CHAPTER I
INTRODUCTION

First generation biofuels derived mainly from food crops serves many problems ranging from net energy losses to greenhouse gas emissions to increased food prices; therefore, second generation biofuels from lignocellulosic materials were developed (Menon *et al*., 2012). In recent years, high crude oil prices and increasing concerns over global warming have renewed the interests in biobutanol production. Biobutanol is an attractive renewable liquid transportation biofuel. It has a better energy density and performance than ethanol and can be made from more sustainable feedstocks than biodiesel. Hence, biobutanol has the potential to substitute for both ethanol and biodiesel.

Thailand is a major agricultural producer with abundant agricultural resources and their by-products. These agricultural by-products could be used as biomass energy that is an alternative with the potential to replace a wide diversity of fossil based products within the energy sector. Lignocellulosic materials mostly from agricultural wastes are a large quantity of raw materials and non-human sources such as bagasse, rice straw, and corncobs. The major component of these wastes is cellulose, which the microbial can change to biobutanol by fermentation.
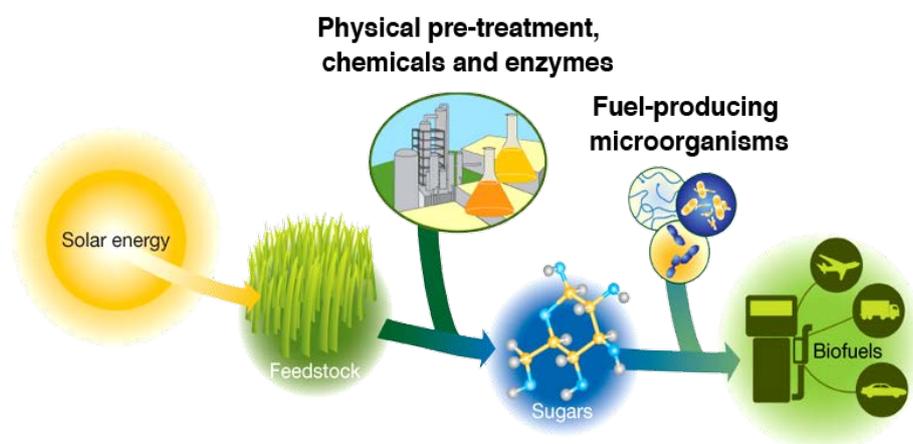
Figure 1.1 Biofuels production from lignocellulosic raw materials. (http://www.eoearth.org/article/Cellulosic_biofuels)

Corncobs are one of the potential lignocellulosic materials that can be used to produce butanol by the Acetone–Butanol–Ethanol (ABE) fermentation. Butanol production from lignocellulosic materials requires the hydrolysis of carbohydrate polymers into monomeric sugars, which is typically performed with enzymes. Pretreatments of lignocellulose using various alkaline or acidic reagents have been evaluated to improve the accessibility of the enzymes to the lignocellulosic material. The purpose of the pretreatment is to remove lignin and hemicelluloses, reduce cellulose crystallinity, and increase the porosity of materials.

Microwave is an alternative method to improve efficiency of the pretreatment due to its high heating efficiency, easy operation, and rapid heating which facilitates the disruption of their recalcitrant structure and microwave could be easily to combine with chemical reaction.

The production of biobutanol from lignocellulosic biomass has four major steps: pretreatment, hydrolysis, fermentation, and separation. Pretreatment step is required to remove lignin and hemicelluloses, reduce cellulose crystallinity, and increase the porosity of the material (Sun and Cheng, 2002). Followed by enzymatic hydrolysis step which carried out by cellulase enzymes, the products of the hydrolysis are usually reducing sugars including glucose. After that, the hydrolysate from pretreatment and hydrolysis step were fermented through acetone-butanol-ethanol (ABE) fermentation using solventogenic clostridia. Finally, the solvent products will be separated.

Dilute acid pretreatment has been applied to a wide range of feedstocks, including softwood, hard wood, etc (Zheng *et al*., 2009). The action mode of dilute acid is to solubilize hemicelluloses, enhance enzyme accessibility in hydrolysis process, and lower degree of polymerization and crystallinity of the cellulose component. In a number of studies, it has been suggested that dilute sulfuric acid and phosphoric acid are advantageous in the pretreatment of cellulosic biomass as it hydrolyzate much of the hemicelluloses (Gómez *et al*., 2010). In addition, there are a number of approaches available to reduce inhibitory effects. Overliming step after pretreatment (hydrolysate) is a technique, which has been noted that yields and productivities in overlimed biomass are higher than or comparable to washing biomass with water (Qureshi *et al*., 2010). Simultaneous saccharification and fermentation (SSF) was carried out for hydrolysis and fermentation step. The advantages of performing the enzymatic hydrolysis together with the fermentation, instead of in a separate step after the hydrolysis are the decrease of

end-product inhibition in enzymatic hydrolysis, and the reduced investment costs. On the other hand, the favorable conditions such as temperature, pH, and time for the enzymatic hydrolysis and the fermentation were required to investigate.

Objectives

The purpose of this work is to investigate the optimal condition of simultaneous saccharification and fermentation (SSF). In this work parameters are time, temperature, and pH by using Response Surface Methodology. In addition the effects of acid type and overliming step at the optimal conditions of pretreatment by dilute sulfuric acid and phosphoric acid on the reducing sugar yield were studied. Moreover, the hydrolysates of corncobs were prepared, employing combination pretreatment using microwave and two-stage pretreatment, including sodium hydroxide pretreatment, followed by dilute sulfuric acid pretreatment and enzymatic hydrolysis. Finally, acetone, butanol, ethanol (ABE) were produced from hydrolysed corncobs using *Clostridium beijerinckii*.

CHAPTER II

LITERATURE REVIEW

2.1 Biobutanol Production

Biobutanol can be produced by Acetone-Butanol-Ethanol (ABE) fermentation process, as shown in Figure 2.1 This process has been improved by using various strains of the bacterium either *Clostridium acetobutylicum*or *Clostridium Beijerinckii* and different substrates such as corn and molasses for many years. However, these substrates have high cost resulting in high price of butanol. Therefore, to produce butanol by using biomass as a feedstock is another choice to reduce butanol price.
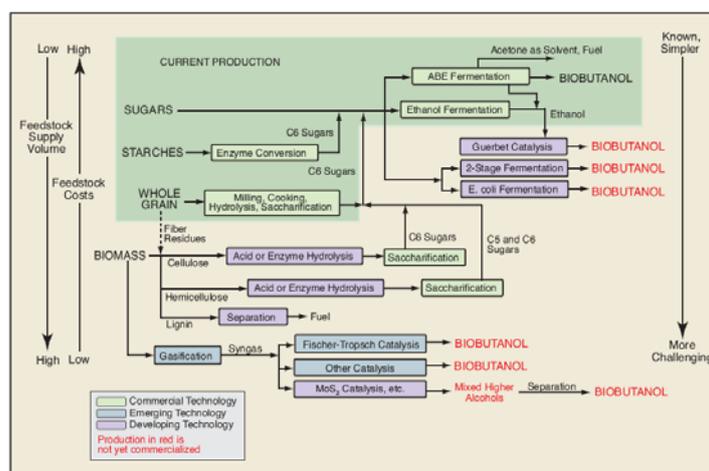

Figure 2.1 ABE fermentation process (Cascone, 2008).

Butanol is a four carbon alcohol. It contains more hydrogen and carbon. Butanol has several advantages. For example, butanol is easier to blend with gasoline and other hydrocarbon products and is safer to handle since butanol, is less volatile and explosive, has high flash point and low vapor pressure. It can be shipped and distributed through existing pipelines and filling stations. An 85% butanol/gasoline blends can be used in unmodified petrol engines and it is cleaner burning than ethanol (Nigam and Singh, 2011).

2.2 Lignocellulosic biomass

Lignocellulosic biomass such as agricultural waste and crop residue resources are one of the major renewable resources for fuels and chemicals. Lignocellulosic biomass consist mainly of cellulose, hemicellulose, and lignin that are closely associated in a complex crystalline structure, as shown in Figure 2.2 These components are complex polymers that are closely associated with each other producing the cellular complex of the vegetal biomass. Basically, cellulose forms a skeleton which is surrounded by hemicellulose and lignin. The complex structure results in limitation of enzymatic hydrolysis accessibility. Table 2.1 shows the composition of various lignocellulosic biomasses.
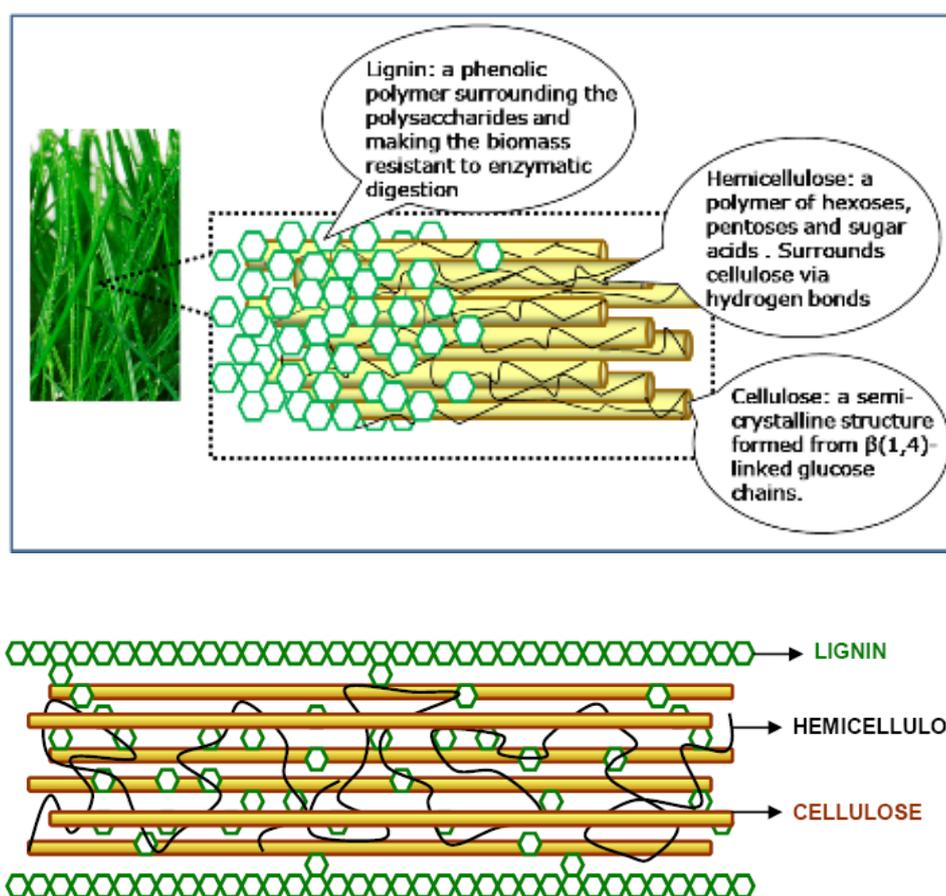

Figure 2.2 Representation of lignocellulose structure showing cellulose, hemicellulose, and lignin fractions (Mussatto *et al.,* 2010).

Table 2.1 Composition of representative lignocellulosicfeedstocks (Menon *et al.*, 2012)

Feedstocks	Carbohydrate composition (% dry wt)		
	Cellulose	Hemicellulose	Lignin
Bamboo	49-50	18-20	23
Banana waste	13	15	14
Corncobs	32.3-45.6	39.8	6.7-13.9
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1
Cotton stalk	31	11	30
Rice straw	29.2-34.7	23-25.9	17-19
Rice husk	28.7-35.6	11.96-29.3	15.4-20
Wheat straw	35-39	22-30	12-16
Grasses	25-40	25-50	10-30
Sugarcane bagasse	25-45	28-32	15-25
Nut shells	25-30	22-28	30-40

2.2.1 Cellulose

Cellulose is the major component of plant biomass including about 30–60% of total feedstock dry matter (Balat, 2011). Cellulose is a high molecular weight linear homopolymer of repeated units of cellobiose that is two anhydrous glucose rings joined via a β-1,4glycosidic linkage. The long-chain cellulose polymers are linked together by hydrogen and van der walls bonds, which cause the cellulose to be packed into microfibrils. The microfibrils are covered by hemicelluloses and lignin. The structure of cellulose is shown in Figure 2.3. By forming these hydrogen bounds, the chains tend to arrange in parallel and form a crystalline structure. Therefore, cellulose microfibrils have both highly crystalline region around 2/3 of the total cellulose, and less-ordered amorphous regions. More ordered or crystalline cellulose is less soluble and less degradable (Taherzadehand Karimi, 2008). The degree of cellulose crystallinity is a major factor affecting enzymatic hydrolysis of the substrate. It has been reported that a decrease in cellulose crystallinity especially influences the initial rate of cellulose hydrolysis. Physical or chemical pretreatment to disrupt the crystalline structure of cellulose is often used to promote the hydrolysis of biomass.
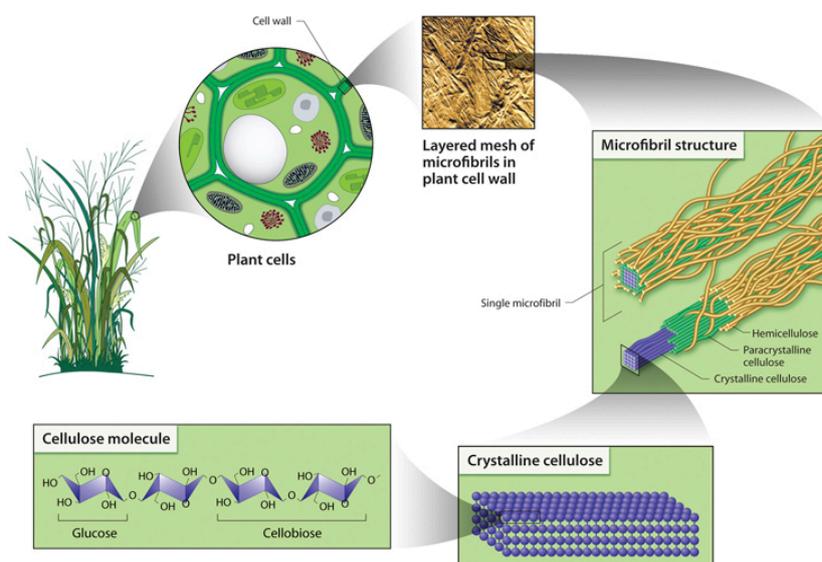
Figure 2.3 The structure of cellulose
(http://www.plantoils.in/portal/ce/blog/2008_12_01_archive.html).

2.2.2 Hemicellulose

The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars which are easy hydrolyzable polymers. Hemicellulose (20–40% of total feedstock dry matter) is a highly branched polymer of five-carbon (pentoses) and six-carbon (hexoses) sugars, as shown in Figure 2.4. Especially, hemicellulose contains xylose, and arabinose for five-carbon sugars andgalactose, glucose, and mannose for six-carbonsugars. Hemicellulose is more readily hydrolyzed compared to cellulose because of its branched, amorphous nature (Lee *et al.,* 2007). The dominant sugars in hemicelluloses are mannose in softwoods and xylose in hardwoods and agriculture residues (Taherzadeh and Karimi, 2008).
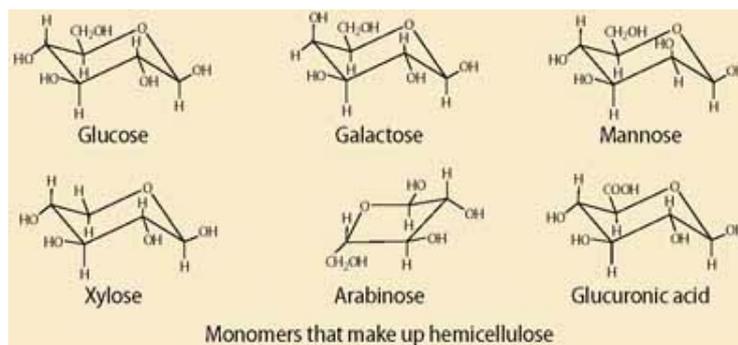
Figure 2.4 Monomers of hemicelluloses (Taherzadeh and Karimi, 2008).

2.2.3 Lignin

Lignin (15–25% of total feedstock dry matter) is an aromatic polymer. More specifically, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol basis are the ones most commonly encountered, as shown in Figure 2.5 (Harmsen *et al.,* 2010).The basic chemical phenyl propane units of lignin are bonded together by a set of linkages to form a very complex matrix (Demirbas, 2008). This matrix comprises a variety of functional groups, such as hydroxyl, methoxyl and carbonyl .Ligninis one of the drawbacks of using lignocellulosic biomass materials in fermentation, as it makes lignocellulose resistant to chemical and biological degradation (Taherzadeh and Karimi, 2008).
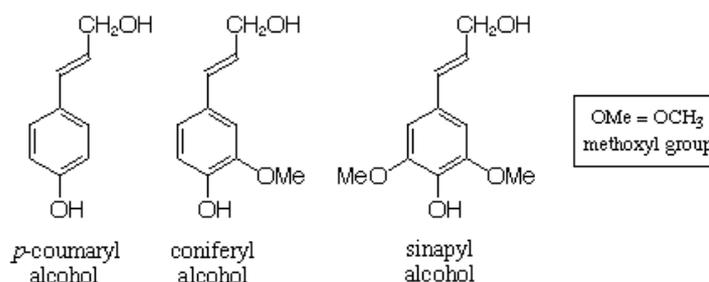

Figure 2.5 Phenyl propane units (Taherzadeh and Karimi, 2008).

2.3 Pretreatment of Lignocellulosic Biomass

Pretreatment is required to alter the structure of lignocellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers (cellulose and hemicelluloses) into fermentable sugars. Pretreatment has great potential for improvement of efficiency and lowering of cost through research and development

(Mosier *et al.,* 2003a, b). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective (Kumar *et al.,* 2009). In general, pretreatment methods can be classified into three categories, including physical, chemical, and biological pretreatment.
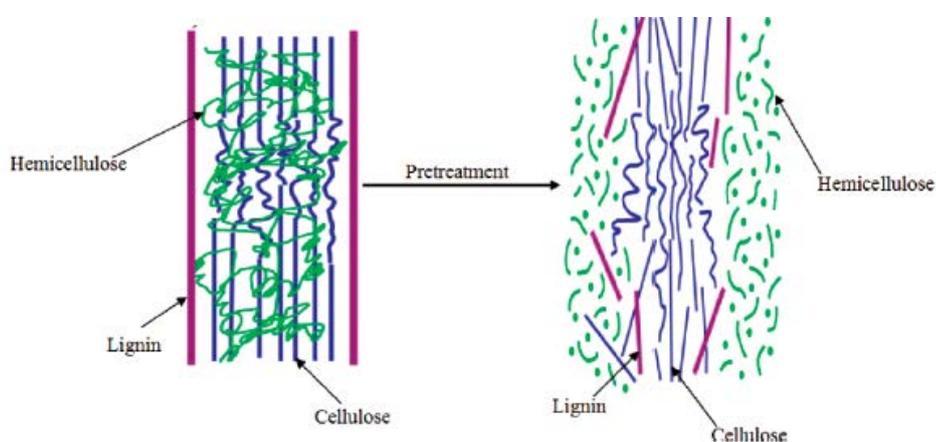

Figure 2.6 Schematic of the role of pretreatment (Kumar *et al*., 2009)

2.3.1 Physical Pretreatment

Lignocellulosic biomass can be comminuted by a combination of chipping, grinding, and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Kumar *et al.,* 2009, Sun and Cheng, 2002, Leustean, 2009). Power requirements of mechanical comminution depend on the final particle size and the biomass characteristics. Power requirements increase rapidly with decreasing particle size. These mechanical pretreatment techniques are time-consuming, energy intensive, or expensive to process (Balat, 2011).

2.3.2 Physico-chemical Pretreatment

2.3.2.1 SteamExplosion (Autohydrolysis)

In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials

undergo an explosive decomposition. Steam explosion is the most commonly used method for the pretreatment of lignocellulosic biomass. Steam explosion increases crystallinity of cellulose by promoting crystallization of the amorphous portions. Moreover, steam explosion hydrolyses hemicelluloses easily. That is evidence that steam explosion promotes delignification (Jeoh, 1998).

2.3.2.2 AmmoniaFiber Explosion

Ammonia fiber explosion (AFEX) is one of the alkaline physico-chemical pretreatment processes. The material is subjected to liquid ammonia at high temperature and pressure, and a subsequent fast decompression, similar to the steam explosion, which causes a fast saccharification of lignocellulosic biomass (Abril *et al.,* 2009). The system does not directly release any sugars but allows hemicellulose and cellulose to be attacked enzymatically and reduced to sugars (Balat, 2011).

2.3.2.3 Liquid Hot–water Pretreatment

Cooking of lignocellulosic biomass in liquid hot water (LHW) is one of the hydrothermal pretreatment methods applied for pretreatment of lignocellulosic biomass (Taherzadeh and Karimi,2008).LHW subjects biomass to hot water in liquid state at high pressure during a fixed period and it presents elevated recovery rates for pentoses and generates low amount of inhibitors (Tomas *et al.,* 2008). If the pH is maintained between 4 and 7, the degradation of monosaccharide sugars can be minimized (Hayes, 2009).

2.3.3 Chemical Pretreatment

Chemical pretreatments were originally developed and have been studied to date have had the primary goal of improving the biodegradability of cellulose by removing lignin and/or hemicellulose, and to a lesser degree of decreasing the degree of polymerization (DP) and crystallinity of the cellulose component. Chemical pretreatment is the most studied pretreatment technique among pretreatment categories. The various commonly used chemical pretreatments include: acid, alkali, organic acids, pH-controlled liquid hot-water, and ionic liquids.

2.3.3.1 AcidPretreatment

Acid pretreatment normally aim for high yields of sugar from lignocellulosic biomass due to this method gives high reaction rate and significantly improves cellulose hydrolysis (Karimi *et al.,* 2006). Acid pretreatment involves the use of

concentrated and diluted acids to break the rigid structure of the lignocellulosic biomass and remove hemicellulose and expose cellulose for enzymatic digestion (Silverstein *et al.,* 2008). The most commonly used acid is dilute sulphuric acid (H_2SO_4), which has been commercially used for a wide variety of biomass types such as switch grass, corn stover, spruce (softwood), and poplar. Other acids have also been studied, such as hydrochloric acid (HCl), phosphoric acid (H_3PO_4), and nitric acid (HNO_3). Acid pretreatments have been used as parts of overall processes in fractionating the components of lignocellulosic biomass due to its ability to remove hemicelluloses (Zhang *et al.,* 2007). The acid addition increases hemicellulose solubilization rate in comparison with the liquid hot water or steam explosion methods; therefore, the enzymatic digestibility of cellulose is enhanced. Acid pretreatment (removal of hemicellulose), followed by alkali pretreatment (removal of lignin) results in relatively pure cellulose (Menon *et al.,* 2012).

The potential of diluteacid pre–hydrolysis as a pretreatment method was studied for sugarcane bagasse, rice hulls, peanut shells, and cassava stalks (Martin *et al*., 2007). The pre–hydrolysis was performed at 122 $^{\circ}$C during 20, 40, or 60 min using 2% H_2SO_4 at a solid-to-liquid ratio of 1:10. Sugar formation increased with increasing reaction time. Xylose, glucose, arabinose, and galactose were detected in all of the pre–hydrolysates, whereas mannose was found only in the prehydrolysates of peanut shells and cassava stalks. The hemicelluloses of bagasse were hydrolyzed to a high-extent yielding concentrations of xylose and arabinose of 19.1 and 2.2 g/l, respectively, and a xylan conversion of more than 80%. High-glucose concentrations (26–33.5 g/l) were found in the prehydrolysates of rice hulls, probably because of hydrolysis of starch of grain remains in the hulls. Peanut shells and cassava stalks rendered low amounts of sugars on pre-hydrolysis, indicating that the conditions were not severe enough to hydrolyze the hemicelluloses in these materials quantitatively.

Cara *et al*., (2008) studied dilute acid pretreatment of olive tree biomass. Pretreatment was performed at 0.2, 0.6, 1.0, and 1.4% (w/w) H_2SO_4 and temperature range 170–210 $^{\circ}$C. It was found that 83% of hemicellulosic sugars in the raw material were recovered in the prehydrolysate obtained at 170 $^{\circ}$C, 1% H_2SO_4; however, the enzyme accessibility of the corresponding pretreated solid was not very high. The maximum enzymatic hydrolysis yield (76.5%) was attained from a pretreated solid at 210 $^{\circ}$C, 1.4 % acid concentration. Moreover, sugar recovery in the prehydrolysate was the poorest one among all the experiments performed. The maximum value (36.3 g sugar/100

g raw material) was obtained when the olive tree biomass at 180 $^{\circ}$C and 1% H_2SO_4 concentration, representing 75% of all sugars in the raw material.

2.3.3.2 AlkalinePretreatment

Alkali pretreatment refers to the application of alkaline solutions to remove lignin and various uronic acid substitutions on hemicellulose that lower the accessibility of enzyme to the hemicellulose and cellulose (Han *et al.,* 2009). These processesare operated at lower temperatures and pressures compared to other pretreatment technologies. Alkali pretreatment may be carried outat ambient conditions, but pretreatment time is measured in terms of hours or days rather than minutes or seconds (Mosier *et al.,* 2005). Sodium, potassium, calcium and ammonium hydroxide are appropriate chemicals for alkaline pretreatment. Of these four, NaOH has been studied the most (Kumar *et al*., 2009). Dilute NaOH treatment of lignocellulosic biomass causes swelling, leading to an increase in the internal surface area,a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure.

Wang (2009) studied NaOH pretreatment of Coastal Bermuda grass. Coastal Bermuda grass was pretreated with NaOH 0.5% to 3% (w/v) from 15 to 90 min at 121 $^{\circ}$C. Pretreatment time of 30 min was sufficient to achieve a significant amount of total lignin removal as long as the NaOH concentration was equal or over 1%. On the other hand, decreasing sodium hydroxide concentration from 1 to 0.5% significantly reduced total lignin removal, but there was no significant difference in lignin removal between 2 and 3% NaOH. Up to 86% lignin removal was observed. The optimal NaOH pretreatment conditions at 121 $^{\circ}$C for total reducing sugars production as well as glucose and xylose yields were 15 min and 0.75% NaOH. The total reducing sugars yield was about 71% of the theoretical maximum, and the overall conversion efficiencies for glucan and xylan were 90.43% and 65.11%, respectively.

Joshua *et al.* (2012) investigated the production of ABE from algae biomass. They found that the pretreatment with acid followed by alkaline produced 8.92 g/l of soluble sugars, whereas non-pretreated algae had only 0.73 g/l of soluble sugar. These data demonstrate the importance of pretreating complex substrates to produce fermentable sugars more efficiently. Additionally, pretreatment increases the surface area, or bio-availability, of the substrate for bacterial enzymes to hydrolyze the biomass more resourcefully (Kumar *et al.,* 2009).

Ponthein and Cheirsilp (2011) studied the pretreatment of palm pressed fiber by hydrothermal, acid and base to remove lignin and obtain high cellulose content fiber. The result indicated that the pretreatment with NaOH, followed by H_2SO_4 gave highest cellulose content and reduced the amount of hemicellulose and lignin more than pretreatment with sodium hydroxide or sulfuric acid alone. Moreover, Zhu *et al.* (2006) also found that the pretreatment of rice straw by alkali and acid increased cellulose content up to 75–80 %. The amount of hemicellulose and lignin content also significantly decreased to 3 and 3–5 % respectively, while the pretreatment with alkaline or acid alone gave similar lignin and hemicellulose content at 7–23 and 7–15 %, respectively.

2.3.3.3 Ozonolysis

Ozonolysis involves using ozone gas to breakdown the lignin and hemicellulose and increase the biodegradability of the cellulose. The pretreatment is usually carried out at room temperature and is effective at lignin removal without the formation of toxic by-products (Vidal *et al.,* 1988). Ozonation has been widely used to reduce the lignin content of both agricultural and forestry wastes. A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive (Kumar *et al*., 2009).

2.3.4 Biological Pretreatment

Biological pretreatment involves microorganisms, such as brown–, white–, and soft–rot fungi that are used to degrade lignin and solubilize hemicellulose. The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment process is very low and requires careful control of growth conditions (Sun *et al.,* 2002).

2.3.5 MicrowavePretreatment

Microwaves (frequencies of 0.3 to 300GHz and wavelengths of 1 m to 1 mm) lie between radio wave frequencies (RF) and infrared (IR) frequencies in the electromagnetic (EM) spectrum, as shown in Figure 8. Microwaves can be reflected, transmitted and/or absorbed. The absorbed microwave energy is converted into heat within the material, resulting in an increase in temperature. Gases, liquids and solids can interact with microwaves and be heated. Under certain conditions, gases can be excited

by microwaves to form plasmas that also can be useful for processing (Clark and Sutton, 1996).
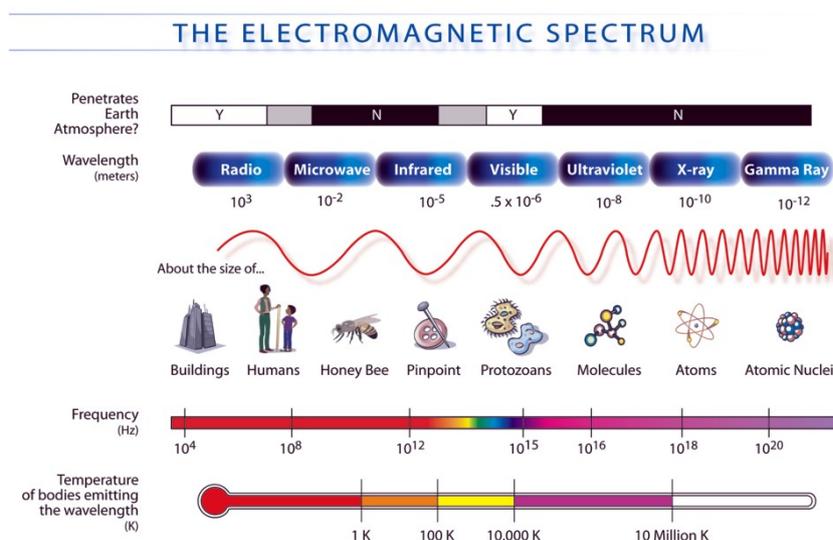

Figure 2.7 The electromagnetic spectrum with applications at various frequencies (https://mynasadata.larc.nasa.gov/ElectroMag.html).

