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ALANINE PRODUCTION BY *Escherichia coli* TRANSFORMED WITH ALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE GENES

Miss Rujirat Hatrongjitt

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อะลานีนดีไฮโดรจิเนส (EC 1.4.1.1) เร่งปฏิกิริยาการดึงหมู่อะมิโนจากแอล-อะลานีนให้ผลิตภัณฑ์คือแอมโมเนียม, ไพรูเวท และ NADH ซึ่งเป็นปฏิกิริยาที่ผันกลับได้และต้องการ NAD^+ เป็นโคเอนไซม์ จากปฏิกิริยาดังกล่าวจึงได้มีการนำเอนไซม์นี้มาใช้ในการสังเคราะห์อะลานีนและสารอนุพันธ์ รวมทั้งนำมาประยุกต์ใช้ในการวินิจฉัยโรค ในการนำเอนไซม์นี้มาใช้ในการผลิตอะลานีนในระดับอุตสาหกรรมประสบกับปัญหาราคาของโคเอนไซม์ที่สูงมาก ดังนั้นจึงได้มีการเพิ่มประสิทธิภาพโดยใช้ระบบการทำปฏิกิริยาร่วมกับเอนไซม์ที่สามารถรีเจนเนอเรตโคเอนไซม์ได้ อะลานีนดีไฮโดรจิเนสที่แยกได้จาก *Aeromonas hydrophila* มีแอคติวิตีและความจำเพาะต่อสับสเตรทสูงเหมาะสมสำหรับใช้ผลิตอะลานีน นอกจากนี้ได้มีการโคลนยีนของเอนไซม์เข้าสู่ *E. coli* JM109 โดยใช้เวคเตอร์ pUC18 เพื่อที่จะเพิ่มประสิทธิภาพในการผลิตอะลานีนโดยทำให้มีแอคติวิตีของอะลานีนดีไฮโดรจิเนสสูงขึ้น และสามารถรีเจนเนอเรตโคเอนไซม์ได้เองภายในเซลล์เพียงเซลล์เดียว การนำเข้ายีน *aladh* และ *fdh* สู่ *E. coli* BL21(DE3) โดย 2 วิธีคือ 1) สร้างโคลนที่มี heterologous gene ของ *aladh* และ *fdh* บนเวคเตอร์ pET-17b (pETAF และ pETFA) 2) ทำการทรานส์ฟอร์มร่วมของยีน *aladh* และ *fdh* ภายใต้โปรโมเตอร์ T7 โดยใช้ เวคเตอร์ pET-17b และ pSY343 (pETAlaDH/pSYFDH) หรือ เวคเตอร์ pMPM-K3 และ pET-17b (pMPMAlaDH/pETFDH) ตามลำดับ พบว่าแอคติวิตีของอะลานีนดีไฮโดรจิเนสและฟอร์เมตดีไฮโดรจิเนสของโคลนที่มี heterologous gene และโคลนที่ได้จากการทรานส์ฟอร์มร่วมสูงขึ้นเมื่อเทียบกับโคลนเดิม (pUCAlaDH และ pUCFDH) แม้ว่าแอคติวิตีของเอนไซม์ทั้งสองจะลดลงเมื่อเทียบกับโคลนที่มียีนเดียว (pUCAlaDH, pMPMAlaDH, pUCFDH) สำหรับการผลิตอะลานีนของทุกโคลนพบว่าไม่แตกต่างกันโดยมีผลผลิตประมาณร้อยละ 50 และอัตราส่วนของอะลานีนรูปแบบ D:L ประมาณ 1.6:1

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RUJIRAT HATRONGJITT: ALANINE PRODUCTION BY *Escherichia coli* TRANSFORMED WITH ALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE GENES. THESIS ADVISOR: ASSISTANT PROFESSOR KANOKTIP PACKDIBAMRUNG, Ph.D., 154 pp. ISBN 974-17-6033-7

Alanine dehydrogenase (EC 1.4.1.1) catalyzes the NAD$^+$-dependent reversible oxidative deamination of L-alanine to form ammonia, pyruvate, and NADH. The enzyme is important as a catalyst for the synthesis of alanine and its derivatives. Moreover, it is therefore applicable to diagnosis of malignant hematopoietic disease. The application of this enzyme to industrial production of L-alanine has been hampered by the cost of coenzymes. A multienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem. Alanine dehydrogenase from *Aeromonas hydrophila* has high activity and high substrate specificity, so it is suitable for L-alanine production. Subsequently, the *aladh* gene was cloned into *E. coli* JM109 by using plasmid vector pUC18. To enhance the enzyme activity and regeneration of NADH in a single cell by co-existence of *aladh* and *fdh* in *E. coli* BL21(DE3) host cell, two methods were performed 1) cloning of heterologous gene of *aladh* and *fdh* in a high expression vector pET-17b (pETAF and pETFA) and 2) co-transformation of plasmids containing *aladh* and *fdh* gene under T7 promoter by two systems using plasmid vector pET-17b and pSY343 (pETAlaDH/pSYFDH) or plasmid vector pMPM-K3 and pET-17b (pMPMAlaDH/pETFDH), respectively. The heterologous gene expression clones and cotransformed clones had alanine dehydrogenase and formate dehydrogenase activities higher than those of their original clones (pUCAlaDH and pUCFDH). However, their activities were less than those of the single gene clones (pETAlaDH, pMPMAlaDH, pETFDH) except for the AlaDH of heterologous gene expression clones which were the same as that expressed by pETAlaDH. Production of alanine by various recombinant clones were not significantly different with about 50% yield and ratio of D:L form about 1.6:1.

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ABBREVIATIONS

A	absorbance, 2’-deoxyadenosine (in a DNA sequence)
AlaDH	alanine dehydrogenase
bp	base pairs
BSA	bovine serum albumin
C	2’-deoxycytidine (in a DNA sequence)
^{O}C	degree Celsius
cm	centrimetre
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	2’-deoxynucleoside 5’-triphosphate
EC	Enzyme Commission
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
HPLC	high-performance liquid chromatography
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid, kilobases in single-standed nucleic acid
KCl	potassium chloride
kDa	kiloDalton
K_m	Michaelis constant
KOH	potassium hydroxide
KPB	potassium phosphate buffer
l	litre
LB	Luria-Bertani
LeuDH	leucine dehydrogenase

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LysDH	lysine dehydrogenase
μg	microgram
μl	microlitre
μmol	micromole
μM	micromolar
M	mole per litre (molar)
mA	milliampere
mg	milligram
min	minute
ml	millilitre
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
pmol	picomole
PMSF	phenyl methyl sulfonyl fluoride
RBS	ribosome-binding site
rpm	revolution per minute
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 biomolecules typically found in all organisms and can be divided into two groups by the ability of rotation the plane of polarized light. They are L-formed and D-formed amino acids. The amino acid in L-formed plays an important role in all life while D-formed is rarely found in organism. L-amino acids serve as building block of enzyme, hormone, antibody and protein. Furthermore, its can also balance buffering capacity in blood (Holum, 1982) and often function as chemical messengers in the cell communication (Bender, 1975). The commercial values of amino acids come from their wide applicability in both food and pharmaceutical industries. The worldwide market value of amino acids is approximately 2 billion dollars annually and the synthesis of optically active amino acids has been extensively studied.

Recently, the using of L-amino acids for many compounds synthesis are spread widely in animal nutrition, human medicine and the pharmaceutical industries. For example, L-leucine, L-valine, L-isoleucine (Gu and Chang, 1990), L-lysine, and L-glutamic acid are used as food and feed additives while L-phenylalanine is one of the essential starting material for an artificial sweetener, aspartame (L-aspartate-L-phenylalanine-1-methyl ester, or NutraSweet) (Ohshima and Soda, 1989). L-alanine, another interesting L-amino acid, is used as food industry and pharmaceutical applications. L-alanine is currently used as a food additive because of its sweet taste, which degree is about 70% of sugar, and used for pharmaceutical applications in which it is incorporated together with several other L-amino acids in standard infusions for parenteral administration in clinical preoperative and postoperative nutrition therapy (Suye *et al*., 1992).

1.2 L-alanine

Alanine, firstly isolated in 1879, is a non-essential amino acid and is used to build protein. The alpha-carbon in alanine is substituted with a levorotatory (I)-methyl group, making it one of the simplest amino acids with respect to molecular structure and is one of the most widely used in protein construction, essential for proper function of the central nervous system and helps form neurotransmitters. Alanine plays a role in the metabolism of several substances, including glucose, tryptophan and pyridoxine. Morever, beta-alanine is a constitutent of vitamin B5 (pantothenic acid) as well as coenzyme A. Alanine has also demonstrated a cholesterol-reducing effect in rats. It has been found that alanine is present in prostate fluid, and it may play a role in the prostate. In one study of men with benign prostatic hyperplasia, it was found that 780 mg of alanine per day for two weeks and then 390 mg for the next two and a half months, taken together in combination with equal amounts of glycine and glutamic acid reduced the symtoms. High levels of alanine along with low levels of tyrosine and phenylalanine have been associated with the Epstein-Barr virus (also sometimes referred to as glandular fever) and chronic Fatigue syndrome. Low levels have been found in patients with hypoglycemia, diabetes and alcohol induced hepatitis (Feinblatt, *et al*., 1958; Damrau, 1962).

In prokaryote, alanine plays many important roles in the growth and physiology of enteric bacteria. It is one of the major amino acids present in proteins and can be used in the biosynthesis of the amino acid valine (Whalen and Berg, 1982), and of the vitamin biotin (Eisenberg and Star, 1968). Both the L- and D-stereoisomers of alanine are major constitutents of the peptidoglycan layer.

In addition, there are many reports described the using of L-alanine in the synthesis of DOPA (dihydroxy-phenylalanine) which is the precursor of many chemicals such as melanin which found in hair and skin, dopamine; the one of chemicals messengers in nervous system, norepinephrine and epinephrine (Reinhold *et al*., 1987) The defect of tyrosine hydroxylase causes the decreasing in DOPA production, subsequently, the defect of melanin will occur. The defect of DOPA also cause Pakinson's disease, however, the using of DOPA drug can release the effect of this disease by reparing the activity of nervous system. The derivative of L-alanine like (*N*-(*p*-chlorobenzoyl)-2-(2-cyanoethyl)) alanine is also used for control immune system and can protect the development of tumor (Nagano *et al*., 1985).

A variety methods has been used for the production of L-amino acid such as chemical synthesis, extraction from protein hydrolysates, fermentative or enzymatic method. L-alanine can also be prepared by chemical synthesis used of the Strecker reaction in which acetaldehyde, prussic acid and ammonium are used as starting material, but this process is not necessarily desirable since L-alanine is used as a food additive and, in the Strecker reaction, toxic cyanogens is used. While L-alanine can also be prepared by the direct fermentation of sugars. The microorganisms involved in this fermentation, such as *Corynebacterium gelatinosium*, *Arthrobacter oxydans*, *Brevibacterium lactofermentum*, *Clostridium* sp. and *Pyrococcus furiosus*, produce D- and L-alanine with a maximum conversion rate of 50 to 60%. L-alanine is produced by culturing a microorganism, *Zymomonas mobilis* that has been genetically modified in such a manner that it expresses an *aladh* gene. The expressed AlaDH converts the glucose in the medium, via conversion into pyruvate, into a mixture of alanine and ethanol, with a maximum alanine yield of 16%, calculated on the amount of glucose converted. (Holes, *et al*., 2003). Several enzymatic processes of L-alanine synthesis have been reported previously. L-alanine can be produced from decarboxylation of L-aspartatic acid catalyzed by L-aspartate-β-decarboxylase of immobilized cells or cell suspension of *Pseudomonas dacunhae* (Yamamoto *et al*., 1980; Takamatsu *et al*., 1981). However, this method has a high substrate (aspartate) cost. Recently, the reductive amination of pyruvate catalyzed by L-alanine dehydrogenase (L-AlaDH) can be an alternative way to produce L-alanine (Suye *et al*., 1992). This method use to produce an L-amino acid from the corresponding keto-acid also applicable to produce various amino acids such as L-leucine (Ohshima, *et al*., 1985), L-valine, L-isoleucine (Gu and Chang, 1990) and L-phenylalanine (Matsunaga, *et al*., 1987).

1.3 Amino acid dehydrogenase

Amino acid dehydroganase (EC 1.4.1-) is a part of the oxidoreductase superfamily which catalyzes the reversible deamination of L-amino acid to their corresponding keto acids in the presence of the pyridine nucleotide coenzymes, NAD^+ and/or $NADP^+$ (Brunhuber and Blanchard, 1994). These enzymes are found generally in an extensive number of diverse prokaryotic and eukaryotic organisms. They are known as important enzymes that exist at the interface of nitrogen and carbon metabolism and provide a route for interconversion of inorganic nitrogen with organic nitrogen (Vancura, *et al.,* 1989). In other words, they serve as a connecting link between amino acid and organic acid metabolism (Vali, *et al*., 1980). The enzymes are considerably different from alcohol and lactate dehydrogenase in their structures and properties. Amino acid dehydrogenases are catagorized base on the specificity that they display toward their amino acid substrate and more than ten kinds of them have been so far found in various kind of organisms as shown in Table 1.1 (Ohshima and Soda, 1989; Ohshima and Soda, 1990; Hummel and Kula, 1989). 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, L-amino acid can be obtained in a lot of yield because the equilibrium for the reaction favors amino acids formation. The general equation for this reaction can be illustrated as shown in figure 1.1 (Brunhuber and Blanchard, 1994). Thus, amino acid dehydrogenases have been extensively studied in the part and have found widespread application in amino acid production for clinical and food analysis.

1.4 Alanine dehydrogenase

One of the most interesting amino acid dehydrogenases is alanine dehydrogenases (L-alanine; NAD^+ oxidoreductase, deaminating, EC 1.4.1.1) (AlaDH), which is a cytoplasmic enzyme that catalyzes the reversible pyridine nucleotide-dependent oxidative deamination of L-alanine to from ammonia, pyruvate and NADH as shown in figure 1.2. L-AlaDH is the first amino acid dehydrogenase that has been studied. This enzyme has been found in vegetative cells (Hong, *et al*., 1959) and spores (Nitta, *et al*., 1974) of various bacilli and in some other bacteria (Germano and Anderson, 1968; Holmes, *et al*., 1961). AlaDH plays an important role in the carbon and nitrogen metabolism of various microorganisms (McCowen, 1974) by providing a link between carbohydrate and amino

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

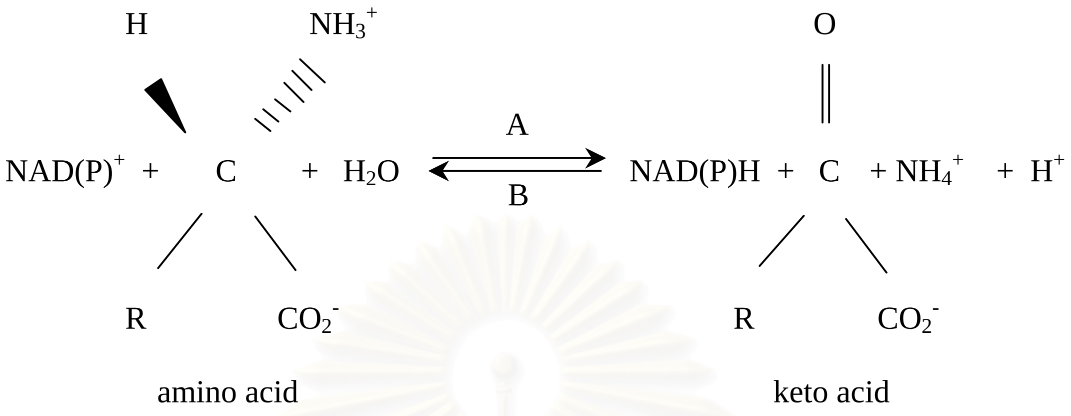
H
NH_3^+
O
A
$NAD(P)^+$ +
C
+ H_2O
NAD(P)H +
C
+ NH_4^+
+ H^+
B
R
CO_2^-
R
CO_2^-
amino acid
keto acid

Figure 1.1 **The general reaction of L-amino acid dehydrogenases**

(A) oxidative deamination (B) reductive amination

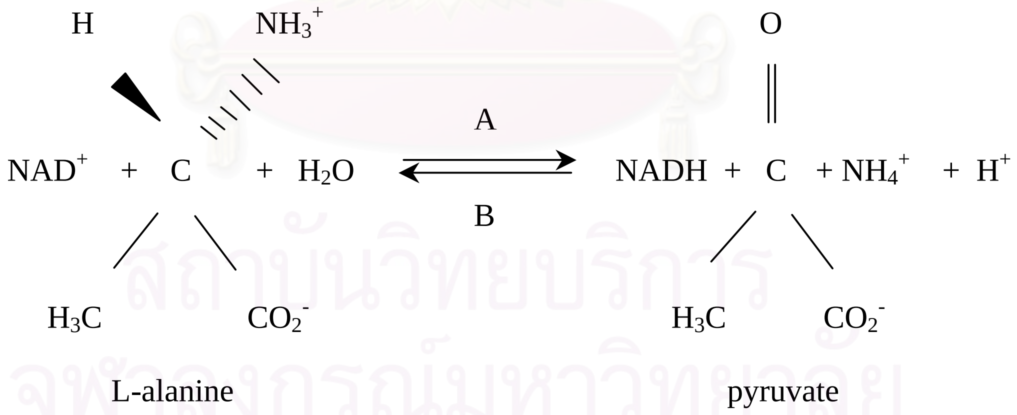
H
NH_3^+
O
A
NAD^+
+
C
+ H_2O
NADH +
C
+ NH_4^+
+ H^+
B
H_3C
CO_2^-
H_3C
CO_2^-
L-alanine
pyruvate

Figure 1.2 **The reaction of L-alanine dehydrogenase**

(A) oxidative deamination (B) reductive amination acid metabolisms. In *Bacillus* strains, the main function of this enzyme is involved in the production of pyruvate from L-alanine as an energy source through the tricarboxylic acid cycle. Like leucine dehydrogenase (LeuDH), AlaDH in spores seems to be responsible for generation of energy during sporulation (Ohshima and Soda, 1990; Siranosian *et al*., 1993). In nitrogen fixing organisms, such as *Anabaena cylindorica* (Neilson and Doudoroff, 1983), *Streptomyces aureofaciens* (Vancurova, *et al*., 1988) and *Rhodobacter capsulatus* (Caballero, *et al*., 1989), AlaDH plays an important role in the incorporation of ammonia into organic compounds.

1.4.1 Properties and characterization of AlaDHs from various sources

In 1955, Wiame and Pierard was firstly identified AlaDH from *Bacillus subtilis*. This enzyme was purified by Yoshida and Freese in 1964 and it was found that its molecular mass 228 kDa. Basic molecular and catalytic properties of various microbial AlaDHs are summarized in Table 1.2. AlaDHs differ with respect to their subunit structures. The majority of L-AlaDHs purified to date exist as homohexamers, This class includes those from a wide range of bacilli, *Geobacillus stearothermophilus, Aeromonas hydrophila*, *Anabaena cylindrica, Enterobacter aerogenes, Phormidium lapideum, Rhodobacter capsulatus*, *Streptomyces phaeochromogenes* and *Thermus thermophilus.* Tetramer has been reported for the L-AlaDH from *Pseudomonas* sp., *Rhizobium japonicum, Streptomyces fradiae* whereas octamer was found in *Streptomyces aureofaciens*. The enzyme of halophilic bacteria and *Streptomyces clavuligerus* are monomer. The substrate specificity of the enzyme for oxidative deamination is high since L-alanine is exclusively deaminated. The specificity for keto acids is lower than that for amino acids. Not only pyruvate, α-ketobutyrate, α-ketovalerate and 3-hydroxypyruvate

Table 1.2 Some properties of AlaDH from various microorganisms

Source	M_r (x10^3) (subunit structure)	Optimum pH	Optimum temperature	K_m values (mM)					Reference
				L-ala	NAD$^+$	pyr	NH$_3$	NADH	
Aeromonas hydrophila	230 (6x40,000)	10.5	37	20.0	0.17	1.33	77	0.25	Phungsangtham, 1997
Anabaena cylindrica	270 (6x43,000)	9.6		0.4	0.01	0.11	8-133	-	Rowell and Stewart, 1976
Bacillus cereus	255 (6x 42,000)	10.5	-	11.3	0.18-1.18	0.48	22-30	0.03-0.32	Porumb, *et al*., 1987
Bacillus japonicum	190	10	-	1	0.2	0.49	8.9	0.08	Brunhuber, *et al*., 1994
Bacillus sphaericus	230 (6x38,000)	10	70	0.01	0.01	1.7	3.8	0.01	Ohshima and Soda, 1979
Bacillus subtilis	228 (6x38,000)	10.1	-	1.73	0.18-0.36	0.54	38	0.02	Yoshida and Freese, 1965
Desulfovibrio desulforicans	-	9.8	-	2	-	5	24	0.05	Germano, *et al*., 1968
Enterobacter aerogenes	245 (6x41,000)	10.9	-	0.47	0.16	0.22	66.7	-	Chowdhury, *et al*., 1998
Geobacillus stearothermophilus	240 (6x40,000)	10.7	-	13.3	1.67	5	0.07		Sakamoto, *et al*., 1990
Halobacterium cultirubrum	72.5 (monomer)	9	-	7	0.5	0.8	0.82	0.2	Kim and Fitt, 1977
Halobacterium salinarum	60 (monomer)	9	-	-	-	-	-	-	Keradjopoulos and Holldorf, 1979
Mycobacterium tuberculosis	-	7-10	-	13.8	0.31	1.45	35.4	0.09	Hutter and Singh,
Phormidium lapideum	240 (6x41,000)	9.2	60	5	0.04	0.33	111	0.02	Sawa, *et al*., 1994
Pseudomonas sp.	217 (4x53,000)	9	-	-	-	4.3	26	0.05	Bellion and Tan, 1987
Rhizobium japonicum	168 (4x42,000)	8.6-10	-	-	-	0.68	-	0.04	Mueller and Werner, 1982
Rhizobium sp.	-	-	-	0.37	-	0.43	5.5	0.02-0.09	Smith, *et al*., 1993
Rhodobacter capsulatus	246 (6x42,000)	10.5	30	1.25	0.15	0.13	16	0.25	Caballero, *et al*., 1989
Rhodopseudomonas capsulata	-	9.8	-	0.45	0.14	0.37	28	0.06	Tolxdorff-Neutzling, *et al*., 1982
Shewanella sp.	-	-	-	7.6	0.24	-	-	-	Irwin, *et al*., 200; Galkin, *et al*., 1999
Streptomyces aureofaciens	395 (8x48,000)	10	75	5	0.11	0.56	6.67	0.02	Vancurova, *et al*., 1988
Streptomyces clavuligerus	92(monomer)	8.4	-	9.1	0.5	1.1	20	0.14	Aharonowitz, *et al*., 1980
Streptomyces fradiae	210 (4x51,000)	10	60	10	0.18	0.23	11.6	0.05	Vancura, *et al*., 1989
Streptomyces phaeochromogenes	240 (6x39,000)	8	60	1.9	0.03	0.29	61	0.04	Itoh, *et al*., 1983
Thermus thermophilus	290 (6x48,000)	10.5	-	0.18-4.2	0.12	0.75	59	0.03	Vali, *et al*., 1980

can be aminated. The optimum pHs for the oxidative deamination and reduction amination are 10 - 10.5 and 8-9, respectively.

1.4.2 Cloning of AlaDH

For gene cloning, there are few reports about AlaDH available. Sakamoto *et al.*, (1990) studied gene cloning, purification and characterization of thermostable AlaDH from *Bacillus stearothermophilus*. The *aladh* gene was cloned and expressed in *E. coli* 600 with a plasmid vector pKK 223-3. The enzyme was purified 30-fold with 46 % yield. It showed immunochemically identical with that of *B. stearothermophilus*. The enzyme had a molecular mass about of 240 kDa and consisted of six subunits (40 kDa). The enzymological properties of AlaDH from *B. stearothermophilus* are very similar to those of the mesophilic *B. sphearicus* enzyme except for thermostability. This research group also reported gene cloning of *B. sphearicus* into *E. coli* 600 with pICD322 as a vector in the same year (Kuroda *et al.*, 1990). Alanine dehydrogenase gene from *B. sphearicus* and *B. stearothermophilus* were analyzed for their nucleotide sequences. It was found that each *aladh* gene consisted of a 1116 bp open reading frame and encoded 372 amino acid residues corresponding to subunits of the hexameric enzyme. The similarity of amino acid sequence between these two AlaDHs is very high (> 70 %). In 1998, Chowdhury *et al*. purified AlaDH from *Enterobacter aerogenes* ICR 0220 and the *aladh* gene was cloned into *E. coli* JM109 cells with pUC18 as a vector and the nucleotides were sequenced. The deduced amino acid sequence was very similar to that of the AlaDH from *B. subtilis*. In 1999, Galkin *et al*. studied the cold-adapted AlaDH from *Shewanella* sp. stain Ac10 (SheAlaDH) and *Carnobacterium* stain St2 (CarAlaDH). The enzyme genes were cloned and expressed in *E. coli* TG1 with a vector plasmid pUC18. The amino acid sequences of SheAlaDH and CarAlaDH were compared with the sequences of AlaDH from other bacterial sources. CarAlaDH exhibited the highest overall levels of identity (58.5 to 62.8 %) with the enzymes from members of the same group of bacteria (the low-G+C-content gram-positive bacteria), such as *B. stearothermophilus*, while SheAlaDH was most similar (level of identity, 76.5 %) to *Vibrio proteolyticus*, that is a mesophilic gram-negative bacterium. SheAlaDH was more stable than CarAlaDH but less stable than all of the AlaDHs from mesophilic (*B. subtilis*) and thermophilic stains (*B. stearothermophilus*).

1.4.3 Application of AlaDH

Nowadays, AlaDH is a useful for many application in industries such as L-alanine and its derivatives production. The importance of L-alanine is described in section 1.2. In addition, L-β-chloroalanine, one of alanine derivative, is used in some pesticides, medicines and natural or unnatural amino acid synthesis (Kato, *et al.*, 1993). Moreover, 3-fluoro-L-alanine has the potential to use in antibacterial, antivirus agents and insecticide production (Ohshima, 1989). AlaDH is also using for the quantitative analysis of L-alanine in sample. In medical applications, it is used for detection of γ-glutamyl cyclotransferase, which is the marker enzyme for malignant hematopoietic disease. This disease is caused by the serious defect in red blood cell production. The pateints who suffered from this disease have abnormal level of this enzyme which can be detected by using AlaDH as shown in figure 1.3 (Ohshima and Soda, 1990).

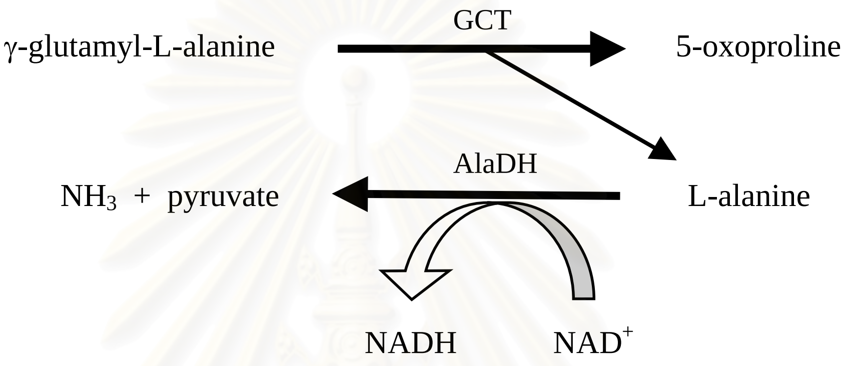
γ-glutamyl-L-alanine
GCT
5-oxoproline
AlaDH
NH_3 + pyruvate
L-alanine
NADH
NAD^+

Figure 1.3 The detection system of γ-glutamyl cyclotransferase

GCT: γ-glutamyl cyclotransferase

AlaDH: alanine dehydrogenase

1.5 Regeneration of NADH

Redox reactions are important steps in the metabolism and energy conversion of living cells. Besides the necessity of enzymes, a number of coenzymes, ferrodoxins, lipoic acid, NAD(H), NADP(H), flavins and cytochromes, are involved in such reaction. The coenzymes differ in their redox potential, the binding constants and the mode of regeneration (Wang and King, 1979). Biosynthetic transformations involving redox reactions also offer a considerable potential for the production of fine chemicals over conventional chemical processes, especially those requiring stereospecificity.

Nicotinamide adenine dinucleotide (NAD) plays a central role in cellular metabolism by function as a cofactor in over 300 redox reactions (Foster *et al,*. 1990). In general, they dissociate easily and need a second reaction with another metabolite for regeneration. These properties are one of the means by which native directs the flow of intermediates in response to biosynthetic needs. Because the spectral properties of the coenzyme moiety, dehydrogenases have been extensively studied in the part (Boyer, 1975) and have found widespread applications in clinical and food analysis (Bergmeyer, 1985).

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 stochiometric amounts of rather expensive coenzymes to perfrom catalytic transformations. The high price of NAD(H) demands its continuous regeneration of nicotinamide coenzymes, including chemical (Ruppert, *et al*., 1988), biological (Morita, *et al*., 1994), electrochemical (Fry, *et al*., 1994; Nakamura, *et al*., 1988), photochemical and enzymatic methods. While the problem of NAD^+ recycling might be reasonably solved by various means, e.g. direct oxidation on carbon or modified electrodes, regeneration of NADH is a more challenging task. Among the methods suggested, enzymic means of

NADH regeneration are most promising.

In recent years, enzyme and whole-cell bioreactor methods as well as cultivation and chemical methods have been developed for L-amino acid production. Continuous production of L-alanine and other aliphatic L-amino acids from their corresponding keto acid analogs and ammonium formate was studied with an ultrafiltration membrane reactor (molecular cut-off at MW 5,000) containing AlaDH, yeast formate dehydrogenase (FDH, MW 80,000) and NADH or NAD bound covalently to polyethylene glycol (PEG, MW 20,000) (Fiolitaktis and Wandrey, 1983). PEG-NADH can not penetrate through the membrane while FDH catalyzes regeneration of the PEG-NADH with formate. It is considerably stable and cheaply available and its reaction is irreversible. Pyruvate and ammonium formate are continuously pumped into the reactor following by production of L-alanine together with CO_2 (figure 1.4). The enzyme membrane reactor system is applicable to the production of L-3-fluoroalanine from β–fluoropyruvate due to the broad sustrate specificity of AlaDH (Ohshima *et al*., 1989). A similar enzyme membrane reactor system with leucine dehydrogenase (LeuDH) has been developed for the continuous production of L-leucine (Buckmann *et al*., 1987), L-methionine (Wichmann *et al*., 1981) and L-tert-leucine due to the broad substrate specificity of LeuDH (Kula and Wandrey, 1985). The efficiency of this reactor system depends mainly on the stability and abandant supply of enzymes. The thermostable LeuDH produced abundantly by *E. coli* cells has a long half-life, and thus is more useful in the reactor system (Ohshima *et al*., 1985). Phenylalanine dehydrogenase (PheDH) is used for the continuous production of L-phenylalanine (Hummel *et al*., 1987; Schmidt *et al*., 1987). The general equation for enzymatic systhesis of L-amino acids by NADH regeneration with coupled reaction of FDH and second enzyme is shown in figure 1.5 (a).

