CHAPTER V
OPTIMIZATION OF SULFURIC PRETREATMENT OF CORNCOB USING RESPONSE SURFACE METHODOLOGY FOR FERMENTATION BY CLOSTRIDIUM BEIJERINCKII TISTR 1461

5.1 Abstract

Corncob is one of the potential feedstocks in Thailand that can be used for fermentable sugar production through dilute sulfuric acid pretreatment and enzymatic hydrolysis. To recover high amount of monomeric sugars from corncob, the sulfuric pretreatment conditions were optimized by the response surface methodology (RSM) with the three independent variables (sulfuric acid concentration, temperature, and time). The highest response of total sugars, 48.70 g/L, was found at 118.36°C, 5.36 min, and 2.89% (v/v) H_2SO_4. With these conditions, total sugars from the experiment was 46.29 g/L, with 4.94% error from the predicted value. The hydrolysate was used as a substrate for the Acetone–Butanol–Ethanol (ABE) fermentation to evaluate its potential for microbial growth. The simultaneous saccharification and fermentation shows that *C. beijerinckii* TISTR1461 can generate Acetone–Butanol–Ethanol products, 11.89 g/L, instantly using hydrolysed corncob from Novozymes® 50013 cellulase enzyme.

Keywords: Butanol · Dilute acid pretreatment · Enzymatic hydrolysis · Corncobs · Response surface methodology

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5.2 Introduction

Increasing demands for fossil fuels and lack of energy sustainability have pushed the scientific community to research alternative and renewable energy sources. Biobutanol can be produced from lignocellulosic materials used as substrates because of the abundance, low cost, and renewability of these materials like ethanol production [1]. Lignocellulosic biomass from corn, cassava, sugarcane, etc. is mainly composed of cellulose (38–50%), hemicellulose (23–32%), and lignin (15–30%) materials, on a dry basis, along with smaller amounts of extractive and ash. Thus these biomass have advantages for utilization in the production of fermentable sugars when used in the acid pretreatment and enzymatic saccharification process of biobutanol production.

Corncob, a byproduct of maize production, is the most abundant agricultural waste around the world. It is one of the top ranked agricultural residues in Thailand and has been estimated at about 3.85 million tons per year. It is considered to be an attractive biomass and low–cost source of sugars for biofuel production and other value–added commodity chemicals [2].

The pretreatment process is an important step required to alter the structure of cellulose to make it more accessible for enzymatic hydrolysis. Thus, the main objective of pretreatment is to remove both hemicellulose and lignin to enhance the surface area of the substrates. Among the pretreatment methods, dilute sulfuric acid hydrolysis is widely used since the fermentable sugar known as xylose is produced, after acid pretreatment, in the aqueous phase. Moreover, this method is effective and inexpensive [1].

The objective of this work was to produce fermentable sugars, xylose, glucose, and arabinose (mainly from hemicellulose and cellulose, respectively) from corncob, by using dilute sulfuric acid pretreatment and enzymatic hydrolysis. To optimize the pretreatment conditions, the response surface method (RSM) was applied to investigate the highest response value by using three independent variables (temperature, time, and sulfuric acid concentration) associated with the pretreatment while the response value was total sugars obtained from the pretreatment and enzymatic hydrolysis. The amount of sugar hydrolysed from these processes was

determined by high performance liquid chromatography (HPLC). Furthermore, the structure of the pretreated corncob after acid pretreatment was also investigated by measuring surface area, particle size, crystallinity from x–ray diffraction (XRD), and scanning electron microscope (SEM) imagery. The obtained fermentable sugars were used as a substrate for ABE fermentation to evaluate the effective route including the simultaneous saccharification and fermentation (SSF), which gave high yield and productivity of ABE [3].

5.3 Materials and Methods

5.3.1 Materials

Corncob was chosen as the raw material and provided by Betagro Public Company Limited, Thailand. It was dried at 105°C, ground and screened to particle size to no.12 mesh (1.68 mm) before the pretreatments. The acid used in the pretreatment was sulfuric acid (H_2SO_4, ≥ 98% purity) purchased from RCI Labscan (Bangkok, Thailand). The cellulase and enzyme was Novozymes® 50013 cellulase enzyme produced by *Trichoderma reesei* purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals (analytical grades) used as standards, enzyme assays, dinitrosalicylic acid analysis (DNSA) and culture medium were obtained from Sigma–Aldrich (St. Louis, MO, USA, Merck (Darmstadt, Germany), Oxoid Ltd. (Hampshire, UK), and Ajax Finechem (New South Wales, Australia) respectively. An Aminex HPX–87H column (300 × 7.8 mm), Deashing cartridge (30 × 4.6 mm) and Cation H micro–guard cartridge (30 × 4.6 mm) were purchased from Bio–Rad Laboratories, Inc., Hercules, CA, USA.

