CHAPTER II

MATERIALS AND METHODS

Equipments

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

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated: 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 Agrarose 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 Brunswicks 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 sterrer and heater: Model IKAMA®GRH, JANKE&KUNKEL GMBH&CO.KG, Japan

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

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

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

Sonicator: Model W375, Heat systems-ultrasonics, U.S.A.

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

Spectrophotometer UV-240, Shimadzu, Japan, and DU Series 650, Beckman, U.S.A.

Thin-wall microcentrifuge tubes 0.2 mL, Axygen Hayward, U.S.A.

UV transluminator: Model 2011 Macrovue, SanGabriel California, U.S.A.

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

Water bath Buchi 461: Switzerland.

Chemicals

Acetonitrile: (Methy cyanide) Sigma, U.S.A.

Acetone: Mallinekrodit, U.S.A.

Acrylamide: Merk, U.S.A.

Agarose: GIBCOBRL, U.S.A.

Aqua sorb: Fluka, Switzerland

[α-^{35}S]-dATP: Amersham, U.S.A.

Ammonium persulphate: Sigma, U.S.A.

Ammonium sulphate: Sigma, U.S.A.

Ampicillin: Sigma, U.S.A.

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

β-mercaptoethanol: Fluka, Switerland

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

Bromphenol blue: Merck, Germany

Chloroform: BDH, England

Coomassie 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 tubing: 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 *Hin*dIII: GIBCOBRL, U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene glycol chitin: Seikaguku Corporation, Japan

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

Flaked chitin: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycine: Sigma, U.S.A.

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

Low molecular weight calibration kit for SDS electrophoresis: Amersham, U.S.A.

100 bp marker: GIBCOBRL, 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 Erbo Reagenti, Italy

Phenol: BDH, England

Polyvinylpyrrolidone: Sigma, U.S.A.

85% Phosphoric acid: Mallinckrodt, 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: Scharlau, Microbiology, England.

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 and Scharlau, Microbiology, England

Enzyme 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 enzyme: GIBCOBRL, U.S.A. and New England BioLabs, Inc., U.S.A.

RNase: Sigma, U.S.A

Taq polymerase: Pacific science, France

Vent polymerase: New England BioLabs, Inc., U.S.A

T_4DNA ligase: New England BioLabs, Inc., U.S.A

Bacterial strains

Bacillus licheniformis strain SK-1, isolated from the central of Thailand, was screened for chitinase and chitinase gene amplification.

Host cells

E. coli DH5α with genotype *F'*, *Φ*80d*lacZ*ΔM15, Δ(*lac*ZYA-*arg*F) U169 *end*A1, RecA1, *hsd*R17(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.

E.coli XL-1-Blue with genotype recA1, relA1, endA1, gyrA96, thi-1, hsdR17, supE44, lac[*F'*, proAB, lac/^{9}ZΔM15Tn10(Tet^r)] (Dower, 1990) 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.

Vectors

Plasmid pBluescriptSK(-) (Stratagene) was used as an alternative vector for cloning and subcloning of chitinase gene into *E. coli*.

pGEM-Teasy (QIAGEN) was used as an alternative vector for PCR cloning and subcloning of chitinase gene into *E. coli*.

Media preparation

Luria-Bertani (LB) medium

LB consisted of 1.0%(w/v) tryptone, 0.5%(w/v) yeast extract, and 1.0% NaCl pH was adjusted to pH 7.5 with NaOH. For solid medium, 2%(w/v) agar was added.

Colloidal chitin minimum medium, CCMM)

Colloidal chitin minimum medium was used for enzyme production. The medium containing 0.02% (w/v, dry weight) colloidal chitin, 0.05%(w/v) yeast

extract, 0.1%(w/v) $(NH_4)_2SO_4$, 0.03%(w/v) $MgSO_4.7H_2O$, 0.6%(w/v) KH_2PO_4 and 1%(w/v) K_2HPO_4 with pH 7.5. For solid medium, 2% agar was added.

