CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Corn cobs from B&C Pulaski Corporation Limited, Thailand

3.1.2 Sodium hydroxide

3.1.3 Citrate (Citric acid/Citric sodium) buffer

3.1.4 Standard glucose, xylose, arabinose, cellubiose, galactose and mannose

3.1.5 Distilled water

3.1.6 Cellulase enzyme

3.1.7 Dinitrosalicylic Acid Reagent Solution *Saccharomyces cerevisiae* yeast from KI Ethanol Company Limited

3.2 Equipment

3.2.1 A CEM (Matthews, NC, USA) MAR-5 HP-500 microwave system

3.2.2 Perkin Elmer Series 200 LC/S/N291N5060508: High Performance Liquid Chromatography (HPLC) with a refractive index detector using an Aminex-HPX 87H column (300 mm x 78 mm, Bio-Rad Lab, USA)

3.2.3 Varian GC-3800 simulated distillation gas chromatograph (Sim-Dist GC)

3.2.4 Scanning Electron Microscope (SEM)

3.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

3.2.6 Thermogravimetric Analyzer (TGA)

3.2.7 Oven

3.2.8 Incubator shaker

3.2.9 No. 4 filter paper

3.3 Methodology

3.3.1 Pretreatment of Corn Cobs by Microwave Assisted Alkali

Microwave solvent extraction lab station was used in this study for a combined of microwave and alkali pretreatment. This process is carried out as follows: 2 g of corn cobs was suspended in 30 mL of different sodium hydroxide concentration (0.75 % to 3 %) and then transferred to microwave oven to treat corn cobs at the desired temperature (60 °C to 120 °C) from 5 to 30 minutes. After this process was completed, the residues would be collected by a filter paper then washed with tap water until the pH of solution reach neutral pH. Then it was dried at 65 °C and weigh (Zhu *et al.*, 2005).

Figure 3.1 A CEM (Matthews, NC, USA) MAR-5 HP-500 microwave system.

3.3.2 Enzymatic Hydrolysis

Hydrolysis experiment consists of 0.5 g of pretreated corn cobs and 15 mL of 0.1 mol L^{-1} citrate buffer (pH 4.8). The mixture was added with 0.1 ml of enzymes and then was adjusted at 50 °C in the incubator shaker at 150 rpm for 48 h. The sample which taken from the hydrolysis solution was heated to 100 °C

immediately for 3 minutes to denature the enzymes, cooled to room temperature, and then centrifuged for 20 minutes at 8000 rpm (Zhu *et al.*, 2005). Then, the sample from the reaction will be stored for sugar analysis.

Figure 3.2 Incubator shaker.

3.3.3 Fermentation

2 mL of active yeast (*Saccharomyces cerevisiae*) was mixed with 20 ml of sugar solution from enzymatic hydrolysis step and then transfer to incubator shaker at 37 ° C for 1 day to 3 day. After this process was complete, the solution was collected to analyze the ethanol concentration by GC instrument.

3.4 Component Analysis of the Biomass Samples

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), and acid insoluble ash (AIA) of corn cobs before and after pretreatment were determined by the Nakhonratchasima Animal Nutrition Research and Development Center (Nakhonratchasima province, Thailand). The difference between NDF and ADF estimates detergent hemicellulose. Detergent cellulose is calculated by subtracting the values for (ADL + AIA) from ADF.

3.5 Crystallinity Measurement

X-ray diffraction (XRD) was used to identify crystallinity present in both untreated and pretreated corn cobs by using a Rigaku/Rint2200 diffractometer equipped with a Ni filtered CuKα radiation source (λ = 1.542 Å) of 40 kV and 30 mV. The sample was pressed into a hollow of glass holder and held in place by glass window. Then, it will be scanned in the 2θ range of 0 to 40° in the continuous mode with the rate of 1°/minute. Biomass crystallinity as expressed by crystallinity index (CrI) was determined according to a method by (Kumar *et al.*, 2009):

$$CrI = 100 \times \left[\frac{I_{002} - I_{amorphous}}{I_{002}} \right]$$

In which, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2h = 22.5 and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2h = 16.6.

3.6 Surface Characteristics

The sample both untreated and treated corncob were observed by a scanning electron microscope (SEM) using a Hitachi S-4800 microscope. Prior to acquiring images, the samples were mounted with double sided carbon tape on precut brass sample stubs and sputter coated with approximately 30 Å of Au/Pd. The representative images of both untreated and treated corn cobs reported here were acquired with a 15 kV accelerating voltage (Li et al., 2004).

