CHAPTER IV

DISCUSSION

4.1 Identification of Lys 6-DH producing bacterium by morphological, biochemical and 16S rRNA gene properties

The historical method for performing bacteria classification was dependent on the comparison of an accurate morphologic and phenotypic description of type strains or typical strains with the accurate morphologic and phenotypic description of the isolate to be identified. The standard references such as *Bergey's Manual of Systematic Bacteriology* or the *Manual of Clinical Microbiology* or compiling results from well-characterized strains such as those found at the Centers for Disease Control and Prevention or the American Type Culture Collection (ATCC) would publish tables summarizing the characteristics of each species of bacteria (Funke *et al.,* 1997, Krieg *et al.,* 1984, Murray *et al.,* 1999).

The molecular approach has been used for bacterial phylogeny and is of major importance for species definition and identification (Clarridge, 2004, Fredricks and Relman, 1996, Raoult *et al.*, 2004 and Rossello-Mora and Amann, 2001). The part of the DNA which now most commonly uses for taxonomic purposes of bacteria is the 16S rRNA gene (Bottger, 1989, Garrity and Holt, 2001, Harmsen, and Karch, 2004, Kolbert, and Persing, 1999, Palys *et al.,* 1997 and Tortoli, 2003). 16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources. Identification based on the 16S rDNA sequence is of interest because ribosomal small subunit exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species level by comparison with databases in the public domain (Vandamme *et al.*, 1996).

The isolated soil bacteria strain K-1 showed Lys 6-DH activity. By using biochemical test and 1.5 kb of the 16S rRNA gene amplification, this strain showed similarity of 98% to that of *Alcaligenes* sp. (Ruger and Tan, 1983). However, in 2003 Coenye and coworkers reclassified *Alcaligenes* sp. to be *Achromobacter* sp. using several techniques including SDS-PAGE of whole-cell proteins, 16S rRNA gene sequencing, DNA–DNA hybridization experiments, determination of DNA base composition, fatty acid methyl ester analysis, phenotypic characterization, and antimicrobial susceptibility testing. From these data, this bacteria strain K-1 was identified to be *Achromobacter denitrificans* K-1.

4.2 Purification of Lys 6-DH from *Achromobactor denitrificans* K-1

The culture conditions for Lys 6-DH production by *A. denitrificans* K-1 were investigated. The enzyme production was induced by the addition of L-lysine into a peptone medium. The highest specific and total activities were obtained by cultivating the cells in a 1.0% peptone medium containing 1.0% L-lysine at 30°C for 20 hours on a reciprocal shaker.

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intened application (Queiroz *et al.*, 2001).

The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility, density and the possession of specific binding sites. Most purification protocols required more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriately

techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham pharmacia biotech, 1999).

The first step in the purification of a protein is the preparation of an extract containing the protein in a soluble form and extraction procedures should be selected according to the source of the protein (Amersham pharmacia biotech, 1999 and Eisenthal and Danson, 1992). In this work, Lys 6-DH, an intracellular enzyme, was extracted from *A. denitrificans* K-1. Mechanical disruption methods are usually necessary to break down cell wall in order to release intracellular protein prior to purification. The cell disintegration technique involved cell lysis by ultrasonication or high pressure sound waves, which causes cell breakage by cavitations and shear forces, so in this work, cell wall was disrupted by ultrasonication. However, several potential problems may be consequent on disruption, due to the destruction of intracellular compartmentation. Lys 6-DH activity can be lost by various reasons. It is essential to consider strategies for protection of the enzyme activity.

First of all, since the disruption of cells results in the release of proteases from subcellular compartment (Cooper, 1977), the effect of the free proteases must be eliminated. In this work, phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were used in the extraction buffer as serine protease inhibitor, and metalloprotease inhibitor, respectively. These reagents protect the desired protein from the degradation of proteolytic enzyme. In addition, a reagent containing a thiol group such as β-mercaptoethanol and also a chelating agent such as EDTA to chelate metal ions in the extraction buffer will minimize the oxidation damage (Bollag *et al.*, 1996). However, acid proteases in cell extracts were not inhibited at all. Acid proteases may not affect Lys 6-DH activity because their reactions occurred only in low pH environment. Furthermore, mechanical cell disruption may cause local overheating with consequent denaturation of protein. To maximize recovery of active enzyme, the equipments and sonication were, therefore, pre-chilled and several pauses of disruption used instead of one long continuous sonication because short interval of

disruption will also minimize foaming and shearing (Harris and Angal, 1989 and Janson and Ryden, 1998).

Solubility differences in salt are frequently exploited to separate proteins in the early stages of purification protocols. Ammonium sulfate was the salt of choice and was used in this work because it combined many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and low price (Bollag *et al.*, 1996 and Creighton, 1993). Preliminary experiment on ammonium sulfate precipitation was also done in this work. Lys 6-DH from *A. denitrificans* K-1 was precipitated by 0-60% saturated ammonium sulfate. In this step, although 46% of protein was removed, less of enzyme activity was lost (97.1% recovery from crude enzyme). Most purification protocols involve some types of chromatography, which has become an essential tool in protein purification. Ion exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity. The difference in charge properties of protein is often considerable. Ion exchange chromatography (IEC) is capable of separating species with very minor differences in properties, such as two proteins differing by only one amino acid. It is a very powerful separation technique indeed (Amersham pharmacia biotech, 1999).

Ion exchange chromatography separates proteins with differences in charge. The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Proteins bind as they are loaded onto a column. Conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by increases in salt concentration or changes in pH.

