PARTIAL CHARACTERIZATION OF CYCLODEXTRIN GLUCANOTRANSFERASE BY *Bacillus* sp. TS1-1

UMMI NOR HUSNA HUSIN

UNIVERSITI MALAYSIA PAHANG

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JUDUL: **PARTIAL CHARACTERIZATION OF CYCLODEXTRIN GLUCANOTRANSFERASE BY *Bacillus* sp. TS1-1**

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UMMI NOR HUSNA HUSIN

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 2009

DECLARATION

I declare that this thesis entitled “Partial Characterization of Cyclodextrin Glucanotransferase by *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.”

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DEDICATION

Special dedication to
Rahana Mahmud my beloved mother and
Husin Ibrahim my beloved father, both of you is important to me
My siblings that always love me

My respective lecturers and tutors those always guide and supporting me
My course mates those are always challenging and helping me

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ABSTRACT

The crude culture that contains cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *Bacillus* sp. TS1-1 has been partially purified by centrifugation and cross-flow filtration. Initial 242.74 U/ml of CGTase was detected in the culture after 24 hours of incubation. The crude supernatant obtained after centrifugation for 5000rpm, 5 minutes and 4^{o}C was subsequently filtered at 15^{o}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. Using cross-flow filtration, the crude enzyme was purified 2.33 fold. The crude and partially purified enzyme was then subjected to gel electrophoresis (SDS-PAGE) to determine the enzyme molecular weight. The partial purified enzyme especially at retentate 50K suggested an initial size of CGTase between 46 to 88 kDa. The crude and partial purified CGTase was then assayed using phenolpthalein method with slight modification for the determination of CGTase optimum pH, pH stability, optimum temperature and thermal stability. Based on this work, the optimum temperature for activity was at 40^{o}C and 80^{o}C, the thermal stability was from 40-70^{o}C and from 60-90^{o}C; the optimum pH for activity was at pH6 and pH10; the pH stability was from pH4 to 6.

ABSTRAK

Kultur mentah yang mengandungi cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) daripada *Bacillus* sp. TS1-1 telah ditulenkan secara separa menggunakan kaedah pengemparan dan penyaringan aliran melintang. Sebanyak 242.74 U/ml CGTase telah dikesan dalam kultur selepas 24 jam pengeraman. Supernatan mentah yang diperolehi selepas pengemparan pada 5000rpm, 5 minit dan 4^{o}C kemudiannya disaring melalui penyaringan aliran melintang pada 15^{o}C menggunakan sistem penyaring Kvick Lab. Dua blok penyaring bersaiz rongga 50K dan 10K bagi tujuan penyaringan tersebut. Baki kultur daripada blok penyaring 50K seterusnya disaring menggunakan blok penyaring 10K. Setiap hasil dan baki daripada setiap blok penyaring kemudian diuji untuk menentukan aktiviti CGTase. Menggunakan kaedah penyaringan aliran melintang ini, kultur mentah telah ditulenkan sebanayak 2.33 kali ganda. Enzim mentah dan enzim separa tulen kemudiannya dianalisis menggunakan elektroforesis gel (SDS-PAGE) untuk menentukan jisim molar enzim. Enzim separa tulen terutama daripada baki penyaring blok 50K menunjukkan saiz awal CGTase adalah antara 46 hingga 88 kDa. CGTase mentah dan separa tulen seterusnya dicerakinkan menggunakan kaedah Fenoftalein dengan sedikit pengubahsuaian untuk menentukan pH optima, kestabilan pH, suhu optima dan kestabilan suhu bagi CGTase. Berdasarkan kajian ini, suhu optima adalah pada 40^{o}C dan 80^{o}C dan kestabilan suhu adalah pada 40-70^{o}C dan dari 60-90^{o}C; pH optima bagi CGTase adalah pada pH6 dan pH10; kestabilan pH adalah pada pH4 – 6.

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α	-	alpha
β	-	beta
γ	-	gamma
CD	-	cyclodextrin
CGTase	-	cyclodextrin glucanotransferase
h	-	hour
K	-	kilo Dalton (molecular weight cut off)
kDa	-	kilo Dalton
mg	-	miligram
mM	-	miliMolar
kg	-	kilogram
mol wt	-	molecular weight
µl	-	microliter
µmol	-	micromole
ng	-	nanogram
nm	-	nanometer
v/v	-	volume per volume
v/w	-	volume per weight
OD	-	optical density
pI	-	isoelectric point
rpm	-	revolution per minute
SDS-PAGE	-	sodium dodecycl sulphate polyacrylamide gel
U	-	unit (enzyme activity)
UV	-	ultraviolet
V	-	volt

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cyclodextrin glucanotransferase (EC 2.4.1.19) (CGTase), is an extracellular enzyme. It degrades starch to form cyclodextrins (CDs). The important sources of CGTases are bacteria. The first reported source of CGTases is *Bacillus macerans*. A variety of bacteria that have been determined as CGTase producers are aerobic mesophilic bacteria, aerobic thermophilic, anaerobic thermophilic and aerobic alkalophilic bacteria (Tonkova, 1998).

Major producers of CGTases are *Bacillus* sp. especially aerobic alkalophilic types. Other psychrophilics, mesophilic and thermophilic microorganims that have been identified able to produce CGTase enzymes are *Bacillus stearothermophilus, Klebsiella pneumoniae, Klebsiella oxytoca* 19-1, *Brevibacterium* sp. and hyperthermophilic archaea-bacteria (Mahat *et al.,* 2004).

1.2 Problem Statement

The isolation of pure enzymes 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 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 characterize partially purify cyclodextrin glucanotransferase (CGTase) from *Bacillus sp*. TS1-1.

1.4 Scope of Study

The scopes of this study are as follows

i. To produce crude CGTase.
ii. To purify CGTase by centrifugation and cross-flow filtration.
iii. To identify pH and temperature effects on stability and activity of the enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Cyclodextrin Glucanotranferase

Cyclodextrin glucanotransferase (EC 2.4.1.19) (CGTase) degrades starch to form cyclodextrins (CDs) through an intramolecular reaction (cyclization). In this specific reaction, closed circular structures are form when the starch is cleaved and the ends are joined together. In addition, in order to catalyzing the reaction on starch and CDs, CGTase is also involved in intermolecular transglycosylation that involves coupling and disproportionation reactions as well as the hydrolytic action.

There are three different types of CDs which mainly exist: α-CD, β-CD and γ-CD, according to the major CD produced (Rahman *et al.*, 2006). The torus-shaped cyclodextrins have hydrophobic CH groups on the inside and hydrophilic hydroxyl groups on the outside of the ring. Del-Rio *et al.* (1997) has proposed that the CGTase play a biological role where it works in concert with a-amylases for the efficient saccharification of starch.