3.7 Reducing Sugar Analysis

The liquid fraction was determined total reducing sugar using DNS (3,5-dinitrosalicylic acid) method (Miller, 1959).

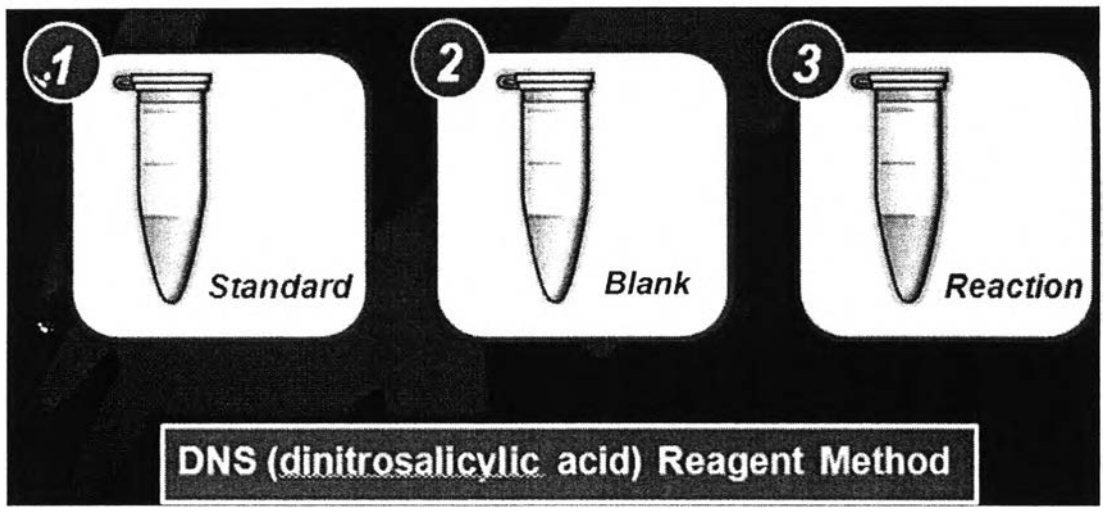
3.8 Monosaccharide Analysis

Monosaccharide such as glucose, xylose, arabinose, mannose, galactose, and cellubiose was determined using an HPLC system equipped with an organic acid column (Lichrospher NH_2) and a refractive index detector. 64%Acetonitrile–16%Water solution was used as the mobile phase at a flow rate of 1.6 mL $minute^{-1}$ and the column temperature was fixed at 25 °C.

