

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Abstract

Biofuels produced from lignocellulosic materials, so called second generation biofuel, showed energetic economic and environmental advantages in comparison to biofuels produced from starch or sugar. There are mainly two processes involved in the conversion routes: hydrolysis of cellulose to produce reducing sugar and fermentation of sugars to biofuels. Therefore, pretreatment of lignocellulosic materials to remove hemicellulose and lignin can significantly enhance the hydrolysis of cellulose. The main goal of research is to increase the enzyme accessibility by improving digestibility of cellulose. Accordingly, dilute sulfuric acid was used to pretreat corn cobs prior to enzymatic hydrolysis. After pretreatment of corn cobs by dilute acid under the optimal condition (120 °C, 5 min), the highest yield of total sugars of 24.73 g/l was obtained. After enzymatic hydrolysis, the highest yield of total sugars of 22.37 g/l was obtained and the final total sugar yield reached 47.11 g/l. It can be concluded that dilute sulfuric acid pretreatment can be successfully applied for corn cobs to achieve high yields of monomeric glucose and xylose.

4.2 Introduction

Due to lacking of recourse sustainability and increasing demand of fossil fuels have forced the global scientific community to intend a research on alternative and renewable energy sources. Bioethanol can be produced from lignocellulosic materials used as substrates for the production of bioethanol because of abundance, cheapness and renewability (Gabhane *et al.*, 2010). Normally, lignocellulosic biomass like corn, cassava, sugarcane etc. is mainly composed of cellulose (38–50%), hemicellulose (23–32%) and lignin (15–30%) % wt. of biomass material on dry basis along with smaller amounts of extractive and ash.

Corn cobs are the most abundant agriculture waste around the world and the amount available in Thailand has been estimated to 3.85 million tons of corn per year. Therefore, corn cobs are considered to be an attractive biomass for bioethanol production (Chen *et al.*, 2011). The most common processing of lignocellulosic biomass to bioethanol consists of four majors; pretreatment of raw materials, enzymatic hydrolysis of the pretreated materials into fermentable sugars, fermentation of the fermentable sugar into bioethanol, and ethanol separation (Lin *et al.*, 2010).

Pretreatment process is an important step for enzymatic hydrolysis required to alter the structure of cellulose and to make it more accessible for enzymatic hydrolysis. Therefore, the main objective of pretreatment is to remove both hemicellulose and lignin and to enhance the surface area of substrates. Pretreatment methods are either physical or chemical. Some methods require both effects. However, among the chemical pretreatment methods, dilute acid hydrolysis is widely used; there is more effective than other pretreatment methods since there is the fermentable sugar known xylose after acid pretreatment in the aqueous phase. Moreover, this method is effective and inexpensive (Gabhane *et al.*, 2010).

The objective of this work is to produce fermentable sugar, both xylose (from hemicellulose) and glucose (from cellulose) of corn cobs, by using dilute sulfuric acid pretreatment and enzymatic hydrolysis. The amount of fermentable sugar yield after pretreatment and enzymatic hydrolysis is determined. Furthermore, the structure of the pretreated corn cobs after acid pretreatment was also investigated by measuring surface area, particle size, crystallinity, and SEM imagery. The influences of acid concentration, reaction temperature, and residence time of pretreatment step on the fermentable sugar yield were also discussed.

4.3 Methods

4.3.1 Biomass Materials

Corn cobs were obtained from the Betagro Company. The collected corn cobs were stored at the ambient room temperature in a large bag. The average particle size of corn cobs was around 1.6 mm homogenized in a single lot and stored until used.

4.3.2 Chemical Analysis of Biomass

Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and acid insoluble ash (AIA) were determined for corn cobs by 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. Carbohydrate content of untreated material was also determined by measuring the hemicellulose (xylan and araban) and cellulose (glucan) derived sugars in supernatants following concentrated acid hydrolysis as described by NREL. Acid-insoluble lignin content of corn cobs and the solid fraction remaining after pretreatment was determined according to NREL methods. Likewise, water and ethanol soluble sugars were extracted from untreated sorghum straw and quantified according to NREL methods (McIntosh *et al.*, 2010).

4.3.3 Pretreatment Process

Dilute acid pretreatment was performed in a laboratory scale stirred autoclave (stainless steel type 316). There is a total volumetric reactor of 1 lit with an electric heater and magnetic agitation. The temperature was controlled by the temperature controller.

The effects of the pretreatment conditions, consisting of reaction temperature, acid concentration and residence time, on the sugar yields in the hydrolyzate liquors were studied. Sulfuric acid concentrations of 2, 5, 7, and 10% (v/v) were examined at temperatures of 100, 120, 140, and 160 °C and residence times of 5, 15, 30, and 60 min.

