CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

3.1.1 1-Ethyl-3-methylimidazolium Acetate ([EMIM][Ac])

3.1.2 Hydrochloric Acid (HCl)

3.1.3 Acetic Acid (CH_3COOH)

3.1.4 Sodium Citrate Buffer

3.1.5 Cellulase from TrichodermaReesei

3.1.6 Standard Glucose, Xylose, Arabinose, HMF and Furfural

3.1.7 Pennisetum Purpureum x P. Alopecuroides (Emperor Napier Grass)

3.1.8 Pennisetum Purpureum x P. Glaucum Pakchong1 (Pakchong1 Napier Grass)

3.2 Equipment

3.2.1 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)

3.2.2 A CEM (Matthews, NC, USA) MAR-5 HP-500 Microwave System

3.2.3 PERICHROM PR2100 Gas Chromatograph with Flame Ionization Detector (GC-FID)

3.2.4 X-ray Diffraction Analyzer (XRD)

3.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

3.2.6 Brunauer–Emmett-Tellet (BET) (Autosorb-1)

3.2.7 Thermogravimetric Analyzer (TGA)

3.2.8 Scanning Electron Microscope (SEM)

3.2.9 Vortex Mixture

3.2.10 Incubator Shaker

3.2.11 Autoclave

3.2.12 pH Meter

3.2.13 Oven

3.2.14 Filter Paper (Whatman No.1)

3.3 Experimental Procedures

3.3.1 Napier Grass/Ionic Liquid Pretreatment

The mixture was prepared by combining dried Napier grass with acidic aqueous-[Emim][Ac] solution at various biomass loadings of 10, 20, 30, 40, 50 g/l, 50 volume%. [Emim][Ac], and volume% of acid 0, 1, 2, 3, 4 (Zhang *et al.*, 2012; Trisinsub, 2013). The mixture was stirred for a few minutes until homogeneous. After that, the mixture of Napier grass and acidic aqueous-[EMIM][Ac] solution was transferred to a microwave oven at various temperatures (100 °C, 120 °C, 140 °C, 160 °C and 180 °C) and pretreatment times (5 min, 20 min, 35 min, 50 min and 65 min). From RSM model, a full five-level factorial design is used to set the condition in 30 experiments as follows:

Table 3.1 Coded and experimental values of the factors for RSM

Independent variables	Symboi	Code levels				
		-2	-1	0	1	2
Temperature (°C)	X_1	100	120	140	160	180
Pretreatment time (min)	X_2	5	20	35	50	65
Biomass loading (g/l)	X_3	10	20	30	40	50
Acid Concentration (%wt.)	X_4	0	1	2	3	4

After cooling, the pretreated Napier grass was filtered from the acidic aqueous-ionic liquid and washed with distilled water until neutral pH. The solid residues were dried in the oven at 105 °C for 24 h. Then, the oven-dried samples were stored in a sealed bag in a desiccator for digestibility evaluation and cellulose crystallinity determination (Fu and Mazza, 2011). The pretreatment procedure flow diagram is shown in Figure 3.1.

Table 3.2 Central composite design (CCD)

Run	Code levels			
	Temperature	**Pretreatment time**	**Biomass loading**	**Acid Concentration**
1	(-1) 120	(-1) 20	(-1) 20	(-1) 1
2	(-1) 120	(-1) 20	(-1) 20	(+1) 3
3	(-1) 120	(-1) 20	(+1) 40	(-1) 1
4	(-1) 120	(-1) 20	(+1) 40	(+1) 3
5	(-1) 120	(+1) 50	(-1) 20	(-1) 1
6	(-1) 120	(+1) 50	(-1) 20	(+1) 3
7	(-1) 120	(+1) 50	(+1) 40	(-1) 1
8	(-1) 120	(+1) 50	(+1) 40	(+1) 3
9	(+1) 160	(-1) 20	(-1) 20	(-1) 1
10	(+1) 160	(-1) 20	(-1) 20	(+1) 3
11	(+1) 160	(-1) 20	(+1) 40	(+1) 3
12	(+1) 160	(-1) 20	(+1) 40	(-1) 1
13	(+1) 160	(+1) 50	(-1) 20	(-1) 1
14	(+1) 160	(+1) 50	(-1) 20	(+1) 3
15	(+1) 160	(+1) 50	(+1) 40	(-1) 1
16	(+1) 160	(+1) 50	(+1) 40	(+1) 3
17	(-2) 100	(0) 35	(0) 30	(0) 2
18	(+2) 180	(0) 35	(0) 30	(0) 2
19	(0) 140	(-2) 5	(0) 30	(0) 2
20	(0) 140	(+2) 65	(0) 30	(0) 2
21	(0) 140	(0) 35	(-2) 10	(0) 2
22	(0) 140	(0) 35	(+2) 50	(0) 2
23	(0) 140	(0) 35	(0) 30	(-2) 0
24	(0) 140	(0) 35	(0) 30	(+2) 4
25-30	(0) 140	(0) 35	(0) 30	(0) 2

