CHAPTER III

EXPERIMENTAL

3.1 Sample collection and isolation of halophilic bacteria

The samples of Pla-ra were collected from markets in Thailand (Table 4.1) Samples collected in sterile plastic containers, diluted in 10% NaCl solution were plated on the JCM no. 377 not later than 24 hours after collection and were incubated at 37 °C for 1-2 weeks. A single colony of the halophilic bacteria were transferred to a JCM medium no.377 (Appendix A-1), and incubated at 37 ^{o}C for 3-7 days. The stock cultures were kept in cold room at 4 ^{o}C and freeze dry at the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.2 Identification methods

Morphological, cultural, physiological, and biochemical characteristics of the isolates were determined using the methods described by Barrow and Feltham (1993), Leifson (1963), Namwong (2005) along with several supplementary tests.

3.2.1 Cell morphology and cultural characteristics

The colonies on the JCM no. 377 containing 10% NaCl after 1 day incubation at 37°C were examined for their characteristics (Barrow and Feltham, 1993).

3.2.1.1 Gram staining. Thin smear of bacterial colony was prepared on a clean slide. Slide was fixed by passing through flame. The smear was covered with crystal violet for 30 sec, then rinsed with water and drained. Next, the smear was covered with iodine for 30 sec, then with rinsed water. Decolourized with ethanol 95% and washed with water, then it was counter stained about 30 sec with safranin.

Blot slide dried and examine under oil immersion (1000x). Colonial appearances were examined after incubated for 3-7 days.

3.2.1.2 Flagella stainning. Standard microscope slide, pre-cleaned only by the manufacturer, were used. The slide was flame briefly, and a thick line was drawn with a wax pencil across its width so that the stain was confined to two-thirds of surface. To this area, 3 drops of sterile distilled water was added. An inoculating needle was touched to the top of an isolated colony from the JCM no.377 agar slant medium and gently mixed in the water. There was no visible opalescence. The suspension was spread over the staining area and then tapped off onto a disinfectant-soaked gauze sponge. The slide was air dried on a level surface. Staining method by Forbes (1981), stainning (Appendix A-13) was timed for 1 min with 1 ml of stain at ambient temperature. The slide was washed in tap water, counterstained with the Hucker modification of Gram crystal violet for 1 min washed, blotted dry, and examined under oil immersion starting near the wax line.

3.2.2 Physiological and biochemical characteristics

3.2.2.1 Oxidase test. Each colony was streaked on the JCM no. 377 containing 10% NaCl and incubated at 37°C for 1 day. A few drops of 1% tetramethyl-p-phenylenediamine were dropped on sterile filter paper disc. The test culture was then smeared across the moist paper disc with sterlie loop. The apperance of dark-purple colour on paper within 30 sec. denoted a positive reaction.

3.2.2.2 Catalase test. A small amount of pure growth was transferred from agar onto the slide. Hydrogen peroxide (H_2O_2) 3% was immediately placed onto a portion of a colony on the slide. The evolution of gas bubbles indicating a positive test was observed.

3.2.2.3 Growth at different temperatures. The JCM no. 377 medium agar was inoculated with bacterial cells and incubated at 37°C , 45°C and 50°C. The growth examination was performed after 7 days.

3.2.2.4 Growth in different NaCl concentration. The JCM no. 377 containing 0, 5, 10, 15, and 20% NaCl was inoculated with the cultures and incubated at 37°C for 7 days, then the growth was observed.

3.2.2.5 Growth at different pH. The JCM no. 377 broth adjusted to pH 5.0, 6.0, 8.0 and 9.0 was used for the growth of cultures incubated at 37°C for 7 days.

3.2.2.6 L-Arginine hydrolysis. Inoculate the arginine agar slant (Appendix A-4) with the cultures and incubated for 7 days. A positive reaction is shown by a colour change of the indicator to red.

3.2.2.7 Aesculin hydrolysis. Inoculated aesculin broth (Appendix A-5) containing 10% NaCl was examined daily up to 5 days for blacking of hydrolysis.

3.2.2.8 Gelatin hydrolysis. Bacterial cells were inoculated on the agar medium JCM no. 377 (Appendix A-6) containing 12% gelatin but omitted casamino acid and incubated at 37°C for 7 days. Flood the surface with 5-10 ml of 30% trichloroacetic acid, and the clear zones areas indicated the hydrolysis.

