การผลิตแอล-ฟีนิลอะลานีนจาก *Escherichia coli* ที่มีเฮเทอโรโลกัสยีนที่กำหนดรหัสฟีนิลอะลานีนดีไฮโดรจิเนสและฟอร์เมตดีไฮโดรจิเนส

นายเมธี ขำดวง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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L-PHENYLALANINE PRODUCTION FROM *Escherichia coli* CONTAINING HETEROLOGOUS GENES ENCODING PHENYLALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE

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เมธี ขำดวง: การผลิตแอล-ฟีนิลอะลานีนจาก *Escherichia coli* ที่มีเฮเทอโรโลกัสยีนที่กำหนดรหัสฟีนิลอะลานีนดีไฮโดรจิเนสและฟอร์เมตดีไฮโดรจิเนสดีไฮโดรจิเนสเข้าสู่ *Escherichia coli*. (L-PHENYLALANINE PRODUCTION FROM *Escherichia coli* CONTAINING HETEROLOGOUS GENES ENCODING PHENYLALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE)
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คณะวิจัยของเราได้ทำการโคลนยีนฟีนิลอะลานีนดีไฮโดรจิเนส จาก *Bacillus badius* BC1 เข้าสู่ *Escherichia coli* JM109 โดยใช้เวคเตอร์ pUC18 เพื่อเพิ่มการผลิตฟีนิลอะลานีนดีไฮโดรจิเนส ให้สูงขึ้น และสามารถรีเจนเนอเรตโคเอนไซม์ $NADH^+$ ได้ภายในเซลล์เพียงเซลล์เดียว งานวิจัยนี้ได้ทำการนำฟีนิลอะลานีนดีไฮโดรจิเนสยีน (*phedh*) และฟอร์เมตดีไฮโดรจีเนสยีน (*fdh*) เข้าสู่ *Escherichia coli* BL21(DE3) และ *Escherichia coli* BL21(DE3)pLysS โดยใช้เวคเตอร์ pET-17b พบว่า แอคติวิตีของฟีนิลอะลานีนดีไฮโดรจิเนส และฟอร์เมตดีไฮโดรจิเนสของโคลนที่มี heterologous gene (pETPF และ pETFP) สูงขึ้นเมื่อเทียบกับโคลนเดิม (pUCPheDH และ pUCFDH) นอกจากนั้นแอคติวิตีที่ได้จากโคลนที่มี heterologous gene ยังไม่มีความแตกต่างจากโคลนที่มียีน *phedh* หรือ *fdh* เพียงยีนเดียว (pETPheDH และ pETFDH) การผลิตฟีนิลอะลานีนของโคลนที่มี heterologous gene สูงกว่าโคลนที่มียีนเดียว (pETPheDH) ประมาณ 2 เท่า โดยฟีนิลอะลานีนที่ผลิตได้อยู่ในรูปแบบ L- ทั้งหมด และชนิดของเซลล์เจ้าบ้านไม่มีผลต่อปริมาณการผลิตฟีนิลอะลานีน นอกจากนี้เซลล์รีคอมบิแนนท์ที่บ่มกับเฮกเซนเป็นเวลา 5 นาที มีการผลิตฟีนิลอะลานีนเพิ่มขึ้น 14–30%

ภาควิชา .....หลักสูตรเทคโนโลยีชีวภาพ...
สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....
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METHEE KHAMDUANG: L-PHENYLALANINE PRODUCTION FROM *Escherichia coli* CONTAINING HETEROLOGOUS GENES ENCODING PHENYLALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE. THESIS ADVISOR: ASSIST. PROF. KANOKTIP PACKDIBAMRUNG, Ph.D., 156 pp. ISBN 974-53-1597-4

Our research group successfully cloned phenylalanine dehydrogenase from *Bacillus badius* BC1 into *Escherichia coli* JM109 by using pUC18 vector. To enhance the enzyme activity and regenerate NAD^+ in a single cell, heterologous genes of phenylalanine dehydrogenase gene (*phedh*) and formate dehydrogenase gene (*fdh*) in a high expression vector pET-17b (pETPF and pETFP) were cloned into two kinds of host cell, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS. The heterologous gene expression clones had higher phenylalanine dehydrogenase and formate dehydrogenase activities than those of their original clones (pUCPheDH and pUCFDH). Moreover, their activities were not different from those of their single gene clones (pETPheDH and pETFDH). For phenylalanine production, both of heterologous gene clones could produce optically pure L-phenylalanine 2 times higher than pETPheDH clone. Type of host cell had no effect on phenylalanine production. In addition, phenylalanine production was increased in the range of 14-30% upon the incubation of recombinant cells with hexane for 5 minutes.

Department …...Biotechnology…

Field of study….Biotechnology… Student's signature…….....

Academic year....2004……. Advisor's signature…….....

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ABBREVIATIONS

A	absorbance, 2´-deoxyadenosine (in a DNA sequence)
bp	base pairs
BSA	bovine serum albumin
C	2´-deoxycytidine (in a DNA sequence)
^{O}C	degree Celsius
cm	centrimeter
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2´-deoxynucleoside 5´-triphosphate
EDTA	ethylene diamine tetraacetic acid
G	gram
FDAA	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide
FDH	formate dehydrogenase
G	2´-deoxyguanosine (in a DNA sequence)
hr	hour
HCl	hydrochloric acid
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid and single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
KOH	potassium hydroxide
KPB	potassium phosphate buffer
l	liter
LB	Luria-Bertani
µg	microgram
µl	microliter
µmol	micromole
µM	micromolar
M	mole per liter (molar)

mA	milliampere
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PheDH	phenylalanine dehydrogenase
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl sulfate
T	2´-deoxythymidine (in a DNA sequence)
TB	Tris-borate buffer
TE	Tris-EDTA buffer
TLC	thin-layer chromatography
T_m	melting temperature, melting point
UV	ultraviolet
V	voltage
v/v	volume by volume
w/w	weight by weight

CHAPTER I

INTRODUCTION

1.1 Amino acids

Amino acids are biomolecule found in all organism. They can be classified into two groups by ability of the polarized light plane rotation: L-formed and D-formed. L-amino acids plays important role in all life by serving as building block of enzyme, hormone, antibody and protein. Furthermore, they can also balance buffering capacity in blood and often function as chemical messengers in cell communication (Holum, 1982). In contrast, D-formed amino acid is rarely found. The optically active amino acids have been extensively studied. They are commonly occurring moiety in the rational design of chiral drugs such as anticancer compounds and viral inhibitors (Taylor *et al.*, 1998).

L-amino acids have stimulated the research on various methods for their synthesis such as chemical reaction, extraction from protein hydrolysates and fermentation (Hummel *et al.*, 1987). However, the products contain both D-formed and L-formed amino acid. Therefore, some researchers have attempted to produce L-amino acid by enzymatic method e.g. L-amino acid transaminase, amino acid racemase (Berberich *et al.*, 1968), L-aminopeptidase (Kamphuis *et al.*, 1992), and L-amino acid-β-decarboxylase (Yamamoto *et al.*, 1980). These methods lead to enantiomerically pure compounds. A recent alternative method is the enzymatic reductive amination of α-keto acid by amino acid dehydrogenase, a route which has the advantage of complete enantioselectivity and up to 100% towards selectivity of

desired product. The enzymatic reaction is performed in a single reaction step and allows synthesis of non-proteinogenic L-amino acids are not available through hydrolysis or fermentation (Krix *et al.*, 1997).

1.2 L-phenylalanine

L-phenylalanine is L-aromatic amino acid, which is essential for human nutrition. It was used as a component of amino acid infusions for medical purposes. L-phenylalanine is also a raw material utilized in the manufacture of a dipeptide sweetener known as aspartame. Its applications range from feed to food and pharmaceutical products. At present, L-phenylalanine biosynthesis genes have been well characterized and the enzymology of L-phenylalanine biosynthesis has been extensively investigated (Liu *et al.*, 2004).

Production of L-aromatic amino acids and derivative compounds is of considerable industrial importance (Bongaerts *et al*., 2001). Various methods have been shown to achieve the production of L-phenylalanine. These include extraction from natural protein, chemical synthesis and production from enzymatic method. Many enzymatic processes are known to produce L-phenylalanine from many substrate including *trans*-cinnamic acid (Yamada *et al.*, 1981), 5-banzylhydantoin and acetamidocinnamic acid (Nakamichi *et al*., 1986) by using L-phenylalanine amino-lyase, from phenylpyruvate by using L-phenylalanine dehydrogenase (Hummel *et al*., 1989) and from phenylpyruvate by using aminotransferase (Calton *et al*., 1986). The production method of L-phenylalanine from phenylpyruvate is very promising as a commercial process because it can be obtained at low cost and the productivity of L-phenylalanine is very high (Nakamichi *et al.*, 1989).

1.3 Phenylalanine dehydrogenase

Amino acid dehydroganase catalyzes the pyridine nucleotide coenzyme-dependent revesible deamination of amino acid to the corresponding keto acid, and provide an important metabolic route for the interconversion between inorganic and organic nitrogen compounds (Oikawa *et al.,* 2001). The metabolic function can be described as the balance of both amino acid and keto acid synthesis. The amino group is firstly removed as free ammonia before the carbon skeleton of an amino acid can be metabolized for energy through the glycolysis and/or TCA cycle. The participation of $NAD(P)^+$ makes these enzyme systems a valuable tool for the analysis of L-amino acids or their corresponding keto acids. By reductive amination of the keto acid, large quantity of L-amino acids are obtained because the equilibrium for the reaction favors amino acid formation. The general equation for this reaction is illustrated in Figure 1.1 (Brunhuber and Blanchard, 1994).

Amino acid dehydrogenase has considerably commercial potential for the chrial synthesis of natural amino acid as well as novel nonproteogenic amino acid using in the pharmaceutical industry. It is also use as diagnostic reagents to monitor the serum levels of amino acid which accumulate in a range of metabolic diseases (Baker *et al.,* 1997). Amino acid dehydrogenases are catagorized based on the specificity they display toward their amino acid substrates. More than ten kinds of them have been found so far in various organisms as shown in Table 1.1 (Ohshima and Soda, 1989; Ohshima and Soda, 1990; Hummel and Kula, 1989).

Phenylalanine dehydrogenase (PheDH) is one of the amino acid dehydrogenase. The distribution of this enzyme is limited to some groups of gram-positive, spore-forming bacteria including actinomycetes (Asano *et al*., 1998).

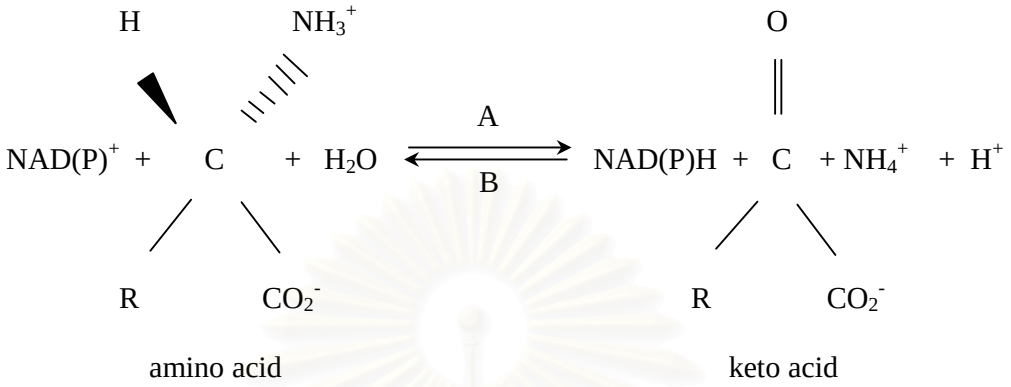


Figure 1.1 The general reaction of L-amino acid dehydrogenases

(A) oxidative deamination (B) reductive amination

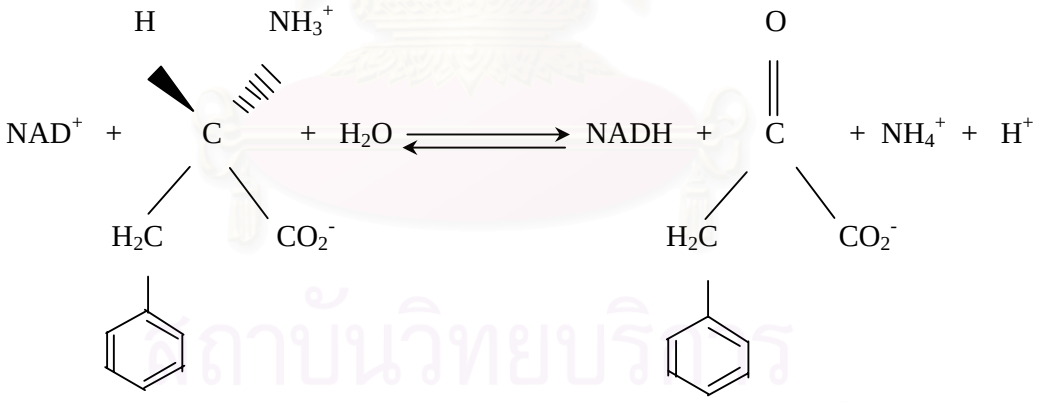


Figure 1.2 The reaction of L-phenylalanine dehydrogenase

Table 1.1 The group of NAD(P)+ - dependent amino acid dehydrogenase

EC number	Enzyme	Coenzymes	Major source
1.4.1.1	AlaDH	NAD^+	Bacteria (*Bacillus, Streptomyces, Anabena, Pseudomonas, Thermus, Rhodobacter, Arthrobacter, Enterobacter, Phormidium), chrorella*
1.4.1.2	GluDH	NAD^+	Plants, fungi, yeasts, bacteria
1.4.1.3	GluDH	$NAD(P)^+$	Animals (bovine liver, chicken liver), tetrahymena, bacteria (*Clostridium, Thiobacillus*)
1.4.1.4	GluDH	$NADP^+$	Plants, *Euglena gracilis, Chrorella sarokiniana*, fungi, yeasts, bacteria
1.4.1.5	L-Amino acidDH	$NADP^+$	Bacteria (*Clostridium sporogenes)*
1.4.1.7	SerDH	NAD^+	Plants (parsley)
1.4.1.8	ValDH	NAD^+, $NADP^+$	Bacteria (*Streptomyces, Alcaligenes faecalis, Planococcus*), plants (pea, wheat)
1.4.1.9	LeuDH	NAD^+	Bacteria (*Bacillus, Clostridium, Thermoactinomyces*)
1.4.1.10	GlyDH	NAD^+	Bacteria (*Mycobacterium tuberculosis*)
1.4.1.11	DAHDH	NAD^+, $NADP^+$	Bacteria (*Clostridium, Brevibacterium*)
1.4.1.12	DAPDH	$NAD(P)^+$	Bacteria (*Clostridium*)
1.4.1.15	LysDH (cylizing)	NAD^+	Human liver
1.4.1.16	DAPMDH	$NADP^+$	Bacteria (*Corynebacterium glutamicum, Brevibacterium sp., Bacillus sphaericus*)
1.4.1.17	MethylalaDH	$NADP^+$	Bacteria (*Pseudomonas sp.)*
1.4.1.18	LysDH(Lys-6-DH)	NAD^+	Bacteria (*Agrobacterium tumefaciens, Klebsiella pneumoniae*)
1.4.1.19	TyrDH	$NAD(P)^+$	Plants (*Nicotiana tabacum,Pisum sativum, Spinacia oleracea*)
1.4.1.20	PheDH	NAD^+	Bacteria (*Sporosarcina ureae, Bacillus sphaericus, Rhodococcus marinas, Thermoactinomyces intermedius*)
1.4.1.-	AspDH	$NADP^+$	Bacteria (*Klebsiella pneumoniae*)

DH, dehydrogenase; $NAD(P)^+$, NAD^+ and $NADP^+$-nonspecific; DAHDH: L-erythro-3,5-diaminohexanoate dehydrogenase; DAPDH, 2,4-diaminopentanoate dehydrogenase; DAPMDH, meso-2,6-diaminopimelate dehydrogenase; MethylalaDH, *N*-methyl-L-alanine dehydrogenase.

Source: Ohshima and Soda, 2000

This enzyme catalyzes the reversible oxidoreduction reactions between L-phenylalanine and phenylpyruvate in the present of NAD[H]. Much attention had been paid to this enzyme because it is useful as an industrial catalyst in the asymmetric synthesis of L-phenylalanine and related L-amino acid from their keto analogs, and as a clinical reagent for the selective determination of L-phenylalanine and phenylpyruvate and NADH as shown in figure 1.2 .

1.3.1 Properties and characterization of phenylalanine dehydrogenase from various sources

The enzyme was first discovered in *Brevibacterium* sp. in 1984 (Hummel *et al.*, 1984) and since then, several other bacterial PheDHs were identified and characterized. *Sporosaccina ureae* enzyme had a molecular weight of 305,000, while that from *Bacillus sphaericus* had a molecular weight of 340,000. Each enzyme was composed of eight identical subunits. The *Sporosarcina ureae* enzyme showed high substrate specificity on L-phenylalanine in the oxidative deamination, while the enzyme from of *Bacillus sphaericus* can use both of L-phenylalanine and L-tyrosine. The enzyme from these 2 sources had lower substrate specificity in the reductive amination on α-keto acid substrate such as phenylpyruvate, *p*-hydroxyphenylpyruvate, and α-keto-γ-methylthiobutyrate (Asano and Nakazawa, 1985). In 1987, Asano and Nakazawa found that phenylalanine dehydrogenase from *Bacillus badius* exhibited higher specific with substrate than those of the previously reported strains. Ohshima *et al.* (1991) purified and characterized phenylalanine dehydrogenase from *Thermoactinomyces intermedius* and found that this enzyme was thermostable which mean, it did not inactivated when incubated at 70^{o}C, pH 7.2 for 60 minutes. This enzyme consisted of six identical, subunit with molecular weigth

of 41,000. The enzyme preferably acts on L-phenylalanine and its keto analog, phenylpyruvate, in the presence of NAD^+ and NADH, respectively. Initial velocity and product inhibition studies showed that the oxidative deamination proceeded through a sequential ordered binary-ternary mechanism. L-methionine utilizing bacteria were isolated and screened for phenylalanine dehydrogenase activity. A bacterial strain showing the enzyme activity was chosen and classified in the genus *Microbacterium*. Its enzyme is composed of eight identical subunits with a molecular weight of approximately 41,000. No loss of enzyme activity was observed upon incubation at 55^oC for 10 min (Asano and Tanetani, 1998). A particularly stable phenylalanine dehydrogenase was identified from *Rhodococcus* sp. M4. It was a tetramer in molecular weight of 39,500 (Hummel *et al*., 1987). The *Rhodococcus* enzyme has limited substrate activity with phenylpyruvate analogues, although phenylketobutyrate is a reasonable good substrate for the enzyme. The properties of various microbial phenylalanine dehydrogenase are shown in Table 1.2

1.3.2 Cloning of phenylalanine dehydrogenase

Though slight PheDH cloning works have been reported, they are useful not only in the study of evolutionary relationship among amino acid dehydrogenase but also for high amount of the enzyme preduction. In 1987, Asano and his colleague cloned PheDH from *Bacillus badius* IAM11059 into *E. coli* RRI by using expression vector pBR322 (Asano *et al*., 1987). After that, the gene from *Thermoactinomyces intermedius* was ligated into the *Bam*HI site of pUC18 and transformed into *E. coli* MV1184 (Takada *et al*., 1991). The *phedh* consists of 1,098 bp and encoded 366 amino acid residues. The level of PheDH in *E. coli* clone was very low about 0.35% of the total soluble protein. In 1994, Norbert and colleague used PCR technique for

Table 1.2 Comparison of properties of microbial phenylalanine dehydrogenases

Properties	Sources			
	B. sphaericus	*S. ureae*	*B. badius*	*R. maris*
Native M_r (subunit M_r), kDa	340 (41 x 8)	310 (41 x 8)	335 (41 x 8)	70 (36 x 2)
Specific activity of final preparation (U/mg protein):	111	84	68	65
Optimum pH:				
Deamination	11.3	10.5	10.4	10.8
Amination	10.3	9.0	9.4	9.8
Thermostability (^oC)	55	<40	<55	35
K_m (mM)				
NAD	0.17	0.14	0.15	0.25
L-Phe	0.22	0.096	0.088	3.80
NADH	0.025	0.072	0.21	0.043
Phenylpyruvate	0.40	0.16	0.106	0.50
Ammonia	78	85	127	70

	Nocardia sp.	*Thermoactinomyces intermedius*	*Microbacterium* sp.
Native M_r (subunit M_r), kDa	42 (42 x 1)	270 (41 x 6)	330 (41 x 8)
Specific activity of final preparation (U/mg protein):	30	86	37
Optimum pH:			
Deamination	10	11	12
Amination	-	9.2	12
Thermostability (^oC)	<53	70	55
K_m (mM)			
NAD	0.23	0.07	0.20
L-Phe	0.75	0.22	0.10
NADH	-	0.025	0.07
Phenylpyruvate	0.06	0.045	0.30
Ammonia	96	106	85

Source: Ohshima and Soda, 2000

Amplify *phedh* from *Rhodococcus* sp., inserted the gene into expression vector pET-3d and then transformed into *E. coli* BL21(DE3). Morever, *phedh* from *B. badius* BC1 was cloned and expressed in *E. coli* JM109 using plasmid vector pUC18. The PheDH activity of *E. coli* clone was about 60 times higher than that of wild type strain (Charoenpanich, 2001). Recently, *phedh* from *B. sphaericus* was cloned and expressed in *E. coli* BL21(DE3) using a vector pET-16b. Expression of *phedh* under T7 promoter was over 140 fold greater than that of the wild type (Omidinia *et al*., 2003)

1.3.3 Application of phenylalanine dehydrogenase

The application of phenylalanine dehydrogenase can be categorized into two major fields: industrial and medical fields. Enzyme-catalyzed reductive amination is potentially useful for the production of optically pure amino acids such as L-phenylalanine, homophenylalanine and allysine ethylene acetal [(*S*)-2-amino-5-(1,3-dioxolan-2-yl)-pentanoic acid]. L-phenylalanine is a main intermediate of the artificial sweetener:aspartame (Asano *et al.*, 1999), while L-homophenylalanine is a component of an angiotensin converting enzyme (ACE) inhibitor used in the treatment of hypertension and heart failure (Hummel *et al*., 1984; and Hummel *et al*., 1987). Allysine ethylene acetal is one of three building blocks used for an alternative synthesis of VANLEV (omapatrilat, BMS 186716), a vasopeptidase inhibitor which is now in clinical trials (figure 1.3). This substance was prepared from the reductive amination of the corresponding keto acid using phenylalanine dehydrogenase from *T. intermedius*. The reaction requires ammonia and NADH as shown in figure 1.4. NAD^+ produced during the reaction was recycled to NADH by the oxidation of formate to carbon dioxide using formate dehydrogenase (FDH). The enzyme is also

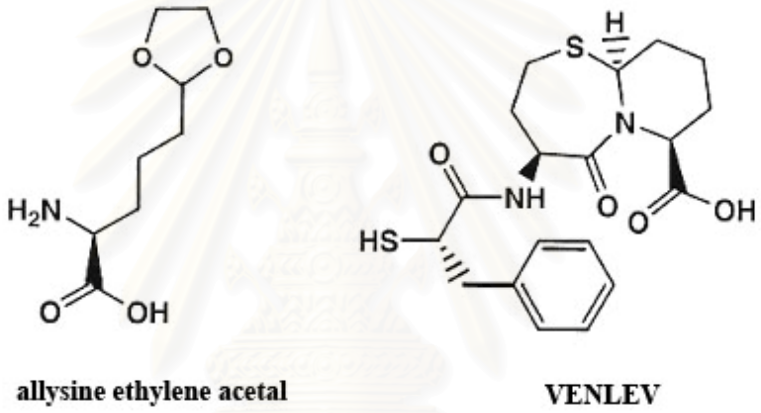
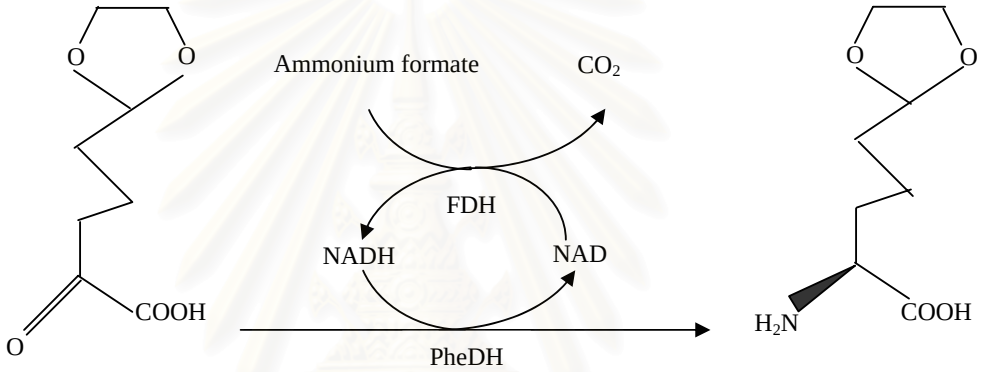


Figure 1.3 Structures of allysine ethylene acetal and VANLEV

Source: Hanson *et al*., 2000



keto acid acetal acetal amino acid

Figure 1.4 Scheme for conversion of the corresponding keto acid acetal to acetal amino acid

Source: Hanson *et al*., 2000

being developed as a biosensor to screen for phenylketonuria (PKU) (Huang *et al*., 1998; and Rivero *et al*., 2000).

1.4 Regeneration of NADH

NAD^+-dependent dehydrogenases have strong potential for practical application such as L-amino acid production in pilot scale. However, their use is still limited because of the requirement for stoichiometric amounts of rather expensive coenzymes (Fry *et al*., 1994; Nakamura *et al*., 1988), photochemical and enzymatic methods. Among those methods suggested, enzymatic means of NADH regeneration are most promising. Concerning the enzymatic regeneration, the use of glucose-(6-P)-dehydrogenase (Wong and Whitesides, 1981), alcohol dehydrogenase (Wong and Whitesides, 1982), lactate dehydrogenase (Davies *et al*., 1974; Wandrey *et al*., 1984), glucose dehydrogenase and formate dehydrogenase (FDH) are well known. The general scheme for enzymatic synthesize of D-, L-amino acids by NADH regeneration with coupled reaction of FDH and second enzyme (Wichmanm *et al*., 1981).

The synthesis of various D-amino acids by a multienzyme system has been developed. In this system, D-amino acids are produced from the corresponding keto acids and ammonia by coupling four enzyme reactions catalyzed by D-amino acid aminotransferase, phenylalanine racemase, phenylalanine dehydrogenase, and formate dehydrogenase illustrated in figure 1.5 (Galkin *et al*., 1997).

Continuous production of L-amino acids from their corresponding keto acid analogs was studied in an ultrafiltration membrane reactor. As shown in figure 1.6, the system contained PheDH, FDH and NAD^+. NAD^+ was covalent binded to

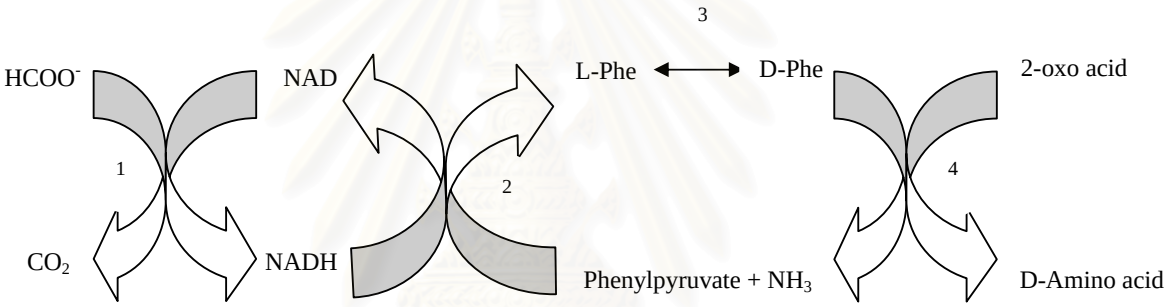
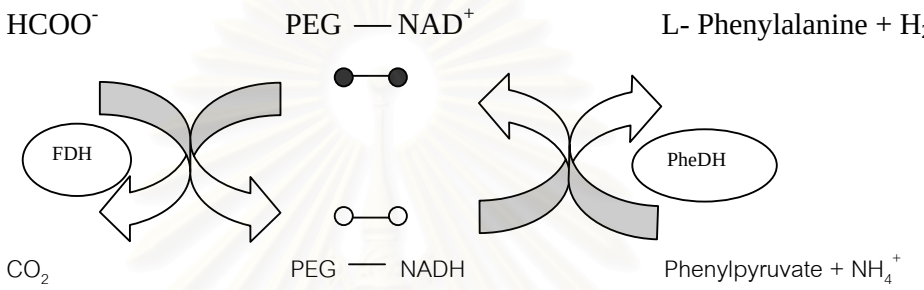


Figure 1.5 Enzymatic synthesis of D-amino acids by a multienzyme system consisting of the coupling reaction of four enzymes.

1 : formate dehydrogenase
2 : phenylalanine dehydrogenase
3 : phenylalanine racemase
4 : D-amino acid aminotransferase

Source: Galkin *et al*., 1997

a)



b)

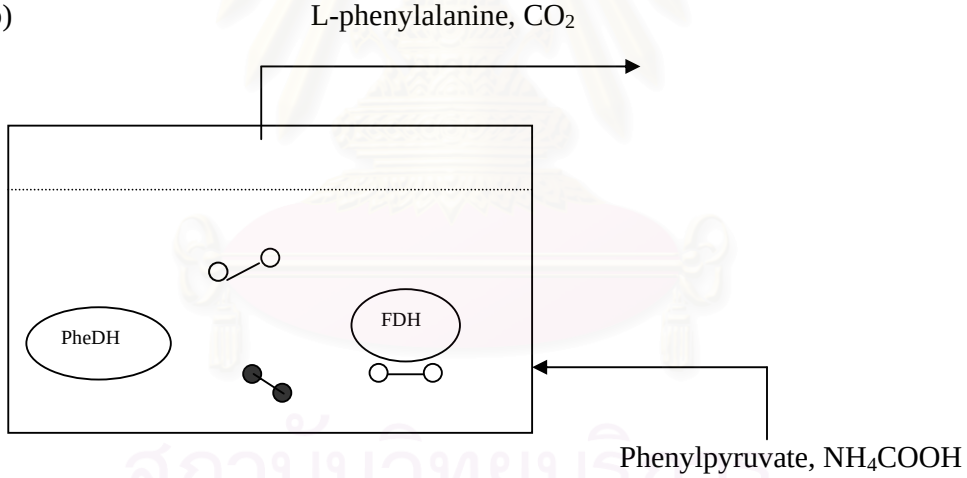


Figure 1.6 Enzymatic synthesis of L-phenylalanine with coenzyme regeneration
(a) Reaction scheme, (b) Reactor scheme

PheDH: phenylalanine dehydrogenase

FDH : formate dehydrogenase

PEG : polyethyleneglycol

Source: Hummel *et al*., 1987

Table 1.3 Synthesis of (*S*)-amino acids from 2-keto acids by using phenylalanine dehydrogenase and formate dehydrogenase

Substrate	Product	Yield, %
Phenylpyruvate	(*S*)-phenylalanine	>99
4-(hydroxyphenyl)-pyruvate	(*S*)-tyrosine	>99
4-(fluorophenyl)-pyruvate	(*S*)-(4-fluorophenyl)alanine	>99
2-oxo-4-phenylbutyrate	(*S*)-2-amino-4-phenylbutyric acid	99
2-oxo-5-phenylvalerate	(*S*)-2-amino-5-phenylvaleric acid	98
2-oxo-3-methyl-3-phenylpropionate	(*S*)-2-amino-3(*RS*)-methyl-3-phenylpropionate	98
2-oxononanoate	(*S*)-2-aminononanoic acid	99

Source: Asano *et al*., 1990

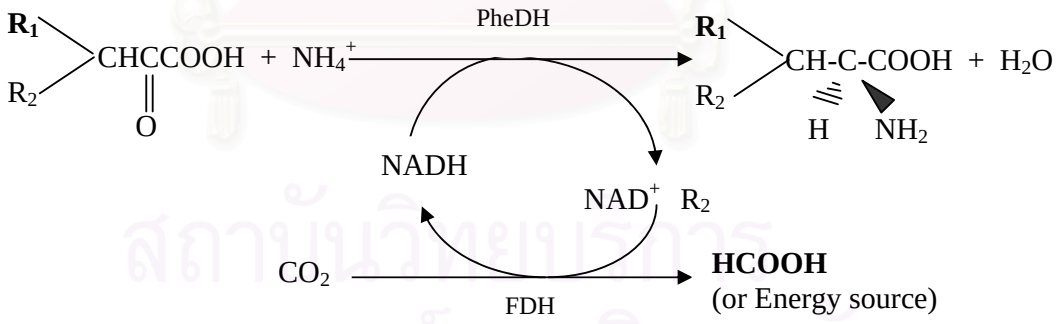


Figure 1.7 Synthesis of (*S*)-amino acid from their 2-keto analogues by phenylalanine dehydrogenase (PheDH) with a regeneration of NADH by formate dehydrogenase (FDH).

Source: Asano *et al*., 1990

polyethyleneglycol (PEG-NAD^+), so the hybrid molecules cannot pass through the membrane. The reaction was started by addition of formic acid and FDH. Then, phenylpyruvate and ammonium formate (NH_4COOH) was continuously passed through the membrane reactor. The products, L-phenylalanine and carbon dioxide, were subsequently separated from the reaction mixture. In addition, optically pure three-substituted pyruvates with bulky substituents, such as *S*-2-amino-4-phenylbutyrate and *S*-2-amino-5-phenylvalerate, were synthesized from their keto analogs in a similar way (Asano *et al*., 1990) (Table 1.3 and Figure 1.7).

