CHAPTER IV
RESULTS AND DISCUSSION

4.1 Abstract

Biobutanol can be produced by Acetone–Butanol–Ethanol (ABE) fermentation of lignocellulosic biomass which can be converted into fermentable sugar such as glucose and xylose. The complex structure of lignocellulosic biomass minimizes the enzymatic hydrolysis accessibility. Therefore, a pretreatment step is necessary to enhance enzymatic accessibility by removing lignin and/or hemicellulose, increasing the biomass porosity or reducing the cellulose crystallinity. The purpose of this work is to optimize the conditions of a combined pretreatment of corn cobs using microwave and sodium hydroxide (0.75 % to 3 % (w/v)) in the temperature range of 60 to 120°C. The pretreated corn cobs were then subjected to enzymatic hydrolysis at 50 °C for 48 hours to produce the reducing sugar prior to ABE fermentation. The highest reducing sugar concentration of 45.6 g/L was obtained from the pretreated corn cobs with 2% of sodium hydroxide at 100 °C for 30 minutes. The results indicate that microwave–assisted alkali treatment was an efficient way to improve the enzymatic hydrolysis accessibility of corn cobs.

Keywords: Corn Cobs/ Microwave Technique/ Enzymatic Hydrolysis/ Alkali Pretreatment

4.2 Introduction

Thailand has had one of the fastest growing economies in Southeast Asia and fuel consumption is rising with an average of 5% per year. Therefore, the renewable energy has become one of possible method for these problems. Corn cobs is one of agricultural waste which can be produced in Thailand approximately 4 million tons annually. In addition, butanol is being an important biofuel which can be produced by fermentation of biomass at the present. However, the conversion of corn cobs into fermentable sugar is difficult because of many enzymatic hydrolysis limiting factors.

The main factors that affect to enzymatic hydrolysis are the crystallinity of cellulose, substrates available area, and the presence of lignin and hemicelluloses [1]. Owing to these factors, pretreatment is the important step to prepare the materials for enzymatic degradation. Different pretreatment method has different effect; therefore, the appropriate pretreatment method and conditions depend on the type of agricultural wastes or lignocellulosic biomass.

There are several pretreatment methods which can be classified into biological, physical, chemical, and physico-chemical pretreatments [2]. Alkali pretreatment, one of chemical pretreatment, can remove lignin and a part of the hemicelluloses which result in increase the accessibility of enzyme to the cellulose [3]. Alkali pretreatment can be operated at low temperature even ambient condition but need long time and high concentration of base. However, lower temperature is more effective because some useful component in lignocellulosic biomass might be decomposed at high temperature [4]. Microwave-based pretreatment might be an alternative pretreatment method to operate at low temperature. Among alkali, sodium hydroxide is a suitable chemical reagent for combining with microwave pretreatment [5].

The purpose of this work is to optimize the condition of combination pretreatment of corn cobs using microwave and sodium hydroxide (0.75 % to 3 % (w/v)) at the temperature range of 60 to 120°C for 5, 10, 20, and 30 minutes that give the highest amount of reducing sugar produced from enzymatic hydrolysis of corn cobs. In addition, the structure of the corn cobs before and after pretreatment was also investigated.

4.3 Experimental

4.3.1 Materials

Corn cobs was obtained from B&C Pulaski Corporation Limited, Thailand. The collected corn cobs was stored at the ambient room temperature in a large bag. The particle size of corn cobs around 1.6 mm homogenized in a single lot and stored until used.

4.3.2 Enzyme Assays

The enzymatic activity was determined according to method developed by Ghose (1987). The substrate (Carboxymethyl cellulase sodium salt for cellulase) 320 μl was added in sample, blank, and standard tube. The sample tubes were heated in a heating box at 50 °C for 10 minute and then added with enzyme samples at 50 °C. Glucose solution (0, 1, 2, 3, 4, 5 mM) was added in standard tubes. After 30 minutes, DNS (dinitrosalicylic acid) reagent 680 μl was added to all tubes. The enzyme samples were also added in the blank tube. All tubes were boiled in water at 100 °C for 5 minutes. The tubes were immediately transferred to cold water. UV-VIS Spectrometer (Thermo Fisher Scientific Inc., USA) at 550 nm was used to measure the color absorbed. The color formed in the blank tube is subtracted from that of the sample tube and translated the absorbance of the sample tube into glucose production during the reaction using standard curve. The sample tube was measured 2 times and the average values were reported. The glucose solution calibration curve was used to calculate the activity of cellulase.

4.3.3 Alkali Pretreatment

A CEM MAR–5 HP–500 microwave system (Matthews, NC, USA) was used in this study for combination of microwave and alkali pretreatment. This process was carried out as follows: 2 g of corn cobs was suspended in 30 mL of different sodium hydroxide concentrations (0.75 % to 3 %) and then transferred to a microwave oven to treat corn cobs at the designed temperature (60 °C to 120 °C) from 5 to 30 minutes. After this process was completed, the residues would be collected by filter paper then washed with tap water until reaching neutral pH, dried at 65 °C and weighted [4].

4.3.4 Enzymatic Hydrolysis

Hydrolysis experiment consists of 0.5 g of pretreated corn cobs and 15 mL of 0.1 mol/L citrate buffer (pH 4.8). The mixture was added with 0.1 mL of enzymes which supported from Novozyme and the activity was 2889.27 unit/mL, and then the temperature was adjusted at 50 °C in the incubator shaker at 150 rpm for 48 hours. The sample which taken from the hydrolysis solution was heated to 100 °C immediately for 3 minutes to denature the enzymes, cooled to room temperature, and then centrifuged for 20 minutes at 8000 rpm [4]. Then, the sample from the reaction was stored for sugar analysis.

4.3.5 Fermentation

2 mL of active yeast (*Saccharomyces cerevisiae*) was mixed with 20 ml of sugar solution from enzymatic hydrolysis step and then transfered to incubator shaker at 37 °C for 1 day to 3 days. After that, the solution was collected to analyze the ethanol concentration by a GC instrument.

4.3.6 Analytical Methods

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), and acid insoluble ash (AIA) of corn cobs before and after pretreatment were determined by the Nakhonratchasima Animal Nutrition Research and Development Center (Nakhonratchasima province, Thailand). The difference between NDF and ADF estimates detergent hemicellulose. Detergent cellulose is calculated by subtracting the values for (ADL + AIA) from ADF.