Microwave pretreatment is an energy–efficient, environmentally–friendly technology. Microwave treatment seems to be similar to steam treatment. However, microwave may have new functions effective for acceleration of reactivity of cellulosic materials. In the conventional steam treatment, the cellulosic materials containing water have been heated by an external heat source, such as the electrical coils surrounding the autoclave, or high pressure steam has been supplied to the cellulosic materials externally. On the other hand, in the microwave, the cellulosic materials are heated internally; therefore, the water, cellulose, hemicelluloses, and the other low molecular compounds such as the organic acid contained in the cellulosic materials absorb the microwave as the kinetic energies when the polar molecules and their neighboring clusters are forced to orient to the specific direction. It thus appears that the microwave gives a direct serious shock to the polar molecules composing cellulosic materials (Ooshima *et al.,* 1984).

Microwave is an alternative method for conventional heating. Compared with conduction/convection heating, which is based on superficial heat transfer; the microwave uses the ability of direct interaction between a heated object and an applied

electromagnetic field to create heat. Therefore, the heating is volumetric and rapid. When microwave is used to treat lignocelluloses, it selectively heats the more polar (lossy) part and creates a "hot spot" with the inhomogeneous materials. It is hypothesized that this unique heating feature results in an "explosion" effect among the particles, and improves the disruption of the recalcitrant structures of lignocellulose. In addition, the electromagnetic field used in microwave might create non-thermal effects that also accelerate the destruction of the crystal structures (Hu *et al.,* 2008).

Compared with conventional heating techniques, microwave heating has the following additional advantages (Jones *et al.,* 2002).

- Higher heating rates
- No direct contact between the heating source and the heated material
- Selective heating may be achieved
- Greater control of the heating or drying process
- Reduced equipment size and waste

However, the microwave technology has been shown possibilities to be an energy efficient technique for chemical processing. The advantages and challenges of microwave processing are summarized in Table 2.2

Table 2.2 Benefits and challenges of microwave processing (Clark and Sutton, 1996)

Benefits	• Cost savings (time and energy, reduced floor space)
	• Rapid heating of thermal insulators (most ceramics and polymers)
	• Precise and controlled heating (instantaneous on/off heating)
	• Selective heating
	• Volumetric and uniform heating (due to deep energy penetration)
	• Short processing times
	• Improved quality and properties
	• Synthesis of new materials
	• Processing not possible with conventional means
	• Reduction of hazardous emissions
	• Increased product yields
	• Environmentally friendly (clean and quiet)
	• Self-limiting heating in some materials
	• Power supply can be remote
	• Clean power and process conditions
Challenges	• Heating low-loss poorly absorbing materials
	• Controlling accelerated heating (thermal runaway)
	• Exploiting inverted temperature profiles
	• Eliminating arcing and controlling plasmas
	• Efficient transfer of microwave energy to workpiece
	• Compatibility of the microwave process with the rest of the process line
	• Reluctance to abandon proven technologies
	• Timing
	• Economics

Antonio *et al.* (2005) studied thermal effect of microwave irradiation. Microwave irradiation is rapid and volumetric, with the whole material heated simultaneously. In contrast, conventional heating was slow and introduced into the sample from the surface. The temperature profile, as shown in Figure 2.8
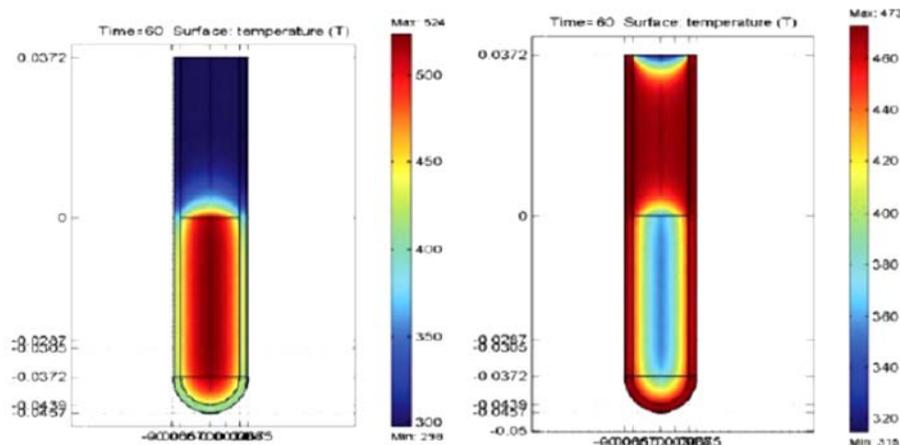

Figure 2.8 The temperature profile after 60 sec as affected by microwave radiation (left) compared to treatment in oil bath (right).

Microwave irradiation raises the temperature of the whole reaction volume simultaneously, whereas in the oil heated tube, the reaction mixture in contact with the vessel wall is heated.

Hu and Wen (2008) studied microwave-based heating pretreated switch grass, which was then hydrolyzed by cellulase enzymes. When switch grass was soaked in water and treated by microwave, total sugar (xylose + glucose) yield from the combined treatment and hydrolysis was 34.5 g/100 g biomass, equivalent to 58.5% of the maximum potential sugars released. This yield was 53% higher than that obtained from conventional heating of switch grass. With alkali loading from 0.05 to 0.3 g alkali/g biomass, microwave pretreatment resulted in a higher sugar yield than from conventional heating, with the highest yield (90% of maximum potential sugars) being achieved at 0.1 g/g of alkali loading. Scanning electron microscope (SEM) images revealed that the advantage of microwave over conventional heating was due to the disruption of recalcitrant structures. At optimal conditions of 190 $^\circ$C, 50 g/l solid content, and 30 min treatment time, the sugar yield from the combined pretreatment and hydrolysis was 58.7 g/100 g biomass, equivalent 99% of potential maximum sugars. The results demonstrate that microwave-

assisted alkali treatment is an efficient way to improve the enzymatic digestibility of switch grass.

Zhu *et al.* (2005) investigated microwave-assisted alkali pretreatment of wheat straw and its enzymatic hydrolysis and compared with the conventional alkali pretreatment process. The results show that the higher microwave power with shorter pretreatment time and the lower microwave power with longer pretreatment time had the same effect on the weight loss and composition at the same energy consumption. It was found that the wheat straw had a weight loss of 48.4% and a composition of cellulose 79.6%, lignin 5.7%, and hemicellulose 7.8% after 25 min microwave assisted alkali pretreatment at 700 W, compared with a weight loss of 44.7% and a composition of cellulose 73.5 %, lignin 7.2%, and hemicellulose 11.2% after 60 min conventional alkali pretreatment. The microwave assisted alkali pretreatment removed more lignin and hemicellulose from wheat straw with shorter pretreatment time compared with the conventional alkali one. Finally, the enzymatic hydrolysis of pretreated wheat straw (substrate concentration 50 g/l, enzyme loading 20 mg/g substrate) was also investigated and the results indicated that the microwave-assisted alkali pre-treated wheat straw had higher hydrolysis rate, reducing sugar concentration and glucose content in the hydrolysate than the conventional alkali pretreated one. Microwave-assisted alkali pretreatment is a potential alternative of wheat straw pre-treatment for it enzymatic hydrolysis.

Zhu *et al.* (2006) also examined three microwave/chemical processes for pretreatment of rice straw that are microwave/alkali, microwave/acid/alkali, and microwave/acid/alkali/H_2O_2 for its enzymatic hydrolysis and for xylose recovery from the pretreatment liquid. They found that xylose could not be recovered during the microwave/alkali pretreatment process, but could be recovered as crystalline xylose during the microwave/acid/alkali and microwave/acid/alkali/H_2O_2 pretreatments. The enzymatic hydrolysis of pretreated rice straw showed that the pretreatment by microwave/acid/alkali/H_2O_2 had the highest hydrolysis rate and glucose content in the hydrolysate.

In our group, Ploypradith P. (2010) studied the NaOH pretreatment with microwave on corncobs. The optimum conditions were found at 2 % NaOH at 100 $^\circ$C for 30 min which could reduce lignin by 66.27 % and increase in surface area by 38.31 %. And the highest glucose concentration can reach up to 32.53 g/l and total sugar of 42.93 g/l

was released. Moreover, microwave assists NaOH can produce total sugar concentration at shorter pretreatment time and lower pretreatment temperature compared with autoclave. In addition, total sugar concentration of microwave was higher than that of conventional heating.

Wanitwattanarumlug B. (2011) also studied the pretreatment of corncobs using microwave and potassium hydroxide. The highest sugar yield of 34.79 g/l was obtained from the corn cobs pretreated by microwave and 2 % KOH at 120 $^{\circ}$C for 25 min. The results indicated that microwave-assisted alkali treatment was an efficient way to improve the enzymatic hydrolysis accessibility.

There are many research work related with pretreatment methods to improve its conversion. Among them, the microwave-assisted chemical pretreatment is a more effective to enhance the enzymatic hydrolysis by accelerating the reaction. In this study, the combined pretreatment of corncobs with microwave was conducted. A two-stage pretreatment used 2 % NaOH at 100 $^{\circ}$C for 30 min with the optimal condition of NaOH from Ploypradith P. (2010), followed by H_2SO_4 pretreatment. In this work NaOH was used to separate lignin in the first stage and the effect of temperature, residence time and solid loading were determined in the second stage of two-stage pretreatment.

2.4 Inhibitors from Biomass Pretreatment

The pretreatment process generates numerous by-products that inhibit the growth of microorganism and fermentation. However, the generation of by-product depends on feedstock and pretreatment method (Jonsson *et al.,* 2013). Especially, acid pretreatment that solubilizes hemicelluloses, leading to the formation of pentoses, hexoses, and inhibitors, such as feruric acid, acetic acid, 2-furaldehyde (furfural), formic acid, and furoic acid. Furthermore, cellulose also degrades hexoses to 5-hydroxymethylfurfural (HMF), and levulinic acid. Other aldehydes and phenol can be formed by degradation of lignin. Figure 2.9 shows the inhibitors that are generated during pretreatment (Liu and Blaschek, 2010). Although more than 100 compounds were detected as inhibitors, many have not been well studied (Liu *et al,* 2004).
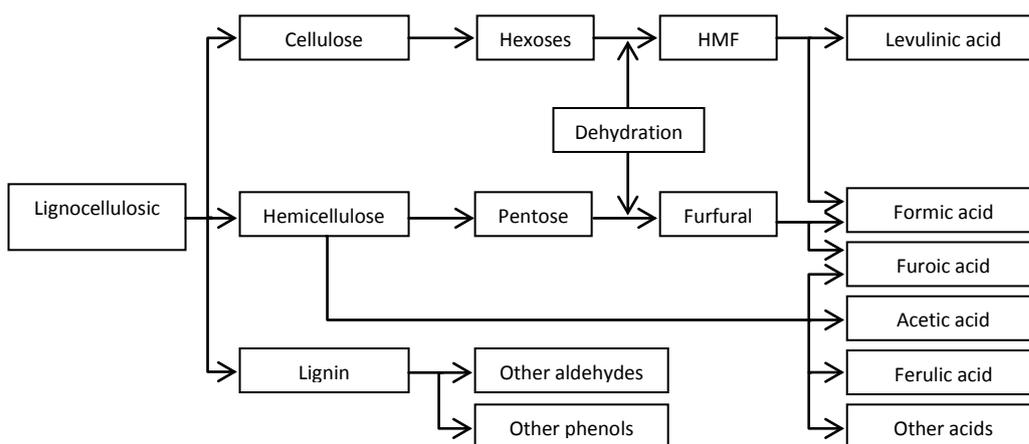
Figure 2.9 The degradation product of lignocellulosic biomass during pretreatment (Liu and Blaschek, 2010).

Inhibitors can be classified base on chemical functional groups into 4 groups as aldehydes, ketones, phenols, and organic acids. Some studies have investigated that the low molecular weight compounds have more toxic to microbes than high molecular weight due to easier to transport (Sierra *et al,* 1991).

2.4.1 Aldehyde inhibitors

Aldehyde inhibitors are compounds with one or more aldehyde functional groups with a furan ring, a benzene ring or a phenol structure. For example, furfural and HMF which contain a furan ring and an aldehyde functional group. Other aldehyde inhibitors include 4-hydroxy-benzaldehyde, vanillin (Klinke *et ai.,* 2002), syringe aldehyde, and other compounds having a benzene ring or a phenol-based structure including isovanillin, ortho-vanillin, and coniferylaldehyde (Liu and Blaschek, 2010). The structure of aldehyde inhibitors are shown in Figure 2.10.
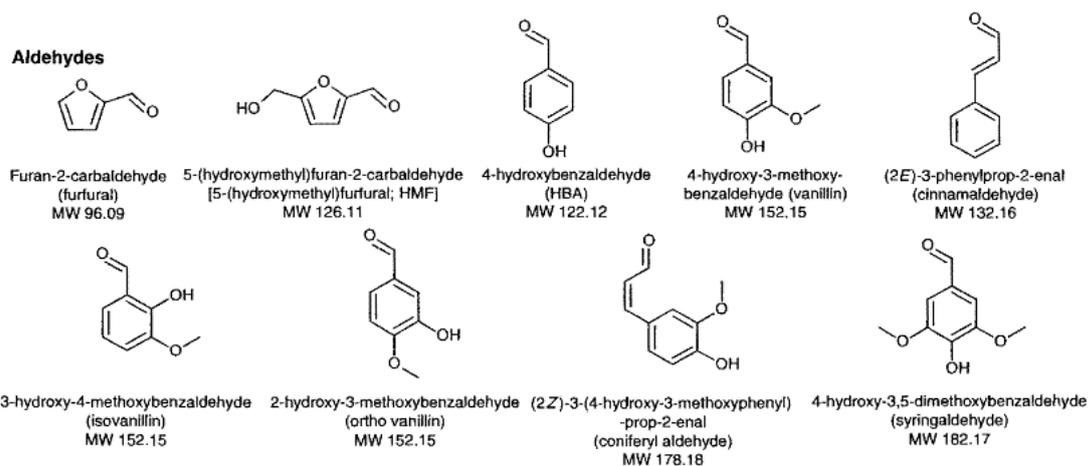
Figure 2.10 The structure of aldehyde inhibitors (Liu and Blaschek, 2010).

2.4.2 <u>Ketone inhibitors</u>

Ketone inhibitors include 4-hydroxyacetopheone and the closely related compounds acetovanillone and acetocsyringone. These compounds all share a common ketone functional group (Klinke *et al.,* 2003). The structure of ketone inhibitors are shown in Figure 2.11.
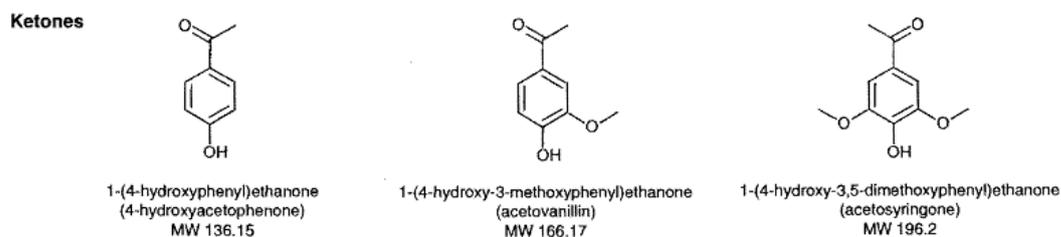

Figure 2.11 The structure of ketones inhibitors (Liu and Blaschek, 2010).

2.4.3 <u>Phenol-based inhibitors</u>

Phenol-based inhibitors are grouped together including phenol, benzene-l,2-diol (catechol), benzene-1,4-diol (hydroquinone), 4-ethylbenzene-l,2-diol (ethylcatechol), 2-methylphenol, 3-methylbenzene-l,2-diol (methy1catechol), 2-methoxyphenol (guaiacol), 4-(hydroxymethyl)-2-methoxyphenol (vanillyl alcohol), and 2,6-dimethoxybenzene-1,4-diol (Klinke *et al.,* 2002). The structure of phonol inhibitors are shown in Figure 2.12.
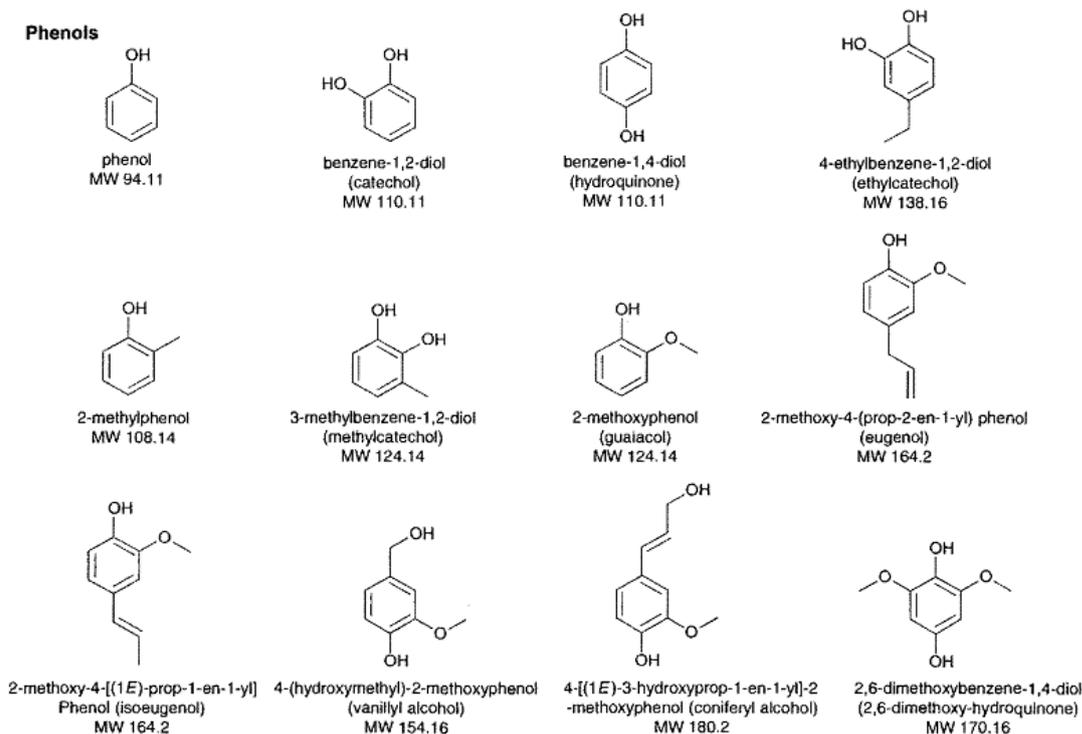
Figure 2.12 The structure of phenols inhibitors (Liu and Blaschek, 2010).

2.4.4 Organic acid inhibitors

Organic acid inhibitors include simple acids as well as furoic acid with a furan ring that was considered as being a furan inhibitor. Moreover, many previously recognized phenolic compounds are now grouped as members of the organic acid inhibitor class based on their functional structure. Inhibitory compounds of this class all contain a carboxyl functional group and include acetic acid, formic acid, levulinic acid, caproic acid, furoic acid, 4-hydroxybenzoic acid,3-hydroxybenzoic acid, 2-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, protocatechic acid, vanillic acid, gallic acid, syringic acid, 4-hydroxycinnarnic acid, ferulic acid, homovanillic acid, guaiaclyglycolic acid, and sinapic acid. These inhibitors are thought to be exerting their inhibitory actions via their carboxyl functional groups. The structures of organic acid inhibitors are shown in Figure 2.13.
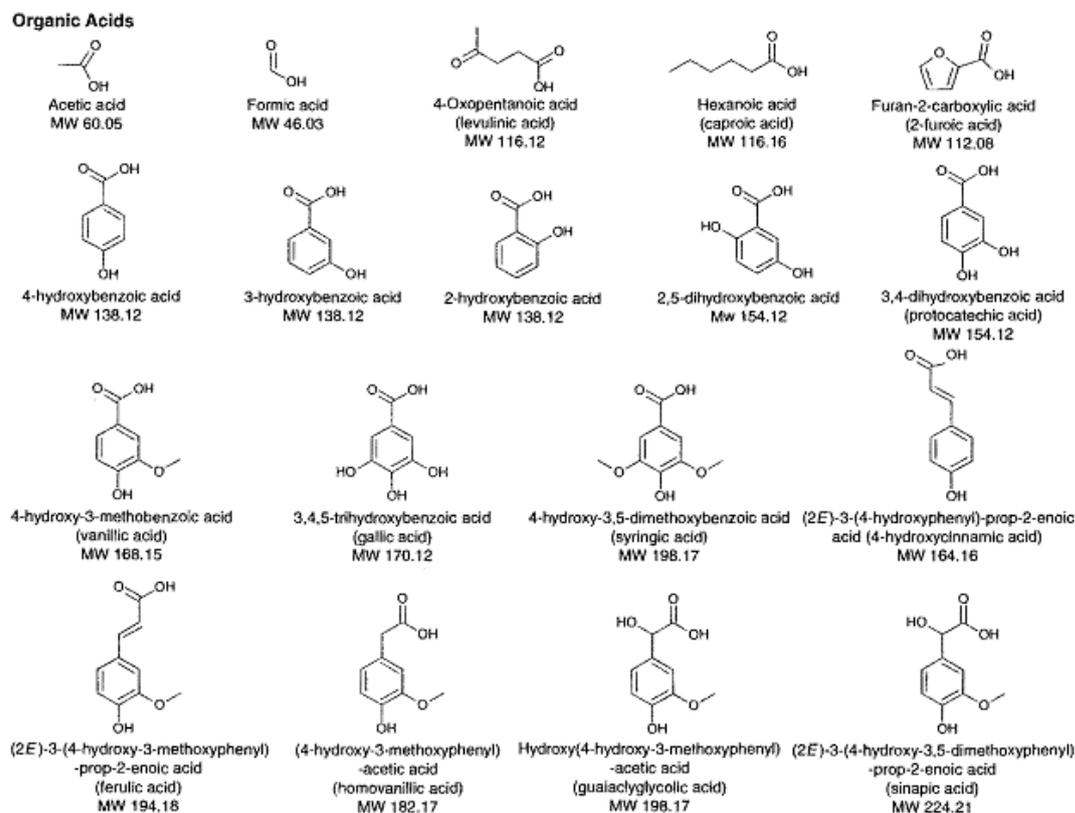
Figure 2.13 The structure of organic acid inhibitors (Liu and Blaschek, 2010).

2.5 Butanol Fermentation Inhibitors

Pretreatment has been seen as a preferred method that make the enzyme in enzymatic hydrolysis step highly digests biomass in order to produce high amount of reducing sugar for further ABE fermentation. However, the toxic compounds including weak acids, furan derivatives, phenolic compounds, vanillic aldehyde, and tannin are generated during pretreatment (Evaand Bärbel, 2000); therefore, no microorganism can efficiently produce butanol from lignocellulosic biomass (Weber *et al.,* 2010) due to the inhibitors affect cell growth and ABE production.

Ezeji *et al.* (2007) studied the impact of inhibitors that generated from H_2SO_4 pretreatment on ABE concentrations. The results showed that syringe aldehyde, ferulic, and ρ-coumaric acids were potent inhibitors of ABE production by *Clostridium beijerinckii* BA101, as shown in Figure 2.14. In general, ferulic and coumaric acids inhibit microorganism by damaging the hydrophobic sites of the bacteria cells because ferulic and coumaric acids are phenolic acids and phenolic compounds that affect membrane

permeability (Heipieper et al., 1994). Furthermore, the authors observed that furfural and HMF (3 g/l) were not inhitory to *Clostridium beijerinckii* BA101. However, the combination of furfural and HMF affects the culture negatively. In addition, the production of salt, sulfate, which is a result from sulfuric acid used for the pretreatment, was also toxic to *Clostridium beijerinckii* BA101.
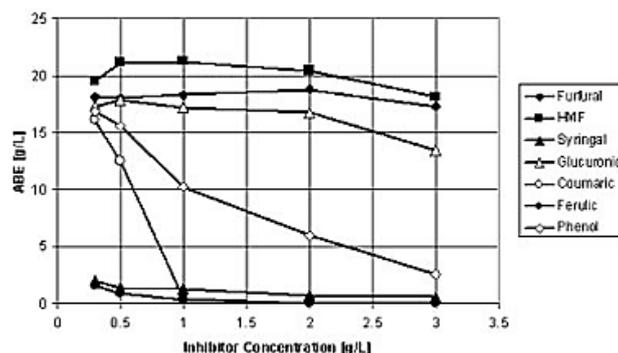

Figure 2.14 The effect of inhibitors generated during 0.5 % H_2SO_4 pretreatment of corn fiber on ABE concentrations (Ezeji *et al.,* 2007).

2.6 **Detoxification Method** (Chandel *et al.,* 2011)

Since inhibitors from pretreatment process can be problematic for fermentation, the removal of inhibitors from hydrolysates is necessary to enhance microbial growth and fermentation efficiency. Nevertheless, inhibitors depend on type of pretreatment and feedstock. The most detoxification methods are physical, chemical, and biological (Chandel *et al.,* 2011).

2.6.1 <u>Physical Methods</u>

2.6.1.1 *Evaporation*

The evaporation under vacuum can remove volatile compounds for example, furfural, acetic acid, and vanillin from hydrolysate of lignocellulosic biomass. However, evaporation retains the non-volatile toxic compounds such as lignin derivatives and extractives in the hydrolysates. A study by Wilson *et al.* (1989) found a decrease in the concentration of furfural, vanillin, and acetic acid by 100 %, 29 % and 54 %, respectively, compared with the concentrations in the hydrolysate. Likewise, Larsson *et al.* (1999) studied the removal of furfural and HMF using vacuum evaporation from wood

hydrolysate. The results showed that furfural and HMF were reduced to 90 %, 4 %, respectively.

2.6.1.2 *Membrane separations*

Adsorptive micro porous membranes have surface functional groups attached to their internal pores that remove the cell wall derived inhibitors from acid hydrolysates. Grzenia *et al.* (2010) applied the membrane extraction for inhibitors removal from sulfuric acid hydrolysate of corn stover. The results showed that acetic acid, formic acid, levulinic acid, HMF, and furfural were eliminated.

2.6.2 <u>Chemical Methods</u>

2.6.2.1 *Neutralization*

The neutralization of acid hydrolysates is required step before fermentation because of low pH. Alkali ($Ca(OH)_2$ or $NaOH$) is used for hydrolysates neutralization (pH in the range of 6-7). Phenolics and furfural may be removed by precipitation.

2.6.2.2 *Overliming*

It was reported that overliming is the most cost effective method for detoxifying soft wood hydrolysates. Detoxification after pretreatment and enzymatic hydrolysis or before fermentation by alkali treatment begins by adding lime ($NaOH$ or $Ca(OH)_2$) to adjust the pH of the hydrolysate to a high value (in the range of 9-11) followed by pH readjustment to 6.6 with H_2SO_4. Adjustment of pH with $Ca(OH)_2$ has been reported to increase the fermentability more than that with $NaOH$. The total amount of phenolic compounds was more efficiently decreased by $Ca(OH)_2$. However, it has been shown that monovalent ions, such as Na^+ affect the ethanol productivity negatively, whereas Ca^{2+} does not. However, acetic acid and sugars were not removed by treatment process with $NaOH$ or $Ca(OH)_2$. Moreover, a heating step in the overliming procedure (leading to some evaporation) improves fermentability (Larsson, 1999). Furthermore, Ethanol productivity was more than twice as high after treatment with $Ca(OH)_2$ compared with $NaOH$. The total concentration of phenolic compounds was affected by overliming detoxification due to phenolics were most efficiently removed with this method (Larsson, 1999).

2.6.2.3 *Activated Charcoal Treatment*

Activated charcoal is a cost effective method with high capacity to absorb compounds without affecting the amount of sugar in hydrolysate (Chandel *et al.,* 2007). The activated charcoal treatment efficiency depends on pH, temperature, contact time, and the activated charcoal taken and the liquid hydrolysate volume ratio (Prakasham *et al.,* 2009).

2.6.2.4 *Ion Exchange Resins*

Ion exchange resins treatment was applied to remove lignin-derived inhibitors, acetic acid and furfurals. The ion-exchange resins based separation of fermentative inhibitors may not be cost effective (Lee *et al.,* 1999).

2.6.3 <u>Biological Methods</u>

The biological methods for detoxification are more feasible, environmental friendly, with fewer side-reactions and less energy requirements (Parawira and Tekere, 2011). The microorganisms and/or the enzymes have potential to alter the chemical nature of inhibitors. However, the slow reaction time and the loss of fermentable sugars make this method unattractive (Yang and Wyman, 2008).

2.7 Response Surface Methodology (RSM) (Carley *et. al.,* 2004)

Response Surface Methodology (RSM) is a statistical and mathematical technique beneficial for developing, improving, and optimizing processes. A low-order polynomial is appropriate to use. In many cases, either a first-order or a second-order model is used. The first-order model is suitable when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variable space. In general, the first-order model is expressed as following equation

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i$$

Where Y_i is the response; x_i is the input variables, which influence the response variable Y_i; a_0 is the offset term; a_i is the *i*th linear coefficient.

The curvature in the true response surface is often strong enough that the first-order model is inadequate. A second-order model will be required. The following

equation was used to correlate the dependent and independent variables of second-order model.

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} x_i x_j$$

Where Y_i is the response; x_i, x_j are the input variables, which influence the response variable Y_i; a_0 is the offset term; a_i is the ith linear coefficient; a_{ii} is the quadratic coefficient and a_{ij} is the ijth interaction coefficient.

The second-order model is widely used in response surface methodology for several reasons:

1. The second-order model is very flexible due to a wide variety of functional forms; therefore it will often work well as an approximation to the true response surface.

2. It is easy to estimate the parameters in the second-order model. The method of least squares can be used for this purpose.

3. There is considerable practical experience indicating that second-order models work well in solving real response surface problems.

CHAPTER III

EXPERIMENTAL

3.1 Materials and Chemicals

3.1.1 Corncob waste was obtained from the Betagro Company. The collected corncob waste was stored at the ambient room temperature in a large bag. The average particle size of corncob waste was around 1.6 mm homogenized in a single lot and stored until used.

