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, USA

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: J-30I, Beckman Instrument Inc., USA

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

Electrophoresis unit: Gelmate 2000, TOYOBO Co., LtD., Japan

Fraction collector: Frac-100, Pharmacia Biotech, Sweden

Freeze-dryer: Model Flexi-Dry^{tm} µp, Stone Rigde, USA

Gene Pulser^{R}/*E. coli* Pulser^{TM} Cuvettes: Bio-Rad, USA

Gel Doc: BioDoc-It^{Tm} Imaging system, Model M20, Cambridge, UK

Heating box: Model RS232 Dri bath incubator, Taiwan

High Performance Liquid Chromatography (HPLC): Model 1050 series, Hewlett-Packard, USA

Incubator, waterbath: Model M20S, Lauda, Germany and BioChiller 2000, FOTODYNE Inc.,USA

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

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

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, USA

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

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

Peristaltic pump: pump p-1, Pharmacia Biotech, Sweden

pH meter: Model S20-K, Schwerzenbach, Switzerland

Power supply: Model POWER PAC 300, Bio-Rad, USA

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., USA

Sonicator: Vibra cell^{Tm}, SONICS & MATERIALS, Inc., USA

Spectrophotometer: DU Series 650, Beckman Instrument Inc., USA

Thermo cycler: Mastercycler gradient, Eppendorf, Germany

Thin-wall microcentrifuge tubes 0.2 ml, Axygen Hayward, USA

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, USA

Vortex: Model K-550-GE, Scientific Industries, Inc., USA

2.2 Chemicals

Acetonitrile (HPLC grade): Merck, Germany

Acrylamide: Merck, Germany

Agar: Merck, Germany

Agarose: SEKEM LE Agarose, FMC Bioproducts, USA

Ammonium persulphate: Sigma, USA

Ammonium sulphate: Carlo Erba Reagenti, Italy

Ampicillin: Sigma, USA

Boric acid: Merck, Germany

Bovine serum albumin: Sigma, USA

5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal): Sigma, USA

Bromphenol blue: Merck, Germany

Butyl-Toyopearl 650M TSK gel: Tosoh, Japan

Chloroform: BDH, England

Coomassie brilliant blue R-250: Sigma, USA

DEAE-Toyopearl 650M TSK gel: Tosoh, Japan

Dialysis tubing: Sigma, USA

DNA marker: Lamda (λ) DNA digested with *Hin*dIII, BioLabs, Inc., USA

100 base pair DNA ladder, Promega Co., USA

Ethidium bromide: Sigma, USA

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

m-Fluoro-DL-phenylalanine: Sigma, USA

o-Fluoro-DL-phenylalanine: Sigma, USA

p-Fluoro-DL-phenylalanine: Sigma, USA

Ficoll type 400: Sigma, USA

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, USA

Glucose: BDH, England

Hydrochloric acid: Carlo Erba Reagenti, Italy

p-Hydroxyphenylactic acid (sodium salt): Sigma, USA

p-Hydroxyphenylpyruvic acid (sodium salt): Sigma, USA

Indole-β-pyruvic acid (sodium salt): Sigma, USA

Isopropanol: Merck, Germany

Isopropylthio-β-D-galactosidase (IPTG): Sigma, USA

α-Keto-n-butyric acid (sodium salt): Sigma, USA

α-Ketocaproic acid (sodium salt): Sigma, USA

α-Ketoglutaric acid (sodium salt): Sigma, USA

α-Ketoisocaproic acid (sodium salt): Sigma, USA

α-Ketoisovaleric acid (sodium salt): Sigma, USA

α-Keto-γ-methiol-butyric acid (sodium salt): Sigma, USA

α-Keto-β-methyl-n-valeric acid (sodium salt): Sigma, USA

α-Ketovaleric acid (sodium salt): Sigma, USA

β- mercaptoethanol: Fluka, Switzerland

Methanol: Lab-Scan, Thailand

Magnesium sulphate 7-hydrate: BDH, England

β-Nicotinamide adenine dinucleotide (NAD^+): Sigma, USA

β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$): Sigma, USA

β-Nicotinamide adenine dinucleotide reduced form (NADH): Sigma, USA

Nicotinamide 1, N^6-ethenoadenine dinucleotide: Sigma, USA

Nicotinamide guanine dinucleotide: Sigma, USA

Nicotinamide hypoxanthine dinucleotide: Sigma, USA

Nicotinic acid adenine dinucleotide: Sigma, USA

N,N-dimethyl-formamide: Fluka, Switzerland

N,N'-methylene-bis-acrylamide: Sigma, USA

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

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

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

Peptone from casein pancreatically digested: Merck, Germany

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol: BDH, England

Phenol reagent: Carlo Erba Reagenti, Italy

Phenylmethylsulfonyl fluoride (PMSF): Sigma, USA

Protein molecular weight marker (MW 14,400-116,000): Fermentas, USA

Phenylpyruvic acid (sodium salt): Sigma, USA

Potassium chloride: Merck, Germany

Potassium di-hydrogen phosphate: Carlo Erba Reagenti, Italy

di-Potassium hydrogen phosphate: Carlo Erba Reagenti, Italy

Potassium hydroxide: Scharlau, Spain

3-Pyridinealdehyde adenine dinucleotide: Sigma, USA

Pyruvic acid (sodium salt): Sigma, USA

Potassium acetate: Merck, Germany

QIA quick Gel Extraction Kit: QIAGEN, Germany

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, USA

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

Sodium hydroxide: Merck, Germany

Standard protein marker for SDS-PAGE: Fermentas Inc., USA

Sucrose: Sigma, USA

Trifluoroacetic acid: BDH, England

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

Yeast extract: Scharlau microbiology, European Union

L-amino acids were from Sigma, USA and D-amino acids were from Nacalai Tesque and Wako, Japan. Other common chemicals were reagent grade from Aldrich; USA, BDH; England, Fluka; Switzerland, Merck; Germany, Scharlau; Spain, Unilab; Australia and Sigma; USA.

