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CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT BACTERIA: SCREENING, OPTIMIZATION, PARTIAL PURIFICATION AND CHARACTERIZATION

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การคัดเลือกแบคทีเรียทนร้อนที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสจากแหล่งดินและน้ำบริเวณบ่อน้ำร้อนในภาคต่างๆของประเทศไทย โดยใช้อาหารเลี้ยงเชื้อ Medium I ที่มีแป้งเป็นส่วนประกอบในการคัดเลือกแบคทีเรียที่สามารถผลิตเอนไซม์อะไมเลส ผลการคัดเลือกระดับ primary บนอาหารแข็ง พบแบคทีเรียที่สร้างวงใสรอบโคโลนีจำนวน 38 ไอโซเลท แล้วนำไอโซเลทเหล่านี้ไปคัดเลือกในขั้นต่อไปเพื่อหาแบคทีเรียที่สามารถผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส โดยใช้อาหาร Horikoshi ซึ่งประกอบด้วยระบบ dyes ที่ใช้ตรวจวัดผลิตภัณฑ์ไซโคลเดกซ์ทริน พบแบคทีเรียที่สามารถผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส จำนวน 2 ไอโซเลท (RB01 และ KB01) จากนั้นนำแบคทีเรียไปศึกษาการเจริญและการผลิตเอนไซม์ที่อุณหภูมิต่างๆ ในช่วง 30-55 องศาเซลเซียส ในอาหารเหลว เพื่อหาสายพันธุ์ที่มีลักษณะเป็น thermotolerant ที่ดีที่สุด พบว่า RB01 เจริญและผลิต CGTase ได้ในช่วง 30-45 องศาเซลเซียส โดยเจริญดีที่สุดที่ 37 องศาเซลเซียส ขณะที่ผลิตเอนไซม์ได้สูงสุดที่ 40 องศาเซลเซียส จากการจัดจำแนกสายพันธุ์ตามลักษณะทางชีวเคมีและสรีระวิทยา พบว่า RB01 คือ *Bacillus circulans* แต่จากการจำแนกสายพันธุ์โดยใช้ 16S rRNA gene พบว่า มี homology 99 % กับ *Paenibacillus campinasensis* (strain 324) ภาวะที่เหมาะสมในการเจริญและการผลิตเอนไซม์คือ เลี้ยงในอาหาร Horikoshi ที่เติม soluble starch 1.0% pH ของอาหารเท่ากับ 10.0 อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 60 ชั่วโมง การทำเอนไซม์ให้บริสุทธิ์บางส่วนโดยวิธีดูดซับด้วยแป้ง ได้เปอร์เซ็นต์ recovery และ purification fold เท่ากับ 57.3 และ 26.8 ตามลำดับ แอคติวิตีจำเพาะเท่ากับ 3,568 U/mg protein ค่า pH และอุณหภูมิที่เหมาะสมของแอคติวิตีของเอนไซม์คือ pH 6.0 และ 55 องศาเซลเซียส ตามลำดับ เอนไซม์เสถียรที่ pH 7.0 และอุณหภูมิ 40 องศาเซลเซียส น้ำหนักโมเลกุลของเอนไซม์หาโดย SDS-PAGE มีค่า 65,000 ผลิตภัณฑ์หลักคือ β-cyclodextrin และสามารถผลิต α- และ γ-cyclodextrins ในอัตราส่วนของ α-, β-, and γ- cyclodextrins เท่ากับ 1.0 : 5.4 : 1.2 อุณหภูมิที่เหมาะสมในการเก็บเอนไซม์คือ –20 องศาเซลเซียส

หลักสูตร เทคโนโลยีทางชีวภาพ ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีทางชีวภาพ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4272459623 : MAJOR BIOTECHNOLOGY
KEY WORD : CYCLODEXTRIN GLYCOSYLTRANSFERASE / THERMOTOLERANT BACTERIA / SCREENING / OPTIMIZATION / CHARACTERIZATION
SOLOS TESANA : CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM THERMOTOLERANT BACTERIA: SCREENING, OPTIMIZATION, PARTIAL PURIFICATION AND CHARACTERIZATION. THESIS ADVISOR : ASSOC. PROF. PIAMSOOK PONGSAWASDI, Ph.D. 107 pp. ISBN 974-17-0244-2

Screening of thermotolerant bacteria producing cyclodextrin glycosyltransferase (CGTase) was performed. Soil and water samples were collected from hot spring areas in different parts of Thailand. Primary screening for amylase using Medium I, which contained starch as enzyme inducer was carried out. Thirty-eight isolates exhibited clear zone when staining with 0.02% I_2 in 0.2% KI were found. Isolates with amylase activity were further screened for CGTase. Selection medium was Horikoshi medium to which dyes system to follow CGTase activity was incorporated. Two isolates (RB01 and KB01) with yellowish orange clear zone on an intense pink background were observed. These isolates were checked for growth and enzyme activity in liquid culture in the temperature range of 30-55^{o}C for selection of the best thermotolerant strain. It was found that RB01 was grown and was able to produce CGTase at temperature range of 30-45^{o}C, while grown best at 37^{o}C but exerted highest activity at 40^{o}C. Biochemical and physiological characterization indicated that RB01 was *Bacillus circulans*. Characterization by16S rRNA gene demonstrated that RB01 was closely related to *Paenibacillus campinasensis* (strain 324) with 99% homology. The optimum conditions for cell growth and enzyme production were culturing RB01 in Horikoshi medium with 1.0% soluble starch at 40^{o}C, pH 10.0 for 60 hours. The enzyme was partially purified by starch adsorption, the % recovery and purification fold were 57.3 and 26.8, respectively. The final specific activity was 3,568 U/mg. The optimum pH and temperature of the enzyme were 6.0 and 55^{o}C, while the pH and temperature stability of the enzyme were 7.0 and 40^{o}C, respectively. The molecular weight was estimated to be 65,000 by SDS-PAGE. The enzyme formed mainly β-cyclodextrin with small amounts of α- and γ-cyclodextrin. The ratio of α-, β- and γ-cyclodextrins was 1.0: 5.4: 1.2. The best condition for storing enzyme was –20^{o}C.

Program……Biotechnology……Student's signature……

Field of study……Biotechnology…… Advisor's signature……

Academic year……2001…… Co-advisor's signature……

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ABBREVIATIONS

A	absorbance
bp	base pair
CDs	cyclodextrins
CGTase	cyclodextrin glycosyltransferase
^{o}C	degree celsius
cm	centimeter
g	gram
hr	hour
HPLC	high performance liquid chromatography
µl	microlitre
µg	microgram
µm	micrometer
min	minute
M	molar
mM	millimolar
MW	molecular weight
ml	milliliter
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
rpm	round per minute
R_t	retention time
R_f	relative mobility
SDS	sodium dodecyl sulfate
Tag	*Thermus aquaticus*
U	unit (s)
w/v	weight by volume

CHAPTER I

INTRODUCTION

Cyclodextrins (CDs)

Cyclodextrins (Schardinger dextrins, Cycloamylose, Cyclomaltoses and Cycloglucans) are the oligomers of anhydroglucose units joined to form a ring structure with α-1,4 glycosidic bond. The main CDs are composed of 6, 7 and 8 glucose units called α- (alpha), β- (beta) and γ- (gamma) cyclodextrin, respectively (Figure 1) (Pulley and French, 1961). They had different physical properties as summarized in Table 1 (Saenger, 1982; Szejtli, T. 1988). CDs with less than 6 glucopyranose units cannot be formed for steric reasons and CDs containing more than 9 glucopyranose units have been shown to be branched (Sundararajan and Rau, 1970). Recently, δ-CD (cyclonanoamylose, composed of 9 α-1,4-linked D-glucose units) and η-CD (cyclododecaamylose, composed of 12 α-1,4-linked D-glucose units) have been reported. They were produced from cyclodextrin powder by the action of β-amylase and pullulanase. Even if these large ringed cyclodextrins are inapplicable for various industrial uses due to instability, they may have some unique characters in comparison with the conventional cyclodextrins. Elucidation of their structures and physicochemical properties may provide much information on a basic knowledge and development of oligosaccharides (Tomohoro *et al*, 1994)

CDs are water-soluble. This fundamental characteristic derives from the location of all free hydroxyl groups of each successive glucose unit on rims of these doughnut-shape molecules-the C_6 primary hydroxyls on the narrower side and the C_2 and C_3 secondary hydroxyls occupying the wider side. These two hydrophilic planes thus confer hydrophilicity upon the molecule, while the inside cavity of CDs is

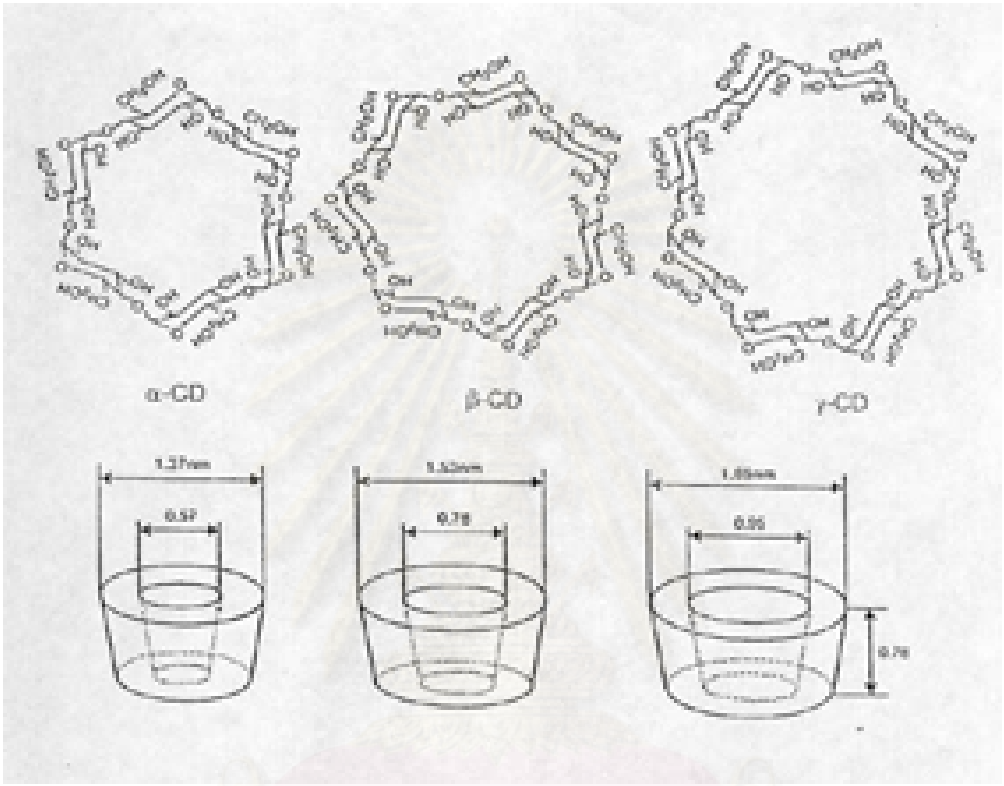


Figure 1. Chemical structure of three kinds of CDs : (A). The molecular dimension structure of CDs : (B). (Szejtli, 1988)

Table 1. Some physical properties of three kinds of CDs (Saenger, 1982; Szejtli, 1988)

Properties	**α-CD**	**β-CD**	**γ-CD**
Amount of glucose units	6	7	8
Molecular weight	972	1,135	1,297
Solubility (%) in water at ambient temperature	14.5	1.85	23.2
Cavity dimensions			
Cavity diameter (A^o)	4.7-5.3	6.0-6.5	7.5-8.3
Cavity depth (A^o)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Cavity volume			
$(A^o)^3$	174	262	472
ml per mol	104	157	256
ml per g	0.10	0.14	0.20
Crystal form (from water)	Hexagonal plates	Monoclinic paralellograms	Quadratic prisms
Coloration with iodine	yellow-orange	lemon-yellow	brown
Melting range (^{o}C)	250-260	255-265	240-245
Appearance	⟵ White crystalline powders ⟶		

hydrophobic because it is lined with C-H groups and glycosidic oxygen bridges (Saenger, 1979,1982), see Figures 1 and 2A.

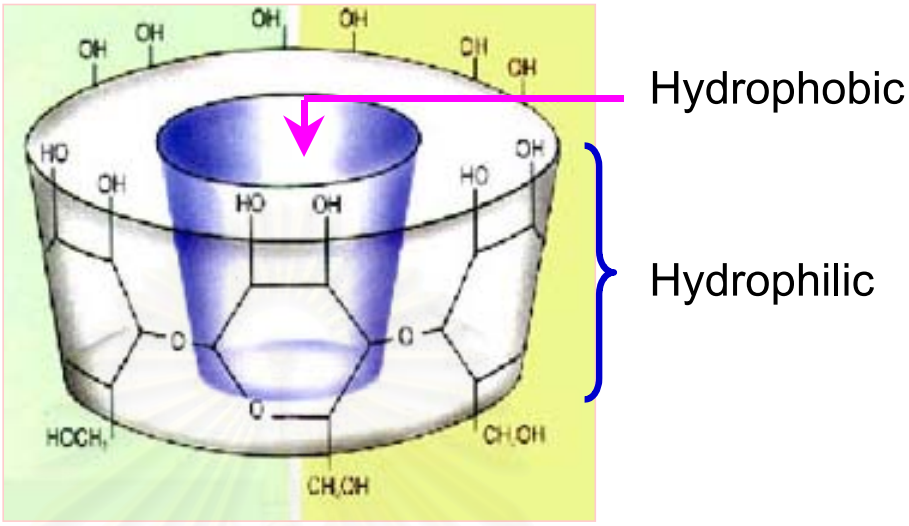
The unique spatial structure of CDs enhances its chemical stability. The chemical properties markedly differ from those of non-cyclic carbohydrates as follows:

- The ring structure has neither a reducing nor non-reducing end group.
- They are not decomposed by hot alkali.
- They are rather resistant to hydrolysis by most organic acids and many common α-amylases, and completely resistant to yeast fermentation and β-amylases.
- They demonstrate the enhanced thermal stability with a decomposition temperature approaching 300 ^{o}C.

The most important consequence of this structure is the ability of CDs to form inclusion complexes with a variety of suitably sized hydrophobic "guest" molecules. The complexation involves Van der Waals interactions and hydrogen bond formation. A guest molecule can be fully or partly included in the cavity. The stability of the complex formed depends both on the size and polarity of the guest molecule. If the guest molecule is too large to fit in the cavity, partial interaction with CD is still possible, with formation of a weaker inclusion complex (Uekama *et al*, 1977). The interactions between CDs and guest molecules are shown in Figure 2B

As mentioned above, CDs can form the inclusion complexes with many kinds of guest molecules. The complexes change the properties of the guest molecule variously, and the guest can be isolated easily from inclusion complex when needed.

A



B

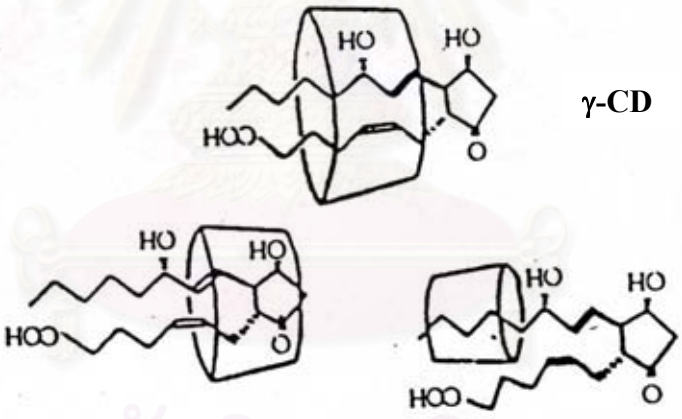


Figure 2. The model structure consisting of the functional group that presented hydrophobic and hydrophilic properties in CD molecules: (A). Schematic representation of prostaglandin inclusion in the cavity of α-, β- and γ-CD: (B).

Complexes can be formed either in solution or in the crystalline state and while water is typically the solvent of choice, inclusion complexation can be accomplished in co-solvent systems and with some non-aqueous solvents (Amaizo, 1993). Inclusion in cyclodextrins can markedly improve the chemical and physical properties of guest molecules as they are temporarily docked or caged within the host cavity, giving rise to the following beneficial modifications of guest molecules :

- Solubility enhancement.
- Stabilization of labile guest against the degradative effects of oxidation, visible or UV light, and heat.
- Control of volatility and sublimation.
- Physical isolation of incompatible compounds.
- Chromatographic separations.

The potential guest list for molecular encapsulation in CDs is quite varied and includes such compounds as straight or branched chain aliphatics, aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, and such polar guest as the halogen and oxy-acids and amines. Additionally, some ionic binding might occur externally. Consequently, considerable interest has been generating to commercially exploit CDs in various industrial sectors. The applications of CDs as solubilizers, emulsifiers, antioxidants, and stabilizing agents have been rapidly increased in the foods, cosmetics, pharmaceuticals, agrochemicals and the others as shown in Table 2 (Nagatomo, 1985).

Table 2. Industrial application of CDs. (Horikoshi, 1982; Bender, 1986; Szejtli, 1988; Cyclodextrin News, 1991)

Functions	Guests	End products
Foods		
1. Emulsification	Oils & Fats	Margarine, Whipping cream, French dressing, Cake, etc.
2. Stabilization	Flavors, Spices, Colors & Pigments	Mustard paste cakes & cookies, Pickled vegetables, Dried vegetables
3. Masking of Taste & Odor		Horse radish paste, Juices, Soy milk, Bone powder, Boiled rice
4. Improvement of Quality		Hard candy, Cheese, Soy sauce, Canned citrus fruits & juices
5. Reduce Volatility	Ethanol	Food preservatives
6. Others		Breath mints
Cosmetics & Toiletries		
1. Emulsification	Oil & Fats	Face creams, Face lotions, Tooth pastes
2. Stabilization	Flavors & Fragrances	Bath refresher crystals
Agrochemicals		
1. Stabilization	Pyrolnitrin	Fungicide
	Pyrethroids	Insecticide
2. Reduce volatility	Organic Phosphates (DDVP)	Insecticide
	Thiocarbamic acid	Herbicide
3. Reduce toxicity	2-Amino 4-methyl-phosphynobutyric acid	Fungicide

Table 2. Industrial application of CDs. (continued)

Functions	Guests & End products
Pharmaceuticals	
1. Improve Solubility	Prostaglandins, Steroids, Cardiac glycosides, Non-steroidal anti-inflammatory agents, Barbiturates, Phenytoin Sulfonamides, Sulfonylureas,
2. Chemical Stabilization	
A) Hydrolysis	Beazodiazepines
B) Oxidation	Prostacyclin, Cardiac glycosides, Aspirin, Atropine, Procaine
C) Photolysis	Aldehydes, epinephrine, Phenothiazines
D) Dehydration	Phenothiazines, Ubiquinones, Vitamins
3. Improve Bioavailability	Prostaglandin E_1, ONO-802
4. Powdering	Aspirin, Phenytoin, Digoxine, Acetohecamide Barbiturates, Non-steroidal antiinflammatories ONO-802, Clofibrate, Benzaldehyde, Nitroglycerin
5. Reduce Volatility	Vitamin K_1, K_2, Methylsalicylate
6. Improve Taste, Smell	Iodine, Naphthalene, d-Camphor, 1-Methylcinnamate
7. Reduce Irritation to Stomach	Prostaglandins, alkylparabens
8. Reduce Hemolysis	Nonsteroidal antiinflammatory agents Phenothiazines, Fluennamic acid, Benzylalcohol, Antibiotics

Other applications for CDs have been continually reported. Applications involved :

- Chemical Industry: CDs can be used as catalyst for the production of 4- hydroxybenzoic acid (Komiyama, 1984).
- Separation Technology: CDs have been utilized in the separation of isomeric hydrocarbon, eliminating organic solvent vapors from air and separation of bitterness from grapefruit juice (Philip, 1985).
- Wastewater Treatment: CDs can be used to reduce unacceptable aromatic compounds such as phenol, benzene and p-chlorophenol from wastewater (Szejtli, 1986) etc.

Cyclodextrin producing enzyme

Cyclodextrin glycosyltransferase (α-1,4-glucan-4-glycosyltransferase, E.C.2.4.1.19, CGTase) is the enzyme which catalyzes the degradation of starch to CDs. The difference between the action of the CGTase and that of other starch-degrading enzymes is that the products of CGTase are cyclic and non-reducing sugar. For CDs production, besides cyclization (the conversion of starch and related α-1,4-glucan into CDs, this enzyme catalyzes a coupling reaction (opening of the rings of CDs and transfer of the linear maltooligosaccharides to the acceptors) and a disproportionation reaction (transfer of linear maltooligosaccharides to acceptors) through intramolecular and intermolecular transglycosylation reactions. Furthermore, the CGTase possesses a weak hydrolyzing activity (Alexandra, 1998). The mechanisms are summarized in Figure 3 and Table 3.

The cyclization is thought to be a special type of disproportionation, the non-reducing end of one chain itself serving as acceptor, whereas the helical conformation of substrate is thought to be a prerequisite for cyclization. It should be mentioned that the acceptor binding sites of enzyme are not absolutely specific for glucose or malto-oligosaccharides (Bender, 1986). The cyclization is efficient for long chain substrates containing 16-80 glucopyranosyl residues. If chain length is greater than 100 units, disproportionation dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 4. High concentration of malto-oligosaccharides or glucose favours the reversed coupling reaction resulting in linear end products with negligible amount of CDs (Kitahara *et al*, 1978). The action of CGTase is different from that of other starch-degrading enzymes in that the products are cyclic and non-reducing.

CGTase are produced by a variety of bacteria including aerobic mesophilic bacteria such as *Bacillus macerans* (Kitahara, 1974), *B. megaterium* (Kitahara, and Okada, 1974), *B. cereus* (Ramakrishna, 1994), *B. obensis* (Jamuna, 1993), *Klebsiella pneumoniae* (Fogarty, 1983), *K. oxytoca* (Wind, 1995), *Micrococcus luteus* (Abelian, 1995), aerobic thermophilic *B. stearothermophilus* (Fujiwara, 1992), aerobic alkalophilic bacteria such as *B. circulans* (Nakamura, 1976), aerobic halophilic *B. halophilus* (Abelian, 1995). In most organisms, CGTases are extracellular enzymes and differ in the amount, type of CDs produced and their properties (Tables 5 and 6).

