CHAPTER III

RESULTS

3.1 Identification of L-Lys 6-DH producing bacteria

A Lys 6-DH producing bacteria strain K-1 was screened from soil in Japan. Its morphological and biochemical properties were characterized by Union Hitech Co., Ltd., Osaka, Japan. The bacterium strain K-1 was a gram negative, rod shape that could move by peritrichous flagella (Figure 3.1). This bacterium could grow at 42°C. The biochemical characteristics of this bacterium were shown in Table 3.1. From these properties, the strain K-1 bacterium was identified as *Alcaligenes denitrificans* (Ruger and Tan, 1983). Moreover, identification of this bacterium was confirmed by 16S rRNA gene sequence. The 16S rRNA gene fragment was PCR amplified with specific primers using genomic DNA of the bacterium strain K-1 as template DNA. Only a single band of PCR amplified product of 1.5 kb was obtained (Figure 3.2). DNA sequence analysis showed that the amplified fragment was 1,523 bp in length (Figure 3.3). By using BLAST program, the 16S rRNA gene of this bacterium K-1 showed 98% identical with 16S rRNA gene of *Alcaligenes* spp. (accession no. AY331576) (Figure 3.4).

According to the reclassification of this organism in 2003 by Tom and his coworkers using several techniques including SDS-PAGE of whole-cell proteins, 16S rRNA gene sequencing, DNA–DNA hybridization experiments, determination of DNA base composition, fatty acid methyl ester analysis, phenotypic characterization, and antimicrobial susceptibility testing, *Alcaligenes denitrificans* was justified to be *Achromobacter denitrificans*. Thus, the bacterium strain K-1 was identified to be *Achromobacter denitrificans*.

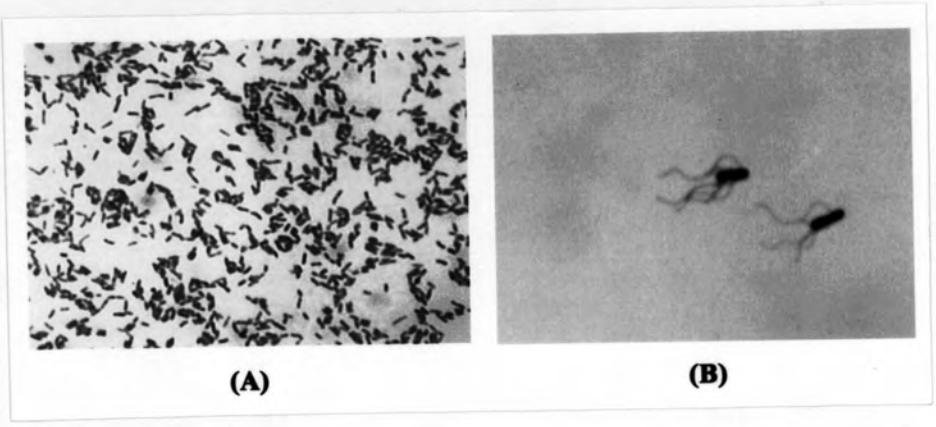

Figure 3.1 Cell morphology of the bacterium strain K-1

(A): Gram staining. The red color indicated that the bacterium was gram negative with rod shape.

(B): The picture of living cell under x1800 objective microscope. This picture showed peritrichous flagella.

Table 3.1 Biochemical characteristics of the bacterium strain K-1

Characteristics	Result
Shape	Rod
Gram staining	negative
Oxidation and Fermentation of glucose	-
Motility	+
Flagella	PE (>2)
Catalase	+
Oxidase	+
Growth at Macconkey agar plate	+
Growth at SS agar plate	+
Growth at cetrimide agar plate	-
Growth at KCN agar plate	+
Utilization of citrate	+
Hydrolysis of casein	-
Hydrolysis of gelatin	-
Hydrolysis of starch	-
Reduction of nitrate	+
Denitrification of nitrate	+
Urease test	-
Lys decarboxylase	-
Arginine dihydrolase	-
Ornithine decarboxylase	-
ONPG	-
LV	-
Lipase	-
Escrine hydrolysis	-
Indole test	-
DNase	-
Acetoamide	-
Growth at 42 °C	+
Acid from Carbohydrate	
Fructose	-
Trehalose	-
Xylose	-
Manitol	-
Cellobiose	-
Lactose	-
Maltose	-
Glucose	-
Erythlose	-
Inositol	-
Sucrose	-
Salicine	-

Remark: (+) = Positive reaction, (-) = Negative reaction

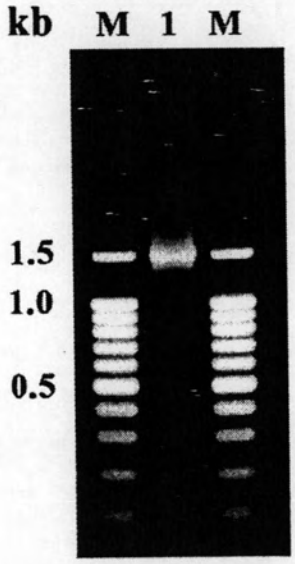

Figure 3.2 16S rRNA gene amplified product from the bacterium strain K-1

Lane M = 100 bp marker

Lane 1 = PCR products of 16S rRNA gene using genomic DNA of bacteria strain K-1 as a template

pA

AGAGTTTGATCCTGGCTCAGATTGAACGCTAGCGGGATGCTTTACACATG 50
CAAGTCGAACGGCAGCGCGGACTTCGGTCTGGCGGCGAGTGGCGAACGGG 100
TGAGTAATGTATCGGAACGTGCCCAGTAGCGGGGGATAACTACGCGAAAG 150
CGTAGCTAATACCGCATACGCCCTTTGGGGGAAAGCGGGGGACCTTCGGG 200
CCTCGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAA 250
AGGCCTACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCA 300
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA 350
ATTTTGGACAATGGGGGCAACCCTGATCCAGCCATCCCGCGTGTGCGATG 400
AAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACGGCCCTGGT 450
TAATACCTGGGGCAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTA 500
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTA 550
CTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCC 600
AGGGCTTAACCTTGGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCA 650
CAAGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGAGATGCGGAG 700
GAACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCA 750
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCT 800
AAACGATGTCAACTAGCTGTTGGGGGCTTCGGCCCTTGGTAGCGCAGCTA 850
ACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAA 900
AGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGA 950
TGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGA 1000
GATTTGGCAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGT 1050
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC 1100
CCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGAC 1150
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT 1200
AGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGCAGCCAACCCGCG 1250
AGGGGGAGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCA 1300
ACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCG 1350
CGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGA 1400
GTGGGTTTTACCAGAAGTAGTTAGCCTAACCGCAAGGGGGGCGATTACCA 1450
CGGTAGGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGA 1500

pH'

AGGTGCGGCTGGATCACCTCCTT 1523

Figure 3.3 Nucleotide sequence of 16S rRNA gene of the bacterium strain K-1

The amplified 16S rRNA gene was sequenced. The result of 1,523 bp was shown above. The primers used for amplification were underlined.

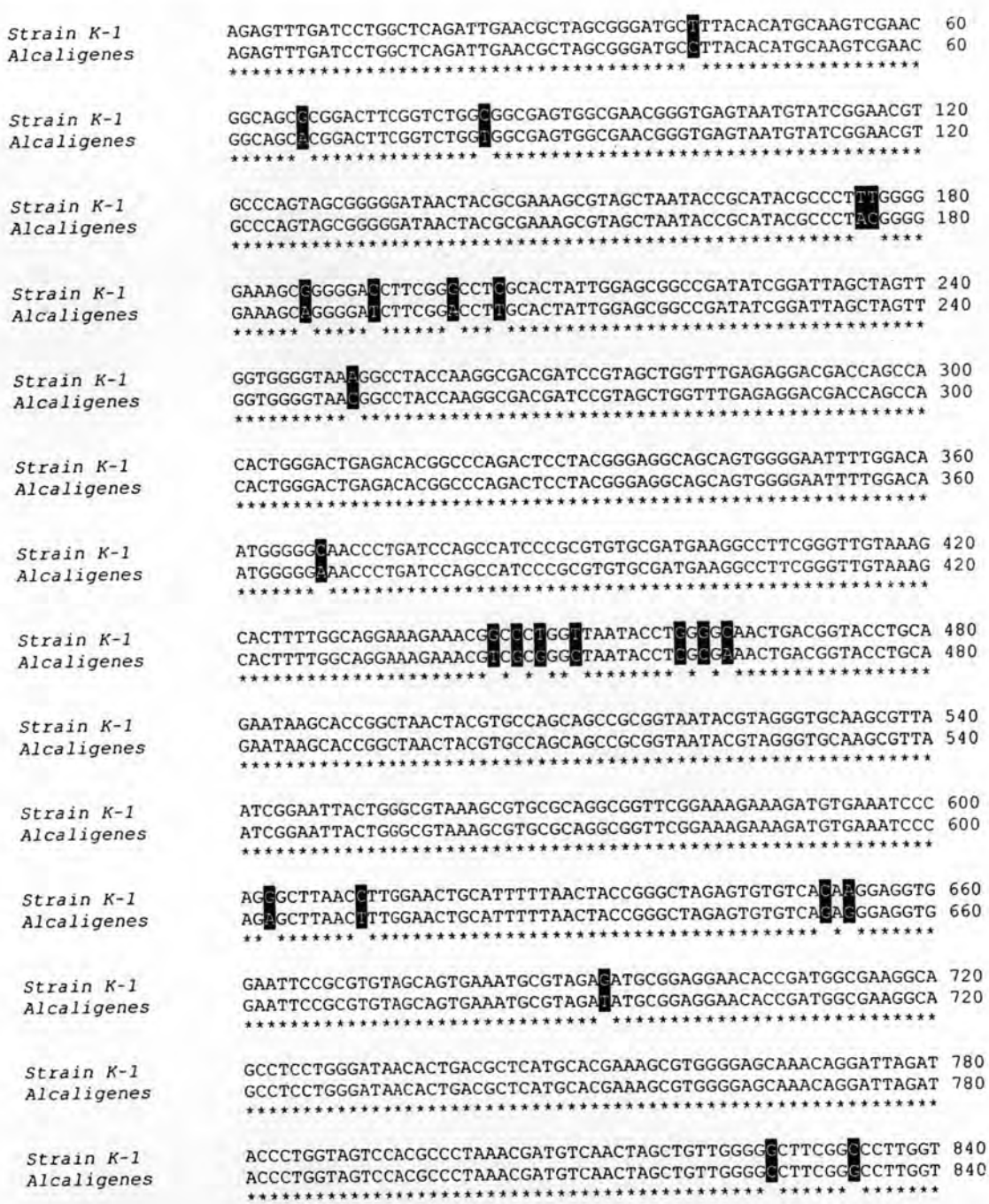

Figure 3.4 **Nucleotide sequence alignment of 16S rRNA gene of the bacterium strain K-1 compared with *Alacligenes* sp. mp-2**

Black colour indicated the different or missing nucleotides.

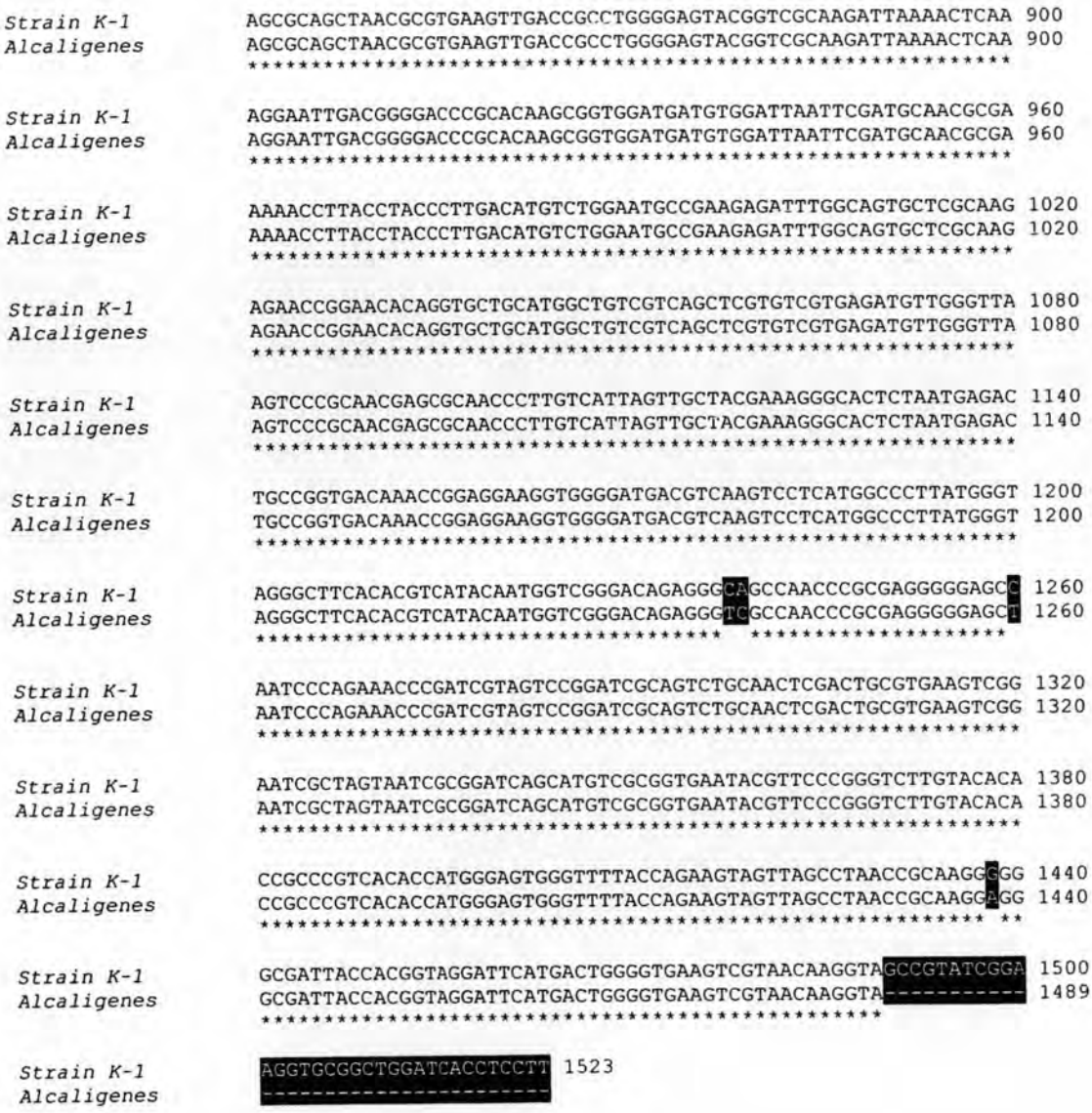

Figure 3.4 **Nucleotide sequence alignment of 16S rRNA gene of the bacterium strain K-1 compared with *Alacligenes* sp. mp-2 (continued)**

3.2 Purification of Lys 6-DH from *A. denitrificans* K-1

Crude lys 6-DH was prepared from 258.9 g of *A. denitrificans* K-1 which was cultivated in total 55 liters of medium, as described in 2.11.1 and 2.11.2. Crude enzyme solution contained 26 g proteins with 838 units of lys 6-DH activity in the total volume of 650 ml. Thus, the specific activity of the enzyme in the crude enzyme preparation was 0.03 unit/mg protein.

3.2.1 0-60% ammonium sulfate precipitation

Crude enzymes solution was further purified by ammonium sulfate precipitation as mentioned in 2.11.3.1. To harvest most of the enzyme, protein fractionation was carried out in the range of 0-60% saturated ammonium sulfate precipitation. The remained proteins were 14 g with enzyme activity of 814 units (97% recovery from the crude enzyme). The specific activity of the enzyme from this step was 0.06 unit/mg protein.

3.2.2 DEAE-Toyopearl 650 M column chromatography

The enzyme precipitated from 0-60% saturated ammonium sulfate precipitation was dissolved and dialyzed against 0.01 M KPB, pH 7.4. The enzyme solution was applied onto DEAE-Toyopearl column as described in 2.11.3.2. The chromatographic profile was shown in Figure 3.5. The unbound proteins were eluted from the column by 0.01 M KPB. The bound proteins were then eluted with stepwise elution of 0.05 M KCl in the same buffer. The enzyme was eluted as indicated in the profile.

The fractions with Lys 6-DH activity were pooled and dialyzed against 0.01 M KPB, pH 7.4. The protein remained from this step was 1.8 g with enzyme activity of 689 units (82.2% recovery from the crude enzyme). The specific activity of the enzyme from this step was 0.37 unit/mg protein.

3.2.3 0-40% ammonium sulfate precipitation

The pooled enzyme from DEAE-Toyopearl column was further purified by ammonium sulfate precipitation as mentioned in 2.11.3.3. To harvest most of the enzyme, protein fractionation was carried out in the range of 0-40% saturated ammonium sulfate precipitation. The remained protein was 389 mg with enzyme activity of 652 units (77.8% recovery from the crude enzyme). The specific activity of the enzyme from this step was 1.68 unit/mg protein.

3.2.4 Sephadex G-150 column chromatography

The enzyme precipitate from 0-40% saturated ammonium sulfate precipitation was dissolved and dialyzed against the 10 mM KPB, pH 7.4. The enzyme solution was applied onto Sephadex G-150 column as described in 2.11.3.4. The chromatographic profile was shown in Figure 3.6. The enzyme was eluted as indicated in the profile. The fractions with Lys 6-DH activity were pooled and dialyzed against 0.01 M KPB, pH 7.4. The remained protein from this step was 365 mg with enzyme activity of 614 units (73% recovery from the crude enzyme). The specific activity of the enzyme from this step was 1.68 unit/mg protein.

3.2.5 Mono Q HR 10/10 column chromatography

The pooled enzyme from Sephadex G-150 column was dialyzed against 0.01 M KPB, pH 7.4. The enzyme solution was applied onto Mono Q column as described in 2.11.3.5. The chromatographic profile was shown in Figure 3.7. The unbound proteins were eluted from the column by 10 mM KPB, pH 7.4. The bound proteins were then eluted with linear gradient of 0-500 mM KCl in the same buffer. The enzyme was eluted at 150 mM KCl as indicated in the profile.

The fractions with Lys 6-DH activity were pooled and dialyzed against 0.01 M KPB, pH 7.4. The remained protein from this step was 115 mg with enzyme activity of 361 units (43% recovery from the crude enzyme). The specific activity of the enzyme from this step was 3.14 unit/mg protein.

3.2.6 Phenyl Sepharose XK 16 column chromatography

The pooled enzyme from Mono Q column was dialyzed against 0.1 M KPB, pH 7.4 and was adjusted to 20% saturation with ammonium sulfate. The enzyme solution was applied onto Phenyl Sepharose column as described in 2.11.3.6. The chromatographic profile was shown in Figure 3.8. The enzyme was eluted by distilled water as indicated in the profile. The fractions with Lys 6-DH activity were pooled and dialyzed against 0.01 M KPB, pH 7.4. The remained protein from this step was 50 mg with enzyme activity of 260 units (33% recovery from the crude enzyme). The specific activity of the enzyme from this step was 5.63 unit/mg protein.

The protein from this step was purified to homogeneity with single band on SDS-PAGE. The summary of purification of Lys 6-DH was shown in Table 3.2. Enzyme purity in each step was determined by SDS-PAGE as described in 2.12.1.

3.3 Amino acid sequence analysis

The purified Lys 6-DH from 2.13.2 was directly used for amino acid sequence analysis by automated Edman degradation with Applied Biosystem model 610A data analysis system for protein sequencing. After digestion of Lys 6-DH with lysyl endopeptidase, the peptides was separated by HPLC, profile of the separation was presented in Figure 3.10. The isolated peptide numbers 2, 5, 13 and 14 were determined for their sequences. The amino acid sequences of the internal peptide fragments were ARNVDYK, SRTQHAITVLGAGK, KPPMEGYETFT LDGVEYEAFNTSGGLGTLPPTLEG and VPPMEGYETF, respectively.

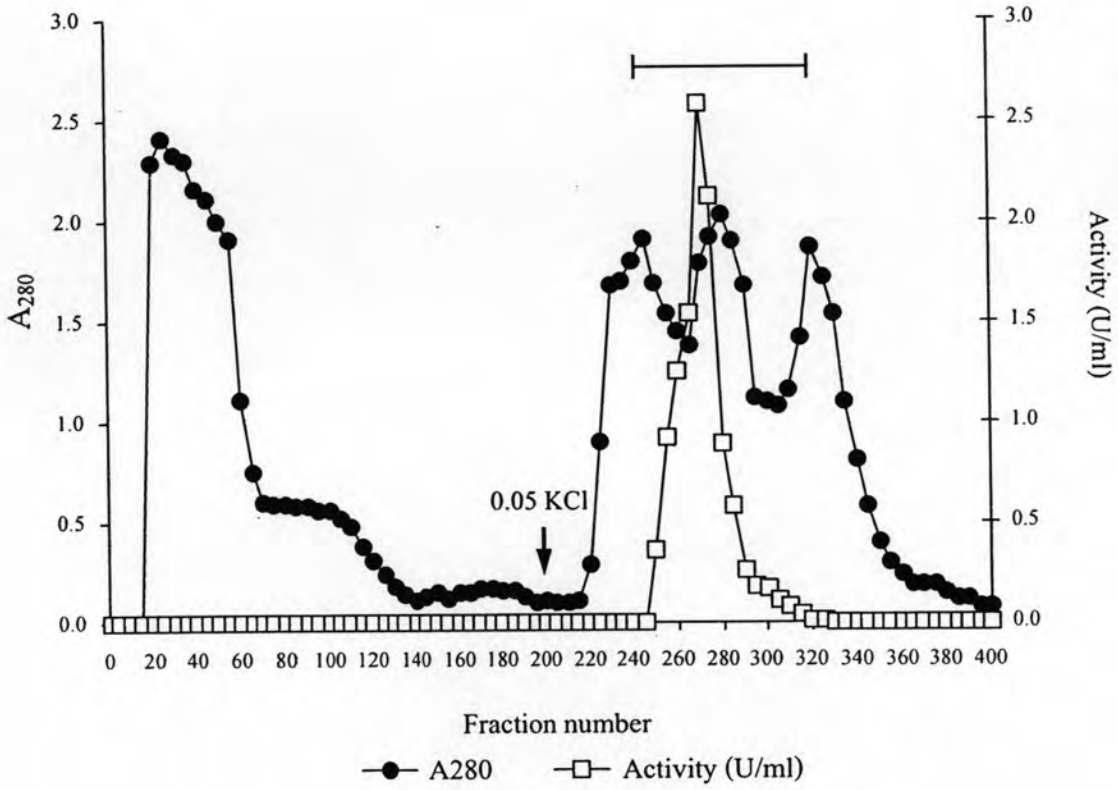

Figure 3.5 Purification of Lys 6-DH from *A. denitrificans* K-1 by DEAE-Toyopearl 650M column chromatography

The enzyme was applied to DEAE-Toyopearl 650 M column (5 x 25 cm) and washed with 0.01 M KPB, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA until the absorbance at 280 nm of eluent decreased to base line. The bound protein elution was made by stepwise of 0.05 M KCl in the same buffer at a flow rate of 1 ml/min. Fractions of 15 ml each were collected. The arrow indicated where stepwise elution was started. Active proteins with activity peaks from the fraction numbers 246-320 were pooled as indicated by (⊢⊣).

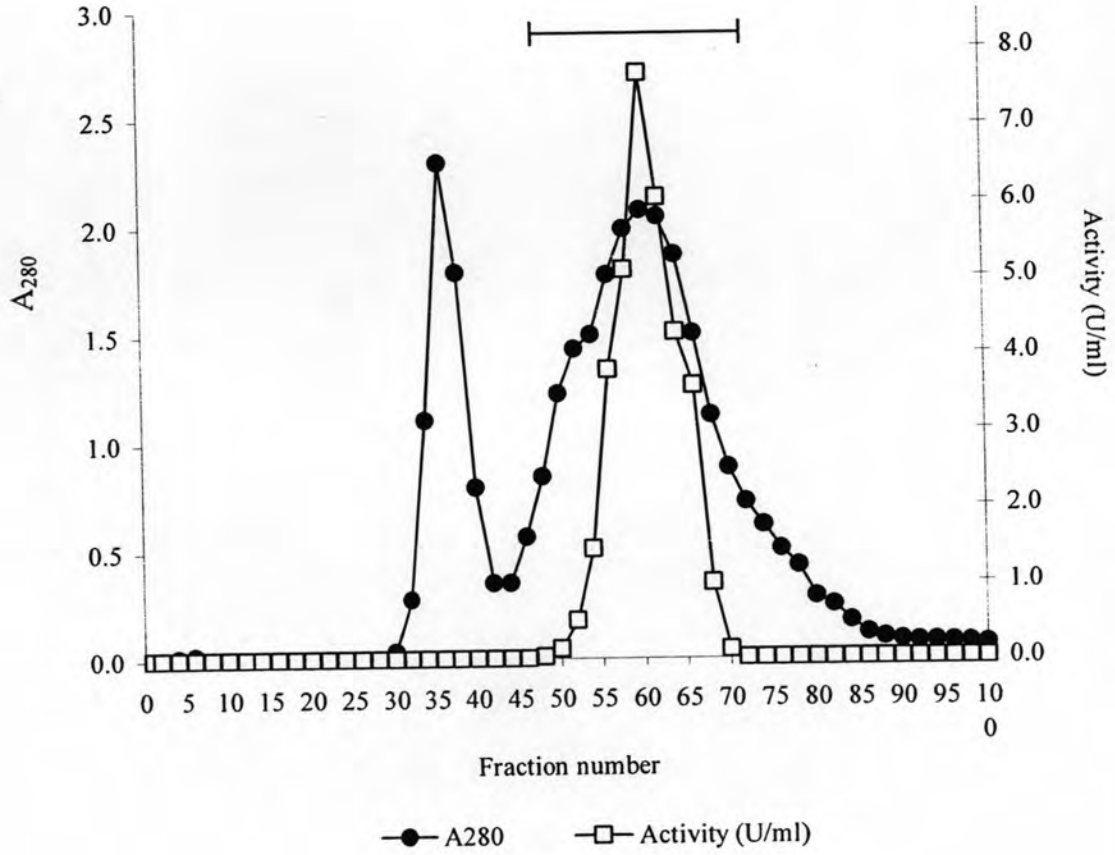

Figure 3.6 **Purification of Lys 6-DH from *A. denitrificans* K-1 by Sephadex G-150 column chromatography**

The enzyme was applied to Sephadex G-150 column (2 x 120 cm) and washed with 0.01 M KPB, pH 7.4 containing 0.1 M KCl, 0.01 % β-mercaptoethanol and 1 mM EDTA until the absorbance at 280 nm of eluent decreased to base line. The bound protein elution was made by stepwise of the same buffer at a flow rate of 0.2 ml/min. Fractions of 5 ml each were collected. Active proteins with activity peaks from the fraction numbers 47-71 were pooled as indicated by (⊢⊣).

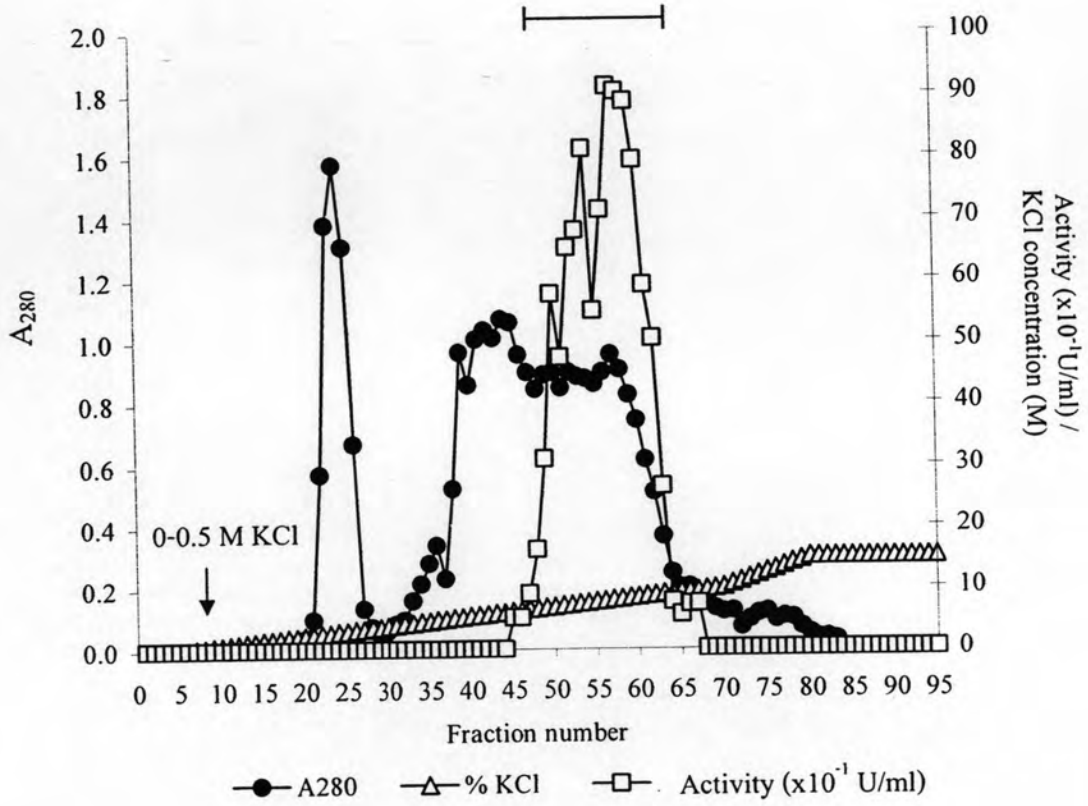

Figure 3.7 **Purification of Lys 6-DH from *A.denitrificans* K-1 by Mono Q HR 10/10 column chromatography**

The enzyme was applied to Mono Q HR 10/10 column (1.5 x 8 cm) and washed with 0.01 M KPB, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA. The bound protein elution was made by a linear gradient of 0-0.5 M KCl in the same buffer a flow rate of 2 ml/min. Fractions of 4 ml each were collected. Active proteins with activity peaks from the fraction numbers 48-63 were pooled as indicated by (⊢⊣).

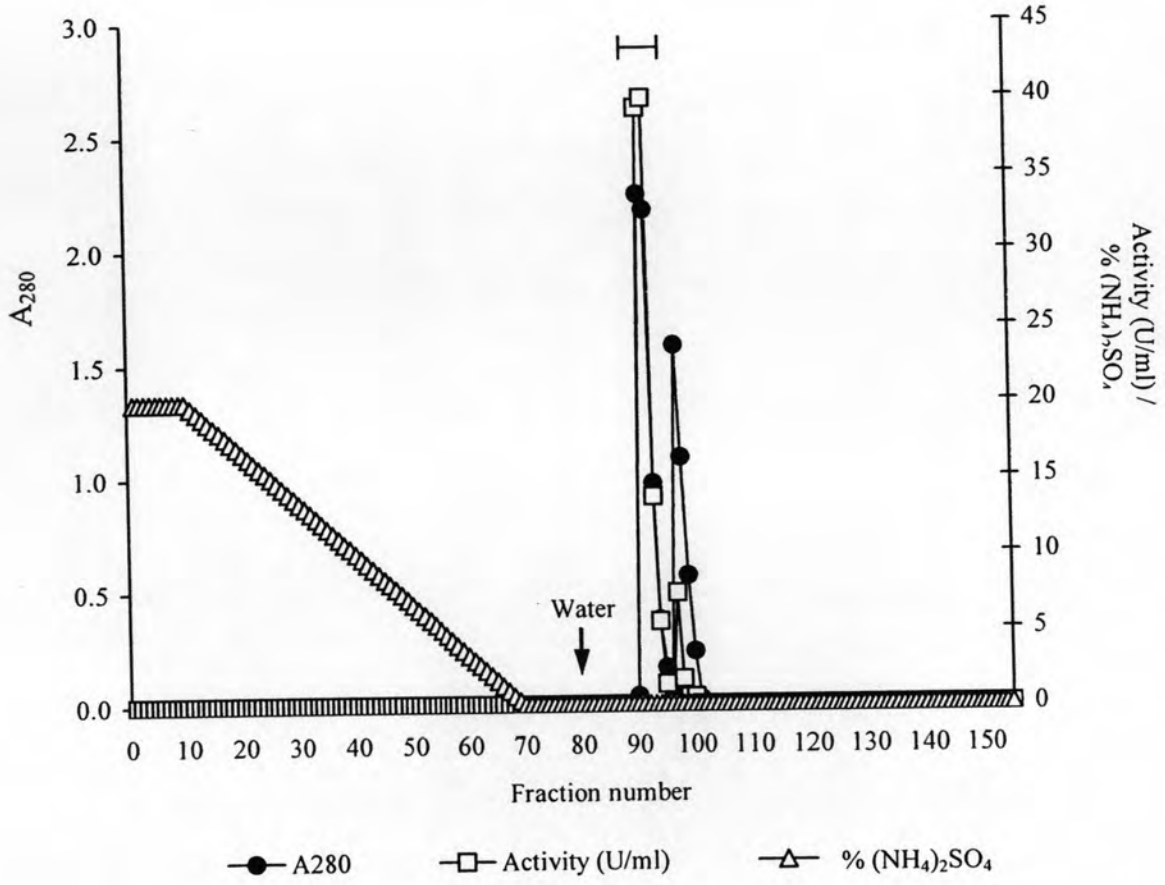

Figure 3.8 Purification of Lys 6-DH from *A.denitrificans* K-1 by Phenyl Sepharose XK 16 column chromatography

The enzyme was applied to Phenyl Sepharose XK 16 column (1.6 x 3 cm) was and washed with 100 mM potassium phosphate buffer, pH 7.4 containing 20% ammonium sulfate, 0.01% β-mercaptoethanol and 1 mM EDTA. The linear gradient of 20-0% ammonium sulfate in the same buffer was made. The bound protein elution was made by distilled water at a flow rate of 2 ml/min. Fractions of 4 ml each were collected. The arrow indicated where distilled water was applied. Active proteins with activity peaks from the fraction numbers 91-95 were pooled as indicated by (⊢⊣).