Most commonly, samples are eluted with salt (NaCl), using a stepwise or continuous gradient elution (Amersham pharmacia biotech, 1999). In this work, DEAE-Toyopearl 650M is an anion exchanger which has negatively charged counter-ions was used. This column matrix is a synthesis polymer containing bound cationic groups. The elution of Lys 6-DH with stepwise 0.05 M KCl indicated that the net charge of this enzyme is negative (anion group) at buffer pH 7.4. Its popularity comes

from the possibility of high resolving power, versatility, reproducibility and ease of performance. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of Lys 6-DH activity compared to the amount of proteins removed.

The 87% of the protein in the step of 0-60% saturated ammonium sulfate precipitation was eliminated. The 82.2% recovery and the purity of the enzyme was increased to 11.4 folds this might be due to the removal of some other proteins. After DEAE-toyopearl column, in order to decrease the volume of enzyme solution and also eliminate the unwanted contaminant proteins, the next step of purification was precipitation by 0-40% ammonium sulfate. This step of purification contributed less loss of Lys 6-DH activity compared to the amount of proteins removed. About 80% of the protein from the step of DEAE-Toyopearl 650 M was eliminated with about 77.8% recovery and the purity of the enzyme was increased to 52 fold.

The next purification step was separation by size exclusion chromatography (SEC) which is a chromatographic method in that particles are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. When an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography. The main application of gel filtration chromatography is the fractionation of proteins and others. When an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography (Amersham pharmacia biotech, 1999).

By using Sephadex G-150, less loss of Lys 6-DH activity compared to the amount of proteins removed was found. However, only 6.2% of the protein in the step of 0-40% ammonium sulfate precipitate was eliminated (73.4% recovery) and the purity of the enzyme was not increased (52.6 fold) from the previous step (0-40% ammonium sulfate precipitation). This should indicate that the size of other proteins contaminated were not different from those of Lys 6-DH therefore they can not

separated by this column. It is not necessary to use this column in this purification process.

The strong ion-exchanger column Mono Q was used in the next step of purification. This column is also anion exchanger but has stronger negatively charged counter-ions than DEAE-toyopearl column. Consequent upon the result, this column contributed greatly to the purification procedures, with less loss of Lys 6-DH activity compared to the amount of proteins removed. About 68% of the protein in the step of Sephadex G-150 gel filtration was eliminated with 43% recovery and the purity of the enzyme was increased to 98 folds.

The last step of Lys 6-DH purification was the hydrophobic interaction chromatography (HIC). HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of reversible hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar regions on the surface of proteins (Queiroz *et al.*, 2001). Phenyl sepharose, of which phenyl groups are chemically bonded on the surface of hydrophilic resin was used. Adsorption of proteins to a HIC adsorbent is favored by a high salt concentration in the mobile phase. The elution of solute is accomplished by decreasing the salt concentration with increasing hydrophobic. In this work, the enzyme was eluted from Phenyl sepharose column by using water. Lys 6-DH was eluted by distilled water that might be the concentration of 20% ammonium sulfate was so high that cause Lys 6-DH tightly bound with the column. This step should be improved by decrease the concentration of ammonium sulfate to be lower than 20%. This might make Lys 6-DH elute easier from this column. This step, the unwanted proteins which were 56% of the protein obtained in Mono Q step was removed. The recovery was 31% and the purity of the enzyme was increased to 175 fold. The specific activity of purified enzyme was 5.6 units/mg protein which was the same of those purified Lys 6-DHs from *A. denitrificans* K-1 (Misono *et al.*, 1989) and *G. stearothermophilus* (Heydari *et al.*, 2004) which were 4.54 and 6.2 units/mg protein, respectively. From all these results, it can be concluded that Lys 6-DH from *Achromobacter denitrificans* K-1 was purified to 175 fold with a 34% yield from the crude extract by a following

procedures involving 0-60% ammonium sulfate precipitation, DEAE-Toyopearl 650 M, 0-40% ammonium sulfate precipitation, Sephadex G-150 column, Mono Q HR 10/10 column and Phenyl Sepharose XK 16 column, respectively.

4.3 Amino acid sequences of Lys 6-DH

Polymerase chain reaction (PCR) is the popular method for *in vitro* synthesis of the interesting gene for cloning. This technique depends on two primers, which are specific to interesting gene. Thus, nucleotide sequence from deduced amino acid sequence of Lys 6-DH was investigated. Lys 6-DH was partially digested with lysyl endopeptidase, the enzyme which cleaves peptide bond between lysine and its next amino acid residue on the C-terminal side. The digested peptides were further separated by reversed-phase high-performance liquid chromatography (HPLC). Then, the amino acid sequences of isolated peptides were determined by automated Edman degradation amino acid sequencer. The sequence at the N-terminus (SRTQHAITVLGAGK) and 4 internal peptide fragments (ARNVDYK, VPPME GYETFTLDGVEYEAFNTSG, GLGTLPPTLEG and VPPMEGYETF) of the enzyme were obtained. From this data, the sets of degenerated primers for amplification of various regions in *lys 6-dh* were designed. Then, PCR amplification of internal *lys 6-dh* gene could be performed.

4.4 Nucleotide sequencing of Lys 6-DH gene

For internal gene fragment amplification, those three restriction enzyme digestion fragments were tested as template using forward primers; EDF1, EDF2 or KN2 coorperated with reverse primers; EDR1, EDR2 or C1 in PCR reaction. The result showed that all restriction enzyme digested fragments gave strong specific products having the size of about 600 bp when using forward primer KN2 and reverse primer C1. Later on, the desired specific band was recovered from gel and sequenced. Once the nucleotide sequence of this amplified fragment had been analyzed, the inner part of gene fragment was obtained and the restriction sites on this fragment could be analyzed.

