PARTIAL PURIFICATION OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM ALKALOPHILIC *Bacillus* sp. TS1-1

MOHD AZIM ABDUL RAHMAN

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

BORANG PENGESAHAN STATUS TESIS♦

JUDUL: **PARTIAL PURIFICATION OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM ALKALOPHILIC *Bacillus* sp. TS1-1**

SESI PENGAJIAN: **2007/2008**

Saya **MOHD AZIM ABDUL RAHMAN**
(HURUF BESAR)

mengaku membenarkan tesis (PSM/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Pahang dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Pahang
2. Perpustakaan Universiti Malaysia Pahang dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (✓)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)
✓	TIDAK TERHAD	

Disahkan oleh

(TANDATANGAN PENULIS)

(TANDATANGAN PENYELIA)

Alamat Tetap:
NO. 13B,
JALAN KG. PAYA,
KG. PAYA DALAM,
86000 KLUANG,
JOHOR.

MISTER ROZAIMI ABU SAMAH
Nama Penyelia

Tarikh: **MAY 2008**

Tarikh: **MAY 2008**

CATATAN:

* Potong yang tidak berkenaan.
** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT atau TERHAD.
◆ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertai bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjans Muda (PSM).

"I hereby declare that I have read this thesis and in
my opinion this thesis is sufficient in terms of scope and
quality for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)"

Signature	:	.....
Supervisor	:	Mister Rozaimi Abu Samah
Date	:	April 2008

PARTIAL PURIFICATION OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM ALKALOPHILIC *Bacillus* sp. TS1-1

MOHD AZIM ABDUL RAHMAN

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

MAY 2008

DECLARATION

I declare that this thesis entitled "Partial Purification of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. TS1-1" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

Signature	:	…….
Name	:	Mohd Azim Abdul Rahman
Date	:	May 2008

DEDICATION

Special dedication to

Khadijah Mustamar my beloved mother and

Abdul Rahman Mohd Seth my beloved father, both of you is everything to me

Baitilalia Ayu Zakaria, I believe in you and me

My siblings that always love me

My coursemates those always are challenging and supporting me

ACKNOWLEDGEMENTS

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

I am so thankful to Allah S.W.T for giving me enough time, good health, spirit, and patient in order to complete this project research. With the mercifulness from Allah, therefore I can produce a lot of useful idea to this project.

I am also would like to express my thankfulness to my beloved family, especially my beloved mother, Madam Khadijah binti Mustamar and my beloved father, Mister Abdul Rahman bin Mohd Seth for their prayers, support, patient, and guidance in my study.

I would like thank my Supervisor, Mister Rozaimi bin Abu Samah, for his continual professional advices, useful guidance, inspiration, patience and enormous support in the effort to complete this project. I am indebted to Universiti Malaysia Pahang (UMP) for their facilities, opportunity as well as financial aid provided in pursuing my project research. I am also grateful to my panels, Miss Asmida binti Ideris and Madam Norashikin binti Mat Zain for their brilliant idea, advice and guidance to me.

I would like to dedicate my appreciation to all the lecturers and associates of Faculty of Chemical and Natural Resources Engineering (UMP) that involve in this project for their invaluable time, guidance and advice especially my former academic advisor, Madam Chua @ Yeo Gek Kee and also post graduate students, Azizul, Fazli and Azrina. Without their cooperation and sacrifices, this research will not be able to complete and published. Last but not least, I would also like to thank you to my lab partner, Zulhelmi, Afnil Danial and Mohd Nizam who have accompanied me throughout this project. Thank you very much.

ABSTRACT

The cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *Bacillus* sp. TS1-1 has been partially purified from crude culture by centrifugation and cross-flow filtration. Initial 72.96 U/ml of CGTase was detected in the culture after 24 hours of incubation. The crude supernatant obtained after centrifugation for 5000 rpm, 5 minutes and 4°C was subsequently filtered at 15°C through cross-flow filtration using Kvick Lab cross-flow system. Two cassettes were used with molecular weight cut off of 50K and 10K. The retentate from 50K cassette was further filtered through 10K cassette. Each permeate and retentate from each cassette were tested for CGTase activity. The highest CGTase activity was detected at retentate of 10K cassette which suggested an initial size of CGTase between 10 kDa and 50 kDa. The crude and partially purified enzyme was then subjected to electrophoresis (SDS-PAGE). It was clear that a large amount of proteins had been removed by cross-flow filtration. Based on this work, the crude enzyme was purified 5.57 fold using cross-flow filtration.

ABSTRAK

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) daripada *Bacillus* sp. TS1-1 telah ditulenkan secara separa daripada kultur mentah menggunakan kaedah pengemparan dan penyaringan aliran melintang. Sebanyak 72.96 U/ml daripada CGTase telah dikesan di dalam kultur selepas 24 jam inkubasi. Supernatan mentah yang diperolehi selepas pengemparan pada 5000 rpm, 5 minit and 4°C yang kemudiannya disaring pada 15°C melalui penyaringan aliran melintang menggunakan sistem penyaring Kvick Lab. Dua blok penyaring yang telah digunakan ialah bersaiz rongga 50K dan 10K. Baki daripada penyaring 50K seterusnya disaring melalui penyaring 10K. Setiap hasil dan baki daripada setiap blok penyaring kemudian diuji untuk aktiviti CGTase. Aktiviti CGTase yang paling tinggi dikesan pada baki penyaring 10K yang mungkin menunjukkan saiz awal CGTase antara 10 kDa dan 50 kDa. Enzim mentah dan enzim yang telah ditulenkan secara separa kemudiannya dianalisis dengan elektroforesis (SDS-PAGE). Adalah jelas bahawa sejumlah besar protein telah disingkirkan semasa penyaringan aliran melintang. Berdasarkan kajian ini, enzim mentah telah ditulenkan sebanyak 5.57 kali ganda menggunakan penyaringan aliran melintang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

Å	-	angstrom
ADP	-	adenosine diphosphate
ATP	-	adenosine triphosphate
CD	-	cyclodextrin
CGTase	-	cyclodextrin glucanotransferase
h	-	hour
K	-	kilo Dalton (molecular weight cut off)
kDa	-	kilo Dalton
mg	-	milligram
min	-	minute
mM	-	millimolar
mol wt	-	molecular weight
µl	-	microliter
µmol	-	micromole
N	-	number of mole
ng	-	nanogram
nm	-	nanometer
OD	-	optical density
pI	-	isoelectric point
rpm	-	revolution per minute
SDS-PAGE	-	sodium dodecyl sulphate polyacrylamide gel
U	-	unit (enzyme activity)
UV	-	ultraviolet
V	-	volt
v/v	-	volume per volume
w/v	-	weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) are able to convert starch into cyclodextrins (CDs), closed-ring structures in which six or more glucose units are joined by means of α-1,4 glycosidic bonds (Hashimoto, 1988). This enzyme is an important enzyme in industries of food, analytical chemistry, agriculture, pharmaceutical field, and toilet articles. Generally, CDs exist as three different types: α-CD, β-CD and γ-CD, containing six, seven and eight glucose residues, respectively.

Villiers first observed CDs in 1891 when in addition to reducing dextrins a small amount of crystalline material was obtained from starch degrades of *Bacillus amylobacter*. The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds. From the X-ray structures it appears that in cyclodextrins the secondary hydroxyl-groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl-groups (C6) on the other edge, and that the apolar C3 and C5 hydrogens and the ether oxygens are at the inside of the torus-like molecules. This results in a molecule with a hydrophilic outside, which can dissolve in water, and an apolar cavity, which provides a hydrophobic matrix, described as a "micro heterogeneous environment" (Saenger, 1980).