1.5 Formate dehydrogenase (FDH)

FDH catalyzes the conversion of formate to carbon dioxide with the concomitant reduction of NAD^+ to NADH as shown in figure 1.8. This enzyme has been found in plants, methylotrophic yeasts, and bacteria. The study of extended to many organisms such as *Pseudomonas* sp.101 (Tishkov *et al*., 1993), *Moraxella* C-2 (EMBL Accession O08375), *Saccharomyces cerevisiae* (EMBL Accession Z75296), *Hansenula polymorpha* (Hollenberg and Janowiez, 1989), *Candida methylica* (Allen and Holbrook, 1995), *Candida boidinii* (Sakai *et al.*, 1997), *Aspergillus nidulans* (Saleeba *et al*., 1992), *Neurospora crassa* (Chow and RajBhandarg, 1993), potato mitochondria (Colas *et al*., 1993) and barley (Suzuki *et al*., 1998). The only commercially available preparation of FDH is the enzyme from *Candida boidinii* which not sufficiently stable. Recently, the bacterial gene from *Mycobacterium vaccae* N10 was cloned and expressed in *E. coli* TG1 with a plasmid pUC119. This enzyme is more stable than the yeast enzyme (Galkin *et al.*, 1997). The use of FDH for coenzyme regeneration has several advantages. Firstly, formate is a substrate for

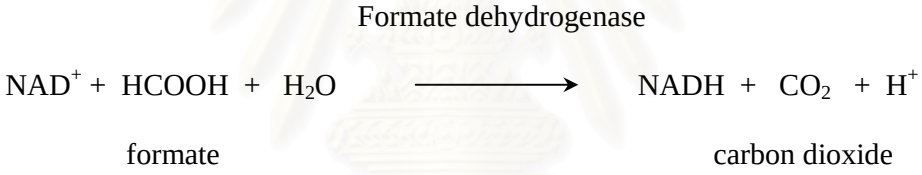


Figure 1.8 The reaction of formate dehydrogenase

FDH, is one of the cheapest hydrogen sources and does not inhibit other dehydrogenases. Secondly, the reaction has a favourable equilibrium strongly shifted towards CO_2 and NADH formation. Thirdly, CO_2 the product from oxidation can be easily removed from the reaction mixture. Finally, this enzyme has a broad pH optimum activity so that it can be easily implemented in coupled enzymatic synthesis (Hummel and Kula, 1989; Drauz and Waldmann, 1995; Peters, 1998). Thus, novel approaches to increase the availability of intracellular NADH through metabolic and genetic engineering by regenerating NADH through the heterologous expression of an NAD^+-dependent FDH have been interesting.

1.6 Phenylalanine dehydrogenase from *Bacillus badius* BC1

Our research group at the Department of Biochemistry screened for thermotolerant bacteria which gave PheDH activity from soil samples collected from temperate and hot spring areas of Thailand (Suriyapanpong *et al*., 2000). *B. badius* BC1 showed high activity of PheDH. Enzyme from this source was further purified and characterized by Leksakorn 2001. This enzyme had molecular weight of about 358,000 which consisted of 8 identical subunits. It showed high substrate specificity in the oxidative deamination on L-phenylalanine while the reductive amination was specific on phenylpyruvate. No loss of the enzyme activity was observed upon incubation at 40^{o}C for 2 hours and 50 % of the activity was retained after incubation at the same temperature for 30 hours.

The phenylalanine dehydrogenase gene was sequenced using cassette-ligation mediated PCR. The whole nucleotide sequence of the structural gene consisted of 1140 nucleotides. The open reading frame encoding a polypeptide of 380 amino acids.

The *phedh* was then successfully cloned and expressed in *E. coli* JM109 cell. The enzyme total activities were found in crude extract of the clones in the range of 0 to 360 units/100 ml culture. The activity was 60 fold higher than that of the enzyme from *B. badius* BC1 (Charoenpanich, 2001).

In this study, L-phenylalanine production of recombinant clone will be improved by coupling reactions between PheDH and FDH. NAD^+-dependent *fdh* from methanol-utilizing bacterium *Mycobacterium vaccae* N10 was a gift from Professor Nobuyoshi Esaki, Kyoto University. The gene was cloned and expressed into *E. coli* TG1 with a plasmid vector pUC119. This gene has an open reading frame of 1200 bp which encoded for 401 amino acid residues. The enzyme product of this gene has molecular mass about 88 kDa and consisted of 2 identical subunits. The K_m values for formate and NAD^+ were 6.0, 0.09 mM, respectively. The methods of *phedh* and *fdh* expression system by heterologous genes (the different genes on a single DNA vector) would be performed in *E. coli* cells for L-phenylalanine production. FDH regenerated NADH, which was a coenzyme of PheDH. The simultaneous expression of both enzymes in a single cell should provide additional benefit for industrial applications since the intracellular pool of NAD^+ (supplied by the cell itself) can be used for NADH regeneration without any additional supplies.

The outline of this research

1. To clone of *phedh* into a high expression vector pET-17b
2. To clone of *fdh* into a high expression vector pET-17b

3. To construct of a heterologous gene expression system of *phedh* and *fdh* on pET-17b and to express the recombinant plasmids in *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS
4. To compare of PheDH and/or FDH activity as well as phenylalanine production efficiency of recombinant clones
5. To produce of L-phenylalanine using organic solvent treated recombinant cells

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.

Centrifuge, microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan,
KUBOTA 1300, KUBOTA, Japan

Centrifuge tube: Nalgene, USA.

Electrophoresis unit: Hoefer™ miniVE, Amersham Pharmacia Biotech.,
U.S.A.; 2050 MIDGET, LKB, Sweden; Mini protein, Bio-Rad,
U.S.A. and submarine agarose gel electrophoresis unit

Gel Document: SYNGENE, England

Gene Pulser®/*E. coli* Pulser™ Cuvettes: Bio-Rad, U.S.A.

Incubator, waterbath: Model M20S, Lauda, Germany; BioChiller 2000;
FOTODYNE Inc.,U.S.A. and ISOTERM 210, Fisher Scientific, U.S.A.

Incubator shaker: Innova™ 4080, New Brunwick Scientific, U.S.A.

Light box: 2859 SHANDON, Shandon Scientific Co., Ltd., England.

Lamina flow: HT123, ISSCO, U.S.A.

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, U.S.A.

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

Orbital incubator: Model 1H-100, Gallenkamp, England

Orbital shaker: Orbital shaker 03, Stuart Scientific, England

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

Shaking waterbath: Model G-76, New Brunswick Scientific Co., Inc., U.S.A.

Sonicator: SONOPULS Ultrasonic homogenizers, BANDELIN, Germany

Spectrophotometer: Spectronic 2000, Bausch & Lomb, U.S.A. and
DU Series 650, Beckman, U.S.A.

Thermal cycler: Gene Amp PCR system 2400, Perkin Elmer Cetus, U.S.A.

Thin-wall microcentrifuge tubes 0.2 ml, Axygen Hayward, U.S.A.

TLC plate (RP-18 F254s, 5 cm x 10 cm) Merck, Germany

TLC plastic sheets (20 cm x 20 cm cellulose) Merck, Germany

Ultrafilter: Suprec$^{Tm-01,Tm-02}$, pore size 0.20 μm and 0.22 μm,
Takara Shuzo Co, Ltd., Japan

UV transluminator: Model 2011 Macrovue, San Gabriel California, U.S.A. and
M-26, UVP, U.S.A.

Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.

2.2 Chemicals

Acrylamide: Merck, Germany

Acetone: Labscan, Ireland

Acetonitrile: (HPLC grade) Labscan, Ireland

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium formate: Wako, Japan

Ammonium persulphate: Sigma, U.S.A.

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, U.S.A.

β- Mercaptoethanol: Fluka, Switzerland

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, U.S.A.

Bromphenol blue: Merck, Germany

Chloroform: BDH, England

Chloramphenicol: Sigma, U.S.A.

Coomassie brilliant blue R-250: Sigma, U.S.A.

Copper sulfate: Merk, Germany

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylene diamine tetra acetic acid: M&B, England

D-phenylalanine: Sigma, U.S.A.

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

100 base pair DNA ladder, Promega Co., U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ficoll type 400: Sigma, U.S.A.

1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey's reagent), Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Glucose: BDH, England

Hexane, Merck, Germany

Hydrochloric acid: Carlo Erba Reagenti, Italy

8- Hydroxychinolin: Merck, Germany

Isoamyl alcohol: Merck, Germany

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactosidase (IPTG): Sigma, U.S.A.

L-phenylalanine: Sigma. U.S.A.

Magnesium sulphate 7-hydrate: BDH, England

Methylalcohol: Merck,Germany

N,*N*-dimethyl-formamide: Fluka, Switzerland

N,*N'*-methylene-bis-acrylamide: Sigma, U.S.A.

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

β-Nicotinamide adenine dinucleotide (oxidized form) (NAD^+): Sigma, U.S.A.

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Ninhydrin: VWR Prolabo RANCE, France

Peptone from casein pancreatically digested: Merck, Germany

70% Perchloric acid: BDH, England

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol: BDH, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, U.S.A.

85% Phosphoric acid: Mallinckrodt, U.S.A.

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium hydroxide: Carlo Erba Reagenti, Italy

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Pyruvate: Sigma, U.S.A.

QIA quick Gel Extraction Kit: QIAGEN, Germany

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, U.S.A.

Sodium hydroxide: Merck, Germany

Sodium formate: Fluka, Switzerland

Standard protein marker: Amersham Pharmacia Biotech Inc., U.S.A.

Triethylamine: Merck, Germany

Trifluoroacetic acid: BDH, England

Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

Yeast extract: Scharlau microbiology, European Union

2.3 Enzymes and restriction enzymes

Restriction enzymes: New England BioLabs, Inc., U.S.A.; Zibenzyme, Sweden, GIBCOBRL, U.S.A. and Amersham Pharmacia Biotech Inc., U.S.A.

RNaseA: Sigma, U.S.A.

Pfu DNA Polymerase: Promega, Co. Ltd., U.S.A.

T_4 DNA ligase: New England BioLabs, Inc., U.S.A. and Zibenzyme, Sweden

2.4 Primers

Oligonucleotides: Bioservice Unit, Thailand., Sigma, U.S.A., Pacific Science. Germany (Table 2.1).

2.5 Bacterial strains

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

Escherichia coli BL21(DE3)pLysS, genotype: F^- *omp*T *hsdS*$_B$ (r^-_B m^-_B) *gal dem*(DE3) pLysS(Cm^R), was used as a host for expression.

2.6 Plasmid

pET-17b, a vector for cloning and expression (Appendix A).

Table 2.1 Nucleotide sequence and T_m of all primers used in *phedh* and *fdh* amplification

No.	Primers	Sequence (5' - 3')	T_m (^oC)
1	PheDHF*Nde*I	GGCATATGAGCTTAGTAGAAAAAACATCCAT	57.2
2	PheDHR*Bam*HI	GGGGATCCTTAATTACGAATATCCCATTTT	56.8
3	PheDHR*Eco*RI	GGGAATTCTTAATTACGAATATCCCATTTTGGCTTAAC	56.0
4	FDHF*Nde*I	GGAATTCCATATGGCAAAGGTCCTGTGCGTTC	59.3
5	FDHR*Bam*HI	CGGGATCCTCAGACCGCCTTCTTGAACTTG	60.0
6	FDHR*Eco*RI	CGGAATTCTCAGACCGCCTTCTTGAACTTG	57.9
7	T7*Bam*HI	CGGGATCC GATCCCGCGAAATTAATACG	60.0

Restriction sites are underlined and T7 promoter is boxed.

2.7 Bacterial growth medium

Luria-Bertani broth (LB medium)

The following medium was used LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.2 with NaOH. For agar plates, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121° C. If needed, selective antibiotic drug was then supplemented.

2.8 Transformation of plasmid

2.8.1 Competent cell preparation (Dower, 1988)

A fresh colony of *E. coli* host cell was cultured as a starter in 10 ml of LB-broth and incubated overnight with 250 rpm at 37 °C. The starter was inoculated to 1 liter of LB-broth and was then incubated at 37 °C with 250 rpm shaking until the optical density at 600 nm reached 0.6 . After that, the culture was chilled on ice for 15 to 30 minutes and the cells were harvested cells by centrifugation at 6,000 xg for 15 minutes at 4 °C. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile water, respectively. The cell pellet was washed with 10 ml of 10% (v/v) ice cold sterile glycerol and finally resuspended in a final volume of 2 - 3 ml of 10% ice cold sterile glycerol. The cell suspension was divided into 40 μl aliquots and stored at -80 °C until used.

2.8.2 Electroporation

The cuvettes and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to 25 μF capacitor, 2.50 kV and the pulse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. One to two microliter of

DNA solution was mixed with 40 μl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette. The cuvette was applied one pulse at the above setting. After that, one milliliter of LB medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette. The cell suspension was transferred to new tube and incubated at 37°C for 1 hour with shaking at 250 rpm. Finally, this suspension was spread onto the LB agar plate containing selective antibiotic drug for the selection of recombinant plasmid.

2.9 Plasmid extraction (Sambrook *et al*., 1992)

Bacteria containing recombinant plasmid was grown in LB-medium (1% peptone, 0.5% NaCl and 0.5% yeast extract, pH 7.2) supplemented with antibiotic drug overnight at 37^{o}C with rotary shaking. The cell culture was collected by centrifugation at 10,000 xg for 5 minutes in each 1.5 ml microfuge tube. Then 100 μl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was resuspended by repeated pipetting and left at room temperature for 10 minutes. After that, the 200 μl of freshly prepared Solution II (0.2 N NaOH and 1% SDS) was added, gently mixed by inverting the tube for five times and placed on ice for 10 minutes. Then, the 150 μl of cold Solution III (3 M sodium acetate, pH 4.8) was added and the tube was placed on ice for 10 minutes. The mixture was centrifuged at 10,000 xg for 10 minutes and then the supernatant was transferred to a new tube. An equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1) was added, mixed and centrifuged at 12,000 xg for 10 minutes. The upper-phased liquid was transferred to a new tube. The plasmid DNA was precipitated with 2 volume of cool absolute ethanol, mixed and placed at –20 ^{o}C

at least 30 minutes. The mixture was centrifuged at 12,000 xg for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. After drying, the pellet was finally dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 μg/ml DNase-free pancreatic RNase.

2.10 Agarose gel electrophoresis

The 0.7 g of agarose powder was added to 100 ml electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in an Erlenmeyer flask and heat until complete solubilization in a microwave oven. The agarose solution was then left at room temperature to 50^{o}C before pouring into an electrophoresis mould. After the gel was completely set, the comb and seal of the mould was carefully removed. When ready, the DNA samples were mixed with one-fifth volume of the gel-loading buffer (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded the mixture into an agarose gel. Electrophoresis had been performed at constant voltage of 10 volt/cm until dye has migrated to approximately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration or molecular weight of DNA sample was compared with the intensity or relative mobility of the standard DNA fragment.

2.11 Extraction of DNA fragment from agarose gel

Extraction of DNA fragment from agarose gel was performed using QIAquick gel extraction kit protocol. DNA fragment was excised from an agarose gel and

transferred to an eppendorf tube. Three volume of QG buffer was then added and incubated for 10 minutes at 50°C. After the gel slice had been dissolved completely, the sample was applied to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded. QG Buffer was added and centrifuged for 1 minute. The column was washed twice with PE buffer and centrifuged for 1 minute. Finally, the elution buffer was added to elute the DNA. Subsequently, the column was left stand for 1 minute, and then centrifuged for 1 minute. The DNA solution was used for cloning in the next experiment.

2.12 Crude extract preparation

Bacterial cells containing the recombinant plasmid were grown in an appropriate condition. The cells were harvested by centrifugation at 8,000 xg for 15 minutes, then washed twice with cold 0.85% NaCl and centrifuged at 8,000 xg for 15 minutes. After that, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01 β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet was stored at -80^{o}C until the next step. For enzyme extraction, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by discontinuous sonication on ice with 5 seconds pulse and 2 seconds stop interval for 15 minutes by sonic dismembrator. Unbroken cell and cell debris were removed by centrifugation at 12,000 xg for 30 minutes. The supernatant was stored at 4^{o}C for enzyme and protein assays**.**

2.13 Enzyme activity assay

2.13.1 Determination of PheDH activity

The PheDH activity was measured by oxidative deamination using L-phenyalanine as a substrate. One milliliter reaction mixture comprised of 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5, 20 μmol of L-phenylalanine, 1 μmol of NAD^+, and enzyme. In a blank tube, L- phenylalanine was replaced by water. Incubation was carried out at 30^{o}C in a cuvette of 1-cm light path. The reaction was started by addition of NAD^+ and monitored by measuring the initial change in the absorbance of NADH at 340 nm.

One unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH in 1 minute. Specific activity was expressed as units per milligram of protein.

2.13.2 Determination of FDH activity

The FDH activity was measured in the reaction mixture of 1 ml contained 200 μmol of potassium phosphate buffer, pH 7.5, 20 μmol of sodium formate, 1 μmol of NAD^+, and enzyme. In a blank tube, sodium formate was replaced by water. Incubation was carried out at 30^{o}C in a cuvette of 1 cm light path. The reaction was started by addition of NAD^+ and monitored by measuring the initial change in the absorbance of NADH at 340 nm.

One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH in 1 minute. Specific activity was expressed as units per milligram of protein.

2.14 Protein measurement

Protein concentration was determined by Lowry's method (Lowry *et al*.,1951) using bovine serum albumin (BSA) as a protein standard. The reaction mixture of 5 ml containing 20-300 μg of protein, 100 μl of solution A and 5 ml of solution B was mixed and incubated at 30^{o}C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C for 20 minutes at room temperature. Preparation of the solutions was described in Appendix B. The protein concentation was derived from the absorbance at 610 nm and calculated from the curve of standard curve of BSA.

2.15 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

2.15.1 Pouring the separating gel (10% acrylamide)

The gel sandwich was assembled to the manufacturer's instruction. For 2 slab gels, the 3.3 ml of solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) and 2.5 ml of solution B (1.5 M Tris-HCl, pH 8.8, 4% SDS) were mixed with 4.2 ml of distilled water. Then 50 μl of 10% ammonium persulfate and 5 μl of TEMED were added and mixed rapidly by swirling. The solution was carefully introduced into gel sandwich using a Pasteur pipette. After the appropriate amount of separating gel solution was added, water was gently layered about 0.5 cm height on top of the separating gel solution. The gel was allowed to polymerize, distinguished by clear interface between the separating gel and the water. The water was poured off before the stacking gel was set.

2.15.2 Pouring the stacking gel (5% acrylamide)

The 0.67 ml of solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) was mixed with 1.0 ml of solution C (0.5 M Tris, pH 6.8, 4% SDS) and 2.3 ml of distilled water. Subsequently, 30 μl of 10% ammonium persulfate and 5 μl of TEMED were added and mixed rapidly. This stacking gel solution was loaded onto separating gel until solution reached the top of short plate. Then, the comb was carefully inserted into gel sandwich. After stacking gel was polymerized, the comb was removed carefully. Then, the gel was placed into electrophoresis chamber. The electrophoresis buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.8) was added into the inner and outer reservoir. The air bubbles, which were occurred in the well, were removed.

2.15.3 Sample preparation

The protein sample was mixed with 5x sample buffer (0.3 mM Tris-HCl, 50% glycerol, 20% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue), boiled for 10 minutes at 95^{o}C and cooled to room temperature. After that, the sample solution was introduced into well.

2.15.4 Gel running

An electrode plugs was attached to proper electrodes. Current flowed towards the anode at constant current (20 mA). The electrophoresis was continued until the dye front reached the bottom of the gel. Then, the gel was removed from glass plates and transferred to a small container.

2.15.5 Staining Procedure

Protein staining

The gel from section 2.15.4 was transferred to Coomassie staining solution (1% Coomassie Blue R-250, 45% methanol, and 10% glacial acetic acid). The gel was agitated for 10 - 20 minutes on a rocking shaker. The stain solution was poured out and the Coomassie destaining solution (10% methanol and 10% glacial acetic acid) was added. The gel was shaked slowly. To complete destaining, the destaining solution was changed many times and agitated until the blue-clear bands of protein were occurred (Appendix E).

2.16 Cloning and expression of *phedh* using pET-17b vector

2.16.1 PCR amplification of *phedh*

The PCR primers were designed and synthesized for amplification of *phedh* using nucleotide sequence obtained from the *phedh* of *Bacillus badius* BC1. The *phedh* was amplified from pUC18 containing *phedh* (pUCPheDH) and overexpressed in *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS with the assistance of T7 promoter of plasmid pET-17b. The forward primer contained *Nde*I site and the 5´ end of *phedh* (PheDHF*Nde*I) and reverse primer contained *Bam*HI site, TAA and the complementary sequence of 3'-end of *phedh* (PheDHR*Bam*HI).

PCR was performed at various annealing temperatures in a 25 µl reaction mixture containing 3.0 U of *Pfu* DNA polymerase, 2.5 mM each dNTPs, 1x PCR buffer with 1.5 mM $MgCl_2$, 10 pmole of each primer and 500 ng of pUC18 containing *phedh* (pUCPheDH) as a DNA template. The PCR condition was predenaturation at 94 ^{o}C for 5 minutes, and 30 cycles of denaturation at 94 ^{o}C for

1 minute, annealing at 49.9 – 56.2 ^{o}C for 1.50 minute, extension at 72 ^{o}C for 2 minutes following by final extension at 72 ^{o}C for 10 minutes.

2.16.2 Cloning of *phedh*

The PCR product was digested by *Nde*I and *Bam*HI. Then a *Nde*I and *Bam*HI digested fragment of *phedh* (1.14 kb) was ligated into the pET-17b, which was digested with the same enzymes. The scheme of plasmid construction is shown in Fig 2.1. The ligation products were transformed into *E. coli* BL21(DE3) by electroporation. The recombinant clones were screened on LB agar plates containing 50 μg/ml ampicillin at 37 ^{o}C. Cells containing the recombinant plasmids, which had *phedh* were picked and their plasmids were isolated and checked by agarose gel electrophoresis.

2.16.3 Crude extracts preparation and enzyme activity assay

Ten *E. coli* BL21(DE3) clones which had pETPheDH were grown in 5 ml of LB-medium supplemented with 50 μg/ml ampicillin at 37^{o}C overnight. After that, 5 % of the cell culture was inoculated into each 200 ml LB medium supplemented 50 μg/ml ampicillin and shaked at 37 ^{o}C, 250 rpm. When the turbidity of the culture at 600 nm reached 0.6, IPTG was added to the final concentration of 0.4 mM in order to induce enzyme production. Then, the cultivation was continued at 37^{o}C for 4 hours before cell harvesting. Finally, crude extracts were prepared, assayed for the enzyme activity and determined the protein content as described in section 2.12, 2.13.1 and 2.14, respectively.

Recombinant plasmid from transformant clone which had the highest total and specific activity was extracted and transformed into *E. coli* BL21(DE3)pLysS by electroporation. Ten recombinant clones were randomly picked

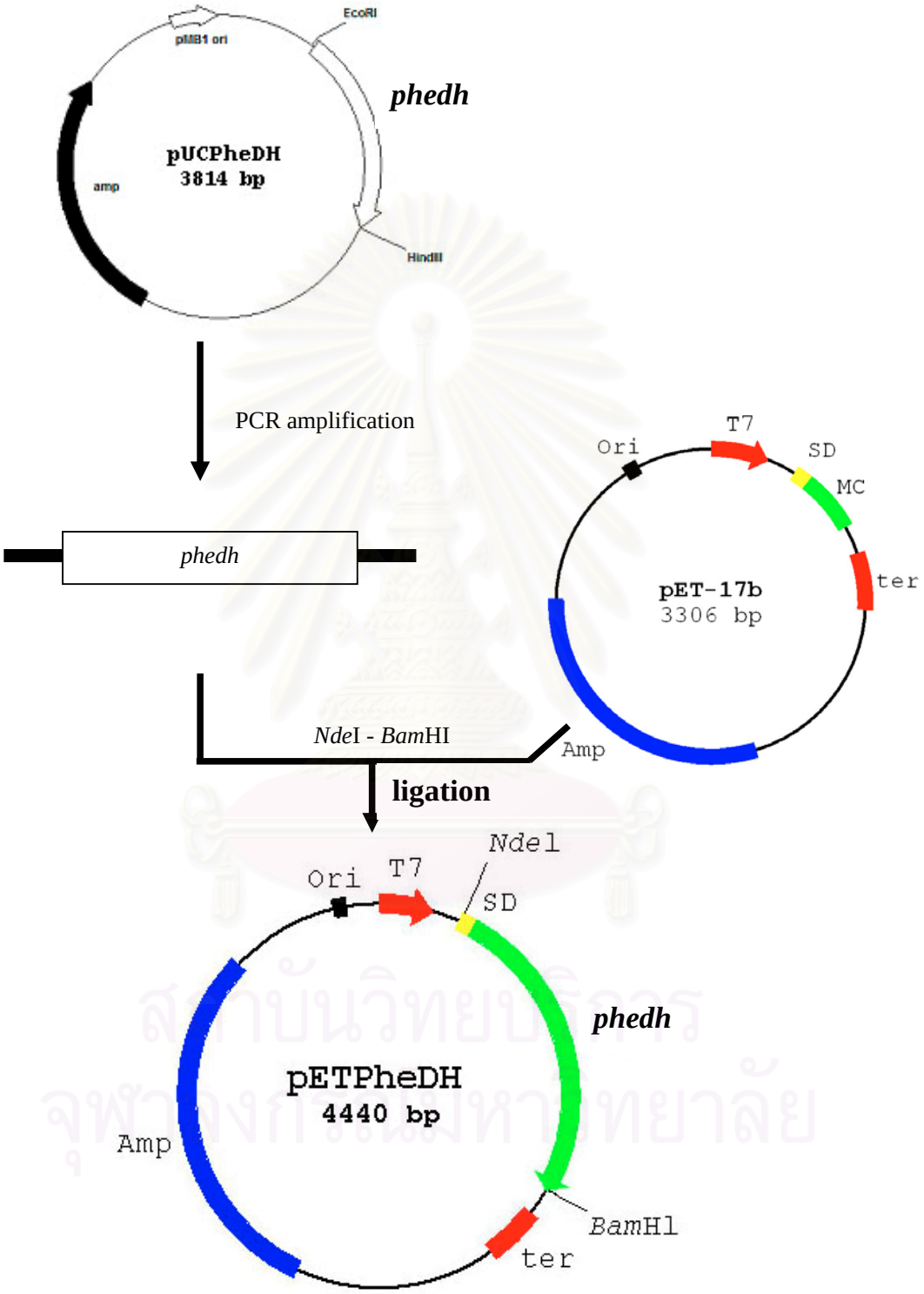


Figure 2.1 Construction of pETPheDH

and grown in LB broth supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. When OD 600 nm of the cell culture reached 0.6, expression of *phedh* gene was induced by 0.4 mM IPTG for 4 hours before cell harvesting. Crude extracts were assayed for PheDH activity and protein concentration.

2.16.4 Optimization of induction time

Since *phedh* structural gene which was cloned into pET-17b did not have its own promoter, T7 promotor on the vector was used. Moreover, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS clones had T7 RNA polymerase gene, controlled under *lac Z* promoter, inserted in their chromosome. Thus, the study about influence of induction time by IPTG was required. *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETPheDH, were grown in 5 ml of LB-medium supplemented with 50 μg/ml ampicillin at 37^{o}C overnight. For *E. coli* BL21(DE3)pLysS, 34 μg/ml chloramphenicol was added. After that, 5 % of the cell culture was inoculated into each 200 ml of the same medium and shaked at 37^{o}C, 250 rpm. When the turbidity of the culture at 600 nm reached 0.6, IPTG at final concentration 0.4 mM was added to induce enzyme production. The cultivations were continued for 0, 1, 2, 4, 8, 16, and 24 hours before the cells were harvested. Finally, crude extracts were prepared and assayed for the enzyme activity and protein as described in section 2.12, 2.13.1 and 2.14, respectively. In addition, cells and crude extracts from induction time couse study were subjected to SDS-PAGE as described in the following section.

2.16.5 Protein patterns of cells and crude extracts

The 1.5 ml of cell culture at various times were harvested by centrifugation. The cell pellets were resuspended in 100 μl of 5x sample buffer. The

7 µl of cell samples or 25 µg proteins of crude extracts was run on 10% gel SDS-PAGE as described in section 2.15.

2.17 Cloning and expression of *fdh* using pET-17b vector

2.17.1 PCR amplification of *fdh*

The PCR primers were designed and synthesized for amplification of *fdh* from pUC119 containing *fdh* (pUCFDH) and overexpressed in *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS with the assistance of T7 promoter of plasmid pET-17b. The primers were designed by using the data of the 5'- and 3'- terminal nucleotide sequence obtained from the *fdh* of *Mycobacterium vaccae* N10. The forward primer contained *Nde*I site and the 5'-end of *fdh* (FDHF*Nde*I) and reverse primer contained *Bam*HI site, TCA and the complementary sequence of 3'-end of *fdh* (FDHR*Bam*HI).

PCR was performed at various annealing temperatures as shown in Table 2.1 in a 25 µl reaction mixture containing 3.0 U of *Pfu* DNA polymerase, 2.5 mM each dNTPs, 1x PCR buffer with 1.5 mM $MgCl_2$, 10 pmole of each primer and 500 ng of pUC119 containing *fdh* (pUCFDH) as a DNA template. PCR condition consisted of predenaturation at 94^{o}C for 5 minutes, and 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 54.2 – 60.5^{o}C for 1.50 minutes, extension at 72^{o}C for 2 minutes following by final extension at 72^{o}C for 10 minutes.

2.17.2 Cloning of *fdh*

The PCR product was digested by both *Nde*I and *Bam*HI. Then a *Nde*I and *Bam*HI digested fragment of *fdh* (1.2 kb) was ligestion into the pET-17b, which was digested with the same enzymes. The scheme of plasmid construction is shown in figure 2.2. The ligation products were transformed into *E. coli* BL21(DE3) by

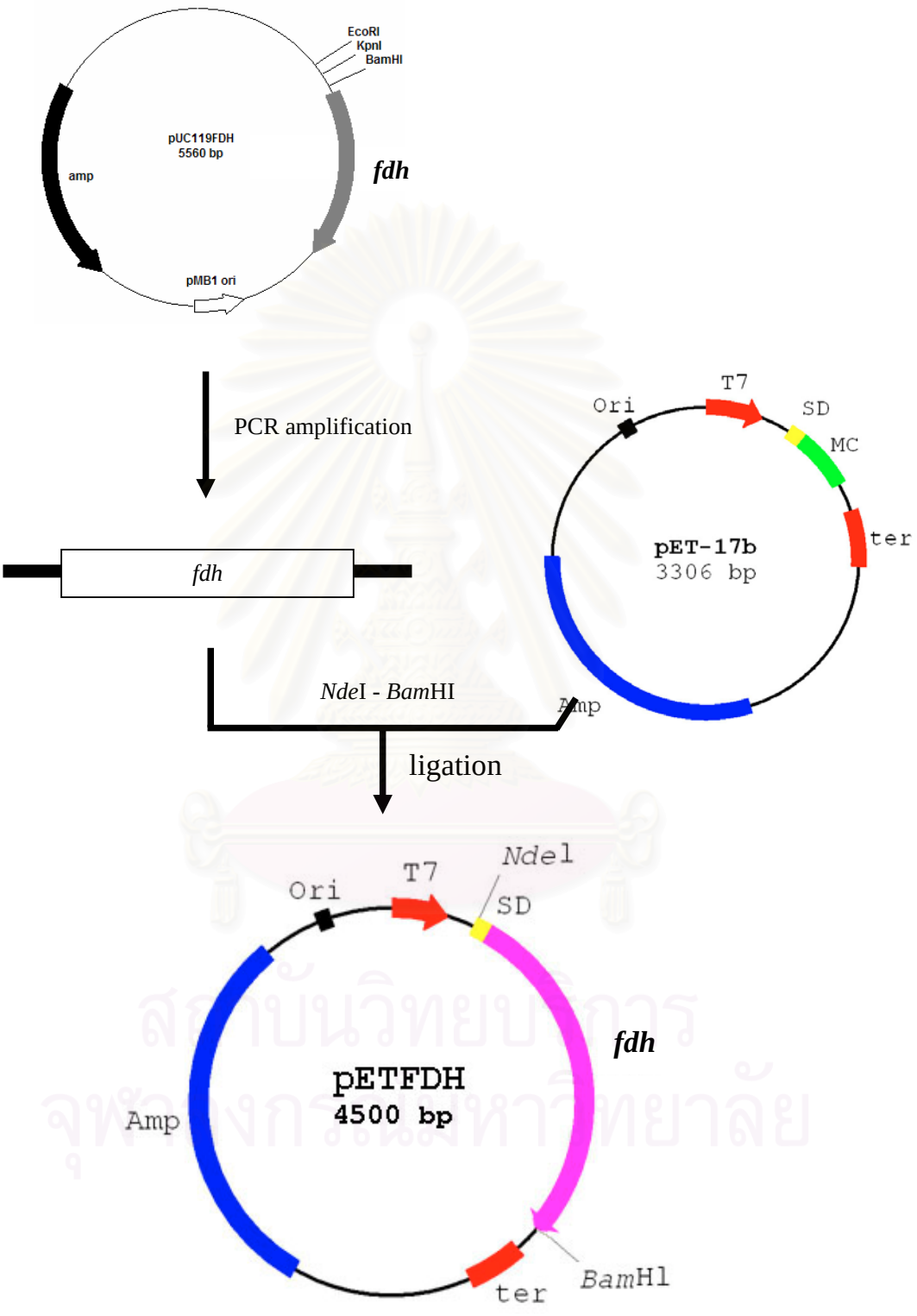


Figure 2.2 Construction of pETFDH

electroporation. The transformed cells were grown on LB agar plates containing 50 μg/ml ampicillin at 37^{o}C for 16 hours. Cells containing the recombinant plasmids, which had *fdh* were picked and their plasmids were isolated and checked by agarose gel electrophoresis.

