CHAPTER III

EXPERIMENTAL

3.1 Objectives

- To evaluate the potential of 4 different Thai grasses as lignocellulosic materials using dilute alkaline/microwave pretreatment and dilute acid/microwave hydrolysis.
- To determine the optimal conditions and a suitable type of *S.cerevisiae* for fermenting monomeric sugar to ethanol.

3.2 Materials and Chemical

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Mission grass (*P.polystachyon*) is obtained from Nakhon Ratchasima Province, Thailand. Kans grass (*S.spontaneum*) is obtained from Bangkok Metropolis, Thailand. Giant reed (*A.donax*) is obtained from Nakorn Pathom Province, Thailand. Tiger grass (*T.Maxima*) is obtained from Lamphun Province, Thailand. Before any pretreatment, grasses (leaves and stems only) are air- and sun-dried before being chopped to small pieces. The grasses are then milled to obtain 60 mesh-size powders. The ground biomass is then stored in sealed plastic bags at room temperature for further use.

Sodium hydroxide (NaOH, Labscan Asia Co., Thailand) and sulfuric acid (H_2SO_4, Merck Co., Germany) are utilized in the pretreatment process. D-(+)-Glucose (G5400), D-(+)-Xylose (X3877), and D-(-)-Arabinose (A6085) standards (Sigma Aldrich Chemicals Co. Inc., USA) are used for sugar quantitative analysis. Acetic acid (CH_3COOH), 5-(hydroxymethyl) furfural and furfural standards (Sigma Aldrich Chemicals Co. Inc., USA) are used for inhibitory chemicals quantitative analysis. Cellulase from *Trichoderma reesei* ATCC 26921 and *Trichoderma viride* C1794 (Sigma Aldrich Chemical Co., USA) were used to hydrolyze cellulose and hemicellulose. Calcium hydroxide ($Ca(OH)_2$, Sigma Aldrich Chemical Co., USA)

and sodium sulfite (Na_2SO_3, Labscan Asia Co, Thailand) were employed during the detoxification process of hydrolyzate. Yeast extract (3 g/L), malt extract (5 g/L), peptone (5 g/L) were obtained from Himedia Laboratories and utilized for the inoculating yeast medium.

3.2.2 Chemical Composition Analysis

The chemical compositions (cellulose, hemicelluloses, lignin, extractive, acetic acid, 5-(hydroxymethyl) furfural, furfural, and ash) of unpretreated and pretreated grasses are analyzed using the method of the National Renewable Energy Laboratory (NREL) (Sluiter and Sluiter, 2011).

3.2.3 Particle Size Analysis

Mechanical pretreatment is a fundamental method for reducing cellulose crystallinity. The reduction in particle size leads to an increase of available specific surface, and a reduction of degree of polymerization (Leustean, 2009). The particle size of grasses after mechanical pretreatment is detected by a particle size analyzer (Malvern/ Mastersizer X) with a 300 mm lens size in the sample detection unit.

3.2.4 Microwave-assisted: Two-stage Microwave/ Chemical Pretreatment Process

3.2.4.1 Microwave-assisted Dilute Sodium Hydroxide (NaOH) Pretreatment

Before microwave pretreatment, grass powders are suspended in NaOH solution (0.5 % (w/v)) using 15:1 liquid-to-solid ratios (LSR) (ml of NaOH solution:g of grass powder). The ratio is reported as the optimal ratio for the maximum release of monomeric sugar yields (Boonmanumsin *et al.*, 2012). The mixture is stirred until homogeneous and then transferred to a Teflon vessel sealed with a Teflon cap. The microwave pretreatment is performed under various temperatures (60–160 °C) for various times (5–60 min). After the optimum temperature and time are obtained, the grass samples are suspended in different

concentrations of NaOH solution (0.1-7 % (w/v)) using 15:1 LSR at the optimum temperature and time to obtain the suitable condition for the maximum release of monomeric sugars.

After the pretreatment, the mixture is filtered. The liquid fractions are collected for monomeric sugars and pH analysis; the solid residues are dried in an oven. The oven-dried solid samples are weighed to compare percentage of solid loss with that of the untreated samples. The amount of monomeric sugars (glucose, xylose, and arabinose) is quantitatively determined using High performance liquid chromatography (HPLC, RID-10A, Shimadzu Corp., Kyoto, Japan) equipped with a refractive index detector, and Aminex-HPX 87H column (300 x 78mm, Bio-Rad Lab. USA) under the following condition: 0.005 M of H_2SO_4 as the mobile phase with the flow rate of 0.60 ml/min. The pretreated solids are neutralized, dried, and stored in sealed containers for further pretreatment studies.