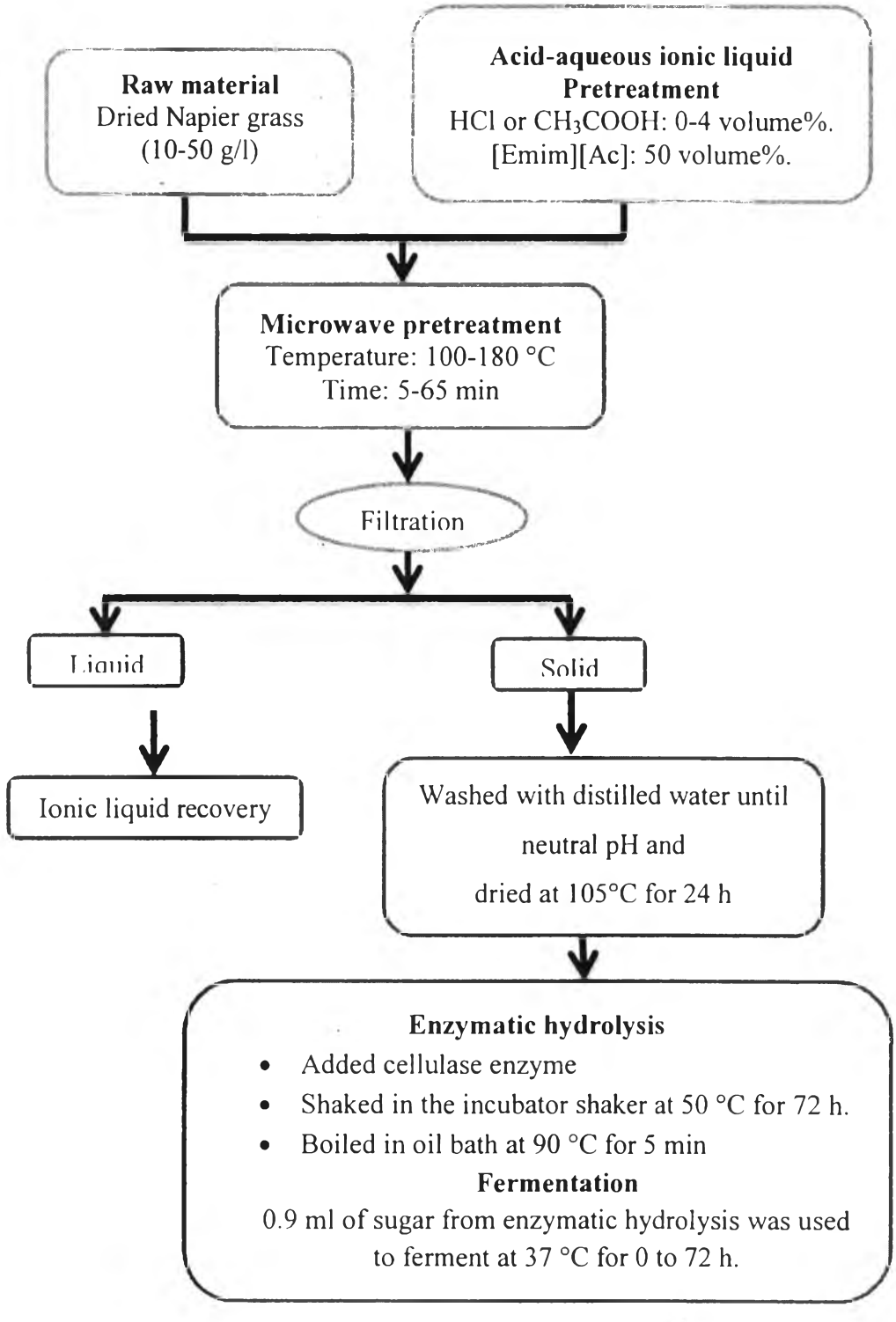

Figure 3.1 Schematic of acidic aqueous ionic liquid pretreatment and hydrolysis procedure flow diagram.

