CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Instruments / Model	Company / country
Autoclave, Model HA-30	Hirayama Manufacturing Corperation, Japan
Autopipette, Pipetman	Gilson, France
Centrifuge, Model J-21C	Beckman Instrument In,c U.S.A.
Gene Amp PCR system, Model 2400	Perkin Elmer, U.S.A.
Incubator	Haraeus, Germany
Incubator shaker, controlled environment: Psycotherm	New Brunswick Scientific Co., U.S.A.
Incubator shaker, Model G76D	New Brunswick Scientific Co., U.S.A.
Laminar flow, Model BVT-124	International Scientific Supply Co.Ltd., U.S.A.
Magnetic stirrer, MS-90	Fargo Instrumetn Ltd., Taiwan
pH meter, PHM 83 Autocal pH meter	Radiometer, Denmark
Spectrophotometer, Du series 1050	Beckman, U.S.A.
Vortex, Model K-550-GE	Scientific Industries, U.S.A.
Water bath	Charles Hearson Co.Ltd., U.K.
Water bath, Shaking	Heto Lab Equipment, Denmark
Transformation apparatus: Gene Pulser™	Biorad, U.S.A.
High performance liquid chromatography	Shimadzu, Japan
Orbital shaker	Gallenkamp, Germany
Power supply: Model EC 135-90	E-C Apparatus Corperation, U.S.A.
Sequencer: model CEQ™8OOO genetic analysis	Beckman Coulter Inc., U.S.A.

2.2 Chemicals

Chemicals	**Company / Country**
Absolute alcohol	Sigma, U.S.A.
Acetonitrile (HPLC grade)	Lab Scan, Ireland.
Acrylamide	Merck, U.S.A.
Agar	Merck, Germany.
Agarose	FMC Bioproducts, U.S.A.
Ampicillin	Sigma, U.S.A
Beef extract	Difco Laboratories, U.S.A.
Boric acid	BDH , England
Bovine serum albumin (BSA)	Sigma, U.S.A.
Bovine serum albumin fraction V	Sigma, U.S.A.
Bromophenol blue	Merck, Germany.
Calcium chloride	Merck, Germany.
Chloroform	Sigma, U.S.A.
Coomassie brilliant blue R-250	BDH , England
α-β- and γ-cyclodextrins	Sigma, U.S.A.
Di-Sodium hydrogenphosphate	Fluka, Switzerland.
DNA marker : Lamda (λ.) DNA digested with *Hin*dIII	Biobasic Inc., Thailand.
Ethidium bromide	Sigma, U.S.A.
Ethylenediamine tetraacetic acid (EDTA)	Fluka, Switzerland.
Glacial acetic acid	BDH, England.
Glucose	Sigma, U.S.A.
Glycerol	Scharlau, Spain.
Hydrochloric acid	Merck, Germany.
Iodine	Baker chemical, U.S.A.
Methanol	Scharlau, Spain.
Peptone from casein	Merck, U.S.A.
Peptone from meat	Merck, U.S.A.
Phenol	BDH, England.
Phenolphthalein	BDH, England

2.2 Chemicals (con't)

Chemicals	Company / Country
Polyethylene glycol 6000	Fluka, Switzerland.
Potassium iodide	Mallinckrodt, U.S.A.
QIAprep Spin Miniprep Kit	Qiagen, Germany.
QIAquick Gel Extraction Kit	Qiagen, Germany.
Sodium carbonate	BDH, England.
Sodium chloride	USB, U S.A.
Sodium citrate	Carlo Erba, Italy.
Sodium dihydrogen orthophosphate	Carlo Erba, Italy.
Sodium dodecyl sulfate	Sigma, U.S.A.
Sodium hydroxide	Carlo Erba, Italy.
Soluble starch (potato)	Sigma, U.S.A.
Soluble starch (potato), synthesis grade	Scharlau, Spain
Standard molecular weight marker protein	Sigma, U.S.A.
Trichloroethylene (TCE)	BDH , England
Tris-base	USB, U S.A.
Tryptone	Merck, Germany.
Xylene cyanol FF	Sigma, U.S.A.
Yeast extract	Scharlau, Spain.

