CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

3.1.1 Corncob waste was obtained from the Betagro Company. The collected corncob waste was stored at the ambient room temperature in a large bag. The average particle size of corncob waste was around 1.6 mm homogenized in a single lot and stored until used.

3.1.2 Sulfuric Acid (H_2SO_4)

3.1.3 Phosphoric Acid (H_3PO_4)

3.1.4 Calcium Hydroxide ($CaOH_2$)

3.1.5 Critic Acid Monohydrate ($C_6H_8O_7.H_2O$)

3.1.6 di-Sodium Hydrogen Orthophosphate Anhydrous (Na_2HPO_4)

3.1.7 pH Meter

3.1.8 Filter Paper

3.1.9 *Clostridium Beijerinckii* TISTR1461

3.1.10 Enzyme (ARR2–7 from Siam Victory, Thailand)

3.1.11 Standard glucose, xylose, arabinose, cellobiose, mannose, rhamnose, galactose, and furfural

3.2 Equipment

3.2.1 High Performance Liquid Chromatograph (HPLC)

3.2.2 Scanning Electron Microscope (SEM)

3.2.3 X-Ray Diffraction Analyzer (XRD)

3.2.4 UV-VIS Spectrometer (UV)

3.2.5 Gas Chromatograph (GC)

3.2.6 Incubator Shaker

3.2.7 Oven

3.2.8 Stainless Steel Reactor

3.2.9 Waterbath

3.2.10 Glassware

3.3 Methodology

3.3.1 Dilute Sulfuric and Phosphoric Acid Pretreatment

Dilute Sulfuric and Phosphoric acid pretreatment of corncobs were performed in a laboratory scale stirred stainless steel reactor. The reactor is an acid resistant stainless steel and has a total volume of 1 L, with an electric heater and mechanic agitation. Corncob waste was suspended in 2% (w/w) H_2SO_4 or H_3PO_4 solution using liquid-to-solid ratios (LSR) of 15:1 and 10:1 for H_2SO_4, but only 10:1 for H_3PO_4 (ml of solution:g of corncob waste). The mixture was stirred until homogeneous before transferring to a stainless steel reactor. The pretreatment of H_2SO_4 was conducted under condition 120 °C for 5 min while H_3PO_4 pretreatment was carried out with condition 140 °C for 10 min. At the end of each run the reactor was removed from the heating jacket and the prehydrolysate agitated until the reactor was cooled to about 40 °C. Then the prehydrolysate was filtered to separate liquid and solid residue. The liquid fraction was collected for monomeric sugar analysis by using HPLC (Perkin Elmer LC200) equipped with a refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.60 ml/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C. While, the solid was washed thoroughly with tab water until no color and neutral pH in the resulting water was obtained. After that, the solid residue was dried for 24 h at 90 °C in an oven. The weight of dried samples was collected and stored in a ziplock bag prior to enzymatic hydrolysis step.

3.3.2 Lime Detoxification

The liquid fraction from pretreatment step was adjusted pH to 10 by $CaOH_2$ and followed by adding Na_2SO_4 1 g/l and heating in water bath at 80 °C for 30 min before being cooled to room temperature as normal step. After that, the product was adjusted pH to 4.8 with the same dilute acid and separated phase by filter paper subsequently and brought to ferment and analyze the composition by HPLC and GC.

3.3.3 Medium Preparation

For DifcoTM Cooked meat medium (CMM) (Qureshi *et al.*, 2008), 0.875 g of CMM pellet and 0.12 g glucose were dissolved with 6 ml of distilled water followed by sterilizing at 121 °C for 15 min and being cooled to room temperature subsequently. After that, one loop of cell spores was put into the prepared solution and heat shock at 80 °C for 2 min to activate and diminish weak cultures. The CMM culture solution was kept in 37 °C without agitation and waited for cells activation within 30 h.

3.3.4 Inoculum Development

Preparing for P2 medium inoculum (Qureshi *et al.*, 1999), 1.8 g of glucose and 0.06 g of yeast extract were dissolved in 53.38 ml of distilled water followed by sterilizing at 121 °C for 15 min and being cooled to room temperature subsequently.

