CHAPTER III
METHODOLOGY

3.1 Materials

3.1.1 Silk Cocoons and Banana Rachises

The *Bombyx mori* silkworm cocoons were obtained from the Queen Sirikit Department of Sericulture (Thailand).The *Musa sapientum Linn* banana rachises were purchased from local banana farm in Ratchaburi province, Thailand.

3.1.2 Yeast Cells

Saccharomyces cerevisiae burgundy KY 11 was purchased from Institute of Food Research and Product Development (IFRPD), Kasetsart University in the form of fresh yeast.

3.1.3 Other Chemicals

Methanol (CH_3OH) and ethanol (C_2H_5OH) (99.5 % purity), analytical grade, were purchased from RCI Labscan., Ltd. Analytical grade calcium chloride dihydrate ($CaCl_2\,2H_2O$) was purchased from Analar®. Sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) pellets, analytical grade, were purchased from RANKEM. Hydrogen peroxide (H_2O_2) was purchased from Fisher Scientific Co., Ltd. D-glucose anhydrous, bacteriogical peptone, and yeast extract powder were purchased from UNIVAR, CONDA, and HimediA, respectively. Sodium potassiam tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$) and 3,5- dinitrosalicylic acid (DNS) were perchased from Sigma Aldrich.

3.2 Experimental Methods

3.3.1 Preparation of CLWs

M. sapientum Linn banana rachis was cut into the length of 100 mm to 300 mm and dried overnight in an oven at 40 °C. Dried banana rachises were then

soaked in a 2 %(w/v) NaOH solution at 80 °C for 2 hours. After thoroughly rinsed with distilled water, the rachises were treated with a 3 % (w/v) H_2O_2 solution at 70 °C for 2 hours. The purified cellulose was hydrolyzed at 60 °C for 4 hours in a 65% (w/v) sulfuric acid solution. The resulting CLWs were diluted with distilled water and centrifuged at 10,000 rpm for 10 min. After the centrifugation, the CLWs suspension was neutralization by dialyzed against distilled water until neutral pH.

3.3.2 Preparation of SF Solution

B. mori silk cocoons were cut into small pieces, washed with water, and dried at 40 °C overnight. The silk degumming process was carried out by boiling the silk cocoons in a 0.05 % (w/v) Na_2CO_3 solution for 15 minutes (repeated for 2 times). The degummed silk was dried in an oven at 40 °C overnight before being dissolved in a polar solvent system containing $CaCl_2$, ethanol, and water at a $CaCl_2$:ethanol:water molar ratio of 1:2:8. The resulting fibroin solution was dialyzed against distilled water until negative test of $AgNO_3$ was found, followed by centrifuged at 10,000 rpm for 10 minutes. The as-prepared SF solution was kept at 4 °C until use.

3.3.3 Preparation of CLWs-Reinforced SF Sponges

The as-prepared SF solution was diluted with distilled water to achieve a desired concentration at 3% (w/v). The CLWs suspension was ultrasonicated for 15 minutes prior to adding into the SF solution at four different CLWs content — 10%, 20 %, 30%, 40% and 50% based on final weight of bionanocomposite sponges — with slow mechanical stirring. The mixture was stirred for 10 minutes and 1 ml of the well-mixed solution was pipetted to each well of COSTAR® 24-multi-wells culture plate and freeze dried at -40°C overnight

3.3.4 Methanol Treatment of CLWs-Reinforced SF Sponges

The SF sponges both with and without CLWs were immersed in a 90% (v/v) methanol solution for 10 minutes. After that the methanol-treated sponges were washed with an excessive amount of distilled water and dried by using a freeze dryer at -40°C for 24 hour.

3.3.5 Inoculums Preparation

One loop of *Saccharomyces cerevisiae* burgundy KY11 was transferred into 10 ml of Yeast Peptone Dextrose (YPD) nutrient broth consisting of glucose, peptone, and yeast extract at a concentration of 20 $g \cdot l^{-1}$, 20 $g \cdot l^{-1}$, and 10 $g \cdot l^{-1}$, respectively The yeast culture was incubated in a shaking at 150 rpm and 30 °C for 16 hours in order to get mature yeast cell which a cell concentration more than 10^8 $cells \cdot ml^{-1}$ for being used in the cell immobilization step.

3.3.6 Cell Immobilization

The methanol-treated CLWs-reinforced SF sponges and 250 ml of YPD broth were autoclaved separately at 121°C for 15 minutes. The inoculum cell suspension was then added to the sterilized medium. To induce natural cell adhesion, the sponges were immersed in the culture medium and then incubated in a shaking incubator at 150 rpm and 30°C for 48 hours following by freeze drying.

3.3.7 Vitalization of Yeast Cell Immobilized in Bionanocomposite Sponges

Immobilized cells of *Saccharomyces cerevisiae burgundy* KY11 in bionanocomposite sponges was revitalized in a 250 ml Erlenmeyer flask containing 100 ml of YPD nutrient medium in a shaking incubator at 150 rpm and 30° C for 24 hour.