CGTase are produced by many microorganisms. *Bacillus circulans* (Hofmann *et al*., 1989), *Bacillus firmus* (Shin *et al*., 2000), *Bacillus ohbensis* (Nishida *et al*., 1997), *Bacillus macerans* (Han *et al*., 1999), *Bacillus stearothermophilus* (Stefanova *et al*., 1999), *Klebsiella pneumoniae* (Gawande *et al*., 2001), *Bacillus* sp. TS1-1 (Mahat, 2004) and *Bacillus* sp. G1 (Sian *et al*., 2005) have been recognized as CGTase producer.

At present at least 38 CGTase enzymes have been identified and purified, and the matching genes cloned, mainly from *Bacillus* species, but also from *Thermoanaerobacterium, Thermoanaerobacter, Micrococcus* species, and from *Klebsiella*, a single Gram-negative source.

1.2 Problem Statement

The isolation of pure enzyme also allows active site studies to be carried out on the homogeneous protein, the characteristics of the enzyme such as kinetic parameters, optimum temperature and pH, temperature and pH stability, the effects of ions and crystallization of the enzyme for X-ray crystallographic analysis.

1.3 Objective

The objective of this research is to partially purify CGTase from alkalophilic *Bacillus* sp. TS1-1 that was isolated from soil.

1.4 Scopes

The scopes of this study are as follows

i. To produce crude CGTase from *Bacillus* sp. TS1-1
ii. To partially purify CGTase by cross-flow filtration
iii. To determine the degree of CGTase purification by SDS-PAGE

CHAPTER 2

LITERATURE REVIEW

2.1 Starch

2.1.1 Structure of starch

Starch has two types of glucan polymers: amylose and amylopectin. Potato starch as example consists of 20 % amylose and 80 % amylopectin. Depending on the origin, plant species, variety within plants, plant organ, age of organ, and growth conditions, this ratio may vary considerably, from 11 to 51 % amylose. Amylose consists mostly of linear chains of α-1,4 linked glucose residues, about 1000 residues long, and branched at a low level (about one branch per 1000 residues) by α-1,6 linkages. Pure amylose forms hydrogen bonds linking the molecules in solution, resulting in rigid gels. After heating this solution it might crystallize and shrink, this process known as retrogradation. The other 80 % of potato starch consists of amylopectin.

Amylopectin is a highly branched α-1,4 glucan polymer (approximately one α-1,6 linkage per 20 glucose residues), which forms organized structures (Figure 2.3). In these structures one can recognize the so called A-chains, which are not substituted at the C6 positions, the inner B-chains which are α-1,6 branched at one (B1-chain), or several points (B2, B3 etc.). There is only one free reducing end per amylopectin

molecule (the C-chain). The branches are clustered at 7-10 nm intervals (approximately 20 glucose residues), 20-40 per molecule, forming an amylopectin molecule 200-400 nm long (approximately 400-800 glucose residues long) and 15 nm wide. In solution amylopectin forms fewer hydrogen bonds than amylose, therefore it remains fluid, with a high viscosity and elasticity (Martin and Smith, 1995).

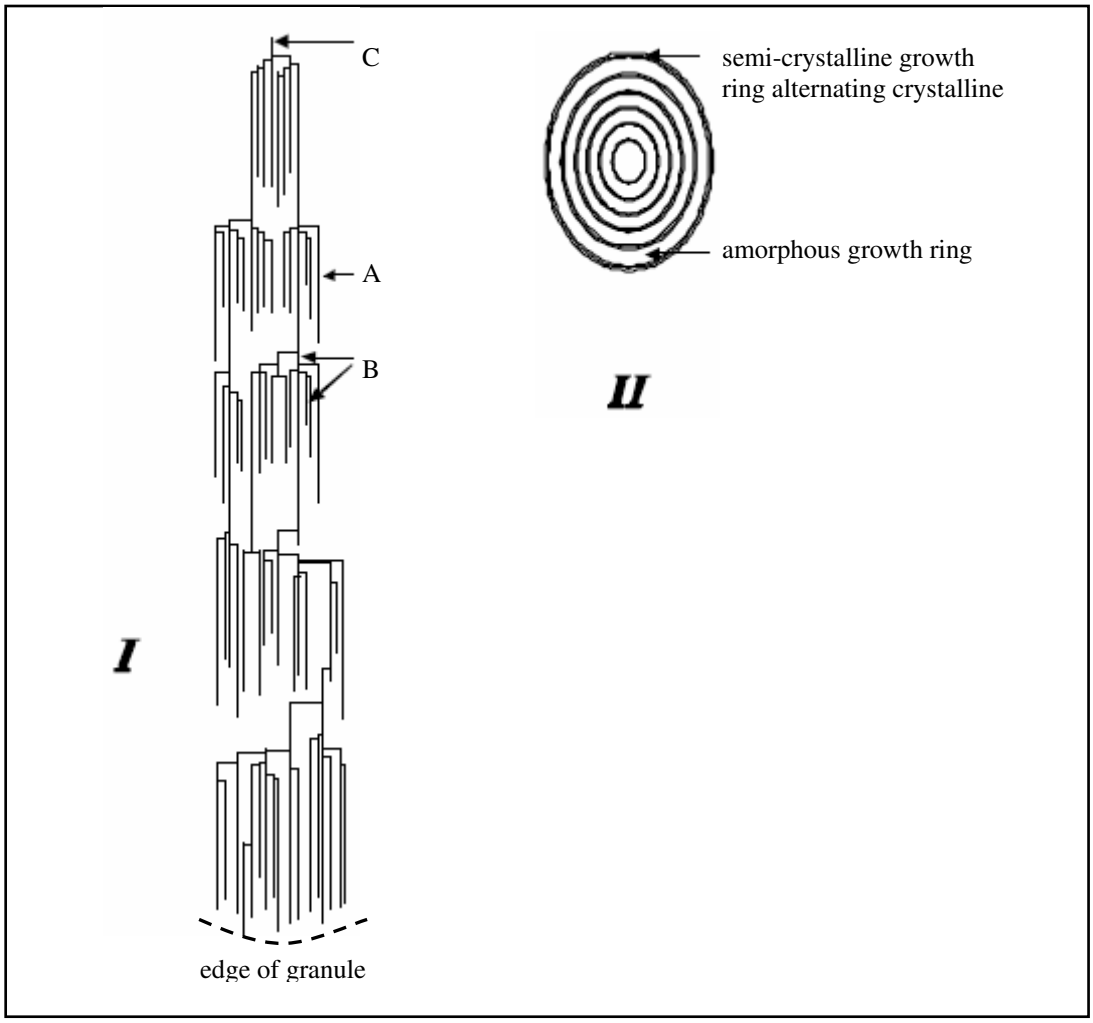

Figure 2.1: Schematic representation of starch.

I: Amylopectin molecule. Indicated are the A-, B-, and C-chains. A-chains are not branched; B-chains contain one branch (B1-chain) or more (B2-, B3-chain, etc). C-chains contain the single reducing end.

II: Starch granule. Show the alternating crystalline and amorphous growth rings.

(Martin and Smith, 1995)

2.1.2 The degradation of starch

The enzymatic process of starch biosynthesis is relatively simple; first an ADP-glucose pyrophosphorylase catalyzes the formation of an ADP-glucose and inorganic pyrophosphate from glucose-1-phosphate and ATP. For potato this takes place in the plant plastids (amyloplasts). Both ATP and glucose-1-phosphate have to be imported from the cytosol. Alternatively, glucose-1-phosphate can be synthesized in the plastid by a phosphoglucomutase from glucose-6-phosphate. The pyrophosphate is removed by the action of an alkaline pyrophosphatase.

The second step involves starch synthase, which catalyzes the formation of an α-1,4 bond between the C1 of the glucose from the ADP glucose and the C4 of the non-reducing glucose of the growing amylose chain. This suggests that an initial primer is needed to start the reaction; the nature of this primer is unknown.

