CHAPTER 2

MATERIALS AND MATHODS

1. Preparation of *Acetobacter xylinum* TISTR 975 preculture and bacterial cellulose (BC) production

1.1 Growing *A. xylinum* TISTR 975 from lyophilized stock

A. xylinum TISTR 975 was preserved in lyophilized form in ampule. This bacteriam was purchased from Thailand Institute of Science and Technology Research (TISTR). It was cultured in Hestrin & Shramm medium (HS medium) (Hestrin and Schramm, 1954). This medium composes of 2% w/v D-glucose, 0.5% w/v peptone, 0.5% w/v yeast extract, 0.27% w/v di-sodium hydrogen phosphate (Na_2HPO_4) and 0.115% w/v citric acid. After aseptic opening, about 0.3 to 0.4 ml of HS medium was added into the ampule. The cells were suspended and transferred to HS broth and incubated at 30^{o}C for 3 days. After 2 subcultures, glycerine was added into the culture to make the final concentration of 20% v/v and mixed homogenously. One milliliter of culture was aliquoted to cryotube and kept at -80^{o}C for long term preservation.

1.2 Preparation of preculture of *A. xylinum* TISTR 975 for bacterial cellulose production

A. xylinum TISTR 975 from -80^{o}C stock culture was grown in HS broth and subcultured two times. The broth culture was spreaded on HS agar and incubated

at 30^{o}C for 3 days. The growing colonies on agar surface were collected and filtered through cotton filter to remove BC fiber which was produced during bacterial growth on HS agar. The turbidity of the cell suspension was adjusted with HS broth (sugar free) equal to McFarland no.1 turbidity standard or about 4.5×10^{7} CFU/ml. The same manner of preculture preparation was used for any further experiments.

1.3 Bacterial cellulose production from different carbon sources

BC was prepared using ingredient similar to HS medium except carbon sources. Seven kinds of carbon sources i.e., 2 sugar alcohols (glycerine, mannitol), 3 monosaccharides (glucose, fructose, arabinose), 2 disaccharides (sucrose, lactose) were used. The medium was contained 8% w/v of each carbon sources. BC was produced in 1,000 ml beaker (cross section area 63.58 cm^{2}). The beaker was added with 50 ml of each production medium and 5 ml of adjusted *A. xylinum* TISTR 975. The beaker was covered and sealed with aluminum foil to control the volume of air. The media containing each carbon sources were done in six beakers. The cultures were incubated at 30^{o}C for 3 days under static condition. Wet BC films or pellicles were produced on the surface of the media. The wet BC films were harvested and washed with running water before immersing in 2% w/v sodium hydroxide (NaOH) for 24 h and followed by 0.1% w/v sodium hypochlorite (NaOCl) for another 24 h and washed with distilled water several times to completely get rid of sodium hypochlorite. The clean BC films were dried at 50^{o}C in hot air oven for 24 h and BC dry films were obtained.

2. Evaluation of bacterial cellulose properties

2.1 Weight of bacterial cellulose dry film

Each of 6 pieces of BC dry films with the area of 63.58 cm^2 was weighted with analytical balance. Average weight and standard derivation were calculated.

2.2 Thickness of bacterial cellulose dry film

The thickness of BC dry films was measured by Teclock caliper. Each piece was measured for 5 positions. The average thickness and standard derivation were calculated.

2.3 Percent yield of bacterial cellulose dry film

Percent yield of BC dry film from each carbon sources was calculated by following equation

$$\text{Percent yield} = \frac{\text{Dry weight of BC film}}{\text{Weight of carbon source used in production medium}} \times 100$$

2.4 Observation of bacterial cellulose film under scanning electron microscope (SEM)

BC dry films produced from different carbon sources were observed under SEM to study morphology and microstructure of cellulose fibers. Prior to

examining, the samples were gently fixed on an aluminum stab with two side adhesive tape and coated with 15-20 mm thick layer of gold. The samples were then examined under scanning electron microscope (JEOL Ltd., Tokyo, Japan).