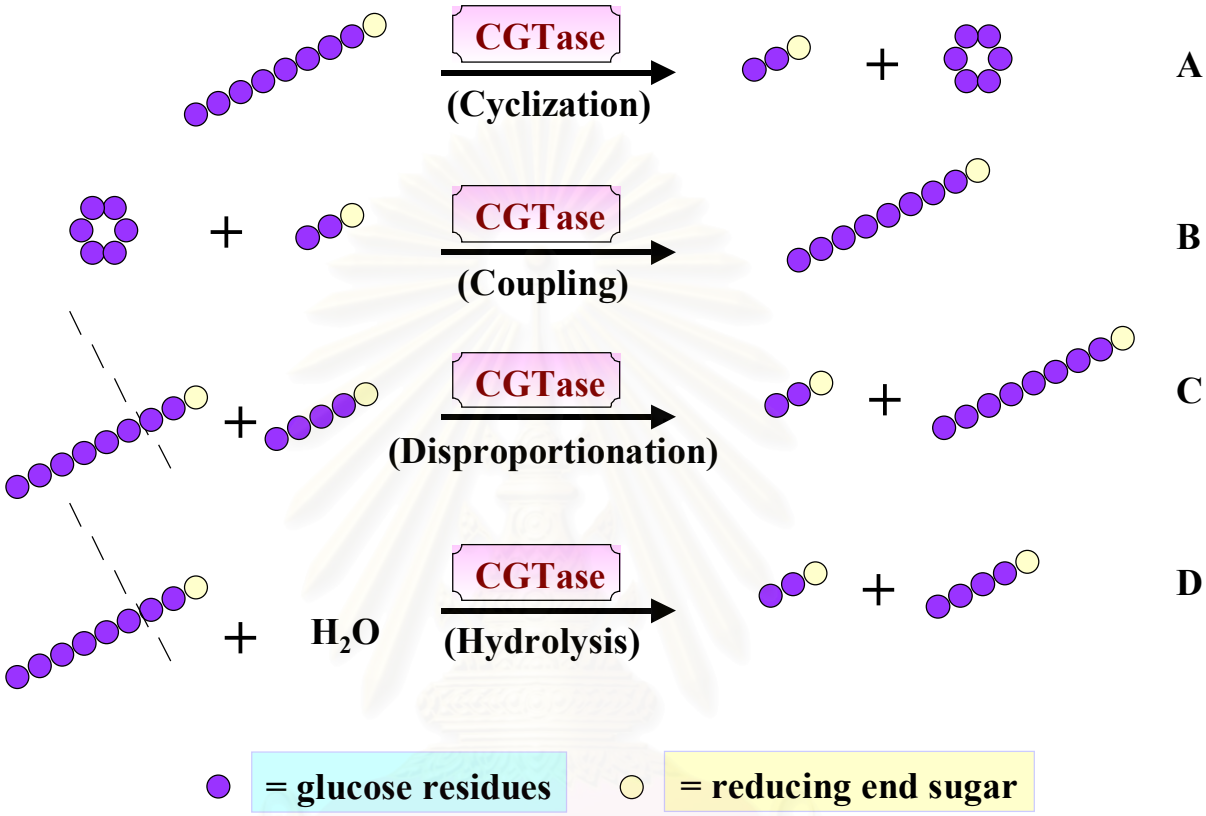


Figure 3. Schematic representation of the CGTase-catalyzed tranglycosylation reactions: (A), cyclization: (B), coupling: (C) disproportionation: and hydrolysis: (D). (Bart *et al*, 2000)

Table 3. Summary of CGTase mechanisms. (Okada and Kitahata, 1975)

Reaction	Action
Cyclization	Starch $\longrightarrow$ cyclodextrin
Coupling	Cyclodextrin + $(glucose)_n$ $\longrightarrow$ oligosaccharide
Disproportionation	$(oligosaccharide)_m + (oligosaccharide)_n$ $\longrightarrow$ $(oligosaccharide)_{m-x} + (oligosaccharide)_{n+x}$

Table 4. Relationship between length of substrate and mechanisms of CGTases. (Szejtli, 1988)

Substrate chain length (residues)	Effect on mechanism of CGTase
1 (D-glucose)	- no catalysis
2-4	- inhibit initial reaction of cyclization
	- substrate for coupling reaction
5-14	- good substrate for coupling reaction
	- poor substrate for disproportionation reaction
16-80	- good substrate for cyclization reaction
> 100	- good substrate for disproportionation

Table 5. List of CGTase producing bacteria and some characteristics of CGTases.

Organism	Predominant product	Optimum pH	Optimum temperature (^{o}C)	MW	pI	Reference
Alkalophilic *Bacillus* 17-1	β-CD	6.0	ND	74,000	ND	Yamamoto, 1972
Bacillus fermus/lentus	γ-CD	6.0-8.0	50	75,000	4.1	Englbrecht, 1990
Bacillus macerans IFO 3490	α-CD	5.0-5.7	55	5,000	4.6	Kitahata, 1974
Bacillus macerans IAM 1243	α-CD	5.5-7.5	60	145,000	ND	Kobayashi, 1978
Bacillus macerans ATCC 8514	α-CD	6.2	ND	139,300	ND	Depinto, 1986
Bacillus megaterium	β-CD	5.0-5.7	55	ND	6.07	Kitahata, 1974
Bacillus stearothermophilus	α-CD	6.0	ND	68,000	4.5	Kitahata, 1982
Klebsiella pneumoniae M5 al	α-CD	6.0-7.2	ND	68,000	4.8	Bender, 1982
Micrococcus sp.	β-CD	6.2	ND	139,300	ND	Yagi, 1980
Brevibacterium sp. No.9605	γ-CD	10.0	ND	75,000	2.8	Mori, 1994

ND = no data

Table 6. Ratio of α-, β- and γ-CDs produced by various microbial CGTases. (Yamamoto, 1994)

Enzyme source	α-CD	:	β-CD	:	γ-CD
B. macerans	5.7	:	1.0	:	0.4
B. megaterium	1.0	:	6.3	:	1.3
B. circulans	1.0	:	6.4	:	1.4
B. sterothermophilus	1.7	:	1.0	:	0.3
Klebsiella oxytoca	1.0	:	1.8	:	0.5
Klebsiella pneumoniae	1.0	:	1.9	:	0.6
Alkalophilic *Bacillus* sp.	1.0	:	11.0	:	1.5
B. ohbensis	0	:	5.0	:	1.0
B. firmus	1.0	:	11.0	:	5.0
B. lentus	1.0	:	67.0	:	1.6

The enzymes from different sources show different characteristics, such as working pH and temperature and molecular weight. Each CGTase enzyme yields different ratio of CD products for example, from Tables 3 and 4 the CGTase from *Bacillus macerans* produces α-, β- and γ-CD in relative amount of 5.7 : 1.0 : 0.4 (Kitahata, 1974) and the CGTase of Alkalophilic *Bacillus* sp. produces CDs in relative ratio of 1.0 : 11.0 : 1.5 (Yamamoto, 1972).

With regard to the isolation of the microorganisms producing CGTase, species that synthesize mainly β-CD are once desirable. Since β-CD is known to be more suitable for practical use because the inclusion complexes are easily prepared and stable due to the size of the apolar cavity being optimum for a large number of molecules such as drugs and preservatives (Horikoshi and Akiba, 1982; Horikoshi, 1979). In addition, β-CD is easily separated from the reaction mixture because of its low solubility in water. However, acquiring a γ-CD producing strain is becoming an attractive point at present. Since γ-CD is more suitable especially in pharmaceutical industry due to its higher solubility and bigger inner cavity compared with α- or β-CD. Moreover, the production of γ-CD is still a problem because very few CGTases produce γ-CD preferentially have been reported (Englbrecht *et al*, 1990) and their enzymatic properties are not suitable for large scale production. Biochemical and genetic characterizations of γ-CGTase have been performed and accumulated in order to compare with those of α- and β-CGTases. Basic knowledge obtained would facilitate construction of a genetically overproduced γ-CGTase strain or an appropriate protein engineered γ-CGTase in the near future.

Various studies have been emphasized on improvement of CD productions. Development of cultivation for CD over-production was carried out under optimized culture condition and with complex nutrient media. A need for thermostable or thermotolerant CGTase which give high CD yield has been recognized. CGTase from an alkalophilic *Bacillus* strain no. 38-2 (ATC 21783) was observed to provide these required properties (Horikoshi and Akiba, 1982). Immobilized CGTase can be utilized in several conversion cycles to steadily increase the volume of production, hence reduce the production cost (Nakamura and Horikoshi, 1976; Kato and Horikoshi, 1984; Yang and Su, 1989). Protein engineering site-directed mutagenesis and gene cloning were also used to increase the yield of enzyme product. These techniques were not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of CDs (Schmid, 1989).

Gene cloning and over-expression of CGTase gene, not only provides satisfactory CD production, but also provides more enzyme for studies on structures and mechanisms including determination of its nucleotide sequence. In such studies, the β-CD synthetase gene from an alkalophilic *Bacillus* sp. #1011 (Kimura *et al*, 1987, 1989, 1990), *Bacillus* sp. strain no. 38-2 (Kaneko *et al*, 1988) and *Klebsiella pneumoniae* M5 al (Binder *et al*, 1986) were cloned and expressed in *E. coli* and *Bacillus subtilis.*

The conventional procedure for the production of CDs includes liquefaction of starch using a thermostable α-amylase at 105 ^{o}C. After the reaction mixture is allowed to cool to ~50 ^{o}C, it is treated with bacterial CGTases (Kitahata, 1974; Nakamura and Horikoshi, 1976; Bender, 1977; Kobayashi, 1978; Kitahata and Okada, 1982; Makela, 1988; Yagi, 1986), which have optimum catalytic reaction

temperatures in the range of 50-65 ^{o}C. However, this procedure is inefficient in many aspects: first, α-amylase should 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.

Problems usually rise in the production of enzyme in the industry when enzymes are purified from strains that grow under normal temperature (mesophiles). An instability of enzymes due to heat produced in the continuous fermentation is likely to be the cause. Researchers have tried to search for bacterial strains that can grow at higher temperature. Thermophiles can grow at temperature above 45°C whereas hyperthermophiles can grow at temperature higher than 80°C (JSPS-NRCT, 2000). However, it is wasteful in terms of energy used and money invested to use microorganisms that can tolerate at 60°C or higher for most of the production of enzymes in industries. Therefore, searching for thermotolerant microorganisms which can be grown at temperature between 30-50°C is quite interesting. It has been defined that thermotolerant microorganisms have evolved together with mesophiles but are able to adapt themselves to higher temperature than that of mesophiles. Moreover, the phylogenic evolution is also different from hyperthermophiles.

Most bacteria that produce CGTase including the ones that are used in industries are mesophilic bacteria, most of them has the optimum temperatures between 30-37°C such as *Bacillus macerans (*Depinto, and Chambell, 1986*), Bacillus lentus* (Englbrecht, 1990), and Alkalophilic *Bacillus* sp. (Nakamura, and Horikoshi, 1976). To date, there are not many reports on the discovery of CGTase producing bacterial strains that are thermophiles or hyperthermophiles. Examples of those

reported are: the thermophilic anaerobic bacteria *Bacillus stearothermophilus* ET1 with the optimum temperature for growth at 65°C. This CGTase had an optimum temperature for 80°C (Chung *et al*, 1998). Recently, hyperthermophilic bacteria which could grow at temperature higher than 90°C had been isolated, and their enzyme had been shown to be extremely thermostable. *Thermococcus* sp. could grow between 60°C and 95°C, the optimum growth temperature was 85°C. While the optimum CD synthesis activity was 90°C to 100°C (Tachibana *et al*, 1999). However, inactivation of the enzyme upon the completion of the process would not be easy due to the thermostability of the enzyme. Nevertheless, the production of CGTase by thermotolerant bacteria has not previously been at the focal point.

This research aims at screening for thermotolerant bacterial strains that produce CGTase in high amount which will be more suitable for industrial applications in the future. Characterization of the enzyme for interesting properties will also be performed in the attempt to look for novel properties of the enzyme.

The objectives of this research are

1. To screen for thermotolerant bacteria that produce CGTase in high quantity.
2. To find optimum condition for high production of the enzyme.
3. To characterize the screened bacteria.
4. To determine for some biochemical properties of the enzyme.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Instruments / Model	Company / Country
-Autoclave, Model HA 30	Hirayama Manufacturing Cooperation, Japan
-Autopipette, Pipetman	Gilson, France
-Centrifuge, Model J-21C	Beckman Instrument Inc, USA
-Electrophoresis unit, Model Mini-protein II Cell	Biorad, USA
-Gene Amp PCR system, Model 2400	Perkin Elmer, USA
-High Performance Liquid Chromatography, Model LC-3A	Shimadzu Cooperation, Japan
-Incubator	Haraeus, Germany
-Incubator shaker, controlled environment, Psyco-Therm	New Brunswick Scientific Co.,USA
-pH meter, PHM 83 Autocal pH meter	Radiometer, Denmark
-Scanning electron microscope JSM-35CF	JEOL, Japan
-Spectrophotometer, JENWAY 6400	UK
-Vortex, Model K-550-GE	Scientific Industries, USA
-Water bath	Charles Hearson Co., Ltd., England
-Water bath, shaker	Heto Lab Equipment, Denmark

2.2 Chemicals

Chemicals	Company / Country
Acrylamide	Merck, USA
Beef extract	Difco Laboratories, USA
Bacto-peptone	Difco Laboratories, USA
Bovine serum albumin fraction V	Sigma Chemical Company, USA
Soluble starch (potato)	Sigma Chemical Company, USA
Standard α-, β- and γ-cyclodextrins	Sigma Chemical Company, USA
Standard molecular weight marker protein	Sigma Chemical Company, USA
Acetonitrile (HPLC grade)	BDH Laboratory Chemical-Division, England
Comassie Blue R-250	BDH Laboratory Chemical-Division, England
Phenolphthalein	BDH Laboratory Chemical-Division, England
Soluble starch	BDH Laboratory Chemical-Division, England
Trichloroethylene (TCE)	BDH Laboratory Chemical-Division, England

Other chemicals used were of reagent grade and were purchased from commercial sources. Raw rice starch (three heads elephant brand), cornstarch (Maizena), cassava starch (dragon fish brand), glutinous rice starch (three heads elephant brand) were locally purchased.

2.3 Media preparation

2.3.1 Medium I

Medium I was consisted of 0.5% beef extract, 0.2% yeast extract, 1.0% peptone, 0.2% NaCl, 1.0% soluble starch and then adjusted to pH 7.2 with 1 N NaOH. For solid medium 1.5% agar was added.

2.3.2 Cultivation medium (Horikoshi's medium)

Medium for enzyme production, slightly modified from Horikoshi (1971) and (Rutchtorn, 1993), contained 1.0% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4\ 7H_2O$, 0.75% Na_2CO_3. The pH of the medium was 10.1-10.2. For solid medium 1.5% agar was added. If for screening purpose, 0.03% phenolphthalein and 0.01% methyl orange were added before solidification.

2.4 Cultivation of bacteria

2.4.1 Starter inoculum

The b**acteria** was streaked on solid Medium I, and incubated for 18 hours at 37°C. Then one loop was put into liquid Medium I at 37°C and grown until A_{660} reached 0.3-0.5.

2.4.2 Enzyme production

For activity checked during the screening step, starter inoculum (1.0%) was transferred into 200 ml Horikoshi's broth in 500 ml Erlenmeyer flask. Cultivation

was at 37^{o}C for 3 days. For optimization experiment, the amount of Horikoshi's broth was 300 ml in 1 liter Erlenmeyer flask. Optimum condition was determined by varying pH, temperature, type and concentration of inducers. After cultivation cells were harvested by centrifugation at 3,000 x g for 30 minutes at 4^{o}C. Culture broth with crude enzyme was collected and kept at 4^{o}C for activity assay and protein content determination.

2.5 Enzyme assay

CGTase activity was determined by assay of starch degrading (dextrinizing) activity and assay of CD product by formation of CD-trichloroethylene complex (CD-TCE).

2.5.1 Dextrinizing activity assay (Iodine Method)

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

Enzyme sample (10-100 μl) was incubated with 0.3 ml starch substrate (0.2 g% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40^{o}C for 10 minutes. The reaction was stopped with 4 ml of 0.2 N 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 control tube, HCl was added before the enzyme sample.

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

2.5.2 Cyclodextrin-Trichloroethylene (CD-TCE) assay

Cyclodextrin-Trichloroethylene (CD-TCE) assay was determined by the method of Nomoto *et al* (1986) with slight modification (Rojtinnakorn, 1994).

Enzyme sample was diluted in serial double dilution by 0.2 M phosphate buffer, pH 6.0. The reaction mixture, containing 0.5 ml of enzyme sample and 2.5 ml of starch substrate (2.0 g% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) was incubated at 40^{o}C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark.

The activity was expressed in terms of the dilution limit ($1:2^{n}$), as the highest dilution that can produce observable CD-TCE precipitate lining between upper starch solution layer and lower TCE layer.

2.5.3 Protein determination

Protein concentration was determined by the Coomassie blue standard method according to Bradford (1976), using bovine serum albumin as standard.(Appendix C)

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm.

2.6 Screening of thermotolerant bacteria producing CGTase

Soil and water samples were collected from hot spring areas in various parts of Thailand. (Northern Part : Chiangmai, Chiangrai, Maehongson, Lumpang,

and Tak provinces; Central Part : Ratchaburi provinces; and Southern Part : Ranong and Krabi provinces). Bacterial screening were as follows.

2.6.1 Primary screening

Suspended 0.5 g soil sample in 5 ml of distilled water. Ten fold serial dilutions (10^{-2} to 10^{-6}) were made and 100 μl of each dilution was spreaded onto an agar plate containing Medium I (water samples were directly spreaded in the same amount on to agar plate). Incubation was made at 37°C for 2 days. Colonies producing amylase were detected by observing clear zone around colonies after pouring iodine reagent (0.02% I_2 in 0.2% KI) on the plate. The ratios of clear zone : colony size were recorded.

2.6.2 Secondary screening

Streaked the isolates that produced clear zone from primary screening onto Medium I agar plate and incubated at 37°C. Selected single colony and cultured on agar plate of Horikoshi medium to which dyes system (0.03% phenolphthalein and 0.01% methyl orange) to follow CGTase activity was added (Yim *et al*, 1997). Incubation was carried out at 37°C for 3 days and colonies with yellowish-orange clear zone on an intense pink background, which were those exhibiting the property of CGTase-producing were recorded.

CGTase producing activity was confirmed by assay of CD product by formation of CD-trichloroethylene complex (CD-TCE) as described in 2.5.2.

2.6.3 Selection for thermotolerant isolate with high CGTase activity

Cultured isolates with positive result from secondary screening in modified Horikoshi liquid medium at the temperature range of 30-55°C. Growth and

CGTase production were measured by the methods of Fuwa and Nomoto every 12 hours of culturing time. Selected the best isolate, which produced highest CGTase activity at or above 40^{o}C.

2.7 Identification of selected bacterial strain

2.7.1 Morphological characterization

The morphology of bacteria was examined by Scanning Electron Microscopy (SEM) (JSM-35CF), using cells from 24 hours culture. The cells were grown on Medium I agar, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at 25^{o}C for 2 hours. After being washed by the same buffer, the samples were treated with 1% osmium acid in the buffer and then dehydrated by using an ethanol series (35, 70, 95 and 100%). After being freeze-dried, the sample was coated with gold (JFC-1100).

2.7.2 Biochemical characterization

Identification was also conducted based on the classification systems presented in Bergey's manual of systematic bacteriology: Gram staining, growth and acid or color production in different sugar medium, and certain enzyme assays were performed. Bacterial strain was sent to analyze at the Thailand Institute of Scientific and Technological Research.

2.7.3 Identification by 16S rRNA gene fragment amplification

2.7.3.1 Extraction of Chromosomal DNA from RB01

Chromosomal DNA of selected bacterial strain was extracted by CTAB method (Sambrook, 1989). RB01 was grown on LB broth at 37^{o}C with shaking

at 250 rpm for 18 hours. Cells were harvested in 1.5 ml microcentrifuge tubes by centrifugation at 5,000 x g for 5 minutes. Cell pellets were resuspended in 567 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) by repeated pipetting. Then, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added and mixed well. The mixture was incubated at 37°C for one hour. One hundred µl of 5 M NaCl was added into the mixture and mixed throughly. After that, 80 µl of CTAB-NaCl solution was added and mixed. The mixture was further incubated at 65°C for 10 minutes. Then, equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed. The mixture was centrifuged at 10,000 x g for 10 minutes. The supernatant was transferred to another tube and added with equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). After mixing by inversion, the mixture was centrifuged at 10,000 x g for 10 minutes. The upper aqueous phase was removed and precipitated by adding 0.6 volume of isopropanol. After gently inversion, fibrous strands of DNA were spooled out and dipped in 1 ml of 70% ethanol to remove excess salt. The DNA was allowed to air-dry and resuspended by 100 µl of TE buffer. The DNA was checked by agarose gel electrophoresis and absorbance ratio of $A_{260/280}$.

2.7.3.2 16S rRNA gene fragment amplification

One µg of bacterial DNA was subjected to PCR in total volume of 100 µl, with 2.5 units of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$, 100 picomoles of forward primers (pA: AGA GTT TGA TCC TGG CTC AG), 100 picomoles of reverse primers (pH: AAG GAG GTG ATC CAG CCG CA) and 200 µM dNTP (Edwards *et al*, 1989). The thermal profile involved 30 cycles of denaturation at 95°C for 1 minutes, primer annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes (Appendix G).

PCR product was checked by agarose gel electrophoresis and was sent for sequencing at the National Science and Technology Development Agency (NSTDA). Sequencing primers were pA: AGA GTT TGA TCC TGG CTC AG, pD: CAG CAG CCG CGG TAA TAA TAC and pF: CAT GGC TGT CGT CAG CTC GT (Edwards *et al*, 1989).