3.2.2.9 Starch hydrolysis. Inoculate in the JCM no. 377 (Appendix A-7) containing 1% soluble starch and incubated plates at 37°C for 7 days. Flood the plate with Lugol's iodine solution then the medium turns blue where starch has not been hydrolysed; clear colourless zones indicated the hydrolysis.

3.2.2.10 Tyrosine hydrolysis. Bacterial cell were inoculated on the JCM no.377 medium agar (Appendix A-8) containing 0.5% tyrosine but omitted casamino acid and incubated at 37°C for 7 days. Clear zone indicate areas of the tyrosine hydrolysis.

3.2.2.11 Tween80 hydrolysis. Bacterial cell were inoculated on the JCM no.377 medium agar (Appendix A-9) containing 0.5% tyrosine but omitted casamino acid and incubated at 37°C for 7 days. Clear zone indicate areas of the tyrosine hydrolysis.

3.2.2.11 Deoxyribonuclease(DNase) activity. Inoculate the cultured on DNase test agar (Difco) (Appendix A-10) for 7 days. After incubation, flood the plates with 1 N HCl then a clear zone around the growth appeared indicated the positive result.

3.2.2.12 Indole test. Inoculate the cultures in tryptone broth (Appendix A-11); and incubated at 37°C for 7 days. After incubation, it was tested by adding 4 drops of Kovacs' reagent (Appendix A14), using *iso*-amyl alcohol as solvent.

3.2.2.13 Nitrate reduction. Inoculate nitrate broth (Appendix A-12) with the cultures and incubated for 7 days. A drop of each Solution A and Solution B reagent (Appendix A15) was added. When nitrite is present a pink colour developed within 5 minutes.

3.2.2.14 Acid from carbohydrate. The acid from carbon sources was performced in marine oxidation-fermentation medium or MOF (Appendix A-2) as described by Leifson (1963). With the addition of appropriates carbon sources at final concentration 1% (w/v). The following 22 different carbon sources were used including L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, *myo*-Inositol, maltose, D-mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose, D-xylose. The media were adjusted to pH 7.2 and phenol red 0.2% solution (w/v) was added as an indicator solution. The 2 ml liquid was dispensed into a 11 mm diameter test tube. The broth was inoculated with two drops of bacterial cell suspensed in saline and incubated at 37°C. Growth was recorded daily for up to 7 days. The positive results were shown by colour change of the indicator from red to yellow.

3.2.2.15 Utilization of carbohydrate. Utilization of various compounds as sole carbon and energy sources was tested in a mineral liquid medium supplemented with 0.2% (w/v) test substrate as described by Romanenko et al., (2003). Growth was determined spectrophotometrically after 2 days cultivation.

3.2.2.16 API ZYM. The efficiency to produce enzymatic reaction to digested 19 substrate were used API ZYM strip (bioMérieux).

3.2.2.17 Sensitivity to antibiotics . The sentivity of antibiotics was tested by spreading cell suspensions onto halophilic medium agar plates and then the antibiotic paper discs (6 mm in diameter) were applied on the medium. Zones of inhibition were measured following 14 days of incubation at 37°C. Sensitivity was considered as strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc (Stan-Lotter et al., 2002).

3.2.3 Chemotaxonomic characteristics

3.2.3.1 Diaminopimelic acid analysis. Dried cells (10 mg) were hydrolyzed with 6N HCl at 100 °C for 18 h. The hydrolyzed solution was filtered and evaporated. The 400 μL of distilled water was added into dried sample. The solution

was loaded onto cellulose HPTLC plateno.5787 and developed with MeOH:H_2O:6N HCl:Pyridine (80:26:4:10). Finally, the cellulose HPTLC plate was sprayed with 0.5% ninhydrin in n-butanol for detection (Komagata and Suzuki, 1987).

3.2.3.2 Cellular fatty acids. Dried cells (40 mg) were put into screw-cap tube and added with 1 mL of reagent 1 (Appendix B-1.1). This suspension was shaken well before heating at 100 °C for 30 min and then cooling to room temperature in water. The reagent 2 (Appendix B-1.2) was added into the suspension and mixed for 5 to 10 sec with vortex mixer. The suspension was heated at 80 °C for 10 min and cooled to room temperature in water. Then, reagent 3 was added (Appendix B-1.3) and the suspension was mixed for 10 min and was transferred the upper layer to another tube. The reagent 4 (Appendix B-1.4) was added into the suspension and mixed for 5 min, if it became to emulsion form, the reagent 5 was added (Appendix B-1.5) into the suspension. The upper layer was transferred to the vial for GC to detect cellular fatty acids (Minnikin et al., 1984).

