การโคลนยีนและลักษณะสมบัติของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจากแบคทีเรียทนร้อน *Paenibacillus* sp. BT01

นายกฤษณ์ ตันตนะรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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GENE CLONING AND CHARACTERIZATION OF THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT *Paenibacillus* sp. BT01

Mr. Krit Tantanarat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	GENE CLONING AND CHARACTERIZATION OF THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT *Paenibacillus* sp. BT01
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Field of Study	Biotechnology
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กฤษณ์ ตันตนะรัตน์ : การโคลนยีนและลักษณะสมบัติของไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจากแบคทีเรียทนร้อน *Paenibacillus* sp. BT01. (GENE CLONING AND CHARACTERIZATION OF THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT *Paenibacillus* sp. BT01) อ. ที่ปรึกษา: รศ. ดร.ทิพาพร ลิมปเสนีย์, 106 หน้า.

ยีน CGTase จาก *Paenibacillus* sp. BT01 ซึ่งคัดเลือกมาจากบริเวณของเสียโรงงานแป้งในประเทศไทย ถูกโคลนเข้าเชื้ออีโคไลสายพันธุ์ BL21(DE3) โดยใช้ pET19b เป็นเวกเตอร์แสดงออก เชื้อที่โคลนได้ให้ชื่อว่า pBT จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของรีคอมบิแนนท์พลาสมิด พบว่าประกอบด้วยนิวคลีโอไทด์ขนาด 2142 คู่เบสซึ่งถอดรหัสได้โปรตีนขนาดกรดอะมิโน 713 ตัวโดย 686 ตัวทำหน้าที่เป็นโครงสร้างของเอนไซม์ ลำดับนิวคลีโอไทด์และลำดับกรดอะมิโนมีความใกล้เคียงกับ CGTase ของ *Paenibacillus* sp. A11 ด้วยค่าความเหมือน 99% เมื่อเลี้ยงเชื้อ pBT และการกระตุ้นด้วย IPTG ความเข้มข้น 0.2 มิลลิโมลาร์ เป็นเวลา 24 ชั่วโมง พบว่า 99.83% ของเอนไซม์ที่สร้างทั้งหมดหลั่งอยู่ในน้ำเลี้ยงเชื้อและมีค่าแอคติวิตี้จำเพาะของเอนไซม์มากกว่าสายพันธุ์ตั้งต้น 6.6 เท่า การทำเอนไซม์ให้บริสุทธิ์ด้วยวิธีดูดซับด้วยแป้ง และผ่านคอลัมน์ DEAE-cellulose พบว่าได้เอนไซม์จาก BT01 คงเหลือ 12.18% และค่าความบริสุทธิ์เพิ่มขึ้น 24 เท่า ถ้าใช้เอนไซม์จากเชื้อ pBT พบว่าได้เอนไซม์ 70.98% และค่าความบริสุทธิ์เพิ่มขึ้น 8 เท่า จากการวิเคราะห์ด้วยอิเล็กโทรโฟเรซิสแบบเสียสภาพพบว่าเอนไซม์จาก BT01 และ pBT มีน้ำหนักโมเลกุลที่เท่ากันประมาณ 71 กิโลดาลตัน เมื่อวิเคราะห์หาค่า pI พบแถบหลักที่ pI 4.74 และแถบรองที่ pI 4.86 และ 4.62 สภาวะที่เหมาะสมในการทำ dextrinizing activity และ cyclization activity คืออุณหภูมิ 50 องศาเซลเซียสและที่ pH 5.0 และ 6.0 ตามลำดับ เอนไซม์ทั้งสองตัวมีความเสถียรที่ pH 6.0 ถึง 10.0 และทนความร้อนได้ถึง 50 องศาเซลเซียส แป้งที่ความเข้มข้น 2% ทำให้เอนไซม์สามารถทนอุณหภูมิได้ถึง 70 องศาเซลเซียส เมื่อทำการวิเคราะห์ไซโคลเดกซ์ทรินด้วย HPLC พบว่าเมื่อบ่มเอนไซม์กับแป้งความเข้มข้น 10% ที่อุณหภูมิ 60 องศาเซลเซียส pH 6.0 เป็นเวลา 24 ชั่วโมงได้ผลิตภัณฑ์ไซโคลเดกซ์ทรินในอัตราส่วน α-:β-:γ-CD เท่ากับ 1.0 : 2.0 : 0.72 โดยได้บีต้าไซโคลเดกซ์ทรินจากเอนไซม์ของ BT01 และ pBT 22.52 และ 19.33 กรัมต่อลิตรตามลำดับ ชนิดของแป้งไม่มีผลอย่างชัดเจนต่ออัตราส่วนการผลิตไซโคลเดกซ์ทริน การศึกษาทางจลพลศาสตร์ของเอนไซม์บริสุทธิ์ด้วยปฏิกิริยา cyclization โดยใช้แป้งเป็นสับสเตรท พบว่าเอนไซม์จาก BT01 มีค่า *Km* เท่ากับ 5.36 มิลลิกรัมต่อมิลลิลิตร และค่า *kcat/Km* เท่ากับ 40.82 (นาที)$^{-1}$(มิลลิกรัม/มิลลิลิตร)$^{-1}$และ pBT จะมีค่า *Km* เท่ากับ 4.90 มิลลิกรัมต่อมิลลิลิตร และค่า *kcat/Km* เท่ากับ 91.35 (นาที)$^{-1}$(มิลลิกรัม/มิลลิลิตร)$^{-1}$

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต กฤษณ์ ตันตนะรัตน์

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา

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KRIT TANTANARAT : GENE CLONING AND CHARACTERIZATION OF THE CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT *Paenibacillus* sp. BT01. THESIS ADVISOR: ASSOC. PROF. TIPAPORN LIMPASENI, Ph.D., 106 pp.

Cyclodextrin glycosyltransferase (CGTase) catalyzes the conversion of starch to a mixture of cyclic oligosaccharides, cyclodextrins (CDs). A cyclodextrin glycosyltransferase gene from *Paenibacillus* sp.BT01 isolated from waste of a starch factory in Thailand, was cloned into *Escherichia coli* BL21(DE3) using pET19b vector. Determination of the nucleotide sequences of both wild type (BT01) and recombinant plasmid showed the presence of 2142 bp open reading frame which encodes a polypeptide of 713 amino acid residues, with 686 amino acids for mature enzyme. The nucleotide and amino acid sequences showed highest homology of 99% to *Bacillus circulans* A11. About 99.83% of total CGTase was secreted into the LB medium after induction with 0.2 mM IPTG for 24 hours and the specific activity was 6.6 folds higher than wild type. The wild type and recombinant CGTase was purified to homogeneity by starch adsorption and DEAE-cellulose with 12.18 %yield, 24 purification fold in wild type and 70.98 %yield, 8 purification fold in recombinant pBT. The molecular weight of both enzymes were estimated to be 71 kDa by SDS-PAGE. Both enzymes showed one major band at pI 4.74 and two minor bands at 4.86 and 4.62 on isoelectric focus gel electrophoresis. The enzymes from BT and pBT exhibited optimum pH and temperature for dextrinizing activity at pH 5.0 at 50ºC. Optimum conditions of cyclization activity were pH 6.0 at 50ºC. The enzymes were stable at pH 6.0-10.0 up to 50 ºC. In the presence of 2% soluble starch the enzymes were stable up to 70 ºC. CGTase catalyzed the conversion of starch to mixture of cyclodextrins with the ratio of α-: β-: γ-CDs at 1.0 : 2.0 :0.72, when incubated with 10% soluble starch at 60 ºC, pH 6.0 for 24 hours. The total β-CD produced were 22.52 g/l in wild type and 19.33 g/l in recombinant. The source of starch did not significantly affect the CGTase action and the ratio of α-: β-: γ-CDs remained constant. Kinetic parameter were determined by used cyclization activity. Calculated the *Km* and *kcat/Km* were 5.36 mg/ml and 40.82 $(min)^{-1}(mg/ml)^{-1}$ in wild type and 4.90 mg/ml and 91.35 $(min)^{-1}(mg/ml)^{-1}$ in recombinant CGTase, respectively.

Field of study Biotechnology Student's signature KRIT TANTANARAT

Academic year 2006 Advisor's signature Tipaporn Limpaseni

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ABBREVIATIONS

A	Absorbance
BSA	bovine serum albumin
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
cm	centimeter
ºC	Degree Celsius
Da	Dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleotide
EDTA	ethylenediamine tetraacetic acid
et al.	Et. Alii (latin),and others
g	gram
IPTG	Isopropylthiogalactoside
Kb	kilobase
kcat	catalytic constant
Km	Michaelis constant
l	litre
µg	Microgram
µl	Microlitre
M	Molar
mA	mililampere
ml	milliliter
mol	mole
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pI	isoelectric point
rpm	revolution per minute
SDS	sodium dodecyl sulfate
V	volt
Vmax	maximal velocity

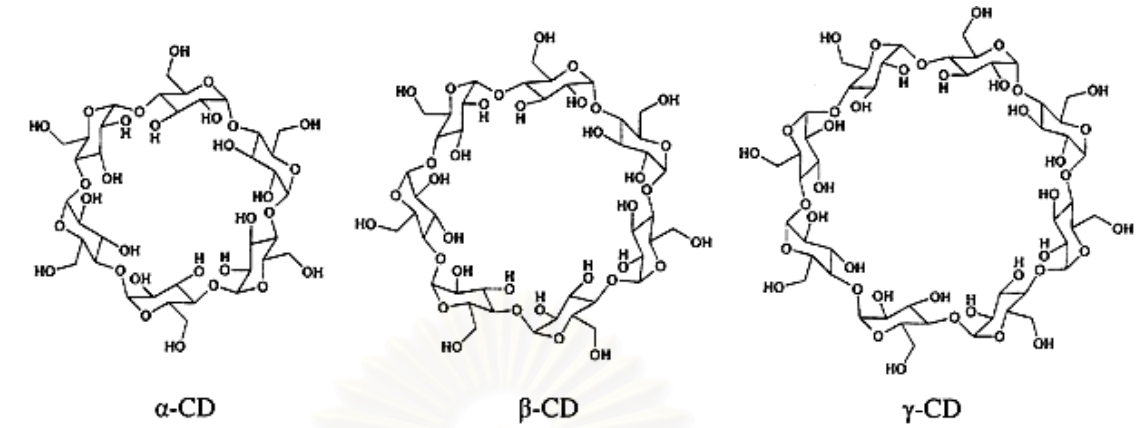
CHAPTER I
INTRODUCTION

Cyclodextrins (CDs)

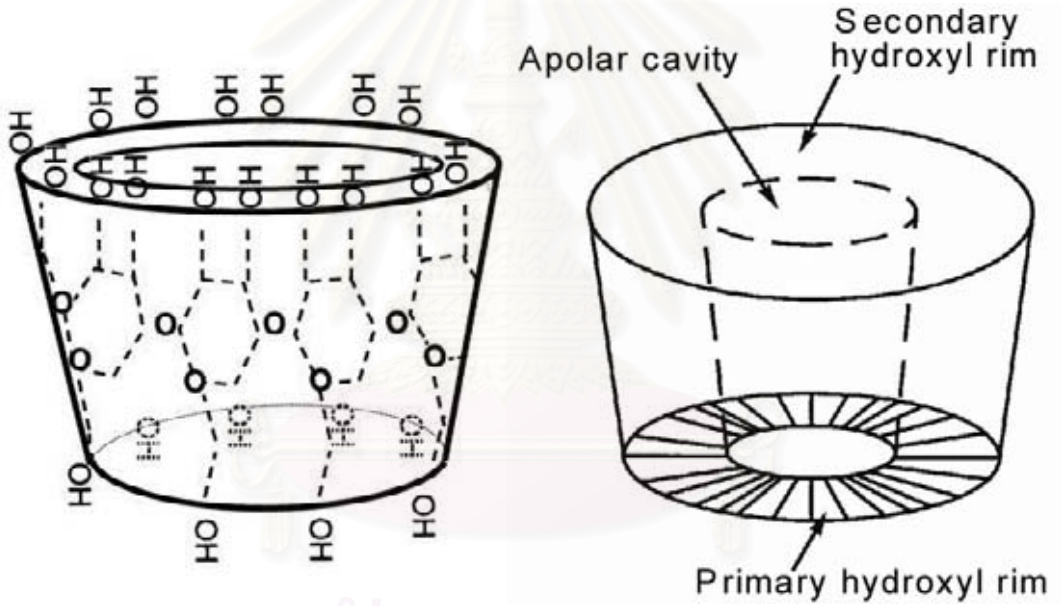
Cyclodextrins (Schardinger dextrin, cycloamyloses, cyclomaltose or cycloglucans) are cyclic oligosaccharides, composed of 6 or more α-D-glucopyranoside units linked by α-1,4 glycosidic bonds. Typical cyclodextrins contain a number of glucose monomers ranging from 6, 7 and 8 glucose units called α-(alpha), β-(beta) and γ-(gamma) cyclodextrins, respectively as shown in Figure 1(a). Cyclodextrins consisting of more than eight D-glucose units were first described by French (1957) but were not abundant. Each of the glucose units is in the rigid $^4C_1$-chair conformation, giving the molecule the shape of a hollow truncated cone. The cone is formed by the carbon skeletons of the glucose units and the glycosidic oxygen atoms in between them, with the primary hydroxyl groups on the outer part of the narrow side of the cone and the secondary hydroxyls at the wide face (Figure 1(b)). The primary and secondary hydroxyl groups on the outside of the cyclodextrin make cyclodextrins water-soluble. Cyclodextrins are insoluble in most organic solvents. The cavity of the cyclodextrin rings consists of a ring of C-H groups, a ring of glycosidic oxygen atoms and a ring of C-H groups. This renders the interior of the cyclodextrin rings less polar. Table 1 shows list of important physical properties and the molecular dimensions of the three most common cyclodextrins.

Cyclodextrin inclusion complexes

In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules that are energetically unfavorable (polar-apolar interaction) and therefore can be readily substituted by appropriate “guest molecule”, which are less polar than water. The dissolved cyclodextrin is the “host” molecule, and part of the



(a)



(b)

Figure 1: Chemical structure of α-(alpha), β-(beta) and γ-(gamma)-cyclodextrin (CD), consisting of 6, 7 or 8 glucose units (Biwer *et al.*, 2002) (a), Structure of cyclodextrin showing glucose molecule arrangement (b).

Table 1: Physical properties of cyclodextrins.

Property	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Number of Glucose	6	7	8
Molecular weight	972	1135	1297
Solubility H_2O [g/100ml]	14.5	1.85	23.2
pKa	12.33	12.2	12.08
Inner diameter [nm]	0.45-0.57	0.62-0.78	0.79-0.95
Outer diameter [nm]	1.37	1.53	1.69
Height [nm]	0.79	0.79	0.79
Cavity volume [nm^3]	0.174	0.262	0.472

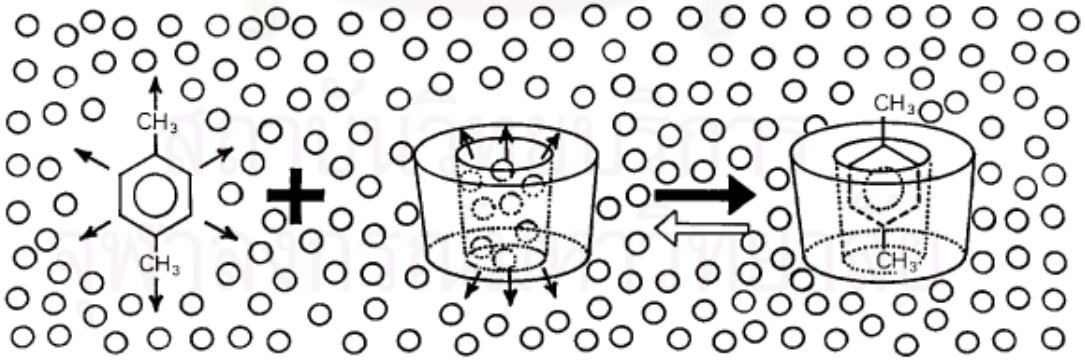


Figure 2: Schematic representation of the association-dissolation of the host (cyclodextrin) and guest (p-xylene) to form guest/host inclusion complex. (Szejtli 2004).

"driving force" of the complex formation is an appropriate "guest" molecule (Figure 2). One, two, or three CD molecules contain one or more entrapped "guest" molecules. The host:guest ratio of 1:1 is the simplest and most frequent case. This is the essence of "molecular encapsulation". However, host:guest ratio of 2:1, 1:2, 2:2 or even more complicated associations and higher-order equilibrium exist, almost always simultaneously. The inclusion complexes formed can be isolated as stable amorphous or microcrystalline substances. Upon dissolving these complexes, an equilibrium is established very rapidly between dissociated and associated species, and this is expressed by the complex stability constant *Ka*. The association of the CD and guest molecules and the dissociation of the CD/guest complex formed is governed by a thermodynamic equilibrium. The beneficial modification of guest molecular properties after the formation of an inclusion compound leads to a large number of industrial applications.

Industrial applications of cyclodextrins

In the food industry, cyclodextrins are used as stabilizers for flavoring agents and to reduce unpleasant odor and taste. In the cosmetic industry, cyclodextrins are included as stabilizers of chemically labile compounds to obtain prolonged action, decrease local irritation and to reduce unpleasant odors. In household and toiletry products, the deodorizing capacity of CDs is utilized. For production of low-cholesterol butter, β-CD is used to remove the cholesterol from butter (Szejtli, 2004). Selected applications of cyclodextrins were shown in Table 2.

Cyclodextrin producing enzyme

Cyclodextrin glycosyltransferase (E.C. 2.4.1.19) (CGTase) catalyzes the production of cyclodextrin from starch and related α-(1, 4)-linked glucose polymers via a transglycosylation reaction. Bacterial CGTase catalyzes four related reactions: cyclization, coupling, disproportionation and hydrolysis (Figure 3). The cyclodextins are produced via an intramolecular transglycosylation reaction in which CGTase cleaves an α(1-4) bond in the starch molecules and concomitantly linking the reducing and non-reducing ends (cyclization) or its transferred to another molecule

Table 2: Selected applications of cyclodextrins (Hedges, 1998)

Industrial	Use	Industrial	Use
Food		**Pharmaceuticals**	
Cinnamon-flavored apples	Stabilize flavor	Itraconazole	Increase solubility
Mint and green tea mints	Stabilize flavor	Piroxicam	Reduce irritation
Peppermint-flavor chewing gum	Flavor delivery	Garlic oil	Mask odor
Mustard oil steak sauce	Improve solubility	Hydrocortisone	Increase solubility
Acetic acid	Convert to a powder	PGE_1	Increase stability
Aloe-containing beverage	Mask bitterness		
Water purifier	Absorb odor	**Cosmetics and Personal care items**	
Lemon-flavored sugar	Flavor stabilization	Skin cleanser	Tocopherol carrier
		Artificial tanning Lotion	Stability, mask Odor
Miscellaneous		Powdered hair bleach	Stability
Laundry drier sheet	Fragrance control	Perfume	Prolonged release
Chromatography column	Separations	Cold cream	Solubility

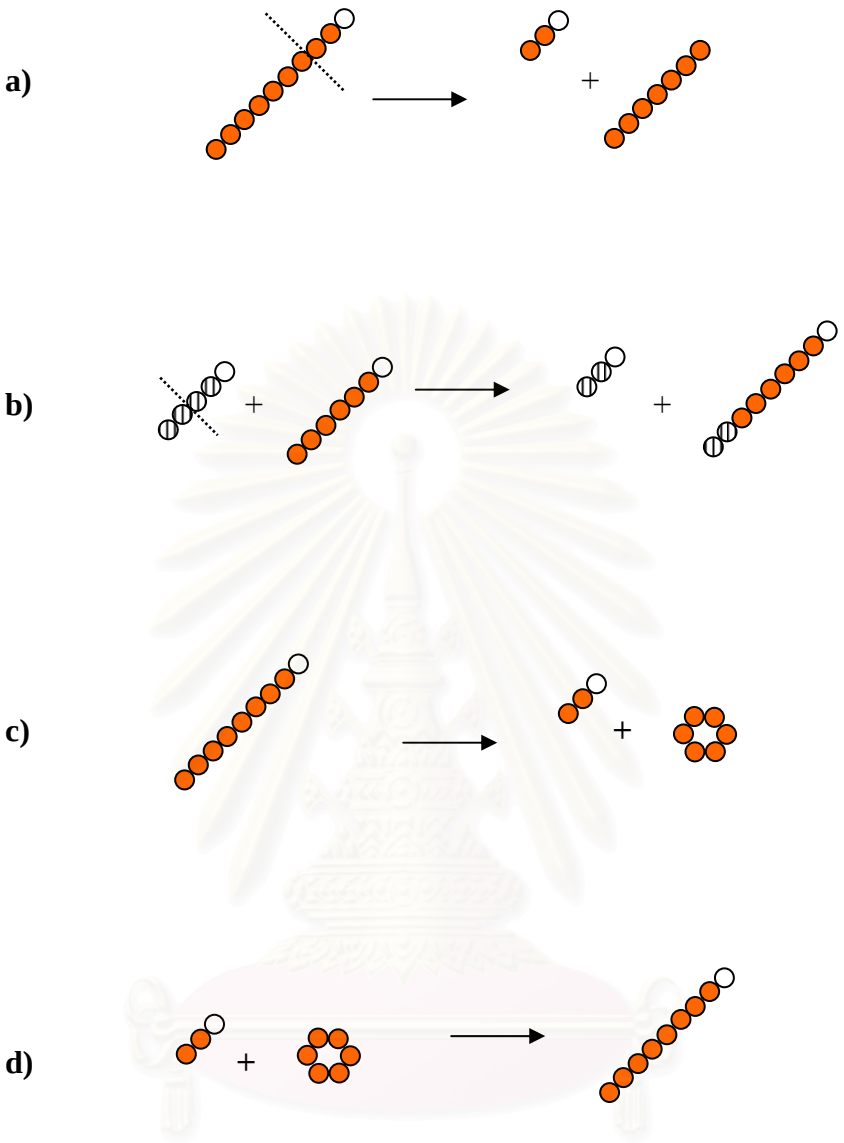


Figure 3: Schematic representation of the CGTase catalyzed reactions. The circles represent glucose residues; the white circles indicate the reducing end sugars. (a) hydrolysis, (b) disproportionation, (c) cyclization, (d) coupling (Van der Veen *et al.*, 2000).

(disproportionation reaction). Cyclodextrin ring can be opened by the enzyme and linearized fragment is transferred to an acceptor (coupling reaction). When reducing end is not transferred to a carbohydrate acceptor but rather to a water molecule, the result is the hydrolysis of amylose or the linearization of cyclodextrin (hydrolysis reaction). The major products of cyclization reaction are α-, β- and γ-cyclodextrins. CGTase is a member of the α-amylase family of glycosyl hydrolases. Kuriki and Imanaka (1999) defined the α-amylase family enzymes as follows: "(i) they act on α-glycosidic bonds, (ii) they hydrolyze or transfer α-glycosidic bonds with the retention of the anomeric configuration of the processed bond, (iii) they contain four conserved sequence motifs in which the catalytic residues and most other residue involved in substrate binding at the catalytic site are located, (iv) they possess Asp, Glu and Asp as catalytic residues". Table 3 shows a list of enzymes with the characteristics of the α-amylase family. The enzymes in this group show a wide diversity in reaction specificities and many of them are active on starch. Whereas amylases generally hydrolyzed glycosidic bonds in the starch molecules, CGTase mainly catalyzes transglycosylation reaction, with hydrolysis being a minor activity (Van Der Veen *et al.,* 2000). The CGTase is an extracellular enzyme. Since the discovery of *Bacillus mercerans* as the first source that capable of producing CGTase (Takano *et al.,* 1986) a large number of microorganism have been identified as CGTase producers which includes; i) aerobic mesophilic bacteria such as *Bacillus macerans, Bacillus megaterium, Klebsiella oxytoca, Klebsiella Pseudomonas, Micrococcus luteus;* ii) aerobic thermophilic *Bacillus stearothermophilus;* iii) anaerobic thermophilic *Thermoanaerobacterium thermosulfurigenes;* iv) aerobic alkalophilic bacteria such as *Bacillus circulans, Bacillus* sp. AL-6 ; and v) aerobic halophilic *Bacillus halophilus* (Tonkova 1998). CGTase is classified into three different types, α-CGTase, β-CGTase and γ-CGTase according to the major CDs produced (Tonkova 1998). The enzyme produced from different sources show different properties. Some properties of bacterial CGTases are shown in Table 4.

Table 3: Enzymes of the alpha-amylase family (Van der Maarel *et al.*, 2002)

Enzyme	EC number	Main substrate
Sucrose phosphorylase	2.4.1.7	Sucrose
Glucan branching enzyme	2.4.1.18	Starch, glycogen
Cyclodextrin glycosyltransferase	2.4.1.19	Starch
alpha-Amylase	3.2.1.1	Starch
alpha-Glucosidase	3.2.1.20	Starch
Amylopullulanase	3.2.1.41 or 3.2.1.1	Pullulan
Cyclomaltodextrinase	3.2.1.54	Cyclodextrins
Isopullulanase	3.2.1.57	Pullulan
Isoamylase	3.2.1.68	Amylopectin
Glucodextranase	3.2.1.70	Starch
Neopullulanase	3.2.1.135	Pullulan

Table 4: Some properties of bacterial CGTases (Tonkova 1998)

Producer	Optimum pH	Optimum Temp(°C)	Molecular mass	Main CD produced	Reference
Bacillus macerans ATCC 8514	6.1-6.2	60 °C	139,000	-	De Pinto, 1968
Bacillus macerans IFO 3490	5.0-5.7	55 °C	-	α-CD	Kitahata, 1974
Bacillus macerans IAM 1243	-	-	74,000	α-CD	Takano, 1986
Bacillus megaterrium No5	5.0-5.7	55 °C	-	β-CD	Kitahata, 1974
Bacillus circutans var. *alkalophilus* ATCC 21783	4.5-4.7	45 °C	88,000	β-CD	Nakamura, 1976
Bacillus sp. AL-6 (alkalophilic strain)	7.0-10.0	60 °C	74,000	γ-CD	Fujita, 1990
Bacillus cereus NCIMB	5.0	40 °C	-	α-CD	Jamuna, 1993
Bacillus sp. INMIA T6 (thermophilic strain)	6.5	55 °C	38,000	α-CD	Abelian, 1994
Bacillus sp. INMIA T42 (thermophilic strain)	6.5	55 °C	35,000	β-CD	Abelian, 1994
Bacillus sp. INMIA A7/1 (alkalophilic strain)	6.0	50 °C	44,000	β-CD	Abelian, 1994
Bacillus sp. INMIA 1919	4.0	50 °C	42,000	α-CD	Abelian, 1994
Bacillus halophilus INMIA 3849	7.0	60-62 °C	71,000	β-CD	Abelian, 1995
Thermoanaerobacterium thermosulfurigene EM1	4.5-7.0	80-85 °C	68,000	β-CD	Wind, 1995

Three dimensional structure of CGTase

The first X-ray crystallographic structure of CGTase analyzed was from *Bacillus circulans* (Hofmann *et al.*, 1989). The protein was subdivided into five domains, designated A–E. The A domain forms a (β/α) 8 barrel or TIM barrel, which was present in all α-amylase family members (Janecek 1994). The catalytic and substrate-binding residues, which are also conserved, are located at the C-termini of the β-strands in the A domain (Klein *et al.*, 1992). The B and C domains are responsible for substrate-binding and the stability of the catalytic domain (Penninga *et al.*, 1996). The C domain contains one of the maltose-binding sites present in CGTases (Penninga *et al.*, 1995). The function of the D domain, which is almost exclusively found in CGTases, was still unknown. In contrast, the E domain is widespread in starch-degrading enzymes and contains several maltose-binding sites. At the active center of CGTases, tandem subsite architecture is found in the substrate-binding groove, with at least nine sugar-binding subsites. These substrate-binding sites are numbered from +2 to –7 (Davies *et al.*, 1997), with the catalytic site between subsites +1 and –2. Asp229, linked by hydrogen-bonding to the glucose residue at subsite –1, has been identified as a residue specific for CGTases and is proposed to be responsible for the cycling reaction. The non-reducing end of the oligosaccharide substrate is bound at subsite –8, which just fits the size of CD8. The glucose residue at subsite –8 is located at the end of the substrate-binding groove. The subsite +1 is the sugar acceptor-binding site, which shows an induced-fit mechanism that activates the transglycosylation activity of CGTase. Furthermore, analysis of the structure of the linear and cyclic oligosaccharide substrates indicates a specific role of the residues in subsite +2 (Figure 4).

