CHAPTER IV
RESULTS AND DISCUSSION

4.1 Chemical Composition of Corncobs

Corncobs is one of the most agricultural waste in Thailand that is mainly consisted of cellulose, hemicellulose, and lignin. Cellulose is the homopolymer of glucose. It is present in both crystalline and amorphous forms. The long–chain cellulose polymers are packed into microfibrils by hydrogen and van der waals bonds. The microfibrils are covered by hemicelluloses and lignin. Therefore, cellulose can obtain a crystalline structure highly resisting to attack by enzymes. Hemicellulose is mainly composed of five carbon sugars such as xylose and arabinose, and six carbon sugars such as glucose, mannose, and galactose. The hemicellulose structure is lack of crystalline structure due to the highly branched structure and amorphous forms that are easy to hydrolysis. Lignin is a very complex polymer which consists of three types of phenolic acids: *p*–coumaryl alcohol, coniferyl alcohol, and synapyl alcohol that links in a three dimensional structure making lignin difficult to hydrolyze.

In this research, corncobs were received from Betagro Corporation Limited, Thailand, the main component was 39.31 % cellulose, 34.46 % hemicellulose, and 10.47 % lignin, as shown in Table 4.1. The others may include some organic compounds (uronic acid and acetyl groups) and other trace components such as minerals, waxes, fats, starches, resins, and gums (Wang *et al.*, 2011).

Table 4.1 Chemical composition of corncobs

Composition	Dry solid (%, w/w)
Cellulose	39.31
Hemicellulose	34.46
Lignin	10.47
Others	15.76

4.2 Optimization of the Glucose Concentration using Response Surface Methodology (RSM)

The first stage pretreatment was carried using microwave/NaOH pretreatment and 2 % NaOH was optimized from the previous work and the conditions were as follows: 100 °C for 30 min and 67:1 SLR (Ploypradith, 2010). In this condition, the highest glucose concentration can reach up to 32.53 g/l and total sugar 45.60 g/l was released. In this research, The second stage of two–stage pretreatment was employed to enhance the efficiency of pretreatment. To simplify the experiment, three variables in second stage pretreatment–temperature, time, and solid loading–were identified as the most significant variables with a range of 80 to 160 °C, 5 to 25 min, and 25 to 125 SLR, respectively. After two–stage pretreatment, the enzymatic hydrolysis was carried out. Enzymes were loaded in the amount of 52 PFU/g substrate for cellulase (celluclast). The mixture (30 ml) was placed into a 125 ml flask and incubated 50 °C for 60 h.

RSM with a central composite design (CCD) was conducted to examine the effect of temperature, time, and SLR of second stage pretreatment on glucose concentration. The experimental design and results of CCD are summarized in Table 4.2.

Table 4.2 Experimental design and results of the central composite design of second stage of two–stage pretreatment

Run	Variables			Response
	Temp. (°C)	Time (min)	SLR (g/l)	Glucose concentration (g/l)
1	140	20	100	45.13
2	140	20	50	31.28
3	140	10	100	47.16
4	140	10	50	27.88
5	100	20	50	21.81
6	100	20	100	32.83
7	100	10	100	37.91
8	100	10	50	24.49
9	160	15	75	37.52
10	80	15	75	30.39
11	120	25	75	28.92
12	120	5	75	30.90
13	120	15	125	35.62
14	120	15	25	14.43
15	120	15	75	37.67
16	120	15	75	40.68
17	120	15	75	38.04
18	120	15	75	38.70
19	120	15	75	37.67
20	120	15	75	37.70

The polynomial equation explains the glucose concentration of second stage pretreatment (Y_1) as a function of temperature, time, and SLR of two–stage pretreatment is shown in the equation below:

$$Y_1 = 39.05 + 2.9625x_1 - 0.2131x_2 + 6.43x_3 - 1.2292x_1^2 - 1.9723x_2^2 - 3.2792x_3^2 + 0.93\ x_1x_2 + x_1x_3 - 0.5075x_2x_3$$

Where x_1 is the second stage pretreatment temperature (°C), x_2 is the second stage pretreatment time (min), and x_3 is the second stage pretreatment SLR (g/l)

Table 4.3 summarizes the statistics for regression including regression coefficient, standard error, t-value, and P-value. The results show the linear coefficients (a_1 and a_3), the quadratic coefficients (a_{11}, a_{22}, and a_{33}) are all significant factors because P-value is very low, which imply that these coefficients in the model significantly influence glucose concentration, whereas coefficient of a_2, a_{12}, a_{13}, and a_{23} do not affect glucose concentration in the study range. In the other word, the temperature and SLR are the most significant factors on the concentration of glucose. The t–test value indicates the significance of the regression coefficient (Wang and Blaschek, 2011).

