CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan
Autopipette: Pipetman, Gilson, France
Autoradiography: Kodak X-Omat™ film
Centrifuge, refrigerated centrifuge: Model J2-21, Beckman Instrument Inc., U.S.A.
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
Film Cassette: sized 8x11 inches, Okamoto, Japan
Gene Pulser™: Bio-Rad, U.S.A.
Gieger counter: Model 900 mini-monitor, Mini-instruments, Ltd.,England
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
Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.
Membrane filter: cellulose nitrate, pore size 0.45μm, Whatman, Japan
pH meter: Model PHM95, Radiometer Copenhegen, Denmark
Spectrophotometer: Spectronic2000, Bausch&Lomb, U.S.A.
Spectrophotometer UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.
Thermolyne dri-bath: Sybron corporation, U.S.A.
Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.
Water bath

2.2 Chemicals

Acetone: Mallinckrodt, U.S.A.
Acrylamide: Merck, U.S.A.
Agarose: GIBCOBRL, U.S.A.
Agua sorb: Fluka, Switzerland
[α-^{35}S]-dATP: Amersham, U.S.A.
Ammonium persulphate: Sigma, U.S.A.
Ammonium sulphate: Carlo Erba Reagenti, Italy
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, Germany
Chloroform: BDH, England
Coomasie brilliant blue R-250: Sigma, U.S.A.
[γ-^{32}P] dATP: Amersham, U.S.A.
DEAE-cellulose resin: Sigma, U.S.A.
Deoxyribonucleic acid from SALMON TESTES: Sigma, U.S.A.
Dialysis Tube: Sigma, U.S.A.
di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy
di-Sodium ethylenediaminetetra acetate: M&B, England
DNA marker: Lamda (λ) DNA digest with *Hind* III: GIBCOBRL, U.S.A.
85% Phosphoric acid: Mallinckrodt, U.S.A.
Ethidium bromide: Sigma, U.S.A.
Ethyl alcohol absolute: Carlo Erba Reagenti, Italy
Ethylene glycol chitin: Seikagaku Corporation, Japan
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.
Hexadecyl trimethyl ammonium bromide: Sigma, U.S.A.
Magnesium sulphate 7-hydrate : BDH, England
Methanol: Merck, Germany
N,N′-methylene-bis-acrylamide: Sigma, U.S.A.
NNN′N′-Tetramethyl-1,2-diaminoethane: Carlo Erba Reagenti, Italy
Phenol: BDH, England
Polyvinylpyrrolidone: Sigma, U.S.A.

Potassium ferricyanide: BDH, England
Potassium phosphate monobasic: Carlo Erba Reagenti, Italy
QIAquick Gel Extraction Kit: QIAGEN, Germany
Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy
Sodium citrate: Carlo Erba Reagenti, Italy
Sodium chloride: Carlo Erba Reagenti, Italy
Sodium dodecyl sulfate: Boehringer Mannheim Gmbtt, Germany
Standard molecular weight marker protein: New England BioLabs, Inc., U.S.A.
Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy
Tryptone: DIFCO, U.S.A.
2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide: Sigma, U.S.A.
Xylene cyanole FF: Sigma, U.S.A.
Yeast extract: DIFCO, U.S.A.

2.3 Enzymes and Restriction enzymes

DNA Polymerase I (Klenow): New England BioLabs, Inc., U.S.A.
Lysozyme: Sigma, U.S.A.
Proteinase K: Sigma, U.S.A.
Restriction enzymes: GIBCOBRL, U.S.A. and New England BioLabs, Inc., U.S.A.
RNase: Sigma, U.S.A.
T4 polynucleotide kinase: New England BioLabs, Inc., U.S.A.
T4 DNA ligase: New England BioLabs, Inc., U.S.A.

2.4 Bacteria

Burkholderia cepacia, isolated from South-East Asian soil, was screened for chitinase.

E.coli DH5α *with genotype F', Φ*80d*lacZΔM15,* Δ(*lac*ZYA-*arg*F) U169 *end*A1, RecA1, *hsdR17*(r_K.m_{K+}), *deo*R, *thi*-1, *sup*E44, λ^-*gyr*A96, *rel*A1 (Liss, L.R., 1987) was purchased from GIBCOBRL, U.S.A.

2.5 Media Preparation

2.5.1 Luria-Bertani (LB) medium

LB consisted of 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl. pH was adjusted to pH 7.2 with NaOH. For solid medium, 2% agar was added. Medium was steriled by autoclaving at 121°C for 15 minutes.

