CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Amino acid analyzer: Beckman system 6360, Na^+ high performance column (0.4x12cm): Beckman company, Data analysis apparatus: Applied Biosystems model 610A, U.S.A.

Autoclave: Model HA-30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Pipetman, Gilson, France

Autoradiography: KodakXAR film or Hyper film MP., Amersham

Centrifuge, bench-top centrifuge: Kokusan Enshinki Co., Ltd., Japan

Centrifuge, refrigerated centrifuge: Model J-21C, Beckman Instrument Inc., U.S.A.

Centrifuge, refrigerator microcentrifuge: Kubota 1300, Japan

Centrifuge, High speed microcentrifuge: HA-30, Memmert Gmbt, Germany

Diaflo Ultrafilter: Stirred Ultrafiltration Cell 8050 Amicon W.R. Grace Cooperation, U.S.A.

Electrophoresis unit: 2050 MIDGET, LKB, Sweden and Mini protein, Bio-Rad, U.S.A.; Submarine Agarose Gel Electrophoresis unit

Film Cassette: sized 8x11 &14x17 inches, Okamoto, Japan

Filter holder: Gelman Sciences, Inc., U.S.A.

Fraction collector: model 2211, Pharmacia LKB, Sweden

Gas phase protein sequencer: Applied Biosystems model 476A, U.S.A.

Gieger counter: U.S.A.

Gel dryer, 583, Bio-Rad Laboratories, U.S.A.

High Performance Liquid Chromatography: Model LC 31 Shimadzu, Japan & Applied Biosystems PTH-C18 reverse phase cartridge column (220x2.1 mm)

Incubator: Haraeus, Germany

Incubator shaker, Controlled environment: Psyco-Therm, New Brunswick Scientific Co., U.S.A.

Magnetic stirrer: 0188 GMS, Scientific Instrument Development and Service Center, Faculty of Science, Chulalongkorn University

Membrane filter: cellulose nitrate, pore size 0.45 μm, Whatman, Japan

Mini Problott^{TM} membrane: Bio-Rad Applied Biosystem company, U.S.A.

Mini Trans-Blot Electrophoresis Transfer cell: Bio-Rad Applied Biosystem company, U.S.A.

NAP-column, Pharmacia, Sweden

pH meter: PHM 83 Autocal pH meter, Radiometer, Denmark

Savelight lamp, Adjustable: 1521541 Kodak 6B, Eastman Kodak Company, U.S.A.

Spectrophotometer: Spectronic 20D, Bauch&Lomb, U.S.A., Spectrophotometer UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.

Syringe and needle, Disposable: Terumo, Japan

Syringe, Glass: Luer Lock interchangable, IPAS, Germany

U.V. transilluminator: 2011 MA crovue, San Gabrial, U.S.A.

Vortex: Model K-550-GE, Scientific Industries, U.S.A.

Water bath: Charles Hearson Co., Ltd., England

Water bath, Shaking: Heto Lab Equipment, Denmark

2.2 Chemicals

Acetonitrile (HPLC grade): J.T. Baker Chemical, U.S.A.

Acrylamide: Merck, U.S.A.

Agarose: Type II, Sigma, U.S.A

Amplicillin: Sigma, U.S.A

CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid): Sigma, U.S.A

Coomasie brilliant blue G-250: Sigma, U.S.A.

Coomasie brilliant blue R-250: Sigma, U.S.A.

[γ-^{32}P] dATP: Amersham, U.S.A.

DEAE-cellulose resin: Sigma, U.S.A

Dialysis tubing: Sigma, U.S.A

DNA marker: Lamda (λ)DNA digest with *Hin*dIII: Biolabs, U.S.A.

Ethidium bromide: Sigma, U.S.A

Glycine: Sigma, U.S.A

Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose: Sigma, U.S.A.

Methylorange: BDH, England

N,N$'$-methylene-bis-acrylamide: Sigma, U.S.A

Nylon membrane: Biorad, U.S.A.

Phenolpthalein: BDH, England

Potato starch: Sigma, U.S.A.

pUC18/BamHI/BAP: Pharmacia, Sweden

Standard α-, β-, and γ-CD : Fluka A.G. Buchs S.G., Switzerland and Sigma, U.S.A.