It is known that the restriction enzymes used for template preparation must not have their sites on the gene otherwise the amplification of this desired gene fragment cannot be occurred. It was implied that *Bam*HI, *Eco*RI and *Hin*dIII restriction sites were not on this internal *lys 6-dh* gene fragment. Since the nucleotide sequence of internal *lys 6-dh* gene fragment from primer KN2 and C1 had been identified. Using Genetyx version 6.0 program, we found the other restriction enzymes including *Apa*I, *Bgl*II, *Eco*RV, *Cla*I, *Kpn*I, *Mlu*I, *Pvu*II, *Sac*I, *Sca*I, *Ssp*I, *Stu*I, *Xba*I and *Xho*I that were also not on *lys 6-dh* gene fragment. These restriction enzymes were used for the 5′-terminal and 3′-terminal gene fragments amplification by inverse PCR.

In this experiment, the strong specific PCR product bands of 5′-terminus of *lys 6-dh* gene (the upstream sequence of *lys 6-dh* gene fragment containing the promoter of this gene) and 3′-terminus of *lys 6-dh* gene (the downstream sequence of *lys 6-dh* gene fragment containing the stop codon of this gene) by EDF1 and EDR1 primers was obtained from the *A. denitrificans* K-1 chromosomal digested with *Stu*I and *Xho*I, respectively. From inverse PCR data, whole nucleotide sequence of 1,382 bp containing one open reading frame of *lys 6-dh* gene which was 1,107 nucleotides and it was encoding a polypeptide of 368 amino acids. The molecular weight of enzyme subunit calculated from deduced amino acid sequence was 39.3 kDa using Genetyx 6.0 program.

The *lys 6-dh* gene was deposited in the EMBL-GenBank-DDBL database in the accession no. DQ165182. The nucleotide sequence was compared with those of the DNA sequences in the EMBL-GenBank-DDBL database. The percentages of sequence similarity between the nucleotide sequences of *lys 6-dh* gene from *A. denitrificans* K-1 and each of those from *Rhizobium leguminosarum*, *Legionella pneumophila,* and *Geobacillus kaustophilus* were 46, 20 and 19%, respectively. When the deduced amino acid sequence of the whole Lys 6-DH was compared to those of the EMBL-GenBank-DDBL database, it was found that the percentages of sequence similarity between the deduced amino acid sequences of Lys 6-DH from *A. denitrificans* K-1 and each of those from *Legionella pneumophila, Rhizobium leguminosarum, Geobacillus stearothermophilus*, *Geobacillus kaustophilus*,

Geobacillus thermodenitrificans NG80-2 and *Oceanobacillus iheyensis* HTE831 were 38, 37, 24, 24, 23 and 18%, respectively. It might be due to the variety of microorganism that produced Lys 6-DH enzyme.

Although the low percentage of amino acid sequence similarity among those enzymes, the conserved glycine-rich NAD^+-binding region with G-X-G-X-X-(G or A), where X was any amino acids, has been found at the N-terminal region of Lys 6-DH from *A. denitrificans* K-1 (amino acid position 12 to 17 in Figure 3.19) (Rik *et al.*, 1986). In contrast, other amino acid dehydrogenase such as PheDH, the NAD^+-binding region was found at the C-terminus of *Bacillus badius* IAM 11059 and *Bacillus lentus* (Yamada *et al.*, 1995 and Thongchuang, 2006.). This indicated the difference of tertiary structure of this enzyme among this group.

4.5 Cloning, expression and stability of *lys 6-dh* gene

A number of researchers attempted to clone *lys 6-dh* gene for many purposes such as to produce enzyme in large amount in order to study of enzyme properties or use the enzyme as biocatalyst for L-amino acids production. However, limited information of *lys 6-dh* gene was obtained because the low activity of Lys 6-DH. Although *Agrobacterium tumefaciens* was screened as Lys 6-DH producer by Misono and his group in 1989, the information of *lys 6-dh* gene and enzyme characterization were rarely limited due to the enzyme stability. Heydari and coworkers (2004) screened for Lys 6-DH from many thermophilic bacteria and hyperthermophilic *archaea* from culture collection such as *Pyrococcus furiosus* DSM 3638, *Pyrococcus horikoshii* OT-3, *Thermococcus litoralis* DSM 5473, *Thermococcus profundus* DSM 9503, *Thermococcus peptonophilus* DSM 10343, *Pyrobaculum islandicum* DSM 4184, *Aeropyrum pernix* JCM 9820, *Sulfolobus tokodaii* JCM 10545, *G. stearothermophilus* DSM 297, DSM 456, DSM 458, DSM 494, IFO 12550, and UTB 1103, *Bacillus sphaericus* DSM 461 and DSM 462, and *Thermus* sp. strain UTB 1104. The Lys 6-DH activity was found only in *G. stearothermophilus* UTB 1103. After cloning, the recombinant had total activity of 995 units/ 2 liter of cell culture that was about 192 fold of the enzyme from *G. stearothermophilus* wild type. The specific

activity from crude extract of recombinant clones was 1.67 units/mg proteins. The highest specific activity was about 800 fold higher than that of the enzyme from *G. stearothermophilus* wild type.

The basic requirements for the successful production of recombinant enzyme are the isolation of the gene encoding enzyme and selection of a suitable expression system. In this research, the *lys 6-dh* gene from *A. denirtificans* K-1 was cloned into *E. coli* BL21(DE3) using plasmid pET-17b. Using pET system, *lys 6-dh* gene was expressed under T7 promoter; moreover, the upstream region of the inserted gene contained highly efficient ribosome binding site from the phage T7 major capsid protein (Novagen, 2002).

