CHAPTER IV
ENHANCEMENT OF ENHYMATIC HYDROLYSIS OF CORNCOB BY MICROWAVE–ASSISTED ALKALI PRETREATMENT AND ITS EFFECT IN MORPHOLOGY

4.1 Abstract

Bioethanol produced from a conventional fermentation process using *Saccharomyces cerevisiae* utilizing pretreated and hydrolyzed corncob as a substrate was studied. It was found that the morphology of corncob was significantly changed after microwave-assisted alkali pretreatment was applied. An increase in the crystallinity index and surface area of the pretreated corncob was also observed. The highest total sugar concentration of 683.97 mg/g of pretreated corncob, or 45.60 g L^{-1}, was obtained from the optimum pretreatment conditions of 2 % NaOH at 100 °C for 30 min in a microwave oven. Microwave-assisted alkali pretreatment was an efficient way to improve the enzymatic hydrolysis accessibility of corncob in a shorter amount of time and at a lower temperature, compared to other methods.

Keywords: corncob · microwave technique · alkali pretreatment · enzymatic hydrolysis

* Accepted by Cellulose

4.2 Introduction

Energy consumption is rising at an average of 5 % per year due to the fast growing economy in Thailand. This increase affects the price of fossil fuels, which are mainly non-renewable resources used for transportation. The use of a renewable energy source such as ethanol has become a promising alternative. Ethanol is an important biofuel which is produced by yeasts using lignocellulosic biomass as a substrate. Corncob is one of the major agricultural byproducts of Thailand. Approximately 4 million tons of this biomass are produced annually. The conversion of corncob into a fermentable sugar for bioethanol production is difficult because of limiting factors in the enzymatic hydrolysis process.

The main factors that adversely affect enzymatic hydrolysis are crystallinity of the cellulose, available substrate surface area, and the presence of lignin and hemicellulose (Alvira et al. 2010). Owing to these factors, pretreatment is an important step to help prepare the material for enzymatic degradation. Different pretreatment methods produce different effects; therefore, an optimal pretreatment method for corncob or lignocellulosic biomass would make it more attractive for ethanol production.

There are several biomass pretreatment methods—biological, physical, chemical, and physico–chemical (Taherzadeh and Karimi 2008). Alkali pretreatment is a chemical process that removes lignin and some parts of hemicellulose, which results in an increase in the accessibility of cellulose to enzymes (Binod et al.2010). Alkali pretreatment can also be operated at low temperatures, even ambient conditions, but requires a long processing time and high concentration of base. Lower temperatures are more effective because some components in the lignocellulosic biomass decompose at high temperatures (Zhu et al. 2005). Microwave-based pretreatment is an alternative method that can be operated at low temperatures. Sodium hydroxide (NaOH), an alkali, is a suitable chemical reagent for use in microwave pretreatment (Keshwani et al. 2007).

Cellulosic ethanol has received much attention in recent years and there have been few studies applying microwave with chemical pretreatment to lignocellulosic biomass (Zhu et al. 2005; Ma et al. 2009). Until now, there has been

no report on the enzymatic saccharification of microwave-assisted pretreatment of corncob. The purpose of this work was to optimize the conditions of microwave–alkali pretreatment to yield fermentable sugars for ethanol production. Sodium hydroxide (NaOH) was used as a base in the alkali pretreatment of corncob. The treated cellulose was subjected to hydrolysis with a commercial cellulase enzyme to determine the highest amount of reducing sugar that could be obtained from corncob. The physical and chemical properties of corncob, before and after pretreatment at the optimal conditions, were investigated with a scanning electron microscope (SEM), BET surface area analyzer, X-ray diffraction (XRD), and thermal gravimetric analysis (TGA). The optimal conditions were also applied to other heating methods (hot plate and autoclave) to compare sugars resulting from enzymatic hydrolysis. Finally, fermentation using *Saccharomyces cerevisiae* was investigated to confirm the possibility of using corncob as a substrate for ethanol production.

4.3 Experimental

4.3.1 Materials

Homogenized corncob was obtained from Betagro Public Company Limited, Thailand. It had a ground particle size of 1.6 mm and was dried in a 105 °C drying oven to a constant weight before use.

