CHAPTER II

MATERIALS AND METHOD

2.1 Equipments

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

Autopipette: Pipetteman, Gilson, France

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

Centrifuge, refrigerated microcentrifuge: Model Kubota 1300, Kubota Corporation, Japan

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

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

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

GeneAmp PCR System 2400: PERKIN-ELMER, U.S.A.

HPLC: Model water 600 system

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker: Model G-76, New Brunswick Scientific Co., Inc., U.S.A.

Incubator, water bath: Model M20S, Lauda, Germany

Lyophilizer Flexi-Dry: Stone Ridge, New York, U.S.A.

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

Magnetic stirrer and heater: Model IKAMA GRH, JANKE&KUNKEL GMBH&CO.KG, Japan

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

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

RapID CB Plus: Remel, U.S.A.

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

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

Water bath: Buchi 451, Switzerland

2.2 Chemicals

Absolute ethanol: Scharlau Chemie, Barcelona

Acetone: Mallinckrodt, U.S.A.

Acetonitrile: Sigma, U.S.A.

Acrylamide: Merck, U.S.A.

Agarose: GIBCOBRL, U.S.A.

Aqua sorb: Fluka, Switzerland

Ammonium acetate: AJAX, Australia

Ammonium persulfate: Sigma, U.S.A.

Ammonium sulfate: Merck, U.S.A.

Ampicillin: Sigma, U.S.A.

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

β-mercaptoethanol: Fluka, Switzerland

Bovine serum albumin: Sigma, U.S.A.

Bromphenol blue: Merck, U.S.A.

Chloroform: BDH, England

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

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

Dialysis tube: Sigma, U.S.A.

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

DNA marker (λ DNA/*Hind*III Fragments): GIBCOBRL, U.S.A.

DNA marker (100bp marker): GIBCOBRL, U.S.A.

Ethidium bromide: Sigma, U.S.A.

Flake chitin: Sigma, U.S.A.

Fluorescent brightener 28: Sigms, U.S.A

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: BDH, England

Glycine: Sigma, U.S.A.

Hydrochloric acid: Merck, U.S.A.

Isopropyl-1-thio-β-D-galactopyranoside (IPTG): Sigma, U.S.A.

Low molecular weight calibration kit for SDS Electrophoresis: Amersham Pharmacia, Germany

Magnesium sulphate-7-hydrate: BDH, England

Methanol: Merck, Germany

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

Phenol: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIAquick gel extraction kit: QIAGEN, Germany

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate (SDS): Sigma, U.S.A.

Sodium hydroxide: Merck, Germany

Tris: Carlo Erba Reagenti, Italy

Triton X-100: Merck, Germany

Tryptic digest of casein (Tryptone): Scharlau Chemie, Barcelona

Yeast Extract: Scharlau Chemie, Barcelona

2.3 Enzymes and Restriction enzymes

Calf Intestile Alkaline Dephosphorylase (CIAP): New England BioLabs, Inc., U.S.A.

*Eco*RI: GIBCOURL, U.S.A.

*Hind*III: GIBCOURL, U.S.A.

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

*Pst*I: GIBCOURL, U.S.A.

RNase: Sigma, U.S.A.

Taq DNA polymerase: Pacific science, France

T_4 DNA Ligase: GIBCOURL, U.S.A.

2.4 Bacterial strain

2.4.1 Chitinase producing bacteria

Chitin sources, shells of prawns, were used for screening bacteria produced chitinase, at first shells of prawns were kept in fabric bag and buried in the ground for one week. After that, chitin sources were brought into sterile distilled water and mixed. The supernatants were diluted in 10-fold serial dilutions and spreaded on 0.02% CCMM and incubated at 30 °C. Bacteria were screened for chitinase.

2.4.2 Host cells

E. coli DH5α with genotype *F'*, *ϕ*80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k-m_{k+}), *pho*A, λ-*thi*-1, *sup*E44, λ$^-$ *gyr*A96, *rel*A1 (Liss, L.R., 1997) was purchased from GIBCOBRL, U.S.A.

E. coli JM109 with genotype *F'*, [*tra*D36, *pro*AB, *lac*lqZM15] λ$^-$ *end*A1, *gyr*A96, *hsd*R17(r_k-m_{k+}), *mcr*B$^+$, *rec*A1, *rel*A1, Δ(*lac-pro*AB), *sup*E44 (Dower, 1990) was purchased from GIBCOBRL, U.S.A.

