CHAPTER III

RESULTS

3.1 DNA Extraction

The chromosomal DNA which was isolated from thermotolerant bacterial strain BC1 by using the method modified from Frederick *et al.* (1995) was determined for its quality and quantity by agarose gel electrophoresis. High molecular weight DNA larger than 23.1 kb was obtained. A_{260}/A_{280} ratio, which was in the range between 1.8-2.0, indicated high purity. The DNA concentration was about 0.2 - 0.5 μg/μl. Thus the quality of obtained DNA was suitable for molecular procedure such as restriction endonuclease digestion and PCR amplification.

3.2 Identification of thermotolerant bacterial strain BC1

3.2.1 Identification of bacterial strain BC1 by morphological and biochemical properties

Morphological and biochemical properties of thermotolerant bacterial strain BC1 which were reported by TISTR showed that thermotolerant bacterial strain BC1 was gram positive bacteria which could produce acid from glycerol, ribose and 5-keto-gluconate while the cultivation of this bacteria in other carbon sources did not give fermentative acid production. Furthermore, enzyme activities of arginine dihydrolase, lysine decarboxylase, ornitine decarboxylase, tryptophane desaminase could not be found as shown in Table 3.1. According to Bergey's manual of determinative bacteriology, TISTR suggested that thermotolerant bacterial strain BC1 was closely similar to *Brevibacillus brevis* (Appendix G). Surprisingly, Bergey's manual of determinative bacteriology indicated that *Brevibacillus brevis* could not catalyze the deamination of phenylalanine. Thus to confirm this result, 16S rRNA gene sequence was analyzed.

Table 3.1 Characteristics of the thermotolerant bacterial strain BC1 by TISTR

Characteristics	Reaction
Gram reaction	+ve
Fermentative production of acid from :	
glycerol	+
ribose	+
5-keto-gluconate	+
erythritol	-
D-arabinose	-
L-arabinose	-
D-xylose	-
L-xylose	-
adonitol	-
galactose	-
β-methyl-D-xyloside	-
glucose	-
fructose	-
mannose	-
L-sorbose	-
rhamnose	-
dulcitol	-
2-keto-gluconate	-
gluconate	-
inositol	-
mannitol	-
sorbitol	-
α-methyl-D-mannoside	-
α-methyl-D-glucoside	-
N-acetyl-glucosamine	-

(continued)

(continued)

Table 3.1 Characteristics of the thermotolerant bacterial strain BC1 by TISTR

Characteristics	Reaction
Fermentative production of acid from :	
amygdalin	-
arbutin	-
esculin	-
salicine	-
cellobiose	-
maltose	-
lactose	-
melibiose	-
saccharose	-
starch	-
inuline	-
melezitose	-
D-raffinose	-
trehalose	-
glycogen	-
xylitol	-
β-gentiobiose	-
D-turanose	-
D-lyxose	-
D-tagatose	-
D-fucose	-
L-fucose	-
D-arabitol	-
L-arabitol	-

(continued)

(continued)

Table 3.1 Characteristics of the thermotolerant bacterial strain BC1 by TISTR

Characteristics	Reaction
β-galactosidase production (*ortho*-nitro-phenyl-galactosidase)	-
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	-
H_2S production	-
Urease production	-
Tryptophane desaminase	-
Indole production of tryptophan	-
Acetoin production (VP test)	-
Hydrolysis of gelatin	+
Fermentation / oxidation of :	
glucose	-
mannitol	-
inositol	-
sorbitol	-
rhamnose	-
sucrose	-
melibiose	-
amygdalin	-
arabinose	-

Remark: + ve = gram positive bacteria
+ = positive reaction
- = negative reaction

3.2.2 Identification of the bacterial strain BC1 by 16S rRNA sequence

Chromosomal DNA and single fresh colony of thermotolerant bacterial strain BC1 were used as sources of DNA template for 16S rRNA whole gene amplification. Primers A and H' designed from 5' and 3' end of a specific gene encoding for 16S rRNA of *Bacillus* species were used. The approximate 1.5 kb amplified PCR product was obtained (Figure 3.1). This product was sequenced by using sense primer: A, D and F and antisense primer: D'. The DNA sequencing profiles were shown in Appendix H. The obtained nucleotide sequence was further compared with available 16S rRNA sequences of *Bacillus* species in the EMBL-GenBank-DDBL database. The highest homology, which found between 16S rRNA gene of thermotolerant bacterial strain BC1 and that of *Bacillus badius*, was 97 %. Comparison of 16S rRNA gene sequences between these two strains is shown in Figure 3.2 whereas Appendix I displays the BLAST results. In addition, multiple sequences obtained from all sequencing primer were aligned (Appendix J), evolutionary distance values were calculated (Appendix K) and a neighbor-joining phylogenetic trees were constructed as shown in Figure 3.3. Phylogenetic trees showed that thermotolerant bacterial strain BC1 gave the closest evolutionary distance values with *Bacillus badius* while *Brevibacillus brevis* was phylogenetically distinct from it. The obtained results indicate that thermotolerant bacterial strain BC1 is *Bacillus badius*. This conclusion is supported by *Tm* determination. The melting temperature of chromosomal DNA from strain BC1 was 50 °C within the range of that from *Bacillus badius* (Appendix L). Bergey' s manual of determinative bacteriology showed that *Bacillus badius* was distinguished from *Brevibacillus brevis* by growth in 5 % NaCl broth. Thus, to confirm this conclusion, BC1 was grown in peptone medium contained various percentage of NaCl: 1, 2, 3, 4, 5 and 6 % at 37 °C overnight. Not surprisingly, BC1 could be grown in each amount of NaCl except in liquid medium contained 6% NaCl. Hence, thermotolerant bacterial strain BC1 was confirmed to be *Bacillus badius*.

3.3 Amino acid sequence of phenylalanine dehydrogenase

Digestion of the purified phenylalanine dehydrogenase with lysyl endopeptidase and isolation of digested peptides by reversed-phase high-performance

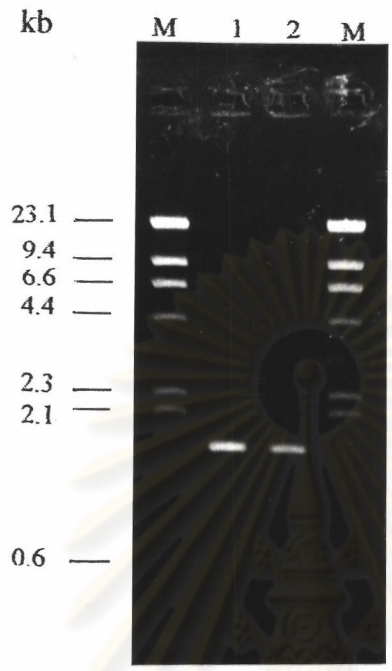

Figure 3.1 PCR products of the 16S rRNA whole gene amplification using primer A and H'

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

Lane 1 = PCR products using chromosomal DNA as the source of DNA template

Lane 2 = PCR products using single fresh colony as the source of DNA template

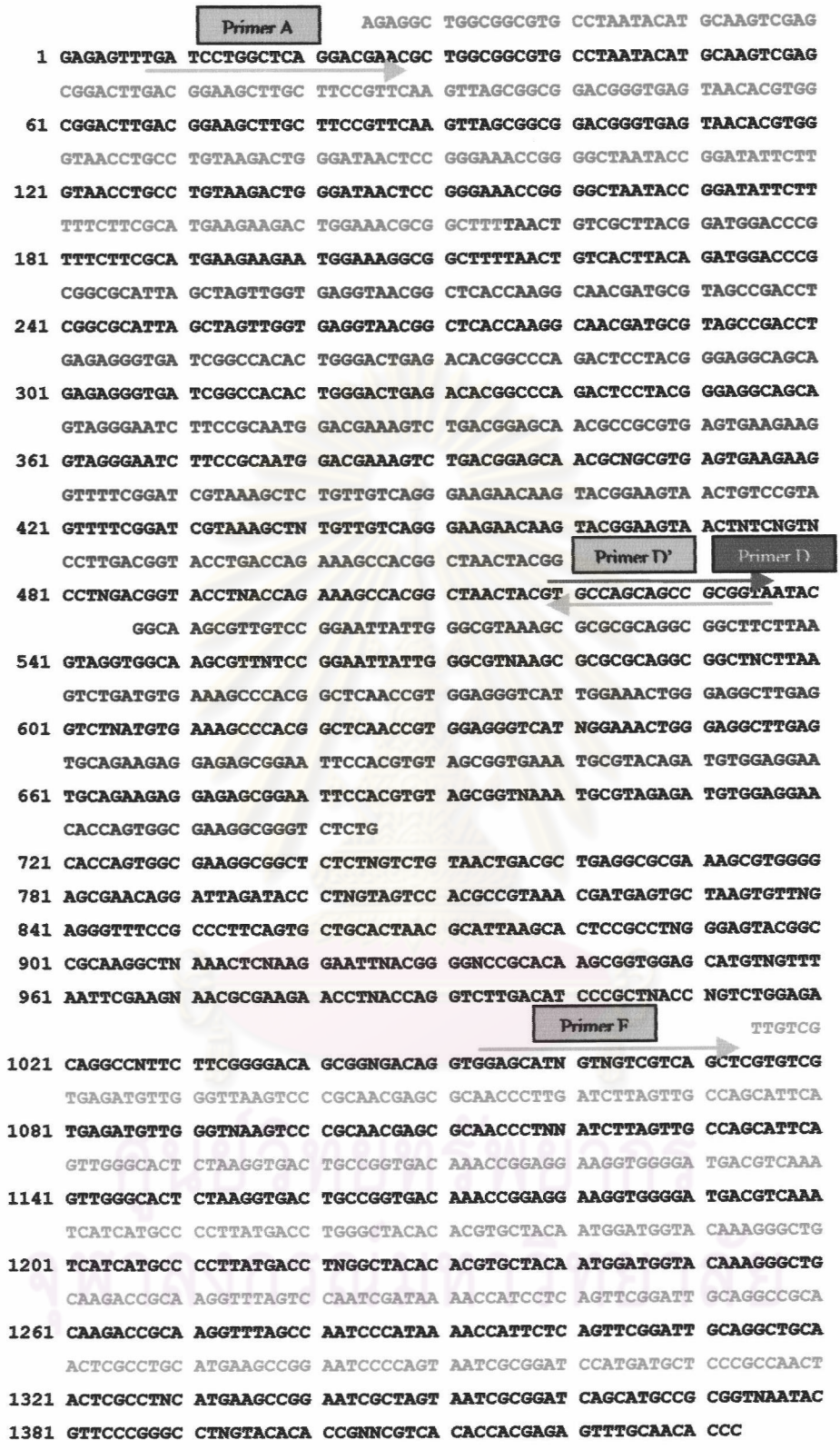

Figure 3.2 Comparison between 16S rRNA gene sequence of *Bacillus badius* and partial 16S rRNA gene sequence of thermotolerant bacterial strain BC1. 16S rRNA gene sequence of *Bacillus badius* = black letter while sequence obtained from primer A, D', D and F = red, blue, green and pink letter, respectively. The arrows show the position of each primer.

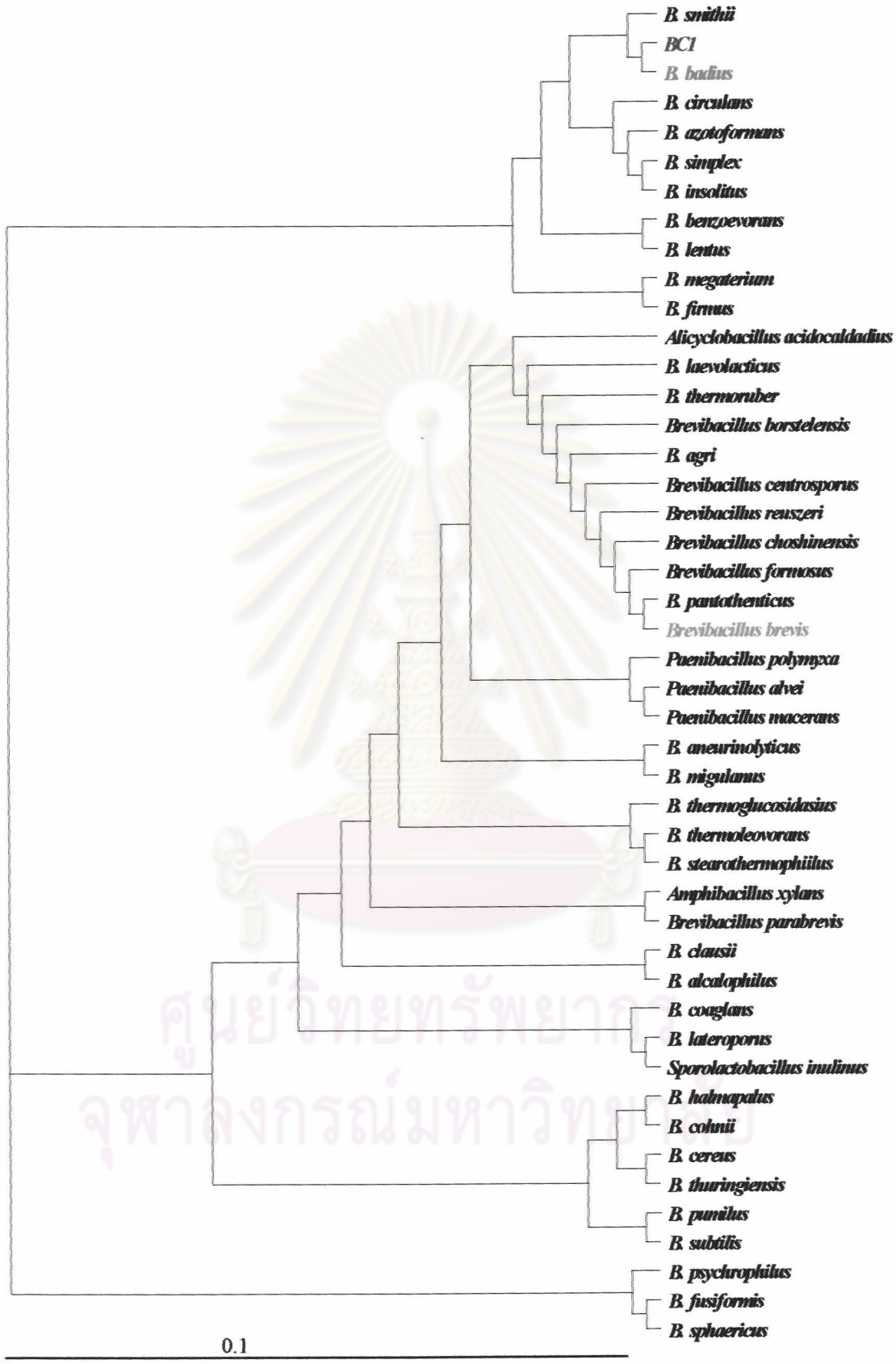

Figure 3.3 Phylogenetic relationship of thermotolerant bacterial strain BC1 and some related organisms based on 16S rRNA gene sequences.

liquid chromatography (HPLC) were carried out as described in section 2.8. The HPLC profile of the separation is presented in Figure 3.4. The isolated peptides at retention time 4.8, 6.7, 10.5, 12.1, 21.6, 26.7 and 44.6 minutes were determined for their amino acid sequences. The peptide at the N-terminus of the protein could be identified as TSIIKDFTLFEKMSEHEQVVFANDPATGLR whereas amino acid sequences of internal peptide fragments were GMTYKXAASDVDFGGGKAVIIGDPQKDKSPELFRAFGQ FVDSLGGRFYTGTDMGTNMEDFIHAMK, ATNK,DDLGGVTYAIQGLGKVGYKV AEGLLEEGAHLFVT, AIAGSANNQLLTEDHGRHL ADK, ERVLAK, and WDIRN.

The CLUSTAL X program was used for alignment of amino acid sequence of phenylalanine dehydrogenase from various sources to indicate the position of each peptide fragment and to use as data for designing degenerated primer in the next step. The alignment is shown in Figure 3.5.

3.4 PCR amplification for the internal gene fragment of phenylalanine dehydrogenase

The chromosomal DNA of *Bacillus badius* BC1 was completely digested with various restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*I, *Spe*I and *Xba*I. The digested products were analyzed by agarose gel electrophoresis (Figure 3.6). Most of the digested DNA fragments still had high molecular weight bands except *Pst*I digested DNA, which gave the smear pattern of DNA lower than 23.1 kb.