2.5 Observation of crystallinity of bacterial cellulose dry film by X-ray diffractometer (XRD)

The X-ray diffraction pattern of BC dry film from different carbon sources was measured at room temperature with a PHILIPS: X' Pert MPD diffractometer (Philips Analytical, Eindhoven, The Netherlands). The X-ray source was a copper–Kα operated at a voltage of 40 kV and with a current of 30 mA. Samples were back-filled into 30 mm holders. The traces were recorded over a range of 5-40° 2 Theta angle with a step size of 0.04° and a count rate of 1 step s^{-1}. The crystallinity index was calculated as the ratio of the area of the resolved crystalline peaks to the total area of a diffraction profile for 5-40°.

2.6 Evaluation of Tensile strength of bacterial cellulose films

The strength of BC films from different carbon sources was presented as tensile strength. The BC films have been equilibrated at 25°C for 24 h in PBS pH 7.4 prior to measurement. BC film with size of 3 x 1.5 cm was put in tensile strength testing machine (Model 4465, Canton, MA, USA) according to ASTM E 18 and the cross-head speed was set at 30 mm/min. BC film was pulled until broken. Five pieces of each sample were used. The force used at the broken point was assigned as maximum load (Newton). The length of BC film at the breaking point was assigned as

extension at break (millimeter). The tensile strength was calculated by following equation

$$\text{Tensile strength} = \frac{\text{Maximum load}}{\text{Cross-section area of the BC film}}$$

2.7 Nitrogen adsorption isotherm and pore size distributions of bacterial cellulose films

Observation of the nitrogen adsorption isotherm and pore size distribution of BC film produced from different carbon sources was determined by nitrogen adsorption isotherms using an automatic surface area and pore size analyzer (Coulter SA3100, Backman Coulter, Inc., Fullerton, CA, USA). Accurately weighted dry samples were put in sample tubes and outgassed at 90ºC for 3 h to rid surface moisture and other contaminants. Prior to the adsorption measurements, sample tube free space was accurately measured using helium gas. The multipoint nitrogen adsorption isotherms of the samples were evaluated using Brunauer-Emmauer-Teller (B.E.T.) method from nitrogen adsorption data. The pore size distributions analysis was calculated using Barret-Joyner-Halenda (B.J.H.) by Coulter software version 2.13.

2.8 Water loss and water readsorption ability of bacterial cellulose wet films

BC wet films were immersed in distilled water at 25^{o}C for 1 h and put on glass surface with 45 degree slope for 20 min to get rid of the excess water before

weighing. BC wet films were then dried at 50°C for 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 h. After each period of drying, they were immersed in distilled water at 25°C for 1 h and get rid of the excess water before weighing. Finally, they were dried at 50°C for 24 h to get the total dry weight. The % loss weight of water and the % water readsorption were calculated by following equation

$$\% \text{ loss weight of water} = \left(\frac{\text{Original wet weight} - \text{Remained water after each period of drying}}{\text{Remained water after each period of drying}} \right) \times 100$$

$$\% \text{ water readsorption} = \left(\frac{\text{Original wet weight} - \text{Dry weight after drying at each periods of time}}{\text{Dry weight after drying at each periods of time}} \right) \times 100$$

2.9 Moisture adsorption isotherm of bacterial cellulose films at various relative humidity (RH)

The moisture adsorption isotherm of BC films produced from different carbon sources was evaluated. Different %RH of atmosphere in dessicator was adjusted by using saturated salt solutions. Saturated salt solutions of potassium hydroxide (KOH), magnesium chloride ($MgCl_2 \cdot 6H_2O$), magnesium nitrate ($MgNO_3 \cdot 6H_2O$), sodium nitrate ($NaNO_2$), sodium chloride (NaCl) and potassium sulfate (K_2SO_4) were prepared at 30°C. Each salt solution was placed in dessicator at 30°C for 24 h to equilibrate the RH of atmosphere before use. The saturated salt