A third step is formation of an α-1,6 branch by a starch-branching enzyme, which cuts an α-1,4 linked glucan chain and forms an α-1,6 linkage between the C1 at the reducing end of a released glucan chain and the C6 of a glucose residue in another chain. Branches are not created randomly, but show a 20 glucan residues periodicity. It is believed that this is due to the fact that the starch-branching enzyme has high affinity for a double helical conformation of glucan chains, which is only formed at a certain minimum chain length (Martin and Smith, 1995).

2.2 Cyclodextrin Glucanotransferase

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) cleaves α-1,4 bonds in a starch molecule, concomitantly linking the reducing and non-reducing ends to produce a cyclic molecule. When starch is used as a carbon source of the organism, CGTase

converts starch into cyclodextrins, which are subsequently degraded by the action of the enzyme cyclodextrinase (Saha and Zeikus, 1990), which is associated with the membrane and is located at the cytosolic site. CGTase is excreted by the bacterium and produces a cyclodextrin (CD) from an amylose chain. A cell-associated CDase converts cyclodextrins into glucose and other oligosaccharides. Glucose (Glu) is subsequently used in glycolysis to produce pyruvate (Pyr) and ATP (Nakamura *et al*., 1993).

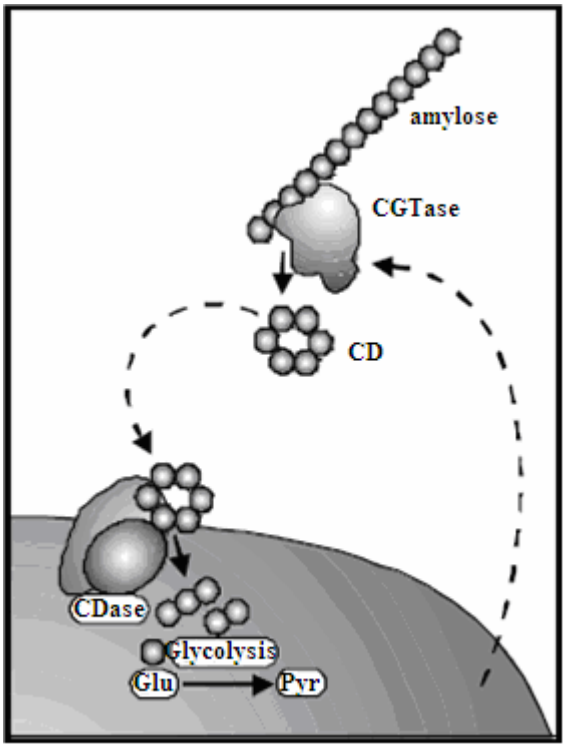

Figure 2.2: Schematic representation of the location and action of CGTase and CDase. Small circles indicate glucose residues (Nakamura *et al*., 1993)

CGTase are enzymes 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 (Figure 2.3). 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. Coupling is the reaction where a cyclodextrin molecule is combined with a linear oligosaccharide

(chain) to produce a longer chain linear oligosaccharide. 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. In hydrolysis (saccharifying activity) the newly made reducing end is transferred to water (Nakamura *et al*., 1993).

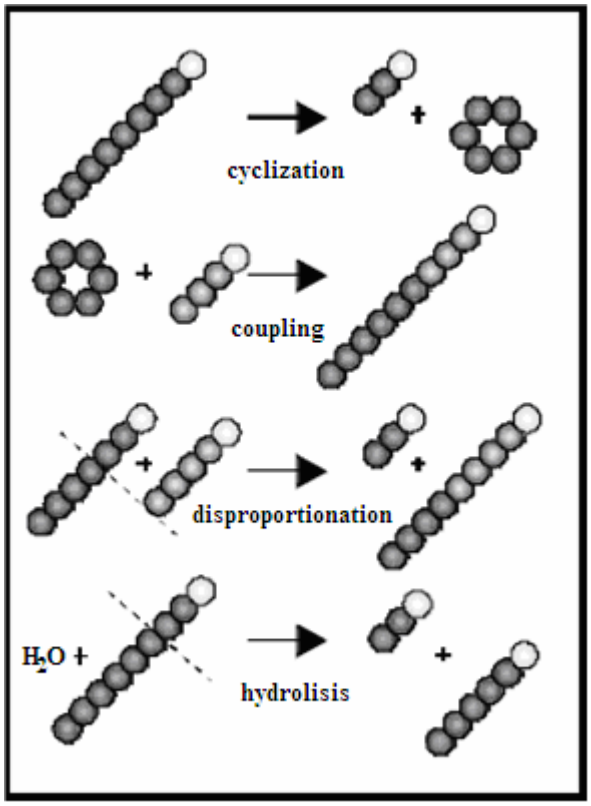

Figure 2.3: Schematic representation of the different activities of CGTase (Nakamura *et al*., 1993).

2.3 Cyclodextrins

2.3.1 Structure of cyclodextrins

Cyclodextrins are cyclic oligosaccharides consisting of six (α-cyclodextrin), seven (β-cyclodextrin), eight (γ-cyclodextrin) or more glucopyranose units linked by α-1,4 bonds (Figure 2.4). They were first discovered in 1891 when in addition to reducing dextrins a small amount of crystalline material was obtained from starch digest of *Bacillus amylobacter* (Villiers, 1891). According to other authors, Villiers probably used impure cultures and the cyclodextrins were produced by a *Bacillus macerans* contamination (Koch, 1891).

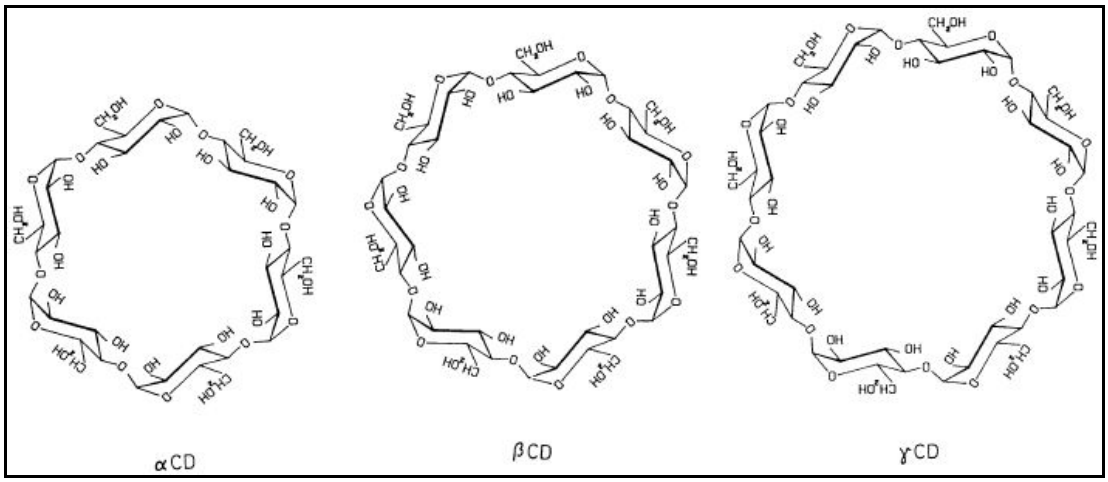

Figure 2.4: The structures of α-, β-, and γ-cyclodextrin (Szejtli, 2004)

The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds. From the X-ray structures it appears that in cyclodextrins the secondary hydroxyl-groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl-groups (C6) on the other edge, and that the apolar C3 and C5 hydrogens and ether like oxygens are at the inside of the torus-like molecules. This results in a molecule with a hydrophilic outside, which can dissolve in

water, and an apolar cavity, which provides a hydrophobic matrix, described as a "micro heterogeneous environment" (Saenger, 1980).

As a result of this cavity, cyclodextrins are able to form inclusion complexes with a wide variety of hydrophobic guest molecules (Figure 2.5). One or two guest molecules can be entrapped by one, two or three cyclodextrins. The most important parameter for complex formation with hydrophobic compounds or functional groups is their three dimensional form and size (Table 2.1). The driving force is the entropic effect of displacement of water molecules from the cavity (Saenger, 1980). Another possibility is that this water causes a strain on the cyclodextrin ring, which is released after complexation, producing a more stable, lower energy state (Saenger, 1980; Szejtli, 1982).

