การวิเคราะห์หาลำดับนิวคลีโอไทด์และการโคลนยีนอะลานีนดีไฮโดรจิเนสจาก *Aeromonas hydrophila*

นางสาว นารถวดี ภูมิภาค

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NUCLEOTIDE SEQUENCING AND CLONING OF ALANINE DEHYDROGENASE GENE FROM *Aeromonas hydrophila*

Miss Natwadee Poomipark

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นารถวดี ภูมิภาค : การวิเคราะห์หาลำดับนิวคลีโอไทด์และการโคลนยีนอะลานีนดีไฮโดรจิเนสจาก *Aeromonas hydrophila* (NUCLEOTIDE SEQUENCING AND CLONING OF ALANINE DEHYDROGENASE GENE FROM *Aeromonas hydrophila*)
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อะลานีนดีไฮโดรจิเนส [EC 1.4.1.1] เป็นเอนไซม์ในกลุ่มอะมิโนแอซิดดีไฮโดรจิเนส เร่งปฏิกิริยาการดึงหมู่อะมิโนจากอะลานีนให้ได้ผลิตภัณฑ์คือไพรูเวทและแอมโมเนีย ซึ่งเป็นปฏิกิริยาที่ผันกลับได้ ปัจจุบันได้มีการนำอะลานีนดีไฮโดรจิเนสมาตรวจสอบวิเคราะห์หาปริมาณอะลานีนและสารอนุพันธ์ รวมทั้งนำมาใช้ในอุตสาหกรรมยาและอาหาร การวิจัยก่อนหน้านี้ได้ทำการศึกษาอะลานีนดีไฮโดรจิเนสจาก *Aeromonas hydrophila* ซึ่งเป็นแบคทีเรียที่ทำการคัดเลือกจากดินในกรุงเทพฯ และพบว่าเอนไซม์นี้ มีน้ำหนักโมเลกุล 230 กิโลดาลตัน ประกอบด้วย 6 หน่วยย่อยที่เหมือนกัน นอกจากนี้พบว่าลำดับกรดอะมิโนทางด้านปลาย N คือ MIIGVPKEIKNHEYRVGM VPASVRELTARNHTVFVQSGAGN และลำดับกรดอะมิโนบางส่วนภายในสายโปรตีนคือ LAEQGYRNLLSDP HLRHGLNVMAGKK ซึ่งในงานวิจัยครั้งนี้ใช้เป็นข้อมูลพื้นฐานในการออกแบบไพรเมอร์เพื่อเพิ่มชิ้นยีนบางส่วนของอะลานีนดีไฮโดรจิเนสด้วยเทคนิคพีซีอาร์ โดยใช้โครโมโซมอลดีเอ็นเอที่ผ่านการย่อยด้วย *Eco*RI หรือ *Hind*III เป็นดีเอ็นเอต้นแบบ จากนั้นนำผลิตภัณฑ์จากพีซีอาร์ที่ได้ไปวิเคราะห์หาลำดับนิวคลีโอไทด์และนำมาเป็นข้อมูลออกแบบไพรเมอร์ในการเพิ่มชิ้นยีนส่วนที่ยังไม่ทราบลำดับนิวคลีโอไทด์ จากการวิเคราะห์หาลำดับนิวคลีโอไทด์พบว่าชิ้นยีนอะลานีนดีไฮโดรจิเนสถอดรหัสพันธุกรรมของสายพอลิเปปไทด์ได้กรดอะมิโน 371 เรซิดิว ลำดับนิวคลีโอไทด์ของยีนอะลานีนดีไฮโดรจิเนส บริเวณปลายด้าน N และ C ได้ถูกนำมาออกแบบไพรเมอร์เพื่อทำการเพิ่มชิ้นยีนทั้งชิ้นและโคลนเข้าสู่ *E. coli* JM109 เมื่อวิเคราะห์สารละลายเอนไซม์ที่ได้จากเซลล์ *E. coli* JM109 ซึ่งได้รับชิ้นยีนอะลานีนดีไฮโดรจิเนสพบว่า เอนไซม์มีค่าแอคติวิตีจำเพาะอยู่ในช่วง 0-10 ยูนิต/มิลลิกรัมโปรตีน โดยโคลนที่มีแอคติวิตีสูงที่สุดจะมีค่าแอคติวิตีจำเพาะสูงกว่าสารละลายเอนไซม์จาก *Aeromonas hydrophila* ประมาณ 50 เท่า

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2543..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NATWADEE POOMIPARK: NUCLEOTIDE SEQUENCING AND CLONING OF ALANINE DEHYDROGENASE GENE FROM *Aeromonas hydrophila*
THESIS ADVISOR : KANOKTIP PACKDIBAMRUNG, Ph.D.,
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Alanine dehydrogenase [EC 1.4.1.1] catalyses the reversible deamination of L-alanine to pyruvate and ammonia. Nowadays, the enzyme is used for quantitating L-alanine and also used for synthesis of L-amino acid and its derivatives, which are used in drug and food industries. *Aeromonas hydrophila*, an isolate from soil in Bangkok, is one of the bacteria, which have high activity of alanine dehydrogenase. Previous work presented that the enzyme had molecular mass of about 230 kDa and consisted of six identical subunits. Amino acid sequence at N-terminus was known as MIIGVPKEIKN HEYRVGMVPASVRELTARNHTVFVQSGAGN and one of the internal amino acid sequences of this enzyme was LAEQGYRNLLSDPHLRHGLNVMAGKK. From the obtained data, the degenerated primers were designed for amplification of one part of alanine dehydrogenase gene by PCR technique using *Eco*RI or *Hin*dIII digested chromosomal DNA as a template. These PCR products were sequenced and the obtained data was used to design primers for amplification of other parts of the gene. The alanine dehydrogenase gene from *Aeromonas hydrophila* encodes for 371 amino acids residues. The whole gene was amplified by using primers designed from N-terminal and C-terminal nucleotide sequences and then cloned into *E. coli* JM109. The specific activity of the crude extracts from recombonant clones were about 0 to 10 units/ mg protein. The specific activity of the clone that has highest activity was about 50 times more than that of *Aeromonas hydrophila*.

Department.....Biochemistry..... Student's signature.....Natwadee Poomipark.....

Field of study.....Biochemistry..... Advisor's signature.....Kanoktip Packdibamrung.....

Academic year.....2000..... Co-advisor's signature.....

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ABBREVIATIONS

A	2'-deoxyadenosine (in a DNA sequence)
alaDH	alanine dehydrogenase
bp	base pair
BSA	bovine serum albumin
C	2'-deoxycytidine (in a DNA sequence)
°C	degree Celsius
cm	centimetre
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
G	2'-deoxyguanosine (in a DNA sequence)
IPTG	isopropyl-thiogalactoside
kb	kilobase
LB	Luria-Bertani
µg	microgram
µl	microlitre
min	minute
ml	millilitre
mM	millimolar
ng	nanogram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
rpm	revolution per minute
SDS	sodium dodecyl sulphate
T	2'-deoxythymidine (in a DNA sequence)
TB	tris-borate buffer
TE	tris-EDTA buffer
UV	ultraviolet

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CHAPTER I

INTRODUCTION

Redox reactions are important steps in the metabolism and energy conversion of living cells. Besides the necessity of enzymes, a number of coenzymes are involved in such reactions: ferrodoxins, lipoic acid, NAD(H), NADP(H), flavins and cytochromes. The coenzymes differ in their redox potential, in the binding constants and the mode of regeneration. NADH/NADPH are the most frequently encountered coenzyme. In general they dissociate easily and need a second reaction with another metabolite for regeneration. These properties are important for the flow of intermediates in response to biosynthetic needs. Because of wide range properties of the coenzyme moiety, dehydrogenases have been extensively studied in the past and have found widespread applications in clinical and food analysis.

The chiral compounds such as hydroxy acids, amino acids or alcohols from prochiral precursors have a high economic value and find direct applications in food and feed, or serve as building block in syntheses of therapeutics, herbicides and insecticides. To develop enzyme-catalyzed synthesis of such chiral compounds further, additional dehydrogenases with a suitable substrate ranges as well as efficient coenzyme regeneration systems are required (Hummel and Kula, 1989).

Amino acids are micromolecules 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 (Lehninger, 1993). The second one is rarely found in organism while the first one play important role in all life. L-amino acids are the building block of enzyme, hormone, antibody and oxygen-transported protein. Furthermore, amino acids can also balance buffering capacity in blood (Holum, 1982) and often function as chemical messengers in the communications between cells (Bender, 1975)

Nowadays, the using of L-amino acid for many compounds synthesis are spread widely in industries. About L-alanine, besides the using in drug, it can be used as food additive because of its sweet taste (Suye *et al.*, 1992). In addition, there are many reports described the using of L-alanine in the synthesis of DOPA (dihydroxyphenylalanine) 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 cause the decreasing in producing of DOPA, subsequently, the defect of melanin will occur. The defect of DOPA also cause Pakinson's disease, but the using of DOPA drug can release the effect of this disease by repairing the activity of nervous system. The derivative of L-alanine like (*N*-(*p*-chlorobenzoyl)-2-(2-cyanoethyl))alanine also used for control immune system and protected the development of tumor (Nagano *et al.*, 1985) while L-phenylalanine was used as precursor in sweetener production e.g. aspartam (Ohshima and Soda, 1979).

From the requirement of L-amino acid in various applications, there were many groups of researchers who were interested in the production of L-amino acid by chemical reaction, however, the products contained equal amount of D-formed and L-formed amino acid (Takai, 1992). Therefore, some of researchers attempted to produce L-amino acid by enzymatic method e.g. L-amino acid transaminase, amino acid racemase (Berberich *et al.*, 1968) and L-amino acid β-decarboxylase (Yamamoto *et al.*, 1980). For L-amino acid dehydrogenases, they are also widely studied and the enzymes, which prepared from microorganism, are widely used for synthesis and measurement of L-amino acid in the samples (Ohshima and Soda, 1979).

Many kinds of nicotinamide-dependent L-amino acid dehydrogenase have been found (Table 1.1). They catalyze the reversible deamination of amino acid to α-keto acid and ammonia. This reaction is specific to L-isomer of amino acid as shown in Figure 1.1. The participation of $NAD(P)^+$ makes these enzyme systems a valuable tool for analysis of L-amino acids or their corresponding oxo acids. By reductive amination of the oxo acid, L-amino acid can be obtained in a lot of yield because the equilibrium of the reaction favours amino acid formation.

Among amino acid dehydrogenases shown in Table 1.1, L-alanine dehydrogenase is the first enzyme that has been studied (Yoshida and Freeze, 1964 cited in Hummel and Kula, 1989). L-alanine dehydrogenase catalyzes the reversible deamination of L-alanine to pyruvate as shown in Figure 1.2. This enzyme has been found in spores and vegetative cells of various bacteria. Some strains of *Bacillus* especially reveal high levels of alanine dehydrogenase and some of these have been used for the preparation of the enzyme. Alanine dehydrogenase is a key enzyme in the degradation of L-alanine ; the resulting pyruvate can easily be metabolized via the tricarboxylic acid cycle. Like leucine dehydrogenase, alanine dehydrogenase in spores seems to be responsible for generation of energy during sporulation. Screening for the distribution

Table 1.1 The group of L-amino acid dehydrogenases

Enzyme	E.C.number	Coenzyme
AlaDH	1.4.1.1	NAD^+
GluDH	1.4.1.2	NAD^+
GluDH	1.4.1.3	$NAD(P)^+$
GluDH	1.4.1.4	$NADP^+$
SerDH	1.4.1.7	NAD^+
ValDH	1.4.1.8	$NAD(P)^+$
LeuDH	1.4.1.9	NAD^+
GlyDH	1.4.1.10	NAD^+
3,5-diamino-hexanoate DH	1.4.1.11	NAD^+
2,4-diamino-pentanoate DH	1.4.1.12	$NAD(P)^+$
LysDH	1.4.1.15	NAD^+
Diamino-pimelate DH	1.4.1.16	$NADP^+$
PheDH	1.4.1.-	NAD^+
TyrDH	1.4.1.-	$NAD(P)^+$

Source : Ohshima and Soda, 1979

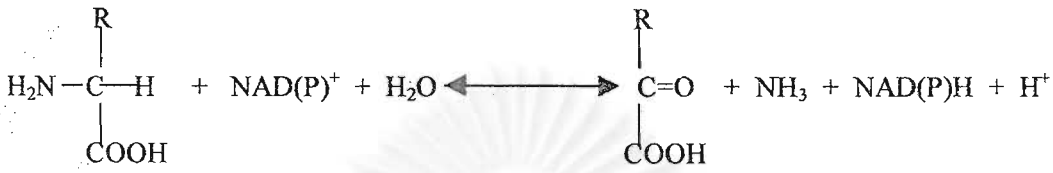


Figure 1.1 The general reaction of L-amino acid dehydrogenase

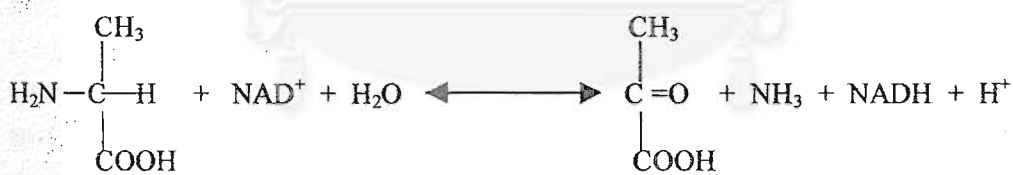


Figure 1.2 The reaction of L-alanine dehydrogenase

of alanine dehydrogenase in bacteria by Ohshima *et al.* (1990) revealed high enzyme activities in *Bacillus sphaericus*, *B. aneurinolyticus*, *B. circulans* and *B. cereus*. The specific activity in the crude extract ranged between 0.15-0.41 units/mg. This homogeneous enzyme from *B. sphaericus* has been prepared after seven purification steps. The enzyme has a molecular mass of 230 kDa and composes of six identical subunits. The enzyme oxidizes quite specifically for L-alanine (K_m 18.9 μM), only slight activity is found for L-2-amino-butyrate, L-serine, L-norvaline, and L-valine. The pH optimum for the reductive amination is around 9.0 and between 10.0-10.5 for the oxidative deamination.

In 1977, Edward and Peter purified and characterized alanine dehydrogenase from *Halobacterium cutirubrum* and found that 100-fold purified enzyme had a molecular mass of about 72.5 kDa. The activity of enzyme was increased when temperature increased, but it was not stable and the enzyme worked well in solution that had positively ions like K^+, Na^+ and NH_4^+. In 1987, Bellion and Tan purified alanine dehydrogenase from methylotrophic bacterium *Pseudomonas* sp. which grown in succinate and ammonium chloride media. The enzyme was purified about 400-fold with a 214 kDa of molecular mass and consisted of four identical subunits. It was specific for NAD^+ and ammonium and showed maximal activity at about pH 9.0 for reductive amination reaction.

In 1988, Vancurova *et al.* purified and characterized the alanine dehydrogenase from *Streptomyces aureofaciens* and found that it had a molecular mass of 395 kDa and consisted of 8 subunits. The suitable pH for the reaction is closely with those from other reports. Caballero *et al.* purified and characterized the enzyme from phototrophic bacterium *Rhodobacter capsulatus* E1F1 in 1989. The enzyme had a molecular mass of about 210 kDa and consisted of 4 subunits which different from *S. aureofaciens*. In 1990, Ohshima *et al.* purified and characterized the enzyme from thermophilic *Bacillus sphaericus* DSM 462 and found that properties of the enzyme was closely with *Bacillus*, but it was more stable at high temperature.

In 1994, Sawa *et al.* purified and characterized the alanine dehydrogenase from cyanobacterium *Phormidium lapideum* and found that the molecular mass and the amount of subunits were closely with that of *Bacillus*, but the suitable pH for reactive amination and oxidative deamination reaction were 8.4 and 9.2, respectively. In 1998, Chowdhury *et al.* found that alanine dehydrogenase from *Enterobacter aerogenes* had molecular mass of about 245 kDa and consisted of 6 identical subunits. The enzyme shows maximal activity at about pH 10.9 for the deamination

of L-alanine and at about pH 8.7 for the amination of pyruvate. The *E. aerogenes* enzyme is stable and has a lower K_m for L-alanine (0.47 mM) than those of the *Bacillus* enzymes. In addition, Chowdhury *et al.* also cloned alanine dehydrogenase from *E. aerogenes* by PCR technique and sequenced the gene and was found that the deduced amino acid sequence had high similarity to the sequences of other alanine dehydrogenases. Recently, in 1999, the cold-adapted alanine dehydrogenase from *Shewanella* sp. was studied by Galkin *et al.* and found that the enzyme had molecular mass of 240 kDa and consisted of 6 identical subunits like *Bacillus* enzymes. They also cloned the gene and compared amino acid sequence of the enzyme with those of other bacterial sources and found the high identity with *Vibrio proteolyticus*, a mesophilic gram-negative bacterium belonging to the same group in the γ-subdivision of class *Proteobacteria* as *Shewanella* sp. The alanine dehydrogenases from various sources have different in properties depending on the kinds of bacteria as shown in Table 1.2.

Alanine dehydrogenase shows pro-R(A-) stereospecificity for hydrogen transfer from C-4 position of the nicotinamide moiety of NADH to the substrate while the other amino acid dehydrogenases show pro-S (B-) stereospecificity except lysine dehydrogenase that is pro-R like L-alanine dehydrogenase as shown in Figure 1.3.