3.2.3.3 Polar lipids. Three ml of MeOH:0.3%NaCl aq. (100:10) and 3 mL of petroleum ether were added to the dried cells (150-300g). The solution was mixed for 15 min. The lower layer was added with 1 mL of petroleum ether and then the solution was mixed for 2-5 min. The lower layer was heated at 100 °C for 5 min and cooled immediately at 37 °C for 5 min. The suspension was added with Chloroform:MeOH:water (90:100:30) and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with Chloroform:MeOH:water (50:100:40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 ml of chloroform and water. The final lower layer was dried with N_2 gas (<37 °C). The polar lipid fraction was dissolved with 60 µL of chloroform:MeOH (2:1) and applied to two-dimensional silica HPTLC no. 1.05633 and was developed with the following solvent systems. The first solvent system: chloroform : MeOH : Water (65:25:4). The second solvent system : chloroform : acetic acid : MeOH : Water (40:7.5:6:2). HPTLC was sprayed with iodine until polar lipid appeared. Subsequently, the first plate was sprayed with Ninhydrin reagent (Appendix B-2.1) and then heated at 110 °C for 10 min. Dittmer and Lester reagent (Appendix B-2.2) was sprayed onto the plate and then blue spots

were detected on the plates containing all phospholipids. The second plate was sprayed with Anisaldehyde reagent (Appendix B-2.3) and then heated at 110 °C for 10 min after spraying. Green-yellow spots and blue spots were detected on plates containing glycolipids and other lipids, respectively. (Sasser et al., 1990; Kämpfer and Kroppenstedt, 1996).

3.2.3.4 Quinone analysis. The isolates were grown in a 300 ml JCM no. 377 broth containing 10%NaCl on a shaking in a rotary shaker (200 rpm) at room temperature for 1 day. Cell were harvested by centrifugulation at 7,000 rpm, washed with water and lyophilized. The isoprenoid quinone were extracted from dried cells by using chloroform-methanol (2:1, V/V) in flask and shaked for 3 hours. The residual cells were separated by filration. The combined filtrate was concentrated to dryness under a reduced pressure on the rotary evaporator. Crude quinone was dissolved in a small amout of acetone. Actone solution was applied to thin-layer chroatography on a siliga-gel glass plate (20x20 cm, E. Merk, Silica gel 60F254, Art.6715) and developed with a solvent system of petroleum and diethyl ether (85:15, V/V). Standard quinones should also be included. The quinone spots can be visualized by UV light at 254 nm. The R_f of menaquinone was 0.4. The band of menaquinone was scraped off and extracted with acetone. The purified quinones were examined by HPLC (Shimadza model LC-3A). μ- Bondapak C_{18} column (water Associates, Milford, Mass., USA) was employed and eluted by methanol-isopropanol (1:1, v/v) with flow rate 1.2 ml/min. The abbreviation (e.q. MK-7, MK-6, etc.) used for menaquinone indicated the number of isoprene unit in the side chain.

3.2.3.5 DNA base composition

Isolation and purification of DNA: Isolation of DNA from bacteria was done according to the method recommened by Saito and Miura (1963). Briefly, the bacterial inoculated in 100 ml JCM. No.377 broth containing 10% NaCl was shaked (200 rpm) for 1 day in order to obtain the cell growth during logarithmic phase. The cell were harvested by centrifugation at 7,000 rpm and washed twice with 10 ml of saline-EDTA. Bacterial cell lysis was done by using lysozyme in Saline-EDTA, 10% sodium dodecyl sulfate (SDS) and Tris-buffer pH 9.0. After well mixing the suspention was heated at 60°C for 10 min. After the cell were lysed, the cell suspention was change from turbid to opalescent and become very viscous. Following