E. coli XL-1 blue with genotype *rec*A1, *rel*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *lac*[*F'*, *pro*AB, *lac*lqZΔM15Tn10(Tet^r)] (Dower, 1990) was purchased from GIBCOBRL, U.S.A.

2.5 Identification of selected strain

2.5.1 Gram-stain and colonial morphology

Gram staining of bacterial cells was carried out using Collins and Patricia methods [45]. Bacteria, grown on different medium, including rich medium; LB agar plate for 1 day and 3 days, and minimum medium; colloidal chitin agar plate for 1 day and 3 days were used.

2.5.2 Biochemical characterization

Biochemical tests were used RapID CB Plus for irregular, gram positive, and non-spore forming bacteria (Figure 11). The IDS RapID CB Plus system includes 18 substrates in RapID CB Plus panels for identification of over 40 organisms.

2.5.3 Identification by 16S rRNA gene

For PCR amplification, universal conserved bacterial 16S rRNA gene primers pA and pH, that have sequences; pA (5-AGAGTTTGATCCTGGCTC AG-3) and pH (5-AAGGAGGTGATCCAGCCGCA-3), were used to amplify the ~1.5 kb 16S rRNA gene fragment (Edwards et al. 1989). The reaction contained 1 μg of PCR template, 10 pmol of each primer, 0.2 mM of deoxynucleoside triphosphate (dNTPs), 2 mM of $MgCl_2$ 2 U of *Taq* DNA polymerase, and 10 μl of 10X*Taq* DNA polymerase buffer (FINENZYMES); reaction volumes were made up to 100 μl with autoclaved ultrapure water. Temperature cycling comprised predenaturation of chromosomal DNA at 95 °C for 5 minutes before amplified, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 2 minutes, and extension at 72 °C for 3 minutes, and then a final extension at 72 °C for 5 minutes (Figure 12).

The PCR products were ligated with pGEM-T easy vector (Promega) and transformed into host cell, *E. coli* JM109, for amplified the PCR products. The PCR products and plasmid contained PCR products were digested simultaneously with restriction enzymes *Eco*RI, and then were analyzed by 1% agarose gel electrophoresis in Tris-acetate-EDTA buffer. After electrophoresis, the gel was visualized on an UV transilluminator. The PCR products were cleaned using a QIAquick spin column (Qiagen) according to the manufacturer's instructions. Purified PCR product was ligated with pGEM-T easy vector (Promega) and analyzed with primers pA (5-AGAGTTTGATCCT GGCTCAG-3), pD (5-CAGCAGCCGCGGTAATAC-3), pF (5-AAGGAGGT GATCCAGCCGCA-3), and pH' (5-TGCGGCTGGATCACCTCCTT-3). The 16S rRNA gene sequences were compared with known sequences in the GenBank using Standard nucleotide-nucleotide BLAST to identify the most similar sequence alignment.

2.6 Media preparation

2.6.1 Luria-Bertani (LB) medium

LB consisted of 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl. The pH medium was adjusted to pH 7.5 with NaOH. For solid medium, 2% agar was added. Medium was steriled by autoclaving at 121°C for 15 minutes.

2.6.2 Colloidal chitin minimum medium (CCMM) and Flake chitin minimum medium (FCMM)

CCMM contained 0.02% colloidal chitin, 0.05% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4 7H_2O$, 0.6% KH_2PO_4, and 1% K_2HPO_4. FCMM contained 10% flaked chitin, 0.05% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4 7H_2O$, 0.6% KH_2PO_4, and 1% K_2HPO_4. For solid medium, 2% agar was added. Medium was steriled by autoclaving at 121°C for 15 minutes.

