การจำแนกแบคทีเรียทนร้อนที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส และลักษณะสมบัติของเอนไซม์

นางสาวพรธิดา แย้มพยนต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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IDENTIFICATION OF THERMOTOLERANT BACTERIA PRODUCING CYCLODEXTRIN GLYCOSYLTRANSFERASE AND ENZYME CHARACTERIZATION

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พรธิดา แย้มพยนต์: การจำแนกแบคทีเรียทนร้อนที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสและลักษณะสมบัติของเอนไซม์ (IDENTIFICATION OF THERMOTOLERANT BACTERIA PRODUCING CYCLODEXTRIN GLYCOSYLTRANSFERASE AND ENZYME CHARACTERIZATION)
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จากการคัดเลือกแบคทีเรียทนร้อนที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรส (CGTase) จากแหล่งดิน น้ำ และของเสียจำพวกแป้งในโรงงานแป้งรวมทั้งตัวอย่างที่มีการปนเปื้อนของคาร์โบไฮเดรตซึ่งเก็บจากบริเวณต่างๆ ในประเทศไทย ในระดับปฐมภูมิพบแบคทีเรียจำนวน 97 โคโลนีที่ทำให้เกิดวงใสบนอาหารแข็ง Medium I ซึ่งมีแป้งเป็นส่วนประกอบเมื่อตรวจด้วย 0.02% I_2 ใน 0.2%KI ในจำนวนนี้พบว่ามี 3 ไอโซเลท (BT01, BD01 และ BO01) ที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสโดยทำให้เกิดวงใสสีเหลืองบนพื้นแดงบนอาหารแข็ง Horikoshi ซึ่งประกอบด้วยฟีนอฟทาลีน นำไอโซเลทเหล่านี้ไปเลี้ยงในอาหารเหลว Horikoshi เพื่อศึกษาการเจริญและการผลิตเอนไซม์ที่อุณหภูมิช่วง 37-50 องศาเซลเซียสและเลือกสายพันธุ์ที่มีลักษณะทนร้อนที่ดีที่สุด พบว่า BT01 เจริญและผลิตเอนไซม์ได้ดีในช่วง 37-45 องศาเซลเซียสและเป็นสายพันธุ์ที่ผลิตไซโคลเดกซ์ทรินไกลโคซิลทรานสเฟอเรสได้ดีที่สุดเมื่อเปรียบเทียบกับ BD01 และ BO01 ดังนั้นจึงเลือก BT01 ในการศึกษาหาภาวะที่เหมาะสมต่อไป จากการจำแนกสายพันธุ์ตามลักษณะทางชีวเคมีและสรีระวิทยาพบว่า BT01 คือ *Bacillus circulans* และจำแนกสายพันธุ์ด้วยวิธี 16S rRNA gene พบว่าเป็น *Paenibacillus* sp. โดยมีความคล้าย 98% BT01 เจริญและผลิตเอนไซม์ได้ดีที่อุณหภูมิ 37-45 องศาเซลเซียส pH และอุณหภูมิที่เหมาะสมในการเลี้ยงเชื้อเพื่อใช้เป็นเชื้อตั้งต้นคือ pH 9.0 และ 37 องศาเซลเซียสในอาหารเหลว Medium I สภาวะที่เหมาะสมในการผลิตเอนไซม์คือเลี้ยงในอาหาร Horikoshi ที่เติม soluble starch 0.5% pH ของอาหารเท่ากับ 9.0 เลี้ยงที่ 40 องศาเซลเซียส เป็นเวลา 72 ชั่วโมง การทำเอนไซม์ให้บริสุทธิ์บางส่วนด้วยวิธีดูดซับด้วยแป้งได้เอนไซม์คงเหลือ 65% และความบริสุทธิ์เพิ่มขึ้น 28 เท่า หาน้ำหนักโมเลกุลโดยวิธี SDS-PAGE มีค่าประมาณ 79 กิโลดาลตัน อุณหภูมิที่เหมาะสมในการวัด dextrinizing activity และ CD-forming activity คือ 70 และ 55 องศาเซลเซียสตามลำดับ ซึ่งทั้งอุณหภูมิ 55 และ 70 องศาเซลเซียสมี pH ที่เหมาะสมในการวัด dextrinizing activity และ CD-forming activity คือ pH 6.0 และ 7.0 ตามลำดับ วัด dextrinizing activity พบว่าเอนไซม์มีความเสถียรที่อุณหภูมิ 30 องศาเซลเซียสและ pH 6.0-9.0 แต่เมื่อวัด CD-forming activity พบว่าเอนไซม์มีความเสถียรที่ 70 องศาเซลเซียสและ pH 6.0-8.0 เมื่อบ่มเอนไซม์ไว้ 1 ชั่วโมง การเติม $CaCl_2$ และ soluble starch มีผลต่อความเสถียรของเอนไซม์เล็กน้อย ผลิตภัณฑ์ไซโคลเดกซ์ทรินที่ได้มีอัตราส่วน α: β-CD เท่ากับ 1:1 จากการศึกษาพบว่า pH และอุณหภูมิมีผลต่อการผลิตไซโคลเดกซ์ทริน และอุณหภูมิที่เหมาะสมต่อการผลิตไซโคลเดกซ์ทริน คือ 55-60 องศาเซลเซียส พบว่าชนิดของแป้งไม่มีผลต่อการผลิตไซโคลเดกซ์ทรินซึ่งมีอัตราส่วนระหว่าง α: β-CD คงที่

ภาควิชา.....หลักสูตรเทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....
สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา.....2545.....ลายมืออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : CLYCLODEXTRIN GLYCOSYLTRANSFERASE /THERMOTOLERANT / BACTERIA/IDENTIFICATION /OPTIMIZATION/CHARACTERIZATION
PORNTHIDA YAMPAYONT: IDENTIFICATION OF THERMOTOLERANT BACTERIA PRODUCING CYCLODEXTRIN GLYCOSYLTRANSFERASE AND ENZYME CHARACTERIZATION.
THESIS ADVISOR: ASST. PROF. TIPAPORN LIMPASENI, Ph.D. 131 pp.
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Thermotolerant bacteria producing cyclodextrin glycosyltransferase (CGTase) were screened from soil, water and starch waste from starch factory including samples contaminated with carbohydrates collected from various areas in Thailand. About ninety- seven colonies exhibited clear zone on Medium I agar plates containing soluble starch when with 0.02% I_2 in 0.2% KI in primary screening. Among them, three isolates (BT01, BD01 and BO01) exhibited CGTase activity by forming yellow clear zone on red background using Horikoshi medium plate phenolphthalene dyes system. These isolates were checked for growth and enzyme activity by culturing in Horikoshi broth at the temperature range of 37-50 ^{o}C for selection of the best thermotolerant strain. It was found that BT01 grew and produced CGTase well at 37-45 ^{o}C. Strain BT01 exhibited highest CGTase compared with BD01 and BO01. Therefore, BT01 was chosen for further optimized condition. Biochemical and physiological characterization showed that BT01 was *Bacillus circulans* and was further identified by 16S rRNA analysis was *Paenibacillus* sp. with 98% homology. BT01 grew and produced CGTase at temperature range 37-45 ^{o}C. The optimum pH and temperature of starter inoculum for cell growth were pH 9.0 and 37 ^{o}C in Medium I. The optimum culturing conditions for highest CD-forming activity were pH 10.0 and 40 ^{o}C for 72 hours in Horikoshi broth containing 0.5% soluble starch. The enzyme was partially purified by starch adsorption, the recovery and purification fold were 65.1% and 28.0, respectively. The molecular weight was estimated to be 79 kDa by SDS-PAGE. The optimum temperature for dextrinizing and CD-forming activity were 70 and 50-55 ^{o}C. At 55 and 70 ^{o}C, the optimum pH for dextrinizing activity were 6.0, while CD-forming activity were 7.0. Dextrinizing activity of enzyme stabilized at temperature up to 30 ^{o}C and at pH range 6.0-9.0, while CD-forming activity retained its full activity up to 70 ^{o}C and at pH range 6.0-8.0 for 1 hour. In presence of $CaCl_2$ and substrate the enzyme activity could be slightly stabilized. The best condition for storing enzyme was -20 ^{o}C in presence of 10 mM $CaCl_2$. Cyclodextrin products from CGTase of BT01 was α:β = 1:1. pH and temperature of reaction mixture can influence the rate of CDs production and the optimum temperature of starch conversion into CDs was 55-60 ^{o}C. The source of starch is not important for CGTase action and the ratio of α:β-CD was remained constant.

Program……Biotechnology……. Student's signature……..…….
Field of study…..Biotechnolohy…….. Advisor's signature…….……...
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ABBREVIATIONS

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

CHAPTER I

INTRODUCTION

Cyclodextrins

Cyclodextrins (Schardinger dextrins, Cycloamyloses, Cyclomaltoses: CDs) are cyclic oligosaccharides produced from starch by the action of the microbial enzyme cyclodextrin glycosyltransferase (CGTase; E.C. 2.4.1.19). The major end-product of the action of CGTases are CD with 6, 7 and 8 glucose units linked by α-1,4-glucosidic bonds, which are known as α-, β- and γ-CD, respectively (French and Rundle, 1942; Freudenberg and Cramer, 1948; Volkova *et al*., 2000) (Figure 1.1). These are the only commercially available CD. Large CD with more than eight glucose units were first described by French (1957). However, because of their low yields and difficulties in purification, only a few of them have been characterized. The most important characteristics of the CDs are summarized in Table 1.1.

The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds. From the X-ray structures of cyclodextrins, it appears that the secondary hydroxyl groups (C_2 and C_3) are located on the wider edge of the ring and the primary hydroxyl groups (C_6) on the other edge. C-H groups and glycosidic oxygen bridge are at the inside of the torus-like molecules. Therefore, CD is water soluble due to the hydrophilic outside while the apolar cavity provides a hydrophobic matrix (Saenger, 1984) (Figure 1.2). The presence of the hydrophobic cavity allows cyclodextrins to form inclusion complexes with a wide variety of hydrophobic guest molecules (e.g. aromatic, alcohol, halides and hydrogen halides, fatty acids, and other esters etc.) as shown in Figure 1.3 (Amaizo, 1993). The guest molecule must satisfy the size criterion of fitting at least partially into the cyclodextrin internal cavity, resulting in an inclusion complex.

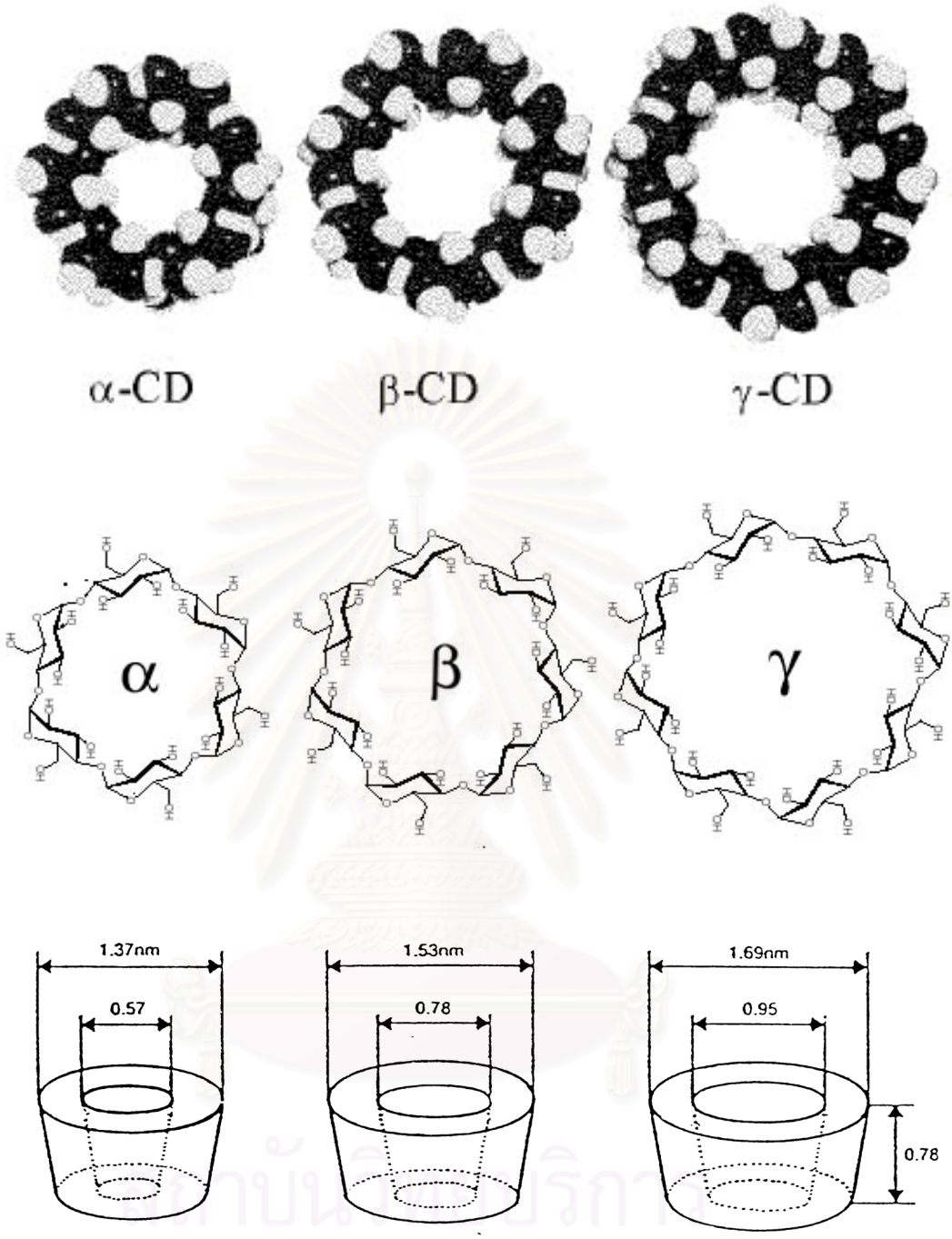


Figure 1.1 Molecular models (Secondary hydroxyl view) : (A), chemical structure : (B) and molecular dimension structure of cyclodextrins : (C)
(Volkova D.A. *et al*., 2000; French and Rundle, 1942)

Table 1.1 Physical properties of cyclodextrins (Szejtli, 1988a)

Properties	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight (Dalton)	972	1,135	1,297
Solubility in water at 25 ^{o}C (%w/v)	14.5	1.85	23.2
Cavity dimensions			
Outer diameter (^{o}A)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Cavity diameter (^{o}A)	4.7 - 5.3	6.0 - 6.5	7.5 - 8.3
Height of torus (^{o}A)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
Approx. cavity volumn ($^{o}A^{3}$)	174	262	427
Crystal form (from water)	hexagonal plates	monoclinic paralellograms	quadratic prisms

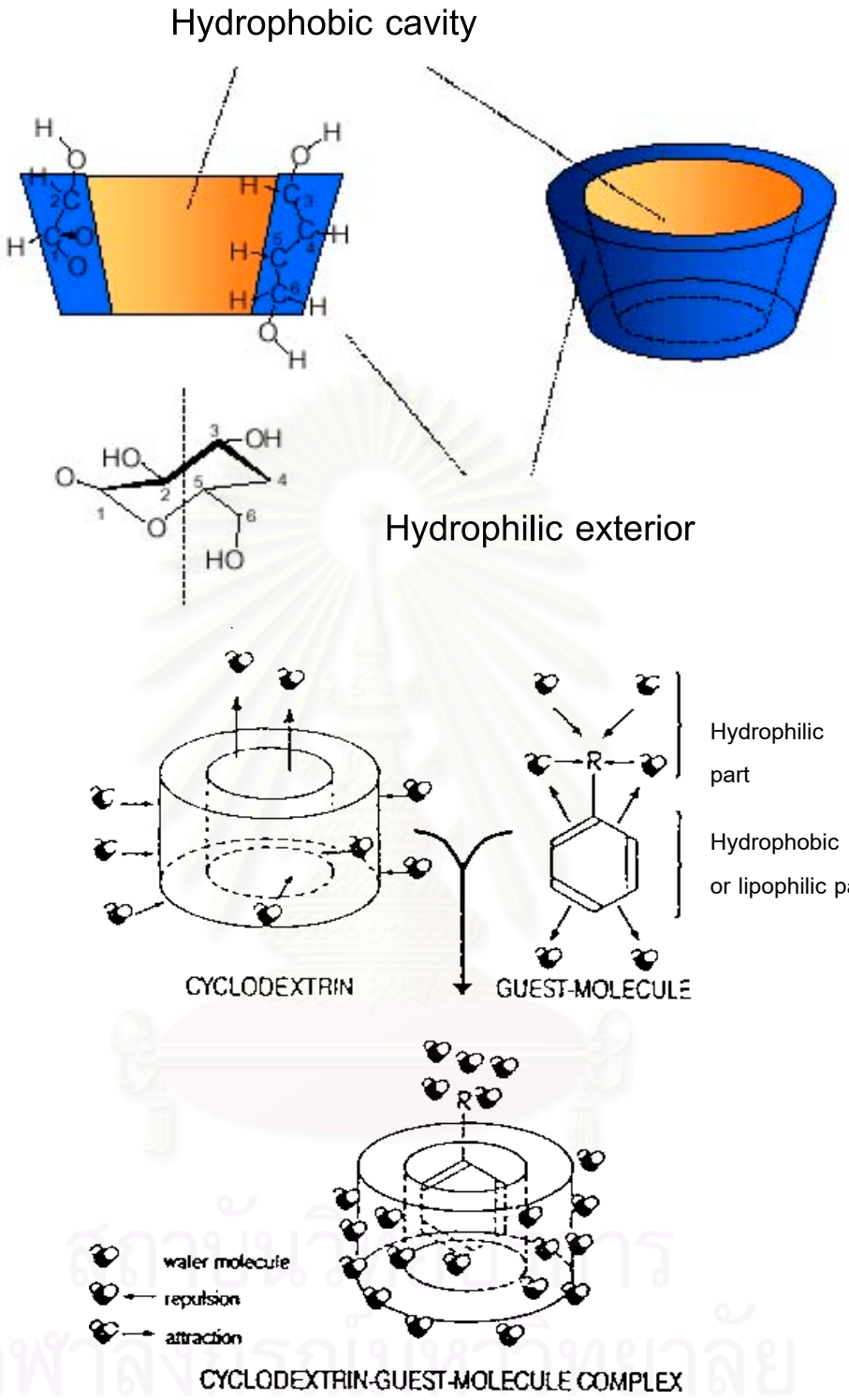


Figure 1.2 Functional structure of cyclodextrin that showed hydrophobic and hydrophilic properties : (A) and the formation of an inclusion complex:(B) (Wacker, 2000; Janssen, 1992)

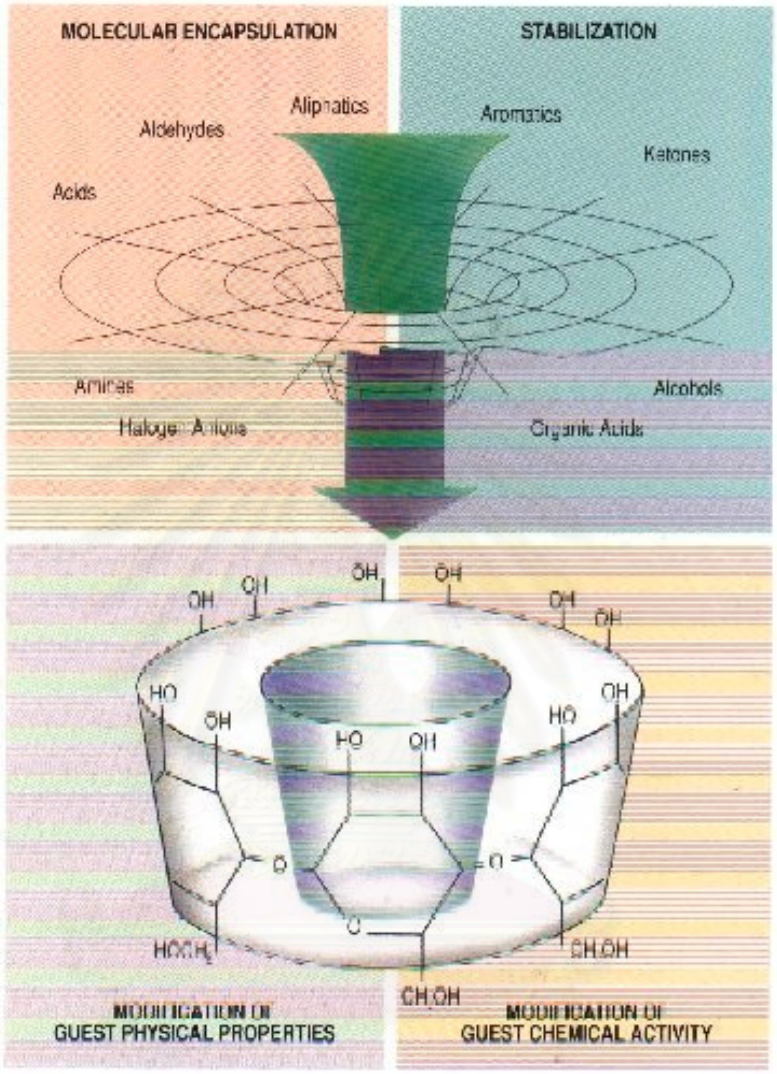


Figure 1.3 Potential guests which can be encapsulated in cyclodextrins

(Amaizo, 1993)

The most important parameters for complex formation between CDs and hydrophobic compounds or functional groups are their three dimensional forms and sizes. The driving force is the entropic effect of displacement of water molecules from the cavity (Saenger, 1980). Other parameters for complexation are charge or polarity of the guest compound or competition with other molecules from the medium. Furthermore, the complexation involves van der Waals interaction, hydrophobic forces and hydrogen bonding. Because the inclusion complexes are quite stable, they can be separated from the medium by crystallization (Starnes, 1990).

Many cyclodextrin derivatives have been synthesized or developed through chemical or enzymatic means in order to obtain CDs with specific desirable properties. These derivatives usually are produced by aminations, esterification or etherifications of primary and secondary hydroxyl groups of the CDs. Depending on the substituent, the solubility of the CD derivatives are usually different from that of their parent CDs. CD derivatives also have a changed hydrophobic cavity volume (Saenger, 1980).

Complex formation of cyclodextrins and guest molecules leads to the change in the physical and chemical properties of guest molecules, giving rise to the following beneficial modifications of guest molecules which have been successfully used in several applications as follow:

- Stabilization of light or oxygen sensitive substance
- Modification of the chemical reactivity of guest molecules
- Fixation of very volatile substances
- Improvement of solubility of substance
- Modification of liquid substances to powders
- Protection against degradation of substances by microorganisms
- Masking of ill smell and taste
- Masking pigments or the color of substances
- Catalytic activity of cyclodextrins with guest molecules

These characteristics of cyclodextrins or their derivatives make them suitable for applications in pharmaceuticals (Figure 1.4), in foods, in cosmetics, chemical and biochemical applications such as enzyme mimicry (Figure 1.5), agricultural, environmental protection, and the others (Table 1.2).

Administered cyclodextrins are quite resistant to starch degrading enzymes, although they can be degraded at very low rates by α-amylases. α-CD is the slowest, and γ-CD is the fastest degradable compounds due to their differences in size and flexibility. Degradation is not performed by saliva or pancreas amylases, but by α-amylases from microorganisms from the colon flora. Adsorption studies revealed that only 2-4% of taken up as glucose. This can explain the low toxicity found upon oral administration of cyclodextrins (Bar and Ulitzur, 1994; Duchene,1988).

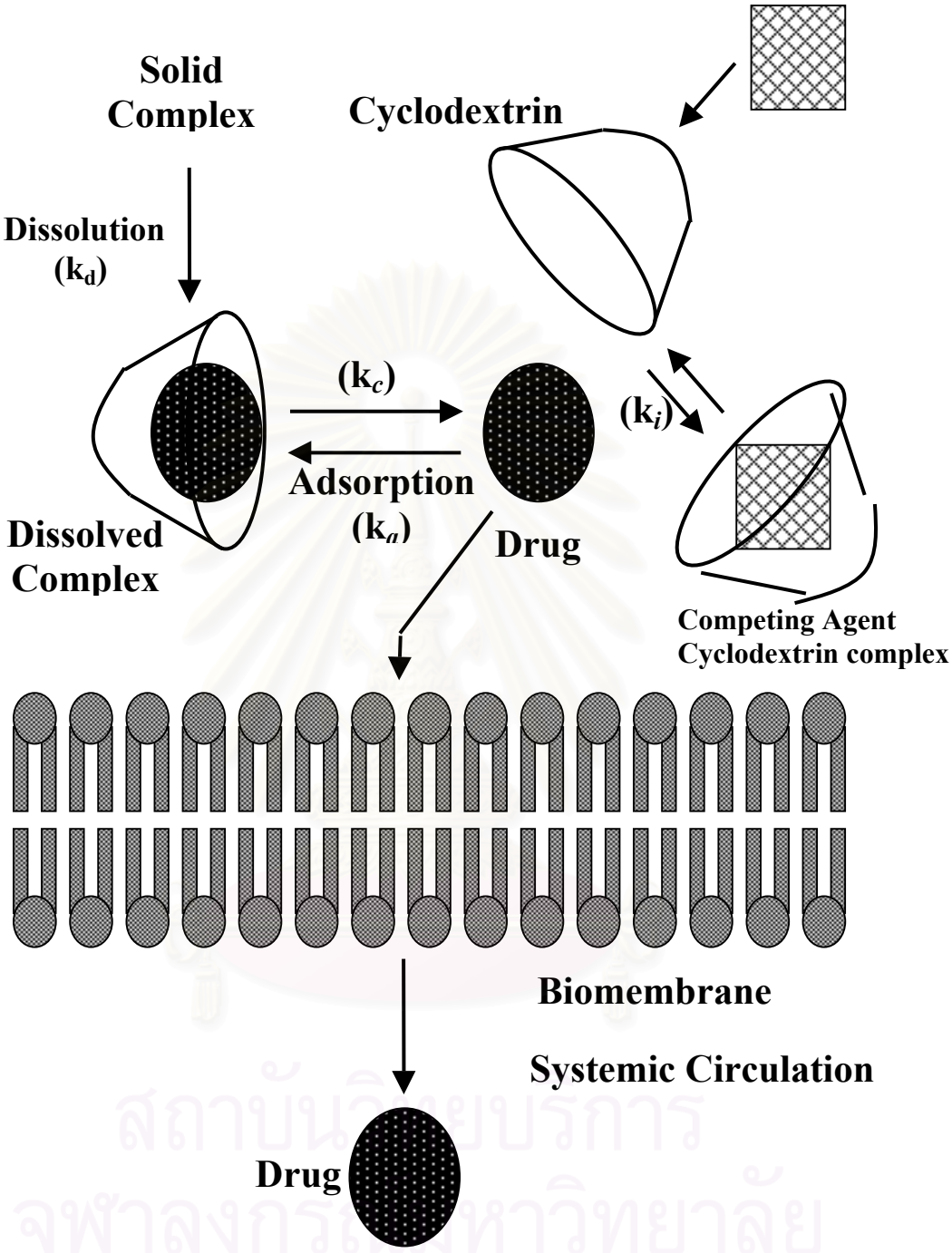


Figure 1.4 Application of cyclodextrins in Phamaceutical industry

(Schneidermann and Stalcup, 2000)

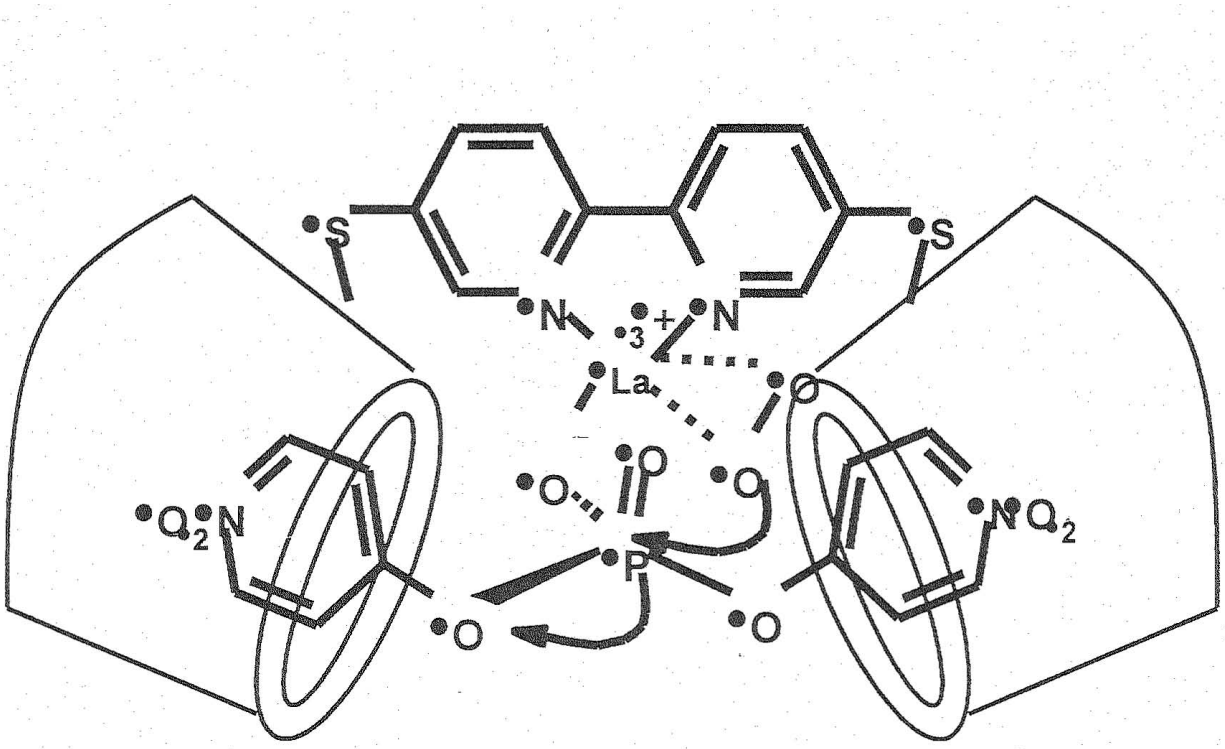


Figure 1.5 Enzyme mimicry: Dimer of β-CD linked on primary side by a metal - binding-group as catalyst of hydrolysis of a phosphate diester

(Breslow, 1995)

Table 1.2 Industrial application of cyclodextrins

(Szejti, 1994; Breslow, 1995; Schneidermann and Stalcup, 2000)

Industries	Utilized properties
1. Phamaceuticals	• Increase the drug' s water solubility and stability • Decrease of irritation • Reduction in volatility of drug molecules • Masking of malodours and bitter tastes • Treatment of inflammation or throat infection (with iodine) • Coronary dilatation (with nitroglycerin) • Anti-ulcerate (with benexate) • Vectors for vitamins or hormones • Reduction of side-effects and increase in efficiency of anti-cancer drugs
2. Foods and Flavours	• Emulsion stabilizer • Increase of foaming power • Stabilization of flavours and seasonings • Taste masking • Elimination of unpleasant tastes • Removal of cholesterol from milk, butter, eggs, etc.
3. Cosmetics and toiletries	• Emulsification • Stabilization of fragrances • Deodoriser (i.e.with peppermint oil) • Removal of dryness wrinkles (with seaweed compounds, Vitamin A and E) • Shampoo industry • Teeth cleaning, anti-plaque compound

Table 1.2 Industrial application of cyclodextrins (continued)

Industries	Utilized properties
4. Chemical and biochemical applications	• Reaction catalyst in adhesives • Use in chromatography (separation of stereoisomers) • Increase in speed of diagnostic test reaction • Enzyme mimicry
5. Agriculture	• Increase the stability and efficiency of herbicides, insecticides, fungicides, repellents, pheromones and growth regulators
6. Environmental protection	• Reduction in oxidizer requirements in paper production • Environmentally friendly oil-spill clean-up • Treatment of tree-wounds (with auxin) • Mobilization of toxins without leaving toxic residues behind (innovative technique) • Removal or detoxification of waste material, esp. aromatic pollutants
7. Others	• Increase the tackness and adhesion of some hot melts and adhesives • Increase in compatibility of paint ingredients • Increase in stability and viscosity of the paint • Increase in the range of colors and in the quality of dyes

Cyclodextrin producing enzymes

Starch can be degraded and formed cyclodextrins by the action of cyclodextrin glycosyltransferase (α-1,4-glucan-4-glycosyltransferase, E.C. 2.4.1.19, CGTase). CGTase is the enzyme capable of several transferase reactions, in which a newly made reducing end of an oligosaccharide is transferred to an acceptor molecule. Depending on the nature of the acceptor molecule, four transferase reactions (cyclization, coupling, disproportionation and hydrolysis) can be distinguished (Nakamura *et al*., 1993).