5.3.2 Filter Paper Assay (FPA)

The activity of Novozymes® 50013 cellulase enzyme was measured by filter paper assay to quantify the precise amount of enzyme loading. This method was done according to Ghose [4] using Whatman No.1 filter paper strips (cut to 1 cm × 6 cm) as a substrate with the enzyme in a pH 4.8 citrate buffer. The enzymatic reaction was conducted in a water bath at 50 °C for 60 min. After that, all samples were added with Dinitrosalicylic (DNS) acid reagent and heated as described by Miller [5]. The reacted samples were then measured with a UV–VIS Spectrometer (Thermo Fisher Scientific Inc., USA) at 540 nm using a standard curve of glucose to convert the obtained optical density back to mg of glucose released from the hydrolysed filter paper. The enzyme dilution (ED), which released 2 mg/ 0.5 mL, was substituted in the following Filter Paper Unit (FPU) equation;

$$FPU = \frac{0.37}{ED}$$

5.3.3 Experimental Design for RSM

The effects of the independent variables (reaction temperature, residence time during pretreatment, and sulfuric acid concentration) on the responses of total sugars (glucose, xylose, and arabinose) were investigated using Response Surface Method (RSM). The central composite design composed of three factors (k = 3) according to the independent variables and each variable contained three levels. The actual factor levels corresponded to the coded factor levels as follows: X_1 = (Temperature-120)/20, X_2 = (Time-15)/10 and X_3 = (H_2SO_4 Conc.-2)/2 (Table 5.1). A second–order polynomial regression model from this experimental design was

$$Y = a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_0$$

where Y = Total sugars (g/L), a_i = the linear coefficients, a_{ii} = the quadratic coefficient, a_{ij} = the cross product coefficients, and a_0 = the model constant. The

program R 2.14 (The R Foundation for Statistical Computing) for Windows was used for the data analysis. All data obtained from duplicate experiments.

5.3.4 Dilute Acid Pretreatment and Enzymatic Hydrolysis

Dilute acid pretreatment was performed in a laboratory scale stirred autoclave (stainless steel type 316). The 1L reactor, with 200 mL working volume, included an electric heater, magnetic agitator, and a temperature controller. For dilute acid pretreatment, the corncob was slurried in diluted H_2SO_4 at 66.67 g/L solid loading (1:15 liquid solid ratio). Water pretreatment was performed in the same condition as acid pretreatment in order to compare the physical characterization. The effects of the reaction temperature, residence time during pretreatment, and sulfuric acid concentration on the sugar yielded in the enzymatic hydrolysis were analyzed through the RSM. After pretreatment, the reactor was cooled to ambient temperature then the hydrolysate was transferred to a 250 mL Erlenmeyer flask. It was adjusted to pH 4.8 with 10 M NaOH to satisfy the condition of cellulase enzyme and then added with Novozymes® 50013 enzyme at the enzyme loading of 10 FPU/g biomass. The flask was then put in an incubator shaker at 50 °C at 150 rpm for 72 h then the hydrolysate was analyzed for sugar content determination.

5.3.5 Overliming Process

The liquid portion obtained from enzymatic hydrolysis to be used for overliming [6] was adjusted to pH 10 using $Ca(OH)_2$. It was added with sodium sulfite (Na_2SO_3) to the final concentration of 1 g/L to reduce the redox potential [7]. The overliming hydrolysate was then heated at 90 °C for 30 min, cooled to room temperature and adjusted to pH 6.6 using concentrated H_2SO_4. It was centrifuged and filtered the same as the previous procedure and kept at -20 °C.

5.3.6 Microorganism and culture maintenance

C. beijerinckii TISTR1461 was provided by Thailand Institute of Scientific and Technological Research (TISTR) as freeze–dried cultures. The culture was stocked by initially inoculating on reinforced clostridial medium agar plate and

incubated at 37 °C in an Anaerojar™ combined with an anaerobic generator and indicator (Oxoid Ltd., Hampshire, UK) and kept at 4 °C for 3 days. The culture stock was revived by an inoculate one loop of colony in 6 mL of cooked meat medium and heat shocked at 80 °C for 2 min. It was then incubated in the anaerobic atmosphere at 37 °C for ~20 h to reach the middle of the log phase. The inoculum continued development by adding 6 mL of actively growing cell in 54 mL of P2 medium [8] supplied with 20 g/L glucose and grown for 6 h for proper seeding the production medium.

5.3.7 Fermentation Experiments

The fermentation of *C. beijerinckii* TISTR1461 in the corncob hydrolysate or P2 medium (a control experiment) was conducted in 100 mL Duran™ screw cap bottles, with 80 mL working volume and 5% (v/v) inoculum size, at 37 °C, and pH 6.6. The mixture was purged with ultra purity N_2 gas to develop the anaerobic condition. P2 medium was added to the final concentration of 40 g/L glucose and sterilized by an autoclave at 121 °C, 15 min prior to fermentation while the hydrolysate was adjusted to pH 6.6 by 10 M NaOH and filter sterilized. Samples were withdrawn at 72 h of fermentation, centrifuged, and passed through a 0.2 μm syringe filter and kept at -20 °C for later analysis of pH, sugar consumption, and products produced (acetone, butanol, and ethanol).

Simultaneous saccharification and fermentation (SSF) was performed, by using washed sulfuric pretreated corncob until reaching the neutral pH. It was dried overnight and then added to 80 mL of citrate–phosphate buffer at the solid loading of 50 g/L. The Novozymes® 50013 was added to 10 FPU/g corncob at the same time of the 5% (v/v) inoculum then it was incubated at 37 °C for 72 h.

5.3.8 Analytical Methods

Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and acid insoluble ash (AIA) content were determined by Nakhonratchasima Animal Nutrition Research and Development Center, Thailand. The

difference between the amount of NDF and ADF was used to estimate the hemicellulose content. Cellulose content was calculated by subtracting the values for (ADL+AIA) from ADF. Carbohydrate content of untreated material was also determined by measuring the hemicellulose (xylan and arabinan) and cellulose (glucan) derived sugars in supernatants following concentrated acid hydrolysis as described by the National Renewable Energy Laboratory (NREL) [9]. Insoluble lignin content of the corncob and the solid fraction remaining after pretreatment were also investigated.

