CHAPTER IV

DISCUSSION

The research group in Starch and Cyclodextrin Research Unit, Chulalongkorn University has been working on several CGTase producing bacteria such as *Paenibacillus* sp A11 (*B. circulan* A11), *Paenibacillus* sp. RB01, *Paenibacillus* sp. T16 and *Paenibacillus* sp. BT. These enzymes were quite extensively studied especially in the aspect of enzyme purification and characterization. However, cloning and sequencing of CGTase gene was performed only in *B. circulans* A11 (**69**). The present study focused on the cloning and expression of CGTase genes from thermotolerant *Paenibacillus* sp.RB01 and *Paenibacillus* sp. T16.

4.1 Monitoring CGTase activities

4.1.1 Screening method on solid medium

During the experiments on cloning, identification of the cells or transformant producing CGTase were performed on the agar plate of either Horikoshi or LB broth medium with added soluble starch at the appropriate pH. Two types of strains were used. Firstly, iodine solution was used to detect the utilization of starch around the positive colonies. The colonies producing CGTase used up starch around the colonies to produce CDs. Iodine stained the unused starch on the plate and appeared as dark blue color. leaving the area around the positive colonies unstained and appeared as clear halo zone. This technique was also positive for enzymes with starch hydrolyzing activities. Secondly, phenolphthalein-methylorange was used to detect β-CD production by CGTase. Phenolphthalein was encapsulated in the cavity of β-CD produced by CGTase, resulting in the formation of colorless dianion of phenolphthalein against the pink background of phenolphthalein on the agar plate. To ensure that the colorless appearance of phenolphthalein around the colonies was not caused by acidic condition produced by

some acidic bacteria, methyl orange was added. Methyl orange will appear reddish in acidic pH. If the area around the positive colonies appeared yellowish, not reddish, the colonies were positive for the presence of β-CD. Throughout our experiments, we employed either iodine and phenolphthalein methods concurrently or the phenolphthalein method alone.

In 1982, Vikmon M.(**89**) used the phenomenon of inclusion complex formation between β-cyclodextrin and phenolphthalein resulting in a colorless complex to develop a spectro-photometric method to measure β-cyclodextrin in solutions (**1, 90**). In 1986, Taguchi reported that phenolphthalein was transformed into a colorless dianion within the cavity of β-CD. On this principle, Park *et al.*, (1989) designed a screening medium to isolate β-CGTase producing bacteria from soil samples (**91**). This method later was developed as a simple and rapid screening by which CGTase could be specifically detected on the basis of the reduction of the color intensity of phenolphthalein under alkaline condition by β-CD product of CGTase. However, a reduction in the color intensity was not observed for α-CD since the complex of α-CD and phenolopthalein was not easily formed. Bromocresol green are generally employed for detection of γ-CD (**92**).

In the present study, we used the modification method of Nomoto (1986) and Park *et al.*, (1989) to screen the positive colony (**91, 93**). The general and non-specific iodine-staining method was indicative of starch hydrolysis; therefore, it could not discriminate between any two amylolytic enzymes (**94**). Moreover, it was known that most of the β-CGTase-secreting bacterial strains also produced other amylolytic enzymes, such as α-amylase (**95**). In these cases, using iodine staining alone could lead to misinterpretation of the result.

4.1.2 Assay of enzyme activity

CGTase activity was also quantitated spectrophotometically by color reaction with iodine or phenolphthalein, based on the same principle as discussed in 4.1.1. Iodine-starch complex was monitored by absorbance at 660 nm and CGTase activity was monitored by utilization of starch resulting in decrease in absorbance of starch-iodine complex. Formation of phenolphthalein- β-CD inclusion complex was monitored by the reduction in absorbance at 540 nm due to the colorless of phenolphthalein-inclusion complexes within β-CD cavity (**1, 90**). The latter technique was called cyclization or CD forming activity. We employed the dextrinizing activity for general assay for CGTase activity. Whenever more specific CGTase characterization was required, cyclization assay was preferably used.

4.2 Cloning and sequencing of CGTase gene

In general, CGTases from different microorganisms produce all three types of cyclodextrins with different ratios. They showed similarity in nucleotide sequences and deduced amino acid sequences ranging from 47% to 99%. Primers A and B used in this experiment was designed from the upstream and downstream sequence of CGTase gene of *Paenibacillus* sp A11, the first CGTase producing bacteria studied in the starch and cyclodextrin research group. Its CGTase gene was fully sequenced and published with accession number AAG31622. The idea to use *Paenibacillus* sp. A11 as template for design these pairs of primers was obtained from the high similarity scores of the small fragments amplified from A11's primers among genomic DNA of A11, RB01, T16 (data not shown). Primer A : 5'- GGCTA TGCT T TCCTT ACCTTACCC -3' and Primer B : 5'-ATAGC ACC TTT CCCCC ACATAACG -3', were designed to cover the whole CGTase gene including the -35 and -10 nucleotide sequences of promoter on the upstream of CGTase gene segment from *Paenibacillus* sp A11. PCR reactions using

these primers produced PCR product of 2.4 Kb-length only with DNA from pVR316 (a plasmid DNA containing A11 CGTase gene), RB01 and T16. The negative control, *B. liceniformis* which was a chitinase producing *bacillus*, did not produce PCR product with our designed primers (Figure 4 (c)). PCR product of each strain was ligated with pGEM® –T easy vector at 16°C for 18 hours and then transformed into *E. coli* JM109 using electrophoration. The bacteria containing ligated plasmids were cultured on LB agar plate at 37°C for 18 hours and stained with iodine solution or with phenolphthalein-methyl orange solution. CGTase producing bacteria produced clear zone (yellowished background) around the positive colony, by the phenomenon of inclusion complex formation between β-cyclodextrin and phenolphthalein.