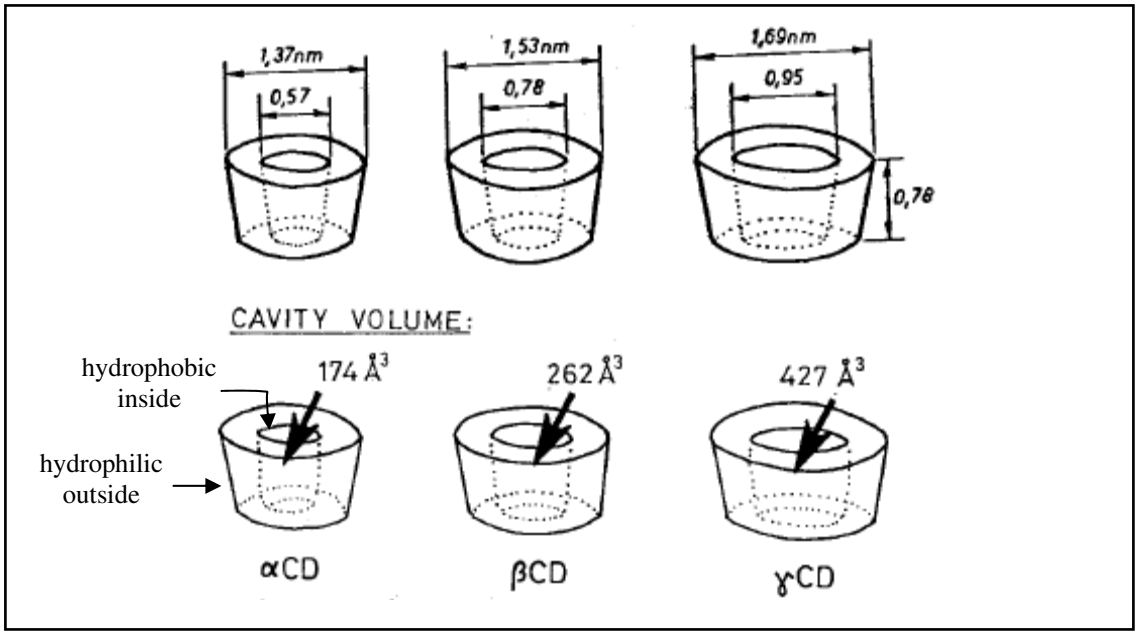

Figure 2.5: Approximate geometric dimension of α-, β-, and γ-cyclodextrin molecules (Szejtli, 1998).

Table 2.1: Properties of α-, β-, and γ-cyclodextrin (Szejtli, 1998).

Properties	**α**	**β**	**γ**
no. of glucose units	6	7	8
mol wt	972	1135	1297
solubility in water, g 100 ml^{-1} at room temperature	14.5	1.82	23.2
cavity diameter, Å	4.7-5.3	6.0-6.5	7.5-8.3
height of torus, Å	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
diameter of outer periphery, Å	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
approx volume of cavity, $Å^3$	174	262	427
approx cavity volume in 1 mol CD (ml)	104	157	256
in 1 g CD (ml)	0.10	0.14	0.20
crystal forms (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms

Administered cyclodextrins are quite resistant to starch degrading enzymes, although they can be degraded at very low rates by α-amylases. α-Cyclodextrin is the slowest, and γ-cyclodextrin is the fastest degradable compound, due to their differences in size and flexibility. Degradation is not performed by saliva or pancreas amylases, but by α-amylases from micro-organisms from the colon flora. Adsorption studies revealed that only 2-4% of cyclodextrins were adsorbed in the small intestines, and that the remainder is degraded and taken up as glucose. This can explain the low toxicity found upon oral administration of cyclodextrins (Duchene, 1987).

2.3.2 Application of cyclodextrins

Since every guest molecule is individually surrounded by a cyclodextrin (derivative) the molecule is micro-encapsulated from a microscopical point of view. This can lead to advantageous changes in the chemical and physical properties of the guest molecules. The characteristics of cyclodextrins or their derivatives that make them suitable for applications in analytical chemistry, agriculture, the pharmaceutical field, in food and toilet articles are stabilization of light- or oxygen-sensitive substances, modification of the chemical reactivity of guest molecules, fixation of very volatile substances, improvement of solubility of substances, 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 and catalytic activity of cyclodextrins with guest molecules.

2.3.2.1 Analytical chemistry

Cyclodextrins are used for the separation of enantiomers by High Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC). The stationary phases of these columns contain immobilized cyclodextrins or derived supra molecular architectures. Other analytical applications can be found in spectroscopic analysis. In Nuclear Magnetic Resonance (NMR) studies they can act as chiral shift agents and in Circular Dichroism as selective (chiral) agents altering spectra.

In some specific cases cyclodextrin-inclusion complexes can be employed as enzyme-substrate complexes. Because these selective complexes facilitate specific steric attack of the organic inclusion compound, isomerization of the products can be accelerated. Another example of high catalytic activity is provided by a β-cyclodextrin-dinicotinamide derivative which catalyzes the reduction of a number of quinones with enzyme like reaction kinetics (Saenger, 1980).

2.3.2.2 Agriculture

Cyclodextrins can be applied to delay germination of seed. In grain treated with β-cyclodextrins some of the amylases which degrade the starch supplies of the seeds are inhibited. Initially the plant grows more slowly, but later on this is largely compensated by an improved plant growth yielding a 20-45% larger harvest. Recent developments involve the expression of CGTases in plants (Saenger, 1980).

2.3.2.3 Food industry

The properties of cyclodextrins have found several applications. Cyclodextrins were reported to have a texture-improving effect on pastry and on meat products. Other applications arise from their ability to reduce bitterness, ill smell and taste, and to stabilize flavors when subjected to long term storage. Emulsions like mayonnaise, margarine or butter creams can be stabilized with β-cyclodextrin. Using β-cyclodextrin one can remove cholesterol from milk, to produce dairy products low in cholesterol (Szejtli, 1998).

2.3.2.5 Toilet articles

Ill smells or irritating properties of compounds in toilet articles are masked with cyclodextrins. The ability of cyclodextrins to stabilize volatile compounds in perfumes is used in perfumed rub sheets in magazines and odor-bags in laundry (Sian *et al.,* 2005)

2.3.2.5 Pharmaceutical field

There are numerous applications for cyclodextrins in the pharmaceutical field. For example the addition of α- or β-cyclodextrin increases the water solubility of several poorly water-soluble substances. In some cases this results in improved bioavailability, increasing the pharmacological effect allowing a reduction in the dose of the drug administered. Inclusion complexes can also facilitate the handling of volatile products. This can lead to a different way of drug administering, e.g. in the form of tablets. Cyclodextrins are used to improve the stability of substances to increase their resistance to hydrolysis, oxidation, heat, light and metal salts. The inclusion of irritating products in cyclodextrins can also protect the gastric mucosa for the oral route, and reduce skin damage for the dermal route. Furthermore, cyclodextrins can be applied to reduce the effects of bitter or irritant tasting and bad smelling drugs (Duchene, 1987).

2.4 Purification of Cyclodextrin Glucanotransferase

There were extensive studies that had been reported on the purification of CGTase. Kim *et al.* (1998) had purified and characterised a CGTase from *Paenibacillus* sp. F8. The molecular weight was estimated to be 72 kDa by SDS-PAGE. The initial production ratio of α-CD, β-CD, γ-CD, and δ-CD from soluble starch was 0.09:1:0.25:0.14. Prolonged incubation times resulted in a decreased ratio of δ-CD and, to a lesser extent, of γ-CD and a increased ratio of α- and β-CD compared to the other CD. Coupling experiments showed that δ-CD was more easily degraded by *Paenibacillus* sp. F8 CGTase compared to α-, β-, and γ-CD.