2.17.3 Crude extract preparation and enzyme activity assay

Ten *E. coli* BL21(DE3) harbouring pETFDH were grown in 5 ml of LB-medium supplemented with 50 μg/ml ampicillin at 37 ^{o}C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 50 μg/ml ampicillin and shaked at 37^{o}C, 250 rpm. When the turbidity of the culture at 600 nm reached 0.6, IPTG was added to 0.4 mM in order to induce enzyme production. Then the cultivation was continued at 37^{o}C for 4 hour before cell harvesting. Finally, crude extracts were prepared, assayed for the enzyme activity and protein determination as described in 2.12, 2.13.2 and 2.14, respectively.

Recombinant plasmid from *E. coli* BL21(DE3) which had the highest total and specific activity was extracted and transformed into *E. coli* BL21(DE3)pLysS by electroporation. Ten colonies were randomly picked and cultured in LB broth supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. When OD 600 nm of the cell culture reached 0.6, expression of *fdh* gene was induced by 0.4 mM IPTG for 4 hours before cell harvesting. Crude extracts were assayed for FDH activity and protein concentration.

2.17.4 Optimization of induction time

The optimum time for induction of FDH production was performed by the procedure described in section 2.16.4.

2.17.5 Protein patterns of cells and crude extracts

Protein pattern of cells and crude extracts was determined by the method described in section 2.16.5.

2.18 Cloning and expression of a heterologous of *phedh* and *fdh* using pET17b

2.18.1 Construction of pETPF

The *fdh* with T7 promoter and Shine-Dargano sequence of pET vector was amplified by the PCR method and inserted to downstream of *phedh* of pETPheDH. The forward primer consisted of *Bam*HI site following by T7 promoter and Shine-Dargano sequence of pET-17b (T7*Bam*HI), while the reverse primer contained *Eco*RI site, TCA and the complementary sequence of 3'-end of *fdh* sequence (FDHR*Eco*RI).

PCR was performed at various annealing temperatures in a 25 µl reaction mixture containing 3.0 U of *Pfu* DNA polymerase, 2.5 mM each dNTPs, 1x PCR buffer with 1.5 mM $MgCl_2$, 10 pmole of each primer and 500 ng of pETFDH as a DNA template. PCR condition was predenaturation at 94^{o}C for 5 minutes, and 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 54.2 – 60.5 ^{o}C for 1.50 minutes, extension at 72^{o}C for 2 minutes following by final extension at 72^{o}C for 10 minutes.

The PCR fragment of *fdh* was digested with *Bam*HI and *Eco*RI, then ligated into pETPheDH, which was digested with the same enzyme. The scheme of plasmid constructed is shown in figure 2.3. The ligation products were transformed

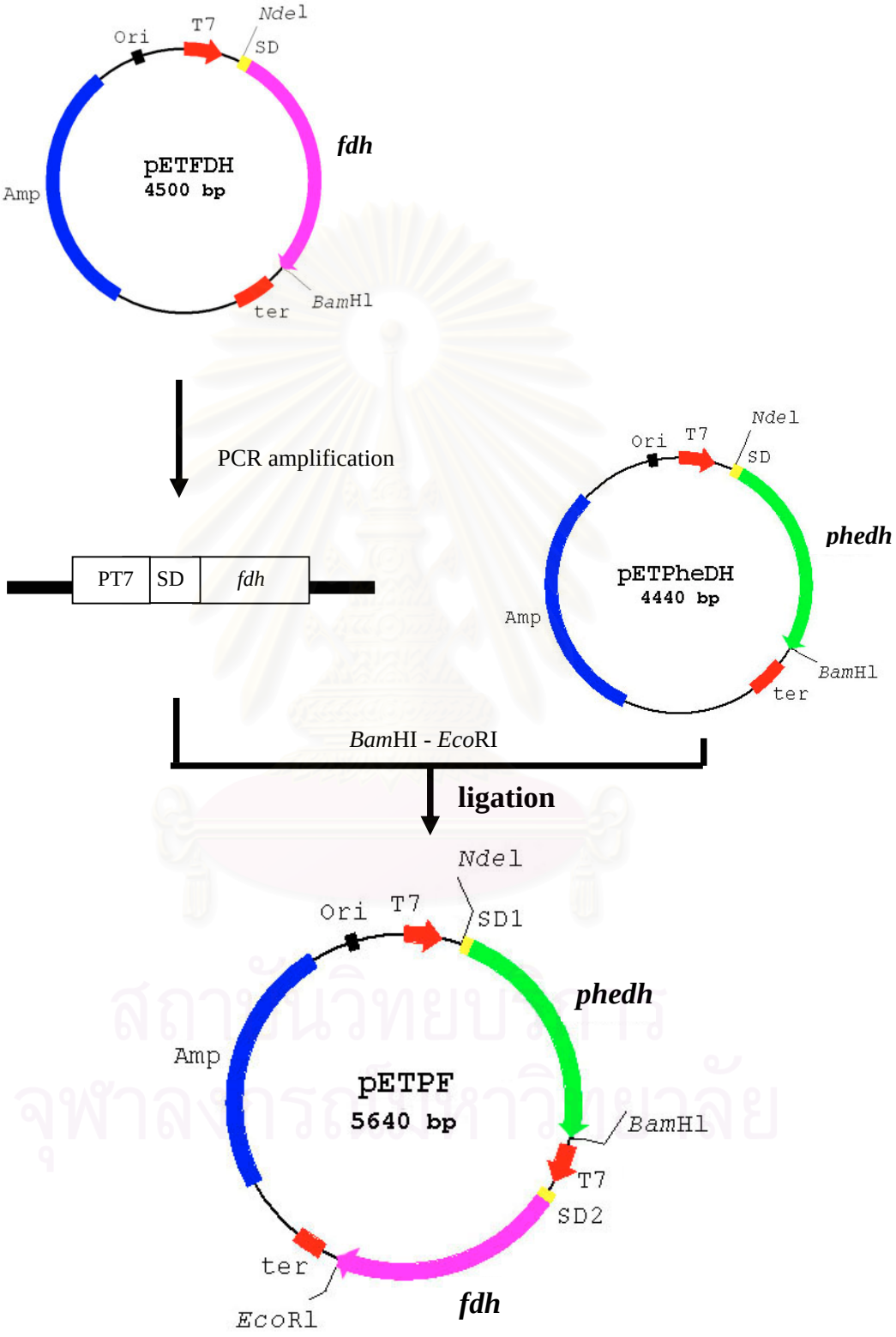


Figure 2.3 Construction of pETPF

into *E. coli* BL21(DE3) by electroporation. The transformed cells were screened on LB agar plates containing 50 µg/ml ampicillin at 37^{o}C for 16 hours. Cells containing the recombinant plasmids, which had *phedh* and *fdh* were picked, and the plasmids were isolated and checked by agarose gel electrophoresis.

After that, ten recombinant clones were assayed for PheDH and FDH activities and selected. Recombinant plasmid from transformant clones which had the highest total and specific activity was extracted and transformed into *E. coli* BL21(DE3)pLysS. Ten transformant clones were randomly picked and grown in LB broth supplemented with 50 µg/ml ampicillin and 34 µg/ml chloramphenicol, assayed for PheDH and FDH activities, optimization and protein determination as described in section 2.13.1, 2.13.2, 2.16.4 and 2.16.5 .

2.18.2 Construction of pETFP

The *phedh* gene which contained T7 promoter and Shine-Dargano sequence was amplified from pETPheDH and inserted to downstream of *fdh* in *Bam*HI and *Eco*RI site of pETFDH. The forward primer contained *Bam*HI site following by T7 promoter and Shine-Dargano sequence of pET-17b (T7F*Bam*HI), while the reverse primer contained *EcoR*I site, TTA and the complementary sequence of the sequence at 3'-end of *phedh* (PheDHR*Eco*RI).

PCR was performed at various annealing temperatures in a 25 µl reaction mixture containing 3.0 U of *Pfu* DNA polymerase, 2.5 mM each dNTPs, 1x PCR buffer with 1.5 mM $MgCl_2$, 10 pmole of each primer and 500 ng of pETFDH as a DNA template. PCR condition was predenaturation at 94 ^{o}C for 5 minutes, and 30 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 54.2 – 59.5 ^{o}C for 1.30 minute, extension at 72^{o}C for 2 minutes following by final extension at 72^{o}C for 10 minutes.

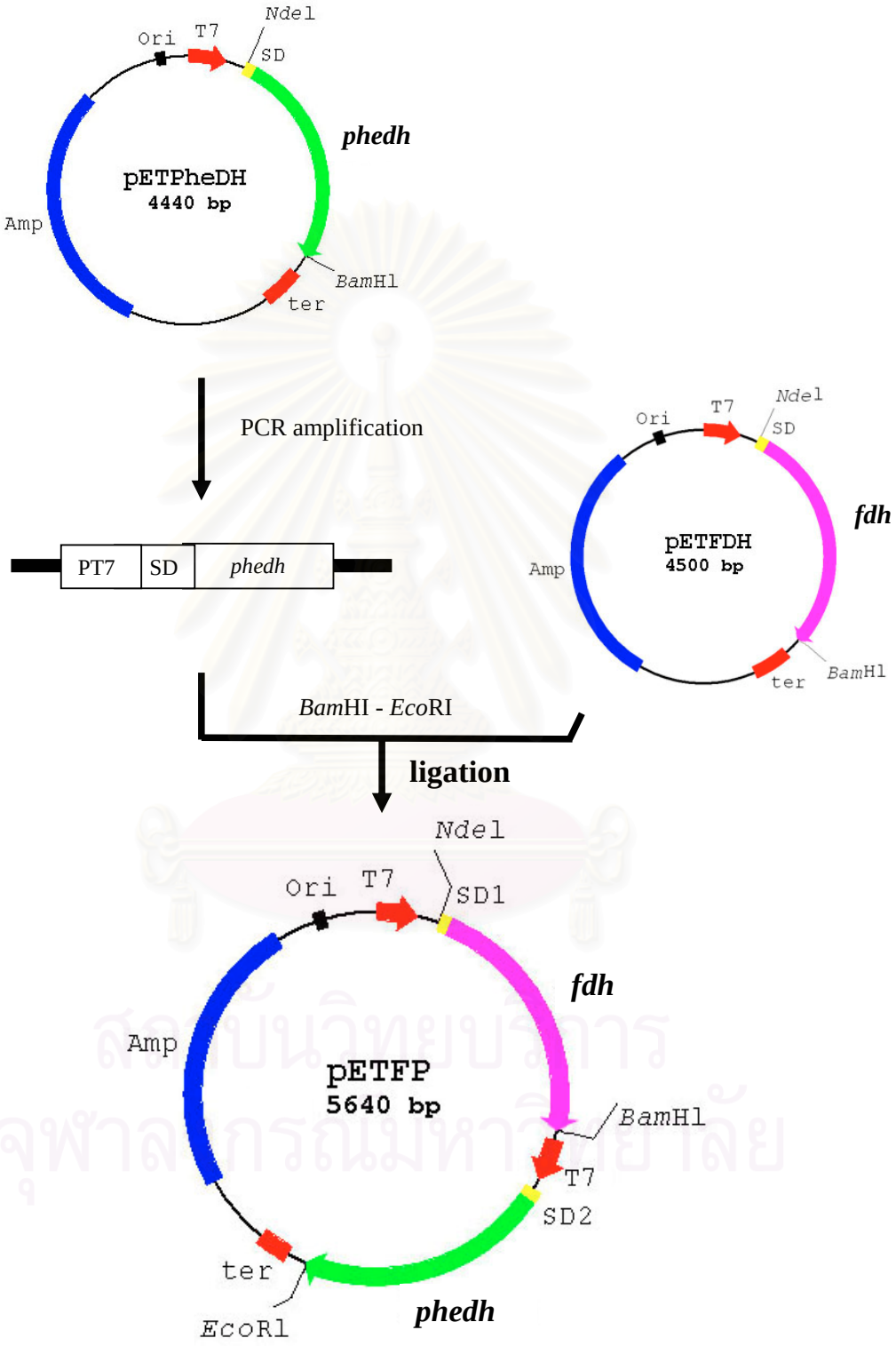


Figure 2.4 Construction of pETFP

The PCR product of *phedh* was digested with *Bam*HI and *Eco*RI site and then ligated into pETFDH, which was digested with the same enzymes. The scheme of plasmid contruction is shown in figure 2.4. The ligation products were transformed into *E. coli* BL21(DE3) by electroporation. The transformants were screened on LB agar plates containing 50 μg/ml ampicillin at 37^{o}C for 16 hours. The recombinant clones, which had *fdh* and *phedh,* were picked and their plasmids were isolated and checked by agarose gel electrophoresis.

After that, ten recombinant clones were assayed for FDH and PheDH activities and selected. Recombinant plasmid from transformant clones which had the highest total and specific activity was extracted and transformed into *E. coli* BL21(DE3)pLysS. The ten transformant clones were randomly picked and grown in LB broth supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, assayed for FDH and PheDH activities, optimization and protein determination as described in section 2.16.4 and 2.16.5 .

All recombinant clones engineered in this study were summarized in Table 2.2.

2.19 Production of phenylalanine by recombinant clones

The *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS containing pETPheDH, pETPF and pETFP were cultured at their optimum condition for the enzyme induction. Cells were harvested by centrifugation at 5,000 x g for 10 minutes, washed and resuspended in 0.1M sodium phosphate buffer pH 7.8 and stored at 4^{o}C .

Table 2.2 Summary of all recombinant clones engineered in this study

Host cell	**Recombinant plasmid**	**Insert gene (kb)**	**Size (kb)**	**Antibiotic drug**
***E. coli* BL21(DE3)**	pETPheDH	*phedh*	4.4	ampicillin
	pETFDH	*fdh*	4.5	
	pETPF	*phedh* and *fdh*	5.6	
	pETFP	*fdh* and *phedh*	5.6	
***E. coli* BL21(DE3)pLysS**	pETPheDH	*phedh*	4.4	ampicillin and chloramphenicol
	pETFDH	*fdh*	4.5	
	pETPF	*phedh* and *fdh*	5.6	
	pETFP	*fdh* and *phedh*	5.6	

2.19.1 Cell permeabilization with organic solvents

The organic solvents used for cell permeabilization were analytical grade including: acetone, benzene, chloroform and hexane. The amount of a cell suspension required to obtain 0.05 g wet weigh was centrifuged at 10000 x g for 1 min, the pellet was resuspended in the 30 µl of organic solvent and vigorously mixed by vortex. The suspension was incubated at room temperature for variable periods of time (1, 2, 5, 10, 15, 30 minute), and then washed with 500 µl of 0.1 M sodium phosphate buffer pH 7.8, and centrifuged at 10000 x g for 1 minute. Cells treated in this way were resuspended in 200 µl of 0.1 M sodium phosphate buffer pH 7.8 prior to analysis of their activity.

2.19.2 Production of phenylalanine

The one milliliter of cell suspension of each clone containing 0.05 g wet weight of washed cell, 0.5 M ammonium formate (pH 7.5) and 0.05 M pyruvate was made. The reactions were performed at 37^{o}C, 250 rpm with reciprocal shaking for 24 hours. The supernatants was separated from the cells for analysis of phenylalanine production by TLC technique.

2.19.3 Analysis of phenylalanine by TLC

2.19.3.1 First analysis

Cellulose TLC plastic sheets (10 cm x 10 cm, Merck, Germany) was used for first analysis. The 2 µl of sample solutions were spotted in parallel with 2 µl of 0.01 M of their standard amino acid on the TLC plate and developed with *n*-butanol : acetic acid: water (4:1:1). After that, the plates were dried in hot air. The detection was carried out by spraying the plates with a 0.5% ninhydrin solution in ethanol and acetone (30:70) then dried in hot air for 5 minute. The sample

solution, which gave the high intensity of the spot in time course study, from each clone was quantified by using reverse phase TLC.

2.19.3.2 Second analysis

The selected samples from the first analysis were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey' s reagent) according to Marfey's methods (cited in Nagata *et al*., 2001). One hundred microgram of standard D- or L- phenylalanine in H_2O or 20 µl of sample and 8 µl of 1 M $NaHCO_3$ were mixed with 400 µg FDAA in 40 µl acetone and incubated at 40^oC for 1 hour with occasional shaking. The reaction was terminated by adding 4 µl of 2 M HCl. The acetone, water and HCl were removed by evaporation under reduced pressure in a centrifugal evaporator. After evaporation, 20 µl of methanol was added to dissolve the FDAA amino acid. FDAA amino acid solution (2 µl) was spotted on a reversed phase RP-18 pre-coated TLC plate (RP-18 F_{254S}) and developed with acetonitrile: 50 mM triethylamine-phosphate buffer, pH 5.0 at ratio 40:60 in a pre-equilibrated glass chamber at room temperature. When the ascending solvent front nearly reached the top margin, the plate was removed from the chamber and dried with a hair-drier. The FDAA amino acid spots were yellow. For quantitative analysis, yellow spots were scraped off after the chromatography, and extracted by 1 ml of methanol: H_2O (1:1). The absorbance of the extract was measured at 340 nm by spectrophotometer. Since FDAA is light sensitive, the FDAA amino acids were protected from exposure to light during all procedures.

CHAPTER III

RESULTS

3.1 Cloning and expression of *phedh* using pET-17b vector

3.1.1 PCR amplification of *phedh*

The *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS were used as host strains for overexpress *phedh* under control of the T7 promoter of plasmid pET-17b. The 1.14 kb of *phedh* fragment was amplified from a pUC18 containing *phedh* (pUCPheDH). The gene were amplified by PCR using the following primers, PheDHF*Nde*I containing *Nde*I site and 5´ end of *phedh* was designed as a forward primer and PheDHR*Bam*HI containing *Bam*HI site, TTA and complementary sequence of the 3´ end of *phedh* was designed as a reverse primer as described in section 2.16.1. Figure 3.1 shows the 1.14 kb PCR product of the *phedh* amplified by various annealing temperatures. The optimum annealing temperatures which gave strong specific PCR product was at 56.2 ^{o}C. The PCR product was purified by agarose gel electrophoresis before using for further cloning.

3.1.2 Cloning of *phedh*

The PCR product of *phedh* was digested with *Nde*I and *Bam*HI as described in section 2.16.2. The *phedh* fragment was ligated to the *Nde*I and *Bam*HI site of 3.3 kb pET-17b vector, and then transformed into *E. coli* BL21(DE3) by electroporation. The recombinant clones were screened on LB plate containing 50 µg/ml ampicillin. Recombinant clones were randomly picked for plasmid extraction and digestion with *Nde*I - *Bam*HI. After digestion with *Nde*I and *Bam*HI, a linear pET-17b and a 1.14 kb of inserted *phedh* fragment (lane 4) which gave the same

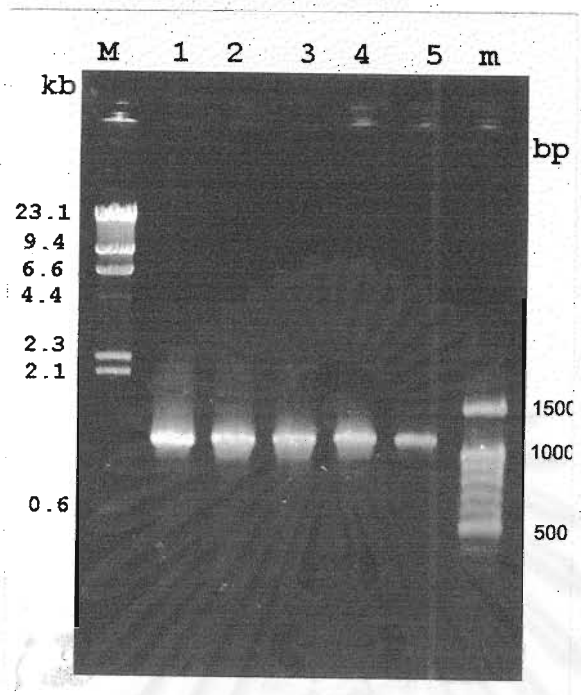


Figure 3.1 PCR product of the *phedh* amplified by various annealing temperatures

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product at annealing temperature 49.9 °C

Lane 2 = amplified product at annealing temperature 52.1 °C

Lane 3 = amplified product at annealing temperature 54.0 °C

Lane 4 = amplified product at annealing temperature 55.0 °C

Lane 5 = amplified product at annealing temperature 56.2 °C

Lane m = 100 bp DNA ladder

mobility with PCR product (lane 5) were obtained as shown in Figure 3.2.

3.1.3 Enzyme activity of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETPheDH

Ten *E. coli* BL21(DE3) clones which had pETPheDH were grown in LB broth supplemented with ampicillin at 50 μg/ml. The clones were assayed for PheDH activity as described in section 2.16.3. The transformants had been induced by IPTG at final concentration of 0.4 mM for 4 hours before cells were harvested. They showed various levels of the enzyme total activity from 629 - 1965 U and specific activity of 11.95 - 34.30 U/mg protein as shown in Table 3.1. Transformant No.4 which had the highest total activity and specific activity of 1965 U and 34.30 U/mg protein, respectively was selected.

Recombinant plasmid from transformant No.4 was extracted and then transformed into *E. coli* BL21(DE3)pLysS by electroporation. The recombinant clones were screened on LB plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Ten transformants were grown and assayed for PheDH activity. They showed various levels of the enzyme total activity from 186 – 632 U and specific activity of 3.93 – 12.71 U/mg protein as shown in Table 3.2 . Transformant plasmid No.5 which had the highest total activity and specific activity of 632 U and 12.71 U/mg protein, respectively was selected.

3.1.4 Optimization of induction time

E. coli BL21(DE3) transformant No.4 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours as described in section 2.16.4. The result shown in Figure 3.3

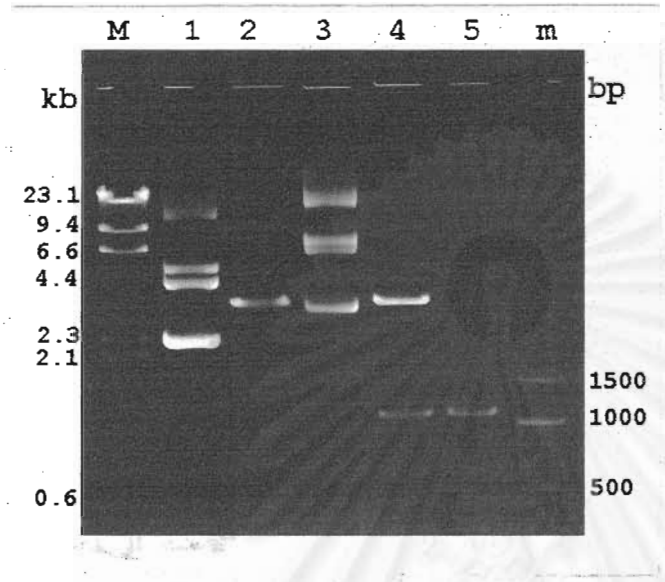


Figure 3.2 Restriction pattern of pETPheDH

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pET-17b

Lane 2 = *Nde*I-*Bam*HI digested pET-17b

Lane 3 = undigested pETPheDH

Lane 4 = *Nde*I-*Bam*HI digested pETPheDH

Lane 5 = amplified product of *phedh*

Lane m = 100 bp DNA ladder

Table 3.1 PheDH activity from crude extract of *E. coli* BL21(DE3) clones harbouring pETPheDH

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCPheDH harbouring *E. coli* JM109	0.95	352	55.32	6.92
1	0.84	1026	42.59	24.10
2	1.12	1039	44.97	23.10
3	1.46	1322	57.20	23.11
4	1.06	1965	57.30	34.30
5	0.89	1244	56.59	21.98
6	0.91	812	55.73	14.57
7	1.26	836	46.57	17.95
8	1.15	754	41.39	18.22
9	1.31	1885	60.11	31.36
10	0.71	629	52.65	11.95

[a] Total activity from 200 ml culture as described in section 2.16.3

Table 3.2 PheDH activity from crude extract of *E. coli* BL21(DE3)pLysS clones harbouring pETPheDH

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pETPheDH harbouring *E. coli* BL21(DE3)	1.44	1536	63.81	24.07
1	1.42	288	52.94	5.45
2	1.24	398	60.70	6.56
3	1.39	484	49.27	9.82
4	1.07	292	41.25	7.09
5	1.31	632	49.75	12.71
6	1.58	445	51.71	8.60
7	1.41	186	47.41	3.93
8	1.57	285	46.46	6.13
9	1.62	217	50.75	4.28
10	1.10	307	41.17	7.46

[a] Total activity from 200 ml culture

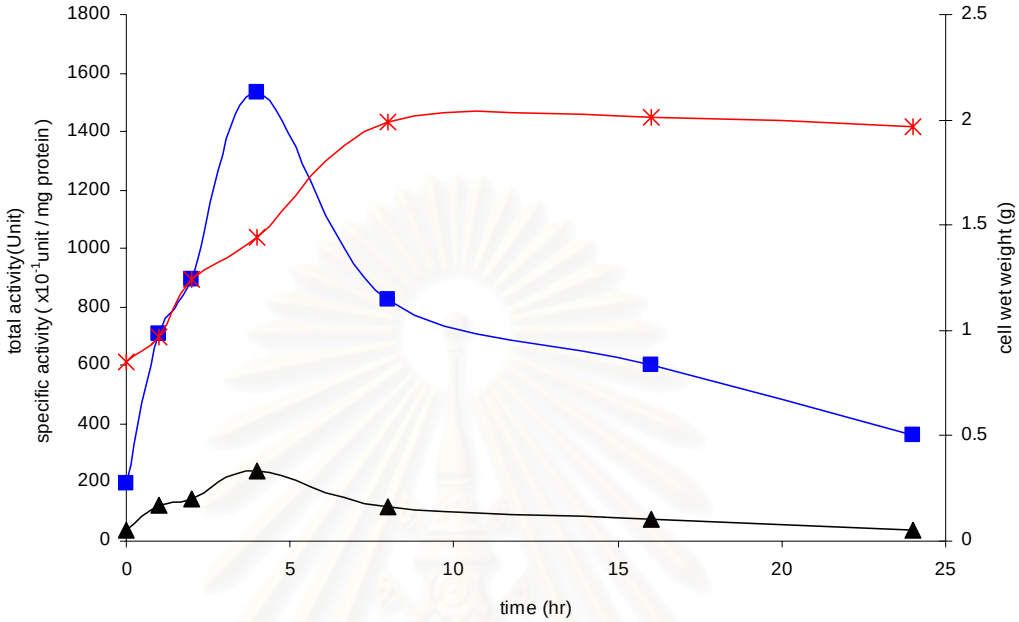


Figure 3.3 Effect of induction time on PheDH production and growth of *E. coli* BL21(DE3) harbouring pETPheDH.

■ = total activity (unit) ▲ = specific activity (x10^{-1}unit/mg protein)

* = cell wet weight (g)

indicated that cell wet weight increased rapidly in the first 8 hours and then slowly decreased. The highest PheDH total activity and specific activity were 1536 U and 24.07 U/mg protein, respectively when the cells were induced for 4 hours and after that the PheDH activity and specific activity were decreased.

E. coli BL21(DE3)pLysS transformant No.5 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours as described in section 2.16.4. The result shown in Figure 3.4 indicated that cell wet weight increased rapidly in the first 8 hours. After that cell wet weight increased slowly untill 16 hours, then slowly decreased. The highest PheDH total activity and specific activity were 1280 U and 15.29 U/mg protein, respectively when the cells were induced for 16 hours and after that the PheDH activity and specific activity were decreased.

3.1.5 Protein patterns of cells and crude extracts

The 1.5 ml of culture of *E. coli* BL21(DE3) transformant No.4 and *E. coli* BL21(DE3)pLysS transformant No.5 were harvested and then centrifuged in eppendorf tube at various times after induction. The cell pellets were resuspended in 100 µl of 5x sample buffer. The 7 µl of cell samples or 15 µg protein of crude extract was run on 10% gel SDS-PAGE. The major protein band from cell and crude extract of transformant No.4 were shown in Figure 3.5 and Figure 3.6, respectively while these of transformant No.5 were shown in Figure 3.7 and 3.8, respectively. The intensity of major protein band from cell and crude extract of both transformants at each induction time corresponded with the level of enzyme activity from crude extract displayed in Figure 3.3 and 3.4 .

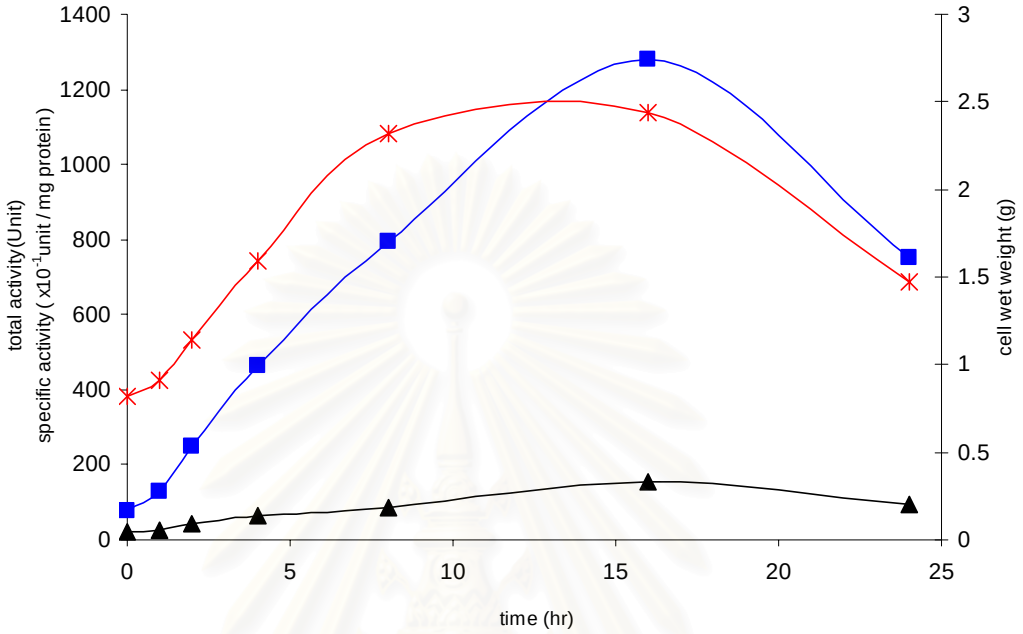


Figure 3.4 Effect of induction time on PheDH production and growth of *E. coli* BL21(DE3)pLysS harbouring pETPheDH

■ = total activity (unit) ▲ = specific activity (x10^{-1}unit/mg protein)

* = cell wet weight (g)

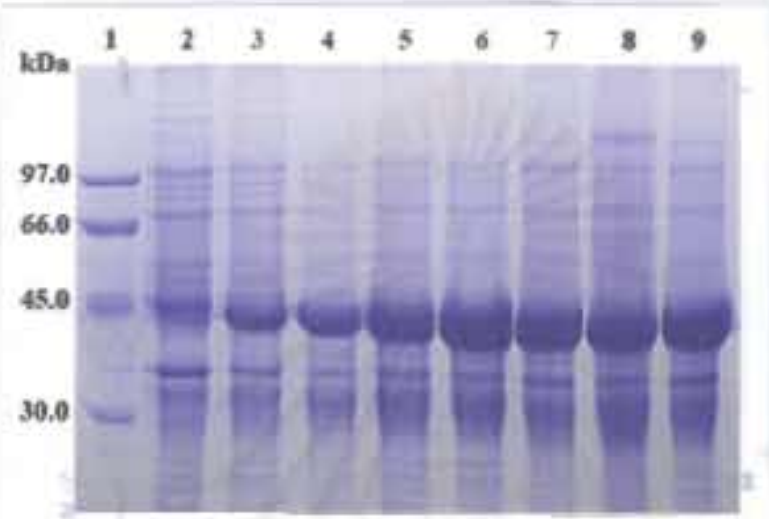


Figure 3.5 Protein pattern of cell of *E. coli* BL21(DE3) harbouring pETPheDH at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

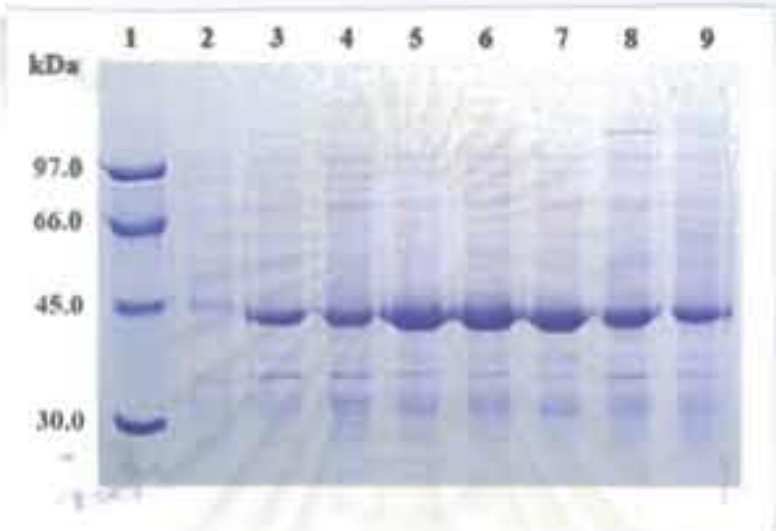


Figure 3.6 Protein pattern of crude extract of *E. coli* BL21(DE3) harbouring pETPheDH at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

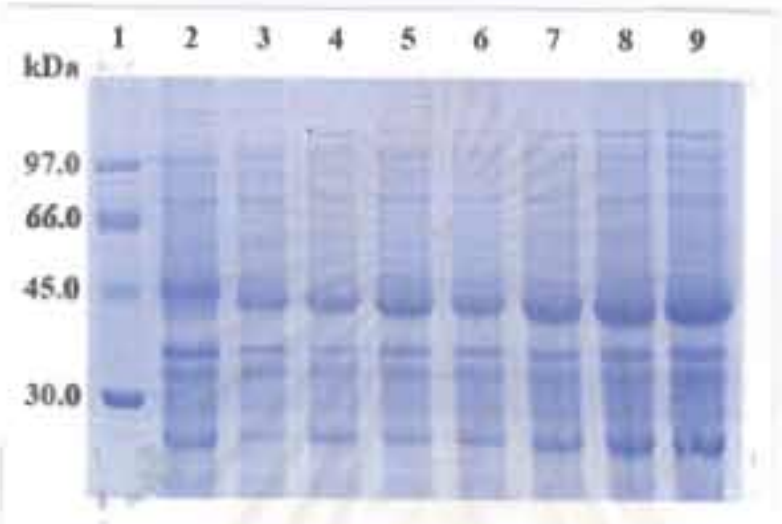


Figure 3.7 Protein pattern of cell of *E. coli* BL21(DE3)pLysS harbouring pETPheDH at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

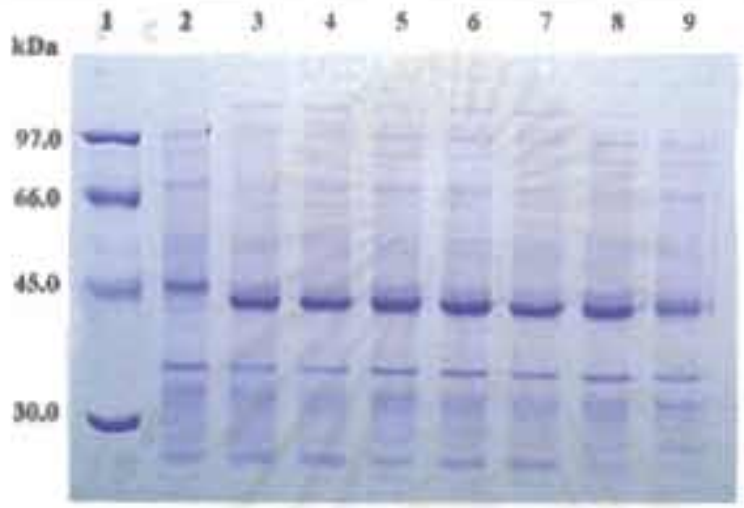


Figure 3.8 **Protein pattern of crude extract of *E. coli* BL21(DE3)pLysS harbouring pETPheDH at various induction times detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = crude extract of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at various induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

3.2 Cloning and expression of *fdh* using pET-17b vector

3.2.1 PCR amplification of *fdh*

The *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS were used as host strains for overexpress of *fdh* under control of the T7 promoter of plasmid pET-17b, the 1.2 kb of *fdh* fragment was amplified from a pUC119 containing *fdh* (pUCFDH). The gene was amplified by PCR using the following primers: FDHF*Nde*I for the forward primer containg *Nde*I site and 5´ end of *fdh* and FDHR*Bam*HI for the reverse primer containing *Bam*HI site, TCA and complementary sequence of 3´ end of *fdh*, as described in section 2.17.1. Figure 3.9 shows the 1.2 kb PCR product of the *fdh* amplified by various annealing temperatures. The optimum annealing temperature was in the range of 56.2 – 60.5 ^{o}C. Thus, annealing at 60.5 ^{o}C was selected for further studies. The PCR product was purified by agarose gel electrophoresis before using for cloning.