Because the pGEM®-T Vector systems were convenient systems for the cloning of PCR products. The pGEM®-T Vector was prepared by cutting the pGEM®-5Zf (+) vector with *Eco*R V and adding a 3′ terminal thymidine to both ends. The single 3′-T overhung at the insertion site greatly improved the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for ligation of PCR products generated by certain thermostable polymerases. These polymerases often added a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of amplified fragments. The multiple cloning site was flanked by recognition sites for the restriction enzyme *Bst*Z I, allowing release of the insert by a single-enzyme digestion. Alternatively, a double digestion may be used to release the insert from the vector. T7 and SP6 RNA polymerase promoters of the vector flanked a multiple cloning region within the α-peptide coding region for β-galactosidase. Insertional inactivation of the α-peptide allowed recombinant clones to be directly identified by color screening on indicator plates. Blue/white colony screening relied on

disruption of the *lac*Z gene (Figure 5). So, the potential transformants with white colonies could be chosen.

In order to test the success of ligation reaction of vector and PCR fragments, an aliquot of linearized, not phosphatase treated plasmid should be transformed and plated on selective medium for checking self-ligation in parallel with the experimental samples. Successful ligation resulted in greater number of colonies on the plate with self-ligated plasmid compared to the cells transformed with linear, unligated plasmid. Moreover, the number of colonies on the linear, unligated plasmid (unligated plasmid background) should be low if the plasmid's linearization was efficient. The number of colonies with the self-ligated plasmid should be comparable to the number of colonies obtained with the uncut plasmid transformation control if the ligation reaction was efficient.

The clones of *Paenibacillus* sp. RB01 and *Paenibacillus* sp. T16 were called pRB and pT, respectively. The plasmids of the clones were prepared and sequenced. The CGTase gene contained its own promoter region, and could be expressed in *E. coli* JM109. The DNA sequence showed an open reading frame of 2194 bp encoding 732 amino acid residues for pRB and an open reading frame of 2139 bp encoding 713 amino acid residues for pT. Amino acid alignment among the CGTase from the *Paenibacillus* sp.RB01 and T16 and other bacteria were performed and shown in Figure 10. The deduced amino acid sequences of pRB and pT showed high homology to Alkalophilic *Bacilli.* The amino acid sequences of pRB and pT showed 97% homology for each and among those *Bacilli* used for alignment. pRB gave 97% identities to the CGTases from A11, 95% to *Bacillus* sp. #1011 and *Bacillus* sp. Strain no.38-2, 85% to *B. circulans* strain 251. pT gave 99% identities to the CGTases from A11, 98% to *Bacillus* sp. #1011, 97% to *Bacillus* sp. Strain no.38-2 and 85% to *B. circulans* strain 251. The four

conserved regions, in all members of the α-amylase family (**96, 97**), were also found in our transformants CGTase. The different amino acid resiudes were found in pRB and pT at residue 95 (shown as glycine (G) and aspartic acid (D), respectively), at residue 340 (shown as asparagine (N) and aspartic acid (D), respectively) and most of the C-terminal sequences of their CGTase sequences, at position from residue 692 to their end of C-terminal. However, the conserved amino acid residues which was specific for all of the CGTase gene sequence on the C-terminal were present such as proline (P) at residue 699, alanine (A) at residue 714 and asparagines (N) at residue 717 (Figure 10).

4.3 CGTase production by recombinant of *Paenibacillus* sp. RB01 and T16

The β-CD synthetase gene from an alkalophillic *Bacillus* sp. #1011(**37**), *Bacillus* sp. Strain no.38-2 (**38**) and *Klebsiella pneumoniae* M5al (**39**) were cloned and expressed in *E. coli* and *Bacillus subtillis*. It was well known that CGTases produced from *Bacillus sp*. were usually secreted into the culture medium. Most of the CGTases expressed in *E. coli*, except those of *K. pneumoniae* (**39**) and *B.* sp.E1 (**98**) were present in the periplasm due to another spatial barrier, the outer membrane. However, the enzyme preparation from the culture supernatant of native CGTase and from the periplasmic fraction of the *E. coli* transformant exhibited the same molecular weight, reaction condition, stability, the production of cyclodextrins and their enzymatic properties (**82, 85, 87**).

From prelimilary test of this study, yields of the excretion of CGTase from the transformants expressed in *E. coli* were calculated from the relative ratios between CGTase activity from the culture media containing bacterial cells and CGTase activity in the homogenate of whole cells separated from the cultured media. The satisfactory levels of 60% of CGTase activity were detected in the culture medium (data not shown). So, the crude enzyme obtained from extracellular CGTase in the culture medium was employed for this study.

4.4 Optimum culture condition for CGTase production

Tesana , 2001 and Pranomitr, 2001 previously reported the optimum conditions for CGTase productions in RB01 and T16, respectively. (**66, 67**). Most wild type CGTase producing bacteria grew well in Horikoshi's medium pH10, containing soluble starch which acted as inducer of CGTase. However, transformants with *E. coli* as host cells cannot grow in such alkaline medium. From our experiments, LB broth at pH 7.0 with added soluble starch seemed to be suitable as medium for growth and CGTase induction in the transformant cells of RB01 and T16. Table 11 showed comparison of the optimum culture conditions for RB01, pRB, T16 and pT.

