CHAPTER II

MATERIALS AND METHODS

1. Equipments

Autoclave: Model HA-30, Hirayama Manufacturing Corporation, Japan.

Autopipette: Pipetman, Gilson, France.

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

Centrifuge: Microcentrifuge High Speed: Model 1110 Mikro 22R, Hettich Zentrifugen, Germany.

Incubator: Model OB-28L, Fisher Scientific Inc., U.S.A.

Magnetic stirrer and heater: Model IKAMA®GRH, Janke & Kunkel Gmbh & Co.KG, Japan.

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

Spectrophotometer: Jenway 6400, England.

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

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

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

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™8000 genetic analysis, Beckman Coulter Inc., U.S.A.

2. Chemicals

Acetonitrile (HPLC grade), Lab Scan, Ireland.

α-, β- and γ-cyclodextrins, Sigma, U.S.A.

Soluble starch, Sigma, U.S.A.

Tris-base, USB, U.S.A.

Boric acid, Merck, Germany.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Sodium dodecyl sulfate, Sigma, U.S.A.

Sodium hydroxide, Carlo Erba, Italy.

Hydrochloric acid, Lab Scan, Ireland.

Sodium chloride, Univar, Australia.

Glacial acetic acid, BDH, England.

Glucose, Sigma, U.S.A.

Calcium chloride, Merck, Germany.

Sodium citrate, Carlo Erba, Italy.

Di-Sodium hydrogenphosphate, Fluka, Switzerland.

Sodium dihydrogen orthophosphate, Carlo Erba, Italy.

Phenol, BDH, England.

Chloroform, Sigma, U.S.A.

Sodium carbonate, BDH, England.

Agarose, SEAKEM LE Agarose, FMC Bioproducts, U.S.A.

Bromophenol blue, Merck, Germany.

Glycerol, Scharlau, Spain.

Xylene cyanol FF, Sigma, U.S.A.

Absolute alcohol, Sigma, U.S.A.

Polyethylene glycol 6000, Fluka, Switzerland.

QIAprep Spin Miniprep Kit, Qiagen, Germany.

Qiaquick Gel Extraction Kit, Qiagen, Germany.

Iodine, Baker chemical, U.S.A.

Potassium iodide, Mallinckrodt, U.S.A.

Ethidium bromide, Sigma, U.S.A.

Ampicillin, Biobasic Inc., Thailand.

DNA marker, Lamda (λ) DNA digested with *Hin*dIII: Biobasic Inc., Thailand.

Bovine serum albumin (BSA), Sigma, U.S.A.

Yeast extract, Scharlau, Spain.

Tryptone, Merck, Germany.

Agar, Merck, Germany.

Methanol, Scharlau, Spain.

3. Bacterial strains

Escherichia coli JM109 (*F', traD36, proA*$^{+}$, *proB*$^{+}$, *lacI*^{q}, *lacZΔM15/recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,Δ(lac-proAB, mcrA*) was used for DNA manipulation.

Escherichia coli BMH 71-18 *mut*S (*F', proA*$^{+}$, *proB*$^{+}$, *lacI*^{q}, *lacZΔM15/mut*S:*Tn*10 *thi, supE, Δ(lac-proAB)* was used for mutagenesis.

4. Plasmid vector

A plasmid pVR328, a pUC119 derivative, containing the β-CGTase gene from *B. circulans* A11 (organism from Pongsawasdi and Yagisawa, 1987), was used for mutagenesis and constructing recombinant plasmids that have mutation regions.

5. Enzymes

5.1 Restriction endonucleases

- *Eco*RV, *Nde*I, *Nsi*I, *Sph*I, *Hin*dIII, *Sac*II, *Sma*I, *Sca*I were purchased from New England Biolabs Inc., U.S.A.
- *Nru*I was purchased from Promega, Co. Ltd., U.S.A.
- *Kpn*I was purchased from SibEnzyme, Russia.

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

5.3 Glucoamylase was purchased from Fluka, Switzerland.

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

6. Media preparation

6.1 Luria-Bertani broth (LB medium)

LB broth consists of 1% Bactotryptone, 0.5% yeast extract and 0.5% NaCl, supplemented with 100 μg/ml ampicillin when needed.