the addition of 4 ml of phenol-chloroform (1:1 v/v), the mixure was vortexed for at least 30 sec. It was then centrifuged at 12,000 rpm for 10 min. The supernatant was then transferred into a small beaker. After adding of cold 95% ethanol into supernatant to precipitate DNA, DNA was spooled with a grass-rod, and rinsed with 70% then 95% (v/v) ethanal and air dried. DNA was then dissolved in 5 ml of 0.1 x SSC. RNase A solution (0.3 ml) was added and the DNA solution was incubated at 37 °C about 20 min for the purification. After adding 0.5 ml of 10 x SSC,2 ml of phenol-chloroform were mixed by vortexed for 1 min and centrifuged at 12,000 rpm for 10 min. The upper layer was transferred to another tube. the DNA was precipitated by adding cold 95% ethanol and DNA was spooled with a grass-rod then rinsed with 70% then 95% (v/v) ethanol. After air dried, DNA was dissolved in 5 ml of 0.1 x SSC. The purity and quality of DNA solution were determined from the ratio between adsorbance value at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur and Doty (1962).

Hydrolysis of DNA: DNA base composition can be determined by Tamaoka and Komagata (1984), DNA was hydrolysed into nucleosides using nuclease P1 (EC 3.1.3.30) and alkaline phosphatase (EC 3.1.3.1) was prepared. Sample of DNA solution; about 0.5-1.0 g of DNA/litre of distilled water (OD_{260}=10-20). Heat the DNA solution in boiling water for 5 min and cool in ice. Take 10 μl of the DNA solution to an eppendorf and incubated with adding 10 μl of nuclease P1 solution (Appendix C-1.9) in water bath at 50 °C for 1 hour. After incaubation, 10 μl of alkaline phosphatase solution (Appendix C-1.10) was added and keep at 37 °C for 1 hour. After DNA hydrolysis, the sample was analyzed by HPLC with condition show in Table 3.1

Table 3.1 Conditions for high-performance liquid chromatography

Detector wave length	270 nm
Column	Nakarai Cosmosil packed column $5C_{18}$(150X4.6 mm)
Column temperature	Room temperature
Eluent	0.2 M $NH_4H_2PO_4$-acetonitrile(20:1, v/v)
Flow rate	1ml / min

3.2.4 DNA-DNA hybridization

DNA labeling probe with photobiotin was started by mixing 10 µl of purified DNA solution (1 mg/mL) and 15 µL of photobiotin solution (1 mg/mL) in an Eppendorf tube and then the mixture was irradiated with sunlamp for 30 min on ice. After irradiation, the excess photobiotins were removed by the addition of 100 µl of 0.1 M Tris-HCl buffer pH 9.0, and 100 µl of n-butanol. The upper layer was removed. A 100 µL of n-butanol was added and mixed well and removed the upper layer. The biotinyiated DNA solution was boiled for 15 min and immediately cooled in ice. The solution was sonicated for 3 min and dissolved with hybridization solution (Appendix C-2.6). DNA-DNA hybridization solution was performed by Ezaki, Hashimoto, and Yabuuchi (1989). 100 µL of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) and fixed by40 incubation at 37 °C for 2 h. After incubation, the DNA solution was removed. 200 µL of a prehybridization solution (Appendix C-2.5) was added to microdilution wells. The microdilution plate was incubated at hybridization temperature, 40°C (Group I-VII), 40°C (GroupVIII), 50°C and (Grou IX) for 1-2 h. The prehybridization solution was removed from the wells and replaced with 100 µL of a hybridization mixture containing biotinylated DNA. The microdilution plate was incubated at hybridization temperature of each Group for 15-18 h. After hybridization, the microdilution wells were washed three times with 200 µl of 0.2xSSC buffer. Then 200 µL of solution I (Appendix C-2.7) was added to microdilution wells and incubated at 30 °C for 10 min. Solution I was removed from the wells and replaced with 100 µL of solution II (Appendix C-2.8). The microdilution plate was incubated at 37 °C for 30 min. After incubation, the microdilution plate was washed for three times with 200 µL of PBS. 100 µL of solution III (Appendix C-2.9) was added, and the plate was incubated at 37 °C for 10 min. The enzyme reaction was stopped with 100 µL of 2M H_2SO_4 (Verlander, 1992). The absorbance was measured at 450 nm with Microplate Reader (Microplate Manager^{R} 4.0 Bio-Rad Laboratories, Inc) and calculated for the value of percentage DNA homology (Tanasupawat et al., 2000).

