CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

3.1.1 Materials

The shell of *Litopeneous vannamei* shrimp was dedicated by Surapon Food Public Co., Ltd. *Acetobacter xylinum* (strain TISTR 975), *Escherichia coli* and *Staphylococcus aureus* were purchased from Microbiological Resource Centre, Thailand Institute of Scientific and Technological Research (TISTR). Air gas used for plasma treatment was obtained from Thai Industrial Gas Co., Ltd.

3.1.2 Chemicals

Analytical grade of anhydrous D-glucose was purchased from Ajax Finechem. Yeast extract, beef extract, peptone and agar powder were bacteriological grade and purchased from HiMedia. Laboratory grade of sodium borohydride was purchased from Carlo Erba (Italy). Analytical grade of sodium hydroxide anhydrate pellet and sodium chloride were purchased from Aldrich Chemical. Amido Black 10B was purchased from Wako Pure Chemical Industries, Co., Ltd. (Japan). Cibacron brilliant red 3B-A (also known as Reactive Red 4) was purchased from Sigma (Italy). Glycine was purchased from Fisher scientific. Analytical grade of glacial acetic acid was purchased from CSL Chemical. Analytical grade of ethanol, sulfuric acid (98%) and acetone were purchased from Labscan (Asia) Co., Ltd. Analytical grade of hydrochloric acid (36.5-38.0%) was purchased from J.T. Baker.

3.2 Equipment

3.2.1 Capillary Viscometry

The viscosity-average molecular weight of chitosan was determined by using Cannon Ubbelohde-type number 75 of capillary viscometer.

3.2.2 Scanning Electron Microscopy (SEM)

SEM will be performed on gold-coated samples, which will be obtained using a polaron sputter coater. Surface morphology of bacterial cellulose

and chitosan-coated bacterial cellulose were investigated by using scanning electron microscope (SEM) (JEOL, JSM-5410LV) at 10 kV.

3.2.3 X-ray Photoelectron Spectroscopy (XPS)

XPS analysis will be conducted to analyze the surface chemical state and compositions of the non-plasma and plasma treated bacterial cellulose and chitosan-coated bacterial cellulose.

3.2.4 Fourier Transformed Infrared Spectroscopy (FTIR)

Qualitative and quantitative Fourier transform infrared spectra of chitosan and bacterial cellulose were obtained from FTIR Spectrophotometer (Thermo Nicolet, Nexus 670) with 32 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed.

3.2.5 UV-Visible Spectroscopy

The dye absorption ability of bacterial cellulose and chitosan-coated bacterial cellulose was measured by Shimazu UV-1800 at λ_{max} 618 nm. The adsorption of amido black 10B, an anionic reactive dye, in the samples were carried out at room temperature (~25 °C) using 20 ml vials containing 15 ml of dye aqueous solutions. The concentration and initial pH value of the dye solution were 0.002 % (w/v) and 8.56, respectively. Bacterial cellulose and chitosan-coated bacterial cellulose (~10 mg) were immersed into each vial and then sampling 1 ml of each solution at a specified immersion period ranging between 0 and 24 h.

3.3 Methodology

3.3.1 Preparation of Bacterial Cellulose

3.3.1.1 Culture Medium

To produce BC, *Acetobacter Xylinum* (TISTR 975) was cultivated in culture medium containing anhydrous D-glucose (40.0 g), yeast extract powder (10.0 g) and distilled water (1.0 L). The 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 vigorously shaken in order to remove active cells embedded in the bacterial cellulose membrane. Then 10 ml 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 4 days as shown in Figure 3.1.

