CHAPTER III

RESULTS

3.1 Amino acid sequence of phenylalanine dehydrogenase

Digestion of the purified PheDH from *Bacillus lentus* with lysyl endopeptidase and isolation of digested peptides by reversed-phase high-performance liquid chromatography (HPLC) were carried out as described in section 2.6. The HPLC profile of the separation is presented in Figure 3.1. The isolated peptides at retention time 27.4, 28.7 and 33.6 minutes were determined for their amino acid sequences. The internal peptide fragments were AVIIGDPQKD, AIAGSANFQLLTEDHGRQ LAD, and GILYAPDYIVNSGGLIQVADELYEVN, respectively.

The CLUSTAL W program was used for alignment of amino acid sequence of PheDH from various sources to indicate the position of each peptide fragment and to use as data for degenerated primer design in the next step. The alignment is shown in Figure 3.2.

3.2 Nucleotide sequencing of phenylalanine dehydrogenase gene

3.2.1 Chromosomal DNA Extraction

The chromosomal DNA was extracted from *Bacillus lentus* according to modified method of Frederick *et al.* (1995). After chromosomal DNA had been achieved, it was run on agarose gel electrophoresis to examine its quality and quantity. It was found that extracted DNA had molecular weight over 23.1 kb and shown high purity which corresponded with its A_{260}/A_{280} ratio of 1.8-2.0. The DNA concentration was about 0.3-0.5 μg/μl. Thus, the quality of obtained DNA was appropriate for digestion with restriction endonuclease for PCR amplification, nucleotide sequencing and further cloning.

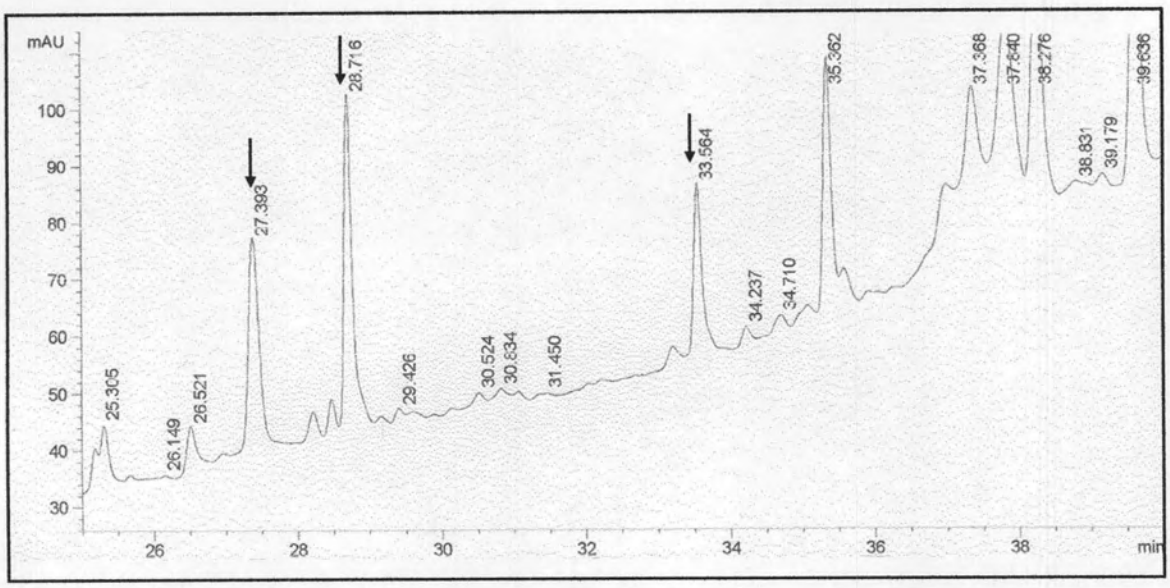

Figure 3.1 The reverse-phase HPLC profile of lysyl endopeptidase digested peptides. The arrows show the isolated peaks that were used for amino acid sequencing.

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CLUSTAL W (1.83) multiple sequence alignment

BLE         ------------------------------------------------------------
BBA         --MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPY 58
BHA         ---MLTKTPTVTSTLDIFTEMAEHEQVLFCHDPSSGLRAIIAIHDTTLGPALGGCRMYPY 57
BSP         -MAKQLEKSSKIGNEDVFQKIANHEQIVFCNDPVSGLQAIIAIHDTTLGPALGGTRMYPY 59
SUR         MILVTLEQTLQDDKASVLDKMVEHEQILFCHDKATGLQAIIAVHDTTMGPALGGCRMAPY 60
TIN         ----------MRDVFEMMDRYG-HEQVIFCRHPQTGLKAIIALHNTTAGPALGGCRMIPY 49
RHO         ------------MSIDSALNWDGEMTVTRFDRETGAHFVIRLDSTQLGPAAGGTRAAQY 47

                                                    F1
                                                ---------->
BLE         --------------------------------AVIIGDP-----QKD---------- 10
BBA         NSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDP-----QKDKSPELFRAFGQFV 113
BHA         QTTEDALRDVLRLSKGMTQKCAAADVDFGGGKAVIIGDP-----AKDKSANLFRAFGQFV 112
BSP         KNVDEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIGDP-----EKDKSPALFRAFGQFV 114
SUR         KTMDLALKDVLRLSKGMTYKCAAADVDFGGGKSVIIGDP-----LKDKTPEKFRAFGQFI 115
TIN         ASTDEALEDVLRLSKGMTYKCSLADVDFGGGKMVIIGDP-----KKDKSPELFRVIGRFV 104
RHO         SQLADALTDAGKLAGAMTLKMAVSNLPMGGGKSVIALPAPRHSIDPSTWARILRIHAENI 107
                                            **   .      .

BLE         ------------------------------------------------------------
BBA         DSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNK 173
BHA         ESLNGRFYTGTDMGTTMEDFVHALKETNGIVGIPKEYGGSGDSSVPTAKGVINSLKAISQ 172
BSP         ESLNGRFYTGTDMGTTMDDFVHAQKETNFINGIPEQYGGSGDSSIPTAQGVIYALKATNQ 174
SUR         ESLNGRFYTGTDMGTTLEDFVHAMKETNYIVGKPVEYGGGGDSSIPTALGVFYGIKATNQ 175
TIN         GGLNGRFYTGTDMGTNPEDFVHAARESKSFAGLPKSYGGKGDTSIPTALGVFHGMRATAR 164
RHO         DKLSGNYWTGPDVNTNSADMDTLNDTTEFVFGRSLERGGAGSSAFTTAVGVFEAMKATVA 167

BLE         ------------------------------------------------------------
BBA         MLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGSV 233
BHA         VVLKDKQFSGRTYAIQGLGKVGFKVAEELLKEGNDLYVSDLQESLPLRLQQLGQRLGRHV 232
BSP         YLFGSDSLSGKTYAIQGLGKVGYKVAEQLLKAGADLFVTDIHENVLNSIKQKSEELGGSV 234
SUR         NLFGDDKVEGRKYSIQGLGKVGYKVAEHIINEGGNVIVTDINEQAIADIQKLG---GSAV 232
TIN         FLWGTDQLKGRVVAIQGVGKVGERLLQLLVEVGAYCKIADIDSVRCEQLKEKY---GDKV 221
RHO         HR-GLGSLDGLTVLVQGLGAVGGSLASLAAEAGAQLLVADTDTERVAHAVALG------H 220

                                                    R2
                                                <----------
BLE         ----------------------------------AIAGSANFQLLTEDHGRQLAD-GI 33
BBA         TVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKGI 293
BHA         EILHGDEIYEAAADVFVPCAQGAILNDATIARLKVKAIAGAANNQLEAERHGQMLHDQGI 292
BSP         TIVKSDDIYSVQADIFVPCAMGGIINDKTIPKLKVKAVVGSANNQLKDLRHANVLNEKGI 294
SUR         RVVSSEEIYSQQADVFVPCAFGGVINDDTLKVLKVRGISGSANNQLAESRHGELLREKGI 292
TIN         QLVDVNRIHKESCDIFSPCAKGGVVNDDTIDEFRCLAIVGSANNQLVEDRHGALLQKRSI 281
RHO         TAVALEDVLSTPCDVFAPCAMGGVITTEVARTLDCSVVAGAANNVIADEAASDILHARGI 280
                                              : *:**  :     .  *   .*
```

(continued)

Figure 3.2 The CLUSTAL W alignment of amino acid sequence of phenylalanine dehydrogenases from various sources. Arrows show the regions of the amino acid sequence that used for degenerated primer design. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (.). (:) means amino acids which have the same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

(continued)

R1

```
BLE             LYAPDYIVNSGGLIQVAD-ELYEVN---------------------------------- 57
BBA             LYAPDYIVNSGGLIQVAD-ELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAANR 352
BHA             WFAPDYIVNSGGLIQVAD-ELYGSNEKRVLSKTNAIYDTILEIFHQAERHHITTLQAANQ 351
BSP             LYAPDYIVNAGGLIQVAD-ELYGPNKERVLLKTKEIYRSLLEIFNQAALDCITTVEAANR 353
SUR             LYAPDYIVNGGGLIQVAD-ELYGTNPARVLAKTENIYTSLLEVFHQAEQDHMTTATAADR 351
TIN             CYAPDYLVNAGGLIQVAD-ELEGFHEERVLAKTEAIYDMVLDIFHRAKNENITTCEAADR 340
RHO             LYAPDFVANAGGAIHLVGREVLGWSESVVHERAVAIGDTLNQVFEISDNDGVTPDEAART 340
                 :***::.*.** *::.. *:

BLE             ----------------------------
BBA             MCEQRMAARGRRNSFFTSSVKPKWDIRN 380
BHA             LCERRIRERARRNNFFVNRIRPKWNLRK 379
BSP             KCQKTIEGQQTRNSFFSRGRRPKWNIKE 381
SUR             MCEKRIADAKNRNSFFTQSNRPKWNFHQ 379
TIN             IVMERLKKLTDIRRILLED--PRNSARR 366
RHO             LAGRRAREASTTTATA------------ 356
```

Figure 3.2 The CLUSTAL W alignment of amino acid sequence of phenylalanine dehydrogenases from various sources. Arrows show the regions of the amino acid sequence that used for degenerated primer design. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (*). (:) means amino acids which have the same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

3.2.2 PCR amplification for the internal fragment of phenylalanine dehydrogenase gene

The chromosomal DNA of *Bacillus lentus* was completely digested with various restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pst*I, *Spe*I and *Xba*I. The agarose gel electrophoresis analysis of digested DNA in Figure 3.3 showed that the pattern resulting from all seven digestions gave the smear pattern of DNA lower than 23.1 kb.

All of digested DNA solutions were used as the templates for amplification of the internal fragment of *phedh* gene using primer F1 and R1, and PCR product of 625 bp was expected. The Figure 3.4 indicated that all of templates gave apparent band about 600 bp. However, nonspecific bands were also appeared. To confirm these PCR products, the PCR product were diluted 100 times and used as templates for second PCR amplification using various annealing temperatures and primer F1 and R2 which should give band of 583 bp. The result (figure not shown) showed that amplification using annealing temperature of 52.8°C and *Bgl*II digested DNA as template gave the strongest specific band. Then, this band was eluted from gel by QIAquick gel extraction kit as shown in Figure 3.5 and nucleotide sequence on both sides of the amplified fragment were determined using primer F1 and R2. After the nucleotide sequence of internal gene fragment had been obtained (Figure 3.6), it was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. The sequence showed high homology (more than 71%) to partial part of *phedh* gene from other published *Bacillus* sp. sources indicated that this amplified fragment should be a part of *phedh* gene (data not shown). Finally, the internal nucleotide sequence was used as data to design antisense primer N1, N2 and sense primer C1, C2 for inverse PCR to determine 5′-terminal and 3′-terminal segment of the gene.

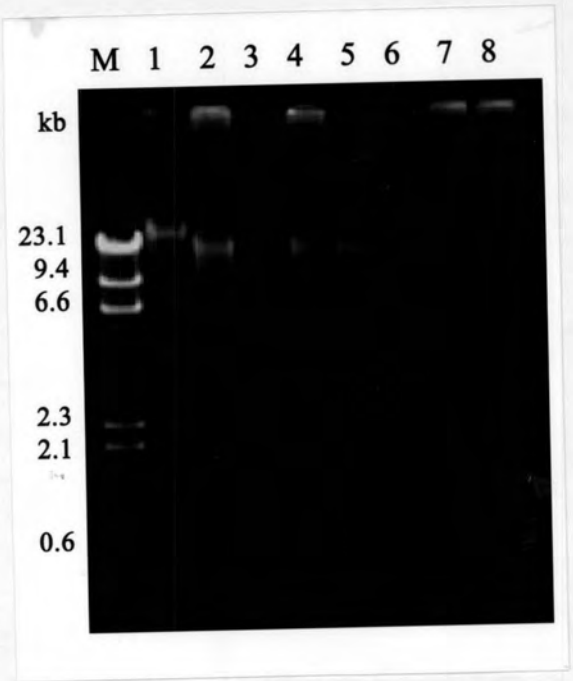


Figure 3.3 Restriction enzyme digested chromosomal DNA of *Bacillus lentus*

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

Lane 1 = undigested chromosomal DNA

Lane 2 = chromosomal DNA digested with *Bam*HI

Lane 3 = chromosomal DNA digested with *Bgl*II

Lane 4 = chromosomal DNA digested with *Eco*RI

Lane 5 = chromosomal DNA digested with *Kpn*I

Lane 6 = chromosomal DNA digested with *Pst*I

Lane 7 = chromosomal DNA digested with *Spe*I

Lane 8 = chromosomal DNA digested with *Xba*I

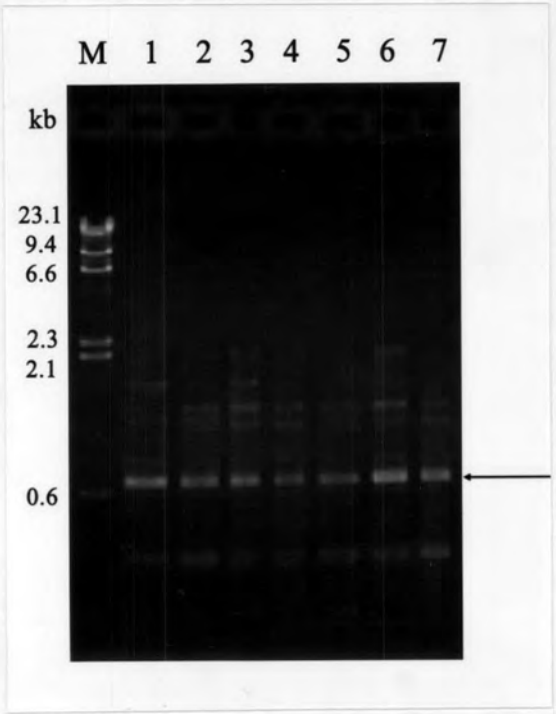


Figure 3.4 PCR products of primer F1 and R1 using various digested DNA templates

The specific product was indicated by arrow.

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

Lane 1 = PCR products using *Bam*HI digested DNA as template

Lane 2 = PCR products using *Bgl*II digested DNA as template

Lane 3 = PCR products using *Eco*RI digested DNA as template

Lane 4 = PCR products using *Kpn*I digested DNA as template

Lane 5 = PCR products using *Pst*I digested DNA as template

Lane 6 = PCR products using *Spe*I digested DNA as template

Lane 7 = PCR products using *Xba*I digested DNA as template

Figure 3.5 Recovered PCR product of the internal fragment of phenylalanine dehydrogenase gene

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

Lane 1 = specific PCR product using primer F1xR2 (583 bp)

Lane m = 100 bp DNA ladder

Note: The template was PCR product from the PCR reaction using *Bgl*II digested DNA as template and F1xR1 as primers.

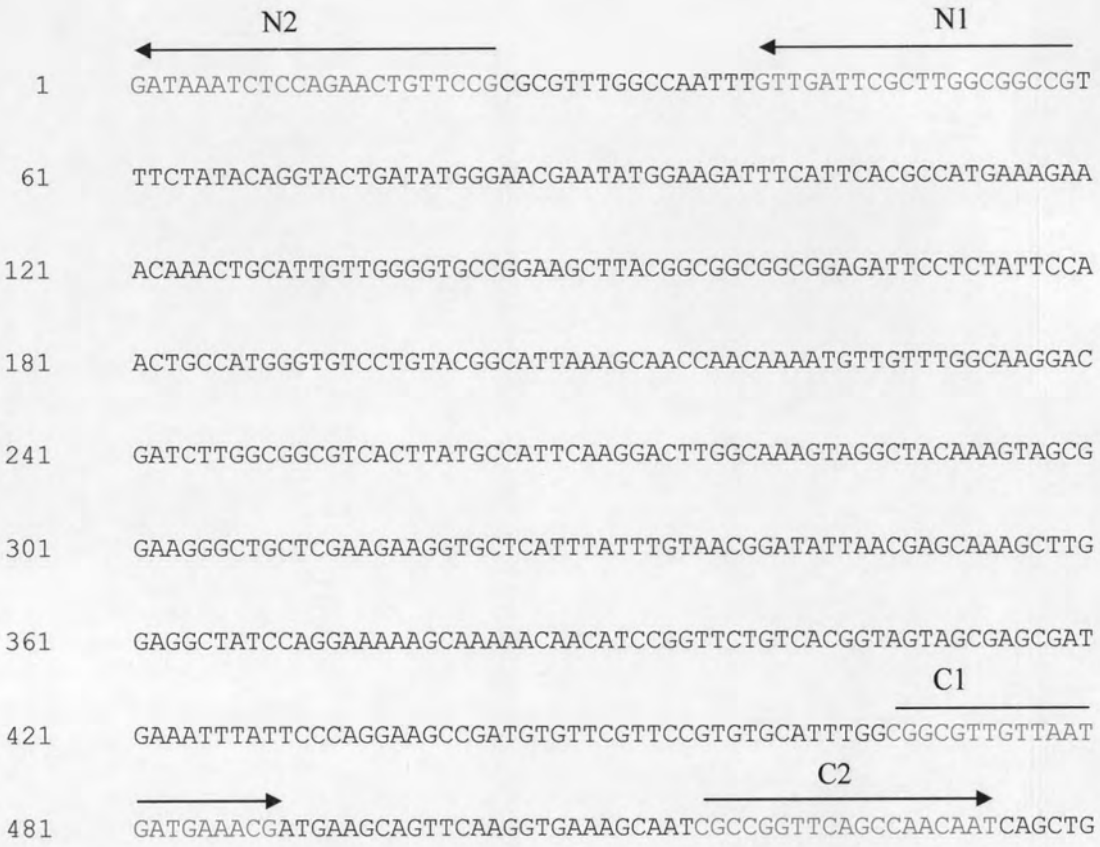


Figure 3.6 The nucleotide sequence of the internal fragment of phenylalanine dehydrogenase gene. The chromatograms were presented in Appendix H. The regions of primers for further inverse PCR were shown by arrow.

3.2.3 PCR amplification for the 5′-terminus and 3′-terminus of phenylalanine dehydrogenase gene (inverse PCR)

3.2.3.1 The 5′-terminus of phenylalanine dehydrogenase gene

For the inverse PCR, the used primers for this part of PCR reaction were designed from the data of known sequence and the templates were prepared by firstly digested with each restriction enzyme (*Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, *Spe*I and *Xba*I) which has no site on known sequence and secondly digested against *Nco*I which has only single site on known sequence to carry out linearized template. The first PCR products which used outer pair of primers (N1xC1) showed multiple and smear bands (Figure 3.7), so those PCR products were used as templates for second PCR which used inner pair of primers (N2xC2). The result (not shown) indicated that only the *Eco*RI digested tamplate gave specific band of 2.3 kb which was just weak band. To overcome this problem, the second PCR product was amplified again by 3 combinations of inner and outer pair of primers (N1xC2, N2xC2 and N2xC1) as shown in Figure 3.8. The result showed that the strongest band occurred when the pair of primers N2xC1 was used. Thus, the specific band was recovered and sequenced. After the obtained nucleotide sequence (Figure 3.9) had been compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database, it was found that this fragment was at 5′-terminal of *phedh* gene which comprised start codon. From the obtained 5′-terminal sequence, the antisense primer N3 and N4 were designed to use in next step.

3.2.3.2 The 3′-terminus of phenylalanine dehydrogenase gene

Due to the result that there was no PCR product occurred when using the previous first PCR products from section 3.2.3.1 as templates and N4xC2 or N3xC2 as primers for second amplification, the different DNA templates for first PCR were prepared, the chromosomal DNA was digested with each restriction enzyme: *Kpn*I, *Nde*I, *Sac*I and *Xho*I, then ligated and digested again with *Nco*I.

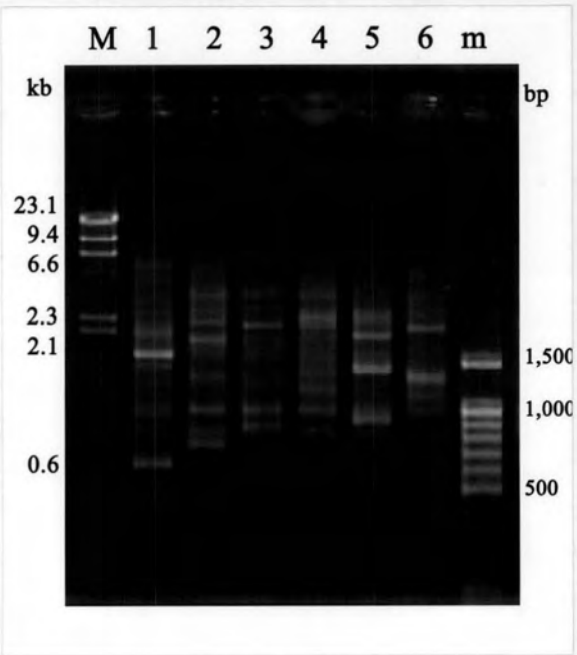


Figure 3.7 The first inverse PCR products using primer N1 and C1 and various digested DNA as templates

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

Lane 1 = PCR products using *Bam*HI digested DNA as template

Lane 2 = PCR products using *Bgl*II digested DNA as template

Lane 3 = PCR products using *Eco*RI digested DNA as template

Lane 4 = PCR products using *Pst*I digested DNA as template

Lane 5 = PCR products using *Spe*I digested DNA as template

Lane 6 = PCR products using *Xba*I digested DNA as template

Lane m = 100 bp DNA ladder

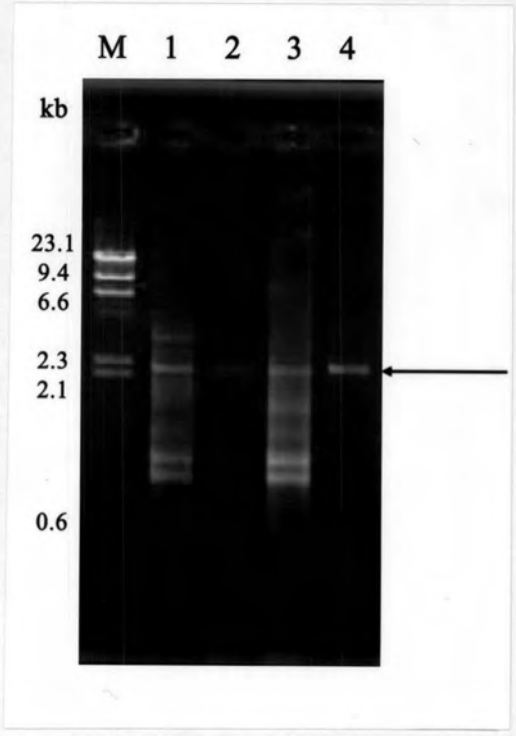


Figure 3.8 The second inverse PCR products at 5′-terminus using various pair of primers

The specific product eluted for sequencing was indicated by arrow.

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

Lane 1 = PCR products using primer N1xC1

Lane 2 = PCR products using primer N2xC2

Lane 3 = PCR products using primer N1xC2

Lane 4 = PCR products using primer N2XC1

Note: The template was PCR product from the first PCR reaction using *Eco*RI digested DNA as template and N1xC1 as primers.