1.) Cyclization is the transfer of the reducing end sugar to another sugar residue in the same oligosaccharide chain, resulting in formation of a cyclic compound.

2.) Coupling is the reaction where a cyclodextrin molecule is combined with a linear oligosaccharide (chain) to produce a longer chain linear oligosaccharide.

3.) Disproportionation is the transfer of part of a linear oligosaccharide chain to another linear acceptor chain. Starting from two molecules of a pure oligosaccharide, this reaction yields a mixture of smaller and longer oligosaccharides.

4.) In hydrolysis (saccharifying activity) the newly made reducing end is transferred to water.

Four mechanisms are shown in Figure 1.6.

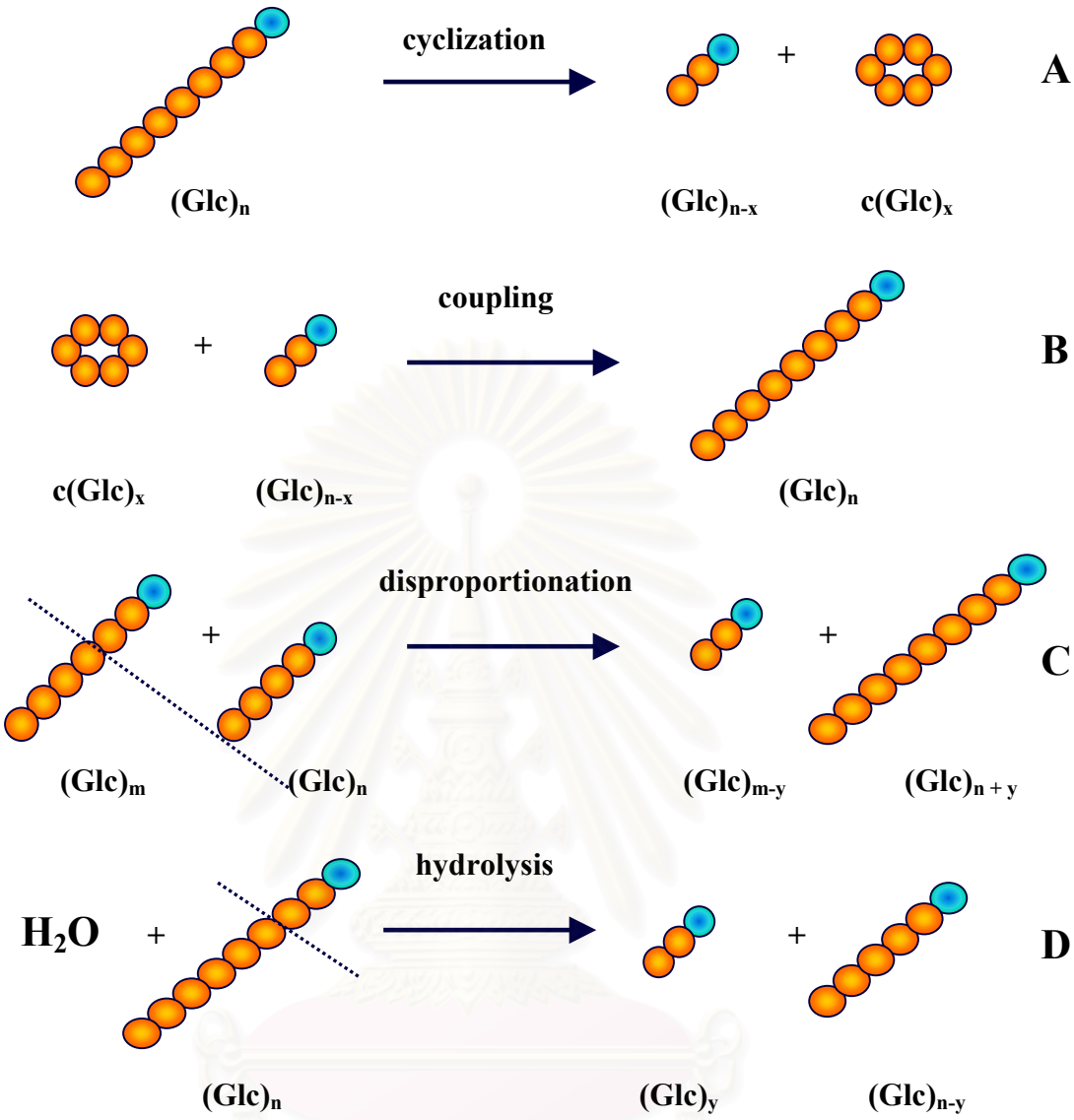


Figure 1.6 Schematic representation of the CGTase catalyzed reactions.

The circle represent glucose residues; the blue circles indicate the reducing end sugars. (A) cyclization, (B) coupling, (C) disproportionation and (D) hydrolysis. (van der Veen *et al*., 2000)

G_m , G_n = α-1,4-D-glucopyranosyl chains

m, n, y = α-D-glucopyranosyl residues

$c(Glc)_x$ = cyclodextrin (x = 6, 7 or 8)

The cyclization reaction 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 maltooligosaccharides (Bender, 1986). The cyclization reaction is efficient for long chain substrates containing 16 – 80 glucopyranosyl residues. If chain length is greater than 100 units, disproportionation reaction dominates. The relationship between chain length of substrate and reaction of CGTase is summarized in Table 1.3. Higher concentration of maltooligosaccharides 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.

CGTases are produced by many bacterial strains e.g. *Klepsiella pneumoniae* (Fogarty, 1983), *Micrococcus* sp. (Yagi *et al*., 1986), *Thermoanaerobacter* sp. (Norman and Jorgensen, 1992), *Brevibacterium* sp. (Mori *et al*., 1994), *Thermoanaerobacterium thermosulfurigenes* (Wind *et al*., 1995), *Paenibacillus sp*. F8 (Larsen *et al*., 1998) and mainly the *Bacillus* sp.. In most cases, the enzymes are extracellular and can be devided into three types: α-, β- and γ- , according to the major type of CD formed (Horikoshi, 1988). The enzymes from different sources show slightly different characteristics such as working pH, temperature, molecular weight and each CGTase yields different ratio of cyclodextrin products.

Table 1.3 Relationship between length of substrate and mechanisms of CGTase

(Szejtli, 1988b)

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

For the industrial production of cyclodextrins, starch is first liquified by a thermostable α-amylase at 105 ^{o}C. After the reaction mixture is allowed to cool to about 50 ^{o}C, it is treated with a bacterial CGTase (Chung *et al.*, 1998), which has an optimum catalytic reaction temperature 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.

A thermostable CGTase with liquifying and cyclizing activities at high temperature would be appropriate for efficient production of CDs. Thermostable CGTases have been found to produce by *Thermoanaerobacter* sp. (Starnes, 1990; Norman and Jorgensen, 1992; Pedersen *et al*., 1995) and *Thermoanaerbacterium thermosulfurigenes* (Wind *et al.*, 1995) both of which are thermophilic microorganisms. Thermophiles can grow at temperature above 45 ^{o}C whereas hyperthermophiles can grow at temperature higher than 80 ^{o}C (JSPS-NRCT, 2000). Hyperthermophilic and thermophilic bacteria require high temperature for culturing and enzyme production, so energy and cost production for culturing these bacteria increased. The use of CGTase from thermotolerant microorganism can solve this problem because thermotolerant microorganisms can be grown at normal temperature as same as mesophiles, but is able to adapt to higher temperatures (between 30-50 ^{o}C) and produce enzymes which are more stable in continuous fermentation than mesophiles.

Thermotolerant bacteria belongs to mesophile which can be discriminated evolutionary and phylogenetically from hypertherm. Some *Bacillus* sp., *Pseudomonas* sp. and *Clostridium* sp. are called thermophile. However, such thermophiles are not phylogenetically identified as a specific bacteria group but included phylogenetically

in the same group as mesophile. Thus, it can be regarded as microorganisms of the phylogenetically same organisms but adapted for higher temperature.

There are not many reports on the discovery of CGTase producing bacterial strain that are thermotolerant but more reports on thermophiles or hyperthermophiles. Some examples of CGTase producing thermophiles are *B. stearothermophilus* ET1 with the optimum temperature for growth at 65 ^{o}C , optimum pH and temperature for CD-forming activity were 6.0 and 80 ^{o}C (Chung *et al.*, 1998) and *B. stearothermophilus* R2 with the optimum for growth at 60 ^{o}C and pH 9.0 and CGTase retained 100% of its initial activity after 30 min at 70 ^{o}C (Kabaivanova *et al.*, 1999). An example of hyperthermophile is *Thermococcus* sp. with the optimum for growth at 85 ^{o}C and pH 7.0. The optimum condition for CD-forming activity were 90-100 ^{o}C and pH 5.0-5.5. This is the first report on the presence of a thermostable CGTase in a hyperthermophilic archaeon (Tachibana *et al.*, 1999).

In Thailand, our research group has been working on mesophile, *Paenibacillus* sp. A11, a strain isolated from South – East Asian soil (Pongsawasdi and Yagisawa, 1987). Recently, a few thermotolerant bacteria have been isolated from hot spring area e.g. *Paenibacillus* sp. strain RB01 from Ratchburi province with the optimum temperature for growth at 37 ^{o}C while exerted highest activity at 40 ^{o}C, pH 10.0. These enzyme formed mainly α- and β-CD with small amounts of γ-CD (Tesana, 2001). Furthermore, *Paenibacillus macerans* strain T16 from Tak province with the optimum temperature for growth at 37 ^{o}C, optimum pH and temperature for CD-forming activity were 10.0 and 37 ^{o}C. The enzyme produced mainly β-CD and quite high α-CD with amounts of γ-CD (Pranommit, 2001).

Since most research reported CGTase production by bacteria only when the starch was contained in the media (Bender, 1981). Therefore, bacteria found around starch factory should have potential in producing CGTase. This research aims at screening for thermotolerant bacterial strains that produce thermostable CGTase from soil, water and starch waste in starch factory. The isolated bacteria would be identified and optimized for the CGTase producing culture conditions. The CGTase produced would also be partially characterized.

The objectives of this research

1. To screen for high CGTase-producing thermotolerant bacteria from soil, water and starch waste in starch factory
2. To identify the isolated bacteria by 16S rRNA gene analysis and biochemical characterization
3. To find optimum condition for CGTase expression
4. To partially purify and characterize 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, U.S.A.
• Electrophoresis unit, Model Mini-protein II Cell	Biorad, U.S.A.
• Gene Amp PCR system, Model 2400	Perkin Elmer, U.S.A.
• High Performance Liquid Chromatography, Model LC-3A	Shimadzu Cooperation, Japan
• Incubator	Haraeus, Germany
• Incubator shaker, controlled environment : Psycotherm	New Brunswick Scientific Co., U.S.A.
• Incubator shaker, Model G76D	New Burnswick Scientific Co., Inc. Edison, N.J., U.S.A.
• pH meter, PHM 83 Autocal pH meter	Radiometer, Denmark
• Scanning electron microscope JSM-35CF	JEOL, Japan
• Spectrophotometer, Du series 1050	Beckman, U.S.A.
• Vortex, Model K-550-GE	Scientific Industries, U.S.A.
• Water bath	Charles Hearson Co. Ltd., U.K.
• Water bath, Shaking	Heto Lab Equipment, Denmark

2.2 Chemicals

Chemicals	Company / Country
• Acetonitrile (HPLC grade)	BDH Laboratory Chemical-Division, England
• Acrylamide	Merck, U.S.A.
• Beef extract	Difco Laboratories, U.S.A.
• Bovine serum albumin fraction V	Sigma Chemical Company, U.S.A.
• Coomassie brilliant blue R-250	BDH Laboratory Chemical-Division, England
• Peptone from meat	Merck, U.S.A.
• Phenolphthalein	BDH Laboratory Chemical-Division, England
• Soluble starch (potato)	Sigma Chemical Company, U.S.A.
• Soluble starch (potato), synthesis grade	Scharlau, Spain
• Standard α-, β- and γ-cyclodextrins	Sigma Chemical Company, U.S.A.
• Standard molecular weight marker protein	Sigma Chemical Company, U.S.A.
• Trichloroethylene (TCE)	BDH Laboratory Chemical-Division, England

The other common chemicals were of reagent grade. Raw rice starch (Three heads elephant brand), corn starch (Maizena), cassava starch (dragon fish brand) and glutinous rice starch (dragon fish brand) were locally purchased.

2.3 Media preparation

2.3.1 Medium I

Medium I consisted of 0.5% beef extract, 0.2% yeast extract, 1.0% polypeptone, 0.2% NaCl, 1.0% soluble starch adjusted to pH 7.2 with 1 N HCl. For solid medium, 1.5% agar was added, Medium I was steriled by autoclaving at 121 ^{o}C for 15 minutes.

2.3.2 Cultivation medium (Horikoshi's medium)

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

2.4 Cultivation of bacteria

2.4.1 Starter inoculum

Bacteria was streaked on solid Medium I, and incubated for 18-24 hours at 37 ^{o}C. Then, isolated colony was transferred into liquid Medium I at 37 ^{o}C with 250 rpm rotary shaking until A_{420} reached 0.5 – 0.8 unit or about 6-8 hours.

2.4.2 Enzyme production

Starter inoculum (1.0%) was transferred into 600 ml Horikoshi' s broth in 2 liter Erlenmeyer flask. Cultivation was performed at 37 ^{o}C for 4 days. Optimum condition was determined by varying pHs, temperatures, types and concentrations of inducers. After cultivation, bacterial cell mass was removed by centrifugation at 3,500 rpm for 15 minutes at 4 ^{o}C. Culture broth with crude enzyme was collected and kept at 4 ^{o}C for activity assay and determination of protein content.

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 and 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 the 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 produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

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 by serial double dilution with 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 [0.2 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 standing overnight at room temperature in the dark.

The activity was expressed as the dilution limit ($1:2^n$), where n is the highest dilution fold that still produces observable CD-TCE precipitate at the interface between upper starch solution layer and lower TCE layer.

2.5.3 Protein determination

Protein concentration was determined by the Coomassie blue 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, water and starch waste samples were collected from starch factory in Bangkok, Nakhon Prathom, Ratchburi, Chachoengsao and Trad provinces. Bacterial screening were as follows.

2.6.1 Primary screening

About 1.0 g of soil, water and starch waste samples were suspended in 99 ml of sterile water and were diluted in serial dilution from 10^{-2} to 10^{-6}. One hundred microlitres of each dilution was spreaded onto Medium I agar plate and incubated at 37 ^{o}C for 1-2 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

The colonies positive for starch hydrolyzing activity were further screened for CD-forming activity on Horikoshi agar plate containing 0.03% (w/v) phenolphthalein and 0.01% (w/v) methyl orange (Yim *et al*., 1997) and incubated at 37 ^{o}C for 3 days. The colonies with yellowish-orange clear zone formed around them on an intense pink background were those exhibiting CGTase-producing property.

CGTase producing activity of the selected colonies were 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

CGTase positive isolates were cultivated in modified Horikoshi broth at the temperature range of 37-55 ^{o}C. Growth and CGTase production were measured by the methods of Fuwa and Nomoto every 12 hours of culturing time. The isolate which produced highest CGTase activity at or above 40 ^{o}C were selected.

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 a series of ethanol concentration (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 such as Gram staining, growth and carbohydrate fermentation using API 50 CHB system (BioMerieux, France). The enzyme assays were also performed.

2.7.3 Identification by 16S rRNA gene fragment amplification

2.7.3.1 Extraction of Chromosomal DNA from bacterial strain

Chromosomal DNA of selected isolate was extracted by SET method (Sambrook and Mainiatis, 1989). It was grown on LB broth at 37 ^{o}C with shaking at 250 rpm for 12-18 hours. Cells were harvested by centrifugation at 3,500 rpm for 15 minutes. Cell pellets (30 µl) were transferred to eppendorf tube and resuspended with 300 µl of SET (Sodium EDTA-Tris). Two hundred microlitres of (5 mg/ml) lysozyme and 100 µl of (10 mg/ml) RNase were added and mixed well. The mixture was incubated at 37 ^{o}C for one hour. 30 µl of 10% SDS and 3 µl of (20 mg/ml) proteinase K were added into the mixture and mixed throughly. The mixture was incubated at 50 ^{o}C for 4-5 hours then 50 µl of 5 M sodium acetate was added and mixed. Equal volume of chloroform-isoamyl (24:1) was added and mixed by inversion. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to another tube and added with equal volume of phenol-chloroform-isoamyl (25:24:1). After gentle mixing inversion, the mixture was centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was removed and precipitated by adding 95% absolute ethanol to the volume of 1 ml, then mix by gentle 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 in 40 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

1.0 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 ^{o}C for 1 minute, primer annealing at 50 ^{o}C for 2 minutes and extension at 72 ^{o}C for 3 minutes.

PCR product was checked by agarose gel electrophoresis and was submitted 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 (http://www.ncbi.nlm.nih.gov). 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 cell culture and CGTase production

Determination of suitable conditions for culturing bacterial isolates with high CGTase production was performed. Parameters such as temperature, pH medium, the type and concentration of starch inducers, and culturing time were varied. The best condition which gave maximum growth and enzyme production was selected for further study.

2.8.1 Optimum pH for growth and CGTase production

Isolated bacteria was cultivated in Horikoshi broth at various pHs (pH adjusted to 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 by HCl or NaOH) at the optimum temperature determined in 2.8.1 with shaking at 250 rpm for 96 hours. Sample was withdrawn every 12 hours for measuring growth and enzyme activity.

2.8.2 Optimum temperature for growth and CGTase production

Starter inoculum (1.0%) of isolated bacteria was transferred into 600 ml of Horikoshi 's broth in 2 liter Erlenmeyer flask and cultivated at the temperature range of 37-45 ^{o}C. Growth and CGTase production were measured by the methods described in 2.5.1, 2.5.2 and 2.5.3 every 12 hours of culturing time.

2.8.3 Effect of various types of starch inducers on CGTase production

Corn starch, cassava starch, rice starch and glutinous rice starch were used to replace soluble starch in Horikoshi medium. Bacterial isolate was cultured in Horikoshi broth, that contained each type of starch at optimum temperature and optimum pH (from 2.8.1 and 2.8.2) with shaking at 250 rpm. Growth and CGTase production were measured every 12 hours of culturing time.

2.8.4 Effect of concentration of starch inducers on CGTase production

The optimum concentration of the suitable starch type was also examined. Horikoshi broth were added with suitable starch type at the concentrations varying from 0.5% to 3.0%. Condition for culturing was the optimum condition as above. 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 the selected bacteria by starch adsorption method of Kato and Horikoshi (1984) with slight modification (Rutchtorn, 1993). Corn starch was oven dried at 100 ^{o}C for 30 minutes and cooled to room temperture. All subsequent steps were conducted at 4 ^{o}C. Corn starch 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 6,000 rpm 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 by centrifugation at 6,000 rpm 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 ^{o}C for further study.

2.10 Biochemical characterization of the enzyme

Partial 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

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

2.10.1.1 Non-denaturing polyacrylamide gel Electrophoresis

Discontinuous PAGE was performed on Mini-Gel Electrophoresis unit (10 x 8 x 7.5 cm) with 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.

2.10.1.2 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.

2.10.2 Coomassie blue staining for protein

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.

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

The electrophoresed 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 ^{o}C for 10 minutes. The gel was then quickly rinsed several times with distilled water and 10 ml of I_2 staining reagent (0.2% I_2 in 2.0% KI) was added for color development at room temperature. The clear zone on the blue background represents starch-degrading activity of the protein.

For SDS-PAGE, the gel was washed with 1.0% Triton X-100 in 0.2 M phosphate buffer, pH 6.0 at 37 ^{o}C with gentle shaking for 3 hours before being soaked in the substrate.

2.10.4 Optimum conditions for enzyme activity

2.10.4.1 Temperature

CGTase was incubated with 0.2% soluble starch in phosphate buffer, pH 6.0 at various temperature 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.

2.10.4.2 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 pHs ranging from 3.0 to 11.0 were used. The buffer solutions used was Universal buffer solutions (Britton and Robinson type), pH 3.0-12.0.

2.10.4.3 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.

2.10.5 Enzyme stability

2.10.5.1 Effect of temperature

Effect of temperature on the stability of enzyme was observed by incubating the enzyme at different temperatures in the range of 20 ^{o}C to 80 ^{o}C, for 60 minutes. The enzyme activity was assayed at the optimal conditions obtained from 2.10.5.

2.10.5.2 Effect of pH

The stability of enzyme at different pHs was investigated by incubating enzyme in various buffers of pH 4.0-11.0 at the optimum temperature determined in section 2.10.5.1) for 60 minutes (buffers used were the same as in 2.10.5.2). The enzyme activity was assayed at the optimal conditions obtained from 2.10.5.

2.10.5.3 Effect of $CaCl_2$

The effect of $CaCl_2$ on enzyme stability was studied by incubating the enzyme sample in the presence of final concentration of 5 mM and 10 mM $CaCl_2$ at optimum condition for enzyme activity for 60 minutes. The enzyme activities were assayed at the optimal conditions obtained from 2.10.5.

2.10.5.4 Effect of substrate

The effect of soluble starch on enzyme stability was also studied by incubating the enzyme sample in the presence of final concentration of 0.2% and 2.0% soluble starch (potato) at optimum condition for enzyme activity for 60 minutes. The enzyme activity was assayed at the optimal conditions obtained from 2.10.5.

2.10.6 Conditions for storage

Partial purified of CGTase was stored at 4 ^{o}C and –20 ^{o}C for 7 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 $CaCl_2$ into enzyme sample before storage.

2.10.7 Identification of cyclodextrin by High Performance Liquid Chromatography

The analysis of cyclodextrins by HPLC was performed (slightly modified from the method of Pongsawasdi and Yagisama, 1987). 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 (0.2%g soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40 ^{o}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 ^{o}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 acetonitrile-water (70:30, v/v) using a flow rate of 1 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) and standard oligosaccharides (G1-G7). For quantitative analysis, peak area corresponded with each cyclodextrin was determined from standard curve.

CHAPTER III

RESULTS

3.1 Screening of thermotolerant bacteria producing CGTase

3.1.1 Primary screening

Starch hydrolyzing microorganisms were screened from soil contaminated with carbohydrates collected from various areas in Thailand and samples from starch factory (soil, water and starch waste). About 97 colonies exhibited clear zone on Medium I agar plates containing soluble starch against dark blue background when stained with I_2 in KI (Table 3.1 and Figure 3.1). Among them, 76 isolates gave high starch hydrolyzing activities with the ratio of clear zone to colony size b/a equal to or greater than 3.0, 20 isolates gave moderate activities with b/a ratio between 2.0 and 3.0 and 1 isolate gave low activity with b/a ratio less than 2.0.

3.1.2 Secondary screening

Amylase positive colonies from the primary screening were checked for CGTase producing capability by inoculated on a Horikoshi medium plate containing 0.03% phenolphthalein and 0.01% methyl orange. After incubation at 37 ^{o}C for 2-3 days, it was found that three isolates (isolate number 50, 92 and 95 in Table 3.1) formed yellow haloed zone on red background indicating CGTase producing activities (Figure 3.2.) The three isolates were named BT01, BD01 and BO01. The bacterial strain BT01 was screened from soil in starch factory (Nakhon Pathom province), BD01 and BO01 were screened from soil contaminated with carbohydrates in Trad province and Pathumthani province, respectively. The CGTase positive isolates were confirmed for cyclodextrin forming activity by the cyclodextrin trichloroethylene (CD-TCE) complex precipitation method described in 2.5.2.

Table 3.1 Amylase and Amylase - CGTase positive isolates screened from soil contaminated with carbohydrates and samples from starch factory.

Province	Site	Temp. (°C)	Isolate number	Diameter (cm.)		
				Colony (a)	Clear zone (b)	(b)/(a)
Bangkok	Starch industry (water)	30	1	0.2	0.3	1.5
	Thai vermicelli industry (water)	40	2	0.2	0.8	4.0
			3	0.2	0.6	3.0
			4	0.3	0.7	2.3
	Bakery industry (water)	30	5	0.2	0.7	3.5
			6	0.1	0.3	3.0
	Bakery industry (soil)	-	7	0.3	1.1	3.7
			8	0.2	0.4	2.0
			9	0.2	0.6	3.0
Ratchaburi (Banpong)	Soil (cassava)	-	10	0.3	0.7	2.3
			11	0.3	0.8	2.7
			12	0.1	0.4	4.0
			13	0.1	0.5	5.0
			14	0.1	0.3	3.0
			15	0.2	0.5	2.5
			16	0.1	0.3	3.0
Rayong	Starch industry (water)	42	17	0.2	0.7	3.5
			18	0.1	0.4	4.0
			19	0.1	0.5	5.0
			20	0.2	0.5	2.5
			21	0.1	0.4	4.0
			22	0.1	0.4	4.0
			23	0.1	0.3	3.0
			24	0.1	0.7	7.0

Table 3.1 Continued

Province	Site	Temp. (°C)	Isolate number	Diameter (cm.)		
				Colony (a)	Clear zone (b)	(b)/(a)
Rayong	Starch industry (water)	42	25	0.1	0.5	5.0
			26	0.1	0.6	6.0
			27	0.2	0.4	2.0
			28	0.1	0.5	5.0
			29	0.1	0.3	3.0
	Starch industry (soil)	-	30	0.3	1.1	3.7
			31	0.4	1.4	3.5
			32	0.2	0.5	2.5
			33	0.2	0.7	3.5
			34	0.1	0.8	8.0
			35	0.1	0.3	3.0
			36	0.1	0.5	5.0
			37	0.1	0.4	4.0
			38	0.2	0.5	2.5
			39	0.1	0.4	4.0
			40	0.8	1.7	2.1
			41	0.1	0.2	2.0
			42	0.1	0.4	4.0
Nakhon Phatom	Starch industry (soil)	-	43	0.1	0.5	5.0
			44	0.4	0.9	2.3
			45	0.3	0.7	2.3
			46	0.3	0.8	2.7
			47	0.1	0.7	7.0
			48	0.1	0.4	4.0
			49	0.2	0.4	2.0
			50*	0.4	0.9	2.3
			51	0.1	0.8	8.0

Table 3.1 Continued

Province	Site	Temp. (°C)	Isolate number	Diameter (cm.)		
				Colony (a)	Clear zone (b)	(b)/(a)
Nakhon Phatom	Starch industry (soil)	-	52	0.1	0.8	8.0
			53	0.4	1.0	2.5
			54	0.2	0.5	2.5
			55	0.1	0.3	3.0
			56	0.1	0.6	6.0
			57	0.1	0.5	5.0
			58	0.1	0.5	5.0
			59	0.1	0.5	5.0
			60	0.1	0.6	6.0
			61	0.1	0.6	6.0
			62	0.2	0.4	2.0
			63	0.1	0.4	4.0
			64	0.1	0.7	7.0
			65	0.1	0.5	5.0
	Starch industry (water)	-	66	0.1	0.4	4.0
			67	0.1	0.8	8.0
			68	0.1	0.9	9.0
			69	0.1	0.4	4.0
			70	0.1	0.4	4.0
			71	0.1	0.4	4.0
			72	0.1	0.4	4.0
			73	0.1	0.9	9.0
			74	0.1	0.4	4.0
			75	0.1	0.3	3.0
			76	0.1	0.4	4.0
			77	0.1	0.3	3.0
			78	0.1	0.5	5.0
			79	0.1	0.3	3.0

Table 3.1 Continue

Province	Site	Temp. (°C)	Isolate number	Diameter (cm.)		
				Colony (a)	Clear zone (b)	(b)/(a)
Nakhon Phatom	Starch industry (starch waste)	-	80	0.1	0.6	6.0
			81	0.1	0.5	5.0
			82	0.1	0.5	5.0
			83	0.1	0.4	4.0
			84	0.1	0.4	4.0
			85	0.1	0.4	4.0
			86	0.1	0.6	6.0
			87	0.1	0.4	4.0
			88	0.1	0.3	3.0
			89	0.1	0.6	6.0
			90	0.1	0.5	5.0
Trad	Soil	-	91	0.2	0.4	2.0
			92*	0.6	1.2	2.0
			93	0.1	0.4	4.0
Pathumthani	Soil (cassava)	-	94	0.1	0.5	5.0
			95*	0.1	0.3	3.0
			96	0.1	0.4	4.0
			97	0.1	0.3	3.0

* indicates isolate with Amylase-CGTase positive

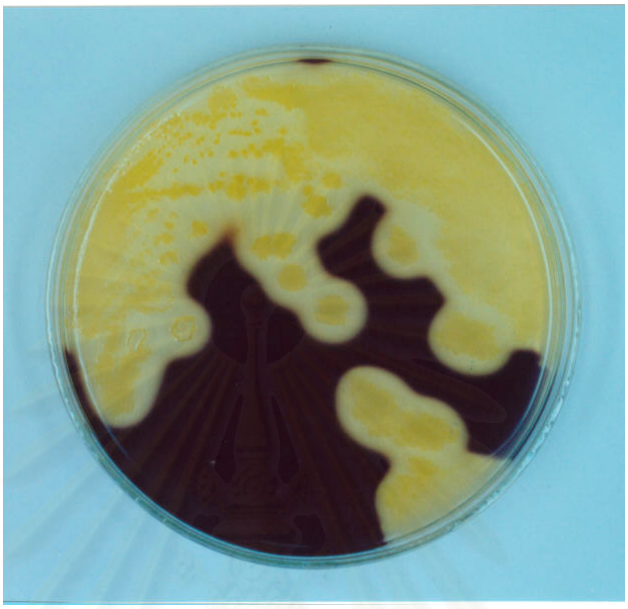


Figure 3.1 Amylase producing bacteria on solid Medium I stained with Iodine solution.