2.3 Enzymes and Restriction enzymes

Lysozyme: Sigma, USA

Proteinase K: Sigma, USA

Restriction enzymes: New England BioLabs, Inc., USA

RNaseA: Sigma, USA

Pfu DNA polymerase: Promega, USA

T_4 DNA ligase: New England BioLabs, Inc., USA

2.4 Primers

Primer synthesis: Bioservice Unit, Thailand

2.5 Bacterial strains and plasmid

Bacillus lentus was used as a source of *phedh* gene.
pET-17b was used as an expression vector for cloning of *phedh* gene (Appendix A). *Escherichia coli* BL21(DE3), genotype: F$^-$ *omp*T *hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3), was used as a host for cloning and expression.

2.6 Amino acid sequence analysis

2.6.1 Sample preparation

Two nanomoles of purified PheDH from *Bacillus lentus* (Inkure, 2005) was lyophilized and then incubated at 37°C with 20 µl of 8 M urea for 1 hour followed by the addition of 60 µl of 0.2 M Tris-HCl buffer, pH 9.0. For digestion, 10 pmol of lysyl endopeptidase was added. The reaction mixture was incubated at 37°C for 2 hours. After incubation, the solution was evaporated and dissolved with 20 µl of ultrapure water. Then, it was filtrated by using Suprec^{Tm-02}, 0.22 µm and the peptide fragments were analyzed on a reverse phase HPLC.

2.6.2 Separation and detection of peptides

The digested peptides from section 2.6.1 were separated with a reversed phase HPLC on a YMC-Pack ODS-AM column (100x4.6 mm ID) equilibrated with 0.12% trifluoroacetic acid. A 60 minutes linear gradient from 0.12% trifluoroacetic acid

in H_2O to 0.07% trifluoroacetic acid in acetonitrile 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 peaks were collected and lyophilized for further analysis.

2.6.3 Amino acid sequencing

The isolated peptides from section 2.6.2 were used directly for amino acid sequence analysis by automated Edman degradation with an Applied Biosystem model 610A data analysis system for protein sequencing.

2.6.4 Computer search for sequence similarities

Amino acid sequences obtained from 2.6.3 were compared with previously published amino acid sequence of PheDHs from the EMBL-GenBank-DDBJ database. Multiple alignment was performed to find the position of peptide sequences by using the CLUSTAL W program and further used as the data for design the degenerated primers in the next step.

2.7 Nucleotide sequencing of phenylalanine dehydrogenase gene

2.7.1 Chromosomal DNA extraction

Chromosomal DNA was isolated from *Bacillus lentus* by the method of Frederick *et al.*, (1995). A single colony was inoculated into 10 ml of peptone medium (1.5% peptone, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.015% $MgSO_4.7H_2O$ and 0.015% yeast extract, pH 7.2) and incubated at 37°C for 24 hours with shaking. Then each 1.5 ml of cell culture was centrifuged in microcentrifuge tube at 8,000xg for 2 minutes. 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 3 µl

of 5 mg/ml lysozyme, 2 µl of 10 mg/ml RNaseA, 30 µl of 10% SDS followed by the addition of 3 µl of 20 mg/ml proteinase K and incubated for 1 hour at 37°C. After incubation, the DNA was extracted with an equal volume of phenol-chloroform (1:1 V/V), mixed gently, and centrifuged at 12,000xg for 10 min. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube. DNA was precipitated by the addition of 5 M NaCl to the final concentration of 1 M and 2 volumes of absolute ethanol, standed at -20°C at least 30 min. Afterwards, the DNA solution was centrifuged at 12,000xg for 10 min. DNA pellet was collected and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer. Finally, DNA concentration was estimated using submarine agarose gel electrophoresis by comparison the intensity of the fluorescence of Ethidium bromide-DNA complex with known amount of λ/*Hin*dIII marker.

2.7.2 Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.8 g of agarose was added to 100 ml of electrophoresis buffer (89 mM Tris-HCl, 8.9 mM boric acid and 2.5 mM EDTA, pH 8.0) in Erlenmeyer flask and heated until complete solubilization in a microwave oven. The agarose solution was left at room temperature to 50°C before pouring into an electrophoresis mould. When the gel was completely set, the DNA samples were mixed with gel loading buffer (0.025% bromphenol blue, 40% ficoll 400 and 0.5% SDS) and loaded into agarose gel. Electrophoresis had been performed at constant voltage of 100 volts until the bromphenol blue migrated to appropiately 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 with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was determined by comparison

of band intensity and relative mobility with those of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

2.7.3 PCR amplification

2.7.3.1 Primers

The first set of primers that used for the PCR amplification of internal *phedh* gene was degenerated primers (F1, R1 and R2) designed by using the data of internal amino acid sequences obtained from the PheDH of *Bacillus lentus*. The example of primer design is shown in Figure 2.1 and the position of each primer is displayed in Figure 2.2. After the nucleotide sequence of the first amplified DNA fragment had been obtained, the next series of primers (N1, N2, N3, N4 and C1, C2) were designed for the sequencing of unknown DNA region at the 5′- and 3′-regions of the structural gene of the enzyme. Finally, to prepare whole *phedh* gene by the PCR method and to overexpression the gene in *E. coli* BL21(DE3) by using expression vector pET-17b, the new pair of primers were designed. The 5′-primer consisted of *Nde*I restriction site (Nde-N), while the 3′-primer consisted of *Bam*HI restriction site (Bam-C). The sequences of all primers are shown in Table 2.1.