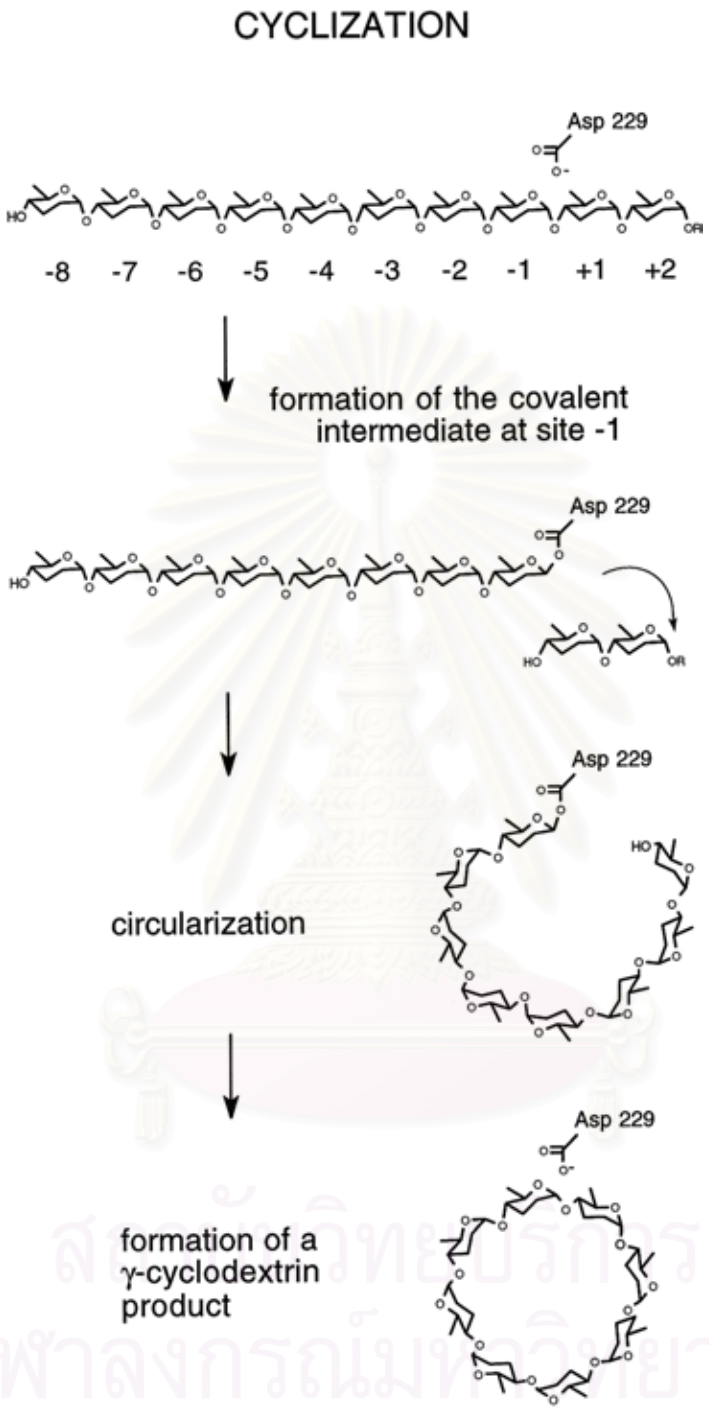


Figure 4: Schematic representation of the cyclization reaction catalyzed by CGTase (Uitdehaag *et al.,* 1999).

Research on cloning and overproduction of CGTase

The objectives of most research on cloning and overproduction of CGTases were to increase the quantity of enzyme and to improve CDs production in industries. In such studies, the CGTase gene from alkalophilic *Bacillus* sp. TS1-1 (Rahman *et al.*, 2006) and *Bacillus mercerans* (Soo-Wan *et al.*, 2001) were cloned and expressed in *E. coli* and *Saccharomyces cerevisiae*. Many researches have been carried out with emphasis on the improvement of CDs production. Development of cultivation for CD overproduction was carried out under optimized culture condition and with complex nutrient media. Thermotolerant CGTase producing bacteria which gives high CD yield have been recognized as another favorable choice. CGTase from an alkalophilic *Bacillus* strain no.38-2(ATC 21783) was observed to provide these required properties. Protein engineering, site-directed mutagenesis and gene cloning have also been applied to increase the yield of enzyme product. These techniques are not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of CDs (Schmid 1989).

Industrial production of CGTase

The conventional procedure for the production of CDs includes liquefaction of starch using α-amylase (around 90°C) followed by the inactivation of α-amylase by heating up to 120°C. Afterwards the dextrin solution is cooled down to the reaction temperature (50°C) of the CGTase (Biwer and Heinzl, 2004) However, this procedure is inefficient in many aspects: first, α-amylase needs be inactivated before the addition of CGTase; second, microbial contamination is possible; third, it is time consuming, conversion of starch to CDs requires an extended reaction time before reasonable yields are achieved. Therefore, a CGTase with liquefying and cyclizing activities at high temperatures would be appropriate for efficient production of CDs. Researchers have tried to search for bacterial strains that can grow at higher temperatures. Thermophiles such as *Thermococcus* sp. and *Anaerobranca gottschalkii* can grow at temperature above

45°C and their enzymes have optimum temperature at 100°C and 65°C (Bertoldo and Antranikian, 2002). Thermophilic archaea bacteria used more energy to produce enzyme. CGTase from this strain was cloned in *E. coli* and obtained as an inclusion body. The enzyme needed to be solubilized in 6 M urea and heated 80°C before use.

Our research group has been working on mesophilic bacteria, *Paenibacillus* sp. A11, a strain isolated from South–East Asian soil. Recently, a few thermotolerant CGTase producing bacteria have been isolated. *Peanibacillus* sp. RB01 was isolated from hot spring area in Ratchburi province with the optimum temperature for growth at 37°C while CGTase showed optimum activity at 40°C pH 10.0 and CDs yield were α-: β-: γ-CD = 1.0 : 5.4 : 1.2 (Yenpetch 2002). *Peanibacillus* sp. T16 (T16) was isolated from Tak province with the optimum temperature for growth at 37°C, optimum pH and temperature for CD-forming activity were 10.0 and 37°C and CD's yield were α-: β-: γ-CD = 0.7 : 1.0 : 0.27 (Pranommit 2001). *Paenibacillus* sp. BT01 was isolated from soil in starch factory in Nakhon Pathom province with the optimum temperature for growth at 40°C, optimum pH and temperature for CD-forming activity were 7.0 and 55 °C. CD's yield were α-: β-: CD = 1.0 : 1.0 (Yampayont *et al.*, 2006). CGTase gene from A11, RB01 and T16 were cloned and analyzed nucleotide sequence whereas BT01 has not been studied. This research aims to clone CGTase gene from *Paenibacillus* BT01 and characterized CGTase properties.

Objectives of this research

1. Cloning of the cyclodextrin glycosyltransferase (CGTase) gene from *Paenibacillus* sp. BT01.
2. Purification and characterization of CGTases from wild type and transformant.

CHAPTER II
MATERIALS AND METHODS

2.1 **Equipments**

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated: Model J-21C, Beckman Instrument Inc, U.S.A.

Electrophoresis unit: Model Mini-protein II Cell, Bio-Rad, U.S.A.

Gene Pulser^{R} / E.coli Pulser^{TM}Cuvettes: Bio-Rad, U.S.A.

Gene Amp PCR system, Model 2400, Perkin Elmer, U.S.A.

Fraction collector: Model 2211 Pharmacia LKB, Sweden

Incubator: Haraeus, Germany

Incubator Shaker, Model SBS30, Control environment: Stuart scientific, U.S.A.

Laminar flow, Model BVT-124, International Sciencetific Supply Co.Ltd.,U.S.A.

Peristaltic pump: Pharmacia LKB, Sweden

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Sonicator : SONOPULS Ultrasonic homogenizers, BANDELIN, Germany

Spectrophotometer UV-240: Shimadzu, Japan, and Du series 650, Beckman, U.S.A.

Vortex: Model K-550-GE, Scientific Industries, U.S.A.

Water bath: Charles Hearson Co., Ltd., England

2.2 **Chemicals**

Acrylamide: Merck, U.S.A.

Ampholine pH 3.5-10.0 for IEF: Amersham Pharmacia Biotech: Sweden

Ampicillin, Sigma, U.S.A.

Agar, Merck, Germany

Agarose, FMC Bioproduct, U.S.A.

Bacto-peptone: Difco Laboratories, U.S.A.

Beef extract: Difco Laboratories, U.S.A.

Bovine serum albumin(BSA), Sigma, U.S.A.

Bromophenol blue, Merck, Germany

Cellobiose: Sigma, U.S.A.

Chloroform, Sigma, U.S.A.

Coomassie brilliant blue G-250: Sigma, U.S.A.

Coomassie brilliant blue R-250: Sigma, U.S.A.

β-cyclodextrin: Nihon shokuhin kako company Ltd., Japan

α-and γ–cyclodextrin: Sigma, U.S.A.

DEAE-cellulose resin: DE 32, Whatman Biosystems Ltd., England

Di-Sodium hydrogenphosphate, Fluka, Switzerland

DNA marker: 1 Kb Ladder, Fermentas

Ethidium bormide, Sigma, U.S.A.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Glacial acetic acid, BDH, England

Glucose, Sigma, U.S.A.

Glycerol, Scharlau, Spain

Glycine: Sigma, U.S.A.

Hydrochloric acid, Merck, Germany

Iodine, Baker chemical, U.S.A.

Maltose: Sigma, U.S.A.

N,*N*′-methylene-bis-acrylamide: Sigma, U.S.A.

Peptone from casein, Merck, U.S.A.

Peptone from meat, Merck, U.S.A.

Phenol, BDH, England

Phenolpthalein: BDH, England

Potassium iodine, Mallinckrodt, U.S.A.

Sodium carbonate, BDH, England

Sodium chloride, USB, U.S.A.

Sodium dodecyl sulfate, Sigma, U.S.A.

Sodium hydroxide, Carlo Erba, Italy.

Soluble starch, potato: Sigma, U.S.A.

Soluble starch,potato: Kanto Chemical Co.,Inc. Japan

Tris-base, USB, U.S.A.

Tryptone, Merck, Germany

Xylene cyanol FF, Sigma, U.S.A.

Yeast extract: Scharlau, Spain

Other chemicals used were of reagent grade and were purchased from commercial sources. Corn starch (Knorr), tapioca starch (New Grade); Modified starch Flo-Max 8 (modified cassava starch from National Starch Food Innovation) was locally purchased.

2.3 **Bacteria strains and plasmids**

Paenibacillus sp. BT01 was isolated from soil in Thai starch factory in Nakhon Pathom province (Porntida *et al.*, 2006).

Escherichia coli DH5α, genotype: Ø80d*lac*ZΔM15Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^- m_k^+)*deo*R *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1, was used as the host for pGEM-T easy vector.

Escherichia coli BL21(DE3), genotype: *omp*T gal dcm lon *hsd*S_B(r_B^- m_B^-) λ(DE3 [*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5]).

pGEM-T easy vector from Promega was used as an cloning vector for cloning of CGTase gene.

pET-17b was used as an expression vector for cloning of CGTase gene.

2.4 **Media preparation**

2.4.1 **Luria-Bertani broth (LB medium)**

LB medium consisted of 1% Bacto tryptone from meat, 0.5% yeast extract and 0.5% NaCl, supplemented with 100 µg/ml ampicillin when needed. The pH of the medium was 7.2.

2.4.2 **LB-starch-agar**

LB-starch agar consisted of 1% Bacto tryptone from meat, 0.5% yeast extract and 0.5% NaCl, 1.5% Agar and 1% soluble starch, supplemented with 100 µg/ml ampicillin when needed.

2.4.3 **LB-IPTG-X-gal Agar**

LB-IPTG-X-gal Agar consisted of 1% Bacto tryptone from meat, 0.5% yeast extract, 0.5% NaCl, 1.5% agar and 1% soluble starch, supplemented with 100 µg/ml ampicillin. One hundred µl of 100mM IPTG and 20 µl of 50 mg/ml X-gal were spread over the surface and allowed to absorb for 30 minutes at 37ºC prior to use.

2.4.4 **Medium I**

Medium I consisted of 0.5% beef extract, 1.0% peptone, 0.2% NaCl, 0.2% yeast extract and 1.0% soluble starch. The pH of the medium was 7.2. For solid medium, 1.5% agar was added.

2.4.5 **Horikoshi Medium**

Horikoshi Medium, slightly modified from (Horikoshi 1971) by (Rutchtorn 1993), contained 1.0% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_20$ and 0.75% Na_2CO_3. The pH of the medium was 10.1-10.2.

2.5 Cultivation of *Paenibacillus* sp. BT01

2.5.1 **Starter inoculum**

Glycerol stock of *Paenibacillus* sp. BT01, isolated from soil in Thai starch factory in Nakhon Pathom province, were streaked on solid Medium I and incubated for 18 hours at 37ºC. One loop was suspended into liquid Medium I at 37ºC and grown until A_{660} reached 0.3-0.5.

2.5.2 **Enzyme production**

Starter inoculum (1.0%) was transferred into 300 ml Horikoshi's broth in 1000 ml Erlenmeyer flask and cultured for 72 hours. Cells were removed by centrifugation at 3,000 x g at 4ºC. Culture broth containing crude CGTase was collected and kept at 4ºC for purification.

2.6 **General techniques in genetic engineering**

2.6.1 **Chromosomal DNA extraction**

Chromosomal DNA was prepared from *Paenibacillus* sp. BT01 cultured on Horikoshi plate prepared as described in section 2.5. A single colony was inoculated into 100 ml of Medium I and incubated at 37ºC for 24 hours with shaking. The cells were harvested by centrifugation at 5,000 rpm for 20 minutes. Cell pellet was dissolved in 30 ml of SET buffer (20% Sucrose, 50 mM Tris-HCl pH 7.6, 50 mM EDTA) then cell suspension was centrifuged at 12,000 rpm for 20 minutes. The cell pellet was resuspended in 2 ml of SET buffer followed by addition of 200 µl lysozyme (5 mg/ml of lysozyme in 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 mM NaCl (TEN buffer)), 100 µl of RNase A (10 mg/ml of RNase A in 0.1 M sodium acetate pH 7.4, 0.3 mM EDTA (RNase A buffer)) and incubated for 30 minutes at 37 ºC. The cell suspension was incubated with 50 µl of 25% SDS at 37ºC for 3 hours followed by the addition 300 µl of proteinase K (2 mg/ml in TEN buffer) and futher incubated for 1 hour at 37ºC. Then, 6 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added and incubated at 37ºC overnight. The DNA was extracted with an addition of an equal volume of phenol-chloroform (1:1 v/v) and mixed gently. The mixture was vigorously vortexed, and then centrifuged at 12,000 rpm for 30 minutes to get phase separation. The upper aqueous layer was carefully transferred to a new tube, avoiding the phenol interface. The 5 M NaCl was added to give final concentration of 0.1 M NaCl. The DNA was precipitated with absolute ethanol, resuspended in TE buffer and kept at 4ºC.

2.6.2 **Agarose gel electrophoresis**

Agarose gel electrophoresis was performed to separate, identify and purify fragments of DNA by using 0.7% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) (Sambrook *et al.*, 1989). DNA samples in 1X gel loading buffer were loaded into the wells. The gel was run at 100 volts for 1 hour, or until bromophenol blue reached the bottom of the gel. After electrophoresis, the gel was stained by immersion in electrophoresis buffer or H_2O containing ethidium bromide (0.5 µg/ml) for 30-45 minutes at room temperature. DNA fragments on agarose gel were visualized under a

long wavelength UV light. The concentration and molecular weight of DNA sample was estimated from the intensity and relative mobility of the standard DNA markers.

2.6.3 **Preparation of competent cells** (Sambrook *et al.,* 1989)

A fresh overnight culture of *E. coli* DH5α or *E. coli* BL21(DE3) was inoculated into 300 ml of LB broth with 1% inoculum size. The cell culture was inoculated at 37ºC with shaking at 250 rpm until OD_{600} reached 0.5 to 0.6. The culture was chilled on ice for 15 minutes and then centrifuged at 6,000 rpm for 15 minutes at 4ºC. The cells were washed with 300 ml of cold water, spun down and washed again with 150 ml of cold water. After centrifugation, the cells were resuspended in approximately 15 ml of 10% glycerol in distilled water and centrifuged at 6,000 rpm for 15 minutes at 4ºC. Finally, the cell pellets were resuspended to final volume of 600 µl in 10% glycerol. This cell suspension was divided into 40 µl aliquots and store at -80ºC until used.

2.6.4 **Transformation into host cell *E. coli*** (Sambrook *et al.*, 1989)

The recombinant plasmids were transformed into competent cells of *E. coli* DH5α or *E. coli* BL21(DE3) by electroporation. The DNA was mixed with cold cell suspension, transferred to a chilled cuvette and placed on ice for 1 minute. The mixture was electroporated in a cold 0.2 cm electrode gap cuvette with the apparatus setting as follows; 25 µF, 200 Ω of the pulse controller unit and 250 kV time constant 4.8-4.6 msec. After electroporation, the cell suspension was transferred into a new microcentrifuge tube containing 1 ml of LB broth and incubated at 37ºC for 1 hour. The cell suspension was spreaded onto the LB agar plated, containing 100 µg/ml ampicillin and spreaded with X-gal IPTG, and incubated at 37ºC overnight.

2.6.5 **Plasmid preparation.**

Plasmid harboring cells were cultured in LB broth (1.5 ml) at 37ºC 250 rpm overnight and harvested by centrifugation at 12,000 rpm at room temperature for 0.5 minutes. The cell pellet was used to plasmid extraction by Aurum Plasmids Mini Kit from Bio-RAD.

2.7 Identification of cyclodextrin glycosyltransferase gene from *Paenibacillus* sp. BT01

2.7.1 **Preparation of the CGTase gene using the PCR technique.**

A pair of primer designed from *Bacillus circulans* A11 (Rimphanitchayakit *et al.,* 2005) was used in the PCR reaction. The forward primer (primer A) was 5′-GGCTATGCTTTCCTTACCTTACCC–3′ and the reverse primer (primer B) was 5′-ATAGCACCTTTCCCCCACATAACG–3′. The melting temperature of primers were 64 and 66ºC, respectively.

The CGTase gene was amplified using gradient PCR method. Fifty microliters reaction mixture contained 1.25 Unit GoTaq Flexi DNA polymerase, 0.2 mM dNTPs, 1x PCR buffer pH 8.5, 4.0 mM $MgCl_2$, 50 ng DNA template and 10 pmole of each primer. Three rounds of temperature cycling for PCR were performed as followed.

The first round : Predenaturation at 95ºC for 2 minutes; 1 cycle.

The second round : Denaturation at 94ºC for 30 seconds
annealing at 45ºC, 50ºC and 55ºC for 45 seconds;
extension at 72ºC for 2 minutes 30 seconds;
30 cycles

The third round : 72ºC for 10 minutes; 1 cycle

The PCR products were analyzed by agarose gel electrophoresis.

2.7.2 **Ligation of the PCR product with the plasmid and transformation**

PCR product (annealing at 45ºC) was ligated with pGEM-T easy Vector. The ligation mixture of 10 µl containing 3 µl PCR product, 50 ng of pGEM-T easy Vector, 1x ligation buffer and 3 units of T_4 DNA ligase, was incubated overnight at 16ºC and then transformed into *E. coli* strain DH5α using electroporation technique. After incubation on LB-ampicillin agar plate at 37ºC for 16 hours, the ampicillin resistance colonies were selected. The transformants were screened for CGTase activity using iodine test. The recombinant plasmids were confirmed to contain CGTase gene inserts by sequencing.

2.8 **Cloning of cyclodextrin glycosyltransferase gene from *Paenibacillus* sp. BT01**

2.8.1 **PCR Amplification**

Primers used for PCR amplification of CGTase gene were designed by using the nucleotide sequence from CGTase gene of *Paenibacillus* sp. BT01. The sequence of 5′-primer (pRBF1) was 5′-CATG**CCATGG**AAAGATTTATGAAACTAACA GCCGTA -3′ with *NcoI* site. The sequence of 3′-primer (pRBR1) was 5′-CCG**CTCGAG**TTAAGGCTGCCAGTTCACATTCATC-3′ with *XhoI* site. The melting temperatures of the primer were 70ºC and 74ºC, respectively.

The CGTase gene was amplified using PCR method. Fifty microliters reaction mixture contained 1 unit of Phusion™ DNA polymerase, 0.2 mM dNTPs, 1x PCR buffer, 1.5 mM $MgCl_2$, 50 ng DNA template and 10 pmole of each primer. The PCR was performed with three rounds of temperature cycling as followed.

The first round : Predenaturation at 95ºC for 2 minutes
add Phusion™ DNA polymerase 1 unit and
predenaturation at 98ºC 30 seconds
; 1 cycle

The second round : Denaturation at 98ºC for 8 seconds, anealing at 70ºC for 20 seconds, extension at 72ºC for 2 minutes 10 seconds
; 32 cycles

The third round : 72ºC for 10 minutes
; 1 cycle

The PCR product was analyzed by agarose gel elctrophoresis.

2.8.2 **Cloning of CGTase gene into plasmid**

The expression vector pET-19b and CGTase gene fragment from method 2.8.1 were linearized with *NcoI* and *XhoI*. The reaction mixture containing of 1 µg pET-19b, 1x NEB buffer 2, 1x BSA solution, 2 U of *NcoI* and 2 U of *XhoI* in total volume of 20 µl was incubated at 37ºC overnight. The restriction enzyme was inactivated at 65ºC for 20 minutes. Digested CGTase gene fragment was ligated to the digested pET-19b vector at molar ratio vector : insert at 1:3. The ligation mixture of 10 µl contained 50 ng of vector DNA, 150 ng of gene fragment, 1X ligation buffer (50 mM

Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 µg/ul bovine serum albumin) and 20 U of T4 DNA ligase was incubated overnight at 16ºC. The recombinant plasmids in this reaction mixture were transformed into *E. coli* BL21 (DE3) using electroporation. The transformed cells were grown in LB-starch agar containing 100 µg/ml of ampicillin at 37ºC overnight.

2.8.3 Selection of positive recombinant

After incubation at 37ºC overnight, the recombinant with CGTase activity produced cyclodextrin from starch which could be detected as clear zone on starch agar plate and detected by staining the dextrinizing activity of CGTase with 0.2% I_2 in 2% KI. The dye was poured on the starch-plate, positive colony appeared as clear zone against dark blue background.

2.9 Expression of CGTase gene from pBT

2.9.1 Starter inoculum

The *E. coli* BL21(DE3) transformant colony (pBT) was transferred into LB medium containing 100 µg/ml ampicillin at 37ºC with rotary shaking overnight.

2.9.2 Enzyme production

Starter inoculums (1%) was transferred into LB medium in Erlenmeyer flask and cultured at 37ºC with shaking. When the turbidity of the culture at 660 nm reached OD. 0.6, IPTG was added to final concentration of 0.2 mM to induce CGTase gene expression and cultivation was continued at 37ºC for 24hours. After cultivation, bacterial cell mass was removed by centrifugation at 3500 rpm for 15 minutes at 4ºC. Culture broth and bacterial cell mass with crude enzyme were collected.

2.9.3 Preparation of intracellular enzyme from pBT (modified from Han and Tao, (1999))

Bacterial cell mass from enzyme section 2.9.2 was suspended in PBS solution (0.1 M sodium phosphate, 0.145 M NaCl, pH7.2) and disrupted by ultrasonication at 25% power and 25% interval setting for 2 minutes. The cell debris was separated by centrifugation at 10,000 rpm for 10 minutes and the supernatant (intracellular crude enzyme) was retained for determination of enzyme activity.

2.10 Purification of CGTase

CGTases were purified from crude extracts from *Paenibacillus* sp. BT01 (section 2.5) and recombinant pBT (section 2.9) by two steps of purification; starch adsorption and DEAE-cellulose column chromatography.

2.10.1 Starch adsorption

Corn starch was oven dried at 120ºC for 30 minutes and cooled to room temperature (Kato and Horikoshi, 1984) according to the starch adsorption method modified by (Kuttiarcheewa 1994). It was then gradually sprinkled into stirring crude CGTase broth to make the concentration of 5%(w/v) concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 5000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with 62.5ml TB1 buffer containing 0.2 M maltose(1liter starting broth), by stirring for 10 minutes. The process was repeated once. The combined CGTase eluted was recovered by centrifugation at 5000 rpm for 30 minutes. The solution was dialyzed against water at 4ºC with 3 changes of 10 mM Tris-HCl pH 8.0 (TB2).

2.10.2 DEAE-cellulose column chromatography

DEAE-cellulose was activated by washing sequentially with excess volume of 0.5 M NaOH followed by distilled water until pH was about 7.0. The activated cellulose was equilibrated with 10 mM Tris-HCl pH 8.0 (TB2). The prepared DEAE-cellulose was packed into the column (15 x 28 cm) and was equilibrated with TB2. The dialyzed protein solution from starch adsorption was applied to DEAE-cellulose column. Unbound proteins were eluted from the column with the elution buffer; washing was continued until the absorbance at 280 nm of eluant decreased to almost zero. After the column was washed thoroughly with the TB2 buffer, the bound proteins were eluted from the column with linear salt gradient of 0 to 0.2 M sodium chloride in the same buffer. Fractions of 4.0 ml were continuously collected. The protein and activity profile of the eluted fractions were monitored by measuring the absorbance at 280 nm and assay of dextrinizing activity (Section 2.11). Fractions with enzyme activity were pooled for further determination.

