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., U.S.A.

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

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

Evaporating Light-Scattering Detector for High Performance Liquid Chromatography (ELSD-HPLC): Alltech Inc, USA

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

Gel Document: SYNGENE, England

High Performance Liquid Chromatography (HPLC): Shimadzu, Japan

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

Lamina flow: HT123, ISSCO, U.S.A.

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

Magnetic stirrer: 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: TRX1500, Turbora International Co., Ltd., Korea

Orbital incubator: 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

Rotary shaker: Orbital shaker 03, Stuart Scientific, England

Sonicator: SONOPULS Ultrasonic homogenizers, Bandelin, Germany

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

Thermo cycler: Mastercycler gradient, eppendorf, Germany

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

Thin layer chromatography (TLC): DC-Plastikfolien cellulose, Merck, Germany

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

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

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

2.2 Chemicals

Acetone: Lab-Scan Ltd, Ireland

Acrylamide: Merck, Germany

Agar: Merck, Germany

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

Ammonium hydroxide: BDH, England

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, USA

Bromphenol blue: Merck, Germany

Chloramphenicol: Nacalai tesque, Inc., Japan

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 tetraacetic acid: M&B, England

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, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

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

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Glucose: BDH, England

Hexane: Merck, Germany

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isoamyl alcohol: Merck, Germany

Isopropanol: Merck, Germany

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

Magnesium sulphate 7-hydrate: BDH, England

Methanol: Lab-Scan, Thailand

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 Reagenti, Italy

Nicotinamide adenine dinucleotide (oxidized form) (NAD^+): Kohjin Co. Ltd., Japan

Nicotinamide adenine phosphate ($NADP^+$): Kohjin Co. Ltd., Japan

Ninhydrin: VWR Prolabo Range, France

Nitroblue tetrazolium: Koch-Light Laboratories Ltd., Japan

Peptone from casein pancreatically digested: Merck, Germany

Perchloric acid: BDH, England

Phenazine methosulfate: Nacalai Tesque, Inc., Japan

Phenol: BDH, England

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

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium dihydrogen phosphate anhydrous : Carlo Erba Reagenti, Italy

Potassium hydroxide: Carlo Erba Reagenti, Italy

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

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, U.S.A.

Sodium hydroxide: Carlo Erba Reagenti, Italy

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

Xylene: BDH, England

Yeast extract: Scharlau microbiology, European Union

2.3 Amino acids

All amino acids used in this research were analytical grade that were purchased from Nacalai tesque Inc., Kyoto, Japan, Wako Pure chemical Industries, Ltd., Japan and Sigma, U.S.A.

2.4 Enzymes and Restriction enzymes

Lysozyme: Sigma, U.S.A.
Proteinase K: Sigma, U.S.A.
Restriction enzymes: New England BioLabs, Inc., U.S.A.
RNaseA: Sigma, U.S.A.
Ex*Taq* DNA polymerase: Takara Shuzo Co., Ltd., Japan
LA *Taq* DNA Polymerase: Takara Shuzo Co., Ltd., Japan
T_4 DNA ligase: New England BioLabs, Inc., U.S.A.

2.5 Primers

All oligonucleotide primers used in this research were synthesized form Bioservice Unit (BSU) of NSTDA and Pacific Science, Thailand.

2.6 Bacterial strains and plasmid

The bacteria strain K-1 used as a source of lysine 6-dehydrogenase (*lys 6-dh*) gene was screened from soil.

Bacillus cereus ATCC 11778 was used as a source of pyrroline-5-carboxylate reductase (*p5cr*) gene was purchased from Thailand Institute of Scientific and Technological Research (TISTR)

pET-17b was used as an expression vector for cloning of *lys 6-dh* gene and *p5cr* gene (Appendix A).

Escherichia coli BL 21(DE3), genotype: F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3), was used as a host for expression.

2.7 Bacterial culture media

2.7.1 1% Peptone medium

Liquid medium consists of 1% peptone from meat, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4.7H_2O$ and 0.01% yeast extract. For solid medium add 1.5% Agar.

2.7.2 1% Peptone, 1% L-lysine medium

Liquid medium consists of 1% peptone from meat, 1% L-lysine, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.01% $MgSO_4.7H_2O$ and 0.01% yeast extract.

2.7.3 Luria-Bertani broth (LB medium)

The following medium was used as LB medium (Sambrook *et. al.*, 1989) containing 1% peptone, 0.5% NaCl and 0.5% yeast extract which was prepared and adjusted pH to 7.2 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121°C. If needed, selective antibiotic drug was then supplemented. All media were adjusted pH to 7.2 with KOH or KCl and steriled at 121 °C for 15 min.

2.8 Lys 6-DH activity assay (Misono, H. and Nagasaki, S. 1982)

The activity of Lys 6-DH for oxidative deamination of lysine was spectrophotometrically assayed. One ml reaction mixture contains 0.2 M glycine-KCl-KOH buffer (pH 9.5), 0.02 M L-lysine, water, 0.002 M NAD^+ and the enzyme. This reaction mixture was incubated at 30°C for 10 minute in an 1-cm light path cuvette. The reaction was started 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 catalyses the formation of 1 μmol of NADH in 1 minute. Specific activity is expressed as units per milligram of protein.

2.9 Protein determination

Protein concentration was determined by the method of Lowry *et al.,* (1956). The reaction mixture 6.1 ml containing 20-300 μg of protein, 5 ml of solution A and 100 μl of solution B were vigorously mixed and incubated at 30 °C for 10 minutes. After that, the solution mixture was added with 0.5 ml of solution C then vigorously mixed and incubated at 30 °C for 20 minutes. The preparation of all solutions was described in Appendix C. The protein concentration was monitored by measuring the absorbance at 610 nm and the amount of protein was calculated from the standard curve of protein standard (BSA).

2.10 Identification of L-lysine 6 dehydrogenase (Lys 6-DH) producing bacteria

2.10.1 Morphological and biochemical properties

The Lys 6-DH producing bacteria was identified by Union Hitech Co., Ltd., Osaka, Japan, according to its morphological and biochemical properties such as gram

staining, fermentative production of acids from various carbon sources and the activity of various enzymes.

