CHAPTER III
EXPERIMENTAL

3.1 Chemical and Materials

3.1.1 Materials

(a) Shells of *Litopeneous vannamei* shrimp were provide by Surapon Food Public Co., Ltd., Thailand.

(b) Polyvinylchloride (PVC) film was purchased from MMP Packaging Group Co., Ltd., Thailand.

(c) Polyethylene (PE) film was purchased from Thantawan Industry Public Co., Ltd., Thailand.

(d) Polypropylene (PP) film was purchased from Danudej Industry Co., Ltd., Thailand.

(e) Polylactic acid (PLA), PLA-2002D pellets were obtained from NatureWorks LLC, USA. PLA film was prepared using a solvent casting method (Rhim, Mohanty, Singh, & Ng, 2006). Eight grams of PLA was dissolved in 100 mL of chloroform while mixing vigorously at room temperature (≈23 °C). The dissolved solution was poured onto petri dish.

3.1.2 Chemicals

Sodium hydroxide (50% w/v aqueous solution) was supplied by KTP Cooperation Co., Ltd., Thailand. Sodium acetate, sodium borohydride, hydrochloric acid 37% w/w were analytical reagent grade purchased from Carlo Erba Co., Ltd., (Italy). Glacial acetic acid 99.9% w/w was analytical reagent grade purchased from Labscan Asia Co., Ltd., Thailand. Sodium hydroxide anhydrous pellets and hydrogen peroxide were purchased from Ajax Finechem Pty Ltd. Amido Black 10B was purchased from Wako Pure Chemical Industries, Co., Ltd. (Japan).

3.1.3 Air Gas for Reaction

Air gas was used for plasma treatment was obtained from Thai Industrial Gas Co., Ltd. (Thailand).

3.2 Equipment

3.2.1 Capillary Viscometer

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

3.2.2 Contact Angle Measurement

Hydrophilicity of the surfaces was evaluated by measuring the contact angle formed between water drop and the surface of the modified sample, by using contact angle analyser system G 10 (KRUSS). The values of the contact angle are the mean value of the water drops on ten different areas.

3.2.3 Lloyd Tensile Tester

Tensile strength and elongation at break were measured from five rectangular films (15 cm ×1 cm) using a Universal Testing Machine (Lloyd, Model LRX) at 25 °C. The polymeric films were equipped with a 500 N load cell. A strain rate of 10 mm min^{-1} and gauge length of 50 mm was employed, according to the ASTM D882-91standard test method.

3.2.4 Thermogravimetric Analyzer (TGA)

The thermal stability and the decomposition temperature of chitosan, polymeric films and chitosan-coated on polymeric films were analyzed by thermogravimetric analysis (TGA) (Dupont Instrument TGA 5.1, model 2950). The temperature range studied of PVC, PE and PP was 30-700 °C. The temperature range studied of PLA was 30-500 °C. TGA patterns were measured at a heating rate of 10 °C/min under a nitrogen gas atmosphere.

3.2.5 Atomic Force Microscopy (AFM)

An Atomic force microscopy (AFM) was used to determine the surface roughness of the film surfaces by using XE-100 Park systems in contact mode. The root mean squared roughness (RMS) and the topographic profiles measured on 10 μm ×10 μm images were evaluated. For each sample, the roughness value was obtained from ten different areas.

3.2.6 X-ray Photoelectron Spectroscopy (XPS)

Chemical compositions on the modified surfaces were determined by an ESACA-3400 X-ray photoelectron spectroscopy (XPS) (Shimadzu, Japan). More chemical information on the modified surface was obtained by fitting the C1s peak.

3.2.7 Fourier Transformed Infrared Spectroscopy (FTIR)

The FTIR spectrum of chitin and chitosan films were recorded with a Thermo Nicolet Nexus 670 FT-IR spectrophotometer, with 32 scans at a spectra resolution of 4 cm^{-1} and continuously purged with dry air. The film samples with the thickness of 10 μm were attached into the sample frames.

