CHAPTER III
EXPERIMENTAL

3.1 Chemicals and Materials

3.1.1 Materials

a.) The shell of *Litopeneous vannamei* shrimps was kindly provided by Surapon Food Public Co., Ltd.

b.) Woven PET fabric was provided by Thai Negoro Co., Ltd. (Thailand).

3.1.2 Chemicals

a.) 1N HCl and 4 % w/w NaOH solution was used to prepare chitin from the shrimp shell.

b.) 50 % w/w NaOH solution and sodium borohydride ($NaBH_4$) was used to produce chitosan from chitin.

c.) Acetic acid was used to dissolve chitosan.

3.1.3 Air Gas for Reaction

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

3.2 Methodology

3.2.1 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 1 N HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was followed by boiling 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. The product obtained was dried at 60°C in a convective oven for 24 h.

3.2.2 Preparation of Chitosan

Chitosan 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. The resulting chitosan flakes were dried in an oven at 60°C for 24 h.

3.2.3 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was determined, based on an IR spectroscopic method reported by Sannan (1978). About 3 mg of chitosan powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide to prepare a KBr disk. An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbances at 2878 cm^{-1} (the C-H band) and 1550 cm^{-1} (the amideII band) were used to quantitate the degree of deacetylation. The degree of deacetylation was calculated from the equation 3.1.

$$D = 98.03 - 34.68\,(A_{1550}/A_{2878}) \tag{3.1}$$

where D = degree of deacetylation (%)

A_{1550} = absorbance at 1550 cm^{-1}

A_{2878} = absorbance at 2878 cm^{-1}

3.2.4 Viscosity-Average Molecular Weight of Chitosan

Chitosan solution of different concentration (0.00, 0.0125, 0.025, 0.050, 0.075, and 0.1 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:

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

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

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

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

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)

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

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

The flow chart of the entire experimental procedure is shown in Figure 3.1:

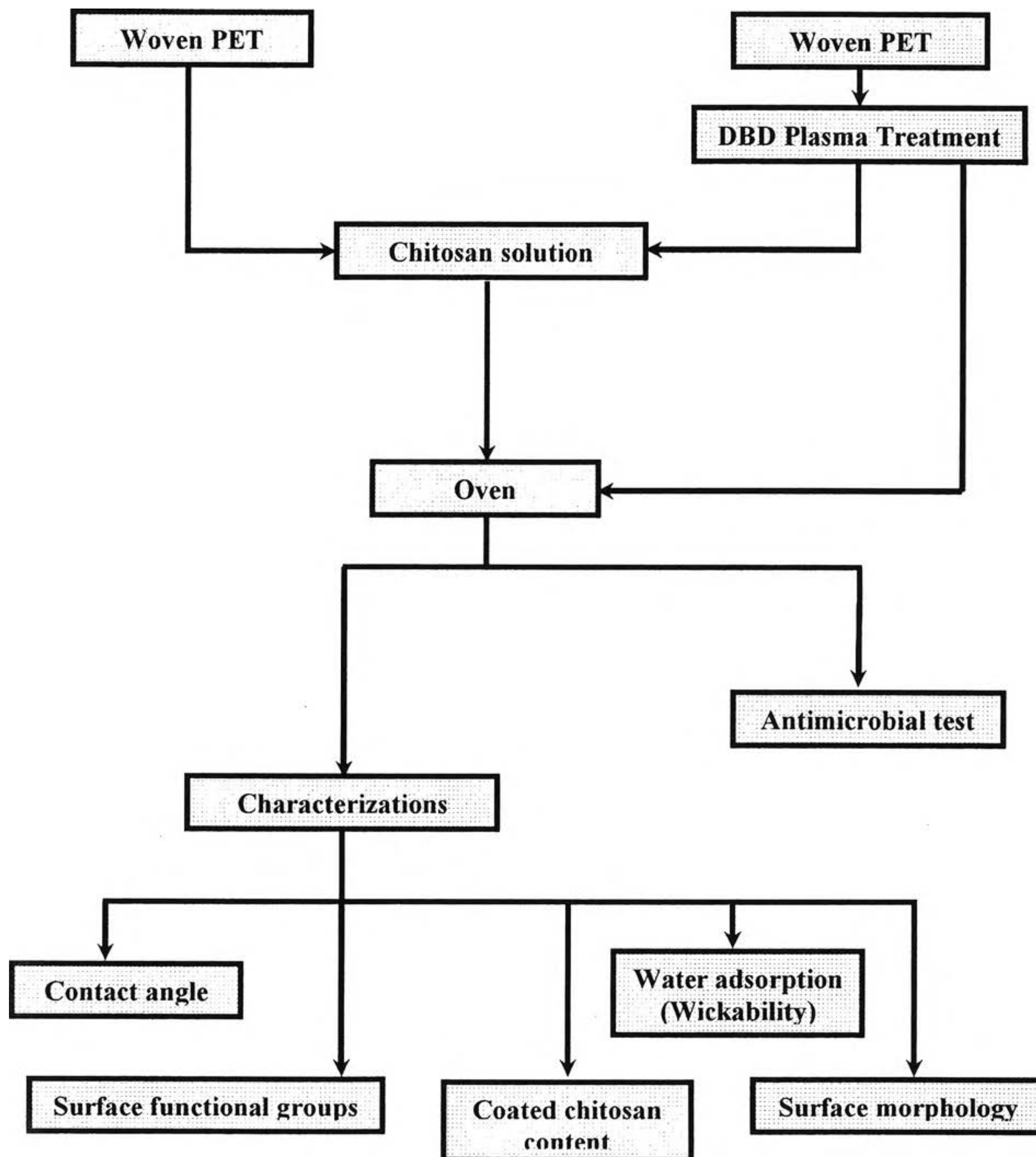

Figure 3.1 Flow chart of the entire experimental procedure.

3.3 Characterizations

3.3.1 Capillary Viscometer

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

3.3.2 Wickability Measurement

The wickability test of woven PET was carried out for hydrophilicity/wettability measurement before and after DBD plasma treatment. The wickability was estimated by testing fabric samples of 1 cm×5 cm area, measuring the time required for distilled water to rise 1 cm up the vertical fabric (25°C temperature, 50% relative humidity, 1 mm initial immersion depth). The values presented were averaged over five measurements for each set of treatment conditions.

3.3.3 Contact Angle Measurement

Hydrophilicity of the surface was evaluated by measuring the contact angle formed between water drops and the surface of the modified samples using contact angle measuring system G 10 (KRUSS). For this purpose, the drops of water were mounted on three different areas of the surface with a microsyringe. The results were mean values of three measurements on different parts of the fabric and film.

3.3.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was performed on gold- coated samples, which were obtained using a polaron sputter coater. A SEM operating condition typically at 10 kV was employed for morphology study. Samples were mounted onto the sample holder, sputter-coated with gold, and finally used for SEM analysis.

3.3.5 X-ray Photoelectron Spectroscopy (XPS)

XPS analysis was conducted to analyze the surface chemical state and compositions of the plasma-treated woven PET.

3.3.6 Fourier Transformed Infrared Spectroscopy (FTIR)

The chitosan and the woven PET were analyzed by a Thermo Nicolet Nexus 670 FTIR spectrometer. Samples were recorded at a spectral resolution and wave number precision of 0.09 and 0.01 cm^{-1}, respectively. The woven PET (5.0×5.0

cm^2) was placed into the Smart Multi-Bounce HATR sample compartment of the spectrometer and continuously purged with dry air. For each spectrum, 64 scans were acquired at a spectra resolution of 4 cm^{-1}.

3.3.7 Amido Black 10B

Amido black 10B was used for the qualitative determination of chitosan coated on the surface of modified woven PET. Amido black 10B is an amino acid staining diazo dye used in biochemical research to stain for total amino group of chitosan on the surface of chitosan coated on woven PET.

3.3.8 Kjeldahl Method

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

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

(b) The ammonium ions were then converted into ammonia gas, heated, and distilled. The ammonia gas was allowed to pass a trapping solution where it dissolved and become an ammonium ion once again.

(c) Finally, the amount of the ammonia that was trapped was determined by titration with a standard solution, and the moles of nitrogen were calculated.