4.3.2 Filter Paper Assay (FPA)

Filter paper assay was used to measure the cellulase activity of Celluclast 1.5 L enzyme (It was produced from *Trichoderma reesei* ATCC 26921 purchased from Sigma–Aldrich (St. Louis, MO, USA)) and to quantify the amount of enzyme loading. The procedure was completed according to the method used by Ghosh (1987). Whatman No. 1 filter paper strips were used as a substrate and the enzyme in a citrate buffer of pH 4.8. Enzymatic hydrolysis took place in a water bath at 50 °C for 60 min. Then all diluted samples were added to 3,5-dinitrosalicylic (DNS) acid reagent and heated as described by DNS analysis (Miller 1959). The reacted samples were then measured with a UV–VIS spectrophotometer (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:

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

4.3.3 Alkali Pretreatment

A CEM Mars 5 microwave system (Matthews, NC, USA) including the HP-500 (500 psig material design pressure and 260 °C) was used in this study for the microwave and alkali pretreatment experiments. This process was carried out as follows: 2 g of corncob was suspended in 30 mL (15:1 liquid solid ratio, LSR) of different NaOH concentrations (0.75–3 %) and then transferred to a microwave oven to treat the corncob at a desired temperature (60–120 °C) for 5–30 min. After this process was complete, the residue was collected on filter paper, then washed with water until reaching neutral pH. Finally, it was dried at 65 °C, and weighed. In a comparison of heating reactors for the pretreatment step, the hot plate and the vertical autoclave were used.

4.3.4 Enzymatic Hydrolysis

In the hydrolysis experiment, a mixture of 1 g of pretreated corncob and 30 mL of 0.1 M citrate buffer (pH 4.8) (15:1 LSR) was added with Celluclast 1.5 L enzyme to 10 FPU/g pretreated corncob, at 50 °C with a shaking rate of 150 rpm in an incubator shaker for 48 h. The sample was taken from the hydrolysis solution and heated to 100 °C for 3 min to denature the enzyme. It was cooled to room temperature, and centrifuged for 20 min at 8,000 rpm (Zhu et al. 2005). The collected samples were stored at −20 °C until sugar analysis.

4.3.5 Fermentation

The pH of the hydrolysate from enzymatic hydrolysis was adjusted with NaOH until the solution reached 6.5. Then, a clear 18 mL sugar solution (without precipitate), in a 100 mL flask, was inoculated with 2 mL of active *S.*

cerevisiae at 35 °C. The positive control used the actively growing cells, which were inoculated with yeast extract–peptone–dextrose (YPD) medium contained 30 g L^{-1}. The fermentation broth samples were collected after 24 h and the ethanol concentration was analyzed by gas chromatography (GC).

4.3.6 Analytical Methods

The composition of the corncob, before and after alkali pretreatment, was determined by the Nakhonratchasima Animal Nutrition Research and Development Center (Nakhonratchasima, Thailand). The difference between the neutral detergent fibre (NDF) and acid detergent fibre (ADF) was calculated to determine the detergent hemicellulose while the difference between ADF and the acid detergent lignin (ADL) determined the detergent cellulose value. The other elements were acid insoluble ash (AIA) and unknown components.

Monosaccharides such as glucose, xylose, arabinose, mannose, galactose, and cellubiose were determined using a high performance liquid chromatography (HPLC) 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). Five millimolar sulfuric acid solution was used as a mobile phase at a flow rate of 0.6 mL/min while the column temperature was fixed at 65 °C. The reducing sugar was also measured by DNS analysis (Miller 1959).

The untreated and treated corncob was observed by 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 corncob were acquired with 15 kV accelerating voltage (Li et al. 2010) at the magnification of 1000×.

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

X-ray diffraction was used to identify crystallinity present in both the untreated and pretreated corncob 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 the hollow of a glass holder and held in place by a glass slide. Then, it was scanned in the 2θ range of 0°–40° in continuous mode at a rate of 1°/min. Biomass crystallinity as expressed by the crystallinity index (CrI), was determined according to the method described:

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

in which, 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° (Segal et al. 1959).

The thermal decomposition of the biomass was investigated by using a Perkin Elmer/Pyris Diamond, thermal gravimetric analyzer (TG–DTG).

4.4 Results and Discussion

4.4.1 Effects of Sodium Hydroxide Pretreatment with Microwave on Weight Loss and Enzymatic Hydrolysis

The main reason for NaOH pretreatment is to remove lignin from the biomass. Lignin can effectively inhibit the cellulase enzymes from hydrolyzing cellulose and hemicellulose into glucose, xylose and other fermentable sugars. Weight loss becomes an important index for determining the effectiveness of pretreatment (Zhu et al. 2005).