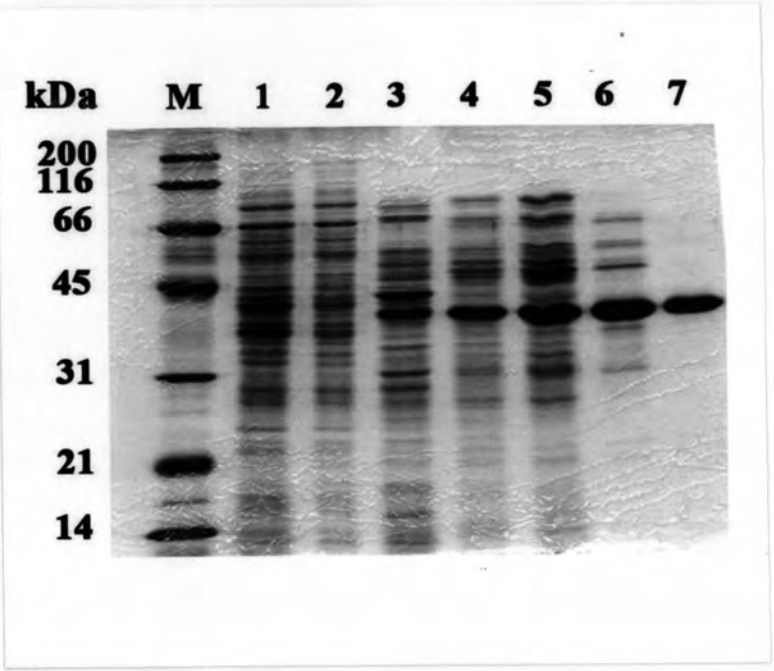

Figure 3.9 **SDS-PAGE of the *A. denitrificans* K-1 Lys 6-DH from each purification step**

Lane M	= Protein MW marker	150 μg
Lane 1	= Crude extract	20 μg
Lane 2	= 0-60% ammonium sulfate precipitation	20 μg
Lane 3	= DEAE-Toyopearl column	20 μg
Lane 4	= 0-40% ammonium sulfate precipitation	20 μg
Lane 5	= Sephadex G-150 column	20 μg
Lane 6	= Mono Q HR 10/10 column	20 μg
Lane 7	= Phenyl Sepharose XK 16 column	20 μg

Table 3.2 Purification of Lys 6-DH from *A. denitrificans* K-1

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	% Recovery
Crude extract[a]	2.6×10^4	838	0.03	1	100
60% ammonium sulfate	14×10^3	814	0.06	1.8	97.1
DEAE-Toyopearl	1885	689	0.37	11.4	82.2
40% ammonium sulfate	389	652	1.68	52.4	77.8
Sephadex G-150	365	614	1.68	52.6	73.3
Mono Q	115	361	3.14	98.1	43.1
Phenyl Sepharose	50	284	5.63	175.8	33.8

[a] Crude extract was prepared from 55 liters (258.9 g cell wet weight) of cell culture.

These amino acid sequences obtained were compared with previously published amino acid sequences of Lys 6-DHs from the EMBL-GenBank-DDBJ database. Multiple sequences were aligned to find the position of peptide sequences using the CLUSTAL W program (Figure 3.11) and further used as the data to design the degenerated primers for PCR amplification (Table 3.3).

3.4 Internal gene amplification of the *lys 6-dh* gene

For PCR amplification, the chromosomal DNA of *A. denitrificans* K-1 was completely digested with each of 3 restriction enzymes; *Bam*HI, *Eco*RI and *Hin*dIII. Agarose gel electrophoresis analysis of all digested DNA showed smear pattern of DNA fragments smaller than 23.1 kb (data not shown). All of digested DNA samples were used as the templates for PCR amplification of partial *lys 6-dh* gene fragment using forward primers; EDF1, EDF2 and KN2 together with reverse primers; EDR1, EDR2 and C1. While the other pair of primers were gaved only nonspecific bands, only the primers KN2 and C1 gave expected PCR product of about 600 bp without nonspecific bands from all three chromosomal DNAs digested with restriction enzymes (Figure.3.12). Then, this amplified DNA fragment was eluted from gel by QIAquick gel extraction kit and its nucleotide sequence was determined from both directions using the primers KN2 and C1.

After the 592-bp sequence of the internal gene fragment had been obtained (Figure 3.13), it was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database by the Blastx program. The alignment showed 43 and 41% nucleotide sequence identity to a part of the *lys 6-dh* genes from *Rhizobium leguminosarum* and *Legionella pneumophila*, respectively. This result indicated that the amplified fragment should be a part of *A. denitrificans lys 6-dh* gene (data not shown). Finally, the internal nucleotide sequence was used to design antisense primers EDF1, EDF2, ADK-U and ADK-U2 and sense primers EDR1, EDR2, ADK-D and ADK-D2 for inverse PCR to amplify the 5′ and 3′-terminal fragments s of the *lys 6-dh* gene (Table 3.4).

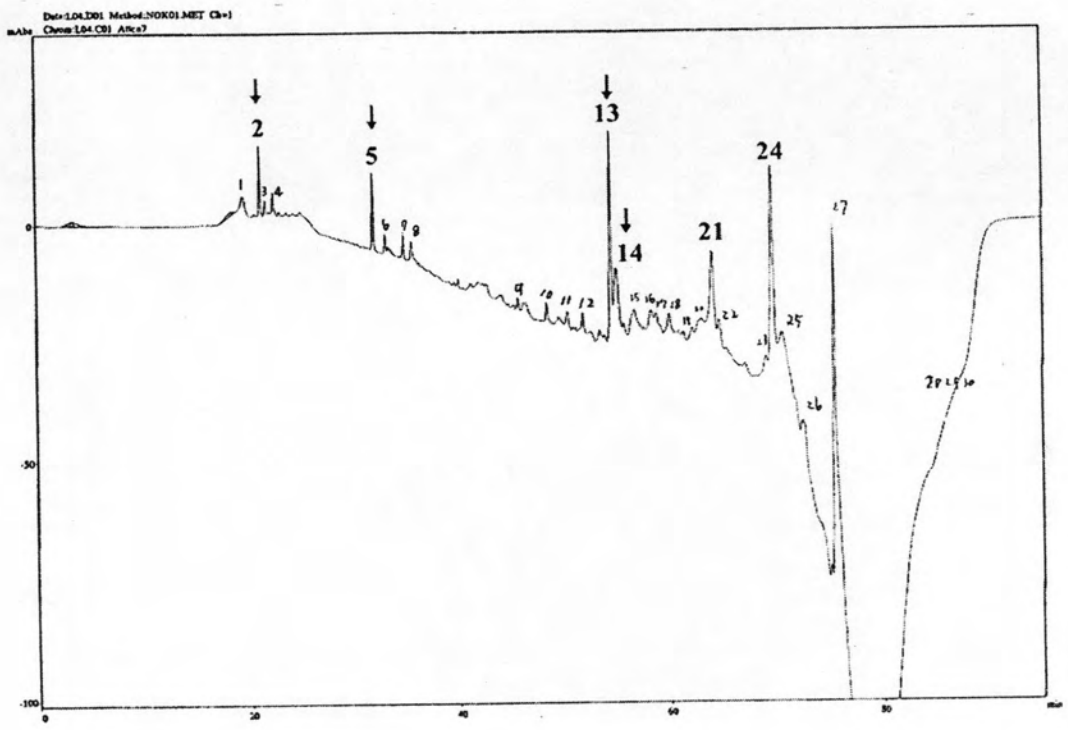

Figure 3.10 The reverse-phase HPLC profile of lysyl endopeptidase-digested peptides.

The arrows show the isolated peaks that were used for amino acid sequencing.

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CLUSTAL W (1.83) multiple sequence alignment

Achromobac      ------------------------------------------------------------
Bacillus        -MKVLVLGAGLMGKEAARDLVQSQDVEAVTLADVDLAKAEQTVRQLHSKKLAAVRVDAGD 59
Oceano          -MKIGVLGSGLMGKEAAVDLTLSEGVKQVGLADIDLLRAQEVCDQLNSQKLTAYQVDASN 59
Agrobact        MKNIVVIGAGNIGSAIAWMLAAS-GDYRITVADR---SADQLANVPAHERVDTEIVDITD 56

Achromobac      ----------------------------------------------------SRTQH 5
Bacillus        PQQLAAAMKGHDVVVNALFYQFNETVAKTAIETGVHSVDLGGHIGHITDRVLELHERAQA 119
Oceano          QLDLAKFMRQFDVIINALFYSFNEVVARTAIEVGVSSVDLGGHIGHVTDKVLTYHEDAKQ 119
Agrobact        RPALEALLKGKFAVLSAAPFHLTAGIAEAAVAVGTHYLDLTEDVESTR----KVKALAET 112
                                                                    ::
Achromobac      A-ITVL----------------GAGK---------------------------------- 14
Bacillus        AGVTIIPDLGVAPGMINILSGYGASQLDEVESILLYVGGIPVRPEPPLEYNHVFSLEGLL 179
Oceano          AQVTVIPDLGVAPGMINILSGYGVSKLDHTKKIKLYVGGIPVKPEPPLEYNHVFSMEGVF 179
Agrobact        AETALIPQCGLAPGFISIVAADLAVKFDKLDSVRMRVGALPQYPSNALNYNLTWSTDGLI 172
                *  :::                 . :      . .::   .:: :*:

Achromobac      ----------------VPPMEGYETFTLDGV-EYEAFNTSGGLGTLPPTLE-GARNVDY 55
Bacillus        DHYTDPALIIRNGQKQEVPSLSEVEPIYFDRFGPLEAFHTSGGTSTLSRSFP-NLKRLEY 238
Oceano          DHYTDPSLIIRNGLVQEVSSLSEVEPIHFEKFGPLEAFHTSGGTSTLSLSYP-YLDTLEY 238
Agrobact        NEYIEPCEGFVEGRLTAVPALEEREEFSLDG-ITYEAFNTSGGLGTLCATLEGKVRTMNY 231
                          ..:.  * : :: .   ***:****   *  :       ::*

Achromobac      K----------------------------------------------------------- 56
Bacillus        KTIRYRGHAEKCKLLVDLTLTRHDVEVEINGCRVKPRDVLLSVLKPLLDLKGKDDVVLLR 298
Oceano          KTIRYPGHAEKFKLLVDLNLTRLDYQVDINGMKVNPREVFLKVLDPIVELGDREDAVLLR 298
Agrobact        RTIRYPGHVAIMKALLN--------DLNLRNRRDVLKDLFENALP-----GTMQDVVIVF 278
                :

Achromobac      ------------------------------------------------------------
Bacillus        VIVGGRKDGKETVLEYETVTFNDREN--KVTAMARTTAYTISAVAQLIGRGVITKRGVYP 356
Oceano          VVVEGEKDGKSTSHIFEMTTYKDKDT--KVTAMARATANTISVVAQMIANGTINTKGVLP 356
Agrobact        VTVCGTRNGRFLQETYANKVYAGPVSGRMMSAIQITTAAGICTVLDLLAEGALPQKGFVR 338

Achromobac      --------------------------------
Bacillus        PEQIVPGDVYMDEMKKRGVLISEKRTVHS---- 385
Oceano          PEKMVPGKQYIDEMKKRGVTIKESVSINKQTSV 389
Agrobact        QEEVALPKFLENRFGRYYGSHEPLARVG----- 366
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Figure 3.11 The CLUSTALW alignment of amino acid sequence of purified Lys 6-DHs from *A. denitrificans* K-1 and those of various sources.

Achromo = *Achromobacter denitrificans*, Agrobact = *Agrobacterium tumefaciens*, Bacillus = *Bacillus sterothermophilus* and Oceano = *Oceanobacillus iheyensis*. Conserved residues were indicated by asterisks (*). (:) indicated amino acids which had the same group of side chains and similar size while (.) means amino acids which had the same group of side chains but different size.

Table 3.3 **The sequences of forward and reverse primers for the internal *lys 6-dh* gene amplification**

Primer	Nucleotide sequence (5′-3′)	Length (bp)	Tm (°C)
Forward primers			
EDF1	5′-CTGGGCGCSGGCAAGATYGGCT-3′	22	72
EDF2	5′-ATYGCSCTGCTGCTGC-3′	16	50
KN2	5′-ACTCARCAYGCTATTCANGTNYT-3′	23	41
Reverse primers			
EDR1	5′-TCRTAYTCCACGCCGTCCAGSGT-3′	23	65
EDR2	5′-GGCCGCCSSWSGTRTTRAASGC-3′	22	55
C1	5′-AATGTYTCRTATCCYTCCATTGGNG GNGGNAC-3′	32	63

S = C or G Y = C or T R = A or G

W = A or T N = A or G or T or C

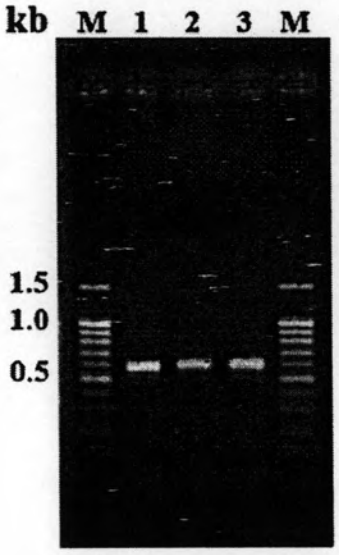


Figure 3.12 The internal fragment PCR products using primer KN2 and C1 with various digested DNA as templates

Lane M = 100 bp DNA ladder
Lane 1 = PCR products using *Bam*HI digested DNA as template
Lane 2 = PCR products using *Hin*dIII digested DNA as template
Lane 3 = PCR products using *Eco*RI digested DNA as template

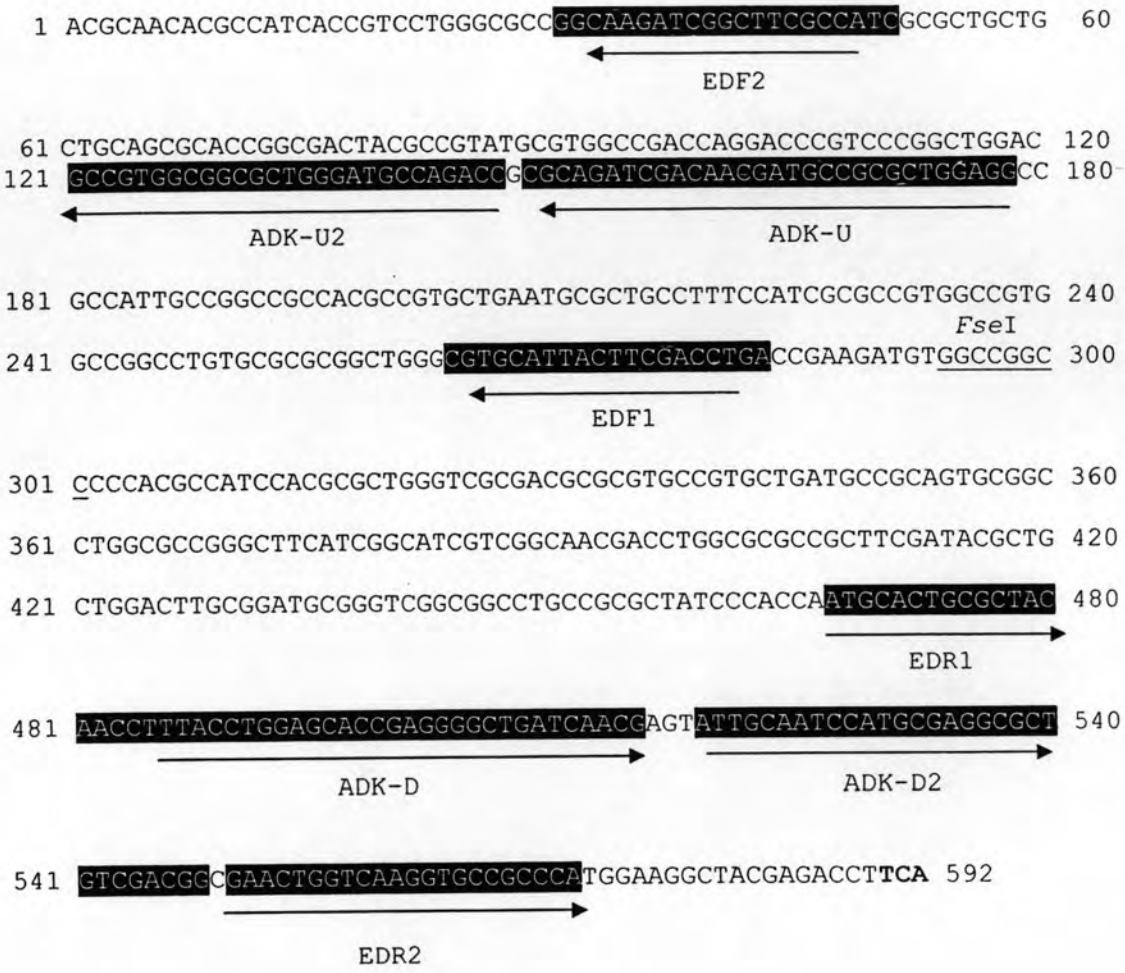


Figure 3.13 The nucleotide sequence of the internal fragment of *lys 6-dh* gene

The region and direction of primers designed for inverse PCR were indicated by highlights and arrows. *Fse*I restriction site (GGCCGG▼CC) was underlined.

Table 3.4 The sequences of forward and reverse primers for inverse PCR amplification

Primer	Nucleotide sequence (5′-3′)	Length (bp)	Tm (°C)
Forward primers			
ADK-U	5′-CCTCCAGCGCGGCATCGTTGTCGA TCTGCG-3′	30	80
ADK-U2	5′-GGTCTGGCATCCCAGCGCCGCCACG GCGTC-3′	30	90
EDF1	5′-CGTGCATTACTTCGACCTGA -3′	20	65
EDF2	5′-GGCAAGATCGGCTTCGCCATC-3′	21	74
Reverse primers			
ADK-D	5′-TCGTTGATCAGCCCCTCGGTGCTCC AGGTA -3′	30	82
ADK-D2	5′-TTGCAATCCATGCGAGGCGCTGGTC GACGG-3′	30	88
EDR1	5′-ATGCACTGCGCTACAACCTT-3′	20	65
EDR2	5′-GAACTGGTCAAGGTGCCTCCAA-3′	22	70

3.5 Inverse PCR amplification for 5′ and 3′-termini of the *lys 6-dh* gene

3.5.1 5′-terminal region of the *lys 6-dh* gene

Inverse PCR was used to amplify 5′-terminal region of the *lys 6-dh* gene as following. The chromosomal DNA was separately digested with each of the 16 restriction enzymes; *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Cla*I, *Hind*III, *Kpn*I, *Mlu*I, *Pvu*II, *Sac*I, *Sca*I, *Ssp*I, *Stu*I, *Xba*I and *Xho*I. All of digested DNA solutions were ligated and subsequently digested with *Fse*I, then used as the templates for inverse PCR amplification with one combination of forward primers; ADK-U2, ADK-U, EDF1 and EDF2 and reverse primers; ADK-D, ADK-D2, EDR1 and EDRII in each reaction.

The amplification result showed that the primers EDF1 and EDR1 gave a PCR product about 600 bp from the chromosomal DNA digested with *Stu*I (Fig.3.14). Thus, the amplified DNA fragment was recovered and sequenced. After that, the obtained nucleotide sequence was compared with the partial *lys 6-dh* gene fragment (Fig. 3.13) and the DNA sequences deposited in the EMBL-GenBank-DDBL database (data not shown). It was found that this fragment was the 5′-terminus of the *lys 6-dh* gene which comprised start codon (Fig.3.15).

Figure 3.14 The inverse PCR products amplified with the primers, EDF1 and EDR1, with chromosomal DNA digested with various restriction enzymes as templates

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = PCR products using *Apa*I digested DNA as template
Lane 2 = PCR products using *Bam*HI digested DNA as template
Lane 3 = PCR products using *Bgl*II digested DNA as template
Lane 4 = PCR products using *Eco*RI digested DNA as template
Lane 5 = PCR products using *EcoR*V digested DNA as template
Lane 6 = PCR products using *Cla*I digested DNA as template
Lane 7 = PCR products using *Hind*III digested DNA as template
Lane 8 = PCR products using *Kpn*I digested DNA as template
Lane 9 = PCR products using *Mlu*I digested DNA as template
Lane 10 = PCR products using *Pvu*II digested DNA as template
Lane 11 = PCR products using *Ssp*I digested DNA as template
Lane 12 = PCR products using *Stu*I digested DNA as template
Lane 13 = PCR products using *Xba*I digested DNA as template

The arrow indicated the 600 bp amplified PCR product.

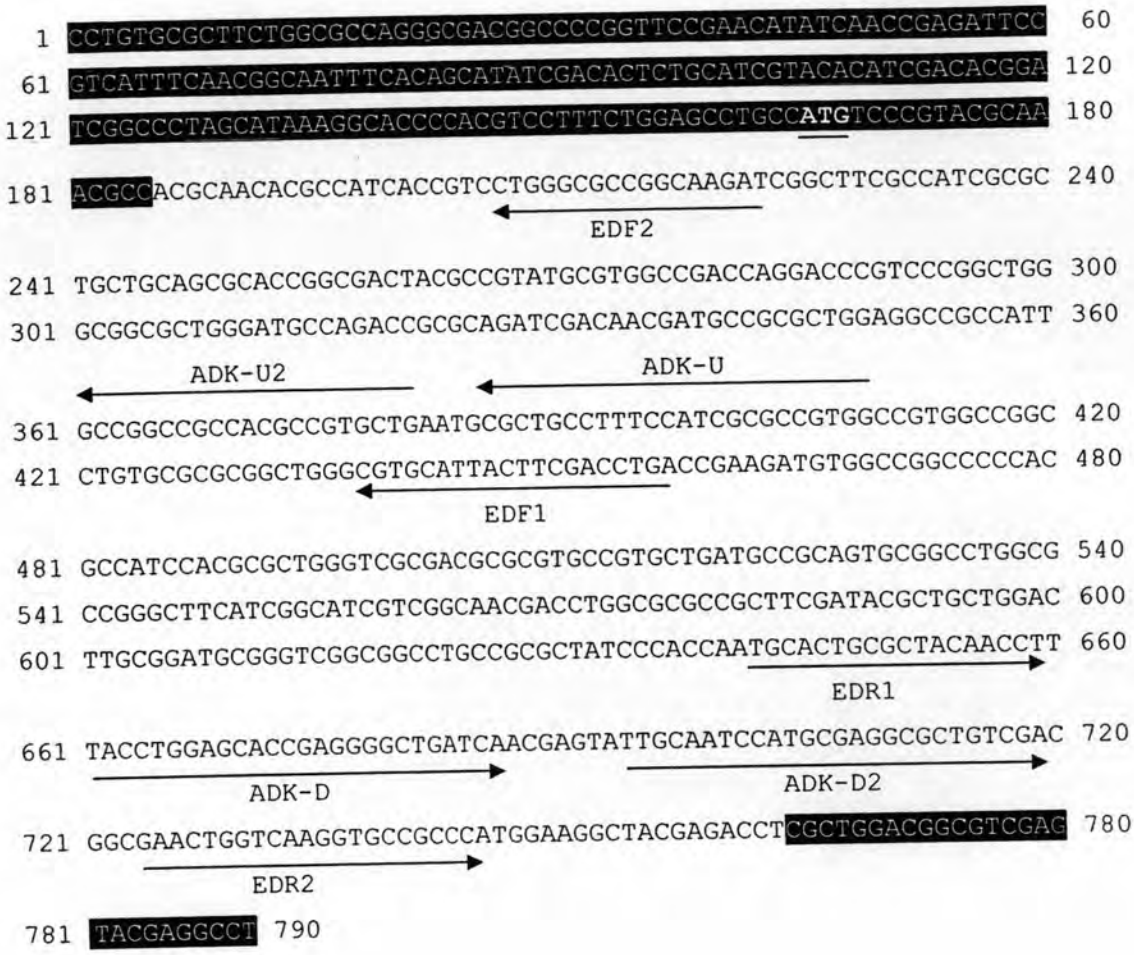


Figure 3.15 Nucleotide sequence of the 5′-terminal region of *lys 6-dh* obtained from inverse PCR using the EDF1 and EDR1 primers

The new nucleotide sequences obtained were highlighted. The start codon was underlined. The region and direction of primers for inverse PCR were indicated by arrows.

3.5.2 3′-terminal region of the *lys 6-dh* gene

The result showed that the primers, EDF1 and EDR1, gave PCR product of about 1.2 kb from the chromosomal DNA digested with *Xho*I (Fig.3.16). Thus, the amplified DNA fragment was recovered and sequenced. After that, the obtained nucleotide sequences were compared with the partial *lys 6-dh* gene fragment (Fig. 3.13) and the DNA sequences deposited in the EMBL-GenBank-DDBL database (data not shown). It was found that this fragment was the 3′-terminus of the *lys 6-dh* gene which consisted of stop codon (Fig.3.17).

3.6 Nucleotide sequence and deduced amino acid sequence of the *lys 6-dh* gene

From the gene walking by inverse PCR, the complete nucleotide sequence of the *lys 6-dh* gene was assembled as shown in Figure 3.18. The structural gene contained 1,107-bp open reading frame, which encoded a polypeptide of 368 amino acids. Molecular weight of the enzyme subunit calculated from the deduced amino acid sequence by Genetyx 6.0 program was 39.3 kDa.

The *lys 6-dh* gene was deposited in the EMBL-GenBank-DDBL database in the accession no. DQ165182. The nucleotide sequence was compared with those of the DNA sequences in the EMBL-GenBank-DDBL database. The percentages of sequence similarity between the nucleotide sequences of *lys 6-dh* gene from *A. denitrificans* K-1 and each of those from *Rhizobium leguminosarum*, *Legionella pneumophila,* and *Geobacillus kaustophilus* were 46, 20 and 19%, respectively (Figure 3.19).

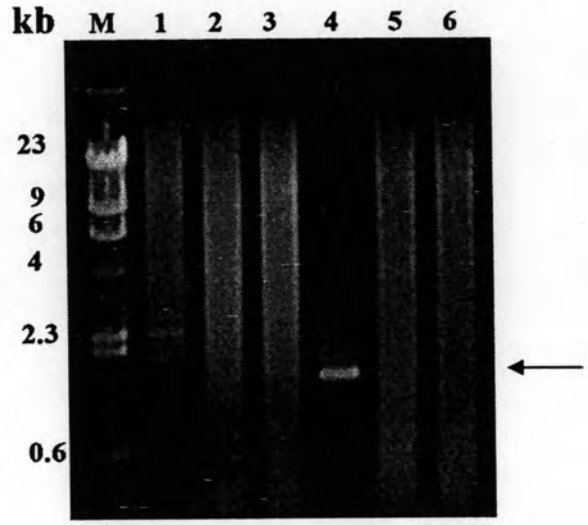


Figure 3.16 The inverse PCR products amplified with the primer EDF1 and EDR1 using chromosomal DNA digested with various restriction enzymes as templates

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = PCR products using *Apa*I digested DNA as template
Lane 2 = PCR products using *Bam*HI digested DNA as template
Lane 3 = PCR products using *Bgl*II digested DNA as template
Lane 4 = PCR products using *Xho*I digested DNA as template
Lane 5 = PCR products using *EcoR*V digested DNA as template
Lane 6 = PCR products using *Cla*I digested DNA as template

The arrow indicated the 1.2 kb amplified PCR product.

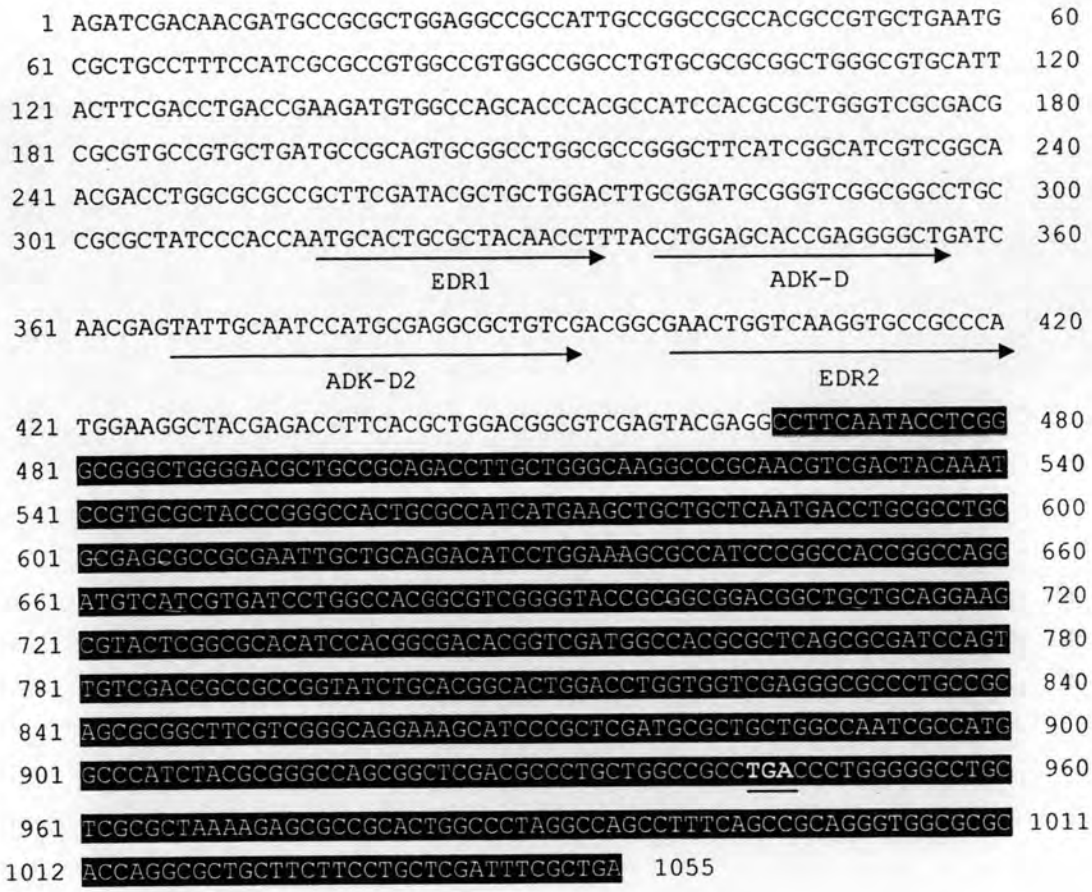


Figure 3.17 Nucleotide sequence at the 3′-terminal region of *lys 6-dh* obtained from inverse PCR using the EDF1 and EDR1 primers

The new nucleotide sequence obtained was highlighted. The stop codon was underlined. The regions of primers for inverse PCR were indicated by arrows.

CCTGTGCGCTTCTGGCGCCAGGGCGACGGCCCCGGTTCCGAACATATCAACCGAGATTCCGTCATTTCAACGGCAA
TTTCACA

-35 -10

GCATATCGACACTCTGCATCGTACACATCGACACGGATCGGCCCTAGCATAAAGGCACCCCACGTCCTTTCTGGAG
CCTGCC

```
1    ATGTCCCGTACGCAACACGCCATCACCGTCCTGGGCGCCGGCAAGATCGGCTTCGCCATC   60
     M  S  R  T  Q  H  A  I  T  V  L  G  A  G  K  I  G  F  A  I
61   GCGCTGCTGCTGCAGCGCACCGGCGACTACGCCGTATGCGTGGCCGACCAGGACCCGTCC  120
     A  L  L  L  Q  R  T  G  D  Y  A  V  C  V  A  D  Q  D  P  S
121  CGGCTGGACGCCGTGGCGGCGCTGGGATGCCAGACCGCGCAGATCGACAACGATGCCGCG  180
     R  L  D  A  V  A  A  L  G  C  Q  T  A  Q  I  D  N  D  A  A
181  CTGGAGGCCGCCATTGCCGGCCGCCACGCCGTGCTGAATGCGCTGCCTTTCCATCGCGCC  240
     L  E  A  A  I  A  G  R  H  A  V  L  N  A  L  P  F  H  R  A
241  GTGGCCGTGGCCGGCCTGTGCGCGCGGCTGGGCGTGCATTACTTCGACCTGACCGAAGAT  300
     V  A  V  A  G  L  C  A  R  L  G  V  H  Y  F  D  L  T  E  D
301  GTGGCCAGCACCCACGCCATCCACGCGCTGGGTCGCGACGCGCGTGCCGTGCTGATGCCG  360
     V  A  S  T  H  A  I  H  A  L  G  R  D  A  R  A  V  L  M  P
361  CAGTGCGGCCTGGCGCCGGGCTTCATCGGCATCGTCGGCAACGACCTGGCGCGCCGCTTC  420
     Q  C  G  L  A  P  G  F  I  G  I  V  G  N  D  L  A  R  R  F
421  GATACGCTGCTGGACTTGCGGATGCGGGTCGGCGGCCTGCCGCGCTATCCCACCAATGCA  480
     D  T  L  L  D  L  R  M  R  V  G  G  L  P  R  Y  P  T  N  A
481  CTGCGCTACAACCTTTACCTGGAGCACCGAGGGGCTGATCAACGAGTATTGCAATCCATG  540
     L  R  Y  N  L  Y  L  E  H  R  G  A  D  Q  R  V  L  Q  S  M
541  CGAGGCGCTGTCGACGGCGAACTGGTCAAGGTGCCGCCCATGGAAGGCTACGAGACCTTC  600
     R  G  A  V  D  G  E  L  V  K  V  P  P  M  E  G  Y  E  T  F
601  ACGCTGGACGGCGTCGAGTACGAGGCCTTCAATACCTCGGGCGGGCTGGGGACGCTGCCG  660
     T  L  D  G  V  E  Y  E  A  F  N  T  S  G  G  L  G  T  L  P
661  CAGACCTTGCTGGGCAAGGCCCGCAACGTCGACTACAAATCCGTGCGCTACCCGGGCCAC  720
     Q  T  L  L  G  K  A  R  N  V  D  Y  K  S  V  R  Y  P  G  H
721  TGCGCCATCATGAAGCTGCTGCTCAATGACCTGCGCCTGCGCGAGCGCCGCGAATTGCTG  780
        A  I  M  K  L  L  L  N  D  L  R  L  R  E  R  R  E  L  L
     CAGGACATCCTGGAAAGCGCCATCCCGGCCACCGGCCAGGATGTCATCGTGATCCTGGCC  840
     Q  D  I  L  E  S  A  I  P  A  T  G  Q  D  V  I  V  I  L  A
841  ACGGCGTCGGGGTACCGCGGCGGACGGCTGCTGCAGGAAGCGTACTCGGCGCACATCCAC  900
     T  A  S  G  Y  R  G  G  R  L  L  Q  E  A  Y  S  A  H  I  H
901  GGCGACACGGTCGATGGCCACGCGCTCAGCGCGATCCAGTTGTCGACCGCCGCCGGTATC  960
     G  D  T  V  D  G  H  A  L  S  A  I  Q  L  S  T  A  A  G  I
961  TGCACGGCACTGGACCTGGTGGTCGAGGGCGCCCTGCCGCAGCGCGGCTTCGTCGGGCAG 1020
     C  T  A  L  D  L  V  V  E  G  A  L  P  Q  R  G  F  V  G  Q
1021 GAAAGCATCCCGCTCGATGCGCTGCTGGCCAATCGCCATGGCCGCATCTACGCGGGCCAG 1080
     E  S  I  P  L  D  A  L  L  A  N  R  H  G  R  I  Y  A  G  Q
1081 CGGCTCGACGCCCTGCTGGCCGCCTGA 1107
     R  L  D  A  L  L  A  A  *
```

CCCTGGGGGCCTGCTCGCGCTAAAAGAGCGCCGCACTGGCCCTAGGCCAGCCTTTCAGCCGCAGGGTGGCGCGCAC
CAGGCGCTGCTTCTTCCTGCTCGATTTCGCTGA

Figure 3.18 Nucleotide and deduced amino acid sequences of the *lys 6-dh* gene from *A. denitrificans* K-1

Bold letter = Nucleotide sequence obtained from inverse PCR using chromosomal DNA digested by *Stu*I with EDF1 and EDR1 primers, Italic letter = Nucleotide sequence obtained from PCR using chromosomal DNA digested by *BamH*I with KN2 and C1 primers, Black highlight = Nucleotide sequence obtained from inverse PCR using chromosomal DNA digested by *Xho*I with EDF1and EDR1 primers.