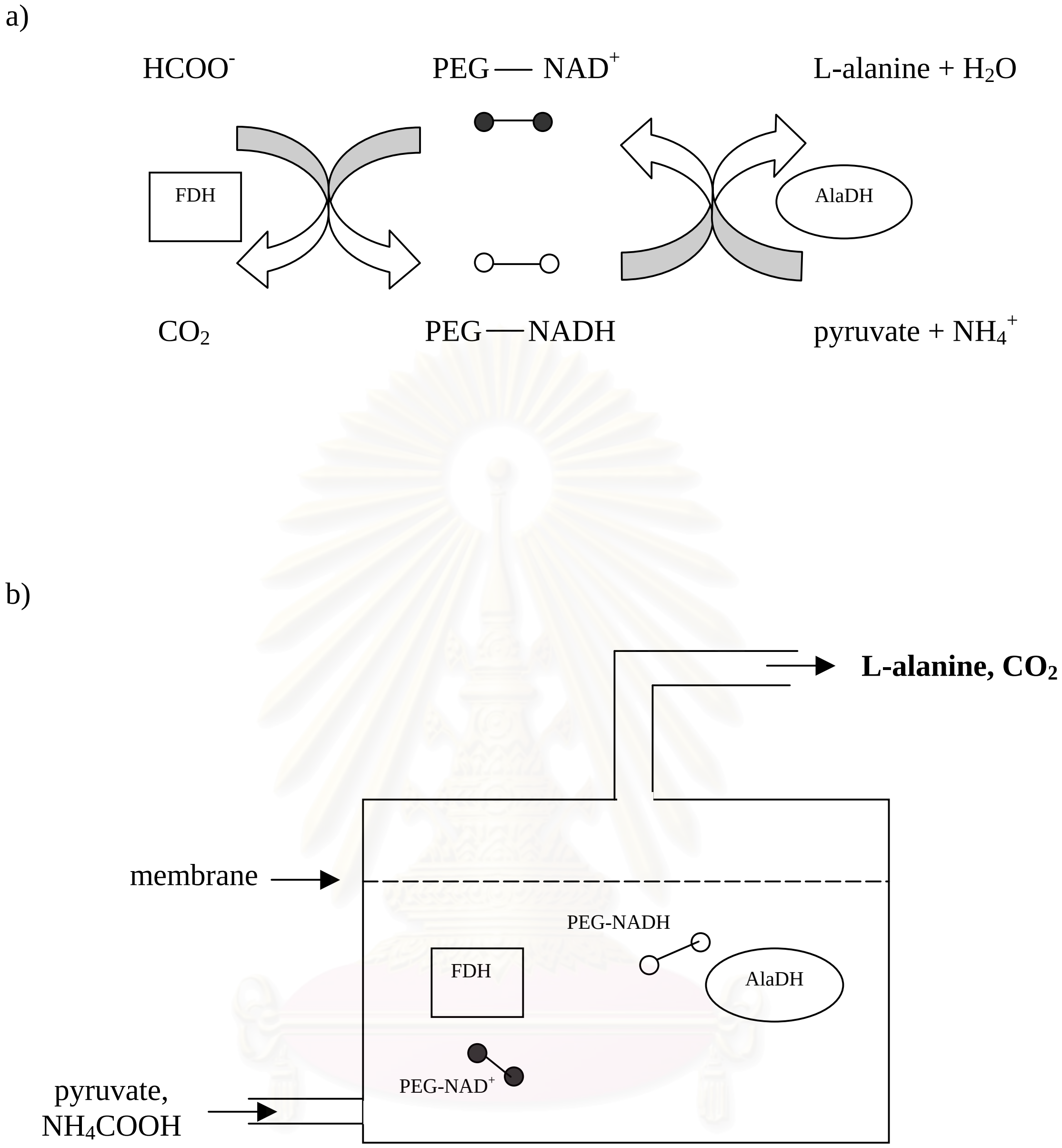
a)
HCOO⁻
PEG — NAD⁺
L-alanine + H₂O
FDH
AlaDH
CO₂
PEG — NADH
pyruvate + NH₄⁺
b)
L-alanine, CO₂
membrane
PEG-NADH
FDH
AlaDH
PEG-NAD⁺
pyruvate,
NH₄COOH

Figure 1.4 Enzymatic reaction system for continuous production of L-alanine with coenzyme regeneration.

(a) Enzyme reaction scheme (b) Enzyme membrane reactor scheme

AlaDH: alanine dehydrogenase

FDH: formate dehydrogenase

PEG: polyethyleneglycol

Source: Hummel *et al.*, 1987

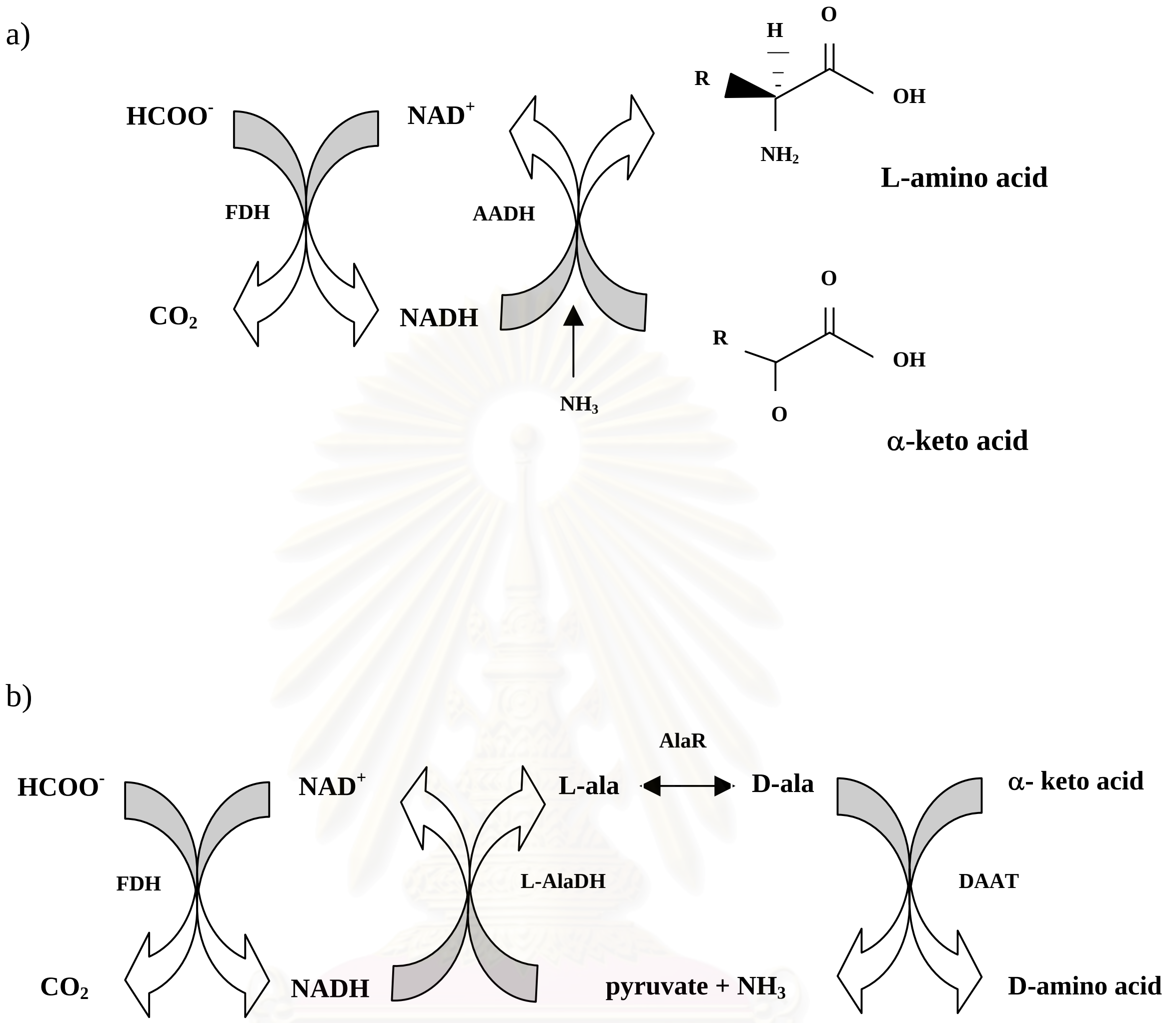
a)
HCOO⁻
NAD⁺
FDH
AADH
CO2
NADH
NH3
H
O
R
OH
NH2
L-amino acid
O
R
OH
O
α-keto acid
b)
HCOO⁻
NAD⁺
AlaR
L-ala
D-ala
α- keto acid
FDH
L-AlaDH
DAAT
CO2
NADH
pyruvate + NH3
D-amino acid

Figure 1.5 Enzymatic synthesis of L-amino acids (a) and D-amino acids (b) by coupling of enzymes reactions.

AADH: L-amino acid dehydrogenase

FDH: formate dehydrogenase

AlaDH: alanine dehydrogenase

AlaR: alanine racemase

DAAT: D-amino acid aminotransferase

Source : Galkin *et al.*, 1997a

The synthesis of various D-amino acids, which are useful for the chemical systhesis of β-lactam antibiotics and bioactive peptides, by a multienzyme system has been developed. In this system, D-amino acids, such as D-glutamate, D-methionine and D-valine, are produced from the corresponding α-keto acids and ammonium formate by coupling four enzyme reactions catalyzed by FDH, AlaDH, alanine racemase (AlaR) and D-amino acid aminotransferase (DAAT) as shown in Figure 1.5 (b) (Galkin, *et al*., 1997a). This procedure is based on the strict stereospecificity and low structural specificity for substrates of D-amino acid aminotransferase and the very high substrate specificity of alanine racemase. A similar system consisting of glutamate dehydrogenase (GluDH) and glutamate racemase is applicable to the effective production of D-alanine, D-aspartate, D-α-aminobutyrate, and D-valine (Nakajima *et al*., 1988). The systhesis of L-β-chloroalanine by using AlaDH, LeuDH, and PheDH in combination with FDH has also been studied (Kato *et al*., 1993). The system mentioned above is also applicable to the synthesis of ^{13}N-labelled D amino acids, which are expected to be valuable in the study of mammalian neural systems.

Suye *et al*., (1992) studied an enzyme reaction system with coenzyme regeneration for L-alanine production. Crude extract of AlaDH from *Corynebacterium flaccumfaciens* AHU-1622 was used as the catalyst for reductive amination of pyruvate to L-alanine. NAD(P)$^+$ -linked malic enzyme from *Pseudomonas diminuta* IFO - 13182 was used for the regeneration of NADH as shown in figure 1.6. The conversion of L-malic acid to L-alanine reached 95 % after 72 hr. of incubation at 30 ^{o}C. One hundred and six mol/m^3 of L-alanine produced was purified in crystal form with 99.4 % purity based on HPLC analysis.

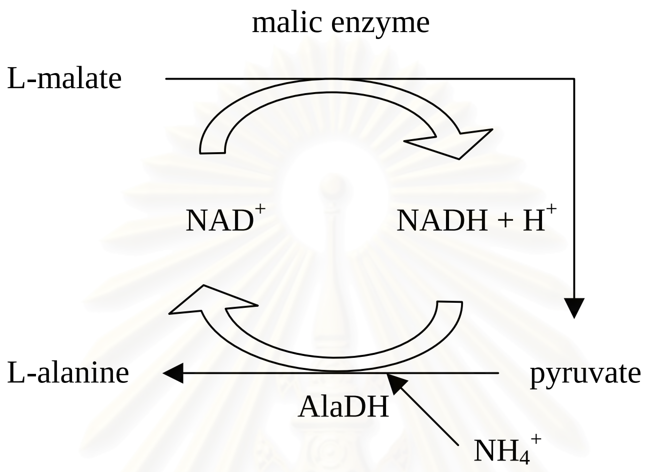
malic enzyme
L-malate
NAD+
NADH + H+
L-alanine
pyruvate
AlaDH
NH4+

Figure 1.6 Production of L-alanine with NADH cycle.

Lin *et al*., (1997) studied a conjugated enzyme system of AlaDH from *Bacillus subtilis* for stereospecific reduction of pyruvate to L-alanine and glucose dehydrogenase (GDH) from *Bacillus* sp. for regeneration of NADH. Both enzymes were coimmobilized in a nanofiltration membrane bioreactor (NFMBR) for the continuous production of L-alanine from pyruvate with NADH regeneration as shown in figure 1.7. The maximum conversion, reactor productivity and NAD regeneration number were 100%, 320 g/litre/d and 20,000 respectively. Since pyruvate was proven to be unstable at neutral pH, it was kept under acidic condition (pH 4.0) and supplied to NFMBR separately from the other substrates. To avoid the effect of pyruvate instability, a consecutive reaction system containing lactate dehydrogenase (L-LDH) from bovine heart and AlaDH was used. In this system, L-LDH provides pyruvate, the substrate for the AlaDH reaction, so the pyruvate could be consumed as soon as it was produced as shown in figure 1.8. The maximum conversion, reactor productivity, and the NAD regeneration number were 100%, 160 g/litre/d, and 20,000 respectively. However, the starting material of this system, L-lactate, is very expensive.

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 are well known. The latter allows an economic regeneration of NADH from NAD^+ with formate.

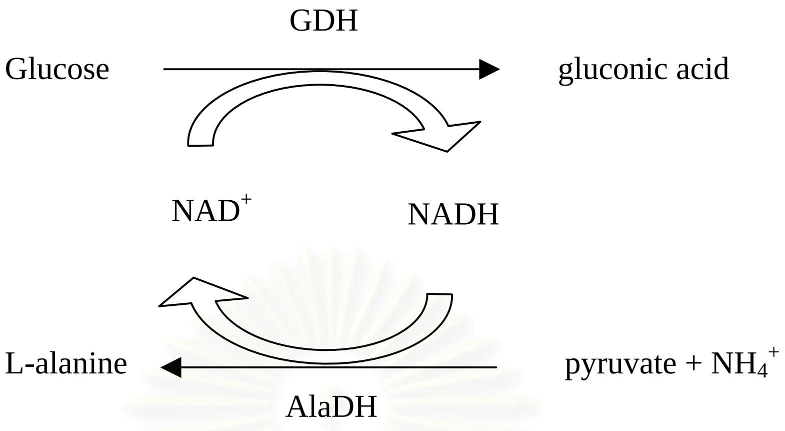
GDH
Glucose
gluconic acid
NAD$^+$
NADH
L-alanine
pyruvate + NH$_4^+$
AlaDH

Figure 1.7 Conjugated enzyme system of AlaDH and GDH for production of L-alanine.

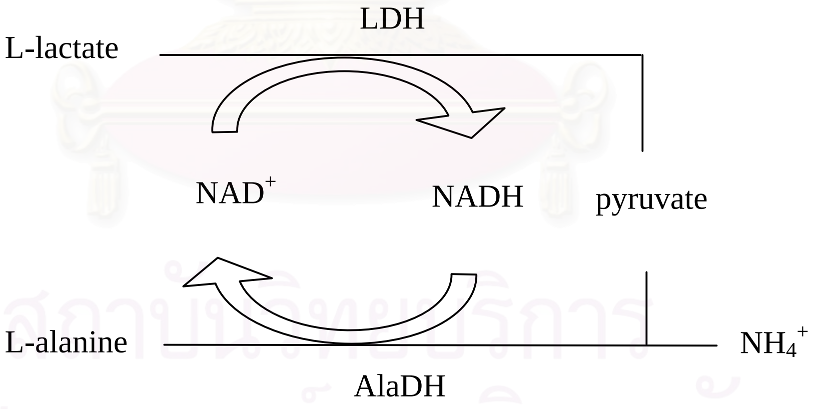
LDH
L-lactate
NAD$^+$
NADH
pyruvate
L-alanine
NH$_4^+$
AlaDH

Figure 1.8 Conjugated enzyme system of AlaDH and L-LDH for production of L-alanine.

1.6 Formate dehydrogenase

Formate dehydrogenase (formate : NAD^+ oxidoreductase, EC 1.2.1.2) (FDH), catalyzes the conversion of formate to carbon dioxide with the concomitant reduction of NAD^+ into NADH as shown in figure 1.9. This enzyme has been found in various organisms such as plants, methylotrophic yeasts, and bacteria. In methylotrophic yeasts and bacteria, FDHs play a key role in the catabolism of C1 compounds such as methanol by catalyzing the final step (Popov and Lamzin, 1994; Ferry, 1990)

NAD^+-dependent formate dehydrogenase is widely spread in nature and the enzyme has extensive studied in many organisms such as *Pseudomonas* sp.101 (Tishkov, *et al*., 1993), *Moraxella* C-2 (EMBL Accession O08375), *Saccharomices 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).

FDH is of great interest from scientific and practical considerations. The enzyme belongs to the superfamily of D-stereospecific 2-hydroxy acid dehydrogenase (Vinals *et al*., 1993) and can be regarded as a model dehydrogenase catalyzing the reaction with the simplest substance: the formate ion. Structure of apo- and holo-FDH from *Pseudomonas* sp.101 have been solved with high resolution (Lamzin *et al*., 1994), and site-directed mutagenesis experiments were carried out to clarify the role of different amino acid residues in the catalytic metabolism of hydride transfer (Tishkov *et al*., 1993, 1996).

From application aspects, FDH is the best catalyst for *in-situ* NADH regeneration in the enzymatic synthesis of amino acids, chiral hydroxy acids and esters, alcohols and other fine chemicals using NAD^+ -dependent dehydrogenase (Hummel and Kula, 1989;

$$NAD^+ + HCOOH + H_2O \longrightarrow NADH + CO_2 + H^+$$

formate   carbon dioxide

Figure 1.9 The reaction of formate dehydrogenase

Drauz and Waldmann, 1995; Peters, 1998). The reaction catalyzed by FDH is practically irreversible and 100 % conversion of products is possible in coupled reactions. The use of FDH for coenzyme regeneration has several advantages. (1) Formate as substrate for the FDH, is one of the cheapest hydrogen sources and does not inhibit most other dehydrogenases. (2) The reaction has a favourable equilibrium strongly shifted towards CO_2 and NADH formation. (3) The oxidation product CO_2 can be easily removed from the reaction mixture. (4) The enzyme has a broad pH optimum of activity so that it can be easily implemented in coupled enzymatic synthesis.

1.7 AlaDH from *Aeromonas hydrophila*

Our research group at the Department of Biochemistry has studied on L-alanine dehydrogenase of *Aeromonas hydrophila* which was screened from soil in Bangkok (Phungsangtham, 1997). AlaDH from this bacterium has molecular mass of about 230 kDa and consists of 6 identical subunits. The enzyme is highly specific for L-alanine and NAD^+. Optimum temperature for reductive amination and oxidative deamination are 45 and 55 ^oC, respectively. Enzyme activity remains high when incubated at 55 ^oC for 16 hours. The optimum pH for reductive amination is 8.0 while the reverse reaction rate is highest at pH 10.5. The steady state kinetic studies including product inhibition on the enzyme reaction indicates that the oxidative deamination proceeds through a sequential ordered binary-ternary mechanism in which NAD^+ binds first to the enzyme followed by L-alanine and products are released in the order of pyruvate, ammonia and NADH, respectively. The K_m values for NAD^+, L-alanine, pyruvate, ammonia and NADH were 0.17, 20, 1.33, 77 and 0.24 mM, respectively. The enzyme gene was cloned and expression into *E. coli* JM109 cells with pUC18 as a vector and the nucleotides were sequenced

(Poomipark, 2000). This gene has an open reading frame of 1,113 bp which encoded for 371 amino acids residues and has GC content about 65 %. Comparison of deduced amino acid sequence with AlaDHs from other bacteria shows over 50 % similarity. The transformant has specific activity 50 times higher than that of the enzyme from wild type.

In this study, L-alanine production of recombinant clone will be improved by coupling reactions between AlaDH and FDH. NAD^+-dependent formate dehydrogenase gene from methanol-utilizing bacterium *Mycobacterium vaccae* N10 is generous a gift from Professor Nobuyoshi Esaki, Kyoto University. The gene was cloned and expressed in to *E. coli* TG1 with a plasmid vector pUC119. This gene has an open reading frame of 1200 bp which encoded for 401 amino acids residues. The enzyme product of this gene has molecular mass about 88 kDa and consists of 2 identical subunits. The K_m values for formate and NAD^+ were 6.0, 0.09 mM, respectively. Two methods of AlaDH and FDH expression system 1) the *aladh* and *fdh* gene on separate DNA vectors were simultaneously introduced into host cells by co-transformation and 2) heterologous genes, the different genes on a single DNA vector, will be performed in *E. coli* cells for L-alanine production. FDH will regenerate NADH, which is a coenzyme of AlaDH (figure 1.10). 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) could be used for NADH regeneration without any additional supplies.

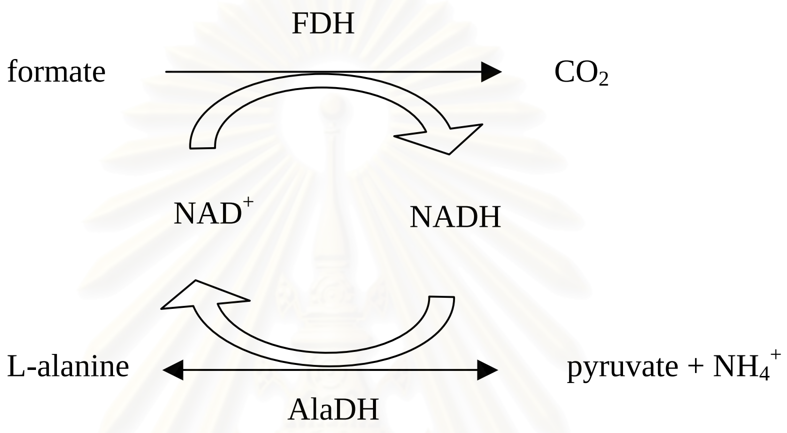
FDH
formate
CO2
NAD+
NADH
L-alanine
pyruvate + NH4+
AlaDH

Figure 1.10 Conjugated enzyme system of AlaDH and FDH for production of L-alanine.

The outline of this research

1. Cloning of *aladh* gene into a high expression vector pET-17b and pMPM-K3.
2. Cloning of *fdh* gene into expression vector pET-17b and pSY343.
3. Co-transformation of pETAlaDH/pSYFDH and pMPMAlaDH/pETFDH into *E. coli* BL21(DE3).
4. Construction of a heterologous gene expression system of *aladh* and *fdh* gene on pET -17b and expression of the recombinant plasmids in *E. coli* BL21(DE3).
5. Comparison of AlaDH and/or FDH activity as well as alanine production efficiency of recombinant clone

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

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

Autopipette: Pipetman, Gilson, France

Camera: Pentax super A, Asahi Opt. Co., Japan

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.

Crownpack CR(+) HPLC column: 0.4 cm x 15 cm, Daicel chemical, Japan

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

Filter paper No.1, Whatman, Japan

Gel Document: SYNGENE, England

Gene Pulser$^{\text{R}}$/*E. coli* Pulser$^{\text{TM}}$ Cuvettes: Bio-Rad, U.S.A.

High Performance Liquid Chromatography (HPLC): SHIMADZU, Japan

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

Incubator shaker: Innova$^{\text{TM}}$ 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.

Membrane filter: cellulose nitrate, pore size 0.45 μm, Whatman, England

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

Ultrafilter: Suprec$^{\text{Tm-01,Tm-02}}$, 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.

5-Bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal): Sigma, U.S.A.

Bromphenol blue: Merck, Germany

Chloroform: BDH, England

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

DL-alanine: 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

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.

Kanamycin: Sigma, U.S.A.

L-Alanine: 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

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 JM109, genotype: F' [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*ZΔM15] *recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ (*lac-proAB*) was used as a host for cloning.

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

2.6 Plasmids

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

2.6.2 pMPM-K3, a vector for cloning and expression (gift from Seiichi Yasuda, Department of Microbial Genetics, National Institute of Genetics, Japan) (Appendix A).

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

No.	Primers	Sequence (5' - 3')	T_m (^oC)
1	AlaDHF*Nde*I	GGAATTCCATATGATTATCGGTGTACCTAAGG	62
2	AlaDHR*Hin*dIII	CCCAAGCTTCAGTTCAGCAGGGTCAGGG	62
3	FDHF*Nde*I	GGAATTCCATATGGCAAAGGTCCTGTG	60
4	FDHR*Bam*HI	CGGGATCCTCAGACCGCCTTCTTGAACTTG	60
5	T7FDHF*Hin*dIII	CGCAAGCTT CGATCCCGCGAAATTAATACG	60
6	FDHR*Eco*RI	CGGAATTCFCAGACCGCCTTCTTGAAC	60
7	T7FDHF*Bam*HI	CGGGATCC GATCCCGCGAAATTAATACG	60

Restriction sites are underlined and T7 promoter in boxed.

2.6.3 pSY343, a vector for cloning and expression (gift from Seiichi Yasuda, Department of Microbial Genetics, National Institute of Genetics, Japan) (Appendix A).

2.6.4 pUCAlaDH, a pUC18 containing *aladh* gene from *Aeromonas hydrophila*. (Poomipark, 2000).

2.6.5 pUCFDH, a pUC119 containing the *fdh* gene from *Mycobacterium vaccae* N10 (gift from Nobuyoshi Esaki, Institute for Chemical Research, Kyoto University, Japan).

2.7 Bacterial growth medium

Luria-Bertani broth (LB medium)

LB medium consisted of 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.2 with NaOH. For solid medium, 1.5% of agar was added. Medium was steriled by autoclaving at 121° C for 20 minutes. If need, selective antibiotic drug was then supplemented.

2.8 Transformation of plasmid

2.8.1 Competent cell preparation (Dower, 1988)

A single colony of *E. coli* host cell was cultured as a starter in 10 ml of LB-broth and incubated at 37 °C with 250 rpm shaking for 24 hours. 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 of the cells reached 0.5 - 0.8 (~3 - 4 hours). After that, the culture was chilled on ice for 15 to 30 minutes and the cells were harvested by centrifugation at 6,000 xg for 15 minutes at 4°C. The supernatant was removed. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile water, respectively. The cells were resuspended and centrifuged at 6,000 xg for 15 minutes at 4°C. The supernatant was discarded. The 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

In the electroporation step, 0.2 cm 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 μl 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. Subsequently, one ml 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 which harboured recombinant plasmid was grown in LB-medium (1% peptone, 0.5% NaCl and 0.5% yeast extract, pH 7.2) containing 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

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 1.0 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 cooled down to 60^{o}C until all air bubbles were completely eliminated. The 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 desired gel-loading buffer (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and slowly loaded the mixture into an appropriate percentage of 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 and photographed through a red filter using Kodak Tri X pan 400 film. 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 according to QIAquick gel extraction kit protocol. Briefly, DNA fragment was excised from an agarose gel and transferred to an eppendorf tube. Three volume of buffer QG 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. Buffer QG was added and centrifuged for 1 minute. The column was washed twice with buffer PE and centrifuged for 1 minute. Finally, the elution buffer was added to the center of the QIAquick membrane to elute the DNA, 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 harboring the recombinant plasmid were grown at appropriated 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 discontinuously 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 AlaDH activity

The activity of AlaDH for oxidative deamination of alanine was spectrophotometrically assayed. Reaction mixture 1 ml comprised of 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5, 20 μmol of L-alanine, 1 μmol of NAD$^+$, and enzyme. In a blank tube, L-alanine 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 was monitored by measuring the initial change in absorbance of NADH at 340 nm.

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

2.13.2 Determination of FDH activity

The activity of FDH was spectrophotometrically assayed. Reaction mixture 1 ml comprised of 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°C in a cuvette of 1-cm light path. The reaction was started by addition of NAD^+ and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

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

2.14 Protein measurement

Protein concentration was determined by the method of Lowry *et al*., (1951) using bovine serum albumin (BSA) as the protein standard. The reaction mixture 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°C for 10 minutes. After that, the solution mixture was incubated with 0.5 ml of solution C at room temperature for 20 minutes. 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 protein standard (BSA).

2.15 Denaturing polyacrylamide gel electrophoresis (SDS PAGE)

2.15.1 Pouring the separating gel (10% acrylamide)

The gel sandwich was assembled according to the manufacturer’s instruction. For 2 slab gels, the 3.30 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 in a 25 ml Erlenmeyer flask and mixed. Then 50 μl of 10% ammonium persulfate and 5 μl of TEMED were added and mixed rapidly by swirling or inverting container gently. The solution was carefully introduced into gel sandwich by using a Pasteur pipette. After the appropriate amount of separating gel solution was added, water was gently layered about 1 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 then poured off.

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 in a 25 ml Erlenmeyer flask and mixed. 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 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 and 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 5 x sample buffer (0.3 mM Tris-HCl, 50% glycerol, 20% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue) in an Eppendorf tube, boiled 10 min at 95^{o}C and cooled to room temperature. Then the sample solution was introduced into well by using syringe.

2.15.4 Running the gel

An electrode plugs were attached to proper electrodes. Current was flowed towards the anode for pH 8.8 gels. The power supply was turned on at constant current (20 mA). Electrophoresis was continued until the dye front reached the bottom of the gel. Power supply was turned off and then the electrode plugs were removed from electrodes. The gel plates were removed from electrophoresis chamber. Then the gel was removed from glass plates and transferred to a small container.

2.15.5 Staining Procedure

Protein staining

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

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

2.16.1 PCR amplification of *aladh* gene

The primers that used for the PCR amplification of *aladh* gene were designed by using the data of the 5'- and 3'- terminal nucleotide sequence obtained from the *aladh* gene of *Aeromonas hydrophila*. The whole *aladh* gene was amplified by the PCR method and was overexpressed in *E. coli* BL21(DE3) with the assistance of T7 promoter of plasmid pET-17b. The 5'- primer contained *Nde*I restriction site and the sequence at end of *aladh* (AlaDHF*Nde*I), while the 3'-primer had *Hin*dIII restriction site and the sequence at the 3'-end of *aladh* as shown in Table 2.1 (AlaDHR*Hin*dIII).

PCR was performed 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 from pUC18 containing *aladh* gene (pUCAlaDH) as a DNA template. The PCR condition was predenaturation at 94 ^{o}C for 5 minutes, denaturation at 94^{o}C for 1 minute, annealing at 55 - 60^{o}C for 1.30 minute, extension at 72^{o}C for 2 minutes and final extension at 72^{o}C for 10 minutes. The number of cycle were 30 cycles.