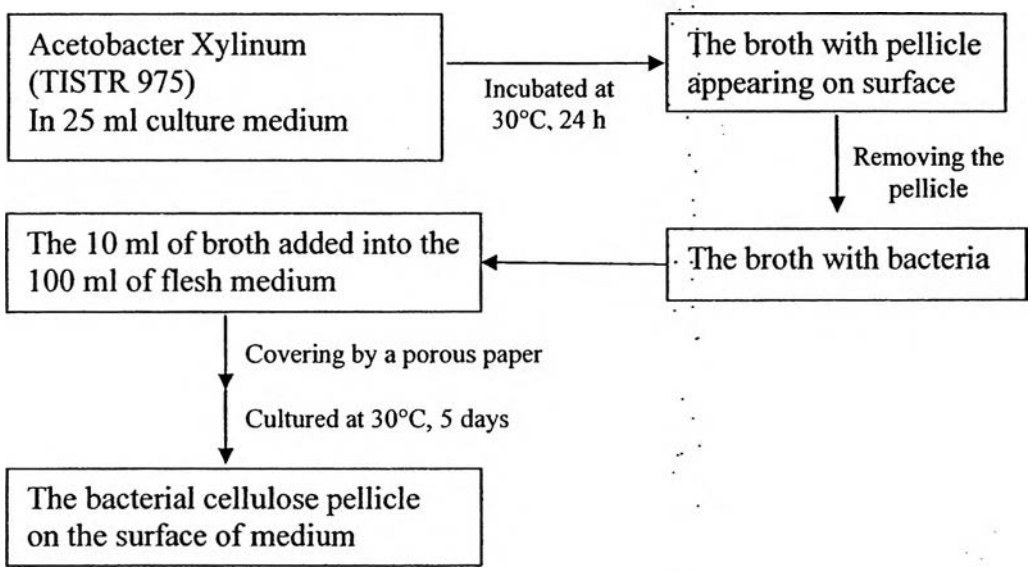

Figure 3.1 Flow chart shows the procedure for preparation of bacterial cellulose.

3.3.1.3 Purification

Bacterial cellulose pellicles were purified by boiled them in sodium hydroxide 1.0 % (w/v) for 2 h many times to eliminate *Acetobacter Xylinum* cells and components of the culture liquid, then neutralized with acetic acid 1.5 % (w/v) and washed with distilled water. The purified pellicles were freeze-dried and stored in a dessicator prior to use.

3.3.2 Preparation of Chitosan

3.3.2.1 Preparation of Chitin

Shrimp shell was decalcified and deproteinized to remove calcium carbonate and protein, respectively. Firstly, the shrimp shells were cleaned and dried under sunlight before grinding into small pieces. Then shrimp shell chips

were treated by immersion in 1N HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was performed by boiling in 4 % (w/w) of NaOH solution at 80-90 °C for 4 hours. After NaOH solution was decanted, the deproteinized sample was washed with deionized water until neutral. The obtained product, called chitin was dried at 60 °C in a convective oven for 24 hours.

3.3.2.2 Preparation of Chitosan

Chitin was deacetylated by heating in 50 % (w/w) NaOH solution containing 0.5 % (w/w) sodium borohydride ($NaBH_4$) to prevent depolymerization. The ratio of chitin to NaOH solution was 1 g of chitin in 10 ml of NaOH solution. Deacetylation was performed in an autoclave at 120 °C for 1 hour. Then the deacetylated product or chitosan was washed with deionized water until neutral. Finally, chitosan was dried in an oven at 60 °C for 24 hours as shown in Figure 3.2.