3.2.5 16S rDNA sequence analysis

3.2.5.1 16S rDNA amplification by PCR.

The PCR was performed in a total volume of 50 μl containing 1 μL of DNA sample, 0.25 μL of *Taq* DNA polymerase, 5 μL of 10x polymerase buffer, 4 μl of dNTP mixture, 2.5 μL of 10 μM forward and reverse primers (Appendix C-3) and 34.75 μL of Milliq water. A DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 3 min at 95 °C followed by 30 cycles of 30 sec at 95°C (denaturing of DNA), 15 sec at 55°C (primer annealimg), and 1 min at 72°C (polymerization) and a final extension for 5 min at 72°C. The PCR amplified products were analyzed by running 5 μL of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution (0.5 mg/mL) and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rDNA band.

3.2.5.2 16S r DNA sequencing

The amplified 16S rDNA was used as templates for sequencing with big dye terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) with a temperature profile of 30 sec at 96°C followed by 25 cycles of 10 sec at 96°C (denaturing of DNA), 5 sec at 50°C (primer annealimg), and 4 min at 60°C (polymerization). Sequencing for each sample was carried out in both forward and reverse directions.

3.2.5.3 Phylogenetic analysis

Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequences at the GenBank/EMBL/DDBJ databases. The sequence was multiply aligned with selected sequences obtained from the three main databases by using the CLUSTAL W version 1.81. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum parsimony methods in the MEGA

program version 2.1 (Kumar et al.; 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program (Thompson et al., 1997). Gaps and ambiguous nucleotides were eliminated from the calculations.

3.3 Protease producing halophilic bacteria

3.3.1 Primary screening of protease-producing halophilic bacteria

The protease-producing halophilic bacteria were screened by using the JCM. no.377 agar plates, containing 5% or 10% (w/v) NaCl with 1% skim milk and incubated at 37°C for 3-7 days. The results of caseinolytic halo-forming colonies on the agar plate was used as the qualitatively test.

Quantitative protease activity assay (caseinolytic activiProtease assay) A loopful of selected strain that had a clear zone on JCM no. 377 medium was inoculated in 250-ml Erlenmeyer flask containing 50 ml of the same medium and incubated at 37°C under shaking condition 200 rpm for 3 days. The cell-free supernatant recovered by centrifugation at the speed of 12,000 g at 4°C for 15 min was used for extracellular protease activity (caseinolytic activity) assay as described by Hiraga et al., (2000). Sample (0.5 ml) was incubated with 1.5 ml of 1.33% (w/v) Hammarsten casein in 25 mM Tris-HCl, pH 7.5 buffer containing 10% NaCl at 37°C for 1 h. The enzymatic reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 3,500 rpm for 10 min to remove undigested substrate. After that, 0.5 ml of the supernatant was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of the Folin-Ciocalteu reagent. After incubation for 20 min at 37°C, the absorbance of the colour developed was measured at 660 nm. A correlation between an absorbance at 660 nm and tyrosine concentration was constructed for an estimation of tyrosine produced. One unit of protease was defined as the amount of the enzyme yielding the equivalent of 1 μmole of tyrosine released per minute under the defined assay conditions. A blank was run in the same manner except the enzyme was added after the addition of TCA solution.

Total protein in the supernatant was measured by the method described by Lowry et al. (1951). The details of the analytical method is described clearly in Appendix D-1

The cell pellet dried at 120°C for 3 h was used for the cell dry weight determination.

3.3.2 Protease production and the effect of various parameters

3.3.2.1 Determination of the growth and protease production kenetics

To determine growth and protease production kinetics, the selected strain was inoculated on JCM no. 377 medium with 5% initial inoculum (A_{660} ~ 0.5, 1.7x10^6 CFU/ml) in 250 ml Erlenmeyer flasks containing 50 ml of medium and incubated at 37°C under shaking condition for 72 h. Samples taken at 2 h interval were assayed for protease activity in cell-free supernatant and monitored for growth by measuring an absorbance at 660 nm.