The digested chromosomal DNA was used as the templates for amplification of the internal gene fragment of phenylalanine dehydrogenase. The specific PCR product of 594, 402 and 372 bp, which corresponds to the size of fragment amplified by N1xC1, N1xC2 and N2xC1, were detected (Figure 3.7, 3.8 and 3.9). Not surprisingly, all of digested DNA templates gave many nonspecific bands pattern. The specific PCR products were eluted from gel by QIAquick gel extraction kit for nucleotide sequencing. The Figure 3.10 displays the results of these elutions. Nucleotide sequences of the three specific products were determined on both sides of the amplified fragment with each sense and antisense primer. The total result of sequencing is shown in Figure 3.11. The nucleotide sequence was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. The sequence showed high homology to partial part of

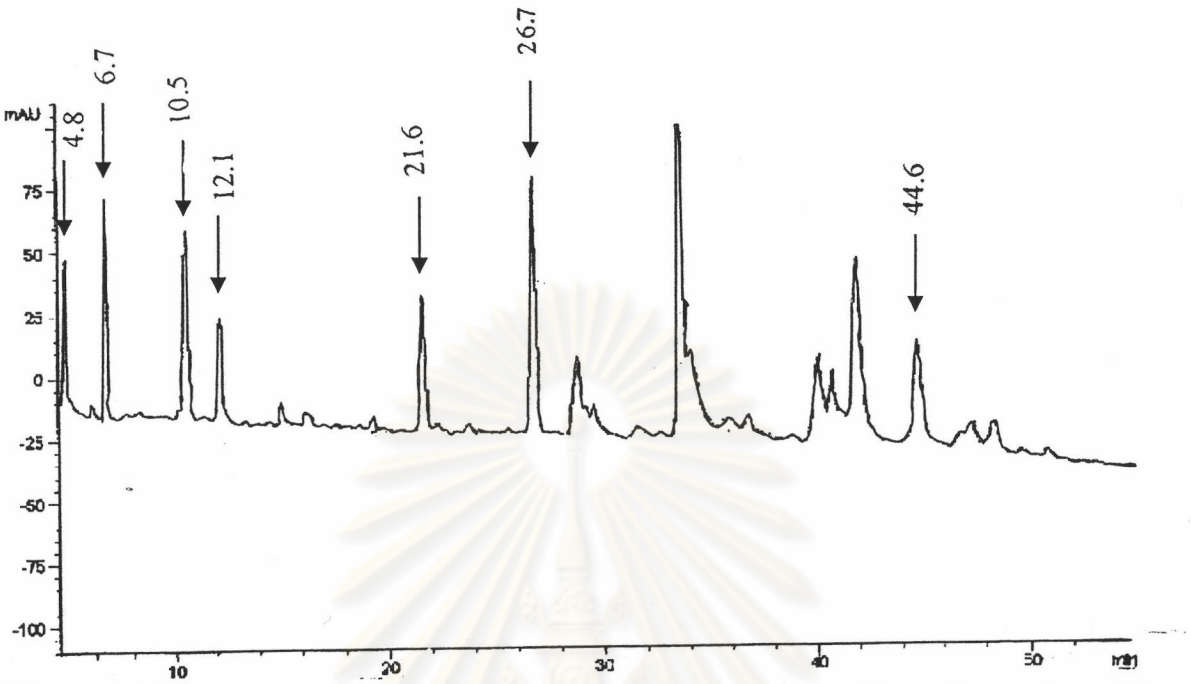

Figure 3.4 The reverse-phase HPLC profile of lysyl endopeptidase digested peptides. The arrows show the isolated peaks that were used for amino acid sequencing.

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CLUSTAL X (1.64 b) multiple sequence alignment
                          N1
                        ------>
BC1          --------TSIIKDFTLFEKMSEHEQVVFANDPATGLR----------------------
BBA          --MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPY
BSP          -MAKQLEKSSKIGNEDVFQKIANHEQIVFCNDPVSGLQAIIAIHDTTLGPALGGTRMYPY
BHA          ---MLTKTPTVTSTLDIFTEMAEHEQVLFCHDPSSGLRAIIAIHDTTLGPALGGCRMYPY
SUR          MILVTLEQTLQDDKASVLDKMVEHEQILFCHDKATGLQAIIAVHDTTMGPALGGCRMAPY
TIN          -----------MRDVFEMMDRYGHEQVIFCRHPQTGLKAIIALHNTTAGPALGGCRMIPY
RHO          -----------MSIDSALN--WDGEMTVTRFDRETGAHFVIRLDSTQLGPAAGGTRAAQY
                                :      *  :   .  :* :
                                      N2
                                    ------>
BC1          ---------------GMTYKXAASDVDFGGGKAVIIG-DPQKDKSPE----LFRAFGQFV
BBA          NSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIG-DPQKDKSPE----LFRAFGQFV
BSP          KNVDEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIG-DPEKDKSPA----LFRAFGQFV
BHA          QTTEDALRDVLRLSKGMTQKCAAADVDFGGGKAVIIG-DPAKDKSAN----LFRAFGQFV
SUR          KTMDLALKDVLRLSKGMTYKCAAADVDFGGGKSVIIG-DPLKDKTPE----KFRAFGQFI
TIN          ASTDEALEDVLRLSKGMTYKCSLADVDFGGGKMVIIG-DPKKDKSPE----LFRVIGRFV
RHO          SQLADALTDAGKLAGAMTLKMAVSNLPMGGGKSVIALPAPRHSIDPSTWARILRIHAENI
                          .** * : ::: :**** **    * :.  .      :*  .. :
                        C1
                      <------
BC1          DSLGGRFYTGTDMGTNMEDFIHAMK------------------------------ATNK
BBA          DSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNK
BSP          ESLNGRFYTGTDMGTTMDDFVHAQKETNFINGIPEQYGGSGDSSIPTAQGVIYALKATNQ
BHA          ESLNGRFYTGTDMGTTMEDFVHALKETNGIVGIPKEYGGSGDSSVPTAKGVINSLKAISQ
SUR          ESLNGRFYTGTDMGTTLEDFVHAMKETNYIVGKPVEYGGGGDSSIPTALGVFYGIKATNQ
TIN          GGLNGRFYTGTDMGTNPEDFVHAARESKSFAGLPKSYGGKGDTSIPTALGVFHGMRATAR
RHO          DKLSGNYWTGPDVNTNSADMDTLNDTTEFVFGRSLERGGAGSSAFTTAVGVFEAMKATVA
               *.*.::**.*:.*.  *:     :
                             C2
                           <------
BC1          -----DDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVT----------------------
BBA          MLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGSV
BSP          YLFGSDSLSGKTYAIQGLGKVGYKVAEQLLKAGADLFVTDIHENVLNSIKQKSEELGGSV
BHA          VVLKDKQFSGRTYAIQGLGKVGFKVAEELLKEGNDLYVSDLQESLPLRLQQLGQRLGRHV
SUR          NLFGDDKVEGRKYSIQGLGKVGYKVAEHIINEGGNVIVTDINEQAIADIQKLG---GSAV
TIN          FLWGTDQLKGRVVAIQGVGKVGERLLQLLVEVGAYCKIADIDS---VRCEQLKEKYGDKV
RHO          HR-GLGSLDGLTVLVQGLGAVGGSLASLAAEAGAQLLVADTDTERVAHAVALG------H
                   .. *    :**:* **  : .   : *    ::

BC1          ------------------------------------AIAGSANNQLLTEDHGRHLADK--
BBA          TVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKGI
BSP          TIVKSDDIYSVQADIFVPCAMGGIINDKTIPKLKVKAVVGSANNQLKDLRHANVLNEKGI
BHA          EILHGDEIYEAAADVFVPCAQGAILNDATIARLKVKAIAGAANNQLEAERHGQMLHDQGI
SUR          RVVSSEEIYSQQADVFVPCAFGGVINDDTLKVLKVRGISGSANNQLAESRHGELLREKGI
TIN          QLVDVNRIHKESCDIFSPCAKGGVVNDDTIDEFRCLAIVGSANNQLVEDRHGALLQKRSI
RHO          TAVALEDVLSTPCDVFAPCAMGGVITTEVARTLDCSVVAGAANNVIADEAASDILHARGI
                                                 : *:*** :  . *  :
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(continued)

Figure 3.5 The CLUSTAL X alignment of amino acid sequence of phenylalanine dehydrogenase from various sources. Arrows show the regions of the amino acid sequence that used for degenerated primer design. BC1 = *Bacillus badius* BC1, 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 acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

(continued)

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BC1          -------------------------ERVLAK----------------------------
BBA          LYAPDYIVNSGGLIQ-VADELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAANR
BSP          LYAPDYIVNAGGLIQ-VADELYGPNKERVLLKTKEIYRSLLEIFNQAALDCITTVEAANR
BHA          WFAPDYIVNSGGLIQ-VADELYGSNEKRVLSKTNAIYDTILEIFHQAERHHITTLQAANQ
SUR          LYAPDYIVNGGGLIQ-VADELYGTNPARVLAKTENIYTSLLEVFHQAEQDHMTTATAADR
TIN          CYAPDYLVNAGGLIQ-VADELEGFHEERVLAKTEAIYDMVLDIFHRAKNENITTCEAADR
RHO          LYAPDFVANAGGAIHLVGREVLGWSESVVHERAVAIGDTLNQVFEISDNDGVTPDEAART
               *   : *