All medium were sterilized by autoclaving at 121 °C for 15 min.

Identification of bacterial strain

Characteristics of *B. licheniformis* SK-1

Culture of *B. licheniformis* SK-1 was characterized in the present study employing morphology, biochemical and physiological characteristics before being used in further experiment.

Morphological characteristics

The exponential phase culture, cultivated in NA after 2-4 days of incubation at 37 °C, was examined for cell form, cell arrangement, spore forming and gram staining for SK-1 using light microscope.

Biochemical characteristics

The hydrolyze of skim milk and starch were carried out by adding 1% skim milk and starch in LB. Catalase production was determined by bubble formation with 3% H_2O_2. Oxidase test and carbohydrate fermentation test were performed as indicated in Appendix B.

Physiological characteristics

The exponential phase culture, cultivated in LB after 12-18 hrs of incubation at 50 °C was used for the starter. Physiological test for SK-1 in various pH and temperature was determined by growing the organism in LB with initial pH adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and at temperatures 30, 35, 40, 45, 50, 55 and 60 °C for 2 hrs. Optimum salt range was determined by observing grow in LB containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 percent NaCl after 2 hrs of incubation at 50 °C. For growth optimization, SK-1 was cultivated in LB in various pH, temperature and salt concentrations.

Scanning electron microscopy

SK-1 cells were observed by scanning electron microscopy (JSM-35CF). The organism, grown on colloidal chitin agar medium, was fixed with 4% paraformal-dehyde in 0.1 M phosphate buffer pH 7.2 at 25 °C for 2 hrs. After being washed with buffer, the samples were treated with 1% osmium acid in buffer then dehydrated by using series of ethanol (35, 70, 95 and 100%). After being freeze-dried, the sample was coated with gold (JFC-1100).

16S rRNA gene comparison

The partial 16S rRNA gene (1.5 kb fragment) of SK-1 was amplified by PCR with forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (8F) and reverse primer 5'-AAGGAGG TGATCCAGCCGCA (1542R). The amplified PCR product was purified by Qiagen quick Gel Extraction Kit (Qiagen, Germany) and sequences by the dideoxy-chain termination method with fluorescent primer (Edwards et al., 1989).

The 16S rRNA gene sequence was aligned with other 16S rRNA bacterial sequence obtained from GenBank by Basic Local alignment search tool (BLAST) program. (http://www.ncbi.nlm.nih.gov/BLAST)

Chitinase assay

Colloidal chitin agar plate for chitinolytic screening

Chitinase producing strains were screened by conventional plate assay using colloidal chitin as a substrate. The principal is based on the capability of chitinase in hydrolyzing insoluble and opaque chitin substrate resulting in clear zone formation around the bacterial colony.

For this study, chitinase activities were determined by the turbidity reduction of a colloidal chitin suspension during chitinolysis and colorimetric reducing sugar producing activity assay, modified Schale's method (Imoto, 1971). Chitinase activity was assayed in 1.5 ml of a reaction mixture containing 0.1% colloidal chitin in 0.1 M phosphate buffer pH 6.0 and 0.1 ml of enzyme solution.

Colorimetric method

Chitinolytic activity was measured quantitatively by detecting the amount of reducing sugar, a product of enzymatic hydrolysis, based on the Schales method.

The enzyme assay was performed as described in the following. A 100 µl of appropriate diluted enzyme solution was added to 75 µl of 2% colloidal chitin (final 1 mg/ml), 150 µl of 1 M phosphate buffer pH 6.0 (final 0.1 M) and adjust volume to 1.5 ml with distilled water. After incubation at 60 °C for 10 min, the reaction was stopped by boiling. Two milliliters of color reagent, 0.5 g of potassium ferricyanide in 1 litre of 1.5 M Na_2CO_3 was added. The mixture was heated in boiling water for 15 mins. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 5,000 g for 10 mins.