3.3.2.2 Optimization of crude protease production

One loopful of selected strain grown in 50 ml of JCM no.377 medium in 250 ml Erlenmyer flask and incubated with shaking (200 rpm) at 37°C for 2 days was used as inoculum. A 2.5 ml (5%) of the inoculum (A_{660nm}~ 0.5; 1.7x10^6 CFU/ml) was inoculated in 50 ml of the same medium in 250 ml Erlenmyer flask and incubated at the same above condition. An optimal condition of prior experiment was used as the basis in the latter experiment to optimize the conditions. Samples were assayed for protease activity in cell-free supernatant and for growth by determination of cell dry weight.

3.3.2.2.1 Effect of different nutrient on protease production: To identify the components for a defined medium yielding a high level of protease activity, ND1-1 was inoculated in the modified JCM no. 377 medium as following in the Table 3.2. After incubation at the same above condition, the growth and enzyme activity was determined.

Table 3.2 The composition of modified medium

Modified medium no.	**Composition of modified medium**
No. 1	JCM no. 377. without casamino acids
No. 2	JCM no. 377. without yeast extract
No. 3	No. 1 supplemented with 0.5% yeast extract
No. 4	No. 1 supplemented with 0.5% skim milk
No. 5	No. 1 supplemented with 0.5% gelatin
No. 6	No. 1 supplemented with 1% gelatin
No. 7	No. 1 supplemented with 2% gelatin
No. 8	No. 1 supplemented with 0.5% soy protein

3.3.2.2.2 Effect of NaCl concentration on protease production: In order to investigate the influence of NaCl concentration on protease production ND1-1 was grown in the suitable modified JCM no. 377 medium (No. 4) containing NaCl at various concentrations (0–20% w/v). After incubation at the same above condition, the growth and enzyme activity was determined.

3.3.2.2.3 Effect of initial pH on protease production: In order to investigate the influence of pH on growth and protease production, ND1-1 was grown in the suitable modified JCM no. 377 medium (No.4) and suitable NaCl concentration at varying initial pH (5.0–10.0). After incubation at the same above condition, the growth and enzyme activity was determined.

3.3.2.2.4 Effect of incubation temperature on protease production: In order to investigate the influence of temperature on growth and protease production, ND1-1 was grown in the suitable modified JCM no. 377 medium (No.4), NaCl concentration and pH. ND1-1 was incubated at varying temperature. After incubation at the same above condition, the growth and enzyme activity was determined.

3.3.3 Purification of protease from ND1-1

3.3.3.1 Enzyme preparation

The selected strain was cultivated in modified JCM no. 377 medium, which omitted 0.5% casamino acids but contained 0.5% (w/v) skim milk(No. 4), pH 8.0 at 37°C. Culture broth after 48 h cultivation was centrifuged at 20,000 g for 20 min at 4°C, and the supernatant was used for enzyme purification.

For enzyme preparation, the supernatant containing the extracellular enzyme were concentrated nearly 50-times using Amicon concentration cell with YM-10 ultrafiltration membrane having molecular weight cut-off 10,000Da and subsequently diafiltered against ten volumes of 50mM Tris-HCl, pH 8.0. The pressure of the cell was kept at 20-30 psi by nitrogen gas.

3.3.3.2. Purification procedure

All of the following purification steps were performed at 4°C by using the ÄKTA explorer purification system (GE Healthcare). The concentrated supernatant was chromatographed on a HiTrap Q XL column (0.7 × 2.5 cm) (GE Healthcare), which was equilibrated with approximately five bed volumes of 50mM Tris-HCl, pH 8.0. The sample was loaded onto the column at a flow rate of 0.5 ml/min. The column was eluted with linear salt gradient (0-2 M NaCl in 50 mM Tris-HCl, pH 8.0). Fractions of 0.5 ml were collected and fractions containing protease activity from the HiTrap Q XL column were pooled and further purified by Superose 12 column. Absorbance at 280 nm (A_{280}) of each fraction was also measured.

Pooled fractions with protease activity from the HiTrap Q XL column were concentrated by ultrafiltration devices with 10,000 MWCO prior to size exclusion chromatography. The sample was applied to a Superose 12 10/300 GL column (1.0 x 30 cm) (GE Healthcare) previously equilibrated with approximately two bed volumes of 50mM Tris-HCl containing 0.15 M NaCl. The sample was loaded onto the column and then eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions (0.5 ml) were collected and the protease active fraction were pooled and used for further studies. The purity of the purified enzyme was analyzed by polyacrylamide gel electrophoresis.