Sian *et al*. (2005) successively purified a CGTase from *Bacillus* sp. G1 by ammonium sulphate precipitation, and affinity chromatography on α-CD (epoxy)-Sepharose 6B column. The specific activity of the CGTase was increased approximately 2200 fold. SDS-PAGE showed that the purified CGTase was homogeneous and the

molecular weight of the purified CGTase was about 75 kDa. The molecular weight of the enzyme that was estimated by gel filtration under native condition was 79 kDa. This has indicated that *Bacillus* sp. G1 CGTase is a monomeric protein. In cyclodextrin production, tapioca starch was found to be the best substrate used to produce CDs. The enzyme produced γ- and β-CD in the ratio of 0.11:0.89 after 24 h incubation at 60 °C, without the presence of any selective agents.

A CGTase from alkalophilic *Bacillus* sp. 7-12 was purified by Cao *et al*. (2005) by of ammonium sulfate precipitation, DEAE–cellulose column chromatography and Sepharose CL-6B column chromatography. The enzyme thus obtained consisted of a single band that did not dissociate into subunits by SDS–PAGE. The molecular weight of the purified enzyme was determined to be 69 kDa by SDS–PAGE. For cyclodextrin production, up to 34% conversion to cyclodextrins was obtained from 10% starch. The enzyme produced α-, β- and γ-cyclodextrins in the ratio of 0.26:1:0.86.

CHAPTER 3

MATERIALS AND METHODS

3.1 Inoculum Preparation

The bacterium was isolated from the soil and has been identified as *Bacillus* sp. TS1-1 (Mahat *et al*., 2004). Bacteria inoculated were grown in 20 ml seed medium containing 2% (w/v) soluble starch, 5% (w/v) yeast extract, 5% (w/v) peptone, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ and 1% (w/v) Na_2CO_3 (autoclave separately). The culture was incubated at 37°C with shaking at 200 rpm for 18 h. Cells were then harvested by centrifugation at 5000 rpm for 5 min and washed once with normal saline solution (0.85% w/v NaCl) to give an optical density (OD) reading of 0.5 at 600 nm (Mahat *et al*., 2004).

3.2 Crude Enzyme Preparation

Ten percent (v/v) of *Bacillus* sp. TS1-1 inoculums was used to cultivate the production media at 37°C for 24 h with continuous shaking in conical flask containing 100 ml of medium. The production medium containing 2% (w/v) soluble starch, 5% (w/v) yeast extract, 5% (w/v) peptone, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ and 1% (w/v) Na_2CO_3 (autoclave separately). Experimental studies were carried out and

after cultivation cells were removed by centrifugation at 5000 rpm for 5 min. The supernatant was used as crude enzyme solution for assaying enzyme activity (Mahat *et al*., 2004).

3.3 Analytical Procedures

3.3.1 Cyclodextrin glucanotransferases assay

CGTase activity was determined using phenolphthalein method assay with slight modification (Kaneko, 1987). Reaction mixture containing 40 mg of soluble starch in 1.0 ml of 0.1M sodium phosphate buffer (pH 6.0) and 0.1 ml of crude enzyme was incubated at 60°C for 10 min. The reaction was stopped by an addition of 3.5 ml of 30mM NaOH subsequently; 0.5 ml of 0.02% (w/v) phenolphthalein in 5mM Na_2CO_3 solution was added into the mixture. The colour intensity was then measured at 550 nm. One unit of the enzyme activity was defined as the amount of enzyme that forms 1μmol of β-cyclodextrin per minute. Standard curve was plotted with β-cyclodextrin concentrations (Mahat *et al*., 2004).

3.3.2 Modified Lowry protein assay

The protein content was predicted by the modified cupric sulfate-tartrate reagent (Pierce, 1999) that replaces two of the three reagents in the original Lowry method with one stable reagent, thus, avoiding the necessity to prepare the fresh reagents daily. There is nearly a 100% correlation of the colour response curves with various proteins between the Pierce Modified Lowry Protein Assay reagents and the original Lowry method.

A reaction mixture containing 1.0 ml Modified Lowry reagent and 0.2 ml sample was incubated at room temperature for 10 minutes. Distilled water was used as blank. At the end of the incubation period, 0.1 ml 1 N Folin-Ciocalteu reagent was added into the reaction mixture and left at room temperature for 30 minutes. The absorbance of the sample was measured at 750 nm. A standard curve was prepared by plotting the average blank corrected 750 nm reading for each BSA (bovine serum albumin) standard versus its concentration in µg/ml. The protein concentration was determined for each sample using standard curve (Rozaimi, 2006).

3.4 Partial Purification by Cross-flow Filtration

The cross-flow filtration was performed using Kvick Lab cross-flow system (Amersham Biosciences). All the procedures were performed at 15°C. Two cassettes were used in this procedure which was 50K and 10K cassettes. The crude enzyme obtained was subjected to the cross-flow system using 50K cassette. The permeate was kept at 4°C for CGTase determination. Meanwhile, the retentate was further subjected to the cross-flow system again, using 10K cassette. Both permeate and retentate were then analyzed for CGTase activity.

3.5 Electrophoresis (SDS-PAGE) Analysis

The molecular weight of the purified enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (1970) method. The SDS-PAGE was performed, in order to check the homogeneity of the enzyme and to determine the molecular weight of the enzyme under denaturing conditions. The partially purified protein was then mixed with an equal volume of 5x sample buffer (20 µl), boiled for 5 minutes and centrifuged prior loaded onto gel. The 5x

sample buffer (10 ml) containing 0.8 ml 1 M Tris-HCl (pH 6.8), 2.3 ml 10% SDS, 4.8 ml 50% glycerol, 0.6 ml distilled water, 0.5 ml 2-mercaptoethanol and 1 ml 1% bromophenol blue.

Two phases of polyacrylamide gel were prepared in advance. The gel can be divided into two parts, a stacking gel for the concentration of the protein samples. The stock solutions and working solutions for both phases are shown in Appendix A4 and A5 respectively. The resolving gel was mixed well, poured between two plates and overlaid with water to keep the gel surface flat and left to polymerize for one hour. After the gel had polymerized, a distinct interface will appear between the resolving gel and the water. The water was rinsed off with fresh distilled water and the stacking gel was prepared. The stacking gel was then poured on top of the resolving gel. A comb was carefully inserted into the top of the stacking gel, so that no bubbles are trapped on the ends of teeth. The gel was allowed to polymerize for 30 minutes. Once polymerized, the gel was attached to electrode assembly of OmniPAGE mini system (Cleaver Scientific) and inserted into electrophoresis tank that filled with 1x Tris-glycine electrophoresis buffer.

Then the comb was removed. Subsequently, the sample solutions, as well as the molecular weight markers consist of 9 precisely sized recombinant proteins, ranging from 10 kDa to 225 kDa, were introduced into wells on the stacking gel using a Hamilton syringe. The electrophoresis was carried out on a vertical slab gel using 15% acrylamide gel at a constant voltage of 150 V for 2 hours at room temperature. The gel was stained with 1% Coomassie Brilliant Blue R-250 to detect the protein band.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Enzyme Purification

Bacillus sp. TS1-1 culture broth (1.7 l) with specific activity determined to be 1,093,023 U/mg proteins was concentrated to a final volume of 150 ml. Tables 4.1 shows that the sample from 10K cassette retentate yielded the most of the target protein (CGTase). By applying cross-flow filtration (10K cassette), CGTase was purified 5.57 fold. The partially purified enzyme exhibited a specific CD synthesis activity of 6,097,653 U/mg of protein.