BC1          ----------------------WDIRN----------------
BBA          MCEQRMAARGRRNSFFTSSVKPKWDIRN----------------
BSP          KCQKTIEGQQTRNSFFSRGRRPKWNIKEBTTMFFRMBTTMFFRM
BHA          LCERRIRERARRNNFFVNRIRPKWNLRK----------------
SUR          MCEKRIADAKNRNSFFTQSNRPKWNFHQ----------------
TIN          IVMERLKKLTDIRRILLEDPRNSARRTPFFRM------------
RHO          LAGRRAREASTTTATA-----------------------------
```

Figure 3.5 The CLUSTAL X alignment of amino acid sequence of phenylalanine dehydrogenase from various sources. Arrows show the regions of the amino acid sequence that used for degenerated primer design. BC1 = *Bacillus badius* BC1, 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 acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

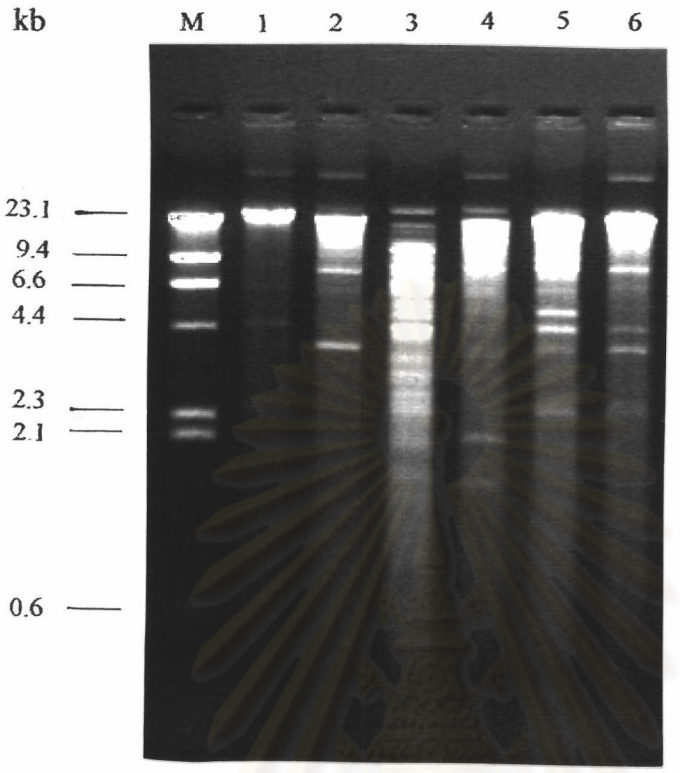

Figure 3.6 Restriction enzyme digested chromosomal DNA of *Bacillus badius* BC1.

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

Lane 1 = undigested chromosomal DNA

Lane 2 = chromosomal DNA digested with *Kpn*I

Lane 3 = chromosomal DNA digested with *Pst*I

Lane 4 = chromosomal DNA digested with *Pvu*I

Lane 5 = chromosomal DNA digested with *Spe*I

Lane 6 = chromosomal DNA digested with *Xba*I

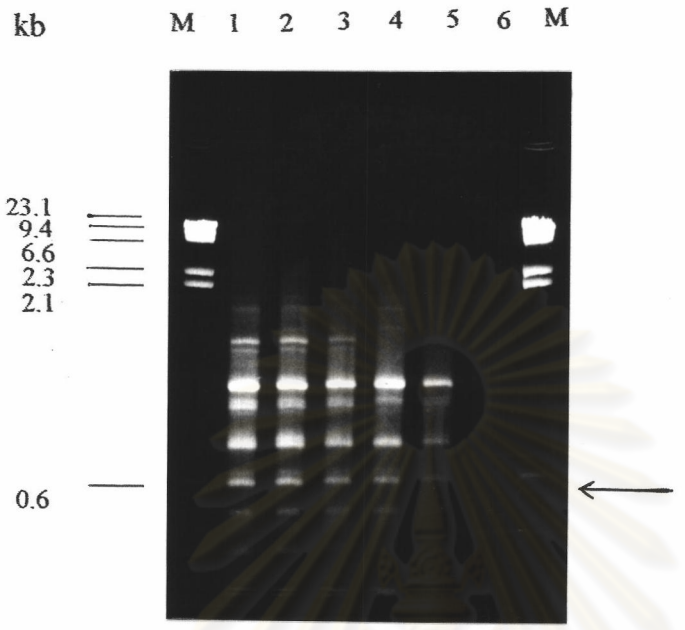

Figure 3.7 PCR products of primer N1 and C1 using various DNA templates. The specific product is indicated by arrow.

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 *Kpn*I 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

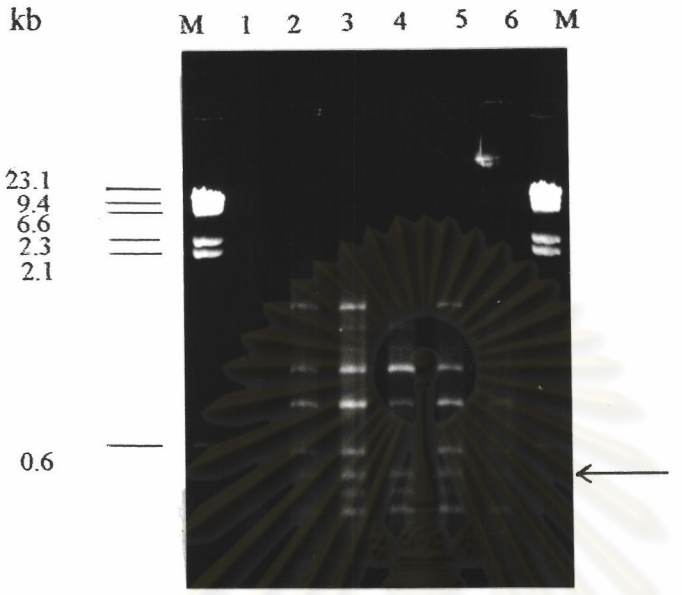

Figure 3.8 PCR products of primer N1 and C2 using various DNA templates. The specific product is indicated by arrow.

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 *Kpn*I 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

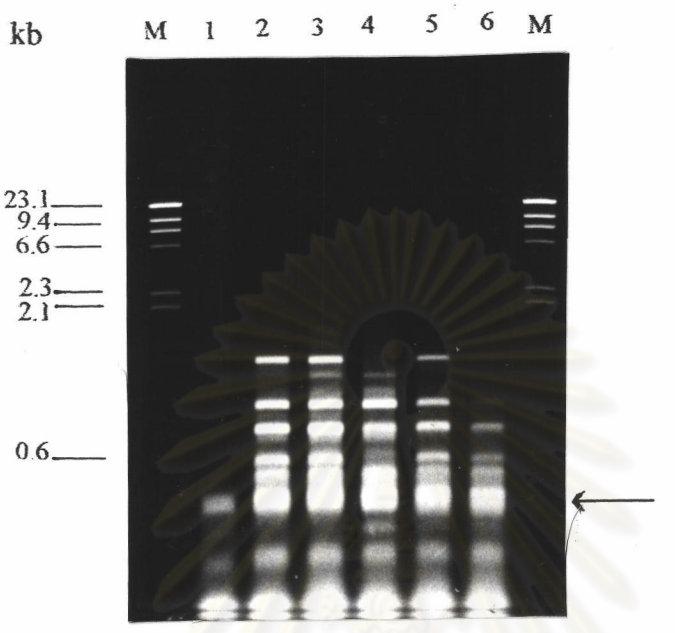

Figure 3.9 PCR products of primer N2 and C1 using various DNA templates. The specific product is indicated by arrow.

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 *Kpn*I 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

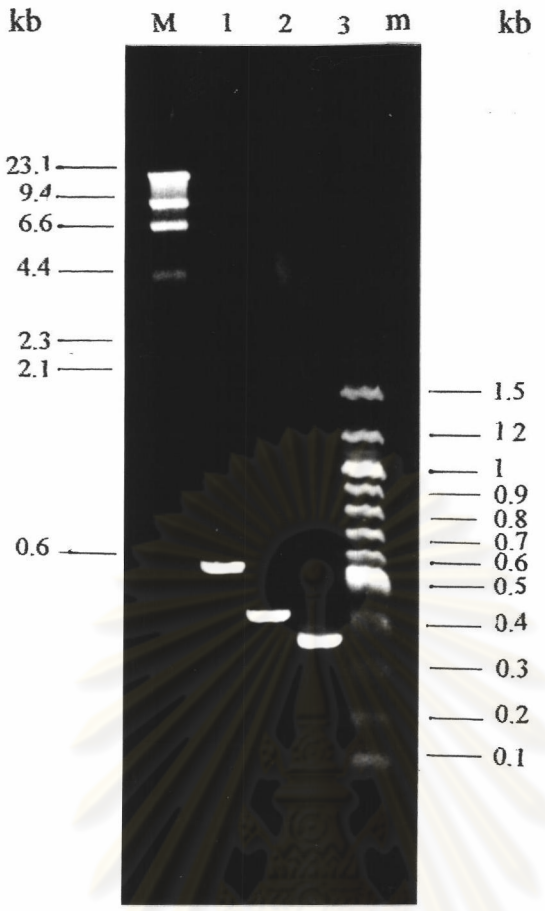

Figure 3.10 Recovered PCR products of the internal gene fragments of phenylalanine dehydrogenase.

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

Lane 1 = specific PCR product using primer N1xC1 (594 bp)

Lane 2 = specific PCR product using primer N1xC2 (402 bp)

Lane 3 = specific PCR product using primer N2xC1 (372 bp)

Lane m = 100 bp DNA ladder

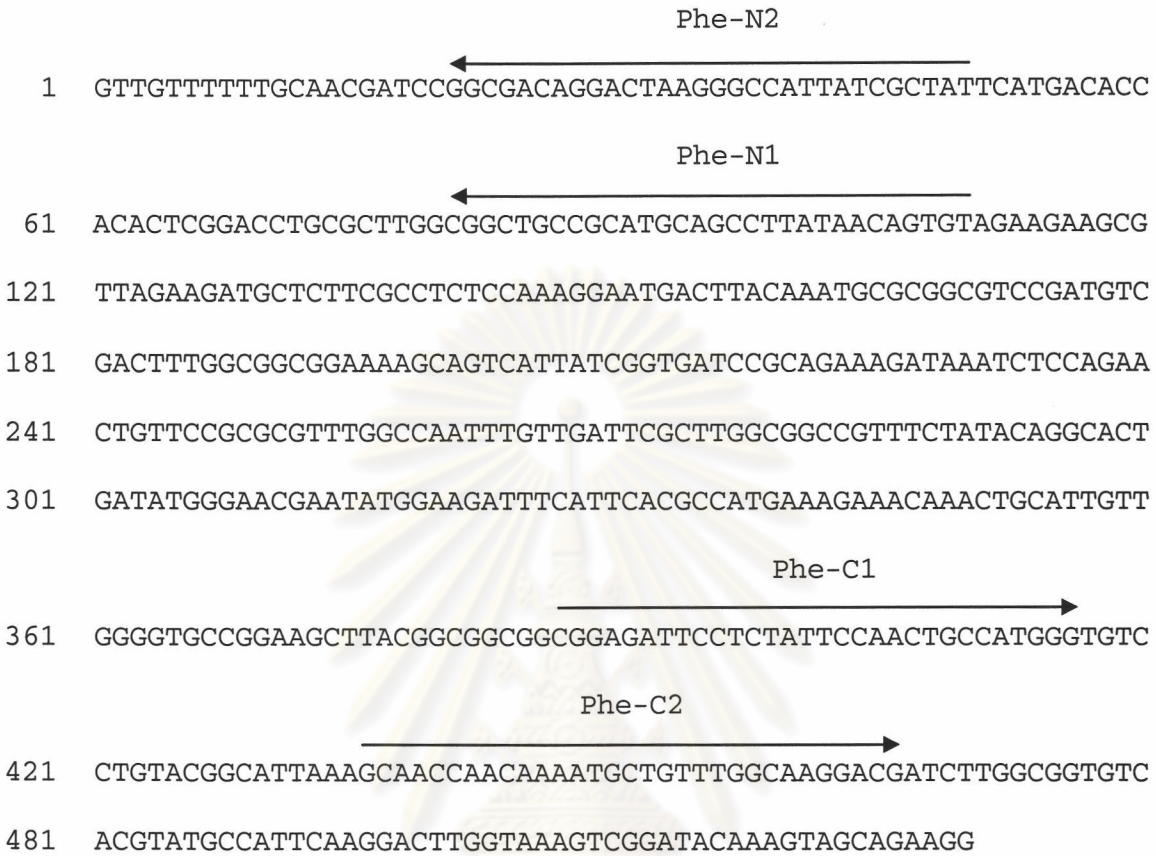

Figure 3.11 Nucleotide sequence of the internal gene fragment of phenylalanine dehydrogenase. The DNA sequencing profiles are presented in Appendix M. The regions for primer design is shown by arrow.

phenylalanine dehydrogenase from other bacterial sources indicated that this amplified fragment was the real phenylalanine dehydrogenase gene (data not shown).

3.5 PCR amplification for the 5'-terminal and 3'-terminal gene fragments of phenylalanine dehydrogenase

To amplify the 5'- and 3'-terminal gene fragments and to sequence the unknown parts of this gene by PCR method, the specific primers, which could anneal to the known sequence of the internal gene, were designed from the nucleotide sequencing data (section 3.4). The cassette primers, which could anneal to one strand of the cassette, were also used. The templates were prepared by digestion of chromosomal DNA with various restriction enzymes and further ligated to one of the cassettes, which possessed either 3' or 5' overhang depending on the corresponding restristion site as described in 2.9.2.2. The PCR products which used outer 5'-terminal primer (Phe-N1 x Cassette C1) showed mutiple bands for all templates (Figure 3.12), while the products from outer 3'-terminal primers (Phe-C1 x Cassette C1) showed only one band when *Pst*I digested chromosomal DNA was used as template (Figure 3.13).

These PCR products were confirmed by being used as templates for second PCR which inner pairs of primers (Phe-N2 x Cassette C2 and Phe-C2 x Cassette C2) were used. The second 5'-terminal PCR products also gave the similar pattern with a few smaller size bands compared with the first ones (Figure 3.14). In addition, products from the second 3'-terminal amplification still gave only one strong band, with a few smaller size bands than the first PCR product (Figure 3.15). All smaller strong bands were chosen and eluted from agarose gel by QIAquick gel extraction kit for nucleotide sequencing. The DNA sequencing profile are shown in Appendix M. The sequencing results suggested that the 5'-terminal gene fragment amplified by using *Spe*I digested DNA as a template is the real PCR product while *Pst*I digested DNA gave the real 3'-terminal gene fragment. Since the direct PCR sequencing method can determine only up to 300 bp from the priming site, a new primer was necessary to be synthesized. Thus, the nucleotide sequence of the Phe-C3 primer, CTCCGGCGGTCTCATCCAAG, was designed and further used as sequencing primer for 3'-terminal gene fragment. The nucleotide sequencing data is shown in Figure 3.16, 3.17 and 3.18.

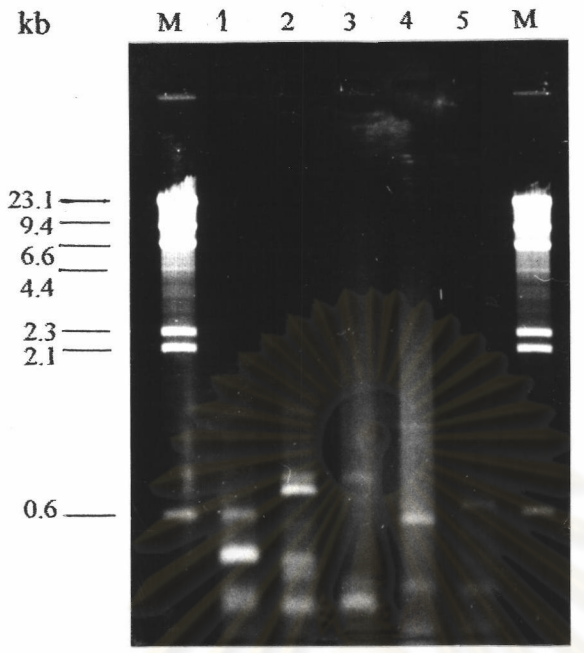

Figure 3.12 The first 5'-terminal amplified products using primer Phe-N1 and Cassette primer C1

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

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

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

Lane 3 = amplified products using *Pst*I digested chromosomal DNA as template

Lane 4 = amplified products using *Spe*I digested chromosomal DNA as template

Lane 5 = amplified products using *Xba*I digested chromosomal DNA as template

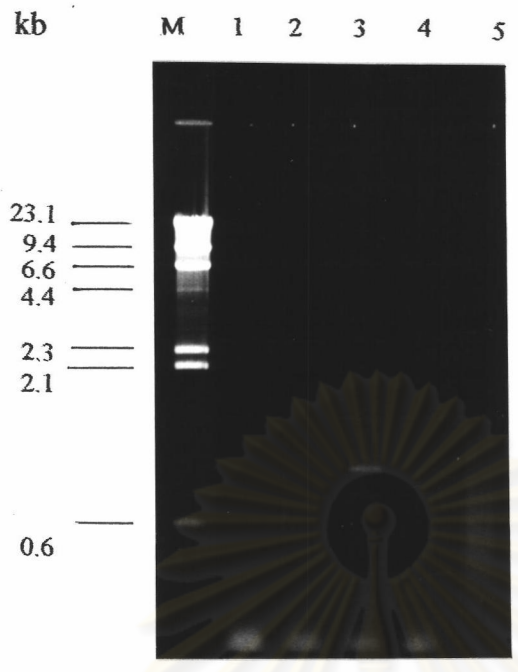

Figure 3.13 The first 3'-terminal amplified products using primer Phe-C1 and Cassette primer C1

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

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

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

Lane 3 = amplified products using *Pst*I digested chromosomal DNA as template

Lane 4 = amplified products using *Spe*I digested chromosomal DNA as template

Lane 5 = amplified products using *Xba*I digested chromosomal DNA as template

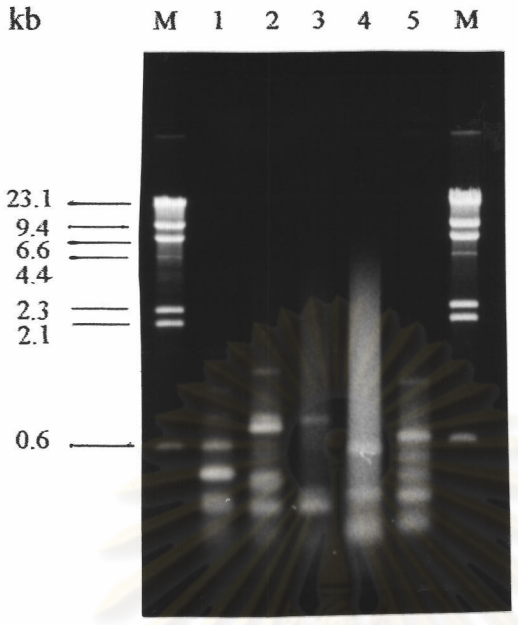

Figure 3.14 The second 5'-terminal amplified products using primer Phe-N2 and Cassette primer C2

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

Lane 1 = amplified products using *Bam*HI digested chromosomal DNA as template for the first 5'-terminal amplified products

Lane 2 = amplified products using *Bgl*II digested chromosomal DNA as template for the first 5'-terminal amplified products

Lane 3 = amplified products using *Pst*I digested chromosomal DNA as template for the first 5'-terminal amplified products

Lane 4 = amplified products using *Spe*I digested chromosomal DNA as template for the first 5'-terminal amplified products

Lane 5 = amplified products using *Xba*I digested chromosomal DNA as template for the first 5'-terminal amplified products

Note: The templates of second 5'-terminal amplified products were PCR products from the reaction using each restriction enzyme digested chromosomal DNA as template and Phe-N1 as well as Cassette primer C1 were used as primers.

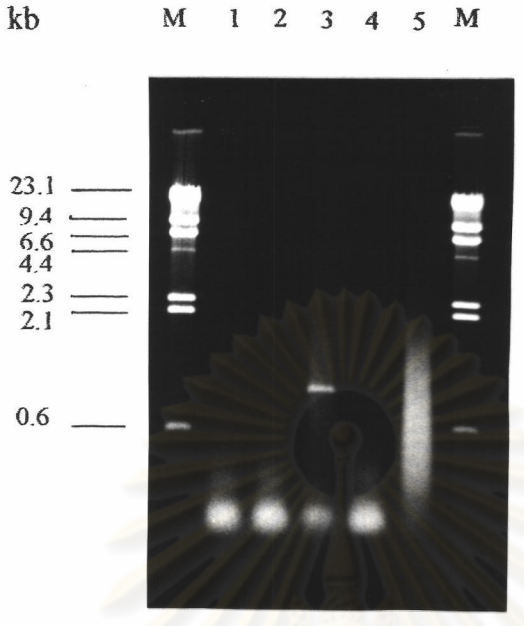

Figure 3.15 The second 3'-terminal amplified products using primer Phe-C2 and Cassette primer C2

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

Lane 1 = amplified products using *Bam*HI digested chromosomal DNA as template for the first 3'-terminal amplified products

Lane 2 = amplified products using *Bgl*II digested chromosomal DNA as template for the first 3'-terminal amplified products

Lane 3 = amplified products using *Pst*I digested chromosomal DNA as template for the first 3'-terminal amplified products

Lane 4 = amplified products using *Spe*I digested chromosomal DNA as template for the first 3'-terminal amplified products

Lane 5 = amplified products using *Xba*I digested chromosomal DNA as template for the first 3'-terminal amplified products

Note: The templates of second 3'-terminal amplified products were PCR products from the reaction using each restriction enzyme digested chromosomal DNA as template and Phe-C1 as well as Cassette primer C1 were used as primers.

```
  1  AAATACCCCAAAATACATACGGGAAGTAAATAAAGGTCTGGAAATTCCCTCATAATGATA
 61  TATATATATATCATTTAACCTTTTATAACTAAAGTGAAGCGTATGCCTGCCGGCGCTGTT
121  ATTGGCGCTCGTTTGAAAGGGCTTACCAAAATTATATAACCAAGGAGCTGACAGATCCTT
181  TTTCTGCGGCTAAATAAAAGCGTTCAACTATTAACGAAAGCAGGGATTAAATATGAGCTT
241  AGTAGAAAAAACATCCATCATAAAAGATTTCACTCTTTTTGAAAAAATGTCTGAACATGA
301  ACAA
```

Figure 3.16 Nucleotide sequence of the 5'-terminal gene fragment of phenylalanine dehydrogenase using antisense primer Phe-N1 and Phe-N2. The DNA sequencing profiles can be seen in Appendix M. The start codon is underlined.

```
  1  GATCTTGGCGGTGTCACGTATGCCATTCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCG
 61  GAAGGGCTGCTCGAAGAAAGCGCTCATTTATTTGTAACGGATATTAACGAGCAAAGCTTG
121  GCGGCCATTCAGGAAAAAGCAAAAACAACATCCGGTTCTGTCACGGTAGTGGCGAGCGAT
181  GAGATTTATTCCCAGGAAGCTGATGTGTTCGTCCCTTGTGCATTTGGCGGTGTTGTTAAT
241  GATGAAACCATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCCAACAATCAGCTG
301  CTCACGGAGGATCACGGCAGACAGCTTGCATACACCGGCATTCTATATGCTCCGGATTAT
                    Phe-C3
                    ------------>
361  ATTATTAACTCCGGCGGTCTCATCCAAGTAGCCGACGAATCGTATGAGGTGAA
```

Figure 3.17 Nucleotide sequence of the 3'-terminal gene fragment of phenylalanine dehydrogenase using sense primer Phe-C1 and Phe-C2. The DNA sequencing profiles can be seen in Appendix M. The region for design further sequencing primer (Phe-C3) is shown by arrow.