The adsorbance of the supernatant (A_1) was measured at 420 nm by a spectrophotometer versus distill water. A blank value (A_0) was obtained when denatured enzyme (heating in boiling water for 20-30 mins) was used instead of the enzyme in the reaction. The difference between A_0 and A_1 was used to determine the reducing property equivalent to amount of N-acetylglucosamine from standard curve (See Appendix D).

One unit (U) of enzyme activity was defined as the amount of an enzyme able to produce reducing property equivalent to1 µmol of N-acetylglucosamine per min. Specific activity was defined as units per mg protein of an enzyme sample.

Chitobiase or N-Acetylglucosaminidase activity was measured quantitatively by detecting the amount of p-nitrophenol, a product of enzymatic hydrolysis when p-nitrophenol-N-acetylglucosamine was used as substrate.

The enzyme assay was performed as described in the following. A 100 µl of appropriate diluted enzyme solution was added to 100 µl of 5 mM p-nitrophenol-N-acetylglucosamine, 25 µl of 1 M citrate buffer pH 5.0 (final 0.1 M) and adjust volume to 0.5 ml with distilled water. After incubation at 60 °C for 60 min, one milliliter of Na_2CO_3 was added, incubated for 5 min at 37°C then measured at 420 nm. The standard curve for p-nitrophenol was showed (See Appendix D).

One unit (U) of enzyme activity was defined as the amount of an enzyme able to liberate 1 μmol product (p-nitrophenol) per min. Specific activity was defined as units per mg protein of an enzyme sample.

Cultivation of bacteria

Starter innoculum

Single colony of *Bacillus licheniformis* strain SK-1 was grown in 2 ml of starter medium LB at 50 °C with 250 rpm rotation shaking for 12-18 hours as an innoculum.

Chitinolytic production and growth curve of *B. licheniformis* SK-1.

To study the correlation between growth curve and enzyme production, the enzyme productions at different time interval of growth were determined. Pure culture of *Bacillus licheniformis* SK-1 was grown by shaking overnight in LB broth at 50 °C. The bacteria was subcultivated in 0.02% colloidal chitin minimum medium and incubated at 50 °C in orbital shaker. The samples of cell culture were randomly taken at 0, 1, 2, 3, 4 and 5 days of culturing time. Cell number at different culturing time was detected by colony forming unit (CFU/ml) and chitinolytic production was assayed by colorimetric method (mU/ml).

Optimization from enzyme production

Opimization temperature

To study the correlation between temperature and enzyme production, the enzyme productions at different time interval were determined. Pure culture of *B. licheniformis* SK-1 was grown by shaking overnight in LB broth at 50 °C. The bacteria was subcultivated in 0.02% colloidal chitin minimum medium and incubated at 30, 40 and 50 °C in orbital shaker. The samples were randomly taken at 0, 1, 2, 3, 4 and 5 days for detect chitinolytic activity.

Effect of various potential inducers

To study the correlation between various source of chitin and enzyme production, the enzyme productions at different time interval were determined. Pure culture of *B. licheniformis* SK-1 was grown by shaking overnight in LB broth at 50 ° C. The bacteria was subcultivated in 0.02% colloidal chitin minimum medium and incubated at 30, 40 and 50 °C in orbital shaker. The samples were randomly taken at 0, 1, 2, 3, 4 and 5 days for detect chitinolytic activity.

Effect of various concentration of colloidal chitin

To study the correlation between %colloidal chitin and enzyme production, the enzyme productions at different time interval were determined. Pure culture of *B. licheniformis* SK-1 was grown by shaking overnight in LB broth at 50 °C. The bacteria was subcultivated in 0.02% colloidal chitin minimum medium and incubated at 30, 40 and 50 °C in orbital shaker. The samples were randomly taken at 0, 1, 2, 3, 4 and 5 days for detect chitinolytic activity.