Standard molecular weight marker protein: Sigma, U.S.A

Trichloroethylene (TCE): BDH Laboratory Chemical company, U.S.A.

The other common chemicals were obtained from Fluka or Sigma. Raw rice starch (three heads elephant brand) and corn starch (Maizena) were locally purchased.

2.3 Enzymes and Restriction enzymes

5'-DNA labeling KIT: Phamacia, Sweden

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction enzymes: Biolabs, U.S.A.

RNase: Sigma, U.S.A.

T4 polynucleotide kinase: Biolabs, U.S.A.

T4 polynucleotide ligase: Pharmacia, Sweden

2.4 Bacteria

*Bacillus sp.*A11, isolated from South-East Asian soil, was screened for CGTase by Pongsawasdi and Yagisawa (1987).

DH5α with genotype, F', 80d*lacZ*ΔM15, *end*A11, *rec*A1, *hsd*R17 (rk-mk-), *sup*E44, *thi*-1, λ, *gyr*A96, *rel*A1, Δ(*lacZYA-arg*F) U169 (Raligh et al., 1981), was purchased from Phamacia, U.S.A.

2.5 Media Preparation

2.5.1 Medium I

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

2.5.2 Cultivation medium (modified from Horikoshi's medium) (Rutchtorn, 1992).

Medium for enzyme production, slightly modified from Horikoshi (1971), contained 1.0% local grade of rice starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7\ H_2O$ and 0.75% Na_2CO_3 with starting pH of 10.1-10.2. Medium was steriled as above.

2.5.3 Luria-Bertani (LB) medium (Maniatis et al., 1982)

LB consisted of 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl. pH was adjusted to pH 7.4 with NaOH. For solid medium 1.5% agar was added. Medium was steriled as above.

2.6 Cultivation of Bacteria

2.6.1 Starter inoculum

A colony of *Bacillus sp.*A11 was grown in 50 ml of starter medium I in 250 ml Erlenmeyer flask at 37 ^{0}C with 250 rpm rotary shaking until A_{420} reached 0.3-0.5 or about 4-6 hours.

2.6.2 Enzyme production

Starter *Bacillus sp.*A11 was diluted 1:100 into 100 ml Horikoshi's broth in 500 ml Erlenmeyer flask and cultivated at 37 ^{0}C with 250 rpm rotary shaking. Cells were separated by centrifugation at 3,000 rpm for 30 minutes at 4 ^{0}C. Culture broth with crude CGTase enzyme was collected and kept at 4 ^{0}C for purification.

2.7 Enzyme assay

For this study, CGTase activities were determined by starch degrading (dextrinizing) activity assay and CD-forming activity (CD-trichloroethylene, CD-TCE) assay.

2.7.1 Dextrinizing activity assay (Fuwa, 1954)

Sample (10-100 μl) was incubated with 0.3 ml of starch substrate (0.2 g% potato starch in 0.2 M phosphate buffer, pH 6.0) at 40 ^{0}C for 10

minutes. The reaction was stopped with 4 ml of 0.2 N HCl. 0.5 ml of iodine reagent (0.02% I_2 in 0.2% KI) was then added. The mixture was adjusted to final volume of 10 ml with distilled water and its absorbance at 600 nm was determined. For a blank tube of each sample, the enzyme sample was added after stopping the reaction with HCl.

One enzymatic unit is the amount of enzyme which produces 10% reduction in the intensity of the blue color of the starch-iodine complex per minute under the described condition.

2.7.2 Determination of cyclodextrin (Nomoto, 1986)

The enzyme sample was diluted in serial double dilution. The reaction mixture, containing 0.5 ml of sample and 2.5 ml of starch substrate (0.2% (w/v) potato starch in 0.2 M phosphate buffer, pH 6.0) was incubated at 4 ^{0}C for 24 hours. The mixture was vigorously mixed with 0.5 ml of trichloroethylene (TCE) and left overnight at room temperature in the dark. The activity was expressed in term of the dilution limit ($1:2^n$) which was the highest dilution that produced observable CD-TCE precipitate between the upper starch solution layer and the lower TCE layer.

2.8 Protein Determination

Protein concentration was determined by the Coomassie blue method according to Bradford (1981), using bovine serum albumin as standard.

