# CHAPTER III
# METHODOLOGY

## 3.1 Materials

### 3.1.1 Silk Cocoon and Banana Rachis

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

### 3.1.2 Yeast Cells

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

### 3.1.3 Other Chemicals

Analytical grade of sodium hydroxide (NaOH) and sodium carbonate ($Na_2CO_3$) pellets were purchased from RANKEM. Calcium chloride dihydrate ($CaCl_2 2H_2O$), analytical grade, was purchased from Analar®. D-glucose anhydrous, bacteriogical peptone, and yeast extract powder were purchased from UNIVAR, CONDA, and HimediA, respectively. Analytical grade of ammonium sulfate ($(NH_4)_2SO_4$) was purchased from LOBA Chemie. Analytical grade of potassium dihydrogen phosphate ($KH_2PO_4$) was purchased from RANKEM. Hydrogen peroxide ($H_2O_2$) was purchased from Fisher Scientific Co., Ltd. 98 % of sulfuric acid ($H_2SO_4$) was purchased from J.T. Baker, Thailand. Methanol ($CH_3OH$) and ethanol ($C_2H_5OH$) (99.5 % purity), analytical grade, were purchased from RCI Labscan., Ltd. Both 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

### 3.2.1 Preparation of SF Solution

*B. mori* silk cocoons were cut into small pieces, washed by water, and dried in oven at 40 °C overnight. Then boil the silk cocoons in 0.05 % (w/v) $Na_2CO_3$ solution for 15 minutes (repeated for 2 times), wash them with boiled water and distilled water in order to obtain degummed silk. The degummed silk was dried in an oven at 40 °C overnight. A polar solvent system containing $CaCl_2$, ethanol, and water at a $CaCl_2$:ethanol:water molar ratio of 1:2:8 was prepared to dissolve the degummed silk. Then the silk fibroin solution was dialyzed in distilled water until negative test of $AgNO_3$ was found and followed by centrifuged at 10,000 rpm for 10 minutes. The SF solution was kept at 4 °C until use.

### 3.2.2 Preparation of CLWs

*M. sapientum Linn* banana rachis was cut into many small pieces about 15 mm to 20 mm of length and dried in oven at 40 °C overnight. The dried small banana rachis pieces were soaked in 4 % (w/v) NaOH at 80 °C for 2 hours to remove non-cellulosic materials to increase susceptibility to its hydrolysis and then thoroughly rinsed with distilled water. Then the banana rachises were bleached by treating with 5% (w/v) $H_2O_2$ solution at 70 °C for 2 hours. This step removed most of the residual lignin and protein. The bleached banana rachis fibers were hydrolyzed by 65% (w/v) $H_2SO_4$ at 60 °C for 4 hours under vigorous stirring. After that, CLWs were diluted with distilled water, centrifuged at 10,000 rpm for 10 minutes for three times and dialyzed until neutral.

### 3.2.3 Preparation of SF/CLWs Bionanocomposite Sponge

The SF solution was diluted with distilled water to get a concentration at 2% (w/v). The CLWs suspension was diluted to be 1% (w/v) and ultrasonicated for 15 minutes before adding into the SF solution at five different CLWs contents (10%, 20%, 30%, 40% and 50% based on weight ratio) with slow mechanical stirring. Then the mixture was stirred for 10 minutes and 1 ml of the mixture was pipetted to each well of COSTAR® 24-multi-wells culture plate and freeze dried at -40°C overnight.

3.2.4 Methanol Treatment of SF/CLWs Bionanocomposite Sponge

The SF and SF/CLWs bionanocomposite sponge were immersed in 90% (v/v) methanol solution for 10 minutes. And then the methanol-treated sponges were washed with an excessive amount of distilled water and dried by freeze drying at -40 °C for 24 hours.

3.2.5 Inoculums Preparation

To prepare an inoculum, 1 loop of yeast cell was added into a test tube containing 10 ml of Yeast Peptone Dextrose (YPD) broth growth medium consisting of D-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 incubator at 150 rpm and 30 °C for 16 hours. Yeast cell concentration more than $10^8$ $cells \cdot ml^{-1}$ was obtained for using in the cell immobilization process.