The quality of the regression was evaluated by Analysis of variance (ANOVA) of Fisher's statistical test, as shown in Table 4.4. The regression statistics indicated that the model represented an accurate representation of the experimental data. The computed F–value (8.6513) is much greater than $F_{0.05,5,23}$ (2.64). In addition, the small P–value for the regression in Table 4.4 also implied the model adequacy.

Table 4.3 Statistics for regression of the optimization model

Coefficients	Value	Standard error	t-value	P-values
a_0	39.0536	0.8602	45.401	$< 2\times10^{-16}$
a_1	2.9625	0.3589	8.254	2.50×10^{-8}
a_2	-0.2131	0.3589	-0.594	0.55845
a_3	6.43	0.3589	17.915	5.26×10^{-15}
a_{12}	0.93	0.5076	1.832	0.07991
a_{13}	1	0.5076	1.97	0.06099
a_{23}	-0.5075	0.5076	-1	0.32781
a_{11}	-1.2292	0.34	-3.615	0.00146
a_{22}	-1.9723	0.34	-5.8	6.56×10^{-6}
a_{33}	-3.2792	0.34	-9.644	1.51×10^{-9}

Table 4.4 ANOVA for the regression

Source of variation	Degree of freedom	Sum of squares	Mean square	F–value	Prob (>F)
Residuals	23	94.82	4.12		
Lack of fit	5	66.96	13.39	8.6513	0.000254
Pure error	18	27.86	1.55		
$R^2 = 0.96$					

The three–dimensional response surface and two–dimensional contour plots for the concentration of glucose are shown in Figures 4.1 and 4.2, respectively. The optimization of temperature, time, and solid loading of second stage pretreatment for glucose concentration were determined at 156 °C, 16 min, and 106 SLR, respectively, which is calculated by setting the partial derivatives of polynomial equation to zero with respect to the corresponding variables. The predicted maximum glucose concentration from the model was 45.66 g/l while from the confirmation experiment the glucose concentration 48.58 g/l and 78.71 g/l of total sugar including glucose, xylose, and arabinose were obtained at the same condition, as shown in Figure 4.3. This result confirmed that the experimental result was in agreement with the model prediction. Figure 4.1 (A) to (C) presents the relative effects of two variables on glucose concentration with the third maintain constant. The interactive effect between temperature and SLR is important at high temperature and high SLR due to more substrate or low dilution of mixture to produce glucose (Cara *et al.*, ; Kim and Lee, 2007). In addition, the high temperature is a technically feasible way to disrupt the biomass structure and resulted in more enzyme accessibility in enzymatic hydrolysis thus glucose concentration will be increased. Nevertheless, At high temperature and long reaction time, carbohydrates are degraded into other compounds, such as furfural and HMF generated in acid condition, resulted in total sugar reduction (Redding *et al.*, 2011). Besides, relatively flat response surface is shown in Figure 4.1 (A), indicating the effect of temperature and time is lower than that of SLR.

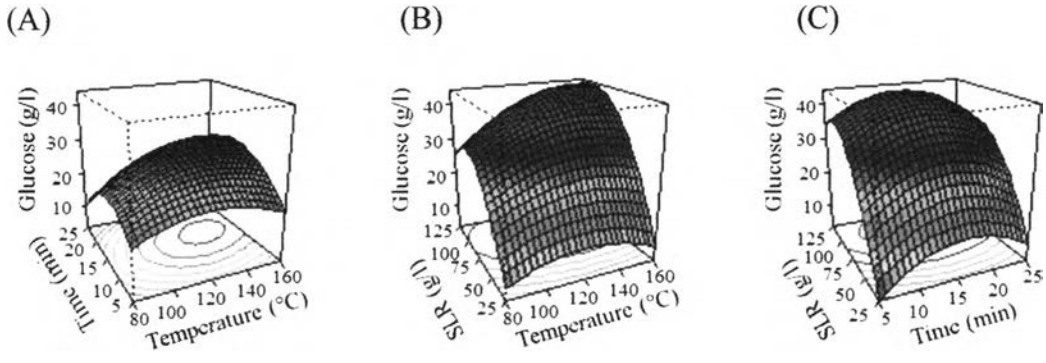

Figure 4.1 Response surface for glucose concentration: effects of temperature and time (A), temperature and SLR (B), and time and SLR (C).