```
   1    GTGAATTATTTATTAAAGGCAGCTGTCAATGACATGGAGCATTTAAACAAGCTGATTGAT

  61    TATTTGATCCGGTTTGGCAGTATTCACACTTCTATCGTGCTGAAATCCCCTGTTCCCGAG

 121    AAGATTATTTTGCCGTCTGATGAAGAACAGGAATGGCCGTGAAGGACGGCGCATCATAGA

 181    AGATGATGTCGAATGATAAGACAAGCCTCCTCTTTCTATTGTCGAAAGAGGAGGCTTTTT

 241    TAGCTTTTATTTACTGGAAATGAAAGCGTTTTACAAAACAAAGATAAATACCAAAAAATG

 301    CTTACGAAAAGTAAATAAAGGTCGGAAAATTCAATCATAATGATGTGTATCTATCTCCTT

 361    TAACCTTTTATAATTAAAGTGAAGCGTATGCCTGCCGGCGCCGTCATTGGCGCTCGTTTG

 421    AAAGGGCTTACAAAAATTATATAACCAAGAAGCTGACAGATCCTTTTTCTGCGGATAAAT

 481    AAAAGCGTTCAACTATTAACGAAAGCAGGGATTAAATATGAGCTTAGTAGAAAAAACATC

 541    CATCATAAAAGATTTCACTCTTTTTGAAAAAATGTCTGAACATGAACAAGTTGTTTTTTG
                                                        N4
 601    CAACGATCCGGCGACAGGACTAAGGGCCATTATCGCTATTCATGACACCACACTCGGACC

 661    TGCGCTCGGCGGCTGCCGCATGCAGCCTTATAACAGTGTGGAAGAAGCATTGGAAGATGC
                                  N3
 721    TCTTCGCCTTTCCAAAGGAATGACTTACAAATGCGCGGCGTCCGATGTCGACTTTGGCGG

 781    CGGAAAAGCAGTCATTATCGGT
```

Figure 3.9 Nucleotide sequence of the 5′-terminal fragment of phenylalanine dehydrogenase gene using antisense primer N2. The chromatogram was shown in Appendix H. The start codon was underlined while the nucleotide sequences of new antisense primers were shown by arrow.

The first PCR products which used outer pair of primers (N1xC1) showed multiple and smear bands (figure not shown). Therefore, those PCR products were used as templates for second PCR which used pair of primers N3xC2 and N4xC2 as shown in Figure 3.10. The result indicated that only the *Nde*I digested tamplate gave specific band of 700 and 600 bp which corresponded to the size of fragment amplified by primer N3xC2 and N4xC2, respectively. The specific band of 600 bp was eluted from gel to sequence by primer C2. After that, the obtained nucleotide sequence (Figure 3.11) was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. It was the 3′-terminal fragment of *phedh* gene which consisted of stop codon.

3.2.4 Nucleotide sequence and deduced amino acid sequence of phenylalanine dehydrogenase gene

From the gene walking by inverse PCR, the complete nucleotide sequence of the whole gene was identified as shown in Figure 3.12. The structural gene contained 1143 bp open reading frame, which was capable for encoding a polypeptide of 380 amino acids. The molecular weight of enzyme subunit was calculated from deduced amino acid sequence to be 41.4 kDa. The nucleotide sequence was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. The percentage of similarity of nucleotide sequences of phenylalanine dehydrogenase from *Bacillus lentus* to those of *Bacillus badius*, *Sporosarcina ureae*, *Bacillus sphaericus*, *Thermoactinomyces intermedius* and *Rhodococcus* sp. M4 were 99, 65, 62, 57 and 10%, respectively. The alignment was shown in Figure 3.13. Comparison of nucleotide sequences between *phedh* gene of *Bacillus lentus* and those of published *Bacillus badius* is shown in Figure 3.14.

The deduced amino acid sequence of PheDH from *B. lentus* was compared with the sequences of PheDH from other bacterial sources. *B. lentus* exhibited the highest overall level of similarity to the enzyme from *B. badius* (99%) as shown in Figure 3.15. Moreover, it showed 67, 66, 62, 53 and 32% similarity to those of *Bacillus sphaericus*, *Sporosarcina ureae*, *Bacillus halodurans*,

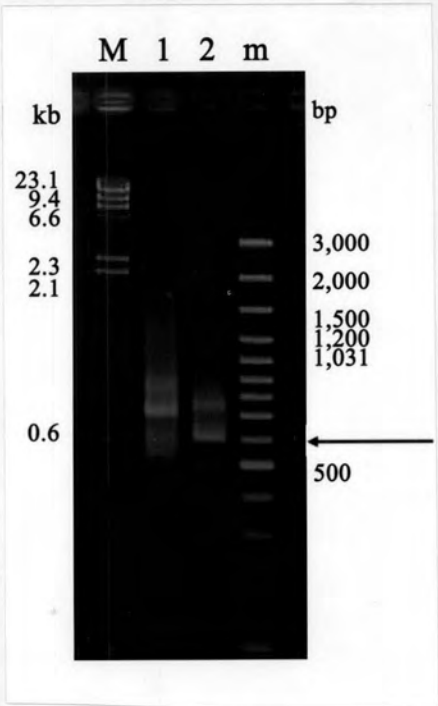


Figure 3.10 **The second inverse PCR products at 3′-terminus**

The specific PCR product indicated by arrow (lane 2), about 600 bp, was recovered for nucleotide sequencing.

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

Lane 1 = PCR products using primer N3xC2 (700 bp)

Lane 2 = PCR products using primer N4xC2 (600 bp)

Lane m = 100 bp DNA ladder

Note: The template was PCR product from the first PCR reaction using *Nde*I digested DNA as template and N1xC1 as primers.

```
  1    CTTGCAGACAAAGGCATTCTGTATGCTCCGGATTATATTGTTAACTCTGGCGGTCTGATC

 61    CAAGTAGCCGACGAATTGTATGAGGTGAACAAAGAACGCGTGCTTGCGAAGACGAAGCAT

121    ATTTACGACGCAATTCTTGAAGTGTACCAGCAAGCGGAATTAGATCAAATCACCACAATG

181    GAAGCAGCCAACAGAATGTGTGAGCAAAGAATGGCGGCAAGAGGCCGACGCAACAGCTTC

241    TTTACTTCTTCTGTTAAGCCAAAATGGGATATTCGCAACTAATACTGTTCGGGGGGATAT

301    CATGAATACTCAATACCCAATCAAAAGAATAATCGATGATGAAGGCAACTTGATAGATGC

361    GTCTTACCAGGATCAGCTGAATGAGCAGCTTGTGA
```

Figure 3.11 Nucleotide sequence at the 3′-terminus of phenylalanine dehydrogenase gene using sense primer C2. The chromatogram was shown in Appendix H. The stop codon was underlined.

```
   1  GTGAATTATTTATTAAAGGCAGCTGTCAATGACATGGAGCATTTAAACAAGCTGATTGAT
  61  TATTTGATCCGGTTTGGCAGTATTCACACTTCTATCGTGCTGAAATCCCCTGTTCCCGAG
 121  AAGATTATTTTGCCGTCTGATGAAGAACAGGAATGGCCGTGAAGGACGGCGCATCATAGA
 181  AGATGATGTCGAATGATAAGACAAGCCTCCTCTTTCTATTGTCGAAAGAGGAGGCTTTTT
 241  TAGCTTTTATTTACTGGAAATGAAAGCGTTTTACAAAACAAAGATAAATACCAAAAAATG
 301  CTTACGAAAAGTAAATAAAGGTCGGAAAATTCAATCATAATGATGTGTATCTATCTCCTT
 361  TAACCTTTTATAATTAAAGTGAAGCGTATGCCTGCCGGCGCCGTCATTGGCGCTCGTTTG
 421  AAAGGGCTTACAAAAATTATATAACCAAGAAGCTGACAGATCCTTTTTCTGCGGATAAAT
 481  AAAAGCGTTCAACTATTAACGAAAGCAGGGATTAAATATGAGCTTAGTAGAAAAAACATC
                                          M  S  L  V  E  K  T  S
 541  CATCATAAAAGATTTCACTCTTTTTGAAAAAATGTCTGAACATGAACAAGTTGTTTTTTG
        I  I  K  D  F  T  L  F  E  K  M  S  E  H  E  Q  V  V  F  C
 601  CAACGATCCGGCGACAGGACTAAGGGCCATTATCGCTATTCATGACACCACACTCGGACC
        N  D  P  A  T  G  L  R  A  I  I  A  I  H  D  T  T  L  G  P
 661  TGCGCTCGGCGGCTGCCGCATGCAGCCTTATAACAGTGTGGAAGAAGCATTGGAAGATGC
        A  L  G  G  C  R  M  Q  P  Y  N  S  V  E  E  A  L  E  D  A
 721  TCTTCGCCTTTCCAAAGGAATGACTTACAAATGCGCGGCGTCCGATGTCGACTTTGGCGG
        L  R  L  S  K  G  M  T  Y  K  C  A  A  S  D  V  D  F  G  G
 781  CGGAAAAGCAGTCATTATCGGTGATCCGCAGAAAGATAAATCTCCAGAACTGTTCCGCGC
        G  K  A  V  I  I  G  D  P  Q  K  D  K  S  P  E  L  F  R  A
 841  GTTTGGCCAATTTGTTGATTCGCTTGGCGGCCGTTTCTATACAGGTACTGATATGGGAAC
        F  G  Q  F  V  D  S  L  G  G  R  F  Y  T  G  T  D  M  G  T
 901  GAATATGGAAGATTTCATTCACGCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGA
        N  M  E  D  F  I  H  A  M  K  E  T  N  C  I  V  G  V  P  E
 961  AGCTTACGGCGGCGGCGGAGATTCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCAT
        A  Y  G  G  G  G  D  S  S  I  P  T  A  M  G  V  L  Y  G  I
1021  TAAAGCAACCAACAAAATGTTGTTTGGCAAGGACGATCTTGGCGGCGTCACTTATGCCAT
        K  A  T  N  K  M  L  F  G  K  D  D  L  G  G  V  T  Y  A  I
```

(continued)

Figure 3.12 The nucleotide sequence and the deduced amino acid of phenylalanine dehydrogenase gene from *Bacillus lentus*. Pink = sequence from the internal gene fragment amplification, green = sequence obtained from the 5′-terminal gene fragment amplification, orange = sequence obtained from the 3′-terminal gene fragment amplification, purple = upstream sequence of *phedh* structural gene, and brown = downstream sequence of *phedh* structural gene.

(continued)

```
1081        TCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCGGAAGGGCTGCTCGAAGAAGGTGCTCA
              Q  G  L  G  K  V  G  Y  K  V  A  E  G  L  L  E  E  G  A  H
1141        TTTATTTGTAACGGATATTAACGAGCAAAGCTTGGAGGCTATCCAGGAAAAAGCAAAAAC
              L  F  V  T  D  I  N  E  Q  S  L  E  A  I  Q  E  K  A  K  T
1201        AACATCCGGTTCTGTCACGGTAGTAGCGAGCGATGAAATTTATTCCCAGGAAGCCGATGT
              T  S  G  S  V  T  V  V  A  S  D  E  I  Y  S  Q  E  A  D  V
1261        GTTCGTTCCGTGTGCATTTGGCGGCGTTGTTAATGATGAAACGATGAAGCAGTTCAAGGT
              F  V  P  C  A  F  G  G  V  V  N  D  E  T  M  K  Q  F  K  V
1321        GAAAGCAATCGCCGGTTCAGCCAACAATCAGCTGCTTACGGAGGATCACGGCAGACAGCT
              K  A  I  A  G  S  A  N  N  Q  L  L  T  E  D  H  G  R  Q  L
1381        TGCAGACAAAGGCATTCTGTATGCTCCGGATTATATTGTTAACTCTGGCGGTCTGATCCA
              A  D  K  G  I  L  Y  A  P  D  Y  I  V  N  S  G  G  L  I  Q
1441        AGTAGCCGACGAATTGTATGAGGTGAACAAAGAACGCGTGCTTGCGAAGACGAAGCATAT
              V  A  D  E  L  Y  E  V  N  K  E  R  V  L  A  K  T  K  H  I
1501        TTACGACGCAATTCTTGAAGTGTACCAGCAAGCGGAATTAGATCAAATCACCACAATGGA
              Y  D  A  I  L  E  V  Y  Q  Q  A  E  L  D  Q  I  T  T  M  E
1561        AGCAGCCAACAGAATGTGTGAGCAAAGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTT
              A  A  N  R  M  C  E  Q  R  M  A  A  R  G  R  R  N  S  F  F
1621        TACTTCTTCTGTTAAGCCAAAATGGGATATTCGCAACTAATACTGTTCGGGGGGATATCA
              T  S  S  V  K  P  K  W  D  I  R  N  *
1681        TGAATACTCAATACCCAATCAAAAGAATAATCGATGATGAAGGCAACTTGATAGATGCGT
1741        CTTACCAGGATCAGCTGAATGAGCAGCTTGTGA
```

Figure 3.12 The nucleotide sequence and the deduced amino acid of phenylalanine dehydrogenase gene from *Bacillus lentus*. Pink = sequence from the internal gene fragment amplification, green = sequence obtained from the 5′-terminal gene fragment amplification, orange = sequence obtained from the 3′-terminal gene fragment amplification, purple = upstream sequence of *phedh* structural gene, and brown = downstream sequence of *phedh* structural gene.