2.11 Enzyme assay

CGTase activity was determined by assay of starch degrading (dextrinizing) activity and cyclization (CD-forming) activity.

2.11.1 Dextrinizing activity

Dextrinizing activity of CGTase was measured by the method of Fuwa (1954) with slight modification (Techaiyakul, 1991).

Sample (10-100 μl) was incubated with 0.3 ml starch substrate (0.2g% soluble potato starch in 0.2 M phosphate buffer, pH 6.0) at 40ºC for 10 minutes. The reaction was stopped with 4 ml of 0.2 M HCl. Then 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and its absorbance at 600 nm was measured. For a control tube, HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described conditions.

2.11.2 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method (modified from Goel and Nene, (1995)). Purified CGTase was added to 1.0 ml of 6.0% soluble starch in 0.2 M acetate buffer, pH 6.0. The reaction mixture was incubated for 30 minutes at 60^{o}C. Reaction was stopped by boiling for 10 minutes. Fifty microliters of the reaction mixture was incubated with 2.0 ml of phenolphthalein solution. Absorption was measured at 550 nm and β-CD formed was calculated using the calibration curve of standard of β-CD- phenolphthalein complex (see Appendix 5). One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the corresponding condition.

Phenolphthalein solution was prepared with 1 ml of 4 mM phenolphthalein solution in absolute ethanol, 100 ml of 125 mM Na_2CO_3 solution in distilled water and 4 ml ethanol. Solution was prepared freshly before starting the experiment. β-CD standard 0-2.5 mM was prepared.

2.11.3 Protein determination

Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard protein (see Appendix 4).

2.12 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE were performed for analysis of enzyme purification according to Bollag *et al.*, (1996), the non-denaturing and denaturing gels. The gels were visualized by coomassie blue staining. For non-denaturing gel, dextrinizing acitivity stain was also undertaken.

2.12.1 Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE)

Discontinuous PAGE was performed on 7.5% (w/v) separating gel, and 5.0% (w/v) stacking gels. Tris-glycine buffer pH 8.3 was used as electrode buffer (see Appendix 1). The electrophoresis was run from cathode towards anode at constant current of 16 mA per slab at room temperature in a Mini-Gel electrophoresis unit (Bio-RAD).

2.12.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was carried out with 0.1% (w/v) SDS in 10.0% (w/v) separating and 5.0%(w/v) stacking gels with Tris-glycine buffer pH 8.0 containing 0.1% SDS as electrode buffer (see Appendix 1). Samples to be analyzed were treated with sample buffer and boiled for 5 minutes prior to application to the gel. The electrophoresis was performed at constant current of 20 mA per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.12.3 Detection of proteins and CGTase

2.12.3.1 Coomassie blue staining

Gels from ND-PAGE and SDS-PAGE were stained with 0.1%(w/v) of coomassie brilliant blue R-250 in 45%(v/v) methanol and 10%(v/v) acetic acid for at least 2 hours. The slab gels were destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours, followed by several changes of destaining solution (10% methanol, 10% glacial acetic acid and 50% distilled water) until gel background was clear.

2.12.3.2 Dextrinizing activity staining

CGTase activity was detected on ND-PAGE using the method slightly modified from Kobayashi *et al.* (1978). The running gel was soaked in 10ml of substrate solution, containing 0.2%(w/v) potato starch in 0.2 M phosphate buffer pH 6.0, at 40^{o}C for 10 minutes. The gel was then quickly rinsed several times with distilled water and 10 ml of I_2 staining reagent (0.2% I_2 in 2% KI) was added for color development at room temperature. The clear zone on the blue background indicated starch degrading activity of the enzyme.

2.13 Determination of the isoelectric point by isoelectric focusing polyacrylamide gel electrophoresis (IEF)

2.13.1 Preparation of gel support film

A few drops of water was pipetted onto the plate. The hydrophobic side of the gel support film was then placed against the plate and flatly rolled with a test tube to force excess water and bubbles. Subsequently, it was placed on the casting tray with the gel support film face down resting on the space bars.

2.13.2 Preparation of the gel

A monomer-ampholyte solution was prepared (Appendix 2) and degassed for 5 minutes under vacuum. A catalyst solution (Appendix 2) was added to the degassed monomer solution and swirled gently. The mixed solution was carefully pipetted to the space between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was lift to polymerize for about 45 minutes, and then lifted from the casting tray using a spatula. The gel was fixed on the gel support film. A template for sample application was placed in the middle of the polymerized gel and ready for used.

2.13.3 Sample application and electrophoresis gel

The samples (1-4 μl) were loaded on the template. Standard protein markers with known pI's in the range 3-10 were included in each run. Samples were allowed to diffuse into the gel for 5 minutes and the template was carefully removed from the gel. The gel with the adsorbed samples was turned upside-down and directly placed on top of the graphite electrodes. Focusing was carried out stepwise under constant

voltage conditions: 100 V for 20 minutes, 200 V for 20 minutes and finally 450 V for an additional 90 minutes. After completing electrofocusing, the gel was stained with coomassie brilliant blue R-250. The pI's of sample proteins were determined using a standard curve constructed from the pI's of the standard proteins and their migrating distance from cathode. The standard proteins consisted of amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydase B (5.85), human carbonic anhydase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.3).

2.14 Optimum conditions for enzyme activity

2.14.1 Effect of pH

Each purified CGTase was used to determine the effect of pH on its activity. The enzyme was assayed as described in section 2.11, replacing the phosphate buffer with various 0.2 M buffer solution in the pH range from 4.0 to 10.0. The buffers used were sodium acetate (pH 4.0-6.0), phosphate (pH 6.0-8.0) and Tris-Glycine NaOH (pH 8.0-10.0) (see Appendix 3).

2.14.2 Effect of temperature

Each purified CGTase was assayed for dextrinizing and cyclization activity as described in section 2.11 at varying temperatures in the range 30-80^{o}C.

2.15 Enzyme stability

2.15.1 pH stability

The stability of each purified CGTase at different pH's was determined by incubating for one hour in various pH's (5.0-10.0) at 50^{o}C. Samples were withdrawn to determine cyclization activity.

2.15.2 Temperature stability

Effect of temperature on the stability of enzyme was studied by incubating the enzyme at different temperatures : 40°C, 50°C, 60°C, 65°C and 70°C in acetate buffer pH 6.0 up to two hours. Samples were taken at intervals and assayed for cyclization activity. To determine the effect of starch on enzyme stability, soluble starch was

added to final concentrations of 2% and 20% at selected temperatures (70°C, 80°C and 90°C) incubated up to two hours and assayed for cyclization activity.

2.16 Determination of kinetic parameters

Kinetic parameters of the cyclization activity were determined by incubating various concentrations of soluble potato starch solution, ranging from 2–60 mg/ml at 50ºC for 15 minutes in 0.1 M acetate buffer pH 6.0. The suitable amount of CGTase used was 50 units dextrinizing activity of each purified CGTase. The reaction was stopped by boiling for 10 minutes. Cyclization activity was assayed as described in section 2.11.2. Kinetic parameters were determined from the Michealis-Menten equation, using Lineweaver-Burk plot.

2.17 Analysis of cyclodextrins by High Performance Liquid Chromatography(HPLC).

The HPLC system was performed on Shimadzu LC-3A machine equipped with Lichrocart-NH_2 column (0.46 x 25 cm) using Shimadzu RID-3A refractometer as detector. For CD analysis, the reaction was performed by incubation of 2 unit cyclization activity of each purified CGTase with 20 ml of various starch substrates (20% of soluble starch (potato), tapioca starch, Flo-Max8 and starch extract from KU50) in 0.2 M acetate buffer, pH 6.0 at 60ºC for 24 hours with shaking and boiled for 10 minutes to stop the reaction. Prior to injection, the mixture was filtered through a 0.45 μm membrane filter. The mixture was injected (10 μl) and eluted with acetronitrile-water (70:30, v/v) using a flow rate of 1.0 ml/min. CD product was identified by comparing Rf with standard CDs injected under the same condition and quantified from the area peak.

CHAPTER III
RESULTS

3.1 Identification of cyclodextrin glycosyltransferase gene *from Paenibacillus* sp. BT01

From previous study on nucleotide sequence of CGTase gene from *Paenibacillus* sp. A11 (*Bacillus circulans* A11), a pair of primers positioned upsteam and downsteam of CGTase gene was designed for amplification of the whole CGTase gene. Chromosomal DNA of *Paenibacillus* sp. BT01 was prepared and the CGTase gene fragment amplified as described in section 2.7.1. One major band at 2.4 Kb, minor band at 1.3 Kb, 0.3 Kb and smear band were observed on agarose gel electrophoresis. Higher amount of PCR product at annealing temperature of 45°C was observed comparing to annealing temperature of 51°C and 55°C (Figure 5). Therefore, the major band of PCR product at annealing temperature 45°C was used for insertion into pGEM-T easy and transformed into *E. coli* DH5α. The transformant appeared as colonies with clear zone on LB-starch plate. The insert fragment in recombinant plasmid was sequenced. The result showed that the insert size was 2425 bp and contained open reading frame of 2142 bp (Figure 6). The nucleotide sequence was compared with the EMBL-GeneBank-DDBL database (Table 5). The analysis showed 98%, 98%, 99%, 98% and 66%homology to CGTase gene of *Bacillus* sp. strain N-227, *Paenibacillus* sp. A11, *Bacillus* sp. strain 38-2, *Bacillus* sp. 1011 and *Bacillus stearothermophilus*, respectively.

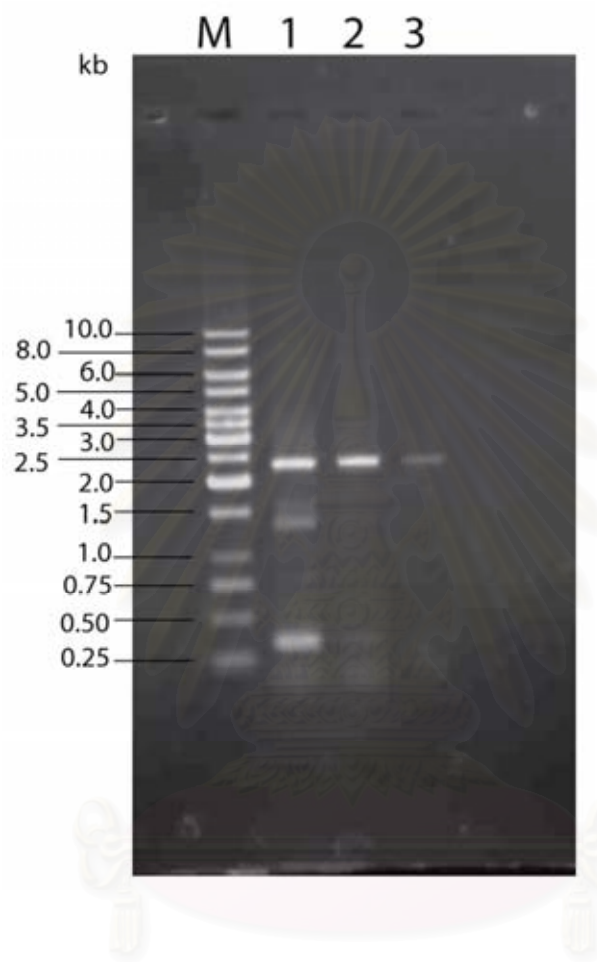


Figure 5: The PCR product from CGTase gene of *Paenibacillus* sp. BT01.

Lane M: GeneRuler^{TM}1 kb DNA Ladder

Lane 1: PCR product using annealing temperature of 45ºC

Lane 2: PCR product using annealing temperature of 50ºC

Lane 3: PCR product using annealing temperature of 55ºC

Primer A

TATGGTCGACCTGCAGGCGGCCGCACTAGTGATTT**GGCTATGCTTTCCTTACCTTACCC**CGGTATGGAA
CAACCCCGGTATCTCTATTAGAGACGCCGGGGTTTCTTATGTAGCCGAGATGAAGGAGGTGATCCCCAA
-10 **SD** **-35**
AGCGACGGACAGGCCTGTTATCCCTAAGCATTGTATACGATGAGGAGGTATAGTATGAAAAGATTTATG
AAACTAACAGCCGTATGGACACTCTGGTTATCCCTCACGCTGGGCCTTTTGAGCCCGGTCCACGCAGCC
CCGGATACCTCGGTATCCAACAAGCAGAATTTCAGCACGGATGTCATATATCAGATCTTCACCGACCGG
TTCTCGGACGGCAATCCGGCCAACAATCCGACCGGCGCGGCATTTGACGGATCATGTACGAATCTTCGC
TTATACTGCGGCGGCGACTGGCAAGGCATCATCAACAAAATCAACGACGGTTATTTGACCGGCATGGGC
ATTACGGCCATCTGGATTTCACAGCCTGTCGAGAATATCTACAGCGTGATCAATTACTCCGGCGTCAAT
AATACGGCTTATCACGGCTACTGGGCGCGGGACTTCAAGAAGACCAATCCGGCCTACGGGACGATGCAG
GACTTCAAAAACCTGATCGACACCGCGCATGCGCATAACATAAAAGTCATCATCGACTTTGCACCGAAC
CATACATCTCCGGCTTCTTCGGATGATCCTTCTTTTGCAGAGAACGGCCGCTTGTACGATAACGGCAAC
CTGCTCGGCGGATACACCAACGATACCCAAAATCTGTTCCACCATTATGGCGGCACGGATTTCTCCACC
ATTGAGAACGGCATTTATAAAAACCTGTACGATCTGGCTGACCTGAATCATAACAACAGCAGCGTCGAT
GTGTATCTGAAGGATGCCATCAAAATGTGGCTCGACCTCGGGGTGGACGGCATTCGTGTGGACGCGGTC
AAGCATATGCCATTCGGCTGGCAGAAGAGCTTTATGGCCACCATTAACAACTACAAGCCGGTCTTCACC
TTTGGCGAATGGTTCCTAGGCGTCAATGAGATCAGTCCGGAATACCATCAATTCGCTAACGAGTCCGGG
ATGAGCCTGCTCGATTTCCGCTTTGCCCAGAAGGCTCGGCAAGTGTTCAGGGACAACACCGACAATATG
TACGGCCTGAAGGCGATGCTGGAGGGCTCTGAAGTAGACTATGCCCAGGTGAATGACCAGGTGACCTTC
ATCGACAATCATGACATGGAGCGTTTCCACACCAGCAATGGCGACAGACGGAAGCTGGAGCAGGCGCTG
GCCTTTACCCTGACTTCACGCGGTGTGCCTGCCATCTATTACGGCAGCGAGCAGTATATGTCTGGCGGG
AATGATCCGGACAACCGTGCTCGGATTCCTTCCTTCTCCACGACGACGACCGCATATCAAGTCATCCAA
AAGCTCGCTCCGCTCCGCAAATCCAACCCGGCCATCGCTTACGGTTCCACACAGGAGCGCTGGATCAAC
AGCGATGTGATCATCTATGAACGCAAATTCGGCAATAACGTGGCCGTTGTTGCCATTAACCGCAATATG
AACACACCGGCTTCGATTACCGGCCTTGTCACTTCCCTCCCGCAGGGCAGCTATAACGATGTGCTCGGC
GGAATTCTAAACGGCAATACGTTAACCGTGGGTGCTGGCGGTGCAGCTTCCAACTTTACTTTGGCTCCT
GGCGGCACTGCTGTATGGCAGTACACAACCGATGCCACAGCTCCGATCATCGGCAATGTCGGCCCGATG
ATGGCCAAGCCAGGGGTCACGATTACGATTGACGGCCGCGGATTCGGCTCCGGCAGGGGAACGGTTTAC
TTCGGTACAACGGCAGTCACTGGCGCGGACATCGTAGCTTGGGAAGATACACAAATCCAGGTGAAAATC
CCTGCGGTCCCTGGCGGCATCTATGATATCAGAGTTGCCAACGCAGCCGGAGCAGCCAGCAACATCTAC
GACAATTTCGAGGTGCTGACCGGAGACCAGGTCACCGTTCGGTTCGTAATCAACAATGCCACAACGGCG
CTGGGACAGAATGTGTTCCTCACGGGCAATGTCAGCGAGCTGGGCAACTGGGATCCGAACAACGCGATC
GGCCCGATGTATAATCAGGTCGTCTACCAATACCCGACTTGGTATTATGATGTCAGCGTTCCGGCAGGC
CAAACGATTGAATTTAAATTCCTGAAAAAGCAAGGCTCCACTGTCACATGGGAAGGCGGCGCGAATCGA
ACCTTCACCACCCCAACCAGCGGCACGGCAACGGTGAATGTGAACTGGCAGCCTTAATAGTCACTTGCA
AGGTAAGCAAGCGGCTCCGGGTAGAGACCCGGGACCGCTTGTTTA**CGTTATGTGGGGGAAAGGTGCTAT**
AATCCCGCGG

Primer B

Figure 6: Nucleotide sequence of the DNA fragment containing the CGTase gene from *Paenibacillus* sp. BT01. The start and stop codons are boxed. Putative promoter elements and the Shine–Dalgarno sequence are shaded.

Table 5: BLAST result of inserted pGEM-T easy fragment (2425 bp)

accession number	source	%identity	score
DQ631916	*Bacillus* sp. N-227	98	4612
AF302787	*Paenibacillus* sp. A11	98	4612
M19880	*Bacillus* sp. 38-2	98	4530
M17366	*Bacillus* sp.1011	98	4508
X59043	*Bacillus sterarothermophilus*	66	256

3.2 Cloning of cyclodextrin glycosyltransferase gene from *Paenibacillus* sp. BT01

3.2.1 PCR amplification of CGTase gene

CGTase gene from BT01 was amplified according to the method described in section 2.8. A pair of primers was designed from the nucleotide sequence at 5′-end and 3′-end of the CGTase gene obtained in section 3.1. To prepare the amplified gene product for insertion into pET-19b, the 5′-end primer (pRBF1) comprised of *Nco*I restriction site and 5′-base sequence of CGTase gene. The 3′-end primer (pRBR1) comprised of *Xho*I restriction site and 3′-base sequence of CGTase gene according to the method described in section 2.8.1. Figure7 showed the 2.4 kb PCR product of CGTase gene fragment amplified from the chromosomal DNA of *Paenibacillus* sp. BT01.

3.2.2 Transformation and selection of positive colony

The 2.4 kb amplified gene fragment was digested with *Nco*I and *Xho*I and ligated to pET-19b vector digested with same restriction enzyme, then transformed into *E. coli* BL21(DE3) by electroporation. The transformed *E. coli* BL21(DE3) with the inserted CGTase gene showed clear zone on the LB-starch plate stained with iodine. The recombinant of *Paenibacillus* sp. BT01 was named pBT. The recombinant plasmid gave two major bands, relaxed and supercoiled bands, on agarose gel as shown in Figure 7.

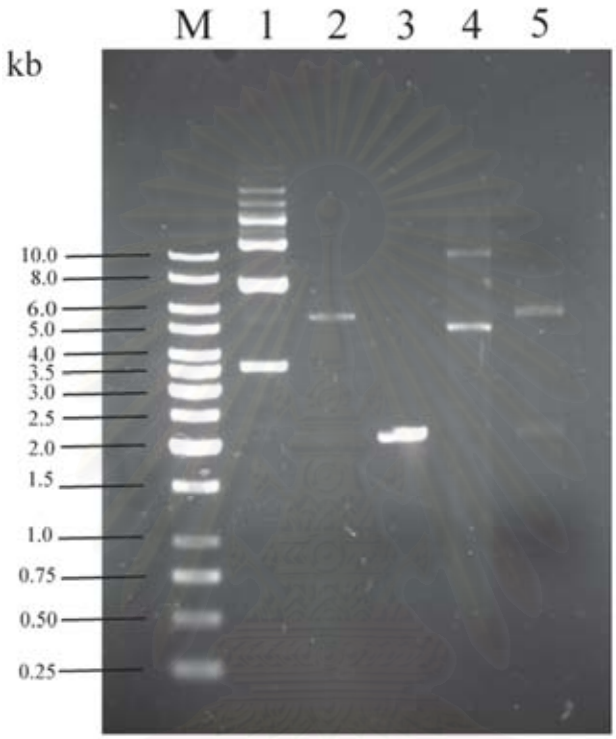


Figure7: PCR product and recombinant plasmid

Lane M: GeneRuler^{TM}1 kb DNA Ladder

Lane 1: pET-19b Vector

Lane 2: pET-19b Vector cut with *Nco*I/*Xho*I

Lane 3: PCR product using pRBF1 and pRBR1 primers

Lane 4: pET-19b vector inserted with CGTase gene (pBT)

Lane 5: Recombinant plasmid digest with *NcoI*/*XhoI*

3.2.3 Nucleotide and deduced amino acid sequences of CGTase gene

The inserted fragment in recombinant plasmid pBT was sequenced. The result showed that it contained a CGTase open reading frame of 2142 base pairs, which encoded a polypeptide of 713 amino acid residues, composed of 27 amino acid residues of signal peptide constituted at the N-terminal and a 686 amino acid mature enzyme (Figure 8). The molecular weight of CGTase calculated from the deduced amino acid sequence was 75 kDa. The nucleotide sequence was compared in the EMBL-GeneBank-DDBL database. It showed 99%, 99%, 98%, 98% and 66% homology to CGTase gene of *Bacillus* sp. N-227, *Paenibacillus* sp. A11, *Bacillus* sp. I-5, *Bacillus* sp. 1011 and *Bacillus stermophilus* (Figure 10, Table 6). The homology of amino acid sequence to *Paenibacillus* sp. A11, *Bacillus* sp. N-227, *Bacillus* sp. 1011 and *Bacillus* sp. I-5 were 97%, 97%, 97% and 96%, respectively (Figure11, Table 7). Tree construction using the neighbor-joining method and bootstrap analysis was performed with ClustalX. Phylogenic tree constructed from sequence alignment with published sequence of various CGTases were shown in Figure 12.

Figure 8: Nucleotide sequences of CGTase of pBT and A11. The nucleotide sequence beginning with the ATG initiator codon and ending with TAA codon. The nucleotide sequences of A11 were shown only at the positions with different base sequence and appeared above the corresponding bases of pBT.

```
 K
MERFMKLTAV WTLWLSLTLG LLSPVHAAPD TSVSNKQNFS TDVIYQIFTD RFSDGNPANN PTGAAFDGSC TNLRLYCGGD WQGIINKIND 90
                                 H
GYLTGMGITA IWISQPVENI YSVINYSGVN NTAYHGYWAR DFKKTNPAYG TMQDFKNLID TAHAHNIKVI IDFAPNHTSP ASSDDPSFAE 180
NGRLYDNGNL LGGYTNDTQN LFHHYGGTDF STIENGIYKN LYDLADLNHN NSSVDVYLKD AIKMWLDLGV DGIRVDAVKH MPFGWQKSFM 270
S
ATINNYKPVF TFGEWFLGVN EISPEYHQFA NESGMSLLDF RFAQKARQVF RDNTDNMYGL KAMLEGSEVD YAQVNDQVTF IDNHDMERFH 360
TSNGDRRKLE QALAFTLTSR GVPAIYYGSE QYMSGGNDPD NRARIPSFST TTTAYQVIQK LAPLRKSNPA IAYGSTQERW INNDVIIYER 450
KFGNNVAVVA INRNMNTPAS ITGLVTSLPQ GSYNDVLGGI LNGNTLTVGA GGAASNFTLA PGGTAVWQYT TDATAPIIGN VGPMMAKPGV 540
TITIDGRGFG SGKGTVYFGT TAVTGADIVA WEDTQIQVKI PAVPGGIYDI RVANAAGAAS NIYDNFEVLT GDQVTVRFVI NNATTALGQN 630
                                                                    M
VFLTGNVSEL GNWDPNNAIG PMYNQVVYQY PTWYYDVSVP AGQTIEFKFL KKQGSTVTWE GGANRTFTTP TSGTATVNVN WQP 713
```

Figure 9: The deduced amino acid residues of pBT and A11. The deduce amino acid sequences of A11 were shown only at the positions with different base sequence and appeared above the corresponding amino acid sequence of pBT. The signal sequence is underlined.

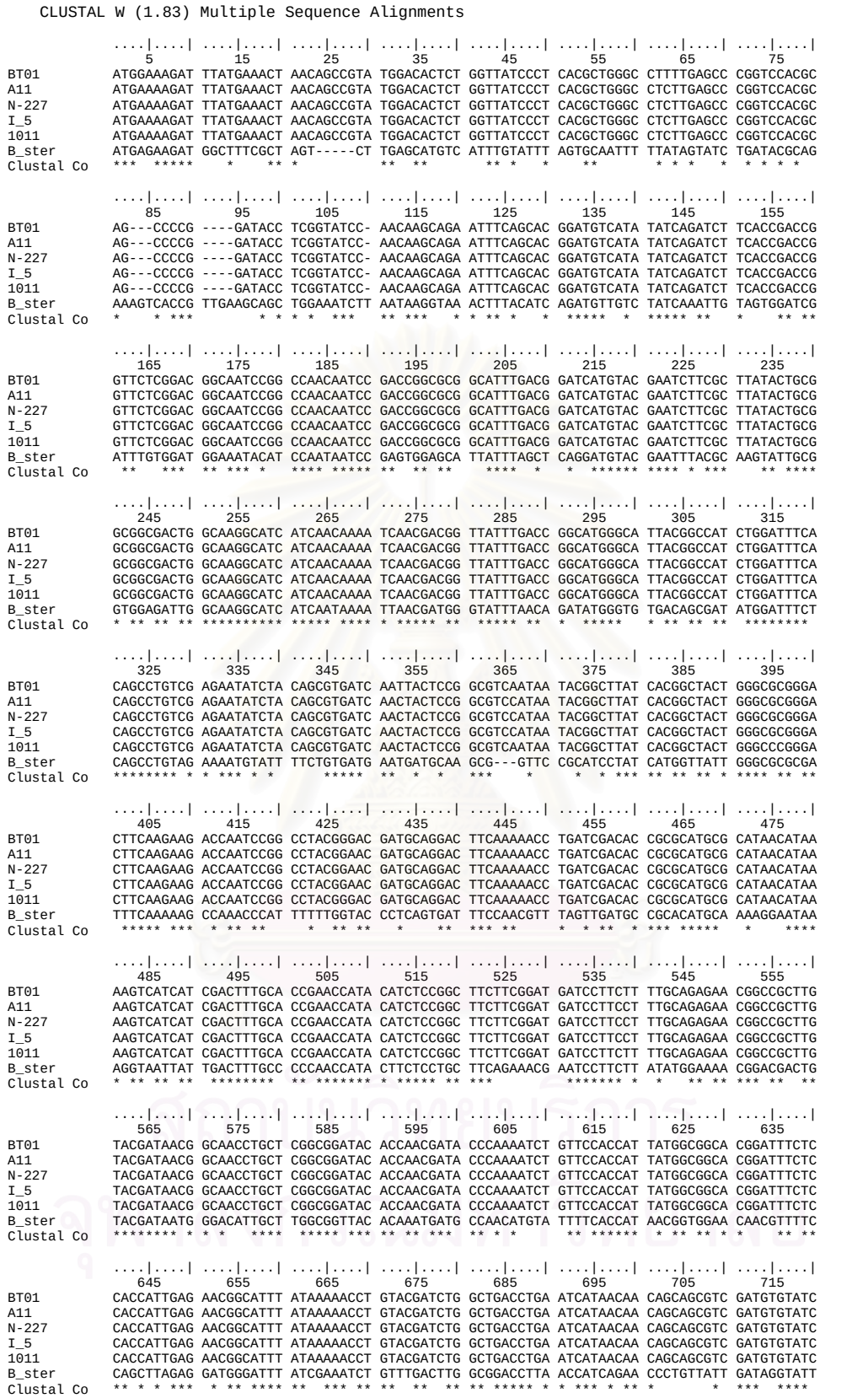


Figure 10: Multiple alignment of the nucleotide sequence of BT01 with other CGTase producing bacteria. The nucleotide sequences indicated by asterisks (*) are completely identical. Dashes (---) denote deleted nucleotide sequence.

