CHAPTER II

MATERIALS AND METHODS

2.1 Equipment

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.

Centrifuge, microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan

Electrophoresis unit: 2050MIDGET, LKB, Sweden and miniprotein, Bio-Rad, U.S.A.; Submarine agarose gel electrophoresis unit

Gene Pulser: Bio-Rad, U.S.A.

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker: Model G-600, New Brunswick Scientific Co., Inc., U.S.A.

Incubator waterbath: Model M20S, Lauda, Germany

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

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

pH meter: Model PHM95, Radiometer Copenhogen, Denmark

Spectrophotometer: Spectronic 2000, Bausch & Lomb, U.S.A.

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

2.2 Chemical

Absolute ethyl alcohol: Carlo Erba Reagenti, Italy

Acetone: Mallinckrodt, U.S.A.

Acrylamide: Merck, U.S.A.

Aqua sorb: Fluka, Switzerland

Ammonium persulfate: Sigma, U.S.A.

Ampicillin: Sigma, U.S.A.

Bacto-agar: DIFCO, U.S.A.

β-mercaptoethanol: Fluka, Switzerland

Bromophenol blue: Merck, Germany

Chloroform: BDH, England

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

Dialysis tube: Sigma, U.S.A.

DNA marker, Lambda (λ) DNA digested with *Hind*III: GIBCOBRL, U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethylene glycol chitin: Sigma, U.S.A.

Ficoll type 400: Sigma, U.S.A.

Flake chitin: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycine: Sigma, U.S.A.

Magnesium sulfate 7-hydrate: BDH, England

Methanol: Merck, Germany

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

Phenol: BDH, England

Potassium ferric cyanide: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Powder chitin: Japan

QIA quick gel extraction kit: QIAGEN, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Strandard molecular weight marker protein: New England BioLabs, Inc., U.S.A.

Tris (hydroxymethyl) – aminomethane: Carlo Erba Reagenti, Italy

Tryptone: Scharlau Microbiology, European Union

Yeast extract: Scharlau Microbiology, European Union

2.3 Enzymes and Restriction Enzymes

Lysozyme: Sigma, U.S.A.

Proteinase K: GIBCOBRL, U.S.A.

Restriction enzymes; GIBCOBRL, U.S.A. and New England BioLabs, Inc., U.S.A.

RNase: Sigma, U.S.A.

T4 DNA Ligase: New England BioLabs, Inc., U.S.A.

2.4 Media and Media Preparation

2.4.1 Starter medium

Luria-Bertani (LB) medium consist of 1% trytone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2.

Terrific medium consist of 1.2% Tryptone, 2.4% yeast extract, 0.23% KH_2PO_4 and 1.25% K_2HPO_4.

2.4.2 Cultivation medium

Colloidal chitin minimum medium consists of 0.05% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4$, 0.06% KH_2PO_4, 1% K_2HPO_4, and 0.2 % colloidal chitin, pH 7.2. 2% agar was added for solid medium.

Powdered chitin minimum medium contains the same ingredients as colloidal chitin minimum medium except 0.2 % powder chitin was used instead of 0.2 % colloidal chitin.

2.4.3 Transformant screening medium

Colloidal chitin medium is used for transformant screening. It has the same ingredients as colloidal chitin minimum medium except for yeast extract, which was increased into 1 %.

2.5 Bacterial Strains

Bacillus circulans PP8 was isolated from PP Islands soil, Krabi, Thailand.

E. coli DH5α with genotype F', ϕ80d *lac*ZΔM15, (Δ*lac*ZYA-argF) U169 *end*A1, RecA1, *hsd*R17(rk^-mk^+), *deo*R, thi-1, supE44, λ^- *gyr*A96, *rel*A1(Liss,L.R., 1987) was purchased from GIBCOBRL, U.S.A.

E. coli JM109, with genotype *F′* [traD36, proAB, laclqZM15], λ^-, endA1, gyrA96, hsdR17($r_{K-}m_{K+}$), $mcrB^+$, recA1, relA1, Δ(lac-proAB), thi, supE44 (Dower, 1990) was purchased from GIBCOBRL, U. S.A.

