CHAPTER III

MATERIALS AND METHODS

Instruments, materials, chemical reagents, and media

Name list of all instruments, materials, chemical reagents, and media were shown in Appendix A.

Methods

1. Screening of protease-producing halophilic bacteria

1.1 Screening of protease-producing halophilic bacteria on agar plate

A total of 65 samples of Pla-ra were collected from markets in Thailand (Table 7). Samples collected in sterile plastic containers, diluted in 10% NaCl solution were plated not later than 24 hours after collection. Protease producing ability of the cultures was screened qualitatively in halobacterium agar medium JCM No. 168. containing 10% or 15% (w/v) NaCl (Appendix B-1), 1% skim milk and incubated at 37 °C for 7-10 days. Colonies with clear zones were selected for the further study.

1.2 Quantitative protease activity assay (caseinolytic activity)

A loopful of 12 strains that have clear zone on halobacterium medium JCM No. 168. containing 15% (w/v) NaCl was inoculated in 125-ml Erlenmeyer flask containing 20 ml of halobacterium medium JCM No. 168 containing 10% or 15% (w/v) NaCl. After 3 days of incubation at 37°C on a rotary shaker operated at 200 rpm, the cell-free supernatant recovered by centrifugation at the speed of 10,000 rpm at 4°C for 20 min was used for extracellular protease activity (caseinolytic activity) assay determined by the method described by Hiraga et al. (2000). Sample (0.5 ml) was incubated with 1.5 ml of 1.33%(w/v) Hammarsten casein in 50 mM Tris-HCl, pH 7.5 buffer containing 10% NaCl at 37° C for 60 min. The enzymatic reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 2,500 rpm for 10 min to remove precipitate. 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. The absorbance

of the color developed at 37° C for 20 min 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 μmol of tyrosine 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.

The cell pellet dried at 120° C for 3 hrs was used for the cell dry weight determination. Total protein in the supernatant was measured by the method described by of Lowry et al. (1951). The details of the analytical method is described clearly in Appendix C.

2. Identification methods

2.1 Cell morphology and cultural characteristics

The colonies on the halobacterium medium JCM No. 168 containing 10% NaCl after 1 day incubation at 37°C were examined for their characteristics (Barrow and Feltham, 1993).

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. Decolorized 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.

2.1.2 Flagella stainning. Standard microscope slide, precleaned 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 halobacterium medium JCM No.168 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 C-3) 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.

2.2 Physiological and biochemical characteristics

2.2.1 Oxidase test . Each colony was streaked on halobacterium medium JCM No. 168 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.

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.

2.2.3 Growth at different temperatures. The halobacterium medium JCM No. 168 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.

2.2.4 Growth in different NaCl concentration. The halobacterium medium JCM No. 168 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.

2.2.5 Growth at different pH. The halobacterium medium JCM No. 168 broth adjusted to pH 5, 6, 8 and 9 was used for the growth of cultures incubated at 37°C for 7 days.

2.2.6 L- Arginine hydrolysis. Inoculate the arginine agar slant (Appendix B-3) with the cultures and incubated for 7 days. A positive reaction is shown by a colour change of the indicator to red.

2.2.7 Aesculin hydrolysis. Inoculated aesculin broth (Appendi B-4) containing 10% NaCl was examined daily up to 5 days for blacking of hydrolysis.

2.2.8 Gelatin hydrolysis. Bacterial cells were inoculated on the halobacterium agar medium JCM No. 168 (Appendi B-5) 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.

2.2.9 Starch hydrolysis. Inoculate halobacterium agar medium JCM No. 168 (Appendi B-6) 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.

2.2.10 Tyrosine hydrolysis. Bacterial cell were inoculated on the halobacterium agar medium JCM No. 168 (Appendi B-7) containing 0.5% tyrosine but omitted casamino acid and incubated at 37°C for 7 days. Clear zone indicate areas of the tyrosine hydrolysis.

2.2.11 Deoxyribonuclease (DNase) activity. Inoculate the cultures on DNase test agar (Difco) (Appendix B-8) for 7 days. After incubation, flood the plates with 1 N HCl then a clear zone around the growth appeared indicated the positive result.

