CHAPTER III
EXPERIMENTAL

3.1 Materials

1. Sodium hydroxide (NaOH, Labscan Asia Co.)
2. Sulfuric acid (H_2SO_4, Merck Co., Germany)
3. Hydrochloric acid (HCl, Labscan Asia Co.)
4. High purity standards: Glucose (Sigma Aldrich Chemical Co. Inc., USA)
5. High purity standards: Xylose (Sigma Aldrich Chemical Co. Inc., USA)
6. High purity standards: Arabinose (Sigma Aldrich Chemical Co. Inc., USA)
7. Calcium carbonate ($CaCO_3$, Sigma Aldrich Chemical Co. Inc., USA)
8. Sodium sulfite (Na_2SO_3, Labscan Asia Co.)
9. Cellulase from *Trichoderma reesei ATCC 26921* (Sigma Aldrich Chemical Co. Inc., USA)
10. Yeast extract (Himedia Laboratories, India)
11. Malt extract (Himedia Laboratories, India)
12. Peptone (Himedia Laboratories, India)
13. Baker's yeast, *Saccharomyces cerevisiae* (Thailand Institute of Scientific and Technological Research (TISTR), Thailand)

3.2 Equipment

1. Ethos Sel: Microwave solvent extraction lab station (Milestone)
2. Shimadzu Corp.: High Performance Liquid Chromatography (HPLC) with a refractive index detector(RID-10A, Shimadzu Corp., Kyoto, Japan) using a chromatography column Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA) under 20 μl injection volume, 0.005 M sulfuric acid (HPLC grade) as

mobile phase, 0.6 ml/min flow rate, 65 °C column temperature, and 20 min run time condition

3. Scanning Electron Microscope (SEM) (Hitachi/S-4800)
4. Agilent Technologies: Gas Chromatography (GC)
5. Microscope
6. Analytical balance
7. Oven
8. Water bath
9. Autoclave
10. Rotary evaporator
11. Shaking Incubator

3.3 Methodology

3.3.1 Mission Grass preparation

Mission grass (*Pennisetum polystachion)* was harvested in March 2013 at Tak Province. The grass (stems and leaves) was left to dry under sunlight for 2 weeks. Then, it was milled into small particles (diameter ~300 μm) by a milling factory. The milled mission grass was stored in a dry container at room temperature.

3.3.2 Pretreatment of Mission Grass

Mission grass was pretreated with the grass optimization method according to Boonmanumsin et al. and Tatijarern et al. The milled grass was mixed with 3% w/v NaOH using liquid-to-solid ratio, 15 mL of NaOH: 1 g of grass (Boonmanumsin *et al.*, 2012, Tatijarern *et al.*, 2013). The mixture was stirred thoroughly until it was homogenous. Then, it was subjected to microwave treatment (300 W) under the

temperature of 120 °C for 10 minutes. After the pretreatment, the mixture was washed until the pH was neutral. The solid residue was oven dry for 24 hours.

3.3.3 Hydrolysis of Mission Grass

The dry solid from the base pretreatment was mixed with 1% v/v H_2SO_4 with liquid-to-solid ratio 15 mL of H_2SO_4: 1 g of pretreated grass. The mixture was stirred thoroughly, and then subjected to microwave treatment (300 W) under the temperature of 200 °C for 5 minutes (Tatijarern *et al.*, 2013). The mixture was left to cool at room temperature. Cellulase from *Trichoderma reesei ATCC 26921* (160 µL/1 g of grass) was added into the liquid hydrolyzate and incubated for 60 hours at 50°C (Qureshi *et al.*, 2008).

3.3.4 Detoxification of Mission Grass Hydrolyzate

Overliming method was used to detoxify the liquid hydrolyzate. The hydrolyzed solution was treated with $Ca(OH)_2$ to increase the pH to pH 8-12, then sodium sulfite (1 g/L) was added into the solution. The overlimed solution was heated for 30 minutes at 90°C, and the pH was adjusted to 6 using 95% H_2SO_4 at room temperature. The condensate in the mixture was filtered. The liquid was stored for fermentation.

3.3.5 Fermentation of Mission Grass Hydrolyzate

Preparation of cell culture

To prepare the yeast medium (YM) for *Saccharomyces cerevisiae,* yeast extract (3 g/L), malt extract (5 g/L), peptone (5 g/L), and glucose (20 g/L) were mixed and stirred until homogenous (Huang *et al.*, 2009). The medium was autoclaved at 121 °C for 15 minutes and cooled down to room temperature.

Agar plate medium was prepared by mixing glucose (10 g/L), yeast extract (3 g/L), malt extract (5 g/L), peptone (5 g/L), agar (20 g/L), and stirred at 100°C until homogeneous. The solution medium was autoclaved at 121°C and then poured into a petri dish under a biosafety cabinet. The agar in the plate was left to cool at room temperature for one day.

A vial of yeast strain obtained from TISTR was resuspended in a small amount of YM medium. A sanitized inoculation loop was dipped into the solution and streaked onto the prepared agar plate. The plate was incubated at 30°C for 24 hours.

A *Saccharomyces cerevisiae* colony from the agar plate was chosen and transferred to the prepared YM medium. The inoculated medium was incubated in a shaker at 30°C for 24 hours. The agitation of the incubator was set to 100 rpm.

Inoculation of *Saccharomyces cerevisiae*

Yeast extract (3 g/L), malt extract (3 g/L), and peptone (5 g/L) were added into the overlimed grass hydrolyzate. No additional sugar was added. The mixture was then autoclaved at 121°C for 15 minutes and allowed to cool down to room temperature. *Saccharomyces cerevisiae* (10% v/v) was added into the autoclaved hydrolyzate. The solution was incubated in the shaker at 30°C for 96 hours. Sugar utilization and ethanol production was detected using HPLC and GC, respectively, every 24 hours.