2.3 Bacterial strains and plasmid

Paenibacillus sp.RB01 was isolated from the hot spring area at Ratchaburi province and screened for CGTase activity by Tesana (2001) (**66**). *Paenibacillus* sp. T16 was isolated from hot spring area at Tak province (T16) and screened for CGTase activity by Pranommit (2001) (**67**).

E. coli strains JM109 [*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17($r_K^- m_K^+$), e14$^-$ (*mcrA*$^-$), *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*$^+$, *lac I*^{q}, *lacZ*ΔM15]] was used as host for gene cloning. Plasmid vector was pGEM® -T Easy Vector from Promega.

2.4 Enzymes

2.4.1 Restriction endonucleases

- *Hin*d III was purchased from New England Biolabs Inc., U.S.A.

2.4.2 T4 DNA ligase was purchased from New England Biolabs Inc., U.S.A.

2.4.3 Glucoamylase was purchased from Fluka, Switzerland.

2.4.4 RNase A was purchased from Sigma, U.S.A.

2.5 Media preparation

2.5.1 Starter medium (Medium I)

Medium I consisted of 0.5 % beef extract, 0.2% yeast extract, 1.0% polypeptone, 0.2% NaCl, 1.0% soluble starch adjusted to pH 7.2 with 1 N HCl. For solid medium, 1.5% agar was added, Medium I was sterilized by autoclaving at 121 ^{0}C for 15 minutes.

2.5.2 Cultivation medium (Horikoshi's medium) (68-70)

Medium used for growing CGTase producing bacteria that was slightly modified. It contained 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 0.75% Na_2CO_3. The pH of the medium was 10.1-10.2. For solid medium 1.5% agar was added. For screening purpose, 0.03% phenolphthalein and 0.01% methyl orange were added before solidification. The medium was sterilized as in 2.5.1.

2.5.3 Luria-Bertani medium (LB medium)

Medium for *E. coli* and transformants contained 0.5% yeast extract, 1.0% tryptone (polypeptone from casein), 1% NaCl and 100 mg/ml ampicillin, with 1.5% agar in solid medium. The medium was sterilized as in 2.5.1 and ampicillin was added after the sterilized medium was cooled down to 40-50°C. The medium containing starch was prepared by adding 1% soluble starch (potato).

2.6 Gene cloning

2.6.1 Bacteria cultivation

RB01 and T16 were streaked on a solid Medium I, and incubated for 18-24 hours at 37^0C. The isolated colony was transferred into Horikoshi's broth at 37^0C with 250 rpm rotary shaking until A_{660} reached 0.3-0.5 unit.

2.6.2 Preparation of chromosomal DNA

The cells were harvested by centrifugation at 3,500 rpm for 15 min and chromosomal DNA preparation was performed. Cell pellet (30 µl) was dissolved in 300 µl of SET (50 mM sucrose, 10 mM EDTA and 25 mM Tris pH 8.0), 200 µl of lysozyme (5 mg/ml in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and 10 µl of RNAse A (10 mg/ml in TE buffer) at 37°C for 1 h. The cell suspension was then incubated with 30 µl of 10% SDS and 3 µl of proteinase K (20 mg/ml in TE buffer) and incubate at 50°C for 4-5 hour (or at 37^0C overnight), after which 50 µl of 3 M sodium acetate was added and inverted gently for 2-3 times. An equal volume of TE-saturated phenol was added to the aqueous sample in a microcentrifuge tube. The mixture was vigorously vortexed, and then centrifuged to get the phase separation. The upper aqueous layer was carefully transfered to a new tube, avoiding the phenol interface and was subjected to two ether extractions to remove residual phenol. An equal volume of water-saturated ether was added to the tube, mixed by vortexing, and centrifuged to allow phase separation. The upper ether layer was removed and discarded, including the phenol droplets at the interface. The extraction was repeated and the DNA was precipitated with 95% ethanol and resuspended in TE buffer and kept at 4 °C.

2.6.3 Preparation of the CGTase gene using the PCR technique

A pair of 24 nucleotide primers were designed from the sequences of the -containing DNA fragment from *Paenibacillus* sp. A11 (**69**) for use in the PCR reaction.

The forward primer (primer A) was 5'- GGCTATGCTTTCCTTACCTTACCC - 3' and the reverse primer (primer B) was 5'-ATAGCACCTTTCCCCCACATAACG -3' DNA template of the positive control was from pVR 316, a plasmid containing CGTase gene from *Paenibacillus* sp. A11 and DNA template of the negative control was from *B. licheniformis* SK-1, the chitinase producing bacteria.

