target gene**	**Base substitution**	**Tm (°C)**
V339AF	GCGGCATAATATTTCGGCAG ACTTAATCACCACG	*lysC*	GTA → GCA	64.4
V339AR	CGTGGTGATTAAGTCTGCCG AAATATTATGCCGC	*lysC*		64.4
E84TF	GGTGCTAACGCTACTGCGAC AGCCATTAGCCTGACGCAGC	*dapA*	GAA → ACA	72.3
E84TR	GCTGCGTCAGGCTAATGGCT GTCGCAGTAGCGTTAGCACC	*dapA*		72.3

2.19 Analysis of gene expression

Each *E. coli* BL21(DE3) clone containing pRSFDuet-1, pD-Y*, pD-D*, pD-Y*D*, or pD-Y*D*LP was cultured in 5 ml LB supplemented with 30 μg/mL kanamycin at 37 °C with shaking at 250 rpm overnight. The seed cultures were transferred into 100 ml of the same medium and shaken at the same condition for 8 h then 10 ml of the cell cultures were inoculated into 200 ml of the same medium. Induction with 0.2mM IPTG was performed when OD_{600} was 0.6. Cell cultures were collected at 4 and 8 h. The collected samples were centrifuged at 8,000 x g for 15 min to separate pellet and supernatant. The pellet was sonicated to obtain crude extract.

2.20 SDS-polyacrylamide gel electrophoresis

The analysis of gene expression was carried out by SDS-PAGE (Bollag *et al*., 1996). The slab gel system consisted of 0.1% SDS (w/v) in 12.5% separating gel and 5% stacking gel. The 10 μl of crude extract from section 2.19 was mixed with 40 μL of the 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (w/v) bromophenol blue) and boiled for 15 min. After centrifugation at 10,000xg for 10 min, 7 μL of each sample was loaded to the gel. The cell extract of *E. coli* BL21(DE3) containing pRSFDuet-1 under induction with 0.2 mM IPTG was loaded as reference of protein pattern. A constant current, 20 mA per gel, was used in the electrophoresis. After electrophoresis, the gel was stained with Coomassie blue solution and then destained by destaining solution.

2.21 AK III activity assay

The activity of AKIII was measured by hydroxamate method (Black and Wright, 1954). Reaction mixture contained 200 mM Tris‑HCl (pH 7.5), 10 mM $MgSO_4 \cdot 6H_2O$, 10 mM aspartate, 10 mM ATP and 160 mM $NH_2OH \cdot HCl$ and crude enzyme in total volume of 1 ml. After incubation at 30 °C for 30 min, the reaction was stopped by mixing with 5% (w/v) $FeCl_3$ solution 1 ml and the absorbance at 540 nm was monitored. The specific activity was expressed as micromoles of aspartyl hydroxamate produced per minute per milligram protein.

2.22 L- AAA production

2.22.1 Shake flask fermentation

E. coli BL21(DE3) with pD-Y*D*LP and pD-LP were cultured in 5 ml LB containing 30 μg/mL kanamycin at 37 °C with shaking at 250 rpm overnight. The seed culture were transferred into 100 ml of the same medium and shaked at the same condition for 8 h after that 10 ml of the seed culture was inoculated into 200 ml minimal medium (Ratchaneeladdajit, 2014) and continued culture. Induction with IPTG was performed when OD_{600} reached 0.6. Samples were collected every 24 h until 168 h and the OD_{600} was continually measured. Cell concentration was calculated from the following formula.

$$OD_{600} \text{ of } 1.0 = 8 \times 10^8 \text{ cells/ml}$$

The collected samples were centrifuged at 8,000 x g for 15 min to separate pellet and supernatant.

2.22.2 TLC analysis

The supernatant from 2.22.1 were used for L-AAA determination by TLC. The 2 μl of the supernatant from the recombinant clones were spotted in 5 cm x 10 cm cellulose TLC plastic sheet along with standard L-AAA an L-lysine. The mobile phase was n-butanol: acetic acid: water (4: 1: 1). After that, the plate was dried and then 0.5% ninhydrin solution in ethanol and acetone (30: 70) was used for color develop. The plate was dried in hot air for 5 min. The ratio between the distance a compound moved from the baseline and the distance of the solvent front moved from the baseline (retardation factor, R_f) was calculate.

2.22.3 HPLC analysis

The supernatants from the minimal medium were analyzed for L-AAA production by HPLC (Shimadzu, Japan) with photodiode array detector using a Chirex 3126(D) penicillamine column (150 mm x 4.6 mm, 5μm). The mobile phase was 90:10 v/v mixture of 2mM copper sulphate and acetonitrile in a constant flow rate of 1 mL/min while being monitored at A_{200} for detection. The L-AAA concentration was quantified by using standard curve of L-AAA. The L-AAA concentration were compared between *E. coli* BL21(DE3) with pD-Y*D*LP and pD-LP.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1 Chromosomal DNA extraction of *E. coli* BL21(DE3)

From agarose gel electrophoresis, the extracted chromosomal DNA from *E. coli* BL21(DE3) showed molecular weight about 23.1 kb without RNA and protein contamination as shown in Figure 3.1. This purity of the extracted chromosomal was measured by *A*260/*A*280 ratio which was around 1.8 to 2.0. The results indicated that the quality of DNA solution was good enough for using in the next experiments.

3.2 Extraction of pRSFDuet-1

pRSFDuet-1 was extracted from *E. coli* TOP10 host cell for using as a cloning and expression vector. The extracted plasmid was mainly in supercoil form. The concentration of the obtained plasmid was examined on agarose gel electrophoresis. Generally, concentration of the obtained pRSFDuet-1 was approximately 50 ng/μL when 5 mL of bacterial cell culture was used together with the elution step by 50 μL of ultrapure water. After digestion with *Bam*HI, the expected DNA fragment of 3,829 bp was observed by agarose gel electrophoresis as shown in Figure 3.2.

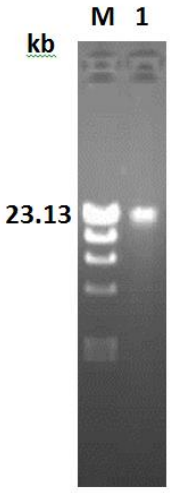

Figure 3.1 Agarose gel electrophoresis of chromosomal DNA from *E. coli* BL21(DE3) Lane M : λ/*Hin*dIII marker (Thermo Scientific™, USA) Lane 1: chromosomal DNA from *E. coli* BL21(DE3)

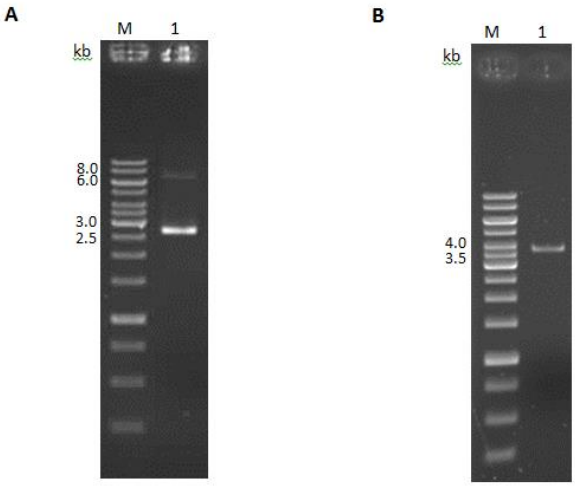

Figure 3.2 Agarose gel electrophoresis of pRSFDuet-1 A. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: extracted pRSFDuet-1 B. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: *Bam*HI digested pRSFDuet-1

3.3 PCR amplification of *lysC* and *dapA* genes

To increase the biosynthesis pool of L-lysine which is a precursor for L-AAA production, L-lysine metabolic flux was aimed to amplify by overexpression of L-lysine feedback resistant AKIII and DHDPS.

The *lysC* encoding AKIII and *dapA* encoding DHDPS were amplified using *E. coli* BL21(DE3) chromosomal DNA from 3.1 as a template. The BamHIlysCF containing *Bam*HI site at 5' end and SacIlysCR containing *Sac*I site at 5' end were used as forward and reverse primers for *lysC* gene amplification. In the same way, BamHIdapAF and NotIdapAR were used as forward and reverse primers, respectively, for *dapA* gene amplification. Agarose gel electrophoresis showed that the PCR product of *lysC* and *dapA* had size around 1,400 bp and 900 bp, respectively (Figure 3.3) which were correlated to the reported size of *E. coli lysC* (accession number CAQ34373.1, 1,399 bp) and *E. coli dapA* (accession number CAQ32849.1, 1947 bp) in NCBI database.

3.4 Construction of pD-Y and pD-D

The PCR products from section 3.3 were double digested with *Bam*HI and *Sac*I for *lysC* and *Bam*HI and *Not*I for *dapA.* Double digested *lysC* was ligated to *Bam*HI and *Sac*I digested pRSFDuet-1 vector to gain pD-Y while double digested *dapA* was ligated to *Bam*HI and *Not*I digested pRSFDuet-1 vector to gain pD-D. After transformation into *E. coli* BL21(DE3), the recombinant plasmid pD-Y and pD-D were extracted and double digested with *Bam*HI and *Sac*I for pD-Y and *Bam*HI and *Not*I for pD-D. The *Bam*HI/*Sac*I digested pD-Y gave 2 DNA bands of pRSFDuet-1 (3,813 bp) and *lysC* gene (1,399 bp). The *Bam*HI/*NotI*I digested pD-D also produced

2 DNA bands of pRSFDuet-1 (3,785 bp) and *dapA* gene (947 bp) as shown in Figure 3.4. After that, DNA sequencing was performed. The sequence of *lysC* and *dapA* with 1,399 bp and 947 bp showed 100% homology to the sequence of *E. coli lysC* and *dapA*, respectively from NCBI database (accession number CAQ34373.1 for *lysC* and CAQ32849.1 for *dapA*). The sequencing chromatograms are shown in Appendix B for *lysC* gene and Appendix C for *dapA* gene. T7 promoter and ribosome binding site of pD-Y and pD-D was also sequence by using lysCsequp-R primer that could sequence upsteam region of nucleotide 142 of *lysC* gene and dapAsequp-R primer that can sequenced upsteam of nucleotide 140 of *dapA* gene. The sequencing chromatograms are shown in Appendix D. The results showed that the sequences of the upstream region of the inserted gene in pD-Y and pD-D including T7 promoter and ribosome binding were 100% homology with that of pRSFDuet-1.

3.5 Construction of pD-Y* and pD-D*

Various mutations of AK III were constructed with the prediction by molecular dynamic simulation and co-evolutionary analysis (Chen *et al*., 2011). The mutation of Val at position 339 to Ala (V339A) can desensitize AK III from lysine inhibition by destroying the hydrogen bonds with lysine which kept the most remaining of enzyme activity when L-lysine concentration was increased when compared with other mutation sites.

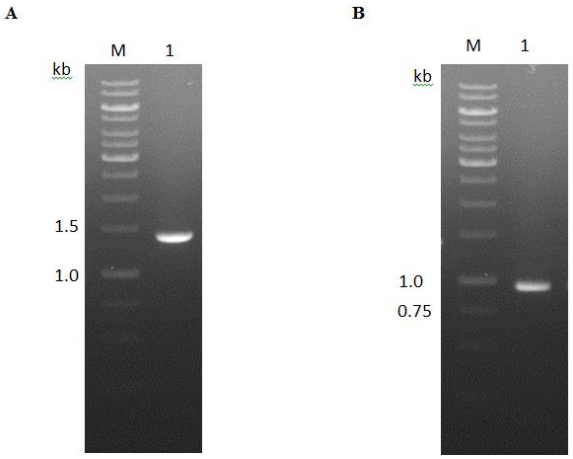

Figure 3.3 PCR product of *lysC* and *dapA* genes A. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *lysC* gene B. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *dapA* gene

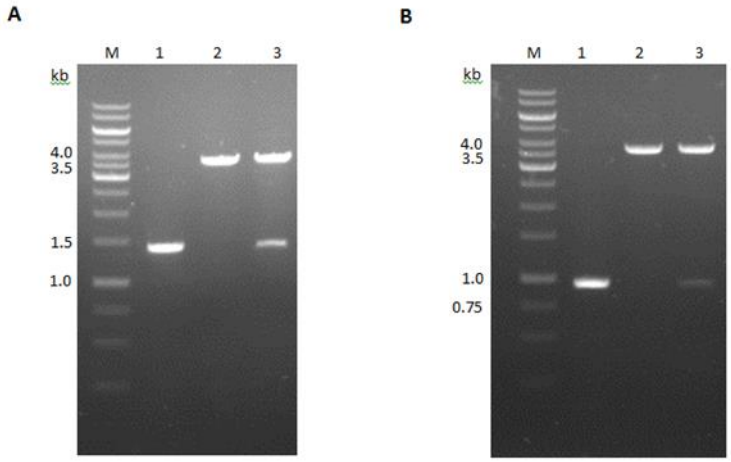

Figure 3.4 Restriction pattern of pD-Y and PD-D A. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *lysC* gene (1,399 bp) Lane 2: *Bam*HI and *Sac*I digested pRSFDuet-1 (3,813 bp) Lane 3: *Bam*HI and *Sac*I digested pD-Y B. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *dapA* gene (947 bp) Lane 2: *Bam*HI and *Not*I digested pRSFDuet-1 (3,785 bp) Lane 3: *Bam*HI and *Not*I digested pD-D

In parallel, mutations of DHDPS were performed by multiple rounds of random mutagenesis or introducing point mutations at regulatory site in DHDPS by site-directed mutagenesis to relieve the feedback inhibition by l-lysine. Mutation of Glu at the position 84 to Thr (E84T) could release the feedback inhibition by destroying the original electrostatic attraction between L-lysine and Glu at position 84. Moreover, kinetic parameters of E84T AK III were similar to those of the wild-type enzyme which indicating that this residue is not directly related to the enzyme catalysis (Geng *et al*., 2012).