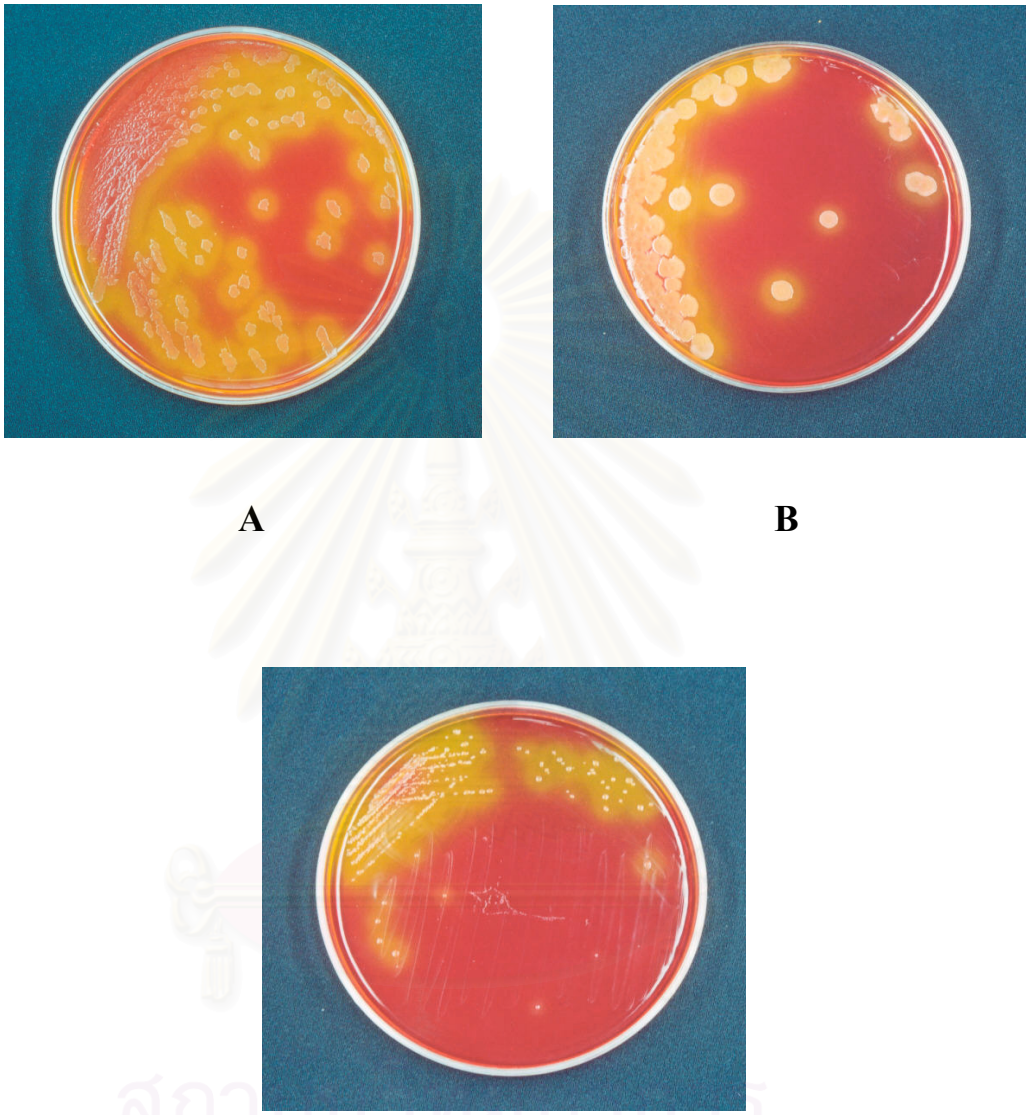


Figure 3.2 CGTase producing bacteria on solid Horikoshi medium : BT01 (A), BD01 (B) and BO01 (C)

3.1.3 Selection of thermotolerant isolates with high CGTase activity.

The three bacterial strains with CGTase producing activity were inoculated in Horikoshi broth incubated at 37, 40 and 45 ^{o}C and monitored for cell growth, dextrinizing activity and cyclodextrin production by CD-TCE as shown in Figure 3.3-3.8. It was found that BT01 grew well at 37, 40 and 45 ^{o}C, BD01 at 37 and 40 ^{o}C but not at 45 ^{o}C and BO01 at 37 ^{o}C only. Only BT01 produced high CGTase from 36 hours onwards at 37, 40 and 45 ^{o}C. BD01 produced CGTase only at 37 and 40 ^{o}C with longer incubation time of 72 hours, while BO01 could only produce CGTase at 37 ^{o}C. Therefore, strain BT01 was chosen for further study because of its high optimal growth temperature and its relatively high CGTase activity compared with other strains.

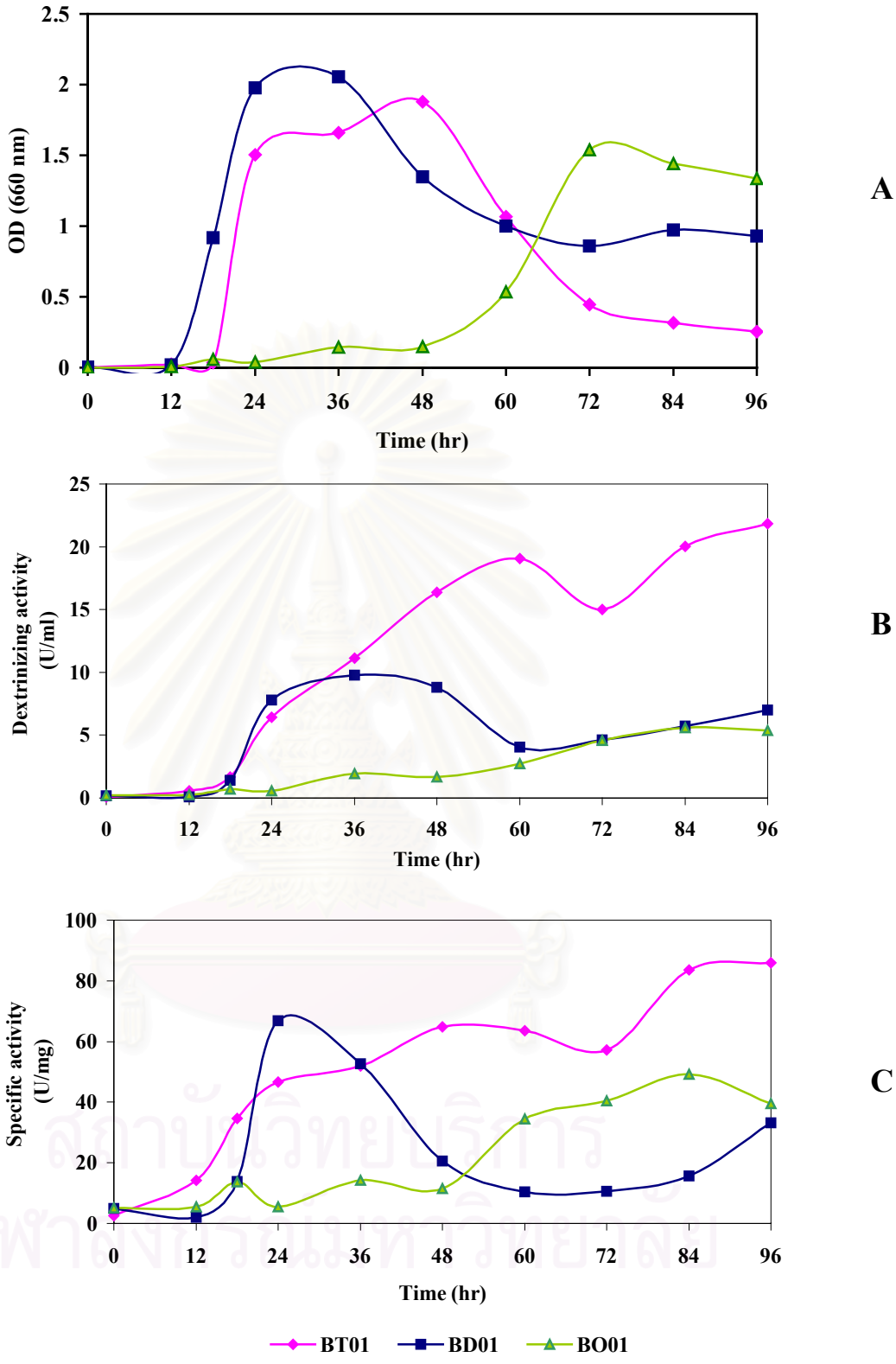


Figure 3.3 Bacterial isolates BT01, BD01 and BO01 cultured at 37 °C, pH 10.0: Growth profile (A), Dextrinizing activity (B) and Specific activity (C)

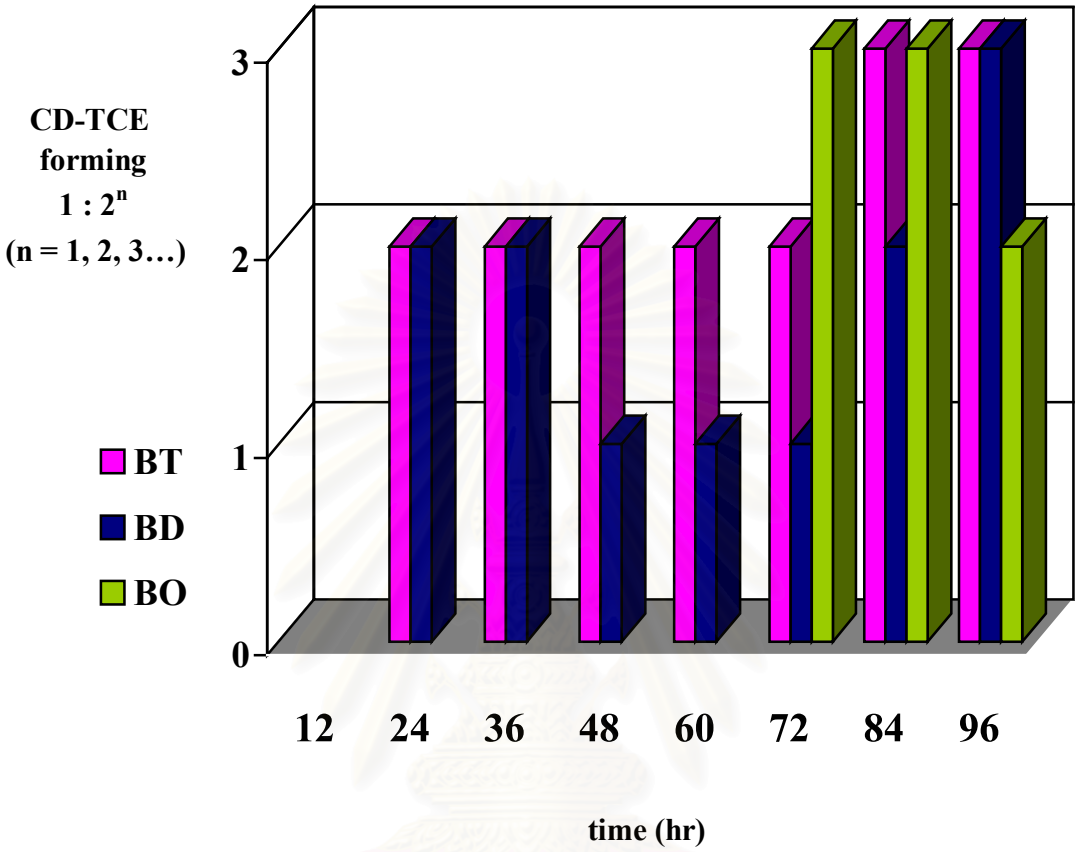


Figure 3.4 CGTase production of BT01, BD01 and BO01 at 37 °C and pH 10.0 monitored by CD-TCE method.

33 μg protein of each crude enzyme sample was used.

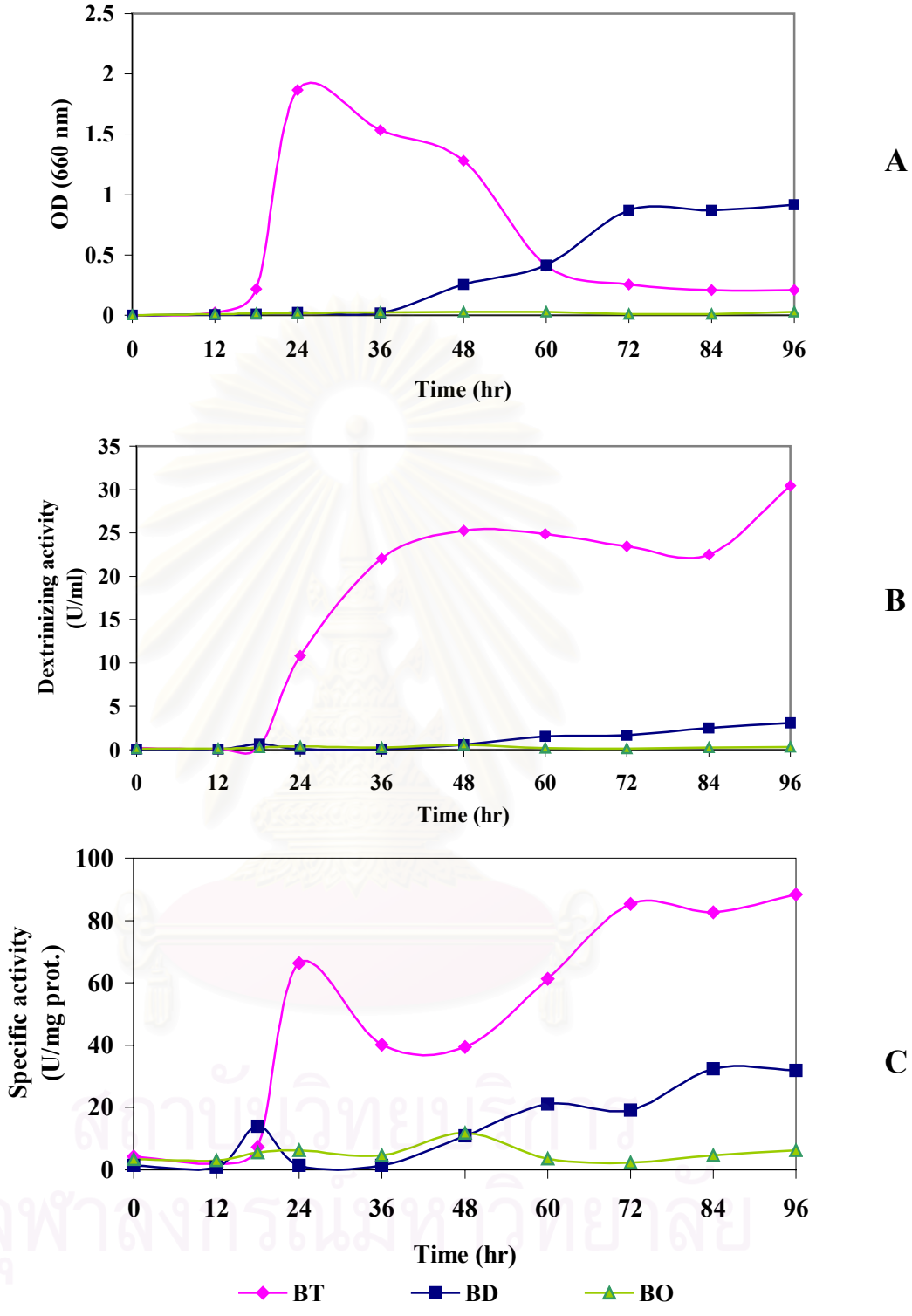


Figure 3.5 Bacterial isolates BT01, BD01 and BO01 cultured at 40 ^{o}C, pH 10.0: Growth profile (A), Dextrinizing activity (B) and Specific activity (C)

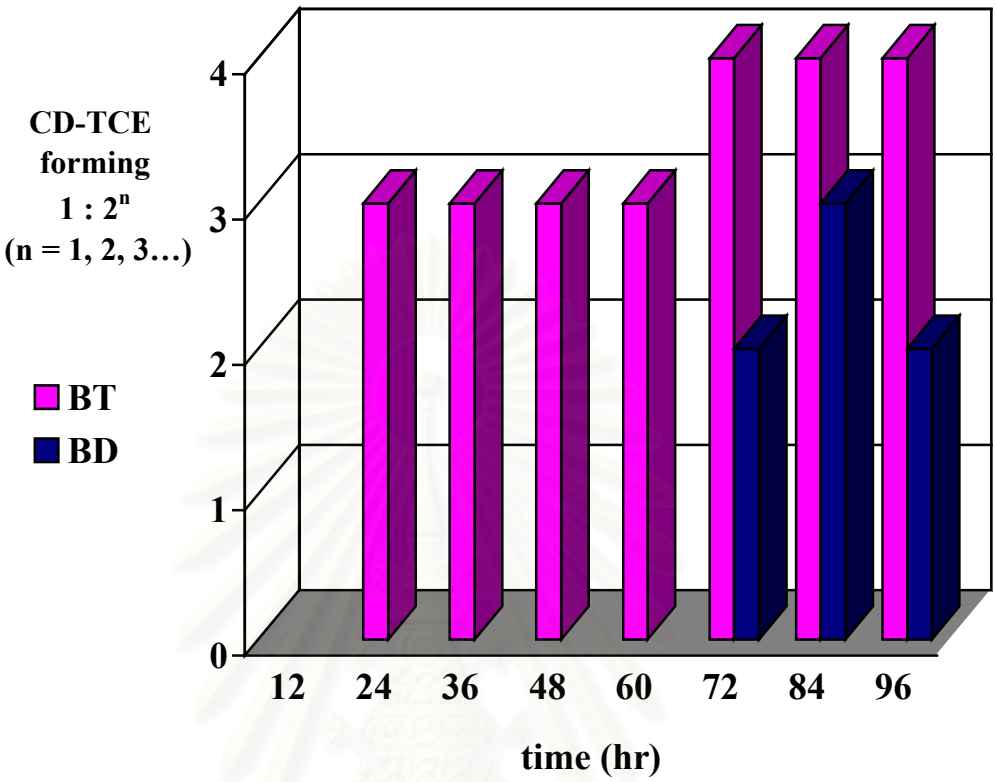


Figure 3.6 CGTase production of BT01, BD01 and BO01 at 40 °C and pH 10.0 monitored by CD-TCE method.

33 μg protein of each crude enzyme sample was used.

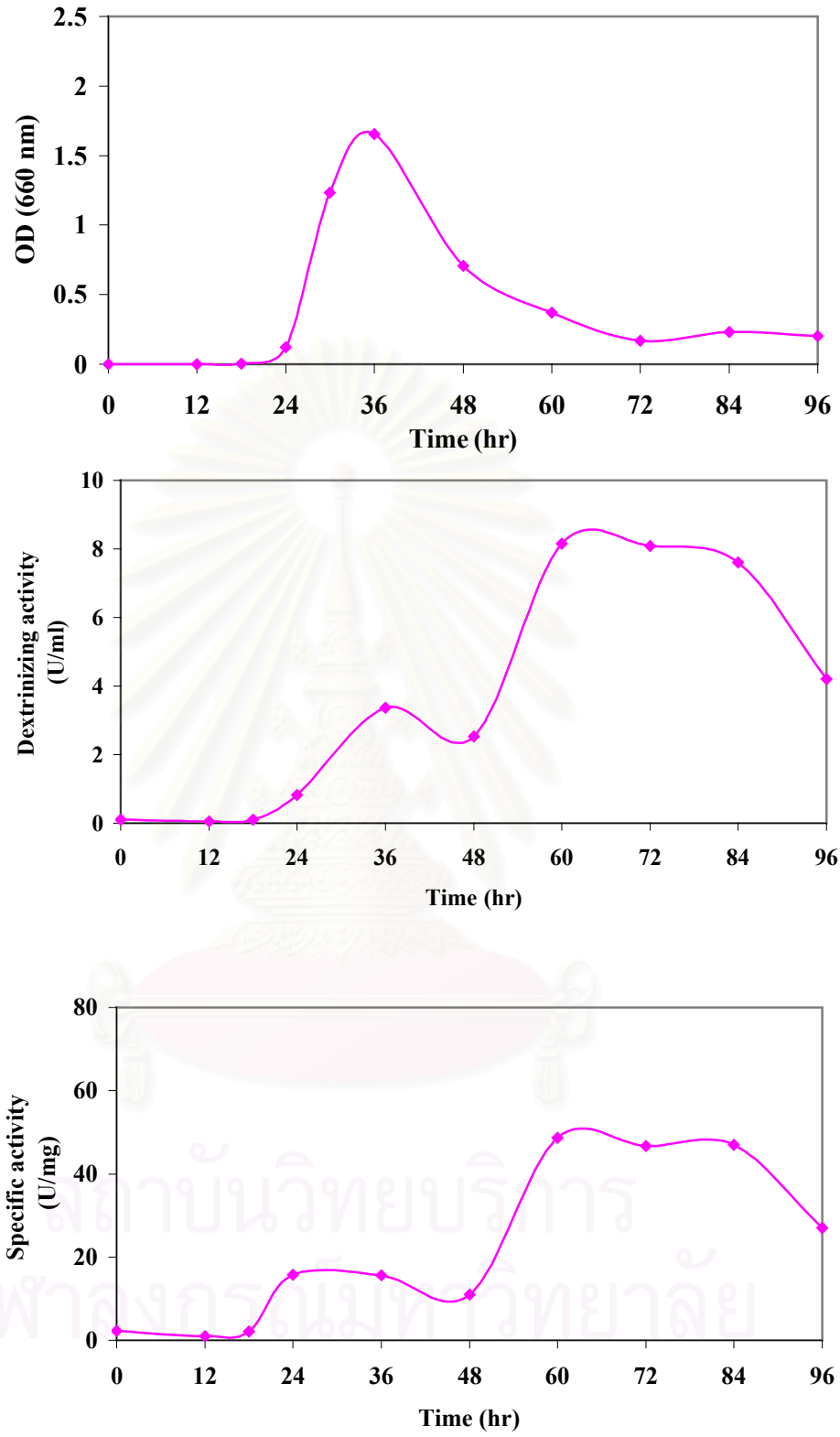


Figure 3.7 Bacterial isolates BT01, BD01 and BO01 cultured at 45 ^{o}C, pH 10.0: Growth profile (A), Dextrinizing activity (B) and Specific activity (C)

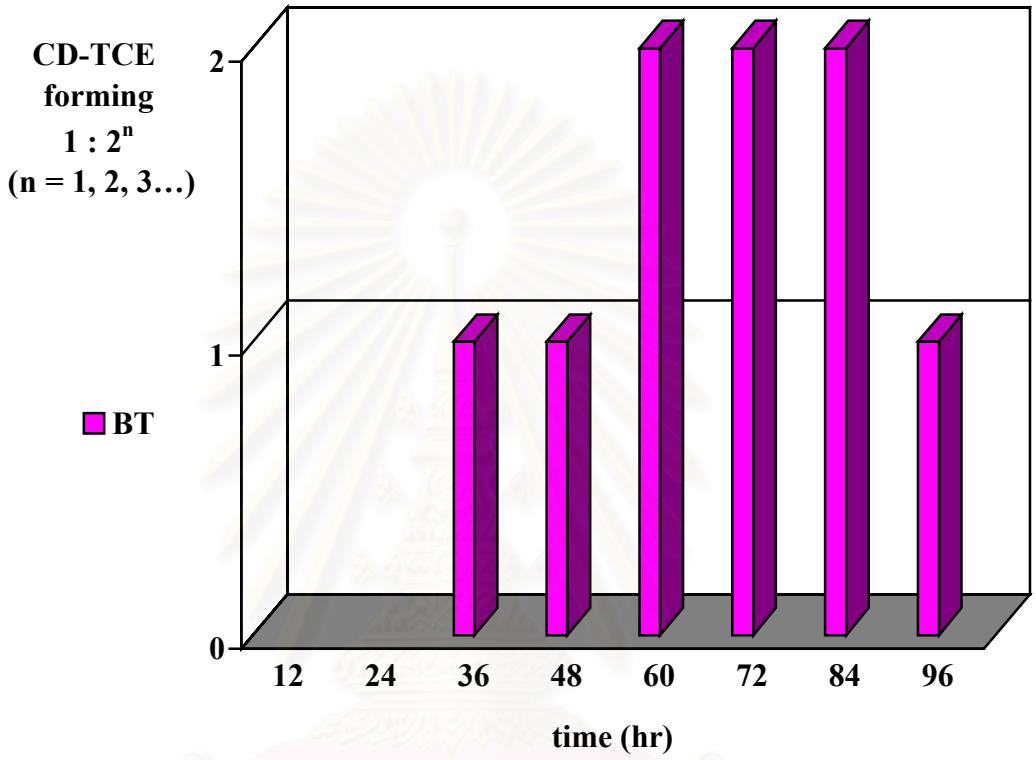


Figure 3.8 CGTase production of BT01, BD01 and BO01 at 45 °C and pH 10.0 monitored by CD-TCE method.

33 μg protein of each crude enzyme sample was used.

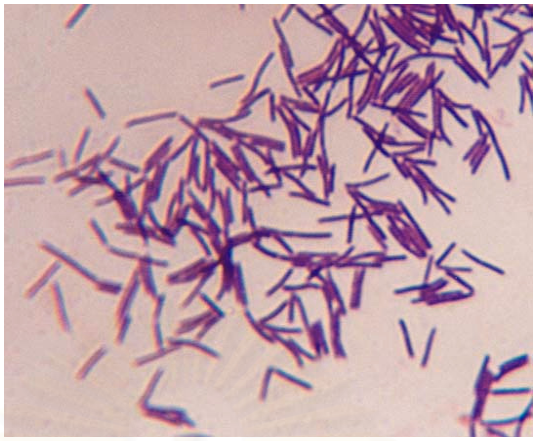
3.2 Identification of the CGTase producing microorganisms

3.2.1 Morphological characterization

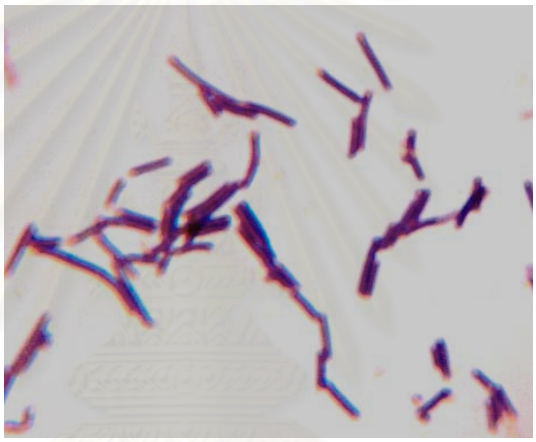
BT01, BD01 and BO01 were screened by gram staining and found to be Gram positive bacilli (Fig 3.9). Scanning electron micrograph showed BT01 as rod shaped cells with length of 2.5-3 μm and width of 0.3-0.5 μm (Fig 3.10A), while BD01 and BO01 were also rod shape with approximate size of 0.4 x 2.3-3.0 and 0.4 x 1.3-2.0 μ m, respectively (Fig 3.10B, 3.10C).

3.2.2 Biochemical characterizations

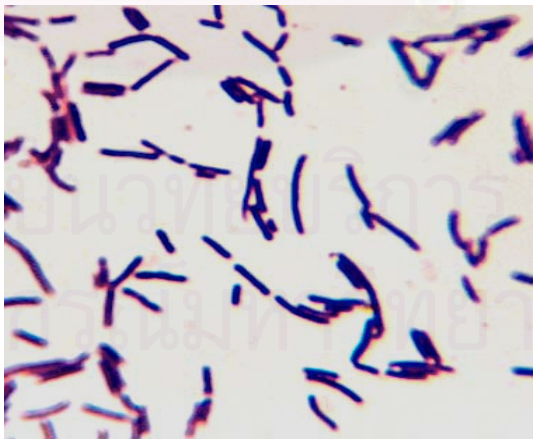
Biochemical characterizations of the three isolates were determined based on the classification systems presented in Bergey's manual of systematic bacteriology. Carbohydrate fermentation using API CHB system (BioMerieux, France) were tested (Fig 3.11) and listed in Table 3.2. BT01 belonged to genus *Bacillus* and was identified as *Bacillus circulans*. BD01 and BO01 were identified as *Brevibacillus brevis*.



A

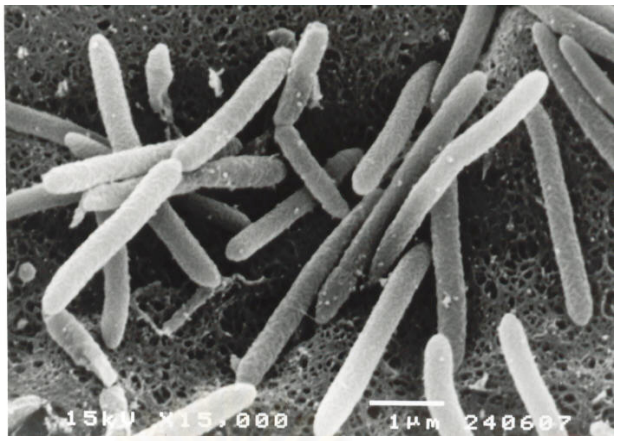


B

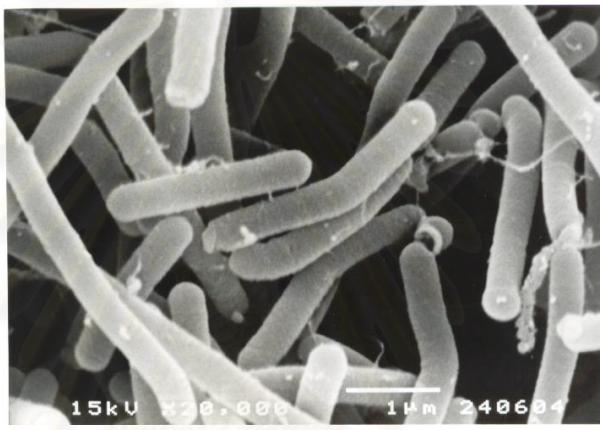


C

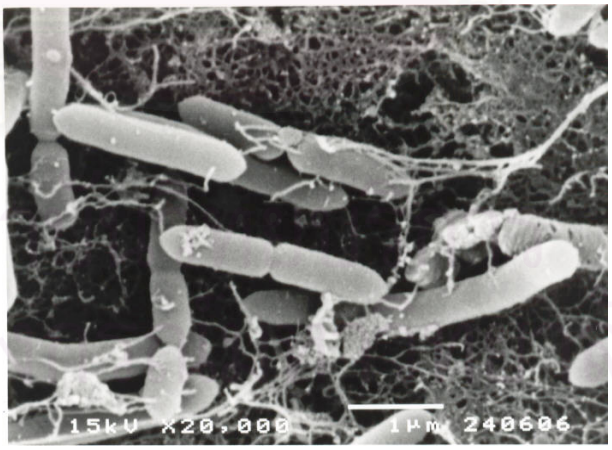
Figure 3.9 Morphology of BT01 (A), BD01 (B) and BO01 (C) were examined by light microscope (x100) using Gram staining



A

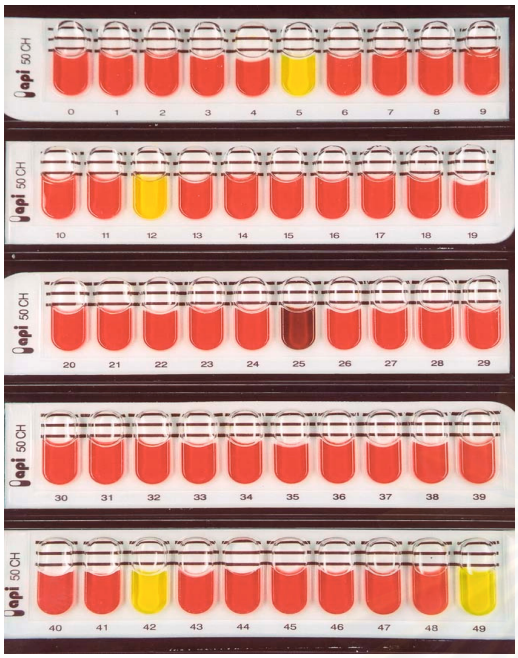


B

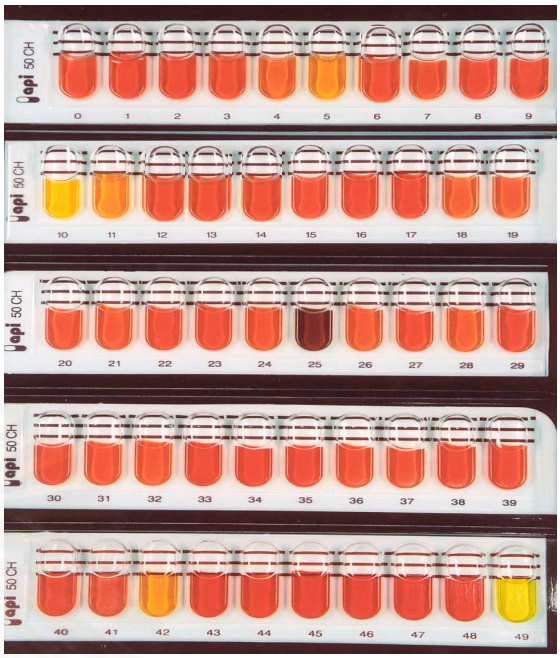


C

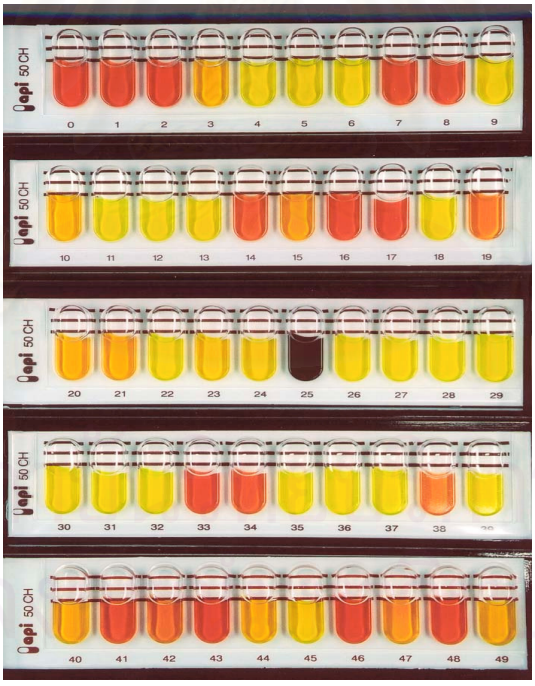
Figure 3.10 Morphology of BT01 (A), BD01 (B) and BO01 (C) were examined by Scanning Electron Microscopy (SEM)



(A)



(B)



(C)

Figure 3.11 Biochemical characteristics of BD01 (A), BO01 (B) and BT01 (C) were examined by API 50 CHB system

Table 3.2 Biochemical characteristics of three CGTase positive isolates

Characteristics	Reaction			Characteristics	Reaction		
	BD	BO	BT		BD	BO	BT
Control	-	-	-	α-Methyl-D-Mannoside	-	-	+
Glycerol	-	-	-	α-Methyl-D-Glucoside	-	-	+
Erythritol	-	-	-	N-Acetyl-Glucosamine	-	-	+
D-arabinose	-	-	+	Amygdalin	-	-	+
L-arabinose	-	-	+	Arbutin	-	-	+
Ribose	+	+	+	Esculin	+	+	+
D-xylose	-	-	+	Salicin	-	-	+
L-xylose	-	-	-	Cellobiose	-	-	+
Adonitol	-	-	-	Maltose	-	-	+
β-Methyl-D-xyloside	-	-	+	Lactose	-	-	+
Galactose	-	+	+	Melibiose	-	-	+
Glucose	-	+	+	Sucrose	-	-	+
Fructose	+	-	+	Trehalose	-	-	+
Mannose	-	-	+	Inulin	-	-	-
Sorbose	-	-	-	Melezitose	-	-	-
Rhamnose	-	-	+	Raffinose	-	-	+
Dulcitol	-	-	-	Amidon	-	-	+
Inositol	-	-	-	Glycogen	-	-	+
Mannitol	-	-	+	Xylitol	-	-	+
Sorbitol	-	-	+	Gentiobiose	-	-	+

Table 3.2 Biochemical characteristics of CGTase positive isolates (continued)

Characteristics	Reaction			Characteristics	Reaction		
	BD	**BO**	**BT**		**BD**	**BO**	**BT**
D-Turanose	-	-	+	D-Arabitol	-	-	+
D-lyxose	-	-	-	L-Arabitol	-	-	-
D-Tagatose	+	+	+	Gluconate	-	-	+
D-Fucose	-	-	-	2-Keto-Gluconate	-	-	-
L-Fucose	-	-	+	5-Keto-Gluconate	+	+	+

3.2.3 Identification by 16S rRNA gene fragment amplification

Bacterial strain BT01 was the only isolate chosen for further study and was further identified by 16S rRNA analysis. Chromosomal DNA was extracted and the 16S rRNA gene of the strain was amplified. PCR products with the size of 1.5 kb was obtained (Figure 3.12) and the sequence was determined by Bioservice Unit, National Science and Technology Development Agency (NSTDA) Thailand (Figure 3.13). The 16S rRNA gene sequence was aligned and the result indicated that BT01 was similar to *Paenibacillus* sp. (98%) (Table 3.3).