The Cyclodextrins are natural cyclic oligosaccharides with doughnut-shaped structure possessing hydrophilic surface and hydrophobic central cavity (Fig. 2.0). Due to this unordinary structure CDs are able to form inclusion complexes with

different guest organic and inorganic molecules and also can change physical and chemical properties of the encapsulated guest compound (Szejtli, 2004).

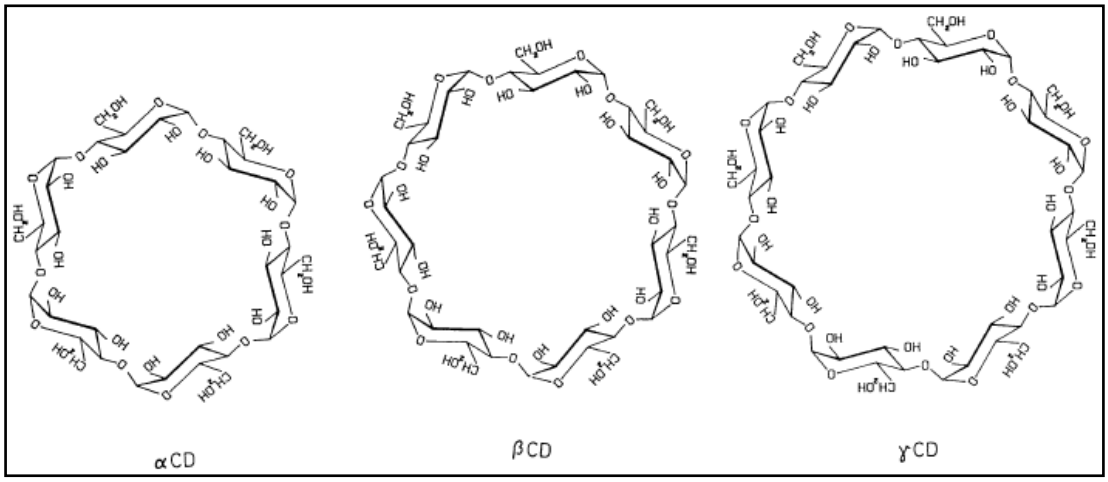

Figure 2.1: Molecular models of α-, β- and γ-CD (Szejtli, 2004).

The origin of the CGTase and the reaction conditions are the two factors that influenced the different of the yields and ratios of α-, β- and γ-CD produced from starch. Commonly, different starches is converted into a mixture of α-, β- and γ-CD by various CGTase but the ratios of α-, β- and γ-CD produced are different. CGTases that can synthesize predominantly one type of CD has great commercial importance. This is different from a separation of one type of CDs from the products mixture, where it is time-consuming, costly and tedious (Sian *et al*., 2005).

Production of CGTase can be optimized by manipulating physio-environmental factors such as the nutrient concentrations and compositions of the production media. Media optimization using statistic experimental design has been cited by many researchers in optimizing either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites (Mahat *et al*., 2004).

Typical modes of CGTase production were conducted by Mahat *et al*. (2004) initially by using shake flash culture and submerge fermentation utilizing selective.

Various types of medium composition mainly carbon and nitrogen source concentration, inoculums size, pH and temperature for fermentation production of CGTase had been studied by Gawande *et al*. (1998), Gawande *et al*. (1999) and Stefanova *et al*. (1999).

Applications of CDs are popular and extensively used in industries such as pharmaceutical, toiletries, agricultural, cosmetic, chemical and food. CDs have several behaviors such as increasing the solubility and stability, reducing volatility, controlling release of drugs and masking odors and tastes (Sian *et al*., 2005).

2.2 Purification of enzyme

Enzyme purification is an extraction of a single enzyme or protein from examples like cells, tissues which may contain more than 1000 different proteins and lots of other biomolecules. There are several objectives of the purification of enzymes. Firstly is to study reactions, kinetics, regulation and others. Secondly is to understand the deviations in normal metabolism or regulation processes, due to abnormal enzymes. Thirdly is to make a rational design of drugs possible, based on the 3D-structure of a protein. Also through enzymes purification, we can identify which enzymes have themselves an added value, as biocatalysts (proteases, lipases, glucose isomerase), therapeutics (insulin, interleukins) and others.

There were various types of enzymes had been purified as reported by previous researchers. The extracellular endoinulinase from *Xanthomonas oryzae* No. 5 which converts inulin into inulooligosaccharides was purified by Cho *et al.* (2002), from the culture broth by ammonium sulphate precipitation, followed by column chromatography on Phenyl-Sepharose and DEAE-Sephacel. The enzyme was purified 29-fold with a yield of 5.5% from the starting culture broth. The purified enzyme gave a single band on polyacrylamide gel electrophoresis, and its molecular

weight was estimated to be 139 kDa. The specific activity of the purified enzyme was 1372 U/mg.

Li and Peeples (2004) was investigated a purification of a recombinant, thermostable α-amylase (MJA1) from the hyperthermophile, *Methanococcus jannaschii*, in the ethylene oxide–propylene oxide random copolymer (PEO–PPO-2500)/$(NH_4)_2SO_4$, and poly(ethylene glycol) (PEG)/$(NH_4)_2SO_4$ aqueous two-phase systems. In the purification, MJA1 partitioned in the top polymer-rich phase, while the remainder of proteins partitioned in the bottom salt-rich phase. It was found that enzyme recovery of up to 90% with a purification factor of 3.31 was achieved using a single aqueous two-phase extraction step.

Tannase (tannin acyl hydrolase EC 3.1.1.20) produced by *Aspergillus awamori nakazawa* was purified and characterized by Mahapatra *et al.* (2005). The acetone-precipitated fraction was further purified using HPLC (GF-250 column with 4.5 mm x 250 mm, 4 mm pore size) at fixed flow rate; 1 ml/min. The solvent system used to elute the protein based on molecular size was 0.2 M acetate buffer (pH 5). Further purification of the partially purified (acetone precipitated fraction) was achieved by using GFC (using G-100 Sephadex) column. HPLC of the partially purified (acetone precipitated) tannase from the new isolate showed a single major peak and the elution time was 6.8 min.