2.16.2 Cloning of *aladh* gene

To clone pETAlaDH (Figure 2.1), the PCR product from 2.16.1 was digested by both *Nde*I and *Hin*dIII. Then a *Nde*I - *Hin*dIII digested fragment of *aladh* gene (1.16 kb) was ligated into the pET-17b, which was digested with the same enzymes. The ligation products were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown on LB agar plates containing 100 µg/ml ampicillin at 37^{o}C for 16 hours. Cells containing the recombinant plasmids, which had *aladh* gene were picked and their plasmids were isolated and checked by agarose gel electrophoresis. After

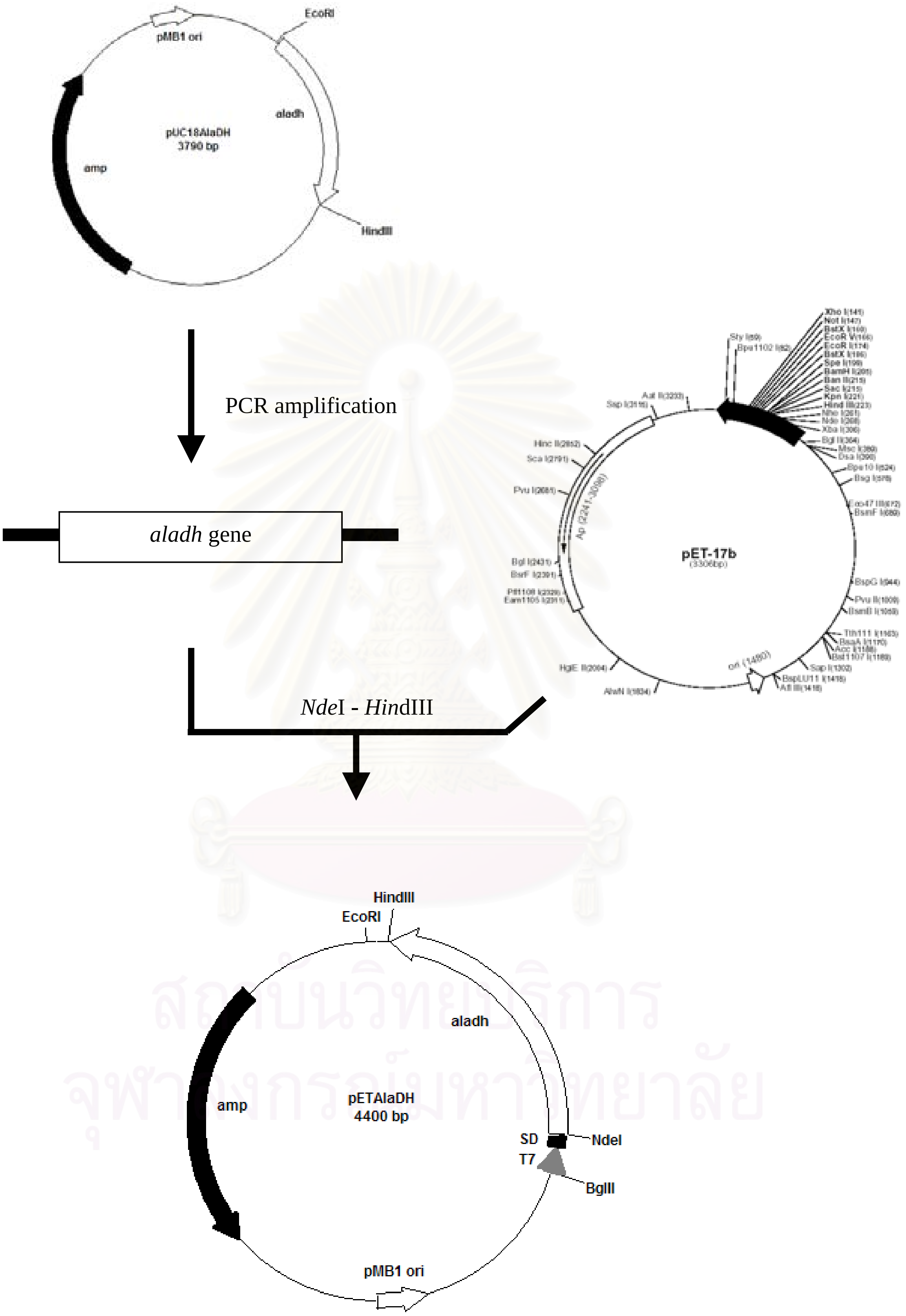
pMB1 ori
EcoRI
aladh
pUC18AlaDH
3790 bp
amp
HindIII
PCR amplification
aladh gene
Sty I(80)
Bpu1102 I(82)
Xho I(141)
Not I(147)
BstX I(160)
EcoR V(166)
EcoR I(174)
BstX I(186)
Spe I(198)
BamH I(205)
Ban II(215)
Sac I(215)
Kpn I(221)
Hind III(223)
Nhe I(261)
Nde I(268)
Xba I(306)
Bgl II(364)
Msc I(380)
Dsa I(390)
Bpe10 I(524)
Bsg I(578)
Eco47 III(672)
BsmF I(680)
BspG I(944)
Pvu II(1009)
BsmB I(1058)
Tth111 I(1163)
BsaA I(1170)
Acc I(1188)
Bst1107 I(1188)
Sap I(1302)
BspLU11 I(1418)
Afl III(1418)
ori (1480)
AlwN I(1804)
HgiE II(2004)
Pfl1108 I(2329)
Eam1105 I(2311)
BsrF I(2391)
Bgl I(2431)
Ap (2241-3098)
Pvu I(2681)
Sca I(2791)
Hinc II(2852)
Sap I(3116)
Aat II(3233)
pET-17b
(3306bp)
NdeI - HindIII
HindIII
EcoRI
aladh
pETAlaDH
4400 bp
amp
SD
T7
NdeI
BglII
pMB1 ori

Figure 2.1 Construction of pETAlaDH

that, the recombinant plasmid which had *aladh* gene was transformed into *E. coli* BL21(DE3).

2.16.3 Crude extracts preparation and enzyme activity assay

E. coli BL21(DE3) cells harboring the recombinant plasmids pETAlaDH were grown in 5 ml of LB-medium supplemented with 100 μg/ml ampicillin at 37 °C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml ampicillin and shaked at 37°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to 0.4 mM in order to induce enzyme production, and the cultivation was continued at 37°C for 4 hour before cell harvesting. Finally, crude extracts were prepared, assayed for the enzyme activity and protein determination as desand 2.14, respectively.

2.16.4 Optimization of induction time

Since *aladh* 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) host cell had T7 RNA polymerase gene, controlled under *lac Z* promoter, inserted in its chromosome. Thus, the study about influence of induction time by IPTG was required.

E. coli BL21(DE3) containing pETAlaDH, was grown in 5 ml of LB-medium supplemented with 100 μg/ml ampicillin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml ampicillin and shaked at 37°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, IPTG at final concentration 0.4 mM was added to induce enzyme production. The cultures 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 cells culture at various times were harvested by centrifugation. The cell pellets were resuspended in 100 µl of 5x sample buffer except 50 µl for 0 hour sample. The 7 µl of cell samples or 25 µg protein 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* gene

The whole *fdh* gene was amplified by the PCR method and overexpressed in *E. coli* BL21(DE3) 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* gene of *Mycobacterium vaccae* N10. The 5'-end of the 5'-primer and 3'-primer contained *Nde*I site (FDHF*Nde*I) and *Bam*HI site (FDHR*Bam*HI), respectively.

PCR was performed 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* gene (pUCFDH) as a DNA template. PCR condition consisted of predenaturation at 94^{o}C for 5 minutes, denaturation at 94^{o}C for 1 minute, annealing at 60^{o}C for 1.30 minute, extension at 72^{o}C for 2 minutes. and final extension at 72^{o}C for 10 minutes. The number of cycle was 30 cycles.

2.17.2 Cloning of *fdh* gene

To cloning of pETFDH (Figure 2.2), the PCR product from 2.17.1 was digested by both *Nde*I and *Bam*HI. Then a *Nde*I – *Bam*HI digested fragment of *fdh* gene (1.2 kb) was ligested into the pET-17b, which was digested with the same enzymes. The ligation products were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown on LB agar plates containing 100 μg/ml ampicillin at 37^{o}C for 16 hours. Cells containing the recombinant plasmids, which had *fdh* gene were picked and their plasmids were isolated and checked by agarose gel electrophoresis. After that, the recombinant plasmid which had *fdh* gene was transformed into *E. coli* BL21(DE3).

2.17.3 Crude extract preparation and enzyme activity assay

E. coli BL21(DE3) cells harboring the recombinant plasmids pETFDH were grown at 37^{o}C in 5 ml of LB-medium supplemented with 100 μg/ml ampicillin overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml ampicillin and shaked at 37^{o}C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to 0.4 mM inorder to induce enzyme production, and the cultivation was continued at 37^{o}C for 4 hour before cell harvesting. Finally, crude extracts were prepared and assayed for the enzyme activity and protein determination as described in 2.12, 2.13.2 and 2.14, respectively.

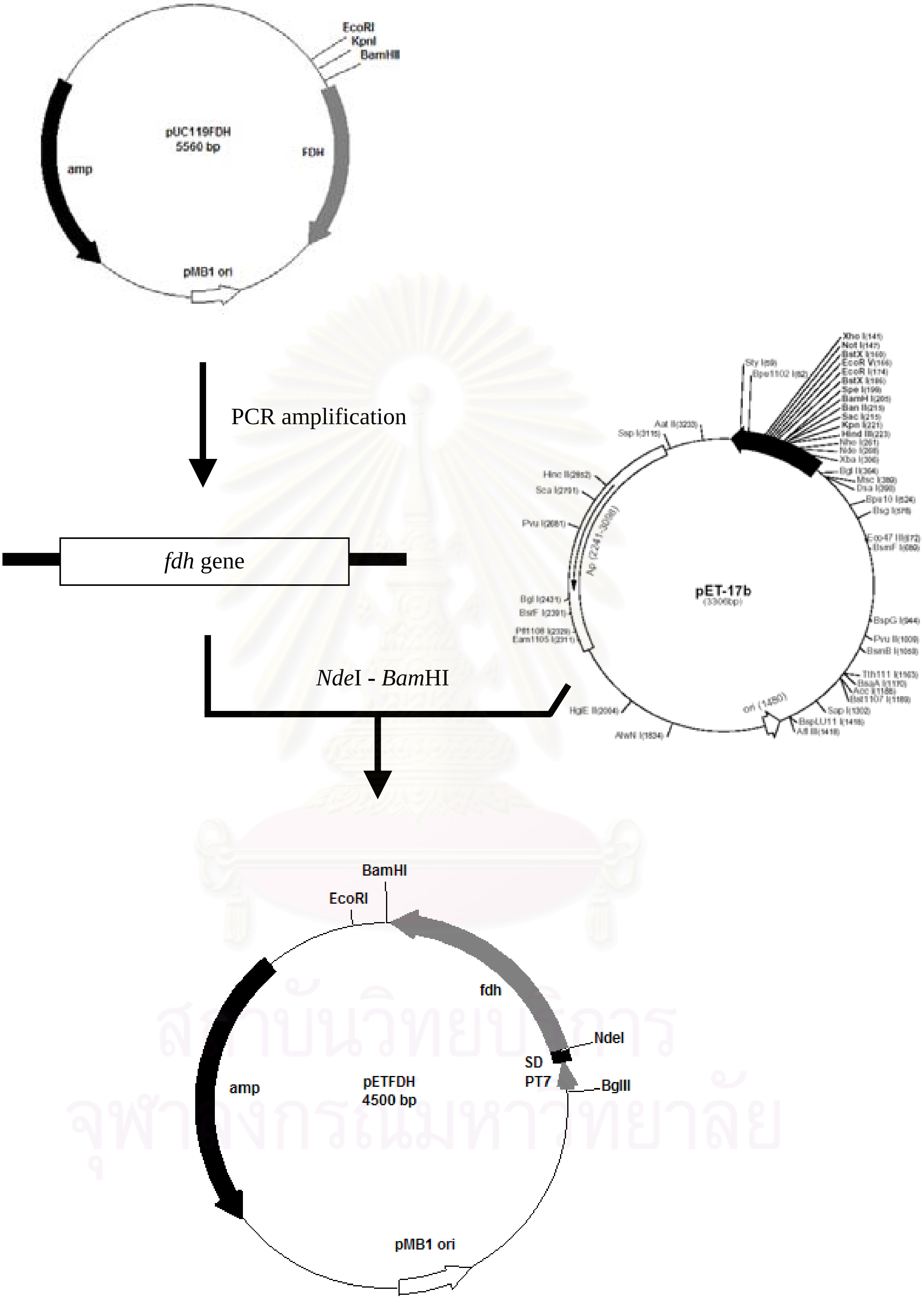
EcoRI
KpnI
BamHII
pUC119FDH
5560 bp
FDH
amp
pMB1 ori
PCR amplification
fdh gene
*Nde*I - *Bam*HI
Xho I(141)
Not I(147)
BstX I(160)
EcoR V(164)
EcoR I(174)
BstX I(184)
Spe I(196)
BamH I(201)
Ban II(215)
Sac I(215)
Kpn I(221)
Hind III(223)
Nhe I(261)
Nde I(268)
Xba I(306)
Sty I(59)
Bpu1102 I(82)
Aat II(3233)
Ssp I(3115)
Hinc II(2852)
Sca I(2791)
Pvu I(2681)
Ap (2241-3098)
Bgl I(2431)
BsiF I(2391)
Pfl1108 I(2329)
Eam1105 I(2311)
HgiE II(2004)
AlwN I(1834)
ori (1480)
BspLU11 I(1418)
Afl III(1418)
Sap I(1302)
Bst1107 I(1189)
Acc I(1188)
BsaA I(1170)
Tth111 I(1163)
BsmB I(1058)
Pvu II(1009)
BspG I(944)
BsmF I(689)
Eco47 III(672)
Bsg I(578)
Bpu10 I(524)
Dsa I(390)
Msc I(389)
Bgl I(364)
pET-17b
(3306bp)
BamHI
EcoRI
fdh
NdeI
SD
PT7
BglII
amp
pETFDH
4500 bp
pMB1 ori

Figure 2.2 Construction of pETFDH

2.17.4 Optimization of induction time

The optimum time for induction of FDH production was performed by the procedure as 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 methode as described in 2.16.5.

2.18 Cloning and expression of a heterologous gene of *aladh* and *fdh* using pET17b

2.18.1 Construction of pETAF

For construction of pETAF (Figure 2.3), the whole FDH gene with T7 promoter and Shine-Dargano sequence of pET vector was amplified by the PCR method and inserted downstream of *aladh* gene of pETAlaDH. The primers were designed upon the sequence of pETFDH. The 5'-primer consisted of *Hin*dIII site following by T7 promoter and Shine-Dargano sequence of pET-17b (T7FDHF*Hin*dIII), while the 3'-primer contained *EcoR*I site and the sequence at 3'-end of *fdh* gene (FDHR*Eco*RI).

PCR was performed 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, denaturation at 94^{o}C for 1 minute, annealing at 60^{o}C for 1.30 minute, extension at 72^{o}C for 2 minutes and final extension at 72^{o}C for 10 minutes. The number of cycle was 30 cycles.

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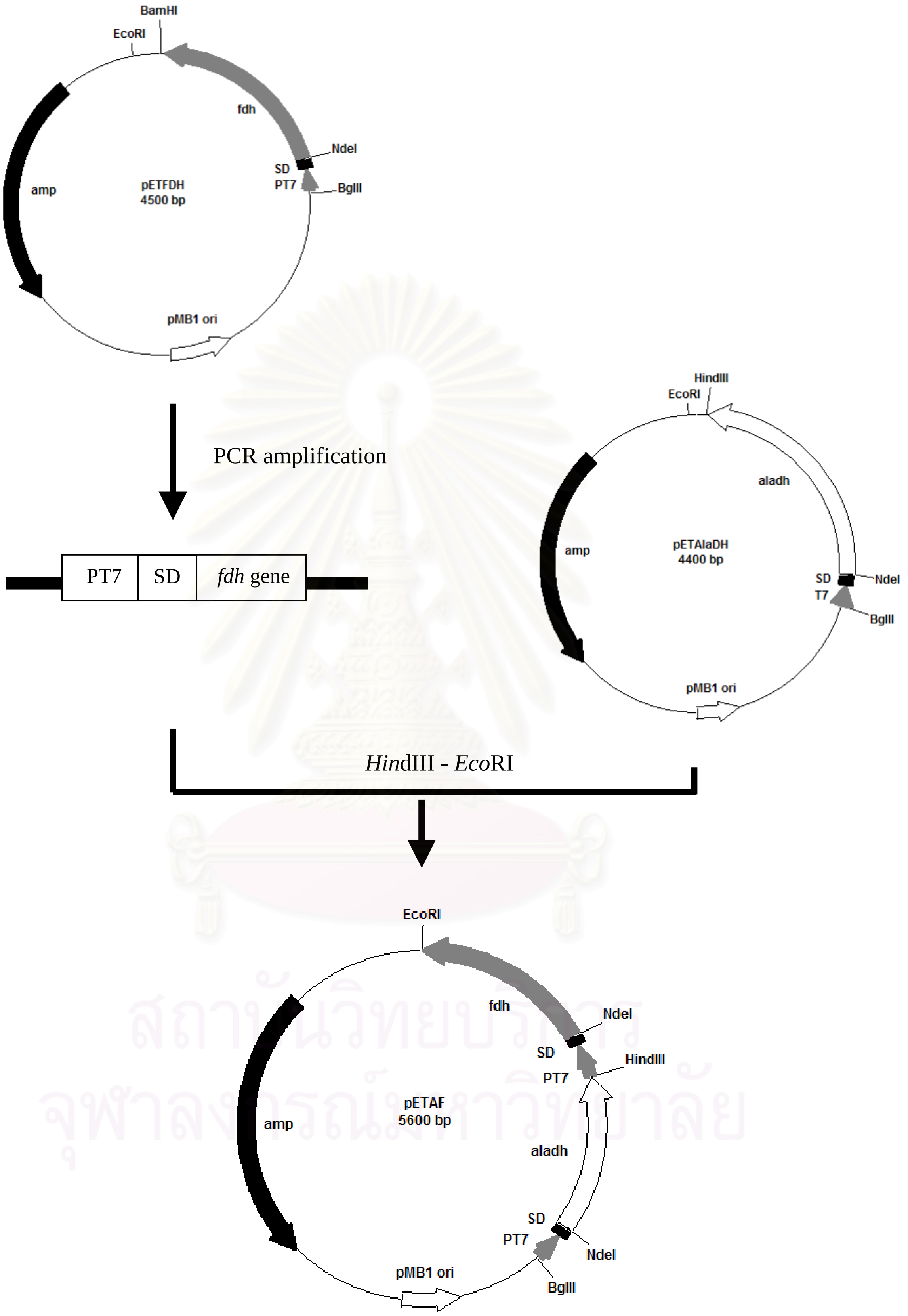

BamHI
EcoRI
fdh
NdeI
SD
PT7
BglII
amp
pETFDH
4500 bp
pMB1 ori
PCR amplification
PT7
SD
fdh gene
HindIII
EcoRI
aladh
amp
pETAlaDH
4400 bp
SD
T7
NdeI
BglII
pMB1 ori
*Hind*III - *Eco*RI
EcoRI
fdh
NdeI
SD
HindIII
PT7
pETAF
5600 bp
amp
aladh
SD
PT7
NdeI
BglII
pMB1 ori

Figure 2.3 Construction of pETAF

The PCR fragment was digested with *Hin*dIII and *Eco*RI and then ligated with *Hin*dIII and *Eco*RI digested pETAlaDH. The ligation products were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown on LB agar plates containing 100 µg/ml ampicillin and were incubated at 37°C for 16 hours. Cells containing the recombinant plasmids, which had *aladh* and *fdh* gene were picked, and the plasmids were isolated and checked by agarose gel electrophoresis. After that, the recombinant plasmid which had *aladh* and *fdh* gene was transformed into *E. coli* BL21(DE3).

2.18.2 Construction of pETFA

The DNA fragment (Figure 2.4), which contained T7 promoter, Shine-Dargano sequence and *aladh* gene was obtained from the digestion of pETAlaDH with *Bgl*II and *Eco*RI. The pETFDH was digested with *Bam*HI and *Eco*RI and then ligated with the *Bgl*II - *Eco*RI fragment of *aladh* gene. The recombinant plasmids were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown in LB agar plates containing 100 µg/ml ampicillin at 37°C for 16 hours. Cells containing the recombinant plasmids, which had *aladh* and *fdh* gene were picked, and the plasmids were isolated and checked by agarose gel electrophoresis. After that, the recombinant plasmid which had *aladh* and *fdh* gene was transformed into *E. coli* BL21(DE3).

The expression of *aladh* and *fdh* in *E. coli* BL21(DE3) containing pETAF or pETFA were performed by the methods described in section 2.16.3, 2.17.3, 2.16.4, 2.17.4, 2.16.5 and 2.17.5.

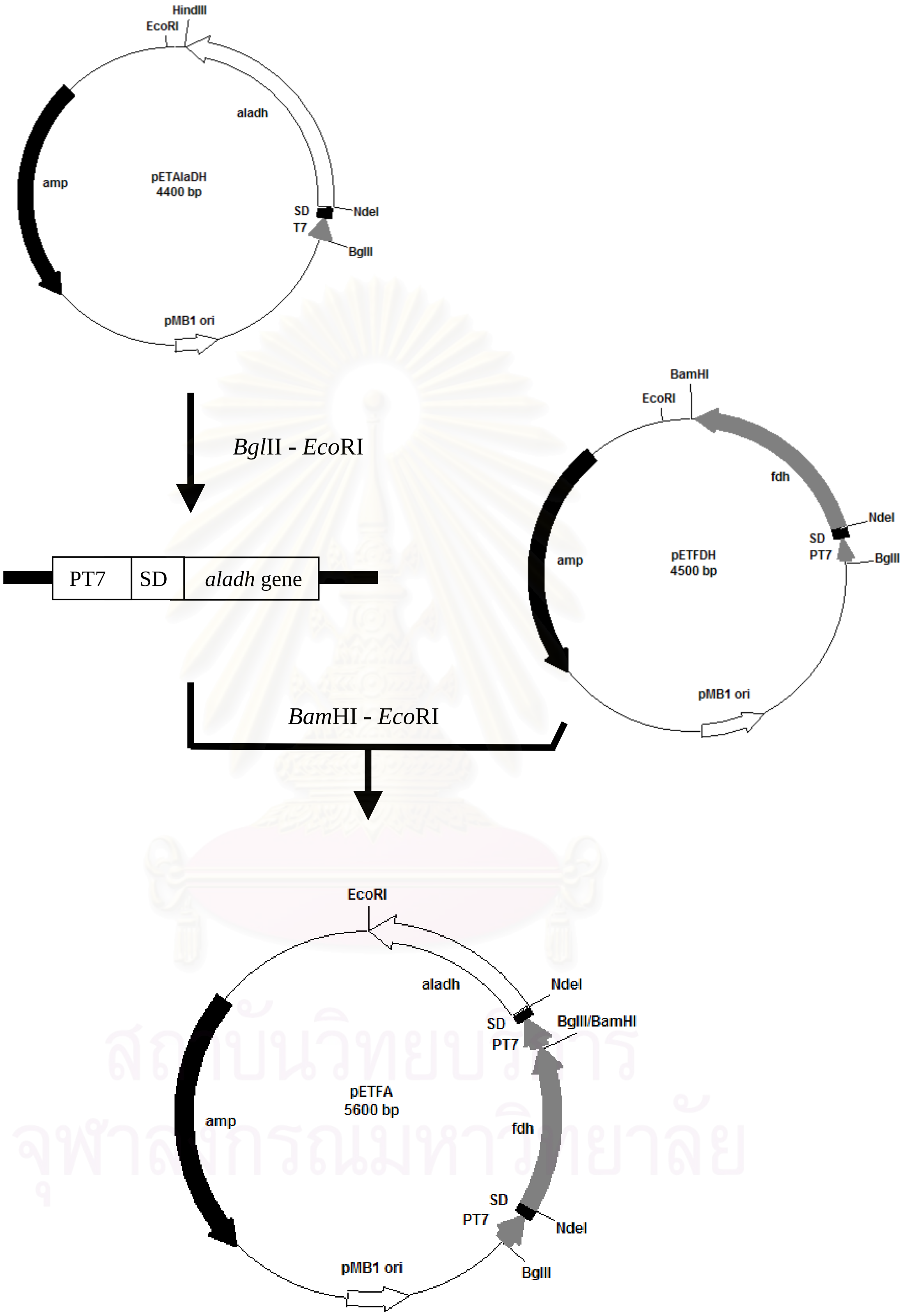

HindIII
EcoRI
aladh
amp
pETAlaDH
4400 bp
SD
T7
NdeI
BglII
pMB1 ori
BglII - EcoRI
PT7
SD
aladh gene
BamHI
EcoRI
fdh
NdeI
SD
PT7
BglII
amp
pETFDH
4500 bp
pMB1 ori
BamHI - EcoRI
EcoRI
aladh
NdeI
SD
BglII/BamHI
PT7
pETFA
5600 bp
amp
fdh
SD
PT7
NdeI
BglII
pMB1 ori

Figure 2.4 Construction of pETFA

2.19 Cloning and expression of *fdh* gene usi6g pSY343 vector

2.19.1 PCR amplification of *fdh* gene

For construction of pSYFDH, the whole *fdh* gene was amplified by the PCR method using pETFDH as a DNA template. The 5'-primer contained *Bam*HI site at its 5'-end and the T7 promoter sequence of pET-17b (T7FDHF*Bam*HI) while the 3'-primer had *Eco*RI site at its 5'-end and the sequence at 3'-end of *fdh* gene (FDHR*Eco*RI).

PCR was performed 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, denaturation at 94^{o}C for 1 minute, annealing at 60^{o}C for 1.30 minute, extension at 72^{o}C for 2 minutes and final extension at 72^{o}C for 10 minutes. The number of cycle was 30 cycles.

2.19.2 Cloning of pSYFDH

To clone pSYFDH (Figure 2.5), the FDH gene PCR product from 2.19.1 was digested by both *Bam*HI and *Eco*RI, and vector pSY343 was digested by *Bam*HI and *Eco*RI. The *Bam*HI–*Eco*RI fragment of *fdh* gene (1.3 kb), which containing T7 promoter and Shine-Dalgarno sequence of expression vector of pET-17b, was ligated to large *Bam*HI-*Eco*RI digested fragment of pSY343. The ligation products were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown in LB agar plates containing 50 µg/ml of kanamycin and were incubated at 37^{o}C for 16 hours. Cells containing the recombinant plasmids, which had *fdh* gene were picked, and the plasmids were isolated and checked by agarose gel electrophoresis. After that, the recombinant plasmid which had *fdh* gene was transformed into *E. coli* BL21(DE3).

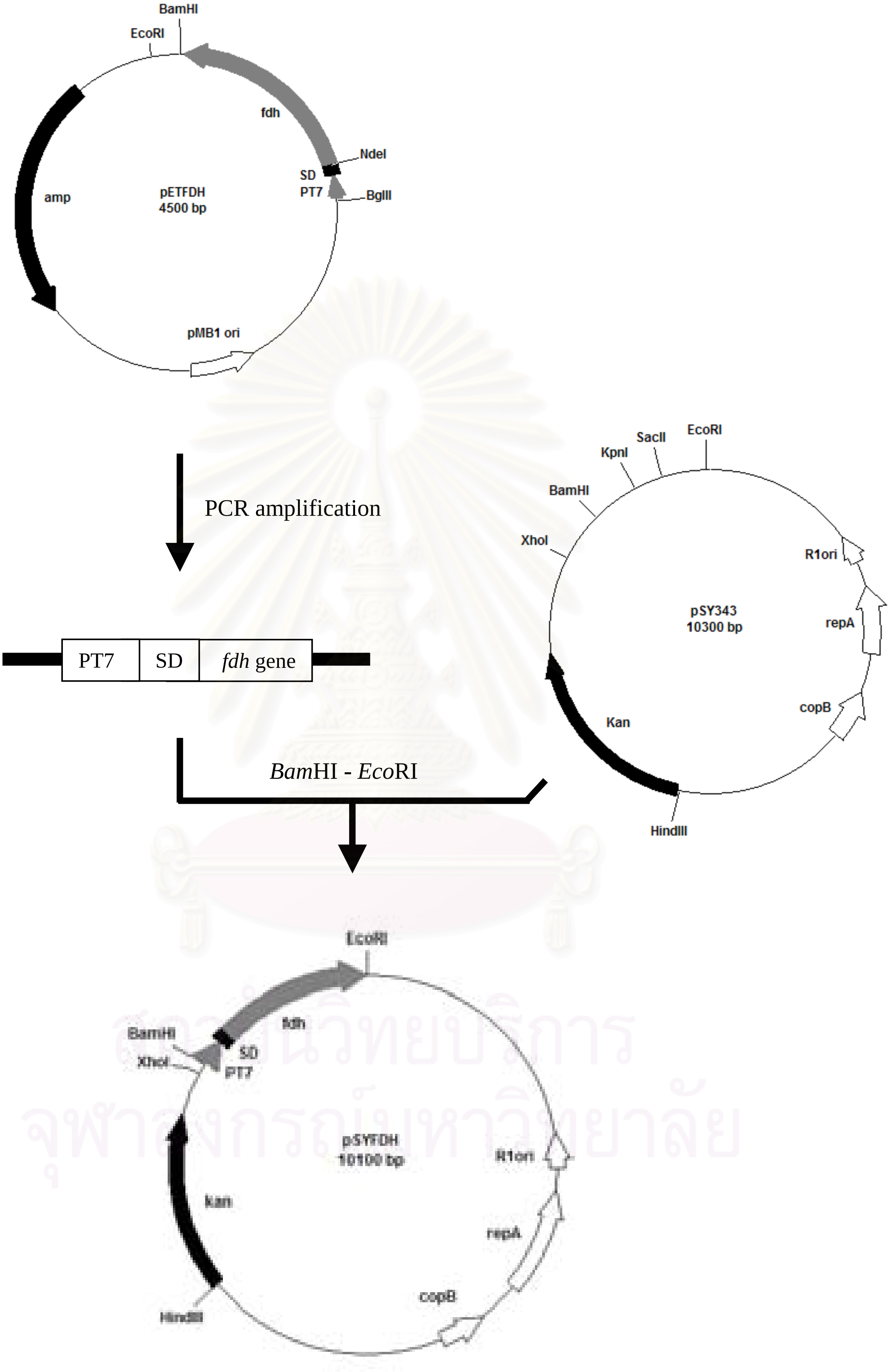

BamHI
EcoRI
fdh
NdeI
SD
PT7
BglII
amp
pETFDH
4500 bp
pMB1 ori
PCR amplification
PT7
SD
fdh gene
*Bam*HI - *Eco*RI
EcoRI
SacII
KpnI
BamHI
XhoI
R1ori
pSY343
10300 bp
repA
copB
Kan
HindIII
EcoRI
fdh
BamHI
XhoI
SD
PT7
pSYFDH
10100 bp
R1ori
kan
repA
copB
HindIII

Figure 2.5 Construction of pSYFDH

2.19.3 Crude extract preparation and enzyme activity assay

E. coli BL21(DE3) cells harboring the recombinant plasmids pSYFDH were grown in 5 ml of LB-medium supplemented with 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 50 μg/ml kanamycin and shaked at 30°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 0.4 mM IPTG was added and the temperature was shifted to 37 °C in order to induce enzyme production before cell was harvested at 4 hours. Finally, crude extracts were prepared and assayed for the enzyme activity and protein determination as described in 2.12, 2.13.2 and 2.14, respectively.

2.19.4 Optimization of induction time

E. coli BL21(DE3) containing pSYFDH was grown in 5 ml of LB-medium supplemented with 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 50 μg/ml kanamycin and shaked at 30°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 0.4 mM IPTG was added and the temperature was shifted to 37°C to induce enzyme production. The cultures were continued for 0, 1, 2, 4, 8, 16, and 24 hours before the cell were harvested. Finally, crude extracts were prepared and assayed for the enzyme activity as described in section 2.12, 2.13.2, and 2.14, respectively. In addition, cells and crude extracts from induction time couse study were subjected to SDS-PAGE as described in 2.16.5.