2.2.12 Indole test. Inoculate the cultures in tryptone broth (Appendi B-9); and incubated at 37°C for 7 days. After incubation, it was tested by adding 4 drops of Kovacs' reagent (Appendix C-4), using *iso*-amyl alcohol as solvent.

2.2.13 Nitrate reduction. Inoculate nitrate broth (Appendix B-10) with the cultures and incubated for 7 days. A drop of each Solution A and Solution B reagent (Appendix C-5) was added. When nitrite is present a pink colour developed within 5 minutes.

2.2.14 Acid from carbohydrate. The acid from carbon sources was performced in marine oxidation-fermentation medium or MOF (Appendix B-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 37ºC. Growth was recorded daily for up to 7 days. The positive results were shown by colour change of the indicator from red to yellow.

2.3. Chemotaxonomy

2.3.1 Quinone analysis. The isolates were grown in a 300 ml halobacterium medium JCM No.168 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.

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2.3.2 Cell wall analysis

2.3.2.1 Whole-cell hydrolysis. Approximately 3 mg of dried cell were hydrolysed with 1 ml of 6 N HCl in a screw-capped tube at 100 ºC for 18 hours. After cooling, the hydrolysate was filtered to dryness by a rotary evaporator. The dried material was dissolved in 1 ml. of water and dried again. The residue was redissolved in 0.3 ml of water.

2.3.2.2 Thin- layer chromatography(TLC). Each sample was applied as 3 μl on the base line of a plastic cellulose TLC plate (Merck No. 5577, E.

Merck, Darmstadt, FRG). As a standard, 1µl of 0.01 M DL-diaminopimelic acid (Sigma Chemical Co., St. Louis, Mo., USA) was also applied for reference. TLC was developed with the solvent system methanol-water-6N HCl-pyridine (80:17.5:1.5:10, V/V) Development will take approximately 3 hours or more. The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-buthanol followed by heating at 100 °C for 5 min. DAP isomers appeared as dark-green spots with R_f 0.29(LL-isomer)and 0.24(*meso*-and DD-isomer). 3-Hydroxy- DAP appeared lower than the *meso*-isomer (R_f approximately 0.02). Spots of other amino acids run faster than DAP (R_f = 0.37-0.08). Spot will gradually disappeared in a few hours.

2.3.3 DNA base composition

2.3.3.1 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 halobacterium medium JCM. No.168 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)

2.3.3.2 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 G) in water bath at 50 °C for 1 hour. After incaubation, 10 µl of alkaline phosphatase solution (Appendix G) was added and keep at 37 °C for 1 hour. After DNA hydrolysis, the sample were analyzed by HPLC with condition show in Table 6.

Table 6. 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
Sample	5-10 µl

2.4 <u>16S rDNA sequence and phylogenetic analysis</u>

The 16S rRNA gene was PCR amplified using the forward primer 9F (5'GAGTTTGATCCTGGCTCAG'3 , *Escherichia coli* numbering) and reverse primer 1541R (5'AAGGAGGTGATCCAGCC'3). Sequencing of amplified 16S rRNA gene was analyzed with an automated DNA sequencer ABI PRISM 377 Genetic analyser (Applied Biosystems) with the following primer: 339F (5'CTCCTACGGGAGGCAGCAG'3), 785F (5'GGATTAGATACCCTGGTAGTC'3),1099F(5'GCAACGAGCGCAACCC'3), 357R (5'CTG CTGCCTCCCGTAG'3) and 802R (5'TACCAGGGTATCTAATCC'3). The sequence was multiply aligned with the CLUSTAL X program (version 1.83; Thompson et al., 1994), then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbour-joining

method (Saito and Nei, 1987) in MEGA program version 2.1 (Kumar et al., 2001). The confidence values of branches of the phylogenetic tree were determined using 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 version 1.83. Gap and ambiguous nucleotides were eliminated from the calculations.