Based on the results obtained, most probably the size of the enzyme was between 10 kDa and 50 kDa. Regarding to Sian *et al*. (2005), most of the previously purified CGTases from various *Bacillus* sp. had a molecular weight ranging from 68 kDa to 88 kDa. However, Wang *et al.* (1995) reported a CGTase from *Bacillus* sp. had a molecular weight of 38 kDa.

Sian *et al*. (2005) obtained a 5 fold purification of CGTase for their first step of purification (ammonium sulfate precipitation). This work yielded a slightly higher fold (5.58) by using cross-flow filtration. This result shows that cross-flow filtration can be used as an alternative to the precipitation process. At the same time, it is known that ammonium sulfate precipitation contributes to higher loss of enzyme activity compared

to filtration (Ghosh, 2006). However, Tachibana *et al*. (1999) obtained higher purification fold by using Resource Q column (5.8 fold). Resource Q column is an ion-exchange type of purification. It is a much specific technique in purification (Ghosh, 2006), but it only can be done once the pI value of the target product is known.

Table 4.1: Summary of partial purification of the CGTase from *Bacillus* sp. TS1-1

Fraction		**Volume (ml)**	**Concentration (mg/ml) x10^{-5}**	**Total protein (mg) x10^{-5}**	**Total activity (U/ml)**	**Specific activity (U/mg)**	**Purification fold**
Crude enzyme		1700	3.927	6.68	72.96	1,093,023	1
Crossflow filtration	Retentate 50K	900	11.969	10.8	71.09	659,953	0.60
	Retentate 10K	150	5.157	1.04	63.61	6,097,653	5.57
	Permeate 10K	650	6.954	3.35	72.96	2,176,793	1.99

4.2 Determination of Purification Degree by SDS-PAGE

The partially purified enzyme was analyzed by SDS-PAGE (Figure 4.1). There were smear bands through lane (b) and almost no band was detected through lane (c). It can be concluded that, an amount of proteins were removed by cross-flow filtration. As the protein concentration at the retentate from 10K cassette was barely detected by Coomassie Brilliant Blue R-250, it is recommended to use silver staining method. The silver staining method can detect as low as 2-5 ng/band protein (Switzer *et al.,* 1979)

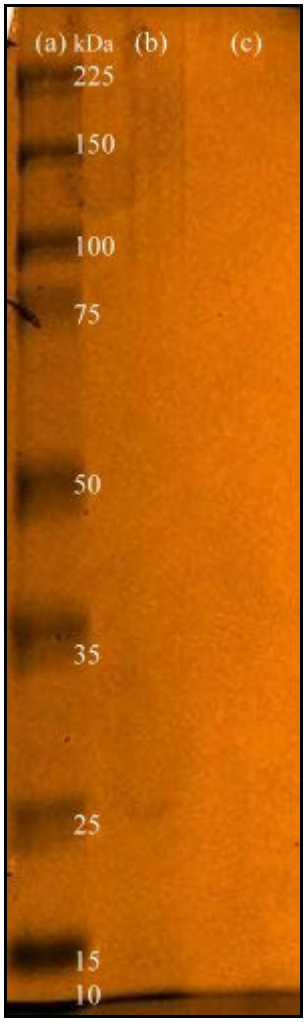

Figure 4.1: SDS-PAGE of the purified enzyme. Lane (a): molecular weight standard; lane (b): crude enzyme; lane (c): partially purified CGTase from retentate of 10K cassette.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

A specific activity of 1,093,023 U/mg of CGTase was observed in crude enzyme. After partial purification by cross-flow filtration, 6,097,653 U/mg was obtained which was 5.57 fold of purification. The predicted size of CGTase from *Bacillus* sp. TS1-1 was in the range of 10 kDa to 50 kDa because the highest specific activity was detected at the retentate from 10K cassette. Based on SDS-PAGE analysis, it was clear that a large amount of proteins had been removed by cross-flow filtration.

5.2 Recommendations

It is recommended to further purify the CGTase by using affinity chromatography such as α-cyclodextrin-bound-epoxy-activated Sepharose 6B, in order to obtain pure CGTase. Characterization of the pure enzyme can be performed which include the kinetics parameters of the enzyme activity and stability as well as the effects of different substrates and ions on the enzyme activity.

REFERENCES

Cao, X., Jin, Z., Wang, X. and Chen, F. (2005). A Novel Cyclodextrin Glycosyltransferase from an Alkalophilic *Bacillus* Species: Purification and Characterization. *Food Research International*. 38: 309–314.

Duchene, D. (Ed.) (1987). Cyclodextrins and Their Industrial Uses. *Paris: Editions de Santé*. 448

Folin, O. and Ciocalteau, V. (1927). Tyrosine and Tryptophan Determination in Proteins. *Journal Biotechnology Chemistry*. 73: 627-650

Gawande, B.N. and Patkar, A.Y., (2001). Purification and Properties of a Novel Raw Starch Degrading-Cyclodextrin Glycosyltransferase from *Klebsiella pneumoniae* AS-22. *Enzyme and Microbial Technology*. 28:735–43.

Ghosh, R. (2006). *Principles of Bioseparations Engineering*. Canada: World Scientific. 1: 68 and 81-82.

Han, N.S. and Tao, B.Y. (1999). Detection of Native and Recombinant *Bacillus macerans* Cyclodextrin Glycosyltransferase Using Microtiter ELISA Techniques. *Enzyme and Microbial Technology*. 24: 36–40.

Hashimoto, H. (1988). *Production and Application of Cyclodextrins. In: The Amylase Research Society of Japan (Ed.). Handbook of Amylase and Related Enzymes.* Tokyo, Japan: Pergamon Press. 233–238.

Hofmann, B.E., Bender, H. and Schulz, G.E. (1989). Three Dimensional Structure of Cyclodextrin Glycosyltransfease from *Bacillus circulans* at 3.4Å Resolution. *Journal Molecular Biology.* 209: 793-800.

Kamalesh, R., Rosli, M.I., Osman, H., Azmi, N.M. and Noor, A.R. (2006). Molecular Cloning of a Cyclodextrin Glucanotransferase Gene from Alkalophilic *Bacillus* sp. TS1-1 and Characterization of the Recombinant Enzyme. *Enzyme and Microbial Technology*. 39: 74–84.

Kaneko, T., Kato, T., Nakamura, N. and Horikoshi, K. (1987). Spectrophotometric Determination of Cyclization Activity of β-cyclodextrin Forming Cyclomaltodextrin Glucanotransferase. *Journal Japan Society Starch Scientific* 34: 45–48.

Kim, L.L., Lene, D.O., Hans, J.S.C., Flemming, M., Lars, H.P. and Wolfgang, Z. (1998). Purification and Characterisation of Cyclodextrin Glycosyltransferase from *Paenibacillus* sp. F8. *Carbohydrate Research*. 310: 211-219.

Koch, R. (1891). Verschiedene Gährungen. *Jahresber. Gährungsorganismen*2. 242-245.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the Assembly. *Journal Molecular Biology.* 209: 793-800.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein Measurement with Folin Phenol Reagent. *Journal Biotechnology Chemistry.* 193: 265–75.

Mahat, K.M., Illias, R.M., Rahman, R.A., Rashid, N.A., Mahmood, N.A., Hassan, O., Aziz, S.A. and Kamaruddin, K. (2004). Production of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. TS1-1: Media Optimization using Experimental Design. *Enzyme and Microbial Technology*. 35(5): 467-473.

Martin, C. and Smith, A.M. (1995). Starch Biosynthesis. *Plant Cell.* 7**:** 971-985.

Nakamura, A., Haga, K. and Yammane, K. (1993). Three Histidine Residues in the Active Center of Cyclodextrin Glucanotransferase from an Alkalophilic *Bacillus* sp. 1011 Effects on the Replacement on pH Dependence and Transition-State Stabilization. *Biochemistry.* 32: 6624-6631.