Even though the alanine dehydrogenase is the first enzyme in the group of amino acid dehydrogenase, which have been studied, very scarce information is available when compared with those of other amino acid dehydrogenases especially in the part of cloning, sequencing and protein structure. Amino acid sequences of alanine dehydrogenase from *B. sphaericus* and *B. stearothermophilus* were compared with those of other amino acid dehydrogenases by Kuroda *et al.* (1990) and were suggested that gly-175, gly-178, gly-181 and asp-198 of alanine dehydrogenase might be the binding region of nicotinamide coenzyme. Furthermore, the chemical modification of alanine dehydrogenase in *B. subtilis* was studied (Delforge *et al*, 1997) and the amino acid sequence was also compared with the enzyme from *Mycobacterium tuberculosis* and *Synechocystis* sp.. From this report, lys-74 was found to be the substrate binding site and the enzyme probably had a different active site from other B-stereospecific amino acid dehydrogenase, since alanine dehydrogenase was A-stereospecific and no sequence similarity was found between alanine dehydrogenase and other B-stereospecific amino acid dehydrogenase such as glutamate dehydrogenase and leucine dehydrogenase, except the coenzyme binding site. The three dimensional

Table 1.2 Some properties of alanine dehydrogenase from different sources

Source	Mr(x10^3 Da) (subunit structure)	Degree of Purification	K_m values(mM)				
			L-ala	NAD^+	pyr	NH_3	NADH
Aeromonas hydrophila	230(6x40,000)	x100	20.0	0.17	1.33	77	0.25
Bacillus subtilis	228(6x38,000)	x356	1.73	0.18	0.54	38	0.023
Bacillus sphaericus	230(6x38,000)	x340	18.9	0.23	1.7	28	0.010
Bacillus cereus	255(6x42,000)	-	-	-	-	-	-
Bacillus stearothermophilus (*Escherichia coli* cloned cell)	235(6x39,465)	x30	-	-	-	-	-
Thermus thermophilus	290(6x48,000)	x85	4.2	0.12	0.75	59	0.035
Streptomyces clavuligerus	92(monomer)	x38	9.1	0.5	1.1	30	0.14
Streptomyces phaeochromogenes	240(6x39,000)	x20	7.1	0.036	0.29	61	0.047
Streptomyces aureofaciens	395(8x48,000)	x714	5.0	0.11	0.56	6.7	0.029
Streptomyces fradiae	205-210(4x51,000)	x1180	10.0	0.18	0.23	12	0.050
Pseudomonas sp. (methylotrophic)	214(4x53,000)	x400	-	-	-	-	-
Desufvibrio desuficans	-	x56	-	-	-	-	-
Halobacterium cutirubrum	72.5(monomer)	x100	K_m values are salt dependent				
Halobacterium salinarium	60(monomer)	x500	K_m values are salt dependent				
Anabaena cylindrica	270(6x43,000)	x700	0.4	0.014	0.11	8-133	-
Rhizobium lupini bacteroids	180(4x41,000)	-	-	-	-	-	-
Rhodobacter capsulatus E1F1 (phototrophic)	246(6x42,000)	x50	1.25	0.15	0.13	16	0.25 (NADPH)
Phormidium lapideum	240(6x41,000)	-	5.0	0.04	0.33	60.6	0.02
Enterobacter aerogenes	245(6x40,000)	x23	0.47	0.16	0.22	66.7	0.067
Shewanella sp.	240(6x40,000)	-	7.6	0.24	-	-	-

Source : modified from Ohshima and Soda, 1979

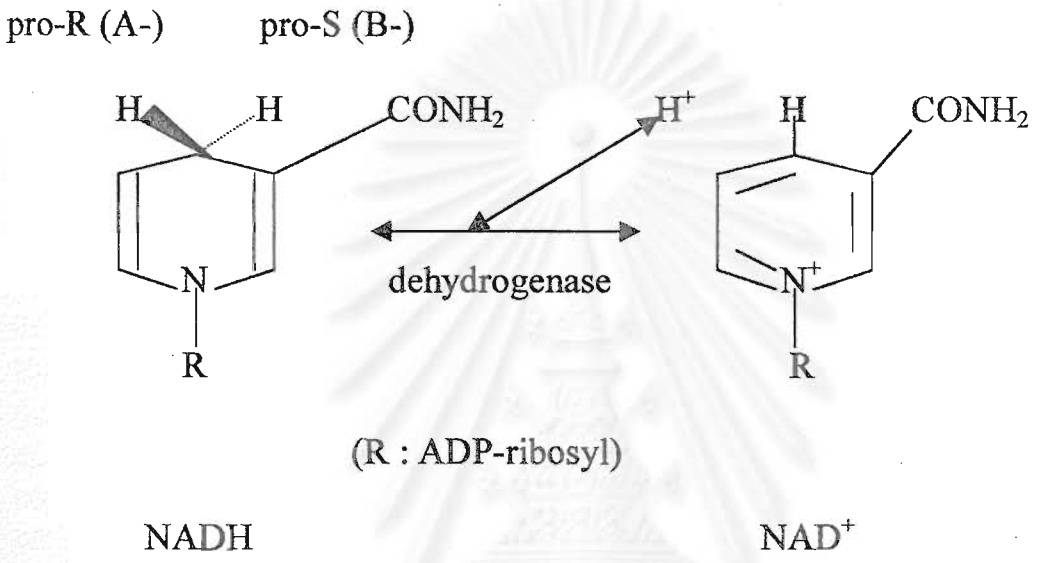


Figure 1.3 Stereospecificity of the transferring of NADH-hydrogen atom catalyzed by alanine dehydrogenase

structure of alanine dehydrogenase was firstly studied by Baker *et al.* (1998). In this report, the alanine dehydrogenase from *Phormidium lapideum* was studied and found that the subunit was constructed from two domains. Each subunit having the common dinucleotide binding fold. The refined structure of the L-alaDH-NAD^+ binary complex reveals that the NAD^+ binds to the C-terminal end of the strands in domain 2 in a manner similar to that seen in other dehydrogenase. From the 27 residues that contact with the NAD^+, 13 residues are identical in all of the alanine dehydrogenase. Baker *et al.* also proposed that all residues involved in binding with pyruvate were conserved residues. About the active site of alanine dehydrogenase, arg-15 may show the property in stabilizing the pyruvate carboxyl, his-95 should be used for the acid/base catalysis and positively charged residue lys-74 should be used for polarize the carbonyl of substrate.

For cloning, there are few reports about alanine dehydrogenase available. Recently, polymerase chain reaction (PCR) technique has been applied for sequencing and cloning of amino acid dehydrogenase gene. Since only two short peptide sequences of the enzyme are required for design the initial primers. It makes more convenience and takes less time when compared with genomic library cloning method. Therefore, PCR cloning and nucleotide sequencing are interesting for this research.

The polymerase chain reaction (PCR) consists of repeated cycles of template denaturation, primer annealing and extension to amplify a segment of DNA located between two primers. The selectivity and specificity of the PCR on two specific primers itself restricts the application of conventional PCR to amplification of unknown genes. A modification of PCR, which is known as 'inverse PCR', has been invented to amplify an unknown gene which juxtaposes a known region. The general application of this technique is shown in Figure 1.4, DNA containing the sequence of interest is digested by a suitable restriction enzyme to produce a 2,000 to 3,000 nucleotide restriction fragment containing the known 'target' sequence flanked by two regions of unknown sequence. After digestion, the DNA is ligated and the ligated DNA circles are then amplified by PCR. Two oligonucleotides are designed which anneal to opposing strands but which direct *Taq* polymerase-catalyzed DNA synthesis away from one another, in contrast to the usual PCR amplification. For this reaction, the method was dubbed 'inverse' PCR. New DNA strands primed by either oligonucleotide primer extends round the circularized DNA, eventually incorporating a complementary copy of the other primer. A linear strand of DNA is

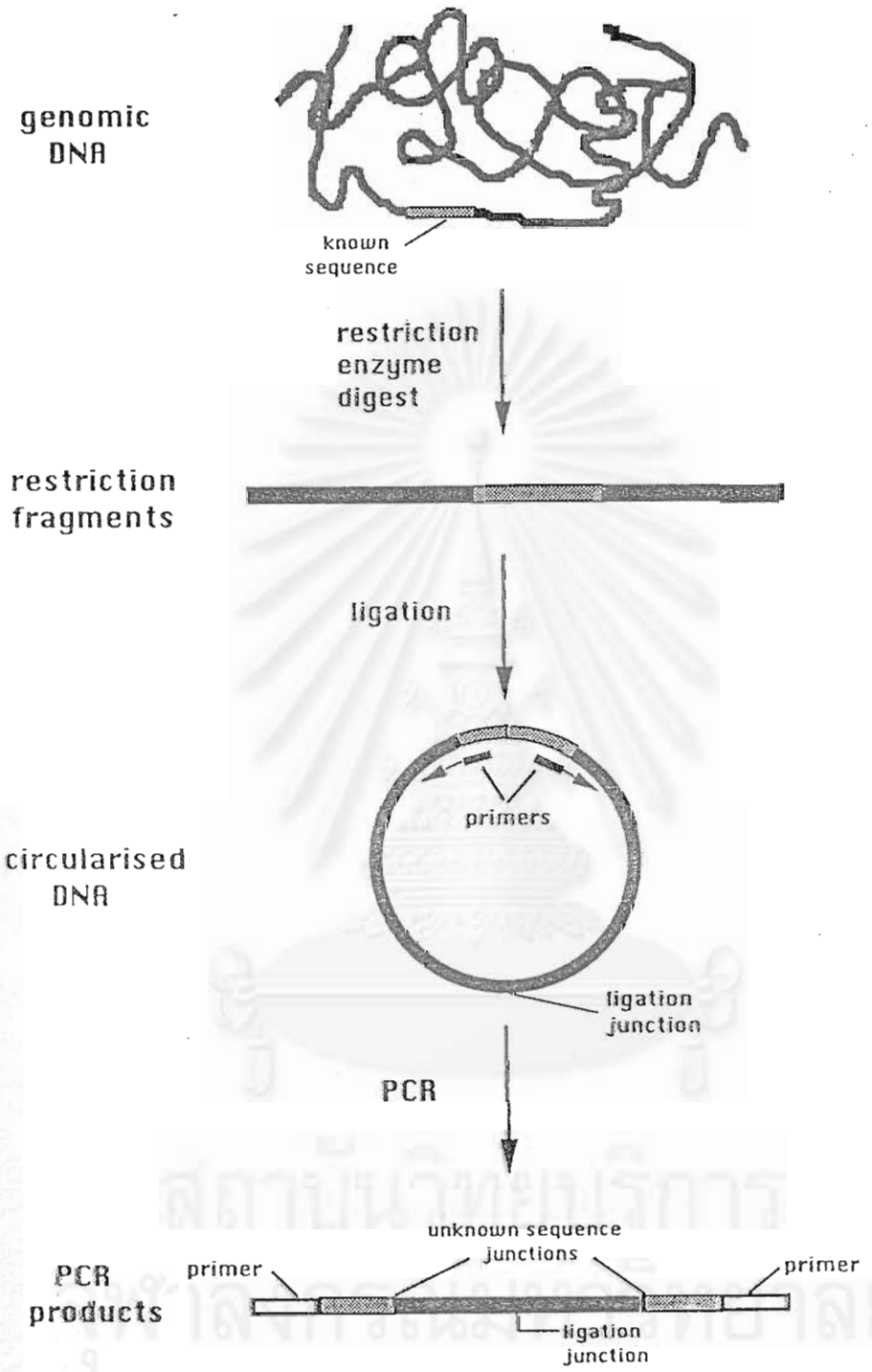


Figure 1.4 Principle of inverse PCR.

produced. This new DNA contains opposing primer sites and is now a new PCR template, which is amplified in the usual way (Eeles *et al.*, 1993)

Alanine dehydrogenase has been used in L-alanine production. The most popular process that used to produce L-alanine is ultrafiltration membrane reactor. The system contains L-alanine dehydrogenase, formate dehydrogenase and NAD^+ which binds with polyethyleneglycol by covalent bond ($PEG\text{-}NAD^+$) so the hybrid molecules can not pass through the membrane. The reaction is started by adding formic acid and formate dehydrogenase which catalyze reaction to form PEG-NADH. The reaction produces more stable NADH and uses low cost in production. Then the reaction is continued by passing pyruvate (pyr) and ammonium formate (NH_4HCO_2) through reactor. L-alanine and carbondioxide gas are taken as products (Yamamoto *et al.*, 1980). The reaction is shown in Figure 1.5.

In addition, L-alanine dehydrogenase can be used with other enzymes for D-amino acid producing called multi-enzyme system. This system contains L-alanine dehydrogenase, formate dehydrogenase, alanine racemase and D-amino acid aminotransferase. The D-amino acid aminotransferase is selected to use because of its high specificity for substrate while L-alanine dehydrogenase and formate dehydrogenase are used for L-alanine and NADH producing, respectively (Galkin *et al.*, 1997). The reaction is shown in Figure 1.6.

According to the system above, many kinds of D-amino acid such as D-glutamate, D-methionine and D-valine can be produced. The D-amino acid products will be further used in chemical synthesis of β-lactam antibiotics and bioactive peptides.

The analysis of amino acid, α-keto acid and ammonia by using of amino acid dehydrogenase are important in medicine, bioprocess control and nutrition. Furthermore, the method is easier and cheaper when compared with ion-exchange high performance liquid chromatography method. The variation in detection of amino acid is depended on type of enzyme. For L-alanine dehydrogenase, besides in the measurement of the amount of L-alanine and pyruvate, 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 patients who suffered from this disease have abnormal level of this enzyme and can be detected the occurred L-alanine by using γ-glutamyl cyclotransferase as shown in Figure 1.7 (Ohshima and Soda, 1990).

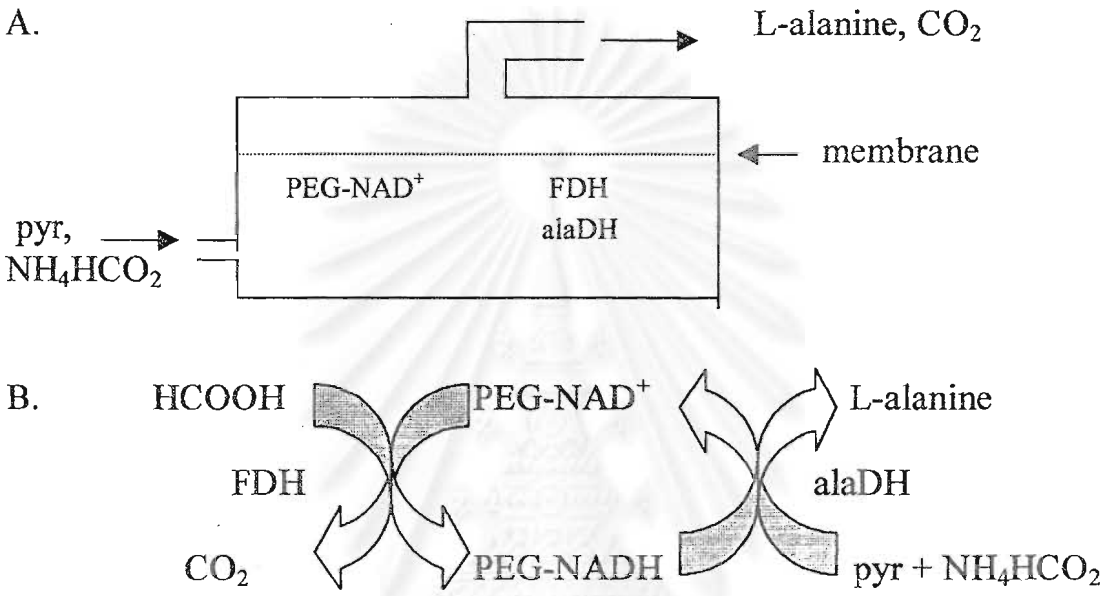


Figure 1.5 The production of L-alanine by ultrafiltration membrane reactor

A. enzyme membrane reactor

B. the reaction of L-alanine producing catalyzed by enzyme

alaDH : alanine dehydrogenase

FDH : formate dehydrogenase

PEG : polyethyleneglycol

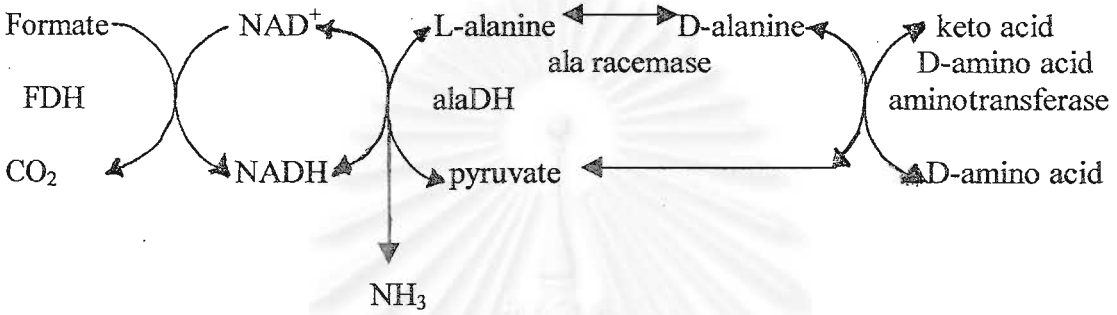


Figure 1.6 Multi-enzyme system in the production of D-amino acid
alaDH : alanine dehydrogenase
FDH : formate dehydrogenase
ala racemase : alanine racemase

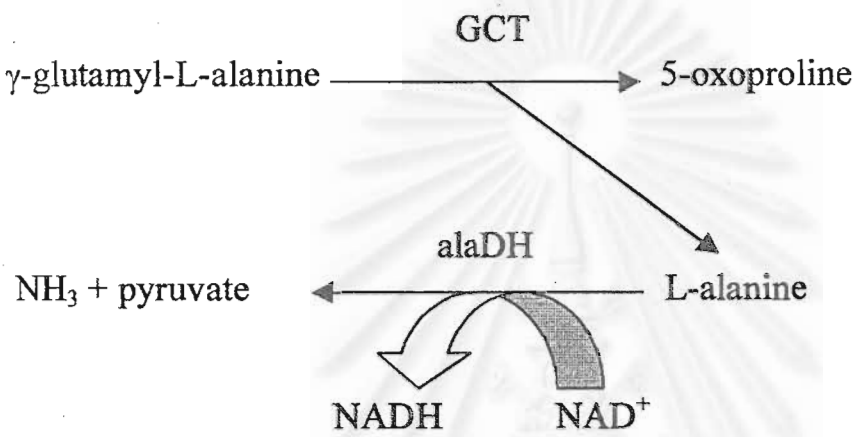


Figure 1.7 The detection system of γ-glutamyl cyclotransferase
GCT : γ-glutamyl cyclotransferase
alaDH : alanine dehydrogenase

According to *Aeromonas hydrophila*, screened from soil in Bangkok (Phungsangthum, 1997), is one of bacteria which have high activity of alanine dehydrogenase. The previous research presented that alanine dehydrogenase 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°C, respectively. Enzyme activity remains high when incubated at 55°C 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. N-terminal amino acid sequence of the enzyme is MIIGVPKEIKNHEYRVGMVPA SVRELTARNHTVFVQSGAGN and one part of the internal amino acid sequences of this enzyme is LAEQGYRNLLSDPHLRHGLNVMAGKK (Phungsangthum, 1997). From the amino acid sequences, they are used as the data for design the degenerated primers for the initial PCR amplification of alanine dehydrogenase gene from *A. hydrophila*. This amplified fragment will be used as template for DNA sequencing and its result will be further used as a primary data for the next amplification, which led to cloning this gene in the final step.

Since alanine dehydrogenase from *Aeromonas hydrophila* has not been reported, the studies about cloning and sequencing of this gene are required for further studies in developing the properties of the enzyme for the suitable process in industries of Thailand.

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
Electrophoresis unit: Hoefer™ miniVE, Amersham Pharmacia Biotech., U.S.A.; 2050 MIDGET, LKB, Sweden ; Mini protein, Bio-Rad, U.S.A. and Submarine agarose gel electrophoresis unit
Gene Pulser™: Bio-Rad, U.S.A.
Heating box: Type 17600 Dri-Bath, Thermolyne, U.S.A.
Incubator: Model 1H-100, Gallenkamp, England
Incubator shaker: Model G-76, New Brunswick Scientific Co., Inc., U.S.A.
Incubator, water bath: Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc., U.S.A.
Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.
Membrane filter: cellulose nitrate, pore size 0.45μm, Whatman, England
Microwave oven: Model TRX1500, Turbora International Co., Ltd., Korea
pH meter: Model PHM95, Radiometer Copenhegen, Denmark
Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.
Sonicator: Model W375, Heat systems-ultrasonics, U.S.A.
Spectrophotometer: Spectronic2000, Bausch&Lomb, U.S.A. ; UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.
Thermal cycler: GeneAmp PCR system 2400, Perkin Elmer Cetus, U.S.A.
UV transluminator: Model 2011 Macrovue, San Gabriel California, U.S.A.
Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.