After pretreatment, the reactor was cooled down to 70 °C within 15 min. The samples were kept until the reactor was cooled to about 40 °C. Then, the samples were filtered for solid and liquid fraction recovery. The liquid phase called hydrolyzate was stored to investigate sugar analysis. And the solid residue was washed by tap water until neutral pH and dried for 24 h at 65 °C. The pretreatment procedure flow diagram is shown in Figure 4.1.

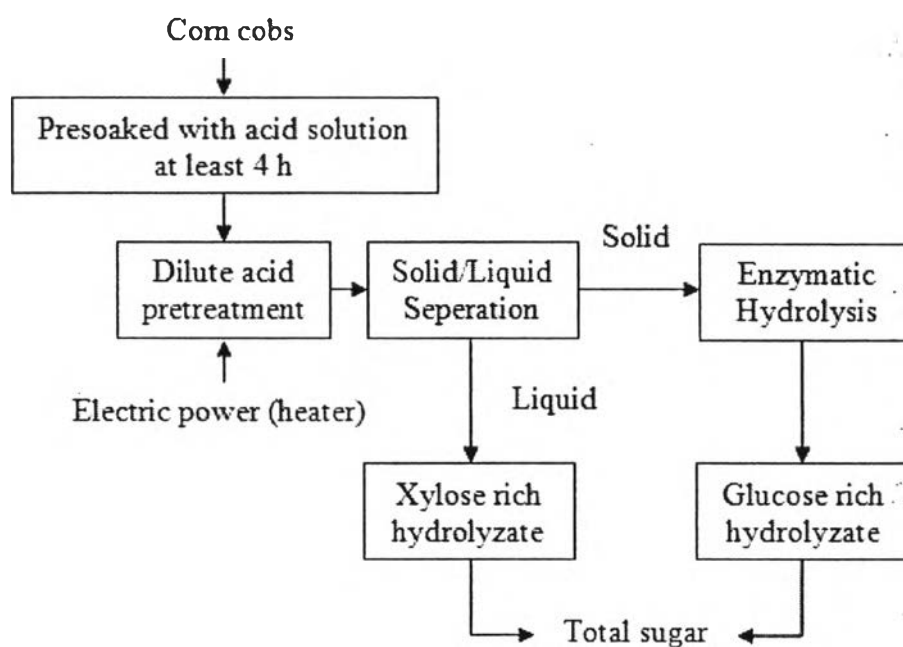


Figure 4.1 Flow diagram of dilute acid pretreatment and enzymatic hydrolysis.

4.3.4 Enzymatic Hydrolysis

The enzyme consisting of a mixture of cellulase, hemicellulase and higher level of beta-glucosidase was gifts from Novozyme and the activity was 2889.27 unit/ml. In this work, the enzyme from Novozyme was used to hydrolyze the solid residue into the fermentable sugar.

The solid residue (about 0.5 g dry weight) was transferred to a 250 ml shake flask and then the pH was adjusted to 4.8 by adding 50 mM citrate buffer at 3% (w/v) solid loading. The flask was put in an incubator shaker. The enzymatic digestion was carried out at 50 °C and 150 rpm for 48 h. Samples were taken every 6 h for the fermentable sugar determination.

4.3.5 Overliming with Calcium Hydroxide

Calcium hydroxide was added to the dilute acid pretreated hydrolyzate to adjust pH to 10 and stirred for 30 min at 90 °C, allowed to cool slowly to room temperature and then adjusted pH of the mixture to 7 by sulfuric acid. The hydrolyzate was filtrated to remove any precipitate formed before using as substrate for fermentation.

4.3.6 Ethanol Production

Active yeast was mixed with sugar solution after adjusting pH from 4.8 to 7 in a ratio of 1:10 and transferred to an incubator shaker at 37 °C for 1 day to 3 days. After this process completed, the solution was stored in order to analyze the ethanol concentration by GC instrument.

4.3.7 Analysis Methods

The composition of the hydrolyzate after pretreatment and enzymatic hydrolysis was cellobiose, glucose, xylose and arabinose, which were filtered through a 0.45 μ m-pore-size filter and measured by high performance liquid chromatography (HPLC) using a packed column (Aminex HPX-87H column, Bio-Rad Lab, USA at 60 °C with 5 mM H₂SO₄ as the eluent at a flow rate of 0.7 mL/min).

BET surface area information of the pretreated residues was measured by N₂ adsorption/desorption measurements (Quantachrome instrument; model: BELSORP-max, BEL Japan INC., Japan)) carried out at 196 °C (77 K). Prior to measurement, all biomass materials were dried at 40 °C for 48 h and then a sample (0.5–1 g) was put into the sample tube of the Quantachrome instrument and degassed using a vacuum for 16 h. The BET surface area and pore volume were obtained from the N₂ adsorption/desorption curves using BELSORP-max software.

