CHAPTER IV

DISCUSSION AND CONCLUSION

In 1993, Surasak constructed recombinant plasmids pCSBC5 and pCSBC8 which contain CGTase gene from *Bacillus* sp. A11 in *E. coli* vector, pUC18 and pSE411, respectively. However, most of the gene product, CGTase, in *E. coli* accumulated in the periplasmic space (Sin *et al.*, 1993). Therefore, in order to produce large amount of CGTase, the *E. coli* system may not be suitable. It is well known that *Bacillus subtilis* secretes a large number of proteins efficiently into the culture medium (Sin *et al.*, 1993), and is a more acceptable host (Schmid, 1989). It is more advantage that CGTase is produced by using the expression and secretion system of *B. subtilis*.

The CGTase gene from the *Bacillus subtilis* strain ATCC21783 had been cloned previously in *E. coli* (Kaneko *et al.*, 1988). The yield of 0.7-fold compared to that of wild type was reported. Other CGTase genes had also been cloned into *B. subtilis* but only one case had the yield being high enough for profitable industrial production (Schmid, 1989). Kimura *et al.* (1987) cloned the CGTase gene from the alkalophilic *Bacillus* sp. 1011 into *E. coli* using pBR322 and *B. subtilis* using pUB110. He reported a 5.7-fold increase in enzyme

yield from *E. coli* and a 0.7-fold yield from *B. subtilis* compared to that from the wild type strain.

The aim of this study was to construct an expression system using *Bacillus subtilis* as host for CGTase production. A shuttle vector pHV33 (Primrose and Ehrlich, 1981; Old and Primrose, 1989) was chosen as a cloning vector. Experiment strategy was as follows: First, the cloned CGTase gene from *Bacillus* sp. A11 in pCSBC5 or pCSBC8 was subcloned into the shuttle vector, pHV33, using an *E. coli* host. Since, transformation into *B. subtilis* host was more complicate to perform than *E. coli* host (Old and Primrose, 1989) and the transformation efficiency was lower. Second, if *E. coli* transformants were positively tested for CGTase activity, a constructed plasmid would be transferred into *B. subtilis* for CGTase production. The experiments reported here showed, however, that subcloning of CGTase gene in pHV33 and *Bacillus* sp. vector, pUB110 were unsuccessful. The reasons for this failure were the followings:

In subcloning experiment of *Hin*dIII and *Sal*I digested CGTase gene from pCSBC8 into 6.2 kb *Hin*dIII and *Sal*I-digested pHV33, the 6.2 kb linear vector DNA of pHV33 could not be prepared. Since pHV33 which had been digested with *Sal*I could not be cleaved with *Hin*dIII (Fig.3.7). While the pHV33 which had been partially digested with *Hin*dIII for 30 min, 1 h and 2 h could not be cleaved with *Sal*I (Fig.3.8 A). However, when digested at longer time with *Hin*dIII, the pHV33 was completely cleaved, two DNA fragments of 4.3 kb and 2.5 kb were obtained (Fig.3.8 B). The *Hin*dIII-digested pHV33 fragment of

4.3 kb was cleaved into 3.7 kb with *Sal*I.

It was found that no transformant,carrying the hybrid plasmid, occurred in subcloning of pC194 segment from pHV33 into *Hin*dIII site of pCSBC8 and pCSBC5 (Table 3.1). The failure of transformation was also found in the experiment of preparation of modified pHV33 by religated *Pvu*II-digested pHV33. In order to remove one *Hin*dIII site adjacent to tetracycline resistance gene and to reduce the size of vector DNA. However,no transformant which carried the modified plasmid vector (4.5 kb) occurred (Table 3.2).

The transformation of not only constructed shuttle vector, pHV33 in our laboratory but also cloned tetracycline resistance gene (about 2 kb) from pBR322 into a shuttle vector pRB374 (Brueckner,1992) could not be done successfully by one of my colleaques (Personal Communication).