Moreover, when the deduced amino acid sequence of the whole Lys 6-DH was compared to those of the EMBL-GenBank-DDBL database, it was found that the percentages of sequence similarity between the deduced amino acid sequences of Lys 6-DH from *A. denitrificans* K-1 and each of those from *Legionella pneumophila, Rhizobium leguminosarum, Geobacillus stearothermophilus, Geobacillus kaustophilus, Geobacillus thermodenitrificans* NG80-2 and *Oceanobacillus iheyensis* HTE831 were 38, 37, 24, 24, 23 and 18%, respectively (Figure 3.20).

3.7 Cloning of *lys 6-dh* gene

3.7.1 Construction of recombinant pET-ADK

To express the *lys 6-dh* gene in *E. coli* under T7 promoter of the expression vector, pET-17b, the gene was amplified using a pair of primers as described in 2.16.2 (Figure 3.21). The 5′-primer (pET-ADKF) comprised *Nde*I restriction site and 5′-end of the *lys 6-dh* gene. The 3′-primer (pET-ADKR) consisted of *Bam*HI site, the TAA translational termination signal and 3′-end of the *lys 6-dh* gene (Table 3.5). Figure 3.22 showed the 1.1 kb PCR product of the *lys 6-dh* gene fragment amplified from *Bam*HI digested DNA template. This PCR product was recovered and used for further cloning.

3.7.2 Transformation

The 1.1-kb amplified gene fragment was digested with *Nde*I and *Bam*HI, ligated with *Nde*I-*Bam*HI digested pET-17b vector, and then transformed into *E. coli* BL21(DE3) by electroporation as described in 2.17. One hundred transformants which could grow on LB plate containing 100 μg/ml ampicillin were randomly picked for plasmid extraction and digestion with *Nde*I-*Bam*HI as described in 2.18.1.

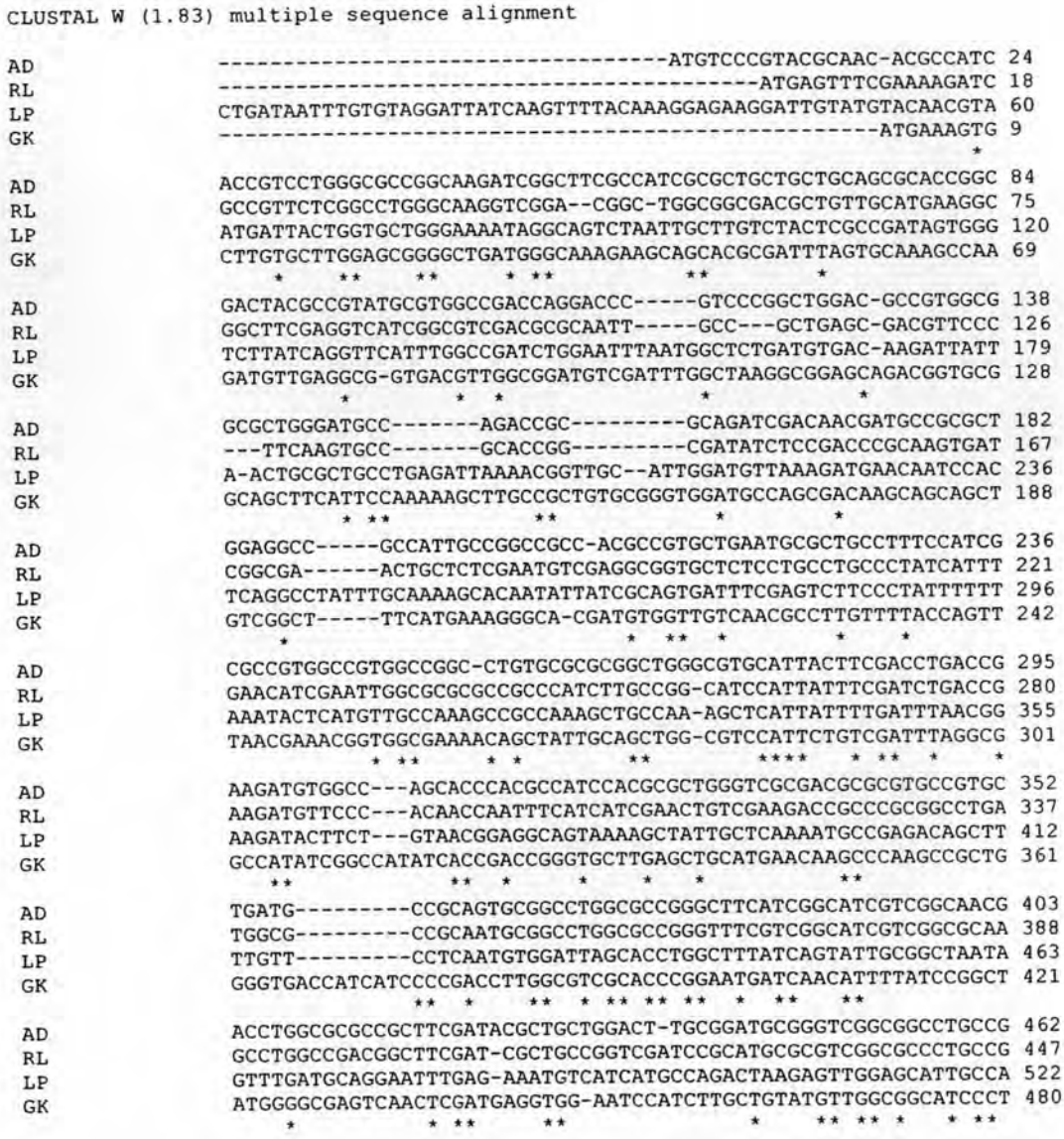


Figure 3.19 Linear alignment of the nucleotide sequence of the *lys 6-dh* gene from *A. denitrificans* K-1 and those from various sources

The linear alignment was made by clustalW program. AD = *A. denitrificans* K-1 (DQ165182), RL = *Rhizobium leguminosarum* (NC_008380.1), LP = *Legionella pneumophila* (NC_002942.5) and GK = *Geobacillus kaustophilus* (NC_006510.1).

Conserved residues are indicated by asterisks (*).

```
AD          CGCTATCCCACCAATGCACTGCGCTACAACCTTTACCTGGAGCACCGAGGGGCTGATCAA 522
RL          CAGCATCCGACCGGGCTGCTCGGCTACG-CCTTCAACTGGTCGCCCGAGGGCGTCGTCAA 506
LP          CAGCGTGCTAATAATGCGCTTCATTATT-CATTAACCTGGTCTACTGATGGGGTTATTAA 581
GK          GTCCGCCCCGAACCGCCGCTGGAGTACAACCAT-GTGTTTTCGCTCGAGGGGCTGCTTGA 539
                  *          **    **   *  *    *        ** **  *  *  *

AD          CGAGTATTGCAATCCATGCGAGGCGCTGTCGACGGCGAACTGGTCAAG---GTGCCGCCC 579
RL          CGAATATCTGAACGACTGCGAGGT--CATCGAGGGCGGTGTGCGCAAGCTCGTCTCGCCG 564
LP          TGAATATGGTAATCCTTGCTATGG--TATTGAAGGTGGTAAAGATGTGGTTATGGCTCCG 639
GK          CCATTACACCGATCCGGCCTTGAT--CATCCGCAACGGCCAAAAGCAGGAAGTGCCGTCC 597
              * **     *     *          *        *          *    *  *  *

AD          ATGGAAGGCTACGAGACCTTCACGCTGGACGGCGTCGAGT---ACGAGGCCTTCAATACC 636
RL          ATGGAATGGCACGAGACCGTCTATGTCGGCGGCGTCAAGC---TCGAAGCCTTCACGACG 621
LP          CTGGAAGGATTGGAGTCAATTCAGATTGATGGTTGTGAAT---ATGAAGCGTTCAACACG 696
GK          CTTTCGGAAGTCGAGCCGATTTATTTCGACCGGTTCGGGCCGCTTGAAGCGTTTCACACC 657
             *       *** *  *     * *   *             ** ** **    **

AD          TCGGGCGGGCTGGGGACGCTG-CCGCAGACCTTGCTGGGCAAGGCCCGCAACGTCGACTA 695
RL          TCGGGCGGCCTTGGCACCATG-TGCGATACGATGCTCGGCAAGATCGACAATCTCGATTA 680
LP          TCCGGTGGTTTGGGAAGCTTGGCTGAACTTTATGCTGGAAAAATACAATCG-ATGAATTA 755
GK          TCAGGCGGGACGTCGACGCTC-TCGCGCTCGTTTCCGAACTTGA--AGCGG-CTCGAGTA 713
            ** ** **       *   *              * *                 *  * **

AD          CAAATCCGTGCGCTACCCGGGCCACTGCGCCATCATGAAGCTGCTGCTCAAT-------- 747
RL          CAAGACCATGCGGTATCCCGGCCATATGGAGCTGATGAACTTCTTCTTCCAC-------- 732
LP          CAAAACCATGCGTTATCCAGGTCATTGTAAAAAAATGCGCTTTTTAATGAAT-------- 807
GK          CAAAACGATCCGCTACCGCGGCCATGCGGAAAAATGCAAGCTGCTTGTCGATTTGAATTT 773
            ***  *  * ** ** *  ** **                 *  *  *  *

AD          GACCTGCGCCTGCGCGAGCGCCG-------------------CGAATTGCTGCAGGACAT 788
RL          GAGCTGTTGATGCGCGACAAGCG-------------------CAAGCTCGCCGGCGAGAT 773
LP          GACTTACGATTAAATGAAGATCG-------------------AGGGACGTTAAAAAGAAT 848
GK          GACGCGCAACGATGTGGAAGTTGAGATCAATGGATGCAGAGTCAAGCCGCGCGATGTGCT 833
            **          *     *                                       *

AD          CCTGGAAAGCGCCATCCCGGCCACCGGCCAGGATGTCATCGTGATCCTGGCCACGGCGTC 848
RL          CCTTACCAATGCCAAGCCGCCGGTTGAAGACGATGTCGTCTATGTTCATGTCGCCGC-CG 832
LP          ACTGGAAAATGCAATTCCAAAAACATATCAGGATATCGTGATAGTTTATGTTACTGT-TG 907
GK          GCTTTCCGTCCTGAAGCCGCTGCTTGATTTGAAAGGAAAAGATGATGTGGT-GTTGCTTC 892
             **          *  **                 *                 *    *

AD          GGGGTACCGCGGCGGACGGCTGCTGCAGGAAGCGTACTCG-GCGCACATCCACGGCGACA 907
RL          AGGGGACCGAGAATGGCAGCCTGCGCCGCAAGGAGTTCGT-GCGCGCCTATTACCCGATC 891
LP          AGGGTATTAAGCAGGGCGAACTAACCGAAAAAAGTTATGTCAAGAAAATTTACCCTGAAG 967
GK          GGGTCATCGTCGGCGGTAGAAAAGATGGAAAAGAAACGGTGCTGGAATACGAAACCGTCA 952
             **  *       *              **                 *          *

AD          CGGTCGATGGCCACGCGCT-CA-----GCGCGATCCAGTTGTCGACCGCCGCCGGTATCT 961
RL          GAGATTGCCGGCGCGCGCCGCA-----CGGCGATCGCCTGGACGACGTCAGCCTCCGTCG 946
LP          TAATTCGAGGTTTGGAGTGGT------CAGCTATTCAAGTCAGTACAGCTTCGGGCGTAT 1021
GK          CGTTCAATGACCGCGAAAATAAGGTGACGGCGATGGCGCGTACGACGGCCTACACCATTT 1012
                      *                 ** **           **  *         *

AD          GCACGGCACTGGACCTGGTGGTCG-AGGGCGCCCTGCCGCAGCGCGGCTTCGTCGGGCAG 1020
RL          TCGCCGTCATCGAGATGGTCCGCG-ACGGTCTGCTGCCGGTGACCGGCTTCCTGCACCAG 1005
LP          GCGCTGTTGTTGATTTGGTATTGGGACAGGGAAATGAATATAAAGGGCTTATTTTGCAAG 1081
GK          CCGCTGTCGCCCAGCTCATCGGCC-GTGGGGTGATCACAAAGCGCGGCGTCTATCCGCCG 1071
             * * *     *  *  *          *     *           *** *         *

AD          GAAAGCATCCCGCTCGATGCGCTGCTGGCCAATCGCCATGGCCGCATCTACGCGGGCCAG 1080
RL          GAGCATATTCCGCTGGAGATGTTCCTGAAAACACCGACCGGCAGCCTCTTCA-AGGCGGG 1064
LP          AAAAATTTAAATTGTCCCACG-TGCTGGAAAATCGCTTCGGCAAATATTATG---CCTAG 1137
GK          GAGCAAATCGTTCCGGGGGATGTA-TATATGGACGAGATGAAAAAACGAGGAGTGGTGAT 1130
             *    *                *  *      *     *

AD          CGGCTCGACGCCCTGCTGGCCGCCTGA-- 1107
RL          CGCCACCAGCCACGGCTGA---------- 1083
LP          ----------------------------
GK          TAACGAGAAGAA-AACGGTTCGCTTATGA 1158
```

Figure 3.19 Linear alignment of the nucleotide sequence of the *lys 6-dh* gene from *A. denitrificans* K-1 and those from various sources (continued)

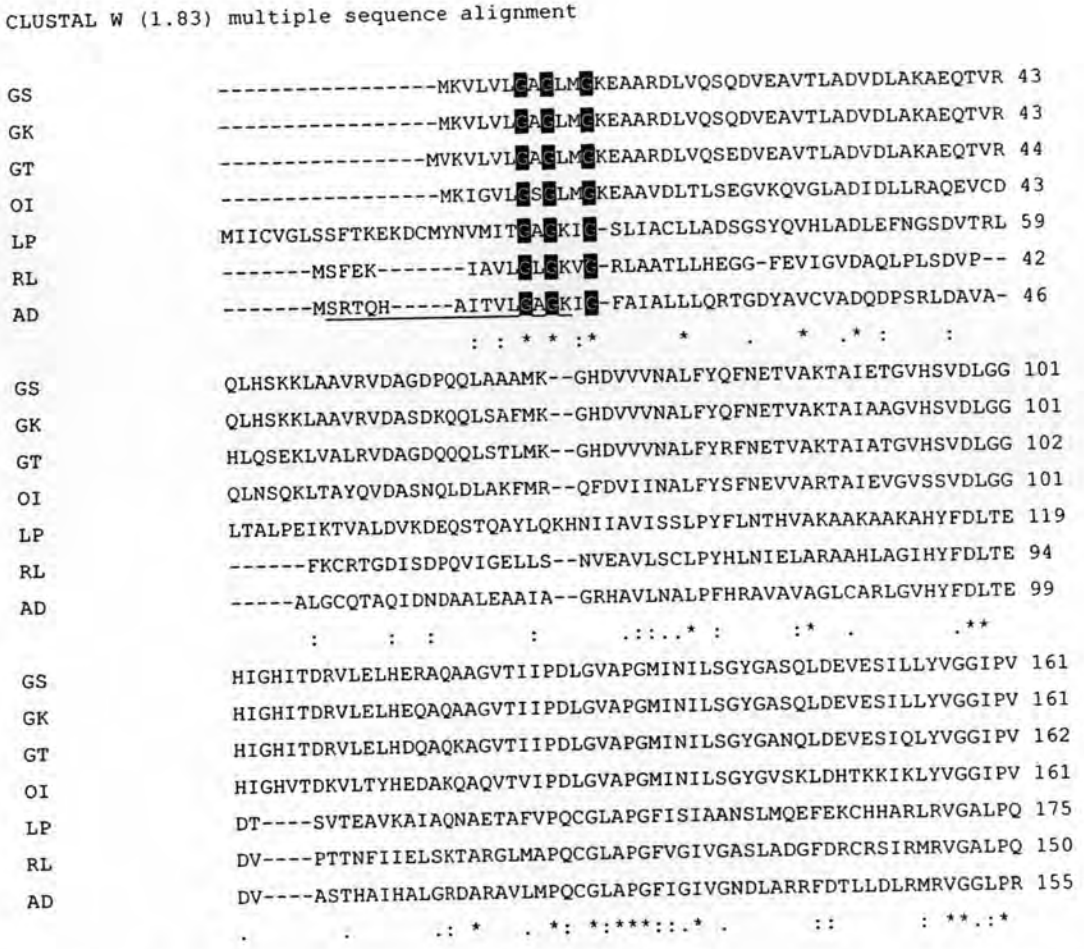


Figure 3.20 Linear alignment of the deduced amino acid sequence of Lys 6-DH from *A. denitrificans* K-1 and those from various sources.

The linear alignment was made by clustalW program.

Amino acid sequence that was determined by automated Edman degradation were underlined. GS = *Geobacillus stearothermophilus* (BAB39707), GK = *Geobacillus kaustophilus* HTA426 (YP_146228), GT = *Geobacillus thermodenitrificans* NG80-2 (YP_001124477), OI =*Oceanobacillus iheyensis* HTE831 (NP_691783), LP = *Legionella pneumophila* (YP_0953709), RL = *Rhizobium leguminosarum* (YP_765983) and AD = *A. denitrificans* K-1 (AAZ94428.1).

Conserved residues were indicated by asterisks (*). (:) meaned amino acids which had the same group of side chains and similar size while (.) meaned amino acids which had the same group of side chains but different size.

```
GS      RPEPPLEYNHVFSLEGLLDHYTDPALIIRNGQKQEVPSLSEVEPIYFDRFGPLEAFHTSG 221
GK      RPEPPLEYNHVFSLEGLLDHYTDPALIIRNGQKQEVPSLSEVEPIYFDRFGPLEAFHTSG 221
GT      QPEPPLEYNHVFSLEGLLDHYTDPSLIIRDGKKQEVPSLSEVEPIYFERFGPLEAFHTSG 222
OI      KPEPPLEYNHVFSMEGVFDHYTDPSLIIRNGLVQEVSSLSEVEPIHFEKFGPLEAFHTSG 221
LP      RANNALHYSLTWSTDGVINEYGNPCYGIEGGKDVVMAPLEGLESIQIDGC-EYEAFNTSG 234
RL      HPTGLLGYAFNWSPEGVVNEYLNDCEVIEGGVRKLVSPMEWHETVYVGGV-KLEAFTTSG 209
AD      YPTNALRYNLYLEHRGADQRVLQSMRGAVDGELVKVPPMEGYETFTLDGV-EYEAFNTSG 214
                .  * *   . * :. :      .*   :..:.  *.. .      *** ***

GS      GTSTLSRSFP-NLKRLEYKTIRYRGHAEKCKLLVDLTLTRHDVEVEINGCRVKPRDVLLS 280
GK      GTSTLSRSFP-NLKRLEYKTIRYRGHAEKCKLLVDLNLTRNDVEVEINGCRVKPRDVLLS 280
GT      GTSTLSRSFP-NLKRLEYKTIRYRGHAEKFKLLVDLNLTRNDVEVEVGGHKVKPRDVLLA 281
OI      GTSTLSLSYP-YLDTLEYKTIRYPGHAEKFKLLVDLNLTRLDYQVDINGMKVNPREVFLK 280
LP      GLGSLAELYAGKIQSMNYKTMRYPGHCKKMRFLMNDLRLN------------EDRGTLKR 282
RL      GLGTMCDTMLGKIDNLDYKTMRYPGHMELMNFFFHELLMR------------DKRKLAGE 257
AD      GLGTLPQTLLGKARNVDYKSVRYPGHCAIMKLLLNDLRLR------------ERRELLQD 262
        * .::          ::**::** **    .::..    .            . *

GS      VLKPLLDLKGKDDVVLLRVIVGGRKDGKETVLEYETVTFNDREN--KVTAMARTTAYTIS 338
GK      VLKPLLDLKGKDDVVLLRVIVGGRKDGKETVLEYETVTFNDREN--KVTAMARTTAYTIS 338
GT      VLTPMLDLRGKDDVVLLRVIVGGRKDGKETVFEYETITFNDSER--KVTAMARTTAYTIS 339
OI      VLDPIVELGDREDAVLLRVVVEGEKDGKSTSHIFEMTTYKDKDT--KVTAMARATANTIS 338
LP      ILENAIPKTY-QDIVIVYVTVEGIKQGELTEKSYVKKIYPEVIRGLEWSAIQVSTASGVC 341
RL      ILTNAKPPVE-DDVVYVHVAAEGTENGSLRRKEFVRAYYPIEIAGARRTAIAWTTSASVV 316
AD      ILESAIPATG-QDVIVILATASGYRGGRLLQEAYSAHIHGDTVDGHALSAIQLSTAAGIC 321
        :*         :* : : . . * . *      :    .         :*:  :*:  :

GS      AVAQLIGRGVITKRGVYPPEQIVPGDVYMDEMKKRGVLISEKRTVHS---- 385
GK      AVAQLIGRGVITKRGVYPPEQIVPGDVYMDEMKKRGVVINEKKTVRL---- 385
GT      VVAQLIGRGLITKRGVYPPEQIVPGNVYIDEMKKRGVVINEKKTVRL---- 386
OI      VVAQMIANGTINTKGVLPPEKMVPGKQYIDEMKKRGVTIKESVSINKQTSV 389
LP      AVVDLVLGQGNEYKGLILQEKFKLSHVLENRFGKYYA-------------- 378
RL      AVIEMVRDGLLPVTGFLHQEHIPLEMFLKTPTGSLFKAGATSHG------- 360
AD      TALDLVVEGALPQRGFVGQESIPLDALLANRHGRIYAGQRLDALLAA---- 368
        .. :::       *.   * :
```

Figure 3.20 **Linear alignment of the deduced amino acid sequence of Lys 6-DH from *A. denitrificans* K-1 and those from various sources. (continued)**

Table 3.5 The sequences of forward and reverse primers for full length *lys 6-dh* gene amplification

Primers	Nucleotide sequence (5′-3′)	Length (bp)	Tm (°C)
Forward primer: pET-ADKF	5′-GGGAATTCCATATGTCCCGTACGCAAC ACGCCATCACCGTC-3′	41	87
Reverse primer: pET-ADKR	5′-CGCGGATCCTCAGGCGGCCAGCAGGGC GTCG AG-3′	33	90

The restriction enzyme sites of forward (*Nde*I) and reverse primers (*Bam*HI) were underlined.

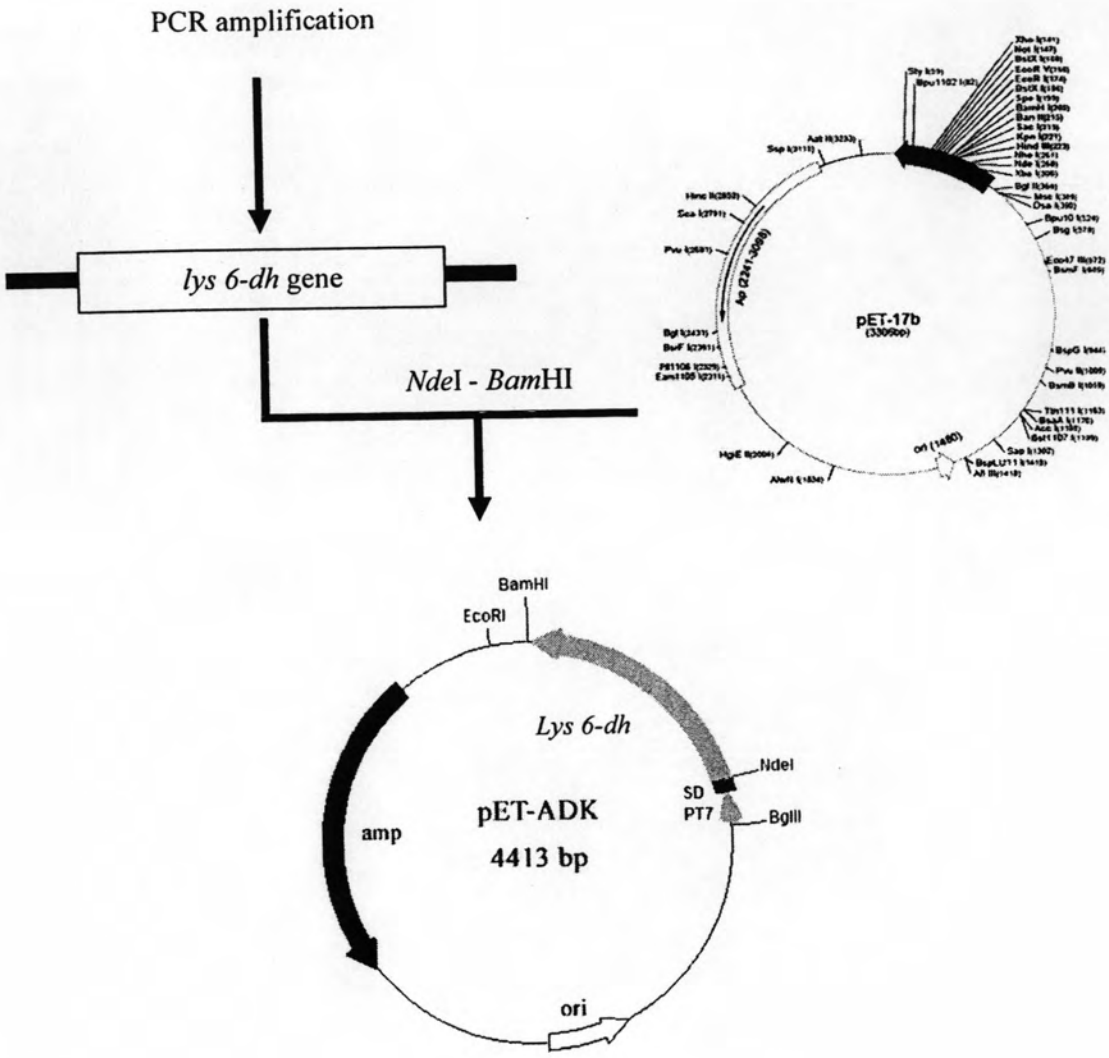


Figure 3.21 Construction of recombinant pET-ADK

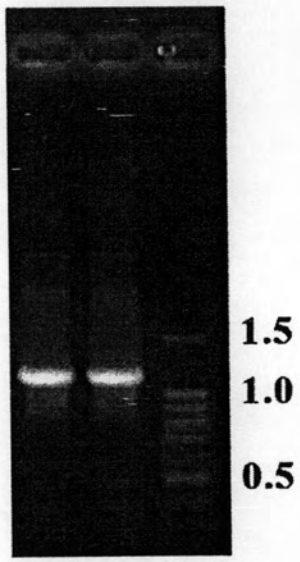


Figure 3.22 PCR amplification of the full-length *lys 6-dh* gene

Lane M = 100 bp DNA ladder

Lane 1-2 = the PCR amplified *lys 6-dh* gene fragment

Note: The template was *BamH*I digested chromosomal DNA and the primers used were pET-ADKF and pET-ADKR.

3.8 Expression of the *lys 6-dh* gene

3.8.1 Recombinant plasmid characterization

The recombinant plasmids (pET-ADK) extracted from *E. coli* BL21 (DE3) were present in two forms, relaxed and supercoiled, as seen on agarose gel (Figure 3.22). After digestion, the linear, 3.3-kb pET-17b and 1.1-kb *lys 6-dh* gene insert were obtained (Figure 3.23). From one hundred transformants containing *lys 6-dh* gene on antibiotic plate, twelve transformants harboured recombinant plasmid (4.4 kb) were randomly selected for Lys 6-DH assay.

3.8.2 Lys 6-DH activity of transformants

The twelve recombinant clones were grown as described in 2.18.2. The crude extracts were prepared and the enzyme activities were measured. *E. coli* BL21(DE3) with and without the plasmid pET-17b were used as control. The recombinant clones showed various levels of the Lys 6-DH specific activity ranging from 1.33-1.93 units/mg protein (Table 3.6). Transformant No. 4 had the highest specific activity at 1.93 units/mg protein, which was 64 fold higher than that of the wild type *A. denitrificans* K-1. Thus, this transformant was used in further experiments.

3.9 Optimization of the *lys 6-dh* gene expression

The transformant No. 4 (pET-ADK) were grown and induced by IPTG at final each of the following concentrations 0, 0.1, 0.2, 0.4 and 0.8 mM. At 0, 1, 2, 4, 6, 8, 16, and 24 hours after induction, the cells were harvested and the Lys 6-DH activity in crude extract was assayed as described in 2.8. When recombinant clone was cultured without IPTG induction, expression of the *lys 6-dh* gene was slightly increase from 0 to maximum value after 24 hours with a specific activity of 0.76 units/mg protein. When induced with 0.2 mM IPTG for 4 hours, the maximal specific activity was 2.9 times higher (2.25 U/mg) than that in the without of IPTG for 24 hours.

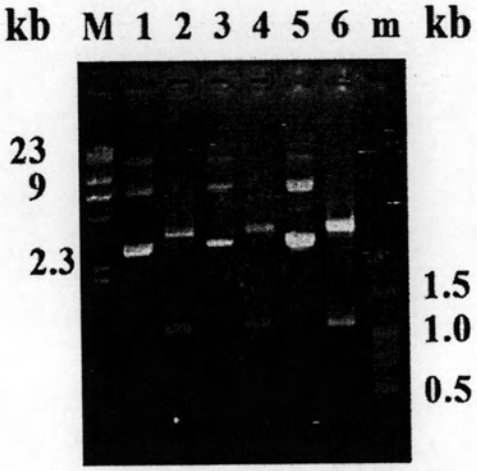


Figure 3.23 Restriction pattern of pET-ADK from selected recombinant clones

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = undigested plasmid
Lane 2 = *Nde*I-*Bam*HI digested plasmid
Lane 3 = undigested plasmid
Lane 4 = *Nde*I-*Bam*HI digested plasmid
Lane 5 = undigested plasmid
Lane 6 = *Nde*I-*Bam*HI digested plasmid
Lane m = 100 bp DNA ladder

Table 3.6 Lys 6-DH activity from the crude extract of 12 selected *E. coli* BL21(DE3) transformants harboring pET-ADK[a]

Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
A. denitrificans K-1	3.35	105	0.03
E. coli BL21(DE3)	0	156	0.00
E. coli BL21(DE3) harbouring pET-17b	0	181	0.00
Transformant No.1	278	166	1.67
Transformant No.2	293	188	1.56
Transformant No.3	224	160	1.40
Transformant No.4	**278**	**144**	**1.93**
Transformant No.5	197	106	1.86
Transformant No.6	204	116	1.76
Transformant No.7	169	100	1.69
Transformant No.8	266	168	1.58
Transformant No.9	234	176	1.33
Transformant No.10	269	192	1.40
Transformant No.11	299	184	1.63
Transformant No.12	244	158	1.54

[a] Crude extracts were prepared from 200 ml of cell culture by induction with 0.1 mM IPTG for 4 hours.