One hundred microlitres of sample was mixed with 5 ml of protein reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent was the mixture of 100 mg Coomassie blue G-250, 50 ml of 95% ethanol, 10 ml of 85% H_3PO_4 and distilled water.

2.9 Purification of CGTase

CGTase was purified from the culture broth of *Bacillus sp.*A11 by various techniques. All steps of purification process were performed at 4 ^{0}C.

2.9.1 Starch adsorption

Corn starch (local grade) was oven dried at 120 ^{0}C for 30 minutes and cooled to room temperature. It was then gradually sprinkled into stirring crude enzyme broth to make 7 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 20 g% ammonium sulphate, and 10 mM $CaCl_2$, pH 8.5 (TB1, see appendix A). The adsorbed CGTase was eluted from the starch cake with TB1 buffer containing 0.2 M maltose (3x150ml for starting broth of 5 l), by stirring for 30 minutes.CGTase eluted was recovered by centrifugation at 3,000 rpm for 30 minutes.

2.9.2 Ammonium sulphate precipitation

Solid ammonium sulphate was slowly added into the starch cake eluant to 40% saturation with gentle stirring for 1 hour. The supernatant obtained after centrifugation at 8,000 rpm for 30 minutes was made to 80% saturatuion of ammonium sulphate and recentrifuged at 12,000 rpm for 30 minutes. The final precipitate was collected and dissolved in a few millilitres of 15 mM Tris-HCl buffer, pH 8.5 (TB2, see Appendix A). The solution was dialyzed for at least 4 hours at 4 ^{0}C against 3 changes of TB2.

2.9.3 DEAE-cellulose column chromatography

DEAE-cellulose (50g) was swollen in water (100ml) and transferred to 0.5 N HCl. The resin was washed several times with distilled water until pH of the wash was 7.0, then suspended in 0.5 N NaOH. The resin was rewashed with distilled water until pH was about 7.0 and finally with TB2 until pH was 8.5. The resin was packed into a column of 2.3x12.0 cm and equilibrated with TB2 buffer at the flow rate of 30 ml/hour.

The 40-80% ammonium sulphate precipitated fraction was loaded onto the column after dialysis. The column was washed with starting buffer until A_{280} was negligible and subjected to 200 ml of 0-0.3 M NaCl linear gradient elution. Fraction of 3 ml were collected for measurement of A_{280}, and conductivity and dextrinizing activity were measured.

2.10 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gel electrophoresis were employed for analysis of the purified protein. Electrophoresis conditions, protein and activity staining were as described by Rojtinnakorn, 1994.

2.11 Amino acid analysis

Amino acid analysis of CGTase was determined using the method described (Beckman, 1988).The dried CGTase samples were hydrolyzed with 6M HCl in evacuated tubes at 110 ^{0}C for 22 hours in the Waters Pico-Tag Workstation. In the determination of tryptophan, 4M methanesulfonic acid was used in inplace of 6 M HCl. When cysteine and methionine were to be analyzed, the samples were hydrolyzed with performic acid reagent (prepared by mixing formic acid and hydrogen peroxide in a ratio of 19:1 (v/v)) for 30 minutes before the addition of 6M HCl. The amino acid mixtures obtained were analyzed on a Na high performance column (0.4x12 cm) and the amino acid compositions were calculated by Beckman system 6300 amino acid analyzer.

2.12 Determination of N-terminal Amino Acid Sequence

2.12.1 Protein Immobilization

Proteins on SDS-polyacrylamide gel were transferred onto Problott™ membrane. The membrane was wetted with methanol for a few seconds, then placed in a dish containing blotting buffer (10 mM CAPS (3-[cyclohexylmino]-1-propanesulfonic acid), 10% methanol, pH 11.0) for 5 minutes.The SDS-gel was sandwiched between pre-wet membrane, three layers of Whatman No.1 paper, and sponges, was assembled on a BioRad Mini Trans-Blot Electrophoretic Transfer Cell and run at constant voltage of 50 volts (170-100 mA) at room temperature for 30 minutes. Problott™ membrane was then removed from transblotting sandwich and rinsed with D.I.water prior to the staining.

2.12.2 Staining and destaining of the membrane

Protein samples blotted onto Problott™ membrane can be detected with conventional staining techniques such as Coomassie brilliant blue.