6.2 LB-starch agar

LB-starch agar consists of 1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar and 1% soluble starch, supplemented with 100 μg/ml ampicillin when needed. LB-starch agar was used for the detection of starch degadating activity of CGTase.

7. General techniques in genetic engineering

7.1 Preparation of competent cells

A single colony of *E. coli* JM109 or JC8679 was cultured as a starter in 5 ml of LB-broth and incubated at 37°C with 250 rpm shaking for 24 hours. The starter was diluted in 500 ml of LB-broth, and the culture was incubated at 37°C with 250 rpm shaking until the optical density at 600 nm of the culture reached 0.5-0.6 (~3-4 hours).

The culture was chilled on ice for 15 minutes and the cells were harvested by centrifugation at 8,000 × g for 15 minutes at 4°C. The supernatant was removed. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile water, respectively. The cells were resuspended and centrifuged at 8,000 × g for 15 minutes at 4°C. The supernatant was discarded. The pellet was washed with 10 ml of ice cold sterile 10% (v/v) glycerol, and finally resuspended in a final volume of 1-2 ml of ice-cold sterile 10% glycerol. The cell suspension was divided into 40 µl aliquots and store at -80°C until used.

7.2 Electroporation

The competent cells were thawed on ice. Fourty microlitres of the cell suspension were mixed with 1-2 µl of the ligation mixture or mutagenesis mixture, mixed well and placed on ice for 1 minute. The mixture was electroporated in a cold 0.2 cm cuvette with the apparatus setting as 2.5 µF, 200 Ω of the pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were resuspended in 1 ml of LB broth and incubated at 37°C for 1 hour with shaking at 250 rpm. One hundred microlitres of the suspension were spread on the LB-starch agar.

7.3 **Plasmid preparation** (Birnboim and Doly, 1979)

Plasmid harboring cells were cultured in LB broth (1.5 ml) and harvested by centrifugation at 8,000 × g, 4°C for 1 minute. The packed cells were resuspended in 100 µl of Solution I (25 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA and 50 mM glucose), mixed by vortexing well. Then 200 µl of Solution II (1% SDS, 0.2 N NaOH) was added, mixed by inversion and kept on ice for 5 minutes. The mixture was neutralized by adding 150 µl of Solution III (3 M sodium acetate, pH 5.2), mixed by inversion and kept on ice for 10 minutes. After centrifugation at 10,000 × g for 10 minutes, 10 mg/ml of RNase A was added to the supernatant to give a final concentration of 10 µg/ml and incubated at 37°C for 20 minutes. The supernatant was

extracted with one volume of phenol:chloroform (1:1). Two volumes of absolute alcohol were added, mixed and stored at –20°C for 30 minutes. The plasmid was pelleted by centrifugation at 10,000 × g, washed with 70% ethanol and vacuum dried for 10 minutes. The pellet was dissolved in TE buffer.

For DNA sequencing, QIAprep Spin Miniprep Kit was used for preparing plasmid DNA and performed according to the kit protocol. Briefly, plasmid-harboring cells were cultured in LB broth (1.5 ml) and harvested by centrifugation at 8,000 × g, 4°C for 1 minute. The pelletted bacterial cells were resuspended in 250 µl Buffer P1 by vortexing until the cell clumps were not visible. Added 250 µl Buffer P2 and gently inverted the tube 4-6 times, the mixture should become viscous and slightly clear. Then added 350 µl Buffer N3, the solution became cloudy, gently inverted the tube 4-6 times and centrifuged for 10 minute at 10,000 × g. The supernatant was applied to the QIAprep Spin column and centrifuged for 1 minute. The flow-though was discarded. Buffer PB was added and centrifuged for 1 minute. The column was washed twice with Buffer PE and centrifuged for 1 minute. Finally, the elution buffer was added to the center of the QIAprep spin column to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

7.4 Agarose gel electrophoresis

To measure the size of DNA using 1% agarose gel in TBE buffer (98 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA, pH 8.2), DNA samples with 1× tracking dye were loaded into the wells. The gels were run at 100 volts for 1 hour or until bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were stained with ethidium bromide solution (2.5 µg/ml) for 2-5 minutes, and the DNA bands were visualized under UV light from the UV transilluminator. The pictures of gels were recorded by using a CCD camera. The sizes of DNA fragments were determined by comparing the relative mobilities with those of standard DNA fragments (λ/*Hin*dIII marker).

