การเพิ่มการผลิตแอล-ฟีนิลอะลานีนโดยการก่อกลายพันธุ์ยีน *pheA* ใน *Escherichia coli*

นายบุรวัชร์ นาคสู่สุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ENHANCEMENT OF L-PHENYLALANINE PRODUCTION BY MUTAGENESIS OF *pheA* GENE IN *Escherichia coli*

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A Thesis Submitted in Partial Fulfillment of the Requirements
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บุรวัชร์ นาคสู่สุข : การเพิ่มการผลิตแอล-ฟีนิลอะลานีนโดยการก่อกลายพันธุ์ยีน *pheA* ใน *Escherichia coli* (ENHANCEMENT OF L-PHENYLALANINE PRODUCTION BY MUTAGENESIS OF *pheA* GENE IN *Escherichia coli*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: กนกทิพย์ ภักดีบำรุง, 84 หน้า.

แอลฟีนิลอะลานีน (L-Phe) เป็นกรดอะมิโนจำเป็น ซึ่งถูกใช้เป็นสารตั้งต้นในการผลิตสารสำคัญในอุตสหกรรมยาและอาหาร ในปัจจุบันความต้องการของ L-Phe เพิ่มมากขึ้นเนื่องมาจากความต้องการที่สูงขึ้นของสารให้ความหวานแอสปาร์แตม การผลิตแอลฟีนิลอะลานีนสามารถทำได้โดยใช้ กระบวนการทางเคมี กระบวนการผลิตโดยเอนไซม์ และ กระบวนการหมักโดยใช้จุลชีพ โดยเฉพาะอย่างยิ่งใน *Escherichia coli* การผลิต L-Phe อยู่ในวิถีการผลิตกรดอะมิโนที่เป็นวงอะโรมาติก ซึ่ง L-Phe ที่ถูกผลิตขึ้นสามารถเกิดการยับยั้งแบบย้อนกลับเอนไซม์ในกระบวนการผลิตได้ โดย chorismate mutase/prephenate dehydratase (CMPD) ซึ่งเข้ารหัสโดยยีน *pheA* เป็นเอนไซม์สำคัญที่ถูกยับยั้งแบบย้อนกลับแบบอัลโลสเตอริกที่บริเวณควบคุมของเอนไซม์ CMPD ประกอบด้วยด้วยบริเวณเร่ง 2 โดเมนสำหรับ CM และ PD ตามลำดับ และบริเวณความคุม 1 โดเมนสำหรับการจับของ L-Phe งานวิจัยนี้มีเป้าหมายที่จะเพิ่มการผลิต L-Phe โดยการกลายพันธุ์ยีน *pheA* เพื่อสร้างยีนที่สามารถต่อต้านการยับยั้งแบบย้อนกลับ (*pheA*^{fbr}) โดยทำการเปลี่ยนกรดอะมิโนในบริเวณควบคุมได้แก่ L300D, M302D, L318D, L344D, L359D และ L359P จากนั้นทำการแสดงออกร่วมของ *pheA*^{fbr} กับยีน *phedh, tktA, glpF, aroB, aroL* และ *yddG* ซึ่งเข้ารหัสให้ phenylalanine dehydrogenase, transketolase, glycerol facilitator, 3-dehydroquinate synthase, shikimate kinase II และ aromatic amino acid exporter ตามลำดับในพลาสมิด pPTFBLYA^{fbr} พบว่าโคลน pPTFBLYA^{L359D} สามารถผลิต L-Phe ในระดับขวดเขย่าขนาด 200 มิลลิลิตรได้สูงสุดที่ 135 มิลลิกรัมต่อลิตรเมื่อใช้ minimal medium ที่มีกลีเซอรอลเป็นแหล่งคาร์บอนโดยสูงกว่าที่ได้จากโคลน pPTFBLYA ถึง 3.78 เท่า เมื่อทำเลี้ยงโคลน pPTFBLYA^{L359D} ในถังหมักขนาด 5 ลิตรแบบกึ่งกะในภาวะควบคุมอุณหภูมิที่ 37 ^{o}ซ อัตราการให้อากาศ 1 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ความเร็วในการกวน 400 รอบต่อนาทีและเติมอาหารหนึ่งครั้งที่ 54 ชั่วโมง พบว่าปริมาณ L-Phe ที่ผลิตได้สูงสุดเท่ากับ 176 มิลลิกรัมต่อลิตร โดยโคลน pPTFBLYA^{L359D} สามารถผลิต L-Phe ในระดับถังหมักได้สูงขึ้นกว่าการผลิตในขวดเขย่า 1.3 เท่า

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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L-Phenylalanine (L-Phe) is an essential amino acid required for dietary meal of human. It is wildly used in chemical synthesis of various pharmaceutical and food industrial products. Nowaday, demand for L-Phe has increased dramatically due to the demand of the low-calorie sweetener aspartame. L-Phe can be synthesized by chemical synthesis, enzymatic synthesis or microbial fermentation. In *Escherichia coli*, the production of L-Phe is under the aromatic amino acid biosynthesis pathway by which accumulation of L-Phe can partially inhibited some enzyme in its own biosynthesis pathway. Bifunctional enzyme chorismate mutase / prephenate dehydratase (CMPD) encoded by *pheA* gene is a key enzyme that is sensitive to feedback inhibition mediated by allosteric binding of L-Phe. CMPD contains two catalytic domains for CM and PD activities as well as one regulation (R) domain for binding with L-Phe. To improve the production of L-Phe by metabolic engineering, feedback-resistant *pheA* (*pheA*^{fbr}) located at L300D, M302D, L318D, L344D, L359D and L359P were obtained by PCR-driven overlap extension mutagenesis technique. Then *pheA*^{fbr} was co-expressed with *phedh, tktA, glpF, aroB, aroL* and *yddG* gene which encoded phenylalanine dehydrogenase, transketolase, glycerol facilitator, 3-dehydroquinate synthase, shikimate kinase II and aromatic amino acid exporter, respectively. Among these mutated clones, pPTFBLYA^{L359D} clone produced the highest concentration L-Phe in 200 mL shake flask culture containing glycerol as a carbon source about 135 mg/L which were calculated as 3.78 times in comparison to the L-Phe content of wild-type (pPTFBLYA). Fed-batch in fermentation a 5 L stirred-tank fermenter (37 ^{o}C, an aeration rate of 1 vvm, an agitation speed of 400 rpm) was with L-Phe production was carried out using pPTFBLYA^{L359D} clone with addition of new medium at 54 h, the yield of L-Phe production of 176 mg/L was obtained. The maximum L-Phe production of pPTFBLYA^{L359D} clone in the fermenter was 1.3-fold higher than that in shake flask.

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A	absorbance, 2'-deoxyadenosine (in a DNA sequence)
AroB	3-dehydroquinate synthase
aroB	3-dehydroquinate synthase gene
AroL	shikimate kinase II
aroL	shikimate kinase II gene
AroF	L-tyrosine-feedback inhibited DAHP synthase
aroF	L-tyrosine-feedback inhibited DAHP synthase gene
aroF^{fr}	L-tryptophan-feedback resistant DAHP synthase gene
AroG	L-phenylalanine-feedback inhibited DAHP synthase
aroG	L-phenylalanine-feedback inhibited DAHP synthase gene
aroG^{fbr}	L-phenylalanine-feedback resistant DAHP synthase gene
AroH	L-tryptophan-feedback inhibited DAHP synthase
aroH	L-tryptophan-feedback inhibited DAHP synthase gene
bp	base pairs
C	2'-deoxycytidine (in a DNA sequence)
^{o} C	degree Celsius
CMPD	chorismate mutase/ prephenate dehydratase
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EC	Enzyme Commission
EDTA	ethylene diamine tetraacetic acid
G	2'-deoxyguanosine (in a DNA sequence)
GlpF	glycerol facilitator

glpF	glycerol facilitator gene
GlpFK	glycerol facilitator and glycerol kinase
glpFK	glycerol facilitator and glycerol kinase genes
GlpK	glycerol kinase
glpK	glycerol kinase gene
HPLC	high-performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactoside
Kb	kilobase pairs in duplex nucleic acid, kilobases in single-stranded nucleic acid
*K*m	Michaelis constant
KOH	potassium hydroxide
L	liter
L-phenylalanine	L-Phe
L-tryptophan	L-Trp
L-tyrosine	L-Tyr
LB	Luria-Bertani
µg	microgram
µL	microliter
µM	micromolar
M	mole per liter (molar)
mg	milligram
min	minute
mL	milliliter
mM	millimolar

MW	molecular weight
N	normal
ng	nanogram
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
pheA	chorismate mutase/ prephenate dehydratase gene
pheA^{fbr}	feedback resistant chorismate mutase/ prephenate dehydratase
PheDH	phenylalanine dehydrogenase
Phedh	phenylalanine dehydrogenase gene
RNase	ribonuclease
T	2'-deoxythymidine (in a DNA sequence)
TE	Tris-EDTA buffer
TktA	transketolase
tktA	transketolase gene
*T*m	melting temperature, melting point
UV	ultraviolet
v/v	volume by volume
vvm	volumes of air per volume of liquid per minute
w/w	weight by weight
YddG	aromatic amino acid exporter
yddG	aromatic amino acid exporter gene

CHAPTER I

INTRODUCTION

1.1 The aromatic amino acids: L-phenylalanine

Aromatic amino acid is classified based upon a structure of aromatic ring existed on a side chain group. The three aromatic amino acids mainly found in protein are L-tryptophan (L-Trp), L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr). Generally, these three aromatic amino acids constitute less than 10% on the protein chain (Sprenger, 2007). The common property of the aromatic core is that it can absorb UV light under maximum excitation of about 260 nm for L-Phe or 280 nm for L-Tyr and L-Trp. Thus, the determination of protein concentrations by spectrophotometric measurement at wavelength 280 nm (A_{280}) is derived from that (Layne, 1957).

L-Phenylalanine (L-Phe, $C_9H_{11}NO_2$, M.W. of 165.19; 2-amino-3-phenylpropanoic acid) is an amino acid that exhibits a benzyl group as a side chain; therefore, it is classified as a neutral and nonpolar type because of the inert and hydrophobic nature of its side chain. Notably, L-Phe is an essential amino acid. It cannot be biochemically synthesized, except intake from dietary meal (*i.e.* meat, fish, milk, cheese and eggs). Apart from being a component of protein structure, L-Phe serves a function of a precursor for the synthesis of other amino acids and various compounds. It is a precursor for biosynthesis of L-Tyr, monoamine neurotransmitters including dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline), and the skin pigment melanin. L-Phenylalanine is converted into L-Tyr which also known as another DNA-encoded amino acid. L-Tyr is straightforwardly converted into L-DOPA and thereafter converted into dopamine, norepinephrine (noradrenaline),

and epinephrine (adrenaline) as shown in Figure 1. Besides, L-Phe can also be a precursor for the synthesis of a neuromodulator dietary 'phenethylamine'. As a monoamine transmitter, L-Phe is of vital importance so that it helps maintain brain's activity (e.g. control cooperation and motion) and extends to regulate the flow through passage way between different areas of the brain (Daniel, Moorhouse, & Pratt, 1976; Fernstrom & Fernstrom, 2007). Some people are born with lacking an enzyme that regulates the catabolism of L-Phe. This will lead to an accumulation of L-Phe, or phenylpyruvic acid, storing up in nervous tissue and subsequently causes severe mental retardation that is known as phenylketonuria, or PKU.

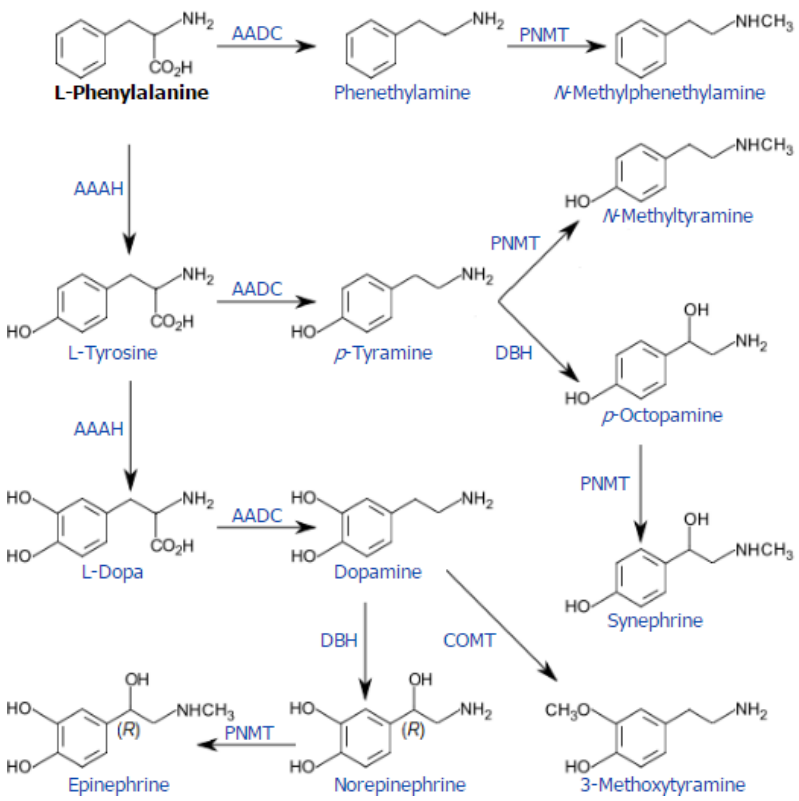

Figure 1 Synthetic and metabolic pathways for endogenous and exogenously administered trace amines and sympathomimetic amines. AAAH, aromatic amino acid hydroxylase; AADC, L-aromatic amino acid decarboxylase; ADH, aldehyde

dehydrogenase; COMT, Catechol O-methytransferase; DBH, dopamine-β-hydroxylase; L-DOPA, L-dihydroxyphenylalanine; MAO, monoamine oxidase; NMT, N-methyl transferase; PNMT, phenylethanolamine N-methyltransferase. *(Broadley,* 2010)

1.2 Medical and industrials uses of L-Phe

For medical use, L-Phe is recognized that L-Phe determines a role in relaxation of neurotransmitter transportation, relating to antagonize of glycine and glutamate within brain's receptors (Glushakov, Dennis, Sumners, Seubert, & Martynyuk, 2003). L-Phe product extends the market availability, in which it is now available in commonplace, as analgesic and antidepressant (Mitchell, 2008). Moreover, the combination of L-Phe and selegiline, a monoamine oxidase inhibitor used for Parkinson's disease patient treatment, strengthens the drug ability. It is noted that approximately 90% of outpatients and 81% of inpatients who took the combination of about 5 to 10 mg/day of seligiline and 250 mg/day of L-Phe are achieved in lowering depression.

Other applications are about food supplements and additives. Aspartame (a low calorie sweetener, LCS with 200 times sweeter than sucrose; L-aspartyl-L-phenylalanine methyl ester) is known as the most important compound harboring L-Phe. The demand of L-Phe is drastically increased because of increasing in the demand for aspartame. Demand is projected to grow 5.1% per year from 2008 to 2015. An estimated world market of aspartame is expected to reach $2.2 billion in 2020 (Sylvetsky & Rother, 2016). As mentioned earlier, aspartame can be found in several diets; for instance, soft drinks, gelatins, yogurt, gum, dessert or even sugar-free candy.

Besides sweetener, L-Phe also serves as an aromatic compound participating in flavor improvement (Smit, 2004).

1.3 Methods for L-Phe production

There are several methods used for the production of L-Phe e.g. chemical synthesis, enzymatic synthesis, microbial production and fermentative process. L-Phe is firstly produced by chemical method but the yields do not reach the standard level for scale-up production. During the past decades, enzymatic and fermentative approaches are being enabled to L-Phe production. By using enzymatic process, phenylalanine ammonia lyase (PAL), aromatic amino acid aminotransferase (AAT) and phenylalanine dehydrogenase (PheDH) have been reported to catalyze a conversion of their substrates into L-Phe (Chao *et al*., 1999 and Hummel *et al*., 1986). Microbial production in small scale of *E. coli* achieves in either biotransformation of substrates, phenylpyruvate and aspartate with the elevated level of related enzymes amino transferase and phosphoenolpyruvate (PEP) carboxykinase. For higher scale production, the method of use is a microbial fermentation performing in a large scale fermenter (Sprenger, 2007). Detail of process on the L-Phe production will be discussed in section 1.6.

1.4 Shikimate pathway: Pathway of aromatic amino acid synthesis

Shikimate pathway, a common route mediating to the production of three aromatic amino acids phenylalanine, tyrosine, and tryptophan, constitutes a part of any metabolisms that are found commonly in myriad microorganisms and plants, but not for animals. This may due to the fact that L-Trp and L-Phe are essential amino acids for human and animals (Sprenger, 2007).

Even though this pathway is named for shikimate, the end product of this pathway is chorismate serving as genuine precursors for those three aromatic amino acids and several other aromatic compounds of primary metabolism. The three aromatic amino acids are precursors to a large variety of plant secondary metabolites as well. Vast intermediates of the main route of the shikimate pathway also serve as starting materials for other biosyntheses of secondary products. The shikimate pathway is therefore of prominent importance regarding to the production of many compounds of commercial interest.

An aromatic amino acid biosynthesis pathway in *E. coli* is illustrated in Figure 2. Initially, the pathway is initiated by the condensation reaction of phosphoenolpyruvate (PEP) from the glycolysis pathway and erythrose 4-phosphate (E4P) from the pentose phosphate pathway to yield 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), catalyzed by three DAHP synthases. These three isoenzymes are encoded by the genes *aroF*, *aroG*, and *aroH*. The three enzymes (AroF, AroG and AroH) are responsible for the overall activity of DAHP synthase in the ratio of 80% from AroG, 15% from AroF and 5% from AroH. They are individually susceptible to feedback inhibition by aromatic amino acids L-Tyr, L-Phe, and L-Trp, respectively. Notably, about 80% of the total DAHP synthase activity can be inhibited by L-Phe

towards DAHP synthase (AroG). AroG is reported to display the highest resistance against specific proteolysis among other DAHP synthases (Bongaerts, Kramer, Muller, Raeven, & Wubbolts, 2001). In the second step, DHQ synthase (encoded by the *aroB* gene) catalyzes a conversion of DAHP to 3-dehydroquinate (DHQ). DHQ dehydratase (encoded by *aroD* gene) subsequently removes the water molecule from DHQ, forming 3-dehydroshikimate (DHS). DHS is then reduced to shikimate (SHIK) by the action of of shikimate dehydrogenase (encoded by *aroE* gene) in these following steps. SHIK is first phosphorylated to produce shikimate 3-phosphate (S3P) by shikimate kinase I and II (encoded by *aroK* and *aroL* genes, respectively). Shikimate kinase II shows higher affinity to its substrate because the K_m value of this enzyme for SHIK is about 100-fold lower from that of the K_m value of shikimate kinase I (DeFeyter & Pittard, 1986). Once the second PEP molecule enters the aromatic amino acid pathway, a condensation of PEP with S3P is subsequently triggered by 5-enolpyruvyl shikimate 3-phosphate (EPSP) synthase (encoded by *aroD*) to yield EPSP.