2.7.4 Template preparation

2.7.4.1 Templates for amplification of internal phenylalanine dehydrogenase gene

The chromosomal DNA of *Bacillus lentus* was prepared according to the method described in 2.7.1 and then was completely digested with various restriction enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pst*I, *Spe*I and *Xba*I. The reaction mixture containing 5 µg of chromosomal DNA, 1 x reaction buffer suggested by the supplier and

Internal amino acid sequence:

AVIIGDPQKD

↓

Amino acid sequence which was used to design primer:

IIGDPQK

(Ile Ile Gly Asp Pro Gln Lys)

↓

Codon usage (sorted by the often usage)

Ile	Ile	Gly	Asp	Pro	Gln	Lys
ATY	ATY	GGN	GAY	CCW	CAA	AAR

↓

Nucleotide sequence of primer (5′⟶3′):

ATY ATY GGN GAY CWW CAA AA

Note: Y = C, T

R = A, G

W = A, T

N = A, C, G, T

Figure 2.1 Flow chart for degenerated primer design

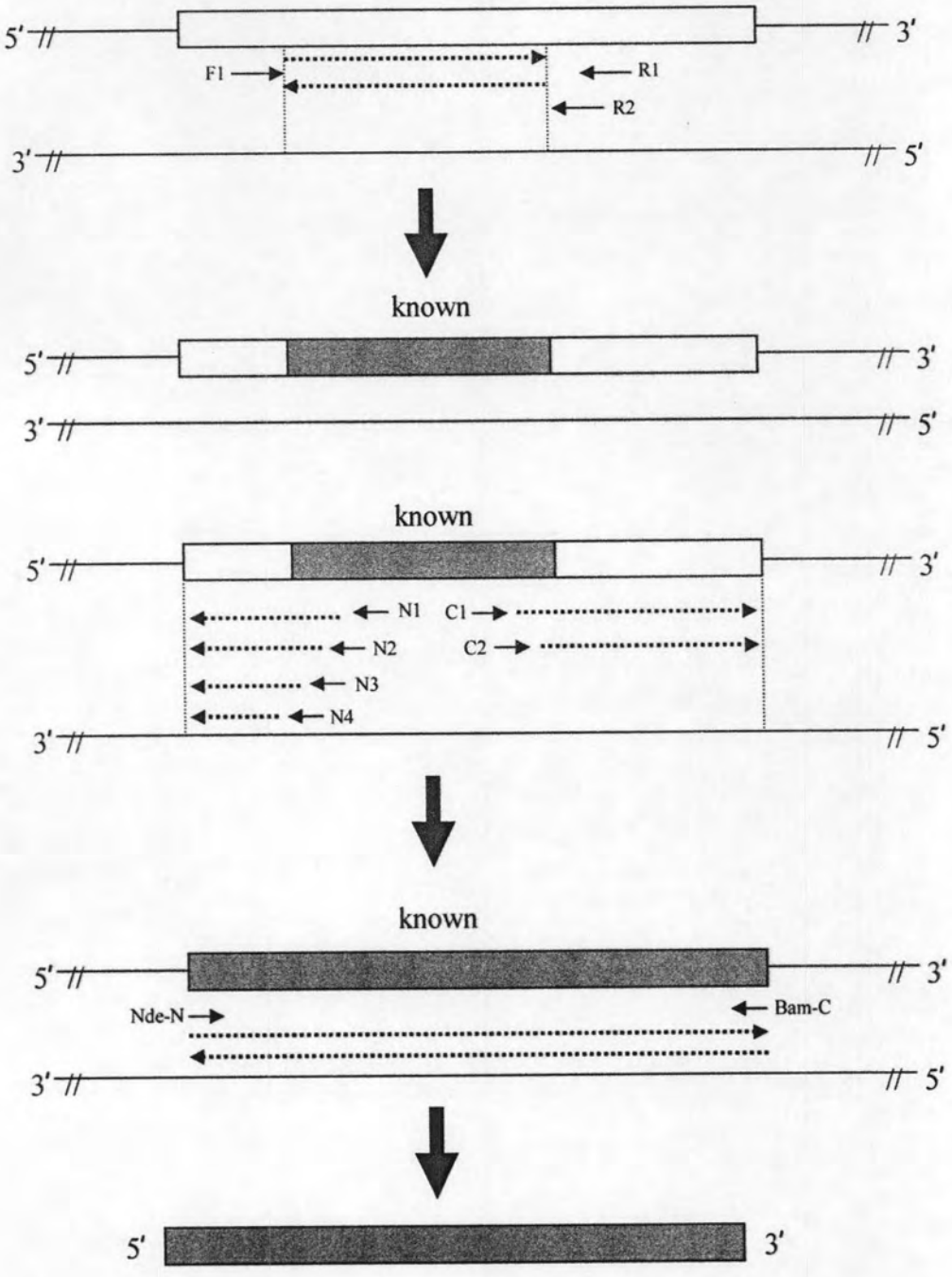

Figure 2.2 Strategy for PCR amplification and sequencing of phenylalanine dehydrogenase gene from *Bacillus lentus*. The phenylalanine dehydrogenase gene is boxed. Thin arrows and doted arrows show the primers for PCR and the sequencing direction, respectively.

