# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Equipments

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

Automated Edman degradation amino acid sequencer with Applied Biosystem model 610A data analysis system for protein sequencing: Perkin Elmer, U.S.A.

Autopipette: Pipetman, Gilson, France

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

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.

Centrifuge, microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan

DNA Sequencer Applied Biosystem 373A with a PRISM kit: Perkin Elmer, U.S.A.

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

Filter paper No.1, Whatman, Japan

Freeze-dryer: Model Flexi-Dry$^{tm}$ µp, Stone Rigde, New York, U.S.A.

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

Heating box: Type 17600 Dri-Bath, Thermolyne, U.S.A.

High Performance Liquid Chromatography (HPLC): Model 1050 series, Hewlett-Packard, U.S.A.

Incubator, waterbath: Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc.,U.S.A.

Light box: 2859 SHANDON, Shandon Scientific Co., Ltd., England.

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

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

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, U.S.A.

Microwave oven: Model TRX1500, Turbora International Co., Ltd., Korea

Orbital incubator: Model 1H-100, Gallenkamp, England

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

Reversed phase HPLC YMC-Pack ODS-AM column (100 x 4.6 mm ID)
Pharmacia LKB, Sweden.

Shaking waterbath: Model G-76, New Brunswick Scientific Co., Inc., U.S.A.

Sonicator: Model 300, Fisher Scientific, U.S.A.

Spectrophotometer: Spectronic 2000, Bausch & Lomb, U.S.A. ;
UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.

Thermal cycler: Gene Amp PCR system 2400, Perkin Elmer Cetus, U.S.A.

Thin-wall microcentrifuge tubes 0.2 ml, Axygen Hayward, U.S.A.

Ultrafilter: Suprec$^{Tm-01,Tm-02}$, pore size 0.20 μm and 0.22 μm,
Takara Shuzo Co, Ltd., Japan

UV transluminator: Model 2011 Macrovue, San Gabriel California, U.S.A.

Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.

**2.2 Chemicals**

Acetonitrile (HPLC grade): Merck, Germany

Acrylamide: Merck, Germany

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium persulphate: Sigma, U.S.A.

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, U.S.A.

β- mercaptoethanol: Fluka, Switzerland

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, U.S.A.

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

Bromphenol blue: Merck, Germany

Chloroform: BDH, England

Coomassie brilliant blue R-250: Sigma, U.S.A.

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylene diamine tetra acetic acid: M&B, England

Dithiothreitol (DTT): Sigma, U.S.A.

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., U.S.A.
100 base pair DNA ladder, Promega Co., U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ficoll type 400: Sigma, U.S.A.

GeneAmp PCR core reagents: FINNZYMES, Inc., Finland; and TAKARA SHUZO Co., Ltd., Japan

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Glucose: BDH, England

Hexadecyl trimethyl ammonium bromide: Sigma, U.S.A.

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isoamyl alcohol: Merck, Germany

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactosidase (IPTG): Sigma, U.S.A.

L-phenylalanine: Koch-Light Laboratories Ltd., Japan

Magnesium sulphate 7-hydrate: BDH, England

Methylalcohol: Merck,Germany

*N*-acetyl-*N,N,N*-trimethylammonium bromide (CTAB): Sigma, U.S.A.

*N,N*-dimethyl-formamide: Fluka, Switzerland

*N,N'*-methylene-bis-acrylamide: Sigma, U.S.A.

*N,N,N',N'*-tetramethyl-1, 2-diaminoethane (TEMED): Carlo Erba Reagent, Italy

Nicotinamide adenine dinucleotide (oxidized form) ($NAD^+$): Sigma, U.S.A.

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Peptone from casein pancreatically digested: Merck, Germany

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol: BDH, England

Phenylmethylsulfonyl fluoride (PMSF): Sigma, U.S.A.

85 % Phosphoric acid: Mallinckrodt, U.S.A.

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium hydroxide: Carlo Erba Reagenti, Italy

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIA quick Gel Extraction Kit: QIAGEN, Germany

Riboflavin: BDH, England

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Sigma, U.S.A.

Sodium hydroxide: Merck, Germany

Standard protein marker: Amersham Pharmacia Biotech Inc., U.S.A.

Sucrose: Sigma, U.S.A.

Trifluoroacetic acid: BDH, England

Tris (hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

Yeast extract: Scharlau microbiology, European Union

**2.3 Enzymes and Restriction enzymes**

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction enzymes: GIBCOBRL, U.S.A., Amersham Pharmacia Biotech Inc., U.S.A., New England BioLabs, Inc., U.S.A. and Zibenzyme, Sweden.

RNaseA: Sigma, U.S.A.

*Taq* DNA Polymerase: FINNZYMES, Inc., Finland and TAKARA SHUZO Co., Ltd., Japan.

$T_4$ DNA ligase: GIBCOBRL, U.S.A., New England BioLabs, Inc., U.S.A. and Zibenzyme, Sweden.

## 2.4 Primers and cassettes

Oligonucleotides: TAKARA SHUZO Co., Ltd., Japan

Oligonucleotide-cassettes and Cassette primers: TAKARA SHUZO Co., Ltd., Japan.

## 2.5 Bacterial strains and plasmids

Thermotolerant bacterial strain BC1 contained phenylalanine dehydrogenase activity was screened from temperate area in Japan (Suriyapanpong *et al.*, 2000).