Table 4.1 shows the weight loss of pretreated corncob with microwave-assisted NaOH pretreatment. Time, temperature, and NaOH concentration had a significant effect on weight loss. The solids loss ranged from 23 % (w/w) under mild pretreatment conditions (0.75 % NaOH for 5 min at 60 °C) up to 61.75 % when the sample was pretreated with 3 % NaOH for 30 min at 120 °C.

Although each variable contributed to the solids loss, temperature was found to have the greatest impact on solids loss. Nevertheless, the microwave pretreatment with 0 % NaOH also showed a weight loss, in the range of 3.75–5.5 %, indicating that the use of hot water has only a slight impact on the corncob structure.

Table 4.1 Effect of pretreatment of corncob on weight loss

NaOH (%)	Time (min)	Weight loss (%)			
		60 °C	80 °C	100 °C	120 °C
0	5	5.50	5.00	5.00	5.25
	10	5.50	4.75	5.00	5.00
	20	5.25	4.75	4.75	4.25
	30	4.00	5.00	4.50	3.75
0.75	5	23.00	25.50	37.25	44.75
	10	24.75	31.75	38.75	47.25
	20	25.75	32.25	40.50	48.75
	30	26.50	32.50	45.00	51.00
1	5	28.00	27.25	39.25	45.75
	10	28.50	33.75	41.25	47.50
	20	28.25	36.25	43.50	48.75
	30	30.00	37.25	45.25	51.50
2	5	33.75	28.75	49.75	58.00
	10	37.00	36.50	50.00	57.25
	20	38.75	45.50	52.75	56.75
	30	41.25	45.75	54.25	58.25
3	5	39.50	29.75	53.25	56.75
	10	37.50	37.75	50.00	60.00
	20	39.75	47.25	54.75	61.00
	30	42.50	47.00	61.25	61.75

Aside from the solubilization of lignin, McIntosh and Vancov (2010) have reported varying amounts of hemicellulose loss following exposure to alkaline substances during the pretreatment process. During NaOH pretreatment, cellulose was more difficult to degrade than hemicellulose. Hemicellulose was more easily solubilized than cellulose, which might contribute to the glucan conversion rate being much higher than the xylan conversion rate (Wang et al. 2010). In this study, we have not found any sugar release as a result of the NaOH pretreatment process.

The optimum conditions for the NaOH pretreatment with microwave on corncob and the total sugar released during hydrolysis needed to be considered. Enzymatic hydrolysis was performed using Celluclast 1.5 L with a filter paper activity of 56.58 FPU/mL. The glucose (Fig. 4.1) and total sugar concentration (Fig. 4.2) obtained from enzymatic hydrolysis of pretreated corncob are shown separately due to different trends in some cases. There is no significant difference in glucose concentration between 5 and 10 min except for the 3 % NaOH pretreatment at 80 °C. As pretreatment time and NaOH concentration increased, glucose concentration slightly increased. Consequently, with an increase in pretreatment temperature, time, and NaOH concentration, enzymatic hydrolysis was improved. Among the variables studied, temperature had the most significant impact on enzymatic hydrolysis. However, higher pretreatment temperatures (100 and 120 °C with 2 % NaOH) gave a higher glucose and total sugar concentration compared with 3 % NaOH. Thus, using higher temperatures (over 100 °C) and a higher NaOH concentration during pretreatment resulted in higher solids loss, which lead to less total sugar being released. In conclusion, pretreatment with 2 % NaOH at 100 °C for 30 min was the optimum condition for the enzymatic saccharification of Celluclast 1.5 L. Under this condition, the hydrolyzed corncob had a 32.53 g L^{-1} glucose concentration and 45.60 g L^{-1} of total sugar (or 683.97 mg/g of pretreated corncob). It should be noted that there was some overlap of the error bars and experimental data from the variations especially from the condition at 120 °C, 20 min which resulting in a greater than 10 % error bars of glucose and total sugars. This may due to the agglomeration of corncob initiated when the treatment heating by microwave above 100 °C and lead to a variation in the enzymatic hydrolysis. It was proven when the pretreatment was operated at 140 °C; it was visible that the corncob was

