CHAPTER III
EXPERIMENTAL

3.1 Materials

Acetobacter xylinum (TISTR 975), Escherichia coli and Staphylococcus aureus were purchased from Microbiological resources centre, Thailand Institute of Scientific and Technological Research (TISTR). Nutrient Broth (Approximate formula*per liter: Beef extract 3.0g and Peptone 5.0g) was purchased from Difco^{TM}. Analytical grade D-Glucose anhydrous was purchased from Ajax Finechem. Yeast extract powder and Agar powder were bacteriological grade and purchased from HiMedia. Laboratory grade Calcium carbonate and analytical grade Silver nitrate were purchased from Fisher Scientific. Laboratory grade Sodium borohydride was purchased from CARLO ERBA. Analytical grade Sodium hydroxide anhydrate pellet and Sodium chloride were purchased from Aldrich Chemical. Analytical grade Glacial acetic acid was purchased from CSL Chemical. Ethanol was commercial grade and used without further purification.

3.2 Equipment

3.2.1 Fourier Transformation Infrared Spectroscopy (FTIR)

Qualitative and quantitative Fourier transform infrared spectra of bacterial cellulose was obtained from Bruker Equinox 55/S with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed using a deuterated triglycinesulfate detector with a specific detectivity, D*, of $1x10^9$ $cm.Hz^{1/2}$ w^{-1}.

3.2.2 Scanning Electron Microscope (SEM)

Cross section and surface morphology of bacterial cellulose was investigated by using JEOL/JSM 5200 scanning electron microscope (SEM) at 15 kV. Moreover, SEM was used to observe the presence of the entrapped-bacteria inside of bacterial cellulose before and after the purification.

3.2.3 UV-visible Spectrophotometer

The surface plasmon resonance of the silver nanoparticle-impregnated bacterial cellulose was investigated by using UV-Visible spectrophotometer (Hitachi U-2010 spectrometer).

3.2.4 X-ray Diffraction (XRD)

The formation of silver nanoparticle from the chemical reduction of silver ions was confirmed by X-ray diffraction (Rigaku, model Dmax 2002) with Ni-filtered CuKα radiation poperated at 40 kV and 30 mA. Sample was scanned from $2\theta = 30°$ to $2\theta = 80°$ at a scanning rate of $5°$ 2θ/min.

3.2.5 Transmission Electron Microscope (TEM)

Transmission electron microscopy (TEM) observations were carried out on a JEOL JEM-2000EX instrument operated at 80 kV accelerating voltage. We prepared the TEM samples on a 400 mesh copper grid coated with carbon. Histogram, mean diameter, and standard deviation were obtained by sampling 200 metal nanoparticles in TEM images of 62000 magnifications, followed by analyses using SPSS14 program.

3.2.6 Atomic Absorption Spectrophotometer (AAS)

Amount of silver ions, which were released from silver nanoparticle-impregnated bacterial cellulose, was measured by Atomic Absorption Spectrophotometer.

3.2.7 Universal Tensile Testing

Tensile strength, Young's modulus and % elongation at break of silver nanoparticle-impregnated bacterial cellulose were determined by using a Lloyd LRX universal testing machine with a 450N load cell at a crosshead speed of 1 mm/min. The specimens were rectangle shaped with a weight of 1.5 cm. and thickness of 1.5 mm.

3.3 Methodology

3.3.1 Production of Bacterial Cellulose

3.3.1.1 Culture Medium

Culture medium used for the fermentation of *Acetobacter xylinum (TISTR 975)* to produce bacterial cellulose contained D-Glucose anhydrous 100.0g, Yeast extract powder 10.0g and Distilled water 1.0L then culture medium was sterilized by autoclaving at 120°C for 15 min.

3.3.1.2 Culture Condition

Pre-inoculum for all experiments was prepared by transferring a single *Acetobacter xylinum (TISTR 975)* colony grown on agar culture medium into a 50-ml Erlenmeyer flask filled with 25 ml of liquid culture medium. After 24 h of cultivation at 30°C, the bacterial cellulose pellicle produced on the surface of the culture medium was either squeezed or vigorously shaken in order to remove active cells embedded in the bacterial cellulose membrane. Ten milliliters of the cell suspension was introduced into a 500-ml Erlenmeyer flask containing 100 ml of a fresh liquid culture medium, covered by a porous paper and kept at 30°C for 5 days as shown in Figure 3.1.