*Escherichia coli* JM109, genotype: F' [*tra*D36 *pro*AB$^+$ *lac*I$^q$ *lac*ZΔM15] *rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ (*lac-proAB*) was used as a host for transformation.

pUC18 was used as a vector for cloning phenylalanine dehydrogenase gene into *E.coli* JM109 (Appendix A).

## 2.6 Chromosomal DNA Extraction

Chromosomal DNA was isolated from thermotolerant bacterial strain BC1 using a protocol modified from Frederick *et al.* (1995). A single colony was inoculated into 10-ml of peptone medium (1 % peptone, 0.2 % $K_2HPO_4$, 0.2 % $KH_2PO_4$, 0.2 % NaCl, 0.01 % $MgSO_4 \cdot 7\ H_2O$ and 0.01 % yeast extract, pH 7.2) and allowed to grow until $A_{600} > 0.7$ (250 rpm rotary shaking at 30 °C for 24 hours). Then each 1.5 ml of cell culture was centrifuged in microfuge tube for 5 minutes at 10,000 rpm. The pellet was resuspended in 550 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. The cell solution was then treated with 30 µl of 10% SDS followed by the addition of 3 µl of 20 mg/ml proteinase K and incubated for at least 2 hours at 37°C. After incubation, 100 µl of 5 M NaCl and 50 µl of CTAB-NaCl solution (10 % CTAB and 0.7 M NaCl) were added, mixed thoroughly, and incubated for 10 minutes at 65 °C. The DNA was extracted with an addition of an equal volume of chloroform-isoamyl alcohol (24: 1 V/V), mixed gently, and centrifuged at 12,000 rpm for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to

a new microcentrifuge tube and further extracted twice with equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 V/V) to ensure the complete extraction of DNA. DNA was precipitated by the addition of 0.6 volume of isopropyl alcohol to the aqueous phase and kept at –20 °C for at least 30 minutes. After that the DNA precipitate was collected by centrifugation at 12,000 rpm for 10 minutes. The DNA-containing pellet was washed with 70 % ethanol. After drying, the pellet was dissolved in an appropriate volume of RNase-TE buffer (1 mg of RNase in 1 ml of TE buffer), and $A_{260}/A_{280}$ was determined. Finally, DNA concentration was estimated by submarine agarose gel electrophoresis compared with known amount of λ/*Hin*dIII marker.

## 2.7 Identification of thermotolerant bacterial strain BC1

2.7.1 Identification of bacterial strain BC1 by morphological and biochemical properties

Thermotolerant bacterial strain BC1 was identified by Thailand Institute of Scientific and Technological Research (TISTR) according to the morphological and biochemical properties such as gram reaction, fermentative production of acid from various carbon sources and activity of various enzymes.

2.7.2 Identification of bacterial strain BC1 by 16S rRNA sequence

Chromosomal DNA was prepared as described in 2.6. PCR amplification of 16S rRNA whole gene from chromosomal DNA was done by using primer pair A and H' that designed from a specific gene coding for 16S rRNA of *Bacillus* species (Ulrike *et al.*, 1989). Approximately 100 ng of chromosomal DNA was subjected to a PCR in a 100 µl reaction mixture containing 2.5 U of TaKaRa LA *Taq* DNA polymerase, 10 µl of 10 x TaKaRa LA *Taq* DNA polymerase buffer, 10 pmole of each primers, 10 mM each deoxynucleoside triphosphate, and 25 mM $MgCl_2$. The thermal profile involved 30 cycles of denaturation at 95 °C for 1 min., primer annealing at 50 °C for 2 min., and extension at 72 °C for 3 min. For parallel experiment, the chromosomal DNA, which used as sources of DNA template, was replaced by single fresh colony. The cell was suspended in 10 µl of sterile distilled water and then heated in boiling water for 10 minutes before used as DNA template in PCR reaction. The expected PCR product (about 1.5 kb) was purified

from agarose gel by using QIA Quick gel extraction kit (Appendix B) and then used for nucleotide sequencing with 4 primers: A, D, D' and F. Primers used for PCR amplification and sequencing analysis are shown in Table 2.1, and the position of all primers on 16S rRNA gene are illustrated in Figure 2.1. Phylogenetic analysis based on the base-sequence homology, alignment of sequence, calculation of evolutionary distance value and construction of a dendogram using the neighbor-joining method was carried out using the GENETYX software package (SOFT-WARE development Co., LTD., Tokyo, Japan).

## 2.8 Amino acid sequence analysis

### 2.8.1 Sample preparation

Two nanomoles of purified phenylalanine dehydrogenase from bacterial strain BC1 (Leksakorn, 2001) was lyophilized and then incubated at 37 °C with 40 µl of 8 M urea for 1 hour followed by adding 120 µl of 0.2 M potassium phosphate buffer, pH 7.2. For digestion, lysyl endopeptidase was added at ratio 1: 50 (w/w) relative to the enzyme. The reaction mixture was incubated at 37 °C for 4 hrs. After incubation, the solution was evaporated and dissolved with 20 µl of ultrapure water. Then, ultrafiltration was performed by using Suprec$^{Tm-02}$, 0.22 µm and the peptide fragments were analyzed on a reverse phase HPLC.