Monosaccharide such as glucose, xylose, arabinose, mannose, galactose, and cellubiose was determined using an HPLC system equipped with an organic acid column (Aminex HPX- 87H column, Bio-Rad Lab, USA) and a refractive index detector (Model 6040 XR, Spectra-Physics, USA). 0.005M sulfuric acid solution was used as a the mobile phase at a flow rate of 0.6 mL minute^{-1} and the column temperature was fixed at 65 °C.

X-ray diffraction (XRD) was used to identify crystallinity present in both untreated and pretreated corn cobs by using a Rigaku/Rint2200 diffractometer equipped with a Ni filtered CuKα radiation source (λ = 1.542 Å) of 40 kV and 30 mV. The sample was pressed into a hollow of glass holder and held in place by glass window. Then, it will be scanned in the 2θ range of 0 to 40° in the continuous mode with the rate of 1°/minute. Biomass crystallinity as expressed by crystallinity index (CrI) was determined according to a method by [7]:

$$CrI = 100 \times \left[\frac{I_{002} - I_{amorphous}}{I_{002}} \right]$$

In which, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2h = 22.5 and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2h = 16.6.

The sample both untreated and treated corn cobs were observed by a scanning electron microscope (SEM) using a Hitachi S-4800 microscope. Prior to acquiring images, the samples were mounted with double sided carbon tape on precut brass sample stubs and sputter coated with approximately 30 Å of Au/Pd. The representative images of both untreated and treated corn cobs reported here were acquired with a 15 kV accelerating voltage [8].

BET surface areas of corn cobs before and after pretreatment were measured by N_2 adsorption/desorption measurements (BELSORP-max, BEL Japan INC., Japan) done at 196 °C. The dried sample (0.5–1 g) was put into the sample tube and degassed using a vacuum for 4 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves.

4.4 Results and Discussion

4.4.1 Chemical Composition of Untreated Corn Cobs

CHON was analyzed according to ASTM D 5373-02 and ASTM D 5291-02 method by using a Perkin-Elmer 2400 Series II CHNS/O Analyzer. The result is shown in the table 4.1

Table 4.1 Elemental of corn cobs

Elements	**%**
Carbon	44.00
Hydrogen	7.00
Oxygen	47.00
Nitrogen	0.40
Trace elements	1.60
Total	100.00

Corn cobs show the potential to be a renewable energy source. The most direct route for energy recovery from biomass is direct combustion. Although it is considered to be a method that utilizes the total energy content of whole biomass, it cannot be expected to be an economically and practically competitive with natural gas or other fuels derived from petroleum. Because the original biomass form has lower density comparing with many solid fuels such as coals. The calorific value of corn cobs is about 16–20 MJ/kg which is approximately half that of lignite and one–quarter that of bituminous coal. Moreover, effective fuels should be in the forms that can be used in conventional engines such as liquid fuels. Therefore, this research is interested in pretreatment of corn cobs for ethanol production.

4.4.2 Thermal gravimetric analysis (TGA)

Thermal decomposition of biomass was investigated by using thermal gravimetric analysis TG-DTG, Perkin Elmer/Pyris Diamond, the result is shown in Figure 4.1.

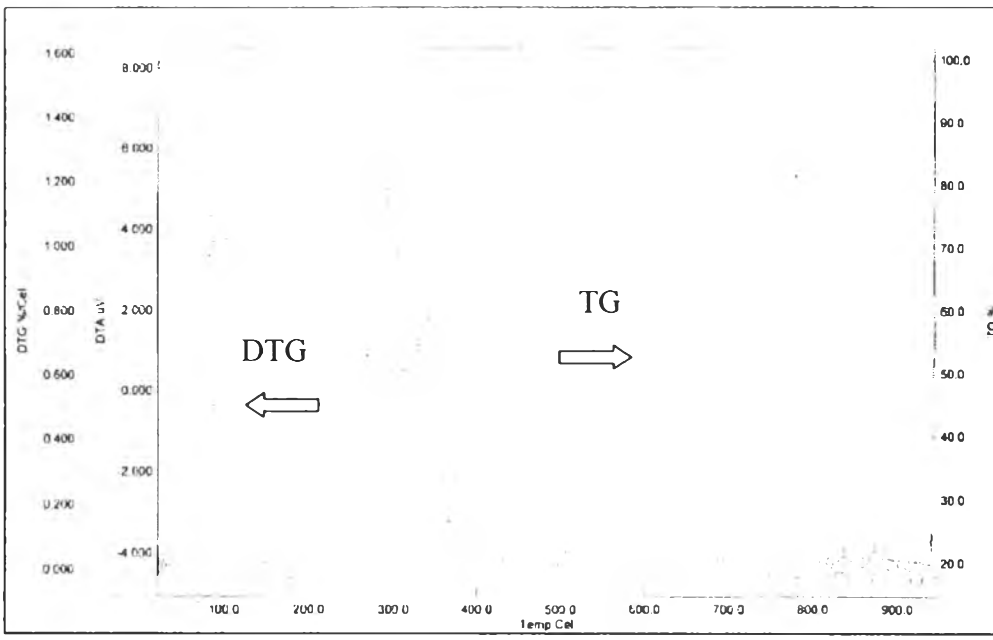


Figure 4.1 TG-DTG curves of corn cobs.

DTA and TGA analyses from room temperature to 1000 °C indicate two main endotherms and mass loss regions, as shown in Figure. 4.1. The first endothermic reaction accompanied by 5% mass loss occurs at about 100 °C due to moisture removal. A second endotherm follows at about 350 °C, as increases in temperature result in depolymerization. Mass loss up to 80% is achieved at about 600 °C due to the decomposition of cellulose and the loss of organics leads to the decrease in specific heat. As temperature is increased from 600 °C to 800 °C, small mass loss is observed with a decrease in specific heat caused by the removal of residual hydrogen atoms. Mass loss is then stabilized as temperature is further increased to 1000 °C [9].

4.4.3 Chemical Composition of Agricultural Waste

Agricultural waste is essentially composed of cellulose, hemicellulose, lignin, extractives, and ash. Table 4.1 represented major chemical composition of corn cobs. Each of these components contributes to fiber properties, which ultimately impact product properties.