```
CLUSTAL W (1.83) multiple sequence alignment

BLE             ------ATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAA 54
BBA             ------ATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAA 54
BSP             ---ATGGCAAAACAGCTTGAAAAGTCATCAAAAATTGGTAATGAGGACGTTTTTCAAAAA 57
SUR             ATGATTTTGGTAACTTTAGAACAGACTTTACAAGACGACAAGGCAAGTGTTTTGGATAAA 60
TIN             ---------------------------------ATGCGCGACGTGTTTGAAATGATGGAC 27
RHO             ---------------------------------ATGAGTATCGACAGCGCACTGAA---- 23
                                                                *

BLE             ATGTCTGAACATGAACAAGTTGTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCATT 114
BBA             ATGTCTGAACATGAACAAGTTGTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCATT 114
BSP             ATAGCGAATCACGAGCAGATTGTGTTCTGTAATGATCCGGTATCCGGCCTGCAAGCTATC 117
SUR             ATGGTCGAGCATGAACAAATTCTATTTTGTCATGATAAAGCAACCGGTCTTCAAGCCATC 120
TIN             CGCTATGGCCACGAGCAGGTCATTTTTTGCCGTCATCCGCAAACCGGTCTCAAAGCGATC 87
RHO             --CTGGGACGGGGAAATGACGGTCACCCGATTCGACCGGGAGACTGGTGCCCATTTCGTC 81
                          **        *    *    *        * **          *

BLE             ATCGCTATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTAT 174
BBA             ATCGCTATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTAT 174
BSP             ATTGCTATCCACGATACAACCCTAGGCCCCGCTTTAGGTGGAACTCGCATGTATCCCTAT 177
SUR             ATTGCAGTCCACGATACGACTATGGGACCTGCACTCGGTGGATGTCGCATGGCGCCTTAT 180
TIN             ATCGCCTTGCATAATACAACCGCGGGGCCGGCTTTGGGTGGATGCCGCATGATCCCGTAT 147
RHO             ATTCGACTCGATTCGACCCAACTCGGACCGGCGGCCGGAGGCACCAGAGCCGCACAGTAC 141
                **     *  *    **        ** ** **    ** **     *       *  **

BLE             AACAGTGTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAAA 234
BBA             AACAGTGTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAAA 234
BSP             AAAAATGTGGATGAAGCTCTGGAAGATGTGCTTCGCCTGTCAGAAGGAATGACGTATAAA 237
SUR             AAAACGATGGATCTCGCATTAAAAGATGTTCTTCGCCTTTCAAAAGGGATGACATATAAA 240
TIN             GCTTCGACGGACGAAGCCTTGGAGGATGTTTTGCGGTTGTCCAAAGGCATGACCTATAAA 207
RHO             TCACAGCTGGCGGACGCCCTCACCGACGCCGGCAAATTGGCGGGGGCGATGACGTTGAAG 201
                       **     **  *    ** *          *  *    *  ***** *  **

BLE             TGCGCGGCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTAT-----CGGTGATC 289
BBA             TGCGCGGCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTAT-----CGGTGATC 289
BSP             TGCGCAGCCGCCGATATCGATTTCGGCGGCGGGAAGGCGGTCATTAT-----CGGAGATC 292
SUR             TGTGCGGCAGCTGATGTAGACTTTGGCGGCGGAAAATCCGTCATCAT-----CGGAGACC 295
TIN             TGCAGTCTGGCGGATGTGGACTTTGGCGGGGGAAAAATGGTTATCAT-----CGGCGATC 262
RHO             ATGGCAGTGAGCAACCTTCCGATGGGCGGGGGCAAATCCGTCATTGCGCTTCCTGCGCCG 261
                         *  *    * ***** ** **    ** **        * * *

BLE             CGCAGAAAGATAAATCTCCAGAACTG----------TTCCGCGCGTTTGGCCAATTTGTT 339
BBA             CGCAGAAAGATAAATCTCCAGAACTG----------TTCCGCGCGTTTGGCCAATTTGTT 339
BSP             CAGAAAAGGATAAATCTCCGGCATTG----------TTCCGTGCATTTGGTCAATTTGTG 342
SUR             CGCTAAAAGATAAAACGCCTGAGAAA----------TTCCGTGCTTTCGGTCAATTCATC 345
TIN             CGAAAAAAGATAAATCGCCGGAGTTG----------TTTCGCGTGATCGGCCGTTTTGTG 312
RHO             CGTCATTCGATCGATCCGAGCACGTGGGCACGCATCCTCCGAATCCACGCCGAGAACATC 321
                *       ***  * *                          * **       *        *
```

(continued)

Figure 3.13 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from various sources. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (*).

(continued)

```
BLE          GATTCGCTTGGCGGCCGTTTCTATACAGGTACTGATATGGGAACGAATATGGAAGATTTC 399
BBA          GATTCGCTTGGCGGCCGTTTCTATACAGGTACTGATATGGGAACGAATATGGAAGATTTC 399
BSP          GAATCACTGAATGGACGATTTTACACAGGTACTGACATGGGGACCACGATGGATGATTTT 402
SUR          GAATCATTGAACGGACGCTTCTATACAGGTACAGACATGGGCACAACGCTTGAAGACTTT 405
TIN          GGCGGGTTAAACGGCCGTTTCTATACCGGAACCGACATGGGAACCAATCCGGAAGATTTT 372
RHO          GACAAGTTGTCCGGCAACTACTGGACCGGACCGGACGTCAACACCAATTCGGCAGACATG 381
             *      *    **    * *  ** **  * **  *    ** *     *  **  *

BLE          ATTCACGCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGC 459
BBA          ATTCACGCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGC 459
BSP          GTCCATGCACAGAAAGAGACGAATTTCATTAACGGAATTCCTGAGCAGTATGGTGGAAGC 462
SUR          GTGCATGCCATGAAAGAAACAAACTACATCGTGGGCAAGCCGGTCGAATATGGTGGCGGT 465
TIN          GTCCATGCCGCCAGGGAATCGAAATCTTTTGCCGGATTGCCGAAATCGTACGGCGGAAAG 432
RHO          GATACTCTGAACGACACCACCGAGTTCGTGTTCGGACGGTCGCTCGAACGCGGCGGCGCG 441
                           * * *   *    **     *          ** **

BLE          GGAGATTCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAA 519
BBA          GGAGATTCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAA 519
BSP          GGCGACTCGTCGATTCCGACCGCCCAGGGAGTCATTTATGCACTGAAGGCTACAAACCAG 522
SUR          GGAGACTCATCGATCCCTACTGCACTCGGAGTCTTCTATGGCATTAAAGCGACAAACCAG 525
TIN          GGGGACACATCCATTCCCACCGCGCTCGGGGTGTTTCACGGAATGCGGGCCACCGCCCGG 492
RHO          GGTTCGAGCGCGTTCACCACCGCCGTTGGCGTGTTCGAGGCGATGAAGGCGACCGTCGCG 501
             **         * * * ** **    ** **  * * *   *    ** **   *

BLE          ATGTTGTTTGGCAAGGACGATCTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAA 579
BBA          ATGTTGTTTGGCAAGGACGATCTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAA 579
BSP          TATTTATTTGGAAGCGATAGCCTTTCAGGTAAAACATATGCTATTCAAGGGCTGGGAAAA 582
SUR          AATCTGTTTGGCGACGACAAAGTAGAAGGCCGAAAATACAGTATCCAAGGTCTTGGGAAA 585
TIN          TTTTTATGGGGGACGGATCAGCTGAAAGGGCGTGTGGTTGCCATCCAAGGAGTCGGCAAG 552
RHO          CACC---GTGGGCTGGGCTCACTCGACGGTTTGACGGTCCTGGTCCAAGGACTGGGGGCA 558
                    **    *      *    **              * *****  * **

BLE          GTAGGCTACAAAGTAGCGGAAGGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGAT 639
BBA          GTAGGCTACAAAGTAGCGGAAGGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGAT 639
BSP          GTAGGGTATAAAGTAGCGGAACAGCTCTTAAAAGCCGGCGCCGATTTATTTGTAACGGAT 642
SUR          GTAGGTTACAAAGTAGCTGAACATATTATCAACGAAGGTGGAAACGTGATCGTCACAGAT 645
TIN          GTGGGAGAGCGCTTGTTGCAGCTTTTGGTCGAAGTGGGGGCTTACTGCAAAATTGCCGAC 612
RHO          GTCGGAGGATCATTGGCATCCCTGGCCGCCGAAGCGGGTGCGCAACTCCTGGTGGCAGAC 618
             ** **        *                  * *  ** *   *        *  * **

BLE          ATTAACGAGCAAAGCTTGGAGGCTATCCAGGAAAAAGCAAAAACAACATC-CGGTTCTGT 698
BBA          ATTAACGAGCAAACGTTGGAGGCTATCCAGGAAAAAGCAAAAACAACATC-CGGTTCTGT 698
BSP          ATACATGAAAATGTCCTCAATTCCATTAAGCAAAAATCAGAAGAGCT-TGGCGGTTCAGT 701
SUR          ATTAATGAGCAAG----CGATTGCA------GATATTCAGAAGCTCGGTGGAAGCGCTGT 695
TIN          ATCGAT----------TCGGTGCGATGCGAACAGCTGAAAGAAAAGTATGGCGACAAGGT 662
RHO          ACCGAC----------------ACCGAGCGAGTAGCGCACGCTGTTGCGTTGGGCCA- 659
             *   *                 *       *
```

(continued)

Figure 3.13 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from various sources. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (•).

(continued)

```
BLE     CACGGTAGTAGCGAGCGATGAAATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGC 758
BBA     CACGGTAGTAGCGAGCGATGAAATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGC 758
BSP     GACCATTGTAAAAAGTGACGATATTTACAGCGTACAAGCGGATATATTTGTTCCGTGTGC 761
SUR     CAGGGTCGTATCAAGTGAGGAGATTTACAGTCAGCAAGCAGATGTTTTTGTTCCTTGTGC 755
TIN     CCAATTGGTGGATGTGAACCGGATTCACAAGGAGAGTTGCGATATTTTCTCGCCTTGCGC 722
RHO     CACAGCGGTTGCCCTCGAGGACGTTCTGTCCACCCCGTGTGATGTCTTCGCACCCTGCGC 719
                ** *  ** *** * **  ** ** **

BLE     ATTTGGCGGCGTTGTTAATGATGAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGG 818
BBA     ATTTGGCGGCGTTGTTAATGATGAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGG 818
BSP     GATGGGTGGTATTATCAATGATAAAACCATTCCTAAGTTAAAGGTGAAGGCTGTTGTGGG 821
SUR     ATTTGGTGGCGTGATCAATGACGACACGCTAAAGGTGCTGAAAGTACGAGGAATCTCCGG 815
TIN     CAAAGGCGGCGTGGTCAATGATGACACCATTGACGAGTTCCGTTGCCTGGCCATTGTCGG 782
RHO     AATGGGCGGCGTCATCACCACCGAGGTGGCGCGAACACTCGACTGTTCCGTCGTGGCCGG 779
           ** **  *  * *     *           *          *  *   **

BLE     TTCAGCCAACAATCAGCTGCTTACGGAGGATCACGGCAGACAGCTTGCAGACAAAGGCAT 878
BBA     TTCAGCCAACAATCAGCTGCTTACGGAGGATCACGGCAGACACCTTGCAGACAAAGGCAT 878
BSP     ATCAGCCAATAACCAGCTCAAAGACCTCCGCCATGCAAATGTACTAAACGAAAAGGGAAT 881
SUR     TTCAGCAAACAATCAGCTCGCGGAAAGCCGCCATGGAGAGCTACTACGTGAAAAGGGTAT 875
TIN     ATCCGCCAACAACCAACTGGTGGAAGACCGGCATGGGGCACTGCTTCAAAAACGGAGCAT 842
RHO     TGCCGCCAACAACGTCATCGCCGACGAGGCCGCCTCGGACATCCTGCACGCACGCGGAAT 839
          * ** ** **     *                          **          * **

BLE     TCTGTATGCTCCGGATTATATTGTTAACTCTGGCGGTCTGATCCAAGTAGCCGAC-GAAT 937
BBA     TCTGTATGCTCCGGATTATATTGTTAACTCTGGCGGTCTGATCCAAGTAGCCGAC-GAAT 937
BSP     TCTATATGCACCCGATTATATCGTCAATGCCGGCGGCTTGATCCAGGTTGCTGAC-GAAC 940
SUR     TTTGTACGCACCAGACTATATCGTCAACGGCGGCGGTTTAATCCAAGTGGCGGAT-GAAT 934
TIN     TTGTTATGCACCCGATTATCTGGTGAATGCCGGCGGGCTGATTCAAGTGGCTGAT-GAAC 901
RHO     TCTGTACGCTCCCGACTTCGTGGCCAACGCCGGCGGTGCCATCCACCTCGTAGGCCGGGA 899
        *   ** ** ** ** *   * *  **    *****    ** **  * *  *   *

BLE     TGTATGAGGTGAACAAAGAACGCGTGCTTGCGAA--GACGAAGCATATTTACGACGCAAT 995
BBA     TGTATGAGGTGAACAAAGAACGCGTGCTTGCGAA--GACGAAGCATATTTACGACGCAAT 995
BSP     TTTATGGGCCGAATAAAGAGCGGGTCTTGCTCAA--AACGAAAGAAATTTACCGTTCTCT 998
SUR     TGTACGGAACGAATCCTGCACGTGTACTCGCTAA--AACTGAAAACATCTATACCTCACT 992
TIN     TGGAAGGCTTCCATGAAGAGAGAGTGCTCGCCAA--AACCGAAGCGATTTATGACATGGT 959
RHO     GGTTCTCGGTTGGTCCGAGTCGGTTGTCCACGAACGAGCAGTTGCCATAGGCGACACCCT 959
                          * *      **    *      **           *

BLE     TCTTGAAGTGTACCAGCAAGCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAACAG 1055
BBA     TCTTGAAGTGTACCAGCAAGCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAACAG 1055
BSP     GCTTGAAATTTTTAATCAGGCAGCCCTTGACTGCATCACAACAGTGGAGGCCGCAAATAG 1058
SUR     GCTTGAAGTATTCCATCAGGCAGAACAGGATCATATGACAACTGCCACTGCCGCAGACCG 1052
TIN     CCTGGATATTTTTCACCGGGCGAAAAATGAGAATATTACCACTTGTGAGGCAGCGGACCG 1019
RHO     GAATCAGGTCTTCGAGATCTCCGACAACGACGGCGTCACCCCGGACGAGGCCGCCCGCAC 1019
             *  * *   *     *       **     * **  *       ** **
```

(continued)

Figure 3.13 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from various sources. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (*).

(continued)

```
BLE             AATGTGTGAGCAAAGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGT 1115
BBA             AATGTGTGAGCAAAGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGT 1115
BSP             GAAGTGTCAAAAGACGATTGAGGGCCAGCAAACCCGTAATAGTTTCTTTTCTAGGGGACG 1118
SUR             TATGTGTGAAAAGCGTATTGCGGATGCCAAGAATCGCAACAGCTTCTTCACACAGTCAAA 1112
TIN             GAT-CGTGATGGAGCGTTTGAAAAAGTTAAC-----CGATATTCGCCGGATCTTGTTGGA 1073
RHO             TCT-CGCTGGACGGCGCGCCCGCGAGGCCTCGACAACGACAGCGACTGCCTAG------- 1071
                     *                                      * *    *


BLE             TAAGCCAAAATGGGATATTCGCAACTAA 1143
BBA             TAAGCCAAAATGGGATATTCGCAACTAA 1143
BSP             CAGGCCGAAGTGGAACATAAAAGAGTAA 1146
SUR             CCGACCGAAATGGAATTTTCATCAGTAA 1140
TIN             GGATCCCCGCAACAGCGCAAGGAGGTAA 1101
RHO             ----------------------------
```

Figure 3.13 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from various sources. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (*).

```
CLUSTAL W (1.83) multiple sequence alignment

BLE         ATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAAATGTCT 60
BBA         ATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAAATGTCT 60
            ************************************************************

BLE         GAACATGAACAAGTTGTTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCATTATCGCT 120
BBA         GAACATGAACAAGTTGTTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCATTATCGCT 120
            ************************************************************

BLE         ATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTATAACAGT 180
BBA         ATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTATAACAGT 180
            ************************************************************

BLE         GTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAAATGCGCG 240
BBA         GTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAAATGCGCG 240
            ************************************************************

BLE         GCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTATCGGTGATCCGCAGAAAGAT 300
BBA         GCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTATCGGTGATCCGCAGAAAGAT 300
            ************************************************************

BLE         AAATCTCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCGCTTGGCGGCCGTTTC 360
BBA         AAATCTCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCGCTTGGCGGCCGTTTC 360
            ************************************************************

BLE         TATACAGGTACTGATATGGGAACGAATATGGAAGATTTCATTCACGCCATGAAAGAAACA 420
BBA         TATACAGGTACTGATATGGGAACGAATATGGAAGATTTCATTCACGCCATGAAAGAAACA 420
            ************************************************************

BLE         AACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGATTCCTCTATTCCAACT 480
BBA         AACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGATTCCTCTATTCCAACT 480
            ************************************************************

BLE         GCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGTTGTTTGGCAAGGACGAT 540
BBA         GCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGTTGTTTGGCAAGGACGAT 540
            ************************************************************

BLE         CTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCGGAA 600
BBA         CTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCGGAA 600
            ************************************************************

BLE         GGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGATATTAACGAGCAAAGCTTGGAG 660
BBA         GGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGATATTAACGAGCAAACGTTGGAG 660
            ************************************************** ******
```

(continued)

Figure 3.14 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from *Bacillus lentus* and published *Bacillus badius*. BLE = *Bacillus lentus*, BBA = *Bacillus badius* Conserved residues are indicated by asterisks (*).

(continued)

```
BLE         GCTATCCAGGAAAAAGCAAAAACAACATCCGGTTCTGTCACGGTAGTAGCGAGCGATGAA 720
BBA         GCTATCCAGGAAAAAGCAAAAACAACATCCGGTTCTGTCACGGTAGTAGCGAGCGATGAA 720
            ************************************************************

BLE         ATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGCATTTGGCGGCGTTGTTAATGAT 780
BBA         ATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGCATTTGGCGGCGTTGTTAATGAT 780
            ************************************************************

BLE         GAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCCAACAATCAGCTGCTT 840
BBA         GAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCCAACAATCAGCTGCTT 840
            ************************************************************

BLE         ACGGAGGATCACGGCAGACAGCTTGCAGACAAAGGCATTCTGTATGCTCCGGATTATATT 900
BBA         ACGGAGGATCACGGCAGACACCTTGCAGACAAAGGCATTCTGTATGCTCCGGATTATATT 900
            ******************* ****************************************

BLE         GTTAACTCTGGCGGTCTGATCCAAGTAGCCGACGAATTGTATGAGGTGAACAAAGAACGC 960
BBA         GTTAACTCTGGCGGTCTGATCCAAGTAGCCGACGAATTGTATGAGGTGAACAAAGAACGC 960
            ************************************************************

BLE         GTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCAGCAAGCGGAA 1020
BBA         GTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCAGCAAGCGGAA 1020
            ************************************************************

BLE         TTAGATCAAATCACCACAATGGAAGCAGCCAACAGAATGTGTGAGCAAAGAATGGCGGCA 1080
BBA         TTAGATCAAATCACCACAATGGAAGCAGCCAACAGAATGTGTGAGCAAAGAATGGCGGCA 1080
            ************************************************************

BLE         AGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAATGGGATATTCGCAAC 1140
BBA         AGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAATGGGATATTCGCAAC 1140
            ************************************************************

BLE         TAA 1143
BBA         TAA 1143
            ***
```

Figure 3.14 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenase genes from *Bacillus lentus* and published *Bacillus badius*. BLE = *Bacillus lentus*, BBA = *Bacillus badius* Conserved residues are indicated by asterisks (•).