3.3 Optimization of conditions for culturing and CGTase production

3.3.1 Starter condition

BT01 was cultivated in Medium I at pH 6.0-10.0 as starter inoculum. The optimum pH giving rapid cell growth (OD 0.5-0.8) was 9.0 after incubated at 37 ^{o}C with shaking at 250 rpm for 4 hours (Figure 3.14).

3.3.2 Culturing condition

3.3.2.1 Optimum pH

The effect of pH on cell growth and enzyme production were studied. BT01 was cultivated in Horikoshi broth at 40 ^{o}C, the temperature which BT01 produced highest CGTase activity (Figure 3.6), at different pHs between 6.0-11.0. It was found that BT01 could grow at pH 6.0-10.0 while not at pH 11.0 and the growth rate at pH 6.0 was lower than pH 7.0-10.0 (Figure 3.15A). Starch dextrinizing activity could be observed from pH 6.0 to pH 10.0 with the highest activity at pH 10.0 (Figure 3.15B and 3.15C). For the CD-forming activity, the highest and significantly different activity was observed at pH 10.0 as shown in Figure 3.16. As a result, the optimum pH of medium for BT01 was 10.0. Hence, this pH was used for further experiments.

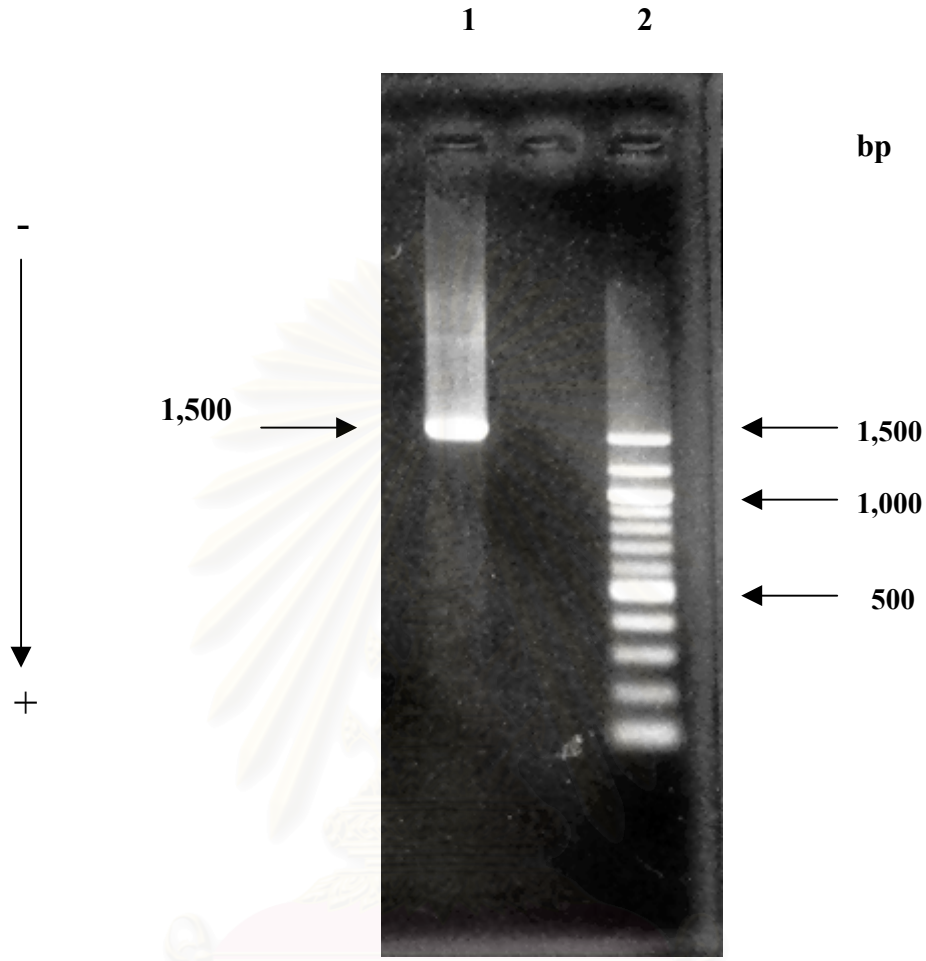


Figure 3.12 PCR product of 16S rRNA gene from BT01

Lane 1 The amplified product of 16S rRNA gene

Lane 2 Standard DNA marker (100 bp)

```
                                        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 CTGCTCTT-- ---------- ----------  480
---------- ---------- -CAGCAGCCG CGGTAATAAT AC-GGGGCAA 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 ---------- ----------  960
---------- ---------- ---------- ---------- ---------- ---------- 1020
---------- ------CATG GCTGTCGTCA GCTCGT---- ---GATGTTG 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 AGCCCTATCG 1500
GAAGGTGCGG CTGGATCACT TCCCTAC                                    1527
```

pA 5′ AGAGTTTGATCCTGGCTCAG 3′

pD 5′ CAGCAGCCGCGGTAATAATAC 3′

pF 5′ CATGGCTGTCGTCAGCTCGT 3′

Figure 3.13 Nucleotide sequence of 16S rRNA from BT01. The primers used for sequencing are shown.

Table 3.3 Blast result of the 16S rRNA gene fragment of BT01 using primer A, D andF

pA

Source	% homology
***Paenibacillus* sp. AG430**	**98**
***Paenibacillus* sp. 38-2**	**98**
***Paenibacillus* sp. strain 324**	**98**

pD

Source	% homology
***Paenibacillus* sp. AG 430**	**97**
***Paenibacillus* sp. 38-2**	**98**
***Paenibacillus* sp. strain 324**	**97**

pF

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

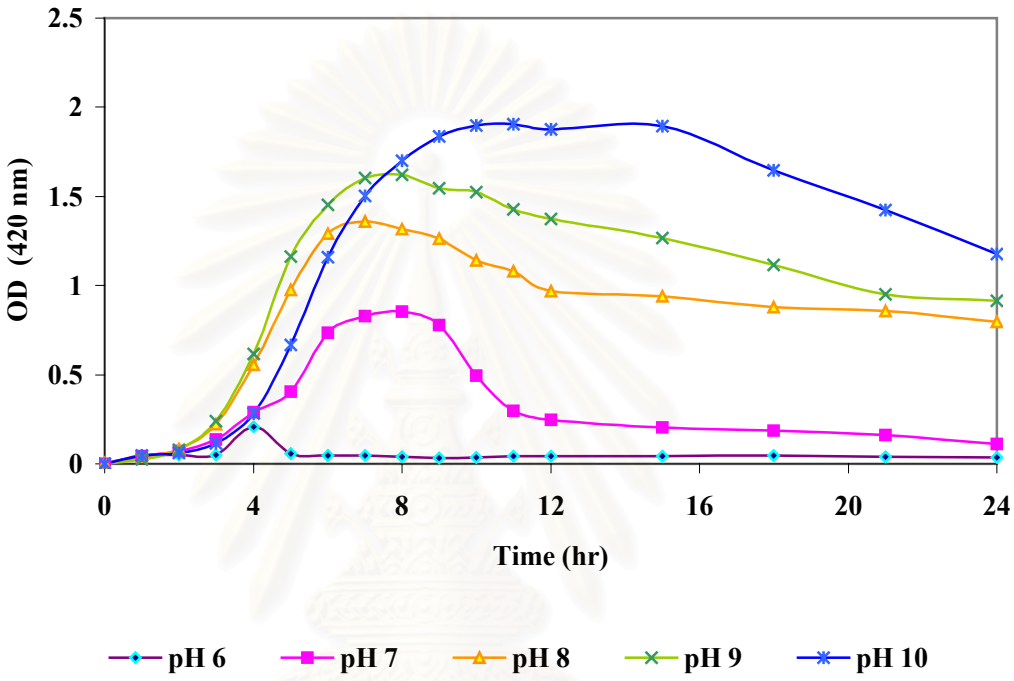


Figure 3.14 Growth profile of BT starter at 37 ^{o}C with various pH range 6-10

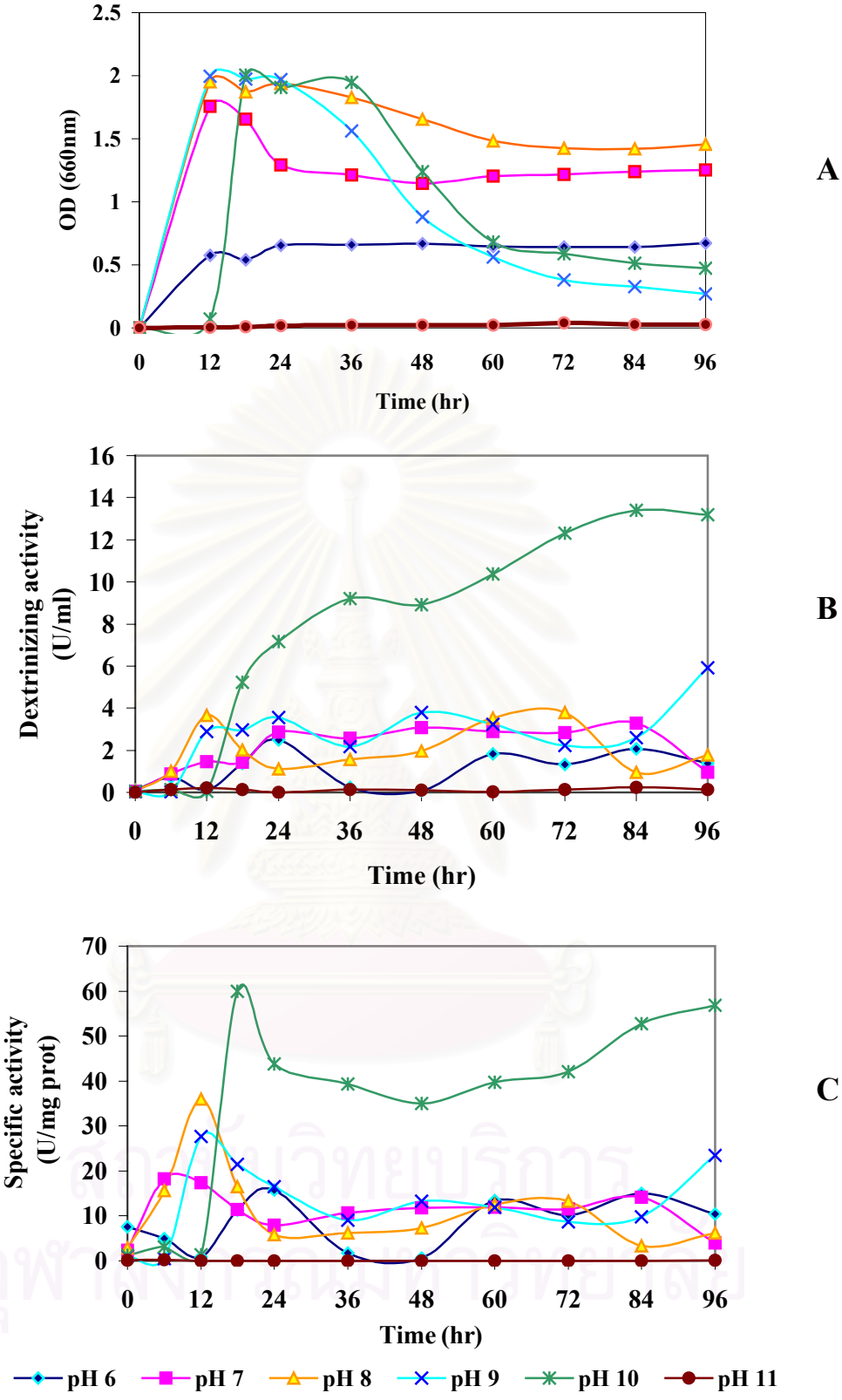


Figure 3.15 Growth profile (A), Dextrinizing activity (B) and Specific activity (C) of BT01 at various pH 6.0-11.0, 40 ^{o}C

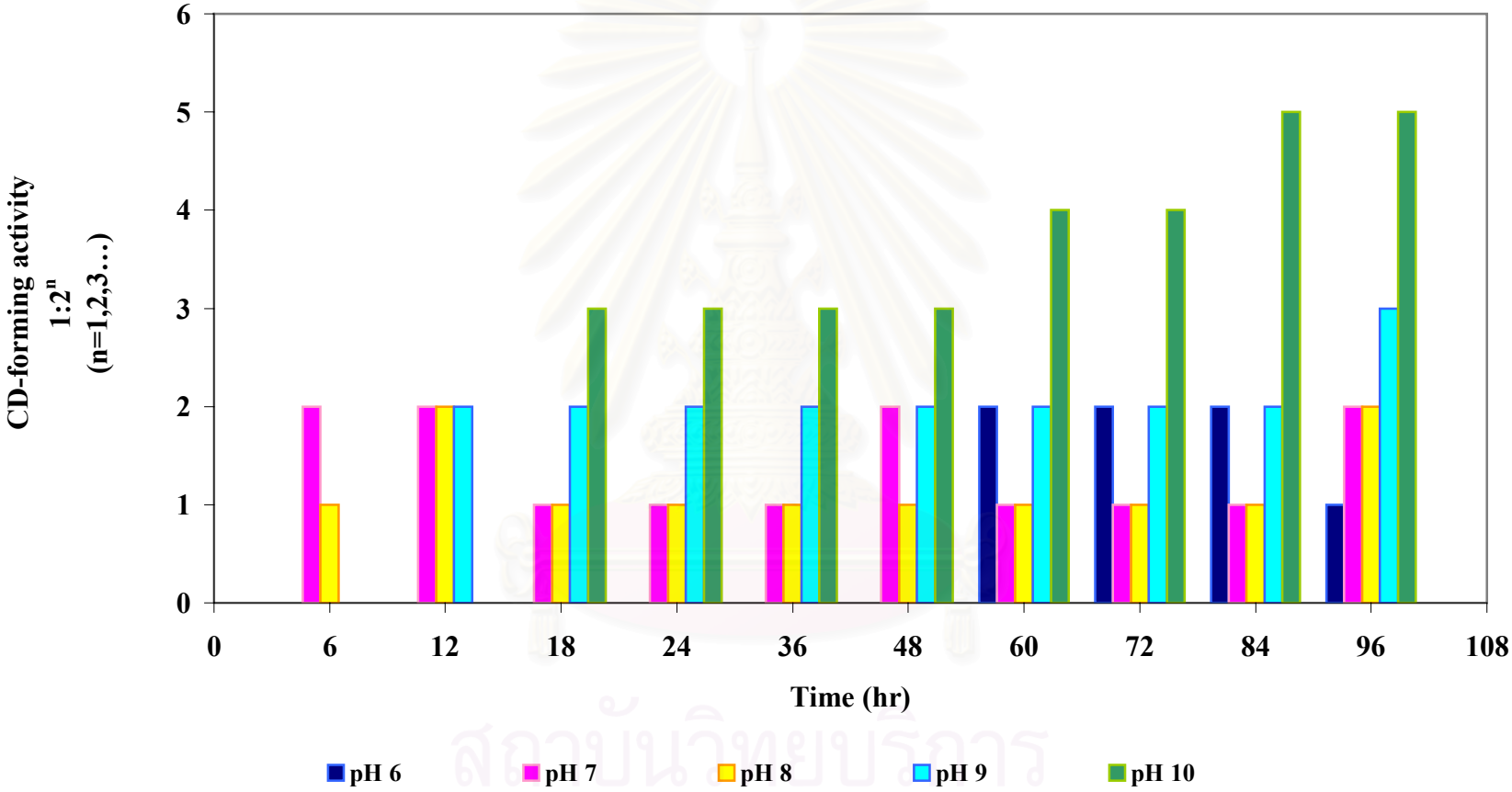


Figure 3.16 CGTase production of BT01 with various pHs at 40 ^{o}C (33 µg protein of each crude enzyme sample was used)

3.3.2.2 Optimum temperature

To confirm the temperature which BT01 produced highest CGTase activity, BT01 was cultivated in Horikoshi broth pH 10.0 (result obtained from section 3.3.2.1) at 37, 40 and 45 ^{o}C with shaking at 250 rpm. Cell growth and enzyme activity were observed. It was found that BT01 grew at temperatures between 37 and 45 ^{o}C. The growth increased sharply and reached stationary phase at 24 hours and decreased slowly after 36 hours. The growth rate was highest at 37 and 40 ^{o}C (Figure 3.17A) with maximum activity of starch dextrinizing activity and specific activity obtained after 84 hours and 24 hours respectively at both temperatures (Figure 3.17B and 3.17C). The CD-forming activity was observed after 24 hours (Figure 3.18) but highest at 72-96 hours.

Considering the results above, the optimum temperature for enzyme production was incubation at 40 ^{o}C for 72 hours. Consequently, this temperature was used for further experiments.

3.3.2.3 Effect of various types of CGTase inducer

The effect of various types of starch on cell growth and induction of CGTase activity were investigated. BT01 was cultivated in Horikoshi broth containing 1.0 % (w/v) soluble starch or replacing soluble starch with different types of starch at 40 ^{o}C and pH 10.0. It was found that BT01 could grow in all types of starch and had the same growth pattern (Figure 3.19A). For the CGTase production, the result suggested that cassava starch and soluble starch were the most suitable sources for starch dextrinizing activity as shown in Figure 3.19B and 3.19C and CD-forming activity (Figure 3.20). Rice starch and glutinous starch were poor sources for inducing enzyme activity whereas corn starch could induce CD-forming activity at longer culture time of 72 hours but gave higher CD-forming activity than rice starch

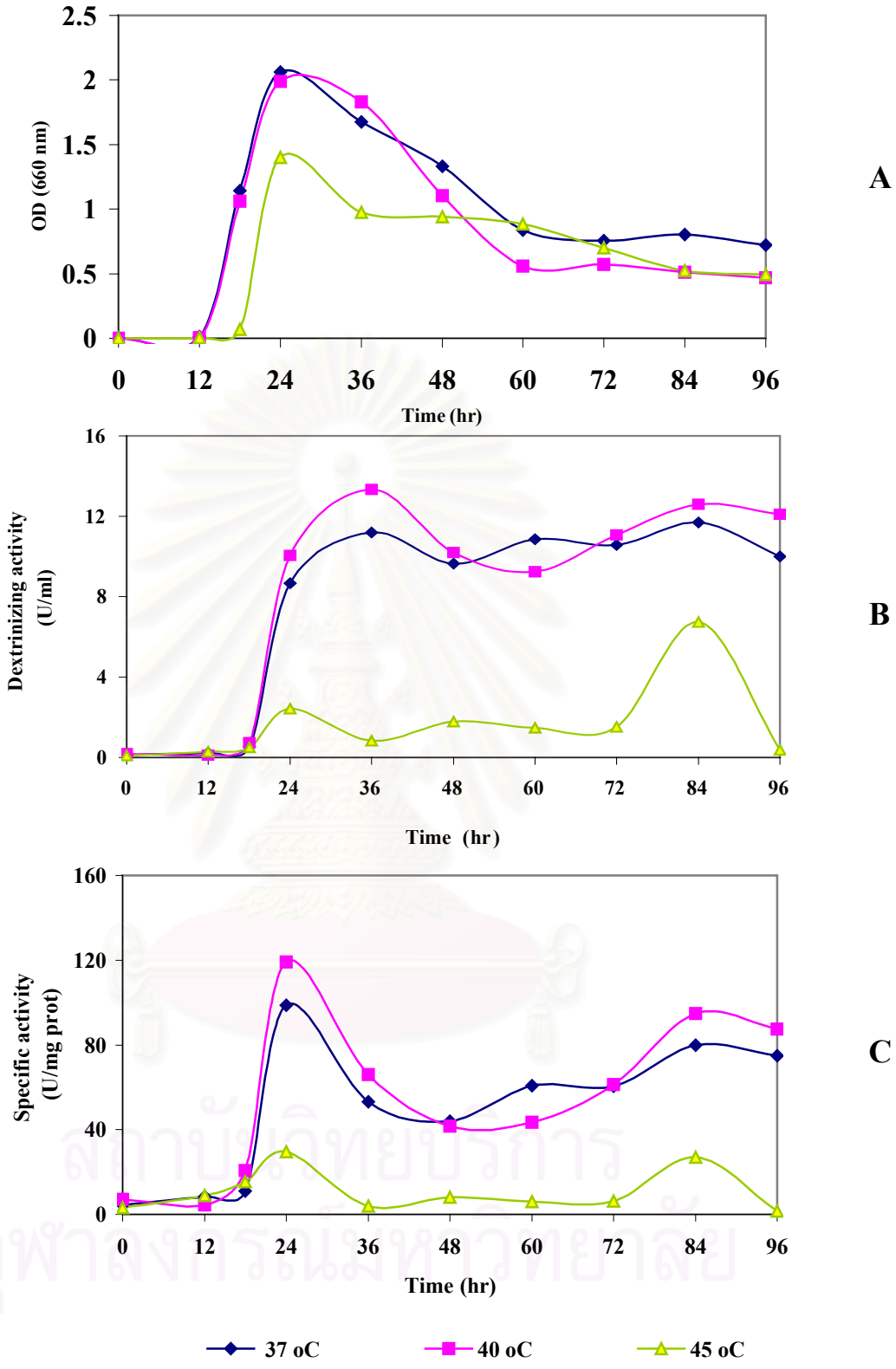


Figure 3.17 Growth profile (A), Dextrinizing activity (B) and Specific activity (C) of BT01 at different temperature, pH 10.0

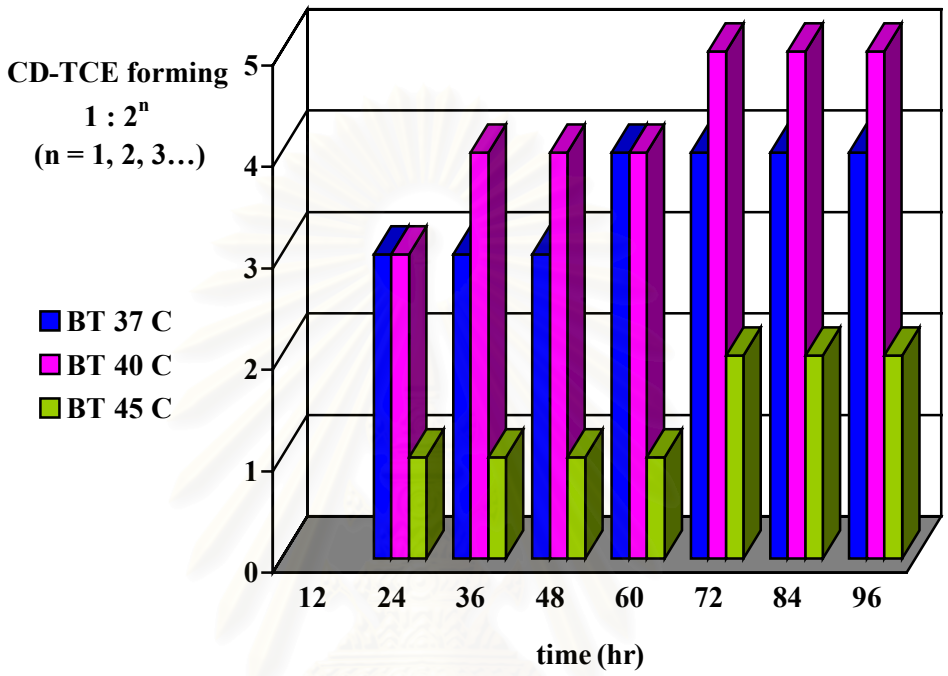


Figure 3.18 CGTase production of BT01 at different temperature and pH 10.0 monitored by CD-TCE method.

33 μg protein of each crude enzyme sample was used.

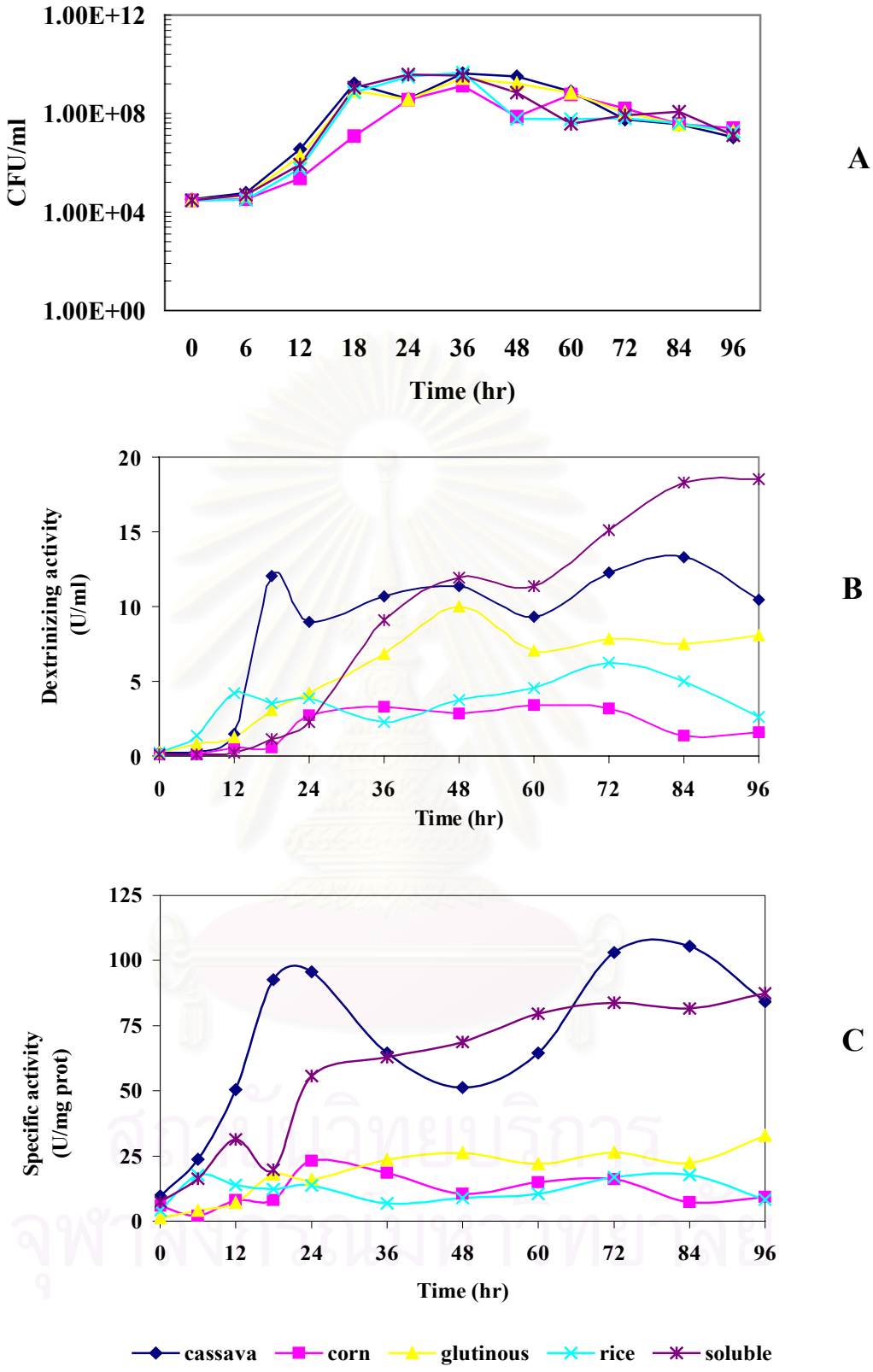


Figure 3.19 Growth profile (A), Dextrinizing activity (B) and Specific activity (C) of BT01 in various types of starch at 40 ^{o}C and pH 10.0

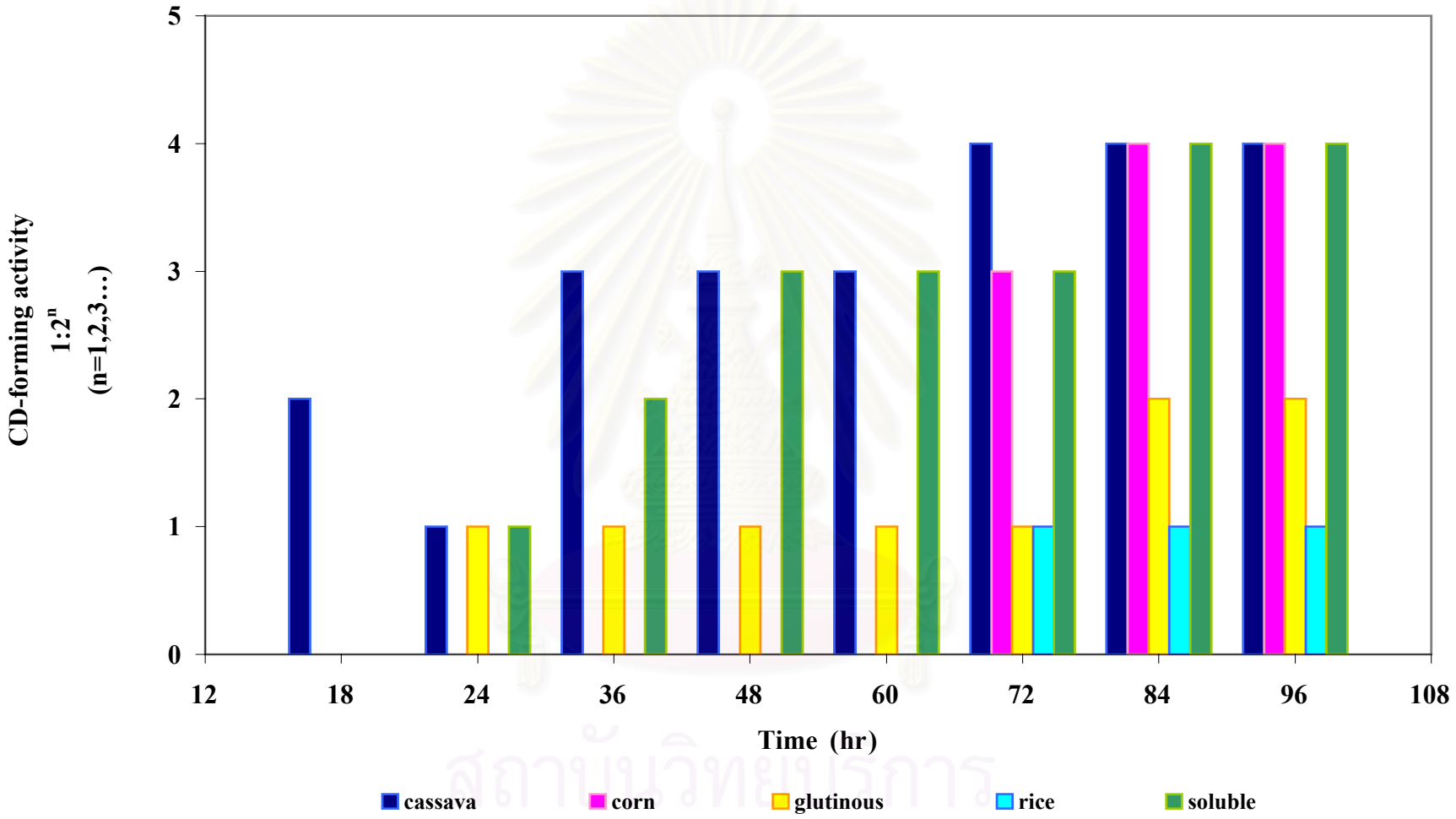


Figure 3.20 CGTase production of BT01 with various types of starch at 40 ^{o}C (33 µg protein of each crude enzyme sample was used)

and glutinous starch. As a result, soluble starch and cassava starch exhibited the same level of CD-forming activity so, they were tested for further experiment.