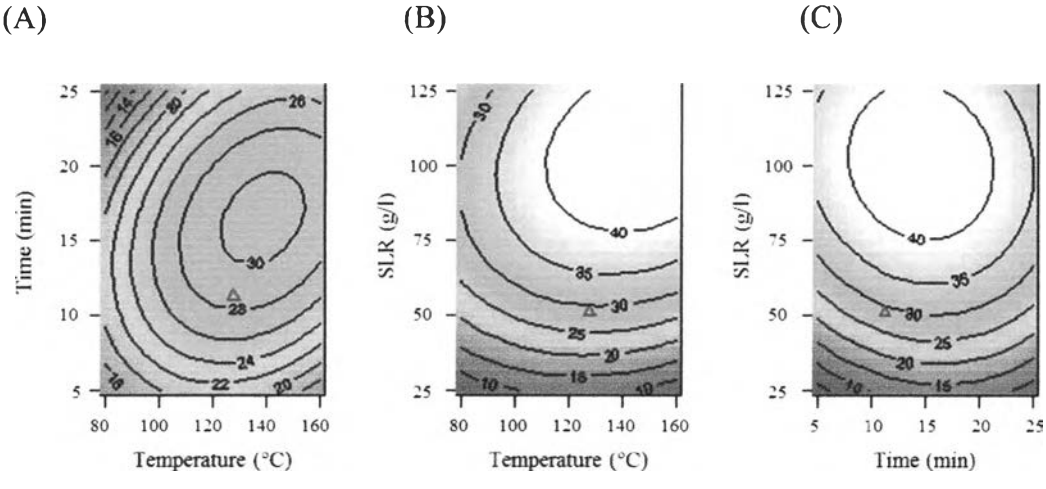

Figure 4.2 Contour plots for glucose concentration: effects of temperature and time (A), temperature and SLR (B), and time and SLR (C).

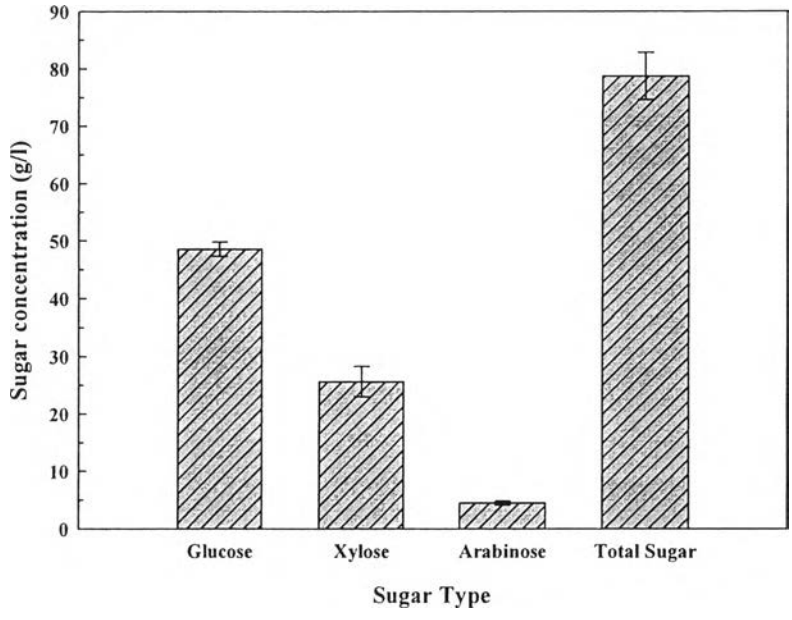

Figure 4.3 The glucose, xylose, arabinose, and total sugar concentration from optimal two–stage pretreatment conditions.