Detection of hydrolytic products produced by chitinolytic enzyme from *B. licheniformis* SK-1

For this experiment, in the total 1.5 ml reaction mixture consisted of 10 mg/ml colloidal chitin, 600 µl of crude chitinase, 150 µl of 1 M citrate pH 5.0 and 750 µL distilled water, then the reaction was incubated at 60 °C for 24 hrs. The digested products at the end of 24 hrs were boiled for 15 mins and centrifuged at 8,000 rpm for 10 mins. Then 300 µl of the supernatant was withdrawn and mixed with 700 µl of acetonitrile, and filtered through a 0.45 micron filter. The products were analyzed by HPLC. The condition use was; Shodex Asahipak NH2P-50 column, mobile phase 300 ml water: 700 mL acetonitrile, sample injected volume 20 µl, flow rate 1.0 ml/min at 25 °C.

Purification of chitinase

Enzyme production

The innoculum was diluted 1:100 into 200 ml of 0.1% CCMM in a 1000 ml

Erlenmeyer flask and cultivated at 50 °C with 250 rpm rotation shaking. Cells were separated by centrifugation at 10,000 rpm for 30 minutes at 4 °C. Crude chitinase in the culture broth was adsorption with colloidal chitin.

Chitin affinity adsorption

The culture medium was stirred gently with fresh colloidal chitin (10 mg/mg of protein) at 0 °C for overnight. The colloidal chitin was then washed three times with 10 mM potassium phosphate buffer (KPB, pH 6.0) and collected by centrifugation. The precipitated colloidal chitin was resuspended in KPB and incubated at 50 °C overnight to digest the colloidal chitin.

Column chromatography

DEAE-cellulose chromatography

DEAE-celllulose (10g) was swelled in 1 liter of distilled water and then washed several times at room temperature to remove the fine particles. The resin was activated by washing sequentially with excess volume of 0.5 M HCl for 30 min followed by distilled water until the pH was equal to 7.0. The activated resin was equilibrated with 25 mM Tris-HCl buffer pH 7.5 overnight. The prepared DEAE-cellulose was packed into a column 2.5 x 20 cm. The column was equilibrated with 25 mM Tris-HCl buffer pH 7.5. The enzyme from colloidal chitin adsorption was dialysed against excess volume of 25 mM Tris-HCl at 4 °C overnight and loaded onto the equilibrated DEAE-cellulose column at flow rate 30 ml/hr. The column was washed with equilibrated buffer until A_{280} was negligible, then the column was subjected to 500 ml of 0-1.0 M NaCl linear gradient elution. Fractions of 3 ml were collected for measurement of protein concentration (A_{280}) and chitinase activity.

Characterization of chitinase enzyme

The chitinase activity was assayed by the colorimetric method as previously described for its properties as the follows:

Optimum pH for chitinase activity

The optimum pH for chitinase activity was determined by incubating the enzyme (0.1 mg protein/ml) in appropriate buffers at different pHs (3-10) using 0.1% colloidal chitin as substrate, and incubated at 50 °C. Citrate buffer (100 mM), phosphate buffer (100 mM) and Tris-HCl (100 mM) were used for pH 3-6, pH 6-8 and pH 8-10, respectively. The chitinase activity was assayed as previously described.

Optimum temperature for chitinase activity

The optimum temperature for the chitinase activity was determined by incubating the reaction mixture, consisted of 150 µl of 1 M phosphate buffer pH 6.0, 75 ml of 2% colloidal chitin and 100 ml of chitinase (0.1 mg protein/ml) at temperature range of 20-80 °C. Then the release of N-acetylglucosamine (GlcNAc) reducing property at 30 mins of the incubation time was measured under standard assay conditions.

Enzyme stability

The stability of chitinase stored in the buffer at various pH values (pH 3-10) at 4 °C was investigated. The remaining chitinase activity was measured at Day 0, Day 1, Day 2, Day 3, Day 4 and Day 5 by the colorimetric method using 0.1% colloidal chitin as substrate.