solutions of KOH, $MgCl_2 \cdot 6H_2O$, $MgNO_3 \cdot 6H_2O$, $NaNO_2$, NaCl, K_2SO_4 gave 6, 32, 51, 62, 73, 97% RH at 30°C, respectively (Rockland, 1960). To prepare the samples, BC wet films after drying for 8 h at 50°C were cut into 5x5 cm and put in dessicator containing silica gel at 30°C for 24 h to absorb the moisture. They were immediately weighted to avoid readsorption of moisture from the air and immediately put in desired atmosphere of dessicator. The samples were weighted at 2, 4, 6, 8, 10, 12 and 24 h of the experiment or until the weigh was stable. The moisture absorption of the films was calculated from the increase weight of sample after equilibrium at given RH to the original weight by following equation.

$$\% \text{ moisture content} = \left(\frac{\text{Weight of BC film in desired atmosphere at the time point} - \text{Initial weight of BC film}}{\text{Initial weight of BC film}} \right) \times 100$$

3. Preparation and evaluation of bacterial cellulose film containing chlorhexidine digluconate

3.1 Antimicrobial activity of chlorhexidine digluconate

Chorhexidine digluconate was tested for antimicrobial activity by agar diffusion method against standards reference strains of bacteria and fungi. It was also test for minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) by microdilution method as following details.

3.1.1 Tested of antimicrobial activity of chlorhexidinedigluconate by agar diffusion method

3.1.1.1 Chlorhexidine digluconate disk preparation

Sterilized paper disk (MACHEREY-NAGEL) with 6 mm diameter was placed on an aluminium wire mesh and 20 µl of 20.2% w/v chlorhexidine digluconate desiccator to prevent contamination of disk surface with foreign matter. Each disk contained 4.04 µg of chlorhexidine digluconate.

3.1.1.2 Preparation of inoculum

Three reference strains of Gram positive bacteria i.e., *Staphylococcus aureus* ATCCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus epidermidis* ATCC 12228, one Gram negative bacterium i.e., *Escherichia coli* ATCC 25922 and one yeast i.e., *Candida albicans* NCPF 3153 were used in this study. Bacteria were grown on Mueller-Hinton agar (MHA) and incubated at 37^{o}C for 24 h and *C. albicans* NCPF 3153 was grown on Sabouraud dextrose agar (SDA) and incubated at 37^{o}C for 24 h. Normal saline was used to wash growing colonies from the agar surface and the turbidity of each tested microorganism was adjusted to obtain a turbidity comparison to 0.5 McFarland turbidity standard (approximately $5x10^{7}$ organisms per ml) by using sterile normal saline.

3.1.1.3 Streaking tested microorganisms on the plate

A sterile cotton swab was dipped into the adjusted inoculum and the excess was removed by rotating the swab several times against inside the wall of the

tube above the fluid level. For bacteria, the surface of MHA plate was inoculated by streaking the swab over the surface. For yeast, the surface of SDA plate was inoculated by the same method. Streaking was repeated 3 times and for each time the plate was rotated 60 degree.

3.1.1.4 Agar diffusion test

The disks were placed on the inoculated agar surface and the plates were incubated at 37°C for 24 h. The inhibition zone diameters around the disks were measured by antibiotic zone reader.

3.1.2 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of chlorhexidine digluconate to reference strains

3.1.2.1 Preparation of inoculum

The tested microorganisms and the preparation of inoculum were the same as in **section 3.1.1.2.** After adjusted turbidity equal to 0.5 McFarland turbidity standard, they were further diluted with MHB 1:10 (0.2 ml in 1.8 MHB). There were about $5x10^6$ organisms per ml.