Nishida, T., Nakamura, A., Masaki, H. and Uozumi, T. (1997). Regulation of Cyclodextrin Glucanotransferase Synthesis in *Bacillus ohbensis*. *FEMS Microbial Letters.* 149: 221–6.

Pierce Chemical Company. (1996). *Modified Lowry Protein Assay*. Rockford (U.S.A.): Instructions.

Rozaimi, A.S. (2006). Production of Pullanase from *Bacillus* flavothermus KWF-1 in Batch Culture: Preliminary Characterization and Medium Development using Experimental Design. *Universiti Teknologi Malaysia: Master's thesis*.

Saenger (1980). *Angewandte Chemie.* England: International Edition. 19: 344.

Saha, B., and Zeikus, J. (1990). Characterization of Thermostable Cyclodextrinase from *Clostridium thermohydrosulfuricum* 39E. *Applied Environmental Microbiology.* 56: 2941-2943.

Shin, H.Y., Park, T.H. and Lee, Y.H. (2000). Site-Directed Mutagenesis and Functional Analysis of Maltose-Binding Site of β-Cyclodextrin Glucanotransferase from *Bacillus firmus* Var. alkalophilus. *Biotechnology Letters*. 22: 115–21.

Sian, H.K. *et al*. (2005). Purification and Characterization of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. G1. *Process Biochemistry.* 40: 1-5.

Stefanova, M.E., Tonkova, A.I., Miteva, V.I. and Dobreva, E.P. (1999). Characterization and Cultural Conditions of a Novel Cyclodextrin Glucanotransferase Producing *Bacillus stearothermophilus* strain. *Journal of Basic Microbiology.* 39(4): 257–63.

Switzer, R.C., 3rd, Merril, C.R., Shifrin, S. (1979). A Highly Sensitive Silver Stain for Detecting Proteins and Peptides in Polyacrylamide Gels. *Analytical Biochemistry*. 98(1): 231-7.

Szejtli, J. (1982). *Cyclodextrins and their inclusion complexes*. Budapest: Akade´miai. Kiado´. 296.

Szejtli, J. (1998). Overview of Cyclodextrins Chemistry. *Chemical Reviews.* 98: 1743-1753.

Szejtli, J. (2004). Past, Present, and Future of Cyclodextrin Research. *Pure Applied Chemistry.* 76(10): 1825–1845.

Tien, S.F. (2001). Production of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus* sp. G1. *Universiti Teknologi Malaysia: Master's thesis.*

Tachibana, Y., Kuramura, A. and Shirasaka, N. (1999). Purification and Characterization of an Extremely Thermostable Cyclomaltodextrin Glucanotransferase from a Newly Isolated Hyperthermophilic Archaeon, a *Thermococcus* sp. *Applied Environmental Microbiology.* Table 1.

Villier, M.A (1891). Sur la Fermentation de la Fecule Par L'action du Ferment Butyrique. *Comptes Rendus Academie Sciences.* 112: 536-538.

Wang, G.S., Chen, P.L., Liu, Y.T. and Wang, L.H. (1995). Purification of Cyclodextrin Glycosyltransferase from a Mutant of *Bacillus* species. *Taiwan Sugar Research Institute*. 150:53–66.

APPENDIX A

MATERIALS AND METHOD

Appendix A1: Buffers Required for Enzyme Assay.

1. Phosphate buffer, 100mM

   Solution A: 2.72 g of K_2HPO_4 per liter (0.2M)

   Solution B: 45.6 g of KH_2PO_4 per liter (0.2M)

   Based on Table A-1, mix the indicated volumes of solutions A and B, then dilute with distilled water to a total of 200 ml.

Table A-1: Preparation of 0.1M phosphate buffers.

Desired pH	Solution A (ml)	Solution B (ml)
6.0	87.7	12.3

Appendix A2: Calculation of CGTase Activity by Phenolphthalein Method

Enzyme activity calculation

$$\% \text{ OD reduction} = \frac{\text{ODcontrol - ODsample}}{\text{ODcontrol}}$$

$$\text{Enzyme activity (U/ml)} = \frac{\% \text{ OD reduction} \times 1\text{x}10^3 \times 100 / \text{standard curve slope}}{\beta\text{-CD molecular weight} \times \text{unit processing time} \times \text{enzyme's volume}}$$

$$= \frac{\% \text{ OD reduction} \times 1\text{x}10^3 \times 100/83.6}{1135.01 \times 10 \times 0.1}$$

Table A-2: Preparation of the diluted β-CD standards.

Volume of β-CD to add (ml)	**Volume of diluent to add (ml)**	**Final β-CD concentration (µg/ml)**
50 of (A)	0	2000 (A)
50 of (A)	66.67	1500 (B)
25 (B)	37.5	1000 (C)
12.5 (C)	25	500 (D)
6.25 (D)	15.625	200 (E)

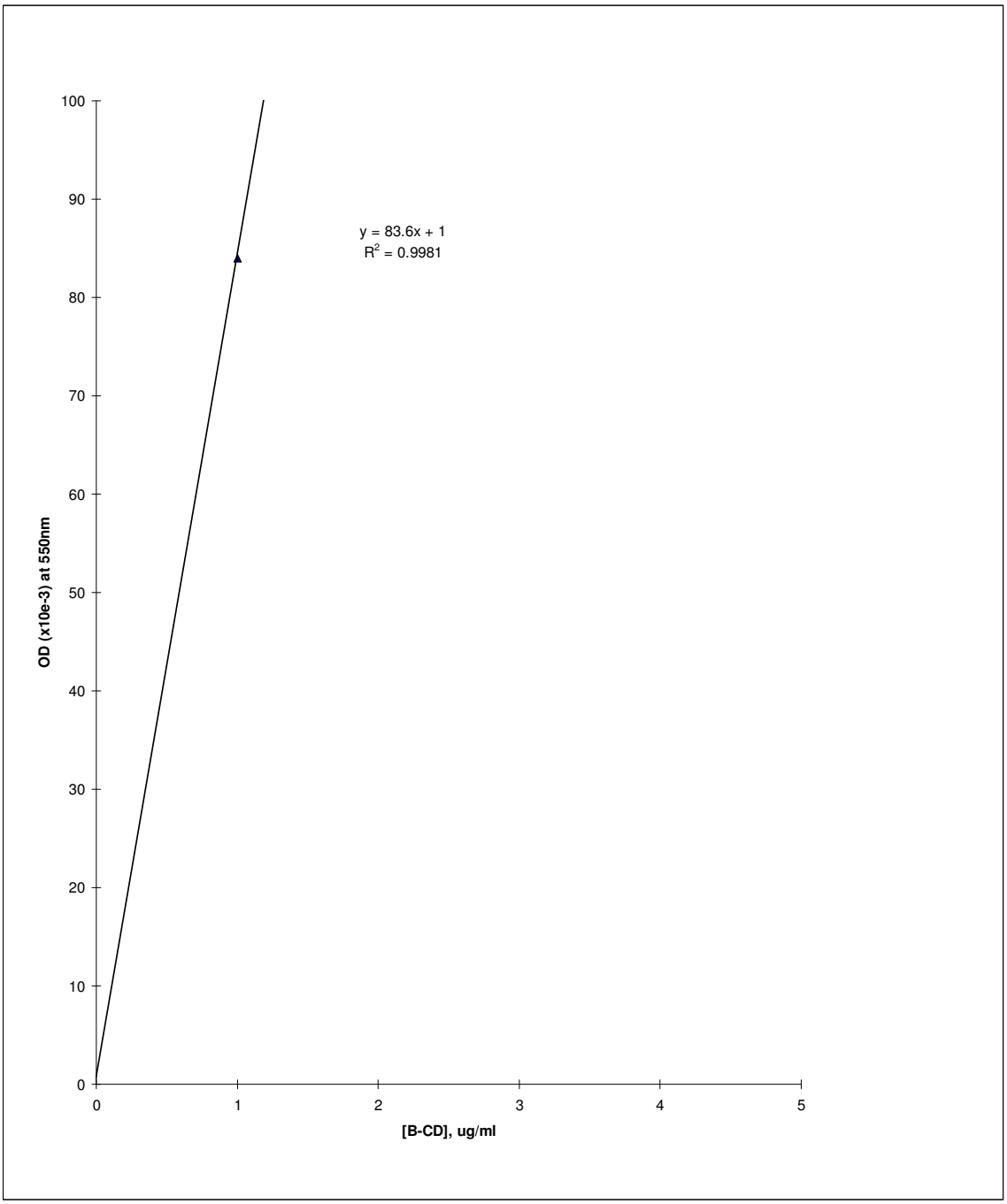

Figure A-1: β-CD concentration standard curve.