3.3 Methodology

3.3.1 Preparation of Chitin and Chitosan

3.3.1.1 Preparation of Chitin

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells were cleaned and dried under sunlight before grinding into small pieces. 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 to pH paper. Deproteinization was followed by boiling the decalcified product in 4 % w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the chips were washed with deionized water until neutral to pH paper. The product obtained was dried at 60°C in a convective oven for 24 h.

3.3.1.2 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. The deacetylation was performed in an autoclave at 110 °C for 1 h. the deacetylated product obtained was washed exhaustedly with deionized water until neutral to paper. The resulting chitosan flakes was dried in an oven at 60 °C for 24 h.

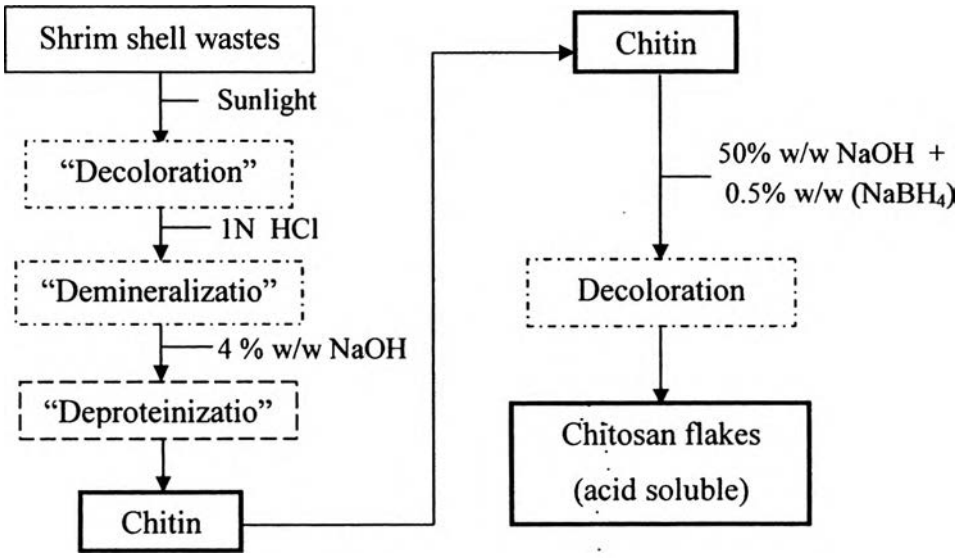

Figure 3.1 Simplified representation of preparation of chitin and chitosan.

3.3.2 Characterization of Chitin and Chitosan

3.3.2.1 Degree of Deacetylation of Chitin and Chitosan

The degree of deacetylation (DD) of chitin and chitosan was determined by FTIR method (Baxter *et al.*, 1998, Sanan *et al.*,1978). The chitosan whiskers from the as-prepared chitosan flakes were dried, mixed with KBr powder and pressed into a pellet. The scanning range was 4000–400 cm^{-1} with 32 scans at a resolution of 4 cm^{-1}. The degree of deacetylation of the as-prepared chitosan was determined using the obtained FTIR spectrum based on the method of Sannan, Kurita, Ogura, and Iwagura (1978). The degree of deacetylation (DD) of chitin and chitosan were calculated by the following equation:

$$D = 98.03\text{-}34.68\ (A_{1550}/\ A_{2878}) \text{ for chitin} \quad (3.1)$$

$$D = 100\text{-}\ [(A_{1655}\ /A_{3450})\ x\ 115] \text{ for chitosan} \quad (3.2)$$

where D = degree of deacetylation (%), A_{1550} = absorbance at 1550 cm^{-1}, A_{2878} = absorbance at 2878 cm^{-1}, A_{1655} = absorbance at 1655 cm^{-1}, A_{3450} = absorbance at 3450 cm^{-1}