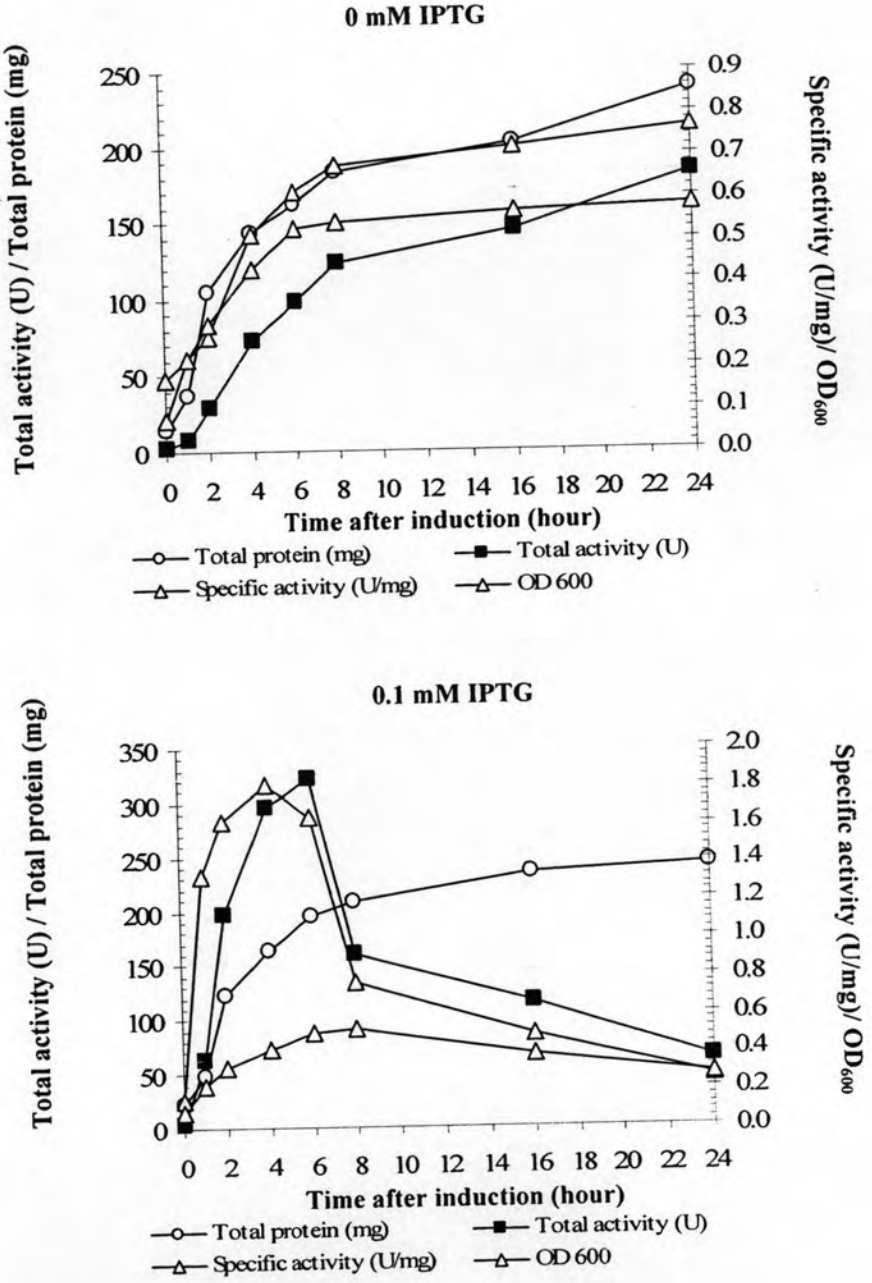


Figure 3.24 **The Lys 6-DH activity in *E. coli* BL21(DE3) clone No.4 induced by various concentrations of IPTG**

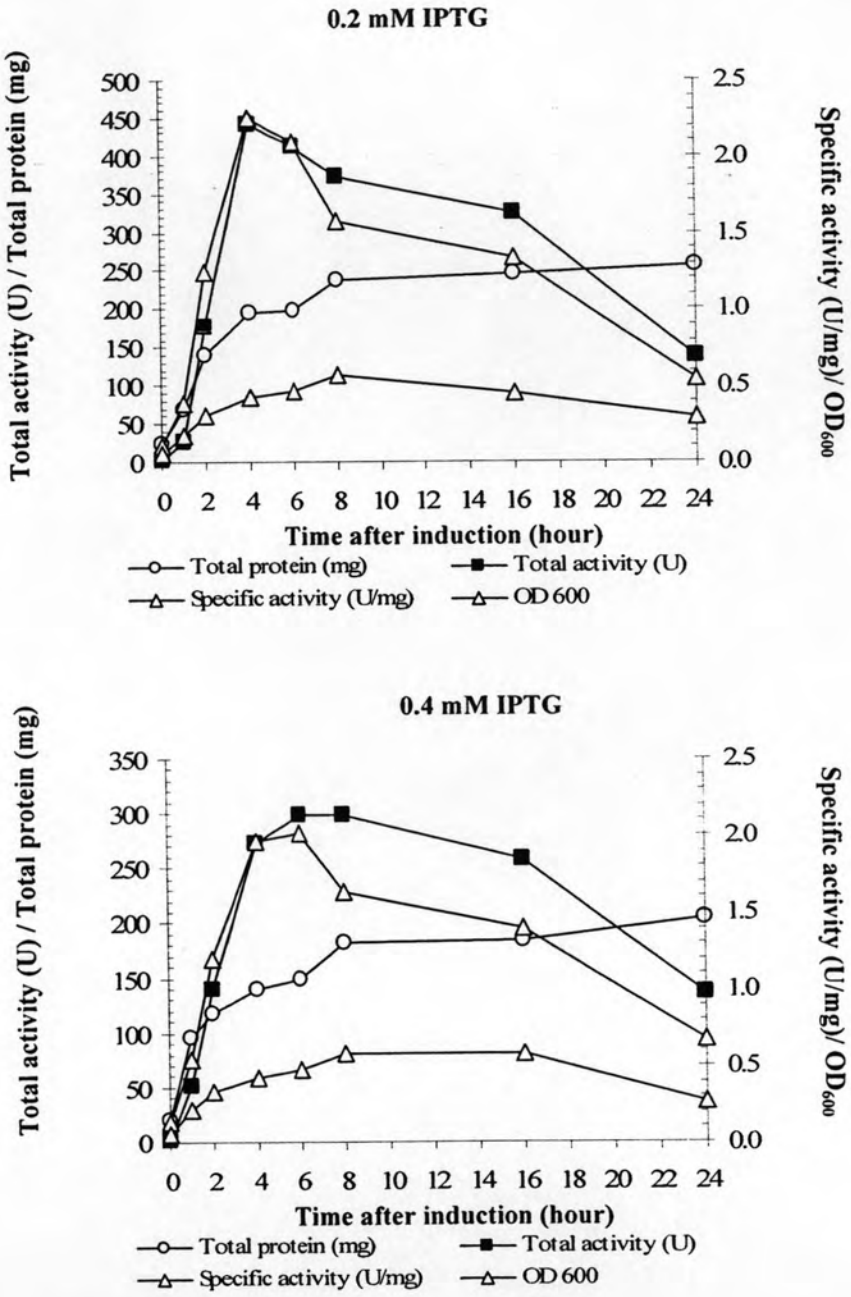


Figure 3.24 The Lys 6-DH activity in *E. coli* BL21(DE3 e No.4 induced by various concentrations of IPTG (continued)

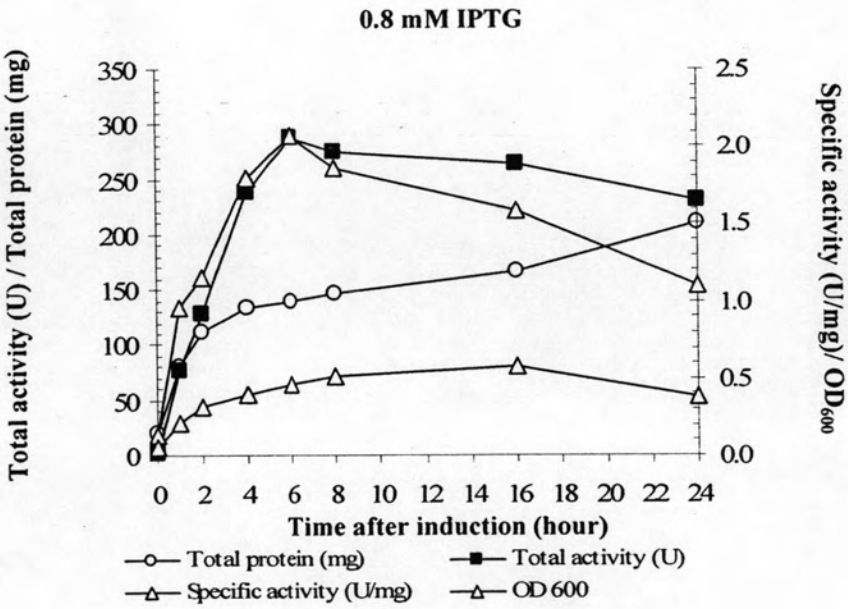


Figure 3.24 The Lys 6-DH activity in *E. coli* BL21(DE3) clone No.4 induced by various concentrations of IPTG (continued)

Highest activity was also found at 6 hours after induction when 0.1, 0.4 and 0.8 mM IPTG were used. However, highest activity induced by 0.1, 0.4 and 0.8 mM IPTG was still lower than that of 0.2 mM at the induction time of 4 hours (Figure 3.24). Hence, the optimum condition for induction of the *lys 6-dh* gene used in further experiments was 0.2 mM IPTG at 4 hours after induction.

3.9.1 Protein patterns of cells and crude extracts

One point five milliliter of transformant No.4 culture induced with various concentrations of IPTG was harvested at various times and was subjected for protein extraction. Ten microliters of cell samples from crude extracts were seperated on 10% SDS-polyacrylamide gel. The results in Figure 3.25-3.29 showed that the intensity of a major protein band of about 40 kDa in cell and crude extracts from each harvested time was corresponded to the level of enzyme activity from its crude extract.

3.10 Stability of the recombinant *lys 6-dh* gene (pET-ADK) in *E. coli* BL21(DE3)

Transformant No. 4 were subcultured for 1, 10, 20, 30, 40, 50, 60, 70 and 80 cycles were picked up to culture and assay for Lys 6-DH activity. The result shown in Table 3.7 indicated that expression level of the *lys 6-dh* gene was not different after 80 cycles of subcultivation. It clearly showed that the recombinant plasmid was stable in this transformant. The protein pattern of whole cell and crude extract were shown in Figure 3.30.

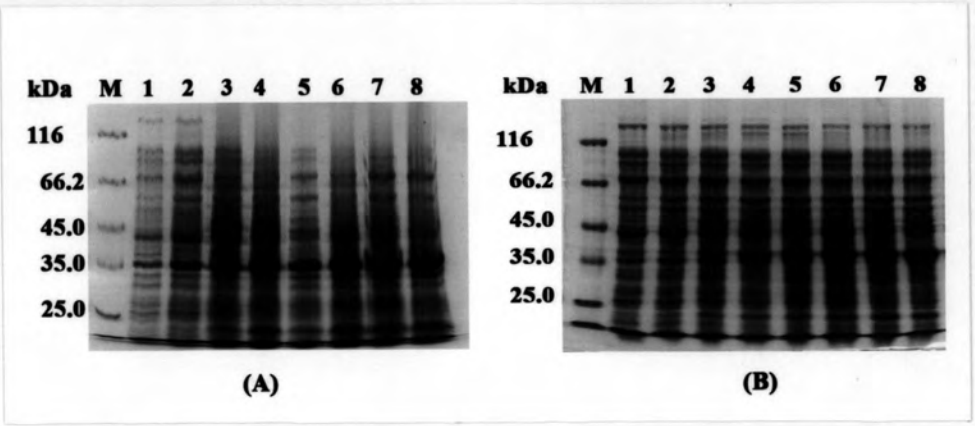


Figure 3.25 **SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformant No. 4 containing pET-ADK induced by 0 mM IPTG at various times**

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-8 = cell and crude extract of pET-ADK clone at 0, 1, 2, 4, 8, 16 and 24 hours after induction, respectively

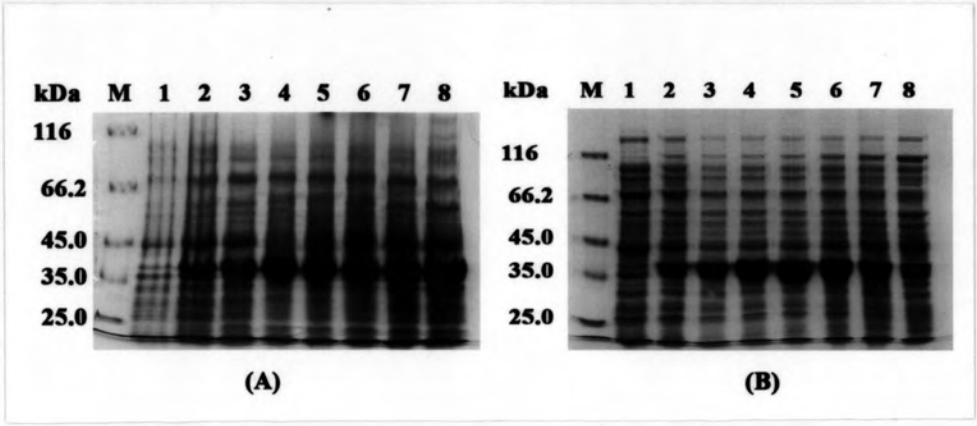


Figure 3.26 SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformant No. 4 containing pET-ADK induced by 0.1 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-8 = cell and crude extract of pET-ADK clone at 0, 1, 2, 4, 8, 16 and 24 hours after induction, respectively

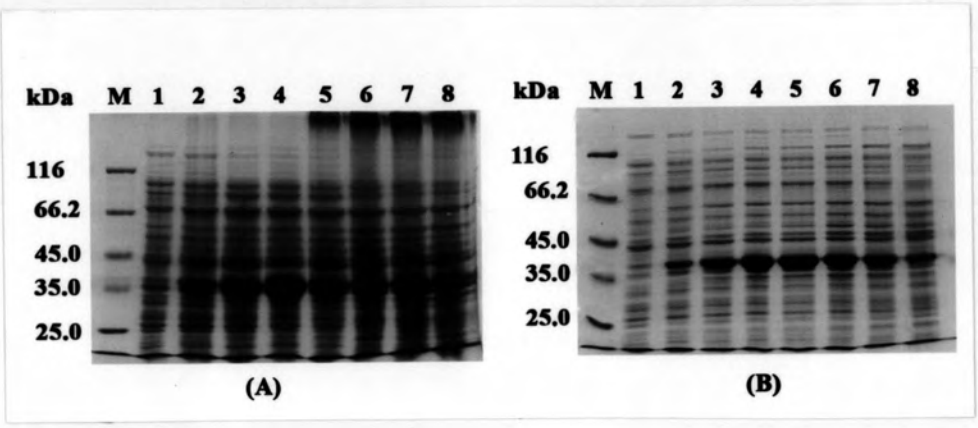


Figure 3.27 SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformant No. 4 containing pET-ADK induced by 0.2 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-8 = cell and crude extract of pET-ADK clone at 0, 1, 2, 4, 8, 16 and 24 hours after induction, respectively

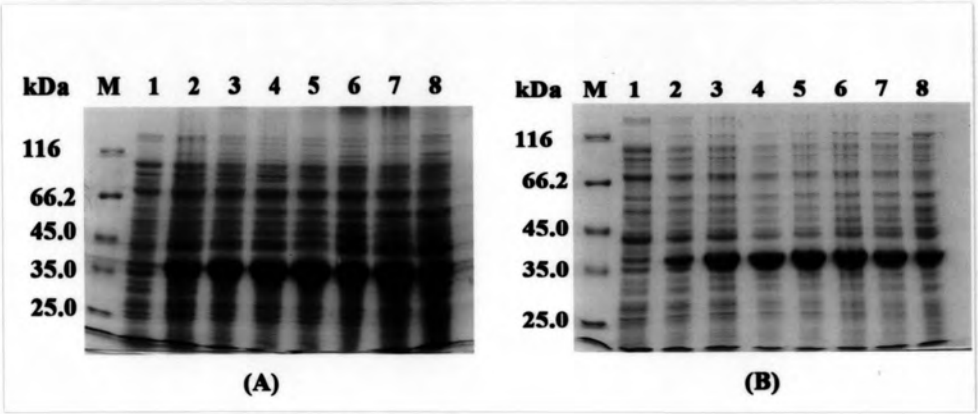


Figure 3.28 SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformant No. 4 containing pET-ADK induced by 0.4 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-8 = cell and crude extract of pET-ADK clone at 0, 1, 2, 4, 8, 16 and 24 hours after induction, respectively

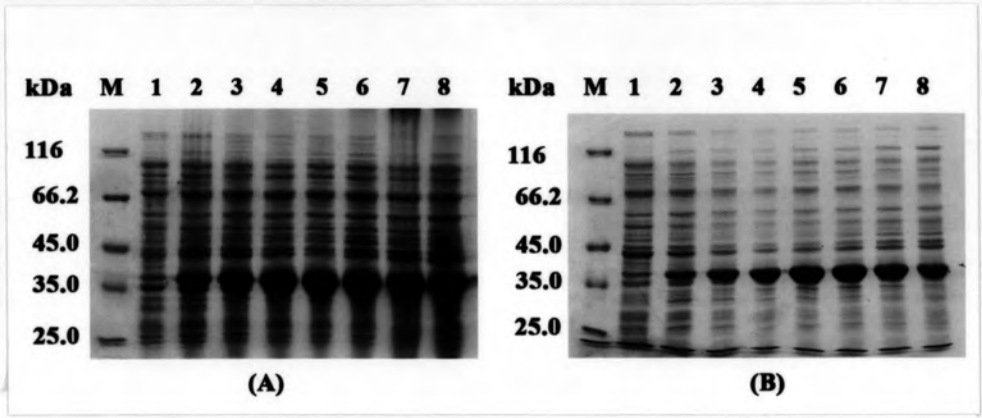


Figure 3.29 SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformant No. 4 containing pET-ADK induced by 0.8 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-8 = cell and crude extract of pET-ADK clone at 0, 1, 2, 4, 8, 16 and 24 hours after induction, respectively

Table 3.7 Stability of the recombinant *lys 6-dh* gene in *E. coli* BL21(DE3)[a]

Cycle of subcultivation	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg)
1	168	454	2.70
10	158	426	2.69
20	165	444	2.69
30	167	440	2.64
40	163	430	2.63
50	173	432	2.49
60	173	442	2.55
70	168	412	2.45
80	175	453	2.59

[a] Crude extracts were prepared from 200 ml of cell culture.

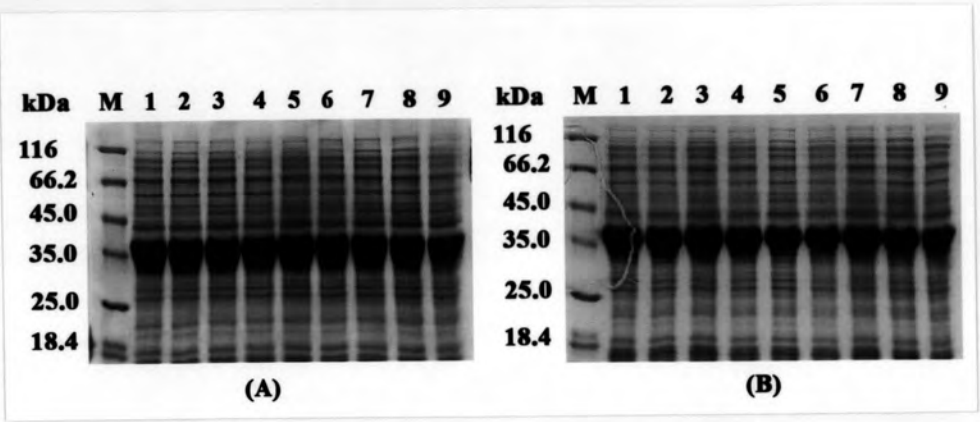


Figure 3.30 SDS-PAGE of whole cell and crude extract of the *E. coli* (BL21) DE3 transformnt No. 4 containing pET-ADK induced by 0.2 mM IPTG for 4 hours from various cycle of subcultivation

(A): whole cell

(B): crude extract

Lane M = protein MW marker

Lane 1-9 = cell and crude extract of the E. coli (BL21) DE3 transformnt No. 4 containing pET-ADK after subcultured for 1, 10, 20, 30, 40, 50, 60, 70 and 80 cycles, respectively

3.11 Purification of Lys 6-DH from recombinant clone

3.11.1 Preparation of crude extract

Crude recombinant Lys 6-DH was prepared from 5 g of *E. coli* BL21(DE3) transformant No. 4 which was cultivated in 600 ml of medium as described in 2.21.1. Ten milliliters of crude enzyme solution contained 880 mg proteins with 1,660 units of the Lys 6-DH activity was used for purification. Specific activity of the enzyme in the crude preparation was 1.89 units/mg protein which was 63 times higher than that of the wild type crude extract.

3.11.2 DEAE-Toyopearl 650 M column chromatography

The recombinant crude enzyme was dialyzed against the 10 mM KPB, pH 7.4. The enzyme solution was applied onto DEAE-Toyopearl column as described in 2.21.2.1. The chromatographic profile was shown in Figure 3.31. The unbound proteins were eluted from the column by 0.01 M KPB, pH 7.4. The bound proteins were then eluted with a linear gradient of 0-0.05 M KCl in the same buffer. The enzyme was eluted at 0.08 M KCl as indicated in the profile. Fractions with the Lys 6-DH activity were pooled and dialyzed against the 0.01 M KPB, pH 7.4.

The protein remained from this step was 459 mg with enzyme activity recovered at 1,090 units. The specific activity of the enzyme from this step was 2.37 unit/mg protein. The enzyme was purified 1.25 fold with 65% recovery.

3.11.3 DEAE-Sephadex A50 column chromatography

The pooled enzyme form DEAE-Toyopearl was dialyzed against 0.01 M KPB, pH 7.4. The enzyme solutions were applied onto DEAE-Toyopearl column as described in 2.21.2.2. The chromatographic profile was shown in Figure 3.32. The

unbound proteins were eluted from the column by 0.01 M KPB, pH 7.4. The bound proteins were then eluted with a linear gradient of 0-0.5 M KCl in the same buffer. The enzyme was eluted at 0.2 M KCl as indicated in the profile. Fractions with the Lys 6-DH activity were pooled and dialyzed against the 0.01 M KPB, pH 7.4.

The protein remained from this step was 97 mg with enzyme activity recovered at 510 units. The specific activity of the enzyme from this step was 5.31 unit/mg protein. The enzyme was purified 2.8 fold with 47% recovery.

The summary of purification of Lys 6-DH was shown in Table 3.8.

3.11.4 Determination of enzyme purity by SDS and Native-PAGE

The enzyme from each step of purification was examined for purity and protein pattern by SDS-PAGE (Figure 3.33) as described in 2.12.2. Single protein band was observed suggesting that Lys 6-DH might consisted of one kind of polypeptide. The molecular weight of Lys 6-DH subunit was calculated to be 40 kDa based on its mobility in SDS-PAGE compared with those of standard proteins (Figure 3.34).

In addition, the enzyme from each step of purification was subjected to non-denaturing PAGE followed by protein and activity staining as described in 2.12.1 and 2.12.2, respectively. The results were shown in Figure 3.34. The purified enzyme in lane 3A on Native-PAGE showed a single protein band which corresponded with a single protein activity in lane 3B. This indicated that Lys 6-DH from DEAE-Sephadex A50 column was highly pure enzyme.

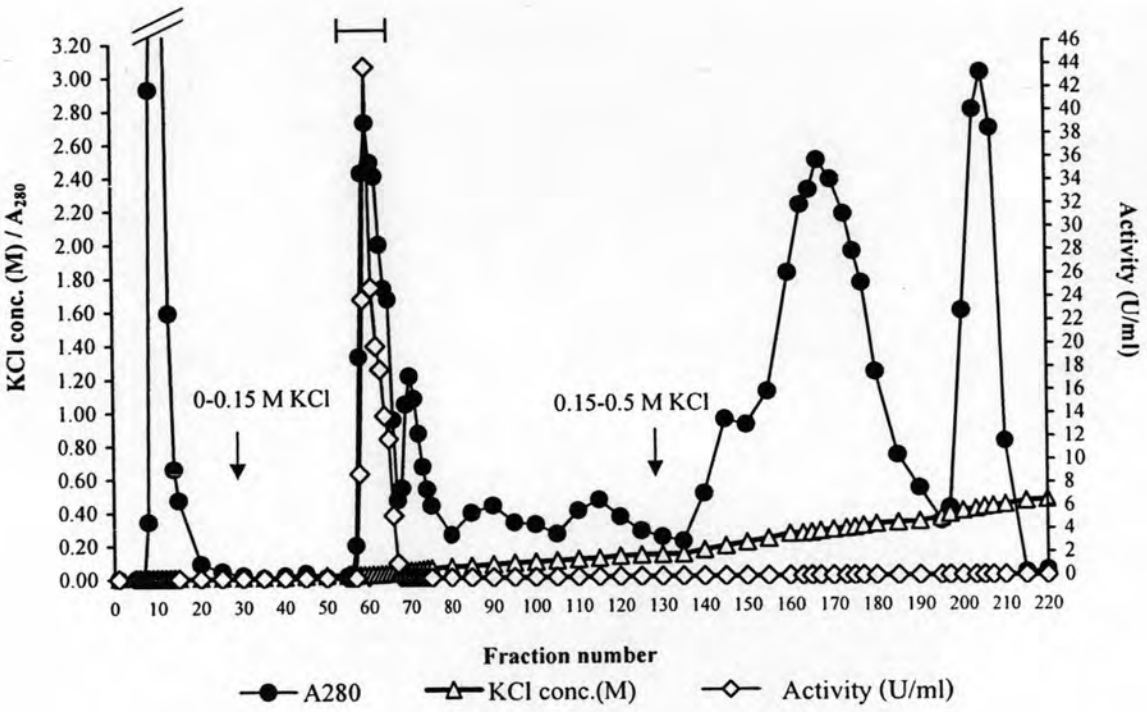


Figure 3.31 Purification profile of Lys 6-DH from the *E. coli* (BL21) DE3 transformant No. 4 by DEAE-Toyopearl 650M column chromatography

The enzyme was applied to DEAE-Toyopearl 650 M column (3 x 10 cm) and washed with 0.01 M KPB, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA until the absorbance at 280 nm of eluent decreased to base line. The bound protein elution was made by a linear gradient of 0-0.15 M and 0.15-0.5 M KCl in the same buffer at a the flow rate of 0.5 ml/min. Fractions of 5 ml each were collected. The arrow indicated where each gradient was started. Active proteins with activity peaks from the fraction number 58-67 were pooled as indicated by (⊢⊣).

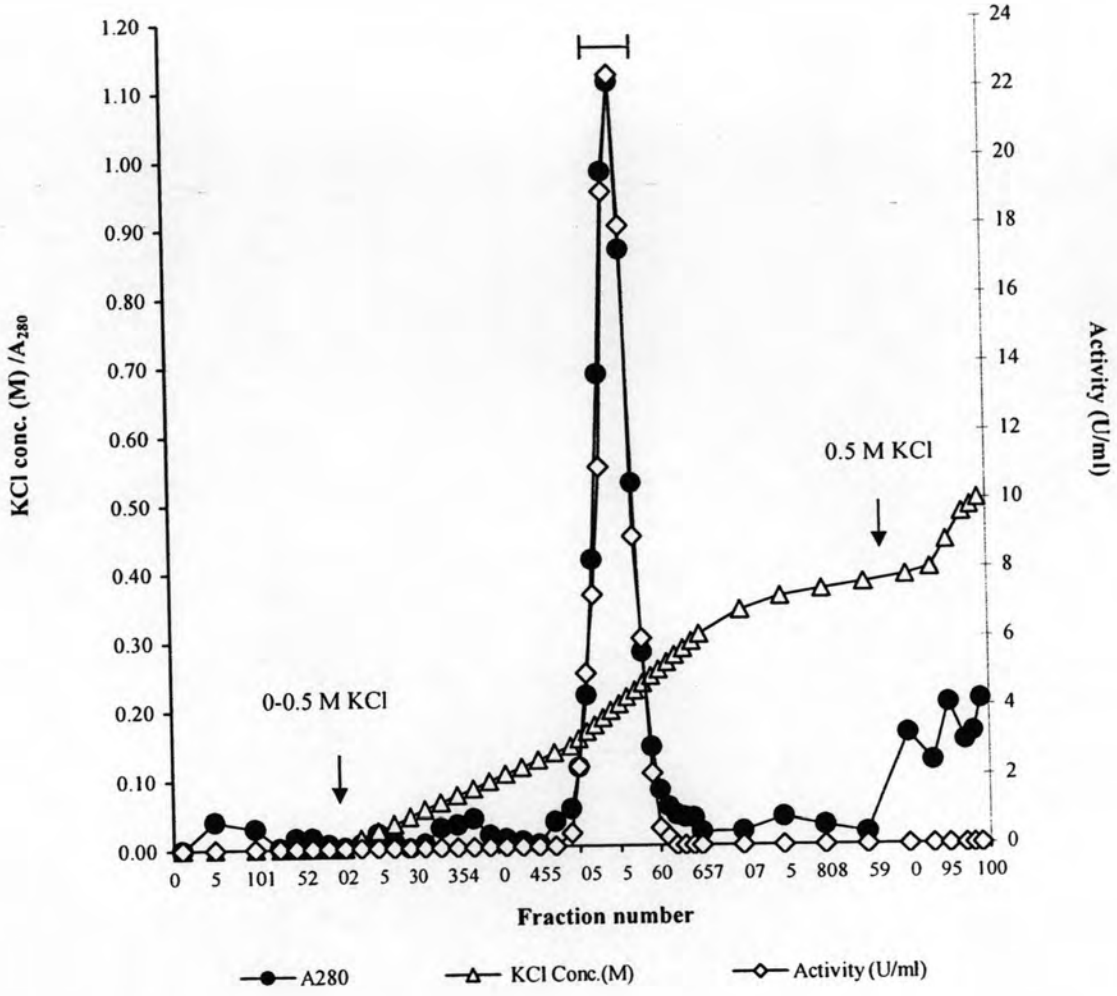


Figure 3.32 Purification profile of Lys 6-DH from the *E. coli* (BL21) DE3 transformant No. 4 by DEAE-Sephadex A50 column chromatography

The enzyme was applied to DEAE-Sephadex A50 column (2 x 20 cm) and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA until the absorbance at 280 nm of eluent decreased to base line. The bound protein elution was made by a linear gradient of 0-0.5 M KCl in the same buffer at a flow rate of 0.5 ml/min. Fractions of 5 ml each were collected. The arrow indicated where each gradient was started. Active proteins with activity peaks from the fraction number 52-60 were pooled as indicated by (⊢⊣).

Table 3.8 Purification of Lys 6-DH from *E. coli* (BL21) DE3 transformant No. 4 [a]

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	% Recovery
Crude extract[a]	880	1,664	1.89	1.0	100
DEAE-Toyopearl	459	1,088	2.37	1.2	65.3
DEAE-Sephadex A50	97	516	5.31	2.8	47.43

[a] Crude extract was prepared from 600 ml (5 g cell wet weight) of cell culture.

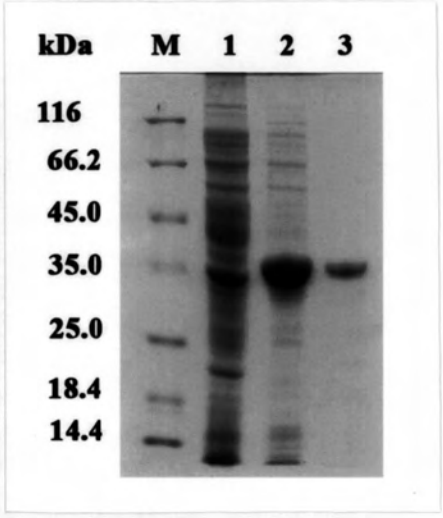


Figure 3.33 SDS-PAGE of the recombinant Lys 6-DH from each purification step

Lane M	= Protein MW marker	150 µg
Lane 1	= Crude extract	20 µg
Lane 2	= DEAE-Toyopearl column	20 µg
Lane 3	= DEAE-Sephadex A50 column	5 µg

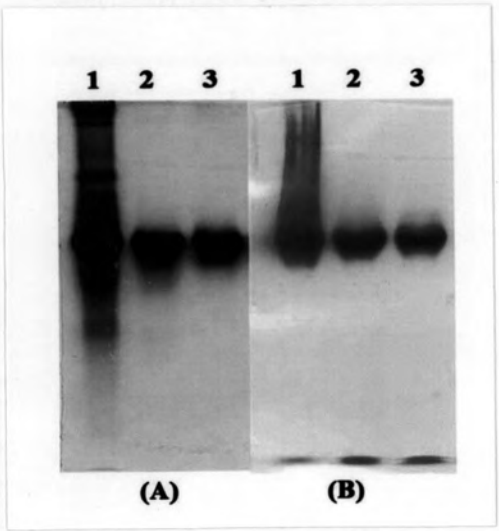


Figure 3.34 Native-PAGE of the recombinant clone Lys 6-DH from each purification step

(A) : protein staining

Lane 1	= Crude extract	20 μg
Lane 2	= DEAE-Toyopearl column	20 μg
Lane 3	= DEAE-Sephadex A50 column	20 μg

(B) : activity staining

Lane 1	= Crude extract	10 U
Lane 2	= DEAE-Toyopearl column	10 U
Lane 3	= DEAE-Sephadex A50 column	10 U

3.12 Characterization of Lys 6-DH

3.12.1 Molecular weight determination of Lys 6-DH

The native molecular weight of recombinant Lys 6-DH was determined from high molecular weight calibration curve obtained by gel filtration on HPLC using TSK gel G3000SW column as mentioned in 2.13.1 (Figure 3.35). The molecular weight of the enzyme after incubated with L-lysine was calculated to be 240,000 Da while the molecular weight of the native enzyme without L-lysine was calculated to be 80,000 Da. SDS-PAGE showed the molecular weight of the enzyme subunit was about 40,000 Da (Figure 3.33 and 3.36). These results suggested that the enzyme possibly consisted of six identical subunits.

3.12.2 Substrate specificity of Lys 6-DH

Substrate specificity of Lys 6-DH in oxidative deamination was studied as mentioned in 2.32.2. The ability of the enzyme to catalyze oxidative deamination of various amino acids and their derivatives was determined at a concentration of 20 mM as shown in Table 3.9. Lysine was the preferred substrate (relative activity, 100%). Various amino acids such as L-alanine, L-glutamate, L-glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-norvaline, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, D-aspartic, D-glutamate, D-phehylalanine, D-serine, D-tryptophan, DL-alanine, DL-isoleucine, L-ornithine, 6-amino-*n*-caproic acid and L-norluecine were not substrate.