Appendix A3: Protein Determination by Modified Lowry Protein Assay.

Table A-3: Preparation of the diluted BSA (bovine serum albumin) standards.

Volume of BSA to add (ml)	Volume of diluent to add (ml)	Final BSA concentration (µg/ml)
1.375 of (Stock)	0	2000
0.75 of (Stock)	0.25	1500 (A)
0.625 of (Stock)	0.625	1000 (B)
0.75 of (A)	0.31	750 (A)
0.75 of (B)	0.625	500 (A)

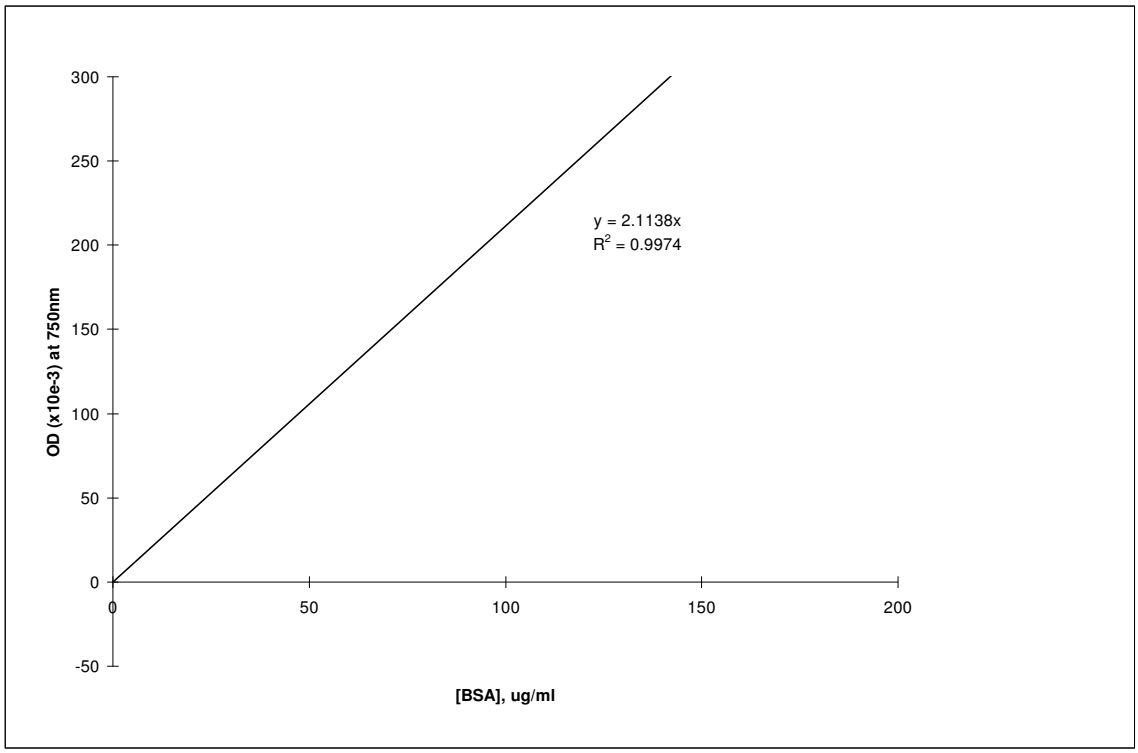

Figure A-2: Protein concentration standard curve.

Appendix A4: Stock Solution for SDS-PAGE.

Table A-4: Preparation of 2 M Tris-HCl (pH 8.8), 100 ml

a	Weigh out 24.2 g Tris base
b	Add to 50 ml distilled water
c	Add concentrated HCl slowly to pH 8.8
d	Add distilled water to a total volume of 100 ml
Store at 4 ˚C	

Table A-5: Preparation of 1 M Tris-HCl (pH 6.8), 100 ml

a	Weigh out 12.1 g Tris base
b	Add to 50 ml distilled water
c	Add concentrated HCl slowly to pH 6.8.
d	Add distilled water to a total volume of 100 ml
Store at 4 ˚C	

Table A-6: Preparation of 10% (w/v) SDS, 100 ml

a	Weigh out 10 g SDS
b	Add distilled water to a total volume of 100 ml
Store at room temperature	

Table A-7: Preparation of 50% (v/v) glycerol, 100 ml

a	Pour 50 ml 100% glycerol
b	Add 50 ml distilled water
Store at 4 ˚C	

Table A-8: Preparation of 1% (w/v) bromophenol blue, 10 ml

a	Weigh out 100 mg bromophenol blue
b	Bring to 10 ml with distilled water, stir until dissolved
Store at 4 ˚C	

Appendix A5: Working Solution for SDS-PAGE.

Table A-9: Preparation of solution A (acrylamide stock solution), 100 ml

a	Required 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide
b	From stock solution with 40% (w/v) acrylamide, withdraw 75 ml
c	Add distilled water to a total volume of 100 ml
Store at 4 ˚C	

Table A-10: Preparation of solution B (4x resolving @ separating gel buffer), 100 ml

a	75 ml of 2 M Tris-HCl (pH 8.8)
b	4 ml of 10% (w/v) SDS
c	21 ml of distilled water
Store at 4 ˚C	

Table A-11: Preparation of solution C (4x stacking gel buffer), 100 ml

a	50 ml of 1 M Tris-HCl (pH 6.8)
b	4 ml of 10% (w/v) SDS
c	21 ml of distilled water
Stable for months in the refrigerator	

Table A-12: Preparation of 10% (w/v) ammonium persulfate, 5 ml

a	0.5 g ammonium persulfate
b	5 ml of distilled water
Stable for months in a capped and aluminium foil covered tube in the refrigerator	

Table A-13: Preparation of electrophoresis buffer, 2 L

a	6 g Tris
b	8.8 g glycine
c	2 g SDS
d	Use distilled water to make up 2 L solution
pH is approximately 8.3, stable indefinitely at room temperature	

Table A-14: Preparation of 5x sample buffer, 10 ml

a	0.6 ml of 1 M Tris-HCl (pH 6.8)
b	5 ml of 50% glycerol
c	2 ml of 10% SDS
d	0.5 ml of 2-mercaptoethanol
e	1 ml of 1% bromophenol blue
f	0.9 ml of distilled water
Stable for weeks in the refrigerator or for months at -20 ˚C	

Table A-15: Preparation of 10% separating gel preparation at 15 ml volume

a	5 ml solution A
b	3.75 ml solution B
c	6.25 ml distilled water
d	75 µl ammonium persulfate
e	15 µl TEMED

Table A-16: Preparation of 5% stacking gel

a	2.3 ml of distilled water
b	0.67 ml of solution A
c	1 ml of solution C
d	30 µl of 10% (w/v) ammonium persulfate
e	15 µl TEMED

Table A-17: Preparation of staining solution, 1 L

a	1 g Coomassie Blue R-250
b	450 ml methanol
c	450 ml distilled water
d	100 ml glacial acetic acid
Store at 4 ˚C	

Table A-18: Preparation of destaining solution, 1 L

a	100 ml methanol
b	100 ml glacial acetic acid
c	800 ml distilled water
Store at 4 ˚C	