CHAPTER I

INTRODUCTION

1.1 Structure and properties of cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides composed of α-(1,4) linked glucopyranose units. Cyclodextrins mainly consist of 6, 7 or 8 glucose residues, designated as α-, β- and γ-cyclodextrins (α-, β- and γ -CDs), respectively (Table 1). Each cyclodextrins is a torus (doughnut-shaped) molecule (Figure 1). The cyclodextrin cavity thus forms a hydrophobic microenvironment in aqueous solutions, making cyclodextrin an ideal host for an unusually wide variety of guest molecules of the appropriate size and polarity.

Table 1 Properties of cyclodextrins (1).

Type of cyclodextrins	α.-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight (g/mol)	972	1135	1297
Solubility in water at 25 °C (% W *N)*	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Inner diameter (Å)	4.7 - 5.3	6.0-6.5	7.5 - 8.3
Height of torus (Å)	7.9	7.9	7.9
Approximate cavity volume (AJ)	174	262	427

1.2 Applications of CDs

CDs are capable of forming inclusion complex with various organic compounds by incorporating them into the cavity of their cyclical structure. This can lead to desirable changes in the physical and chemical properties of the incorporated compounds.

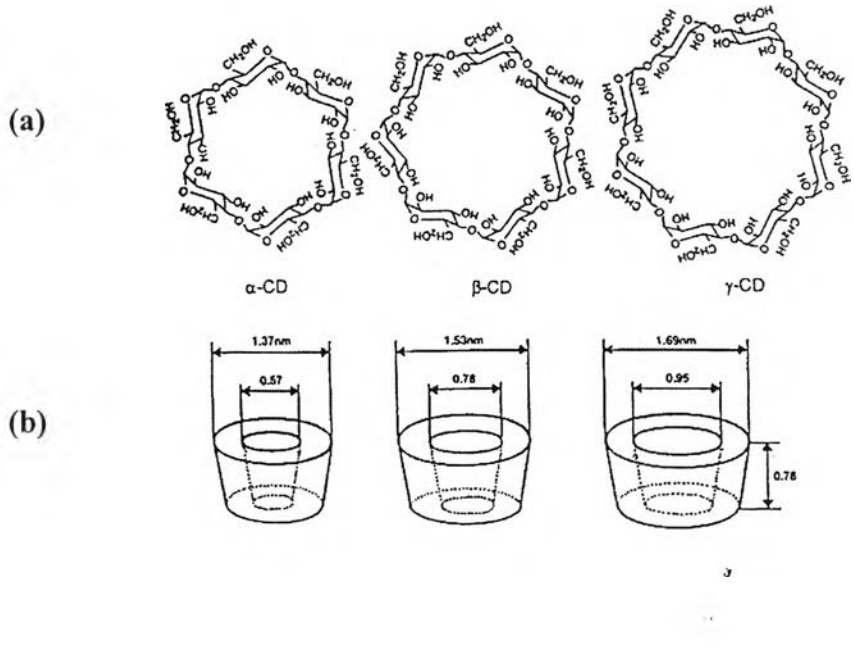

Figure 1 Structures of cyclodextrins.

(a) Chemical structure of three kinds of the CDs

(b) Molecular dimension of the CDs

Cyclodextrin inclusion is a stoichiometric molecular phenomenon in which usually only one guest molecule interacts with the cavity of the CD molecule to become entrapped. A variety of non-covalent forces, such as van der Waals forces, hydrophobic interactions and other forces, are responsible for the formation of a stable complex. Generally, one guest molecule is included in one CD molecule, although in the case of some low molecular weight molecules, more than one guest molecule may fit into the cavity, and in the case of some high molecular weight molecules, more than one CD molecule may bind to the guest. In principle, only a portion of the molecule fits into the cavity to form a complex. As a result, one-to-one molar ratios are not always achieved, especially with high or low molecular weight guests molecules. Inclusion in CD exerts a profound effect on the physicochemical properties of guest molecules as they are temporarily locked or caged within the host cavity giving rise to beneficial modifications of guest molecules, which are not achievable otherwise (**2**). Examples of such changes are solubility enhancement of highly insoluble guests, stabilization of labile guests against the degradative effects of oxidation, visible or UV light and heat, control of volatility and sublimation, physical isolation of incompatible compounds, chromatographic separations, taste modification by masking off flavors, unpleasant odors and controlled release of drugs and flavors. As a result of molecular complexation phenomenon, CDs are widely used in many industrial products, technologies and analytical methods. The negligible cytotoxic effects of CDs are an important attribute in applications such as drug carrier (**3**), food and flavors (**4**), cosmetics (**5**), packing (**6**), textiles (**7**), separation processes (**8**), environment protection (**9**), fermentation (**10**) and catalysis fields (**11**). Some applications of CDs shown in Table 2.