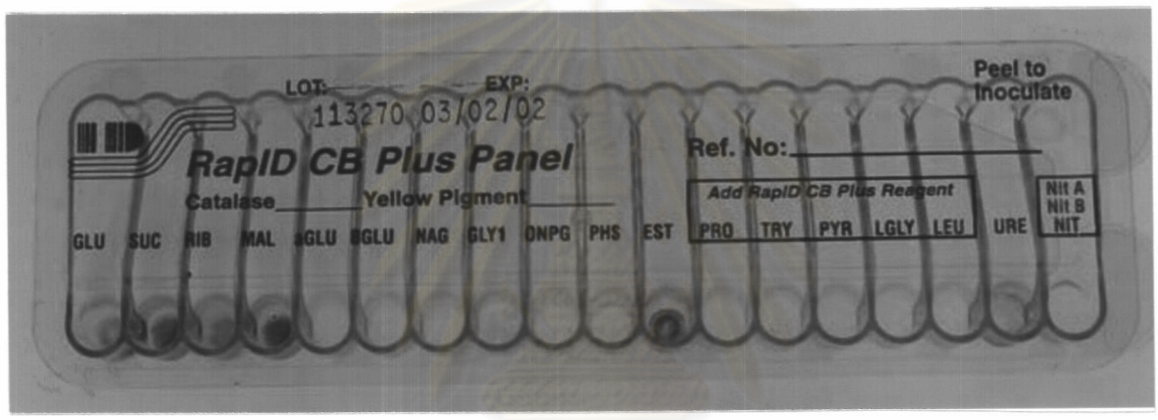

Figure 11 RapID CB Plus panels for identification irregular, gram positive, and non-spore forming bacteria .

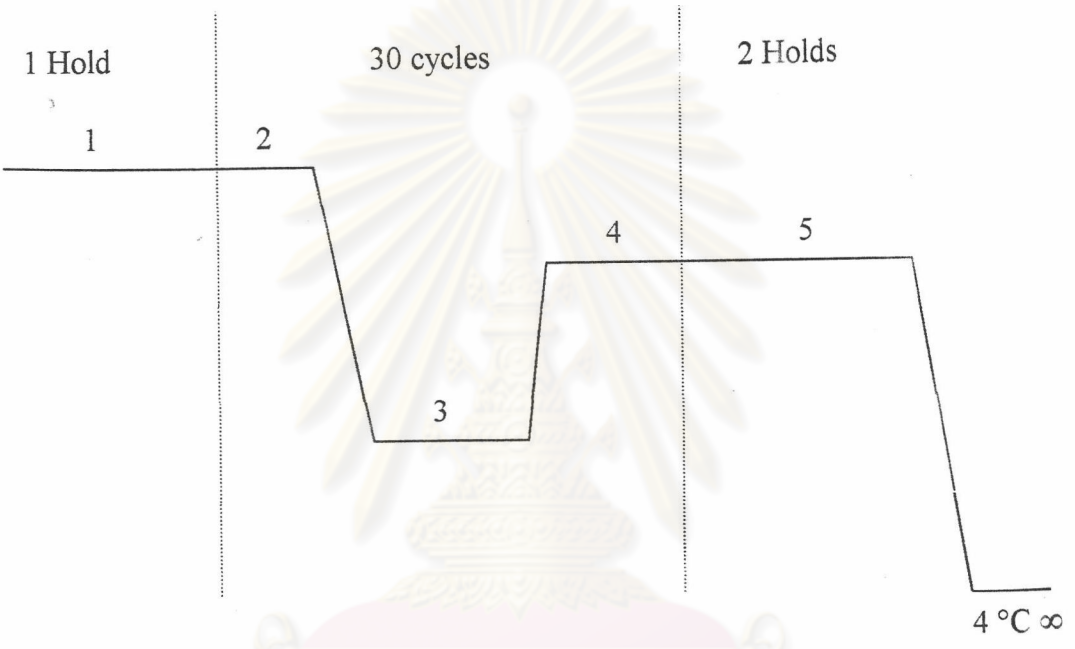

Figure 12 Diagram for 16S rRNA PCR amplification

1: predenaturation, 95 °C, 5 minutes
2: denaturation, 95 °C, 1 minute
3: annealing, 55 °C, 2 minutes
4: extension, 72 °C, 3 minutes
5: final extension, 72 °C, 5 minutes

2.7 Cultivation of *Microbacterium* sp. TU05 and enzyme production

2.7.1 Starter inoculum

Microbacterium sp. TU05 was isolated from soil in Thailand. On colloidal chitin minimum medium (CCMM) agar plate, a single colony that formed a large clear zone was selected and picked into LB broth, 2 ml. *Microbacterium* sp. TU05 was grown at 30 °C with 250 rpm shaking for 12 to 16 hours.