4.3 The Effect of Pretreatment on Chemical Composition of Corncobs

The untreated corncobs used in this research was composed of 39.31 % cellulose, 34.46 % hemicellulose, 10.47 % lignin, and others. As seen in Table 4.5, The Microwave/NaOH or first stage pretreatment decreased lignin to 3.98 %. Lignin is a major effect of NaOH pretreatment to remove lignin from lignocellulosic biomass in order to improve the enzymatic efficiency due to lignin inhibits enzymatic hydrolysis. After that, The microwave/NaOH followed by microwave/H_2SO_4 or two–stage pretreatment was applied. That reduced hemicellulose to 0.48 % because acid pretreatment solubilizes hemicellulose from solid residual in soluble form (Binod *et al.*, 2012). In addition, two–stage pretreatment increased cellulose content to 88.74 % that readily to hydrolize into sugars. The microwave/NaOH followed by water pretreatment decreased hemicellulose to 17.37 %; therefore, this can be concluded that H_2SO_4 can significantiy improve hemicellulose solubilization more than water.

Table 4.5 Composition of corncobs before and after pretreatment

Method	Chemical Composition (%)		
	Cellulose	Hemicellulose	Lignin
Untreated	39.31	34.46	10.47
Microwave/NaOH	75.54	19.37	3.98
Microwave/NaOH followed by H_2SO_4	88.74	0.48	9.13
Microwave/NaOH followed by water	77.48	17.37	4.27

4.4 Surface Morphology by Scanning Electron Microscope (SEM)

The images of scanning electron microscope (SEM) of the untreated and the pretreated corncobs in different techniques are shown in Figure 4.4. Figure 4.4 (A) shows the SEM image of the untreated corncobs has smooth and continuous surface that some pore was observed. While, applying 2 % NaOH pretreatment at 100 °C for 30 min, residual lignin was eliminated, as shown in Figure 4.4 (B). The structure was damaged like a sieve or hole at the surface. This indicates that microwave/NaOH pretreatment removed external fibers which increase surface area. Moreover, after the samples were pretreated by microwave/NaOH followed by H_2SO_4 at 156 °C for 16 min, as shown in Figure 4.4 (C), the structure was loose and irregular and has a very rough surface because it is easy to damage and solubilize hemicellulose due to the loss of lignin in first stage. The image of sample pretreated by microwave/NaOH followed by water pretreatment is shown in Figure 4.4 (D). The SEM results show that the surface of sample pretreated by microwave/NaOH followed by water changed less radically than two–stage pretreatment that used H_2SO_4, indicating that H_2SO_4 can improve the pretreatment efficiency. In addition, it increases the solubilization of hemicellulose; therefore, cellulose becomes more accessible to the enzymes and gives high reducing sugar.