Shibusawa *et al.* (2007) has conducted a study where a histone deacetylase from *Escherichia coli* cell-lysate was purified by counter-current chromatography (CCC) using aqueous two-phase system. Aqueous–aqueous two-phase (AATP) systems composed of polyethylene glycol (PEG) (molecular mass, *M*r:1000–8000) and dextran (*M*r:40,000) were evaluated for purification of maltose binding protein tagged-histone deacetylase (MBP-HDAC). CCC purification of an MBP-HDAC was demonstrated with a 7.0% PEG 3350–10% dextran T40 system containing 10mM potassium phosphate buffer at pH 9.0. The collected fractions containing target protein were analyzed by an HPLC-based *in vitro* assay and then by sodium dodecyl

sulfate polyacrylamide gel electrophoresis. MBP tag was digested from fusion HDAC during the CCC separation and native HDAC was purified by one-step operation with well preserved deacetyl enzyme activity.

The effect of several metal ions and calcium on purified paraoxonases (PON1 and PON3) from rat liver was studied by Pla *et al.* (2007). In this study, PON1 and PON3 were purified by hydroxyapatite adsorption, chromatography on DEAE-Sepharose CL-6B and non-specific affinity chromatography on Cibacron Blue 3GA. Chromatography on Cibacron Blue rendered two separated peaks: M1 containing PON3 and M2 that contained PON1. PON1 was then further purified by anion exchange on Mono Q HR 5/5. SDS-PAGE of the final preparation indicated a single protein-staining band at 45 kDa. This enzyme was purified 415-fold to apparent homogeneity with a final specific activity of 1370 μmol/(min mg) and an overall yield of 6%. The pooled fractions from Cibacron Blue containing PON3 were chromatographed twice on DEAE-cellulose and a final affinity chromatography step was applied on Concanavalin A-Sepharose. The purity checked by SDS-PAGE showed a single band at about 43 kDa. The overall purification factor was about 177 with a final specific activity of 461μmol/(min mg) and a yield of 0.4%.

Chen *et al.* (2009) had purified and characterized exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. Three exoinulinases (Exo-I, Exo-II, and Exo-III) and two endoinulinases (Endo-I and Endo-II) were purified from culture broth of *A. ficuum* JNSP5-06. The purification methods involved were ammonium sulphate precipitation, DEAE-cellulose column chromatography, and Sepharose CL-6B column chromatography. The molecular weights of Exo-I, Exo-II, Exo-III, Endo-I and Endo-II were determined to be 70 kDa, 40 kDa, 46 kDa, 34 kDa, and 31 kDa, respectively. The results from thin-layer chromatography analysis of the hydrolysis products of inulin by four active fractions (A,B,C,D) indicated that the fraction of A, B, and C were exo-type inulinases, whereas D was an endo-type inulinase. It was also found that the I/S ratio of D toward inulin and sucrosewas higher than those of A, B, and C.

Wang *et al.* (2009) successively purified a halostable cellulase from *Salinivibrio* sp. strain NTU-05 using ion exchange chromatography and gel filtration chromatography using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech). The crude enzyme obtained as a cell-free supernatant was precipitated using ammonium sulfate to 80% saturation. The protein was purified by ion exchange resources Q chromatography and Sephadex G-200 gel filtration chromatography. The purified enzyme cellulase exhibited 32.4 U/mg specific activity. Overall levels of recoveries and purification of cellulase was observed to be 18.9% with 29.5-fold. The purified enzyme showed a single protein band on SDS-PAGE with an estimated molecular mass of 29 kDa. The zymogram of the cellulase exhibited a significant activity band that corresponded to 29 kDa.

2.3 Purification of Cyclodextrin Glucanotransferase

There were several studies that had been reported on the purification of CGTase. Kim *et al.* (1998) had purified and characterized 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 lesser extent, of γ-CD and an increased ratio of α-CD and β-CD compared to the other CD. Coupling experiments showed that δ-CD was more easily degraded by from *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. SDS-PAGE showed that the purified CGTase was homogeneous and the molecular weight of the purified CGTase was about 75kDa. The molecular weight of the enzyme that was estimated by gel filtration under native condition was 79kDa. In cyclodextrin production, tapioca starch was found to be the

best substrate used to produce CDs. The enzyme produced α- and β-CD in the ratio of 0.11:0.89 after 24 h incubation at 60^{o}C, without the presence of any selective agents.

A CGTase from alkalophilic *Bacillu*s sp. 7-12 was purified by Cao *et. al* (2005) by ammonium sulphate 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. For cyclodextrin production, up to 34% conversion to cyclodextrins was obtained from 10% starch. The enzyme produced α-, β- and γ-CD in the ratio of 0.26:1:0.86.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strain

The bacterium identified as *Bacillus* sp. TS1-1 was obtained from culture stock in university's laboratory. According to Zain *et al.*, (2007), the bacteria innocula was 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 (mixed and autoclaved) in a conical flask. The culture was incubated at 40^{o}C with shaking at 200 rpm for 18 h. Cells were then harvested by centrifugation at 5000 rpm for 5 min and is washed once with distilled water to give the optical density (OD) reading 0.5 at 600 nm.

3.2 Preparation of crude enzyme

Preparation of crude enzyme was carried out according to Mahat *et al.*, (2004). 10% (v/v) of *Bacillus* sp. TS1-1 inoculum was used to cultivate the production media at 37^{o}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 (mixed and autoclaved). Experimental studies were carried out and after cultivation cells are removed by centrifugation at 5000 rpm for 2 min. The supernatant was used as crude enzyme solution for assaying enzyme activity.

3.3 Assay of CGTase

CGTase assay was carried out according to Kaneko *et al.*, (1998). The reaction mixtures containing 40 mg of soluble starch in 1.0 ml of 0.1M sodium phosphate buffer (pH 6.0) and 0.1 ml supernatant was incubated at 60^{o}C for 10 min. The reaction was stopped by adding 3.5 ml of 30mM NaOH, followed by 0.5 ml of 0.02% (w/v) phenolphthalein in 5mM Na_2CO_3 solution (red in colour), and left for 15 min at room temperature. The colour intensity of the samples was measured at 550 nm. A blank solution (fresh medium) was prepared for each batch of assay. The definition of one unit of enzyme activity was the amount of enzyme that forms 1 μmol β-CD/min.

3.4 Modified Lowry protein assay

The protein content was estimated by the modified cupric sulfate-tartrate reagent (Pierce, 1999). 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).

3.5 Purification by Cross-flow Filtration

The cross-flow filtration was performed using Kvick Lab cross-flow system (Amersham Biosciences) at temperature 15^{o}C. Two cassettes were used which were 50K and 10K cassettes. The crude enzyme was subjected to the cross-flow system using 50K cassette. The permeate was kept at 4^{o}C for CGTase determination while the retentate was then further subjected to the cross-flow system using 10K cassette. Both the permeate and retentate were then analyzed for CGTase activity.