The 16S rRNA sequence was aligned by using Clustal W software. Reference sequences were obtained from the GenBank database (Altschul *et al*, 1997). Percent homology obtained from comparison of the data with the reference base sequences of 16S rRNA gene of different bacterial strains leads to indication of the strain.

2.8 Optimization of conditions for culturing and CGTase production

Determination of suitable conditions for culturing isolates with high CGTase activity was performed. Variable parameters were: the type and concentration of enzyme inducer, medium pH, temperature, and culturing time. The best condition which gave maximum growth and enzyme production was selected for further study.

2.8.1 Optimum temperature for growth and CGTase production

Starter inoculum (1.0%) of RB01 was transferred into 300 ml of Horikoshi's broth in 1 liter Erlenmeyer flask cultivated at the temperature range of 30-45^{o}C. Growth and CGTase production were measured by the methods as described in 2.5.1, 2.5.2 and 2.5.3 every 12 hours of culturing time.

2.8.2 Optimum pH for growth and CGTase production

Strain RB01 was cultivated in Horikoshi medium of various pHs (adjusted pH to 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 by HCl or NaOH) at optimum temperature from 2.8.1, 250 rpm for 72 hours. Sample was withdrawn every 12 hours for measuring growth and enzyme activity.

2.8.3 Effect of various types of starch inducer on CGTase production

Cornstarch, cassava starch, rice starch and glutinous rice starch were replaced soluble starch in Horikoshi medium. RB01 was cultured in Horikoshi liquid medium that contained each type of starch at optimum temperature and pH from 2.8.1 and 2.8.2, 250 rpm. Growth and CGTase production were measured every 12 hours of culturing time.

2.8.4 Effect of concentration of starch inducer on CGTase production

The optimum concentration of the suitable starch type was also examined. Horikoshi liquid medium were added with suitable starch type at the concentrations varying from 0.5% to 3.0%. Condition for culturing was optimum temperature and pH from 2.8.1 and 2.8.2, 250 rpm. Sample was withdrawn every 12 hours for measuring growth and enzyme activity.

2.9 Partial purification of CGTase

CGTase was partially purified from the culture broth of RB01 by starch adsorption method of Kato and Horikoshi (1984) with slight modification (Rutchtorn, 1993). Cornstarch was oven dried at 100^{o}C for 30 minutes and cooled to room temperature. All subsequent steps were conducted at 4^{o}C. Cornstarch was then

gradually sprinkled onto stirring crude enzyme broth to make 5 g% concentration for 3 hours. The corn starch cake which adsorbed the enzyme was collected by centrifugation at 3,000 x g for 30 minutes and washed twice with 200 ml of 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake by stirring for 30 minutes with 125 ml of TB1 buffer containing 0.2 M maltose twice. CGTase was recovered from the supernatant by centrifugation at 3,000 x g for 30 minutes. Purity of the enzyme was checked by polyacrylamide gel electrophoresis. Protein and activity staining were compared. The partial purified enzyme was again checked for enzyme activity and protein content. The enzyme was kept at 4^oC for further study.

2.10 Biochemical characterization of the enzyme

Partially purified enzyme was characterized for its biochemical properties. Molecular weight was checked by SDS-polyacrylamide gel electrophoresis. Optimum pH and temperature for activity and stability were studied.

2.10.1 Polyacrylamide gel electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gel, were employed for analysis of the partially purified enzyme.

2.10.1.1 Types of PAGE

- **Non-denaturing polyacrylamide gel electrophoresis**

Discontinuous PAGE was performed as described by Rojtinnakorn (1994) on slab gel (10 x 8 x 7.5 cm) of 7.5% (w/v) separating gel, and 5.0% (w/v) stacking gel. Tris-glycine buffer, pH 8.3 was used as electrode buffer (Appendix A).

The electrophoresis was run from cathode towards anode, at constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit.

- **SDS-PAGE**

The denaturing gel was performed according to Bollag and Edelstein (1991). The gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating gel, and 5.0% (w/v) stacking gel and Tris-glycine buffer, pH 8.3 containing 0.1% SDS was used as electrode buffer (Appendix A). Samples to be analyzed were treated with sample buffer (Appendix A) 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 in a Mini-Gel Electrophoresis unit from cathode towards anode.

2.10.1.2 Detection of protein

After electrophoresis, protein in the gel was visualized by Coomassie blue staining. For non-denaturing gel, dextrinizing activity staining was also undertaken.

- **Coomassie blue staining**

Gels were stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (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 until gel background was clear.

- **Dextrinizing activity staining** (slightly modified from the method of Kobayashi *et al*, 1978)

The running gel was soaked in 10 ml of 2.0% substrate solution (2.0% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0), incubated at 40°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 2.0% KI) was added for color development at room temperature. The clear zone on the blue background represents starch-degrading activity of the protein.

2.10.2 Determination of cyclodextrin by High Performance Liquid Chromatography

The analysis of cyclodextrin by HPLC was performed as described by Ruchtorn (1993). The HPLC system was a Shimadzu LC-3A equipped with Spherisorb-NH_2 column (0.46 x 25 cm) and using Shimadzu RID-3A refractometer as detector. For CD analysis, the reaction was performed by incubating 0.5 ml of enzyme sample with 2.5 ml of starch substrate (2.0 g% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40°C for 24 hours. The reaction was stopped by boiling in water for 5 minutes. After cooling, the mixture was treated with 20 units of β-amylase at 25°C for an hour, and the reaction was stopped by heating in boiling water. Prior to injection, the mixture was filtered through a 0.45 μm membrane filter. The mixture was injected and eluted with acetronitrile-water (65:35, v/v) using a flow rate of 2 ml/min. The CD peak was identified by comparing the retention time with that of standard CDs, which was composed of α-, β- and γ- CD mixture (20 mg/ml). For quantitative analysis, peak area corresponded with each cyclodextrin was determined from standard curve. (Appendices D-F)

2.10.3 Optimum conditions for enzyme activity

2.10.3.1 Temperature

CGTase was incubated with 0.2% soluble starch in phosphate buffer, pH 6.0 at various temperatures from 0-100^{o}C, for 10 minutes for the dextrinizing activity. Detail procedure was as described in 2.5.1. For CD-forming activity, experiment was performed as mentioned in 2.5.2 except incubation temperature was varied from 30-70^{o}C.

2.10.3.2 pH

To determine the optimum pH for the dextrinizing activity and CD-forming activity of CGTase, the reactions were as described in 2.5.1 and 2.5.2 except that pH of the reaction was varied between 3.0-11.0. The 0.2 M buffer solutions of various pH ranging from 3.0 to 11.0 and 5.0-11.0 were used for dextrinizing activity and CD-forming activity, respectively. The buffer solutions used were potassium acetate (pH 3.0, 4.0 and 5.0), phosphate (pH 5.0, 6.0 and 7.0), Tris-HCl (pH 7.0, 8.0 and 9.0) and Tris-Glycine NaOH (pH 9.0, 10.0 and 11.0). (Appendix B)

2.10.3.3 Incubation time

The incubation time of dextrinizing activity was varied from 0 to 30 minutes and the assay was as described in 2.5.1 and 2.5.2.

2.10.4 Enzyme stability

All enzyme used in this experiment was 0.7 mg protein/ml.

2.10.4.1 Effect of temperature

Effect of temperature on the stability of enzyme was observed by incubating the enzyme at different temperatures in the range of 40°C to 55°C, for 60 minutes. The enzyme activity was assayed at the optimal conditions obtained from 2.10.3.

2.10.4.2 Effect of pH

The stability of enzyme at different pHs was investigated by incubating enzyme in various buffers of pH 6.0-9.0 at the temperature which enzyme gave high activity (obtained from 2.10.3.2) for 60 minutes (buffers used were the same as in 2.10.3.2). The enzyme activity was assayed at the optimal conditions obtained from 2.10.3.

2.10.4.3 Effect of $CaCl_2$

The effect of $CaCl_2$ on enzyme stability was studied by incubating the enzyme samples in the presence of final concentrations of 10 and 20 mM $CaCl_2$ at 55°C for 60 minutes. The enzyme activities were assayed at the optimal conditions obtained from 2.10.3.

2.10.4.4 Effect of substrate

The effect of substrate on enzyme stability was also studied by incubating the enzyme sample in the presence of final concentration of 0.2% soluble starch (potato) at 55°C for 60 minutes. The enzyme activity was assayed at the optimal conditions obtained from 2.10.3.

2.10.5 Conditions for storage enzyme

CGTase (0.7 mg protein/ml in 10 mM Tris-HCl buffer, pH 8.5) was stored at 4^{o}C and -20^{o}C for 5 weeks. Enzyme was withdrawn for measuring dextrinizing activity at the suitable time points. The effect of 10 mM $CaCl_2$ on enzyme stability was also studied by adding them into the enzyme sample before storage.

CHAPTER III

RESULTS

3.1 Screening of thermotolerant bacteria producing CGTase

3.1.1 Primary screening

The screening for extracellular starch-degrading microorganisms, from hot spring areas in various parts of Thailand, was conducted on Medium I plate containing soluble starch. About 38 amylase-producing isolates from 57 soil and water samples exhibited large halo zone and are shown in Table 7. Figure 4A shows the typical clear zone formed on starch agar plate after 2 days of incubation at 37^{o}C and made better visualized by staining with I_2 in KI. Among 38 isolates, 5 isolates gave very high starch degrading activity with the ratio of clear zone to colony size b/a of $\geq$ 3.0. Sixteen isolates gave moderate activity with 2.0 $\leq$ b/a < 3.0, while 17 isolates produced low activity with 1.0 $\leq$ b/a < 2.0.

3.1.2 Secondary screening

These clear zone forming colonies from primary screening were checked for CGTase producing ability by inoculated on Horikoshi medium plate supplemented with 0.03% phenolphthalein and 0.01% methyl orange following the method described by Park *et al* (1989). By this screening medium, bacteria that produce CGTase showed yellowish orange clear zone on an intense pink background (Figure 4B). From this step, two bacterial strains (isolates number 10 and 15 in Table 7), were named RB01 and KB01, exhibited CGTase activity. They were both screened from hot spring areas in Ratchaburi and Krabi province, respectively.

CGTase producing activity was confirmed by CD-TCE assay of the culture filtrate by the method described in 2.5.2. Maximum CD-forming activity of RB01 and KB01 were 2^6 and 2^5, respectively.

3.1.3 Selection for thermotolerant isolate with high CGTase activity

$RB0_1$ and KB_{01} were checked for growth and enzyme activity in Horikoshi liquid culture, in the temperature range of 30-55°C for selection of the best thermotolerant strain. KB_{01} was grown and able to produce CGTase but lower than RB_{01} in the same condition and KB01 was not stable after subcultured for many times. RB01 could grow and produce CGTase at temperature range of 30-45°C, with optimum growth best at 37°C but produced highest CGTase activity at 40°C (Figure 5). Thus, RB01 had thermotolerant characteristic, it could grow and produce CGTase at rather broad mesophilic temperature range (30-45°C).

Table 7 Isolates with amylase activity screening from hot spring areas in different parts of Thailand.

Central Thailand

Site	Isolate number	Diameter (cm)		(b)/(a)
		colony (a)	clear zone (b)	
KANCHANABURI (Hindad)	1	0.4	0.8	2.0
	2	1.1	2.0	1.8
	3	0.6	1.2	2.0
	4	0.9	1.3	1.4
	5	0.9	1.8	2.0
RATCHABURI (Bo-kloeng)	6	1.0	2.0	2.0
	7	0.7	0.8	1.1
	8	0.6	1.4	2.3
	9	0.7	1.4	2.0
	10*	0.6	0.8	1.3
(Pongkrating)	11	1.0	1.3	1.3
	12	1.2	1.8	1.5
	13	1.0	1.4	1.4
	14	0.7	1.6	2.3

Southern Thailand

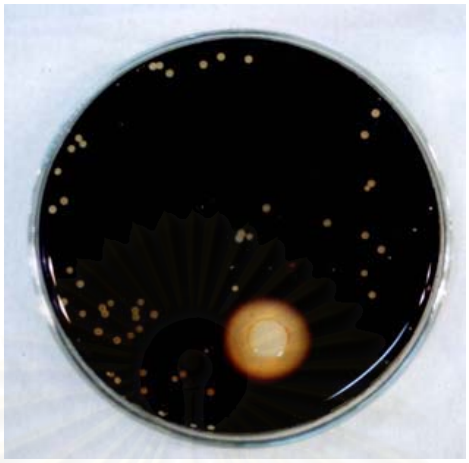
Site	Isolate number	Diameter (cm)		(b)/(a)
		colony (a)	clear zone (b)	
KRABI	15*	0.5	1.4	2.8
	16	0.5	1.7	3.4
	17	0.6	1.5	2.5
RANONG	18	1.1	1.6	1.5
	19	1.2	1.5	1.3

* indicates isolate with Amylase-CGTase activity.

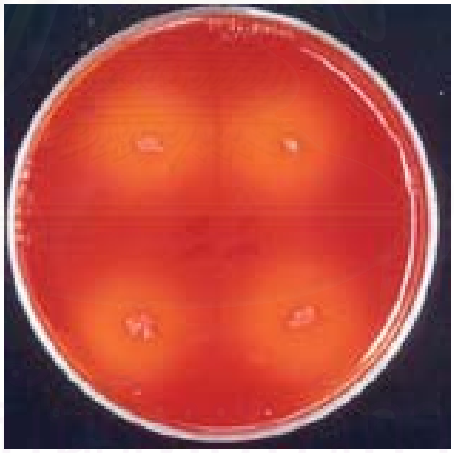
Table 7 Continue

Northen Thailand

Site	Isolate number	Diameter (cm)		(b)/(a)
		colony (a)	clear zone (b)	
CHIANG MAI (Sankampang)	20	0.3	2.3	7.7
	21	0.5	0.7	1.4
	22	0.9	1.5	1.7
(Pong-daod)	23	0.9	1.5	1.7
	24	1.3	2.2	1.7
	25	1.0	1.6	1.6
CHIANG RAI (Banpongnamron)	26	1.0	2.0	2.0
	27	0.2	0.5	2.5
	28	0.5	1.2	2.4
	29	0.3	0.7	2.3
(Thamachat)	30	0.4	0.9	2.5
	31	0.4	1.2	3.0
	32	1.0	2.0	2.0
	33	0.7	1.3	1.9
	34	0.3	1.5	5.0
(Huaysai)	35	0.3	0.5	1.7
(Toongtaewee)	36	1.1	1.7	1.5
	37	0.6	1.4	2.3
MAE HONG SON (Pongnamdaod)	38	0.2	1.0	5.0



A



B

Figure 4 Colonies producing amylase formed clear zone on blue background: (A) and colonies producing CGTase formed yellow color clear zone on red background: (B).

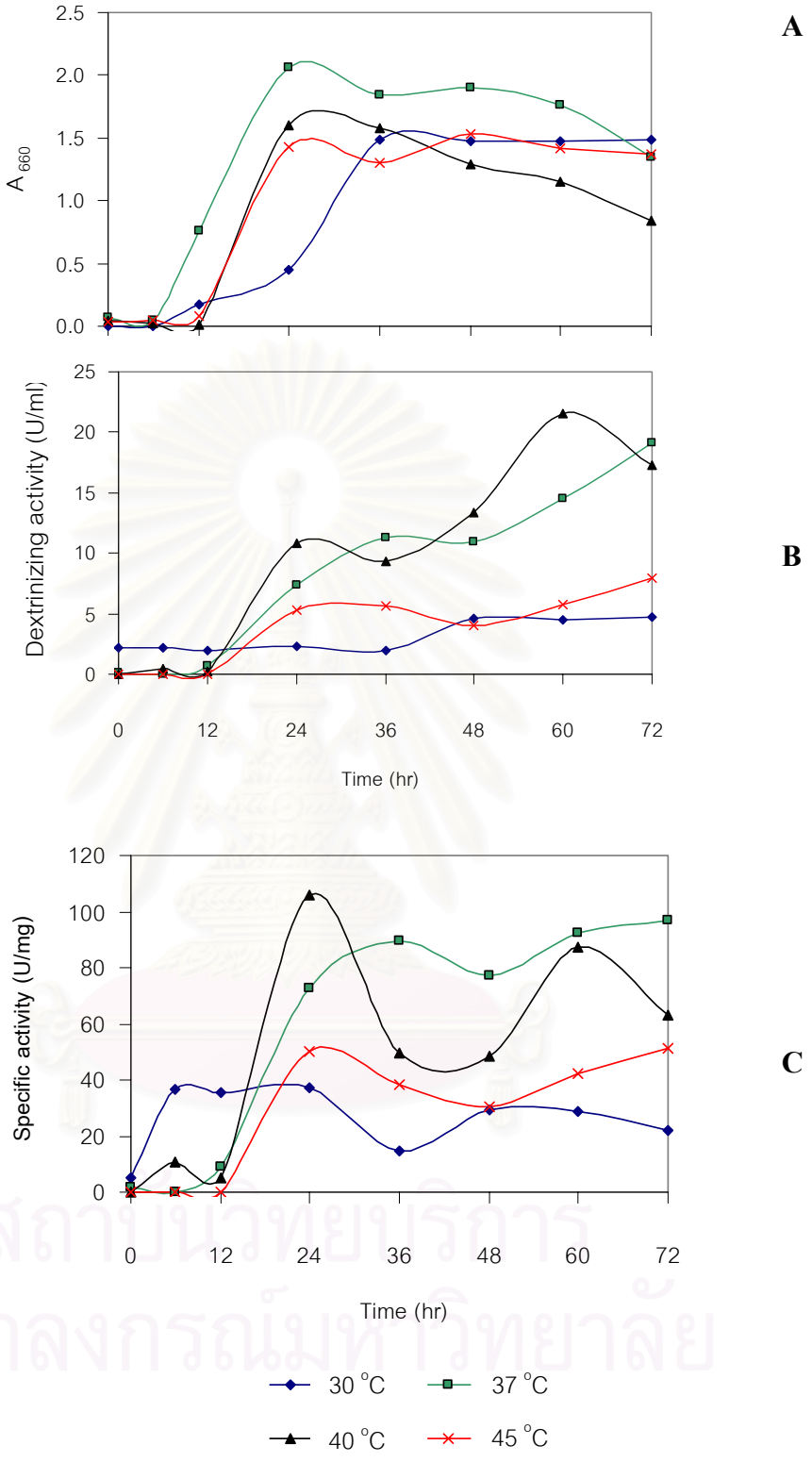


Figure 5 Growth profile: (A). CGTase production, dext ıctivity: (B) and specific activity: (C) of RB01 at various temperatures between 30- 45°C.

3.2. Identification of selected bacterial strain

3.2.1 Morphological characterization

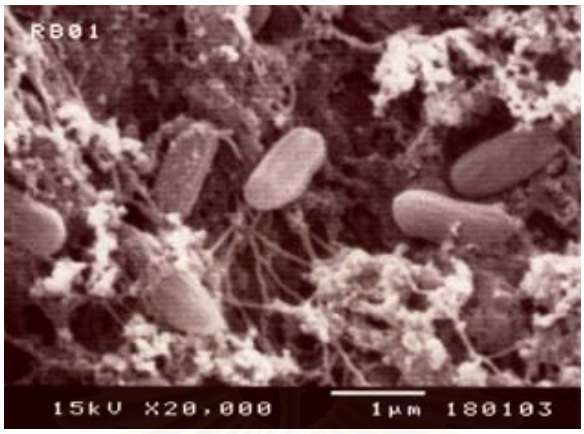
From scanning electron microscopy, RB01 cells were rod-shaped. The size was approximately 0.3 x 1.0 μm (Figure 6A). Colonies on Medium I agar plate had white color, flat, irregular and spreading (Figure 6B)

3.2.2 Biochemical characterization

RB01 was Gram-positive. Biochemical characteristics determined by fermentation of different sugars were tested and listed in Table 8. When compared the result with Bergey's manual systematic identification, RB01 was identified as *Bacillus circulans*.

3.2.3 Identification by 16S rRNA gene fragment amplification

Chromosomal DNA of RB01 was checked by agarose gel electrophoresis and spectrophotometry ($A_{260/280}$). The absorbance ratio was 1.95. DNA obtained was larger than 23.1 kb. From the gel pattern and the absorbance ratio, the extracted DNA was good enough for amplification. After amplification, PCR products of 1.5 kb was obtained and shown in Figure 7. The 16S rRNA gene sequence was run by 3 primers (pA, pD and pF as described in 2.7.3.2). Each primer gave more than 500 bp. All sequences were extended and searched for overlapping region. The resulted sequence was 1508 bp as shown in Figure 8. The alignment of this 16S rRNA gene of RB01 was blasted with those deposited in EMBL/Gen Bank database. The result indicated that RB01 showed 99% homology with *Paenibacillus* sp. strain 324 (*Paenibacillus campinasensis*) (Figure 9).



A

B

Figure 6 Morphology of RB01. Scanning electron micrograph (SEM): (A).

On Medium I at 40 hours of incubation: (B).