### 2.8.2 Separation and detection of peptide

The digested peptides from section 2.8.1 were separated with a reversed- phase HPLC on a YMC-Pack ODS-AM column (100x4.6 mm ID) equilibrated with 0.1 % trifluoroacetic acid. A 60-min linear gradient from 0 % acetonitrile in 0.1 % (v/v) trifluoroacetic acid to 100 % (v/v) acetonitrile in 0.07 % (v/v) trifluoroacetic acid was used to elute peptides at a flow rate of 1.0 ml/min. The absorbency of the eluted peptide at 210 nm was continuously monitored. The isolated peak were collected and lyophilized for further analysis.

Table 2.1 Nucleotide sequence of all primers used in 16S rRNA gene amplification

| Primer | Sequence(5'→3') | Position of primers (from start codon) |
|---|---|---|
| Primer A | AGAGTTTGATCCTGGCTCAG | 8 to 28 |
| Primer D | CAGCAGCCGCGGTAATAC | 518 to 536 |
| Primer D' | GTATTACCGCGGCTGCTG | 536 to 518 |
| Primer F | CATGGCTGTCGTCAGCTCGT | 1053 to 1073 |
| Primer H' | AAGGAGGTGATCCAGCCGCA | 1542 to 1522 |

Source: Ulrike *et al.*, 1989.

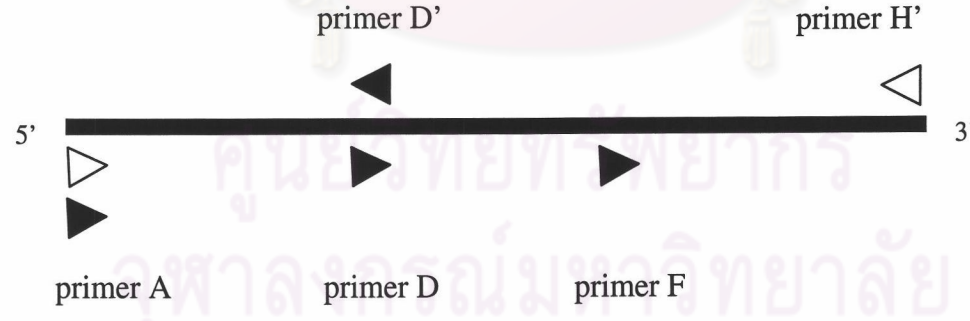


Figure 2.1 Position of primers for 16S rRNA gene amplification (white arrowheads) and sequencing (black arrowheads).

Source: Ulrike *et al.*, 1989.

2.8.3 Amino acid sequencing

The isolated peptides from section 2.8.2 were used directly for amino acid sequence analysis by automated Edman degradation with an Applied Biosystem model 610A data analysis system for protein sequencing.

2.8.4 Computer search for sequence similarities

Amino acid sequence obtained from 2.8.3 was compared with previously published amino acid sequence of phenylalanine dehydrogenases from the EMBL-GenBank-DDBJ database. Multiple sequence were aligned to find the position of peptide sequences by using the CLUSTAL X program and further used as the data for design the degenerated primers in the next step.

**2.9 PCR Amplification**

2.9.1 Primers

The first set of primers that used for the PCR amplification of internal phenylalanine dehydrogenase gene was degenerated primers (N1, N2, C1 and C2) designed by using the data of N-terminal and internal amino acid sequence obtained from the phenylalanine dehydrogenase of bacterial strain BC1. The example of primer design is shown in Figure 2.2 and the position of each primer is illustrated in Figure 2.3. After nucleotide sequences of the first amplified DNA fragment was obtained, the next two series of primers (Phe-N1, Phe-N2 and Phe-C1, Phe-C2) were designed. For the sequencing of unknown DNA region at the 5'- and 3'- end of the structural gene for the enzyme, cassette-ligation mediated PCR was performed. Since the direct PCR sequencing method can determine only up to 300 bp from the priming site, a new primer (Phe-C3) was synthesized according to the sequence information. Finally, to prepare whole phenylalanine dehydrogenase gene by the PCR method and to overexpression the gene in *E. coli* with the assistance of *lac* promoters of plasmid pUC18, the new pair of primers was designed. The 5'-primer included a Shine-Dalgarno sequence of expression vector p*Trc*99, and an *Eco*RI restriction site was added directly to the 5'-end of the desired sequence (Phe-Eco), while the 3'-primer had *Bam*HI restriction site (Phe-Bam). The sequences of all primers are shown in Table 2.2.

N-terminal amino acid sequence:

TSIIKDFTLFEKMSEHEQVVFANDPATGLR

↓

Amino acid sequence which was used to design primer

FEKMSEHEQV

(Phe Glu Lys Met Ser Glu His Glu Gln Val)

↓

Codon usage (sorted by the often usage)

| Phe | Glu | Lys | Met | Ser | Glu | His | Glu | Gln | Val |
|---|---|---|---|---|---|---|---|---|---|
| TTT | GAR | AAR | ATG | TCT | GAR | CAY | GAR | CAR | GTN |

↓

Nucleotide sequence of primer (5'→3') :