The culturing time to obtain maximum yield of CGTase production for RB01 and T16 were 60 and 72 h. while the transformants required only 24 h. Both of the transformants required shorter culture time, about one-third of the wild types in producing CGTase. Enzyme induction of the wild type was best at pH 10, whereas the neutral pH 7.0 was suitable for enzyme production in the transformant expressed in *E. coli*. This was different from other wild types such as *B. amylolyticus* 11149 (**99**) and *Thermococcus sp.* (**100**) which required optimum pH for growth and the enzyme production at 6.0 and 7.0, respectively. Our results especially of the wild type RB01 and T16 were compatable to those reported by Tesana (2001) and Pranomitr (2001).

The bacterial growth rates and enzyme production were greatly influenced by pH of the environment, growth temperature and culturing time. Most of bacteria producing CGTase were mesophiles (30-40°C) and were reported to grow well in the similar condition as *Paenibacillus* sp. RB01 and *Paenibacillus* sp. T16. Usual conditions for other CGTase producing bacteria were in the range of pH 7-10, 30-40°C and 24-90 h (Table 13).

Table 11 Comparison of growth and CGTase production profiles between wild-type and transformant CGTase.

	RB01	pRB	T16	pT16
Growth	30-45°C, pH 10	37 °C, pH 7	37-50°C, pH 10	37°C, pH 7
Condition of enzyme production	40 °C, pH 10	37 °C, pH 7	37°C, pH 10	37°C, pH 7
Time of cultivation (h)	60	24	72 h	24 h
Optimum condition Dextrinizing Cyclization	 60 °C, pH 5-6 60-70 °C, pH 6.5	 50-70°C, pH 5-9 50-60°C, pH 6.5	 60°C, pH 5-9 60-70°C, pH 6.5	 37-70°C, pH 5-9 50-70°C, pH 6.5
Stability * Thermostability pH	 40-65 °C, pH 6.5 pH 6-10	 40-65 °C, pH 6.5 pH 6-10	 40-55°C, pH 7.0 40-55°C, pH 7.0	 40-65°C, pH 7.0 40-60°C, pH 7.0

* Cyclization activity was retained to 60% relative activity after incubation for 1 hour.

4.5 Purification and Characterizations of CGTase from transformants

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 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 intended applications. The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility and the possession of specific binding sites. Most purification protocols require 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 for the enzyme to perform and maintain activity. The key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of step required (**101**).

One common step in the purification of CGTase reported by different laboratories was the use of starch adsorption (**47**). The interaction between starch and CGTase involves not only adsorption but also substrate-enzyme affinity type binding. The advantage of this step was that CGTase could be eluted in a small volume, which allowed a good recovery of the enzyme. Moreover, the elution of CGTase which was quite specific resulted in high purification fold since many other proteins were removed.

Most of purification schemes involve some forms of chromatography, which has become an essential tool in every laboratory where protein purification is needed. Ion-exchange chromatography separates proteins with differences in charge to give a very high resolution with high sample loading capacity (**101**). DEAE-cellulose is an anion exchanger and was used in the purification of CGTase from many sources. Its popularity

stems from the possibility of high resolving power, versatility, reproducibility and ease of performance.

Another types of column was also used in purification of CGTase, one of which was hydrophobic interaction chromatography (HIC) (**102, 103**). HIC takes advantage of the hydrophobicity of proteins promoting its separation on the basis of hydrophobic interaction between immobilized hydrophobic ligands on chromatographic medium and non-polar region on the surface of proteins. Phenyl-Sepharose CL-4B is the resin with the chemically bonded phenyl groups on the surface of Sepharose. Prior to HIC column, the sample ionic strength should be adjusted with salt to increase hydrophobicity of protein (**104**). Thus, care must be taken in the aspect of the salt concentration used for adsorption. It should be lower than the concentration used in precipitation of our enzyme. Usually, the salt concentration of 1M was used for increase hydrophobicity of protein. The elution of proteins is achieved by decreasing the salt concentration in order to increase hydrophobicity. Different kind of salt gives rise to difference in the strength between proteins and the HIC adsorbent. Some purification guidebooks suggest that HIC is ideal for used immediately after salt precipitation where the ionic strength of the sample will enhance hydrophobic interaction and also for avoiding the desalting step (**101, 104**).

4.5.1 Purification of CGTase from RB01 and pRB

CGTases from wild type RB01 and T16 and their transformants pRB and pT were purified in parallel to compare their biochemical properties. RB01 and pRB enzymes were purified by starch adsorption and DEAE-cellulose column chromatography. From Table 11, total enzyme activities of pRB, was much higher while total proteins was lower resulting in much higher specific activity of CGTase from pRB. In the subsequently steps, substantial amount of proteins in RB01 preparation was removed

with little loss of dextrinizing activity while in pRB little protein was removed with high loss of dextrinizing activity. This resulted in the apparent higher purification and higher yield in RB01. The purification of CGTase using starch adsorption followed by ion-exchange chromatography column lead to the desired level of enzyme purity since one band on native gel gel electrophoresis was observed. Further purification by ion-exchange, DEAE-cellulose column showed decreasing of % yield for both in RB01 and pRB.

Starch adsorption seemed to be the crucial step in purification of CGTase since it removed high percentage of proteins from the crude enzyme with much lower percentage of loss of enzyme activity. pRB and pT seemed to substantially lost their dextrinizing activity in this step. (Table 12 (b)). The non-absorbed dextrinizing activity which were lost may be from other amylolytic enzymes produced by the host cell *E. coli* which had low binding capacity to the starch cake. However, the purification folds of all four enzymes through this step were still at high level except pRB.