The Problott™ membrane was saturated with 100% methanol for a few seconds and stained with 0.1% Coomassie blue R-250 in 40% methanol, 1% acetic acid. The Problott™ membrane was removed from staining solution and destained with 50% methanol. The membrane was rinsed extensively with D.I. water. The protein bands of interest were excised and stored at -20 ^{0}C until N-terminal amino acid sequencing.

2.12.3 N-terminal amino acid sequencing

Amino acid sequencing was performed by the method described below (Applied Biosystems company, 1993). Automated cycles of Edman degradation were performed by gas phase protein sequencer (Applied Biosystems model 476A) and Phenylthiohydantion (PTH) amino acid derivatives were automatically identified by data analysis apparatus (Applied Biosystems model 610A). The membrane sample from electroblotting was dried by a SC100A Speedvac Plus and placed into the blott cartridge. A zitex seal was placed between the two blocks and the cartridge assembly in the cartridge holder. The resulting PTH amino acids were analyzed on an Applied Biosystems PTH-C18 reversed phase cartridge column (220x2.1 mm).

2.13 Oligonucleotide Design and Synthesis

The oligonucleotides were designed by decoding the N-terminal amino acid sequence of CGTase from *Bacillus sp.*A11 and also comparing with the published nucleotide sequences of CGTase gene from other microorganisms. The synthetic oligonucleotides were 19-23 bp in length and had no potential annealing within and between the molecules of oligonucleotides. The oligonucleotides, used as probes for the detection of CGTase gene from *Bacillus sp.*A11, were synthesized commercially from Bio-synthesis, USA.

2.14 Labeling of Synthetic Oligonucleotide Probe with [γ-^{32}P]

One hundred pmol of the oligonucleotide was end-labelled with 5 µl of [γ-^{32}P]-ATP (50 µCi), 5 unit of T4 polynucleotide kinase, 5 µl of exchange buffer (250mM imidazole-HCl, pH 6.4, 60mM $MgCl_2$, 350µM ADP, 5mM 2-mercaptoethanol) in 25 µl reaction. The reaction mixture was incubated at 37 ^{0}C for 30 minutes. Then the reaction was stopped by incubating at 60 ^{0}C for 10 minutes and chilled on ice before the oligonucleotide was purified.

2.15 Purification of Labeled Synthetic Oligonucleotide Probes

The 5' end labelled oligonucleotide probe was purified by NAP-10 columns. After equilibrating the NAP-10 column with 10 mM sodium phosphate buffer, pH 6.8, the reaction mixtures was loaded into the column and eluted with 1.5 ml of 10mM sodium phosphate buffer, pH 6.8. Fractions of 0.5 ml were collected and the radioactivity was measured by using geiger counter. Fractions with high radioactivity were pooled and kept at -20 ^{0}C until to be used.

2.16 Extraction and Purification of Chromosomal DNA from *Bacillus sp.*A11(modified from Rodriguez and Tsit, 1983)

*Bacillus sp.*A11 was grown on LB broth (100 ml) at 37 ^{0}C with shaking at 250 rpm for 24 hours. Cells were harvested by centrifugation at 5000xg for 10 minutes at 4 ^{0}C and washed once with SET buffer

(20% sucrose, 50mM Tris-Cl, and 50mM Na_2EDTA ,pH 7.6). The packed cells were frozen at -70 ^{0}C for 10 minutes, thawed at 65 ^{0}C before resuspending in 2 ml of SET buffer and put on ice. 200µl of 10mg/ml lysozyme and 100ul of 10mg/ml RNase (10mg/ml RNAse in 0.1M sodium acetate and 0.3mM Na_2EDTA) was added to the cells and incubated at 37 ^{0}C for 30 minutes. Then 50 µl of 25% SDS was added and mixed by inversion. The mixture was incubated at 37 ^{0}C with gently shaking for 3-6 hours. Then 300 µl of 2 mg/ml proteinase K and 1.5 ml of chloroform/isoamylalcohol solution (24:1) were added.The mixture was further incubated at 37^{0}C with gently shaking for 15 hours. Then 1 ml of sterile distilled water and 10 ml of chloroform/isoamylalcohol solution (24:1) were added into the mixture. After mixing by inversion for 5 minutes, the mixture was centrifuged at 4000xg for 20 minutes. The upper aqueous phase was removed and reextracted twice with chloroform/isoamylalcohol solution (24:1). The DNA in upper aqueous phase was precipitated by adding 200 µl of 5M NaCl and 2 volumes of absolute alcohol. After gently inversion, the content was placed at -20^{0}C for 10 minutes. Fibrous strands of DNA were spooled out with a glass rod and dipped in 70% alcohol to remove excess salt. The DNA was allowed to air-dry, redissolved in 2 ml TEN buffer (10mM Tris-HCl, 1mM Na_2EDTA , and 10mM NaCl, pH 7.6) plus 100µl chloroform and stored at 4^{0}C.