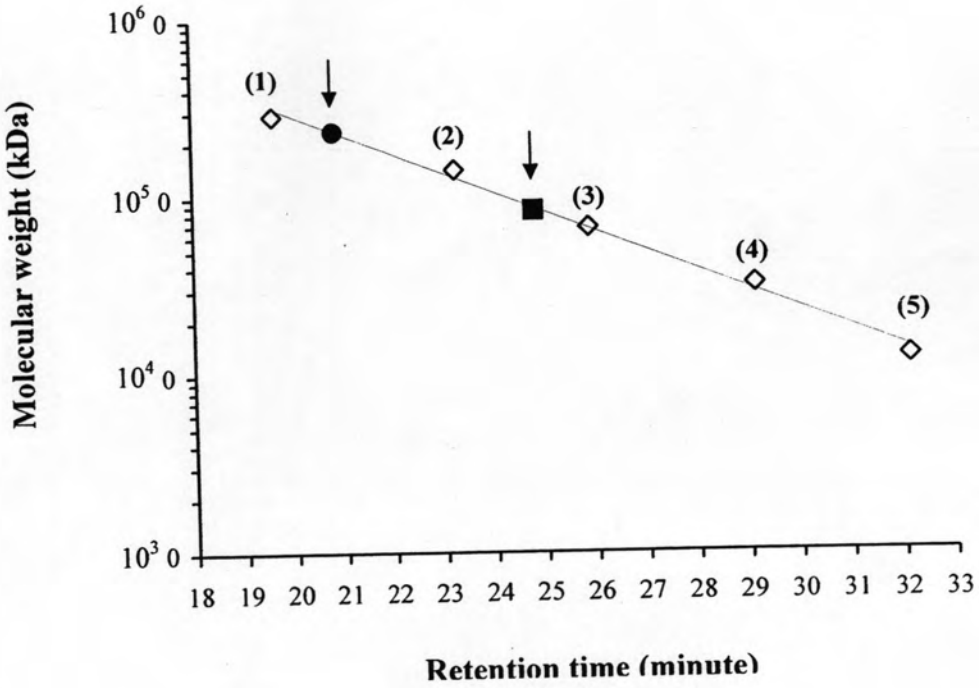


Figure 3.35 Calibration curve for molecular weight estimation of Lys 6-DH by gel filtration on HPLC

(1) =	Glutamate dehydrogenase	(290,000 Da)
(2) =	Lactate dehydrogenase	(142,000 Da)
(3) =	Enolase	(67,000 Da)
(4) =	Myokinase	(32,000 Da)
(5) =	Cytochrome c	(12,400 Da)

Arrow indicated the estimated molecular weight of Lys 6-DH

● = with 10 mM L-lysine ■ = without 10 mM L-lysine

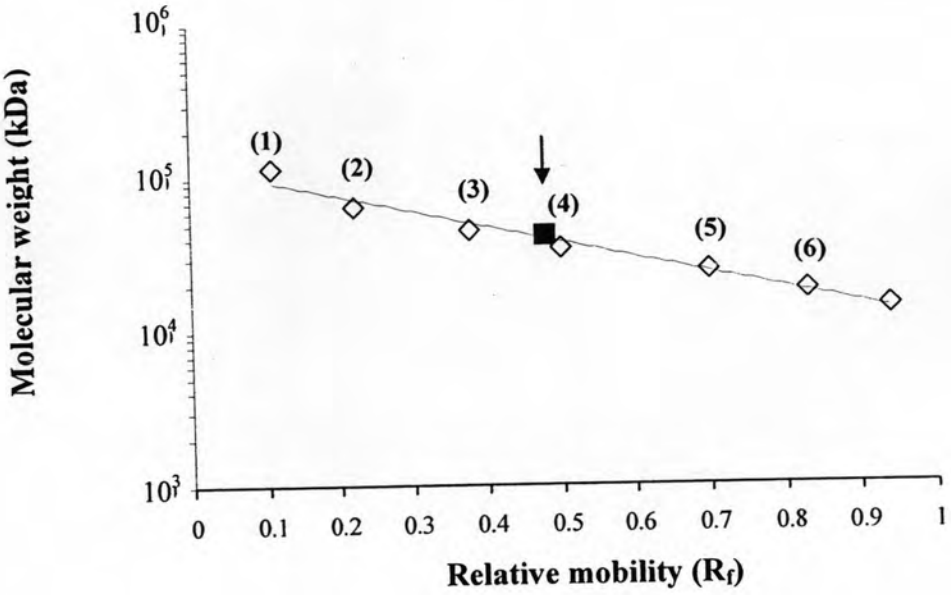


Figure 3.36 Calibration curve for molecular weight estimation of Lys 6-DH by SDS-PAGE

(1) =	β-galactosidase	(MW 116,000 Da)
(2) =	Bovine serum albumin	(MW 66,200 Da)
(3) =	Ovalbumin	(MW 45,000 Da)
(4) =	Lactate dehydrogenase	(MW 35,000 Da)
(5) =	REase Bsp98I	(MW 25,000 Da)
(6) =	β-lactoglobulin	(MW 18,400 Da)
(7) =	Lysozyme	(MW 14,400 Da)

Arrow indicated the estimated molecular weight of Lys 6-DH

Table 3.9 **Substrate specificity of the recombinant Lys 6-DH from recombinant clone**

Substrate[a]	Relative activity[b] (%)
L-lysine	100
L-alanine	0
L-glutamate	0
L-glycine	0
L-histidine	0
L-isoleucine	0
L-leucine	0
L-methionine	0
L-norvaline	0
L-proline	0
L-serine	0
L-threonine	0
L-tryptophan	0
L-valine	0
D-aspartic	0
D-glutamate	0
D-phehylalanine	0
D-serine	0
D-tryptophan	0
DL-alanine	0
DL-isoleucine	0
L-ornithine	0
6-amino-*n*-caproic acid	0
L-norluecine.	0

[a] Final concentration of each substrate was 20 mM.

[b] Relative activity of various amino acids were compared with the activity of L-lysine (100%)

3.12.3 Inhibitory effect of various amino acids on Lys 6-DH activity

To investigate the effect of various amino acids on the Lys 6-DH binding site, L-lysine was combined with various amino acids prior to enzyme assay. In control experiment, L-lysine alone was used. The decrease in Lys 6-DH activity may be caused by the inhibitory effect of these amino acids on the enzyme binding site. When mixed with L-lysine which was the preferred substrate, three L-amino acids, L-ornithine, 6-amino-*n*-caproic acid and L-norluecine, were found to adversely effect the oxidative deamination of L-lysine 70, 86 and 60% relative activity, respectively (Table 3.10).

3.12.4 Coenzyme specificity of Lys 6-DH

Coenzyme specificity of Lys 6-DH was investigated as described in 2.23.4. Lys 6-DH required NAD^+ as a natural coenzyme for oxidative deamination (100% relative activity). Some analogs of NAD^+ could serve as a coenzyme as shown in Table 3.11. Nicotinamide guanine dinucleotide contributed higher Lys 6-DH activity (133%) than that of NAD^+. Deamino NAD^+, 3-acetylpyridine adenine dinucleotide and nicotinamide $1,N^6$-ethano adenine dinucleotide could act as coenzyme as well since it showed 64, 58 and 80% relative activity to that of NAD^+. On the contrary, no activity was detected when each of β-$NADP^+$, deamido NAD^+, 3-pyridine aldehyde adenine dinucleotide, and thionicotinamide adenine dinucleotide was added instead of NAD^+.

Table 3.10 Inhibitory effect of various amino acids on Lys 6-DH activity

Amino acid[a]	Relative activity[b] (%)		
	20 mM	10 mM	1 mM
None	100	100	100
L-ornithine	**70**	**72**	**100**
6-amino-*n*-caproic acid	**86**	**87**	**100**
L-norluecine	**60**	**83**	**100**
L-alanine	100	100	100
L-glutamate	100	100	100
L-glycine	100	100	100
L-histidine	100	100	100
L-isoleucine	100	100	100
L-leucine	100	100	100
L-methionine	100	100	100
L-norvaline	100	100	100
L-proline	100	100	100
L-serine	100	100	100
L-threonine	100	100	100
L-tryptophan	100	100	100
L-valine	100	100	100
D-aspartic	100	100	100
D-glutamate	100	100	100
D-phehylalanine	100	100	100
D-serine	100	100	100
D-tryptophan	100	100	100
DL-alanine	100	100	100
DL-isoleucine	100	100	100

[a] Final concentration of each amino acid was 20 mM.

[b] Relative activity of various amino acids were compared with the activity of the reaction containing only L-lysine (100%).

Table 3.11 Coenzyme specificity of Lys 6-DH

Coenzyme[a]	Relative activity (%)
β-NAD^+	100
β-$NADP^+$	0
Deamino NAD^+	64
Deamido NAD^+	0
3-Pyridine aldehyde adenine dinucleotide	0
3-Acetylpyridine adenine dinucleotide	58
Nicotinamide 1,N^6-ethano adenine dinucleotide	80
Nicotinamide guanine dinucleotide	133
Thionicotinamide adenine dinucleotide	0

[a] Final concentration of each coenzyme analog was 2.0 mM.

3.12.5 Effect of pH on Lys 6-DH activity

Effect of pH on the enzyme activity for oxidative deamination was examined. The enzyme was assayed at various pHs ranging from 6.0 to 12 as mentioned in 2.23.5. The result was shown in Figure 3.37. The enzyme exhibited maximal activity at pH 9.3. At pH 7.5 and 9.5 of relative activities of the enzyme were more than 50% while the other pH that less than 6.5 and more than 10.0, the activities of the enzyme were loss.

3.12.6 Effect of pH on Lys 6-DH stability

Effect of pH on Lys 6-DH stability was studied as described in 2.23.6. The enzyme was preincubated in various 0.1 M buffers at various pHs ranging from 6.5 to 10.0 at 30 °C for 10 minutes. The result was shown in Figure 3.38. The enzyme was stable with relative activity higher than 80% over the pH ranging from 6.0 to 7.5 and 8.5 in KPB and glycine-KCl-KOH buffer, respectively.

3.12.7 Effect of temperature on Lys 6-DH activity

Effect of temperature on enzyme activity was investigated as described in 2.23.7. The temperature was varied from 30°C to 75°C. The result was shown in Figure 3.39. The enzyme performed the highest activity at 50 °C.

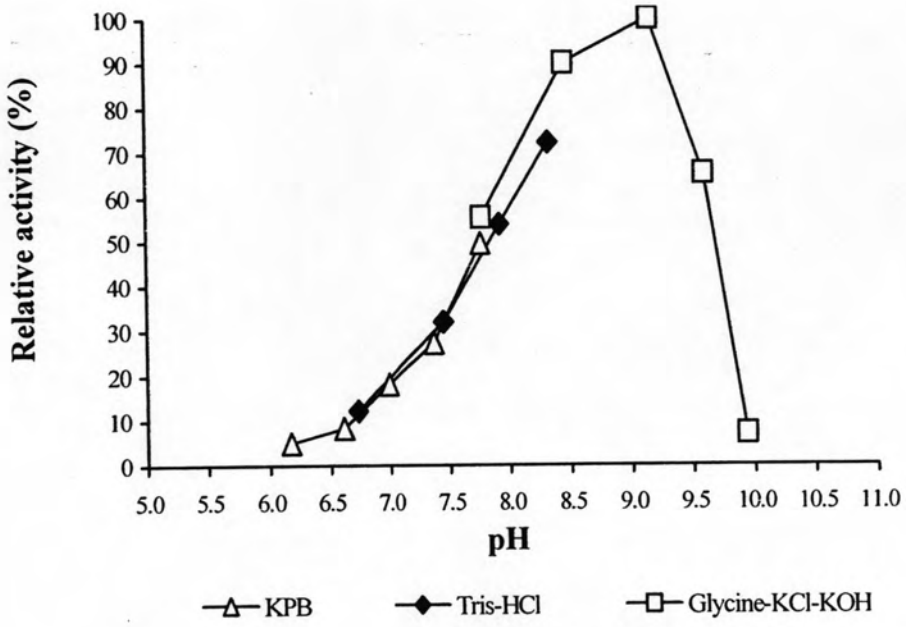


Figure 3.37 Effect of pH on Lys 6-DH activity

The reactions were assayed followed by the standard enzyme assayed condition in each various pHs at 30 °C for 10 minutes. Relative activity of various pHs were compared with the maximum activity (100%)

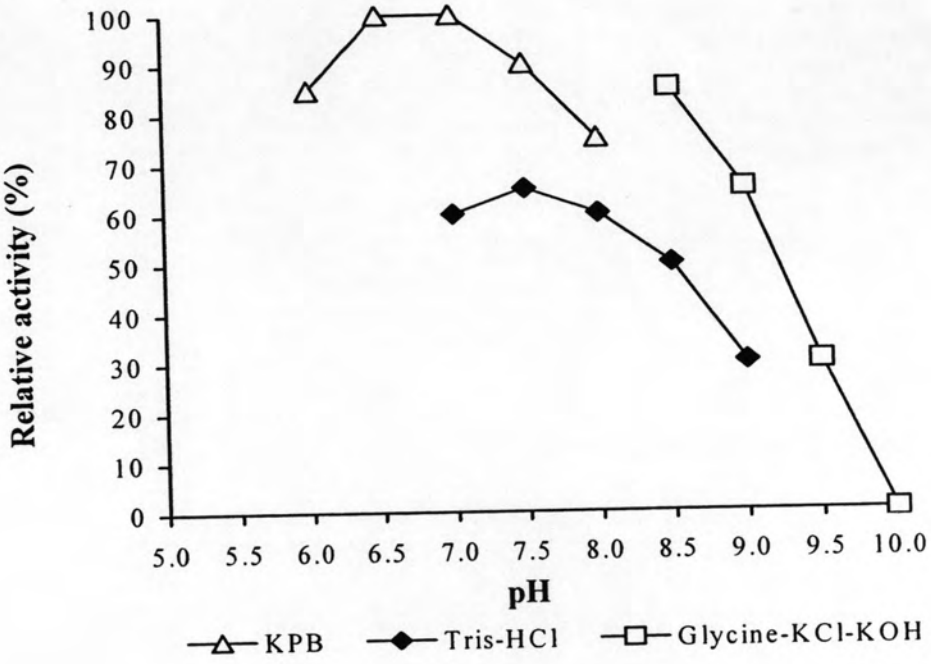


Figure 3.38 Effect of pH on Lys 6-DH stability

The reactions were assayed followed by the standard enzyme assayed condition after incubated free enzyme with each various pHs at 30 °C for 10 minutes.Relative activity of various pHs were compared with the maximum activity (100%)

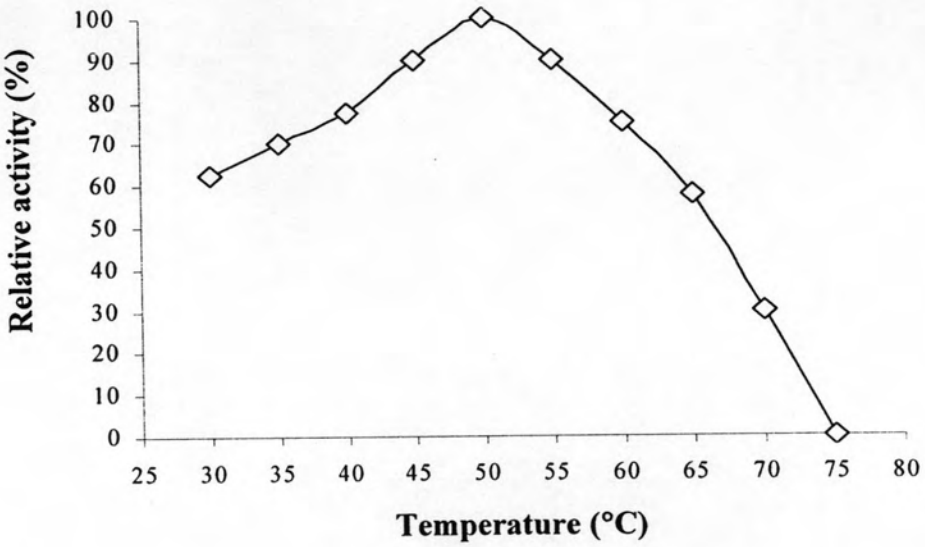


Figure 3.39 Effect of temperature on Lys 6-DH activity

The reactions were assayed followed by the standard enzyme assayed condition at various temperatures ranging from 30 °C to 75 °C. Relative activity of various temperatures were compared with the maximum activity (100%)

3.12.8 Effect of tempeature on Lys 6-DH stability

Thermostability of Lys 6-DH was studied as described in 2.23.8. The 5 mg of the enzyme was incubated in 0.01 M KPB, pH 7.4. Because for the next experiment for L-pipecolic production would be done at 30 °C, the stability of Lys 6-DH at this temperature was performed. The enzyme was incubated at 30 °C for 14 days. The remaining activities were expressed as percentage of the original activity. The result was shown in Figure 3.40. The enzyme was fully stable at 30 °C for 3 days and retained 50% of its activity after at day 9. The Lys 6-DH activity was completely lost after incubation for 14 days.

3.12.9 Kinetic parameter studies of Lys 6-DH

3.12.9.1 Initial velocity analysis for the dimeric form

A series of steady-state kinetic analysis was carried out to investigate the kinetic parameters. First, initial velocity studies for the dimeric form of Lys 6-DH was performed. The concentration of L-lysine was varied in the presence of several fixed concentrations of NAD^+. Double-reciprocal plots of initial velocity against L-lysine concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in Figure 3.41(A).

The apparent K_m value for NAD^+ was calculated to be 0.14 mM. From the secondary plot of intercept at the ordinate versus reciprocal concentrations of NAD^+, the apparent K_m value for L-lysine was calculated to be 11.11 mM as shown in Figure 3.41(B).

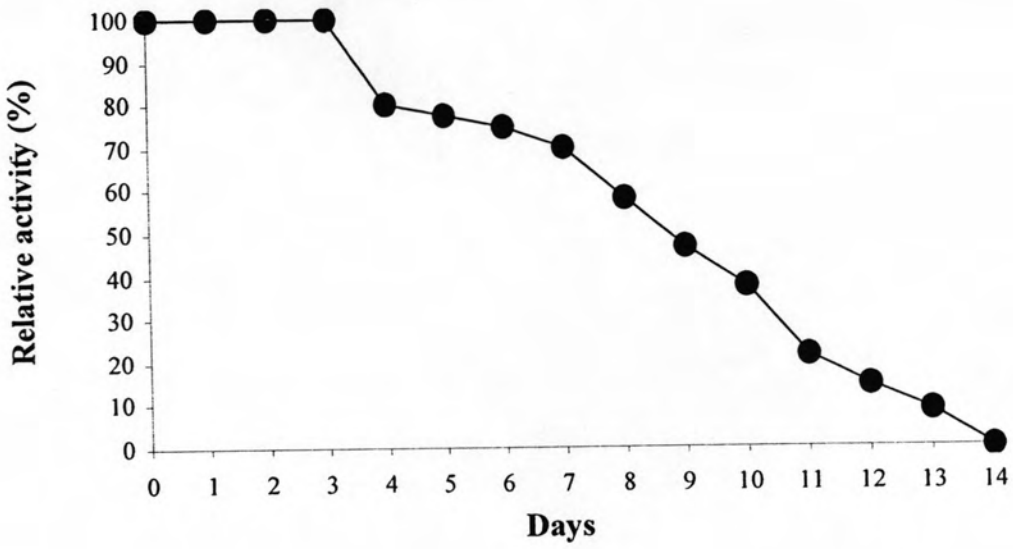


Figure 3.40 Effect of temperature at 30 °C on Lys 6-DH stability

The reactions were daily assayed followed by the standard enzyme assayed condition. Relative activity of enzyme from each day were compared with the first day (100%).

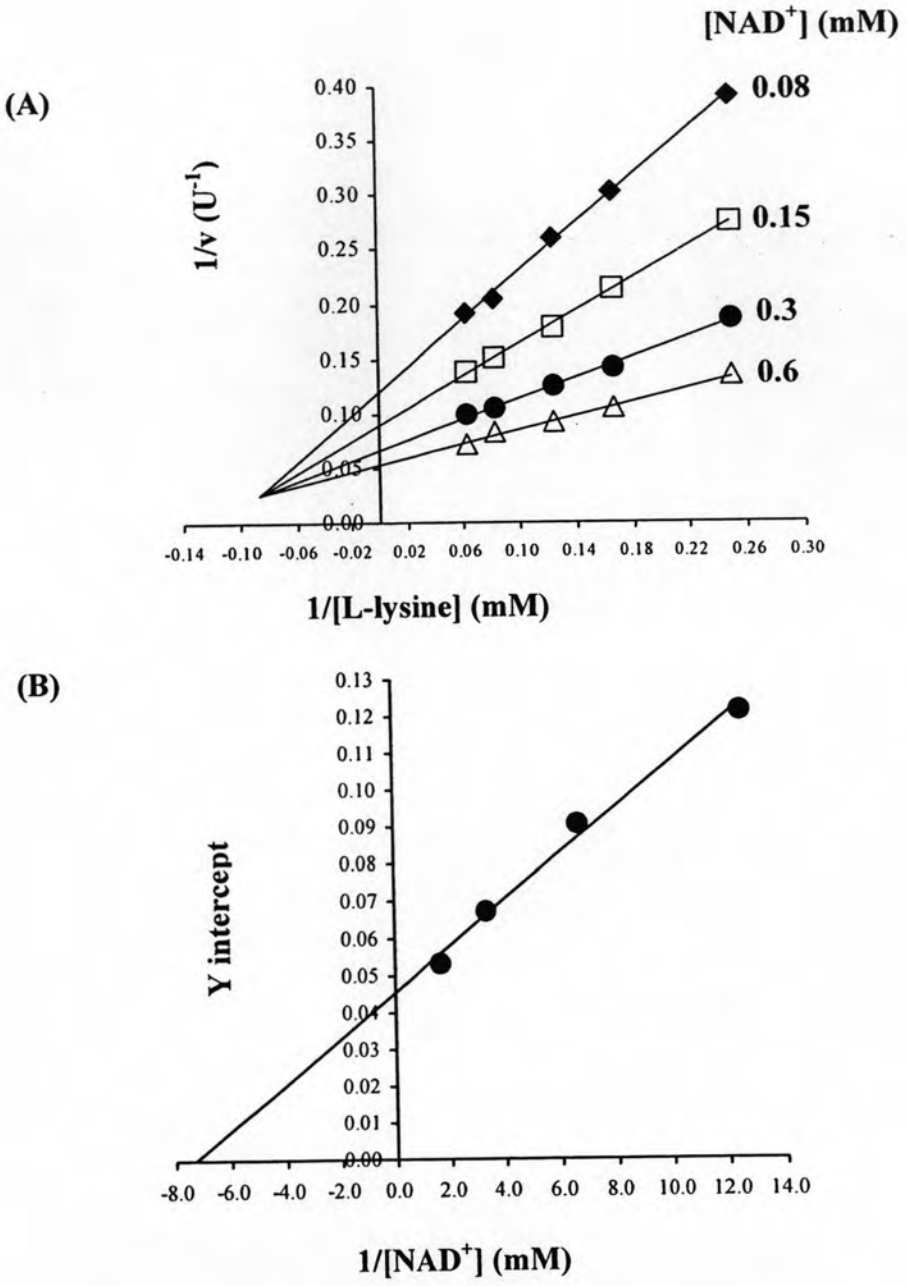


Figure 3.41 Initial velocity patterns for dimeric Lys 6-DH

(A) Double-reciprocal plots of initial velocities versus L-lysine concentrations at a series of fixed concentrations of NAD^+. Concentrations of NAD^+ were 0.08, 0.15, 0.3 and 0.6 mM, respectively.

(B) Secondary plot of y intercepts versus reciprocal NAD^+ concentrations.

3.12.9.2 Initial velocity analysis for the hexameric form

Because this enzyme was possibly hexameric when preincubated with 10 mM of L-lysine, a series of steady-state kinetic analysis for hexameric form was carried out to investigate the kinetic parameters. The initial velocity studies for the hexamer form of Lys 6-DH was performed. After preincubated Lys 6-DH with 10 mM of L-lysine for 20 minutes at 30 °C, the enzyme activity was performed. The concentration of L-lysine was varied in the presence of several fixed concentrations of NAD^+. Double-reciprocal plots of initial velocity against reciprocals of L-lysine concentrations gave a family of straight lines, which intersected in the upper left quadrant as shown in Figure 3.42(A).

The apparent K_m value for NAD^+ was calculated to be 0.09 mM. From the secondary plot of intercept at the ordinate versus reciprocal concentrations of NAD^+, the apparent K_m value for L-lysine was calculated to be 8.62 mM as shown in Figure 3.42(B).

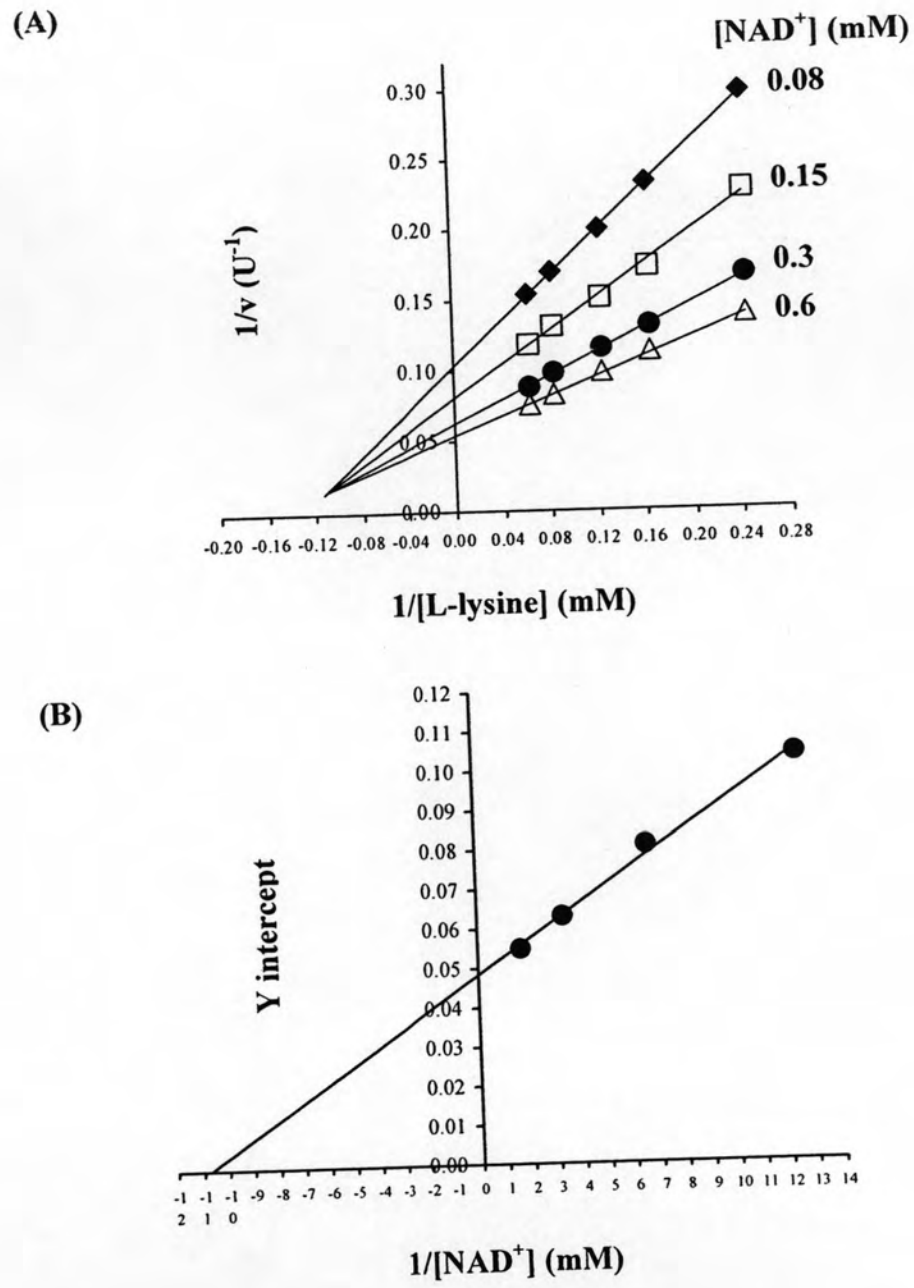


Figure 3.42 Initial velocity patterns for hexameric Lys 6-DH

(A) Double-reciprocal plots of initial velocities versus L-lysine concentrations at a series of fixed concentrations of NAD^+. Concentrations of NAD^+ were 0.08, 0.15, 0.3 and 0.6 mM, respectively.

(B) Secondary plot of y intercepts versus reciprocal NAD^+ concentrations.

3.13 PCR amplification of the full length pyrroline-5-carboxylate reductase (*p5cr*) gene

To express the *p5cr* gene in *E. coli* under T7 promoter of expression vector, pET-17b, a specific primer pair used for full length amplification of the *p5cr* gene of *Bacillus cereus* ATCC 11778 was designed as described in 2.24.2. The 5′-primer, P5CR-BCpETF, comprised *Nde*I restriction site and 5′-end of the *p5cr* gene. The 3′-primer, P5CR-BCpETR, consisted of *Eco*RI site, the TAA translational termination signal and 3′-end of the *p5cr* gene. The nucleotide sequences of forward and reverse primers were shown in the Table 3.12. Figure 3.43 showed the 0.8-kb PCR product of the *p5cr* gene fragment amplified from *B. cereus* ATCC 11778 DNA template. These PCR products were recovered and used for further cloning (Figure 3.44).

3.14 Expression of the *p5cr* gene

3.14.1 Recombinant plasmid characterization

The amplified gene fragment from 3.36 was digested with *Nde*I and *Eco*RI, and then ligated with *Nde*I-*Eco*RI-digested pET-17b vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3) by electroporation as described in 2.24. The transformants which could grow on LB plate containing 100 μg/ml ampicillin were randomly picked for plasmid extraction and digestion with *Nde*I-*Eco*RI as described in 2.25.1.

The recombinant plasmids (pET-P5CR) in *E. coli* BL21 (DE3) were presented in two forms, relaxed and supercoiled, as seen on agarose gel (Figure 3.45). After digestion the linear 3.3-kb pET-17b and 0.8-kb *p5cr* gene insert were obtained. From one hundred transformants containing *p5cr* gene on antibiotic plate, twelve transformants harboured recombinant plasmid were randomly selected for P5CR assay.

Table 3.12 The sequences of forward and reverse primers for full length *p5cr* gene amplification

primers	Nucleotide sequence (5′-3′)	Length (bp)	Tm (°C)
Forward primers: P5CR-BCpETF	5′-GGGAATTCCATATGGATAAACAA ATTGGATTCATCGGATGCGG-3′	43	83
Reverse primers: P5CR-BCpETR	5′-CGGAATTCTTACTTTTTCGTTTG ACCAGATAGTTCAACAGACTTT TG-3′	47	78

The restriction enzyme sites of forward (*Nde*I) and reverse primers (*Eco*RI) were underlined.

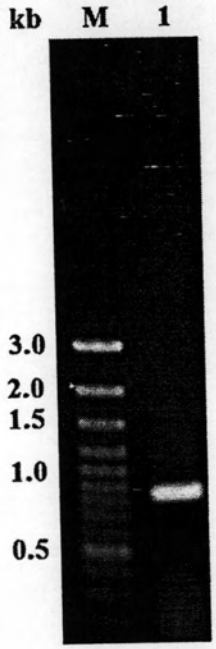


Figure 3.43 Putative *p5cr* gene fragment amplification

Lane M = 100 bp Plus DNA marker

Lane 1 = PCR product of the putative *p5cr* gene fragment

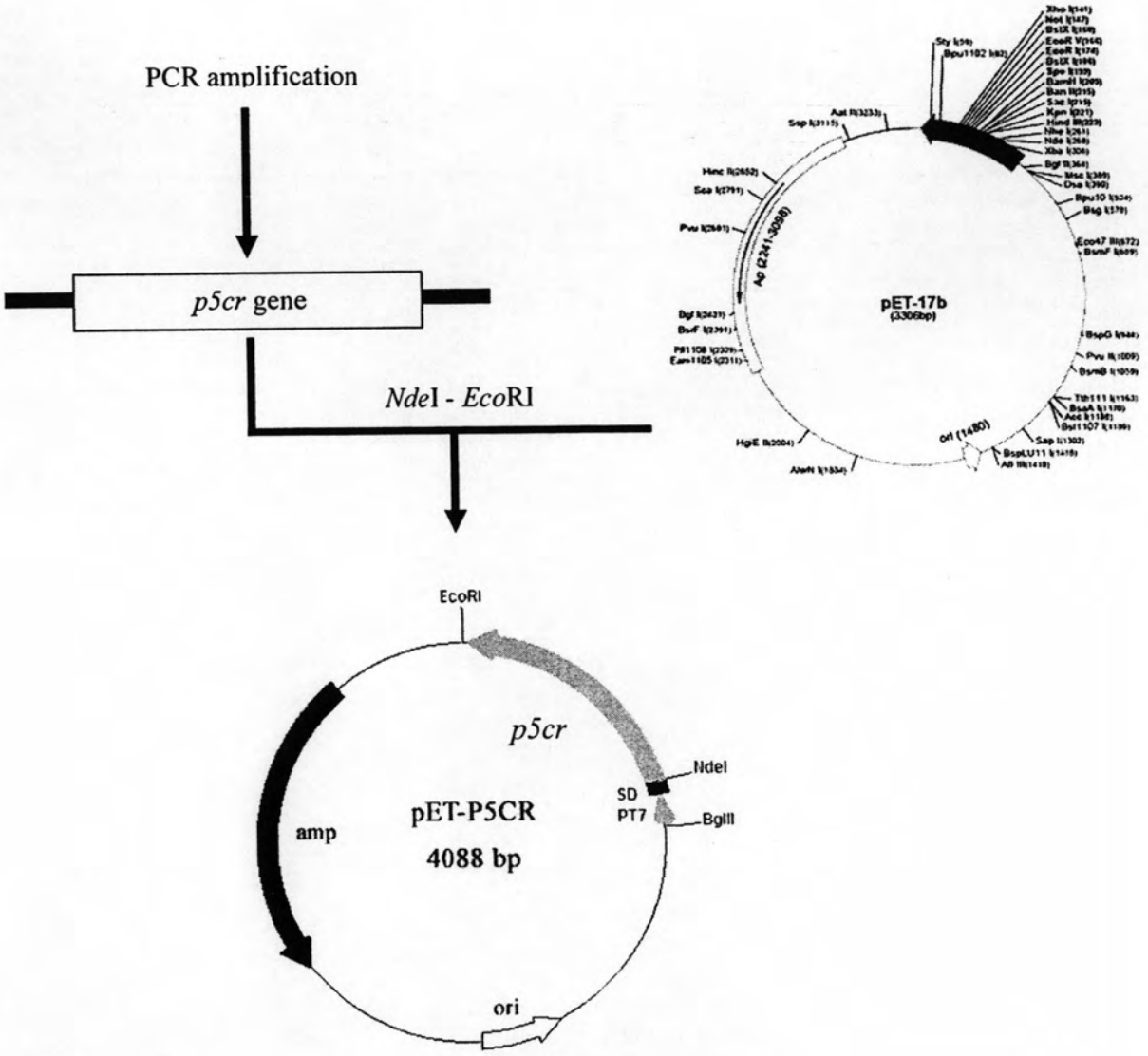


Figure 3.44 Construction of recombinant pET-P5CR

3.8.2 P5CR activity of transformants

Twelve recombinant clones were grown as described in 2.25.1. The crude extracts were prepared and the enzyme activities were measured. *E. coli* BL21(DE3) with and without the plasmid pET-17b were used as control. The recombinant clones showed various levels of the P5CR specific activity ranging from 1.39-5.28 units/mg protein (Table 3.13). Transformant No. 6 had the highest specific activity at 1.24 4.75 units/mg protein, which was 95 fold higher than that of the wild type *B. cereus* ATCC 11778. Thus, this transformant was used in further experiments.

3.15 Nucleotide sequence and deduced amino acid sequence of the *p5cr* gene

DNA insert of recombinant plasmid (pET-P5CR) was sequenced. The complete nucleotide sequence obtained was shown in Figure 3.46. The structural gene contained 782-bp open reading frame, which encoded a polypeptide of 260 amino acids. Molecular weight of the enzyme subunit calculated from the deduced amino acid sequence by Genetyx 6.0 program was 28.4 kDa.

When the percentage of similarity of nucleotide sequences of the whole *p5cr* gene was compared to those in the EMBL-GenBank-DDBL database, the percentage of nucleotide sequence identity between the *p5cr* gene from *Bacillus cereus* ATCC 11778 and each of those from *Bacillus cereus* ATCC 14579, *Bacillus cereus* ATCC 10987, *Bacillus cereus* E33L, *Bacillus thuringiensis*, *Bacillus anthracis* and *Escherichia coli* W3110 DNA were 3, 80, 3, 3, 3 and 2%, respectively (Figure 3.47).

Moreover, The percentages of similarity between the deduced amino acid sequence of P5CR from *B. cereus* ATCC 11778 and each of those from *Bacillus thuringiensis* ATCC 35646, *Bacillus cereus* ATCC 14579, *Bacillus weihenstephanensis* KBAB4, *Bacillus cereus* ATCC 10987 and *Escherichia coli* CFT073 were 55, 56, 53, 53 and 31%, respectively (Figure 3.48).