Table 8 Biochemical characteristics of the bacteria strain RB01

Characteristics	Reaction	Characteristics	Reaction
Gram reaction	+	**Fermentative production of acid from**	
Fermentative production of acid from		salicine	+
Glycerol	-	cellobiose	+
Erythritol	-	maltose	+
D-arabinose	+	lactose	+
L-arabinose	+	melibiose	+
Ribose	+	sucrose	+
D-xylose	+	trehalose	+
L-xylose	-	inuline	-
Adonitol	-	melezitose	-
β-methyl –D-xylose	+	D-raffinose	+
Galactose	+	starch	+
D-glucose	+	glycogen	+
D-fructose	+	xylitol	+
D-mannose	+	β-gentiobiose	+
L-sorbose	-	D-turanose	+
Rhamnose	-	D-lyxose	-
Dulcitol	-	D-tagatose	-
Inositol	-	D-fucose	-
Mannital	+	L-fucose	+
Sorbitol	+	D-arabitol	+
α-methyl-D-mannoside	+	L-arabitol	-
α-methyl-D-glucoside	+	gluconate	+
N-acetyl-glucosamine	+	2-keto-gluconate	-
Amygdaline	+	5-keto-gluconate	+
Arbutine	+	esculine	+

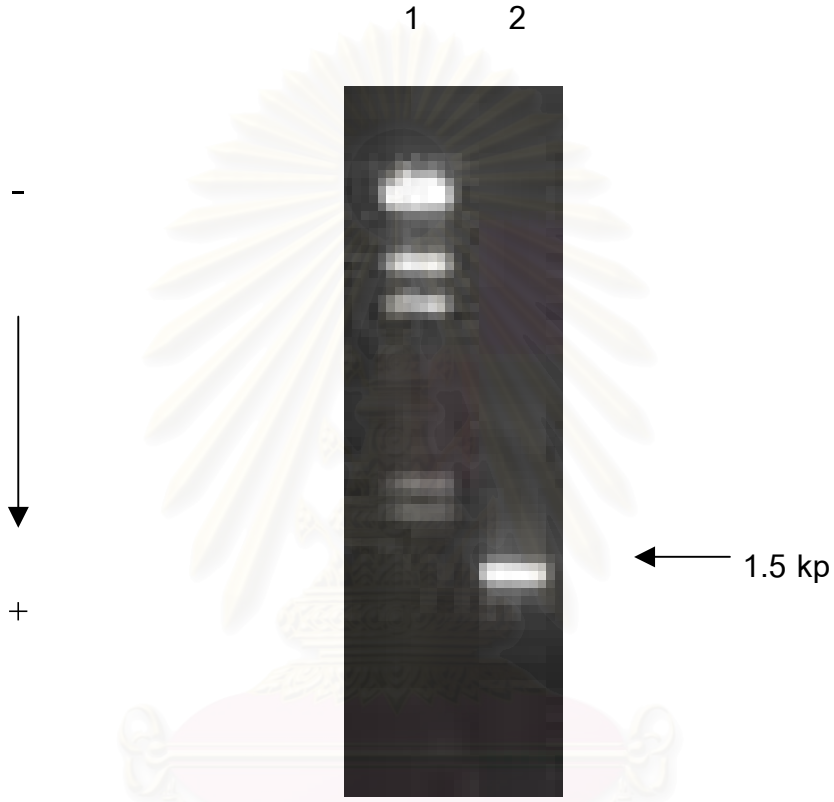


Figure 7 PCR product 16S rRNA gene of RB01.

Lane 1 Standard DNA marker (λ/*Hind* III)

Lane 2 the amplified product of 16S rRNA gene

AGAGTTTGAT CCTGGCTCAG----------

GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC GGAATCGATG GAGTGCTTGC 60

ACTCCTGAGA TTTAGCGGCG GACGGGTGAG TAACACGTAG GCAACCTGCC CTCAAGACTG 120

GGATAACTAC CGGAAACGGT AGCTAATACC AGATAGGATA TTTGGCTGCA TGGCCGNATA 180

TGGAAAGGCG GAGCAATCTG TCACTTGAGG ATGGGCCTGC GGCGCATTAG CTAGTTGGTG 240

GGGTAATGGC CTACCAAGGC GACGATGCGT AGCCGACCTG AGAGGGTGAA CGGCCACACT 300

GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG TAGGGAATCT TCCGCAATGG 360

ACGAAAGTCT GACGGAGCAA CGCCGCGTGA GTGATGAAGG TTTTCGGATC GTAAAGCTCT 420

GTTGCCAGGG AAGAACGCCA GANAGAGTAA CTGCTCTTTG GGTGACGGTA CCTGAAAANA 480

AAGCCCCGGC TAACTACNTG CCAGCAGCCG CGGTAATAAT ACGGGGGCAA GCGTTGTCCG 540

GAATTATTGG GCGTAAAGCG CGCGCAGGCG GTTCTTTAAG TCTGGTGTTT AAACCCGGAG 600

CTTAACTTCG GGACGCACTG GAAACTGGGG AACTTGAGTG CAGAAGAGGA GAGTGGAATT 660

CCACGTGTAG CGGTGAAATG CGTAGATATG TGGAGGAACA CCAGTGGCGA AGGCGACTCT 720

CTGGGCTGTA ACTGACGCTG AGGCGCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT 780

GGTAGTCCAC GCCGTAAACG ATGAATGCTA GGTGTTAGGG GTTTCGATAC CCTTGGTGCC 840

GAAGTTAACA CATTAAGCAT TCCGCCTGGG GAGTACGGTC GCAAGACTGA AACTCAAAGG 900

AATTGACGGG GACCCGCACA AGCAGTGGAG TATGTGGTTT AATTCGAAGC AACGCGAAGA 960

ACCTTACCAG GTCTTGACAT CCCTNTGACC CCTCTAGAGA TAGAGGTTTC CTTCGGGACA 1020

GAGGANACAG GTGGTGCATG GCTGTCGTCA GCTCGTGTCG TGAGATGTTG GGTTAAGTCC 1080

CGCAACGAGC GCAACCCTTG ATCTTAGTTG CCAGCACGTA ATGGTGGGCA CTCTAAGGTG 1140

ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AATCATCATG CCCCTTATGA 1120

CCTGGGCTAC ACACGTACTA CAATGGCTGG TACAACGGGA AGCGAAGCCG CGAGGTGGAG 1260

CCAATCCTAA AAAGCCAGTC TCAGTTCGGA TTGCAGGCTG CAACTCGCCT GCATGAAGTC 1320

GGAATTGCTA GTAATCGCGG ATCAGCATGC CGCGGTGAAT ACGTTCCCGG GTCTTGTACA 1380

CACCGCCCGT CACACCACGA GAGTTTACAA CACCCGAAGT CGGTGGGGTA ACCGCAAGGA 1440

GCCAGCCGCC GAAGGTGGGG TAGATGATTG GGGTGAAGTC GTAACAAGGT AGCCGTATCG 1500

GAAGGTGC 1508

pA	5’	AGAGTTTGATCCTGGCTCAG	3’
pD	5’	CAGCAGCCGCGGTAATAATAC	3’
pF	5’	CATGGCTGTCGTCAGCTCGT	3’

Figure 8 Nucleotide sequence of the 16S rRNA gene of RB01. The primers used for sequencing are shown.

```
RB01   GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGC  60
324    GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGAATCGATGGAGTGCTTGC  60
       ************************************************************

RB01   ACTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTG 120
324    ACTCCTGAGATTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCTCAAGACTG 120
       ************************************************************

RB01   GGATAACTACCGGAAACGGTAGCTAATACCAGATAGGATATTTGGCTGCATGGCCGNATA 180
324    GGATAACTACCGGAAACGGTAGCTAATACCRGATAGGATATTTGGCTGCATGGCYGAATA 180
       ***************************** ********************** * ***

RB01   TGGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTG 240
324    TGGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAGTTGGTG 240
       ************************************************************

RB01   GGGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACT 300
324    GGGTAATGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACT 300
       ************************************************************

RB01   GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 360
324    GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG 360
       ************************************************************

RB01   ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT 420
324    ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCT 420
       ************************************************************

RB01   GTTGCCAGGGAAGAACGCCAGANAGAGTAACTGCTCTTTGGGTGACGGTACCTGAAAANA 480
324    GTTGCCAGGGAAGAACGCCAGAGAGAGTAACTGCTCTTTGGGTGACGGTACCTGAGAAGA 480
       ******************** ****************************** ** *

RB01   AAGCCCCGGCTAACTACNTGCCANCAGCCGCGGTAATACCTAGGGGGCAAGCGTTGTCCG 540
324    AAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCG 540
       ***************** ***** ************** ********************

RB01   GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAG 600
324    GAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTGGTGTTTAAACCCGGAG 600
       ************************************************************
```

```
RB01   CTTAACTTCGGGACGCACTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT 660
324    CTTAACTTCGGGACGCACTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAGAGTGGAATT 660
       ************************** **************************

RB01   CCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCT 720
324    CCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCT 720
       ************************************************************

RB01   CTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
324    CTGGGCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT 780
       ************************************************************
```

Figure 9 Alignment of 16S ribosomal RNA sequence of RB01 with that of *Paenibacillus* sp. strain 324 (AF021924).

* denote identical base

```
RB01   GGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCC 840
324    GGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCC 840
       ************************************************************

RB01   GAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG 900
324    GAAGTTAACACATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG 900
       ************************************************************

RB01   AATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGA 960
324    AATTGACGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCGAAGA 960
       ************************************************************

RB01   ACCTTACCAGGTCTTGACATCCCTNTGACCCCTCTAGAGATAGAGGTTTCCTTCGGGACA 1020
324    ACCTTACCAGGTCTTGACATCCCTTTGACCCCTCTAGAGATAGAGGTTTCCTTCGGGACA 1020
       ********************** ******************************

RB01   GAGGANACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
324    GAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC 1080
       ***** ************************************************

RB01   CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGT-AATGGTGGGCACTCTAAGGT 1139
324    CGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACGTGAATGGTGGGCACTCTAAGGT 1140
       ************************************ ******************

RB01   GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG 1199
324    GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG 1200
       ************************************************************
```

```
RB01    ACCTGGGCTACACACGTACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGTGGA
1259 324
        ACCTGGGCTACACACGTACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGTGGA
1260
        ************************************************************

RB01    GCCAATCCTAAAAAGCCAGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGT 1319
324     GCCAATCCTAAAAAGCCAGTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGT 1320
        ************************************************************

RB01    CGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTAC 1379
324     CGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTAC 1380
        ************************************************************

RB01    ACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCGCAAGG
1439 324
        ACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGGGGTAACCGCAAGG
1440
        ************************************************************

RB01    AGCCAGCCGCCGAAGGTGGGGTAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATC
1499 324        AGCCAGCCGCCGAAGGTGGG-
TAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTATC 1499
        ********** ************************************

RB01    GGAAGGTGC 1508
324     GGAAGGTGC 1508
        *********
```

Figure 9 Continue.

3.3. Optimization of conditions for culturing and CGTase production

3.3.1 Optimum temperature

RB01 was cultivated in Horikoshi medium pH 10.0 at various temperatures between 30°C to 45°C. Samples were shaken at 250 rpm and withdrawn at every 12 hours for measuring growth and enzyme activity. It was found that RB01 could grow at temperatures between 30°C to 45°C. Highest growth was observed at

37°C. The cultures at temperature 37°C- 45°C reached stationary phase at 24 hours, while at 30°C lag phase was 12 hours longer (Figure 5A). For enzyme production, RB01 began to produce enzyme at 12 hours when grown at 37°C- 45°C. The highest dextrinizing activity was highest observed when cultivated at 40°C for 60 hours (Figure 5B). Specific activity was at 40°C, 24 hours (Figure 5C). Therefore, the suitable temperature for growth and enzyme production was 40°C.

3.3.2 Optimum pH

The effects of pH on bacterial growth and enzyme production were investigated. Strain RB01 was cultivated in Horikoshi medium of various pHs from 6.0 to 11.0 at 40°C, 250 rpm for 60 hours. Samples were withdrawn at every 12 hours for measuring growth and enzyme activity. It was found that RB01 could grow at pH 6.0-10.0, at pH 6.0 growth was less than at pH 7.0-9.0 but time for reaching stationary phase was shorter. At pH 10.0, growing was about the same as at pH 7.0-9.0 but longer lag phase was observed. It was clear that RB01 could not grow in Horikoshi medium, pH 11.0 (Figure 10A). Growth and enzyme productions were about the same at culturing pH of 7.0-9.0. Enzyme production at pH 6.0 was low while highest activity was observed at pH 10.0 (Figure 10B). From Figure 10C, specific activity at pH 10.0 was the highest and significantly different from other

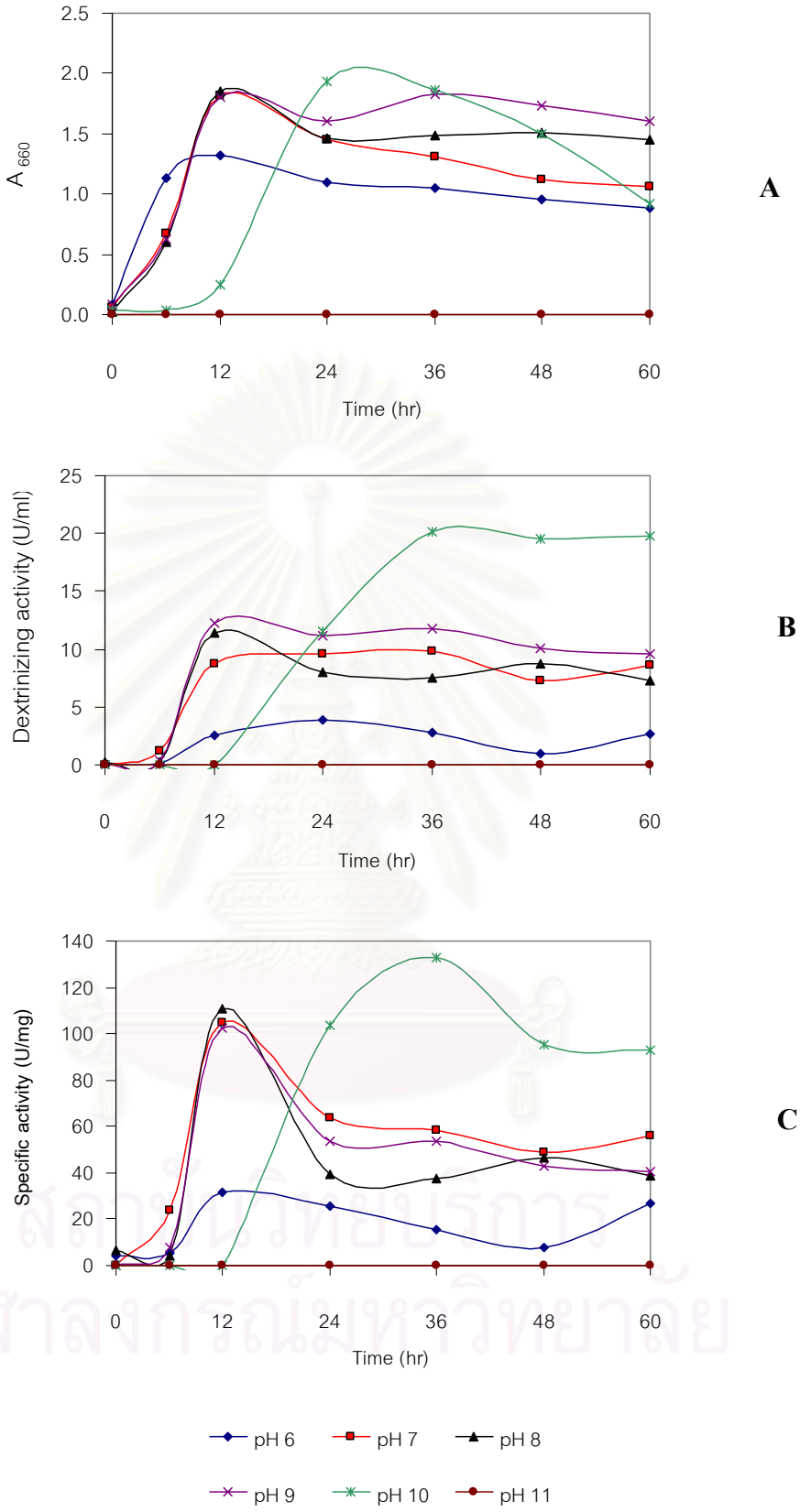


Figure 10 Growth profile: (A). CGTase production, dextrinizing activity: (B) and specific activity: (C) of RB01 at different pHs when temperature was fixed at 40^0C

pHs. Therefore, pH 10.0 was chosen as optimal pH for both cell growth and enzyme production.

3.3.3 Effect of various types of starch inducer

The effect of types of starch; cornstarch, cassava starch, rice starch and glutinous rice starch, on growth and CGTase production was investigated. The basic medium used was Horikoshi medium but soluble starch was replaced by other types of starch. RB01 was cultivated in Horikoshi medium pH 10.0 at 40^{o}C, 250 rpm for 60 hours. It was found that all types of starch investigated were consumed rapidly by RB01, while glutinous rice starch was an exception. Lag phase was longer but the same maximum cell growth was reached at 48 hours (Figure 11A). As demonstrated in Figure 11B, enzyme production when added rice starch, cornstarch and glutinous rice starch was not different. Soluble starch gave the highest enzyme production at 36-60 hours of culturing time. Soluble starch also gave the highest specific activity at 36 hours (Figure 11C).

3.3.4 Effect of concentration of starch inducer

The optimum concentration of soluble starch was investigated by varying concentration from 0.5% to 3.0%. The data in Figure 12A shows that at 1.5, 2.0, 2.5 and 3.0% of soluble starch in medium, RB01 exhibited the same level of growth. While 0.5% and 1.0% of soluble starch had the same growth pattern and enzyme production (Figures 12A and 12B). For enzyme production, 1.0% of soluble starch gave the highest dextrinizing activity at 60 hours (Figure 12B). Specific activity was highest at 1.5% of soluble starch after culturing for 36 hours. However, 1.0% soluble starch was chosen here as optimum condition since it gave highest activity and relatively high specific activity at 60 hours.

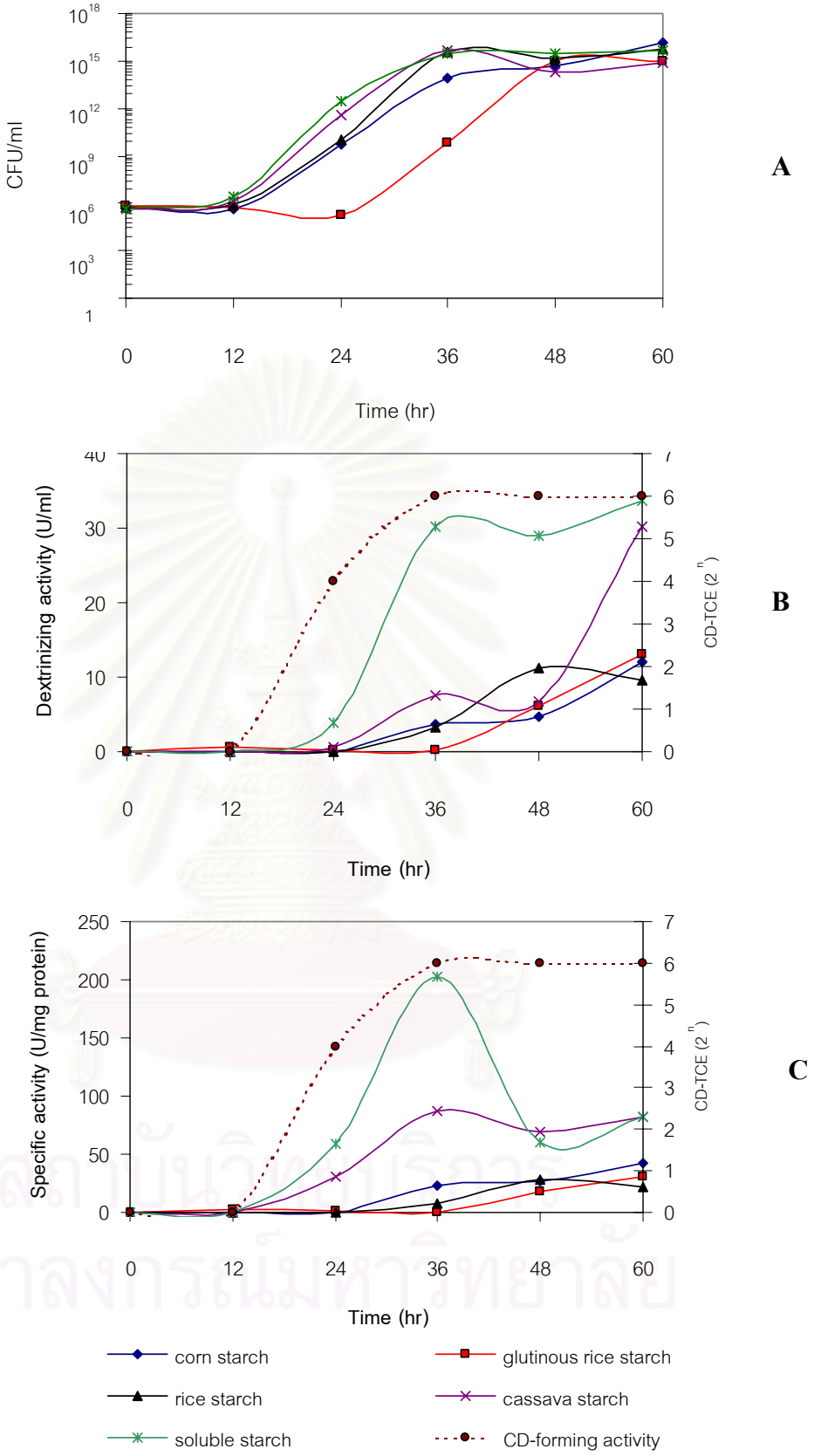


Figure 11 Growth profile: (A). CGTase production, dextrinizing activity: (B) and specific activity: (C) of RB01 in various types of starch at pH 10.0, 40^{0}C. CD-forming activity was measured when soluble starch was used as inducer.