2.5.2 Cultivation medium (Colloidal chitin minimum medium)

Medium for enzyme production contained 1% colloidal chitin, 0.05% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4.7H2O$, 0.6% KH_2PO_4 and 1% K_2HPO_4 with pH 7.2. For solid medium, 2% agar was added. Medium was steriled as above.

2.6 Cultivation of Bacteria

2.6.1 Starter inoculum

A colony of *Burkholderia cepacia* was grown in 2 ml of starter medium at 30°C with 250 rpm rotation shaking about 12-16 hours.

2.6.2 Enzyme production

Starter *Burkholderia cepacia* was diluted 1:100 into 100 ml of colloidal chitin medium in 250 ml Erlenmeyer flask and cultivated at 30°C with 250 rpm rotary shaking. Cells were separated by centrifugation at 5,000 rpm for 20 minutes at 4°C. Culture broth with crude chitinase enzyme was concentrated by agua sorb and then dialyzed with 10 mM phosphate buffer (pH7.0). Concentrated crude chitinase enzyme was kept at 4°C for characterization.

2.7 Enzyme assay

For this study, chitinase activities were determined by the turbidity reduction of a colloidal chitin suspension during chitinolysis and reducing sugar-producing activity assay (modified Schale's method, Imoto, 1971)

2.7.1 Determination of chitinase activity by measuring turbidity reduction

Chitinase activities were assayed by measuring the turbidity reduction of a colloidal chitin suspension during chitinolysis. An enzyme sample was incubated at 37 °C with 6 μg/ml colloidal chitin in citrate buffer (pH 7.0). %Transmitance at 650 nm was measured by a spectrophotometer versus water at 0, 15, 30 and 60 minutes, respectively. One unit (U) of enzyme activity was defined as the increase of % transmitance at 650 nm in one minute. Specific activity was defined as units per mg of an enzyme sample.

2.7.2 Determination of chitinase activity by measuring reducing sugar

Chitinase activity was assayed by measuring reducing sugar (Imoto and Yagishita, 1971) produced from 2.0 ml mixture containing 0.2 g/ml of substrate and the desired amount of enzyme in 0.2 M citrate buffer pH 7.0. The mixture was incubated at 37 °C for 15 minutes. The reaction was stopped by boiling for 15 minutes. Small particles were removed from the mixture by centrifugation at 10,000xg for 10 minutes. An appropriate amount of supernatant was adjust to the final volume of 1.5 ml with buffer then mixed with 2.0 ml of color reagent, made by dissolving 0.5 g of potassium ferricyanide in 1 litre of 1.5 M Na_2CO_3. The mixture was heated in boiling water for 15 minutes. After cooling to room temperature, the absorbance (A1) at 420 nm was measured by a spectrophotometer versus water. A blank value (A0) was obtained when denatured enzyme was used instead of the enzyme in the reaction. The difference between A0 and A1 was used to determine the amount of N-acetylglucosamine from 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. Specific activity was defined as units per mg or ml of an enzyme sample.

2.8 Protein Determination

Protein concentration was determined by dye binding method (Bradford, 1976), using bovine serum albumin as standard.

Eight hundred microliter of sample was mixed with 200 µl of Bradford working solution (5x) and left 20 minutes before measuring the absorbance at 595 nm. Two hundred milliliter of Bradford working solution (5x) contains 100 mg Coomassie Brilliant Blue G-250, 50 ml of 95% ethanol, 100 ml of 85% phosphoric acid and 50 ml of distilled water.

2.9 Chitinase production of *Burkholderia cepacia*

Starter *Burkholderia cepacia* was diluted 1:100 into 50 ml of minimum medium containing either 0.2% colloidal chitin or 10 g chitin. The culture was cultivated at 30 °C with 250 rpm shaking for 6 days. Everyday, the culture medium from both minimum medium containing 0.2% colloidal chitin and 10 g chitin, were collected and assay for chitinase activity by determination of turbidity reduction.

2.10 Characterization of crude Chitinase

2.10.1 Optimum pH

The optimum pH of crude chitinase was measured after incubation in buffer at pH range of 2-10. The buffers used were 0.1 M sodium citrate buffer (pH from 2-7), 0.1 M phosphate buffer (pH from 6.5-7.5) and 0.1 M Tris-HCl buffer (pH from 7,8-10). The reaction mixture containing 500 µl of 0.012 mg/ml colloidal chitin, 1.2 ml of buffer and 300 µl of concentrated crude enzyme was incubated at 37 °C for 30 minutes. The enzyme activities were assayed by the turbidity reduction of a colloidal chitin suspension during chitinolysis.

