CHAPTER III
EXPERIMENTAL

3.1 Materials and Equipment

3.1.1 Materials

1. Rice straw
2. Cellulase from TrichodermaReesei
3. Standard glucose, xylose, arabinose
4. 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac])
5. Sodium citrate buffer
6. Sodium hydroxide
7. Sulfuric acid
8. Nitric acid

3.1.2 Equipment

1. A CEM (Matthews, NC, USA) MAR-5 HP-500 microwave system
2. Perkin Elmer Series 200 LC/S/N291N5060508: High Performance Liquid Chromatography (HPLC) with a refractive index detectorusing an Aminex–HPX 87H column (300 mm x 78 mm, Bio-Rad Lab, USA)
3. PERICHROM PR2100 Gas chromatography (GC)
4. Scanning Electron Microscope (SEM)
5. Brunauer–Emmett-Tellet (BET) Surface Area Analyzer
6. X-Ray Diffraction (XRD)
7. Fourier Transform Infrared Spectroscopy (FTIR)
8. Thermogravimetric Analysis (TGA)
9. Oven
10. Incubator shaker
11. Filter paper
12. Vortex mixture

3.2 Experimental Procedures

3.2.1 Microwave/Ionic Liquid Pretreatment

Dried rice straw at a biomass loading of 5% (w rice straw/w ionic liquid) was pretreated via 50% concentrations of aqueous-[EMIM][Ac] solution (Fu *et al.*, 2011). The mixture was stirred until homogeneous for a few minutes. After that, the mixture of rice straw and aqueous-[EMIM][Ac] solution was transferred to a microwave oven to treat rice straw at various temperatures (140 °C, 150 °C, and 160 °C) and times (25 min, 40 min, and 55 min). From RSM model, a full three-level factorial designs is used to set the condition in 9 experiments from the following.

Table 3.1 Code levels of each variables

Independent variable	Unit	Code Levels		
		-1	0	1
Temp	°C	140	150	160
Time	min	25	40	55

Table 3.2 Pretreatment condition of each experimental runs

Run	Temp		Time	
1	(-1)	140	(-1)	25
2	(-1)	140	(0)	25
3	(-1)	140	(+1)	25
4	(0)	150	(-1)	40
5	(0)	150	(0)	40
6	(0)	150	(+1)	40
7	(+1)	160	(-1)	55
8	(+1)	160	(0)	55
9	(+1)	160	(+1)	55

After cooling, the mixture was filtered to separate the recovered straw samples. The solid residues were thoroughly washed with distilled water to remove ionic liquid and 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.