randomly burnt. Likewise, these results were not produced at the optimal conditions, suggesting proper temperature for NaOH pretreatment in microwave irradiation. It could be concluded that lower NaOH pretreatment temperatures were not favorable for enhanced total sugar release because the crosslink between lignin and the carbohydrates was not interrupted sufficiently to reach high sugar production. Moreover, higher solids loss resulted in less total sugar production. Figure 4.3 indicates glucose concentration and total sugar concentrations from enzymatic hydrolysis under optimum conditions. After 48 h, there was no significant difference in glucose levels while there was only slightly increased total sugar concentration. Thus, 48 h was chosen as the optimal period for enzymatic saccharification.

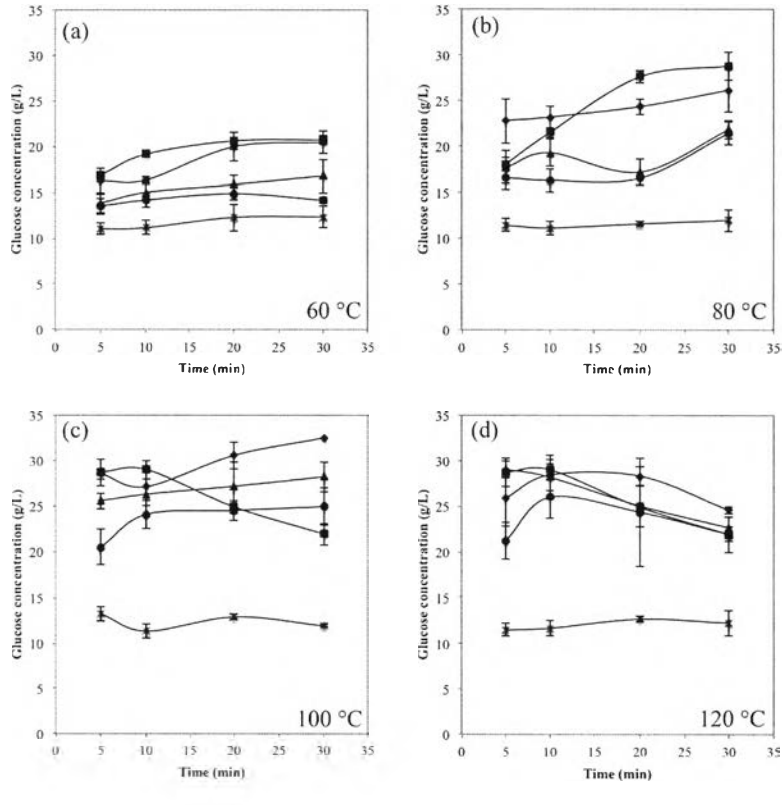

Figure 4.1 Glucose concentrations obtained from enzymatic hydrolysis of pretreated corncob at different times, temperatures, (a) 60 °C, (b) 80 °C, (c) 100°C and (d) 120 °C and NaOH concentrations, (∗) 0%, (●) 0.75%, (▲) 1%, (◆) 2%, (■) 3%.