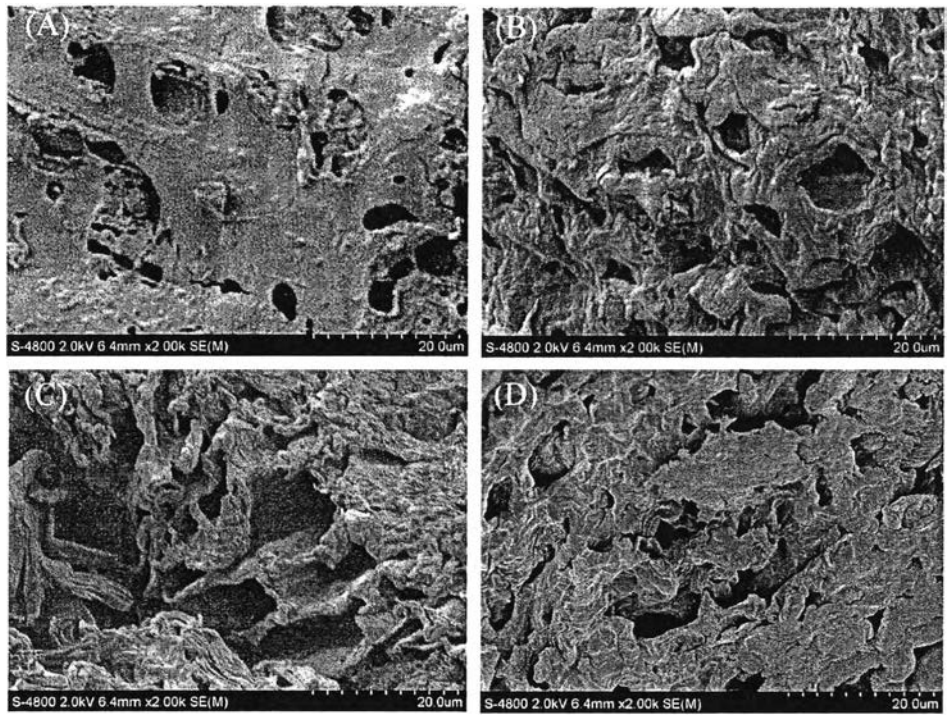

Figure 4.4 SEM images of untreated corncobs (A), pretreated corncobs with microwave/NaOH (B), microwave/NaOH followed by H_2SO_4 (C), and microwave/NaOH followed by water (D).

4.5 BET Surface Area

From the SEM images, pretreated corncobs structure was featured by fragmentation and swelling. The fragmentation released small components and enlarged surface area of biomass. On the other hand, the swelling behavior reduced the surface area and scooped the particles interior (Chen *et al.*, 2011). From Table 4.6, the surface area of sample pretreated by microwave/NaOH pretreatment was increased from 3.8 to 4.1 m^2/g, it is likely that the increase in surface area caused by fragmentation. The fragmentation increased the surface area of corncobs due to the more small particles formation. In contrast, the surface area of sample pretreated by microwave/NaOH followed by H_2SO_4 pretreatment was reduced from 4.1 to 2.0 m^2/g because of swelling behavior that decreased the surface area of corncobs and average pore diameter was increased from 55.1 to 111.5 Å. The small

holes merged into large holes was observed and resulted in the inside of the particles scooped. Similarly, Chen et al. (2011) employed microwave radiation and dilute H_2SO_4 1.56 % to pretreat sugarcane bagasse at 190 °C for 10 min and observed a decrease in surface area. The authors suggested that the swelling behavior becomes more drastic.

From the above results, it can be concluded that the optimization of two–stage pretreatment conditions combined with microwave radiation can open the structure of corncobs cell wall that allow cellulase to access the surface area of cellulose microfibrils in order to increase enzymatic hydrolysis digestibility.

Table 4.6 BET surface area and average pore diameter of corncobs

Method	Surface area (m^2/g)	Average pore diameter (Å)
Untreated	3.8	66.7
Microwave/NaOH	4.1	55.1
Microwave/NaOH followed by H_2SO_4	2.0	111.5
Microwave/NaOH followed by water	2.1	69.8

4.6 X–Ray Diffraction Analysis

The crystallinity index (CrI) of cellulose has been used for more than five decades to describe the relative amount of crystalline material in cellulose and to interpret changes in cellulose structure (Park *et al.*, 2010). Biomass crystallinity as expressed by crystallinity index (CrI) was determined according to a method by Segal *et al.* (1959) as follows:

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

where, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about $2\theta = 22.5°$ and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about $2\theta = 18.6°$.

The x-ray diffraction profiles of untreated and pretreated corncobs is shown in Figure 4.5 and the crystallinity index is shown in Table 4.7. The crystallinity index of untreated corncobs was less (22.88 %) compared to other pretreated samples. Microwave/NaOH followed by H_2SO_4 pretreatment gave the highest crystallinity index (82.19 %). In the lignocellulosic biomass structure, cellulose microfibrils are protectively surrounded by hemicellulose and lignin. Therefore, the disruption of hemicellulose and lignin by NaOH and H_2SO_4 pretreatment have opened up the cellulose and cellulose crystallinity can be increased. Bak *et al.*, 2009 found that the crystalline portion of untreated rice straw increased from 54.5 % to 58.0% and 57.2% after pretreatment by electron beam irradiation (EBI) at 80 and 90 kGy, respectively, due to exposure of the crystalline portion of the rice straw in response to pretreatment and cellulose portion became more exposed than the amorphous portion due to the effects of EBI. Moreover, the main reason for increased crystallinity index is solubilization of hemicellulose and lignin together with less ordered cellulose (Xiao *et al.*, 2011). Therefore, amorphous area will decrease when crystallinity index increases, as shown in Table 4.7.