TTT GAR AAR ATG TCT GAR CAY GAR CAR G



Figure 2.2 Flow chart for degenerated primer design

Y = C,T

R = A,G

N = A,C,G,T

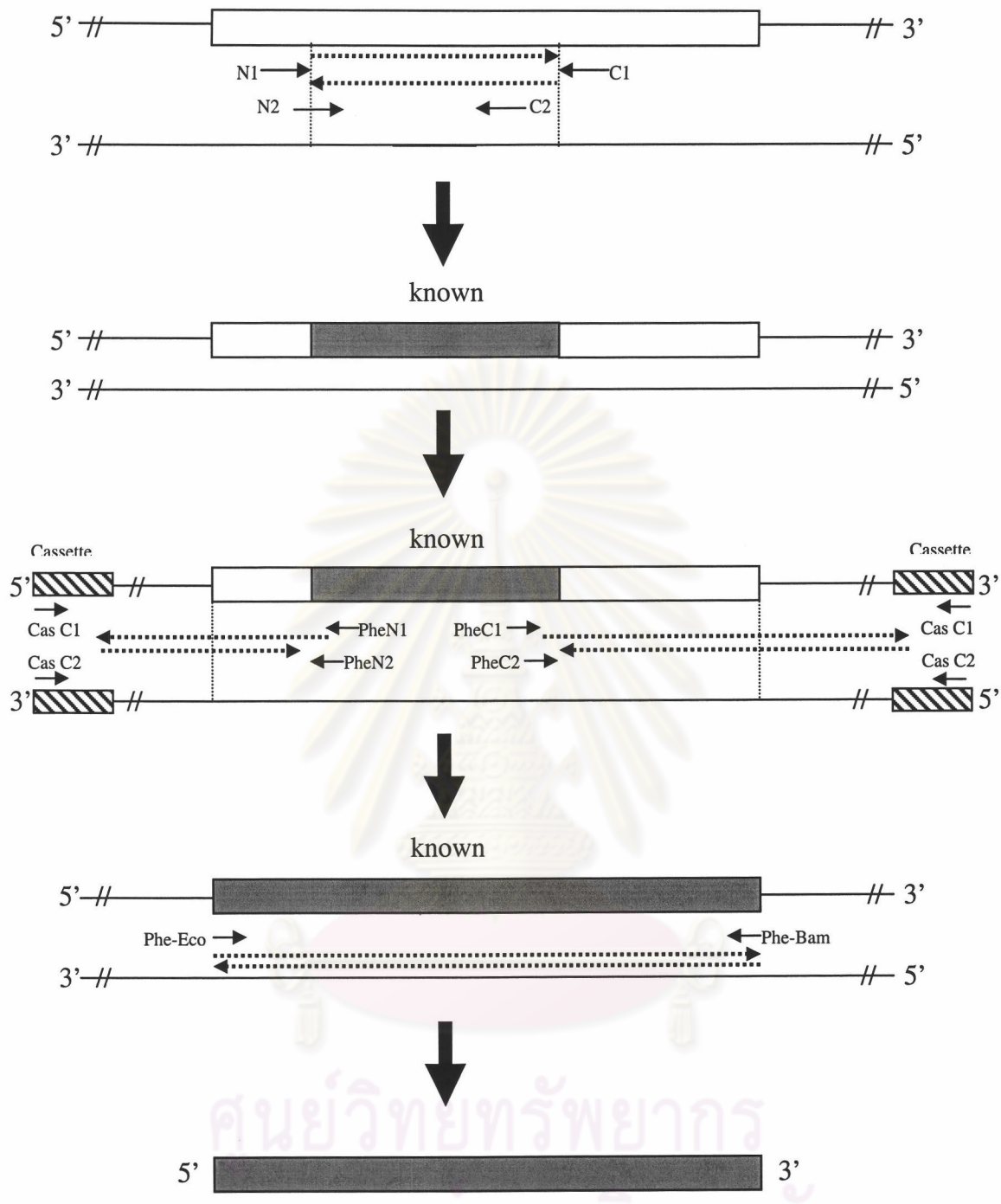


Figure 2.3 Strategy for PCR amplification and sequencing of phenylalanine dehydrogenase gene of thermotolerant bacterial strain BC1. The phenylalanine dehydrogenase gene is boxed. Thick arrows and doted arrows show the primers for PCR and the sequencing strategy, respectively.

Table 2.2 Nucleotide sequence and $T_m$ of all primers used in phenylalanine dehydrogenase amplification

| Primer | Sequence (5' 3') | $T_m$ (°C) | Remark |
|---|---|---|---|
| N1 | TTTGARAARATGTCTGARCAYGARCARG | 58.2 | For the initial internal gene fragment amplification and sequencing |
| N2 | GCTGTTATTATHGGTGAYCCTCARAARGAYAA | 60.1 | "---------" |
| C1 | CCYTCTGCTACYTTRTATCCNACYTTNCC | 62.5 | "---------" |
| C2 | TTCATTGCRTGTATRAARTCYTCCATRTTNGT | 58.4 | "---------" |
| PheN1 | ACACTGTTATAAGGCTGCATGCGGCAGCCG | 66.5 | For N-terminal and C-terminal gene fragment amplification and sequencing |
| PheN2 | ATAGCGATAATGGCCCTTAGTCCTGTCGCC | 65.1 | "---------" |
| PheC1 | CGGAGATTCCTCTATTCCAACTGCCATGGG | 65.1 | "---------" |
| PheC2 | GCAACCAACAAAATGCTGTTTGGCAAGGAC | 62.4 | "---------" |
| PheC3 | CTCCGGCGGTCTCATCCAAG | 53.0 | "---------" |
| Cassette Primer C1 | d(GTACATATTGTCGTTAGAACGCGTAATACGACTCA) | 98.0 | "---------" |
| Cassette Primer C2 | d(CGTTAGAACGCGTAATACGACTCACTATAGGGAGA) | 102.0 | "---------" |
| Phe-Eco | GGGAATTCAGGAAACAGACCATGAGCTTAGTAGAAAAAACATCCA | 108.0 | For the whole gene fragment amplification |
| Phe-Bam | GGGGATCCTTAATTACGAATATCCCATTTTGGCTTAACA | 93.0 | "---------" |