Twenty five microliters of the PCR reaction mixture was prepared with the following compositions: 100 ng of genomic DNA, 10 pmol of primer 1, 10 pmol of primer 2, 1 x (Mg^{2+} free) Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 units Taq. The following three rounds of temperature cycling were performed.

The first round : 94°C for 5 min; 1 cycle.

The second round : 94°C for 1 min, 45°C for 2 min, 72°C for 3 min; 30cycles.

The third round : 72°C for 5 min; 1 cycle.

2.6.4 DNA fragment determination

The amplified fragment was determined by using agarose gel electrophoresis with the standard DNA markers, λ *Hin*d III. The PCR product was run on a 0.7% agarose gel in 1x TAE buffer (40 mM Tris-HCl, 40 mM acetate and 1 mM Na_2EDTA, pH 8.0), under the current of 100 volts until the tracking dye (glycerol 50%, bromophenol blue 0.1% and xylene cyanol-FF 0.1%) reach the buttom margin. The agarose gel was stained with 0.5% ethidium bromine for 10 min, destained in distilled water for 30 min and the fluorescence band of DNA was observed under ultraviolet light.

2.6.5 Ligation of the PCR product with the plasmid (71)

Standard cloning techniques were used as described by Sambrook *et.al.*,1989. PCR product of each strain were ligated with pGEM -T Easy Vector at 16°C for 18 hours and then transformed into JM109 using Short Gun technique. After incubation at 37°C

for 18 hours, the ampicillin resistant colonies were selected. The transformants were screened for CGTase activity using iodine test.

The PCR was precipitated with ethanol before recombining with the vector pGEM^{TM}-T Easy. The reaction volume was 5 µl, containing 0.5 µl DNA + vector, 2.5 µl 2x ligation buffer, 5 unit T4 DNA ligase and 1.5 µl distilled water and incubate at 16 °C overnight

2.6.6 Competent cells preparation (71)

The competent *E.coli* strain JM109 was prepared according to the method of Sambrook *et al.*, 1989. A single colony of *E. coli* JM109 was cultured as the starter in 12 ml of LB broth and incubated at 37°C with 250 rpm shaking for overnight. One percent of starter was inoculated into 200 ml LB broth in 1,000 ml Erlenmeyer flask and cultivated at 37°C with 250 rpm shaking for about 3 hours until the absorbance at 650 nm of the cells reached 0.4-0.5.

The cells were chilled on ice for 14-30 min and harvested by centrifugation at 8,000 x g for 15 min at 4°C. The supernatant was removed as much as possible. The cell pellet was washed with 800 ml of cold steriled water, resuspended by gentle mixing and centrifuged at 8,000 x g for 15 min at 4°C. The supernatant was discarded. The pellet was then wash and centrifuged with 400 ml of cold steriled water, followed by 20 ml of ice cold steriled 10% (v/v) glycerol, and finally resuspended in a final volume of 1.6-2.0 ml ice cold steriled 10% (v/v) glycerol. The cell suspension was divided into 40 µl aliquots and stored at -80°C until used. Usually, these competent cells were good for at least 6 months under these conditions.

2.6.7 Transformation into host cell (*E. coli*)

The *E. coli* cells suspended in 10% glycerol at a concentration of 3 x 10^{10} cells/ml were thawed. Electroporation was carried out at low temperatures (0-4°C). The DNA

was mixed with the cold cell suspension, transferred to a chilled cuvette and the electroporation apparatus was set to deliver an electrical pulse of 25 μF capacitance, 2.5 kV, and 200 ohm resistance. After applying the very high field strengths, *E. coli* cells were then transferred into the new microcentrifuge containing 1 ml of LB broth, incubated at 37°C for 1 h, spreaded onto the agar plate containing 50 μg/μl ampicillin, 0.002% x-gal and 0.0025%IPTG, and incubated at 37°C overnight.

2.6.8 Selection of positive recombinant

After incubation at 37°C for 18 hours, the ampicillin resistant colonies (the white colonies) were selected. The transformant was streaked on the replica plates of LB agar (containing 1% soluble potato starch and 50 μg/μl of ampicillin) incubated at 37°C overnight. The transformant with CGTase activity could be detected by staining with appropriate dyes. There were 2 types of dye staining for CGTase activity:

2.6.8.1 Iodine stain for dextrinizing activity (48)

The dye used to stain the dextrinizing activity of CGTase was 0.2% I_2 in 0.2% KI. The dye was poured onto the plate from 2.7.7. Positive colony appeared as clear zone against dark blue background.