3.3.1.3 Purification

The bacterial cellulose pellicles were purified by boiled them in sodium hydroxide (1.0 %w/v) for 2 h (2 times) to eliminate *Acetobacter Xylinum* cells as well as components of the culture liquid, then neutralized with acetic acid (1.5 %w/v) and washed extensively with water prior to use.

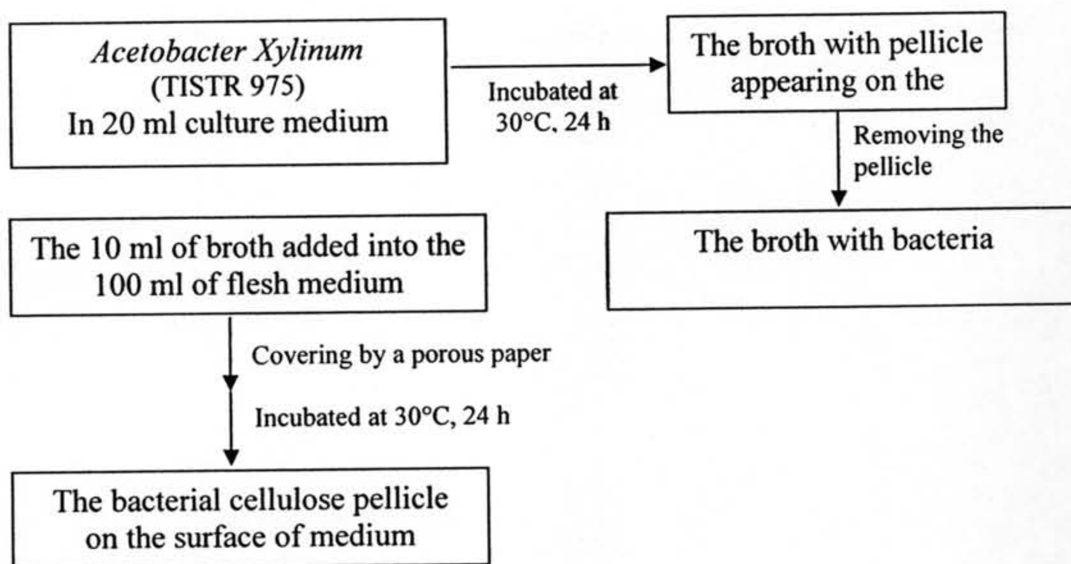

Figure 3.1 Flow chart shows the experimental procedure for production of bacterial cellulose.

3.3.2 Impregnation of Silver Nanoparticles into Bacterial Cellulose

Silver nanoparticles were impregnated into the bacterial cellulose fiber by immersing the bacterial cellulose pellicles in 0.001 M of the aqueous $AgNO_3$ for 1 h, followed by rinsing with ethanol for ca. 30 s. After then the silver ion-saturated bacterial cellulose pellicles were reduced in 0.001, 0.01 and 0.1 M of the aqueous $NaBH_4$ for 10 min and rinsed with large amount of pure water for 10 min, the obtained specimens were frozen at -40°C and dried in a vacuum at -52°C.

3.3.3 Silver Content and Releasing Behavior

Sample prepared from the molar ratio of $NaBH_4$: $AgNO_3$ were cut into a disc shape with 1.5 cm diameter and then digested by heating in a concentrated HNO_3 to break down the bacterial cellulose matrix and to release and dissolve all of the silver present. The digest was then filtered and diluted with deionized water. Total silver in the aqueous samples was determined by Atomic Absorption Spectrophotometer (AAS). The total extractable silver content of each dressing was then determined and expressed in ppm (mg/L).

Silver nanoparticle-impregnated bacterial cellulose was cut into a disc shape with 1.5 cm of diameter, and then 8 pieces of sample were immersed in 50mL

of the deionized water 1 day at 37°C. The next day they were removed, blotted free of excess fluid, and transferred to a fresh 50mL of the deionized water. The process was continued for 5 days. The suspending fluids from days 1, 2, 3, 4, 5 and 6 were then analyzed for silver ion by Atomic Absorption Spectrophotometer (AAS).

3.3.4 Swelling

Bacterial cellulose with silver nanoparticles dried to constant weight was immersed in distilled water for the certain time at room temperature. Re-swelling was calculated as follows

$$\text{Swelling} = (G_{s,t} - G_i) / G_i$$

Where G_i s the initial weight of dried sample and $G_{s,t}$ is the weight of sample in swollen state.