```
CLUSTAL W (1.83) multiple sequence alignment

BLE             MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPYNS 60
BBA             MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPYNS 60
                ************************************************************

BLE             VEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKDKSPELFRAFGQFVDSLGGRF 120
BBA             VEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKDKSPELFRAFGQFVDSLGGRF 120
                ************************************************************

BLE             YTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNKMLFGKDD 180
BBA             YTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNKMLFGKDD 180
                ************************************************************

BLE             LGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQSLEAIQEKAKTTSGSVTVVASDE 240
BBA             LGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGSVTVVASDE 240
                ************************************:***********************

BLE             IYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRQLADKGILYAPDYI 300
BBA             IYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKGILYAPDYI 300
                ************************************************:***********

BLE             VNSGGLIQVADELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAANRMCEQRMAA 360
BBA             VNSGGLIQVADELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAANRMCEQRMAA 360
                ************************************************************

BLE             RGRRNSFFTSSVKPKWDIRN 380
BBA             RGRRNSFFTSSVKPKWDIRN 380
                ********************
```

Figure 3.15 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases from *Bacillus lentus* and published *Bacillus badius*. BLE = *Bacillus leentus*, BBA = *Bacillus badius*. Conserved residues are indicated by asterisks (*). (:) means amino acids which have the same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

Thermoactinomyces intermedius and *Rhodococcus* sp. M4, respectively. The alignment was shown in Figure 3.16.

3.3 Cloning of phenylalanine dehydrogenase gene

3.3.1 PCR amplification of the whole gene fragment

To express *phedh* gene in *E. coli* under T7 promoter of expression vector, pET-17b, the *phedh* gene was amplified using a pair of primers as described in section 2.7.3.1. The 5′-primer (Nde-N) comprised *Nde*I restriction site and 5′-end of *phedh* gene. The 3′-primer (Bam-C) consisted of *Bam*HI site, the TAA translational termination signal and 3′-end of *phedh* gene. Figure 3.17 showed the 1.1 kb PCR product of the *phedh* gene fragment amplified from *Nde*I digested DNA template at various annealing temperatures. All of annealing temperatures gave strong band of *phedh* gene without non-specific DNA fragment, so these PCR products were recovered and used for further cloning.

3.3.2 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 2.8.1.2, 2.8.1.3, 2.8.1.4 and 2.8.2.2, respectively. The eighty-two 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 2.8.1.1 and 2.8.1.2, respectively. The recombinant plasmids (pBLPheDH) in *E. coli* BL21(DE3) gave three bands, relaxed, linearized and supercoiled bands, on agarose gel electrophoresis. After digestion, a linear pET-17b with 3.3 kb and 1.1 kb of inserted *phedh* gene fragment were obtained as shown in Figure 3.18. From eighty-two transformants, sixteen transformants harboured recombinant plasmid (4.4 kb) which contain *phedh* gene. Therefore, these transformants were assayed for PheDH activity.

```
CLUSTAL W (1.83) multiple sequence alignment

BLE             -MSLVEKTSIIKD--FTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQP 57
BBA             -MSLVEKTSIIKD--FTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQP 57
BSP             MAKQLEKSSKIGN--EDVFQKIANHEQIVFCNDPVSGLQAIIAIHDTTLGPALGGTRMYP 58
BHA             ---MLTKTPTVTS-TLDIFTEMAEHEQVLFCHDPSSGLRAIIAIHDTTLGPALGGCRMYP 56
SUR             -MILVTLEQTLQDDKASVLDKMVEHEQILFCHDKATGLQAIIAVHDTTMGPALGGCRMAP 59
TIN             ---MRDVFEMMDR---------YGHEQVIFCRHPQTGLKAIIALHNTTAGPALGGCRMIP 48
RHO             --MSIDSALNWDG-----------EMTVTRFDRETGAHFVIRLDSTQLGPAAGGTRAAQ 46
                                        *  :   .  :* : :* :..*  *** ** *

BLE             YNSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKD-----KSPELFRAFGQF 112
BBA             YNSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKD-----KSPELFRAFGQF 112
BSP             YKNVDEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIGDPEKD-----KSPALFRAFGQF 113
BHA             YQTTEDALRDVLRLSKGMTQKCAAADVDFGGGKAVIIGDPAKD-----KSANLFRAFGQF 111
SUR             YKTMDLALKDVLRLSKGMTYKCAAADVDFGGGKSVIIGDPLKD-----KTPEKFRAFGQF 114
TIN             YASTDEALEDVLRLSKGMTYKCSLADVDFGGGKMVIIGDPKKD-----KSPELFRVIGRF 103
RHO             YSQLADALTDAGKLAGAMTLKMAVSNLPMGGGKSVIALPAPRHSIDPSTWARILRIHAEN 106
                *     ** *. :*: .** * : ::: :**** **   . :.     . .  :*  ..

BLE             VDSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATN 172
BBA             VDSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATN 172
BSP             VESLNGRFYTGTDMGTTMDDFVHAQKETNFINGIPEQYGGSGDSSIPTAQGVIYALKATN 173
BHA             VESLNGRFYTGTDMGTTMEDFVHALKETNGIVGIPKEYGGSGDSSVPTAKGVINSLKAIS 171
SUR             IESLNGRFYTGTDMGTTLEDFVHAMKETNYIVGKPVEYGGGGDSSIPTALGVFYGIKATN 174
TIN             VGGLNGRFYTGTDMGTNPEDFVHAARESKSFAGLPKSYGGKGDTSIPTALGVFHGMRATA 163
RHO             IDKLSGNYWTGPDVNTNSADMDTLNDTTEFVFGRSLERGGAGSSAFTTAVGVFEAMKAT- 165
                :  *.*.::**.*:.*.  *:      :: . * .   ** *.::..** **: .::*

BLE             KMLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQSLEAIQEKAKTTSGS 232
BBA             KMLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGS 232
BSP             QYLFGSDSLSGKTYAIQGLGKVGYKVAEQLLKAGADLFVTDIHENVLNSIKQKSEELGGS 233
BHA             QVVLKDKQFSGRTYAIQGLGKVGFKVAEELLKEGNDLYVSDLQESLPLRLQQLGQRLGRH 231
SUR             QNLFGDDKVEGRKYSIQGLGKVGYKVAEHIINEGGNVIVTDINEQAIADIQKLG---GSA 231
TIN             RFLWGTDQLKGRVVAIQGVGKVGERLLQLLVEVGAYCKIADIDS---VRCEQLKEKYGDK 220
RHO             VAHRGLGSLDGLTVLVQGLGAVGGSLASLAAEAGAQLLVADTDTERVAHAVALG------ 219
                       .. *    :**:* **  : .   : *    ::* .

BLE             VTVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRQLADKG 292
BBA             VTVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKG 292
BSP             VTIVKSDDIYSVQADIFVPCAMGGIINDKTIPKLKVKAVVGSANNQLKDLRHANVLNEKG 293
BHA             VEILHGDEIYEAAADVFVPCAQGAILNDATIARLKVKAIAGAANNQLEAERHGQMLHDQG 291
SUR             VRVVSSEEIYSQQADVFVPCAFGGVINDDTLKVLKVRGISGSANNQLAESRHGELLREKG 291
TIN             VQLVDVNRIHKESCDIFSPCAKGGVVNDDTIDEFRCLAIVGSANNQLVEDRHGALLQKRS 280
RHO             HTAVALEDVLSTPCDVFAPCAMGGVITTEVARTLDCSVVAGAANNVIADEAASDILHARG 279
                   :  : : .  .*:* *** *.::.  .   :    : *:*** :     .  *  :.
```

(continued)

Figure 3.16 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases. Amino acid sequences that were determined by automated Edman degradation were underlined. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus* sp. Conserved residues are indicated by asterisks (*). (:) means amino acids which have the same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

(continued)

```
BLE             ILYAPDYIVNSGGLIQVAD-ELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAAN 351
BBA             ILYAPDYIVNSGGLIQVAD-ELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAAN 351
BSP             ILYAPDYIVNAGGLIQVAD-ELYGPNKERVLLKTKEIYRSLLEIFNQAALDCITTVEAAN 352
BHA             IWFAPDYIVNSGGLIQVAD-ELYGSNEKRVLSKTNAIYDTILEIFHQAERHHITTLQAAN 350
SUR             ILYAPDYIVNGGGLIQVAD-ELYGTNPARVLAKTENIYTSLLEVFHQAEQDHMTTATAAD 350
TIN             ICYAPDYLVNAGGLIQVAD-ELEGFHEERVLAKTEAIYDMVLDIFHRAKNENITTCEAAD 339
RHO             ILYAPDFVANAGGAIHLVGREVLGWSESVVHERAVAIGDTLNQVFEISDNDGVTPDEAAR 339
                * :***::.*.** *::.. *:       *  ::  *   : :::. :  . :*.  **