3.6 Molecular weight determination

The molecular weight and homogeneity of the partial purified enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, according to Laemmli (1970). The crude and the series of partial purified cyclodextrin glucanotransferase (CGTase) were mixed with 5x sample buffer (4:1), boiled for 5 minutes and centrifuged prior loaded onto gel.

Two phases of polyacrylamide gel; stacking and resolving gel were used in this procedure. The resolving gel was prepared and poured between assembled two plates and overlaid with distilled water to keep the gel surface flat and left to be polymerized for 1 hour. After the resolving gel had polymerized, a distinct interface will appear between the resolving gel and the water. Then the distilled water was drained and the stacking gel was prepared. Immediately (before the gel begin to polymerize), the stacking gel was poured on top of the resolving gel and a comb was inserted into the stacking gel. The gel was allowed to be polymerized for 30 minutes. Once polymerized, the comb was taken off and the assembled plates

were then inserted into electrophoresis tank. 1x Tris-glycine electrophoresis buffer was poured at the center of the two assembled plates and allowed to be overflow until the marked maximum level was reached. Then the samples were loaded into the selected wells and the electrodes were attached to the apparatus to allow the electrophoresis process to be run at a constant voltage of 150 V for 2 h at 25^{o}C. The molecular weight markers consist of nine precisely sized recombinant proteins, ranging from 10 kDa to 225 kDa, were used as standard protein molecular weight markers. The gel was stained with 1% Coomassie Brilliant Blue R-250.

3.7 Optimum pH and temperature of CGTase

Optimum pH and temperature were determined according to Sian *et al*., (2005). 0.1 M phosphate buffer pH 6.0 was replaced in the CGTase assay with the following buffers: sodium acetate buffer, 0.1 M (pH 4–5), sodium phosphate buffer, 0.1 M (pH 6–7) and 0.1M glycine–NaOH buffer (pH 9-10). The reaction was carried out using the CGTase assay procedure as above. The optimum temperature of the pure enzyme was determined by incubating the reaction mixture of the CGTase assay in temperatures from 40 to 90^{o}C for 10 min. The reaction was done according to the CGTase assay mentioned.

3.8 The pH and thermal stability of CGTase

Optimum pH and thermal activity were determined according to Sian *et al*., (2005). 0.1 ml pure enzyme was incubated with 0.2 ml of 0.1 M sodium acetate buffer (pH 4–5), 0.1 M sodium phosphate buffer (pH 6–7) and 0.1 M glycine–NaOH

buffer (pH 9–10), respectively at 60^{o}C, without substrate for 30 min. The remaining activity of the enzyme was assayed by the standard assay method. The temperature stability of the enzyme was measured by incubating 0.1 ml pure enzyme with 0.2 ml buffer (0.1 M sodium phosphate buffer, pH 6.0) without substrate at different temperatures (40–90^{o}C) for 30 min. Standard CGTase assay was performed to determine their residual activity.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Enzyme Purification

Bacillus sp. TS1-1 culture broth with specific activity determined to be 129.12 U/mg proteins was concentrated to a final volume of 170 ml. Table 4.1 shows that the sample from permeate 10K cassette yielded the most of the target protein (CGTase). By applying cross-flow filtration (50K and 10K cassettes), CGTase was purified 2.33 fold. The partially purified CGTase exhibited a specific CD synthesis activity of 300.77 U/mg of protein.

Larsen *et al*. (1998) obtained a 0.72 purification fold of CGTase while Sian *et al.* (2005) obtained 5.0 fold for first step of purification process (ammonium sulphate precipitation). This work yielded medium fold of purification (2.33) by using cross-flow filtration. This result shows that cross-flow filtration can be used as an alternative to the precipitation process. Ammonium sulphate precipitation contributes to higher loss of enzyme activity compared to filtration (Ghosh, 2006). However, Volkova *et al.* (2000) obtained approximately 3.0 fold for first step of purification process by affinity chromatography on β-CD Sepharose 4B. Sian *et al.* (2005) also reported to obtain higher purification fold (2238.59) by affinity chromatography but it was used in the second step of purification process.

Fraction		Volume (ml)	Concentration (mg/ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mg)	Purification fold)
Crude enzyme		1200	1.88	21.3	242.74	129.12	1
Cross-flow filtration	Retentate 50K	610	1.59	12.6	257.25	161.79	1.25
	Retentate 10K	400	1.21	17.3	227.36	275.11	2.13
	Permeate 10K	170	0.52	14.6	578.41	300.77	2.33

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

4.2 Determination of Purification Degree by SDS-PAGE

The partially purified CGTase was analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Based on the results obtained, most probably the size of the enzyme was between 46 kDa to 88 kDa (Figure 4.1). Regarding to Volkova *et al*., (2000), most of the previously purified CGTases from various *Bacillus* sp. had a molecular weight of 40 to 68 kDa. Martins *et al.*, (2002) reported that the CGTase from *Bacillus agaradhaerens* had ranging in molecular weight in between 110 to 112 kDa. CGTase from *Paenibacillus* sp. F8 have molecular weight of 72 kDa as reported by Larsen *et al*. (1998). However, Rahman *et al.* (2006) and Sian *et al.* (2005) reported a CGTase from *Bacillus* sp. had a molecular weight of 75 kDa.

Due to smear bands barely detected at the retentate from 50K cassette by Coomasie Brilliant Blue R-250, the silver staining method is suggested to detect the molecular weight of the partially purified CGTase. The silver staining method can detect as low as 2-5 ng/band protein (Switzer *et al*., 1979).

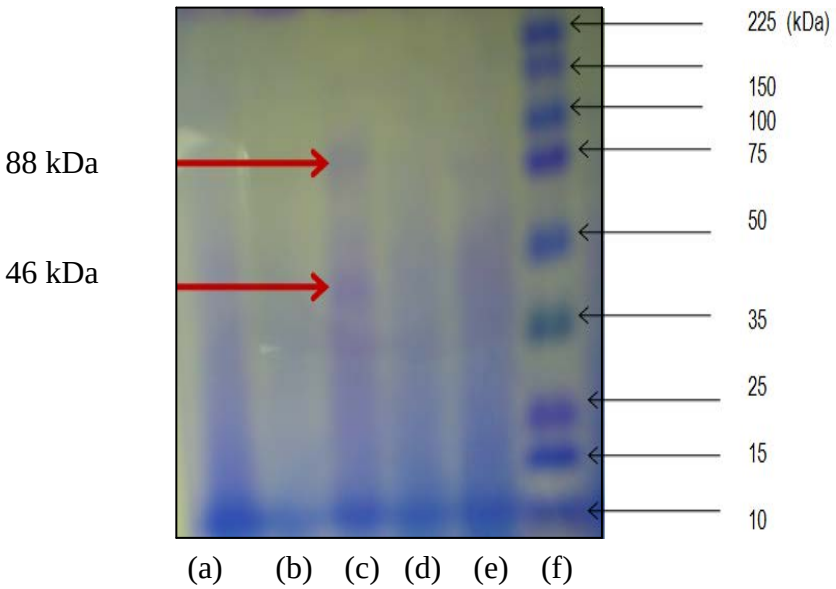

Figure 4.1: SDS-PAGE of partial purified CGTase. Lane (a): retentate 10K; lane (b):permeate 10K; lane (c):retentate 50K; lane (d): permeate 50K; lane (e):crude CGTase; lane (f): molecular weight markers.