2.7.2 Enzyme production

For maximum production of chitinase, the starter of *Microbacterium* sp. TU05 was grown as discribed as above, then 1 ml culture was inoculated into 100 ml of 1% CCMM in an Erlenmeyer flask (250 ml). The cultured was grown for 2 days at 30 °C with 250 rpm rotary shaking. The culture broth was centrifuged for 20 min, 4 °C at 5,000xg and the supernatant was kept at 4 °C for characterization.

When concentrated crude enzyme was used, Crude chitinase was concentrated by lyophillization. At first, protein pellets were resuspended in 10 mM phosphate buffer (pH 7.0) with minimum volume, and then dialyzed with the same buffer at 4 °C. Concentrated crude chitinase was kept at 4 °C.

2.8 Assay of chitinase activity

In this study, chitinase activity was determined by colorimetric method, which assayed for reducing sugar that was produced in the chitinolytic reaction.

Chitinase activities were assayed by measuring reducing sugar produced in the reaction mixture by the modified Schales method [48]. The reaction mixture contained 0.1% colloidal chitin in 0.1M buffer and 0.4 ml

of crude enzyme in 1.5 ml total volume. The reaction was incubated at 37 °C for 1 hour and then stopped by boiling at 100 °C. The small particles were removed from the reaction mixture by centrifugation at 3,500xg for 15 minutes, the supernatant was mixed with ferric cyanide 2.0 ml and heated in boiling water for 15 minutes. When the reaction mixture cooled down to room temperature, reducing sugar was measured at A_{420} (A1). A blank value (A0) of each reaction used denatured enzyme instead of the enzyme in reaction. The different between A0 and A1 was used to estimate the amount of *N*-acetylglucosamine that was produced by chitinase in each reaction from a standard curve. One unit (U) of enzyme activity was defined as the amount of an enzyme able to liberate 1 μmol product (as *N*-acetylglucosamine equivalent) per minute.

2.9 Protein Determination

Protein in enzyme solution was determined by dye binding method [49], using bovine serum albumin as standard.

In the reaction, 800 μl of sample was mixed with 200 μl of Bradford working solution (5x) and incubated for 20 minutes before measuring the absorbance at wavelength 595 nm. In 200 ml of Bradford working solution (5x) contain 100 mg Coomasie Brilliant Blue G-250, 50 ml of 95% Ethanol, 100 ml of 85% phosphoric acid, and 50 ml of distilled water.

2.10 Chitinase production of *Microbacterium* sp. TU05

Productions of chitinase in different chitin sources were detected, the activity of chitinase was checked in two different culture supernatants, including 1% colloidal chitin minimum medium (CCMM) and 10% flaked chitin minimum medium (FCMM), both of them were shaken at 30 °C, chitinase activities were checked everyday for 10 days.

2.11 Characterization of crude chitinase

2.11.1 Optimum pH

The chitinase activity was measured at various pH from 2 to 10 using colloidal chitin as a substrate. The enzyme was incubated in 0.1M Sodium citrate buffer at pH 2.0 to 6.0, 0.1M Sodium phosphate buffer pH 6.0 to 8.0, and 0.1M Tris-HCl pH 8.0 to 10.0. Chitinase activity was assayed by colorimetric method.

2.11.2 Optimum temperature

The reaction mixture was incubated in 0.1M Citrate buffer pH 5.0 at different temperature from 20 °C to 70 °C for 1 hour. Chitinase activity was assayed by colorimetric method.

2.11.3 Substrate specificity

Chitinase activity was assayed on chitin-related compounds, including regenerated chitin, colloidal chitin, 45% deacetylated chitosan, 80% deacetylated chitosan, 90% deacetylated chitosan, powder chitin, and flake chitin. All experiments were done under the same condition. In each reaction, 10 mg/ml of substrate was incubated with 0.1 U of chitinase in 0.1M citrate buffer, pH 5.0 at 40 °C for 30 minutes. The reactions were stopped by heated in boiling water for 15 minutes. Chitinase activity on chitin-related substrates was measured by colorimetric method.

2.11.4 Estimation of chitinase molecular weight

The molecular weight of chitinase was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done by the

method of Trudel and Asselin [38] using 10% separating gel with 0.01% (W/V) glycol chitin. Sample solution of crude chitinases were denatured by heating at 100 °C with 15% (W/V) sucrose, 2% (V/V) β-mercaptoethanol, and 25% (W/V) SDS in 125mM Tris-HCl (pH 6.7), which contained 0.1% (W/V) Bromphenol blue as the tracking dye, for 10 minutes. After electrophoresis, proteins were stained with 0.25% Commassie Brilliant Blue R-250 at room temperature for 1 hour, and then destaining with a mixture of 10% (v/v) acetic acid and 25% methanol.