7.5 Extraction of the DNA fragment from the agarose gel

The QIAquick gel extraction kit was used for extracting DNA fragment from the agarose gel, and performed according to the kit protocol. Briefly, a gel piece containing the DNA fragment was excised from an agarose gel, added 3 volume of buffer QG and incubated for 10 minutes at 50°C. After the gel slice had dissolved completely, the sample was applied to the QIAquick column, and centrifuged for 1

minute. The flow-through was discarded. Buffer QG was added and centrifuged for 1 minute. The column was washed twice with buffer PE and centrifuged for 1 minute. Finally, the elution buffer was added to the center of the QIAquick membrane to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

7.6 Preparation of the single stranded plasmid

Single-straned plasmid was prepared from pVR328, a pUC119 plasmid carrying the β-CGTase gene from *B. circulans* A11, by using helper phage M13KO7. A single colony of *E. coli* JM109, containing pVR328, was inoculated in LB broth containing ampicillin 50 μg/ml and incubated with shaking at 37°C for 2-3 hours. Then, the culture was added the helper phage at 10^7-10^8 pfu/ml and grown at 37°C for 1-2 hours with vigorous shaking. The culture was added kanamycin to 70 μg/ml to select for infected cells and further grown at 37°C for 12-18 hours with vigorous shaking. The 1.5 ml of the cell culture was centrifuged for 5 minutes at 10,000 × g in a microcentrifuge. The supernatant was removed to a fresh tube. The supernatant was added 150 μl of a solution containing 20% PEG6000 and 2.5 M NaCl, mixed and incubated to allow the phage particles to precipitate on ice for 30 minutes. The mixture was centrifuged for 15 minutes at 10,000 × g to collect the phage precipitate. The pellet was resuspended in 90 μl of TE buffer and 10 μl of 3 M NaOAc. The supernatant was extracted with one volume phenol:chloroform (1:1). Two volumes of absolute alcohol were added, mixed and stored at –20°C for 30 minutes. The single stranded plasmid was pelleted by centrifugation at 10,000 × g, washed with 70% ethanol and vacuum dried for 10 minutes. The pellet was dissolved in TE buffer.

7.7 Phosphorylation of the oligonucleotides

Three mutagenic oligonucleotides, purchased from Biobasic Inc., Thailand, were phosphorylated before use in the mutagenesis procedure. Approximately 100-200 pmol of a oligonucleotide was mixed with 10 U of kinase,1 μl of 10X kinase buffer and 1 μl of 10 mM of ATP and incubated at 37°C for 1 hour. Then, the reaction mixture was heated at 70°C for 15 minutes to stop the reaction. Then the reaction was spun for 1 minute and stored at -20°C.

8. Mutagenesis of β-CGTase gene in pVR328 by using USE procedure

Three mutagenic primers were designed according to the amino acid regions in β-CGTase that are different from those of γ-CGTase. The nucleotide sequences of the

three primers are shown in Figure 17. For the screening of the mutants, primers 1, 2 and 3 were also designed to create *Sma*I, *Eco*RV and *Nru*I sites at the mutation sites, respectively.

PRIMER 1 (46mer)

*Sma*I

5′ GCCATGATAAGCCGTGAAGCCTCCCGGGTGCACGCTGTAGATATTC 3′

PRIMER 2 (40mer)

*Eco*RV

5′ CAAGCGGCCGTTCTCGATATCGACCGGAGATGTATGGTTC 3′

PRIMER 3 (36mer)

*Nru*I

5′ GATGTCCGCGCCAGTTGTCGCGAAGTAAACCGTTCC 3′

Figure 17. Oligonucleotides used in the USE procedure. The restriction recognition sites are shaded.

