# CHAPTER III
# EXPERIMENTAL

## 3.1 Materials

*A. xylinum* TISTR 975, *E. coli* TISTR 780 and *S. aureus* TISTR 1466 were purchased from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR). Chitosan A (MW 34,500 and 80 % Deacetylation) and Chitosan B (MW 100,000 and 80 % Deacetylation) were purchased from Seafresh. D-glucose anhydrous (analytical grade) was purchased from Ajax Finechem. Yeast extract powder (bacteriological grade) was purchased from Himedia. Sodium hydroxide anhydrous pellets (analytical grade) were purchased from Ajax Finechem. Glacial acetic acid (analytical grade) was purchased from Merck. *B.mori* silk cocoons were obtained from Queen Sirikit Sericulture Center (Thailand).

## 3.2 Methodology

### 3.2.1 Bacterial Cellulose Pellicle Preparation

#### *3.2.1.1 Culture Medium*

4% D-glucose and 2% Yeast extract powder were mixed in distilled water as a culture medium for the production of bacterial cellulose by *A. xylinum* (TISTR 975) fermentation, then culture medium was sterilized by autocleaving at 115°C for 15 min.

#### *3.2.1.2 Cultivation of Bacterial Cellulose Producing*

Pre-inoculum was prepared by adding *A. xylinum* TISTR 975 in a 20 mL of culture medium. After a static incubation at 30°C for 2 days, the bacterial cellulose pellicle appeared on the surface of culture medium. After that, the stock culture was prepared by adding a 20 mL *A. xylinum* in culture medium into a 250 mL of new culture medium. Then, the stock culture was static incubated at 30°C for 4 days and kept it in refrigerator prior to use. The stock culture was activated in

an incubator at 30°C for 30 min before used. A 10 mL of stock culture was transferred to a 500 mL Erlenmayer flask containing 100 mL of culture medium, followed by a static incubation at 30°C for 4 days (Maneerung *et al.*, 2007).The bacterial cellulose pellicle with an appropriate thickness was received.

*3.2.1.3 Bacterial Cellulose Purification*

The bacterial cellulose pellicles were purified by boiled them in 1.0 %(w/v) sodium hydroxide solution at 90°C for many times to remove bacterial cells and to deproteinize protein from yeast extract powder, the neutralized with 1.5 %(v/v) acetic acid at room temperature for 30 min and washed with distilled water until pH becomes neutral and kept in distilled water prior to use.

3.2.2 Sericin Solution Preparation.

Silk cocoons were cut into small pieces, then washed in distilled water and dried in an oven at 65°Cfor 24 hours. 10 grams of silk cocoons were boiled in 100 mL of distilled water in an autocleaving at 121°C for 30 min. After that, silk cocoons were filtered out to get sericin solution. Finally, sericin solution was dried by freeze drying process for 24 hours.

3.2.3 Chitosan and Sericin-incorporated Bacterial Cellulose

Chitosan A (CTSN A) and chitosan B (CTSN B) were dissolved in 1.0 %(v/v) acetic acid and 2.0 %(v/v) acetic acid, respectively. Sericin was dissolved in water by autoclave at 121 °C for 10 min. The chitosan and sercin solution were blended at different chitosan/sericin blend ratio as follows 100/0, 75/25, 50/50, 25/75 and 0/100. After that, freeze-dried bacterial cellulose was immersed in 15 mL of blend solution and heated in an autoclave for 2 hours to obtain bacterial cellulose containing chitosan and silk sericin. Finally, it was dried by freeze drying process for 48 hours.

## 3.3 Characterization and Testing

3.3.1 Fourier Transformation Infrared Spectroscopy (FTIR)

The chemical structure and conformation of bacterial cellulose, chitosan, sericin, chitosan and sericin-incorporated bacterial cellulose were

characterized by a Thermo Nicolet Nexus 671 FTIR spectrophotometer. The spectra were collected at a resolution of 4 $cm^{-1}$ and 64 scans in the wavenumber range of 4000 $cm^{-1}$ to 650 $cm^{-1}$

3.3.2 Field Emission Scanning electron Microscopy (FE-SEM)

A HITACHI S4800 FE-SEM microscope was used to observed surface and cross sectional morphology of the bacterial cellulose, chitosan and sericin-incorporated bacterial cellulose at an operating voltage of 5.0 kV. The specimens were coated with platinum by using sputtering equipment operated for 200 seconds before the SEM observation.