Table 4.2 Chemical composition of corn cobs

Constituents	Dry solid (%, w/w)
Cellulose	42.6
Hemicellulose	39.04
Lignin	7.56

Table 4.2 shows that the biomass contents consisted of 42.6% cellulose, 39.04% hemicelluloses, and 7.59% lignin. The other may include ash, some organic compounds (uronic acid and acetyl groups), and other trace components (minerals, waxes, fats, starches, resin and gums) [10]. Cellulose, the major chemical component on fiber wall and contributing 42.6% of the corn cobs's dry weight, is the most abundant organic compound in nature, and composed of glucose units joined together in the form of the repeating unit of the disaccharide cellobiose with numerous cross linkages. Hemicellulose, contributing 39.04% of the corn cobs's 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), and sugar acid. Lignin, contributing 7.56% of the corn cobs's dry weight, is amorphous heteropolymer consisting of three different phenylpropane unit (p–coumaryl, coniferyl, and sinapyl alcohol), which are held together by different kinds of linkages. The mian propose of having lignin was to provide structural support, impermeability, and resistance against microbial attack and oxidative stress to the plant.

4.4.4 Effects of Sodium Hydroxide Pretreatment with Microwave on Enzymatic Hydrolysis

The major effect of NaOH pretreatment is to remove lignin in biomass. The removal of lignin is the important part because lignin can effectively inhibit the cellulase enzymes to hydrolyzed cellulose and hemicellulose into glucose and xylose, respectively. Thus, to produce higher sugar, we need higher lignin reduction. The weight loss was become important indices for effectiveness of its pretreatment [4].

Table 4.3 shows the weight loss of pretreated corn cobs with NaOH and microwave. The result shown that time, temperature, and NaOH concentration have a significant effect on weight loss. Solid losses ranged from 23% (w/w) under mild pretreatment conditions (0.75% NaOH for 5 minutes at 60 °C) up to 61.75% when the sample was pretreated with 3% NaOH for 30 minutes at 120 °C. Although each variable contributed to solid loss, the temperature was found to be the greatest impact on solid loss.

The solid loss can be accounted for by analysis of isolated component by analysis of isolated components solubilised in the hydrolysates. Aside from the solubilisation of lignins several studies have reported varying amounts of hemicellulose loss following exposure to alkaline substances during the pretreatment process [11]. Because of during NaOH pretreatment, cellulose was more difficult to degrade than hemicellulose so that hemicellulose was easier to be lost in the pretreatment than cellulose, which might contribute to the fact that glucan conversion rate was much higher than xylan conversion rate [10]. But in this study we have not found any sugar release from pretreatment process, which might good to avoid sugar loss such as xylose during pretreatment.

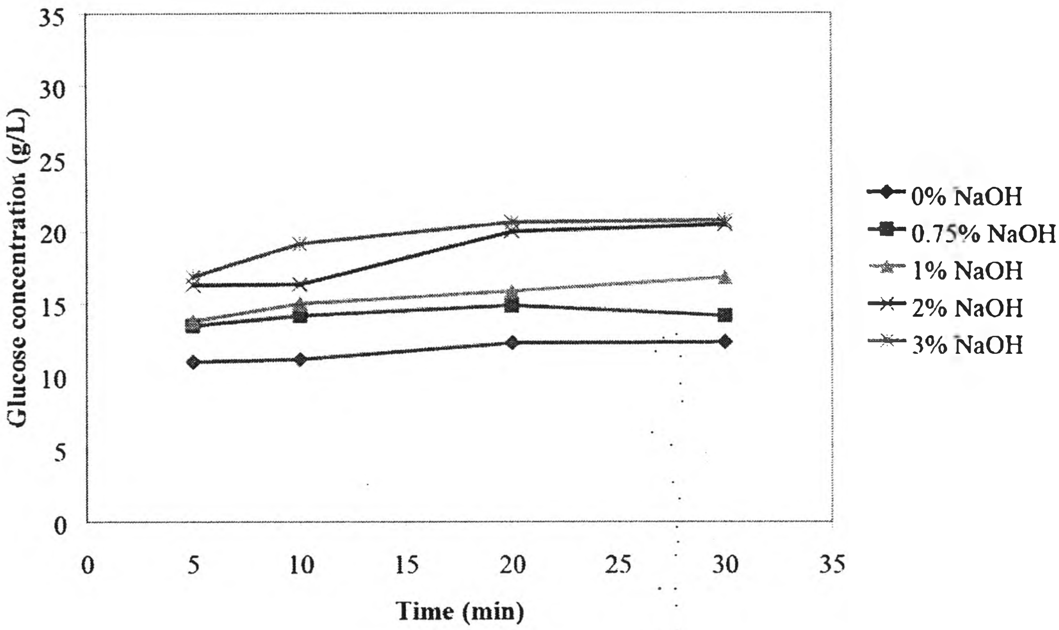
Table 4.3 Effect of pretreatment of corn cobs on weight loss

%NaOH	Time(min)	% Weight loss			
		60 °C	80 °C	100 °C	120 °C
0	5	5.5	5	5	5.25
	10	5.5	4.75	5	5
	20	5.25	4.75	4.75	4.25
	30	4	5	4.5	3.75
0.75	5	23	25.5	37.25	44.75
	10	24.75	31.75	38.75	47.25
	20	25.75	32.25	40.5	48.75
	30	26.5	32.5	45	51
1	5	28	27.25	39.25	45.75
	10	28.5	33.75	41.25	47.5
	20	28.25	36.25	43.5	48.75
	30	30	37.25	45.25	51.5
2	5	33.75	28.75	49.75	58
	10	37	36.5	50	57.25
	20	38.75	45.5	52.75	56.75
	30	41.25	45.75	54.25	58.25
3	5	39.5	29.75	53.25	56.75
	10	37.5	37.75	50	60
	20	39.75	47.25	54.75	61
	30	42.5	47	61.25	61.75