Because *lys 6-dh* gene was under T7 promoter, expression of *lys 6-dh* gene can be induced the T7 RNA polymerase on the *E. coli* BL21 (DE3) genome by IPTG. Therefore, the studies of induction time and final concentration of IPTG were required for maximum expression. pET-ADK clone which showed the highest Lys 6-DH activity was induced at various conditions. Even in absence of IPTG, the expression of *lys 6-dh* gene was occurred because there is some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 from *E. coli* genome (Novagen, 2002).

The variation of expression level of recombinant Lys 6-DH from various bacteria may be obtained from the promoters, mRNA construction, and condition of expression e.g. optimization of IPTG induction, type of cell line, media, and incubation circumstances. For example, 3 hours of induction with 1 mM IPTG maximized the expression of *phedh* gene from *Rhodococcus* sp. M4 which was cloned into *E. coli* BL21(DE3) using pET-3d plasmid (Brunhuber *et al.*, 1994). Moreover, alanine dehydrogenase gene from *Aeromonas hydrophila,* expressed under T7 promoter of pET-17b in *E. coli* BL21(DE3), showed the highest expression at 4 hours of induction with 0.4 mM IPTG (Hatrongjitt, 2004). In addition, Kim and coworkers (2002) cloned and expressed bovine brain glutamate dehydrogenase gene using pET-

15b in *E. coli* BL21 (DE3). The highest expression was found at 3 hours after induction.

In this study, the final concentration of 0.2 mM IPTG was the suitable concentration that capable for maximum enzyme production at 4 hours after induction. Usually the expression of pET plasmid carrying the T7 promoter was induced with a final concentration of 0.4 mM IPTG is recommended by the manufacturer (Novagen, 2002). However, different optimum conditions have been reported for some inserted genes among amino acid dehydrogenases using pET plasmids. The specific activity of crude Lys 6-DH from *A. denitrificans* in recombinant clone (1.89 U/mg protein) was higher than Lys 6-DH from G. *stearothermophilus* recombinant clone (1.67 U/mg protein).

The stability of the *lys 6-dh* gene from recombinant clone no. 4 showed the highest enzyme activity was studied by daily subculturing for 80 days. The crude enzyme of 1, 5, 10, 20, 30, 40, 45, 50, 60, 70 and 80 subcultured were extracted and Lys 6-DH activities were determined in order to compare with the activity of their parent. The result indicated that the *lys 6-dh* gene showed high consisitancy of expression even though it was subcultured for 80 days. This indicated that the copy number of recombinant plasmid was not lost during subculture.

4.6 Purification of Lys 6-DH from recombinant clone

Lys 6-DH was extracted from pET-ADK clone cell by using ultrasonication to break down cell wall in order to release intracellualr protein prior to purification. In the ammonium sulfate precipitation step, the solution to which salt is added should be provided with a stirring system which must be regular and gentle because it may cause protein denature as evidenced by foaming. pH may be important in precipitation. It is best to operate at a neutral pH value (6-7.5). Ammonium sulfate has a slight acidifying action, so around 50 mM buffer should be present (Scopes, 1987).

In this work, Lys 6-DH was a major protein from recombinant clone, therefore the purification step did not include ammonium sulfate precipitation. The purification began with DEAE-Toyopearl 650 M. Using this column, about 52% of the other bulk proteins were eliminated with 65.6% recovery of Lys 6-DH and the purity of the enzyme was increased to 1.3 fold. DEAE-Sephadex A-50 column was used in the next purification step. This column was also the anion exchanger and molecular sieve with mixture of strong and weak anion exchangers. The ion exchange group is diethylaminoethyl which remains charged and maintains consistently high capacity over the entire working range at pH 2-9. This column is a good choice for the high molecular weight proteins ranging from 30,000 to 100,000. High molecular weight molecules, which are excluded from the bead, may be adsorbed to some extent on the outer surface (Amersham pharmacia biotech, 1999).

The Lys 6-DH from recombinant clone (2.7 U/ml cultured medium) produced 180 folds higher amount of Lys 6-DH than *A. denitrificans* K-1 wild type (0.015 U/ml culture medium). After purification, about 89% of other proteins from DEAE-toyoperal 650 M were eliminated with 47% recovery and the purity of the enzyme was increased to 2.8 fold. The purified enzyme was determined by SDS-PAGE and activity staining. The specific activity of purified recombinant enzyme was also the same as the purified enzyme from wild type.

When compared to purification of Lys 6-DH from wild type, *Achromobacter denitrificans* K-1, which was purified by a procedure involving 0-60% ammonium sulfate precipitation followed by DEAE-Toyopearl, 0-40% ammonium sulfate precipitation, Sephadex G-150 column, Mono Q HR 10/10 column and Phenyl Sepharose XK 16 column chromatography, respectively. This indicated that the purification steps of cloned enzyme was faster and convenience.

4.7 Characterization of Lys 6-DH

The Lys 6-DH from *E. coli* harbouring pET-ADK was characterized. The enzyme properties such as the molecular weight, the specificity on substrate, effect of pH and temperature on its activity and kinetic mechanism had been performed.

4.7.1 Molecular weight determination of Lys 6-DH

The molecular weight of the recombinant enzyme determined by gel filtration on TSK Gel G3000 SW column was approximately 240,000 Da when incubated Lys 6-DH with L-lysine and 80,000 Da for the native enzyme without L-lysine. The molecular weight of subunits was calculated to be 40,000 Da on SDS-PAGE by comparing the mobility to that of standard proteins. The molecular of enzyme subunit was corresponded to calculated value from the deduced amino acid sequence of the enzyme (39,331 Da). Thus, the enzyme probably associated to hexamer (six identical subunits) in the presence of L-lysine substrate, while in nature form the enzyme existed as dimer (two identical subunits). This phenomenon was similar to the Lys 6-DH from *Agrobacterium tumefaciens* and *Geobacillus stearothermophilus* which their subunits were 39,000 Da and 42,239 Da and these enzymes were associated into a tetramer and hexamer in the presence of L-lysine substrate, respectively (Misono *et al.*, 1989 and Heydari *et al.*, 2004). The enzyme from various sources were considered variable in the quaternary structures (tetramer and hexamer).