The column used in HPLC was a packed column (Aminex HPX–87H column, Bio–Rad Lab, USA at 60 °C with 5 mM H_2SO_4 as the eluent at a flow rate of 0.7 mL/min). Acetone, butanol, and ethanol concentration was determined by a gas chromatograph (GC; Perichrom; PR2100) equipped with a DB–FFAP column and a flame ionization detector. The initial and final temperatures were 60 °C and 157 °C with a heating rate of 15 °C/min. The detector and injector temperatures were 220 °C.

BET surface area of the pretreated residues was measured by N_2 adsorption/desorption measurements (Quantachrome instrument; model: BELSORP–max, BEL Japan INC., Japan) and carried out at -196 °C (77 K). Before measurement, all biomass materials were dried at 40 °C for 48 h and then a sample (0.5 g to 1 g) was put into the sample tube of the Quantachrome instrument and degassed in a vacuum for 16 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves using BELSORP–max software.

An SEM (HitachiS–4800 SEM instrument operated at 10–15 kV accelerated voltage) was used to explore surface morphology and corncob size before and after pretreatment.

The structure of the pretreated solid residues was characterized by XRD with a Rigaku/Rint2200 diffractometer equipped with a Ni filtered CuKα radiation source (λ = 1.542 Å) of 40 kV and 30 mV. The scanning was performed between 2θ = 5° and 60° with a step size of 0.02° at a speed of 1°/min. Data from the XRD results were used to calculate the corncob crystallinity index (CrI) from the formula :

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

where I_{002} is the maximum intensity for the crystalline portion of the biomass (i.e., cellulose) at ~2θ = 22.6° and I_{am} is the intensity of the background scatter measured at 2θ = 18.7° [10].

5.4 Results and Discussion

5.4.1 Composition of Corncob

The corncob was examined for cellulose, hemicellulose, lignin, and ash content [11]. Cellulose, the major chemical component in fiber walls of plants and contributing 39.04% of corncob dry weight, is the most abundant organic compound in nature. Hemicellulose, contributing 42.60% of the corncob dry weight, is a complex polysaccharide structure that contains different carbohydrate polymers like pentoses (e.g. xylose and arabinose) and hexoses (e.g. mannose, glucose, and galactose). Lignin, contributing 7.56% of corncob dry weight, is an amorphous heteropolymer consisting of three different phenylpropane units (**ρ**–coumaryl, coniferyl, and sinapyl alcohol) that are held together by different kinds of linkages. Extractive biomass and ash contained 7.84% and 2.19% of corncob dry weight, respectively. The remaining portion was considered as protein and trace elements.

5.4.2 Optimization of Total Sugars from Hydrolysed Corncob Using RSM

Xylan is the major polysaccharide present in the largest hemicellulose structures of corncob, so the amount of xylose contained in the filtrate can be used as a marker of the solubilization level of hemicellulose [2]. The highest concentration of xylose measured in the hydrolysate, 17.41 g/L, was generated after a 15 min pretreatment at 120 °C, with 2% (v/v) H_2SO_4 (Table 5.1). However, the maximum response value for xylose was estimated at 17.24 g/L after setting the partial derivatives to zero with respect to the corresponding variables, which were 114.06 °C, 5.76 min, and 2.70% (v/v) H_2SO_4 (analyzed statistical data is not shown here) meaning that the maximum value of xylose was similar to the condition of the center point. These findings were similar to reported results, which indicated that the cellulose and

xylan conversion obtained under these conditions were achieved in low–temperature pretreatments [12]. In this work, the optimum conditions were considered as a milder condition (lower hydrolysed temperature) as compared to a previous study, where the optimal pretreatment temperature was between 160 °C and 170 °C [13].

Table 5.1 Optimization of total sugars from acid pretreatment and enzymatic hydrolysis using RSM

Run	Code values			Real values			Xylose after acid pretreatment (g/L)	Total sugars after acid pretreatment and enzymatic hydrolysis (g/L)
	Temperature	Time	H_2SO_4 Conc.	Temperature (°C)	Time (min)	H_2SO_4 Conc. (%)		
1	1	1	1	140	25	4	3.23	26.27
2	1	1	-1	140	25	0	0.48	6.14
3	1	-1	1	140	5	4	7.20	32.98
4	1	-1	-1	140	5	0	0.42	6.34
5	-1	1	-1	100	25	0	0.23	5.93
6	-1	1	1	100	25	4	12.58	45.36
7	-1	-1	1	100	5	4	12.13	37.38
8	-1	-1	-1	100	5	0	0.17	2.89
9	1	0	0	140	15	2	10.71	38.03
10	-1	0	0	100	15	2	12.87	33.79
11	0	1	0	120	25	2	11.36	34.86
12	0	-1	0	120	5	2	17.05	47.12
13	0	0	1	120	15	4	13.01	40.51
14	0	0	-1	120	15	0	0.20	5.96
15	0	0	0	120	15	2	17.41	47.09
16	0	0	0	120	15	2	17.32	46.37
17	0	0	0	120	15	2	16.87	47.53

The increase of sugars in enzymatic hydrolysis, due to hemicellulose removal from the corncob in the pretreatment step, can be seen after comparing the result of total sugars between the runs which sulfuric concentration is 4% and 2% (v/v) in Table 5.1 (eg. run 1 and 2 or run 3 and 4). During pretreatment either xylose or furfural generation lead to high glucose yield in the hydrolysate after enzymatic hydrolysis. The success of the acid pretreatment is due to hemicellulose removal in order to maximize sugars yield after enzymatic hydrolysis. Since both furfural and xylose levels are substantially influenced by temperature and acid concentration, it would be expected that the sugars in the hydrolysate would be induced most substantially by temperature and acid concentration [14]. The purpose of pretreatment is not only to break down hemicellulose to xylose, but also to increase the rate of enzymatic hydrolysis of cellulose to glucose.