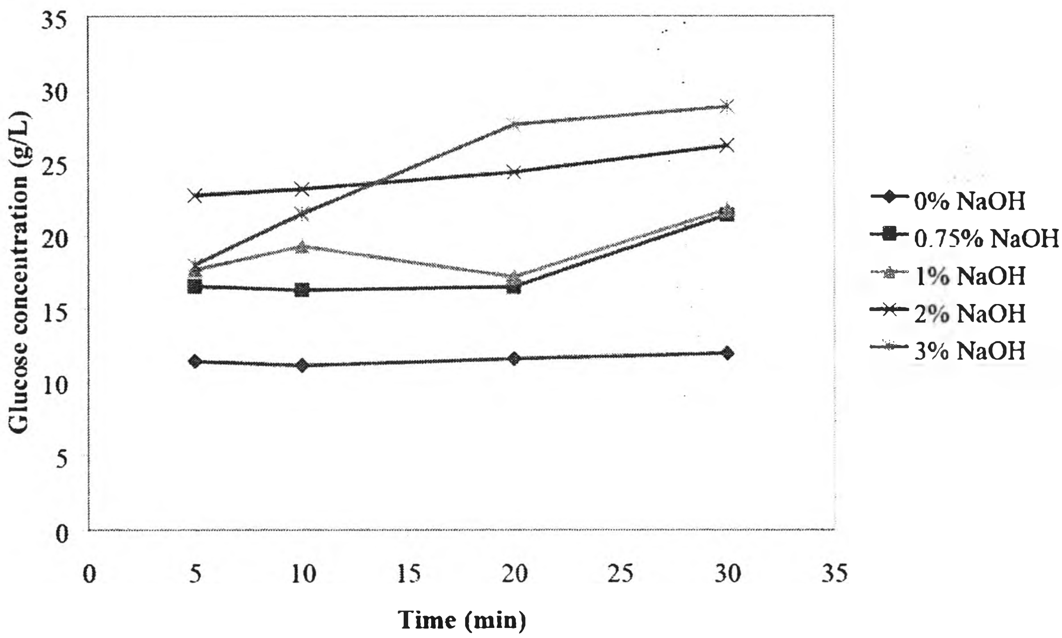
4.4.5 Total Sugar in Enzymatic Hydrolysis

To investigate the optimum conditions for the pretreatment of NaOH with microwave on corn cobs, total sugar released from hydrolysis part need to be considered. The enzymatic hydrolysis was performed at pH 4.8 with citrate buffer for 48 h. Although commercial cellulase enzymes contain β–endoglucanases and cellobiohydrolyase activity, they characteristically lack adequate β–glucosidase and xylanase activities required for efficient monomeric sugar release [11].

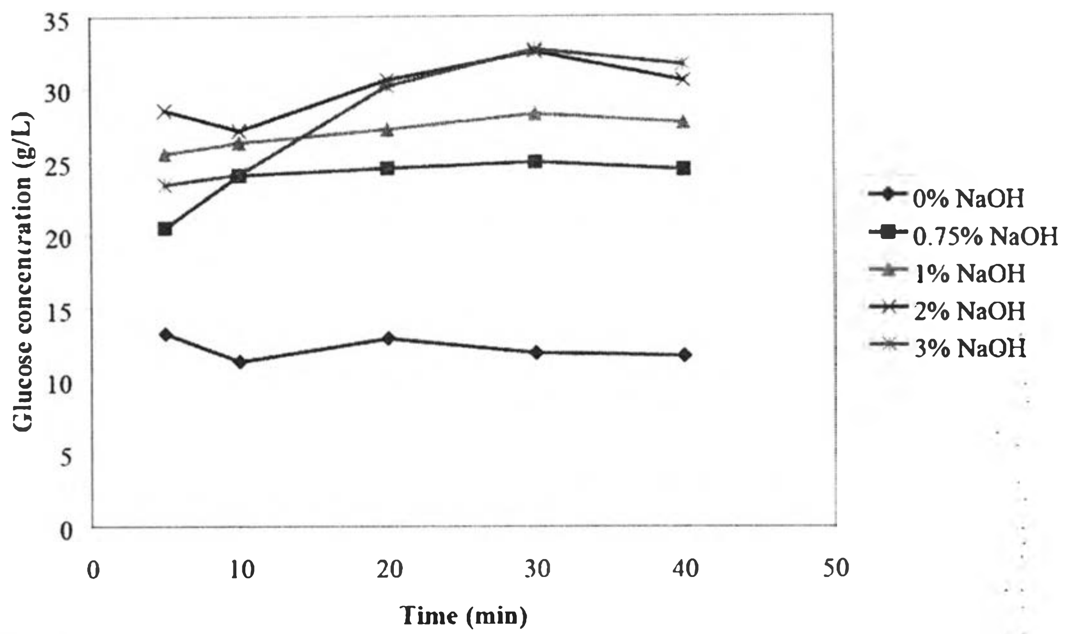
(A)


Glucose concentration (g/L)
Time (min)
0% NaOH
0.75% NaOH
1% NaOH
2% NaOH
3% NaOH

(B)


Glucose concentration (g/L)
Time (min)
0% NaOH
0.75% NaOH
1% NaOH
2% NaOH
3% NaOH

(C)



(D)