4.7.2 Substrate specificity of Lys 6-DH

Substrate specificity is the ability of enzyme to discriminate between a substrate and competing molecules. If an enzyme active site has functional groups arranged optimally to form a variety of weak interactions with a given substrate in the transition state, the enzyme will not be able to interact to some degree with any other molecules. Specificity is derived from the formation of multiple weak interactions between the enzyme and its specific substrate molecule (Lehninger, 2000). Basically, a substrate binding site consists of an indentation or cleft on the surface of an enzyme

molecule that is complementary in shape to the substrate. Moreover, the amino acids residues that form the binding site are arranged to interact specifically with the substrate in an attractive manner. Molecules that differ in shape or functional group distribution from the substrate did not productively bind to the enzyme that means they can not form enzyme-substrate complexes that lead to the formation of products.

Lys 6-DH catalyzes the irreversible NAD^+-dependent oxidative deamination of L-lysine. The activities of other amino acids as substrates were examined. the recombinant enzyme from pET-ADK exhibited a significant specificity on only L-lysine and showed no activity with other L-amino acids such as aromatic amino acid (L-phehylalanine, L-tyrosine and L-tryptophan), positively charged amino acids (L-arginine and L-histidine), negatively charged amino acids (L-aspartate and L-glutamate), uncharged amino acids (L-serine, L-threonine, L-cysteine and L-asparagine) and other aliphatic amino acids (glycine, L-alanine, L-proline and L- methionine).

This result indicated that size of aliphatic side chain with increasing size of amino acid from small to large seemed to have no affect on specificity of enzyme. The length of a straight chain of L-amino acids also had no significantly influence on the enzyme specificity. Thus, the side chain of these amino acids may not fit on the active site of the enzyme. D-amino acids can not be the substrate of this enzyme. The Lys 6-DH from *A. denitrificans* K-1 was highly stereo-specificity for L-lysine similar to the enzymes from *A. tumefaciens* (Misono *et al.*, 1989) and *G. stearothermophilus* (Heydari *et al.*, 2004) which can applied to biochemical and medical applications such as determination of L-lysine in serum (Hashimoto *et al.*, 1990) and mouse brain (Murthy and Janardanasarma, 1999).

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

After the enzyme specificity was obtained, the inhibitory effects of various amino acids which did not act as substrates were performed. To investigate the effect on the Lys 6-DH binding site, various amino acids were combined with L-lysine, and

then the Lys 6-DH activity compared with absence of various amino acids had been performed. The decreased Lys 6-DH activity may cause the result of inhibitory effect of enzyme binding site. L-lysine analogs, L-norluecine, (whose side chain similar to L-ornithine but contains one methylene group in replace of amino group at the end of its side chain), 6-amino-*n*-caproic acid, (whose side chain contains one methylene group more than L-lysine and replaced amino group at the end of R-group by carboxylic group) and L-ornithine, (whose side chain contains one methylene group less than L-lysine) (Appendix K) affected Lys 6-DH activity on L-lysine. The relative activities of L-lysine combined separately with L-ornithine, 6-amino-*n*-caproic acid and L-norluecine decreased to 70%, 86% and 60%, respectively. This result suggested that three L-lysine analogs had inhibitory effects with the enzyme binding site which obstruct L-lysine reached to the enzyme binding site. This effect gave low activities after those analogs were separately added into the reaction.

4.7.4 Coenzyme specificity of Lys 6-DH

Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a cofactor. Cofactors that are small organic molecules are called coenzymes. NAD^+ which is coenzyme of the Lys 6-DH is replaced by some of the NAD^+ analogs. According to the result, NAD^+ could not be replaced by $NADP^+$ which differs from NAD^+ only by the addition of a phosphoric group at C-2 position of its adenosyl ribose. The NAD^+ analogs modified at an amino group in the adenine moiety, deamino-NAD^+, nicotinamide guanine dinucleotide and nicotinamide-1,N^6-ethenoadenine dinucleotide, could be used as coenzymes of the Lys 6-DH but only nicotinamide guanine dinucleotide gave more activity than NAD^+. This suggests that changing of amino group in the adenine moiety of NAD^+ was low effected the coenzyme specificity.

NAD^+ analog modified at the amino group of the nicotinamide moiety, 3-acetylpyridine adenine dinucleotide, thionicotinamide adenine dinucleotide, 3-pyridinealdehyde adenine dinucleotide and deamido NAD^+ were

tested. Only 3-acetylpyridine adenine dinucleotide, exhibited some activity but lower than NAD^+ while the others were inerts. This suggests that replacement of the amino group of the nicotinamide moiety by methyl group can used for Lys 6-DH activity but the activity was quit low. These effects had been reported among amino acid dehydrogenases such as LeuDH from *B. sphaericus* (Ohshima *et al.*, 1978), LeuDH from *Corrynebacterium pseudodiphtheriticum* (Misono *et al.*, 1990), and PheDH from *Rhodococus marls* K-18 (Misono *et al.*, 1989). In addition, the enzyme could not use deamido-NAD^+ and 3-pyridinealdehyde adenine dinucleotide, which their amino groups in the nicotinamide moiety are substituted by OH group and H atom, respectively. This might involve polarity of the substituents which might affect the binding mechanism in the area of the conserved hydrophobic residues of the enzyme. Moreover, no activity from thionicotinamide adenine dinucleotide, this suggests that the amino group of the nicotinamide moiety is essential for the enzyme activity and the type of substituted groups of NAD^+ analogs also has influence on enzyme activity. The structure of NAD^+ and NAD^+ analogs are shown in Appendix L.