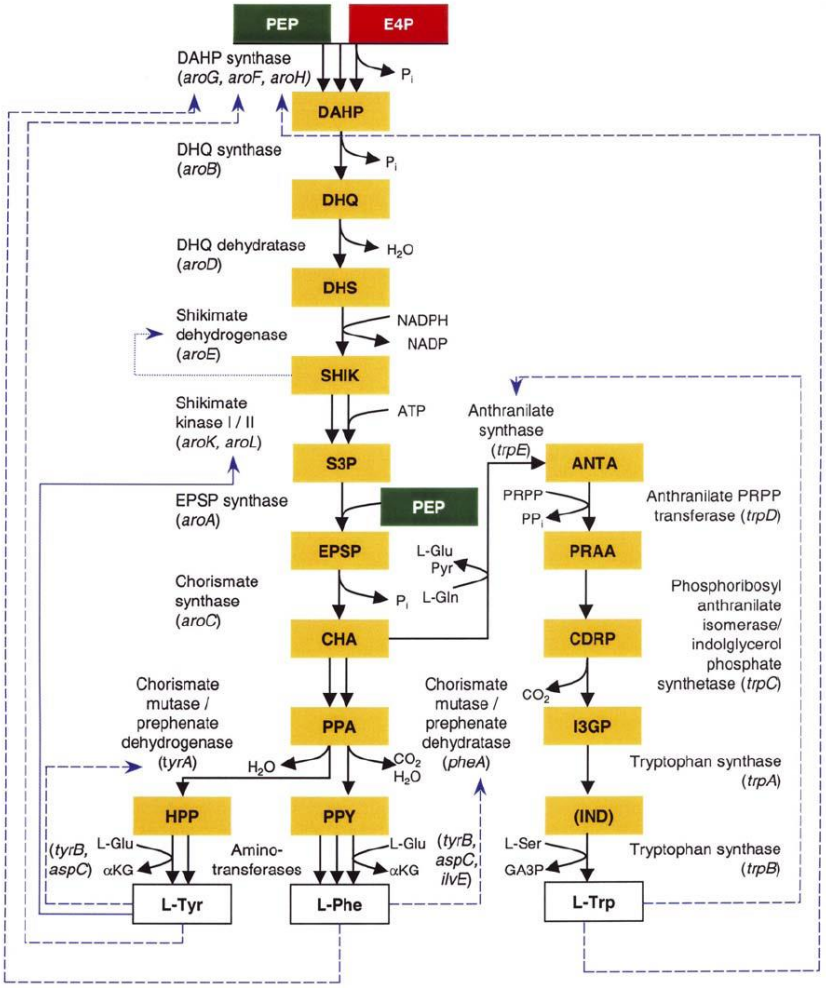

Figure 2 Overview of aromatic amino acid biosynthesis pathway and its regulation in E. coli. Each type of lines represents: – – –, allosteric control by the aromatic amino acid end products; —, transcriptional control. Abbreviations used: ANTA, anthranilate; αKG, α-ketoglutarate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate; CHA, chorismate; DAHP, 3-deoxy-D-arobino-heptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; EPSP, 5 enolpyruvoylshikimate 3-phosphate; E4P, erythrose 4-phosphate; HPP,4-hydroxyphenlypyruvate, I3GP,

indole 3-glycerolphosphate; IND, indole; L-Gln, L-glutamine; L-Glu, L-glutamate; L-Phe, L-phenylalanine; L-Ser, L-serine; L-Trp, L-tryptophan; L-Tyr, L-tyrosine; PEP, phosphoenolpyruvate; PPA, prephenate; PPY, phenylpyruvate; PRAA, phosphoribosyl anthranilate; PRPP, 5-phosphoribosyl-α-pyrophosphate; Pyr, pyruvate; SHIK, shikimate; S3P, shikimate 3-phosphate (Source: Bongaerts et al., 2001)

Finally, removal of phosphate on EPSP is done by chorismate synthase (encoded by *aroC* gene) in order to yield chorismate (CHA). As analyzed by intermediate metabolite accumulation method, the rate-limiting enzymes for an aromatic amino acid pathway of *E. coli* are successfully identified: DHQ synthase (encoded by *aroB*) and shikimate kinase (encoded by *aroL* or *aroK*) (Dell & Frost, 1993). The end product CHA is subjected to distinct pathways aiming to produce those relevant aromatic amino acids (L-Phe or L-Tyr or L-Trp). Further explanation on L-Phe biosynthesis pathway will be described in the next section.

1.5 L-Phe biosynthesis pathway

The steps in L-Phe production can be followed as shown in Figure 2. The biosynthesis pathway of L-Phe or L-Tyr is branched at prephenate (PPA) by the activity of the bifunctional enzymes chorismate mutase/prephenate dehydratase for L-Phe (encoded by *pheA*) and chorismate mutase/prephenate dehydrogenase for L-Tyr (encoded by *tyrA*). In the pathway of L-Phe biosynthesis, PheA catalyzes a conversion of chorismate to phenylpyruvate (PPY) through prephenate (PPA) intermediate. It is suggested that this is the key step in determining the L-Phe production in which the activity of PheA can also be inhibited by L-Phe itself (Hudson & Davidson, 1984). Of

about 90% and 55% of prephenate dehydratase and chorismate mutase activity are feedback-inhibited by L-Phe, respectively. To increase the yield of L-Phe contents, the construction of feedback-resistant mutant for AroG and PheA were reported in the aim to be used in biotechnology for the L-Phe production instead of using the wild-type (WT). Eventually, the transamination reaction onto the phenylpyruvate (α-keto acids) via amino donor glutamate is done in order to yield L-Phe. This reaction is catalyzed by three aminotransferases encoded by *tyrB, aspC* and *ilvE* genes. They differ in terms of catalytic mechanisms. The aminotransferase encoded by *tyrB* is the pacemaker enzyme for L-Phe (and L-Tyr) biosynthesis from PPY (and 4-hydroxyphenylpyruvate (HPP)) under normal conditions. When the metabolic pools of phenylpyruvate and 4-hydroxyphenylpyruvate pools are highly accumulated, the aspartate aminotransferase, encoded by *aspC,* regulates to the biosynthesis of these amino acids in charge. The third one, isoleucine aminotransferase encoded by *ilvE,* normally catalyzes the synthesis of L-isoleucine, L-valine, and L-leucine. It is also found that isoleucine aminotransferase switches its role to perform the synthesis of L-Phe, but not L-Tyr under the *aspC* and *tyrB* deletion mutants (Bongaerts et al., 2001; Kramer et al., 2003; Muller et al., 2006; Yakandawala, Romeo, Friesen, & Madhyastha, 2008). Three mechanisms of regulation can be determined during the L-Phe production as follows: feedback inhibition towards the key enzyme, repression at the transcriptional level and attenuation at the transcriptional/translational interface. The first regulating step relates to DAHP synthase and chorismate mutase/prephenate dehydratase (PheA) that are dictated by feedback inhibition by its own end product, L-Phe. The intracellular level of the three DAHP synthases in *E. coli* is maintained by transcriptional repression through the repressors TyrR and TrpR. Deletion of the *tyrR* and *trpR* genes alleviates the

transcriptional regulation. Attenuation is a regulatory element in the biosynthesis of L-Phe where *pheL* gene encoding a leader region is found upstream of *pheA* (Sprenger, 2007).

1.6 Improvement of L-Phe production via metabolic engineering

Aromatic amino acid are a group where production efficiency by fermentation remain relatively low. The current production yields towards sugar (wt%) are 20-30 for L-Trp and 25 For L-Phe while for higher yield have been reported for other amino acid, e.g., L-Lys 40-50, L-Glu 45-55 and L-Ala 45-55 (Leuchtenberger, Huthmacher, & Drauz, 2005). Classical mutagenesis was previous attempts at strain improvement for amino acid production strain by deletion of competing pathways and elimination of feedback regulation. However, by classical approach, complete deregulation and enhancement of an appropriate biosynthetic enzyme activity are difficult to achieve. Therefore, recombinant DNA techniques have been introduced for strain improvement (Ikeda, 2006). This strategy provided the improvement of production via the amplification of possible rate-limiting enzyme(s) and/or the first enzyme in the common pathway. *E. coli*, *C. glutamicum* and *Brevibacterium* strains, rational designed by metabolic engineering are commonly used for the production of L-Phe (de Boer & Dijkhuizen, 1990). Sugimoto and coworkers used a temperature controllable expression vector to conditionally express the deregulated $aroF^{fbr}$ and $pheA^{fbr}$ genes in *E. coli* and obtained a maximal L-Phe titer of 16.8 g/L at the optimal temperature of 38.5 °C (Sugimoto et al., 1987). This process was further developed leading to the enhancement of titer up to 46 g/L of L-Phe (Konstantinov, Nishio, Seki, & Yoshida, 1991). Backman and coworkers also engineered *E. coli* for L-Phe production with feedback-resistant

PheA (*pheA*^{fbr}) and feedback-resistant AroF (*aroF*^{fbr}) genes, with which a titer of 50 g/L L-Phe with a yield of 0.25 (g L-Phe/g glucose) was obtained after 36 h (Backman, 1990).

Central metabolism was also modified for improving L-Phe production. For example, PEP carboxylase (*ppc*) disruption provided other effective way for L-Phe production in *E. coli*, even though such unwanted by-products, such as acetate and pyruvate, were obtained (Miller, Backman, O'Connor, & Hatch, 1987). Tatarko and Romeo reported an approach to regulate the global network of central carbon metabolism for improvement of L-Phe production. After the *csrA* gene, one of the global regulatory networks was impaired, the alteration of metabolic flux tended to increase for gluconeogenesis, but downwards for glycolysis. This resulted in an elevation of the PEP intermediates; thus, the L-Phe product was 2-fold increase (Tatarko & Romeo, 2001). Moreover, the construction of strains for L-Phe production can be approached through a precise chromosomal deletion. The gene cluster *pheA-aroF-tyrA*, in conjunction with was the TyrR repressor site and *pheL* attenuator region, were removed and then served a function of host (named F4 strain) for L-Phe production. In order to enhance L-Phe production, two genes (*aroF*^{fbr} and *pheA*^{fbr}) were introduced onto the vector under control of a *lacI*q/Ptacl promoter, and this plasmid was named pJF119EH (Furste et al., 1986). The trial experiment under 20-L fermenter (initial volume of 7.5 L; 37 °C) revealed that shikimic acid and 3-dehydroshikimic acid were obtained as by-products (Gerigk et al., 2002). This could be stated as a hindrance of metabolic flux through the aromatic pathway by the insufficient activity of the shikimate kinases. The *aroL* gene (encoding shikimate kinase) was therefore cloned as the third gene under the same promoter, namely plasmidpF46 (*aroF*^{fbr}-*pheA*^{fbr}-*aroL*). It

was found that the reduction of shikimic acid and 3-dehydroshikimic acid was observed but L-Phe yields were not improved at all. This might be due to pointing to still other limitations in the pathway. Later, when DAHP and its dephosphorylated derivative DAH were detected in the culture, *aroB* gene (coding for dehydroquinate synthase) was provided onto the gene construct, aiming to reduce the formation of DAHP and DAH as well (Ruffer et al., 2004). To sum up, the significantly lowered formation of by-products did not get along with the increment of L-Phe contents (Sprenger, 2007).

The new method was done by using expression plasmid carrying the wild-type *aroF* (*aroF*^{wt}) instead of that feedback-resistant enzyme (AroF^{fbr}), namely plasmid pF69 (*aroF*^{wt}-*pheA*^{fbr}-*aroL*). In fed-batch fermentation under controlling of L-Tyr, wild-type *aroF* (*aroF*^{wt}) successfully enhanced the L-Phe content (34 g/L) than that of *aroF*^{fbr} strain (28 g/L) (Gerigk et al., 2002). When the *aroB* gene was also combined (plasmid pF81: *aroF*^{wt}-*pheA*^{fbr}-*aroL*-*aroB*), a higher L-Phe titer (up to 38 g/L) could be obtained with no by-products from the aromatic biosynthesis pathways and, in particular, low acetate formation during 50 h time course production (Ruffer et al., 2004). To investigate whether the increase of aromatic amino acid exporter affected the aromatic amino acid production, *E. coli* strain DV1060 with overexpressing *yddG* gene was constructed by Doroshenko and coworkers (Doroshenko et al., 2007). Notably, the accumulation of L-Tyr or L-Phe in the media was increased by 3-fold, and 1.5-fold, respectively.

Figure 3 illustrates an example of L-Phe production in *E. coli* via metabolic engineering (Bang, Lee, Kim, Sung, & Jeong, 2016). To obtain the accumulated L-Phe pool, *E. coli* W3110 was rational designed based upon available metabolic and regulatory information as follows:

(i) deletion of the *crr* gene that encodes EIIA^{Glc} protein in order to moderate the substrate uptake rate and decrease the metabolite overflow.

(ii) deletion of the *tyrR* gene to attenuate the strict regulation of the TyrR regulon that contains aromatic amino acid (AAA) synthesis genes.

(iii) deletion of the *trpE* (anthranilate synthase component) and *tyrA* (chorismate mutase/prephenate dehydrogenase) genes to prevent the occurrence of competing pathways, and

(iv) deletion of the *pykA* (pyruvate kinase A) gene to enrich the precursor and balance the flux between growth and L-phenylalanine production.

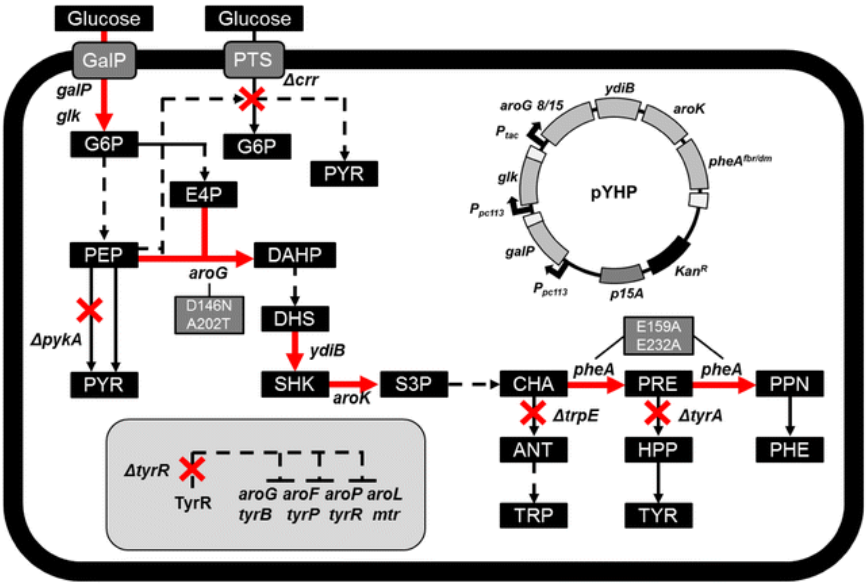

Figure 3 Schematic representation of train engineering to increase the pool of L-phenylalanine in *E. coli* W3110. The symbol "*X*" indicates the corresponding gene deletion. The *red-color arrows* indicate overexpression of the relevant genes (*galP, glk, aroG, ydiB, aroK, pheA*), (Bang et al., 2016).

1.7 An introduction to feedback-resistant *pheA* gene by mutagenesis

Once talking about feedback resistance of *pheA* gene, it is focusing on the modification of the bifunctional enzyme chorismate mutase / prephenate dehydratase (PheA). PheA encoded by *pheA* gene is known as the second rate-limiting step in the L-Phe biosynthesis which can convert chorismate into phenylpyruvate. PheA contains 386 amino acids that can be separated into two catalytic domains (CM and PDT) and one regulatory (R) domain contains. The CM domain is located at residues 1 to 109 while residues 101 to 285 constitute the PDT domain. The R domain involved in L-Phe binding and feedback inhibition mediated by allosteric binding of L-Phe encompasses residues at 286 to 386 (Zhang et al., 1998). The PDT activities of the PheA are almost inhibited under L-Phe concentration of 1mM (Nelms, Edwards, Warwick, & Fotheringham, 1992). To deal with this feedback inhibition, many feedback-resistant CM–PDT are generated and characterized; for example, by modifying Trp226 and Trp338 (Gething & Davidson, 1978). The recombinant *E. coli* carrying $pheA^{fbr}$ gene identified as a substitution of Thr residue 326 into Pro on PheA exhibited strong resistance to feedback inhibition of CM–PDT up to 200 mM of L-Phe (Nelms et al., 1992).

1.8 Use of glycerol as carbon source

Glycerol is byproduct (about 10% w/v) of biodiesel production which is increasing as demand of biodiesel. It was projected that the world biodiesel market will reach 37 billion gallons by 2016, which suggested that approximately 4 billion gallons of crude glycerol would be produced (Yang, Hanna, & Sun, 2012). Biodiesel is selected to be the first alternative energy which can be used as a fuel in diesel engines. Biodiesel

is the abundant resources from vegetable oils or animal fats via transesterification process. Although high purity glycerol is used as an important feed stock in food, pharmaceutical, cosmetic and many other industries (Wang, Zhuge, Fang, & Prior, 2001) but crude glycerol, which is derived from the transesterification reaction of fat and vegetable oils (triglycerides) to produce biodiesel, contains methanol, salts, soaps and water as the main contaminants could not be used. Consequently, finding alternative ways to apply raw glycerol from biodiesel production by using as carbon- and energy-source in microorganisms like *E. coli*. Aerobic glycerol metabolism in *E. coli* is shown in Figure 4. The early step of glycerol utilization is the uptake of glycerol molecule into the by glycerol facilitator (GlpF) encoded by the *glpF* gene. Then Glycerol is trapped by an ATP-dependent glycerol kinase (GlpK) to glycerol- 3-phosphate (G3P) which is then oxidized by a membrane-bound ubiquinone-8 (UQ8)-dependent G3P dehydrogenase (GlpD) to dihydroxyacetone phosphate (DHAP) that enters glycolysis (Gottlieb, Albermann, & Sprenger, 2014). Both DHAP and G3P intermediates are further metabolized in glycolysis to supply phosphoenol pyruvate, an initial precursor, for the common aromatic amino acid biosynthetic pathway. Moreover, DHAP and G3P are metabolized via gluconeogenesis to form fructose-1,6-bisphosphate (F1,6BP) which is dephosphorylated by fructose-1,6-bisphosphatase to yield fructose 6-phosphate (F6P). F6P can be shunted from glycolysis to the non-oxidative branch of the pentose phosphate pathway to supply erythrose 4-phosphate, another initial precursor, of the common aromatic amino acid biosynthetic pathway.

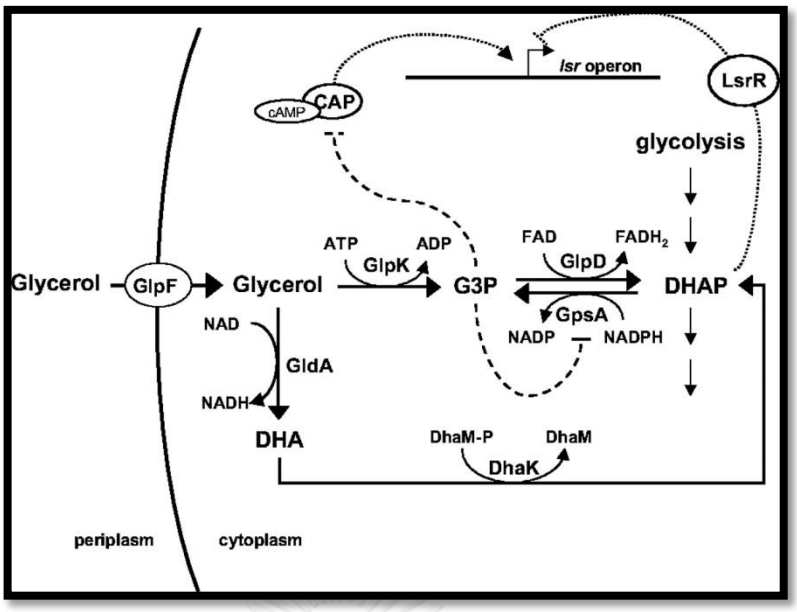

Figure 4 Aerobic glycerol and G3P metabolism in *E. coli* Glycerol enters the cytoplasm through the glycerol facilitator (GlpF) and can be phosphorylated to G3P by the glycerol kinase (GlpK). In the presence of oxygen, G3P is oxidized by the G3P dehydrogenase (GlpD) to DHAP, which is further metabolized through the glycolytic pathway. Source: Xavier and Bassler, 2005

Thongchuang and coworkes (2011) overexpressed L-Phe by cloning of *phedh* gene (P) into *E. coli* combination with key enzyme in aromatic amino acid biosynthetic pathway using glycerol as carbon source as show in Figure 5 .