2.2 Chemicals

Acrylamide: Merck, Germany
Agar: Merck, Germany
Agarose: SEKEM LE Agarose, FMC Bioproducts, U.S.A.
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
Coomasie brilliant blue R-250: Sigma, U.S.A.
di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy
di-Sodium ethylenediaminetetra acetate: M&B, England
DNA marker: Lamda (λ) DNA digested with *Hind* III, GIBCOBRL, U.S.A.
85% Phosphoric acid: Mallinckrodt, U.S.A.
Ethidium bromide: Sigma, U.S.A.
Ethyl alcohol absolute: Carlo Erba Reagenti, Italy
Ficoll type 400: Sigma, U.S.A.
Glacial acetic acid: Carlo Erba Reagenti, Italy
Glycine: Sigma, U.S.A.
Glucose: BDH, England
GeneAmp PCR core reagents: Perkin Elmer Cetus, U.S.A.; GIBCOBRL, U.S.A. and TAKARA SHUZO Co., Ltd., Japan
Hexadecyl trimethyl ammonium bromide: Sigma, U.S.A.
Hydrochloric acid: Carlo Erba Reagenti, Italy
Isopropylthio-β-D-galactosidase(IPTG): Sigma, U.S.A.
Isoamyl alcohol: Merck, Germany
Isopropanol: Merck, Germany
L-alanine: Sigma. U.S.A.
Magnesium sulphate 7-hydrate : BDH, England
N,N-dimethyl-formamide: Fluka, Switzerland
N,N'-methylene-bis-acrylamide: Sigma, U.S.A.
NNN'N'-tetramethyl-1,2-diaminoethane (TEMED): Carlo Erba Reagenti, Italy

Nicotinamide adenine dinucleotide (oxidized form) (NAD^+): Sigma, U.S.A.
Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan
Peptone from casein pancreatically digested: Merck, Germany
Phenazine methosulfate: Nacalai Tesque, Inc., Japan
Phenol: BDH, England
Potassium chloride: Merck, Germany
Potassium phosphate monobasic: Carlo Erba Reagenti, Italy
QIAquick Gel Extraction Kit: QIAGEN, Germany
Riboflavin: BDH, England
Sodium acetate: Merck, Germany
Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy
Sodium citrate: Carlo Erba Reagenti, Italy
Sodium chloride: Carlo Erba Reagenti, Italy
Sodium dodecyl sulfate: Sigma, U.S.A.
Sodium hydroxide: Merck, Germany
Sucrose: Sigma, U.S.A.
Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy
Yeast extract: Scharlau microbiology, European Union

2.3 Enzymes and Restriction enzymes

Lysozyme: Sigma, U.S.A.
Proteinase K: Sigma, U.S.A.
Restriction enzymes: GIBCOBRL, U.S.A. and New England BioLabs, Inc., U.S.A.
RNase: Sigma, U.S.A.
Taq DNA Polymerase: GIBCOBRL, U.S.A. and TAKARA SHUZO Co., Ltd., Japan
T_4 DNA ligase: GIBCOBRL, U.S.A.

2.4 Bacterial strains and plasmids

Aeromonas hydrophila was screened from soil in Bangkok (Piyarat, 1997).

Escherichia coli JM109, genotype: F' [*tra*D36 *pro*AB^+ *lac*I^q *lacZ*ΔM15] *recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) was used as a host for transformation.

pUC18 was used as a vector for cloning and transformation into *E. coli* JM109 (Appendix A).

2.5 Chromosomal DNA Extraction

Chromosomal DNA of *Aeromonas hydrophila* was prepared by using the method modified from Frederick *et al.* (1995). A single colony of the bacterium was grown in 10 ml of 1% peptone medium (1% peptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4.7H_2O$ and 0.01% yeast extract, pH 7.2) with 250 rpm rotary shaking at 30°C for 24 hrs. The cell culture was centrifuged in each 1.5 ml microfuge tubes for 5 min at 10,000 rpm. Then cell pellet was collected and resuspended in 567 µl of TE buffer pH 8.0 (10 mM Tris-HCl and 1 mM EDTA) by repeated pipetting. The cell solution was then treated with 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K, gently mixed and incubated at 37°C for at least 2 hrs. After incubation, it was added with 100 µl of 5 M NaCl and mixed thoroughly. Subsequently, 80 µl of CTAB-NaCl solution (10% CTAB and 0.7 M NaCl) was added, then mixed and incubated at 65°C for 10 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added and gently mixed by inversion. The mixture was centrifuged at 12,000 rpm for 10 min. The upper-phased liquid was transferred to a new tube. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, then mixed and centrifuged for 10 min at 12,000 rpm. The upper-phased liquid was transferred to a new tube and extracted with phenol-chloroform-isoamyl alcohol as above again. The upper-phased liquid was transfered to a new tube and then 0.6 volume of isopropanol was added to precipitate DNA. The tube was inverted several times and then placed at –20°C for at least 30 min. The mixture was centrifuged at 12,000 rpm for 10 min, then supernatant was discarded and the pellet was washed with 100 µl of 70% ethanol. The tube was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was allowed to air-dry. The DNA was finally dissolved in an appropriate volume of RNase-TE buffer (1 mg of RNase in 1 ml of TE buffer). The amount of DNA was estimated by submarine agarose gel electrophoresis compared with known amount of λ DNA/*Hin*dIII marker.

2.6 PCR Amplification

2.6.1 Primers

The first set of primers that used for the first internal-part gene fragment amplification were degenerated primers designed by using the data of N-terminal and internal amino acid sequence (see

introduction) of alanine dehydrogenase. The example of primer design is shown in figure 2.1. The next two series of primers were designed from the nucleotide sequences. The sequences of all primers are shown in Table 2.1.

2.6.2 Template

2.6.2.1 Templates for amplification of internal alanine dehydrogenase gene

Chromosomal DNA was digested with *Eco*RI or *Hin*dIII. The reaction mixture containing 5 μg of chromosomal DNA, 1x reaction buffer for *Eco*RI or *Hin*dIII and 10 U of *Eco*RI or 10 U of *Hin*dIII in total volume of 50 μl was incubated at 37°C for overnight. After incubation, the 5 μl of 3 M sodium acetate, pH 5.2 was added, then mixed and an equal volume of isopropanol was added for DNA precipitation. The tube was centrifuged at 12,000 rpm for 10 min. Then supernatant was discarded and the 70% ethanol was added to pellet for washing. The tube was centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was dried briefly. The DNA was finally dissolved in 10 μl of sterile distilled water. A 0.5 μl (250 ng) of the DNA solution was used as template in each reaction of PCR.

2.6.2.2 Templates for amplification of N-terminal and C-terminal region of alanine dehydrogenase gene (inverse PCR)

Chromosomal DNA was completely digested with each restriction enzyme : *Bgl*II, *Bst*EII, *Eco*RI, *Hin*dIII, *Nde*I, *Pst*I, *Pvu*II, *Spe*I and *Sph*I. The reaction mixture containing 5 μg of chromosomal DNA, 1x reaction buffer for each enzyme and 10 U of each enzyme in total volume of 50 μl was incubated at 37°C overnight, except for *Bst*EII digestion, the reaction was incubated at 60°C. After incubation, the 5 μl of 3 M sodium acetate (pH 5.2) was added, then mixed and an equal volume of isopropanol was added for DNA precipitation. The tube was centrifuged at 12,000 rpm for 10 min. Then supernatant was discarded and the 70% ethanol was added to pellet for washing. The tube was centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was dried briefly. The DNA was finally dissolved in 10 μl of sterile distilled water. Then the DNA solution was ligated by T_4 DNA ligase. The reaction mixture containing 10 μl of DNA solution, 1x ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5%(w/v) polyethylene glycol-8000)

N-terminal amino acid sequence :
MIIGVPKEIKNHEYRVGMVPASVRELTARNHIVFVQSGAGNGIGF
SDADYLAAGAEILA

Amino acid sequence which used for design primer :
KEIKNHEY (Lys Glu Ile Lys Asn His Glu Tyr)

Codon usage of *A. hydrophila* (sorted by the often usage) :

Lys	Glu	Ile	Lys	Asn	His	Glu	Tyr
AAG	GAG	ATC	AAG	AAC	CAC	GAG	TAC
AAA	GAA	ATT	AAA	AAT	CAT	GAA	TAT
		ATA					

Nucleotide sequence of primer (5'→3') :
AAR GAR ATT AAR AAY CAY GAR TA (64 combinations)

Note : Y = C,T R = A,G N = A,C,G,T

Figure 2.1 Flow chart for degenerated primer design

Table 2.1 Nucleotide sequence and T_m of all primers used in alanine dehydrogenase amplification

Primer	Sequence (5'→3')	T_m (°C)	Remark
NF1	AARGARATTAARAAYCAYGARTA	49.2	For the first internal gene fragment amplification and sequencing
NF2	CCSAARGARATCAARAAYCAYGA	47	"---------"
NF3	AAYCAYACTGTTTTYGTNCAR	52.7	"---------"
CR1	CCTGCCATTACRTTTARNCCRTG	62.3	"---------"
CR2	SGCGTTGCGRTASCCCTGYTCNGC	60	"---------"
CR3	GCRTTTCTRTATCCYTGYTCNGC	61.9	"---------"
NF4	GCCAGTGTACGTGAACTGACAGCACGAAA CCATACC	67.7	For the second internal gene fragment amplification and sequencing
NF5	GCCAAGGCGGAGATGATCGTCAAG	63	"---------"
NF6	AAGGTCAAGGAGCCCCAGGCGGTC	66.4	"---------"
CR4	CTTGATGATGAAGGGCAGGGTGGCGTTGT TCAGGGC	69.9	"---------"
NR0	GCCCGGACGCAGCATGGCGCGCTCGACCG CCTGGGG	79	For N-terminal and C-terminal gene fragment amplification and sequencing
NR	GGCAGCCAGATAATCTGCGTCACTGAACC CAATGCC	68.8	"---------"
NR2	GAACCCAATGCCATTTCCTGCGCCGCTTT GGACGAA	68.8	"---------"
CF0	GTGGACGTGGCCATCGATCAGGGCGGCTG CGTCGAG	74.5	"---------"
CF	ACCTTCATTGTCGACGACGTGGTGCACTA CTGCGTG	68.8	"---------"
CF2	GCCGTGGCACGCACCTCCACCGTGGCCCT GAACAAC	74.5	"---------"
N-ECO	GGGAATTCAGGAAACAGACCATGATTATC GGTGTACCTAAGG	66.5	For the whole gene fragment amplification
C-HIN	GGAAGCTTCAGTTCAGCAGGGTCAGGGGA TCCGTGTAGGCCA	72.4	"---------"

Note : N = A, C, G, T
R = A, G
Y = C, T

and 10 U of T_4 DNA ligase in a total volume of 20 µl was incubated at 16°C for 16 hours, then precipitated and dissolved in sterile water as the direction above. After that, the DNA product in each reaction was completely digested with *Kpn*I. The reaction mixture containing DNA solution, 1x reaction buffer for *Kpn*I and 10 U of *Kpn*I in total volume of 20 µl was incubated at 37°C overnight, then precipitated and dissolved in 10 µl sterile water as above. The 2 µl of DNA solution was used for each reaction of PCR.

2.6.2.3 Templates for amplified the whole gene fragment

Chromosomal DNA was digested with *Hin*dIII. The reaction mixture containing 5 µg of chromosomal DNA, 1x reaction buffer for *Hin*dIII and 10 U of *Hin*dIII in total volume of 50 µl was incubated at 37°C overnight. The 5 µl of 3 M sodium acetate, pH 5.2 was added, then mixed and an equal volume of isopropanol was added for DNA precipitation. The tube was centrifuged at 12,000 rpm for 10 min. After centrifugation, the supernatant was discarded and the pellet was washed with 70% ethanol. Then the tube was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was dried briefly. The DNA pellet was finally dissolved in 10 µl of sterile distilled water. The 1 µl (500 ng) of DNA solution was used as template for each reaction of PCR.

2.6.3 PCR condition

The 4 parts of alanine dehydrogenase gene were amplified by 4 steps of PCR as shown in Figure 2.3 and the conditions of each part of PCR was described in Table 2.2.

The 50 µl of reaction mixture contained 2.5 U of *Taq* DNA polymerase, 2.5 mM each dNTPs, 1X PCR buffer, 1.0-2.0 mM $MgCl_2$, DNA template and 10 pmole of each primer, except in the first internal gene fragment amplification, 100 pmole of each primer was used.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate DNA fragments on the basis of their molecular sizes. Appropriate amount of agarose was weighed and mixed with TBE buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) and

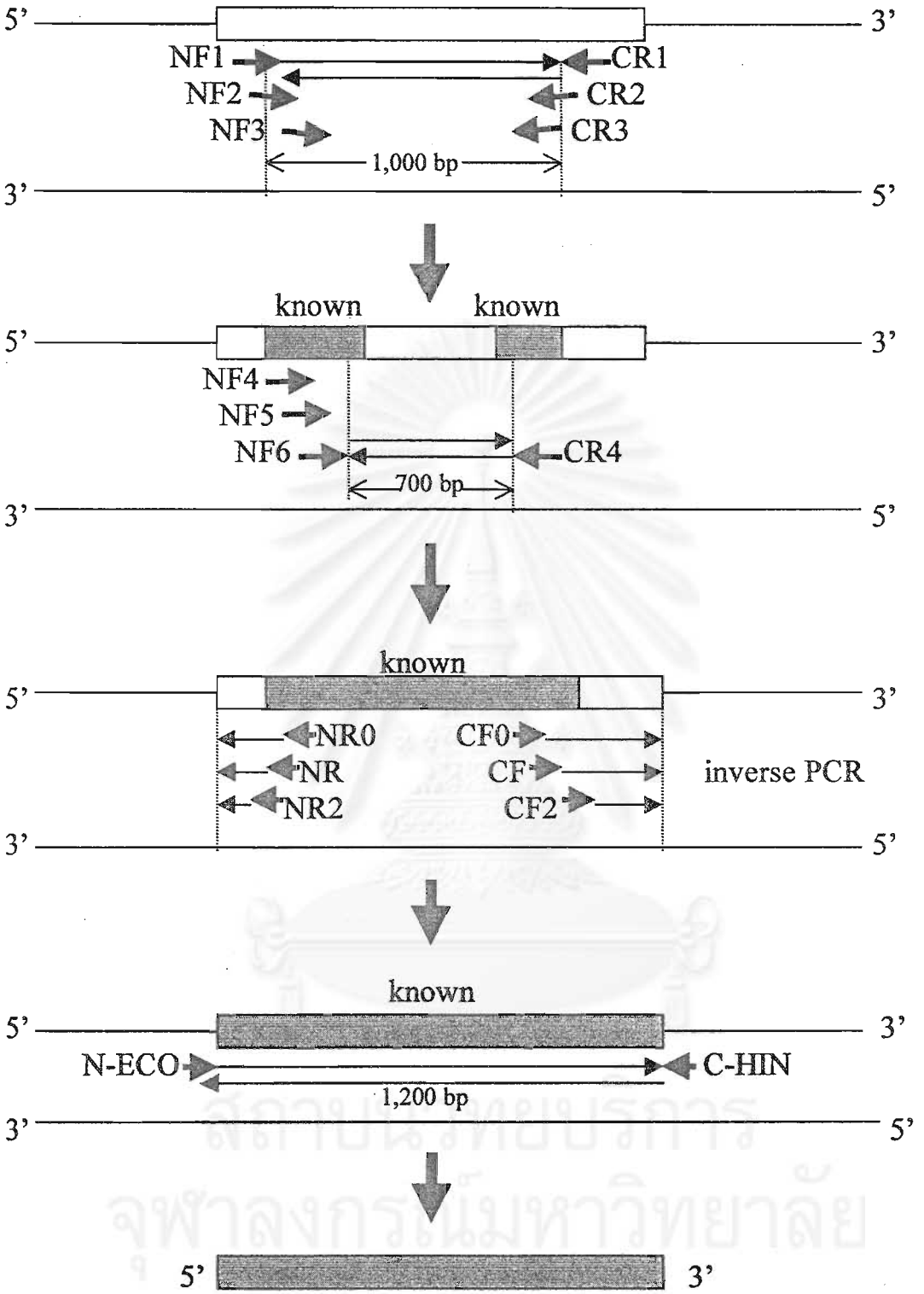


Figure 2.2 Strategy for PCR amplification and sequencing of alanine dehydrogenase gene of *Aeromonas hydrophila*

The alanine dehydrogenase gene is boxed. The primers for PCR are shown by thick arrows. The sequencing strategy is indicated by thin arrows.

Table 2.2 PCR condition in each step

The region of gene fragment which amplified	Primer pairs	Predenaturation	Denaturation	Annealing	Extension	Final extension	Number of cycle
The first internal gene fragment	NF1xCR1, NF1xCR3, NF3xCR1, NF3xCR3 and NF2xCR2	94°C for 10 min.	94°C for 1 min.	37°C for1 min.	72°C for2 min.	72°C for 10 min.	40
The second internal gene fragment	NF4xCR4, NF5xCR4 and NF6xCR4	94°C for 10 min.	94°C for 1 min.	60°C for1 min.	72°C for2 min.	72°C for 10 min.	40
N-terminal and C-terminal gene fragment	NR0xCF0, NRxCF and NR2xCF2	94°C for 10 min.*	94°C for 1 min.	50°C for 30 sec.	72°C for2 min.	72°C for 7 min.	30
The whole gene fragment	N-ECOxC-HIN	94°C for 10 min.*	94°C for 1 min.	50°C for 30 sec.	72°C for2 min.	72°C for 7 min.	30

* After predenaturation for 10 min., the *Taq* DNA polymerase was added.

heated until complete solubilization in a microwave oven. The agarose solution was incubated at 60°C until all air bubbles were completely eliminated. The solution was then left at room temperature to 50°C before pouring into an electrophoresis mould. After the gel was completely set, it was prechilled in a refrigerator for at least 30-60 minutes before using. The comb and seal of the mould were carefully removed. When ready, one-fifth volume of the loading dye (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) was mixed with each DNA sample before loading into the well. Electrophoresis was operated at 100 V until bromphenol blue migrated to approximately 2 cm from the bottom of the gel. The gel was stained with a 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 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 and relative mobility of the standard DNA fragment.

2.8 Nucleotide sequencing

The specific bands of amplified DNA fragment were purified from agarose gel by using QIAQuick gel extraction kit and then used for nucleotide sequencing. The nucleotide sequence of the amplified DNA fragment was determined after dideoxynucleotide chain termination using an Applied Biosystems 373A DNA sequencer with a PRISM kit (Perkin Elmer, U.S.A.)

2.9 Recombinant DNA preparation

2.9.1 Vector DNA preparation

The plasmid pUC18 was digested with *Eco*RI and *Hin*dIII. The reaction mixture containing 5 μg of pUC18, 1x reaction buffer of *Eco*RI (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of *Eco*RI and 10 U of *Hin*dIII in total volume of 100 μl. The reaction mixture was incubated at 37°C for overnight and then harvested only linear-formed pUC18 from agarose gel electrophoresis by using QIAQuick gel extraction kit.

2.9.2 The alanine dehydrogenase gene fragment preparation

After the amplification of the whole gene, the 4 reactions of PCR product were pooled (200 µl) and cleaned up by adding 1 µl of 10% SDS and 1 µl of 20 mg/ml proteinase K, mixed and incubated at 65°C for 15 min. The 20 µl of 3 M sodium acetate was added and mixed. After that, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added for protein extraction and mixed thoroughly. The tube was centrifuged at 12,000 rpm for 10 min and the upper-phased liquid was transferred to new tube. Then 2 volume of absolute ethanol was added and placed at –20°C for precipitation at least 30 min. The tube was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol, then centrifuged again as above. The pellet was dried and dissolved in 50 µl of sterile distilled water.

According to the amplification of this fragment, the primer N-ECO which contained *Eco*RI site and the primer C-HIN which contained *Hin*dIII site were used, therefore the above cleaned gene fragment was further digested with *Eco*RI and *Hin*dIII. The reaction containing 50 µl of gene fragment, 1x reaction buffer for *Eco*RI (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of *Eco*RI and 10 U of *Hin*dIII in a total volume of 100 µl was incubated at 37°C overnight and then the gene fragment was purified from agarose gel by using QIAQuick gel extraction kit.

2.9.3 Ligation of vector DNA and the gene fragment

The vector DNA from 2.9.1 and the gene fragment from 2.9.2. were ligated. The reaction mixture containing 50 ng of vector DNA, 150 ng of the gene fragment, 1x ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5%(w/v) polyethylene glycol-8000) and 10 U of T_4 DNA ligase (GIBCOBRL) in a total volume of 20 µl was incubated at 16°C for 16 hours and further used for transformation.