A 6 ml of active growing cells (from liquid CMM) was inoculated into prepared P2 medium solution following by adding 500 µl of buffer (KH_2PO_4 50 g/l, K_2HPO_4 50 g/l, ammonium acetate 220 g/l), 100 µl of mineral ($MgSO_4 \cdot 7H_2O$ 20 g/l, $MnSO_4 \cdot H_2O$ 1 g/l, $FeSO_4 \cdot 7H_2O$ 1 g/l, NaCl 1 g/l) and 20 µl of Vitamins (para-amino-benzoic acid 0.1 g/l, thiamin 0.1 g/l, biotin 0.001 g/l) to solution. After keeping P2 medium solution in 37 °C without agitation for 8 h, cells were ready for simultaneous saccharification and fermentation step.

3.3.5 Simultaneous Saccharification and Fermentation

First of all, Simultaneous Saccharification and Fermentation was performed by using the washed water-insoluble residue of pretreated corn cobs with 2% (w/w) H_2SO_4 120 °C 5 min and 10:1 LSR for 4 g mixed with 73.90 ml of Citrate-Phosphate buffer solution by varying pH 4.8, 5.7, and 6.6, respectively, and adding initial glucose sugar for 20 g/l. Secondly, mixed solution was brought to sterile in an autoclave reactor (121 °C for 15 min) then cooled until room temperature. Then, 4 ml of P2 medium was mixed with following supplementations: 0.8 ml of sterilized yeast stock 1g/l, 666.8 µl of ammonium acetate 220 g/l, 133.2 µl of mineral (

$MgSO_4 \cdot 7H_2O$ 20 g/l, $MnSO_4 \cdot H_2O$ 1 g/l, $FeSO_4 \cdot 7H_2O$ 1 g/l, NaCl 1 g/l) and 26.8 µl of (Vitamins: para-amino-benzoic acid 0.1 g/l, thiamin 0.1 g/l, biotin 0.001 g/l). All components were mixed and softly shaken for making homogeneous solution. Next, the mixture was brought to the prepared fermentation broth followed by adding enzyme of 10.36 FPU/g and purged with nitrogen gas for 10 min accordingly. After that, mixed solution will be shaken in an incubator at different temperatures (37 °C, 43.5 °C, and 50 °C) and different time (24 h, 48 h, and 72 h). At the end of reaction time, the product solution was filtered to separate liquid and solid residue. The liquid was determined the quantity of monomeric sugars yield by HPLC (Perkin Elmer LC200) equipped with a refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.30 ml/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C and determined the quantity of Acetone-Butanol-Ethanol yield by GC.

3.3.6 Response Surface Methodology (RSM) for Experimental Design

RSM with a full factorial central composite design (CCD) was employed in this work. The variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (i = 1, 2, 3, \ldots, k)$$

Where x_i is the code value of the independent variable; X_i is a real value; X_0 is its real value at the centre point; and ΔX_i is the step change in the variable X_i. pH (X1), temperature (X2), and reaction time (X3) were chosen as the three independent factors. Their values at different coded and actual levels were shown in Table 3.1. The code and uncoded level of variables were used in experimental design are listed in Table 3.2.

A second order polynomial regression model was used to express Y_i as a function of the independent variables as follows:

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} x_i x_j$$

Where Y_i is the response; x_i, x_j are the input variables, which influence the response variable Y_i; k is the number of variables; a_0 is the constant or offset term; a_i represents the coefficients of the linear parameters; a_{ii} represent the coefficients of interaction parameters and a_{ij} represents the coefficients of quadratic parameters. Analysis of variance (ANOVA) program was used for regression analysis of the data and to estimate the coefficients of the regression equation.