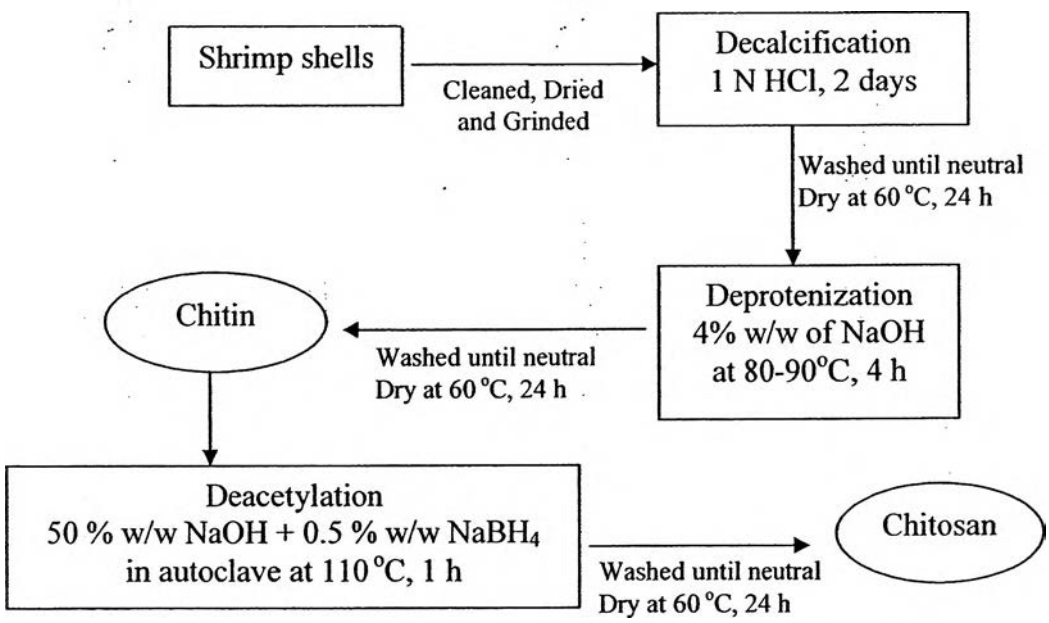

Figure 3.2 Flow chart shows the procedure for preparation of chitosan.

3.3.2.2 Characterization of Chitosan

The influence of chitosan on the properties of final products will be studied by varying chitosan solution concentrations, degree of deacethylation and molecular weight of chitosan.

a.) Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was determined by IR spectroscopy. Chitosan films were prepared by 1 % (w/v) of different molecular weight chitosan solution in 2 % (v/v) acetic acid, neutralized with 1 M NaOH, then washed with distilled water. Chitin film was prepared by 1 % (w/v) of chitin in formic acid, neutralized and then washed with distilled water. An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbances at 3450 cm^{-1} (the OH band) and 1655 cm^{-1} (the amide I band) were used to quantitate the degree of deacetylation. The degree of deacetylation was calculated from equation (3.1) (Baxter *et al.*, 1991).

$$\%DD = 100 - 115\ (A_{1655}/ A_{3450}) \qquad (3.1)$$

where D = degree of deacetylation (%)

A_{1655} = absorbance at 1655 cm^{-1}

A_{3450} = absorbance at 3450 cm^{-1}

b.) Viscosity-Average Molecular Weight of Chitosan

Chitosan solution of different concentration (0.00, 0.025, 0.050, 0.075, and 0.100 g/100 ml) in 0.2 M acetic acid: 0.1 M sodium acetate was prepared. An Ubbelohde viscometer was filled with 10 ml of sample, which maintained the temperature at 30°C. The sample was passed through the capillary once before the running times were measured. Each sample was measured 3 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity. The reduced viscosity was plotted against the concentration and the intrinsic viscosity determined from the intercept. The corresponding equations are:

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \quad (3.2)$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s) - 1 \quad (3.3)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \quad (3.4)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c \to 0} \quad (3.5)$$

where t is the flow time in seconds of chitosan solution, t_s is the flow time in seconds of solvent and C is the concentration of chitosan solution in g/100 ml.

The viscosity average molecular weight of chitosan was determined based on the Mark-Houwink equation (Wang *et al.*, 1991):

$$[\eta] = 6.59 \times 10^{-5} M_v^{0.88} \quad (3.6)$$

where $[\eta]$ is the intrinsic viscosity and M_v is viscosity average molecular weight.

3.3.3 Plasma Treatment

3.3.3.1 Power Supply Unit

The block diagram of the power supply unit is shown in Figure 3.3. For the first step, the alternating current (AC) input of 220 V and 50 Hz will be converted to direct current (DC) of about 70-80 V by DC power supply converter. For the second step, the DC current will be supplied through a 500-W power amplifier, which is connected to the Instek function generator to generate waveform and to amplify voltage and frequency. The signal of alternative current is a sinusoidal waveform. For the third step, the modified current will be passed through the transformer to convert to 230 V AC. Thereafter, the variable output will be finally transmitted to a high voltage current by nominal factor 130 times of low side voltage (input). An Extech® series 380801 power analyzer will be used to measure power, power factor, current, frequency, and voltage at the low side of the power supply unit.

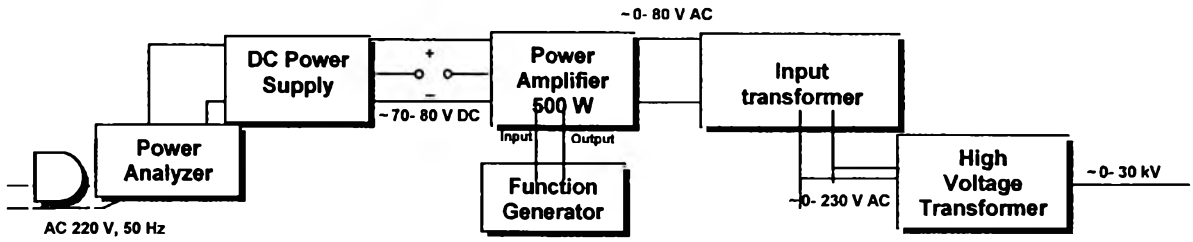

Figure 3.3 Block diagram of the power supply unit.