```
  1  CAAAGAACGCGTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCA
 61  GCAAGCGGAATTAGATCAAATTACCATAATGGAAGCAGCCAACAGAATGTGTGAGCAAAG
121  AATGGCGGCCAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAATGGGA
181  TATTCGTAATTAATACTTGTTCGGGGGGATATCATGA
```

Figure 3.18 Nucleotide sequence of the 3'-terminal gene fragment of phenylalanine dehydrogenase using sense primer Phe-C3. The DNA sequencing profile can be seen in Appendix M.

From the gene walking and sequencing by cassette-ligation mediated PCR, the complete nucleotide sequence of the whole gene fragment was identified as shown in Figure 3.19. The nucleotide sequence of the phenylalanine dehydrogenase structural gene contains 1140 nucleotides open reading frame, which is capable for encoding a polypeptide of 380 amino acids. This gene had GC content about 40 %. The nucleotide sequence was compared with the DNA sequences deposited in the EMBL-GenBank-DDBL database. The percentage of identical nucleotide sequences of the enzyme compared with phenylalanine dehydrogenase from *Bacillus badius*, *Thermoactinomyces intermedius*, *Sporosarcina ureae*, and *Bacillus sphaericus* were 96, 85, 83, and 81%, respectively. The alignment was shown in Figure 3.20. Comparison of nucleotide sequences between phenylalanine dehydrogenase gene of *Bacillus badius* BC1 and those of published *Bacillus badius* is shown in Figure 3.21.

3.6 Deduced amino acid sequence comparison with phenylalanine dehydrogenase from other sources

Deduced amino acid sequence of phenylalanine dehydrogenase from *Bacillus badius* BC1 was compared with the sequences of phenylalanine dehydrogenase from other bacterial sources. *Bacillus badius* BC1 exhibited the highest overall levels of identity with the enzyme from *Bacillus badius* (98 %) as shown in Figure 3.22. The percentage of identical amino acids of the enzyme compared with phenylalanine dehydrogenase from *Thermoactinomyces intermedius*, *Bacillus sphaericus*, *Sporosarcina ureae*, *Bacillus halodurans*, and *Rhodococcus sp.* were 75, 70, 70, 62 and 35%, respectively. The alignment was shown in Figure 3.23. In addition, Lys-78 and Asp-118, which involved in the active site (Brunhuber *et al.*, 1999), were identical in all the phenylalanine dehydrogenase sequences including *Bacillus badius* BC1 enzyme.

3.7 PCR amplification of the whole gene fragment

To overexpress phenylalanine dehydrogenase gene in *E. coli* with the assistance of *lac* promoters of plasmid pUC18, the whole gene fragment was amplified by using the new pair of primers. The 5'-primer (Phe-Eco) containing (i) an *Eco*RI

Figure 3.19 The nucleotide sequence and the deduced amino acid of phenylalanine dehydrogenase gene from *Bacillus badius* BC1. Blue = sequence from the internal gene fragment amplification, violet = the overlap sequence between internal gene fragment and the first 3'-terminal gene fragment amplification, green = sequence from the 5'-terminal gene fragment amplification, red = sequence from the first 3'-terminal gene fragment amplification and pink = sequence from the second 3'-terminal gene fragment amplification.

```
CLUSTAL X (1.64b) multiple sequence alignment

BC1             ------------------------------------------------------------
BBA             GCATGCCTTCTATCGTGCTGAAATCCCCTGTTCCCGAGAAGATTATTTTGCCGTCTGATG
SUR             ------------------------------------------------------------
BSP             ------------------------------------------------------------
TIN             ------------------------------------------------------------
RHO             ------------------------------------------------------------

BC1             ------------------------------------------------------------
BBA             AAGAACAGGAATGGCCGTGAAGGACGGCGCATCATAGAAGATGATGTCGAATGATAAGAC
SUR             ------------------------------------------------------------
BSP             ------------------------------------------------------------
TIN             ------------------------------------------------------------
RHO             ------------------------------------------------------------

BC1             ------------------------------------------------------------
BBA             AAGCCTCCTCTTTCTATTGTCGAAAGAGGAGGCTTTTTTAGCTTTTATTTACTGGAAATG
SUR             ------------------------------------------------------------
BSP             ------------------------------------------------------------
TIN             ------------------------------------------------------------
RHO             ------------------------------------------------------------

BC1             ------------------------------------------AAATACCCCAAAATACAT
BBA             AAAGCGTTTTACAAAACGAAGATAAATACCAAAAAATACATACGAAAAGTAAATAAAGGT
SUR             ----------------------------------------------------------CCGGC
BSP             ------------------------------------------------------------
TIN             ---------------------------------------GGAACTTCCCTTGGAACACGGT
RHO             ------------------------------------------------------------

BC1             ACGGGAAGTAAATAAAGGTCTGGAAATTCCCTCATAATGATATATATATATATCATTTAA
BBA             CTGAAAATTCAATCATAATGATAT---ATAT-ATATATCATTTAACCTTTTATAATT---
SUR             TCCAATATCAGCACACAATTATTTTCTGCCT-GGTTTTTATTAATTCGTTACCTTGC---
BSP             --CAAT----GAGCACGATGAAA----GC---GGTATTCGTTCCATGATGAACGTG----
TIN             TTGAAT-TTGGAACACAATGCTCTTTTCCTGCTCTTTTCTTCCGAAGATCAAAGTGAGGG
RHO             ------------------------------------------------------------

BC1             CCTTTTATAACTAAAGTGAAGCGTATGCCTGCCGGCGCTGTTATTGGCGCTCGTTTGAAA
BBA             -AAAGTGAAGCGTATGCCT-----------GCCGGCGCCGTCATTGGCGCTCGTTTGAAA
SUR             -ACCGGTAGCTATTCTCAT-----------CGTGTTGCAGACGTACTTGGACTATTGAAC
BSP             -ACAATGACGTTTGACCAT-----------CGTGTCGCCGACGG---TGGAACAGTCAAT
TIN             AATGCGGGCGTTTTTGGAAAAAAGAAAACCGGTTTTTCGGGGGGAATAAGATCACACTTC
RHO             ----------------------------------------------------CTGCAGAGA

BC1             GGGCTTACCAAAATTATATAACCAAGGAGCTGACAGATCCTTTTTCTGCGGCTAAATAAA
BBA             GGGCTTACAAAAATTATATAACCAAGAAGCTGACAGATCCTTTTTCTGCGGCTAAATAAA
SUR             TTCTT---------GAAATAAATAGATAGATTGCTGATACCCTCATATAGGTTCAC-AGC
BSP             TGCTTTTACAAACCGATTTAAATCCTTGAATTGAAGATCCCAAAAAATTAATGCTGGAGC
TIN             CGGTTTTGGGCA-TATGATGAGTTGAAAGATTTTCAGGCTTGTCAGTCAACTTGTTCAAA
RHO             CGTTTT-CG-----CCAAAGACTGGTCACACTATGAAGACGCACCCTCCGTCGCCGCGGA
```

(continued)

Figure 3.20 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from various sources. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO= *Rhodococcus sp.* Conserve residues are indicated by asterisks.

(continued)

```
BC1             AGCGTTCAACTATTAACGAAAGCAGGGATTAAATATGAGCTTA---GTAGAAAAAACATC
BBA             AGCGTTCAACTATTAACGAAAGCAGGGATTAAATATGAGCTTA---GTAGAAAAAACATC
SUR             A--------ATAAAATAGA--GGAGGAAATGATTTTGGTAACT---TTAGAACAGACTTT
BSP             TT--GTCTGATAAGATTGAATGGAGGAAAAAGAAATGGCAAAACAGCTTGAAAAGTCATC
TIN             GTGGAGATGATAAGAATGGGAAGCATGAAAATCAATGAACAAAAGCGGGGATCTTTGTTG
RHO             CC-------CGAATCGGGACTGCTCGGAGTTGTCTGCGTCCACTGCATCCATCGAACATC
                                                                  *      *

BC1             CAT-CATAAAAGATTT-CACTCTTTTTGAAAAAATGTC------TGAACATGAACAAGTT
BBA             CAT-CATAAAAGATTT-CACTCTTTTTGAAAAAATGTC------TGAACATGAACAAGTT
SUR             ACA-AGACGACAAGGC-AAGTGTTTTGGATAAAATGGT------CGAGCATGAACAAATT
BSP             AAA-AATTGGTAATGA-GGACGTTTTTCAAAAAATAGC------GAATCACGAGCAGATT
TIN             GAGGAAGCGAAGATGCGCGACGTGTTTGAAATGATGGACCGCTATGGCCACGAGCAGGTC
RHO             AAGGGGTACATCATGAGTATCGACAGCGCACTGAACTG------GGACGGGGAAATGACG
                          *                 *                 **

BC1             GTTTTTTGCAACGATCCTCGCACAGGACTAAGGGCCATTATCGCTATTCATGACACCACA
BBA             GTTTTTTGCAACGATCCGGCGACAGGACTAAGGGCCATTATCGCTATTCATGACACCACA
SUR             CTATTTTGTCATGATAAAGCAACCGGTCTTCAAGCCATCATTGCAGTCCACGATACGACT
BSP             GTGTTCTGTAATGATCCGGTATCCGGCCTGCAAGCTATCATTGCTATCCACGATACAACC
TIN             ATTTTTTGCCGTCATCCGCAAACCGGTCTCAAAGCGATCATCGCCTTGCATAATACAACC
RHO             GTCACCCGATTCGACCGGGAGACTGGTGCCCATTTCGTCATTCGACTCGATTCGACCCAA
                 *     *     *        * **           * **     *  *    **

BC1             CTCGGACCTGCGCTTGGCGGCTGCCGCATGCAGCCTTATAACAGTGTAGAAGAAGCGTTA
BBA             CTCGGACCTGCGCTCGGCGGCTGCCGCATGCAGCCTTATAACAGTGTGGAAGAAGCATTG
SUR             ATGGGACCTGCACTCGGTGGATGTCGCATGGCGCCTTATAAAACGATGGATCTCGCATTA
BSP             CTAGGCCCCGCTTTAGGTGGAACTCGCATGTATCCCTATAAAAATGTGGATGAAGCTCTG
TIN             GCGGGGCCGGCTTTGGGTGGATGCCGCATGATCCCGTATGCTTCGACGGACGAAGCCTTG
RHO             CTCGGACCGGCGGCCGGAGGCACCAGAGCCGCACAGTACTCACAGCTGGCGGACGCCCTC
                   ** ** **    ** **    *       *  **          *     **  *

BC1             GAAGATGCTCTTCGCCTCTCCAAAGGAATGACTTACAAATGCGCGGCGTCCGATGTCGAC
BBA             GAAGATGCTCTTCGCCTTTCCAAAGGAATGACTTACAAATGCGCGGCGTCCGATGTCGAC
SUR             AAAGATGTTCTTCGCCTTTCAAAAGGGATGACATATAAATGTGCGGCAGCTGATGTAGAC
BSP             GAAGATGTGCTTCGCCTGTCAGAAGGAATGACGTATAAATGCGCAGCCGCCGATATCGAT
TIN             GAGGATGTTTTGCGGTTGTCCAAAGGCATGACCTATAAATGCAGTCTGGCGGATGTGGAC
RHO             ACCGACGCCGGCAAATTGGCGGGGGCGATGACGTTGAAGATGGCAGTGAGCAACCTTCCG
                   ** *        *  *    *  ***** *  **                *  *

BC1             TTTGGCGGCGGAAAAGCAGTCATTAT-----CGGTGATCCGCAGAAAGATAAATC-----
BBA             TTTGGCGGCGGAAAAGCAGTCATTAT-----CGGTGATCCGCAGAAAGATAAATC-----
SUR             TTTGGCGGCGGAAAATCCGTCATCAT-----CGGAGACCCGCTAAAAGATAAAAC-----
BSP             TTCGGCGGCGGGAAGGCGGTCATTAT-----CGGAGATCCAGAAAAGGATAAATC-----
TIN             TTTGGCGGGGGAAAAATGGTTATCAT-----CGGCGATCCGAAAAAAGATAAATC-----
RHO             ATGGGCGGGGGCAAATCCGTCATTGCGCTTCCTGCGCCGCGTCATTCGATCGATCCGAGC
                 * ***** ** **    ** **       * * *   *      ***  * *

BC1             -----TCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCGCTTGGCGGCCGTTTC
BBA             -----TCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCGCTTGGCGGCCGTTTC
SUR             -----GCCTGAGAAATTCCGTGCTTTCGGTCAATTCATCGAATCATTGAACGGACGCTTC
BSP             -----TCCGGCATTGTTCCGTGCATTTGGTCAATTTGTGGAATCACTGAATGGACGATTT
TIN             -----GCCGGAGTTGTTTCGCGTGATCGGCCGTTTTGTGGGCGGGTTAAACGGCCGTTTC
RHO             ACGTGGGCACGCATCCTCCGAATCCACGCCGAGAACATCGACAAGTTGTCCGGCAACTAC
                       *        * **       *         * *      *    **    *
```

(continued)

Figure 3.20 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from various sources. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO= *Rhodococcus sp.* Conserve residues are indicated by asterisks.

(continued)

```
BC1             TATACAGGCACTGATATGGGAACGAATATGGAAGATTTCATTCACGCCATGAAAGAAACA
BBA             TATACAGGTACTGATATGGGAACGAATATGGAAGATTTCATTCACGCCATGAAAGAAACA
SUR             TATACAGGTACAGACATGGGCACAACGCTTGAAGACTTTGTGCATGCCATGAAAGAAACA
BSP             TACACAGGTACTGACATGGGGACCACGATGGATGATTTTGTCCATGCACAGAAAGAGACG
TIN             TATACCGGAACCGACATGGGAACCAATCCGGAAGATTTTGTCCATGCCGCCAGGGAATCG
RHO             TGGACCGGACCGGACGTCAACACCAATTCGGCAGACAT-GG--ATACTCTGAACGACACC
                *  ** **  * **  *    ** *     *  **  *     *  *    *  **  *

BC1             A---ACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGATTCCTCTATTCCA
BBA             A---ACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGATTCCTCTATTCCA
SUR             A---ACTACATCGTGGGCAAGCCGGTCGAATATGGTGGCGGTGGAGACTCATCGATCCCT
BSP             A---ATTTCATTAACGGAATTCCTGAGCAGTATGGTGGAAGCGGCGACTCGTCGATTCCG
TIN             A---AATCTTTTGCCGGATTGCCGAAATCGTACGGCGGAAAGGGGGACACATCCATTCCC
RHO             ACCGAGTTCGTGTTCGGACGGTCGCTCGAACGCGGCGGCGCGGGTTCGAGCGCGTTCACC
                *   * *   *    **     *          ** **    **         * *  *

BC1             ACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGCTGTTTGGCAAGGAC
BBA             ACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGTTGTTTGGCAAGGAC
SUR             ACTGCACTCGGAGTCTTCTATGGCATTAAAGCGACAAACCAGAATCTGTTTGGCGACGAC
BSP             ACCGCCCAGGGAGTCATTTATGCACTGAAGGCTACAAACCAGTATTTATTTGGAAGCGAT
TIN             ACCGCGCTCGGGGTGTTTCACGGAATGCGGGCCACCGCCCGGTTTTTATGGGGGACGGAT
RHO             ACCGCCGTTGGCGTGTTCGAGGCGATGAAGGCGACCGTCGCGCACC---GTGGGCTGGGC
                ** **    ** **  *  * *   *    ** **   *            **    *

BC1             GATCTTGGCGGTGTCACGTATGCCATTCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCG
BBA             GATCTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAAGTAGGCTACAAAGTAGCG
SUR             AAAGTAGAAGGCCGAAAATACAGTATCCAAGGTCTTGGGAAAGTAGGTTACAAAGTAGCT
BSP             AGCCTTTCAGGTAAAACATATGCTATTCAAGGGCTGGGAAAAGTAGGGTATAAAGTAGCG
TIN             CAGCTGAAAGGGCGTGTGGTTGCCATCCAAGGAGTCGGCAAGGTGGGAGAGCGCTTGTTG
RHO             TCACTCGACGGTTTGACGGTCCTGGTCCAAGGACTGGGGGCAGTCGGAGGATCATTGGCA
                    *    **              * *****  * **    ** **        *

BC1             GAAGGGCTGCTCGAAGAAAGCGCTCATTTATTTGTAACGGATATTAACGAGCAAAGCTTG
BBA             GAAGGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGATATTAACGAGCAAACGTTG
SUR             GAACATATTATCAACGAAGGTGGAAACGTGATCGTCACAGATATTAATGAGCAAG----C
BSP             GAACAGCTCTTAAAAGCCGGCGCCGATTTATTTGTAACGGATATACATGAAAATGTCCTC
TIN             CAGCTTTTGGTCGAAGTGGGGGCTTACTGCAAAATTGCCGACATCGATTCGGTG-----C
RHO             TCCCTGGCCGCCGAAGCGGGTGCGCAACTCCTGGTGGCAGACACCGACACCGAG------
                          * *   * *   *        *  * ** *   *

BC1             GCGGCCATTCAGGAAAAAGCAAAAACAACATC-CGGTTCTGTCACGGTAGTGGCGAGCGA
BBA             GAGGCTATCCAGGAAAAAGCAAAAACAACATC-CGGTTCTGTCACGGTAGTAGCGAGCGA
SUR             GATTGCAG------ATATTCAGAAGCTCGGTGGAAGCGCTGTCAGGGTCGTATCAAGTGA
BSP             AATTCCATTAAGCAAAAATCAGAAGAGCT-TGGCGGTTCAGTGACCATTGTAAAAAGTGA
TIN             GATGCGAAC-----AGCTGAAAGAAAAGTATGGCGACAAGGTCCAATTGGTGGATGTGAA
RHO             ------------CGAGTAGCGCACGCTGT-TGCGTTGGGCCACACAGCGGTTGCCCTCGA
                           *              *                  **       *

BC1             TGAGATTTATTCCCAGGAAGCTGATGTGTTCGTCCCTTGTGCATTTGGCGGTGTTGTTAA
BBA             TGAAATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGCATTTGGCGGCGTTGTTAA
SUR             GGAGATTTACAGTCAGCAAGCAGATGTTTTTGTTCCTTGTGCATTTGGTGGCGTGATCAA
BSP             CGATATTTACAGCGTACAAGCGGATATATTTGTTCCGTGTGCGATGGGTGGTATTATCAA
TIN             CCGGATTCACAAGGAGAGTTGCGATATTTTCTCGCCTTGCGCCAAAGGCGGCGTGGTCAA
RHO             GGACGTTCTGTCCACCCCGTGTGATGTCTTCGCACCCTGCGCAATGGGCGGCGTCATCAC
                     **              *** * **    ** ** **    ** **  *  * *
```

(continued)

Figure 3.20 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from various sources. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO= *Rhodococcus sp.* Conserve residues are indicated by asterisks.

(continued)

