CHAPTER VI

EFFECT OF ACID PRETREATMENT OF CORNCOBS TO THE FERMENTABILITY OF *CLOSTRIDIUM BEIJERINCKII* TISTR 1461 FOR BIOBUTANOL PRODUCTION

6.1 Abstract

Corncobs pretreated with H_2SO_4, HNO_3, and H_3PO_4 were being examined to evaluate the fermentability of *C. beijerinckii* TISTR 1461 to produce biobutanol. It was found that H_2SO_4 pretreatment gave the best total sugars result (97.49% of theoretical yield) while H_3PO_4 pretreatment proved that the residues can be used as a substrate without any inhibitor removing methods. Response surface methodology (RSM) was applied to optimize enzymatic hydrolysis of pretreated corncobs. The result of the optimization reduces the consumption of enzyme and hydrolysis time to 7.68 FPU/g biomass and 63.88 h, respectively. In addition, the solventogenic products yield dramatically increased by adding supplementary glucose.

Keywords: Biobutanol, ABE fermentation, *Clostridium beijerinckii*, Acid pretreatment, Corncobs

6.2 Introduction

Biobutanol production from lignocellulosic biomass is a second–generation biofuel generated via the fermentation of *Clostridium beijerinckii*, by Acetone–Butanol–Ethanol (ABE) fermentation or the Weizmann process [1]. This process is far different than butanol production with a petrochemical process; it has no potential for carcinogenic carryover [2]. Biobutanol is considered to be a sustainable energy due to the use of non–food crop byproducts as a substrate. It has gained more interest recently because of the fluctuation of fossil fuel prices. There are several advantages in using butanol over ethanol, which is a practical fuel additive used nowadays: higher energy density (29.2 MJ/L and 19.6 MJ/L), more miscible with gasoline or diesel and lower Reid Vapor Pressure (RVP). In the separation process, butanol is more immiscible with water and does not require high energy amounts as ethanol does. Moreover, biobutanol produced from solventogenic bacteria, *Clostridium beijerinckii*, has the ability to utilize both C5 and C6 sugars from hydrolysed lignocellulosic biomass without any genetic modification as *Saccharomyces cerevisiae* requires.

Corncobs, a byproduct from corn production, which is an abundant biomass in Thailand. Thai makes corncobs an attractive feedstock to be converted to biofuels and other value-added products. Its biomass composition contains mostly cellulose (~39%) and hemicellulose (~43%) with a low percentage lignin (~8%) [3]; therefore, it has high potential for biofuels production from enzymatic saccharification. There have been previous studies, where other parts of corn such as corn stover [4], corn fiber [5,6], corn steep water [7], and degermed corn [8], have been used for butanol production but there is no report of using various type of acids to pretreat corncob for fermentation. The above studies suggest that corn residues can be efficiently used as a substrate for butanol production. Dilute acid pretreatment of corncobs is used to adjust its structure properly for enzymatic hydrolysis. From a wide range of acids, sulfuric acid was frequently been chosen due to its low price and high impact to the biomass structure. At high temperatures, sulfuric acid can solubilize the hemicellulose well; however, inhibitory compounds such as furfural, hydroxymethyl furfural (HMF), and acetic, ferulic, glucuronic, ρ–coumaric acids [5], from the

degradation of digested biomass under this condition were reported. Thus other types of acids, nitric and phosphoric acid, have been studied in the present work. Pretreatment of sugar cane bagasse with phosphoric acid compared with sulfuric acid has been reported to have lower sugar concentrations [9], but has high fermentability when treating ryegrass straw as a substrate for *Candida utilis*. [10] On the other hand, pretreatment with nitric acid to corn stover has an environmental advantage [11].

In the present work, a comparison of dilute acid pretreatment methods that increase the material digestibility was presented. Pretreated corncobs with sulfuric, nitric, and phosphoric acid were characterized with x–ray diffraction (XRD) and scanning electron microscope (SEM) to inspect the possibility of enzymatic saccharification. The fermentability of *Clostridium beijerinckii* was investigated in detail for butanol production both from mock sugars as well as from pretreated corncobs. Then this work focuses on acids used in the pretreatment of corncob for ABE fermentation. Another variable that affects the cost of operation, enzyme loading, was also studied. To reduce the amount of cellulase enzyme, Celluclast 1.5L, due to its high cost, and to lower operation time, the Response Surface Methodology (RSM) was applied to optimize the enzyme loading and reduce time needed for the enzymatic saccharification. The loading amount of cellobiase enzyme, Novozymes 188, was also investigated to create the proper cocktail of the enzyme mixture. To assure that the investigated condition is consistent, the activities of the enzymes (filter paper activity, carboxymethyl cellulase, β–glucosidase, xylanase) were quantified by enzyme assays to prevent the variation of different batch enzymes. The optimum condition was used as a comparison to a non–commercial enzyme. Enzymatic hydrolysis conditions obtained from RSM were further applied to investigate the properly hydrolysate used as a substrate for ABE fermentation from *C. beijerinckii* TISTR 1461.