A scanning electron microscope (SEM) (HitachiS-4800 SEM instrument operated at 10–15 kV accelerated voltage) was used to observe surface morphology and size of the corn cobs before and after pretreatment.

The structure of the pretreated residues was characterized by powder X-Ray diffraction (XRD) (Bruker D8 Advance) in order to investigate the crystallographic structure, chemical composition, and physical properties of materials of the corn cobs before and after pretreatment.

4.4 Results and Discussion

4.4.1 Biomass Characterizations

4.4.1.1 Chemical Composition of Corn Cobs

Corn cobs were examined for cellulose, hemicellulose, lignin and ash content according to McIntosh *et al.*, (2010) with the resulting dry weight shown in Table 4.1.

Table 4.1 Chemical composition of corn cobs

Chemical components	Dry solid (%)
Cellulose	39.04
Hemicellulose	42.60
Lignin	7.56
Ash	2.19

Table 4.1 shows that the biomass contents consisted of 39.04% cellulose, 42.6% hemicellulose, 7.56% lignin, and 2.19% ash, respectively. Firstly, cellulose, the major chemical component on fiber wall and contributing 39.04% of corn cobs, 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. Secondly, hemicellulose, contributing 42.60% of corn cobs, is a complex polysaccharide structure that contains different carbohydrate polymers like pentoses (e.g. xylose and arabinose), hexoses (e.g. mannose, glucose, and galactose), and sugar acid. Thirdly, lignin, contributing 7.56% of corn cobs, 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 main propose of having lignin was to provide structural support, impermeability, and resistance against microbial attack and oxidative stress to the plant. Finally, ash, contributing 2.19% of corn cobs, is the amount of the mineral content and other inorganic matter in biomass.

4.4.1.2 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 4.2.

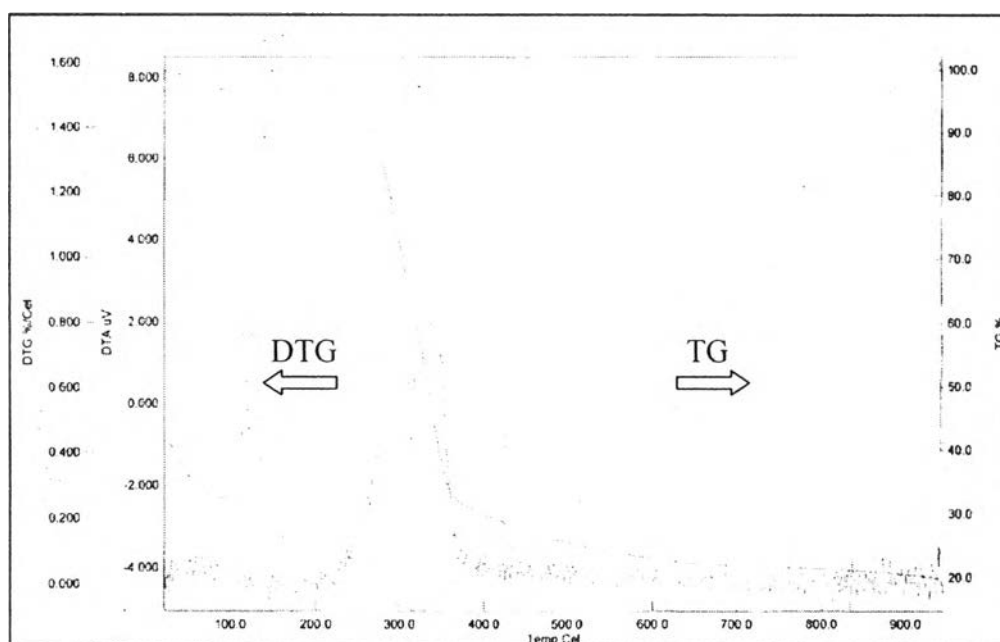


Figure 4.2 TGA curve of corn cobs.

The pyrolysis characteristics, both TG (in wt. %) and DTG (in wt. % °C) curves of the three components, are shown in Figure 4.2. Great differences were found among the pyrolysis behaviors of the three components. Hemicellulose started its decomposition easily, with the weight loss mainly happened at 220–315 °C. Cellulose pyrolysis was focused at a higher temperature range (315–400 °C). Among the three components, lignin was the most difficult one to decompose. Its

decomposition happened slowly under the whole temperature range from ambient to 900 °C.