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

Primer	Sequence (5'⟶ 3')	T_m (°C)	Remark
F1	ATYATYGGNGAYCCWCAAAA	-	For the initial internal gene fragment amplification and sequencing
R1	ACRATATARTCWGGNGCRTA	-	"---------- "
R2	TGNCGHCCATGRTCYTCHGT	-	"---------- "
N1	CGGCCGCCAAGCGAATCAAC	74.40	For 5'-terminal and 3'-terminal regions amplification and sequencing
N2	CGGAACAGTTCTGGAGATTTATC	69.98	"---------- "
N3	CGCCGCGCATTTGTAAGTCATT	71.32	"---------- "
N4	CCGAGTGTGGTGTCATGAATAG	71.32	"---------- "
C1	CGGCGTTGTTAATGATGAAACG	69.45	"---------- "
C2	CGCCGGTTCAGCCAACAATC	72.35	"---------- "
Nde-N	GGAATTCCATATGAGCTTAGTAGAAAAAAC ATCCATCATA	77.95	For the whole gene fragment amplification
Bam-C	CGCGGATCCTTAGTTGCGAATATCCCATTTT GGCTTAA	82.08	"---------- "

Note: R = G, T

Y = C, T

W = A, T

H = A, C, T

N = A, C, G, T

10 U of each restriction enzyme in total volume of 50 µl was incubated at 37°C for 18 hours. After incubation, the 5 M NaCl was added to the final concentration of 1 M. The digested chromosomal DNA was precipitated with 2 volumes of absolute ethanol, standed at -20°C at least 30 minutes, and centrifuged at 12,000xg for 10 minutes. The DNA pellet was washed with 70% ethanol, collected by centrifugation at 12,000xg 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.

2.7.4.2 Templates for amplification of 5′-terminal and 3′-terminal regions of phenylalanine dehydrogenase gene (inverse PCR)

The complete digestion of chromosomal DNA with each restriction enzyme: *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Nde*I, *Pst*I, *Sac*I, *Spe*I, *Ssp*I, and *Xba*I were prepared as described in section 2.7.4.1. Later on, the DNA fragments from each restriction enzyme digestion was ligated by T_4 DNA ligase. The reaction mixture containing 2 µl (1 µg) of DNA solution, 1x ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (W/V) polyethylene glycol-8000) and 10 U of T_4 DNA ligase in a total volume of 20 µl was left at 16°C for 18 hours. and then precipitated and dissolved in sterile water as the direction above. The ligated DNA product in each reaction was completely digested with *Nco*I which has only one site on known sequence to obtain linearized DNA templates. The reaction mixture containing DNA solution, 1x reaction buffer of *Nco*I and 10 U of *Nco*I in total volume of 20 µl was incubated at 37°C for 18 hours, and then precipitated and dissolved in 10 µl sterile water as above. The 2 µl of DNA solution was used as template of each PCR reaction.

2.7.4.3 Templates for amplified the whole gene fragment

The chromosomal DNA of *Bacillus lentus* was completely digested with *Nde*I as the same condition as described in section 2.7.4.1. Then 5 M NaCl was added to the reaction tube 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.

2.7.5 PCR condition

Three parts of *phedh* gene were amplified by 3 steps of PCR as shown in Figure 2.2 and the conditions of each PCR were described in Table 2.2.

The 50 µl of reaction mixture contained 1.5 U of *Pfu* DNA polymerase, *Pfu* DNA Polymerase 1x buffer with $MgSO_4$, 0.2 mM each dNTPs, DNA template and 10 pmole of each primer, except 100 pmole of each degenerated primer in the internal gene fragment amplification.

2.7.6 Nucleotide sequencing

After purification of PCR products from agarose gels by using QIA Quick gel extraction kit, the DNAs were sent to Macrogen, Korea for determination of nucleotide sequence in both directions. PCR products were sequenced directly by cycle sequencing. The sequencing primers were the same as primers of PCR amplification.

Table 2.2 PCR condition in each step

The region of gene fragment which was amplified	Primer pairs	Predenaturation*	Denaturation	Annealing	Extension	Final extension	Number of cycle
The internal gene fragment	F1xR1 F1xR2	94°C for 10 min.	94°C for 1 min.	42°C for 30 sec. 52.8°C for 30 sec.	72°C for 3 min.	72°C for 7 min.	35
The first 5′-terminal and 3′- terminal regions	N1xC1, N2xC2, N1xC2 and N2xC1	94°C for 10 min.	94°C for 1 min.	50°C for 30 sec.	72°C for 3 min.	72°C for 7 min.	35
The second 5′-terminal and 3′- terminal regions	N4xC2and N3xC2	94°C for 10 min.	94°C for 1 min.	55°C for 30 sec.	72°C for 3 min.	72°C for 7 min.	35
The whole gene fragment	Nde-NxBam-C	94°C for 10 min.	94°C for 1 min.	56.5°C for 30 sec.	72°C for 2 min.	72°C for 7 min.	35

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

2.8 Cloning of phenylalanine dehydrogenase gene

2.8.1 Recombinant DNA preparation

2.8.1.1 Plasmid extraction

The *E. coli* BL21(DE3), which harboured pET-17b plasmid was grown in 5 ml LB medium containing 100 μg/ml ampicillin at 37°C for 18 hours with shaking. The cell culture was collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000xg for 1 minute. Then 100 μl of 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. 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 and placed on ice for 5 minutes. Then 150 μl of cooled solution III (3 M sodium acetate, pH 4.8) was added and the tube was placed on ice for 5 minutes. The mixture was centrifuged at 10,000xg for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then DNA solution was extracted with equal volume of phenol-chloroform (1: 1 V/V). The plasmid DNA was precipitated by the addition of 2 volumes of absolute ethanol to the aqueous phase, collected by centrifugation at 10,000xg for 10 minutes and washed with 70% ethanol. After drying, the pellet was dissolved in an appropiate volume of TE buffer, pH 8.0 containing 20 μg/ml RNase.