3.2.2 Cloning of pETFDH

The PCR product of *fdh* was digested with *Nde*I and *Bam*HI as described in section 2.17.2. The *fdh* fragment was ligated to the *Nde*I-*Bam*HI site of 3.3 kb pET-17b vector, and then transformed into *E. coli* BL21(DE3) by electroporation. The recombinant clone were screened on LB plate containing 50 µg/ml ampicillin. Recombinant clones were randomly picked for plasmid extraction and digestion with *Nde*I and *Bam*HI. After digestion with *Nde*I and *Bam*HI, a linear pET-17b and a 1.2 kb of inserted *fdh* fragment (lane 4) which gave the same mobility with PCR product (lane 5) were detected as shown in Figure 3.10.

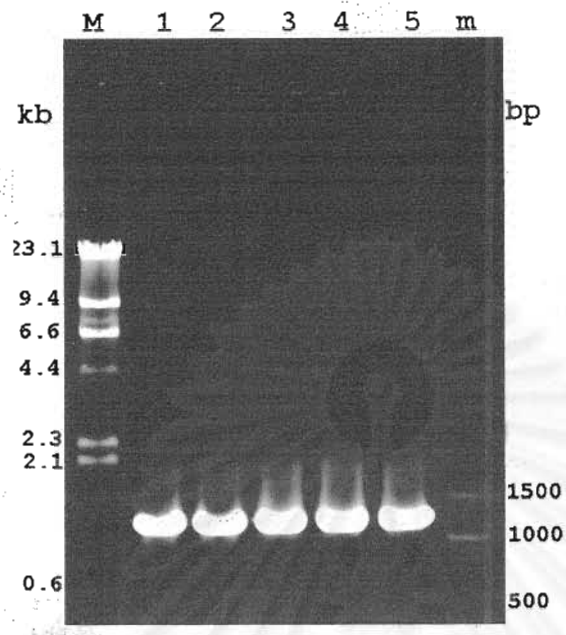


Figure 3.9 PCR product of the *fdh* amplified by various annealing temperatures

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product at annealing temperature 54.2 °C

Lane 2 = amplified product at annealing temperature 56.2 °C

Lane 3 = amplified product at annealing temperature 57.3 °C

Lane 4 = amplified product at annealing temperature 58.4 °C

Lane 5 = amplified product at annealing temperature 60.5 °C

Lane m = 100 bp DNA ladder

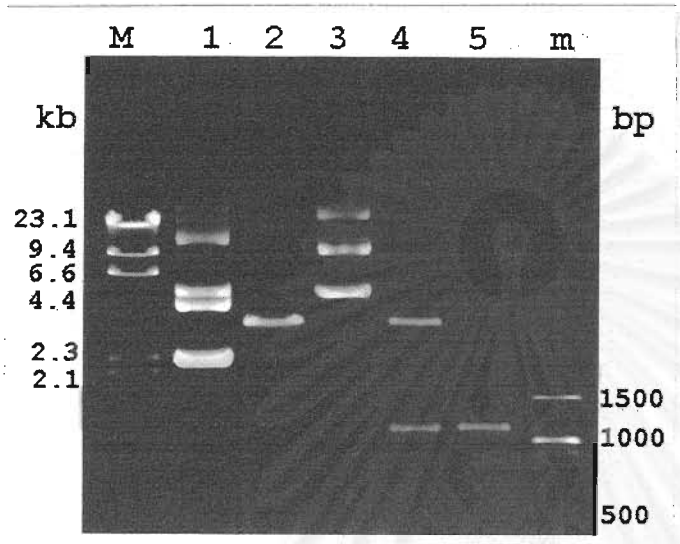


Figure 3.10 Restriction pattern of pETFDH

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pET-17b

Lane 2 = *Nde*I-*Bam*HI digested pET-17b

Lane 3 = undigested pETFDH

Lane 4 = *Nde*I- *Bam*HI digested pETFDH

Lane 5 = amplified product of *fdh*

Lane m = 100 bp DNA ladder

3.2.3 Enzyme activity of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETFDH

Ten *E. coli* BL21(DE3) clones which had *fdh* inserted were grown in LB broth supplemented with ampicillin at 50 μg/ml. The clones were assayed for FDH activity as described in section 2.17.3. The transformants was induced by IPTG at final concentration of 0.4 mM before cell was harvested at 4 hours. They showed various levels of the enzyme total activity from 22 - 275 U and specific activity of 0.34 – 5.87 U/mg protein as shown in Table 3.3 . Transformant No.4 which had the highest total activity and specific activity of 275 U and 5.87 U/mg protein, respectively was selected.

Recombinant plasmid from transforment No.4 was extracted and then transformed into *E. coli* BL21(DE3)pLysS by electroporation. The recombinant clones were screened on LB plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Ten transformants were grown and assayed for FDH activity. They showed various levels of the enzyme total activity from 24 – 48 U and specific activity of 0.42 – 0.90 U/mg protein as shown in Table 3.4 . Transformant plasmid No.3 which had the highest total activity and specific activity of 48 U and 0.90 U/mg protein, respectively was selected.

3.2.4 Optimization of induction time

E. coli BL21(DE3) transformant No.4 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours as described in section 2.17.4 . The result shown in Figure 3.11 indicated that cell wet weight increased rapidly in the first 4 hours. After that cell wet weight slowly increased untill 16 hours, then slowly decreased. The highest FDH

Table 3.3 FDH activity from crude extract of *E.coli* BL21(DE3) harbouring pETFDH

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCFDH harbouring *E. coli* JM109	0.76	20	48.95	0.41
1	1.01	38	55.88	0.67
2	1.43	189	67.48	2.80
3	0.97	22	66.81	0.34
4	0.88	275	46.88	5.87
5	1.12	251	48.88	5.13
6	1.39	31	45.48	0.68
7	1.54	218	54.74	3.98
8	1.19	211	55.57	3.80
9	1.69	209	55.79	3.75
10	0.93	140	63.74	2.20

[a] Total activity from 200 ml

Table 3.4 FDH activity from crude extract of *E.coli* BL21(DE3)pLysS clones harbouring pETFDH

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
E. coli BL21(DE3) harbouring pETFDH	1.74	173	75.33	2.29
1	0.91	27	40.90	0.66
2	1.26	41	55.81	0.73
3	1.37	48	53.67	0.90
4	1.15	24	56.62	0.42
5	1.42	47	48.86	0.96
6	1.61	40	51.14	0.77
7	1.54	46	51.10	0.90
8	1.39	30	59.76	0.50
9	0.88	37	41.52	0.89
10	1.20	39	45.29	0.85

[a] Total activity from 200 ml

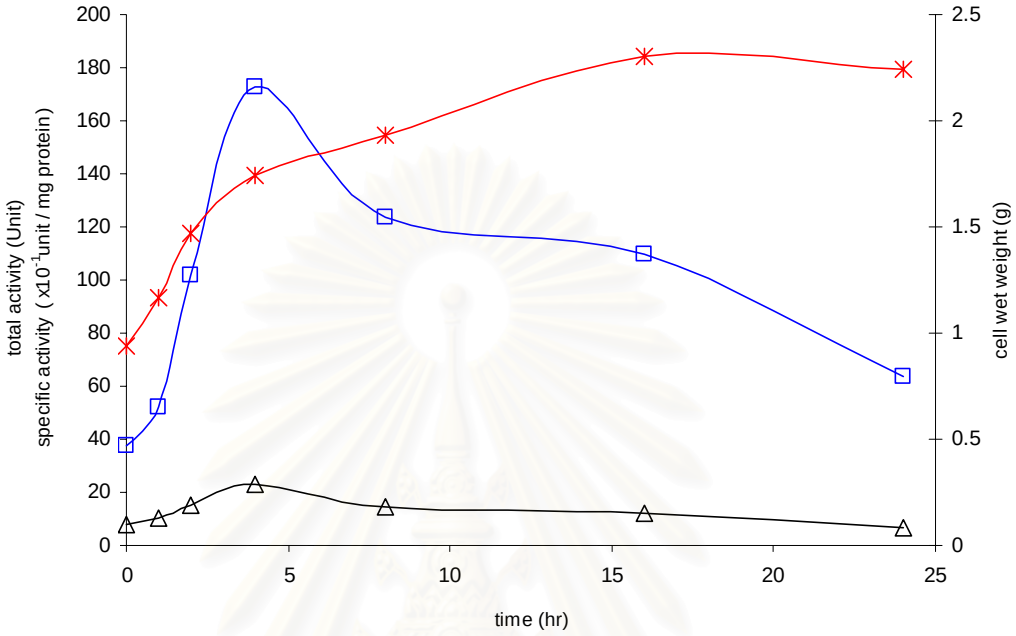


Figure 3.11 Effect of induction time on FDH production and growth of *E. coli* BL21(DE3) harbouring pETFDH

□ = total activity (unit) Δ = specific activity ($x10^{-1}$unit/mg protein)

∗ = cell wet weight (g)

total activity and specific activity were 173 U and 2.29 U/mg protein, respectively when the cells were induced for 4 hours and after that the PheDH activity and specific activity were decreased.

E. coli BL21(DE3)pLysS transformant No.3 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours as described in section 2.17.4. The result shown in Figure 3.12 indicated that cell wet weight increased rapidly in the first 8 hours. After that cell wet weight was slowly decreased. The highest FDH total activity and specific activity were 127 U and 1.86 U/mg protein, respectively when the cells were induced for 16 hours and after that the FDH activity and specific activity were decreased.

3.2.5 Protein patterns of cells and crude extracts

The 1.5 ml cell culture of *E. coli* BL21(DE3) transformant No.4 and *E. coli* BL21(DE3)pLysS transformant No.3 were harvested at various times after induction. The cell samples was run on 10% gel SDS-PAGE. The protein band from cell and crude extract of *E. coli* BL21(DE3) were showed in Figure 3.13 and Figure 3.14 . The intensity of major protein band from cells was increased after induction because number of cell per volume of cell culture increased though the enzyme activity decreased. However, band intensity of major protein from crude extract at various times corresponded with their total enzyme activity shown in Figure 3.11. For *E. coli* BL21(DE3)pLysS transformant No.3, the major protein band from both cell (Figure 3.15) and crude extract (Figure 3.16) at each induction time corresponded with their total activity in crude extract (Figure 3.12).

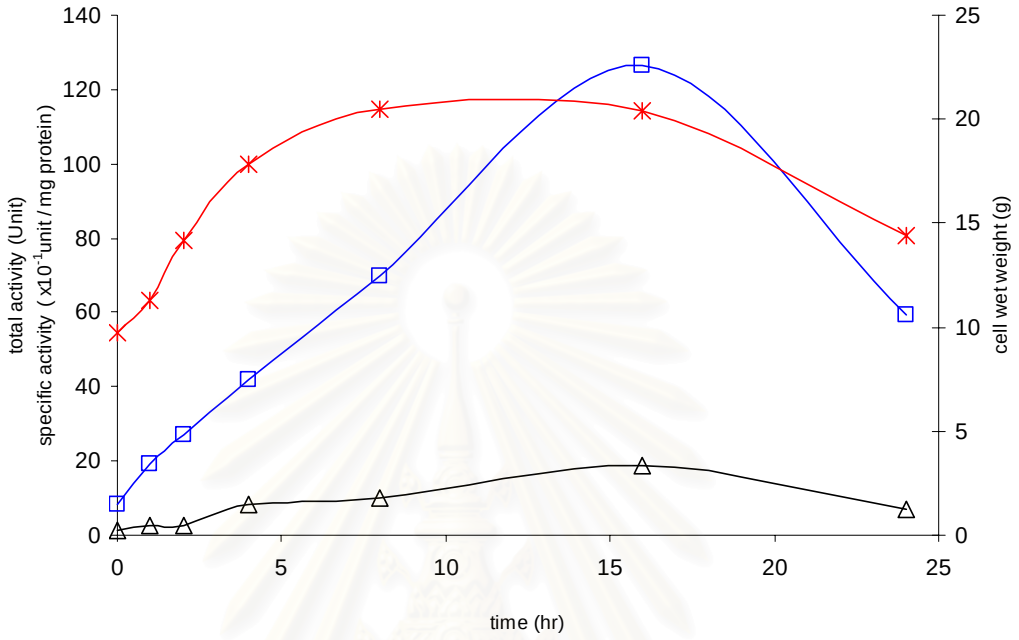


Figure 3.12 Effect of induction time on FDH production and growth of *E. coli* BL21(DE3)pLysS harbouring pETFDH

□ = total activity (unit) Δ = specific activity (x10^{-1}unit/mg protein)

* = cell wet weight (g)

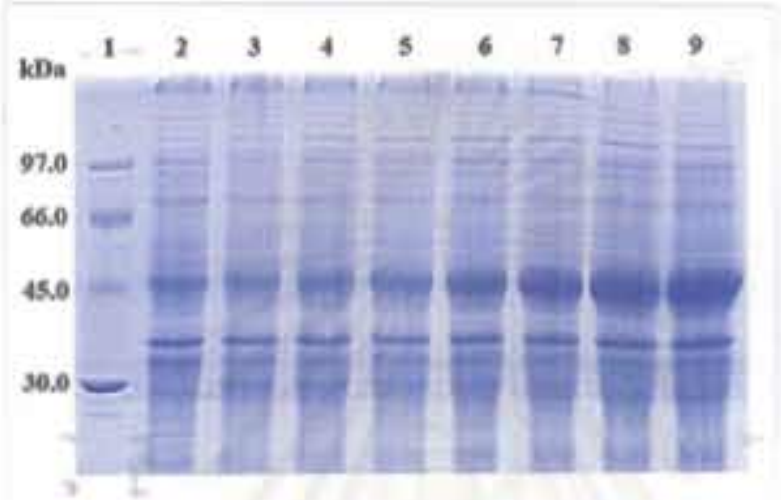


Figure 3.13 **Protein pattern of cell of *E. coli* BL21(DE3) harbouring pETFDH at various induction times detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

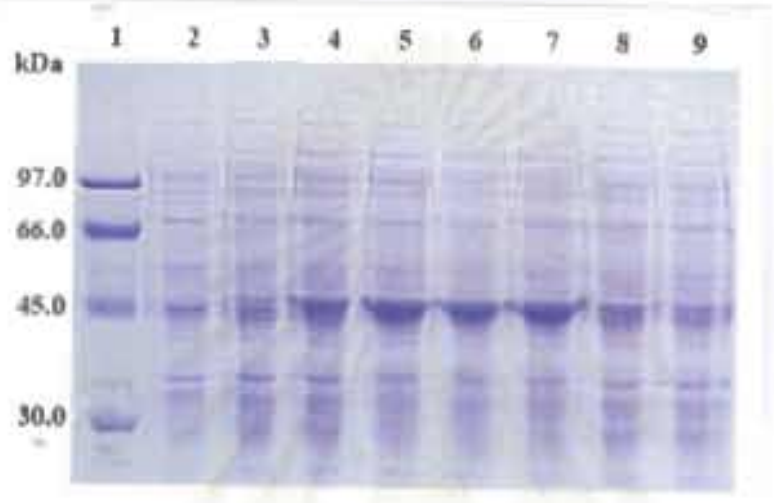


Figure 3.14 **Protein pattern of crude extract of *E. coli* BL21(DE3) harbouring pETFDH at various induction times detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = crude of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = crude of transformant at t_0

Lane 4-9 = crude of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

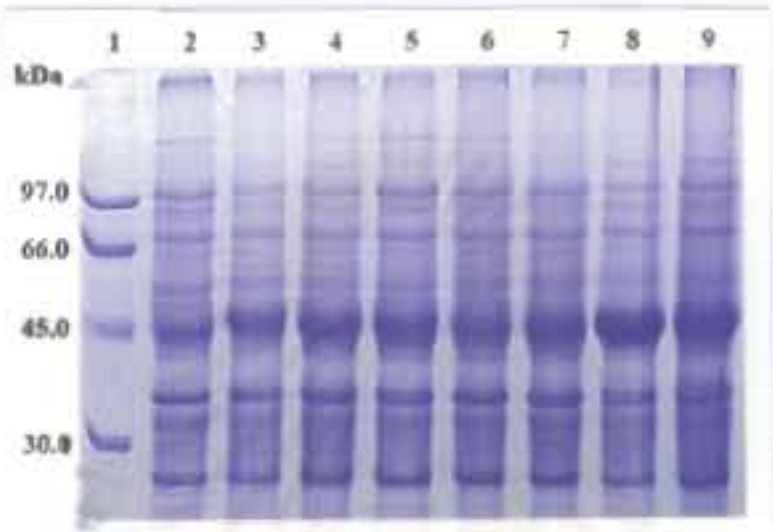


Figure 3.15 Protein pattern of cell of *E. coli* BL21(DE3)pLysS harbouring pETFDH at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

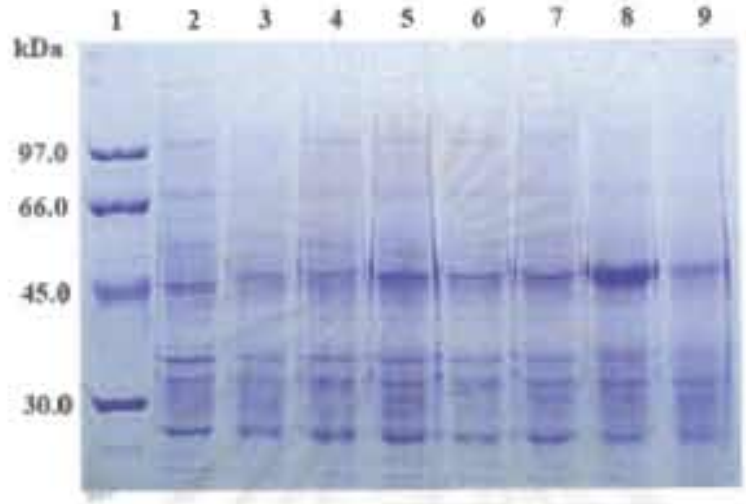


Figure 3.16 Protein pattern of crude extract of *E. coli* BL21(DE3)pLysS harbouring pETFDH at various induction times detected by SDS-PAGE

Lane1 = protein marker

Lane2 = crude extract of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at various induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

3.3 Cloning and expression of a heterologous gene of *phedh* and *fdh* using pET-17b vector

3.3.1 PCR amplification of *fdh*

The 1.2 kb of *fdh* fragment was amplified using a pETFDH containing *fdh* as a template. The gene were amplified by PCR using the following primers, T7*Bam*HI which contained *Bam*HI site and T7 promoter of expression vector of pET series as a forward primer and FDHR*Eco*RI, reverse primer, containing *Eco*RI site, TCA and complementary sequence of the 3´ end of *fdh* as described in section 2.18.1. Figure 3.17 shows the 1.2 kb PCR product of the *fdh* amplified by various annealing temperatures. The optimum annealing temperatures which gave strong specific PCR product was at 60.5 ^{o}C. The PCR product was purified by agarose gel electrophoresis before using for further cloning.

3.3.2 Construction of pETPF

The PCR product of *fdh* which was contained T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b as described in section 3.3.1 was digested with *Bam*HI and *Eco*RI. The *fdh* fragment was ligated to the *Bam*HI - *Eco*RI site of 4.4 kb pETPheDH, and then transformed into *E. coli* BL21(DE3) by electroporation. The recombinant clone were screened on LB plate containing 50 µg/ml ampicillin. When recombinant plasmid was digested with *Nde*I, *Eco*RI and *Bam*HI, three fragments were detected: a 3.3 kb of linear pET-17b vector, a 1.2 kb of *fdh*, and a 1.14 kb of *phedh*, respectively as shown in Figure 3.18.

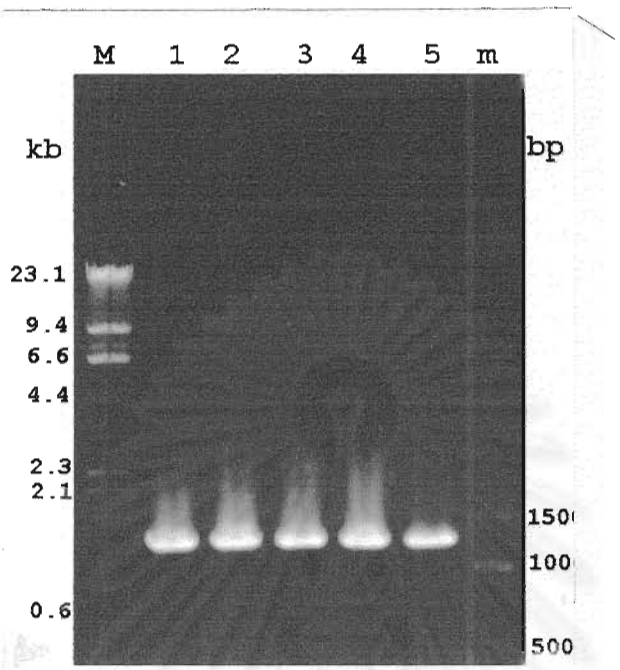


Figure 3.17 PCR product of the *fdh* with T7 promoter and SD sequence amplified by various annealing temperatures

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product at annealing temperature 54.2 °C

Lane 2 = amplified product at annealing temperature 56.2 °C

Lane 3 = amplified product at annealing temperature 57.3 °C

Lane 4 = amplified product at annealing temperature 58.4 °C

Lane 5 = amplified product at annealing temperature 60.5 °C

Lane m = 100 bp DNA ladder

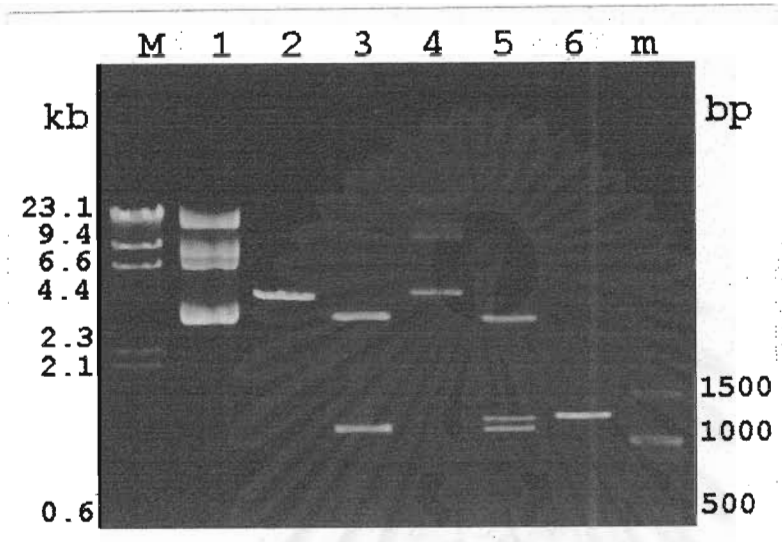


Figure 3.18 **Restriction pattern of pETPF**

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pETPheDH

Lane 2 = *Bam*HI-*Eco*RI digested pETPheDH

Lane 3 = *Nde*I-*Bam*HI digested pETPheDH

Lane 4 = undigested pETPF

Lane 5 = *Nde*I –*Eco*RI– *Bam*HI digested pETPF

Lane 6 = amplified product of *fdh* with T7 promoter and SD sequence

Lane m = 100 bp DNA ladder

3.3.3 Enzyme activities of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETPF

Ten *E. coli* BL21(DE3) harbouring pETPF were grown for enzyme assay of PheDH and FDH activity as described in 2.13.1 and 2.13.2 The transformant was induced by IPTG at final concentration of 0.4 mM before cell was harvested at 4 hours. They showed various levels of the enzyme total activity and specific activity of PheDH from 256 – 1531 U and 5.02 – 27.30 U/mg protein as well as FDH total activity and specific activity from 17 - 192 U and 0.25 - 3.43 U/mg protein, respectively, as shown in Table 3.5. The transformant plasmid No.2, that had the highest total activities and specific activities of both PheDH and FDH: 1531 U, 27.30 U/mg protein, 192 U, 3.43 U/mg protein, respectively was selected.

Recombinant plasmid from transformant No.2 was extracted and then transformed into *E. coli* BL21(DE3)pLysS by electroporation. The recombinant clones were screened on LB plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Ten transformants were grown and assayed for PheDH activity. They showed various levels of the enzyme total activity and specific activity of PheDH from 242 - 681 U and 3.70 – 12.69 U/mg protein as well as FDH total activity and specific activity from 37 - 68 U and 0.71 - 1.27 U/mg protein, respectively, as shown in Table 3.6 . The transformant plasmid No.6 had the highest total activities and specific activities of both PheDH and FDH with 681 U, 12.69 U/mg protein, 68 U, 1.27 U/mg protein, respectively was selected.

3.3.4 Optimization of induction time

E. coli BL21(DE3) transformant No. 2 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1,

Table 3.5 PheDH and FDH activities from crude extract of *E. coli* BL21(DE3) harbouring pETPF

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		PheDH	FDH		PheDH	FDH
pETPheDH	1.44	1536	-	63.81	24.07	-
pETFDH	1.74	-	173	75.33	-	2.29
1	1.21	300	190	51.19	5.87	3.71
2	1.32	1531	192	56.10	27.30	3.43
3	0.98	718	17	66.67	10.77	0.25
4	1.10	542	178	61.81	8.77	2.88
5	1.42	388	184	44.33	8.75	4.14
6	1.23	256	161	50.95	5.02	3.17
7	1.04	1091	49	49.76	21.92	0.98
8	0.92	976	125	54.19	18.00	2.31
9	1.27	451	162	50.57	8.92	3.20
10	1.37	844	98	58.81	14.35	1.67

[a] Total activity from 200 ml culture

Table 3.6 PheDH and FDH activities from crude extract of *E. coli* BL21(DE3)pLysS harbouring pETPF

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		PheDH	FDH		PheDH	FDH
pETPheDH	1.50	1683	-	64.62	26.04	-
pETFDH	1.50	-	189	64.62	-	2.72
1	1.47	242	42	65.48	3.70	0.65
2	1.11	628	61	59.00	10.64	1.03
3	1.22	489	56	58.10	8.42	0.95
4	1.53	466	56	50.62	9.20	1.11
5	1.41	649	46	55.90	11.61	0.82
6	1.07	681	68	53.67	12.69	1.27
7	1.32	573	37	51.62	11.10	0.71
8	1.12	559	51	49.19	11.37	1.04
9	1.51	403	49	68.81	5.86	0.71
10	0.97	615	54	59.24	10.38	0.91

[a] Total activity from 200 ml culture

2, 4, 8, 16, and 24 hours. As shown in Figure 3.19, the result indicated that cell wet weight increased untill 16 hours after that it slowly decreased. After induction for 4 hours, the highest total activity and specific activity of PheDH and FDH were obtained at 1682 U, 26.04 U/mg protein, 189 U, 2.73 U/mg protein, respectively. After 4 hours, the total activity and specific activity of PheDH and FDH were decreased.

The transformant No. 6 from *E. coli* BL21(DE3)pLysS was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours. As show in figure 3.20, the result indicated that cell wet weight increased untill 16 hours after that it slowly decreased. When induction time 16 hours were used, the highest total activity and specific activity of PheDH and FDH were obtained at 1547 U, 22.26 U/mg protein, 168 U, 2.42 U/mg protein, respectively, and after 16 hours the total activity and specific activity of PheDH and FDH were decreased.