3.1.2 Sulfuric Acid (H_2SO_4)

3.1.3 Phosphoric Acid (H_3PO_4)

3.1.4 Calcium Hydroxide ($CaOH_2$)

3.1.5 Critic Acid Monohydrate ($C_6H_8O_7.H_2O$)

3.1.6 di-Sodium Hydrogen Orthophosphate Anhydrous (Na_2HPO_4)

3.1.6 pH Meter

3.1.5 Filter Paper

3.1.6 *Clostridium Beijerinckii* TISTR1461

3.1.7 Enzyme (ARR2–7 from Siam Victory, Thailand)

3.1.8 Standard glucose, xylose, arabinose, cellobiose, mannose, rhamnose, galactose, and furfural

3.2 Methodology

3.2.1 Dilute Sulfuric and Phosphoric Acid Pretreatment

Dilute Sulfuric and Phosphoric acid pretreatment of corncobs were performed in a laboratory scale stirred stainless steel reactor. The reactor is an acid resistant stainless steel and has a total volume of 1 L, with an electric heater and mechanic agitation. Corncob waste was suspended in 2% (w/w) H_2SO_4 or H_3PO_4 solution using liquid-to-solid ratios (LSR) of 15:1 and 10:1 for H_2SO_4, but only 10:1 for H_3PO_4 (mL of solution:g of corncob waste). The mixture was stirred until homogeneous before transferring to a stainless steel reactor. The pretreatment of H_2SO_4 was conducted under condition 120 °C for 5 min while H_3PO_4 pretreatment was carried out with condition 140

°C for 10 min. At the end of each run the reactor was removed from the heating jacket and the prehydrolysate agitated until the reactor was cooled to about 40 °C. Then the prehydrolysate was filtered to separate liquid and solid residue. The liquid fraction was collected for monomeric sugar analysis by using HPLC (Perkin Elmer LC200) equipped with a refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.60 mL/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C. While, the solid was washed thoroughly with tab water until no color and neutral pH in the resulting water was obtained. After that, the solid residue was dried for 24 h at 90 °C in an oven. The weight of dried samples was collected and stored in a ziplock bag prior to enzymatic hydrolysis step.

For two-stage pretreatment, dried corncobs was pretreated with 2 % (w/v) NaOH solution using a solid–to–liquid ratio (SLR) 67:1, g of corncobs: L of solution. A CEM (Matthews, NC, USA) MAR–5 HP–500 microwave system was used in this research. Then, the mixture of corncobs and NaOH solution was transferred to a microwave oven to treat corncobs at 100 °C for 30 min. After that the solid residues were thoroughly washed with tap water to neutral pH and dried in the oven at 105 °C for 24 h (Chen et al., 2011). The dried solid was treated with 1 % H_2SO_4 using different SLR (25:1 to 125:1). Then, it was transferred to a microwave oven (at 80 °C to 160 °C) for 5 to 25 min.

3.2.2 Lime Detoxification

The liquid fraction from pretreatment step was adjusted pH to 10 by $CaOH_2$ and followed by adding Na_2SO_4 1 g/l and heating in water bath at 80 °C for 30 min before being cooled to room temperature as normal step. After that, the product was adjusted pH to 4.8 with the same dilute acid and separated phase by filter paper subsequently and brought to ferment and analyze the composition by HPLC and GC.

3.2.3 Medium preparation

For DifcoTM Cooked meat medium (CMM) (Qureshi et al., 2008), 0.875 g of CMM pellet and 0.12 g glucose were dissolved with 6 ml of distilled water followed by sterilizing at 121 °C for 15 min and being cooled to room temperature subsequently. After that, one loop of cell spores was put into the prepared solution and heat shock at 80 °C for 2 min to activate and diminish weak cultures. The CMM culture solution was kept in 37 °C without agitation and waited for cells activation within 30 hours.

3.2.4 Inoculum Development

A 6 ml of active growing cells (from liquid CMM) was inoculated into development P2 medium, prepared in 100 ml screw-capped bottle. The inoculums development P2 medium contained glucose (30 g/l), yeast extract (1g/l), and stock solution [(buffer: KH_2PO_4 50 g/l; K_2HPO_4 50 g/l; ammonium acetate 220 g/l), (vitamin: para-amino-benzoic acid 0.1 g/l; thiamin 0.1 g/l; biotin 0.001 g/l), mineral: $MgSO_4 \cdot 7H_2O$ 20 g/l; $MnSO_4 \cdot H_2O$ 1 g/l; $FeSO_4 \cdot 7H_2O$ 1 g/l; NaCl 1 g/l)] (Qureshi et al., 1999). The solution containing 1.8 g of glucose and 0.06 g of yeast extract were dissolved in 53.38 ml of distilled water and sterilized at 121 °C for 15 min and being cooled to room temperature subsequently. Next, the filter sterile of buffer, vitamin, and mineral were added by 500 μl, 20 μl, and 100 μl, respectively. The P2 medium solution was kept in 37 °C without agitation for 8 h; cells were ready for simultaneous saccharification and fermentation step.

3.2.5 Simultaneous Saccharification and Fermentation

First of all, Simultaneous Saccharification and Fermentation was performed by using the washed water-insoluble residue of pretreated corn cobs with 2% (w/w) H_2SO_4 120 °C 5 min and 10:1 LSR for 4 g mixed with 73.90 ml of Critic-Phosphate buffer solution by varying pH 4.8, 5.7, and 6.6 respectively and adding initial glucose sugar for 20 g/l. Secondly, mixed solution will be brought to sterile in autoclave reactor (121 °C for 15 min) then cooled until room temperature. Then, 4 ml of P2 medium was mixed with following supplementations: 0.8 ml of sterilized yeast stock 1g/l, 666.8 μl of ammonium acetate 220 g/l, 133.2 μl of mineral ($MgSO_4 \cdot 7H_2O$ 20 g/l, $MnSO_4 \cdot H_2O$ 1 g/l, $FeSO_4 \cdot 7H_2O$ 1 g/l, NaCl 1 g/l) and 26.8 μl of (Vitamins: para-amino-benzoic acid 0.1 g/l, thiamin 0.1 g/l, biotin 0.001 g/l). All components were mixed and softly shaken for making homogeneous solution. Next, the mixture was brought to the prepared fermentation broth followed by adding enzyme of 10.36 FPU/g of biomass and purged with nitrogen gas for 10 minute accordingly. After that, mixed solution will be shaken in incubator at different temperatures (37 °C, 43.5 °C and 50 °C) and different time (24 h, 48 h and 72 h). At the end of reaction time, the product solution was filtered to separate liquid and solid residue. The liquid was determined the quantity of monomeric sugars yield by HPLC (Perkin Elmer LC200) equipped with a refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.30 ml/min, mobile phase 0.005 M of

H_2SO_4 and column temperature was fixed at 60 °C and determined the quantity of Acetone-Butanol-Ethanol yield by GC.

3.2.6 Response Surface Methodology (RSM) for Experimental Design

RSM with a full factorial central composite design (CCD) was employed in this work. The variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (\ i = 1,2,3,...,\ k\ )$$

Where x_i is the code value of the independent variable; X_i is a real value; X_0 is its real value at the centre point; and ΔX_i is the step change in the variable X_i. pH (X1), temperature (X2), and reaction time (X3) were chosen as the three independent factors. Their values at different coded and actual levels were shown in Table 3.1. The code and uncoded level of variables were used in experimental design are listed in Table 3.2.

A second order polynomial regression model was used to express Y_i as a function of the independent variables as follows:

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} x_i x_j$$

Where Y_i is the response; x_i, x_j are the input variables, which influence the response variable Y_i; k is the number of variables; a_0 is the constant or offset term; a_i represents the coefficients of the linear parameters; a_{ii} represent the coefficients of interaction parameters and a_{ij} represents the coefficients of quadratic parameters. Analysis of variance (ANOVA) program was used for regression analysis of the data and to estimate the coefficients of the regression equation.

Table 3.1 Design of experiment for statistical analysis. Coded and experimental values of the factors for RSM

Independent variables	Symbol	Coded		
		-1	0	1
pH	X1	4.8	5.7	6.6
Temperature (°C)	X2	37	43.5	50
Reaction time (h)	X3	24	48	72

Table 3.2 Central composite design (CCD)

Run	Coded values			Real values		
	pH	Temperature	Time	pH	Temperature ($^{\mathbf{o}}$C)	Time (h)
1	1	1	1	6.6	50	72
2	1	1	-1	6.6	50	24
3	1	-1	1	6.6	37	72
4	1	-1	-1	6.6	37	24
5	-1	1	1	4.8	50	72
6	-1	1	-1	4.8	50	24
7	-1	-1	1	4.8	37	72
8	-1	-1	-1	4.8	37	24
9	1	0	0	6.6	43.5	48
10	-1	0	0	4.8	43.5	48
11	0	1	0	5.7	50	48
12	0	-1	0	5.7	37	48

Table 3.2 Central composite design (CCD) (cont.)

Run	Coded values			Real values		
	pH	Temperature	Time	pH	Temperature ($^{\text{o}}$C)	Time (h)
13	0	0	1	5.7	43.5	72
14	0	0	-1	5.7	43.5	24
15	0	0	0	5.7	43.5	48
16	0	0	0	5.7	43.5	48
17	0	0	0	5.7	43.5	48
18	0	0	0	5.7	43.5	48
19	0	0	0	5.7	43.5	48
20	0	0	0	5.7	43.5	48

3.3 Analysis Method

3.3.1 High Performance Liquid Chromatography (HPLC)

The quantity of monomeric sugars yield was determined by High Performance Liquid Chromatography (HPLC) equipped with a refractive index detector (Series 200 LC/S/N291N5060508, Perkin Elmer) using an Aminex-HPX 87H column (300 mm x78 mm, Bio-Rad Lab, USA) and a guard column (30 mm x 4.6 mm, Bio-Rad Lab, USA) under these following conditions: flow rate 0.60 ml/min, mobile phase 0.005 M of H_2SO_4 and column temperature was fixed at 60 °C.

3.3.2 UV-VIS Spectrometer (UV)

After pretreatment process, the product in liquid phase, prehydrolysate, was measured the total sugar yield by UV-VIS (1800) equipped with the photometric detector mode and UV wavelength of 540 nm.

3.3.3 Scanning Electron Microscope (SEM)

The surface morphology and porosity of untreated corncobs pretreated sample after pretreatment process was observed by Scanning Electron Microscope (SEM) (HitachiS–4800 SEM instrument operated at 10–15 kV accelerated voltage). Besides, samples were dried in oven (80 ℃) overnight before analysis.

3.3.4 X-Ray Diffraction (XRD)

Cellulose crystallinity was determined by X-ray diffraction (Bruker AXS Model D8 Discover). Samples of each size fraction were analyzed. All samples were scanned from 2θ = 5° to 40° with a step size of 0.02°. Determination time was 0.5 s/0.02°. In addition, the chemical structure of lignocellulosic biomass changing between fresh and treated corncob waste were compared.

3.3.5 Surface Area Analysis

BET surface area information of the pretreated residues and untreated were measured by N_2 adsorption/desorption measurements (Quantachrome instrument; model: BELSORP–max, BEL, Japan) done at 100 ℃ (373 K). Prior to measurement, all biomass materials were dried at 90 $^{\circ}$C for 48 h and then 1 g of sample was put into tube of the Quantachrome instrument and degassed using a vacuum for 24 h. The BET surface area and pore volume were obtained from the N_2 adsorption/desorption curves using BELSORP–max software.

3.3.6 Gas Chromatography (GC)

The acetone-butanol-ethanol yield produced from fermentable sugars that got from ABE fermentation step was measured concentration by a gas chromatograph (Series Perichrom 2100) equipped with a flame ionization detector using Innowax column length 30 m under these following conditions: N_2 flow rate 45 ml/min, detector temperature 240℃, injection Temperature 240℃, column temperature 170℃, and volume injection 0.5 μl.

3.3.7 Fibertect M6

The methods to determine cellulose, hemicelluloses, lignin, and ash in solid residue after pretreatment and enzymatic hydrolysis were amylase neutral detergent fiber (NDF), acid detergent fiber (ADF), and cellulose acid detergent lignin (ADL). Amylase

neutral detergent fiber (NDF) method used to measure the quantity of cellulose, hemicelluloses, and lignin. After that, the hemicelluloses were detected by using acid detergent fiber (ADF) method. Then, cellulose was measured by using cellulose acid detergent lignin (ADL) method. The quantity of cellulose was measured when suspended with sulfuric acid. After this process was completed, the sample was burned at 500 °C for 2 h in order to determine lignin's quantity. The solid residue after this process is ash.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Chemical Composition of Corncobs

Corncobs were measured the quantities of cellulose, hemicellulose, and lignin. The results are shown in Table 4.1 as the percentage of dry weight unit.

Table 4.1 Chemical composition of corncobs

Chemical components	Dry solid (%)
Cellulose	39.31
Hemicellulose	34.46
Lignin	10.47
Others	15.76

Table 4.1 presents the composition of corncobs, which consists of 39.31% cellulose, 34.46% hemicelluloses, and 10.47% lignin respectively. Commonly lignocellulosic biomass such as corn, cassava, sugarcane etc. is mainly comprised of cellulose (38–50%), hemicelluloses (23–32%) and lignin (15–30%) % wt. of biomass material on dry basis along with smaller amounts of extractive and ash. Cellulose is a main structural component in plant cell walls, which its structure is a homopolymer consists of repeating β–D–glucose units. This linear structure is connected by β–1–4 glycosidic bonds. The cellulose in a lignocellulosic biomass can be divided into two parts: a crystalline structure and amorphous structure. Crystalline cellulose is a major proportion of cellulose while a small proportion is amorphous cellulose. Cellulose is a highly crystalline material which mainly effects to resist enzymatic hydrolysis accessibility. Hemicellulose represents as the connection between cellulose and lignin which results in the network of cellulose-hemicellulose-lignin more rigidity. Hemicellulose is a heteropolymers of carbohydrate which consists of five-carbon sugars (e.g. xylose and arabinose) and six-carbon sugars (e.g. mannose, glucose, and galactose). Hemicellulose has a lower molecular weight than cellulose and the structure is random, amorphous, and

branched that causes hemicellulose can hydrolyze easily. In addition, lignin made up of three types of phenolic acids (p–coumaryl, coniferyl, and sinapyl alcohol) and they linked in a three dimensional structures affected lignin particularly difficult to hydrolyzed (Kumar *et al.,* 2009).

4.2 One-stage Pretreatment Using Acid

4.2.1 A Comparison between Dilute Sulfuric Acid and Phosphoric Acid at an Optimal Pretreatment Condition

The purposes of the pretreatment process are to remove lignin and hemicellulose, disrupt the cellulose crystalline structure, and increase the porosity of the materials. Dilute acid pretreatment helps to break down the crystalline cellulose and hemicellulose polymers from lignocellulosic biomass to form individual sugar molecules, which can be fermented into biofuels (Wyman 1994). It would be recognized that the structure of hemicellulose is branches with short lateral chains, helps them be simple to hydrolyze than cellulose. Furthermore, pretreatment process could increase surface area and porosity of lignocellulosic structure in order to increase enzymatic digestibility of cellulose in enzymatic hydrolysis process; therefore, the enzyme can be easy to hydrolyze polysaccharide to monosaccharide. Corncobs were pretreated with dilute acid under these following conditions: 2% w/w at 120 °C for 5 min and 15:1 liquid-to-solid ratio (LSR) in case of sulfuric acid (Tangmanasakul, 2011), while phosphoric acid is 2% w/w at 140 °C for 10 min and 10:1 liquid-to-solid ratio (LSR) (Satimanont, 2012). However, liquid-to-solid ratio (LSR) in sulfuric acid (H_2SO_4) optimal condition has not been investigated in the previous work. Therefore, comparison of sulfuric condition between 10:1 liquid-to-solid ratio (LSR) and 15:1 under the same condition will be investgated. When this process was completed, the monomeric sugar yield was measured from the product in liquid phase, prehydrolysate.

Table 4.2 and Figure 4.1 illustrate monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatments by using 2% (w/w) with different pretreatment times, temperatures, and LSRs and it was found that the monomeric sugar yield was increased with decreased pretreatment LSR. The major component in prehydrolysate from pretreatment process was xylose. Owing to the structure of hemicellulose which is a random, amorphous, and branches, therefore, xylose

presented as the part that can be readily to hydrolyze than glucose. In term of monomeric sugar (glucose, xylose, and arabinose) yield for sulfuric acid condition, the trend clearly presents that it increased with pretreatment LSR from 15:1 to 10:1. These results were supported by previous works, which reported that the appropriate ratio is 10:1 which acid and corncobs are being well-mixed with the highest monomeric sugar yield (Jeevan *et al*, 2011, Satimanont, 2012). When LSR was too high, it caused loss volume and reduced total monomeric sugar. Sufficient liquid is required to dissolve hemicelluloses, lignin, and their degradation products; too small LSR decreases the solubility because less liquid is present, and deposition of dissolved hemicellolses, leading to a decrease in total monomeric sugar (Jeevan *et al*, 2011). However, furfural formation at a 10:1 LSR higher 15:1 LSR because of high amount of xylose yield which presented in prehydrolysate can be converted to higher furfural (Satimanont, 2012). When comparing with phosphoric acid condition, monomeric sugar contents are lower than sulfuric condition with 10:1 LSR according to higher temperature and longer time from 120 ℃ to 140 ℃ and 5 min to 10 min respectively. Satimanont, 2012 reported that increasing pretreatment temperatures and times can drive xylose degradation into furfural. Under severity pretreatment conditions (> 120 ℃), the xylose yield decreased owing to xylose degradation into furfural which can obstacle the micro-organism growing in fermentation process (Ezeji *et al.,* 2007). The result of total monomeric sugar of 10:1 LSR in sulfuric acid condition is greater than in phosphoric acid condition with lower temperature and time. The highest total monomeric sugar (35.72 g/l) was obtained in pretreated corncobs with 2% H_2SO_4 at 120 ℃ for 5 minutes and 10:1 LSR. However, the glucose, xylose, and arabinose concentrations were not much different from reducing yield of phosphoric acid pretreatment. While, the highest furfural content can be found in pretreated corncobs with 2% H_3PO_4 at 140 ℃ for 10 min and 10:1 LSR (0.37 g/l).

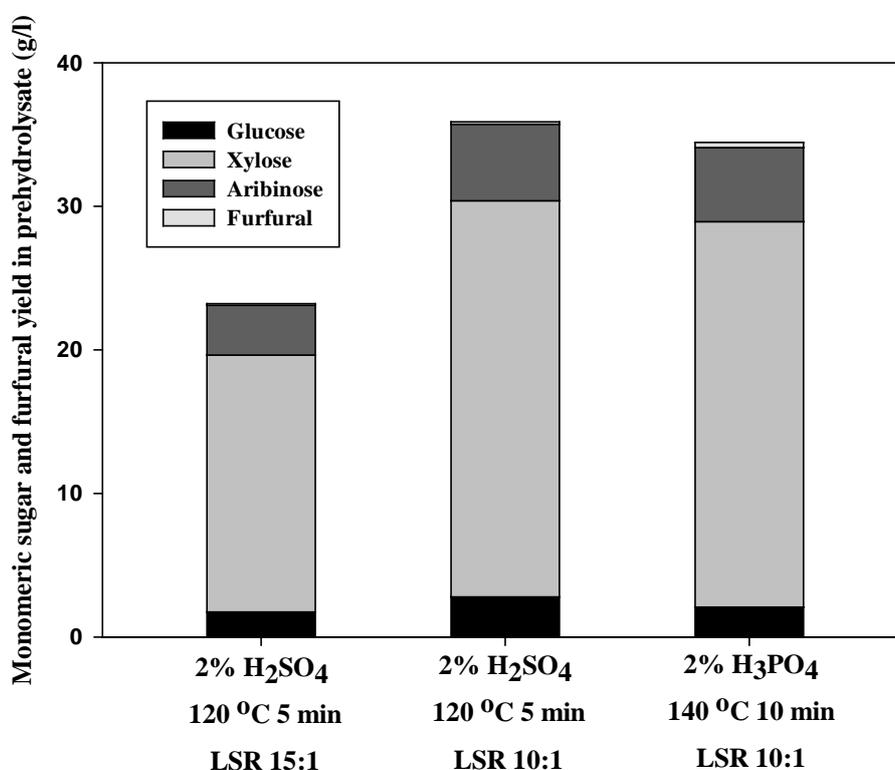**Table 4.2** Total sugar yield of corncobs in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment using 2% (w/w) with different pretreatment times, temperatures and LSRs

Conditions	Glucose (g/l)	Xylose (g/l)	Arabinose (g/l)	Total sugar (g/l)	Furfural (g/l)
2% H_2SO_4 at 120 °C for 5 min and 15:1 LSR	1.74	17.89	3.47	23.10	0.11
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR	2.79	27.60	5.33	35.72	0.17
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR	2.08	26.86	5.15	34.09	0.37

Figure 4.1 Monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment by using 2% (w/w) with different pretreatment times, temperatures and LSRs.

4.2.2 <u>Surface Area Analysis</u>

Surface area, pore volume and pore diameters of corncobs were measured and the results are shown in Table 4.3.

Table 4.3 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 corncobs	3.83	6.20×10^{-3}	64.7
Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 15:1	4.41	1.36×10^{-2}	123.4
Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 10:1	4.58	1.20×10^{-2}	104.8
Pretreated corncobs with 2% H_3PO_4 at 140 °C for 10 min and LSR 10:1	3.90	1.32×10^{-2}	116.1

Accessible cellulose surface area is one of the main factors which have influenced to the ease of enzymatic hydrolysis of pretreated lignocellulosic biomass. Table 4.3 shows the physical properties of dilute acid pretreated corncob compared with untreated corncob. It was found that the surface area and total pore volume of pretreated corncobs were higher than the untreated corncobs after dilute sulfuric and phosphoric acid pretreatments. In case of sulfuric acid pretreatment conditions at 2% H_2SO_4 at 120 °C for 5 min, it indicated that surface area of the substrates pretreated at a 15:1 LSR was lower than the substrates pretreated with 10:1 LSR. The highest surface area of 4.58 m^2/g and total pore volume of 1.36×10^{-2} were obtained under the pretreatment condition at 120 °C, 5 min pretreatment times, 2% (w/w) H_2SO_4, with 10:1 LSR. On the other hand, surface area of pretreated corncobs with 2% (w/w) H_3PO_4 at 140 °C for 10

min with 10:1 LSR was not much different from untreated corncobs. For average pore diameter, all pretreated samples have higher diameter than unpretreated sample which benefit to contact of the enzyme (Mercier and Pinnavaia, 1997).

4.2.3 X-ray Diffraction (XRD) Analysis

The degree of cellulose crystallinity is a major factor affecting enzymatic hydrolysis of the substrate (Yoshida *et al*., 2008). Crystallinity is believed as an important feature affecting enzymatic saccharification of cellulose. CrI refers to the fraction of crystalline material in the sample and CrI value depends on the compositions in lignocellulosic materials. Crystallinity refers as a measure of ordered orientation of cellulose crystallites and it would be increased when removing of amorphous component (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 (Kumar *et al*., 2009). Biomass crystallinity as expressed by crystallinity index (CrI) was determined according to:

$$CrI = 100 \times [(I_{002} - I_{amorphous})/I_{002}]$$

in which, 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 $2\theta = 18.7°$ (Kumar *et al*., 2009). Crystallinity increased with changing in pretreatment temperature, time, acid type, and LSR, as compared with the untreated corncob and the results are illustrated by XRD pattarns in Figure 4.2. Table 4.4 presents crystallinity index of pretreated corncob with dilute sulfuric and phosphoric acid under various pretreatment conditions and untreated corncob. There are three peaks of cellulose at the 2θ of 15.61°, 22.09°, and 34.53°, respectively. The higher intensity peak indicates that more crystalline part of pretreated corncobs. Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 10:1 gives the highest crystallinity index, 39.98. In the meantime, the CrI index of pretreated corncobs with 2% H_3PO_4 at 140 °C for 10 min and LSR 10:1 is almost the same as the previous one. Kim *et al.,* (2003) reported that the crystallinity index increased with pretreatment reflecting removal of the amorphous portion of biomass. Besides, Yoshida *et al*., (2008) discovered that the crystallinity will be

increased after pretreatment process because the removal of lignin which is considered to be amorphous covering cellulose in lignocellulosic biomass.
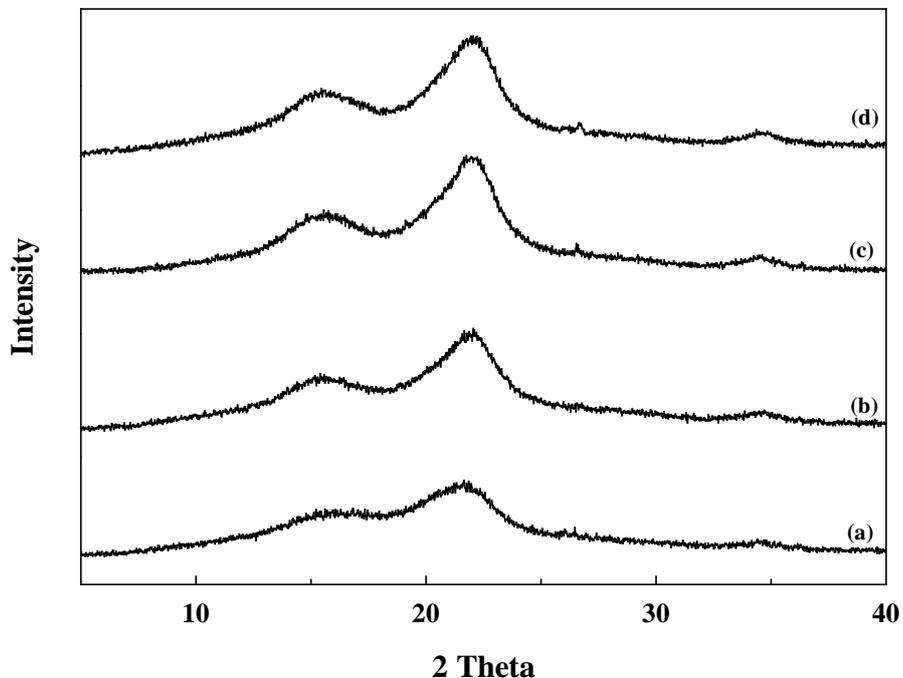

Figure 4.2 XRD patterns of the corncob. Symbols; (a) fresh corncob, (b) pretreated corncob at 120 °C, 5 min of pretreatment time by using 2% (w/w) H_2SO_4 at a 15:1 LSR, (c) pretreated corncob at 140 °C, 10 min pretreatment time by using 2% (w/w) H_3PO_4 at a 10:1 LSR, (d) pretreated corncob at 120 °C, 5 min pretreatment time by using 2% (w/w) H_2SO_4 at a 10:1 LSR.

Table 4.4 Crystallinity index (%) of untreated and treated corncobs

Sample	Crystallinity index
Untreated corncobs	26.94
Pretreated corncobs with 2% H_2SO_4 at 120°C for 5 min and LSR 15:1	34.63
Pretreated corncobs with 2% H_2SO_4 at 120°C for 5 min and LSR 10:1	39.98
Pretreated corncobs with 2% H_3PO_4 at 140°C for 10 min and LSR 10:1	39.25

4.2.4 <u>Scanning Electron Microscope (SEM)</u>

The pretreatment process can disorganize the crystalline cellulose in dilute acid pretreated corncob to an amorphous form. The damaged structure of dilute acid pretreated corncob has high surface area which can increase enzymatic accessibility, as shown in Figure 4.3, presenting the structure of dilute acid pretreated corncob after pretreatment process at condition of 2% (w/w) H_2SO_4 and H_3PO_4, under different pretreatment temperatures, times, and LSRs by using SEM. Figure 4.3 (a–d) shows the SEM images of fresh corncob, pretreated samples with sulfuric acid with different LSRs, and pretreated samples with phosphoric acid, respectively. The SEM pictures show that fresh corncob is non-porous, bulging, smooth and uniform surfaces. In contrast, SEM images of the dilute acid pretreated corncob shows significant collapse and destruction structures. For dilute sulfuric pretreatment with 15:1 LSR, Figure 4.3 (b), many of bulges have oval characteristic which become hole and the average shape size is 61 μm wide and 156 μm long while, at a 10:1 LSR. Figure 4.3 (c) presents more porous and deeply cracks which could increase the surface area of the samples than that of 15:1 LSR and has average diameter 150 μm. In addition, pretreated corncobs with phosphoric acid cause circle shape crack with 132 μm of average diameter. The dilute acid pretreated corncob had a rougher surface and more porous than fresh corncob. This kind of cracks was essential for enzymatic hydrolysis of cellulose because they can increase surface area and porosity of lignocellulose (Gabhane *et al.*, 2011).
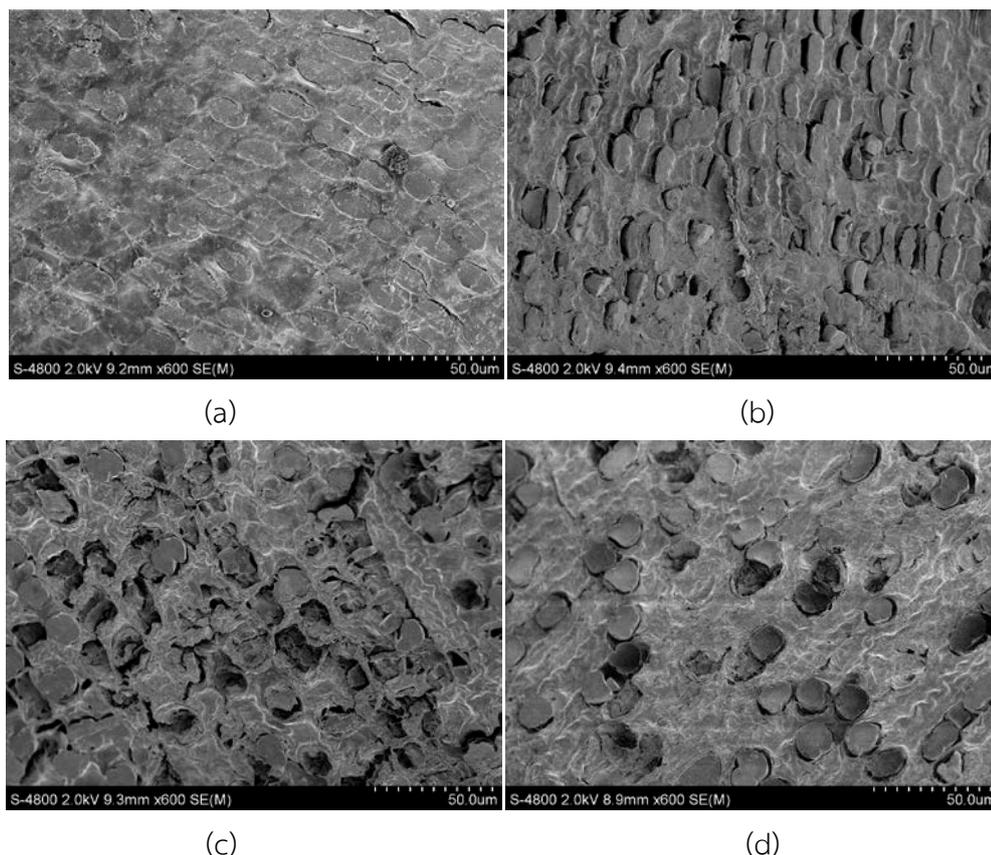
Figure 4.3 SEM images of samples: (a) untreated, (b) after pretreated with 2% (w/w) H_2SO_4 with 15: 1 LSR at 120°C for 5 min, (c) after pretreated with 2% (w/w) H_2SO_4 with 10: 1 LSR at 120°C for 5 min, and (d) after pretreated with 2% (w/w) H_3PO_4 with 15: 1 LSR at 140°C for 10 min.

