CHAPTER IV

DISCUSSION

The commercial value of amino acids come from their wide applicability in both food and pharmaceutical industries. The world wide market value of amino acid is approximately two billion dollar annually. L-alanine is currently used as a food additive because of its sweet taste and used for pharmaceutical application in which it is incorporated together with several other L-amino acids in standard infusion for parenteral administration in clinical preoperative and postoperative nutrition therapy (Suye *et al.*, 1992).

Large-scale industial production of L-alanine from pyruvate with NAD^+ dependent alanine dehydrogenase has been proposed. However, the application of this method to industrial production of L-alanine has been hampered by the cost of coenzymes. In 2004, a multienzyme reaction system approach for L-alanine production was attempted in *Escherichia coli* BL21(DE3) by the heterologous gene expression between formate dehydrogenase gene (*fdh*) and alanine dehydrogenase gene (*aladh*). The two method were performed 1) cloning of heterologous gene of *aladh* and *fdh* in high expression vector pET-17b and 2) co-transformation of plasmid containing *aladh* and *fdh* under T7 promoter. The coupling reaction of these two enzymes was shown in Figure 1.1. However, the production of alanine by various recombinatnt clones were not significantly different with ratio of D:L form about 1.6:1 because of the activity of alanine racemase (Hatrongjitt, 2004). In *E. coli*, two genes of alanine racemase are present. The *alr* gene encodes the constitutively expressed biosynthetic enzyme, sufficient to provide enough D-alanine for cell wall

biosynthesis. The catabolic *dadX* gene encodes a second alanine racemase isozyme whose expression is subject to induction by L-alanine and thus is most active when L-alanine is used as a carbon and energy source (Lobocka *et al.*, 1994). In this research, both alanine racemase genes were inactivated by using group II intron, which has advantages on insertional mutagenesis, because it is minimally dependence on host factors and highly specific into target site.

4.1 Creation and confirmation of *alr* gene knockout mutant

To target group II intron into *alr* gene, the *alr* gene sequence was submitted to Intron Prediction Program. In the entired 1,080 bp of *alr* gene, two recognition sited for the intron insertion were predicted. The E-values (expected number of false positives) ranged from 0.06-0.491. To complete inactivation of *alr* gene, the site 636/637, with E-value 0.060 was chosen for construction of pACD4K-C-Alr.

PCR analysis by using *alr* gene specific primers showed *alr* gene inactivation band of 3,368 bp that come from 1,080 bp of *alr* gene and 2,288 bp of intron. Since the mutant will be applied for L-alanine production under T7 system, the intactness of T7 RNA polymerase gene was confirmed by T7 For and T7 Rev primers. The 3,557 bp PCR product indicated that the T7 RNA polymerase gene should not be disrupted. Nucleotide alignment of *alr* gene and *dadX* gene showed 45% similarity so the unwanted insertion of pACD4K-C-Alr into *dadX* gene was investigated. The *dadX* gene still showed 1,071 bp PCR product like that of wild type. Thus, the *dadX* gene was intacted. In Southern blot analysis, *Bam*HI, *Hin*dIII and *Xho*I digested DNA from the *alr* gene knockout mutant gave only one hybridized band indicative single intron insertion.

4.2 Construction and confirmation of *dadX* gene knockout mutant

As described in section 4.1, PCR analysis suggested that *dadX* gene of *E. coli* BL21(DE3) was specifically inactivated by group II intron insertion whilst T7 RNA polymerase gene and *alr* gene were still intact.

From Southern blot analysis, *Hin*dIII digested DNA showed single hybridized band that revealed one intron insertion while *Bam*HI and *Kpn*I digested DNA gave 2 bands. However, the lower hybridized band of *Bam*HI and *Kpn*I digested DNA correlated well with those obtained from double knockout mutant (*dadX* and *alr* genes). Thus, the upper hybridized bands should come from uncomplete digested chromosomal DNA. Therefore, it can be concluded that the group II intron was inserted only a desired site.

4.3 Construction and confirmation of *dadX* and *alr* genes knockout mutant

To sequentially disrupt multiple gene in the same bacterial cell, an antibiotic resistant gene cassette from the first plasmid must be excised from host chromosomal DNA before the second gene inactivation. By using the application of Cre/loxP system, the pACD4K-C-loxP was used for creation of *dadX* and *alr* genes knockout mutant.

Firstly, pACD4K-C-loxP-Dad was constructed and transformed into *E. coli* BL21(DE3). PCR analysis confirmed that the *dadX* gene was disrupted by intron insertion of 2,355 bp intron and T7 RNA polymerase as well as *alr* genes were undamaged. Moreover, Southern blot analysis by using 350 bp retargeting PCR product of *dadX* gene as a probe clearly showed single intron insertion.

Secondly, the kanamycin resistant gene cassette (1,367 bp) was excised from *dadX* gene knockout mutant by using Cre/loxP system from pACD4K-C-loxP and p706-Cre. The 2,059 bp of PCR product was obtained in this step

Finally, the *alr* gene inactivation was consequently performed. pACD4K-C-loxP-Alr was constructed and introduced into *dadX* gene knockout mutant by electroporation. PCR analysis by using *alr* gene specific primers gave a 3,435 bp PCR product resulted from 1,080 bp of *alr* gene and 2,355 bp of intron. T7 RNA polymerase gene was confirmed to be intact. Southern blot analysis by using *dadX* and *alr* probes showed only one by hybridized band for all of restriction enzyme digested DNA. This result indicated that intron specifically inserted into *dadX* and *alr* genes.