Table 2 Selected applications of CDs (7)

Industries	Applications
Food	
Cinnamon-flavored apples	Stabilize flavor
Mint and green tea mints	Stabilize flavor
Peppermint-flavor chewing gum	Flavor delivery
Mustard oil steak sauce	Improve solubility
Acetic acid	Convert to a powder
Aloe-containing beverage	Mask bitterness
Water purifier	Absorb order
Lemon-flavored sugar	Flavor stabilization
Pharmaceuticals	
Itraconazole	Increase solubility
Piroxicam	Reduce irritation
Garlic oil	Mask order
Hydrocortisone	Increase solubility
PGE1	Increase stability
Cosmetics and personal care items	
Skin cleanser	Tocopherol carrier
Artificial tanning Lotion	Stability, mask order
Powdered hair bleach	Stability
Perfume	Prolonged release
Cold cream	Solubility
Miscellaneous	
Laundry drier sheet	Fragrance control
Chromatography column	Separations

CDs can form inclusion complexes with fats, flavors and colors. They are used for the removal and masking of undesirable components and controlled release of desired food constituents (**12**). Cosmetic preparation is another area which demands a lot of CD use, mainly in the volatility suppression of perfumes, room fresheners and detergents by the controlled release of fragrances from the inclusion compounds. The major benefits of cyclodextrins in this sector are the stabilization, odor control, process improvement upon conversion of a liquid ingredient to a solid form, flavor protection and flavor delivery in lipsticks, water solubility and enhanced thermal stability of oils (**5**). Some of the other applications include the uses in toothpaste, skin creams, liquid and solid fabric softeners, paper towels, tissues and underarm shields (**1**).

CDs serve as an ideal selector by molecular recognition and further enhance the complex forming ability and selectivity in various types of separations. CDs are used as chemically bonded or sorbed ligands in stationary phase or in mobile phase (**13**). Currently, chiral separations are one of the most important areas of the application of CDs and their derivatives (**8**). Hydrophilic CDs have been frequently used in capillary electrophoresis as buffer modifiers to effect chiral separation of drugs and specialty chemicals (**14**). Furthermore, CDs are also extensively used in high-performance liquid chromatography (HPLC) as stationary phases bonded to solid support or as mobile phase additives in HPLC and in capillary electrophoresis for the separation of chiral compounds (**15**). CDs are used in gel electrophoresis (**16**), capillary zone electrophoresis (**17, 18**) isotachophoresis (**19**), capillary gas chromatography (**20**), electrokinetic chromatography (**21**), microdialysis (**22**), ion exchange (**23**), affinity chromatography (**24**), thin layer chromatography (**25**) and separation through membranes (**26**).

Fabrics can be imbued with novel properties by means of cyclodextrins. In order to permanently transfer the versatile properties of cyclodextrins to textiles, Wacker-

Chemie (the world's largest producer of γ-CDs) covalently attached reactive CD derivative with monochlorotriazinyl (MCT) substituents to the fiber. This substituted CD provided excellent textile finishing to cottons, blended materials and woolens (7).

1.3 Cyclodextrin glycosyltransferase

Cyclodextrin glycosyltransferase (CGTase EC 2.4.1.19) is a unique enzyme capable of converting starch into cyclodextrins (CDs). The systematic name of CGTase is 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing). It is also called cyclodextrin glucanotransferase, cyclomaltodextrin glucanotransferase or cyclomaltodextrin glycosyltransferase.