The differences in the inherent structures and chemical nature of the three components possibly account for the different behaviors observed. Hemicellulose is consisted of various saccharides (xylose, mannose, glucose, galactose, etc.), it appears a random, amorphous structure, rich of branches, which are very easy to remove from the main stem and to degrade to volatiles evolving out (CO, CO₂, and some hydrocarbon, etc.) at low temperatures. However, cellulose is consisted of a long polymer of glucose without branches, its structure is in a good order and very strong, and the thermal stability of cellulose is higher than hemicellulose. Lignin is full of aromatic rings with various branches, the activity of the chemical bonds in lignin covered an extremely wide range, which led to the degradation of lignin occurring in a wide temperature range (100–900 °C) (Yan *et al.*, 2007).

4.4.1.3 Elemental Analysis (CHON)

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 Table 4.2.

Table 4.2 Elements of corn cobs

Elements	% of Element
Carbon	44.00
Hydrogen	7.00
Oxygen	47.00
Nitrogen	0.40
Trace elements	1.60

The elemental contents of C, H, O, N, and trace elements listed in Table 4.2, showed clearly that these biomass fuels contain higher proportion of hydrogen and oxygen, compared with carbon, which reduces the energy value. It was

reported that carbon–oxygen and carbon–hydrogen bonds contain lower energy than in carbon–carbon bonds. Therefore, the calorific value of corn cobs is in the range of 16 to 20 MJ/kg. However, the calorific value of bituminous coal achieves 34 MJ/kg, which is much higher than that of biomass. Moreover, corn cobs, containing 10% of moisture with high moisture content, is not more suited for thermal conversion technology. Therefore, corn cobs and other biomasses are not popular to be used as solid fuel (Cuiping *et al.*, 2004).

4.4.2 Dilute Acid Pretreatment

Xylan is the major component present in the largest hemicelluloses structure of corn cobs, so the presence of xylose in the filtrate can be used as an indicator of the solubilization level of hemicellulose, which is easily utilized by the fermentation process (Chen *et al.*, 2011). The highest yield of xylose measured in the hydrolyzate, 17.58 g/l, was generated after a pretreatment at 5% acid, 120 °C, and 5 min residence time. However, xylose yields at 2% acid, 120 °C, and 5 min and 2% acid, 120 °C, and 15 min were found to be statistically similar to the highest yield.

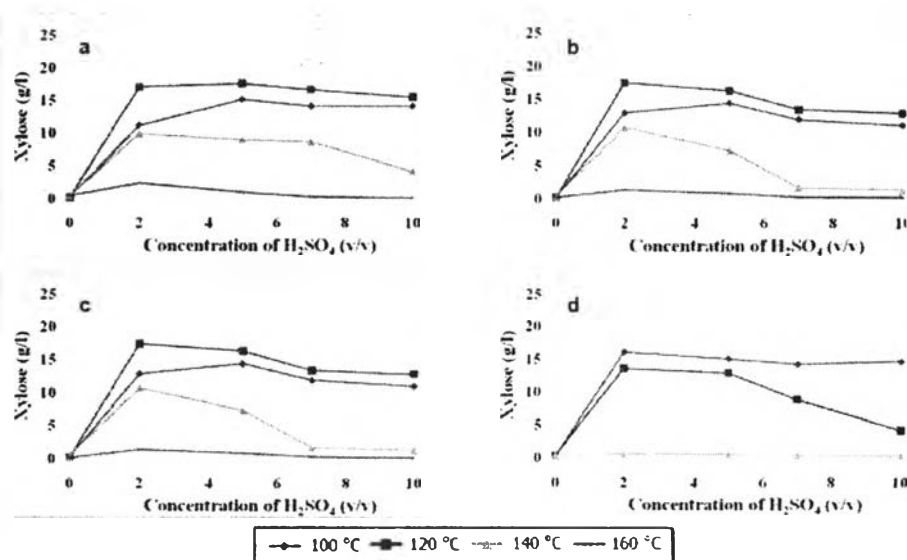


Figure 4.3 Monomeric xylose measured in the prehydrolyzate after pretreatment as a function of sulfuric acid concentration (v/v) and temperature for (a) 5 min (b) 15 min, (c) 30 min, and (d) 60 min residence time.

Figure 4.3 contains four graphs corresponding to each of the four residence time. Each graph shows the monomeric xylose measured in the hydrolyzate for each combination of temperature and acid concentration. The effects of temperature and acid concentration offer the greatest influence, about 25% (Redding *et al.*, 2011). Normally, the importance of temperature, acid concentration, time, and interactions between these factors is not too surprising because the relationship has already been investigated using modified Arrhenius equations in other studies. They have proposed these equations as rate constants in kinetic models to describe the acid hydrolysis of hemicellulose into xylose (Carrasco and Roy, 1992; Baugh and McCarty, 1988).