BLE             RMCEQRMAARGRRNSFFTSSVKPKWDIRN 380
BBA             RMCEQRMAARGRRNSFFTSSVKPKWDIRN 380
BSP             RKCQKTIEGQQTRNSFFSRGRRPKWNIKE 381
BHA             QLCERRIRERARRNNFFVNRIRPKWNLRK 379
SUR             RMCEKRIADAKNRNSFFTQSNRPKWNFHQ 379
TIN             RIVMERLKKLTDIRRILLED--PRNSARR 366
RHO             TLAGRRAREASTTTATA------------ 356
                         .
```

Figure 3.16 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases. Amino acid sequences that were determined by automated Edman degradation were underlined. BLE = *Bacillus lentus*, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae*, TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus sp.* Conserved residues are indicated by asterisks (*). (:) means amino acids which have the same group of side chains and similar size while (.) means amino acids which have the same group of side chains but different size.

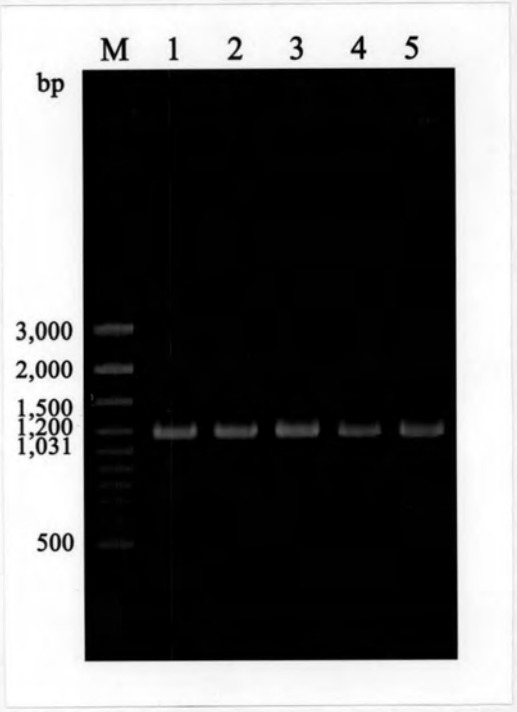


Figure 3.17 Whole *phedh* gene amplification at various annealing temperatures

Lane M = 100 bp DNA ladder

Lane 1 = PCR product using annealing temperature at 53.2°C

Lane 2 = PCR product using annealing temperature at 55.3°C

Lane 3 = PCR product using annealing temperature at 56.5°C

Lane 4 = PCR product using annealing temperature at 57.5°C

Lane 5 = PCR product using annealing temperature at 58.4°C

Note: The template was *Nde*I digested chromosomal DNA and primers were Nde-N and Bam-C.

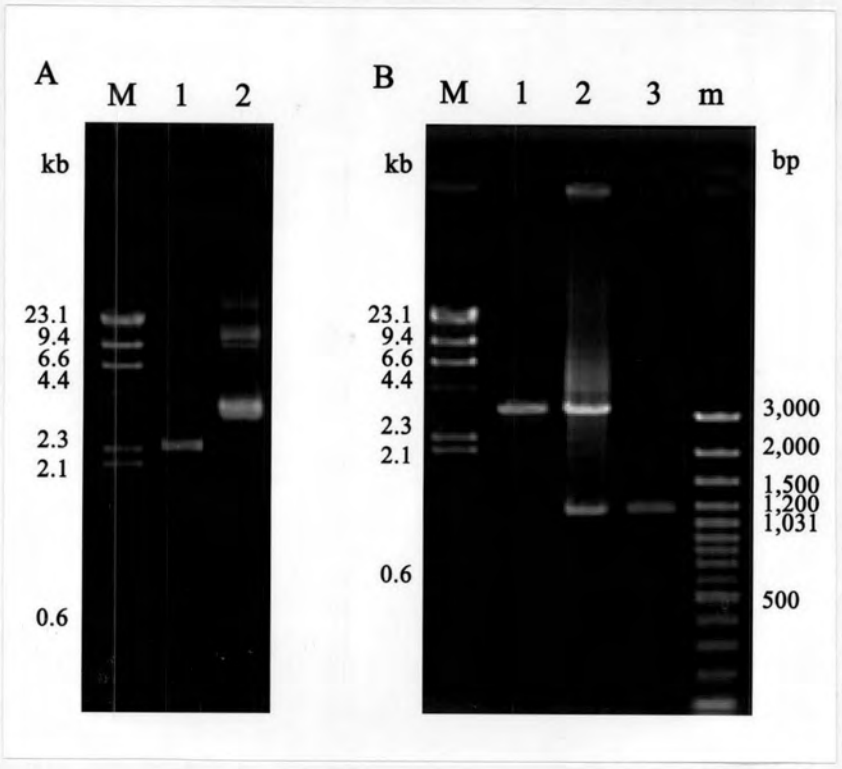


Figure 3.18 Electrophoretic pattern of pBLpheDH

A: Extracted plasmid pattern

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

Lane 1 = undigested pET-17b

Lane 2 = extracted recombinant plasmid

B: Restriction pattern of pBLpheDH

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

Lane 1 = *Nde*I-*Bam*HI digested pET-17b

Lane 2 = *Nde*I-*Bam*HI digested pBLpheDH

Lane 3 = PCR product of *phedh* gene

Lane m = 100 bp DNA ladder

3.3.3 Phenylalanine dehydrogenase activity of transformants

The sixteen recombinant clones were grown as described in 2.9.2 to prepare crude extract for determination of the enzyme activity. *E. coli* BL21(DE3) with and without plasmid pET-17b were used as references. The result was shown in Table 3.1. The clones showed various levels of the specific activity from 9.34-92.0 units/mg protein. The highest specific activity, 92.0 fold higher than that of *Bacillus lentus* (Inkure, 2005), was produced by *E. coli* BL21(DE3) transformant No. 2. Thus, this recombinant clone was used in the further experiments.

3.4 Optimization of phenylalanine dehydrogenase gene expression

3.4.1 Optimization of phenylalanine dehydrogenase gene expression

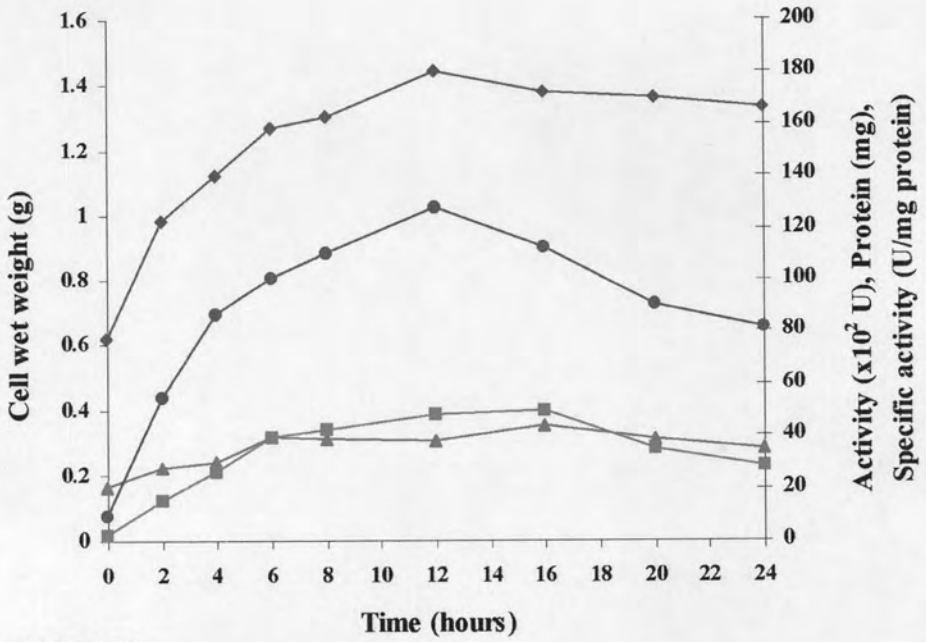
The transformant No. 2 giving the highest PheDH activity had been grown and induced by IPTG at final concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM at various times (0, 2, 4, 8, 12, 16, 20 and 24 hours) before the cells were harvested as described in 2.10. The results were displayed in Figure 3.19. When recombinant clone was cultured without IPTG induction, the expression of *phedh* gene was slightly increased until 16 hours with specific activity of 44.0 units/mg protein after that expression of gene tended to decrease. When using 0.2 mM IPTG, the maximal specific activity was 2 times higher than that of absence of IPTG. When transformant was induced by various final concentrations of IPTG (0.2, 0.4, 0.6, 0.8 and 1 mM), the expression was highest at induction time of 8 hours using 0.2 mM IPTG. Although, other each final concentration (0.4, 0.6 and 0.8 mM) shown highest the expressions at induction time less than 8 hours, they had the expression less than that of induction with 0.2 mM at induction time 8 hours. Hence, the optimum condition for induction of *phedh* gene was 0.2 mM IPTG at 8 hours of induction. The obtained specific activity of PheDH was about 111 units/mg protein.

Table 3.1 Phenylalanine dehydrogenase activity from crude extract of *E. coli* BL21(DE3) transformants[a]

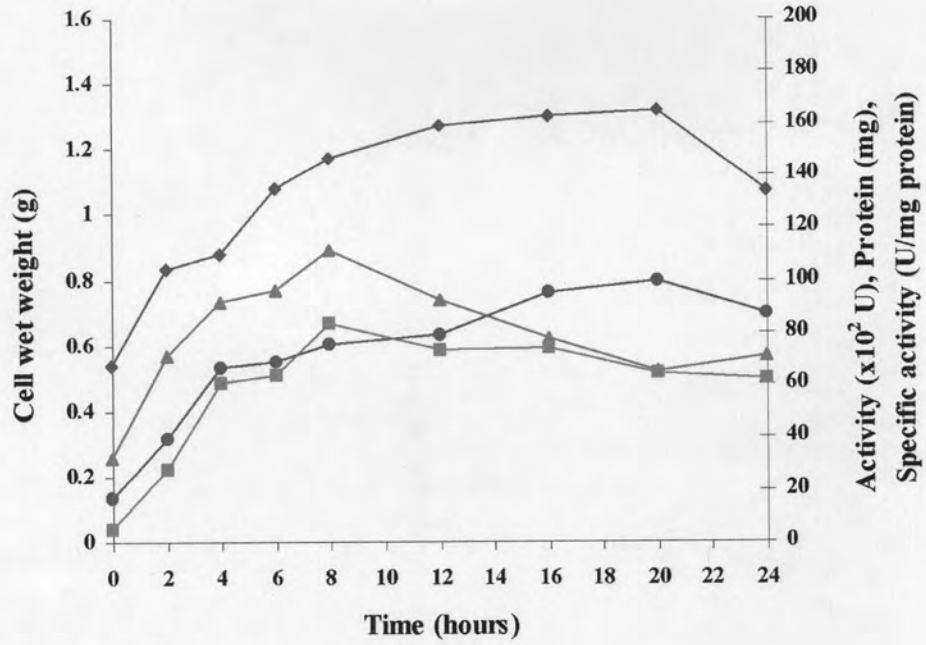
Sources	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
Bacillus lentus	223	217	1.0
E. coli BL21(DE3)	0	78.2	0
E. coli BL21(DE3) harbouring pET-17b	0	90.4	0
Transformant No.1	4,180	76.9	54.4
Transformant No.2	9,080	98.7	92.0
Transformant No.3	2,180	80.2	27.2
Transformant No.4	2,880	76.5	37.6
Transformant No.5	1,490	56.1	26.6
Transformant No.6	2,390	46.4	51.5
Transformant No.7	853	50.3	17.0
Transformant No.8	2,500	86.5	28.9
Transformant No.9	7,200	98.2	73.3
Transformant No.10	6,720	96.4	69.7
Transformant No.11	6,530	103	63.4
Transformant No.12	5,970	79.0	75.6
Transformant No.13	2,500	78.3	31.9
Transformant No14	1,890	93.1	20.3
Transformant No.15	786	84.2	9.34
Transformant No.16	7,850	103	76.3

[a] Crude extracts were prepared from 200 ml of cell culture by induction with 0.4 mM IPTG for 4 hours.

0 mM IPTG



0.2 mM IPTG



(continued)

Figure 3.19 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) clone No.2 at various final concentrations of IPTG

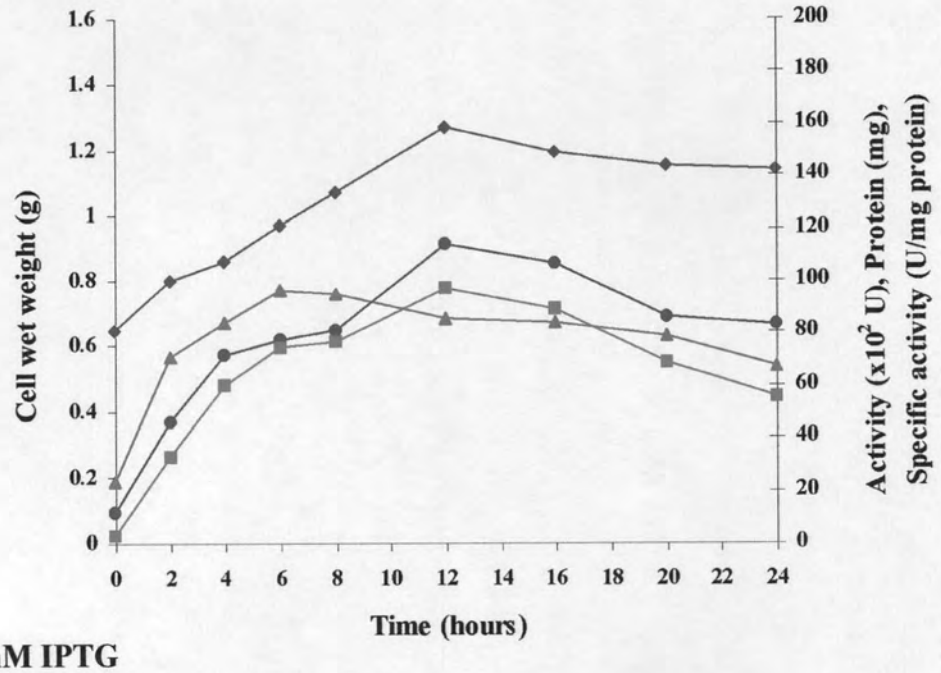
◆ = cell wet weight

■ = total acitivity

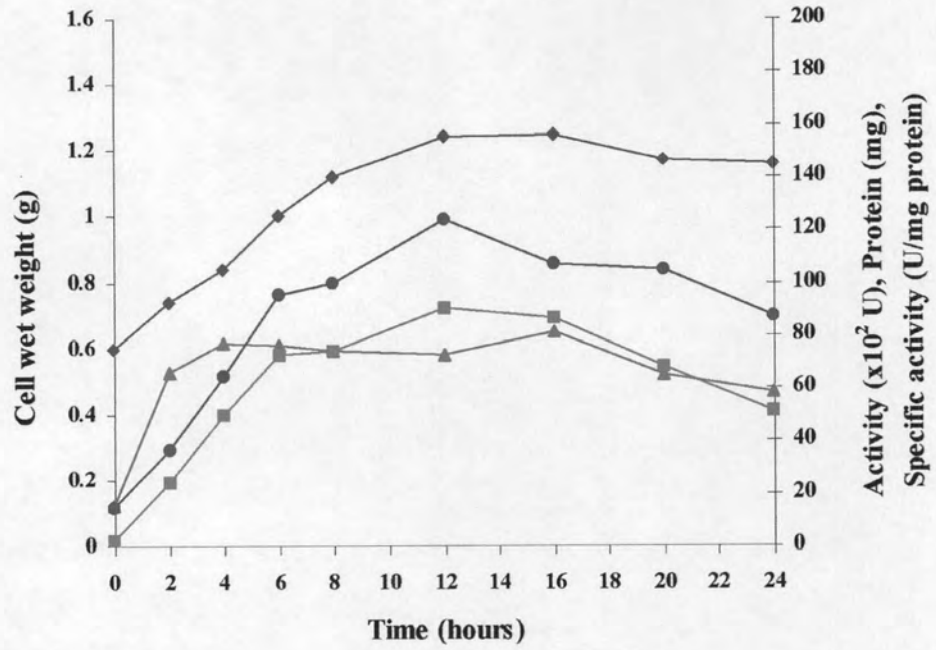
▲ = specific activity

● = protein

0.4 mM IPTG



0.6 mM IPTG



(continued)

Figure 3.19 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) clone No.2 at various final concentrations of IPTG

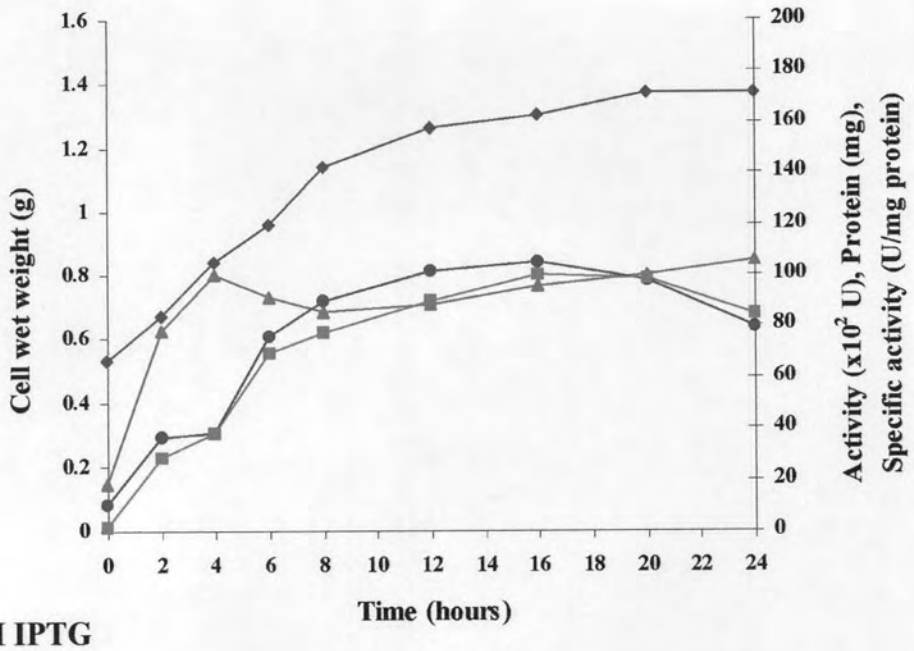
◆ = cell wet weight

■ = total acitivity

▲ = specific activity

● = protein

0.8 mM IPTG



1.0 mM IPTG

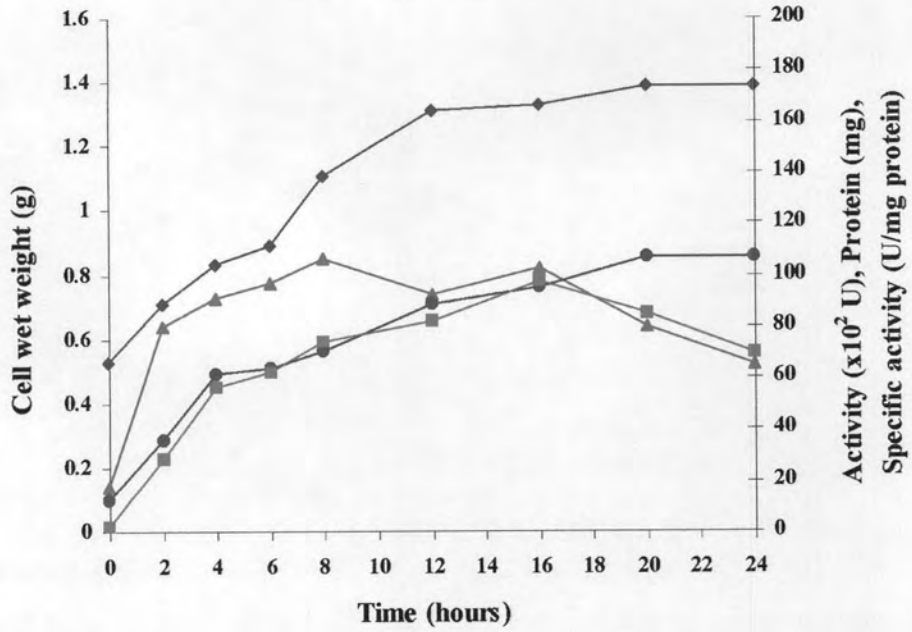


Figure 3.19 Expression of phenylalanine dehydrogenase gene in *E. coli* BL21(DE3) clone No.2 at various final concentrations of IPTG

◆ = cell wet weight

■ = total acitivity

▲ = specific activity

● = protein

3.4.2 Protein pattern of cells and crude extracts

The 1.5 ml of transformant No.2 cultures which had grown at various concentrations of IPTG and various times as described in 2.10 were harvested and centrifuged in microcentrifuge tube. The cell pellets were resuspended in 100 µl of 5x sample buffer. Seven microliters of cell samples or 20 µg protein of crude extracts were subjected to electrophoresis on 10% SDS-polyacrylamide gel. The results in Figure 3.20-3.25 showed that the intensity of major protein band at 42 kDa of cell and crude extracts at each induction time was quite corresponded to the level of enzyme activity from its crude extract.

3.5 Stability of phenylalanine dehydrogenase gene (pBLPheDH) in *E. coli* BL21(DE3)

The 1^{st}, 5^{th}, 10^{th}, 15^{th}, 20^{th}, 25^{th}, 30^{th}, 35^{th}, 40^{th}, 45^{th} and 50^{th} subcultured colonies were picked up to culture and assay for oxidative deamination activity. The result shown in Table 3.2 indicated that each generation also showed high expression level of *phedh* gene when compared to that of 1^{st} generation. The parent showing highest expression level gave specific activity of 93.2 units/mg protein in crude extract. Later on, expression of *phedh* gene was gradually decreased with increasing number of generation. Despite the fact that the transformant No.2 was daily subcultured for 50 days, the *phedh* gene expression in *E. coli* BL21(DE3) remained 57.7% of that of the parent. It was implied that this transformant has high stability of recombinant plasmid.