- *aroB* (B) encodes rate-limiting enzyme, 3-dehydroquinate synthase, in the common aromatic amino acid biosynthesis pathway

- *aroL* (L) encodes rate-limiting enzyme, shikimate kinase II, in the common aromatic amino acid biosynthesis pathway

- *glpF* (F) encodes glycerol facilitator for glycerol uptake

- *glpK* (K) encodes rate-limiting enzyme, glycerol kinase, in glycerol utilization

- *pheA* (A)encodes branch point enzyme, chorismate mutase/prephenate dehydratase, in L-Phe biosynthesis pathway

- *tktA* (T)encodes transketolase for increase in E4P availability

- *yddG* (Y)encodes aromatic amino acid exporter for L-Phe excretion

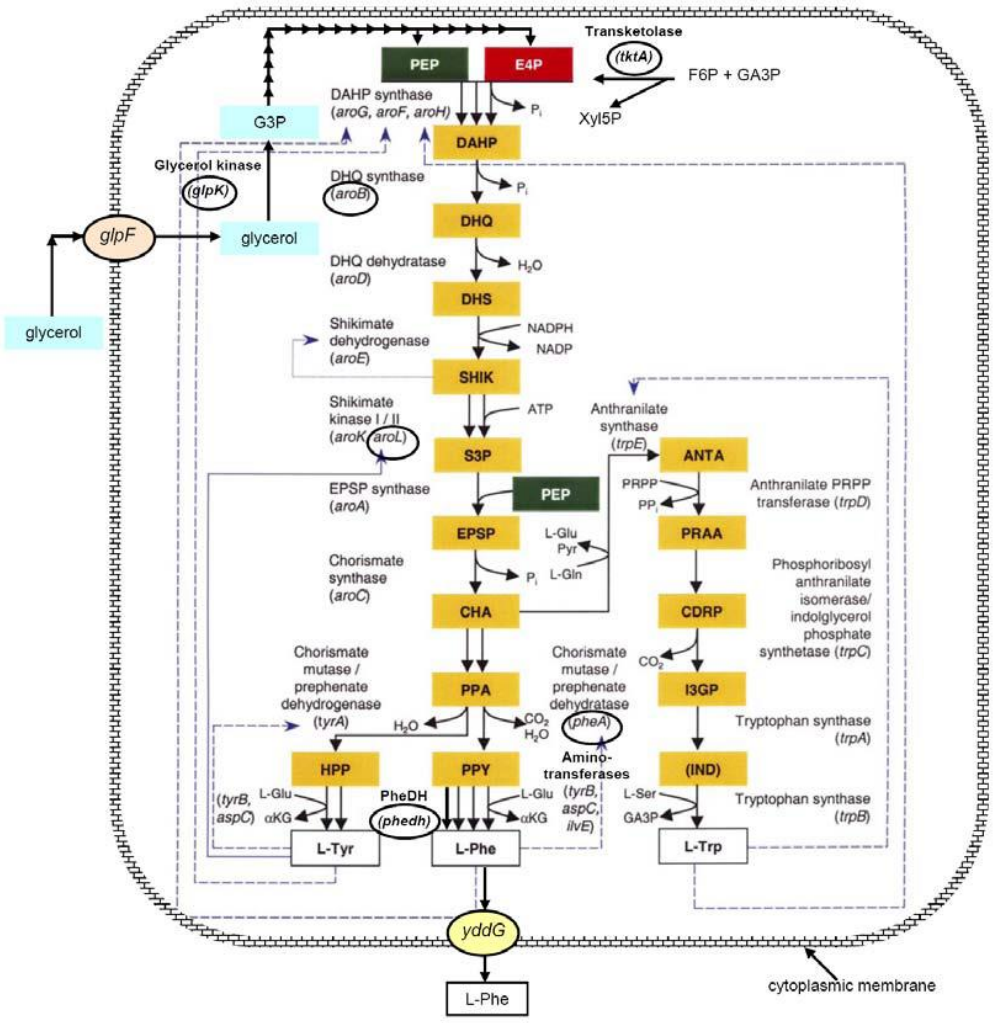

Figure 5 Aromatic amino acid biosynthetic pathway using glycerol as carbon source in *E. coli* BL21(DE3) Source: Thongchuang, 2011

The L-Phe production of *E. coli* BL21 (DE3) using glycerol as the substrate in different clones are shown in Figure 6. The combination of key enzyme genes in amino acid biosynthetic pathway can increased the production rates, which the highest clone was pPTFBLY. Although, pPTFBLYA had more key enzyme genes but *pheA* gene could not effect to improve L-Phe production, cause by feedback inhibition with PheA (Thongchuang, 2011)

Table 1 Comparison of L-Phe production in shake flask cultivation of different recombinant *E. coli* BL21(DE3) Source: Thongchuang, 2011

No.	Recombinant clone	L-Phe production		L-Phe production (mg/L) at 240 h of induction (IPTG (mM))
		Rate* (mg/L h) (IPTG (mM))	Fold**	
1	pPheDH	1.61 (0)	1.0	230 (0.5)
2	pPY	2.33 (0.5)	1.4	255 (1)
3	pPYF	2.86 (1)	1.8	280 (1)
4	pPYFK	2.15 (0.25)	1.3	268 (0.5)
5	pPTY	2.94 (0.5)	1.8	345 (0.5)
6	pPTFY	2.21 (0.25)	1.4	345 (0.25)
7	pPTFKY	2.32 (1)	1.4	340 (1)
8	pPTFBY	2.90 (1)	1.8	316 (0)
9	**pPTFBLY**	**3.36 (0)**	**2.1**	**429 (0)**
10	pPTFBLYA	2.76 (1)	1.7	389 (0)

*The L-Phe production rate was considered within 120 h after induction.

**Calculated by comparison to L-Phe production rate of pPheDH clone.

1.9 Objective of this research

In our previous works, Thongchuang and coworkes (2011) successfully constructed pPTFBLYA plasmid harboring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and *pheA* genes and expressed in *E. coli* BL21(DE3). Unexpectedly, the amount of L-Phe produced by pPTFBLYA clone was 389mg/L whereas 429 mg/L was obtained from pPTFBLY clone that lacking of *pheA* gene. This could be due to the feedback inhibition of L-Phe towards PheA. Thus, mutagenesis of PheA was carried out in the aim to overcome this incidence. This research expected that the construction of $pheA^{fbr}$ gene might lead to the higher production of L-Phe as well.

The objectives of this research

1. To construct $pheA^{fbr}$ genes by site directed mutagenesis.
2. To construct $pPTFBLYA^{fbr}$ plasmid sand to express in *E. coli* BL21(DE3).
3. To select $pPTFBLYA^{fbr}$ clone that exemplified the highest L-Phe production and determine the L-Phe production of the mutant in 5 L bioreactor.

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)

Bioreactor (B.E. Marubishi, Japan)

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

High performance liquid chromatography (1100, Agilent, Germany)

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 (Midisart 2000, 0.22µm, 64 mm, PTFE, sterile, Sartorius Stedim Biotech S.A., Germany)

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, 5 mL and 20 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)

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

Agar, Bacteriological grade (Criterion, USA)

Agarose (FMC Bioproducts, USA)

Ammonium sulphate (Carlo Erba, Italy)

Antifoam Y-30 Emulsion (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)

Glacial acetic acid (Carlo Erba, Italy)

Glucose (Carlo Erba, Italy)

Glycerol (Ajax Finechem, Australia)

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)

L-Phenylalanine (Sigma, 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)

Zine sulfate (BDH, England)

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

The bacterial strains and plasmids used are shown in Table 1. *E. coli* BL21 (DE3) was the host strain used to overexpress all genes throughout this work. *E. coli* TOP10 was used for replication of plasmid in this study.

2.8 Media

2.8.1 Luria-Bertani broth

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

2.8.2 Minimum medium

The basic culture medium for L-Phe production contained carbon source, nitrogen source, salts, vitamins, and trace elements. In this work, glycerol and $(NH_4)_2SO_4$ were used as carbon and nitrogen sources, respectively. This medium pH 7.4 contained (g/L): glycerol 30; $(NH_4)_2SO_4$ 50; $MgCl_2 \cdot 6H_2O$ 0.81; KH_2PO_4 2.43; K_2HPO_4 2.43; yeast

extract 0.085; thiamine-HCl 0.0085; $FeSO_4 \cdot 7H_2O$ 0.002; $MnSO_4 \cdot H_2O$ 0.002; $CaCl_2 \cdot 2H_2O$ 0.05; $ZnSO_4 \cdot 7H_2O$ 0.01. Glycerol, $(NH_4)_2SO_4$, $MgCl_2 \cdot 6H_2O$, KH_2PO_4, K_2HPO_4, yeast extract, $MnSO_4 \cdot H_2O$ and $CaCl_2 \cdot 2H_2O$ were sterilized together by autoclaved at 121 °C for 15 min. While $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and thiamine-HCl were sterilized by filtration. After that these two potions were mixed together at room temperature.

2.9 Molecular docking

The docking of the CMPD (EC 5.4.99.5/4.2.1.51) with L-Phe was carried out using Discovery Studio 3.1 (DS 3.1) to identify interactions between L-Phe and R domain of CMPD in order to propose amino acid residues for site-directed mutagenesis. The protein structure of CMPD was prepared from Protein Homology / Analogy Recognition Engine V 2.0 (Pyre2) (http://www.sbg.bio.ic.ac.uk/phyre2/). The structure was then optimized using DS 3.1. LigandFit and CDOCKER programs implemented in DS 3.1 were used in this study.

2.10 Plasmid extraction of pPheA

The *E. coli* BL21(DE3) harboring pPheA plasmid was grown in 5 mL LB medium containing 30 µg/mL of kanamycin at 37 ^{O}C for 18 hours with shaking at 250 rpm. After that, cell pellet was collected by centrifugation at 5,000 x g for 2 min at room temperature. Then, plasmid was isolated using High-Speed Plasmid Mini kit (Geneaid Biotech).

Table 2 Plasmids used in this work

Plasmids	Characteristics	reference
pPheA	*E. coli* TOP10 *pheA* gene inserted under T7*lac* promoter of pRSFDuet-1	Thongchuang, 2011
pLY	Each *aroL* and *yddG* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	Thongchuang, 2011
pPTFB	Each *phedh*, *tktA*, *glpF* and *aroB* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	Thongchuang, 2011
pPTFBLY	Each *phedh*, *tktA*, *glpF*, *aroB*, *aroL* and *yddG* preceded by T7*lac* promoter inserted into pRSFDuet-1	Thongchuang, 2011
pPTFBLYA	Each *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and *pheA* preceded by T7*lac* promoter and ribosome binding site inserted into pRSFDuet-1	Thongchuang, 2011
pPheA_L300D	*pheA* gene, carried a mutation L300D, inserted under T7*lac* promoter of pRSFDuet-1	This work
pPheA_M302D	*pheA* gene, carried a mutation L302D, inserted under T7*lac* promoter of pRSFDuet-1	This work
pPheA_L318D	*pheA* gene, carried a mutation L318D, inserted under T7*lac* promoter of pRSFDuet-1	This work

Plasmids	Characteristics	reference
pPheA_L344D	*pheA* gene, carried a mutation L344D, inserted under T7*lac* promoter of pRSFDuet-1	This work
pPheA_L359D	*pheA* gene, carried a mutation L359D, inserted under T7*lac* promoter of pRSFDuet-1	This work
pPheA_L359P	*pheA* gene, carried a mutation L359P, inserted under T7*lac* promoter of pRSFDuet-1	This work
pLYA_L300D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{L300D}$ gene	This work
pLYA_M302D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{M302D}$ gene	This work
pLYA_L318D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{L318D}$ gene	This work
pLYA_L344D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{L344D}$ gene	This work
pLYA_L359D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{L359D}$ gene	This work
pLYA_L359D	pRSFDuet-1, carring *aroL*, *yddG* and $pheA^{L359P}$ gene	This work
pPTFBLYA _L300D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{L300D}$ gene	This work

Plasmids	Characteristics	reference
pPTFBLYA_M302D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{M302D}$ gene	This work
pPTFBLYA_L318D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{L318D}$ gene	This work
pPTFBLYA_L344D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{L344D}$ gene	This work
pPTFBLYA_L359D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{L359D}$ gene	This work
pPTFBLYA_L359D	pRSFDuet-1, carring *phedh*, *tktA*, *glpF*, *aroB*, *aroL*, *yddG* and $pheA^{L359P}$ gene	This work

2.11 Agarose gel electrophoresis

The pPheA samples 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 in a microwave oven. The solution was cooled to about 55-50 ^{o}C and then 1 µl of red safe dye: 20 mL of buffer solution was added before pouring into the casting tray inserted with proper comb. The DNA samples were mixed with 6x loading buffer (30% glycerol and 0.25% bromphenol blue), then loaded into the gel. Electrophoresis was performed at 100 volt. The DNA bands were detected by exposing the gel to UV light. The concentration and molecular weight of DNA sample was compared with the standard DNA markers (GeneRuler 1 kb DNA Ladder).

2.12 Separation of *pheA* gene

2.12.1 Double digestion of pPheA

Plasmid pPheA was digested with restriction enzyme *Hin*dIII and *Afl*II according to the restriction site in Figure 7. The 20 µL of mixture for double digestion contained 1 µg of plasmid DNA, 1X of NEBuffer 4, 100 µg/mL of BSA, 10 U of *Hin*dIII, and 10 U of *Afl*II. The mixture was incubated at 37 °C for 3 h. DNA fragments were separated by agarose gel electrophoresis.

2.12.2 Extraction of DNA fragment from agarose gel

Extraction of DNA fragment from agarose gel was performed using Gel/PCR DNA fragment extraction kit (Geneaid Biotech).

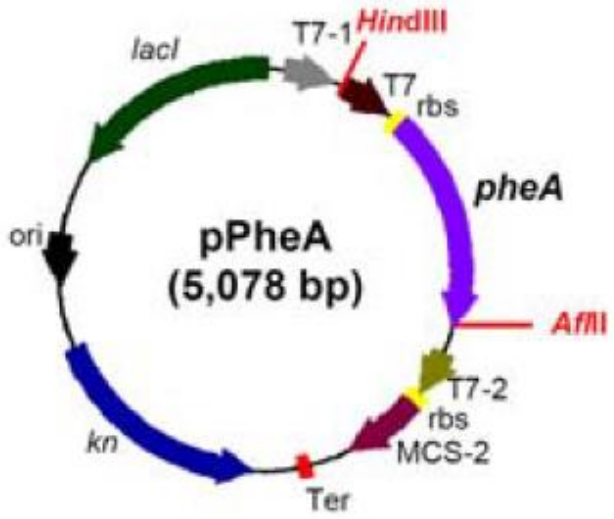

Figure 6 Schematic representation of pPheA plasmid (modified from Thongchuang, 2011)

2.13 Mutagenesis of *pheA* gene

pheA^{fbr} gene was constructed by PCR-driven overlap extension method (Heckman & Pease, 2007) as shown in Figure 7 using the recombinant *pheA* as a DNA template. In this method, two flanking master primers, T7-*Hin*dIII (a) and pheA-*Afl*II (d), were used to mark as 5' ends of both strands (Table 4) and two internal primers, forward (b) and reverse (c) mutant primers, were used to introduce a base-substitution mutation (Table 5). For PCR, 50 microliter of PCR reaction mixture contained 50 ng of recombinant *pheA* used as template, 0.2 mM each dNTPs, 10 pmole of each primer, 1xbuffer with $MgSO_4$ and 2.5 Unit of Pfu DNA polymerase. Condition for amplified gene was predenature (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 last final extention (72 °C, 5 min).

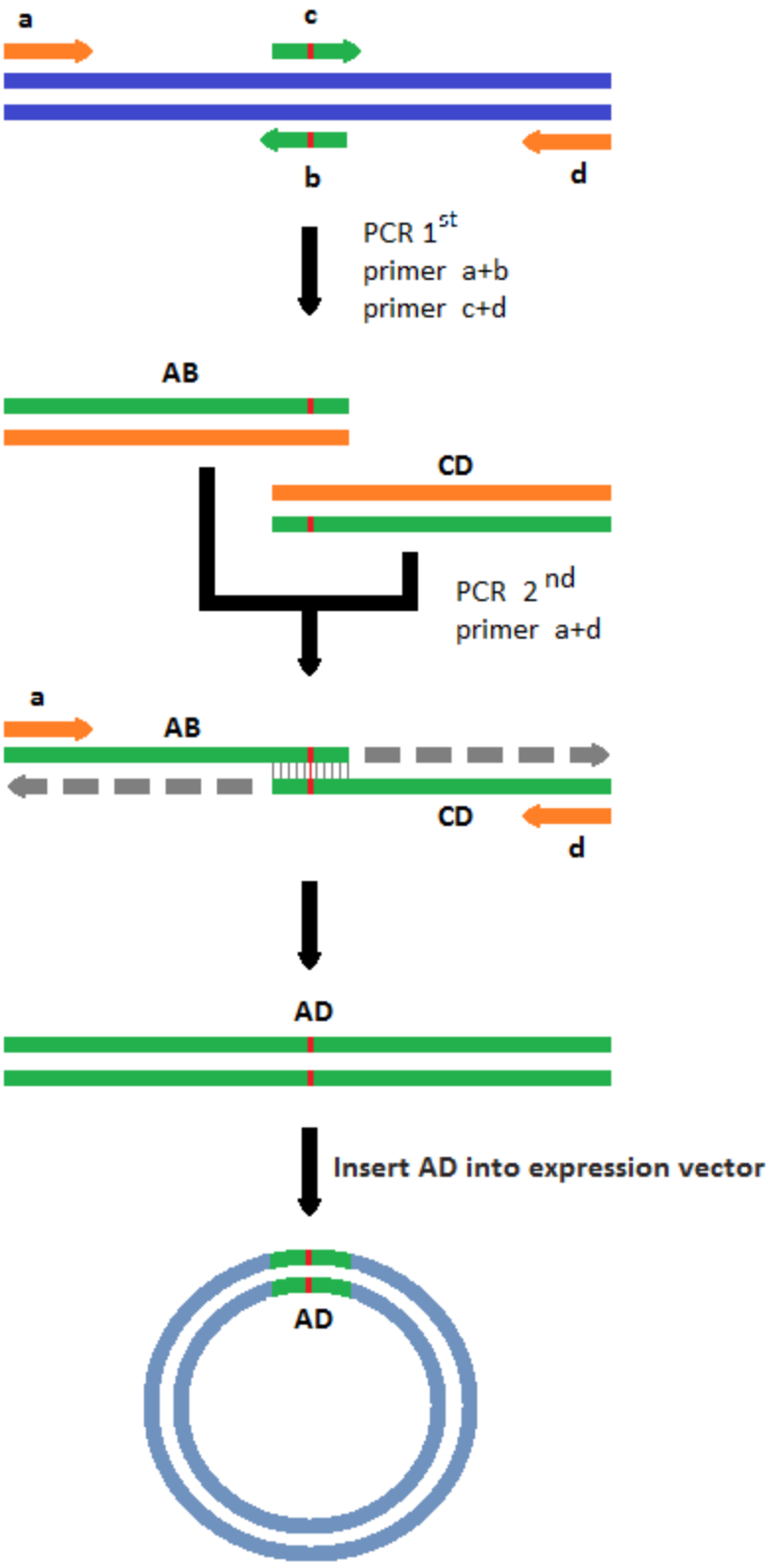

Figure 7 Site-directed mutagenesis by PCR-driven overlap extension

Table 3 Oligonucleotide primers used for DNA sequencing in this work

Primer	**Sequence (5' to 3')**	**Target gene**	**Direction**	**Tm (°C)**
ACYC Duet Up1	GGA TCT CGA CGC TCT CCC T	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Forward	55
Duet Down1	GAT TAT GCG GCC GTG TAC AA	Gene inserted into multiple cloning sites-1 of pRSFDuet-1	Reverse	52

Table 4 *Oligonucleotide primers used for PCR-driven overlap extension*

Primer	**Sequence (5' to 3')**	**Restriction site**	**Tm (°C)**
T7-*Hin*dIII	CCCAAGCTTCGATCCCGCGAAATTAATACGACTC	*Hin*dIII	65.6
pheA-*Afl*II	CCCCTTAAGTCAGGTTGGATCAACAGGCACTACG	*Afl*II	66.8

Table 5 Mutagenic primers used for PCR-driven overlap extension

Primer	Sequence (5’ to 3’)	Base substitution	Tm (°C)
Leu300AspF	CAGGTTCCGGCGAAAACCACGGACTTAATGGCGACCGGGCAACAAGC	TTG → GAC	75
Leu300AspR	GCTTGTTGCCCGGTCGCCATTAAGTCCGTGGTTTTCGCCGGAACCTG	CAA → GTC	75
Met302AspF	GGTTCCGGCGAAAACCACGTTGTTAGACGCGACCGGGCAACAAGCCGG	ATG → GAC	76.5
Met302AspR	CCGGCTTGTTGCCCGGTCGCGTCTAACAACGTGGTTTTCGCCGGAACC	CAT → GTC	76.5
Leu318AspF	GCCGGTGCGCTGGTTGAAGCGTTGGACGTACTGCGCAACCACAATCTG	CTG → GAC	75.7
Leu318AspR	CAGATTGTGGTTGCGCAGTACGTCCAACGCTTCAACCAGCGCACCGGC	CAG → GTC	75.7
Leu344AspF	GCCGGTGCGCTGGTTGAAGCGTTGGACGTACTGCGCAACCACAATCTG	CTG → GAC	75.7
Leu344AspR	CAGATTGTGGTTGCGCAGTACGTCCAACGCTTCAACCAGCGCACCGGC	CAG → GTC	75.7

Primer	Sequence (5’ to 3’)	Base substitution	Tm (°C)
Leu359AspF	CTTGAATCAGCGGAAATGCAAAAAGCAGACAAAGAGTTAGG GGAAATCACCCGTTC	TTG → GAC	71.2
Leu359AspR	GAACGGGTGATTTCCCCTAACTCTTTGTCTGCTTTTTGCATTTC CGCTGATTCAAG	CAA → GTC	71.2
Leu359ProF	CTTGAATCAGCGGAAATGCAAAAAGCACCGAAAGAGTTAGGG GAAATCACCCGTTC	TTG → CCG	71.9
Leu359ProR	GAACGGGTGATTTCCCCTAACTCTTTCGGTGCTTTTTGCATTTC CGCTGATTCAAG	CAA → CGG	71.9

2.14 PCR product cleaning

PCR product was purified using Gel/PCR DNA fragment extraction kit (Geneaid Biotech) according to the protocol.