3.3.3.3 Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenol blue). The samples (15 μg) were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using an ATTO AE-6530 Dual mini-slab system. After electrophoresis, the gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 7% acetic acid.

Native-PAGE was performed using 12.5% separating gels in a similar manner with SDS-PAGE, except that the addition of SDS and reducing agent was omitted (Appendix D-2).

3.3.3.4 Activity staining

The sample were subjected to electrophoresis and stained for protease activity by the modified method of Garcia'-Carreno et al., 1993. After electrophoresis, the gel was immersed in 100 ml of 2% w/v casein Hammarsten in 50 mM Tris-HCl, pH 8 for 1 h at 0°C to allow the substrate to penetrate into the gels. Then, gel was immersed in 100 ml of 2% w/v casein Hammarsten in 50mM Tris-HCl, pH 8 containing 10% w/v NaCl and incubate at 55°C for 30 min under gentle shaking. The gel was fixed and stained with 0.125% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 7% acetic acid. The development of clear zone on the blue background indicated proteolytic activity.

3.3.4 Characterization of the purified protease from strain ND1-1

3.3.4.1 Determination of molecular weight

The molecular weight of the purified protease was determined using size exclusion chromatography on Superose 12 10/300 GL column (1.0 × 30 cm) (GE Healthcare), at 4°C using the ÄKTA explorer purification system (GE Healthcare). The protein solution was passed through the column equilibrated with approximately two bed volumes of 50 mM Tris-HCl, pH 8 containing 0.15 M NaCl at a flow rate of 0.1 ml/min and then eluted with the same buffer. A flow rate of 0.1 ml/min was maintained throughout.

The protease from ND1-1 separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the purified histamine dehydrogenase. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included thyroglobulin (bovine) (M_r 670,000), γ-globulin (bovine) (M_r 158,000), ovalbumin (chicken) (M_r 44,000), myoglobin (horse) (M_r 17,000) and vitamin B_{12} (M_r 1,350).

3.3.4.2 Effect of temperature on activity and stability

Activity of the purified enzyme was assayed at different temperatures in the range of 30-80°C as previously described for the enzyme assays. To study the effect of temperature on enzyme stability, the purified enzyme previously was incubated at various temperatures ranging from 30-80°C for 1 h after that the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions.

3.3.4.3 Effect of pH on activity and stability

The activity of the purified enzyme was assayed at 55°C over the pH range of 4.0–11.0 by using universal buffer (Britton and Robinson, 1931) which consists of 40 mM of 40 mM H_3PO_4, 40 mM acetic acid, 40 mM H_3BO_3, the different volume of 0.2 NaOH. The pH stability experiment was conducted by incubating the enzyme without substrate in universal buffer with pH values ranging from 4.0-11.0 at 4°C for 24 h and measuring residual activity under the standard assay conditions.

3.3.4.4 Effect of salt concentration on activity and stability

To study the effect of salt, the purified enzyme was dialyzed against 25 mM Tris-HCl buffer, pH 8.0 at ratio of 1:100 v/v at 4°C. Then, the activity was measured in various NaCl concentrations (0-30% NaCl) at 55°C, pH 8.0 . For the effect of NaCl on stability, the dialyzed purified enzyme was incubated at various concentrations of NaCl concentrations at 30°C for 24 h. Then the NaCl concentration was adjust to 10% NaCl w/v and remaining activity was determined.

3.3.4.5 Effect of inhibitors on the purified protease activity

The effect of inhibitors on the purified protease activity was determined by incubating the purified enzyme with an equal volume of enzyme inhibitor solution to obtain the final concentration designated (0.1 mM chymostatin, 10 μM E-64, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), 0.1 mM leupetin, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM pepstatin A, 10 μM phosphoramidon, and 0.02 mg/ml soybean trypsin inhibitor (SBTI)). The mixture was allowed to stand at 37°C for 1 h. Thereafter, the remaining activity was measured and the percent inhibition was calculated. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

3.3.4.6 Effect of metal ion on the purified protease activity

The effect of metal ion were investigated by using $CuSO_4$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeSO_4$, and $ZnCl_2$. The purfied enzyme was pre-incubated in absence and the presence of cation such as Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} with a final concentration of 1 mM in 50mM Tris-HCl, pH 8.0 for 1 h at 37°C. The relative protease activity was measured at optimum condition.