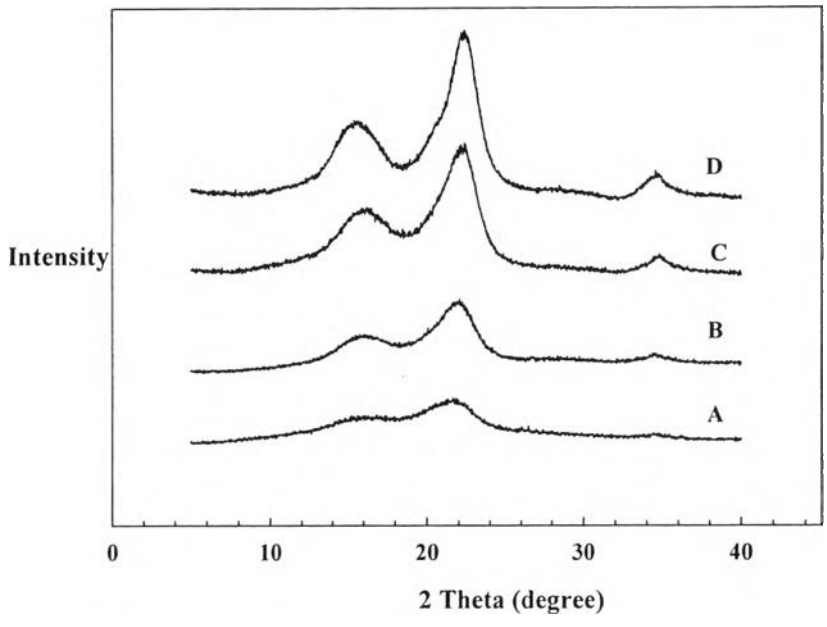

Figure 4.5 XRD patterns of untreated and pretreated corncobs. untreated corncobs (A), microwave/NaOH pretreated (B), microwave/NaOH followed by water pretreated (C), and microwave/NaOH followed by H_2SO_4 pretreated (D).

Table 4.7 Crystallinity index, crystalline area, and amorphous area of corncobs

Method	Crystallinity index (%)	Crystalline area	Amorphous area
Untreated	22.88	2853	7738
Microwave/NaOH	48.26	5630	7688
Microwave/NaOH followed by H_2SO_4	82.19	21439	4645
Microwave/NaOH followed by water	80.41	20049	4885

The lowest value for crystallinity index was found in the case of untreated corncobs which was not subjected to any kind of chemical pretreatment; therefore, the amorphous area including hemicellulose and lignin was higher than pretreated samples. The microwave/NaOH pretreatment sample that has low amount of lignin due to delignification of alkaline pretreatment so, the amorphous area was slightly decreased compared to untreated sample. In addition, the sample from microwave/NaOH followed by H_2SO_4 pretreatment which both lignin and hemicellulose were eliminated. Hence, the amorphous area was significantly decreased that exposed cellulose accessible to the enzyme and high sugars were produced. And microwave/NaOH followed by water pretreatment sample has amorphous area higher than microwave/NaOH followed by H_2SO_4 pretreatment sample. It is indicated that H_2SO_4 can improve the solubilization of hemicellulose which makes cellulose more accessible to the enzyme. From this results can be concluded that crystallinity is affected by biomass composition. Hemicellulose and lignin are considered to be amorphous while cellulose is considered to be crystalline (Jeoh *et al.*, 2007). Increase in crystallinity can be indicated that pretreatment was effective due to the removing of hemicellulose and lignin expose all crystalline cellulose available and increase the rate of enzymatic hydrolysis (Bak *et al.*, 2009, Chang and Holtzapple, 2002, Gabhane *et al.*, 2011, Kim and Holtzapple, 2006, Liu *et al.*, 2009).

4.7 Effect of Fermentation Techniques and Fermentation Time on the Acetone–Butanol–Ethanol (ABE) Concentration

After two–stage pretreatment and enzymatic hydrolysis, the liquid fraction was sent to the fermentation step to produce Acetone–Butanol–Ethanol (ABE) using *Clostridium beijerinckii* TISTRI1461. In this research, there are 7 fermentation experiments using 10 ml hydrolysate as a substrate at 37 °C, as shown in Table 4.8. The result showed that the highest ABE concentration of all fermentation techniques was obtained at 48 h of fermentation time, as shown in Figure 4.6.