2.15 Ligation

Ligation mixture (20 µL) contained 2 µL of 60 ng/µL of plasmid vector, 10 µL of 70 ng/µL of PCR product, 2 µL of 10x T4 DNA ligase buffer, 5 µL ultrapure water and 1 µL of 5 Unit of T4 DNA ligase. Ligation mixture was mixed and incubated at 16 °C for 16 h.

2.16 Transformation of plasmid

2.16.1 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 at 37 °C with shaking speed 250 rpm for 24 h. The 50 mL of starter was inoculated to 1 liter of LB medium and continued incubation. When the optical density at OD_{600} of cell culture reached 0.3-0.4, the culture was chilled on ice for 30 min and centrifuged at 2,500xg for 15 min at 4 °C. The cell pellets were washed with 1.5-2 medium volume of cold autoclaved distilled water. Finally, the cells were washed with 10 mL of cold 10% glycerol, centrifuged at 2,500xg for 15 min at 4 °C and resuspended with cold 10% glycerol to the final volume of 2-3 mL. Then 40 µL of cell suspension was aliquoted to 1.5 mL microcentrifuge tube and stored at -80 °C.

2.16.2 Electroporation

In the electroporation step, 0.1 cm cuvette and holder were chilled on ice. Competent cells were thawed on ice. After that, 4 µL of ligation products were mixed

with 40 µL competent cells and replaced 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, 1 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 30 µg/mL of kanamycin and incubated at 37 °C for 18 h

2.17 Nucleotide sequencing

The plasmids were sent to Bioneer, Korean for sequencing. The obtained nucleotide sequences were compared with that of wildtype using ClustalW2 Multiple Sequence Alignment tools (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The nucleotide sequences were translated to protein sequence using ExPASy translate tool (http://web.expasy.org/translate/)

2.18 Construction of recombinant plasmid pPTFBLYA^{fbr}

2.18.1 Construction of pLYA^{fbr}

As shown in Figure 8, the recombinant plasmid pLYA^{fbr} containing *aroL, yddG* and *pheA^{fbr}* gene was constructed using the genes from pPheA^{fbr} and pLY. pPheA^{fbr} and pLY were digested with restriction enzyme *Hin*dIII and *Bsr*GI. The 20 µL of mixture for double digestion contained 1 µg of plasmid DNA, 1X of NEBuffer 4, 100 µg/mL of BSA, 10 U of *Hin*dIII, and 10 U of *Bsr*GI. The mixture was incubated at 37 °C for 3 h. DNA fragments were separated by agarose gel electrophoresis. The 1,296 bp DNA fragment of pPheA^{fbr} digestion and the 5,376 bp DNA fragment of pLY digestion were harvested by Gel/PCR DNA fragment extraction kit (Geneaid Biotech). Both of them

were ligated to produce recombinant plasmid pLYA^{fbr} (6,672 bp). After ligation, pLYA^{fbr} was transformed into *E. coli* Top10.

2.18.2 Construction of pPTFBLYA^{fbr}

The construction of a recombinant plasmid pPTFBLYA^{fbr} containing *phedh, tktA, glpF, aroB, aroL, yddG* and *pheA*^{fbr} genes preceded by each own T7*lac* promoter and ribosome binding site was carried out by the ligation of the DNA fragments obtained from the digestion of pPTFB and pLYA^{fbr} with *Bam*HI and *Afl*II (Figure 9).

The longer DNA fragment (8,904 bp) carrying *phedh, tktA, glpF* and *aroB* genes attaching to each own T7*lac* promoter and ribosome binding site obtained from the digestion of pPTFB with *Bam*HI and *Afl*II was used as DNA vector for ligation. The short DNA fragment (2,900 bp) containing *aroL, yddG* and *pheA*^{fbr} genes closed to each T7*lac* promoter and ribosome binding site obtained by the digestion of pLYA^{fbr} with *Bam*HI and *Afl*II was used as inserted DNA for ligation.

Both DNA fragments were ligated by DNA ligase as described in section 2.15 leading to the recombinant plasmid pPTFBLYA^{fbr} (11,804 bp) and then pPTFBLYA^{fbr} was transformated into *E. coli* BL21 (DE3) described in section 2.16.2.

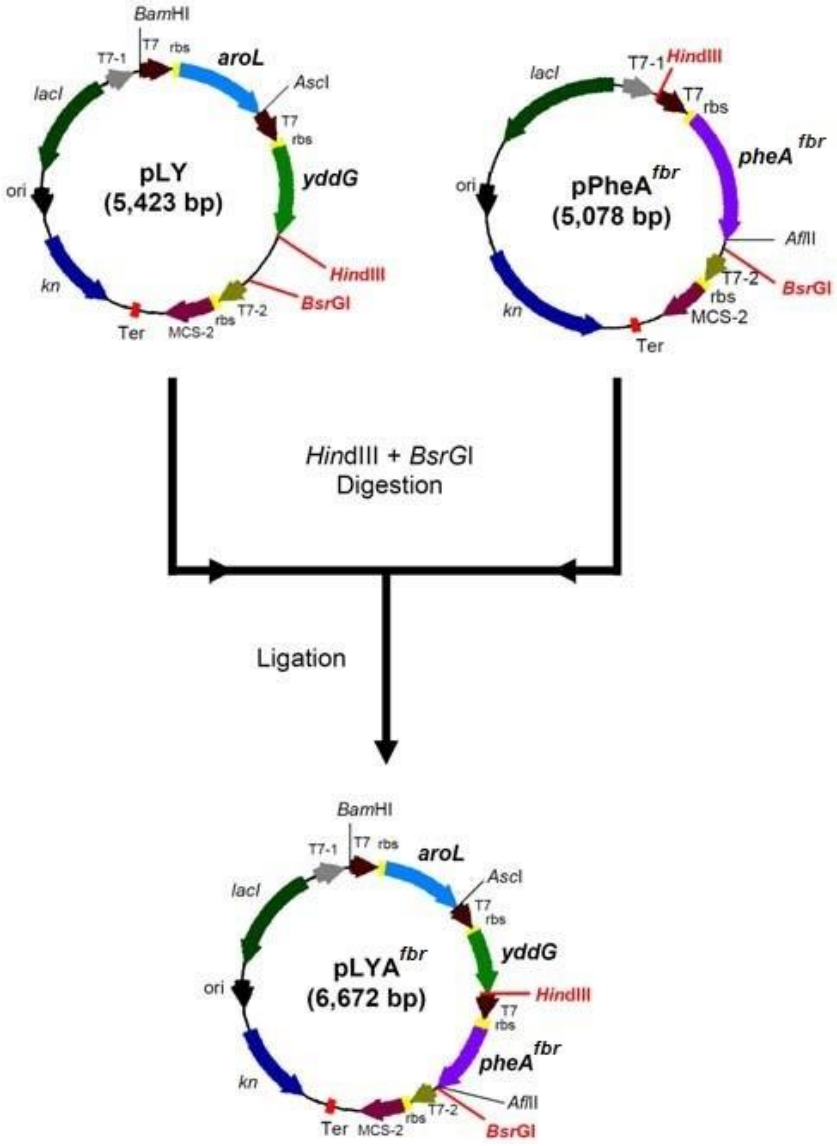

Figure 8 Schematic representation of pLYA^{fbr} construction

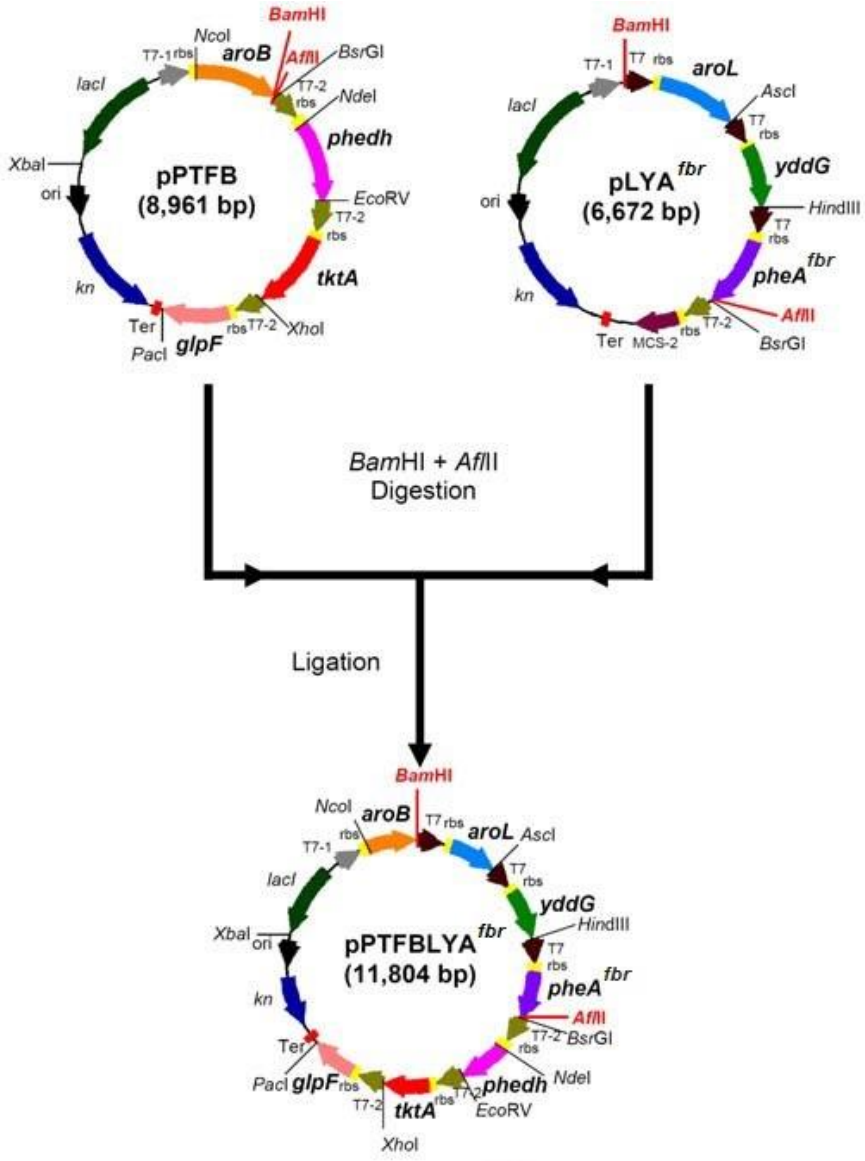

Figure 9 Schematic representation of pPTFBLYA^{fbr} construction

2.19 L-Phenylalanine production

2.19.1 Shake flask fermentation

L-Phe production of 6 pPTFBLYA^{fbr} clones in minimum medium were studied in comparison to the pPTFBLYA and pPTFBLY clone.

Shake flask culture was used to observe the L-Phe production of the *E. coli* BL21 (DE3) harboring recombinant plasmid. A fresh single colony from LB agar plate was cultured in 50 mL of LB medium (pH 7.4, supplemented with 30 µg/mL of kanamycin) and continue incubated at 37 ^{o}C, 18 hours with 250 rpm shaking. The seed culture (10 mL) was inoculated to 200 mL of the production medium, pH 7.4 containing (g/L): glycerol 30; $(NH_4)_2SO_4$ 50; $MgCl_2{\cdot}6H_2O$ 0.81; KH_2PO_4 2.43; K_2HPO_4 2.43; yeast extract 0.085; thiamine-HCl 0.0085; $FeSO_4{\cdot}7H_2O$ 0.002; $MnSO_4{\cdot}H_2O$ 0.002; $CaCl_2{\cdot}2H_2O$ 0.05; $ZnSO_4{\cdot}7H_2O$ 0.01 and 30 µg/mL of kanamycin and was then incubated at 37 ^{o}C, 250 rpm. Samples were collected at every 24 h until 240 h, and OD_{600} was measured. For measurement of L-Phe concentration, 1 mL of each culture broth was centrifuged at 8,000xg for 15 min to remove the cells. The remaining supernatant was collected and subjected to HPLC analysis for determination of L-phenylalanine.

2.19.2 HPLC analysis for determination of L-phenylalanine

Supernatant from 2.20.1 was filtrated through 0.2 µm syringe filter set. The sample was analyzed by HPLC technique using Chirex 3126 (D) - penicillamine column. A mixture of 2 mM copper sulfate (Appendix A) and methanol in the ratio of 70:30 was used as a mobile phase. The column was operated at a constant flow rate of 1

mL/min and A_{254} was detected. The injected sample volume was 20 µL. L-Phe was quantified by comparing to the calibration curve (Appendix B).

2.19.3 Fed batch fermenter

2.19.3.1 Inoculum preparation

One fresh colony of *E*. coli BL21 (DE3) recombinant clone grown on 30 µg/mL of kanamycin LB agar plate was cultured in 100 mL of LB medium, pH 7.4, containing 30 µg/mL of kanamycin. Incubation was at 37 ºC, 250 rpm for 18 h. After that 35 mL of the overnight culture was transfered to fresh LB medium containing 30 µg/mL of kanamycin (final volumn of 350 mL) and the incubation was continued at 37 ºC, 250 rpm until OD_{600} of about 0.6 to 0.8. The culture was used as the inoculum for the fermenter culture.

2.19.3.2 Fed batch cultivation condition

Fed batch fermentation was used to produce L-phenylalanine in the 5 L glass stirred tank bioreactor. The inoculum from 2.20.3.1 (10% of working volume) was transferred to the bioreactor that had been filled to 3.5 L of production medium as described in the section 2.19.1. The aeration and agitation rate were set at 1 vvm (3.5 L/min) and 400 rpm respectively. The incubation temperature was 37 ºC. The dissolved oxygen concentration was monitored using a Polarographic Oxygen Sensor. The pH was automatically controlled at 7.4 by 5 M NaOH which detected from the pH electrode. The sterile silicon antifoam agent was used to control foam formation. When OD_{600} of cell culture reached 0.5-0.6, IPTG was added to the final concentration of 1 mM. Samples were taken at every 6 h after induction in order to measure growth at

OD_{600}. For the other analyses, the samples were centrifuged at 8,500xg for 15 min and the supernatants were subjected to determining of L-Phe content. When the growth was under stationary phase, new feeding medium of 200 mL was then provided. The feeding medium contained 200 g/L of glycerol and other media components were prepared using the same component ratio of shake flask medium as described in section 2.19.1. After the feeding medium (200 mL) was added to the fermenter, the culture was collected from the bioreactor at every 6 hours until 96 hours. The samples were analyzed L-Phe production.

CHAPTER III

RESULTS AND DISCUSSIONS

3.1. Molecular docking of CMPD-L-Phe complex

Due to, the 3D structure of CMPD from *E. coli* was not found in database. Consequently, Protein Homology / Analogy Recognition Engine V 2.0 (Pyre2) was used to simulate CMPD. The result showed confidence in the model: 378 residues (98%) modeled at >90% accuracy when aligned to known protein structures in Database with crystal structure of chorismate mutase from *Bartonella henselae* and prephenate dehydratase from *Arthrobacter aurescens*.

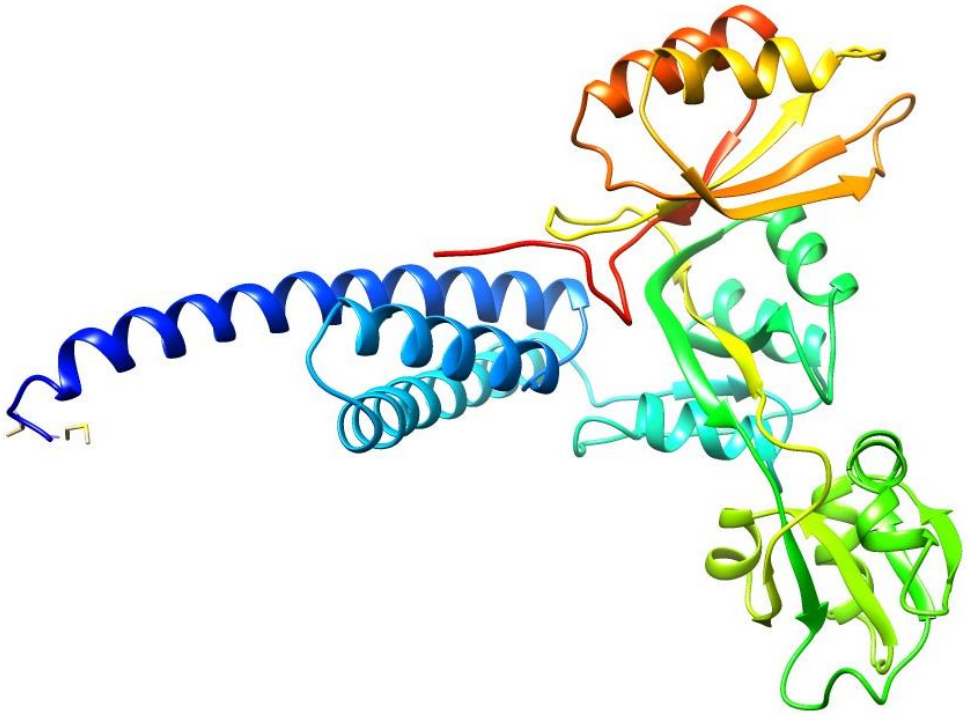

Figure 10 *3D structure of CMPD protein from Pyre2*

L-Phe was docked to the specified ligand binding site. This step was well-analyzed under program's criteria such as conformational searching for ligand, site shape matching to select ligand conformation, positioning ligand conformation into the binding site and minimizing the energy of the candidate ligand pose. As shown in Figure 11, there amino acid residues are in R domain of PheA, based upon distance within 5.0 Angstrom from L-Phe molecule, which might be participated in L-Phe binding.

According to the previously reviews, many feedback resistant were generated in R domain such as modifying Trp226 and Trp338 (Gething & Davidson, 1978). Especially substitution of Thr 326 to Pro provided strong feedback resistance (Nelms et al., 1992). However, the previously mentioned residues were not present in the docking area as shown in Figure 11.