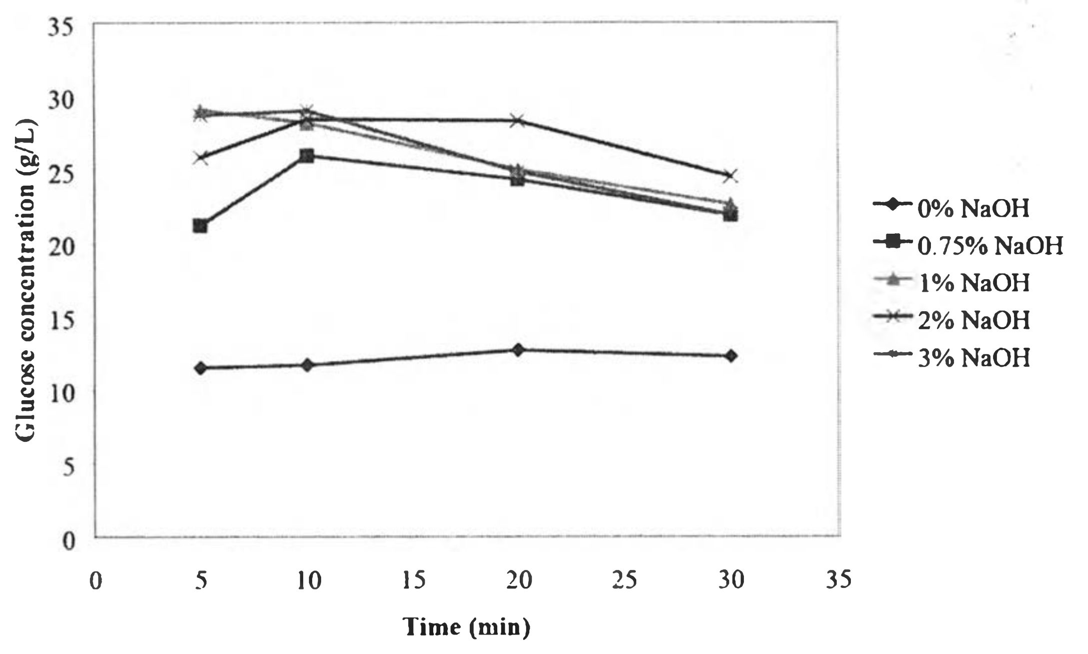
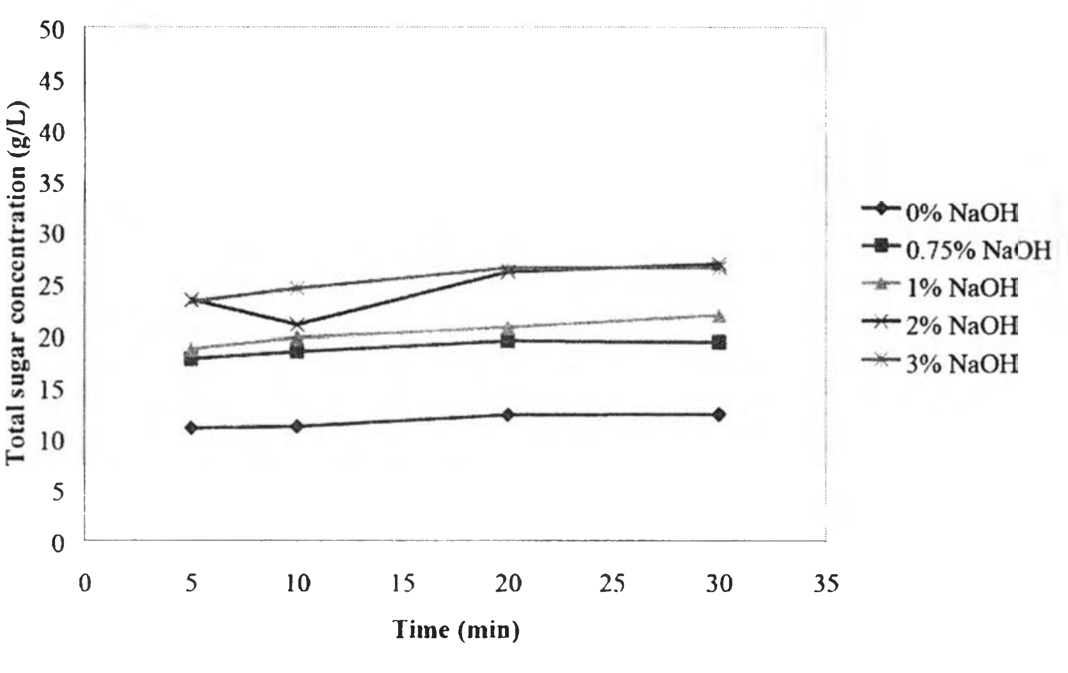


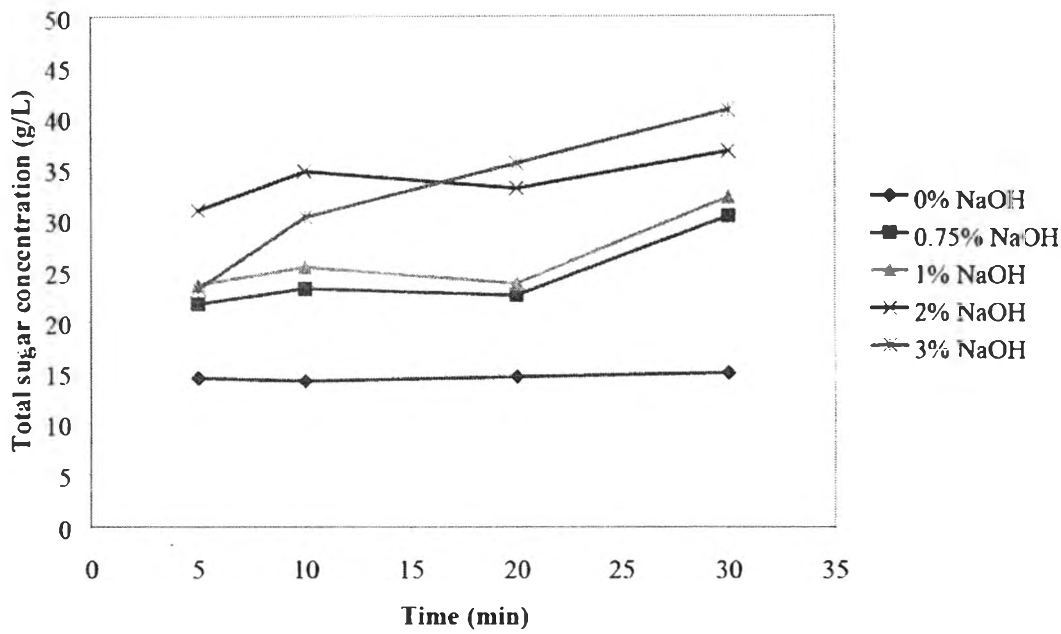
Figure 4.2 Glucose concentration obtained from enzymatic hydrolysis of pretreated corn cobs at different conditions. (A) 60°C; (B) 80°C; (C) 100°C; (D) 120°C.

Figures 4.2 and 4.3 show the glucose and total sugar concentration obtained from enzymatic hydrolysis of pretreated corn cobs. The results indicated that there was no significant difference in glucose concentration between 5 and 10 min. And as pretreatment time and NaOH concentration increased, glucose concentration slightly increased. According to an increase in pretreatment temperature, pretreatment time, and NaOH concentration, enzymatic hydrolysis improved. Among the variables studied, the pretreatment temperature had the most significant impact on enzymatic hydrolysis. However, higher pretreatment temperature (100 °C and 120 °C) pretreated with NaOH of 2% gave higher glucose concentration than that pretreated with NaOH of 3%. As a result of higher temperature and higher NaOH concentration during pretreatment, result in higher solid loss which leading to less total sugar released. In addition, the pretreatment with 2% NaOH at 100°C for 30 min is good enough for maximized glucose concentration. And the highest glucose concentration can reach up to 32.53 g/L and total sugar was released 45.60 g/L.