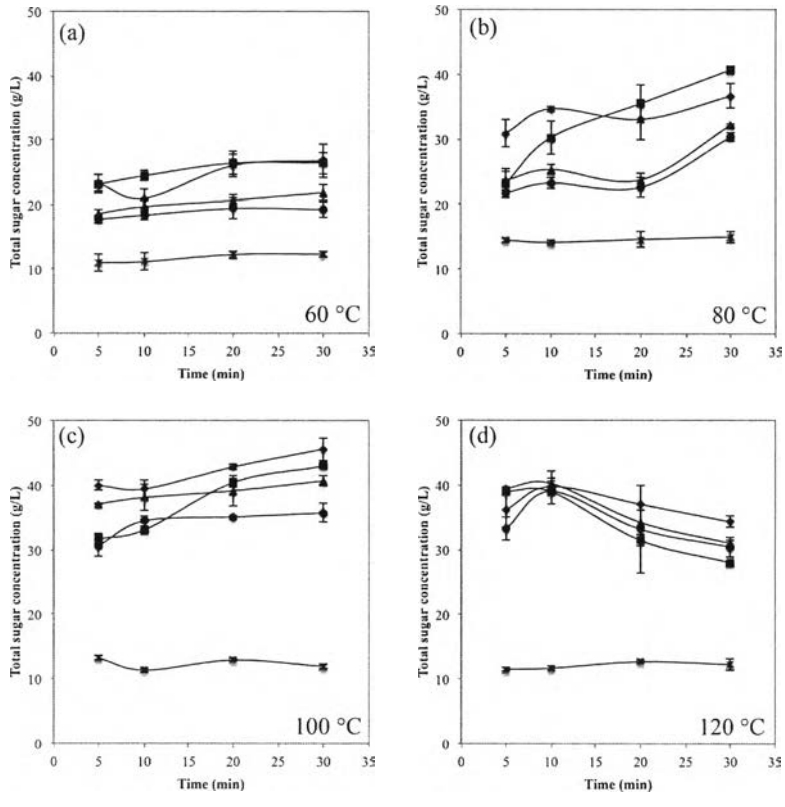

Figure 4.2 Total sugar concentrations obtained from enzymatic hydrolysis of pretreated corncob at different times, temperatures, (a) 60 °C, (b) 80 °C, (c) 100°C and (d) 120 °C and NaOH concentrations, (∗) 0%, (●) 0.75%, (▲) 1%, (◆) 2%, (■) 3%.

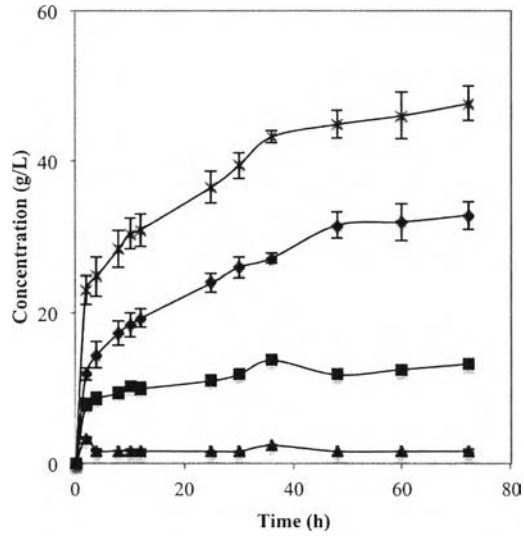

Figure 4.3 Glucose (◆), xylose (■), arabinose (▲) and total sugar (∗) concentration from enzymatic hydrolysis of pretreated corncob with 2% NaOH 30 min at 100°C (optimum condition).

4.4.2 Composition of Corncob After Pretreatment and Enzymatic Hydrolysis

The main composition of corncob is cellulose, hemicellulose, and lignin. The other constituents include ash, organic compounds (uronic acid and acetyl groups), and other trace components (minerals, waxes, fats, starches, resin and gums) (Wang et al. 2010). The pretreated corncob, after hydrolysis by cellulase enzyme, was analyzed for chemical components. As seen in Table 4.2, the amount of cellulose in the pretreated corncob increased significantly while more than 60 % of the lignin content was removed. The NaOH pretreatment reduced lignin efficiently due to solubilization in the NaOH. On the other hand, after enzymatic hydrolysis, the corncob still contained cellulose, hemicellulose and lignin, implying that the corncob was not completely hydrolysed by the cellulase enzyme.

Table 4.2 Composition of corncob

	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

4.4.3 Corncob Morphology, Surface Area, Crystallinity Index and Thermogravimetric Analysis

The physical structure of the pretreated corncob at different conditions was captured by SEM. As shown in Fig. 4.4a, the texture of raw corncob seems to be rigid with no pores present. After pretreatment with 0.75 % NaOH at 60 °C for 20 min (Fig. 4.4b), the pretreated corncob looks soft and pores appear. After pretreatment with 0.75 % NaOH at 100 °C for 20 min (Fig. 4.4c), the pretreated corncob is more porous, meaning that enzymatic accessibility is enhanced by increased surface area of the biomass material.