Figure 4.3 also shows xylan oligomer content at the point of the highest total yields when corn cobs were pretreated with dilute sulfuric acid. The amount of xylan oligomers as a fraction of the total solubilized xylose was greatest at short pretreatment times, coinciding with the breakdown of the fast xylan fraction (Esteghlalian *et al.*, 1997). After the optimal point each condition was under harsh conditions, which were too long residence time and too high reaction temperature, carbohydrates are degraded into furfural and HMF which in turn are degraded into levulinic acid and formic acid, respectively (Redding *et al.*, 2011).

The hydrolyzate also contains glucose and arabinose. Table 4.3 shows the total sugar measured, glucose, and arabinose monomers liberated after the acid pretreatment. A clear trend is visible, showing that the increase of temperature or acid concentration results in the increase of glucose in the hydrolyzate. This follows the logic that as the pretreatment conditions become harsher, the hemicelluloses is hydrolyzed faster and more completely, exposing the cellulose to acid hydrolysis as well. Similar trends were reported in a study on the dilute acid pretreatment of corn stover (Lloyd and Wyman 2005).

Table 4.3 Glucose and arabinose in the prehydrolyzate after the dilute sulfuric acid pretreatment of corn cobs

Sulfuric acid concentration % (v/v)	Temperature (°C)	Time (min)	Glucose (g/l)	Arabinose (g/l)	Total sugar (g/l)
2	100	30	2.16	6.21	24.93
2	120	5	3.34	3.17	24.74
2	120	15	3.42	3.58	25.97
5	100	30	2.97	5.31	25.73
5	120	5	3.58	3.46	25.53
5	120	15	4.49	3.49	25.17
10	120	60	2.63	1.39	8.23

4.4.3 Enzymatic Hydrolysis

After the enzymatic hydrolysis, the hydrolyzate was analyzed for glucose, xylose and arabinose monomers. Figure 4.4 shows the monomeric glucose measured in the hydrolyzate as a function of temperature, acid concentration, and residence time. The highest yield of glucose and total sugar measured in the hydrolyzate was 14.48 g/l and 22.38 g/l, respectively with 2% sulfuric acid at 120 °C for 5 min.

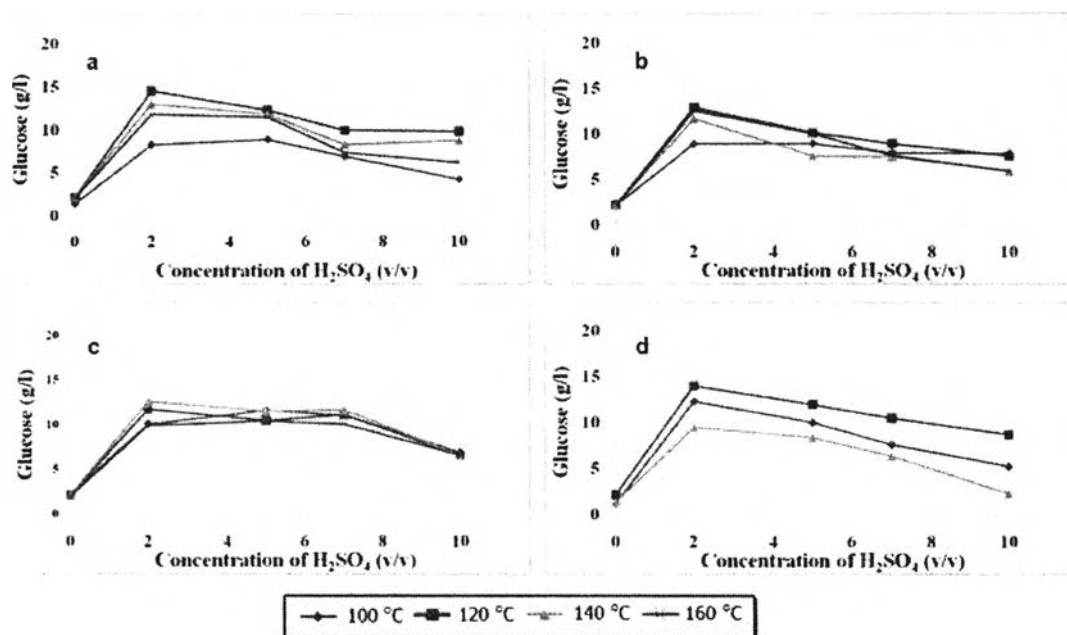


Figure 4.4 Monomeric glucose measured in the prehydrolyzate after pretreatment as a function of sulfuric acid concentration (v/v) and temperature for (a) 5 min (b) 15 min (c) 30 min and (d) 60 min residence time.