2.20 Co-transformation of *aladh* and *fdh* using pET-17b and pSY343 vectors

2.20.1 Co-transformation of pETAlaDH/pSYFDH into *E. coli* BL21(DE3)

The pSYFDH from 2.19 was transformed into *E. coli* BL21(DE3) containing pETAlaDH by electroporation (Figure 2.6). The transformed cells were grown on LB agar plates containing 100 μg/ml amplicillin and 50 μg/ml kanamycin at 37°C for 16 hours. Cells containing both pETAlaDH/pSYFDH were screened by colony PCR. The co-transformant was grown in 5 ml of LB-medium supplemented with 100 μg/ml amplicillin and 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml amplicillin and 50 μg/ml kanamycin, shaked at 30°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 0.4 mM IPTG was added and the temperature was shifted to 37°C to induce enzyme production, and the cultivation was continued at 37°C for 4 hour before cell harvesting. Finally, crude extracts were prepared and assayed for the enzyme activity of AlaDH and FDH and protein as described in section 2.12, 2.13 and 2.14, respectively.

2.20.2 Optimization of induction time for co-transformant of pETAlaDH/pSYFDH

The co-transformant was grown in 5 ml of LB-medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and shaked at 30 °C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 0.4 mM IPTG was added and the temperature was shifted to 37 °C to induce enzyme production, and the cultivation was continued at 37°C for various times: 0, 1, 2, 4, 8, 16 and 24 hours before cell harvesting. Finally, crude extracts were

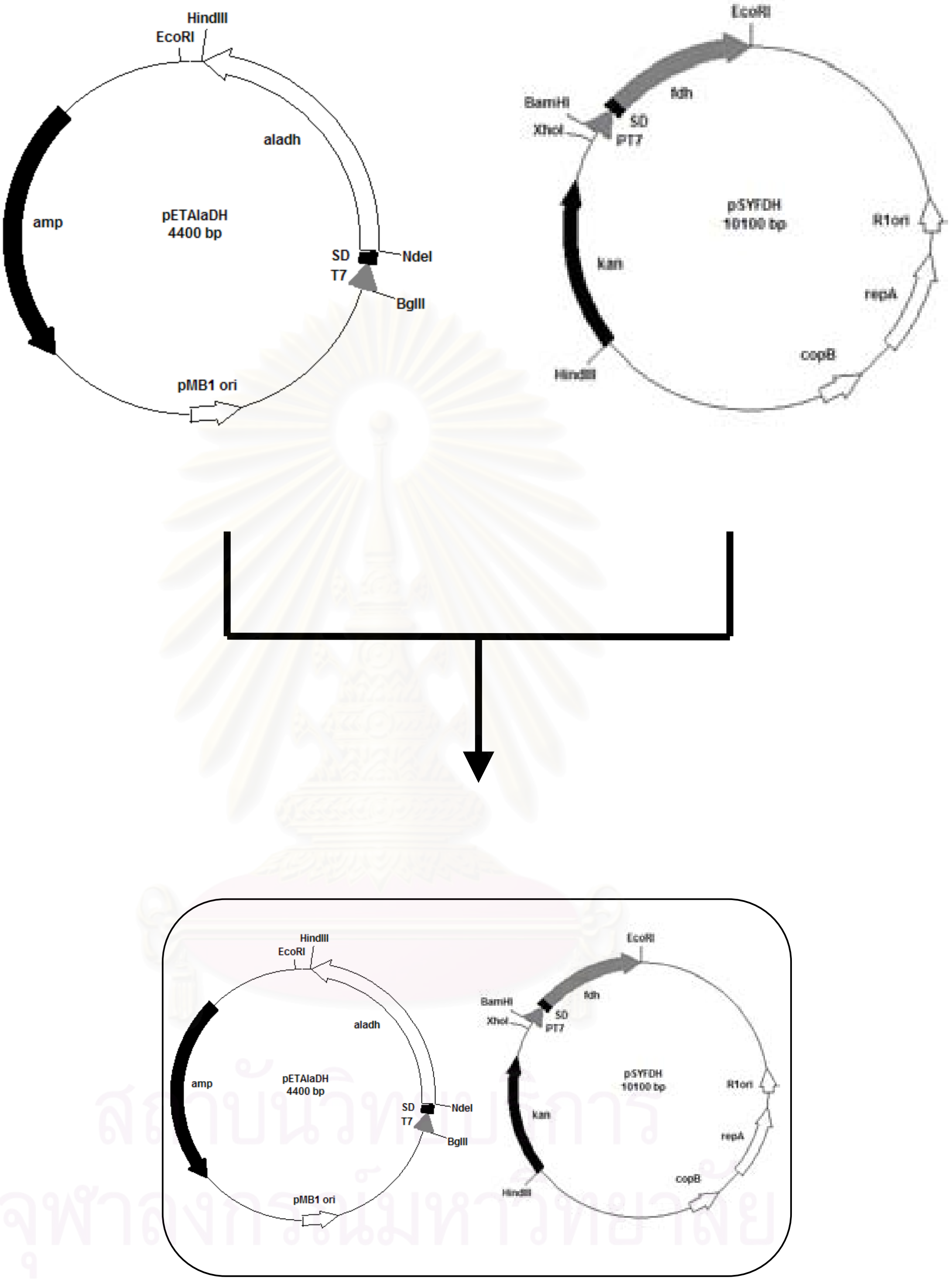

HindIII
EcoRI
aladh
amp
pETAlaDH
4400 bp
SD
T7
NdeI
BglII
pMB1 ori
EcoRI
BamHI
XhoI
fdh
SD
PT7
pSYFDH
10100 bp
R1ori
kan
repA
copB
HindIII
HindIII
EcoRI
aladh
amp
pETAlaDH
4400 bp
SD
T7
NdeI
BglII
pMB1 ori
EcoRI
BamHI
XhoI
fdh
SD
PT7
pSYFDH
10100 bp
R1ori
kan
repA
copB
HindIII

Figure 2.6 **Co-existence of pETAlaDH/pSYFDH in *E. coli* BL21(DE3)**

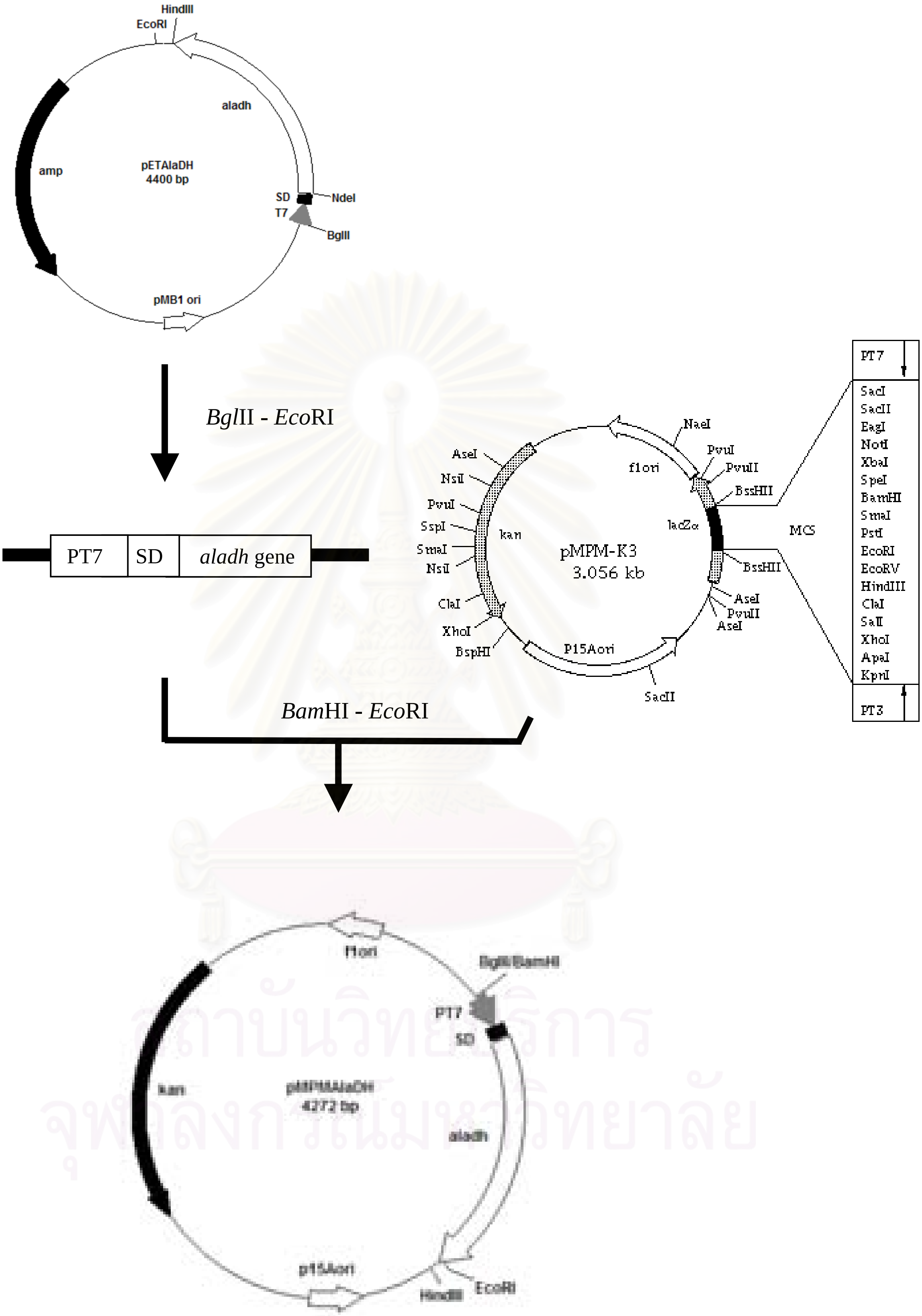
prepared and assayed for the enzyme activity and protein as described in section 2.12, 2.13 and 2.14, respectively. Cells and crude extracts from induction time couse study were subjected to SDS-PAGE as described in section 2.16.5.

2.21 Cloning and expression of *aladh* gene using pMPM-K3 vector

2.21.1 Cloning of pMPMAlaDH

The DNA fragment containing T7 promoter as well as Shine-Dalgarno sequence of pET-17b and the whole *aladh* gene, was obtained from the digestion of pETAlaDH with *Bgl*II and *Eco*RI. After that the *Bgl*II-*Eco*RI fragment (1.2 kb) was ligated to the *Bam*HI-*Eco*RI sites of pMPM-K3 vector (Figure 2.7). The ligation products were transformed into *E. coli* JM109 by electroporation. The transformed cells were grown on LB agar plates containing 50 μg/ml kanamycin at 37^{o}C for 16 hours. Cells containing the recombinant plasmids were picked, and the plasmids were isolated and checked by agarose gel electrophoresis. After that, the recombinant plasmid which had *aladh* gene was transformed into *E. coli* BL21(DE3).

E. coli BL21(DE3) cells harboring the recombinant plasmids pMPMAlaDH were grown in 5 ml of LB-medium supplemented with 50 μg/ml kanamycin at 37^{o}C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 50 μg/ml kanamycin and shaked at 37^{o}C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, or 0.4 mM IPTG was added to induce enzyme production, and the cultivation was continued at 37^{o}C for 4 hour before cell harvesting. Finally, crude extracts were prepared and assayed for the enzyme activity and protein of AlaDH a described in section 2.12, 2.13.1 and 2.14, respectively.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EcoRI
aladh
amp
pETAlaDH
4400 bp
SD
NdeI
T7
BglII
pMB1 ori
BglII - EcoRI
PT7
SD
aladh gene
NaeI
PvuI
PvuII
BssHII
AseI
NsiI
PvuI
SspI
SmaI
NsiI
ClaI
XhoI
BspHI
f1ori
kan
lacZα
pMPM-K3
3.056 kb
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AseI
PvuII
AseI
P15Aori
SacII
MCS
PT7
SacI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
KpnI
PT3
BamHI - EcoRI
f1ori
BglII/BamHI
PT7
SD
kan
pMPMAlaDH
4272 bp
aladh
p15Aori
HindIII
EcoRI

Figure 2.7 Construction of pMPMAlaDH

2.21.2 Optimization of iduction time

E. coli BL21(DE3) containing pMPMAlaDH, was grown in 5 ml of LB-medium supplemented with 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 50 μg/ml kanamycin and shaked at 37°C, 250 rpm. The optimum induction time was carried out by the procedure as described in section 2.16.4 and 2.16.5.

2.22 Co-transformation of *aladh* and *fdh* using pET-17b and pMPM-K3 vectors

2.22.1 Co-transformation of pMPMAlaDH/pETFDH into *E. coli* BL21(DE3)

The pETFDH from 2.17 were transformed into *E. coli* BL21(DE3) containing pMPMAlaDH by electroporation (Figure 2.8). The transformed cells were grown on LB agar plates containing 100 μg/ml amplicillin and 50 μg/ml kanamycin at 37°C for 16 hours. Cells containing both pMPMAlaDH/pETFDH were screened by colony PCR. The co-transformant was grown in 5 ml of LB-medium supplemented with 100 μg/ml amplicillin and 50 μg/ml kanamycin at 37°C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml amplicillin and 50 μg/ml kanamycin, shaked at 37°C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 0.4 mM IPTG was added to induce enzyme production, and the cultivation was continued at 37°C for 4 hour before cell harvesting. Finally, crude extracts were prepared and assayed for the enzyme activity of AlaDH and FDH and protein as described in section 2.12, 2.13 and 2.14, respectively.

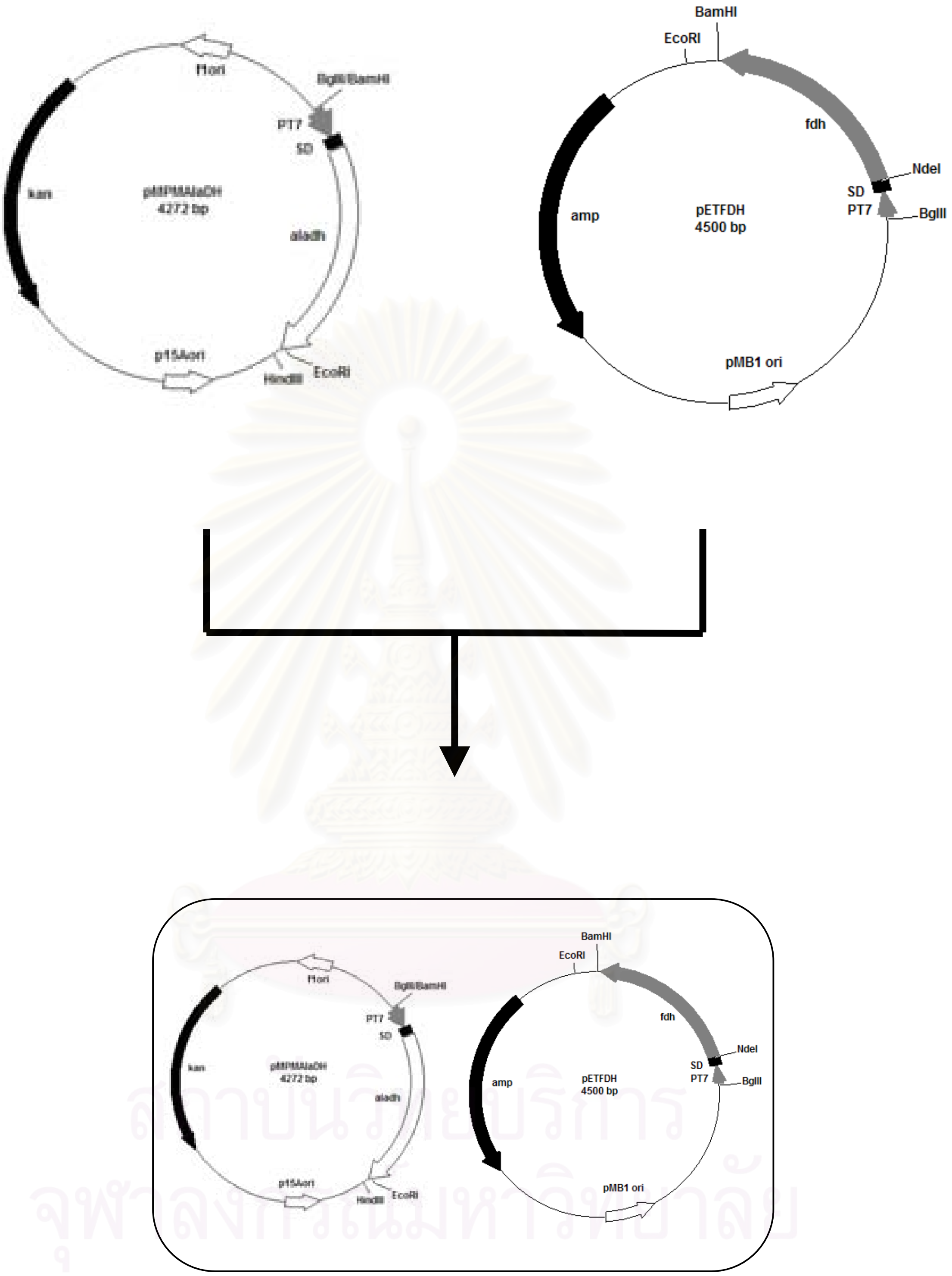

f1ori
BglII/BamHI
PT7
SD
kan
pMPMAlaDH
4272 bp
aladh
p15Aori
HindIII
EcoRI
BamHI
EcoRI
fdh
NdeI
SD
PT7
BglII
amp
pETFDH
4500 bp
pMB1 ori

Figure 2.8 Co-existence of pMPMAlaDH/pETFDH in *E. coli* BL21(DE3)

2.22.2 Optimization of induction time for co-transformant of pMPMAlaDH/pETFDH

The co-transformant was grown in 5 ml of LB-medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37^{o}C overnight. After that, 1% of the cell culture was inoculated into each 200 ml LB-medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and shaked at 30 ^{o}C, 250 rpm. The optimum induction time was carried out by the procedure as described in section 2.16.4 and 2.16.5.

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

2.23 Production of alanine by recombinant clones

2.23.1 Production of alanine

The *E. coli* BL21(DE3) containing pETAlaDH, pETAF, pETFA, pMPMALaDH, or pMPMAlaDH/pETFDH were cultured at their optimum condition for the enzyme induction. Cells were harvested by centrifugation at 8,000 xg for 20 minutes. The cell pellets were washed twice with cold 0.85% NaCl and centrifuged at 8,000 xg for 15 minutes. After that, the cell pellet was washed once with cold 10 mM phosphate buffer pH 7.4 and centrifuged again. The two ml of cell suspension of each clone containing 0.1 g wet weigh of washed cell, 0.5 M ammonium formate (pH 7.5) and 0.3 M pyruvate was made. The reactions were performed at 37^{o}C, 250 rpm with reciprocal shaking for 12 hours. The supernatants were separated from the cells for analysis of alanine production by TLC technique.

Table 2.2 **Summary of all recombinant clones engineered in this study**

Recombinant plasmid	**Vector (kb)**	**Insert gene (kb)**	**Size (kb)**	**Antibiotic drug**
pETAlaDH	pET-17b	*aladh*	4.4	ampicillin
pETFDH	pET-17b	*fdh*	4.5	ampicillin
pETAF	pET-17b	*aladh* and *fdh*	5.6	ampicillin
pETFA	pET-17b	*aladh* and *fdh*	5.6	ampicillin
pSYFDH	pSY343	*fdh*	10.1	kanamycin
pETAlaDH/pSYFDH	pETAlaDH and pSYFDH	*aladh* and *fdh*	4.4, 10.1	ampicillin and kanamycin
pMPMAlaDH	pMPM-K3	*aladh*	4.3	kanamycin
pMPMAlaDH/pETFDH	pMPMAlaDH and pETFDH	*aladh* and *fdh*	4.3, 4.5	ampicillin and kanamycin

2.23.2 Analysis of alanine by TLC

The supernatant were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey' s reagent) according to Marfey's methods (cite in Nagata, *et al.*, 2001). One hundred μg of standard D- or L- alanine in 20 μl of H_2O or sample 20 μl and 8 μl of 1 M $NaHCO_3$ was mixed with 400 μg FDAA in 40 μl acetone and incubated at 40^oC for 1 hours 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 pre-coated TLC plate (RP-18 F_{254S}, 5 cm x 10 cm) and developed with acetonitrile:50 mM triethylamine-phosphate buffer, pH 5.0 at ratio 35:65 in a pre-equilibrated glass chamber at 25 ^oC. The FDAA amino acid spots were yellow. When the ascending solvent front nearly reached the top margin, the plate was removed from the chamber and dried with a hair-drier. For quantitative analysis, yellow spots were scraped off the plate after the chromatography, and extracted with methanol:H_2O (1:1). The absorbance of the extracts was measured at 340 nm with a 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 *aladh* gene using pET-17b vector

3.1.1 PCR amplification of *aladh* gene

To overexpress *aladh* gene in *E. coli* BL21(DE3) with the assistance of T7 promoter of plasmid pET-17b, the 1.1 kb of *aladh* gene fragment was amplified from a pUC18 containing *aladh* gene (pUCAlaDH), using primers AlaDHF*Nde*I and AlaDHR*Hin*dIII as described in section 2.16.1. The 5'-primer (AlaDHF*Nde*I) containing *Nde*I restriction site and 5'-end of *aladh* gene sequence was designed. The 3'-primer contained 3'-end of *aladh* gene, the TGA translational termination signal followed by the restriction site for *Hin*dIII. Figure 3.1 shows the 1,116 bp PCR product of the *aladh* gene amplified by various annealing temperatures. The optimum annealing temperatures which gave strong specific PCR product were broad, ranging from 55.1- 62.4^{o}C, while the others gave the same product with lower band intensity and had non-specific bands. The PCR product was purified by agarose gel electrophoresis before using for further cloning.

3.1.2 Cloning of *aladh* gene

As described in section 2.16.2, the 1.1 kb PCR product of *aladh* gene was digested with *Nde*I and *Hin*dIII. The *Nde*I-*Hin*dIII fragment of *aladh* gene was ligated to the *Nde*I-*Hin*dIII site of 3.3 kb pET-17b vector, and then transformed into *E. coli* JM109 by electroporation. The clones were randomly picked for plasmid extraction and digestion with *Nde*I - *Hin*dIII. The recombinant plasmids gave two strong bands, relaxed

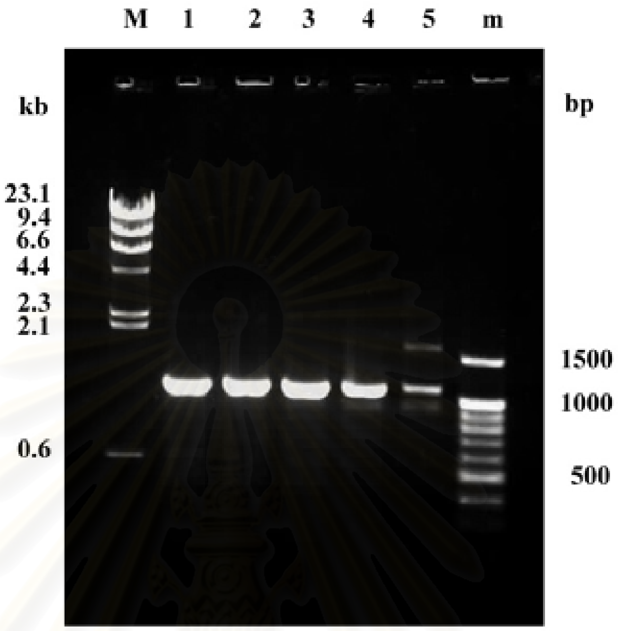


Figure 3.1 **PCR product of the *aladh* gene amplified by various annealing temperatures**

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

Lane 1 = amplified product at annealing temperature 55.1 °C

Lane 2 = amplified product at annealing temperature 59.9 °C

Lane 3 = amplified product at annealing temperature 62.4 °C

Lane 4 = amplified product at annealing temperature 64.6 °C

Lane 5 = amplified product at annealing temperature 66.5 °C

Lane m = 100 bp DNA ladder

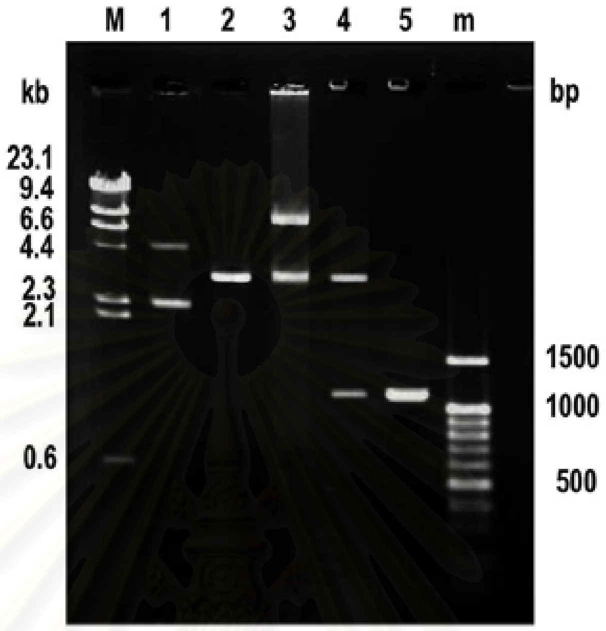
and supercoiled bands on agarose gel electrophoresis. After digestion with *Nde*I and *Hin*dIII, a linear pET-17b and a 1.1 kb of inserted *aladh* gene fragment (lane 4) which gave the same mobility with PCR product (lane 5) were obtained as shown in Figure 3.2.

3.1.3 Enzyme activity of *E. coli* BL21(DE3) harboring pETAlaDH

Ten recombinant plasmids which had inserted *aladh* gene were transformed into *E. coli* BL21(DE3) and the clones were assayed for AlaDH activity as described in section 2.16.3. The transformants showed various levels of the enzyme total activity from 835.66 - 1881.64 U and specific activity of 16.31 - 32.71 U/mg protein as shown in Table 3.1. Transformant plasmid No. 8 which had the highest total activity and specific activity of 1881.64 U and 32.71 U/mg protein, respectively, was chosen for the next experiment.

3.1.4 Optimization of induction time

For induction time course study, the transformant No. 8 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 indicated that cell wet weight increased rapidly in the first 4 hours. After that cell wet weight increased slowly untill 16 hours, then slowly decreased. The highest AlaDH total activity and specific activity were 1831.82 U and 17.76 U/mg protein, respectively, when the cells were induced for 4 hours and after that the AlaDH activity and specific activity were decreased.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m
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4.4
2.3
2.1
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bp
1500
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500

Figure 3.2 Restriction pattern of pETAlaDH

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

Lane 1 = undigested pET-17b

Lane 2 = *Nde*I-*Hin*dIII digested pET-17b

Lane 3 = undigested pETAlaDH

Lane 4 = *Nde*I-*Hin*dIII digested pETAlaDH

Lane 5 = amplified product of *aladh* gene

Lane m = 100 bp DNA ladder

Table 3.1 AlaDH activity from crude extract of pETAlaDH clones

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCAlaDH in *E. coli* JM109	0.75	300.00	44.16	6.79
1	0.74	1002.71	55.14	18.18
2	0.82	835.66	51.21	16.31
3	0.66	1047.65	54.68	19.16
4	0.76	1840.15	66.42	27.70
5	0.79	1493.30	51.80	28.83
6	0.81	1564.44	67.33	23.24
7	0.76	1138.70	63.45	17.95
8	0.80	1881.64	57.52	32.71
9	0.91	1769.81	54.71	32.35
10	0.86	1564.44	56.82	27.53

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

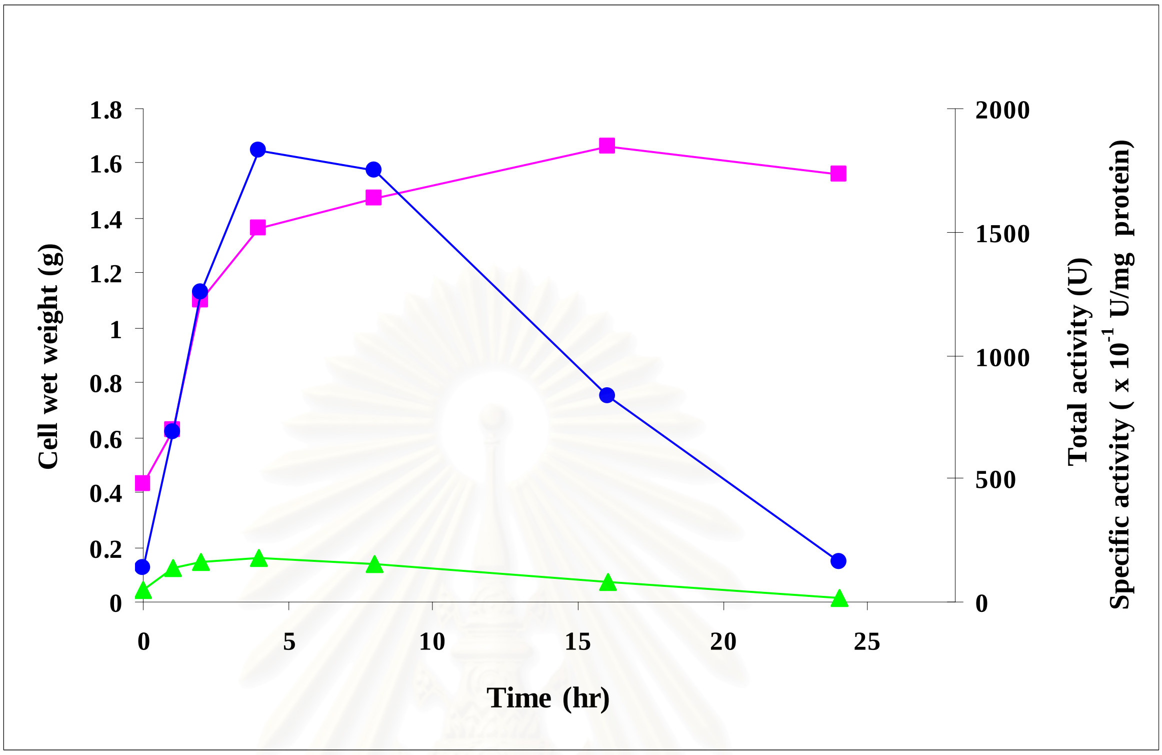

Figure 3.3 **Effect of induction time on AlaDH production and growth of pETAlaDH clone.**

🟪 Cell wet weight ● Total activity ▲ Specific activity

3.1.5 Protein patterns of cells and crude extracts

The 1.5 ml of the culture of transformant No.8 was harvested and then centrifuged in eppendorf tube at various times after induction. The cell pellets were resuspended in 100 µl of 5x sample buffer except 50 µl for 0 hour sample. The cell samples 7 µl or 25 µg protein of crude extract was run on 10% gel SDS-PAGE. The major protein band from cell (Figure 3.4) and crude extract (Figure 3.5) of each induction time corresponded to the level of enzyme activity from crude extract. The intensity of major protein band from cells at 16 hours after induction was higher than that of 8 hour because number of cell per volume of cell culture increased though the enzyme activity decreased.