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

2.9.2 Template

2.9.2.1 Templates for amplification of internal phenylalanine dehydrogenase gene

The chromosomal DNA of thermotolerant bacterial strain BC1 was prepared according to the method described in 2.6 and then 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 reaction mixture containing 5 μg of chromosomal DNA, 1 x reaction buffer suggested by the supplier and 10 U of each restriction enzyme in total volume of 50 μl was incubated at 37 °C for 18 hours. After incubation, the samples were extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 10 minutes. The upper phase was then transferred to a new tube and the 5 M NaCl was added to the final concentration of 1 M. The digested chromosomal DNA was precipitated with 2 volume of absolute ethanol, standed at –20 °C for at least 30 minutes, and centrifuged at 12,000 rpm for 10 minutes. The DNA-pellet was washed with 70 % ethanol, collected by centrifugation at 12,000 rpm for 10 minutes, and dried briefly. The DNA was finally dissolved in 10 μl of sterile distilled water. A 1 μl (about 500 ng) of the DNA solution was used as template in each reaction of PCR.

2.9.2.2 Templates for amplification of 5'-terminal and 3'-terminal region of phenylalanine dehydrogenase gene

The complete digestion of chromosomal DNA with each restriction enzyme: *Bam*HI, *Bgl*II, *Pst*I, *Spe*I and *Xba*I were prepared as described in section 2.9.2.1. The digested DNA was ligated with one of the oligonucleotide-cassettes DNA possessing a cohesive end that is compatible with sticky end of digested DNA. List of cassette DNA is shown in Table 2.3. The ligation mixture contained 5 μg of chromosomal DNA, 20 ng of cassette DNA, 1 x ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5 % (w/v) polyethylene glycol- 8000) and 2 U of $T_4$ DNA ligase in a total volume of 20 μl. The ligation was carried out at 16 °C for 16 hours and then the reaction mixture was precipitated and dissolved in 10 μl of sterile water. Each two microliters (1μg) of ligated fragments were used as the DNA template for PCR of the unknown 5'-end and 3'-end region. In order to increase the primer specificity, the product of the first PCR with the cassette primer C1 was used as the template DNA for the second

Table 2.3 Compatible recognition sites of the oligonucleotide-cassettes DNA.

| Cassette DNA | Recognition site |
|---|---|
| *Pst*I Cassette | *Pst*I site |
| *Sau*3AI Cassette | *Bam*HI site and *Bgl*II site |
| *Xba*I Cassette | *Spe*I site and *Xba*I site |

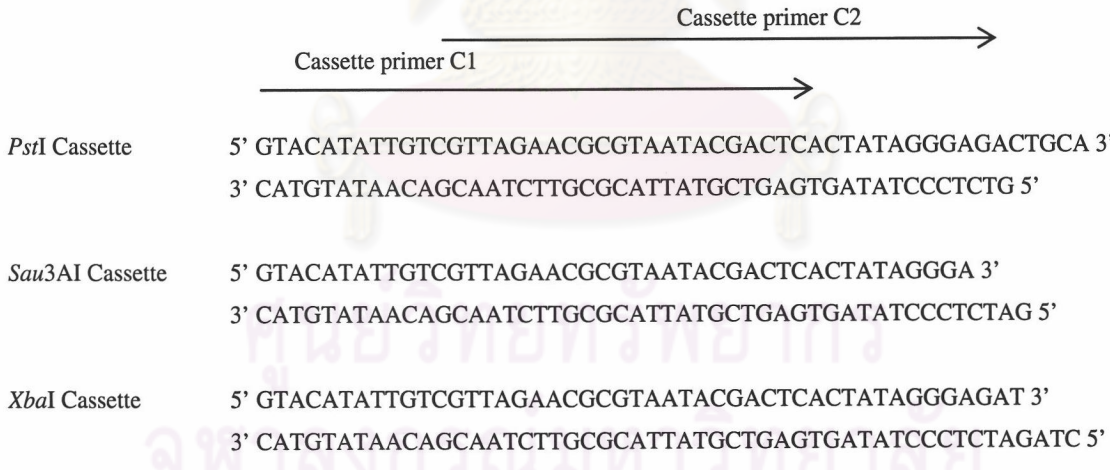


Figure 2.4 The sequences of oligonucleotide cassettes used in this research. These cassettes are 44-51 bp long and posses either 3' or 5' overhang (2-4 nucleotides).

PCR with the cassette primer C2. The nucleotide sequences of the cassette used in this research are shown in Figure 2.4.

2.9.2.3 Templates for amplified the whole gene fragment

Chromosomal DNA was completely digested with each restriction enzyme: *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*I, *Spe*I, *Xba*I and *Xho*I as the same condition as described in section 2.9.2.1. Then phenol-chloroform extraction was done. Subsequently, 5 M NaCl was added to the reaction tubes to final concentration of 1 M. The DNA was then precipitated and dissolved in 10 µl of sterile distilled water. The 1 µl (500 ng) of DNA solution was used as template for each PCR reaction.

2.9.3 PCR condition

Three parts of phenylalanine dehydrogenase gene were amplified by 3 steps of PCR as shown in Figure 2.3 and the conditions of each PCR was described in Table 2.4.