3.35 Protein patterns of cells and crude extracts

The protein patterns of cells and crude extracts of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS from induction time course study of PheDH and FDH activity were subjected to SDS-PAGE. The intensity of protein bands from crude extract (Figure 3.22 and Figure 3.24) of each induction time was corresponded with their total activity in crude extract (Figure 3.19 and Figure 3.20). Though, the intensity of bands from cell seemed to be increased with increasing time because number of cell per volume of cell culture increased (Figure 3.21 and Figure 3.23). The result showed that the protein pattern of cells and crude extracts from pETPF clones

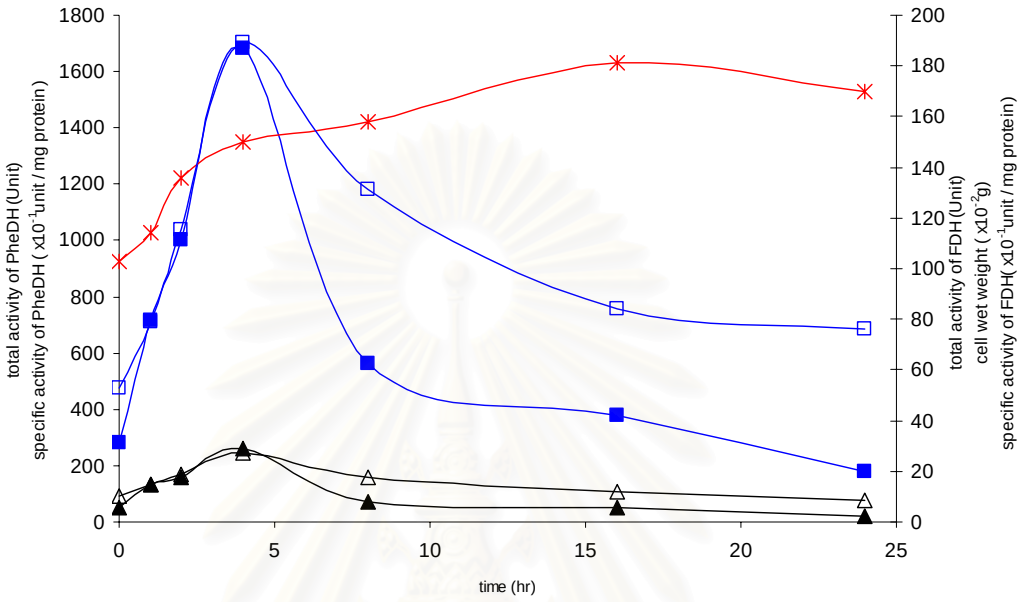


Figure 3.19 Effect of induction time on PheDH and FDH production and growth of *E. coli* BL21(DE3) harbouring pETPF

■ = total activity of PheDH (unit) ▲ = specific activity of PheDH ($x10^{-1}$unit/mg protein)

□ = total activity of FDH (unit) Δ = specific activity of FDH ($x10^{-1}$unit/mg protein)

* = cell wet weight ($x10^{-2}$ g)

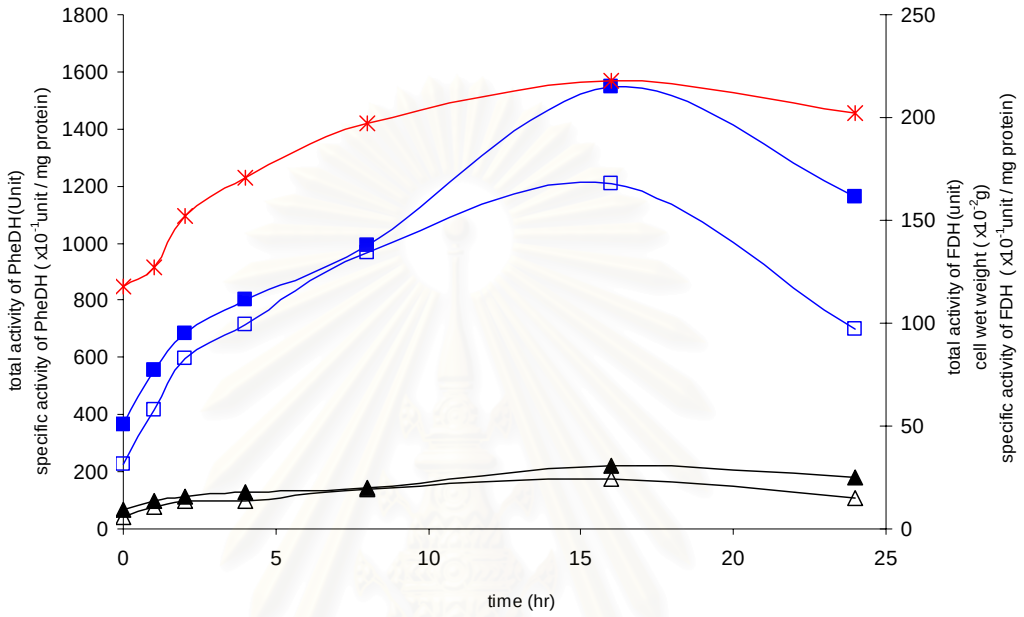


Figure 3.20 Effect of induction time on PheDH and FDH production and growth of *E. coli* BL21(DE3)pLysS harbouring pETPF

■ = total activity of PheDH (unit) ▲ = specific activity of PheDH ($x10^{-1}$unit/mg protein)

□ = total activity of FDH (unit) Δ = specific activity of FDH ($x10^{-1}$unit/mg protein)

∗ = cell wet weight ($x10^{-2}$ g)

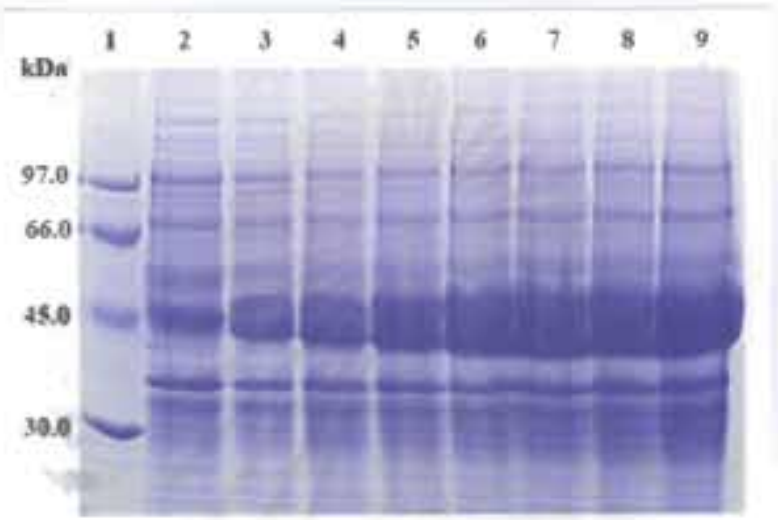


Figure 3.21 **Protein pattern of cell of *E. coli* BL21(DE3) harbouring pETPF at various induction time detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

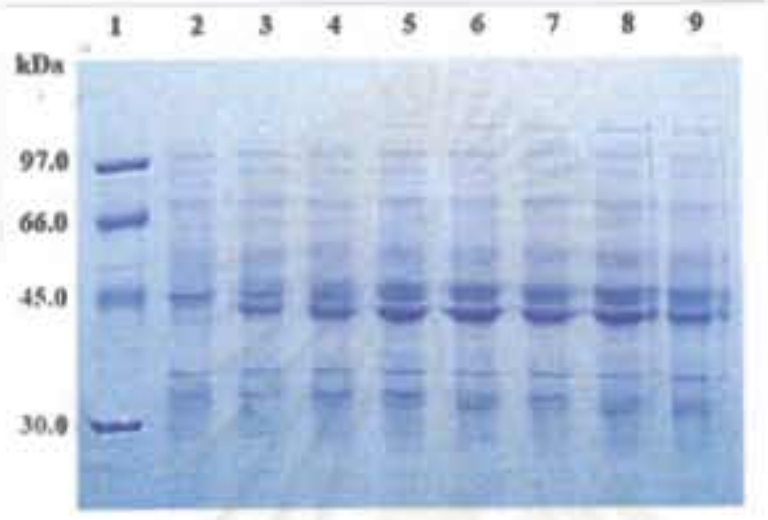


Figure 3.22 **Protein pattern of crude extract of *E. coli* BL21(DE3) harbouring pETPF at various induction times detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

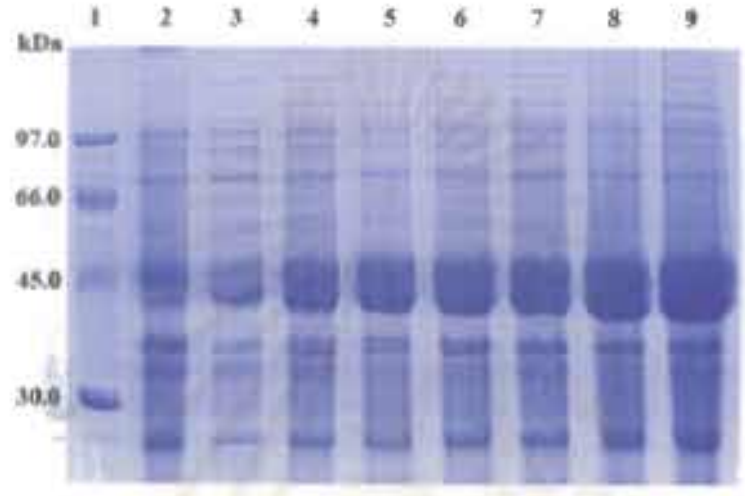


Figure 3.23 **Protein pattern of cell of *E. coli* BL21(DE3)pLysS harbouring pETPF at various induction times detected by SDS-PAGE**

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

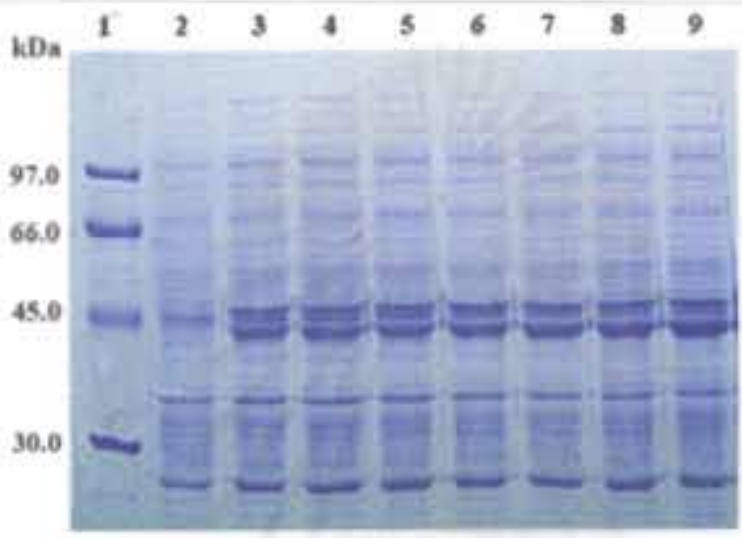


Figure 3.24 **Protein pattern of crude extract of *E. coli* BL21(DE3)pLysS harbouring pETPF at various induction times detected by SDS-PAGE**

Lane1 = protein marker

Lane2 = crude extract of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at induction times:

1, 2, 4, 8, 16 and 24 hours, respectively

had two adjacent major bands. The upper band was FDH and the other was PheDH since FDH (44 kDa) had a larger size than PheDH (35.8 kDa).

3.4 Cloning and expression of a heterologous gene of *fdh* and *phedh* using pET-17b vector

3.4.1 PCR amplification of *phedh*

The 1.14 kb of *phedh* fragment was amplified from a pETPheDH containing *phedh* by PCR using the following primers: T7*Bam*HI a forward primer, containing *Bam*HI site and T7 promoter of expression vector of pET series as well as PheDHR*Eco*RI, a reverse primer, containing *Eco*RI site, TTA and complementary sequence of the 3´ terminus sequence of *phedh* as described in section 2.18.2. Figure 3.25 shows the 1.14 kb PCR product of the *phedh* amplified by various annealing temperatures. The optimum annealing temperatures which gave strong specific PCR product was 59.5 ^{o}C. The PCR product was purified by agarose gel electrophoresis before using for further cloning.

3.4.2 Construction of pETFA

The PCR product of *phedh* which contained T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b as described in section 3.4.1 was digested with *Bam*HI and *Eco*RI. The *phedh* fragment was ligated to the *Bam*HI - *Eco*RI site of 4.5 kb pETFDH vector, and then transformed into *E. coli* BL21(DE3) by electroporation. The recombinant clone were screened on LB plate containing 50 µg/ml ampicillin. When digested recombinant plasmid with *Nde*I, *Eco*RI and *Bam*HI, three fragments were appeared: a 3.3 kb of linear pET-17b vector, a 1.14 kb of *phedh*, and a 1.2 kb of *fdh*, respectively as shown in Figure 3.26.

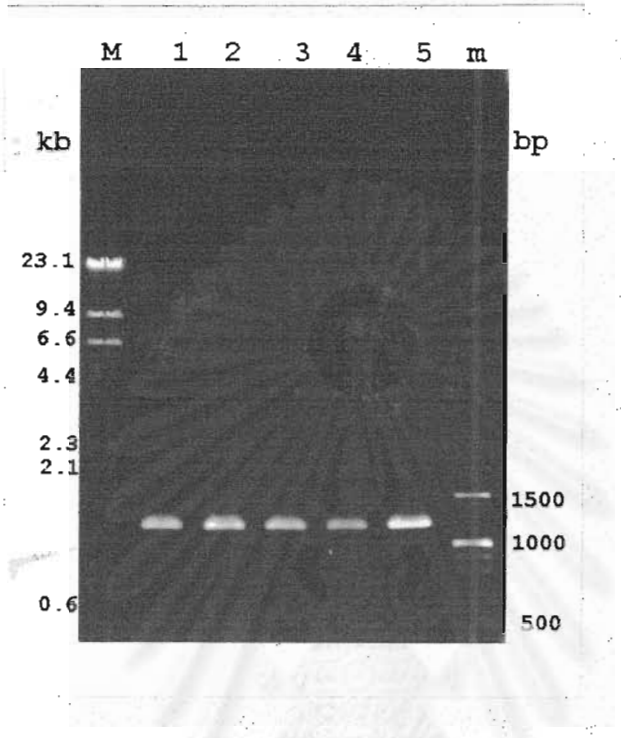


Figure 3.25 PCR product of the *phedh* with T7 promoter and SD sequence amplified by various annealing temperatures

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product at annealing temperature 54.2 °C

Lane 2 = amplified product at annealing temperature 55.3 °C

Lane 3 = amplified product at annealing temperature 56.2 °C

Lane 4 = amplified product at annealing temperature 57.3 °C

Lane 5 = amplified product at annealing temperature 59.5 °C

Lane m = 100 bp DNA ladder

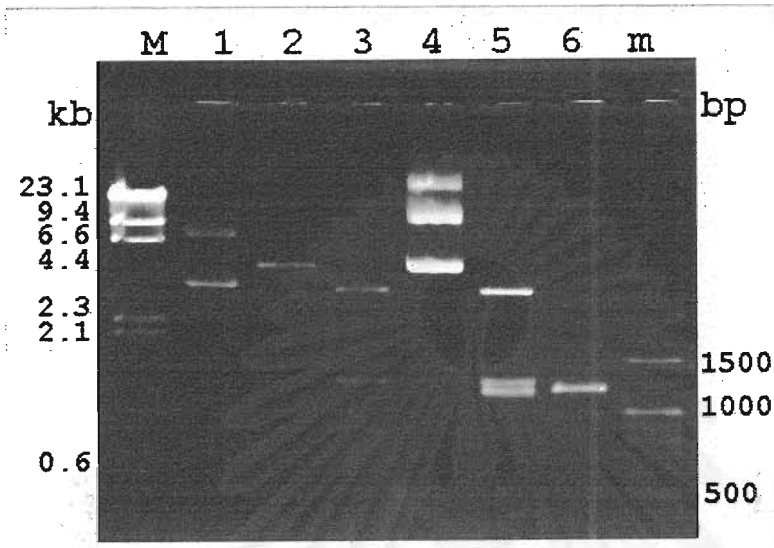


Figure 3.26 Restriction pattern of pETFP

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = undigested pETFDH

Lane 2 = *Bam*HI-*Eco*RI digested pETFDH

Lane 3 = *Nde*I-*Bam*HI digested pETFDH

Lane 4 = undigested pETFP

Lane 5 = *Nde*I –*Eco*RI, *Bam*HI digested pETFP

Lane 6 = amplified product of *phedh* with T7 promoter and SD sequence

Lane m = 100 bp DNA ladder

3.4.3 Enzyme activities of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETFP

Ten *E. coli* BL21(DE3) harbouring pETFP were grown for assay of FDH and PheDH activities. The transformants were induced by IPTG at final concentration of 0.4 mM before cells were harvested at 4 hours. They showed various levels of total activity and specific activity of FDH from 22 - 94 U and 0.47 – 1.68 U/mg protein as well as total activity and specific activity of PheDH from 827 – 1277 U and 15.82 – 22.91 U/mg protein, respectively, as shown in Table 3.7. The transformant No.5 had the highest total activities and specific activities of both PheDH and FDH with 1277 U, 22.91 U/mg protein, 94 U, 1.68 U/mg protein, respectively was selected.

Recombinant plasmid from transformant No.5 was extracted and then transformed into *E. coli* BL21(DE3)pLysS by electroporation. The recombinant clones were screened on LB plate containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Ten transformants were grown and assayed for FDH and PheDH activity. They showed various levels of the enzyme total activity and specific activity of FDH from 59 - 96 U and 1.13 - 1.55 U/mg protein as well as total activity and specific activity of PheDH from 52 - 1513 U and 0.89 – 24.58 U/mg protein, respectively, as shown in Table 3.8. The transformant No.6 had the highest total activities and specific activities of both PheDH and FDH: 1513 U, 24.58 U/mg protein, 96 U, 1.55 U/mg protein, respectively was selected.

3.4.4 Optimization of induction time

E. coli BL21(DE3) transformant No. 5 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1,

Table 3.7 FDH and PheDH activities from crude extract of *E.coli* BL21(DE3) harbouring pETFP

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		FDH	PheDH		FDH	PheDH
pETFDH	1.74	173	-	75.33	2.29	-
pETPheDH	1.44	-	1536	63.81	-	24.07
1	1.44	43	947	54.57	0.78	17.35
2	1.51	71	892	56.38	1.27	15.82
3	0.92	58	1175	58.14	0.99	20.20
4	1.21	90	1050	45.67	1.97	23.00
5	1.26	94	1277	55.71	1.68	22.91
6	1.34	71	956	47.71	1.49	20.04
7	1.49	22	913	46.86	0.47	19.49
8	0.89	59	1059	49.43	1.19	21.43
9	1.14	48	960	58.67	0.83	16.36
10	1.33	58	827	51.29	1.12	16.12

[a] Total activity from 200 ml culture

Table 3.8 FDH and PheDH activities from crude extract of *E.coli* BL21(DE3)pLysS harbouring pETFP

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		FDH	PheDH		FDH	PheDH
pETFDH	1.81	185	-	65.81	2.81	-
pETPheDH	1.81	-	1644	65.81	-	24.98
1	0.95	77	52	58.46	1.31	0.89
2	1.31	80	577	87.57	0.91	6.59
3	1.07	91	747	82.64	1.10	9.03
4	1.24	73	398	53.54	1.36	7.44
5	1.13	77	1473	65.54	1.17	22.48
6	1.47	96	1513	61.57	1.55	24.58
7	0.92	59	446	52.24	1.13	8.54
8	1.67	62	258	54.71	1.13	4.71
9	1.20	75	549	71.67	1.05	7.65
10	1.18	69	736	63.76	1.08	11.54

[a] Total activity from 200 ml culture

2, 4, 8, 16, and 24 hours. As shown in Figure 3.27, the result indicated that cell wet weight increased rapidly until 4 hours after that cell slowly increased until 16 hours, then slowly decreased. After induction for 4 hours, the highest total activity and specific activity of PheDH and FDH were obtained at 1644 U, 24.98 U/mg protein, 185 U, 2.80 U/mg protein, respectively. After 4 hours the total activity and specific activity of PheDH and FDH were decreased.

The *E. coli* BL21(DE3)pLysS transformant No.6 was grown and induced by IPTG at final concentration of 0.4 mM before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours. As show in figure 3.28, the result indicated that cell wet weight increased untill 16 hours after that their slowly decreased. When induction time 16 hours were used, the highest total activity and specific activity of PheDH and FDH were obtained at 1602 U, 27.88 U/mg protein, 178 U, 2.86 U/mg protein, respectively, and after 16 hours the total activity and specific activity of PheDH and FDH were decreased.

3.4.5 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS for induction time course study of FDH and PheDH activity were analyzed by SDS-PAGE. The intensity of major protein bands from cell (Figure 3.29 and Figure 3.31) and crude extract (Figure 3.30 and Figure 3.32) of both recombinant clones increased rapidly in the first 2 hours after that the band intensity of major bands were constant until 24 hours. The protein pattern of cells and crude extracts from pETFP had two adjacent major bands. The upper band was FDH and the other was PheDH since FDH (44 kDa) had a size larger than PheDH (35.8 kDa).

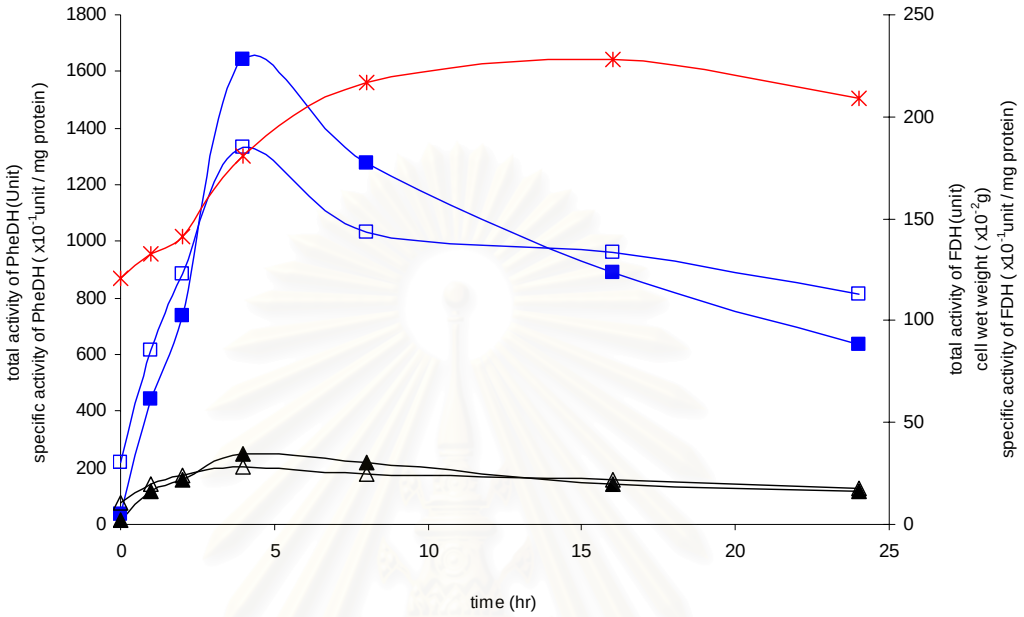


Figure 3.27 Effect of induction time on FDH and PheDH production and growth of *E. coli* BL21(DE3) harbouring pETFP

■ = total activity of PheDH (unit) ▲ = specific activity of PheDH ($x10^{-1}$unit/mg protein)

□ = total activity of FDH (unit) Δ = specific activity of FDH ($x10^{-1}$unit/mg protein)

∗ = cell wet weight ($x10^{-2}$ g)

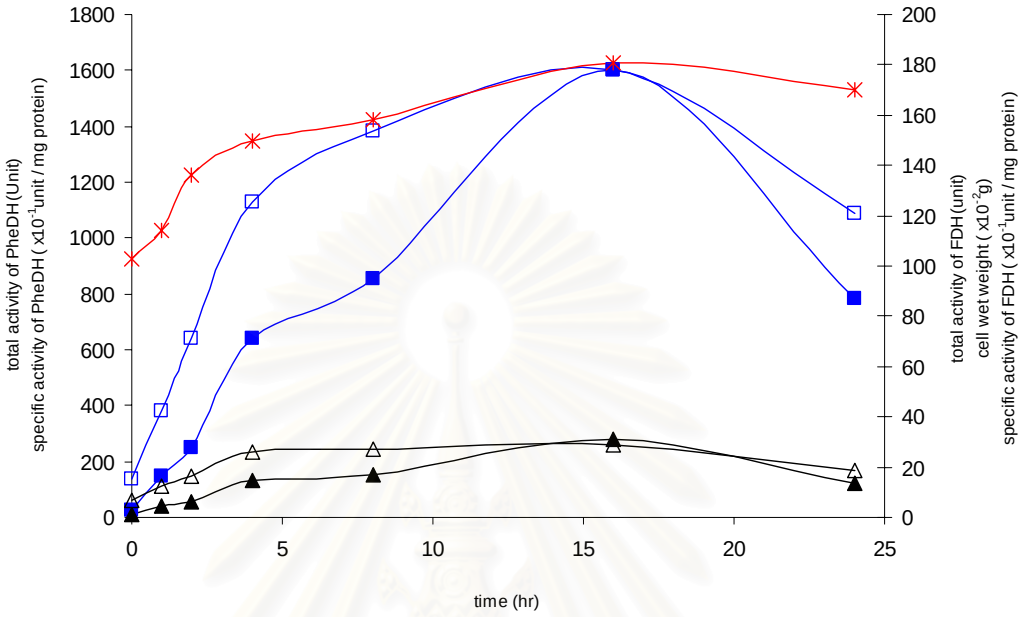


Figure 3.28 Effect of induction time on FDH and PheDH production and growth of *E. coli* BL21(DE3)pLysS harbouring pETFP

■ = total activity of PheDH (unit) ▲ = specific activity of PheDH ($x10^{-1}$unit/mg protein)

□ = total activity of FDH (unit) Δ = specific activity of FDH ($x10^{-1}$unit/mg protein)

∗ = cell wet weight ($x10^{-2}$ g)

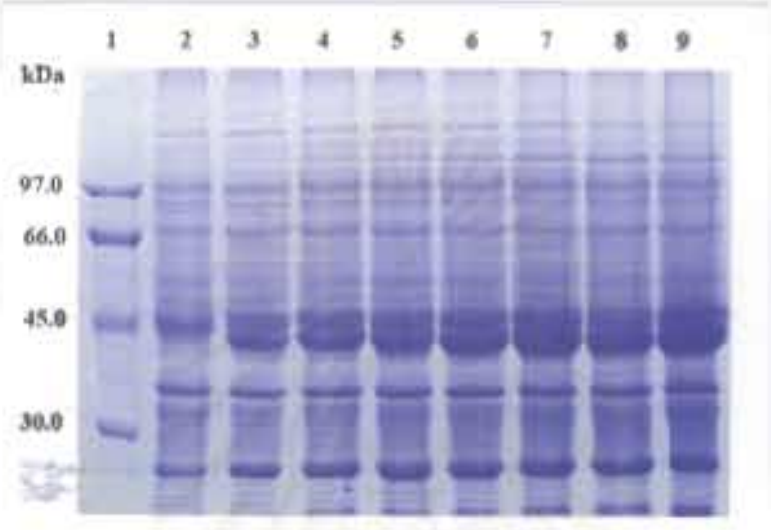


Figure 3.29 Protein pattern of cell of *E. coli* BL21(DE3) harbouring pETFP at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at inducion times:

1, 2, 4, 8, 16 and 24 hours, respectively

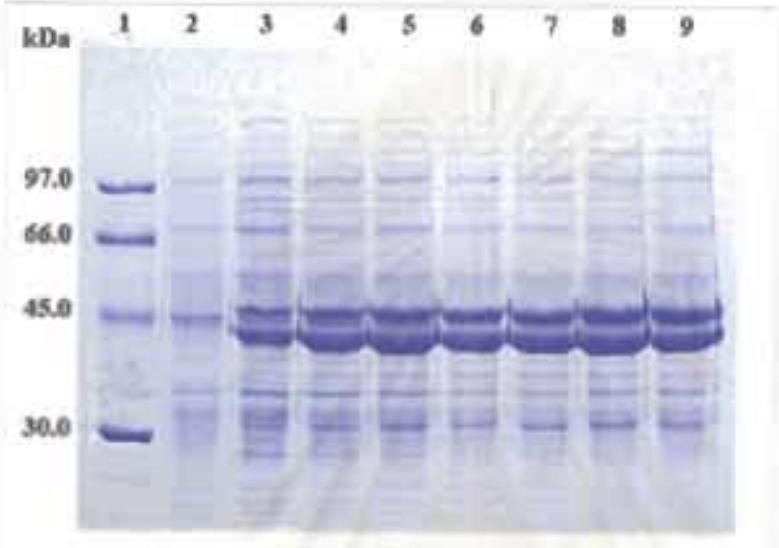


Figure 3.30 Protein pattern of crude extract of *E. coli* BL21(DE3) harbouring pETFP at various induction times detected by SDS-PAGE

Lane1 = protein marker

Lane2 = crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at inducion times:

1, 2, 4, 8, 16 and 24 hours, respectively

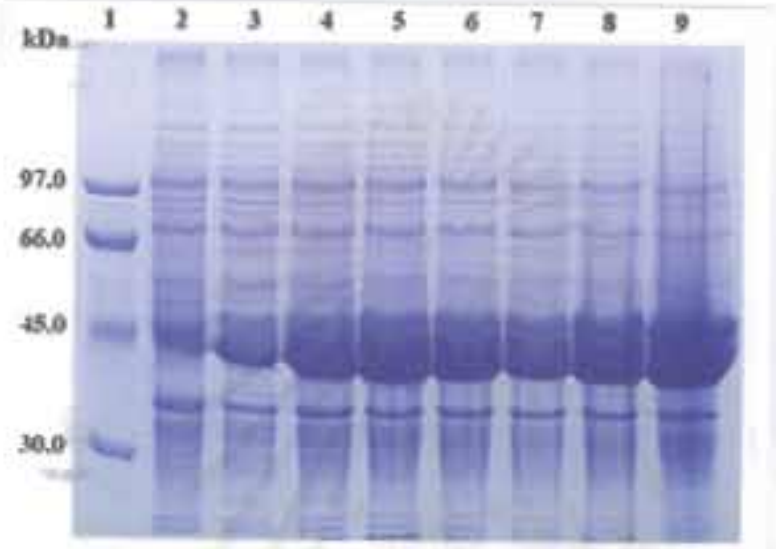


Figure 3.31 Protein pattern of cell of *E. coli* BL21(DE3)pLysS harbouring pETFP at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = cell of transformant at t_0

Lane 4-9 = cell of transformant at inducion times:

1, 2, 4, 8, 16 and 24 hours, respectively

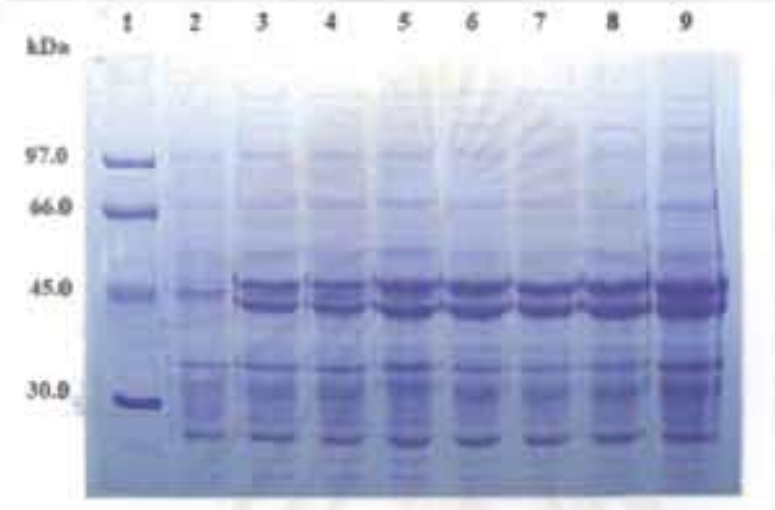


Figure 3.32 Protein pattern of crude extract of *E. coli* BL21(DE3)pLysS harbouring pETFP at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of *E. coli* BL21(DE3)pLysS harbouring pET-17b

Lane 3 = crude extract of transformant at t_0

Lane 4-9 = crude extract of transformant at inducion times:

1, 2, 4, 8, 16 and 24 hours, respectively

Specific activity of PheDH / FDH of all recombinant clones were summarized in Table 3.9 .

3.5 The production of phenylalanine by recombinant clones

As described in 2.19, the *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring pETPheDH, pETPF and pETFP grown at their optimum condition for the enzyme production were used to produce phenylalanine. For cell permeabilization in an attempt to develop a commercially attractive biocatalysis method, four different organic solvents including acetone, benzene, chloroform and hexane were used. The 0.05 g of cell was resuspended in the required 30 µl of organic solvent for variable period of time. After treated with benzene and chloroform, *E. coli* cell was immediately lysed. Therefore, only acetone and hexane treated cells were used for phenylalanine production experiment.

3.5.1 The first analysis

Since TLC on cellulose plastic plate can be used for identification of phenylalanine, the sample were separated on the plate using an *n*-butanol, acetic acid and water mixture in the ratio 4:1:1 as the mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone mixture (70:30), the purple spot of amino acid could be seen. As shown in Figure 3.33 – 3.38, all recombinant clones could produce phenylalanine (lane1). For acetone treated cells, all types of *E. coli* BL21(DE3)pLysS clone lose their ability to produce phenylalanine while *E. coli* BL21(DE3) harbouring pETPF and pETFP could produce decreased amount of phenylalanine with increasing incubation time but *E. coli* BL21(DE3) harbouring pETPheDH could not produce phenylalanine. In all hexane treated clones, the production of phenylalanine increased

Table 3.9 Summary of PheDH/FDH specific activity of all recombinant clones

Plasmid	PheDH specific activity (U/mg protein)			FDH specific activity (U/mg protein)		
	E. coli JM109	*E. coli* BL21(DE3)	*E. coli* BL21(DE3) pLysS	*E. col* JM109	*E. coli* BL21(DE3)	*E. coli* BL21(DE3) pLysS
none	-	-	-	-	-	-
pET-17b	-	-	-	-	-	-
pUCPheDH	6.92	-	-	-	-	-
pUCFDH	-	-	-	0.41	-	-
pETPheDH	-	24.07	15.29	-	-	-
pETFDH	-	-	-	-	2.29	1.86
pETPF	-	26.04	22.26	-	2.72	2.42
pETFP	-	24.98	27.88	-	2.81	2.86

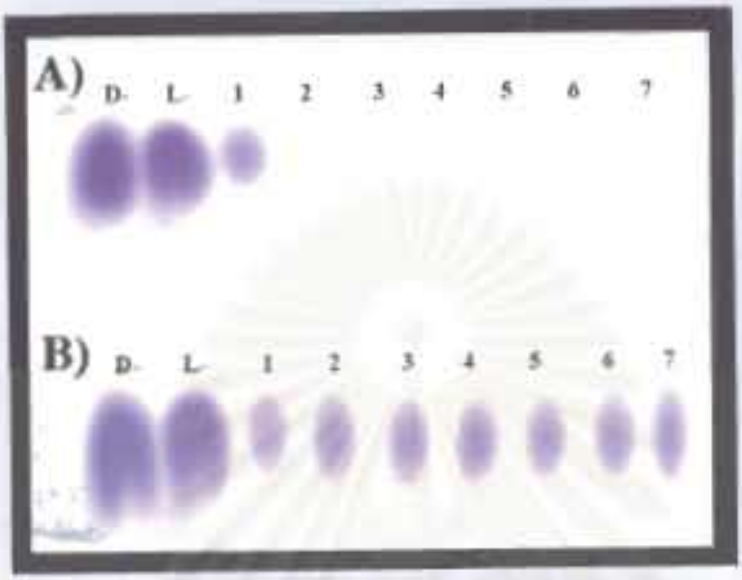


Figure 3.33 Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3) harbouring pETPheDH.