4.2.5 <u>Effect of Overliming Step in Prehydrolysate</u>

During pretreatment of lignocellulosic substrates, various toxic compounds are formed that can inhibit microorganism in fermentation step. Furfural and 5-hydroxymethyl furfural (HMF) are two furan derivatives which are formed by the further hydrolysis of the sugars (pentoses and hexoses). Furfural is mostly found after pretreatment and other inhibitors such as 5-hydroxymethy furfural or 5-HMF and acetic acid are found secondly. These furans are available in relatively high concentration in the hydrolyzates and known as serious inhibitors to many other microorganisms (Purwadi *et al*., 2004). To enhance the efficiency of the fermentation, many detoxification techniques are performed; for example, alkaline detoxification, activated charcoal etc. Lime detoxification or overliming is widely known as one of inhibitor removal technique which gives high efficiency and most economical choice (Larsson *et al*., 1999). Ca(OH)$_2$ adjustment of pH has been reported the result in better fermentability than NaOH

adjustment due to the precipitation of toxic compounds (Palmqvist and Hahn-Hägerdal, 2000).
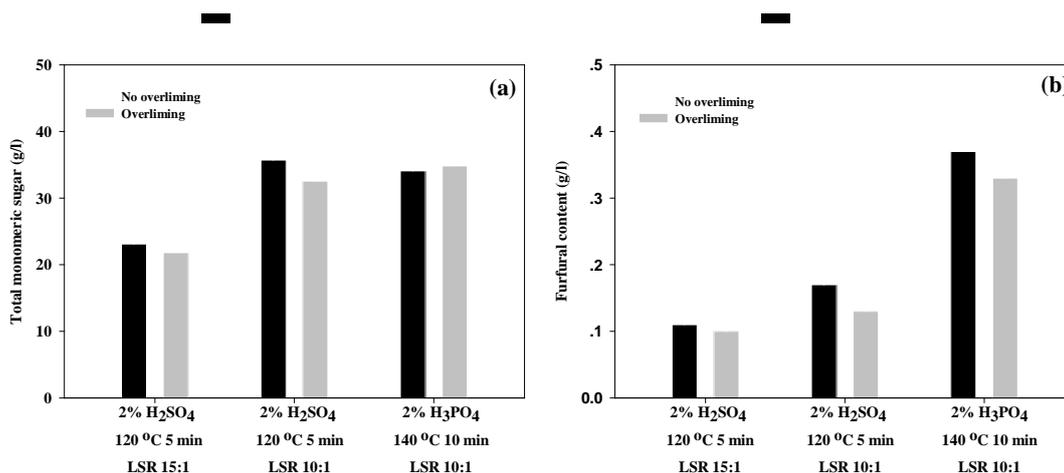
Table 4.5 and Figure 4.4 show monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment by using 2% (w/w) with different pretreatment times, temperatures and LSRs with and without overliming step.

Table 4.5 Total sugar yield of corncobs in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment using 2% (w/w) and different pretreatment times, temperatures and LSR with and without overliming step

Conditions	Total monomeric sugar (g/l)	Furfural (g/l)
2% H_2SO_4 at 120°C for 5 min and 15:1 LSR	23.10	0.11
2% H_2SO_4 at 120°C for 5 min and 15:1 LSR (with overliming step)	21.81	0.10
2% H_2SO_4 at 120°C for 5 min and 10:1 LSR	35.72	0.17
2% H_2SO_4 at 120°C for 5 min and 10:1 LSR (with overliming step)	32.53	0.13
2% H_3PO_4 at 140°C for 10 min and 10:1 LSR	34.09	0.37
2% H_3PO_4 at 140°C for 10 min and 10:1 LSR (with overliming step)	34.82	0.33

Figure 4.4 Comparison of monomeric sugar and furfural yields in prehydrolysate after dilute sulfuric and phosphoric acid pretreatments using 2% (w/w) with different pretreatment times, temperatures, and LSRs with and without overliming step.

From Figure 4.4 (a), after overliming step the concentration of total monomeric sugar of sulfuric pretreated were reduced slightly (5–9%). Larsson *et al.*, 1999 have indicated that small decreases in concentrations of fermentable sugar were observed owing to dilution with either acid or $Ca(OH)_2$ when adjusted to 10. Moreover, heating step in the overliming procedures leading to some sugar decompose at high temperature. Pentose sugars are less stable than hexose sugars and the observed decline resulted primarily from a reduction in xylose, the most abundant sugar (Martinez *et al.*, 2000). However, degradation of fermentable sugars in lime detoxification has the lowest percentage compared to other method (Larsson *et al.*, 1999). For furfural content as well known as a volatile compound can cause lower membrane permeability (Palmqvist *et al.*, 1999). Figure 4.4 (b) presents furfural content obtained from with and without overliming step and the treatment of hydrolysate with $Ca(OH)_2$ resulted in a decline in furfural in every condition. For sulfuric acid condition with 15:1 LSR has 0.01 g/l of furfural reduction. While, sulfuric acid and phosphoric acid condition with 10:1 LSR can reduce 0.04 g/l of furfural content. Some furfural has evaporated in heating step of overliming. In addition, $Ca(OH)_2$ over-liming involved precipitation of toxic components and instability of a few inhibitors at high pH (Chandel *et al.*, 2011). Agblevor *et al.*, 2004 used Carbon-13 Nuclear Magnetic Resonance Spectroscopy (^{13}C-NMR) to elucidate the functional groups involved in the overliming reaction has descript that overliming process can remove aliphatic and

aromatic acids or esters, and other aromatic and aliphatic compounds functional group. The result in inhibitors removal leads to improve the ABE fermentability.

4.2.6 Acetone-Butanol-Ethanol Production

From the result of mononeric sugar in Table 4.6, high monomeric sugar content which was prehydrolysate from pretreated corncobs with 2% H_2SO_4 10:1 LSR at 120 °C for 5 min and 2% H_3PO_4 10:1 LSR at 140 °C for 10 min also with and without overliming step were investigated for ABE production. The *C. beijerinckii* culture was growing in glucose-based P2 medium (control) and liquid part of prehydrolysate from the previous condition which previously mentioned above. According to Table 4.6, pretreatment with overliming step shows an increase in ABE yield while the pretreatment without overliming step has low ABE yield. The highest ABE yield, 23% belongs to liquid prehydrolysate fermentation of pretreated corncobs with 2% H_2SO_4 10:1 LSR at 120 °C for 5 min followed by overliming step. On the other hand, ABE yield from liquid prehydrolysate fermentation obtained from pretreated corncobs with 2% H_3PO_4, 10:1 LSR at 140 °C for 10 min followed by overliming step has found high secondly, 17%. However, furfural content shows slightly decrease in overliming step and the ABE yields were increasing significantly. This can implied that furfural is not the major inhibitor affecting to ABE fermentation.

Table 4.6 Acetone-Butanol-Ethanol production between dilute sulfuric acid and phosphoric acid at an optimal pretreatment condition of corncobs followed by with and without overliming step

Samples	Total ABE (g/l)	ABE yield (%)	ABE productivity
Control	8.59	23	0.12
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR	0.05	3	0.01
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR with overliming step	5.21	23	0.07
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR	0.06	4	0.01
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR with overliming step	3.15	17	0.02

4.2.7 <u>Optimization of Acetone-Butanol-Ethanol Production Employing Response Surface Methodology (RSM)</u>

4.2.7.1 Development of a Model for ABE Production

Factors affecting the statistical analysis were pH, temperature, and reaction time. The ranges were approximately 4.8–6.6 for pH, 37–50 °C for temperature, and 24–72 h for reaction time. Table 4.7 presents the RSM experimental design matrix with three factors at three levels and experimental results.

Table 4.7 Three level central composite design (CCD) design and response of dependent variable (ABE production)

Run	Coded values			Real values			Total ABE production (g/l)
	pH	Temperature	Reaction time	pH	Temperature (°C)	Reaction time (h)	
1	1	1	1	6.6	50	72	0.58
2	1	1	-1	6.6	50	24	0.13
3	1	-1	1	6.6	37	72	11.50
4	1	-1	-1	6.6	37	24	8.79
5	-1	1	1	4.8	50	72	0.18
6	-1	1	-1	4.8	50	24	0.14
7	-1	-1	1	4.8	37	72	11.64
8	-1	-1	-1	4.8	37	24	2.74

9	1	0	0	6.6	43.5	48	0.16
10	-1	0	0	4.8	43.5	48	0.15
11	0	1	0	5.7	50	48	0.21
12	0	-1	0	5.7	37	48	8.80
13	0	0	1	5.7	43.5	72	0.17
14	0	0	-1	5.7	43.5	24	0.14
15	0	0	0	5.7	43.5	48	0.17
16	0	0	0	5.7	43.5	48	0.21
17	0	0	0	5.7	43.5	48	0.17
18	0	0	0	5.7	43.5	48	0.21
19	0	0	0	5.7	43.5	48	0.16
20	0	0	0	5.7	43.5	48	0.19

The ANOVA was carried out to determine the significance of the model equation and the model term. Table 4.8 shows statistical analysis of the coefficient. The following polynomial equation was obtained using multiple regression analysis.

$$Y = 0.16+0.62X_1-4.19X_2+1.2X_3-0.02X_{11}+4.28X_{22}-0.02X_{33}-0.68X_{12}-0.71X_{13}-1.38X_{23}.......(1)$$

where the code variables were: Y = Total ABE (g/l); X_1 = pH; X_2 = temperature (°C), and X_3 = reaction time (h).

Table 4.8 Statistics for the regression of the optimization model

Coefficient	Value	Standard error	t-value	P-value
a_0	0.16	0.3396	0.475	0.6450
a_1	0.62	0.3124	1.995	0.0741
a_2	-4.19	0.3124	-13.402	<0.0001
a_3	1.20	0.3124	3.851	0.0032
a_{12}	-0.68	0.3492	-1.958	0.0787
a_{13}	-0.71	0.3492	-2.037	0.0690
a_{23}	-1.38	0.3492	-3.948	0.0027
a_{11}	-0.02	0.5956	-0.039	0.9697
a_{22}	4.28	0.5956	7.189	<0.0001
a_{33}	-0.02	0.5956	-0.039	0.9697

Table 4.9 ANOVA for the regression

Source of variation	Degree of freedom	Sum of Square	Mean Square	F-static	Prob>F
Model	5	9.754	1.951	6361.604	<0.0001
Residual	5	0.002			
Total	10	9.756	0.976		
R^2	0.97				

Table 4.8 shows t-value and the corresponding P-value along with the parameter estimate. The P-values indicate the significance of the regression coefficient, which the

significance will be higher with smaller *P*-values. Therefore, the linear effect of temperature (a_2) and reaction time (a_3), the quadratic effect of temperature (a_{22}) and the interaction between temperature and reaction time (a_{23}) are the most influential factors (P < 0.05). On the contrary, the linear effect of pH (a_1) as well as the quadratic effect of pH and reaction time (a_{11} and a_{33}), and the interactive effect of pH with temperature and reaction time (a_{12} and a_{13}) on ABE production were all slight as indicated by the large P-values. In order of significance of three variables on ABE production, the most importance is temperature followed by reaction time and pH, which can be remarked from the *P*-values, respectively.

   The quality of the regression was evaluated by Fisher's statistical test for analysis of variance (ANOVA) as shown in Table 4.9. The regression statistics showed that the model represented an accurate representation of the experimental data, as the computed $F_{statistic}$ (6361.604) is much larger than $F_{0.05,5,5}$ (5.05). The coefficient of determination or R^2 value of 0.97 is a measure of the amount of variation around the mean, explained by the model indicated that only 3% of all variation for response could not be explained by the model and expresses enough fit. Generally, a regression model with R^2 higher that 0.9 is considered to have a very high correlation (Haaland, 1989). Moreover, it is suggested that R^2 value should be at least 0.8 for a good fit of a model (Joglekar and may, 1987).

4.2.7.2 Effect of Parameters on ABE Production

   Figure 4.5 presents the three-dimension response surfaces and two-dimensional contour plots with ABE production as the response. The response models are mapped against two experimental factors while the other factor is maintained constant at its central value. Figure 4.5 shows that the variation of temperature influenced the ABE production greatly across the test range, while the effect of pH has small effect. ABE production increased to a peak with decreased in temperature and pH. The effect of pH and reaction time was shown in Figure 4.6; it can indicate that total ABE production was high with increased pH and reaction time. Figure 4.7 demonstrated the variation of temperature and reaction time which can be indicated that the variation of temperature is very important than reaction time. Figure 4.7(b) illustrates two-dimensional contour plot with changing of time, and ABE production time presents almost constant with raised temperature. The results of the effects show that temperature has the most significant of

variable followed by pH and reaction time, respectively. This trend is reasonable and corresponding with P-value in the previously mention.

The temperature of the medium has been recognized for a long time to be very important for optimum solvent production. The previous results showed that the most preferable temperature of microorganism, *Clostridium species,* in ABE fermentation step is 35–37 °C (Mcwell and Kristiansen, 1985, Voget *et al*, 1985) as the highest yield of produced ABE. The results presented that the sensitivity of microorganism to temperature higher than 37 °C leading to lower ABE production significantly. On the other hand, effect of pH has found secondly significant. Maddox *et al*, 2000 has described that if the pH decreases below 4.5 before sufficient acids are produced, "acid crash" which is solventogenesis may not take place. Whereas the pH of ABE fermentation initiated at the value near 6.8 and after that the pH was decreased to lower value (5.0–5.5), which encourage solventogenesis and resulted in a proper butanol production (Qureshi *et al*., 2008). ABE fermentation is composed of two main phase such as acidogenesis and solventogenesis. The batch fermentation can be completed within 2–6 days depending on the condition and the type of substrate employed. The final total concentration of solvents was produced in batch fermentation with ranges from 12 to 20 g/l, which was separated from the fermentation broth by distillation (Lee *et al*, 2008).
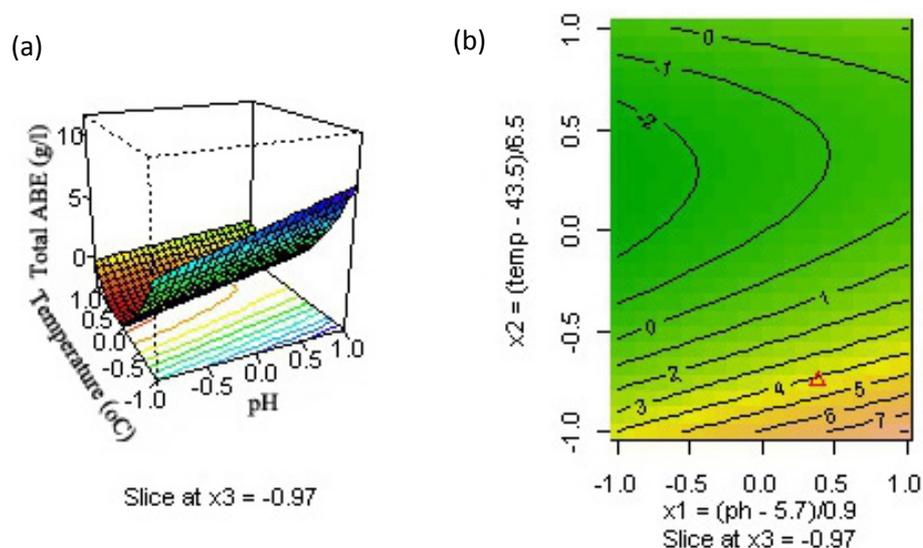

Figure 4.5 Response surface and contour plot for ABE production: effects of temperature and pH.
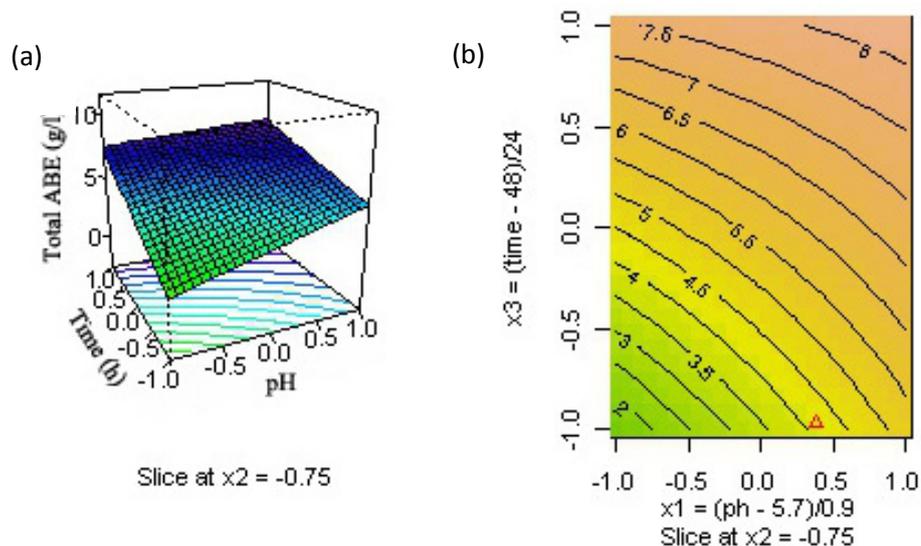
(a)

(b)

Slice at x2 = -0.75

x1 = (ph - 5.7)/0.9
Slice at x2 = -0.75

Figure 4.6 Response surface and contour plot for ABE production: effects of pH and reaction time.

(a)

(b)

Slice at x1 = 0.38

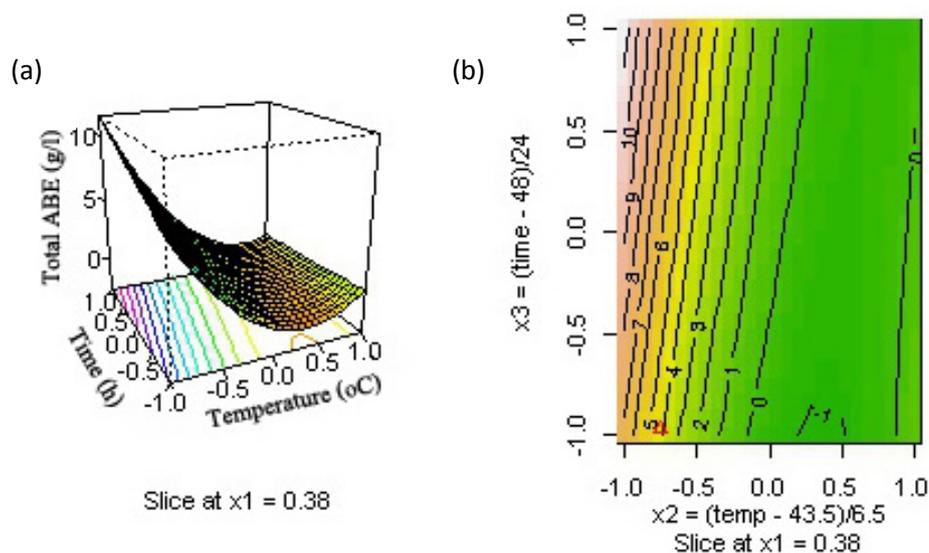x2 = (temp - 43.5)/6.5
Slice at x1 = 0.38

Figure 4.7 Response surface and contour plot for ABE production: effects of temperature and reaction time.

4.2.7.3 Optimization of ABE Production and Confirmation Experiment

The optimum conditions for maximum ABE concentration, calculated by setting the partial derivatives to zero of the eq. 1. The results showed the conditions of pH 6.2, 47.3 °C temperature, and 54.8 h reaction time. However, the predictable result of total ABE concentration was -0.707 g/l which is the unpractical value and shows minimum response of the model. As mention previously, temperature effect has the highest significant than pH and reaction time. Results in Table 4.7 demonstrated

that high total ABE concentration (2.74–11.64 g/l) was observed at 37 °C temperature condition. In contrast, condition of 43.5 °C and 50 °C show low total ABE concentration (0.16–0.58 g/l). Voget *et al*, 1985 and Mcwell and Kristiansen, 1985 have described that microorganism species of *clostridium* to produce butanol has high effective at temperature range of 35–37 °C. Referring to the previous work (Boonsombuti *et al*., unplublished data), using 60 g/l of glucose as a substrate *C. beijerinckii* TISTR1461 can produce 4.84, 7.98, 0.26, and 13.07 g/L of acetone, butanol, ethanol, and total ABE, respectively and the highest utilized glucose concentration of 46 g/L was gained. New optimal condition experiment will be selected base on partial derivative of Eq.(1) of 13.07 g/l (the assumption of maximum total ABE production from *C. beijerinckii* TISTR1461) using 0.1 for step size from the center in lower temperature path, as shown in Table 4.10. Experiment number 10, the distance of 1.8, was suitable and corresponded with the assumption condition that was predicted the total ABE resulting of 13.578 g/l.

Table 4.10 Experiment design and results for RSM

No.	Dist	Coded values			Real values			Predicted total ABE (g/l)
		X1	X2	X3	pH	Temp	Time	
1.	0.0	0.551	0.579	0.284	6.254	40.848	58.368	-0.707
2.	1.0	0.616	-0.408	0.432	6.254	40.848	58.368	3.698
3.	1.1	0.622	-0.507	0.447	6.260	40.205	58.728	4.626
4.	1.2	0.628	-0.606	0.462	6.265	39.561	59.088	5.643
5.	1.3	0.635	-0.704	0.477	6.272	38.924	59.448	6.738
6.	1.4	0.641	-0.803	0.492	6.277	38.281	59.808	7.932
7.	1.5	0.648	-0.902	0.506	6.283	37.637	60.144	9.213
8.	1.6	0.654	-1.000	0.521	6.289	37.000	60.504	10.570
9.	1.7	0.661	-1.099	0.536	6.295	36.357	60.864	12.030
10.	1.8	0.667	-1.198	0.551	6.300	35.713	61.224	13.578
11.	1.9	0.673	-1.296	0.566	6.306	35.076	61.584	15.197
12.	2.0	0.680	-1.395	0.581	6.312	34.433	61.944	16.922

In order to confirm the validity of the optimization strategy, confirmation experiments were conducted with two replicates under selected previously mention condition. Total ABE concentration of 11.82 g/l was obtained in condition of pH 6.30, 35.7 °C and 61.2 h (Table 4.11). Qureshi *et al.,* 2008 has recommended that temperature of 35 °C and a pH 5.0–6.5 could enhance the rate of hydrolysis for simultaneous saccharification and fermentation (SSF).

Table 4.11 Acetone-Butanol-Ethanol production at the condition of pH 6.30, 35.7 °C and 61.2 h

Samples	Total ABE (g/l)	ABE yield	ABE productivity
Control (20 g/l of glucose as a substrate)	7.43	0.38	0.14
SSF with sugar supplementation	11.82	0.43	0.22
SSF with non-sugar supplementation	4.81	0.62	0.09

The fermentation profile of the experiment is shown in Figure 4.8. During 24 h of process period, ABE production was fast, but it considerably slowed down during the next 61.2 h. However, it was noticed that the SSF process continued during this period, though at a very slow rate. The initial amounts of sugars present in the system were 21.95 g/l of glucose and 0.12 g/l of cellobiose. During fermentation, monomeric sugars were produced by 6.87 g/l of glucose, 0.77 g/l of xylose, and 0.09 g/l of arabinose, respectively. At the end of fermentation, 0.72 g/l glucose, 0.56 g/l xylose, 6.26 g/l butanol, 5.43 g/l acetone, and 0.13 g/l ethanol were measured. As result mention previously, it clearly presented that the limitation of resulting in maximum of produced total ABE (11.82 g/l) was the deficiency of sugar at the end of fermentation (Qureshi *et al*., 2008). Furthermore, one of the most critical problems in ABE fermentation is butanol toxicity. Jones and Woods, (1986) has reported the minimum concentration of butanol which no observable decrease in growth occurred was 4–4.8 g/l, while, 7–13 g/l of butanol to

culture medium resulted in a 50% inhibition of growth and the addition of acetone and ethanol up to 40 g/L reduced growth by 50%.
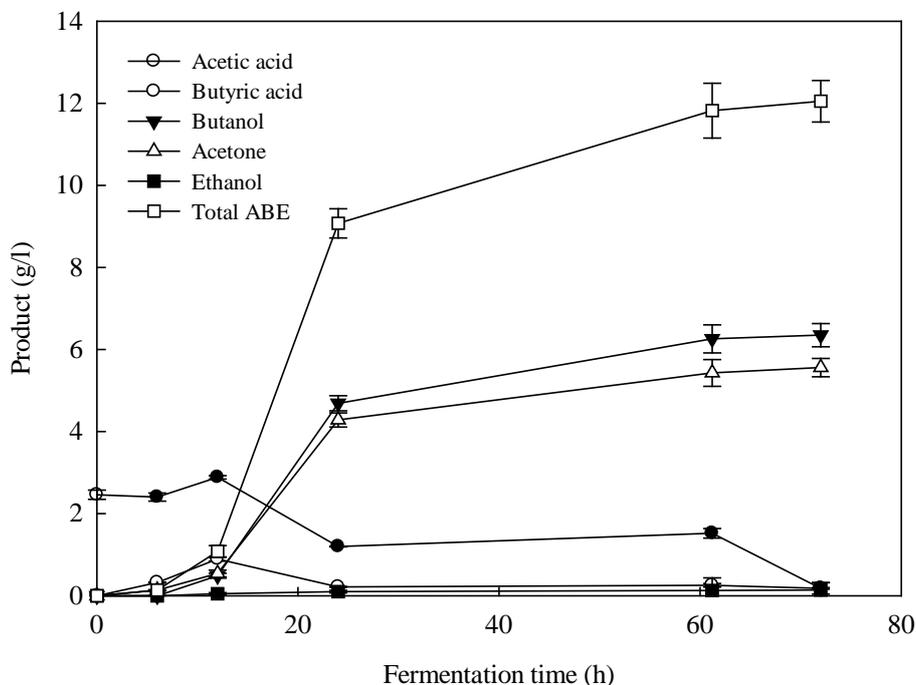

Figure 4.8 Simultaneous saccharification and fermentation (SSF) of corncobs using *C. beijerinckii* TISTR1461 at the condition of pH 6.30, 35.7 °C, and 61.2 h with 20 g/l glucose as sugar supplementation.

To confirm whether fermentation was inhibited by sediment present in the hydrolysate, Figure 4.9 shows chromatogram of GC-MS analysis, comparing between the controlled experiment (20 g/l of glucose as a substrate) in Figure 4.9(a) and SSF conditions of pH 6.30, 35.7 °C, and 61.2 h in Figure 4.9(b). The retention time of 1.91 min, 4.17 min, 6.94 min, 8.20 min, and 11.96 min refer to acetone, butanol, acetic acid, butyric acid and glycerin, respectively. The results of chemical compounds in both of experiment were mainly found acetone, butanol, acetic acid, butyric acid. While SSF resulting in glycerin compound which degraded from membrane phospholipids in corncob cells plant and has no inhibit effect to the culture of *C. beijerinckii*.
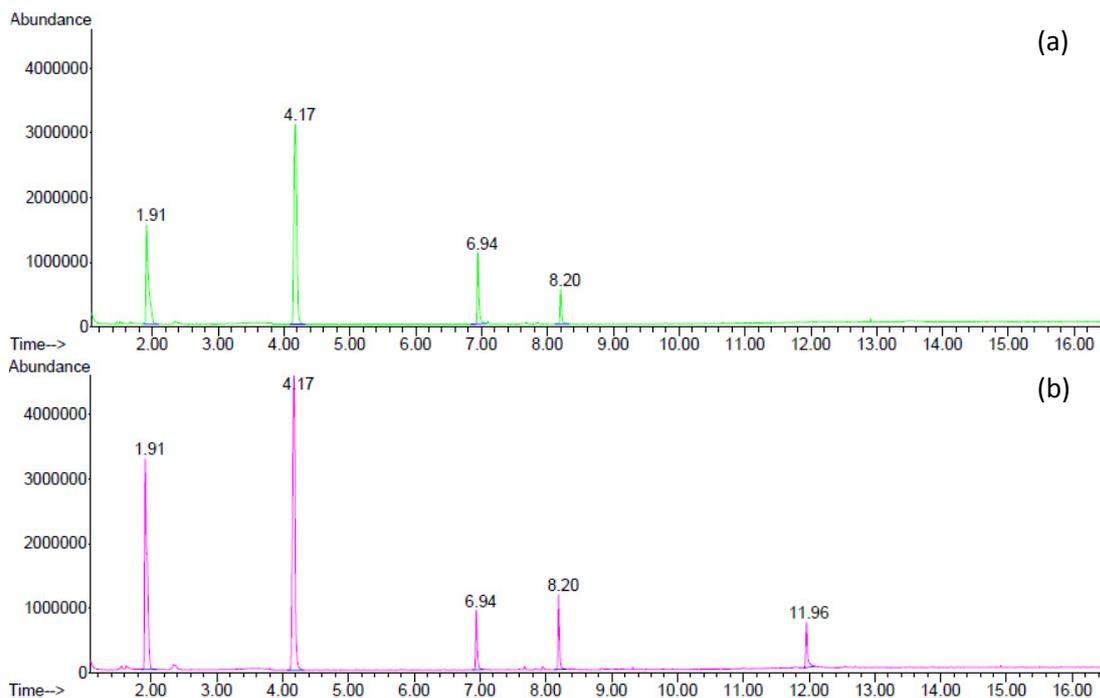
Figure 4.9 Chromatogram for GC-MS analysis of (a) control experiment (20 g/l of glucose as a substrate); (b) conditions of pH 6.30, 35.7 °C, and 61.2 h with 20 g/l glucose as sugar supplementation.

The productivity and yield were also compared in control experiment, with sugar and non-sugar supplementation of SSF batch as shown in Figure 4.10. When SSF batch was supplemented with 20 g/l glucose, the productivity of 0.22 g/l·h and a yield of 0.43 were recorded, while non-sugar supplementation batch resulted in a productivity of 0.09 g/l·h and a yield of 0.62. In the control experiment using glucose as a substrate, a productivity of 0.14 g/l·h and a yield 0.38 were obtained.