CGTase is produced by a variety of bacteria including aerobic mesophilic bacteria, aerobic thermophilic bacteria, anaerobic thermophilic, and aerobic halophilic bacteria. The examples of these strains were listed in Table 9.

Bacillus is one of the several species of bacteria mostly found to produce cyclodextrin glycosyltransferase. When these bacteria were grown in the presence of starch, CGTase production is induced and excreted into the starch medium. These CGTases convert starch into CDs, which are subsequently transported back into the cells and degraded by the action of another enzyme cyclodextrinase located at the cytosolic side.

Table 3 Examples of bacteria producing cyclodextrin glycosyltransferases (CGTase) (**27**).

Aerobic mesophilic bacteria
Pseudomonas spp.
Bacillus cereus
B. megaterium
B. ohbensis
Paenibacillus macerans
Klebsiella oxytoca
K. pneumoniae
Micrococccus luteus
Brevibacillus brevis
Aerobic thermophilic bacteria
B. stearothermophilus
Anaerobic thermophilic bacteria
Thermoanaerobacterium thermosulfunigens
Thermoanaerobacter
Aerobic halophilic bacteria
B. halophilus

There are two possible explanations for the existence of this complicated system:

i) The organism can build up an external storage form of glucose, not accessible to most other organisms because they are not able to metabolize CDs (**28**).

ii) CDs are used to form inclusion complexes with toxic compounds in the environment or with compounds needed for growth (**29**).

The bacterial CGTase is a multifunctional enzyme which catalyzes four related reactions: cyclization, coupling, disproportionation and hydrolysis (**30**). By means of the cyclization activity, CGTases convert starch and related substrates into CDs (Figure 9).

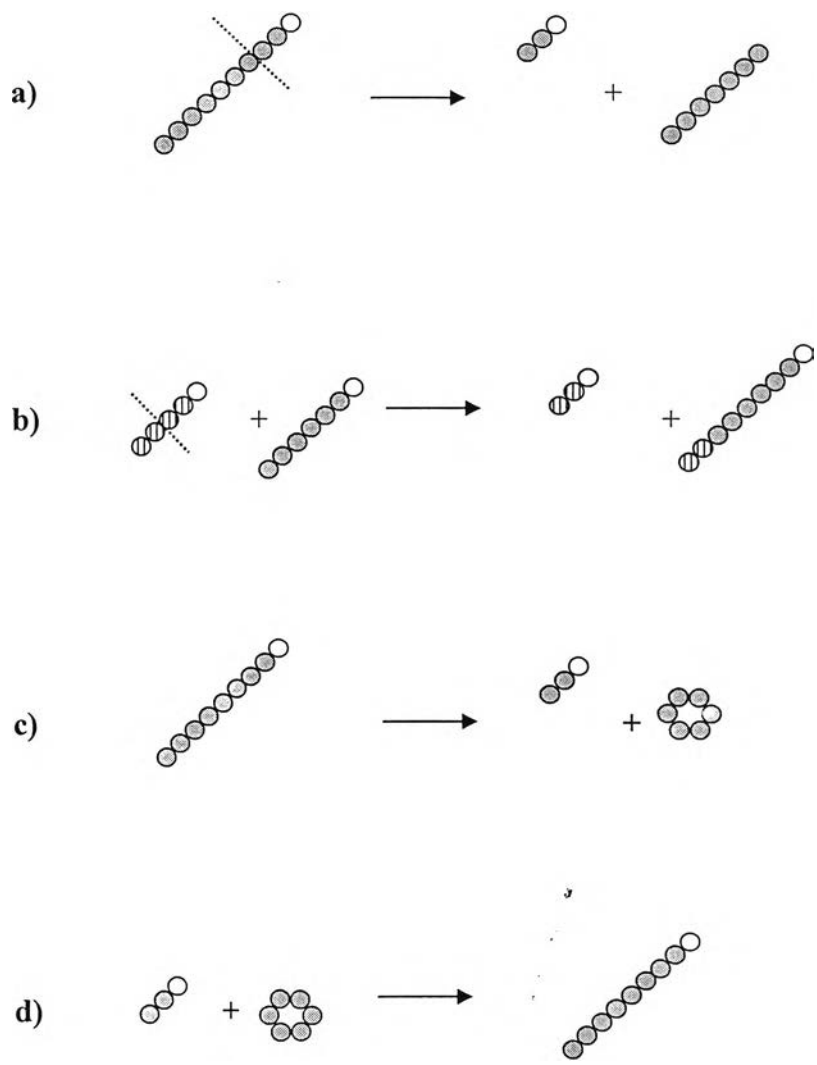

Figure 2 Schematic representation of the CGTase catalyzed reactions (**31**).