2.10.2 Molecular genetic properties

2.10.2.1 Chromosomal DNA extraction

Chromosomal DNA was isolated from *Achromobacter denitrificans* K-1 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 30 °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 30 µl of 10% (w/v) SDS followed by the addition of 3 µl of 20 mg/ml proteinase K and incubated for 1 hour at 37 °C. After incubation, 100 µl of 5 M NaCl and 50 µl of CTAB-NaCl solution (10 % (w/v) CTAB and 0.7 M NaCl) were added 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 10,000xg for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube and extracted 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 isopropanol to the aqueous phase and collected by centrifugation at 10,000xg for 10 minutes. The DNA was washed with 70% ethanol. After drying, the pellet was dissolved in an appropriated volume of TE buffer. Finally, DNA concentration was estimated by submerged agarose gel electrophresis in comparison with known amount of λ/*Hin*dIII marker.

2.10.2.2 Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.7 g of agarose 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 Erlenmeyer flask and heated until complete solubilization. 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 was performed at constant voltage of 10 volt/cm 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 compared with the intensity and relative mobility of the standard DNA markers (λ/*Hin*dIII and 100 bp ladder).

2.10.2.3 16S rRNA gene amplification

Chromosomal DNA from *Achromobacter denitrificans* K-1 was used as a template for the 16S rRNA sequence amplification. The amplification procedure followed the method described by Woese and coworkers (1985, 1987). The sense pA and antisense pH′ primer sequences were 5′-AGAGTTTGAT CCTGGCTCAG-3′ and 5′-TGCGGCTGGATCACCTCCTT-3′, respectively. PCR was performed with Ex*Taq* DNA polymerase following this procedure. Predenaturation at 94 °C for 5 minutes following by 30 cycles of denaturation at 94 °C for 1 minutes, annealing at 55 °C for 2 minutes and extension at 72 °C for 3 minutes. Then, 1 cycle of final extension at 72 °C for 5 minutes was added. After final extension, the PCR products were kept at 4 °C. The nucleotide sequence of the

amplified DNA fragment (~1500 bp) was determined using the PRISM kit with an Applied Biosystems 373A DNA sequencer.

2.10.2.4 Computer search for sequence similarities

Nucleotide sequence obtained from 2.10.2.3 was compared with previously published nucleotide sequences from the EMBL-GenBank-DDBJ database. Multiple sequences were aligned to find the position of nucleotide sequences by using the CLUSTAL W program.

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

2.11.1 *A. denitrificans* K-1 culture

Starter inoculum was prepared by inoculation 1 ml of *A. denitrificans* K-1 from the starter into 1 L 1% peptone medium, pH 7.2 and cultivated for 20 hours with 250 rpm shaking at 30 °C. The 1 L of cell culture was transferred to 10 L of 1% peptone, 1% L-lysine medium, pH 7.2 and cultivated at the same condition as described previously. The total cultivation of *A. denitrificans* K-1 was 55 L.

2.11.2 Cell collection and crude extract preparation

Cultivated cells were harvested by centrifugation at 10,000x*g* for 10 minutes, and then washed twice with cold 0.85 % NaCl and subsequently washed once with 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). Then, the cells were harvested by centrifugation. The total cells of 258.9 g were resuspended in 650 ml of cold extraction buffer then disrupted by discontinuous sonication on ice for 2 minutes (100% pulse, stop 2 minutes with 10 cycles) using sonicator model SONOPLUS Ultrasonic homogenizers (T13). Unbroken cells and cell debris were removed by centrifugation at 12,000x*g* for 30 minutes. The supernatant was dialyzed against 0.01

M KPB (0.01 M potassium phosphate buffer, pH 7.4 containing 0.01% β-mercaptoethanol and 1.0 mM EDTA) before determination of enzyme activity and protein concentration as described in 2.8 and 2.9, respectively.

2.11.3 Enzyme purification steps

The crude extract from 2.11.2 was purified by the following steps. All operations were carried out at 4 °C. The buffer used in all steps was 0.01 M KPB, pH 7.4 containing 0.01 % β-mercaptoethanol and 1 mM EDTA.

2.11.3.1 0-60% ammonium sulfate precipitation

The precipitation of crude extract was carried out by slowly adding solid ammonium sulfate to 60% saturation with gentle stirring by magnetic stirrer. The solution was left for 30 minutes on ice with continuous stirring and was subsequently centrifuged at 10,000x*g* for 30 minutes. The precipitate was dissolved in 0.01 M KPB. The protein solution was dialyzed against the same buffer at least 4 hours and repeated 3 times before determination of enzyme activity and protein concentration.

2.11.3.2 DEAE-Toyopearl 650 M column chromatography

DEAE-Toyopearl was activated by washing with 0.5 N NaOH twice before rewashing with deionized water until pH was 8.0. The activated DEAE-toyopearl was resuspended in 0.01 M KPB buffer and packed into 5 x 25 cm column followed by washing with the same buffer for 10 column volume at a flow rate of 1 ml/min. The 230-ml dialyzed protein solution (14 g proteins) from 2.11.3.1 was applied into the column followed by equilibrating with the same buffer for 5-10 column volume at a flow rate of 1 ml/min. After the unbound proteins had been eluted, the column was continuously washed until the absorbance at 280 nm of eluent

decreased to base line value. After that, the bounded proteins were eluted stepwise from the column with 0.01 M KPB containing 50 mM KCl. The fractions of 15 ml were collected by fraction collector. The protein elution profile was monitored by measuring the absorbance at 280 nm and the enzyme activity was detected as described in 2.8. The KCl concentration was investigated by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against 0.01 M KPB before determination of the enzyme activity and protein concentration.

2.11.3.3 0-40% ammonium sulfate precipitation

The precipitation of protein solution from 3.11.3.2 was done by slowly adding solid ammonium sulfate to 40% saturation with gentle stirring by magnetic stirrer. The solution was left for 30 minutes on ice with continuous stirring and was subsequently centrifuged at 10,000x*g* for 30 minutes. The protein pellet was dissolved in KPB buffer. The protein solution was dialyzed against the same buffer at least 4 hours for 3 times before determination of enzyme activity and protein concentration.