```
BC1             TGATGAAACCATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCCAACAATCAGCT
BBA             TGATGAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCCAACAATCAGCT
SUR             TGACGACACGCTAAAGGTGCTGAAAGTACGAGGAATCTCCGGTTCAGCAAACAATCAGCT
BSP             TGATAAAACCATTCCTAAGTTAAAGGTGAAGGCTGTTGTGGGATCAGCCAATAACCAGCT
TIN             TGATGACACCATTGACGAGTTCCGTTGCCTGGCCATTGTCGGATCCGCCAACAACCAACT
RHO             CACCGAGGTGGCGCGAACACTCGACTGTTCCGTCGTGGCCGGTGCCGCCAACAACGTCAT
                     *              *          *   *    **  * ** ** **     *

BC1             GCTCACGGAGGATCACGGCAGACAGCTTGCATACACCGGCATTCTATATGCTCCGGATTA
BBA             GCTTACGGAGGATCACGGCAGACACCTTGCAGACAAAGGCATTCTGTATGCTCCGGATTA
SUR             CGCGGAAAGCCGCCATGGAGAGCTACTACGTGAAAAGGGTATTTTGTACGCACCAGACTA
BSP             CAAAGACCTCCGCCATGCAAATGTACTAAACGAAAAGGGAATTCTATATGCACCCGATTA
TIN             GGTGGAAGACCGGCATGGGGCACTGCTTCAAAAACGGAGCATTTGTTATGCACCCGATTA
RHO             CGCCGACGAGGCCGCCTCGGACATCCTGCACGCACGCGGAATTCTGTACGCTCCCGACTT
                                  **           * ***   ** ** ** ** *

BC1             TATTATTAACTCCGGCGGTCTCATCCAA---GTAGCCGACGAATCGTATGAGGTGAACAA
BBA             TATTGTTAACTCTGGCGGTCTGATCCAA---GTAGCCGACGAATTGTATGAGGTGAACAA
SUR             TATCGTCAACGGCGGCGGTTTAATCCAA---GTGGCGGATGAATTGTACGGAACGAATCC
BSP             TATCGTCAATGCCGGCGGCTTGATCCAG---GTTGCTGACGAACTTTATGGGCCGAATAA
TIN             TCTGGTGAATGCCGGCGGGCTGATTCAA---GTGGCTGATGAACTGGAAGGCTTCCATGA
RHO             CGTGGCCAACGCCGGCGGTGCCATCCACCTCGTAGGCCGGGAGGTTCTCGGTTGGTCCGA
                  *    **    *****    ** **    ** *     **       *

BC1             AGAACGCGTGCTTGCG-AAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCAGC
BBA             AGAACGCGTGCTTGCG-AAGACGAAGCATATTTACGACGCAATTCTTGAAGTGTACCAGC
SUR             TGCACGTGTACTCGCT-AAAACTGAAAACATCTATACCTCACTGCTTGAAGTATTCCATC
BSP             AGAGCGGGT-CTTGCTCAAAACGAAAGAAATTTACCGTTCTCTGCTTGAAATTTTTAATC
TIN             AGAGAGAGTGCTCGCC-AAAACCGAAGCGATTTATGACATGGTCCTGGATATTTTTCACC
RHO             GTCGGTTGTCCACGAA-CGAGCAGTTGCCATAGGCGACACCCTGAATCAGGTCTTCGAGA
                       ** *  *       *      **           *     *  * *   *

BC1             AAGCGGAATTAGATCAAATTACCATAATGGAAGCAGCCAACAGAAT-GT--GT--GAGCA
BBA             AAGCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAACAGAAT-GT--GT--GAGCA
SUR             AGGCAGAACAGGATCATATGACAACTGCCACTGCCGCAGACCGTAT-GT--GT--GAAAA
BSP             AGGCAGCCCTTGACTGCATCACAACAGTGGAGGCCGCAAATAGGAA-GT--GT--CAAAA
TIN             GGGCGAAAAATGAGAATATTACCACTTGTGAGGCAGCGGACCGGATCGT--GATGGAGCG
RHO             TCTCCGACAACGACGGCGTCACCCCGGACGAGGCCGCCCGCACTCTCGCTGGACGGCGCG
                   *      **     * **          ** **          *   *

BC1             AAGAATGGCGGCCAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAA-GCCAAAAT
BBA             AAGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAA-GCCAAAAT
SUR             GCGTATTGCGGATGCCAAGAATCGCAACAGCTTCTTCACACAGTCAAACCG-ACCGAAAT
BSP             GACGATTGAGGGCCAGCAAACCCGTAATAGTTTCTTTTCTAGGGGACGCAG-GCCGAAGT
TIN             TTTGAAAAAGTTAACCGATATTCGCCGGATCTTGTTGGAGGATCCCCGCAACAGCGCAAG
RHO             CCCGCGAGGCCTCGACAACGACAGCGACTGCCTAGT-AATCGATCTCGGAGTCTGGCGAT
                                     *        *  *

BC1             GGGATATTCGTAATTAAT---ACTT-GTTCGGGGGGATATCATGA---------------
BBA             GGGATATTCGCAACTAAT---ACT--GTTCGGGGGGATATCATGAATA-CTCAATACCCA
SUR             GGAATTTTCATCAGTAAT---AAAAATAGCTGAA--------------------------
BSP             GGAACATAAAAGAGTAAT---ATTGAAAGCGTAAACATTGGAAGAGGAGCTGAACATATG
TIN             GAGGTAAAAATCATTGATGAAACTGATGACCGAGGAGGATGTCCGCCATCTTGCCCCGGA
RHO             CGACCATCGGTCCCC-ATCTGGCACGGACGGTCATGCGAGGGTCGGCGTCCCGTCCAGTC
                              **
```

(continued)

Figure 3.20 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from various sources. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO= *Rhodococcus sp.* Conserve residues are indicated by asterisks.

(continued)

```
BC1             ------------------------------------------------------------
BBA             ATCAAAAGAATAATCGATGATGAAGGCAACTTGATAGATGCGTCTTACCAGGATCAGCTG
SUR             ------------------------------------------------------------
BSP             AACAAATATGAAACGATTGATCTTATGGAGGTGGCCAATAATGGGGCCACTCCTCCAAAT
TIN             TGGCCGTTTGACGGAATCGGGAAAGGAAATCTGGAACCGGTTGAGTGTGGAAAAGAAAAA
RHO             CGCCTCGGTCCT---------------------------------------------

BC1             ------------------------------------------------------------
BBA             AATGAGCAGCTTGTGAAAGACCTTTATTACCATATGCATCGAATTAGAACATTTGATAGA
SUR             ------------------------------------------------------------
BSP             TGTGATCTTACCTTGCAAATCCAGCCTGTTCATGCAAAGGATGGAAAATCAAAAGGAATT
TIN             AGACTTTTACCGGTGGATG--------------------------------------
RHO             ------------------------------------------------------------

BC1             --------------
BBA             AAGGCGATCAGCC-
SUR             --------------
BSP             TGGGAGGTAGATGA
TIN             --------------
RHO             --------------
```

Figure 3.20 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from various sources. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO= *Rhodococcus sp.* Conserve residues are indicated by asterisks.

```
CLUSTAL X (1.64b) multiple sequence alignment

BC1             ------------------------------------------------------------
BBA             GCATGCCTTCTATCGTGCTGAAATCCCCTGTTCCCGAGAAGATTATTTTGCCGTCTGATG

BC1             ------------------------------------------------------------
BBA             AAGAACAGGAATGGCCGTGAAGGACGGCGCATCATAGAAGATGATGTCGAATGATAAGAC

BC1             ------------------------------------------------------------
BBA             AAGCCTCCTCTTTCTATTGTCGAAAGAGGAGGCTTTTTTAGCTTTTATTTACTGGAAATG

BC1             ----------------------AAATACCCCAAAATACATACGGGAAGTAAATAAAGGT
BBA             AAAGCGTTTTACAAAACGAAGATAAATACCAAAAAATACATACGAAAAGTAAATAAAGGT
                                *******  ************  **************

BC1             CTGGAAATTCCCTCATAATGATATATATATATATCATTTAACCTTTTATAACTAAAGTGA
BBA             CTGAAAATTCAATCATAATGATATATATATATATCATTTAACCTTTTATAATTAAAGTGA
                *** ******  ************************************* ********

BC1             AGCGTATGCCTGCCGGCGCTGTTATTGGCGCTCGTTTGAAAGGGCTTACCAAAATTATAT
BBA             AGCGTATGCCTGCCGGCGCCGTCATTGGCGCTCGTTTGAAAGGGCTTACAAAAATTATAT
                ****************** ** ************************** **********

BC1             AACCAAGGAGCTGACAGATCCTTTTTTCTGCGGCTAAATAAAAGCGTTCAACTATTAACGA
BBA             AACCAAGAAGCTGACAGATCCTTTTTTCTGCGGCTAAATAAAAGCGTTCAACTATTAACGA
                ******* ************************************************

BC1             AAGCAGGGATTAAATATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTT
BBA             AAGCAGGGATTAAATATGAGCTTAGTAGAAAAAACATCCATCATAAAAGATTTCACTCTT
                ************************************************************

BC1             TTTGAAAAAATGTCTGAACATGAACAAGTTGTTTTTTGCAACGATCCTCGCACAGGACTA
BBA             TTTGAAAAAATGTCTGAACATGAACAAGTTGTTTTTTGCAACGATCCGGCGACAGGACTA
                ********************************************    *********

BC1             AGGGCCATTATCGCTATTCATGACACCACACTCGGACCTGCGCTTGGCGGCTGCCGCATG
BBA             AGGGCCATTATCGCTATTCATGACACCACACTCGGACCTGCGCTCGGCGGCTGCCGCATG
                ****************************************** ***************

BC1             CAGCCTTATAACAGTGTAGAAGAAGCGTTAGAAGATGCTCTTCGCCTCTCCAAAGGAATG
BBA             CAGCCTTATAACAGTGTGGAAGAAGCATTGGAAGATGCTCTTCGCCTTTCCAAAGGAATG
                **************** ******** ** ***************** ************

BC1             ACTTACAAATGCGCGGCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTATCGGT
BBA             ACTTACAAATGCGCGGCGTCCGATGTCGACTTTGGCGGCGGAAAAGCAGTCATTATCGGT
                ************************************************************

BC1             GATCCGCAGAAAGATAAATCTCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCG
BBA             GATCCGCAGAAAGATAAATCTCCAGAACTGTTCCGCGCGTTTGGCCAATTTGTTGATTCG
                ************************************************************

BC1             CTTGGCGGCCGTTTCTATACAGGCACTGATATGGGAACGAATATGGAAGATTTCATTCAC
BBA             CTTGGCGGCCGTTTCTATACAGGTACTGATATGGGAACGAATATGGAAGATTTCATTCAC
                ********************** ***********************************
```

(continued)

Figure 3.21 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from *Bacillus badius* BC1 and published *Bacillus badius*. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius* Conserve residues are indicated by asterisks.

(continued)

```
BC1             GCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGAT
BBA             GCCATGAAAGAAACAAACTGCATTGTTGGGGTGCCGGAAGCTTACGGCGGCGGCGGAGAT
                ************************************************************

BC1             TCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGCTG
BBA             TCCTCTATTCCAACTGCCATGGGTGTCCTGTACGGCATTAAAGCAACCAACAAAATGTTG
                ************************************************************ **

BC1             TTTGGCAAGGACGATCTTGGCGGTGTCACGTATGCCATTCAAGGACTTGGCAAAGTAGGC
BBA             TTTGGCAAGGACGATCTTGGCGGCGTCACTTATGCCATTCAAGGACTTGGCAAAGTAGGC
                ********************** ***** ******************************

BC1             TACAAAGTAGCGGAAGGGCTGCTCGAAGAAAGCGCTCATTTATTTGTAACGGATATTAAC
BBA             TACAAAGTAGCGGAAGGGCTGCTCGAAGAAGGTGCTCATTTATTTGTAACGGATATTAAC
                ***************************** * **************************

BC1             GAGCAAAGCTTGGCGGCCATTCAGGAAAAAGCAAAAACAACATCCGGTTCTGTCACGGTA
BBA             GAGCAAACGTTGGAGGCTATCCAGGAAAAAGCAAAAACAACATCCGGTTCTGTCACGGTA
                *******  **** *** ** ************************************

BC1             GTGGCGAGCGATGAGATTTATTCCCAGGAAGCTGATGTGTTCGTCCCTTGTGCATTTGGC
BBA             GTAGCGAGCGATGAAATTTATTCCCAGGAAGCCGATGTGTTCGTTCCGTGTGCATTTGGC
                ** ************ ***************** ************ ** ************

BC1             GGTGTTGTTAATGATGAAACCATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCC
BBA             GGCGTTGTTAATGATGAAACGATGAAGCAGTTCAAGGTGAAAGCAATCGCCGGTTCAGCC
                ** ****************** ****************************************

BC1             AACAATCAGCTGCTCACGGAGGATCACGGCAGACAGCTTGCATACACCGGCATTCTATAT
BBA             AACAATCAGCTGCTTACGGAGGATCACGGCAGACACCTTGCAGACAAAGGCATTCTGTAT
                ************** ******************** ****** ***  ******** ***

BC1             GCTCCGGATTATATTATTAACTCCGGCGGTCTCATCCAAGTAGCCGACGAATCGTATGAG
BBA             GCTCCGGATTATATTGTTAACTCTGGCGGTCTGATCCAAGTAGCCGACGAATTGTATGAG
                *************** ******* ******** ******************* *******

BC1             GTGAACAAAGAACGCGTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTG
BBA             GTGAACAAAGAACGCGTGCTTGCGAAGACGAAGCATATTTACGACGCAATTCTTGAAGTG
                ************************************************************

BC1             TACCAGCAAGCGGAATTAGATCAAATTACCATAATGGAAGCAGCCAACAGAATGTGTGAG
BBA             TACCAGCAAGCGGAATTAGATCAAATCACCACAATGGAAGCAGCCAACAGAATGTGTGAG
                *************************** **** ****************************

BC1             CAAAGAATGGCGGCCAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAA
BBA             CAAAGAATGGCGGCAAGAGGCCGACGCAACAGCTTCTTTACTTCTTCTGTTAAGCCAAAA
                ************** ******************************************

BC1             TGGGATATTCGTAATTAATACTTGTTCGGGGGGGATATCATGA------------------
BBA             TGGGATATTCGCAACTAATACT-GTTCGGGGGGGATATCATGAATACTCAATACCCAATCA
                *********** ** ******* ********************

BC1             ------------------------------------------------------------
BBA             AAAGAATAATCGATGATGAAGGCAACTTGATAGATGCGTCTTACCAGGATCAGCTGAATG
```

(continued)

Figure 3.21 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from *Bacillus badius* BC1 and published *Bacillus badius*. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius* Conserve residues are indicated by asterisks.

(continued)

```
BC1             ------------------------------------------------------------
BBA             AGCAGCTTGTGAAAGACCTTTATTACCATATGCATCGAATTAGAACATTTGATAGAAAGG


BC1             ---------
BBA             CGATCAGCC
```

(continued)

Figure 3.21 Linear alignment of the nucleotide sequence of phenylalanine dehydrogenases gene from *Bacillus badius* BC1 and published *Bacillus badius*. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius* Conserve residues are indicated by asterisks.