The circles represent glucose residues; the white circles indicate the reducing end sugars. (a): hydrolysis, (b): disproportionation, (c): cyclization, (d): coupling

1.4 Limitation of industrial production of CDs

There are several problems in CD production by CGTase. Firstly, the CGTase which is used for the commercial production of CDs (**32**) is poorly active on native starch due to the well organized structure of the granules held together by internal hydrogen bonds. Liquefaction by α-amylase treatment or heating in water to weaken the hydrogen bonds away from starch molecules is needed. The α-amylase used for liquefaction produces maltodextrins, which can act as acceptor molecules in the coupling reaction of the CGTase, severely reducing the yield of CDs. However, many thermostable CGTases have been isolated and characterized from the thermophilic bacteria. These CGTases are active and stable at high temperature, and are able to solubilize starch, thereby eliminate the need for α–amylase pretreatment (**33**). Secondly, all CGTases usually produce a mixture of α-, β- and γ-CD and are sensitive to product inhibition (**31, 34**).

Two different industrial approaches are used to purify the produced CDs, solvent and non-solvent processes (Figure 3). The solvent process is the selective crystallization of complexes of CDs with organic solvents. Toluene and cyclohexane are commercially used for the complexation and selective precipitation of β-CD. For α-CD, I-decanol can be used, but this compound is difficult to remove from aqueous solution because of its high boiling point (229°C). For γ-CD, cyclododecanone is used but this solvent is very expensive. Further, disadvantages of the use of organic solvents are their toxicity, which limits the applications to human consumption, their flammability and the need for a solvent recovery process (**31**). The non-solvent process was first developed for β-CD production. Due to its low solubility, β-CD can be easily purified by crystallization. The purification of α- and γ-CD is achieved via complex and expensive chromatography with low yields and a wide range of by-products. Lower yield of the enzymatic products, a more complex purification process, ineffective crystallization, a higher energy demand

and a large number of byproducts are the major disadvantages of the non-solvent process (**27**). Clearly, the availability of CGTase enzymes capable of producing an increased ratio of one particular type of CD with reduced product inhibition would help to avoid the disadvantages described above.

Thirdly, The conventional procedures for the production of CDs include liquefaction of starch using a thermostable α-amylase at 105°C. After the reaction mixture is allowed to cool down to about 50°C, it is treated with bacterial CGTase, which has an optimum catalytic reaction temperature of 50-65°C (**35, 36**). The high temperature in the fermenter tank can destroy the enzyme activity. Therefore, a CGTase with liquifying and cyclization activities at high temperatures (optimal reaction temperatures of thermostable CGTases are between 60 and 90°C) is desirable. It would be beneficial for efficient production of CDs; reducing the temperature gap between the use of α-amylase and CGTase, reducing risk of the microbial contamination, improving reaction rate of enzymatic conversion, lower viscosity and higher solubility of substrates.