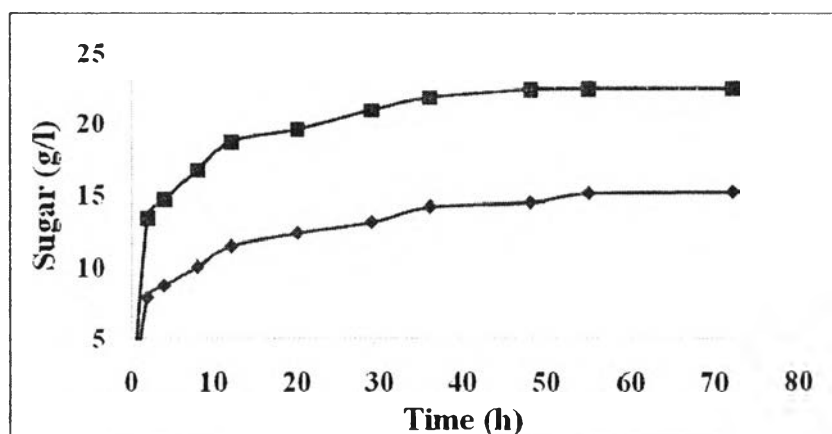


Figure 4.5 Enzymatic releasing of sugars from pretreated corn cobs. Symbols; (\blacklozenge) for glucose and (\blacksquare) for total sugar.

Figure 4.5 shows glucose content from cellulose using commercial enzyme consisting of cellulase, hemicellulase and higher level of beta-glucosidase enzyme activities. The corn cobs were individually pretreated with 2% sulfuric acid at 120 °C for 5 min). The result is summarized in Figure 4.5 in which the largest of total sugar released after 48 h. After that, the total sugars hardly changed so the optimal time to hydrolyze of enzymatic hydrolysis was 48 h, which agrees well with the results reported by Sumphanvanich *et al.*, (2008).

The glucose yield in the enzymatic hydrolysis step is also increased because of hemicellulose removal from corn cobs. During the pretreatment either xylose or furfural generation can lead to high glucose yield in the hydrolyzate after the enzymatic hydrolysis. On the other words, the success of the acid pretreatment is based around the concept of hemicellulose removal in order to maximize glucose yield after enzymatic hydrolysis. Since furfural levels are substantially influenced by temperature and xylose levels are influenced by temperature and acid concentration, it would be expected that glucose in the hydrolyzate would be influenced most substantially by temperature, acid concentration (Redding *et al.*, 2011).

The purpose of pretreatment is not only to remove xylose from hemicellulose but also to increase the rate of enzymatic hydrolysis of cellulose to glucose. Thus, it is necessary to estimate pretreatment efficiency by combining total xylose and glucose conversion yield after both the pretreatment and enzymatic hydrolysis processes.

4.4.4 Total Sugar

The total sugars called the fermentable sugar were the summation of the xylose and glucose monomers found in both the prehydrolyzate and the hydrolyzate. For total sugars both after acid pretreatment and after enzymatic hydrolysis results were summarized in Table 4.4. The pretreatment conditions that maximized the total yield for each acid/temperature combination are reported. The highest total glucose and xylose yields were obtained with the pretreatment at 120 °C with a sulfuric acid concentration of 2% and a reaction time of 15 min or at 120 °C with a sulfuric acid concentration of 5% and a reaction time of 5 min (the highest of total sugar after acid pretreatment) and at 120 °C with a sulfuric acid concentration

of 2% and 5 min reaction time (the highest of total sugar after enzymatic hydrolysis and total yield). Overall, we found that the optimum condition is at 120 °C with a sulfuric acid concentration of 2% and 5 min reaction time. This condition was released the total sugar around 47.11 g/l.

Table 4.4 Summary of total acid pretreatment and enzymatic hydrolysis results for best overall combined yields for each acid/temperature time series

Conc. Sulfuric Acid % (v/v)	Temp. (°C)	Time (min)	Acid Pretreatment Total sugar (g/l)	Enzymatic Hydrolysis Total sugar (g/l)	Total Sugar (g/l)
2	120	5	24.73	22.37	47.11
2	120	15	25.52	18.79	44.32
5	120	5	25.97	21.11	47.09

4.4.5 Physical Characterization of Pretreated Corn Cobs

Examination of dry weight loss for the treated samples indicated that the weight loss was due to partial removal of the amorphous materials (hemicellulose, lignin, and pectin). The removal and weight loss in the case of acid treatment was high compared to the water treatment at the same elevated temperature. The result is shown in Table 4.5. Amounts of cellulose content of fibers after the acid treatments, indicated that the cellulose molecules were relatively unaffected by acid treatment. Similar observations were also originated from an investigation on the influence of boiling temperature on chemical composition of hemp fibers (Kostic *et al.*, 2008). Complete removal of hemicellulose by acid treatments was usually achieved because the proton ions of sulfuric acid should be able to penetrate into the corn cobs surface and further catalyze the de-acetylation and hydrolysis; this will break the beta (1,4)-glycosidic bonds of the hemicelluloses (Chen *et al.*, 2011). However, lignin was not totally removed because of the presence of chemically resistant carbon-carbon bonds in lignin molecules (Pejic *et al.*, 2008).

