CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals

- Barium chloride ($BaCl_2$) (Fluka, Switzerland)
- Calcium chloride ($CaCl_2$) (E. Merck, Germany)
- Galacturonic acid (Fluka-Biochemika, Slovakia)
- Gentamicin sulfate (Sigma Chemical Co. Ltd., U.S.A.)
- Hydrochloric acid (HCl) 36.5-38%, analytical grade (J.T. Baker, U.S.A.)
- Magnesium chloride ($MgCl_2$) (E. Merck, Germany)
- Mueller hinton agar (E. Merck, Germany)
- Mueller hinton broth (E. Merck, Germany)
- Potassium chloride (E. Merck, Germany)
- Sodium chloride (NaCl) (Merck, Germany)
- Sodium hexametaphosphate (Carlo. ERBA, Germany)
- Sodium hydrogencarbonate (E. Merck, Germany)
- Tryptic soy agar (TSA) (E. Merck, Damsatadt, Germany)
- Tryptic soy broth (TSB) (E. Merck, Damsatadt, Germany)
- Zinc chloride ($ZnCl_2$) (Unilab, Australia)

2. Equipments

- Analytical balance (Mettler Toledo, PL602-5, Switzerland)
- Autoclave HA-3D (Hirayama Manufacturing Coorperation, Japan)
- Blender (Moulinex 327, Spain)
- Cylinder (Pyrex, U.S.A.)
- Digital camera (Cannon Power shot S50)
- Hot air oven (Mammert, Becthai Co., Ltd., Thailand)
- Hot plate (E.G.O., Germany)

- Incubator Model 6 (Thelco, U.S.A.)
- Magnetic stirrer (Model SP 46920-26, Barnstead/Hermodyne, U.S.A.)
- Micropipette (Pipetman, France)
- pH meter (MP 230, Mettler Toledo, LE413, ME 51340 251, Switzerland)
- pH paper (pH 0-14) (E. Merck, Germany)
- Refrigerator (Sharp TH-8903, Thailand)
- Rotary evaporator (Buchi R-200, Switzerland)
- Stirrer (KMO2, Janke and Kenkel GMBC and Co. KG, Germany)
- Suction apparatus (Buchner Funnel, Aspirator, SIBATA circulating aspirator WJ-20, Japan)
- Vertical laminar air flow carbinet (LFV-60, MSSP, Thailand)
- Viscometer (Brookfield, Model LVDV-I+, Brookfield Engineering Laboratories INC., USA)
- Vortex mixer (Vortex-2 genie, U.S.A.)

Methods

1. Preparation of polysaccharide gel (PG) from Durian rinds

1.1 PG isolation

A polysaccharide gel (PG) was isolated from dried fruit-hulls of durian (*Durio zibethinus* Murr.). Waste of fresh durian fruit-hulls was collected, washed, blended and dried in hot air oven at 70°C. A process of PG isolation was performed based on the method previously described by Pongsamart and Panmuang (1998). PG was purified by reprecipitating of crude PG solution using acidic solution of ethanol. The precipitate of PG was dried and powdered.

1.2 Preparation of test solution of PG

Solution of polysaccharide gel (PG) was freshly prepared by dissolving PG powder in sterile distilled water overnight and sterilized by autoclaving. The 5% w/v PG solution was diluted to make a series of two fold dilutions of various concentrations of polysaccharide gel in sterile distilled water and added to the agar and broth media to make the desire concentrations for microbiological test.

2. Test microorganisms and preparations of inoculum

All microorganisms of cow mastitis isolates were obtained from cause of bovine mastitis in which *Staphylococcus* spp., *Streptococcus* spp. *Escherichia coli*, *Klebsiella* spp. and *Pseudomonas* spp. Department of Veterinary Medicine, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok has been collected. Isolates of *Staphylococcus* spp. and *Streptococcus* spp. was used as test gram positive bacteria and *Escherichia coli, Pseudomonas* spp. and *Klebsiella pneumoniae* were used as test gram negative bacteria.