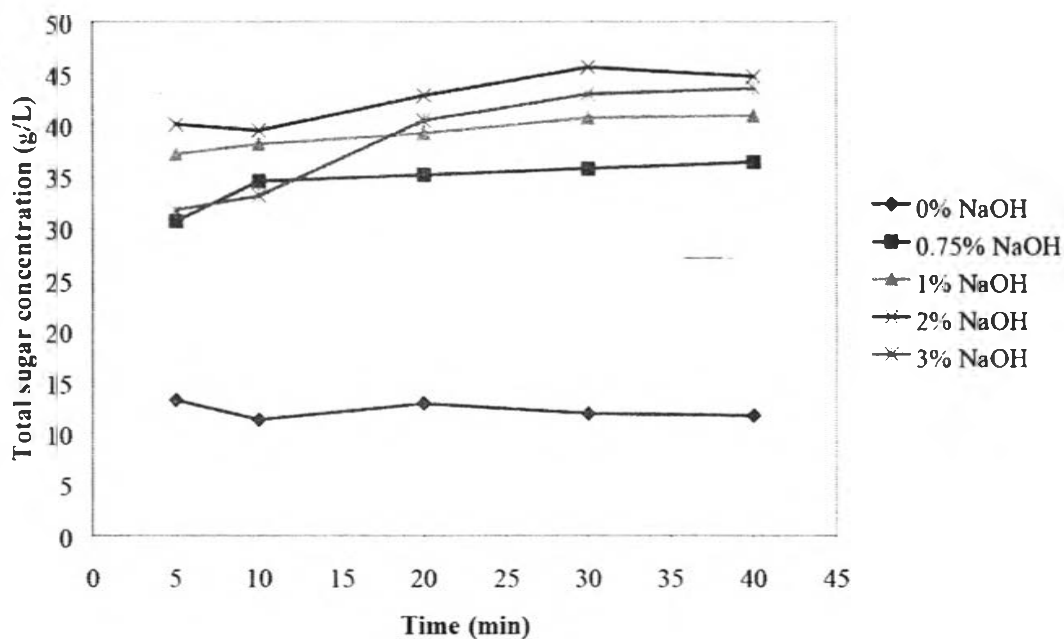
(A)



(B)


Total sugar concentration (g/L)
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
Time (min)
0% NaOH
0.75% NaOH
1% NaOH
2% NaOH
3% NaOH

(C)


Total sugar concentration (g/L)
50
45
40
35
30
25
20
15
10
5
0
0
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10
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20
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40
45
Time (min)
0% NaOH
0.75% NaOH
1% NaOH
2% NaOH
3% NaOH

(D)

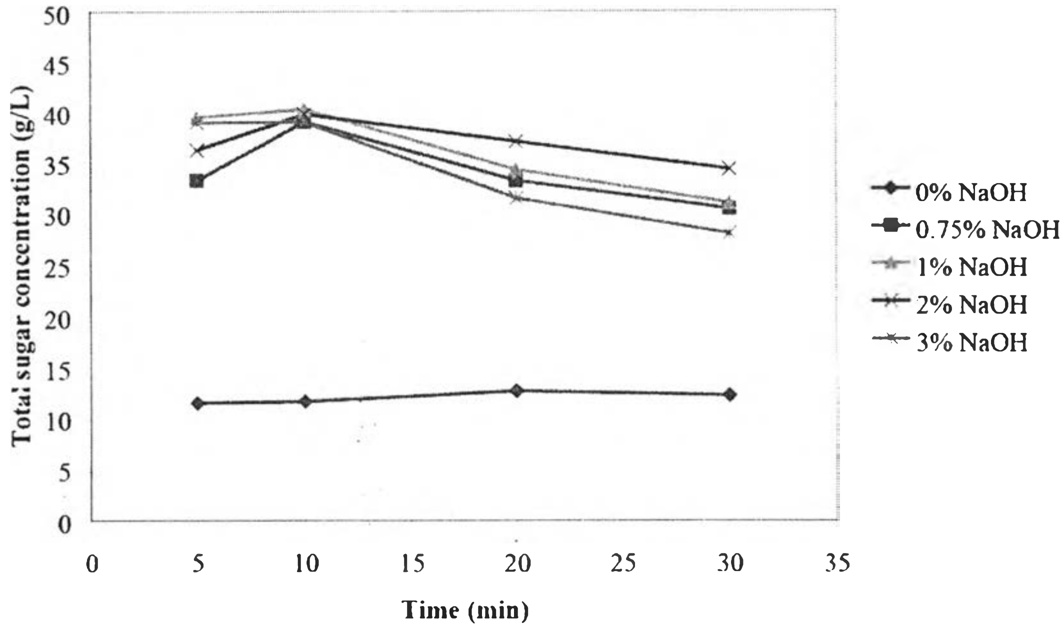

Figure 4.3 The total sugar concentration obtained from enzymatic hydrolysis of pretreated corn cobs at different conditions. (A) 60°C; (B) 80°C; (C) 100°C; (D) 120°C.

It can be concluded that lower temperatures for NaOH pretreatment was not favorable to enhanced total sugar released because the crosslink between lignin and carbohydrates were not interrupted sufficiently to reach high sugar production. Moreover, higher solid loss resulted in leading to less total sugar production.

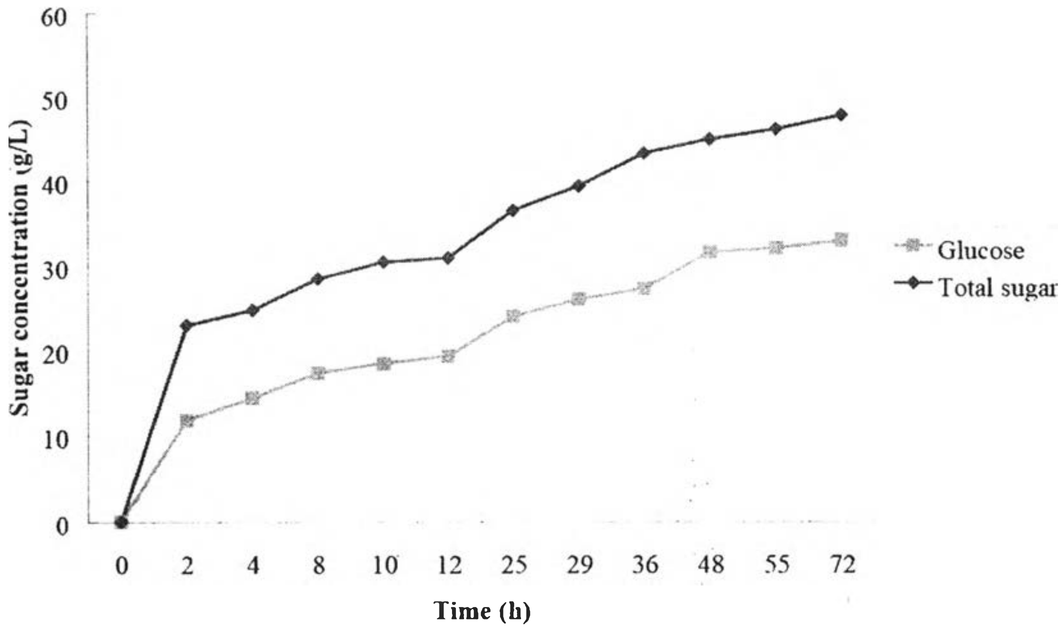

Figure 4.4 Glucose concentration and total sugar concentration from enzymatic hydrolysis of pretreated corn cobs with 2% NaOH 30 minutes at 100°C (optimum condition).