2.11.3.4 Sephadex G-150 column chromatography

Sephadex G-150 had been washed with approximately 500 ml of 0.01 M KPB containing 0.1 M KCl before it was packed into 2 x 120 cm column followed by equilibrating with the same buffer for 10 column volume at a flow rate of 0.2 ml/min. The 10-ml dialyzed protein solution (389 mg proteins) from 2.11.3.3 was applied into column followed by equilibrating with the same buffer for 5-10 column volume at a flow rate of 0.2 ml/min. The fractions of 5 ml were collected, and the protein elution profile was monitored by measuring the absorbance at 280 nm whereas the enzyme activity was determined as described in 2.8. The active fractions were

pooled. The pooled protein solution was dialyzed against 0.01 M KPB before determination of the enzyme activity and protein concentration.

2.11.3.5 Mono Q HR 10/10 column chromatography

The 10-ml dialyzed protein solution (365 mg proteins) from 2.11.3.4 was applied into the Mono Q HR 10/10 column followed by equilibrating with 0.01 M KPB buffer for 5-10 column volume at a flow rate of 2 ml/min. The unbound proteins were eluted from the column with the same buffer. The column was continuously washed until the absorbance at 280 nm of eluent decreased to base line value. After that, the bound proteins were eluted from the column with 0.01 M KPB containing linear salt gradient 0 to 0.5 M KCl. The fractions of 4 ml were collected and the elution profile was monitored for protein by measuring the absorbance at 280 nm and the enzyme activity was determined as described in 2.8. The KCl concentration was detected by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against 0.01 M KPB before determination of the enzyme activity and protein concentration.

2.11.3.6 Phenyl Sepharose XK 16 column chromatography

Before applied the protein solution from 2.11.3.5 into the phenyl sepharose XK16 column, the solution was dialyzed in 0.1 M KPB then adjusted to 20% saturation with ammonium sulfate and stirred gently for at least 30 minutes. The protein solution of 10 ml (115 mg proteins) was then applied into column at a flow rate of 2 ml/min. The unbound proteins were eluted from the column with 0.1 M KPB buffer containing 20% ammonium sulfate. The column was washed until the absorbance at 280 nm of eluent decreased to base line value. After that, the bound proteins were eluted from the column with a negative salt gradient, from 20% to 0% saturated ammonium sulfate in the buffer. The fractions of 4 ml were collected, the protein elution profile was monitored by measuring the absorbance at 280 nm and the

enzyme activity was determined as described in 2.8. The ammonium sulfate concentration (percentage) was detected by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against the 10 mM KPB before determination of the enzyme activity and protein concentration.

2.12 Determination of enzyme purity by polyacrylamide gel electrophoresis

The proteins from each step of purification were analyzed for enzyme purity by SDS-PAGE to determine the denature protein pattern.

2.12.1 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Bollag *et al.*, 1996. The slab gel system consisted of 0.1% SDS (W/V) in 10 % separating 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 proteins were 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 a ratio of 5: 1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at a constant current (30 mA) at room temperature. The standard molecular weight markers were phosphorylase B (MW 97,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), trypsin inhibitor (MW 20,100) and α-lactalbumin (MW 14,400). After electrophoresis, proteins in the gel were visualized by coomassie blue staining.

2.12.2 Gel staining

2.12.2.1 Coomassie blue staining

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

2.13 Amino acid sequence analysis

2.13.1 Sample preparation

Two nanomoles of purified Lys 6-DH from *A. denitrificans* K-1 was lyophilized and then incubated at 37 °C with 20 µl of 8 M urea for 1 hour followed by adding 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 using Suprec^{Tm-02}, 0.22 µm and the peptide fragments were analyzed on a reverse phase HPLC.

2.13.2 Separation and detection of peptides

The digested peptides from 2.14.1 were separated with a reversed phase HPLC on a YMC-Pack ODS-AM column (100 x 4.6 mm ID) equilibrated with 0.12% trifluoroacetic acid. A 60-min 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 absorbance of the eluted peptide at 210 nm was continuously monitored. The isolated peaks were collected and lyophilized for further analysis.

2.13.3 Amino acid sequencing

The isolated peptides from 2.13.2 were directly used for amino acid sequence analysis by Automated Edman Degradation with Applied Biosystem model 610A data analysis system for protein sequencing.

2.13.4 Computer search for sequence similarities

Amino acid sequence obtained from 2.13.3 was compared with previously published amino acid sequence of Lys 6-DH from the EMBL-GenBank-DDBJ database. Multiple sequences were aligned to find the position of peptide sequences using the CLUSTAL W program and further used as the data for design the degenerated primers in the next step.

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

2.14.1 Preparation of templates for PCR amplification

Chromosomal DNA of *A. denitrificans* K-1 was prepared by the method of Frederick *et al.*, (1995) and then it was completely digested with each restriction enzyme: *Bam*HI, *Hin*dIII and *Eco*RI. The each 20 µl reaction mixture contained 1 µg of chromosomal DNA, 1X reaction buffer, 1X BSA solution (supply from the manufacture) and 10 U of each restriction enzyme. The reaction mixtures were incubated at 37°C for 18 hours. One microliter (about 500 ng) of the DNA solution was used as template in each PCR mixture.

2.14.2 Primers design

The primer pairs used for partial amplification of internal *lys 6-dh* gene of *A. denitrificans* K-1 were designed from the amino acid sequence from 2.13.3.

2.14.3 PCR amplification of partial *lys 6-dh* gene

The partial *lys* 6-*dh* gene was amplified using PCR. Twenty five microliters reaction mixture contained 2.5 U of EX *Taq* DNA polymerase, 0.2 mM dNTPs, 1x PCR buffer (100 mM Tris-HCl, pH 8.8 , 500 mM KCl and 1 % Triton X-100), 2 mM $MgCl_2$, 500 ng DNA template and 10 pmole of each primer. The thermocycle consisted of predenaturation at 96 °C for 20 minutes, and 30 cycles of denaturation at 96 °C for 1 minute, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds following by final extension at 72 °C for 7 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative partial *lys 6-dh* gene fragment was harvested from the agarose gel by QIA Quick gel extraction kit (Appendix B). All PCR products were sequenced and compared with other sequences in Genbank database using BLAST program.