The RSM experimental design results shown in Table 5.1 are composed of three factors (temperature, time, and acid concentration) at three levels (-1, 0 and 1) and the result of total sugars after acid pretreatment and enzymatic hydrolysis. The regression coefficient, standard error, t values, and P value are shown in Table 5.2. The t–test value and P value indicates the significance of the regression coefficient. The smaller the value of P, the more significant the coefficient. The linear coefficients a_3 and the quadratic coefficient a_{33} are significant at least 5% significance level. Thus, the most important factor is the linear effect of sulfuric acid concentration (a_3) and the quadratic effect of the sulfuric acid concentration (a_{33}) while the temperature, time, and the interaction between temperature, time, and sulfuric acid concentration has a negative effect on total sugars due to their negative coefficient value. Moreover, the large P value on temperature (a_1) and time (a_2) implied that these factors are not important to the obtained total sugars. The quality of the regression was well qualified via the analysis of variance (ANOVA), as shown in Table 5.3. The $F_{statistic}$ from this regression was 105.74 which is much higher than $F_{0.05,9,10}$ (5.79) or even $F_{0.001,9,10}$ (37.2), meaning that the model can represent an accurate representation of the experimental data which coincides with the small P–value for the regression.

Table 5.2 Statistics for the regression of the optimization model

Coefficient	Value	Standar d error	t value	P
a_0	43.7627	2.2726	19.257	$2.54e^{-07}$
a_1	-1.559	1.6795	-0.928	0.384162
a_2	-0.815	1.6795	-0.485	0.642304
a_3	15.524	1.6795	9.243	$3.59e^{-05}$
a_{11}	-5.4522	3.2447	-1.68	0.136776
a_{22}	-0.3722	3.2447	-0.115	0.911899
a_{33}	-18.1272	3.2447	-5.587	0.000827
a_{12}	-2.2413	1.8777	-1.194	0.27151
a_{13}	-3.3938	1.8777	-1.807	0.113649
a_{23}	-0.1963	1.8777	-0.105	0.919692

Table 5.3 ANOVA for the regression

Source of variation	Degree of freedom	Sum of squares	Mean square	$F_{statistic}$	Prob > F
Model	5	196.7	39.34	105.74	0.0093946
Residual	2	0.74	0.37		
Total	7	197.45	28.21		
$R^2 = 0.958$					

Figure 5.1 shows the two–dimensional contour plots (Fig. 5.1a–c) and the three–dimensional response surfaces (Fig. 5.1d–f) while total sugars were the response. A relationship between two of the three factors (temperature, time, and sulfuric acid concentration) was observed from each plot while one was kept constant. In relation to the coefficient results, the plots were consistent since the interaction between temperature and time did not have much of an effect on the total sugars, as noticed in Fig. 5.1a, d which are nearly a plain surface. The obvious changes in surface are shown in the interaction that was composed of sulfuric acid concentration (Fig.

5.1b–c, e–f)). The total sugars increased to a high value with an increase in sulfuric acid concentration and temperature while time did not have much effect (Fig. 5.1 d–f). However, total sugars decreased with further increase in sulfuric acid concentration and temperature, thus the optimal conditions of total sugars were defined. The two–dimensional contour plots confirm the effect of sulfuric acid concentration since it influences the total sugars along the test range. The order of importance of the three factors on total sugars is: sulfuric acid concentration>temperature>time as indicated from the P value and plots.

After analysis of the total sugar concentration (glucose, xylose, and arabinose) from acid pretreatment and enzymatic hydrolysis, the optimum conditions obtained from RSM were similar to the previous maximum response value of xylose after acid pretreatment (114.06 °C, 5.76 min, and 2.70% (v/v) H_2SO_4) as they were 118.36 °C, at 5.36 min, and 2.89% (v/v) H_2SO_4 while the maximum response value for total sugars was 48.70 g/L.

The influence of temperature, time, sulfuric acid concentration, and interactions have already been proven by using modified Arrhenius equations from other studies [15, 16]. These studies have proposed equations as rate constants in kinetic models to describe the acid hydrolysis of hemicellulose into xylose. The amount of xylan oligomers as a fraction of the solubilized xylose was greatest at short pretreatment times [17]; it was consistent with both models of using xylose after acid pretreatment and total sugars after acid pretreatment and enzymatic hydrolysis as the response surface since the optimal times were 5.76 and 5.36 min, respectively. Similar trends are reported in a study on the dilute acid pretreatment of corn stover [18]. Esteghlalian *et al.*, reported that the monomeric yields of 80% to 90% could be achieved at temperatures of 170 °C to 180 °C with sulfuric acid concentrations higher than 1.0% (w/w) [17].