2.6.8.2 Phenolpthalein-methyl orange staining (68)

The recombinant colonies on the 1% soluble starch-LB agar plate were stained with 0.03% phenolphthalein and 0.01% methyl orange in 1% Na_2CO_3 and stood for 5 minutes at room temperature. The colony producing CGTase appeared as yellow zone on reddish pink background.

2.7 Nucleotide sequencing

The plasmid of recombinant colonies was extracted by alkaline extraction method (72) and then sequencing analysis of the gene fragment was performed by first using primers M13 forward, M13 reverse, 1-5 and B. The obtained DNA sequences were

then used to design the additional primers for further DNA sequencing by the chain termination method with an automated DNA sequencer (Beckman Coulter, CEQ2000), and the results were analyzed using GENETYX-WIN software.

The designed primers were 1, 2, 3, 4 and 5 as shown below;

Primer 1	5′- TGATCCCCAAAGCGACGG – 3′
Primer 2	5′-GACTTCAAGAAGACCAATCC – 3′
Primer 3	5′-GGCGAATGGTTCCTTGGC – 3′
Primer 4	5′-TATCAAGTCATCCAAAAGC – 3′
Primer 5	5′-GGGTAGAGGCTCGGGG – 3′

The primers from a set of five primers (Biogenomed Inc., Thailand), designed for the sequencing of the whole CGTase gene were used in the sequencing. According to the CEQ2000 Dye Terminator Cycle Sequencing with Quick Start Kit protocol, approximately 200 ng of plasmid was mixed with approximately 10 ng of DNA primer and amplified by thermal cycling (96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes for 30 cycles) and finally reserved at 4°C. The DNA obtained was cleaned up by ethanol precipitation. The DNA sample was added with 4 µl stop solution (1.5 M NaOAc + 50 mM EDTA) and 1 µl of 2 mg/ml glycogen to each reaction tube, the sequencing reaction was then mixed with 60 µl cold 95% ethanol and centrifuged at 12,000 x g for 20 minutes. The pellet was rinsed twice with 200 µl 70% ethanol and centrifuge at 12,000 x g for 5 minutes. The pellet was vacuum dried and resuspended in 40 µl of the sample loading solution. The sample was transferred to the CEQ sample plate and overlaid with one drop of light mineral oil. The sample was sequenced with the CEQ sequencer.

2.8 Protein determination

Protein concentration was determined by the Coomassie blue method according to Bradford (**73**), using bovine serum albumin as a standard (Appendix 4).

One hundred microlitres of sample was mixed with 1 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm.

2.9 Enzyme assay

CGTase activity can be determined by different types of reactions: assay of starch degrading (dextrinizing) activity, assay of CD product by formation of CD-trichloroethylene complex (CD-TCE), cyclization activity assay, and coupling activity assay.

2.9.1 Dextrinizing activity assay

Dextrinizing activity of CGTase was measured by the method of Fuwa (**74**) with slight modification (**48**).

Enzyme sample (10-1000 µl) was incubated with 0.3 ml starch substrate (0.2 g% soluble potato starch in 0.2 M phosphate buffer, pH 6.0) at 40°C for 10 minutes. The reaction was stopped with 4 ml of 0.2 N HCl and 0.5 ml of iodine reagent (0.02% I_2 in 0.2%KI) was added. The mixture was adjusted to a final volume of 10 ml with distilled water and the absorbance at 600 nm was measured. For the control tube, HCl was added before the enzyme sample.

One unit of enzyme was defined as the amount of enzyme which produced 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

2.9.2 Cyclization activity assay

Cyclization activity was determined by the phenolphthalein method of Goel and Nene ,1995 (**75**). To 1.25 ml of 4.0% soluble starch, 0.25 ml purified CGTase was

added. The reaction mixture was incubated for 30 minutes at 60°C. The reaction was stopped by boiling 5 minutes and 1.0 ml of the reaction mixture was incubated with 4.0 ml of phenolphthalein solution. Absorption was measured at 550 nm and the β-CD forming was calculated using the calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 μmole of β-CD per minute under the appropriate condition.