6.3 Experimental

6.3.1 Materials and Chemicals

Corncobs were provided by Betagro Group, Thailand, using as received. Corncobs composition was determined using the NREL (National

Renewable Energy Laboratory) method of the determination of structural carbohydrates and lignin in biomass (% dry weight): glucan 34.25 ± 0.48, xylan 23.14 ± 0.06; arabinan 4.06 ± 0.24. It was ground and screened to the particle size of 12 mesh (1.68 mm), then dried at 105 °C overnight prior to pretreatment. The acids used in the pretreatment step were sulfuric acid (H_2SO_4, ≥98% purity), nitric acid (HNO_3, ≥70% purity), and phosphoric acid (H_3PO_4, ≥85% purity) purchased from RCI Labscan (Thailand). Two commercial enzyme solutions, Celluclast 1.5L and Novozymes 188, were used. Celluclast 1.5L was produced from *Trichoderma reesei* ATCC 26921, and Novozymes 188 was produced from *Aspergillus niger*. They were purchased from Sigma–Aldrich (St. Louis, MO, USA) while the non–commercial enzyme, ARR2–7, was a generous gift from Siam Victory (Thailand). Other chemicals (analytical grades) used as standards, enzyme assays, dinitrosalicylic acid (DNS) method and culture medium were obtained from Sigma–Aldrich (St. Louis, MO, USA), or Merck (Darmstadt, Germany), Oxoid Ltd. (Hampshire, UK), or Ajax Finechem (New South Wales, Australia), respectively.

6.3.2 Pretreatment of Corncobs

Dilute acid pretreatments performed at a corncob loading of 10% (w corncob/v dilute acid) with 30 mL of 1% acid (H_2SO_4 or HNO_3 or H_3PO_4) slurried in 100 mL Erlenmeyer flasks. The corncob slurry was presoaked at 50 °C, shaken at 150 rpm for 30 min before being pretreated at 121 °C in an autoclave for 1 h. The loss of water was measured and then sterile water was added to obtain the initial weight. The pH of pretreated corncob was adjusted with NaOH to pH 4.8 for the enzymatic hydrolysis. All pretreatments were performed in triplicate. For the determination of solid recovery, the pretreated corncob was filtered through Whatman No.40 filter paper and dried at 105 °C overnight prior the weighing.

6.3.3 Enzymatic Hydrolysis of Pretreated Corncobs

The enzyme loading and hydrolysis period were 8 FPU/g corncob and 120 h, respectively, for each type of acid pretreatment [12]. Then they were optimized by RSM while other parameters were fixed. The 10 % (w/v) loading of acid

pretreated corncob, after adjusting to pH 4.8, was added with one of these, Celluclast 1.5L or ARR2–7 or mixed with Novozymes 188 (as a cocktail enzyme), and incubated at 50 °C with a 150 rpm shaking rate. After hydrolysis, the liquid samples (1 mL) were passed through a 0.2 μm syringe filter and kept at -20 °C for further analysis. The pH of remaining liquid was adjusted to pH 6.6 using 10 M NaOH for the early phase of the ABE fermentation. The samples were taken from each flasks, centrifuged (10,000 rpm, 10 min), filtered with Whatman No.40 to remove the sediment, and measured for total reducing sugars. Finally, it was kept at -20 °C to be filter sterilized later, and used as a substrate for the ABE fermentation.

6.3.4 Enzyme Assays

The activity of cellulase, β–glucosidase, and xylanase was quantified by various methods for Celluclast 1.5L, Novozymes 188, and ARR2–7 enzyme. The filter paper activity was assayed to express the cellulase activity in terms of a filter paper unit (FPU). Other than filter paper assay, the cellulase activity was expressed in terms of carboxymethyl cellulase activity using 1% (w/v) of carboxymethyl cellulose (CMC) in 50 mM citrate buffer as a substrate instead of filter paper. Glucose acquired from these methods was measured by DNS method using for the calculation of the activity. The β–glucosidase activity followed a similar assay to the carboxymethyl cellulase activity but the substrate was changed to cellobiose. Glucose obtained from the β–glucosidase enzyme could not be detected with DNS method due to the noise of cellobiose sugar, HPLC was used to measure it instead. These three methods were detailed by Ghose [13]. For the xylanase activity, the Somogyi reducing sugar assay was modified and 1% (w/v) xylan from beech wood in acetate buffer was used as a substrate [14].