After acid pretreatment, the pretreated corn cobs were characterized by XRD to identify the crystallinity change. The crystallinity is influenced by the composition of the biomass, in which hemicellulose and lignin are considered to be amorphous while cellulose is considered to be crystalline (Gabhane *et al.*, 2010). The result shown Table 4.5 indicated that the increasing in crystallinity can be viewed as the indication of effective pretreatment by which the way of removing amorphous components, hemicelluloses and lignin. In addition, expose all crystalline cellulose available and disruption of hydrogen bonding within and in between cellulose chains, which occur in a high energy treatment process such as steaming (Mosier *et al.*, 2005) as well as increasing the rate of enzymatic hydrolysis.

Scanning Electron Microscope (SEM) showed the details on tissue damage and ultra structure changes in corn cobs tissue after acid pretreatment. Untreated corn cobs were non-porous, the smooth surface of substrate, and uniform and mostly linear in nature (as shown in Figure 4.6). The corn cobs treated by sulfuric acid also shows significant destruction derived structures. For the removal of surface impurities, non-cellulosic materials, inorganic substances and waxes was found to result in rougher surfaces and better fiber separation as shown in Figure 4.7 (Saha *et al.*, 2010). This kind of cracks can increase the surface area and porosity to facilitate disruption of lignocelluloses, a prerequisite for enzymatic hydrolysis of cellulose (Gabhance *et al.*, 2010).

Furthermore, it was found that under the pretreatment condition, the surface area and the total pore volume of pretreated corn cobs were higher than that of untreated corn cobs and the average pore diameter of pretreated corn cobs was less than untreated corn cobs. The results shown in Table 4.5 indicated that most of the hemicellulose in acid pretreatment has been solubilized and removed after the integrated pretreatment process, which increases the surface area and makes enzymatic hydrolysis easier (Chen *et al.*, 2011).

Table 4.5 Effect of acid pretreatment on the compositions, physical properties and enzymatic hydrolysis of the pretreated residues

Sample	Weight loss (%)	Composition (Acid Pretreatment) ^b			Physical properties			Composition (Enzymatic Hydrolysis) ^d		
		Cellulose (%)	Hemicellulose (%)	Lignin (%)	CrI ^c (%)	BET (m ² /g)	Particle size (nm)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	–	39.04	42.6	7.56	29.07	0.99	7.34	–	–	–
Acid Pretreatment ^a	65	67.63	4.06	25.53	33.19	3.87	5.85	38.38	3.18	38.53
Water Pretreatment ^a	14.85	42.01	40.36	9.63	31.42	1.36	7.12	38.93	38.33	12.36

^a The sample were pretreated at 120 °C 5 min 2 % of H₂SO₄

^b Each value is the average of three replicates and calculated on the basis of dry weight.

^c CrI (%) = $(I_{002}-I_{am})/I_{002} * 100$ where I_{002} is intensity of the 002 peak at $2\Theta = 22.4^\circ$ and I_{am} is intensity of the background scatter at $2\Theta = 18.7^\circ$ (Kim and Holtzapple, 2006).

^dAfter 48 h of enzymatic hydrolysis

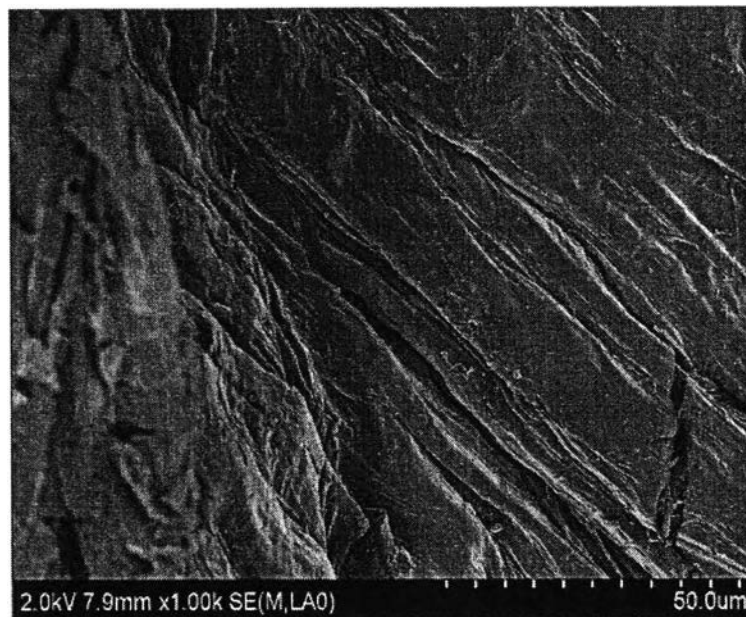


Figure 4.6 SEM micrographs of before corn cobs.