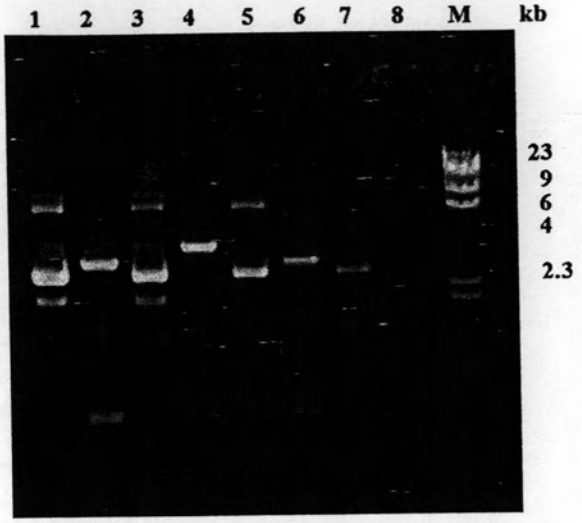


Figure 3.45 Restriction pattern of pET-P5CR from selected recombinant clones

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = undigested plasmid
Lane 2 = *Nde*I-*Eco*RI digested plasmid
Lane 3 = undigested plasmid
Lane 4 = *Nde*I-*Eco*RI digested plasmid
Lane 5 = undigested plasmid
Lane 6 = *Nde*I-*Eco*RI digested plasmid
Lane 7 = undigested plasmid
Lane 8 = *Nde*I-*Eco*RI digested plasmid

Table 3.13 P5CR activity from the crude extract of 12 selected *E. coli* BL21(DE3) transformants harboring pET-P5CR[a]

Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
B. cereus ATCC 11778	6	116	0.05
E. coli BL21(DE3)	0	110	0.00
E. coli BL21(DE3) harbouring pET-17b	0	132	0.00
Transformant No.1	104	66	1.58
Transformant No.2	200	50	4.00
Transformant No.3	84	68	1.24
Transformant No.4	166	66	2.52
Transformant No.5	125	86	1.45
Transformant No.6	**266**	**56**	**4.75**
Transformant No.7	194	70	2.77
Transformant No.8	281	60	4.68
Transformant No.9	198	88	2.25
Transformant No.10	95	76	1.25
Transformant No.11	194	52	3.73
Transformant No.12	210	82	2.56

[a] Crude extracts were prepared from 200 ml of cell culture by induction with 0.1 mM IPTG for 4 hours.

```
1   ATGGAATTCTTCGAAGGCGGAATTATGGGAAGGGCTATGATGGGCGAAATGATAAACAAA   60
    M  E  F  F  E  G  G  I  M  G  R  A  M  M  G  E  M  I  N  K
61  ATTATATGGCCTCCAAATCAAATTATTGGTCCAAATTTAACCCCGGCTAATTTAAAAAAG  120
    I  I  W  P  P  N  Q  I  I  G  P  N  L  T  P  A  N  L  K  K
121 GCTAGGGAAAAATATGGACTACCTCCACCTCCCGACAACATTGAAGTACCTAAAAAGGCT  180
    A  R  E  K  Y  G  L  P  P  P  P  D  N  I  E  V  P  K  K  A
181 AATATTTTAATTTTATCAACCAACCCAAACCTATCCCCGCCATTAATTAAGGAAATAAAA  240
    N  I  L  I  L  S  T  N  P  N  L  S  P  P  L  I  K  E  I  K
241 AAAGTAACCAAAACCAATCCTATCACCGAACCAATCCCTGCTGGAAAAAGTATTGAAAGT  300
    K  V  T  K  T  N  P  I  T  E  P  I  P  A  G  K  S  I  E  S
301 CCTGAAAATGCCTTTAATAAAAAAGTAAAGGTTGAAAAAGTAAGGCCTAATCCCCCGGCC  360
    P  E  N  A  F  N  K  K  V  K  V  E  K  V  R  P  N  P  P  A
361 CTGGTTGAAAAGGAAATGCCTGCTTTATGCCCTATTGAAAGGGTGACTGAAAAAAATTTA  420
    L  V  E  K  E  M  P  A  L  C  P  I  E  R  V  T  E  K  N  L
421 AAAAATGTGCTAACCTTTTCCAATAGTTTGGGCCAACCAAAAATCGTAAGTAAAAAATTA  480
    K  N  V  L  T  F  S  N  S  L  G  Q  P  K  I  V  S  K  K  L
481 AGGAAGGTTGTACCTCCGGTAAGGGGTCCTCCCCCACCTAATGTATATATAATTATAAAA  540
    R  K  V  V  P  P  V  R  G  P  P  P  P  N  V  Y  I  I  I  K
541 CCAATGGCAAATCCTGCTGTCCTAAATGGAATGCCAAGAAACCAACCATATAAACCCCCT  600
    P  M  A  N  P  A  V  L  N  G  M  P  R  N  Q  P  Y  K  P  P
601 GCTCAACCTGTGTTAGGCTCTGCAAAAATGGTCCTAAAACCAGGATTCCTTCCAGGTGAA  660
    A  Q  P  V  L  G  S  A  K  M  V  L  K  P  G  F  L  P  G  E
661 TTGAAAAATATGGTTGGTCCCCCTGGCGAACCAACATTAAAACCTGTACCACCTTTAAAG  720
    L  K  N  M  V  G  P  P  G  E  P  T  L  K  P  V  P  P  L  K
721 GAAAAAGGACTGAAATACACCATTTATTCCACCTATCCACCGTTGTCCGCAAAAGCCTGT  780
    E  K  G  L  K  Y  T  I  Y  S  T  Y  P  P  L  S  A  K  A  C
781 TG  782
```

Figure 3.46 Nucleotide and the deduced amino acid sequence of the *p5cr* gene from *Bacillus cereus* ATCC 11778

Start and stop codon of the *p5cr* gene were underlined.

```
CLUSTAL W (1.83) multiple sequence alignment

BT              -TTATTTCCCCCTAGCTTTTTTGTTTGTTGCTTCTACTGCTTTTTGAATT--GCCTGTGA 57
BA              ------------------------------------------------------------
BC3             -TTATTTCCCCCTAGCTTTTTTGTTTGTTGCTTCTACTGCTTTTTGAATT--GCCTCTGA 57
BC1             -TTATTTCCCCCTAGCTTTTTTGTTTGTTGCTTCTACTGCTTTTTGAATT--GCCTCTGA 57
P5CR            -----ATGGATAAACAAATTGGATTCATCGGATGCGG---------------GGCTATGA 40
BC2             -----ATGGATAAACAAATTGGATTCATCGGATGCGGAAATATGGGGAT---GGCTATAA 52
EC              TCAGGATTTGCTGAGTTTTTCTGATTTTTCCATACACTTCGTCATCGCTTCGATCACTGC 60

BT              AAAATTATA-----T-TCATATAAAGCTTTGAGACCTTCAGCTGTGGAACCACCTGGCGT 111
BA              -------------T-TAATATAAAGCTTTGAGACCTTCAGCTGTGGAACCACCTGGCGT 45
BC3             AAAATTATA-----T-TCATATAAAGCTTTGAGACCTTCAGCTGTGGAACCACCTGGCGT 111
BC1             AAAATTATA-----T-TCGTATAAAGTTTTGAGACCTTCAGCTGTGGAACCACCTGGCGT 111
P5CR            TGGGCGAAA-----TGATAAACAAAATTATATGGCCTCCAA--ATCAAATTATTGGTCCA 93
BC2             TTGGCGGGA-----TGCTAAACAAAAAGATAGTGTCTTCAA--ATAAAATCATGTGTTCA 105
EC              AGCACGGAAGCCTTTCTCTTCCAGTACGCGTACCGCTTCAATGGTGGTGCCTCCCGGTGA 120
                             *          *          ** **    *          *

BT              T---GTTACTTGTTCACTTAGGTTAGCTGGATCTTGTGTTTGTTCAAGCATAGAAGCAGA 168
BA              T---GTTACTTGTTCACGGAGGTTAGCTGGATCTTGTGTTTGTTCAAGCATAGAAGCAGA 102
BC3             T---GTTACTTGTTCACGGAGGTTAGCTGGATCTTGTGTTTGTTCAAGCATAGAAGCAGA 168
BC1             T---GTTACTTGTTCGCGAAGGTTAGCTGGATTTTGCGTTTGTTCAAGCATAGAAGCAGA 168
P5CR            A---ATT-TAACCCCGGCTAATTTAAAAAAGGCTAGGGAAAAATATGGACTACCTCCACC 149
BC2             G---ATT-TAAACACGACGAATTTAGAAAATGCTAGTGAAAAGTACGGAATAACTATAAC 161
EC              GCAGACCATATCTTTCAGTGCCCCCGGATGTTCTCCCGTTTCCAGCACCATTTTTGCGGA 180
                                                 *   *          *

BT              GCCAGAAATCATTTGAATGAC--AAGGTGTT----TGGCGGTTGCTTCATCGACTCCATA 222
BA              GCCAGAAATCATTTGAATGAC--AAGGTGTT----TTGCGGTTGCTTCATCGACTCCATA 156
BC3             GCCAGAAATCATTTGAATGAC--AAGGTGTT----TGGCGGTTGCTTCATCGACTCCATA 222
BC1             GCCAGAAATCATTTGAATGAC--AAGATGTT----TTGCGGTTTCTTCATCAACTCCATA 222
P5CR            TCCCGACAACATTGAAGTACCTAAAAAGGCTAATATTTTAATTTTATCAACCAACCCAAA 209
BC2             TACCGACAACAATGAAGTCGCTAAAAATGCTGATATTTTAATTTTATCAATTAAACCAGA 221
EC              ACCCATTACCGCCTGAGCGGC--AAATTTATACGCCTGGGCGCGTGGCATCCCGCCCAGC 238
                  *   * *    *   * **    *                    **     ***

BT              ACTTTTTGTTGCTTCAATTAA-ACTTTCAGCAAAGTAATAAAGG--AAGGCTGGTGCACT 279
BA              ACTTTTTGTTGCTTCAATTAA-ACTTTCAGCAAAGTAATAAAGG--AAGGCTGGTGCACT 213
BC3             ACTTTTTGTTGCTTCAATTAA-ACTTTCAGCAAAGTAATAAAGG--AAGGCTGGTGCACT 279
BC1             ACTTTTTGTTGCTTCAATTAA-GCTTTCAGCAAAGTAATAAAGG--AAGGCTGGTGCACT 279
P5CR            CCTATCCCCGCCATTAATTAAGGAAATAAAAAAAGTAACCAAAACCAATCCTATCACCGA 269
BC2             CTTATACCCATTAGTAATTAACGAAATTAAAGAAGTAATAAAAAACGATGCTATCATCGT 281
EC              ACG----GCGGCGTCGGCCATCGCTTCGATAAACATAAATACGT---AGGCTGGCGAAGA 291
                          *          *   *  ***  *       *  **

BT              TCCAGTAACTGCAGT--AAGTTGATGAACTTCTTCCTCGGTACAAAGTTGCGAAGTACCA 337
BA              TCCAGTAACTGCAGT--AAGTTGATGAACTTCTTCCTCGGTACAAAGTTGCGAAGTACCA 271
BC3             TCCAGTAACTGCAGT--AAGTTGATGAACTTCTTCCTCGGTACAAAGTTGCGAAGTACCA 337
BC1             TCCAGTAACTGCAGT--AAGCTGATGAACTTCTTCCTCAGTACAAAGTTGCGAAGTACCA 337
P5CR            ACCAATCCCTGCTGGAAAAAGTATTGAAAG---TCCTGAAAATGCCTTTAATAAAAAAGT 326
BC2             TACGATCGCTGCTGGTAAAAGTATTGAAAG---TACTGAAAATGCCTTTAATAAAAAATT 338
EC              ACCGCTCACACCGAC--CACCGGGTGGATCATCGGCTCAGCAATTACTTCCGCTTCGCCA 349
                  *  *  *  *       *     ** *        **     *     **

BT              ATGCCCCTTAAAAGCAATTGCAGGGTTTCTTGATGAGTCTCGTTTACGAATTGTCCCATC 397
BA              ATGCCCCTTAAAAGCAATTGCAGGGTTTCTTGATGAGTCTCGTTTACGAATTGTCCCATC 331
BC3             ATGCCCCCTAAAAGCAATTGCAGGGTTTCTTGATCAGTCTCGTTTACGAATTGTCCCATC 397
BC1             ATACCTTTTAAAAGTAGTTGCAGGGTTTCTTGATGTATCTCGTTTACTGATTGTCCCATT 397
P5CR            AAAGGTTGAAAAAGTAAGGCCTAATCCCCCGGCCCTGGTTGAAAAGGAAATGCCTGCTTT 386
BC2             AAAAGTTGTACGAGTAATGCCTAATACTCCTGCTCTTGTTGGAGAAGGAATGTCTGCATT 398
EC              AAGCAGCGGAA----AATATTCAGCACATCAGCGGTATCTTCTGGGGTTACCAGCGCGTT 405
                *        *     *                *        *        *      * *
```

Figure 3.47 Linear alignment of the nucleotide sequence of the *p5cr* gene from *Bacillus cereus* ATCC 11778 and those from various sources

BT = *Bacillus thuringiensis* (CP000485.1), BA = *Bacillus anthracis* (AE017334.2), BC3 = *Bacillus cereus* E33L (CP000001.1), BC2 = *Bacillus cereus* ATCC 10987 (AE017194), BC3 = *Bacillus cereus* ATCC 14579 (AE016877.1), P5CR = *Bacillus cereus* ATCC 11778 and EC = *Escherichia coli* W3110 (AE014075). Conserved residues are indicated by asterisks (*).

```
BT      GTGT----ATAAGGAGATAGACTTTCCAATTTCAGCAGCTGTATTTGGCATAATCCAAGC 453
BA      GTGT----ATAAGGAGATAGACTTTCCAATTTCAGCAGCTGTATTTGGCATAATCCAAGC 387
BC3     GTGT----ATAAGGAGATAGACTTTCCAATTTCAGCAGCTGTATTTGGCATAATCCAAGC 453
BC1     GTAT----ATAAGGAGATAGACTTTCCAATTTCAGCAGCTGTATTAGGCATAATCCAAGC 453
P5CR    ATGCCCTATTGAAAGGGTGACTGAAAAAAATTTAAAAAATGTGCTAACCTT--TTCCAAT 444
BC2     ATGCCCAAATGAAATGGTGACAGAAAAGGATTTAGAAGATGTGCTAAACAT--TTTCAAT 456
EC      TGGCGTTACGGAGGTCATCCCGGCATTAACCAGTGCGGGAGTGTTCGGCATGGCGCGGAT 465
                  *    *                        **  *   * *

BT      AACAGGCGTCCCCCCGGGAAGTCTTTCTTCTAAATAAGATGGGCCGATGCCAGCAGCTAC 513
BA      AACAGGCGTCCCCCCGGGAAGTCTTTCTTCTAAATAAGATGGGCTGATGCCAGCAGCTAC 447
BC3     AACAGGCGTTCCCTCGGGAAGTCTTTCTTCTAAATAAGATGGGCCGATGCCGGCAGCTAC 513
BC1     AACAGGCGTGCCTTTTGGGAGTCTAGCTTCTAAATAAGATGGTCCAATGCCAGCTGCTAC 513
P5CR    AGTTTGGGC-CAACCAAAAATCGTAAGTAAAAAATTAAGGAAGGTTGTACCTCCGGTAAG 503
BC2     AGTTTTGGT-CAATCAGAGATCGTAAGTGAAAAGTTAATGGATGTTGTAACATCTGTAAG 515
EC      AATTTTCCGGTCATGGCCCAGCGCGCGGGCAAGCTGGTCGAGCGTGACACCTGCAGCAAT 525
        *                 *              * *                 * * * *

BT      CGTTACGACAAGTTGATTCGATAGTAA---CGGAGATAACTCTGCTAATAATTCT--TCA 568
BA      CGTTACGACAAGTTGATTCGATAGTAA---CGGAGATAACTCTGCTAATAATTCT--TCA 502
BC3     CGTTACGACAAGTTGATTCGATAGTAA---CGGAGATAATTCTGCTAATAATTCT--TCA 568
BC1     TGTTACGACTAGTTGATTTGATATTAA---CGGAGATAGCTCAGCTAATAACTCT--TCA 568
P5CR    GGGTCCTCCCCCACCTAATG-TATATA---TAATTATAAAACCAATGGCAAATCC--TGC 557
BC2     TGGCTCTTCACCAGCATACG-TATATA---TGATTATAGAAGCGATGGCAGATGC--TGC 569
EC      AGAAACGACCAGAGAGTCTT-TATTCAGGCTGGAGGTGATTTCGCTAAGCACTTTAATCA 584
         *   *  *            **   *          *         *      *    *

BT      TG-TGCAGAAGGCGGCATT-GCTAAAACAATCGTATCAACAGATGTAACATGTTGCTTCC 626
BA      TG-TGCAGAAGGCGGCATT-GCTAAAACAATCGTATCAACAGATGTAACATGTTGTTTCC 560
BC3     TG-TGCAGAAGGCGGCATT-GCTAAAACAATCGTATCAACAGATGTAACATGTTGCTTCC 626
BC1     TG-AGCCGAAGGTGGCATT-GCTAAAACAACCGTATCAACAGATGTAACATGCTGTTTCC 626
P5CR    TG-TCCTAAATGGAATGCC-AAGAAACCAACCATATAAACCCCCTGCTCAACCTGTGTTA 615
BC2     TG-TACTAGATGGCATGCC-AAGAAATCAAGCATATAAATTCGCTGCTCAAGCAGTATTA 627
EC      TGATGCCAGGTTTAACGGCAGCAAAAATGATGTCGGCGATTTGCGCCACTTCTTGCGCCG 644
        **   *              ***   *        *         *      *

BT      AA-TCTGTTGTAGTAGACACATTATATCGCGCTCGT--AATTGATCTAGCTTTTCTACGT 683
BA      AA-TCTGTTGTAGTAGACACATTATATCGCGCTCGT--AATTGATCTAGCTTTTCTACGT 617
BC3     AA-TCTGTTGTAGTAGACACATTATATCGCGCTCGT--AATTGATCTAGCTTTTCTACGT 683
BC1     AA-TCTGTTGTAGTAGACACATTATATTGATGTTGT--AACTGTTTTAGCTTTTCTATGT 683
P5CR    GGCTCTGCAAAAATGGTCCTAAAACCAGGATTCCTTCCAGGTGAATTGAAAAATATGGTT 675
BC2     GGCTCTGCAAAAATGGTACTAGAAACAGGAATACATCCAGGTGAATTGAAAGATATGGTT 687
EC      AT-TCTGCGGCGTTG--------ATGCCGAACTGGTC-ATGCAGGGCGGCGACTTTATCC 694
          ****      *          *    *      * *                * *

BT      TACTTCGGTTA-GAAACGATAATCTCTTCGATGTATTCTTTGCTTGTTTTAAGTAATCCG 742
BA      TACTTCGGTTA-GAAACGATAATCTCTTCGATGTATTCTTTGCTTGTTTTAAGTAATCCG 676
BC3     TACTTCGGTTA-GAAATGATAATCTCTTCGATGTATTCTTTGCTTGTTTTAAGTAATCCA 742
BC1     TGCTTCGGTTA-GAGACGATAATTTCTTCGATGTACTCTTTGCTTGTTTTAAGTAATCCG 742
P5CR    GGTCCCCCTGGCGAACCAACATTAAAACCTGTACCACCTTTAAAGGAAAAAGG---ACTG 732
BC2     TGTTCTCCTGGCGGAACAACGATAGAAGCTGTAGCAACTTTAGAGGAAAAAGGCTTACGA 747
EC      GGGGAGGGGGTGTATACCCAGATTTGCCCTGGAAGCACCTGACCGCTGGCAATCAGACCG 754
                        *     *      * *          *      *

BT      GAAAATATAGCTTCTGCCATACGACCA-----GCACCAATAAATAGAATTCGATGTTTAG 797
BA      GAAAATATAGCTTCTGCCATACGACCA-----GCACCAATAAATAGAATTCGATGTTTAG 731
BC3     GAAAATATAGCTTCTGCCATACGACCA-----GCACCAATAAATAGAATTCGATGTTTAG 797
BC1     GAAAATATAGCTTCTGCCATACGACCG-----GCACCAATAAATAAAATTCGATGTTTAG 797
P5CR    AAATACACCAT----------------------CAAAAGTCTGTTGAACTATCTGGTCAA 770
BC2     ACAGCCATCATTTCAGCTATGCAACGTTGTACACAAAAGTCTGTTGAACTATCTGGTCAA 807
EC      CCGAGAATGGCTTTTCCCATATTGCCGC-----AGCCAATAAAACCGATTTTCTTTTCCA 809
           *                                * *      * *   *  *

BT      TTAGCAT----- 804
BA      TTAGCAT----- 738
BC3     TTAGCAT----- 804
BC1     TTAGCAT----- 804
P5CR    ACGAAAAAGTAA 782
BC2     ACGAAAAAGTAA 819
EC      T----------- 810
```

Figure 3.47 Linear alignment of the nucleotide sequence of the *p5cr* gene from *Bacillus cereus* ATCC 11778 and those from various sources. (continued)

```
CLUSTAL W (1.83) multiple sequence alignment

BT              -----------------------------------MDKQIGFIGCGNMGMAMIGGMLNK 24
BC2             -----------------------------------MDKQIGFIGCGNMGMAMIGGMINK 24
BW              -----------------------------------MNKQIGFIGCGNMGMAMIGGMINK 24
BC1             -----------------------------------MDKQIGFIGCGNMGMAIIGGMLNK 24
P5CR            ------------------------------------MEFFEGGIMGRAMMGEMINK 20
EC              MLGNPLLCRAFAFRHSSVYASASDYQNNEFHGRSEAMEKKIGFIGCGNMGKAILGGLIAS 60
                                                     : *:  * ** *::* :: .

BT              KIVSSNHIICSDLNTANLKNASEKYGLTTTTDNNKVAKNADILILSIKPDLYASIINEIK 84
BC2             NIVSSNQIICSDLNTANLKNASEKYGLTTTTDNNEVAKNADILILSIKPDLYASIINEIK 84
BW              NIVSSNKIICSDLNTTNLKNASEKYGLTITTDNNEVAKNADILILSIKPDLYASIINEIK 84
BC1             KIVSSNKIMCSDLNTTNLENASEKYGITITTDNNEVAKNADILILSIKPDLYPLVINEIK 84
P5CR            IIWPPNQIIGPNLTPANLKKAREKYGLPPPPDNIEVPKKANILILSTNPNLSPPLIKEIK 80
EC              GQVLPGQIWVYTPSPDKVAALHDQFGINAAESAQEVAQIADIIFAAVKPGIMIKVLSEIT 120
                   ..:*     .. ::    :::*:  . .  :*.: *:*:: : :*.:   ::.**.

BT              EVIKNDAIIVTIAAGKSIESTENAFNKKLKVVRVMPNTPALVGEGMSALCPNEMVTEKDL 144
BC2             EIIKNDAIIVTIAAGKSIESTENAFNKKVKVVRVMPNTPALVGEGMSALCPNEMVTEKDL 144
BW              EVIKNDVIIVTIAAGKSIKSTEDAFDKKLKVVRVMPNTPALVGEGMSALCPNEMVTEKDL 144
BC1             EVIKNDAIIVTIAAGKSIESTENAFNKKLKVVRVMPNTPALVGEGMSALCPNEMVTEKDL 144
P5CR            KVTKTNPITEPIPAGKSIESPENAFNKKVKVEKVRPNPPALVEKEMPALCPIERVTEKNL 140
EC              SSLNKDSLVVSIAAGITLDQLARALGHDRKIIRAMPNTPALVNAGMTSVTPNALVTPEDT 180
                .  :.: :  .*.** ::..   *:.:. *: :. **.****   *.:: *   ** ::

BT              EDVLNIFNSFGQTEIVSEKLMDVVTSVSGSSPAYVYMIIEAMADAAVLDGMPRNQAYKFA 204
BC2             EDVLNIFNSFGQTEIVSEKLMDVVTSVSGSSPAYVYMIIEAMADAAVLDGMPRNQAYKFA 204
BW              EDVLILFNSFGQTEIVSEKLMDVVTSVSGSSPAYVYMIIEAMADAAVLDGMPRNQAYKFA 204
BC1             EDVLNIFNSFGQSEIVSEKLMDVVTSVSGSSPAYVYMIIEAMADAAVLDGMPRNQAYKFA 204
P5CR            KNVLTFSNSLGQPKIVSKKLRKVVPPVRGPPPPNVYIIIKPMANPAVLNGMPRNQPYKPP 200
EC              ADVLNIFRCFGEAEVIAEPMIHPVVGVSGSSPAYVFMFIEAMADAAVLGGMPRAQAYKFA 240
                 :** : ..:*:.::::: : . *  * *..*. *:::*:.**:.***.**** *.** .

BT              AQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAVATLEEKGLRTAIISAMQRCTQKSV 264
BC2             AQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAVATLEEKGLRTAIISAMQRCTQKSV 264
BW              AQAVLGSAKMVLETGMHPGALKDMVCSPGGTTIEAVATLEEKGLRTAIISAMQRCTQKSV 264
BC1             AQAVLGSAKMVLETGIHPGELKDMVCSPGGTTIEAVATLEEKGLRTAIISAMQRCTQKSV 264
P5CR            AQPVLGSAKMVLKPGFLPGELKNMVGPPGEPTLKPVPPLKEKGLKYTIYSTYPPLSAKAC 260
EC              AQAVMGSAKMVLETGEHPGALKDMVCSPGGTTIEAVRVLEEKGFRAAVIEAMTKCMEKSE 300
                **.*:*******:.*  ** **:** .** .*::.*  *:***:: :: .:      *:

BT              ELSGQTKK 272
BC2             ELSGQTKK 272
BW              ELSGQTKK 272
BC1             ELSGQTKK 272
P5CR            --------
EC              KLSKS--- 305
```

Figure 3.48 The CLUSTAL W alignment of the deduced amino acid sequence of P5CR from various sources

BT= *Bacillus thuringiensis* ATCC 35646 (ZP_00744228.1), BC2 = *Bacillus cereus* ATCC 14579 (NP_832723.1), BW = *Bacillus weihenstephanensis* KBAB4 ZP_01186549.1|, BC1 = *Bacillus cereus* ATCC 10987 (NP_979334.1), P5CR = *Bacillus cereus* ATCC 11778 and EC = *Escherichia coli* CFT073 (NP_752427.1). Conserved residues are indicated by asterisks (*). (:) means amino acids which have same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

3.16 Optimization of the *p5cr* gene expression

The transformant No.6 (pET-P5CR) were grown and induced by IPTG at each of the following final concentrations 0, 0.1, 0.2, 0.4 and 0.8 mM. At 0, 1, 2, 4, 6, 8, 16, and 24 hours after induction, the cells were harvested and the P5CR activity in crude extract was assayed as described in 2.10. The results were shown in Figure 3.47. When the recombinant clone was cultured without IPTG induction, the total activity was increased until stable at 8 hours after induction. The specific activity was increased after 0 to 4 hours after induction and slightly increased after 4 to 24 hours after induction.

After induction with 0.2 mM IPTG for 8 hours, the maximal specific activity was 19.5 times (8.8 U/mg) higher than that in the without of IPTG for 24 hours. The activity was highest at induction time of 8 hours after induction when 0.2 mM IPTG was used. Highest activity was also found at 8, 6 and 6 hours after induction were found when 0.1, 0.4 and 0.8 mM IPTG were used, respectively. However, the highest activity induced by 0.1, 0.4 and 0.8 mM IPTG was still lower than that of 0.2 mM at 8 hours after induction (Figure 3.49). Hence, the optimum condition for induction of the *p5cr* gene for further experiment was 0.2 mM IPTG at 8 hours after induction.

One point five milliliter of transformant No.6 culture induced with various concentrations of IPTG was harvested at various times and was subjected for protein pattern determination. Ten microliters of cell samples from crude extracts were seperated on 12.5% SDS-polyacrylamide gel. The results in Figure 3.50-3.54 showed that the intensity of a major protein band of about 26 kDa in cell and crude extracts from each harvested time was corresponded to the level of enzyme activity from its crude extract. The molecular weight of P5CR subunit as estimated by SDS-PAGE was 26,400 Da (Figure 3.55).

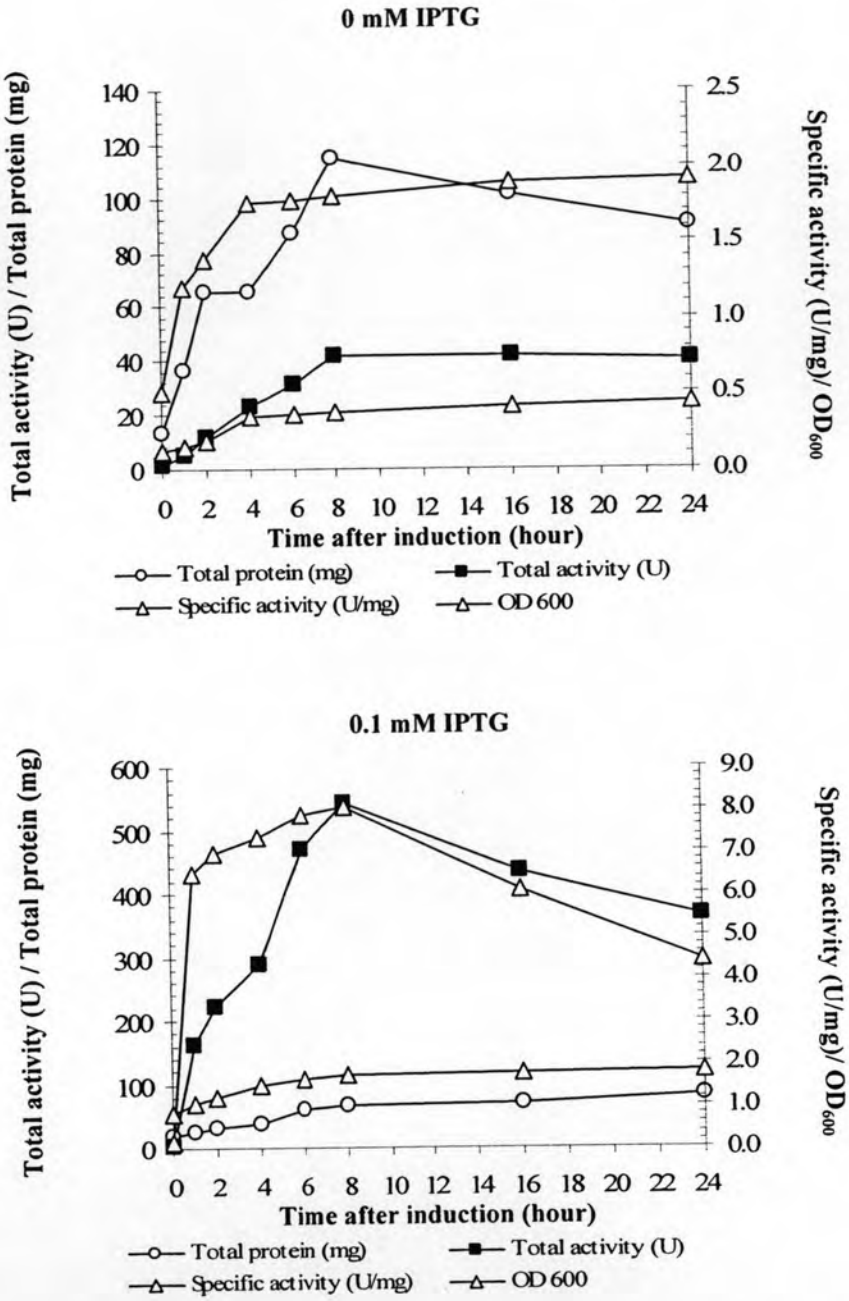


Figure 3.49 **P5CR activity in *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR after induction with various final concentrations of IPTG**

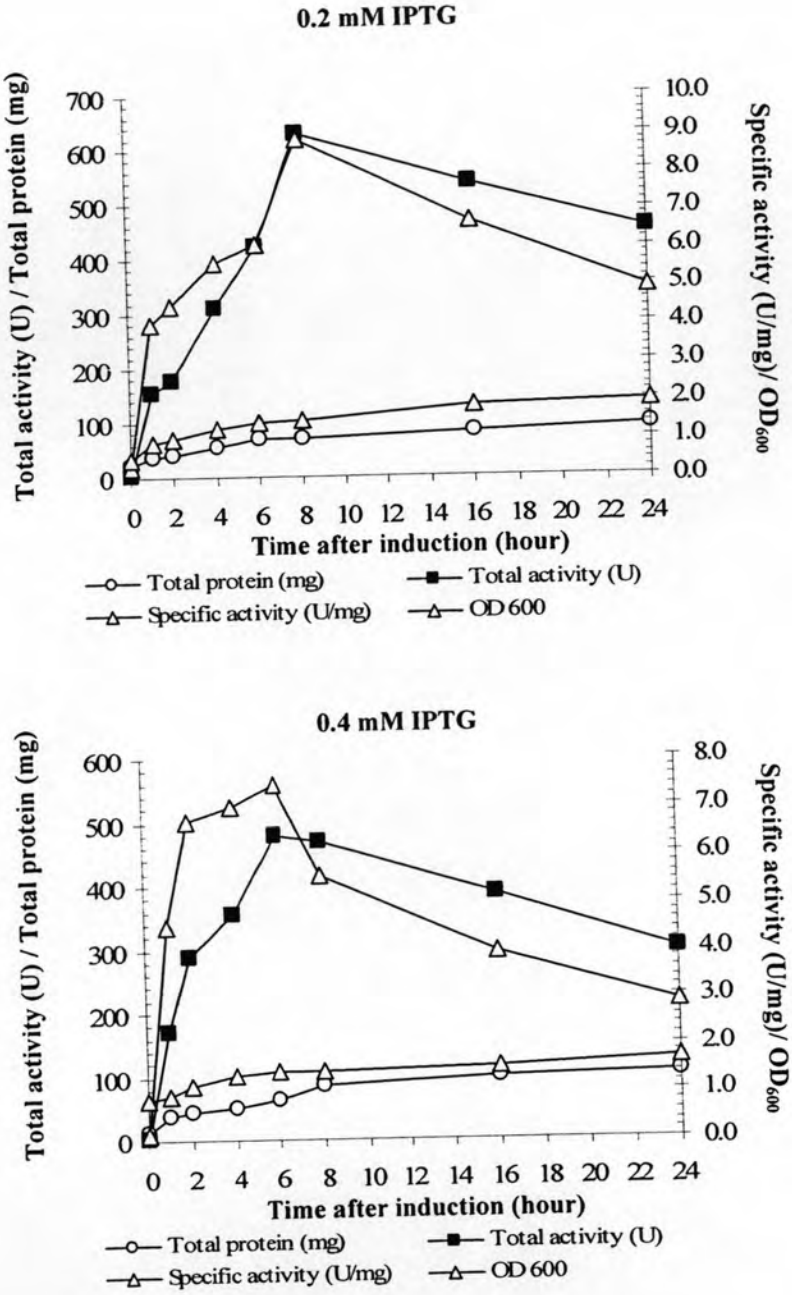


Figure 3.49 **P5CR activity in *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR after induction with various final concentrations of IPTG (continued)**