2.15 Inverse PCR amplification for the 5′-terminus and 3′-terminus Of the *lys 6-dh* gene

The inverse PCR is a rapid and reliable technique for obtaining sequences flanking a known region of DNA. The chromosomal DNA containing the sequence of target gene was digested, using a suitable restriction enzyme to produce a nucleotide restriction fragment containing the known target sequence flanked by two regions of unknown. Consequently, this DNA was re-ligated as circularized DNA. The re-ligated DNA is amplified by PCR. Since polymerase works more efficiently with linear than circular DNA, the circular fragments should be linearized before amplification by digestion with a restriction enzyme which cuts only one position between the two primers in the known target DNA sequence (Rosalind *et al.*, 1993). Normally, the inverse PCR was done for two times in which the PCR products from the first PCR using outer pair of primers was then used as templates for the second PCR, and the inner pair of primers was used to produce more specific products.

2.15.1 Templates for Inverse PCR amplification

5′ and 3′-terminal fragments of the *lys 6-dh* gene was isolated using inverse PCR from the partial sequence of the *lys 6-dh* gene obtained from 2.14.3. The chromosomal DNA was separately digested with 16 restriction enzymes; *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Cla*I, *Hin*dIII, *Kpn*I, *Mlu*I, *Pvu*II, *Sac*I, *Sca*I, *Ssp*I, *Stu*I, *Xba*I and *Xho*I. All of digested solutions were religated and linearized by *Fse*I, then used as the templates for inverse PCR amplification.

2.15.2 Primers design for Inverse PCR amplification

The primer pairs used for Inverse PCR were designed from the partial sequence of *lys 6-dh* gene obtained from 2.14.3.

2.15.3 PCR amplification of 5′ an 3′ end of *lys 6-dh* gene

The lys 6-*dh* gene was amplified using PCR method. Twenty five microliters reaction mixture contained 2.5 U of EX *Taq* DNA polymerase, 0.2 mM dNTPs, 1x PCR buffer, 2 mM $MgCl_2$, 500 ng DNA template from 2.16.1 and 10 pmole of each primer. The thermocycle consisted of predenaturation at 96 °C for 20 minutes, and 30 cycles of denaturation at 96 °C for 1 minute, annealing at 60 °C for 1 minute, extension at 72 °C for 3 minutes following by final extension at 72 °C for 7 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative *lys 6-dh* gene fragment was harvested from agarose gel by QIA Quick gel extraction kit. All PCR products were sequenced and compared both with other sequences in Genbank database and the partial *lys 6-dh* nucleotide fragment using the BLAST program.

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

2.16.1 Template preperation

Chromosomal DNA of *A. denitrificans* was prepared according to the method described in 2.14.1.

2.16.2 Primers design

From the nucleotide sequence of *lys 6-dh* gene, the primer pairs used for full length amplification of *lys 6-dh* gene of *A. denitrificans* were designed with two difference restriction sites for directional cloning into pET-17b with the same restriction sites. The sequence of forward primer (ADK-pETF) contained *Nde*I site (underline) whereas that of reverse primer (ADK-pETR) contained *Bam*HI site (underline).

2.16.3 PCR condition

The complete *lys* 6-*dh* gene was amplified using PCR method. Twenty five microliters reaction mixture contained 2.5 U of LA *Taq* DNA polymerase, 0.3 mM dNTPs, 1x PCR buffer, 2 mM $MgCl_2$, 500 ng DNA template and 10 pmole of each primer. The thermocycle consisted of was predenaturation at 96 °C for 10 minutes, and 30 cycles of denaturation at 96 °C for 1 minute, annealing at 65 °C for 1 minute, extension at 72 °C for 2 minutes following by final extension at 72 °C for 7 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative full length *lys 6-dh* gene fragment was recovered from agarose gel by QIA Quick gel extraction kit.

2.17 Recombinant DNA preparation

2.17.1 Vector DNA preparation (modified from Miniprep in Sambrook *et al.*, 1989)

The *Escherichia coli* BL21(DE3), which harboured pET-17b plasmid was grown in 5 ml LB medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.2) containing 100 μg/ml ampicillin at 37 °C for 16 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 and 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 10,000x*g* for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then, DNA solution was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25: 24: 1 V/V). The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol to the aqueous phase and then kept at -70°C for 2 hours. The DNA pellet was 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 DNase-free pancreatic RNase.

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

2.17.2 The *lys 6-dh* gene fragment preparation

The *lys 6-dh* gene fragment from 2.16.3 was digested with *Nde*I and *Bam*HI. The reaction mixture (1 μg of gene fragment, 1x *Bam*HI reaction buffer, 1x BSA solution, 2 U of *Nde*I and 2 U of *Bam*HI in total volume of 20 μl) was incubated at 37°C for 18 hours. The DNA fragment was harvested from agarose gel by QIA Quick gel extraction kit.

2.17.3 Ligation of vector DNA and the *lys 6-dh* gene fragment

The *Nde*I and *Bam*HI digested *lys 6-dh* gene fragment was ligated to the *Nde*I and *Bam*HI digested pET-17b vector at a molar ratio of 1: 5 (DNA vector: inserted DNA). The ligation mixture of 20 μl contained 50 ng of vector DNA, 250 ng of the gene fragment, 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 1 U of T_4 DNA ligase. The mixture was incubated overnight at 16°C. The recombinant plasmids in this reaction mixture were further used for transformation.

2.17.4 Transformation

2.17.4.1 Preparation of competent cells

A fresh overnight culture of *E. coli* BL21(DE3) was inoculated into 1 liter of LB medium with 1% inoculum size. Cells were grown to log phase at 37°C with vigorous shaking until OD_{600} was about 0.5. The culture was chilled on ice for 15 to 30 minutes and then centrifuged at 8,000x*g* for 15 minutes at 4 °C. The cells were washed with 1 liter of cold distilled water, spun down and washed again with 0.5 liter of cold distilled water. After centrifugation, the cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged at 8,000 *g* 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 (Sambrook *et al.*, 1989).