6.3.5 Microorganism and Culture Maintenance

Freeze–dried cultures of *C. beijerinckii* TISTR 1461 (purchased from Thailand Institute of Scientific and Technological Research, mutant strain of *C. beijerinckii* DSM 6422) were inoculated on a reinforced clostridial medium (RCM) agar plate and incubated at 37 °C in an Anaerojar™ coupled with Anaerogen™ and an

anaerobic indicator (Oxoid Ltd., Hampshire, UK) for 3 days, then kept as stock at 4 °C. Prior to fermentation, one loop of colony was inoculated in 6 mL of cooked meat medium (CMM) and heat shocked at 80 °C for 2 min, then it was incubated in an anaerobic atmosphere at 37 °C until it reached the middle of the log phase (~20 h). After that, 6 mL of actively growing cells were inoculated in the P2 medium supplied with 20 g/L glucose, and grown for 6 h [15]. The medium was then used to seed the production medium.

6.3.6 Fermentation Experiments

The fermentation experiments were performed in 100 mL Duran™ screw capped bottles, with 80 mL working volume and 5% (v/v) inoculum size, at 37 °C, with an initial pH of 6.6 initially under the same anaerobic condition as in the Anaerojar™. When using hydrolysate from sulfuric, nitric and phosphoric pretreatment and enzymatic hydrolysis as a substrate, prior to fermentation, the hydrolysate was adjusted with 10 M NaOH to pH 6.6 and filter sterilized, while the control experiments with P2 medium, supplied with 40 g/L glucose, were sterilized via an autoclave at 121 °C for 15 min. In the fermentation experiments using phosphoric acid pretreatment plus the enzymatic hydrolysis condition obtained from RSM, hydrolysates were adjusted with 10 M NaOH as previously described. In addition, six fermentations were conducted in order to investigate the most suitable hydrolysate media to *C. beijerinckii* TISTR 1461 for butanol production [CH was corncob hydrolysate from phosphoric acid pretreatment and enzymatic hydrolysis using Celluclast 1.5L while CHP2 was CH supplied with P2 medium nutrients. CHP2A, and CHP2G was CHP2 added with ammonium acetate to 70 mM, and 10 g/L glucose, respectively. CHCP2 was CHP2 added with Novozymes 188 as a combination enzyme in the enzymatic hydrolysis. P2 was mock sugars, 40 g/L glucose, supplied with P2 medium nutrients]. All fermentations were conducted in three replicates.

6.3.7 Experimental Design for RSM

There have been several reports on the enzyme loading based on dry weight or amount of glucan in the biomass [16–19]. It indicates that the variation in

biomass type and enzyme lots have a significant effect on the enzymatic hydrolysis condition. Then amounts of enzyme loading and time used in the hydrolysis were optimized by using RSM. ARR2–7 was used to assure the optimum conditions. The experiment was compared with the results of the reducing sugar from enzymatic hydrolysis using commercial enzyme, Celluclast 1.5L. With 2 variables, hydrolysis time and enzyme loading (k = 2), central composite designs were composed of 13 experiments; 4 from factorial points (2^k), 4 from star points (2^k), and 5 from center points (5 levels). The upper and lower limits were chosen from data in prior research [17–18, 20–21]. A second–order polynomial regression model was

$$Y = a_0 + a_1x_1 + a_2x_2 + a_{12}x_1x_2 + a_{11}x_1^2 + a_{22}x_2^2$$

where Y = reducing sugar (g/L), x_1 = (time - 48)/12, x_2 = (enzyme loading - 6)/2, a_0 = the constant value, a_i = the linear coefficients, a_{ii} = the quadratic coefficients and a_{ij} = the cross product coefficients. The program R 2.14 (The R Foundation for Statistical Computing) was used for the data analysis.