3.2.4.2 Microwave-assisted Dilute Sulfuric Acid (H_2SO_4) Pretreatment

The solid residues from the microwave/ NaOH pretreatment are treated with microwave/ dilute H_2SO_4. The alkaline-pretreated grasses are mixed with dilute acid solution (0.5 % (w/v)) using 15:1 LSR. The pretreatment temperature is varied between 80–200 °C for reaction time range of 5–60 min. After the optimum temperature and time conditions are obtained, the microwave/dilute H_2SO_4 pretreatment is conducted at different concentrations of H_2SO_4 solution (0.5-3 % (w/v)) to maximize the release of monomeric sugars.

After the two-stage pretreatment process, the liquid fractions are collected for monomeric sugar analysis by HPLC. All liquid fractions collected are measured pH value. The solid residues are oven-dried and weighed to measure % solid loss. Then, the residues are kept in sealed bags for further characterization.

3.2.5 Hydrolysis

The pH of the hydrolyzate was adjusted to 4.8 using 40 % w/v NaOH. Cellulase from *T. reesei* ATCC 26921 (160 μL/1 g of grass) was added into the liquid hydrolyzate. The hydrolyzate was incubated for 60 h at 50 °C (Qureshi *et al.*, 2008).

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3.2.6 Total Monomeric Sugar Analysis

Monosaccharides are measured using HPLC with a pulsed refractive index detector. Sugar standard curves are plotted to calculate the sugar concentrations. The relationship between sugar concentrations and their peak areas is represented by the following equations: y = 4E-06x + 0.0421 for glucose, y = 4E-06x + 0.0952 for xylose, and y = 4E-06x + 0.0396 for arabinose (y = sugar concentration (g/l), x = peak area). The monomeric sugar yields in the unit of g/100 g biomass can be calculated from the equation below (Boonmanumsin *et al.*, 2012).

Monomeric sugar yields (g/100 g biomass)

= [Sugar concentration (g/l) x **V** (ml) x 100 g] / [1000 (ml) x **M** (g)]

Where **V** is volume of acid or alkaline added (ml) and **M** is the amount of biomass added (g) in the pretreatment process.

3.2.7 Detoxification

A combined detoxification method of evaporation, overliming, addition of sodium sulfite, and heating was carried out to remove inhibitory products from the MG hydrolyzate. Approximately 60 % of the hydrolyzate was evaporated using a rotary evaporator. The evaporated hydrolyzate was treated with $Ca(OH)_2$ to increase the pH to 8–12. Sodium sulfite (1 g/L) was then added to the solution. The overlimed solution was heated at 90 °C for 30 min. The condensate in the mixture was filtered, and the pH was adjusted to 6 using 95 % v/v H_2SO_4 at room temperature. The liquid was stored in a closed bottle at ambient temperature for fermentation.

3.2.8 Inoculation of Baker's Yeast (*Saccharomyces Cerevisiae*)

Yeast extract, malt extract, peptone, and glucose were mixed together to make a yeast medium. The medium was autoclaved at 121 °C for 15 min and was left to cool at room temperature. One inoculation loop of *S. cerevisiae* was transferred to the sterile medium and incubated for 24 h at 30 °C. The standard deviation obtained is in triplicates.

3.2.9 Fermentation of MG Hydrolyzate

Yeast extract, malt extract, and peptone were added to the overlimed grass hydrolyzate. No additional glucose was added. The mixture was sterilized at 121 °C for 15 min and allowed to cool at room temperature. *S. cerevisiae* (2–10 % v/v) from the previous step was added to the hydrolyzate, and the solution was incubated in a shaker at 30 °C for 96 h. Samples from the solution were taken out every 24 h for glucose and ethanol detection.

3.3 Characterization

The chemical structures of all grasses before and after the pretreatment processes are compared and characterized using fourier transform infrared spectrometer (FTIR, Nicolet nexus 670) using 64 scans with a resolution of 1 cm^{-1}. The spectra are run under the range of 400–4000 cm^{-1}. The physical structures of untreated and pretreated grasses are obtained using scanning electron microscope (SEM, Hitachi/S-4800) at 2 kV accelerating voltages. Ethanol production was detected by gas chromatography (GC, Agilent Technologies, USA) equipped with TCD Detector, using 0.5 μL injection volume, 55 kPa of helium as a carrier gas, 200 °C oven and injector temperatures. Both glucose and ethanol concentrations were detected every 24 h of incubation.