The stability of chitinase at various temperatures was examined with the maintenance of the chitinase enzyme was stored in the 50 mM phosphate buffer pH 6, pH 7 for crude enzyme and partial purified enzyme, respectively, at the temperature range of 40, 50 and 60 °C.

The chitinase activity on different substrates

The enzyme was incubated with each of the following substrate; 0.1% (w/v) colloidal chitin and regenerated chitin or 0.01% (w/v) 80% chitosan, partially N-acetyl chitin (PNAC) or 1% (w/v) β-chitin powdered chitin, flake chitin at 60 °C for 30 mins. The release of N-acetylglucosamine hydrolysis of the substrate was measured by the colorimetric method for determination of its activity.

The kinetic of enzyme

The enzyme was incubated with varies concentration of colloidal chitin in 0.1 M phosphate buffer at 55 °C for 10 mins. The release of N-acetylglucosamine hydrolysis of the substrate was measured by the corlorimetric method for determination of its activity.

Native isoelectric focusing (IEF) gel electrophoresis

The native isoelectric focusing polyacrylamide gel on mini gel system was used for determination of the pI value of chitinase. The gel (10 x 9.4 x 0.075 cm) was prepared as described in Appendix C. The IEF system was ran at pH range 3.0-10.0. The enzyme from DEAE column chromatography step was mixed with sample buffer (60% glycerol, 4% ampholyte) at ratio of 2:1. The IEF condition was performed by the method of Robertson et al., the cathode solution was 25 mM NaOH and the anode solution was 20 mM acetic acid. Electrophoresis was performed at 4 ° C for 1.5 hrs at 200V constant voltage, then increased to 400V constant voltage for additional 1.5 hrs. After electrophoresis was completed, the gel was fixed by immersion in 10% trichloroacetic acid (TCA) for 10 min followed with 1% TCA for at least 2 hrs to remove ampholyte. The fixed gel was stained for protein. The standard pI calibration kit (pI 3.0-10.0) was used as standard pI markers.

Glycoprotein staining

Periodic acid and Schiff's (PAS) stain is used to stain glycoprotein, haparin, chondroitin sulphate, polysaccharide, and other materials with a high carbohydrate content.

The purified enzyme was analyze on SDS-PAGE, then the gel slab was submerged in 10% TCA for 30 min and then washed in distilled water for 15 min. The gel slab was then soaked in the 1% periodic (IO_3) in 3% acetic acid for 50 min. Next, the gel slab was washed with distilled water to remove the excess IO_3. The excess IO_3 were checked by adding 0.1 N $AgNO_3$ in washed water (the brown precipitates will occurred in the presence of IO_3). Then the gel was immersed in Schiff's reagent and left in the dark at room temperature for 50 min. The excess dye was washed with 0.5% metabisulfite for 10 mins 3 times, then washed with distilled

water until the background was clear. The gel was then stored the gel in 7% acetic acid.

General methods used in cloning and subcloning of chitinase gene

Extraction of plasmid DNA from *E. coli*

Plasmid vectors and recombinant plasmids from *E. coli* were extracted by rapid alkaline lysis method (Maniatis et al., 2001)

E. coli cells were grown overnight with shaking at 37 °C in 2 ml LB broth supplemented with 50 μg/ml ampicillin. Cell pellet was collected from each 1.5 ml culture by centrifugation at 8,000 rpm for 2 min. The cells were washed once with solution I (25 mM Tris-HCl pH 8.0, 10 mM EDTA and 15% sucrose). The pellet was resuspended in 100 μl of solution I containing 1 mg/ml of lysozyme and left on ice bath for 10 min. Two hundred microliters of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added, immediately mixed by inverting the tube and incubated on ice for 10 min. The lysate was subsequently neutralized by mixing with 150 μl of chilled solution III (3 M potassium acetate pH 5.2) and maintained on ice bath for 10 min. The plasmid DNA in the supernatant was collected after centrifugation at 12,000 rpm for 10 min. A 0.6 volume of chilled isopropanol or 2.5 volume of ethanol was added to precipitate plasmid DNA which was kept at 0 °C for 30 min. The DNA was collected as a precipitate after centrifugation at 12,000 rmp for 10 min. The precipitate DNA was dissolved in 50 μl TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA). RNA was removed by adding 0.5 μl of boiled 10 mg/ml RNase and traces of ethanol was removed by incubating at 65 °C for 10 min. The yield and quality of DNA at this step was sufficient for further used.