Table 3.1 Design of experiment for statistical analysis. Coded and experimental values of the factors for RSM

Independent variables	Symbol	Coded		
		-1	0	1
pH	X1	4.8	5.7	6.6
Temperature (°C)	X2	37	43.5	50
Reaction time (h)	X3	24	48	72

Table 3.2 Central composite design (CCD)

Run	Coded values			Real values		
	pH	Temperature	Time	pH	Temperature (°C)	Time (h)
1	1	1	1	6.6	50	72
2	1	1	-1	6.6	50	24
3	1	-1	1	6.6	37	72
4	1	-1	-1	6.6	37	24
5	-1	1	1	4.8	50	72
6	-1	1	-1	4.8	50	24
7	-1	-1	1	4.8	37	72
8	-1	-1	-1	4.8	37	24
9	1	0	0	6.6	43.5	48
10	-1	0	0	4.8	43.5	48
11	0	1	0	5.7	50	48
12	0	-1	0	5.7	37	48

Table 3.2 Central composite design (CCD) (cont.)

Run	Coded values			Real values		
	pH	Temperature	Time	pH	Temperature (°C)	Time (h)
13	0	0	1	5.7	43.5	72
14	0	0	-1	5.7	43.5	24
15	0	0	0	5.7	43.5	48
16	0	0	0	5.7	43.5	48
17	0	0	0	5.7	43.5	48
18	0	0	0	5.7	43.5	48
19	0	0	0	5.7	43.5	48
20	0	0	0	5.7	43.5	48

3.4 Analysis Method

3.4.1 High Performance Liquid Chromatography (HPLC)

The quantity of monomeric sugars yield was determined by High Performance Liquid Chromatography (HPLC) equipped with a refractive index detector (Series 200 LC/S/N291N5060508, Perkin Elmer) using an Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA) and a guard column (30 mm x 4.6 mm, Bio-Rad Lab, USA) under these following conditions: flow rate 0.60 ml/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C.

3.4.2 UV-VIS Technique (UV)

After pretreatment process, the product in liquid phase, prehydrolysate, was measured the total sugar yield by UV-VIS (1800) equipped with the photometric detector mode and UV wavelength of 540 nm.

3.4.3 Scanning Electron Microscope (SEM)

The surface morphology and porosity of untreated corncobs pretreated sample after pretreatment process was observed by Scanning Electron Microscope

(SEM) (HitachiS–4800 SEM instrument operated at 10–15 kV accelerated voltage). Besides, samples were dried in oven (80 °C) overnight before analysis.

3.4.4 X-Ray Diffraction (XRD)

Cellulose crystallinity was determined by X-ray diffraction (Bruker AXS Model D8 Discover). Samples of each size fraction were analyzed. All samples were scanned from $2\theta = 5°$ to 40° with a step size of 0.02°. Determination time was 0.5 s/0.02°. In addition, the chemical structure of lignocellulosic biomass changing between fresh and treated corncob waste were compared.

3.4.5 Surface Area Analysis (BET)

BET surface area information of the pretreated residues and untreated were measured by N_2 adsorption/desorption measurements (Quantachrome instrument; model: BELSORP–max, BEL, Japan) done at 100 °C (373 K). Prior to measurement, all biomass materials were dried at 90 °C for 48 h and then 1 g of sample was put into tube of the Quantachrome instrument and degassed using a vacuum for 24 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves using BELSORP–max software.

3.4.6 Gas Chromatography (GC)

The acetone-butanol-ethanol yield produced from fermentable sugars that got from ABE fermentation step was measured concentration by a gas chromatograph (Series Perichrom 2100) equipped with a flame ionization detector using Innowax column length 30 m under these following conditions: N_2 flow rate 45 ml/min, detector temperature 240°C, injection Temperature 240°C, and volume injection 0.5 µl.

3.4.7 Fibertect M6

The methods to determine cellulose, hemicelluloses, lignin, and ash in solid residue after pretreatment and enzymatic hydrolysis were amylase neutral detergent fiber (NDF), acid detergent fiber (ADF), and cellulose acid detergent lignin (ADL). Amylase neutral detergent fiber (NDF) method was used to measure the

quantity of cellulose, hemicelluloses, and lignin. After that, the hemicelluloses were detected by using acid detergent fiber (ADF) method. Then, cellulose was measured by using cellulose acid detergent lignin (ADL) method. The quantity of cellulose was measured when suspended with sulfuric acid. After this process was completed, the sample was burned at 500 °C for 2 h in order to determine lignin's quantity. The solid residue after this process is ash.