3.3.2.2 Viscosity-Average Molecular Weight of Chitosan

The different concentration (0.00, 0.0125, 0.025, 0.050, 0.075, and 0.1 g/100 ml) of chitosan dissolved in suitable solvents were prepared in 0.2 M acetic acid: 0.1 M sodium acetate. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which maintained the temperature at 30 °C. The sample solution was passed through the capillary once before the running times were measured. Each sample was measured three 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:

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \tag{3.3}$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s) - 1 \tag{3.4}$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \tag{3.5}$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c \rightarrow 0} \tag{3.6}$$

where t is the running time of chitosan solution, t_s is the running time 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). The K and a values were dependent on kind of solvent and measured temperature.

$$[\eta] = 6.59 \times 10^{-5} M^{0.88} \tag{3.7}$$

where $[\eta]$ = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.3 Plasma Treatment and Sample Preparation

3.3.3.1 Dielectric Barrier Discharge (DBD)

Figure 3.2 presents the schematric show of the experiment setup of DBD plasma treatment. The dielectric discharge has the thickness of 2 mm. The two parallel electrodes are stainless steel. The polymeric films were cut into square shape with the dimension of 6 cm × 6 cm and then were put into the parallel-plate dielectric barrier discharge (DBD) reactor for plasma treatment before chitosan coating. The experiment was operated with the condition of voltage of 50 kV, frequency of 325 Hz and the electrode gap of 4 mm. The flowing air gas was introduced directly through the gab of electrode.

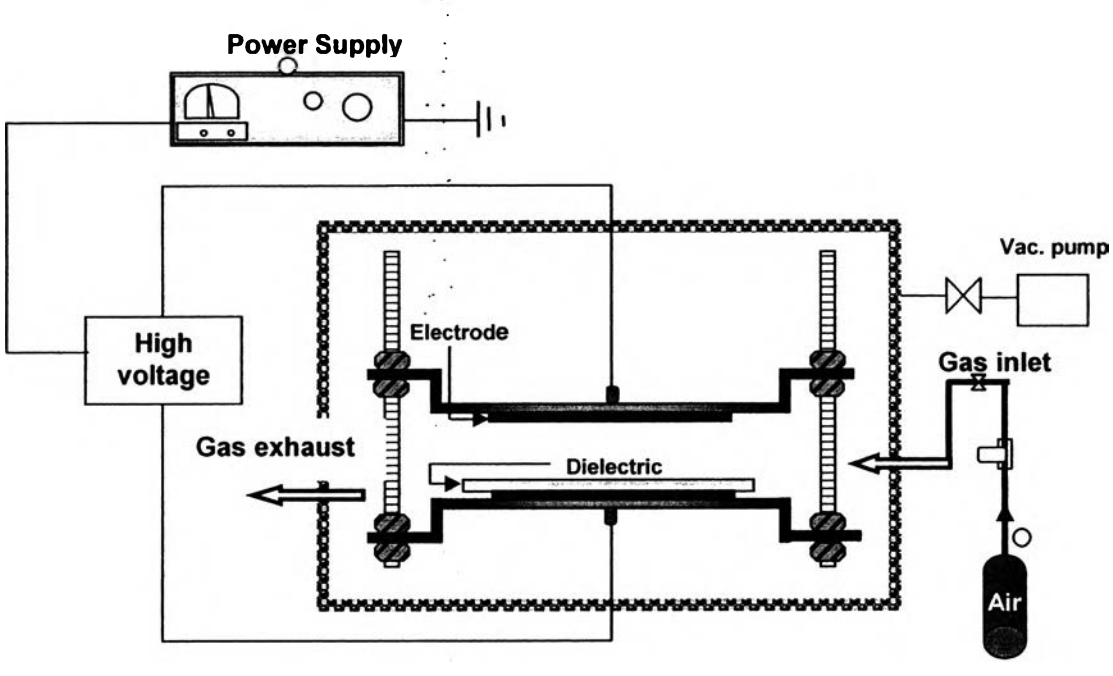

Figure 3.2 Schematic show of the experimental setup of DBD plasma

3.3.3.2 Preparation of Chitosan-Coated Films

To make the polymeric films hydrophilic and chemically active, the polymeric films were first treated with DBD plasma for the subsequent chitosan coating. Chitosan was dissolved in 1% acetic acid solution to attain different concentrations of chitosan (0.125, 0.25, 0.5, 0.75, 1.0 and 2.0%). After plasma treatment, all plasma-treated films were immediately immersed in the chitosan

solution with constant stirring for 1 min, followed by washing with deionized water for three times to accomplish pH neutralization.