Table 4.8 ABE fermentation techniques

Fermentation techniques	Description
C (Control)	2–stage pretreatment without dilution and overliming
CO	2–stage pretreatment + Overliming
D2	2–stage pretreatment + Diluted 2 times
D2O	2–stage pretreatment + Overliming + Diluted 2 times
D4	2–stage pretreatment + Diluted 4 times
D4O	2–stage pretreatment + Overliming + Diluted 4 times
W	Microwave/NaOH followed by water pretreatment

From Figure 4.6, The amount of ABE concentration in 7 fermentation techniques are less than that observed in a P2 medium batch fermentation which contained 40 g/l synthetic glucose and nutrients without inhibitors. Moreover, ABE cannot produce in the control experiment (C), which is the hydrolysate obtained from two–stage pretreatment without dilution and overliming process. The results show that the best condition for conditioning step of hydrolysate is the diluted 4 times hydrolysate combined with overliming (D4O) technique at 48 h. The level of ABE concentration is improved to 8.43 g/l, which promising the substitution of hydrolysate to P2 medium.

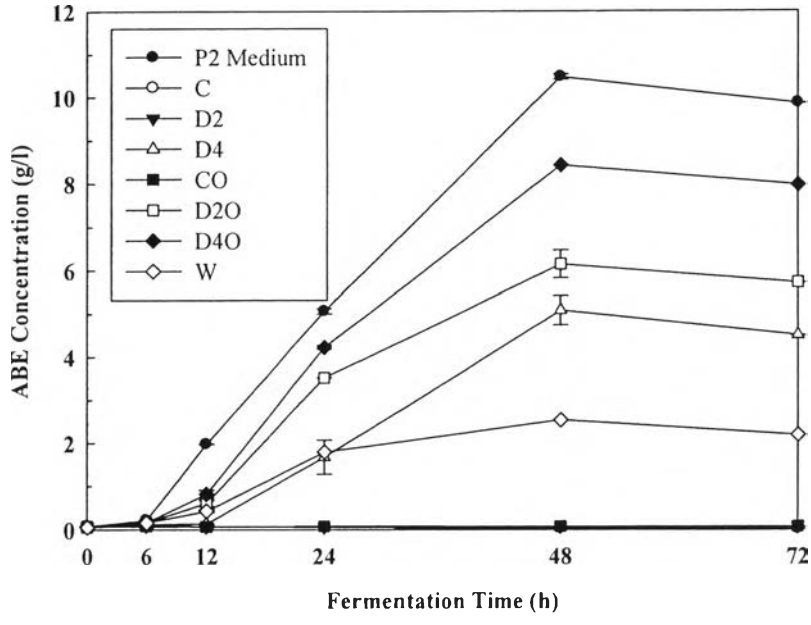

Figure 4.6 The effect of fermentation time on ABE concentration.

The overliming process performed by adding $Ca(OH)_2$ to adjust the pH of the hydrolysate to a high pH range and is heated to a temperature range of 90 °C. The purpose of the present work was to determine the appropriate conditioning step. The results showed that the overlimed hydrolysate conditioned gave ABE yield higher than non overlimed hydrolysate, as shown in Figure 4.7. It indicated that overliming process positively affects the ABE yield due to the removal of inhibitors that highly toxic to microorganism. As a result, overliming treatment can significantly improve the ABE concentration. Therefore, The removal of inhibitors prior to fermentation is essential for successful ABE fermentation. Normally, higher initial sugar concentrations cannot be consumed owing to butanol toxicity (Qureshi and Maddox, 2005). Therefore, the dilution is the necessary step to increase ABE yield in fermentation. The results showed that, the dilution of hydrolysate also improved ABE yield because the diluted hydrolysate highly reduced fermentation inhibitors and 4 times dilution gave the ABE yield higher than 2 times dilution.