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

3.2.1 PCR amplification of *fdh* gene

As shown in figure 3.6 lane 1, the 1.2 kb of *fdh* gene fragment was amplified from a pUC119 containing *fdh* gene (pUCFDH), using primers FDHF*Nde*I and FDHR*Bam*HI as described in section 2.17.1. The 5'-primer (FDHF*Nde*I) containing *Nde*I restriction site and 5'-end of *fdh* gene sequence was designed. The 3'-primer (FDHR*Bam*HI) contained 3'-end of *fdh* gene, the TGA translational termination signal followed by the restriction site for *Bam*HI. The optimum annealing temperature for this PCR condition was 60 ^{o}C.

3.2.2 Cloning of pETFDH

As described in 2.17.2, the 1.2 kb PCR product of *fdh* gene was digested with *Nde*I and *Bam*HI. The *Nde*I - *Bam*HI fragment of *fdh* gene was ligated to *Nde*I and

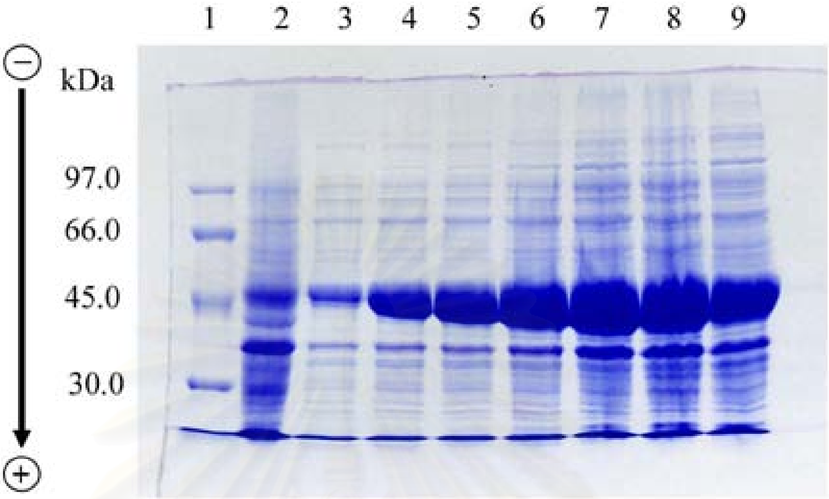

Figure 3.4 Protein pattern of cell harboring pETAlaDH clone at various induction times detected by SDS-PAGE

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

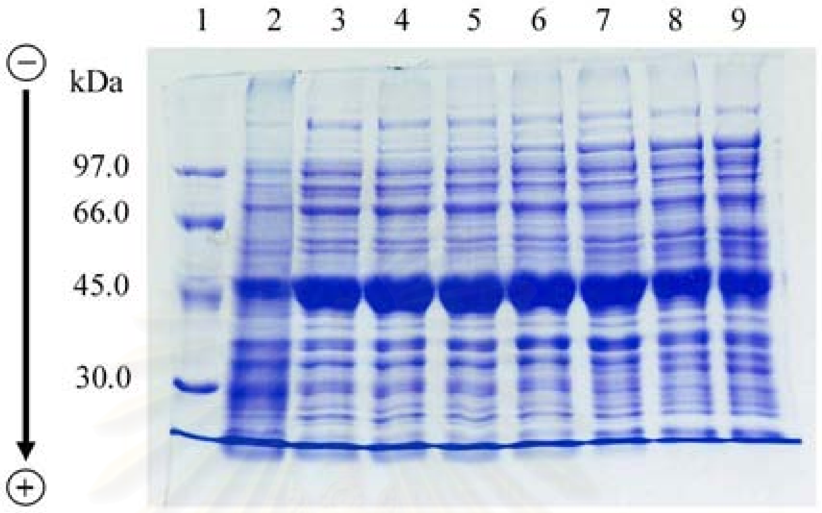

Figure 3.5 Protein pattern of crude extract of pETAlaDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

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

Lane 3 = crude extract of transformant at t_0

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

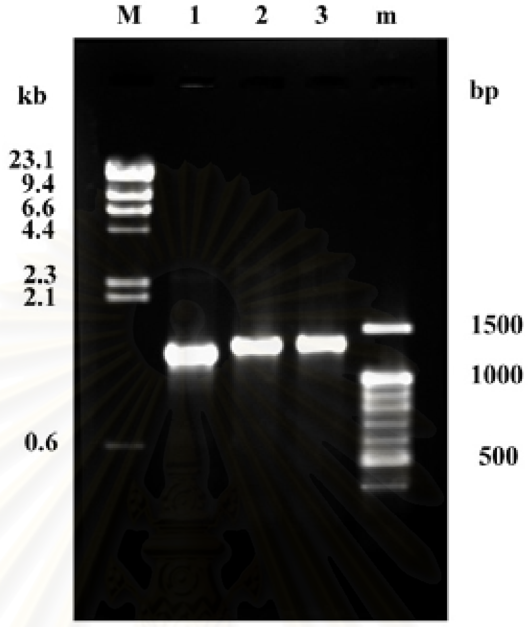
M
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Figure 3.6 PCR production of the *fdh* gene using various primers

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

Lane 1 = PCR product using primers FDHF*Nde*I and FDHR*Bam*HI

Lane 2 = PCR product using primers T7FDHF*Hin*dIII and FDHR*Eco*RI

Lane 3 = PCR product using primers T7FDHF*Bam*HI and FDHR*Eco*RI

Lane m = 100 bp DNA ladder

*Bam*HI site of 3.3 kb pET-17b vector, and then transformed into *E. coli* JM109 by electroporation. The clones were randomly picked for plasmid extraction and digestion with *Nde*I - *Bam*HI. The recombinant plasmids gave three strong bands, relaxed, linear and supercoil bands on agarose gel electrophoresis. After digestion with *Nde*I and *Bam*HI, a linear of 3.3 kb pET-17b and a 1.2 kb of inserted *fdh* gene fragment (lane 4), which was corresponded with size of PCR product (lane 5) were detected as shown in Figure 3.7.

3.2.3 Enzyme activity of *E. coli* BL21(DE3) harboring pETFDH

Ten of recombinant plasmid which had inserted *fdh* gene, were transformed into *E. coli* BL21(DE3) and assayed for FDH activity as described in 2.12 and 2.13.2. The transformants showed total activities varied from 44.80 - 142.18 U and specific activity 0.92 - 2.61 U/mg protein as shown in Table 3.2. The transformant plasmid No. 8 which showed the highest total activity and specific activity about 142.18 U and 2.61 U/mg protein, respectively was chosen for the next experiment.

3.2.4 Optimization of induction time

For induction time course study, the transformant No. 8 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 3.8 indicated that cell wet weight increased rapidly in the first 2 hours. After that cell wet weight increased slowly until 16 hours, then slowly decreased. The highest FDH total activity and specific activity were 150.60 U and 2.85 U/mg protein, respectively,

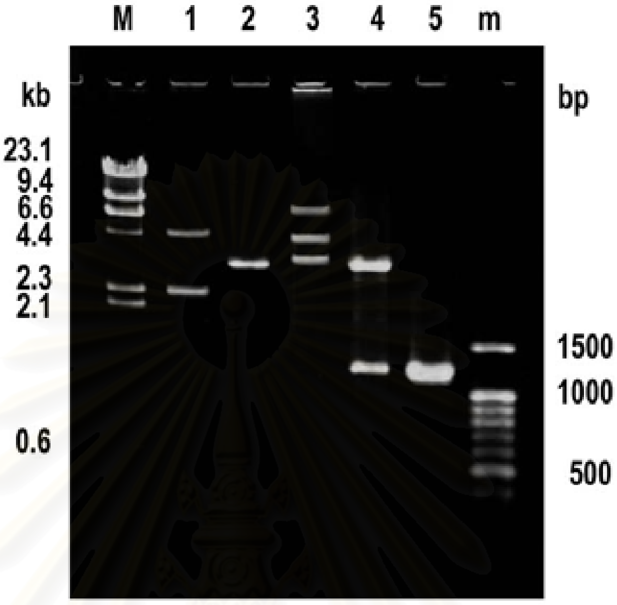
M
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Figure 3.7 **Restriction pattern of pETFDH**

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* gene

Lane m = 100 bp DNA ladder

Table 3.2 FDH activity from crude extract of pETFDH clones

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCFDH in *E. coli* JM109	0.76	20.00	48.95	0.41
1	0.91	89.64	57.32	1.56
2	0.73	59.78	47.26	1.26
3	0.67	44.80	48.80	0.92
4	0.84	90.78	50.45	1.80
5	0.82	122.30	67.33	1.82
6	0.79	111.12	60.12	1.84
7	0.84	78.46	58.32	1.35
8	0.79	142.18	54.56	2.61
9	0.89	100.79	51.16	1.97
10	0.81	95.46	56.73	1.63

[a] Total activity from 200 ml culture as described in section 2.17.3.

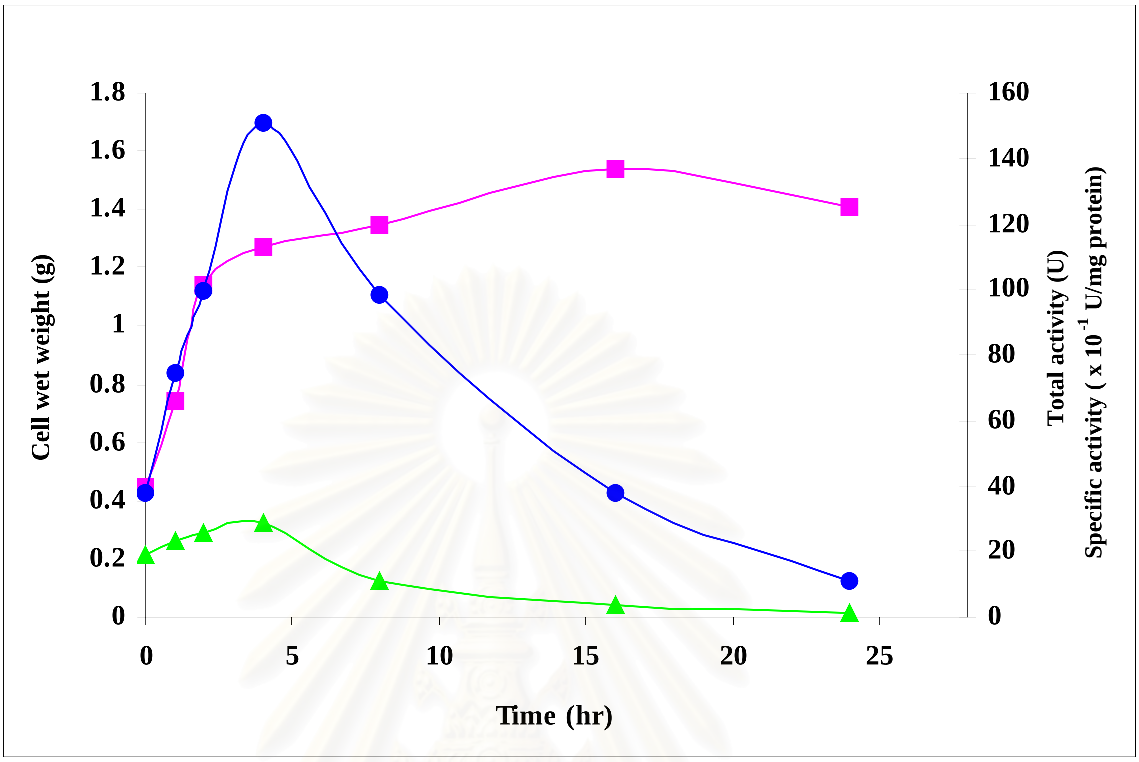

Figure 3.8 Effect of induction time on FDH production and growth of pETFDH clone

■ Cell wet weight ● Total activity ▲ Specific activity

when the cells were induced for 4 hours and after that the FDH activity and specific activity were decreased.

3.2.5 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of FDH activity were subjected to SDS-PAGE. The major protein band from cell (Figure 3.9) and crude extract (Figure 3.10) of pETFDH of each induction time corresponded to the level of enzyme activity from crude extracts. The intensity of major protein bands, from cells at 24 hours after induction was higher than that of 16 hours because number of cell per volume of cell culture increased though the enzyme activity decreased.

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

3.3.1 PCR amplification of *fdh* gene

As shown in Figure 3.6 lane 2, the 1.3 kb of *fdh* gene fragment was amplified from pETFDH using primers T7FDHF*Hin*dIII and FDHR*Eco*RI as described in 2.18.1. The 5'-terminus of *fdh* gene fragment containing *Hin*dIII restriction site, T7 promoter and Shine-Dalgarno sequence of expression vector of pET series. The 3'-terminus contained 3'-end of *fdh* gene, the TGA translational termination signal followed by the restriction site for *Eco*RI. The optimum annealing temperature for this PCR condition was 60 ^{o}C.

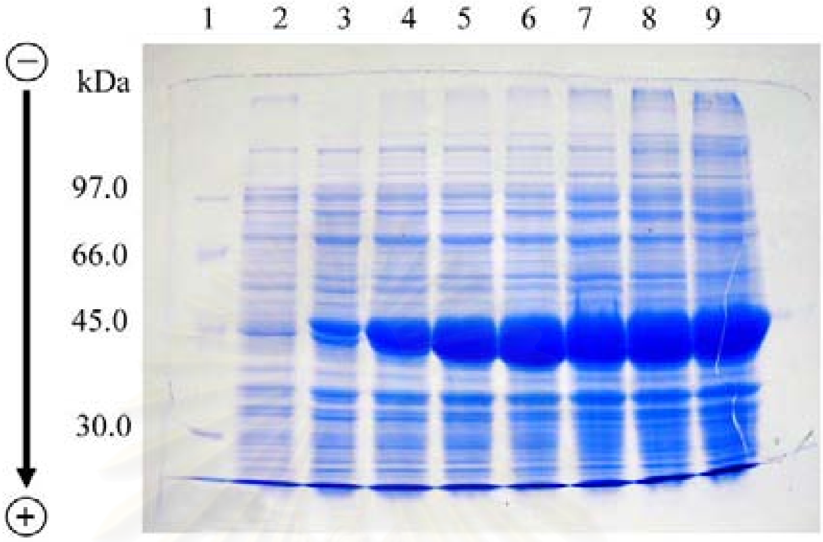

Figure 3.9 Protein pattern of cell harboring pETFDH clone at various induction times detected by SDS-PAGE

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

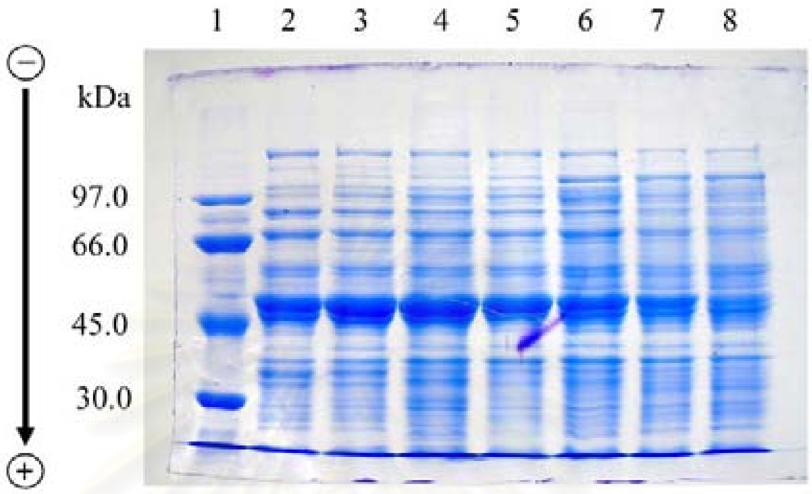

Figure 3.10 Protein pattern of crude extract of pETFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant at t_0

Lane 3-8 = crude extracts of transformant at various induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

3.3.2 Cloning of pETAF

As described in 3.3.1, the 1.3 kb PCR product of *fdh* gene, which containing T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b, was digested with *Hin*dIII and *Eco*RI. The *Hin*dIII-*Eco*RI fragment of *fdh* gene was ligated to the *Hin*dIII-*Eco*RI site of 4.4 kb pETAlaDH vector. As shown in Figure 3.11, the recombinant clone was confirmed by *Hin*dIII and *Eco*RI digestion. The digestion product showed two DNA bands, upper band was a 4.4 kb of linear pETAlaDH and the other was a 1.3 kb of *fdh* gene which contained T7 promoter sequence and Shine-Dalgarno sequence (lane 4). When digested recombinant plasmid with *Nde*I-*Eco*RI, three DNA fragments were appeared: a 3.3 kb of linear pET-17b vector, a 1.2 kb of *fdh* gene, and a 1.1 kb of *aladh* gene, respectively (lane 5).

3.3.3 Enzyme activities of *E. coli* BL21(DE3) harboring pETAF

Ten of recombinant clones were also grown for enzyme assay of AlaDH and FDH activity as described in 2.12 and 2.13. The transformant clones showed various levels of the enzyme total activity and specific activity of AlaDH from 751.13-1802.69 U and 14.37-30.74 U/mg protein as well as FDH total activity and specific activity from 28.41-60.02 U and 0.51-1.04 U/mg protein, respectively, as shown in Table 3.3. The transformant plasmid No. 4 that had the highest total activities and specific activities of both AlaDH and FDH: 1802.69 U, 30.74 U/mg protein, 58.71 U, 1.00 U/mg protein, respectively was chosen for the next experiment.

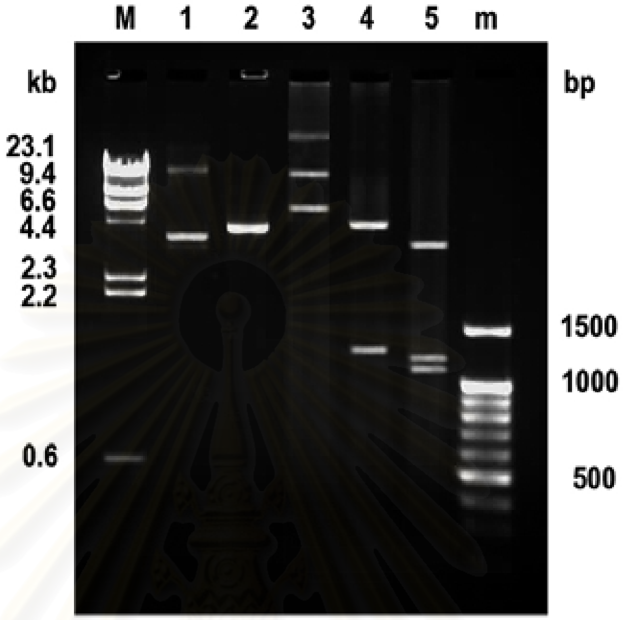
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Figure 3.11 Restriction pattern of pETAF

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

Lane 1 = undigested pETAlaDH

Lane 2 = *Hin*dIII-*Eco*RI digested pEAlaDH

Lane 3 = undigested pETAF

Lane 4 = *Hin*dIII-*Eco*RI digested pETAF

Lane 5 = *Nde*I- *Eco*RI digested pETAF

Lane m = 100 bp DNA ladder

Table 3.3 AlaDH and FDH activities from crude extract of pETAF clones

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		AlaDH	FDH		AlaDH	FDH
pETAlaDH	0.80	1800.00	-	55.00	32.72	-
pETFDH	0.90	-	140.00	44.00	-	3.18
1	0.70	1024.21	42.73	49.42	20.72	0.86
2	0.84	751.13	45.51	50.10	14.99	0.91
3	0.87	938.70	39.32	65.32	14.37	0.60
4	0.89	1802.69	58.71	58.65	30.74	1.00
5	0.80	1422.20	60.02	57.58	24.70	1.04
6	0.76	1365.34	39.28	52.04	26.24	0.75
7	0.94	853.34	28.41	55.19	15.46	0.51
8	0.90	1165.78	36.45	55.37	21.05	0.66
9	0.87	1732.64	51.23	62.71	27.63	0.82
10	0.91	1516.17	48.56	64.28	23.59	0.76

[a] Total activity from 200 ml culture as described in section 2.16.3 and 2.17.3.

3.3.4 Optimization of induction time

For induction time course study, the 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 shown in Figure 3.12, the result indicated that cell wet weight increased untill 16 hours after that their slowly decreased. When induction time 8 hours were used, the highest total activity and specific activity of AlaDH and FDH were obtained at 1795.89 U, 15.19 U/mg protein, 32.50 U, 0.28 U/mg protein, respectively, and after 8 hours the total activity and specific activity of AlaDH and FDH were decreased.

3.3.5 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of AlaDH and FDH activity were subjected to SDS-PAGE. The protein bands from cell (Figure 3.13) and crude extract (Figure 3.14) of pETAF of each induction time was corresponded with the level of enzyme activity from crude extracts. The result showed that the major protein bands of cells and crude extracts from pETAF had two adjacent bands, the upper band was FDH and the other was AlaDH since FDH (44 kDa) had a size larger than AlaDH (40 kDa).

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

3.4.1 Construction of pETFA

The 1.1 kb fragment of *aladh* gene which contained T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b was obtained from the

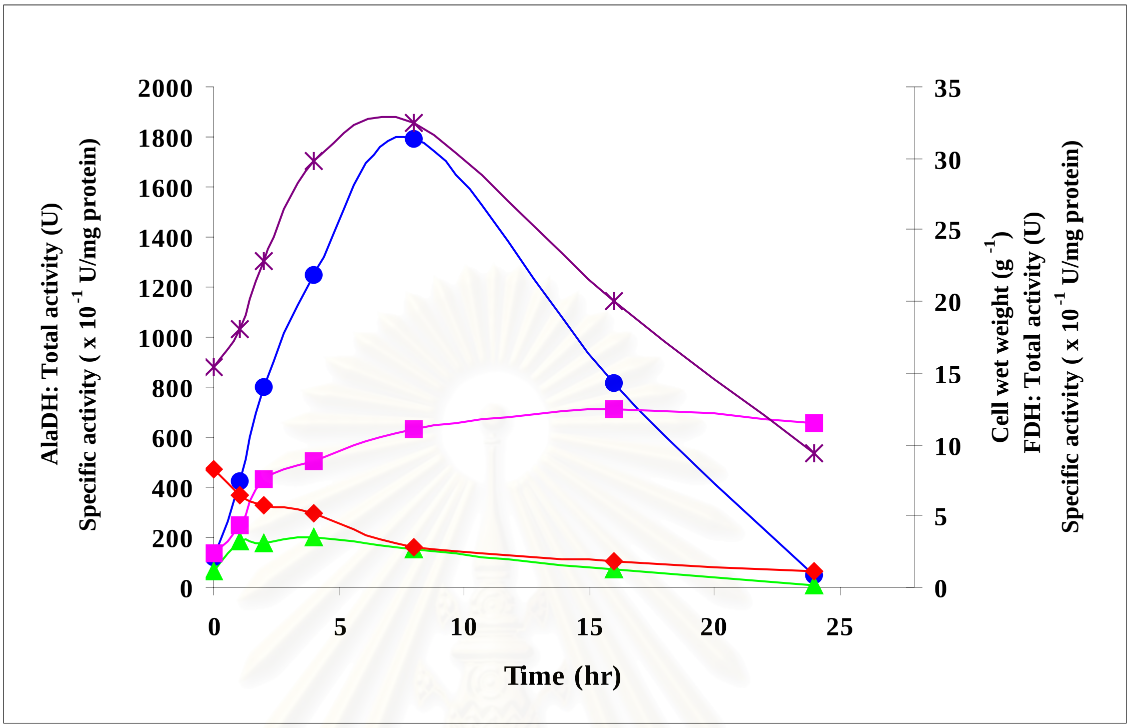

Figure 3.12 **Effect of induction time on AlaDH and FDH production and growth of pETAF clone**

■ Cell wet weight ● AlaDH activity ▲ AlaDH specific activity

* FDH activity ◆ FDH specific activity

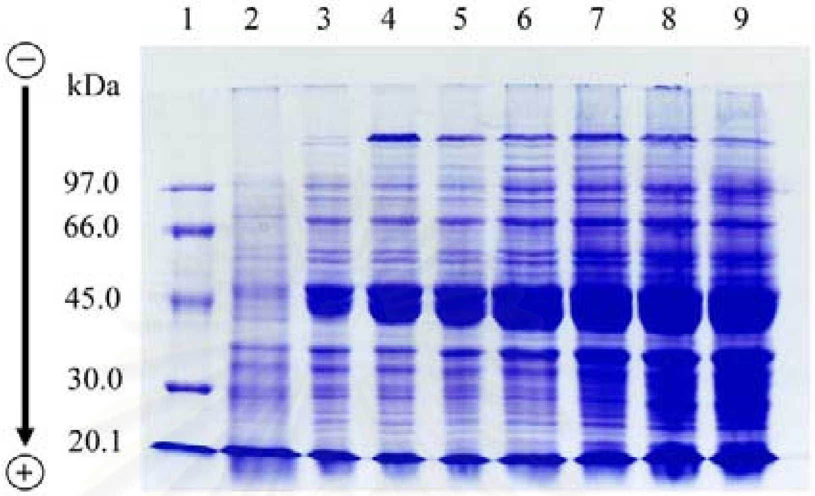
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Figure 3.13 Protein pattern of cell harboring pETAF clone at various induction time detected by SDS-PAGE

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

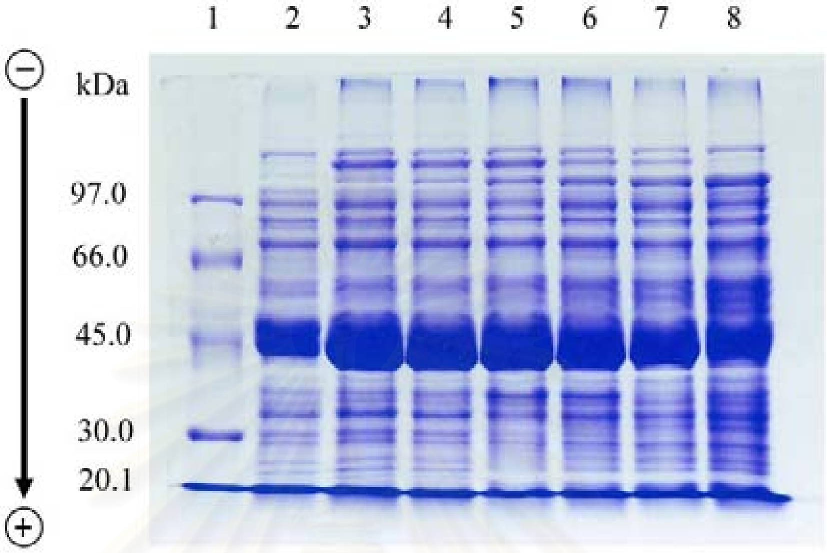
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Figure 3.14 Protein pattern of crude extract of pETAF clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant at t_0

Lane 3-8 = crude extracts of transformant at induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

digestion of pETAlaDH with *Bgl*II and *Eco*RI. The *Bgl*II-*Eco*RI fragment of *aladh* gene (1.2 kb) was ligated to the *Bam*HI-*Eco*RI sites of 4.5 kb pETFDH vector. As shown in Figure 3.15, the recombinant clones was confirmed by *Nde*I and *Eco*RI digestion. The three fragments were appeared, a 3.3 kb of linear pET-17b vector, a 1.2 kb of *fdh* gene, and a 1.1 kb of *aladh* gene, respectively (lane 4).

3.4.2 Enzyme activities of *E. coli* BL21(DE3) harboring pETFA

Ten of recombinant clones were grown for enzyme assay of AlaDH and FDH activity as described in 2.12 and 2.13. The transformant clones showed various levels of the total activity and specific activity of AlaDH from 1005.67-1726.72 U and 17.38-24.51 U/mg protein and FDH total activity and specific activity from 18.52-57.00 U and 0.34-0.92 U/mg protein, respectively, as shown in Table 3.4. The transformant plasmid No.2 which had the highest total activities and specific activities of both AlaDH and FDH: 1726.72 U, 24.51 U/mg protein, 57.00 U, 0.81 U/mg protein, respectively was chosen for the next experiment.

3.4.3 Optimization of induction time

For induction time course study, the transformant No. 2 was grown and induced by IPTG at final concentration of 0.4 before cell was harvested at various times: 0, 1, 2, 4, 8, 16, and 24 hours. As shown in Figure 3.16, the highest total activity and specific activity of AlaDH (1747.14 U, 14.88 U/mg protein, respectively,) and FDH (32.54 U, 0.28 U/mg protein, respectively) were found at 8 hours and after that the total activities and specific activities of AlaDH and FDH were decreased.

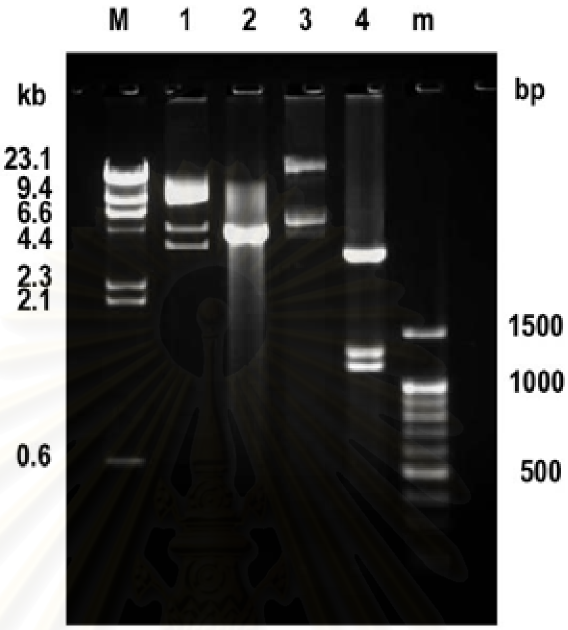
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Figure 3.15 Restriction pattern of pETFA

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

Lane 1 = undigested pETFDH

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

Lane 3 = undigested pETFA

Lane 4 = *Nde*I- *Eco*RI digested pETFA

Lane m = 100 bp DNA ladder

Table 3.4 AlaDH and FDH activities from crude extract of pETFA clones

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		AlaDH	FDH		AlaDH	FDH
pETAlaDH	0.80	1800.00	-	55.00	32.72	-
pETFDH	0.90	-	140.00	44.00	-	3.18
1	0.72	1638.41	54.23	67.20	24.38	0.81
2	0.84	1726.72	57.00	70.43	24.51	0.81
3	0.90	1194.76	18.52	55.26	21.62	0.34
4	0.86	1493.35	39.10	68.08	21.94	0.57
5	0.87	1308.48	51.24	67.58	19.36	0.76
6	0.94	1365.32	46.27	76.03	17.96	0.61
7	0.91	1005.67	53.18	57.85	17.38	0.92
8	0.85	1245.53	47.32	62.34	19.93	0.76
9	0.79	1619.64	49.78	68.52	23.64	0.72
10	0.89	1516.17	50.64	65.48	23.27	0.77

[a] Total activity from 200 ml culture as described in section 2.16.3 and 2.17.3.