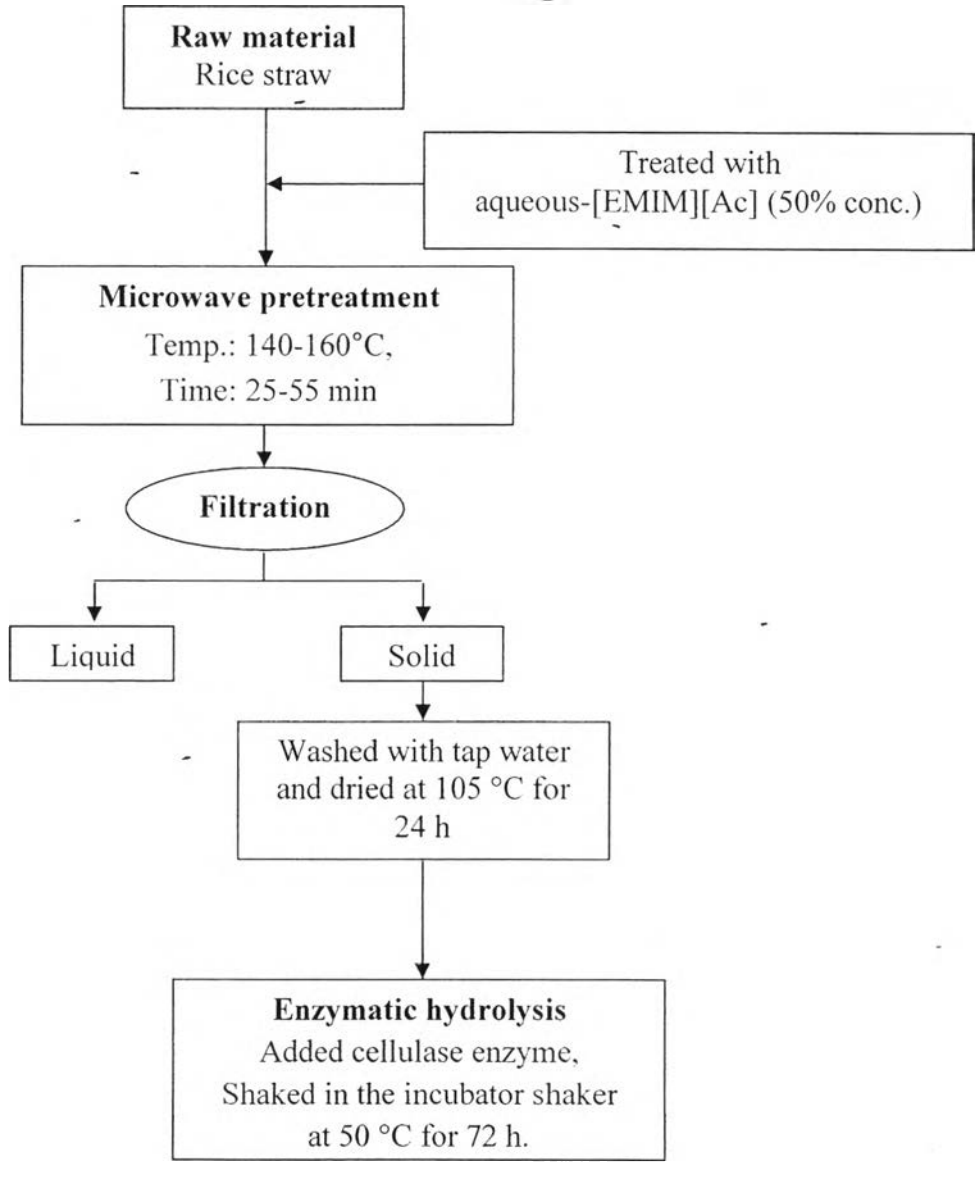

Figure 3.1 Schematic of aqueous ionic liquid pretreatment and hydrolysis procedure flow diagram.

3.2.2 Microwave/Alkali Pretreatment

0.5 g of dried rice straw was pretreated with 10 mL of 0.5% concentrations of NaOH solution. The mixture was stirred until homogeneous for a few minutes and heated in the microwave oven at 140 °C for 15 min (Cheng *et al.*, 2011). After that, the pretreated rice straw was filtered from the supernatant and washed by distilled water until neutral pH. The sample was dried overnight to remove water and collected in a seal bag for hydrolysis.

3.2.3 Microwave/Dilute Acid Pretreatment

1 g of dried rice straw was suspended in 10 mL of 2% concentrations of HNO_3 solution and vortexed until homogeneous solution. Then the mixture was treated in the microwave oven at 100 °C for 7 min (Chittibabu *et al.*, 2012). After the pretreatment, similarly for alkali pretreatment process, the biomass was filtered and washed by distilled water until neutral pH was reached. The sample was dried overnight and kept in a seal bag for hydrolysis.

3.2.4 Enzymatic Hydrolysis

The recovered rice straw of 0.15 g from aqueous-[EMIM][Ac], NaOH and HNO_3 pretreatment were added with 5 mL of sodium citrate buffer to adjust pH 4.8 and then was added with Cellulase 150 µl (cellulase; Sigma Chemicals, 52 FPU). After that the sample was shaked in an incubator shaker at 50 °C for 72 h. The hydrolysates were filtered to separate solid residues out. Then, the liquid fraction was collected for sugar analysis by using HPLC. The amount of sugar in each aqueous-[EMIM][Ac] experiment were analyzed by RSM in order to find the optimal condition for fermentation step. And the best condition of ionic liquid pretreated sample was compared the sugar results to NaOH and HNO_3 pretreated samples.

3.2.5 Fermentation

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

The mixture of DifcoTM Cooked meat medium (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.

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

Yeast extract, 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 8 mL of hydrolysate and this solution was kept in 37 °C without agitation for 8 h. After that, these cells would be used in fermentation step.

3.2.5.3 Fermentation Step

The 8 mL liquid fraction of three chemicals pretreatment and untreated rice straw 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.

3.3. Analytical Methods

3.3.1 High Performance Liquid Chromatography (HPLC)

Glucose and xylose which were the main composition of C6 sugar and C5 sugar (Li *et al.*, 2010), respectively, 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.

3.3.2 Scaning Eletron Microscopy (SEM)

The physical structure changes of the untreated and pretreated of rice straws were imaged by scanning electron microscope (SEM) using a Hitachi TM-3000 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 rice straw were obtained with 15 kV accelerating voltage.

3.3.3 BET Surface Area Analysis

A BET surface area of rice straws 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 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.

3.3.4 X-ray Diffraction (XRD)

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

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

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°.

3.3.5 Fourier transform infrared spectroscopy (FTIR)

FTIR was also used for crystallinity index of rice straw. Detection of the functional group on the rice straw samples was done with Thermo Nicolet/NEXUS 670 FT-IR. Rice straw 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) (Nelson and O'Connor, 1964).

3.3.6 Thermogravimetric Analysis (TG-DTA)

The thermal decomposition profiles of several pretreatment conditions of rice straw were presented by using TG-DTA (TG/Q50). The 2-5 mg of pretreated and untreated samples in a platinum pan were introduced into the furnace and the temperature was ramped from room temperature to 900 °C at a heating rate of 10 °C/min in a N_2 gas. The initial mass of the rice straw samples was determined.

3.3.7 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 flow rate of 45 mL/min.