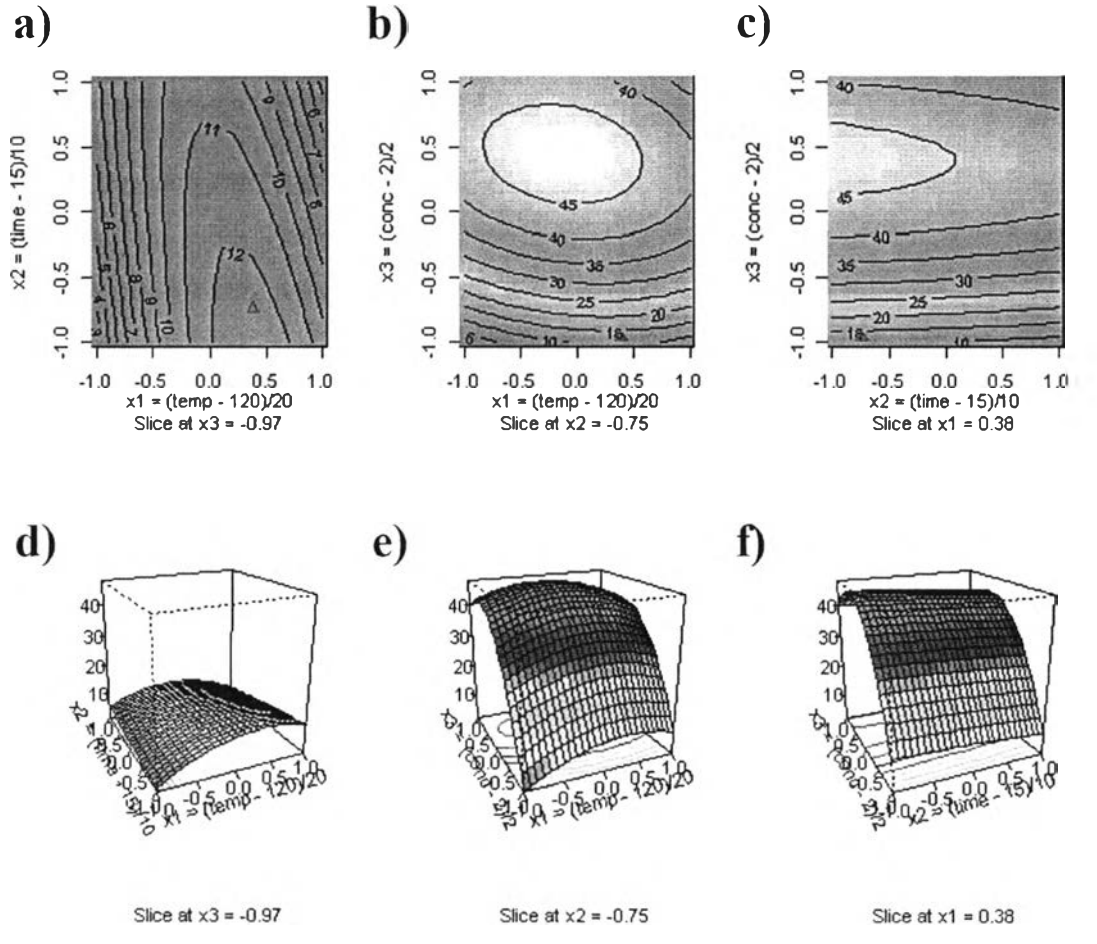

Figure 5.1 Contour plots (a–c) and response surface (d–f) for total sugars from hydrolysed corncob under the effects of temperature and time (sulfuric concentration was fixed at 2%), temperature and sulfuric concentration (time was fixed at 15 min), and sulfuric concentration and time (temperature was fixed at 120 °C) respectively.

5.4.3 Sulfuric Acid Pretreatment and Enzymatic Hydrolysis at the Optimum Condition

The optimum conditions from the RSM were applied to the experiment to confirm the calculated result of total sugars (48.70 g/L) after pretreatment and enzymatic hydrolysis. The total sugar from the experiment was 46.29 ± 1.01 g/L, which was similar to the predicted value (48.70 g/L). The total sugars, composed of 22.14 g/L glucose and 17.05 g/L xylose, were estimated at 86.4% and 97.1%, respec-

tively, proving the recovery of monomeric sugars in corncob. The recovery of glucose and xylose was calculated from the maximum theoretical sugar yield obtained from the method of NREL. However, the result of 46.29 g/L total sugars from the optimum conditions was close to run 12 (Table 5.1), (120 °C, 5 min, and 2% (v/v) H_2SO_4) as it was 47.12 g/L. Time course of enzymatic saccharification from the corncob pretreated at the optimum condition shown in Fig. 5.2 indicates monomeric sugars released from the biomass. The xylose concentration is slightly increased since it nearly completes hydrolysis in the pretreatment step while the glucose concentration is twice increased at 72 h. The total sugars did not dramatically rise after 24 h of hydrolysis.

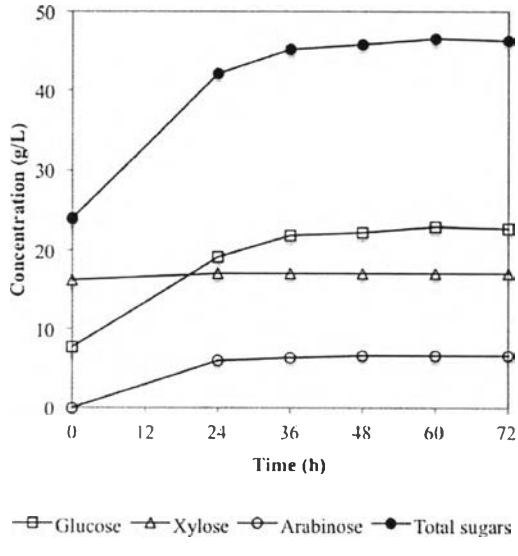

Figure 5.2 Time courses of enzymatic saccharification (45 °C, pH 4.8) of the optimum conditions of sulfuric acid pretreated corncob (2.89% H_2SO_4, 118.36 °C, and 5.36 min) using a Novozymes 50013 cellulase enzyme.