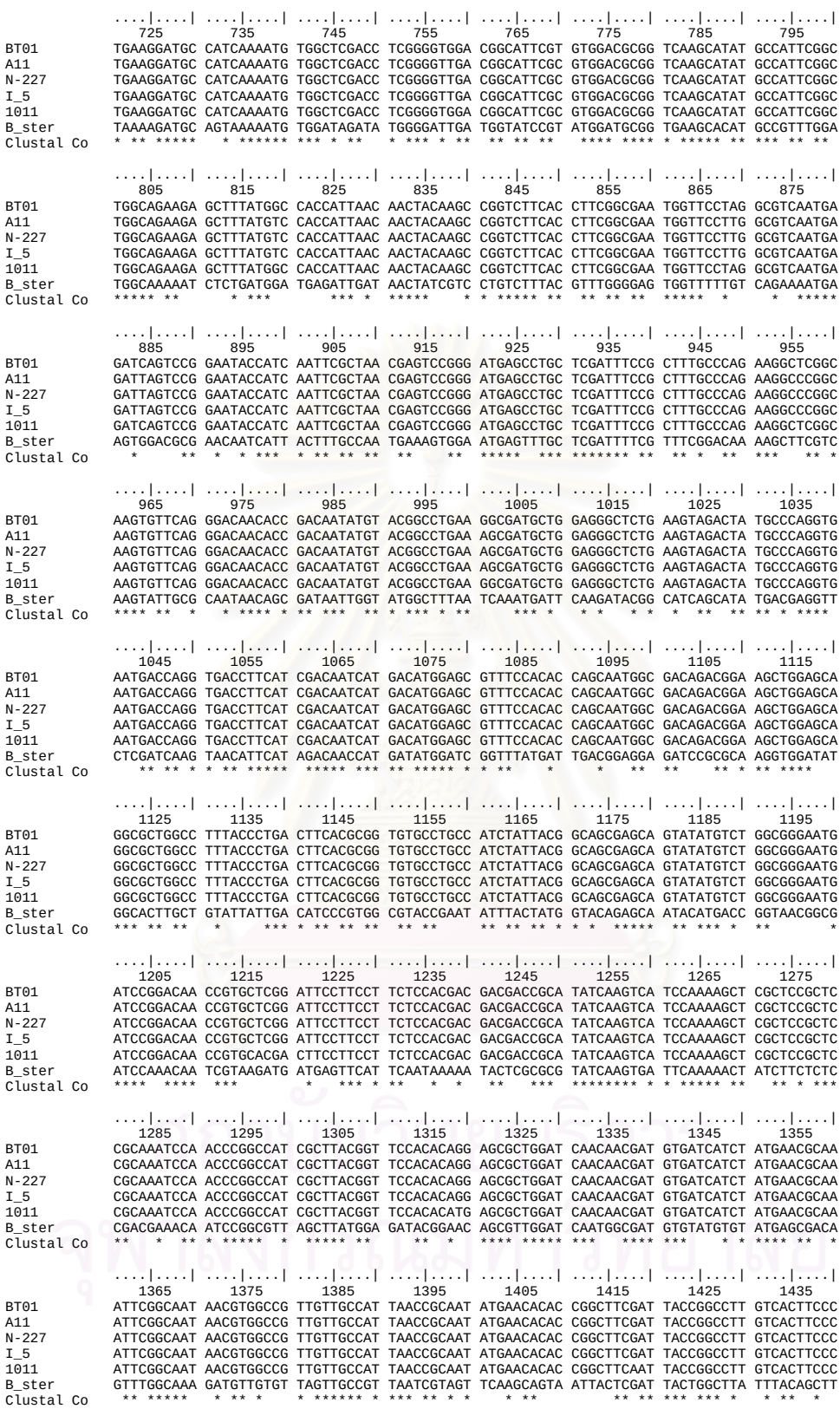


Figure 10(continued)

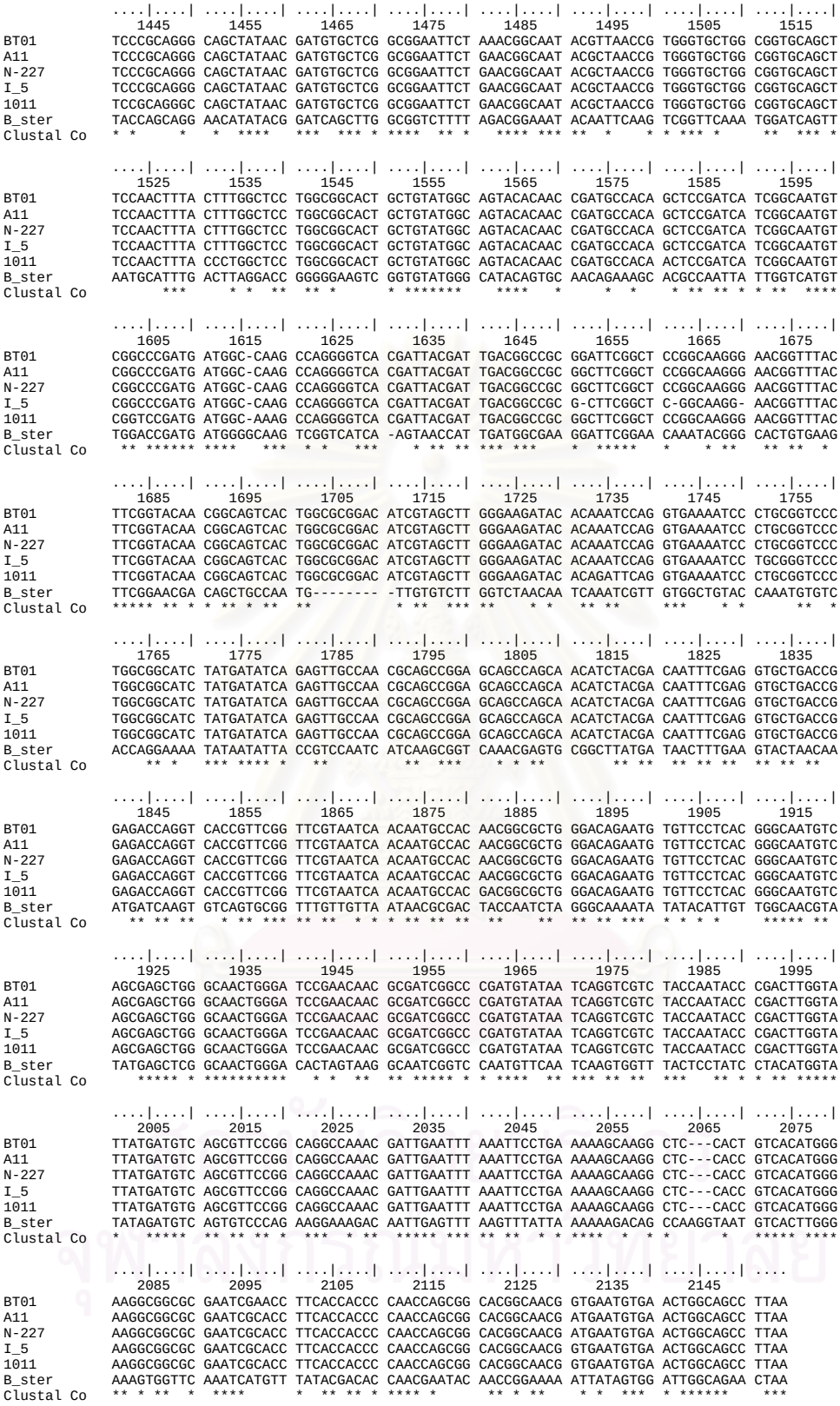


Figure 10 (continued)

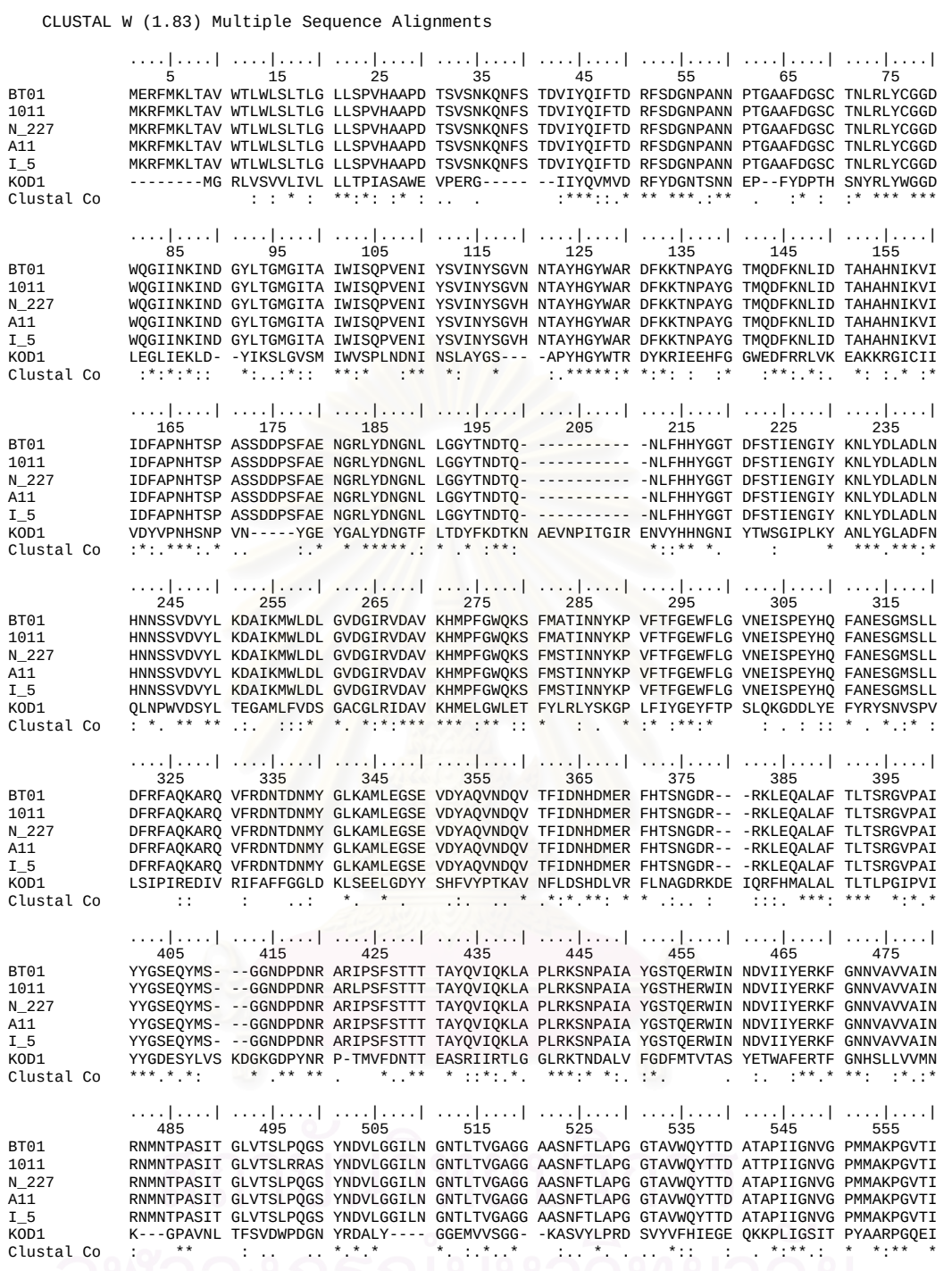


Figure 11: Comparison of deduced amino acid sequence for pBT. The amino acid residues indicated by asterisks (*) were completely identical , (:) conserved substitutions (.) semi-conserved substitutions Dashes (---) denote deleted amino acids.

```
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                565        575        585        595        605        615        625        635
BT01         TIDGRGFGSG KGTVYFGTTA VTGADIVAWE DTQIQVKIPA VPGGIYDIRV ANAAGAASNI YDNFEVLTGD QVTVRFVINN
1011         TIDGRGFGSG KGTVYFGTTA VTGADIVAWE DTQIQVKIPA VPGGIYDIRV ANAAGAASNI YDNFEVLTGD QVTVRFVINN
N_227        TIDGRGFGSG KGTVYFGTTA VTGADIVAWE DTQIQVKIPA VPGGIYDIRV ANAAGAASNI YDNFEVLTGD QVTVRFVINN
A11          TIDGRGFGSG KGTVYFGTTA VTGADIVAWE DTQIQVKIPA VPGGIYDIRV ANAAGAASNI YDNFEVLTGD QVTVRFVINN
I_5          TIDGR-ASAR QGTVYFGTTA VTGADIVAWE DTQIQVKILR VPGGIYDIRV ANAAGAASNI YDNFEVLTGD QVTVRFVINN
KOD1         VIGGAGFGKG GKVIIGGR-- --EAKVLSWE DGKIVVEVPR LETSAAWVNV TVVSDGGRSP PRPLRYYSGN DVPALIALNA
Clustal Co   .*.*   .     .:  *       *.::** * :* *::   :  .   :.* : .:... .     :.  :*: :*.. :.:*

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                645        655        665        675        685        695        705        715
BT01         ATT-ALGQNV FLTGNVSELG NWDPNNAIGP MYNQVVYQYP T-------WY YDVSVPAGQT IEFKFLKKQG STVTWEGGAN
1011         ATT-ALGQNV FLTGNVSELG NWDPNNAIGP MYNQVVYQYP T-------WY YDVSVPAGQT IEFKFLKKQG STVTWEGGAN
N_227        ATT-ALGQNV FLTGNVSELG NWDPNNAIGP MYNQVVYQYP T-------WY YDVSVPAGQT IEFKFLKKQG STVTWEGGAN
A11          ATT-ALGQNV FLTGNVSELG NWDPNNAIGP MYNQVVYQYP T-------WY YDVSVPAGQT IEFKFLKKQG STVTWEGGAN
I_5          ATT-ALGQNV FLTGNVSELG NWDPNNAIGP MYNQVVYQYP T-------WY YDVSVPAGQT IEFKFLKKQG STVTWEGGAN
KOD1         SLVGEVSGTL WLSGDLPELG EPRPLLKSSM GYYFTVAPLP EGVPFSVRLY EGKAWGALRP LNLTLYGVGN RTVTLTEKPP
Clustal Co   : .  :. .: :*:*::.*** :  *    .   *  .*   *          *  . :  * :. :::.:    .  ***    .

             ....|....| ....|....| ....|....| ....|
                725        735        745        755
BT01         RTFTTPTSG- ---------- ---TATVNVN WQP--
1011         RTFTTPTSG- ---------- ---TATVNVN WQP--
N_227        RTFTTPTSG- ---------- ---TATMNVN WQP--
A11          RTFTTPTSG- ---------- ---TATMNVN WQP--
I_5          RTFTTPTSG- ---------- ---TATVNVN WQP--
KOD1         GVSEGQKAGQ KDVALYALSV VMIAALIAVV WKRKG
Clustal Co    .    .:*              :* : *  *:
```

Figure 11 (continued)

Table 6: BLAST result of nucleotide sequence of CGTase gene. (2142 bp)

Abbreviation	Accession number	Source	%identity	Score
BT01		*Paenibacillus* sp. BT01		
A11	AF302787	*Paenibacillus* sp.A11	99	4188
N_227	DQ631916	*Bacillus* sp.N-227	99	4188
I_5	AY478421	*Bacillus* sp.I-5	98	4150
1011	M17366	*Bacillus* sp. 1011	98	4148
B_ster	X59043	*Bacillus stermophilus*	66	250

Table 7: BLAST result of deduce amino acid sequence of CGTase gene. (713 aa)

Abbreviation	Accession number	Source	%identity	Score
BT01		*Paenibacillus* sp. BT01		
A11	Q9F5W3	*Paenibacillus* sp. A11	97	3707
N_227	Q197W1	*Bacillus* sp. N-227	97	3707
1011	P05618	*Bacillus* sp. 1011	97	3688
I_5	Q6S3E3	*Bacillus* sp. I-5	96	3650
KOD1	Q8X268	*Bacillus stermophilus*	66	250

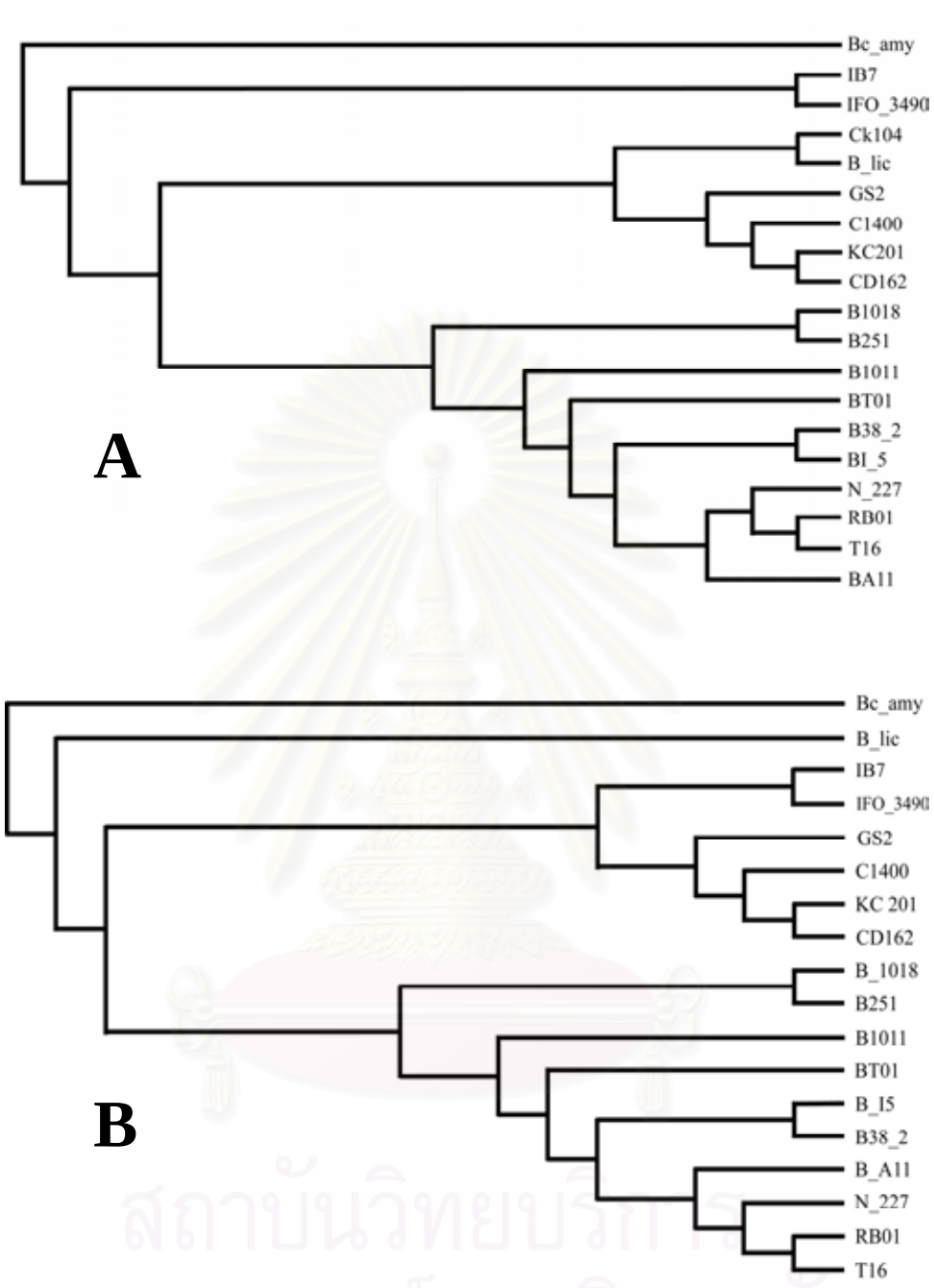


Figure12: Neighbor-joining tree based on nucleotide sequence (A) and amino acid (B) similarities among CGTase genes. See appendix 8 for list of CGTase used in construction of phylogenic tree.

3.3 Expression of CGTase in recombinant pBT.

Recombinant cells (pBT) which gave largest clear zone on LB-starch plate was selected for expression of CGTase. The selected pBT colony was grown in LB medium containing IPTG at final concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mM. Cell growth, total activity, total protein and specific activity were determined. The results were shown in Figure 13. When no IPTG was added, expression of CGTase gene was increased until 24 hours with total activity (300ml) of 3.89 x 10^4 unit dextrinizing. When the culture medium was added with various concentrations of IPTG, CGTase expression was increased up to 1.7 times at 24 hours at all concentration of IPTG tested compared to non-induced condition. Therefore, the concentrations of IPTG at 0.2 mM was sufficient for induction of CGTase. The optimum condition for induction of CGTase gene was 0.2 mM IPTG at 24 hours induction time (Figure 13).

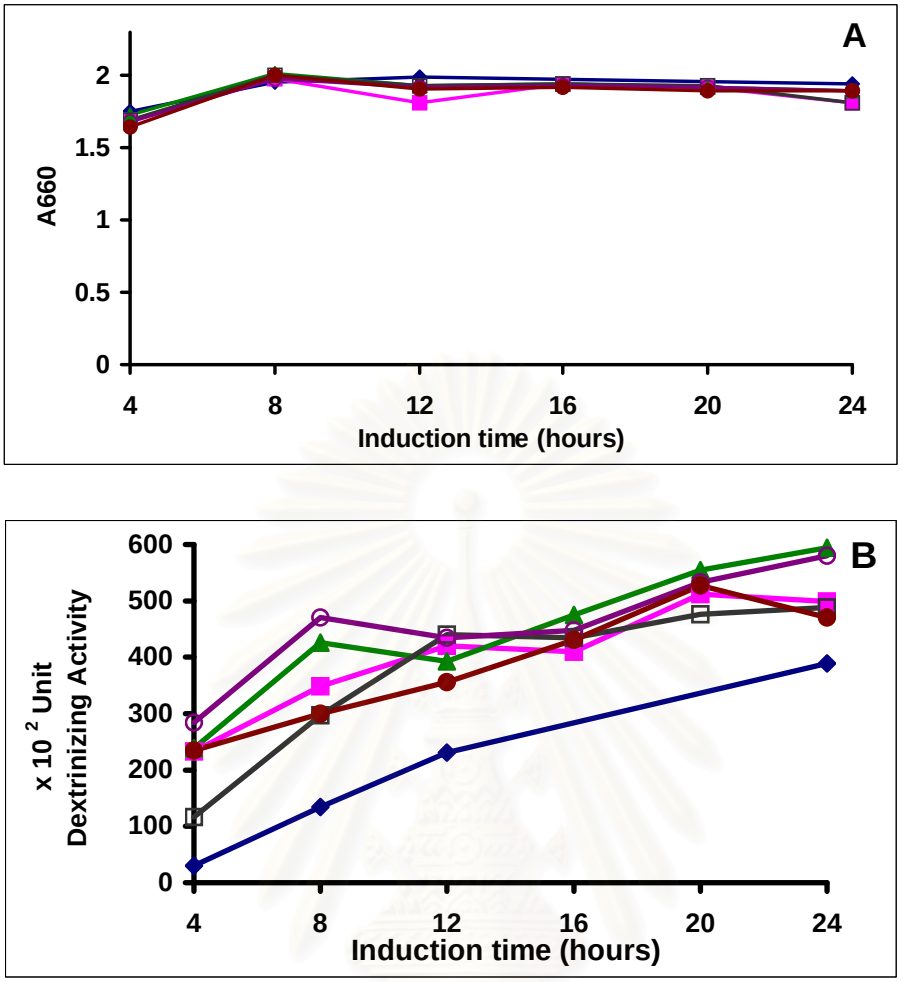


Figure 13: Expression of CGTase gene in recombinant cell by various IPTG concentrations

A) Growth profile B) Total Dextrinizing Unit.

= 0 mM IPTG
= 0.6 mM IPTG
= 0.2 mM IPTG
= 0.8 mM IPTG
= 0.4 mM IPTG
= 1.0 mM IPTG

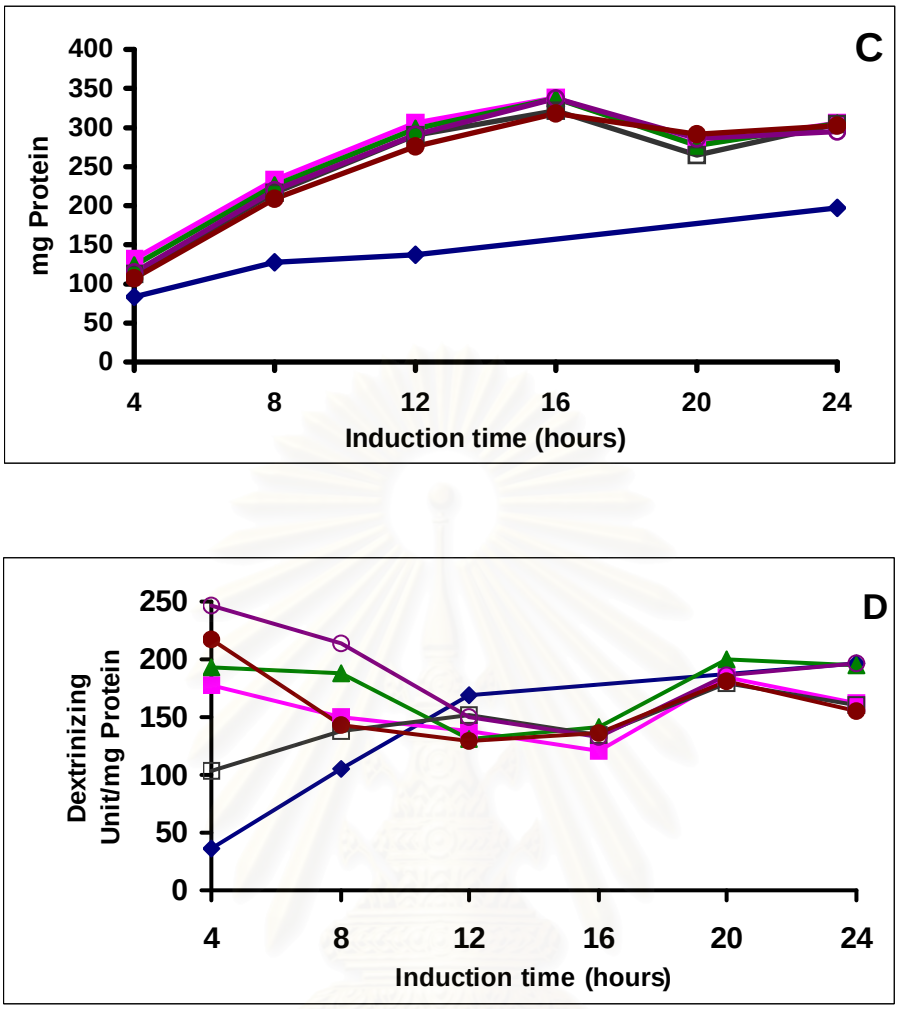


Figure 13 (continued)**:** Expression of CGTase gene in recombinant cell by various IPTG concentrations

C) Total Protein D) Specific activity(Dextrinizing Activity).