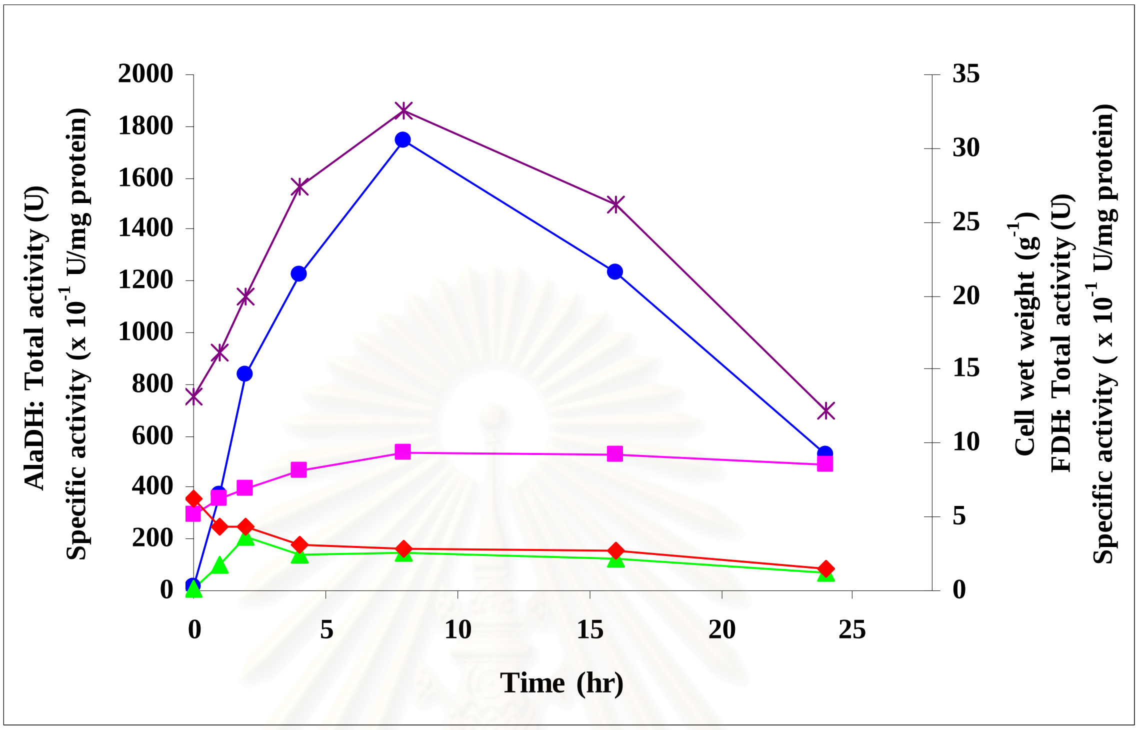

Figure 3.16 Effect of induction time on AlaDH and FDH production and growth of pETFA clone

■ Cell wet weight ● AlaDH total activity ▲ AlaDH specific activity

* FDH activity ◆ FDH specific activity

3.4.4 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of AlaDH and FDH activity were subjected to SDS-PAGE. The protein bands from cell (Figure 3.17) and crude extract (Figure 3.18) of pETFA of each induction time was corresponded with the level of enzyme activity from crude extracts. The result showed that the major protein bands of cells and crude extracts from pETFA had two adjacent bands, FDH and AlaDH.

3.5 Cloning and expression of *fdh* gene using pSY343

3.5.1 PCR amplification *fdh* gene

As shown in Figure 3.6 lane 3, the 1.3 kb of *fdh* gene fragment was amplified from pETFDH, using primers T7FDHF*Bam*HI and FDHR*Eco*RI as described in section 2.19.1. The 5'-primer containing *Bam*HI restriction site, T7 promoter sequence of expression vector of pET-17b. The 3'-primer contained 3'-end of *fdh* gene, the TGA translational termination signal followed by the restriction site for *Eco*RI. The optimum annealing temperature for this PCR condition was 60 ^{o}C.

3.5.2 Cloning of pSYFDH

As described in 3.5.1, the 1.3 kb PCR production of *fdh* gene, which contained T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b, was digested with *Bam*HI and *Eco*RI. The *Bam*HI-*Eco*RI fragment of *fdh* gene was ligated to the same site of large fragment of pSY343 vector (8.8 kb). As shown in Figure 3.19, the recombinant clones were confirmed by *Bam*HI and *Eco*RI digestion. Two fragments were appeared as a 8.8 kb of linear pSY343 vector and 1.3 kb of *fdh* gene.

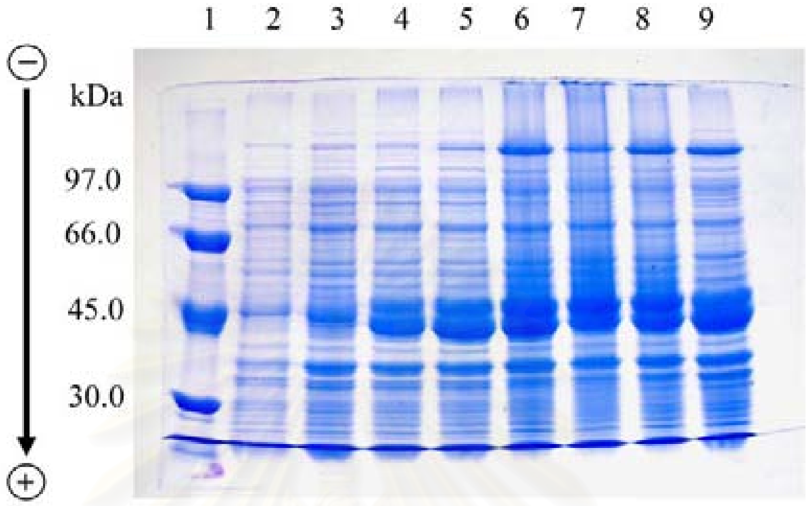

Figure 3.17 Protein pattern of cell harboring pETFA various induction times detected by SDS-PAGE

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

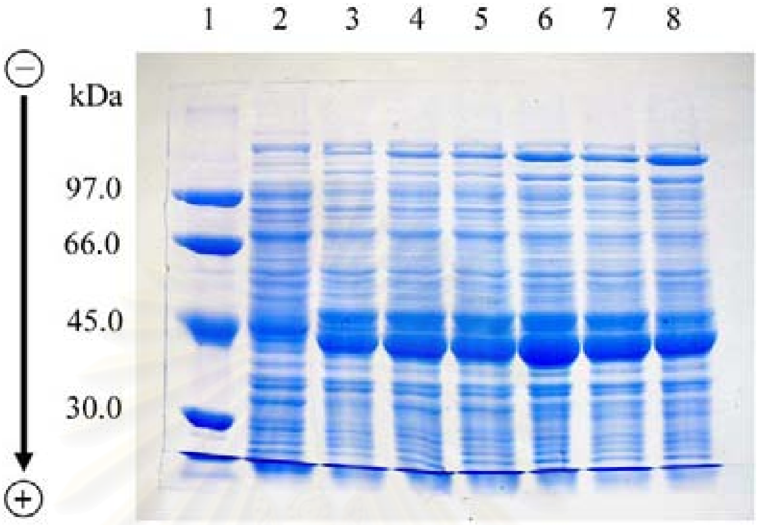

Figure 3.18 Protein pattern of crude extract of pETFA at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant at t_0

Lane 3-8 = crude extracts of transformant at induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

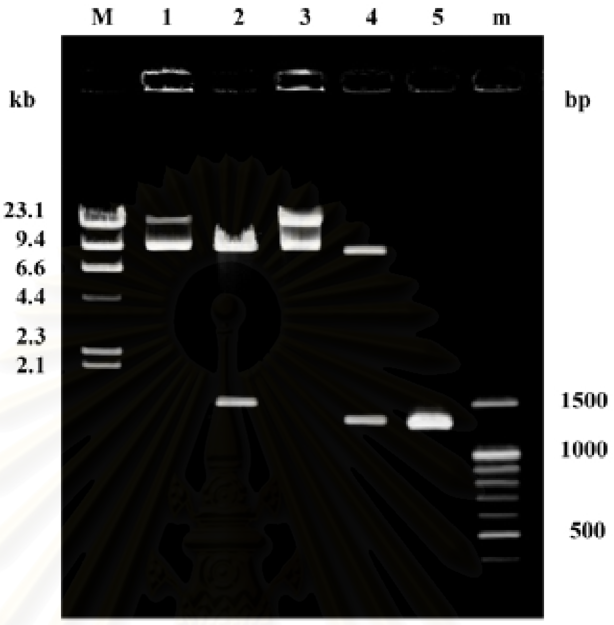
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Figure 3.19 Restriction pattern of pSYFDH

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

Lane 1 = undigested pSY343

Lane 2 = *Bam*HI-*Eco*RI digested pSY343

Lane 3 = undigested pSYFDH

Lane 4 = *Bam*HI-*Eco*RI digested pSYFDH

Lane 5 = amplified product of *fdh* gene

Lane m = 100 bp DNA ladder

3.5.3 Enzyme activity of *E. coli* BL21(DE3) harboring pSYFDH

Ten of recombinant clones were grown for enzyme assay of FDH activity as described in 2.12 and 2.13.2. The transformant clones showed low level of the enzyme total activity from 5.40 – 8.49 U and specific activity from 0.081 – 0.140 U/mg protein as shown in Table 3.5. The transformant plasmid No. 7 which had the highest total activity and specific activity of FDH (8.49 U, 0.137 U/mg protein) was chosen for the next experiment.

3.5.4 Optimization of induction time

For induction time course study of FDH activity, the transformant No. 7, 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 shown in Figure 3.20, the result indicated that the highest FDH total activity and specific activity were 19.95 U and 0.35 U/mg protein, respectively, when induced at 8 hours and after that the FDH activity and specific activity were decreased.

3.5.5 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of FDH activity were subjected to SDS-PAGE. The protein bands expected to be FDH from cell (Figure 3.21) and crude extract (Figure 3.22) of each induction time of pSYFDH had low intensity band (44 kDa) which were corresponded to the level of enzyme activity from crude extracts.

Table 3.5 FDH activity from crude extract of pSYFDH clones

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCFDH in *E. coli* JM109	0.76	20.00	48.95	0.41
1	1.00	5.40	54.84	0.081
2	0.95	6.52	53.21	0.123
3	0.87	7.15	58.74	0.123
4	0.99	6.94	55.63	0.125
5	0.77	6.84	54.38	0.126
6	0.86	7.58	60.24	0.125
7	0.95	8.49	61.15	0.137
8	0.89	8.19	59.87	0.137
9	0.78	7.64	57.61	0.133
10	0.94	8.12	57.89	0.140

[a] Total activity from 200 ml culture as described in section 2.19.3.

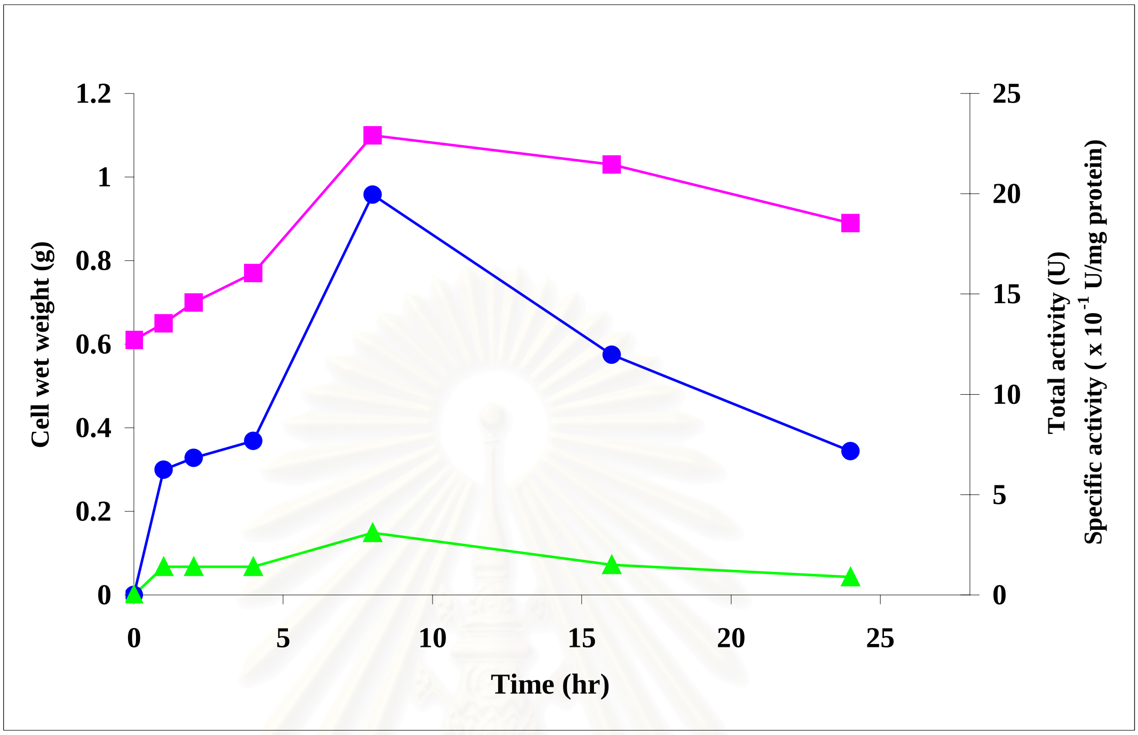

Figure 3.20 Effect of induction time on FDH production and growth of pSYFDH clone

■ Cell wet weight ● Total activity ▲ Specific activity

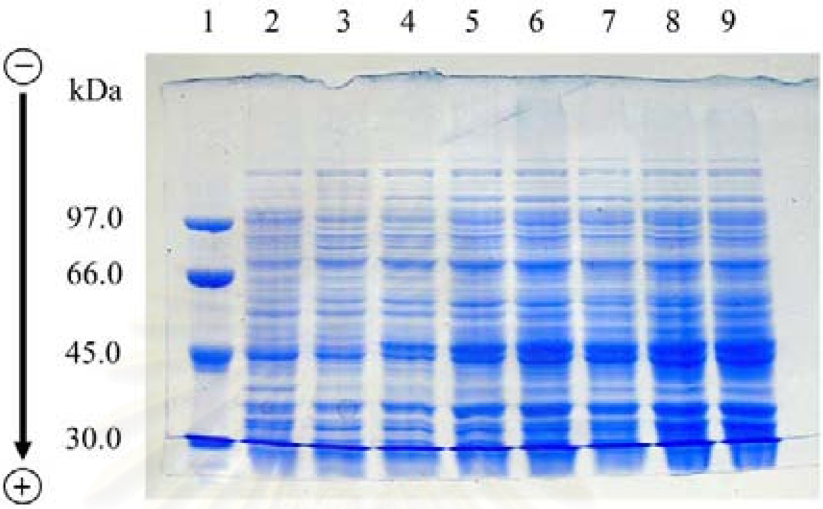

Figure 3.21 Protein pattern of cell harboring pSYFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pSY343

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

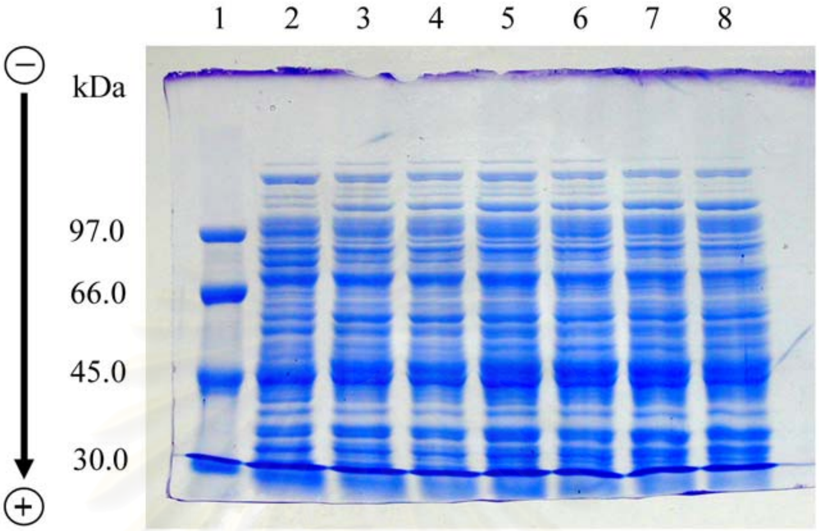

Figure 3.22 Protein pattern of crude extract of pSYFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant t_0

Lane 3-8 = crude extracts of transformant at induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

3.6 Co-transformation of pETAlaDH/pSYFDH into *E. coli* BL21(DE3)

The pETAlaDH was transformed into *E. coli* BL21(DE3) containing pSYFDH. The co-existence of both plasmids was confirmed by growing the clone in LB medium with contained 100 µg/ml amplicilin and 50 µg/ml kanamycin, PCR and digested with *Eco*RI. As shown in Figure 3.23, the uncut plasmids had 7 bands (lane 3). When the plasmids were digested with *Eco*RI, which had one site on both pETAlaDH and pSYFDH, 2 bands of 8.8 kb pSYFDH and 4.4 kb of pETAlaDH were appeared (lane 4). The PCR product of each gene showed the fragment of 1.3 kb for *fdh* gene (lane 5) and 1.2 kb for *aladh* gene (lane 6).

3.6.1 Enzyme activities of *E. coli* BL21(DE3) containing pSYFDH/pETAlaDH

Ten of recombinant clones were grown for enzyme assay of AlaDH and FDH activity as described in 2.12 and 2.13. The transformant clones showed the total activity and specific activity of AlaDH from 124.65-270.00 U and 2.41-6.69 U/mg protein, however, the activity of FDH could not be detected as shown in Table 3.6. The transformant plasmid No.4 which had the highest total activity and specific activity of AlaDH was chosen for the next experiment.

3.6.2 Optimization of induction time

When induction for 2 hour, the highest total activity and specific activity of AlaDH were obtained about 336.00 U, 7.77 U/mg protein, respectively, and after that the AlaDH activity and specific activity were decreased. While no FDH activity was detected (data not shown).

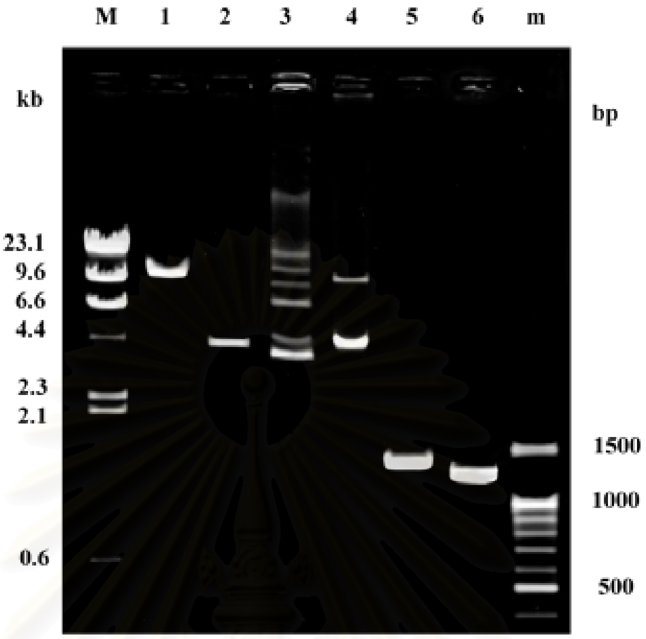
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Figure 3.23 Restriction pattern of co-transformed pETAlaDH/pSYFDH

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

Lane 1 = *Eco*RI digested pSYFDH

Lane 2 = *Eco*RI digested pETAlaDH

Lane 3 = undigested plasmids of co-transformed pSYFDH/pETAlaDH

Lane 4 = *Eco*RI digested plasmids of co-transformed pSYFDH/pETAlaDH

Lane 5 = amplification of *fdh* gene

Lane 6 = amplification of *aladh* gene

Lane m = 100 bp DNA ladder

Table 3.6 AlaDH and FDH activities from crude extract of pETAlaDH/pSYFDH co-transformant

Number	Cell wet weight (g)	Total activity (U) [a]		Total protein (mg)	Specific activity (U/mg protein)	
		AlaDH	FDH		AlaDH	FDH
pETAlaDH	0.80	1800.00	-	55.00	32.72	-
pSYFDH	0.90	-	19.00	54.00	-	0.35
1	0.75	153.60	-	47.00	3.27	-
2	0.77	149.76	-	52.87	2.83	-
3	0.90	124.98	-	51.76	2.41	-
4	0.82	270.00	-	40.36	6.69	-
5	0.74	128.00	-	46.74	2.74	-
6	0.80	124.65	-	45.23	2.76	-
7	0.81	150.98	-	50.97	2.96	-
8	0.79	137.84	-	49.78	2.77	-
9	0.76	199.21	-	44.46	4.48	-
10	0.85	182.36	-	47.78	3.82	-

[a] Total activity from 200 ml culture as described in section 2.20.

3.6.3 Protein patterns of cells and crude extracts

The protein pattern from cells (Figure 3.24) and crude extracts (Figure 3.25) of co-transformant of pETAlaDH/pSYFDH at each induction time showed the major band of AlaDH while FDH had low intensity band in lane 6-8.

3.7 Cloning and expression of *aladh* gene using pMPM-K3 vector

3.7.1 Preparation of *aladh* gene fragment

The fragment of *aladh* gene, which contained T7 promoter sequence and Shine-Dalgarno sequence of expression vector of pET-17b, was obtained from the digestion of pETAlaDH with *Bgl*II and *Eco*RI. The *Bgl*II-*Eco*RI fragment of *aladh* gene (1.2 kb) was ligated to the sites of 3.05 kb pMPM-K3 vector. As shown in Figure 3.26, *Eco*RI digested pMPMAlaDH fragment was 4.2 kb (lane 4) shifted from 3.0 kb of *Bam*HI-*Eco*RI digested pMPM-K3 (lane 2).

3.7.2 Enzyme activity of *E. coli* BL21(DE3) harboring pMPMAlaDH

Ten of recombinant clones were also grown for enzyme assay of AlaDH activity as described in 2.12 and 2.13.1. The transformant clones showed various levels of the total activity and specific activity of AlaDH from 477.87-1382.40 U and 6.66-16.72 U/mg protein, respective, as shown in Table 3.7. The transformant clone No.6 which had the highest total activity and specific activity of AlaDH about 1382.40 U, 16.72 U/mg protein, respectively was chosen for the next experiment.

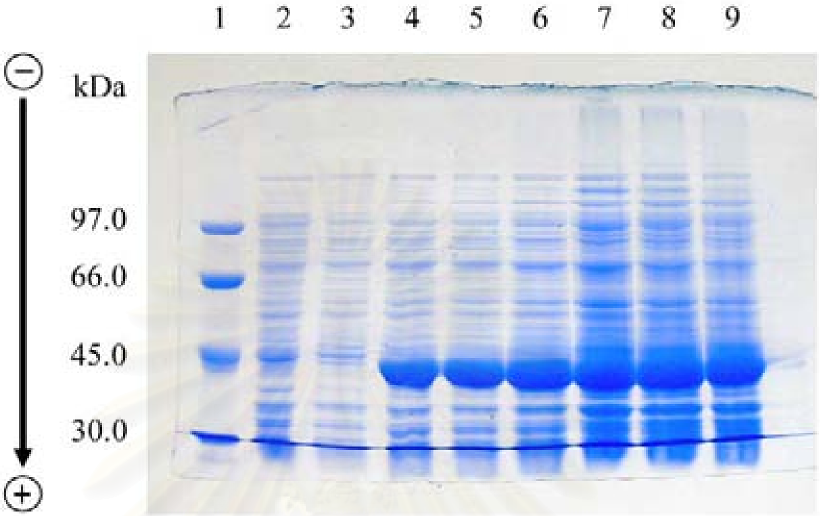

Figure 3.24 Protein pattern of cell harboring pETAlaDH/pSYFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pETAlaDH and pSY343

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

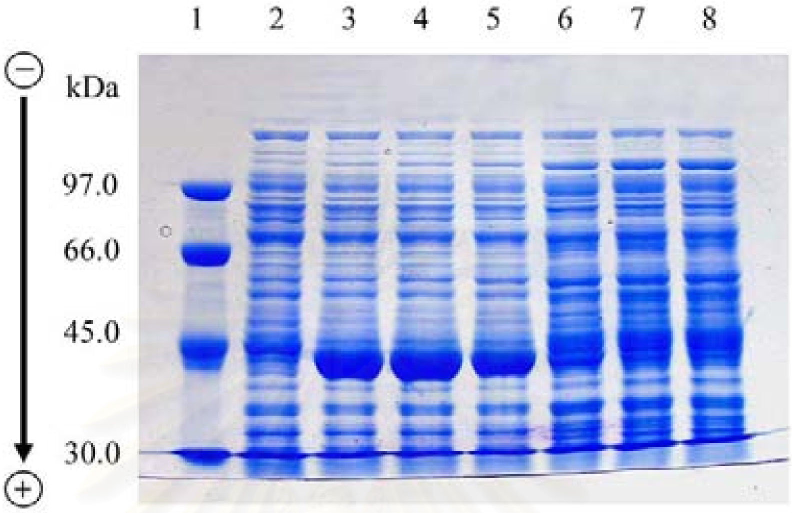

Figure 3.25 Protein pattern of crude extract of pETAlaDH/pSYFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant t_0

Lane 3-8 = crude extracts of transformant at induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

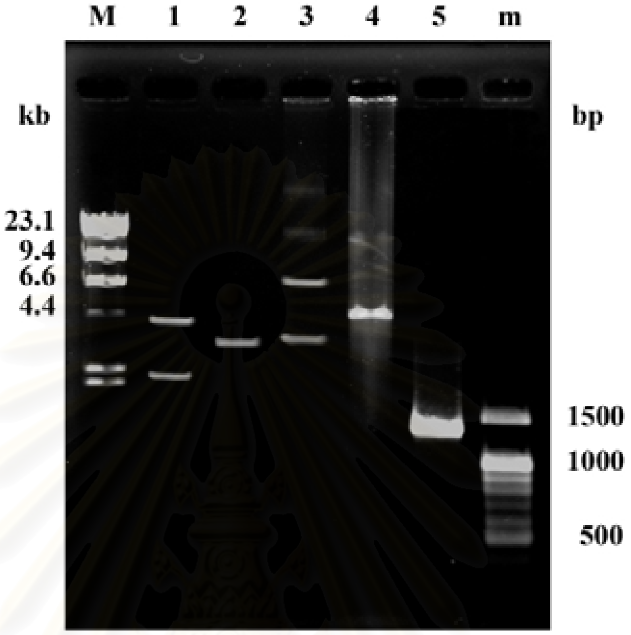
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Figure 3.26 Restriction pattern of pMPMAlaDH

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

Lane 1 = undigested pMPM-K3

Lane 2 = *Bam*HI-*Eco*RI digested pMPM-K3

Lane 3 = undigested pMPMAlaDH

Lane 4 = *Eco*RI digested pMPMAlaDH

Lane 5 = amplified PCR product of *aladh* gene

Lane m = 100 bp DNA ladder

Table 3.7 AlaDH activity from crude extract of pMPMAlaDH clones

Number	Cell wet weight (g)	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
pUCFDH in *E. coli* JM109	0.76	20.00	48.95	0.41
1	1.16	1046.75	89.38	11.71
2	1.11	798.72	69.28	11.53
3	1.08	704.00	93.11	7.56
4	0.94	477.87	71.71	6.66
5	0.90	1072.92	94.20	11.39
6	1.04	1382.40	82.69	16.72
7	0.98	1072.92	81.38	13.18
8	1.09	819.20	66.06	12.40
9	0.91	1042.50	74.71	13.95
10	0.86	975.63	76.82	12.70

[a] Total activity 200 ml culture as described in section 2.21.

3.7.3 Optimization of induction time

For induction time course study, the 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 shown in figure 3.27, when cultivation times 4 hours were used, the highest total activity and specific activity of AlaDH of 1392.54 U, 16.28 U/mg protein, respectively were obtained. After 4 hours, the total activity and specific activity of AlaDH were decreased.

3.7.4 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of AlaDH activity were subjected to SDS-PAGE. The protein major band from cell (Figure 3.28) and crude extract (Figure 3.29) of each induction time of pMPMAlaDH was corresponded with the level of enzyme activity from crude extracts.

3.8 Co-transformation of pMPMAlaDH/pETFDH into *E. coli* BL21(DE3)

The pMPMAlaDH was transformed into *E. coli* BL21(DE3) containing pETFDH. The co-existance of both plasmids was confirmed by growing the clone in LB medium with contained 100 µg/ml amplicilin and 50 µg/ml kanamycin, PCR, and digested with *Eco*RI, which had one site on both pETAlaDH and pSYFDH. As shown in Figure 3.30, the uncut plasmids had 5 bands. When the plasmids were digested with *Eco*RI, the bands of pETFDH/pMPMAlaDH were appeared (lane 4). The PCR of each gene showed the fragment of *fdh* gene (lane 5) and aladh gene (lane 6).