PCR was performed in a 25-µl reaction mixture containing 2.5 U of *Taq* DNA polymerase, 2.5 mM each dNTPs, 1 X PCR buffer, 1 mM $MgCl_2$, DNA template and 10 pmole of each primer, except in the internal gene fragment amplification, 100 pmole of each primer was used.

**2.10 Agarose gel electrophoresis**

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.7% of agarose powder was added to 100 ml electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in an Erlenmeyer flask and heat until complete solubilization in a microwave oven. The agarose solution was cooled down to 60 °C until all air bubbles were completely eliminated. The solution was then left at room temperature to 50 °C before pouring into an electrophoresis mould. After the gel was completely set, the comb and seal of the mould was carefully removed. When ready, the DNA samples were mixed with one-fifth volume of the desired gel-loading buffer (0.025 % bromphenol blue, 40 % ficoll 400 and 0.5 % SDS) and slowly loaded the mixture into an appropriate percentage of agarose gel.

Table 2.4 PCR condition in each step

| The region of gene fragment which amplified | Primer pairs | The 1st Predenaturation | The 2nd Predenaturation | Denaturation | Annealing | Extension | Final extension | Number of cycle |
|---|---|---|---|---|---|---|---|---|
| The internal gene fragment | N1xC1, N2xC2 and N1xC2 | 94°C for 10 min.* | 94°C for 10 min**. | 94°C for 1 min. | 42°C for 1 min. | 72°C for 2 min. | 72°C for 7 min. | 40 |
| The first 5'-terminal and 3'-terminal gene fragment | Phe-N1xCassetteC1 and Phe-C1xCassetteC1 | 94°C for 10 min*. - | 94°C for 10 min**. - | 94°C for 9 min. 94°C for 9 min. | 65°C for 1 min. 65°C for 1 min. | 65°C for 9 min. 65°C for 9 min 15 sec. | - 65°C for 7 min. | 14 and 16 |
| The second 5'-terminal and 3'-terminal gene fragment | Phe-N2xCassetteC2 and Phe-C2xCassetteC2 | 94°C for 10 min*. - | 94°C for 10 min**. - | 94°C for 9 min. 94°C for 9 min. | 65°C for 1 min. 65°C for 1 min. | 65°C for 9 min. 65°C for 9 min 15 sec. | - 65°C for 7 min. | 14 and 16 |
| The whole gene fragment | Phe-EcoxPhe-Bam | 94°C for 10 min*. | 94°C for 10 min**. | 94°C for 1 min. | 50°C for 30 sec. | 72°C for 2 min. | 72°C for 7 min. | 30 |

* = After 1st predenaturation for 10 min., the PCR reaction mixture were added.

** = After 2nd predenaturation for 10 min., the *Taq* DNA polymerase were added.

Electrophoresis had been performed at constant voltage of 10 volt/cm until the faster migration dye (bromphenol blue) has migrated to approximately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide in distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light and photographed through a red filter using Kodak Tri X pan 400 film. The concentration or molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA fragment.

**2.11 Nucleotide sequencing**

After purification of PCR products from agarose gels by using QIA Quick gel extraction kit, the nucleotide sequence was determined in the both directions by the dideoxynucleotide chain termination method of Sanger *et al.*, using an Applied Biosystems 373 A DNA sequencer with a PRISM kit (Perkin Elmer, U.S.A.). PCR products were sequenced directly by cycle sequencing. The sequencing primers were the same as for PCR amplification primers.

**2.12 Recombinant DNA preparation**

2.12.1 Vector DNA preparation

The vector pUC18 was linearized with *Eco*RI and *Bam*HI. The reaction mixture containing of 5 μg pUC18, 1 x *Eco*RI reaction buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of *Eco*RI and 10 U of *Bam*HI in total volume of 100 μl was incubated at 37°C for 18 hours. The linear-formed pUC18 was harvested from agarose gel electrophoresis by using QIA Quick gel extraction kit.

2.12.2 The phenylalanine dehydrogenase gene fragment preparation

After the whole gene fragment amplification, the 4 reactions of PCR product were pooled (200μl) and cleaned up by addition of 1 μl of 10 % SDS and 1 μl of 20 mg/ml proteinase K, mixed and incubated at 65 °C for 15 minutes. After that, 20 μl of 3 M sodium acetate was added to precipitate proteins from nucleic acids.

Phenol-chloroform extraction and DNA precipitation was done as described in 2.9.2.1. The DNA-pellet was dissolved in 50 µl of sterile distilled water.

Due to the amplification of whole gene fragment, the primer Phe-Eco that contained *Eco*RI site and the primer Phe-Bam which contained *Bam*HI site were used. The cleaned gene fragment was completely digested by both *Eco*RI and *Bam*HI. The reaction mixture containing 50 µl of gene fragment, 1 x reaction buffer for *Eco*RI (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 100 mM NaCl), 10 U of *Eco*RI and 10 U of *Bam*HI in total volume of 100 µl was incubated at 37 °C for 18 hours and then extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1). The DNA-pellet was precipitated with 2 volume of absolute ethanol and further dissolved in 100 µl of sterile distilled water. Finally, the gene fragment was concentrated with Ultrafilter suprec$^{Tm-01}$.