= 0 mM IPTG **= 0.6 mM IPTG**

= 0.2 mM IPTG **= 0.8 mM IPTG**

= 0.4 mM IPTG **= 1.0 mM IPTG**

3.4 Cellular localization of CGTase

CGTase activity was checked for its intracellular and extracellular distributions by separating the cells from culture medium and breaking the cells as described in section 2.9.3. Dextrinizing activity was measured both in the culture media (extracellular) and in the sonicated cells (intracellular). Table 8 showed that 99.83% of the dextrinizing activity was found in the culture medium. Figure 14 showed the electrophoresis pattern of CGTase from both fractions which were similar.

3.5 Purification of Cyclodextrin glycosyltransferase

Crude enzymes from BT01 and pBT were purified by starch adsorption followed by DEAE-cellulose chromatography column.

3.5.1 Starch adsorption

Crude CGTases from BT01 and pBT were prepared as described in sections 2.5 and 2.9 and subjected to starch adsorption as described in section 2.10.1. The specific activities of the enzymes from this step were 2.67×10^3 and 5.74×10^3 unit/mg protein for wild type BT01 and recombinant pBT, respectively. The purification fold and recovery of CGTase obtained were 21 folds and 54.7% yield for wild type BT01, 7 folds and 89.0% yield for recombinant pBT.

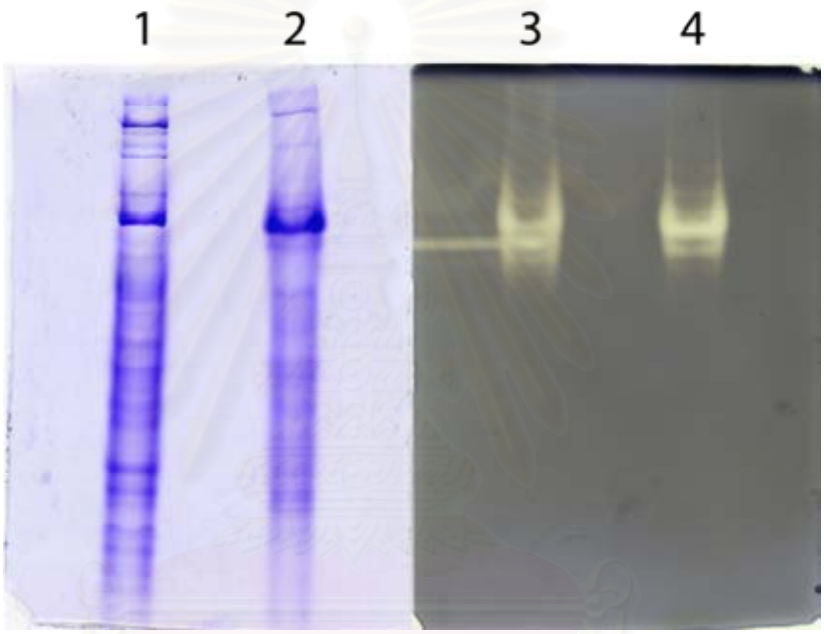


Figure 14: Crude CGTase from recombinant pBT on 7.5% Native PAGE

Coomasie blue staining

Lane 1: Intracellular Enzyme (45 μg Protein)

Lane 2: Extracellular Enzyme (45 μg Protein)

Iodine staining

Lane 3: Intracellular Enzyme (0.3 unit)

Lane 4: Extracellular Enzyme (0.3 Unit)

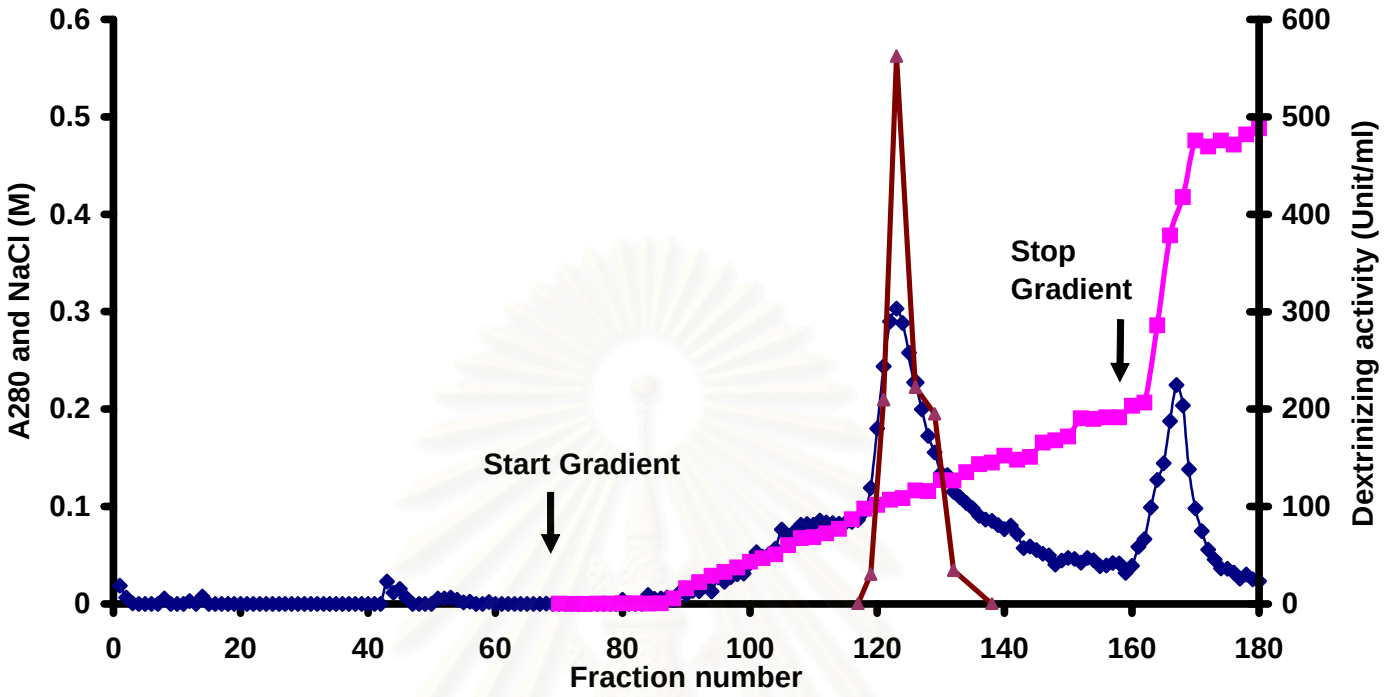


Figure 15: DEAE-cellulose column profile of CGTase from *Paenibacillus sp.* BT01 separation at pH 8.0. Column, elution was by 0 - 0.2 M NaCl in TB2 buffer. Fractions of 4 ml were collected.

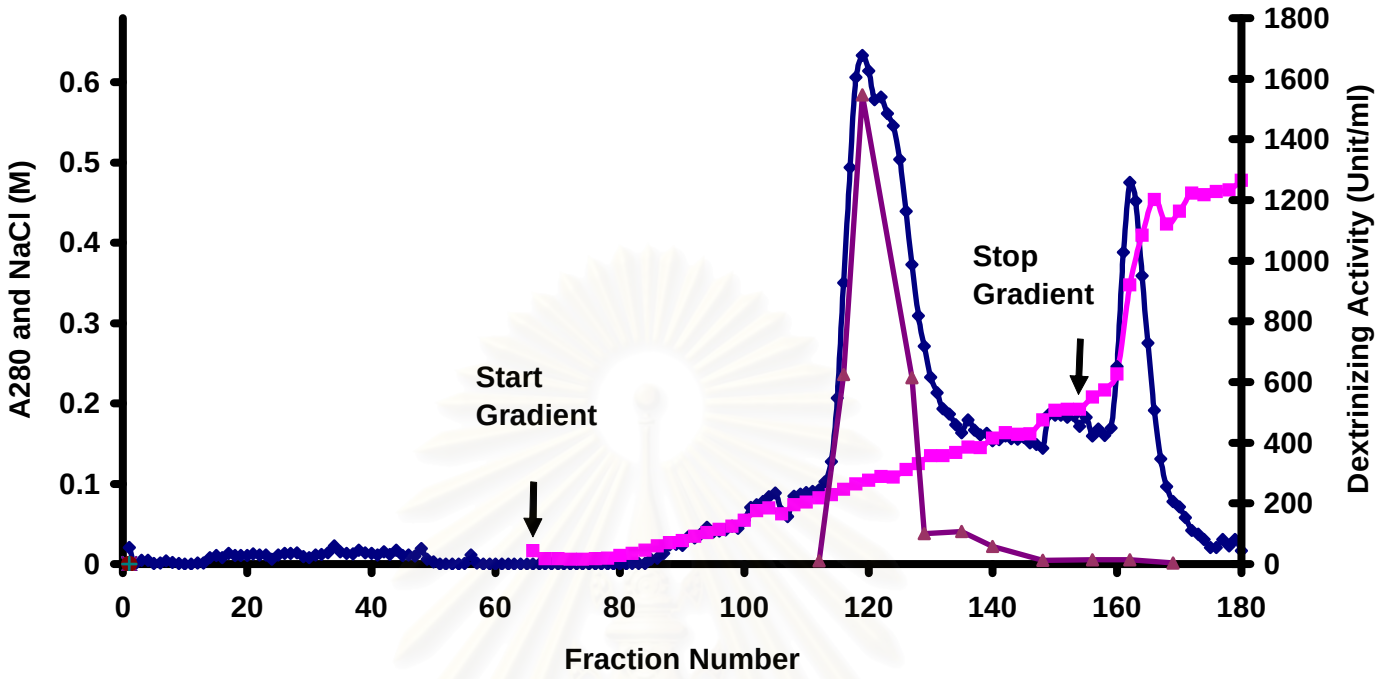


Figure 16: DEAE-cellulose column profile of CGTase from recombinant pBT separation at pH 8.0. Column, elution was by 0 - 0.2 M NaCl in TB2 buffer. Fractions of 4 ml were collected.

A_{280} , Dextrinizing activity , Conductivity

3.5.2 DEAE-cellulose column chromatography.

Each enzyme from starch adsorption was dialyzed to get rid of maltose and loaded to DEAE-cellulose column (section 2.10.2). The chromatographic profiles were shown in Figures 15 and 16. Unbound proteins were eluted from the column by the elution buffer. The bound proteins were eluted by linear salt gradient of 0 to 0.2 M sodium chloride in the same buffer. Both enzymes were eluted at approximately 0.1 M. The fractions with dextrinizing activity were pooled, dialyzed against distilled water and lyophilized. This step yielded 1.98 mg protein with and $6.00x10^3$ dextrinizing activity units for wild type enzyme, 9.53 mg protein and $6.35x10^4$ dextrinizing activity units for recombinant enzyme. The specific activity and purification fold of the enzyme from this step was $3.02x10^3$ units/mg protein and 24.4 folds for BT01, $6.66x10^3$ units/mg protein and 8.2 folds for recombinant pBT. The enzyme from this step was kept at 4ºC for future experiments. Table 9 summarized the results of overall purification process.

Table 8: Localization of CGTase activity in pBT

Total dextrinizing activity (Unit)		Specific activity (Unit/mg protein)		% Total Activity	
Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular
105.32	6.26×10^4	3.30	2.06×10^3	0.17	99.83

Table 9: Purification of CGTase from *Paenibacillus sp.* BT01 and transformant pBT

Step	Total Activity (Units)		Total Protein (mg)		Specific activity (Unit/mg)		Purification Fold		Yield (%)	
	BT01	pBT	BT01	pBT	BT01	pBT	BT01	pBT	BT01	pBT
Crude	4.66×10^4	8.94×10^4	375.20	109.52	1.24×10^2	8.16×10^2	1.00	1.00	100	100
Starch adsorption	2.55×10^4	7.96×10^4	9.54	13.87	2.67×10^3	5.74×10^3	21	7	54.7	89.0
DEAE-cellulose	6.00×10^3	6.35×10^4	1.98	9.53	3.02×10^3	6.66×10^3	24	8	12.9	71.0

3.6 Electrophoretic pattern of the CGTase

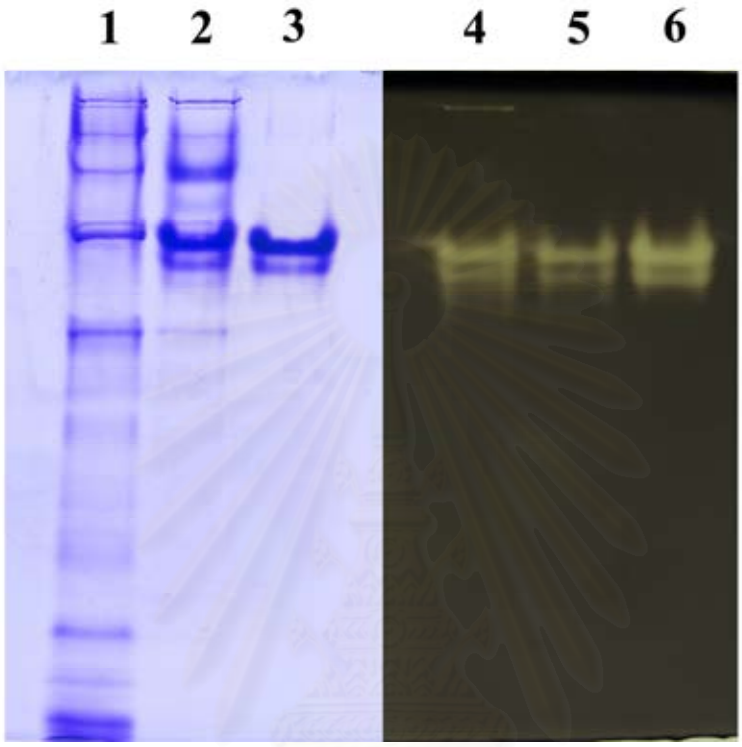
The CGTases from each step of purification were monitored by two types of electrophoresis.

3.6.1 Non denaturing polyacrylamide gel electrophoresis

Purified CGTases from BT01 and pBT were electrophoresed on 7.5% non denature-PAGE and stained for both protein and dextrinizing activity as described in section 2.12.1. Lanes 1-3 in Figures 17 and 18 showed protein staining of sample from each step of purification of BT and pBT, respectively. DEAE-cellulose column chromatography yielded one major band and 2 faint bands in both BT01 and pBT. The bands corresponded to those appeared in dextrinizing activity stain in lanes 4-6 of Figure 17 and 18, which were isoform of CGTase. Therefore, DEAE-cellulose column yielded pure CGTases from BT01 and pBT.

3.6.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples from each step of purification of CGTases from BT01 and pBT were treated and electrophoresed on 10% SDS-PAGE with standard molecular weight proteins as described in section 2.12.2. From Figure 19, CGTase from DEAE-cellulose column showed only single band in both BT01 and pBT and their molecular weight were calculated from the standard curve in Figure 20 to be 71 kDa.



Coomassie blue staining **Dextrinizing activity staining**

Figure 17: Non-denaturing PAGE (7.5% gel) of BT01-CGTase from different step of purification.

Coomassie blue staining

Lane 1: Crude enzyme (30 μg protein)

Lane 2: Concentrated Starch adsorbed enzyme (20 μg protein)

Lane 3: Concentrated DEAE cellulose column (10 μg Protein)

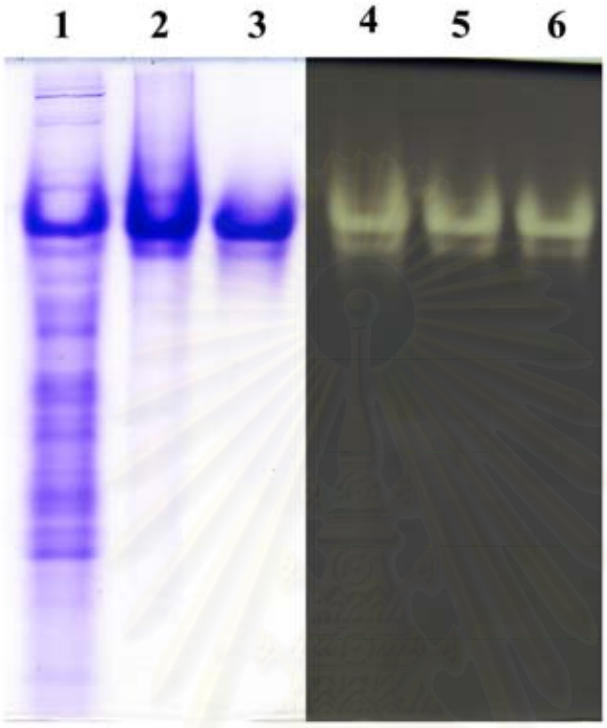
Dextrinizing activity staining

Lane 4: Crude enzyme

Lane 5: Starch adsorbed enzyme

Lane 6: DEAE cellulose column

0.3 Units of dextrinizing activity was loaded to each well



Coomassie blue staining **Dextrinizing activity staining**

Figure 18: Non-denaturing PAGE (7.5% gel) of recombinant pBT from different step of purification

Coomassie blue staining

Lane 1: Crude enzyme (30 μg protein)

Lane 2: Concentrated Starch adsorbed enzyme (20 μg protein)

Lane 3: Concentrated DEAE cellulose column (10 μg Protein)

Dextrinizing activity staining

Lane 4: Crude enzyme

Lane 5: Starch adsorbed enzyme

Lane 6: DEAE cellulose column

0.3 Units of dextrinizing activity was loaded to each well

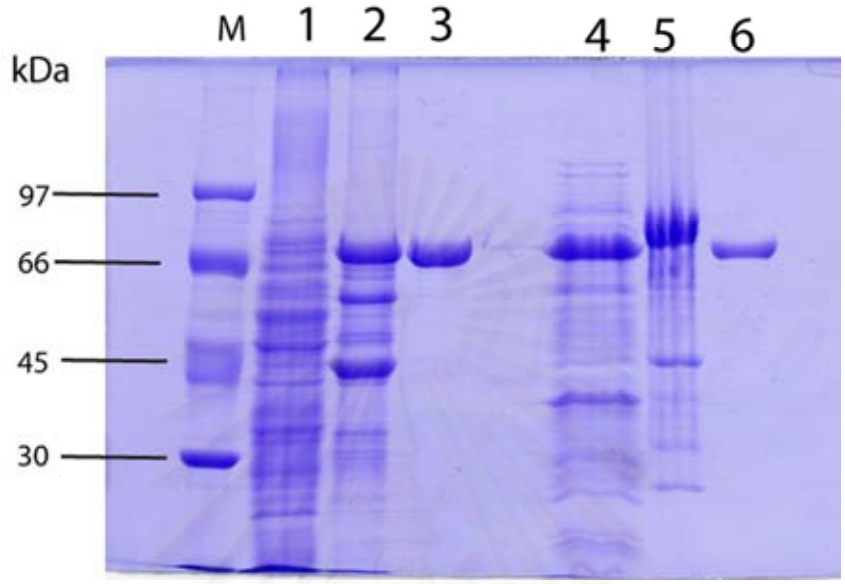


Figure 19: SDS-PAGE (10 % gel) of CGTase from each purification steps

Coomassie blue staining

Lane M: Protein molecular weight Markers

Phosphorylase b	97	kDa
Albumin	66	kDa
Ovalbumin	45	kDa
Carbonic anhydrase	30	kDa

Lane 1-3 Purification of CGTase from wild type

Lane 1: Crude enzyme (20 μg)

Lane 2: Concentrated starch adsorbed enzyme (10 μg)

Lane 3: Concentrated DEAE cellulose column (3 μg)

Lane 4-6 Purification of CGTase from recombinant pBT

Lane 4: Crude enzyme (20 μg)

Lane 5: Concentrated starch adsorbed enzyme (10 μg)

Lane 6: Concentrated DEAE cellulose column (3 μg)

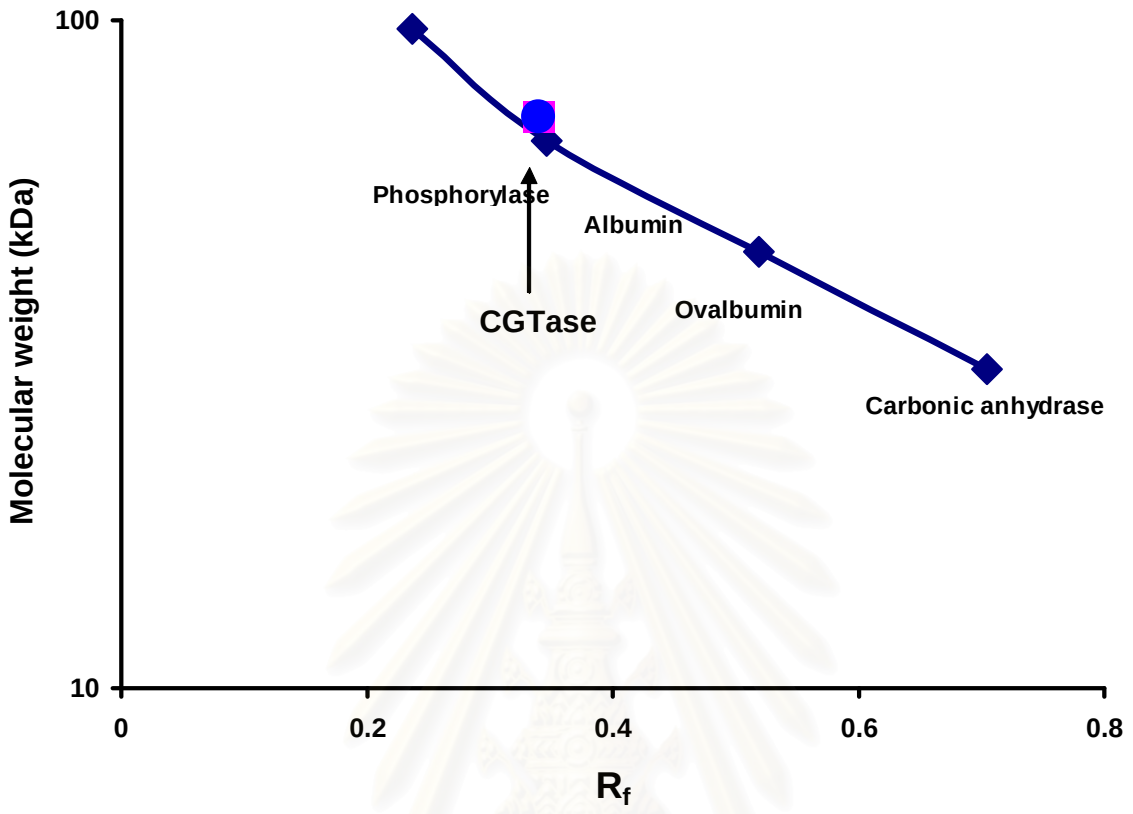


Figure 20: Molecular weight calibration curve of standard protein by SDS-PAGE

Phosphorylase b	97	kDa
Albumin	66	kDa
Ovalbumin	45	kDa
Carbonic anhydrase	30	kDa

● = BT

■ = pBT

3.6.3 Determination of pI

Purified CGTases were analyzed for their isoelectric points by separation on IEF gel electrophoresis (section 2.13), comparing to standard pI markers. Ampholine pH range 3-10 was used and relative mobility against pI was plotted (Figures 21, 22). Samples from both *Paenibacillus* sp. BT01 and pBT showed one major band at pI 4.74 and two faint bands at pI 4.86 and 4.62.

3.7 Optimum conditions for CGTase activity

Experiments were carried out to determine optimum pH and temperature for dextrinizing activity and cyclization activity of CGTase.

3.7.1 Optimum pH

Dextrinizing and cyclization activities of CGTase from BT01 and pBT were assayed in reaction mixtures at different pH's as described in section 2.14.1. The results were calculated as relatively enzyme activity, taking the pH with highest activity as 100%. Figure 23 showed the pH-activity profiles of dextrinizing activity and cyclization activity from *Paenibacillus* sp. BT01 and pBT which were similar. The optimum pH's for dextrinizing activity and cyclization activity were at acetate buffer pH 5.0 and acetate buffer pH 6.0, respectively.

3.7.2 Optimum temperature

The effect of temperature on the dextrinizing and cyclization activities were investigated by incubating the reaction mixtures at various temperatures as described in section 2.14.2. The highest enzyme activity at any temperature was taken as 100% and enzyme activity at other temperatures were calculated as relatively enzyme activity. The optimum temperature for dextrinizing activity of CGTase from BT01 and pBT were 50°C. The optimum temperatures for cyclization activity of CGTase from BT01 and pBT were similar 50°C. (Figure 24).