2.17.4.2 Electroporation

The recombinant plasmids from 2.17.3 were transformed into the competent cells of *E. coli* BL 21(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. One microliter 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 with one pulse of electric shock at the above setting. Subsequently, 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 shaking. Finally, this suspension was spreaded onto the LB agar plates containing 100 µg/ml ampicillin and incubated at 37 °C for 10 hours. Cells containing the recombinant plasmids which could grow on selective plate were picked and the plasmids were isolated.

2.18 Expression of the *lys 6-dh* gene

2.18.1 Recombinant plasmid characterization

The recombinant *E. coli* BL21(DE3) clones were grown in LB medium containing 100 µg/ml ampicillin at 37 °C for 16 hours with shaking. The cell cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 8,000x*g* for 2 minutes. Then, the plasmid from individual clone was extracted as described in 2.17.1. After that, the plasmids DNA were completely digested with *Nde*I and *Bam*HI. The size of recombinant plasmids and the inserted DNA were estimated by

submerged agarose gel electrophoresis compared with the λ/*Hin*dIII marker. Finally, the inserted DNA fragments in the recombinant plasmids were confirmed to be *lys 6-dh* gene by sequencing.

2.18.2 Crude extract preparation

The *E. coli* BL21(DE3) transformants were grown overnight at 37 °C in 5 ml of LB medium containing 100 μg/ml ampicillin. After that, 1.0 % of the cell culture was inoculated into 100 ml of the same medium and was cultured at 37 °C with shaking. When the turbidity at 600 nm had reached 0.5, IPTG was added to the final concentration of 0.2 mM to induce *lys 6-dh* gene expression, and cutivation was continued at 37 °C for 4 hours. The cells were harvested by centrifugation at 10,000x*g* for 10 minutes, and 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 was stored at –80 °C until used for crude extract preperation.

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) then disrupting cells by sonication on ice. Unbroken cells and cell debris were removed by centrifugation at 17,500x*g* for 30 minutes. The supernatant was collected for enzyme activity assay and protein concentration.

2.18.3 Enzyme activity assay

The activity of Lys 6-DH in the recombinant clones was determined by the method described in 2.8.

2.18.4 Protein determination

Protein concentration of Lys 6-DH from the recombinant clones was determined by Lowry's method as described in 2.9.

2.19 Optimization for the *lys 6-dh* gene expression

The transformants of *E. coli* BL21(DE3) were grown overnight at 37 °C in 2 ml of LB medium containing 100 μg/ml ampicillin. After that, 1.0 % of the cell culture was inoculated into 100 ml of the same medium and was cultured at 37 °C with shaking at 250 rpm. When the turbidity at 600 nm had reached 0.5, the transformant was induced by IPTG at the final concentration of 0, 0.1, 0.2, 0.4 and 0.8 mM. At various times after induction (0, 1, 2, 4, 6, 8, 16, and 24 hours), the cells were harvested by centrifugation at 8,000x*g* for 10 minutes, and then were washed with cold 0.85 % NaCl. Then, the cell pellet was washed once in cold extraction buffer and centrifuged again. The cell pellet was stored at -20°C until used. For crude extract preparation, 1 g of the cell pellet was resuspended in 2 ml of cold extraction buffer and then disrupted on ice by sonication. Unbroken cell and cell debris were removed by centrifugation at 8,000x*g* for 30 minutes. The supernatant was stored at 4°C for enzyme activity assay and protein determination.

2.19.1 Protein patterns of cells and crude extracts

One point five milliliter of cell culture were harvested at various time (0, 1, 2, 4, 6, 8, 16, and 24 hours after induction) by centrifugation. The cell pellets were resuspended in 100 μl of 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (v/v) bromophenol blue). Ten micro liter of cell samples and 20 □g proteins of crude extracts were used for the protein pattern determination by SDS-PAGE as described in 2.12.2.

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

The transformant was daily subcultured by streaking on LB agar plate containing 100 μg/ml ampicillin for 80 days. Then the 1, 10, 20, 30, 40, 50, 60, 70 and 80 subcultured were picked up and then culture in the optimum condition obtained from 2.19 and assayed for enzyme activity and determined protein concentration.

2.21 Purification of Lys 6-DH from recombinant clone

2.21.1 Preparation of crude extract

The transformant was grown in 600 ml of LB medium at the optimum condition obtained from 2.19. The cell cultivation, crude extract preparation, enzyme activity and protein concentration were carried out.

2.21.2 Enzyme purification steps

The crude extract from 2.21.1 was purified by the following steps. All operations were carried out at 4 °C. The buffer used in all steps was 10 mM potassium phosphate buffer, pH 7.5 containing 0.01 % β-mercaptoethanol and 1 mM EDTA.

2.21.2.1 DEAE-Toyopearl 650 M column chromatography

The 10 ml of crude enzyme (880 mg proteins) was loaded into column followed by elution with the same buffer for 5-10 column volume at a flow rate of 0.5 ml/min. The unbound proteins were eluted from the column with the buffer. After that, the bounded proteins were eluted from the column with 0.01 M KPB containing linear salt gradient of 0 to 0.15 M KCl. The fractions of 5 ml were

collected by fraction collector. The protein elution profile was monitored by measuring the absorbance at 280 nm and the enzyme activity was determined as described in 2.8. The KCl concentration was investigated by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against the buffer before determination of the enzyme activity and protein concentration.

2.21.2.2 DEAE-Sephadex A50 column chromatography

DEAE-Sephadex A50 was resuspended in the 0.01 M KPB and packed into a column (15 x 1.5 cm) followed by equilibrating with the same buffer for 5-10 column volumes at a flow rate of 0.5 ml/min. The protein solution (459 mg proteins) from 2.21.2.1 was applied into the column. The unbound proteins were eluted from the column with 0.01 M KPB, and the column was washed until the absorbance at 280 nm of eluent decreased to base line value. After that, the bound proteins were eluted from the column with 0.01 M KPB containing linear salt gradient of 0 to 0.5 M KCl. The fractions of 5 ml were collected by fraction collector. The elution profile was monitored for protein by measuring the absorbance at 280 nm and the enzyme activity was determined as described in 2.8. The KCl concentration was investigated by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against the KPB buffer before determination of the enzyme activity and protein concentration.

2.22 Determination of enzyme purity by SDS and Native-PAGE

The proteins from each step of purification were analyzed for enzyme purity by SDS-PAGE to determine the denature protein as described in 2.12.1 and native PAGE for native protein pattern as described below.