3.3.8 Fermentation

Batch fermentation of both suspended cells (used as control) and immobilized cells of *Saccharomyces cerevisiae burgundy* KY11 in CLWs-reinforced SF sponges was used in an equivalent number of yeast cells. Suspended cell and immobilized cell were transferred to a cotton plugged 250 ml Erlenmeyer flask containing 100 ml of YPD nutrient broth. The culture medium was sampled at a specific time interval for 48 hours and was then centrifuged at 10,000 rpm for 10 minutes to remove cell pellets. The clear supernatant was further subjected the reducing sugar concentration, ethanol content, and cell viability analyses by DNS method, Gas chromatography (GC) and direct counting using Neubaeur Precicolor HBG heamacytometer counting chamber, respectively.

3.3 Characterizations and Testing

3.3.1 Fourier Transformed Infrared (FTIR) Spectroscopy

A Thermo Nicolet Nexus 671 FTIR spectrophotometer was used to characterize the chemical structure and conformation of SF, CLWs, and CLWs-reinforced SF sponges. The spectra were collected at a resolution of 4 cm^{-1} and 64 scans in the wavenumber range of 4000 cm^{-1} to 400 cm^{-1}

3.3.2 Field Emission Scanning Electron Microscopy (FE-SEM)

A HITACHI S4800 FE-SEM microscope was used to observe both surface and cross-section morphology of the CLWs-reinforced SF sponges at an operating voltage of 2 kV. The specimens were coated with platinum by using a sputtering equipment operated for 200 seconds before the SEM observation.

3.3.3 Transmission Electron Microscopy (TEM)

The TEM image of cellulose whiskers were taken by a JEOL JEM 2100 TEM microscope at an operating voltage of 200 kV. Samples for TEM observation were prepared by staining the diluted cellulose whiskers suspension with 1 % uranyl acetate aqueous solution. The sample was dropped on a carbon-coated copper grid and air-dried.

3.3.4 Shrinkage of Bionanocomposite Sponges

The volume of the bionanocomposite sponges before and after methanol treatment was investigated to calculate the shrinkage (%) after treated with methanol.

$$\text{Shrinkage (\%)} = \frac{(V_i - V_f)}{V_i} \times 100$$

Vi : the volume of the sponges before methanol treatment

Vf : the volume of the sponges after methanol treatment

3.3.5 Lloyd Instrumental

To evaluate the compression modulus, the compression test was performed at crosshead speed 1 mm/minute at room temperature. The compressive modulus was

calculated from the initial slope of the linear portion of the stress–strain curve. The sample number was five for each experimental group.

3.3.6 Weight Loss

To identify the stability of bionanocomposite sponge in water, both non-methanol treated and methanol treated bionanocomposite sponges were immersed in distilled water and incubated at 30°C 150 rpm for 72 hours. Weight loss was calculated from this equation;

$$\% \text{ weight loss} = \frac{(W_i - W_f)}{W_i} \times 100$$

W_i : initial dry weight of bionanocomposite sponge

W_f : final dry weight of bionanocomposite sponge

3.3.7 Ultraviolet-Visible (UV-vis) Spectroscopy

An Tecant Infinite® 200 PRO UV-Vis spectrophotometer was used to examine the utilization of reducing sugar by yeast cells during fermentation process. To determine the immobilization efficiency, the utilization of reducing sugar during fermentation process was evaluated by using the DNS method (Miller, 1959) based on the precipitation of residual sugar. The tests were made with 3 ml of DNS reagent added to 3 ml of sample solution. The color intensities in terms of absorbance were measured at a wavelength of 575 nm. The sugar concentration was then determined from the glucose standard curve prepared in the concentration range of 0.1 mg/ml to 1 mg/ml.

3.3.8 Gas Chromatography (GC)

The bioethanol concentration was determined by using a Shimazu GC-7AG instrument equipped with a flame ionization (FID) detector. A steel gas chromatograph column packed with Porapak Q was used. Temperature of the column and injector were fixed constant at 170 °C and 220 °C, respectively. Nitrogen (N_2) gas with the flow rate of 45 ml min^{-1} was used as a carrier gas. Peak areas in the GC chromatograms were compared with ethanol with known concentration of 0.01% to 20% (v/v) in order to calculate bioethanol concentration in the test sample.

3.2.9 Optical Microscopy (OM)

Sampling the suspension of ferment then centrifuged at 10000 rpm 4°C for 10 minutes in order to separate the cell pellets out of the supernatant. The cell pellets were resuspended in a 0.85 % NaCl solution before dropped to a Neubaeur Precicolor HBG heamacytometer counting chamber. The number of yeast cells was counted directly under an Olympus CX31 OM microscope in order to evaluate the cell growth during fermentation process.