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D : D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

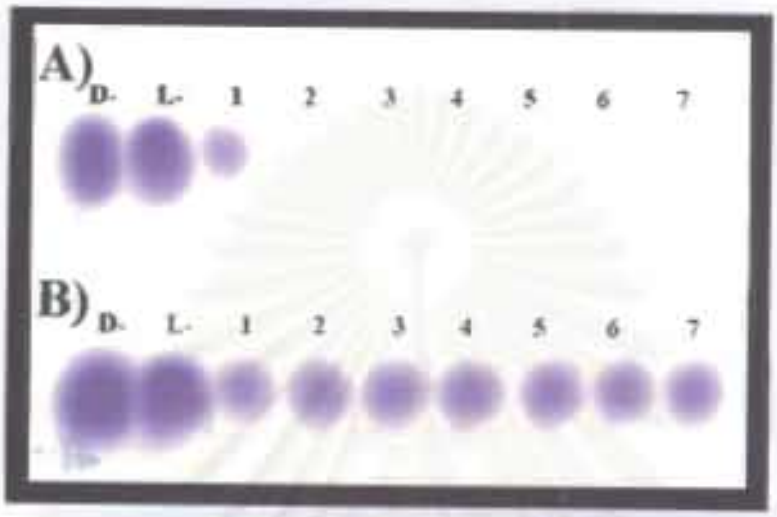


Figure 3.34 **Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3)pLysS harbouring pETPheDH.**

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D : D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

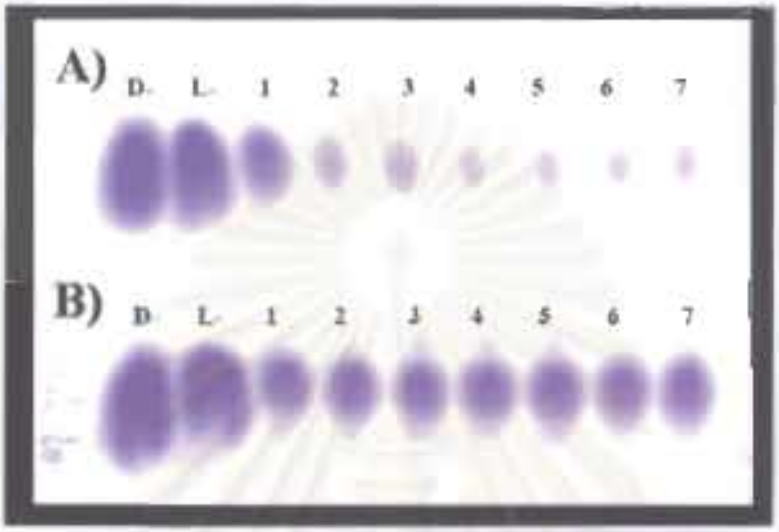


Figure 3.35 Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3) harbouring pETPF

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D : D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

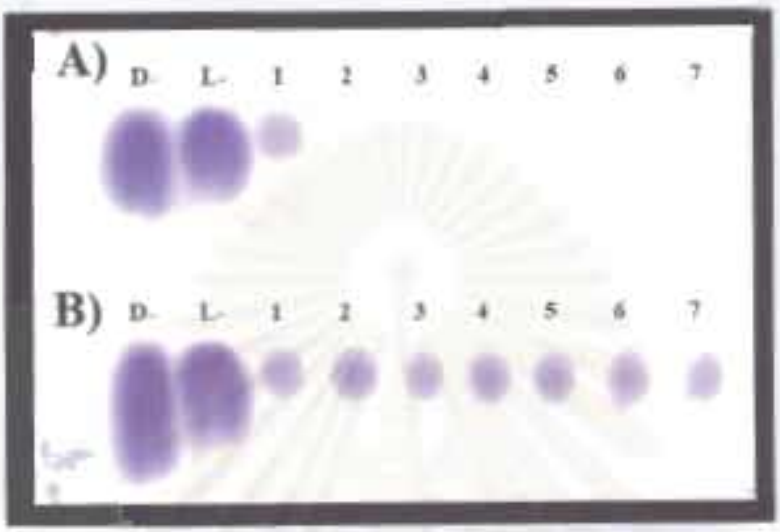


Figure 3.36 **Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3)pLysS harbouring pETPF**

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D : D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

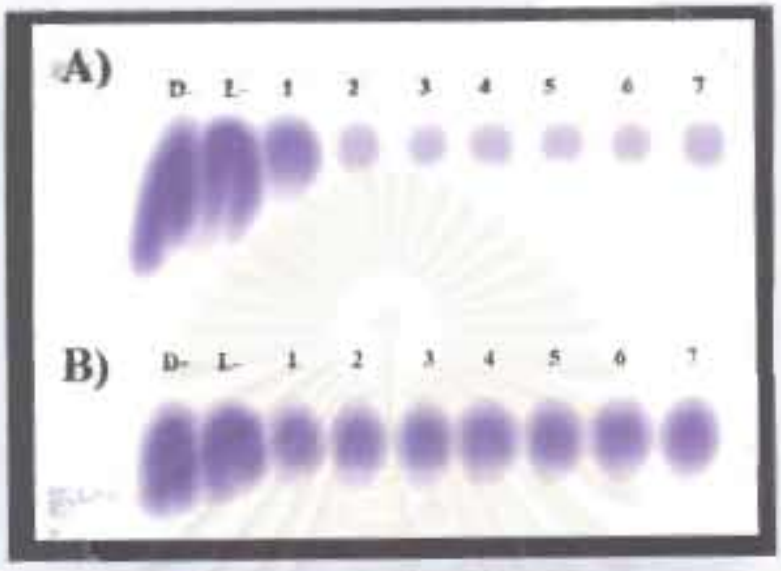


Figure 3.37 **Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3) harbouring pETFP**

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D : D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

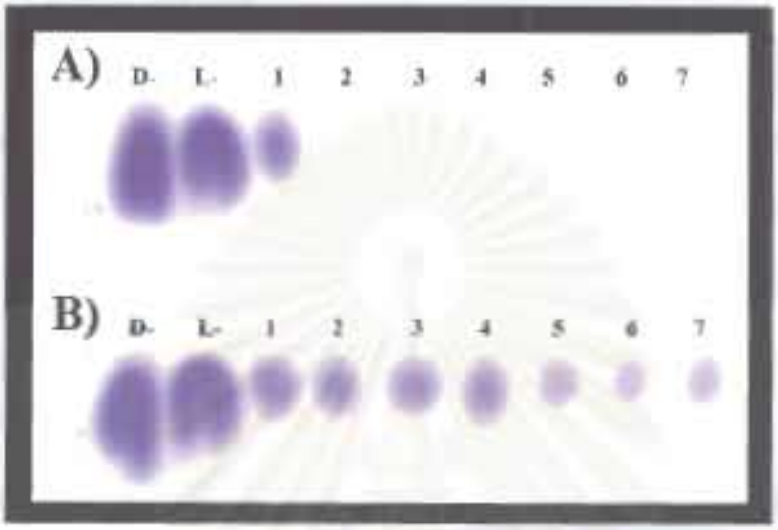


Figure 3.38 **Effect of permeabilization time on phenylalanine production of *E. coli* BL21(DE3)pLysS harbouring pETFP**

Samples were spoted on cellulose TLC plate and separated by using an *n*-butanol: acetic acid: water (4:1:1) mixture a mobile phase. After developed with 0.5% ninhydrin in ethanol : acetone (30:70), the plate was dried by hot air.

Lane D: D-phenylalanine

Lane L : L-phenylalanine

Lane 1 : untreated cell

Lane 2-7 : treated cell for 1, 2, 5, 10, 15 and 30 min, respectively

A) acetone treated cell

B) hexane treated cell

until 5 minutes and then slowly decreased. The phenylalanine product from hexane treated cells at 5 minutes was further quantified by using reverse phase TLC.

3.5.2 The second analysis

In this experiment, the identification and quantification of optical isomers of phenylalanine was performed using the modified Marfey's method which is sample and rapid without expensive impregnated plates or a chiral mobile phase. Figure 3.39 – 3.44 show the chromatograms of FDAA phenylalanine developed with acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). The mobility of yellow spot of 10 µg standard D and L-phenylalanine gave no difference at the first development. After repeating the chromatography for ten times, the result showed that the mobility of all sample spots were similar to that of L-phenylalanine.

For quantitative determination of L-phenylalanine, the yellow spots were scraped off the plate after chromatography and extracted with methanol: water (1:1, v/v). The absorbance of the extracts was measured at 340 nm with a spectrophotometer and the quantitative analysis could be performed by comparison with a standard calibration curve. Hexanes treated cells showed higher yield than untreated cells in a range of 14-30%. The phenylalanine product of heterologous gene clones was higher than that of single gene clone about 2 fold while type of host cell did not effect L-phenylalanine production. Summary of phenylalanine production is shown in Table 3.10

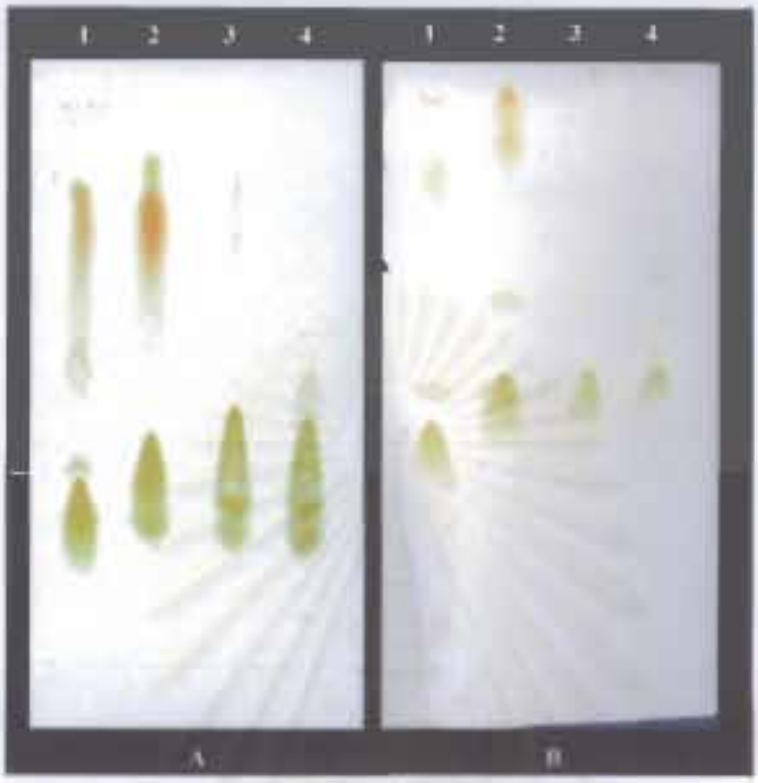


Figure 3.39 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3) contained pETPheDH on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

Figure 3.40 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3)pLysS contained pETPheDH on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

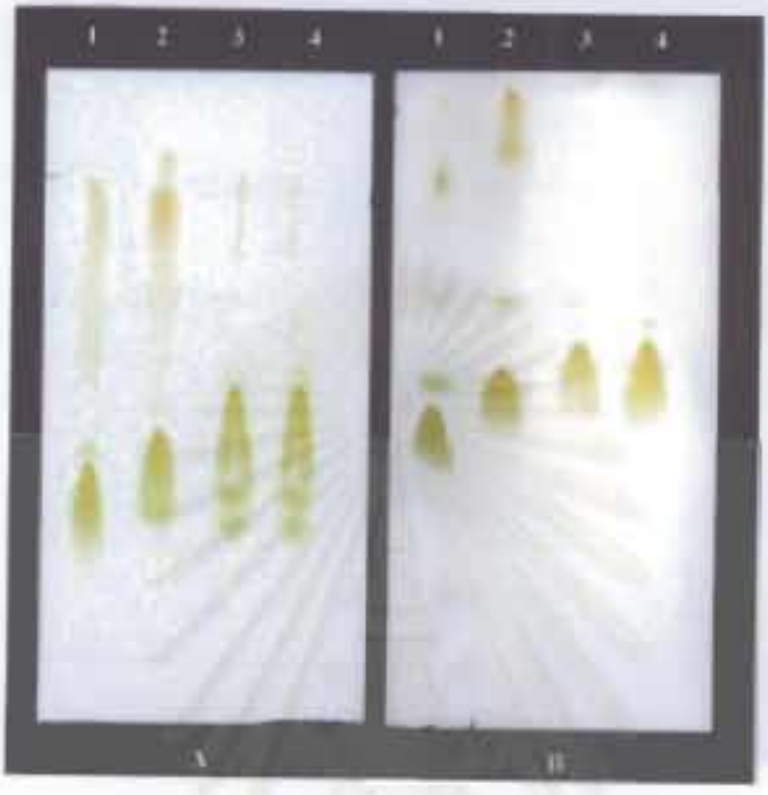


Figure 3.41 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3) contained pETPF on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

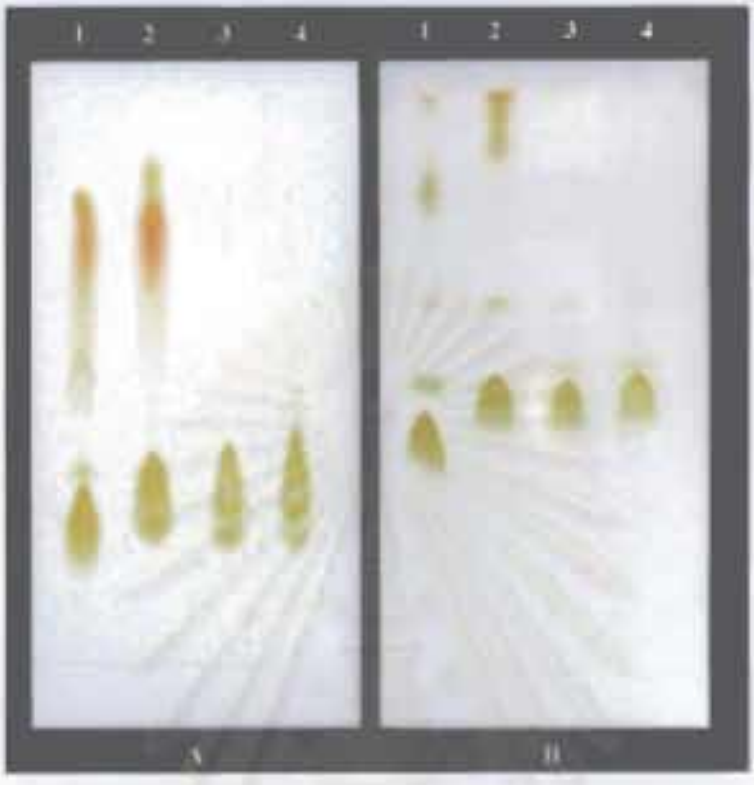


Figure 3.42 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3)pLysS contained pETPF on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

Figure 3.43 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3) contained pETFP on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

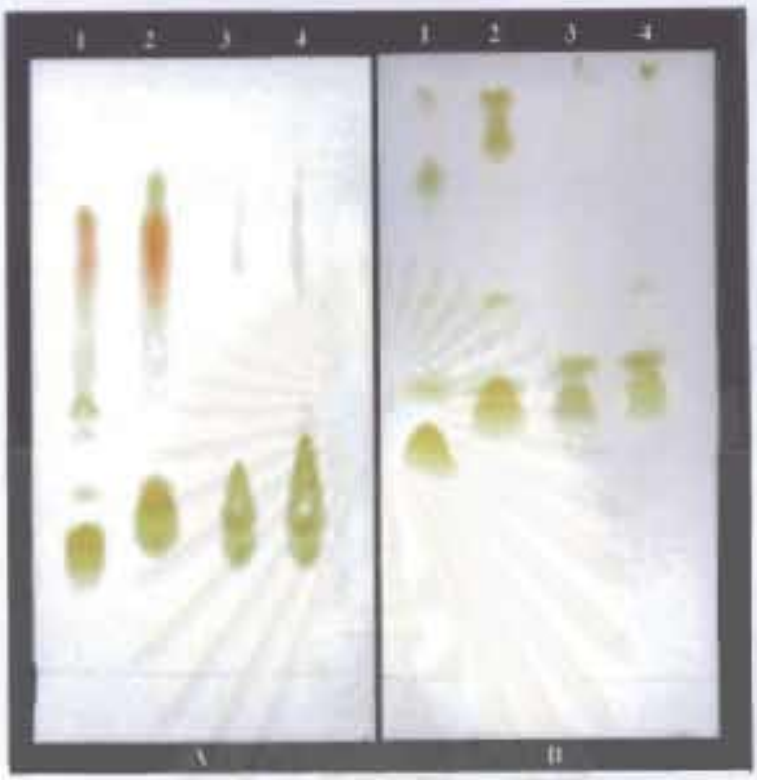


Figure 3.44 Separation of optical isomer of FDAA phenylalanine from *E. coli* BL21(DE3)pLysS contained pETFP on reversed phased TLC plates.

Each sample of FDAA phenylalanine was spotted and developed in solvent acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (40:60). Lane 1: D-phenylalanine, Lane 2: L-phenylalanine, Lane 3: product from untreated cell, Lane 4: product from cell treated with hexane for 5 min.

A) The first development B) The tenth development

Table 3.10 Phenylalanine production[a] by recombinant clones

Host	plasmid	D-phenylalanine (mg)		L-phenylalanine (mg)		Yield (%)	
		Untreated cell	Treated cell	Untreated cell	Treated cell	Untreated cell	Treated cell
E. coli BL21(DE3)	pETPheDH	0	0	1.34	1.74	32.42	42.03
	pETPF	0	0	2.69	3.07	65.04	74.35
	pETFP	0	0	2.58	3.17	62.39	76.69
E. coli BL21(DE3)pLysS	pETPheDH	0	0	1.35	1.61	32.62	38.90
	pETPF	0	0	2.74	3.15	66.37	76.19
	pETFP	0	0	1.99	2.34	48.11	56.72

[a] One ml of the reaction mixture containing 0.05 g wet weight of cell, 0.5 M ammonium formate (pH 7.5) and 0.05 M phenylpyruvate was incubated at 37 ^{o}C, 250 rpm for 24 hours as described in section 2.19 .

CHAPTER IV

DISCUSSION

The commercial values of essential amino acid come from their wide applicability in both pharmaceutical and food industries. With a stricter demand on the quality of human life, many food products with the emphasis on good test as well as the benefit to health emerge enormously in the last two decades. Aspartame, an artificial sweetener, appears to be such a product. Traditionally, the synthesis of amino acid has been carried out by chemical methods. However, the pressing need for the optically active forms of amino acids has called for the investigation on the bioprocessing (Chao *et al*., 2000).

Several enzymatic methods have been developed for the synthesis of L-phenylalanine. Because enzymatic method usually provide and optically pure product without consuming so much energy as in chemical synthesis and without yielding by-products as in fermentation, they will probably become more important in amino acid industries (Cho and Soda, 1997). The industrial use of such systems mentioned above depends chiefly on the cost of the enzymes. Intact cells of microorganism containing enzyme is also used as catalysts in order to decrease cost.

Our research group at the Department of Biochemistry has studied on L-phenylalanine dehydrogenase of *Bacillus badius* BC1 in thermotolerant bacteria, isolated from soil samples collected from temperate and hot spring areas (Suriyapanpong *et al*., 2000). Catalytic propoties of PheDH from this bacterial strain are suitable for industrial application such as L-phenylalanine and its derivative

production. In addition, the *phedh* was already clone and expression in *E. coli* JM109 cell using pUC18 as a vector (Charoenpanich, 2001).

The scale production of L-phenylalanine by PheDH has been hampered by the cost of coenzyme. A mutienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem. In this research, the L-phenylalanine production efficiency of recombinant clone was attempted to improve by co-existence of *phedh* and NAD^+ regenerating enzyme gene.

In general, one or more homologous or heterologous genes coding for the phenylalanine-forming activity can be brought to overexpression in the microorganism used in a well-known manner, for example under the control of a conventional homologous or heterologous promoter that is active in the microorganism used. For such purpose, one or more structural genes are actively combined with the promoter, for example by introducing the coding gene in the right orientation and the right reading frame into a plasmid or another vector, which already contains the promoter.

Expression of *phedh* and/or *fdh* under T7 promoter of high expression vector pET-17b as well as phenylalanine production of *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS were shown in Figure 4.1 and 4.2 .

In single gene expression system, *E. coli* BL21(DE3) harbouring pETPheDH and *E. coli* BL21(DE3)pLysS harbouring pETPheDH showed PheDH activity 4.36 and 3.64 fold higher than those of original clone, *E. coli* JM109 harbouring pUCPheDH. While *E. coli* BL21(DE3) harbouring pETFDH and *E. coli* BL21(DE3)pLysS harbouring pETFDH showed FDH activity 8.65 and 6.35 fold higher than the activity of their original clone *E. coli* JM109 harbouring pUCFDH.

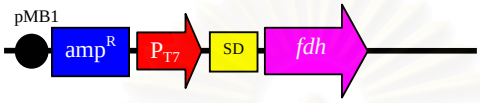
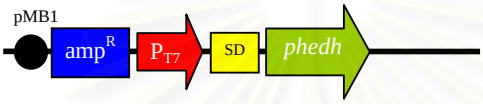
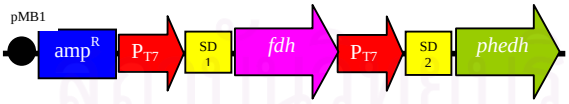
		Specific activity (U)	L-phenylalanine (mg)[a]	
		FDH / PheDH	hexane untreated cell /	hexane treated cell
Single system				
pETFDH		2.29 / -	ND	
pETPheDH		- / 24.07	1.34 / 1.74	
Heterologous gene system				
		Specific activity (U)	L-phenylalanine (mg)[a]	
		FDH / PheDH	hexane untreated cell /	hexane treated cell
pETPF		2.72 / 26.04	2.69 / 3.07	
pETFP		2.81 / 24.98	2.58 / 3.17	

Figure 4.1 Summary of gene expression and L-phenylalanine production in *E. coli* BL21(DE3)

[a] The phenylalanine production from 1 ml reaction mixture determined by TLC technique.

ND, not determined.

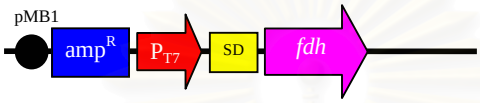
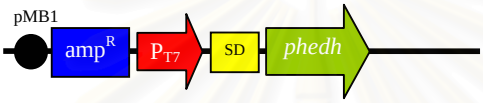
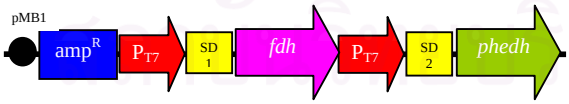
		Specific activity (U)	L-phenylalanine (mg)[a]	
		FDH / PheDH	hexane untreated cell /	hexane treated cell
Single system				
pETFDH		1.86 / -	ND	
pETPheDH		- / 15.29	1.35 / 1.61	
Heterologous gene system				
		Specific activity (U)	L-phenylalanine (mg)[a]	
		FDH / PheDH	hexane untreated cell /	hexane treated cell
pETPF		2.42 / 22.26	2.74 / 3.15	
pETFP		2.86 / 27.88	1.99 / 2.34	

Figure 4.2 Summary of gene expression and L-phenylalanine production in *E. coli* BL21(DE3)pLysS

[a] The phenylalanine production from 1 ml reaction mixture determined by TLC technique.

ND, not determine

Since, pETPheDH and pETFDH were controlled under T7 promoter which is stronger than *lac* promoter of pUC18, the expression of inserted gene were well elevated.

In heterologous gene expression system of *phedh* and *fdh*, pETPF and pETFP which had T7 promoter upstream of each were constructed. Specific activities of PheDH and FDH of pETPF and pETFP in *E. coli* BL21(DE3) clones were 26.04, 2.72, 24.98 and 2.81 U, respectively and in *E. coli* BL21(DE3)pLysS clones were 22.26, 2.42, 27.88 and 2.86 U, respectively as shown in Figure 4.1 and 4.2 . The result showed that with their own T7 promoter, the position of both *phedh* and *fdh* on pET-17b vector had no effect on their expression. Furthermore, expression of *phedh* and *fdh* in heterologous gene clones were not different form their expression in single gene clone (pETPheDH and pETFDH).

The induction time of each recombinant clones were varied from 0-24 hours. The highest PheDH total activity of *E. coli* BL21(DE3) containing pETPheDH, pETPF and pETFP clones were found at 4 hours while those of *E. coli* BL21(DE3)pLysS clones containing pETPheDH, pETPF and pETFP were at 16 hours. For induction of *fdh* expression, similar result with *phedh* expression was obtained. The variation of induction time between the hosts may be caused by the difference in control of expression of these hosts. *E. coli* BL21(DE3)pLysS has pLysS plasmid for production of T7 lysozyme, which is an inhibitor of the production of T7 RNA polymerase, and leads to further inhibition of expression in absence of IPTG. The presence of pLysS increases both the tolerance and stability of *E. coli* BL21(DE3) for separate plasmid, which encodes potentially toxic gene insert (pET system manual, 10^{th} edition).

Phenylalanine production from *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS containing plasmid pETPheDH, pETPF and pETFP were determined. According to report of Galkin *et al.* (1997), L-phenylalanine were synthesized by *E. coli* TG1 which contained pFDHPheDH. The α-keto acid was added to the reaction mixture stepwise in several portions to keep its concentration not higher than 50 mM because PheDH suffers from substrate inhibition at high concentration of phenylpyruvate. A major disadvantage used cells as biocatalysts is the diffusional limitation of substrates and products. When the enzyme is located in the periplasmic space or in the cytoplasm, permeabilization is recommended to produce biocatalysts with high enzyme activities. The cell permeabilization using organic solvents, detergents, CTAB, deoxycholate or bile salts has been reported to reduce the limitations due to insufficient permeation of substrate and product through the external membrane. Organic solvents are less expensive than detergents and may be eliminated by simple evaporation. Physicochemical properties of the organic solvents used such as dielectric constant, hydrophobicity and density correlated with the increase of productivity and could be useful to select the most convenient permeabilization.

The determination of phenylalanine production using thin layer chromatography (TLC) for qualitative and quantitative analysis has a privileged position, due to the simplicity of apparatus (Simion *et al.*, 2001). Enantiomeric resolution of amino acids has been achieved using chiral TLC plates.

A simple and rapid method of separating optical isomers of amino acids was achieved on a reversed-phase TLC plate, without using expensive impregnated plates or chiral mobile phase. In Marfey's method, amino acids are derivatized with

1-fluoro-2,4-dinitrophenyl-5-l-alanine amide (FDAA) to form diastereomer of amino acid. The spots of standard L-amino acid move faster than those of the D-amino acid because the FDAA D-amino acid has greater affinity for the C_{18} silica gel than the FDAA L-phenylalanine. FDAA amino acid can then be extracted from the plate with methanol/water (1/1, v/v), and quantitative analysis was made by applying several different amounts of standard amino acid to the plate. The yellow spots were scraped off the plate after the chromatography. The absorbance of the extracts was measured at 340 nm with a spectrophotometer (Marfey, 1984).

In this work, recombinant *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS containing PheDH and FDH were permeabilized with benzene, chloroform, acetone and hexane. The biocatalytic improvement of phenylalanine production by cell permealization using hexane 30 µl per 0.05 mg wet weight of cell for 5 minutes gave amount of phenylalanine 14-30% larger than the product obtained from intact cells. The product from all *phedh* expression clones were optically pure L-phenylalanine. This method is simple, inexpensive, rapid and easy to scale up.

Flores and coworkers in 1994, analyzed the effect of permeabilization of *Kluyveromyces lactis* cells with chloroform, toluene and ethanol on β-galactosidase activity and found that pure chloroform or toluene were more efficient than ethanol. Krishnan and colleague 2000, reported a three-fold increase in the lactate dehydrogenase activity of *Lactobacillus plantarum* cells permeabilized with 0.1 ml of 1% (v/v) diethyl ether per milligram biomass. Yin and colleague (2000) reported that D-hydantoinase activity increased up to 80% in recombinant *E. coli* cells permeabilized with 1.5% dimethyl sulfoxide.

The production of L-phenylalanine from *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS containing pETPF and pETFP showed double yield to the clone containing pETPheDH without effect of host cell. This result indicated that the regeneration of NAD^+ by formate dehydrogenase enhanced phenylalanine production efficiency of the heterologous gene expression clones

Galkin *et a*l.(1997) studied an enzyme synthesis of L-phenylalanine by *E. coli* TG1 cells which expressed heterologous genes of *phedh* and *fdh*. They inserted *phedh* from *B. sphaericus* at downstream of *fdh* from *Candida boidinii* under the tandem *lac-tac* promoter and used a plasmid vector pUC19 (pFDHPheDH). They found that amount of the specific activity of clone cell extract were 1.0 U/mg for FDH and 6.7 U/mg for PheDH.

In this research, recombinant clones contained phenylalanine dehydrogenase gene from *B. badius* BC1 could produced a high yield of L-phenylalanine when treated with organic solvents. However, concentration of solvent, ratio of solvent per cell wet weight and duration of cell treatment should be further studied.

CHAPTER V

CONCLUSIONS

1. *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring *phedh* in pET-17b (pETPheDH) had PheDH specific activity 3.48 and 2.21 fold of the specific activity obtained from the original clone, *E. coli* JM109 harbouring *phedh* in pUC18 (pUCPheDH).
2. *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS harbouring *fdh* in pET-17b (pETFDH) had FDH specific activity 5.59 and 4.54 fold of the specific activity obtained from the original clone, *E. coli* JM109 harbouring *fdh* in pUC119 (pUCFDH).
3. The level of PheDH and FDH in heterologous gene clones (pETPF and pETFP) were similar to those of single gene clones (pETPheDH and pETFDH).
4. Production of phenylalanine from recombinant clones of heterologous gene system (pETPheDH and pETFDH) were 2 times higher than pETPheDH clone.
5. No host effect between *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS on L-phenylalanine production.
6. % Yield of L-phenylalanine production was increased in the range of 14 – 30% when recombinant cells treated with hexane for 5 minutes were used.
7. The phenylalanine product from all recombinant clones were optically pure.

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Wong, C.-H., and Whitesides, G. M. (1981) Enzyme-catalyzed organic synthesis: NAD(P)H cofactor regeneration by using glucose 6-phosphate and the glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides J. Am. Chem. Soc*. 103: 4890-4899

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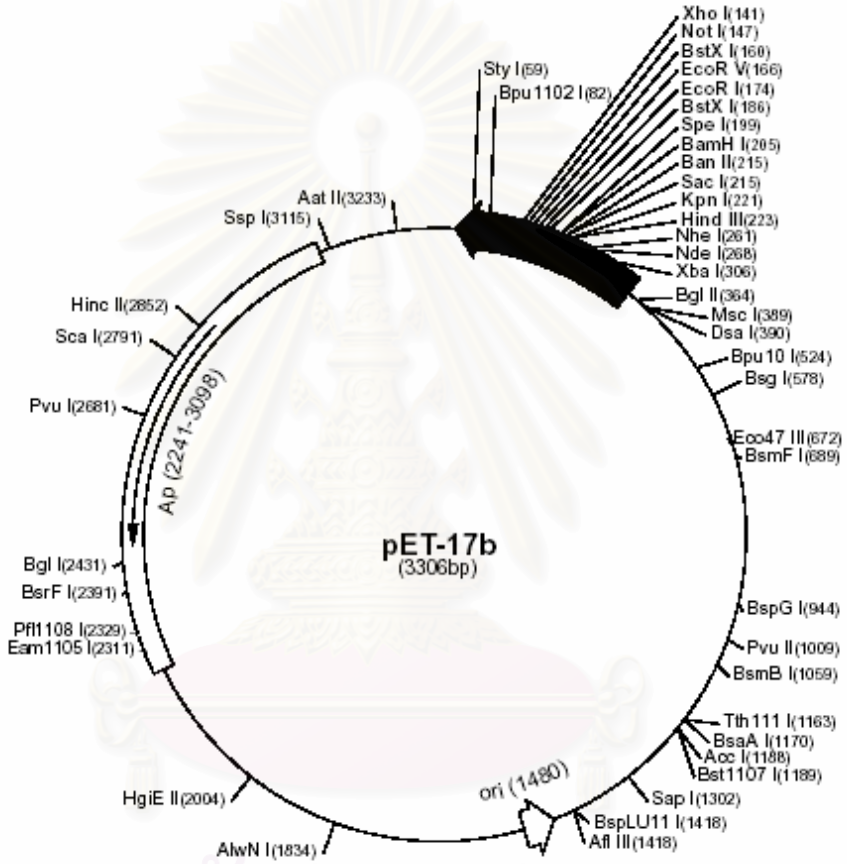
APPENDICES

APPENDIX A

Restriction map of pET-17b



pET-17b cloning/expression region

APPENDIX B

Preparation for protein determination

Reagent for determination of protein concentration (modified from Lowry *et al.*, 1951)

Solution A (0.5% copper sulfate, 1% potassium tartate, pH 7.0)

Potassium tartate	1.0	g
Copper sulfate	0.5	g

Adjust pH to 7.0 and adjust the solution volume to 100 ml

Solution B (2% sodium carbonate, 1N sodium hydroxide)

Sodium carbonate	20.0	g
Sodium hydroxide	4.0	g

Dissolved in distilled water to 1 liter.

Solution C (phenol reagent)

Sodium tungstate	50.0	g
Sodium molybdate	12.5	g
85% phosphoric acid	25.0	g
Distilled water	350	ml
Concentrated hydrochloric acid	50	ml

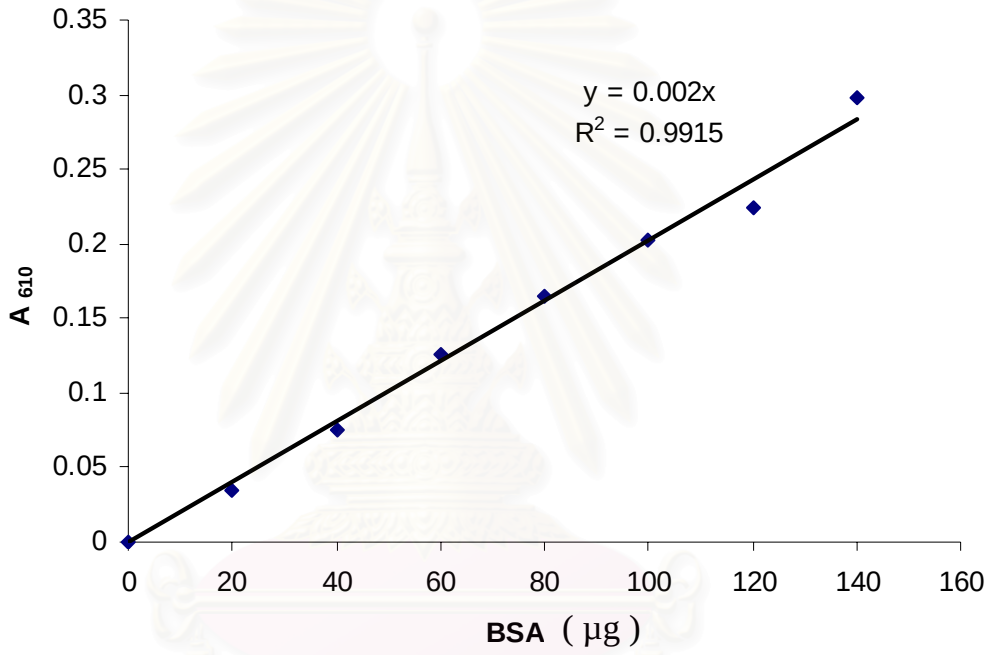
Reflux for 10 hour

Lithium sulphate	75.0	g
Distilled water	25	ml
Bromine solution	2-3	drops

Boil the solution to reduce excess bromine for 15 minutes, then adjust volume to 500 ml with distilled water and store at 4 °C. Dilute the stock solution with distilled water in ratio 1:1 (V/V) before using.