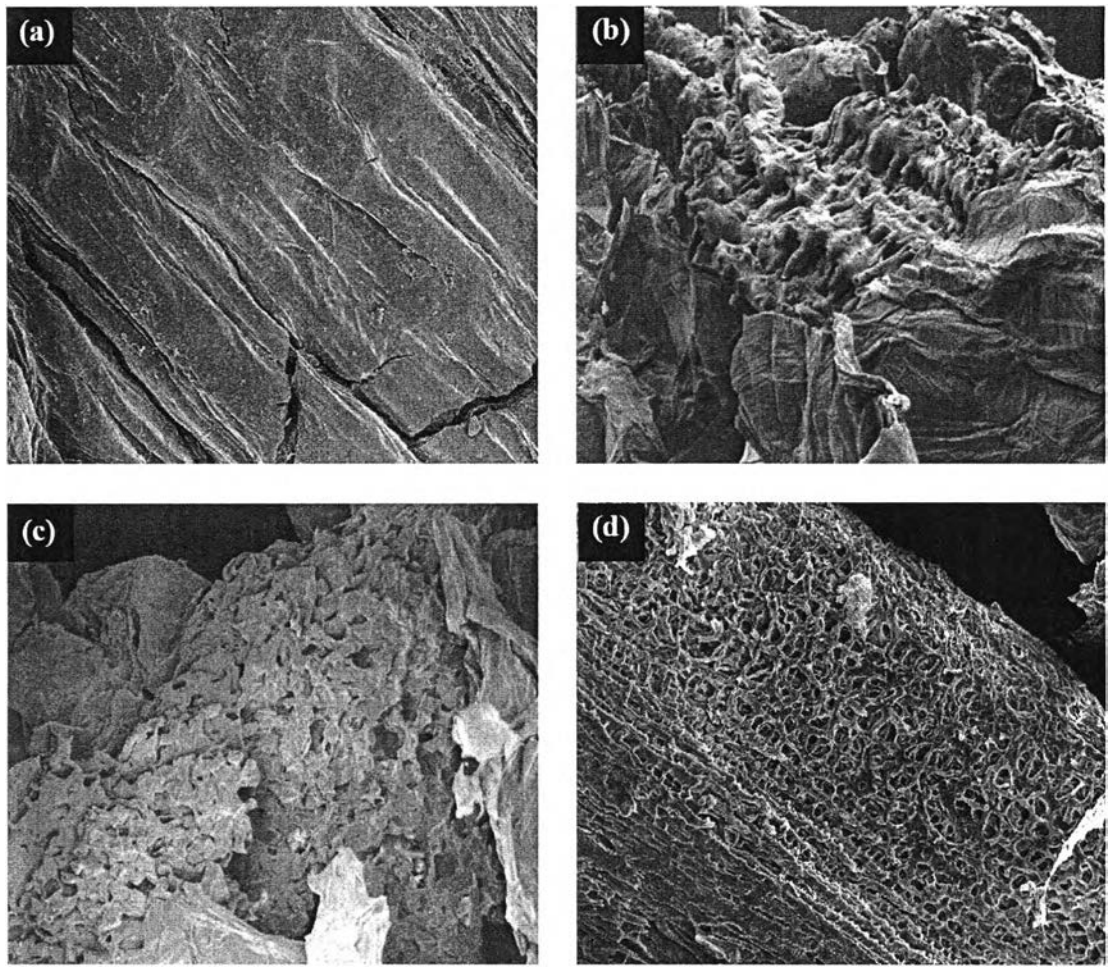

Figure 4.4 Scanning electron microscope images of (a) raw corncob without pretreatment; (b) corncob after pretreatment with 0.75% NaOH at 60 °C for 20 min; (c) after pretreatment with 0.75% NaOH at 100 °C for 20 min and (d) after pretreatment with 2% NaOH at 100 °C for 30 min.

Crystallinity is believed to be an important feature affecting enzymatic saccharification of cellulose. Table 4.3 shows the CrI of corncob at different conditions. The CrI of untreated corncob was 32.70 %, which is relatively consistent with the work of Sahare et al. (2012). The results indicated that microwave-assisted NaOH pretreatment of corncob had an influence on the CrI due to the removal of lignin and hemicellulose. At the optimum condition, the CrI of the treated corncob was much higher compared to the untreated corncob. The effect of NaOH pretreatment on the CrI at the optimum condition was clearly seen when compared to

the other NaOH concentrations, and times. A lower pretreatment NaOH concentration, and time when compared to the optimum conditions, led to an increase in CrI compared to the untreated corncob. In some cases, the CrI was much different even from the same biomass and the method of calculation (French and Cintrón 2013; Reddy and Yang 2007; Samayam and Schall 2010). It has been reported by Hu and Yu (2012) that cattail pretreated by microwave irradiation has a lower CrI compared to conventional heat. This indicates that, without NaOH, heating by microwave can break down the crystalline structure of cellulose aided for enzymatic hydrolysis. A decrease in CrI also observed when using ionic liquid pretreatment of switchgrass (Li et al. 2010) and acid pretreatment of wheat straw at 190 °C (Dhabhai et al. 2013). The hypothesis of this phenomenon is the transformation of cellulose I_β to cellulose II, which had lower crystalline when treated with ionic liquid (Sun et al. 2009). The other reason was the thermal degradation of the components occurred under severe conditions (Dhabhai et al. 2013).