3.4 Power Supply Unit

The block diagram of the power supply unit is shown in Figure 3.2. For the first step, the alternating current (AC) input of 220 Volt and 50 Hz was converted to direct current (DC) of about 70-80 Volt by DC power supply converter. For the second step, the DC current was supplied through a 500-Watt 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 was passed through the transformer to convert to 230 Volt AC. Thereafter, the variable output was finally transmitted to a high voltage current by nominal factor 130 times of low side voltage (input). An Extech® series 380801 power analyzer was used to measure power, power factor, current, frequency, and voltage at the low side of the power supply unit.

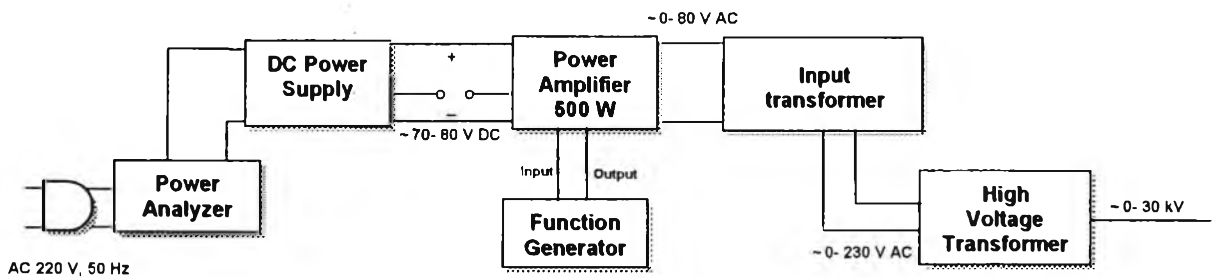

Figure 3.2 Block diagram of the power supply unit.

3.5 Experimental Setup

A schematic diagram of the plasma configuration is depicted in Fig. 3.3. A dielectric barrier discharge or "silent discharge" could be obtained between 2 electrodes. Two electrodes were placed within vacuum chamber, one of electrodes was covered with a glass plate (thickness=2 mm) and the distance between the plates was 4 mm. Plasma reactor was connected via electrical line to power supply with frequency and voltage of 325 Hz and 50 V. The chamber was then connected with air gas input and vacuum pump was also connected to desiccators for evacuating air inside chamber out.

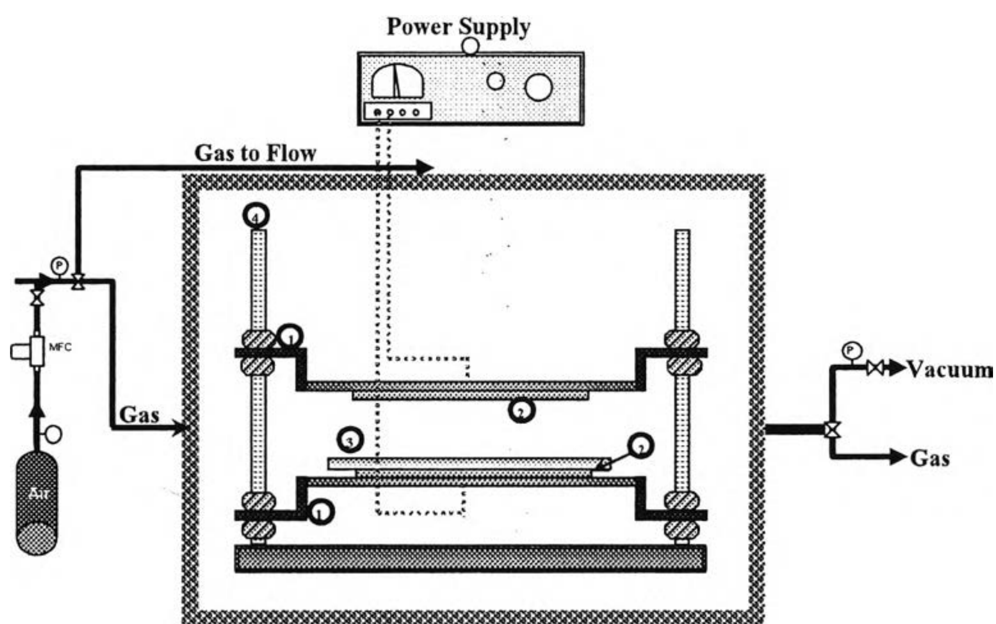

Figure 3.3 Schematic of experimental setup for dielectric-barrier discharge system.

