SCREENING OF POTENTIAL MICROORGANISMS FOR THE PRODUCTION OF NOVEL CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTase) ENZYME

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CERTIFICATE

This is to certify that the dissertation entitled "Screening of Potential Microorganisms For The Production of Novel Cyclodextrin Glycosyltransferase (CGTase) Enzyme" is the bonafide record of research work done by Mr. Mohd. Nazrul Anuar bin Ali during the period from July 2008 to October 2008 under my supervision.

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ABSTRACT

Ten soil samples were collected from the vicinity of restaurant's drain where the wastewater contain remaining starch food constituent was filtered and drained away, housing estate ditch and trash waste disposal area around Kubang Kerian. They were screened for potential CGTase enzyme producer by culturing in media containing cassava starch and nutrient agar. A total of 40 colonies have been successfully isolated in this study by detecting the production of clearance zone around them, and those colonies were selected as CGTase producing bacteria. The CGTase producing bacteria were identified as *Bacillus* species by microscopy observation. The bacteria was grown in production medium containing 1.5% (w/v) cassava starch, 0.4% (w/v) $(NH_4)_2SO_4$, 0.1M phosphate buffer (pH 7.0), 0.002% (w/v) $MgSO_4$ and 0.002% (w/v) $FeSO_4$ for the production of CGTase enzyme. The supernatant obtained after centrifugation at 3000 g for 15 minutes was used as crude enzyme. The activity of CGTase production was evaluated qualitatively by observing the production of clearance zone around the colony and quantitatively by determining the dextrinizing activity based on the method described by Fuwa, (1954) and Matsuzawa *et al.*, (1975) with slight modification.

ABSTRAK

Sepuluh jenis sampel tanah telah diambil dari kawasan berdekatan sekitar restoran, di mana sisa-sisa makanan berkanji ditapis sebelum dialirkan dan tempat pembuangan sampah di kawasan perumahan sekitar Kubang Kerian. Sampel tanah ini telah disaring untuk mengesan kehadiran mikroorganisma yang menghasilkan enzim CGTase dengan mengkulturkannya di atas media yang mengandungi kanji ubi kayu dan agar nutrien. Dalam kajian ini, sejumlah 40 koloni telah berjaya dipencilkan dengan mengesan kehadiran zon cerah di sekelilingnya dan ia telah dipilih sebagai mikroorganisma penghasil enzim CGTase. Bakteria ini telah dikenalpasti sebagai spesis *Bacillus* melalui pemerhatian di bawah mikroskop. Pertumbuhan bakteria dilakukan di atas media penghasilan yang mengandungi 1.5% (b/i) kanji ubi kayu, 0.4% (b/i) $(NH_4)_2SO_4$, 0.1M larutan penampan fosfat (pH 7.0), 0.002% (b/i) $MgSO_4$ dan 0.002% (b/i) $FeSO_4$ untuk menghasilkan enzim CGTase. Supernatan yang diperoleh selepas media tersebut diemparkan pada 3000 g selama 15 minit digunakan sebagai enzim CGTase mentah. Aktiviti enzim CGTase ini telah dinilai secara kuantitatif dengan melihat penghasilan zon cerah di sekeliling koloni dan secara kualitatif dengan menentukan aktiviti pendekstrinan enzim CGTase berdasarkan prosedur yang telah dicadangkan oleh Fuwa, (1954) dan Matsuzawa *et al.*, (1975) dengan sedikit pengubahsuaian.

CHAPTER 1 INTRODUCTION

1.1 GENERAL OVERVIEW OF CYCLODEXTRIN GLYCOSYLTRANSFERASE (CGTase) PRODUCER

Bacillus species constitute the major contributor of industrially important enzymes (Starnes, 1990). Cyclodextrin Glucanotransferase (CGTase) is an enzyme commonly produced by many bacterial species. Examples of CGTase producer are in the species of *Bacillus* such as *Bacillus macerans, Bacillus circulans,* alkalophilic *Bacillus* sp., *Bacillus coagulans, Bacillus polymyxa* and *Bacillus lentus* (Lee *et al.*, 1992; Nakamura and Horikoshi, 1976; Schmid, 1989). In fact, industrial production of CGTase was made attractive only when alkalophilic *Bacillus* species were introduced as production organism (Savergave, *et al.*, 2007). However, production by other species such as *Klebsiella, Thermoactinomyces, Micrococcus, Brevibacterium, Aspergillus and* thermophilic *Archea* has also been reported (Rita and Rajni, 2002).