2.17 Large Scale Preparation of Plasmid by Rapid Alkaline Extraction (Birnboim and Doly, 1979)

Plasmid harboring cells from LB broth (100 ml)were harvested by centrifugation at 5000xg, 4^{0}C for 10 minutes and washed once with TE buffer (Tris-HCl and 50mM Na_2EDTA, pH8.0). The packed cells were resuspended in 2 ml of solution I (Lysis buffer; 25mM Tris-HCl, 10mM Na_2EDTA, 50mM glucose, and 20mg/ml lysozyme), mixed by inversion and kept on ice for 30 minutes. 4 ml of freshly prepared solution II (0.2M NaOH and 1% SDS) was added into the lysate, mixed by inversion and kept on ice for 30 minutes. The mixture was neutralized by adding 3 ml of solution III (3M sodium acetate, pH 4.8), mixed by gently inversion for several times and kept on ice for 30 minutes. After centrifugation at 5000xg for 20 minutes, the supernate was extracted twice with phenol:chloroform (1:1). Trace amount of phenol was removed by extracting the aqueous phase with 5 ml of diethylether. Excess diethylether was removed by incubating at 37 ^{0}C for an hour. Two volumes of cold absolute alcohol was added into the plasmid solution and stored at -20 ^{0}C for 3 hours. The DNA was pelleted by centrifugation at 12000xg, 4 ^{0}C for 10 minutes and then redissolved in 1ml TE buffer. 50mg/ml of RNase was added to the final concentration of 5 µg/µl and was incubated at 37 ^{0}C for 1 hour.The plasmid DNA was recovered by adding 1/10 volume of 3M sodium acetate and 2 volumes of absolute alcohol and stored at -20 ^{0}C for 3 hours. After centrifugation, the DNA pellet was redissolved in 100 µl TE buffer and quantified by measuring absorbance at 260 nm (1 A_{260}=50

μg/ml of nucleic acids). The purity of the DNA was checked by measuring the ratio of A_{260}/A_{280} which should be more than 2.0 for pure DNA.

For mini-preparation, cells were grown in 1 ml LB broth and treated as in Maxi-preparation but the amount of solution I, II, III added were 100, 200, and 300 μl, respectively.

2.18 DNA Digestion by Restriction Enzymes (Maniatis *et al.*, 1982 and Blakesley, 1983)

Conditions for the complete digestion of DNA by restriction enzymes were as described by New England Biolab. Reaction mixtures of 20μl consisted of 0.5 μg DNA, 10xbuffer (5 mM Tris-HCL, 1 mM $MgCl_2$, 10 μg/ml BSA, pH 7.5), and 5 units of enzyme, were performed as recommended by New England Biolab. Reaction mixtures were incubated at 37^{0}C for 3 hours, stopped by adding with 4 μl tracking dye solution (50% glycerol, 0.1% bromophenol blue, and 0.1% Xylene cyanol FF) and analysed by agarose gel electrophoresis.

2.19 Agarose Gel Electrophoresis

To measure the sizes and amounts of DNA in samples using 0.7-1.5% agarose gel in TBE buffer (98mM Tris-HCl, 89mM Boric acid, and 2.5mM EDTA, pH 8.3), DNA samples with 4 μl tracking dye were loaded into the wells. The gels were run at 100 volts for 2 hours, or until bromophenol blue tracking dye reached the bottom of the gel. After electrophoresis, the gels were stained with ethidium bromide solution

(2.5μg/ml in water) for 5-10 minutes and the DNA bands were visualized under UV light from UV transluminater (UVP). The gels were photographed through a red filter onto Kodak Tri-X pan 400 film. The concentrations and molecular weight of DNA samples were determined by comparing with the intensity and relative mobility of the standard DNA fragments (λ/*Hin*dIII).