2.22.1 Non-denaturing gel electrophoresis

Discontinuous PAGE was performed on the slab gel of a 7.7 % separating 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 proteins were mixed with 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50 % glycerol and 0.05 % bromophenol blue) by a ratio of 5: 1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at a constant current (30mA). For activity staining, the experiment was conducted at 4 °C. After electrophoresis, the gel was developed by coomassie blue staining and enzyme activity staining.

2.22.2 Enzyme activity staining

After electrophoresis at 4 °C, the gel was transferred to a small box containing activity staining solution (4.25 mM of Tris-HCl, pH 8.5, 40 μM of L-lysine, 50 μmol of NAD^+, 25 μg/ml of phenazine methosulfate and 250 μg/ml of nitroblue tetrazolium) for 5 minutes at room temperature and then quickly rinsed several times with ionized water to stop the reaction.

2.23 Characterization of Lys 6-DH

2.23.1 Molecular weight determination of Lys 6-DH

Molecular weight of the purified Lys 6-DH was determined by gel filtration on TSK Gel G3000 SW column (0.75 x 60 cm) with 0.1 M potassium phosphate buffer, pH 7.0 containing 150 mM NaCl at a flow rate of 1 ml/min. The molecular weight marker proteins consisted of glutamate dehydrogenase (290,000 Da), lactate dehydrogenase (142,000 Da), enolase (67,000 Da), myokinase (32,000 Da) and cytochrome c (12,400 Da) were used.

2.23.2 Substrate specificity of Lys 6-DH

The ability of the enzyme to catalyse the oxidative deamination of various amino acids and L-lysine analogs was determined at a final substrate (L-lysine) concentration of 20 mM. Substrate, L-lysine, was replaced by various amino acids and L-lysine analogs for the substrate specificity test. The enzyme activity of oxidative deamination was determined as described in 2.8. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100 %.

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

The ability of the enzyme to catalyse the oxidative deamination of L-lysine combining with various amino acids and L-lysine analogs was determined at a final substrate (L-lysine) concentration of 20 mM. Substrate, L-lysine was combined with various amino acids and L-lysine analogs to determine the inhibitory effect of binding site of substrate and enzyme. The enzyme activity of oxidative deamination was determined as described in 2.8. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100 %.

2.23.4 Coenzyme specificity of Lys 6-DH

The purified Lys 6-DH was used to study coenzyme specificity. NAD^+ was replaced by various NAD^+ analogs at a final concentration of 2 mM for oxidative deamination. Assays with NAD^+ analogs were conducted by measuring the increase in absorbance of various compounds at the following wavelengths: 3-acetylpyridine adenine dinucleotide, 363 nm (ε = 9.1 x 10^3 $M^{-1}.cm^{-1}$); β-nicotinamide adenine dinucleotide phosphate (NADP), 340 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$); nicotinic acid adenine dinucleotide, 338 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$); 3-pyridinealdehyde adenine dinucleotide, 358 nm (ε = 9.3 x 10^3 $M^{-1}.cm^{-1}$); nicotinamide 1, N^6- ethenoadenine dinucleotide, 334 nm (ε = 6.9 x 10^3 $M^{-1}.cm^{-1}$); nicotinamide hypoxanthine dinucleotide, 338 nm (ε = 6.2 x 10^3 $M^{-1}.cm^{-1}$) and thionicotinamide adenine dinucleotide, 395 nm (ε = 11.3 x 10^3 $M^{-1}.cm^{-1}$) (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.

2.23.5 Effect of pH on Lys 6-DH activity

The effect of pH on the Lys 6-DH activity was determined under the standard assay conditions for the oxidative deamination as described in 2.8 but at various pHs. The following buffers were used; 0.2 M 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 12.0 were used. The pH of each reaction mixture was measured with a pH meter at 30 °C after the reaction was ended. The result was expressed as a percentage of the relative activity. The maximum activity was set as 100%. The percentage of relative activity was plotted against the final pH.

2.23.6 Effect of pH on Lys 6-DH stability

The purified Lys 6-DH was used to study pH stability. After the enzyme had been incubated at 30°C for 10 minutes in each of the 0.1 M buffer at various pHs, an aliquot of the enzyme solution was withdrawn and the remaining activity of the enzyme was measured under the standard assay condition for the oxidative deamination as described in 2.8. The used 0.01 M buffers were potassium phosphate for pH 6.0 to 8.0 and Tris-HCl buffer for pH 6.5 to 8.5 and glycine-KCl-KOH buffer for pH 7.5 to 10.0. The percentage of Lys 6-DH relative activity was plotted against the incubated pH.

2.23.7 Effect of temperature on Lys 6-DH activity

The effect of temperature on the Lys 6-DH activity was examined. The purified enzyme was determined for its activity as described in 2.8 but at various temperatures of 30-60 °C. The result was expressed as a percentage of the relative

activity. The maximum activity was set as 100%. The percentage of relative activity was plotted against the temperature.

2.23.8 Effect of temperature on Lys 6-DH stability

The effect of temperature on the stability of the enzyme was determined at 30°C. The purified Lys 6-DH was incubated at 30°C and then the enzyme activity was collected to assay enzyme activity every day as described in 2.8. The result was expressed a percentage of the relative activity. The highest activity was defined as 100%. The percentage of relative activity was plotted against the temperature.

2.23.9 Kinetic parameter studies of Lys 6-DH

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

2.23.9.1 Initial velocity analysis for the dimeric form

Initial velocity studies for the dimeric form reactions were carried out under the standard reaction condition as described in 2.8, except that various amounts of L-lysine and NAD^+ were used. The concentrations of L-lysine used in the reaction mixture were 4, 6, 8, 12 and 16 mM, and those of NAD^+ were 0.08, 0.15, 0.3 and 0.6 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-lysine concentrations at a series of fixed concentrations of NAD^+ and the secondary plots of y intercepts against reciprocal concentrations of NAD^+ were made. K_m values of L-lysine and NAD^+ were calculated from these two plots, respectively.