3.2.6 Yeast Cells Immobilization

The methanol-treated SF/CLWs bionanocomposite sponge and 100 mL of YPD broth were autoclaved at 121 °C for 15 minutes. The sterilized sponges were immersed in the sterilized medium for 10 minutes. After that the inoculum yeast cells suspension were added to the sterilized medium. Then it was incubated in a shaking incubator at 150 rpm and 30 °C for 48 hours. After yeast cells were immobilized in the methanol-treated SF/CLWs bionanocomposite sponge, sponges were freeze dried at -40 °C overnight.

3.2.7 Continuous Ethanol Fermentation

The column containing the immobilized yeast cells was a tubular column that its inner diameter and height were about 3 and 40 cm, respectively. A tubular column was wrapped by jacket column in order to control the temperature within column (30 °C) during fermentation. The cooling bath circulator was used to control water temperature flowed within jacket column. The fresh nutrient medium was fed to the bottom of column and the effluent from column after fermentation was collected at the top of column. Both flow rate of feed in and feed out of medium in column were controlled by two peristaltic pumps. The 120 pieces of SF/CLWs bionanocomposite sponge containing yeast cells were contained in the column. The system was precultured and revitalized by feeding the medium at 0.6 ml/min of flow

rate consisting of (in g/l): D-glucose, 20; peptone, 20; yeast extract, 10; $(NH_4)_2SO_4$, 1; $KH_2PO_4$, 1 at 30 °C for 24 hours. The working volume and height of the column were 247.30 ml and 35 cm, respectively.

The flow rates for the feed into the column with hydraulic retention time (H) 6.87, 5.15 and 4.12 hr were 0.6, 0.8, 1.0 ml/min, respectively. At the hydraulic retention time (H) 6.87, 5.15 and 4.12 hr corresponded to 0.15, 0.20 and 0.25 $hr^{-1}$ of dilution rate (D=1/H). The concentrations of 100, 150 and 200 g/l D-glucose were used to be carbon sources for continuous ethanol fermentation. Furthermore, the feeding medium comprised of (in g/l): peptone, 20; yeast extract, 10; $(NH_4)_2SO_4$, 1; $KH_2PO_4$, 1. The effluent from column was collected every three hours. The effluent sample was centrifuged at 10,000 rpm at 4°C for 10 minutes to remove cell pellets. The clear supernatant was determined the residual sugar concentration and ethanol content by DNS method and gas chromatography (GC), respectively.

### 3.3 Equipments and Characterizations

#### 3.3.1 Fourier Transformed Infrared (FTIR) Spectroscopy

The chemical structure of SF, CLWs, and SF/CLWs bionanocomposite sponges were characterized by a Thermo Nicolet Nexus 671 FTIR spectrophotometer. The spectra were collected at a resolution of 4 $cm^{-1}$ and 64 scans from 4000 $cm^{-1}$ to 400 $cm^{-1}$ of the wavenumber.

#### 3.3.2 Transmission Electron Microscopy (TEM)

The TEM image of cellulose whiskers were detected 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.

3.3.3 Field Emission Scanning Electron Microscopy (FE-SEM)

Both surface and cross-section morphology of the sponges were observed by a HITACHI S4800 FE-SEM microscope at an operating voltage of 2 kV. The sponges were coated with platinum by using a sputtering equipment operated for 200 seconds before the SEM observation.

3.3.4 Lloyd Instrumental

The compression test was achieved at crosshead speed 1 mm/minute at room temperature. The compressive modulus of SF/CLWs bionanocomposite sponge was reported.

3.3.5 Optical Microscopy (OM)

The number of yeast cells was counted directly under an Olympus CX31 OM microscope by the cell pellets resuspended in a 0.85 % NaCl solution before dropped to a Neubaeur Precicolor HBG heamacytometer counting chamber.