4.5.2 Purification of CGTase from T16 and pT

Pranomitr (2001) reported that CGTase from T16 did not bind to ion exchange column and β-CD affinity column was used to purify the enzyme (**67**). In our experiment, T16 and pT did not bind to ion exchange column either. We subjected the enzymes to phenyl sepharose column and was able to use it to purify the enzymes. The column was eluted with 1 M to 0 M ammonium sulfate in 10 mM Tris-HCl pH 8.5. Both enzymes were eluted at very low concentrations of ammonium sulfates, suggesting that they were very hydrophobic. The difference in column binding abiligy of CGTase from RB and T16 and then transformants were in contrast of their high similarity in nucleotide and amino acid sequences. The possible explanation was the occurrence of some

differences in cellular environments such as pH, cellular compositions which caused the different folding of the protein resulting in different surface changes of the enzymes.

Purification of CGTase from *Bacillus circulans* was 30% recovery by adsorption on corn starch followed by size-exclusion chromatography (**47**). Purification of CGTase from alkaliphilic *Bacillus* sp by adsorption on corn starch eluted with 18.6% ammonium sulphate followed by chromatography on Q-Sepharose, achieved a 51% yield (**105**). Purification of CGTase from *Klebsiella pneumoniae* by an affinity matrix with gelatinized corn starch was 68% yield (**106**). CGTase from *Bacillus agaradhaerens* was purified with a yield of 50% by adsorption to corn starch in the presence of 1 M ammonium sulphate followed by elution with β-CD solution (**107**). CGTase from *B. circulans* was isolated and purified with α-cyclodextrin - derivatised Sepharose 4B affinity chromatography (**108**). Another method for purified the CGTase was Aqueous two-phase systems (ATPS). This method allowed selective partitioning with high yields and a good cost-benefit ratio (**109**). CGTase from *Bacillus circulans* DF 9R. was purified with two methods, affinity precipitation with starch and aqueous two-phase partition. The first method, optimized by a factorial design, gave an 80% CGTase adsorption at 11% starch eluted with 1.6% ammonium sulphate, and a 65% recovery after elution with 10 mM α-cyclodextrin. The purification fold was 17. Aqueous two-phase partition yielded a 72% CGTase recovery in a two-step procedure; CGTase was obtained in the bottom phase with a purification factor of 37 (**110**).

Table 12 Comparison of the purification profiles between wild-type and transformant CGTase.

(a)

Fraction	Total Protein (mg)				Dextrinizing activity (Units/ml)			
	RB01	pRB	T16	pT	RB01	pRB	T16	pT
Crude	388.9	293.9	465.6	188.7	60	183	28	161
Starch Adsorption	9.66	20.7	8.8	1.1	504	189	68	140
Column Chromatograhpy*	5.59	0.8	0.98	0.9	85	306	452	3509

(b)

Fraction	Total Dextrinizing activity (Units)				Specific activity (U/mg)			
	RB01	pRB	T16	pT	RB01	pRB	T16	pT
Crude	59500	210200	44750	181900	153	715	96	964
Starch Adsorption	42840	47250	27320	35000	4435	2283	3105	31818
Column Chromatograhpy*	30770	14688	9486	80700	5504	18360	9680	89667

(c)

Fraction	Purification fold				%Yield			
	RB01	pRB	T16	pT	RB01	pRB	T16	pT
Crude	1	1	1	1	100	100	100	100
Starch Adsorption	29	3	32	33	72	22	61	19
Column Chromatograhpy*	36	26	101	93	52	7	21	44

* DEAE-cellulose for RB01 and pRB, Phenyl Sepharose for T16 and pT

Preliminary experiment was performed by direct application of the enzyme solution from starch adsorption step to phenyl-Sepharose column after adding ammonium sulfate to final concentration of 1 M. It was found that even though great removal of other proteins was observed but elution time was too long about 10- 12 h which caused the unacceptable great loss of CGTase activity. The long operation time of the conventional method was solved by using the application of FPLC technique. The Phenyl-sepharose column was connected to the peristaltic pump controller that made the high pressure of the buffer pushed the unbound- and bound-protein and the process was finished within 2 hours.

Total enzyme activity in pT was much higher than T16, resulting in much higher specific activity of CGTase from pT (Table 12 (b)). In the subsequently steps, substantial amount of proteins in T16 preparation was removed with little loss of dextrinizing activity while in pT little protein was removed with high loss of dextrinizing activity. This resulted in the apparent higher yield in T16 in starch adsorption step. However, At the last step of purification, in T16 little protein was removed with high loss of dextrinizing activity. Therefore, the % yield in pT was higher than T16 (Table 12 (c)). The purification fold from subsequently steps of T16 and pT were similar increasing pattern.

The purification of CGTase using starch adsorption followed by DEAE-cellulose or Phenyl Sepharose column lead to the desired level of enzyme purity since one band on native gel gel electrophoresis was observed. These procedures gave acceptable yield and purification fold and high purity of CGTase (summarized in Table 12 (a-c)). All purified enzymes were subjected to SDS-PAGE and non-denaturing PAGE to determine their purity. The enzymes from the last step of purification showed high purity with only minute contamination observed in SDS-PAGE (Figures 16-18).

Considering the overall purification processes of all four enzymes, some interesting points were noted. The total amount of proteins in the crude extract or the extracellular proteins in the culture medium was much lower in the preparations from transformants. The culture medium contained proteins from 0.5% yeast extract and 0.5% polypeptone from meat in case of Horikosh, 0.5% yeast extract and 1% polypeptone from casein for LB. The total protein contents from each formula were 40 mg% and 80 mg% respectively. This might contribute partially to the protein content of crude enzyme extract but not at significant level, especially when protein in LB medium was twice that of Horikoshi medium. The low protein content in transformant cells may indicate low protein excreted extracellularly from *E. coli* cells. High level of CGTase were detected in crude preparation of transormant even though we had calculated that the CGTase in the medium was about 60% of the total CGTase produced by the cell culture. Perhaps, the CGTase expression system in the transformants were more efficient than the wild types.

