CHAPTER III
EXPERIMENTAL

3.1 Materials and Equipment

Equipment:

1) Hot air oven: Model UC 30, Memmert GmbH and Co., KG., Western Germany.
2) MFC grinder.
3) Autoclave: Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan.
4) Cold room: Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K.
5) 4-Digit precision weighting balance: Model AG 204, Mettler Toledo, Switzerland.
6) High speed refrigerated centrifuge: Beckman Coulter TM Avanti J-30I, Palo Alto, California, U.S.A.
7) Hot plate stirrer: Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand.
8) Incubator: Model 800, Memmert GmbH and Co., KG., Western Germany.
9) Incubator shaker: Model SK-737, Amerex Instruments, Inc., U.S.A.
10) Kubota refrigerated microcentrifuge 6500: Kubota Corporation, Tokyo, Japan.
11) Laminar flow 'clean': Model V6, Lab Service Ltd., Thailand.
12) Microscope: Model CH 30RF200, Olympus Optical Co., Ltd., Japan.
13) Orbital shaker: Innova Model, New Brunswick Co., Inc., U.S.A.
14) Pipetteman: Gilson, France.
15) pH meter: Mettler-Toledo International Inc., New York, U.S.A.
16) Spectrophotometer: Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, U.S.A.

17) Vortex mixer: Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, U.S.A.
18) Water bath: Model WB14, Memmert GmbH and Co. KG., Western Germany.
19) High pressure liquid chromatography: Model 200LC, Perkin Elmer.

Chemicals:

1) Bagaase from Thai Roong Rueng Sugar Group and Corncob, Kanchanaburi, Thailand.
2) Carboxymethyl cellulose (CMC) purchased from Fluka, Sigma-Aldrich Co., Inc., Singapore.
3) Malt Extract purchased from Lab Scan Analytical Sciences, Thailand.
4) Yeast extract purchased from Bio Springer, France.
5) Sodium hydroxide (NaOH) purchased from Merck KGaA, Germany.
6) Ethanol absolute (C_2H_5OH) Analytical grade, purchased from Scharlau Chemie S.A., Spain.
7) Standard sugars (glucose, xylose, arabinose, mannose and galactose) for HPLC analysis.
8) Sulfuric acid (H_2SO_4) purchased from Merck KGaA, Germany.

3.2 Experimental Procedures

3.2.1 Biomass Preparation and Composition Analysis

Bagasse and corncob were dried in an oven at 105 °C for 12 h. Then, the dried bagasse and corncob were milled and extensively homogenized by using a MFC grinder to obtain small particles. The dried bagasse and corncob were stored in a sealed plastic bag. Then, they were milled to small size particles and sieved to sizes between 20 to 80 mesh.

To determine the amount of extractives in the bagasse and corncob, solvent extraction (60 mL acetone for 1 g of dried biomass sample) was used, and the temperature was held at 90 °C for 2 h. After that, the sample was dried at 105 °C

until a constant weight was obtained. The weight difference before and after the extraction is the amount of the extractives (Blasi *et al.*, 1999). To determine the amount of hemicellulose, 10 mL of sodium hydroxide solution (0.5 mol/L) was added to 1 g of extractive-freedried biomass, and the temperature was held at 80 °C for 3.5 h. After that, the sample was washed using DI water until pH value of the solution approach 7. Then, it was dried to a constant weight. The difference between the sample weight before and after this treatment is hemicellulose content (Blasi *et al.*, 1999).

To determine the amount of lignin, 30 mL of 98 wt% sulfuric acid was added for each extractive-free dried biomass. After the sample was held at ambient temperature for 24 h, it was boiled at 100 °C for 1 h. The mixture was filtered, and the residue was washed until the sulfate ion in the filtrate was undetectable (via titration of a 10% barium chloride solution); it was then dried to a constant weight. The weight of the residue was recorded as the lignin content (Blasi *et al.*, 1999).

The content of cellulose was calculated by difference, assuming that extractives, hemicellulose, lignin, and cellulose were the only components of the entire biomass. (Blasi *et al.*, 1999; Li *et al.*, 2004).

The elemental analysis of dry biomass was obtained by means of a C/H/N 2000 LECO analyser.