3.3.2.4 Effect of concentration of starch inducer

Further optimization of the cultivation medium for a higher enzyme yield was performed by varying concentrations of cassava starch and soluble starch from 0.5% to 3.0%. For cassava starch, it was found that the growth rate at 0.5 and 1.0% exhibited the same pattern, while at 1.5, 2.0, 2.5 and 3.0% had the similar pattern (Figure 3.21A). For enzyme production, BT01 grown in 0.5% of cassava starch exhibited the highest dextrinizing activity after 72 hours as shown in Figure 3.21B and 3.21C. The data shown in Figure 3.22 indicated that CD-forming activity at 0.5 and 1.0% were at similar level and the maximum activity obtained at 96 hours in 0.5% cassava starch was 2^6. In soluble starch, it was found that BT01 showed the same growth at pattern at 0.5 and 1.0% whereas growth at 1.5, 2.0, 2.5 and 3.0% had the same pattern (Figure 3.23A). Maximum activity of dextrinizing activity as found in medium which contained 0.5% soluble starch after about 72 hours (Figure 3.23B and 3.23C). CD-forming activity at 0.5 and 1.0% were at the same level and increased corresponding with increasing time of culturing. On the other hand, CD-forming activity at 1.5, 2.0, and 2.5% decreased as shown in Figure 3.24. Comparison of the result from 0.5% cassava starch and 0.5% soluble starch suggested that 0.5% soluble starch was the most suitable sources for enzyme production.

Therefore, the optimum conditions for culturing BT01 for high production of CGTase enzyme were using Horikoshi medium at pH 10.0, containing 0.5% soluble starch and incubated at 40 ^{o}C for 72 hours.

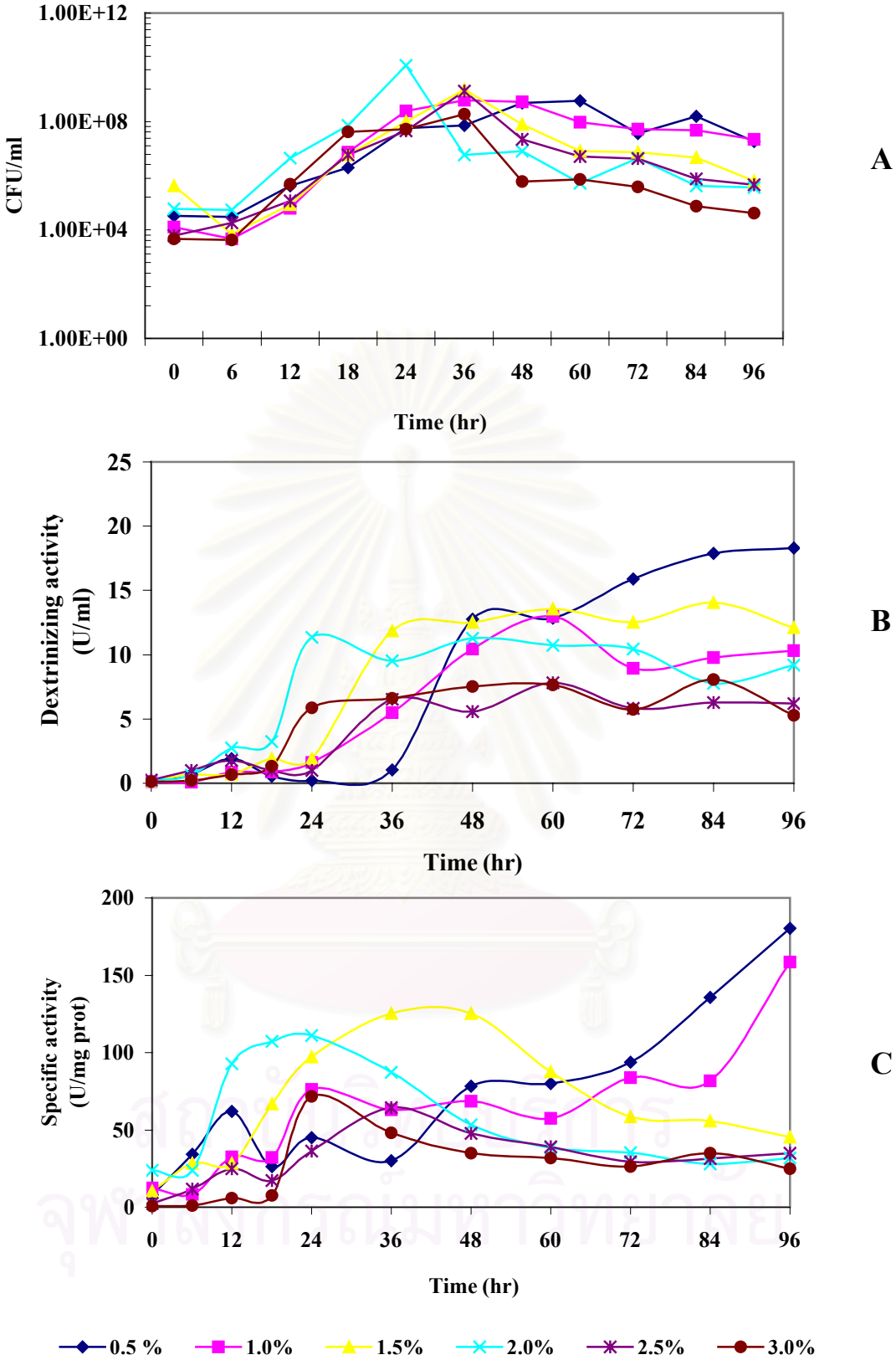


Figure 3.21 Growth profile (A), Dextrinizing activity (B) and Specific activity (C) of BT01 in various concentrations of cassava starch at 40 ^{o}C and pH 10.0

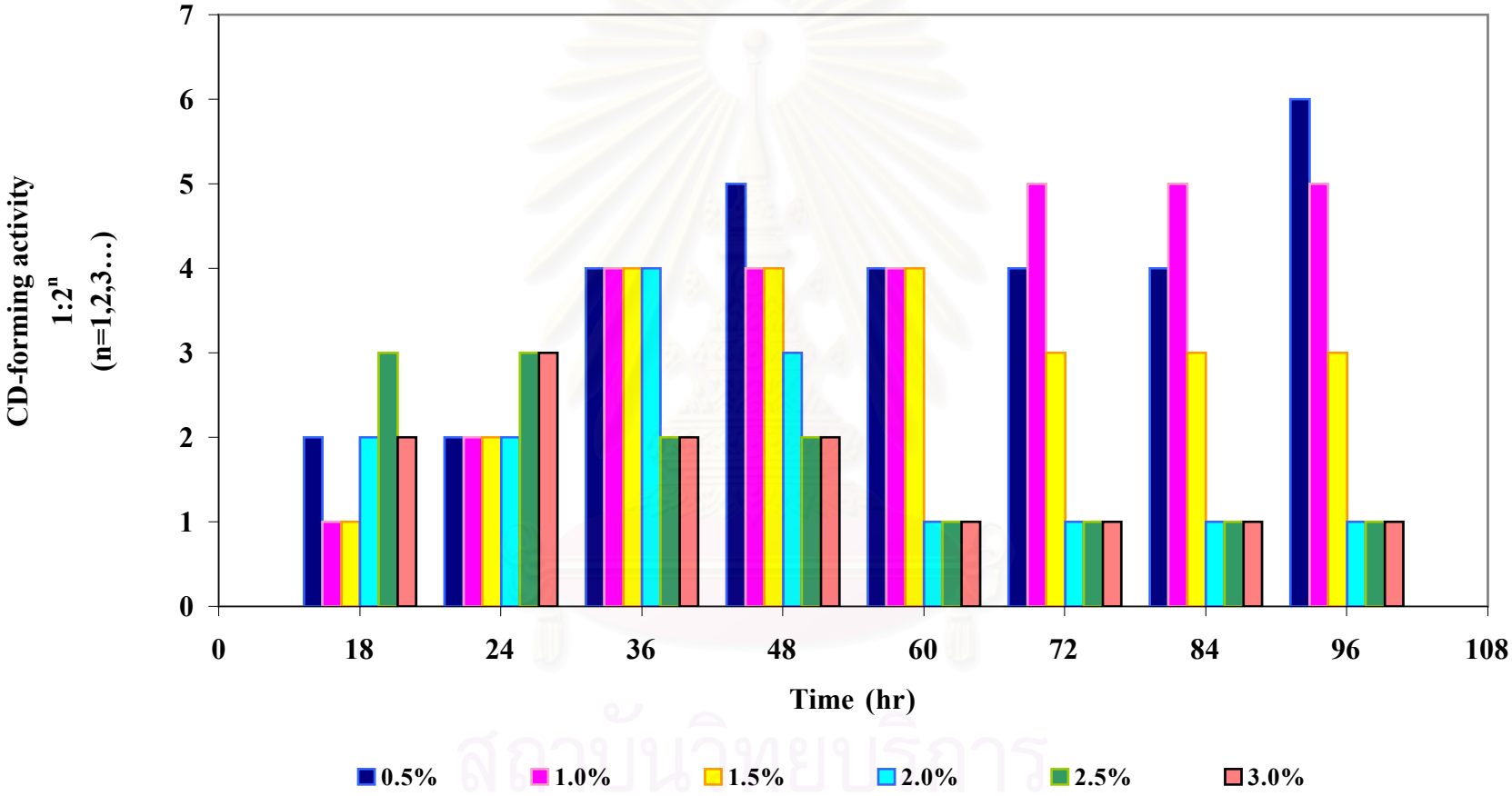


Figure 3.22 CGTase production of BT01 in various concentrations of cassava starch at 40 ^{o}C and pH 10.0 (33 μg protein of each crude enzyme sample was used)

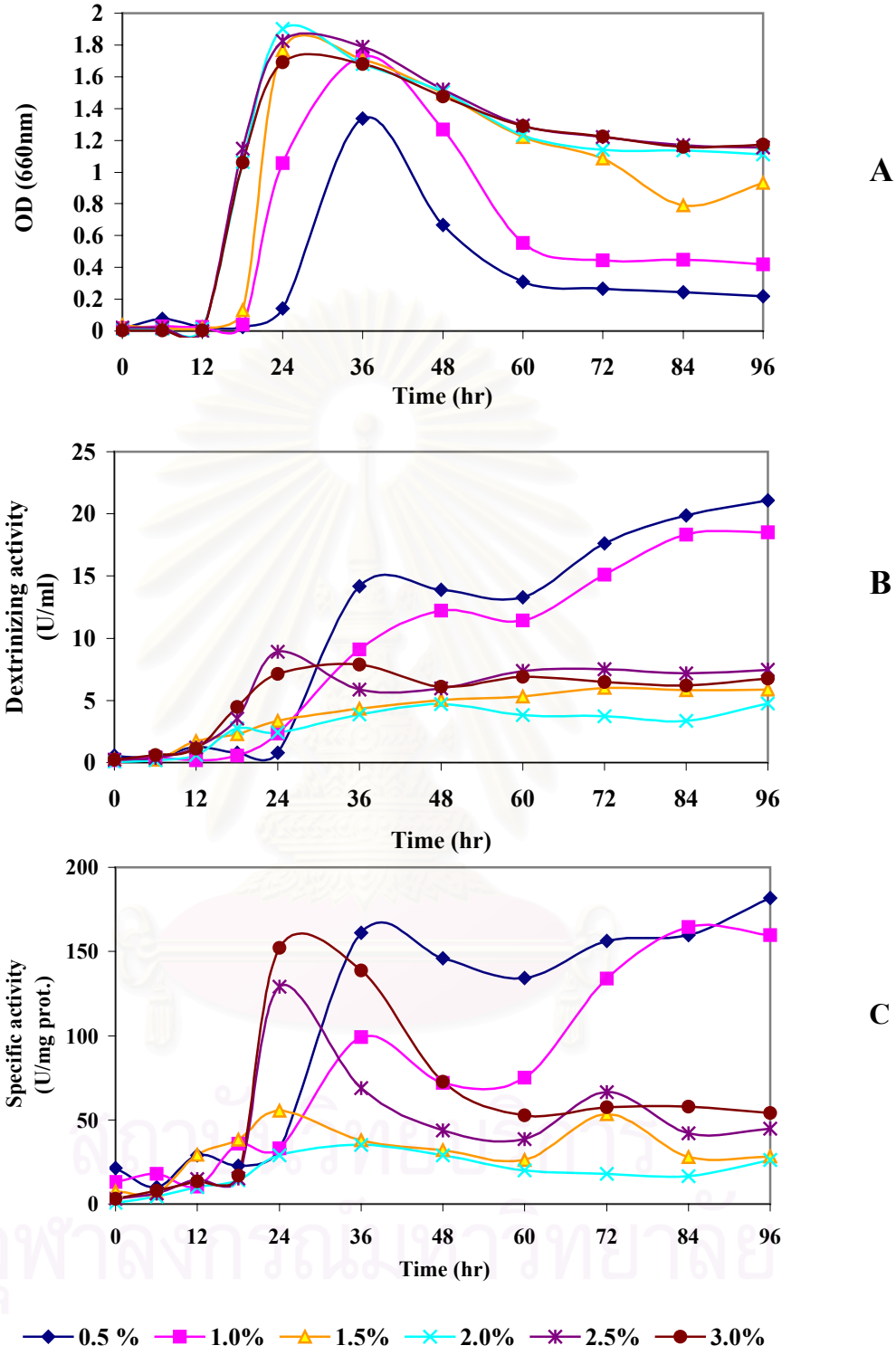


Figure 3.23 Growth profile (A), Dextrinizing activity (B) and Specific activity (C) of BT01 in various concentrations of soluble starch at 40 °C and pH 10.0

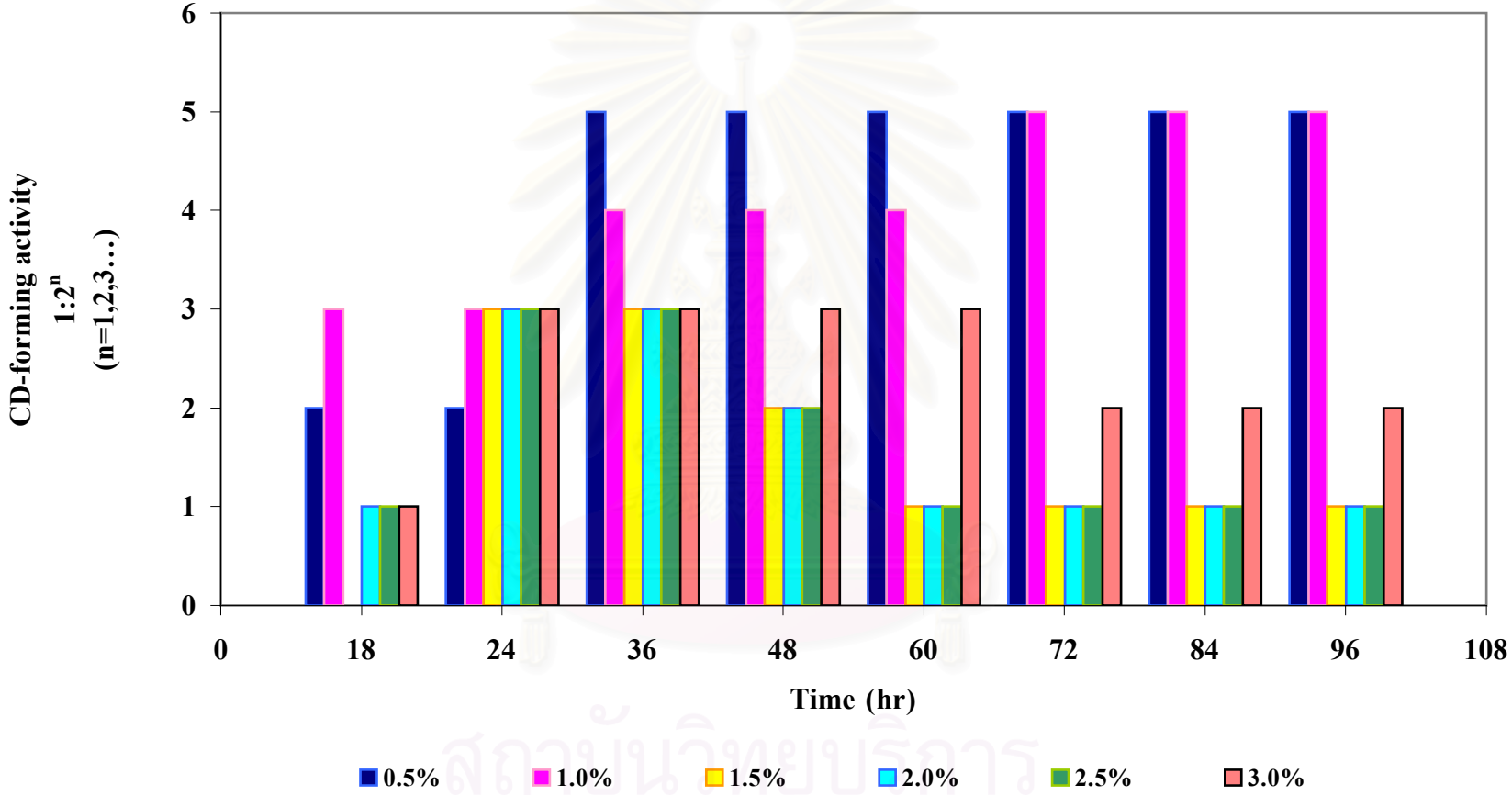


Figure 3.24 CGTase production of BT01 in various concentrations of soluble starch at 40 °C and pH 10.0 (33 μg protein of each crude enzyme sample was used)

3.4 Partial purification and Biochemical characterization of CGTase

3.4.1 Partial purification of CGTase from BT01

The bacterial strain BT01 was cultivated in Horikoshi broth containing 0.5% soluble starch pH 10.0 at 40 ^{o}C with continuous shaking at 250 rpm for 72 hours. The culture was centrifuged to remove cells and supernatant which contained crude enzyme was collected. Crude enzyme was partially purified by starch adsorption as described in methods section 2.9. The BT01 enzyme was sufficiently purified for primary characterization using the starch adsorption as the sole purification step. The purification fold and recovery of CGTase obtained were 28 and 65.1% respectively as shown in Table 3.4. The partial purified CGTase was used for further experiments on primary characterization of the enzyme.

The enzyme from partial purification by starch adsorption was analyzed for purity and protein pattern by non-denaturing polyacrylamide gel electrophoresis. The activity staining was performed to compare with protein staining. The result shown in Figure 3.25 showed many protein bands in the crude enzyme but only 2-3 bands in the partial purified form. The amylolytic activity stain indicated that the CGTase might contain at least 3 isoforms. All amylolytic activity bands gave only a single band in SDS-PAGE as presented in Figure 3.26. From the mobility on SDS-PAGE, the molecular weight of CGTase was estimated to be 79 kDa (Figure 3.27).

Table 3.4 Purification of CGTase from bacterial strain BT01

Step	Volume (ml)	Dextrinizing activity (U/ml)	Total activity ($Ux10^3$)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yeild (%)	CD-TCE ($1:2^n$)
Crude enzyme	1200	18.50	22.2	255.6	86.85	1.0	100	2^5
Starch adsorption	250	57.81	14.45	6.0	2408.75	28	65.10	2^9

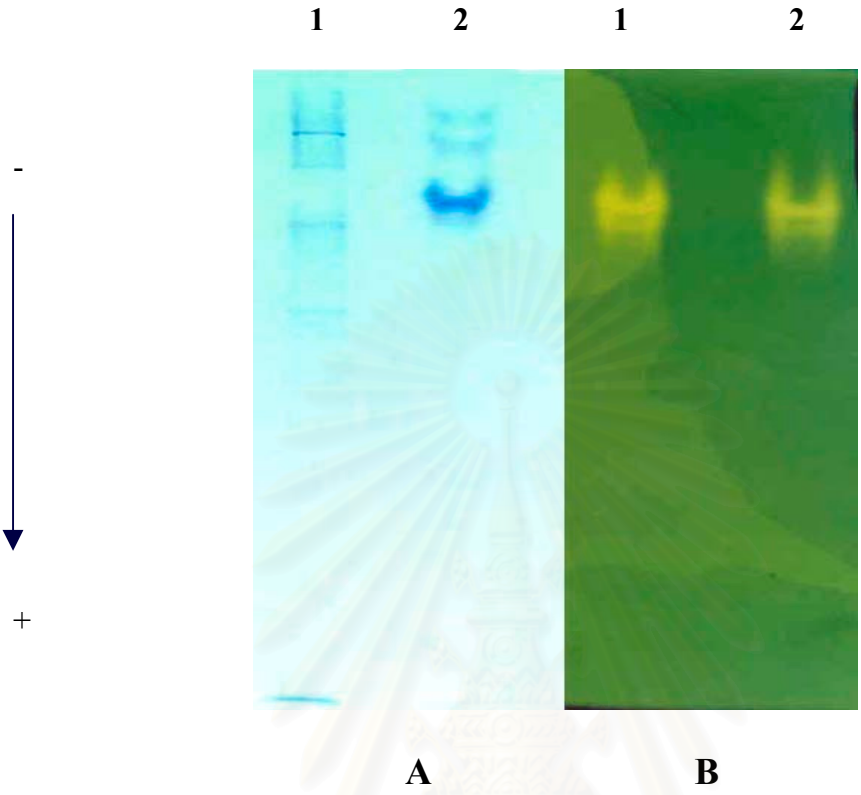


Figure 3.25 Non-denaturing PAGE (7.5% gel) of CGTase from different steps of purification

A: Coomassie blue staining (described in 2.10.2)

Lane 1 : Crude enzyme (20 μg)

Lane 2 : Concentrated starch adsorbed enzyme (20 μg)

B: Amylolytic activity staining by iodine reagent (described in 2.10.3)

Lane 1-2, as in A

0.2 units of dextrinizing activity was loaded to each well

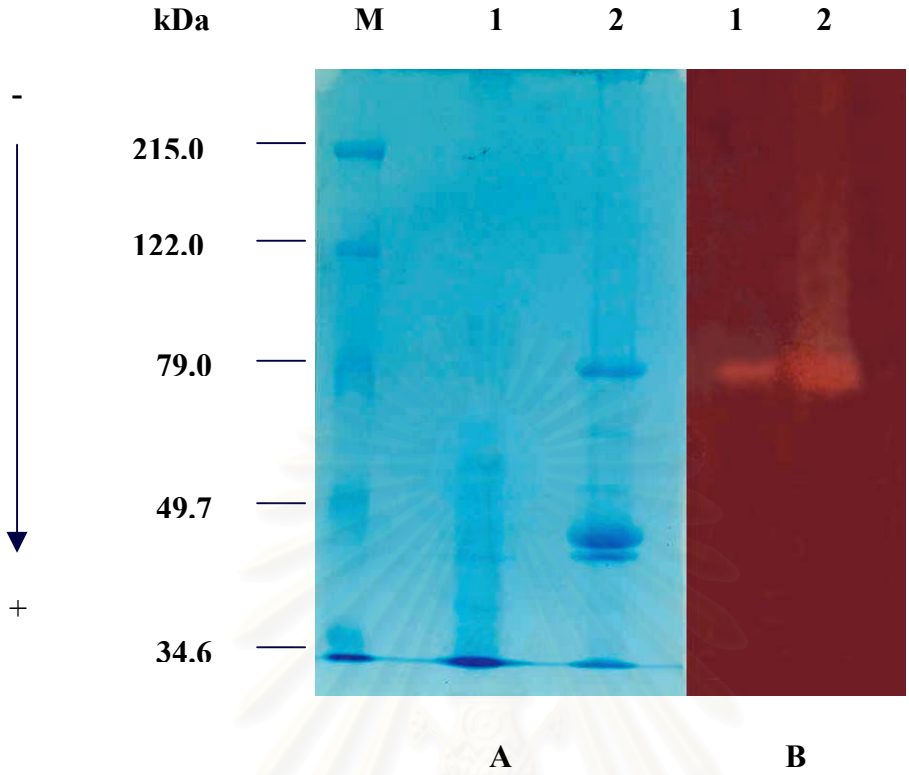


Figure 3.26 SDS-PAGE (7.5% gel) of CGTase from steps of partial purification

A: Coomassie blue staining (described in 2.10.2)

Lane M : Prestained protein molecular weight markers

Myosin	**215.0**	**kDa**
β-galactosidase	**122.0**	**kDa**
Bovine serum albumin	**79.0**	**kDa**
Ovalbumin	**49.7**	**kDa**
Carbonic anhydrase	**34.6**	**kDa**

Lane 1 : Crude enzyme (40 μg)

Lane 2 : Concentrated starch adsorbed enzyme (20 μg)

B: Amylolytic activity staining by iodine reagent (described in 2.10.3)

Lane 1 : Crude enzyme (20 U)

Lane 2 : Concentrated starch adsorbed enzyme (20 U)

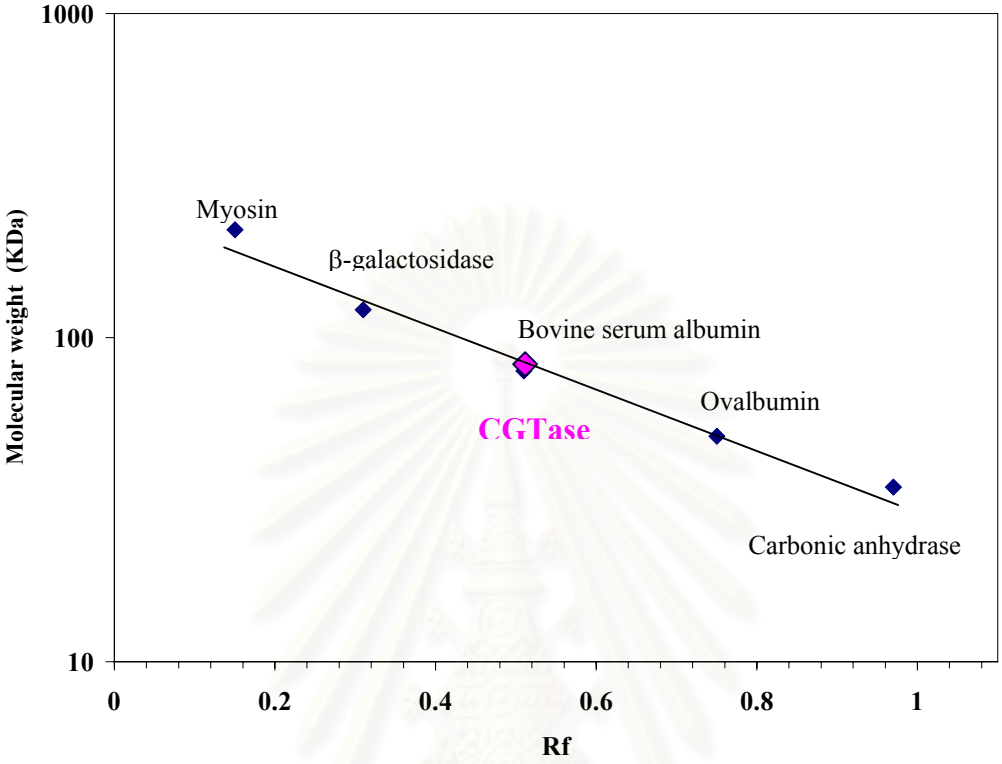


Figure 3.27 Molecular weight calibration curve of standard protein by SDS-PAGE

Myosin	**215.0**	**kDa**
β-galactosidase	**122.0**	**kDa**
Bovine serum albumin	**79.0**	**kDa**
Ovalbumin	**49.7**	**kDa**
Carbonic anhydrase	**34.6**	**kDa**
CGTase	**79**	**kDa**

3.4.2 Effect of temperature on CGTase activity

The optimum temperature of the partially purified enzyme was investigated by incubating the reaction mixture at various temperatures as mentioned in section 2.10.3.1. The enzyme activities were determined for both the starch dextrinizing activity and the CD-forming activity as described in section 2.5.1 and 2.5.2. The result was shown in Figure 3.28A, the optimum temperature for dextrinizing activity was 70 ^{o}C, while the optimum temperature for CD-forming activity was highest at 50 to 55 ^{o}C. The dextrinizing activity rapidly declined above and below 70 ^{o}C. CD-forming activity slowly declined above and below 50-55 ^{o}C with approximately 50% activity retained at 25 ^{o}C and 65 ^{o}C. The maximum activity was defined as 100%.