Extraction of chromosomal DNA

B. licheniformis SK-1 was precultured with shaking at 50 °C in 3 ml of LB broth for overnight. One milliliter of this culture was transferred to 100 ml of LB broth and incubated further for 12-18 hr. The cells were collected by centrifugation at 10,000 rpm for 5 min and washed once with SET buffer (25 mM Tris, pH8.0 containing 10 mM EDTA and 50 mM Sucrose). The cell suspension was then treated

with 30 mg of lysozyme and 2 mg RNase at 37 °C for 1 hr. A 0.6 ml of 10% SDS was subsequently added to the suspension following by immediate mixing by racking the tube back and forth. The suspension was treated with 0.12 mg of proteinase K at 50 °C for overnight. The debris were separated from the supernatant by centrifugation at 12,000 rpm for 10 min. Then, equal volume of phenol-chloroform-isoamyl alcohol (24:24:1, v/v/v) was added and mixed. The remaining protein contaminants were removed by centrifugation at 12,000 rpm for 10 min. The process was repeated several times till no white precipitate was observed between the 2 phases. The upper aqueous phase was transferred to a new tube and added 0.6 volume of chilled isopropanol or 2.5 volumes of chilled absolute ethanol was added to precipitate the DNA. DNA was collected by centrifugation at 12,000 rpm for 10 min. Then washed with chilled 70% ethanol, air dried and dissolved in TE buffer.

The purity of DNA was calculated from absorbance at 260 nm divide by adsorbance at 280 nm (A_{260}/A_{280}), range between 1.8-2.0 was good for further used.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze DNA or DNA fragments. The latter obtained form restriction enzyme digestion was analyzed according to their sizes. The size was estimated from standard curve of molecular weight markers, i.e., fragments of Lamda DNA digested with *Hin*dIII. The calibration curve was plotted between logarithmic molecular massed and their relative mobilities. The concentration of agarose gel was used varies with the size of the DNA fragment to be separated. Generally 0.7-1.5% gel in Tris-acetate-EDTA (TAE) buffer was used.

A solution of gel was mixed with 10-20% (v/v) of 6x loading buffer [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 15 % (w/v) Ficoll type 400 in distilled water]. The mixture was loaded into slots of the gel which was submerged in electrophoretic chamber filled with TAE. Electrophoresis was carried out at constant 50-100 volts. The duration of the run depended on the size of DNA. Generally, the gel was run until the tracking dye reached near the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 3-5 mins and then destained with distilled water 2-3 times.

The resolved DNA bands were visualized on an UV transilluminator and photographed.

Restriction enzyme digestion

Restriction endonuclease was used to cut DNA based on its specific binding property and cleaving double-stranded DNA at a specific sequence. The condition of digestion was performed as recommended by the enzyme manufacturer. Typically, a reaction contains about 0.5-1 μg of DNA in a final volume of 10 μl containing 1X enzyme reaction buffer and 2-5 units of restriction enzyme. The digested DNA was analyzed by agarose gel electrophoresis as described above.

Partial digestion of chromosomal DNA

The chromosomal DNA of *B. licheniformis* SK-1 was partially digested with *Pst*I for 30 mins under conditions recommended by the manufacturer and separated on 1% agarose gel in TAE buffer (0.004M Tris-acetate and 0.001M EDTA) at 100 volts. Lamda DNA cut with *Hin*dIII (λ/*Hin*dIII) was used as size standard. The gel segment corresponding to the size between 3 and 9 kb was cut out, DNA fragments in the gel were recovered by using QIA quick Gel Extraction Kit (Qiagen, Germany).