Stock solution of 4 mM phenolphthalein was prepared by mixing 100 ml of 125 mM Na_2CO_3 solution with 4 ml phenolphthalein in 95% ethanol just before starting the experiment. Standard curve was prepared from 0-2.5 mM β-CD.

2.9.3 Coupling activity assay

Coupling reaction was determined by incubating various concentrations of α-, β- or γ–cyclodextrin or their derivatives as donor (0.5-15 mM) with 10 mM cellobiose as glucosyl acceptor at 55°C. Total volume of reaction mixture was adjusted to 0.25 ml by 50 mM acetate buffer, pH 6.0. Cyclodextrin and cellobiose were pre-incubated for 5 minutes at 55°C. The reaction was started with 10 μl of 200 units of purified CGTase. the reaction was stopped by boiling for 5 minutes. Subsequently, 0.2 units of *Aspergillus niger* glucoamylase (10 μl) was then added to convert the linearized oligosaccharides to glucose. The released reducing sugar was measured by the dinitrosalicylic acid method (Appendix 6)

2.10 Enzyme production

Starter inoculum (1%) was transferred into 600 ml LB broth containing 1% soluble potato starch in 2 liter Erlenmeyer flask. Cultivation was performed at 37°C for 72 hours for wild type and for 24 hours for transformant. After cultivation, bacterial cell mass was removed by centrifugation at 4,500 rpm for 15 minutes at 4°C. Culture broth

with crude enzyme was collected and kept at 4°C for activity assay and determination of protein content.

2.11 Purification of CGTase

CGTase was purified from the culture broth of wild types and transformants by two steps of purification; starch adsorption, DEAE-cellulose column chromatography or Phenyl sepharose column chromatography.

2.11.1 Starch adsorption (**76**), modified by Kuttiarcheewa, 1994) (**53**). Corn starch was oven dried at 120°C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude CGTase broth to make 5 g% concentration. After 3 hours of continuous stirring, the starch cake was collected by centrifugation at 3,000 rpm for 30 minutes and washed twice with 10 mM Tris-HCl containing 10 mM $CaCl_2$, pH 8.5 (TB1). The adsorbed CGTase was eluted from the starch cake with TB1 buffer containing 0.2 M maltose (2 x 125 ml for starting broth of 1 liter), by stirring for 30 minutes. CGTase eluted was recovered by centrifugation at 3,000 rpm for 30 minutes. The solution was dialyzed against water at 4 ^{0}C with 3 changes of water.

2.11.2 DEAE-cellulose column chromatography

DEAE-cellulose was activated by washing sequentially with excess volume of 0.5 N HCl for 3 hours followed by distilled water until pH was about 7.0 and then washed with 0.5 N NaOH for 3 hours followed by distilled water to obtain pH about 8.0. The activated cellulose was equilibrated with Tris-HCl pH 8.0 (TB2). The prepared DEAE-cellulose was packed into the column (1.5 x 28 cm) and was equilibrated with TB2 at around 25°C.

The dialyzed protein solution from starch adsorption section was applied to DEAE-cellulose column. The unbound proteins were eluted from the column with the elution buffer, washing was continued until the absorbance at 280 nm of eluant decreased

to almost nil. After the column was washed thoroughly with the buffer TB2, the bound proteins were eluted from the column with linear salt gradient of 0 to 0.3 M sodium chloride in the same buffer. Fractions of 4.0 ml were continuously collected. The protein profiles of the eluted fractions were monitored by measuring the absorbance at 280 nm. The dextrinizing activity was determined as described in section 2.9.1. The fractions showing dextrinizing activity were pooled for further determination.

2.11.3 Phenyl Sepharose column chromatography

Phenyl Sepharose high performance column (1.7 x 7 cm.) was washed with 2 column volumes of sodium hydroxide 0.5 N followed with 500 ml distilled water. The column was then equilibrated with 50 mM Tris-buffer, pH 8.5 containing 1 M ammonium sulfate ($(NH_4)_2SO_4$). The sample from starch adsorption step was added with $(NH_4)_2SO_4$ to make final concentration of 1 M and centrifuged to remove undissolved particles before filtrated through the 0.45 μm membrane filter. The supernatant was loaded onto the column and eluted with 10 column volumes of elution buffer at flow rate of 20 ml/hr controlled by peristaltic pump. The enzyme was eluted with gradient of 1 M to 0 M of $(NH_4)_2SO_4$ in elution buffer. Fractions of 2 ml were collected using fraction collector. The eluted fractions were monitored for protein by measuring the absorbed at 280 nm using spectrophotometer (DU series650, Beckman, USA). The enzyme activity was detected by the method described in section 2.10.1 The fractions with enzyme activity were pooled in dialysis bag (molecular weight cut off 10 kDa) and concentrated with aquarsorb to reduce volume for further determination.