E. coli XL1-blue, with genotype recA1, relA1, endA1, gyrA96, thi-1, hsdR17, supE44, lac[*F'*, proAB, lacI^{q}ZΔM15Tn10(Tet^{r})] (Dower, 1990) was purchased from GIBCOBRL, U. S.A.

2.6 Bacterial Cultivation

2.6.1 Starter inoculum

A single colony was grown in LB medium at 37°C with 250 rpm shaking.

2.6.2 Enzyme Production

Starter culture was inoculated into cultivation medium at the ratio of 1:100 and cultivated at 37° C with 250 rpm shaking. Cells were separated by centrifugation at 5,000 rpm for 10 minutes 4° C. Crude enzyme was concentrated by Aqua sorb.

2.7 Screening of High Crystalline Chitin Degrading Bacteria

2.7.1 Isolation of chitinase producing bacteria

Chitinase producing bacteria were isolated from 4 soil sources in Thailand: Pattaya, Mahachai, Chachengsao, and PP Islands by using colloidal chitin minimum medium plates. All colonies with clear zone were picked to study further.

2.7.2 Screening for high crystalline degrading chitinase

All colonies with clear zone were picked into LB and colloidal chitin minimum medium for assay enzyme activity. Crude enzymes were incubated with 2 substrates, colloidal chitin and powdered chitin. After assay activity the ratio of activity with powder chitin (PC) and activity with colloidal chitin (CC), % PC/CC was

calculated to determine the ability of chitinase for high crystalline chitin degrading ability.

2.7.3 Calculation of % PC/CC

Crude enzymes were seperately incubated with colloidal chitin and powdered chitin for 1 and 3 hours respectively. The Relative activity (%PC/CC) was calculated from the activity when powdered chitin was used as substrate divided with activity when colloidal chitin was used as substrate and multiplied with 100.

2.8 Enzyme Assay

Chitinase activity was determined by measuring reducing sugar method. The principle of thismethod is measuring the increasing of the reducing sugar product by a color reagent. Enzyme samples were incubated with 1mg/ml colloidal chitin or 10 mg/ml powdered chitin final concentration, in total volume of 1.5 ml for 1 and 3 hours respectively. Heating to 100°C for 10 minutes to stop the reaction. The remaining substrate was removed by centrifugation at 5,000 rpm for 10 minutes. Color reagent, Potassium ferric cyanide, was added and developed by heating to 100°C for 15 minutes. Then stop the reaction by chilling in ice cool water, immediately. The color change was measured by spectrophotometer at 420 nm. The reducing sugar was detemined using a standard curve of GlcNAc. The enzyme activity was determined from the absorbance difference between a blank and the reaction mixture, where substrate incubated with heat-treated enzyme. One unit of enzyme activity was defined as the amout of the enzyme able to liberate 1μmol product per minute.

2.9 Enzyme Production of *Bacillus circulans* PP8

Starter *Bacillus circulans* PP8 was diluted 1:100 into 500 ml of colloidal chitin and powdered chitin minimum medium, respectively then cultivated at 37°C with 250 rpm shaking for 6 days. Crude enzymes were collected and assayed for activity with 3 different substrates; colloidal chitin, powdered chitin, and chitosan in every 12 hours.

2.10 Characterization of Crude Enzymes

Crude enzyme was collected at 12 and 24 hours for crude chitinase and 84 hours for crude chitosanase.

2.10.1 Optimum pH

Crude enzyme was incubated in a buffer of pH 3-10 and assayed for activity by measuring reducing sugar product. The buffers used were McIlvain for the pH range of pH 3-7 and Tris-HCl for the range of pH 8-10. The reaction mixture for chitinase assay consist of 300 µl of crude enzyme, 500 µl of buffer, and 1mg/ml colloidal chitin or 10 mg/ml powdered chitin, final concentration, in total volume of 1.5 ml. For chitosanase assay, 300 µl of crude enzyme was mixed with buffer and 1 mg/ml chitosan.

2.10.2 Optimum Temperature

The optimum temperature was determined in the range of 30-70°C at pH 7. The components in the reaction mixture were the same as in the experiment determining the optimum pH.

2.11 Estimating of Molecular Weight of chitinase

The molecular weight of chitinase was estimated by using activity staining on SDS-polyacrylamide gel electrophoresis using the method of Trudel and Asselin, 1995. Proteins were refolded in appropriate buffer containing tritonX-100 and stained with Calcofluor white M2R in water. After destained, activity bands were visualized under UV light.