3.3.6 Ultraviolet-visible (UV-vis) Spectroscopy

An Tecant Infinite® 200 PRO UV-Vis spectrophotometer was used to analyze the utilization of reducing sugar by yeast cells during continuous ethanol fermentation process. The DNS method (Miller, 1959) based on the precipitation of residual sugar was used in order to determine the utilization of reducing sugar during fermentation process. At a wavelength of 575 nm, the color intensities in terms of absorbance were measured. The sugar concentration was then determined from the D-glucose standard curve that was prepared in the concentration range of 0.2 to 1.0 (g/l).

3.3.7 Gas Chromatography (GC)

A Shimazu GC-7AG instrument equipped with a flame ionization (FID) detector was used to determine ethanol concentration. 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 of the GC chromatograms of ethanol were compared with peak areas of the ethanol standard that was prepared in the concentration range of 0.01 to 15 (%v/v). Then ethanol concentration in the test sample was calculated.

## 3.4 Analytical Measurements

### 3.4.1 Weight Loss of SF/CLWs Bionanocomposite Sponge

Non-methanol and methanol-treated SF/CLWs bionanocomposite sponge were immersed in distilled water and incubated at 30 °C 150 rpm. The stability of bionanocomposite sponges in water was identified at various times (16, 24, 48, 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

### 3.4.2 Shrinkage of SF/CLWs Bionanocomposite Sponge

The volume of the SF/CLWs bionanocomposite sponge before and after methanol treatment was calculated to identify the shrinkage (%) of bionanocomposite sponges after methanol treatment. Shrinkage (%) was calculated from this equation:

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

$V_i$ : The volume of the sponges before methanol treatment

$V_f$ : The volume of the sponges after methanol treatment

### 3.4.3 Immobilization and Leakage of Yeast Cells

A number of yeast cell contained SF/CLWs bionanocomposite sponge were counted. Then bionanocomposite sponge containing yeast cells were immersed in distilled water and incubated at 30°C 150 rpm for 24 hr in order to count a number of yeast cells which leaked out. The number of yeast cells in distilled water was counted by Olympus CX31 OM microscope.

Immobilization efficiency (%) was calculated from this equation:

$$\text{Immobilization efficiency (\%)} = \frac{X_i \times 100}{X_t}$$

Leakage (%) was calculated from this equation:

$$\text{Leakage (\%)} = \frac{X_f \times 100}{X_t}$$

$X_i$ = Immobilized yeast cell concentration in sponges (cells/1 g of sponge) after immersing in distilled water for 24 hr.

$X_f$ = Leaked free cell concentration in distilled water (cells/1 g of sponge) after immersing in distilled water for 24 hr.

$X_t$ = Total initial immobilized yeast cell concentration in sponges (cells/1 g of sponge)

3.4.4 Sugar Consumption during Fermentation

The sugar concentration was determined from the D-glucose standard curve that was prepared in the concentration range of 0.2 to 1.0 (g/l). The % sugar consumption was calculated by this equation:

$$\text{Sugar consumption (\%)} = \frac{(S_0 - S) \times 100}{S_0}$$

S = Residual glucose concentration (g/l) at outlet

$S_0$ = Glucose concentration (g/l) in feed

3.4.5 Ethanol Production during Fermentation

Peak area of the GC chromatograms of the ethanol from fermentation was compared with peak areas of the ethanol standard prepared in the concentration range of 0.01 to 15 (%v/v).

3.4.5.1 *Ethanol Production was calculated by this equation:*

$$\text{Ethanol production (g/l)} = P - P_0$$

P = Ethanol concentration (g/l) at outlet

$P_0$ = Initial ethanol concentration (g/l) = 0

3.4.5.2 *Volumetric Ethanol Productivity ($Q_p$) was also calculated by this equation:*

$$\text{Volumetric ethanol productivity } (gl^{-1}hr^{-1}) = P/H \text{ or } PD$$

P = Ethanol concentration (g/l) at outlet

H = Hydraulic retention time (hr)

D = Dilution rate ($hr^{-1}$) = 1/H

3.4.5.3 *Ethanol Yield ($Y_{P/S}$) was determined from this equation:*

$$\text{Ethanol yield} = P/S_0\text{-}S$$

P = Ethanol concentration (g/l) at outlet

$S_0$-S = Sugar consumption (g/l)