2.12 Identification and characterization of CGTase

2.12.1 Polyacrylamide Gel Electrophoresis (PAGE) (77)

Two types of PAGE, non-denaturing and denaturing gels, were employed for analysis of the purified protein. The gels were visualized by coomassie blue staining. For non-denaturing gel, dextrinizing activity staining was also undertaken.

2.12.1.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

Discontinuous PAGE was performed on slab gels (10 x 8 x 0.75 cm), of 7.5% (w/v) separating gel, and 5.0 % (w/v) stacking gels. Tris-glycine buffer, pH 8.3 was used as electrode buffer (see Appendix 1). The electrophoresis was run from cathode towards anode at constant current of 20 mM per slab at room temperature in a Mini-Gel Electrophoresis unit.

2.12.1.2 SDS-polyacrylamide gel electrophoresis (SDS -PAGE)

The denaturing gel was carried out with 0.1% (w/v) SDS in 7.5% (w/v) separating and 5.0% (w/v) stacking gels and Tris-glycine buffer, pH 8.0 containing 0.1% SDS was used as electrode buffer (see Appendix 1). Sample to be analyzed were treated with sample buffer and boiled for 5 minutes prior to application to the gel. The electrophoresis was performed at constant current of 20 mM per slab, at room temperature on a Mini-Gel electrophoresis unit from cathode towards anode.

2.12.1.3 Detection of CGTase

2.12.1.3.1 Coomassie blue staining

Gels were stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 2 hours. The slab gels were destained with a solution of 10% methanol and 10% acetic acid for 1-2 hours, followed by several changes of destaining solution unit gel background was clear.

2.12.1.3.2 Dextrinizing activity staining

The non-denaturing gel was stained for dextrinizing activity or amylolytic activity by the slightly modified technique of Kobayashi et al., 1978 (**78**) in 10 ml of substrate solution, containing 0.2% (w/v) potato starch in 0.2 M phosphate buffer, pH 6.0, at 40°C for 10 minutes. It was then quickly rinsed several times with distilled water and immersed in 10 ml I_2 staining reagent (0.2% I_2 in 2% KI) and left for color development at room temperature. The clear zone on the blue background represents starch degrading activity of the protein.

2.12.2 Determination of the isoelectric point by isoelectric focusing polyacrylamide gel electrophoresis (IEF)

2.12.2.1 Preparation of gel support film

A few drops of water were pipetted onto the plate. The hydrophobic side of the gel support film was then placed against the plate and flatly rolled with a test tube to force excess water and bubbles. Subsequently, it was placed down on the casting tray with the gel support film facing down so that it rested on the space bars.

2.12.2.2 Preparation of the gel

The gel solution composed of 30% acrylamide, 1% bis-acrylamide, 50% sucrose, 10% ammonium persulfate, ampholite and TEMED (see Appendix 2) was carefully pipetted between the glass plate and casting tray with a smooth flow rate to prevent air bubbles. The gel was left about 45 minutes to allow polymerization, and then lifted from the casting tray using spatula. The gel was fixed on the gel support film and ready for used.

2.12.2.3 Sample application and running the gel

The sample was loaded on a small piece of filter paper which was placed on the gel to allow diffusion into the gel for 5 minutes and the filter paper was carefully

removed. Standard protein markers with known pI's in the range 3-10 were run in parallel. The standards consist of amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydase B (5.85), human carbonic anhydase B (6.55), myoglobin-acidic band (6.85), myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.3).

The gel with the adsorbed samples was turned upside-down and directly placed on top of the graphite electrodes. Focusing is carried out under constant voltage conditions in a stepwise fashion. The gel was firstly focused at 100 V for 15 minutes, followed by an increase in voltage up to 200 V for 15 minutes and finally run at 450 V for and additional 60 minutes. After the completion of electrofocusing, the gel was stained with coomassie blue. The pI's of sample proteins were determined by the standard curve constructed from the pI's of the standard proteins and their migrating distance from cathode.