According to the reports of Chen and coworkers (2011) and Geng and coworkers (2012), mutation at Val 339 to Ala of AKIII and Glu 84 to Thr of DHDPS were selected to produce L-lysine feedback resistant enzymes in this study.

pD-Y and pD-D from section 3.4 were used as DNA templates for creating of mutated *lysC* and *dapA* genes, respectively by QuikChange Site-Directed Mutagenesis. V339AF and V339AR were designed for a base substitution of T to C at nucleotide 1016 of *lysC* to create V399A AKIII. For *dapA* mutagenesis, E84TF and E84TR primers provided 2 bases substitution of G to A and A to C at nucleotide 250 and 251, respectively. After QuikChange Site-Directed Mutagenesis were performed, pD-Y* containing mutated *lysC* (*lysC**) and pD-D* containing mutated *dapA* (*dapA* *) were confirmed by digestion pD-Y* with *Bam*HI and *Sac*I and pD-D* with *Bam*HI and *Not*I (Figure 3.5). After that, both mutations were verified by DNA sequencing using ACYC Duet Up1 and Duet Down primers. The nucleotide sequences and mutated site of *lysC** and *dapA** are shown in Figure 3.6 and 3.7, respectively. The sequencing chromatograms are shown in Appendix E for V339A *lysC** and Appendix

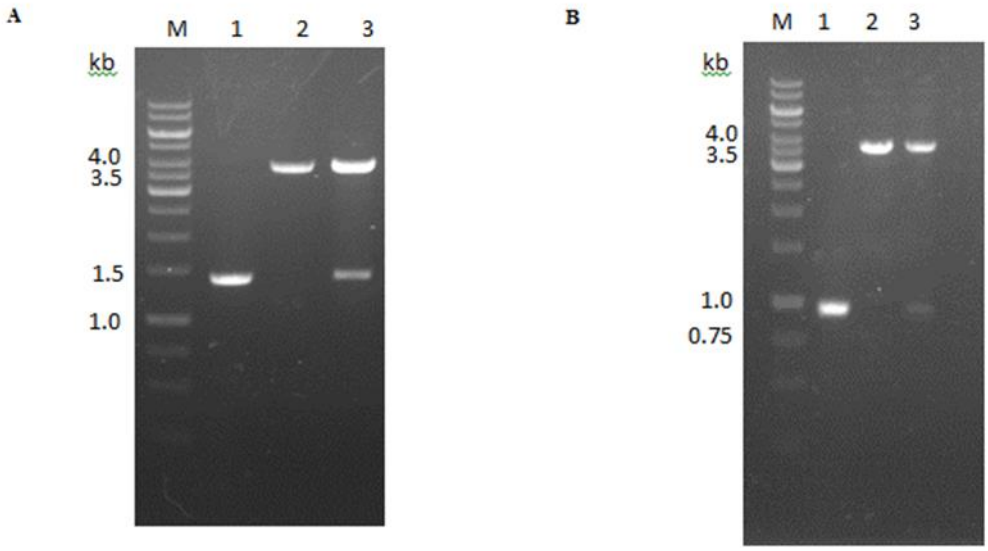

Figure 3.5 Restriction pattern of pD-Y* and PD-D* A. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *lysC* gene (1,399 bp) Lane 2: *Bam*HI and *Sac*I digested pRSFDuet-1 (3,813 bp) Lane 3: *Bam*HI and *Sac*I digested pD-Y* B. Lane M: 1kb marker (Thermo Scientific™, USA) Lane 1: PCR product of *dapA* gene (947 bp) Lane 2: *Bam*HI and *Not*I digested pRSFDuet-1 (3,785 bp) Lane 3: *Bam*HI and *Sac*I digested pD-D*

A

```
   1 ATGTCTGAAATTGTTGTCTCCAAATTTGGCGGTACCAGCGTAGCTGATTTTGACGCCATG
   1 M  S  E  I  V  V  S  K  F  G  G  T  S  V  A  D  F  D  A  M
  61 AACCGCAGCGCTGATATTGTGCTTTCTGATGCCAACGTGCGTTTAGTTGTCCTCTCGGCT
  21 N  R  S  A  D  I  V  L  S  D  A  N  V  R  L  V  V  L  S  A
 121 TCTGCTGGTATCACTAATCTGCTGGTCGCTTTAGCTGAAGGACTGGAACCTGGCGAGCGA
  41 S  A  G  I  T  N  L  L  V  A  L  A  E  G  L  E  P  G  E  R
 181 TTCGAAAAACTCGACGCTATCCGCAACATCCAGTTTGCCATTCTGGAACGTCTGCGTTAC
  61 F  E  K  L  D  A  I  R  N  I  Q  F  A  I  L  E  R  L  R  Y
 241 CCGAACGTTATCCGTGAAGAGATTGAACGTCTGCTGGAGAACATTACTGTTCTGGCAGAA
  81 P  N  V  I  R  E  E  I  E  R  L  L  E  N  I  T  V  L  A  E
 301 GCGGCGGCGCTGGCAACGTCTCCGGCGCTGACAGATGAGCTGGTCAGCCACGGCGAGCTG
 101 A  A  A  L  A  T  S  P  A  L  T  D  E  L  V  S  H  G  E  L
 361 ATGTCGACCCTGCTGTTTGTTGAGATCCTGCGCGAACGCGATGTTCAGGCACAGTGGTTT
 121 M  S  T  L  L  F  V  E  I  L  R  E  R  D  V  Q  A  Q  W  F
 421 GATGTACGTAAAGTGATGCGTACCAACGACCGATTTGGTCGTGCAGAGCCAGATATAGCC
 141 D  V  R  K  V  M  R  T  N  D  R  F  G  R  A  E  P  D  I  A
 481 GCGCTGGCGGAACTGGCCGCGCTGCAGCTGCTCCCACGTCTCAATGAAGGCTTAGTGATC
 161 A  L  A  E  L  A  A  L  Q  L  L  P  R  L  N  E  G  L  V  I
 541 ACCCAGGGATTTATCGGTAGCGAAAATAAAGGTCGTACAACGACGCTTGGCCGTGGAGGC
 181 T  Q  G  F  I  G  S  E  N  K  G  R  T  T  T  L  G  R  G  G
 601 AGCGATTATACGGCAGCCTTGCTGGCGGAGGCTTTACACGCATCTCGTGTTGATATCTGG
 201 S  D  Y  T  A  A  L  L  A  E  A  L  H  A  S  R  V  D  I  W
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 221 T  D  V  P  G  I  Y  T  T  D  P  R  V  V  S  A  A  K  R  I
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 241 D  E  I  A  F  A  E  A  A  E  M  A  T  F  G  A  K  V  L  H
 781 CCGGCAACGTTGCTACCCGCAGTACGCAGCGATATCCCGGTCTTTGTCGGCTCCAGCAAA
 261 P  A  T  L  L  P  A  V  R  S  D  I  P  V  F  V  G  S  S  K
 841 GACCCACGCGCAGGTGGTACGCTGGTGTGCAATAAAACTGAAAATCCGCCGCTGTTCCGC
 281 D  P  R  A  G  G  T  L  V  C  N  K  T  E  N  P  P  L  F  R
 901 GCTCTGGCGCTTCGTCGCAATCAGACTCTGCTCACTTTGCACAGCCTGAATATGCTGCAT
 301 A  L  A  L  R  R  N  Q  T  L  L  T  L  H  S  L  N  M  L  H
 961 TCTCGCGGTTTCCTCGCGGAAGTTTTCGGCATCCTCGCGCGGCATAATATTTCGGTACGAC
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1021 TTAATCACCACGTCAGAAGTGAGCGTGGCATTAACCCTTGATACCACCGGTTCAACCTCC
 341 L  I  T  T  S  E  V  S  V  A  L  T  L  D  T  T  G  S  T  S
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 361 T  G  D  T  L  L  T  Q  S  L  L  M  E  L  S  A  L  C  R  V
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1201 GGCGTTGGCAAAGAGGTATTCGGCGTACTGGAACCGTTCAACATTCGCATGATTTGTTAT
 401 G  V  G  K  E  V  F  G  V  L  E  P  F  N  I  R  M  I  C  Y
1261 GGCGCATCCAGCCATAACCTGTGCTTCCTGGTGCCCGGCGAAGATGCCGAGCAGGTGGTG
 421 G  A  S  S  H  N  L  C  F  L  V  P  G  E  D  A  E  Q  V  V
1321 CAAAAACTGCATAGTAATTTGTTTGAGTAA
 441 Q  K  L  H  S  N  L  F  E  *
```

Figure 3.6 The nucleotide sequence and the deduced amino acid sequence of mutated V339A *lysC* gene in pD-Y*(B) compared with those of wild type (A). The mutation site is shown in □. All sequencing chromatogram of mutated V339A *lysC* are shown in Appendix E.

B

```
   1 ATGTCTGAAATTGTTGTCTCCAAATTTGGCGGTACCAGCGTAGCTGATTTTGACGCCATG
   1 M  S  E  I  V  V  S  K  F  G  G  T  S  V  A  D  F  D  A  M
  61 AACCGCAGCGCTGATATTGTGCTTTCTGATGCCAACGTGCGTTTAGTTGTCCTCTCGGCT
  21 N  R  S  A  D  I  V  L  S  D  A  N  V  R  L  V  V  L  S  A
 121 TCTGCTGGTATCACTAATCTGCTGGTCGCTTTAGCTGAAGGACTGGAACCTGGCGAGCGA
  41 S  A  G  I  T  N  L  L  V  A  L  A  E  G  L  E  P  G  E  R
 181 TTCGAAAAACTCGACGCTATCCGCAACATCCAGTTTGCCATTCTGGAACGTCTGCGTTAC
  61 F  E  K  L  D  A  I  R  N  I  Q  F  A  I  L  E  R  L  R  Y
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  81 P  N  V  I  R  E  E  I  E  R  L  L  E  N  I  T  V  L  A  E
 301 GCGGCGGCGCTGGCAACGTCTCCGGCGCTGACAGATGAGCTGGTCAGCCACGGCGAGCTG
 101 A  A  A  L  A  T  S  P  A  L  T  D  E  L  V  S  H  G  E  L
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 121 M  S  T  L  L  F  V  E  I  L  R  E  R  D  V  Q  A  Q  W  F
 421 GATGTACGTAAAGTGATGCGTACCAACGACCGATTTGGTCGTGCAGAGCCAGATATAGCC
 141 D  V  R  K  V  M  R  T  N  D  R  F  G  R  A  E  P  D  I  A
 481 GCGCTGGCGGAACTGGCCGCGCTGCAGCTGCTCCCACGTCTCAATGAAGGCTTAGTGATC
 161 A  L  A  E  L  A  A  L  Q  L  L  P  R  L  N  E  G  L  V  I
 541 ACCCAGGGATTTATCGGTAGCGAAAATAAAGGTCGTACAACGACGCTTGGCCGTGGAGGC
 181 T  Q  G  F  I  G  S  E  N  K  G  R  T  T  T  L  G  R  G  G
 601 AGCGATTATACGGCAGCCTTGCTGGCGGAGGCTTTACACGCATCTCGTGTTGATATCTGG
 201 S  D  Y  T  A  A  L  L  A  E  A  L  H  A  S  R  V  D  I  W
 661 ACCGACGTCCCGGGCATCTACACCACCGATCCACGCGTAGTTTCCGCAGCAAAACGCATT
 221 T  D  V  P  G  I  Y  T  T  D  P  R  V  V  S  A  A  K  R  I
 721 GATGAAATCGCGTTTGCCGAAGCGGCAGAGATGGCAACTTTTGGTGCAAAAGTACTGCAT
 241 D  E  I  A  F  A  E  A  A  E  M  A  T  F  G  A  K  V  L  H
 781 CCGGCAACGTTGCTACCCGCAGTACGCAGCGATATCCCGGTCTTTGTCGGCTCCAGCAAA
 261 P  A  T  L  L  P  A  V  R  S  D  I  P  V  F  V  G  S  S  K
 841 GACCCACGCGCAGGTGGTACGCTGGTGTGCAATAAAACTGAAAATCCGCCGCTGTTCCGC
 281 D  P  R  A  G  G  T  L  V  C  N  K  T  E  N  P  P  L  F  R
 901 GCTCTGGCGCTTCGTCGCAATCAGACTCTGCTCACTTTGCACAGCCTGAATATGCTGCAT
 301 A  L  A  L  R  R  N  Q  T  L  L  T  L  H  S  L  N  M  L  H
 961 TCTCGCGGTTTCCTCGCGGAAGTTTTCGGCATCCTCGCGCGGCATAATATTTCGGCAGAC
 321 S  R  G  F  L  A  E  V  F  G  I  L  A  R  H  N  I  S  A  D
1021 TTAATCACCACGTCAGAAGTGAGCGTGGCATTAACCCTTGATACCACCGGTTCAACCTCC
 341 L  I  T  T  S  E  V  S  V  A  L  T  L  D  T  T  G  S  T  S
1081 ACTGGCGATACGTTGCTGACGCAATCTCTGCTGATGGAGCTTTCCGCACTGTGTCGGGTG
 361 T  G  D  T  L  L  T  Q  S  L  L  M  E  L  S  A  L  C  R  V
1141 GAGGTGGAAGAAGGTCTGGCGCTGGTCGCGTTGATTGGCAATGACCTGTCAAAAGCCTGC
 381 E  V  E  E  G  L  A  L  V  A  L  I  G  N  D  L  S  K  A  C
1201 GGCGTTGGCAAAGAGGTATTCGGCGTACTGGAACCGTTCAACATTCGCATGATTTGTTAT
 401 G  V  G  K  E  V  F  G  V  L  E  P  F  N  I  R  M  I  C  Y
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 421 G  A  S  S  H  N  L  C  F  L  V  P  G  E  D  A  E  Q  V  V
1321 CAAAAACTGCATAGTAATTTGTTTGAGTAA
 441 Q  K  L  H  S  N  L  F  E  *
```