Recombinant DNA construction

Both 3-9 kb chromosomal DNA fragments and dephosphorylated *Pst*I-digested pBluescriptSK$^-$ were mixed together in a ratio of DNA: vector from 2:1. In a total of 20 μl, 2 μl of 10x ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP) was added to the mixture, the volume was adjusted to 20 μl with steriled distilled water. One microlitre of T4 DNA ligase was added (10 units) into the reaction mixture. The reaction was incubated for18-20 hours at 18-20 °C. Ligation products were kept at –20 °C.

Competent cells preparation

Competent *E. coli* JM109 was prepared according to the method of Sambrook et al. (1989). A single colony of *E. coli* JM109 was cultured as the starter in 12 ml of LB broth [1 % (w/v) tryptone, 0.5% (w/v) yeast extract, and 1 % (w/v) NaCl] and incubated at 37 °C with shaking 250 rpm for overnight. One percent of starter was innoculated into 1,000 ml of LB broth [1 % (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5 % (w/v) NaCl] and the culture was incubated at 37 °C with shaking 250 rpm for 2-3 hours until the optical density at 600 or 660 nm (OD_{600} or OD_{660}) of the cells reached 0.5-0.6 or 0.4-0.5 respectively.

The cells were chilled on ice for 15-30 mins and harvested by centrifugation at 8,000 rpm for 15 mins at 4 °C. The supernatant was removed as much as possible. The cell pellet was washed with 1 volume of cultured broth (800 ml) of cold steriled water, resuspended by gently mixing and centrifugated at 8,000 rpm for 15 mins at 4 ° C. The supernatant was discarded. The pellet was washed and centrifugation further with 400 ml of cold steriled water, followed by 20 ml of ice cold steriled 10 % (v/v) glycerol, and finally resuspended in a final volume of 1.6-2.0 ml ice cold steriled 10 % (v/v) glycerol. This cell suspension was divided into 40 µl aliquots and stored at -80 ° C until used.

These competent cells were good for at least 6 months under these conditions.

Electrotransformation

The competent cells were thawed on ice. Forty microlitres of the cell suspension was mixed with 1.0 µl of the ligation mixture, mixed well and placed on ice for 1 min. The mixture of cell and DNA was electroporated in a cold 0.2 cm cuvette with apparatus setting as follows; 25 µF of the Gene pulser, 200 Ω of the Pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were immediately resuspended with 1 ml of LB medium [1.0 % (w/v) tryptone, 0.5 % (w/v)yeast extract and 1.0 % (w/v) NaCl] and transferred to a steriletest tube. The cell suspension was incubated at 37 °C with shaking at 250 rpm for 1 hour. All of the cells in the suspension was spread on the

colloidal chitin agar plates, which contained 100 mg/ml ampicillin, 25 μl of 25 mg/ml iso-1-thio-β-D-galactopyranoside (IPTG) and 25 μl of 25 mg/ml chloro-3-indolyl-β-D-galactopyranoside (X-gal), incubated at 37 °C for 1 week.

The positive transformants with insertions were screened for plasmid harboring chitinase gene by phenotype screening described below.

Detection of chitinase gene (Phenotype screening)

Transformants harboring chitinase gene were detected by the formation of clearing zone around the colonies on screening medium which consisted of 0.25% yeast extract, 0.02% colloidal chitin, 100 μg/ml (w/v) ampicillin, 25 μl of 25 mg/ml (w/v) IPTG and 25 μl of 25 mg/ml X-gal.

Transformants, which produced clear zone, were cultured for plasmid extraction. The plasmid was then cut with *Pst*I to determine size of inserted fragment.

PCR Amplification

Primers

The first set of primers that used for partial chitinase gene amplificaiton were degenerate primers designed from N-terminal amino acid sequence of family 18 chitinase from *B. circulans* chi41, *B. licheniformis* TP, *B. circuland* chi1, *B. circulans* A1, *B. thuringiensis* and *B. cereus* chiB. All constructed primer see Table 4.