The outcome in the lowest productivity of SSF non-sugar supplementation batch due to lack of sugar which negatively affected to ABE production (Qureshi *et al.*, 2008). In addition, the total ABE production in sugar supplementation batch was greater than control experiment by 159%, and SSF with sugar supplementation improved total ABE concentration from 4.81 g/l to 11.82 g/l comparing to without sugar supplementation batch. Qureshi *et al.*, 2008 suggested that at the beginning of fermentation, the culture was deficient in sugar because it used sugar faster than released by enzymatic hydrolysis. Moreover, the appropriate sugar concentration without inhibit enzyme was lower than 60 g/l.
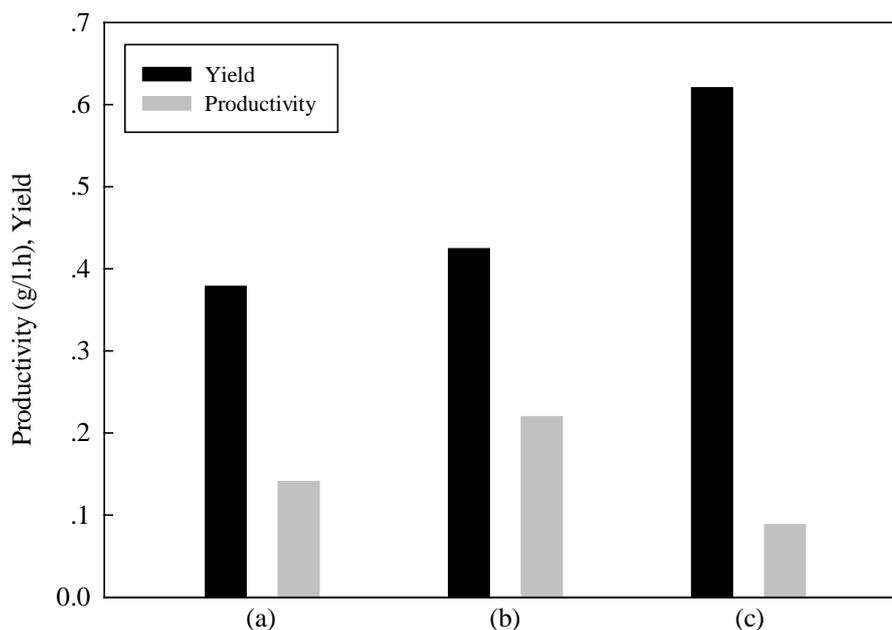
Figure 4.10 Productivity and yield of ABE production at the condition of pH 6.30, 35.7 ℃, and 61.2 h in (a) control experimental (20 g/l of glucose as a substrate); (b) SSF with sugar supplementation (20 g/l glucose); (c) SSF non-sugar supplementation.

4.3 Two-stage Pretreatment Method

4.3.1 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 ℃ 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 ℃, 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 ℃ 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 4.12.

Table 4.12 Experimental design and results of the central composite design of second stage of two–stage pretreatment

Run	Variables			Response
			SLR	Glucose concentration
	Temp. (°C)	Time (min)	(g/l)	(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:

$$Y_1 = 39.05 + 2.9625x_1 - 0.2131x_2 + 6.43x_3 - 1.2292x_1^2 - 1.9723x_2^2 - 3.2792x_3^2 +$$
$$0.93\, x_1x_2 + x_1x_3 - 0.5075x_2x_3 \qquad\qquad \text{......................(2)}$$

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

Table 4.13 summarizes the statistics for regression including regression coefficient, standard error, t value, and significant level. 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 due to '*Prob > t*' less than 0.01 ,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).

Table 4.13 Statistics for regression of the optimization model

Coefficients	Value	Standard error	t value	*Prob*[a] $(>|t|)$
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}

[a] Probability values (P–values).

The quality of the regression was evaluated by Analysis of variance (ANOVA) of Fisher's statistical test, as shown in Table 4.14. 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 4.14 also implied the model adequacy. Furthermore, the model F–value of 8.6513 and the value of the '$Prob > t$' (0.000254) for the overall model are also significant.

Table 4.14 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 4.11 and 4.12, 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 the confirmation experiment was obtained the glucose concentration 48.58 g/l and 78.71 g/l of reducing sugar including glucose, xylose, and arabinose at the same condition, as shown in Figure 4.13. This result confirmed that experimental result was in agreement with the model prediction. Figure 4.11 (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, Under severe conditions (high temperature and long 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 4.11 (a) indicating the effect of temperature and time is lower than that of SLR.
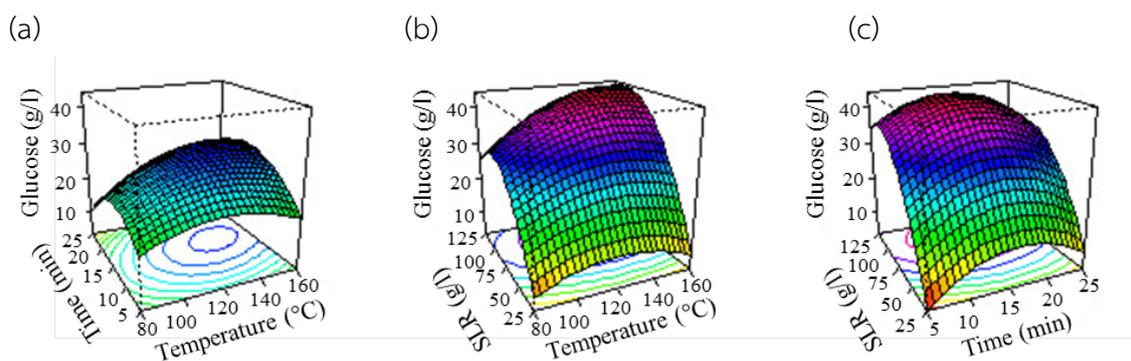
(a) (b) (c)

Figure 4.11 Response surface for glucose concentration: effects of temperature and time (a), temperature and SLR (b), and time and SLR (c).

(a) (b) (c)
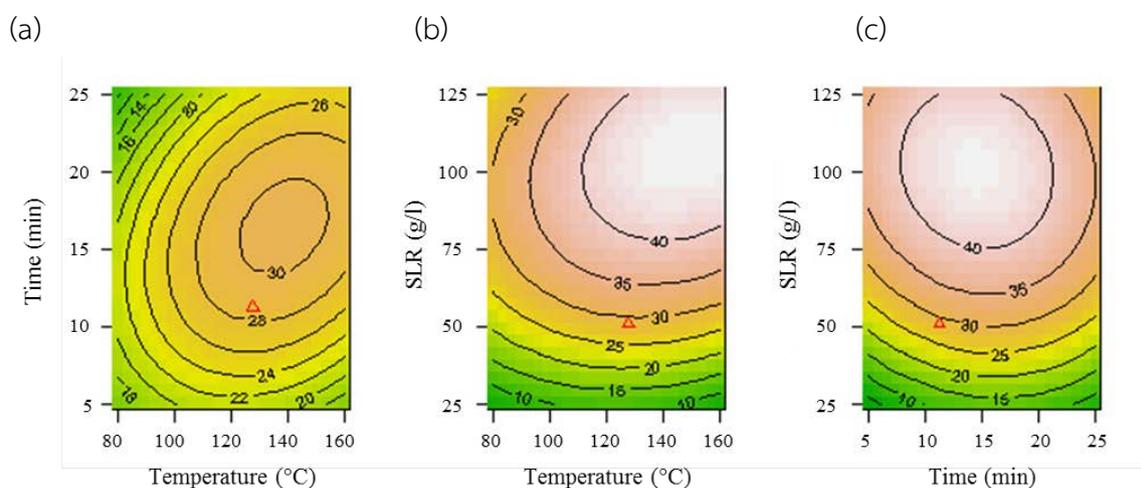
Figure 4.12 Contour plots for glucose concentration: effects of temperature and time (a), temperature and SLR (b), and time and SLR (c).
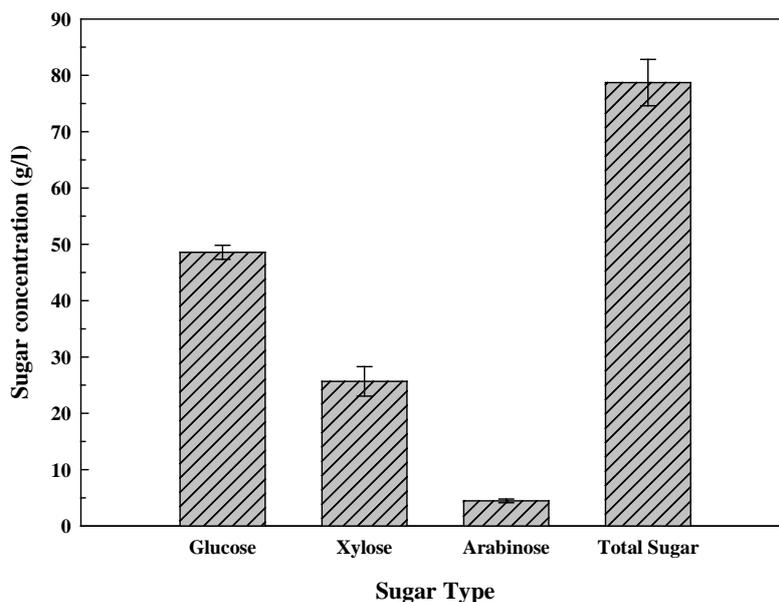
Figure 4.13 The glucose, xylose, arabinose and total sugar concentration from optimal two–stage pretreatment conditions.

4.3.2 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 4.15, The Microwave/NaOH or first stage pretreatment decreased lignin by 61.99 % compared to untreated corncobs that is the 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 H_2SO_4 or Two–stage pretreatment was applied. That reduced hemicellulose by 97.52 and 98.61 % compared to fist stage pretreatment and untreated corncobs, respectively, because acid pretreatment solubilizes hemicellulose from solid residual in soluble form (Binod *et al.,* 2012). In addition, Two–stage pretreatment increased cellulose by 97.10 % that readily to hydrolize into reducing sugar. The Microwave/NaOH followed by water pretreatment decreased only 10.33 % of hemicellulose compared to first stage pretreatment therefore, this can conclude that H_2SO_4 improves hemicellulose solubilization more than water.

Table 4.15 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

4.3.3 <u>Surface Morphology by SEM</u>

The images of scanning electron microscope (SEM) of the untreated and the pretreated corncobs in different techniques are shown in Figure 4.14. Figure 4.14 (a) shows the SEM image of the untreated corncobs has smooth and continuous surface that no pore was observed which may be due to high residual of lignin content. While, applying 2 % NaOH pretreatment at 100 °C for 30 min, residual lignin was eliminated, as shown in Figure 4.14 (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 microwave/NaOH followed by H_2SO_4 at 156 °C for 16 min, as shown in Figure 4.14 (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 4.14 (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.
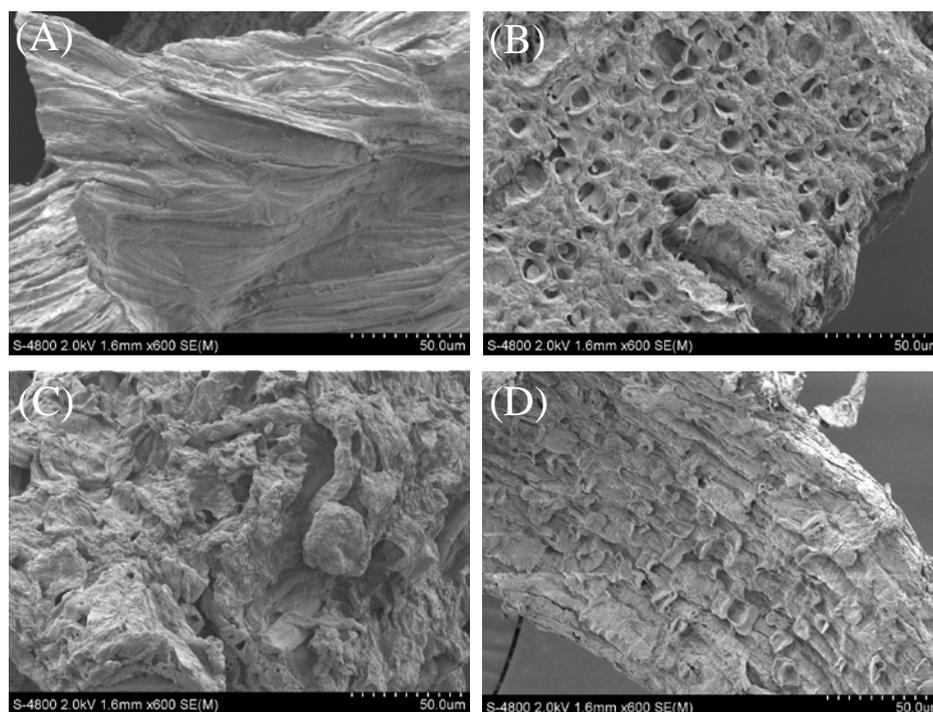
Figure 4.14 SEM images of untreated corncobs (A), Pretreated corncobs with Microwave/NaOH (B), Microwave/NaOH followed by H_2SO_4 (C), and Microwave/NaOH followed by water (D).

4.3.4 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 4.16, 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 due to swelling behavior that also increased the average pore diameter 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 conclude 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 4.16 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

4.3.5 XRD Analysis

The XRD profiles of untreated and pretreated corncobs are shown in Figure 4.8 and the crystallinity index is shown in Table 4.17. The crystallinity index of untreated corncobs was less (26.94 %) 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 increase 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 increase, as shown in Table 4.17.
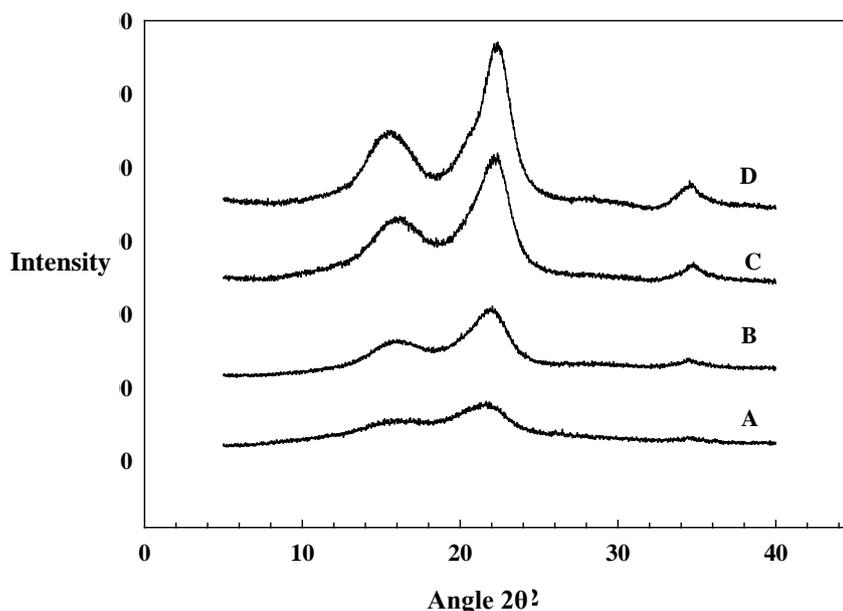
Figure 4.15 XRD patterns of untreated and pretreated corncobs. Untreated corncobs (A), Microwave/NaOH pretreated (B), Microwave/NaOH followed by water pretreated (C), and Microwave/NaOH followed by H_2SO_4 pretreated (D).

Table 4.17 Crystallinity index, crystalline area, and amorphous area of corncobs

Method	Crystallinity index (%)	Crystalline area	Amorphous area
Untreated	26.94	2853	7738
Microwave/NaOH	42.28	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 the untreated corncob 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).

4.4.7 Effect of Fermentation Techniques and Fermentation Time on the 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*. In this research, there are 7 fermentation experiments using 10 ml hydrolysate as a substrate at 37 °C, as shown in Table 4.18. The result showed that the highest ABE concentration was obtained at 48 h of fermentation time, as shown in Figure 4.16.

Table 4.18 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 4.16, 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.
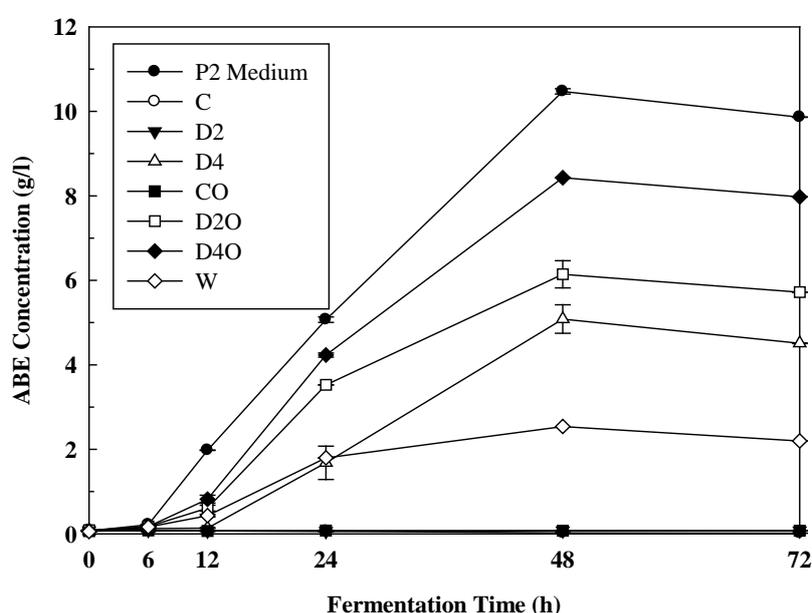
Figure 4.16 The effect of fermentation time on ABE concentration

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 4.17. 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.
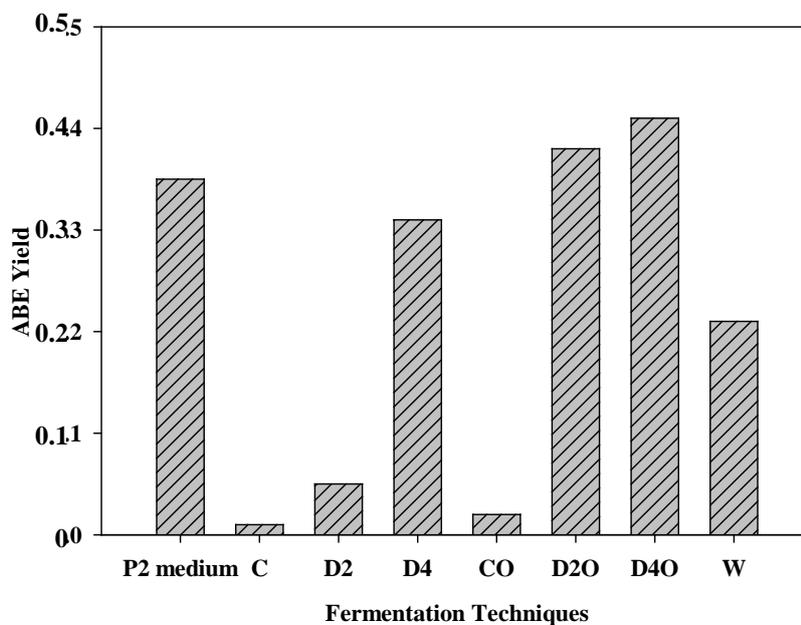

Figure 4.17 The effect of fermentation techniques on ABE yield at 37 °C for 48 h.

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 4.19. 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 4.19 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 ℃ 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.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Dilute sulfuric acid and phosphoric acid pretreatments contributed to the successful method for improving enzymatic hydrolysis. The highest total monomeric sugar, surface area, and cystallinity index were obtained in the corncobs pretreated with 2% (w/w) H_2SO_4 120 °C for 5 min and 10:1 LSR. While corncobs pretreated with 2% (w/w) H_3PO_4 140 °C 10 min and 10:1 LSR gave total monomeric sugar and cystallinity index which are similar to the highest condition obtained from sulfuric acid pretreated sample. In term of furfural formation, an inhibitor, sulfuric acid pretreatment gives lower content than phosphoric acid pretreatment. In order to remove inhibitors, lime detoxification or overliming has been recognized as an effective procedure. Acetone-Butanol-Ethanol (ABE) yield and productivity were increase significantly with a slightly decrease of furfural. Therefore, furfural might not be the major effect to inhibit culture. Simultaneous saccharification and fermentation (SSF) has main benefit of performing enzymatic hydrolysis together with fermentation, instead of in a separate step after hydrolysis, is the reduced inhibition of enzymatic hydrolysis. To enhance ABE yield, optimization by using Response Surface Methodology (RSM) with important variables such as pH, temperature, and time has been performed. A high ABE concentration was obtained under an optimal condition of pH 6.30, 35.7 °C, and 61.2 h. Moreover, SSF with sugar supplementation can improve ABE concentration comparing with non-sugar supplementation.

The two-stage pretreatment of corncobs has been well performed with the response surface methodology (RSM). The model equation indicated that temperature and solid-to-liquid in second stage were significant factors for glucose concentration. The predicted value of glucose concentration was 45.66 g/l while the confirmation experiment was obtained the glucose concentration 48.58 g/l under the optimal condition. The maximum ABE concentration that was produced from ptreated corncobs hydrolysate was 8.43 g/l, ABE yield 0.41, and productivity 17.56 g/l·h by using the diluted 4 times hydrolysate combined with overliming technique. It is concluded that the dilution and overliming process can improve ABE fermentation ability due to the reducing of inhibitors.

The results indicate that via ABE fermentation, corncobs can be used as a successful substrate because ABE yield from this technique was higher than that from synthetic sugar.

5.2 Recommendations

Alternative design the ABE fermentation process to avoid the problem caused by inhibitors was using Simultaneous Saccharificiaton and Fermentation (SSF) or by using fed-batch or continuous cultivation rather than batch process (Olofesson *et al.,* 2008). Moreover, detoxification by using other method, like activated carbon, should be performed to compare with the overliming step.

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APPENDICES

INTERNATIONAL PUBLICATIONS

1. Sirikarn Satimanont, Apanee Luengnaruemitchai, and Sujitra Wongkasemjit, "Effect of Temperature and Time on Dilute Acid Pretreatment of Corn Cobs", International Journal of Chemical and Biological Engineering, 6 (2012), 333-337.

2. Boonyisa Wanitwattanarumlug, Apanee Luengnaruemitchai, and Sujitra Wongkasemjit, "Characterization of Corn Cobs from Microwave and Potassium Hydroxide Pretreatment", Internaional Journal of Chemical and Biological Engineering, 6 (2012), 354-358.

3. Akarin Boonsombuti, Apanee Luengnaruemitchai, Sujitra Wongkasemjit, "Enhancement of enzymatic hydrolysis of corncob by microwave-assisted alkali pretreatment and its effect in morphology", Cellulose, 20 (2013), 1957-1966

4. Akarin Boonsombuti, Kittinan Komolpis, Apanee Luengnaruemitchai, Sujitra Wongkasemjit, "Enhancement of ABE fermentation through regulation of ammonium acetate and D–xylose uptake from acid-pretreated corncobs", Annual Microbiology, In press.

Effect of Temperature and Time on Dilute Acid Pretreatment of Corn Cobs

Sirikarn Satimanont, Apanee Luengnaruemitchai, and Sujitra Wongkasemjit

Abstract—Lignocellulosic materials are new targeted source to produce second generation biofuels like biobutanol. However, this process is significantly resisted by the native structure of biomass. Therefore, pretreatment process is always essential to remove hemicelluloses and lignin prior to the enzymatic hydrolysis. The goals of pretreatment are removing hemicelluloses and lignin, increasing biomass porosity, and increasing the enzyme accessibility. The main goal of this research is to study the important variables such as pretreatment temperature and time, which can give the highest total sugar yield in pretreatment step by using dilute phosphoric acid. After pretreatment, the highest total sugar yield of 13.61 g/L was obtained under an optimal condition at 140°C for 10 min of pretreatment time by using 1.75% (w/w) H_3PO_4 and at 15:1 liquid to solid ratio. The total sugar yield of two-stage process (pretreatment+enzymatic hydrolysis) of 27.38 g/L was obtained.

Keywords—Butanol production, Corn cobs, Phosphoric acid, Pretreatment

I. INTRODUCTION

THE unavoidable shortage of the world petroleum crisis and the increasing problem of the greenhouse gas are strongly effect the worldwide interest in an alternative fuels. A viable alternative for improving energy security and reducing greenhouse gas emission is conversion lignocellulosic biomass to biofuels. Biobutanol is one type of biofuels, which can be used for mixing with gasoline in order to decrease the amount of pollutants emitted from motor vehicles [1].

In order to produce biobutanol from lignocellulosic material involves 4 steps: pretreatment, hydrolysis, fermentation, and butanol separation/purification. Pretreatment is essential to remove lignin and separate hemicelluloses and improve enzymatic accessibility. After pretreatment process, cellulose will be less crystalline, allowing enzyme to hydrolyze it into fermentable sugars which mainly consist of six carbon sugar like glucose in hydrolysis step.

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After that, the hydrolysated cellulose and hemicelluloses were fermented to ABE (acetone, butanol, and ethanol) by using an anaerobic bacterium in fermentation step. Then the products were sent to butanol separation step.

Since many physicochemical structural and compositional factor in lignocellulosic biomass structure like a complex mixture of cellulose, hemicelluloses, and lignin [2]. Therefore, pretreatment process is a necessary process in order to achieve biobutanol yield. Various pretreatment techniques have been used to improve physical and chemical of lignocellulosic biomass such as physical, chemical, and biological pretreatment [3]. However, in this research focus on chemical pretreatment with dilute acid, which can modify the crystalline poly-saccharides form to a more reactive amorphous form [3]. In addition, dilute acid can solubilize hemicellulose and remain lignin and cellulose. The advantages of this step are enhancing the enzymatic digestibility of cellulose and significantly increasing value-added production yield [4]. Sulfuric and hydrochloric acids are widely used in dilute acid hydrolysis. In contrast to these acids, phosphoric acid is less toxic than other acids. Moreover, after neutralization of hydrolysate, the salt can be used as nutrient by microorganism. Therefore, the filtration process is not needed [5]. Dilute phosphoric acid, on hydrolysates from sugarcane bagasses, fermentable sugars with 21.4 g/L with less than 4 g/L of inhibitors was obtained at operating conditions of 6% acid concentration at 100°C for 300 min [6]. In this present work, dilute phosphoric acid was used to remove hemicellulose and lignin in order to increase reducing sugar.

II. EXPERIMENTAL

A. Material

Corn cobs was obtained from the Betagro Company. The collected corn cobs was stored in a sack bag at ambient room temperature. Prior to pretreatment process, it was dried in an oven at 65°C for 24 h and was ground to particle size of 1.6 mm homogenized in a single lot. The resulting ground biomass was stored in a sealed plastic bag at ambient temperature. Standard of glucose, xylose, arabinose, and furfural were purchased from Sigma Chemical Co., Ltd., Thailand.

B. Corn cobs Pretreatment

To study the important variables of pretreatment process such as pretreatment temperature and pretreatment time on the sugar yield in both the prehydrolysate and the hydrolysate as well as the generation of xylose degradation like furfural, which presented in prehydrolysate. Phosphoric acid 1.75%

(w/w) was examined at temperatures of 100, 120, 140 and 160°C and pretreatment time of 5, 10, 30 and 60 min.

Dilute phosphoric acid pretreatment was performed in a laboratory scale stirred Stainless Steel reactor. The reactor is an acid resistant alloy and has a total volumn of 1 L, with an electric heater and mechanic agitation. A 5 g of corn cobs were placed in a 250 mL glass beaker and mixed well with 75 mL of 1.75% (w/w) phosphoric acid at desired pretreatment temperature and time. The pretreatment time was counted when the temperature reached set point.

After pretreatment process, the prehydrolysate was filtrated to separate liquid and solid phase by using a vacuum pump. A vacuum pump was used to pull a vacuum across a Whatman 40 filter paper in a standard vacuum flask and Buchner filter funnel setup. After that, the solid residues were thoroughly washed with tab water to neutralize pH. Then, the solid residues were dried at 65°C oven for 24 h and collected in a sealed plastic bag at ambient room temperature. While the liquid fraction was collected for monomeric sugar analysis by using HPLC (Perkin Elmer LC200) equipped with a refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.30 mL/min, mobile phase 0.005 M of H_2SO_4, and column temperature of 65°C.

C. Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted with solid residues using a commercial enzyme, donated by Novozyme (Cellulase). Enzyme contains a mixture of cellulase, hemicellulase and higher level of betaglucosidase enzyme activities. They are a brown liquid. Enzymatic hydrolysis was performed by using the solid residues of pretreated corn cobs 0.5 g with 15 mL of 0.05 N citric acid–sodium citrate buffer (pH 4.8) at 50°C on an incubator shaker at 150 rpm for 48 h. After enzymatic hydrolysis, the hydrolysate was taken in the water baht at 50 °C to inhibit growing enzyme. Then, the hydrolysate was filtered to separate liquid and solid residue by using a vacuum pump. The liquid was determined the quantity of monomeric sugars yield by HPLC (Perkin Elmer LC200) using refractive index detector and Aminex HPX-87H column under these following conditions: flow rate 0.30 mL/min, mobile phase 0.005 M of H_2SO_4, and column temperature of 65°C.

III. RESULTS AND DISCUSSION

A. Chemical Composition of Corn cobs

Quantity of cellulose, hemicellulose and lignin in corn cobs was analyzed and the results are shown in Table I as a dry weight unit. Table I shows the composition of corn cobs which composed of 41.27% cellulose, 46.00 % hemicelluloses, and 7.4% lignin respectively. Cellulose is a main structural component in plant cell walls. The structure of cellulose is a homopolymer consists of β–D–glucose repeating units that are linear connected by β–1–4 glycosidic bonds. The cellulose in a lignocellulosic biomass comprises of parts with a crystalline structure and amorphous structure [2]. Crystalline cellulose is a major proportion of cellulose while a small

proportion is amorphous cellulose. Cellulose is a highly crystalline material, which is a major effect to resist enzymatic hydrolysis accessibility. Hemicellulose acts as a connection between cellulose and lignin which gave cellulose-hemicellulose-lignin network more rigidity. Hemicellulose is a heteropolymers of carbohydrate which consists of five-carbon sugars (e.g. xylose and arabinose) and six-carbon sugars (e.g. mannose, glucose, and galactose). Hemicellulose has a lower molecular weight than cellulose. The structure of hemi-celluloses is random, amorphous, and branched that help hemicellulose is easily to hydrolyze [2]. Lignin is a very complex molecule made up of three types of phenolic acids (p–coumaryl, coniferyl, and sinapyl alcohol) [2], which linked in a three dimensional structure that make lignin particularly difficult to hydrolyze [7].