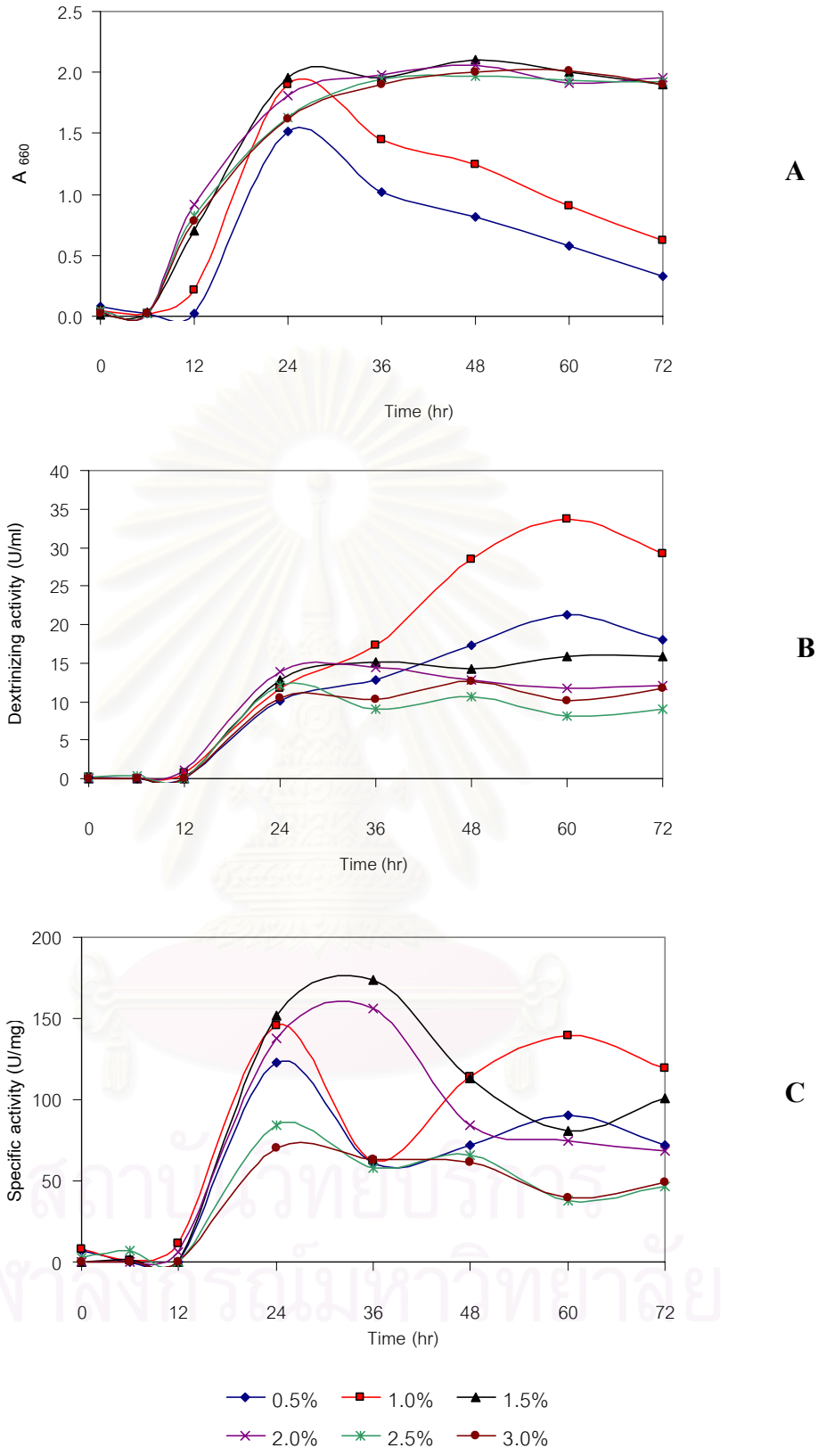


Figure 12 Growth profile: (A). CGTase production, dextrinizing activity: (B) and specific activity: (C) of RB01 at various concentrations of soluble starch, pH 10.0 and 40°C.

Therefore, the best condition for culturing RB01 for high production of CGTase enzyme were culturing the strain in Horikoshi medium with 1.0% soluble starch at pH 10.0, 40^{o}C for 60 hours.

3.4 Partial purification and Biochemical characterization of CGTase

3.4.1 Partial purification of CGTase from RB01.

RB01 was cultivated in Horikoshi medium pH 10.0 with 1.0% soluble starch, incubation at 40^{o}C, 250 rpm for 60 hours (from optimization conditions). The culture was centrifuged to remove cells and crude CGTase in the supernatant was collected. Crude enzyme was partially purified by starch adsorption as described in Methods section 2.9. The purification fold and recovery of CGTase obtained at each step are shown in Table 9. The % recovery of CGTase and purification fold were 57.3% and 26.8 respectively. Specific activity expressed in terms of dextrinizing activity per mg protein was increased though this step. These corresponded to the increased in the CD-product, which was determined by CD-TCE dilution limit. The final specific activity was 3,568 U/mg. The partially purified CGTase was then used for the study on some characteristics of the enzyme.

Crude CGTase showed many protein bands on native polyacrylamide gel (Figure 13A). A few were observed in the partially purified form, which corresponded with amylolytic activity bands. As presented in Figure 13B, this enzyme had 4 isoform patterns. The major form was the slowest moving band. The fastest moving band was hardly seen in the picture. The ratio of the amount of the four forms was approximately 10: 4: 1: 0.2. From protein bands on SDS-polyacrylamide gel (Figure 14), molecular weight of CGTase was estimated to be 65,000 (Figure 15).

Table 9 Purification of CGTase from RB01

Step	Volume (ml)	Dextrinizing activity (U/ml)	Total activity ($U \times 10^3$)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)	CD-TCE (2^n)
Crude enzyme	2500	53.3	133.8	1,000	133	1.0	100	2^6
Cornstarch adsorption	300	255.4	76.6	21.6	3,568	26.8	57.3	2^9

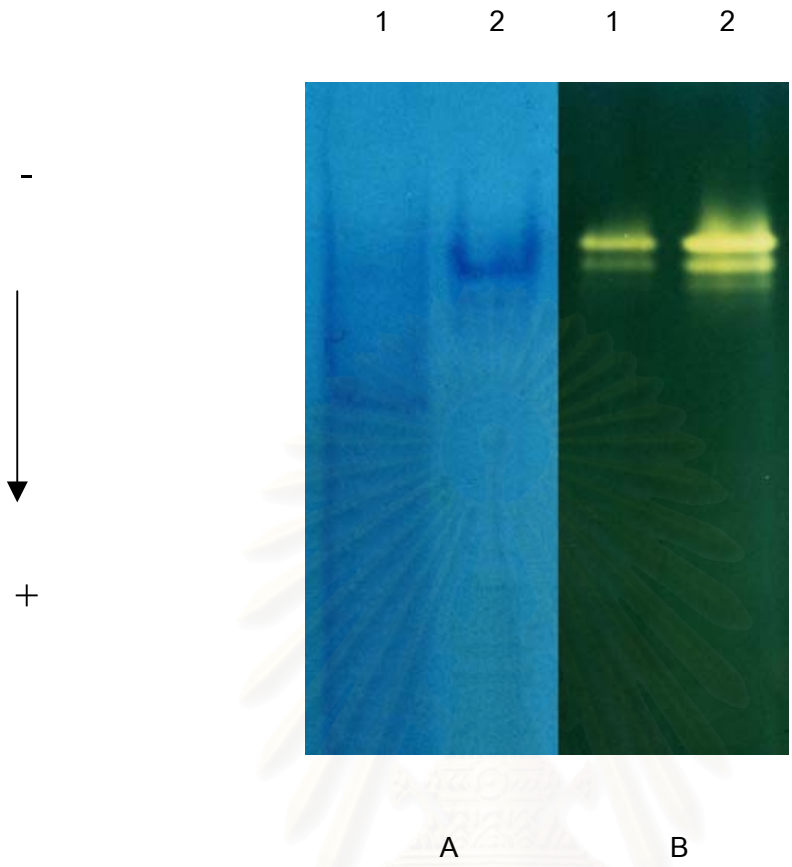


Figure 13 Non-denaturing PAGE of CGTase from different steps of purification.

A: Coomassie blue staining.

Lane 1 : Crude enzyme (45 μg)

Lane 2 : Concentrated starch adsorbed enzyme (20 μg)

B: Amylolytic activity staining by iodine solution.

Lane 1-2, as in A, 0.2 units of dextrinizing activity was loaded to each well

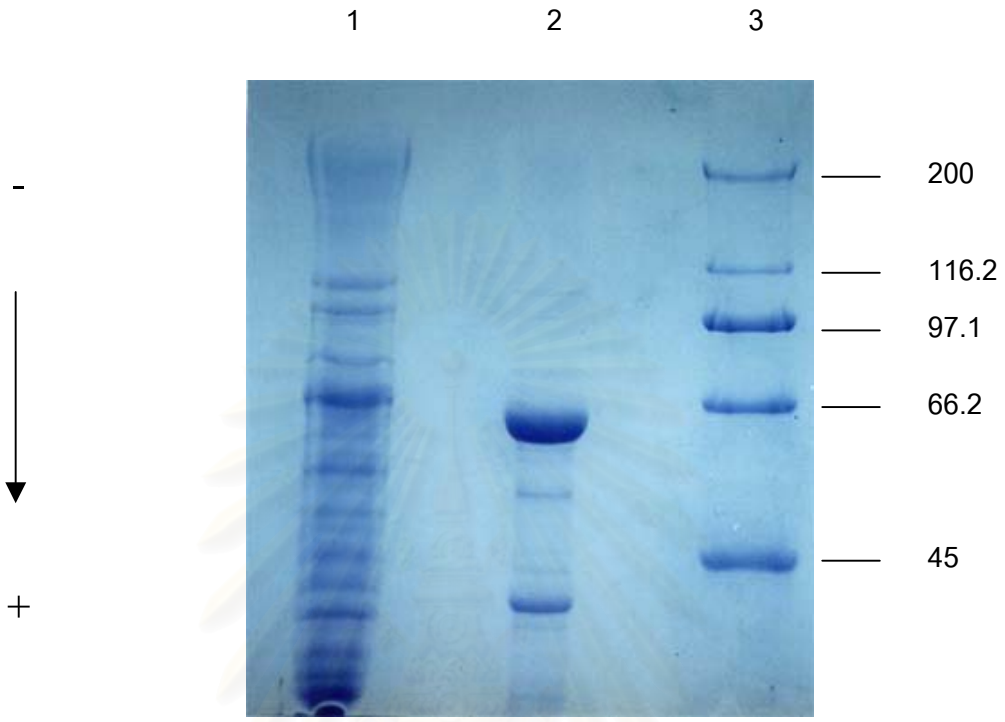


Figure 14 SDS-PAGE of CGTase from steps of partial purification.

Lane 1 : Crude enzyme (50 μg)

Lane 2 : Concentrated starch adsorbed enzyme (20 μg)

Lane 3 : Protein molecular weight markers.

{myosin (200), β-galactosidase (116.2),

phosphorylase b (97.1), BSA (66.2), and

ovalbumin (45)}

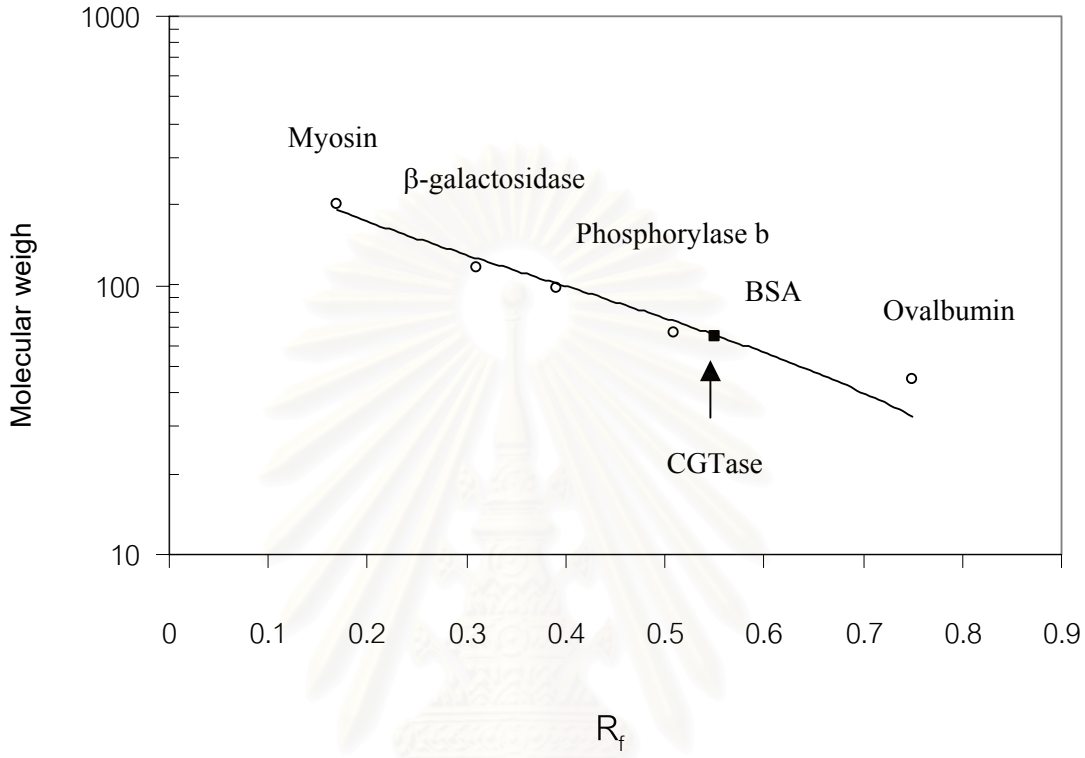


Figure 15 Molecular weight calibration curve of standard protein by SDS-PAGE

Myosin	MW	200
β-galactosidase	MW	116.2
Phosphorylase b	MW	97.1
BSA	MW	66.2
Ovalbumin	MW	45
CGTase	MW	65

3.4.2 Determination of cyclodextrin product by HPLC.

One ml of the reaction mixture containing 2.0% soluble starch in 0.2 M phosphate buffer (pH 6.0) was incubated with CGTase from RB01 at 40^{o}C. After 24 hours incubation, sample was transferred into boiling water-bath to stop the reaction, and the CDs formed were quantified by HPLC. Prior to HPLC, the sample was filtered through 0.45 μm membrane. A main product, β-CD and two minor products, α- and γ-CDs, were observed. The ratio of α : β : γ -CD calculated was 1.0 : 5.4 : 1.2 respectively (Figure 16).

3.4.3 Effect of temperature for enzyme activity and stability.

The effect of temperature on enzyme activity was investigated by incubating the enzyme with 0.2% soluble starch in phosphate buffer, pH 6.0 at various temperatures from 0^{o}C to 100^{o}C, for 10 minutes for the dextrinizing activity. For CD-forming activity, the enzyme was incubated with 2.0% soluble starch in phosphate buffer, pH 6.0 at various temperatures between 30^{o}C to 70^{o}C, for 24 hours. As shown in Figure 17A, the optimum temperature for dextrinizing activity was 65^{o}C while the optimum temperature for CD-forming activity was 55^{o}C.

For thermal stability test, the enzyme was incubated at various temperatures ranging from 40^{o}C to 55^{o}C for 60 minutes, and 10 μl was withdrawn to assay the residual activity as usual. It was found that enzyme was rather stable at 40^{o}C. At 55^{o}C, where maximum activity was, enzyme activity was lost about 50% after 60 minutes of incubation. (Figure 18A).

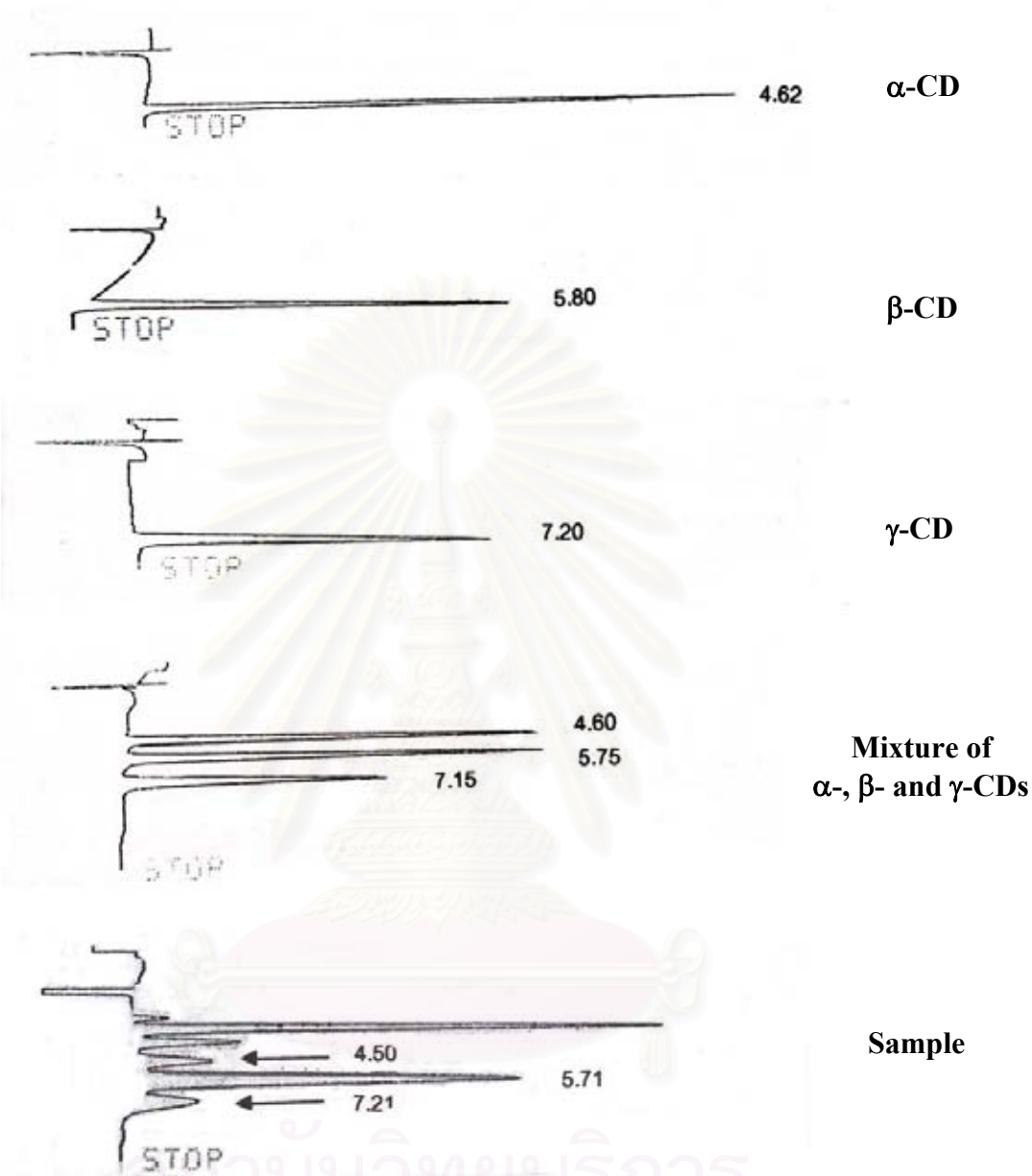


Figure 16 HPLC chromatogram of standard cyclodextrins (α-CD, β-CD and γ-CD) and CDs produced by CGTase from RB01. Spherisorb-NH_2 column was used. Acetonotrile : water (65 : 35, v/v) was used as eluent at 2 ml/min flow rate.

3.4.4 Effect of pH for enzyme activity and stability.

The activity of CGTase from RB01 as a function of pH was investigated. The reaction mixture containing the enzyme and soluble starch substrate in buffer solution (0.2 M) of various pHs ranging from 3.0 to 11.0 was incubated at 55^{o}C. Different buffers were used as mentioned in Methods section 2.10.3.2. The result is shown in Figure 17B. The enzyme had broader range of pH for optimal CD-forming activity and had maximum pH at 6.0. For dextrinizing activity, the maximum pH was 7.0 while not much difference in activity was observed at pH 8.0. However, the enzyme had no activity at acidic pH (pH 3.0 to 4.0) and basidic pH (pH 11.0).

The stability of enzyme in buffers at different pH values was also investigated by dialyzing enzyme in 50 mM of each buffer pH 6.0 to 9.0 until the final pHs of the enzyme were 6.0 to 9.0, respectively. Samples were then incubated at 55^{o}C for 60 minutes before the enzymic activity was assayed in phosphate buffer pH 6.0 at 55^{o}C. Figure 18B shows that the enzyme was most stable at pH 7.0, 15% of the activity was lost after 60 minutes of incubation. While at pH 8.0 to 9.0, about 30% of the enzyme activity were lost.

3.4.5 Effect of incubation time for enzyme activity

CGTase and soluble starch substrate were incubated for different time periods (0-30 minutes) and dextrinizing activity was assayed as described in Methods section 2.5.1. As shown in Figure 17C, linearity was observed from 0 to 15 minutes of incubation time. Therefore, the suitable time incubation for enzyme activity was 10 minutes.