4.8.4 Effect of pH on Lys 6-DH activity and stability

The enzyme activity is often limited to a relatively narrow pH range. Beyond this range, the enzyme activity is dramatically decreased. A pH change affects a reaction by altering the state of ionization of the enzyme, the substrate, or an intermediate or causing a change in the conformation of the enzyme or the substrate, or both. The enzyme forming an enzyme-substrate complex requires electrostatic attraction between oppositely charged groups of the enzyme and the substrate. Within a relatively narrow pH range, the charges of these groups remain essentially the same so that the rate, a function of substrate binding, does vary greatly. At a sufficiently low or high pH, some of these groups lose their charge, thereby impairing binding. Additionally, previously uncharged groups may acquire a charge as the pH increases or decreases. Large shifts in pH lead to denaturation of the enzyme, due to interference with the many weak noncovalent bonds maintaining its three-dimensional structure (Segal, 1976).

At some pH values, complementary regions of the enzyme and the substrate may carry charges, of the same sign and repel each other, leading to a drop in rate. Altering the charges of functional groups leads to altered ionic interactions involving these groups. These new interactions may produce a conformational change at the active site, affecting the site's capacity to bind substrate. The enzyme may undergo conformational changes and lose their capacity to bind to the substrate.

The pH which Lys 6-DH showed maximum activity was 9.3 for the oxidative deamination of L-lysine, this was similar to those of the enzymes from *A. tumefaciens* (pH 9.7) (Misono *et al.,* 1989) and *G. stearothermophilus* (pH 10.0) (Heydari *et al.,* 2004). In addition, KPB and Tris-HCl buffer were not suitable for the activity of cloned Lys 6-DH in alkaline pH due to the capacity of these buffers. The glycine-KCl-KOH buffer was an appropriate buffer for the Lys 6-DH. This kind of buffer is widely used for the assay of amino acid dehydrogenases.

Although the optimum pH of Lys 6-DH was in the glycine-KCl-KOH buffer pH 9.5, the enzyme stability upon incubation at 30°C for 10 minutes was in a pH range of 6.0 to 7.5 and 8.5 in KPB and glycine-KCl-KOH buffer, respectively. While pH stability of other Lys 6-DHs were between 5.0 to 9.0. Lys 6-DH from *G. stearothermophilus* was stable in pH range 6.0 to 9.0 after incubation at 50°C for 30 minutes (Heydari *et al.,* 2004) and Lys 6-DH from *A. tumefaciens* was stable in pH range 5.0 to 7.5 after incubation at 30°C for 10 minutes (Misono *et al.,* 1989). The pH stability of an enzyme depends on many factors including temperature, ionic strength, chemical nature of the buffer, concentration of various stabilizers and concentration of substrates or cofactors of the enzyme and enzyme concentration (Segal, 1976).

4.8.5 Effect of temperature on Lys 6-DH activity and stability

The influence of temperature on an enzymatic reaction was from two opposing effects, an increase in rate and increase in denaturation. Increasing of thermal energy of the substrate molecules increases the proportion of molecules with sufficient energy to overcome the activation barrier and hence increases the rate of the reaction.

In addition, increasing of the thermal energy of the molecules which make up the protein structure of the enzyme itself will increase the chance of breaking the multiple weak noncovalent interactions holding the three-dimensional structure (Segal, 1976). The optimum temperature of Lys 6-DH for the oxidative deamination was 50 °C while Lys 6-DH from *A. tumefaciens* (Misono *et al.,* 1989) and *G. stearothermophilus* (Heydari *et al.,* 2004) were 40 and 60 °C, respectively. After incubated this enzyme at 50 °C for 10 minutes before activity assay, the enzyme was completely loss its activity (data not shown). This enzyme was not stable at high temperature. Lys 6-DH from *A. tumefaciens* lost 100% activity after incubated at optimum temperature 40 °C more than 10 minutes (Misono *et al.,* 1989).

Due to the L-pipecolic acid production at 30 °C, time course of the Lys 6-DH stability at 30 °C was performed. It was found that the Lys 6-DH activity was not lost upon incubation for 3 days and about half the enzyme activity was lost when incubated for 9 days.

4.9 Kinetic studies of Lys 6-DH

In initial velocity studies, the apparent K_m values for L-lysine and NAD^+ in dimeric and hexameric form were 11.11, 0.14, 8.62 and 0.09 mM, respectively, The coexistence of L-lysine significantly increased its activity and decreased K_m values, suggesting that the hexameric enzyme was more effective than the dimeric enzyme and the substrate, L-lysine, acted as an effector that increased Lys 6-DH activity similar to that of the *A. tumefaciens* Lys 6-DH (Misono *et al.,* 1989).

4.10 Cloning and expression of *p5cr* gene

Pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) catalyzes the final step of L-pipecolic acid production. In this work, the *p5cr* gene fragment was amplified from genomic DNA of *B. cereus* ATCC 11778. The sense primer contained *Nde*I site, while the antisense primer consisted of *Eco*RI site for further cloning purpose. The

amplified whole gene fragment (782 bp) was ligated with *Nde*I-*Eco*RI site of pET-17b and then transformed into *E. coli* BL 21(DE3). Twelve transformants harboured recombinant plasmid which contain *p5cr* gene showed various levels of the specific activity of P5CR from 2.5-9.5 units/mg protein. The highest specific activity obtained was 95 fold higher than that of *B. cereus* wild type with the molecular weight of subunit around 26.4 kDa was obtained.

