CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Corncobs from Betagro Corporation Limited, Thailand

3.1.2 Cellulase from Trichoderma Reesei

3.1.3 Standard glucose, xylose, arabinose

3.1.4 Sodium hydroxide

3.1.5 Sulfuric acid

3.1.6 Calcium hydroxide

3.1.7 Sodium Sulphite

3.1.8 Hydroxymethylfurfural (HMF)

3.1.9 Furfural

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 x78 mm, Bio–Rad Lab, USA)

3.2.3 PERICHROM PR2100 Gas chromatography (GC)

3.2.4 Scanning Electron Microscope (SEM)

3.2.6 Brunauer–Emmett–Tellet (BET) Surface Area Analyzer

3.2.7 X–Ray Diffraction (XRD)

3.2.8 Oven

3.2.9 pH meter

3.2.10 Incubator shaker

3.2.11 Filter paper

3.2.12 Vortex mixture

3.3 Methodology

3.3.1 First Stage: Microwave/Alkali Pretreatment

Dried corncobs was pretreated with 2 % (w/v) NaOH solution using a solid–to–liquid ratio (SLR) 67:1, g of corncobs: L of solution. The mixture was stirred until homogeneous. Then, the mixture of corncobs and NaOH solution was transferred to a microwave oven to treat corncobs at 100 °C for 30 min. The picture of microwave oven is shown in Figure 3.1. After that the mixture was filtered to separate solid residues out. The solid residues were thoroughly washed with tap water to neutral pH and dried in the oven at 105 °C for 24 h (Chen *et al.*, 2011). Then, the oven–dried samples were stored for further dilute acid pretreatment in second stage of two–stage pretreatment.

3.3.2 Second Stage: Microwave/Acid Pretreatment

The solid residues from the microwave/alkaline pretreatment were treated with 1 % H_2SO_4 using different SLR (25:1 to 125:1). The mixture was stirred until homogeneous. Then, it was transferred to a microwave oven (at 80 °C to 160 °C) for 5 to 25 min. The pretreatment procedure flow diagram is shown in Figure 3.2.