```
CLUSTAL X (1.64b) multiple sequence alignment

BC1             MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPRTGLRAIIAIHDTTLGPALGGCRMQPYNS
BBA             MSLVEKTSIIKDFTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQPYNS
                ****************************** ****************************

BC1             VEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKDKSPELFRAFGQFVDSLGGRF
BBA             VEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIGDPQKDKSPELFRAFGQFVDSLGGRF
                ************************************************************

BC1             YTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNKMLFGKDD
BBA             YTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATNKMLFGKDD
                ************************************************************

BC1             LGGVTYAIQGLGKVGYKVAEGLLEESAHLFVTDINEQSLAAIQEKAKTTSGSVTVVASDE
BBA             LGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGSVTVVASDE
                *************************.***********:* ********************

BC1             IYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRQLAYTGILYAPDYI
BBA             IYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKGILYAPDYI
                ***********************************************:** .*********

BC1             INSGGLIQVADESYEVNKERVLAKTKHIYDAILEVYQQAELDQITIMEAANRMCEQRMAA
BBA             VNSGGLIQVADELYEVNKERVLAKTKHIYDAIL-------LDQITTMEAANRMCEQRMAA
                :*********** ********************       ***** **************

BC1             RGRRNSFFTSSVKPKWDIRN
BBA             RGRRNSFFTSSVKPKWDIRN
                ********************
```

Figure 3.22 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenase gene from *Bacillus badius* BC1 and published *Bacillus badius*. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*. Conserve residues in these enzymes are indicated in asterisks. **:** means amino acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

```
CLUSTAL X (1.64b) multiple sequence alignment

BC1             -MSLVEKTSIIKD--FTLFEKMSEHEQVVFCNDPRTGLRAIIAIHDTTLGPALGGCRMQP
BBA             -MSLVEKTSIIKD--FTLFEKMSEHEQVVFCNDPATGLRAIIAIHDTTLGPALGGCRMQP
SUR             -MILVTLEQTLQDDKASVLDKMVEHEQILFCHDKATGLQAIIAVHDTTMGPALGGCRMAP
BSP             MAKQLEKSSKIGN--EDVFQKIANHEQIVFCNDPVSGLQAIIAIHDTTLGPALGGTRMYP
BHA             ---MLTKTPTVTS-TLDIFTEMAEHEQVLFCHDPSSGLRAIIAIHDTTLGPALGGCRMYP
TIN             ----------MRD-VFEMMDRYG-HEQVIFCRHPQTGLKAIIALHNTTAGPALGGCRMIP
RHO             --------MSIDS----ALNWDG--EMTVTRFDRETGAHFVIRLDSTQLGPAAGGTRAAQ
                         : .    :      *  :   .  :* : :* :..*  *** ** *

BC1             YNSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIG-DPQK--DKS--PELFRAFGQF
BBA             YNSVEEALEDALRLSKGMTYKCAASDVDFGGGKAVIIG-DPQK--DKS--PELFRAFGQF
SUR             YKTMDLALKDVLRLSKGMTYKCAAADVDFGGGKSVIIG-DPLK--DKT--PEKFRAFGQF
BSP             YKNVDEALEDVLRLSEGMTYKCAAADIDFGGGKAVIIG-DPEK--DKS--PALFRAFGQF
BHA             YQTTEDALRDVLRLSKGMTQKCAAADVDFGGGKAVIIG-DPAK--DKS--ANLFRAFGQF
TIN             YASTDEALEDVLRLSKGMTYKCSLADVDFGGGKMVIIG-DPKK--DKS--PELFRVIGRF
RHO             YSQLADALTDAGKLAGAMTLKMAVSNLPMGGGKSVIALPAPRHSIDPSTWARILRIHAEN
                *     ** *. :*: .** * : ::: :**** **    * :  * :  .  :*  ..

BC1             VDSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATN
BBA             VDSLGGRFYTGTDMGTNMEDFIHAMKETNCIVGVPEAYGGGGDSSIPTAMGVLYGIKATN
SUR             IESLNGRFYTGTDMGTTLEDFVHAMKETNYIVGKPVEYGGGGDSSIPTALGVFYGIKATN
BSP             VESLNGRFYTGTDMGTTMDDFVHAQKETNFINGIPEQYGGSGDSSIPTAQGVIYALKATN
BHA             VESLNGRFYTGTDMGTTMEDFVHALKETNGIVGIPKEYGGSGDSSVPTAKGVINSLKAIS
TIN             VGGLNGRFYTGTDMGTNPEDFVHAARESKSFAGLPKSYGGKGDTSIPTALGVFHGMRATA
RHO             IDKLSGNYWTGPDVNTNSADMDTLNDTTEFVFGRSLERGGAGSSAFTTAVGVFEAMKATV
                :  *.*.::**.*:.*.  *:      :: . * .   ** *.::..** **: .::*
```

(continued)

Figure 3.23 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases. Protein sequences that were determined by automated Edman degradation are underlined. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus sp.* Conserve residues in these enzymes are indicated in asterisks. **:** means amino acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

(continued)

```
BC1             KMLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEESAHLFVTDINEQSLAAIQEKAKTTSGS
BBA             KMLFGKDDLGGVTYAIQGLGKVGYKVAEGLLEEGAHLFVTDINEQTLEAIQEKAKTTSGS
SUR             QNLFGDDKVEGRKYSIQGLGKVGYKVAEHIINEGGNVIVTDINEQAIADIQKLG---GSA
BSP             QYLFGSDSLSGKTYAIQGLGKVGYKVAEQLLKAGADLFVTDIHENVLNSIKQKSEELGGS
BHA             QVVLKDKQFSGRTYAIQGLGKVGFKVAEELLKEGNDLYVSDLQESLPLRLQQLGQRLGRH
TIN             RFLWGTDQLKGRVVAIQGVGKVGERLLQLLVEVGAYCKIADIDSVRCEQLKEKY---GDK
RHO             AHR-GLGSLDGLTVLVQGLGAVGGSLASLAAEAGAQLLVADTDTERVAHAVALG------
                      .. *    :**:* **  : .   : .    ::* .

BC1             VTVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRQLAYTG
BBA             VTVVASDEIYSQEADVFVPCAFGGVVNDETMKQFKVKAIAGSANNQLLTEDHGRHLADKG
SUR             VRVVSSEEIYSQQADVFVPCAFGGVINDDTLKVLKVRGISGSANNQLAESRHGELLREKG
BSP             VTIVKSDDIYSVQADIFVPCAMGGIINDKTIPKLKVKAVVGSANNQLKDLRHANVLNEKG
BHA             VEILHGDEIYEAAADVFVPCAQGAILNDATIARLKVKAIAGAANNQLEAERHGQMLHDQG
TIN             VQLVDVNRIHKESCDIFSPCAKGGVVNDDTIDEFRCLAIVGSANNQLVEDRHGALLQKRS
RHO             HTAVALEDVLSTPCDVFAPCAMGGVITTEVARTLDCSVVAGAANNVIADEAASDILHARG
                   :  : : .  .*:* *** *.::.  .   :    : *:*** :     .  *   .
```

(continued)

Figure 3.23 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases. Protein sequences that were determined by automated Edman degradation are underlined. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus sp.* Conserve residues in these enzymes are indicated in asterisks. **:** means amino acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

(continued)

```
BC1             ILYAPDYIINSGGLIQ-VADESYEVNKERVLAKTKHIYDAILEVYQQAELDQITIMEAAN
BBA             ILYAPDYIVNSGGLIQ-VADELYEVNKERVLAKTKHIYDAILEVYQQAELDQITTMEAAN
SUR             ILYAPDYIVNGGGLIQ-VADELYGTNPARVLAKTENIYTSLLEVFHQAEQDHMTTATAAD
BSP             ILYAPDYIVNAGGLIQ-VADELYGPNKERVLLKTKEIYRSLLEIFNQAALDCITTVEAAN
BHA             IWFAPDYIVNSGGLIQ-VADELYGSNEKRVLSKTNAIYDTILEIFHQAERHHITTLQAAN
TIN             ICYAPDYLVNAGGLIQ-VADELEGFHEERVLAKTEAIYDMVLDIFHRAKNENITTCEAAD
RHO             ILYAPDFVANAGGAIHLVGREVLGWSESVVHERAVAIGDTLNQVFEISDNDGVTPDEAAR
                * :***:: *.** *: *. *       *  ::  *   : :::. :  . :*   **