For activity staining, after electrophoresis, the gel was incubated in 0.1M Phosphate buffer pH 7.0 and 1% (V/V) Triton x-100 at 37 °C for 10 hours. The gel was transferred into 0.01% (W/V) fluorescent reagent, Calcofluor white M2R, in 0.5M Tris-HCl (pH 8.9) for 5 min, then the gel was washed with distilled water. Chitinase activity in the gel was detected under UV light.

2.11.5 Hydrolysis products of colloidal chitin

Chitinase from *Microbacterium* sp. TU05 was used to digest colloidal chitin. The reaction mixture was 0.1g/ml of colloidal chitin in 0.1M citrate buffer (pH 5.0) and 0.20 unit crude chitinase. In this experiment, the reaction mixture was incubated at 40 °C for 24 hours. After incubation, the reaction was boiled and centrifuged at 3,500xg for 10 minutes. The product, in supernatant phase, was diluted 10 folds and then 300 µl of diluted product was withdrawn and mixed with 700 µl of acetonitrile before centrifuged at 3,500xg for 10 minutes in a bench-top centrifuge. The mixture was filtered through 0.45 micron filter. Then the product was separated by HPLC. The condition used was; Shodex Ashipak NH2P-50 AE column, mobile phase was solution mixture of acetonitrile and water with ratio 70 acetonitrile: 30 water, flow rate 1.0ml/minute, and temperature 25 °C.

2.12 Partial purification by DEAE-column

2.12.1 Activate DEAE-cellulose

DEAE-celllulose, Diethylaminoethyl cellulose, 10 g was swelled in 1 liter of distilled water and then washed with distrilled water at room temperature to removed fine particles. The resin was activated before used. First, 100 ml of 0.5M HCl was added to the resin and stirred for 30 minutes followed by washes with distilled water to neutral pH. Then, the resin was washed with 100 ml of 0.5N NaOH and stirred for 30 minutes, followed by water until neutral pH was attained. The resin was stored in water at 4 °C.

2.12.2 DEAE-cellulose chromatography

The resin was swelled in 0.5M Tris-HCl buffer pH7.0 overnight. Then the resin was packed into a column, diameter and height were 1.8 and 20 cm, respectively. The column was equilibrated with 0.01M Tris-HCl buffer pH 7.0, and then concentrated crude chitinase was loaded onto DEAE-cellulose column at flow rate 1 ml/minute. The unbound protein was washed with equilibrated buffer until A_{280} was flow 0.05. Bound protein was eluted by 0-1M NaCl gradient elution. Fractions of 5 ml were collected for measurement of protein concentration at A_{280} and chitinase activity.

2.13 Characterization of partial purified chitinase

2.13.1 Optimum pH

The chitinase activity was measured at various pHs from 2 to 10 using colloidal chitin as a substrate. The enzyme was incubated in 0.1M Sodium citrate buffer from pH 2.0 to 6.0, 0.1 M Sodium phosphate buffer from pH 6.0

to 8.0, and 0.1 M Tris-HCl pH 8.0 to 10.0. Chitinase activity was assayed by colorimetric method.

2.13.2 Optimum temperature

The reaction mixture was incubated in 0.1M Citrate buffer pH 5.0 at different temperature from 20 °C to 70 °C for 1 hour. Chitinase activity was assayed by colorimetric method.

2.13.3 Substrate specificity

Chitinase activity was assayed on chitin-related compounds as assayed in crude enzyme. All experiments were done under the same condition. In each reaction, 10 mg/ml of substrate was incubated with 0.1 U of chitinase in 0.1M citrate buffer, pH 5.0 at 40 °C for 30 minutes. The reaction was stopped by heated in boiling water for 15 minutes. Chitinase activity on chitin-related substrates was measured by colorimetric method.