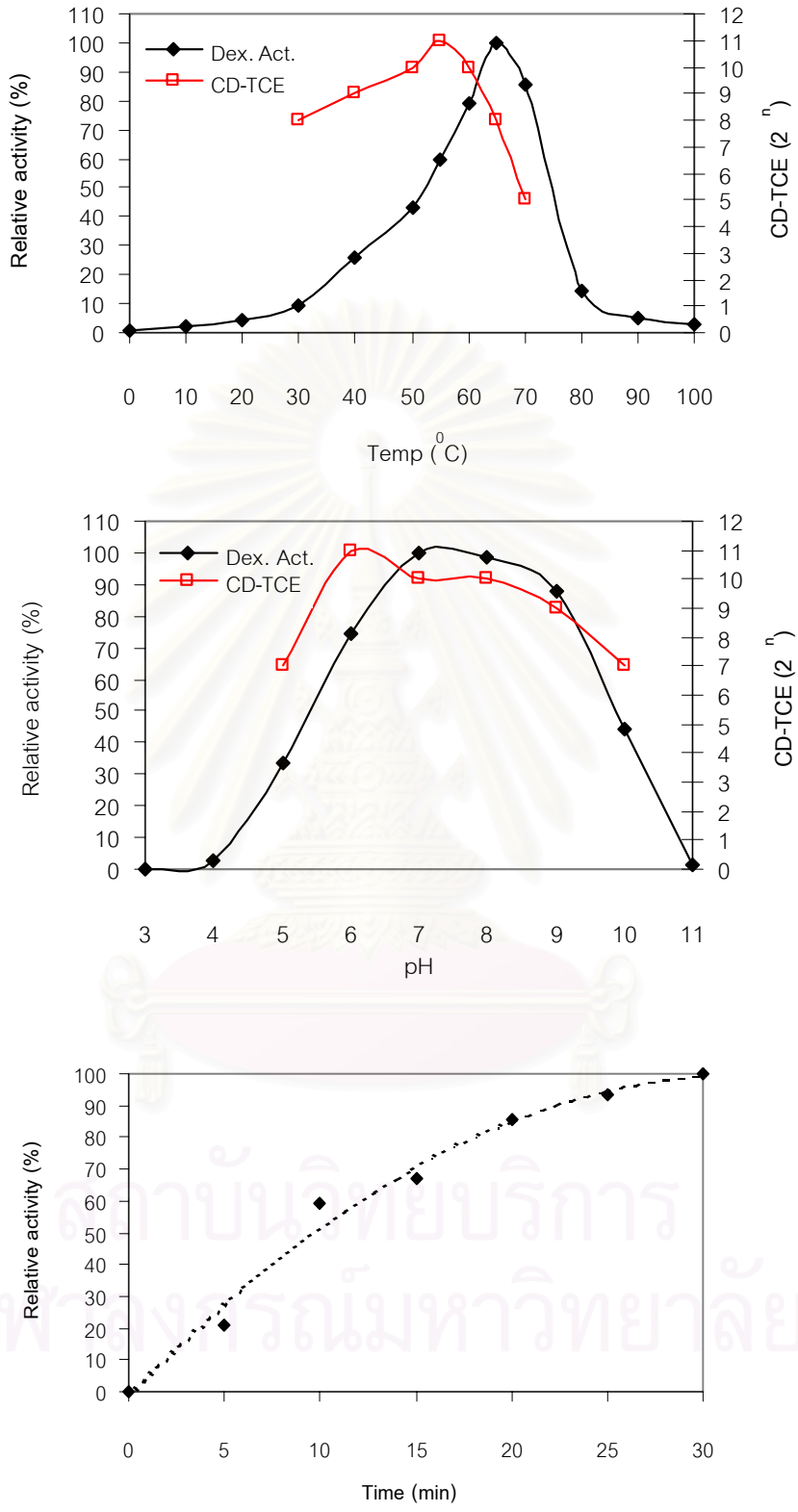


Figure 17 Effect of temperature: (A), pH: (B) and incubation time: (C) on enzyme activity.

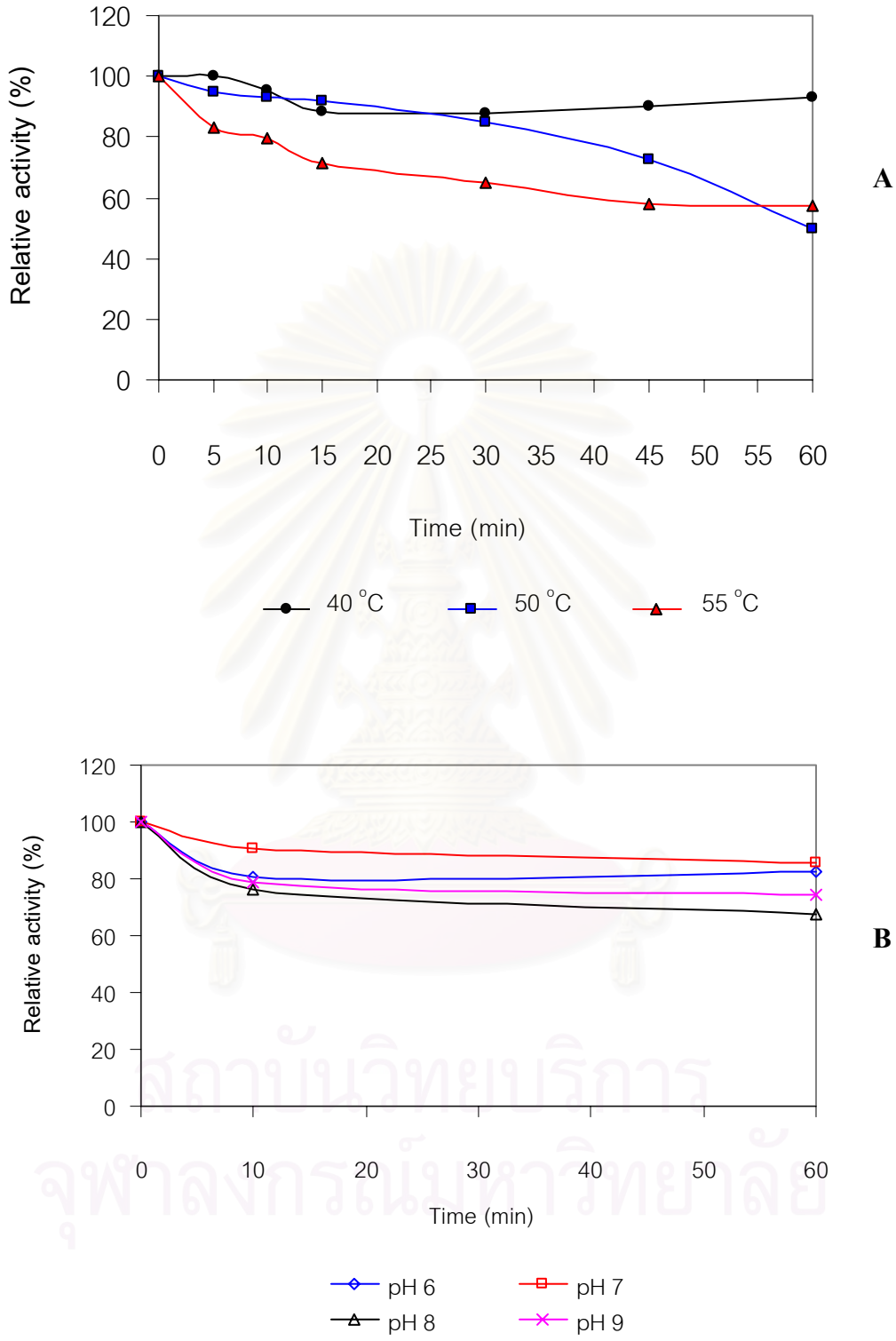


Figure 18 Effect of temperature: (A), pH: (B) on enzyme stability.

3.4.6 Effect of $CaCl_2$ on enzyme stability

The effect of $CaCl_2$ on enzyme stability was investigated by incubating the enzyme with 10 mM and 20 mM $CaCl_2$ in phosphate buffer, pH 6.0 at 55^{o}C, for 60 minutes. Samples were withdrawn at intervals to measure for enzyme activity. It was found that adding $CaCl_2$ at 10 or 20 mM in the enzyme sample did not have any effect on enzyme stability (Figure 19A).

3.4.7 Effect of substrate on enzyme stability

The effect of substrate on enzyme stability was determined by similar experiment as described in 3.4.6. As shown in Figure 19B, in the presence of 0.2% soluble starch (potato), no effect on enzyme stability was observed.

3.4.8 Condition for storage enzyme

Enzyme samples (0.7 mg protein/ml in10 mM Tris-HCl buffer, pH 8.5) were stored at 4^{o}C and -20^{o}C for 5 weeks. Ten mM $CaCl_2$ was also added in the enzyme sample to compare with control enzyme. Sample was withdrawn every weeks for measuring enzyme activity by dextrinizing assay. Figure 20 demonstrated no loss in enzyme activity after stored at –20^{o}C for 4 weeks, while stored at 4^{o}C, 15% of the activity was lost. Adding $CaCl_2$ in the enzyme sample did not have an effect on stability at both storage temperatures. The most suitable condition for storing enzyme was to keep the enzyme at –20^{o}C.

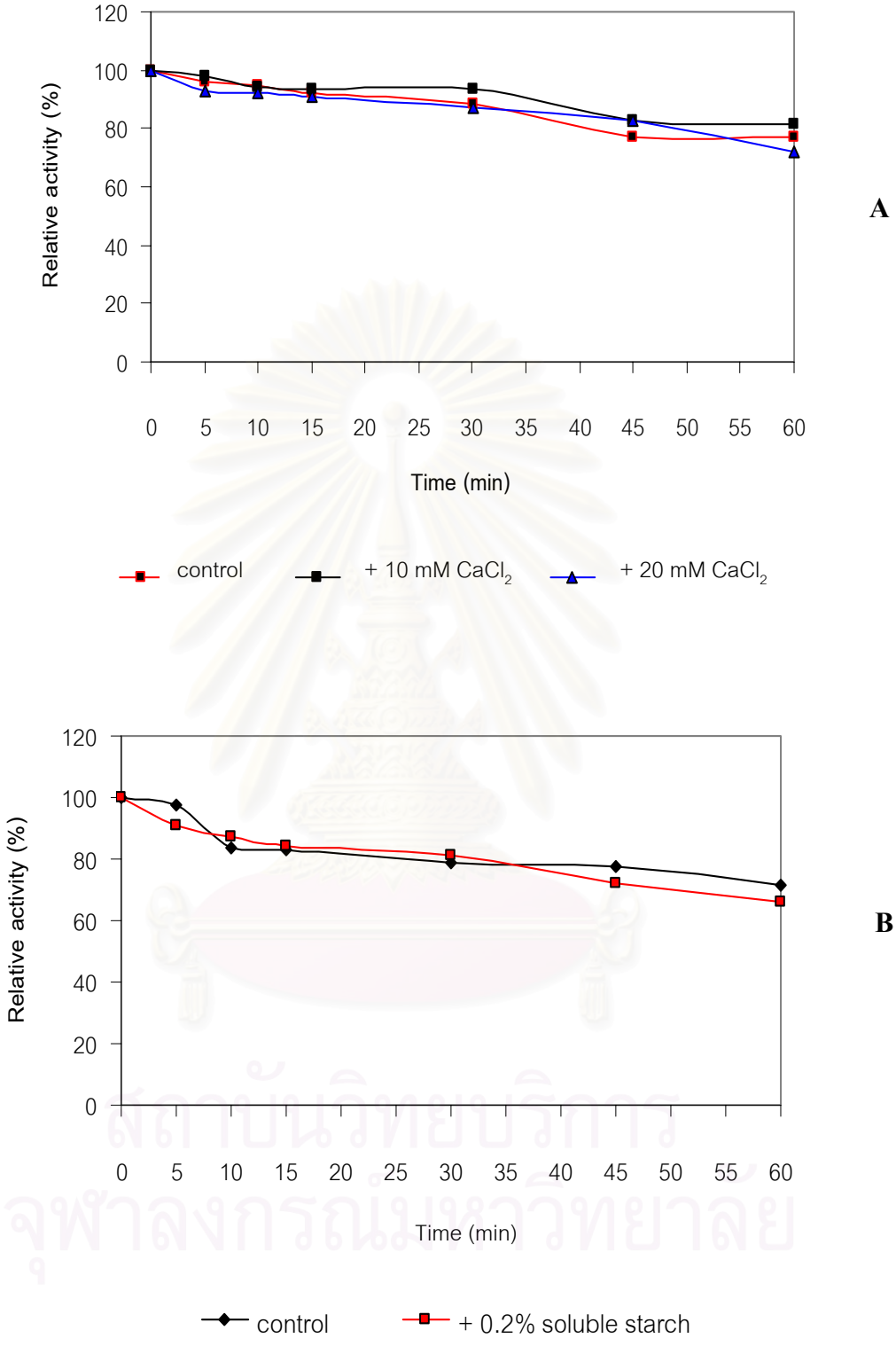


Figure 19 Effect of $CaCl_2$ (A) and substrate (B) on enzyme stability at 55°C.

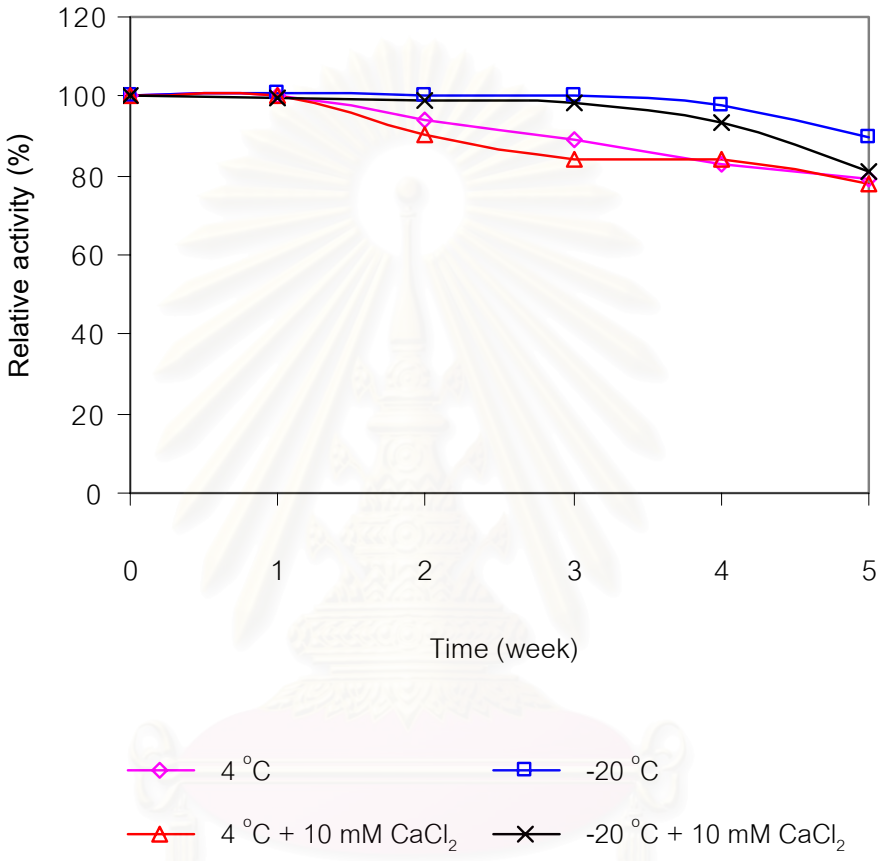


Figure 20 Condition for storing enzyme at 4°C and –20°C.

CHAPTER IV

DISCUSSION

Screening of thermotolerant bacteria producing CGTase

For screening purpose, because CGTase degrades α-1,4-glycosydic bond, of which the reaction specificity depends on length and complexation of glycosyl groups in substrate (Fuwa, 1954), CGTase activity was determined by dextrinizing activity method (Iodine method) similar to amylase. This method is rapid and simple so it was used in this study for primary screening.

Since, CGTase activity was indistinguishable from amylase activity in primary screening, so additional method, specific for CGTase activity, such as HPLC (Sato, 1985), Tilden-Hudson method (Tilden and Hudson, 1942), dye-binding method (Park, 1989; Siripornadulsilp, 1993) or tricloroethylene method (CD-TCE) (Nomoto, 1986) were necessary for CGTase screening. In this work, a simple and rapid screening method by which CGTase can specifically be detected on agar medium was performed. Phenolphthalein was transformed into a colorless dianion within the cavity of CDs (Taguchi, 1986). For this reason we used this method to determine CGTase activity in secondary screening. The possibility that acid-producing microorganisms can change the color of phenolphthalein to colorless by producing acids was examined by adding methyl orange to the medium. The color of methyl orange will change from orange to red with acid-forming bacteria, thus CGTase activity can be distinguished from the action of acids produced by microorganisms (Park, 1989).

From the result of primary screening, 38 isolates gave positive results while in the next step (secondary screening), only 2 isolates RB01 and KB01 gave CGTase activity. It should be noted that the 2 isolates had relatively low activity of

amylase. The b/a ratios were 1.3 and 2.8, respectively (Table 7). Those strains with high amylase were not CGTase producing, this finding was in agreement with many previous reports (Alexandra, 1998; Techaiyakul, 1991).

CD-TCE assay was further used to confirm CGTase production. RB01 had higher CD-TCE activity than KB01. It was found that RB01 could grow over a wide temperature range from 30^{o}C up to 45^{o}C. However, maximum growth occurred at 37^{o}C whereas CGTase production was highest at 40^{o}C. At temperature 30^{o}C to 37^{o}C good growth was observed but CGTase production was lower than at 40^{o}C. The result indicated that RB01, not only gave higher CGTase activity than KB01, but also had thermotolerant property, so we selected RB01 for further studies.

Identification of selected bacterial strain

Morphological characterization was performed by scanning electron microscopy (SEM). It was found that RB01 was rod-shaped, measuring 0.3 x 1.0 μm. From Table 8, RB01 was Gram positive, belonged to genus *Bacillus* and was identified as *Bacillus circulans* from biochemical characteristics. Identification by 16S rRNA gene fragment amplification is the technique that is lately used to confirm bacterial classification since comparison is at molecular level (Fox *et al*, 1977; Stackebrandt, 1987; Edwords *et al*, 1989; Ash, 1993; Yoon *et al*, 1998). In this study, the forward primer pA (AGA GTT TGA TCC TGG CTC AG) and reverse primer pH (AAG GAG GTG ATC CAG CCG CA) were used to amplify the gene. Then primers pA: AGA GTT TGA TCC TGG CTC AG, pD: CAG CAG CCG CGG TAA TAA TAC and pF: CAT GGC TGT CGT CAG CTC GT were used for sequencing. Edwards (1989) had used these primers to determine the nucleotide sequence of 16S rRNA gene of *Mycobacterium kansasii*, which was found to be 98.7% homologous to

that of *M. bovis* BCG. This is the first report on a contiguous sequence information of an entire amplified gene spanning 1.5 kb without any subcloning procedures. These primers have been used to identify various bacteria due to the conserved sequence property of 16S rRNA gene (Ash, 1991; Yoon *et al*, 1998). After 16S rRNA gene of RB01 was amplified, PCR product about 1.5 kb was sequenced. The 16S rRNA sequence of RB01 was 1508 bp and showed 99% homology with *Paenibacillus campinasensis* strain 324 (description shown in Appendix K) whereas 88% homology was found with *Bacillus circulans*. Ninety-nine percents homology was also observed with *Paenibacillus* sp. 38-2 and *Paenibacillus* sp. AG 430 (Appendix I). The 99% homology were obtained from the identity ratio of 1495/1509, 1479/1491 and 1478/1491 for *Paenibacillus campinasensis* strain 324, *Paenibacillus* sp. 38-2 and *Paenibacillus* sp. AG 430, respectively. The score (bits) calculated were 2886, 2878 and 2870 for the three respectively strains (Appendix J). From this result, RB01 may be closer to *Paenibacillus campinasensis* (strain 324) rather than *Paenibacillus* sp. 38-2, *Paenibacillus* sp. AG 430 and *Bacillus circulans*. Thus identification of RB01 by two different techniques gave different result. Figure 20 shows phylogenetic tree demonstrating the position of strain 324 in relation to *Paenibacillus* sp. and other rod-shaped, endospore-forming bacteria of other related genus based on 16S rRNA gene sequence.

Rod-shaped, aerobic, endospore-forming bacteria have generally been assigned to the genus *Bacillus*, a systematically diverse taxon (Claus and Berkeley, 1986). 16S rRNA oligonucleotide cataloguing (Fox *et al*, 1977 ; Stackebrandt, 1987) and more recently comprehensive 16S rRNA gene sequence analysis (Ash, 1991) have shown that the genus *Bacillus* is phylogenetically very heterogeneous, at least 10

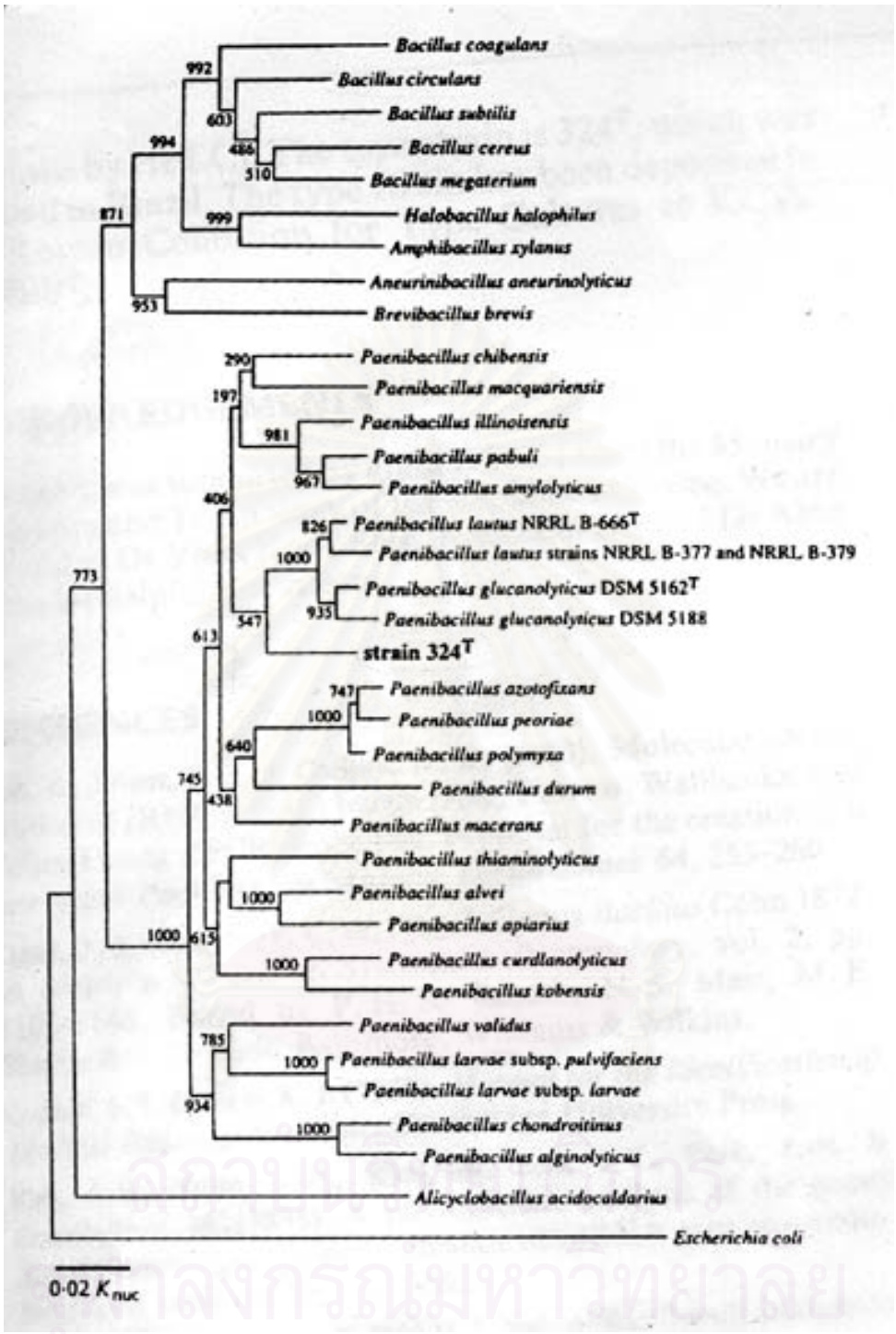


Figure 21 Phylogenetic tree showing the position of strain 324 in relation to *Paenibacillus* sp. and other rod-shaped, endospore-forming bacteria of other related genus based on 16S rRNA gene sequence. Bootstrap values are indicated Bar, 2 nucleotide substitutions per nucleotide. (Yoon *et al*, 1998)

phylogenetic groups have been identified in the genus *Bacillus* (Ash *et al*, 1993 ; Ash e*t al*, 1991; Farrow, 1992 ; Teather and Wood, 1982 ; Wallace and Brammall, 1985). Five of the groups have been reclassified as the new genera *Alicyclobacillus*, *Paenibacillus*, *Halobacillus*, *Brevibacillus* and *Aneurinibacillus* (Shida *et al*, 1997).