3.3.2 Enzymatic Hydrolysis

The recovered Napier grass of 20 mg from acidic-aqueous-[EMIM][Ac] pretreatment were added with 1 ml of 0.05 M sodium citrate buffer to adjust pH 4.8 and then was added with Cellulase 13.2 µl (cellulase; Sigma Chemicals, 52 FPU). After that the sample was shaken in an incubator shaker under the condition of 150 rpm and 50 °C for 72 h and boiled in water bath at 90 °C for 5 min. The hydrolysates were filtered to separate solid residues out. Then, the liquid fraction was collected for composition analysis by using HPLC. The experiments were conducted in triplicate. The amount of sugar in each acid-aqueous-[EMIM][Ac] experiment were analyzed by RSM in order to find the optimal condition for fermentation step.

3.3.3 Fermentation

The 0.9 ml of sugar which were produced from enzymatic hydrolysis was fermented by using *Clostridium beijerinckii* TISTR1461 at 37 °C for 0 to 72 h. After that, the ABE products from each pretreatment conditions were measured by HPLC and GC.

3.4 Analytical Method

3.4.1 High Performance Liquid Chromatography (HPLC)

Glucose and xylose which were the main composition of C_6 and C_5 sugar, respectively, with small amounts of arabinose and galactose (Li *et al.*, 2010) 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 of 60 °C. A 150 µl sample volume was injected.

3.4.2 Scanning Electron Microscopy (SEM)

The physical structure changes of the untreated and pretreated of Napier grass were imaged by scanning electron microscope (SEM) using a Hitachi S-

3400N microscope. The samples were located on a specimen holder by using carbon tape, which was sputter-coated with Pt for reducing electrostatic charging. The surface structure images of the untreated and pretreated samples were obtained with a 15 kV accelerating voltage.

3.4.3 BET Surface Area Analysis

A BET surface area of samples before and after pretreatment was measured by N_2 adsorption/desorption measurements (Quantachrome/Autosorb1). The dried sample (0.1-0.2 g) was put into the sample tube and outgassed to remove the humidity and volatile adsorbents adsorbed on surface under vacuum at 100 °C for 24 h prior to 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.

3.4.4 X-ray Diffraction (XRD)

X-ray diffraction (XRD) was used for phase identification of a crystalline of the untreated and pretreated sample. 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

$$CrI = \frac{I_{002} - I_{amorphous}}{I_{002}}$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ = 22.5° and $I_{amorphous}$ is minimum intensity between the 101 and 002 lattice plane (i.e., cellulose, hemicellulose, and lignin) at about 2θ = 18.7°.

3.4.5 Fourier Transform Infrared Spectroscopy (FTIR)

To characterize the structure of the cellulose, FTIR spectra were recorded using an absorbance spectrometer equipped with computer operated by OMNIC program. FTIR was also used for crystallinity index of sample. The samples were dried and mixed with potassium bromide (KBr) which was also dried for 24 h before using. After that, mixture was pressed into discs and scanned in the range 4000-400 cm^{-1} with a resolution of 4 cm^{-1}.

The infrared ratio $A_{1373cm^{-1}}/A_{2900cm^{-1}}$, which is known as the total crystallinity index (TCI). It is used to measure crystallinity of cellulose I and II. $A_{1430cm^{-1}}/A_{897cm^{-1}}$ is lateral order index (LOI) which measure cellulose I fraction in cellulose structure. The last, $A_{1510cm^{-1}}/A_{897cm^{-1}}$ measures the ratio of lignin and cellulose.

3.4.6 Simultaneous Thermal Analysis (STA)

The thermal decomposition profiles of several pretreatment conditions of sample were presented by using STA (NETZSCH/TG449 F3 Jupitor). The 4-10 mg of pretreated and untreated samples in a alumina pan were used to heat in a furnace at 30-800 °C at a heating rate of 10 °C/min in a N_2 gas and then cool down until 30°C.