As summarized in Fig. 18, the USE mutagenesis procedure involved the use of single stranded plasmid containing the target gene and mutagenic primers. Approximately 50 ng of the kinased mutagenic primers 1, 2 and 3 along with 50 ng of the kinased reference mutagenic *Sca*I primer that in the process eliminates the *Sca*I site in ampicillin resistant gene were annealed to 40 ng of the single-stranded plasmids in 10 µl reaction containing annealing buffer (200 mM of Tris-HCl, 100 mM of $MgCl_2$ and 500 mM NaCl) at 37°C for 15 minutes. After cooled to room temperature, the mixture was placed on ice. Then, 6 ul of an enzyme mixture containing with T7 polymerase in the presence of dNTPs and ligated with T4 ligase in the present of ATP were added and incubated at 37°C for 1-2 hours. Upon transformation into an *E. coli* strain BMH71-18 *mut*S, a mixture of plasmids was generated by culturing the transformation mixture in the presence of 50 µg/ml ampicillin overnight, pelleting the cells and preparing the plasmids. The plasmid mixture was digested with *Sca*I and transformed into an *E. coli* strain JM109 to separate individual clones. Colonies were cultured for plasmid miniprep and screened for the plasmids with added restriction sites by using restriction enzyme digestion.

The DNA sequences around the mutation sites were sequenced and subcloned into the same location in the original plasmid (pVR328) to avoid possible other mutated sequenced in the mutated plasmids.

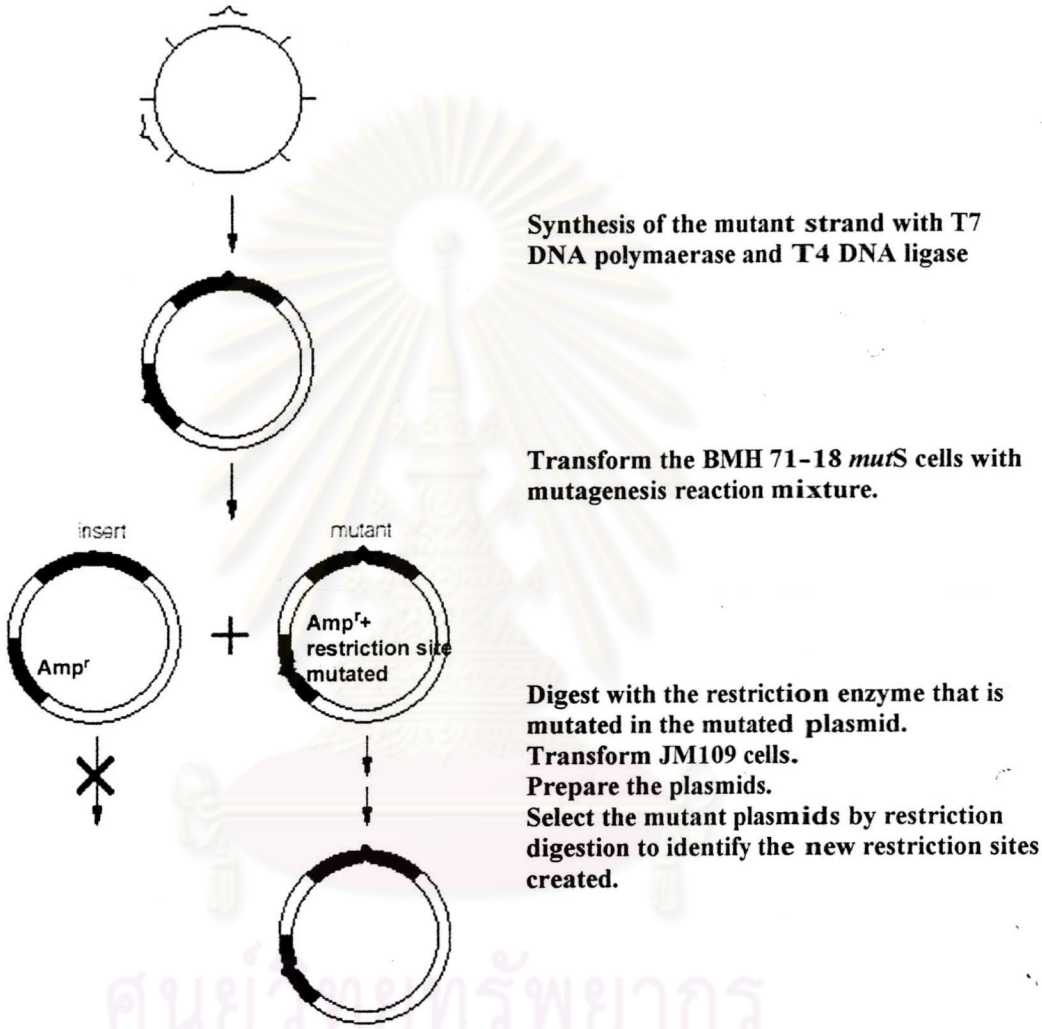

Figure 18. General schematic representation of USE mutagenesis protocol.