2.12.3 Optimum Conditions for enzyme activity

2.12.3.1 Effect of pH

The purified CGTases from 2.11 were used to study the effect of pH on their activities by incubate in buffer at various pH's for 60 minutes at 4°C. Each enzyme was assayed as described in section 2.9.1-2.9.2. The 0.2 M buffer solution was in the pH range from 4.0 to 11.0. The buffer used were universal buffer, pH 4.0-11.0 (Appendix 3)

2.12.3.2 Effect of temperature

Each purified CGTase (20 units) was incubated with 0.2% potato starch in phosphate buffer, pH 6.0 at various temperatures from 20-100°C for 10 minutes, and its dextrinizing and cyclization activity was assayed as described in section 2.9.1-2.9.2.

2.12.4 Enzyme stability

2.12.4.1 Effect of pH

The stability of purified CGTase at different pHs was determined by incubating each enzyme (20 units) in various buffers of pH 4.0-10.0 at the optimum temperature determined in 2.13.2.2 for 60 minutes (buffers used were the same as in 2.13.2.1). Samples were withdrawn from reaction mixture at 5 minutes interval and. the enzyme activity was determined as described in section 2.9.1 and 2.9.2.

2.12.4.2 Effect of temperature

Effect of temperature on the stability of enzyme was studied by incubating the enzyme (20 units) at different temperatures in the range 40-70°C, for 60 minutes. The reaction mixture was taken at 5 minutes interval and cyclization activity was assayed at the optimal conditions obtained from 2.9.2

2.12.4.3 Effect of substrate

The effect of substrate on enzyme stability was also studied by incubating the enzyme sample in the presence of final concentration of 4% soluble starch (potato) at 70°C for 60 minutes. The enzyme activity was then assayed as in section 2.12.2.

2.13 Substrate specificity of CGTase

The cyclization activity of purified CGTase was determined as described in section 2.9.2 with varying substrates: soluble starch, amylose, amylopectin, pullulans, glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and dextrins at 6.0%W/V were used, except for amylopectin of which 1.5% in acetate buffer, pH 6.0 was used.

2.14 Determination of kinetic parameters

Kinetic parameters of the coupling reaction were determined by incubated various concentrations of α-, β- or γ–cyclodextrins and their derivatives (0.5-15 mM) with 10

mM cellobiose as glucosyl acceptor at 55°C. 50 mM acetate buffer, pH 6.0 was added to make the total volume of reaction mixture 0.25 ml. Cyclodextrin and cellobiose were pre-incubated for 5 minutes at 55°C. The reaction was started with 10 ml of 0.88 mg/ml of purified CGTase. Boiling for 5 minutes stopped the reaction. Subsequently, 0.2 units of *Aspergillus niger* glucoamylase (10 μl) was then added to convert linearized oligosaccharides to glucose. The released reducing sugars was measured by the dinitrosalicylic acid method as described in section 2.9.3. Kinetic parameter, K_m and V_{max}, were determined from the Michealis-Menten equation using nonlinear least square regression analysis of the EZ-FIT V1.1 Computer program.

2.15 Analysis of cyclodextrins by High Performance Liquid Chromatography (52)

The HPLC system was performed on a Shimadzu LC-3A machine equipped with Lichrocart-NH_2 column (0.46 x 25 cm) using Shimadzu RID-3A refractometer as detector. For CD analysis, the reaction was performed by incubating 0.5 ml of enzyme sample with 1.5 ml of starch substrate (2.0 g% soluble starch (potato) in 0.2 M phosphate buffer, pH 6.0) at 40 ^{o}C for 24 hours and boiled in hot water for 5 minutes to stop the reaction. After cooling, the mixture was treated with 20 units of β–amylase at 25^{o}C for 3 hours, and the reaction was stopped by heating in boiling water. Prior to injection, the mixture was filtered through a 0.45 μm membrane filter. The mixture was injected and eluted with acetronitrile-water (70:30, v/v) using a flow rate of 1.0 ml/min. The CD peak was identified by comparing the retention time with that of standard α-, β- and γ-CDs (20 mg/ml).