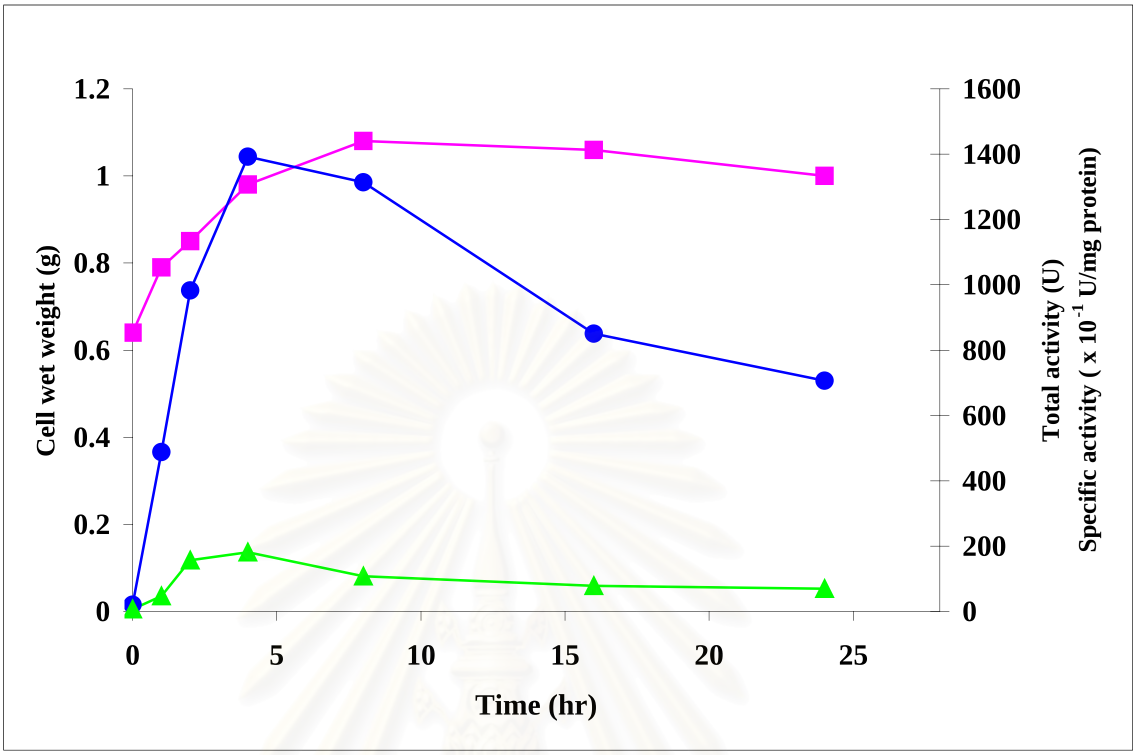

Figure 3.27 Effect of induction time on AlaDH production and growth of pMPMAlaDH clone

■ Cell wet weight ● Total activity ▲ Specific activity

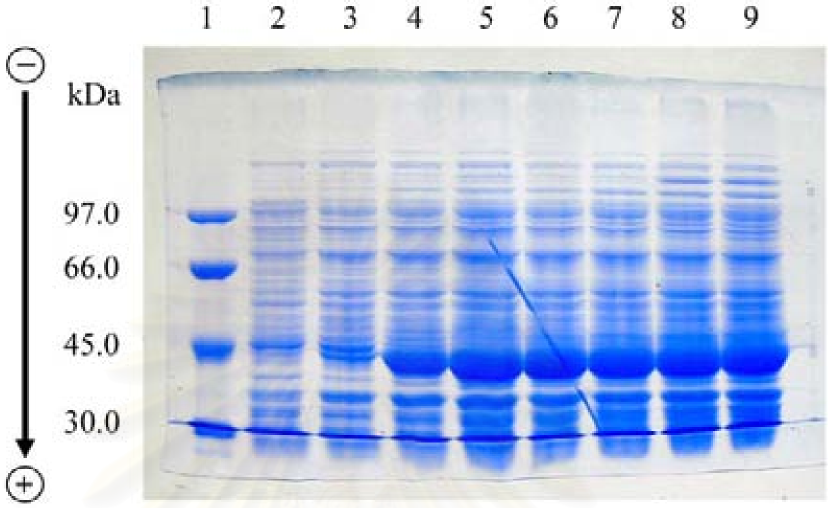

Figure 3.28 Protein pattern of cell harboring pMPMAlaDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pMPM-K3

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

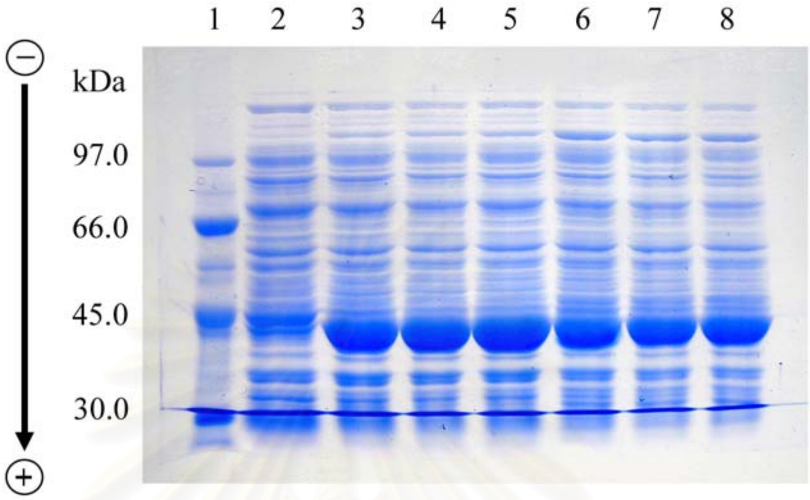

Figure 3.29 Protein pattern of crude extract of pMPMAlaDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformant t_0

Lane 3-8 = crude extracts of transformant at induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

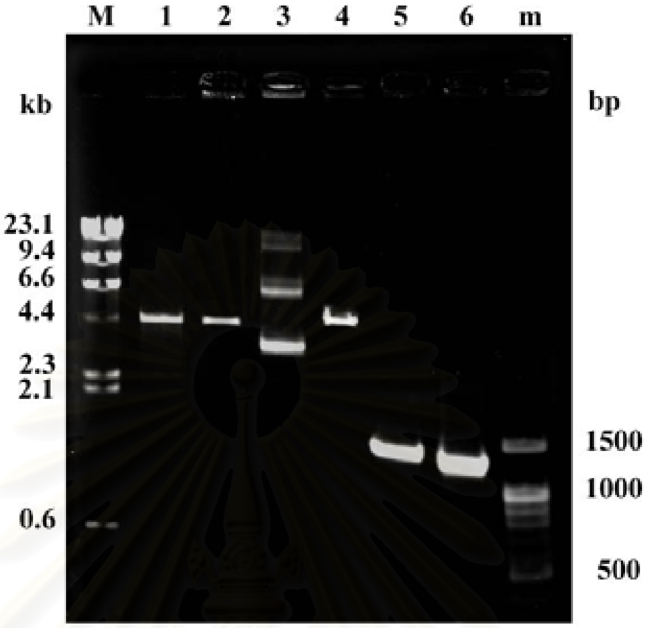
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Figure 3.30 Restriction pattern of co-transformed pMPMAlaDH/pETFDH

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

Lane 1 = *Eco*RI digested pETFDH

Lane 2 = *Eco*RI digested pMPMAlaDH

Lane 3 = undigested plasmids of co-transformed pETFDH/pMPMAlaDH

Lane 4 = *Eco*RI digested plasmids of co-transformed pETFDH/pMPMAlaDH

Lane 5 = amplified of *fdh* gene

Lane 6 = amplified of *aladh* gene

Lane m = 100 bp DNA ladder

3.8.1 Enzyme activities *E. coli* BL21(DE3) containing pMPMAlaDH/pETFDH

Ten of recombinant clones were grown for enzyme assay of AlaDH and FDH activity as described in 2.12 and 2.13. The transformant clones showed various levels of the total activity and specific activity of AlaDH from 58.03-160.20 U and 1.02-3.54 U/mg protein and FDH total activity and specific activity from 16.03-50.45 U and 0.29-0.80 U/mg protein, respective as shown in Table 3.8. The co-transformant No.7 which had the highest total activity and specific activity of both AlaDH and FDH: 160.20 U, 2.54 U/mg protein, 50.45 U, 0.80 U/mg protein, respectively was chosen for the next experiment.

3.8.2 Optimization of induction time

The induction time of AlaDH and FDH activity were varied from 0 - 24 hours after induced with 0.4 mM IPTG. As shown in Figure 3.31, when induction for 4 hours, the highest total activity and specific activity of AlaDH were 165.35 U, 2.70 U/mg protein, respectively were obtained. After that the total activity and specific activity of AlaDH were decreased. The highest FDH total activity and specific activity were 61.93 U, 1.09 U/mg protein, respectively were also found at 4 hours of induction.

3.8.3 Protein patterns of cells and crude extracts

Protein patterns of cells and crude extracts from induction time course study of AlaDH and FDH activity were subjected to SDS-PAGE. The major protein bands from cells (Figure 3.32) and crude extracts (Figure 3.33) of co-transformant had

Table 3.8 AlaDH and FDH activities from crude extracts of pETFDH/pMPMAlaDH co-transformant

Number	Cell wet weight (g)	Total activity (U)		Total protein (mg)	Specific activity (U/mg protein)	
		AlaDH	FDH		AlaDH	FDH
pMPMAlaDH	0.89	1392.54	-	85.50	16.28	-
pETFDH	0.90	-	140.00	44.00	-	3.18
1	1.16	195.13	16.03	55.16	3.54	0.29
2	1.24	96.26	31.58	59.60	1.62	0.53
3	1.00	96.26	32.34	59.80	1.61	0.54
4	1.11	58.03	23.94	56.87	1.02	0.42
5	0.94	86.36	18.77	37.33	2.31	0.50
6	1.04	112.30	17.56	50.38	2.23	0.35
7	1.06	160.20	50.45	63.07	2.54	0.80
8	0.98	106.56	32.23	48.54	2.19	0.66
9	1.12	98.05	36.48	55.41	1.77	0.65
10	0.92	130.25	38.21	58.87	2.21	0.65

[a] Total activity 200 ml culture as described in section 2.22.

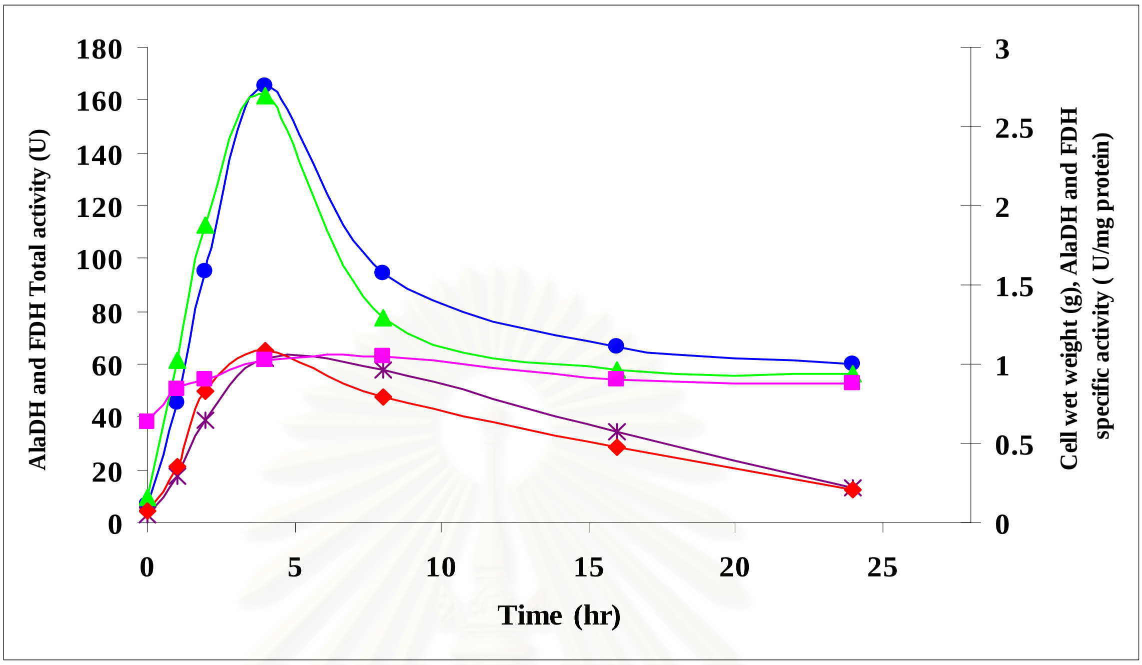


Figure 3.31 Effect of induction time on AlaDH and FDH production and growth of pMPMAlaDH/pETFDH co-transformant

■ Cell wet weight ● AlaDH activity ▲ AlaDH specific activity

* FDH activity ◆ FDH specific activity

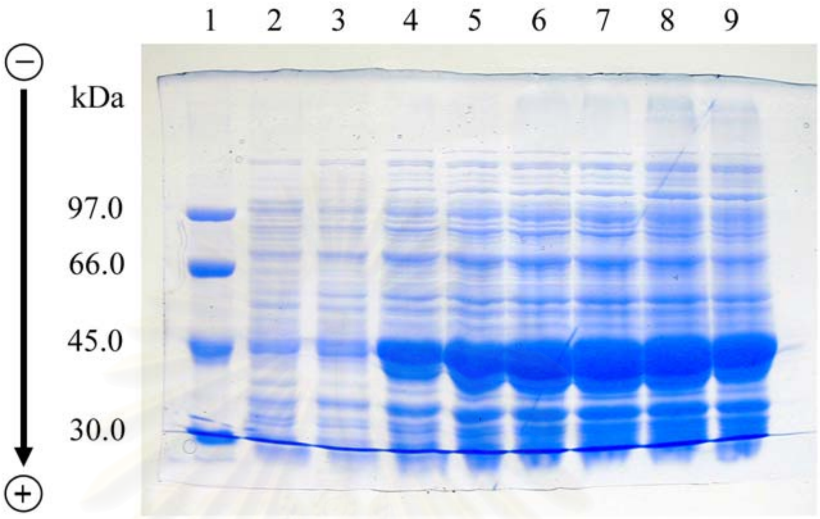


Figure 3.32 Protein pattern of cell harboring pMPMAlaDH/pETFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = cell of *E. coli* BL21(DE3) harbouring pMPMAlaDH and pETFDH

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

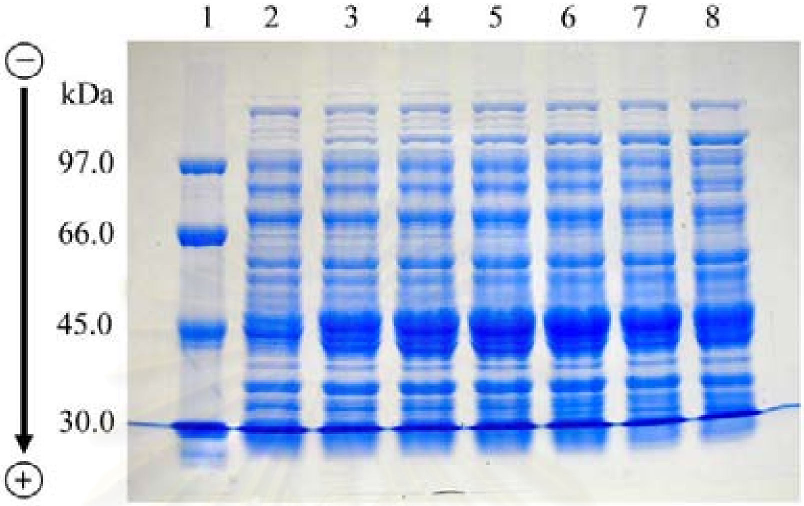


Figure 3.33 Protein pattern of crude extract of pMPMAlaDH/pETFDH clone at various induction times detected by SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract of transformants at t_0

Lane 3-8 = crude extracts of transformant at various induction times: 1, 2, 4, 8, 16 and 24 hours, respectively

two adjacent major bands. The upper band was FDH (44 kDa) and the other was AlaDH (40 kDa).

The sumarry of total activity of all recombinant clones were showed in Table 3.9.

3.9 The production of alanine by recombinant clones

As described in 2.23.1, the *E. coli* BL21(DE3) containing plasmid pETAlaDH, pETAF, pETFA, pMPMAlaDH and pMPMAlaDH/pETFDH were used to produce alanine. Alanine can be detected on a reversed-phase TLC plate, which can separate optical isomers of amino acid. This method is simple and rapid without using expensive impregnated plates or a chiral mobile phase. Figure 3.34 shows the chromatograms of FDAA alanine developed with acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (35:65). The yellow spot of D- and L-alanine of samples were not separated by this condition. So the R_f values for each L- and D-enantiomer of alanine could not be measured. A better separation was obtained after repeating the chromatography by putting the developed and dried plate back into the glass chamber for further development. The enantiomers were well-separated after the third development. The spots of L-enantiomers move faster than those of the D-enantiomer because the FDAA D-enantiomers have greater affinity for the C_{18} silica gel than the FDAA L-enantiomers.

For quantitative determination of alanine enantiomers, 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. D- and L-alanine production from *E. coli* BL21(DE3) containing pETAlaDH, pMPMAlaDH, pETAF, pETFA or pMPMAlaDH/pETFDH were shown in Table 3.9.

Table 3.9 Summary of AlaDH/FDH activity of all recombinant clones

Recombinant clones	**AlaDH activity (U)** [a]	**FDH activity (U)** [a]
E. coli BL21(DE3)	-	-
E. coli BL21(DE3) harboring pET-17b, pSY343, pMPM-K3	-	-
pUCAlaDH in *E. coli* JM109	300	-
pUCFDH in *E. coli* JM109	-	20
pETAlaDH	1850	-
PMPMAlaDH	1390	-
pETFDH	-	145
pSYFDH	-	19
pETAF	1800	44
pETFA	1770	44
pETAlaDH/pETFDH	336	-
pMPMAlaDH/pETFDH	160	55

[a] Total activity from 200 ml culture

All recombinant clones could produce alanine in the same level with about 50% yield. Ratio of D- : L-alanine produced by each clone was about 1.6:1.

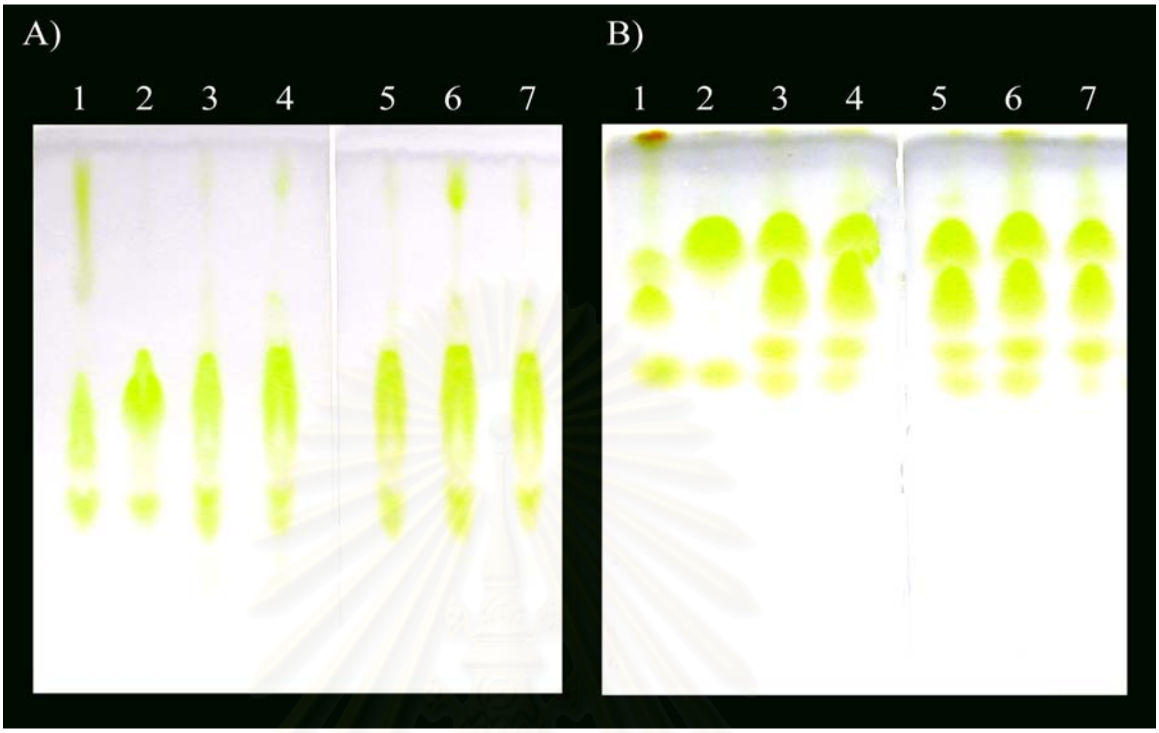


Figure 3.34 Separation of optical isomers of FDAA alanine on reversed phase TLC plates. Each sample of FDAA alanine was spotted and developed in acetronitrile: 50 mM triethylamine-phosphate buffer, pH 5.5 (35:65) for 20 min. Lane 1: D-alanine, Lane 2: L-alanine, Lane 3: pETAlaDH, Lane 4: pMPMAlaDH. Lane 5: pETAF, Lane 6: pETFA, and Lane 7: pMPMAlaDH/pETFDH

A) The first development B) The fifth development

Table 3.10 Alanine production[a] by recombinant clones

Recombinant clones	**D-alanine (mg)**	**L-alanine (mg)**	**Yield (%)**	**Ratio of D:L enantiomer**
pETAlaDH	19.5	12.0	50.00	1.63
pMPMAlaDH	19.3	11.6	49.05	1.66
pETAF	21.0	13.0	53.97	1.62
pETFA	22.4	13.5	56.98	1.62
pMPMAlaDH/pETFDH	22.0	14.0	57.14	1.57

[a] Two ml of the reaction mixture containing 0.1 g wet weigh of washed cell, 0.5 M ammonium formate (pH 7.5) and 0.3 M pyruvate was incubated at 37^{o}C, 250 rpm for 12 hours as described in section 2.23.

CHAPTER IV

DISCUSSION

The commercial values of amino acids come from their wide applicability in both food and pharmaceutical industries. The worldwide market value of amino acids is approximately 2 billion dollars annually. L-alanine, one of interesting amino acids, is used as food industry and pharmaceutical applications. L-alanine is currently used as a food additive because of its sweet taste and used for pharmaceutical applications in which it is incorporated together with several other L-amino acids in standard infusions for parenteral administration in clinical preoperative and postoperative nutrition therapy (Suye *et al*., 1992).

In Thailand, there is no production of L-alanine at an industrial scale. Our research group at the Department of Biochemistry has studied on L-alanine dehydrogenase of *Aeromonas hydrophila* which was screened from soil in Bangkok (Phungsangtham, 1997). Catalytic properties of AlaDH from this bacterial strain are suitable for industrial application such as L-alanine production or clinical diagnosis. In addition, the *aladh* gene was already cloned and expression in *E. coli* JM109 cells using pUC18 as a vector (Poomipark, 2000).

Large-scale industrial production of L-alanine from pyruvate with NAD^+-dependent alanine dehydrogenase has been proposed. However, the application of this method to industrial production of L-alanine has been hampered by the cost of coenzymes. A multienzyme reaction system for simultaneous coenzyme regeneration has been proposed to overcome this problem.

In this research, the alanine production efficiency of recombinant clone was attempted to improve by co-existence of *aladh* and NAD^+ regenerating enzyme gene.

FDH was selected as a NAD^+ regenerator to developing the system for L-alanine production because 1) Formate, as a substrate for the FDH, is one of the cheapest hydrogen sources and does not inhibit most other dehydrogenases. 2) The reaction has a favourable equilibrium strongly shifted towards CO_2 and NADH formation. 3) The oxidation product CO_2 can be easily removed from the reaction mixture. 4) The enzyme has a broad pH optimum of activity.

Production of L-alanine can be carried out by 1) chemical synthesis 2) direct fermentation of sugar in batchwise or continuous process to produce L-alanine into the medium 3) using the microorganism in an immobilized form on a suitable carrier, for example in an appropriate cell reactor or in a column or 4) using of cell free extract. However, the use of intact cells of the microorganism is generally preferred according to the invention over the use of cell extracts. Thus many microorganisms have been reported for the preparation of L-alanine by fermentation of glucose such as *Corynebacterium gelatinosium, Arthrobacter oxydans, Brevibacterium lactofermentum, Clostridium* sp. (Oerlgysson, *et al*., 1995) *Pyrococcus furiosus* (Hols, *et al*., 2003) and *Escherichia coli* (Galkin, *et al*., 1997) or immobilized cells or cell suspension of *Pseudomonas dacunhae* (Yamamoto, *et al*., 1980; Takamatsu, *et al*., 1981).

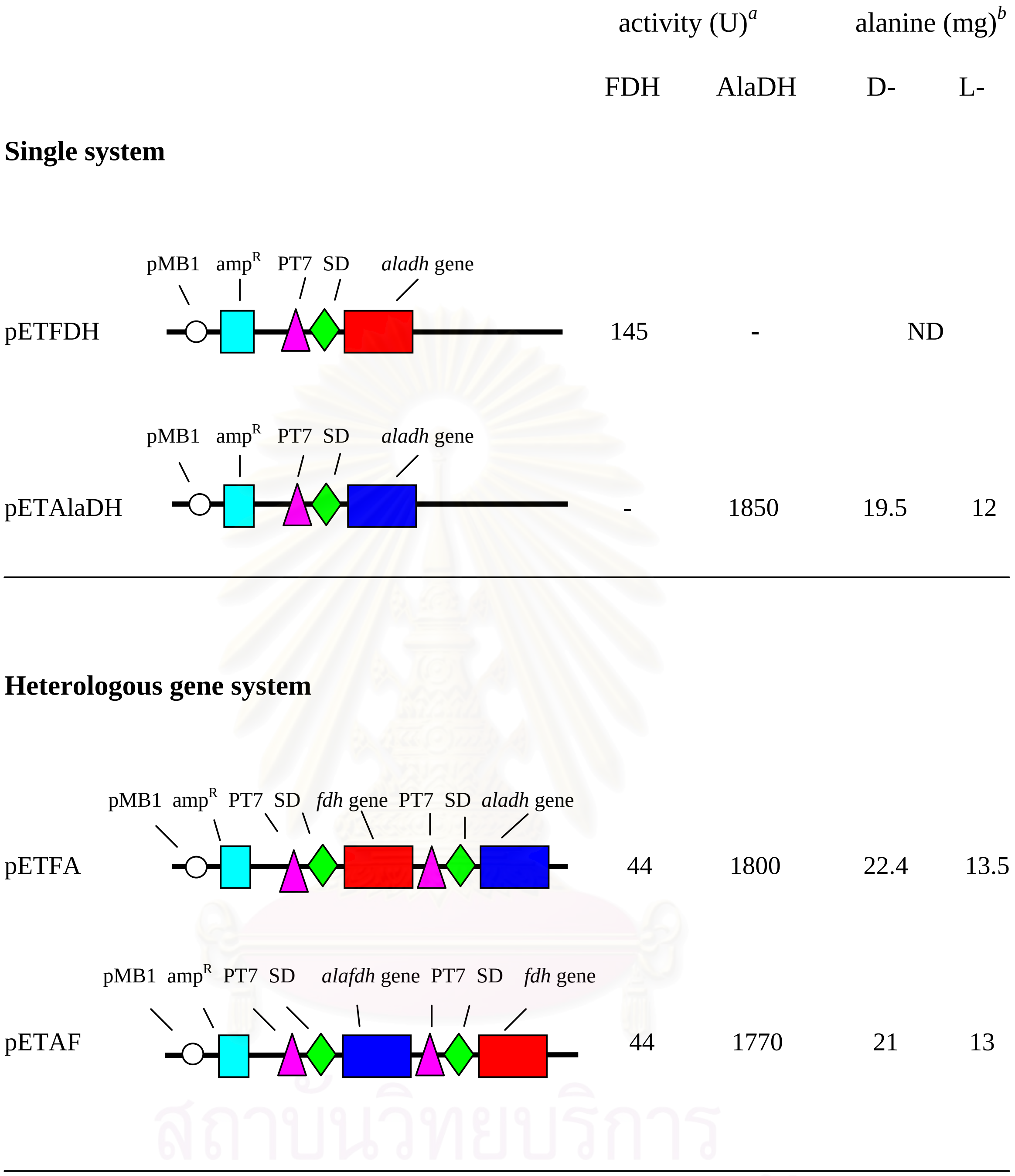
In general, the one or more homologous or heterologous genes coding for the alanine-forming activity can be brought to overexpression in the microorganism used in a well-known manner, for example under the control of a conventional homologous or heterologous promoter that is active in the microorganism used. For thus purpose, the 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.

To co-existence high expression of *aladh* and *fdh* in *E. coli* host cell, two methods were performed 1) cloning of heterologous gene of *aladh* and *fdh* under T7 promoter of high expression vector pET-17b and 2) co-transformation of plasmids containing each desired gene by 2 systems a) using plasmid vector pET-17b and pSY343 containing *aladh* and *fdh* gene with T7 promoter, respectively and b) using plasmid vector pMPM-K3 and pET-17b containing *aladh* and *fdh* gene under T7 promoter, respectively. A summary of plasmid construction is shown in Figure 4.1 and 4.2.

As summarized in Figure 4.1 and 4.2, clones of *aladh* in high expression vector pET-17b (pETAlaDH) and pMPM-K3 (pMPMAlaDH) showed total activity about 1,850 U and 1,390 U, respectively which were about 6.2 and 4.6 folds of the activity obtained from their original clone pUCAlaDH (300 U) (Poomipark, 2000) because the pETAlaDH and pMPMAlaDH are controlled under T7 promoter of pET-17b that is stronger than *lac* promoter of pUC18.

The copy number of pET-17b vector (pMB1 ori) in the *E. coli* host cell is known to be higher than that of pMPM-K3 (p15A ori). Therefore, pETAlaDH clone had higher activity than pMPMAlaDH clone.

When cloning the *fdh* gene into pSY343 and pET-17b vector, the pETFDH clone had total activity about 145 U which was greater than pSYFDH clone (19 U) and its original recombinant pUCFDH clone (20 U). This suggested that the T7 promoter of pET-17b could well elevate the expression of *fdh* gene. Although pSYFDH contained T7 promoter at 5' upstream of *fdh* gene which was similar to pETFDH, its expression was very lower than that of pETFDH. The result indicated that the difference in copy number of these vectors greatly affected on *fdh* expression. Moreover, expression of *fdh* under *lac*



	activity (U)[a]		alanine (mg)[b]	
	FDH	AlaDH	D-	L-
Single system				
pETFDH	145	-	ND	
pETAlaDH	-	1850	19.5	12
Heterologous gene system				
pETFA	44	1800	22.4	13.5
pETAF	44	1770	21	13

pMB1
amp^R
PT7
SD
aladh gene
pMB1
amp^R
PT7
SD
aladh gene
pMB1
amp^R
PT7
SD
fdh gene
PT7
SD
aladh gene
pMB1
amp^R
PT7
SD
alafdh gene
PT7
SD
fdh gene

Figure 4.1 Summary of plasmid construction (Heterologous system)

[a] The total activity obtained from 200 ml cultured

[b] The alanine production from 2 ml reaction mixture determined by TLC technique

ND, not determined

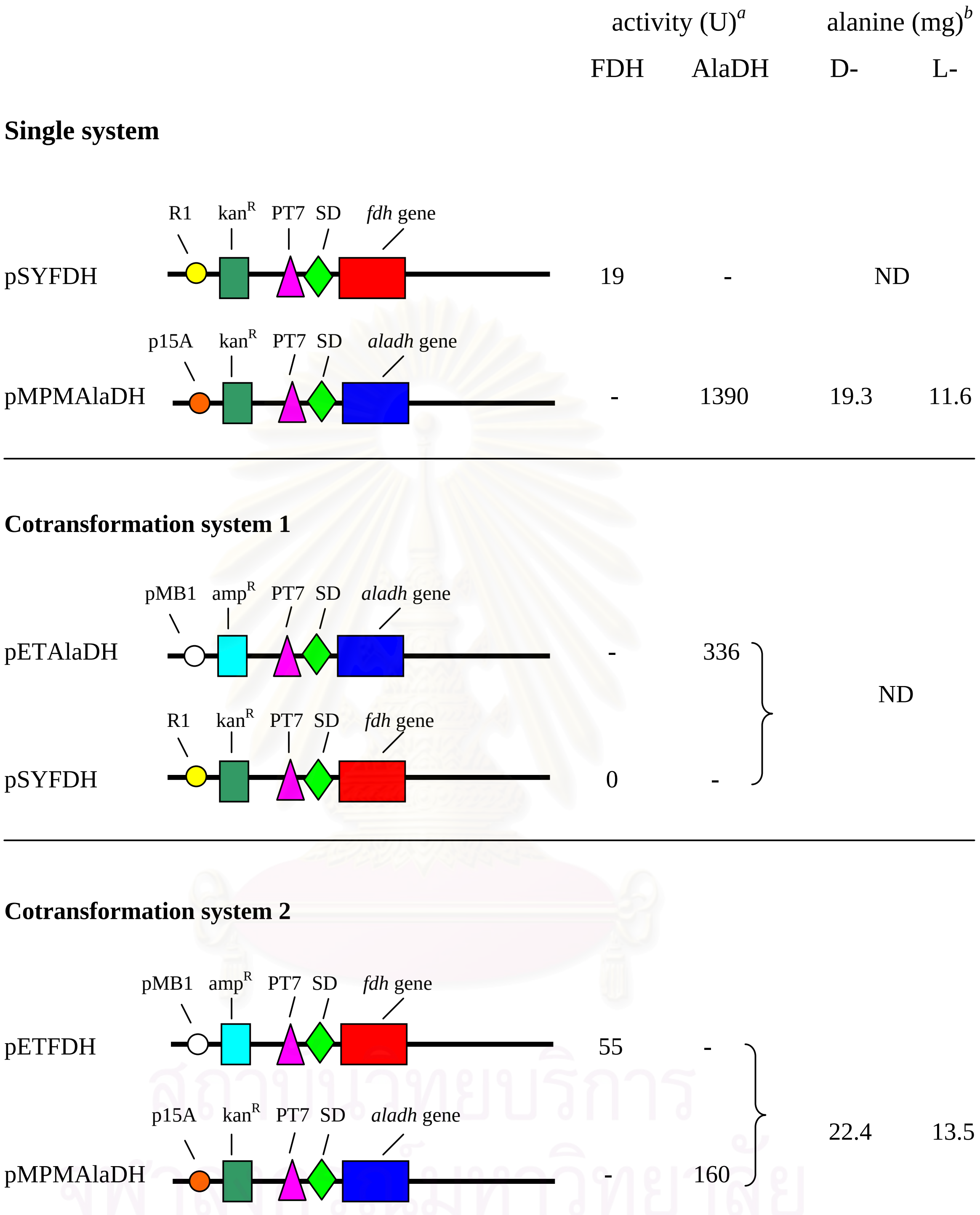

activity (U)[a]
alanine (mg)[b]
FDH
AlaDH
D-
L-
Single system
R1
kanR
PT7
SD
fdh gene
pSYFDH
19
-
ND
p15A
kanR
PT7
SD
aladh gene
pMPMAlaDH
-
1390
19.3
11.6
Cotransformation system 1
pMB1
ampR
PT7
SD
aladh gene
pETAlaDH
-
336
ND
R1
kanR
PT7
SD
fdh gene
pSYFDH
0
-
Cotransformation system 2
pMB1
ampR
PT7
SD
fdh gene
pETFDH
55
-
p15A
kanR
PT7
SD
aladh gene
pMPMAlaDH
-
160
22.4
13.5

Figure 4.2 Summary of plasmid construction (Co-transformation)

[a] The total activity obtained from 200 ml cultured

[b] The alanine production from 2 ml reaction mixture determined by TLC technique

ND, not determined

promoter of high copy number plasmid pUC119 (pUCFDH) was a same of low copy pSYFDH which contains stronger promoter, T7.