TABLE I
CHEMICAL COMPOSITION OF CORN COBS

Chemical components	Dry solid (%)
Cellulose	41.27
Hemicellulose	46.00
Lignin	7.40

B. Thermal Gravimetric Analysis

TG-DTG, Perkin Elmer/Pyris Diamond is an equipment to analyze thermal decomposition of biomass. The thermal decomposition of corn cobs is shown in Fig. 1.
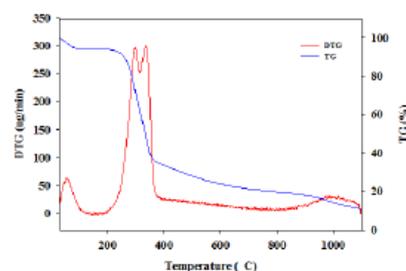

Fig. 1 Thermal gravimetric analysis curve of corn cobs

From thermal gravimetric analysis, it has four decomposition temperature range, implying corn cobs consist of four components. The different in decomposition temperature range depends on the physical and chemical structure of each component in corn cobs. The first decomposition presented around 100°C, implying that in this decomposition temperature was moisture contained in the corn cobs. The second decomposition generated in the range of 200-315°C, which was a hemicellulose. Decomposition of hemicellulose presented in the low temperature range because its structure is a random, amorphous, and branches. Cellulose was the third de-composition that presented in the range of 315-380°C. The structure of cellulose is mainly composes of both crystalline cellulose and the small percentage is

amorphous cellulose. Therefore, the cellulose decomposition temperature is higher than hemicelluloses. Among the four components in corn cobs, lignin was the most difficult component to decompose. The decomposition temperature range of lignin was higher than 900°C, owing to its structure that consists of aromatic rings with various branches.

C. Effect of Temperature and Time on Pretreatment Step

Corn cobs was pretreated with dilute H_3PO_4 under these following conditions: 1.75% (w/w) H_3PO_4, 15:1 of liquid to solid ratio, temperature in the range of 100-160°C and 5-60 min of pretreatment time. The highest yield of total sugar in prehydrolysate, 13.61 g/L, was measured after a pretreated at condition of 1.75% (w/w) H_3PO_4, 15:1 of liquid to solid ratio, 140°C, and 10 min of pretreatment time. Moreover, under the condition at 1.75% (w/w) H_3PO_4, 140°C for 5 min and 160°C for 5 min were found to have similar highest total sugar yield (as shown in Table II and Fig. 2).

TABLE II
THE TOTAL SUGAR YIELD OF CORN COBS IN PREHYDROLYSATE AFTER DILUTE PHOSPHORIC ACID PRETREATMENT BY USING 1.75% (W/W) H_3PO_4 15:1 LSR, AND DIFFERENT PRETREATMENT TIMES AND TEMPERATURES

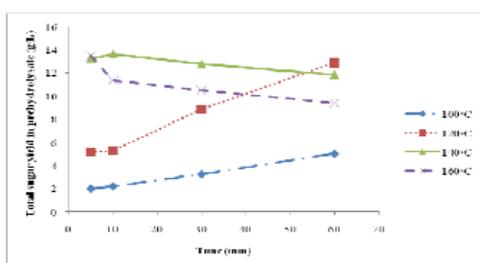| Temperature (°C)	Pretreatment time (min)	Total monomeric sugars (g/L)
100	5	2.01
	10	2.23
	30	3.27
	60	5.03
120	5	5.14
	10	5.29
	30	8.87
	60	12.86
140	5	13.24
	10	13.61
	30	12.77
	60	11.85
160	5	13.48
	10	11.37
	30	10.48
	60	9.38

Fig. 2 The total sugar yield of corn cobs in prehydrolysate after dilute phosphoric acid pretreatment using 1.75% (w/w) H_3PO_4 15:1 LSR, and different pretreatment times and temperatures

Table III shows monomeric sugar yield of corn cobs in prehydrolysate after dilute H_3PO_4 pretreatment using 1.75% (w/w) H_3PO_4 15:1 LSR, and different pretreatment times and temperatures. The major component in prehydrolysate from pretreatment process was xylose. Since the structure of

hemicellulose which consists of short lateral chain as mentioned previously; therefore, xylose presented in hemicelluloses can be readily to hydrolyze than glucose. In term of xylose yield, a clear trend is observed, showing that it increased with pretreatment time from 5 min to 60 min and pretreatment temperature from 100°C to 120°C. While harsher pretreatment conditions (> 120°C), the xylose yield decreased owing to xylose degradation into furfural which can interfere the micro-organism growing in fermentation process [8]. The presence of furfural at 140 °C and 160 °C was first obtained at 10 min and the furfural yield was 0.17 g/L and 0.50 g/L, respectively (as shown in Table IV). The highest xylose yield of 11.40 g/L was obtained at 140°C for 10 min of pretreatment time. From table IV, the xylose lost reversed with the furfural generation. Moreover, the results showed that pretreatment temperature and time can drive xylose degradation into furfural and at higher temperatures and times there is faster furfural formation which is in a good agreement with previous results [9]. The mechanism and toxicity of furfural was reported in previous work [10]. The arabinose production was obtained as a function of temperature and time which is similar to the xylose trend under mild pretreatment conditions. After 140°C and 30 min of pretreatment time, the arabinose yield decreased since the mass of solid residue was burned under severity pretreatment conditions. For glucose yield, we can not detect the glucose yield because the retention of phosphoric acid is exactly match with glucose. However, same research groups reported that the glucose yield that formed in prehydrolysate has a small amount [5], [11].

TABLE III
THE MONOMERIC SUGAR YIELD OF CORN COBS IN PREHYDROLYSATE AFTER DILUTE PHOSPHORIC ACID PRETREATMENT USING 1.75% (W/W) H_3PO_4, 15:1 LSR, AND DIFFERENT PRETREATMENT TIMES AND TEMPERATURES

Temperature (°C)	Pretreatment time (min)	Xylose (g/L)	Arabinose (g/L)
100	5	1.24	0.77
	10	1.26	0.97
	30	1.82	1.45
	60	3.25	1.78
120	5	3.68	1.46
	10	3.72	1.57
	30	7.15	1.72
	60	10.9	1.96
140	5	11.26	1.98
	10	11.40	2.21
	30	10.51	2.26
	60	9.61	2.24
160	5	11.34	2.14
	10	9.25	2.12
	30	8.59	1.89
	60	7.80	1.58

TABLE IV
FURFURAL LEVELS IN PREHYDROLYSATE UNDER VARIOUS PRETREATMENT CONDITIONS

Temperature (°C)	Pretreatment time (min)	Average furfural (g/L)
140	10	0.17
	30	0.31
	60	0.52
160	10	0.50
	30	1.00
	60	1.40

amorphous cellulose. Therefore, the cellulose decomposition temperature is higher than hemicelluloses. Among the four components in corn cobs, lignin was the most difficult component to decompose. The decomposition temperature range of lignin was higher than 900°C, owing to its structure that consists of aromatic rings with various branches.

C. Effect of Temperature and Time on Pretreatment Step

Corn cobs was pretreated with dilute H_3PO_4 under these following conditions: 1.75% (w/w) H_3PO_4, 15:1 of liquid to solid ratio, temperature in the range of 100-160°C and 5-60 min of pretreatment time. The highest yield of total sugar in prehydrolysate, 13.61 g/L, was measured after a pretreated at condition of 1.75% (w/w) H_3PO_4, 15:1 of liquid to solid ratio, 140°C, and 10 min of pretreatment time. Moreover, under the condition at 1.75% (w/w) H_3PO_4 140°C for 5 min and 160°C for 5 min were found to have similar highest total sugar yield (as shown in Table II and Fig. 2).

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	60	5.03
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	10	5.29
	30	8.87
	60	12.86
140	5	13.24
	10	13.61
	30	12.77
	60	11.85
160	5	13.48
	10	11.37
	30	10.48
	60	9.38

Fig. 2 The total sugar yield of corn cobs in prehydrolysate after dilute phosphoric acid pretreatment using 1.75% (w/w) H_3PO_4 15:1 LSR, and different pretreatment times and temperatures

Table III shows monomeric sugar yield of corn cobs in prehydrolysate after dilute H_3PO_4 pretreatment using 1.75% (w/w) H_3PO_4 15:1 LSR, and different pretreatment times and temperatures. The major component in prehydrolysate from pretreatment process was xylose. Since the structure of

hemicellulose which consists of short lateral chain as mentioned previously; therefore, xylose presented in hemicelluloses can be readily to hydrolyze than glucose. In term of xylose yield, a clear trend is observed, showing that it increased with pretreatment time from 5 min to 60 min and pretreatment temperature from 100°C to 120°C. While harsher pretreatment conditions (> 120°C), the xylose yield decreased owing to xylose degradation into furfural which can interfere the micro-organism growing in fermentation process [8]. The presence of furfural at 140 °C and 160 °C was first obtained at 10 min and the furfural yield was 0.17 g/L and 0.50 g/L, respectively (as shown in Table IV). The highest xylose yield of 11.40 g/L was obtained at 140°C for 10 min of pretreatment time. From table IV, the xylose lost reversed with the furfural generation. Moreover, the results showed that pretreatment temperature and time can drive xylose degradation into furfural and at higher temperatures and times there is faster furfural formation which is in a good agreement with previous results [9]. The mechanism and toxicity of furfural was reported in previous work [10]. The arabinose production was obtained as a function of temperature and time which is similar to the xylose trend under mild pretreatment conditions. After 140°C and 30 min of pretreatment time, the arabinose yield decreased since the mass of solid residue was burned under severity pretreatment conditions. For glucose yield, we can not detect the glucose yield because the retention of phosphoric acid is exactly match with glucose. However, same research groups reported that the glucose yield that formed in prehydrolysate has a small amount [5], [11].

TABLE III
THE MONOMERIC SUGAR YIELD OF CORN COBS IN PREHYDROLYSATE AFTER DILUTE PHOSPHORIC ACID PRETREATMENT USING 1.75% (W/W) H_3PO_4, 15:1 LSR, AND DIFFERENT PRETREATMENT TIMES AND TEMPERATURES

Temperature (°C)	Pretreatment time (min)	Xylose (g/L)	Arabinose (g/L)
100	5	1.24	0.77
	10	1.26	0.97
	30	1.82	1.45
	60	3.25	1.78
120	5	3.68	1.46
	10	3.72	1.57
	30	7.15	1.72
	60	10.9	1.96
140	5	11.26	1.98
	10	11.40	2.21
	30	10.51	2.26
	60	9.61	2.24
160	5	11.34	2.14
	10	9.25	2.12
	30	8.59	1.89
	60	7.80	1.58

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Temperature (°C)	Pretreatment time (min)	Average furfural (g/L)
140	10	0.17
	30	0.31
	60	0.52
160	10	0.50
	30	1.00
	60	1.40

D.Effect of Temperature and Time on Enzymatic Hydrolysis Step

After the enzymatic hydrolysis, the major component in hydrolysate was glucose and the others were xylose and arabinose. In pretreatment process, hemicellulose was removed in order to maximize glucose yield in hydrolysate after enzymatic hydrolysis step. Table V and Fig. 3 show the levels of total sugar yield measured in the hydrolysate after enzymatic hydrolysis of corn cobs under different pretreatment conditions. The maximum total sugar yield obtained after enzymatic hydrolysis was found to be 13.77 g/L at an optimum condition of 1.75% (w/w) H_3PO_4, 15:1 liquid to solid ratio, 140°C, and 10 min of pretreatment time. Table VI shows the monomeric sugar yield in the hydrolysate after enzymatic hydrolysis of corn cobs under different pretreatment conditions. The maximum glucose contained in the hydrolysate was 11.46 g/L at 1.75% (w/w) H_3PO_4, 15:1 liquid to solid ratio, 160 °C, and 60 min. The glucose yield increased with an increase of pretreatment temperature and time, due to hemicellulose removal during the pretreatment in the form of xylose and xylose degradation (furfural). Furfural formation is influenced by temperature and acid concentration so it can imply that the glucose in hydrolysate would be influenced by temperature and acid concentration as well. In addition, the pretreatment process can disorganize the crystalline cellulose to an amorphous form which made it was easily to hydrolyze by enzyme (as shown in Fig. 4). Fig. 4 shows the structure of corn cobs after pretreatment process under different pretreatment conditions by using scanning electron microscope. Fig. 4 (a) shows the corn cobs without pretreated with dilute phosphoric acid. Fig. 4 (b), (c) were the results of pretreated samples in different temperatures. Fig. 4 (c), (d) the samples pretreated in different times. It was found that high temperature and pretreatment time can disrupt crystalline structure in corn cobs. The minor products in hydrolysate were xylose and arabinose with small amount. Increasing pretreatment times and temperatures will decrease the yield of xylose and arabinose since the furfural formation and the pretreated xylose and arabinose during pretreatment step. It has been reported that 1 g/L furfural could inhibit yeast growing work [12]-[14].
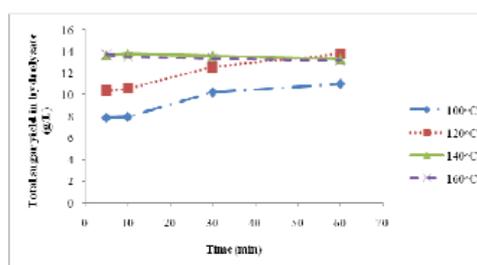
TABLE V
THE TOTAL SUGAR YIELD OF CORN COBS IN HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS UNDER THE CONDITION OF 1.75% (W/W) H_3PO_4, 15:1 LSR, AND DIFFERENT PRETREATMENTTIMES AND TEMPERATURES

Temperature (°c)	Pretreatment time (min)	Total monomeric sugars (g/L)
100	5	7.89
	10	7.96
	30	10.22
	60	11.00
120	5	10.38
	10	10.55
	30	12.55
	60	13.76
140	5	13.64
	10	13.77
	30	13.59
	60	13.31
160	5	13.71
	10	13.52
	30	13.34
	60	13.14

Fig. 3 The total sugar yield of corn cobs in hydrolysate after enzymatic hydrolysis under the condition of 1.75% (w/w) H_3PO_4, 15:1 LSR, and different pretreatment times and temperatures

TABLE VI
THE MONOMERIC SUGAR YIELD OF CORN COBS IN HYDROLYSATE AFTER ENZYMATIC HYDROLYSIS UNDER THE CONDITION OF 1.75% (W/W) H_3PO_4, 15:1 LSR, AND DIFFERENT PRETREATMENT TIMES AND TEMPERATURES

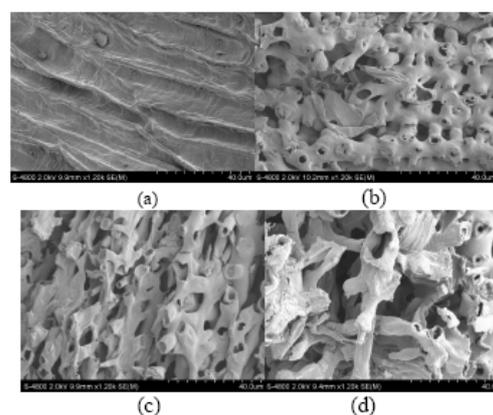| Temperature (°C)	Pretreatment time (min)	Glucose (g/L)	Xylose+Arabinose (g/L)
100	5	4.31	3.58
	10	4.52	3.44
	30	6.79	3.43
	60	7.84	3.16
120	5	7.29	3.09
	10	7.52	3.03
	30	9.80	2.75
	60	11.19	2.57
140	5	11.10	2.54
	10	11.30	2.47
	30	11.40	2.19
	60	11.43	1.88
160	5	11.34	2.37
	10	11.38	2.14
	30	11.41	1.93
	60	11.46	1.68

Fig. 4 Scanning electron microscope of samples after pretreated with dilute phosphoric acid under the condition of 1.75% (w/w) H_3PO_4, 15:1 LSR: (a) untreated, (b) 100°C 10 min, (c) 140°C 10 min, and (d) 140°C 30 min

IV. CONCLUSIONS

High temperature is an achievable way to disorder the complex structure of corn cobs for enzymatic hydrolysis step. However, an optimal condition in pretreatment step: pretreatment times, temperatures and the formation of furfural needs to be accounted. In this research, the overall highest total sugar yield in both pretreatment and enzymatic hydrolysis was 27.38 g/L under an condition at 140°C for 10 min of pretreatment time using 1.75% (w/w) H_3PO_4 and 15:1 liquid to solid ratio. In this optimal condition, furfural 0.17 g/L was obtained.

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Characterization of Corn Cobs from Microwave and Potassium Hydroxide Pretreatment

Boonyisa Wanitwattanarumlug, Apanee Luengnaruemitchai, and Sujitra Wongkasemjit

Abstract—The complexity of lignocellulosic biomass requires a pretreatment step to improve the yield of fermentable sugars. The efficient pretreatment of corn cobs using microwave and potassium hydroxide and enzymatic hydrolysis was investigated. The objective of this work was to characterize the optimal condition of pretreatment of corn cobs using microwave and potassium hydroxide enhance enzymatic hydrolysis. Corn cobs were submerged in different potassium hydroxide concentration at varies temperature and resident time. The pretreated corn cobs were hydrolyzed to produce the reducing sugar for analysis. The morphology and microstructure of samples were investigated by Thermal gravimetric analysis (TGA, scanning electron microscope (SEM), X-ray diffraction (XRD). The results showed that lignin and hemicellulose were removed by microwave/potassium hydroxide pretreatment. The crystallinity of the pretreated corn cobs was higher than the untreated. This method was compared with autoclave and conventional heating method. The results indicated that microwave-alkali treatment was an efficient way to improve the enzymatic hydrolysis rate by increasing its accessibility hydrolysis enzymes.

Keywords—Corn cobs, Enzymatic hydrolysis, Microwave, Potassium hydroxide, Pretreatment.

I. INTRODUCTION

IN the last several decades, there has been an argument over the energy policy in many countries. Due to energy consumption has increased and many countries have become industrialized countries; therefore, the world energy consumption is projected to increase by 50%, by the year 2020. In addition, they are not a renewable energy resource and the combustion of fossil fuels results in greenhouse gas emissions and accelerates the global climate change. Therefore, the reduction of greenhouse gas emissions has become a primary focus of environmental solution in countries around the world.

There are many types of biofuel produced from lignocellulosic biomass such as bioethanol and biobutanol. However, butanol has several advantages over ethanol. It is used in industrial feedstock not only for use as a solvent, but also as a fuel that can replace gasoline. Moreover, butanol is less corrosive and more suitable for distribution through existing pipelines.

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Corn is one of the most important agricultural crops in Thailand and it produce corn cob residue around 1.1 million ton per year, which it is normally used as animal feed and the elimination of residue leads to an environmental problem. In order to increase its value, the most attractive is to convert it to fermentable sugar. Corn cob is mainly composed of cellulose, hemicellulose, and lignin. This natural structure makes it difficult to hydrolyze into fermentable sugar.

The enzymatic hydrolysis is an interesting way to produce sugars from cellulose wastes because of its mild operating condition, regarding pH and temperature, and the absence of by-products [1]. Therefore, efficient of pretreatment method has improved enzymatic hydrolysis to remove lignin and hemicellulose, disordered the crystalline structure of cellulose, and increased the porosity of the materials to make cellulose more accessible to the enzyme for a maximal reducing sugar production.

There are many research work related with pretreatment methods; the microwave irradiation is widely used because of high heating efficiency, easy operation, and taking short time. Microwave heating pretreatment can break down lignin-hemicellulose complex and expose more accessible surface area of cellulose with cellulase enzyme [2]. Microwave irradiation can be easily combined with chemical reaction and, in some case, accelerate the chemical reaction [3]. Recently, a few reports combined microwave with alkali pretreatment, which could operate process at low temperature. Alkali pretreatment removed lignin and increased the biodegradability of cellulose owing to cleavage of the lignin bond. In my studies, the chemical composition, morphology and microstructure were catalyzed by HPLC, TG-DTA, SEM, XRD and BET.

The objective of this work was to characterize the pretreated corn cobs from microwave and potassium hydroxide pretreatment.

II. EXPERIMENTAL

A. Pretreatment of Corn Cobs by Microwave Assisted Alkali

Microwave radiation system was used in this study and combine with alkali pretreatment. This process used 2 g of corn cobs suspended in 30 mL of different potassium hydroxide concentrations (0.75 % to 3 %) and then transferred to microwave oven to treat corn cobs at 60-120 °C for 10 min to 30 min. After this process was completed, the residues were collected by filter paper and washed with tap water until neutral pH, dried at 65 °C.

B. Enzymatic Hydrolysis

A hydrolysis mixture consisted of 0.5 g of pretreated corn cobs and 15 mL of 0.1 mol L^{-1} citrate buffer (pH 4.8). The mixture was added with 0.1 mL of the commercial cellulese enzymes that was incubated at 50 °C in an incubator shaker

at 150 rpm for 48 h. Thereafter 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 min at 8000 rpm [3]. Then, the sample from the reaction was stored for sugar analysis.

C. Component Analysis of the Biomass Samples

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 estimated detergent hemicellulose. Detergent cellulose was calculated by subtracting the values for (ADL + AIA) from ADF.

D. Thermal Gravimetric Analysis

For TG-DTA work, the untreated corn cobs were loaded with approximately 5 mg in high purity alumina pan in Perkin Elmer/Pyris Diamond. Nitrogen was used as a carrier gas for creating the inert environment. The heating rate was 10 °C/min from 50 °C to 1000 °C. In general, weight change of a sample was recorded as a function of time or temperature and characterized by a TG curve. DTG emphasized the zone of reaction where various reaction steps are taking place over the entire temperature range [4].

E. Monosaccharide Analysis

Glucose, xylose, and arabinose were determined using an HPLC system equipped with a refractive index detector (Model 6040 XR, Spectra-Physics, USA). An organic acid column (Aminex HPX- 87H column, Bio-Rad Lab, USA) was used with 0.005 M sulfuric acid solution as a mobile phase. The flow rate was controlled at 0.6 mL min^{-1} and the column temperature was 65 °C.

F. Crystallinity Measurement

X-ray diffraction (XRD) was used for phase identification of the untreated and pretreated corn cobs. Samples were scanned and recorded by using Rigaku X-Ray Diffractometer system (RINT-2200) with Ni filter and Cu K$_\alpha$ radiation (1.5406 Å) that generated at 30 mA and 40 kV. The scan speed of 5° (2θ)/min with scan step of 0.02 (2θ) was used for the continuous run in 5 to 90 °C (2θ) range.

The crystalline indices of cellulose samples were calculated from the X-ray diffraction patterns by the following equation [5]:

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

Where I_{002} was the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2h = 22.5 and $I_{amorphous}$ was the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2h = 19.

G. BET Surface Area Analysis

BET surface area of corn cobs before and after pretreatment was measured by N$_2$ adsorption/desorption measurements (Quantachrome/Autosorb1). The dried sample (0.1-0.5 g) was put into the sample tube and outgassed to remove the humidity and volatile adsorbents

adsorbed on surface under vacuum at 150 °C for 4 h prior to the analysis. Then, N$_2$ was purged to adsorb on surface, and the quantity of gas adsorbed onto or desorbed from their solid surface at some equilibrium vapor pressure by static volumetric method will be measured. The solid sample was maintained at a constant temperature of the sample cell until the equilibrium is established. The BET surface area and pore volume were obtained from the N$_2$ adsorption/desorption curves.

III. RESULT AND DISCUSSION

A. Chemical Composition of Biomass

The main component of corn cobs was 41.27% cellulose, 46% hemicellulose and 7.4% lignin as shown in Table I. 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 [6]. Cellulose is the β-1,4-polyacetal of cellobiose, which is considered as the polymer of the glucose. The common polymer of hemicellulose is xylan that is mainly composed of five carbon sugar monomers such as xylose and arabinose, and six carbon sugar monomers such as glucose, mannose and galactose. Lignin is a complex polymer which consists of three types of phenolic acids: *p*-coumaryl alcohol, coniferyl alcohol, and synapyl alcohol. It plays an important role in the cell's endurance and development, as it affects the transport of water, nutrients, and metabolites in the plant cell [7].

TABLE I
CHEMICAL COMPOSITION OF CORN COBS

Composition	Dry solid (%, w/w)
Cellulose	41.27
Hemicellulose	46.00
Lignin	7.40

B. Thermal Gravimetric Analysis (TG-DTA)

The thermal decomposition behavior of untreated corn cobs was investigated by thermal gravimetric analysis TG-DTG, as shown in Fig. 1.

The first step of decomposition begins with moisture which was removed by 5.5% mass loss occurs at about 100 °C. The second mass loss step was hemicellulose starting decomposition at 200-320 °C by 32% mass loss. Hemicellulose was constructed an amorphous structure and linear polymer structure with short side chains which were easier to remove from the main stem than cellulose and degraded to volatiles such as CO, CO$_2$, and some hydrocarbon at the lower temperature. The third thermal decomposition of cellulose took place from 320 °C to 700 °C by 56.6% mass loss. Since cellulose was mainly consisted of semicrystalline arrangement chains associated with other which its structure was strong and produced it thermally. As for lignin, it was steadily decomposed at 250 to 500 °C. However, some groups reported that decomposition of heavier volatiles such as lignin occurs from temperature range 150 up to 900°C since it is more thermally stable in contrast to cellulose and hemicelluloses [4]. Lignin was the most difficult to decompose because it was complex structure of phenolic polymer covering the polysaccharides of the cell walls which made strong and durable composite material.
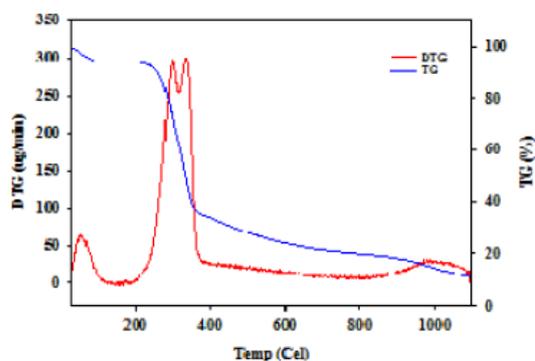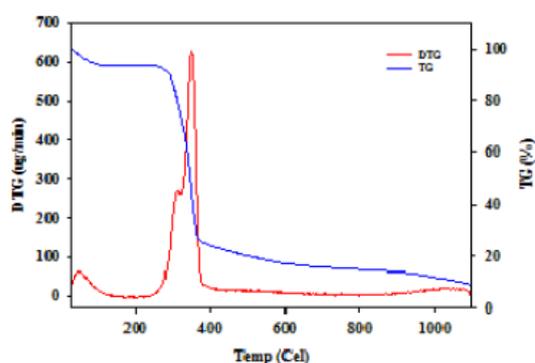
Fig. 1 TG-DTG curves of corn cobs

Fig. 2 TG-DTG curves of pretreated corn cobs
(2% KOH 120 °C 25 min)

Fig. 2 shows that the microwave/KOH pretreatment could remove hemicellulose and lignin. The weight loss of hemicellulose decreased from 32.1% to 19.2 %. Due to the overlapped peak of lignin involved with another peak that could not prove the percentage of lignin removal. For DTG peak of cellulose degradation temperature of pretreated corn cobs had left shifted comparing to the untreated control because of the lower crystallinity [8].

C. Total Sugar in Enzymatic Hydrolysis

The corn cobs were treated with microwave and KOH solution. Hydrolysis experiment contained a mixture of buffer solution and pretreated corn cobs. The enzyme used in this work is Novozyme 50013. The enzyme solution consisted of three components: endo-β-glucanase (EG) which attach to regions of low crystallinity in the cellulose fiber, generating free chain ends; exo-β-glucanase or cellobio-hydrolase (CBH) which degrade cellulose molecules further by removing cellobiose units from the free chain; and, β-glucosidase that hydrolyze cellobiose to produce glucose [9].

The maximum total sugar concentration in each pretreatment temperature obtained from enzymatic hydrolysis of pretreated corn cobs is shown in Fig. 3. At low temperature, 3% KOH gave the highest total sugar concentration. And as pretreatment time and KOH concentration increased, glucose concentration slightly

increased. On the other hand, high sugar concentration was obtain by 2% KOH at high temperatures. At high temperature and high KOH concentration during pretreatment resulted in higher solid loss, which leading to less total sugar released.

These results indicated that the optimal condition for enzymatic hydrolysis was 2% KOH at 120 °C for 25 min.
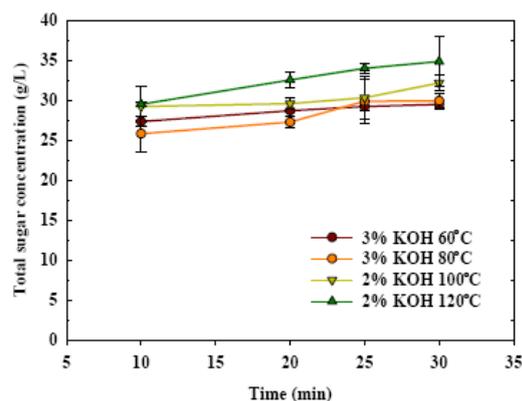

Fig. 3 The total sugar concentration obtained from enzymatic hydrolysis of pretreated corn cobs at different conditions.

D. Surface Morphology by SEM

SEM images of the untreated and the pretreated corn cobs in different conditions are shown in Fig. 4. The untreated corn cobs were clear and no pore (Fig. 4a). After the pretreatment with 0.75% KOH at 60°C for 25 min, the structure was damaged, looked soft and pores were appeared (Fig. 4b). When the temperature was 120 °C, the structure become more porous (Fig. 4c). This temperature would certainly increase the external surface area. With the KOH concentration was 2%, the surface area of corn cobs had many micropores which were favorable for cellulase to hydrolyze the cellulose into reducing sugar.

From the SEM result, the surface area of corn cobs increased when pretreatment temperature, pretreatment time and KOH concentration increased that was consistent with BET analysis, as shown in Table II. At optimal pretreatment condition surface area of corn cobs was increased from 3.926 m^2/g to 5.719 m^2/g.