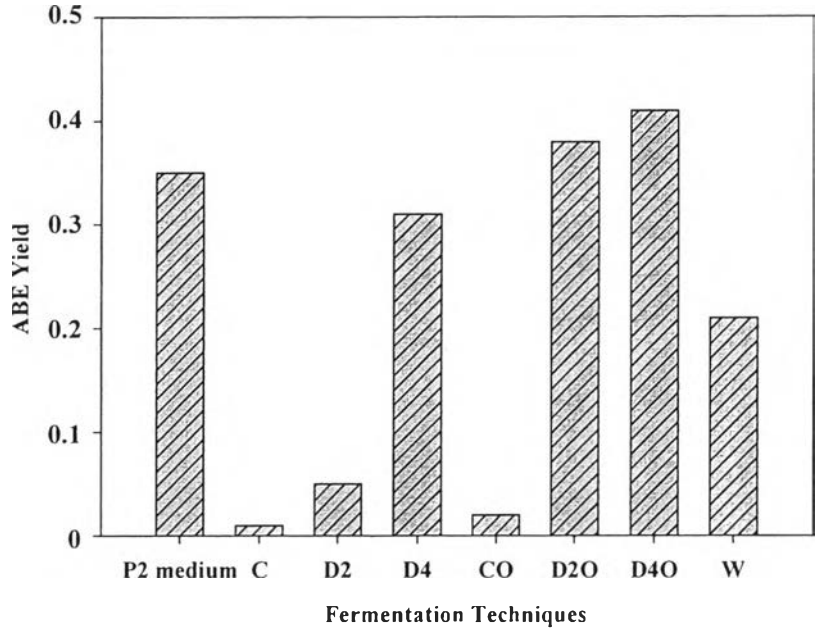

Figure 4.7 The effect of fermentation techniques on ABE yield at 37 °C for 48 h.

Furthermore, the diluted 4 times hydrolysate combined with overliming (D4O) gave the highest ABE yield, 0.41, and % productivity, 17.56 g/l·h, compared to other techniques, as shown in Table 4.9. It can be concluded that overliming and dilution enhanced the ABE yield. ABE yield was calculated as total ABE produced divided by the total sugar utilized and productivity was calculated as total ABE produced divided by fermentation time. In addition, D4O technique gave the highest total sugar which consumed in fermentation process. It revealed that microorganism can utilize high amount of total sugar in order to produce high ABE yield. The unconsumed total sugar was monitored due to toxicity in fermentation process (Qureshi et al., 1988).

Table 4.9 The effect of fermentation techniques on ABE yield and productivity at 48 h

	Fermentation Techniques							
	P2 medium	C	D2	D4	CO	D2O	D4O	W
ABE Yield	0.35	0.01	0.05	0.31	0.02	0.38	0.41	0.21
ABE/Sugars (g/g)	0.27	2.3×10^{-4}	1.7×10^{-3}	0.19	1.0×10^{-3}	0.14	1.19	0.06
% Productivity (g/l·h)	21.82	0.04	0.16	10.59	0.15	12.80	17.56	5.29
Utilized sugars (g/l)	29.81	1.12	1.66	16.45	3.43	15.95	20.70	11.86
Remaining sugars (g/l)	8.75	68.95	43.22	9.94	69.29	27.43	7.08	52.17

The optimum technique that could be used in ABE fermentation was D4O technique at 37 °C for 48 h, that gave the highest ABE yield of 0.41, and % productivity of 17.56 g/l·h. The dilution and overliming process can reduce fermentation inhibitors, increase cell growth and improve ABE yield. The ABE yield from D4O technique was higher than that P2 medium batch fermentation that contained 0.35 ABE yield. It implied that pretreated corncobs can be used as a carbon source in ABE fermentation. Moreover, it can reduce the cost of the ABE process by using corncobs instead of food crops such as corn, cassava and sugarcane that have a high price and caused food price to go up due to the high demand of food crops. Furthermore, D4O technique can produce the highest g of ABE from 1 g of sugars. Therefore, the results can be concluded that using pretreated corncobs as a substrate with overliming and 4 times dilution of hydrolysate at 48 h of fermentation time can enhance ABE fermentation efficiency compared to control batch which gave lower ABE. However, the microorganisms cannot utilize corncobs directly. Therefore, pretreatment process is an important step to produce reducing sugar from corncobs.