3.4.3 Effect of pH on CGTase activity

The effect of pHs on the enzyme activity was studied at various pHs, as mentioned in section 2.10.3.2 at 55 and 70 ^{o}C, the optimum temperature for CD-forming and dextrinizing activity. Enzyme activity at different pHs were shown in Figure 3.28B. At 55 ^{o}C, maximum dextrinizing activity was observed at pH 6.0 with activity above 80% in the pH range 5.0-9.0, while CD-forming activity was highest at pH 7.0. At 70 ^{o}C, CD-forming activity had a broad pH optimum in the range of pH 6.0-8.0 with optimum pH of dextrinizing activity at 6.0.

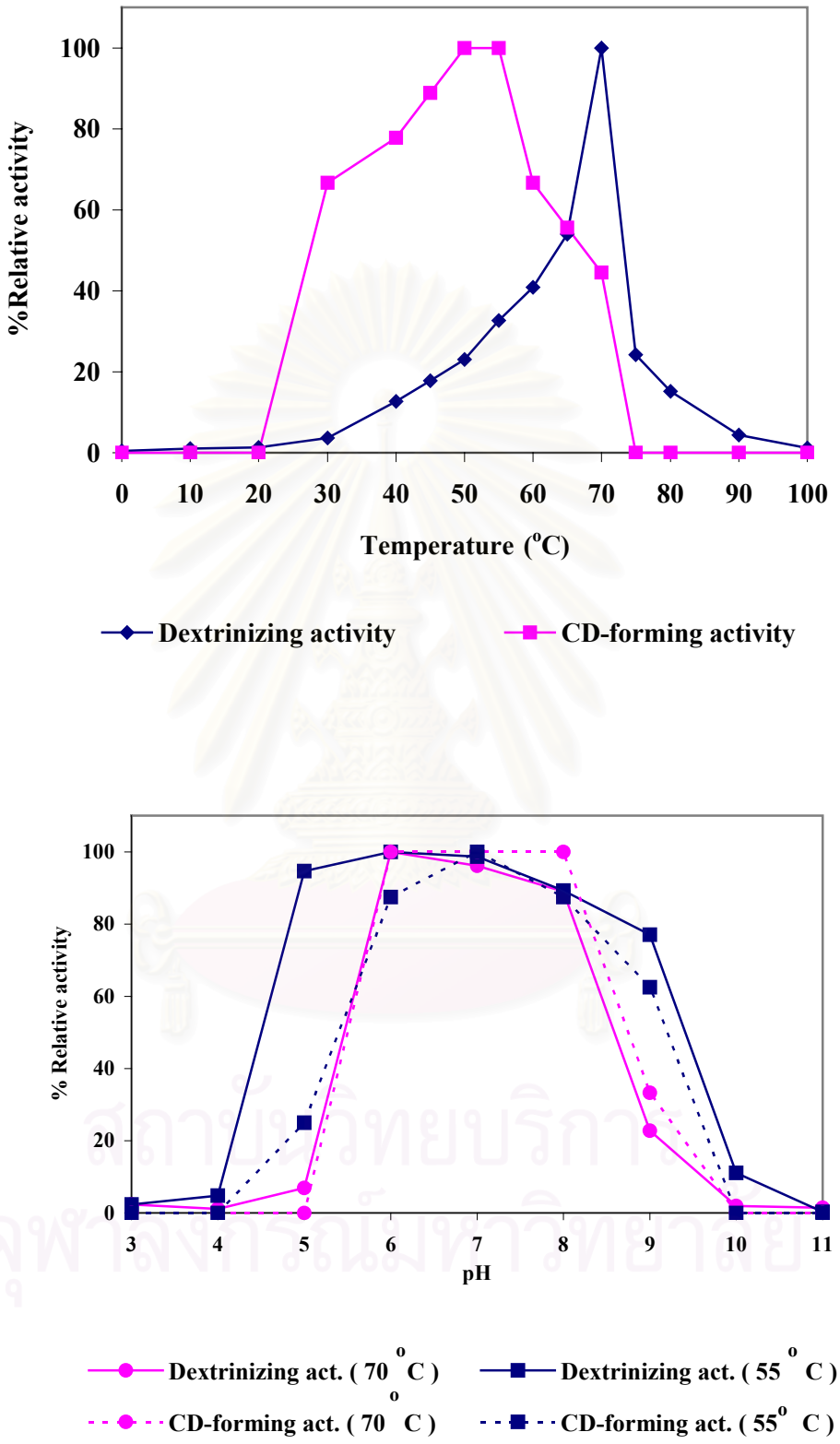


Figure 3.28 Effect of temperature (A) and pH (B) on enzyme activity

3.4.4 Effect of incubation time on CGTase activity

Effect of incubation time on dextrinizing activity was investigated by incubating the enzyme with 0.2% soluble starch, pH 6.0 at 70 ^{o}C for different time intervals (0-30 minutes) while the incubation condition of 2.0% soluble starch at pH 7.0 at 55 ^{o}C for different time intervals (0-48 hours) was employed for CD-forming activity. It was found that the linearity of dextrinizing activity was observed from 0-10 minutes as shown in Figure 3.29A. For CD-forming activity, the shortest incubation time which gave the maximum dilution limit (shown as % Relative activity) was observed at 6 hours (Figure 3.29B). Therefore, the most suitable incubation time for dextrinizing activity and CD-forming activity were 10 minutes and 6 hours, respectively.

3.4.5 Effect of temperature on CGTase stability

Partial purified CGTase was preincubated at various temperatures for 1 hour before its activity was assayed as described in section 2.10.4.1. The result was shown in Figure 3.30. The dextrinizing activity of the enzyme could be maintained at temperature up to 30 ^{o}C, whereas loss of about 35% activity was observed at 70 ^{o}C and totally loss when the temperature was raised up to 80 ^{o}C. The CD-forming activity retained its full activity up to 70 ^{o}C, but lost about 45% at 80 ^{o}C.

Cyclodextrin products from the reactions above were confirmed by analyzed with HPLC (Appendix D).

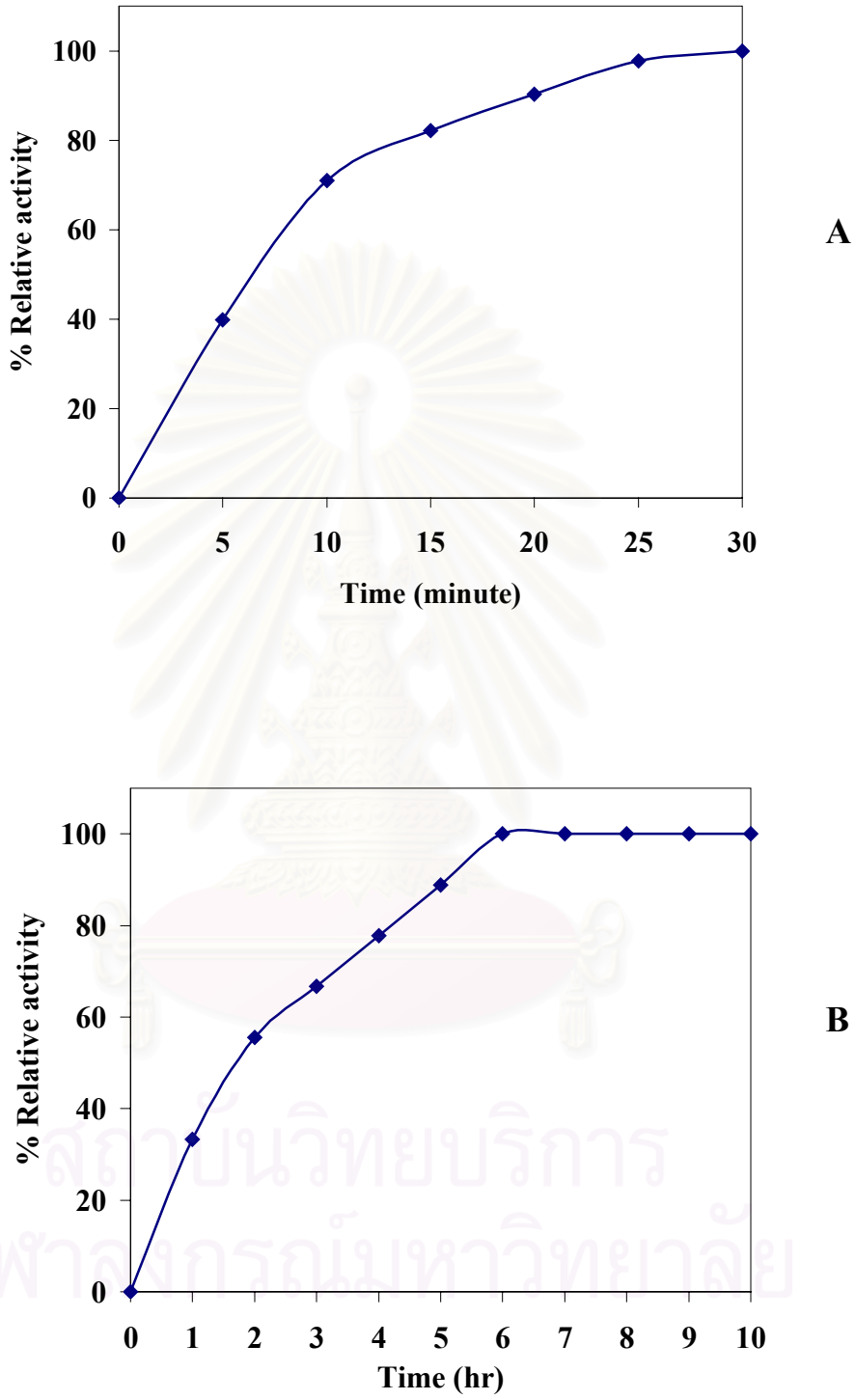


Figure 3.29 Effect of incubation time of dextrining activity (A) and CD-forming activity (B)

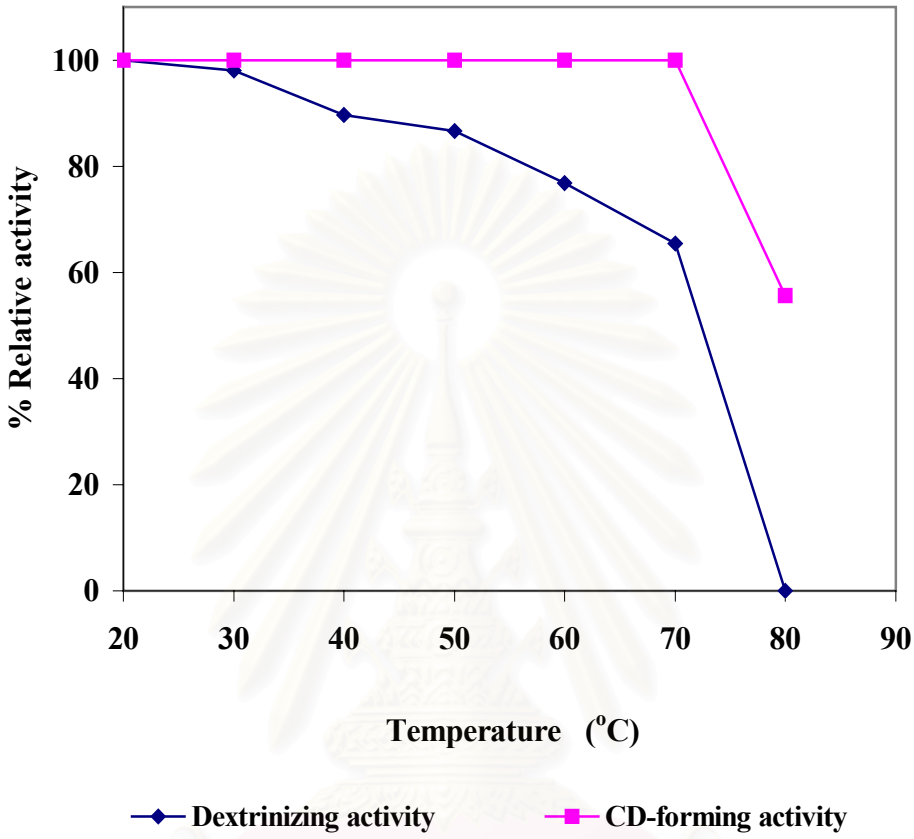


Figure 3.30 Effect of temperature on enzyme stability

3.4.6 Effect of pH on CGTase stability

The effect of pH on enzyme stability was investigated by incubating the enzyme for 1 hour at 55 and 70 ^{o}C under varying pH condition prior to determination of relative activity under standard assay conditions. As shown in Figure 3.31, it was found that, the dextrinizing activity remained stable between pH 6.0 and 9.0 activity and between pH 6.0 and 8.0 for CD-forming activity at 55 ^{o}C. Treatment of the enzyme at same pH range showed that, both enzyme activities were most stable at pH 6.0 at 70 ^{o}C.

3.4.7 Effect of $CaCl_2$ on CGTase stability

The enzyme in 0.2 M Tris-HCl buffer, pH 7.0 containing 5 mM and 10 mM $CaCl_2$ was incubated at 75 ^{o}C and dextrinizing activity was assayed at intervals. As shown in Figure 3.32A, the thermostability of the enzyme was slightly enhanced by addition of 5 mM and 10 mM $CaCl_2$. At 60 minute, the enzyme was the most stable in the presence of 10 mM $CaCl_2$. CD-forming activity was also slightly stabilized during 60 minutes incubated in the presence of $CaCl_2$. The effect of 5 mM and 10 mM $CaCl_2$ were similar to dextrinizing activity (Figure 3.32B).

3.4.8 Effect of substrate on enzyme stability

The effect of substrate on enzyme stability was investigated by incubating the enzyme in 0.2 M Tris-HCl buffer, pH 7.0 containing 0.2% and 2.0% soluble starch at 75 ^{o}C. The dextrinizing and CD-forming activity was more stable in the presence of starch substrate. The result was shown in Figure 3.33A and 3.33B. In presence of 2.0% starch substrate, the enzyme was slightly stable than 0.2% starch substrate.

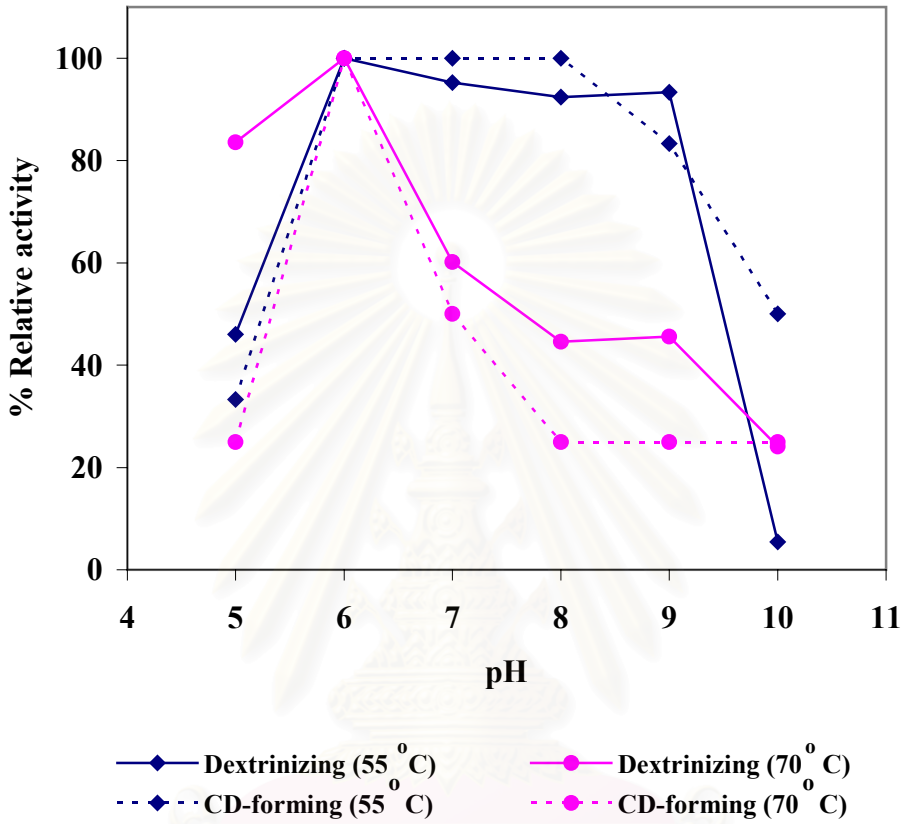


Figure 3.31 Effect of pH on enzyme stability

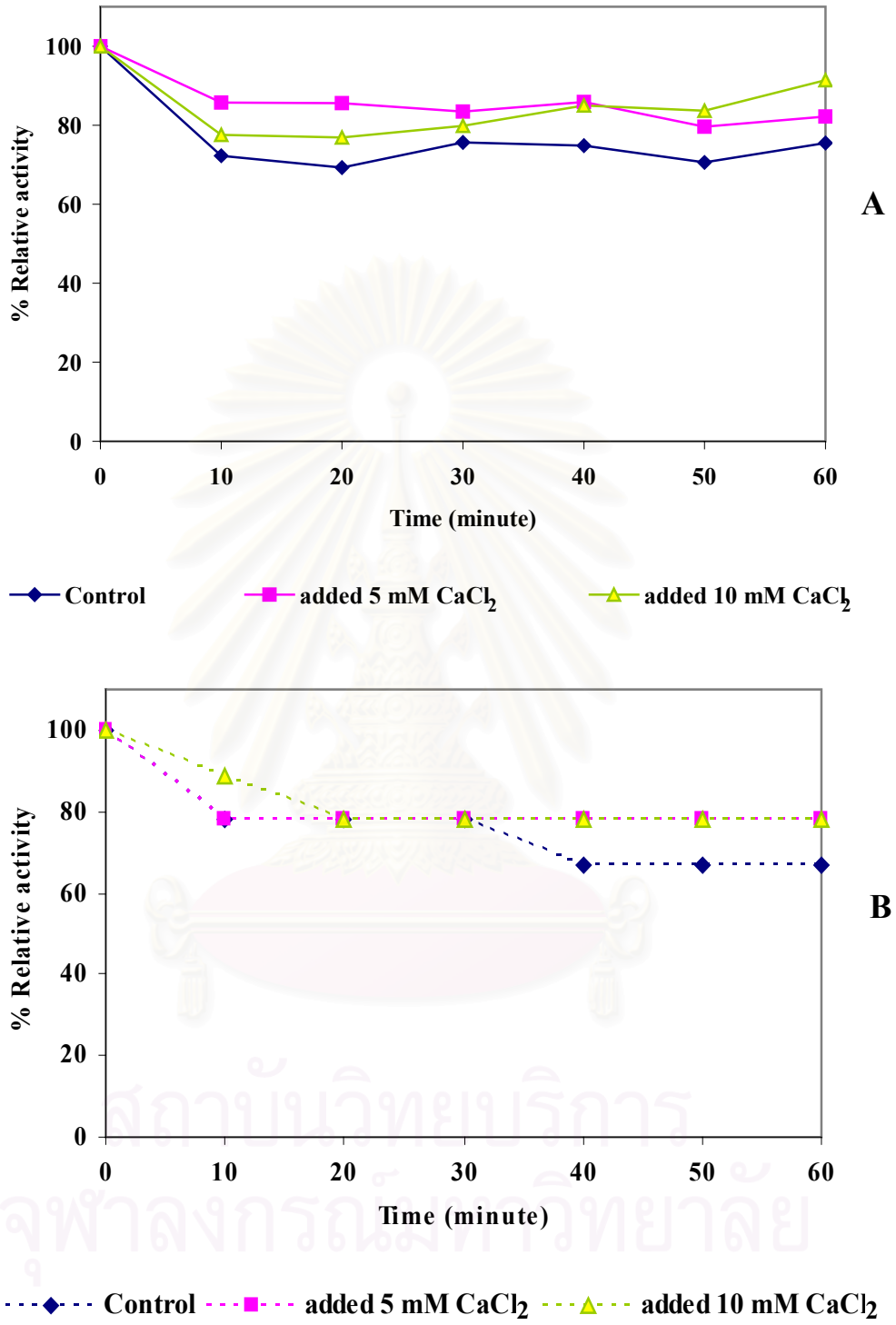


Figure 3.32 Effect of $CaCl_2$ on enzyme stability at 75 ^{o}C:

dextrinizing activity (A) and CD-forming activity (B)

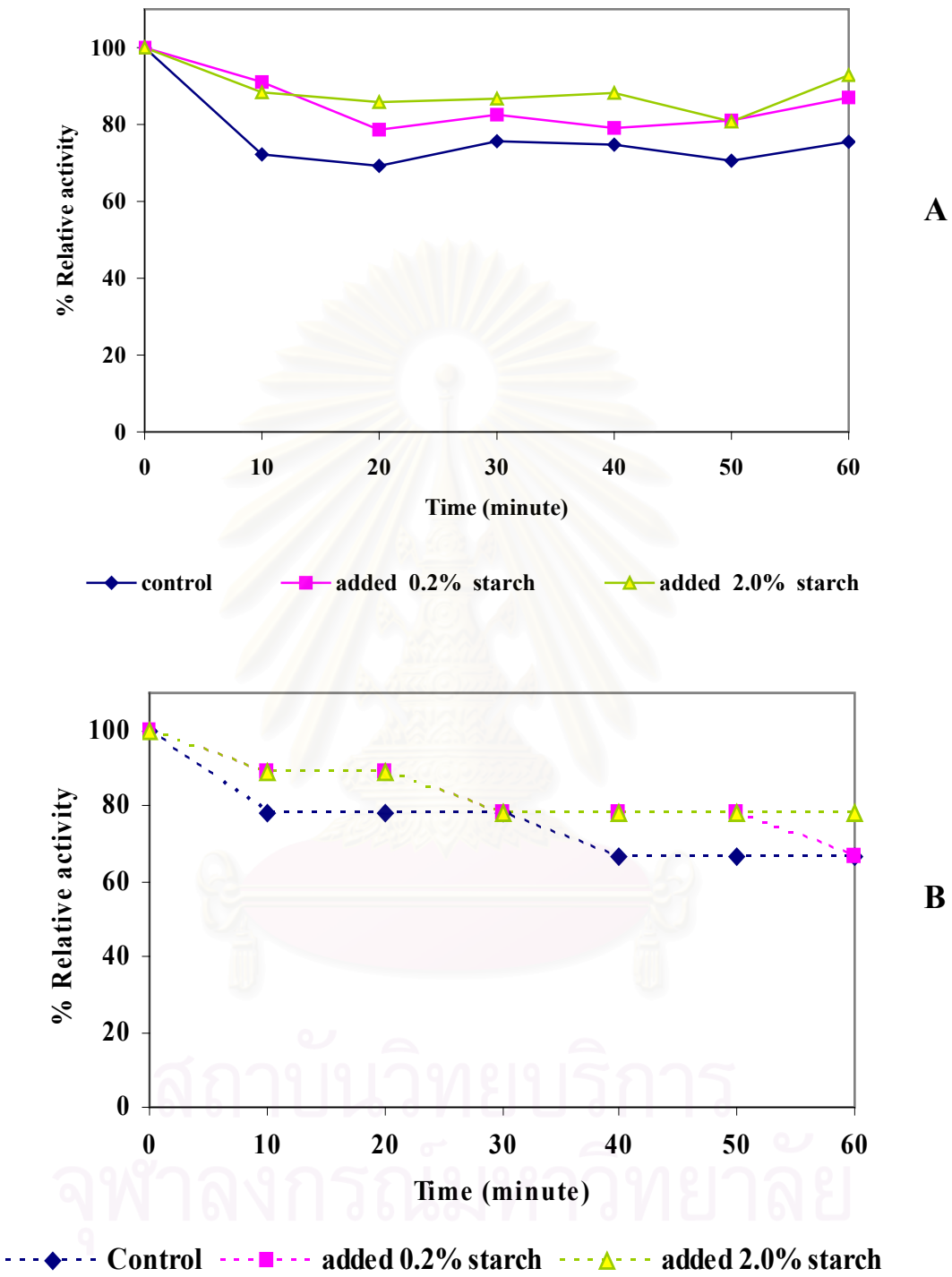


Figure 3.33 Effect of starch on enzyme stability at 75 ^{o}C:

dextrinizing activity (A) and CD-forming activity (B)

3.4.9 Condition for storage enzyme

The enzyme were stored at 4 ^{o}C and –20 ^{o}C for 7 weeks. CGTase sample was withdrawn every week for measuring dextrinizing activity and CD-forming activity. The result was shown in Figure 3.34A. At 4 ^{o}C, 10% of dextrinizing activity and CD-forming activity was lost when the enzyme was stored without 10 mM $CaCl_2$ for 2 and 3 weeks, respectively. At –20 ^{o}C, over 90% of both activities remained after storing for 3 weeks and 10% of CD-forming activity was lost at 6 weeks as shown in Figure 3.34B. It was found that 100% of CD-forming activity remained for 7 weeks in the presence of 10 mM $CaCl_2$ at both storage temperatures.

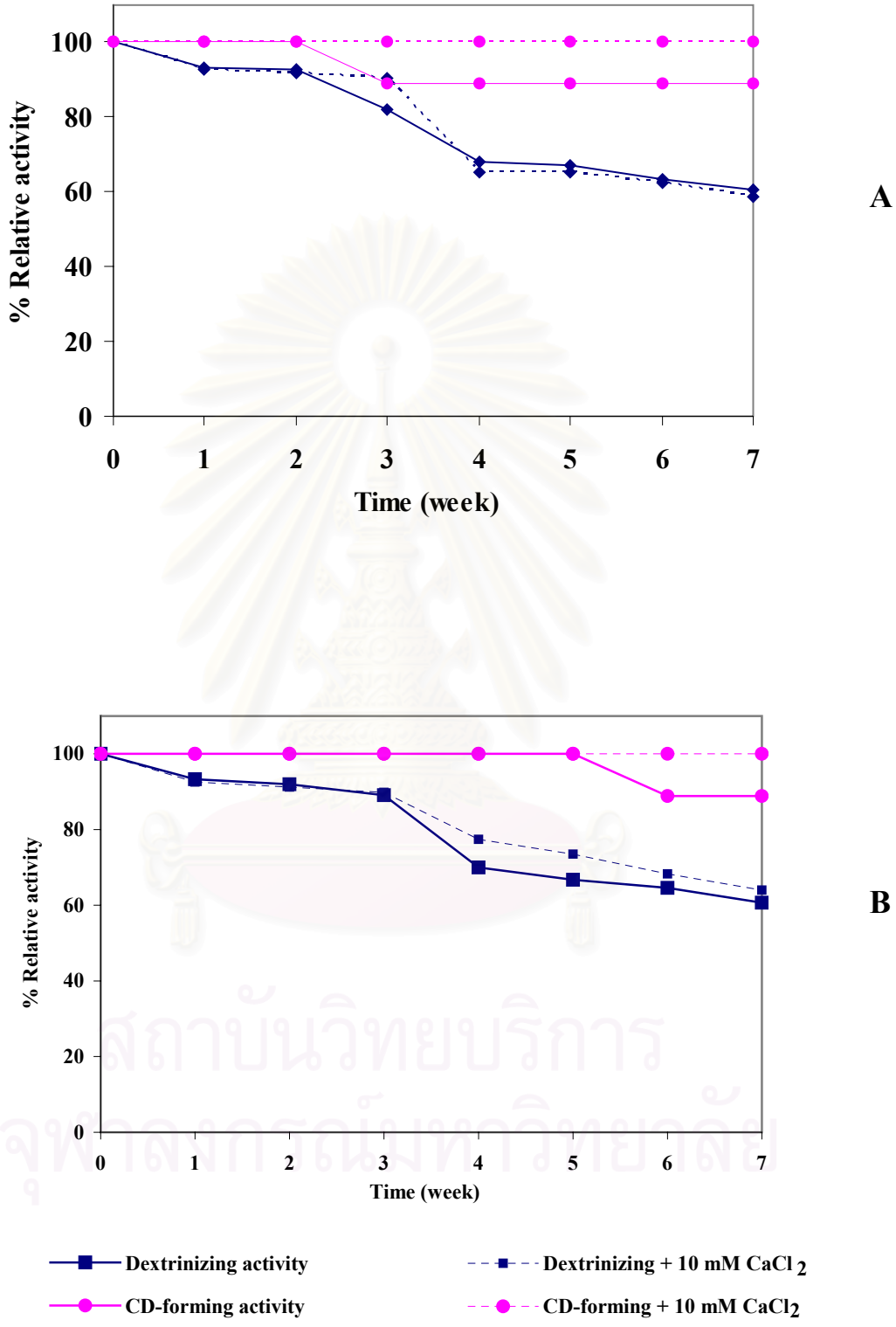


Figure 3.34 Condition for storing enzyme at 4 °C (A) and –20 °C (B)

3.5 Analysis of cyclodextrin products by HPLC

3.5.1 Identification of cyclodextrin products

The reaction mixture containing 2.5 ml of 2.0% soluble starch in 0.2 M phosphate buffer, pH 6.0 was incubated with 0.5 ml (100 U/ml) enzyme at 40 ^{o}C for 24 hours and reaction was stopped by incubation in boiling water bath for 10 min. β-amylase (20 U/ml) was added to the aliquot and the mixture was incubated at 25 ^{o}C for 3 hours. The HPLC analysis of the resulted mixture revealed the presence of mainly 2 types of CDs. It was found that CGTase from BT01 produced α-CD at the same level as β-CD and the ratio of α : β- CD was 1.04 : 1.0 (Figure 3.35).

3.5.2 Effect of pH on cyclodextrin products

The profile of CDs product at pH 6.0 and 7.0 which was the optimum pH for dextrinizing and CD-forming activities at 40 ^{o}C were investigated. The result was shown in Figure 3.37. At pH 6.0, the enzyme converted the starch into 64.2% of short chain oligosaccharide and CDs. The CD products were 18.3% of α-CD and 17.5% of β- CD but no γ-CD was detected (Figure 3.36A). When performing the same reaction at pH 7.0, the yield of short chain oligosaccharides were 63.0% and the CD products was increased to 18.2% of α- CD, 18.0% of β- CD and 0.8% of γ-CD (Figure 3.36B).