Figure 3.6 (continued) The nucleotide sequence and the deduced amino acid sequence of mutated V339A *lysC* gene in pD-Y*(B) compared with those of wild type (A). The mutation site is shown in □. All sequencing chromatogram of mutated V339A *lysC* are shown in Appendix E.

A

```
  1 ATGTTCACGGGAAGTATTGTCGCGATTGTTACTCCGATGGATGAAAAAGGTAATGTCTGT
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 61 CGGGCTAGCTTGAAAAAACTGATTGATTATCATGTCGCCAGCGGTACTTCGGCGATCGTT
 21 R  A  S  L  K  K  L  I  D  Y  H  V  A  S  G  T  S  A  I  V
121 TCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTGATGTGGTG
 41 S  V  G  T  T  G  E  S  A  T  L  N  H  D  E  H  A  D  V  V
181 ATGATGACGCTGGAGCTGGCTGACGGGCGCATTCCGGTGATTGCCGGGACTGGTGCTAAC
 61 M  M  T  L  E  L  A  D  G  R  I  P  V  I  A  G  T  G  A  N
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 81 A  T  A  E  A  I  S  L  T  Q  R  F  N  D  S  G  I  V  G  C
301 CTGACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTTCAAA
101 L  T  V  T  P  Y  Y  N  R  P  S  Q  E  G  L  Y  Q  H  F  K
361 GCCATCGCTGAGCATACTGACCTGCCGCAAATTCTGTATAATGTGCCGTCCCGTACTGGC
121 A  I  A  E  H  T  D  L  P  Q  I  L  Y  N  V  P  S  R  T  G
421 TGCGATCTGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGAATC
141 C  D  L  L  P  E  T  V  G  R  L  A  K  V  K  N  I  I  G  I
481 AAAGAGGCAACAGGGAACTTAACGCGTGTAAACCAGATCAAAGAGCTGGTTTCAGATGAT
161 K  E  A  T  G  N  L  T  R  V  N  Q  I  K  E  L  V  S  D  D
541 TTTGTTCTGCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCATGCAATTAGGCGGTCAT
181 F  V  L  L  S  G  D  D  A  S  A  L  D  F  M  Q  L  G  G  H
601 GGGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGGCCCAGATGTGCAAACTG
201 G  V  I  S  V  T  A  N  V  A  A  R  D  M  A  Q  M  C  K  L
661 GCAGCAGAAGGGCATTTTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGCCATTACAC
221 A  A  E  G  H  F  A  E  A  R  V  I  N  Q  R  L  M  P  L  H
721 AACAAACTATTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAGGAACTGGGT
241 N  K  L  F  V  E  P  N  P  I  P  V  K  W  A  C  K  E  L  G
781 CTTGTGGCGACCGATACGCTGCGCCTGCCAATGACACCAATCACCGACAGTGGTCGTGAG
261 L  V  A  T  D  T  L  R  L  P  M  T  P  I  T  D  S  G  R  E
841 ACGGTCAGAGCGGCGCTTAAGCATGCCGGTTTGCTGTAAAGTTTAGGGAGATTTGATGGC
281 T  V  R  A  A  L  K  H  A  G  L  L  *
```


Figure 3.7 The nucleotide sequence and the deduced amino acid sequence of mutated E84T *dapA* gene in pD-D*(B) compared with those of wild type (A). The mutation site is shown in □. All sequencing hromatogram of mutated E84T *dapA* are shown in Appendix F.

B

```
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  61 CGGGCTAGCTTGAAAAAACTGATTGATTATCATGTCGCCAGCGGTACTTCGGCGATCGTT
  21 R  A  S  L  K  K  L  I  D  Y  H  V  A  S  G  T  S  A  I  V
 121 TCTGTTGGCACCACTGGCGAGTCCGCTACCTTAAATCATGACGAACATGCTGATGTGGTG
  41 S  V  G  T  T  G  E  S  A  T  L  N  H  D  E  H  A  D  V  V
 181 ATGATGACGCTGGAGCTGGCTGACGGGCGCATTCCGGTGATTGCCGGGACTGGTGCTAAC
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 301 CTGACGGTAACCCCTTACTACAATCGTCCGTCGCAAGAAGGTTTGTATCAGCATTTCAAA
 101 L  T  V  T  P  Y  Y  N  R  P  S  Q  E  G  L  Y  Q  H  F  K
 361 GCCATCGCTGAGCATACTGACCTGCCGCAAATTCTGTATAATGTGCCGTCCCGTACTGGC
 121 A  I  A  E  H  T  D  L  P  Q  I  L  Y  N  V  P  S  R  T  G
 421 TGCGATCTGCTCCCGGAAACGGTGGGCCGTCTGGCGAAAGTAAAAAATATTATCGGAATC
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 161 K  E  A  T  G  N  L  T  R  V  N  Q  I  K  E  L  V  S  D  D
 541 TTTGTTCTGCTGAGCGGCGATGATGCGAGCGCGCTGGACTTCATGCAATTAGGCGGTCAT
 181 F  V  L  L  S  G  D  D  A  S  A  L  D  F  M  Q  L  G  G  H
 601 GGGGTTATTTCCGTTACGGCTAACGTCGCAGCGCGTGATATGGCCCAGATGTGCAAACTG
 201 G  V  I  S  V  T  A  N  V  A  A  R  D  M  A  Q  M  C  K  L
 661 GCAGCAGAAGGGCATTTTGCCGAGGCACGCGTTATTAATCAGCGTCTGATGCCATTACAC
 221 A  A  E  G  H  F  A  E  A  R  V  I  N  Q  R  L  M  P  L  H
 721 AACAAACTATTTGTCGAACCCAATCCAATCCCGGTGAAATGGGCATGTAAGGAACTGGGT
 241 N  K  L  F  V  E  P  N  P  I  P  V  K  W  A  C  K  E  L  G
 781 CTTGTGGCGACCGATACGCTGCGCCTGCCAATGACACCAATCACCGACAGTGGTCGTGAG
 261 L  V  A  T  D  T  L  R  L  P  M  T  P  I  T  D  S  G  R  E
 841 ACGGTCAGAGCGGCGCTTAAGCATGCCGGTTTGCTGTAAAGTTTAGGGAGATTTGATGGC
 281 T  V  R  A  A  L  K  H  A  G  L  L  *
```


Figure 3.7 (continued) The nucleotide sequence and the deduced amino acid sequence of mutated E84T *dapA* gene in pD-D*(B) compared with those of wild type (A). The mutation site is shown in □. All sequencing hromatogram of mutated E84T *dapA* are shown in Appendix F.

F for E84T *dapA**. The results from this part indicated that the mutated *lysC* and *dapA* were successfully construction.

3.6 Construction of pD-Y*D*LP

In previous work, *E. coli* BL21(DE3) harboring pET-LP which contains *lysdh* gene for LysDH from *A. denitrificans* and *pcd* gene for P6CDH from *P. putida* ADH 3 was constructed (Pamorn, 2011). Twenty five mM of L-AAA could be detected after incubated resting cells of the recombinant *E. coli* BL21(DE3) clone in the presence of 200 mM L-lysine for 24 hours.

It means that L-AAA could be produced in *E. coli* possessing heterologous expression of *lysdh* and *pcd* genes. Therefore, the *lysdh* and *pcd* genes from pET-LP were used for L-AAA production along with *lysC**, *dapA** with the expectation for more L-AAA production.

Firstly, pD-Y*D* was constructed. The DNA fragment from T7 promoter to *dapA** gene was amplified by PCR technique using pD-D from section 3.4 as a template. The PCR product was digested with *Sac*I and *Not*I and then ligated with *Sac*I and *Not*I digested pD-Y* to gain pD-Y*D* (6265 bp). To construct pD-Y*D*LP, pET-LP was double digested with *Bgl*II and *Not*I. The *lysdh* - *pcd* fragment was then ligated with *Bgl*II and *Not*I digested pD-Y*D*. The obtained pD- Y*D*LP was confirmed by *Bgl*II and *Not*I digestion. The digested products consisted of the 6,265 bp fragment of pD-Y*D* and 2,868 bp fragment of *lysdh-pcd* (Figure 3.8). L-AAA production of recombinant clone harboring pD-Y*D*LP is shown in Figure 3.9.

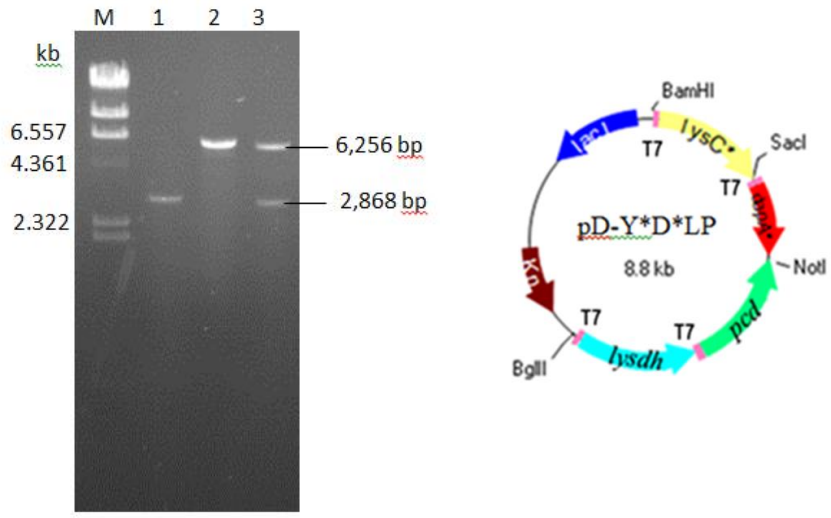


Figure 3.8 Restriction pattern of pD- Y*D*LP Lane M: λ/*Hin*dIII marker (Thermo Scientific™, USA) Lane 1: *Bgl*II and *Not*I digested of *lysdh-pcd* fragment from pET-LP Lane 2: *Bgl*II and *Not*I digested pD-Y*D* Lane 3: *Bgl*II and *Not*I digested pD-Y*D*LP