2.10.2 Optimum temperature

The optimum temperature of crude chitinase was determined at pH 7.0, in the range of 10-65°C. The reaction mixture containing 500 µl of 0.012 mg/ml colloidal chitin, 1.2 ml of 0.1 M sodium citrate buffer (pH 7.0) and 300 µl of concentrated crude enzyme was incubated for 30 minutes. The enzyme activities were assayed by the turbidity reduction of a colloidal chitin suspension during chitinolysis.

2.10.3 Substrate specificity

The activity of chitinase was assayed on chitin-related compounds, including crab shells, flaked chitin, colloidal chitin, glycol chitin, 78% deacetylated chitosan and 90% deacetylated chitosan. The activity was assayed by measuring reducing sugar produced from a mixture composed of 0.2 g/ml of substrate and 0.06 U of crude enzyme in 0.2 M citrate buffer pH7.0 (2.0ml). All experiments were done under the same condition.

2.10.4 Estimation of molecular weight

The molecular weight of chitinase was estimated by SDS-polyacrylamide gel electrophoresis followed by activity staining of chitinase. SDS-PAGE was performed by the method of Trudel and Asselin (1995) using a 12.5% acrylamide gel containing 0.01% (W/V) glycol chitin. Sample solution of enzyme were denatured by heating at 100 °C for 5 minutes with sample loading buffer containing 15% (W/V) sucrose, 2.5 % (W/V) SDS in 125 mM Tris-HCl (pH 6.7), 2% (V/V) β-mercaptoethanol, and bromphenol blue (0.01% W/V). Gels were run using the Davis system. Prestained protein marker, broad range 6-175 kDa, was used. After electrophoresis, proteins were stained with 0.25% Commassie Brilliant Blue R-250 at room temperature for one hour then destained with a mixture of 10% (V/V) acetic acid and 25% (V/V) methanol. The protein band containing chitinase activity after SDS-PAGE was detected by incubating gel overnight at 37°C with reciprocal shaking in 100 mM sodium citrate buffer (pH7.0) containing 1%(V/V) Triton X-100. The gel was stained with 0.01% (W/V) Calcofluor white M2R in 500 mM Tris-HCl (pH8.9) and destained with distilled water. Lytic zones in gel were visualized under UV light.

2.11 Extraction of Chromosomal DNA from *Burkholderia cepacia* (Sambrook, Fritsch, and Miniatis, 1989)

Burkholderia cepacia was grown on LB broth (5 ml) at 37 °C with shaking at 250 rpm for 24 hours. Cells were harvested in 1.5 ml microcentrifuge tube by centrifugation at 5000xg for 5 minutes. Cell pellets were resuspended in 567 µl of TE buffer (10 mM Tris-HCl and

1 mM EDTA, pH8.0) by repeated pipetting. Then, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added and mixed well. The mixture was incubated at 37 °C for one hour. 100 µl of 5 M NaCl was added into the mixture and mixed thoroughly. After that, 80 µl of CTAB-NaCl solution was added and mixed. The mixture was further incubated at 65 °C for 10 minutes. Then, equal volume of chloroform-isoamyl (24:1) alcohol was added and mixed. The mixture was centrifuged at 10,000xg for 10 minutes. The supernatant was transferred to a fresh tube and added with equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). After mixing by inversion, the mixture was centrifuged at 10,000xg for 10 minutes. The upper aqueous phase was removed and precipitated by adding 0.6 volume of isopropanol. After gently inversion, 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 100 µl of TE buffer.

2.12 DNA Digestion by Restriction Enzymes

The chromosomal DNA from *Burkholderia cepacia* was completely digested by each restriction enzyme (*Eco*R I, *Bam*H I, *Hin*d III and *Pst* I) under the conditions recommended by GIBCOBRL and New England BioLabs.

2.13 Agarose Gel Electrophoresis

To detect the DNA fragment containing chitinase gene by Southern blot analysis, DNA samples completely digested with *Eco*R I, *Bam*H I, *Hin*d III or *Pst* I were loaded into the wells of 1% agarose gel in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA). λ/*Hin*d III was labeled in 20 µl reaction mixture containing 1 µl of [α-^{35}S]-dATP (10 mCi/ml, specific activity at reference date 1,250 Ci/mmol, Amershame, USA), 5 unit of Large Klenow Fragment of DNA Polymerase I, 2 µl of buffer (100 mM Tris-HCl (pH7.5), 50 mM $MgCl_2$ and 15 mM dithiothreitol) and used as DNA standard marker. The gel was run at 50 volts overnight. After electrophoresis, the gel was stained with ethidium bromide solution (10 µg/ml in distilled water) for 15 minutes and the DNA bands were visualized under UV light from UV

transluminator (UVP). The gel was photographed through a red filter onto Kodak Tri-X pan 400 film.