2.20 DNA cloning

Six reactions of 0.5 μg of DNA from *Bacillus sp.*A11 were partially digested with 10 units of *Sau*3AI and incubated at 37^{0}C for 10, 20, 30, 60, 120, and 180 minutes, respectively. Reaction mixtures were stopped by adding tracking dye. Digested DNAs were subjected to electrophoresis in 0.7% agarose gel at 100 volts until bromophenol blue tracking dye reached the bottom of the gel. λ/*Hin*dIII was used as size standard. DNA fragments of 2-6 kb were recovered from the gel by electroelution. The gel under the size of 2 kb was removed by cutting into a well and dialysis tubing was inserted under the well. The gel was rerun at 100 volts until 2-6 kb DNA fragments migrated into the well. Solution in the well was removed to a microcentrifuge tube, and 1/10 volume of 3M sodium acetate was added. The solution was extracted with phenol:chloroform (1:1) and diethylether. Two volumes of cold absolute alcohol was added and stored at -20 ^{0}C for 3 hours. The DNA was pelleted by centrifugation at 12000xg, 4 ^{0}C for 10 minutes and then redissolved in TE buffer.

2.21 Ligation

The pUC18 digested with *Bam*HI and dephosphorylated by bacterial alkaline phosphatase was used as vector. The DNA ligation mixture contained 50 ng of vector, 100 ng of the digested inserted DNA, 1x ligation buffer (10mM Tris-HCL, pH 7.5, 50mM NaCl, 10mM $MgCl_2$), 1 µl of ATP (10mM), 1 µl DTT (50mM), and 1-7 unit of T4 polynucleotide ligase in a total volume of 20 µl. All ingredients except T4 polynucleotide ligase were mixed in a 0.5 ml microcentrifuge tube and heated at 65^{0}C for 15 minutes. The tube was left until it was cooled to room temperature and T4 polynucleotide ligase was added.The reaction was incubated at 12-15^{0}C for 20 hours (Sambrook et al., 1989).

2.22 Transformation

A portion of the ligation mixture was transformed into host cell *E. coli* strain DH5α. Competent cells were prepared as described by Mandel and Higa, (1970) which used $CaCl_2$ to change the cell membrane permeability. DH5α was grown to log phase in 10 ml LB broth at 37^{0}C (2-3 hours) and kept on ice for 10 minutes. After centrifugation at 4000xg for 10 minutes, cells were washed once with cold 0.01M $CaCl_2$ and finally resuspended in 0.6-0.7 ml of cold 0.1M $CaCl_2$. The cell suspension was kept on ice for an hour before used in transformation.

30 µl of competent cells were aliquoted individually into prechilled tubes. 1 µl of ligation mixture was added in each tube. The mixtures were incubated on ice 30 minutes. Cells were then heat shocked at 42^{0}C for 45

seconds and chilled on ice immediately. 1 ml of LB medium was added and incubated with shaking at 37 ^{0}C for an hour. The content was spreaded onto the LB agar plates containing 50 μg/ml ampicillin. Cells were grown at 37 ^{0}C overnight. The recombinant colonies were counted and transferred to new LB agar plates containing 50 μg/ml of ampicillin. After incubating at 37 ^{0}C overnight, the colonies were screened for transformants harboring CGTase gene by colony hybridization as described below.

2.23 Colony Hybridization

The selective recombinant colonies were transferred to a nylon membrane by placing the nylon membrane on the surface of agar plate in contact with the bacterial colonies until it was completely wet. Marked the membrane in two or three asymmetric locations with black drawing ink. Placed the nylon membrane with colony side up in 1 ml of 0.5M NaOH for 2-3 minutes. Blotted the nylon membrane on a dry 3MM paper and repeated this step with fresh solution. Blotted the nylon membrane on a dry 3MM paper and transfered into 1 ml of 0.5M Tris-HCl, pH 7.4. After 5 minutes, blotted dry and repeated this step with fresh solution. Transferred the nylon membrane into 1 ml of 0.5M Tris-HCl, pH7.4 containing 1.5M NaCl. After 5 minutes , blotted dry and repeated this step with fresh solution. The nylon membrane was dried at room temperature for 30-60 minutes, sandwiched the nylon membrane between 3 MM papers and baked for 1-2 hours in 80 ^{0}C oven.