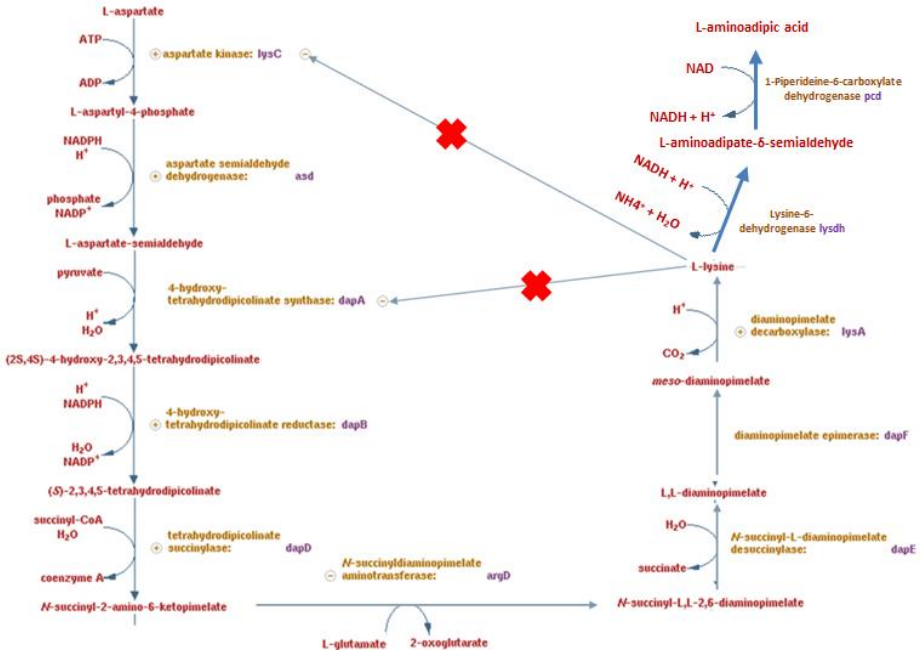


Figure 3.9 L-AAA production of recombinant clone harboring pD-Y*D*LP

3.7 Protein expression

After induction the inserted genes under T7 promoter of pRSFDuet-1 in *E. coli* clones harboring pD-Y*, pD-D*, pD-Y*D*, pD-LP and pD-Y*D*LP by IPTG for 8 h in LB medium, the clones were harvested and sonicated. The protein expression in crude extract was observed by SDS-PAGE. The protein pattern of the recombinant clones was compared with that of the host cell containing pRSFDuet-1. Protein bands of LysDH and P6CDH which are about 39.4 KDa and 54 KDa, respectively, were clearly seen on the gel, however, the bands of AKIII* and DHDPS* at 48.5 and 31.2 KDa could not be detected due to the interference of proteins from host cell that had similar size as AKIII and DHDPS as shown in Figure 3.10.

Differ from Michelis-Menten enzyme, protein expression of recombinant allosteric enzymes are quite low. Rastegari and coworker (2012) cloned and expressed allosteric regulation aspartokinase from L-lysine resistance *Corynebacterium glutamicum* ATCC21799 under Ptac promoter of pEKEx2 into L-lysine sensitive *C. glutamicum*. SDS-PAGE was performed to detect the protein expression in minimal medium containing glucose as a carbon source. The recombinant enzyme of the collected cell at different time points after incubation (24, 48, 68, 72, 74, 76 h) also showed very faint band, however, L-lysine titer increased about 2 times even during the growing and stationary phases.

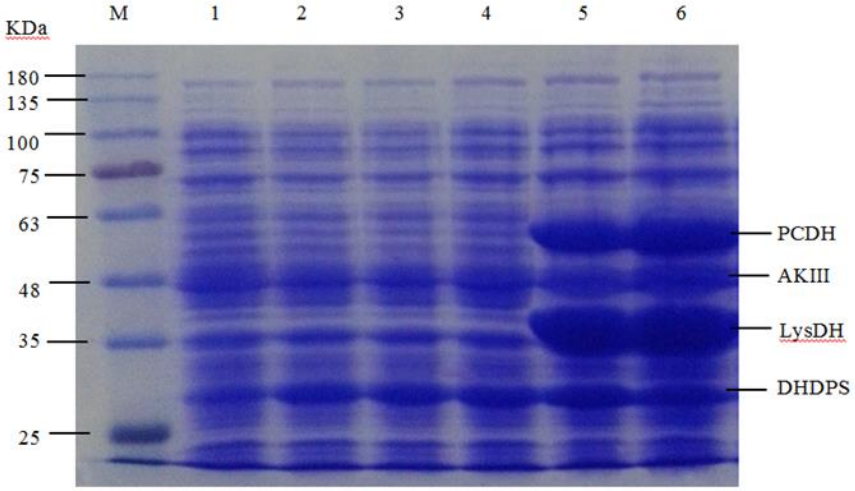


Figure 3.10 SDS-PAGE analysis of the crude extract of *E. coli* clones at 8 h after induction. Lane M: TriColor protein ladder marker (Biotechrabbit, Germany) Lane 1: *E. coli* BL21(DE3) with pRSFDuet-1 Lane 2: pD-Y* (*lysC**) Lane 3: pD-D* (*dapA**) Lane 4: pD-Y*D* (*lysC** and *dapA**) Lane 5: pD-LP (*lysdh* and *pcd*) Lane 6: pD-Y*D*LP (*lysC**, *dapA**, *lysdh* and *pcd*)

3.8 AK III activity assay

The recombinant clones with pRSFDuet-1, pD-Y*, pD-Y*D*, pD-Y*D*LP were collected after induction with 0.2 mM IPTG in LB medium for 4 and 8 h for AK III activity assay. The AK III activity of pD-Y* at 4 h was 1.64 fold higher that of control, *E. coli* containing pRSFDuet-1. In contrast, the recombinant clone with pD-Y*D*LP and pD-Y*D* had lower AK III activity than the control at both 4 and 8 h. Therefore, the time course study of AK III activity in all recombinant clones should be finely repeated.

Unfortunately, the activity of DHDPS could not be determined in this study since the assay procedure (Geng *et al*, 2013) requires a coupling enzyme dihydrodipicolinate reductase (DHDPR) which is not a commercial reagent. However, from the correct sequence of *dapA*, T7 promoter and ribosome binding site, DHDPS in pD-Y*, pD-Y*D* and pD-Y*D*LP should be overexpressed.

3.9 L- AAA production

3.9.1 Shake flask fermentation

To determine the production of L-AAA by the constructed clones, *E. coli* containing pD-Y*D*LP and pD-LP were cultivated in minimal medium from Ratchaneeladdajit (2014). This medium was chosen as an initial recipes because the recipes was developed for the fermentation for L-Phe production by recombinant *E. coli* BL21(DE3) containing L-Phe production plasmid using glycerol, the low value by-product from biodiesel production, as a carbon source. The recombinant clones were cultivated in the minimal medium for 7 days after induction by 0.2 mM IPTG.

Table 3.1 The activity of AK III in *E. coli* recombinant clones

Clone	**Time after induction (h)**	**Specific activity** ($x10^{-2}$ units/mg of protein)**	**Fold**
pRSFDuet-1	4	2.44	1
	8	3.51	1
pD-Y *	4	3.99	1.64
	8	3.52	1.00
pD-Y*D*	4	2.28	0.93
	8	2.73	0.78
pD-Y*D*LP	4	1.42	0.58
	8	1.10	0.31

** The data obtained from 2 independent experiments using the same samples.

The cell concentration of the recombinant clones showed that growth of pD-Y*D*LP and pD-LP clones were similar (Figure 3.11). Thus, addition of *lysC** and *dapA** expressions did not affect the growth of pD-LP clone.

3.9.2 Thin layer chromatography

After induction with IPTG for 4 days in shake flask fermentation, the culture medium of recombinant clones harboring pRSFDuet-1, pD-Y*D*LP and pD-LP were collected. The supernatants were obtained by centrifugation and then applied on TLC plate. L-AAA dissolved in the minimal medium was used as a standard. The R_f of L-AAA was 0.3. The spots with R_f around 0.3 were observed from recombinant clone harboring pD-LP and pD-Y*D*LP but did not found in pRSFDuet-1 (Figure 3.12). The spot intensity from pD-Y*D*LP was slightly higher than that of pD-LP.

3.9.3 HPLC analysis

To confirm the result from TLC, HPLC using a Chirex 3126(D) column was performed. The peak of L-AAA standard dissolved in minimal medium had the retention time at 1.766 min. Chromatogram of the supernatant from recombinant clones harboring pRSFDuet-1, pD-Y*D*LP and pD-LP are shown in Figure 3.13. The peak with similar retention time with L-AAA standard were observed from samples of pD-Y*D*LP and pD-LP. L-AAA concentration from the recombinant clones harboring pD-LP and pD-Y*D*LP was 0.86 mg/ml and 0.93 mg/ml, respectively.

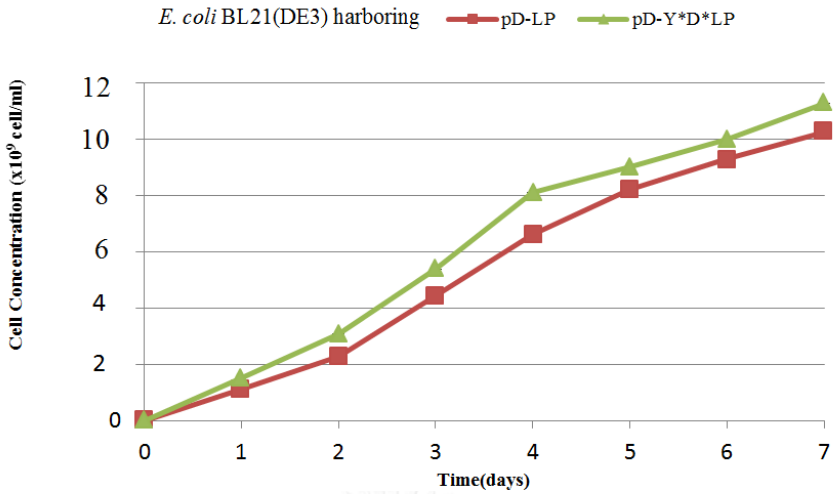


Figure 3.11 Cell concentrations of recombinant clones in minimal medium. The samples were collected after induction with 0.2mM IPTG for 7 days. The data were obtained from 2 independent experiments.

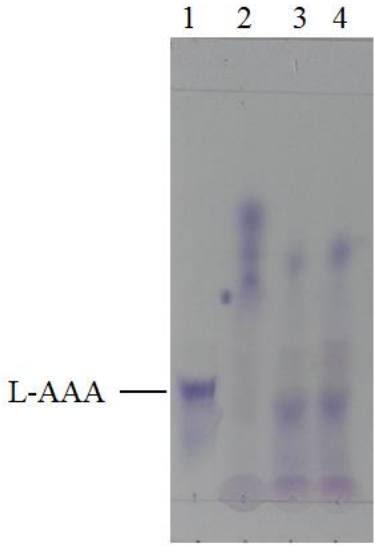
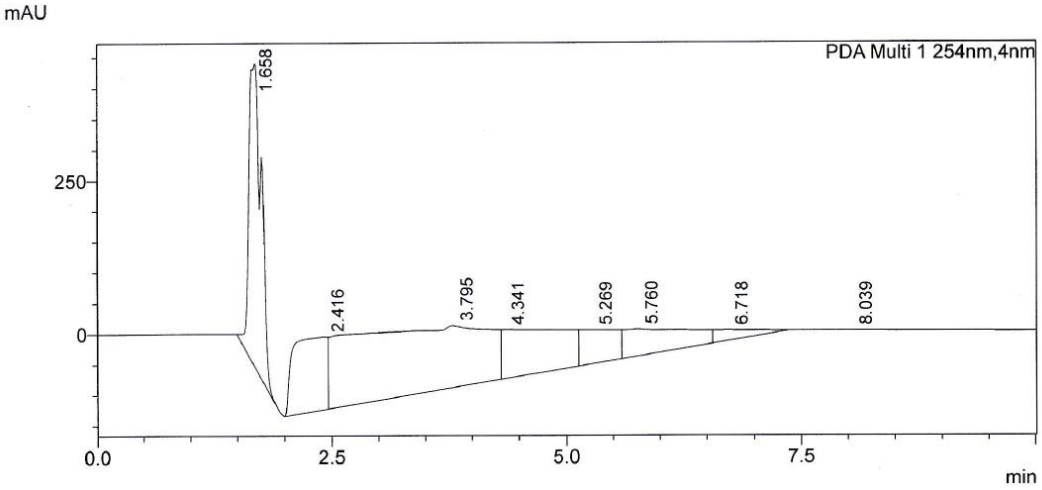


Figure 3.12 TLC analysis of L-aminoadipic acid production from *E. coli* BL21(DE3) harboring pD-Y*D*LP and pD-LP Lane 1: standard L-AAA Lane 2: supernatant from *E. coli* BL21(DE3) harboring pRSFDuet-1 Lane 3: supernatant from *E. coli* BL21(DE3) harboring pD-LP Lane 4: supernatant from *E. coli* BL21(DE3) harboring pD-Y*D*LP

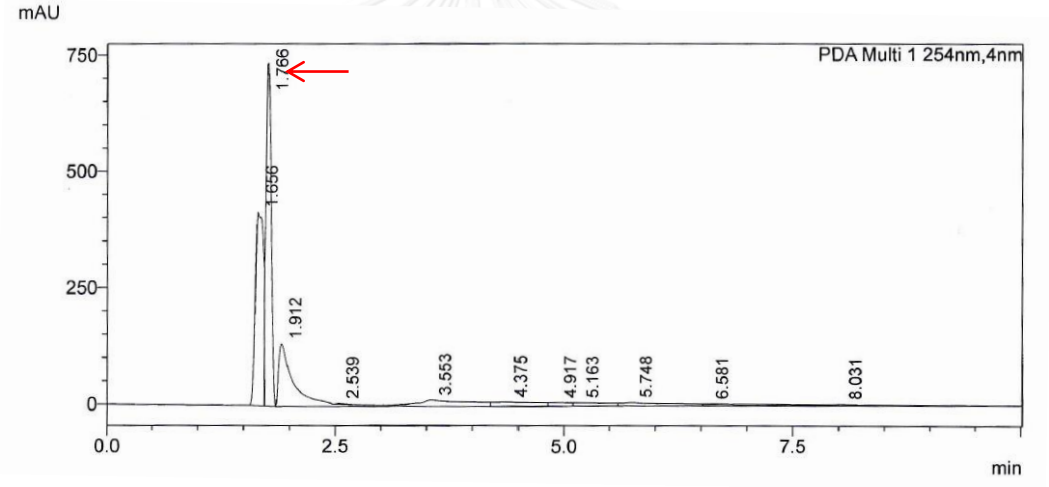
From the above results, we can conclude that the overexpression of *lysC** and *dapA** can elevate the L-AAA titer in fermentation broth. The similar result was reported by Ying and coworkers (2017) who developed a metabolically engineered strain of *E. coli* for the overproduction of L-pipecolic acid from glucose by amplification of L-lysine production. They overexpressed *dapA*, *lysC*, and *lysA* encoding for DHDPS, AK III and diaminopimelate decarboxylase, respectively and found that over-expression of *dapA* and *lysC* could increase L-lysine production and the L-pipecolic production was also increased when L-lysine production increased.

