CHAPTER IV

DISCUSSION

Amino acids are known as biomolecules, which play important roles in all life. Bacteria are one of organisms, which can metabolize amino acid as the energy sources through the glycolytic or TCA cycle reactions by using three enzymes. The first enzyme is pyridoxyl phosphate-dependent transaminases, which transfers the amino group of amino acid to keto acid and results in new amino acid such as glutamate. The second one is to employ deaminases in the way to remove amino group from amino acid in the form of ammonia. The last is to use an amino acid dehydrogenase. This enzyme has the advantages of producing the amino group as free ammonia and other important metabolize molecules such as pyruvate and α-ketoglutarate (Nobert *et al.*, 1994). Phenylalanine dehydrogenase is one of the enzymes used for producing phenylalanine and phenylpyruvate, which are of industrial importances. Moreover, this enzyme can be applied for diagnosis of neonatal hyperphenylalaninaemia and phenylketonuria (Hummel *et al.*, 1984). Since thermotolerant bacterial strain BC1 has been reported to produce high phenylalanine dehydrogenase activity (Suriyapanpong *et al.*, 2000), this bacteria was suitably used for further studies in molecular genetic of phenylalanine dehydrogenase gene.

Although phenylalanine dehydrogenase from strain BC1 was characterized by Leksakorn in 2001, the bacterial strain has never been identified. Therefore, strain BC1 was sent to Thailand Institute of Scientific and Technological Research (TISTR) to be identified by using morphological and biochemical properties such as gram reaction, fermentative production of acid from various carbon sources and activity of various enzymes. The results suggested that strain BC1 was closely similar to *Brevibacillus brevis*. However, Bergey's manual of determinative bacteriology indicated that *Brevibacillus brevis* could not catalyze the deamination of phenylalanine. Thus, to confirm TISTR suggestion, 16S rRNA gene sequence was analyzed.

PCR amplification of 16S rRNA gene fragments is useful for identification of bacterial strain with specific primers. This method is rapid, simple and efficient. From these advantages, it is possible to be used in taxonomic studies of aerobic,

endosporeforming rods including thermotolerant bacterial strain BC1. Chromosomal DNA of thermotolerant bacterial strain BC1 was easily prepared by miniprep of bacterial genomic DNA method. Its molecular weight was found to be greater than 23.1 kb and A_{260}/A_{280} ratio was also greater than 1.8 indicating high purity (Manchester, 1995). This chromosomal DNA was the template for amplification the 16S rRNA whole gene fragments by PCR method using primer A and H' (Ulrike *et al.*, 1989). Single fresh colony was also used as sources of DNA template. The PCR product of approximately 1.5 kb was sequenced in both directions and further compared with available 16S rRNA sequences of *Bacillus* species in the EMBL-Genbank-DDBL database. The highest homology was found between 16S rRNA gene of thermotolerant bacterial strain BC1 and *Bacillus badius*. Additionally, phylogenetic tree showed that BC1 gave the closest evolutionary distance values with *Bacillus badius* while *Brevibacillus brevis* was phylogenetically distinct from it. These results indicated that BC1 should be *Bacillus badius*. The conclusion is supported by *Tm* determination. The melting temperature of chromosomal DNA from strain BC1 was 50 °C in the same range with that from *Bacillus badius*. These results confirmed that bacterial strain BC1 belongs to *Bacillus badius* specie. Bergey' s manual of determinative bacteriology showed that *Bacillus badius* was distinguished from *Brevibacillus brevis* by growth in 5 % NaCl broth. Thus, to confirm this conclusion, BC1 was grown in peptone medium contained various percentage of NaCl: 1, 2, 3, 4, 5 and 6 % at 37 °C overnight. Bacterial strain BC1 could be grown in the medium containing NaCl upto 5 %. Hence, thermotolerant bacterial strain BC1 was identified as *Bacillus badius*.

Basic study in molecular genetic level was the nucleotide sequencing of interesting gene. PCR amplification, the popular method, was applied. This technique depends on two primers, which are specific to interesting gene. Although the specific primer could be designed from the conserve region of the gene from various sources, the similarity was low. Thus, deduced nucleotide sequence from amino acid sequence of phenylalanine dehydrogenase was interesting. Phenylalanine dehydrogenase was partially digested with lysyl endopeptidase, the enzyme which cleavage peptide bond between lysine and it next amino acid residue. The digested peptides were further purified 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 peptide and internal amino acid sequences could be identified. After alignments, the positions of each peptide fragments which overlapped with amino acid sequences of the other phenylalanine dehydrogenases were indicated. From this data, the degenerated primers used for the PCR amplification of internal phenylalanine dehydrogenase gene were designed. It is known that templates used in PCR reaction should be single strand in the purpose of annealing with primers. The heat denatured chromosomal DNA was previously used as templates, however, it gave poor PCR amplification due to effect of its viscosity. So, the chromosomal DNA was digested by restriction enzyme before using. From various restriction enzyme digestions, the chromosomal DNA fragment cut by *Pst*I gave the smear DNA pattern of low molecular weight DNA while the digestion with *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Pvu*I, *Spe*I and *Xba*I still had a high molecular weight DNA longer than 9 kb. It can be implied that chromosomal DNA of *Bacillus badius* BC1 has more *Pst*I sites than the others.