2.5 DNA-DNA hybridization labelled with photobiotin

2.5.1 DNA-DNA hybridization ; Under optimal condition, 100 µl portion of heat-denatured, purified DNA solution of unknown and type strains (10µg of DNA /1ml) in phosphate-buffered saline comtaining 0.1 M $MgCl_2$ were incubated for 2 hours at 37ºC in microdilution plates (Nunc Corp., Denmark). Photobiotinylation of DNA was performed, by meaning that 10 µg /1 ml of photobiotin and an equql volume of DNA solution (10 µg of DNA / 1 ml) were mixed in an eppendorf tube and then irradiated with sunlamp(500W) for 25 min. After irradiation, free photobiotin was removed by n-butanal extraction. The biotanylation of DNA was used immediately for hybridization experiment.

For quantitative detection of biotinylated DNA in microdilution wells, 200 µl of a prehybridize solution (20xSSC, 5%Denhadt solution, 50% formamide) containing 10 µg of denatured salmon sperm DNA / ml was added to microdilution plates and then incubated at 39°C for 1 hr. The prehybridize solution was discarded and replaced with 100 µl portions of hybridization mixture (20xSSC, 5% Denhadt solution, 3%dextrane sulfate, 50% formamide, 10 µg ofdenatured salmon sperm DNA / ml) contraining 10 µg of bitinytlated DNA. The microplates were then covered with aluminium foil, and incubated for overnight (16 hours) at 39°C. After hybridzation, the microdilution well were washed three times 200 µl of 0.2 x SSC buffer. A 100 µl of streptavidin peroxidase conjugate solution (Boehringer Mannheim Germany) was added to the wells, and the preparations were incubated at 37°C for 30 min. After incubation, the wells were washed three times with PBS-buffer. Then the enzyme solution was discarded and 100 µl of substrate 3,3',5.5' – tetramethyl benzidine – H_2O_2 solution (Wako,Japan) was added to each well. The plates were inculated at 37°C for

10 min. The reaction was stopped with 2 M H_2SO_4 and the colour intensity was measured with Microplate Reader Model 3350 (Bio-Rad, CA,USA) at a wavelength 450 nm. The homology values for the DNA-DNA hybridization were calculateted. In practice, a DNA-homology above 70% indicates a relationship in the species level reported by Wayne et al., 1987.

3. Optimization of crude protease production

One loopful of strain NB2-1 grown in 200 ml of halobacterium medium JCM No. 168 (Appendix B-1) containing 15% (w/v) NaCl in 1000 ml Erlenmyer flask and incubated with shaking (200 rpm) at 37°C for 2 days was used as inoculum.

A 2.5 ml of the inoculum was inoculated in 50 ml of the same medium in 250 ml Erlenmyer flask and incubated at the same above condition. The influence of NaCl concentration, cultivation time, initial pH, incubation temperature and the medium composition on protease production was determined by varying each parameter. An optimal condition of prior experiment was used as the basis in the latter experiment to optimize the conditions. Samples taken at different times were assayed for protease activity in cell-free supernatant and monitored for growth by measuring an absorbance at 660 nm.

4. Characterization of crude protease

The protease activity was determined by the method described by Hiraga et. al. (2000). Hammarsten casein (Research organics, Germany) dissolved in 50 mM Tris-HCl, pH 7.5 containing 10% (w/v) NaCl, at 1.33% (w/v) was use as substrate. A 0.5 ml of crude protease was incubated with 1.5 ml of substrate solution at 37° C for 60 min. The reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid (TCA), and then centrifuged at 2,500 rpm for 10 min to remove precipitate. After that 0.5 ml of the resulting supernatant was mixed with 2.5 ml of 0.44 M sodium carbonate and 0.5 ml of the Folin-Ciocalteu reagent and incubated at 37° C for 20 min. The amount of tyrosine occurred was quantified by measuring an absorbance at 660 nm. Protein concentration was analyzed by the method described by lowry et. al. (1951) using bovine serum albumin as standard. The influence of reaction pH, incubation temperature, and NaCl

concentration on protease activity was determined by varying each parameters. The effect of various kind of protease inhibitors including 20 μM E-64, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 2 μM pepstatin and 0.2 mg/ml trypsin on protease activity was analyzed after incubation of an equal volume of crude protease and the above protease inhibitors at room temperature (26° - 28°C) for 30 min.

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