To increase L-AAA production by pD-Y*D*LP clones, optimization of medium compositions and induction time should be studied.

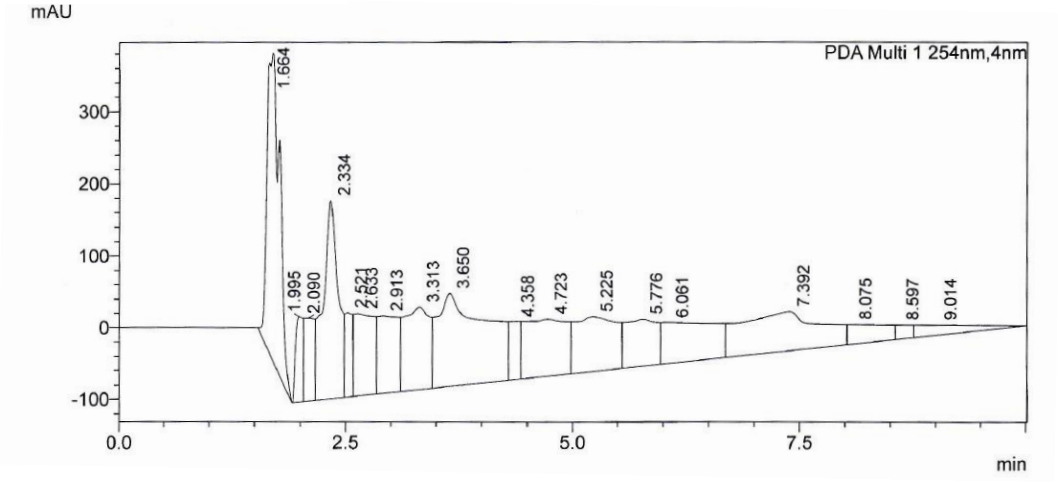
A)


mAU
PDA Multi 1 254nm,4nm
1.658
2.416
3.795
4.341
5.269
5.760
6.718
8.039
250
0
0.0
2.5
5.0
7.5
min

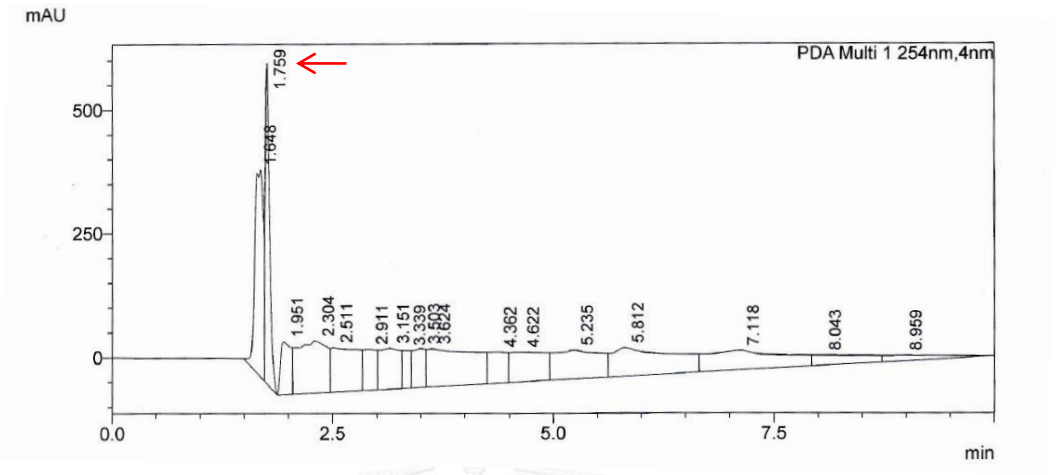
B)


mAU
PDA Multi 1 254nm,4nm
1.766
1.656
1.912
2.539
3.553
4.375
4.917
5.163
5.748
6.581
8.031
750
500
250
0
0.0
2.5
5.0
7.5
min

C)


mAU
PDA Multi 1 254nm,4nm
1.664
1.995
2.090
2.334
2.521
2.633
2.913
3.313
3.650
4.358
4.723
5.225
5.776
6.061
7.392
8.075
8.597
9.014
300
200
100
0
-100
0.0
2.5
5.0
7.5
min

D)



E)

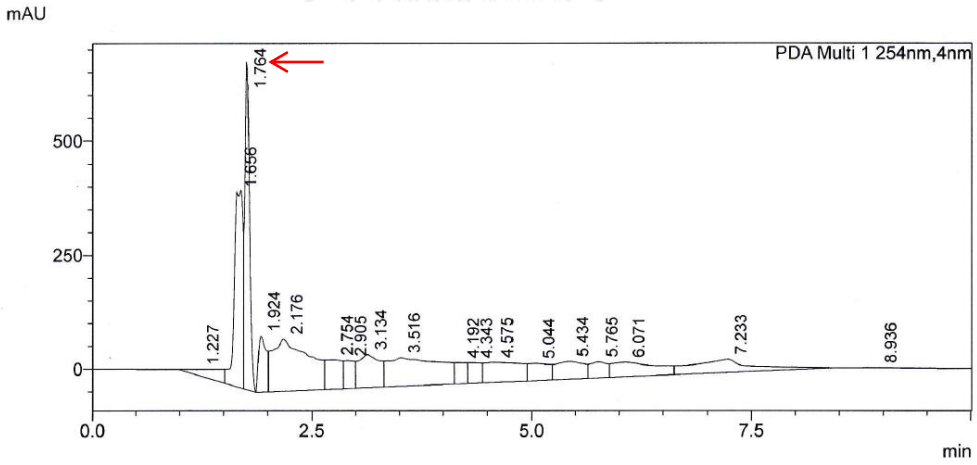


Figure 3.13 HPLC chromatogram of L-AAA (A) minimal medium (B) 10 µg of standard L-AAA in minimal medium (C) supernatant of *E. coli* BL21(DE3) harboring pRSFDuet-1 (D) supernatant of *E. coli* BL21(DE3) harboring pD-LP (E) supernatant of *E. coli* BL21(DE3) harboring pD-Y*D*LP. The red arrow indicates the peak of L-AAA.

CHAPTER IV

CONCLUSIONS

1) The pD-Y*D*LP containing V339A mutated *lysC*, E84T mutated *dapA*, *lysdh* and *pcd* genes was successfully constructed.
2) Expression of *lysdh* and *pcd* genes from the pD-Y*D*LP clone were clearly observed by SDS-PAGE. However, the expression of V339A *lysC* and E84T *dapA* could not be detected because of the interference of host cell proteins.
3) The L-AAA production could be observed from recombinant clone harboring pD-Y*D*LP and pD-LP by TLC and HPLC analysis. L-AAA produced from the recombinant clones harboring pD-LP and pD-Y*D*LP were 0.86 mg/ml and 0.93 mg/ml, respectively.

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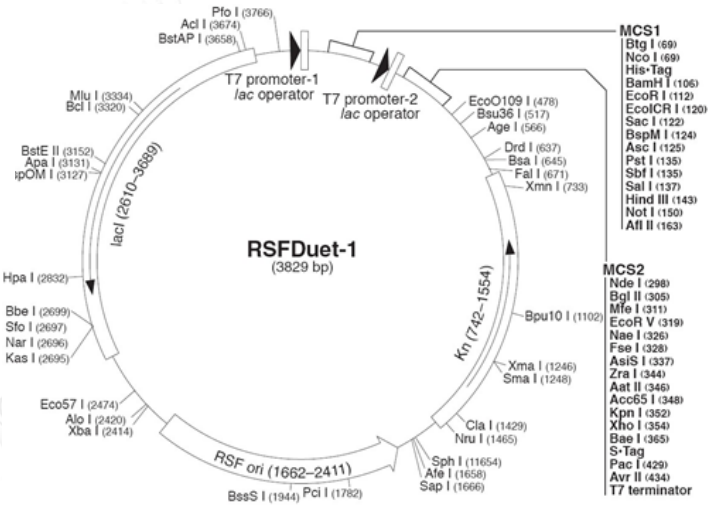
APPENDIX

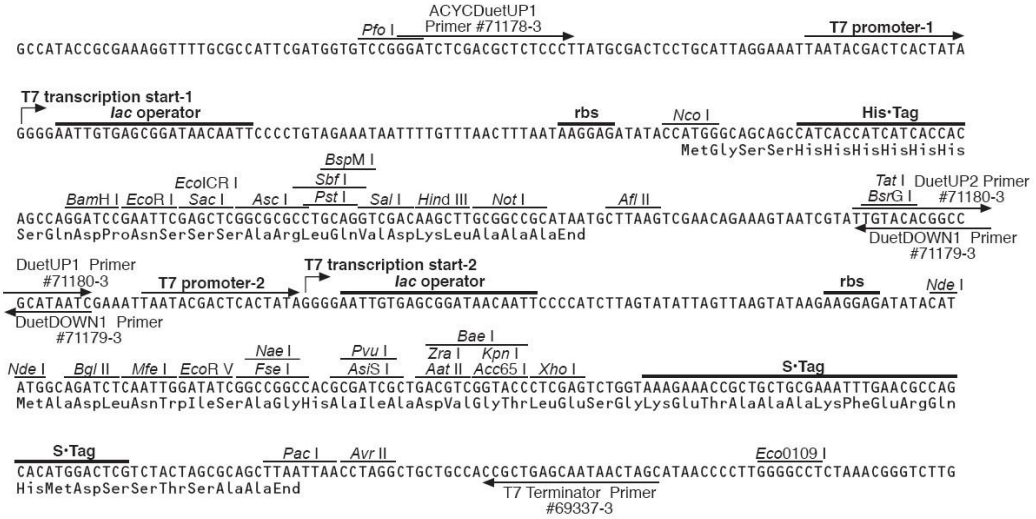
APPENDIX A

Restriction map of pRSFDuet-1

pRSFDuet-1 Vector

	Cat. No.
pRSFDuet-1 DNA	71341-3
pRSFDuet-1 sequence landmarks	
T7 promoter-1	3582–3598
T7 transcription start-1	1
His•Tag® coding sequence	83–100
Multiple cloning sites-1 (*Nco* I–*Afl* II)	69–168
T7 promoter-2	214–230
T7 transcription start-2	231
Multiple cloning sites-2 (*Nde* I–*Avr* II)	297–438
S•Tag™ coding sequence	366–410
T7 terminator	462–509
kan (Kn^R) coding sequence	742–1554
RSF origin	1662–2411
lacI coding sequence	2610–3689