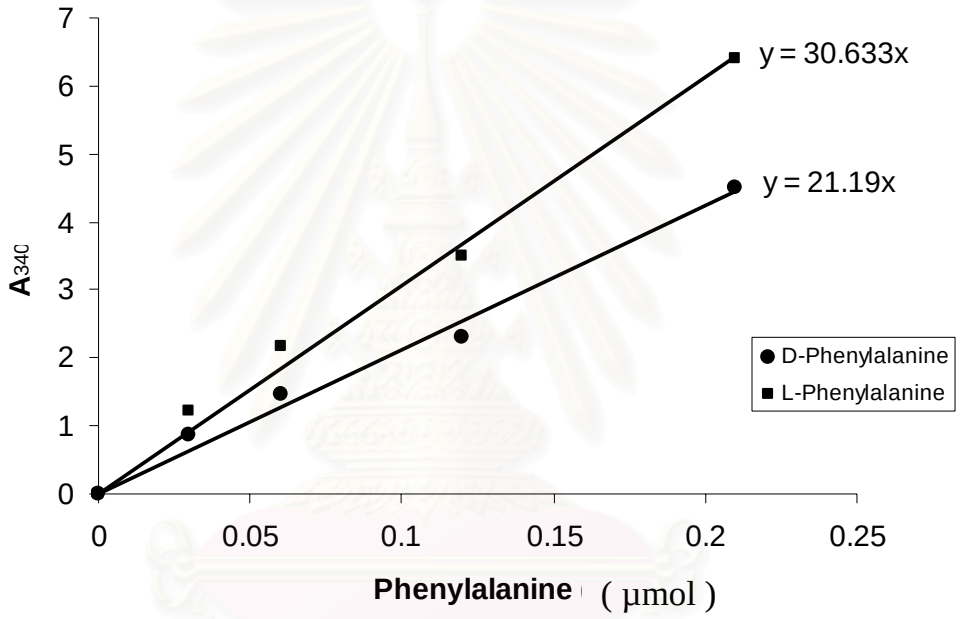
APPENDIX C

Standard curve for protein determination by Lowry's Method (1951)



APPENDIX D

Standard curve for D- and L-alanine determination by Marfey' s method



APPENDIX E

Preparation for denaturing polyacrylamide gel electrophoresis

1. stock solutions

2 M Tris-HCl pH 8.8

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

1 M Tris-HCl pH 6.8

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10 % SDS (W/V)

Sodium dodecyl sulfate (SDS) 10 g

Added distilled water to a total volume of 100 ml.

50 % Glycerol (W/V)

100 % Glycerol 50 ml

Added 50 ml of distilled water.

1 % Bromophenal blue (W/V)

Bromophenol blue 100 ml

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

Filtration will remove aggregated dye.

2. Working solutions

Solution A (30 % (W/V) acrylamide, 0.8 % (W/V) bis-acrylamide)

Acrylamide 29.2 g

N, N´-methylene-bis-acrylamide 0.8 g

Adjusted volume to 100 ml with distilled water and stirred until completely dissolved.

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

2 M Tris-HCl pH 8.8 75 ml

10 % SDS 4 ml

Distilled water 21 ml

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

1 M Tris-HCl pH 8.8 50 ml

10 % SDS 4 ml

Distilled water 46 ml

10 % Ammonium persulfate

Ammonium persulfate 0.5 g

Distilled water 5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS

Tris (hydroxymethyl)-aminomethane 3.0 g

Glycine 14.4 g

SDS 1.0 g

Adjusted volume to 1 liter with distilled water (pH should be approximately 8.3).

5 x Sample buffer (60 mM Tris-HCl pH 6.8, 25 % glycerol, 2 % SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl pH 6.8 0.6 ml

50 % Glycerol 5.0 ml

10 % SDS 2.0 ml

2-Mercaptoethanol 0.5 ml

1 % Bromophenol blue 1.0 ml

Distilled water 0.9 ml

3. SDS-PAGE

10 % Separating gel

Solution A 3.3 ml

Solution B 2.5 ml

Distilled water 4.2 ml

10 % Ammonium persulfate 50 µl

TEMED 5 µl

5.0 % Stacking gel

Solution A 0.67 ml

Solution C 1.0 ml

Distilled water	2.3 ml
10 % Ammonium persulfate	30 µl
TEMED	5 µl

4. Protein staining solution

Staining solution, 1 liter

Coomassie brilliant blue R-250	1.0 g
Methanol	450 ml
H_2O	450 ml
Glacial acetic acid	100 ml

Destaining solution, 1 liter

Methanol	100 ml
Glacial acetic acid	100 ml
H_2O	800 ml

APPENDIX F

The DNA sequencing profiles of the phenylalanine dehydrogenase gene and formate dehydrogenase gene from recombinant plasmids pETPheDH, pETFDH, pETPF and pETFP

(a) = The DNA sequencing profile of *phedh* in pETPheDH amplification using T7 forward X T7 reverse as PCR primers; sense sequencing primer T7 forward

(b) = The DNA sequencing profile of *phedh* in pETPheDH amplification using T7 forward X T7 reverse as PCR primers; antisense sequencing primer T7 reverse

(c) = The DNA sequencing profile of *fdh* in pETFDH amplification using T7 forward X T7 reverse as PCR primers; sense sequencing primer T7 forward

(d) = The DNA sequencing profile of *fdh* in pETFDH amplification using T7 forward X T7 reverse as PCR primers; antisense sequencing primer T7 reverse

(e) = The DNA sequencing profile of *phedh* in pETPF amplification using PheDHF*Nde*I X PheDHR*Bam*HI as PCR primers; sense sequencing primer PheDHF*Nde*I

(f) = The DNA sequencing profile of *phedh* in pETPF amplification using PheDHF*Nde*I X PheDHR*Bam*HI as PCR primers; antisense sequencing primer PheDHR*Bam*HI

(g) = The DNA sequencing profile of *fdh* in pETPF amplification using FDHF*Bam*HI X FDHR*Eco*RI as PCR primers; sense sequencing primer FDHF*Nde*I

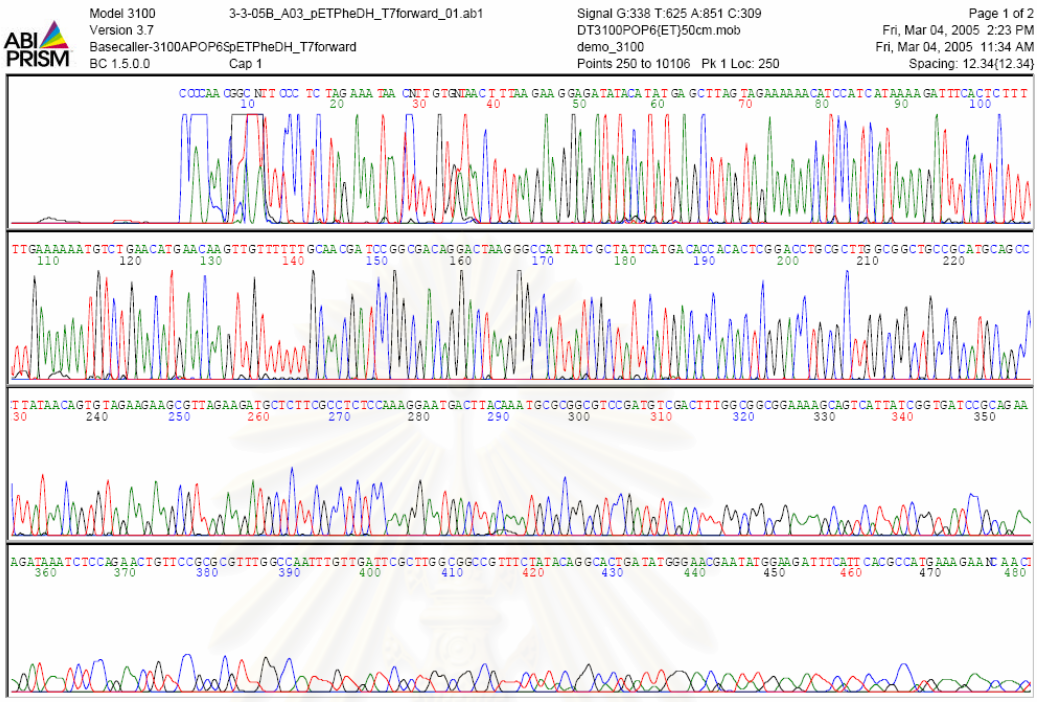
(h) = The DNA sequencing profile of *fdh* in pETPF amplification using FDHF*Bam*HI X FDHR*Eco*RI as PCR primers; antisense sequencing primer FDHR*Bam*HI

(i) = The DNA sequencing profile of *fdh* in pETFP amplification using FDHF*Nde*I X FDH*Bam*HI as PCR primers; sense sequencing primer FDHF*Nde*I

(j) = The DNA sequencing profile of *fdh* in pETFP amplification using FDHF*Nde*I X FDH*Bam*HI as PCR primers; antisense sequencing primer FDH*Bam*HI

(k) = The DNA sequencing profile of *phedh* in pETFP amplification using PheDHF*Bam*HI X PheDH*Eco*RI as PCR primers; sense sequencing primer PheDHF*Bam*HI

(l) = The DNA sequencing profile of *phedh* in pETFP amplification using PheDHF*Bam*HI X PheDH*Eco*RI as PCR primers; antisense sequencing primer PheDH*Eco*RI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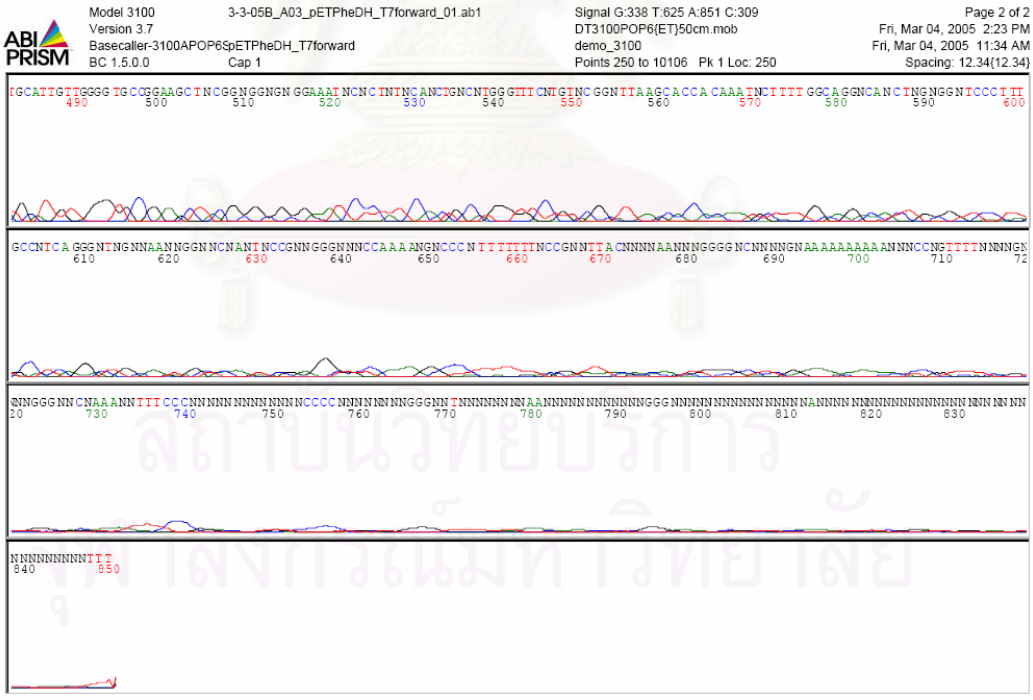
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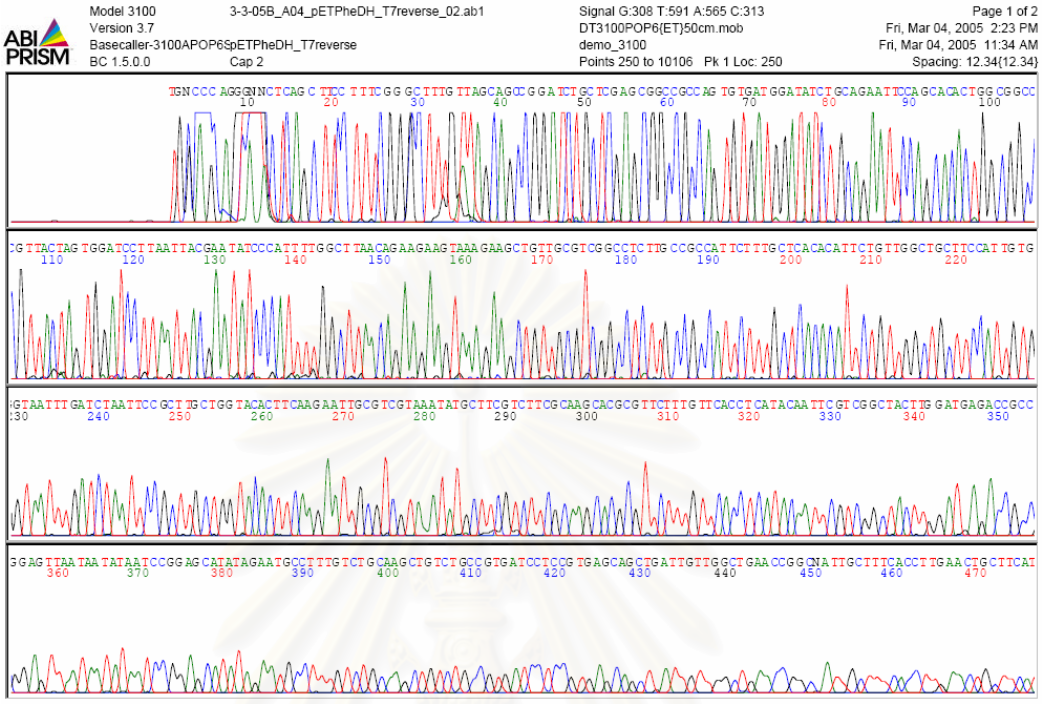
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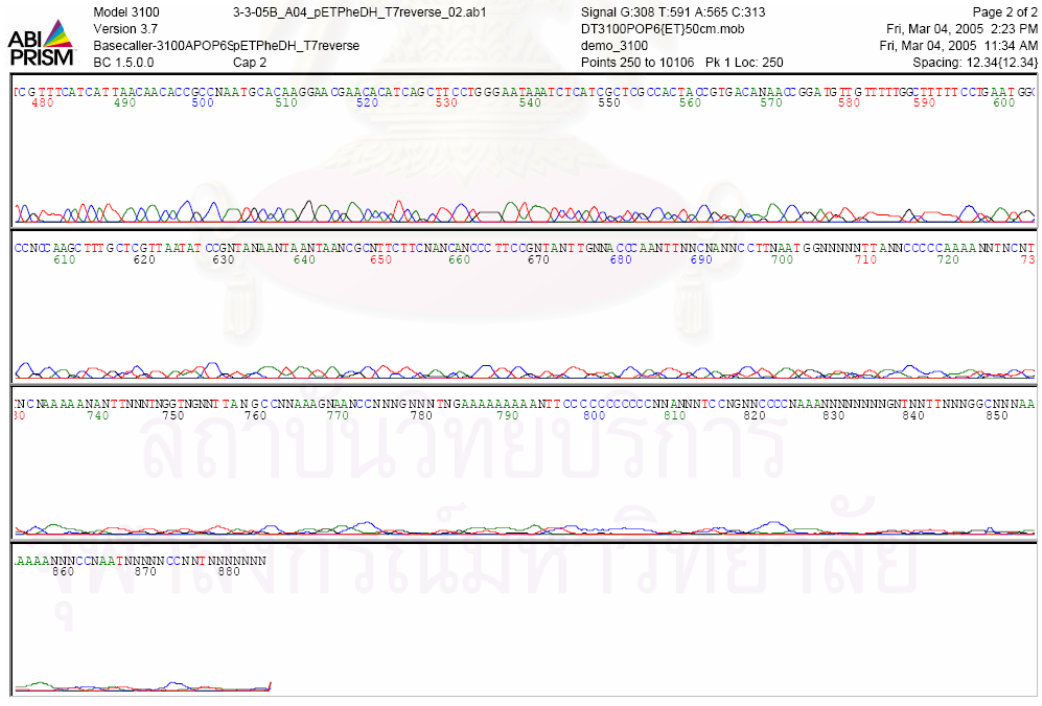
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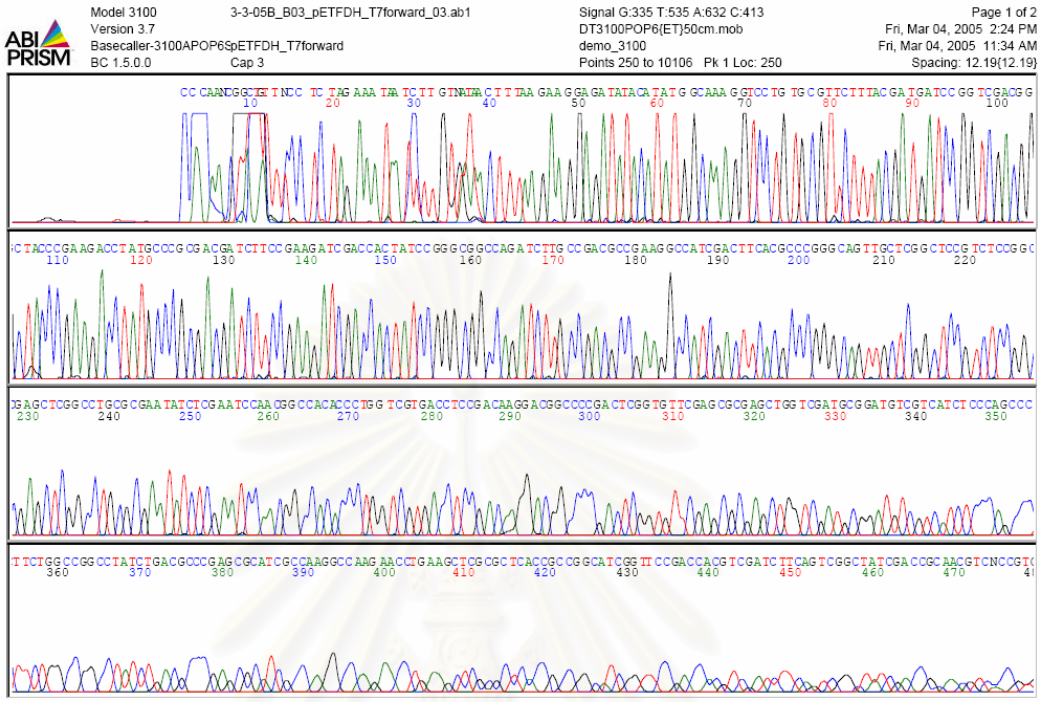
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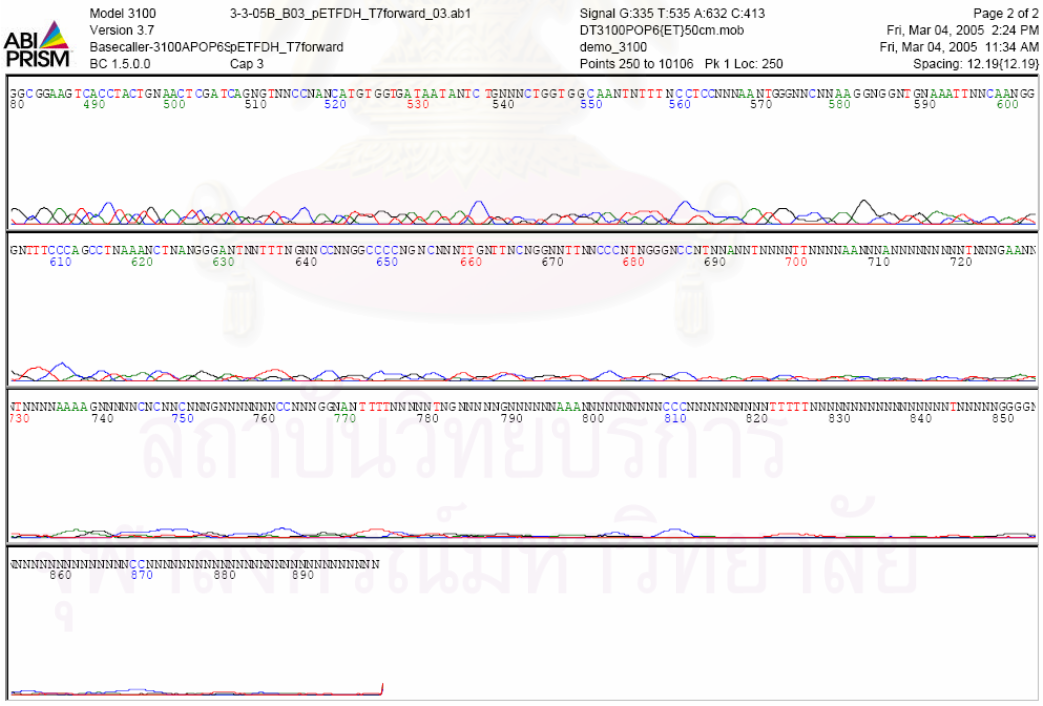
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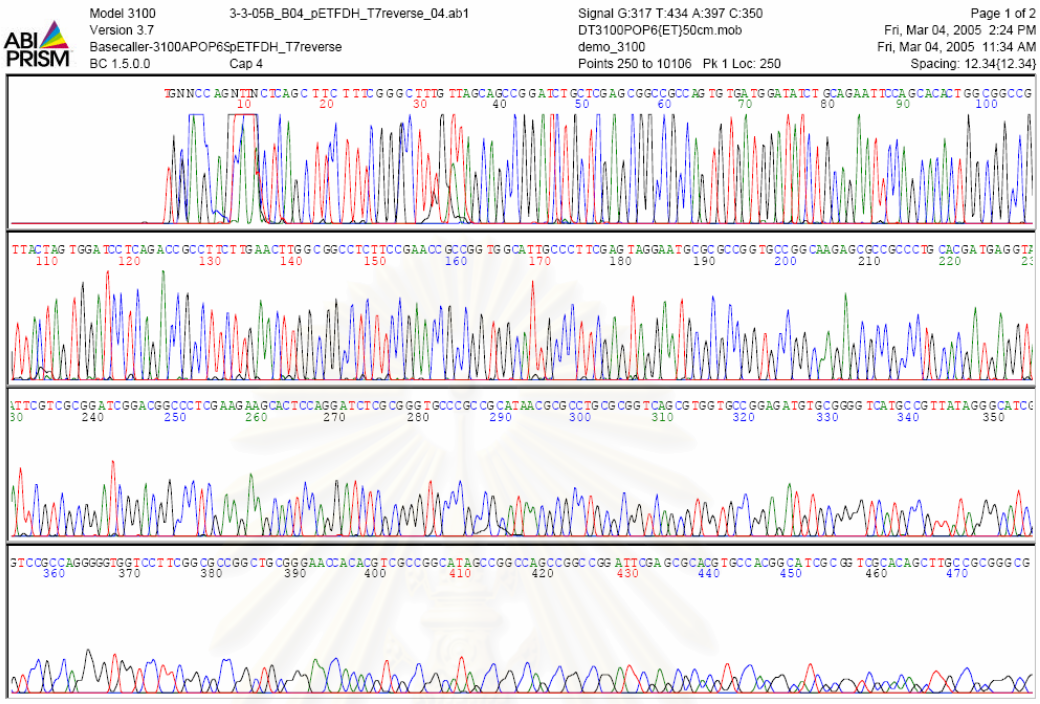
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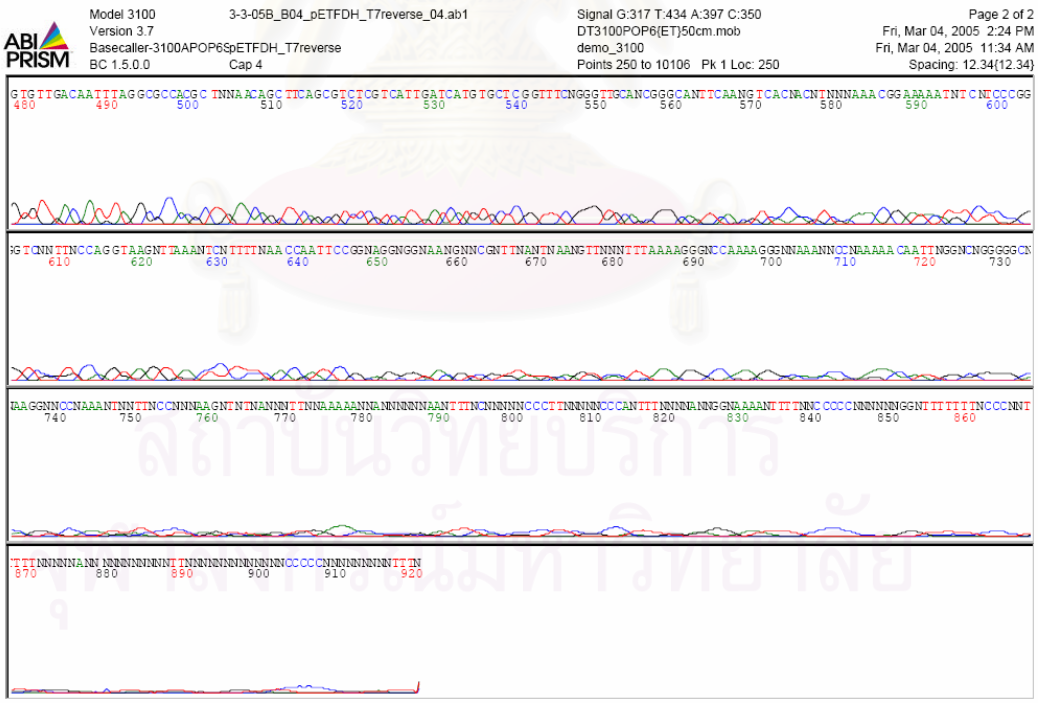
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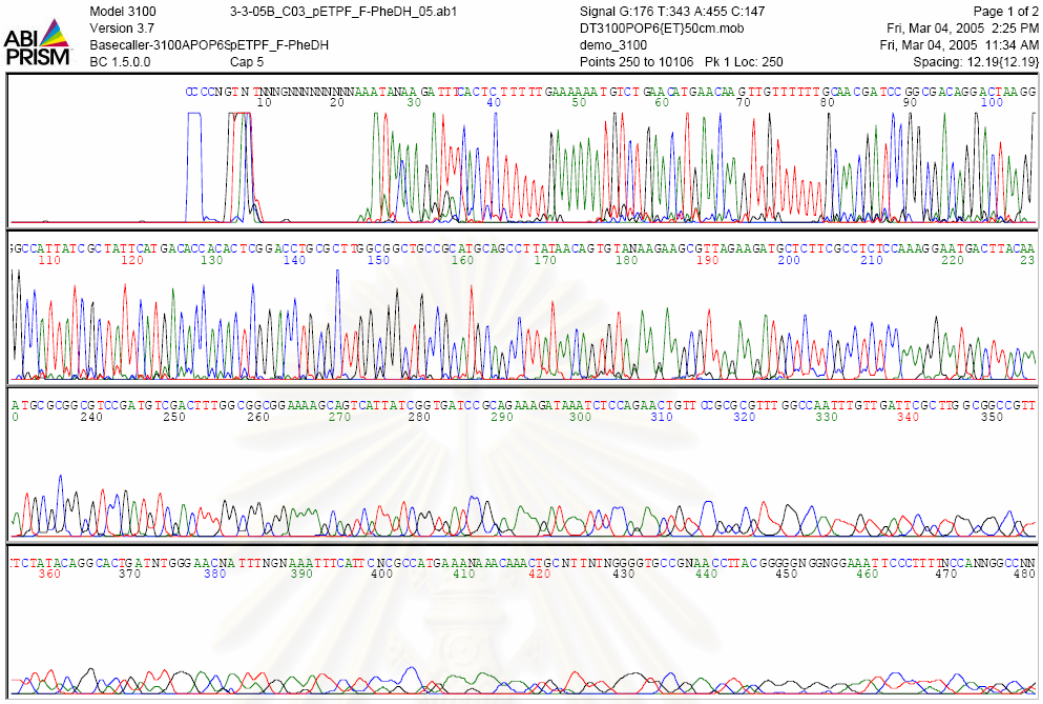
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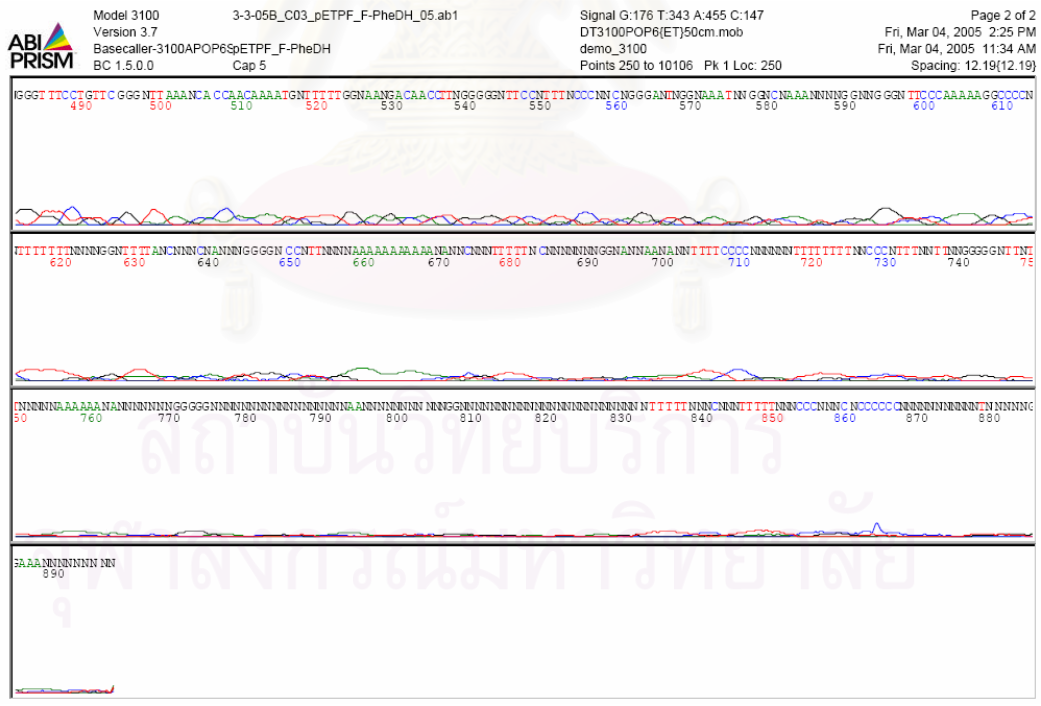
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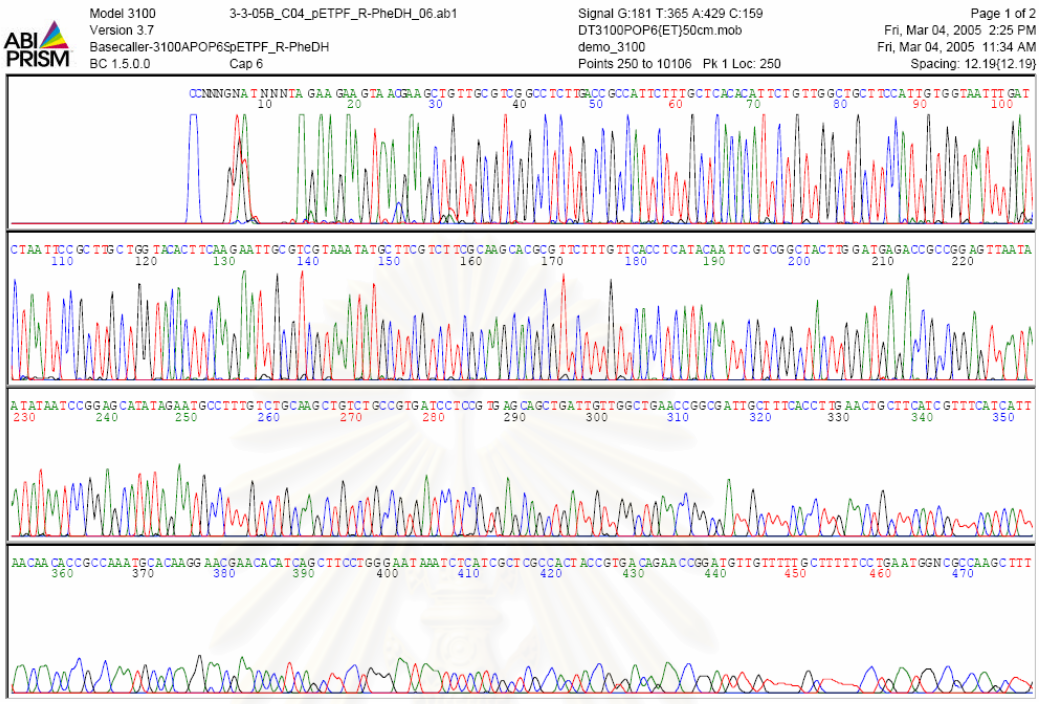
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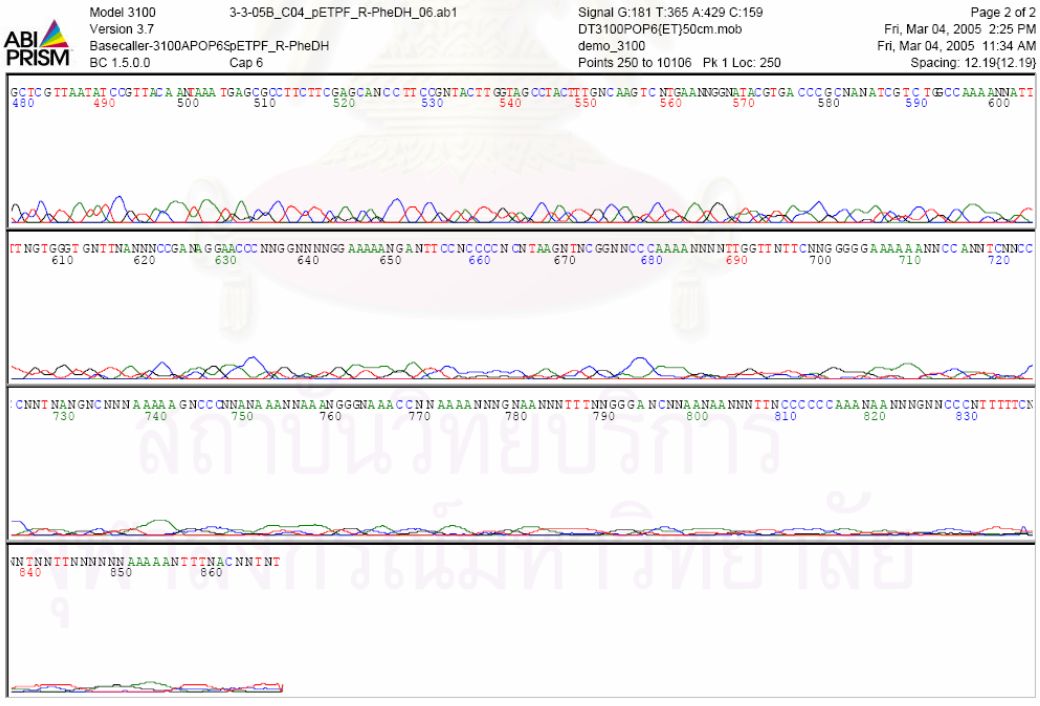
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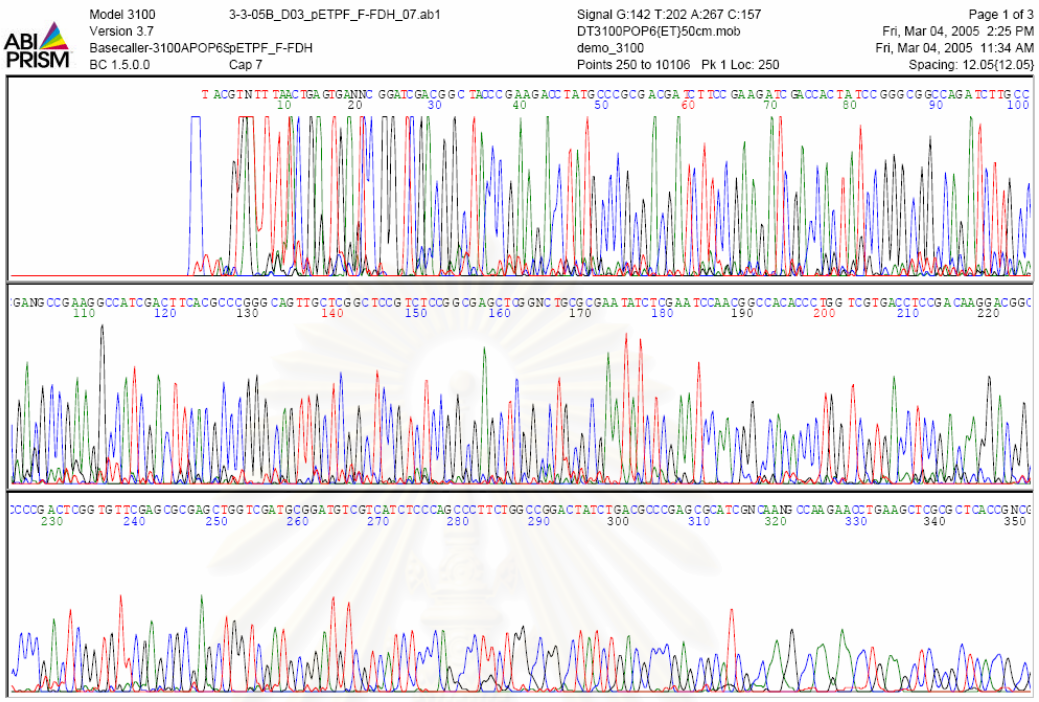
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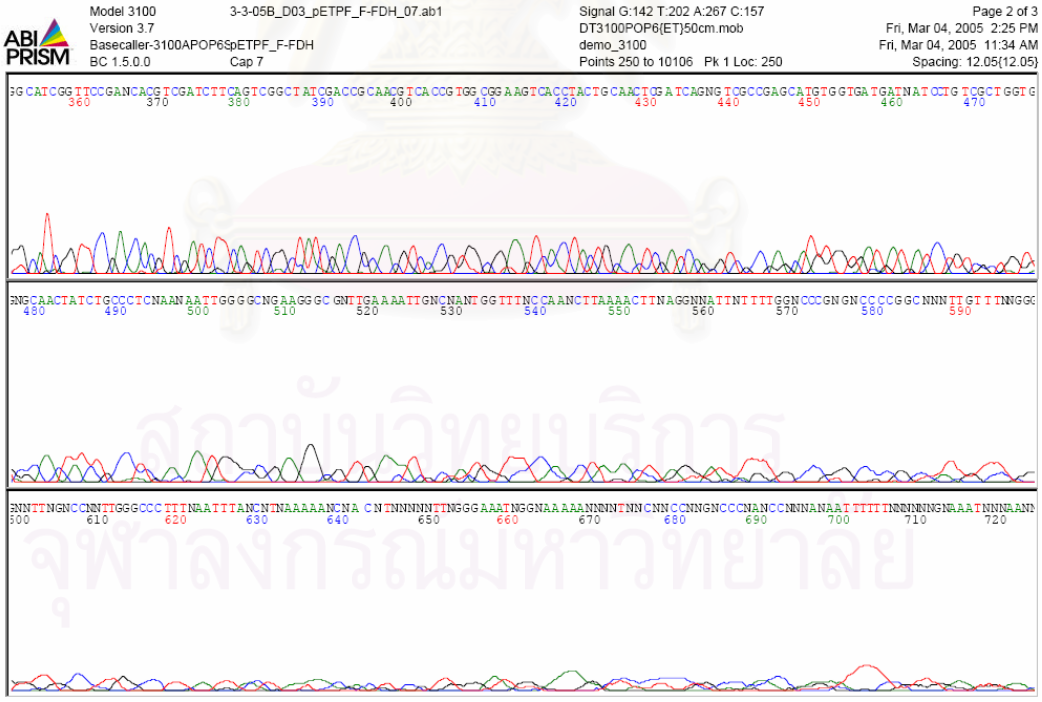
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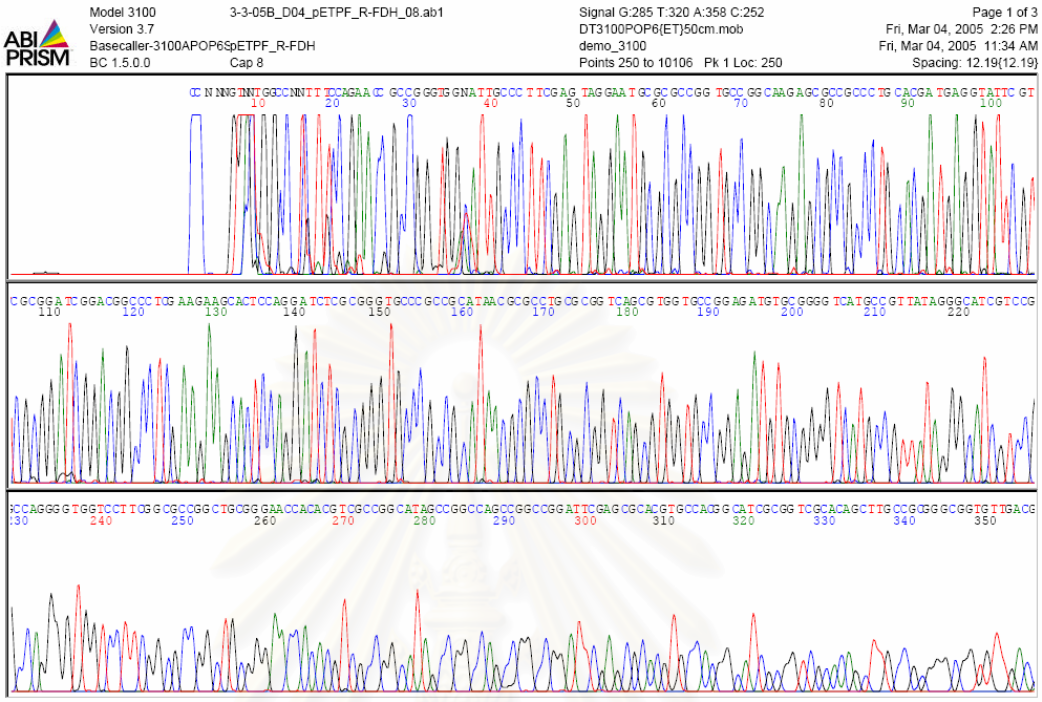
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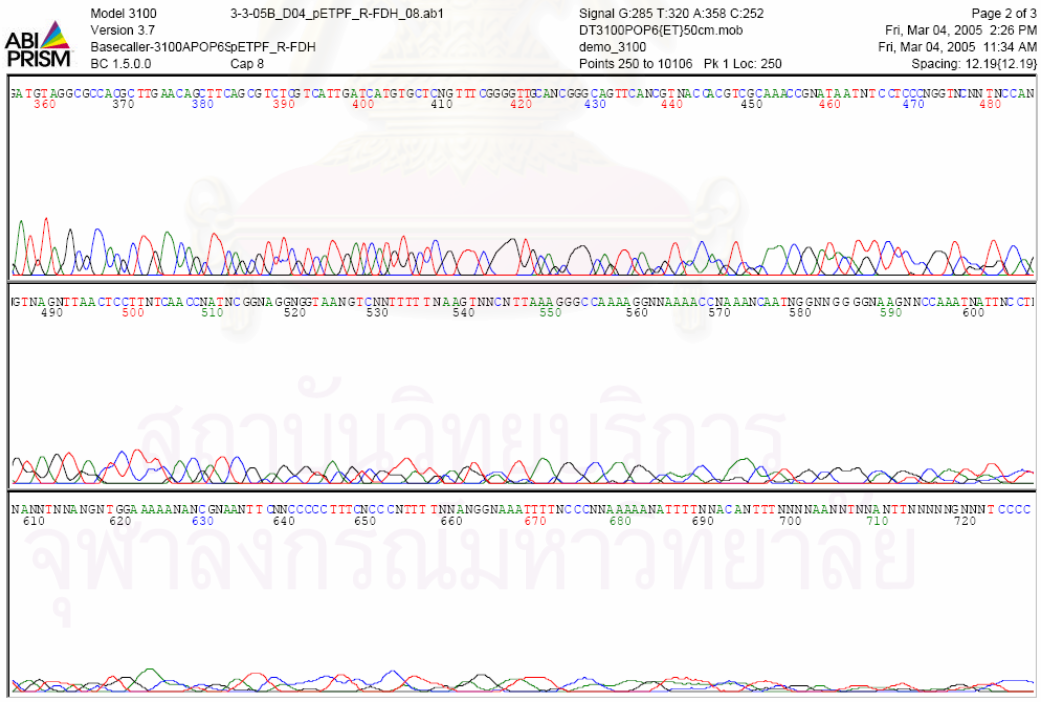
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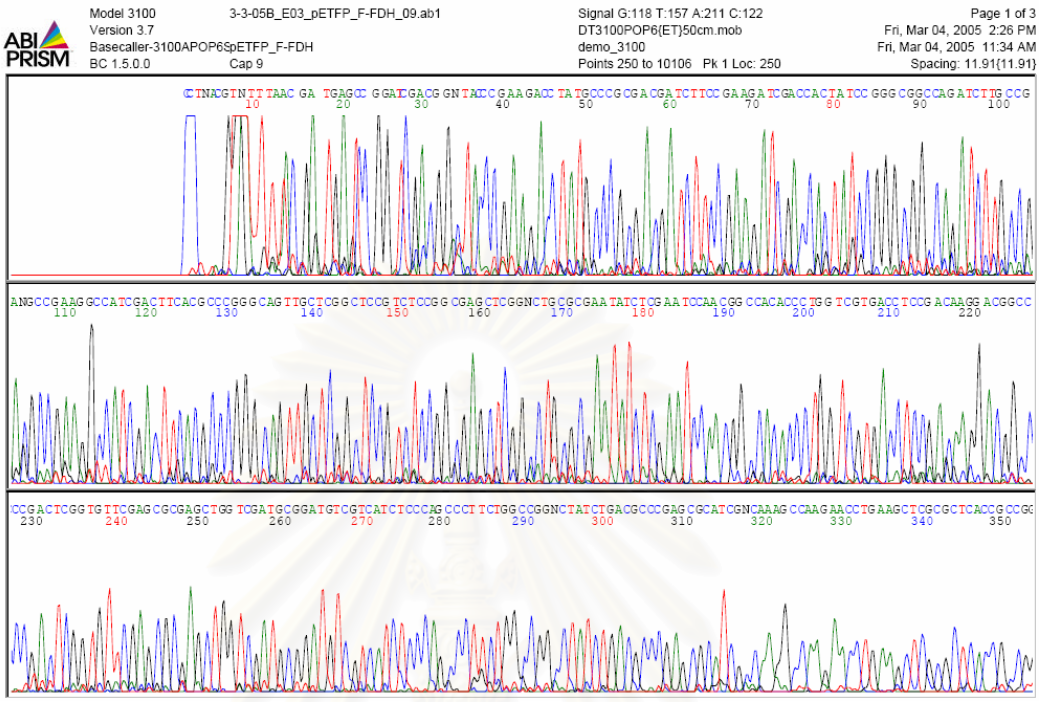
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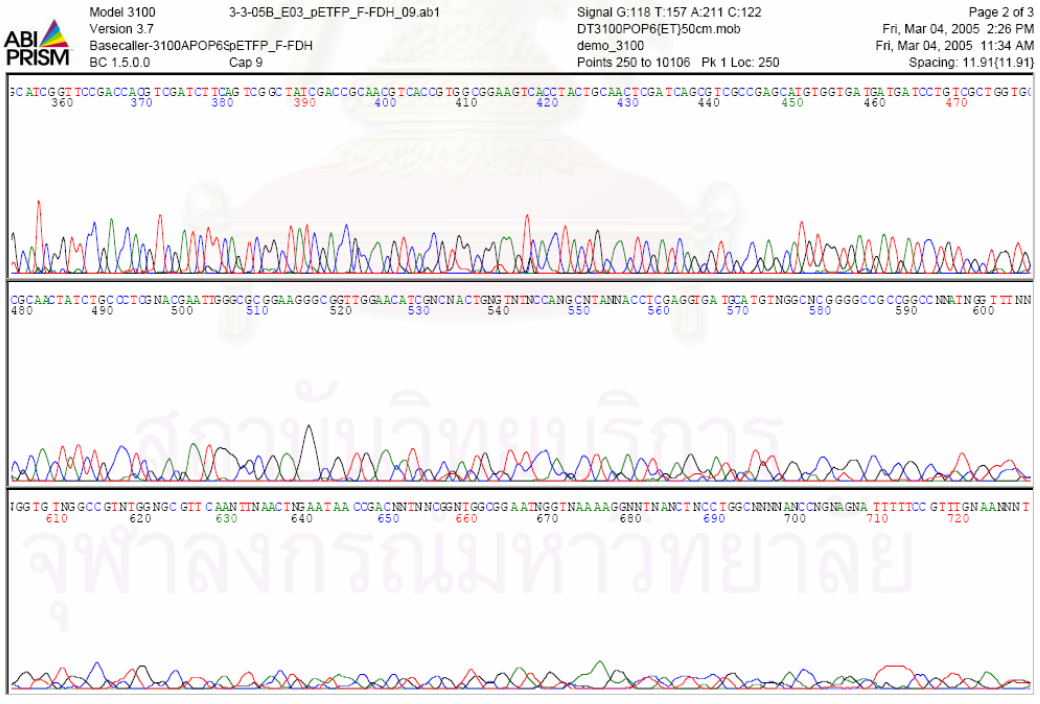
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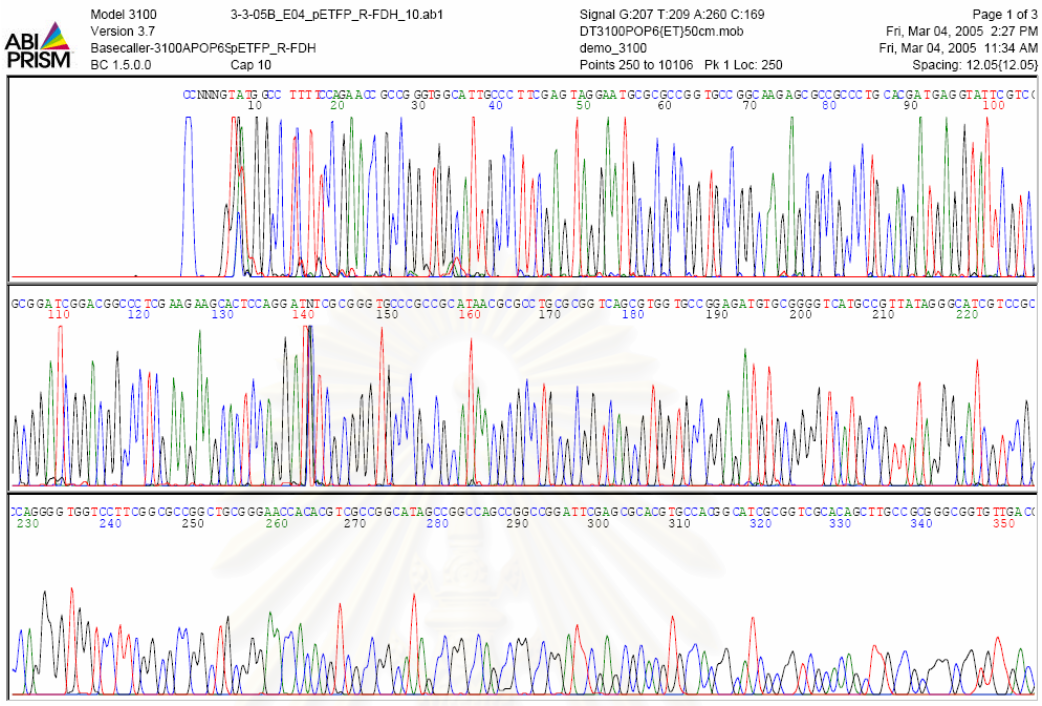
3-3-05B_E03_pETFP_F-FDH_09.ab1
Cap 9
Signal G:118 T:157 A:211 C:122
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 2 of 3
Fri, Mar 04, 2005 2:26 PM
Fri, Mar 04, 2005 11:34 AM
Spacing: 11.91{11.91}