All of restriction enzyme digested DNA fragments were also used for testing as the templates in PCR reaction. The PCR products were detected by relative mobility on agarose gel electrophoresis compared with standard marker. Due to primers used for this step were degenerated primers, all PCR reactions gave different non-specific band patterns. The specific product bands, determined by size, were extracted for nucleotide sequencing. Although *Xba*I and *Bam*HI digested DNA fragment did not give the specific product by using primer N1 x C1 and N2 x C1, respectively, specific product of primer N1 x C2 (402 bp) was detect when all restriction enzyme digested DNA fragments were used as templates. It is widely known that the restriction enzyme used for template preparation must not have their sites on the gene; otherwise, the amplified whole gene fragment cannot be occurred. Thus, it was implied that *Bam*HI, *Bgl*II, *Kpn*I, *Pst*I, *Spe*I and *Xba*I restriction sites were not on the phenylalanine dehydrogenase gene fragments. The PCR product from this step was analyzed for nucleotide sequence using ABI prism sequencer. The internal gene about 530 bp was obtained, so the 5'- and 3'- terminal gene fragment amplification were further done by using the next two series of primers, designed from the internal nucleotide sequencing data. For sequencing of unknown DNA region at the 5' and 3' end of the structural gene, cassette-ligation mediated PCR was performed (Rosenthal and Jones, 1990).

The cassette-ligation mediated PCR is a rapid and reliable method for obtaining sequences flanking a known region of DNA. The source of the DNA may be anything from chromatin to cDNA including the purified insert of a lambda bacteriophage vector (Rosalind *et al.*, 1993). DNA containing the target sequence and its unknown flanking regions is digested with the restriction enzyme specified by the sticky end of the cassette. After that, it was further ligated to an excess double-stranded DNA cassette possessing a corresponding restriction site. The ligated products are then amplified by PCR using the specific and cassette primers. The specific primer is designed to prime synthesis from the known sequence whereas the cassette primer anneals to one strand of the cassette. In this experiment the PCR was done for two times in which the products from the first PCR, which used outer pair of primers, were then used as the templates for the second PCR and the inner pair of primers were used to produce more specific products. Because both cassette primers overlapped to each other and the distance between each outer and inner primer was approximate 60 bp, the second PCR products, which gave smaller band than the first PCR products were purified and used for nucleotide sequencing. Since the direct PCR sequencing method can determine only up to 300 bp from the priming site, a new primer was necessary to be synthesized according to the obtained sequence information. From the basic of gene walking, the complete nucleotide sequence of the whole gene fragment was identified. Unfortunately, although the nucleotide sequencing was done for several times with various primers, the upstream sequence of this gene was so confused. Thus, the ribosome binding site could not be obtained. The nucleotide sequence of the phenylalanine dehydrogenase gene contains 1140 nucleotides open reading frame, which is capable of encoding a polypeptide of 380 amino acids that matched with other phenylalanine dehydrogenase. This gene had GC content about 40 %.

The deduced amino acid sequence was matched well with amino acid sequence analyzed from 7 peptides, which were obtained by lysyl endopeptidase digestion. Amino acid sequences of all phenylalanine dehydrogenases were aligned by using the CLUSTAL X (1.64 b). The overall similarity scores of phenylalanine dehydrogenase from *Bacillus badius* BC1 compared with *Bacillus badius*, *Thermoactinomyces intermedius, Bacillus sphaericus*, *Sporosarcina ureae*, *Bacillus halodurans* and *Rhodococcus sp.* were calculated to be 98, 75, 70, 70, 62 and 35 %,

respectively. According to Brunhuber *et al.* (1999), Lys-78 and Asp-118 were necessary for stabilizing and binding of phenylalanine in the active site of the *Rhodococcus* enzyme. Both of residues were identical in all the phenylalanine dehydrogenase sequences including *Bacillus badius* BC1 enzyme. In addition, conserved residues in the catalytic domains G-G-(G or S or A)-K-X-(V or G)-X-X-X-(D or N)-(P or L) at the N-terminal (amino acid position 87 to 97 in Figure 3.23), and the glycine-rich nucleotide binding domain G-X-G-X-X-(G or A) at the C-terminal (amino acid position 190 to 195 in Figure 3.23) were also found (Yamada *et al.*, 1995). However, its can be noted that some disparity of amino acid sequences may cause different properties of *Bacillus badius* BC1 phenylalanine dehydrogenase from published *Bacillus badius* enzyme (Leksakorn, 2001).