Placed the membrane into a clean dry petri-dish with colony side up and added 5 ml of prehybridization solution (Appendix C). Swirled to

completely wet the filter, covered and incubated at 37 ^{0}C for 1 hour. Poured off prehybridization solution and added 4 ml of probe solution. Swirled to cover filter with probe and incubated at 23 ^{0}C for 1-3 hours. Removed probe solution (Appendix C) with a pipetman and placed filter into a 1 litre beaker with 100 ml 6xSSC (Appendix C). Swirled for 1 minute and poured off solution. Repeated with 100 ml 6xSSC, swirled for 5 minutes and then poured off. Repeated the step if gieger counter could detect radioactivity in the washes. Briefly blotted onto Whatmam 3MM then wraped filter in Saran wrap and put in film cassette. Autoradiographed (Kodak XAR film or HyperfilmMP, Amersham) for 3 hours with screen at -70 ^{0}C. If the radioactivity was high, higher temperature wash was required. Carefully removed the Saran wrap from the membrane. Preheated 100 ml of 6xSSC in a 2 litre beaker to 40 ^{0}C. Covered the beaker with Saran wrap to maintain the temperature. When temperature was reached, dropped the membrane into the beaker. Swirled for 2 minutes and removed the membrane with forceps, blotted dry, wrapped in Saran wrap and then autoradiographed. The wash could be repeated if required at 50-62 ^{0}C.

Individual positive clones which gave strong hybridization signal were picked up from the master plate using a sterile toothpick and regrew in 2 ml of LB broth containing 100 mg/ml ampicillin at 37 ^{0}C for 4-6 hours. Added 3 ml of 87% glycerol to 1 ml of cultures and kept at -20 ^{0}C as preservative stock culture. The remaining of the cultures were subjected to plasmid preparation.

2.24 Detection of CGTase Gene

2.24.1 Starch Hydrolytic activity (Iodine test)

The procedure is for the detection of starch hydrolytic enzymes such as amylase, glucosidase, xylulinase as well as CGTase. These enzymes hydrolyzed long chain starch into short chain starch, Thus resulted in a gradual decrease in the blue color of starch-I_2 complex.

The recombinant colonies from the master plate were replicated onto LB-starch agar (LB broth contained 1% soluble starch and 1.5% agar) and grown at 37 ^{0}C for 60-72 hours. Iodine solution (0.203% I_2 and 5.202% KI) was added into the plate. Positive colonies showed surrounding clear zones.

2.24.2 Phenol red Inclusion Complex Test (PICT) (modified from Park et al.,1989)

Colonies with starch hydrolytic activity were further tested by PICT. Cells were grown in 5 ml of PM medium (LB broth containing 1% soluble starch, 50 μg/ml ampicillin, 0.02% phenol red, and 0.01 methyl orange) at 37 ^{0}C for 3 days. The color of PM medium changes from red to yellow when CGTase-producing cells were grown. This is because CD produced from the action of CGTase from the cells traps phenol red into CD-phenol red complex which is colorless. The remaining color is that of methyl orange which is yellow. The yellow color also indicates the color change is not the result of acidic condition in the medium. Methyl orange changes to

red in acidic condition which counter-balances the color change of phenol red under the same condition.

2.24.3 Cyclodextrin-trichloroethylene assay (CD-TCE)

Positive clones with positive iodine test and PICT were grown in LB-starch medium at 37 ^{0}C for 72 hours. After centrifugation at 4000xg for 30 minutes, supernatants were tested for CD production by CD-TCE method.