3.3.4 Characterization of Chitosan-Coated on Polymeric Films

3.3.4.1 Kjeldahl Method

Chemically modified chitosan-coated polymeric films were precisely weighed and then suspended in concentrated sulfuric acid (5 ml) small amount of copper sulfate (0.05-0.1g) in the presence of a catalyst, which helps in the conversion of the amine nitrogen to ammonium ions. Five drops of hydrogen peroxide (35%) was added to the suspension and the mixture was heated until the solution became transparent and colorless. The resulting solutions were subjected to Kjeldahl nitrogen analysis. A 20 ml of 0.1 mole of hydrochloric acid aqueous solution was added to 200 ml of erlenmeyer flask and set to the condenser end after mixing of a few drops of indicator (phenolphthalein) and water (50 ml). A 40% sodium hydroxide aqueous solution was added to bring pH of solution alkaline (This procedure would accelerate the volatile of ammonia). The ammonium ions in the clear solution will be then converted into ammonia gas, heated, and distilled. The ammonia gas will be allowed to pass a trapping solution into 0.01 mole hydrochloric acid solution where it dissolves and becomes an ammonium ion once again. Finally, the amount of the ammonia that is trapped will be determined by titration with a standard solution (0.01 mole of NaOH). The chitosan content in the polymeric films were calculated from the nitrogen percentage on the basis of the calibration curve for the weight of chitosan and titration value.

3.3.4.2 Staining of Chitosan Deposited on Plasma-Treated Polymeric Surfaces

To investigate the dispersion of chitosan deposition on polymeric films, chitosan-coated on polymeric films were immersed in 0.01% w/v Amido Black 10B (an amino acid staining diazo dye) aqueous solution for 12 h in order to stain the polymeric films after chitosan coated on the method of Watthanaphanit *et al.*, 2009. The polymeric films were then washed with distilled water to remove excess dye and later observed for the dispersion and distribution of the deposited chitosan by an optical microscope.

3.3.5 Antimicrobial Evaluation

Antibacterial property of the control and the chitosan-coated polymer films was evaluated based on the colony count method ASTM E 2149-01. Chitosan-coated polymeric films were tested against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. Briefly, a broth solution was prepared by mixing 0.3 g beef extract and 0.5 g peptone in 100 ml water. An inoculum was prepared by transferring one colony of each microorganism into 20 mL of a broth solution. The mixture was cultured at 37 °C in a shaking incubator for 24 h. About 1 mL of the cell suspension of each microorganism was added into several vials of 9 mL of 0.85% sterile NaCl aqueous solution. Standard serial dilution method was used, i.e., 10^{-6} for S. aureus and 10^{-5} for E. coli. Chitosan-coated polymer fims will be cut into asquare shape of 3.0×3.0 cm^2. Each of the chitosan-coated polymer fims was added into the mixture. The suspensions were shaken at 150 rpm. After the contact time period of 3 h, 100 μL of these suspensions was dipped and spread on sterilized agar in Petri dishes. Bacterial growth was visualized after an overnight incubation at 37 °C in a incubator for 24 h (Watthanaphanit, *et al.*). The percentage reduction was determined as follows:

$$\text{Bacterial reduction}(\%) = \frac{(C - A)}{C}$$

Where, *C* and *A* are the colonies counted from the plate of the control and chitosan-coated polymeric films, respectively (Gubta and Haile, 2007). The experiments were carried out in triplicate.