Generally, CGTase enzyme producer can be classified into following groups:

a) Mesophilic aerobic bacteria such as *Bacillus macerans* (Depinto and Campbell, 1968; Takano *et al.*, 1986), *Bacillus megaterium* (Fogarty, 1983; Ramakrishna *et al.*, 1994), *Bacillus cereus* and *Bacillus ohbensis* (Jamuna *et al.*, 1993), *Klebsiella pneumonia* (Fogarty, 1983), *Klebsiella oxytoca* (Wind *et al.*, 1995) and *Micrococcus luteus* (Abelian *et al.*, 1995b).

b) Thermophilic aerobic bacteria such as *Bacillus sterothermophilus* (Abelian *et al.*, 1995b; Fujiwara *et al.*, 1992; Wind *et al.*, 1995).

c) Thermophilic anaerobic bacteria *Thermoanaerobacterium thermosulfurigenes* (Wind *et al.*, 1995).

d) Alkalophilic aerobic bacteria such as *Bacillus circulans* (Nakamura and Horiskoshi, 1976a, 1976b, 1977) and *Bacillus* sp AL-6 (Fujita *et al.*, 1990).

e) Halophilic aerobic bacteria such as *Bacillus halophilus* (Abelian *et al.*, 1995b).

1.2 CYCLODEXTRIN GLYCOSYLTRANSFERASE ENZYME (EC 2.4.1.19)

1.2.1 General Background of Cyclodextrin Glycosyltransferase (CGTase)

Cyclodextrin glycosyltransferase [α-1,4-glucan 4-glycosyltransferase] or CGTase is a bacterial enzyme belongs to the same family of glycosyl-hydrolase of α-amylase. This special enzyme is also known as Cyclodextrin glucanotransferase or Cyclomaltodextrin glucanotransferase (Akimaru *et al.*, 1991) as it other official name. Historically, this CGTase enzyme was accidently found by Tilden and Hudson in *Aeromonas (Bacillus) macerans* culture filtrate (Tilden and Hudson, 1939; Kitahata *et al.*, 1974).

The fermentation media used are different in composition but, most of them utilize starch as the main ingredient in the production of CGTase (Tonkova, 1998). However, the research done by Jamuna *et al.* (1993) revealed that *Bacillus cereus* was capable to produce CGTase with maximum yield by utilizing xylose as the carbon source. On the other hand, *Bacillus stearothermophilus* was also able to yield CGTase when lactose, glycerol, sorbitol or sucrose was being used as the carbon source (Bong *et al.*, 1990).

Nitrogen source is also important in the production of CGTase. Most of the complex nitrogenous sources such as corn steep liquor, yeast extract, bacteriological pepton and plant based protein source such as Pharmamedia™ and hydrolysed plant protein such as Proflo™ could highly controlled the production of CGTase as well as supporting the growth of the particular microorganism (Pongsawasdi and Yagisawa,

1987; Gawande *et al.*, 1998). *Bacillus lentus* on the other hand, is the only exclusive bacteria which able to produce optimum CGTase activity when none of nitrogen source being used. (Sabioni and Park, 1992).

1.2.2 Functions of Cyclodextrin Glycosyltransferase

The reaction of CGTase and α-amylase upon starch can be distinguished as shown in Figure 1.1:

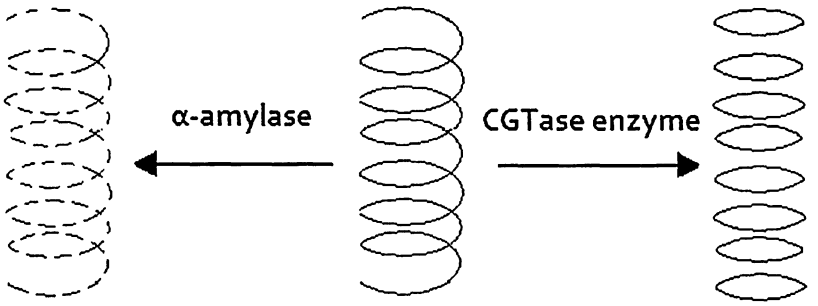


Figure 1.1: Comparison between CGTase and α-amylase action

CGTase is a unique enzyme with multifunctional features capable in catalyzing several reactions. The enzyme can catalyze up to four main reactions which are cyclization, coupling, disproportination and hydrolysis. All these activities share the same catalytic mechanisms which are common to all glycosyl-hydrolases group.