2.8.1.2 Vector DNA preparation

The expression vector pET-17b was linearized with *Nde*I and *Bam*HI. The reaction mixture containing of 2 μg pET-17b, 1x *Bam*HI reaction buffer, 1x BSA solution, 20 U of *Nde*I and 20 U of *Bam*HI in total volume of 50 μl was incubated at 37°C for 18 hours. The linear formed pET-17b was harvested from agarose gel by QIA Quick gel extraction kit.

2.8.1.3 The phenylalanine dehydrogenase gene fragment preparation

After the whole gene fragment had been amplified and then cleaned by QIA Quick gel extraction kit, the putative *phedh* gene fragment was digested with *Nde*I and *Bam*HI. The reaction mixture containing of 2 μg of gene fragment, 1x *Bam*HI reaction buffer, 1x BSA solution, 20 U of *Nde*I and 20 U of *Bam*HI in total volume of 50 μl was incubated at 37°C for 18 hours. The DNA fragment was colleted from agarose gel by QIA Quick gel extraction kit.

2.8.1.4 Ligation of vector DNA and the gene fragment

The gene fragment (2.8.1.3) was ligated to the pET-17b vector (2.8.1.2) at vector: insert molar ratio of 1: 5. The 20 μl of ligation mixture contained 100 ng of vector DNA, 500 ng of the gene fragment, 1x ligation buffer and 10 U of T_4 DNA ligase was incubated overnight at 16°C. The recombinant plasmids were further used for transformation.

2.8.2 Transformation

2.8.2.1 Preparation of competent cells

A fresh overnight culture of *E. coli* BL21(DE3) was inoculated into 1 liter of LB broth (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.2) with 1% inoculum size. Cells were grown to log phase at 37°C with vigorous shaking until OD_{600} was about 0.5 to 0.8. The culture was chilled on ice for 15 to 30 minutes and then centrifuged at 8,000 xg for 15 minutes at 4°C. The cells were washed with 1 liter of cold water, spun down and washed again with 0.5 liter of cold water. After centrifugation, the cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged at 8,000xg for 15 minutes at 4°C. Finally, the cell pellets were resuspended to

a final volume of 2 to 3 ml in 10% glycerol. This suspension was divided into 40 µl aliquots and stored at -80°C until used.

2.8.2.2 Transformation

The recombinant plasmids from 2.8.1.4 were transformed into competent cells of *E. coli* BL21(DE3) by electroporation. In the electroporation step, cuvette 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. Two microliters of recombinant plasmid was mixed with 40 µl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette and the cuvette was applied one pulse at the above setting. Subsequently, 1 ml 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, incubated at 37°C for 1 hour with shaking and spun down to retain 200 µl. Finally, this suspension was spread onto the LB agar plates containing 100 µg/ml ampicillin and incubated at 37°C for 10 hours. Cells containing the recombinant plasmid which could grow on selective plate were picked and the plasmids were extracted.

2.9 Expression of phenylalanine dehydrogenase gene

2.9.1 Recombinant plasmid preparation

E. coli BL21(DE3) recombinant clones were grown in LB medium containing 100 µg/ml ampicillin. The growing condition was 37°C for 18 hours with shaking. The cell cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000xg for 2 minutes. Then the plasmid from individual clone was extracted as described in 2.8.1.1. After that, the plasmids were completely digested with *Nde*I and

*Bam*HI. The sizes of recombinant plasmids were estimated by submarine agarose gel electrophoresis compared with λ/*Hin*dIII marker. Finally, the recombinant plasmids were confirmed for *phedh* gene inserts by sequencing.

2.9.2 Crude extract preparation

The *E. coli* BL21(DE3) transformants were grown overnight at 37°C for 18 hours in 5 ml of LB medium, pH 7.2, containing 100 μg/ml ampicillin. After that, 2.5% of the cell culture was inoculated into 200 ml of the same medium and was cultured at 37°C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, IPTG was added to final concentration of 0.4 mM to induce *phedh* gene expression, and cutivation was continued at 37°C for 4 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then washed with cold 0.85% NaCl. 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% β-mercaptoethanol and 1.0 mM EDTA) and centrifuged again. The cell pellet had been stored at -80°C until it was sonicated.

Preparation of crude extract was performed by resuspending cell pellet in 5 ml of cold extraction buffer (0.1 M potassium phosphate buffer, pH 7.4 containing 0.1 mM PMSF, 0.01% β-mercaptoethanol and 1.0 mM EDTA). Cell suspension was sonicated on ice. Unbroken cells and cell debris were removed by centrifugation at 17,500xg for 30 minutes. The enzyme had been kept at 4°C until it was used to assay activity and protein concentration.