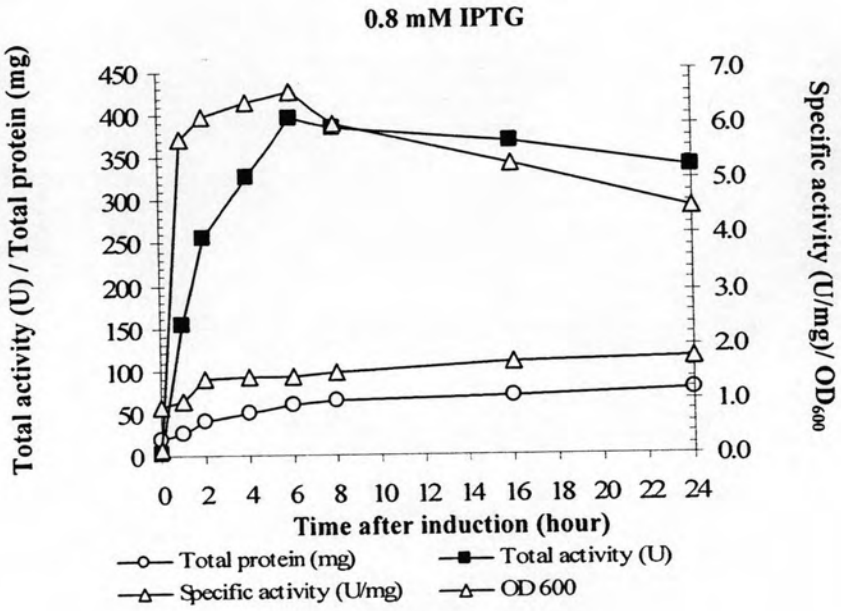


Figure 3.49 **P5CR activity in *E. coli* BL21(DE3) transformant No.6 containing pET-P5CR after induction with various final concentrations of IPTG (continued)**

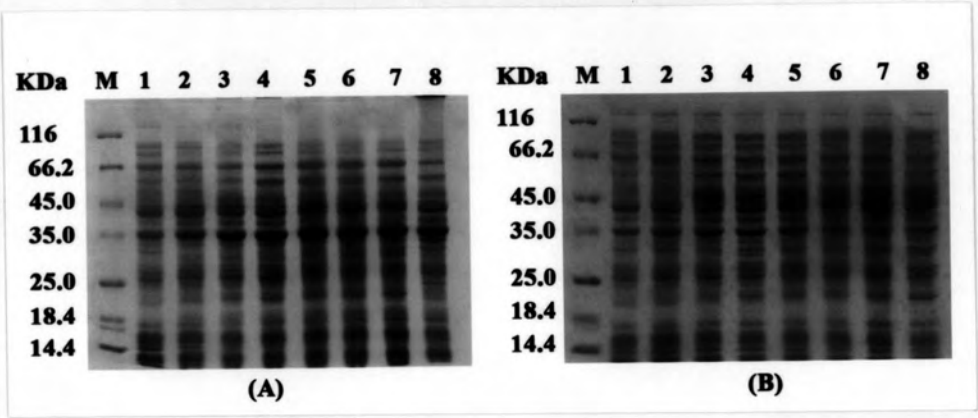


Figure 3.50 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21 (DE3) transformant No.6 containing pET-P5CR induced by 0 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-P5CR clone at 0, 1, 2, 4, 6, 8, 16 and 24 hours after induction, respectively

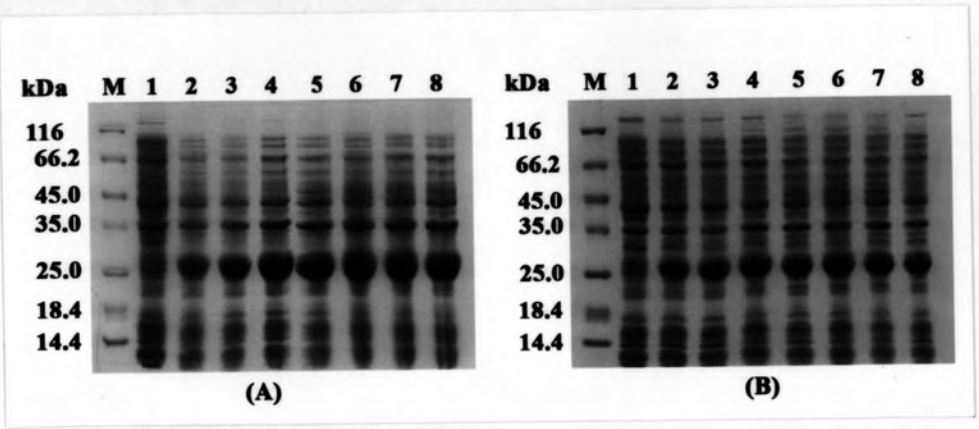


Figure 3.51 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21 (DE3) transformant No.6 containing pET-P5CR induced by 0.1 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-P5CR clone at 0, 1, 2, 4, 6, 8, 16 and 24 hours after induction, respectively

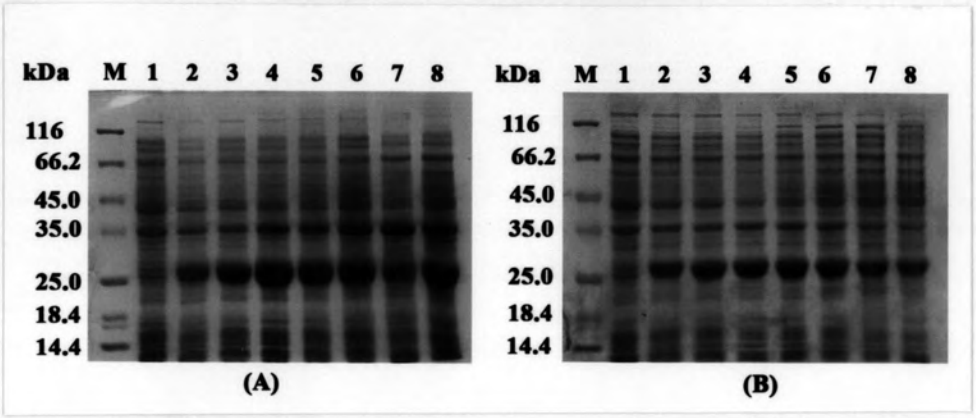


Figure 3.52 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21 (DE3) transformant No.6 containing pET-P5CR induced by 0.2 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-P5CR clone at 0, 1, 2, 4, 6, 8, 16 and 24 hours after induction, respectively

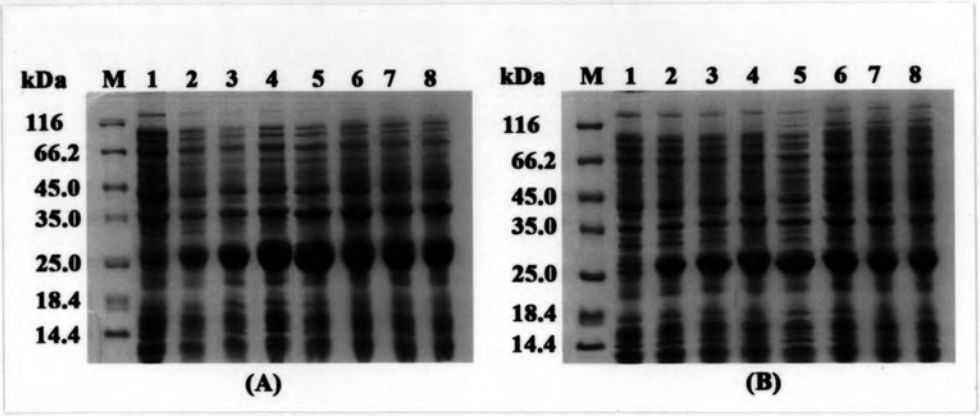


Figure 3.53 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21 (DE3) transformant No.6 containing pET-P5CR induced by 0.4 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-P5CR clone at 0, 1, 2, 4, 6, 8, 16 and 24 hours after induction, respectively

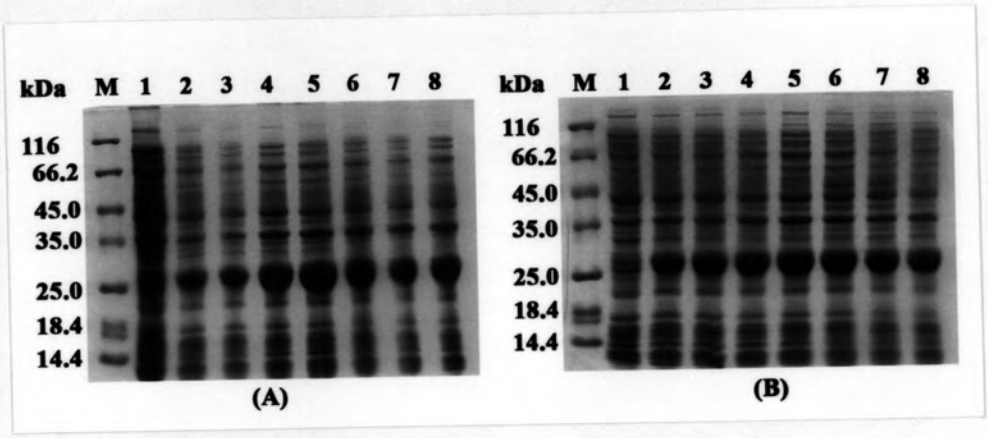


Figure 3.54 SDS-PAGE of whole cell and crude extract of the *E. coli* BL21 (DE3) transformant No.6 containing pET-P5CR induced by 0.8 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-P5CR clone at 0, 1, 2, 4, 6, 8, 16 and 24 hours after induction, respectively

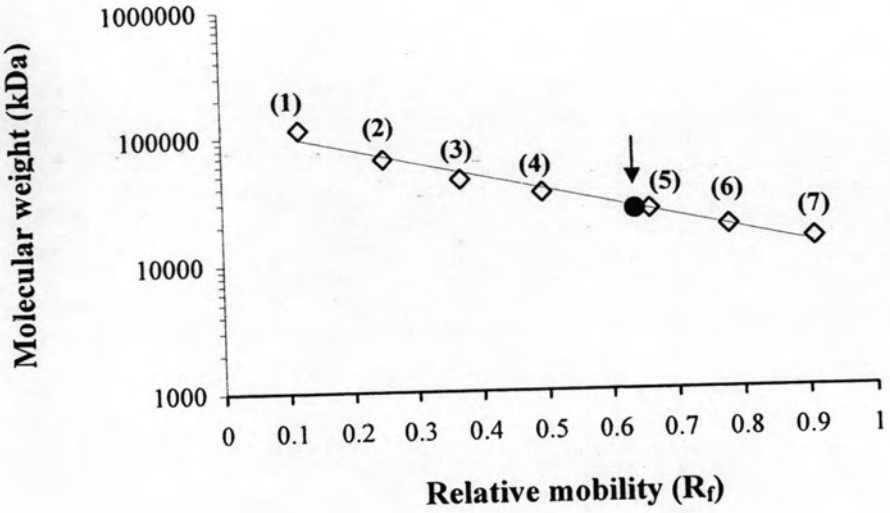


Figure 3.55 Calibration curve for molecular weight of P5CR by SDS-PAGE

(1) =	β-galactosidase	(MW 116,000 Da)
(2) =	Bovine serum albumin	(MW 66,200 Da)
(3) =	Ovalbumin	(MW 45,000 Da)
(4) =	Lactate dehydrogenase	(MW 35,000 Da)
(5) =	REase Bsp98I	(MW 25,000 Da)
(6) =	β-lactoglobulin	(MW 18,400 Da)
(7) =	Lysozyme	(MW 14,400 Da)

Arrow indicated the estimated molecular weight of P5CR

3.17 Cloning and expression of the *lys 6-dh* and *p5cr* heterologous genes in *E. coli* BL21(DE3) using pET-17b

3.17.1 Construction of pET-ADK-P5CR

To express the *lys 6-dh* and *p5cr* genes in *E. coli* under separated T7 promoters of expression vector pET-17b, the pET-ADK (containing the whole *lys 6-dh* gene with T7 promoter and Shine-Dargano sequence of pET vector) was digested by restriction enzymes *Bam*HI and *Eco*RI. Then 4.4-kb linear pET-ADK containing the *lys 6-dh* gene was recovered from agarose gel. Similary, the whole *p5cr* gene with T7 promoter and Shine-Dargano sequence (878 bp) excised from pET-P5CR was recovered from agarose gel after digestion with *Bgl*II and *Eco*RI (Figure 3.56). The 878 bp fragment of the *p5cr* gene with T7 promoter and Shine-Dargano sequence was then inserted at downstream of the *lys 6-dh* gene in pET-ADK. The obtained recombinant plasmid (pET-ADK-P5CR) with a size of 5.29 kb was transformed into *E. coli* BL21(DE3) by electroporation (Figure 3.57).

3.17.1 Recombinant plasmid characterization

One hundred transformants which could grow on LB plate containing 100 μg/ml ampicillin were randomly picked for plasmid extraction and digestion with each *Eco*RI or *Nde*I to screene for recombinant plasmids containing the heterologous genes as described in 2.27.2. After digestion with *Eco*RI, the 5.2-kb linear pET-17b with the *lys 6-dh* and *p5cr* gene insert fragment was obtained while after digestion with *Nde*I, the 4-kb linear pET-17b and 1.2-kb of *p5cr* and *lys 6-dh* insert were obtained, respectively as shown in Figure 3.58. Twelve transformants harboured recombinant plasmid (5.2 kb) which contain both the *lys 6-dh* and *p5cr* genes were assayed for Lys 6-DH and P5CR activity.

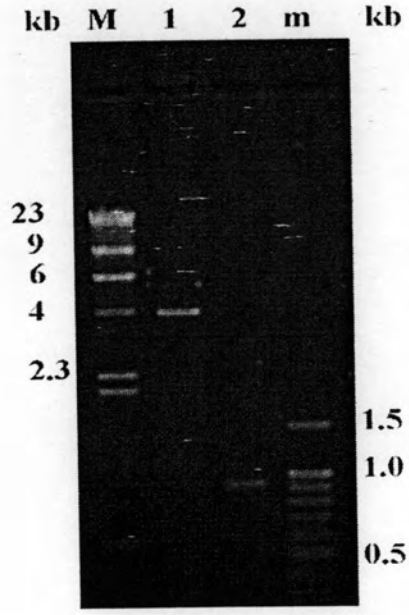


Figure 3.56 The *lys 6-dh* gene in pET-ADK and *p5cr* gene fragment

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

Lane 1 = Linear pET-ADK containing the *lys 6-dh* gene after digested with *Bam*HI and *Eco*RI

Lane 2 = The *p5cr* gene fragment after digested with *Bgl*II and *Eco*RI

Lane m = 100 bp DNA marker

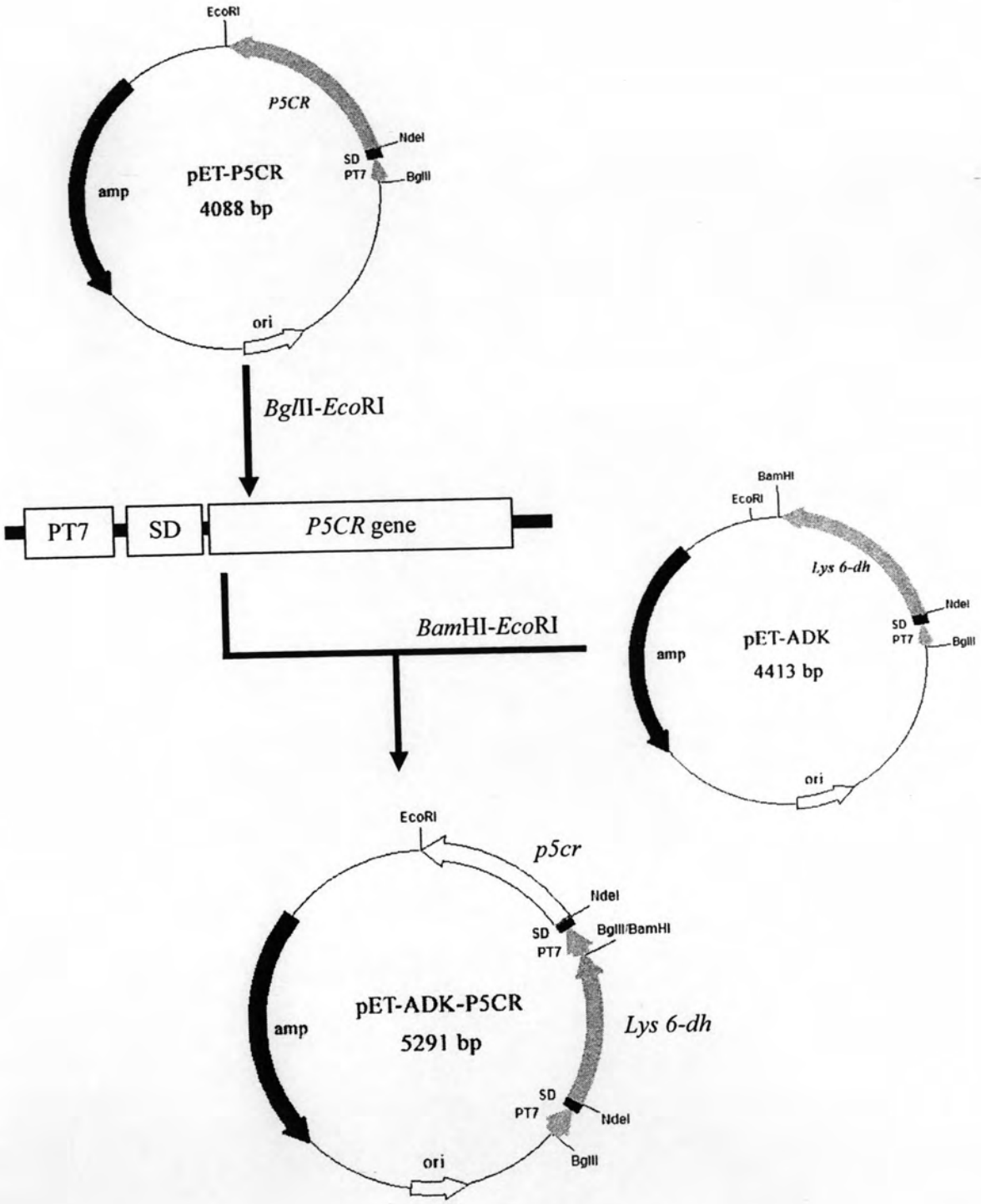


Figure 3.57 Construction of pET-ADK-P5CR

3.17.2 Heterologous activity of transformants

Twelve recombinant clones were grown and the crude extracts were prepared as described in 2.18.2. The activity of Lys 6-DH and P5CR were assayed using these crude extracts. Crude extracts from *E. coli* BL21 (DE3) with and without plasmid pET-17b were used as control. The result was shown in Table 3.14. The transformants showed various levels of the specific activity of Lys 6-DH and P5CR, ranging from 0.27-0.75 and 0.10-0.54 units/mg protein, respectively. Transformant no. 4 showed the highest specific activity of Lys 6-DH (0.75 units/mg protein) and P5CR (0.54 units/mg protein) which were 25 and 11 fold higher than that of *A. denitrificans* K-1 and *B. cereus* ATCC 11778 wild type, respectively. However, the specific activity of Lys 6-DH and P5CR in heterologous transformant No. 4 were decreased 2.4 .and 8.8 fold, respectively when compared with those from pET-ADK transformant No.4 and pET-P5CR transformant No. 6. Nevertheless, the heterologous transformant No. 4 was used in the further experiments.

3.18 Optimization of the *lys 6-dh* and *p5cr* gene expression

The transformant No.4 (pET-ADK-P5CR) was grown and induced by IPTG at each of the following final concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 mM. At 0, 30 min, 1, 2, 3, 4, 6 and 8 hours after induction. The cells were harvested and crude extract were prepared for Lys 6-DH and P5CR activity assay as described in 2.28. The results were showed in Figure 3.59.

When the recombinant clone was cultured without IPTG induction, the activity of Lys 6-DH was slightly increased and reached its maximum at 3 hours after induction with a specific activity of 0.54 units/mg protein while P5CR specific activity never raised above 0.03 units/mg protein.

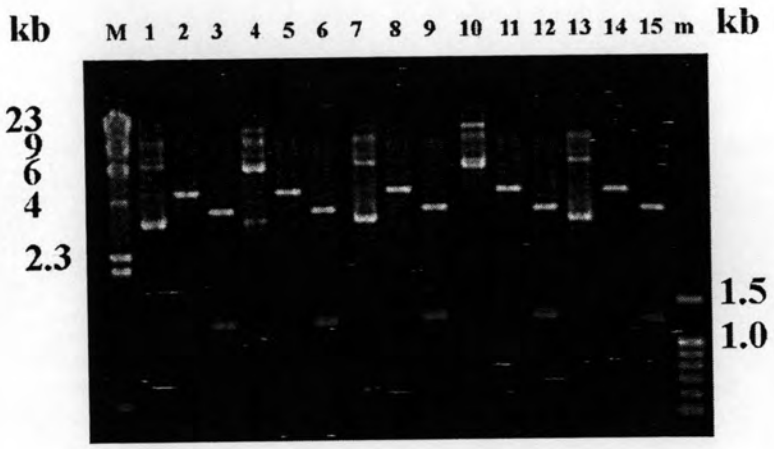


Figure 3.58 Restriction pattern of pET-P5CR from selected recombinant clones

Lane M = λ/*Hin*dIII standard DNA marker
Lane 1 = undigested plasmid
Lane 2 = *Eco*RI digested plasmid
Lane 3 = *Nde*I digested plasmid
Lane 4 = undigested plasmid
Lane 5 = *Eco*RI digested plasmid
Lane 6 = *Nde*I digested plasmid
Lane 7 = undigested plasmid
Lane 8 = *Eco*RI digested plasmid
Lane 9 = *Nde*I digested plasmid
Lane 10 = undigested plasmid
Lane 11 = *Eco*RI digested plasmid
Lane 12 = *Nde*I digested plasmid
Lane 13 = undigested plasmid
Lane 14 = *Eco*RI digested plasmid
Lane 15 = *Nde*I digested plasmid

Table 3.14 Lys 6-DH and P5CR activity from crude extracts of selected *E. coli* BL21(DE3) transformants harboring pET-ADK-P5CR[a]

Sources	Total activity (U)		Total protein (mg)	Specific activity (U/mg protein)	
	Lys 6-DH	P5CR		Lys 6-DH	P5CR
E. coli BL21(DE3)	0	0	92	0.00	0.00
E. coli BL21(DE3) harbouring pET-17b	0	0	124	0.00	0.00
A. denitrificans K-1	3.35	0	105	0.03	0.00
B. cereus ATCC 11778	0	6	116	0.00	0.05
E. coli pET-ADK No.4	278	0	144	1.93	0.00
E. coli pET-P5CR No. 6	0	266	56	0.00	4.75
Transformant No.1	40	16	96	0.42	0.17
Transformant No.2	120	32	158	0.76	0.20
Transformant No.3	110	42	190	0.58	0.22
Transformant No.4	**130**	**89**	**166**	**0.78**	**0.54**
Transformant No.5	60	12	224	0.27	0.05
Transformant No.6	82	30	124	0.66	0.24
Transformant No.7	30	54	110	0.27	0.49
Transformant No.8	84	28	204	0.41	0.14
Transformant No.9	94	56	134	0.70	0.42
Transformant No.10	79	92	172	0.46	0.53
Transformant No.11	42	66	152	0.28	0.43
Transformant No.12	66	21	208	0.32	0.10

[a] Crude extracts were prepared from 200 ml of cell culture after induction with 0.1 mM IPTG for 4 hours.

After induction of gene expression with various final concentrations of IPTG (0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 mM). The highest specific activity was observed at 3 hours after induction using 0.1 mM IPTG with 1.6 and 42 fold of Lys 6-DH and P5CR specific activity. Moreover, induction with IPTG at the final concentrations of 0.025 and 0.3 mM showed the highest activity of Lys 6-DH at 6 hours after induction while the highest activity of Lys 6-DH at the IPTG final concentrations of 0.05 and 0.2 mM were 4 hours after induction. In addition, the highest P5CR activity was detected at 6 hours after induction with 0.025 mM while the highest P5CR activity was detected at 2 hours after induction with 0.05 and 0.2 mM IPTG. When induced with 0.3 and 0.4 mM, the highest P5CR activity was detected at 5 hours after induction. However the level of specific activity when induced with other final concentration of IPTG was lower than that of induction with 0.1 mM at 3 hours after induction. Hence, the optimum condition for induction of both *lys 6-dh* and *p5cr* genes were 0.1 mM IPTG at 3 hours after induction. The obtained specific activity of Lys 6-DH and P5CR were 0.86 and 1.24 units/mg protein, respectively.

3.18.1 Protein pattern of cells and crude extracts

One point five milliliter of transformant No.4 culture induced with various concentrations of IPTG was harvested at various times and was subjected for protein pattern determination. Ten microliters of cell samples or 20 μg proteins from crude extracts were seperated on 12.5% SDS-polyacrylamide gel. The results in Figure 3.60-3.66 showed that the intensity of a major protein band of about 40 kDa in cell and crude extracts from each harvested time was corresponded to the level of Lys 6-DH activity from its crude extract. However, the protein band of P5CR at the molecular size of 26 kDa was not found as the major band in all analysis.

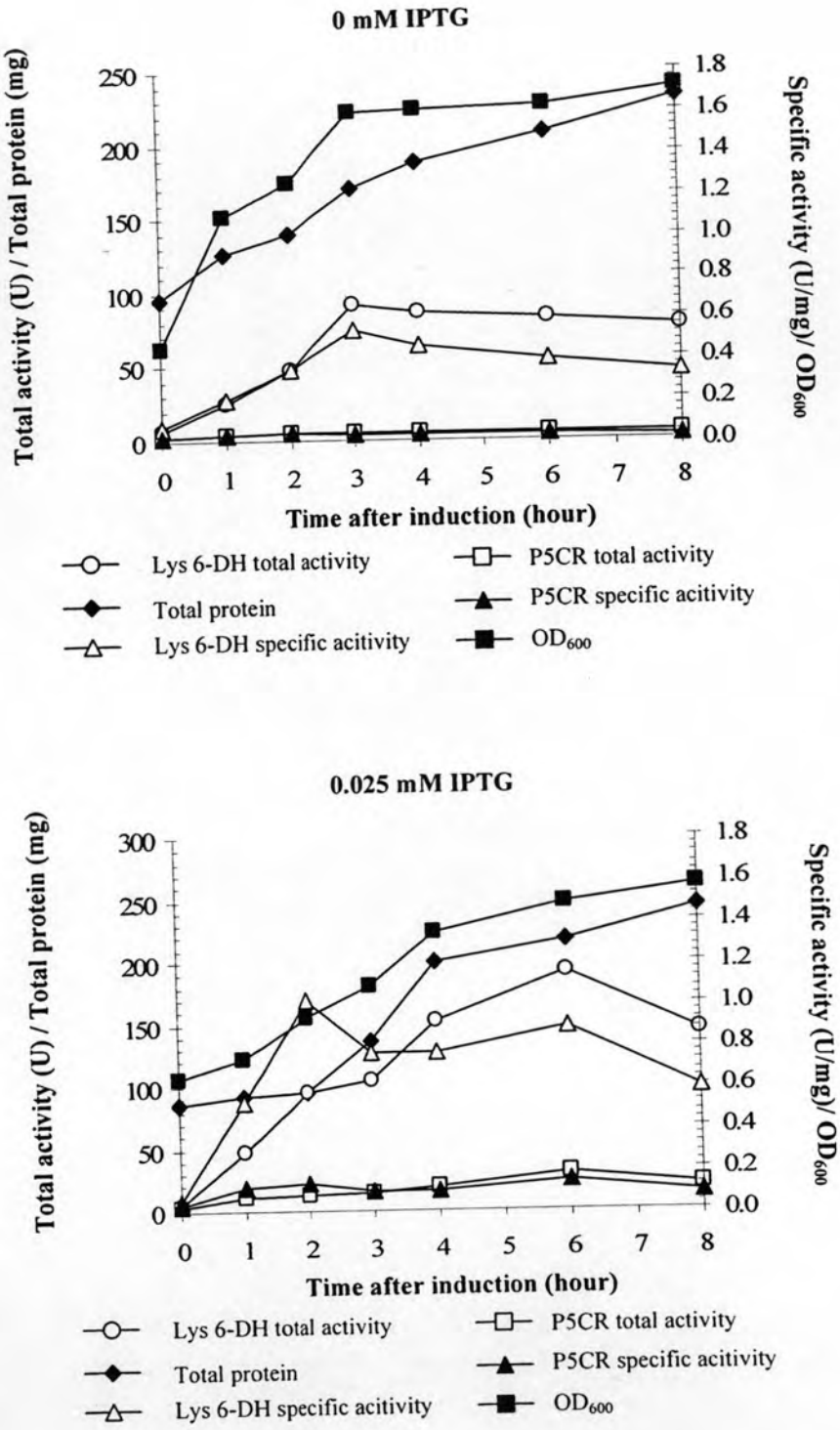
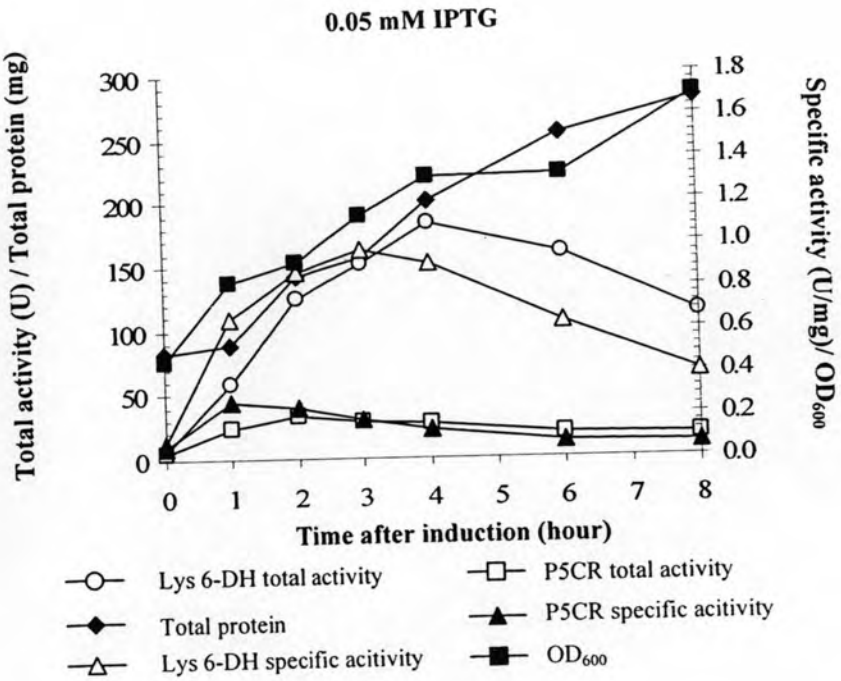


Figure 3.59 Lys 6-DH and P5CR activities in *E. coli* BL21(DE3) transformant No.4 after induction with various final concentrations of IPTG



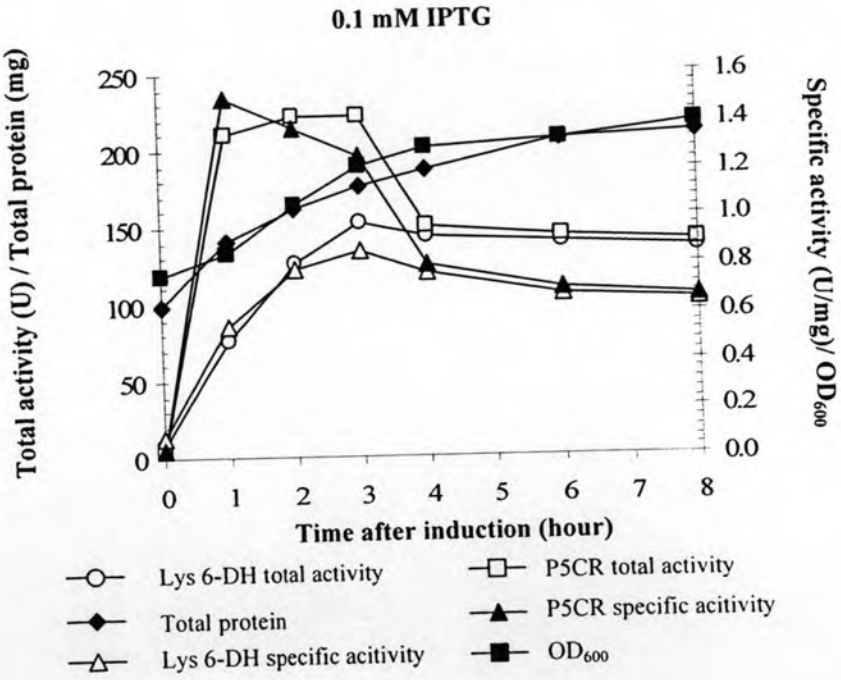


Figure 3.59 **Lys 6-DH and P5CR activities in *E. coli* BL21(DE3) transformant No.4 after induction with various final concentrations of IPTG (continued)**