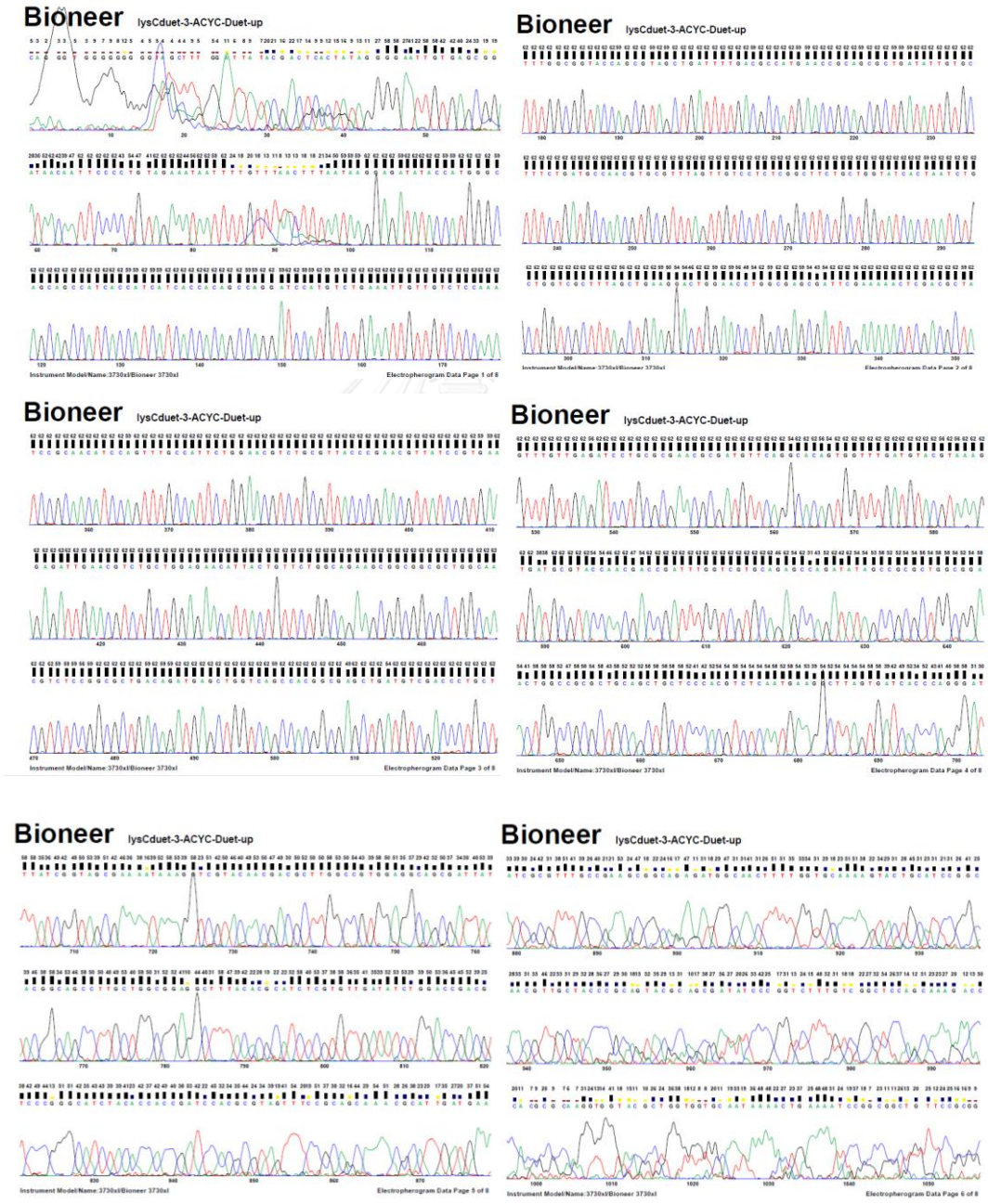


pRSFDuet-1 cloning/expression regions

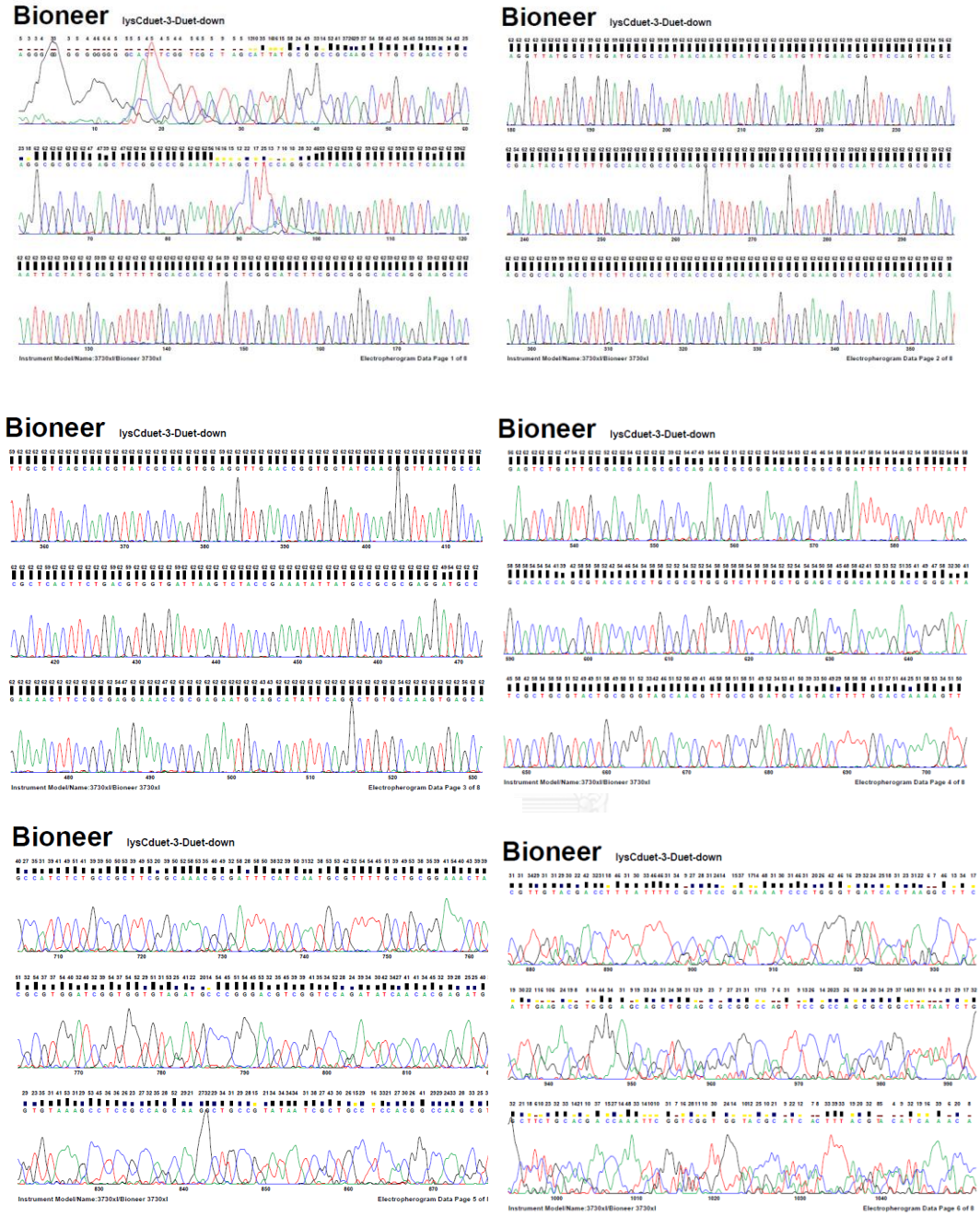
APPENDIX B

The sequencing chromatogram of *lysC* gene from pD-Y using ACYCDuetUP1 primer (A) and DuetDOWN1 primer (B)

A.



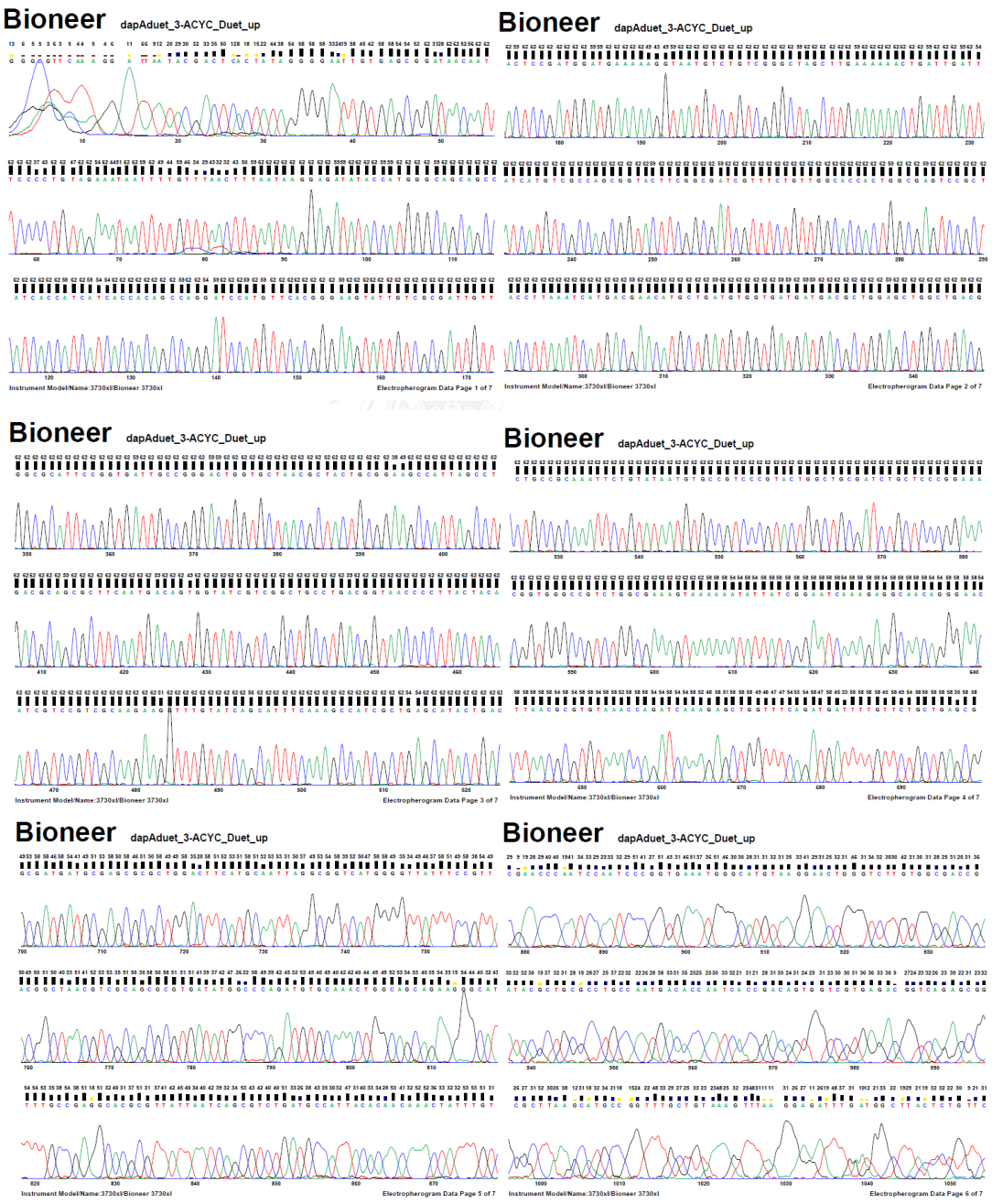
B.


Bioneer
lysCduet-3-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 1 of 8
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 2 of 8
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 3 of 8
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 4 of 8
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 5 of 8
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 6 of 8