In this research, we focused on Leu and Met which are classified as non-polar amino and share the same properties with L-Phe. Thus, feedback inhibition of L-Phe on R domain of CMPD could be mainly stated that L-Phe was stabilized within CMPD via hydrophobic interaction. Therefore, Leu300, Met302, Leu318, Leu344, Leu359 were selected for site-direct mutagenesis. To disrupt the interaction between L-Phe and hydrophobic residues, a negatively charged amino acid Asp should be introduced instead and Pro was candidate to interfere the interaction by disrupt regular secondary structures.

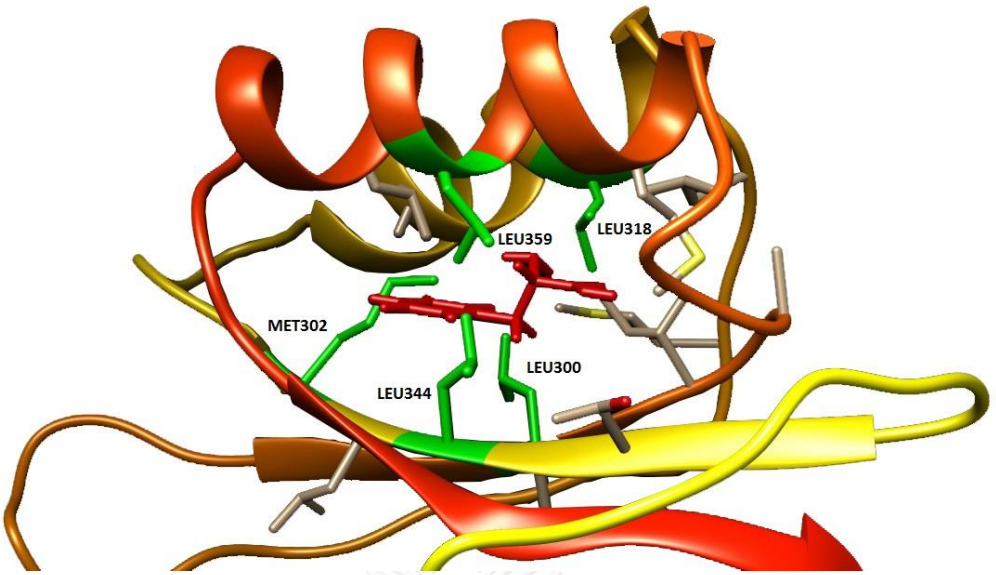

Figure 11 *3D docking model of CMPD-L-Phe complex*

3.2 Plasmid Extraction

For mutation of *pheA* gene, pPheA (5,078 bp) were extracted from *E. coli* Top10 host cell and were digested with *Hin*dIII and *Afl*II, then electrophoresed on agarose gel. The DNA fragments of 3,809 bp and 1,269 bp were obtained as shown in Figure 12. Then the shorter fragment (1,269 bp) of *pheA* gene was isolated by Gel/PCR DNA fragment extraction kit for use as template in PCR-driven overlap extension.

3.3 PCR amplification of *pheA* feedback resistance gene

The *pheA* feedback resistance gene was amplified using *pheA* gene as a template. The site-directed mutagenesis segments of the target gene were amplified from the template DNA using two flanking master primers (T7-*Hin*dIII and pheA-*Afl*II) that marked the 5' ends of both strands and two internal mutant primers (F and R) that

introduced the mutation of interest. The 1st PCR generated long fragment AB and short fragment CD of each mutant gene. Both fragments were used as 2nd PCR template. The *pheA*^{fbr} genes were completely synthesized in 2nd PCR as AD fragments which was the same length as *pheA* gene template (1,269 bp) as shown in Figure 13. In addition, the 1,269 bp PCR product of 1st PCR was obtained when primer D was used.

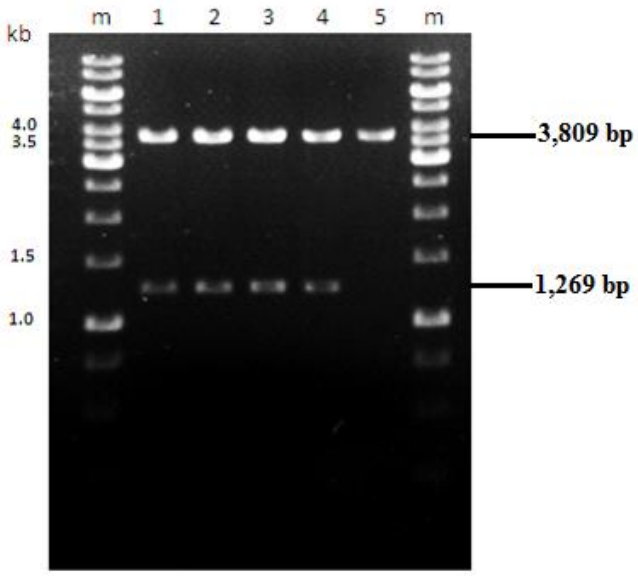

Figure 12 Restriction pattern of pPheA

Lane m	=	Gene Ruler 1 kb DNA ladder
Lane 1-4	=	*Hin*dIII/*Afl*II -digested pPheA
Lane 5	=	*Hin*dIII/*Afl*II -digested pRSFDuet-1
Lane m	=	Gene Ruler 1 kb DNA ladder

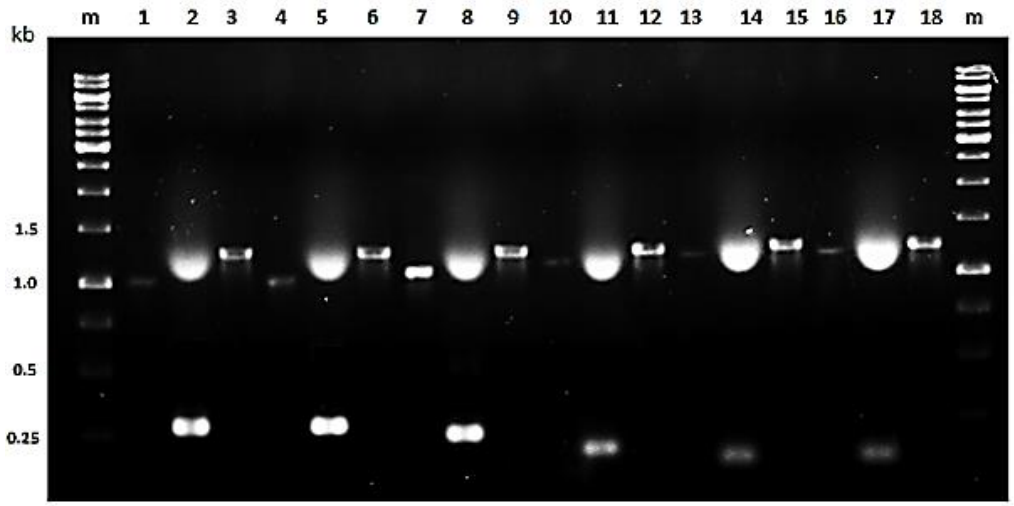

Figure 13 Electrophoretic patterns of PCR products from site-directed mutagenesis

Lane m = Gene Ruler 1 kb DNA ladder

Lane 1 = AB of *pheA*^{L300D}

Lane 2 = CD of *pheA*^{L300D}

Lane 3 = AD of *pheA*^{L300D}

Lane 4 = AB of *pheA*^{M302D}

Lane 5 = CD of *pheA*^{M302D}

Lane 6 = AD of *pheA*^{M302D}

Lane 7 = AB of *pheA*^{L318D}

Lane 8 = CD of *pheA*^{L318D}

Lane 9 = AD of pheA^{L318D}

Lane 10 = AB of *pheA*^{L344D}

Lane 11 = CD of *pheA*^{L344D}

Lane 12 = AD of *pheA*^{L344D}

Lane 13 = AD of *pheA*^{L359D}

Lane 14 = AD of *pheA*^{L359D}

Lane 15 = AD of *pheA*^{L359D}

Lane 16 = AD of *pheA*^{L359P}

Lane 17 = AD of *pheA*^{L359P}

Lane 18 = AD of *pheA*^{L359P}

Lane m = Gene Ruler 1 kb DNA ladder

3.4 Construction of pPheA^{fbr} clone

The 2nd PCR product was digested with *Hin*dIII and *Afl*II. Then the PCR fragment (1,269 bp) was ligated into the pRSFDuet-1 which was digested same restriction enzyme to produce pPheA^{fbr} plasmid (5,078 bp). The pPheA^{fbr} was transformed into *E. coli* top10. pPheA^{fbr} clone was selected and its plasmid was confirmed by cutting with *Hin*dIII and *Afl*II as shown in Figure 14.

All pPheA^{fbr} were verified of substitution in R domain by DNA sequencing at Bioneer, Republic of Korea using primers ACYC Duet Up1 and Duet Down1 due to the insertion of *pheA^{fbr}* at multiple cloning sites-1 of pRSFDuet-1 vector (Figure 15-20).

The result showed that, the expected nucleotide substitution was only residue which was changed .These Six of *PheA^{fbr}* gene were used for combination with other genes in further experiments.

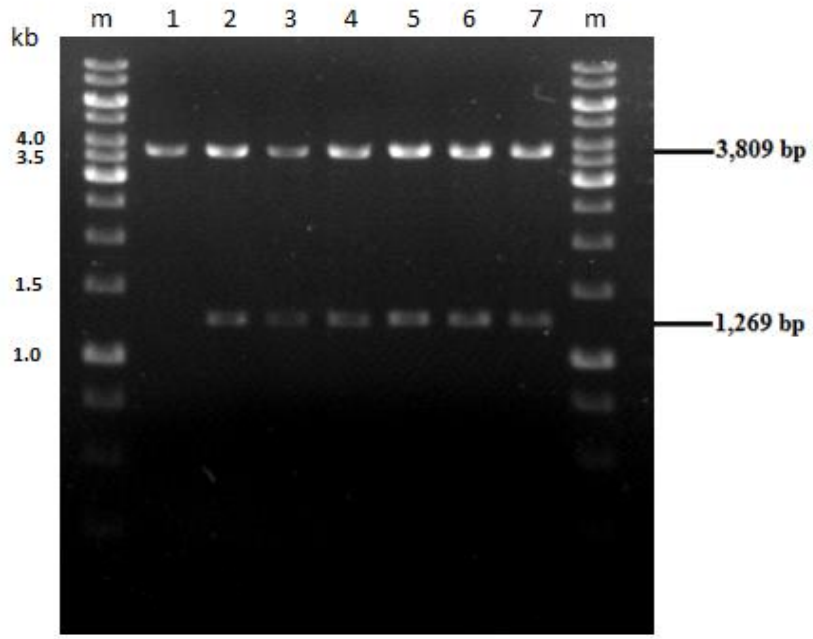

Figure 14 Restriction pattern of pPheA^{fbr}

Lane m = Gene Ruler 1 kb DNA ladder

Lane 1 = *Hin*dIII/*Afl*II-digested pRSFDuet-1

Lane 2 = *HindIII/AflII* -digested pPheA^{L300D}

Lane 3 = *Hin*dIII/*Afl*II -digested pPheA^{M302D}

Lane 4 = *Hin*dIII/*Afl*II -digested pPheA^{L318D}

Lane 5 = *Hin*dIII/*Afl*II -digested pPheA^{L344D}

Lane 6 = *Hin*dIII/*Afl*II -digested pPheA^{L359D}

Lane 7 = *Hin*dIII/*Afl*II -digested pPheA^{L359P}

Lane m = Gene Ruler 1 kb DNA ladder

```
-180  ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACCGGAC
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  D
901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
       L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA
       M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  L  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTAGTCCGACAGTAGTTCCCC
       V  P  V  D  P  T  *
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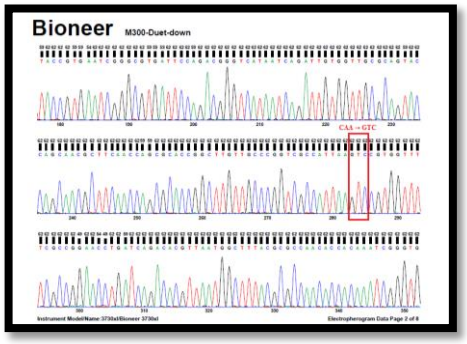

Figure 15 The nucleotide sequence and the deduced amino acid sequence of pheA^{L300D} gene in recombinant plasmid pPheA^{L300D}. The mutation site was shown in box. Complete chromatogram of PheA^{L300D} was shown in Appendix D.

```
180   ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
901   TTAGACGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
       L   D  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA
       M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  L  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTAAGTCGACAGCATGGCCG
       V  P  V  D  P  T  *
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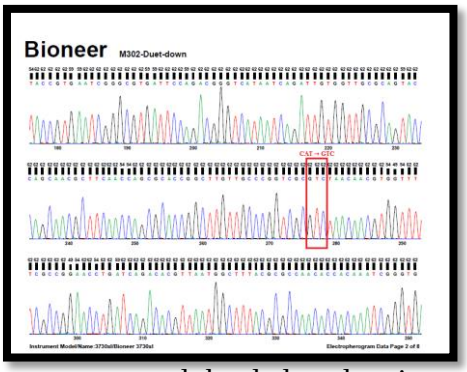

Figure 16 The nucleotide sequence and the deduced amino acid sequence of pheA^{M302D} gene in recombinant plasmid pPheA^{L302D}. The mutation site was shown in box. Complete chromatogram of PheA^{L302D} was shown in Appendix E.

```
-180  ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTAGACCGCAAC
       L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  D  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA
       M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  L  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTAAGTCGAACAGCATTGGGCG
       V  P  V  D  P  T  *
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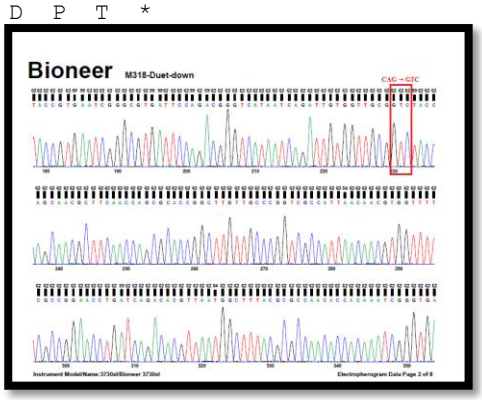

Figure 17 The nucleotide sequence and the deduced amino acid sequence of pheA^{M318D} gene in recombinant plasmid pPheA^{L318D}. The mutation site was shown in box. Complete chromatogram of PheA^{L318D} was shown in Appendix F.

```
-180  ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
       L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATGACGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCATTGAAA
       M  F  Y  D  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  L  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTTAAGTCGAACAGAAAGTAAT
       V  P  V  D  P  T  *
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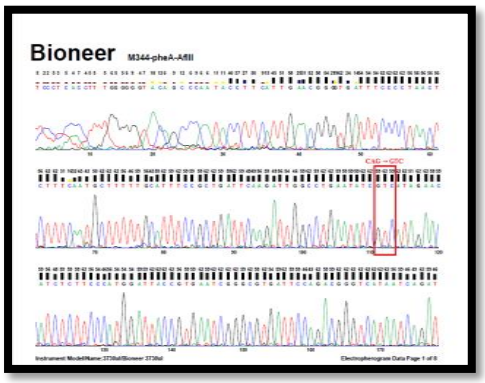

Figure 18 The nucleotide sequence and the deduced amino acid sequence of pheA^{M344D} gene in recombinant plasmid pPheA^{L344D}. The mutation site was shown in box. Complete chromatogram of PheA^{L344D} was shown in Appendix G.

```
-180  ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
       L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCAGACAAA
       M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  D  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTTAAGTCGAA
       V  P  V  D  P  T  *
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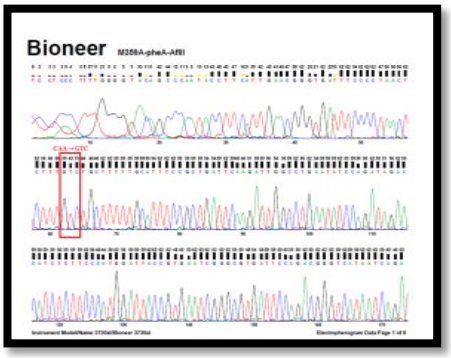

Figure 19 The nucleotide sequence and the deduced amino acid sequence of pheA^{M359D} gene in recombinant plasmid pPheA^{L359D}. The mutation site was shown in box. Complete chromatogram of PheA^{L359D} was shown in Appendix H.