The plasmid DNAs could be ligated, but no transformant occurred. The reason for this was still unknown. However, the control transformation process of plasmids pBR322, pHV33 and self-religated of *Hin*dIII-digested pBR322 and *Bam*HI-digested pHV33 could be done successfully (Table 3.1 and 3.2). Therefore, the unsuccessful constructed shuttle vector may be effect of transformation efficiency. Since the competent cells *E. coli* were prepared by $CaCl_2$, the transformant efficiency of pBR322 was about 10^5-10^6 transformants/μg DNA. From Table 3.1 and 3.2, the transformation efficiency of pBR322 into *E. coli* was about 7-8x10^5 transformants /μg DNA , but pHV33 into *E. coli* was just 6x10^3 transformts/μg DNA. In order to increase

the transformation efficiency, the competent cells were prepared by $RuCl_2$ (Hanaharn,1983), it was found that the transformation efficiency was the same as the $CaCl_2$ method.However the transformation efficiency of this experiment may be improved by preparing competent cells by DMSO (Fani *et al.*, 1986), which has been reported to yield the transformation efficiency of about 10^7-10^8 transformants/μg DNA, should be done.

The subcloning of CGTase gene containing fragment from pCSBC8 and pCSBC5 into the shuttle vector pHV33 as described above could not be done successfully. Therefore, CGTase gene containing fragment from pCSBC5 was subcloned into pUB110, which there were limited cloning sites, at *Eco*RI and *Pvu*II sites. CGTase gene containing DNA fragment from pCSBC5 was prepared by digestion pCSBC5 with *Eco*RI and *Hin*cII (*Sal*I site), whereas, the vector pUB110 was prepared by cleaving with *Eco*RI and *Pvu*II. *Hin*cII and *Pvu*II digestions produced blunt-ended DNA,which can be ligated to each other. The expected DNA fragments from the digestion of pCSBC5 with *Eco*RI and *Hin*cII should be of 4.7 kb (CGTase gene containing fragment) and 3.2 kb. In contrast to the result (Fig.3.17) obtained, two DNA fragments of about 3.9 kb and 2.7 kb, which were not target DNA fragments, were obtained. Recognition sequence of *Hin*cII, *Sal*I and *Acc*I are 5'-GTCorT/AorGAC-3', 5'-G/TCGAC-3' and 5'-GT/(AorC)(GorT)AC-3', respectively. It indicated that not only *Sal*I but also *Acc*I sites on CGTase gene of pCSBC5 could be cleaved with restriction endonuclease *Hin*cII.

However,the shuttle vector pHV23 was used as a cloning vector

by Paloheimo *et al.*(1992). The *Kpn*I fragment containing CGTase gene from *Bacillus circulans* var. *alkalophilus* ATCC21783 was ligated into *Kpn*I-cleaved pHV23, resulting recombinant plasmid pALK153 and then was transformed into *B. subtilis*. The clones obtained did not show any amylolytic activity but produced some CGTase activity,and a faint band corresponding to CGTase was detected on Western blot of the culture supernatant after 2-3 days growth in CGTase expression medium.

Unfortunately,about 1 year later *E. coli* contained both pCSBC5 and pCSBC8 had lost their CGTase activity as assayed by iodine test and CD-TCE complex formation. It suggested that the CGTase gene in pCSBC5 and pCSBC8 were unstable in terms of their activities. However, the sizes and restriction maps were found to be the same as reported by Surasak (Personal communication).

Attempt was made to reclone the CGTase gene from *Bacillus* sp. A11 to plasmid vector pUC18 in order to construct a new stable clone. Therefore, CGTase gene containing DNA fragment from pCSBC5 was used as DNA probe for the isolation of CGTase gene from chromosomal DNA of *Bacillus* sp. A11 via DNA-DNA hybridization technique.

The labeled CGTase gene probe was prepared by using random primed labeling system in the presence of DIG-11-dUTP (Boehringer, 1993). This reaction is by the ELISA principle (enzyme linked immunosorbent assay) (Kassler *et al.*, 1992; Lion and Hass, 1990; Martin *et al.*,1987) and catalyzed by Klenow polymerase. The steroid hapten digoxigenin is linked with uracil at position C5 of

the deoxyribonucleotide by an 11-atom spacer. The denatured single stranded templates are annealed to the random mixture of hexanucleotides. The synthesis is started by adding Klenow polymerase and all deoxynucleotides, one is the hapten modified substrated (DIG-dUTP), Klenow polymerase incorporates not only the nonmodified deoxynucleotide but also DIG-dUTP. The primers are able to bind all possible target sequences (both of two single strands may act as templates). However, a small amount of DNA can be used and gives high specific activity (Boehringer, 1993). Hybridization was detected by using chemiluminescent detection method. The emission of light was catalyzed by alkaline phosphatase (linked to digoxigenin antibody) using Lumigen PPD as a substrate.