Table 13 Properties of CGTases from different *Bacillus* sp.

Strain	Molecular Weight (kDa)	Optimum pH	pH stability	Optimum temperature (°C)	Temperature stability (°C)	Ratio of CD formed a:b:g	Reference
Paenibacillus sp. RB01	65	6.5	7-9	60-70	40-60	0.57 : 1 : 0.13	This study
pRB	66	6.5	7-9	40-70	40-60	0.21 : 1 : 0.05	This study
Paenibacillus sp. T16	76	6.5	7-9	60-70	40-60	0.95 : 1 : 1.57	This study
pT	77	7.5	7-9	50-70	40-60	0.25 : 1 : 0.51	This study
Paenibacillus sp. A11	72	5-6	—	60	—	1.0 : 4.1 : 1.1	**(47, 69)**
B. macerans IFO 3490	ND	5.2–5.7	8.0–10.0	55	55	71% α-CD	**(111, 112)**
B. megaterium	75	5.0–5.7	7.0–10.0	55	55	02:01.1	**(113)**
B. stearothermophilus	66.8	6	—	80	—	4.2:5.9:1	**(114)**
B. circulans E 192	78	5.5–5.8	7.0–8.0	60	45	1:07:03	**(115)**
B. circulans DF9R	78	5.5	6.5–8.5	60	—	1:0.9:0.4	**(116)**
B. amyloquefaciens AL35	ND	4.0–7.0	8.0–10.0	70	60	up to 95% α-CD	**(117)**
Alkaliphlic *B. sp.* ATCC21783	88	4.5–5.0	6.0–10.0	45	65	2:22:03	**(118)**
Alkaliphlic *B. sp.* ATCC21783	85–88	7.0 , 8.–9	6.0–9.0	50	60	0.4:14:2.5	**(119)**
B. coagulans A-147	ND	6.5	6.0–9.0	65	50	02:06.6	**(112)**
B. coagulans	36	6.5	5.0–10.0	—	65	8.1:8.9:1	**(120)**
B. lentus	33	6.5–7.5	6.5–8.5	45–55	55	07:01.6	**(121)**
B. firmus	ND	7.5–8.5	6.5–9.0	65	55	1:11:05	**(122)**
	ND	—	—	—	—	0:40:08	**(75)**
B. autolyticus	70	5.0–6.0	5.0–9.0	60	40	10:30:05	**(99)**
B. obhensis	ND	5.5	—	50	—	0:04:01	**(123)**
B. licheniformis	72	5.0–6.0	6.0–8.0	—	60	5:01:00	**(124)**
B. subtilis	64	8	6	65	50	mainly γ-CD	**(125)**
B. agaradhaerens LS-3C	110	9	5.0–11.4	55	40	0.12:1:0	**(107)**

4.5.3 Characterizations of CGTases from RB01, pRB, T16 and pT

4.5.3.1 Molecular weight Determination

All purified CGTases were subjected to SDS-PAGE to determine their molecular weight. From the calibration curves in Figure 19 (a) and (b). The molecular weight of CGTase from pRB and pT were 65 kDa and 77 kDa, respectively. Table 13 showed comparison of our CGTases to those previously reported.

Our results were the same as reported by Tesana (2001), and Pranomitr (2001). However, they did not agree from the data on deduced amino acid sequences in figures 8 and 9. From the deduced amino acid sequences, the approximate molecular weight of pRB and pT enzymes should be around 73200 and 71300 Da respectively. This indicated that in the expression processes, these enzymes should have been subjected to some modifications especially the enzyme from pRB, resulting in the lower molecula weights on SDS-PAGE.

The CGTase from *B. circulans* E192 showed 2 isozymes with different subunits of 33.5 kDa and 48.5 kDa, respectively (**126**). There were reported that CGTase from *B. macerans* IAM 1243 and *B.* megaterium NO.5 were dimeric protein of identical subunit size of 66 kDa and 145 kDa, respectively (**113**) (**78**). The extremely thermophilic anaerobic archaeon, a *Thermococcus* sp. strain B1001 was purified 1,750-fold, and the molecular mass was determined to be 83 kDa by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (**100**). Molecular weight of *Paenibacillus* sp.A11 was determined to be 72 kDa by SDS-PAGE (**47, 69**).

4.5.3.2 Determination of Isoelectric point

The isoelectric point of *Paenibacillus* sp. RB01 and T16 were determined by isoelectrofocusing gel in the ampholine pH range 3-10, comparing with standard pI marker (pI 3.5-9.3). The enzyme from pRB showed major band at pI 4.7 and other bands

at pI 5.85, 5.74 and 5.64, which all bands except pI 5.85 were observed in RB01. This was different from previous report by Yenpet (2002) that the enzyme from RB01 showed 3 bands of CGTase on ampholine gel, two major bands were found at pI 5.2 and 5.3 with one minor band at 5.1. For pT, it showed major band at pH 5.85 with faint bands at 5.55 and 5.35 while T16 showed protein bands at pI 5.74, 5.54, 5.34, 4.75 and 4.60 this result was different from previous report by Pranormit (2001) that CGTase from T16 showed 3 bands with the CGTase activity of pI 5.7, 5.2 (major band) and 4.77, repectively. In our experiment, only the major band belonged to pI 5.85 was observed for pT. There were also other bands appeared for RB01, pRB and T16 (except pT), outside the range of pI's of those reported by Tesana (2001) (**66**); Pranormit (2001) (**67**) and Yenpetch (2002) (**138**). These may be some contaminating proteins or may be some minor forms of CGTase.