In this study, group II intron was specifically inserted into target alanine racemase genes of *E. coli* BL21(DE3) at frequencies of 1.25-3.5%. In *Staphylococcus aureus*, intron insertion frequencies to disrupt the *Seb* gene and *Has* gene was 37 and 100%, respectively (Yao *et al.*, 2006) whereas intron insertion frequencies of alpha toxin gene (*plc*) in *Clostridium perfringens* was 5.3% (Chen *et al.*, 2005). In *E. coli*, many publications were reported for intron insertion with various frequencies for example 1-80% in the group of DNA helicase gene (Perutka *et al.*, 2004) and 0.1-22% in *thyA* gene (Kerberg *et al.*, 2001). Thus, the efficient of group II intron insertion may differ for different genes and different organism.

Because of its high specificity, group II intron is applied in many fields. *C. perfringens*, an intestinal microorganism, produced alpha toxin that is responsible for its pathogenesis. To use this bacteria a safe vaccine vector, group II intron was applied into alpha toxin gene (Chen *et al.*, 2005). In addition, *S. aureaus*, a facultative

pathogen which is responsible for most of the infections suffered by hospital patient. Group II intron was applied to disruption *Has* and *Seb* genes, to understand the pathobiology of this organism and to develop new antibacterial agent to counter (Yal *et al.*, 2007)

4.4 Growth of *E.coli* BL21(DE3) and mutants

In general, D-alanine was directly synthesized by alanine racemase, encoded by *alr* gene, for cell wall biosynthesis. In this experiment, the *alr* gene knockout mutant showed growth rate constant 2.0 times lower then that of wild type. Contrarily, *dadX* gene knockout mutant gave an equal growth rate constant to that of wild type. It can be explained that *alr* gene in *dadX* knockout mutant was still intact so the level of alanine racemase encoded by *alr* gene, when growing the cell in LB medium, should not differ from that of wild type. The growth of *dadX* and *alr* genes knockout mutant could not performed in this experiment because its showed slow growth in LB medium supplemented with 10 mM D-alanine.

4.5 Alanine racemase activity assay from *E. coli* BL21(DE3) and mutants

In this research, alanine dehydrogenase was prepared for alanine racemase activity assay. Alanine dehydrogenase from *Aeromonas hydrophila* was inserted into pUC18 (pUCAlaDH) and introduced to *E. coli* JM109 (Poomipark, 2003). Consequently, the gene was subcloned into high expression vector pET17b (pETAlaDH) and transformed into *E. coli* BL21(DE3) by Hatrongjitt (2004). Thus, *E. coli* BL21(DE3) harbouring pETAlaDH was used as a source of alanine dehydrogenase. From 5 gram of cell wet weight (1,000 ml of culture medium),

15,100 unit of crude enzyme was obtained. It was 3.2 times higher than the total activity of crude extract from *E. coli* JM109 harbouring pUCAlaDH since the gene was expressed under T7 promoter. The purified enzyme showed no activity of alanine racemase so it was suitable for determination of alanine racemase from the alanine racemase gene knockout mutants.

The *alr* gene knockout mutant, *dadX* gene knockout mutant as well as *dadX* and *alr* genes knockout mutant were analyzed for alanine racemase activity. The crude extract from *alr* gene knockout mutant and *dadX* gene knockout mutant had lower specific activity than that of wild type 3.3 and 1.1 times, respectively. In addition, *dadX* and *alr* genes knockout mutant grew very slowly in LB medium supplemented with 10 mM D-alanine with OD_{600} = 0.2 upon the cultivation at 37°C for 48 hours. Moreover, the latest mutant could not grow on LB plate supplemented with 10 mM D-alnine. Thus its alanine racemase activity could not be performed.

D-alanine is an essential component in a peptidoglycan layer of bacterial cell wall. The essentiality of D-alanine stems from the key role of the dipeptide D-alanyl-D-alanine in the cross-linking of peptidoglycan strand. *Corynebacterium glutamicum* 13032*Δalr91*, Gram-positive bacteria displayed a strict dependence on the presence of D-alanine on LB medium suggesting that the 75 bp deletion within the *alr* gene resulted in a D-alanine auxotrophic phenotype. In general, the D-isomer of alanine is not presence in common complex media. Therefore, LB medium was routinely supplement with 0.4 g/l D-alanine to support growth of the *alr* mutant strain. Furthermore, cell were plated on minimal medium without D-alanine or L-alanine supplement. The growth assay revealed that D-alanine was still nessary for growth. This result rule out the presence of a second alanine racemase inducible by L-alanine.

Consequently, these result suggested that D-alanine biosynthesis in *C. glutamicum* is dependent on a single alanine racemase (Tauch, 2002). In contrast, this study showed that D-alanine was not necessary for growth of the *dadX* gene knockout mutant and *alr* gene knockout mutant. The result corresponded to that explained by Wasserman *et al.*, (1983) that the *dadX* gene encodes for a second source of D-alanine for cell wall biosynthesis by L-alanine inducible in *Salmonella typhimurium*.

4.6 Stability of intron insertion in *E. coli* BL21(DE3) and mutants

At the 20th subculture, the *alr* gene knockout mutant and *dadX* gene knockout mutant showed stable insertion. The *plc* gene knockout mutant in *C. perfringen* was also reported to be stable the throughout the 10 day of daily subculture (Chen *et al.*, 2005). Since the ORF encoding the IEP is deleted, the intron is unable to splice after insertion at a target location (Zhong *et al.*, 2003) In double alanine racemase gene inactivation mutant, its stability could not be determined because the mutant could not growth on LB plated containing 10 mM D-alanine.

From the result obtained from this work, the *alr* gene knockout mutant should be a good alternative for L-alanine production by *E. coli* BL21(DE3) under T7 promoter since its alanine racemase activity is 3.3 and 2.2 fold lower than that of *E. coli* BL21(DE3) and *dadX* gene knockout mutant, respectively.