3.6 Purification of phenylalanine dehydrogenase

3.6.1 Preparation of crude extract

Crude recombinant PheDH was prepared from 5 g of transformant No. 2 which was cultivated from 1.6 liter of medium as described in section 2.12.1. Crude

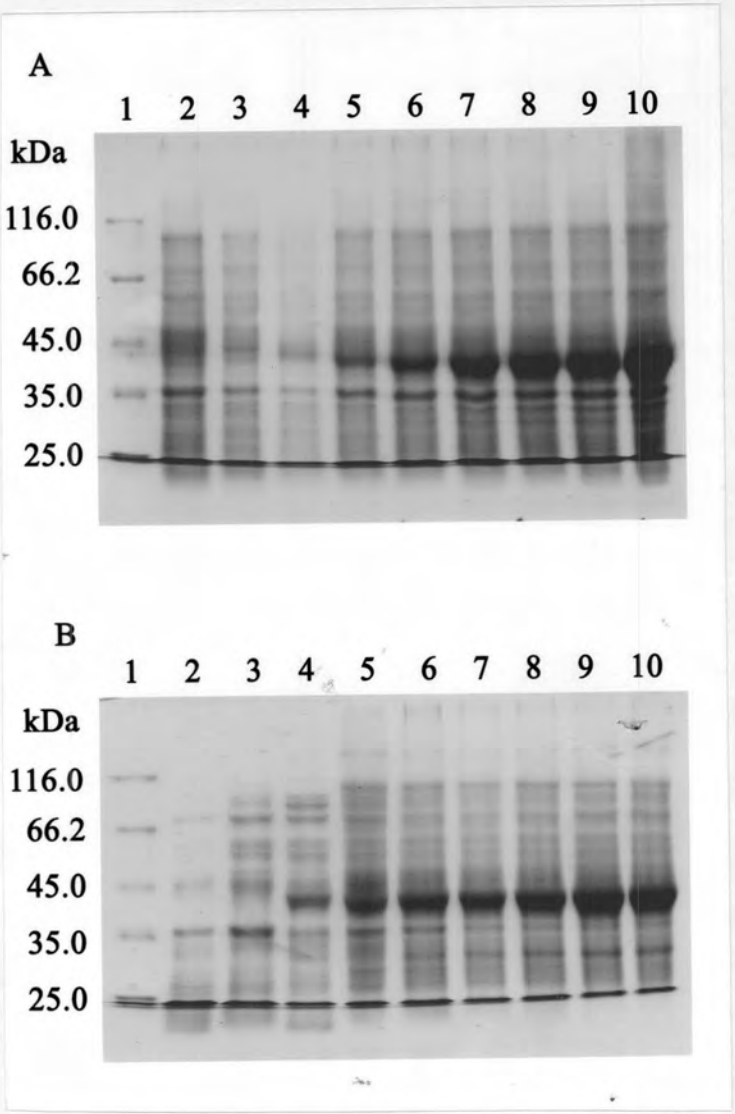


Figure 3.20 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 0 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

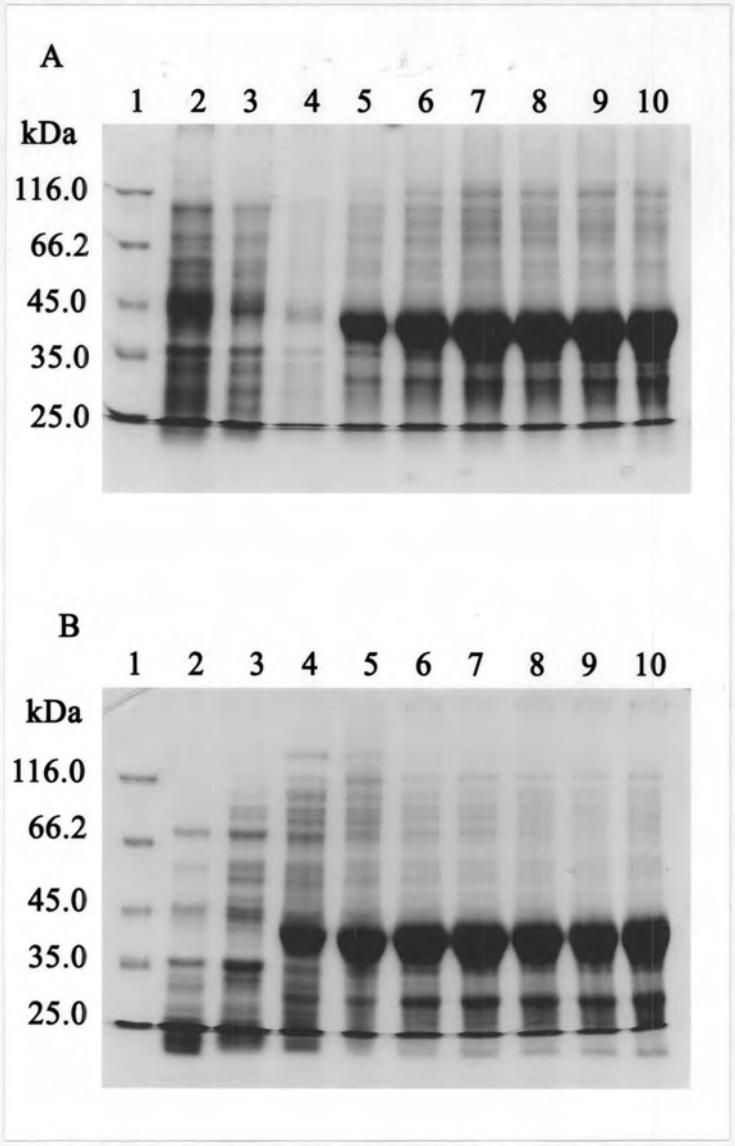


Figure 3.21 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 0.2 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

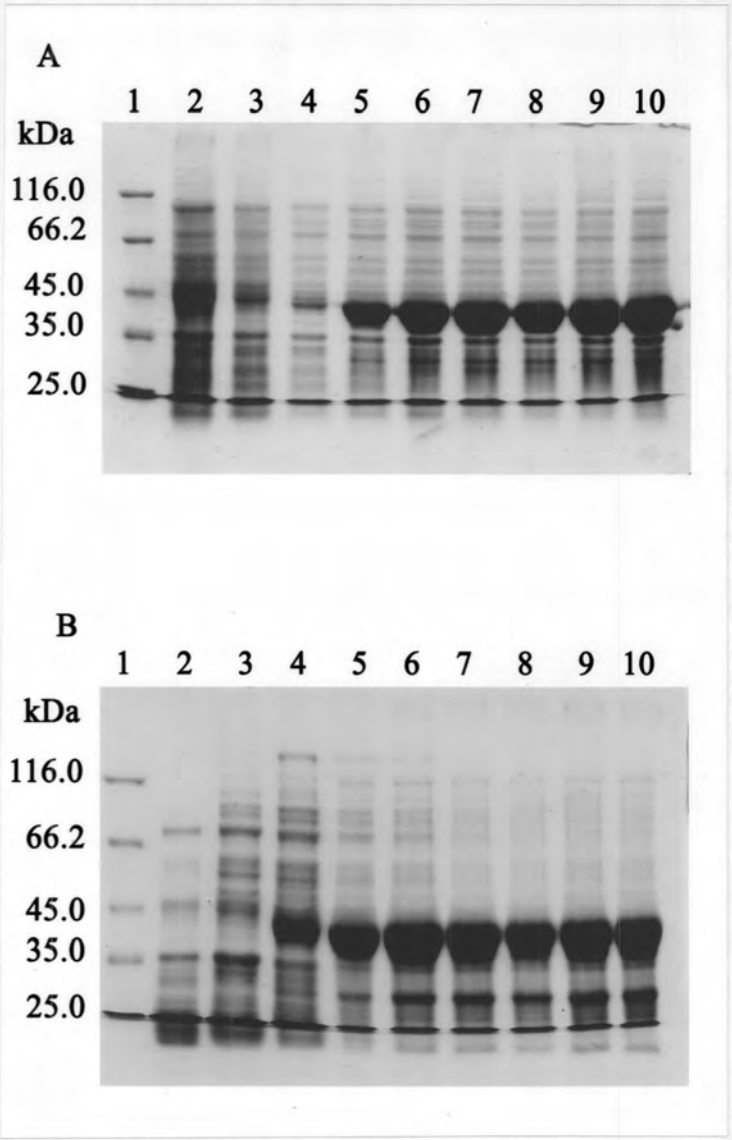


Figure 3.22 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 0.4 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

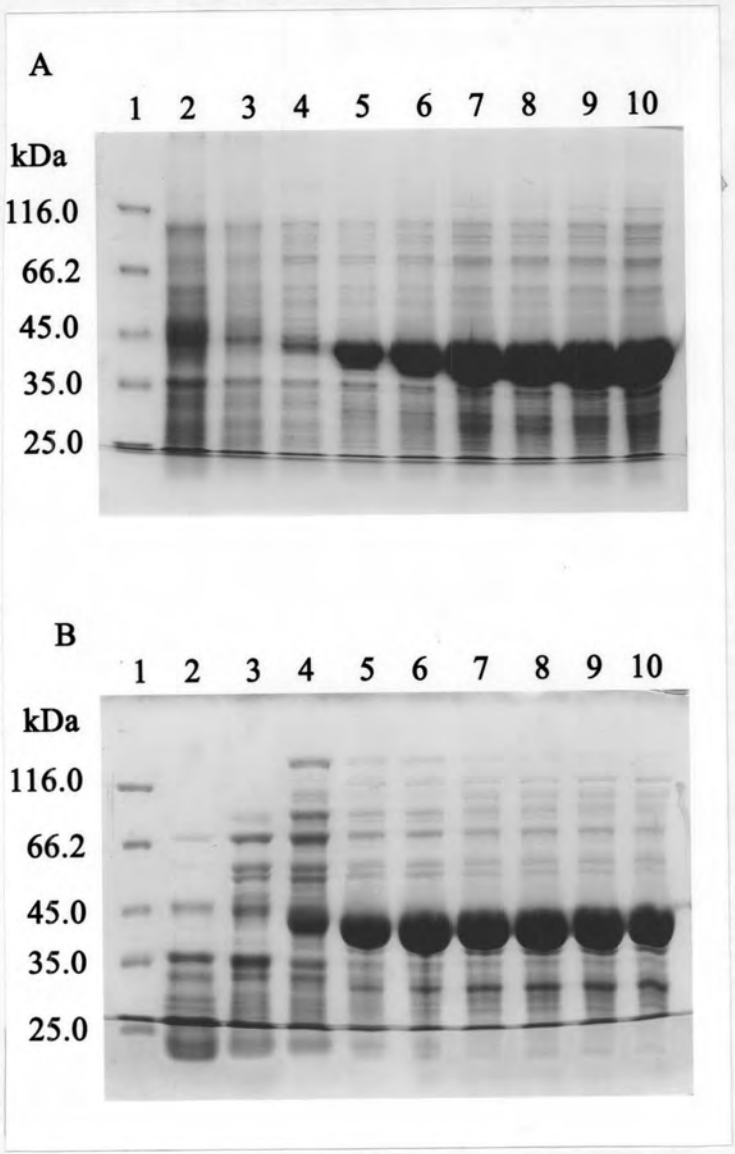


Figure 3.23 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 0.6 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

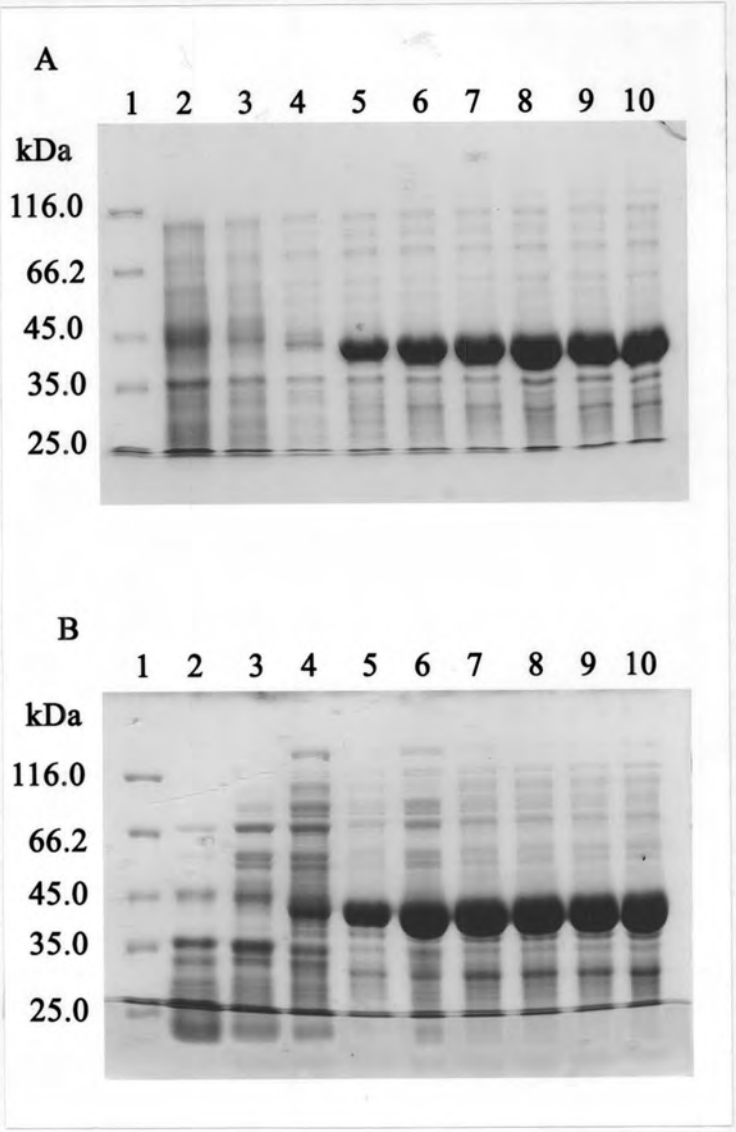


Figure 3.24 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 0.8 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

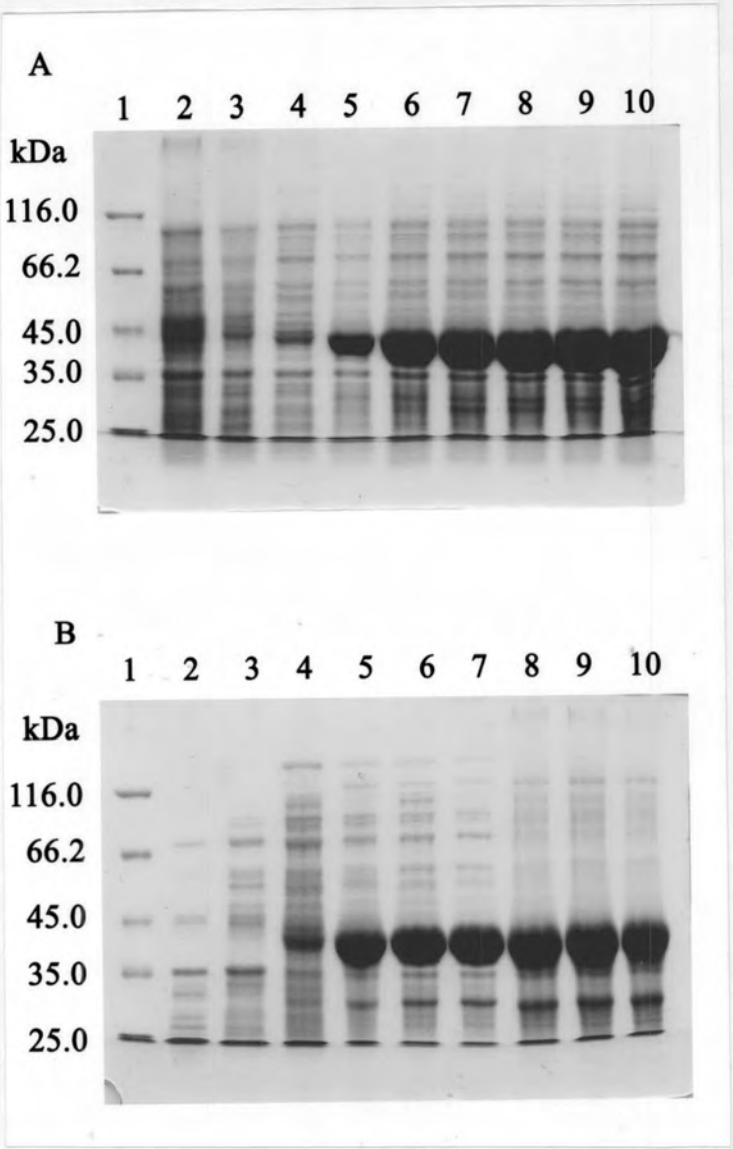


Figure 3.25 SDS-PAGE of whole cell and crude extract of pBLPheDH clone induced by 1.0 mM IPTG at various times

A: whole cell

B: crude extract

Lane 1 = protein marker

Lane 2 = cell and crude extract of *E. coli* BL21(DE3)

Lane 3 = cell and crude extract of *E. coli* BL21(DE3) harbouring pET-17b

Lane 4-10 = cell and crude extract of pBLPheDH clone at various induction times: 0, 2, 4, 8, 12, 16 and 20 hours, respectively

Table 3.2 Stability of phenylalanine dehydrogenase gene expression in pBLPheDH clone[a]

Number of subculture	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
1	10,900	117	93.2
5	9,250	104	89.2
10	8,260	129	75.5
15	7,840	119	71.3
20	7,090	103	68.5
25	7,370	108	68.2
30	7,280	110	66.2
35	7,930	124	64.0
40	7,960	128	62.0
45	7,750	125	62.0
50	6,360	118	53.9

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

The data represent the mean values of three independent experiments.

extract contained 712 mg proteins and 55,500 units of PheDH activity. Thus, the specific activity of the enzyme in the crude preparation was 77.9 units/mg protein which was 78 times higher than that of wild type crude extract.

3.6.2 Ammonium sulfate precipitation

First step of purification, crude extract was purified by ammonium sulfate precipitation as described in section 2.12.2.1. To determine the proper ammonium sulfate concentration for enzyme precipitation, the precipitation using 0-30%, 30-40%, 40-50% and 50-60% ammonium sulfate were performed in preliminary experiment. The result showed that most of enzyme activity was found in the 40-50% fraction. Therefore, protein from 40-50% saturated ammonium sulfate fraction was collected and dialysed against the buffer. The recovered protein and enzyme activity were 350 mg and 28,800 units, respectively. The specificity activity of the enzyme from this step was 82.3 units/mg protein.

3.6.3 DEAE-Toyopearl column chromatography

The enzyme from 40-50% saturated ammonium sulfate precipitation was loaded into DEAE-Toyopearl column as described in section 2.12.2.2. The chromatographic profile was shown in Figure 3.26. The unbound proteins were eluted from column by the phosphate buffer, whereas the bound proteins were eluted by linear salt gradient of 0 to 0.5 M potassium chloride in the buffer. PheDH was eluted at about 0.25 M potassium chloride as indicated in the profile. PheDH fractions were pooled, dialysed against the buffer, concentrated by aquasorb to reduce enzyme volume. This operation obtained the enzyme with 153 mg proteins and 21,400 activity units. The specificity activity of the enzyme from this step was 140 units/mg protein. The enzyme was purified 1.80 fold with 38.6% recovery.

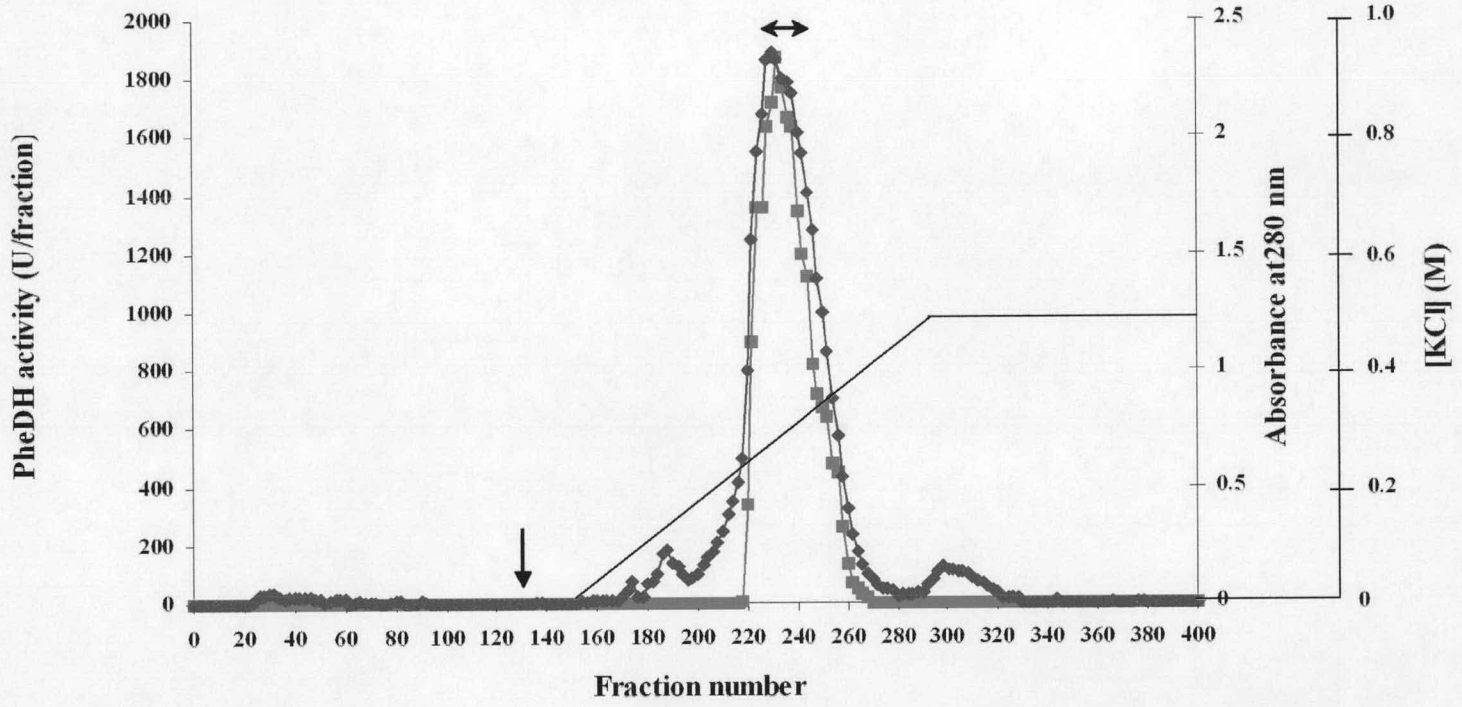


Figure 3.26 Purification of phenylalanine dehydrogenase from pBLPheDH clone by DEAE - Toyopearl column

The enzyme solution was applied to DEAE -Toyopearl column and washed with 10 mM potassium phosphate buffer, pH 7.4 containing 0.01%(v/v) β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The bound proteins were eluted by 0-0.5 M KCl in the same buffer at the flow rate of 1 ml/min. The fractions of 3 ml were collected. The arrow indicates where gradient started. The protein peak from fraction number 222 to 256 was pooled (⟷). ◆ A_{280}, ■ PheDH activity, — [KCl]

3.6.4 Butyl-Toyopearl column chromatography

The pooled active fraction from DEAE-Toyopearl column was applied to the Butyl-Toyopearl column as described in section 2.12.2.3. The chromatographic profile was shown in Figure 3.27. The unbound proteins were eluted from column with buffer containing 25% saturated ammonium sulfate and then the bound proteins were eluted with 22.5% saturated ammonium sulfate in the buffer. The enzyme was eluted immediately with the buffer containing 22.5% salt saturation. The pooled fraction containing PheDH activity was dialyzed against the buffer and concentrated by aquasorb. The proteins remained from this step was 105 mg with 17,200 units of PheDH activity. The specific activity of the enzyme was 164 units/mg protein. The PheDH was purified 2.11 fold with about 31.0% recovery. The purified enzyme from this step was kept at 4°C for further experiments.

The summary of purification of PheDH was shown in Table 3.3.

3.6.5 Determination of enzyme purity and protein pattern on non-denaturing polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis

The enzyme from each step of purification was examined for purity and protein pattern by SDS-PAGE as described in section 2.13.2. In addition, purified enzyme from the last step of purification was run on non-denaturing PAGE followed by protein and activity staining as described in 2.13.1.1 and 2.13.1.2, respectively. The results are shown in Figure 3.28. The purified enzyme in lane 5A on SDS-PAGE showed a single band which corresponded with a single protein band in lane 1B and its activity staining in lane 2B on native-PAGE. It indicated that PheDH from Butyl-Toyopearl column was a pure enzyme. The molecular weight of PheDH subunit was calculated to be 42 kDa by its mobility in SDS-PAGE compared with those of standard proteins.

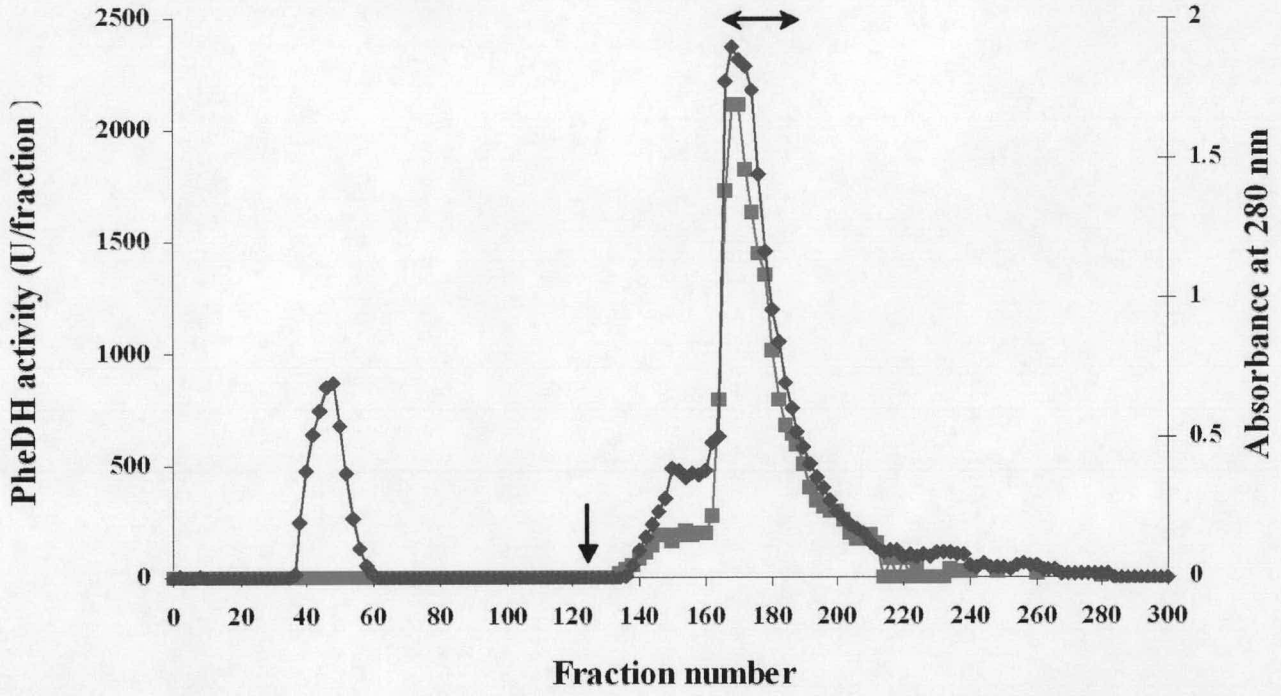


Figure 3.27 Purification of phenylalanine dehydrogenase from pBLPheDH clone by Butyl-Toyopearl column

The enzyme solution was applied to Butyl-Toyopearl column and washed with 25% saturated ammonium sulfate in 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA until A_{280} decreased to base line. The enzyme was eluted by 22.5% saturated ammonium sulfate in the same buffer at the flow rate of 1 ml/ min. The fractions of 2 ml were collected. The arrow indicates where 22.5% saturated ammonium sulfate in the same buffer begun. The protein peak from fraction number 164 to 192 was pooled (⟷). ◆ A_{280}, ■ PheDH activity

Table 3.3 Purification of phenylalanine dehydrogenase from pBLPheDH clone[a]

Purification steps	Total activity (unit)	Total protein (mg)	Specific activity (units/mg protein)	% Recovery	Purification fold