3.3.3 Kjeldahl Analysis

A Kjeldahl analysis was used to determine the amount of chitosan and sericin incorporated in bacterial cellulose pellicle. The sample was digested by using 10 mL of concentrated sulfuric acid and 0.1 g of copper sulfate as a catalyst which expedited the conversion of amine nitrogen to ammonium sulfate solution. After that, the mixture was heated by using heating mantle at 400°C for 1 hour. Then, 35 %(v/v) hydrogen peroxide solution was dropped to the mixture until getting a clear solution, followed by subjecting the digested sample to the distillation unit. A 250 mL Erlenmeyer flask containing 30 mL of 0.1 M hydrochloric acid and 3 drops of phenolphthalein, used as an indicator, was set to the end of the unit. Therefore, the conversion of ammonium sulfate solution to ammonia gas was done by dropping a 40 %(v/v) sodium hydroxide solution to the digested sample. After that, in the distillation step, the ammonia gas was trapped with hydrochloric receiving solution by boiling and condensation. The amount of ammonia entrapped in hydrochloric solution was determined by back titration with a 0.1 M sodium hydroxide solution by using pH meter to determine the end point of titration. The chitosan and sericin content ware calculated based on nitrogen percentage by using the following equation (1):

$$\text{Nitrogen Content (mg)} = (M_{HCl} \times V_{HCl}) - (M_{NaOH} \times V_{NaOH}) \times 14 \times 1000 \quad (1).$$

3.3.4 Water Vapor Transmission Rate

The water vapor transmission rate (WVTR) was determined according to European pharmacopiae (Razzak *et al.*). A water container with diameter of 33 mm was covered firmly on the top with a sample of BC containing chitosan and sericin firmly. Then, the container was placed in an incubator at a temperature of 35.0 °C for 24 hour. The samples were weighed before and after incubating in an incubator. The WVTR was calculated according to equation (2).

$$\text{WVTR } (g/m^2/day) = \frac{(W_i - W_t)}{A} \times 10^6 \quad (2).$$

where, $W_i$ and $W_t$ are the weight of bottle before and after place in an incubator respectively, A is area of bottle mouth ($mm^2$)

3.3.5 Antioxidant Activity

The antioxidant activity of the chitosan and sericin-incorporated bacterial cellulose samples was determined based on the 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity method. Each specimen was cut into disc shape with a diameter of 1.5 cm and then put the sample in 3.5 mL of methanolic solution containing DPPH reagent at a concentration of $1 \times 10^{-4}$ mol/L, and the mixture was vortexed at a high speed. Then, the mixture was kept in the dark at room temperature for 30 min. After that, the free radical scavenging activity of the clear supernatant was determined using a UV/Vis spectrophotometer at a wavelength of 517 nm and compared with a blind control containing DPPH and distilled water instead the presence of sericin was also assayed, The scavenging activity of samples at different Chitosan/Sericin blend ratio was calculated by using the following equation:

$$\text{Scavenging activity } (\%) = \frac{(A_0 - A_x)}{A_0} \times 100 \quad (4).$$

where $A_x$ and $A_0$ refer to the absorbance of the DPPH solution with and without sericin, respectively.

3.3.6 Antibacterial Activity

Antibacterial activities of the chitosan and sericin-incorporated bacterial cellulose were evaluated in a comparison with those of the pure BC based on the colony forming count method (Watthanaphanit *et al.*). *E. coli* and *S. aureus* were selected as the representatives of gram-negative and gram–positive bacteria, respectively. The bacterial suspension was prepared by transferring one colony of microorganisms to 20 ml of nutrient broth (0.3% beef extract and 0.5% peptone) solution before being incubated at 37 °C in a shaking incubator at 120 rpm for 24 h. Next, 2 ml of bacterial suspension was added to 198 mL of 0.85% sterile saline solution. After that, the flask was incubated at 37 °C in a shaking incubator at 120 rpm for 24 h. The sterilized specimen was added into a vial containing the 10 mL bacterial suspension before being incubated at 37 °C in a shaking incubator at 120 rpm for 24 h. Then, 0.1 ml of bacterial suspension was serially diluted until the optimum bacterial concentration and withdrawn from the vial and subsequently spread on an agar plate. After incubation at 37 °C for 22 h, the bacterial reduction rate (BRR) was calculated by using the following equation:

$$\text{Bacterial reduction rate (\%)} = \frac{(N_1 - N_2)}{N_1} \times 100 \qquad (2)$$

where, $N_1$ is the number of colonies of the blank and $N_2$ is the number of colonies after incubation with the chitosan and sericin-incorporated bacterial cellulose samples.

3.3.7 Chitosan and Sericin Releasing Behavior

The chitosan and sericin-incorporated bacterial cellulose samples were cut into a circular shape with a diameter of 1.5 cm and amount of chitosan and sericin released from bacterial cellulose matrix was determined by a modified Franz diffusion cell. Chitosan and sericin-incorporated bacterial cellulose samples were place on cellulose acetate membrane (Pore size 0.45 μm). The two layers were clamped between the donor and receptor chamber of a modified Franz diffusion cell. The receptor chamber was filled with acetic/acetate buffer at pH 5.5 and the receptor medium was stirred by a magnetic bar and placed on a magnetic stirrer in a water

bath maintained at 32 °C. Then, the releasing medium was sampling at a specific time interval for 72 hours. The protein and chitosan releasing concentration in the medium was determined based on the BCA assay reagent (Pierce) with the use of a UV/Vis spectrophotometer at a wavelength of 562 nm and Ninhydrin assay (M.M. Leane *et al.*, 2004) with the use of a UV/Vis spectrophotometer at a wavelength of 570 nm, respectively. The amount of released sericin and chitosan were calculated from a calibration curve of bovine serum albulmin standard in the concentration range of 20 μg/mL to 2000 μg/mL.