2.9.3 Enzyme activity assay

This enzyme catalyzes the reversible reaction. Thus, the enzyme activity can be determined in two directions. The forward reaction is oxidative deamination

of L-phenylalanine, while the reverse reaction is the reductive amination of L-phenylpyruvate .

$$\text{L-Phe} + NAD^+ + H_2O \rightleftharpoons \text{phenylpyruvate} + NH_3 + NADH + H^+$$

2.9.3.1 Oxidative deamination

The activity of phenylalnine dehydrogenasee for oxidative deamination of L-phenylalanine was spectrophotometrically assayed. The 1 ml of standard reaction mixture contained 200 μmol of glycine-KCl-KOH buffer, pH 11.5, 20 μmol of L-phenylalanine, 1 μmol of NAD^+, and the enzyme. The reaction was incubated at 30°C for 5 minutes in a cuvette of 1-cm light path. The reaction was begun by addition of NAD^+ and 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.

2.9.3.2 Reductive amination

The activity of PheDH for reductive amination of phenylpyruvate was spectrophotometrically assayed. The reaction mixture of 1 ml contained of 100 μmol of glycine-KCl-KOH buffer, pH 9.0, 500 μmol of NH_4Cl, 10 μmol of L-phenylpyruvate, 0.2 μmol of NADH, and the enzyme. Incubation was carried out at 30°C for 5 minutes in a cuvette of 1-cm light path. The reaction was started by addition of NADH 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 decrement of 1 μmol of NADH in 1 minute. Specific activity is defined as units per milligram of protein.

2.9.4 Protein measurement

Protein concentration was determined by the modified method of Lowry *et al.,* (1956). The reaction mixture 3.05 ml containing 20-100 μg of protein, 50 μl of solution A, 2.5 ml of solution B was mixed and incubated at 30°C for 10 minutes. After that, the solution mixture was incubated with 0.25 ml of solution C at room temperature for 20 minutes. Then, the protein concentration was monitored by measuring the absorbance at 610 nm and calculated from the standard curve of protein standard (BSA). The preparations of all solutions were described in Appendix C.

2.10 Optimization for phenylalanine dehydrogenase gene expression

The *E. coli* BL21(DE3) transformant which showed the highest PheDH activity from 2.9 was grown overnight at 37°C in 5 ml of LB medium containing 100 μg/ml ampicillin. After that, 2.5% of the cell culture was inoculated into 200 ml of the same medium and was cultured at 37°C with shaking. When the turbidity of the culture at 600 nm had reached 0.6, the *phedh* gene was induced by IPTG at final concentration of 0-1.0 mM at various induction times: 0, 2, 4, 6, 8, 12, 16, 20 and 24 hours. The cells were harvested by centrifugation at 10,000xg for 10 minutes, then washed with cold 0.85% NaCl. After that, The cell pellet was washed once in cold extraction buffer and centrifuged again. The cell pellet was stored at -80°C until the next step. In crude extract preparation, the cell pellet was resuspended in 5 ml of cold extraction buffer and then broken by sonication on ice. Unbroken cell and cell debris were removed by centrifugation at 17,500xg for 30 minutes. The supernatant was stored at 4°C for enzyme and protein assays as described in 2.9.3.1 and 2.9.4, respectively.

2.11 Stability of phenylalanine dehydrogenase gene expression

The transformant was daily subcultured by streaking on LB plate contained 100 μg/ml ampicillin for 50 days. Then the 1st, 5th, 10th, 15th, 20th, 25th, 30th, 35th, 40th, 45th and 50th subcultured colonies were picked up to culture at the optimum condition obtained from 2.10 and assayed for enzyme activity and protein as described in 2.9.3.1 and 2.9.4, respectively.

2.12 Purification of phenylalanine dehydrogenase

2.12.1 Preparation of crude extract

The transformant was grown in 1.6 liter of LB medium, pH 7.2, containing 100 μg/ml ampicillin at the optimum condition from 2.10. The cell cultivation, crude extract preparation, assay for activity and protein determination were performed as described in 2.9.2, 2.9.3.1 and 2.9.4.

2.12.2 Enzyme purification procedures

The crude extract from 2.12.1 was purified by the following steps. All operations were done at 4°C. The buffer used in all steps was 10 mM potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1 mM EDTA.

2.12.2.1 Ammonium sulfate precipitation

The precipitation of crude extract was done by slowly adding fine solid ammonium sulfate to 40% saturation with gentle stirring by magnetic stirrer. After 1 hour, the supernatant was collected by centrifugation at 17,500xg for 30 minutes and then adjusted to final concentration of 50% saturation with solid ammonium sulfate.

The solution was left for 1 hour on ice with continuous stirring and centrifuged again. The precipitate was dissolved in the buffer. The protein solution was dialyzed against 100 volumes of the buffer at least 4 hours for 3 times before determination of the enzyme activity and protein concentration as described in 2.9.3.1 and 2.9.4, respectively.

2.12.2.2 DEAE-Toyopearl column chromatography

The activated DEAE-Toyopearl was prepared by washing with 0.5 N NaOH for 2-3 times, and rewashed by deionized water until the pH reached 8.0. The activated DEAE-Toyopearl was resuspened in the buffer, packed into 2.3 x 19.5 cm column and equilibrated with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The crude enzyme solution from section 2.12.2.1 was applied to the DEAE-Toyopearl column. The unbound proteins were eluted from the column with the buffer until the absorbance at 280 nm was nearly zero. After that, the buffer was changed by making linear salt gradient of 0 to 0.5 M KCl in the same buffer in order to elute the bound proteins from the column. The 3 ml fractions were collected using a fraction collector. The protein profile was determined by measuring the absorbance at 280 nm. PheDH activity was assayed using the method described in section 2.9.3.1. The KCl concentration was investigated by measuring its conductivity. The fractions containing PheDH activity was pooled and dialyzed against the buffer. The PheDH activity and protein concentration of pooled fraction were measured as described in section 2.9.3.1 and 2.9.4, respectively.