2.23.9.2 Initial velocity analysis for the hexameric form

Initial velocity studies for the hexameric form were carried out. The enzyme was preincubated with 10 mM of L-lysine before starting the reaction as

described in 2.8. Various amounts of L-lysine and NAD^+ were used. The concentrations of L-lysine used in the reaction mixture were 4, 6, 8, 12 and 16 mM, and those of NAD^+ were 0.08, 0.15, 0.3 and 0.6 mM. The Lineweaver-Burke plots (double-reciprocal plots) of initial velocities against L-lysine concentrations at a series of fixed concentrations of NAD^+ and the secondary plots of y intercepts against reciprocal concentrations of NAD^+ were made. K_m values of L-lysine and NAD^+ were calculated from these two plots, respectively.

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

2.24.1 Template preperation

Chromosomal DNA of *Bacillus cereus* ATCC 11778 was prepared according to the method described in 2.14.1. One microliter (about 500 ng) of the DNA solution was used as template in each PCR reaction mixture.

2.24.2 Primers design

The primer pairs used for full length amplification of *B. cereus* ATCC 11778 *p5cr* gene were designed from 5′ and 3′ of *p5cr* gene of *B. cereus* ATCC 10987 from the genetic data bank. Forward primer contained *Nde*I site (underline) and reverse primer contained *Eco*RI site (underline).

2.24.3 PCR amplification of *p5cr* gene

The *p5cr* gene was amplified using PCR method. Twenty five microliters reaction mixture contained 2.5 U of LA *Taq* DNA polymerase, 0.3 mM dNTPs, 1x PCR buffer (100 mM Tris-HCl, pH 8.8 , 500 mM KCl and 1 % Triton X-100), 2 mM $MgCl_2$, 500 ng DNA template and 10 pmole of each primer. The thermocycle

consisted of predenaturation at 96 °C for 10 minutes, and 30 cycles of denaturation at 96 °C for 1 minute, annealing at 65 °C for 1 minute, extension at 72 °C for 2 minutes following by final extension at 72 °C for 7 minutes. The PCR products were electrophoresed through agarose gel. Finally, the *p5cr* gene fragment was recovered from agarose gel by QIA Quick gel extraction kit.

2.24.4 Recombinant DNA preparation

2.24.4.1 Vector DNA preparation

The expression vector pET-17b from 2.17.1 was linearized with *Nde*I and *Eco*RI. The reaction mixture containing 1 µg pET-17b, 1x *Eco*RI reaction buffer, 2 U of *Nde*I and 2 U of *Eco*RI in total volume of 20 µl was incubated at 37 °C for 18 hours. The linear-formed pET-17b was recovered from agarose gel by QIA Quick gel extraction kit.

2.24.4.2 The *p5cr* gene fragment preparation

The *p5cr* gene fragment from 2.24.3 was digested with *Nde*I and *Eco*RI. The reaction mixture containing 1 µg of gene fragment, 1x *Eco*RI reaction buffer, 2 U of *Nde*I and 2 U of *Eco*RI in total volume of 20 µl, was incubated at 37°C for 18 hours. The DNA fragment was recovered from agarose gel by QIA Quick gel extraction kit.

2.24.4.3 Ligation of vector DNA and the gene fragment

The gene fragment (2.24.4.2) was ligated to the pET-17b vector (2.24.4.1) at a molar ratio of 1:5 (vector: insert). The ligation mixture of 20 µl containing 50 ng of vector DNA, 250 ng of the gene fragment, 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 1 U of T_4 DNA ligase was incubated overnight at 16°C. The recombinant plasmids in this reaction mixture were further used for transformation.

2.24.5 Transformation

2.24.5.1 Preparation of competent cells

The competent cell was prepared as described in 2.17.4.1.

2.24.5.2 Electroporation

The recombinant plasmid was introduced into *E.coli* (BL21) DE3 as described in 2.17.4.2.

2.25 Expression of the *p5cr* gene

2.25.1 Recombinant plasmid characterization

The recombinant plasmids were extracted from the recombinant clones by the procedure described in 2.18.1. After that, the plasmids were completely digested with *Nde*I and *Eco*RI. The size of recombinant plasmids was estimated by submerged agarose gel electrophoresis compared with λ/*Hin*dIII marker. Finally, the inserted DNA fragment was confirmed to be the *p5cr* gene by sequencing.

2.25.2 Crude extract preparation

The P5CR crude enzyme was prepared by the procedure described in 2.18.2.

2.25.3 P5CR activity assay

The activity of P5CR with proline as the substrate was spectrophotometrically assayed by measuring the amount of NADH produced in one minutes. One ml of reaction mixture contained 200 mM of glycine-KCl-KOH buffer, pH 10.0, 20 mM of L-proline, water and the enzyme. This reaction mixture was incubated at 30 °C for 5 minutes in an 1-cm light path cuvette. The reaction was started by addition of 2 μmol of NAD^+ and the initial reduction of NAD^+ to NADH, was monitored by measuring the absorbance at 340 nm.

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

2.25.4 Protein determination

Protein concentration was determined by the method of Lowry *et. al.* (1956) as described in 2.9.

2.26 Optimization of the *p5cr* gene expression

Optimization of the *p5cr* gene expression was performed by the same method in 2.19. The enzyme activity and protein concentration were determined as described in 2.25.3 and 2.9, respectively.

2.26.1 Protein patterns of cells and crude extracts

One point five milliliter of cell culture were harvested at various time (0, 1, 2, 4, 6, 8, 16, and 24 hours after induction) by centrifugation. The cell pellets were resuspended in 100 μl of 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (v/v) bromophenol blue). Ten micro liter of cell samples and 20 μg

proteins of crude extracts were used for the protein pattern determination by SDS-PAGE as described in 2.12.

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

2.27.1 Construction of pET-ADK-P5CR

For construction of pET-ADK-P5CR (Figure 2.3), the whole *p5cr* gene with T7 promoter and Shine-Dargano sequence was isolated from pET-P5CR by digestion with *Bgl*II and *Eco*RI. This fragment was inserted downstream of *lys 6-dh* gene at *Bam*HI and *Eco*RI of pET-ADK. After that, the heterologous recombinant plasmid was transformed into *E. coli* BL21(DE3) by electroporation.