3.3.3.2 Experimental Setup

The experimental setup for surface modification of bacterial cellulose by using dielectric barrier discharge is shown below.

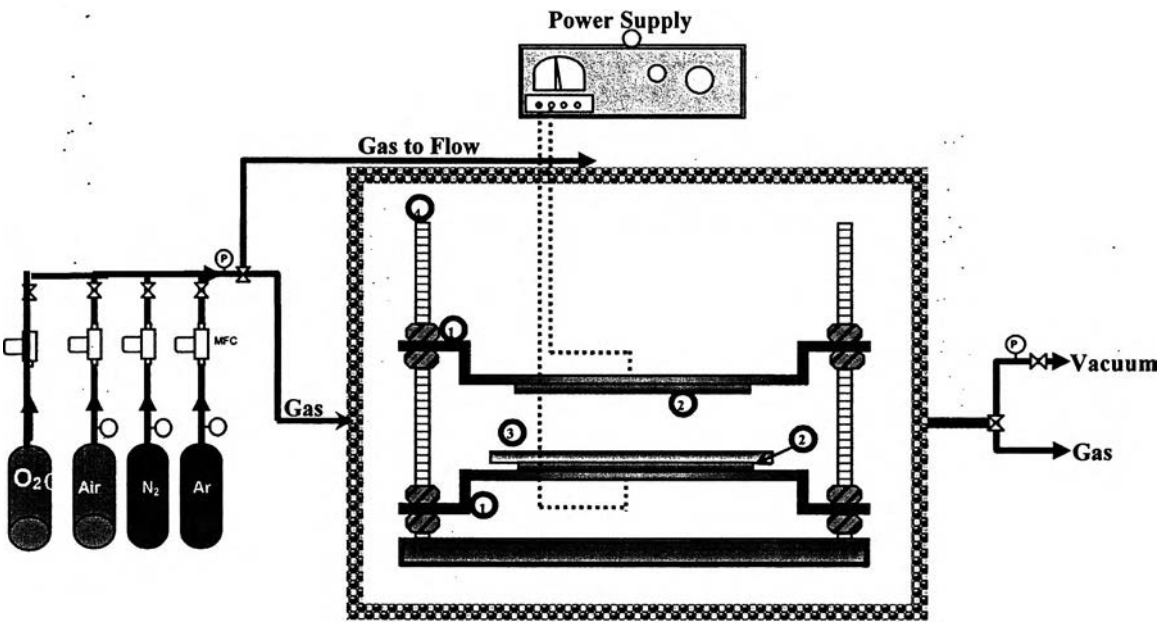

Figure 3.4 Schematic of experimental setup for dielectric-barrier discharge system where 1. Acrylic Structure

2. Electrode Plate (Stainless steel plate)
3. Glass Plate (Dielectric insulator)
4. Acrylic Screw.

3.3.3.3 Studied Conditions

The experiments will be carried out in air atmosphere. The plasma conditions are electrode gap distance = 4 mm, applied voltage = 40 V, input frequency = 325 Hz, time for plasma treatment = 10 s in atmospheric pressure.

3.3.4 Chitosan Coating on Bacterial Cellulose

Chitosan was coated on bacterial cellulose by immersing the freeze-dried pellicle into chitosan solution at room temperature for 24 h, followed by washing with distilled water for 2 times, then freeze-drying. The concentrations of chitosan solution were varied from 0.25 %, 0.50 %, 0.75 %, 1.00 % and 2.00 % by diluting chitosan flake in 2.0 % acetic acid solution.

3.3.5 Swelling Property

Bacterial cellulose was dried to constant weight before immersing in distilled water for the certain time at room temperature. Re-swelling was calculated as equation (3.7).

$$\text{Swelling} = (G_{s,t} - G_i) / G_i \qquad (3.7)$$

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

3.3.6 Chitosan Content and Releasing Behavior

Kjeldahl method was used for the quantitative determination of total amount of chitosan coated on the surface of bacterial cellulose. The Kjeldahl method consists of three steps, which have to be carefully carried out in sequence:

1. The sample was first digested in strong sulfuric acid in the presence of a catalyst, which helps in the conversion of the amine nitrogen to ammonium ions.