All test bacteria were cultivated overnight on tryptic soy agar (TSA) slant at 37°C except *Streptococcus* spp. was cultivated overnight on 5% sheep blood

TSA. The bacterial suspension were prepared by washing bacteria from a surface of the agar slant with sterile normal saline solution (0.9% NaCl, NSS). The culture suspension was then adjusted to match turbidity of standard McFarland no. 0.5 (10^8 CFU/ml) before used.

3. Preparation of agar and broth media

All agar and broth media were dissolved in water and sterilized in autoclave for 20 minute at 15 pounds pressure (121° C). Mueller hinton agar (MHA) and Mueller hinton broth (MHB) were used for agar diffusion susceptibility test and broth microdilution susceptibility test, respectively. All test bacteria were tested on MHA and MHB excepted *Streptococcus* spp. was tested on Tryptic soy agar (TSA) and Tryptic soy broth (TSB).

4. Determination of antimicrobial activity of polysaccharide gel

4.1 Agar diffusion susceptibility test

The antibacterial activity against *Staphylococcus* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Streptococcus* spp. were tested as described in the standard guideline technique (Lorian, 1991). Agar diffusion testing was performed as follows: serial two - fold dilutions of PG concentrations 50 to 3.12 mg/ml in sterile distilled water were freshly prepared. MHA with 1% bacterial suspension (the bacterial colonies from TSA slant were suspended in sterile NSS, diluted in NSS and adjusted to match turbidity of standard Mcfarland no. 0.5 before used) was seeded over the solidified base layer of the same medium in petri dishes (Petri dishes with internal diameter of 100 mm containing 20 ml bottom layer of enriched agar media were inoculated using 5 ml agar media with 1% microorganism suspension as top layer by seed layer method (Lorian, 1991). Sterile stainless steel cylinder cup (6 mm internal diameter 8.5mm external diameter and 10 mm height) were placed on the inoculated agar surface. The various concentrations of PG were filled into the cylinders (300 µl/cylinder). After pre–diffusion at room temperature for 1 h, the inoculated plates were incubated at 37° C overnight. The results were

obtained by measuring the diameters of inhibition zone by Vernia (mm). The determination were carried out in triplicate and distilled water was used as control.

4.2 Broth microdilution susceptibility test

4.2.1 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimal inhibitory concentration (MIC) values were determined by a broth microdilution test as described by NCCLS (1990). Fresh cultures of each strain, *Stapphylococcus* spp., *Escherichia coli, Klebsiella* spp., *Pseudomonas* spp. and *Streptococcus* spp., at a final inoculum of $5x10^5$ colony forming units per ml (CFU/ml) by adjusting turbidity to match McFarland No.0.5 were used to inoculate each well (100 µl) of a 96–well microtitre plate. Serial two-fold dilutions of PG concentration 50-3.12 mg/ml were made, the microtitre plates were incubated at 37°C overnight. After 24 h incubation the presence or absence of growth was investigated. The MIC was defined as the lowest concentration of PG that inhibited growth of bacteria compared with the growth control plate. MBC was determined by subculturing from wells showing no turbidity of bacterial growth onto PG-free agar media, MBC was the lowest concentration of PG showing no growth on PG-free agar media after 24h incubation. Media without PG were used as a culture control. Gentamicin sulfate at concentrations ranging from 64-0.003 µg/ml was used as a positive control.

Antibiotic susceptibility test was determined as a positive control to ensure that the test system is functioning properly. Determination of the antimicrobial activity of antibiotic was performed by the same procedure of broth microdilution susceptibility test as described for PG testing. Gentamicin sulfate was used as antibacterial agent against all test bacteria. Gentamicin sulfate powder was completely dissolved in sterile water and serial doubling dilutions were freshly prepared in sterile MHB to obtain concentrations ranging from 64-0.003 µg/ml.