```
-180  ATGGGCAGCAGCCATCACCATCATCACCACAGCCAGGATCCGAATTCGAGCTCGGCGCGC
-120  CTGCAGGTCGACAAGCTTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTG
-60   AGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT
1     ATGACATCGGAAAACCCGTTACTGGCGCTGCGAGAGAAAATCAGCGCGCTGGATGAAAAA
       M  T  S  E  N  P  L  L  A  L  R  E  K  I  S  A  L  D  E  K
61    TTATTAGCGTTACTGGCAGAACGGCGCGAACTGGCCGTCGAGGTGGGAAAAGCCAAACTG
       L  L  A  L  L  A  E  R  R  E  L  A  V  E  V  G  K  A  K  L
121   CTCTCGCATCGCCCGGTACGTGATATTGATCGTGAACGCGATTTGCTGGAAAGATTAATT
       L  S  H  R  P  V  R  D  I  D  R  E  R  D  L  L  E  R  L  I
181   ACGCTCGGTAAAGCGCACCATCTGGACGCCCATTACATTACTCGCCTGTTCCAGCTCATC
       T  L  G  K  A  H  H  L  D  A  H  Y  I  T  R  L  F  Q  L  I
241   ATTGAAGATTCCGTATTAACTCAGCAGGCTTTGCTCCAACAACATCTCAATAAAATTAAT
       I  E  D  S  V  L  T  Q  Q  A  L  L  Q  Q  H  L  N  K  I  N
301   CCGCACTCAGCACGCATCGCTTTTCTCGGCCCCAAAGGTTCTTATTCCCATCTTGCGGCG
       P  H  S  A  R  I  A  F  L  G  P  K  G  S  Y  S  H  L  A  A
361   CGCCAGTATGCTGCCCGTCACTTTGAGCAATTCATTGAAAGTGGCTGCGCCAAATTTGCC
       R  Q  Y  A  A  R  H  F  E  Q  F  I  E  S  G  C  A  K  F  A
421   GATATTTTTAATCAGGTGGAAACCGGCCAGGCCGACTATGCCGTCGTACCGATTGAAAAT
       D  I  F  N  Q  V  E  T  G  Q  A  D  Y  A  V  V  P  I  E  N
481   ACCAGCTCCGGTGCCATAAACGACGTTTACGATCTGCTGCAACATACCAGCTTGTCGATT
       T  S  S  G  A  I  N  D  V  Y  D  L  L  Q  H  T  S  L  S  I
541   GTTGGCGAGATGACGTTAACTATCGACCATTGTTTGTTGGTCTCCGGCACTACTGATTTA
       V  G  E  M  T  L  T  I  D  H  C  L  L  V  S  G  T  T  D  L
601   TCCACCATCAATACGGTCTACAGCCATCCGCAGCCATTCCAGCAATGCAGCAAATTCCTT
       S  T  I  N  T  V  Y  S  H  P  Q  P  F  Q  Q  C  S  K  F  L
661   AATCGTTATCCGCACTGGAAGATTGAATATACCGAAAGTACGTCTGCGGCAATGGAAAAG
       N  R  Y  P  H  W  K  I  E  Y  T  E  S  T  S  A  A  M  E  K
721   GTTGCACAGGCAAAATCACCGCATGTTGCTGCGTTGGGAAGCGAAGCTGGCGGCACTTTG
       V  A  Q  A  K  S  P  H  V  A  A  L  G  S  E  A  G  G  T  L
781   TACGGTTTGCAGGTACTGGAGCGTATTGAAGCAAATCAGCGACAAAACTTCACCCGATTT
       Y  G  L  Q  V  L  E  R  I  E  A  N  Q  R  Q  N  F  T  R  F
841   GTGGTGTTGGCGCGTAAAGCCATTAACGTGTCTGATCAGGTTCCGGCGAAAACCACGTTG
       V  V  L  A  R  K  A  I  N  V  S  D  Q  V  P  A  K  T  T  L
901   TTAATGGCGACCGGGCAACAAGCCGGTGCGCTGGTTGAAGCGTTGCTGGTACTGCGCAAC
       L  M  A  T  G  Q  Q  A  G  A  L  V  E  A  L  L  V  L  R  N
961   CACAATCTGATTATGACCCGTCTGGAATCACGCCCGATTCACGGTAATCCATGGGAAGAG
       H  N  L  I  M  T  R  L  E  S  R  P  I  H  G  N  P  W  E  E
1021  ATGTTCTATCTGGATATTCAGGCCAATCTTGAATCAGCGGAAATGCAAAAAGCACCGAAA
       M  F  Y  L  D  I  Q  A  N  L  E  S  A  E  M  Q  K  A  P  K
1081  GAGTTAGGGGAAATCACCCGTTCAATGAAGGTATTGGGCTGTTACCCAAGTGAGAACGTA
       E  L  G  E  I  T  R  S  M  K  V  L  G  C  Y  P  S  E  N  V
1141  GTGCCTGTTGATCCAACCTGACTTAAGTCGAACAG
       V  P  V  D  P  T  *
```

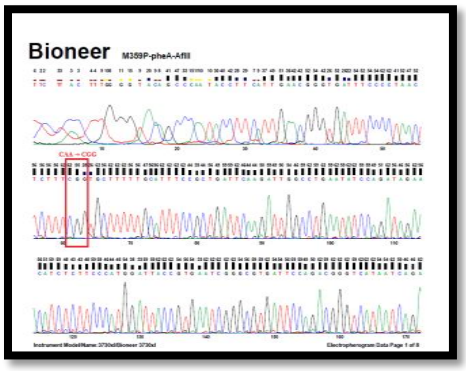

Figure 20 The nucleotide sequence and the deduced amino acid sequence of pheA^{M359P} gene in recombinant plasmid pPheA^{L359P}. The mutation site was shown in box. Complete chromatogram of PheA^{L359P} was shown in Appendix I.

3.5 Cloning of *pheA^{fbr}* gene into a recombinant plasmid pPTFBLY

3.5.1 Cloning of *pheA^{fbr}* gene into a recombinant plasmid pLY

The *pheA^{fbr}* gene fragment from pPheA^{fbr} was inserted into pLY at the recognition sites of *Hin*dIII and *Bsr*GI. The pPheA was digested with *Hin*dIII and *Bsr*GI to collect the fragment of 1,296 bp containing T7*lac* promoter followed by the *pheA^{fbr}* gene. In parallel, pLY was digested with the same restriction enzymes leading to the 5,376 bp potential vector containing *aroL* and *yddG* genes. The *pheA^{fbr}* fragment was then ligated into the vector to produce pLYA^{fbr} (6,672 bp) as shown in Figure 8. After transformation, *E. coli* top10 containing pLYA^{fbr} was selected and its plasmid was confirmed by cutting with *Bam*HI and *Afl*II as shown in Figure 21.

3.5.2 Cloning of *AroL, yddG* and *pheA^{fbr}* genes into a recombinant plasmid pPTFB

The pLYA^{fbr} was cut with *Bam*HI and *Afl*II to collect the 2,900 bp DNA fragment containing *aroL, yddG* and *pheA^{fbr}* genes and the unwanted fragment of 3,772 bp was discharged. The target fragment of 2,900 bp was purified from the gel. The pPTFB was digested with the same restriction enzymes leading to the 8,904 bp fragment containing p*hedh, tktA, glpF* and *aroB* genes. The LYA^{fbr} fragment was ligated into the vector to produce pPTFBLYA^{fbr} (11,804 bp) as shown in Figure 9. Then, each pPTFBLYA^{fbr} vector was introduced into host cell to produce *E. coli* BL21(DE3)/ pPTFBLYA^{fbr} clones. Each pPTFBLYA^{fbr} was confirmed by cutting with *Bam*HI and *Afl*II. The restriction pattern analysis of six pPTFBLYA^{fbr} were shown in Figure 22.

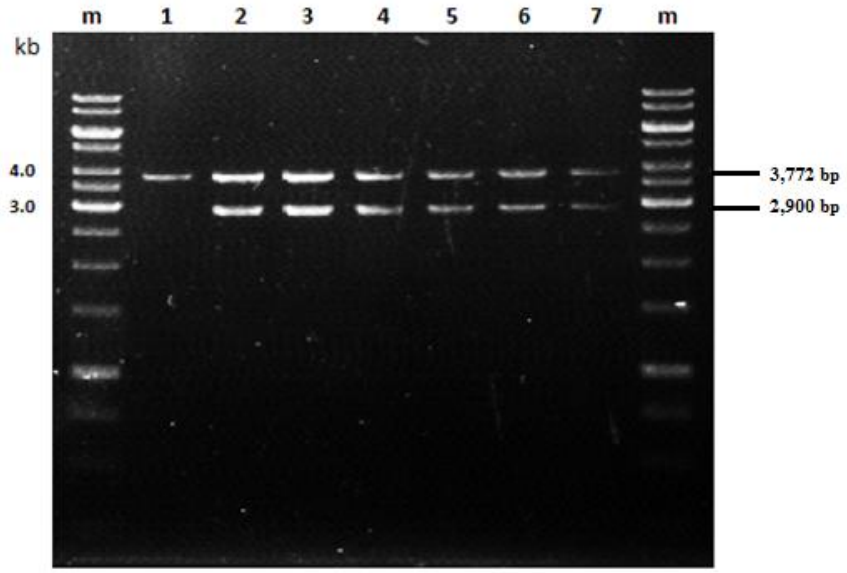

Figure 21 Restriction pattern of pLYA^{fbr}

Lane m = Gene Ruler 1 kb DNA ladder

Lane 1 = *Bam*HI/ *Afl*II -digested pRSFDuet-1

Lane 2 = *Bam*HI/ *Afl*II -digested pLYA^{L300D}

Lane 3 = *Bam*HI/ *Afl*II -digested pLYA^{M302D}

Lane 4 = *Bam*HI/ *Afl*II -digested pLYA^{L318D}

Lane 5 = *Bam*HI/ *Afl*II -digested pLYA^{L344D}

Lane 6 = *Bam*HI/ *Afl*II -digested pLYA^{L359D}

Lane 7 = *Bam*HI/ *Afl*II -digested pLYA^{L359P}

Lane m = Gene Ruler 1 kb DNA ladder

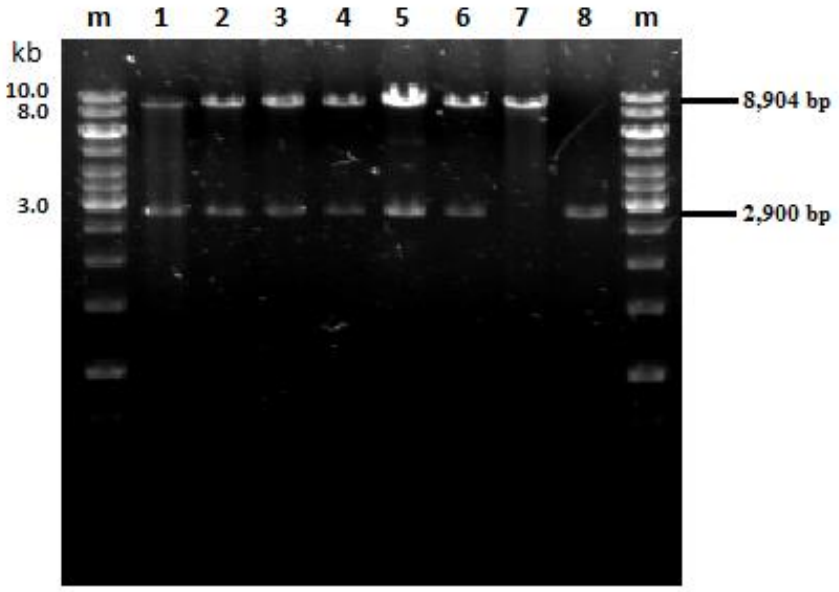

Figure 22 Restriction pattern of of pPTFBLYA^{fbr}

Lane m = Gene Ruler 1 kb DNA ladder

Lane 1 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{L300D}

Lane 2 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{M302D}

Lane 3 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{L318D}

Lane 4 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{L344D}

Lane 5 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{L359D}

Lane 6 = *Bam*HI/ *Afl*II -digested pPTFBLYA^{L359P}

Lane 7 = *Bam*HI/ *Afl*II -digested pPTFB

Lane 8 = *Bam*HI/ *Afl*II -digested LYA fragment

Lane m = Gene Ruler 1 kb DNA ladder

3.6 L-Phenylalanine production

3.6.1 Shake flask fermentation

L-Phe production of 6 pPTFBLYA^{fbr} clones in the optimized minimum medium for L-Phe of pPheDH clone obtained from Thongchuang (2012) were studied in comparison to the pPTFBLYA and pPTFBLY clone.

From the results in Figure 23, the growth of the recombinant clones were monitored by measuring OD at 600 nm. The growth pPTFBLYA^{L300D}, pPTFBLYA^{M302D} and wild type pPTFBLY were dramatically increased from 0-96 h of cultivation and then their growth were increased slowly until OD_{600} about 8 to 9. In contrast, the growth of mutant clone pPTFBLYA^{L344D}, pPTFBLYA^{L359D}, pPTFBLYA^{L359P} and the wild type clone pPTFBLYA were increased at first 72 h and then remained constant as shown in Figure 23. The pPTFBLYA^{L318D} clone showed different patterns when compared to the other clones.

From the result in Figure 24, almost of the L-Phe production trend were increased until 120 h and then the amount of L-Phe were constant because the kanamycin were stable at 37 °C for approximately 5 days after that plasmid were discarded. The mutated clone pPTFBLYA^{L359D} could produce L-Phe approximately 135 mg/L which was the highest amount among the tested clone and was 3.78 fold of that of pPTFBLYA clone.

The mutant harboring feedback resistance *pheA*^{L359D}gene which Leu359 in regulatory (R) domain was substitution by Asp (Zhang et al., 1998) could improve the L-Phe production. According to the result, the changes of amino acid group, might successfully disrupt the hydrophobic interaction between PheA and L-Phe in L-Phe binding site.

The pPTFBLYA^{L359D} clone was selected for further applied in large-scale production using bioreactor.

Although the concentration of L-Phe was relatively low compared with the highest concentration of 389mg/L reported previously by Thongchuang (2012), the production of L-Phe by *E. coli* was remarkably improved by co-expression of the *pheA*^{fbr} in the plasmid.

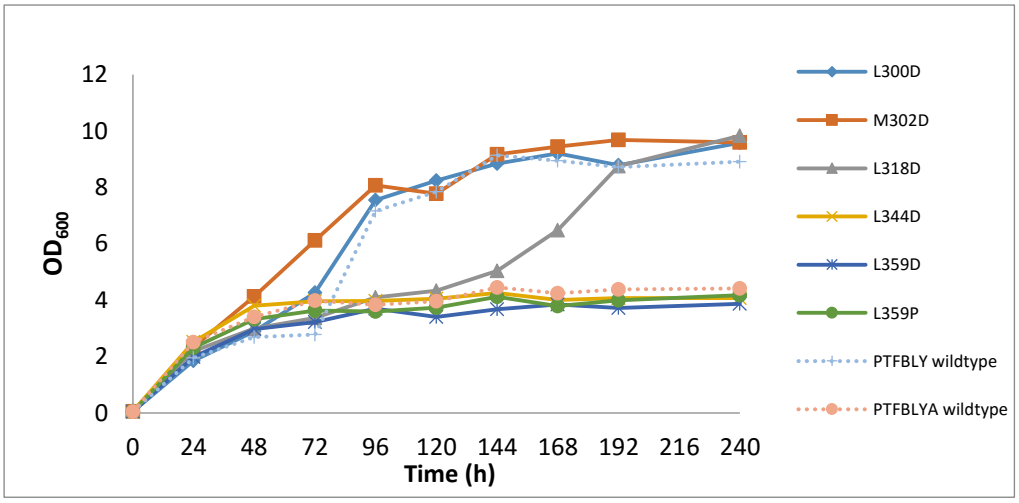

Figure 23 Growth curve of recombinant clones in 200 mL of minimum media

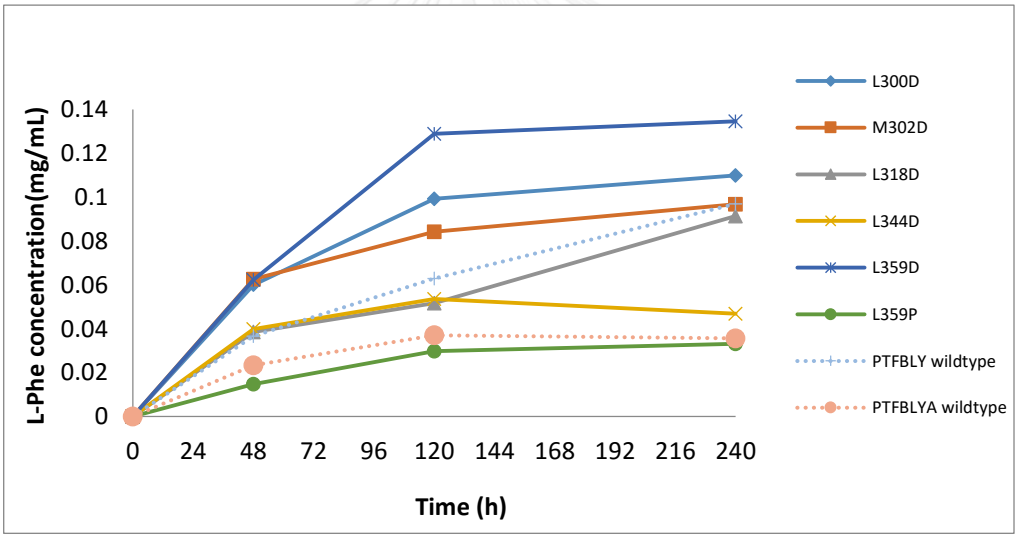

Figure 24 L-Phenylalanine production of recombinant clones in 200 mL of minimum media

3.6.2 Fed-batch fermentation

As reported in section 3.6.1, pPTFBLYA^{L359D} clone was selected for cultivation in 5 L bioreactor and compared with pPTFBLYA clone, using the same condition as reported by Thongchuang (2011).

For fed batch operation, an air flow rate of 1 vvm with agitation speed of 400 rpm was controlled during the experiment. The medium from the bioreactor was continuously withdrawn every 6 h interval until 96 h. The growth, L-Phe production and dissolved oxygen were shown in Figure 3.25 (A), (B) and (C), respectively.

The growth of pPTFBLYA^{L359D} clone cultured in bioreactor was 2.0 times higher than the shake flask culture because of highly aerobic condition. In contrast, the pPTFBLYA clone shared the growth pattern similar to that of shake flask culture. According to the result shown by Figure 3.25 (B), the L-Phe content of pPTFBLYA^{L359D} from fermented culture (176 mg/L) was nearly 1.3 fold as compared to the shake flask culture (134 mg/L) in section 3.6.1. Little production of L-Phe by pPTFBLYA^{L359D} might be from inappropriate medium composition and cultivating condition. The cultivating condition used in this experiment was optimized by Thongchuang (2011), but for pPYF, a clone carrying three essential genes for L-Phe production. Thus, the composition of glycerol medium should be adjusted and the cultivating condition should also be optimized for pPTFBLYA^{L359D}, the clone carrying seven essential genes.

As a result, L-Phe product of pPTFBLYA clone in fermenter (143 mg/L) was increased more than in shake flasks (35mg/L) about 4.1-fold. A better supply of oxygen in the bioreactor contributed its higher productivity compared to the shake flasks. L-Phe production of pPTFBLYA^{L359D} clone, however, was still more than that of

pPTFBLYA, although the mutant clone had a less increase of L-Phe production compared to shake flask culture.

Even though the fed-batch fermentation was done, the L-Phe product was quite lower than those previously reported on other fermentations. For example, fermentation of L-Tyr auxotrophic *E. coli* strain of which the high level of L-Phe was obtained from glucose substrate. Several factors including glucose feeding, L-Tyr feeding and oxygen supply were reported to improve L-Phe production (Gerigk et al., 2002; Konstantinov et al., 1991). Under fermentation process, the carbon flow through the L-Phe production pathway could be partially utilized to the production of acetic acid. This caused lowering in the amount of L-Phe product. Prevention of acetic acid excretion therefore can be achieved by control of DO. The total amount of tyrosine fed was used to provide an optimal balance between biomass synthesis and that of phenylalanine. Manipulation of glucose feeding was performed to deplete acetic acids which were produced by overfeeding, and at the same time, to restrict the CO_2 evolution caused by glucose limitation. The L-Phe production was successfully improved via those of optimized factors resulted in the L-Phe final concentration of 46 g*/L* with 17% yield. In this work, L-Phe production might be improved by considering all mentioned parameters.

Another strategy was done by using expression plasmid carrying the wild-type aroF ($aroF^{wt}$) instead of that feedback-resistant enzyme ($AroF^{fbr}$) under fed-batch fermentation. The L-Phe content of $AroF^{wt}$ was enhanced up to 34 g/L from that of $aroF^{fbr}$ strain (28 g/L) (Gerigk et al., 2002).

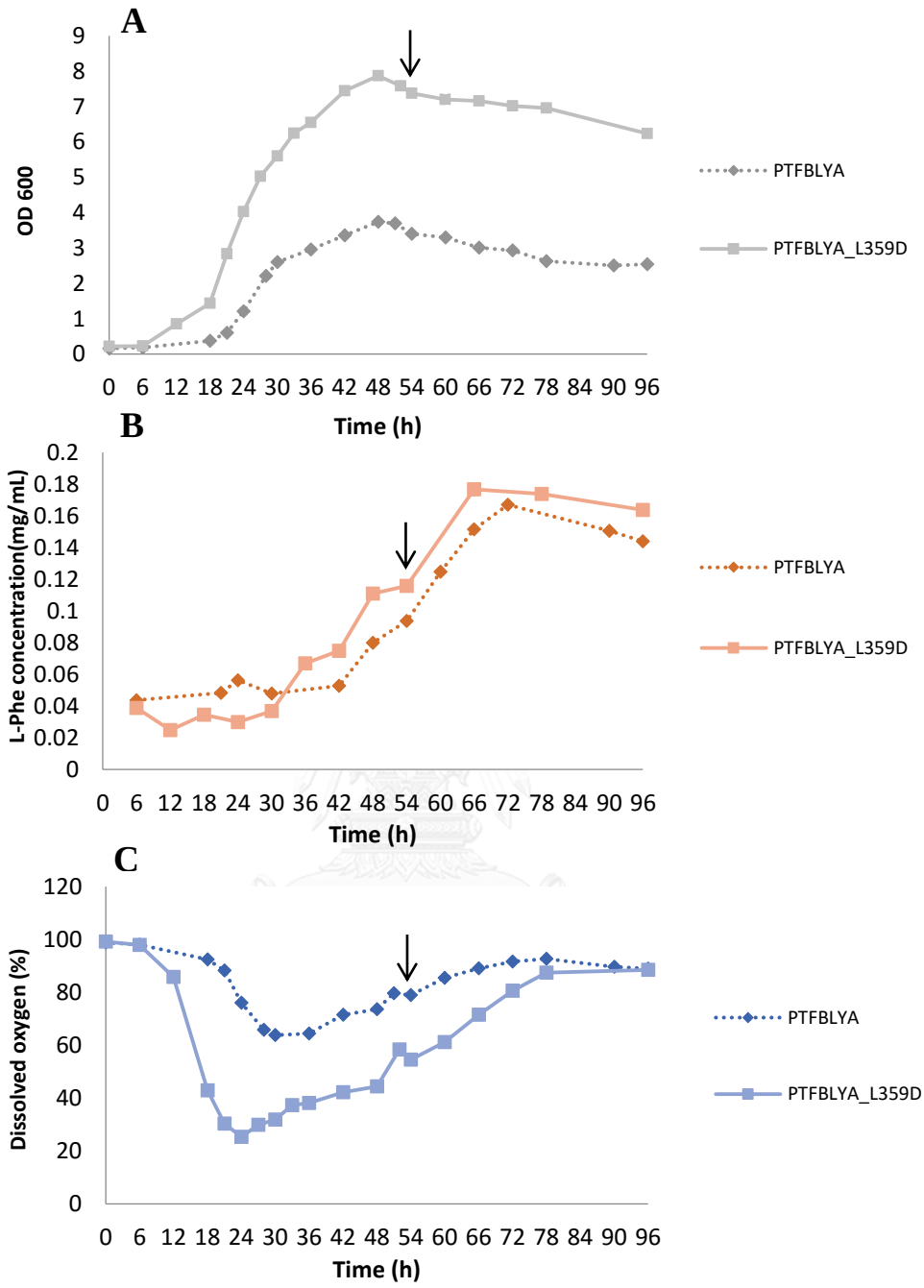

Figure 25 (A) Growth profiles, **(B)** L-Phe production profiles and **(C)** Dissolved oxygen profiles of *E. coli* BL21(DE3) harbouring pPTFBLYA and pPTFBLYA^{L359D} in 3.5 L fermentation at an aeration rate of 3.5 L/min and an impeller speed of 400 rpm. The feeding of new glycerol medium is indicated by arrow ⬇.

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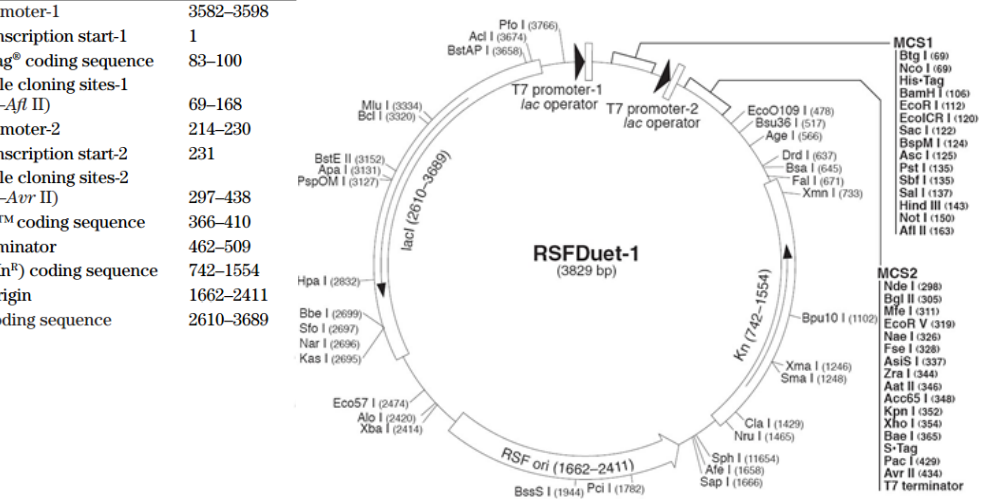
APPENDICES

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



ACYCDuetUP1 Primer #71178-3 (*Pfo* I) — T7 promoter-1