2.10 Transformation

A portion of the ligation mixture was transformed into host cell *E.coli* strain JM109. Competent cells were prepared for electroporation as described by Dower (1988). A fresh overnight culture of *E.coli* JM109 was inoculated into 1 litre of LB broth with 1 volume of overnight culture to 100 volume of LB broth. Cells were grown to log phase (OD_{600} of 0.5 to 0.8) at 37°C with vigorous shaking. To harvest,

cells were kept on ice for 15 to 30 minutes, and then centrifuged at 4,000xg for 15 minutes at 4 °C. The cell pellet was resuspended in 1 litre of cold water and centrifuged as above. Then, the cell pellets were resuspended in 0.5 litre of cold water and centrifuged again. After the centrifugation, cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged as above. The cell pellets were resuspended to a final volume of 2 to 3 ml in 10% glycerol. This suspension was stored at –70 °C. In the electro-transformation process, cuvettes and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to the 25 μF capacitor, 2.5 kV, and the Pulse controller unit was set to 200 Ω. The competent cells were gently thawed on ice. One to five microlitre of DNA from 2.9.3 was mixed with 40 μl of the competent cells. This mixture was transferred to a cold, 0.2 cm electroporation cuvette, and shaken to the bottom of the cuvette. The cuvette was applied one pulse at the above settings. One millilitre of LB medium was added immediately to the cuvette. The cells were quickly resuspended with a Pasteur pipette. The cell suspension was transferred to polypropylene tube and incubated at 37°C for 1 hour with 225 rpm shaking. This suspension was spread onto the LB agar plates containing 100 μg/ml ampicillin, 25 μg/ml isopropylthio-β-D-galactosidase (IPTG) and 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). Cells were grown at 37°C overnight. Bacterial clones carrying recombinant plasmids which formed white colonies were picked and selected for transformants harbouring alanine dehydrogenase gene by measuring of the enzyme activity.

2.11 Plasmid extraction (Sambrook *et al*, 1992)

The *E. coli* JM 109, which had recombinant plasmids was grown in LB medium (1% peptone, 0.5% NaCl and 0.5% yeast extract, pH 7.2) containing 100 μg/ml ampicillin at 37°C overnight. The cell culture was centrifuged in each 1.5-ml microfuge tube for cell pellet collecting at 10,000 rpm for 5 min. Then 100 μl of solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) which containing 5 mg/ml lysozyme was added and the pellet was resuspended by repeated pipetting and left at room temperature for 10 min. After that, the 200 μl of solution II (0.2 N NaOH and 1% SDS) was added, gently mixed and placed on ice for 10 min. Then the 150 μl of cooled solution III (3 M sodium acetate, pH 4.8) was added, gently mixed and placed on ice for 10

min. The mixture was centrifuged at 10,000 rpm for 10 min and then the supernatant was transferred to new tube. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, then mixed and centrifuged at 12,000 rpm for 10 min. The upper-phased liquid was transferred to a new tube. For DNA precipitation, the 2 volume of cool 95% ethanol was added, then mixed and placed at –20°C at least 30 min. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was dried briefly. The DNA was finally dissolved in an appropiate volume of RNase-TE buffer (1 mg of RNase in 1 ml of TE buffer). Plasmids were completely digested with *Eco*RI and *Hin*dIII. The size of recombinant plasmid was estimated by submarine agarose gel electrophoresis compared with relative mobility of λ DNA/*Hin*dIII marker.

2.12 Crude extract preparation

The *E. coli* JM 109, which contained recombinant plasmids was grown in 10 ml LB-medium containing 100 μg/ml ampicillin at 37°C overnight. After that, 10 % of the cell culture was inoculated into 100 ml LB-medium containing 100 μg/ml ampicillin and 75 μg/ml isopropylthio-β-D-galactosidase (IPTG). Cell culture was grown at 37°C for 20 hrs. The cell pellet was collected by centrifuged at 8,000 rpm for 15 min, then washed with 0.85% NaCl and centrifuged as above. After that the cell pellet was washed with extraction buffer (0.1 M potassium phosphate buffer, pH 7.4, 0.1 mM PMSF (phenyl methyl sulfonyl fluoride), 0.01 % DTT (dithiothreitol) and 1.0 mM EDTA (ethylene diamine tetraacetic acid) and centrifuged again. The cell pellet was placed at –80°C until the next step. Before sonication, the cell pellet was resuspended with 5 ml of extraction buffer. The sonication was done for 5-min pulse at the combination of 5-sec pulse and 2-sec stop condition. The crude extract was collected by centrifuged at 10,000 rpm for 45 min and store at 4°C.

2.13 Activity Assay

The assay system for the deamination contained 20 μmole of L-alanine, 1 μmole of NAD^+, 200 μmole of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5 and enzyme in a final volume of 1.0 ml. Incubation was done at 30°C in a cuvette with a 1-cm length path.

The reaction was started by addition of NAD^+ and monitored by measuring the initial changes in the absorbance at 340 nm.

One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmole of NADH per minute.

2.14 Protein measurement

Protein solution (maximum 100 μl) was pipetted into tube. Distilled water was added to make a total volume of 100 μl. Then 1 ml of Bradford working buffer (see Appendix B) was added and mixed by vortex. A_{595} was read after 2 minutes but before 1 hour. Bovine serum albumin was used as standard protein.

2.15 Polyacrylamide gel electrophoresis

2.15.1 Pouring the separating gel (7.7% acrylamide)

The gel sandwich was assembled according to the manufacturer's instruction. For 2 slabs gel, the 2 ml of solution A (0.23%TEMED(*N,N,N',N'*-tetramethylethylene diamine), 1.5 M Tris-HCl, pH 8.9) was combined with 4 ml of solution C (30.8% acrylamide) and 10 ml of distilled water in a small flask. The 22.4 mg of ammonium persulphate was added and mixed by swirling or inverting container gently. Carefully introduced solution into gel sandwich using a pipet. When the appropriate amount of separating gel solution has been added, gently layered about 1 cm of water on top of the separating gel solution. The gel was allowed to polymerize about 1 hour. The water covering the separating gel was poured off.

2.15.2 Pouring the stacking gel

The 0.5 ml of solution B (0.46% TEMED and 0.5 M Tris-HCl, pH 6.7) was combined with 1.0 ml of solution D (12.5% acrylamide), 0.5 ml of solution E (0.04% Riboflavin) and 2.0 ml of solution G (40% sucrose) in a small flask. The solution was mixed by gently swirling or inverting the container. This stacking gel solution was loaded onto separating gel until solution reached top of front plate. Then the comb was carefully inserted into gel sandwich. Fluorescence lamp was used for the polymerization of stacking gel about 1 hour. After stacking gel had been polymerized, the comb was removed carefully. Then the gel was placed into electrophoresis chamber. Solution F (0.05 M Tris and 0.384 M glycine, pH 8.3) was added into the reservoir. The air bubbles, which were occured in the well should be removed.

2.15.3 Sample preparation

The protein sample was combined with 5X tracking dye (0.05% bromphenol blue and 40% sucrose). Then the sample solution was loaded into well by using syringe.

2.15.4 Running the gel

An electrode plugs were attached to proper electrodes. Current should be flowed towards the anode for pH 8.8 gels. The power supply was turned on to 100-200 V (constant current). For activity staining electrophoresis, the experiment was done at 4°C. Electrophoresis should be continued until the dye front migrated to the bottom of the gel. Power supply was turn off and then the electrode plugs were removed from electrodes. The gel plates were removed from electrode assembly. Then a spacer was removed and inserted the spacer in one corner between the plates.

2.15.5 Staining Procedure

Protein staining

The gel was picked up and transferred to a small container containing Coomassie Stain (0.04% coomassie brilliant blue G-250 and 3.5% $HClO_4$).The gel was agitated for 5-10 minutes on a slow rotary or rocking shaker. The stain solution was poured out and the Coomassie destain solution (7% acetic acid) was added. The gel was continued slow shaking. To complete destain, the destain solution should be changed and agitated overnight or until the blue-clearly bands of protein were occurred.

Activity staining (Gabriel, 1971 cited in Bollag *et al.*, 1993)

After electrophoresis at 4°C was done, the gel was transferred to a small container containing activity staining solution (4.25 mmole of Tris-HCl, pH 8.5, 40 μmole of L-alanine, 50 μmole of NAD^+, 250 μg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium) and agitated for 15 minutes. The purple band of alanine dehydrogenase would be occurred and then destained with distilled water.

CHAPTER III

RESULTS

3.1 *Aeromonas hydrophila* DNA Extraction

Total DNA was extracted by using the method of Federick *et al.*(1995). DNA was determined for its quality and quantity by agarose gel electrophoresis (Figure 3.1). It was found that extracted DNA had molecular weight greater than 23.1 kb. The $OD_{260/280}$ ratio was in the range of about 1.8-2.5 indicating high purity. The DNA concentration was about 0.1-0.4 μg/μl. The quality of obtained DNA was suitable for molecular procedure, e.g. restriction endonuclease digestion and being a template for PCR amplification.

3.2 Preparation of DNA as templates for PCR amplification

The chromosomal DNA was separately digested with each of 5 restriction enzymes; *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Sal*I. The agarose gel electrophoresis analysis of digested DNA in Figure 3.2 showed that the pattern resulted from *Bgl*II, *Eco*RI and *Hin*dIII digestion still had a high molecular weight band, while *Bam*HI and *Sal*I gave the smear pattern of DNA lower than 23.1 kb.

All of five digested DNA solutions were used as the templates for testing PCR amplification of internal alanine dehydrogenase gene fragment (using primer NF1 and CR1 which gave estimated PCR product of 1,000 bp). The Figure 3.3 showed that only *Eco*RI and *Hin*dII digested DNA templates gave strong specific PCR product. *Bgl*II digested DNA template also gave a similar one but at lower concentration.

3.3 Optimization of PCR condition

For amplification of internal alanine dehydrogenase gene fragment using degenerate primer NF1 and CR1 was performed as described in 2.6. $MgCl_2$ concentration was optimized at a constant concentration of 2.5 U enzyme, 250 ng of *Hin*dIII digested-DNA template and 100 pmole of each primers. PCR-amplified DNA was obtained only at 1.0 mM $MgCl_2$ as shown in Figure 3.4. There was no product when other concentration of $MgCl_2$ was used, therefore an optimal $MgCl_2$ concentration was 1.0 mM

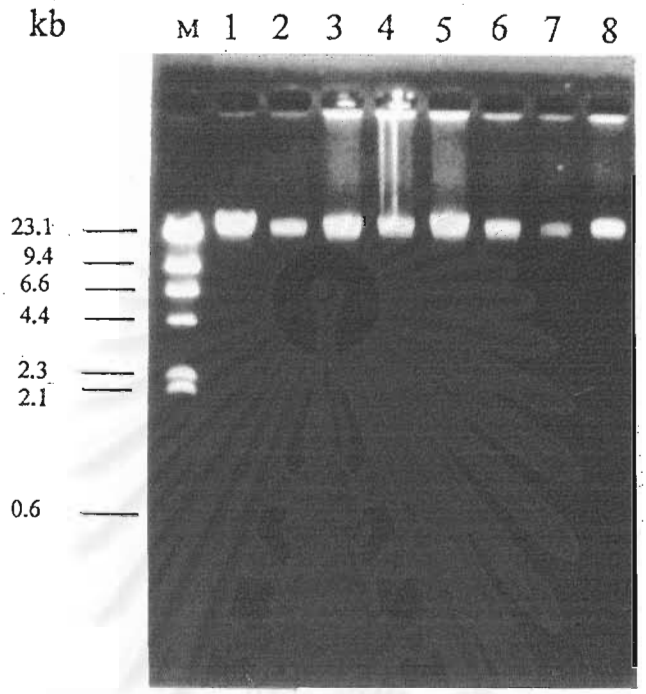


Figure 3.1 Agarose gel electrophoresis showing DNA extracted from *Aeromonas hydrophila*
Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = standard λ DNA (1 μg)
Lane 2-8 = total DNA from *Aeromonas hydrophila*

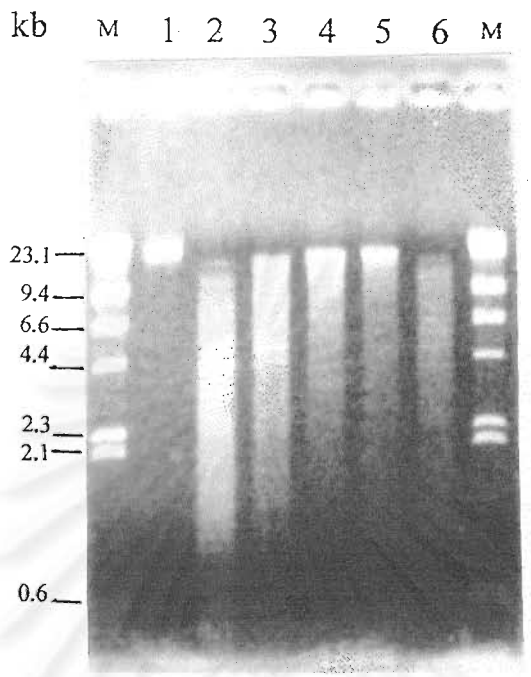


Figure 3.2 Restriction enzyme digested *Aeromonas hydrophila* DNA detected by agarose gel electrophoresis

Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1 = undigested *A. hydrophila* chromosomal DNA
Lane 2 = *A. hydrophila* DNA digested with *Bam*HI
Lane 3 = *A. hydrophila* DNA digested with *Bgl*II
Lane 4 = *A. hydrophila* DNA digested with *Eco*RI
Lane 5 = *A. hydrophila* DNA digested with *Hin*dIII
Lane 6 = *A. hydrophila* DNA digested with *Sal*I

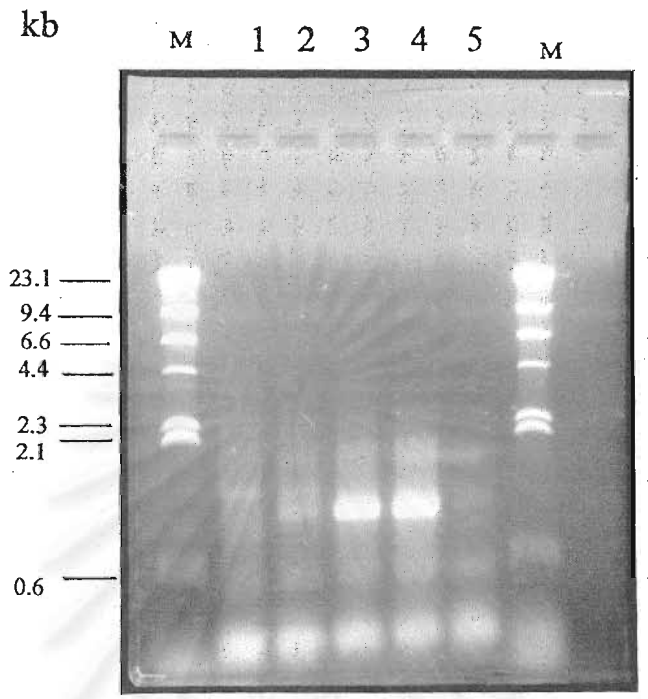


Figure 3.3 PCR products of primer NF1x CR1 using various templates
Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = PCR products using *Bam*HI digested DNA as template
Lane 2 = PCR products using *Bgl*II digested DNA as template
Lane 3 = PCR products using *Eco*RI digested DNA as template
Lane 4 = PCR products using *Hin*dIII digested DNA as template
Lane 5 = PCR products using *Sal*I digested DNA as template

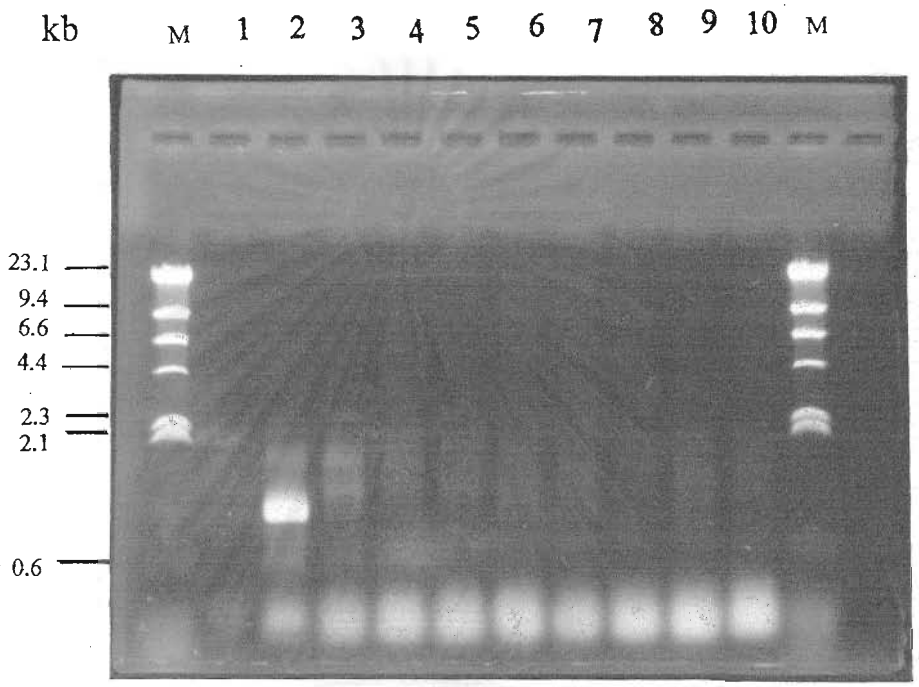


Figure 3.4 Optimization of $MgCl_2$ concentration
Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1-10 =amplified product in a series of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mM of $MgCl_2$, respectively

For amplification of internal alanine dehydrogenase gene fragment which used degenerated primer NF1 and CR1, *Taq* DNA polymerase was also optimized by varying the concentration between 1-3 U at a constant concentration of 1.0 mM $MgCl_2$, 250 ng of *Hin*dIII digested-DNA template and 100 pmole of each primers. PCR-amplified DNA was appeared in all lanes. As shown in Figure 3.5, the products had strong band at more than 2.0 U of enzyme. Therefore an appropriated concentration of enzyme was 2.5 U.

3.4 Nucleotide sequencing for the first fragment of alanine dehydrogenase gene

As shown in Figure 3.3, 3.4 and 3.5, the specific PCR product (about 1,000 bp) was obtained in each reactions. In addition, PCR amplification by using other pairs of primers (NF1xCR1, NF1xCR3, NF2xCR2, NF3xCR1 and NF3xCR3) also gave the specific products as shown in Figure 3.6. Therefore, the specific band of NF1xCR1 PCR products were extracted from gel by QIAQuick gel extraction kit for nucleotide sequencing. The DNA was sequenced on both sides of the 1,000 bp fragment with sense primer (NF1) and antisense primer (CR1). The result of sequencing were shown in Figure 3.7 and 3.8 and the nucleotide sequences of 253 bp at the 5'end and 390 bp at 3'end were determined by compared with the GENBANK deposited DNA sequences using BLAST (Basic Local Alignment Search Tool) at the website http://www.ncbi.nlm.nih.gov. The 5'and 3' sequences were homologous to partial part of *B. subtilis* alanine dehydrogenase gene (data not shown).