2.12 *Bacillus circulans* PP8 Chromosomal DNA Extraction

Bacillus circulans PP8 was grown on LB at 37°C for 24 hours. After harvested cells, resuspended cells with TE buffer with 10 mg/ml lysozyme for cell lysis. The mixture was incubated at 37°C for one hour. Then, 30 µl of 10% SDS and 3 µl of 20mg/ml proteinase K was added and mixed well for cell wall lysis. One hundred microlitres of 5M NaCl was added for DNA precipitation. After that, equal volumn of phenol-chloroform-isoamyl alcohol, 25-24-1, was added and mixed for DNA extraction. The mixture was centrifuged at 10,000xg for 10 minutes and removed the upper aqueous to a new test tube. This procedure was repeated twice. Then, the mixture was added with 0.6 volumn of isopropanol. After gentle inversions, fibrous strands of DNA were spooled out and dipped in 1 ml of 70% ethanol to remove excess salt. The DNA was allowed to air-dry and resuspended in 50 µl of TE buffer.

2.13 DNA Digestion by Restriction Enzymes

The chromosomal DNA of *Bacillus circulans* PP8 was partially digested with

*Pst*I for 10 and 15 minutes under the condition recommended by GIBCOBRL and New England BioLabs.

2.14 Preparation of Plasmid by Rapid Alkaline Extraction

E. coli with pBluescript II KS ⁻ was grown in terrific broth with 100 μg/ml ampicillin at 37 °C overnight. Cells were harvested by centrifugation at 5000xg for 10 minutes. Preparation of plasmid was accomplished by alkaline lysis with 10% SDS, minipreparation method (Sambrook and Russell, 2000).

2.15 Competent Cell Preparation

E. coli strain DH5α was used as host cell and prepared for electro competent cells using standard method (Sambrook and Russell, 2000).

2.16 DNA Cloning

Genomic DNA of *Bacillus circulans* PP8 was partially hydrolysed with *Pst I*. DNA fragments of 2-9 kb in size were harvested, ligated with pBluescript SK⁻ and cloned into *E.coli* DH5α by shot gun method.

2.17 Transformation

Recombinant DNA was transformed into host cells by electrotransformation. The gene pulser apparatus was set at the follow settings, capacitance 25 μF, voltage to 2.5

KV, and resistance to 200 Ω. After transformation, LB medium was added immediately to the cuvette to resuspend cells. Cell suspension was transferred to sterile tube and incubated at 37 °C, 250 rpm shaking for 1 hour. Next, the cell suspension was spreaded on LB plate containing 100 μg/ml of ampicillin, 20 μl of 20 μg/ml X-gal, and 25 μl of 25 μg/ml IPTG and incubated at 37 °C overnight.

2.18 Screening of Transformant Cells

Phenotype screening was used. After transformation, white colonies on LB medium were transferred to colloidal chitin medium. Colonies, which show visible clear zone on colloidal chitin medium, indicating that they might have DNA fragments containing chitinase gene, were picked.

2.19 Determination of Recombinant Clones

White colonies, which had clear zone on colloidal chitin medium, were grown in terrific broth at 37 °C with 250 rpm shaking overnight. After harvested cell, recombinant plasmid was extracted by alkaline lysis with 10%SDS. These recombinant plasmids were retransformed to other hosts to confirm that visible clear zone is the phenotype conferred by the recombinant plasmid, and cut with *Pst*I to determine sizes of insert fragments.

2.20 Detection of Chitinase Activity of Recombinants by SDS-PAGE

The two recombinant clones were cultured on colloidal chitin medium for 10

days. Crude enzyme was added with 1 fold of acetone for protein precipitation. Then, added sample loading buffer and boiled for 10 minutes. After run SDS-PAGE, protein was refolded by adding Triton-X 100. Gel was stained with Calcofluor M2R for 15 minutes. After destained with distilled water, chitinase activities bands were visualized under UV light.

2.21 Detection of Chitinase Activity of Recombinant Clones by Colorimetric Method

Recombinant clones were cultured on colloidal chitin medium at 37 °C with shaking for a week. Crude enzymes were collected for chitinase assay with two substrates, powdered chitin and colloidal chitin by using colorimetric method as described above.