In heterologous gene expression system of *aladh* and *fdh* gene, pETAF clone and pETFA clone which had T7 promoter upstream of each gene were constructed. Total activities of AlaDH and FDH of pETAF and pETFA clones were 1800, 44, 1770 and 44 U, respectively. The result showed that with their own T7 promoter, the position of both *aladh* and *fdh* on pET-17b vector had no effect on their expression. In addition, before the construction of pETAF and pETFA was performed, only a structural gene of *fdh* was inserted to 3' downstream of *aladh* gene in pETAlaDH. The recombinant clone showed 1700 U of AlaDH total activity without FDH activity (data not shown).

For co-transformation of plasmid containing *aladh* and *fdh* in *E. coli* BL21(DE3), the system of pETAlaDH/pSYFDH was firstly performed. The clone had total activity of AlaDH about 336 U with no FDH activity. Since, pSYFDH itself had low level of expression in the single gene system, cloning of *fdh* in pET-17b and *aladh* in pMPM-K3 was carried out and 160 U of AlaDH as well as 55 U of FDH were obtained. Co-transformation of pMPMFDH/pETAlaDH could not be performed because the clone of pMPMFDH had no activity of FDH.

The induction time of each recombinant clones were varied from 0 - 24 hours. The highest AlaDH total activities of clones containing pETAlaDH, pMPMAlaDH and pMPMAlaDH/pETFDH were found at 4 hours while those of pETAF and pETFA were at 8 hours. For FDH activity, the highest total activities of pETFDH, pMPMAlaDH/pETFDH clones were obtained at 4 hours, whereas those of pSYFDH, pETAF and pETFA were 8 hours. The variation of inductime time between the single gene system and the heterologous gene expression system may be caused by sharing of the materials required for transcription and translation steps because the heterologous gene system had T7 promoter upstream to both *aladh* and *fdh* gene. Not only change in the induction time, total activity of FDH also dropped about 3 times in heterologous gene system. For the co-transformant system, clone of pMPMAlaDH/pETFDH had optimum induction time at 4 hours similar to its single gene system, however, total activities of both AlaDH and FDH were greatly decreased.

Alanine production from *E. coli* BL21(DE3) containing plasmid pETAlaDH, pMPMAlaDH, pETAF, pETFA or pMPMAlaDH/pETFDH was determined. As shown in Figure 3.35, all recombinant clones produced both D- and L-enantiomer when detected by TLC technique. For the stereoselective production of L-alanine, the microorganism must not contain any appreciable alanine racemase activity. Unexpectedly, two types of alanine racemase, constitutive and inducible enzymes, had been reported in *E. coli*. To overcome this nonstereospecificity, the alanine racemase activities should be suppressed by using *E. coli* that had been genetically manipulated to express both native alanine racemase activities in a greatly reduced or defective way or not at all. However, Kato, *et al*. (1993) reported that L-β-chloroalanine, a derivative of L-alanine used in some pesticides, medicines and natural or unnatural amino acid synthesis, could be synthesized with a 90% yield from β-chloropyruvate with purified preparations of AlaDH and FDH. Since alanine racemase is efficiently inactivated by β-chloroalanine (Badet, *et al.*, 1984), the clone of *E. coli* BL21(DE3) containing pETAF, pETFA or pMPMAlaDH/pETFDH may be probably used as an efficient tool for the production of optically pure L-β-chloroalanine.

Not only alanine racemase, the host cell should be preferably deficient in activity or activities of enzyme that complete with the alanine dehydrogenase for the avialable substrate sach as lactate dehydrogenase (Hols, *et al*., 2003).

The quantitative of alanine production as shown in Table 3.9 indicated that *E. coli* BL21(DE3) containing plasmid pETALaDH, pMPMALaDH, pETAF, pETFA or pMPMAlaDH/pETFDH produced alanine in the same level with 50.0, 49.05, 53.97, 56.98 and 57.14% yield, respectively and ratio of D:L alanine was 1.6:1. Quantitative analysis of alanine production was previously performed by HPLC using CROWNPAK CR(+) column with solvent system of perchloric acid aqueous solution (pH 1.5). D- and L-isomers of alanine were clearly separated. Unfortunately, the peak of D-alanine product from all samples was interfered by its substrates as shown in Appendix F. So, amount of produced alanine could not be determined.

Eventhough various kinds of recombinant clone were used, the level of alanine production was not significantly different. Uhlenbusch *et al*. (1991) studied the expression of an L-alanine dehydrogenase gene in *Zymomonas mobilis* and excretion of L-alanine. They concluded that alanine efflux is specific and the excretion of alanine appears to be saturable. In addition, uptake of pyruvate in *E. coli* K-12 is via a specific active transport system in which lactate and alanine are competitive inhibitors (Lang *et al*., 1987). Alanine and lactate exhibited a proportional increase in percent inhibition with increasing concentration. Thus, upon 12 hours of the alanine production, the excretion of alanine of all recombinant clones might reach their saturation which pyruvate uptake was almost completely inhibited by alanine.

Galkin *et al*., (1997) studied an enzymatic synthesis of L- and D-alanine by *E. coli* TG1 cells which expressed heterologous genes of *aladh* and *fdh*. They inserted *aladh* gene from *B. stearothermophilus* at downstream of *fdh* gene from *Mycobacterium vaccae* N10 under the tandem *lac-tac* promoter and used a plasmid vector pUC119 (pFDHAlaDH). They found that amount the specific activities in the clone cell extract were 1.2 U/mg for FDH and 7.3 U/mg for AlaDH. The resting cells of transformed *E. coli* TG1 was used as the catalyst for L-alanine production from pyruvate and ammonium formate. The amount of L-alanine produced increased as the concentration of pyruvate increased as the pyruvate concentration increased. Moreover, the optical purity of L-alanine decreased with prolonged incubation; the enatiomeric excess of L-alanine was 88% after 3 hours, while it was only 80% after 10 hours. This was probably due to the action of the alanine racemase produced by the host cells.

The level of alanine dehydrogenase in recombinant cells and the formation of L-alanine seem to be separately controlled by the addition of the inducing factor for promotion or concentration of substrate and inhibitor in the reaction. Nevertheless, *E. coli* BL21(DE3) containing co-existence of *aladh* and *fdh* (pETAF and pETFA) still has the advantage over pETAlaDH clone because the simultaneous of two enzymes in a single cell should provide additional benefit for industrial production of cell-free extract of the recombinant enzymes.

CHAPTER V

CONCLUSIONS

1. Clones of *aladh* gene in high expression vector pET-17b (pETAlaDH) and pMPM-K3 (pMPMAlaDH) had AlaDH activity about 6.2 and 4.6 fold of the activity obtained from the original clone in pUC18.
2. Clones of *fdh* gene in a vector pET-17b (pETFDH) had greater FDH activity than the original clone in pUC119 about 7.2 fold, while those of the clone containing *fdh* gene in pSY343 (pSYFDH) had the similar level of FDH activity as their original clones.
3. The level of FDH in the clones constructed by a heterologous gene expression system (pETFA and pETAF) was lower than those in the single gene system (pETFDH) about 3.3 fold, while AlaDH was not significantly different from that of the single gene system (pETAlaDH).
4. Clones containing pETAlaDH/pSYFDH constructed by cotransformation technique had less activity of AlaDH than pETAlaDH clone about 5.5 fold, while FDH could not be detected. In pMPMAlaDH/pETFDH clones the activity of AlaDH and FDH showed 8.7 and 2.6 fold lower than those activities from clones of single gene, pMPMAlaDH and pETFDH, respectively.
5. Production of alanine by various recombinant were not significantly different with about 50% yield and ratio of D:L alanine about 1.6:1.

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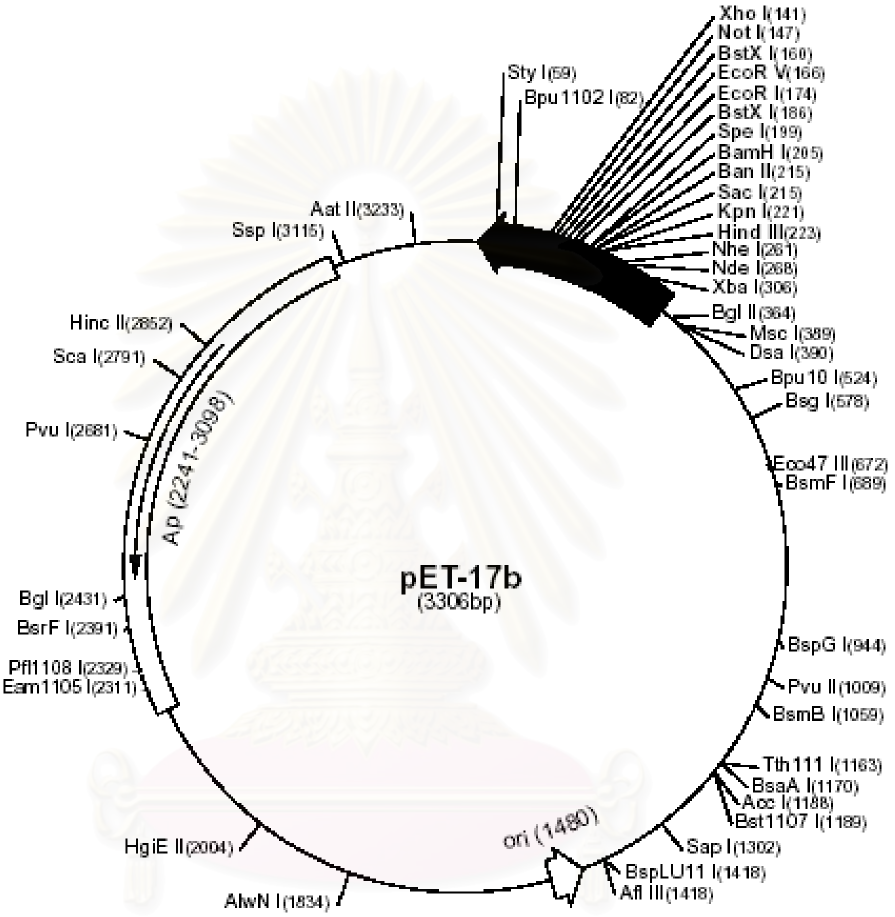
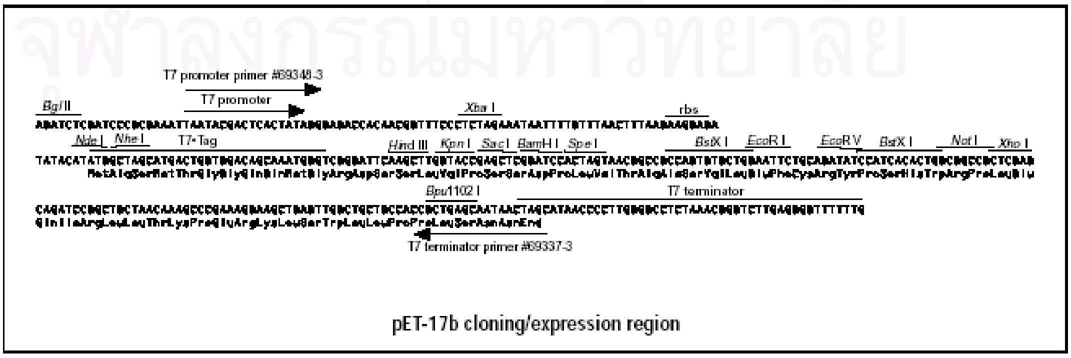
APPENDICES

APPENDIX A

Restriction map of pET-17b


Xho I(141)
Not I(147)
BstX I(160)
EcoR V(166)
EcoR I(174)
BstX I(186)
Spe I(199)
BamH I(205)
Ban II(215)
Sac I(215)
Kpn I(221)
Hind III(223)
Nhe I(261)
Nde I(268)
Xba I(306)
Bgl II(364)
Msc I(389)
Dsa I(390)
Bpu10 I(524)
Bsg I(578)
Eco47 III(672)
BsmF I(689)
BspG I(944)
Pvu II(1009)
BsmB I(1059)
Tth111 I(1163)
BsaA I(1170)
Acc I(1188)
Bst1107 I(1189)
Sap I(1302)
BspLU11 I(1418)
Afl III(1418)
ori (1480)
AlwN I(1834)
HgiE II(2004)
Eam1105 I(2311)
Pfl1108 I(2329)
BsrF I(2391)
Bgl I(2431)
Pvu I(2681)
Sca I(2791)
Hinc II(2852)
Ap (2241-3098)
Ssp I(3115)
Aat II(3233)
Sty I(59)
Bpu1102 I(82)
pET-17b
(3306bp)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T7 promoter
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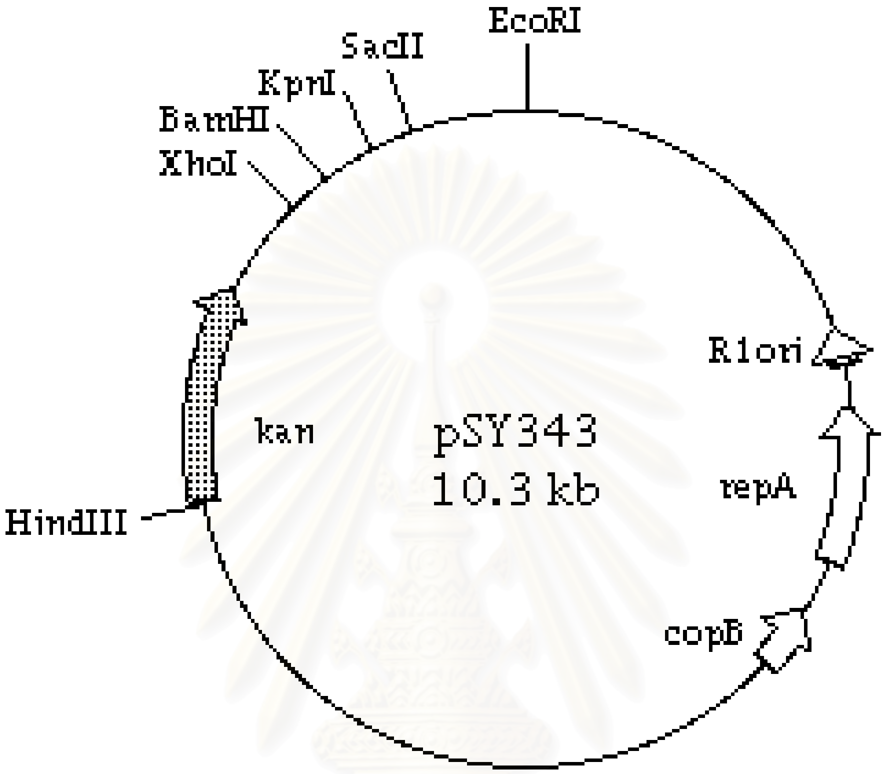
Xba I
rbs
Nde I
Nhe I
T7•Tag
Hind III
Kpn I
Sac I
BamH I
Spe I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Bpu1102 I
T7 terminator
T7 terminator primer #69337-3

pET-17b cloning/expression region

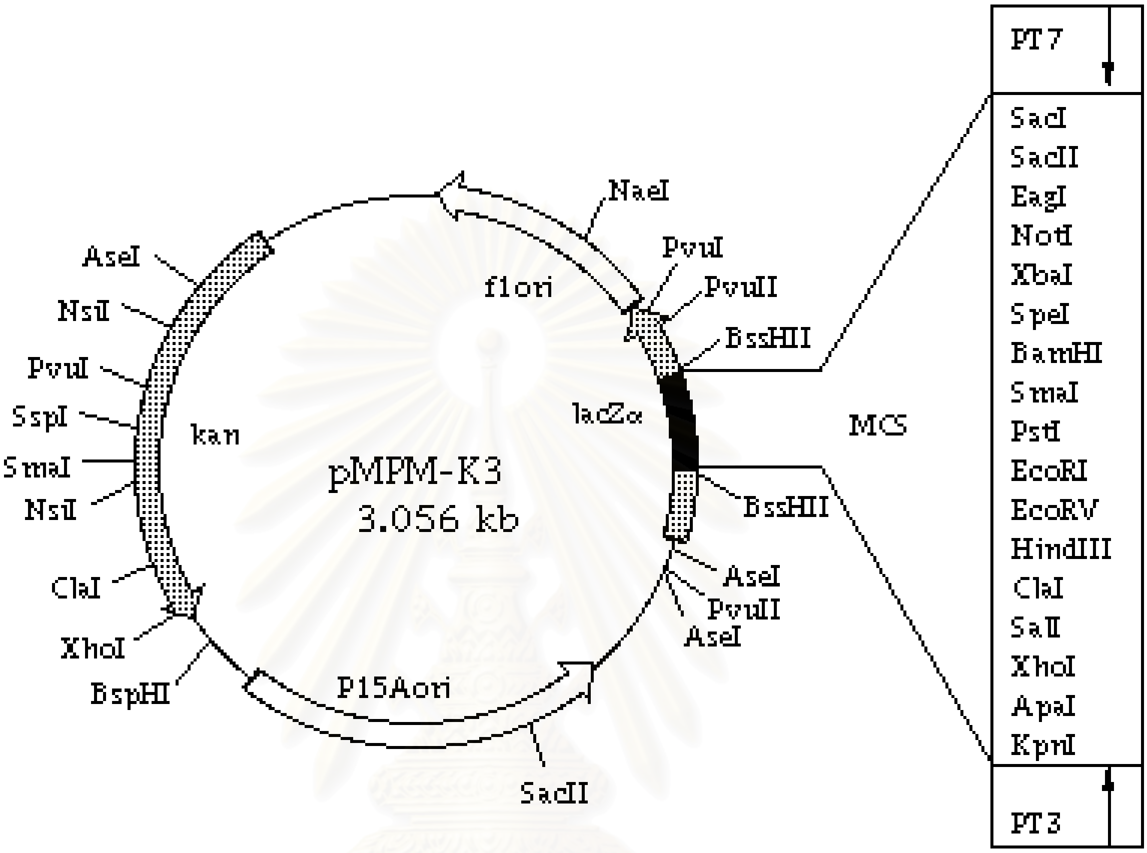
Restriction map of pSY343


EcoRI
SacII
KpnI
BamHI
XhoI
R1ori
kan
pSY343
10.3 kb
repA
HindIII
copB

Full List

Name	pSY343
Replicon	R1(copy mutant)
Size	10.3
Purpose	High Copy Number
Selectable Marker	kan
Cloning Site	BamHI EcoRI HindIII XhoI
Reference	Journal of Bacteriology 154, 1153-1161 (1983)
Source	S. Yasuda
Map	pSY343.gif

Restriction map of pMPM-K3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lacZα
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kan
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PvuI
SspI
SmaI
NsiI
ClaI
XhoI
BspHI
P15Aori
SacII
BssHII
AseI
PvuII
AseI
MCS
PT7
SacI
SacII
EagI
NotI
XbaI
SpeI
BamHI
SmaI
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
KpnI
PT3

Full List

Name	pMPM-K3
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Size	3.056
Purpose	Expression
Selectable Marker	kan
Other Genes	lacZalpha
Promoter	Plac, PT3, PT7
Cloning Site	ApaI BamHI EagI EcoRI EcoRV HindIII KpnI NotI PstI SacI SalI SpeI XbaI
Reference	Gene 163, 41-46 (1995)
Source	M. P. Mayer
Map	pMPM-K3.gif

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

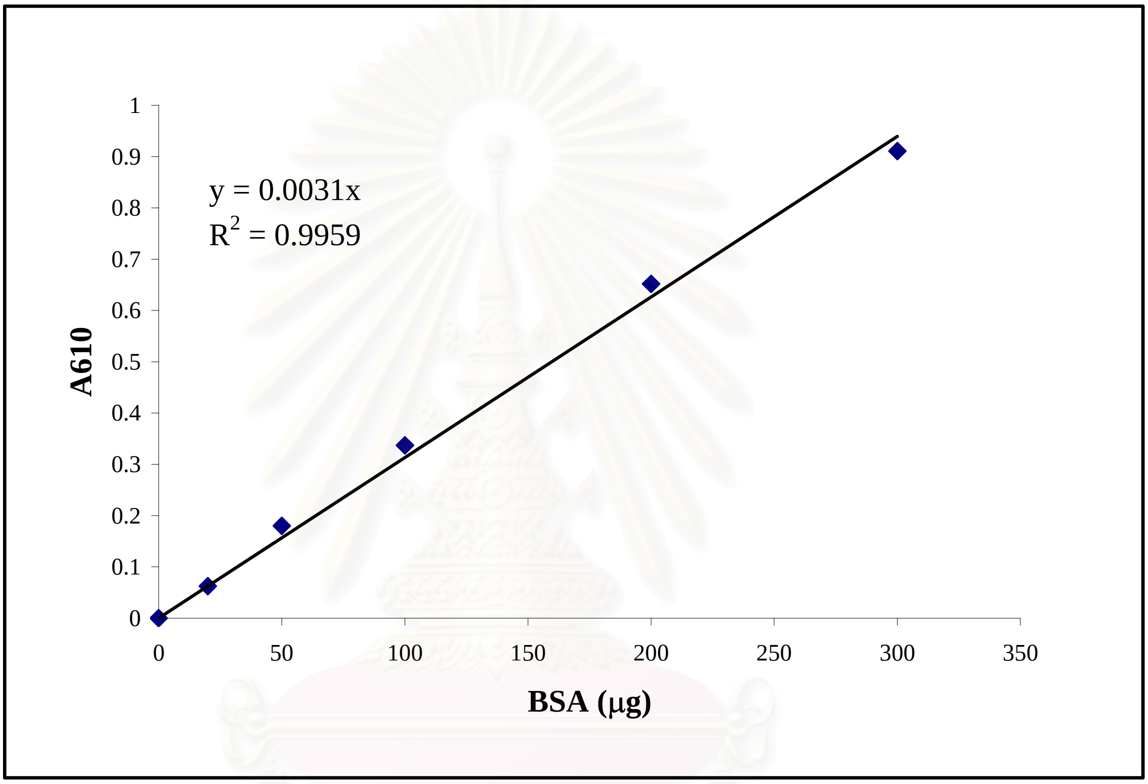
Reflex for 10 hour

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: (V/V) before using.

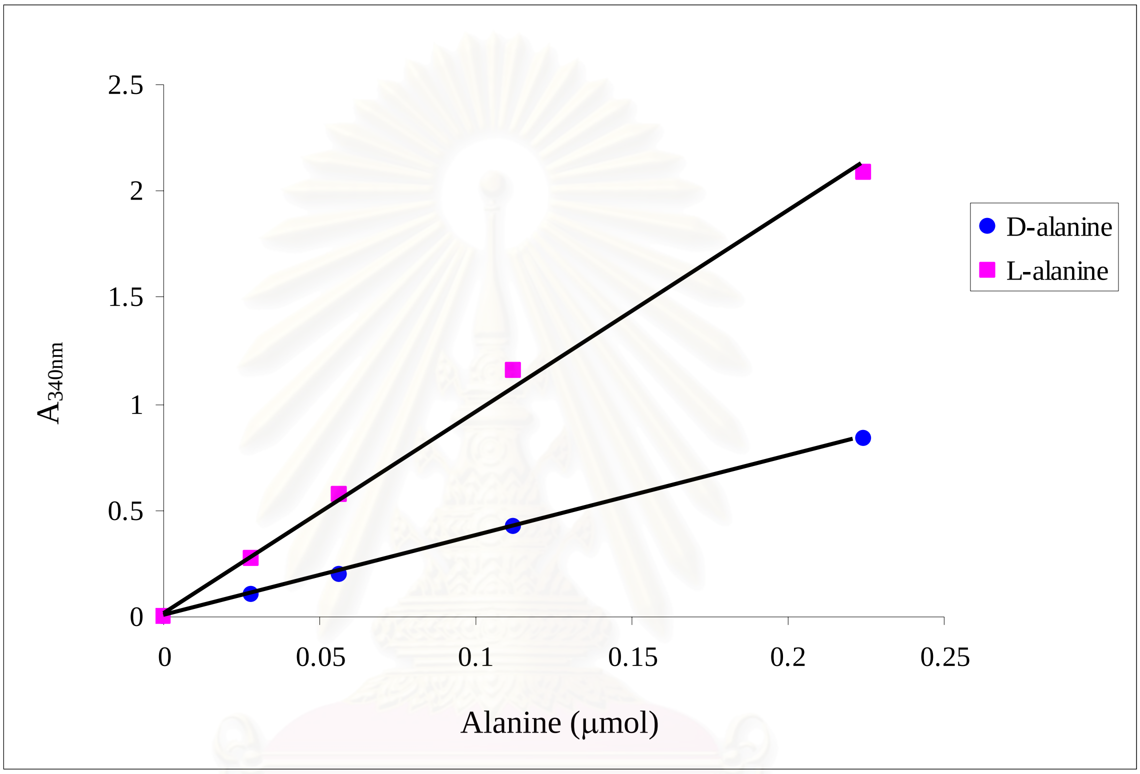
APPENDIX C

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



APPENDIX D

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



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)

Bromophenal 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 glycin, 0.1 % SDS

Tris (hydroxymethyl)-aminomethane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre 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 litre

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

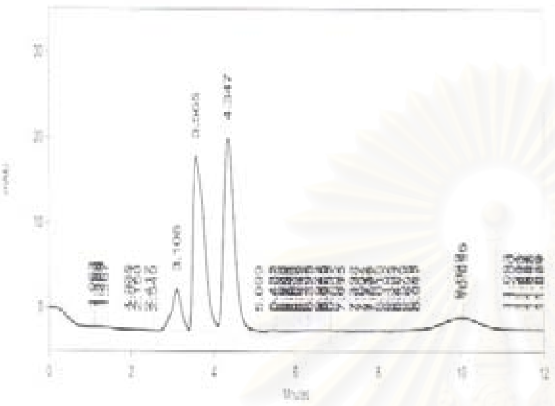
Destaining solution, 1 litre

Methanol	100	ml
Glacial acetic acid	100	ml
H_2O	800	ml

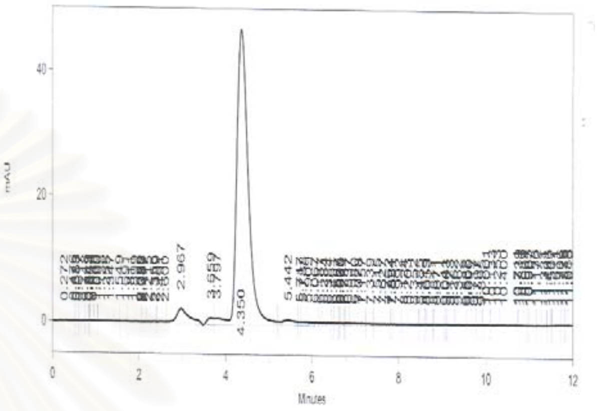
APPENDIX F

HPLC profile of D- and L-Alanine

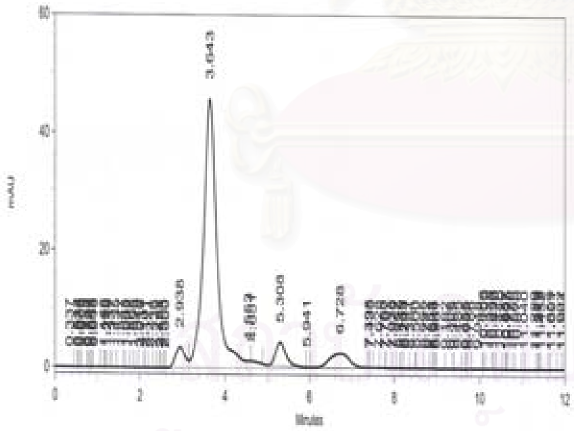
A) DL-alanine standard



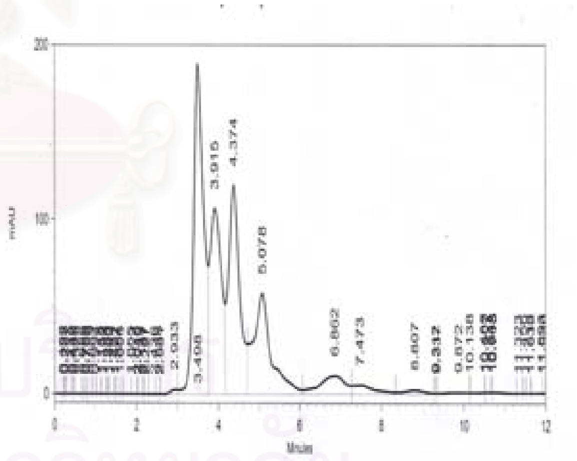
B) L-alanine standard



C) substrates (pyruvate, ammonium formate)



D) Sample



Condition: CROWNPAK CR(+) column

Eluent: perchloric acid aqueous solution (pH 1.5).

Absorbance 200 nm

BIOGRAPHY

Miss Rujirat Hatrongjitt was born on July 25, 1979 in Udonthani. She finished High school at Satri Rachinutit school, Udonthani. She graduated with the degree of Bachelor of Science from the department of Biochemistry at Kasetsart University in 2000. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 2001.