2.27.2 Recombinant plasmid characterization

Recombinant plasmid containing the heterologous genes was extracted form *E. coli* by the procedure described in 2.18.1. After that, the plasmid was completely digested with *Nde*I and *Eco*RI. The size of recombinant plasmid and the inserted DNA fragment was estimated by submerged agarose gel electrophoresis compared with λ/*Hin*dIII marker. Finally, sequence of the heterologous genes in the recombinant plasmid was confirmed by sequencing.

2.27.3 Crude extract preparation

Crude enzymes of the heterologous genes were prepared by the procedure described in 2.18.2.

2.27.4 Enzyme activity assay

Crude enzyme activity of Lys 6-DH and P5CR were measured as described in 2.8 and 2.25.3, respectively.

2.27.5 Protein determination

Protein concentration was determined by the method of Lowry *et al.*, (1956) as described in 2.9.

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

Optimization of the heterologous genes expression was performed by the same method described in 2.19. The two enzyme activities and protein concentration were determined as described in 2.8, 2.25.3 and 2.9, respectively.

2.28.1 Protein patterns of cells and crude extracts

One point five milliliter of cell culture were harvested at various time (0, 30 min., 1, 2, 3, 4, 6 and 8 hours after induction) by centrifugation. The cell pellets were resuspended in 100 µl of 5x sample buffer (312.5 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 1% (v/v) bromophenol blue). Ten micro liter of cell samples and 20 µg proteins of crude extracts were used for the protein pattern determination by SDS-PAGE as described in 2.12.

2.29 Preliminary production of L-pipecolic acid

2.29.1 L-pipecolic acid production in *E.coli* containing pET-ADK-P5CR

The *E. coli* BL21 (DE3) harboring pET-ADK-P5CR was determined for its ability to biotransform L-lysine into L-pipecolic acid. Ten organic solvents, hexane, methanol, ethanol, pyridine, xylene, toluene, isopropyl, acetone, chloroform and benzene, were used to increase permeability of the cell membrane. After *E. coli* transformant was grown in LB medium, the expression of both Lys 6-DH and P5CR was induced using optimum condition obtained from 2.28. Thirty milligram of cell pellet each was treated separately in 100 µl of various organic solvents for variable period of time (5, 10, 15, 20, 30, 45 and 60 minutes). Water was used as a control. After the treatment, cells were collected by centrifugation and the organic solvents were removed. Each 500 µl of biotransformation mixture containing 200 mM L-lysine, 200 mM Glycine-KCl-KOH (pH 9.5), distilled water and each organic solvent treated cell was shaken at 250 rpm for 24 hours at 30 °C. Then, L-lysine and L-pipecolic acid from all samples were detected by TLC (Nelson and Cox, 2000).

2.29.2 Effect of pH on production of L-pipecolic acid in *E.coli* containing pET-ADK-P5CR

The *E. coli* BL21 (DE3) harboring pET-ADK-P5CR was determined for the L-pipecolic acid production in various pHs. After *E. coli* transformant was grown in LB medium, the expression of both Lys 6-DH and P5CR was induced using optimum condition obtained from 2.28. Thirty milligram of cell pellet was treated in the 100 µl of xylene and distilled water for 5 minutes. Non-treated cells were used as control. Treated cells were collected by centrifugation and the organic solvent was removed. The biotransformation was carried out as described in 2.29.1 but with various buffers (distilled water, glycine-KCl-KOH (pH 9.5), KPB (pH 7.0), Tris-HCl (pH 7.0) and

Tris-HCl (pH 9.0) at the same concentration (200 mM) as the standard buffer used for L-pipecolic acid production in *E. coli* BL21(DE3) harbouring pET-ADK. After 24 hours, L-lysine and L-pipecolic acid from all samples were detected by TLC (Nelson and Cox, 2000).

2.29.3 Optimization of L-pipecolic acid production in *E.coli* containing pET-ADK-P5CR

The *E. coli* BL21 (DE3) harboring pET-ADK-P5CR was used to determine the optimal condition for production of L-pipecolic acid. Xylene was used to increase permeability of the cell membrane with various treated times. The 500 µl of biotransformation mixture containing 200 mM L-lysine, 200 mM Tris-HCl (pH 9.0) and 30 mg of each xylene treated cell was shaken at 30 °C for 48 h. The supernatants were collected in the interval time for 0, 1, 2, 4, 6, 8, 12, 16, 24, 30, 36, 42 and 48 hours. Then L-lysine and L-pipecolic acid from all samples were detected on TLC (Nelson and Cox, 2000) and their concentrations were determined by ELSD-HPLC (Michelle *et al.*, 2000).

2.29.4 Determination of L-lysine and L-pipecolic acid

2.29.4.1 Analysis of L-lysine and L-pipecolic acid by TLC (Nelson and Cox, 2000)

The biotransformation mixtures were preliminarily analyzed by TLC. The 0.5 µl of the mixtures and commercial L-lysine and L-pipecolic acid standards were subjected to 10 cm x 10 cm cellulose TLC plastic sheets (MERCK, Germany) in parallel with standard. The TLC plates were developed with *n*-butanol: acetic acid: H_2O (4:1:1) and after that the plates were dried in hot air. The detection was carried out by dipping the plate in 0.5% ninhydrin in acetone solution and then drying at 110 °C for 5 minutes. Each spot of the samples and standards, which gave

high intensity in time course study, were quantified by Gel Document. The ratio of the distance a compound moves from the baseline to the distance of the solvent front from the baseline is defined as the retardation factor (R_f)

2.29.4.2 Quantity deterination of L-lysine and L-pipecolic acid by ELSD-HPLC (Michelle *et al.*, 2000)

Selective samples of L-pipecolic acid produced in various times (0 to 24 hours) and standards were subjected to HPLC using Prevail C18 (250 x 4.6 cm) column and evaporative light scattering detector (ELSD). The solvent system of 0.1% trifluoroacetic acid in H_2O (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) were used. The ultrafiltrated supernatants or standards were injected into the column equilibrated with solvent A at a flow of rate 0.6 ml/min. The gradient used was 0% solvent B for 10 min, following by 0-60% solvent B for 5 minutes and then it was hold at 60% solvent B. The yield of the enzyme reaction is defined as mole of L-pipecolic acid per mole of L-lysine.