BC1             RMCEQRMAARGRRNSFFTSSVKPKWDIRN
BBA             RMCEQRMAARGRRNSFFTSSVKPKWDIRN
SUR             RMCEKRIADAKNRNSFFTQSNRPKWNFHQ
BSP             RKCQKTIEGQQTRNSFFSRGRRPKWNIKE
BHA             QLCERRIRERARRNNFFVNRIRPKWNLRK
TIN             RIVMERLKKLTDIRRILLED--PRNSARR
RHO             TLAGRRAREASTTTATA------------
```

Figure 3.23 Linear alignment of the deduced amino acid sequence of phenylalanine dehydrogenases. Protein sequences that were determined by automated Edman degradation are underlined. BC1 = *Bacillus badius* BC1, BBA = *Bacillus badius*, BSP = *Bacillus sphaericus*, BHA = *Bacillus halodurans*, SUR = *Sporosarcina ureae* , TIN = *Thermoactinomyces intermedius* and RHO = *Rhodococcus sp.* Conserve residues in these enzymes are indicated in asterisks. **:** means amino acid which have the same group of side chains and similar size while **.** means amino acid which have the same group of side chains but different size.

restriction site, (ii) a Shine-Dalgarno sequence of plasmid p*Trc*99, the expression vector for *E. coli* JM105 and (iii) 5'-end of the desired sequence was designed. The 3'-primer (Phe-Bam) contained 3' end of phenylalanine dehydrogenase gene, the TAA translational termination signal followed by the restriction site for *Bam*HI. Figure 3.24 shows the 1171 bp PCR product of the whole gene fragment amplified from the various templates. Only *Cla*I, *Pvu*I and *Spe*I digested DNA templates gave strong specific PCR product, while the others also gave a similar product with lower band density. After the nucleotide sequences of all products were confirmed, the PCR product of *Cla*I digested DNA template was used for further cloning.

3.8 Transformation

The whole gene fragment was treated with *Eco*RI and *Bam*HI, ligated with *Eco*RI - *Bam*HI digested pUC18 vector, and then transformed into *E. coli* JM109 by electroporation. Twenty-five white colonies expected to contain the recombinant plasmids were ramdomly picked for plasmid extraction and digestion with *Eco*RI-*Bam*HI. Four types of plasmid were obtained. In details, twenty-two selected colonies had the first type of plasmid, which gave two strong bands, supercoil and relaxed forms, on agarose gel electrophoresis (Figure 3.25, lane 2-6). Moreover, it could be double digested by *Eco*RI-*Bam*HI to linear pUC18 (about 2.7 kb) and inserted phenylalanine dehydrogenase gene fragment (1.17 kb) (Figure 3.26, lane 4-8). For the left three colonies, they were one colony of second, third and fourth type of plasmids. The second type (Figure 3.25, lane 8) gave 3 bands which 2 of them were similar to those of the first type (supercoil and relaxed form), while the other one was unknown, supposed not to be linear form base on its mobility compared with λ/*Hin*dIII standard marker. Surprisingly, *Eco*RI-*Bam*HI digested products of this type were composed of linear pUC18 and inserted gene fragment similar to the products of the first type (Figure 3.26, lane 10). The third type of plasmid had higher molecular weight than the first type (Figure 3.25, lane 7), however, it also gave a band of inserted gene after double digestion. The result suggested that its part of vector was changed. The last type showed 2 main strong bands, which gave their mobility of cut and uncut plasmid similar to those of pUC18 (Figure 3.25, lane 9 and Figure 3.26, lane 11). Thus, it may suggested to be pUC18 monomer.

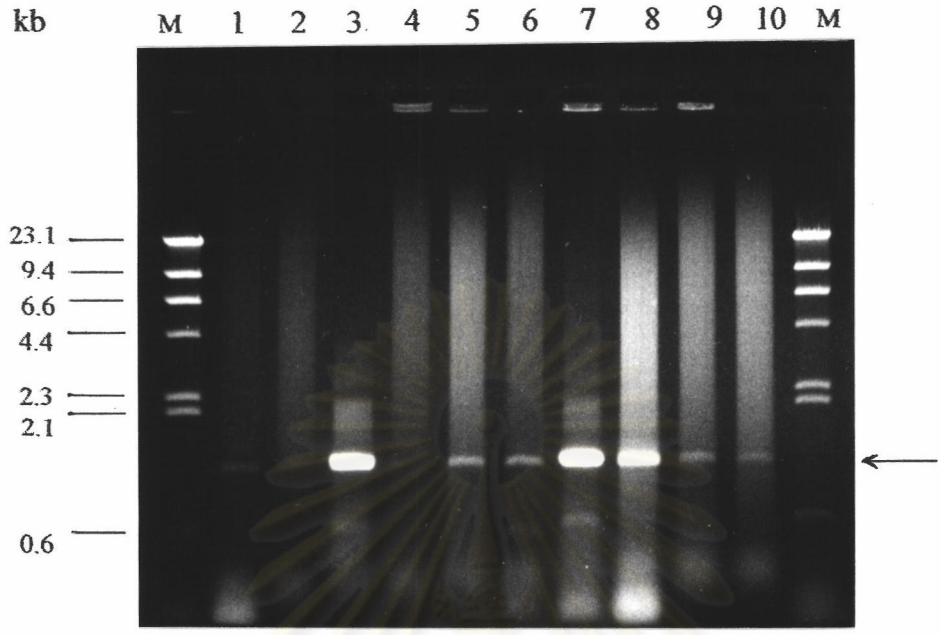

Figure 3.24 PCR product of the whole phenylalanine dehydrogenase gene amplification using primers Phe-Eco and Phe-Bam (1171 bp). The specific product is indicated by arrow.

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

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

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

Lane 3 = amplified products using *Cla*I digested chromosomal DNA as template

Lane 4 = amplified products using *Eco*RI digested chromosomal DNA as template

Lane 5 = amplified products using *Kpn*I digested chromosomal DNA as template

Lane 6 = amplified products using *Pst*I digested chromosomal DNA as template

Lane 7 = amplified products using *Pvu*I digested chromosomal DNA as template

Lane 8 = amplified products using *Spe*I digested chromosomal DNA as template

Lane 9 = amplified products using *Xba*I digested chromosomal DNA as template

Lane 10 = amplified products using *Xho*I digested chromosomal DNA as template

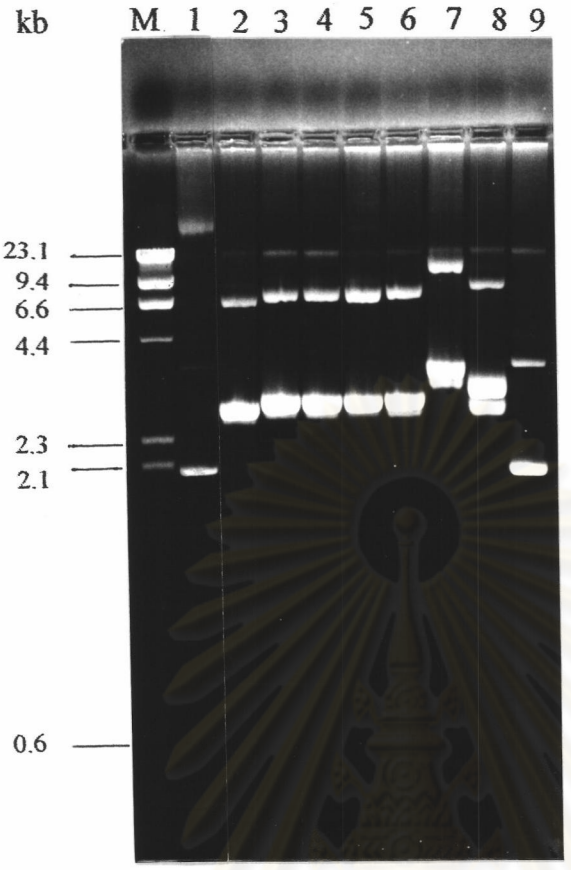

Figure 3.25 Extracted plasmid of transformants

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

Lane 1 = undigested pUC18

Lane 2-9 = extracted plasmids of transformant No 4, 5, 6, 10, 15, 19, 20 and 22, respectively

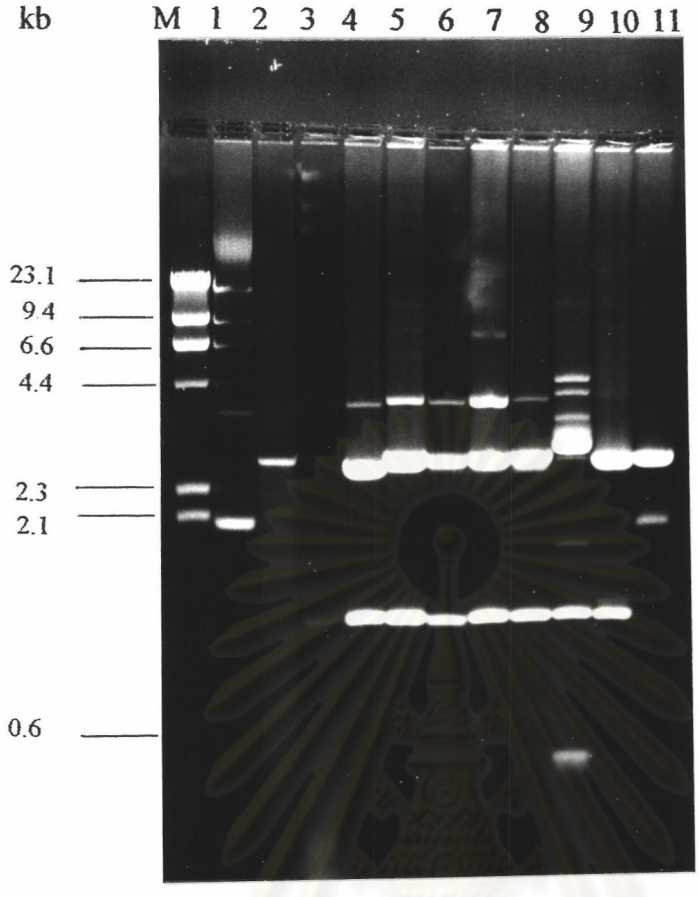

Figure 3.26 *Eco*RI - *Bam*HI digested plasmid of transformants

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

Lane 1 = undigested pUC18

Lane 2 = *Eco*RI – *Bam*HI digested pUC18

Lane 3 = amplified product of the whole phenylalanine dehydrogenase gene

Lane 4-11 = *Eco*RI - *Bam*HI digested plasmid of transformants No 4, 5, 6, 10, 15, 19, 20 and 22, respectively

In addition to 2 strong bands, this type of plasmid also gave very weak bands corresponded with the pattern of the first type.

3.9 Phenylalanine dehydrogenase activity of transformant

The 25 recombinant clones were also grown for enzyme assay as described in 2.15 and 2.16. *E. coli* JM109 and *E. coli* JM109 containing plasmid pUC18 were used as references. The transformants possessed the first type of plasmid showed various levels of the enzyme total activities from 0-338 Units. This may be caused by error in PCR amplification of the whole gene, which led to the changing of essential amino residues of the enzyme. The highest total activity with 60- fold higher than that of *Bacillus badius* BC1 was produced by transformant No. 22 which seemed to harbour only vector plasmid. It was noted that transformant No. 19 which had abnormal part of vector showed very low enzyme activity. The result showed in Table 3.2.

3.10 Protein and activity patterns of crude extract from transformants

Crude extracts from 7 transformants that had high total enzyme activity, *E. coli* JM109, *E. coli* JM109 harbouring pUC18 and purified phenylalanine dehydrogenase from *Bacillus badius* BC1 (Leksakorn, 2001) were subjected to the native-PAGE. The intensity of recombinant protein bands and activity staining bands (Figure 3.27 and 3.28) corresponded with the level of enzyme assayed from crude extracts (Table 3.2). From these results, the crude extracts from both *E. coli* JM109 and *E. coli* JM109 containing pUC18 had neither the protein nor the activity band of phenylalanine dehydrogenase, while crude extracts from the transformants exhibited identical mobility bands with the purified enzyme from *Bacillus badius* BC1 wild type.

3.11 Induction time determination

For induction time course study, the transformant No.15, which showed the highest phenylalanine dehydrogenase activity among the transformant containing the first type of plasmid, was grown and induced by IPTG at final concentration of 1 mM at

Table 3.2 Phenylalanine dehydrogenase activity from crude extracts of transformants

Sources of crude extract	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
E.coli JM109	0	3.27	0
E.coli JM109 with pUC18	0	2.90	0
Bacillus badius BC1	6.40	3.20	2.00
Transformant No.1	25.03	3.20	7.82
Transformant No.2	0	2.40	0
Transformant No.3	1.92	2.40	0.80
Transformant No.4	269.0	2.52	106.75
Transformant No.5	66.13	2.56	25.83
Transformant No.6	249.0	2.88	86.46
Transformant No.7	3.38	2.72	1.24
Transformant No.8	3.0	2.77	1.08
Transformant No.9	0.08	2.70	0.03
Transformant No.10	173.0	2.47	70.04
Transformant No.11	3.91	2.53	1.55
Transformant No.12	1.42	2.77	0.51
Transformant No.13	1.71	2.53	0.68
Transformant No.14	0	2.57	0
Transformant No.15	338.0	2.52	134.0
Transformant No.16	2.55	3.10	0.82
Transformant No.17	30.22	2.87	10.53
Transformant No.18	0.46	2.53	0.18
Transformant No.19	0.98	2.43	0.40
Transformant No.20	78.22	2.43	32.19
Transformant No.21	0.10	2.50	0.04
Transformant No.22	360.0	2.68	134.0
Transformant No.23	1.69	2.80	0.60
Transformant No.24	8.10	3.33	2.43
Transformant No.25	2.24	3.47	0.65

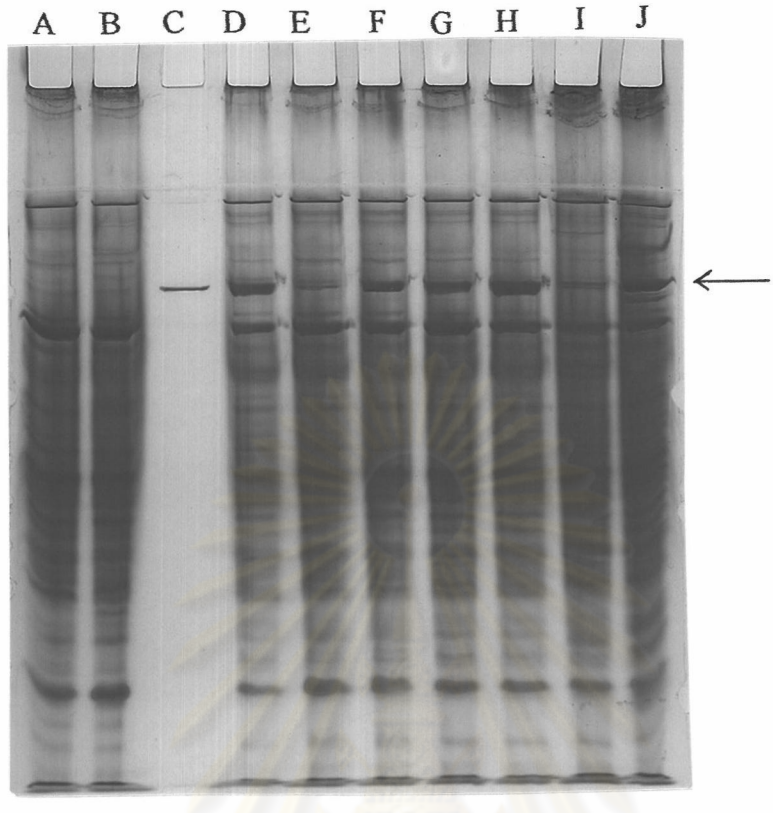