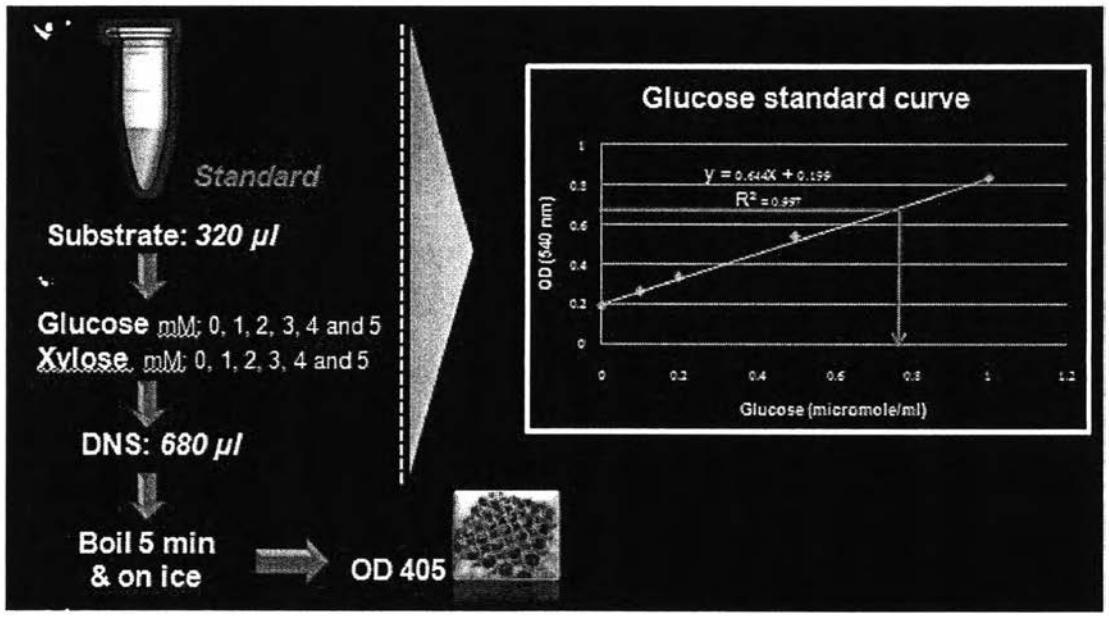
3.9 Cellulase Activity Analysis

The enzymatic activity was determined according to the method developed by Ghose (1987). The substrate (Carboxymethyl cellulase sodium salt for cellulase) (320 µl) was added in sample, blank, and standard tubes. The sample tubes were heated in a heating box at 50 °C for 10 minutes and then added with enzyme samples at 50 °C. Glucose solution (0, 1, 2, 3, 4, 5 mM) was added in standard tubes. After 30 minutes, DNS (dinitrosalicylic acid) reagent (680 µl) was added to all tubes. The enzyme samples were also added in a blank tube. All tubes were boiled in water at 100 °C after 5 minutes. The tubes were immediately transferred to cold water. UV-VIS Spectrometer (Thermo Fisher Scientific Inc., USA) at 550 nm was used to measure the color absorbed. The color formed in the blank tube is subtracted from that of the sample tube and translated the absorbance of the sample tube into glucose production during the reaction using standard curve. The sample tube was measured 2 times and the average values were reported. The glucose solution calibration curve was used to calculate the activity of cellulase. A calibration curve was established when glucose (0–1 g/L) and the activities of the cellulase was expressed.

(a)


1
Standard
2
Blank
3
Reaction
DNS (dinitrosalicylic acid) Reagent Method

(b)


Standard
Substrate: 320 µl
Glucose mM: 0, 1, 2, 3, 4 and 5
Xylose mM: 0, 1, 2, 3, 4 and 5
DNS: 680 µl
Boil 5 min
& on ice
OD 405
Glucose standard curve
y = 0.644x + 0.199
R² = 0.997
OD (540 nm)
Glucose (micromole/ml)

(c)

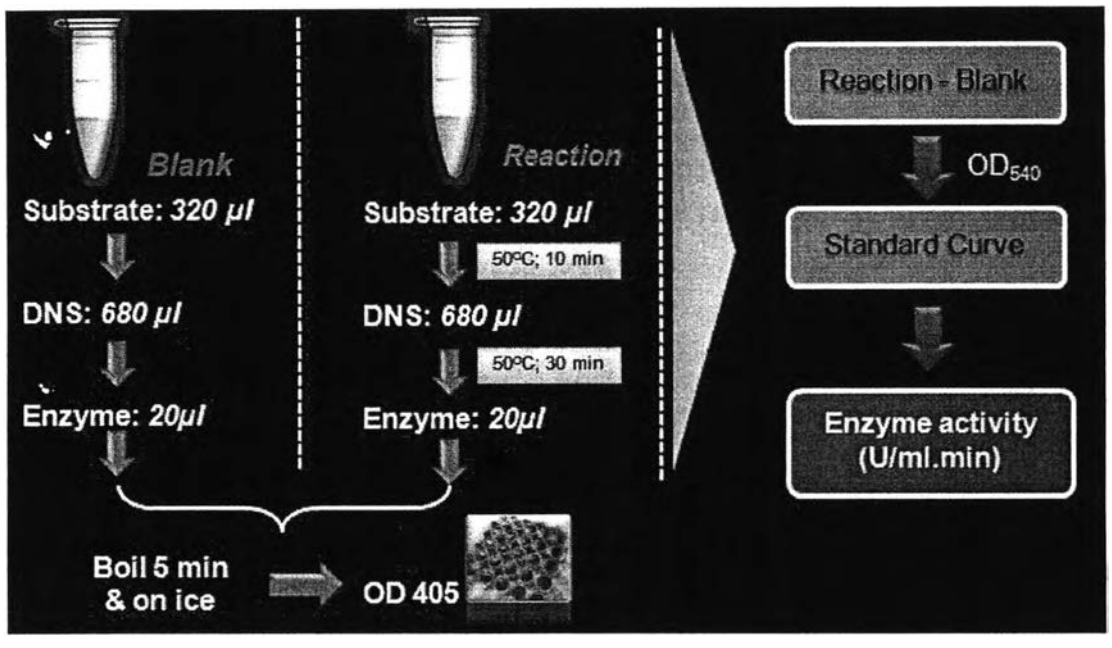


Figure 3.3 Cellulase activity measurement: (a) The substrate was added in sample, blank and standard tube, (b) Preparation of standard curve, and (c) The sample tube was measured to calculate the activity of cellulase.