Table 4.3 Crystallinity index (%) of untreated and treated corncob

NaOH	Time (min)	Temp (°C)	CrI (%)
Untreated			32.70
0 %	30	100	28.78
0.75 %	30	100	39.91
2 %	30	100	57.44
2 %	5	100	50.19

Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) results are shown in Fig. 4.5. Three endothermic and mass loss areas are indicated. Both samples, untreated corncob and treated corncob with NaOH at the optimal conditions, have an endothermic peak from the reaction at ~100 °C, recognized by the removal of moisture when heated (Rhim et al. 2009). The other

two peaks are related hemicellulose and cellulose. The hemicellulose seemed to decompose easier than cellulose since it pyrolyzed at 220–315 °C, while the cellulose pyrolyzed at 315–400 °C (Yang et al. 2007). The differences between untreated and treated of corncob were clearly seen. A shift of hemicellulose and cellulose peaks occured from the residual NaOH used in the pretreatment (Jenkins et al. 1998). In addition, the mass loss rate of cellulose was higher than the untreated sample indicating a greater amount of cellulose was present. Mass loss above ~400 °C of untreated and treated corncob happened at the same time but the treated corncob had lower solid residue mass (wt%) due to delignification since lignin is difficult to pyrolyze. The effect of lignin was also shown in the DTA results. There was a cancellation of a cellulose peak in comparison to the treated sample (Yang et al. 2007). When compared to untreated corncob, alkali pretreatment at optimal conditions was shown to be effective for the removal of lignin and increase in amount of cellulose.