2.12.2.3 Butyl-Toyopearl column chromatography

Butyl-Toyopearl was washed with deionized water for 2-3 times, and then resuspended in the buffer containing 25% saturated ammonium sulfate and packed

into 2.3 x 18.5 cm column followed by equilibrating with the same buffer for 5-10 column volume at flow rate 1 ml/min.

The pooled active fraction from section 2.12.2.2 was slowly adjusted to 25% saturation with fine ammonium sulfate and stirred gently at least 30 minutes. The protein solution was applied to the column at flow rate 1 ml/min. The unbound proteins were eluted from the column with the buffer containing 25% saturated ammonium sulfate until the absorbance to 280 nm was nearly zero. The bounded protein was eluted from the column with 22.5% saturated ammonium sulfate in the buffer. Two milliliter fractions were collected by using fraction collector and assayed for both protein concentration and PheDH activity. Protein profile was determined by measuring an absorbance at 280 nm. PheDH activity was assayed by using the method as described in section 2.9.3.1. The fractions containing PheDH activity were pooled and dialyzed against the buffer. After desalting, the enzyme was concentrated with aquasorb. The PheDH activity and protein concentration were determined as described in section 2.9.3.1 and 2.9.4, respectively.

2.13 Polyacrylamide gel electrophoresis

The enzyme from each step of purification was analyzed by native PAGE and SDS-PAGE to determine the native protein and denature protein pattern, respectively.

2.13.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel of a 7.7% seperating gel and a 5% stacking gel. Tris-glycine buffer, pH 8.3 (25 mM Tris and 192 mM glycine) was used as electrode buffer. Preparation of solution and polyacrylamide gels was

described in Appendix F. The enzyme was mixed with 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 50% glycerol and 0.05% bromophenol blue) by ratio 5: 1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current (20 mA). For activity staining, the experiment was done at 4°C. After electrophoresis, the gel was developed by protein and activity staining.

2.13.1.1 Protein staining

The gel was transferred to a small box containing Coomassie staining solution (1% Coomassie Blue R-250, 45% methanol, and 10% glacial acetic acid). The gel was agitated for 30 minutes on the shaker. The stain solution was poured out and the Coomassie destaining solution (10% methanol and 10% glacial acetic acid) was added. The gel was gently destained for several times until gel background was clear.

2.13.1.2 Activity staining

After electrophoresis at 4°C, the gel was moved to a small box which consisted of 10 ml solution containing 4.25 mmol of Tris-HCl buffer, pH 8.5, 40 µmol of L-phenylalanine, 50 µmol of NAD^+, 250 µg of phenazine methosulfate and 2.5 mg of nitroblue tetrazolium. The gel was gently shaked at room temperature for 5 min. After the brown band had appeared, the staining reaction was stopped by pouring off the staining solution The gel was then quickly rinsed several times with deionized water until gel background was clear.

2.13.2 SDS-polyacrylamide gel electrophoresis

The SDS-PAGE system was performed according to the method of Bollag *et al.*, 1996. The slab gel system consisted of 0.1% SDS (W/V) in 10% seperating gel and 5% stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% SDS),

pH 8.3 was used as electrode buffer. The gel preparartion was described in Appendix G. The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol) by ratio 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current (20 mA) at room temperature. The molecular weight marker proteins were β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endonuclease Bsp98I (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da). After electrophoresis, the gel was stained with Coomassie blue as described in section 2.13.1.1.

2.14 Characterization of phenylalanine dehydrogenase

2.14.1 Molecular weight determination of phenylalanine dehydrogenase

The molecular weight of purified PheDH was determined by gel filtration on TSK Gel G3000 SW column (0.75x60 cm ID) with 0.1 M potassium phosphate buffer, pH 7.0 containing 0.15 M NaCl at flow rate 1 ml/min. The molecular weight marker protein consisted of alcohol dehydrogenase (669,000 Da), bovine serum albumin (150,000 Da) and thyroglobulin (66,000 Da) was used.

2.14.2 Substrate specificity of phenylalanine dehydrogenase

The ability of the enzyme catalysis in the oxidative deamination of various amino acids and L-phenylalanine analogs was determined at a final substrate concentration of 20 mM. L-Phenylalanine was replaced by various amino acids and L-phenylalanine analogs as substrate for the oxidative deamination as described in section 2.9.3.1. In the same way, the ability of the enzyme catalysis in the reductive

amination of various keto acids and phenylpyruvate analogs was determined at a final concentration of 10 mM. Phenylpyruvate was replaced by various keto acids and phenylpyruvate analogs as substrate for the reaction. The reductive activities were determined as described in section 2.9.3.2. The results were expressed as a percentage of the relative activity.