2.14 Capillary Transfer of DNA to Nylon membrane

(Sambrook *et al.*, 1989)

DNA fragments in agarose gel were transferred to nylon membrane (positively charged, Boehringer Mannheim, Germany) by capillary transfer method (Southern, 1975). The DNA fragments were denatured by soaking the gel for 45 minutes in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant and gentle agitation. After that, the gel was rinsed briefly in deionized water and neutralized by soaking for 30 minutes in several volumes of solution of 1 M Tris (pH7.4), 1.5 M NaCl at room temperature with constant and gentle agitation. Capillary transfer platform was set by placing a glass plate to form a support on a large baking dish, filled with transfer buffer (10XSSC). The wet 3MM papers were put on the support, smooth out all air bubbles with a glass rod. The nylon membrane, cut 1 mm larger than gel in both dimensions, was floated on the surface of a dish of deionized water until it wets completely from beneath, and then the membrane was immersed in transfer buffer for at least 5 minutes. The gel was removed from the neutralization solution and inverted so that gel's underside is now uppermost. The inverted gel was placed on the support so that it is centered on the wet 3MM papers. The wet nylon membrane was placed on top of the gel. Two pieces of 3MM paper were wet with 2xSSC and then placed on the top of the wet nylon membrane. The paper towels were placed on the 3 MM papers. A glass plate was put on the top of the stack and weighed the paper towel down with a 500-g weight. The DNA fragments were allowed to transfer for 8-24 hours. As the paper towels become wet, they should be replaced. After transfer process, the membrane was peeled from the gel and soaked in 6xSSC for 5 minutes at room temperature. The membrane was removed from 6xSSC and allowed excess fluid to drain away. Then, the membrane was sandwiched between two sheets of dry 3MM paper to dry for at least 30 minutes at room temperature. The DNA fragments were fixed to the membrane by UV crosslinking. The membrane was kept in dry place prior hybridization.

2.15 Labelling of degenerate probe specific for chitinase gene with [γ-^{32}P]

The oligonucleotide, used as a degenerate probe specific for chitinase gene, was designed by decoding the amino acid sequence conserved in family 18 chitinase genes, FDGLDLDWEYP. The degenerate probe, CB1, was 33 oligonucleotides in length, 5′-TTYGAYGGNCTNGAYCTNGAYTGGGARTAYCCN-3′. Ten picomol of the degenerate probe was end-labelled with 5 µl of [γ-^{32}P]-ATP (10 mCi/ml, specific activity at reference date 5,000 Ci/mmol, Amershame, USA), 10 unit of T4 DNA Polynucleotide kinase, 2 µl of buffer (100 mM Tris-HCl (pH7.5), 50 mM $MgCl_2$ and 15 mM dithiothreitol) in 20 µl reaction. The reaction mixture was incubated at 37 °C for 30 minutes. Then the reaction was stopped by incubation at 65 °C for 10 minutes.

2.16 Southern Hybridization

Nylon membrane containing the target DNA was floated on the surface of a tray of 6xSSC until it becomes thoroughly wetted from beneath. The membrane was submerged for 2 minutes and then transferred to a tray containing 0.2 ml of prehybridization solution (6xSSC, 5x Denhardt's reagent, 0.5%SDS and 100 µg/ml of denatured, fragmented salmon sperm DNA) for each square centimeter of nylon membrane. The nylon membrane was incubated in prehybridization solution at 65 °C for 2 hours. Then, [γ-^{32}P] labeled degenerate probe specific for chitinase gene was added into the prehybridization solution. The nylon membrane was incubated with [γ-^{32}P] labeled specific probe at 50°C overnight. After that, the nylon membrane was transferred to a fresh try containing 2XSSC and 0.2% SDS and incubated for 20 minutes at 50°C with gentle agitation. The membrane, covered with Saran Wrap, was exposed to Kodak X-Omat film to obtain autoradiographic image. The exposure time was determined empirically.