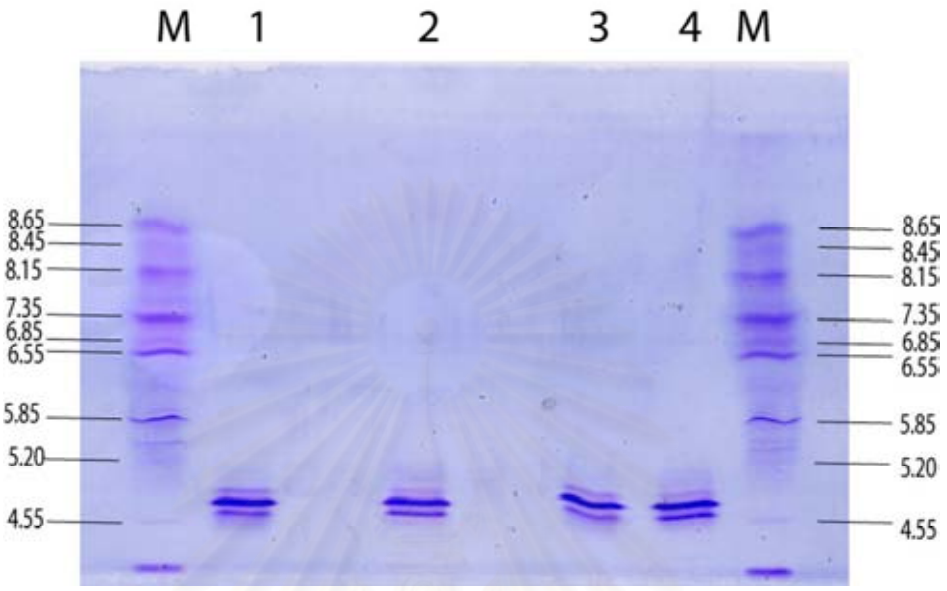


Figure 21: Isoelectric focusing gel with ampholyte solution (pH 3.0-10.0) of purified CGTase

Lane M:	Standard pI markers	pI value
	Soybean trypsin inhibitor	4.55
	B-lactoglobulin	5.20
	Bovine carbonic anhydrase B	5.85
	Human carbonic anhydrase B	6.55
	Myogloblin-acidic band	6.85
	Myoglobin-basic band	7.35
	Lentil lectin-acid band	8.15
	Lentil lectin-middle band	8.45
	Lentil lectin-basic band	8.65
Lane 2, 4: Purified CGTase from BT01 (4 μg, 3 μg protein)		4.86, 4.74, 4.62
Lane 1, 3: Purified CGTase from pBT (4 μg, 3 μg protein)		4.86, 4.74, 4.62

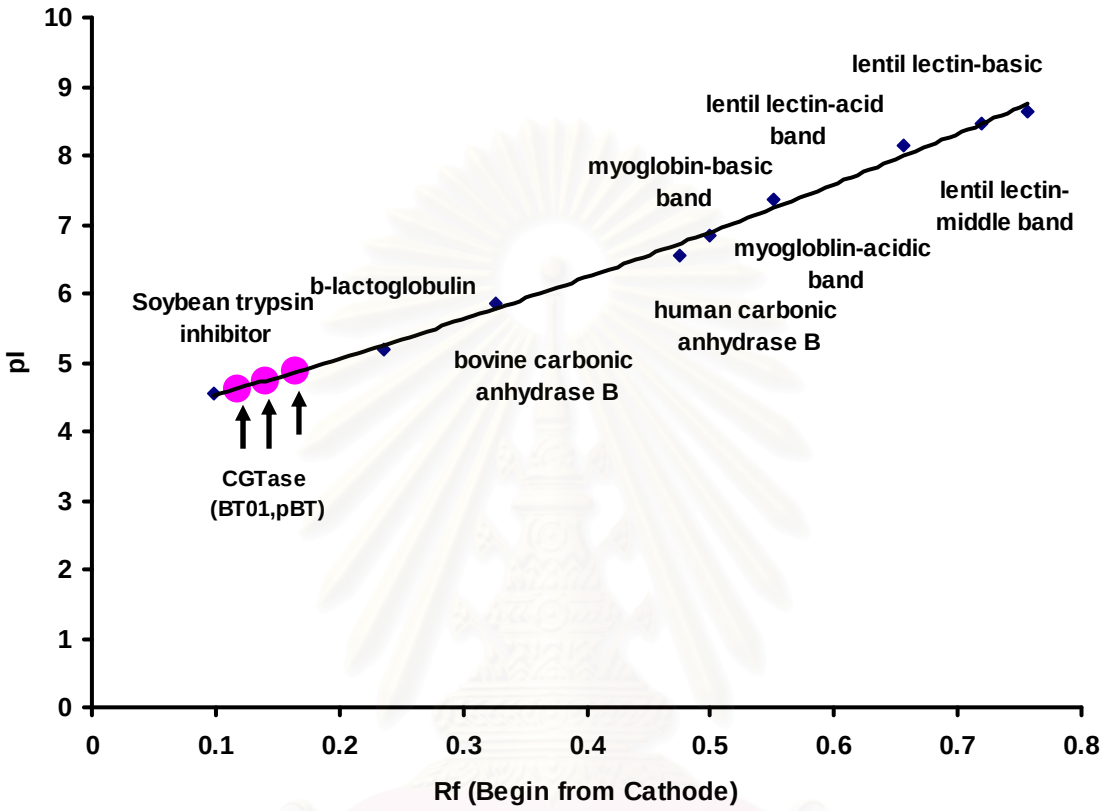


Figure 22: Standard curve of pI determination

◆ - **Standard Proteins** ● - **CGTase (BT01, pBT)**

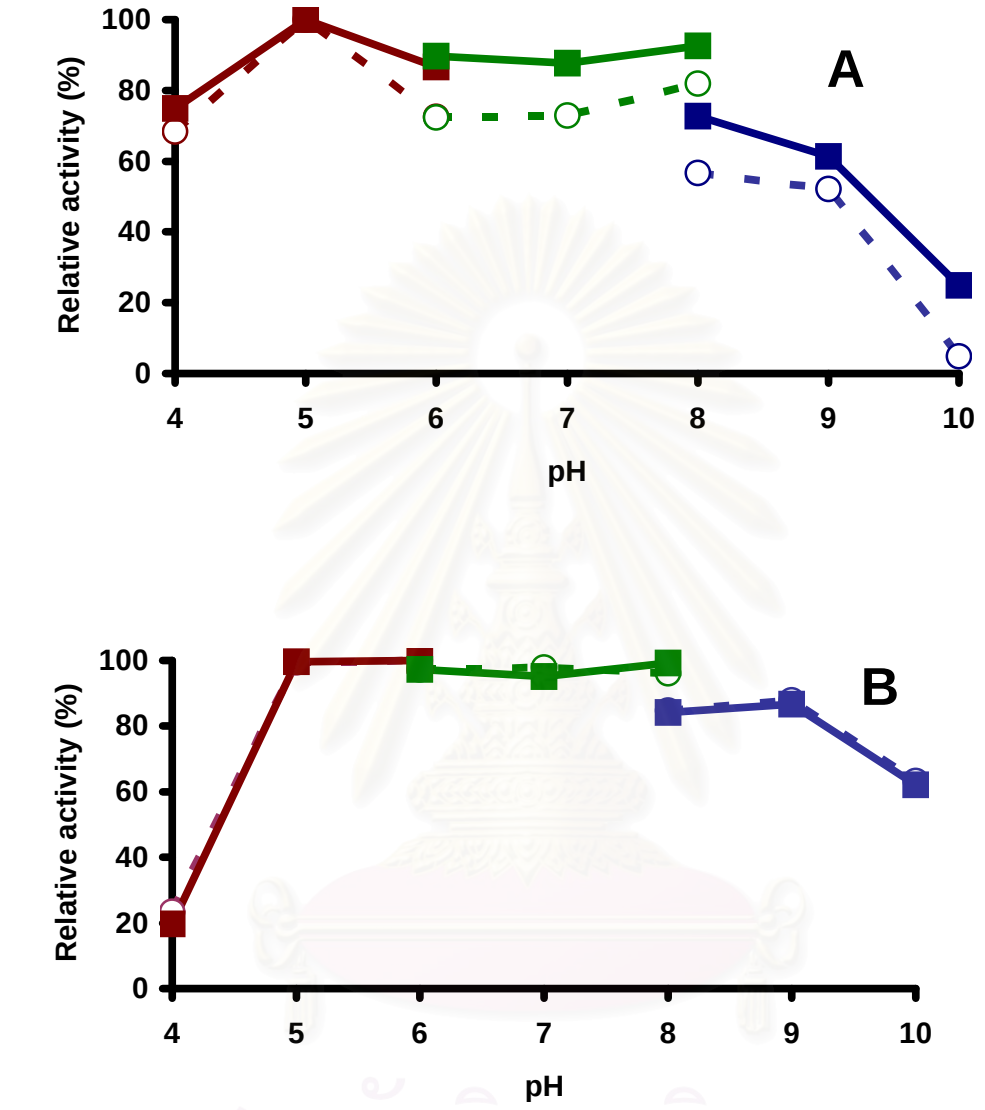


Figure 23: Optimum pH on CGTase Activity from BT01 and pBT

Dextrinizing Activity performed at 40°C (A)

Cyclization Activity performed at 60°C (B)

BT01 pBT

Acetate buffer Phosphate buffer Glycine buffer

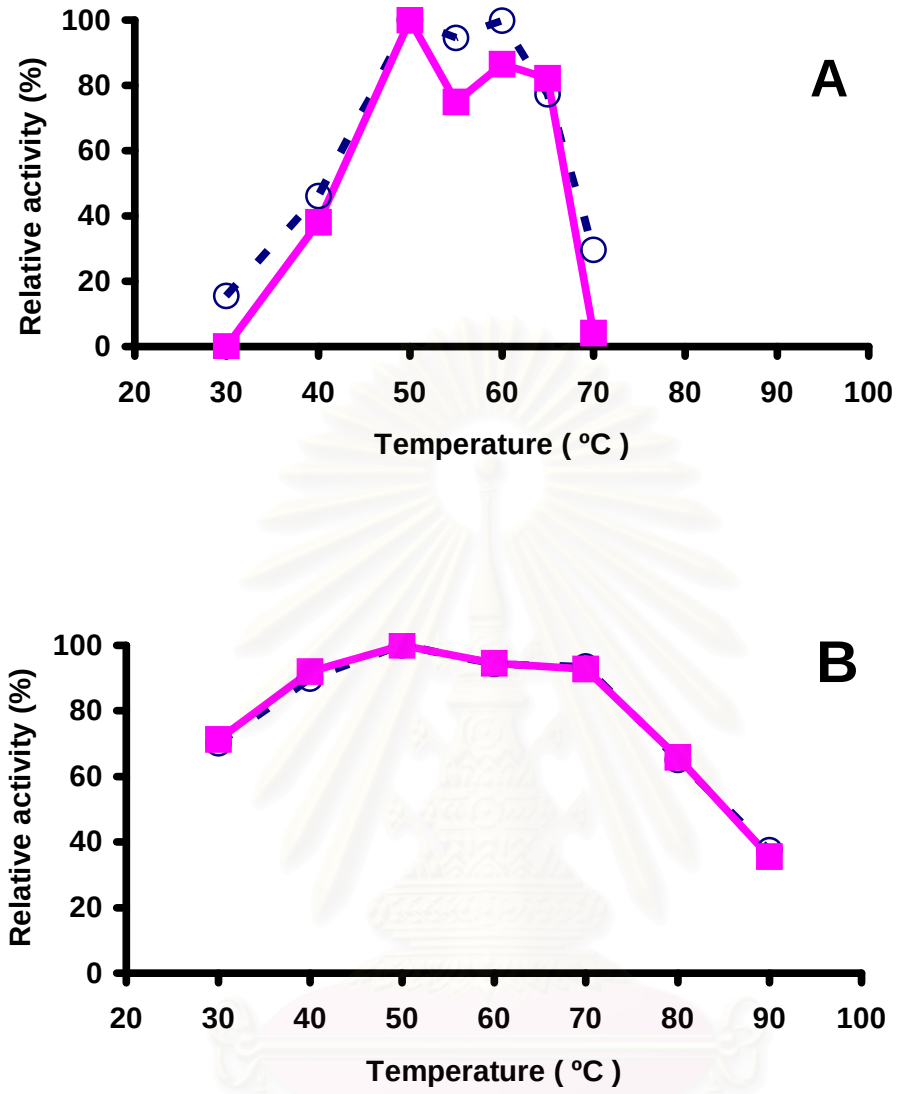


Figure 24: Optimum Temperature on CGTase activity from BT01 and pBT
Dextrinizing Activity performed in acetate buffer pH 5.0 (A)
Cyclization Activity performed in acetate buffer pH 6.0 (B)

= Wild Type BT01 = recombinant pBT

3.8 Stability of CGTase activity

The purified CGTases from BT01 and pBT were studied for their stability at various pH's and temperatures.

3.8.1 pH stability

The cyclization activity of CGTases from BT01 and pBT were studied by incubated for one hour at 50°C in buffer of varying pH's prior to determination of cyclization activity as described in section 2.15.1. The highest activity was defined as to 100% activity. Activity at other pH's were expressed as relative activity to the highest activity. The cyclization activity remained stable between pH 6.0 to 10.0 at 50°C (Figure 25). At pH 5.0 the cyclization activity of pBT was at 60% more stable than BT01 which dropped to 10% activity.

3.8.2 Temperature stability

The purified CGTases were preincubated at 40°C, 50°C, 60°C, 65°C and 70°C in acetate buffer pH 6.0 up to two hours. Samples were taken at regular intervals and assayed for cyclization activity as described in section 2.15.2. The CGTase activity at zero time was taken as 100% relative activity and the activity at other time point were expressed as relative activity CGTases from BT01 and pBT retained their cyclization activities up to 90% or more at 40–50°C up to two hours (Figure 26). At 60°C, CGTases from BT01 and pBT retained 58% and 45%, respectively. At 65°C, cyclization activity was retaining to 50% relative activity around 20 minutes. At 70°C, cyclization activity completely lost after 10 minutes. When 2% and 20% soluble starch was added, enzymes stabilized at 60°C, 65°C and 70°C. At 80°C, cyclization activity in both enzymes were stabilized around 50-100% at 20 minutes but declined to lower than 50% at 30 minutes and completely lost activity at 120 minutes. At 90°C, both enzymes completely lost activity (Figure 27). The stabilization effect of 2% and 20% were not different.

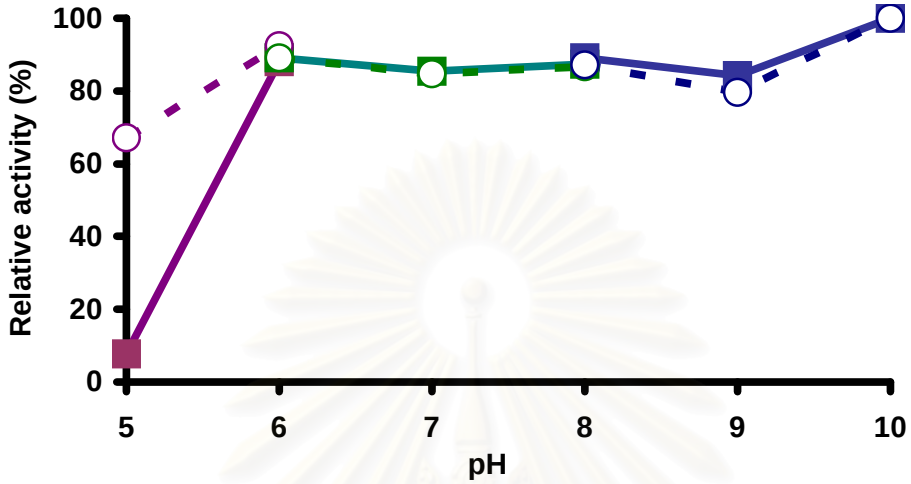


Figure 25: pH stability of CGTase on cyclization activity from BT01 and pBT

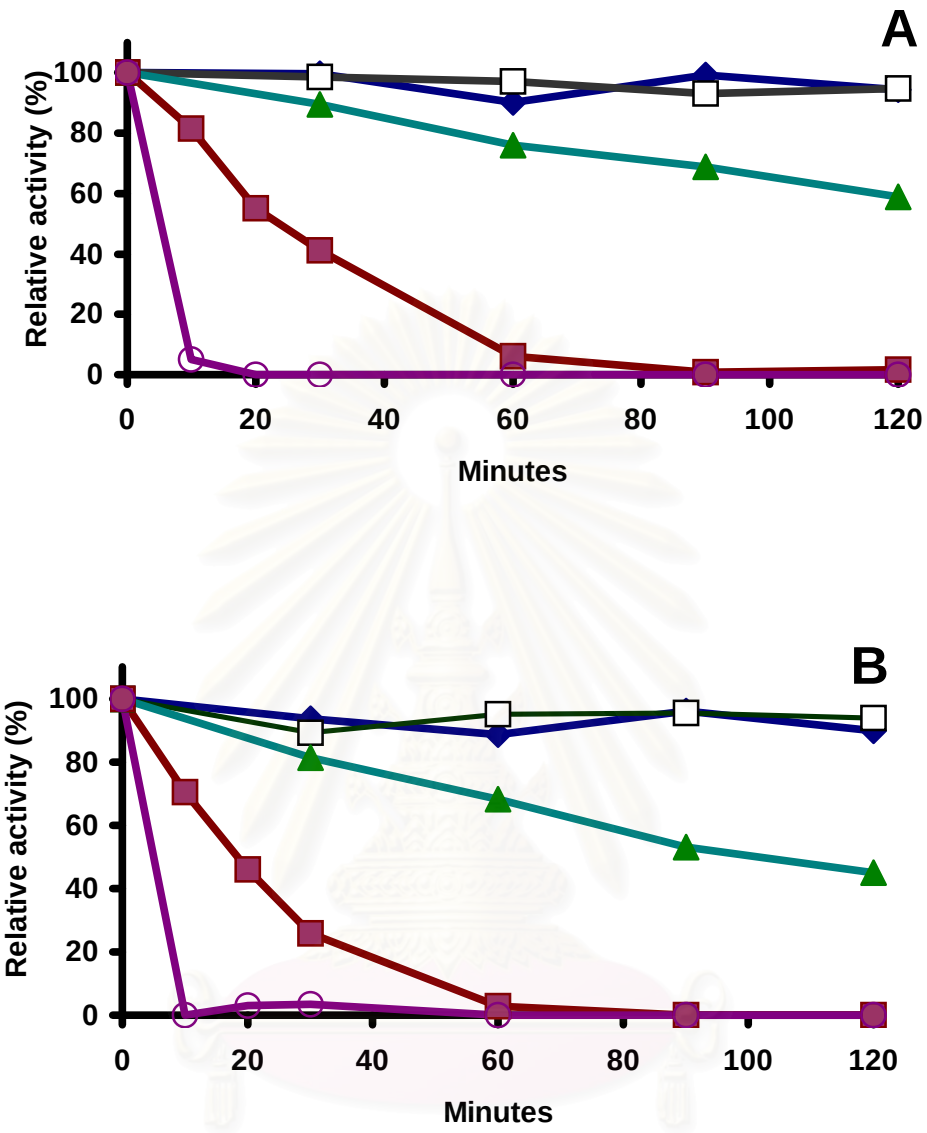


Figure 26: Temperature stability of CGTase on cyclization activity from BT01 and pBT

Paenibacillus sp. BT01 (A) Recombinant pBT (B)

= 40 °C

= 50 °C

= 60 °C

= 65 °C

= 70 °C

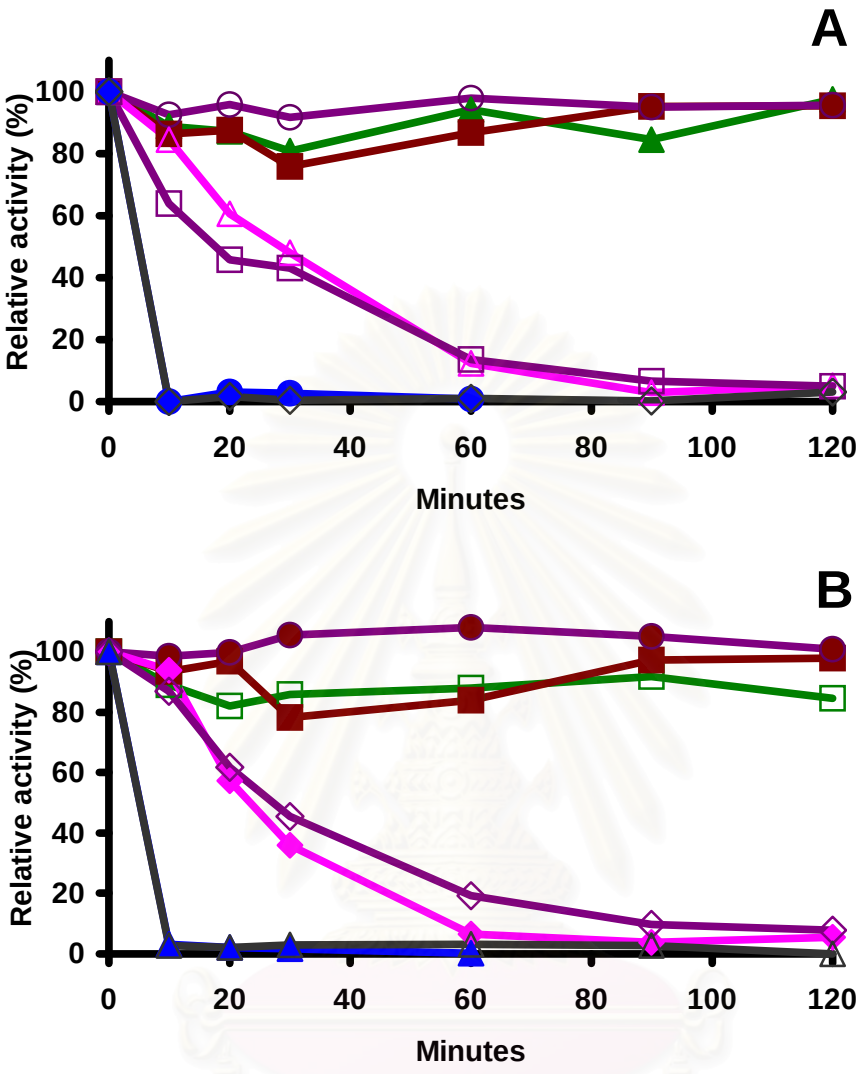


Figure 27: Temperature stability of CGTase on cyclization activity from BT01 (A) and pBT (B) in the presence of 2% and 20% soluble starch

= 60 °C+ 2 % soluble starch

= 65 °C+ 2 % soluble starch

= 70 °C+ 2 % soluble starch

= 80 °C+ 2 % soluble starch

= 90 °C+ 2 % soluble starch

= 80 °C+ 20% soluble starch

= 90 °C+ 20 % soluble starch

3.9 Kinetic study of purified CGTases.

CGTase Purified from DEAE-cellulose were studied for kinetic parameters of cyclization activity. The experiments were performed with 2-50 mg/ml soluble starch as substrate in 0.2M acetate buffer pH 6.0 for 15 minutes at 50°C. The Lineweaver-Burk plot was performed (Figure 28) and kinetic parameters of cyclization reaction of CGTases from BT01 and pBT were shown in Table 10. The *kcat* and *kcat/Km* were significantly higher than BT01. *Vmax* values were not different for both enzymes.

3.10 Product analysis by High Performance Liquid Chromatography

Purified CGTases were incubated with soluble potato starch, topioca starch, Flo-max 8 and starch extract from cassava KU50 tubers as described in section 2.17. The reaction mixtures filtered through 0.45 μm membrane were injected to HPLC column and eluted with acetronitrile-water (70:30, v/v) using a flow rate of 1.0 ml/min. The CD peaks were identified by comparing the retention time with that of standard α-, β- or γ-CDs (10 mg/ml). After conversion of the peak area to CD concentration, it was found that CGTase from wild type and recombinant produced mainly β-CD from all substrates. The ratio of α-: β-: γ-CDs was shown in figures 29, 30, 31 and Table 11. The β-CD yield obtained from various starch substrates was determined by phenolphthalein method, the result was not significantly different as shown in Table 12. Percent conversion of starch substrate to β-CD was around 18-22%.

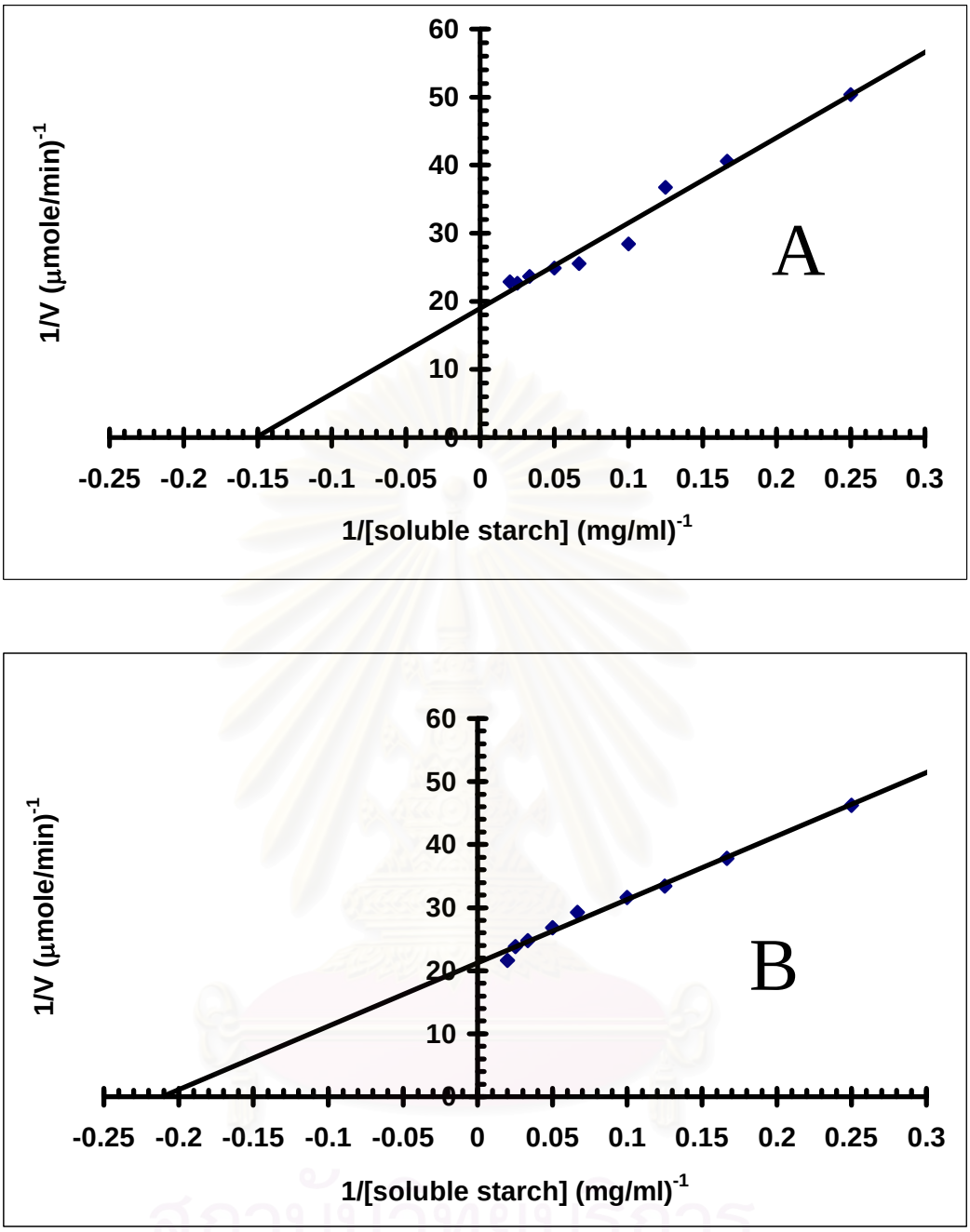


Figure 28: Lineweaver-Burk plot of CGTase with soluble starch as substrate *Paenibacillus* sp. BT01 (A) Recombinant pBT (B)

Table 10: Kinetic parameters of cyclization activity of CGTases from *Paenibacillus* sp. BT01 and pBT.

