CHAPTER III
METHODOLOGY

3.1 Equipment

- CEM Corporation Mars 5 version 049104 : Microwave Accelerated reaction Systems
- Shimadzu Corporation: High Performance Liquid Chromatography (HPLC) with a refractive index detector (RID-10A, Shimadzu Corp., Kyoto, Japan) using an Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA)
- FTIR spectrometer (Nicolet nexus 670)
- UV-Visible spectrophotometer
- Analytical balance
- Oven
- Water bath
- Autoclave
- Muffin furnace
- Vacuum oven
- Soxhlet extraction
- Rotaty evaperation
- Filtration setup

3.2 Materials

- Sulfuric acid (H_2SO_4, Merck Co., Germany)
- Phosphoric acid (H_3PO_4, Labscan Asia Co.)
- Sodium hydroxide (NaOH, Labscan Asia Co.)
- Ammonium hydroxide (NH_4OH, Panreac Quimica Sau)
- Ethanol (C_2H_5OH, J.T. Baker)
- High purity standards : glucose (Sigma Aldrich Chemicals Co. Inc.,USA)
- High purity standards : xylose (Sigma Aldrich Chemicals Co. Inc.,USA)
- High purity standards : arabinose (Sigma Aldrich Chemicals Co. Inc.,USA)

3.3 Methodology

3.3.1 Biomass Preparation

Pennisetum purpureum (*Napier grass*), harvested in April 2011, was obtained from Supunburi province, Thailand. Harvest strips were taken randomly from each quarter of the field. *Napier grass* (only leaves and stems) was washed with tap water, and dried under sunlight. It was then milled to obtain small particles using herb grinder. The ground biomass was then stored in sealed plastic bags at room temperature until further use.

3.3.2 Compositional Analysis of Raw Biomass and Pretreated Biomass

The chemical composition of raw *Napier grass* was measured using a two-step acid hydrolysis method developed by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2006). All analytical determinations were performed in triplicate and averaged results.

Prior to other determinations, raw material was extracted consecutively with water and ethanol (two-step extraction procedure) to determine extractive in

biomass. Sample (2 g) was added to an extraction thimble. The thimble was inserted into Soxhlet tube. Water (HPLC grade, 190 mL) was added to the receiving flask equipped with the Soxhlet apparatus. A minimum 4–5 siphon cycles per hour was set before refluxing for 24 h. Ethyl alcohol (190 mL) was added and the mixture was continued refluxing for 12 h. When the reaction was complete, extracted solid was filtered and allowed to dry using air dry. All filtrates were combined with any solvent from the upper section of the Soxhlet apparatus. Solvent was evaporated off using a rotary evaporator with a controlled temperature at 45 °C. After all solvent was removed, the flask was placed in a vacuum oven (75–100 torr) at 40 °C for 24 h. The flask was then weighed to determine extractive.

The extracted sample (0.3 g) was taken through a 72% sulfuric acid hydrolysis at 30 °C for 60 min using water bath, followed by a second 4% sulfuric acid hydrolysis at 121 °C for 60 min using autoclave. The autoclaved hydrolysis solution was vacuum filtered through a filtering crucible; the filtrate was collected for further analysis.

Amounts of monomeric sugars (glucose, xylose, and arabinose) were determined by HPLC (Shimadzu) equipped with refractive index detector using the following condition: 20 µl injection volume, 0.005 M sulfuric acid (HPLC grade) as a mobile phase and a flow rate of 0.60 ml/min.

Amount of acid soluble lignin (ASL) was determined by UV–visible spectroscopy. The absorbance of the filtrate was measured at 205 nm, using a 1-cm light path cuvette and a 4% solution of H_2SO_4 as a reference blank. Amount of acid insoluble lignin (AIL) content was defined as the difference between the weight of filter cake (oven-dried at 105 °C to constant weight) and the weight of ash. Total lignin content was the sum of ASL and AIL. Amount of ash content was determined by placing the dried filter cake in the muffle furnace at 575 °C for 24 h.

The composition of solid fraction was analyzed for hemicellulosic sugars, glucose, acid-soluble lignin (ASL), and acid-insoluble lignin (AIL) contents,

using the same procedure described in the raw biomass, except that no extraction was used.

Moreover, the changes of the chemical structure were characterized using Fourier transform infrared (Nicolet nexus 670 FTIR) with a resolution of 1 cm^{-1} and over the wavelength range of 400–4000 cm^{-1}. The solid samples were dried and then pressed into a disc with KBr.

3.3.3 Pretreatment

Microwave heating was used to digest *Napier grass* using different catalysts, as follows:

3.3.3.1 Dilute Alkaline Pretreatment

Prior to microwave pretreatment, *Napier grass* was suspended in different concentrations of alkaline solution (0.5–5 % (w/v)) using different liquid-to-solid ratios (LSR, 15:1–40:1, mL of solution: g of *Napier grass*). For alkaline pretreatment comparison, NaOH and NH_4OH pretreatments were performed. The mixture was stirred until homogeneous before transferring to a Teflon-vessel sealed with a Teflon cap. The microwave (1200 W) pretreatment was conducted under various reaction temperatures (40–160 °C) and times (5–60 min). After the pretreatment, the mixture was filtered to separate solid residues from filtrate fraction. The liquid fraction was collected for monomeric sugar analysis. Measurement of pH of liquid fraction before and after pretreatment was performed. The solid residues were thoroughly washed with distilled water to neutral pH and dried in the oven. Then, the oven-dried samples were weighed to compare with unpretreated sample and were stored in valve bags for further dilute acid pretreatment in the two-stage pretreatment study.

3.3.3.2 Dilute Acid Pretreatment

To optimize the pretreatment, *Napier grass* was mixed with different concentrations of acid solution (0.5–5.0 % (w/v)) using 15:1–45:1 LSR. The pretreatment temperature and time were varied from 60–160 °C (1200W microwave power) and 5–60 min. For acid pretreatment comparison, different types of acid, namely, H_2SO_4 and H_3PO_4 were used. After the pretreatment, the liquid fraction was collected

for monomeric sugar analysis using HPLC. Measurement of pH of liquid fraction before and after pretreatment was performed. The solid residues were thoroughly washed with distilled water to neutral pH and dried in the oven. Then, the oven-dried samples were weighed to compare with unpretreated sample and stored in valve bags for further analysis.

3.3.3.3 Two-Stage Pretreatment (Dilute Alkaline Followed By Dilute Acid Pretreatment)

The solid residues from the alkaline pretreatment, having the highest monomeric sugar yield, were treated with dilute acid, which resulted in the highest monomeric sugar yield, using the optimal conditions from the dilute acid pretreatment. The obtaining solution mixture was filtered to collect the liquid part, and solid residues were thoroughly washed with distilled water to neutral pH before drying in the oven for further characterization. Measurement of pH of liquid fraction and the weight of the solid sample before/after pretreatment were performed.

3.3.4 Composition Analysis of The Prehydrolysates

The liquid fraction from the pretreatment was analyzed for monomeric sugars. The monomeric sugar content (glucose, xylose, and arabinose) was determined by HPLC using 20 µl injection volume, 0.005 M sulfuric acid (HPLC grade) as mobile phase, 0.6 mL/min flow rate, 65 °C column temperature, and15 min run time. Since H_3PO_4 provided its peak at the same retention time as the glucose peak, therefore, it is necessary to analyze H_3PO_4 at the same condition as the prehydrolysate of microwave-assisted H_3PO_4 sample. The difference between two values was applied to determine the real glucose content in the prehydrolysate (see in Appendices).