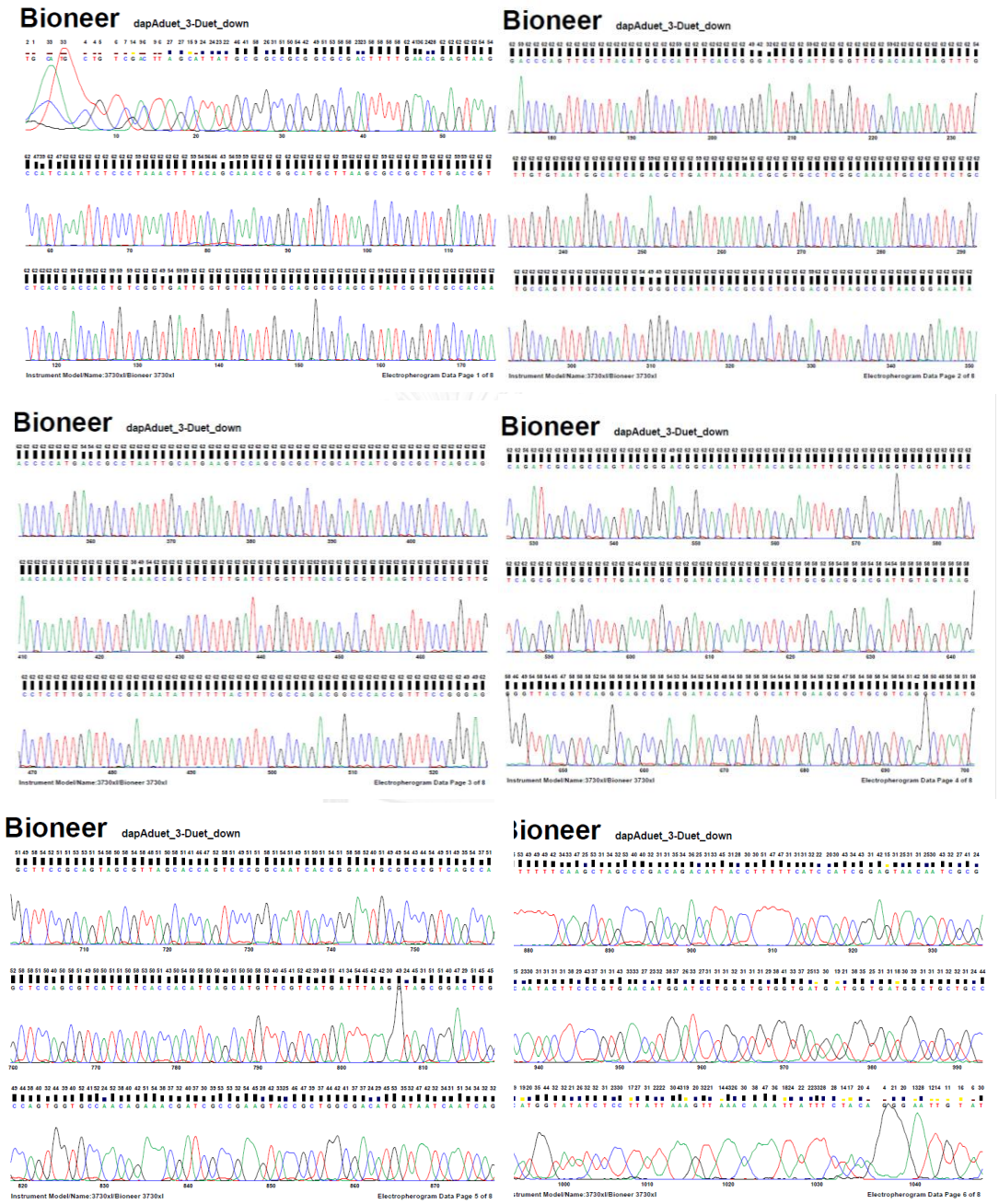
APPENDIX C

The sequencing chromatogram of *dapA* gene from pD-D using ACYCDuetUP1 primer (A) and DuetDOWN1 primer (B)

A.



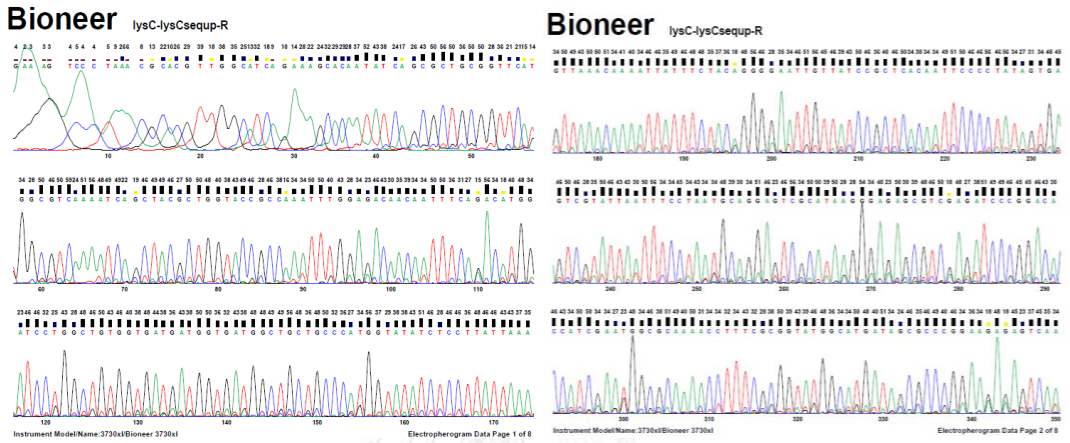
B.


Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 1 of 8
Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 2 of 8
Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 3 of 8
Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 4 of 8
Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 5 of 8
Bioneer dapAduet_3-Duet_down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 6 of 8

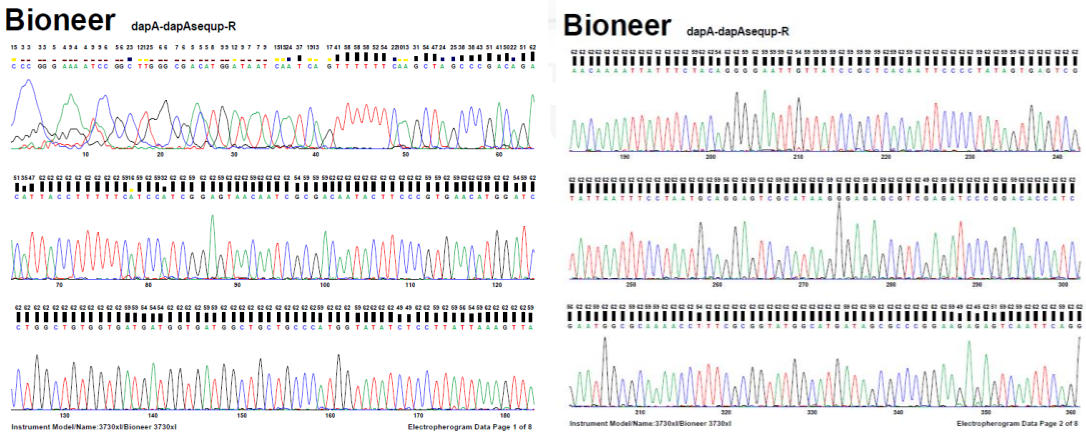
APPENDIX D

The sequencing chromatogram of the T7 promoter and ribosome binding site from pD-Y using lysCsequp-R primer (A) and pD-D using dapAsequp-R primer (B)

A.



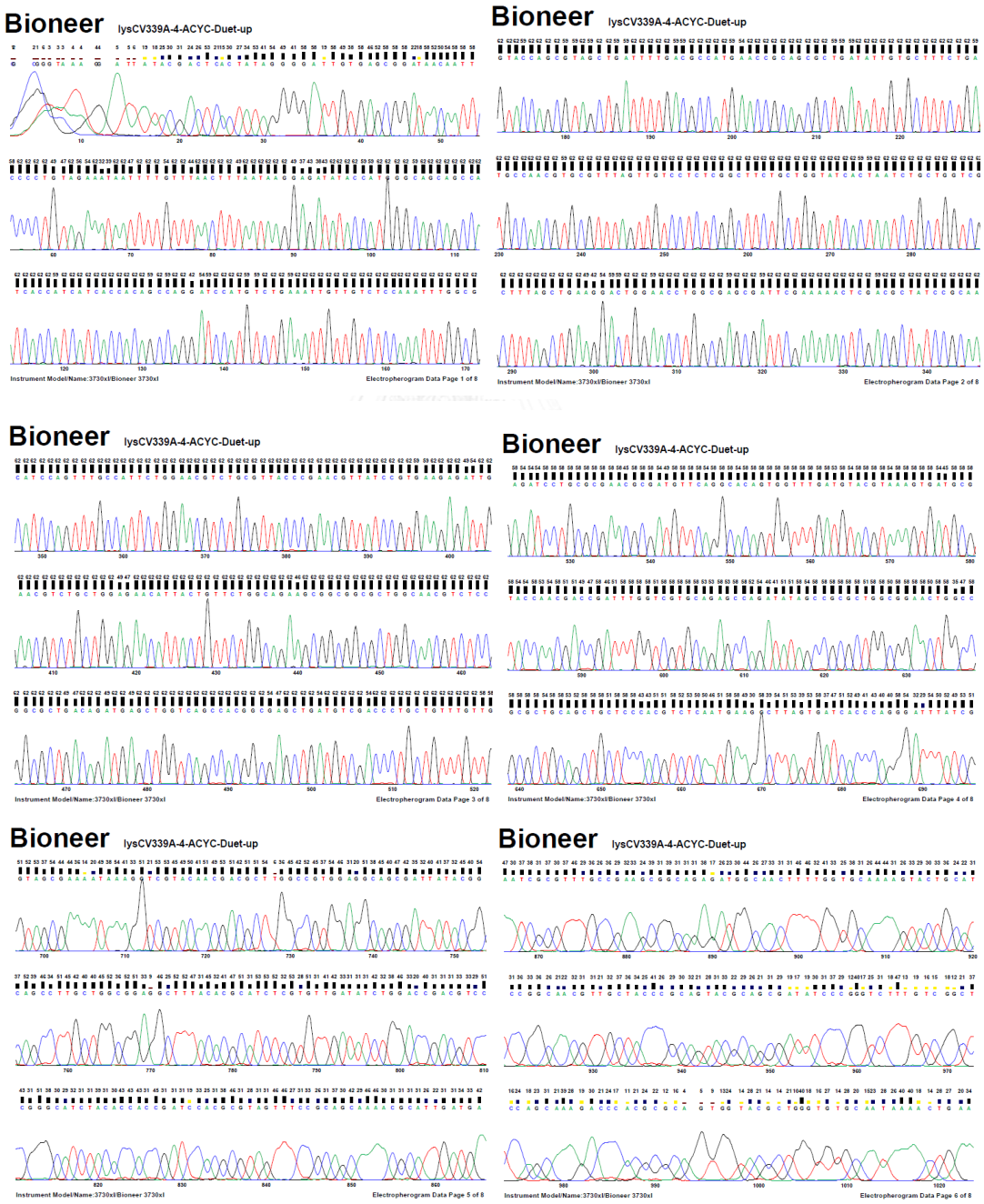
B.



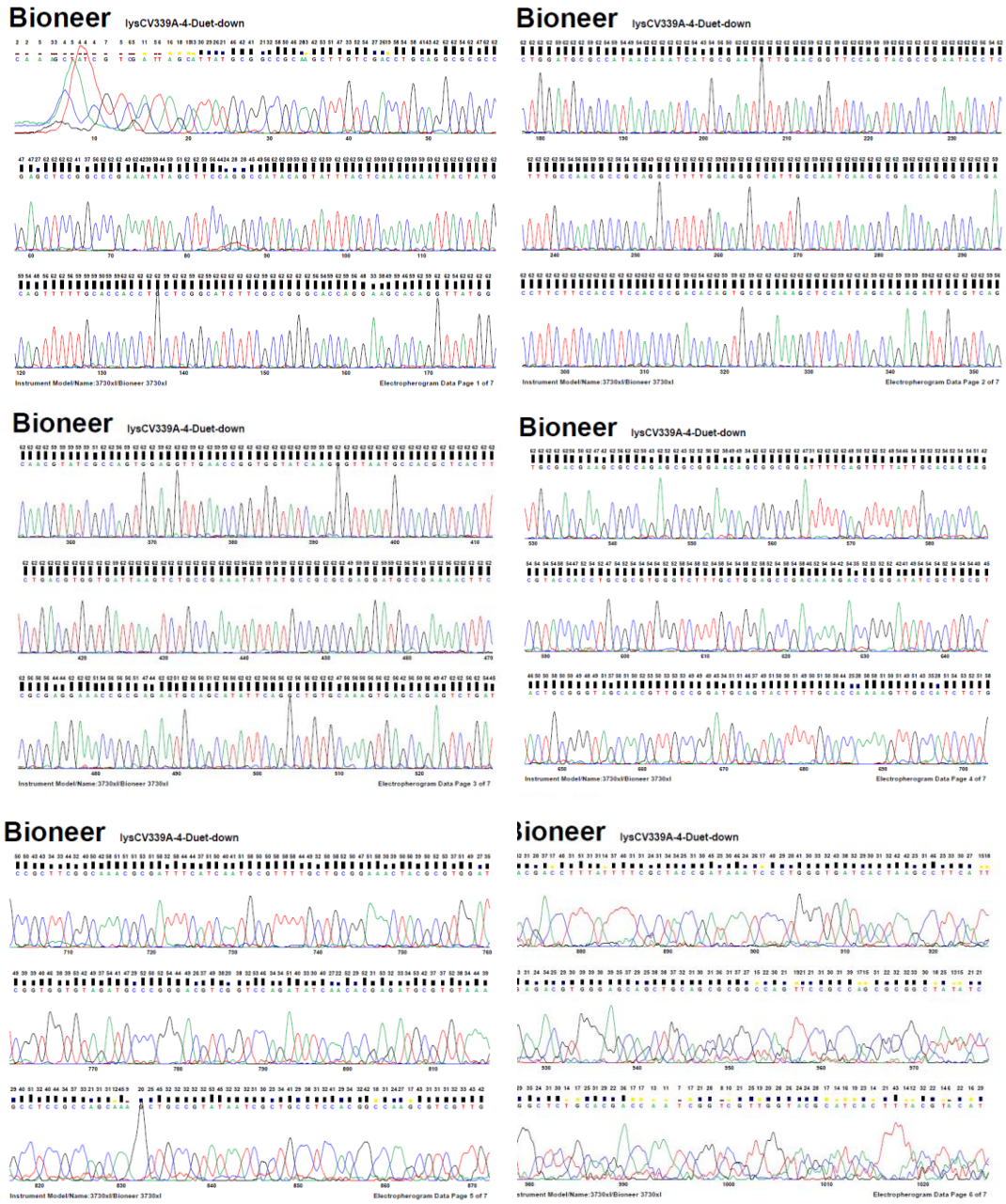
APPENDIX E

The sequencing chromatogram of mutated V339A *lysC* gene from pD-Y* using ACYCDuetUP1 primer (A) and DuetDOWN1 primer (B)

A.