2.14.3 Coenzyme specificity of phenylalanine dehydrogenase

The purified PheDH was used to study coenzyme specificity. NAD^+ was replaced by various NAD^+ analogs at final concentration of 2 mM for oxidative deamination. Assays with NAD^+ analogs were conducted by measuring the increase in absorbance at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm ($\varepsilon = 9.1x10^3\ M^{-1}.cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm ($\varepsilon = 6.2x10^3\ M^{-1}.cm^{-1}$); nicotinic acid adenine dinucleotide, 338 nm ($\varepsilon = 6.2x10^3\ M^{-1}.cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm ($\varepsilon = 9.3x10^3\ M^{-1}.cm^{-1}$); nicotinamide 1, N^6-ethenoadenine dinucleotide, 334 nm ($\varepsilon = 6.9x10^3\ M^{-1}.cm^{-1}$); nicotinamide hypoxanthine dinucleotide, 338 nm ($\varepsilon = 6.2x10^3\ M^{-1}.cm^{-1}$) and thionicotinamide adenine dinucleotide, 395 nm ($\varepsilon = 11.3x10^3\ M^{-1}.cm^{-1}$) (Misono *et al.*, 1989). The reactions were carried out at pH 9.5 to avoid the degradation of NAD^+ analogs at a more alkaline pH. The result was expressed as a percentage of the relative activity.

2.14.4 Effect of pH on phenylalanine dehydrogenase activity

The effect of pH on the activity was determined under the standard assay conditions for the oxidative deamination and reductive amination as described in section 2.9.3.1 and 2.9.3.2 at various pHs. The 200 mM of potassium phosphate for pH 6.0 to 8.5, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.5 were used. The pH of each reaction mixture was measured with a pH meter at room

temperature after the reaction. To compare the effect of pH on PheDH activity, The percentage of relative activity was plotted against the final pH.

2.14.5 Effect of temperature on phenylalanine dehydrogenase activity

The effect of temperature on the activity was examined under the standard assay condition for both of the oxidative deamination and reductive amination as described in section 2.9.3.1 and 2.9.3.2 at various temperatures from 25°C to 75°C. The percentage of relative activity was plotted against the temperature used for the activity assay.

2.14.6 Effect of pH on phenylalanine dehydrogenase stability

The purified PheDH was used to study pH stability. After the enzyme had been incubated at 30°C for 20 minutes in each of the 10 mM buffer at various pHs, an aliquot of the enzyme solution was withdrawn and the remaining activity of enzyme was measured under the standard assay condition for the oxidative deamination as described in section 2.9.3.1. The 10 mM buffers used were acetate buffer for pH 4.0 to 6.0, potassium phosphate for pH 6.0 to 8.5, Tris-HCl buffer for pH 7.0 to 9.0 and glycine-KCl-KOH buffer for pH 8.5 to 12.5. The percentage of PheDH relative activity was plotted against the incubated pH.

2.14.7 Effect of temperature on phenylalanine dehydrogenase stability

The effect of temperature on the stability of the enzyme was determined from 30°C to 75°C. The purified PheDH was incubated at various temperatures for 10 minutes before determination of enzyme activity under the standard assay condition in the oxidative deamination according to the method described in section 2.9.3.1. Afterward, the enzyme was incubated at the highest temperature which the enzyme

activity still remained full activity and daily collected for enzyme activity assay as described previously for 15 days. The result was shown as a percentage of the relative activity.

2.15 Kinetic studies of phenylalanine dehydrogenase

A series of steady-state kinetic analyzes were carried out in order to investigate the kinetic parameters as described below.

2.15.1 Initial velocity analysis for the oxidative deamination

Initial velocity studies for the oxidative deamination were carried out under the standard reaction condition as described in section 2.9.3.1, except that various amounts of L-phenylalanine and NAD^+ were used. The concentrations of L-phenylalanine used were 0.075, 0.1, 0.125 and 0.2 mM, and those of NAD^+ used were 0.2, 0.3, 0.4, 0.6 and 0.8 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-phenylalanine concentrations at a series of fixed concentrations of NAD^+ and the secondary plots of y intercepts against reciprocal concentrations of NAD^+ were made from the data. K_m of L-phenylalanine and NAD^+ were determined from these two plots, respectively.

2.15.2 Initial velocity analysis for the reductive amination

Initial velocity studies for the reductive amination were carried out under the standard reaction condition as described in section 2.9.3.2, except that various amounts of phenylpyruvate, NH_4Cl and NADH were used in the experiments as described below.

2.15.2.1 The enzyme was assayed in the reductive amination by using phenylpyruvate as a variable substrate (0.1, 0.15, 0.2, 0.25 and 0.4 mM) at several fixed

concentrations of NH_4Cl (20, 40, 60, 80 and 100 mM) in the presence of a high and saturating concentration of NADH (0.2 mM). The double-reciprocal plots of initial velocities against phenylpyruvate concentrations at a series of fixed concentrations of NH_4Cl and the secondary plots of y intercepts against reciprocal concentrations of NH_4Cl were made for K_m determination of phenylpyruvate and NH_4Cl.

2.15.2.2 The enzyme was assayed in the reductive amination by using NH_4Cl as a variable substrate (60, 80, 100 and 120 mM) at several fixed concentrations of NADH (0.1, 0.125, 0.15 and 0.2 mM) in the presence of a high and saturating concentration of phenylpyruvate (10 mM). The double-reciprocal plots of initial velocities against NH_4Cl concentrations at a series of fixed concentrations of NADH and the secondary plots of y intercepts against reciprocal concentrations of NADH were made for K_m determination of NH_4Cl and NADH.

2.15.2.3 The enzyme was assayed in the reductive amination by using NADH as a variable substrate (0.08, 0.1, 0.15 and 0.2 mM) at several fixed concentrations of phenylpyruvate (0.01, 0.2, 0.4 and 0.5 mM) in the presence of a high and saturating concentration of NH_4Cl (500 mM). The double-reciprocal plots of initial velocities against NADH concentrations at a series of fixed concentrations of phenylpyruvate and the secondary plots of y intercepts against reciprocal concentrations of phenylpyruvate were made for K_m determination of NADH and phenylpyruvate