2.14 Extraction of chromosomal DNA from *Microbacterium* sp. TU05

Microbacterium sp. TU05 was grown on LB broth, 2 ml, at 37 °C with 250 rpm rotation shaking for 12 to 16 hours. The culture was centrifuged at 5000xg for 5 minutes, supernate was removed and bacterial cell pellet was resuspended in 400 µl of SET buffer and 100 µl of 20 mg/ml lysozyme in SET buffer was added. The reaction mixture was incubated at 37 °C for 1 hour. After that, 5 µl of 10% SDS and 3 µl of 20 mg/ml proteinaseK were added and mixed thoroughly before incubated at 50 °C for 3 hours. After incubation, 50 µl of 3 M Sodium acetate was added and mixed. The mixture was added with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mixed by inversion, then centrifuged at 12,000xg for 10 minutes. The upper liquid

phase was transferred to a fresh tube. Chromosomal DNA was precipitated by adding 0.6 volume of isopropanol and gently mixed, fibrous strands of Chromosomal DNA was found, then spooled out and washed with 1 ml of 70% ethanol to remove excess salt. The last step, DNA pellet was dried with open air and dissolved in TE buffer contains 20 μg/ml RNase A. DNA solution was incubated at 37 °C for 3 hours and stored at 4 °C.

2.15 Preparation of plasmid DNA by rapid alkaline extraction

E. coli containing plasmid DNA was grown on LB broth with 50μg/ml ampicillin 2 ml at 37 °C with 250 rpm rotation shaking for 16 to 18 hours. The culture was centrifuged at 5000xg for 5 minutes and the supernatant was removed. The bacterial cell pellet was resuspended in 100 μl of ice-cold solution I (50mM Glucose, 25mM Tris-HCl, and 10mM EDTA, pH 8.0) containing 5 mg/ml lysozyme. The reaction mixture was stored on ice for 5 minutes, and then 200 μl of solution II (1% SDS and 0.2N NaOH) was added and mixed followed by stored on ice for 10 minutes. The next step, 150 μl of solution III (3M Potassium, 5M Acetate) was added to the reaction mixture and mixed thoroughly. The mixture was left on ice for 30 minutes before centrifuged at 12,000xg for 10 minutes. The supernatant was transferred to a fresh tube and added with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mixed, then centrifuged at 12,000xg for 10 minutes. The upper liquid phase was transferred to a fresh tube and added with 2 volume of cold absolute ethanol and mixed. The reaction mixture was kept at -20 °C for 30 minutes, after that, plasmid DNA was recovered by centrifugation at 12,000xg for 10 minutes. The supernatant was removed and plasmid DNA pellet was washed with 70% ethanol. The plasmid DNA pellet was air dried and dissolved in TE buffer contains 20 μg/ml RNase A. Plasmid DNA solution was incubated at 37 °C for 3 hours and stored at -20 °C.

2.16 Agarose gel electrophoresis

DNA was detected on agarose gel with various % agarose depended on sizes of DNA samples, the gel was run at 100 volts. After electrophoresis, the gel was stained with ethidium bromide solution (10 μg/ml in distilled water) for 15 minutes then destained in distill water, DNA samples were detected under UV light from UV transluminater (UVP).

2.17 DNA cloning

2.17.1 Partial digestion of chromosomal DNA

The chromosomal DNA from *Microbacterium* sp. TU05 was partial digested by 0.5 Unit of *Pst* I (GIBCOBRL) for 5, 10, 15, and 20 minutes at 37 ° C. After partial digestion, DNA fragment products at various time point were subjected to electrophoresis in 1 % agarose gel at 50 volts. Lambda phage DNA cut with *Hind* III, λ/*Hind* III, was used as standard molecular weight marker. DNA fragments, range of 2 to 9 kb, were harvested by QIAquick Gel Extraction Kit (QIAGEN).

2.17.2 Plasmid preparation and dephosphorylated by CIAP

E. coli, containing plasmid, pBluescriptII SK$^-$, was grown on 5 ml of LB broth with 0.1 mg/ml ampicillin at 37 °C with 250 rpm rotation shaking for 16 to 18 hours. Cells were centrifuged at 5,000xg for 5 minutes and plasmid DNA was extracted as previously described. Plasmids were completely digested with *Pst*I (plasmids/*Pst*I), and the reaction mixtures were incubated at 37 °C for 3 hours. After incubation, 0.1 volume of 3M sodium acetate was added and extracted with phenol: chloroform: isoamyl alcohol (25:24:1) there centrifuged at 12,000xg for 10 minutes. Then, plasmids/*Pst*I were recovered as previously described. For dephosphorylated by alkaline phosphatase, CIAP, 90 μl of

plasmids/*Pst*I were added with CIAP 1 unit and incubated at 37 °C for 30 minutes, after that, the reaction mixture was added with another 1 unit of CIAP and incubated for another 30 minutes. Then, the reaction mixture was subjected to electrophoresis in 1.5 % agarose gel at 50 volts. Lambda phage DNA cut with *Hind* III, λ/*Hind* III, was used as standard. The linear form of Plasmids/*Pst*I were harvested by QIAquick Gel Extraction Kit (QIAGEN). Plasmids/*Pst*I treated with CIAP (pBluescriptII SK⁻/*Pst*I/CIAP) were stored at -20 °C and used as a vector for DNA cloning.