5.4.4 Physical Characterization of Pretreated Corncob

Examination of dry weight loss for the treated samples indicates that weight loss occurred due to partial removal of the amorphous materials (hemicellulose and lignin). The amorphous material removal and weight loss during acid treatment is obviously high compared to the water treatment at the same elevated temperature (Table 5.4). Cellulose content of the corncob after the acid treatments, indicates that the molecules are relatively unaffected by acid treatment. Similar observations were observed in an investigation on the influence of boiling temperatures on chemical composition of hemp fibers [19]. Complete removal of hemicellulose by acid treatments can be achieved because the proton ions of the sulfuric acid is able to penetrate into the corncob surface and further catalyze the de–acetylation and hydrolysis; this breaks the beta (1,4)–glycosidic bonds of the hemicelluloses [2]. However, lignin was not totally removed due to the presence of chemically resistant carbon–carbon bonds in the lignin molecules [20]. The digestibility of H_2SO_4–treated corncob was found to increase with a decrease of hemicellulose content from 42.60% to 4.06%, while water–treated corncob had a hemicellulose content of 40.36%.

After sulfuric acid pretreatment, the pretreated corncob was characterized by XRD to identify the crystallinity index. The crystallinity index indicates the composition of the biomass as its increased value referred to high content of cellulose which considered to be crystalline [1]. Thus, uncovering all crystalline cellulose available and disruption of hydrogen bonding within and in between cellulose chains can increase the rate of enzymatic hydrolysis [21].The results in Table 5.4 show that the crystallinity index increased in the order of untreated corncob < water pretreatment < acid pretreatment. Thus, sulfuric acid pretreatment had more potential of removing amorphous components compared to water pretreatment. This was consisted with the report in the enhancement of enzymatic digestion as the acid pretreatment affects an increase in crystallinity index and hydrolysed more sugars after enzymatic hydrolysis [22]. However, the crystallinity index is not the most exact factor as the ionic liquid pretreatment left highly amorphous structure on switchgrass with far low crystallinity index compared with acid pretreatment but yielded more sugars from cellulase hydrolysis [23].

Table 5.4 Effect of sulfuric pretreatment of corncob at the optimum conditions on the composition, physical properties and enzymatic hydrolysis of the pretreated residues compared with hot water pretreatment (Each value is the average of triplicate experiments and calculated on the basis of dry weight)

Sample	Weight loss (%)	Furfural		Composition			Physical properties			Composition (after enzymatic hydrolysis)[e]		
		Untreated (g/L)	Overliming (g/L)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	CrI[d] (%)	BET (m^2/g)	Particle size (nm)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	ND[c]	ND	ND	39.04	42.6	7.56	29.07	0.99	7.34	ND	ND	ND
Acid Pretreatment[a]	65	0.11	0.1	67.63	4.06	25.53	33.19	3.87	5.85	38.38	3.18	38.53
Water Pretreatment[b]	14.85	ND	ND	42.01	40.36	9.63	31.42	1.36	7.12	38.93	38.33	12.36

[a] The samples were pretreated at 2.89% H_2SO_4, 118.36°C, and 5.36 min

[b] The samples were pretreated at 118.36°C, and 5.36 min slurried in water

[c] not detected

[d] CrI (Crystallinity Index) (%) = $(I_{002}-I_{am})/I_{002}$*100 where I_{002} is intensity of the 002 peak at $2\theta = 22.6°$ and I_{am} is intensity of the background scatter at $2\theta = 18.7°$

[e] After 72 h of enzymatic hydrolysis

Images of SEM showed the details of structural surface changes of corncob after acid pretreatment. The untreated corncob was nonporous, uniform, and mostly linear in nature (Fig. 5.3). The corncob pretreated with sulfuric acid shows dramatically changed structures. The pretreatment with sulfuric acid, at the optimal conditions, could break down the structural rigidity of the corncob matrices and create micropores. The removal of surface impurities, non–cellulosic materials, inorganic substances, and waxes has also been found to result in rougher surfaces and better fiber separation [24]. These cracks can increase the surface area and porosity to facilitate disruption of lignocelluloses, a prerequisite for enzymatic hydrolysis of cellulose [1].