6.3.8 Analytical Methods

Sugars (glucose, xylose, arabinose, and cellobiose) and inhibitor compounds (furfural and HMF) from acid pretreatments and enzymatic hydrolysis were measured by high performance liquid chromatography (HPLC; Shimadzu) equipped with an Aminex HPX–87H column (Bio–Rad Lab, USA). The column was operated at 60 °C at a flow rate of 0.6 mL/min, eluted with 5 mM H_2SO_4 and detected by the refractive index. The yield of total sugars (glucose, xylose, arabinose) was calculated by the NREL (National Renewable Energy Laboratory) method of the determination of structural carbohydrates and lignin in biomass [22]. The reducing sugar was quantified with DNS method using UV–VIS spectrophotometer (Thermo Fisher Scientific Inc., USA) at a wavelength of 540 nm. The other wavelength used in the spectrophotometer was 520 nm for the reducing sugar developed in the xylanase assay and 600 nm for cell concentration. The fermentation broths containing acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed by gas chromatography (GC; PR2100; Perichrom) equipped with a flame ionization detector and a 30m×0.25mm

HP–INNOWAX capillary column. Productivity and yield of total ABE was calculated as ABE produced (g/L) divided by the fermentation time (h) or sugar utilized (g/L), respectively.

The characterization techniques applied to the pretreated corncob were XRD analysis and SEM. The XRD was conducted with a Rigaku/Rint2200 diffractometer equipped with a Ni filtered CuKα radiation source (λ = 1.542 Å) of 40 kV and 30 mV. Scanning was performed between 2θ = 5° and 60° with a step size of 0.02° at a speed of 1°/min. Data from XRD were used to calculate the corncob crystallinity index (CrI) with the formula [23]:

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

In which, I_{002} is the intensity for the crystalline portion of biomass at 2θ = 22.6°, (002) lattice plane and I_{am} is the peak for the amorphous portion at 2θ = 18.7°, (101) lattice plane.

SEM images were acquired using a Hitachi S–4800 microscope at 15 kV. All samples were sputter coated with gold/palladium (Au/Pd), 30 Å before analysis. Images of corncob samples before and after pretreatment were taken at 5000x magnification.

6.4 Results and Discussion

6.4.1 Activity Assay of Enzymes

The activity of cellulase (FPU), carboxymethyl cellulase, β–glucosidase, and xylanase in each of these enzymes are given in Table 6.1. The Celluclast 1.5L and ARR2–7 enzyme have high activity in both cellulase and xylanase and low in cellobiase compared with Novozymes 188. The filter paper activity (FPU) of Celluclast 1.5L and ARR2–7 were much higher than that of Novozymes 188. The Novozymes 188 added to enhance the hydrolysis of exocellulase product inspected to increase monomeric sugars. However, Novozymes 188 could not be solely used in

enzymatic hydrolysis due to its low value of filter paper unit and carboxymethyl cellulase (6.29 and 119.87 U/mL, respectively). At the same volume, the Celluclast 1.5L commercial enzyme has higher activity almost 2 times compared with ARR2–7. Compared with other studies of Celluclast 1.5L and Novozymes 188, it is seen that the activity has a different value between lots, as measured with the same method [12, 24].

Table 6.1 Activity level of enzymes used in pretreated corncob enzymatic hydrolysis

Enzyme activity (U/mL)[a]	Celluclast 1.5L	Novozymes 188	ARR2–7
Filter paper activity (FPA)	53.94	6.29	40.88
Carboxymethyl cellulase	1151.32	119.87	703.82
β–glucosidase	26.57	683.39	14.08
Xylanase	2295	375	1219

[a] At pH 4.8 and 50 °C.

6.4.2 Acid Pretreatment and Enzymatic Hydrolysis of Corncobs

Three types of acids used in the pretreatment step— H_2SO_4, HNO_3, and H_3PO_4—were applied to investigate the digestive performance using corncob as a raw material and Celluclast 1.5L in the enzymatic hydrolysis step. The results of glucose, xylose, arabinose, and acetic acid in acid pretreatment and enzymatic saccharification are shown in Table 6.2. In the biomass pretreatment, it was reported that the inhibitory compounds present in the hydrolysate of lignocellulosic biomass are major problems affecting fermentation process. For the sugar degradation products, our study showed that no detectable quantities of HMF were observed in the hydrolysate. In the other study, HMF is commonly present in the hydrolysate from the dilute H_2SO_4 pretreatment of wheat straw, corn stover, barley straw, and switch grass [16]. Nevertheless, this work is consistent with the report using H_2SO_4 (up to 2%) to pretreat corncobs produced a low level of HMF in the hydrolysate (<0.2 g/L)

[25]. The sulfuric acid pretreated sample showed the best solubilization of hemicellulose due to the high concentration of xylose in the hydrolysate (28.16 ± 0.64 g/L) and yielded a half of the maximum theoretical sugar yield of corncobs. In comparison, the dilute HNO_3 and H_3PO_4 pretreatment of corncobs released 14.92 ± 0.89 g/L and 5.59 ± 0.02 g/L xylose, respectively. This is the reason why the combination of pretreatment and enzymatic hydrolysis should be applied to prevent the loss of sugar content. However, a small amount (0.23 g/L) of furfural was found in the H_2SO_4 pretreatment.