Hybridization of CGTase gene probe with chromosomal DNA from *Bacillus* sp. A11, digested with *Hin*dIII, as shown in Fig.3.20 resulted in intense bands signal at about 2.5-4.4 kb. These 2.5-4.4 kb DNA fragments were chosen for recloning into pUC18 vector. The DNA fragment was ligated to *Hin*dIII-digested pUC18 and transformed into *E. coli* JM101 competent cells. 26 white colonies were tested for dextrinizing activity by iodine test (Table 3.3). It appeared only just one white colony, namely no.17 which showed clear zone around the colony. In order to find more positive clones, many cloning experiments as mentioned above had been repeated several times. More than 1,000 white colonies derived from the cloning into pUC18 of *Hin*dIII-digested chromosomal DNA were obtained, but all were tested negative for halo formation. The result indicated that the

transformant no.17 may contain CGTase gene. To test whether plasmid isolated from transformant no.17 contained the same DNA fragment as that in pCSBC5, DNA hybridization with CGTase gene containing probe was carried out using the insert fragment from pCSBC5 as a probe. From the result shown in Fig.3.21 and 3.22, the recombinant plasmid no.17, digested with *Hin*dIII, could hybridize with 1.7 kb labeled CGTase gene fragment probe and 3 kb labeled CGTase gene fragment probe. The size of the signal band was 3.54 kb. The result suggested that the recombinant no.17 may carry CGTase gene from *Bacillus* sp. A11. Unfornately, the transformant no.17 lost its dextrinizing activity upon subculture. The plasmid stability has been studied in *B. subtilis* by Paloheimo *et al.* (1992). The *B. subtilis* CGTase producing clones ALKO2189 and ALKO2279 were grown for 50 generations without antibiotic selection. All the colonies tested were kanamycin-resistant and also showed halo-formation on starch plates after iodine treatment. Both segregational and structural instability were thus under 0.5%, indicating the two strains to be sufficiently stable for large scale fermenter culture. Thus, systematic search for more stable CGTase clone still remained to be done.

CONCLUSIONS

1. The linear form of shuttle vector pHV33 6.2 kb could not be prepared by particular cleaving the *Hin*dIII site of tetracycline resistance gene, therefore subcloning of CGTase gene from pCSBC8 to the shuttle vector pHV33 at *Hin*dIII site (on tetracycline resistance gene) and *Sal*I site were not successful.

2. It was found that no transformant, carrying the hybrid plasmid, was observed in subcloning of pC194 segment from pHV33 into *Hin*dIII site of pCSBC8 and pCSBC5.

3. The pHV33 was digested with *Pvu*II and religated in order to remove one *Hin*dIII site adjacent to tetracycline resistance gene and to reduce the size of vector (4.5 kb). However, no transformant which carried the modified plasmid vector was observed. Since, the vector DNA could not be prepared, subcloning of CGTase from pCSBC5 to the vector DNA could not be done successfully.

4. The complete CGTase gene could not be separated from pCSBC5 by cleaving with *Eco*RI and *Hin*cII. The subcloning of CGTase gene from pCSBC5 to vector plasmid pUB110 could not be done successfully.

5. Cloning of 2.5-4.4 kb DNA fragments of the *Hin*dIII-digested chromosomal DNA of *Bacillus* sp. A11 into *Hin*dIII-digested pUC18, it was found that only one transformant, namely no.17 showed dextrinizing activity (clear zone around the colony). The DNA fragment 3.54 kb from the *Hin*dIII-digested the recombinant plasmid

no.17 could hybridize to both labeled CGTase gene fragment 1.7 kb and 3 kb probes from pCSBC5. After subcultured, the transformant no.17 could not produce clear zone around the colony upon iodine test. It indicated that the transformant no.17 was not a stable clone.