The genus *Paenibacillus* can be thus readily distinguished from other *Bacillus* groups using a battery of phenotypic characters and highly specific gene probe based on 16S rRNA. Over the past decade, great advances have been made in elucidating the natural interrelationships of bacilli. The genus *Paenibacillus* was successfully differentiate from the other taxa belonging to the *Bacillaceae* by using PAEN 515 F at the detection primer (Ash, 1993).

Although, RB01 was similar to *Paenibacillus campinasensis* (strain 324), it has some different properties, for example RB01 grows at pH 6.0-10.0 while *Paenibacillus campinasensis* (strain 324) cannot grow at pH 7.0 but grows in the range of pH 7.5-10.5 (Yoon *et al*, 1998). In addition, one of the main characteristics of *Paenibacillus campinasensis* (strain 324) is its ability to grow in the presence of 7% NaCl. When we checked growth of our strain, it was found that growth was observed at $\leq$ 3 % NaCl (Appendix M).

Optimization of conditions for culturing and CGTase production

The optimum temperature of RB01 was 40^{o}C. Although at 37^{o}C growth of RB01 was higher than at 40^{o}C, but for enzyme production, 40^{o}C was better. Though RB01 was screened from hot spring area at which water temperature is 55^{o}C (Appendix L), but the temperature of soil sample around the water is less than 55^{o}C. We found that RB01 could not grow at temperature higher than 45^{o}C. At 45^{o}C, profile

of enzyme production was decreased when comparing with at 40^{o}C, whereas the growth profile was the same.

This strain was grown immensely at pH 6.0 to 10.0 and constantly produced the CGTase over this pH range. Enzyme production was highest at pH 10.0. RB01 was different from *Paenibacillus campinasensis* (strain 324) which could not grow at pH 7.0 but grew at pH 7.5-10.5 (Yoon *et al*, 1998).

CGTase produced from RB01 was the starch inducible enzyme. The various types of starch used in this study were different in the percentage of amylose and amylopectin content (Table 10). The results in the Figure 11 demonstrated that all of the starches investigated were good for RB01, except for glutinous rice starch. This may be because glutinous rice starch has high % of amylopectin (92%(w/w)), which may not be suitable for consumption by the cells whereas other starches had % of amylopectin in the range of 76%-83% (w/w).

Significant enzyme production was induced by soluble starch. This form of starch is highly soluble and should be easily used by bacteria. The enzyme production in various concentrations of soluble starch was investigated. It was found that low yield of CGTase production was obtained from cell cultivation in high concentration of soluble starch. It could be suggested that at higher concentration of this starch might provide the higher amount of reducing sugar. This reducing sugar resulted from hydrolysis of normal starch with α-amylase was known to repress the CGTase activity (Szejtli, 1988). Another explanation is that at higher concentration, higher viscosity of starch substrate is faced. The contacting of cells to substrate was lowered and thus caused the reduction of substrate consumption. From the optimization steps, it was also observed that CGTase of RB01 was produced during

Table 10 Amylose and amylopectin contents in starch (Swinkels, 1985; Nilmanee, 2000)

Type of Starch	Amylose (%)	Amylopectin (%)
Rice starch	18	82
Corn starch	24	76
Glutinous rice starch	8	92
Cassava starch	17	83
Potato starch	24	76

the early exponential growth and reached maximum during the stationary phase (12-36 hours). This was typical with other CGTase-producing organisms (Alexandra, 1998). It should also be noted that the pattern of enzyme production as observed from Figures **5, 10, 11 and 12 demonstrated biphasic curves. This might be explained by the fact that CGTase activity was determined by dextrinizing activity method (Iodine method), which measured degradation of α-1,4-glycosydic bonds. Other enzymes such as amylase or glycoamylase were also detected by this method. Therefore, biphasic curves might be due to combination of CGTase with other amylolytic enzymes. In this aspect, tricloroethylene assay (CD-TCE method) which is specific for CGTase was also performed. The activity observed were 2^4, 2^5 and 2^6 at 24, 48 and 60 hours of culturing time, respectively (Figure 11). This suggests that CGTase production mainly occurred at stationary phase, which should be referred to the second peak of the biphasic curve.**

Partial purification and Biochemical characterization of CGTase from RB01

One common step in the purification of CGTase reported by different laboratories was the use of starch adsorption (Lloyed *et al*, 1984; Pongsawasdi and Yagisawa, 1988). Considering that the interaction between starch and CGTase involves not only adsorption but also substrate-enzyme affinity type binding. The advantage is that CGTase can be eluted in a small volume, which allows a good recovery of the enzyme. Moreover, the specific activity of the elution of CGTase resulted in high purification fold since starch adsorption step could get rid of many other proteins. Purity of the enzyme was confirmed by SDS-PAGE followed by Coomassie blue staining. A major protein band with molecular weight of 65,000 was observed. Molecular weight of CGTases isolated from various bacteria varies greatly from

64,000 to 200,000 (Hofman *et al*, 1989; Kitahata *et al*, 1974; Kitahata and Okada, 1982; Kobayashi *et al,* 1978; Makela *et al*, 1988; Nakamura and Horikoshi, 1976; Tomita *et al*, 1993; Yaki *et al*, 1986; Marechal *et al*, 1996). When compared RB01 with *Paenibacillus* sp. A11, the strain being used for CGTase studies by our group, molecular weight of *Paenibacillus* sp. A11 was 72,000 (Techaiyakul, 1991).

The CDs formation ability of CGTase was determined by HPLC. It was found that CGTase from RB01 produced α-, β- and γ-CD in proportion of 1.0 : 5.4 : 1.2. RB01 was thus a β-CGTase producer. To check for other biochemical characteristics, effect of temperature and pH on enzyme activity was performed. pH 6.0 was the best for CD-forming activity while pH 7.0 gave highest dextrinizing activity. The optimum starch degrading activity was observed at 65^{o}C, and the optimum CD-forming activity was noted at 55^{o}C. To explain this, the reaction mechanism of CGTase has to be taken into account. Since the catalytic residues of CGTase are proposed to be equivalent to those of α-amylase, CGTase cleaves the α-1,4-glycosidic bond of amylose in the same way as α-amylase does. The transglycosylation reaction of CGTase is operated by Ping-Pong mechanism (Nakamura *et al*, 1994). In this mechanism, the transglycosylation occurs after the reducing side of the cleaved amylose is released from the enzyme. Then the enzyme transfers the newly formed reducing end of the substrate either to the non reducing end of a separate linear acceptor molecule or glucose (the dispropotionation reaction) or to its own non reducing end (the cyclization reaction or CD synthesis reaction). The hydrolysis reaction (the starch-degrading reaction) will occur when this intermediate is attacked nucleophilically by a water molecule. For preferential CD synthesis, the efficient formation of the helical structure of amylose in the active-site cleft of enzyme is required (Fujiwara *et al*, 1992; Nakamura, 1994). In a crystal

structure, amylose can occur as a single helix with 6 to 8 glucose molecules in one helical turn (Kubik *et al*, 1996). The most widely accepted hypothesis describes amylose in solution having an interrupted coil-like structure composed of helical and non-helical segments (Szejtli, 1991). Therefore, the formation of CD by CGTase can be explained as a consequence of preferential helical structure of amylose in solution. However, the high temperature will destabilize the helical structure of the amylose, resulting in a shift to random structure. Accordingly, it is considered that the reaction at high temperature by RB01 CGTase resulted in a shift towards the starch-degrading reaction. Another explanation is that CGTase was contaminated by other amylolytic enzymes. Therefore, optimum starch degradation activity was shifted from optimum CD-forming activity. The optimum temperature for CGTase of RB01 was at 55^{o}C, the temperature which gave the highest CD-product. When compared with *Paenibacillus* sp. A11, the optimum temperature for CGTase of *Paenibacillus* sp. A11 was at 40^{o}C (Techaiyakul, 1991).

When stability was concerned, the enzyme was rather stable at 40^{o}C while at 55^{o}C, where maximum activity was, enzyme activity was lost about 50% after 60 minutes of incubation. However, the optimum time of incubation for assaying was 10 minutes and only 20% of the enzyme activity was lost (Figure 19A). $CaCl_2$ had no effect on enzyme stability both at 55^{o}C and at storage condition of 4 or -20^{o}C. This is contradict to previous reports by Horikoshi (1971) and Akimura *et al* (1991). Horikoshi (1971) demonstrated that calcium ion was effective to stabilize the enzyme CGTase from *Bacillus* no.A-40-2 especially at high temperature. Akimura *et al* (1991) observed that saturation of CGTase from *Bacillus coagulans* with Ca^{2+} resulted in an increase of heat stability. However, it is believed that the crude enzyme with other interacting proteins should be more stable than the purified enzyme. Then CGTase of

RB01 could be suitable for industrial CD production. In the industrial production of CDs, the first step is generally liquefaction of starch with thermostable α-amylase prior to incubation with CGTase (Wind *et al*, 1995). This step can be omitted if CGTase of RB01 is used, since it is sufficiently thermostable to solubilize and degrade starch, and form CDs at fermentation temperature of 40-50 ^{o}C.

CHAPTER V

CONCLUSIONS

1. Thirty-eight isolates produced amylase activity with b/a ratio ≥ 1.0 were screened. Among these, 2 strains (RB01 and KB01) were found to exhibit CGTase activity.

2. RB01 could grow and produce CGTase at rather broad mesophilic temperature range (30-45^{o}C), thus had thermotolerant characteristic.

3. From scanning electron microscopy, RB01 cells were rod-shaped. The size was approximately 0.3 x 1.0 μm. Colonies on Medium I agar plate had white color, flat, irregular and spreading.

4. From biochemical characterization, RB01 was Gram-positive and was identified as *Bacillus circulans*.

5. In the identification by 16S rRNA gene fragment amplification, the sequence obtained had 1508 bp which showed 99% homology with that of *Paenibacillus campinasensis* (strain 324). While only 88% homology was found between sequence of RB01 and *Bacillus circulans*.

6. The best condition for culturing RB01 for high production of CGTase enzyme was culturing the strain in Horikoshi medium with 1.0 % soluble starch at pH 10.0, 40^{o}C for 60 hours.

7. CGTase of RB01 was partially purified by starch adsorption. The % recovery and purification fold were 57.3% and 26.8, respectively. The final specific activity was 3,568 U/mg.

8. This enzyme exhibited 4 isoform patterns with amylolytic activity. The molecular weight was 65,000 from SDS- PAGE.

9. A main product, β-CD and two minor products, α-and γ-CDs, were produced from starch, the ratio of α : β : γ was 1.0 : 5.4 : 1.2 .

10. Optimum condition for measuring enzyme activity was at 55^{o}C, pH 6.0 for 10 minutes.

11. Enzyme was stable at 40^{o}C, pH 7.0 for 60 minutes.

12. The best condition for storing enzyme was –20^{o}C.

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APPENDICES

APPENDIX A: 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 with distilled water

- 1.5 M Tris-HCl pH 8.8

  Tris (hydroxymethyl)-aminometane 18.17 g

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

- 2 M Tris-HCl pH 8.8

  Tris (hydroxymethyl)-aminometane 24.2 g

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

- 0.5 M Tris-HCl pH 6.8

  Tris (hydroxymethyl)-aminometane 6.06 g

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

- 1 M Tris-HCl pH 6.8

  Tris (hydroxymethyl)-aminometane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 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) Non-denaturing PAGE

- 7.5% separating gel

30% acrylamide solution	2.5 ml
1 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

- Sample buffer

1 M Tris-HCl pH 6.8	3.1 ml
glycerol	5.0 ml
1% bromphenol blue	0.5 ml
distilled water	1.4 ml

One part of sample buffers was added to four parts of sample.

- Electrophoresis buffer, 1 litre

(25 mM Tris, 192 mM glycine)

Tris (hydroxymethyl)-aminometane	3.0 g
Glycine	14.1 g

Dissolved in distilled water to 1 litre (final pH should be 8.8)

3) SDS-PAGE

- 7.5% separating gel

30% acrylamide solution	2.5 ml
solution B	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

- 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% bromphenol 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 before loading to the gel.

- Electrophoresis buffer, 1 litre

Tris (hydroxymethyl)-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)	

APPENDIX B: Preparation for buffer solution

- 0.2 M Potassium Acetate pH 3.0,4.0,and 5.0

  CH_3COOK 1.96 g

  Adjusted to pH 3,4 or 5 by 0.2 M acetic acid and adjusted volume to 100 ml with distilled water.

- 0.2 M Phosphate pH 6.0

  KH_2PO_4 2.27 g

  K_2HPO_4 0.58 g

  distilled water 100 ml

- 0.2 M Phosphate pH 7.0

  KH_2PO_4 0.91 g

  K_2HPO_4 2.32 g

  distilled water 100 ml

- 0.2 M Tris-HCL pH 8.0 and 9.0

  Tris (hydroxymethyl)-aminometane 2.42 g

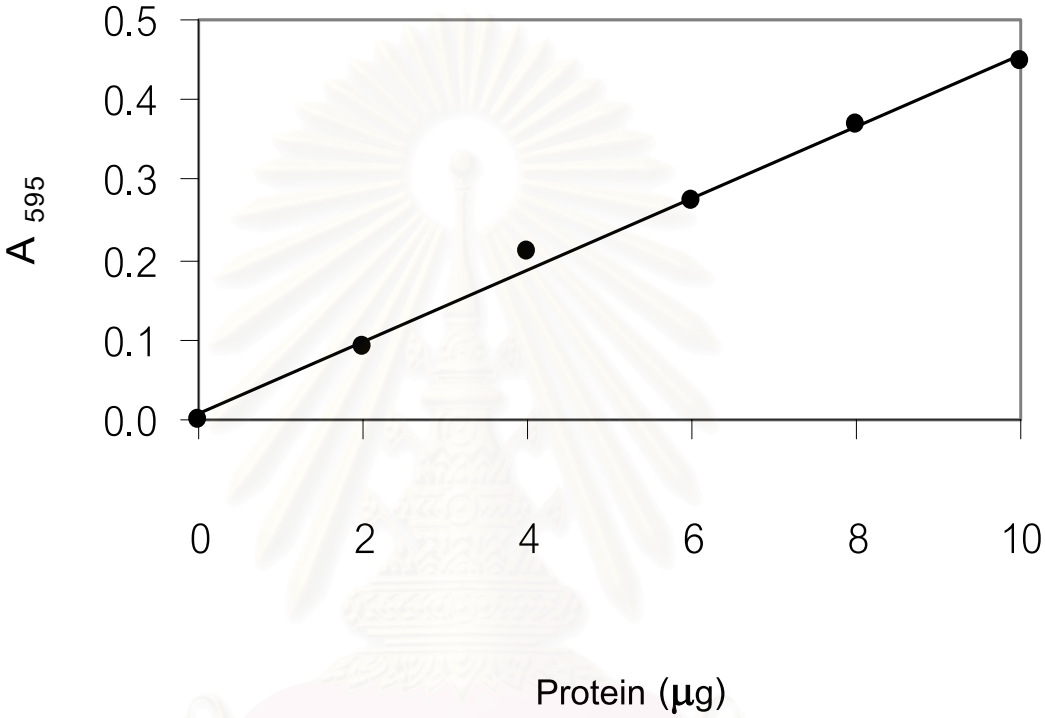
  Adjusted to pH 8.0 or 9.0 by 1 M HCl and adjusted volume to 100 ml with distilled water.

- 0.2 M Tris-Glycine NaOH pH 10.0 and 11.0

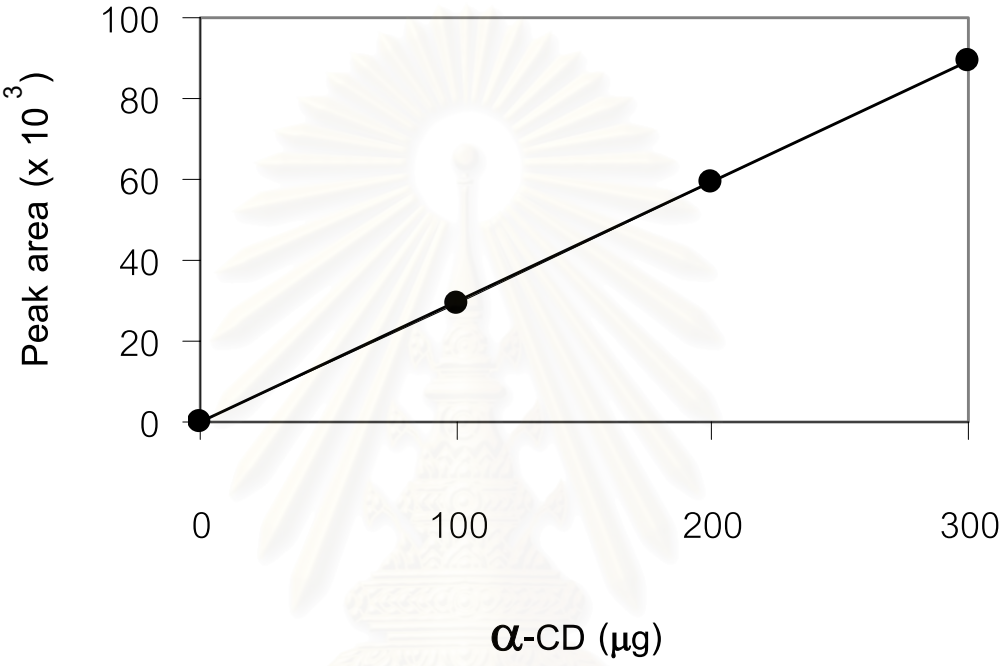
  Glycine 1.5 g

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

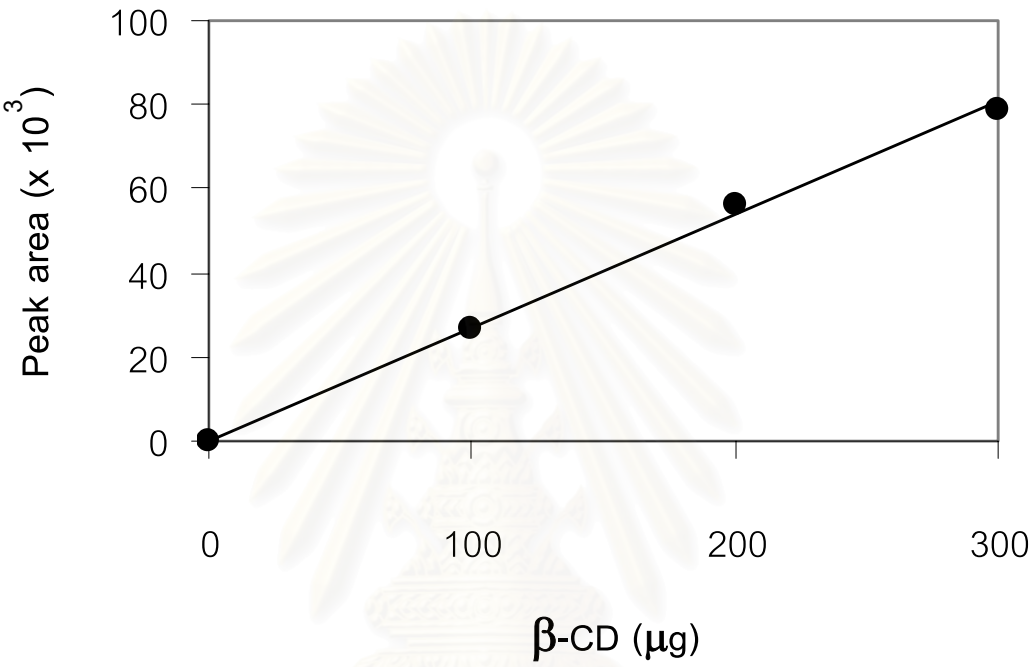
APPENDIX C: Standard curve for protein determination by Bradford's method



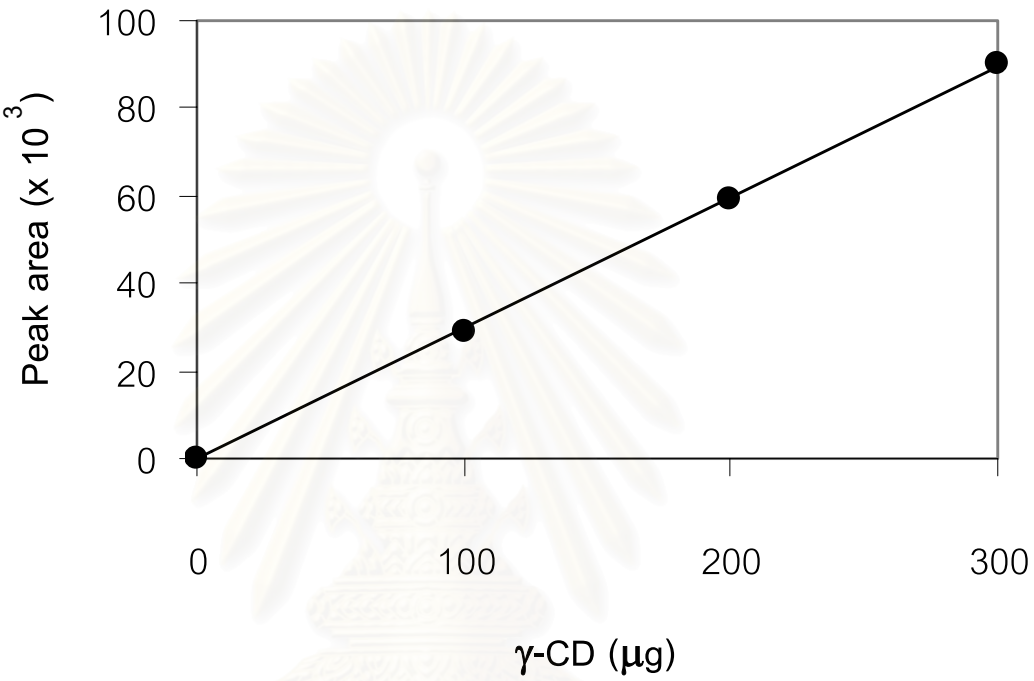
APPENDIX D: Standard curve of α-cyclodextrin by HPLC method



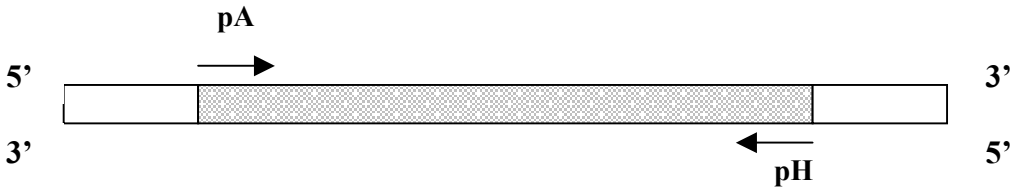
APPENDIX E: Standard curve of β-cyclodextrin by HPLC method



APPENDIX F: Standard curve of γ-cyclodextrin by HPLC method



APPENDIX G: Condition of 16S rRNA amplification.