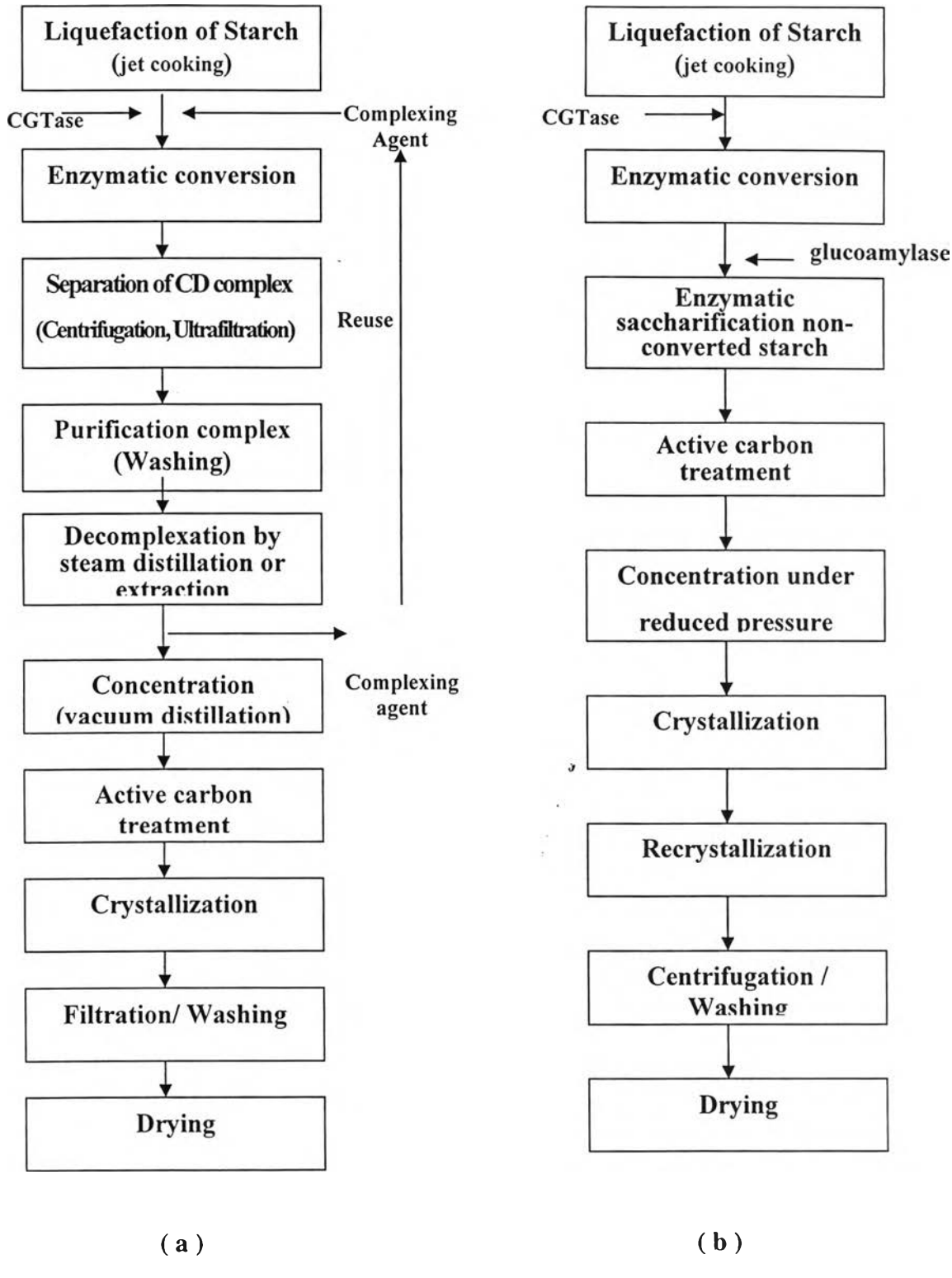

Figure 3 Industrial separation of CDs.

a) Solvent process for CD production.

b) Non-solvent process for CD production

1.5 Research on overproduction and application of CGTase

Gene cloning and overexpression of CGTase gene, not only provides satisfactory CD production, but also provides more enzymes for studies on structures and mechanisms including determination of its nucleotide sequence. In such studies, the β–CGTase genes from an alkalophilic *Bacillus* sp. #1011 (**37**), *Bacillus* sp. strain no. 38-2 (**38**) and *Klebsiella pneumoniae* M5 a1 (**39**) were cloned and expressed in *E. coli* and *Bacillus subtilis.*

Various studies have been emphasized on the improvement of CD productions. Development of cultivation for CD over-production was carried out under optimized culture condition and with complex nutrient media. A need for thermostable or thermotolerant CGTase which gives high CD yield has been recognized. CGTase from an alkalophilic *Bacillus* strain no. 38-2 (ATC 21783) was observed to provide these required properties (**40**). Immobilized CGTase can be utilized in several conversion cycles to steadily increase the volume of production, hence reduced the production cost (**41, 42**). Protein engineering by site-directed mutagenesis and gene cloning are also used to increase the yield of enzyme product. These techniques are not only based partly on an assumption of lower production costs, but also on the trends towards greater acceptability of CDs (**43**).