Finally, to overexpress the phenylalanine dehydrogenase gene in *E. coli* under the regulation of *lac* promoter of plasmid pUC18, the whole gene fragment was amplified by using the 5' end primers that contained a Shine-Dalgarno sequence of plasmid p*Trc*99, the expression vector for *E. coli* JM105. The amplified whole gene fragment was purified before the digestion *Eco*RI and *Bam*HI. Then, the whole gene fragments were ligated to *Eco*RI-*Bam*HI site of pUC18 and transformed into *E. coli* JM109 host cells. White colonies, expected to contain phenylalanine dehydrogenase gene, were selected.

Recombinant clones were grown in the medium containing ampicillin and further determined for phenylalanine dehydrogenase activity. Because the gene fragments did not have their own promoter, so they were expressed under *lac*Z promoter on the plasmid pUC18. Then IPTG was added to induce phenylalanine dehydrogenase gene expression. From 25 randomly selected colonies, there were four types of insert plasmid. The first type of plasmid was the ideal target recombinant plasmid, contained phenylalanine dehydrogenase gene joined with plasmid vector. Moreover, extracted recombinant plasmid showed supercoil or/and relaxed forms on agarose gel electrophoresis. Twenty-two colonies harboured this type of plasmid. Their phenylalanine dehydrogenase total activities were found between 0-338 units/ 100 ml culture. High expression was the result from 1) The gene was expressed under *lac*Z promotor which can be induced by IPTG. 2) The upstream region of the inserted plasmid contained Shine Dalgarno sequence of expression vector p*Trc*99 which supported the translation in *E. coli* host cell. However, low enzyme activities were also observed from crude extracts

of transformant carried this type of plasmid since the inserted gene fragments were prepared by PCR under the reaction contained *Taq* DNA polymerase which lacked of proof reading activity. The mutation may be occurred at the nucleotide coding for essential amino acid residue of the enzyme. In order to decrease the possibility of mutation DNA polymerase consists of proof reading activity such as *Vent* DNA polymerase should be use in whole gene amplification. The second type of plasmids, found in one recombinant clone, also contained one piece of pUC18 connected with one piece of inserted gene fragment as description for the first type, but its extracted plasmid contained another band between supercoil and relaxed form when electropheresed on agarose gel. This band was supposed not to be linear form based on its mobility compared with λ/*Hin*dIII standard marker. This type of plasmid expressed the moderate level of phenylalanine dehydrogenase. The third type of plasmid had higher molecular weight than the first type, however, it also gave a band of inserted gene after double digestion. It can be suggested that its part of DNA vector was changed. The third type appeared in only one clone and its transformant produced very low level of the enzyme. The last type showed 2 main strong bands, which gave their mobility of cut and uncut plasmid similar to those of pUC18. Thus, it may suggested to be pUC18 monomer. In addition to 2 strong bands, this type of plasmid also gave very weak bands corresponded with the pattern of the first type. After digestion with *Eco*RI and *Bam*HI, the result showed no main phenylalanine dehydrogenase gene fragment. In contrast, the highest total activity with 60 - fold higher than that of *Bacillus badius* BC1 was produced by transformant No. 22, an only one clone that showed the last type plasmid. Thus, transformant No.22 may contain mixed colonies of recombinant clone and clone containing only pUC18 vector. To prove this hypothesis, ten single colonies of transformant No.22 were selected for plasmid extraction. All of transformant No.22 colonies gave the same pattern corresponded with the forth type of plasmid (data not shown). It was clearly to say that transformant No.22 was quickly received some effects from host cell deletion process. Thus, inserted gene fragment was hardly detected by plasmid extraction.