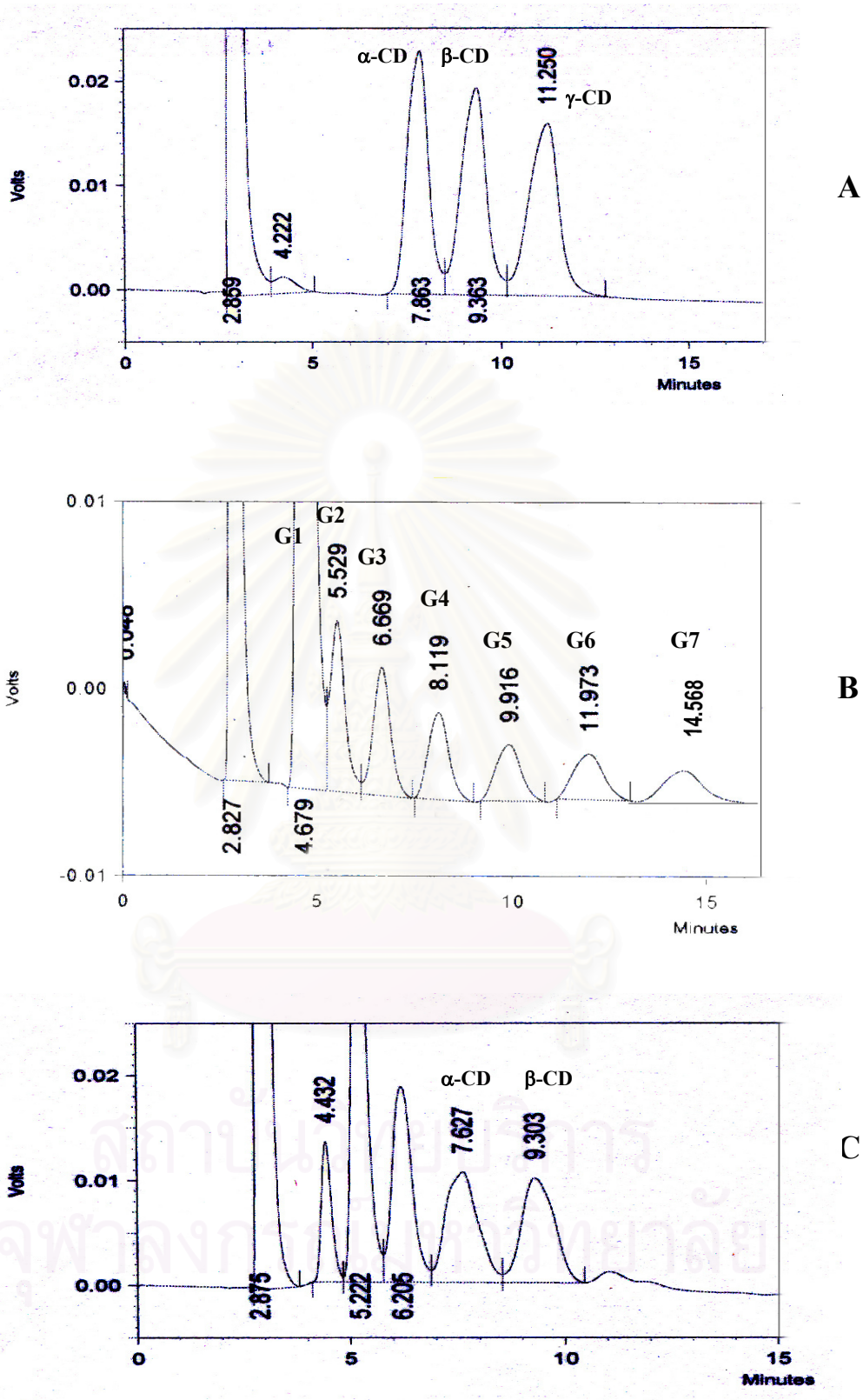


Figure 3.35 HPLC chromatograms of standard cyclodextrin (A), standard oligosaccharides (B) and cyclodextrin products from CGTase BT01 (C).

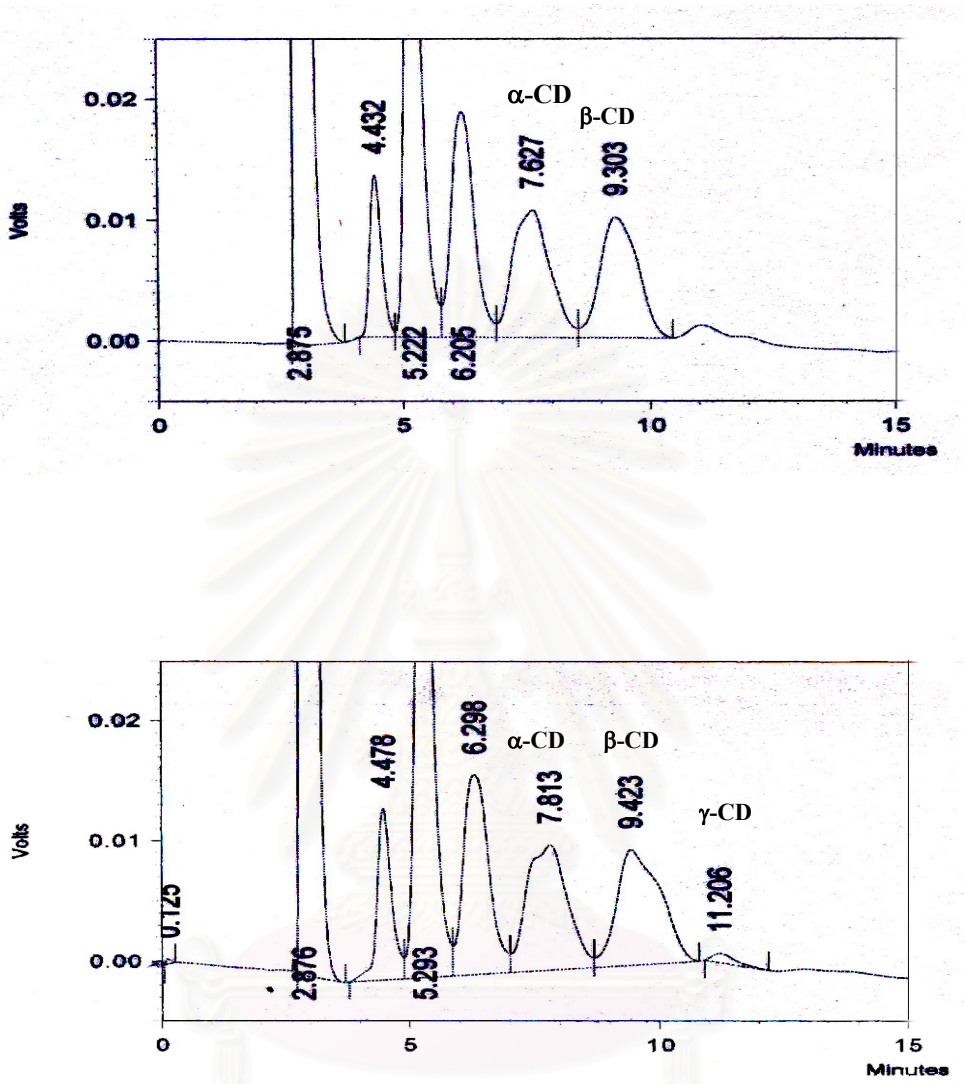


Figure 3.36 HPLC chromatogram of cyclodextrin products when the reaction mixture was incubated at 40 ^{o}C with pH 6.0 (A) and pH 7.0 (B)

3.5.3 Efffect of temperature on cyclodextrin products

The CDs formation ability of CGTase was determined at various temperature between 40 and 80 ^{o}C at pH 7.0. As shown in Figure 3.37, the CD conversion rate increased corresponding with increasing temperature of reaction until the temperature reached 60 ^{o}C and declined at 70 and 80 ^{o}C. At 40 ^{o}C (Figrure 3.36B), the ratio of α : β- CD was about 1:1 similar to the result at 55 ^{o}C (Figure 3.37A) and 60 ^{o}C (Figure 3.37B), but the maximum total CDs yield obtained at 55 ^{o}C was 38.2% and 37.9% at 60 ^{o}C. At 70 ^{o}C (Figure 3.37C), the product consisted of 13.8% of α-CD, 17.3% of β-CD and 1.8% of γ-CD (32.9% of total CDs yield) while at 80 ^{o}C, only α-CD could be detected at 7.4% (Figure 3.37D).

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3.5.4 Effect of types of starch on cyclodextrin products

Conversion of various types of starch to CD by CGTase was tested using soluble starch, cassava starch, corn starch, glutinous starch and rice starch. The reaction mixture was incubated at 55 ^{o}C, pH 7.0 for 24 hours. The result was shown in Figure 3.38. It was found that the most appropriate substrate for the production of highest CD using the enzyme was corn starch which produced more β than α-CD products. The production profile was 20.4% of α-CD and 21.6% of β-CD, but no γ-CD was detected.

The result of pH, temperature and types of starch on cyclodextrin product were summarized in Table 3.5. % Yield was calculated as

$$\% \text{ yield} = \frac{\text{Area peak of each product } (\alpha, \beta, \gamma \text{ and oligosaccharides})}{\text{Total area peaks on the HPLC profiles (except buffer peak)}}$$

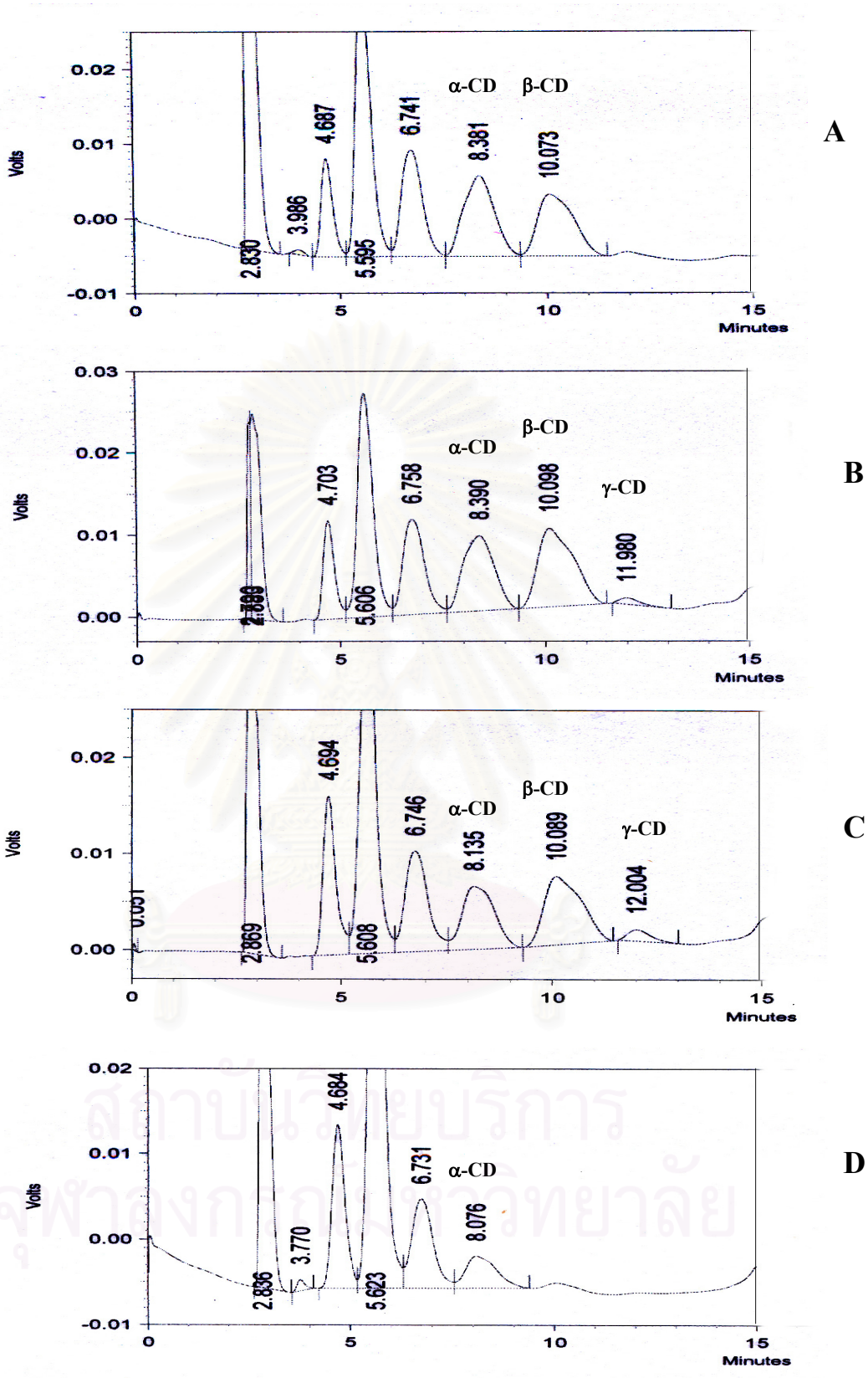

Figure 3.37 HPLC chromatogram of cyclodextrin products when the reaction mixture was incubated in pH 7.0 at 55 ^{o}C (A), 60 ^{o}C (B), 70 ^{o}C (C) and 80 ^{o}C (D).

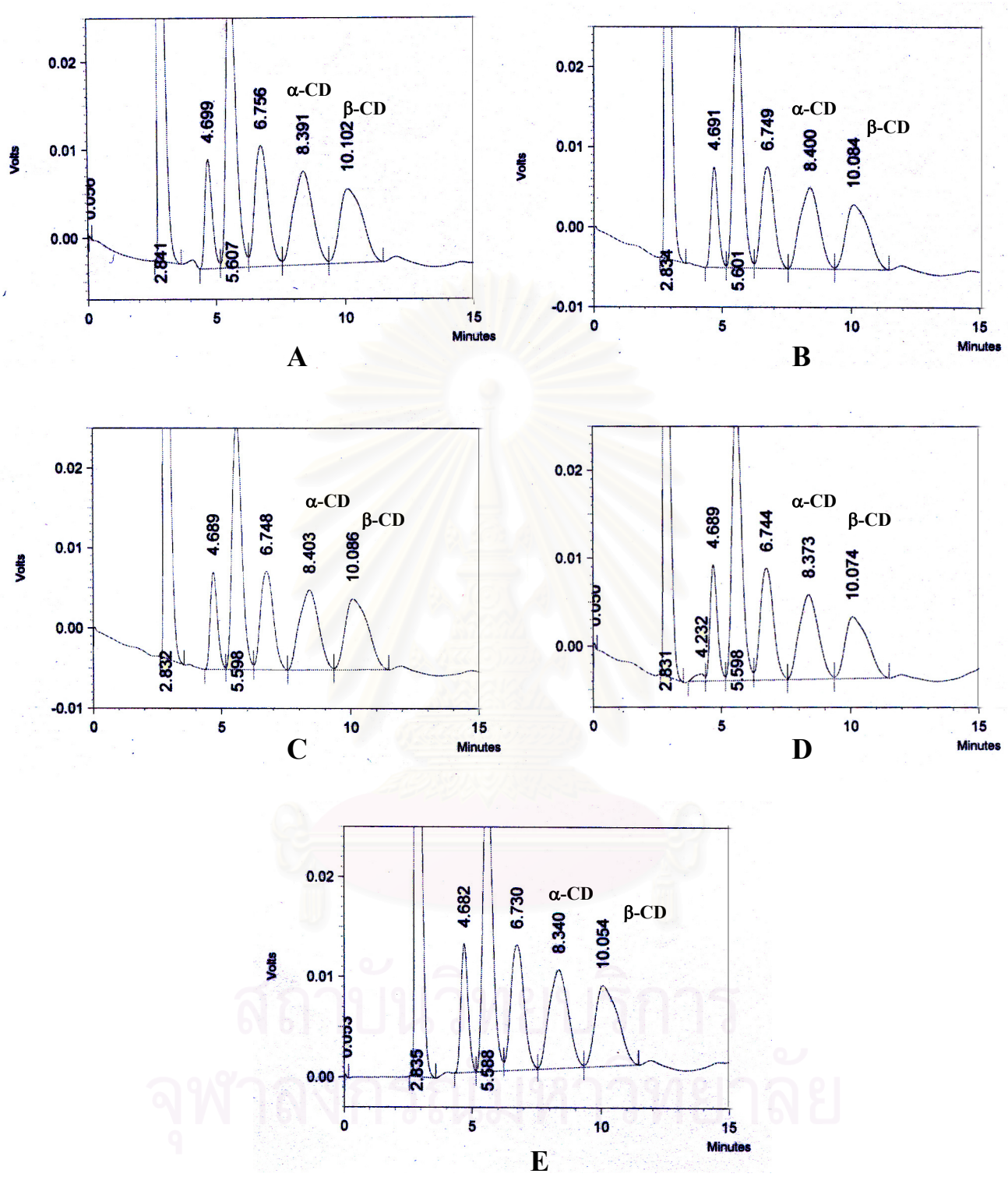

Figure 3.38 HPLC chromatogram of cyclodextrin products when the reaction mixture was incubated at 55 ^{o}C, pH 7.0 with soluble starch (A), cassava starch (B), corn starch (C), glutinous starch (D) and rice starch (E).

Table 3.5 Effect of pH, temperature and types of starch on the production of CDs by BT01 CGTase

Condition	% Yield				
	α-CD	β-CD	γ-CD	Total CD	Short chain oligosaccharides
pH (40 °C)					
6.0	18.3	17.5	-	35.8	64.2
7.0	18.2	18.0	0.8	37.0	63.0
Temperature (°C) (pH 7.0)					
40	18.2	18.0	0.8	37.0	63.0
55	20.0	18.2	-	38.2	61.8
60	16.8	20.2	0.9	37.9	62.1
70	13.8	18.3	1.8	32.9	67.1
80	7.4	-	-	7.4	92.6
Types of substrate (pH 7.0, 55 °C)					
Soluble	20.5	19.1	-	39.6	60.4
Cassava	20.7	19.5	-	40.2	59.8
Corn	20.4	21.6	-	42.0	58.0
Glutinous	19.9	17.3	-	37.2	62.8
Rice	20.4	19.8	-	40.2	59.8

CHAPTER IV

DISCUSSION

There are a number of enzymes that can convert starch to various products of industrial value. One example of such an enzyme is CGTase which catalyzes cyclodextrins production from starch, amylose, and other polysaccharides by different transglycosylation steps. Firstly, the intermolecular coupling and disproportionation, reaction which occurs at the α-1,4-glycosidic bond of oligosaccharides. Secondly, the reactions which modifies the length of non-cyclic dextrins. Thirdly, the intermolecular cyclization forms CDs from oligosaccharides of the appropriate chain length. Cyclodextrins have a wide range of applications in foods, pharmaceuticals, chemical industries, agricultures and environmental industries (Biwer *et al*., 2002). Industrial process involved in CD production encounter temperature changes which affect the growth and metabolism of bacteria. Temperature control system was required to maintain temperature of the process in favorable range for bacterial growth and CGTase production. Thermostable CGTase is very useful in liquefaction and then in CD formation in replacement of amylase for industrial utilization.

A need for thermotolerant bacteria producing a thermostable CGTase which gives high CD yield has been recognized. The understanding of growth profile, CGTase production and CD formation of the selected bacteria can simplify the production process and increase the selectivity of the product for efficient production of CDs.

Screening of thermotolerant bacteria producing CGTase

The screening was carried out for the purpose of finding CGTase producing microorganisms which can grow at ambient temperature and can tolerate higher

temperature up to 40 ^{o}C or higher and culture conditions that led to abundant enzyme production were examined in this work.

Most research on CGTase producing bacteria reported CD production only when starch was present in the culture media (Bender, 1981). Therefore, bacteria found around starch factory should have potential in producing CGTase. This research aims at screening thermotolerant bacterial strains that produce thermostable CGTase from soil, water and starch waste. Various methods have been reported for the determination of CGTase activity. Similar to amylases, one reaction catalyzed by CGTase is starch hydrolyzing activity. Therefore, in this study, starch-dextrinizing method (Iodine method of Fuwa) was one of the assay chosen as primary screening of CGTase activity. This method is a simple and rapid for screening CGTase activity but indistinguishable from amylase activity. Additional methods which were specific for CGTase activity were necessary after the selection of isolates. A rapid screening method for CGTase producing bacteria by agar plate containing phenolphthalein-methyl orange dyes was used for secondary screening. Phenolphthalein was transformed into a colorless dianion within the cavity of β-CD and CGTase activity was determined on the basis of the reduction of the color intensity of phenolphthalein by CGTase under alkaline condition (Taguchi, 1986) in presence of CD. The possibility that acid-producing bacteria can change the color of phenolphthalein to colorless by producing acids was cross-checked by adding methyl orange to the medium. The color of methyl orange will change from orange to red in acidic condition i.e. when acid forming bacteria is present. Thus, CGTase activity can be distinguished from the action of acids produced by microorganisms (Park *et al.*, 1989). The reduction in the color intensity was not observed for α-CD, since the complex of α-CD and phenolphthalein was not easily formed.

For the primary screening, it was found that 97 colonies exhibited clear zone on Medium I agar plates containing 1.0% soluble starch when with I_2 in KI, while the secondary screening showed 3 colonies indicating CGTase activity named BT01, BD01 and BO01. Among them, BT01 and BD01 gave moderate starch hydrolyzing activities (the b/a ratio were 2.3 and 2.0, respectively), whereas BO01 gave high activity with b/a ratio equal 3.0. Other strains with high amylase did not produce CGTase. This finding was in agreement with many previous reports (Alexandra, 1998; Techaiyakul, 1991). The CGTase positive isolates were confirmed for cyclodextrin forming activity by CD-TCE complex precipitation method. The difference in the amount of precipitate in the two neighboring dilutions was so distinguishable that it was easy to define the level of CGTase activity capable of producing sufficient amounts of observable CD precipitate. Although the values obtained was semi-quantitative, the CD-TCE method was direct and also efficient as a screening test for CGTase-producing bacteria. Only BT01 could grow well at 37 to 45 ^{o}C and produced highest CGTase activity compared with BD01 and BO01, therefore BT01 was chosen for further study.

Identification of the CGTase producing microorganism

There are two principles used in this study for identifying the bacteria namely phenotypic and phylogenetic characteristics. The first one relies on the phenotypic and biochemical characteristics, i.e. gram staining, scanning electron microscopy (SEM) and carbohydrate fermentation. It was found that BT01, BD01and BO01 were gram positive rod-shaped. BT01 belonged to genus *Bacillus* and was identified as *Bacillus circulans* using API CHB system which was commonly used at Thailand Institute of Science and Technology Research (TISTR), while BD01 and BO01 were

identified as *Brevibacillus brevis*. The second method was 16S rRNA gene analysis. Identification by 16S rRNA gene fragment amplification is the technique which is widely used at present to confirm bacterial classification and has gained wide acceptance (Edwards *et al*., 1989). In this study, only BT01 was identified by 16S rRNA. The forward primer (pA) and reverse primer (pH) were used to amplify the gene. Then primers pA, pD and pF were used for sequencing. These primers have been used to identify various bacteria due to the conserved sequence of 16S rRNA gene (Ash *et al*., 1991; Yoon *et al*., 1998). After 16S rRNA gene of BT01 was amplified, PCR products of about 1.5 kb was sequenced. It was found that 16S rRNA gene sequence of BT01 was about 1312 bp and not overlapped. The sequence missing was about 188 bp at the end sequence using primer A to beginning sequence of primer D and the end sequence using primer D to beginning sequence primer F. However, the sequence using each primer (pA, pD and pF) was blasted with those deposite in EMBL/Gen Bank database. The result of each primer sequencing indicated that BT01 showed 98% similarity to *Paenibacillus* sp., especially with *Paenibacillus* sp.AG 430, *Paenibacillus* sp. 38-2 and *Paenibacillus* sp. strain 324. It was therefore not possible to definitely identify strain BT01 as one of the described species within the *Paenibacillus* group. However, this result was not in correlation with the biochemical characteristic of this isolate.

16S rRNA gene sequence analysis have shown that the genus *Bacillus* is phylogenetically very heterogeneous, at least 10 phylogenetic groups have been identified in the genus *Bacillus* (Ash *et al*., 1993; Ash *et al*., 1991; Farrow, 1992; 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). From this result, BT01 should belong to

Paenibacillus sp. and was named *Paenibacillus* sp. BT01. Figure 4.1 shows phylogenetic tree of *Bacillus* sp., *Paenibacillus* sp., and other rod shaped endospore forming bacteria related genus based on 16S rRNA gene sequence.

Optimization of conditions for culturing and CGTase production

For starter inoculum, BT01 could grow well in wide pH range (7.0-10.0) at 37 ^{o}C in Medium I. The optimum pH for starter inoculum was pH 9.0. Although the growth rate at pH 10.0 was highest, but the time taken to reach mid- log phase was 6 hours which was longer than culturing at pH 9.0 (4-5 hours). Therefore, to save time and energy, pH 9.0 was chosen to prepare starter inoculum. When growth and CGTase production profiles of BT01 was observed when cultured in Horikoshi's medium, it could grow well in pH range 7.0-10.0 at 37-40 ^{o}C, with highest CGTase production at pH 10.0 at 40 ^{o}C. Therefore, BT01 had thermotolerant characteristics because it could grow and produce CGTase at rather broad mesophilic temperature range (30-45 ^{o}C).

Starch was the inducer and substrate for CD synthesis and CGTase production was induced by starch (Bender, 1981). When various types of starch which contain different percentage of amylose and amylopectin content (Table 4.1) was added in Horikoshi's medium, the result showed that cassava starch and soluble starch could induce highest CGTase production, while glutinous rice starch, corn starch and rice starch gave low CGTase production. However, the result cannot be correlated with the level of amylose or amylopectin content (Grull and Stifter, 2001). Other factors such as starch granule size, structure and starch solubility may have to be taken into account. By varying concentrations of cassava starch and soluble starch, it was found that 0.5% soluble starch was most suitable for CGTase production. Adding 0.5-1.0%

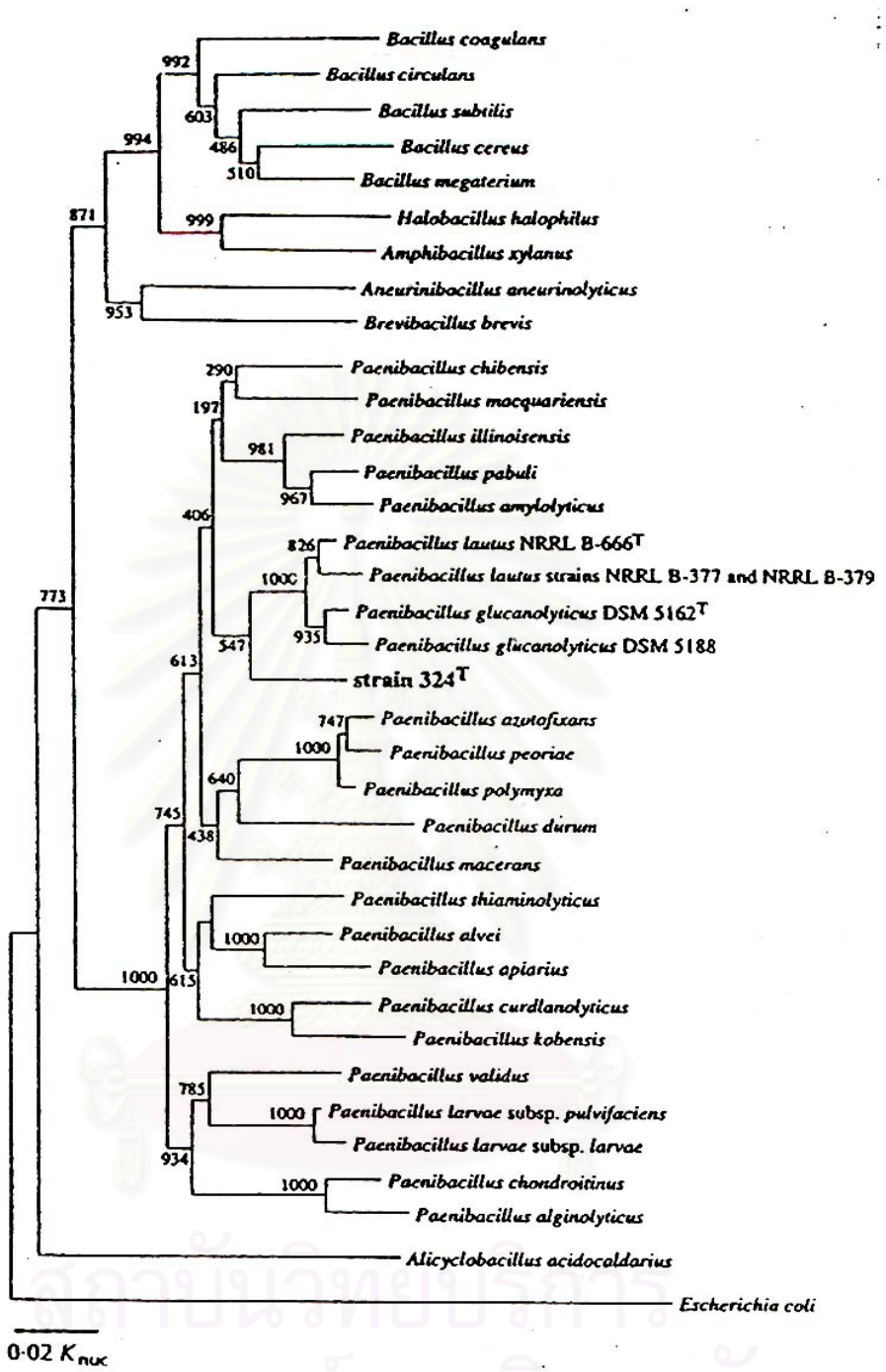

Figure 4.1 Phylogenetic tree showing the position of *Bacillus* sp., *Paenibacillus* sp., and other rod shaped endospore forming bacteria related genus based on 16S rRNA gene sequence. Bootstrap values are indicated Bar, 2 nucleotide position substitutions per 100 nucleotide. (Yoon *et al*., 1998)

Table 4.1 Amylose and amylopectin contents in starch (Swinkels, 1985; Nilmanee, 2000)

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

cassava starch can be selected as alternative for replacing soluble starch because of the low price and its high CGTase induction. Starch concentrations at higher than 1.0% did not lead to higher CGTase synthesis but on the contrary, inhibited it. This was probably due to substrate inhibition. Furthermore, 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. Production of CGTase began as the cell entered early exponential phase and the rate was greatest after 72 hours. The result correlated with *Paenibacillus macerans* strain T16 (Pranommit, 2001) and *Paenibacillus* sp. RB01 (Tesana, 2001) but, different from *Bacillus* sp. A11 of which the enzyme synthesis started from early stationary phase and reached maximum during 72-120 hours (Techaiyakul, 1991).

Partial purification and Biochemical characterization of CGTase from BT01

The global aim of a protein purification process is not only the removal of unwanted contaminations, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intended application (Queiroz *et al*., 2001). For precise and thorough study of the properties of a protein, highest purification should be achieved. However, in our study we only aimed at partial characterizations of the CGTase from BT01 to obtain informations for further planning of detail study on the enzyme. Therefore, partial purification was performed.

One common step in the purification of CGTase reported by the different laboratories was the use of starch adsorption and the adsorbed enzyme was eluted with buffer containing maltose (Lloyd *et al*., 1984; Pongsawasdi and Yagisawa, 1988). The interaction between starch and the CGTase involves not only adsorption

but also substrate-enzyme affinity type binding. When CGTase from BT01 was subjected to starch adsorption, the purification fold and recovery of CGTase achieved were 28.0 and 65.1%, respectively (Table 3.4). The amylolytic activity stain in native PAGE showed at least 2-3 isoforms (Figure 3.25). Purity of the enzyme was confirmed by SDS-PAGE stained with Coomassie blue and iodine stain detect amylolytic activity. Figure 3.26 showed only one intense protein band on Coomassie blue stain which corresponded to the amylolytic activity stain. The molecular weight of this protein band was estimated to be 79 kDa. The result was different from *Paenibacillus* sp. RB01 (65 kDa) (Tesana, 2001), *Paenibacillus* sp. T16 (76 kDa) (Pranommit, 2001) and *Paenibacillus* sp. A11 (72 kDa) (Techaiyakul, 1991). Molecular weight of CGTase reported usually vary between 60 and 110 kDa (Prowe, 1996; Starnes, 2001).

Biochemical characterization of CGTase from BT01 were studied. The result showed that the optimum temperature for dextrinizing activity was 70 ^{o}C, while highest CD-forming activity was exhibited at 50-55 ^{o}C (Figure 3.28A). To explain this result, 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-amylases, CGTase will cleave the α-1,4-glucosidic bond of amylose in the same way as α-amylase. The transglycosylation reaction of CGTase is operated by a ping-pong mechanism (Nakamura *et al*., 1994a). 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 disproportionation 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 *et al*., 1994b). In a crystal structure, amylose can occur as a single helix with six to eight 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. 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 the activity of CGTase in BT01 may shift towards the starch-degrading reaction.

When CGTase was preincubated at various temperatures for 1 hour before both activities was assayed. CD-forming activity of CGTase was stable at temperature range of 20 ^{o}C to 70 ^{o}C. Loss of about 35% dextrinizing activity was observed at 70 ^{o}C and total lost at 80 ^{o}C. The result of CD-TCE assays were further confirmed by analysis of CDs with HPLC (Appendix D). The result indicated that the enzyme produced rather constant ratio of α-CD to β-CD at 7% and 11%, respectively.

Studies on effect of pH on enzyme activity were performed at 55 ^{o}C and 70 ^{o}C which were determined to be optimum temperature for CD-forming and amylolytic activity (Figure 3.28B). The pH optimum for dextrinizing activity and CD-forming activity were observed at 6.0 and 7.0 respectively at both 55 ^{o}C and 70 ^{o}C. At 70 ^{o}C, the enzyme had a broad pH optimum for dextrinizing in pH range of 5.0-9.0, where as the CD-forming activity was optimum in pH range of 6.0-9.0. It is also possible that

the dextrinizing activity found at pH 5.0 might be caused by the other starch dextrinizing enzymes also produced by BT01.