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

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3.6 Antimicrobial Activity Testing

The antimicrobial activities of chitosan-coated woven PET fabrics were tested against gram-negative bacteria *Escherichia coli* and gram-positive bacteria *Staphylococcus aureus* by using two methods.

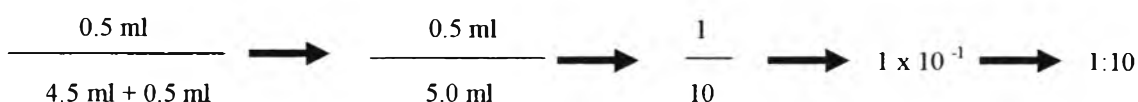
3.6.1 The Agar Diffusion Test

This method was performed in Luria–Bertani (LB) medium solid agar Petri dish. The chitosan-coated woven PET was cut into a disc shape of 2.0×2.0 cm^2, sterilized by autoclaving 15 min at 120°C, and placed on *Escherichia coli*-cultured and *Staphylococcus aureus*-cultured agar plates, which were then incubated for 24 h at 37°C. The zone of inhibition was then measured and recorded.

3.6.2 The Colony Counting Method

Chitosan-coated woven PET was cut into a disc shape of 1.0×1.0 cm^2. Before inoculation of the bacteria, the pieces of sample were sterilized by autoclaving at 120°C for 15 min. Sample was divided into three groups, and every group was contained 1 ml nutrient broth containing either 10^7 colony forming units per ml (cfu/ml) of *Escherichia coli* or *Staphylococcus aureus*. Ten-fold serial dilutions of bacterial suspension were then made as described below. The first group was referred as control. One piece of woven PET or chitosan-coated woven PET was then added to second or third group, respectively. The 0.05 ml of bacterial suspension was drawn from each of tube, spread on to a nutrient agar plate, and then incubated at 37°C for 24 h. The colony counting was calculated to obtain a number of living (see below). The experimental design is shown below, where the mathematical reasoning for performing the serial dilutions is:

Tube 1 contains 4.5 ml of sterile media; adding 0.5 ml of the undiluted bacterial suspension to yield a total volume of 5.0 ml.



$$\frac{0.5\ \text{ml}}{4.5\ \text{ml} + 0.5\ \text{ml}} \rightarrow \frac{0.5\ \text{ml}}{5.0\ \text{ml}} \rightarrow \frac{1}{10} \rightarrow 1 \times 10^{-1} \rightarrow 1{:}10$$

Tube 2 contains 4.5 ml of sterile media; adding 0.5 ml of the 1:10 diluted bacterial suspension to yield a total volume of 5.0 ml

$$\frac{0.5\text{ ml}}{4.5\text{ ml} + 0.5\text{ ml}} \longrightarrow \frac{0.5\text{ ml}}{5.0\text{ ml}} \times \frac{1}{10} \longrightarrow \frac{1}{100} \longrightarrow 1 \times 10^{-2} \longrightarrow 1{:}100$$

Plate the following dilutions: 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} and 1×10^{-10}.

For the cell suspension that was plated onto the agar plate labeled as 1×10^{-1} 0.05 ml of the diluted suspension was pipetted from the appropriately diluted test tube onto the surface of the plate.

To calculate the number of bacteria per ml of diluted sample, one should use the following equation:

$$\frac{\text{Number of CFU}}{\text{Volume plated (ml)} \times \text{total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{ml}} \qquad (3.6)$$

And, reduction percentage was calculated using the following the formula:

$$R = \frac{B-A}{B} \times 100\% \qquad (3.7)$$

where R is the reduction percentage, A is the number of bacteria recovered from the inoculated treated test sample swatches in the jar incubated over the desired contact period (24 h) and B is the number of bacteria recovered from the inoculated treated test sample swatches in the jar immediately after inoculation, i.e. at zero contact time.