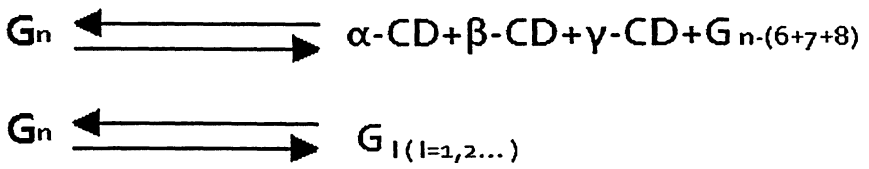
a) Cyclization: formation of cyclic in a process called intramolecule transglycosylation. The linear polysaccharide chain is cleaved and the two ends of the cleaved fragment are joined to produce a circular dextrin or cyclodextrin (bioconversion of related starch and α-1,4 glucan substance into CD). Cyclodextrin can be distinguished as α-CD, β-CD and γ-CD based on the number of six, seven or eight sugar residues respectively.

b) Coupling: known as a reverse process of cyclization through which the CGTase cleaves a cyclodextrin to produce a linear dextrin which subsequently joined to a linear oligosaccharide (cleavage of cyclic structure accompany by transferring of generated linear maltooligosaccharide to the recipient).

c) Disproportination: a process in which linear maltooligosaccharide is transferred to recipient in intermolecule transglycosylation reaction. It is very similar to coupling, but the cleaved dextrin is a linear oligosaccharide instead of cyclodextrin, that is then joined to a second oligosaccharide.

d) Hydrolysis: a process by which starch and related substance is hydrolysed (Akimaru *et al.*, 1991a; Kim *et al.*, 1997; Wind *et al.*, 1995). This weak hydrolyzing activity possessed by CGTase enables it to cleave the longer polysaccharide chains into shorter fragments.

CGTase could also act on pullulan to generate branches of pullulan (α-1,4 pullulan). The following figure show a reaction scheme catalyzed by CGTase :



G = glucose unit

CD = cyclodextrin

n,i = numbers of glucose unit (G)

Figure 1.2: Reaction scheme of Cyclodextrin glycosyltransferase enzyme

1.2.3 Characteristics of Cyclodextrin Glycosyltransferase (EC 2.4.1.19)

Cyclodextrin glycosyltransferase (CGTase) is an extracellular enzyme that converts starch into non-reducing, cyclic malto-oligosaccharide called cyclodextrin (CDs). It is an important hydrolytic enzyme that carries out reversible intermolecular as well as intramolecular transglycosylation and performs cyclization, coupling and disproportionation of maltooligosaccharides.

CGTase acquired from different microorganisms will produce different yield ratios of major type of CD (Pongsawasdi and Yagisawa, 1987; Akimaru *et al.*, 1991a). All the CGTase enzymes produce α-CD, β-CD and γ-CD from starch in different ratios. However, this enzyme was not suitable for industrial scale application because of instability at high temperature (Norman and Jorgensen, 1992). Industrial production of CGTase became feasible only when alkalophilic and thermophilic *Bacillus* species were introduced as production organism. Enzymes that could synthesize predominantly one type of CD are preferred for industrial application because expensive cost in separation of individual CDs (Gawande *et al.*, 1999).