```
GCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA
```

T7 transcription start-1 — *lac* operator — rbs — *Nco* I — His•Tag

```
GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACTTTAATAAGGAGATATACCATGGGCAGCAGCCATCACCATCATCACCAC
                                                                MetGlySerSerHisHisHisHisHisHis
```

BspM I, *Sbf* I, *EcoI*CR I, *BamH* I, *EcoR* I, *Sac* I, *Asc* I, *Pst* I, *Sal* I, *Hind* III, *Not* I, *Afl* II, *Tat* I, DuetUP2 Primer #71180-3, *BsrG* I, DuetDOWN1 Primer #71179-3

```
AGCCAGGATCCGAATTCGAGCTCGGCGCGCCTGCAGGTCGACAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCC
SerGlnAspProAsnSerSerSerAlaArgLeuGlnValAspLysLeuAlaAlaAlaEnd
```

DuetUP1 Primer #71180-3 — T7 promoter-2 — T7 transcription start-2 — *lac* operator — rbs — *Nde* I — DuetDOWN1 Primer #71179-3

```
GCATAATCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACAT
```

Nde I, *Bgl* II, *Mfe* I, *EcoR* V, *Nae* I, *Fse* I, *Pvu* I, *AsiS* I, *Bae* I, *Zra* I, *Aat* II, *Kpn* I, *Acc65* I, *Xho* I, S•Tag

```
ATGGCAGATCTCAATTGGATATCGGCCGGCCACGCGATCGCTGACGTCGGTACCCTCGAGTCTGGTAAAGAAACCGCTGCTGCGAAATTTGAACGCCAG
MetAlaAspLeuAsnTrpIleSerAlaGlyHisAlaIleAlaAspValGlyThrLeuGluSerGlyLysGluThrAlaAlaAlaLysPheGluArgGln
```

S•Tag — *Pac* I, *Avr* II, *Eco*0109 I

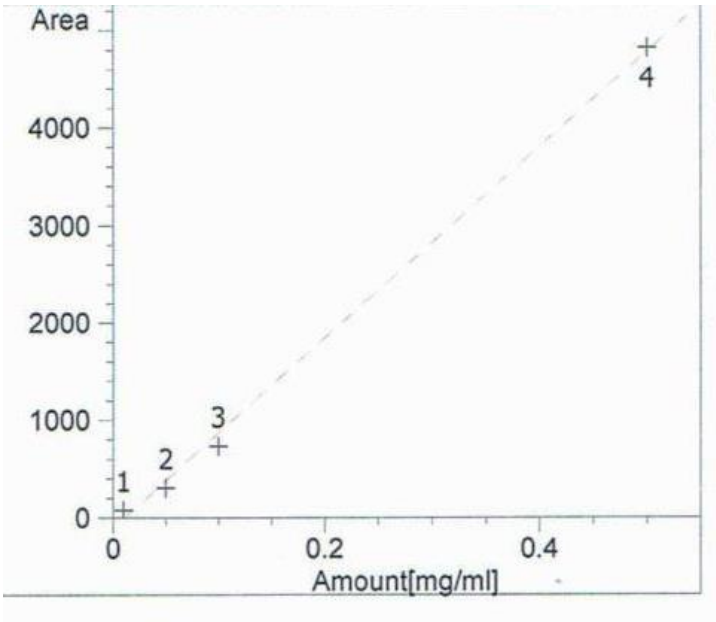
```
CACATGGACTCGTCTACTAGCGCAGCTTAATTAACCTAGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTG
HisMetAspSerSerThrSerAlaAlaEnd
```

T7 Terminator Primer #69337-3

pRSFDuet-1 cloning/expression regions

APPENDIX B

Calibration curve for determination of L-Phe concentration

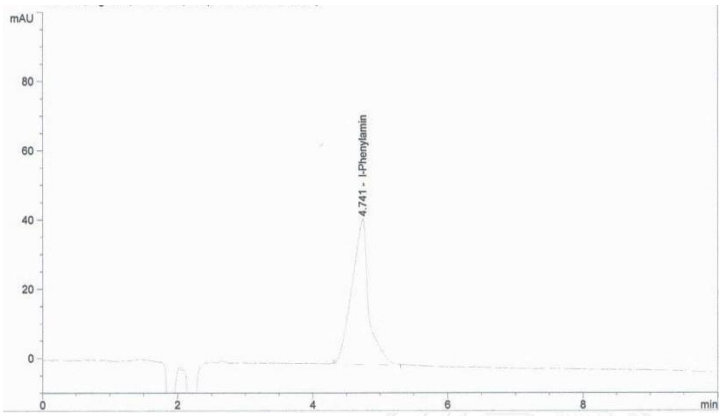