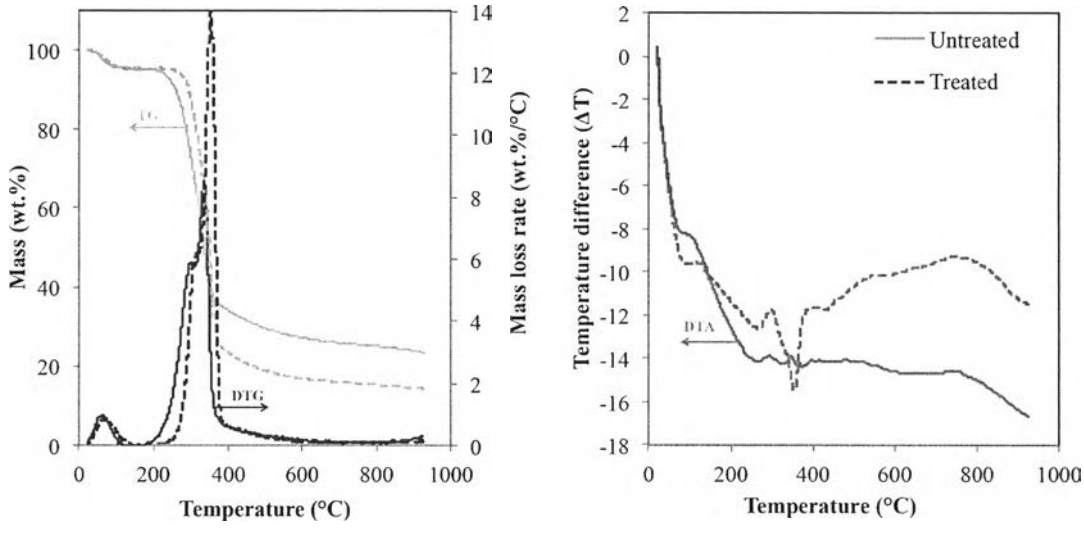

Figure 4.5 TG–DTG curves of corncob before and after pretreatment

4.4.4 Comparison of Total Sugars Obtained from Enzymatic Hydrolysis with Different Pretreatment Methods

The comparison of total sugars obtained from enzymatic hydrolysis of pretreated corncob between the optimum conditions of microwave-assisted NaOH pretreatment, hot plate (conventional heating) and autoclave are shown in Table 4.4. The results indicated that microwave irradiation had more heating efficiency than the hot plate and autoclave. Compared to the hot plate, at the same conditions, a total sugar obtained from microwave pretreatment was much higher and also enhanced the glucose/xylose ratio. The pretreatment with an autoclave was performed as a comparison between the conventional pretreatment method used in many studies since it could operate at temperatures above 100 °C (Sumphanwanich et al. 2008; Wang et al. 2010; Zhang et al. 2010). Microwave irradiation was achieved at a lower temperature in less time. Likewise, microwave-assisted pretreatment was more suitable for enzymatic hydrolysis than by direct heat.

Table 4.4 Comparison of reducing sugar concentration obtained from enzymatic hydrolysis with different pretreatment methods

Method	NaOH (%)	Temp (°C)	Time (min)	Glucose (gL^{-1})	Xylose (gL^{-1})	Reducing Sugar (gL^{-1})
Microwave	2	100	30	32.52	10.41	45.60
Hot plate	2	100	30	22.44	13.30	37.46
Autoclave	2	121	60	33.75	11.42	46.85

4.4.5 Ethanol Production

The ethanol profile of hydrolysate from alkali pretreatment and enzymatic hydrolysis using *S. cerevisiae* was compared with the semi-synthetic medium (YPD) as shown in Fig. 4.6. The hydrolysate contained 32.52 and

10.41 g L^{-1} glucose and xylose (Table 4.4), respectively, while YPD medium contained 30 g L^{-1}glucose. The ethanol production profiles were supposed to be the same because they had similar initial glucose concentrations; yet, the results show that the ethanol concentration in the hydrolysate dropped ~10 % after 36 h and again after 48 h of fermentation while it continued to increase in the YPD medium. The decrease in ethanol was consistent with the work of Xin et al. (2010) who used hydrolysate from newspaper as a substrate. They found that the maximum ethanol concentration at 8 h reduced over 10 % after 22 h of fermentation. It was likely due to the diauxic growth shift that occurred in *S. cerevisiae* when they encounter the exhaustion of glucose. Then they turn to utilize ethanol. Though, in this present study, there was 11.69 gL^{-1} remaining glucose in the hydrolysate. This indicates that hexose transportation was irreversibly arrested due to nitrogen depletion (Buglass 2011) because there was no additional nitrogen source like $(NH_4)_2SO_4$ or yeast extract in the hydrolysate (Sumphanwanich et al. 2008; Xin et al. 2010). That is why the YPD broth contained yeast extract and peptone as a source of vitamins, minerals, and particularly nitrogen, which further produced ethanol (O'Connor-Cox et al. 1991; Albers et al. 1996).

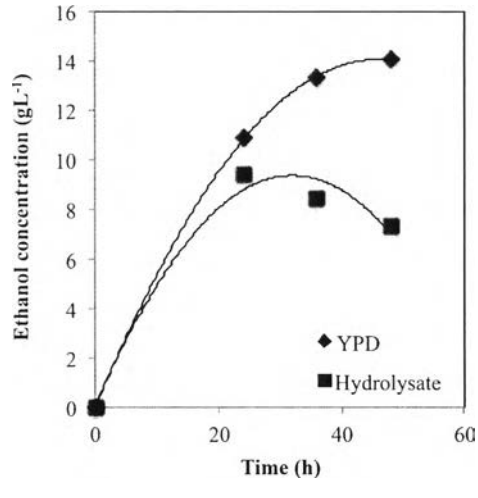

Figure 4.6 Ethanol profile of YPD (◆) and corncob hydrolysate (■) using *Saccharomyces cerevisiae*

4.5 Conclusions

Microwave–alkali pretreatment with sodium hydroxide on corncob was effective in improving enzymatic hydrolysis accessibility. The optimum conditions were found at 2 % NaOH at 100 °C for 30 min, which reduced lignin by 66.27 % and increased surface area by 38.31 %. The highest glucose and total sugar concentrations from enzymatic hydrolysis reached 32.53 and 45.60 g L^{-1}, respectively. Moreover, microwave assisted NaOH hydrolysis produced a high concentration of total sugars in a shorter time and at a lower temperature when compared to conventional methods using an autoclave or hot plate.

4.6 Acknowledgements

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