CHEPTER III
EXPERIMENTAL

3.1 Materials

Acetobacter xylinum strain TISTR 975 was purchased from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR). D-glucose anhydrous (analytical grade) was purchased from Ajax Finechem. Yeast extract powder (bacteriological grade) was purchased from Biobasic. Sodium hydroxide anhydrous pellet (analytical grade) was purchased from Ajax Finechem. Glacial acetic acid (analytical grade) was purchased from RCI Labscan. Nylon mesh was purchased from NBC meshtec inc, Japan. And cotton mesh was purchased from Santext fabric, Thailand.

3.2 Methodology

3.2.1 Production of Bacterial Cellulose and Bacterial Cellulose Composites

3.2.1.1 Culture Medium

The culture medium used for bacterial cellulose synthesis of *Acetobacter xylinum* contained 4.0 % w/v D-glucose and 2.0 % w/v Yeast extract powder in distilled water. Then, the culture medium was sterilized by autoclaving at 121 °C for 15 min.

3.2.1.2 Culture Condition

Pre-inoculum was prepared by adding *Acetobacter xylinum* TISTR 975 in a 100 mL Erlenmeyer flask containing 200 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, 10 mL of stock culture medium was transferred to a 500 mL Erlenmeyer flask containing 100 mL of culture medium, followed incubation at 30 °C for 4 days. For production of bacterial cellulose composites, after 10 mL of stock culture medium was transferred to a 600 mL beaker

containing 100 mL of culture medium, immersed porous supporting fabric on the surface of culture medium, followed by incubation at 30 °C for 2 days.

3.2.1.3 Proparation of Pure Bacterial Cellulose

Acetobacter xylinum TISTR 975 Inoculum was added 10 mL in a 100 mL Erlenmeyer flask containing 200 mL of culture medium. After that moved into static incubation at 30 °C for 2 days the bacterial cellulose pellicle appeared on the surface of culture medium.

3.2.1.4 Proparation of BC/Cotton Composite Mon-Immobilization

Acetobacter xylinum TISTR 975 Inoculum was added 10 mL in a 100 mL Erlenmeyer flask containing 200 mL of culture medium. After that immersed cotton fabric on surface of culture medium. Then moved into static incubation at 30 °C for 2 days, the bacterial cellulose pellicle appeared on the surface of cotton fabric.

3.2.1.5 Preparation of BC/Cotton Composite by Using Immobilization

Acetobacter xylinum TISTR 975 Inoculum was dropped on cotton fabric 3 mL after that immersed cotton fabric on 100 mL of culture medium. After that moved into a static incubation at 30 °C for 2 days, the bacterial cellulose pellicle appeared on the surface of cotton fabric.

3.2.1.6 Purification of Bacterial Cellulose and Bacterial Cellulose Composites

After incubation, bacterial cellulose pellicles produced on the surface of culture medium were harvested and purified by boiling them in 1.0 % w/v sodium hydroxide solution at 90 °C for 2 hrs (repeated 3 times) to remove bacterial cells and culture medium, followed by neutralized with 1.5 % w/v acetic acid solution at room temperature for 30 min and then immersed in distilled water until pH become neutral. The bacterial cellulose pellicles were kept in distilled water prior to use.

3.2.2 Surface Treatment

The dielectric discharge has the thickness of 2 mm. The two parallel electrodes are stainless steel. The porous supporting fabrics were cut into square shape with the dimension of 10 x 10 cm and were put into the parallel plate dielectric

barrier discharge (DBD) plasma reactor for plasma treatment before being immersed in culture medium to produce bacterial cellulose composites. The experiment was operated with the condition of voltage of 50 kV, frequency of 325 Hz and the electrode gap of 5 mm. The flowing air gas was introduced directly through the gap of electrodes. In case of acid pre-treatment of cotton fabrics, citric acid and acetic acid were used. Cotton fabrics were cut into a square shape and then immersed in the acid solutions having different concentrations of 1, 2, 3, 4, and 5 % v/v overnight. The plasma and acid pre-treated cotton fabrics were dried prior to cultivation with *Acetobacter xylinum*

3.3 Characterization

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used to investigate the chemical functional groups of bacterial cellulose and fabrics. The FTIR spectra were measured at wavenumbers ranging from 4000 to 650 cm^{-1} with a Thermo Nicolet Nexus 670.

3.3.2 Scanning Electron Microscope (SEM)

The surface and cross sectional morphology of bacterial cellulose, bacterial cellulose composites and fabrics were observed by using scanning electron microscope (JEOL JSM 5410LV) operating at 15 kV.

3.3.4 Water Absorption and swelling Capacity

To determine the WAC, the freeze dried materials were immersed in distilled water at room temperature until equilibration. After that the materials were removed from water and the excess water at the surface of materials was blotted out with tissue paper. The weights of swollen material were measured and the procedure was repeated until no further weight change was observed. The WAC was calculated with the following equation:

$$WAC = \frac{W_h - W_d}{W_d}$$

Where W_h and W_d were the hydrate and dry weight, respectively.

3.3.5 Water Vapor Transmission Rate (WVTR)

Samples were cut into disc shape with diameter of 33 mm. The bottle containing 25 ml distilled water was weighted, after that the samples were placed and sealed on a mouth as a cap of bottle and placed in incubator at 35 °C for 24 hours. The WVTR was calculated according to the following equation:

$$WVTR = \frac{W_i - W_f}{A}$$

Where W_i and W_f were the weight before and after being placed in an incubator, respectively. A was area of the bottle mouth (m^2).

3.3.8 MTT Cytotoxicity Test

This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The MTT assay1 is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded into 6-well tissue culture dish on the sample's surface at a density of 100,000 cells/dish/3 ml, and incubated for 48 hours in fresh medium and then tested with MTT assay. Briefly, 500 µl of MTT in PBS at 5 mg/ml was added to the medium in each dish and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the dishes, and formazan solubilized with 1 ml of DMSO and 50 µl of Sorensen's Glycine buffer, pH10.5. The solution was then seeded into 96-well plate for the reading. The optical density was read with a microplate reader (Molecular

Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The experiments were done 3 times to get the values and standard deviation. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the %survival for each sample compared to controls.

3.3.8.1 Cell Culture

The target cells were human dermal skin fibroblast cell lines (HDF, neonatal, C-004-5C). The dermal skin fibroblast cells (L929) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 unit/ml penicillin and 100 ug/ml streptomycin. The cells were incubated at 37 °C in a fully humidified, 5% CO_2 : air atmosphere.

3.3.5 Cytocomplatibility

Samples were cut $1^{x}1$ cm and then immersed into culture medium of dermal skin fibroblast cells (L929) overnight. Next removed samples out of culture medium, add fibroblast cells L929 on surface of samples. After that incubation in static system 24, 48, 96 house. Cells behavior were evaluated by scanning electron microscope (JEOL JSM 5410LV) operating at 15 kV.