3.2.2 Steam Explosion Pretreatment of Biomass

Before pretreatment, corncob and sugarcane bagasse residue were soaked for 18 h at 45 °C in 0.9% w/w diluted sulfuric acid solution or water (solid-liquid ratio: 1/10). The soaked material was vacuum filtered to approximately 20% solids content and then steam-exploded in an autoclave reactor (Autoclave ES-315) followed by sudden depressurization by fully opening the steam exhaust valve of autoclave. The pretreatment was performed by high temperature (120 °C) for either 1,2, and 3 h respectively. After pretreatment, the material was recovered, cooled to approximately room temperature and filtered to recover two fractions: (1) the water insoluble solid (WIS) fraction and (2) the filtrate or pre-hydrolyzate. After separating the filtrate, the WIS fraction was thoroughly washed with water, weighted, and dried at room temperature for storage (Ballesteros *et al.*, 2006).

3.2.3 Bacteria Preparation for Microbial Hydrolysis

For the preparation of bacteria cells, an inoculum was prepared by transferring a loop of colonies, which was prepared by streak plate technique into a 250 mL Erlenmeyer flask containing 50 mL of 65 modified DSMZ broth medium 2, pH 7.2. The culture was incubated at 37 °C in a shaking incubator at 180 rpm for 12 h. Then, 50 mL of the prepared inoculum was transferred into a 500 mL bottle with a screw cap containing 450 mL of the production medium (65 modified DSMZ broth medium 2, pH 7.2) and incubated at 37 °C in a shaking incubator at 180 rpm for 12 h. After that, the cells were harvested by centrifugation (8000 rpm, 4 °C for 10 min).

3.2.4 Microbial Hydrolysis

Before the hydrolysis experiments, the reactors, production medium (65 modified DSMZ broth medium 2 without CMC, pH 7.2), and biomass powder were autoclaved under aseptic conditions.

3.2.4.1 Configuration of batch reactor

Figure 3.1 illustrates the configuration of the batch reactor for microbial hydrolysis. The reactor was a two-layer round-bottom glass vessel with a total volume of 2,000 mL and a working volume of 1,000 mL. The operating temperature was controlled by circulating hot water through the water jacket by using a circulating water bath. Compressed air was filtered through a 0.2 μm pore size filter to prevent all contaminants. The air was also passed distilled water to increase the humidity before being entered the hydrolysis reactor, in order to maintain aerobic condition and ensure homogeneity of the system.

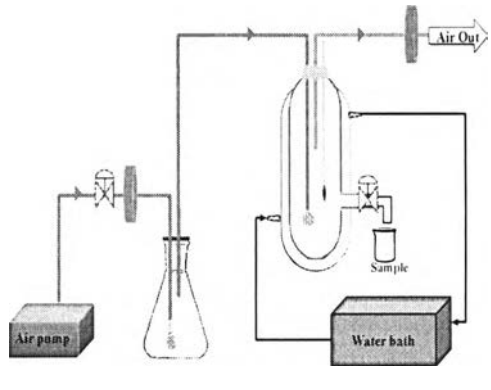

Figure 3.1 Configuration of batch reactor.

3.2.4.2 Batch reactor start-up and operating conditions

Before running the microbial hydrolysis experiment, the reactor system, production medium, and ground biomass sample were sterilized in the autoclave at 121 °C for 15 min. The sterilized biomass and bacterial cells were approximately loaded at 1 g and 7-9 g (wet basis) respectively, per liter of the production medium. The reactor temperature was controlled at 37°C. Simultaneously, compressed air was filtered through 0.2 μm pore size to the reactor to maintain complete mixing and aerobic condition inside the reactor.

3.2.5 Determination of Sugar and Bacteria Concentrations

Glucose was analyzed by a high performance liquid chromatography (HPLC) (Shimadzu, Japan) with a refractive index detector (Model 6040 XR, Spectra-Physics, USA) and an organic acid column (BIORAD HPX-87H). 0.005M H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. The column temperature was fixed at 65 °C and a sample running time of 25 min. The concentration of bacteria was determined by the total nitrogen test kit (DR 2700, spectrophotometer).

3.2.6 Scanning Electron Microscope (SEM) Analysis

Micrographs of corncob and sugarcane bagasse powder after pretreatment process were carried out on a scanning electron microscope (SEM), Hitachi/S-4800. The specimens to be coated were mounted on a conductive tape and coated with gold palladium using JEOL JFC-1200 fine coater and observed using a voltage of 10 to 15 kV.

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