```
Residual Std. Dev.:  123.01205
Formula: y = mx + b
      m:   9780.30856
      b:   -103.94639
      x: Amount[mg/ml]
      y: Area
```

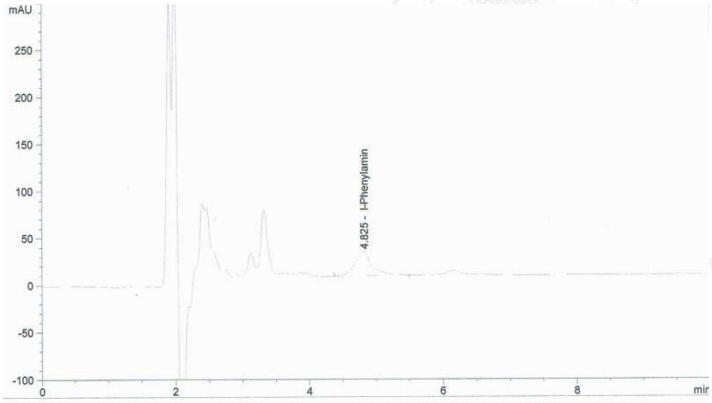
APPENDIX C

Chromatograms of L-phenylalanine

A) L-Phenylalanine



B) Sample

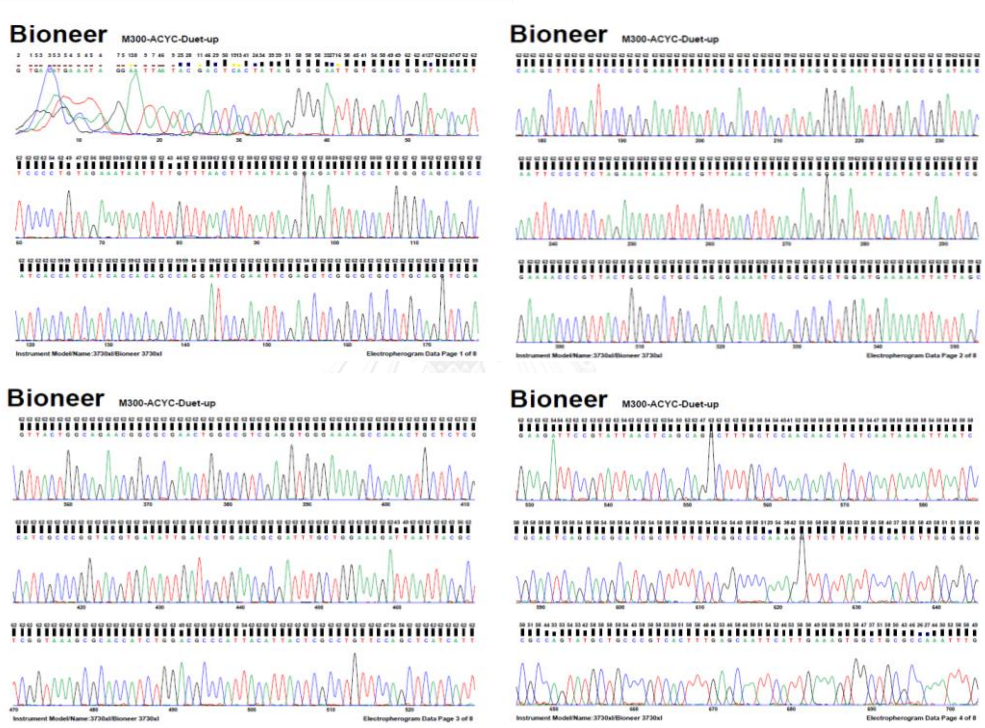


HPLC technique using Chirex **3126** (D) - penicillamine column
Mobilephase: 2 mM copper sulfate : methanol ratio (70:30)
flow rate: 1 mL/min
peaks were detected : 254 nm.

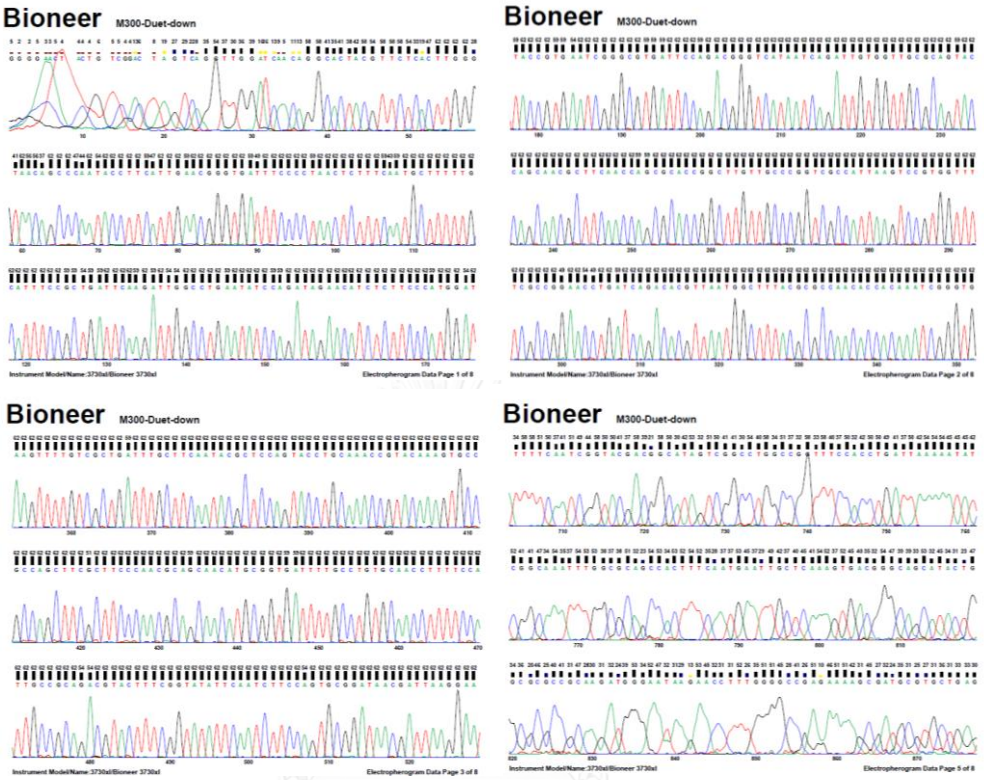
APPENDIX D

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{L300D} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



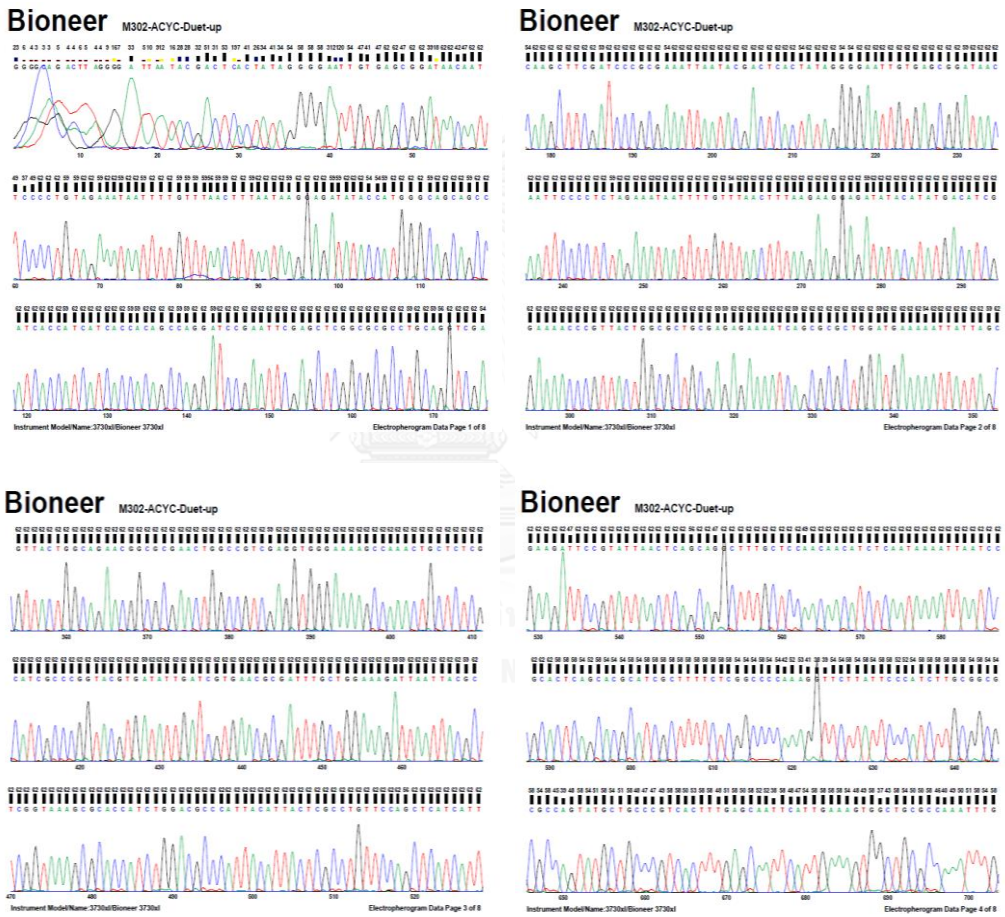
(B)



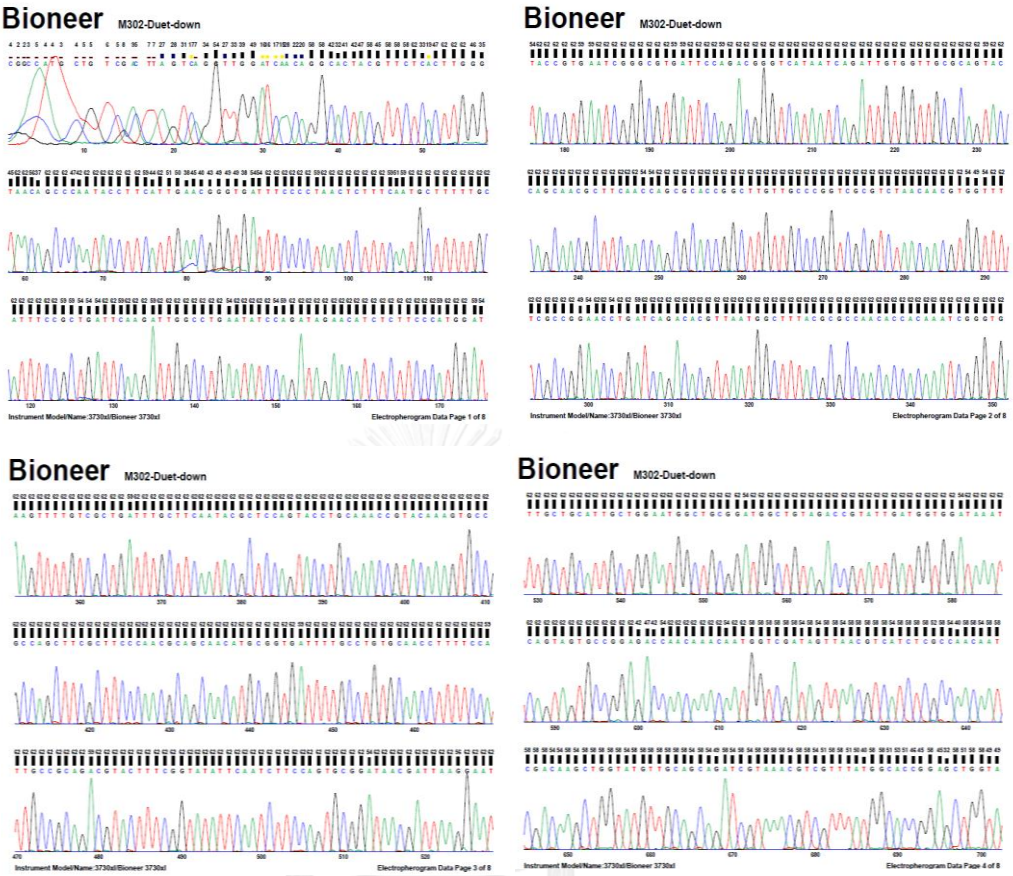
APPENDIX E

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{M302D} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



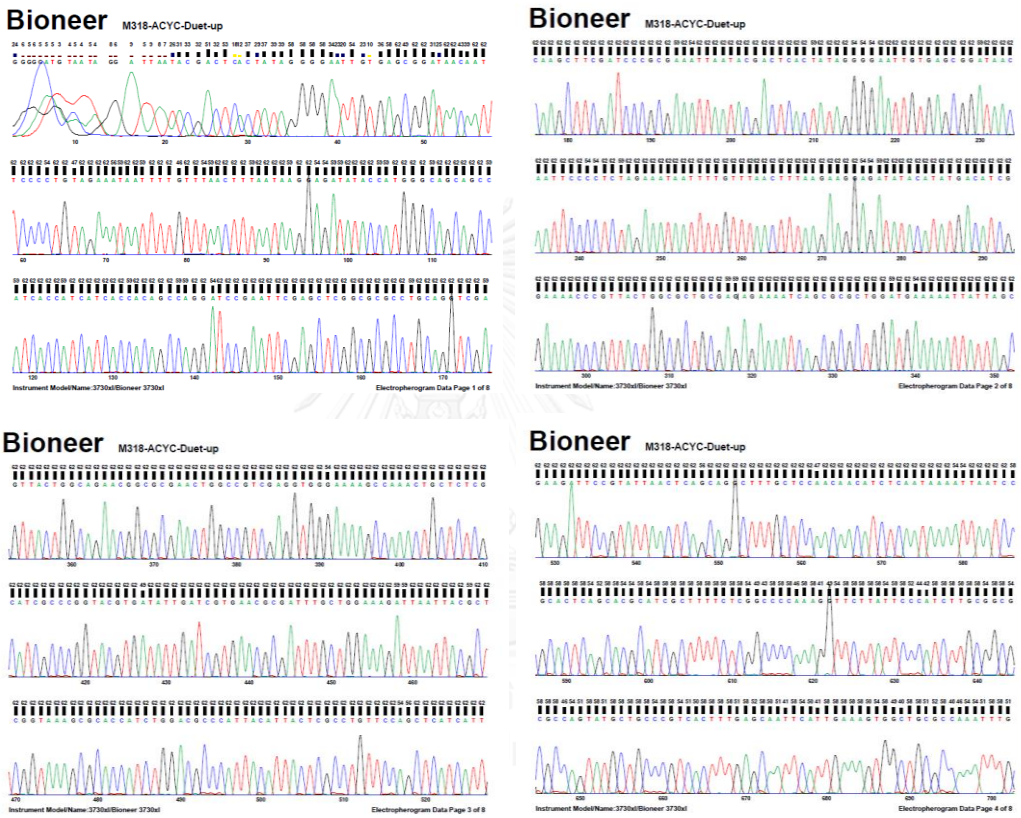
(B)



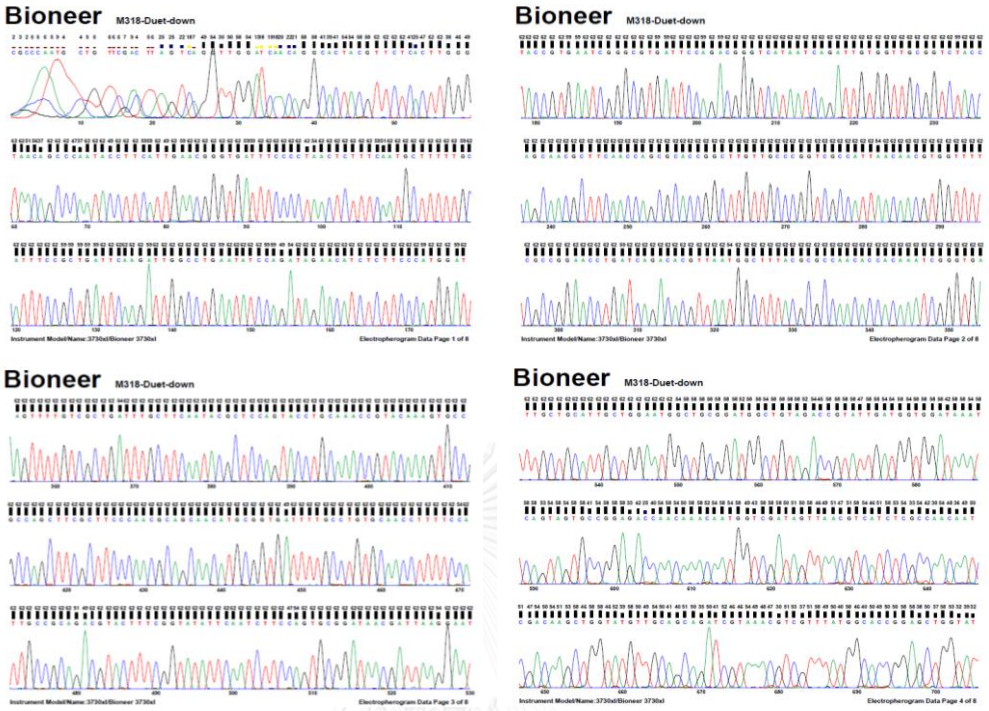
APPENDIX F

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{L318D} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



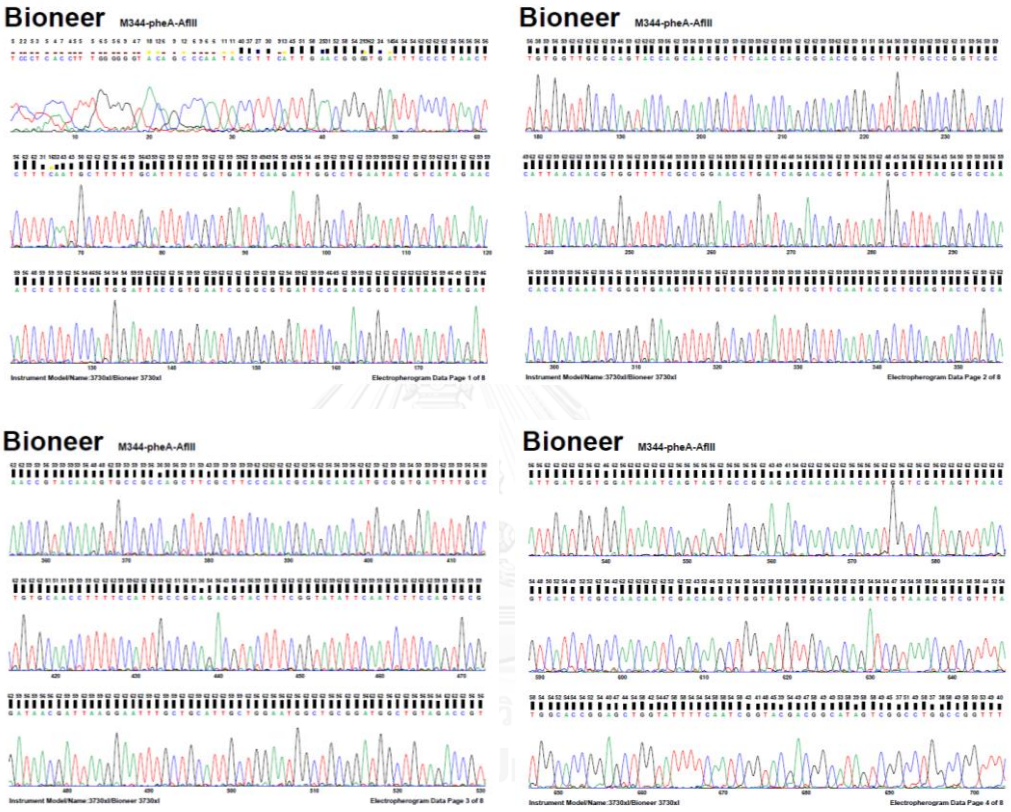
(B)



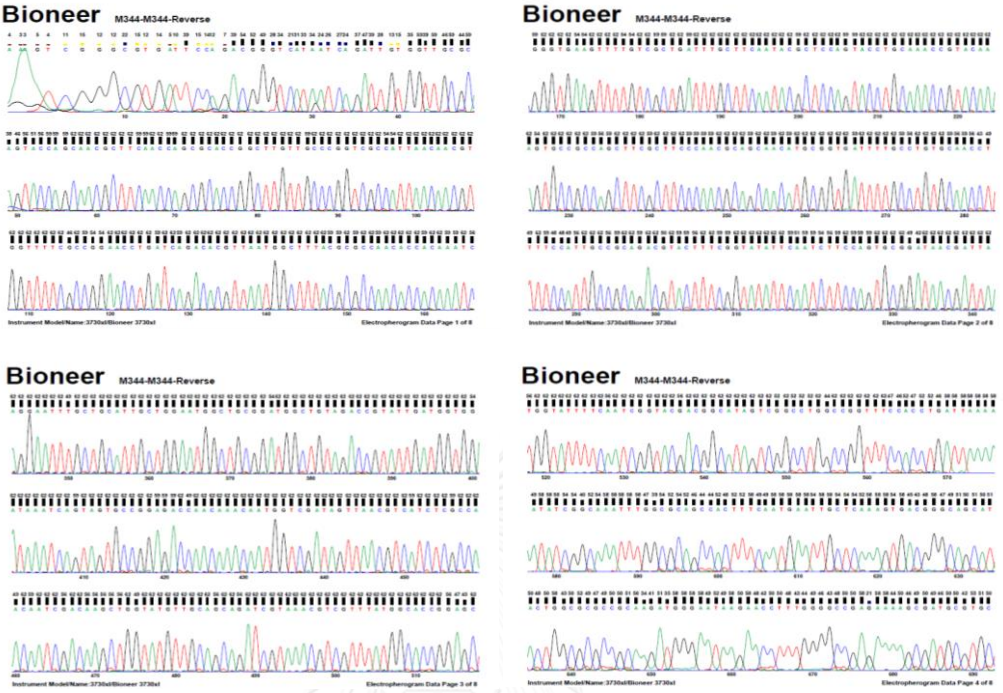
APPENDIX G

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{L344D} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



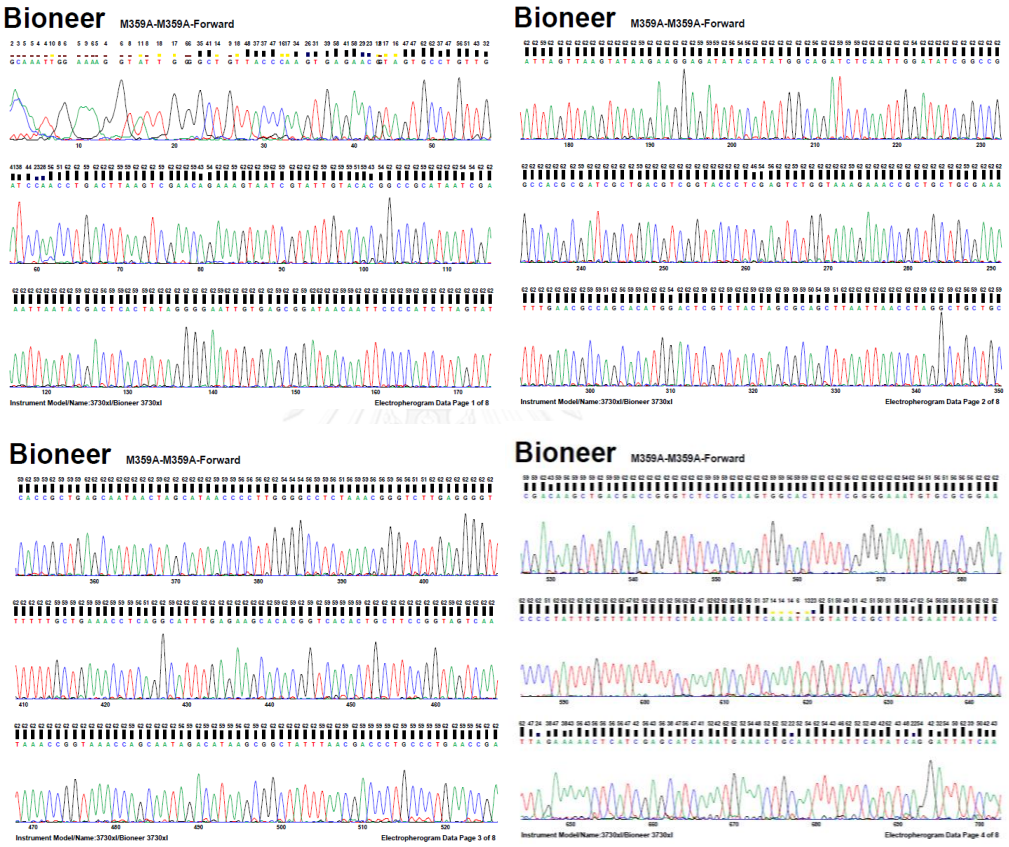
(B)



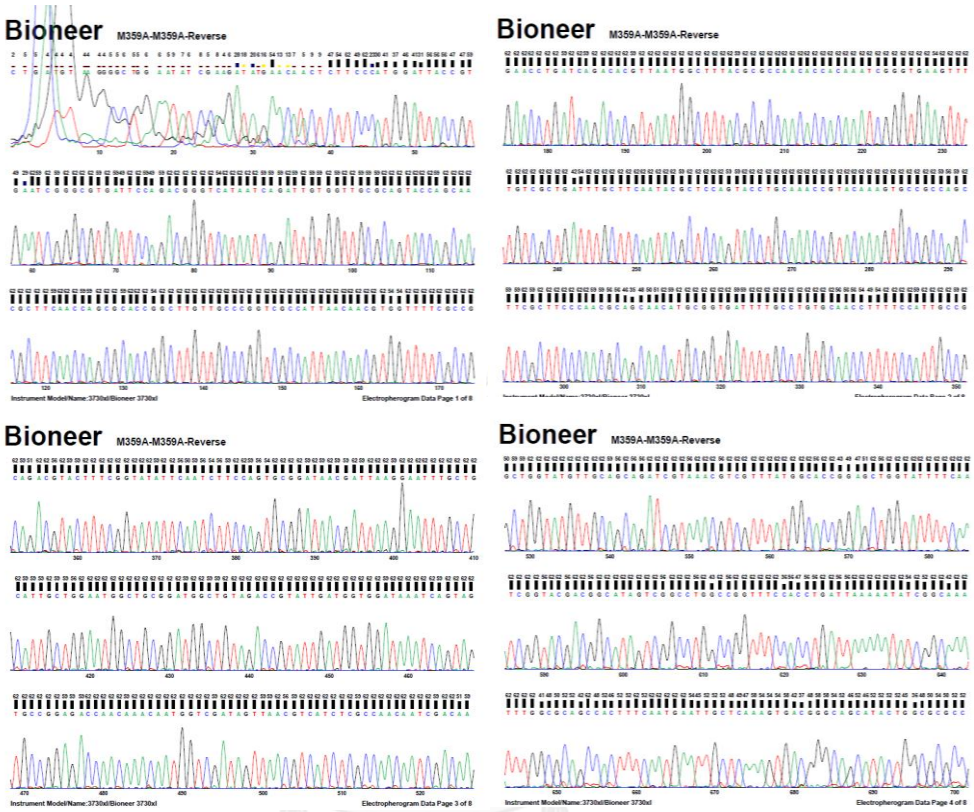
APPENDIX H

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{L359D} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



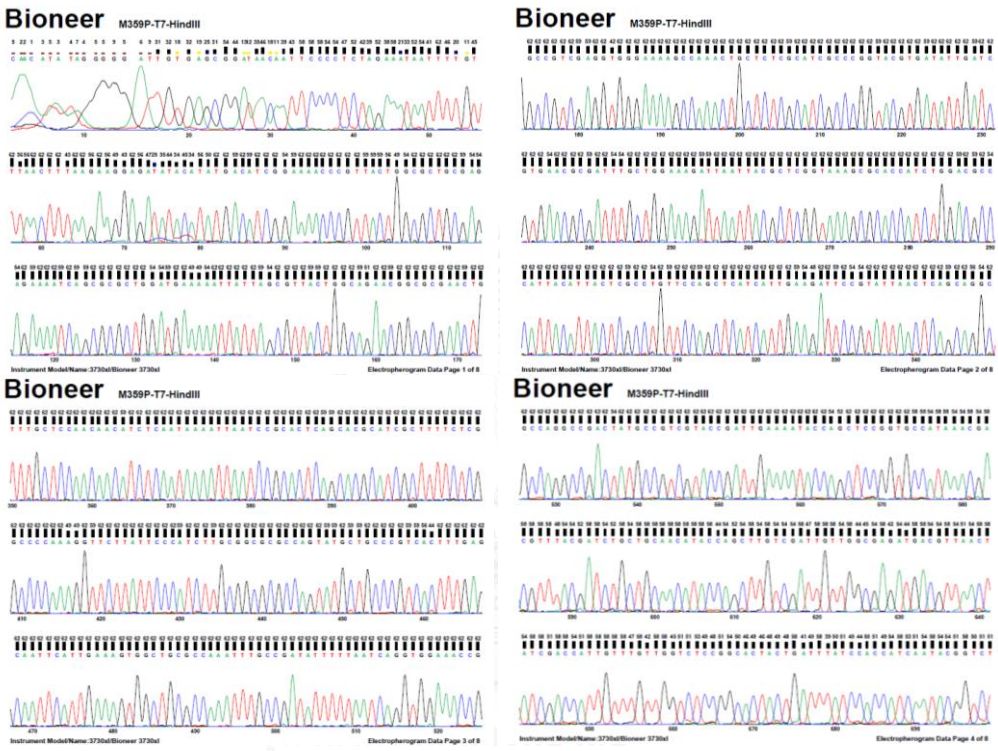
(B)



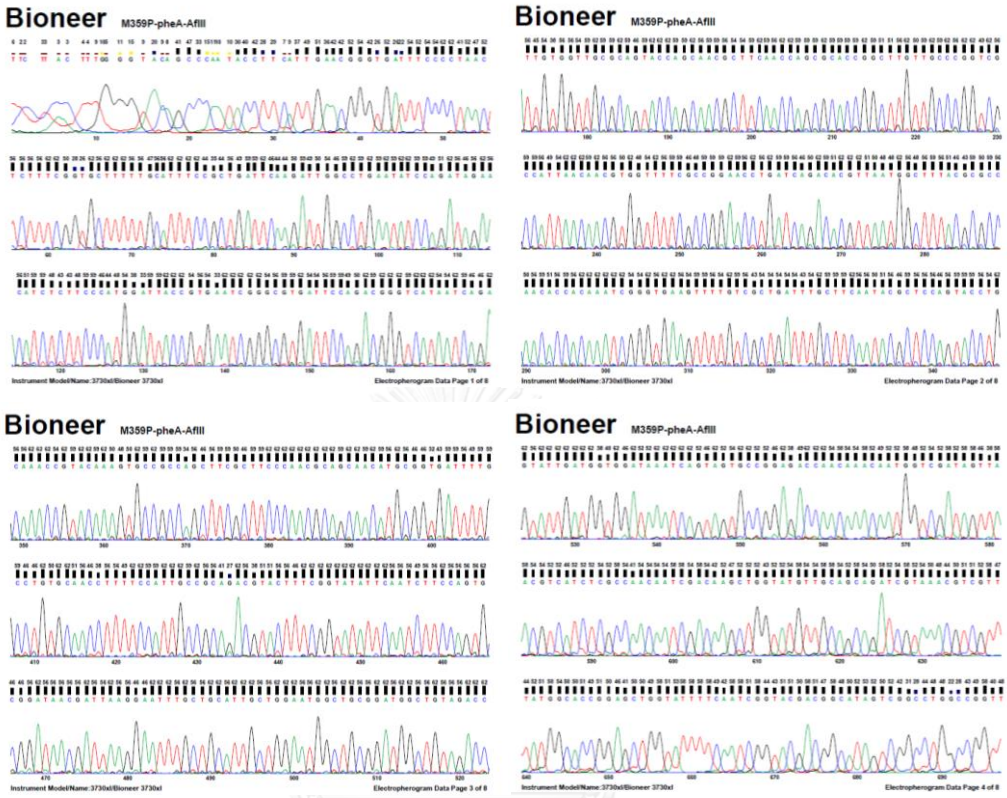
APPENDIX I

The sequencing chromatogram of *pheA*^{fbr} gene of pPheA^{L359P} using ACYCDuetUP1 (A) and DuetDOWN1 (B) primers.

(A)



(B)



VITA

Mr. Burawat Naksusuk was born on November 2, 1989 in Bangkok. He graduated with the degree of Bachelor of Science from the Department of Biochemistry, Faculty of Science, Chulalongkorn University in 2011. He has studied for the degree of Master of Science from Program in Biotechnology, Faculty of Science, Chulalongkorn University since 2012.