Template Chromosomal DNA from was used as templates.

PCR reaction

The 25 ml of reaction mixture contained 1.5 U of *Taq* or *Vent* DNA polymerase, 200 μM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, DNA fragment and 10 pmole of each primer, and 50 pmole of chromosomal DNA.

All PCR products were analyzed by agarose gel electrophoresis and sequenced as describe in chapter III.

The conditions for 16S rRNA and chitinase gene amplification is described in Table 5.

Chitinase gene amplification

Primer BP-F and BP-R were used to amplify the full-length chitinase gene from *Bacillus licheniformis* SK-1 as described above.

Analysis of chitinase gene

DNA Sequencing

Primers BP-II, BP-V, BPVI, BPVIII and M13 universal primers were used for sequencing by automate sequencer at Bioservice unit (BSU), Thailand.

Analysis of *chi66* gene

Homology search of *chi66* gene using Basic Local Alignment Search Tool (BLAST)

The sequence of Chi66 was analyzed using BLAST (Altschul et al., 1997 and Zang and Madden, 1997) program from GenBank and CLUSTAL W (Peitsch, 1995 and Peitsch, 1996)to search for homologues of *chi66* gene and protein in other bacteria.

DNA Sequence manipulation

GENETYX-WIN version 3.1 was used to analyze *chi66* gene in the following; find origin of replication (ORF), translated to amino acid sequence and calculated molecular weight including isoelectric point of the translated protein.

Table 4. Nucleotide sequence and Tm (°C) of all primers used in chitinase gene amplification.

Forward Primer	Sequence (5'→ 3')	Tm (°C) [2°(A+T)+4°(G+C)]	Remark
FPSK⁻	GGTGGCGGCCCGCTCTAGAAC	68	For sequencing
BP-I	AAYTAYGCDTTYGCDGAYATHTGYTGGRANGG	84	For partial gene amplification
BP-II	TTYGAYGGNGTNGAYYTNGAYTGGGARTA	76	For partial gene amplification and sequencing
BP-VIII	AAYATCATGACNTAYGAYTTYAAYGGNGGNTGG	86	For partial gene amplification
BP-F	GTTTTCCCTTGTTGTCTTC	54	For whole gene amplification
BP-IX	CATGGGAAAAGGGCG	48	For gene sequencing
Reverse Primer	**Sequence (5'→ 3')**	**Tm (°C) [2°(A+T)+4°(G+C)]**	**Remark**
BP-VII	NCCNTYCCARCADATRTCHGCRAAHGCRTARTT	86	For partial gene amplification
BP-V	RTAYTCCCARTCNARRTCNACNCCRTCRAA	76	For partial gene amplification
BP-VI	CCANCCNCCRTTRAARTCRTANGTCATGATRTT	86	For partial gene amplification
BP-R	CTCTTTATCGTTTTCTATCC	54	For whole gene amplification

Abbreviations; **Y** = C,T **R** = A,G **D** = A,G,T

H = A,C,T **N** = A,C,G,T

Table 5. PCR condition in each step

Corporated primers	Predenaturation	Denaturation	Annealing	Extension	Final extension	Number of cycle
16s rRNA pA+pH'	94 °C, 5 mins	94 °C, 1 min	55 °C, 2 mins	72 °C, 3 mins	72 °C, 5 mins	25-30
Partial chitinase gene BP-I+BP-V BP-II+BP-VI BP-I+BP-VI	94 °C, 4 mins	94 °C, 1 min	60 °C, 2 mins	72 °C, 3 mins	72 °C, 5 mins	25-30
Whole chitinase gene BP-F+BP-R	94 °C, 4 mins	94 °C, 1 min	50 °C, 2 mins	72 °C, 2 mins 30 sec	72 °C, 5 mins	25-30

After final extension, the PCR products were kept at 4 °