The property of the IEF using in this study was the native-IEF. This technique had some disadvantage that was available for estimation the approximate net charge of the sample, the IEF patterns may be too complicated to be analyzed when it was used to distinguish glycosylation variants (**127**). The major pI band of CGTases from transformant were 5.85 which were not observed in wild type. The characteristic of CGTase pI implied that the total net charge of amino acid component of CGTase could be negative when the environment was in alkaline condition. So, the DEAE-cellulose column equilibrating with Tris-buffer, pH 8.5 should be appropriated to bind protein bound forms of all four CGTases which their total net charges were negative. Although the pI and the amino acid sequences of RB01, pRB, T16 and pT were quite similar, different molecular weight patterns on SDS-PAGE were observed by fast moving of protein bands of RB01 and pRB comparing to T16 and pT. In addition, only CGTase from RB and pRB could be purified by DEAE-cellulose, at pH 8.5 but CGTase from T16

and pT could not be purified by this column. The explanation of this phenomenone could be emphasis on the surface net charge of these CGTases. The similarity of pI and nucleotide sequences reflexed to the same total net charge of the amino acid contents. There might be different arrangement of the charged amino acids at the surface of CGTase of RB01, T16 and their transformants. This could affect T16 and pT enzymes ability to bind the DEAE-cellulose or CM-cellulose matrix and reflected on the preference binding to the strong hydrophobic column (Phenyl Sepharose) and their elutions at low concentrations of ammonium sulfate (0.1 M).

Yamagata et al. (1995) and Hirano et al. (2006) reported that the molecular mass of the purified enzyme determined by SDS-PAGE did not agree with the calculated molecular mass from the deduced amino acid sequence in spite of several trials of SDS-PAGE. Some alkaline proteases from alkalophilic *Bacillus*, including *Bacillus* sp. G-825-6, showed larger molecular masses on SDS-PAGE than those from the calculated data (**128, 129**). There might be some characteristic structure in those alkaline enzymes (**130**). Some studies reported that the glycosylation or glycoproteins interaction might be one of the causes of the retarded migration on SDS-PAGE (**131**). The products of *touABCDEF* gene were analyzed by SDS-PAGE. The expected molecular masses from sequencing data were catagorised into 3 groups. First, *tou A, B, C* and *E* were estimated corresponsed to the expected value. Second, *tou* F was slightly higher than analyzed on gel and might be due to the acidic negatively charged N-terminal region, which can retard migration, as previously observed with other chloroplast-type ferredoxins (**132**). Third, the estimated molecular mass of *tou D* was more than twice the expected mass. A similar overestimation of molecular mass in SDS-PAGE was also observed with the TmoC peptide (**133**). The low molecular mass along with the acidity of the peptide (pI

4.45) (**134**) or the incomplete reduction of a dimeric form might account for this result.(**135**).

4.5.3.3 Effect of pH and temperature on enzyme activity

All enzymes showed optimum activity at certain pH and temperature which may be a single point or at wide range. Most CGTases from mesophelic or alkalophilic bacteria had optimum pH at basidic side and optimum temperature around 40-50°C.

Optimum conditions for purified CGTases from RB01 and T16 were reported by Tesana (2001) (**66**) and Pranomitr (2001) (**67**). In our study, comparison of optimum pH of RB01, pRB. T16 and pT showed the same pattern for dextrinizing activity of both RB01 and pRB. pT seemed to have a wider range of optimum temperature than T16, around 40-75°C, while the cyclization activity remained the same. Table 13 summarized the comparative data of the optimum pH and temperature of CGTase in this study with those from some other *Bacillus* sp.

Pranomitr (2001) and Kaulpiboon J. (2003) reported that the types of buffer affected the enzyme activity (**65, 67**). Tris-HCl buffer was not suitable for CGTase since low enzyme activity was observed. Potassium phosphate buffer was more suitable and has been used widely as a buffer for the assay and purification of CGTase. However, Tris-HCl buffer, pH 8.5 or TB buffer was found to have no inhibitory effect on the CGTase activities of both pRB and pT and was used during the purification steps. Potassium phosphate buffer was also used as an assay buffer in this study. The optimum temperature for CGTase of RB01 was at 55°C, the temperature which gave the highest CD-product, comparing to *Paenibacillus* sp. A11 which showed optimum temperature at 40°C (**48**).

pH 6.0 was the best for CD-forming activity while pH 7.0 gave highest dextrinizing activity. The optimum starch degrading activity was observed at 65°C, and

the optimum CD-forming activity was at 55°C. To explain this, the reaction mechanism of CGTase has to be taken into account. Since the catalytic residues of CGTase are proposed to be equivalent to that of α-amylase. The transglycosylation reaction of CGTase is operated by Ping-Pong mechanism (**136**). In this mechanism, the transglycosylation occurs after the reducing side of the amylase cleaved product is released from the enzyme. Then the enzyme transfers the newly formed reducing end of the substrate either to the non reducing end of a separate linear acceptor molecule or glucose (the dispropotionation reaction) or to its own non-reducing end (the cyclization reaction or CD synthesis reaction). The hydrolysis reaction (the starch-degrading reaction) will occur when this intermediate is attacked nucleophilically by a water molecule. For preferential CD synthesis, the efficient formation of the helical structure of amylase in the active–site cleft of enzyme is required (**41, 137**). In a crystal structure, amylase can occur as a single helix with 6 to 8 glucose molecules in one helical turn (**138**). The most widely accepted hypothesis describes amylase in solution having an interrupted coil-like structure composed of helical and non-helical segments (**1**). Therefore, the formation of CD by CGTase can be explained as a consequence of preferential helical structure of amylase in solution. However, the high temperature will destabilize the helical structure of the amylase, resulting in a shift to random structure. Accordingly, it is considered that the reaction at high temperature by RB01 CGTase resulted in a shift towards the starch-degrading reaction (**67**).