Attempt to clone *p5cr* gene began in 1982, Deutch and coworkers reported that the *E. coli* gene coding for *p5cr* gene was contained on a 1.2 kb DNA fragment, which encoded for 269 amino acid residues, bounded by restriction sites for *Hinc*II and *Hae*II with the expression vector pGW7 under the control of the highly active $_{\lambda}P_{L}$ promoter, was cloned into *E. coli*. BRL1945. The P5CR activity was 200 fold higher than wild type with subunit molecular weight of 26,500 Da as determined by SDS-PAGE. In addition, Kenklies and his groups (1999) cloned *p5cr* gene from *Clostridium sticklandii* by genomic DNA. Genomic DNA was partially digested with the restriction enzymes *Sau*3A and *Eco*RI. The fragments size of 2-3 kb and 4-6 kb were ligated into pBluescript II SK and transformed into *E. coli* XL2-Blue. After *p5cr* gene in recombinant plasmid was identified and sequenced, the full length of *p5cr* gene fragment (798 bp) was obtained which encoded for 266 amino acids, the calculated molecular weight of subunit was 28.9 kDa. Recently, Fujii and coworkers (2002a) constructed *E. coli* JM109 *p5cr* gene, using primers EX1 (5′-ATGGATCCGAAATC GGTTTTATTGGCT-3′) with *Bam*HI site and primer EX2 (5′-TCGAATTCCAGGA TTTGCTGAGTTTTTCT-3′) with *Eco*RI site, ligated with pTrcHis2A plasmid and transformed into *E. coli* TOP 10. After purified P5CR and characterized, the molecular weight of subunit was 32 kDa on SDS-PAGE.

Because of *p5cr* gene was under T7 promoter, expression of *p5cr* gene can be induced the T7 RNA polymerase on the *E. coli* BL21 (DE3) genome by IPTG. Therefore, the studies of induction time and final concentration of IPTG were required for maximum expression. The result showed that 0.2 mM final concentration of IPTG which was the suitable concentration to be capable for induction. The expression of the *p5cr* gene reached the maximum at 8 hours after induction.

4.11 Cloning and expression of *lys 6-dh* and *p5cr* using pET-17b

In general, the one or more homologous or heterologous genes coding for the cooperative activities can be brought to over expression in the microorganism used in a well-known manner, For thus purpose, the one or more structural genes are actively combined with the promoter, for example by introducing the coding gene in the right orientation and the right reading frame into a plasmid or another vector, which already contains their own promoter.

To co-existence high expression of *lys 6-dh* gene and *p5cr* gene in *E. coli* host cell, cloning of heterologous gene of *lys 6-dh* gene and *p5cr* gene under separated T7 promoter of high expression vector pET-17b has been performed. Twelve recombinant clones showed various levels of the specific activity of Lys 6-DH and P5CR from 0.27-0.75 and 0.10-0.54 units/mg protein, respectively. The highest specific activity of Lys 6-DH and P5CR were 24.5 and 10.7 fold higher than that of *A. denitrificans* K-1 and *B. cereus* ATCC 11778 wild type, was produced by *E. coli* BL21(DE3) transformant No. 4. When compare the specific activity of Lys 6-DH and P5CR with *E. coli* BL 21(DE3) harboring pET-ADK and pET-P5CR, the activities were decreased 2.6 and 8.8 fold, respectively. The low intensity of protein bands might due to the decreased of specific activity of both Lys 6-DH and P5CR on SDS-PAGE.

The variation of induction time between cloning of the single gene system and two genes expression system may secured because of sharing the materials required for transcription and translation steps because this two genes were under T7 promoter (*lys 6-dh* and *p5cr* gene). Not only change in the induction time, total activity of crude Lys 6-DH and P5CR at the optimum condition also dropped about 3.4 and 7.1 times in two genes system, respectively. For the co-transformant system, clone harbouring pET-ADK-P5CR had optimum induction time shorter at 3 hours with 0.1 mM IPTG, however, total activities of both Lys 6-DH and P5CR were greatly decreased. The T7 promoter was the strong promoter that may caused the competitive of mRNA in the transcription level in the *E. coli* cell. The greatly decreased of P5CR

may occurred with the start codon of the *p5cr* gene that was located near the stop codon of the *lys 6-dh* gene (less than 30 bp) may caused the RNA polymerase hardly to attach and regulated the *p5cr* gene expression.

4.12 Production of L-pipecolic acid

Current methods to obtain a pure enantiomer of L-pipecolic acid involve chemical resolution (Hasegawa *et al.*, 2000), stereo-selective transformation (Eichhorn *et al.*, 1997), the derivatization of natural amino acids (Fujii and Miyoshi, 1975), and enzymatic reactions (Fujii, *et al.*, 2002a, 2002b, Nazabadioko, *et al.*, 1998, Ng-Youn-Chen, *et al.*, 1994, and Sánchez-Sancho and Herradon, 1998), but most of these methods fail to provide a satisfactory solution for the synthesis of chiral pipecolic acid on an industrial scale due to certain limitations, such as tedious procedures, low yields, and unavailability of starting materials. Hence, new and convenient methods for the preparation of optically active L-pipecolic acid are required.

Although Fujii and coworkers (2000) found that the *p5cr* gene encoded P5CR was located in *E. coli* chromosome. The increasing the *p5cr* gene copy number had no effected of L-pipecolic acid production. In contrast, L-pipecolic acid production was 4 folds higher than *E. coli* BL21 (DE3) harboring pET-ADK by *E. coli coli* BL21 (DE3) harboring pET-ADK-P5CR indicated that the expression of both Lys 6-DH and P5CR was essential and the increasing of *p5cr* gene copy number affected the L-pipecolic acid production. In addition, it was possible that in this biotransformation system of L-lysine to L-pipecolic acid, the reduction of P-6-C to L-pipecolic acid was not the rate-limiting step. The limitation of L-pipecolic acid production was the L-lysine uptake into *E. coli* cell. Fujii and coworkers (2002) found that after increased the L-lysine uptake into *E. coli* cell by cloning the *lysP* gene which was responsible to L-lysine transportation into the cell, L-pipecolic acid production was increased 6 folds than non adding the *lysP* gene.