3.3.5 Mechanical Properties Testing

Silver nanoparticle-impregnated bacterial cellulose, whose thickness estimated 1.5 mm, was cut into the rectangle shape with a width of 1.5 cm and height of 5.0 cm. These samples were divided into two groups; each group consists of five pieces of sample. The first group was immersed in the deionized water at 37°C for 24 hours or until they completely swelled, which was tested the mechanical properties as silver nanoparticle-impregnated bacterial cellulose in the hydrate state. The last group was tested as silver nanoparticle-impregnated bacterial cellulose in the dry state. Experiment of the each group was run at 1 mm/s cross head speed. Stress (σ) is calculated by F/A where A is the cross sectional area and F is force in Newton. Strain (ε) is calculated by D_L/L_0 where D_L is exerted extension from starting point L_0. Young's modulus is calculated by stress/strain.

3.3.6 Antimicrobial Activity Testing

Antimicrobial activities of silver nanoparticle-impregnated bacterial cellulose have been investigated against *Escherichia coli* as the model gram-negative bacteria and *Staphylococcus aureus* as the model gram-positive bacteria. The antimicrobial activities of silver nanoparticle-impregnated bacterial cellulose were carried out by two methods.

3.3.6.1 The Disk Diffusion Method

This method was performed in Luria–Bertani (LB) medium solid agar Petri dish. The silver nanoparticle-impregnated bacterial cellulose was cut into a disc shape with 1.5 cm diameter, sterilized by autoclaving 15 min at 120°C, and was placed on *Escherichia coli*-cultured agar plate and *Staphylococcus aureus*-cultured agar plate which were then incubated for 24 h at 37°C and inhibition zone was monitored.

3.3.6.2 The Colony Forming Count Method

Silver nanoparticle-impregnated bacterial cellulose was cut into a disc shape with 1.5 cm diameter. Before inoculation of the bacteria, the pieces of sample were sterilized by autoclaving at 120°C for 15 min. The experimental design is shown in Figure 3.2. Sample was divided into two groups; each group consists of eight pieces. The first group was seeded with 1mL sterile nutrient broth as sterility control. The second group was seeded with fresh *Escherichia coli* or *Staphylococcus aureus* culture at a concentration of 10^5 colony forming units per mL (cfu/mL), then incubated in shaking incubator at 37°C for 24 h. After incubation, 50mL saline was added to each of groups and then all tubes were vortexed. The 50μL of bacterial suspension was drawn from each of tube, spread on to a nutrient agar plate and incubated at 37°C for 48 h for colony forming counts. The same procedure was performed on pure bacterial cellulose. The percentage reduction in bacterial count was calculated by the formula (Y. Li et al, 2006):

$$\frac{\text{(Viable count at 0 h - Viable count at 24 h)}}{\text{Viable count at 0 h}} \text{ X } 100\%$$

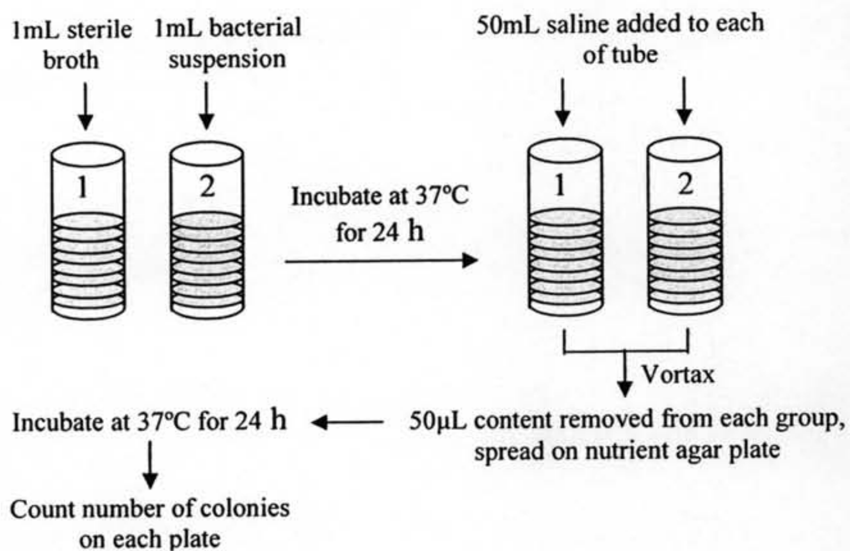

Figure 3.2 Flow chart showing the experimental procedure for antimicrobial activity study.