Table 6.2 Sugars and degradation products from acid pretreatment and enzymatic hydrolysis

Sugars and degradation products	Acid pretreatment (g/L)			Enzymatic hydrolysis (g/L)		
	H_2SO_4	HNO_3	H_3PO_4	H_2SO_4	HNO_3	H_3PO_4
Glucose	2.75 ± 0.03	0.88 ± 0.07	0.40 ± 0.00	31.42 ± 0.08	23.38 ± 0.93	21.99 ± 0.48
Xylose	28.16 ± 0.64	14.92 ± 0.89	5.59 ± 0.02	29.59 ± 0.04	24.75 ± 0.89	23.40 ± 0.52
Arabinose	4.55 ± 0.07	5.42 ± 0.56	3.04 ± 0.01	6.23 ± 0.02	5.98 ± 0.26	5.19 ± 0.04
Total sugars (yield)	35.46 ± 0.74 (51.42)	21.22 ± 1.51 (30.76)	9.03 ± 0.03 (13.09)	67.24 ± 0.14 (97.49)	54.11 ± 2.07 (78.45)	50.59 ± 1.04 (73.34)
Acetic acid	3.81 ± 0.06	2.64 ± 0.32	1.25 ± 0.00	3.91 ± 0.01	3.34 ± 0.06	3.09 ± 0.12
Furfural	0.23 ± 0.00	ND[a]	ND	0.12 ± 0.00	ND	ND
HMF	ND	ND	ND	ND	ND	ND

[a] not detected

The order of acids on the solubilization of the structural corncob is: $H_2SO_4 > HNO_3 > H_3PO_4$, which related with the sugars and acetic acid found in the hydrolysates. Dilute H_2SO_4 pretreatment and enzymatically saccharified hydrolysates contained 3.81 ± 0.06 and 3.91 ± 0.01 g/L acetic acid. In comparison with the other two dilute acid pretreatments, acetic acid obtained in the acid pretreated hydrolysates was much lower than that obtained in the enzymatic hydrolysis step. The result of total sugars found in enzymatic hydrolysis again shows the same trend; however, the

total sugars from the HNO_3 pretreatment was not much different from that of H_3PO_4 pretreatment (54.11 and 50.59 g/L, respectively). It implies that the benefit from the structural change in H_3PO_4 pretreatment is sufficient when compared with HNO_3 pretreatment. As expected, the H_2SO_4 pretreatment showed the best result of both acid pretreatment and enzymatic hydrolysis, especially the later step which yielded 97.49% of maximum theoretical sugar. Generally, the removal of inhibitory compounds from hydrolysate was required to improve the butanol production. However, the hydrolysates from the present work were instantly used as a fermentation substrate.

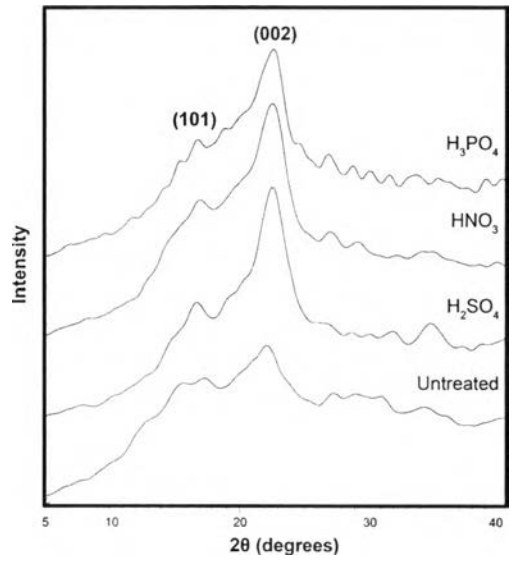

Figure 6.1 XRD patterns for untreated and pretreated corncob with H_2SO_4, HNO_3 and H_3PO_4.