Figure 4.4 indicates glucose concentration and total sugar concentration from enzymatic hydrolysis of pretreated corn cobs with 2% NaOH 30 minutes at 100°C (optimum condition). The result shown that when hydrolysis after 48 h, there was no significant different in glucose concentration and total sugar concentration slightly increased. Thus, it can be concluded that 48 h is an appropriate time to produce glucose and other sugars.

4.4.6 <u>Composition of Corn Cobs After Pretreatment and Enzymatic Hydrolysis</u>

Pretreated corn cobs by 2% NaOH at 100 °C for 30 minutes was analyzed for chemical components. As seen in Table 4.4, pretreated corn cobs consisted of 42.6% cellulose, 39.04% hemicelluloses, and 7.59% lignin. Compared with the chemical components of untreated corn cobs, it showed that NaOH pretreatment reduced lignin by 66.27% because of its solubilization in the NaOH aqueous. Therefore, it is clear that the major effect of NaOH pretreatment is to reduce lignin. On the other hand, corn cobs after enzymatic hydrolysis consisted of 39.96% cellulose, 26.37% hemicelluloses, and 10.96% lignin. The result show that after enzymatic hydrolysis, cellulose content decreased compared with after pretreatment because it is converted into glucose.

Table 4.4 Content of cellulose, hemicellose, and lignin in corn cobs

	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Before pretreatment	42.6	39.04	7.56
After pretreatment	75.73	19.47	2.55
After enzymatic hydrolysis	39.96	26.37	10.96

4.4.7 Surface Morphology by SEM

The physical structure changes of the corn cobs pretreated by different methods were imaged by scanning electron microscope (Figure 4.5). As shown in Figure 4.5A, the texture of raw corn cobs seem to be rigid and no pore was presented. After pretreatment with 0.75% NaOH at 60°C for 20 minutes (Figure 4.5B), the pretreated corn cobs look soft and pores were appeared. And after pretreatment with 0.75% NaOH at 100°C for 20 minutes (Figure 4.5C), the pretreated corn cobs has more porous meaning that pretreatment step can help enzymatic accessibility by increasing surface area of the biomass material.

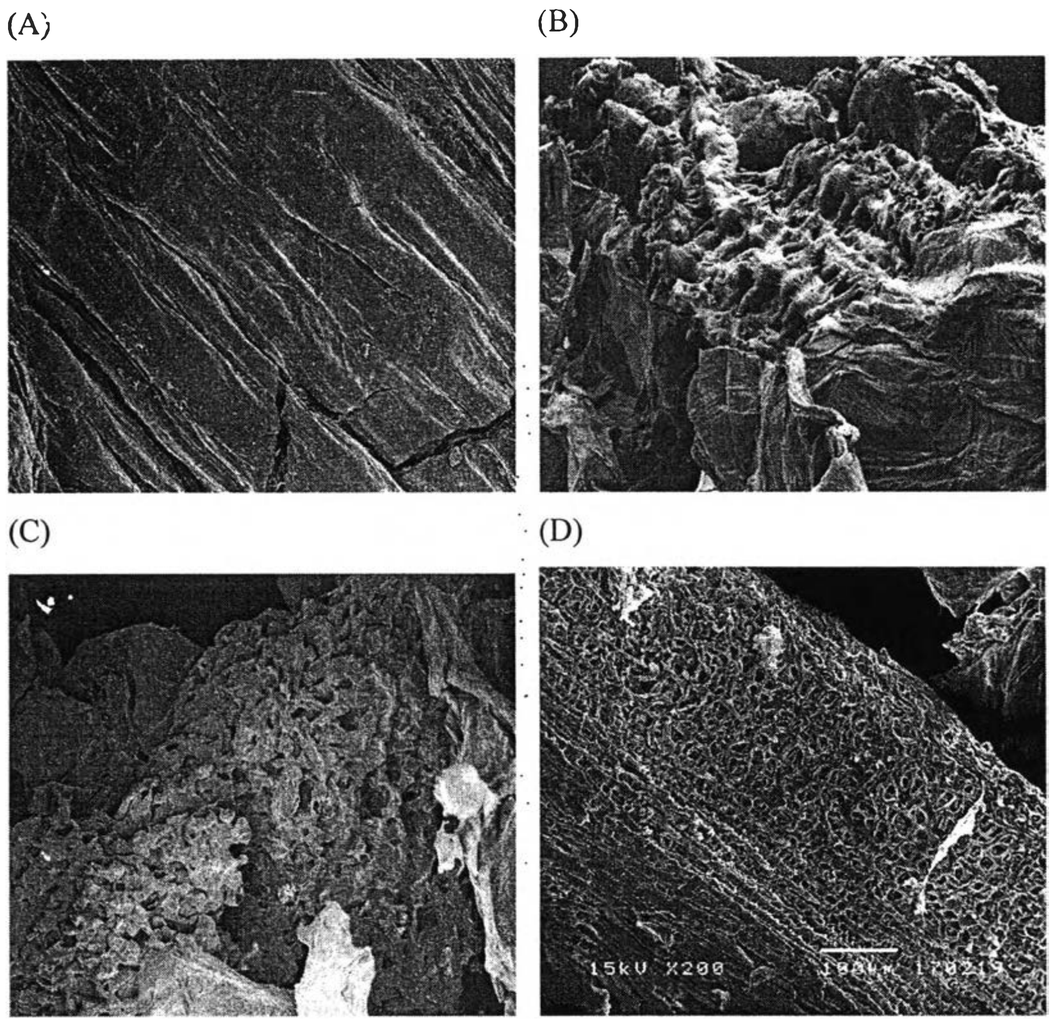

Figure 4.5 Scanning electron microscope images of corn cobs. (A) Raw corn cobs without treatment; (B) Corn cobs after pretreatment with 0.75% NaOH at 60°C for 20 minutes; (C) Corn cobs after pretreatment with 0.75% NaOH at 100°C for 20 minutes; (D) Corn cobs after pretreatment with 2% NaOH at 100°C for 30 minutes.