TABLE II
BET SURFACE AREA, TOTAL PORE VOLUME, AND AVERAGE PORE DIAMETER OF SAMPLES

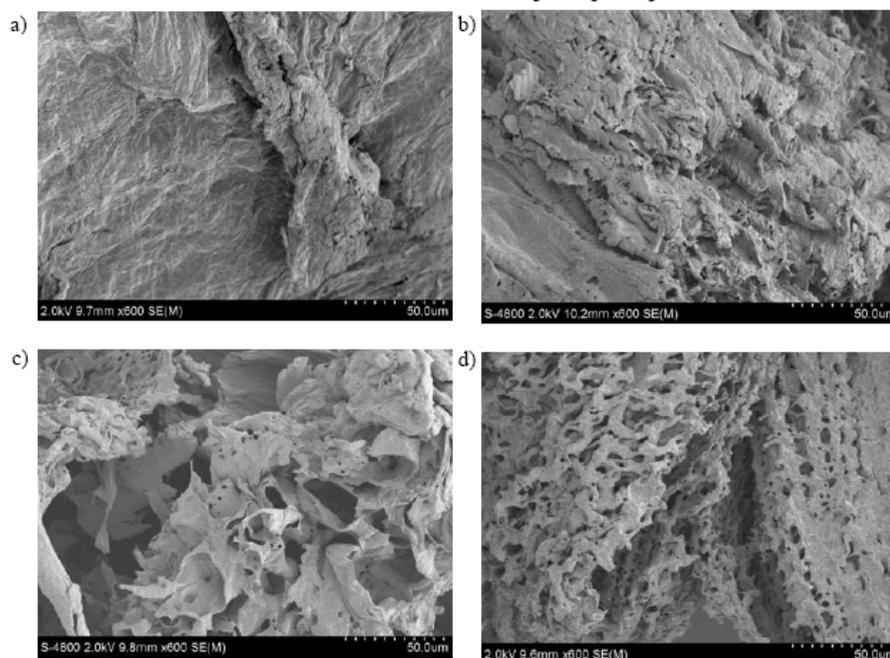| Sample	Surface area (m^2/g)	Total pore volume (cm^3/g)	Average pore diameter (nm)
Untreated corn cobs	3.926	0.0078	7.930
Pretreated corn cobs with 2% KOH at 120 °C for 25 min	5.719	0.0096	8.158

Fig. 4 Scanning electron microscope images of corn cobs. (a) Raw corn cobs without treatment; (b) Corn cobs after pretreatment with 0.75% KOH at 60°C for 25 min; (c) Corn cobs after pretreatment with 0.75% KOH at 120°C for 25 min; (d) Corn cobs after pretreatment with 2% KOH at 120°C for 25 min.

E. X–ray Diffraction Analysis

Crystallinity of cellulose is one of the main factors influencing enzymatic hydrolysis [10]. The cellulose crystallinity value of untreated sample was 24.5 % while the pretreated sample was up to 57.28 %. The crystallinity index in pretreated corncobs with KOH and microwave increased due to lignin and hemicellulose removal, which was good evidence that the amorphous portion of the corn cobs was more removed than the crystalline portion. Moreover, higher KOH concentration, pretreatment temperature and time which leaded to higher lignin removal, resulting in more crystallinity index which raised the enzymatic digestibility.

TABLE III
CRYSTALLINITY INDEX (%) OF UNTREATED AND TREATED CORN COBS

Sample	Crystallinity index
Untreated corn cobs	24.50
Pretreated corn cobs with KOH of 0% for 25 min at 120 °C	39.78
Pretreated corn cobs with KOH of 0.75% for 25 min at 120 °C	40.07
Pretreated corn cobs with KOH of 2% for 25 min at 60 °C	45.39
Pretreated corn cobs with KOH of 2% for 10 min at 120 °C	56.06
Pretreated corn cobs with KOH of 2% for 25 min at 120 °C	57.28

F. Comparison of Total Sugar Concentration Obtained from Different Pretreatment Methods

This part, the pretreatment by microwave and KOH solution was compared with other pretreatment methods. From Table IV shown that the total sugar obtained from enzymatic hydrolysis of pretreated corn cobs with 2% KOH with microwave 120 °C for 25 min was 34.79 g/L much was higher than total sugars of pretreated corn cobs with 2% KOH with autoclave 121 °C for 60 min and 2% KOH with conventional heating 120°C for 25 min. Because microwave radiation can heat uniformly inside the sample, it breaks down the lignin and hemicellulose structure. Furthermore, the partial crytallinity of cellulose was destroyed and hemicellulose was degraded into reducing sugar. Microwave also saved the pretreatment time and increased its accessibility to hydrolytic enzymes.

TABLE IV
COMPARISON OF TOTAL SUGAR CONCENTRATION OBTAINED FROM DIFFERENT PRETREATMENT METHODS

Method	Glucose (g/L)	Total Sugar (g/L)
2% KOH with microwave 120°C for 25 min	19.49	34.79
2% KOH with autoclave 121°C for 60 min	15.88	27.64
2% KOH with conventional heating 120°C for 25 min	15.59	27.24

IV. CONCLUSION

The results showed that the potassium hydroxide pretreatment with microwave on corn cobs was an effective method in improving enzymatic hydrolysis accessibility. The optimum conditions were found at 2% KOH at 120 °C for 25 minutes which could increase in surface area by 45.67% and the cellulose crystallinity index up to 57.28 %. And the highest glucose concentration can reach up 19.49 g/L and total sugar 34.79 g/L was released. Moreover, microwave assists KOH can produce high total sugar concentration at shorter pretreatment time compared with autoclave and conventional methods.

ACKNOWLEDGMENT

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ORIGINAL PAPER

Enhancement of enzymatic hydrolysis of corncob by microwave-assisted alkali pretreatment and its effect in morphology

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Sujitra Wongkasemjit

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

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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.

Experimental

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.

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:

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

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.

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.

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).

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/Rint 2200 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:

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

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).

Results and discussion

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 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

Table 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.

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. 1) and total sugar concentration (Fig. 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

Cellulose
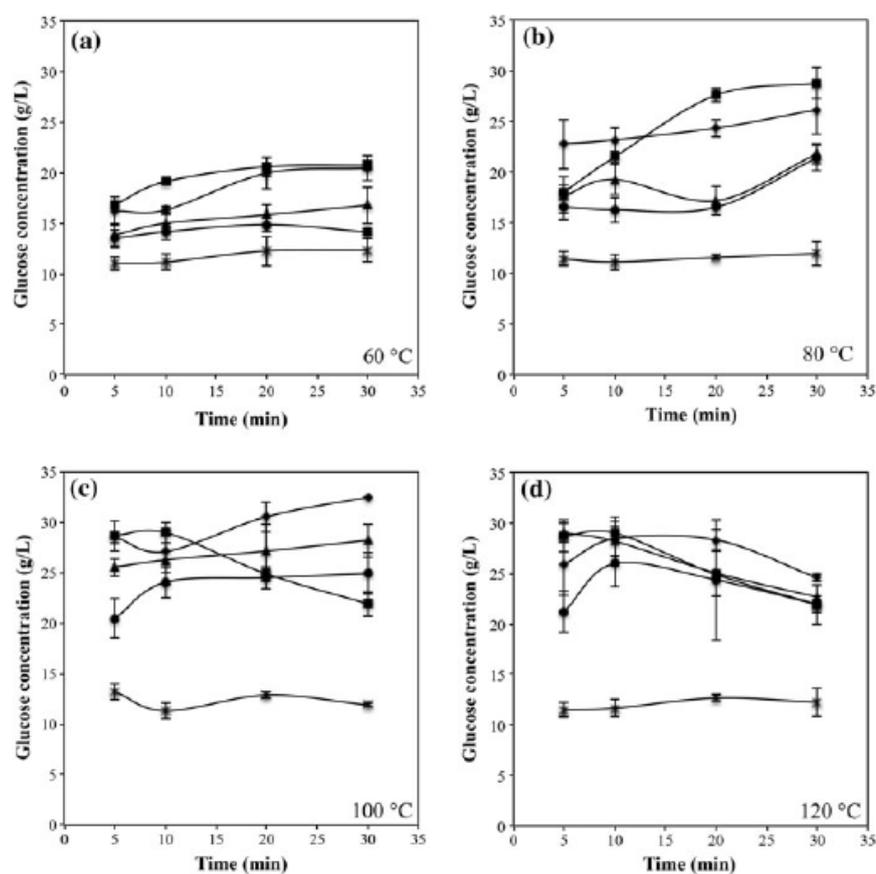
high sugar production. Moreover, higher solids loss resulted in less total sugar production. Figure 3 indicates glucose concentration and total sugar concentrations from enzymatic hydrolysis under optimum conditions. After 48 h, there was no significant difference in glucose levels while there was only slightly increased total sugar concentration. Thus, 48 h was chosen as the optimal period for enzymatic saccharification.

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 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.

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. 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. 4b), the pretreated

Fig. 2 Total sugar concentrations obtained from enzymatic hydrolysis of pretreated corncob at different times, temperatures of **a** 60 °C, **b** 80 °C, **c** 100 °C and **d** 120 °C and NaOH concentrations: 0 % (*asterisk*), 0.75 % (*circle*), 1 % (*triangle*), 2 % (*diamond*), 3 % (*square*)
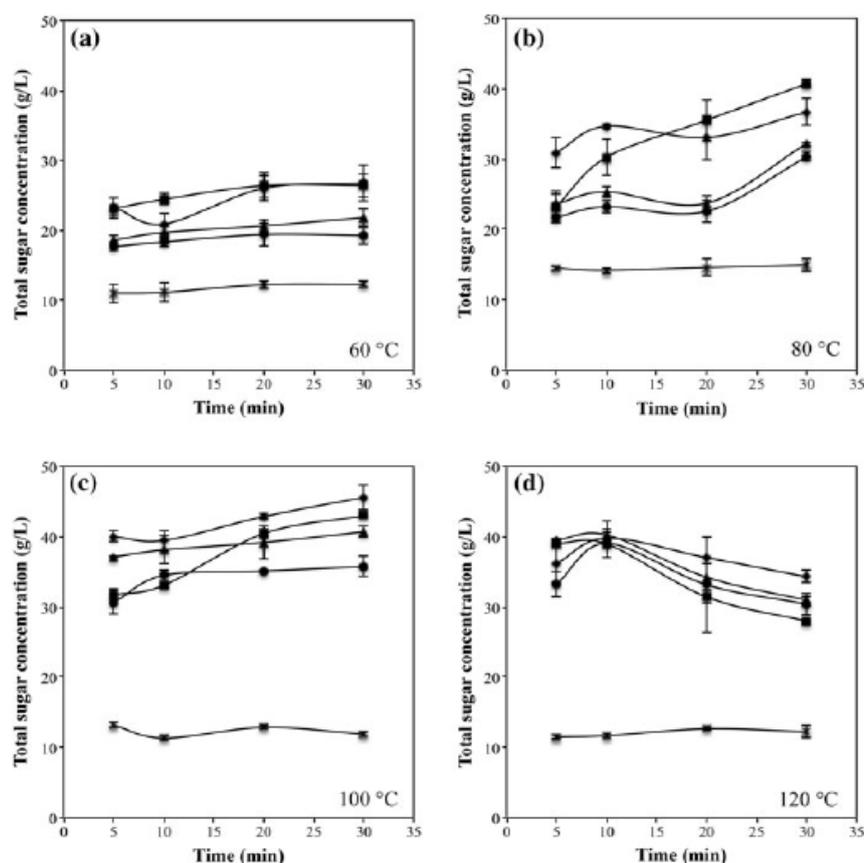
corncob looks soft and pores appear. After pretreatment with 0.75 % NaOH at 100 °C for 20 min (Fig. 4c), the pretreated corncob is more porous, meaning that enzymatic accessibility is enhanced by increased surface area of the biomass material.

Therefore, pretreatment temperature, time, and NaOH concentration increased the surface area. The SEM results were also consistent with the BET surface area results. Under optimum pretreatment conditions (Fig. 4d), there was ~40 % increase in surface area of corncob from 0.994 to 1.375 m^2/g and total pore volume from 0.0018 to 0.0025 cm^3/g while the average pore diameter decreased from 7.34 to 7.11 nm.

Crystallinity is believed to be an important feature affecting enzymatic saccharification of cellulose. Table 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

Fig. 2 Total sugar concentrations obtained from enzymatic hydrolysis of pretreated corncob at different times, temperatures of **a** 60 °C, **b** 80 °C, **c** 100 °C and **d** 120 °C and NaOH concentrations: 0 % (*asterisk*), 0.75 % (*circle*), 1 % (*triangle*), 2 % (*diamond*), 3 % (*square*)

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Therefore, pretreatment temperature, time, and NaOH concentration increased the surface area. The SEM results were also consistent with the BET surface area results. Under optimum pretreatment conditions (Fig. 4d), there was ~40 % increase in surface area of corncob from 0.994 to 1.375 m^2/g and total pore volume from 0.0018 to 0.0025 cm^3/g while the average pore diameter decreased from 7.34 to 7.11 nm.

Crystallinity is believed to be an important feature affecting enzymatic saccharification of cellulose. Table 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
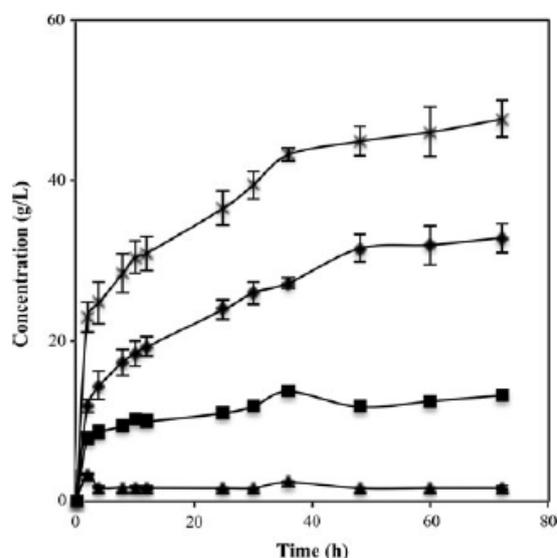
Fig. 3 Glucose (*diamond*), xylose (*square*), arabinose (*triangle*) and total sugar (*asterisk*) concentrations from enzymatic hydrolysis of pretreated corncob with 2 % NaOH for 30 min at 100 °C (optimum condition)

Table 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

acid pretreatment of wheat straw at 190 °C (Dhabhai et al. 2013). The hypothesis of this phenomenon is the transformation of cellulose I$_\beta$ 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).

Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) results are shown in Fig. 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.

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. 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.

Ethanol production

The ethanol profile of hydrolysate from alkali pretreatment and enzymatic hydrolysis using *S. cerevisiae*

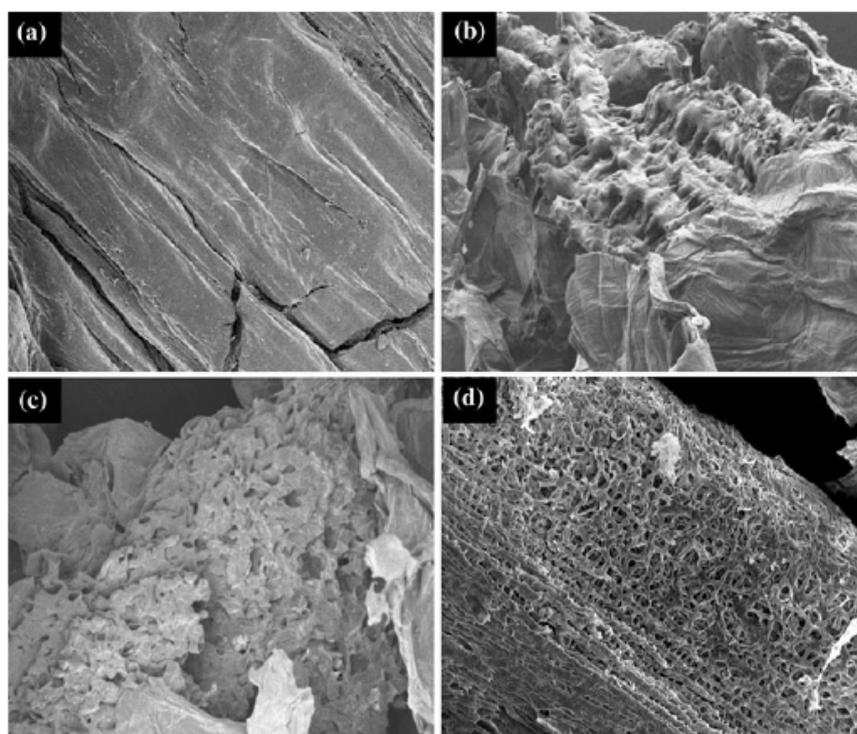

Fig. 4 Scanning electron microscope images of **a** raw corncob without pretreatment; **b** corncob after pretreatment with 0.75 % NaOH at 60 °C for 20 min; **c** after pretreatment with 0.75 % NaOH at 100 °C for 20 min, and **d** after pretreatment with 2 % NaOH at 100 °C for 30 min

Table 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

was compared with the semi-synthetic medium (YPD) as shown in Fig. 6. The hydrolysate contained 32.52 and 10.41 g L^{-1} glucose and xylose (Table 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 g L^{-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).
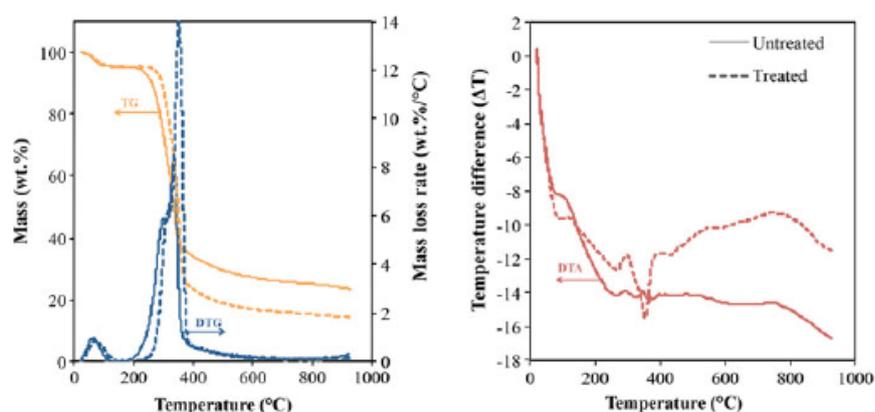
Fig. 5 TG–DTG curves of corncob before and after pretreatment

Table 4 Comparison of reducing sugar concentration obtained from enzymatic hydrolysis with different pretreatment methods

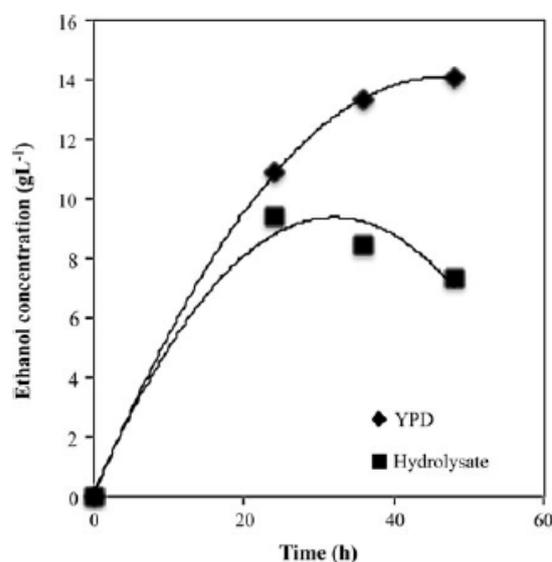| Method	NaOH (%)	Temp (°C)	Time (min)	Glucose (g L^{-1})	Xylose (g L^{-1})	Reducing sugar (g L^{-1})
Microwave	2	100	30	32.52	10.41	45.60
Hot plate	2	100	30	22.44	13.30	37.46
Autoclave	2	121	60	33.75	11.42	46.85

Fig. 6 Ethanol profile of YPD (*diamond*) and corncob hydrolysate (*square*) using *S. cerevisiae*

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.

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ORIGINAL ARTICLE

Enhancement of ABE fermentation through regulation of ammonium acetate and D–xylose uptake from acid-pretreated corncobs

Akarin Boonsombuti · Kittinan Komolpis ·
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Abstract *Clostridium acetobutylicum* TISTR 1462 and *Clostridium beijerinckii* TISTR 1461 were chosen to optimize acetone–butanol–ethanol (ABE) fermentation by using glucose as a carbon source. The enhancement in its productivity by adding various concentrations of ammonium acetate was studied. Then, the variation of glucose/xylose ratios in the pre-grown medium was investigated. The results showed that both increased ammonium acetate in the production medium and D–xylose in the pre-grown medium could produce more ABE. With these conditions, using corncob hydrolysate as a substrate, 20.58 g/L ABE was produced from *C. beijerinckii* TISTR 1461 with 0.44 g/L/h and 0.45 of ABE productivity and yield, respectively.

Keywords Butanol · *Clostridium acetobutylicum* · *Clostridium beijerinckii* · Corncobs · Fermentation

Introduction

The fluctuation of oil prices and worldwide environmental regulatory concerns have led researchers to seek alternative energy resources that are less dependent on fossil fuels. In recent years, ethanol production has been increasing because it can be blended with gasoline to reduce gasoline consumption, while at the same time helping to reduce greenhouse gas emissions (Talebnia et al. 2010). Among various alternative fuels, butanol has been recognized as one of the most promising and can be produced via fermentation or petrochemical processing in the same way as ethanol. However, butanol has additional advantages due to its low Reid vapor pressure (RVP) and higher energy (Ladisch 1991). In addition, it can be blended with gasoline at a higher concentration than ethanol, and less energy is required to separate butanol from water than to separate ethanol from water. Moreover, without necessitating any modifications of vehicles, butanol can compete with ethanol because it is not corrosive like ethanol (Lee et al. 2008).

The solventogenic products—acetone, butanol, and ethanol (ABE)—produced from ABE fermentation have a conventional ratio of 6:3:1, respectively (Jones and Woods 1986). *Clostridium acetobutylicum* and *C. beijerinckii* are among the prominent solventogenic species capable of ABE formation via fermentation, with *C. acetobutylicum* being the species that is most often used. In some cases, the productivity of the fermentation can be enhanced by adding ammonium acetate (Gu et al. 2009), or strain degeneration can be prevented by adding acetate or butyrate to *C. beijerinckii* NCIMB 8052 (Lee et al. 2008; Chen and Blaschek 1999b), *C. beijerinckii* BA101 (Chen and Blaschek 1999a), or *C. acetobutylicum* strain 77 (a mutant from *C. acetobutylicum* ATCC 824) (Matta-el-ammouri et al. 1987). It has been reported that butyrate and acetate pathways play important roles in the energy metabolism of various strains of solventogenic products; therefore, solventogenesis is triggered, which appears to be essential for butanol production. So far, however, there have been no studies of the two strains provided by the Thailand Institute of Scientific and Technological Research (TISTR), *C. acetobutylicum* TISTR 1462 and *C. beijerinckii* TISTR 1461, in terms of how they might enhance ABE production. In this study, we chose these two strains as the representatives for *C. acetobutylicum* and *C. beijerinckii* (Keis et al. 2001).

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One of the limitations of ABE fermentation is the cost of substrates, to the extent that substrate costs have been identified as a major factor affecting the economic viability of industrial ABE fermentation. In the past, production facilities have even been shut down due to the high price of molasses (Jones and Woods 1986). Therefore, the possibility of using abundant and renewable sources of lignocellulosic residues, such as corncob waste, as sources of monomeric sugars for conversion into biobutanol holds great promise. Indeed, it makes this waste more attractive for producing a variety of valuable products, including biofuels. In Thailand, corncobs are considered agricultural waste, with over 35 million kg generated each year. Corncobs consist mainly of cellulose (39 %), hemicellulose (43 %), and lignin (7.6 %), with xylose being the major constituent of hemicellulose.

To increase the value of corncobs, they have been used to produce fermentable sugars via acid pretreatment and enzymatic saccharification. Among the acids studied for the pretreatment step, diluted sulfuric acid is the most commonly used. Through the pretreatment step, small amounts of cellulose and most of the hemicellulose can be hydrolyzed into monomeric sugars, which can then be further fermented into butanol.

Therefore, the present study deals with the optimization of fermentation conditions for butanol production by *C. acetobutylicum* TISTR 1462 and *C. beijerinckii* TISTR 1461 with respect to their ability to produce ABE, in addition to considering product enhancement with ammonium acetate in batch fermentation with glucose as the carbon source, since, until now, there has been no report on the supplementation of ammonium acetate to the hydrolysate of biomass. Since sugars from biomass often contain mixed C_5 and C_6 sugars, modification of the medium in the preculture step with various ratios of glucose and xylose has also been studied (Ounine et al. 1985; Wayman and Yu 1985). Finally, the selected strain was employed, under suitable conditions, to produce ABE from acid-pretreated corncobs.

Materials and methods

Strain and inoculum development

The *C. acetobutylicum* TISTR 1462 and *C. beijerinckii* TISTR 1461 strains were obtained from the Thailand Institute of Scientific and Technological Research. The spores were maintained in cooked meat medium (CMM; Difco Laboratories, Detroit, MI, USA) at 4 °C. The spores (1.8 mL) were transferred to cooked meat medium (CMM) and heat-shocked at 75 °C for 2 min (Qureshi et al. 2008b). The heat-shocked spores were then transferred to 16.2 mL CMM for spore germination at 37 °C until the optical density (600 nm) reached 0.8. Then, 8 mL of active growth cells were

transferred into 72 mL of tryptone glucose yeast (TGY) medium (Areesirisuk 2007) for 6 h at 37 °C.

Corncob pretreatment and overliming

Corncobs were supplied as complimentary samples by the Betagro Company, Thailand. The composition was determined using the NREL (National Renewable Energy Laboratory) method for the determination of structural carbohydrates and lignin in biomass (Sluiter et al. 2008). The average particle size of the corncob was 1.6 mm when homogenized in a single lot. The corncobs were dried in a 65 °C oven for 2 days. Then, 34 g of the corncobs were presoaked in 0.5 L of 0.75 % (v/v) H_2SO_4 in a 1-L glass screw-cap bottle (1 g of biomass per 15 mL of solution) at 50 °C for 30 min. For pretreatment, 0.5 L of the suspended corncobs in diluted acid were heated in an autoclave at 121 °C for 1 h, then left to cool to room temperature. Dilute acid pretreatment is generally well known to produce a significant amount of fermentation inhibitors during the process; therefore, the overliming process was also conducted by using $Ca(OH)_2$ to detoxify the inhibitors contained in dilute acid hydrolysates of lignocelluloses prior to fermentation (Martinez et al. 2001). The hydrolysate was adjusted with $Ca(OH)_2$ to pH 10, which was followed by the addition of 1 g/L of $NaSO_3$ to reduce the redox potential, and then heating to 90 °C for 30 min (Qureshi et al. 2010). After cooling to room temperature, the pH was adjusted to 6.6 by H_2SO_4. Before being used as a substrate, the hydrolysate was filtered twice to remove sediments and sterilized with a 0.2-μm pore size membrane. Samples with and without overliming treatment were analyzed for sugar composition and furfural content.

Fermentation

Fermentations were performed in a 100-mL screw cap Duran® glass bottle with 2 stainless tubes inserted for purging ultra-high nitrogen gas (to provide anaerobic conditions), sample collecting, and pressure release. The incubation temperature was maintained at 37 °C with a 150-rpm orbital shaking rate without pH control. The P2 medium was chosen as the production medium (Qureshi and Blaschek 1999). Glucose, xylose, and corncob hydrolysates were utilized as the carbon source at various concentrations. To prepare the P2 medium, sugar-containing distilled water and 80 g/L of yeast extract were autoclaved separately to prevent a Maillard reaction (Jaeger et al. 2010). The fermentation process was initiated by mixing all the substances to reach an exact concentration: 5 % (v/v) actively grown cells obtained from TGY, 1 g/L yeast extract, acetate buffer (0.5 g/L K_2HPO_4 and KH_2PO_4 and 2.2 g/L ammonium acetate), vitamins (0.001 g/L p–amino–benzoic acid and thiamine and 0.00001 g/L biotin), minerals (0.01 g/L $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, and NaCl and 0.2 g/L $MgSO_4 \cdot 7H_2O$) and glucose or xylose at various

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Fig. 1 Production of ABE, acid concentration, ABE productivity, yield, and percentage of utilized glucose where (*I*) *C. acetobutylicum* TISTR 1462, 2 % initial glucose, (*II*) *C. beijerinckii* TISTR 1461, 2 % initial glucose, (*III*) *C. beijerinckii* TISTR 1461, 4 % initial glucose, and (*IV*) *C. beijerinckii* TISTR 1461, 6 % initial glucose collected at 48 h of fermentation
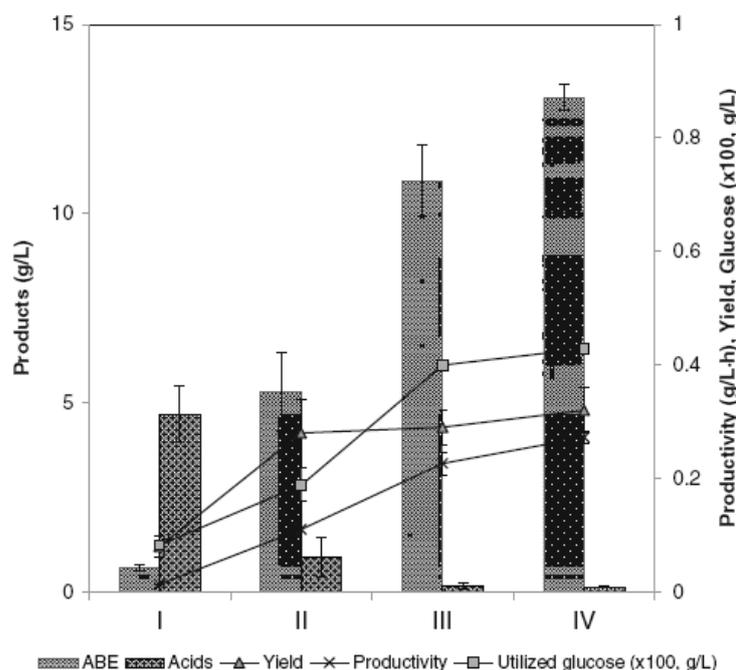
13.07 g/L of acetone, butanol, ethanol, and total ABE, respectively, with an A:B:E ratio at 3:6:1. Then, the proper amount of initial glucose for *C. beijerinckii* TISTR 1461 was indicated for solventogenic enhancement by ammonium acetate since the excessive glucose combined with acetate may alter the acetone/butanol ratio (Jones and Woods 1986).