A particular CGTase that was successfully isolated from *Thermoanaerobacter thermosulfurigenes* which showed a unique feature of stability at high temperature since the optimal temperature is about 95^0C. This feature enables the enzyme to be used for industrial production of CGTase by the reason of lack in contamination problem and easily sterilized (Wind *et al.*, 1995). All CGTase are produced extracellularly except from

CGTase enzyme is classified based on its optimum pH. Most of the microorganisms produce an enzyme with a single pH optimum. However, *Bacillus* sp. No. 38-2 produce three types of CGTase with acidic, alkaline or neutral pH simultaneously. Acidic CGTase is the most thermostable among of them (Nakamura and Horikoshi, 1976c). Table 1.2 showed the characteristics of CGTase enzyme from *Bacillus* sp. No 38-2.

Table 1.2: Characteristics of Cyclodextrin glycosyltransferase from *Bacillus* sp. No. 38-2

Characteristics	**Acidic**	**Neutral**	**Alkaline**
Optimum pH	4.5-4.7	7.0	8.0-9.0
Optimum temperature (°C)	45	50	-
Molecular weight	88 000	85 000-88 000	85 000-88 000
Isoelectric point	5.4	-	-
pH stability*	6.0-10.0	6.0-9.0	-
Thermal stability#	approaching 65°C	approaching 60°C	-
Dominant product	β-CD	β-CD	β-CD

*: incubation for 30 minutes at 60°C

#: incubation for 30 minutes

1.3 CYCLODEXTRIN

1.3.1 General background of Cyclodextrin

Cyclodextrins (CDs) are crystallize, non-reducing, cyclic oligosaccharides, homogenous, non hygroscopic with torus ring structure doughnut shape-like (French, 1957; Nakamura and Horikoshi, 1976). CD composed of D-glucose residue units linked by α-1,4 glycosidic bond (Kim *et al.*, 1997). Cyclodextrin containing six up to twelve D-glucose unit are the most common and produced by enzymatic reaction of Cyclodextrin glyscosyltransferase (CGTase) from starch and α-1,4 glucan substances such as amylase, amylopectin, maltooligosaccharide and glycogen (Abelian *et al.*, 1995; Nakamura and Horikoshi, 1976; Szejtli, 1988). CDs are named based on its cyclic shape. It was also known as Schardinger dextrin (first founder) and cyclomylose (cyclic shape and originated from amylose) [Lee *et al.*, 1992].

There are 3 major types of CDs being produced at industrial scale which known as α-CD, β-CD and γ-CD with six, seven and eight D-glucose residual units respectively (Szejtli, 1988). Each of the CDs possesses different interior ring size or cavity space at centre depends on the type of CDs. Following figure shows different shape of α-CD, β-CD and γ-CD.

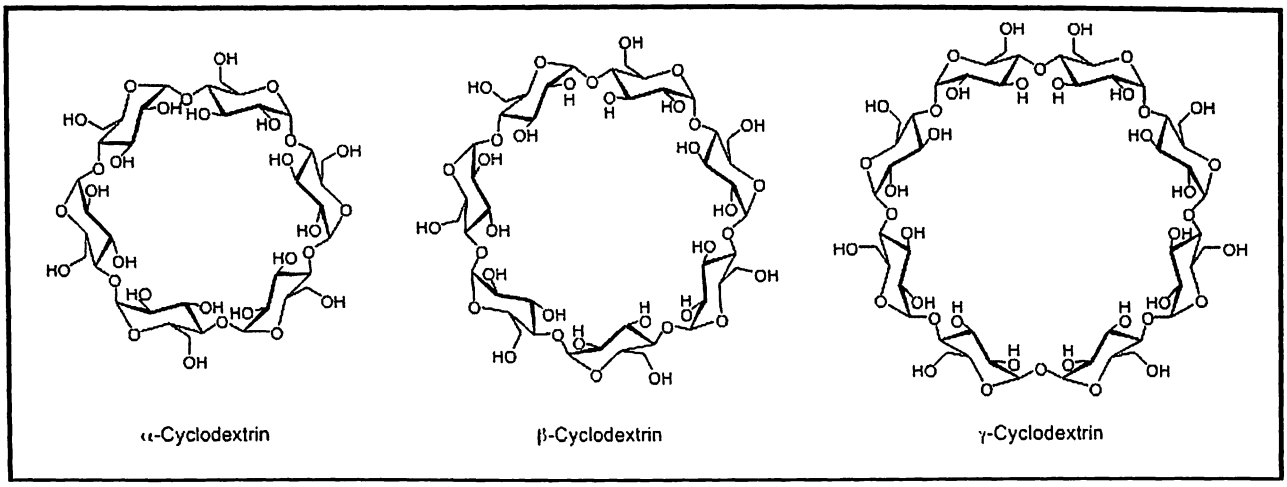


Figure 1.3: Different shape between α-CD, β-CD and γ-CD.