Concentration of ingredients; In 1 reaction consist of

Template (100 ng)	10 µl	
Primer A (1 pmol/µl)	10 µl	(10 pmol)
Primer H (1 pmol/µl)	10 µl	(10 pmol)
10x buffer	10 µl	(1x)
dNTPs (2.0 mM/µl)	10 µl	(20 mM)
$MgCl_2$ (25 mM/µl)	8 µl	(200 mM)
Tag (5u/µl)	0.5 µl	
Ultrapure claved water	41.5 µl	
Total	100 µl	

Condition for amplification (30 cycles)

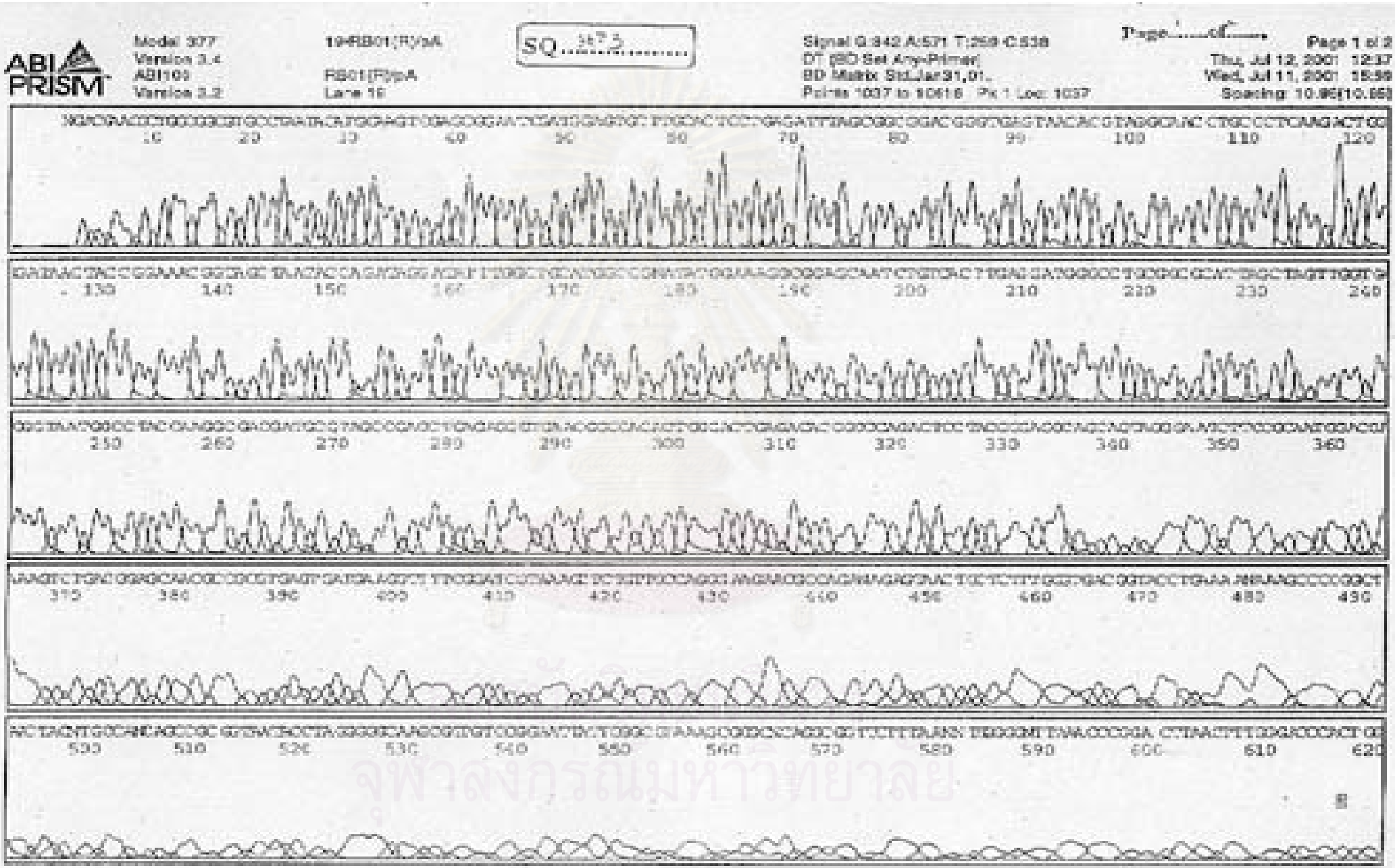
95 ^{o}C 1 minute
50 ^{o}C 2 minutes
72 ^{o}C 3 minutes
} 1 cycle

APPENDIX H: The DNA sequencing profile of 16S rRNA gene fragment from RB01

(A) = The DNA sequencing profile of 16S rRNA gene fragment from RB01 using the sense primer A

(B) = The DNA sequencing profile of 16S rRNA gene fragment from RB01 using the sense primer D

(C) = The DNA sequencing profile of 16S rRNA gene fragment from RB01 using the sense primer F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Version 3.2
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BD Matrix Std_Jan31,01_
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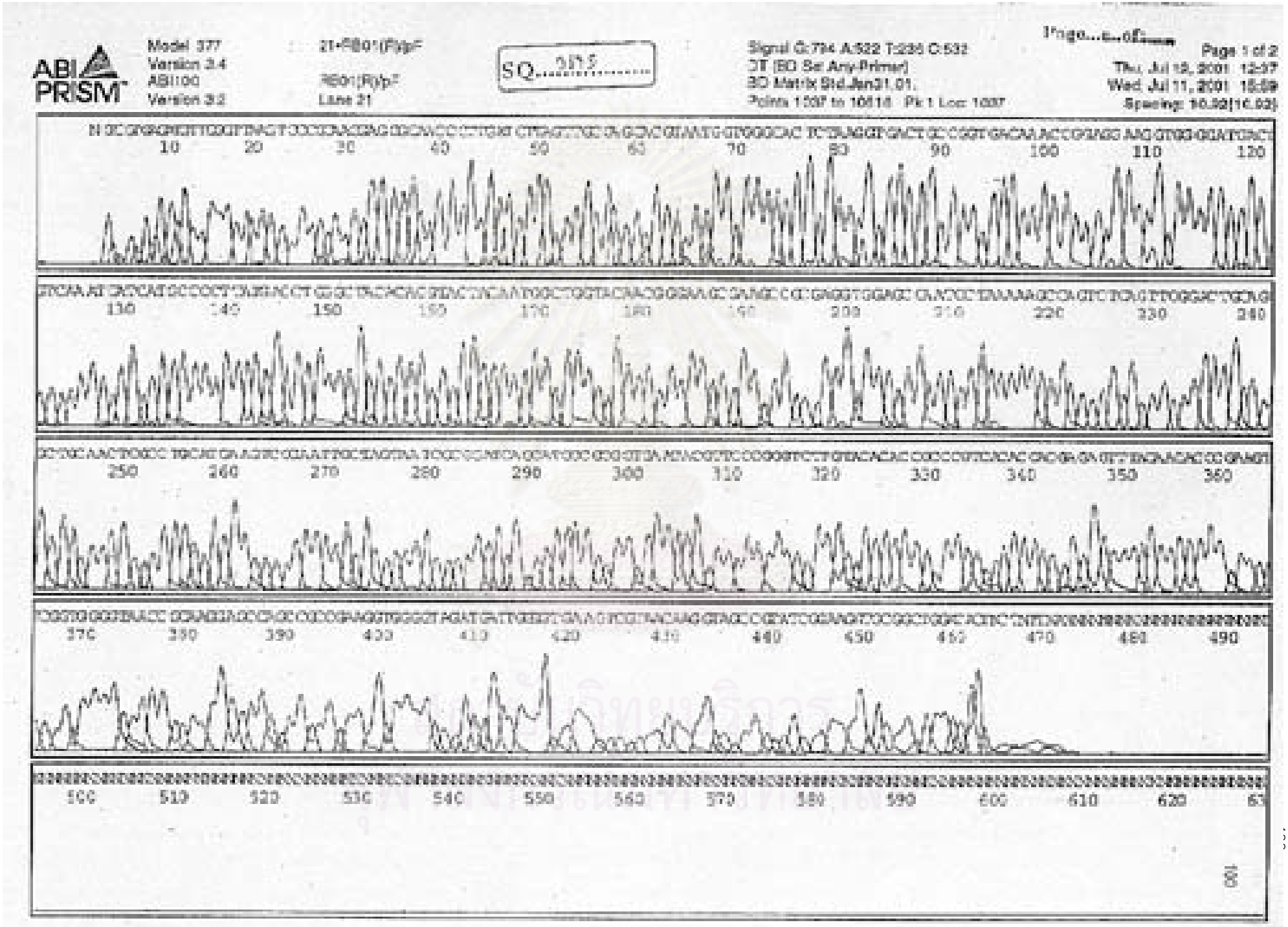
Page 1 of 2
Thu, Jul 12, 2001 12:37
Wed, Jul 11, 2001 15:59
Spacing: 10.86[10.65]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ABI100
Version 3.2
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Lane 21
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BD Matrix Std Jan31.01.
Points 1597 to 10818 Pk 1 Loc 1597
Page 1 of 2
Thu, Jul 12, 2001 12:37
Wed, Jul 11, 2001 15:59
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APPENDIX I: Blast result of the 16S rRNA gene fragment of RB01 using primers A, D and F

pA

Source	% homology
Paenibacillus sp. AG 430	97
Paenibacillus sp. 38-2	96
Paenibacillus sp. strain 324	96

pD

Source	% homology
Paenibacillus sp. 38-2	97
Paenibacillus sp. S39	97
Paenibacillus sp. strain 324	97

pF

Source	% homology
Paenibacillus sp. strain 324	99
Paenibacillus sp. S39	99
Paenibacillus sp. 38-2	99

All primers (pA, pD and pF)

Source	% homology
Paenibacillus sp. strain 324	99
Paenibacillus sp. 38-2	99
Paenibacillus sp. AG 430	99

APPENDIX J: Taxonomy reports from BLAST program using all primers (A, D and F)

Sequences producing significant alignments:	Score (bits)	E Value
gi|3582525|gb|AF021924.1|AF021924 Paenibacillus sp. strain ...	2886	0.0
gi|11414979|dbj|AB043866.1|AB043866 Paenibacillus sp. 38-2 ...	2878	0.0
gi|11414982|dbj|AB043869.1|AB043869 Paenibacillus sp. AG430...	2870	0.0
gi|11414980|dbj|AB043867.1|AB043867 Paenibacillus sp. S39 g...	2863	0.0
gi|2895561|gb|AF039409.1| Bacillus vortex 16S ribosomal RNA...	2296	0.0
gi|6900431|emb|AJ271157.1|PJA271157 Paenibacillus jamilae p...	2284	0.0
gi|4468642|emb|AJ011687.1|PBU011687 Paenibacillus burgondia...	2226	0.0
gi|2073371|dbj|D78473.1|D78473 Paenibacillus lautus DNA for...	2212	0.0
gi|3328015|gb|AF071860.1|AF071860 Paenibacillus popilliae s...	2204	0.0
gi|3328014|gb|AF071859.1|AF071859 Paenibacillus popilliae s...	2185	0.0
gi|2769591|emb|Y16129.1|PS16SC168 Paenibacillus sp. C-168 1...	2149	0.0
gi|3929897|emb|AJ131119.1|PSP131119 Paenibacillus sp. 16S r...	2147	0.0
gi|457638|dbj|D16276.1|BAC16SRR11 Bacillus polymyxa 16S rRN...	2143	0.0
gi|7209530|dbj|AB021183.1|AB021183 Bacillus chitinolyticus ...	2131	0.0
gi|2760290|dbj|D78472.1|D78472 Paenibacillus lautus DNA for...	2131	0.0
gi|3328016|gb|AF071861.1|AF071861 Paenibacillus lentimorbus...	2121	0.0
gi|3646401|emb|AJ011326.1|PSP011326 Paenibacillus borealis ...	2119	0.0
gi|3646397|emb|AJ011322.1|PSP011322 Paenibacillus borealis ...	2119	0.0
gi|7110405|gb|AF227827.1|AF227827 Paenibacillus sp. 61724 1...	2115	0.0
gi|3646400|emb|AJ011325.1|PSP011325 Paenibacillus borealis ...	2115	0.0
gi|3646399|emb|AJ011324.1|PSP011324 Paenibacillus borealis ...	2115	0.0
gi|3646398|emb|AJ011323.1|PSP011323 Paenibacillus borealis ...	2115	0.0
gi|5701912|emb|AJ223989.1|PS16SCF43 Paenibacillus polymyxa ...	2115	0.0
gi|5701911|emb|AJ223988.1|PS16SPMD2 Paenibacillus polymyxa ...	2115	0.0
gi|3646402|emb|AJ011327.1|PSP011327 Paenibacillus borealis ...	2111	0.0
gi|7160077|emb|AJ272249.1|PSP272249 Paenibacillus sp. CM1 p...	2111	0.0
gi|11064494|emb|AJ250319.1|BFU250319 Bacterium LMG 18437 16...	2101	0.0
gi|11064491|emb|AJ250317.1|BFU250317 Bacterium LMG 18419 16...	2101	0.0
gi|2077922|dbj|D85609.1|D85609 Paenibacillus lautus DNA for...	2091	0.0
gi|2077921|dbj|D85394.1|D85394 Paenibacillus lautus DNA for...	2091	0.0

gi|12060407|dbj|AB042938.1|AB042938 Paenibacillus sp. DS-1 ... 2079 0.0

gi|14537949|gb|AF385540.1|AF385540 Paenibacillus sp. oral c... 2066 0.0

gi|10443301|emb|AJ297715.1|PSP297715 Paenibacillus sp. 16S ... 2054 0.0

gi|10334692|gb|AF181573.1|AF181573 Paenibacillus cf. polymy... 2050 0.0

gi|12044264|gb|AF309811.1|AF309811 Uncultured synthetic was... 2036 0.0

gi|1089784|dbj|D78318.1|PBB16SRRJ Paenibacillus azotofixans... 2030 0.0

gi|11414981|dbj|AB043868.1|AB043868 Paenibacillus sp. 7-5 g... 2028 0.0

gi|12006264|gb|AF273740.1|AF273740 Paenibacillus sp. 172 16... 2016 0.0

gi|9392286|dbj|D85396.2|D85396 Paenibacillus amylolyticus D... 2016 0.0

gi|7209531|dbj|AB021184.1|AB021184 Bacillus ehimensis gene ... 2012 0.0

APPENDIX K: Description of *Paenibacillus sp.* and *Paenibacillus campinasensis*

Description of *Paenibacillus* gen.nov. (pae. ba. cillus., L. adv. paene, almost; bacterial name, bacillus; N>L>mas.n; *Paenibacillus* almost bacillus, because it derives from this genus but phylogenetically distinct.) The description is based upon data taken from several sources (Claus and Berkeley, 1986)

Description of *Paenibacillus campinasensis* (cam. pi. na. sen sis. M.L. adj. Campinasensis refering to Campinas, the city where the College of Food Engineering, State University of Campinas, Brazil, is located).

Cells are rods measuring 0.6-0.9 by 3.0-6.0 μm and motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat, smooth and opaque. Forms motile microcolonies on wet agar plates. Facultative anaerobic and gram variable. Catalase-positive and oxidase- and urease negative. Growth occurs in the presence of 7% NaCl. Gelatin, casein, aesculin and starch are hydrolyzed. Utilitizes L-arabinose, β-cyclodextrin, D-fructose, D-glucose, melibiose, 3-methylglucose, psicose, D-ribose, D-sorbiyal, tagatose, D-xylose, Tween 40, acetic acid, propionic acid, pyruvic acid, fructose 6-phosphate and glucose 6-phosphate as sole carbon sources for respiration. Grows at 10 and 45^{o}C, but not at 5 and 50^{o}C; optimum temperature is 40^{o}C. Alkaliphilic. Does not grow at pH 7.0. Grows at pH

7.5-10.5; optimum pH is 10.0. Cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major isoprenoid quinone is menaquinone, MK-7. The major fatty acid is anteiso-$C_{15:0}$. The G + C content is 50.9 mol % (determinatiom by HPLC). The type strain is 324^T, which was isolated in Brazil. The type strain has been deposited in the Korean Collection for Type Cultures as KCTC 0364BP^T.

APPENDIX L: Data on collected samples from hot springs.

Site	Isolate number	Source of each isolate	Water temp. (^oC)
KANCHANABURI (Hindad)	1 2 3 4 5	soils around the wells	41
RATCHABURI (Bo-kloeng)	6 7 8 9 10*	soils around the wells	55
(Pongkrating)	11 12 13 14	soils around the wells	45
KRABI	15* 16 17	water from the well water from the well soil from the bottom of the well	45
RANONG	18 19	soil, 5 m from the well soil, 25 m from the well	65

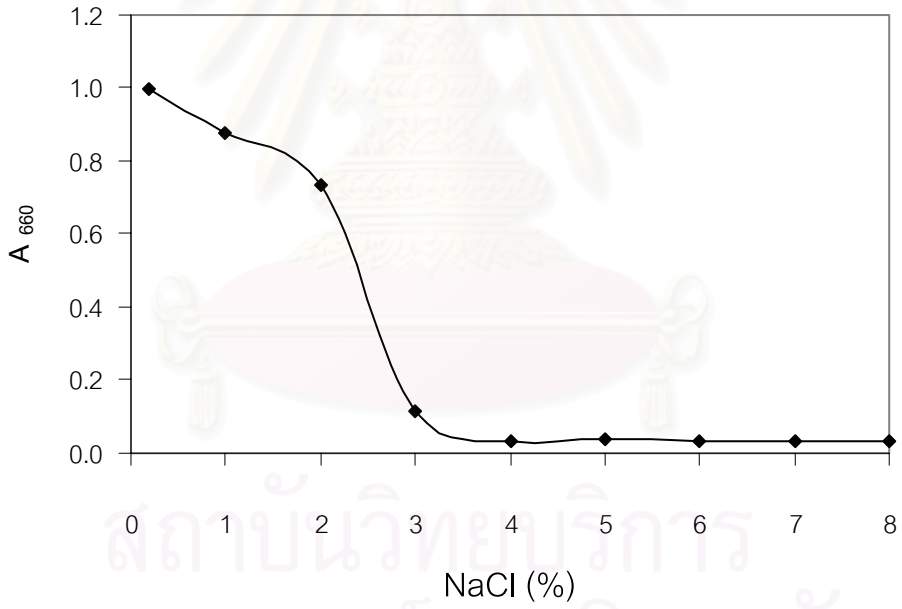
* indicates isolate with Amylase-CGTase activity.

APPENDIX L: Continue.

Site	Isolate number	Source of each isolate	Water temp. (°C)
CHIANG MAI	20	soil, 30 m from the well	69
(Sankampang)	21	soil, 5 m from the well	71
	22	soil around the well	92
(Pong-daod)	23	soil from the rim of the well	
	24	soil, 1 m from the well	94
	25	soil, 2 m from the well	
CHIANG RAI	26	soil, 7 m from the well	85
(Banpongnamron)	27	soil from the rim of the well	85
	28	soil from the rim of the well	85
	29	soil, 2 m from the well	80
(Thamachat)	30	soil from the well	83
	31	soil from the well	83
	32	soil, 2 m from the well	75
	33	soil, 2 m from the well	75
	34	soil, 2 m from the well	75
(Huaysai)	35	soil, 2 m from the well	50
(Toongtaewee)	36	soil, 1 m from the well	60
	37	soil, 2 m from the well	60
MAE HONG SON			

(Pongnamdaod)	38	soil from the rim of the well	76

APPENDIX M: Growth of RB01 in the presence of NaCl



Note: RB01 was cultured in Medium I until A_{660} was around 0.3-0.5, then 1.0 % transfer was made to Medium I in the presence of various concentrations of NaCl at 37 ^{o}C for 12 hours. Then A_{660} was measured.

BIOGRAPHY

Miss Solos Tesana was born on October 27th, 1977 in Uthaithani. She graduated with the Bachelor Degree of Science in Department of Plantpathology, Faculty of Agriculture, Kasetsart University in 1999 and continued studying for Master course in Biotechnology program, Faculty of Science, Chulalongkorn University.