3.5 PCR amplification for the second fragment of alanine dehydrogenase gene

From the nucleotide sequencing data of the first internal fragment of the gene, the new set of primers were designed from the obtained nucleotide sequence and used for amplified inner part of gene. The second internal gene fragment was amplified by using primer NF4xCR4, NF5xCR4 and NF6xCR4 (the expected PCR products size about 700 bp). No specific band was obtained from amplification with NF4xCR4 and NF5xCR4 (data not shown). The Figure 3.9 showed that there were smeared products which had strong band at about 700 bp size when using primer NF6xCR4. Therefore the 700 bp band was extracted from gel by QIAQuick gel extraction kit for nucleotide sequencing. The

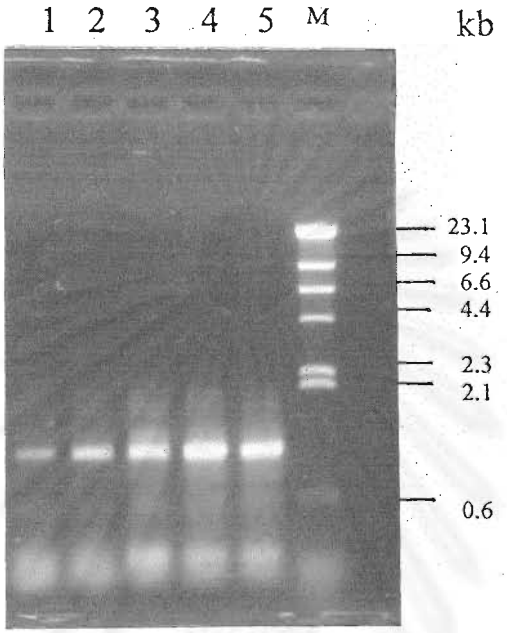


Figure 3.5 Optimization of *Taq* DNA polymerase concentration
Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1-5 = amplified product in series of 1.0, 1.5, 2.0, 2.5 and 3.0 U of *Taq* DNA polymerase respectively

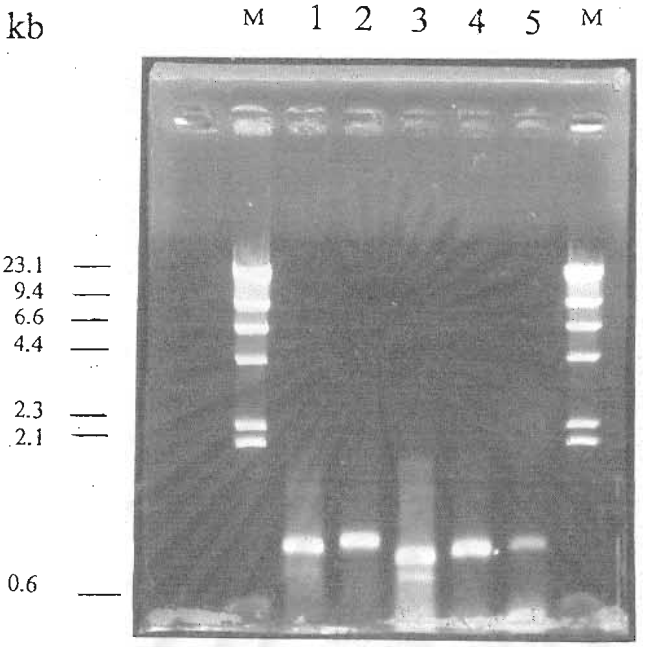
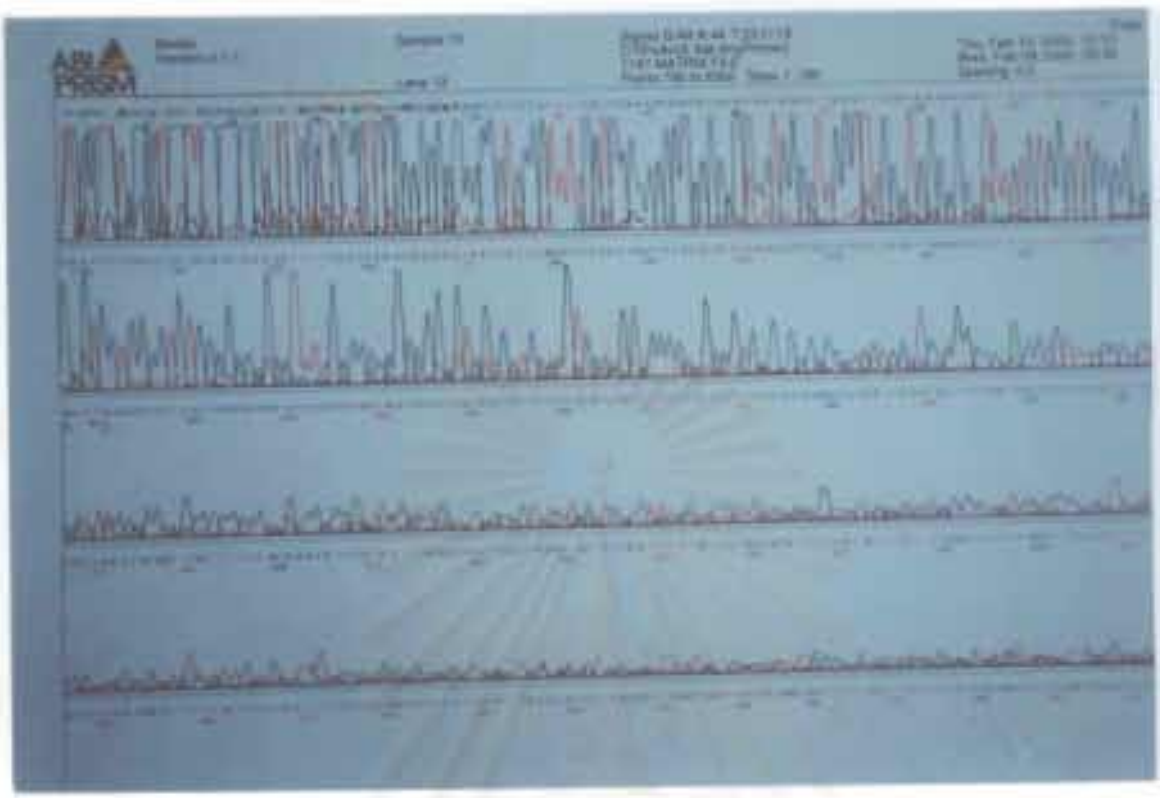


Figure 3.6 Agarose gel electrophoresis showing PCR products of the internal gene fragment amplification by using various pairs of primers

Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1 = amplified products using primer NF1xCR1
Lane 2 = amplified products using primer NF1xCR3
Lane 3 = amplified products using primer NF3xCR3
Lane 4 = amplified products using primer NF3xCR1
Lane 5 = amplified products using primer NF2xCR2

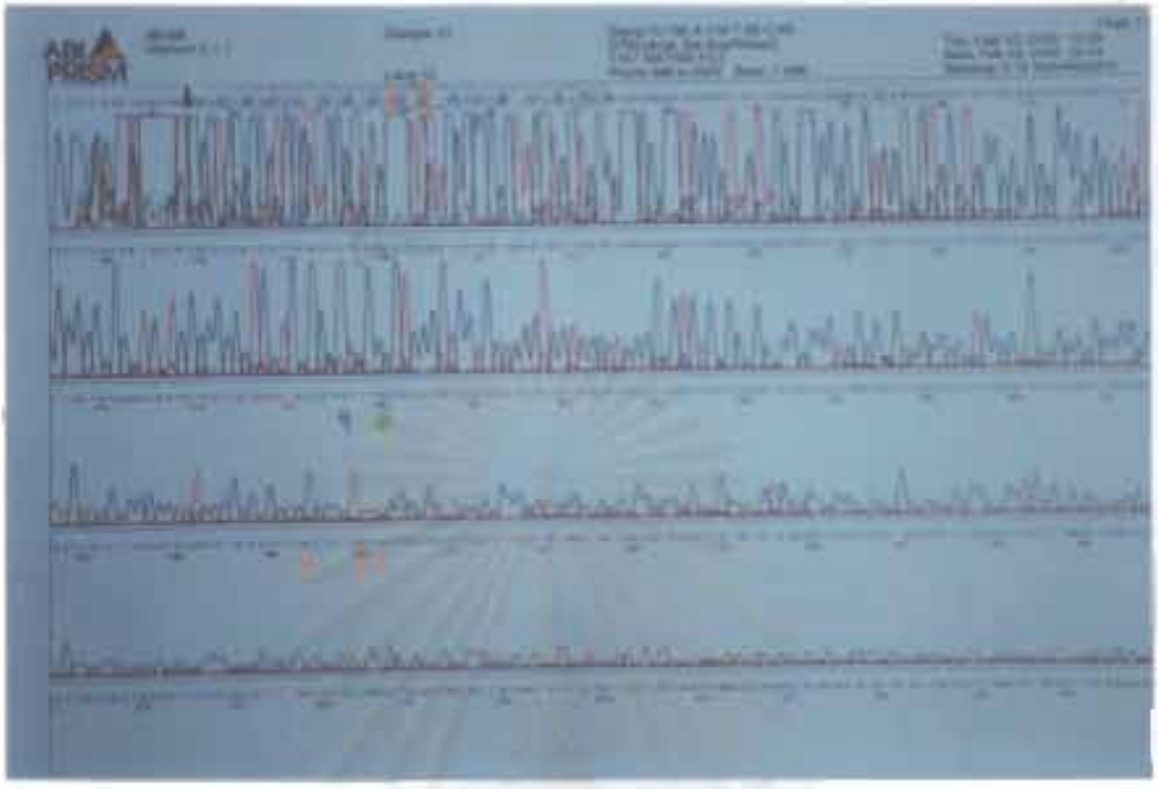


(a)

```
  1  GTAGGCATGGTTCCGGCCAGTGTACGTGAACTGACAGCACGAAACCATACCGTTTTCGTC
 61  CAAAGCGGCGCAGGAAATGGCATTGGGTTCAGTGACGCAGATTATCTGGCTGCCGGAGC
121  GAGATCCTGGCCTCTGCGGCAGACGTTTTCGCCAAGGCGGAGATGATCGTCAAGGTCAG
181  GAGCCCCAGGCGGTCGAGCGCGCCATGCTGCGTCCGGGCCAGACCCTCTTTACCTACCG
241  CACCTGGCGCCAG
```

(b)

Figure 3.7 Sequence of the first internal gene fragment of alanine dehydrogenase using sense primer NF1
(a) DNA sequencing result
(b) nucleotide sequence of 253 bases
The regions for design further primers are shown by underline.



(a)

```
  1  GTCTCGACAGCGAGTTCCAGGGTGCCGCCAAGGTGGTTTACTCCAACCGCGAGACCCTG
 61  GAGCGCCATTTGCTGGCGGCAGACCTGGTTATCGGTGGCGTGCTGGTACCGGGCGCCAC
121  TGCGCCCAAACTTGTCAGCCGTGACCACATTGCCCGCATGAAGCCGGGGTCGGCCATTG
181  TGGCCATCGATCAGGGCGGCTGCGTCGAGACCTCCCATGCCACCACCCATGAGGATCCC
241  ACCTTCATTGTCGACGACGTGGTGCACTACTGCGTGGCCAACATGCCGGGCGCCGTGGC
301  ACGCACCTCCACCGTGGCCCTGAACAACGCCACCCTGCCCTTCATCATCAAGCTGGCCG
361  AACAGGGCTATCGCAACGCACTGCTCAGCG
```

(b)

Figure 3.8 Sequence of the first internal gene fragment of alanine dehydrogenase using antisense primer CR1
(a) DNA sequencing result
(b) complementary nucleotide sequence of 390 bases
The region for design further primers are shown by underline.

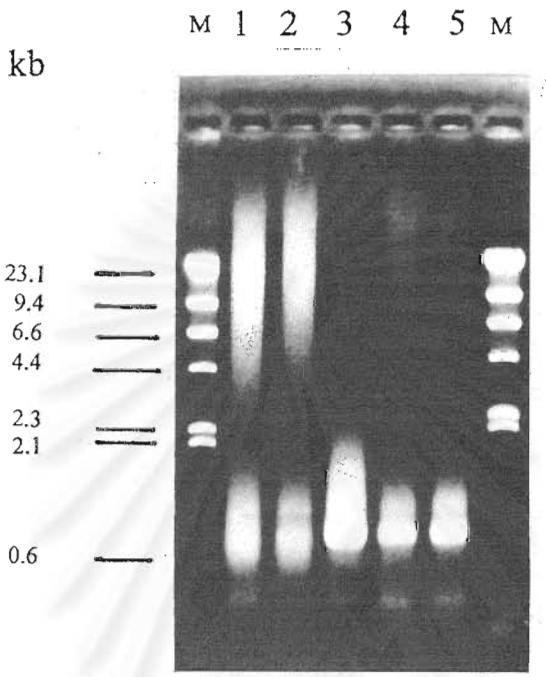


Figure 3.9 PCR products of primers NF6 and CR4
Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1-5 = amplified products using *Hin*dIII digested chromosomal DNA as template

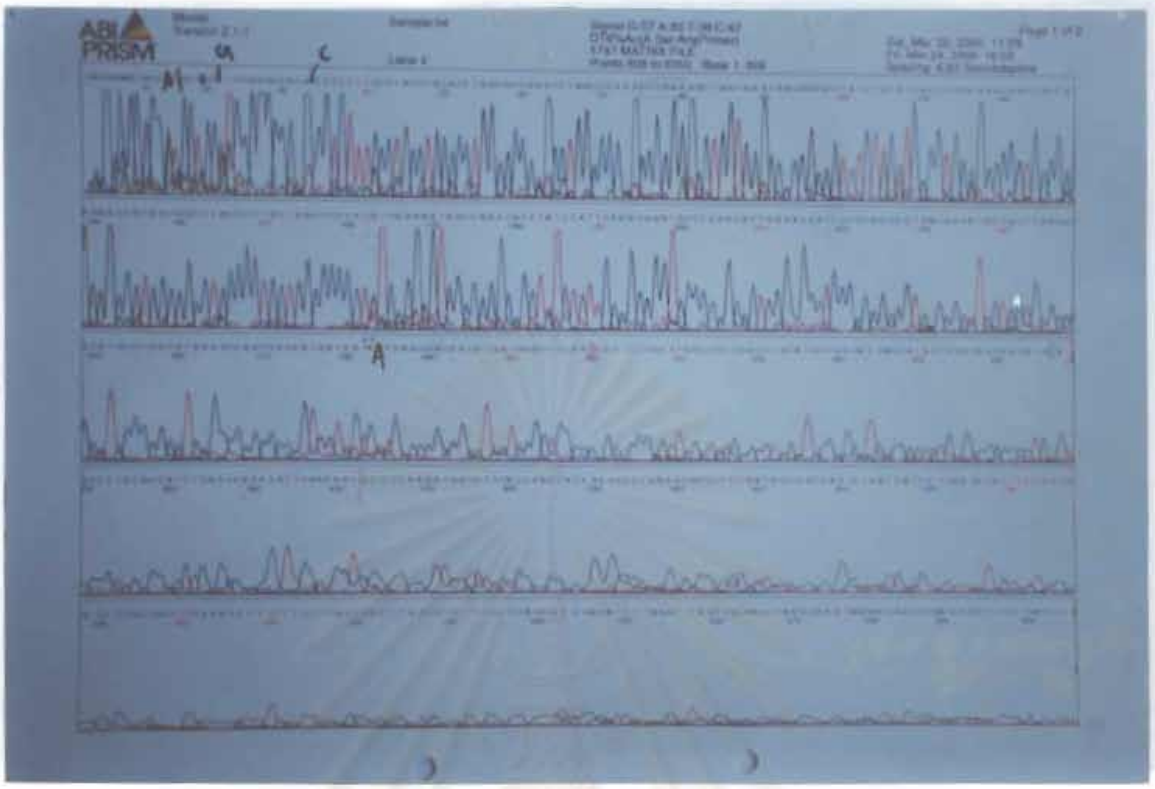
DNA was sequenced on both sides of the 700 bp fragment with sense primer (NF6) and antisense primer (CR4). The result of sequencing were shown in Figure 3.10 and 3.11 and the nucleotide sequences of 378 bp at the 5'end and 327 bp at 3'end were determined by compared with the GENBANK deposited DNA sequences using BLAST at the website http://www.ncbi.nlm.nih.gov. The results of comparing the 5' and 3' sequences were homologous to the partial part of *B. subtilis* alanine dehydrogenase gene (data not shown). All internal nucleotide sequences of the gene were analyzed and called core sequence.

3.6 PCR amplification for the N-terminal and C-terminal gene fragment of alanine dehydrogenase

For the inverse PCR, the primers that used for this part of PCR reaction were designed from the data of core sequence and the templates were prepared by first digested with restriction enzymes, which have no site on core sequence. The first PCR products which used outer pair of primers (NR0 and CF0) showed multiple and smear bands (data not shown) so those PCR products were used as templates for second PCR which used inner pair of primers (NR and CF). The second PCR gave more specific product than those of the first PCR. Figure 3.12 showed that products from lane 2 and 9 gave almost one strong band so these strong bands were chosen and extracted from gel by QIAQuick gel extraction kit for nucleotide sequencing. The DNA was sequenced on both sides of the fragment with sense primer (NR2) and antisense primer (CF2). The nucleotide sequencing data is shown in Figure 3.13 and 3.14. The sequences of 96 bp at the 5'end and 332 bp at 3'end were determined by compared with the GENBANK deposited DNA sequences using BLAST at the website http://www.ncbi.nlm.nih.gov. The results of comparing the 5' and 3' sequences were homologous to the partial part of *B. subtilis* alanine dehydrogenase gene (data not shown). From two walk in and one inverse PCR technique, the nucleotide sequences of the whole gene fragment were identified as shown in Figure 3.15.