9. Sequencing of mutant plasmids

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 of the mutant sites as well as the nearby DNA sequences, according to the CEQ2000 Dye Terminator Cycle Sequencing with Quick Start Kit protocol. Briefly, 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 held at 4°C. The DNA was cleaned up by ethanol precipitation by adding 4 µl stop solution (1.5 M NaOAc + 50 mM EDTA) and 1 µl of 2 mg/ml glycogen to each reaction tube, transferring the sequencing reaction to the microfuge tube and adding 60 µl cold 95% ethanol and centrifuged at 12,000 x g for 20 minutes. The pellet was rinsed with 200 µl 70% ethanol and centrifuge at 12,000 x g for 5 minutes for 2 times. 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 plate was loaded into the CEQ sequencer, and the sample was sequenced by starting the desired instrumental method.

10. **Construction of recombinant plasmids containing various combination of the mutant sites**

The mutated sites in the mutated plasmids were subcloned into the same sites in pVR328. The plasmids were digested with appropriate restriction enzymes to remove DNA fragments containing the mutant sites. The DNA fragments were gel purified using agarose gel electrophoresis and eluted with Qiaquick gel extraction kit. The eluted fragments were ligated to the pVR328 at the same restriction sites that flank the mutant sites. The ligation reactions were incubated for 16-24 hours at 16°C. The ligation products were transformed into the *E. coli* JM109 and plated on the LB agar containing 100 µg/ml of ampicillin at 37°C for an overnight. Each transformant was grown on LB-broth for plasmid preparation. The recombinant plasmids were first screened for the presence of new restriction enzymes. The cloning sites were also checked by restriction enzyme digestion. The recombinant plasmids were constructed such that they contained all possible combinations of the three mutant sites.

11. Detection of mutant CGTase activity

11.1 Dextrinizing activity

11.1.1 Halo zone on LB-starch agar

E. coli JM109 cells, containing a CGTase plasmid, were plated on a LB-starch agar plate and incubated at 37°C for 24 hours. The halo formation was observed after iodine solution (0.02% I_2 in 0.27% KI) was sprayed.

11.1.2 Dextrinizing activity assay (Fuwa, 1954)

CGTase sample (100 µl) was incubated with 0.3 ml of 1% soluble starch in 0.2 M phosphate buffer, pH 6.0 at 37°C for 10 minutes. The reaction was stopped by adding 4 ml of 0.2 N HCl. Then 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 its absorbance at 600 nm was measured.

One unit of enzyme is defined as 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.

11.2 CD forming activity

E. coli JM109 cells, containing the CGTase plasmid, were grown at 37°C for 16 hours in LB broth and pelleted. Supernatant (200 µl) was incubated with 0.5 ml of 1% soluble starch at 37°C for 8 and 12 hours. After boiled, the mixture was treated for an overnight with about 30 U of glucoamylase to digest the remaining starch and oligomers formed from CGTase. After stopping the glucoamylase reaction by boiling for 10 minutes, the reaction was clarified by centrifugation. The cyclodextrin samples were filtered through the 0.45 µ membrane filters. The samples were injected into the HPLC sherisorb-NH_2 column (0.46 × 25 mm) and detected by RI detector. The eluent was 70% (v/v) acetonitrile in water and the flow rate was 1 ml/minute. The α-, β- and γ-cyclodextrins were identified by comparing the retention times to those of the standard mixture of α-, β- and γ-cyclodextrins (20 mg/ml each). For quantitive analysis, peak area corresponding to each cyclodextrin was used to calculate the cyclodextrin product ratios.

12. Protein determination (Lowry *et al.*, 1951)

The protein sample was mixed with 2.5 ml of solution C (Appendix A), and stood at room temperature for 5-10 minutes. Then 0.25 ml of solution D (Folin-Ciocalteu phenol reagent) was added and mixed. After 20-30 minutes, the wavelength of 750 nm was measured. The protein concentration was calculated from a standard curve of bovine serum albumin.