Effect of ammonium acetate on production

The two most important parameters affecting butanol formation are the pH condition and the concentrations of the acids produced. There have been studies on the addition of acids to promote solventogenic products for some strains (Chen and Blaschek 1999b; Ezeji et al. 2007), and on the addition of ammonium acetate to improve the solvent yield when using cassava as a fermentation medium (Gu et al. 2009). In this study, ammonium acetate was present in the P2 medium at 30 mM, which is an optimum concentration. However, the effect of ammonium acetate on the production of ABE fermentation by *C. beijerinckii* TISTR 1461 has not previously been reported. Therefore, testing was carried out on the effect of ammonium acetate on product enhancement by varying concentrations of ammonium acetate from 30 to 80 mM with 6 % glucose concentration. The acidity of the fermentation broth is shown in Fig. 2, where each ammonium acetate concentration is significantly different due to a high buffer capacity (Bryant and Blaschek 1988).

A pH of 5.0 is usually suitable for ABE fermentation; however, the pH range may vary widely depending on the type

of strain and the conditions used. The results showed that 30 mM ammonium acetate may lack the buffer capacity necessary since the pH decreased during the fermentation period. In order to maintain the pH at an optimum level, the addition of ammonium acetate in P2 medium was monitored. The addition of ammonium acetate could maintain the pH above 5.0 after the exponential phase, suggesting that acids produced from the culture did not have much effect on the broths. It has been reported that the addition of ammonium acetate can increase transcription of acidogenic (*ask* and *buk*)
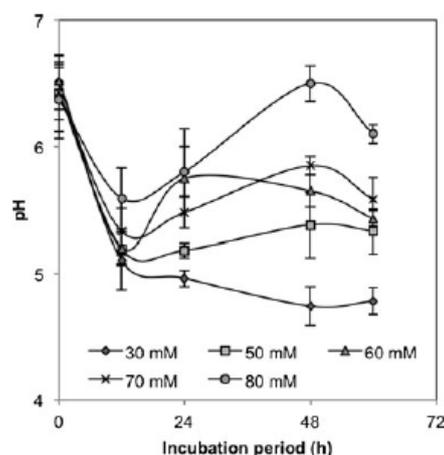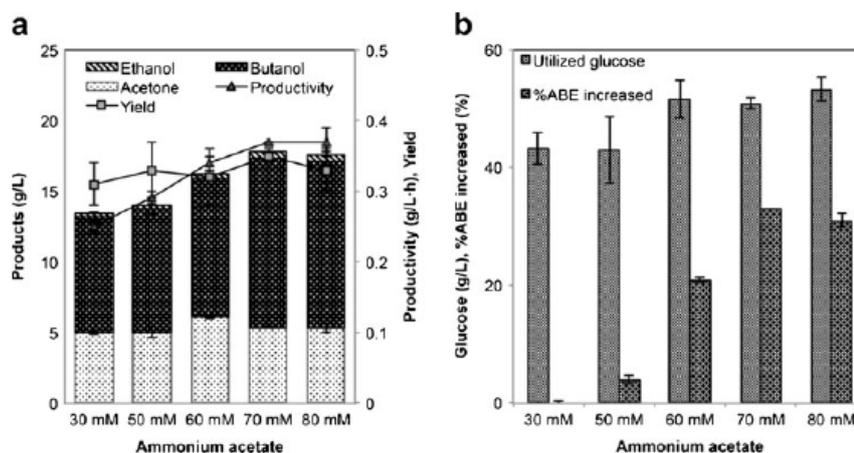

Fig. 2 The effect of the addition of ammonium acetate on broth acidity

Ann Microbiol

Fig. 3 The effect of the
addition of ammonium acetate
on a ABE concentration, ABE
productivity, and yield (for each
ammonium acetate addition);
and b utilized glucose and
percentage of product increase

and solventogenic genes (*adc*, *ctfAB*, *adhE* and *bdhB*) observed by reverse transcription–PCR analysis (Gu et al. 2009).

When ammonium acetate was introduced, the ABE concentration, ABE productivity, and yield increased as the ammonium acetate concentration increased, as shown in Fig. 3a. It can be suggested that the supplementation of ammonium acetate into hydrolysate is required. However, further increases in the ammonium acetate concentration did not result in a higher yield of solvent. The optimum ammonium acetate concentration of 70 mM was confirmed by statistical analysis using one-way analysis of variance (ANOVA, $P < 0.05$). For ABE product concentration, the addition of 70 mM of ammonium acetate was considered as the same group of homogeneous subset of 80 mM ammonium acetate in both Tukey HSD (honestly significant difference) and Scheffe methods. In the experiment, the addition of 70 mM ammonium acetate elevated the ABE concentration, total solvent productivity, and yield to 17.82 g/L, 0.37 g/L/h, and 0.35, respectively, after 48 h of fermentation.

At 80 mM of ammonium acetate, the ABE concentration, total solvent productivity, and yield were 17.63 g/L, 0.37 g/L/h, and 0.33, respectively, which were approximately the same as for 70 mM of ammonium acetate. The *C. beijerinckii* TISTR 1461 in this work can produce butanol at a rate of 12.01 g/L compared with 13.90 g/L for cultures grown in MP2 medium containing 60 mM sodium acetate and *C. beijerinckii* NCIMB 8052 (Chen and Blaschek 1999b). The

highest butanol yield (0.24) and productivity (0.25) was obtained after 48 h of fermentation by *C. beijerinckii* TISTR 1461. Meanwhile, Chen and Blaschek (1999b) used *C. beijerinckii* NCIMB 8052 with the addition of 60 mM sodium acetate for butanol production which led to butanol yield and productivity of 0.39, and 0.29, respectively.

There was an improvement in glucose utilization and also an increase in the level of the products, except at 80 mM, as displayed in Fig. 3b. The %ABE value increased rapidly during the initial addition of ammonium acetate. At higher concentrations of ammonium acetate, the utilized glucose in the presence of 80 mM ammonium acetate was comparable to that observed for 70 mM ammonium acetate. Yet, this result was inconsistent with the results of studies of *C. acetobutylicum* EA 2018 (Gu et al. 2009) and *C. beijerinckii* NCIMB 8052 (Lee et al. 2008). For *C. acetobutylicum* EA 2018, either the sodium acetate or ammonium sulfate addition alone is not sufficient to enhance solvent production in cassava medium, which is different from *C. beijerinckii* NCIMB 8052, which uses only acetate. Other researchers state that the acetate addition can change the ratio of A:B (conventionally 1:2) to 2:3 due to the metabolic shift toward acetone production/ since the improvement in CoA–transferase activity consequently positively affects the conversion of acetate to acetyl–CoA and further converts to acetone by a metabolic pathway (Nölling et al. 2001).

Table 1 Effects of different glucose/xylose (*G/X*) ratios in preculture medium (TGY) on production medium (P2)

G/X ratio	Total ABE (g/L)	ABE productivity (g/L·h)	ABE yield	Utilized glucose (g/L)	Utilized xylose (g/L)	Sugar uptake (g/L·h)	A:B:E ratio
100/0	17.80±1.22	0.37±0.00	0.35±0.00	29.55±1.25	21.79±1.64	1.07±0.06	36:59:05
70/30	18.96±0.24	0.39±0.01	0.38±0.01	29.60±0.26	20.31±0.56	1.04±0.02	36:58:06
50/50	17.84±1.30	0.37±0.00	0.38±0.00	29.89±0.94	17.58±2.09	0.99±0.06	37:56:07
30/70	6.20±0.13	0.13±0.00	0.20±0.00	29.44±0.04	1.24±0.09	0.64±0.00	0:0:100

Table 2 Sugar, acetic acid, and furfural concentrations before and after lime treatment of corncob hydrolysate

	Before lime treatment (g/L)	After lime treatment (g/L)
Glucose	1.60	1.96
Xylose	15.85	14.68
Arabinose	3.43	4.46
Total sugar	20.88	21.10
Acetic acid	2.16	2.85
Furfural	0.04	0.01

Effect of sugar composition in preculture medium (TGY) on ABE fermentation

Sugars obtained from pretreated biomass usually contain both C_5 (mainly from hemicellulose) and C_6. Many studies of sugar uptake from glucose and xylose have been reported. One prior study observed diauxic growth by means of glucose consumption until depletion, after which xylose utilization occurred (Kanouni et al. 1998). There have also been studies about the effect of xylose–pregrown cells in production medium (Soni et al. 1982) that differed from the finding with *C. beijerinckii* TISTR 1461 in the present study. Since growth in P2 medium was relatively low, an adjustment of the amount of xylose in the preculture step (TGY) by varying the ratio of the carbon sources (glucose/xylose) and using mixed glucose (30 g/L) and xylose (30 g/L) as a substrate was investigated. The results of ABE production, shown in Table 1, indicate that ABE fermentation occurred in all the samples except for the medium inoculated with glucose–xylose–pregrown cells at the ratio of 30/70. Although there was not much difference under the 100/0 and 70/30 conditions, the latter produced a higher total ABE concentration and higher accuracy for replication results. Moreover, the utilization of glucose and xylose in the fermentation broth was also observed. Since glucose was completely utilized, it was found that glucose was a preferable sugar for *C. beijerinckii* TISTR 1461 compared to xylose in all cases. With the fixed incubation period of 24 h, the fastest glucose utilization rate occurred in the pregrown cells

inoculated with glucose/xylose at the ratio of 100/0. For the 30/70 condition, the highest amount of xylose concentration in preculture medium resulted in a low rate of sugar uptake and produced mainly ethanol. In the batch fermentation containing a mixture of glucose and xylose, the highest amount of fermentable sugars of 50 g/L was obtained. This result is comparable to the work of Ounine et al. (1985) and Wayman and Yu (1985). It has been reported that the suppression of glucose in xylose utilization may be caused by glucose competition with xylose in the transport system (Zyl et al. 1993) and catabolite repression in the xylose metabolism pathway (Blencke et al. 2003).

Moreover, this study indicates the amount of butanol that can inhibit the growth of *C. beijerinckii* TISTR 1461. A previous study (Ounine et al. 1985) reported that glucose and xylose permease were inhibited when butanol concentration reached 12 and 8 g/L, respectively. As found in this study, *C. beijerinckii* TISTR 1461, with an ammonium acetate 70 mM supplement, can tolerate butanol toxicity to 12 and 11 g/L for glucose and xylose permease, respectively.

ABE fermentation using acid pretreated corncobs

The substrate obtained from acid-pretreated corncobs contains mainly glucose (1.60 g/L), xylose (15.85 g/L), and arabinose (3.43 g/L) as a carbon source with a sugar concentration of 20.88 g/L (Table 2). Detoxification by the overliming process was implemented in order to lower the amount of furfural. Previous reports suggest that furfural and hydroxymethyl furfural (HMF) at a level less than 3 g/L have no effect as an inhibitor (Ezeji et al. 2007). However, in this study, *C. beijerinckii* TISTR 1461 was not able to grow in the acid-pretreated corncobs without the overliming process (no ABE was detected after 48 h of fermentation). Table 2 shows the amount of sugars, acetic acid, and furfural of the pretreated corncobs before and after the overliming process. Lime treatment can lower furfural concentration twofold and slightly increase other substances

Table 3 Butanol production from various types of acid/alkaline-pretreated biomass

Substrate	Hydrolysis	Culture	ABE (g/L)	ABE yield	ABE productivity	References
Rice straw	Alkaline	*C. saccharoperbutylacetonicum* ATCC 27022	20.40	0.35	0.24	Soni et al. 1982
Defatted rice bran	Dilute sulfuric acid	*C. beijerinckii* NCIMB 8052	13.19	0.44	0.37	Jieun et al. 2009
Wheat bran	Dilute sulfuric acid	*C. beijerinckii* ATCC 55025	11.18	0.32	0.16	Liu et al. 2010
Corn fiber	Dilute sulfuric acid	*C. beijerinckii* BA101	9.30	0.39	0.10	Qureshi et al. 2008a
Corncobs	Dilute sulfuric acid	*C. beijerinckii* TISTR 1461	13.09	0.26	0.27	Present study
Corncobs[a]	Dilute sulfuric acid	*C. beijerinckii* TISTR 1461	20.58	0.45	0.44	Present study

[a] With a studied condition of glucose/xylose ratio at 70/30 in pregrown medium and the addtion of ammonium acetate to 70 mM

Fig. 4 The results of ABE fermentation using corncobs as the substrate, including a solventogenic and acid products from acid pretreatment plus 30 g/L glucose and b acidity level and sugar consumption
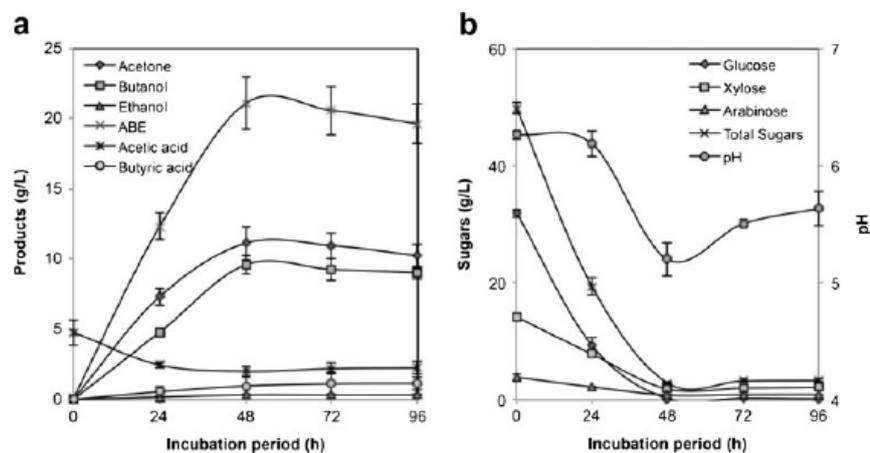
due to the high temperature of the reaction and loss of water. Acetic acid contained in the hydrolysate occurs from the deacetylation of xylan, which also has a significant effect on microbial fermentation (Martinez et al. 2000). In the initial phase of the fermentation, there is a high amount of acetic acid, 4.80 ± 0.93 g/L, compared with that found in glucose/xylose fermentation.

To investigate the ABE fermentation capability of *C. beijerinckii* TISTR 1461 with acid-pretreated corncobs, a glucose addition of up to 50 g/L total sugars was applied. At 48 h of fermentation, a productivity and yield of ABE at 0.44 g/L/h and 0.45 were found to have significantly higher level compared to the control experiment (Table 3). Butanol toxicity produced in ABE fermentation is often encountered in butanol production. Therefore, the amount of utilized sugar (with approximately 3 g/L total sugars) at 96 h of fermentation (Fig. 4) may be due to butanol toxicity. It was lower compared with the effect of sugar composition in the preculture medium (TGY) on ABE fermentation since the culture can tolerate only 9.61 g/L butanol compared to 11 g/L as previously mentioned. According to the literature, the best results ever obtained for ABE fermentation in terms of butanol concentration have been less than 20 g/L since butanol can significantly inhibit cell growth and fermentation. Liu et al. (2010) found that 6.3 ± 0.5 g/L of butanol were produced in 72 h by *C. beijerinckii* 550251. On the other hand, *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* BA101 grown in semidefined P2 medium containing 6 % glucose produced 9.2 and 18.8 g/L of butanol, respectively, in batch fermentation (Formanek et al. 1997). *C. beijerinckii* BA101 was developed from *C. beijerinckii* NCIMB 8052; therefore, *C. beijerinckii* BA101 produces a higher concentration of total solvents.

Although *C. beijerinckii* TISTR 1461 can produce ABE at a rate of up to 20 g/L, the main component is acetone (11.14 g/L), which is affected by the ABE ratio changing from 3:6:1 to 5:4:1. Nevertheless, there was an enhancement

of butanol production since butanol yield and productivity were elevated from 0.16 and 0.17 to 0.20 and 0.20, respectively, compared to the control experiment. This was confirmed after comparison to the butanol yield and productivity, using the reducing sugar content of $Ca(OH)_2$-detoxified corncob residue hydrolysate, as they were 0.16 and 0.17, respectively (Zhang et al. 2012). The high concentration of acetone may be due to the acetate from the hydrolysate and ammonium acetate since the acetate can reversibly change in the solventogenic phase. Moreover, the carbon recovery, shown in Table 4, indicates that the amount of carbon source was not limited.

Table 4 Carbon recovery from ABE fermentation by *C. beijerinckii* TISTR 1461 using acid pretreated corncobs and 30 g/L of glucose as substrates

Substrate or product	Total carbon (mol/L)
Substrates	
Glucose utilized	1.05
Xylose utilized	0.41
Arabinose utilized	0.10
Acetate	0.16
Products	
Butyrate	0.04
Acetate	0.07
Ethanol	0.02
Butanol	0.52
Acetone	0.58
Total products	1.22
Total substrates	1.72
Recovery (%)	70.84
% Recovery (+33 % theoretical CO_2[a])	103.84

[a] Theoretical CO_2 value was obtained under a specific condition where *Clostridium beijerinckii* BA101 was grown in MP2 medium containing 8 % glucose and 60 mM sodium acetate (Chen and Blaschek 1999a)

Conclusions

In efforts to eliminate the obstacle of high substrate prices, corncobs present a promising alternative due to their high sugar content. The present study found that acid-pretreated corncobs are a potential substrate for ABE fermentation since they can produce total sugars up to 20 g/L (294 mg sugar/g corncobs) and be used practically as a carbon source for *C. beijerinckii* TISTR 1461. Both the glucose and xylose in the pregrown medium and the addition of ammonium acetate have a significant effect on the ABE concentration, ABE productivity, and yield. Butanol toxicity remains a major problem when the concentration reaches ~10 g/L. Future work should concentrate on enhancing extraction efficiency to increase the amount of sugar obtained from the biomass by using both acid pretreatment and enzymatic saccharification, or on investigating techniques to collect butanol during fermentation.

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ประวัติคณะผู้วิจัย

</div>

<u>**หัวหน้าโครงการวิจัย**</u>

1. **ชื่อ - สกุล (ภาษาไทย)** นางสุจิตรา วงศ์เกษมจิตต์

   (ภาษาอังกฤษ) Ms. Sujitra Wongkasemjit

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3. **ตำแหน่งปัจจุบัน** รองศาสตราจารย์

4. **หน่วยงานที่อยู่ที่ติดต่อได้สะดวก พร้อมหมายเลขโทรศัพท์ โทรสาร และ E-mail**

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5. **ประวัติการศึกษา**

ปริญญา	สาขาวิชา	มหาวิทยาลัย	ปี พ.ศ. ที่ได้รับ
B.S.	วิทยาศาสตร์ทั่วไป	จุฬาลงกรณ์มหาวิทยาลัย	2523
M.S.	Organic Chemistry	Rochester Institute of Technology/USA	2526
Ph.D.	Organic Chemistry	West Virginia University/USA	2531
Post Doctoral	Organic Chemistry	Food and Drug Administration/ USA	2532

6. **สาขาวิชาการที่มีความชำนาญพิเศษ**

   การสังเคราะห์ การตรวจสอบโครงสร้าง และการประยุกต์สารอินทรีย์ สารโลหะอินทรีย์ และพอลิเมอร์

7. **ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุ สถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วม วิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น**

   7.1 **หัวหน้าโครงการวิจัย :**

      7.1.1 ชื่อโครงการ: Development of Carbon Aerogel via Functionalized Polybenzoxazine for Catalyst Support Applications

      แหล่งทุน: ศูนย์นาโนเทคโนโลยี 737,000 บาท

      ระยะเวลาดำเนินการ: 23 กันยายน 2551 – 22 กันยายน 2553 (เสร็จสมบูรณ์)

      7.1.2 ชื่อโครงการ: Study of a Synthetic Method and Application of SBA-1

Synthesized Directly from Silatrane

แหล่งทุน: TRF-RGJ   3,550,000 บาท

ระยะเวลาดำเนินการ: มิถุนายน 2548 - กรกฎาคม 2552 (เสร็จสมบูรณ์)

7.1.3   ชื่อโครงการ: การสังเคราะห์และศึกษาสมบัติของสารเพื่อใช้เป็นคาโทดสำหรับ
เซลล์เชื้อเพลิงชนิดออกไซด์ของของแข็งที่ใช้ในช่วงอุณหภูมิปานกลาง

แหล่งทุน: สำนักงานคณะกรรมการวิจัยแห่งชาติ   350,000 บาท

ระยะเวลาดำเนินการ: 1 ตุลาคม 2550-30 กันยายน 2551 (เสร็จสมบูรณ์)

7.1.4   ชื่อโครงการ: Understanding Particle Morphology and Size Control of
Catalyst Solid Support

แหล่งทุน: บริษัทปูนซิเมนต์ไทย จำกัด   437,000 บาท

ระยะเวลาดำเนินการ: 23 สิงหาคม 2549-22 มิถุนายน 2550 (เสร็จสมบูรณ์)

7.1.5   ชื่อโครงการ: Synthesis and Applications of Organometallics Reseach
Unit

แหล่งทุน: กองทุนรัชดาภิเษกสมโภช จุฬาฯ   3,500,000 บาท

ระยะเวลาดำเนินการ: 1 สิงหาคม 2546–31 กรกฎาคม 2551 (เสร็จสมบูรณ์)

7.1.6   ชื่อโครงการ: Study of a Synthetic Method and Application of TiO_2-SiO_2

as Mixed Matrix Membrane

แหล่งทุน: TRF-RGJ   3,550,000 บาท

ระยะเวลาดำเนินการ: ธันวาคม 2545-พฤศจิกายน 2548 (เสร็จสมบูรณ์)

7.2   ผู้ร่วมโครงการวิจัย : ชื่อโครงการวิจัย

7.2.1   ชื่อโครงการ: Development of Ceramic Membrane for Micro- and
Ultrafiltration and Zeolite Membrane for Ethanol Separation

แหล่งทุน: ทบวงฯ ภายใต้โครงการ RBD   6,900,000 บาท

ระยะเวลาดำเนินการ: สิงหาคม 2546-กรกฎาคม 2551 (เสร็จเรียบร้อยแล้ว)

7.3   ผลงานวิจัย (5 ปีย้อนหลัง)

7.3.1   การจดสิทธิบัตร

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7.3.2 การตีพิมพ์ในวารสารนานาชาติ

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Corresponding Author

7.3.3 การตีพิมพ์ในวารสารภายในประเทศ

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7. นพพร ธนบดีกิจ และ สุจิตรา วงศ์เกษมจิตต์ "กระบวนการโซล-เจลสารประกอบสไปโรซิลิเกต" วารสารวิจัยวิทยาศาสตร์ (Section T) คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ฉบับที่ 1, 57-71 (2546)

8. มาธาวี สาธุปัญญา และ สุจิตรา วงศ์เกษมจิตต์ "ซีโอไลท์ชนิด ANA และ GIS" วารสารวิทยาศาสตร์ สมาคมวิทยาศาสตร์แห่งประเทศไทย ฉบับที่ 1 มกราคม-กุมภาพันธ์ 2546

7.3.4   การเสนอผลงานวิจัยในที่ประชุมวิชาการนานาชาติ

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Composites", Int'l Conference on Nanotechnologies (NN2003),

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ผู้ร่วมโครงการวิจัย

1.1 ผู้ร่วมโครงการวิจัย 1

1. **ชื่อ-นามสกุล**   (ภาษาไทย) นางอาภาณี เหลืองนฤมิตชัย

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2. **เลขหมายประจำตัวประชาชน**   3-1001-00293-19-4

3. **ตำแหน่งปัจจุบัน**   รองศาสตราจารย์

4. **หน่วยงานที่อยู่ที่สามารถติดต่อได้สะดวก พร้อมหมายเลขโทรศัพท์ โทรสาร และ e-mail**

วิทยาลัยปิโตรเลียมและปิโตรเคมี จุฬาลงกรณ์มหาวิทยาลัย

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5. **ประวัติการศึกษา**

B.Sc. (Chemistry)	Prince of Songkla University	1990
M.Sc. (Chem Tech)	Chulalongkorn University	1994
Ph.D. (Petrochem Tech)	Chulalongkorn University	2003

6. **สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ**

การสังเคราะห์ตัวเร่งปฏิกิริยาและการประยุกต์ใช้งานทางด้านการผลิตก๊าซ

ไฮโดรเจน การกำจัดก๊าซคาร์บอนมอนนอกไซด์ การผลิตน้ำมันดีโซฮอล์ /ไบโอดีเซล

7. **ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ   โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น**

7.1 หัวหน้าโครงการวิจัย

7.1.1 ชื่อโครงการ: ตัวเร่งปฏิกิริยาที่ใช้ในการกำจัดคาร์บอนมอนนอกไซด์โดยใช้

two-stage reactor สำหรับการผลิตไฮโดรเจนเพื่อเซลเชื้อเพลิงประเภทพอลิ

เมอร์อีเล็คโตรไลท์เมมเบรน

แหล่งทุน: สกว. ร่วมกับ สกอ

ระยะเวลาดำเนินการ: 1 มิถุนายน 2550-30 พฤษภาคม 2552 (เสร็จสมบูรณ์)

7.1.2 ชื่อโครงการ: การใช้ไบโอดีเซลเป็นสารเติมแต่งในเชื้อเพลิงผสมระหว่าง

ดีเซล-เอทานอล และดีเซล-บิวทานอล

แหล่งทุน: TRF

ระยะเวลาดำเนินการ: 1 ตุลาคม 2549-30 พฤศจิกายน 2550 (เสร็จสมบูรณ์)

7.1.3 ชื่อโครงการ: A Study of Heterogeneous Catalyst for Biodiesel Production via Transesterification

แหล่งทุน: สำนักงานนโยบายและแผนพลังงาน กระทรวงพลังงาน

ระยะเวลาดำเนินการ: 1 มิถุนายน 2549–30 พฤษภาคม 2550 (เสร็จสมบูรณ์)

7.1.4 ชื่อโครงการ: พัฒนาและสาธิตการผลิตและการจัดเก็บไฮโดรเจน

แหล่งทุน: กรมพัฒนาพลังงานทดแทนและอนุรักษ์พลังงาน กระทรวงพลังงาน

ระยะเวลาดำเนินการ: 17 กุมภาพันธ์ 2549– 12 มกราคม 2550 (เสร็จสมบูรณ์)

7.1.5 ชื่อโครงการ: การศึกษาตัวเร่งปฏิกิริยาในการกำจัดคาร์บอนมอนนอกไซด์ใน บรรยากาศ ไฮโดรเจนสำหรับเซลล์เชื้อเพลิงประเภทพอลิเมอร์อีเล็คโตรไลท์ เมมเบรน

แหล่งทุน: สกว. ร่วมกับ สกอ

ระยะเวลาดำเนินการ: 1 มิถุนายน 2548-30 พฤษภาคม 2550 (เสร็จสมบูรณ์)

7.1.6 ชื่อโครงการ: ผลของการเติมสาร oxygenated additive และลักษณะทาง อีมัลชันของการผสมน้ำมันดีเซลกับเอทานอล

แหล่งทุน: สำนักงานนโยบายและแผนพลังงาน กระทรวงพลังงาน

ระยะเวลาดำเนินการ: 1 สิงหาคม 2548 – 30 เมษายน 2549 (เสร็จสมบูรณ์)

7.1.7 ชื่อโครงการ: การใช้ไบโอดีเซลเป็นสารเติมแต่งในดีโซฮอล์

แหล่งทุน: สำนักงานนโยบายและแผนพลังงาน กระทรวงพลังงาน

ระยะเวลาดำเนินการ: 1 มิถุนายน 2548–30 พฤษภาคม 2549 (เสร็จสมบูรณ์)

7.1.8 ชื่อโครงการ: การผลิตไฮโดรเจนจากปฏิกิริยารีฟอร์มมิงมีเทนด้วยก๊าซ คาร์บอนไดออกไซด์บนตัวเร่งปฏิกิริยานิเกิลที่มีเค-เอชซีโอไลท์เป็นตัวรองรับ

แหล่งทุน: สกว

ระยะเวลาดำเนินการ: 1 ตุลาคม 2547-30 พฤศจิกายน 2548 (เสร็จสมบูรณ์)

7.1.9 ชื่อโครงการ: ตัวเร่งปฏิกิริยาชนิดใหม่สำหรับปฏิกิริยาออกซิเดชันของ คาร์บอนมอนอกไซด์ในบรรยากาศไฮโดรเจน

แหล่งทุน: กองทุนรัชดาภิเษกสมโภช จุฬาลงกรณ์มหาวิทยาลัย

ระยะเวลาดำเนินการ: 24 มีนาคม 2547-23 มีนาคม 2548 (เสร็จสมบูรณ์)

7.1.10 ชื่อโครงการ: การผลิตไฮโดรเจนจากปฏิกิริยามีเทนรีฟอร์มมิงด้วยตัวเร่ง ปฏิกิริยานิเกิล

7.2 ผลงานวิจัย (5 ปีย้อนหลัง)

7.2.1 **บทความวิชาการตีพิมพ์ในวารสารนานาชาติ**

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1.2 ผู้ร่วมโครงการวิจัย 2

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Polymer Composites, Hybrid Organic-Inorganic Composites, Nanocomposites, Functionalization of Polymers

7.   ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย เป็นต้น

7.1 หัวหน้าโครงการวิจัย

7.1.1   ชื่อโครงการ: การพัฒนาเมมเบรนที่มีคุณสมบัติพิเศษในการแยกก๊าซผสมจากพอลิเบนซอกซาซีนและโลหะออกไซด์ไฮบริดคอมพอสิท
แหล่งทุน: สกว
ระยะเวลาดำเนินการ: 15 พฤษภาคม 2551–14 พฤษภาคม 2553 (เสร็จสมบูรณ์)

7.1.2   ชื่อโครงการ: การแยกสารผสมเอทธานอลและน้ำโดยการใช้พอลิเบนซอกซาซีนเมมเบรนเพื่อใช้สำหรับก๊าซโซฮอล
แหล่งทุน: สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
ระยะเวลาดำเนินการ: 15 พฤษภาคม 2551–14 พฤษภาคม 2553 (เสร็จสมบูรณ์)

7.2 ผู้ร่วมโครงการวิจัย: ชื่อโครงการวิจัย

7.2.1 ชื่อโครงการ: การพัฒนาคาร์บอนแอโรเจลจากพอลิเบนซอกซาซีนสำหรับใช้เป็นสารรองรับตัวเร่งปฏิกิริยา
แหล่งทุน: ศูนย์นาโนเทคโนโลยี
ระยะเวลาดำเนินการ: 23 กันยายน 2551 – 22 กันยายน 2553 (เสร็จสมบูรณ์)

7.3 ผลงานวิจัย

7.3.1 บทความวิชาการตีพิมพ์ในวารสารนานาชาติ

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