(i)


Model 3100
Version 3.7
Basecaller-3100APOP6SpETFP_R-FDH
BC 1.5.0.0
3-3-05B_E04_pETFP_R-FDH_10.ab1
Cap 10
Signal G:207 T:209 A:260 C:169
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 1 of 3
Fri, Mar 04, 2005 2:27 PM
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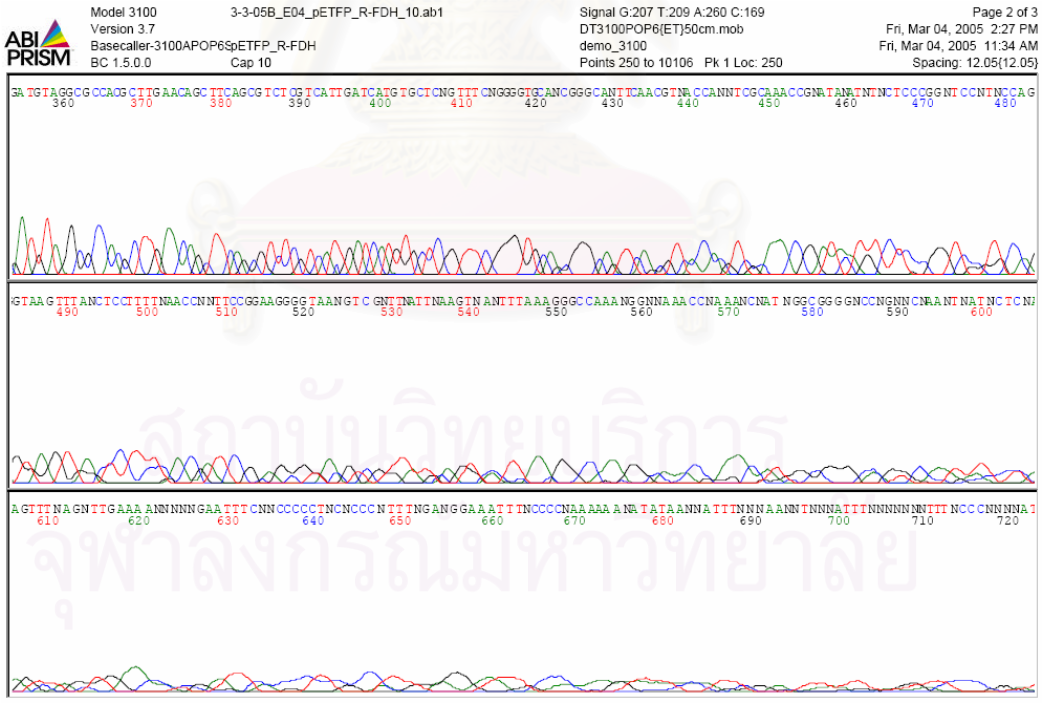
Spacing: 12.05{12.05}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Version 3.7
Basecaller-3100APOP6SpETFP_R-FDH
BC 1.5.0.0
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Cap 10
Signal G:207 T:209 A:260 C:169
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 2 of 3
Fri, Mar 04, 2005 2:27 PM
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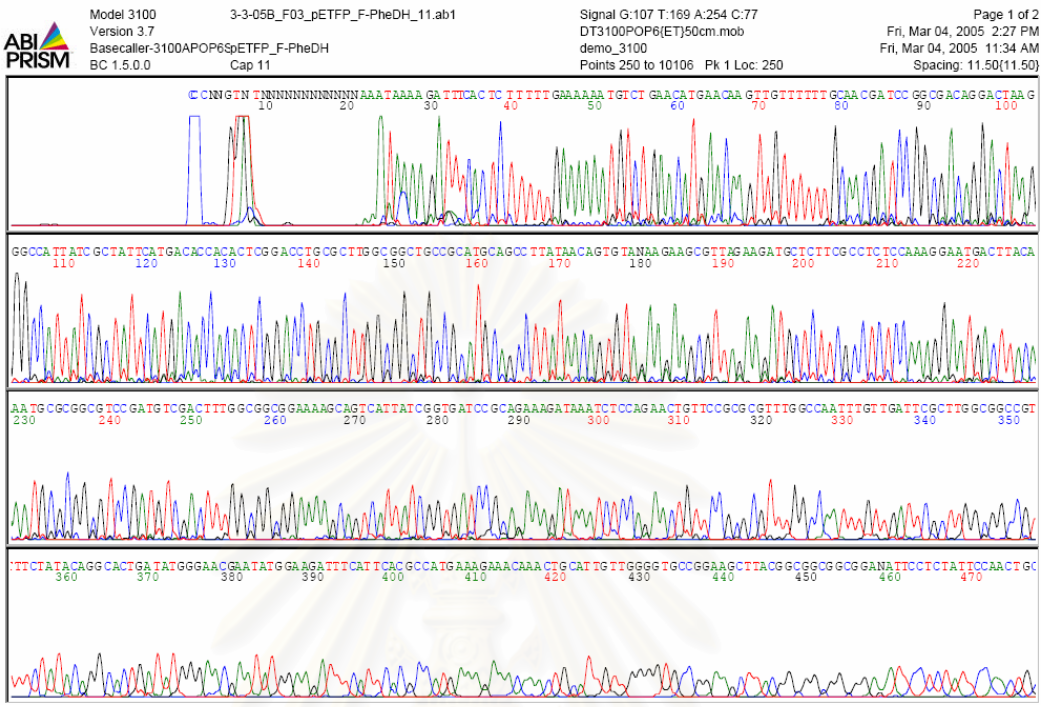
(j)


Model 3100
Version 3.7
Basecaller-3100APOP6SpETFP_F-PheDH
BC 1.5.0.0
3-3-05B_F03_pETFP_F-PheDH_11.ab1
Cap 11
Signal G:107 T:169 A:254 C:77
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 1 of 2
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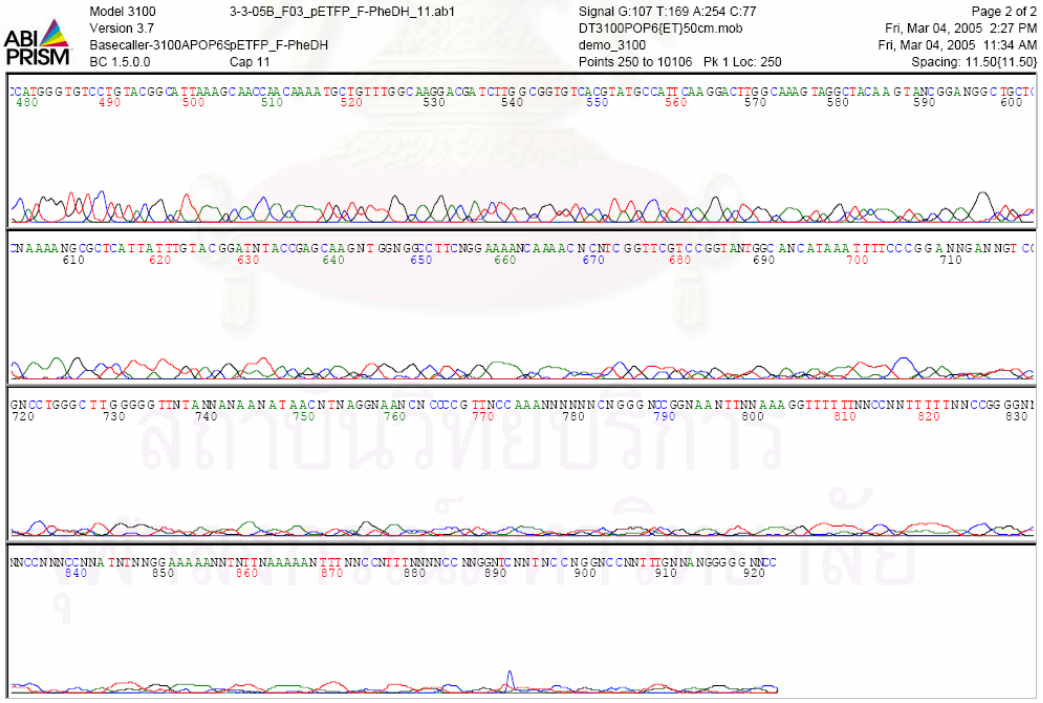
Spacing: 11.50{11.50}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Version 3.7
Basecaller-3100APOP6SpETFP_F-PheDH
BC 1.5.0.0
3-3-05B_F03_pETFP_F-PheDH_11.ab1
Cap 11
Signal G:107 T:169 A:254 C:77
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 2 of 2
Fri, Mar 04, 2005 2:27 PM
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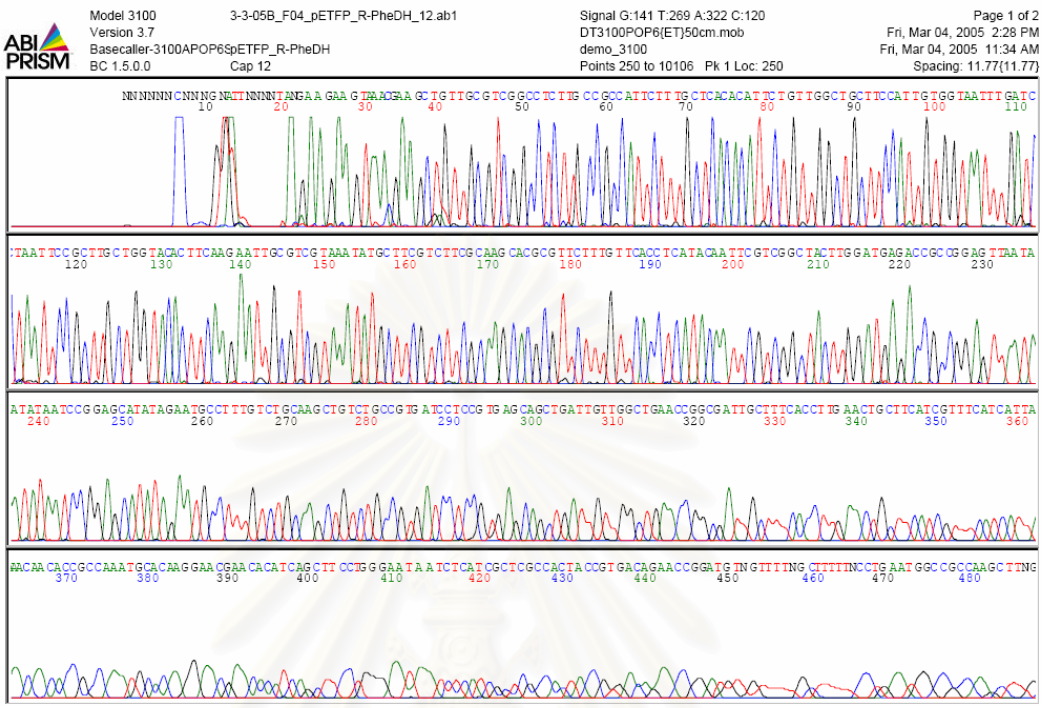
(k)


Model 3100
Version 3.7
Basecaller-3100APOP6
BC 1.5.0.0
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SpETFP_R-PheDH
Cap 12
Signal G:141 T:269 A:322 C:120
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 1 of 2
Fri, Mar 04, 2005 2:28 PM
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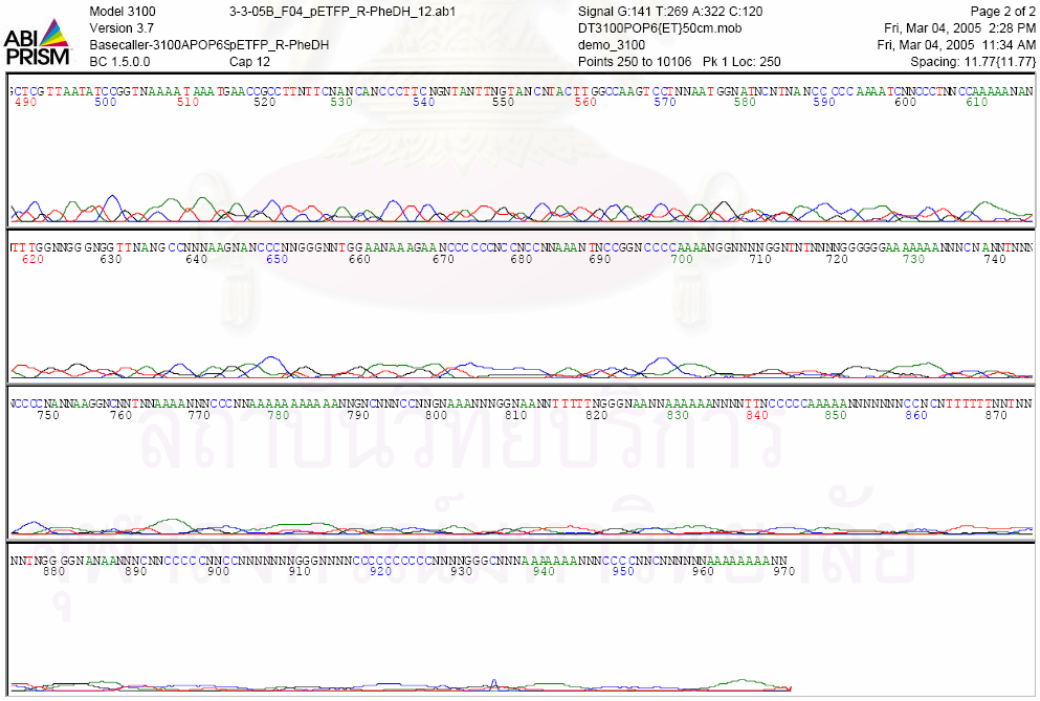
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Basecaller-3100APOP6
BC 1.5.0.0
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SpETFP_R-PheDH
Cap 12
Signal G:141 T:269 A:322 C:120
DT3100POP6{ET}50cm.mob
demo_3100
Points 250 to 10106 Pk 1 Loc: 250
Page 2 of 2
Fri, Mar 04, 2005 2:28 PM
Fri, Mar 04, 2005 11:34 AM
Spacing: 11.77{11.77}

(l)

BIOGRAPHY

Mr. Methee Khamduang was born on June 19, 1979 in Angthong. He finished high school at Pibulwitthayalai school, Lopburi. He graduated with the degree of Bachelor of Science from the Department of Botany, Major of Genetics at Chulalongkorn University in 2001. He has studied for the degree of Master of Science at the Department of Biotechnology, Chulalongkorn University since 2002.