4.3 Determination of Optimum pH and Temperature of CGTase

The activity of the partial purified CGTase was measured in different buffers at pH4-5 (0.1M sodium acetate), pH6-7 (0.1M phosphate) and pH9-10 (0.1M Glyciene-NaOH) at temperature 60^{o}C using standard assay method. The optimum pH for CGTase from *Bacillus* sp. TS1-1 was at pH6 and pH10 with soluble starch as substrate (Figure 4.2). This enzyme was completely inactive at pH 4. Studies done by other researchers on CGTase from other strain like *Bacillus agaradhaerens* (Martins *et al*., 2002) discovered pH9 as the optimum pH while at pH7.5 for CGTase from *Bacillus macerans* ATCC 8244 (Arya *et al*., 2006). The CGTase exhibited a peak at pH10 which in agreement with CGTase produced from *Brevibacterium* sp. no. 9605 (Mori *et al*., 1994).

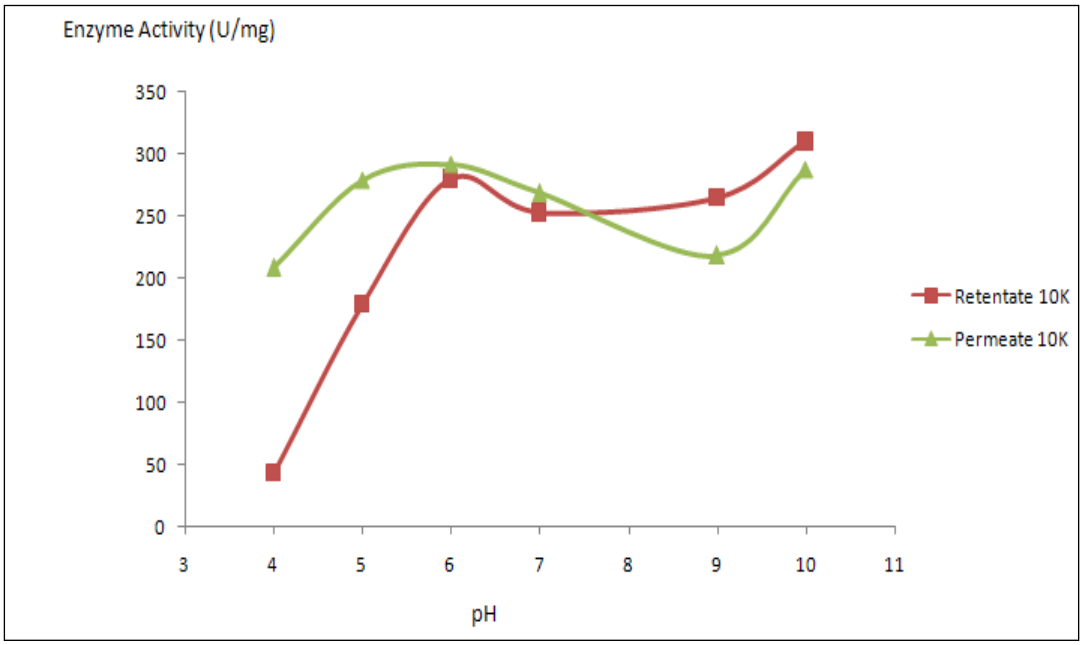

Figure 4.2: Optimum pH of partially purified CGTase

Meanwhile, at pH6 (0.1M Phosphate buffer) with varying temperature ranging from 40^{o}C to 90^{o}C with soluble starch as substrate, (Figure 4.3), the enzyme has an optimum temperature at 40^{o}C (permeate 10K) and at 80^{o}C (retentate 10K). Sian *et al.* (2005) has reported that there was a peak at 80^{o}C, which satisfied with this result. This enzyme (retentate 10K) also exhibited a peak at 50^{o}C which as reported by Qi *et al.* (2004), but using strain of *Bacillus macerans*. There was also a peak exhibited by permeate 10K at 70^{o}C. It is interesting to note that this enzyme (permeate 10K) exhibited a peak at 90^{o}C, which was never reported before.