Crude enzyme	55,500	712	77.9	100	1
40-50% saturated ammonium sulfate precipitation	28,800	350	82.3	51.9	1.06
DEAE-Toyopearl column	21,400	153	140	38.6	1.80
Butyl-Toyopearl column	17,200	105	164	31.0	2.11

[a] Crude extract was prepared from 1.6 liters (5 g cell wet weight) of cell culture.

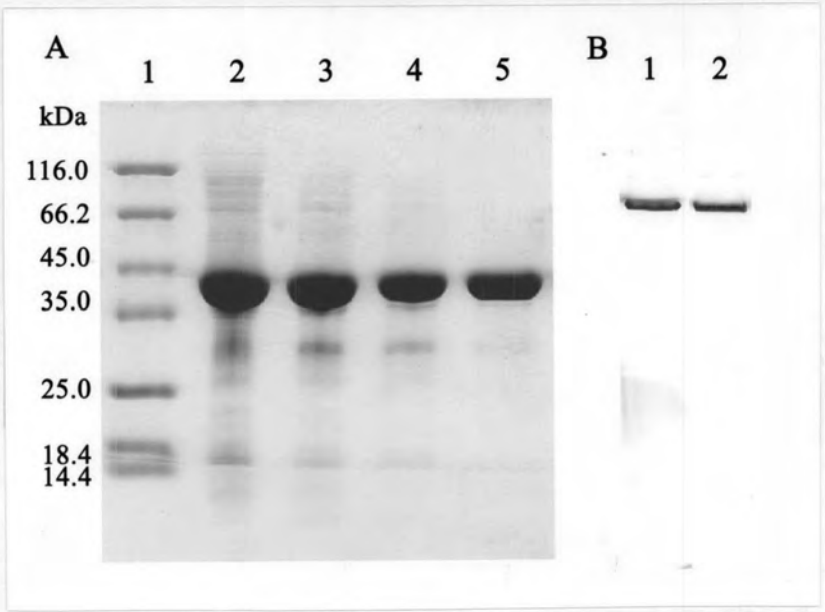


Figure 3.28 Protein pattern from each step of purification investigated by SDS-PAGE and the purified PheDH at last step examined by native-PAGE

A: SDS-PAGE

Lane 1 = protein marker

Lane 2 = crude extract

Lane 3 = 40-50% saturated ammonium sulfate precipitation

Lane 4 = DEAE-Toyopearl column

Lane 5 = Butyl-Toyopearl column

B: native-PAGE

Lane 1 = Butyl-Toyopearl column (protein staining)

Lane 2 = Butyl-Toyopearl column (activity staining)

3.7 Characterization of phenylalanine dehydrogenase

3.7.1 Molecular weight determination of phenylalanine dehydrogenase

The native molecular weight of recombinant PheDH was determined from high molecular weight calibration curve obtained by gel filtration on HPLC by TSK gel G3000SW column as mentioned in section 2.14.1 (Figure 3.29). The molecular weight of the native enzyme was estimated to be about 340,000 Da. The subunit molecular weight of the enzyme was estimated to be about 42,000 Da by SDS-PAGE, as previously described in section 3.6.5. The result indicated that the enzyme was consisted of eight identical subunits.

3.7.2 Substrate specificity of phenylalanine dehydrogenase

Substrate specificity of PheDH in the oxidative deamination and amination directions were studied as mentioned in section 2.14.2. The ability of the enzyme to catalyze the oxidative deamination of various amino acids and their derivatives was determined at a concentration of 20 mM as shown in Table 3.4. In addition to L-phenylalanine (relative activity, 100%), which was the preferred substrate, various L-amino acids served as substrates (relative activities given in parentheses): *p*-fluoro-DL-phenylalanine (30), *m*-fluoro-DL-phenylalanine (16), *S*-methyl-L-cysteine (13), L-norleucine (11), L-methionine (8), L-ethionine (7), *o*-fluoro-DL-phenylpyruvate (5), L-tryptophan (5), L-valine (4), L-norvaline (4), L-leucine (3), L-alloisoleucine (3), L-isoleucine (1) and L-histidine (1). The tested amino acids, which were not substrate for the oxidative deamination, were D-phenylalanine, L-alanine, and L-proline.

The reductive amination of the keto acids (10 mM) was shown in Table 3.5. Among the substrate tests, the highest activity occurred when phenylpyruvate was used as substrate. The relative activities for α-ketocaproate, α-ketoisocaproate, α-ketovalerate, α-ketoisovalerate, α-keto-β-methyl-n-valerate, α-keto-γ-methiol-

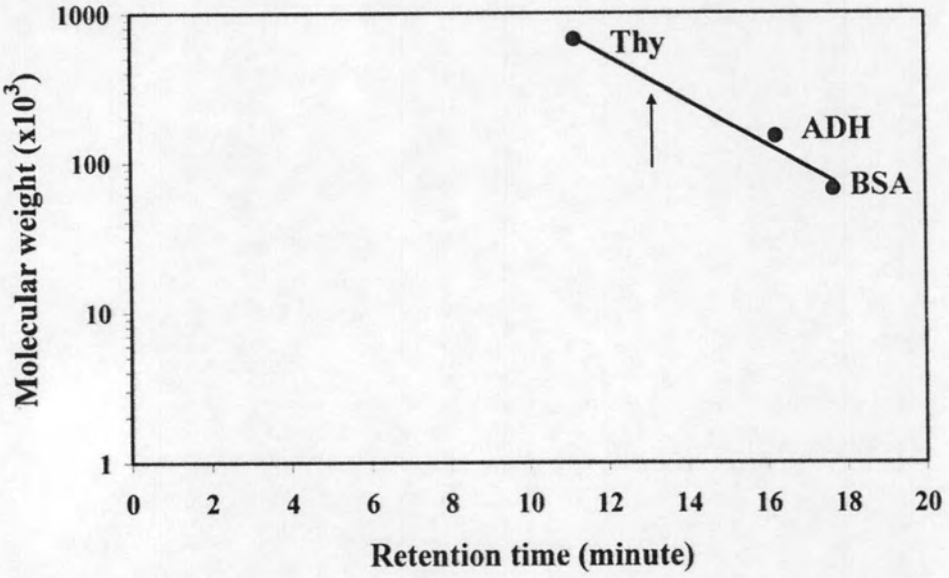


Figure 3.29 Calibration curve for molecular weight of phenylalanine dehydrogenase by gel filtration on HPLC

Thy	=	thyroglobulin	(669,000 Da)
ADH	=	alcohol dehydrogenase	(150,000 Da)
BSA	=	bovine serum albumin	(66,000 Da)

Arrow indicated a determined molecular weight of PheDH.

Table 3.4 Substrate specificity of phenylalanine dehydrogenase in oxidative deamination[a]

Substrate[b]	Relative activity (%)
L-phenylalanine	100
p-fluoro-DL-phenylalanine	30
m-fluoro-DL-phenylalanine	16
o-fluoro-DL-phenylpyruvate	5
S-methyl-L-cysteine	13
L-norleucine	11
L-ethionine	7
L-methionine	8
L-histidine	1
L-leucine	3
L-tryptophan	4
L-valine	5
L-alloisoleucine	3
L-isoleucine	1
L-norvaline	4

[a] The data represent the mean values of three independent experiments.

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

Table 3.5 Substrate specificity of phenylalanine dehydrogenase in reductive amination[a]

Substrate[b]	Relative activity (%)
phenylpyruvate	100
α-ketocaproate	31
α-ketoisocaproate	11
α-ketovalerate	12
α-ketoisovalerate	9
α-keto-β-methyl-n-valerate	4
α-keto-γ-methiol-butyrate	1

[a] The data represent the mean values of three independent experiments.

[b] Final concentration of keto acids was 10 mM.

butyrate were 31, 11, 12, 9, 4 and 1%, respectively when compared with the activity for phenylpyruvate. The tested keto acids which was not substrate for the reductive amination was α-keto-n-butyrate.

3.7.3 Coenzyme specificity of phenylalanine dehydrogenase

Coenzyme specificity of PheDH was investigated as described in section 2.14.3. PheDH required NAD^+ as a natural coenzyme for oxidative deamination. Some analogs of NAD^+ could serve as a coenzyme as shown in Table 3.6. 3-Acetylpyridine-NAD^+ (167%) was much better coenzyme than NAD^+. Nicotinamide-1, N^6-ethenoadenine dinucleotide, nicotinamide hypoxanthine dinucleotide, nicotinamide guanine dinucleotide and thionicotinamide adenine dinucleotide showed 75, 89, 77 and 52% relative activity to that of NAD^+, while $NADP^+$, nicotinic acid adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide showed no activity.

3.7.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the enzyme activity in both of the oxidation deamination and reduction amination was examined at various pHs of buffers ranged from 6.0 to 12.5 as mentioned in section 2.14.4. The result was shown in Figure 3.30. The enzyme exhibited maximal activity at pH 10.7 for oxidative deamination (Figure 3.30 A) while the maximal activity for reductive amination was at pH 7.8 (Figure 3.30 B).

3.7.5 Effect of temperature on phenylalanine dehydrogenase activity

The effect of temperature on enzyme activity was investigated as described in section 2.14.5. The temperature was varied from 25°C to 75°C. The result was shown in Figure 3.31. The enzyme performed the highest activity at 45°C for oxidative deamination and at 50°C for reductive amination.

Table 3.6 Coenzyme specificity of phenylalanine dehydrogenase[a]

Coenzyme[b]	Relative activity (%)
β-nicotinamide adenine dinucleotide	100
3-acetylpyridine adenine dinucleotide	167
nicotinamide-1, N^6-ethenoadenine dinucleotide	75
nicotinamide hypoxanthine dinucleotide	89
nicotinamide guanine dinucleotide	77
thionicotinamide adenine dinucleotide	52
nicotinic acid adenine dinucleotide	0
β-nicotinamide adenine dinucleotide phosphate	0
3-pyridinealdehyde adenine dinucleotide	0

[a] The data represent the mean values of three independent experiments.

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

The assay was conducted at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm ($\varepsilon = 9.1 \times 10^3$ $M^{-1}.cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), 340 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide-1, N^6-ethenoadenine dinucleotide, 334 nm ($\varepsilon = 6.9 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinamide guanine dinucleotide, 340 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); nicotinic acid adenine dinucleotide (deamido-NAD^+), 338 nm ($\varepsilon = 6.2 \times 10^3$ $M^{-1}.cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1}.cm^{-1}$); and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3 \times 10^3$ $M^{-1}.cm^{-1}$). The reaction was carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH.

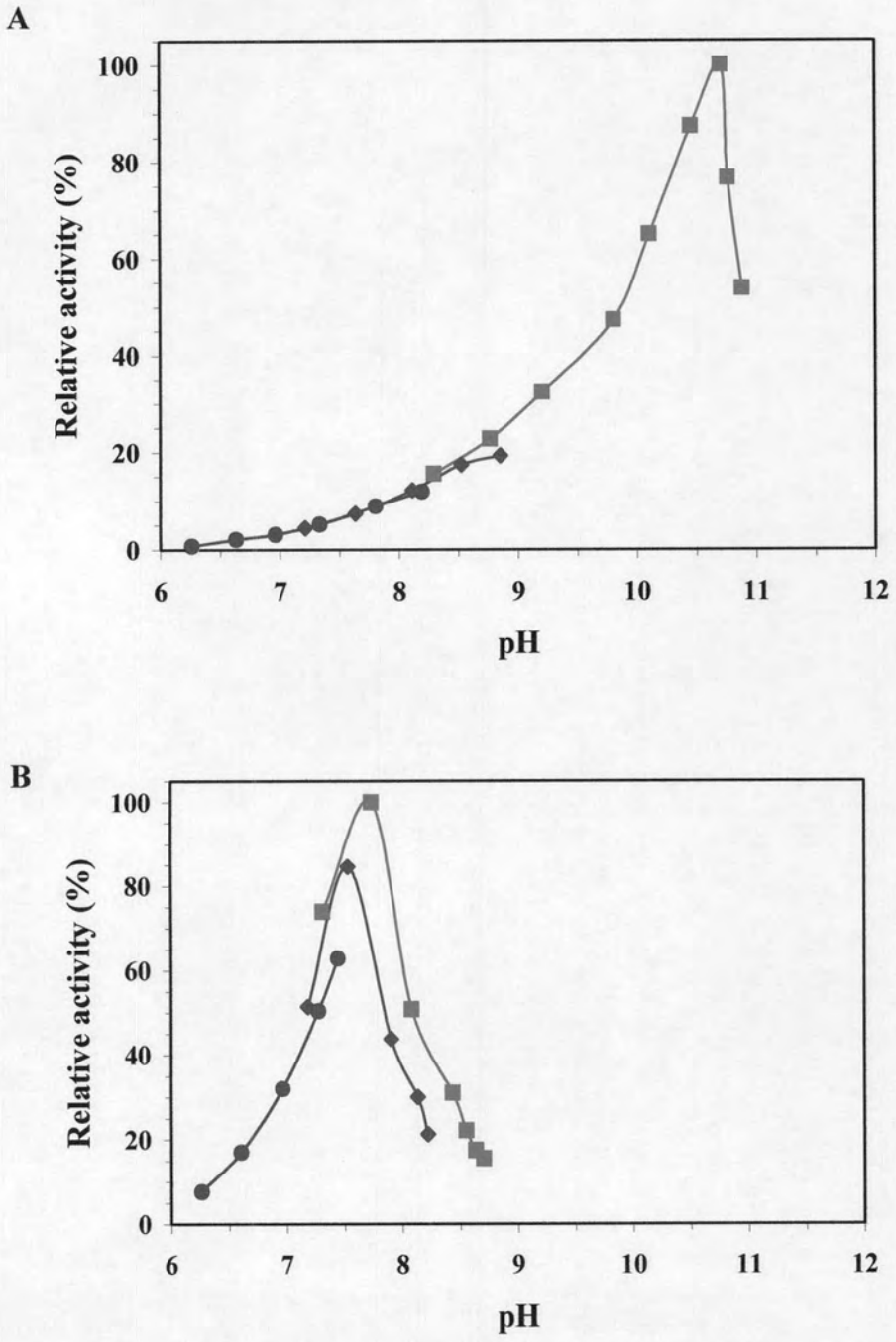


Figure 3.30 Effect of pH on phenylalanine dehydrogenase activity