3.7 Amino acid sequence comparison with alanine dehydrogenase from other sources

Deduced amino acid sequence of alanine dehydrogenase from *Aeromonas hydrophila* was aligned with the dehydrogenases by using Clustal X (1.64 b) (Figure 3.16). The over all similarity scores of

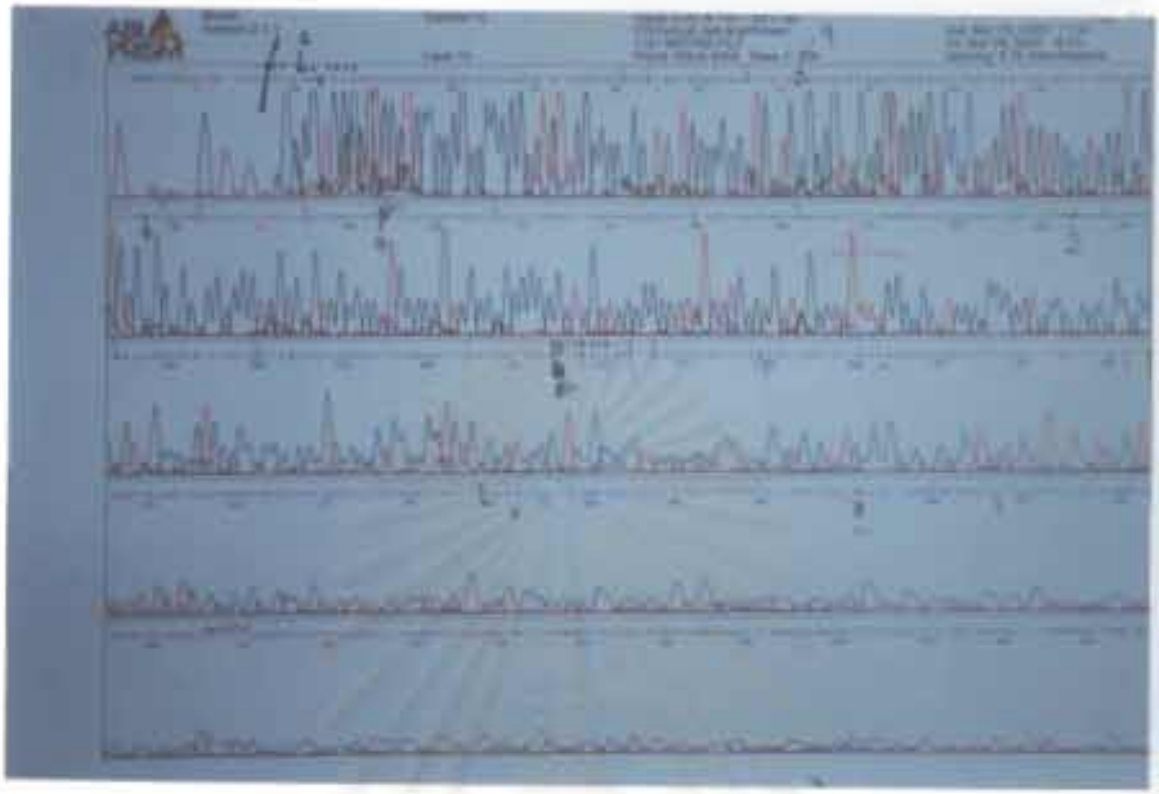


(a)

```
  1  CGCGCCATGCTGCGTCCGGGCCAGACCCTCTTTACCTACCTGCACCTGGCGCCAGACCTG
 61  GCCCAGACCCGGGAGCTGGTGGACAGCGGCGCTATCTGCATCGCCTACAAAACCGTCACC
121  GACGGCCGTGGCGGCCTGCCCCTGCTGGCCCCCATGTCGGAGGTGGCCGGACGCATGTCT
181  ATTCAGGCGGGTGCCCAGGCGCTGGAAAAATCCCGCGGCGGTAGCGGCGTGCTGCTCGGC
241  GGCGTGCCCGGCGTGGAACCGGCCAAGGTGGTGATCATCGGCGGCGGCGTGGTGGGCTCC
301  AACGCAGCCCGCATGGCCATCGGCCTGCGTGCCGACGTCACCATACTCGACAACAACATC
361  GATACCCTGCGTCTCGAC
```

(b)

Figure 3.10 Sequence of the second internal gene fragment of alanine dehydrogenase using sense primer NF6
(a) DNA sequencing result
(b) nucleotide sequence of 378 bases



(a)

```
  1  GATACCCTGCGCCGTCTCGACAGCGAGTTCCAGGGTGCCGCCAAGGTGGTTTACTCCAAC
 61  CGCGAGACCCTGGAGCGCCATTTGCTGGCGGCAGACCTGGTTATCGGTGGCGTGCTGGTA
121  CCGGGCGCCACTGCGCCCAAACTTGTCAGCCGTGACCACATTGCCCGCATGAAGCCGGGG
181  TCGGCCATTGTGGACGTGGCCATCGATCAGGGCGGCTGCGTCGAGACCTCCCATGCCACC
241  ACCCATGAGGATCCCACCTTCATTGTCGACGACGTGGTGCACTACTGCGTGGCCAACATG
301  CCGGGCGCCGTGGCACGCACCTCCACC
```

(b)

Figure 3.11 Sequence of the second internal gene fragment of alanine dehydrogenase using antisense primer CR4
(a) DNA sequencing result
(b) complementary nucleotide sequence of 327
The regions for design further primers are shown by underline

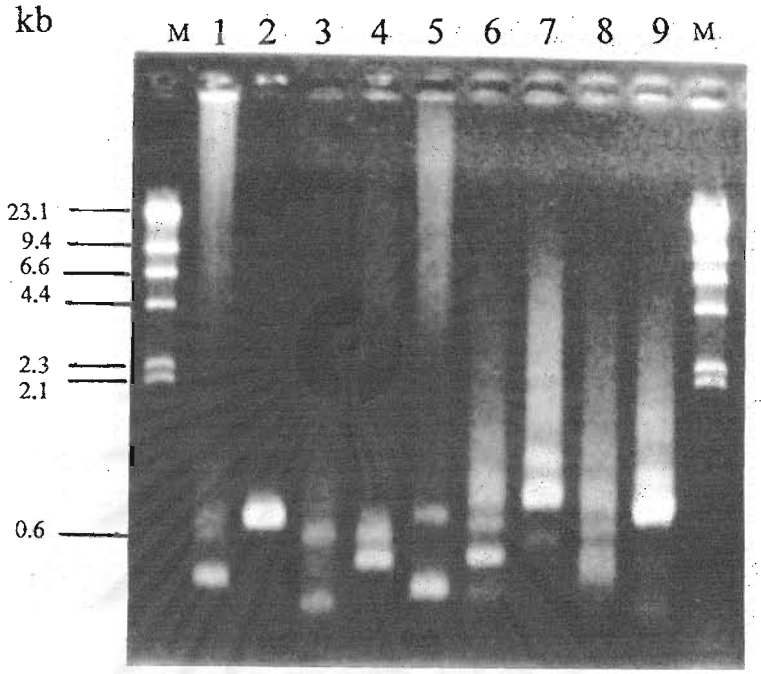


Figure 3.12 The second inverse PCR products using primer NR and CF

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

Lane 1 = amplified products using *Bgl*II digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 2 = amplified products using *Bst*EII digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 3 = amplified products using *Eco*RI digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 4 = amplified products using *Hin*dIII digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 5 = amplified products using *Nde*I digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

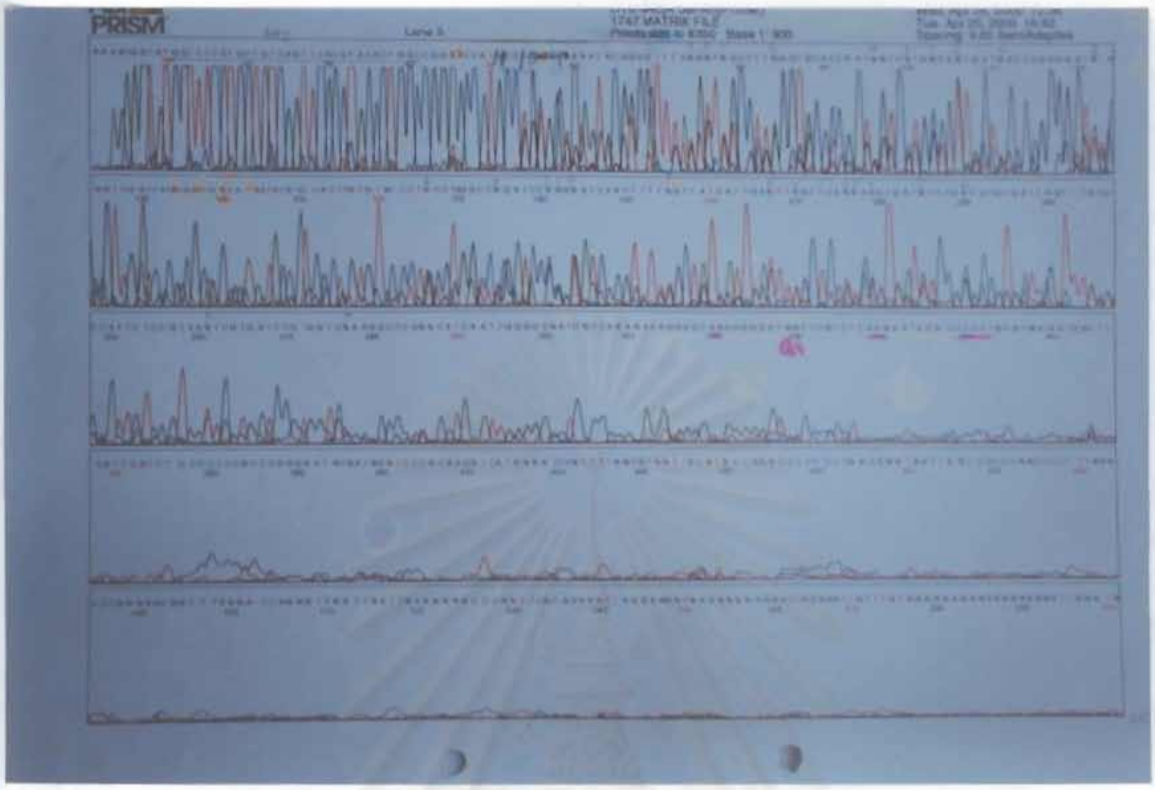
Lane 6 = amplified products using *Pst*I digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 7 = amplified products using *Pvu*II digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 8 = amplified products using *Spe*I digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Lane 9 = amplified products using *Sph*I digested chromosomal DNA as template for the first inverse PCR (NR0xCF0)

Note : The templates of second inverse PCR were PCR porducts from the reaction using each restriction enzyme digested chromosomal DNA as template and NR0 as well as CF0 were used as primers



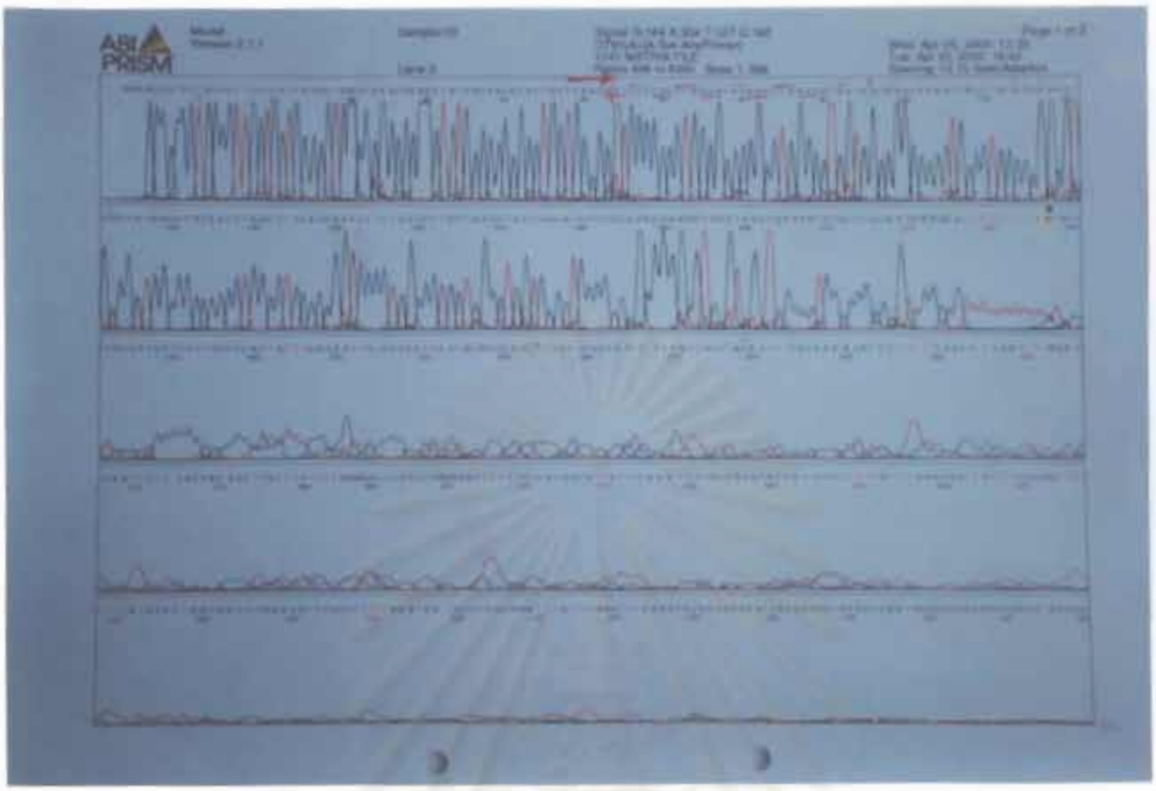
(a)

```
1   ATGATTATCGGTGTACCTAAGGAAATCAAAAACCACGAATATCGCGTAGGCATGGTTCCG

61  GCCAGTGTACGTGAACTGACAGCACGAAACCATACC
```

(b)

Figure 3.13 Sequence of the N-terminal gene fragment of alanine dehydrogenase using antisense primer NR2
(a) DNA sequencing result
(b) complementary nucleotide sequence of 96 bases



(a)

```
  1  ACCCTGCCCTTCATCATCAAGCTGGCCGAACAGGGCTATCGCAACGCACTGCTCAGCGAT

 61  CCCCACCTGCGGCACGGCCTGAACGTGATGGCGGGCAAAATCACCTGCAAGGAGGTCGCC

121  GTGGCCCACAACCTGGCCTACACGGATCCCCTGACCCTGCTGAACTGATCGCATCCCCGC

181  GAGGGGAGGTAGATGAGTGAAAATCCGCCCGCGATGGGCGGTTTTTTTTTTATGCCTGCC

241  ATCCCCCCTGCCCCTCTCTTTGGCAATTGCCCCAATCAATGCGCCGGCCTTTGACCACGG

301  ATCACAAATTACCTAAATCGCGCAAAGTGATC
```

(b)

Figure 3.14 Sequence of the C-terminal gene fragment of alanine dehydrogenase using sense primer CF2
(a) DNA sequencing result
(b) nucleotide sequence of 332 bases

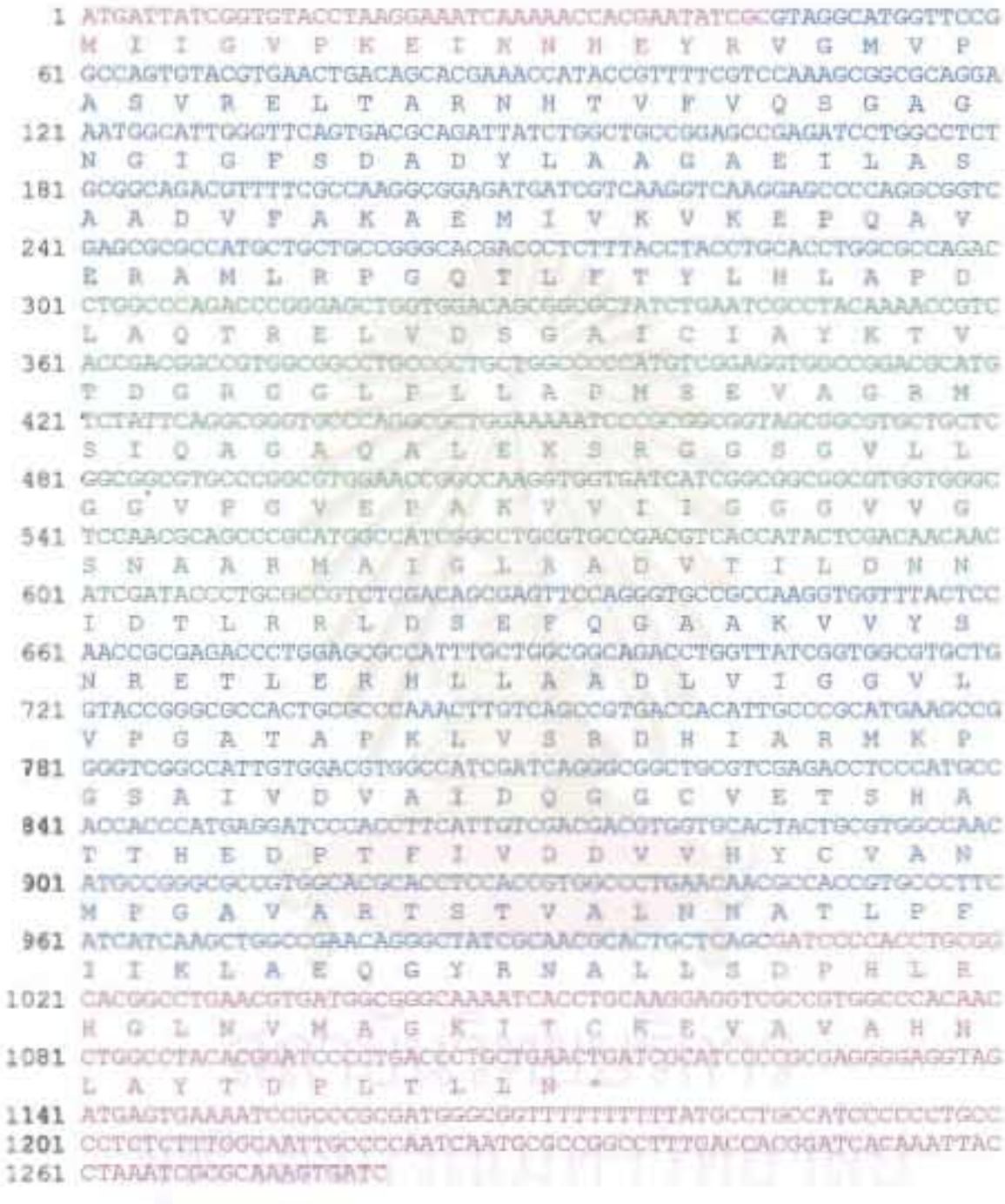


Figure 3.15 The nucleotide sequence and the deduced amino acid of alanine dehydrogenase from *Aeromonas hydrophila*

Note : blue = sequence from the first walk in PCR
green = sequence from the second walk in PCR
pink = sequence from inverse PCR

```
                                  (1)
AHY     MIIGVPKEIKNHEYRVGMVPASVRELTARNHT-VFVQSGAGNGIGFSDADYLAAGAEILA-SAADVFAKAEMIVKVKEPQ 78
BST     MKIGIPKEIKNNENRVAITPAGVMTLVKAGHE-VYVETEGGAGSGFSDSEYEKAGAADRCRTWRDAWT-AEMVLKVKEPL 78
BSP     MKIGIPKEIKNNENRVAMTPAGVVSLTHAGHERLAIETGGGIGSSFTDAEYVAAGAAYRC-IGKEAWA-QEMILKVKEPV 78
EAE     MIIGVPKEIKNNENRVAMTPAGVVHLLNAGHK-VIIETNAGLGSGFTNEEYKQAGAEIIE-SASDVWTKADMIMKVKEPL 78
BSU     MIIGVPKEIKNNENRVALTPGGVSQLISNGHR-VLVETGAGLGSGFENEAYESAGAEIIA-DPKQVWD-AEMVMKVKEPL 77
PLA     MEIGVPKEIKNQEFRVGLSPSSVRTLVEAGHT-VFIETQAGIGAGFADQDYVQAGAQVVP-SAKDAWS-REMVVKVKEPL 77
MTU     MRVGIPTETKNNEFRVAITPAGVAELTRRGHE-VLIQAGAGEGSAITDADFKAAGAQLVG-TADQVWADADLLLKVKEPI 78
        *  * * * ** * **   *  *  *    *         * *          ***                  *****

                                                                        (2)
AHY     AVERAMLRPGQTLFTYLHLAPDLAQTRELVDSGAICIAYKTVTDGRGGLPLLAPMSEVAGRMSIQAGAQALEKSRGGSGV 158
BST     AREFRYFRPGLILFTYLHLAAAERVTKAVVEQKVVGIAYETVQLANGSL-LLTPMSEVAGRMSVQVGAQFLEKPHGGKGI 157
BSP     ASEYDYFYEGQILFTYLHLAPRAELTQALIDKKVVGIAYETVQLANGSLPLLTPMSEVAGKMATQIGAQYLEKNHGGKGI 158
EAE     ASEYGYFRKGLILFTYLHLAAEPELTKALVDSEVIAIAYETVTVNR-TLPLLSPMSEVAGRMAAQVGAQFLEKTQGGKGI 157
BSU     PEEYVYFRKGLVLFTYLHLAAEPELAQALKDKGVTAIAYETVSEGR-TLPLLTPMSEVAGRMAAQIGAQFLEKPKGGKGI 156
PLA     PAEYDLMQKDQLLFTYLHLAAARELTEQLMRVGLTAIAYETVELPNRSLPLLTPMSIIAGRLSVQFGARFLERQQGGRGV 157
MTU     AAEYGRLRHGQILFTFLHLAASRACTDALLDSGTTSIAYETVQTADGALPLLAPMSEVAGRLAAQVGAYHLMRTQGGRGV 158
          *         *** ****                  *** **      * ** ***  **    * **  *    ** *

                  (3)                          (4)
AHY     LLGGVPGVEPAKVVIIGGGVVGSNAARMAIGLRADVTILDNNIDTLRRLDSEFQGAAKVVYSNRETLERHLLAADLVIGG 238
BST     LLGGVPGVRRGKVTIIGGGTAGTNAAKIGVGLGADVTILDINAERLRELDDLFGDHVTTLMSNSYHIAECVRESDLVVGA 237
BSP     LLGGVSGVHARKVTVIGGGIAGTNAAKIAVGMGADVTVIDLSPERLRQLEDMFGRDVQTLMSNPYNIAESVKHSDLVVGA 238
EAE     LLSGVPGVKRGKVTIIGGGMVGTNAAKIAVGLGADVTIIDLNPDRLRQLEDIFGTSVQTLMSNPYNIAEAVKESDLVIGS 237
BSU     LLAGVPGVSRGKVTIIGGGVVGTNAAKMAVGLGADVTIIDLNADRLRQLDDIFGHQIKTLISNPVNIADAVAEADLLICA 236
PLA     LLGGVPGVKPGKVVILGGGVVGTEAAKMAVGLGAQVQIFDINVERLSYLETLFGSRVELLYSNSAEIETAVAEADLLIGA 237
MTU     LMGGVPGVEPADVVVIGAGTAGYNAARIANGMGATVTVLDINIDKLRQLDAEFCGRIHTRYSSAYELEGAVKRADLVIGA 238
        *  ** **    *   * *  *  **    * * *  *     * *  *        *             **
```