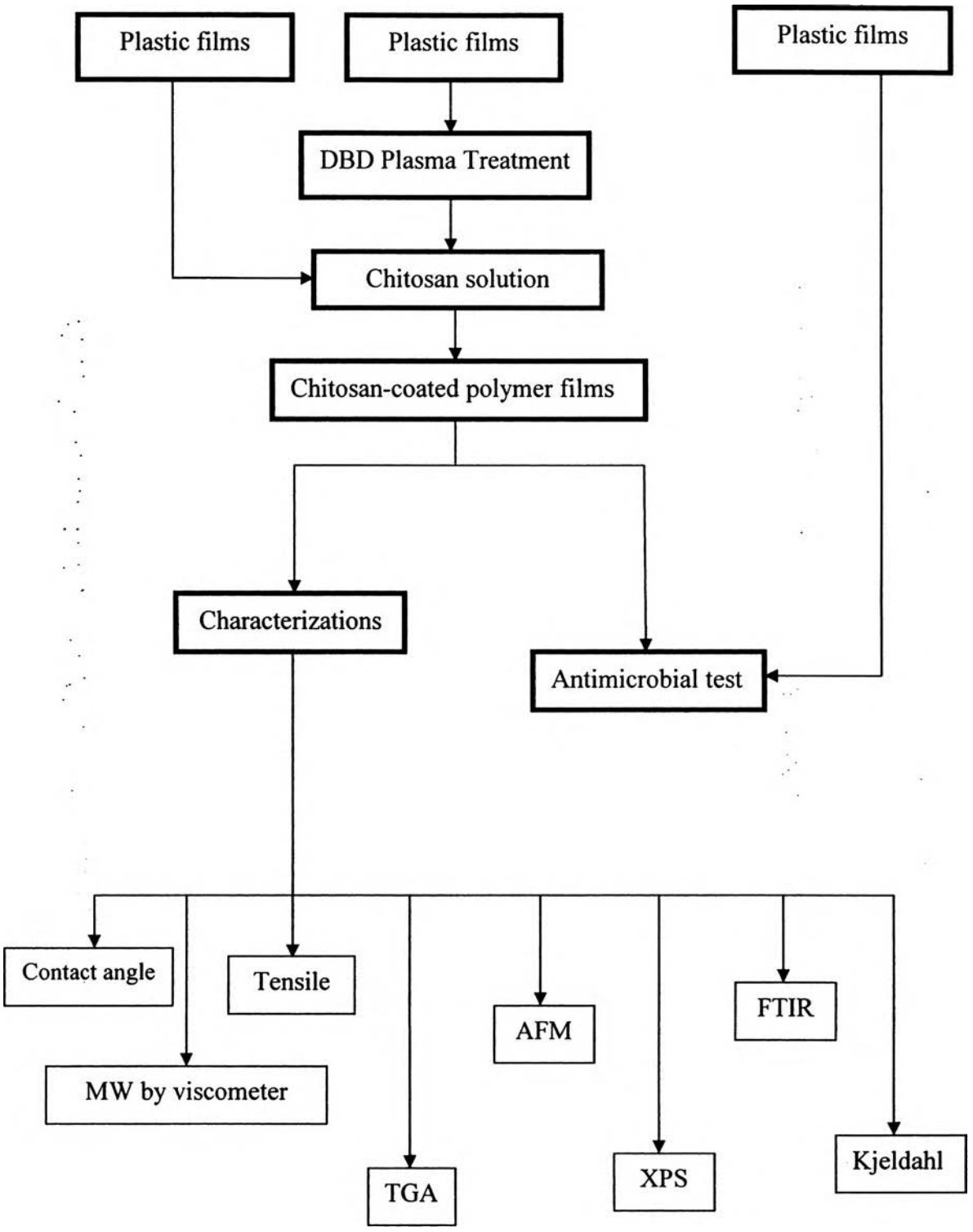

Figure 3.3 Flow chart of the entire experimental procedure.