The PheDH activities for oxidative deamination (A) and reductive amination (B) were measured at different pHs with 200 mM potassium phosphate buffer (●), Tris-HCl buffer (◆) and glycine-KCl-KOH buffer (■).

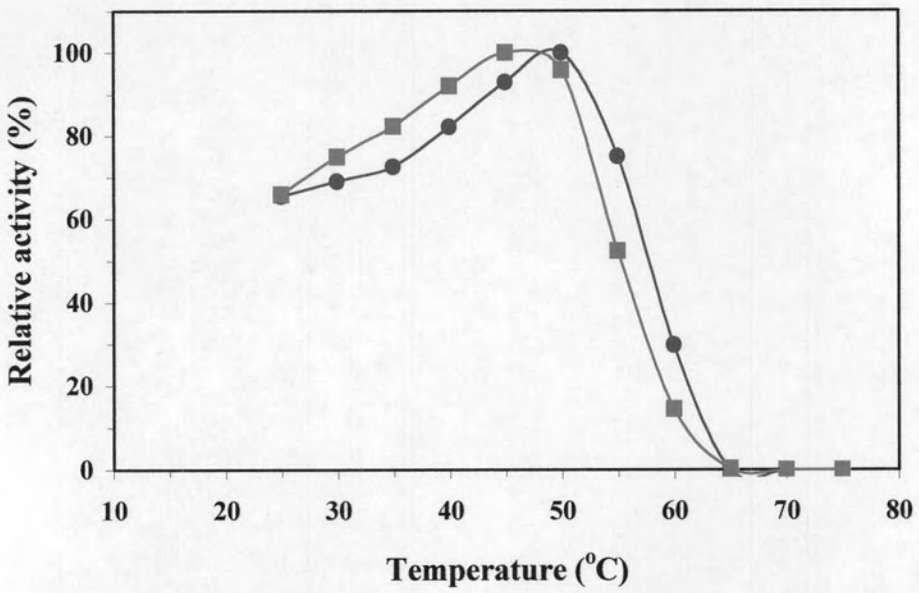


Figure 3.31 Effect of temperature on phenylalanine dehydrogenase activity

The PheDH activities for oxidative deamination (■) and reductive amination (●) reactions were measured at various temperatures varying from 25°C to 75°C.

3.7.6 Effect of pH on phenylalanine dehydrogenase stability

The pH stability of PheDH was studied as described in section 2.14.6. The enzyme was preincubated at 30°C for 20 minutes in various 10 mM buffers at various pHs ranging from 4.0 to 12.5. The result was shown in Figure 3.32. The enzyme was stable over the pH ranged from 7.0 to 11.0.

3.7.7 Effect of temperature on phenylalanine dehydrogenase stability

The thermostability of PheDH was studied as described in section 2.14.7. The enzyme was preincubated at various temperatures ranged from 30°C to 70°C for 10 min. The enzyme activity of non-preincubated enzyme was defined as 100% relative activity. The result was shown in Figure 3.33 A. The enzyme retained its full activity at temperature up to 45°C and lost about half of its activity at about 55°C. At 60°C, PheDH absolutely lost its activity. The enzyme stability was tested at 45°C by incubation for 0 to 15 days and investigated its activity everyday. The remained deamination activities were expressed as the percentage of the original activity. The result was shown in Figure 3.33 B. The enzyme was fully stable at 45°C only 1 hour and retained 50% of its activity after treatment for 3 days. The enzyme activity was relatively decreased with increasing of incubation time and the activity was completely lost after incubation for 14 days.

3.8 Kinetic studies of phenylalanine dehydrogenase

3.8.1 Initial velocity studies for oxidative deamination

A series of steady-state kinetic analysis was carried out to investigate the kinetic parameters. First, initial velocity studies for oxidative deamination were performed. The concentration of L-phenylalanine was varied in the presence of several fixed concentration of NAD^+. Double-reciprocal plots of initial velocity against reciprocals of L-phenylalanine concentrations gave a family of straight lines,

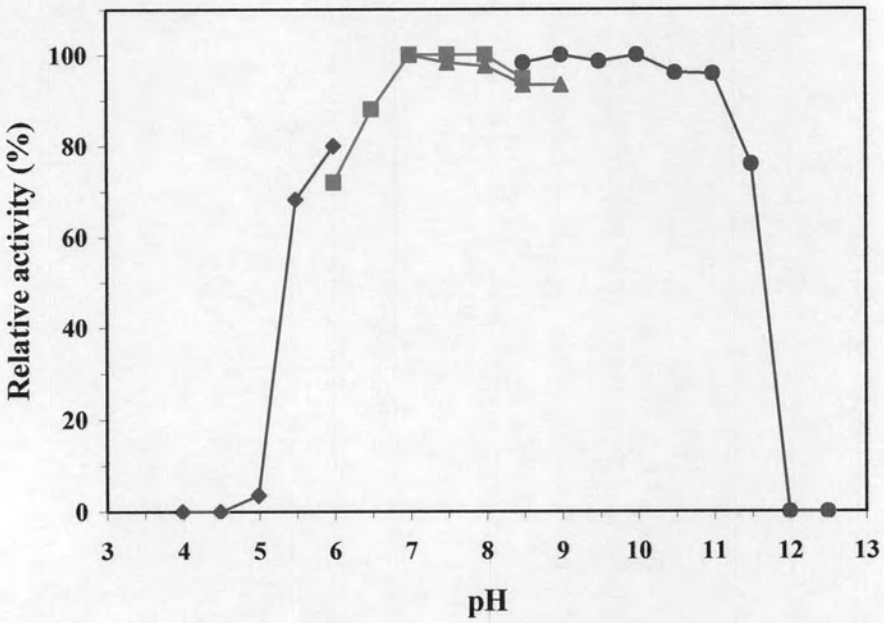
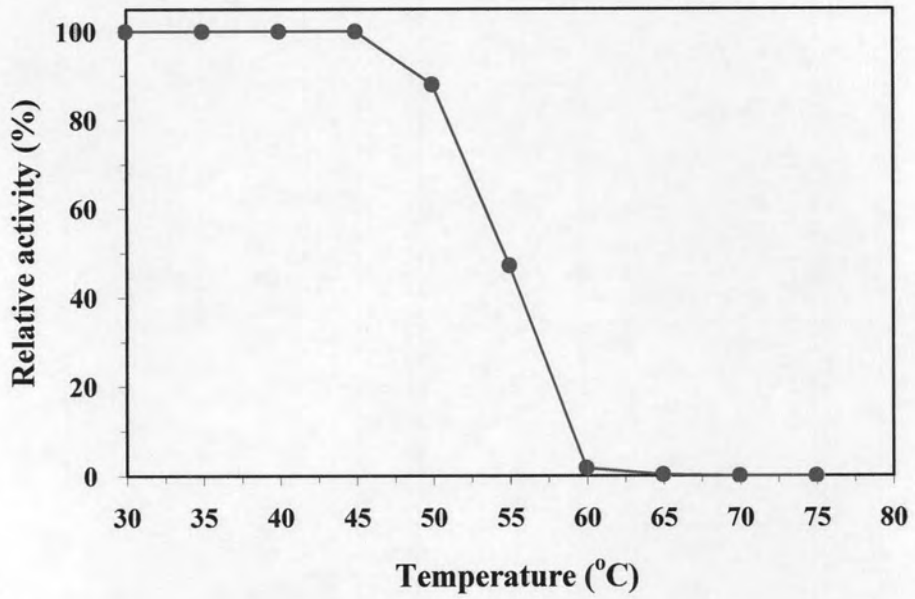


Figure 3.32 Effect of pH on phenylalanine dehydrogenase stability

The enzymes in buffers at various pHs ranged from 4.0 to 12.5 were incubated at 30°C for 20 minutes and then the relative activities were assayed for the oxidative deamination. The 10 mM buffers used were acetate buffer (pH 4.0-6.0; ◆), potassium phosphate buffer (pH 6.0-8.5; ■), Tris-HCl buffer (pH 7.0- 9.0; ▲) and glycine-KCl-KOH buffer (pH 8.5-12.5; ●).

A



B

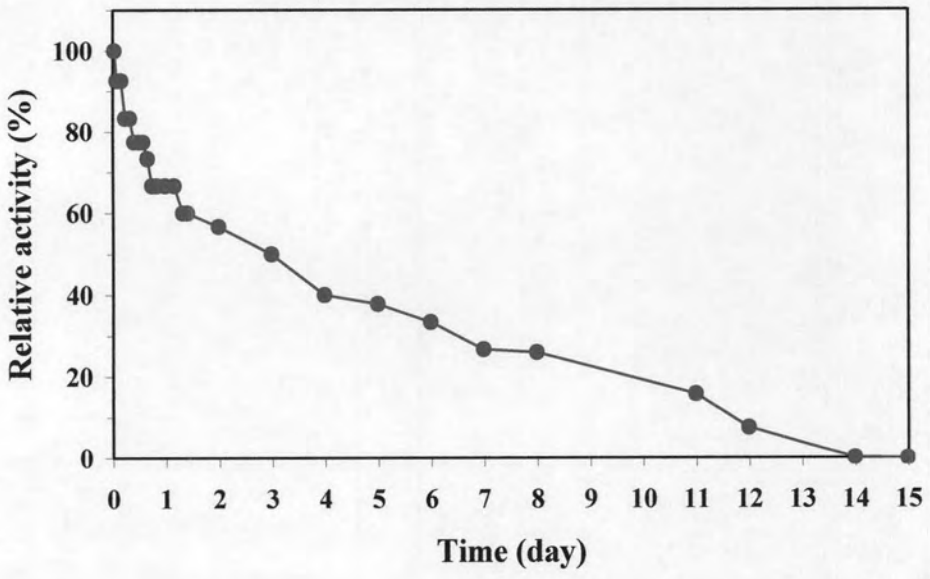


Figure 3.33 Effect of temperature on phenylalanine dehydrogenase stability

A The effect of temperature on stability of the enzyme activity was performed at 30 to 75°C for 10 minutes before the residual oxidative deamination activity was determined under standard condition at 30°C.

B The enzyme stability was tested at 45°C and the residual oxidative deamination activity was determined under standard condition at 30°C.

which intersected in the upper left quadrant as shown in Figure 3.34 A. These results showed that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD^+ and L-phenylalanine (Cleland, 1971). The apparent K_m value for L-phenylalanine was calculated to be 0.45 mM. From the secondary plots of intercept at the ordinate versus reciprocal concentrations of NAD^+, the apparent K_m value for NAD^+ was calculated to be 0.40 mM as shown in Figure 3.34 B.

3.8.2 Initial velocity studies for reductive amination

A kinetic analysis of reductive amination was performed to investigate possible reaction mechanism.

3.8.2.1 At a saturating concentration of NADH (0.2 mM), the double reciprocal plots of initial velocities versus phenylpyruvate concentrations at several fixed concentrations of NH_4Cl gave straight intersecting lines as shown in Figure 3.35 A. The apparent K_m value for phenylpyruvate was calculated to be 0.15 mM from this figure. The apparent K_m value for NH_4Cl was calculated to be 48 mM from the secondary plots of intercept at the ordinate versus reciprocal concentrations of NH_4Cl as shown in Figure 3.35 B.

3.8.2.2 At a saturating concentration of phenylpyruvate (10 mM), the double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentrations of NADH gave parallel straight lines as shown in Figure 3.36.

3.8.2.3 At a saturating concentration of NH_4Cl (500 mM), the double reciprocal plots of initial velocities versus NADH concentration at several fixed concentrations of phenylpyruvate gave straight intersecting lines as shown in Figure 3.37 A. The apparent K_m of NADH was calculated to be 0.15 mM. The secondary plots of intercept at the ordinate versus reciprocal concentrations of phenylpyruvate, as shown in Figure 3.37 B, gave the same apparent K_m of 0.15 mM with that in Figure 3.35 A.

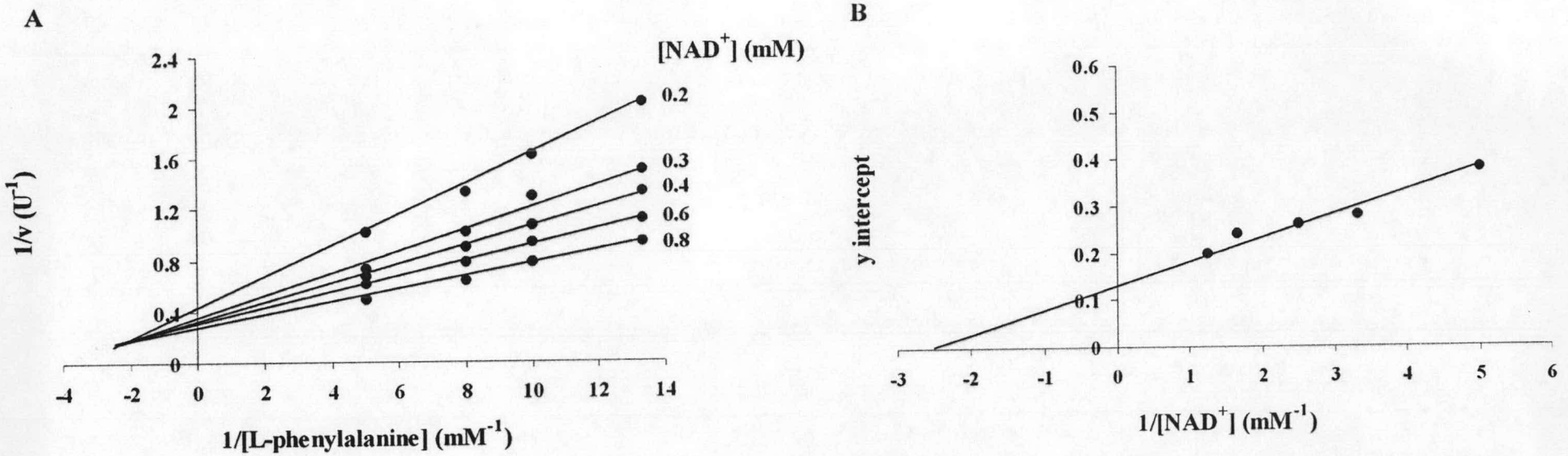


Figure 3.34 Initial velocity patterns for oxidative deamination

A Double-reciprocal plots of initial velocities versus L-phenylalanine concentrations at a series of fixed concentrations of NAD^+. Concentrations of NAD^+ were 0.2, 0.3, 0.4, 0.6 and 0.8 mM, respectively.

B Secondary plots of y intercepts versus reciprocal NAD^+ concentrations

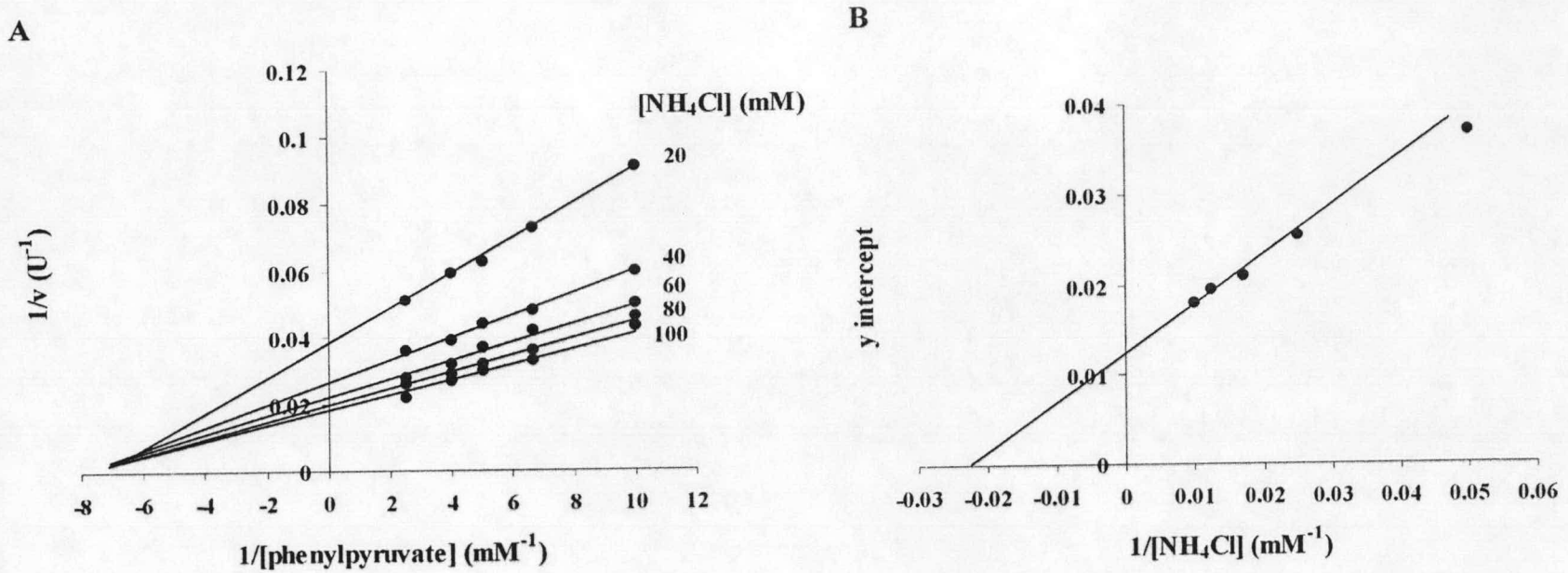


Figure 3.35 Initial velocity patterns for reductive amination (phenylpyruvate versus NH_4Cl)

A Double-reciprocal plots of initial velocities versus phenylpyruvate concentrations at several fixed concentrations of NH_4Cl in the presence of a high concentration (0.2 mM) of NADH. The concentrations of NH_4Cl were 20, 40, 60, 80 and 100 mM, respectively.

B Secondary plots of y intercepts versus reciprocal NH_4Cl concentrations

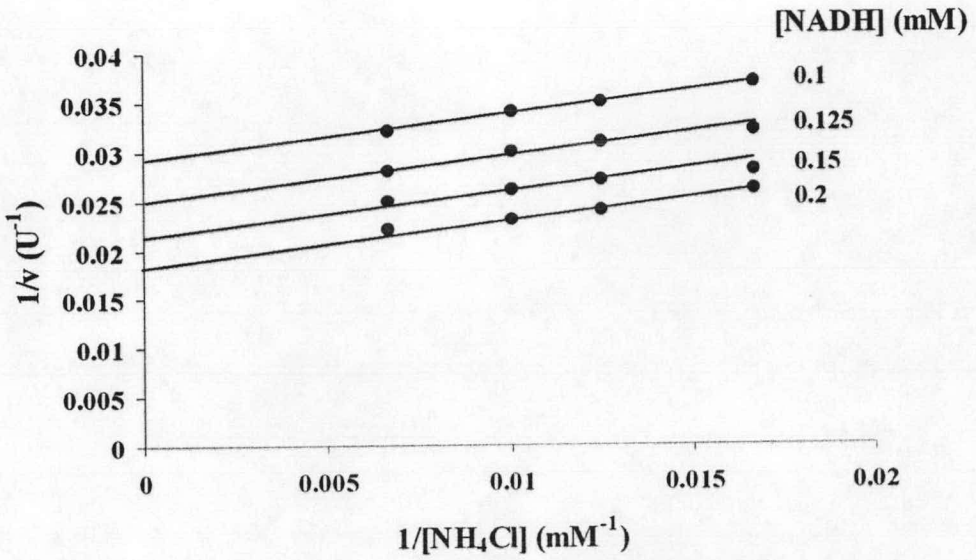


Figure 3.36 Initial velocity patterns for reductive amination (NH_4Cl versus NADH)

Double-reciprocal plots of initial velocities versus NH_4Cl concentration at several fixed concentrations of NADH in the presence of a high concentration (10 mM) of phenylpyruvate. The concentrations of NADH were 0.1, 0.125, 0.15 and 0.2 mM, respectively.

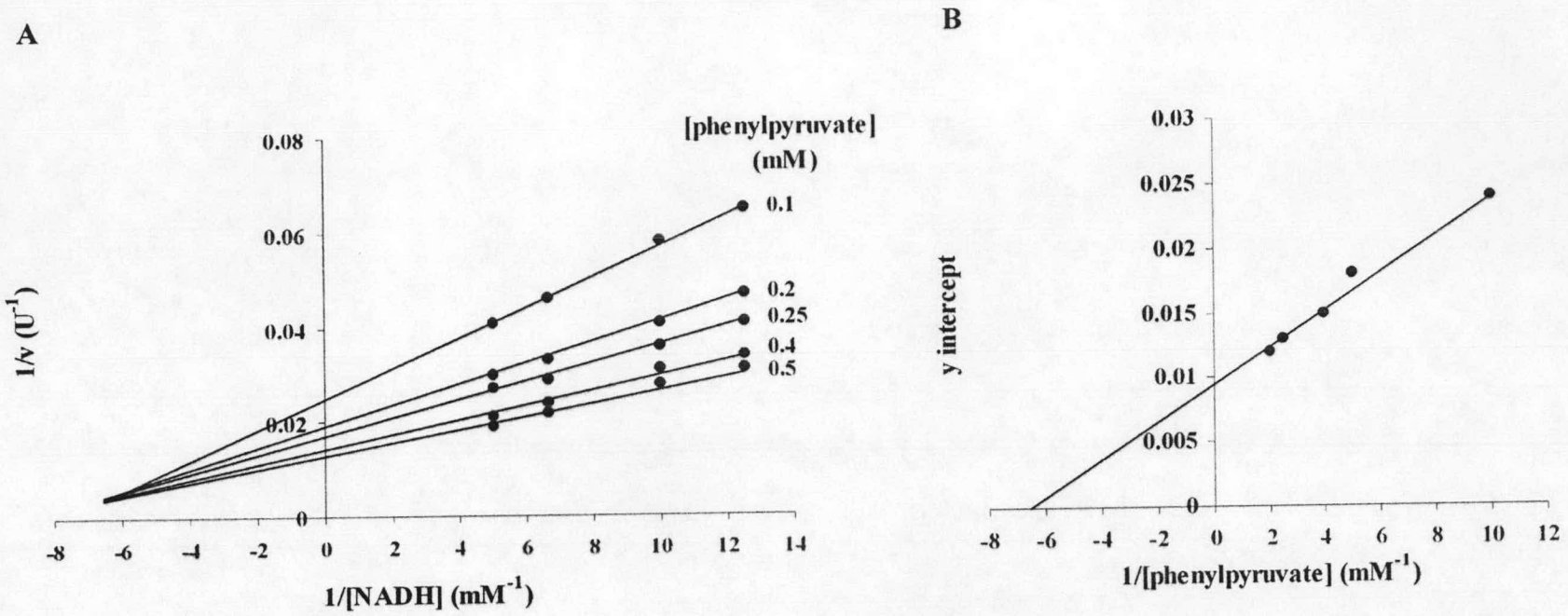


Figure 3.37 Initial velocity patterns for reductive amination (NADH versus phenylpyruvate)

A Double-reciprocal plots of initial velocities versus NADH concentrations at several fixed concentrations of phenylpyruvate in the presence of a high concentration (500 mM) of NH_4Cl . The concentrations of phenylpyruvate were 0.1, 0.2, 0.25, 0.4 and 0.5 mM, respectively.

B Secondary plots of y intercepts versus reciprocal phenylpyruvate concentrations

The apparent K_m values of the substrates of PheDH were summarized in Table 3.7.

Table 3.7 The apparent K_m values of substrates of phenylalanine dehydrogenase from *E. coli* BL21 (DE3) harbouring pBLPheDH

Substrate	K_m (mM)
L-phenylalanine	0.45
NAD^+	0.40
phenylpyruvate	0.15
NH_4Cl	48
NADH	0.15