The effectiveness of the type of acids was examined through the changes in crystallinity of the cellulose, which was calculated from the maximum diffraction intensity at peak position $2\theta = 22.6°$ and $2\theta = 18.7°$ in the XRD patterns (Figure 6.1). A change in pattern among samples was observed, especially a peak at $2\theta = 22.6°$ which defined the crystalline cellulose structure. The dilute acid pretreatment significantly enhanced corncobs crystallinity, especially the sample treated with H_2SO_4 had higher crystallinity compared to others. The calculated results, shown in Table 6.3, show a significant increase in crystallinity of samples after acid pretreat-

ment when compared to that of untreated. The high crystallinity index shows high cellulose content (compared to the other amorphous components lignin and hemicellulose), suggesting that removal of more hemicellulose and leaving the cellulose fraction. A small difference in crystallinity index between HNO_3 and H_3PO_4 indicates a similar result in structural alternation. The percentage of corncob recovered indicated the potential of solubilization of hemicellulose and lignin from the acids.

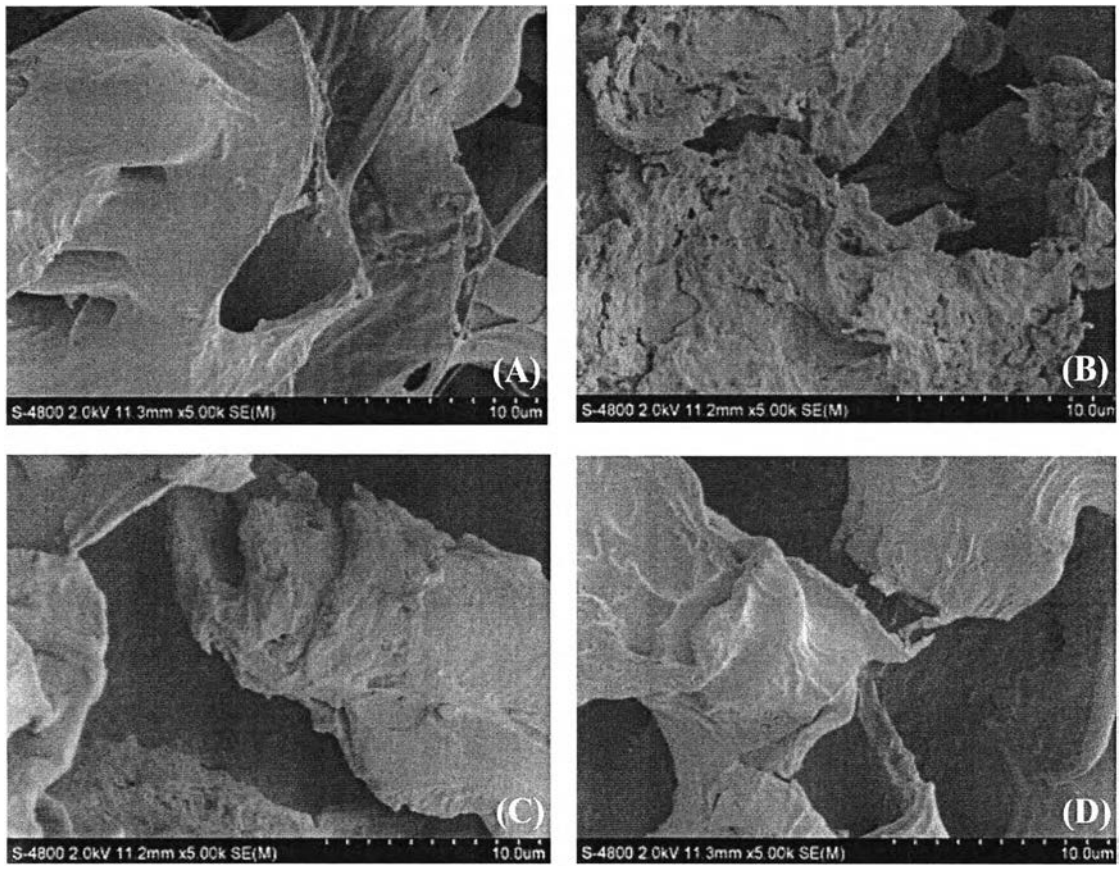

Figure 6.2 SEM images of (A) untreated corncob, (B) pretreated corncob with H_2SO_4, (C) HNO_3, and (D) H_3PO_4.

Physical changes after acid pretreatments are revealed in Figure 6.2. Compared to the untreated corncobs, all acid treated samples damaged the surfaces. A cracked surface was obviously seen. Other than the solubilization of acids to hemicellulose, the rough surface revealed high accessibility to enzyme [26]. However, the

change in the structure when using HNO_3 and H_3PO_4 was indiscernible and was consistent with the small difference in the crystallinity index (Table 6.3).