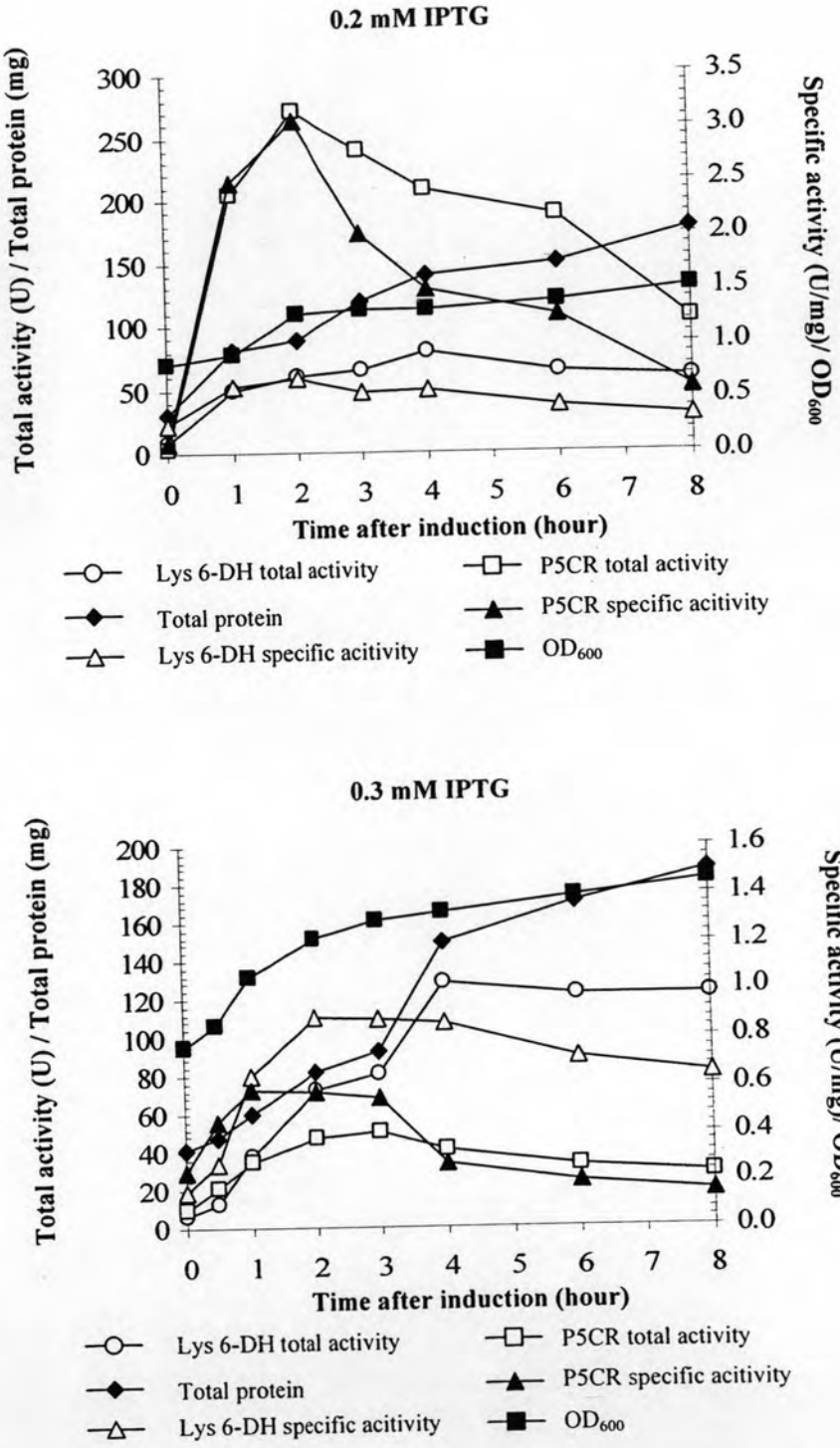


Figure 3.59 **Lys 6-DH and P5CR activities in *E. coli* BL21(DE3) transformant No.4 after induction with various final concentrations of IPTG (continued)**

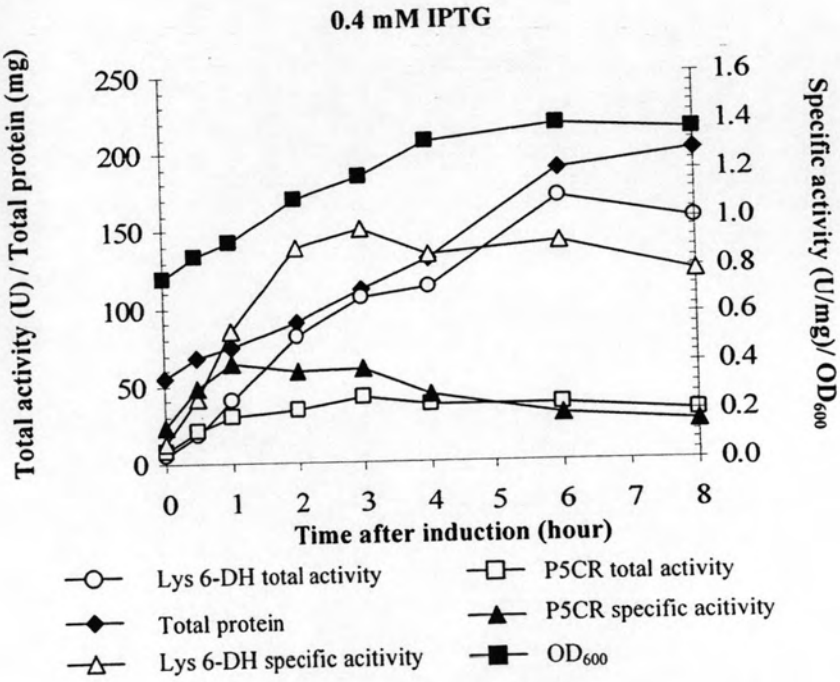


Figure 3.59 **Lys 6-DH and P5CR activities in *E. coli* BL21(DE3) transformant No.4 after induction with various final concentrations of IPTG (continued)**

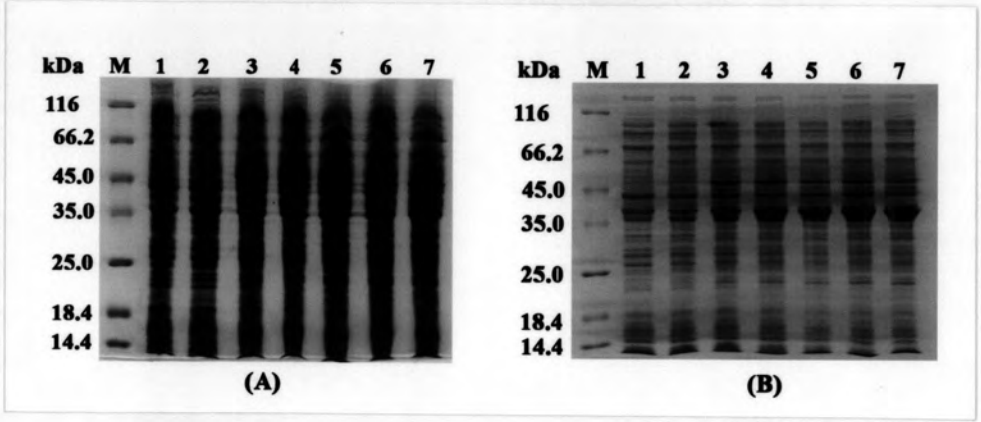


Figure 3.60 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-ADK-P5CR clone at 0, 1, 2, 3, 4, 6 and 8 hours after induction, respectively

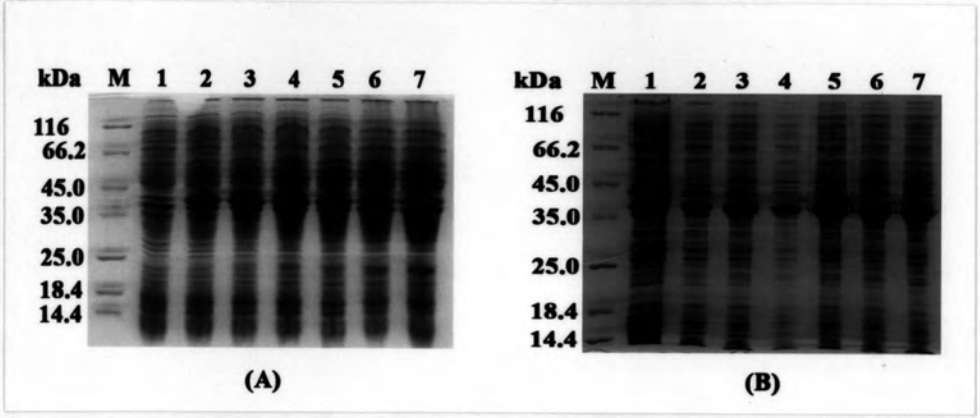


Figure 3.61 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.025 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-ADK-P5CR clone at 0, 1, 2, 3, 4, 6 and 8 hours after induction, respectively

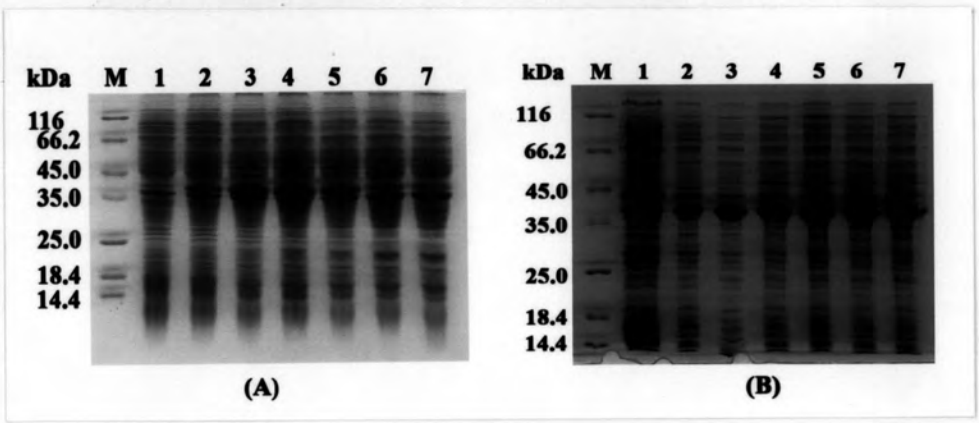


Figure 3.62 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.05 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-ADK-P5CR clone at 0, 1, 2, 3, 4, 6 and 8 hours after induction, respectively

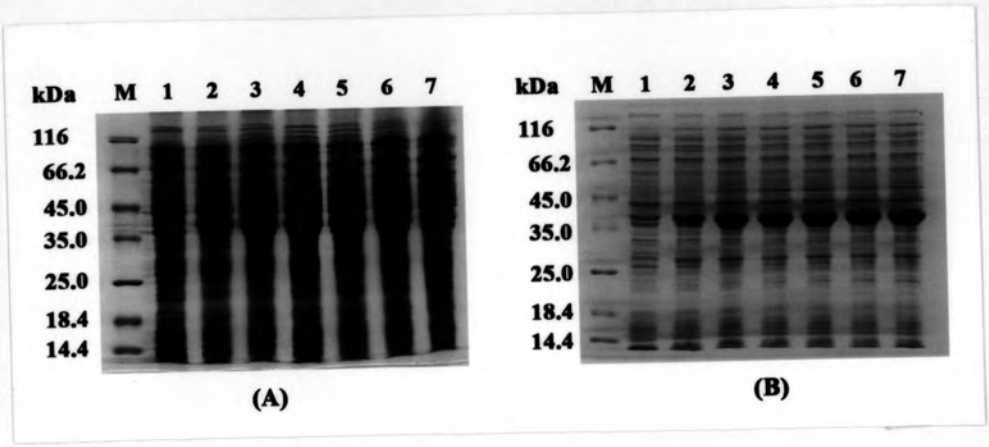


Figure 3.63 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.1 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-ADK-P5CR clone at 0, 1, 2, 3, 4, 6 and 8 hours after induction, respectively

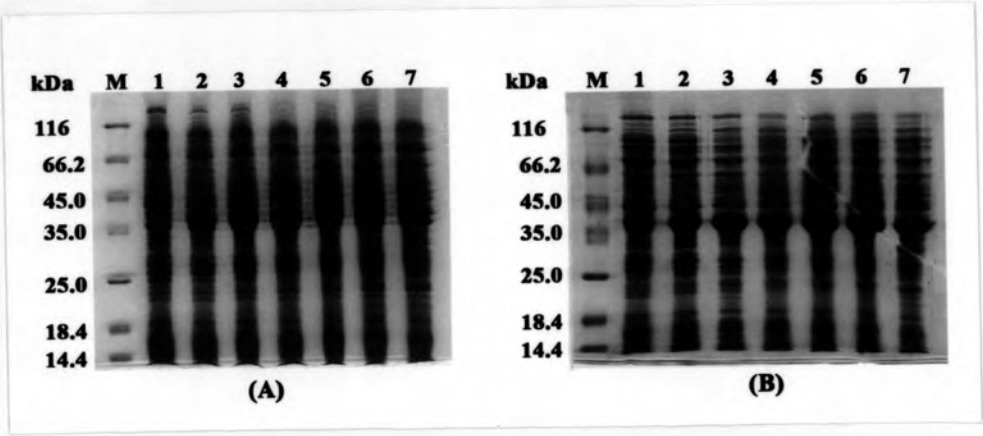


Figure 3.64 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.2 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-ADK-P5CR clone at 0, 1, 2, 3, 4, 6 and 8 hours after induction, respectively

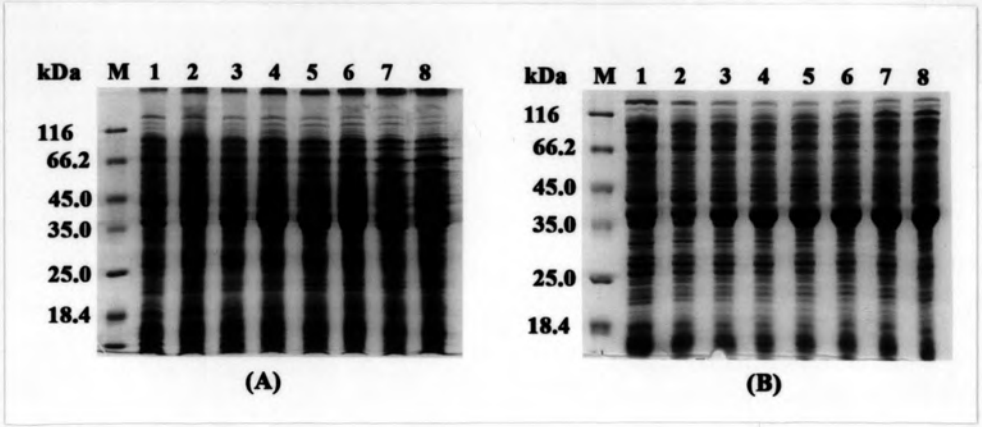


Figure 3.65 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.3 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-7 = cell and crude extract of pET-ADK-P5CR clone at 0, 30 min.,1, 2, 3, 4, 6 and 8 hours after induction, respectively

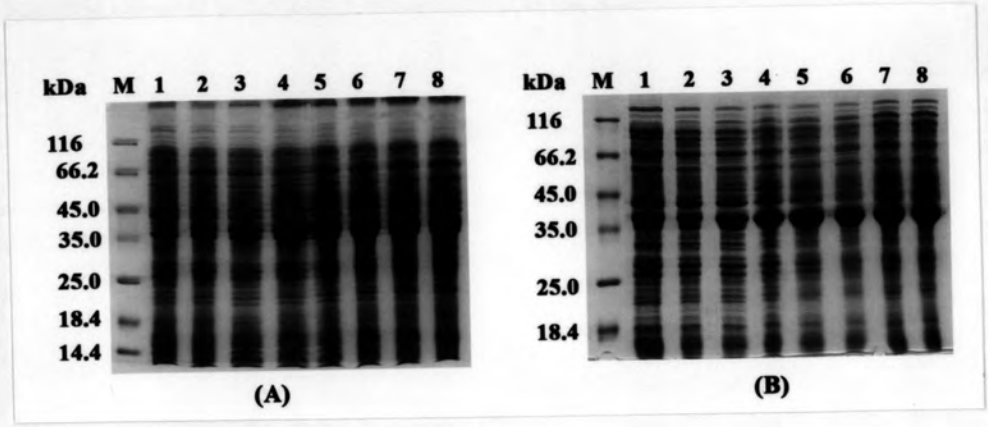


Figure 3.66 SDS-PAGE of whole cell and crude extract of the *E. coli* BL 21 (DE3) transformant No.4 containing pET-ADK-P5CR induced by 0.4 mM IPTG at various times

(A): whole cell

(B): crude extract

Lane M = protein marker

Lane 1-8 = cell and crude extract of pET-ADK-P5CR clone at 0, 30 min.,1, 2, 3, 4, 6 and 8 hours after induction, respectively

3.19 Production L-pipecolic acid

3.19.1 The L-pipecolic acid production from *E. coli* BL 21 (DE3) harboring pET-ADK-P5CR

E. coli BL21(DE3) harbouring pET-ADK-P5CR was used to produce L-pipecolic acid. In an attempt to increase cell permeability for L-lysine uptake into the cell, ten different organic solvents including hexane, methanol, ethanol, pyridine, xylene, toluene, isopropyl alcohol, acetone, chloroform and benzene were separately used to treat *E. coli* transformant using treating cell with distilled water as control. After *E. coli* transformant was grown in LB medium, the expression of both Lys 6-DH and P5CR were induced using optimum condition obtained from 3.19 (0.1 mM IPTG for 3 hours). Thirty milligram of cell pellet was treated in 100 µl of each organic solvent for variable periods of time (5, 10, 15, 20, 30, 45 and 60 minutes). After the cell had been treated, various organic solvents were removed. L-pipecolic production was started in 500 µl reaction consisted of 200 mM L-lysine, 200 mM glycine-KCl-KOH, pH 9.5 and distilled water as described in 2.29.1. Firstly, the time of L-pipecolic production was fixed at 24 hours. Then L-lysine and L-pipecolic acid from all samples were detected on TLC plate.

L-lysine and L-pipecolic acid can be detected on a TLC plate. The mobile phase consisted of butanol: acetic acid: distilled water (4:1:1) by volume. The relative motility (R_f) value of L-lysine and L-pipecolic were 0.04 and 0.3, respectively. The intensity of the spots was analyzed in comparison with that of the standard in each TLC plate. Figure 3.67-3.70 showed the effect of various organic solvents and various treated times on L-pipecolic acid production.

The hexane-treated cells showed an increasing the intensity of L-pipecolic acid spot from 5 to 30 min, after that the intensity was stable as shown in Figure 3.67. In contrast, when cells were treated with methanol, ethanol, pyridine (Figure 3.68) and isopropyl alcohol (Figure 3.69), L-pipecolic acid could not be detected.

Eventhough, acetone, chloroform and benzene treated cells (Figure 3.70) could produce L-pipecolic acid but the production was very low when compare to that of distilled water treated cells. In the Figure 3.69, toluene-treated cells could also produce L-pipecolic acid.

The best L-pipecolic acid production was from xylene-treated cells using the treated time of 5 to 10 minutes, which the intensity of L-pipecolic acid using Gel Document was 2.2 fold higher than that of distilled water-treated cells. When treated the cells longer than 10 minutes, the L-pipecolic acid production was decreased (Figure 3.69). Therefore, xylene treated cell for 5 min would be further used to produce L-pipecolic acid in the next experiments.

3.19.2 The effect of pH on production of L-pipecolic acid from pET-ADK-P5CR clone

Because of the first experiment was done by using glycine-KCl-KOH, pH 9.5 as the buffer, glycine in the buffer was also detected on TLC plate closed to L-lysine. To avoid this interference, another buffer without glycine should be used. Moreover, the effect of pH on L-pipecolic acid production was also performed by using glycine-KCl-KOH (pH 9.5), KPB (pH 7.0), Tris-HCl (pH 7.0) and Tris-HCl (pH 9.0) as buffers and distilled water as control for L-pipecolic acid production. The results were shown in Figure 3.71. The best buffer of this experiment was Tris-HCl, pH 9.0 which gave more L-pipecolic acid yield than that of glycine-KCl-KOH, pH 9.5 and did not interfere with L-lysine detection on TLC. While KPB pH 7.0 and Tris-HCl, pH 7.0 gave the same results with that of water. In contrast, a little amount of L-pipecolic acid was found in all reactions from the *E. coli* BL21(DE3) harbouring pET-ADK. From this result, Tris-HCl, pH 9.0 was used for the next experiment.

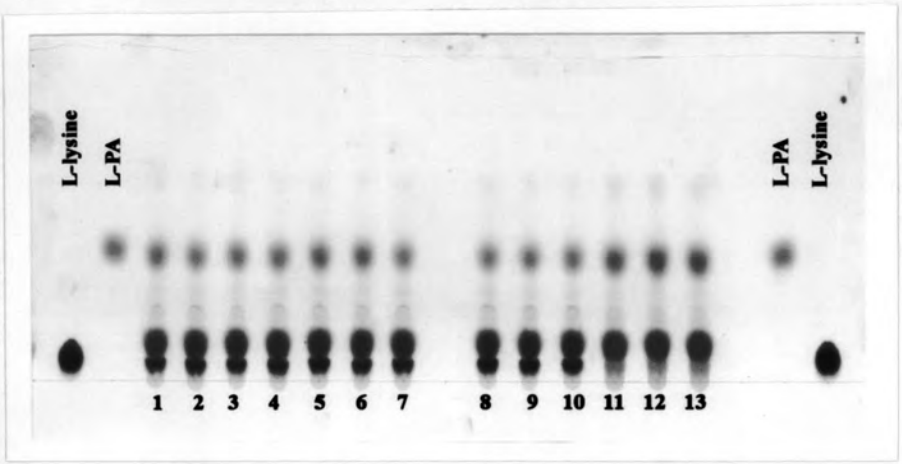


Figure 3.67 Effect of organic solvents an incubation time on permeability and L-pipecolic acid production of *E. coli* BL21 (DE3) harboring pET-ADK-P5CR[a]

Lane 1-7 = Treated cell with distilled water for 5, 10, 15, 20, 30, 45 and 60 min, respectively

Lane 8-13 = Treated cell with hexane for 5, 10, 20, 30, 45 and 60 min, respectively

[a] The 0.5 µl of sample after 24 hours of production time in each organic solvents and various incubation times were spot on TLC and detected by 0.5% ninhydrin in acetone.

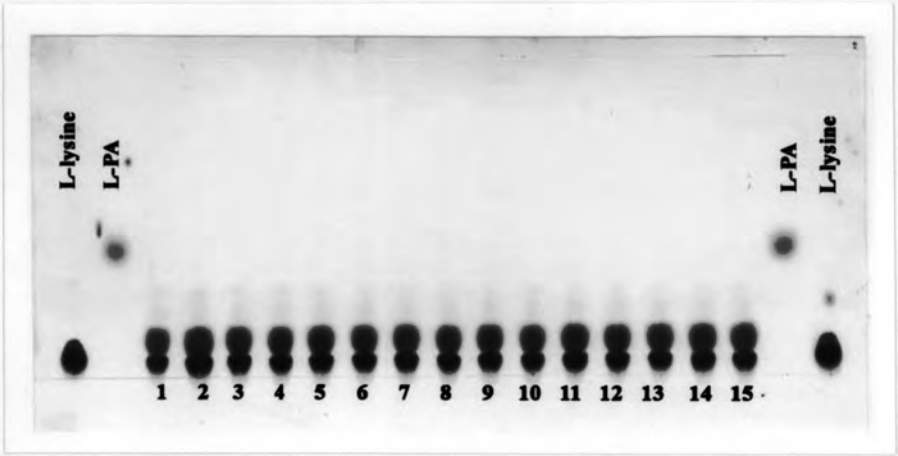


Figure 3.68 **Effect of organic solvents an incubation time on permeability and L-pipecolic acid production of *E. coli* BL21 (DE3) harboring pET-ADK-P5CR[a]**

Lane 1-5 = Treated cell with methanol for 5, 10, 15, 20 and 30 min, respectively

Lane 6-10 = Treated cell with ethanol for 5, 10, 15, 20 and 30 min, respectively

Lane 11-15 = Treated cell with pyridine for 5, 10, 15, 20 and 30 min, respectively

[a] The 0.5 µl of sample after 24 hours of production time in each organic solvents and various incubation times were spot on TLC and detected by 0.5% ninhydrin in acetone.

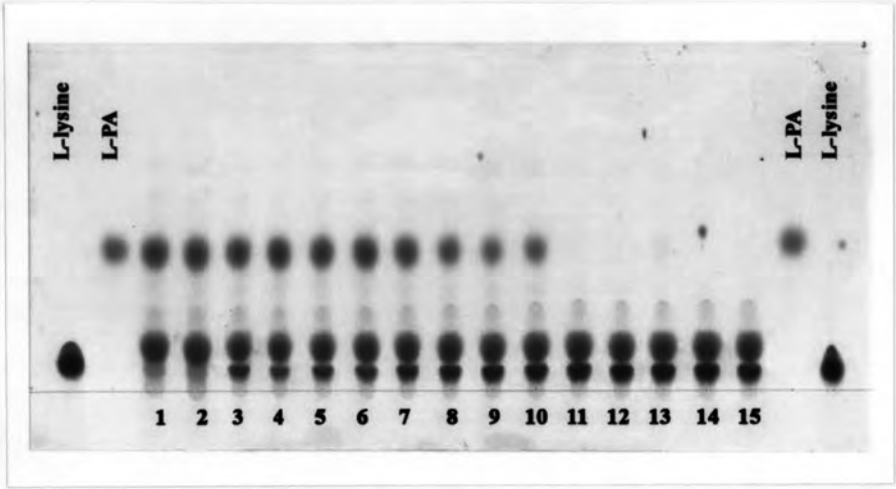


Figure 3.69 **Effect of organic solvents an incubation time on permeability and L-pipecolic acid production of *E. coli* BL21 (DE3) harboring pET-ADK-P5CR[a]**

Lane 1-5 = Treated cell with xylene for 5, 10, 15, 20 and 30 min, respectively

Lane 6-10 = Treated cell with toluene for 5, 10, 15, 20 and 30 min, respectively

Lane 11-15 = Treated cell with isopropyl alcohol for 5, 10, 15, 20 and 30 min, respectively

[a] The 0.5 µl of sample after 24 hours of production time in each organic solvents and various incubation times were spot on TLC and detected by 0.5% ninhydrin in acetone.

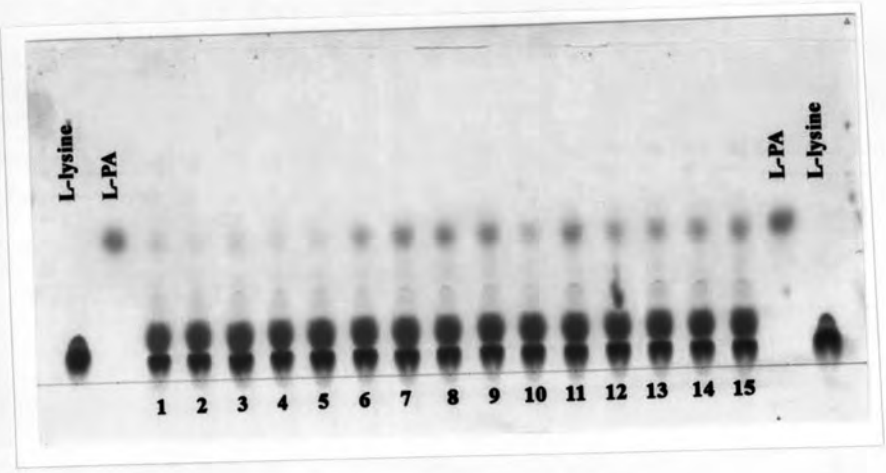


Figure 3.70 Effect of organic solvents an incubation time on permeability and L-pipecolic acid production of *E. coli* BL21 (DE3) harboring pET-ADK-P5CR[a]

Lane 1-5 = Treated cell by acetone for 5, 10, 15, 20 and 30 min, respectively

Lane 6-10 = Treated cell by chloroform for 5, 10, 15, 20 and 30 min, respectively

Lane 11-15 = Treated cell by benzene for 5, 10, 15, 20 and 30 min, respectively

[a] The 0.5 µl of sample after 24 hours of production time in each organic solvents and various incubation times were spot on TLC and detected by 0.5% ninhydrin in acetone.

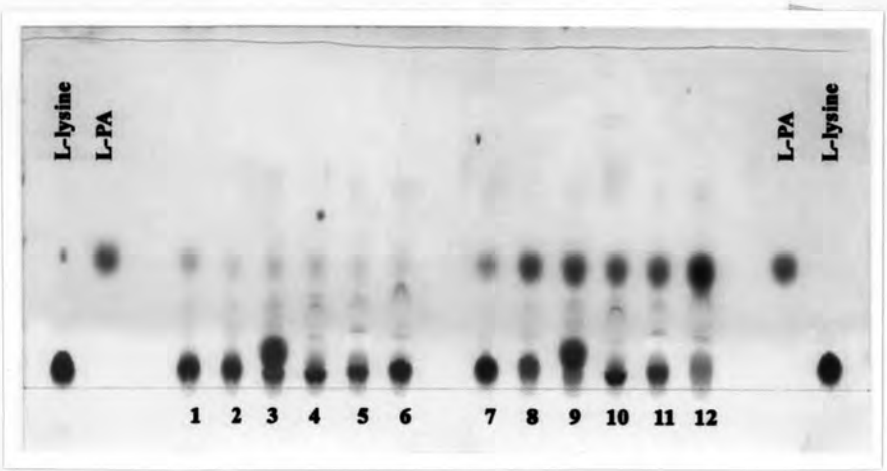


Figure 3.71 TLC analysis of effect of pH on L-pipecolic acid production by pET-ADK-P5CR clone[a]

Lane 1 = pET-ADK non-treated cell[b]

Lane 2 = pET-ADK treated cell with distilled water for 5 min[b]

Lane 3-6 = pET-ADK treated cell with xylene for 5 min[c]

Lane 7 = pET-ADK-P5CR non-treated cell[b]

Lane 8 = pET-ADK-P5CR treated cell with distilled water for 5 min[b]

Lane 9-12 = pET-ADK-P5CR treated cell with xylene for 5 min[c]

[a] The 0.5 µl of sample after the production time for 24 hours in all treated cells were spot on TLC and detected by 0.5% ninhydrin in acetone.

[b] The water was used for L-pipecolic acid production.

[c] The Glycine-KCl-KOH (pH 9.5), KPB (pH 7.0), Tris-HCl (pH 7.0) and Tris-HCl (pH 9.0) were used as buffer for L-pipecolic acid production, respectively.

3.19.3 The optimization of L-pipecolic acid production from pET-ADK-P5CR clone

To study the production time for maximum L-pipecolic acid production, the *E. coli* BL21 (DE3) harbouring pET-ADK-P5CR was treated by xylene for 5 min and used Tris-HCl (pH 9.0) as buffer as described in 2.29.3. After that the various production times for the L-pipecolic acid production were determined. The results were shown in Figure 3.72. L-Pipecolic acid was slightly increased after 1 hour and reaching the maximum production at 24 hours of production time.

The amount of L-pipecolic acid in each production time from 0 to 48 hours was determined by ELSD-HPLC using C_{18} reverse phase column. The retention time of L-lysine and L-pipecolic acid were around 5.4-5.6 and 13-13.4 minutes, respectively (Appendix N). The concentration of L-lysine and L-pipecolic acid in each production time was calculated by compared with L-lysine and L-pipecolic acid standard curves (Appendix O). The amount of L-pipecolic acid could be detected after 1 hour of production time, and then slightly increased to the maximum amount of 77 mM (9.9 g/L) with 32.5 % yield of L-lysine converted to L-pipecolic acid after 24 hours production time (Figure 3.73).

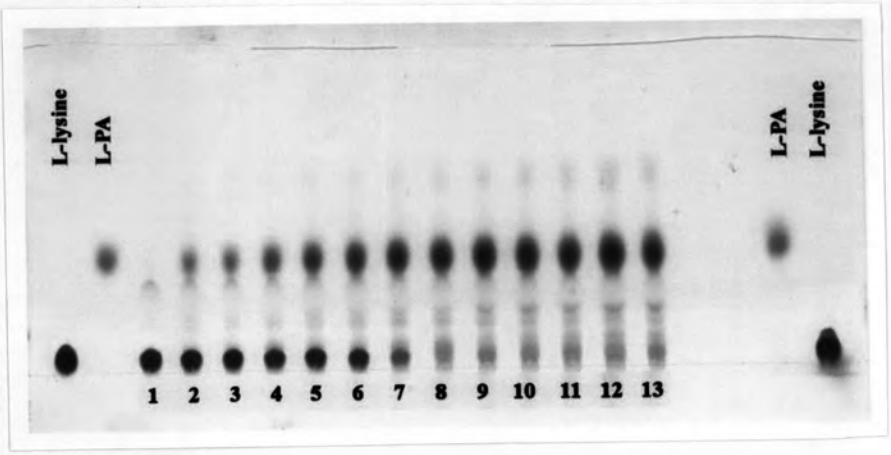


Figure 3.72 TLC analysis of optimization of L-pipecolic acid production by pET-ADK-P5CR clone [a]

Lane 1-13 = The production time were 0, 1, 2, 4, 6, 8, 12, 16, 24, 30, 36, 42 and 48 hours, respectively.

[a] Treated cell by xylene for 5 min and using Tris-HCl (pH 9.0) for L-pipecolic production. The 0.5 µl of sample after the production time for 24 hours in all treated cells were spot on TLC and detected by 0.5% ninhydrin in acetone.

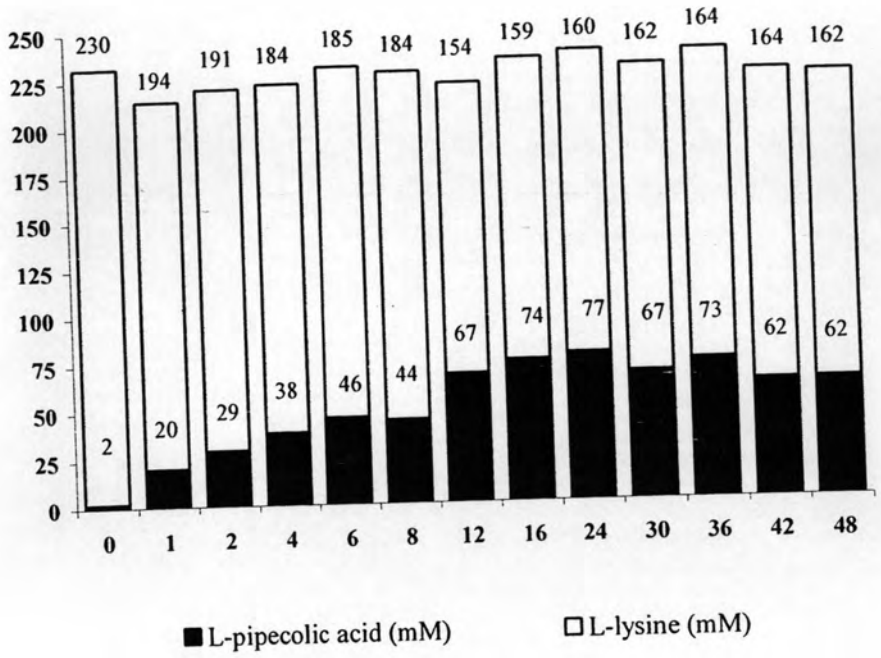


Figure 3.73 Concentration of L-lysine and L-pipecolic acid from *E. coli* BL21 (DE3) harboring pET-ADK-P5CR at the end of various production times[a]

[a] Concentration of L-lysine and L-pipecolic acid was determined by ELSD-HPLC compared with L-lysine and L-pipecolic acid standard curves (Appendix O).