(continued)

Figure 3.16 Linear alignment of the amino acid sequence of alanine dehydrogenases

Tryptic peptides, as well as the N-terminal region, for which sequences were determined by automated Edman degradation, are underlined. *Enterbactor aerogenes* (EAE), *Bacillus subtilis* (BSU), *Bacillus sphearicus* (BSP), *Bacillus stearothermophilus* (BST), *Phormidium lapideum* (PLA), *Mycobacterium tuberculosis* (MTU) and *Aeromonas hydrophila* (AHY)

(continued)

```
                                                    (5)
AHY       VLVPGATAPKLVSRDSIARMKPGSAIVDVAIDQGGCVETS-HATTHEDPTFIVDDVVHYCVANMPGAVARTSTVALNNAT 317
BST       VLIPGAKA-KLVTEEMVRSMTPGSVLVDIAIDQGGIFETTDRVTTHDDPTYVKHGVVHYAVANMPGAVPRTSTFALTNVT 316
BSP       VLIPGAKAPKLVSEEMIQSMQPGSVVVDIAIDQGGIFATSDRVTTHDDPTYVKHGVVHYAVANMPGAVPRTSTIALTNNT 318
EAE       VLIPGAKAPKLVTEEMVKSMQPGSVIVDVAIDQGGNFETVDHITTHDDPTYVKHGVVHYAVANMPGAVPRTATIALTNVT 317
BSU       VLIPGAKAPTLVTEEMVKQMKPGSVIVDVAIDQGGIVETVDHITTHDQPTYEKHGVVHYAVANMPGAVPRTSTIALTNVT 316
PLA       VLVPGRRAPILVPASLVEQMRTGSVIVDVAVDQGGCVETL-HPTSHTQPTYEVFGVVHYGVPNMPGAVPWTATQALNNST 316
MTU       VLVPGAKAPKLVSNSLVAHMKPGAVLVDIAIDQGGCFEGS-RPTTYDHPTFAVHDTLFYCVANMPASVPKTSTYALTNAT 317
          ** **  *  **       *  *   ** * ****       *   *          * * ***  *  * * ** * *

            (6)                                    (7)
AHY       LPFIIKLAEQGYRNALLSDPHLRHGLNVMAGKITCKEVAVAHNLAYTDPLTLLN-------- 371
BST       IPYALQIANKGYRAGCLDNPALLKGINTLDGHIVYEAVAAAHNMPYTDVHSLLHG-------- 371
BSP       IPYALQIANKGYKQACIDNPALKKGVNALEGHITYKAVAEAQGLPYVNVDELIQ-------- 372
EAE       IPYAVQIATKGVVKAVNDNPAIKAGVNVANGHVTFEAVANDLGYKYVTVEEAISKEAINA-- 377
BSU       VPYALQIANKGAVKALADNTALRAGLNTANGHVTYEAVARDLGYEYVPAEKALQDESSVAGA 378
PLA       LPYVVKLANQGLKALETDD-ALAKGLNVQAHRLVHPAVQQVFPDLA---------------- 361
MTU       MPYVLELADHGWRAACRSNPALAKGLSTHEGALLSERVATDLGVPFTEPASVLA-------- 371
           *     *  *             *           *
```

Figure 3.16 Linear alignment of the amino acid sequence of alanine dehydrogenases

Tryptic peptides, as well as the N-terminal region, for which sequences were determined by automated Edman degradation, are underlined. *Enterbactor aerogenes* (EAE), *Bacillus subtilis* (BSU), *Bacillus sphearicus* (BSP), *Bacillus stearothermophilus* (BST), *Phormidium lapideum* (PLA), *Mycobacterium tuberculosis* (MTU) and *Aeromonas hydrophila* (AHY)

alanine dehydrogenase from *Aeromonas hydrophila* compared with *Bacillus stearothermophilus*, *Enterbactor aerogenes*, *Bacillus subtilis*, *Phormidium lapideum*, *Mycobacterium tuberculosis* and *Bacillus sphaericus* were calculated to be 53.91%, 53.64%, 53.10%, 53.10%, 52.56% and 50.67%, respectively.

3.8 PCR amplification of the whole gene fragment

For further cloning, the whole gene fragment was amplified by using the primers that had Shine Dalgano Sequence of plasmid p*Trc*99, the expression vector for *E. coli* JM105, and *Eco*RI restriction site for the sense strand (N-ECO), while the antisense strand was the primer that had *Hin*dIII restriction site (N-HIN). About 1,200 bp fragment was produced as shown in Figure 3.17.

3.9 Transformation

The whole gene fragments were ligated with *Eco*RI-*Hin*dIII site of pUC18 and transformed into *E. coli* JM109 by electroporation. The white colonies were picked for plasmid extraction. From 24 selected colonies, there were three patterns of plasmid as shown in Figure 3.18. Twenty selected colonies had the first pattern of plasmid (lane 3). For the left four colonies, there were each two colonies of second (lane 4) and third pattern (lane 5) of plasmids. All plasmid were digested with *Eco*RI and *Hin*dIII, the result in Figure 3.19 showed that the first pattern of plasmid were the recombinant DNA that had alanine dehydrogenase gene fragment.

3.10 Enzyme assay

The 20 colonies that had recombinant DNA containing PCR product were grown for enzyme assay as described in 2.12 and 2.13. The specific activities of the crude extracts were about 0 to 10 Units/mg protein as shown in Table 3.1. The host cell *E. coli* JM109, *E. coli* JM109 harbouring vector DNA, pUC18, and *A. hydrophila* were also grown for comparison of enzyme activity. From 20 transformants, there were 10 transformants that had enzyme activity.

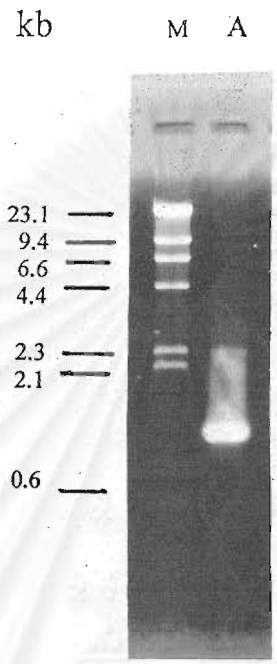


Figure 3.17 Agarose gel electrophoresis showing PCR product of the whole alanine dehydrogenase gene amplification using primer N-ECO and C-HIN (about 1,200 bp)
Lane M = λ/ *Hin*dIII standard DNA marker
Lane A = amplified product using *Hin*dIII digested chromosomal DNA as template

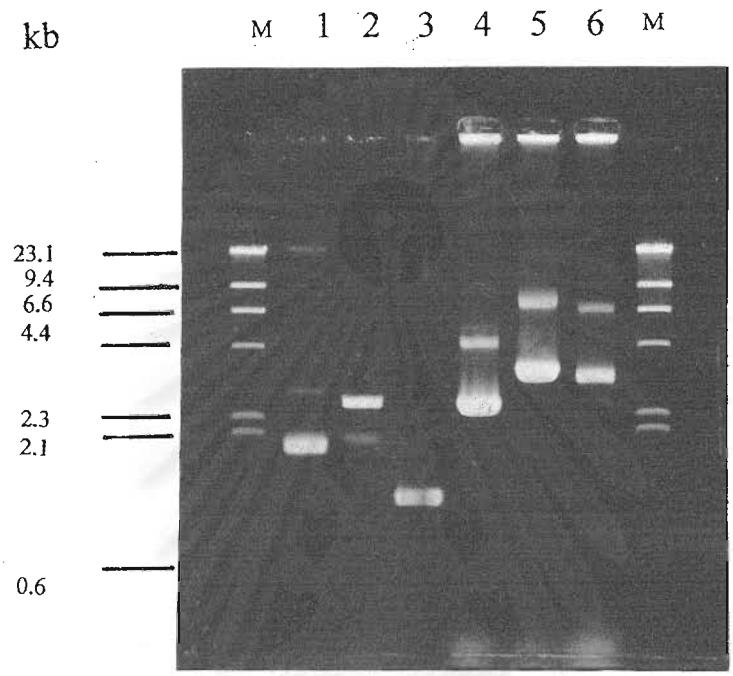


Figure 3.18 Agarose gel electrophoresis showing extracted plasmids of transformants

Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1 = uncut pUC18
Lane 2 = *Eco*RI/*Hin*dIII digested pUC18
Lane 3 = amplified product of the whole alanine dehydrogenase gene
Lane 4 = the 1st pattern of extracted plasmids
Lane 5 = the 2nd pattern of extracted plasmids
Lane 6 = the 3rd pattern of extracted plasmids

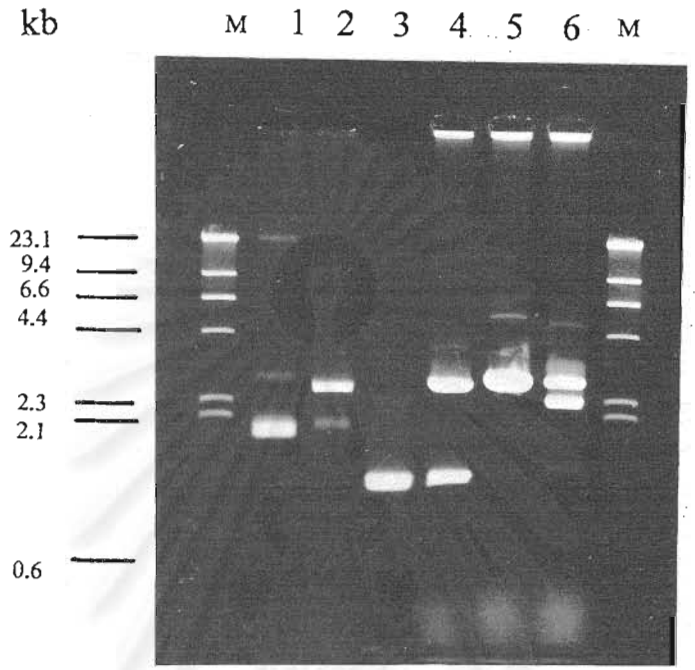


Figure 3.19 Agarose gel electrophoresis showing *Eco*RI/*Hin*dIII digested plasmids of transformants

Lane M = λ/ *Hin*dIII standard DNA marker
Lane 1 = uncut pUC18
Lane 2 = *Eco*RI/*Hin*dIII digested pUC18
Lane 3 = amplified product of the whole alanine dehydrogenase gene
Lane 4 = the 1st pattern of *Eco*RI/*Hin*dIII digested extracted plasmids
Lane 5 = the 2nd pattern of *Eco*RI/*Hin*dIII digested extracted plasmids
Lane 6 = the 3rd pattern of *Eco*RI/*Hin*dIII digested extracted plasmids

Table 3.1 Comparison of the activity of crude extracts

Sources of crude extract	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
E. coli JM109	-	11.00	-
E. coli JM109 with pUC18	-	11.35	-
A. hydrophila	8.0	41.16	0.19
Transformant No. 1	-	12.00	-
Transformant No. 2	144.0	18.25	7.89
Transformant No. 3	20.0	15.39	1.30
Transformant No. 4	48.0	13.39	3.58
Transformant No. 5	88.0	17.36	5.07
Transformant No. 7	3.3	19.16	0.17
Transformant No. 9	-	11.02	-
Transformant No. 10	-	13.68	-
Transformant No. 11	-	16.09	-
Transformant No. 12	198.4	22.19	8.94
Transformant No. 13	-	22.72	-
Transformant No. 14	-	16.46	-
Transformant No. 16	222.7	23.45	9.50
Transformant No. 19	-	12.06	-
Transformant No. 20	70.0	10.07	6.95
Transformant No. 21	-	19.94	-
Transformant No. 22	-	11.84	-
Transformant No. 23	4.2	13.27	0.32
Transformant No. 24	184.6	19.19	9.62

3.11 Polyacrylamide gel electrophoresis

Crude extracts from 7 chosen transformants that had enzyme activity, *E. coli* JM109, *E. coli* JM109 harbouring pUC18 and purified alanine dehydrogenase marker from *A. hydrophila* were loaded on gels. Figure 3.20 showed the protein staining and Figure 3.21 showed activity staining. From the two figures, only crude extract from the transformants had alanine dehydrogenase band, while the crude extract from both *E. coli* JM109 and *E. coli* JM109 harbouring pUC18 had neither the protein nor the activity band of alanine dehydrogenase.

In addition, the crude extracts from the transformants that did not have enzyme activity were also loaded on the native-PAGE and compared with purified alanine dehydrogenase from *A. hydrophila* as shown in Figure 3.22. All of them did not have alanine dehydrogenase protein band.

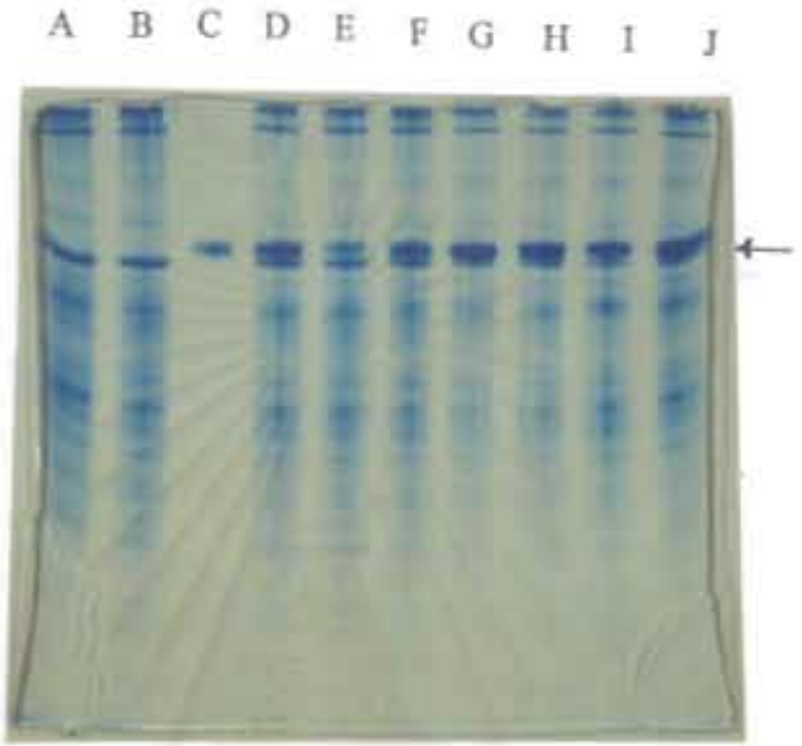


Figure 3.20 Protein pattern of crude extracts of alanine dehydrogenase producing transformants detected by native-PAGE
Lane A = crude extract of *E. coli* JM109
Lane B = crude extract of *E. coli* JM109 horbouring pUC18
Lane C = purified alanine dehydrogenase from *A. hydrophila*
Lane D-I = crude extracts of transformant No. 2, 4, 5, 12, 16, 20 and 24, respectively.
Bands corresponding to alanine dehydrogenase are indicated by an arrow.

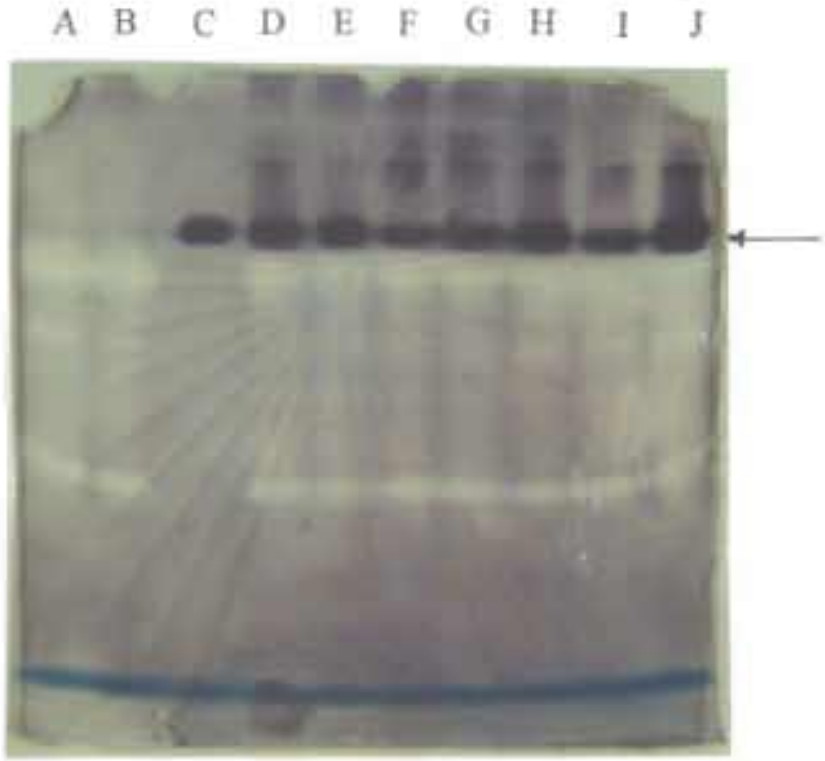


Figure 3.21 Alanine dehydrogenase activity staining of the enzyme producing transformant

Lane A = crude extract of *E. coli* JM109

Lane B = crude extract of *E. coli* JM109 harbouring pUC18

Lane C = purified alanine dehydrogenase from *A. hydrophila*

Lane D-I = crude extracts of transformant No. 2, 4, 5, 12, 16, 20 and 24, respectively.

Bands corresponding to alanine dehydrogenase are indicated by an arrow.

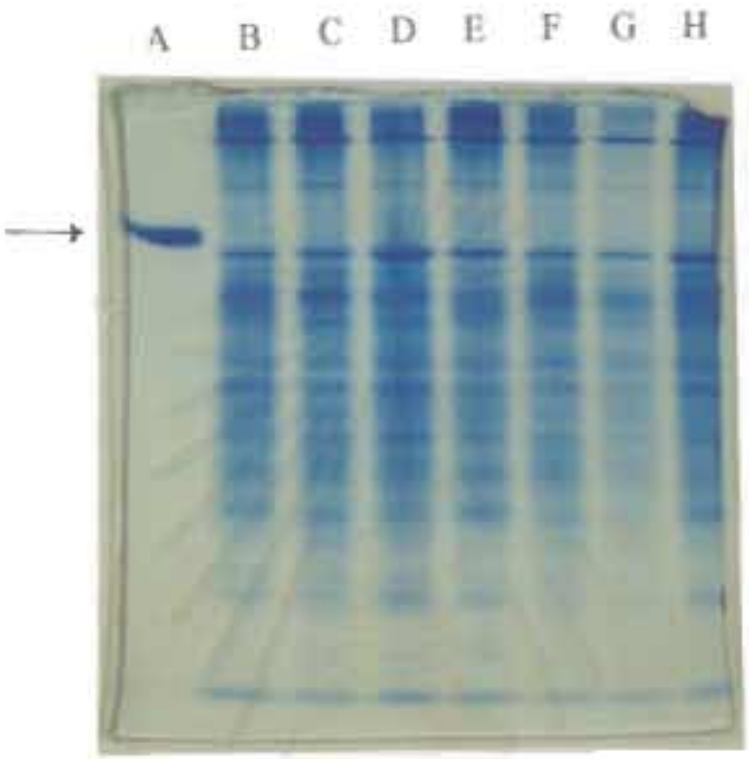


Figure 3.22 Protein pattern of crude extracts of transformants that had no alanine dehydrogenase activity detected by native-PAGE
Lane A = purified alanine dehydrogenase from *A. hydrophila*
Lane B-H = crude extracts of transformant No. 1, 9, 10, 11, 13, 14, and 19, respectively.
Bands corresponding to alanine dehydrogenase are indicated by an arrow.