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

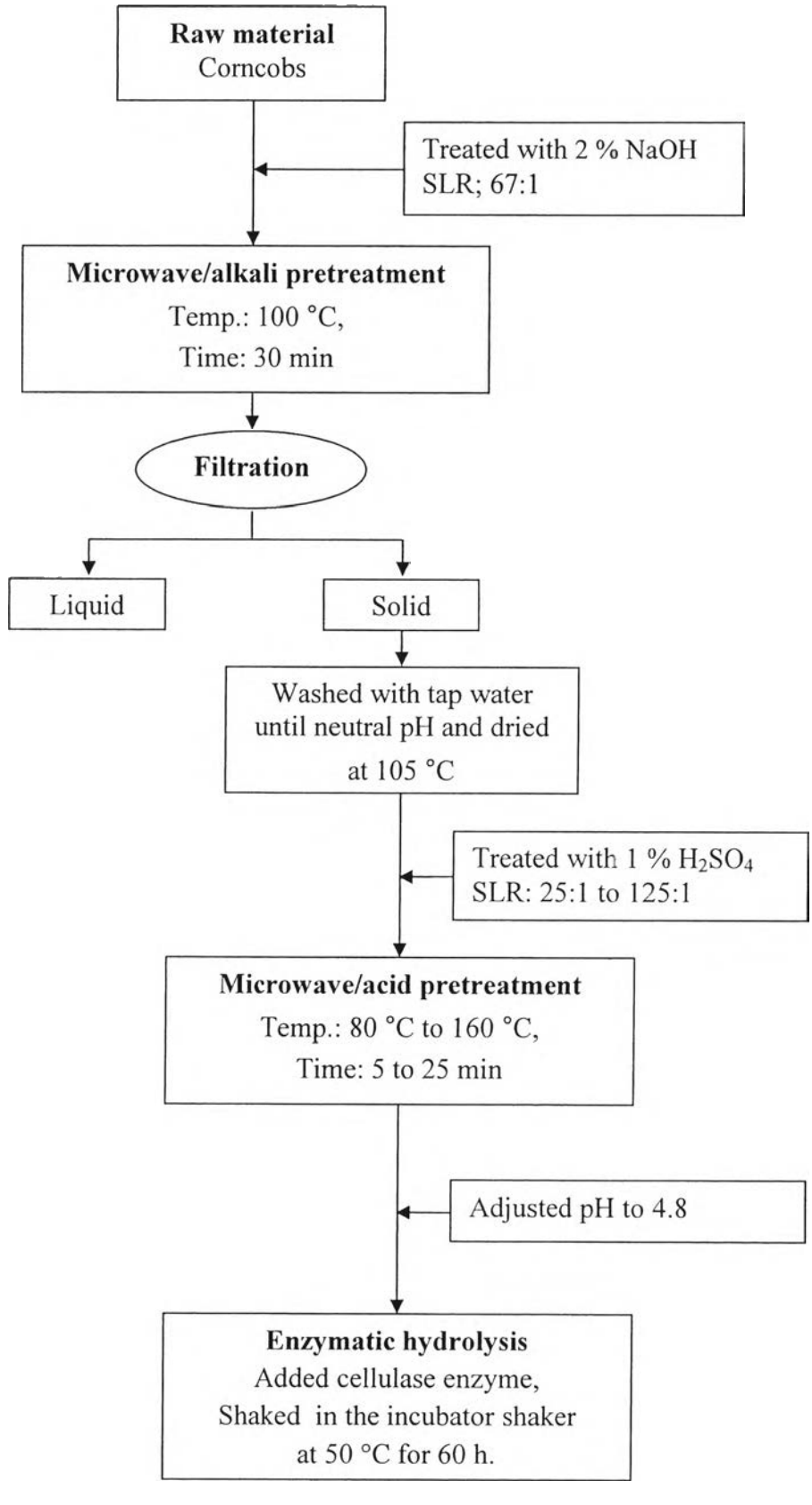

Figure 3.2 Schematic of pretreatment and hydrolysis procedure flow diagram.

3.3.3 Enzymatic Hydrolysis

The mixture from two–stage pretreatment was added with NaOH to adjust pH 4.8 and then was added with Celluclast 160 µl/g pretreated corncobs (cellulase; Sigma Chemicals, 52 FPU). After that the sample was shaked in an incubator shaker at 50 °C for 60 h. The hydrolysate was filtered to separate solid residues out. Then, the liquid fraction was collected for sugar analysis using HPLC.

3.3.4 Response Surface Methodology (RSM)

Response Surface Methodology (RSM) was adopted to determine the optimal condition of second stage pretreatment. RSM is more economical approach because a small number of experiments are performed for monitoring the interaction of the independent variables on the response. RSM with a full factorial central composite design (CCD) was employed in this study. The series of experiments designed and conducted are shown in Table 3.1. The metrix corresponding to CCD is presented in Table 3.2. Twenty experiments were carried out with three variables, and each variable varied at five level for glucose concentration; therefore, the concentration of glucose was the dependent variables.

Table 3.1 Independent variables and their levels in the experimental design

Independent variable	Symbols	Unit	Code Levels				
			-2	-1	0	1	2
Temperature	x_1	°C	80	100	120	140	160
Time	x_2	min	5	10	15	20	25
SLR	x_3	g/l	25	50	75	100	125

CCD was conducted to determine the individual and interactive effects of three parameters on glucose concentration. The following response equation was used to correlate the dependent and independent variables.

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} x_i x_j$$

Where Y_i is the response; x_i, x_j are the input variables, which influence the response variable Y_i; a_0 is the offset term; a_i is the *i*th linear coefficient; a_{ii} is the quadratic coefficient and a_{ij} is the *ij*th interaction coefficient.

Table 3.2 Experimental design of the central composite design

Run	Coded values			Real values		
	Temp.	Time	SLR	Temp. (°C)	Time (min)	SLR (g/l)
1	1	1	1	140	20	100
2	1	1	-1	140	20	50
3	1	-1	1	140	10	100
4	1	-1	-1	140	10	50
5	-1	1	-1	100	20	50
6	-1	1	1	100	20	100
7	-1	-1	1	100	10	100
8	-1	-1	-1	100	10	50
9	2	0	0	160	15	75
10	-2	0	0	80	15	75
11	0	2	0	120	25	75
12	0	-2	0	120	5	75
13	0	0	2	120	15	125
14	0	0	-2	120	15	25
15	0	0	0	120	15	75
16	0	0	0	120	15	75
17	0	0	0	120	15	75
18	0	0	0	120	15	75
19	0	0	0	120	15	75
20	0	0	0	120	15	75

3.3.5 Overliming process

The hydrolysate from enzymatic hydrolysis was adjusted to pH 10 with $Ca(OH)_2$ and then added Na_2SO_3 1 g/l. After that the mixture was heated at 90 °C for 30 min, and was cooled to room temperature. The mixture was filtered to separate solid residues out.