4.5.3.4 Stablility of the enzymes

Enzyme stability was one of the important factors in considering the suitability of any enzyme to be used in industries. Ability of the thermotolerant bacteria to grow and produce enzymes at moderately high temperature must also be accompanied by the stability of the enzyme at higher temperatures for an appropriate period of time. From Figure 29-30 and 33-34, the stability of pRB enzyme in term of pH and temperature was quite the same as enzyme from wild type. However, enzymes from pT seemed to be more stable than T16 at all pH's, especially at acidic pH and temperatures higher than 50°C. At 70°C, the stability of all 4 enzymes improved by greater than 40% of in the presence of starch. The result was corresponded with those of reports that starch (substrate) enhanced the thermostability of CGTase from *Thermoanaerobic thermosulfurigenes* EM1 at 80-85°C and *Bacillus firmus* at higher than 30°C (**106**) (**139**). Furthermore, other reports showed that the stability of CGTase from *Bacillus* no A-40-2 at high temperature was increased when calcium ion was present (**68**).

Although CGTase was capable of hydrolyzing and cyclizing reactions, the two activities occurred in different proportion. It depends on pH and temperature. For RB01, the optimum pH for dextrinizing, cyclizing and CD-TCE activity were 5.0, 7.0 and 7.0-9.0 and the temperature were 65°C, 70°C and 55°C, respectively. The optimum pH and temperature of *Paenibacillus* sp. F8 on dextrinizing activity were 8.0 and 60°C and cyclizing activity were 7.5 and 50°C (**92**). CGTase from extremely thermophilic anaerobic archaeon strain B1001 had 110°C and pH 5.0-5.5 for optimum starch degrading while for cyclodextrin synthesis the optimum were 90-100°C and pH 5.0 (**100**).

When stablility was concerned, the enzyme was rather stable at 40°C while at 55°C, where maximum activity was, enzyme was almost totally loss at 30 minutes.

However, incubation time for assaying was only 10 minutes and no significant lost of activity was observed in the presence of substrate (Figure 32-35) or in the presence of $CaCl_2$ (**68**).

The stabilizing effect of Ca^{2+} on CGTase had also been previously reported. Calcium ion was effective to stabilize the enzyme CGTase from *Bacillus* no.A-40-2 especially at high temperature (**68**). The saturation of CGTase from *B. coagulans* with Ca^{2+} resulted in an increase of heat stability (**120**). However, our purified CGTase was less affected from the Ca^{2+} in the sample solution. During the starch adsoption step, the CGTase adsorbent on starch was washed with TB1 which was Tris-HCl buffer, pH 8.5 containing 10 mM $CaCl_2$ and during the elution of CGTase from the starch cake by 0.2 M of maltose in TB1. The solution of CGTase eluted was recovered by centrifugation and then concentrated with the ultrafiltration (Viva-Flow 50) from 250 ml to 50 ml before dialyzed against 100x volume of water at 4 ^{0}C with 3 changes of water. The final concentrations of $Ca2^+$ in each purified CGTase samples after dialysis were less than 1 nanomolar (1 nM).

Besides $CaCl_2$, starch substrate (0.2% (W/V)) was found to enhance the thermal stability of the enzyme at 55°C. This behavior of the enzyme could provide advantage to the CD industry. In the industrial production of CDs, the first step is generally liquefaction of starch with thermostable α-amylase prior to incubation with CGTase (**140**). This step can be omitted if CGTase of RB01, T16 and their transformants (pRB and pT) are used, since it is sufficiently thermostable to solubilize and degrade starch, and form CDs at fermentation temperature of 40-50°C.

4.5.4 Kinetics studies of the coupling reaction

In the coupling activity of CGTase, The CD molecule was opened by the reaction with glucosyl acceptor, resulting in the increase open chain oligosaccharides. Treatments

of the reaction mixture with glucoamylose release the glucose units. The reducing sugars were determined by 3, 5-dinitrosalicylic acid reaction (**141**). To determine the kinetic parameters of CGTase, parental CDs, α-, β- and γ-CDs; and derivatived CDs; glucosyl-α-CD (G-α-CD), glucosyl-β-CD (G-β-CD) and hydroxypropyl-β-CD (HP-β-CD) were used as substrates. Cellobiose was used as glucosyl acceptors. Preliminary experiments with other sugars such as maltose, sucrose, lactose, raffinose, trehalose showed that they either did not react or poorly reacted with β-CD in the coupling reaction and cellobiose was most reactive among them.

When analyzing the structures of these disaccharides, it could be interpreted that β linkage between C1 and C4 of glycosyl residues in the disaccharides was important for acceptor specificity. Maltose, isomaltose, trehalose, and sucrose, all possess α-1, 4 linkage while cellobiose and lactose contained the β-linkage. The acceptor binding site of CGTase form alkalophilic *Bacillus* sp. can recognize at least two glucopyranose units whereas the fructofuranosyl ring of sucrose inhibited binding to the acceptor binding site of CGTase (**136**). It had been reported that most of transglycosylation yield of disaccharides showed much higher values compared to monosaccharides (**142**). This indicated that the CGTase has high affinity for disaccharides and the acceptor binding site of CGTase can recognize at least two glucopyranose molecules.