2.24.4 Analysis of Cyclodextrins by High Performance Liquid Chromatography

The sample solutions were analyzed for cyclodextrins by HPLC using Supelco-NH_2 column (0.46x25 cm) and detected by RI detector (Rutchtorn, 1992). Prior to injection, the samples were filtered through 0.45 μm membrane filters. The eluant was a mixture of 75% acetonitrile and 25% water by volume, and the flow rate was 1 ml/minute. Cyclodextrins (α-, β-, and γ- CD) were analyzed by comparing the retention times to those of standard CDs and quantitated. Cyclodextrins formed will be expressed as % conversion from starch.

$$\% \text{ Conversion} = \frac{\text{Concentration of cyclodextrins detected (g/l)}}{\text{Concentration of starch substrate (g/l)}} \times 100$$

2.24.5 Detection of CGTase activity in recombinant colonies (Nomoto et al., 1984)

Cells were grown in LB-starch medium at 37^0C for 72 hours. After centrifugation at 4000xg for 10 minutes, supernatant, called S1 (extracellular enzyme), was collected. The cell pellet was resuspended and incubated with 2 ml of Solution I (25mM Tris-HCl, pH 8.0, 10mM EDTA, and 2μg/ml lysozyme) at 0^0C for 2-3 hours. S2 (periplasmic space enzyme) was removed by centrifugation at 4000xg for 10 minutes. The resuspended cells were called S3. S1, S2, and S3 were tested for dextrinizing activity and CD-TCE followed by HPLC.

2.24.6 Analysis of CGTase of host and recombinant cells by PAGE

S1, S2, and S3 of recombinant colonies were analyzed and compared with CGTase from host cells by non-denaturing PAGE and SDS-PAGE.

2.25 Determination of size and restriction sites of recombinant colonies

Recombinant plasmids from CGTase-producing clones were analyzed by restriction enzyme digestion.

2.26 DNA sequencing

Nucleotide sequence of the CD-producing clones were determined by Sequenase Version 2.0 DNA sequencing kit (Amersham, 1997), as described below.

An extracted plasmid DNA was denatured by adding 0.1 volume of 2M NaOH, 2 mM EDTA, and incubated for 30 minutes at 37 ^{0}C. Then the content was neutralized by adding 0.1 volume of 3 M sodium acetate (pH 4.8) and precipitated with 2 volumes of absolute ethanol, mixed well and placed at -80 ^{0}C for 15 minutes. The precipitate DNA was centrifuged at 10,000 rpm for 10 minutes. The pellet was washed with 70% ethanol and recentrifuged for 10 minutes. The pellet was redissolved in 7 µl of distilled water. 2 µl of sequenase reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), and 1 µl of primer (5 pmol/µl of primer) was added. The resuspended template can be either immediately annealed with the primer or stored at 4 ^{0}C for later use.

The resuspended template was incubated at 65 ^{0}C for 5 minutes, at 37 ^{0}C for 10 minutes or 30 minutes, placed at room temperature for at least 5 minutes, and centrifuged briefly before proceeding to the sequencing reactions.

Labelling reaction was made by mixing annealed primer-template mixture with 1 µl of DTT, 2 µl of diluted Labelling Mix [(15 µM dITP, 7.5 µM dCTP, 7.5 µM dTTP) , 0.5 µl of [α-^{35}S] dATP (>1000 Ci/mmol)], and diluted T7 DNA polymerase, respectively, before adding 3.5 µl of the reaction solution (annealed primer-template solution). The tube was incubated at room temperature for 5 minutes. Then 3.5 µl of the reaction

was transferred into each of the four pre-warmed sequencing mixes (100 μM each of dGTP, dATP, dCTP, and dTTP in 50 mM NaCl) and incubated at 37 ^{0}C for 5 minutes. 4 μl of stop solution (10 mM NaOH, 99% formamide, 0.1% bromophenol blue, and 0.1% Xylene cyanol) was added to each tube and mixed gently.

Sequencing products were separated in 8% denaturing polyacrylamide gel electrophoresis containing 7.8 M urea (Appendix E), using 1xTBE buffer (90mM Tris-HCl, 90 mM boric acid, 2 mM EDTA pH 8.3). The gel was run at 1200 volts for approximately 2.5 hours. Sequencing gel was fixed in fixing solution (10% methanol and 10% acetic acid) for at least 30 minutes before transferring onto Whatmann 3 MM paper for vacuum drying with a gel dryer for 2 hours. Dried gel was exposed to autoradiography film without intensifying screen overnight at room temperature.

The film was soaked in developer solution for a minute and washed with water for a minute. The film was then soaked in fixer solution for 3-5 minutes, rewashed with water, and dried. Nucleotide sequences were read directly from the film.