3.10 BET Surface Area Analysis

BET surface areas of corn cobs before and after pretreatment were measured by N_2 adsorption/desorption measurements (BELSORP-max, BEL Japan INC., Japan) at 196 °C. The dried sample (0.5–1 g) was put into the sample tube and degassed using a vacuum for 4 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves.

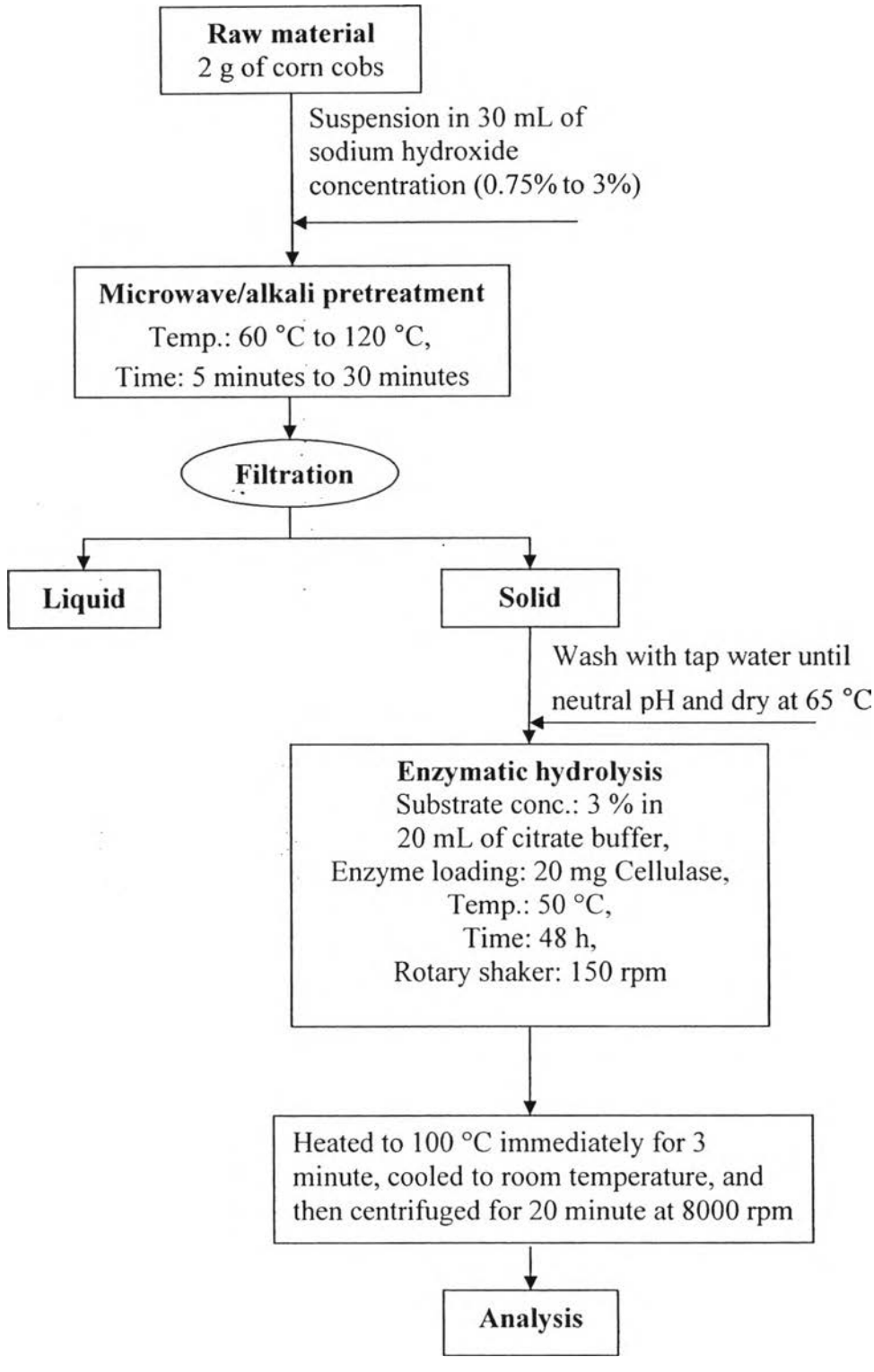


Figure 3.4 Schematic of pretreatment and hydrolysis procedure flow diagram.