2.17.3 Ligation

The pBluescriptII SK⁻/*Pst*I/CIAP was used as a cloning vector. The ligation mixture contained approximately 200 ng of vector, 200 ng of 2 to 9 kb DNA fragments from *Microbacterium* sp. TU05, 1x ligation buffer, and 3 units of T4 DNA Ligase (GIBCOURL) in a total volume of 20 µl. The reaction mixture was incubated at 20 °C for 16 to 18 hours.

2.17.4 Preparation of competent cells

Competent cells, *E. coli* JM109, were prepared by the method of Sambrook *et al.* A single colony was inoculated in 15 ml of LB broth and cultivated at 37 °C with shaking at 250 rpm for overnight. The starter was inoculated into 1,000 ml of LB broth with ratio 1:1000, and the culture was incubated at 37 °C with 250 rpm rotary shaking for 2 - 3 hours until the optical density at 600 or 660 nm (OD_{600} or OD_{660}) of the culture was 0.5-0.6 or 0.4-0.5 respectively. After that, the culture was chilled on ice for 15-30 minutes and the cells were harvested by centrifugation at 8,000 rpm for 15 minutes at 4 °C. The supernatant was removed, and the cell pellet was washed in 2 steps, first with 1 volume of cold sterile distilled water and second with 0.5 volume of cold sterile distilled water. The cells were harvested and added with 20 ml of

ice-cold sterile 10% (V/V) glycerol in water and centrifuged again. Finally, the cells were resuspended in a final volume of 1.0-2.0 ml of ice-cold sterile 10% (V/V) glycerol in water. The cell suspension was divided into 40 µl aliquots and stored at -80 °C.

2.17.5 Transformation

A portion of the ligation products was transformed into host cell *E coli* JM109 by electrotransformation [46]. Shotgun cloning of plasmid vector, contained 2 to 9 kb DNA fragments, was accomplished by using Gene Pulser and controller setting at 25 µF capacitor, 2.5 kV, and 200 Ω. After transformation, the host cells were incubated at 37 °C for 1 hour in 1 ml of LB medium. The host cells were spreaded on LB agar plate, contained 0.1 mg/ml ampicillin, 20 µl of 20 µg/ml iso-1-thio-β-D-galactopyranoside (IPTG), and 20 µl of 20 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) per plate. Cells were grown at 37 °C for overnight. Transformants with recombinant plasmids, white colonies, were screened for plasmid harboring chitinase gene by phenotype screening.

2.17.6 Detection of chitinase gene

After the transformants were spreaded on LB agar plate, contained 0.1mg/ml ampicillin, 40µg/ml IPTG, and 40µg/ml X-gal per plate. Cells were grown at 37 °C for overnight. Then, white colonies were selected and transferred to replicate plates, one is LB agar plate contained 0.1mg/ml ampicillin, 40µg/ml IPTG, and 40µg/ml X-gal and the other is colloidal chitin minimum medium (CCMM) agar plate contained 0.1mg/ml ampicillin, 40µg/ml IPTG, 0.02% colloidal chitin, 0.25% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4 7H_2O$, 0.6% KH_2PO_4, and 1% K_2HPO_4. The LB agar plates were incubated at 37 °C overnight, and then stored at 4 °C. The CCMM agar

plates were incubated at 37 °C for 1 to 14 days. The transformants carrying chitinase gene were detected by formation of clear zone around the colony on CCMM agar plate.