3.1.2.2 MIC test of chlorhexidine digluconate to reference strains

Ninety well microtiter plate was used and 50 µl of MHB was inoculated into well 1-12. The chlorhexidine digluconate solution with 512 µg/ml was added into well 1 and made 2-fold serial dilution with MHB until well 10 and at well

10, 50 µl of the mixture was removed and discarded. From well 1-11, 50 µl of adjusted microorganisms was added and the final concentration of chlorhexidine digluconate form well 1 to 10 equal to 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml, respectively and each well contained 2.5×10^5 organisms/well. Well 11 contained MHB and tested microorganism and well 12 contained only MHB without chlorhexidine digluconate and microorganism. They were used as positive and negative control, respectively. The microtiter plates were incubated at 37°C for 16-20 h. Each experiment was done duplicate. The MIC was read from the well with the lowest concentration of chlorhexidine digluconate that showed no growth of tested organism or no turbidity of the medium.

3.1.2.3 MBC test of chlorhexidine digluconate to reference strains

According to the results from MIC test, 100 µl of content in the well without visible growth was inoculated on MHA surface and spreaded across the agar surface with sterile bent glass rods. The plated were incubated overnight at 37°C for the MBC test. Determine the MBC from the plate with colony count that represent 0.1% of original inoculum (i.e., 99.9% reduction).

3.1.3 Tested of antimicrobial activity of bacterial cellulose disk containing chlorhexidine digluconate

The BC was dry in hot air oven for 8 h. The sheet was cut with paper puncher with 6 mm diameter to make the BC disk. The BC disk was soaked in 20.2% w/v chlorhexidine digluconate solution. The release of chlorhexidine digluconate from the disk was observed by performing sensitivity test using agar diffusion method

with *E. coli* ATCC 25922*, S. aureus* ATCCC 25923, *S. epidermidis* ATCC 12228*, P. aeruginosa* ATCC 27853 and *C. albicans* NCPF 3153. The inhibition zone was read by antibiotic zone reader. Each BC disk contained 42.04 µg of chlorhexidine digluconate.

3.2 Preparation of bacterial cellulose films containing chlorhexidine digluconate

The BC wet films from different carbon sources after drying for 8 h at 50°C was cut into a circle shape with the area of 28.26 cm^2. The BC films were applied with chlorhexidine digluconate solution and dried in hot air oven at 50^oC for 24 h. Finally, each film contained 0.2% (w/w) chlorhexidine digluconate. The chlorhexidine digluconate content of each film was evaluated. The circle film was cut into small pieces and soaked in 12 ml PBS pH 7.4 at 37^oC for 24 on 200 rpm shaking water bath. The sample released to PBS solution was collected and measured for ultraviolet light absorption at the wavelength of 289 nm using spectrophotometer (Spectronic® GENESYS™ 5). The content of chlorhexidine digluconate from each film was read from the standard curve of chlorhexidine digluconate.

3.2.1 *In vitro* chlorhexidine digluconate release using Franz diffusion cell

The films containing chlorhexidine digluconate were used to study for their chlorhexidine digluconate release from BC film by using Franz diffusion cell (The Hanson Model 57-6M). The films were put on the receptor of the cell. The receptor compartment was filled with 12 ml PBS pH 7.4. The diffusion cell was

thermoregulated with a water jacket at 37^{o}C and receptor compartment was stirred with magnetic stirrer. The sample (2 ml) was withdrawn at 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. The equal volume of fresh PBS was immediately added to the receptor cell after each sampling. Chlorhexidine digluconate released from each time point was measured spectrophotometrically at 289 nm and its concentration was read from the standard curve of chlorhexidine digluconate.

3.2.2 Determination of chlorhexidine digluconate content in the bacterial cellulose film

Chlorhexidine digluconate content in each BC films was evaluated. The film with the area of 1x1 cm was cut into small pieces and soaked in PBS pH 7.4 and incubated in shaking incubator (200 rpm) at 37°C for 24 h. The PBS was collected and measured for chlorhexidine digluconate concentration by spectrophotometer at the wave length 289 nm.

3.2.3 Observation of bacterial cellulose film containing chlorhexidine digluconate under SEM

From previous study, BC films produced from glycerine containing 0.2% (w/w) chlorhexidine digluconate was observed under SEM (JEOL Ltd., Tokyo, Japan) to study morphology and microstructure by using the method described in **section 2.4.**