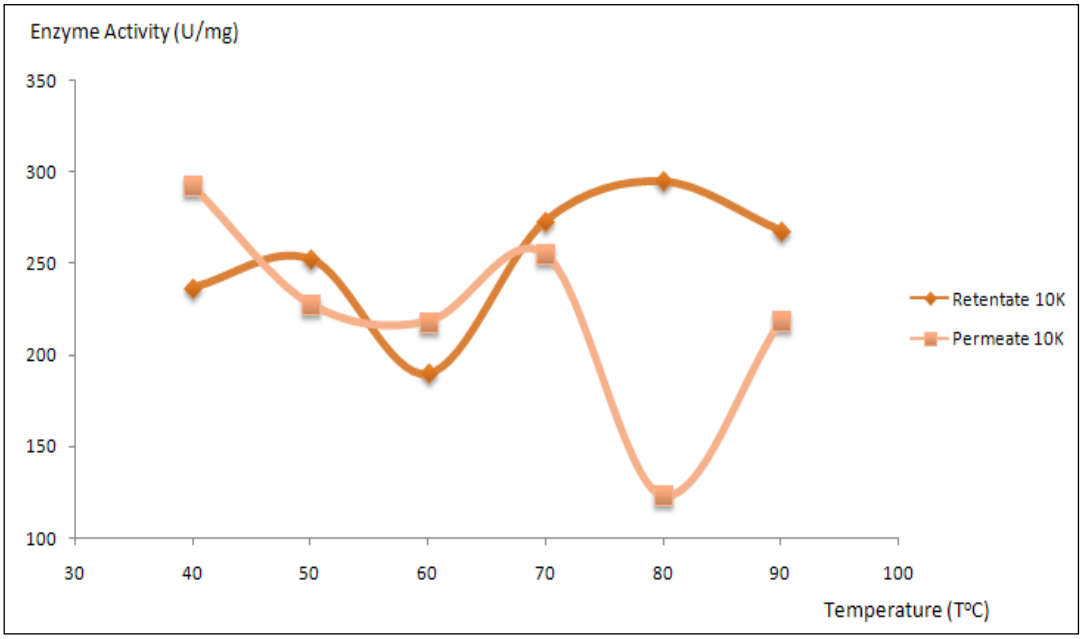

Figure 4.3: Optimum temperature of partially purified CGTase

4.4 Determination of pH and Thermal Stability of CGTase

Enzyme activity stability was measured in different buffers at pH4-5 (0.1M sodium acetate), pH6-7 (0.1M phosphate) and pH9-10 (0.1M Glyciene-NaOH) at 60^{o}C with 30 minutes incubation using standard assay method, but this time without a substrate (soluble starch). The enzyme was stable from pH 4 to 6 (permeate 10K) and from pH 4 to 5 (retentate 10K). The enzyme activity declined rapidly when incubated at pH 6 to 7 (Figure 4.4). CGTase from *Bacillus* sp. TS1-1 has narrower pH span for stability compared to CGTase from *Bacillus agaradhaerens* (pH5-11), *Bacillus autoliticus* (pH5-9) and *Bacillus coagulans* (pH5-10) as reported by Martins *et al*. (2002).

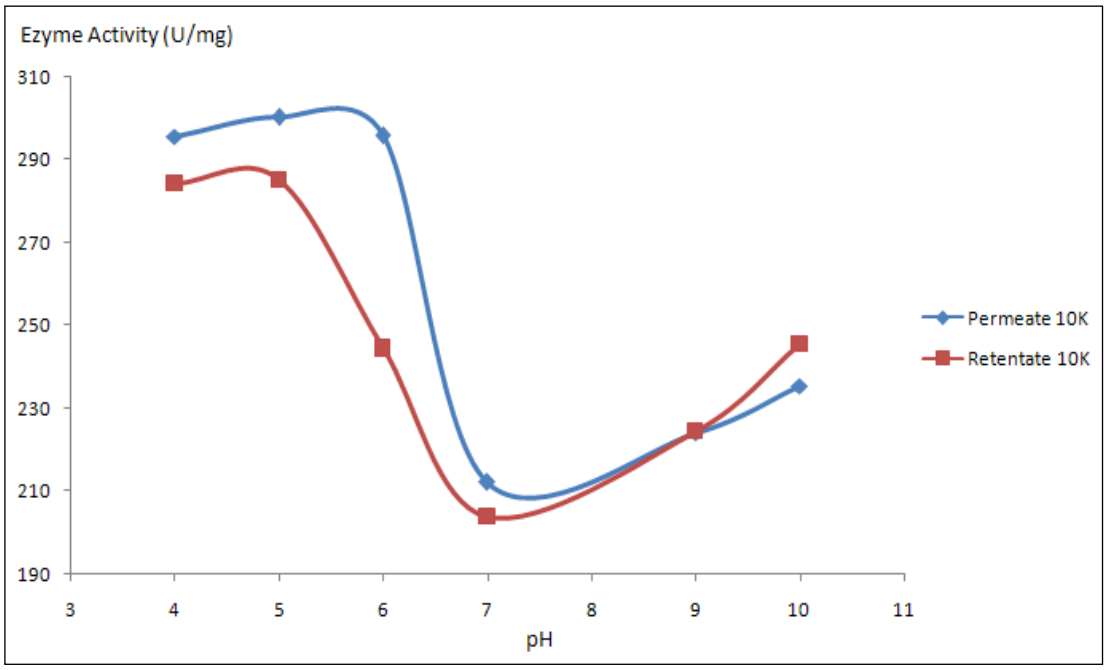

Figure 4.4: pH stability of partially purified CGTase

Meanwhile, at pH6 (0.1M Phosphate buffer) with different temperatures (40-90^{o}C), the CGTase showed (Figure 4.5) a maximum thermal stability at 70^{o}C (permeate 10K) and at 90^{o}C (retentate 10K). The enzyme activity of retentate 10K declined when incubated at temperature 50-60^{o}C and then inclined when incubated to 90^{o}C. However, the enzyme activity of permeate 10K inclined rapidly when incubated at 40-90^{o}C. This result was totally different compared to other researches done on CGTases; as for example, the thermal stability investigated on CGTase from *Bacillus* sp. G1 (Sian *et al*., 2005) was from 40-70^{o}C and then declined rapidly to 90^{o}C, from *Bacillus agadhaerens* (Martins et al., 2002) was from 20-50^{o}C and from *Paenibacillus* sp. F8 (Larsen *et al*., 1998) was from 30-60^{o}C.

The possible errors that result the data to be like this are the samples are not incubated within required time (less than 30 minutes) that caused the enzyme unable to show its real stability at certain temperature; overdose of buffer amount (more than 0.2 ml compared to other stage of temperature) added to sample lead to different rate of reaction for the enzyme to fully react with the buffer; the sample is highly contaminated

with other bacteria/enzymes/particles that caused the stability of the required enzyme at certain temperatures are different from the theory as well as reported by previous researches.