2.17.7 Partial sequence of chitinase gene by PCR

Degenerated primers designed from conserved amino acid sequence of *Bacillus* sp. family 18 chitinase gene, including forward primer, primer BP-I (32 mers 5-AAYTAYGCDTTYGCDGAYATHTGYTGGRANGG-3) and primer BP-II (29 mers 5-TTYGAYGGNGTNGAYYTNGAYTGGGARTA-3), and reverse primer, primer BP-V (30 mers 5-RTAYTCCCARTCNARRTCN ACNCCRTCRAA-3) and primer BP-VI (33mers 5-CCANCCNCCRTTRAAR TCRTANGTCATGATRTT-3), were used for partial chitinase gene amplification. The reaction contained 50 ng of DNA template, 10 pmol of each primer, 200μM of deoxynucleoside triphosphate (dNTPs), 1.5mM of $MgCl_2$, 1 U of *Taq* DNA polymerase, and 1X*Taq* DNA polymerase buffer (FINENZYMES); reaction volumes were made up to 20 μl with autoclaved ultrapure water. Temperature cycling was described in Figure 13 comprised 30 cycles of denaturation at 95 °C for 5 minutes and annealing at 55 °C for 3 minutes followed by extension at 72 °C for 3 minutes (Figure 13). The PCR products were analyzed by 1.5% agarose gel electrophoresis in Tris-acetate-EDTA buffer, then visualized on an UV transilluminator.

The PCR product from primers BP-I and primer BP-VI was ligated with pGEM-T easy vector (Promega) and transform into host cell, JM109. The PCR product and plasmid contained PCR product were digested simultaneously with restriction enzymes *Eco*RI, and then were analyzed by 1.5% agarose gel electrophoresis in Tris-acetate-EDTA buffer, then visualized on an UV transilluminator. The PCR product was cleaned using a QIAquick spin column (Qiagen) according to the manufacturer's instructions. Purified PCR products were ligated with pGEM-T easy vector (Promega) and 16S rRNA gene sequence was analyzed. The partial chitinase gene sequences were compared

with known sequences in the GenBank using Standard nucleotide-nucleotide BLAST to identify the most similar sequence alignment.

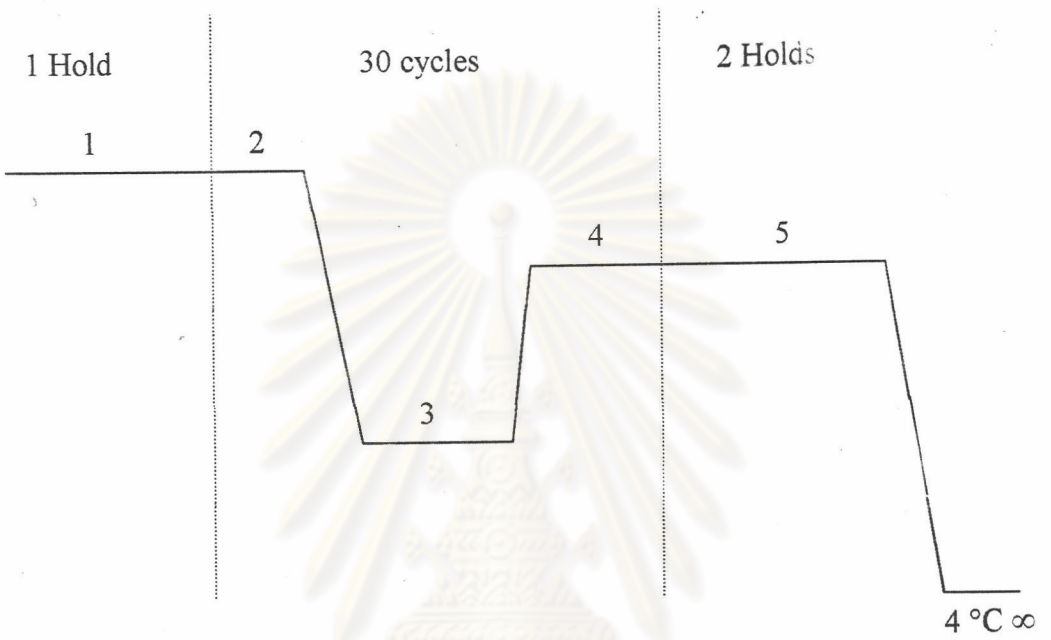

Figure 13 Diagram for partial chitinase gene PCR amplification

1: predenaturation, 95 °C, 5 minutes
2: denaturation, 95 °C, 1 minute
3: annealing, 55 °C, 3 minutes
4: extension, 72 °C, 3 minutes
5: final extension, 72 °C, 5 minutes