Tables 8 and 9 summarized kinetic parameters of purified CGTase from pRB and pT in present study comparing with CGTase from their wild types, RB01 and T16. From the substrate binding affinity (K_m) and the rate of CD ring-opening (k_{cat}) of both transformant enzymes showed the highest efficiency (k_{cat} / K_m) in hydrolysis on HP-β-CD (0.039 and 0.019 $mM^{-1}sec^{-1}$, respectively). On the other hand, different result was observed with RB01 and T16 which showed the highest efficiency in hydrolysis on β-CD and γ-CD (0.015 and 0.044 $mM^{-1}sec^{-1}$, respectively).

In 2000, Bart *et al.*, reported that K_m values for α–, β- and γ-CD were 1.09 ± 0.26, 8.5 ± 2.2 and 15.7 ± 6.1 mM and V_{max} values were 192 ± 5.7, 294 ± 7.6 and 150 ± 9.0 U/mg, respectively for coupling reaction of CGTase from *Bacillus circulans* (strain 251) when methyl α-D-glucopyranoside was the acceptor molecule. In the absence of acceptor, K_m values of α–, β- and γ-CD were 0.45 ± 0.05, 18.1 ± 1.4 and 16.6 ± 3.0 mM, respectively (**143**).

From the K_m values, CGTase from both RB01 and pRB preferred β-CD and its derivatives. This was also reflected in the k_{cat}/K_m which reflected the enzyme efficiency. Both transformant CGTase showed improved affinity for the β-CD and their derivatives (Tables 8 and 9). Both wild type RB01 and T16 were reported to have preference for β-CD (**66, 138, 67**). The improved affinity for these substrates may be enhanced by some changes occurred to the enzymes when expressed in *E. coli*. From the deduced amino acid sequences of the transformed CGTases and its apparent molecular weight on SDS-PAGE, there seemed to be some modification occurred during the expression processes. In addition, the difference in culture environment such as culture medium and the pH's may lead to some difference in the changes or arrangement of the active sites.

The recombinant CGTases especially from pT seemed to have some advantageous improvement of their properties such as pH and temperature stability and catalytic activities in the coupling reactions. The changes showed preference towards β-CD and its derivative. The transformed cells also showed improved production of the CGTase in term of higher yield, shorter culture time and milder culture condition. Perhaps, the CGTase expression and post translation modification in the transformants may be more favorable for CGTase production. Posttranslational system in the host cells may provide some factors or environment which affected the active site of the enzymes such that their catalytic activity was improved.

4.5.5 Substrate specificity

The cyclization activity which was the CD producing reaction was studied on its substrate specificity for all four purified CGTase. Among high molecular weight polysaccharides, the sequence of substrate preference of RB01 and pRB was starch > amylopectin > dextrin > amylase > pullulan. Among the oligosaccharides with chain length from 2-7 glucose units, the substrate preference increased with chain length up to 6 glucose units for all four enzymes. For enzymes from RB and pRB, the results indicated lower capacity of the transformant than the wild type enzyme in utility all tested substrates, while pT seemed to be more capable of catalyzing the reactions with all tested substrates. The enzyme had specificity for substrates with α-1,4 glycosidic bond with minimum 3 glucose units, so glucose and maltose could not act as substrate. In addition, this CGTase could not use pullulans as substrate because its structure is linked by β-1,6 glycosidic bond. This finding agreed with CGTase mechanisms and corresponded with studies on different bacterial CGTases from previous studies. (**1, 139, 144, 145**). They reported on the degradation of soluble starch, glycogen, and pullulan by CGT_{Tk} and $CGT_{Tk\Delta C}$ from a hyperthermophilic archaeon *T. kodakaraensis* KOD1(**145**). They exhibited identical substrate specificities. Both proteins hydrolyzed soluble starch and glycogen to form oligosaccharides of various lengths but failed to utilize pullulan as a substrate (data not shown).

The results in the present study correspond with experiment investigated on the substrate specificity of CGTase which showed a relatively high rate of hydrolysis for soluble starch and amylopectin. Beta-CGTase catalyzed predominantly the formation of β-CD, with smaller amounts of α- and γ-CDs, from starch (**51, 60, 146**).

4.5.6 HPLC analysis of CDs produced by the CGTases.

Both RB01 and T16 were β-CGTase producers. From the previous studies, partially purified CGTase from starch adsorption step, RB01 prepared by Tesana (2001) produced a ratio of α- : β- : γ-CD as 1.0 : 5.4 : 1.2 (**66**) while crude and purified CGTase from RB01 prepared by Yenpet (2002) showed the average ratio of 1.0 : 1.8 : 0.4 (**147**). While CGTase from T16 prepared by Pranomitr (2001) produced α–, β- and γ-CDs in proportion of 0.74 : 1 : 0.27 (**67**). The difference may arise from different buffer used in enzyme preparation. There was previous report that crude CGTase from *Bacillus* sp. 1070 produced 0.6 : 5.7 : 0.6 while purified enzyme gave 0.6 : 9.0 : 0.0 of α- : β- : γ-CD (**148**). It was known that different ratio of CDs production may be performed by varying incubation time. In present study, it was shown that wild types and their transformants gave the same trend of products ratio with the major product as β-CD (Table 10). However, it was noticeably observed that the transformants gave the higher β-CD ratio to 3 times than the wild types.

From the studies on the expressions and characteristics of transformant CGTases from RB01 and T16, the transformant seemed to possess improved CGTase production activities such as shorter culture time and mild pH of the media which should be beneficial for industrial applications. The products of the enzymes from pRB also shifted towards more β-CD which could facilitate purification of β-CD. The data on coupling reaction should also bring attention to further imvestigation on using the tranformants to producing new oligosaccharides for industrial uses.