3.3.6 Fermentation

The liquid fraction which was taken from enzymatic hydrolysis or overliming was adjusted to pH 6.6 by 98 % H_2SO_4 and 10 ml of hydrolysate was fermented by using *Clostridium beijerinckii* TISTR1461 at 37 °C for 0 to 72 h.

3.3.6.1 Medium Preparation (Qureshi *et al.*, 2008)

To prepare the DifcoTM Cooked meat medium (CMM). The mixture of CMM pellet 0.875 g, glucose 0.12 g, and distilled water 6 ml was sterilized at 121 °C for 15 min and cooled to room temperature. After that, one loop of cell spores was put into the prepared solution and heat shock at 80 °C for 2 min. The CMM culture solution was kept in 37 °C and waited for cells activation within 30 h.

3.3.6.2 Inoculum Development (Qureshi *et al.*, 1999)

Yeast extract 100 µl, 0.5 of active growing cells from CMM solution, buffer (KH_2PO_4, K_2HPO_4, and CH_3COONH_4), mineral ($MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl), and vitamins (para-amino-benzoic acid, thiamin, biotin) was added in 9.3 ml of hydrolysate.

3.4 Analytical Methods

3.4.1 Component Analysis of the Biomass Samples

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

3.4.2 Monosaccharide Analysis

Glucose, xylose, and arabinose were determined using an HPLC system equipped with a refractive index detector (Model 6040 XR, Spectra–Physics, USA). An organic acid column (Aminex HPX–87H column, Bio–Rad Lab, USA) was used with 0.005 M sulfuric acid solution as a mobile phase. The flow rate was controlled at 0.6 ml/min and the column temperature was 60 °C.

3.4.3 Surface Characteristics

The physical structure changes of the untreated and pretreated of corncobs were imaged by scanning electron microscope (SEM) using a Hitachi S–4800 microscope. The sample was located on a specimen holder by using carbon tape, which was sputter–coated with Au–Pd for reducing electrostatic charging. The surface structure images of the untreated and pretreated corncobs were obtained with a 2 kV accelerating voltage.

3.4.4 BET Surface Area Analysis

A BET surface area of corncobs before and after pretreatment was measured by N_2 adsorption/desorption measurements (Quantachrome/Autosorb1). The dried sample (0.1–0.5 g) was put into the sample tube and outgassed to remove the humidity and volatile adsorbents adsorbed on surface under vacuum at 150 °C for 4 h prior to the analysis. Then, N_2 was purged to adsorb on surface, and the quantity of gas adsorbed onto or desorbed from their solid surface at some equilibrium vapor pressure by static volumetric method will be measured. The solid sample was maintained at a constant temperature of the sample cell until the equilibrium is established. The BET surface area and pore volume was obtained from the N_2 adsorption/desorption curves.

3.4.5 Crystallinity Measurement

X–ray diffraction (XRD) was used for phase identification of a crystalline of the untreated and pretreated corncobs. Samples were scanned and recorded by using Rigaku X–Ray Diffractometer system (RINT–2200) with Ni filter and Cu K_α radiation (1.5406 Å) that generated at 30 mA and 40 kV. The scan speed of 5° (2θ)/min with scan step of 0.02 (2θ) was used for the continuous run in 5 to 90°C (2θ) range.

The crystalline index of cellulose samples were calculated from the X–ray diffraction patterns by the following equation (Xiao *et al.*, 2011):

$$CrI = \frac{I_{002} - I_{amorphous}}{I_{002}} \times 100\%$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ = 22.5° and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2θ = 18.6°.

3.4.6 Acetone–Butanol–Ethanol (ABE) Analysis

Acetone, butanol, and ethanol were measured by a gas chromatograph (Series Perichrome) equipped with a flame ionization detector using Innowax column. The column was maintained at 170 °C and a 0.5 µl of sample was injected. The injection and detector temperature were 240 °C and N_2 was used as carrier gas at a constant flowrate of 45 ml/min.