Cyclization Activity	BT01	pBT
Km (mg/ml)	5.36 ± 0.44	4.90 ± 0.18
Vmax (nmole/min)	50.80 ± 0.36	47.28 ± 0.23
kcat $(min)^{-1}$	218.09 ± 1.54	447.24 ± 2.13
kcat/Km $(min)^{-1}(mg/ml)^{-1}$	40.82 ± 3.06	91.35 ± 3.22

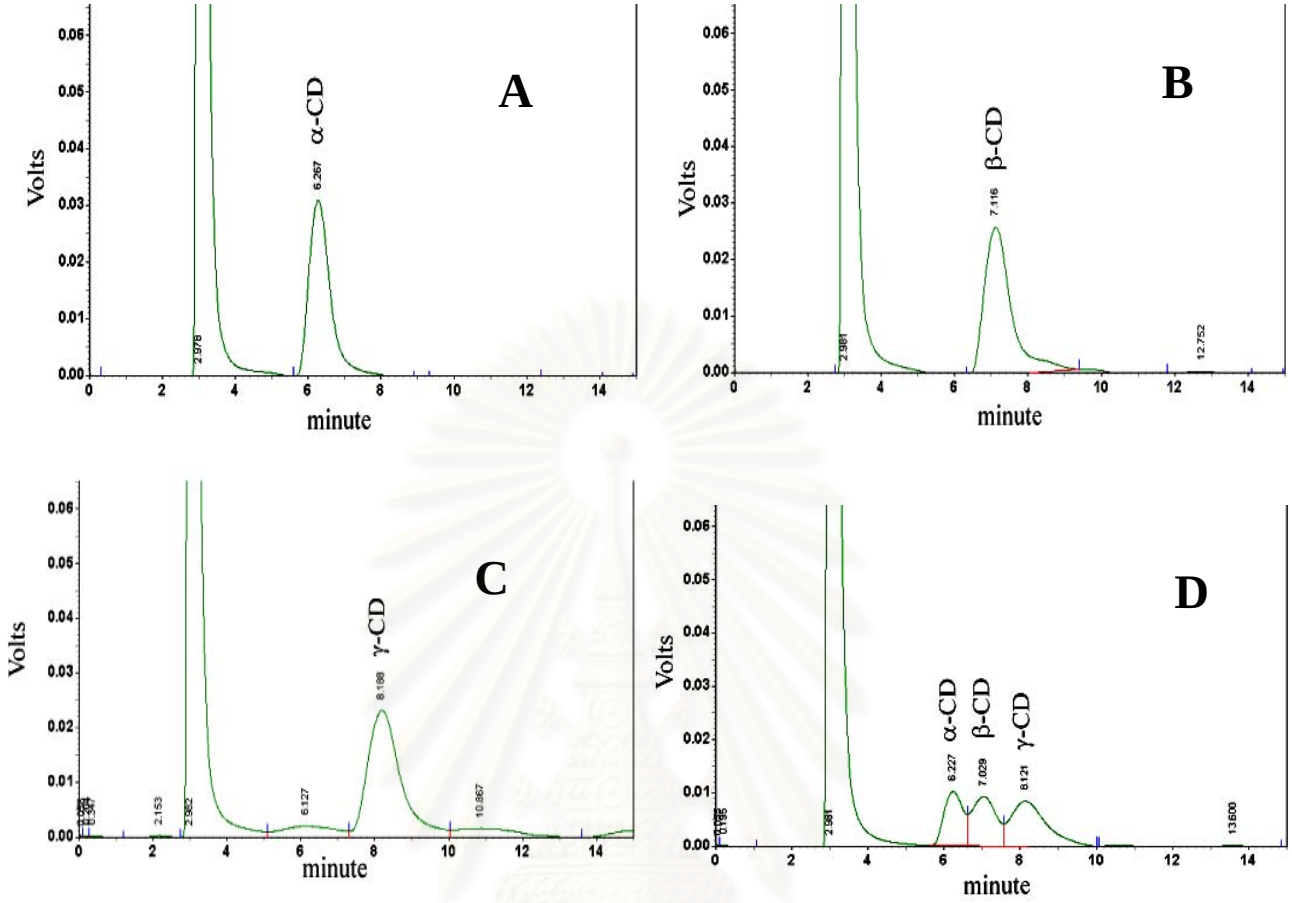


Figure 29: HPLC chromatogram of standard CDs (10 mg/ml)

α-CD (A) β-CD (B) γ-CD(C)

Mixture of α-CD, β-CD, γ-CD (D)

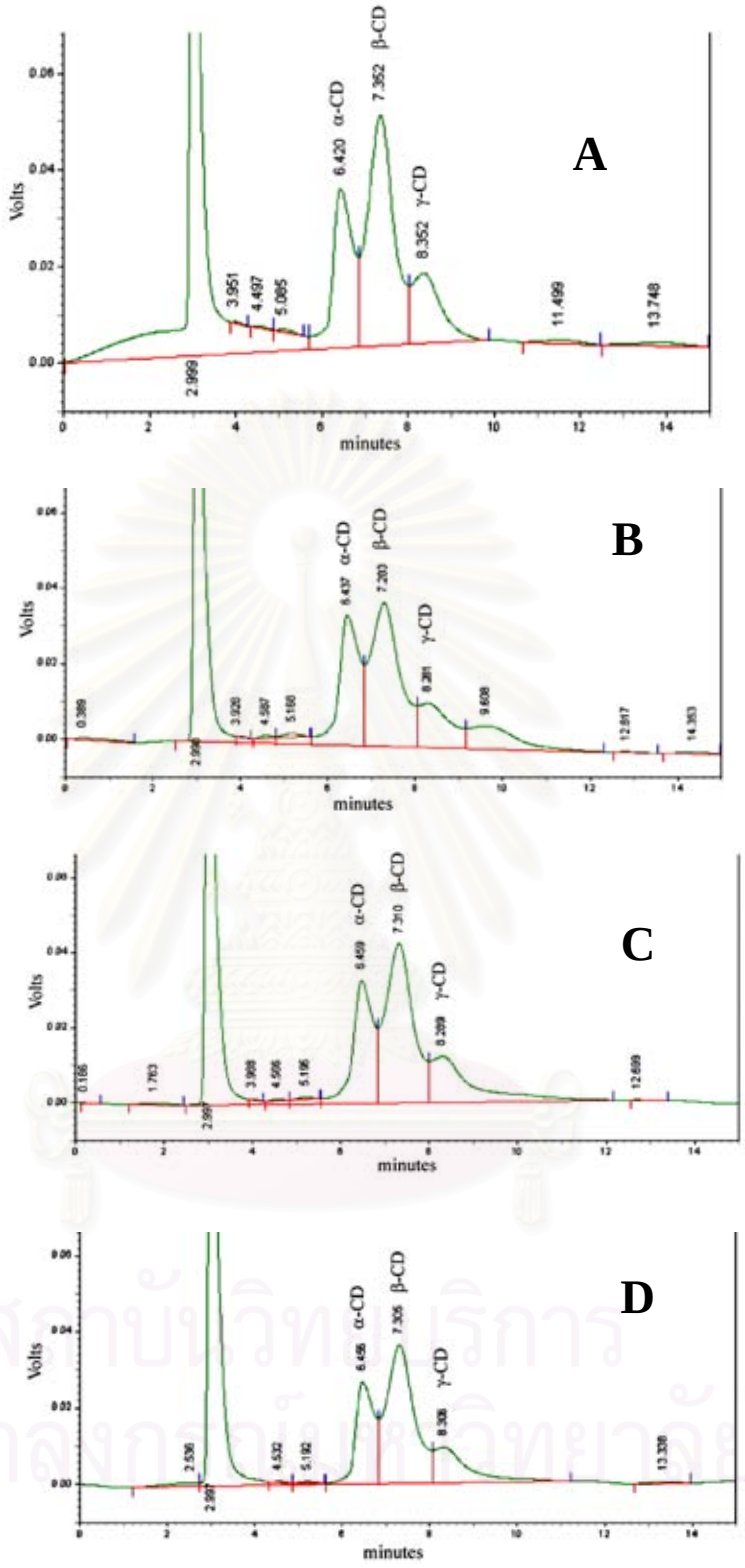


Figure 30: HPLC chromatogram of CDs produced from various starch substrates by purified CGTase from BT01. Starch from tubers of cassava (A), soluble starch (B), tapioca starch (C) and Flo-max 8 (D)

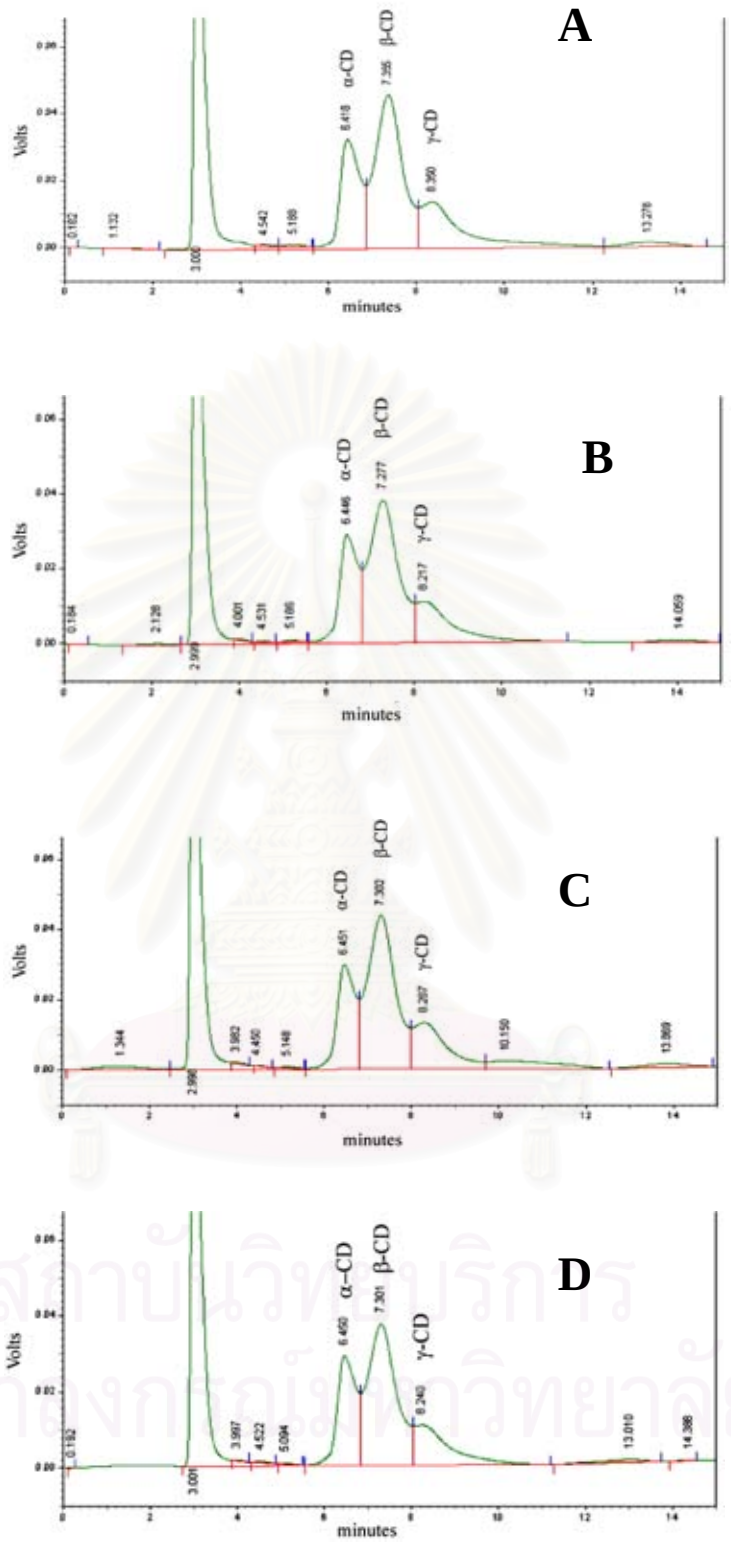


Figure 31: HPLC chromatogram of CDs produced from various starch substrates by purified CGTase from pBT. Starch from tubers of cassava (A), soluble starch (B), tapioca starch (C) and Flo-max 8 (D)

Table 11: The Ratio of CD production from purified CGTase on the various starch substrates determined by HPLC

Substrate	Wild Type			Clone		
	α-CD	β-CD	γ-CD	α-CD	β-CD	γ-CD
Starch from tubers of cassava	1.00	1.88	0.65	1.00	1.96	0.92
Flo-MAX 8	1.00	2.02	0.65	1.00	1.94	0.69
Tapioca starch	1.00	1.75	0.75	1.00	2.22	0.82
Soluble starch	1.00	1.52	0.51	1.00	2.00	0.72

Table 12: β-CD production on various starch substrates.

Starch substrate 100 g/l	β-CD (g/l)	
	Wild Type	Clone
Starch from tubers of cassava	22.04	16.37
Flo-max 8	20.18	18.45
Tapioca starch	24.40	18.73
Soluble starch	22.52	19.33

CHAPTER IV
DISCUSSION

Since cyclodextrins still show great potential for industrial usage, improved CD production process including new discovery of CD-producing bacteria is still needed. Bacteria producing large amount of CD-producing enzyme (CGTase) which can tolerate extreme conditions such as acid or basic pH's, high temperature etc. are still being screened. Molecular cloning has also been used to produce new clones of bacteria with the desired CD-producing capacity. Our research group had successfully isolated a few strains of thermotolerant bacteria and performed cloning of their CGTase genes into *E. coli.* This work aims at cloning of CGTase gene from thermotolerant bacteria isolated from soil contaminated with waste of starch factory using a vector for overexpression of the gene.

4.1 Identifcation of CGTase gene from *Paenibacillus* sp. BT01

Several techniques have been tried for identification of CGTase gene from *Paenibacillus* sp. BT01. In Southern blot analysis, (Sambrook *et al.*, 1989) genomic DNA was digested with one or more restriction enzymes and the resulting fragments were separated according to size by electrophoresis on agarose gel. The DNA on agarose gel was denatured in situ and transferred from the gel to solid support (Nylon membrane). The DNA attached to the membrane was hybridized to a labeled probe (designed from conserved sequence gene), and DNA band complementary to the probe were located by fluorescence. The size and number of bands of the genomic DNA digested with different restriction enzymes was determined and the bands of digested genomic DNA with the estimated size were ligated to the plasmid and transformed into competent cell. The gene in recombinant cell was located by colony hybridization. The disadvantage of this technique was that the interested gene may not be ligated into plasmid. In addition, high amount of recombinant colonies obtained made it very hard to identify the interested gene. Finally, the cost of reagents used in southern blot analysis was high. Another technique used to identify gene was inverse PCR. In this method, genomic DNA was digested by restriction enzymes to prepare DNA containing the known sequence and its flanking region. The restriction

fragments were converted into circles by ligation to circularized DNA and used as the template in PCR. The unknown sequence was amplified by two primers (designed to extend outward from the known sequence). However, inverse PCR remains one of the more problematic techniques in molecular cloning and success is not guaranteed. The restriction enzyme may cut at unknown sequence or yielded long flanking DNA which cannot be amplified with PCR reaction. The technique employed to identify CGTase gene from *Paenibacillus* sp. BT01 was PCR technique using oligonucleotides (primers) from known sequence of other CGTase genes. Primers from *Paenibacillus* sp. A11 (PrimerA (5′– GGCTATGCTTTCCTTACCTTACCC–3′) and (Primer B (5′–ATAGCACCTTTCCCCCACATAACG–3′)) were selected to be used to amplify CGTase gene from *Paenibacillus* sp.BT01. The result (Figure 5) showed that PCR products at annealing temperature 45°C produced higher amount of gene fragment at 2.4kb and 1.4kb with some smear band, suggesting that PrimerA and PrimerB were not very specific to CGTase gene from *Paenibacillus* sp.BT01. At annealing temperatures of 50°C, 55°C, the band PCR products at annealing temperature 45°C were higher than at 50, 55°C and were used for insertion into pGEM-T easy vector and transformed into *E. coli* DH5α. PCR product were not purified to one band at 2.4 Kb because of CGTase gene was easy to identified by detected activity on starch plate. Only a single colony produced starch hydrolysis activity on LB-agar plate and was used for DNA purification and sequence determination. The sequence from inserted pGEM-T easy vector gave high homology to CGTase gene from *Paenibacillus* sp. A11.

4.2 Cloning of cyclodextrin glycosyltransferase gene from *Paenibacillus* sp.BT01

Expression system using the bacteriophage T7 promoter was first developed by Tabor and Richardson (1985) and Studier and Moffat (1986), employing transcription signals derived from the bacteriophage T7 genome. The system had the following advantages (Sambrook *et al.*, 1989). First, Bacteriphage T7 RNA polymerase, unlike *E. coli* RNA polymerase, is not inhibited by rifampicin. The antibiotic can therefore be used to extinguish transcripts of host cell gene. Second, the bacteriophage-encoded enzyme recognizes only bacteriophage T7 promoters which

are not present in the *E. coli* chromosomal DNA. Finally, bacteriophage T7 RNA polymerase a processive enzyme, will transcribe around a circular plasmid several times and may therefore produce genes that are not efficiently transcribed by *E. coli* RNA polymerase. Therefore, T7 promoter was suitable for expression of CGTase gene and pET19b vector was selected. *E. coli* strain BL21(DE3) was used for host expression because this strain had gene encoding bacteriophage T7 RNA polymerase, which is integrated into the chromosome of BL21. To insert the CGTase gene fragment into pET19b at the right position, both the gene fragment and pET 19b must have similar restriction sites. In this experiment *Nco*I and *Xho*I restriction sites were selected. The gene fragment was amplified with the 5' end primer containing *Nco*I restriction site and 3'end primer that containing *Xho*I restriction site. The whole gene fragment amplified was digested with *Nco*I and *Xho*I, then ligated to *Nco*I-*Xho*I sites of pET19b and transformed into *E. coli* BL21(DE3) host cells. Stain hydrolytic activity on LB-Ampicillin plate containing starch, indicating the presence of cyclodextrin glycosyltransferase allowed selection of CGTase producing transformants.

4.3 Nucleotide sequence of CGTase gene and its deduced amino acid sequence.

Nucleotide sequence of PCR product from primers pRBF1 and pRBR1 was compared with inserted fragment in pET19b to check error of which may occurred from DNA polymerase. In cloning of DNA, DNA polymerase with 3'-5' exonuclease proof reading was used to reduce the error in PCR product. In our experiment, Phusion^{TM} DNA polymerase with the error rate of 4.4×10^{-7} errors per base pair, lower than that of Taq DNA polymerase (1×10^{-4} to 2×10^{-5} errors per base pair), was employed. The signal peptide and mature enzyme were identified by using sequence alignment analysis with reported gene structure such as *Bacillus circulans* A11 (Rimphanitchayakit, V. *,et.al.* 2005). DNA sequence from pBT contained signal peptide of 81 base pairs and CGTase gene encoded mature enzyme 2058 base pairs. Amino acid and nucleotide sequence of cyclodextrin glycosyltransferase were BLAST in the EMBL-GeneBank-DDBL database. The nucleotide sequence showed highest homology 99% to *Paenibacillus* sp. A11 (Table 6). The highest homology in

amino acid sequence was to *Paenibacillus* sp. A11 with 97% homology (Table 7). Phylogenic tree from sequence alignment with various published CGTases were shown in Figure 12. The phylogenic tree can be separated into four major groups. *Paenibacillus* sp.BT01 was in the group of alkalophilic CGTase and major produce was β-cyclodextrin. Nucleotide and deduce amino acid sequence of BT01 was compare with A11 (Figure 8) which showed difference in 17 nucleotides and one amino acid in signal peptide and 3 amino acid in mature enzyme.

4.4 Expression of CGTase in recombinant pBT

Recombinant *E. coli* was grown in the medium containing ampicillin and determined for CGTase activity. Since the gene fragments did not have their own promoter, they were expressed under T7 promoter on the plasmid pET19b. In the pET system, T7 RNA polymerase gene was under the control of the lacUV5 promoter, and the plasmid vector equipped with a bacteriophage T7 promoter upstream of the gene. Both promoters contain the *lac* operator (*lacO*) in such position that binding of a lac repressor to the operator site blocks transcription. IPTG can bind to the repressor which resulted in the loss of its affinity for the lac operator. Therefore, adding IPTG should allow transcription of cyclodextrin glycosyltransferase gene in pET system vector. Without induction by IPTG, the expression of CGTase gene also occurred because there was some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 lysogen from E. coli genome. Transformants, which showed the highest cyclodextrin glycosyltranferase gene expression on LB-ampicillin starch plate, were grown in LB-ampicillin broth attending density of cell mass at mid-log growth phase (OD660 = 0.6-0.8) at about 3 hours, after which 0.2 mM IPTG was added to culture cells and continuously grew for 24 hours before harvested. Specific activity from pBT was 816.39 unit dextrinizing /mg protein while Ratiya *et al.,* (2007) reported pRB01, expression in pGEM-T easy vector had specific activity at 715 unit dextrinizing /mg protein. Some of CGTases expressed in *E.coli* were reported to be located in the cell (intracellular enzyme) such as the enzyme from *Bacillus* sp. KC201 (Kitamoto *et al.,* 1992), while some were located out side the cells (extracellular enzyme) such as *Brevibacillus brevis* CD162 (Kim *et al.,* 1998), *Bacillus* sp. TS1-1

(Rahman *et al.,* 2006), *Bacillus circulans* A11. Localization of CGTase from pBT showed that 99.83 % of dextrinizing activity was found extracellularly (Table 8). In recombinant cell *E. coli* of *Brevisbacillus* CD162, 74% of the total CGTase activity was found in the extracellular space (Kim *et al.,* 1998). They proposed that the recombinant enzyme can be excreted because the enzyme contained the intrinsic structural properties of an enzyme designed for excretion from the cells.

4.5 Purification of cyclodextrin glycosyltransferase

The characteristics of proteins and other biomolecules that were utilized in their various separation procedures were differences in solubility (salting in, salting out), ionic charge (ion exchange chromatography, electrophoresis, isoelectric focusing), polarity (adsorption chromatography, paper chromatography, reverse-phase chromatography, hydrophobic interaction chromatography), molecular size (dialysis, ultrafiltration, gel electrophoresis, gel filtration chromatography, ultracentrifugation) and binding specificity (affinity chromatography) (Voet *et al.*, 2004).

Many methods have been used to purify CGTase such as 70% ammonium sulfate precipitation and Phenyl separose HR 5/5 column (Kim *et al.,* 1998). Rahman *et al.*,(2006) used ammonium sulfate precipitation at 70% saturation and α-cyclodextrin-bound-epoxy-activated Sepharose 6B affinity column and Rojtinnakorn *et al,* (2001) used immunoaffinity column. Ammonium sulfate precipitation was based on the differences in solubility but starch adsorption was separated on the basis of substrate-enzyme binding specificity. To purify CGTase from BT01 and pBT, starch adsorption and DEAE-cellulose column were used. Starch adsorption was selected because it results in higher purification fold and %yield than ammonium sulfate precipitation. After this step, the enzyme was purified to homogeneity with 21 purification fold and 54 % yield in BT01, 7 purification fold and 89% yield in pBT. Yenpetch (2002) reported 28 fold purification and 65.10% yield of CGTase from *Paenibacillus* sp. RB01. Charoensakdi *et al*. (2007a ,2007b) reported 3 fold purification and 22% yield, 3 fold purification and 19% yield in recombinants from *Paenibacillus* sp. RB01 and *Paenibacillus* sp. pT, respectively. Martins and Hatti-Kaul (2002) reported 43 fold purification and 50% yield in *Bacillus agaradhaerens*

LS-3C. Purification fold of pBT enzyme was lower than BT01 at the same purification step because crude pBT contained higher amount of enzyme and less proteins (higher specific activity) than BT01. However, ND-PAGE and SDS-PAGE (Figures 17, 18, 19) still revealed many bands, so the enzymes were not purified to homogeneity yet. Second step of purification was performed on DEAE-cellulose, an anion exchange chromatography. The advantages of DEAE-cellulose column were high sample volume can be applied and lower cost than affinity and immunoaffinity columns. The chromatograms (Figures 14, 15) showed that significant amount of unwanted proteins were separated from the CGTase peak. After this step, the enzymes showed only one band on SDS-PAGE (Figure 19) and one major and two minor bands on native PAGE. Therefore, DEAE-cellulose column can purify the enzymes to homogeneity, with 24 purification fold and 13 % yield in BT01 and 8 purification fold and 71 % yield in pBT.

4.6 Characterization of CGTase from BT01 and pBT.

Several characteristics of purified CGTase from BT01 and pBT were compared.

4.6.1 Molecular weight determination

Purified CGTase from BT01 and pBT were determined for their molecular weight by SDS-PAGE. From the calibration curve in Figure 19, the molecular weight of CGTases from BT01 and pBT were the same at 71 kDa. The result from SDS-PAGE and the approximate molecular weight from deduced amino acid of 75 kDa suggested that both CGTases were monomeric enzymes. Table 4 showed the reported molecular weight of CGTase in several bacteria studied.

4.6.2 Isoelectric point determination.

The isoelectric points of these CGTases were determined by isoelectrofocusing gel in the ampholine pH range 3-10 along with with standard pI markers (pI 3.5-9.3). Both enzymes showed major band at pI 4.74 and faint bands at 4.86 and 4.62. Kaskangam (1998) reported four isoforms from *Bacillus circulans* sp. A11 with pI's of 4.73, 4.49, 4.40 and 4.31. The pI values obtained were rather acidic while some microorganism such as *Bacillus* sp. G1 produced CGTase with basic pI of 8.0 (Sian *et al.*, 2005).

4.6.3 Effect of pH on enzyme activity and stability.

Each enzyme has an optimum pH at which the rate of the reaction it catalyzes is at its maximum. Small deviations in pH from the optimum value lead to decrease activity, due to changes in the ionization of groups at the active site of the enzymes. Larger deviations in pH lead to the denaturation of the enzyme protein itself, due to interference with many weak noncovalent bonds maintaining its three-dimensional structure (Hames *et al.,* 2000).

CGTase from BT01 and pBT were stable at pH range 6-10 when incubated for 60 minutes at 50°C, activity was retained above 80%, suggesting that this CGTase may be an alkalophilic enzyme. The optimum pH for dextrinizing and cyclization activity were 5.0 and 6.0 in 0.2M acetate buffer, respectively. At pH 5.0 the stability of cyclization activity were lost (decrease to 7% in BT01 and 67% in pBT) (Figure 25). The activity of CGTases in the study of optimum temperature were monitored in the reaction mixture which included starch cyclization activity was at about 99% relative activity. This may suggest that soluble starch substrate may have significant effect in maintaining enzyme activity. Binding of starch to active site may protect the site from pH effect.