4.3 Time-kill analysis

Time-kill analysis is an extension of the MBC and give information on the rates at which organisms are killed. Cultivation of bacteria in NSS and media without PG were used as culture control to observe bacterial survival pattern. The inoculum was prepared by adjusting the turbidity to match McFarland No. 0.5 of an actively growing broth culture in Mueller Hinton Broth (MHB) to give the desired initial inoculum of 10^5 CFU/ml. Samples (50 µl) were removed at time points 0, 1, 2, 4, 6, 8, 10 and 24 h from each reaction, visible counts were performed by preparing 10-fold serial dilution of the initial sample in NSS, and 25 µl drop on MHA. Plates were incubated at 37°C for 24 h and colony counts (CFU/ml) were determined. The result were presented as the logarithmic change from the initial starting value determined at the start of the experiment with a negative sign indicating a decrease in colony counts. All time-kill experiments were conducted in duplicate. Killing curves were constructed by plotting $\log_{10}$CFU/ml against time over 24 h. The inoculated media with PG were incubated at 37° C for 24 h. At the indicated time, the culture of serial dilution in NSS was counted by drop plate method (Lorian, 1991) on agar media (MHA). Visible colonies were observed and calculated as colony forming unit (CFU) per milliliter. Survival was plotted with time against the logarithm of the visible count. The sample were vortex mixed and incubated for 24 h at 37°C.

4.4 Effect of various factors on antibacterial activity of PG

The objective of these experiments was to examine the effect of several environmental factors on the antibacterial activity of PG against the represent gram positive and gram negative bactria, *Staphylococcus* spp. and *E. coli*, respectively. The factors examined in this assay included pH, ionic strength, metal ions and temperature. Four main bacteria, *S. aureus* (ATCC 25923), *E. coli* (ATCC 25923) and 1-2 species of *S.aureus* and *E. coli* isolates from cow mastitis were tested to evaluate the potential of PG as natural antibacterial agent.

4.4.1 Effect of pH on antibacterial activity of PG

Tests were conducted in two sets: a test set with PG and control set without PG. In the test set with PG, PG was dissolved in distilled water and pH was adjusted with 1M HCl and 1M NaOH from 1 to 9. The above operation was carried out under sterile conditions *S. aureus*, *E. coli* and 2 isolates were separately inoculated into the PG solution at concentration 10^7 CFU/ml. The control sets were prepared using the same method except that no PG was added.

4.4.2 Effect of ionic strength on antibacterial activity of PG

PG was dissolved in distilled water to make 5% w/v and diluted with nutrient broth (MHB) 50 ml. In the test set, the ionic strength of PG solution was adjusted to 25 and 100 mM respectively, by adding a NaCl aqueous solution. The entire operation was conducted under sterile conditions. *S. aureus*, *E. coli* and 2 isolates were separately inoculated into PG solution at a concentration of 10^7 CFU/ml. At 24 h, two samples were collected from each cell suspension, and cell numbers were measured using the drop plate count method.

4.4.3 Effect of metal ions on antibacterial activity of PG

PG was dissolved in distilled water. The PG solution was mixed with nutrient broth (MHB) then 0, 10 and 20 mM of metal solution of $BaCl_2$, $CaCl_2$, $MgCl_2$ and $ZnCl_2$ was separately added to the PG solution. *S. aureus*, *E. coli* and 2 isolates were separately inoculated into PG solution at a concentration of 10^7 CFU/ml. The control sets (PG solution without metal ion) were prepared and the same metal ions concentration as the test set was adjusted. Triplicate experiments were prepared for each test set and control set and incubated at 37°C for 24 h. Two samples were collected from each cell suspension, and cell numbers were calculated using the drop plate count method.

4.4.4 Effect of temperature on antibacterial activity of PG

PG was dissolved in distilled water. The PG solution was mixed with nutrient broth (MHB). *S. aureus*, *E. coli* and 2 isolates were separately inoculated into PG solution at a concentration of 10^7 CFU/ml and incubated at 37°C and 25°C for 24 h., Triplicate experiments were prepared for each test set and control set. Two samples were collected from each cell suspension, and cell numbers were calculated using the drop plate count method.