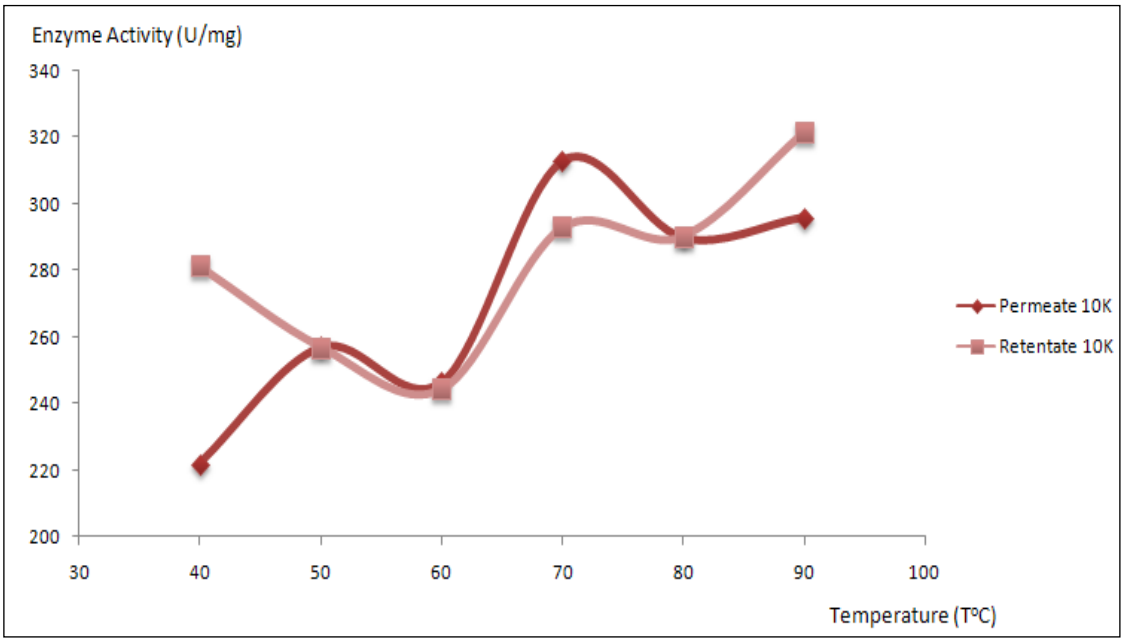

Figure 4.5: Thermal stability of partially purified CGTase

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

A specific activity of CGTase; 129.12 U/mg was detected in crude enzyme. After a partial purification by cross-flow filtration, 300.77 U/mg was obtained which is 2.33 purification fold. The predicted size of CGTase from Bacillus sp. TS1-1 was in the range 46 kDa to 88 kDa. Based on CGTase activity and stability analysis, the optimum pH is at pH6 and pH10; the optimum temperature is at 40^{o}C and 80^{o}C; the pH stability is from pH4 to 6 and the thermal stability is from 40-70^{o}C and from 60-90^{o}C.

5.2 Recommendations

It is recommended to further purify the CGTase by using affinity chromatography method such as purification system, in order to obtain pure CGTase. The silver staining method is suggested to detect the molecular weight of the partially purified CGTase. Full characterization of the pure CGTase can be performed which include the effect of ion, effect of solvent, effect of reagent, isoelectric point, kinetic parameters, comparison on coupling and hydrolytic activity as well as the analysis of the reaction products.

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APPENDIX A

MATERIALS AND METHOD

Appendix A.1 Protein Determination by Modified Lowry Protein Assay.

Mass of BSA (mg)	Volume of distilled water to add (ml)	Final BSA concentration (µg/ml)
2.0	10	200
5.0		500
10.0		1000
15.0		1500
20.0		2000

Table A.1: Preparation of Bovine Serum Albumin (BSA) Stock Solutions

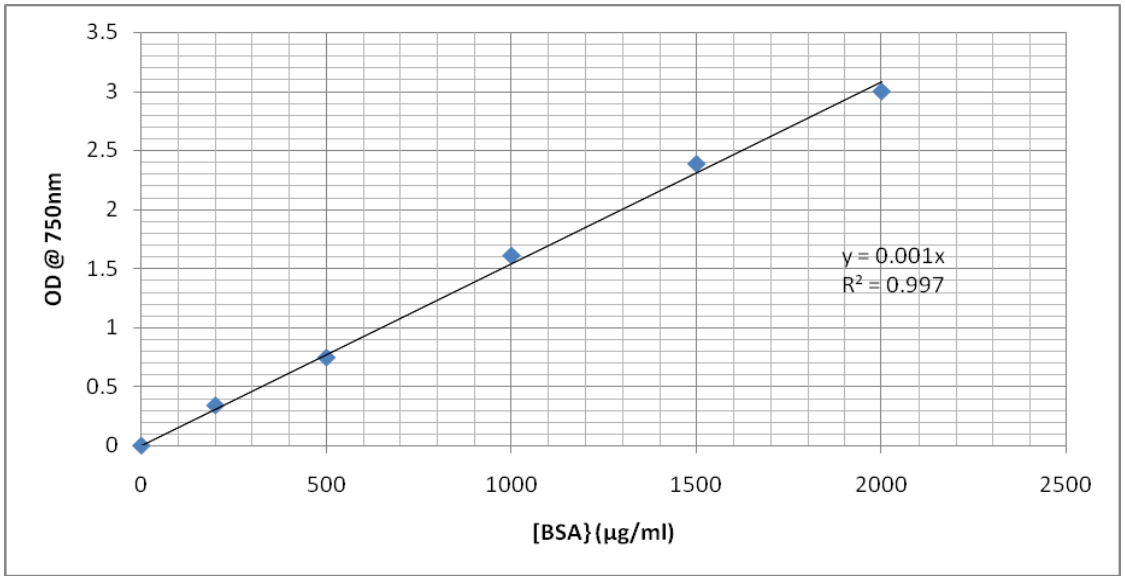

Figure A.1: Protein Concentration Standard Curve

Appendix A.2 Protein Determination by Phenolphtalein Method with Slight Modification.

Volume of 2mg/ml β-CD stock solution to add (ml)	Volume of 0.1M Phosphate buffer (pH6) to add (ml)	Final concentration of β-CD stock solutions (mg/ml)
0.1	0.9	0.2
0.2	0.8	0.4
0.3	0.7	0.6
0.4	0.6	0.8
0.5	0.5	1.0
0.6	0.4	1.2
0.7	0.3	1.4
0.8	0.2	1.6
0.9	0.1	1.8
1.0	0.0	2.0

Table A.2: Preparation of Beta-cyclodextrin glucanotransferase (β-CD) stock solutions