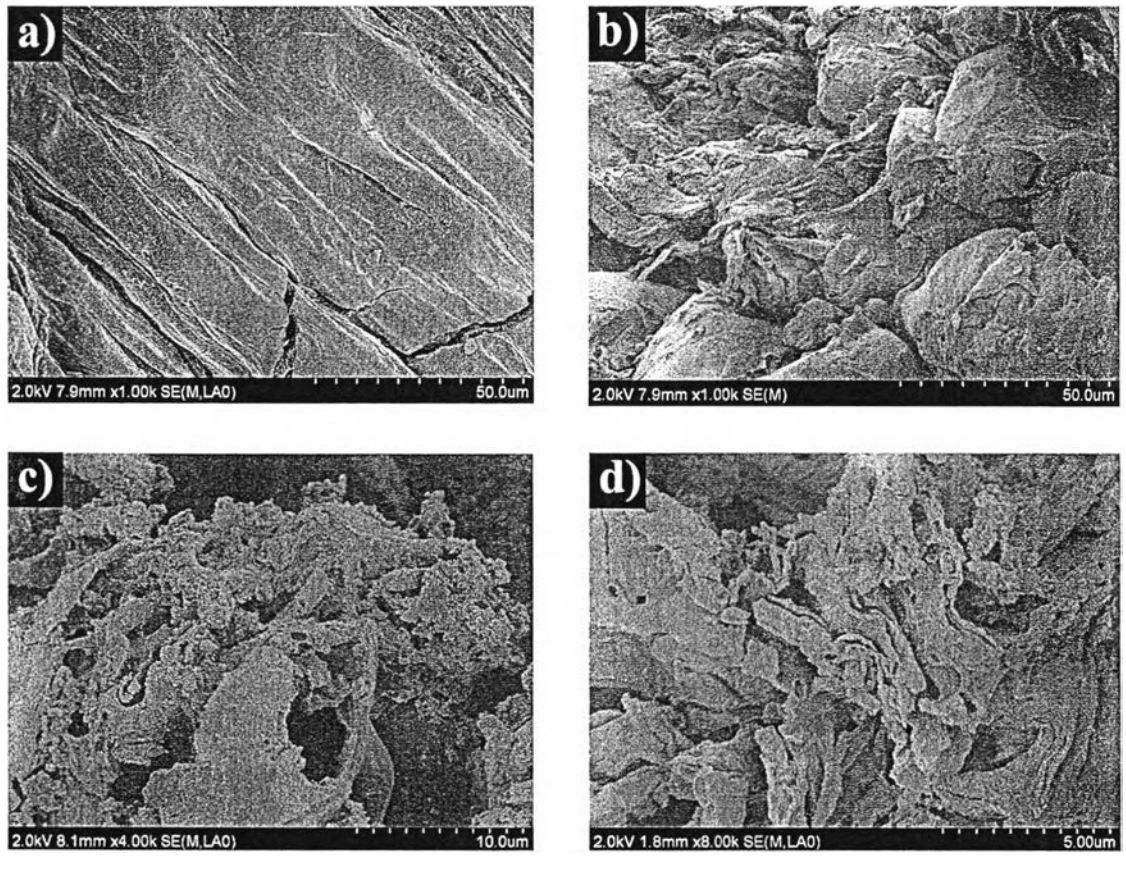

Figure 5.3 SEM micrographs of (a) untreated (1000×) and (b) pretreated corncobs at the optimum conditions and magnification of 1000×, (c) 4000×, and (d) 8000×.

Furthermore, it was found that under the optimum conditions of pretreatment, the surface area and total pore volume of the pretreated corncob was higher than that of untreated corncob and the average pore diameter of pretreated corncob was less than untreated corncob. The results indicate that most of the hemicellulose from acid pretreatment has been solubilized and removed, which increases the surface area and makes enzymatic hydrolysis effortlessly occur (Table 5.4) [2].

5.4.5 Butanol Production

The hydrolysate of corncob (after the sulfuric acid pretreatment at optimum conditions and enzymatic hydrolysis) was used as a substrate for butanol production from *C. beijerinckii* TISTR1461. It was reported that the inhibitor like furfural in the hydrolysate was found after acid pretreatment at a high temperature, the hydrolysate then passed the overliming process to eliminate the toxicity [6]. To evaluate the proper condition for fermentation, six different experiments were conducted. From Table 5.5, the hydrolysates which had not removed furfural by the overliming process (Experiment I, III) could not be used as a substrate for *C. beijerinckii* TISTR1461 since the products (acetone, butanol, and ethanol) were not detected and no cell growth observed. Then, the overliming process was applied to the hydrolysate produced from sulfuric pretreatment (Experiment II, IV) resulting in the elimination of furfural from 0.11 g/L to 0.1 g/L (Table 5.4). The positive result was observed in Experiment IV as microbes tend to grow and excrete products at the end of the fermentation gave an ABE yield of 0.26 similar to 0.28 of the control experiment (Experiment VI). Nevertheless, the hydrolysate obtained from the combination step of acid pretreatment and enzymatic hydrolysis in Experiment II required alternative treatment prior used as a substrate. These results indicate that hydrolysate from acid pretreatment of corncob can use as a substrate after detoxify by overliming process like wheat bran [25]. However, this hydrolysate was not proper for *C. beijerinckii* TISTR1461 after hydrolysed further with cellulase enzyme as shown in the Experiment II which is not found in barley straw [6], corn fiber [26], and wheat straw [3]. To investigate whether or not the negative effect came from the cellulase enzyme (such as a preservative), SSF was applied to Experiment V with the initial addition of

20 g/L glucose to support bacterial growth. The evidence of SSF was proven by the high production of ABE since the initial total sugars of 20 g/L should supply 6 g/L approximately of ABE (at yield of 0.30) but Experiment V contained 11.89 g/L of total ABE at 72 h of fermentation where the additional sugars from enzymatic saccharification should be used when the initial sugars ran out. The SSF step further investigated the condition used to enhance the ABE production and enzymatic hydrolysis with the applied conditions of 37 °C incubation and initial pH adjustment to 6.6 which was preferred for the bacterial growth while the proper temperature and pH of enzymatic hydrolysis was 50 °C and pH 4.8 [3].