Therefore, pretreatment temperature, pretreatment time, and NaOH concentration affected on an increasing surface area. And in the optimum pretreatment conditions, it can increase the surface area of corn cobs from 0.994 m^2/g to 1.375 m^2/g.

Table 4.5 BET surface area, total pore volume, and average pore diameter of samples

Sample	Surface area (m^2/g)	Total pore volume (cm^3/g)	Average pore diameter (nm)
Untreated corn cobs	0.9940	0.0018	7.3363
Pretreated corn cobs with 2% NaOH at 100 °C for 30 min	1.3748	0.0025	7.1126

4.4.8 X-ray Diffraction Analysis

Crystallinity is believed to be an important feature affecting enzymatic saccharification of cellulose [8]. Crystallinity index refers as a measure of ordered orientation of cellulose crystallites and it would be increased when removing of amorphous components (hemicellulose and lignin) as well as disruption of hydrogen bonding within and in between cellulose chains, which occur in a high energy treatment process such as steaming [12]. Because of lignin removal, crystallinity index in pretreated corn cobs with NaOH and microwave could increase. Moreover, higher NaOH concentration and higher pretreatment temperature leading to higher lignin removal result in more crystallinity index.

Table 4.6 Crystallinity index (%) of untreated and treated corn cobs

Sample	Crystallinity index
Untreated corn cobs	29.06
Pretreated corn cobs with NaOH of 0% for 30 min at 100 °C	16.39
Pretreated corn cobs with NaOH of 2% for 30 min at 60 °C	29.67
Pretreated corn cobs with NaOH of 0.75% for 30 min at 100 °C	32.26
Pretreated corn cobs with NaOH of 2% for 5 min at 100 °C	32.92
Pretreated corn cobs with NaOH of 2% for 30 min at 100 °C	36.01

4.4.9 Comparison of Total Sugar Concentration Obtained from Different Pretreatment Methods

This part is to compare total sugar obtained from the pretreated corn cobs at the optimum conditions of microwave assists NaOH with other methods. Table 4.7 shows total sugars obtained from enzymatic hydrolysis of pretreated corn cobs with 2% NaOH with autoclave 121 °C for 60 min were 45.17 g/L , which close to total sugar of pretreated corn cobs with 2% NaOH with microwave 100 °C for 30 min. Therefore, it can be concluded that microwave assists NaOH can be produced total sugar concentration at shorter pretreatment time and lower pretreatment temperature. On the other hand, microwave assists NaOH can be produced higher total sugar concentration than conventional heating.

Table 4.7 Comparison of total sugar concentration obtained from different pretreatment methods

Method	Glucose (g/L)	Xylose (g/L)	Total Sugar (g/L)
2% NaOH with microwave 100 °C for 30 min	32.52	10.41	45.60
2% NaOH with autoclave 121 °C for 60 min	33.75	11.42	46.85
2% NaOH with conventional heating 100 °C for 30 min	22.44	13.30	37.46

4.4.10 Ethanol Production

For corn cobs pretreated under optimal conditions, the hydrolysate from enzymatic hydrolysis was adjust pH with $Ca(OH)_2$ until the pH of solution reach neutral pH. Then, clear sugar solution (without precipitate) was fermented at 37 °C for 1 to 3 days. The *saccharomyces cerevisiae* yeast was used in this study. After that, the solution was collected to analyze the ethanol concentration by GC instrument. Figure 4.5 shows the hydrolyzate of corn cobs pretreated under optimal conditions, which contained total sugar 45.60 g/L, can produce the maximum ethanol of 9.41 g/L. Moreover, longer fermentation time leads to less ethanol production, which caused by some inhibitors during fermentation.

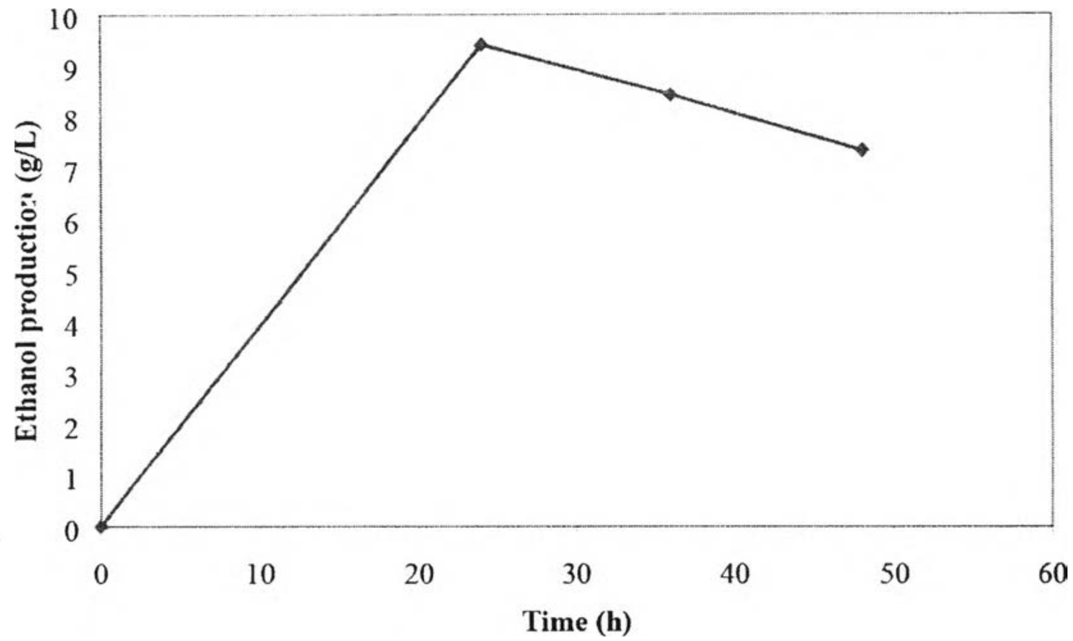

Figure 4.6 Ethanol production from hydrolyzate of corn cobs pretreated under optimal conditions.

4.5 Conclusions

The results showed that the sodium hydroxide pretreatment with microwave on corn cobs was effective in improving enzymatic hydrolysis accessibility. The optimum conditions were found at 2% NaOH at 100 °C for 30 minutes which could reduce lignin by 66.27% and increased in surface area by 38.31%. And the highest glucose concentration can reach up to 32.53 g/L and total sugar 45.60 g/L was released. Moreover, microwave assists NaOH can produce high total sugar concentration at shorter pretreatment time and lower pretreatment temperature compared with conventional method.

4.6 Acknowledgements

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