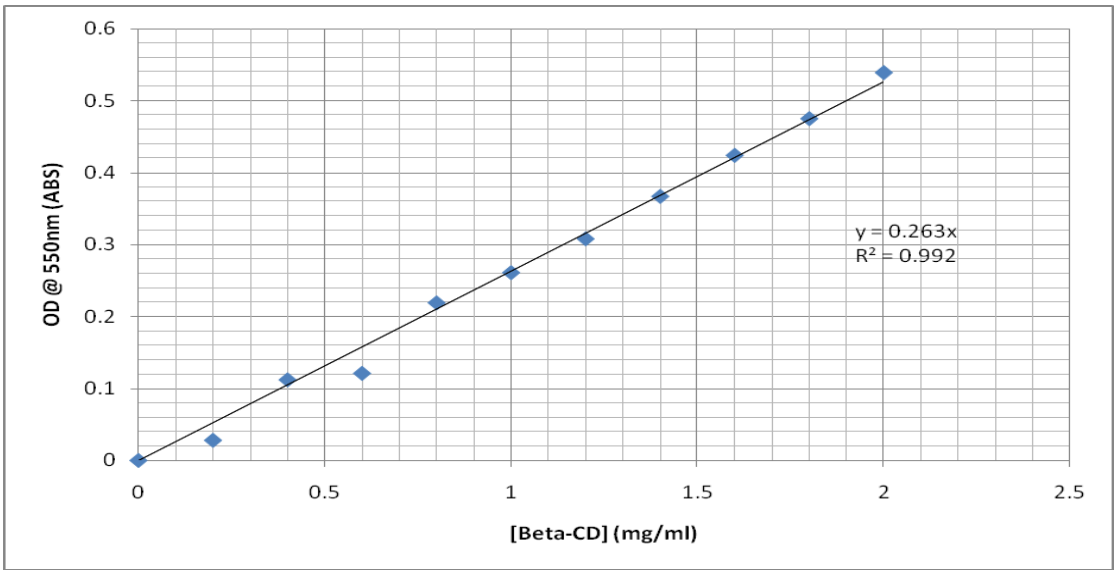

Figure A.2: β-CD Concentration Standard Curve

Appendix A.3 Buffers Required for Enzyme Activity Determination.

1. Sodium Acetate buffer, 0.1M
   Solution A: 2.72g of Sodium Acetate Trihydrate per 100ml (0.2M)
   Solution B: Glacial Acetic Acid

2. Phosphate buffer, 0.1M
   Solution A: 0.272g of K_2HPO_4 per 100ml (0.2M)
   Solution B: 4.56g of KH_2PO_4 per 100ml (0.2M)

3. Glycine-NaOH buffer, 0.1M
   Solution A: 0.751g of Glycine per 80ml (0.2M)
   Solution B: 4.0g of NaOH per 100ml (1.0M)

   Based on Table A.3, for each buffer, mix the indicated volumes of Solution A and B, then dilute with distilled water to a total of 100ml solutions.

Type	Desired pH	Solution A (ml)	Solution B (ml)
Sodium Acetate buffer	4	40	1.5
	5	40	0.2
Phosphate buffer	6	36.1	13.9
	7	10.1	39.9
Glycine-NaOH buffer	9	80	3.0
	10	80	10.0

Table A.3: Preparation of buffers

Appendix A.4 Calculation of CGTase Activity by Phenolphtalein Method

Enzyme Activity Calculation

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

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

$$= \frac{\%\text{OD reduction} \times 1\text{x}10^{3} \times 100 / 0.263}{1135.01 \times 10 \times 0.1}$$

Appendix A.5 Stock Solutions for SDS-PAGE

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.5-1: Preparation of 2 M Tris-HCl (pH 8.8), 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.5-2: Preparation of 1 M Tris-HCl (pH 6.8), 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.5-3: Preparation of 10% (w/v) SDS, 100 ml

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

Table A.5-4: Preparation of 50% (v/v) glycerol, 100 ml

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

Table A.5-5: Preparation of 1% (w/v) bromophenol blue, 10 ml

Appendix A.6: Working Solutions for SDS-PAGE.

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.6-1: Preparation of solution A (acrylamide stock solution), 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.6-2: Preparation of solution B (4x resolving@separating gel buffer), 100 ml

a	50 ml of 1 M Tris-HCl (pH 6.8)
b	4 ml of 10% (w/v) SDS
c	46 ml of distilled water
Stable for months in the refrigerator	

Table A.6-3: Preparation of solution C (4x stacking gel buffer), 100 ml

a	0.5 g ammonium persulfate
b	5 ml of distilled water
Must be prepared freshly	

Table A.6-4: Preparation of 10% (w/v) ammonium persulfate, 5 ml

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.6-5: Preparation of electrophoresis buffer, 2 L

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.6-6: Preparation of 5x sample buffer, 10 ml

a	5 ml solution A
b	3.75 ml solution B
c	6.25 ml distilled water
d	75 µl of 10% (w/v) ammonium persulfate
e	15 µl TEMED
Do not prepare until gel sandwich has been assembled	

Table A.6-7: Preparation of 10% separating gel preparation at 15 ml volume

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
Do not prepare until separating gel has already polymerized	

Table A.6-8: Preparation of 5% stacking gel

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^{o}C	

Table A.6-9: Preparation of staining solution, 1 L

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

Table A.6-10: Preparation of destaining solution, 1 L

Appendix A.7 Data for Optimum pH and pH stability, optimum temperature and thermal stability of CGTase

		Temperature (^{o}C)	pH	OD @ 550nm (ABS)	Enzyme Activity (U/mg)
(Retentate 10K)	**Enzyme activity – Optimum pH & Temperature (with substrate)**	**Optimum pH**			
		60	**4**	0.027	43.23
			5	0.673	179.51
			6	0.255	280.02
			7	0.406	252.82
			9	0.112	264.93
			10	0.106	310.10
		Optimum Temperature			
		40	**6**	0.386	236.74
		50		0.426	252.56
		60		0.673	189.92
		70		0.279	273.10
		80		0.168	294.89
		90		0.347	267.65
	Enzyme activity – Optimum pH & Thermal Stability (without substrate)	**Optimum pH**			
		60	**4**	0.187	284.07
			5	0.262	285.05
			6	0.606	244.33
			7	0.700	203.85
			9	0.546	224.28
			10	0.433	245.29
		Optimum Thermal Stability			
		40	**6**	0.357	281.35
		50		0.512	256.82
		60		0.606	244.33
		70		0.253	293.15
		80		0.234	290.31
		90		0.091	321.61

Table A.7-1: Activity of CGTase from Retentate 10K

		Temperature (^{o}C)	pH	OD @ 550nm (ABS)	Enzyme Activity (U/mg)
(Permeate 10K)	**Enzyme activity – Optimum pH & Temperature (with substrate)**	**Optimum pH**			
		60	**4**	0.040	208.58
			5	0.241	279.32
			6	0.201	291.66
			7	0.329	268.40
			9	0.186	218.38
			10	0.201	287.79
		Optimum Temperature			
		40	**6**	0.168	292.23
		50		0.554	227.78
		60		0.543	217.94
		70		0.360	255.13
		80		0.886	123.45
		90		0.601	218.35
	Enzyme activity – Optimum pH & Thermal Stability (without substrate)	**Optimum pH**			
		60	**4**	0.145	295.51
			5	0.183	300.11
			6	0.263	295.65
			7	0.656	212.09
			9	0.548	223.87
			10	0.481	235.35
		Optimum Thermal Stability			
		40	**6**	0.753	221.83
		50		0.511	256.98
		60		0.592	246.42
		70		0.134	312.85
		80		0.236	289.93
		90		0.266	295.85

Table A.7-2: Activity of CGTase from Permeate 10K