2. The ammonium ions was then converted into ammonia gas, heated, and distilled. The ammonia gas will be allowed to pass a trapping solution where it dissolves and becomes an ammonium ion once again.

3. Finally, the amount of the ammonia that is trapped was determined by titration with a standard solution, and calculated into moles of nitrogen in the samples.

Releasing of chitosan from the BC surface was carried out based on the colorimetric quantification assay (Muzzarelli, 1998). Chitosan was absorbed in a monochlorotriazine dye, Cibacron Brillient Red (CBR) 3B-A. Solution of the dye was prepared by dissolving 150 mg of the dye powder in 100 ml of distilled water. Aliquots of the dye solution (5 ml) were filled with 0.1 M glycine hydrochloride buffer to fill the final volume of 100 ml. The final concentration of the dye solution was 0.075 g/l (pH = 3.2). The buffer solution was prepare by dissolving glycine (1.87 g) and sodium chloride (1.46 g) and made up to 250 ml with distilled water. Aliquots (81 ml) of this solution were made up to 100 ml with 0.1 M HCl until the final pH of 3.2 (Muzzarelli, 1998; Watthanaphanit *et al.*, 2009).

The release characteristic of chitosan from the bacterial cellulose (about 30 mg) was immersed in 10 ml of acetate buffer solution (pH = 5.5, pH of skin) and phosphate buffered saline, PBS (pH = 7.4, pH of extrudates) at temperature of 37°C and shaking speed of 50 rpm. At a specified immersion period from 0 and 72 h, 1 mL of the medium was withdrawn and an equal amount of the fresh medium was refilled. The amount of chitosan within the sample solution was determined by drying the sample solution, then adding the 0.3 ml of 0.1 M glycine hydrochloride buffer and 3 ml of the dye solution, respectively. The absorbance at λ_{max} 575 nm was measured by Shimazu UV-1800.

3.3.7 Antimicrobial Activity Testing

Antimicrobial activities of chitosan-coated bacterial cellulose have been investigated against *Escherichia coli (E. coli)* as the model gram-negative bacteria and *Staphylococcus aureus (S. aureus)* as the model gram-positive bacteria by the colony forming counting method. The bacterial suspension was prepared by transferring one colony of microorganism into 20 ml of nutrient broth (Approximate formula per liter: beef extract 3.0 g and peptone 5.0 g), then cultured in a shaking incubator at 37 °C for 24 h. The 1 ml of bacterial suspention was then added to a vial contains 9 ml of 0.85 % sterile saline solution (to control the osmotic pressure of bacteria). The solution was diluted in serial dilutions to other vials:

Vial a.) contains 9.0 ml of 0.85 % sterile saline; adding 1.0 ml of the undiluted bacterial suspension to yield a total volume of 10.0 ml.

$$\frac{1\ ml}{9\ ml + 1\ ml} \rightarrow \frac{1}{10} \rightarrow 1 \times 10^{-1} \rightarrow 1{:}10$$

Vial b.) contains 9.0 ml of .85 % sterile saline; adding 1.0 ml of the 1:10 diluted bacterial suspension to yield a total volume of 10.0 ml

$$\frac{1\ ml}{9\ ml + 1\ ml} \rightarrow \frac{1\ ml}{10\ ml} \times \frac{1}{10} \rightarrow \frac{1}{100} \rightarrow 1 \times 10^{-2} \rightarrow 1{:}100$$

Until reached the optimum concentration, 10^{-6} colony units per ml (cfu/ml) for *E. coli* and 10^{-5} colony units per ml (cfu/ml) for *S. aureus.*, each vial was added with samples (sterilized under UV light over a period of 20 min). Then all bottles are shaked in shaking incubater at 37°C with the speed 150 rpm for contact time of 3 h. The 100 μL of bacterial suspension was drawn from each of vial, spread on 3 nutrient agar plates and incubated at 37 °C for 24 h for colony forming counts. The bacterial reduction rate (BRR) was calculated as equation (3.8).

$$BRR = (N_1 - N_2) / N_1 \times 100 \qquad (3.8)$$

Where N_1 are the Number of colonies of blank control and N_2 are Number of colonies of samples.