The enzyme was found to be stable between pH 6.0 and 9.0 for dextrinizing activity and between pH 6.0 and 8.0 for CD-forming activity upon incubation at 55 ^{o}C for 1 hour. Treatment of the enzyme at various pHs at 70 ^{o}C resulted in stability of both enzyme activities at pH 6.0.

Earlier reports have shown that CGTase was more resistant to thermal denaturation in the presence of its calcium ions and substrate (Bovetto *et al*., 1992; Yim *et a*l., 1997; Larsen *et al*., 1998). In the present study, adding of $CaCl_2$ had effect on the enzyme stability at 75 ^{o}C for both dextrinizing activity and CD-forming activity. Furthermore, the enzyme could be stored in presence of 10 mM $CaCl_2$ at 4 ^{o}C and -20 ^{o}C for 7 weeks with minimum loss of CD-forming activity. Calcium ions had been reported to affect the activity of CGTase. It could stimulate α-amylase activity and stabilized the enzyme structure. The results were supported by Wind *et al*., 1995; Chung *et al*., 1998; Larsen, 1998; Martins and Hatti-Kaul, 2002. Akimura *et al*. (1991) who observed that saturation of CGTase with Ca^{2+} resulted in an increase of heat stability and the optimum temperature of the enzyme shifted from 60 to 75 ^{o}C. Mori (1994) suggested that the enzyme activity was enhanced by $CaCl_2$. However, Bovetto (1992) reported that $CaCl_2$ did not increase the reaction rate, they only acted as a protective agent. An additive effect was obtained when the medium contained all the factors (Ca^{2+}, substrate and reaction product). In this studied, the stability of the enzyme at 75 ^{o}C for 60 minutes was slightly enhanced by addition of 0.2% and 2.0% soluble starch.

Analysis of cyclodextrin products by HPLC

Identification of CD products was first performed using the condition used in *Paenibacillus* sp. A11. The cyclodextrin products of CGTase from BT01 were analyzed by HPLC. CGTase from BT01 produced mainly 2 types of CDs, α-CD and β-CD, at pH 6.0, 40 ^{o}C for 24 hours. The ratio of α- : β-CD was approximately 1:1. It seems that BT01 produced α- and β-CD as major products in equal amount.

The production yield and ratio of the different CDs formed by CGTases is dependent not only on the microbial source producing the enzyme but also on the nature of the substrate and the bioconversion conditions (such as temperature, pH and time) (Martins and Hatti-Kaul, 2002). When optimum conditions were established for CGTase of BT01, the profile of CDs product between pH 6.0 and 7.0 incubated at 40 ^{o}C were studied. The result showed that at pH 7.0 which is the optimum condition of CD-forming activity, the enzyme converted starch into CDs more than at pH 6.0 but the ratio of α- : β-CD was constant. This shows that the pH of reaction mixture can influence the rate of production CDs. When the production of CDs was studied by varying the temperature at pH 7.0 and incubated for 24 hour, it was found that starch conversion rate increased correspondingly with increasing temperatures until the temperature reached 60 ^{o}C and slightly decreased at 70 and 80 ^{o}C. It should be noted that the optimum temperature was about 55-60 ^{o}C for CD production. Interestingly, at 80 ^{o}C the product detected was mostly β-CD.

Cyclodextrin productions was investigated using various sources of starch. The enzyme could utilize all the starches tested (Figure 3.38). This suggests that the source of starch is not important for CGTase action and the ratio of α- : β-CD remained constant. However, the yield varied with the type of starch used. The CD forming activity of the enzyme was found to be very stable when stored at both 4 ^{o}C

and -20 ^{o}C, indicating that it was a very stable enzyme. Addition of $CaCl_2$ could stabilze the CGTase for up to 7 weeks similar to that reported by other researchers.

From our screening of bacteria from samples of soil, water and starch waste from different sites with carbohydrate contamination in the area, we could select one strain of thermotolerant bacteria which can produce high level of CGTase. Primary study on the properties of partial purified CGTase suggested a CGTase produced by the bacteria with potential industrial applications. More detailed study on the enzyme should be carried out to establish the most favorable conditions for the use of the enzyme in the industrial process.

CHAPTER V

CONCLUSIONS

1. Ninety-seven amylase-producing isolates were screened from soil and waste in the areas contaminated with carbohydrate. Among them, 3 strains (BT01, BD01 and BO01) exhibited CGTase activity.
2. A bacterial strain exhibiting highest CGTase activity was BT01 with was screened from soil in Thai starch factory in Nakhon Pathom province.
3. From biochemical characterization, BT01 was Gram-positive and was identified as *Bacillus circulans.*
4. In the identification by 16S rRNA gene fragment amplification, the sequence obtained had 1312 bp which was identified as *Paenibacillus* sp. BT01 with 98% homology.
5. Optimum conditions for CGTase production by BT01 were culturing in Horikoshi medium with 0.5% soluble starch at pH 10.0, 40 ^{o}C for 72 hours.
6. CGTase from BT01 was partially purified by starch adsorption with purification fold of 28 and yield of 65.1%.
7. The partially purified CGTase had the molecular weight of 45 kDa from SDS-PAGE and exhibited at least 3 isoforms with amylolytic activity stain.
8. Optimum temperature for dextrinizing activity and CD-forming activity were 70 ^{o}C and 55 ^{o}C, respectively. Optimum pH for dextrinizing activity and CD-forming activity were 6.0 and 7.0, repectively. The most suitable incubation time for dextrinizing activity was 10 minutes and CD-forming activity was 6 hours.

9. Dextrinizing activity remained stable between pH 6.0-9.0 and between pH 6.0-8.0 for CD-forming activity at 55 ^{o}C. At 70 ^{o}C, both activities were most stable at pH 6.0 for 1 hour.
10. The presence of 5mM and 10 mM $CaCl_2$ slightly enhanced the stability of enzyme similar to addition of 0.2% and 2.0% starch substrate.
11. The best condition for storing enzyme was -20 ^{o}C in presence of 10 mM $CaCl_2$.
12. Cyclodextrin products from CGTase of BT01 was α : β = 1:1
13. The pH of reaction mixture can influence the rate of CDs production and the optimum temperature of starch conversion into CDs was 55-60 ^{o}C.

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APPENDICES

APPENDIX A: Preparation for polyacryamide gel electrophoresis

1. Stock reagents

1.) 30 % Acrylamide, 0.8% bis-acrylamide, 100 ml

acrylamide 29.2 g

N,N' – methylene-bis-acrylamide 0.8 g

Adjusted volume to 100 ml with distilled water.

2.) 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 ml with distilled water.

3.) 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 ml with distilled water.

4.) 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 ml with distilled water.

5.) 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 ml with distilled water.

6.) Solution B (SDS-PAGE)

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

7.) 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

1.) 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

2.) 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

3.) Sample buffer

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

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

4.) 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

1.) 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

2.) 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

3.) Sample buffer

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

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

4.) 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

1.) 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.

2.) 0.2 M Phosphate pH 6.0

KH_2PO_4 2.27 g

K_2HPO_4 0.58 g

distilled water 100 ml

3.) 0.2 M Phosphate pH 7.0

KH_2PO_4 0.91 g

K_2HPO_4 2.32 g

distilled water 100 ml

4.) 0.2 M Tris-HCl pH 8.0 and 9.0

Tris (hydroxymethyl)-aminometane 2.42 g

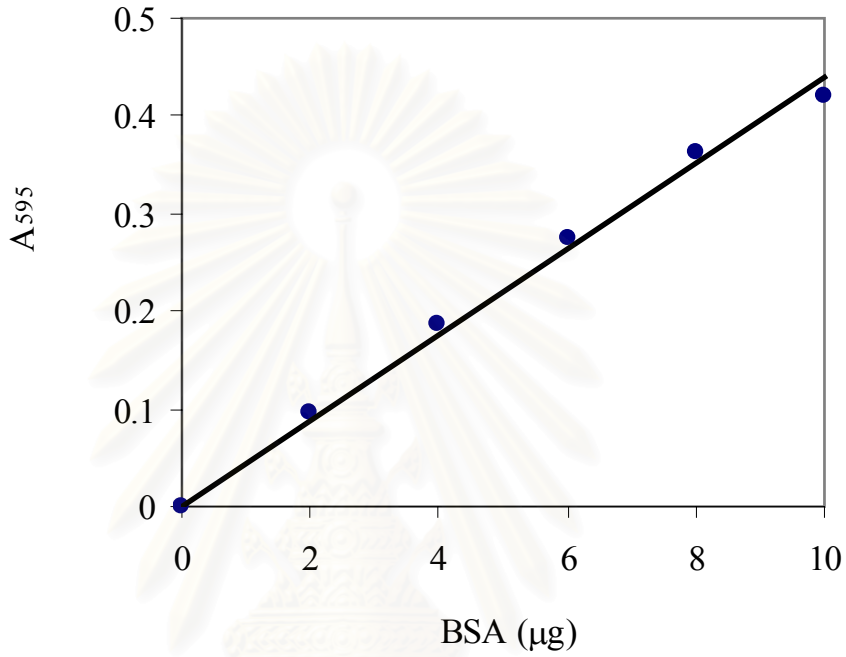
Adjusted to pH 8.0 or 9.0 by 1 M HCl and adjusted volume to 100 ml with distilled Water.

5.) 0.2 M Tris-Glycine NaOH pH 10.0 and 11.0

Glycine 1.5 g

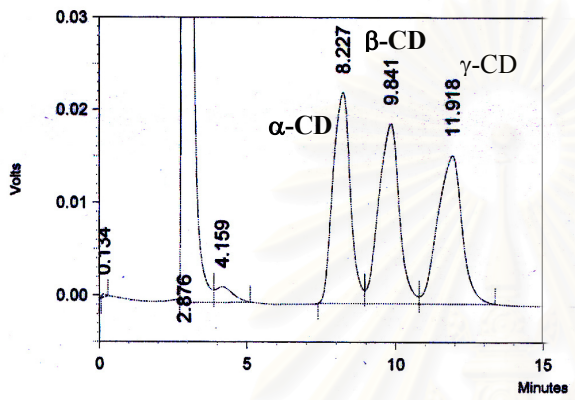
Adjusted to pH 10.0 or 11.0 by 1 M NaOH and adjusted volume to 100 ml with distilled Water.

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



APPENDIX D: Analysis of cyclodextrin products

Chromatogram of standard cyclodextrins (A) and standard linear oligosaccharides (B). CGTase was preincubated at temperature 30 ^{o}C (C), 50 ^{o}C (D), 70 ^{o}C (E) and 80 ^{o}C (F) for 1 hour.



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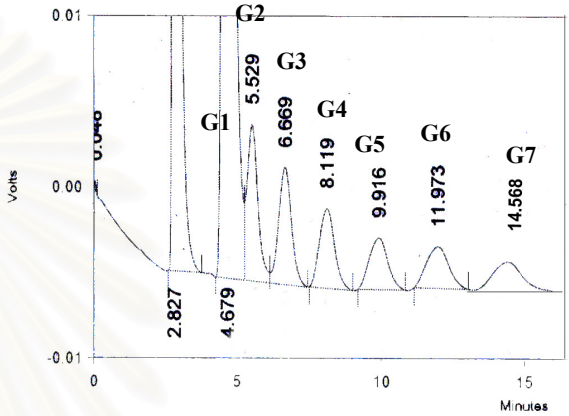
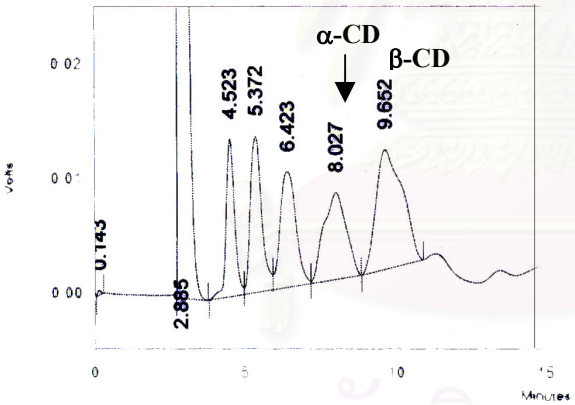


B



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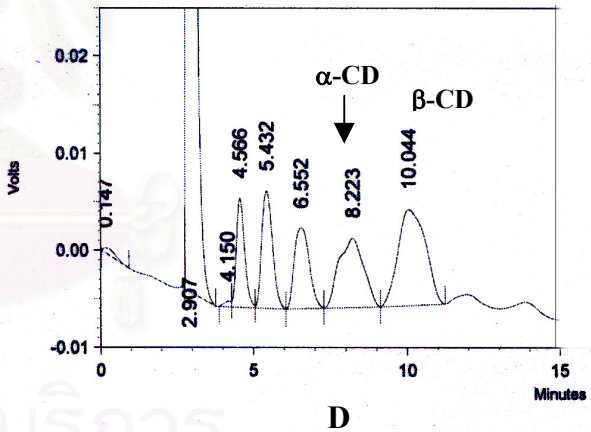
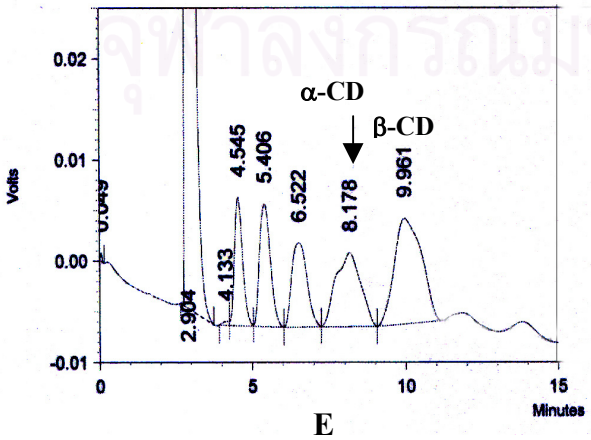
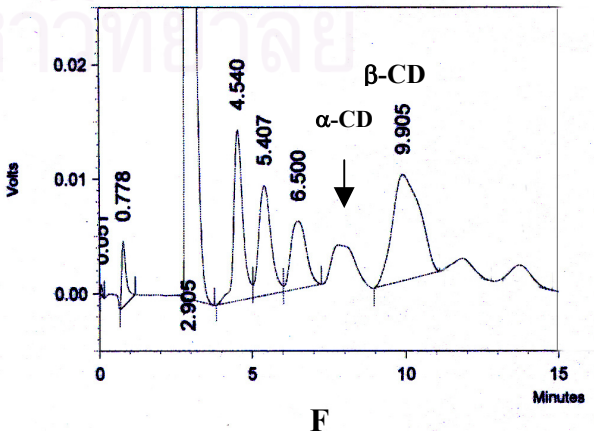


D



E



F

APPENDIX E: 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)
$MgCl_2$ (25mM/µl)	8 µl	(200 mM)
Taq (5u/µl)	0.5 µl	(25U)
Ultrapure claved water	41.5 µl	
Total	100 µl	

Condition for amplification (30 cycle)

95 °C	1 minute	1 cycle
50 °C	2 minute	
72 °C	3 minute	

APPENDIX F: The DNA sequencing profile of 16S rRNA gene fragment from BT01

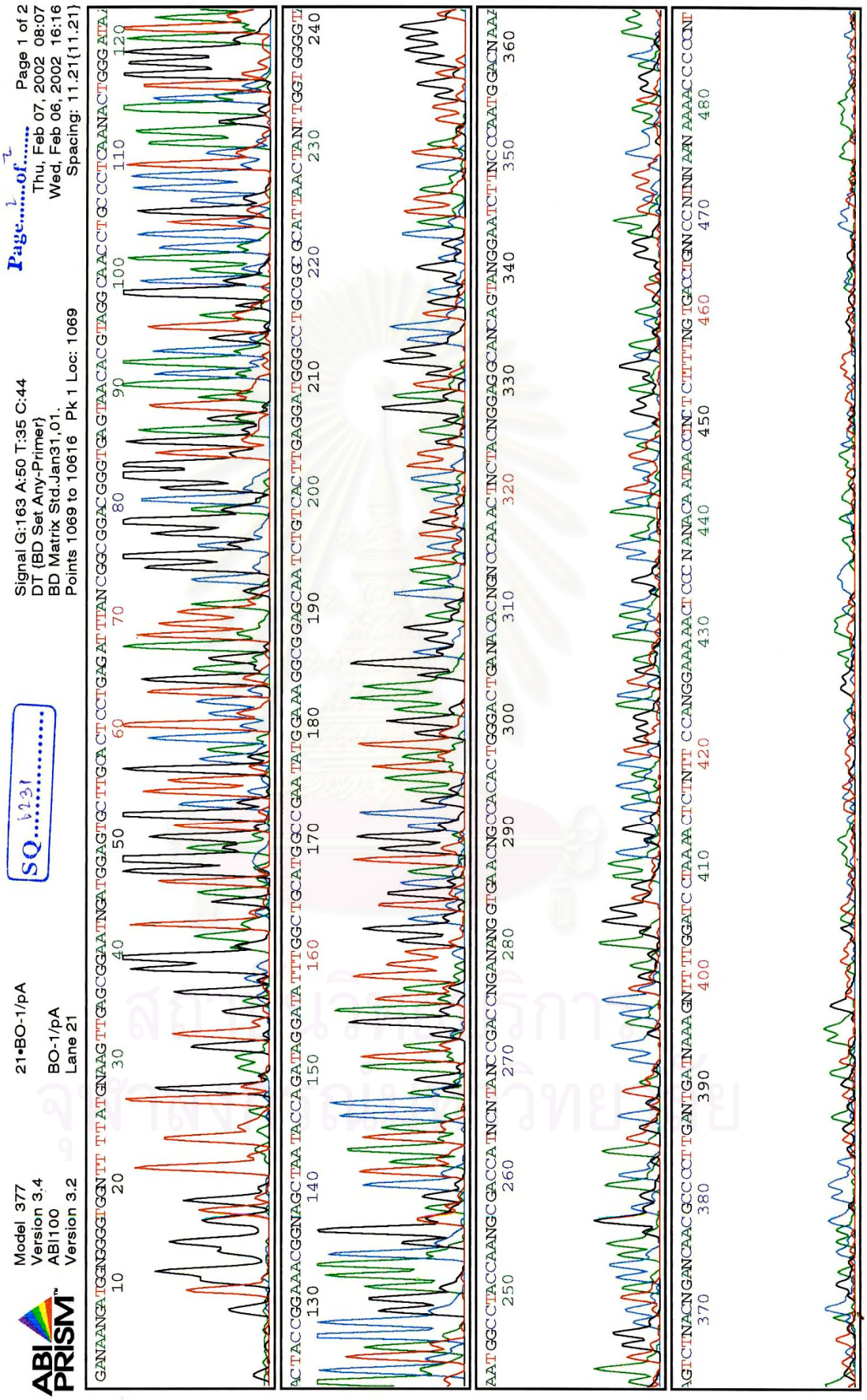
(A): The DNA sequencing profile of 16S rRNA gene fragment from BT01 using the sense primer A

(B): The DNA sequencing profile of 16S rRNA gene fragment from BT01 using the sense primer D

(C): The DNA sequencing profile of 16S rRNA gene fragment from BT01 using the sense primer F

(D): The DNA sequencing profile of 16S rRNA gene fragment from BT01 using the sense primer D’

(A)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Model 377
Version 3.4
ABI100
Version 3.2
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BO-1/pA
Lane 21
SQ.....
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Page 1 of 2
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Wed, Feb 06, 2002 16:16
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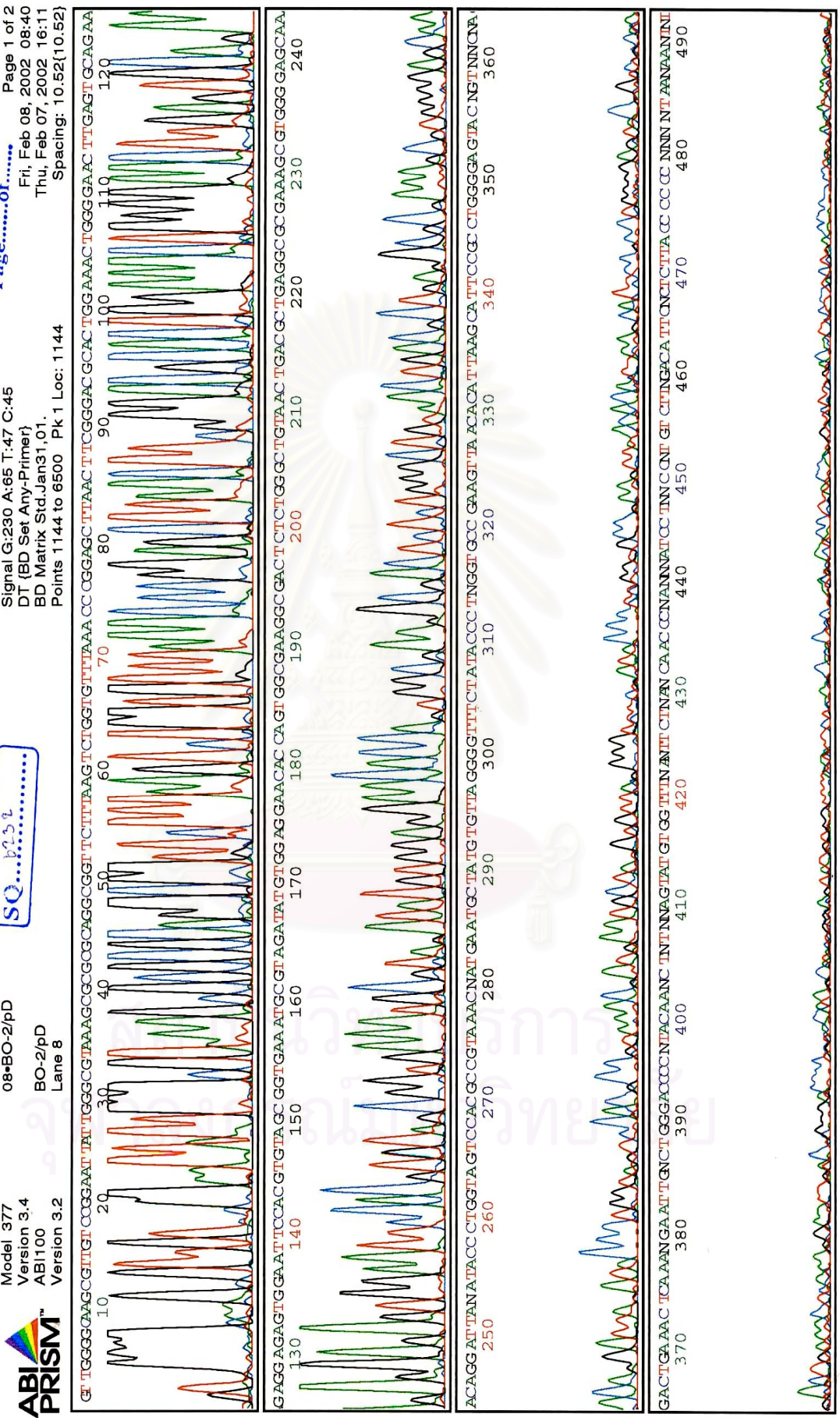
(B)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Version 3.4
ABI100
Version 3.2
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BO-2/pD
Lane 8
SQ...b23t
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BD Matrix StdJan31.01.
Points 1144 to 6500 Pk 1 Loc: 1144
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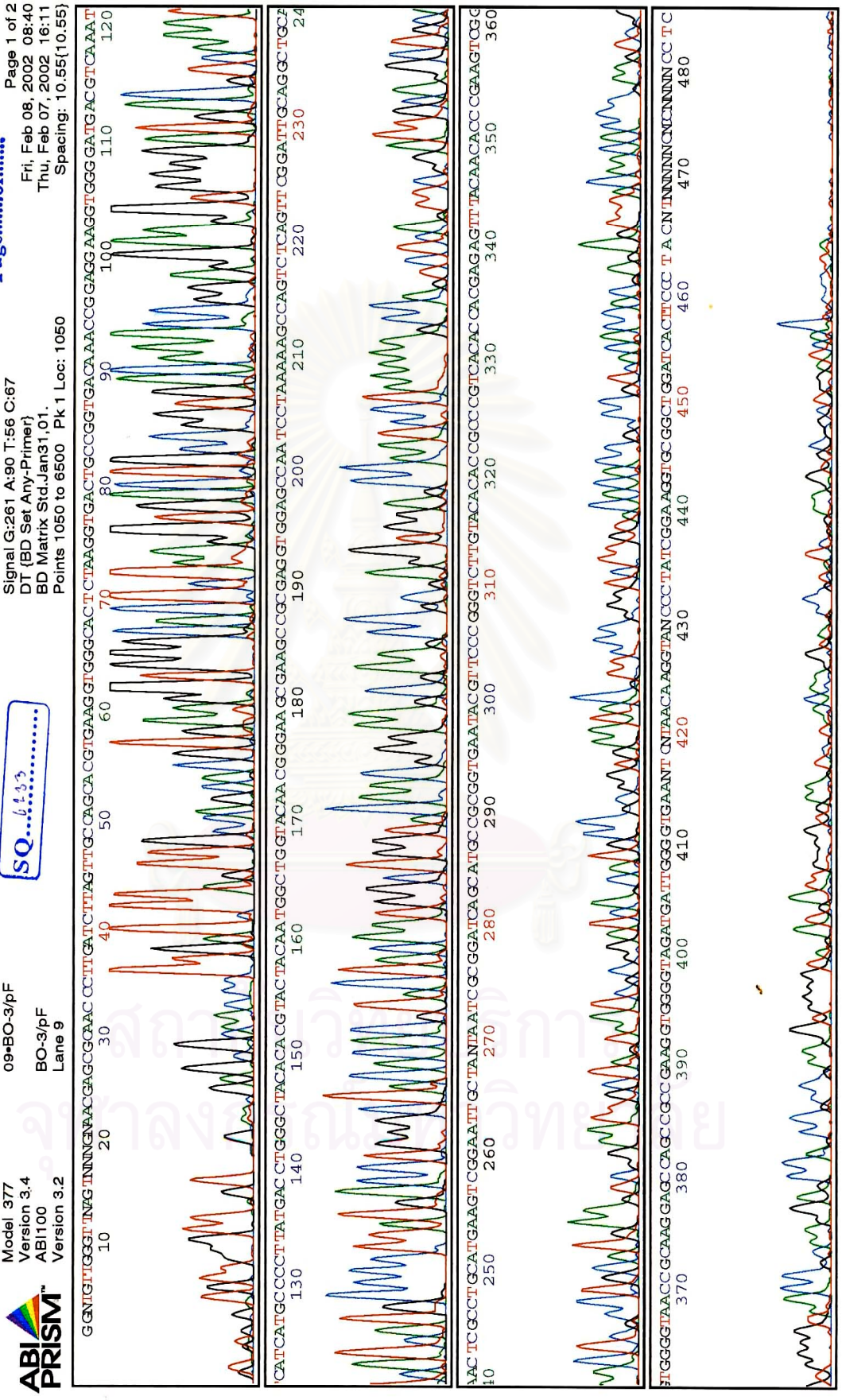
Page 1 of 2

(C)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Version 3.4
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Version 3.2
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BO-3/pF
Lane 9
SQ...
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BD Matrix Std.Jan31,01.
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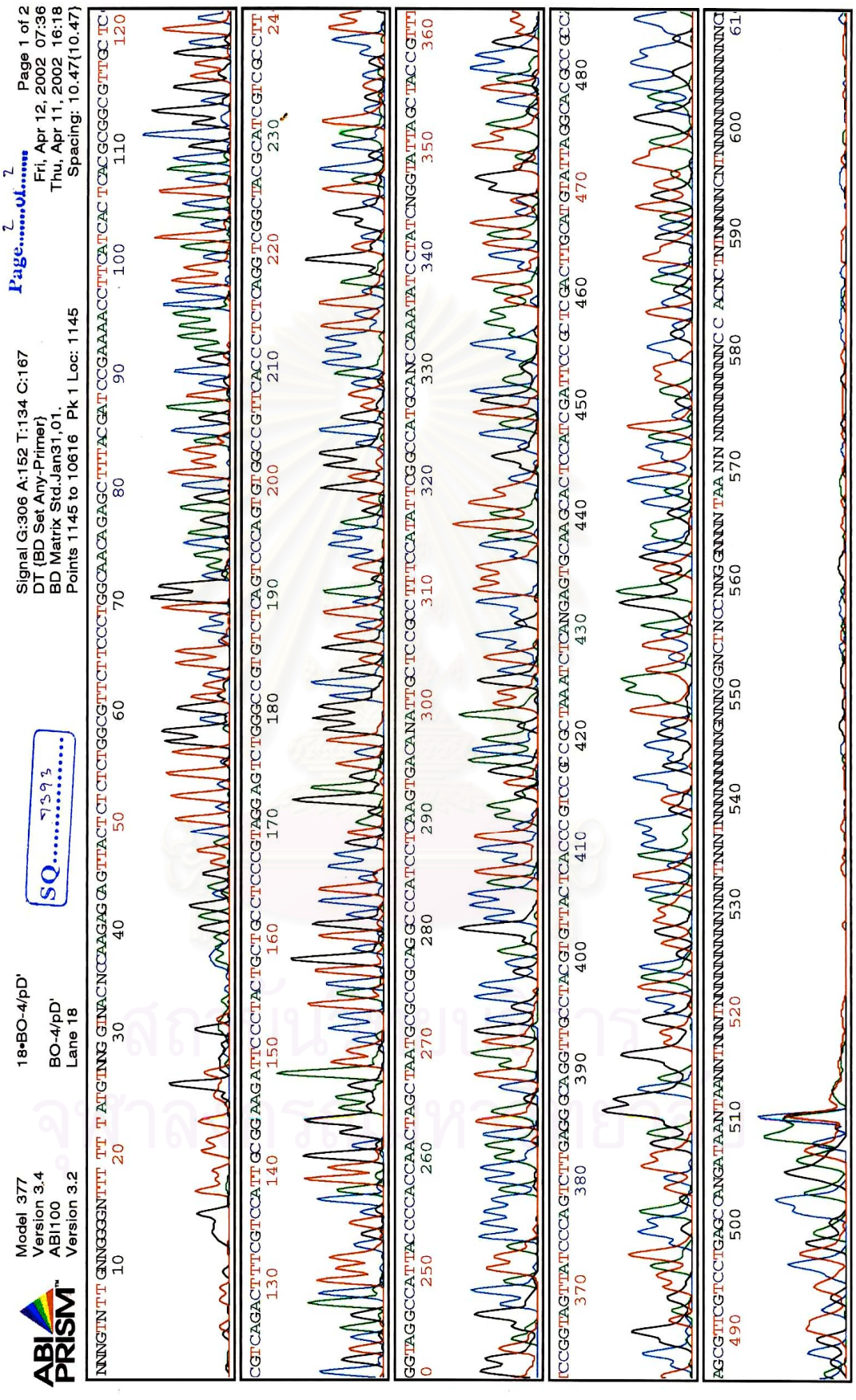
(D)


ABI PRISM
Model 377
Version 3.4
ABI100
Version 3.2
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BO-4/pD'
Lane 18
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BD Matrix Std.Jan31.01.
Points 1145 to 10616 Pk 1 Loc: 1145
Page.....of.....
Fri, Apr 12, 2002 07:36
Thu, Apr 11, 2002 16:18
Spacing: 10.47{10.47}
Page 1 of 2

BIOGRAPHY

Miss Pornthida Yampayont was born on March 14th, 1978 in Bangkok. She graduated with the Bachelor Degree of Science in Department of Microbiology, Faculty of Science, Chulalongkorn University in 2000 and continued studying for Master course in Biotechnology program, Faculty of Science, Chulalongkorn University.