CHAPTER IV

DISCUSSION

Bacteria can metabolize amino acid as the energy source via the tricarboxylic acid cycle by (1) using pyridoxal phosphate-dependent transaminase for transferring amino group of amino acid to keto acid and resulting in new amino acid (2) using deaminase in the way to remove amino group from amino acid in the form of ammonia and (3) using amino acid dehydrogenase for producing the free ammonia and other important metabolize molecules such as pyruvate and α-ketoglutarate (Nobert *et al.*, 1994). Alanine dehydrogenase is one of the enzyme used for producing alanine and pyruvate which are industrial importance. *Aeromonas hydrophila*, isolated from soil in Bangkok, was found to have high alanine dehydrogenase activity (Phungsangthum, 1997). Thus, this bacteria was used for further study in molecular genetic of alanine dehydrogenase gene.

Chromosomal DNA of *Aeromonas hydrophila* was easily extracted by miniprep of bacterial genomic DNA method and found to have molecular weight greater than 23.1 kb. The ratio of A_{260}/A_{280} which was greater than 1.8 indicating its high purity. According to PCR reaction, the templates should be easily denatured in the purpose of annealing with primers. The heat denatured *A. hydrophila* chromosomal DNA was previously used as templates, however, it gave poor PCR amplification. Therefore, the chromosomal DNA was digested by restriction enzyme before using. From various restriction enzyme digestions, the chromosomal DNA fragment those were digested with *Eco*RI or *Hin*dIII gave almost high MW DNA of more than 9 kb. On the other hand, the digestion with *Bam*HI, *Bgl*II and *Sal*I gave the smear DNA patterns. It can be assumed that chromosomal DNA of *A. hydrophila* has more restriction sites of *Bam*HI, *Bgl*II and *Sal*I than those of *Eco*RI and *Hin*dIII. Those five restriction enzyme digested DNA fragments were also used for testing as templates in PCR reaction and the result showed that only *Eco*RI and *Hin*dIII digested DNA fragments gave strong specific products having the size of about 1,000 bp as determined from relative mobility on agarose gel electrophoresis compared with λ/*Hin*dIII marker, while the *Bgl*II digested DNA gave weak specific product. For *Bam*HI and *Sal*I digested DNA, both of them did not give the specific product. It is widely known that the restriction enzyme used

for template preparation must not have their sites on the gene, otherwise, the amplified complete gene fragment can not be occurred. Thus, it was implied that *Bgl*II, *Eco*RI and *Hin*dIII restriction sites were not on the alanine dehydrogenase gene fragments, while the site of *Bam*HI and *Sal*I may be on the gene fragment. Therefore, *Eco*RI and *Hin*dIII digested DNA was chosen as suitable template for alanine dehydrogenase gene cloning. This assumption was strongly supported by recognition sites of restriction enzyme on gene sequence obtained from this work. PCR condition was also optimized for optimum concentration of $MgCl_2$ and *Taq* DNA polymerase. The data showed that an appropriated concentration of $MgCl_2$ and the enzyme were 0.1 mM and 2.5 U, respectively.

About the first internal gene fragment amplification, degenerated primers which designed from the amino acid sequence data of alanine dehydrogenase and had 64 combinations of oligonucleotides in one set of primer were used. Therefore using more amount of primers should be required. In PCR condition, starting with annealing at 37°C was done because the lowest T_m of the primers was 49°C. For the possibility in binding with template of primers, the temperature of annealing step should be lower than the T_m of primers. Although the non-specific products can be obtained, all five pairs of primers also gave one specific products, so all of the primer could be used for nucleotide sequencing. The PCR product from this step (about 1,000 bp) was analyzed for nucleotide sequence using ABI prism sequencer. Unfortunately, the whole fragment could not be sequenced, but about 600 bp from both 5'end and 3'end was known, so the second internal gene fragment amplification was further done. The inner part of the first fragment was amplified by using the primer, which designed from the nucleotide sequence data of the first amplified fragments.

The second internal gene fragment amplification was done with the same condition of the first one, except that annealing step was done at 60°C and the new inner set of primer (NF4, NF5, NF6 and CR4) was used. Although the three pairs of primer had been tried, only one pair (NF6xCR4) gave the specific products (about 700 bp). The nucleotide sequence of this amplified gene fragment was identified. From the two times walk in amplification, the inner part of gene fragment was obtained and restriction map of this part was also analyzed.

For the N-terminal and C-terminal gene fragment amplification for sequencing, inverse PCR was used. Inverse PCR is a rapid and reliable method for obtaining sequences flanking a known region of DNA. The source of the DNA may be anything from chromatin

to cDNA to the purified insert of a lambda bacteriophage vector. After digestion, the DNA is diluted to a low concentration. This favors the formation of monomeric, circular molecules when the 150 ng of DNA fragment is ligated, as it is in the next step. The ligated DNA circles are then amplified by PCR. New DNA strands primed by either oligonucleotide primer extends round the circularized DNA, eventually incorporating a complementary copy of the other primer. A linear strand of DNA is thus produced, as the *Taq* enzyme is unable to displace the annealed primer as it attempts to make a second round of the circular template. The first PCR cycle thus effectively cuts the circularized molecule between the two primer sites, producing an amplified DNA fragment consisting of the two 'unknown' flanking regions joined end-to-end. Since *Taq* polymerase works slightly more efficiently with linear than circular DNA, the circular fragments may be linearized before amplification by digestion with a restriction enzyme which cuts only at a position between the two primers in the known target DNA sequence (Eeles *et al*, 1993). In this experiment the inverse PCR was done for two times in which the products from the first PCR which used outer pair of primers were then used as the templates for the second PCR and the inner pair of primers were used to produce more specific products. Nucleotide sequencing of this gene fragment was analyzed by using the innermost primers and the nucleotide of both N-terminal and C-terminal gene fragments were known. Unfortunately, although several times of sequencing were done with various primers, the signal of upstream sequence of this gene was so confused and could not be read. Therefore the ribosomal binding site was not obtained and the known sequence was started up only at open reading frame.

The ABI prism sequencer was used for nucleotide sequencing. From the result, the open reading frame of alanine dehydrogenase gene comprised 1,113 bp encoding 371 amino acids that matched with other alanine dehydrogenase and had GC content about 65%. More than 50% of the entire amino acid sequence was also confirmed by amino acid sequence analyses of 7 peptides obtained by lysyl endopeptidase and purification by HPLC (unpublished data) and found that the deduced amino acid matched well with those amino acid sequence. These peptides are shown at their positions in the amino acid sequence (Figure 3.16), Thus, the 1,113-bp open reading frame was identified as the translational unit for alanine dehydrogenase. On the basis of the deduced amino acid sequence (371 residues), the molecular weight of the enzyme subunit was calculated as 40,810 which corresponded to

the value (about 40,000) determined by sodium lauryl salfate-polyacrylamide gel electrophoresis of the protein previously report (Phungsanthum, 1997). Amino acid sequence of all alanine dehydrogenases were aligned by using Clustal X (1.64b) as shown in Figure 3.16. The over all similarity scores of alanine dehydrogenase from *Aeromonas hydrophila* compared with *Bacillus stearothermophilus*, *Enterbactor aerogenes*, *Bacillus subtilis*, *Phormidium lapideum*, *Mycobacterium tuberculosis* and *Bacillus sphaericus* were calculated to be 53.91%, 53.64 %, 53.10%, 53.10%, 52.56% and 50.67%, respectively. Sawa *et al.* (1994, cited in Chowdhury *et al.*,1998) showed that lys-74 was a catalytically important residue by chemical modification of the *P. lapideum* enzyme with pyridoxal 5'-phosphate and by site-directed mutagenesis. Delforge *et al.* (1997) also reported that lys-74 was necessary for the binding of pyruvate in the active site of the *B. subtilis* enzyme. This lysine residue and the surrounding region (KVKEP) are also conserved in the *A. hydrophila* enzyme. The side chain amino group of lys-74 may interact with both the carbonyl oxygen of the pyruvate and one of its carboxyl oxygens. A hydrogen bond may be also made between carbonyl oxygen of the pyruvate and the side chain of his-95, and NH_2 from the side chain of asn-299 may interact with one of the carboxylate oxygens of the pyruvate. All of the above residues are identical in all the L-alanine dehydrogenase sequences (Baker *et al.*, 1998) and also in *A. hydrophila.* For the L-alanine dehydrogenase-NAD^+ binary complex, the presence of conserved hydrophobic residues, the G-X-G-X-X-(G/A) fingerprint sequence and the conserved aspartate residue involved in ribose hydroxyl hydrogen bonding suggest that the amino acid dehydrogenases bind dinucleotides in a manner analogous to other structurally characterized dehydrogenase (Norbert *et al.*, 1994). In addition, the *syn* conformation of this bond is precluded by a potential steric clash with met-132 and ser-133. As a consequence, the 4-pro-S hydrogen of the nicotinamide ring is buried against the enzyme surface allowing the 4-pro-R hydrogen to be involved in the hybrid transfer thus explaining the stereospecificity seen in L-alanine dehydrogenase (Baker *et al.*, 1998).

For the whole gene fragment amplification, the new pair of primers was designed. The sense primer had contained *Eco*RI site and Shine Dalgano Sequence, while the antisense primer had *Hin*dIII site for further cloning purpose. The amplified whole gene fragment was purified before the digestion at the end of both sides in the purpose of eliminating of bound protein, which may inhibit the restriction enzyme activity. The whole gene fragments were ligated with *Eco*RI-*Hin*dIII site of pUC18

and transformed into *E. coli* JM109 host cells. The blue-white colony screening method was used to select the colony that has recombinant plasmid.

The clones that have recombinant plasmid were determined for alanine dehydrogenase activity. The clones were grown in the medium containing ampicillin and IPTG. Because the gene fragments which were cloned into host cell did not have their own promoter, therefore *lacZ* promoter on the plasmid pUC18 was used and IPTG was added to induce alanine dehydrogenase gene expression. From 24 clones, there were 3 patterns of inserted plasmids (Figure 3.18). The plasmid pattern no. 1 contained alanine dehydrogenase gene joined with 1 piece of plasmid vector pUC18, while the plasmid pattern no.2 was 2 pieces of pUC18 which joined to each other. The plasmid pattern no.3 consisted of blunt-end ligated DNA fragment of two single digested PCR products, *Eco*RI digested whole gene product and *Hin*dIII digested whole gene product, joined with 1 piece of *Eco*RI-*Hin*dIII digested plasmid. From the 20 clones harboured plasmids pattern no.1, only 10 colonies presented alanine dehydrogenase activity. The specific activity of transformants were ranging from of 0-10 U/mg protein and the transformant which had highest activity had specific activity almost 50 times higher than that of the enzyme from *A. hydrophila*. This high expression may be occurred because the gene fragment which was inserted into *E. coli* JM 109 contained the Shine Dalgano Sequence of expression vector p*Trc*99 and the multicopy plasmid, pUC18, was also used as a cloning and expression vector. Another reason is the nucleotide sequence of the gene fragment, which obtained from PCR may be mutated from the native alanine dehydrogenase gene of *A. hydrophila* to the others, which gave higher activity. The crude extracts from those transformants were determined by native-PAGE compared with the crude extract of host cell, *E. coli* JM109, *E. coli* JM109 which has only vector DNA and purified alanine dehydrogenase from *Aeromonas hydrophila*. The result showed that the crude extract from transformants had high intensity of alanine dehydrogenase protein band and also had alanine dehydrogenase activity, while *E. coli* JM109 and *E. coli* JM109 which has only vector pUC18 did not have the enzyme activity at all. The mobility of the enzyme band in native-PPAGE of tranformants were similar to that of *A. hydrophila* enzyme, thus, its physical properties may not be changed. However, studies of these transformant enzymes compare with that of *A. hydrophila* should be done in the future. On the other hand, the transformants, which did not show the enzyme activity presented in native-PAGE that, they did

not produce protein band at the position of the alanine dehydrogenase. It may due to the error in transcription or translation level.

In this research, the sequence of alanine dehydrogenase gene from *Aeromonas hydrophila* was determined and it was cloned into *E.coli* JM109. The obtained information can be used as primary data for further development of alanine dehydrogenase. For further studies, properties of transformant enzymes should be investigated firstly.

CHAPTER V

CONCLUSION

1. Alanine dehydrogenase gene from *Aeromonas hydrophila* was sequenced and cloned into *E. coli* JM109. This gene has open reading frame of 1,113 bp which encoded for 371 amino acids and made good agreement with about a 40,000-Da subunit of purified alanine dehydrogenase from *Aeromonas hydrophila* (Phungsangthum, 1997).
2. The alanine dehydrogenase gene from *Aeromonas hydrophila* has GC content about 65%.
3. The over all similarity scores of alanine dehydrogenase from *Aeromonas hydrophila* compared with *Bacillus stearothermophilus*, *Enterbactor aerogenes*, *Bacillus subtilis*, *Phormidium lapideum*, *Mycobacterium tuberculosis* and *Bacillus sphearicus* were calculated to be 53.91%, 53.64%, 53.10%, 53.10%, 52.56% and 50.67%, respectively.
4. The transformants that showed alanine dehydrogenase activity had specific activity in the range of about 0-10 U/mg protein. The highest activity was 50 times more than the enzyme from *Aeromonas hydrophila*.

REFFERENCES

ภาษาไทย

ปิยะรัตน์ พึ่งแสงธรรม. 2540. การคัดเลือกแบคทีเรียที่ผลิตแอล-อะลานีนดีไฮโดรจิเนสที่ต้องการไพริดีนนิวคลีโอไทด์เป็นโคเอนไซม์ และการทำให้บริสุทธิ์. วิทยานิพนธ์ปริญญามหาบัณฑิต ภาควิชาชีวเคมี บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย.

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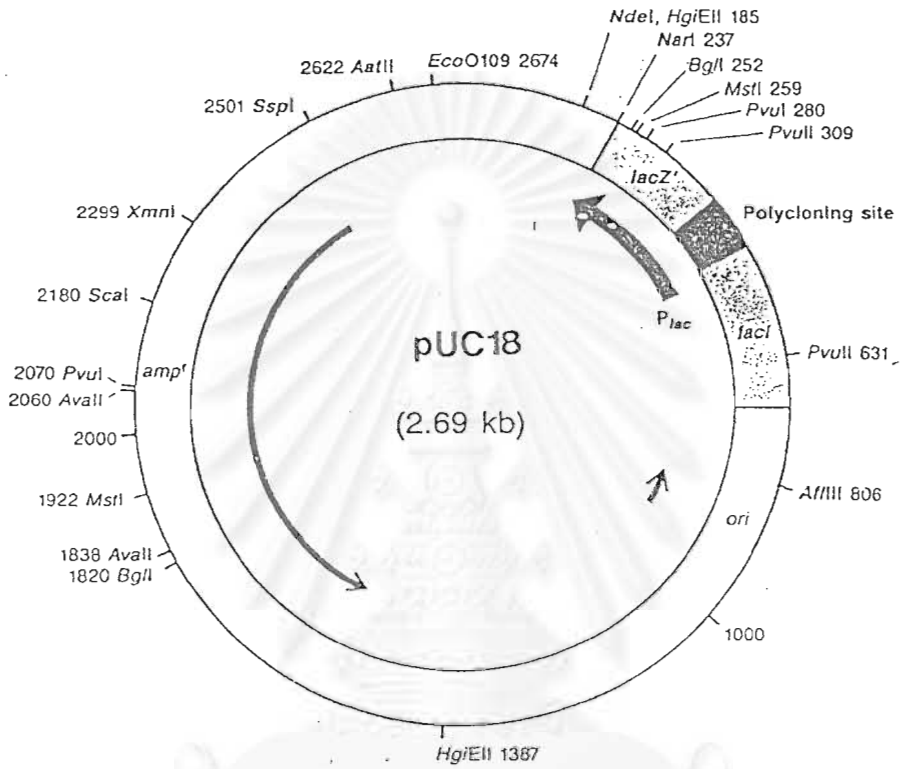
APPENDICES

APPENDIX A

Restriction map of pUC18



Polycloning Sites
pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC
							EcoRI		*SacI*		*KpnI*		*SmaI* *XmaI*		*BamHI*		*XbaI*		*SalI* *AccI* *HincII*		*PstI*		*SphI*		*HindIII*	

APPENDIX B

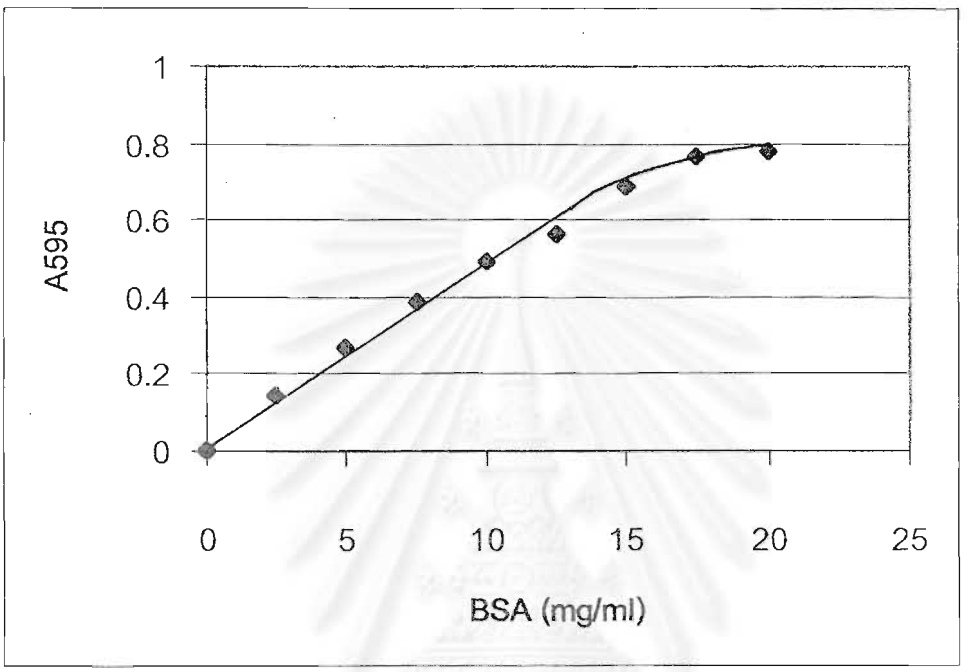
Bradford Solution

1. Bradford Stock Solution
   100 ml of 95% ethanol
   200 ml of 85% phosporic acid
   350 mg Serva Blue G

2. Bradford Working Buffer
   425 ml of distilled water
   15 ml of 95% ethanol
   30 ml of 88% phosphoric acid
   30 ml of Bradford Stock Solution

APPENDIX C

Standard curve for protein determination by Bradford's method



BIOGRAPHY

Miss Natwadee Poomipark was born on February 27, 1976. She graduated with the degree of Bachelor of Science from the department of Biochemistry at Chulalongkorn University in 1997. She has studied for the degree of Master of Science at the Department of Biochemistry, Chulalongkorn University since 1998.