Table 5.5 Butanol production from dilute acid and enzymatic hydrolysis of corncob by *C. beijerinckii* TISTR1461

Experiment	Hydrolysate			Initial total sugars (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	ABE Yield (g ABE/g utilized sugars)
	Dilute sulfuric	Enzymatic hydrolysis	Overliming					
I	+	+	-	46.29 ± 1.01	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.00
II	+	+	+	41.66 ± 2.28	ND	0.14 ± 0.00	ND	0.00
III	+	-	-	23.93 ± 1.15	ND	ND	ND	0.00
IV	+	-	+	20.45 ± 1.88	1.94 ± 0.15	3.20 ± 0.27	0.07 ± 0.00	0.26
V (SSF)	+	+	-	20.33 ± 0.69	5.62 ± 0.12	6.11 ± 0.05	0.16 ± 0.00	ND
VI	Control (Glucose)			41.97 ± 0.58	2.44 ± 0.18	7.15 ± 0.43	0.22 ± 0.00	0.28

ND: not detected

5.5 Conclusions

In this work, dilute acid hydrolysis pretreatment was carried out to break down the structure of the corncob in order to enhance enzymatic hydrolysis and to maximize sugar conversion by RSM. Our study shows that the mainly factor that effect the process optimization is a concentration of sulfuric acid while a temperature range of 100–140 °C has a minor effect. It can be concluded that the hydrolysate from acid pretreatment of corncob, after detoxified by overliming process, was promised to use as a substrate. Moreover, this research shows the possibility of butanol production from fermentable sugars found in corncob using SSF since it reduces time of enzymatic saccharification and does not require the detoxification process. The further work should focus on the optimization of conditions for SSF.

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5.7 References

1. Gabhane J, William SPMP, Vaidya AN, Mahapatra K, Chakrabarti T (2011) Influence of heating source on the efficacy of lignocellulosic pretreatment - A cellulosic ethanol perspective. Biomass Bioenerg 35:96–102
2. Chen WH, Pen BL, Yu CT, Hwang WS (2011) Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. Bioresource Technol 102:2916–2924
3. Qureshi N, Saha BC, Hector RE, Hughes SR, Cotta MA (2008) Butanol production from wheat straw by simultaneous saccharification and fermentation using Clostridium beijerinckii: Part I—Batch fermentation. Biomass Bioenerg 32:

168–175

4. Ghosh T (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268
5. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
6. Qureshi N, Saha BC, Dien B, Hector RE, Cotta MA (2010) Production of butanol (a biofuel) from agricultural residues: Part I – Use of barley straw hydrolysate. Biomass Bioenerg 34:559–565
7. Liu Z, Ying Y, Li F, Ma C, Xu P (2010) Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. J Ind Microbiol Biot 37:495–501
8. Qureshi N, Blaschek H (1999) Butanol recovery from model solution/fermentation broth by pervaporation: evaluation of membrane performance. Biomass Bioenerg 17:175–184
9. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. U.S. Department of Energy, Colorado
10. Segal L, Creely JJ, Martin AE, Conrad CM (1959) An empirical method for estimating the degree of crystallinity of native cellulose using the X-Ray diffractometer. Text Res J 29(10):786–794
11. McIntosh S, Vancov T (2010) Enhanced enzyme saccharification of Sorghum bicolor straw using dilute alkali pretreatment. Bioresource Technol 101:6718–6727
12. Schell DJ, Farmer J, Newman M, McMillan JD (2003) Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. Appl Biochem Biotech 105:69–85
13. Wang GS, Lee JW, Zhu JY, Jeffries TW (2010) Dilute acid Pretreatment of corncob for efficient sugar production. Appl Biochem Biotech 163:658–668
14. Redding AP, Wang Z, Keshwani DR, Cheng JJ (2011) High temperature dilute acid pretreatment of coastal Bermuda grass for enzymatic hydrolysis. Bioresource Technol 102:1415–1424
15. Carrasco F, Roy C (1992) Kinetic study of dilute-acid prehydrolysis of xylan-containing biomass. Wood Sci Technol 26:189–208
16. Baugh KD, McCarty PL (1988) Thermochemical pretreatment of lignocellulose

to enhance methane fermentation: I. Monosaccharide and furfurals hydrothermal decomposition and product formation rates. Biotechnol Bioeng 31:50–61

17. Esteghlalian A, Hashimoto A, Fenske J, Penner M (1997) Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. Bioresource Technol 59:129–136
18. Lloyd TA, Wyman CE (2005) Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresource Technol 96:1967–1977
19. Kostic TS, Stojkov NJ, Janjic MM, Maric D, Andric SA (2008) The adaptive response of adult rat Leydig cells to repeated immobilization stress: The role of protein kinase A and steroidogenic acute regulatory protein. Stress 11:370–380
20. Pejic BM, Kostic MM, Skundric PD, Praskalo JZ (2008) The effects of hemicelluloses and lignin removal on water uptake behavior of hemp fibers. Bioresource Technol 99:7152–7159
21. Mosier N (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol 96:673–686
22. Hsu TC, Guo GL, Chen WH, Hwang WS (2010) Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresource Technol 101:4907–4913
23. Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. Bioresource Technol 101:4900–4906
24. Saha BC, Cotta MA (2010) Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol. New Biotechnol 27:10–16
25. Liu Z, Ying Y, Li F, Ma C, Xu P (2010) Butanol production by Clostridium beijerinckii ATCC 55025 from wheat bran. J Ind Microbiol Biot 37:495–501
26. Qureshi N, Ezeji TC, Ebener J, Dien BS, Cotta MA, Blaschek HP (2008) Butanol production by Clostridium beijerinckii. Part I: Use of acid and enzyme hydrolyzed corn fiber. Bioresource Technol 99:5915–5922