2.17 Preparation of Plasmid by Rapid alkaline Extraction

E. coli, containing pBluescript II KS$^+$, was grown on LB broth (5 ml) at 37 °C with shaking at 250 rpm for 24 hours. Cells were

harvested in 1.5 ml microcentrifuge tube by centrifugation at 5000xg for 5 minutes. Bacterial cell pellets were resuspended in 100 µl of GET buffer (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH8.0) containing 5 mg/ml lysozyme by repeated pipetting. The mixture was stored on ice for 10 minutes before 200 µl of solution II (1% SDS and 0.2 N NaOH) was added and mixed by inversion. The mixture was kept on ice for 10 minutes. One hundred and fifty microlitre of 7.5 M ammonium acetate, pH 4.8 was added into the mixture, mixed thoroughly and left on ice for 10 minutes. After centrifugation at 12,000xg for 10 minutes, the supernatant was extracted twice with equal volume of phenol-chloroform-isoamyl alcohol (24:24:1). The mixture was centrifuged at 12,000xg for 10 minutes. The supernatant was transferred to a fresh tube and added 2.5 volume of ice-cold absolute ethanol. After gently inversion, the plasmid was recovered by centrifugation at 12,000xg for 10 minutes. The plasmid pellet was washed with 200 µl of 70% ethanol and then resuspended in 25 µl of TE buffer containing 20 µg/ml RNase A.

2.18 DNA fragments for DNA Cloning

Chromosomal DNA from *Burkholderia cepacia*, completely digested with *Eco*R I or *Bam*H I, were subjected to electrophoresis in 1% agarose gel at 50 volts overnight. Lamda phage DNA cut with *Hin*d III (λ/*Hin*d III) was used as size standard. DNA fragments with appropriate size were harvested using QIAquick Gel Extraction Kit (QIAGEN, Germany). Briefly, the DNA fragments were excised from the agarose gel and placed in 1.5 ml microcentrifuge tube. Three volumes of Buffer QX1 were added to 1 volume of gel (100 mg~100 µl). The mixture was incubated at 50 °C until the gel slice has completely dissolved. After that, 1 volume of isopropanol was added to the sample. To bind DNA, the sample was applied to the QIAquick column and centrifuged at 12,000xg for 1 minute. Flow-through was discarded and 0.75 ml of Buffer PE was added to the column. After centrifugation at 12,000xg for 1 minute, 30 µl of TE buffer was added to elute DNA from the column.

2.19 Ligation

The pBluescript II KS^+, digested with *Eco*R I or *Bam*H I and dephosphorylated by alkaline phosphatase, was used as a cloning vector. The DNA ligation mixture contained approximately 200 ng of vector, 200 ng of DNA fragments from *Burkholderia cepacia* (2.18), 1xligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 μg/ml bovine serum albumin) and 5 unit of T4 DNA Ligase in a total volume of 20 μl. The reaction mixture was incubated at 14-16 °C for 16-18 hours.

2.20 Transformation

A portion of the ligation mixture was transformed into host cell *E.coli* strain DH5α F′. Competent cells were prepared for electro-transformation (Dower, 1988). A fresh overnight culture of *E.coli* DH5α F′ was inoculated into 1 litre of LB broth with 1 volume of overnight culture to 100 volume of LB broth. Cells were grown to log phase ($O.D_{600}$ of 0.5 to 0.8) at 37°C with vigorous shaking. To harvest, cells were kept on ice for 15 to 30 minutes, and then centrifuged at 4,000xg for 15 minutes at 4 °C. The cell pellet was resuspended in 1 litre of cold water and centrifuged as above. Then, the cell pellets were resuspended in 0.5 litre of cold water and centrifuged again. After the centrifugation, cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged as above. The cell pellets were resuspensed to a final volume of 2 to 3 ml in 10% glycerol. This suspension was frozen in aliquots and stored at –70 °C. In the electro-transformation process, cuvettes and sliding cuvette holder were chilled in ice. The Gene Pulser apparatus was set to the 25 μF capacitor, 2.5 kV, and the Pulse controller unit was set to 200 Ω. The competent cells were gently thawed on ice. One to five microlitre of DNA was mixed with 40 μl of the competent cells. This mixture was transferred to a cold, 0.2 cm electroporation cuvette, and placed at the bottom of the cuvette. The cuvette was applied one pulse at the above settings. One millilitre of LB medium was added immediately to the cuvette. The cells were quickly resuspensed with a Pasteur pipette. The cell suspension was transferred to polypropylene tube and incubated at 37°C for 1 hour with 225 rpm shaking. This suspension was spreaded onto the LB agar plates

containing 100 μg/ml ampicillin for final concentration, 20 μl of 25 mg/ml isopropylthio-β-D-galactosidase (IPTG) and 20 μl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) per plate. Cells were grown at 37 °C overnight. Bacteria carrying recombinant plasmids form white colonies which were picked and screened for transformants harboring chitinase gene by hybridization and phenotype screening described below.