The use of organic chemicals to increased permeabilization was cost effective and cause selective of the outer cell wall barriers allowing large-scale preparation of periplasmic enzymes in a relatively pure form (Bansal-Mutalik and Gaikar, 2003 and De Leon *et al.*, 2003). The cell envelope of bacteria basically consists of cell wall and one or two lipid membranes. For the bacteria, two major groups can be discerned on the basis of their cell envelope composition: the gram-positive and gram-negative bacteria. Gram-positive bacteria have only one membrane, the cyto-plasmic membrane, which is surrounded by a thick, rigid cell wall. Gram-negative bacteria have, in addition to the cytoplasmic cell membrane, an outer membrane that consists of phospholipids and lipopolysaccharides. Between the outer membrane and the cytoplasmic membrane is a thin peptidoglycan layer, forming the support for the cell envelope (Sikkema *et al.*, 1995). The periplasmic space in gram-nagative bacteria in between the plasma membrane and the outer membrane, mainly between the plasma membrane and peptidoglycan layer. This space contains enzymes and proteins functioning in detection of the environment, acquisition of nutrients into the cell, enzymatic hydrolysis and detoxification of harmful compounds (Madigan *et al.*, 2003).

There are many reports in which the physicochemical properties of solvents in the reaction mixture related to various effects on the activity and stability of enzymes (Affleck, *et al.*, 1992, Yoshida, *et al.*, 1997 and Halling, 1987). It has been reported that electrostatic forces affect protein structures and their functionality (Affleck, *et al.*, 1992, Yoshida, *et al.*, 1997 and Perutz, 1976). First, the dielectric constant represents the dipolar moment of the solvent molecules and directly affects the flexibility of proteins (Guinn, *et al.*, 1991). Enzymes are usually very flexible in solvents with high dielectric constants such as alcohols capable of inducing the denaturation of many enzymes (Guinn, *et al.*, 1991, Arroyo, *et al.*, 1999 and Mozhaev, *et al.*, 1989). Another solvent property that affects enzyme activity was hydrophobicity, commonly measured as log P, where P is the partition coefficient of the solvent between 1-octanol and water. Organic solvents had been used for improvement of the production in many reports. Flores and coworkers (1994) found that pure chloroform or toluene

was more efficient than ethanol for permeabilization of *Kluyveromyces lactis* cells. While NADP production by acetone treated cell produced 16 mM (14 g/l) of NADP after 24 hours (Shigeyuki *et al.*, 2001).

To improve the yield of L-pipecolic acid production by co-existence of *lys 6-dh* and *p5cr* genes. Increasing permeabilization of L-lysine uptake into *E. coli* transformant cells by treated with various organic solvents was observed. Treatment of *E. coli* recombinant cell with water was included as the control to determine whether the osmotic pressure caused by water influx had any effect on L-pipecolic acid production. Interestingly, the study showed that the physicochemical properties of the organic solvents such as dielectric constant, and hydrophobicity (Appendix M) correlated with increase in L-pipecolic acid production. Hexane, toluene and xylene increased L-pipecolic acid production, whereas chloroform, acetone and benzene had drastically decreased the production. In contrast, solvents such as pyridine and alcohols (ethanol, methanol and isopropyl alcohol) completely inactivated the enzyme. From this result, organic solvents with a dielectricity was less than 2.5 and hydrophobicity of log *P* more than 2, xylene was more effective in increasing L-pipecolic acid production in this research.

The maximum L-pipecolic acid production by this research was 77 mM (10 g/L of reaction mixture) after 24 hours of L-pipecolic acid production. Compare with the other productions with untreated cell in the fermenter, Fujio and Maruyama (1997) used *E. coli* MM294/pUCK3 and MM294/pCC41 cells, which expressed a choline kinase from *Saccharomyces cerevisiae* (CKIase) and a cholinephosphate cytidylyltransferase from *S. cerevisiae* (CCTase) respectively, were added to CTP-producing reaction system, produced 7.7 g/L (15.1 mM) of CDP-choline after 23 hours of the reaction. Aiba and coworkers (1982) constructed a genetically engineered strain by introducing a plasmid containing the *trp* operon with deregulated *trpE* and *trpD* genes into an *E. coli trpR* and *tnaA* mutant. By cultivating the strain in glucose medium to which anthranilic acid was continuously fed, they obtained 6.2 g/L of L-tryptophan after 27 hours of production. When compare the L-pipecolic acid production of Fujii and coworkers (2000 and 2002), the maximum production was

3.9 g/L after 159 hours of reaction after increased the L-lysine uptake the maximum production was 16 g/L within 110 hours of reaction, xylene *E. coli* BL 21 (DE3) containing pET-ADK-P5CR treated cells in this research produced 9.9 g/L of the reaction mixture after 24 hours that was production time saving than that of Fujii and coworkers in the time course production. An additional potential advantages in this research were xylene was easy to remove by a simple evaporation or centrifugation, low cost for substrate L-lysine was used and no contamination in the production solution with other substances. The reaction mixture was easy to purified by commonly used methods, such as ion exchange chromatography and crystallization. (Rodwell, 1971). In conclusion, as all the results obtained in this work, Lys 6-DH and P5CR in the *E. coli* BL21 (DE3) transformant cell had potentially use and apply in the industrial fields.