Table 6.3 Corncob recovery and crystallinity index after acid pretreatment

Acid types	Crystallinity Index (CrI, %)	Percentage of corncob recovered (%)
Untreated	20.21	100.00
H_2SO_4	48.18	57.70
HNO_3	40.87	64.84
H_3PO_4	40.45	76.03

6.4.3 Fermentability of *Clostridium beijerinckii* TISTR 1461 to Hydrolysate from Acid Pretreatment and Enzymatic Hydrolysis of Corncobs

The sugar concentration in each substrate was diluted to 40 g/L of reducing sugar to prevent slow growth on excessive carbon source. The result of ABE concentration and utilized sugars at 48 h of fermentation is shown in Figure 6.3. Cell growth in hydrolysate from H_2SO_4 and HNO_3 pretreatment and enzymatic saccharification was inhibited while the hydrolysate from H_3PO_4 pretreatment had a utilization of sugars and a generation of solventogenic products. Furthermore, *C. beijerinckii* TISTR 1461 growing in P2 medium produces ~11.53 ± 0.57 g/L ABE during the course of fermentation. Unfortunately, the ABE concentrations lower than 1 g/L were obtained from H_2SO_4 and HNO_3 pretreated hydrolysate, indicating low potential of pretreated H_2SO_4 and HNO_3 samples as a substrate. There was no significant difference in ABE concentration and pH of both hydrolysates. Microbial growth was also measured by the pH change in the fermentation broth. From the initial value of pH 6.6, the pH value of H_3PO_4 pretreated hydrolysate has a similar value to the control (5.19 and 5.14, respectively) while the other H_2SO_4 and HNO_3 pretreated hydrolysates still have high basicity at pH 5.99 and 5.97. This result was consistent with the previous work that the treated corn fiber hydrolysis has no fermentability to *C. beijerinckii* BA101 due to salts generated during the dilute sulfuric pretreatment

especially from ρ–coumaric and ferulic acids [5]. It was found that there is no furfural or HMF in the HNO_3 pretreated hydrolysates; however, other inhibitors could affect to the metabolism of the microorganism. Due to the result of fermentability, it can be implied that the H_3PO_4 pretreated hydrolysate is promising to be used as a substrate in the fermentation in *Clostridium beijerinckii* TISTR 1461 without being treated with overliming or other methods such as XAD–4 resin [6] or nanofiltration [27]. In addition, *C. beijerinckii* TISTR 1461 has higher utilized reducing sugar using H_3PO_4 pretreated hydrolysate than other two acid hydrolysates.

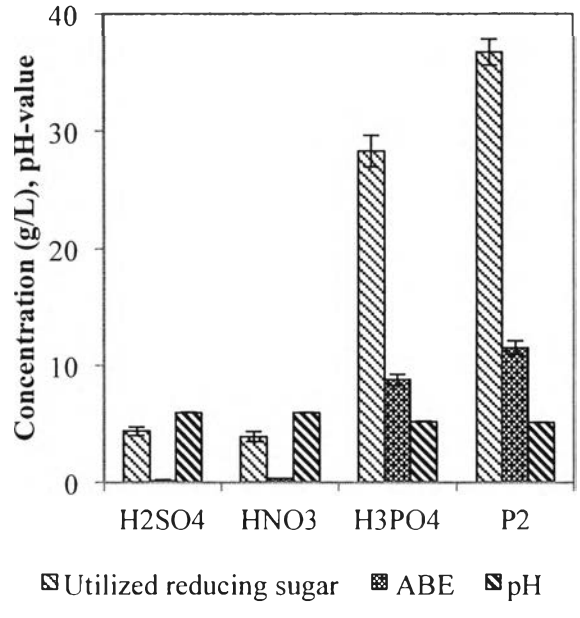

Figure 6.3 Concentration of utilized reducing sugars, ABE, and pH in fermentation broths at 48 h using hydrolysate of corncob from H_2SO_4, HNO_3, and H_3PO_4 pretreatment and enzymatic hydrolysis as a substrate. The P2 medium were added with glucose as a substrate for control experiments.