2.21 Detection of Chitinase Gene

2.21.1 Hybridization screening

The selected recombinant colonies were grown on LB broth containing 100 μg/ml ampicillin at 37 °C with shaking at 250 rpm for 24 hours. Cells of each colonies were harvested by centrifugation at 5000xg for 5 minutes. The pellets were resuspended in 200 μl STET (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 5% Triton X-100) and 20 μl of lysozyme was added into the mixture. Then, the mixture was boiled in water bath for 90 seconds. After centrifugation at 12,000xg for 10 minutes, the supernatant was removed to a fresh tube and 200 μl of isopropanol was added. The mixture was centrifuged at 12,000xg for 10 minutes and then the pellets were washed with 70% ethanol. Dry pellets were resuspended in 30 μl of TE buffer containing 20 μg/ml RNase A. Plasmids were denatured by addition of 200 μl of dilution buffer (0.25 N NaOH/0.5 M NaCl) before dot onto nylon membrane. Nylon membranes containing plasmids were prehybridized at 65 °C and then hybridized with [γ-^{32}P] labeled specific DNA probe for family 18 chitinase gene, CB1, at 50 °C, 6xSSC. After that, nylon membranes were washed with 2XSSC, 0.2% SDS for 20 minutes at 50°C with gentle agitation and exposed to Kodak X-Omat film to obtain autoradiographic image. Plasmids which gave positive signal were selected for further confirmation by Southern blot hybridization. For Southern blot analysis, the selected plasmids from *Eco*R I libraries were digested with *Eco*R I and *Pst* I while plasmids from *Bam*H I libraries were digested with *Bam* H I and *Pst* I. Completely digested plasmids were subjected to electrophoresis in 1% agarose gel. λ/*Hin*d III labeled with [α-^{35}S] was used as marker. The target plasmids on agarose gel were transferred to nylon membranes and hybridized with [γ-^{32}P] labeled

specific DNA probe for chitinase gene at 50 °C, 6xSSC. The nylon membranes were washed with 2XSSC, 0.2% SDS at 50 °C and exposed to Kodak X-Omat film. Plasmids which gave autoradiographic image were further tested by phenotype screening.

2.21.2 Phenotype screening

Colonies with positive result on Southern blot analysis were grown onto LB agar plates containing 100 μg/ml ampicillin overlayed with 20 μl of 25 mg/ml IPTG and 100 μl of 10 mg/ml glycol chitin per plate. Colonies were grown at 37 °C for 3 days and then stained with Congo red according to the method of Watanabe *et al* (1990). Production of chitinase by the transformed *E. coli* cells was judged by the formation of a visible clearing zone around the colony.

2.22 Mapping of recombinant plasmid containing Chitinase Gene

Plasmid fragment containing chitinase gene was digested with restriction enzymes *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Hin*d III, *Kpn* I, *Nde* I, *Pst* I, *Sal* I, *Sma* I, *Spe* I and *Xba* I. The plasmid was also double-digested with *Bam*H I-*Eco*R I, *Bgl* II-*Eco*R I, *Sal* I-*Bam*H I, *Sal* I-*Hin*d III, *Sal* I-*Eco*R I, *Sal* I-*Cla* I, *Cla* I-*Bam*H I, *Cla* I-*Hin*d III, *Hin*d III-*Bam*H I, *Hin*d III-*Eco*R I, *Hin*d III-*Pst* I, *Hin*d III-*Nde* I, *Eco*R I-*Cla* I, *Pst* I-*Cla* I, *Pst* I-*Eco*R I, and *Pst* I-*Sal* I. All digestion products were subjected to agarose gel electrophoresis for analysis and determination of the positions of the restriction sites on the plasmid.

2.23 Detection of Chitinase activity in recombinant colonies

Transformants with positive results in Southern blot analysis or phenotype screening were cultivated into LB-colloidal chitin and LB medium at 37°C with 250 rpm rotary shaking overnight. Crude chitinase in cultured medium was assayed by modified Schale's method.

2.24 Analysis of Chitinase Gene

Nucleotides of Chitinase gene were sequenced by method of Sanger *et al.* (1977).