There were several reports on the thermostable CGTases. *Thermoanaerobacter* was a thermophilic anaerobe. Its CGTase had a temperature optimum of 90°C and produced CDs more efficiently than the CGTase isolated from *Bacillus macerans* with a temperature optimum of 55°C (**44**). *Thermanaerobacter thermosulfurigenes* and *Anaerobranca gottschalkii* were reported to suit for these above proposes (**45**). However, it was sometime wasteful in terms of energy to use microorganisms that tolerated 60°C or higher for most of the production of enzymes in industries. In addition,

inactivation of the enzyme upon the completion of the process would not be easy due to the thermostability of the enzyme. Therefore, it was advantageous to perform overexpression of these thermostable enzymes in mesophilic hosts such as *Bacillus* sp., *yeast (Pichia* sp.), filamentous fungi or, more recently, *Staphylococcus* (**46**) and to optimize the cultivation process. Alternatively, searching for thermotolerant microorganisms which could be grown at temperature between 30-50°C was quite interesting.

Our research group in the Starch and Cyclodextrin Research Unit has been working on β-CGTase of *B. circulans* A11. Its enzyme was quite extensively studied. It was isolated from South-East Asian soil (**47**). The enzyme was purified and characterized in its properties such as molecular weight, working pH and temperature and the enzyme activity on various substrates were characterized (**48**). Effect of some carbohydrates on the induction of CGTase to produce higher CD-products mainly γ-CD was also studied (**49**). The enzyme was purified by chromatofocusing column and analysis on native-PAGE suggested that it composed of 4 isozymes with different isoelectric points in the range of 4.40-4.90 (**50**) and was used in enzyme purification through immunoaffinity column chromatography (**51**). Optimization of CGTase production in a 5 litre-fermenter and cyclodextrin production from rice starch by using immobilized CGTase in both batchwise and continuous processes and also free CGTase were studied (**52-54**) Molecular cloning techniques, gene expression, mapping and partial nucleotide sequence determination (**55-57**), synthesis of oligonucleotide probes for CGTase gene (**58**) and studied on the mutation of *Bacillus* sp. A11 for the production of higher CGTase activity were reported (**59**). CGTase isozymes from this strain were isolated and characterized (**60**). Production of cyclodextrins from cassava starch was studied (**61**) and specificity of glycosyl acceptor in coupling and transglycosylation

reactions of CGTase from *Bacillus circulans* A11 were reported (**62**). Cloning and sequencing of genes coding for CGTase (**63**) (**64**)and CDase (**65**) were also studied.

Recently, our group started to be interested in thermotolerant bacteria. Tesana, 2001 and Pranommit, 2001 had recently screened a few CGTase producing thermotolerant bacteria from soil around the hot spring area. *Paenibacillus* sp.RB01 from Ratchaburi (**66**)and *Paenibacillus* sp.T16 from Tak (**67**) were selected. The optimum temperatures for growth of RB01 and T16 were 30-45°C and 37-50°C, respectively, while the optimum temperature for enzyme production was 40°C and 37°C, respectively. The pH and temperature for optimum dextrinizing activity was 7-9 and 45-55°C for RB01, and 8 and 65°C for T16. Their CD's yield were α : β : γ-CD = 1 : 5.4 : 1.2 and 0.74 : 1: 0.27, respectively.

Since RB01 and T16 showed potential of producing thermotorelant CGTase, cloning of CGTase gene from these thermotolerant bacteria were planned in order to obtain transformants with improved CGTase producing capacity CGTase for use in CDs production at industrial scale.

1.6 Objectives of this research

1. Cloning of the cyclodextrin glycosyltransferase (CGTase) gene from thermotolerant bacteria, *Paenibacillus* sp.RB01 from Ratchaburi province and *Paenibacillus* sp. T16 from Tak province
2. Selection of the transformant with the highest CGTase producing activity and expression of the enzymes
3. Purification and Characterization of CGTases produced by the selected transformants in comparison with wild type enzymes.