2.12.3 Ligation of vector DNA and the gene fragment

The gene fragment from 2.12.2 was ligated to the pUC18 vector from 2.12.1 at a vector: insert molar ratio of 1: 8. The ligation mixture that contained 50 ng of vector DNA, 400 ng of the gene fragment, 1 x ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5 % (w/v) polyethylene glycol - 8000) and 10 U of $T_4$ DNA ligase in a total volume of 20 µl was incubated at 16 °C for 16 hours. The recombinant plasmids were further used for transformation.

**2.13 Transformation**

The recombinant plasmids from 2.12.3 were introduced into a competent *rec*A, *end*A *E. coli* strain JM 109 by electroporation. The competent cells for electroporation were prepared by the method of Dower (1988) (see Appendix C). In the electroporation step, 0.2 -cm cuvettes and sliding cuvette holder were chilled on ice. The Gene Pulser apparatus was set to the 25 µF capacitor, 2.5 kV, and the pulse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. One to five microlitre of recombinant plasmid from 2.12.3 was mixed with 40 µl of the competent cells and then placed on ice for 1 minutes. This mixture was transferred to a cold cuvette. The cuvette was applied one pulse at the above settings. Subsequently, one millitre of

LB medium was added immediately to the cuvette. The cells were quickly resuspended with a Pasteur pipette. Then the cell suspension was transferred to new tube and incubated at 37 °C for 1 hour with 225 rpm shaking. Finally, this suspension was spread onto the LB agar plates containing 100 μg/ml ampicillin, 25 μg/ml IPTG and 20 μg/ml X-gal, and was incubated at 37 °C for 16 hours. Cells containing the recombinant plasmids, which formed white colonies, were picked and the plasmids were further isolated.

**2.14 Plasmid extraction**

The *E. coli* JM 109, which harboured recombinant plasmid was grown in LB-medium (1 % peptone, 0.5 % NaCl and 0.5 % yeast extract, pH 7.2) containing 100 μg/ml ampicillin overnight at 37 °C with rotary shaking. The cell culture was collected by centrifugation at 10,000 rpm for 5 minutes in each 1.5-ml microfuge tube. Then 100 μl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was resuspended by repeated pipetting and left at room temperature for 10 minutes. After that, the 200 μl of freshly prepared Solution II (0.2 N NaOH and 1 % SDS) was added, gently mixed by inverting the tube for five times and placed on ice for 10 minutes. Then the 150 μl of cooled Solution III (3 M sodium acetate, pH 4.8) was added and the tube was placed on ice for 10 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes and then the supernatant was transferred to a new tube. An equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1) was added, mixed and centrifuged at 12,000 rpm for 10 minutes. The upper-phased liquid was transferred to a new tube. The plasmid DNA was precipitated with absolute ethanol and washed with 70 % ethanol. After drying, the pellet was finally dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 μg/ml DNase-free pancreatic RNase. Then plasmids were completely digested with *Eco*RI and *Bam*HI. The size of recombinant plasmid was estimated by submarine agarose gel electrophoresis compared with relative mobility of DNA marker.

## 2.15 Crude extract preparation

*E. coli* JM109 cells harboring the recombinant plasmid were grown at 37 °C in 10 ml of LB-medium supplemented with 100 μg/ml ampicillin overnight. After that, 10 % of the cell culture was inoculated into 100 ml LB-medium containing 100 μg/ml ampicillin and cultured at 37°C with shaking speed 250 rpm. When the turbidity of the culture at 600 nm had reached 0.6, 1 mM IPTG was added to induce enzyme production, and the cultivation was continued at 37 °C for 5 hours. The cells were harvested by centrifugation at 8,000 rpm for 15 minutes, then washed with cold 0.85 % NaCl and centrifuged at 8,000 rpm for 15 minutes. After that, the cell pellet was washed once in cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01 % DTT and 1.0 mM EDTA) and centrifuged again. The cell pellet was stored at –80 °C until the next step. For enzyme extraction, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by discontinuously sonication on ice with 5 seconds pulse and 2 seconds stop interval for 15 minutes by sonic dismembrator. Unbroken cell and cell debris were removed by centrifugation at 10,000 rpm for 45 minutes. The supernatant was stored at 4 °C for enzyme and protein assays.

## 2.16 Enzyme activity assay

The activity of phenylalanine dehydrogenase for oxidative deamination of phenylalanine was spectrophotometrically assayed. Reaction mixture 1 ml comprised 200 μmol of glycine-potassium chloride-potassium hydroxide buffer, pH 10.5, 20 μmol of L-phenylalanine, 1 μmol of $NAD^+$, and enzyme. In a blank tube, L-phenylalanine was replaced by water. Incubation was carried out at 30 °C in a cuvette of 1-cm light path. The reaction was started by addition of $NAD^+$ and was monitored by measuring the initial change in absorbance of NADH at 340 nm.

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

## 2.17 Protein measurement

Protein concentration was determined by the method of Bradford (1976). Protein solution (maximum 100 µl) was pipetted into tube. Distilled water was added to make a total volume of 100 µl. Then 1 ml of Bradford working buffer (see Appendix D) was added and mixed. After 2 minutes but before 1 hour, $A_{595}$ was read. Bovine serum albumin was used as standard protein. Standard curve was shown in Appendix E.