Many researchers attempted to clone phenylalanine dehydrogenase gene by various techniques. In detail, Asano (1987) recovered phenylalanine dehydrogenase gene fragment from estimated size of digested chromosomal DNA isolated from *Bacillus*

badius by electroelution, and then ligated with *Eco*RI-digested pBR322. *E.coli* RR1 was used for host cell. Next, Takada (1991) used the same method to prepared gene fragment from *Thermoactinomyces intermedius*, ligated into the *Bam*HI site of pUC18 and transformed to host cell *E.coli* MV 1184. Finally, Norbert (1994) constructed oligonucleotide probe by PCR method, using genomic *Rhodococcus* DNA as the template and degenerated primer designed from amino acid sequence of purified enzyme. The probe was used to screen phenylalanine dehydrogenase gene fragment from cosmid library of *Rhodococcus* genomic DNA. Gene fragment was cloned into pET-3d vector, transformed to *E.coli* BL21 (DE3) and induced phenylalanine dehydrogenase production by 1 mM IPTG. However, expression level of these enzyme was rather low about 1-2 fold of wild type. It was inappropriately for advance study such as the structure and function of the enzyme as well as its applications. It was known that the basic requirements for the successful production of recombinant enzyme are the isolation of the gene encoding that enzyme and the development of suitable expression system for the gene (Price and Stevens, 2000). The gene to be overexpressed is generally incorporated in a plasmid under the control of a strong promoter, so that the expression of the gene can be induced by addition of an inducer or some other change in the culture medium. In this case, the gene was expressed under *lac*Z promoter and can be induced by IPTG. Moreover, Shine Dalgarno sequence of expression vector p*Trc*99 was designed at upstream region of phenylalanine dehydrogenase gene. Thus, the highest total enzyme activity of recombinant clones was about 60 times higher than that of *Bacillus badius* BC1 wild type.

Due to the phenylalanine dehydrogenase gene fragments, which were cloned into host cell did not have their own promoter, so *lac*Z promoter on the plasmid pUC18 was used and IPTG was added to induce phenylalanine dehydrogenase gene expression. Hence, the study about influence of induction time of IPTG was required. Transformant No.15, which showed the highest phenylalanine dehydrogenase activity among the transformants containing the first type of plasmid, was grown, induced by 1 mM IPTG and continuously grown for various periods of times before cell harvested. The result showed that 120 minutes was optimum time to induce phenylalanine dehydrogenase production. In addition, the enzyme production could not induce at 300 minutes. From the results, it can be noted that phenylalanine dehydrogenase activity which

produced from the 25 recombinant clones as described in section 3.9 should not present the optimum activities since the induction time was 120 minutes.

Stability of phenylalanine dehydrogenase gene from recombinant clones that showed high phenylalanine dehydrogenase activity was studied by daily subculturing for 15 days and retransformation into host cell. Plasmid of the 15^{th} subcultured transformants gave the same patterns with their original plasmids and can be digested with *Eco*RI and *Bam*HI. The crude extracts of the 5^{th}, 10^{th} and 15^{th} subcultured clone were prepared and enzyme activity was determined and compared with the activity of their parents. The result indicated that each clone showed vary expression level of phenylalanine dehydrogenase gene. Variation of phenylalanine dehydrogenase activity in each clone may cause from copy number of recombinant plasmid during cultivation step. Enzyme activity of five transformants, No. 4, 6, 10, 15 and 20 were still remained upon subculturing for 15 times while the enzyme activity of transformant No. 22, which harboured the last type of plasmid, decreased rapidly since the crude extract of the 5^{th} subcultured clone was detected. It can be explained that at the beginning, recombinant plasmid of transformant No. 22 had the properties similar to the first type of plasmid since the weak bands corresponded to the pattern of the first type were detected. Moreover, the express of the phenylalanine dehydrogenase gene of its original plasmid should be very high because the total activity of phenylalanine dehydrogenase was highest among all transformants though its original plasmid bands were very weak. The high expression of phenylalanine dehydrogenase which was nonessential protein in *E. coli* may disturb the common metabolism of the host cell so the inserted phenylalanine dehydrogenase gene was eliminated rapidly as we could detect the majority of pUC18 band when agarose gel electrophoresis of transformed plasmid was done. This result indicated that all of recombinant plasmids except No.22 were stable without host cell deletion process.

After retransformation and plasmid extraction, the same patterns of recombinant plasmids were obtained. In addition, all of retransformant clones were grown and crude extracts were prepared. Subsequently, they were subjected to native-PAGE. The result showed that phenylalanine dehydrogenase activity of all retransformants was still constantly remained.

From two experiments for assay stability of phenylalanine dehydrogenase gene, its can be noted that phenylalanine dehydrogenase gene in transformant No.22 was possibly removed. Plate culture or plasmid form was acceptable method to keep the recombinant clones.

In this study, the sequence of phenylalanine dehydrogenase gene from *Bacillus badius* BC1 was determined and the structural gene has been successfully cloned and overproduced by PCR method. The obtained information can be used as primary data for further development of phenylalanine dehydrogenase production.