Figure 3.27 Protein pattern of crude extracts of phenylalanine dehydrogenase producing transformants detected by native-PAGE

Lane A = crude extract of *E. coli* JM109 (65 μg)

Lane B = crude extract of *E. coli* JM109 harbouring pUC18 (60 μg)

Lane C = purified phenylalanine dehydrogenase from *Bacillus badius* BC1

Lane D = crude extracts of transformant No.4 (50 μg)

Lane E = crude extracts of transformant No.5 (50 μg)

Lane F = crude extracts of transformant No.6 (50 μg)

Lane G = crude extracts of transformant No.10 (50 μg)

Lane H = crude extracts of transformant No.15 (50 μg)

Lane I = crude extracts of transformant No.20 (65 μg)

Lane J = crude extracts of transformant No.22 (65 μg)

An arrow indicates bands corresponding to phenylalanine dehydrogenase.

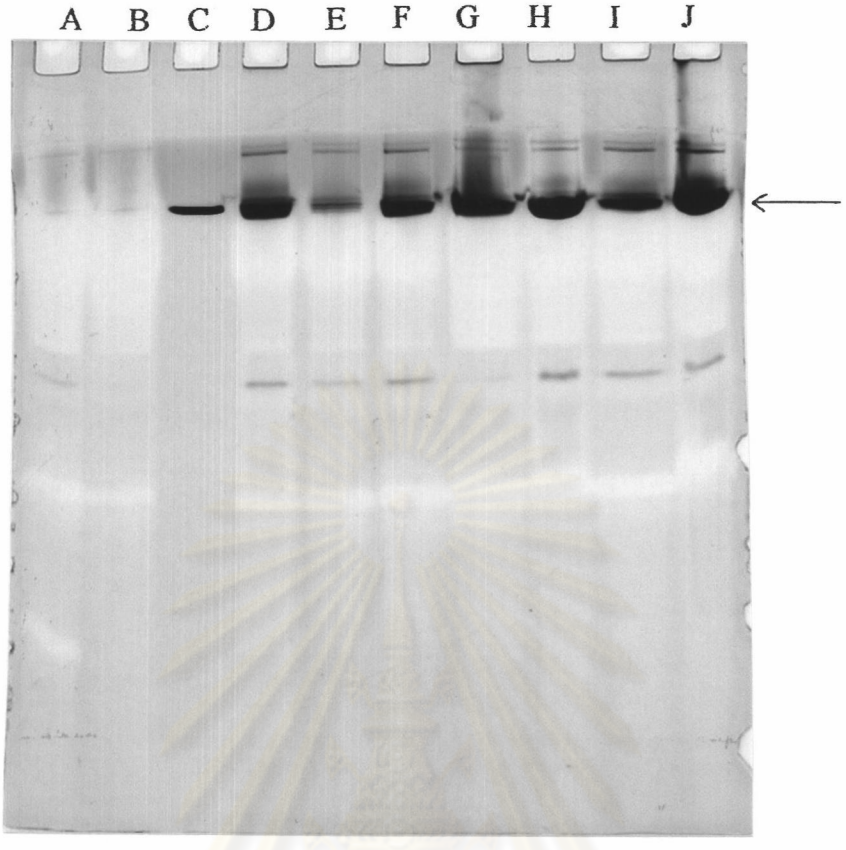

Figure 3.28 Phenylalanine dehydrogenase activity staining of crude extracts of phenylalanine dehydrogenase producing transformants

Lane A = crude extract of *E. coli* JM109 (65 μg)

Lane B = crude extract of *E. coli* JM109 harbouring pUC18 (60 μg)

Lane C = purified phenylalanine dehydrogenase from *Bacillus badius* BC1

Lane D = crude extracts of transformant No.4 (50 μg)

Lane E = crude extracts of transformant No.5 (50 μg)

Lane F = crude extracts of transformant No.6 (50 μg)

Lane G = crude extracts of transformant No.10 (50 μg)

Lane H = crude extracts of transformant No.15 (50 μg)

Lane I = crude extracts of transformant No.20 (65 μg)

Lane J = crude extracts of transformant No.22 (65 μg)

An arrow indicates bands corresponding to phenylalanine dehydrogenase.

various times before cell was harvested as described in 2.19. The result showed in Figure 3.29 indicated that 120 minutes was optimum time to induce phenylalanine dehydrogenase production. However, the enzyme induction did not occurred after 300 minutes.

3.12 Stability of phenylalanine dehydrogenase gene in host cell *E. coli* JM109

Stability of phenylalanine dehydrogenase gene from recombinant clones that showed high phenylalanine dehydrogenase activity was studied by daily subculturing for 15 days as described in 2.20.1. Plasmid of the 15^{th} subcultured transformants gave the same patterns with their original plasmids and can be digested with *Eco*RI and *Bam*HI (Figure 3.30 and 3.31). Phenylalanine dehydrogenase activities in crude extracts of the 5^{th}, 10^{th} and 15^{th} subcultured clone were determined as shown in Figure 3.32. Polyacrylamide gel electrophoresis for protein and activity staining were performed (Figure 3.33 and 3.34). The result suggested that each clone showed vary expression level of phenylalanine dehydrogenase gene. Enzyme activity of five transformants, No. 4, 6, 10, 15 and 20 were still remained upon subculturing for 15 times while the enzyme activity of transformant No. 22 decreased rapidly since the crude extract of the 5^{th} subcultured clone was detected. These results indicated that all of recombinant plasmids except No.22 were stable without host cell deletion process.

After retransformation of recombinant plasmids, which represented each type of plasmid pattern into *E. coli* JM109, pattern of recombinant plasmids and enzyme activities were confirmed. All of retransformed plasmids gave the same patterns with their original plasmids and also can be digested with *Eco*RI and *Bam*HI (Figure 3.35 and 3.36). Phenylalanine dehydrogenase activities in crude extracts of each retransformant were determined as shown in Table 3.3 while protein patterns were shown in Figure 3.37. The results presented that phenylalanine dehydrogenase activity of all retransformants were still constantly remained. In detail, retransformant No. 6, which had the first type of plasmid still gave high enzyme activity whereas retransformant No.20 and No.19 showed moderate and low enzyme activity from the second and third type of plasmid, respectively. Enzyme activity of retransformant No. 22 that showed the fourth type of plasmid matched well with that of daily subculturing.

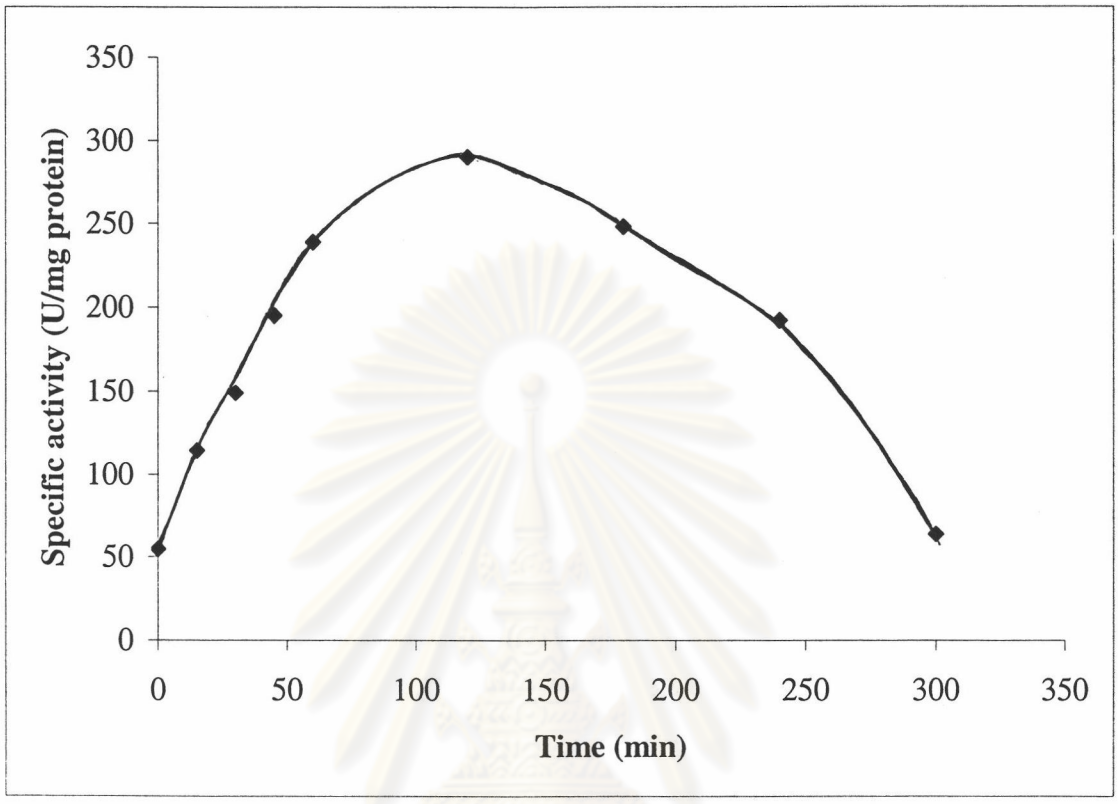

Figure 3.29 Induction time course studies of the transformant No.15

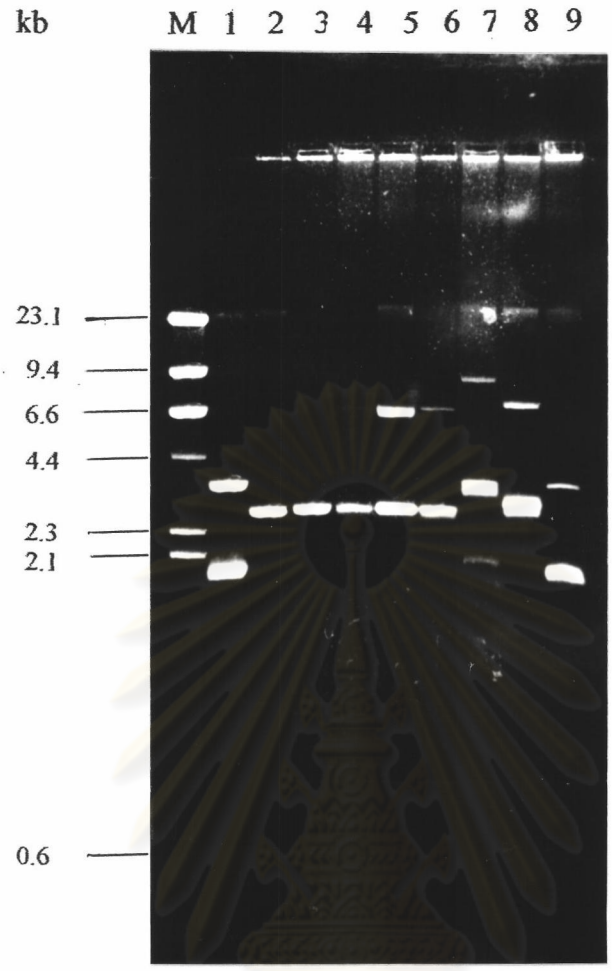

Figure 3.30 Extracted plasmid of the 15th subcultured transformants

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

Lane 1 = undigested pUC18

Lane 2 - 9 = extracted plasmid of the 15th subcultured transformants No.4, 5, 6, 10, 15, 19, 20 and 22, respectively.

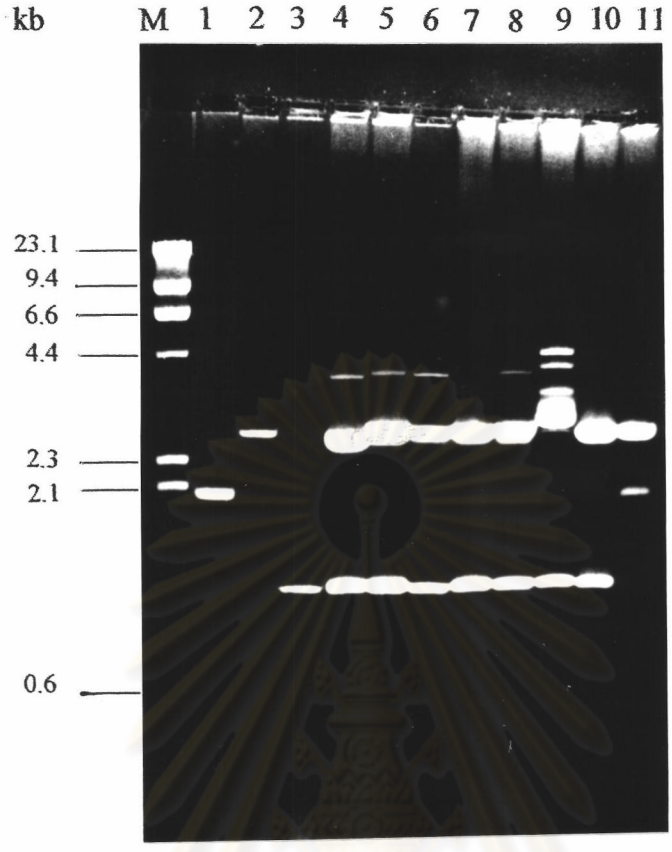

Figure 3.31 *Eco*RI – *Bam*HI digested plasmid of the 15th subcultured transformants

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

Lane 1 = undigested pUC18

Lane 2 = *Eco*RI – *Bam*HI digested pUC18

Lane 3 = amplified product of the whole phenylalanine dehydrogenase gene

Lane 4 – 11 = *Eco*RI – *Bam*HI digested plasmid of the 15th subcultured transformants No.4, 5, 6, 10, 15, 19, 20 and 22, respectively.

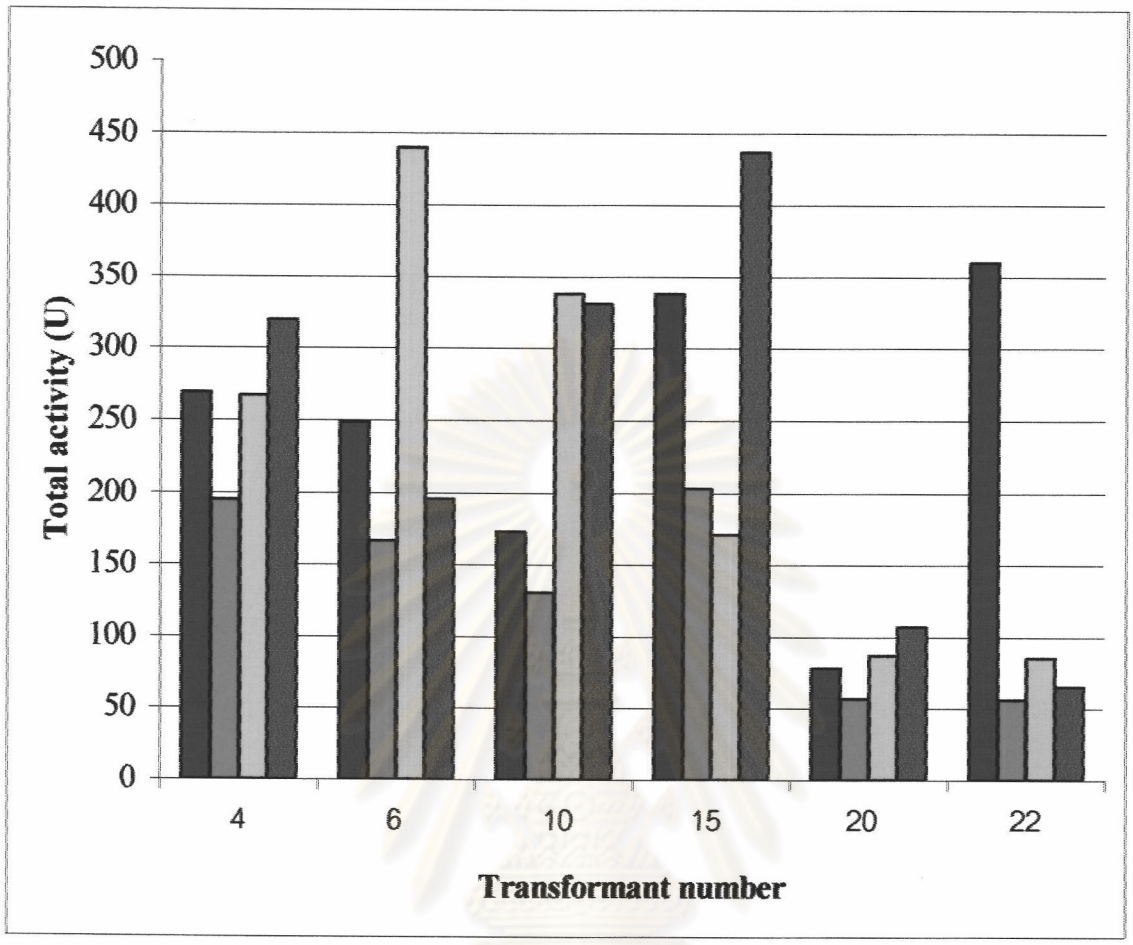

Figure 3.32 Comparison of phenylalanine dehydrogenase activity of subcultured clones

Note: blue = parent

red = the 5th subcultured colony

yellow = the 10th subcultured colony

green = the 15th subcultured colony

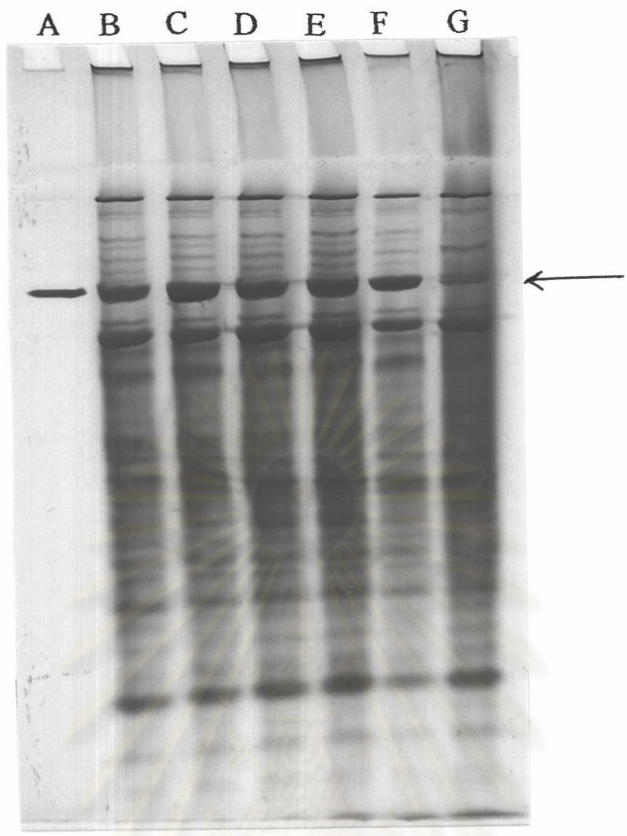

Figure 3.33 Protein pattern of crude extracts of the 15th subcultured transformants detected by native-PAGE

Lane A = purified phenylalanine dehydrogenase from *Bacillus badius* BC1

Lane B = crude extracts of the 15th subcultured transformant No.4 (68 μg)

Lane C = crude extracts of the 15th subcultured transformant No.6 (78 μg)

Lane D = crude extracts of the 15th subcultured transformant No.10 (82 μg)

Lane E = crude extracts of the 15th subcultured transformant No.15 (89 μg)

Lane F = crude extracts of the 15th subcultured transformant No.20 (60 μg)

Lane G = crude extracts of the 15th subcultured transformant No.22 (86 μg)

An arrow indicates bands corresponding to phenylalanine dehydrogenase.

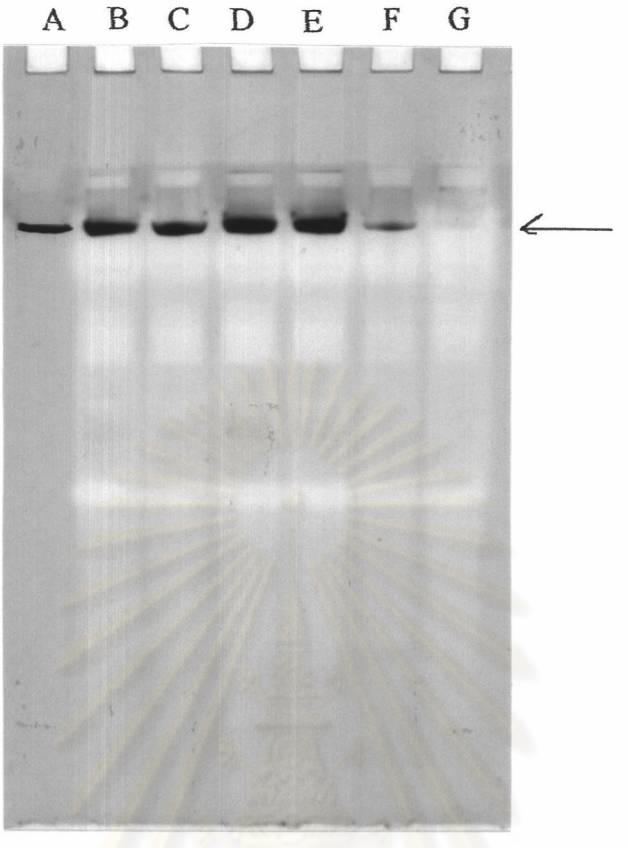

Figure 3.34 Phenylalanine dehydrogenase activity staining of crude extracts of the 15th subcultured transformants

Lane A = purified phenylalanine dehydrogenase from *Bacillus badius* BC1

Lane B = crude extracts of the 15th subcultured transformant No.4 (68 μg)

Lane C = crude extracts of the 15th subcultured transformant No.6 (78 μg)

Lane D = crude extracts of the 15th subcultured transformant No.10 (82 μg)

Lane E = crude extracts of the 15th subcultured transformant No.15 (89 μg)

Lane F = crude extracts of the 15th subcultured transformant No.20 (60 μg)

Lane G = crude extracts of the 15th subcultured transformant No.22 (86 μg)

An arrow indicates bands corresponding to phenylalanine dehydrogenase.

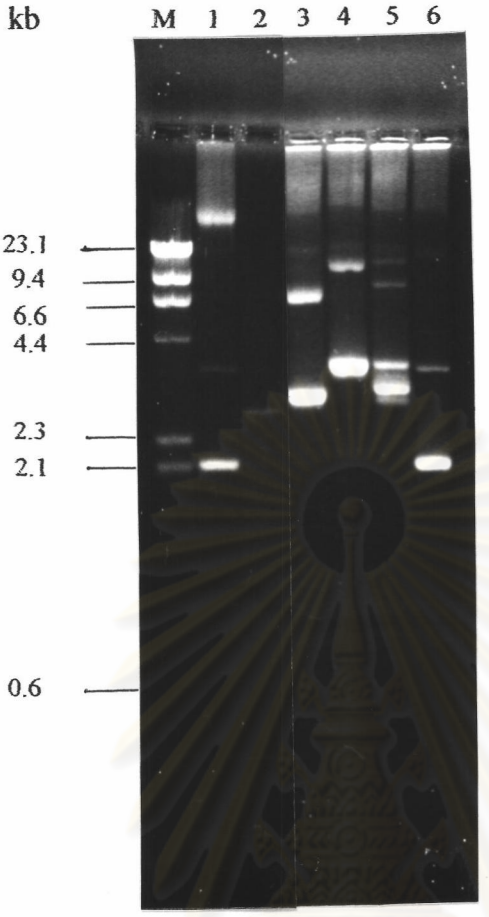

Figure 3.35 Extracted plasmid of retransformants

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

Lane 1 = undigested pUC18

Lane 2 = *Eco*RI – *Bam*HI digested pUC18

Lane 3 – 6 = extracted plasmid of retransformant No.6, 19, 20 and 22, respectively.

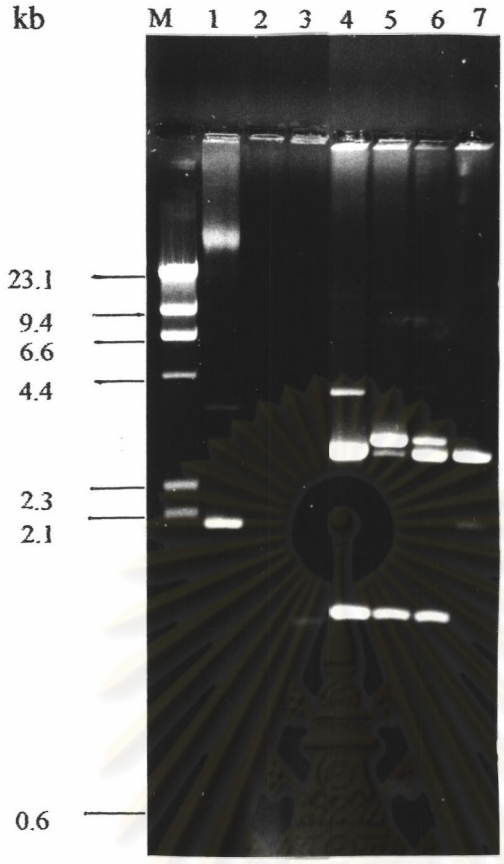

Figure 3.36 *Eco*RI – *Bam*HI digested plasmid of retransformants

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

Lane 1 = undigested pUC18

Lane 2 = *Eco*RI – *Bam*HI digested pUC18

Lane 3 = amplified product of the whole phenylalanine dehydrogenase gene

Lane 4 – 7 = *Eco*RI – *Bam*HI digested plasmid of retransformant No.6, 19, 20 and 22, respectively.

Table 3.3 Phenylalanine dehydrogenase activity from crude extracts of retransformant clones

Sources of crude extract	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)
Retransformant No.6	352.00	2.55	138.04
Retransformant No.19	7.80	2.50	3.12
Retransformant No.20	128.88	2.44	52.82
Retransformant No.22	24.53	2.60	9.44

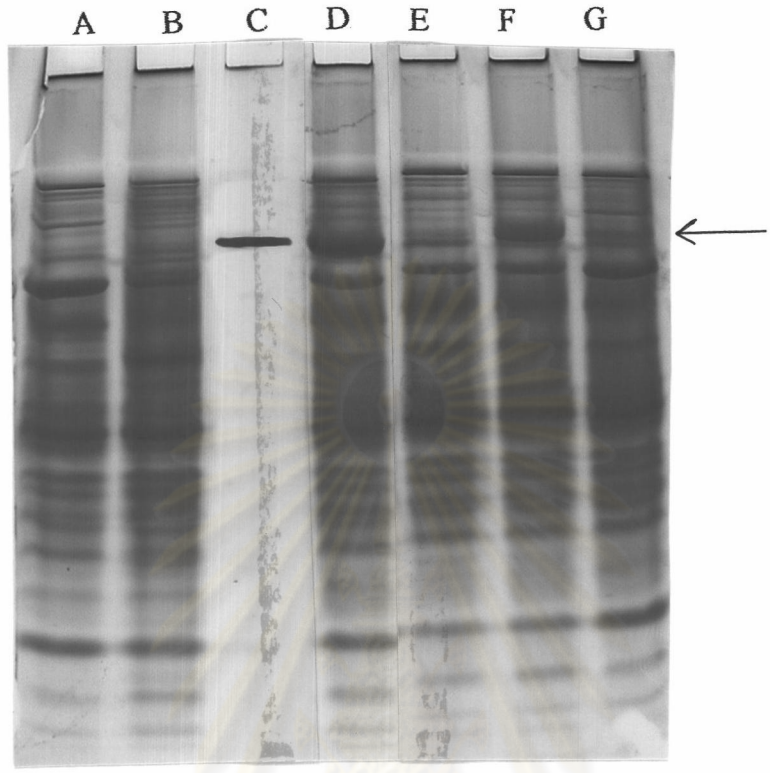

Figure 3.37 Protein pattern of crude extracts of retransformants detected by native-PAGE

Lane A = crude extract of *E. coli* JM109 (60 μg)

Lane B = crude extract of *E. coli* JM109 harbouring pUC18 (65 μg)

Lane C = purified phenylalanine dehydrogenase from *Bacillus badius* BC1

Lane D = crude extracts of transformant No.6 (66 μg)

Lane E = crude extracts of transformant No.19 (60.0 μg)

Lane F = crude extracts of transformant No.20 (63 μg)

Lane G = crude extracts of transformant No.22 (65 μg)

An arrow indicates bands corresponding to phenylalanine dehydrogenase.