## 2.18 Polyacrylamide gel electrophoresis

2.18.1 Pouring the separating gel (7 % acrylamide)

The gel sandwich was assembled according to the manufacturer's instruction. For 2 slab gels, the 2.33 ml of solution A (30 % (w/v) acrylamide, 0.8 % (w/v) Bis-acrylamide) and 2.5 ml of solution B (1.5 M Tris-HCl, pH 8.8) were mixed with 5.17 ml of distilled water in a 25 ml Erlenmeyer flask and mixed. Then 50 µl of 10 % ammonium persulfate and 10 µl of TEMED were added and mixed rapidly by swirling or inverting container gently. Carefully introduced solution into gel sandwich by using a Pasteur pipette. After the appropriate amount of separating gel solution was added, gently layered about 1 cm height of water on top of the separating gel solution. The gel was allowed to polymerize, distinguished by clear interface between the separating gel and the water. The water was then poured off.

2.18.2 Pouring the stacking gel (5 % acrylamide)

The 0.67 ml of solution A (30 % (w/v) acrylamide, 0.8 % (w/v) Bis-acrylamide) was mixed with 1.0 ml of solution C (0.5 M Tris, pH 6.8) and 2.3 ml of distilled water in a 25 ml Erlenmeyer flask and mixed. Subsequently, 30 µl of 10 % ammonium persulfate and 5 µl of TEMED were added and mixed rapidly. This stacking gel solution was loaded onto separating gel until solution reached top of short plate. Then the comb was carefully inserted into gel sandwich. After stacking gel was polymerized, the comb was removed carefully. Then the gel was placed into electrophoresis chamber. The electrophoresis buffer ( 25 mM Tris and 192 mM glycine, pH 8.8) was added into the

inner and outer reservoir. The air bubbles, which were occurred in the well, were removed.

2.18.3 Sample preparation

The protein sample was mixed with 5 x sample buffer (0.3 mM Tris-HCl, 50 % glycerol and 0.05 % bromophenol blue) in an Eppendorf tube and mixed. Then the sample solution was introduced into well by using syringe.

2.18.4 Running the gel

An electrode plugs were attached to proper electrodes. Current was flowed towards the anode for pH 8.8 gels. The power supply was turned on at constant current (30 mA). For activity staining electrophoresis, the experiment was done at 4°C. Electrophoresis was continued until the dye front reached the bottom of the gel. Power supply was turned off and then the electrode plugs were removed from electrodes. The gel plates were removed from electrophoresis chamber. Then the gel was removed from glass plates and transferred to a small container.

2.18.5 Staining Procedure

Protein staining

The gel from 2.18.4 was transferred to a small container containing Coomassie stain solution (1 % Coomassie Blue R-250, 45 % methanol, and 10 % glacial acetic acid). The gel was agitated for 10-20 minutes on a shaker. The stain solution was poured out and the Coomassie destain solution (10 % methanol and 10 % glacial acetic acid) was added. The gel was shaked slowly. To complete destain, the destain solution was changed many times and agitated overnight or until the blue-clearly bands of protein were occurred.

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

After electrophoresis at 4°C was done, the gel was transferred to a small container containing activity staining solution (4.25 mmole of Tris-HCl, pH 8.5, 40 μmole of L-phenylalanine, 50 μmole of $NAD^+$, 25 μg/ml of phenazine methosulfate

and 250 μg/ml of nitroblue tetrazolium) and agitated for 30 minutes at room temperature. After purple bands were occurred, the gel was then destained with distilled water.

**2.19 Induction time determination**

According to the phenylalanine dehydrogenase gene fragment, which was cloned into host cell, did not have its own promoter, *lac*Z promoter on the plasmid pUC18 was used and IPTG was added to induce phenylalanine dehydrogenase gene expression. Thus, the studies about influence of induction time of IPTG were required. Tranformant cell, which showed the high phenylalanine dehydrogenase activity, was grown at 37 °C in 10 ml of LB-medium supplemented with ampicillin (100 μg/ml) overnight. After that, 1 % of the cell culture was inoculated into each 100 ml LB-medium containing 100 μg/ml ampicillin and shaked at 37 °C, 250 rpm. When the turbidity of the culture at 600 nm had reached 0.4, 1 mM IPTG was added to induce enzyme production, and the cultivation was continued at 37 °C for various times: 0, 15, 30, 45, 60, 120, 180, 240 and 300 minutes before cell harvesting. Finally, crude extracts were prepared and assayed for the enzyme activities as described in section 2.15.

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

2.20.1 Continuous subculture

Six recombinant clones, which showed high phenylalanine dehydrogenase activity, were daily subcultured for 15 days. Then the $5^{th}$, $10^{th}$ and $15^{th}$ subcultured colonies were picked up to culture and assay for phenylalanine dehydrogenase activity as described in 2.15. Moreover, the plasmids of the $15^{th}$ subcultured colonies were extracted and completely digested with *Eco*RI and *Bam*HI. The size of recombinant plasmid was estimated by submarine agarose gel electrophoresis compared with relative mobility of DNA marker.

2.20.2 Retransformation

Four plasmids from recombinant clones, which represented each type of plasmid pattern, were extracted as described in 2.14 and then retransformed into

*E. coli* JM109 host cell by electroporation once again. White colonies that contained the recombinant plasmid were selected and the plasmids were extracted. After that, the retransformant plasmids were completely digested with *Eco*RI and *Bam*HI, and estimated the size by agarose gel electrophoresis. Finally, retransformants were cultured for crude extract preparation, assayed for the enzyme activities as described in section 2.15 and subjected to native-PAGE as described in 2.18.