The orientation of the CDs molecule is unique since the hydrophilic hydroxyl groups are on the outside of the ring structure, and the interior of the cavity contains the hydrophobic CH groups and glycosidic oxygens (Pedersen *et al.*, 1995). The dimensions of the ring depend on the number of glucose units. One of the main applications of CDs makes use of this hydrophobic center. Owing to their apolar cavity, they are able to form inclusion complexes with variety of small hydrophobic compounds. Inclusion complexes are formed by encapsulating various kinds of compound such as flavors and medical products interior of the cavity on the center of the ring structure (Wind *et al.*, 1995). This process changes the physicochemical properties, such as solubility and stability, of the guest compounds as well as stabilized the active compounds (Szetjli, 1990). Furthermore, CDs molecule is relatively stable compared to linear maltooligosaccharide due to absence of reducing and non-reducing ending. Table 1.3 simplified the properties of α-CD, β-CD and γ-CD.

Table 1.3: Properties of major Cyclodextrins (Pedersen *et al.*, 1995)

Characteristics	α -CD	β-CD	γ –CD
No. of glucose unit	6	7	8
Molecular weight	973	1135	1297
Cavity depth	7.9-8.0	7.9-8.0	7.9-8.0
Diameter of cavity	4.7-5.2	6.0-6.4	7.5-8.3
Outer diameter	14.6	15.4	17.5
Cavity volume	174	262	427
Solubility g/100 ml*	14.5	1.85	23.2

*at 25°C.

On the other hand, there are also several types of uncommon CDs being produced beside the 3 types of major CDs. They are also known as homolog CDs such as δ^-, η^-, ς^-, θ^- and ϵ^-. For instance, δ^- composed of nine glucose unit while the remaining η^-, ς^-, θ^- and ϵ^- are not being identified thoroughly due to their inclusion complexes formation weakness, isolation difficulty from the production medium, low recovery and purity through chromatography techniques. Theoretically, it is difficult to obtain CDs that possess less than six glucose molecule because of steric restriction (Sundararajan and Rao, 1970).

1.3.2 The Uses of Cyclodextrin

Owing to apolar cavity of CDs, they are suitable to be used in food, pharmaceutical, cosmetic, agricultural and chemical industries. In addition, it could function as emulsifiers, antioxidant substance and stabilizing agent in maintaining any stuff for long term storage (Horikoshi and Akiba, 1982). Apart from that, some researchers classified CDs as diet fibre that could benefit as calories replacement substance in order to control body weight (Suzuki and Sato, 1985). Current study found that α-CD, β-CD, γ-CD as well as the other derivation of CDs could possibly encapsulate cytotoxin produced by *Helicobacter pylori*. The cytotoxin is believed to be the major cause of ulcer and gastric ulcers among the patients, thus lead to gastric carcinoma type cancer (Marchini *et al.*, 1995). The uses of CDs are:

a) β-CD is widely used in a process to eliminate cholesterol from chicken eggs and milk (Lin and Yang, 1999) as well as unwanted natural components in foods (i.e caffeine, naringin and teobromine), bitter taste of citrus juice and add onto fruits to maintain the freshness and avoid oxidation.

b) β-CD is applied in encapsulation process of flavors and dye such as d-limonene (obtained from lemon oil), antocyanin pigment (exist on plant polysaccharide), allicine (active ingredient be found in garlic) and periodic timed release substance also replacement for high cost arabic gum (Lee *et al.*, 1992).

c) CD play major part in encapsulation and emulsification process of linoleic acid (Reichenbach and Min, 1997) and therapeutic compound exist inside chlorella extract (Testa, 1999).

d) Modified CD being used in pesticide contamination detector chromatography system in water (Shea *et al.*, 1999) and capillary electrophoresis (Perez *et al.*, 1998).