4.6.4 Effect of temperature on enzyme activity and stability.

Temperatures affect the rate of enzyme-catalyzed reactions in two ways. First, a rise in temperature increases the thermal energy of the substrate molecules with sufficient energy to overcome the ΔG^0 (Gibbs free energy of activation) and hence increases the rate of the reaction. However, a second effect comes into play at higher temperatures. Increasing the thermal energy of the molecules which make up the protein structure of the enzyme itself will result in breaking of the multiple weak non-covalent interactions which hold the three-dimensional structure of the enzyme together. Ultimately, this will lead to denaturation (unfolding) of the enzyme, even small changes in the three-dimensional shape of the enzyme can alter the structure of the active site and lead to a decrease in catalytic activity. The overall effect of a rise in temperature on the reaction rate of the enzyme is the balance between these two opposing effects. (Hames *et al.,* 2000)

For temperature stability of BT01 and pBT CGTase, when the enzyme was incubated at temperature range of 40 to 70°C for 2 hours, 100% activity was retained at 40 to 50°C and dropped to about 50% activity at 60°C and completely lost activity at 65, 70°C. At high temperature range (65-70°C), which the enzymes lost activity, the solutions changed to white colloidal solution. This suggested that the enzymes were denatured. On the other hand, the addition of substrate (2%, 20% soluble starch) to the enzyme solution resulted in an increased stability at higher temperature as shown in Figure 27. At 70°C, the enzymes retained 90% activity for longer than 120 minutes and lost about 50% activity when incubated at 80°C for 30 minutes, suggesting prolong temperature stability in the presence of substrate. The result corresponded with those CGTases from *Bacillus firmus* (Gawande *et al.,* 1999), alkaliphilic *Bacillus agaradhaerens* strain LS-3C (Martins and Hatti-Kaul, 2002) and *Bacillus* sp. RB01 (Yenpetch 2002) reported improved thermal stability in the presence of substrate and $CaCl_2$. Optimum temperature for dextrinizing and cyclization activity were both at 50°C.

4.7 Kinetic study of purified CGTase

Cyclization activity was employed to determine the kinetic parameter. Soluble starch was used as substrate to produce the mixture of cyclodextrins. β-CD the major product was determined by phenolphthalein method as described in section 2.11.2**.** The initial reaction rate of the purified CGTase was measured at various concentrations of soluble starch and the results were analyzed using Lineweaver-Burk plot. The *Km* values calculated were 5.36 mg/ml for wild type and 4.90 mg/ml for recombinant CGTase. The *Vmax* value calculated were 50.80 nmol/min in wild type and 47.28 nmol/min in recombinant CGTase. *Km* values for several CGTase have been reported such as CGTase from *Bacillus circulans* E192 (Bovetto *et al.,* 1992) exhibited *Km* value for soluble starch of 5.7 mg/ml,CGTase from *Bacillus* TS1-1 (Rahman *et al.,* 2006) exhibited *Km* value for soluble starch of 0.52 mg/ml while CGTase from *Bacillus firmus* (Gawande *et al.,* 1999) had a *Km* value for soluble stacrh of 1.21 mg/ml. On the other hand, CGTase from *Bacillus agaradhaerens* had a *Km* value for soluble starch of 21.2 mg/ml. This data indicated that *Paenibacillus* sp.

BT01 enzyme had relatively moderate affinity for the substrate. pBT had a *kcat/Km* higher than BT01 suggeted that enzyme from recombinant pBT was more efficient in converting starch to β-CD than BT01.

4.8 Analysis of cyclodextrin products

The purified CGTase of BT01 from DEAE-cellulose produced cyclodextrins at the α:-β:-γ:-CDs ratio of 1.0 : 1.5 : 0.51 when incubated with 10%(w/v) soluble starch at pH 6.0, 60ºC while partial purified enzyme from starch adsorption step produced a ratio of α:-β:-CD as 1:1 at pH 6.0, 40ºC (Yampayont *et al.*, 2006). One of the possible explanation for the different ratio of CDs reported by Yampayont *et al.* (2006) and in our result was the presence of other proteins in partial purified CGTase might affect CGTase catalysis. Another reason was the different substrate concentration and temperature of the reaction. Martins and Hatti-Kaul (2002) proposed that the production yield and ratio of the different CDs formed by CGTase is dependent not only on the microbial source producing the enzyme but also on the nature of the substrate and the bioconversion conditions (such as temperature, pH and time.). Yampayont *et al.* (2006) suggested that the ratio of CDs seemed to be unaffected by change in pH but at higher temperatures (60-70ºC) more β-CD was produced with trace amount of γ–CD observed, which agreed with our findings in Table 11. Starch from tubers of cassava was extracted from tubers of KU50 in Rayong province. Soluble starch used in our study was modified from potato starch, tapioca starch was a food grade cassava starch from local market, Flo-max 8 modified starch from cassava purchased from National Starch Food Innovation. The approximate amylose:amylopectin ratios from tapioca and potato were 16.7:83.3 and 20:80, respectively (Young 1984). The cyclodextrin products from the four types of substrates used yielded almost similar α-: β-: γ:-CDs ratio, most likely due to the starch were from similar amylose:amylopectin ratios sources i.e. cassava and potato. The amount of β-CD reported in Table 11 was calculated from HPLC peak area which is more suitable for semi-quantitative data but may not be accurate quantitatively due to overlapping of all CD peaks. Data in Table 12 was calculated

from cyclization activity which monitored quite specificly for β-CD and quantitation was obtained from standard curve, therefore, should be more accurate.

From the result obtained, pBT produced enzyme about 2 times by total activity and 7 times by specific activity more than BT01. Induction time of pBT was 27 hours to produce CGTase while BT01 incubated 72 hours. Although pBT appeared to give slightly lower β-CD yield than BT01 (Table 12), the higher amount of CGTase and shorter incubation time rendered pBT to be more beneficial for industrial use in β-CD production. Table 14 summarized the properties of thermotolerant bacteria isolated by our group and their transformants. They were all in the *Paenibacilli* strain. The recombinant pRB and pT16 were cloned using pGEM-T Easy vector and *E. coli* JM109 as host cells.

Table 14: Comparison of the properties of CGTase thermotolerant bacteria isolated by our group.

Producer	Optimum pH	Optimum Temp(°C)	Molecular mass	Ratio of CD formed α–:β–:γ-CD	Reference
Paenibacillus sp. RB01	6.5	60-70	65,000	1.0:1.8:0.2	Charoensakdi *et al.*, 2005, Yenpetch, 2002
pRB	6.5	40-70	66,000	1.0:4.8:0.2	Charoensakdi *et al.,* 2005
Paenibacillus sp. T16	6.5	60-70	76,000	1.0:1.1:1.7	Charoensakdi *et al.,* 2007 (b), Pranommit 2001
pT	7.5	50-70	77,000	1.0:4.0:2.0	Charoensakdi 2007 (b)
Paenibacillus sp. BT01	6.0	50-70	71,000	1.0:1.9:0.65	This study
pBT	6.0	50-70	71,000	1.0:2.0:0.92	This study

CHAPTER V
CONCLUSIONS

1. CGTase gene from *Paenibacillus* sp. BT01 were ligated to pET19b and transformed into *E. coli* BL21(DE3). This recombinant cell was name pBT.
2. CGTase gene consisted of an open reading frame of 2142 bp which encoded for a polypeptide of 713 amino acid residues, with 686 amino acids for mature enzyme.
3. CGTase gene was expressed under T7 promoter from pET19b.
4. The nucleotide and deduced amino acid sequences showed 99% homology with CGTase of *Paenibacillus* sp. A11.
5. 99.83% of total CGTase from pBT was secreted into the LB medium after induction with 0.2 mM IPTG for 24 hours
6. The CGTase from BT01 was purified to homogeneity by starch adsorption and followed by DEAE-cellulose column chromatography with final 2.18%yield and 24.35 purification fold.
7. The CGTase from pBT was purified to homogeneity by starch adsorption and followed by DEAE-cellulose column chromatography with final 70.98 %yield, 8.16 purification fold.
8. The molecular weight of CGTase from BT01 and pBT were estimated to be 71 kDa by SDS-PAGE.
9. Isoelectric focus gel electrophoresis gel showed one major band at pI 4.74 and two minor bands at 4.86 and 4.62.
10. The enzyme from BT and pBT exhibited optimum pH and temperature for dextrinizing activity at pH 5.0 and 50 to 60ºC.
11. The enzyme from BT and pBT exhibited optimum conditions of cyclization activity were pH 6.0 and 50 to 70ºC.
12. CGTase were stable at pH 6.0-10.0 upto 50ºC and stable up to 70ºC in the presence of 2% soluble starch or 20% soluble starch.
13. CGTase catalyzed the conversion of starch to mixture cyclodextrins with a ratio of α-: β-: γ-CDs of 1.00: 2.00:0.72, when incubated with 10% soluble starch at 60ºC pH 6.0 for 24 hours.

14. The total β-CD produced were 22.52 g/l in wild type and 19.33 g/l in recombinant when incubated with 10% soluble starch at 60ºC, pH 6.0, 24 hours.
15. For cyclization activity, *Km* for soluble starch were 5.36 mg/ml in wild type and 4.90 mg/ml in recombinant CGTase. The *Vmax* value calculated were 50.80 nmol/min in wild type and 47.28 nmol/min in recombinant CGTase.

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APPENDICES

Appendix 1: Preparation for polyacrylamide gel electrophoresis

1) Stock reagents

30% Acrylamide, 0.8% bis-acrylamide, 100 ml

acrylamide	29.2 g
N, N'-methylene-bis-acrylamide	0.8 g

Adjusted volume to 100 ml with distilled water

1.5 M Tris-HCl pH 8.8

Tris(hydroxymethyl)-aminomethane	18.17 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

2 M Tris-HCl pH 8.8

Tris(hydroxymethyl)-aminomethane	24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

0.5 M Tris-HCl pH 6.8

Tris(hydroxymethyl)-aminomethane	6.06 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl pH 6.8

Tris(hydroxymethyl)-aminomethane	12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

Solution B (SDS-PAGE)

2 M Tris-HCl pH 8.8	75 ml
10% SDS	4 ml
distilled water	21 ml

Solution C (SDS-PAGE)

1 M Tris-HCl pH 6.8	50 ml
10% SDS	4 ml
distilled water	46 ml

2) Denaturing PAGE (SDS-PAGE)

10.0 % Separating gel

30% acrylamide solution	2.50 ml
Solution B (SDS-PAGE)	2.50 ml
distilled water	2.39 ml
10% $(NH_4)_2S_2O_8$	100 µl
TEMED	10 µl

5.0% stacking gel

30% acrylamide solution	0.84 ml
Solution C (SDS-PAGE)	1.0 ml
distilled water	3.1 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

Sample buffer

1 M Tris-HCl pH 6.8	0.6 ml
50% glycerol	5.0 ml
10% SDS	2.0 ml
2-mercaptoethanol	0.5 ml
1% bromophenol blue	1.0 ml
distilled water	0.9 ml

One part of sample buffers was added to four parts of sample. The mixture was heated 5 minutes in boiling water loading to the gel.

Electrophoresis buffer, 1 litre

Tris (hydroxylmethyl)-aminometane	3.0 g
Glycine	14.4 g
SDS	1.0 g

Adjusted volume to 1 litre with distilled water (pH should be approximately 8.3)

3) Non-denaturing PAGE

7.5% Separating gel

30% acrylamide solution	2.5 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
distilled water	5.0 ml
10% $(NH_4)_2S_2O_8$	50 µl
TEMED	10 µl

5.0% Stacking gel

30% acrylamide solution	0.67 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
distilled water	2.3 ml
10% $(NH_4)_2S_2O_8$	30 µl
TEMED	5 µl

Appendix 2: Preparation for isoelectric focusing gel electrophoresis

1) Stock reagent

Momomer concentrate solution

24.25% (w/v) acrylamide

0.75% (w/v) bis (*N*,*N*′-Methylene-bis-acrylamide)

Dissolve 24.25 g acrylamide and 0.75 g bis (*N*,*N*′-methylene-bis-acrylamide) in water, bring to a final volume of 100 ml, and filter through a 0.45 μm filter. Store protected from light at 4^{o}C. This solution may be stored up to 1 month.

0.1 % (w/v) riboflavin-5′-phosphate (FMN)

50 mg riboflavin-5′-phosphate

50 ml water

The solution was heated 5 minutes in boiling water bath.

2.) Polyacrylamide electrofocusing gel

Monomer-ampholyte solution

distilled water	2.75 ml
momomer concentrate solution	1.0 ml
25% glycerol	1.0 ml
Ampholyte(pH 3.5-10, 0.4g/ml)	0.25 ml

Catalyst solution

10% (w/v) $(NH_4)_2S_2O_8$	15 μl
0.1% (w/v) FMN	50 μl
TEMED	5 μl

3.) Fixative and staining solution

Fixative solution, 100 ml

sulfosalicylic acid	4 ml
trichloroacetic acid	12.5 ml
methanol	30 ml

immerse gels in this solution for 30 minutes.

Staining solution, 100 ml

Ethanol	27 ml
acetic acid	10 ml
Coomassie brilliant blue R-250	0.04 g
$CuSO_4$	0.5 g
distilled water	63 ml

Dissolved the $CuSO_4$ in water before adding the alcohol. Either dissolved the dye in alcohol or added it to the solution at the end. Immersed the gel in stain for approximately 1-2 hours.

4.) Destaining solutions

First destaining solution (100 ml)

Ethanol	12 ml
glacial acetic acid	7 ml
$CuSO_4$	0.5 g
distilled water	81 ml

Dissolved the cupric sulfate in water before adding the alcohol. Immersed the gel in two of three 500 ml changes of this solution until the background was nearly clear. Gentle agitation and slight heating will speed the destaining process.

Second destaining solution (100 ml)

ethanol	25 ml
glacial acetic acid	7 ml
distilled water	68 ml

Immersed the gel in this solution to removed the least traces of stain and $CuSO_4$.

Note : Prolong soaking of gels with gel support film backings in acetic solutions may cause the gel to separate from the backing. Staining and destaining steps should be no longer than 3-4 hours.

Appendix 3: Preparation for buffer solution

0.2 M Sodium Acetate pH 4.0, 5.0 and 6.0

CH_3COONa	1.21 g

Adjusted volume to 100 ml with distilled water. Adjusted to pH 4, 5 or 6 by 0.2 M acetic acid

0.2 M Phosphate pH 6.0

KH_2PO_4	3.28 g
K_2HPO_4	0.16 g
distilled water	100 ml

0.2 M Phosphate pH 7.0

KH_2PO_4	1.35 g
K_2HPO_4	1.67 g
distilled water	100 ml

0.2 M Phosphate pH 8.0

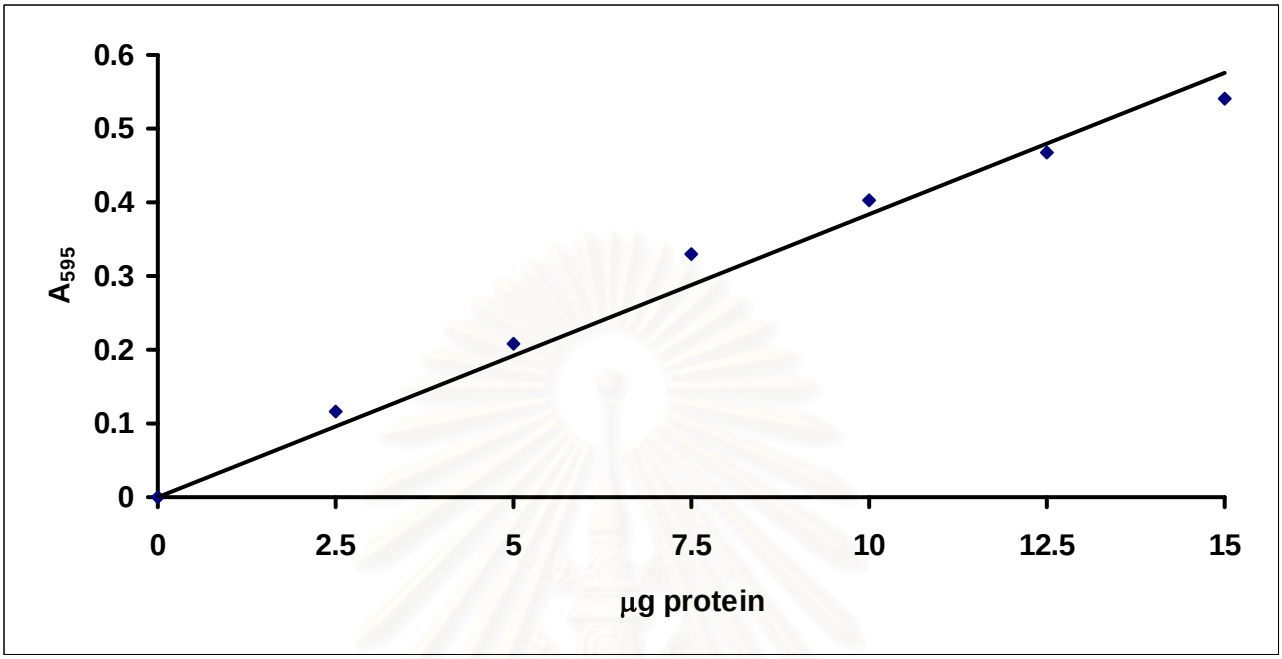
KH_2PO_4	0.48 g
K_2HPO_4	2.34 g
distilled water	100 ml

0.2 M Tris-Glycine NaOH pH 8.0, 9.0 and 10.0

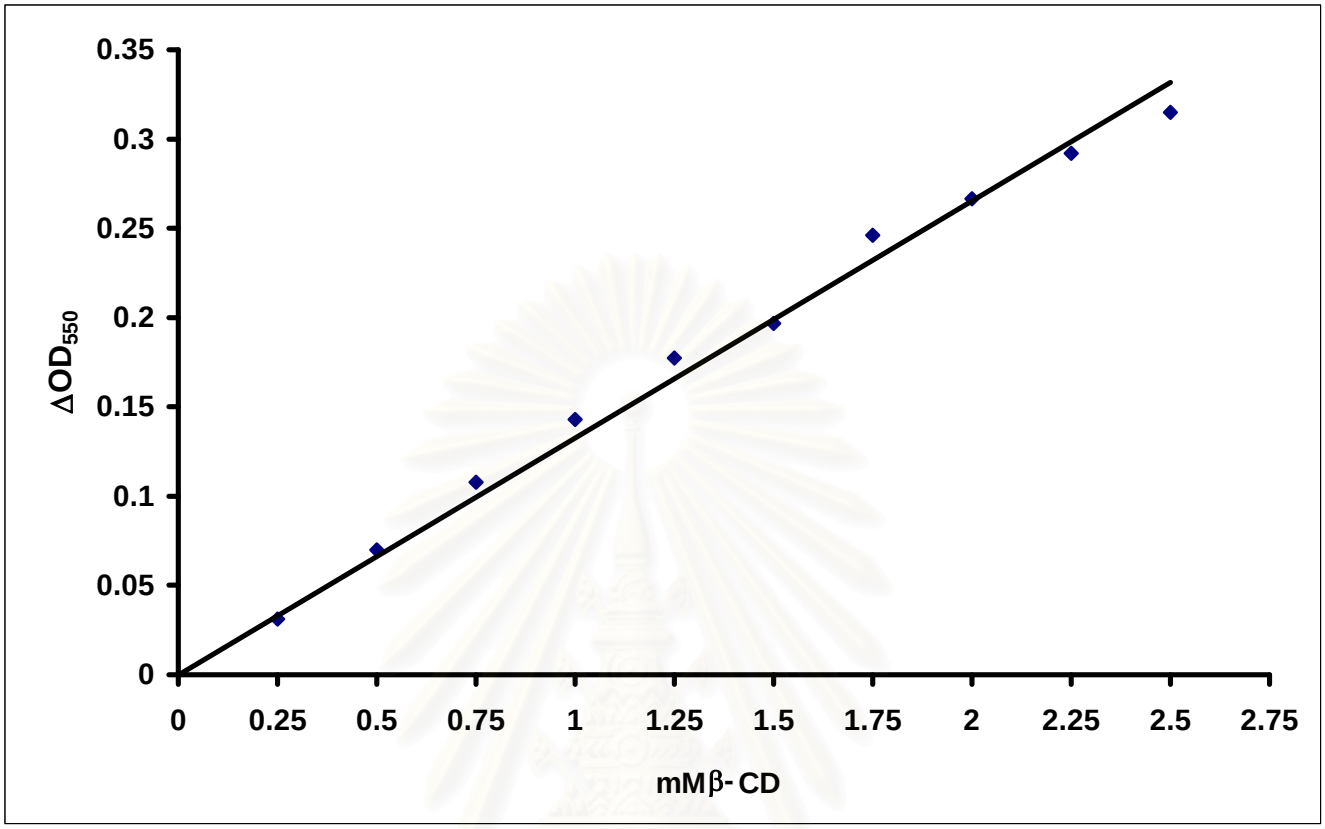
glycine	1.5 g

Adjusted to pH 8.0, 10.0 and 11.0 by 1 M NaOH and adjusted volume to 100 ml with distilled water.

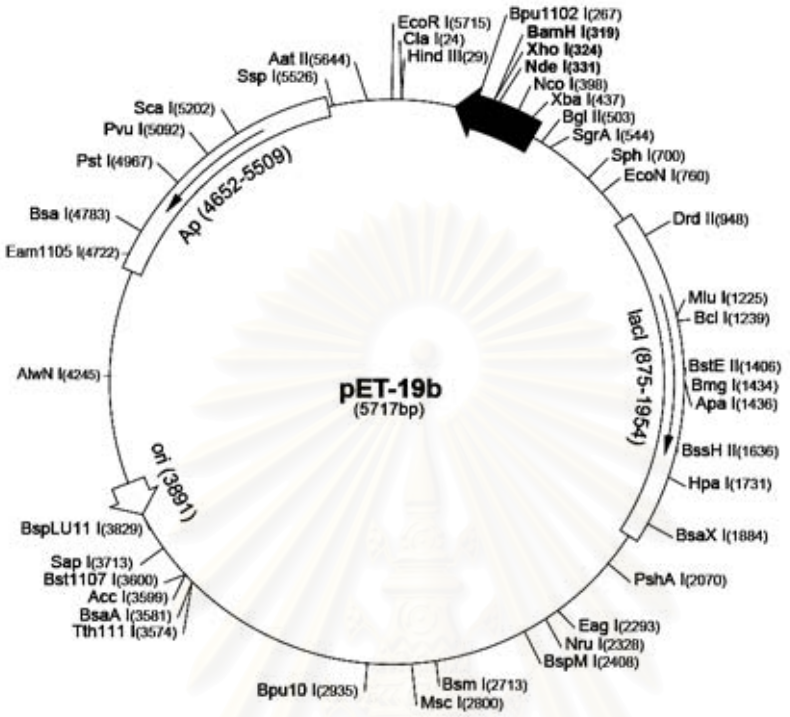
Appendix 4: Standard curve for protein determination by Bradford's method

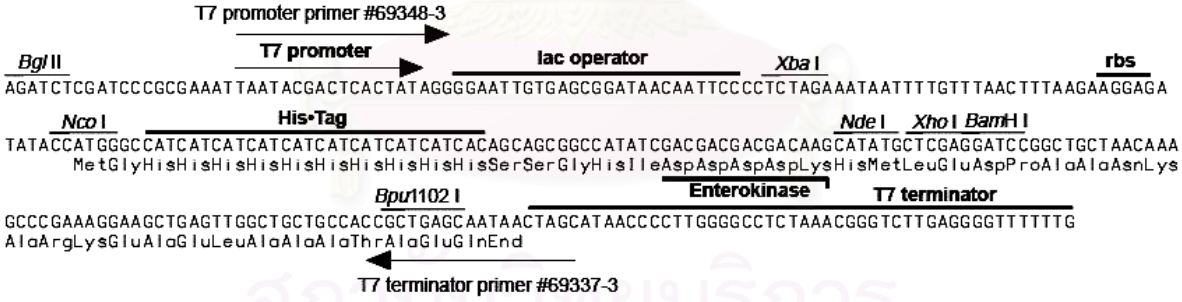


Appendix 5: Standard curve of β–cyclodextrin by phenolpthalein method



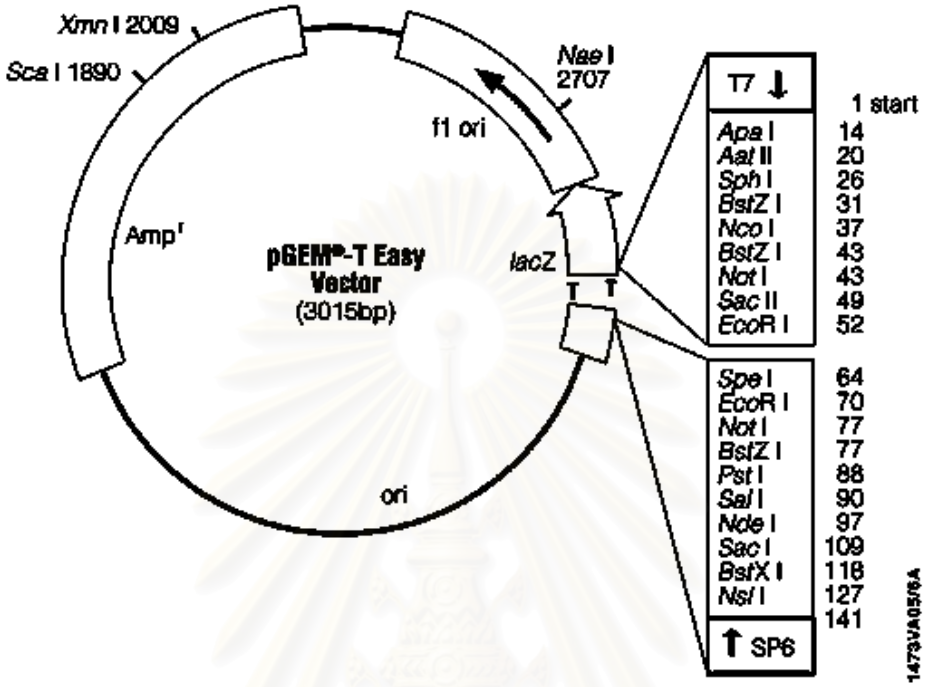
Appendix 6: Restriction map of pET19b





pET-19b cloning/expression region

Appendix 7: Restriction map of pGEM-T easy



Appendix 8: List of CGTase used in phylogenic tree construction

Abbreviation	Primary Accession Number	Source	Main product
KC201	D13068	*Bacillus* sp. KC201	β
GS2	X59044	*Geobacillus stearothermophilus*	α
CD162	AF011388	*Brevibacillus brevis* strain CD162	γ/β
C1400	D90243	*Bacillus ohbensis* strain C-1400	β
Ck104	L25256	*Bacillus* sp. Q	
IB7	AF047363	*Paenibacillus macerans* strain IB7	α
IFO_3490	X59045	*Paenibacillus macerans* strain IFO 3490 (NRRL B-388)	α
B_lic	X15752	*Bacillus licheniformis*	α/β
Bc_amy	M16657	*Bacillus circulans* amylase gene	
B1018	D90112	*Bacillus* sp. B1018	β
B251	X78145	*Bacillus circulans* strain 251	β
B38_2	D00129	*Bacillus* sp. 38-2	β
BI_5	AY478421	*Bacillus* sp. I-5	β
B1011	M17366	*Bacillus* sp. strain 1011	β
BA11	AF302787	*Bacillus circulans* strain A11	β
N_227	DQ631916	*Bacillus* sp. N-227	β
RB01	-	*Paenibacillus* sp. RB01	β
BT01	-	*Paenibacillus* sp. BT01	β
T16	-	*Paenibacillus* sp. T16	β

BIOGRAPHY

Mr. Krit Tantanarat was born on April 3, 1981. He graduated with the degree of Bachelor of Science from the Department of Biochemistry at Chulalongkorn University in 2003. He has studied for the degree of master of Science at the program of Biotechnology, Faculty of Science, Chulalongkorn University since 2004.