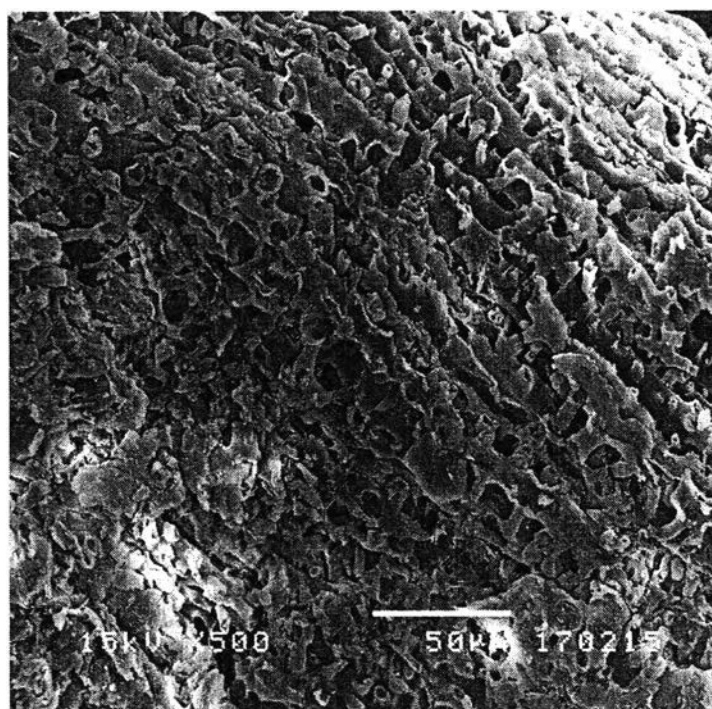


Figure 4.7 SEM micrographs of pretreated corn cobs.

4.4.6 Ethanol production

The results of fermentation of dilute sulfuric acid pretreated and enzyme hydrolyzed corn cobs by *Saccharomyces Cerevisiae* are shown in Figure 4.8. At 24 h, the highest ethanol yield of 5.6 and 7.0 g/l for prehydrolyzate sugar and hydrolyzate sugar were obtained, respectively.

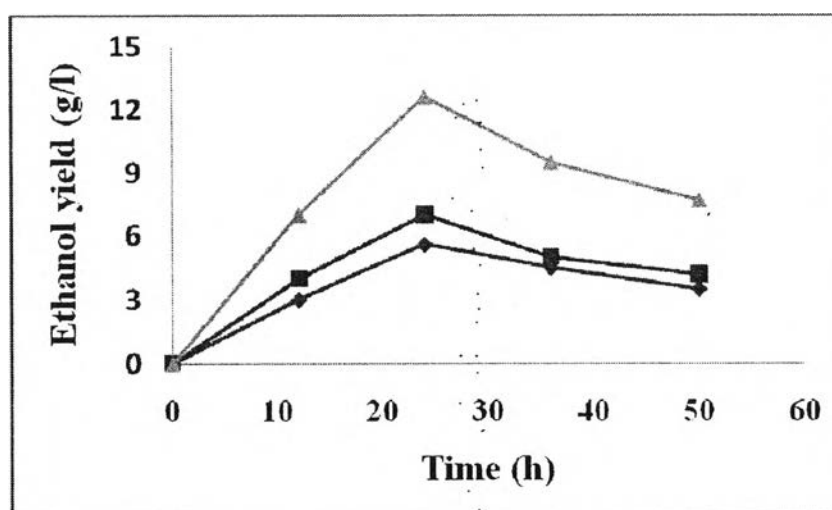


Figure 4.8 Time courses of ethanol production by the *Saccharomyces Cerevisiae* from corn cobs. The data presented ethanol production from both dilute sulfuric acid pretreated and enzyme saccharified. Symbol (\blacktriangle), total sugar; (\blacksquare), dilute sulfuric pretreated and (\blacklozenge), enzyme saccharified.

4.5 Conclusions

The dilute acid pretreatment studies described above satisfy and meet some of the more important requisites for an effective pretreatment process. This dilute acid hydrolysis is to maximize the xylose conversion and to destroy the corn cobs structure in order to enhance enzymatic hydrolysis. Our study shows that there may be opportunities for further process optimization, finding the right pretreatment temperature, reaction time, concentration of sulfuric acid and enzyme combinations. Moreover, this research also shows the ethanol production from the fermentable sugar.

4.6 References

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