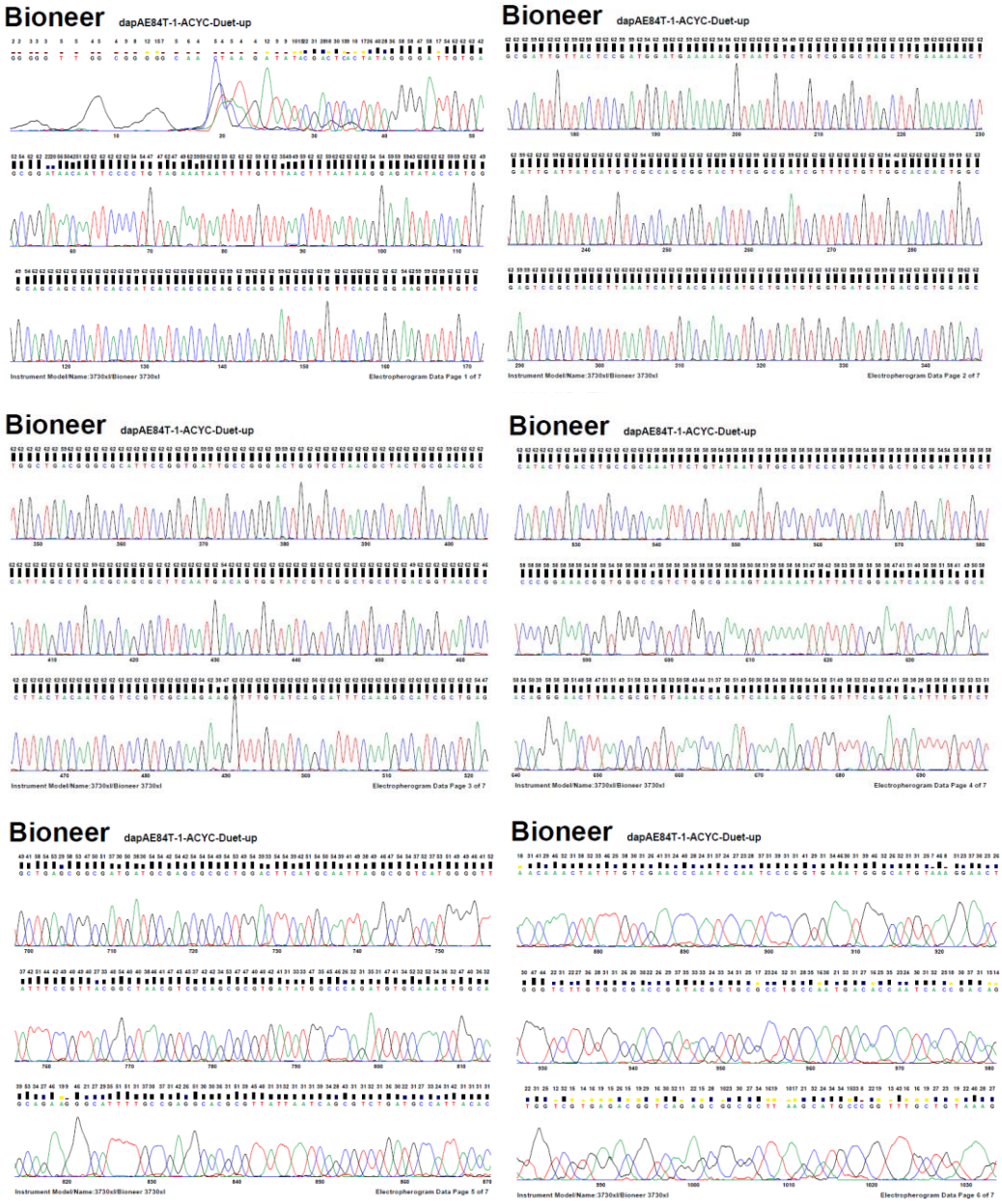
B.


Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 1 of 7
Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 2 of 7
Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 3 of 7
Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 4 of 7
Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 5 of 7
Bioneer lysCV339A-4-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 6 of 7

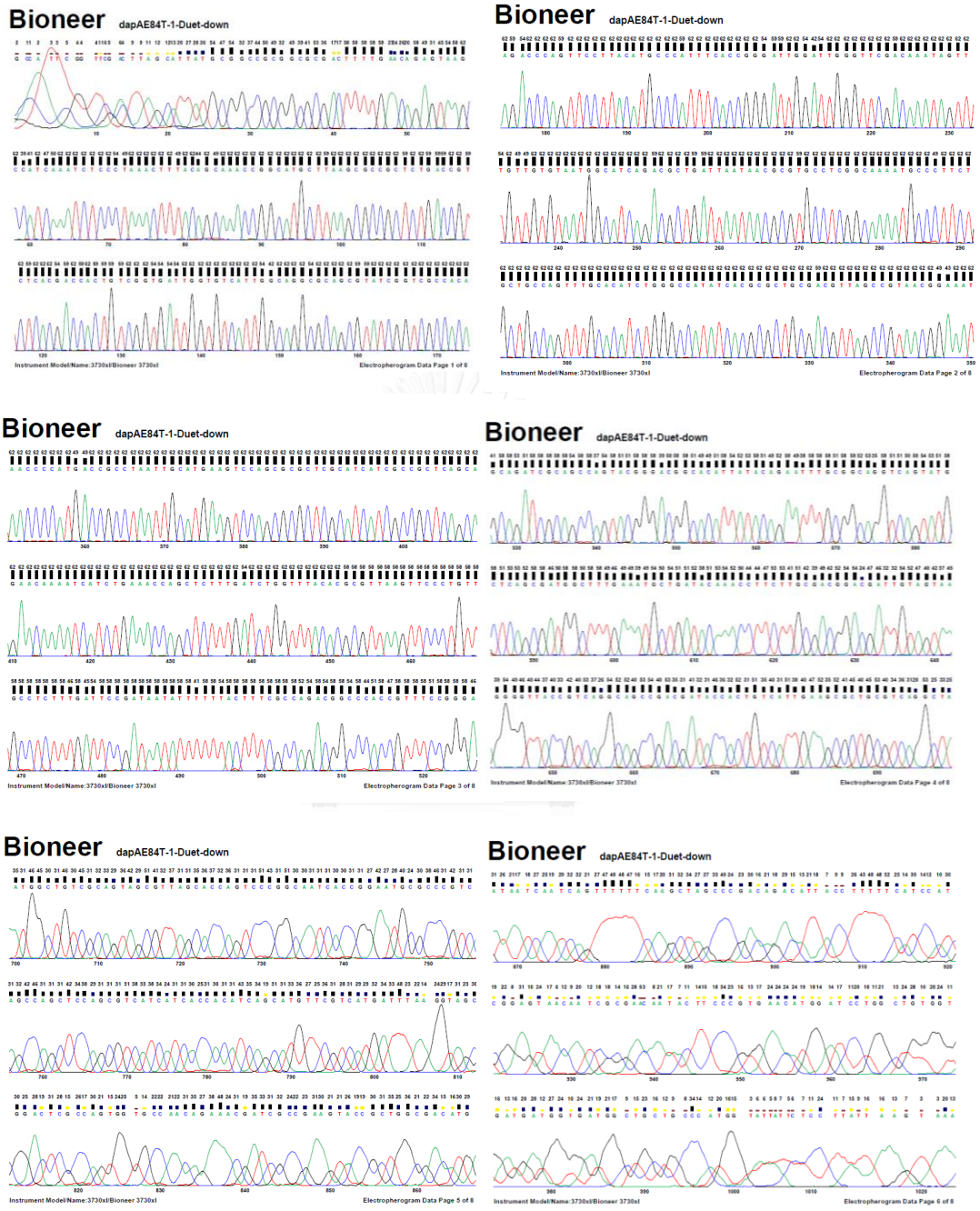
APPENDIX F

The sequencing chromatogram of mutated E84T *dapA* gene from pD-D* using ACYCDuetUP1 primer (A) and DuetDOWN1 primer (B)

A.

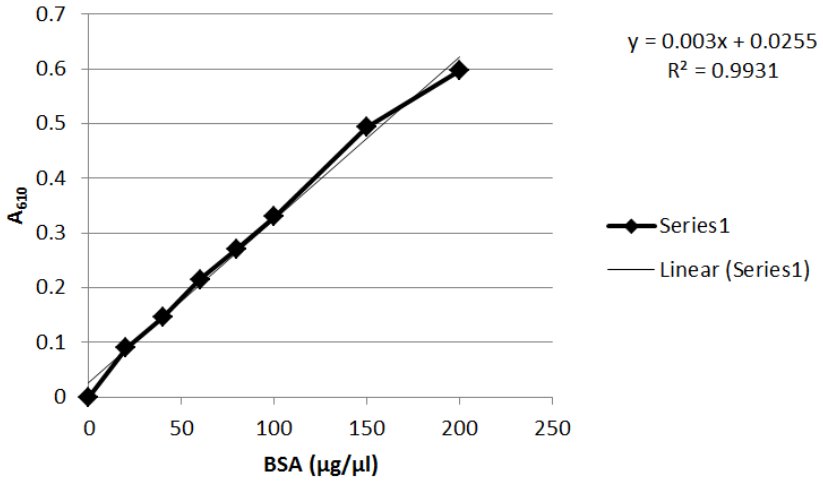


B.


Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 1 of 8
Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 2 of 8
Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 3 of 8
Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 4 of 8
Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 5 of 8
Bioneer
dapAE84T-1-Duet-down
Instrument Model/Name:3730xl/Bioneer 3730xl
Electropherogram Data Page 6 of 8

APPENDIX G

Standard curve for protein determination by Lowry's method



APPENDIX H

Preparation for denaturing polyacrylamide gel electrophoresis

1.Stock solution

2M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1N HCl and adjusted volumn to 100 ml with distilled water

1M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1N HCl and adjusted volumn to 100 ml with distilled water

10% SDS

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volumn of 100 ml

50% Glycerol

100% Glycerol 50 ml

Added distilled water to a total volumn of 100 ml

1% Bromophenol blue

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved. The aggregated dye was removed by filtration.

2. Working solution

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 volumn to 100 ml with distilled water. Filtered and stored in dark (brown bottle) at 4 °C

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

2 M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

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

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% (w/v) Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5.0 ml

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

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

Dissolved and adjusted to total volumn to 1 liter with distilled water (final pH should be approximate 8.3)

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

1M 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
B-mercaptoethanol	0.5 ml
Distilled water	1.4 ml

SDS-PAGE

12.5% Separating gel

Solution A	4.2 ml
Solutionn b	2.5 ml
Distilled water	3.3 ml
10% (w/v) Ammonium persulfate	50 µl
TEMED	5 µl

5% stacking gel

Solution A	0.67 ml
Solutionn b	1.0 ml
Distilled water	2.3 ml
10% (w/v) Ammonium persulfate	30 ul
TEMED	5 µl

3. Protein staining solution

Staining solution, 1 liter

Coomassie brilliant blue R-250	1.0 g
Methanol	450 ml
Distilled water	450 ml

Destaining solution, 1liter

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

APPENDIX I

Personal information

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2011 B.Sc. (Biochemistry), Khonkaen University,Thailand

Previous Research:

Tophangtiam, K., Norasetsingh, T., Pimphumee, J. and Lomthaisong, Khomsorn. 2011. Total carotenoid, total phenolic contents and antioxidant capacity of five local fruits found in northeast region of Thailand. Senior Project. Department of Biochemistry, Faculty of Science, Khonkaen University.

Activity:

2017 Participant and poster presentation, The 13rd Asian Congress on Biotechnology (ACB 2017) Pullman Khon Kaen RaJa Orchid Hotel, Khon Kaen, Thailand.

Poster Presentation:

Norasetsingh, T. and Packdibamrung, K. 2017. Expression of feedback resistant *lysC* gene for L-aminoadipic acid production in *Escherichia coli*. The 13rd Asian Congress on Biotechnology (ACB 2017) Pullman Khon Kaen RaJa Orchid Hotel, Khon Kaen, Thailand.

VITA

Mr. Teerapat Norasetsingh was born on March 16, 1990 in Surin. He graduated with the degree of Bachelor of Science from the Department of Biochemistry, Faculty of Science, Khonkaen University in 2011. He has studied for the degree of Master of Science from Program in Biochemistry and Molecular biology, Faculty of Science, Chulalongkorn University since 2013.