6.4.4 Determination of Enzyme Loading and Hydrolysis Time from RSM

As a result of high fermentability using H_3PO_4 in the pretreatment step followed by the enzymatic hydrolysis compared to other two acids, H_3PO_4 was chosen for further optimize the enzyme loading and hydrolysis time using RSM. The data from the 13 experiments, done in triplicate were analyzed using R 2.14 for

Windows (Table 6.4). The analysis of variance (ANOVA) shown in Table 6.5 indicates that the model of the reducing sugar hydrolysed from Celluclast 1.5L was highly significant ($P < 0.05$) except the term of interation (TWI) as its F calc was lower than F listed. The model was confirmed by Fisher's statistical test as the F calc (16.78) had higher value than the F listed (3.97) at the degree of freedom of 5 and 7 (not shown in Table). This implies that this model is accurate to represent the experimental data. The goodness of fit of the model was expressed by the R^2 value of 0.868, which implied that 86.80% of the total variations in the observed response values could be explained by this model while the rest (13.12%) were not defined. The mathematical model is well fitted to the experimental data implied from the lack of fit test as the F calc of the lack of fit has lower than F listed. The coefficients estimated in the regression model (Table 6.6) were substituted in the response equation:

$$Y = 48.1341 + 1.9536x_1 + 2.2968x_2 - 0.3318x_1x_2 - 0.633x_1^2 - 1.1074x_2^2$$

to predict the reducing sugar levels from hydrolysis at various conditions. In addition, the P value of each coefficient was used to indicate its significance ($P < 0.05$). As shown in Table 6.6, all P values from a_1, and a_2 have a small value (< 0.01) which shows that the two variables, time of hydrolysis and enzyme loading, have significance for this model. In the comparison of the effect of the two variables, enzyme loading has more of an effect than time, as observed from the lower value of P (0.000465 and 0.001205). However, the interaction between two variables has low significant due to its p value (0.623417) which is higher than 0.05 consisted with the ANOVA result in the term of interaction (TWI).

Table 6.4 Central composite design (CCD) and response results of the reducing sugar from enzymatic hydrolysis

Run	Code values		Real values		Reducing sugar (g/L)
	Time	Enzyme loading	Time (h)	Enzyme loading (FPU/ g corncob)	
1	1	1	60	8	48.31
2	1	-1	60	4	44.92
3	-1	1	36	8	46.57
4	-1	-1	36	4	41.86
5	2	0	72	6	50.75
6	-2	0	24	6	41.43
7	0	2	48	10	49.06
8	0	-2	48	2	39.33
9	0	0	48	6	47.97
10	0	0	48	6	48.63
11	0	0	48	6	49.72
12	0	0	48	6	47.78
13	0	0	48	6	48.53

Table 6.5 ANOVA for the models of reducing sugar yield

Source of variation	Degree of freedom	Sum of squares	Mean square	F calc[d]	F listed[e]
FO[a] (x1, x2)	2	109.10	54.55	32.66	4.74
TWI[b] (x1, x2)	1	0.44	0.44	0.26	5.59
PQ[c] (x1, x2)	2	30.63	15.31	9.17	4.74
Residuals	7	11.70	1.67		
Lack of fit	3	9.40	3.13	5.44	6.59
Pure error	4	2.30	0.58		

[a] first-order

[b] two-way interaction

[c] pure quadratic

[d] F test for statistical significance and lack of fit

[e] F Listed to 95% of confidence.

Table 6.6 Statistics for the regression of the optimization model

Coefficient	**Value**	**Standard error**	**t value**	**P**
a_0	48.1341	0.5367	89.691	5.64E-12
a_1	1.9536	0.3731	5.236	0.001205
a_2	2.2968	0.3731	6.156	0.000465
a_{12}	-0.3318	0.6462	-0.513	0.623417
a_{11}	-0.633	0.27	-2.344	0.05152
a_{22}	-1.1074	0.27	-4.101	0.004565

The effect of the interaction of time and enzyme loading on the reducing sugar was evaluated by plotting three–dimensional response curves against these two variables, as shown in Figure 6.4(A). It indicates positive results of the reducing sugar from enzymatic hydrolysis were obtained when using time and enzyme loading over than "0" level (48 h and 6 FPU/g corncob, respectively) with a decrease at the end, thus it shows a stationary point on this response surface plot. The optimum conditions for the reducing sugar from enzymatic hydrolysis, 63.88 h of incubation time and 7.68 FPU/g corncob enzyme loading, can calculated by setting the partial derivatives of the response equation to zero with respect to the corresponding variables. With these conditions, the maximum response value of reducing sugar, was 50.39 g/L. A symmetrical shape in the two–dimensional contour plot in Figure 6.4(B,C) indicates a significant interactive effect between time and enzyme loading. The peak of the reducing sugar was found around the level of "1" meaning that further increase in time or enzyme loading cannot enhance enzymatic saccharification.

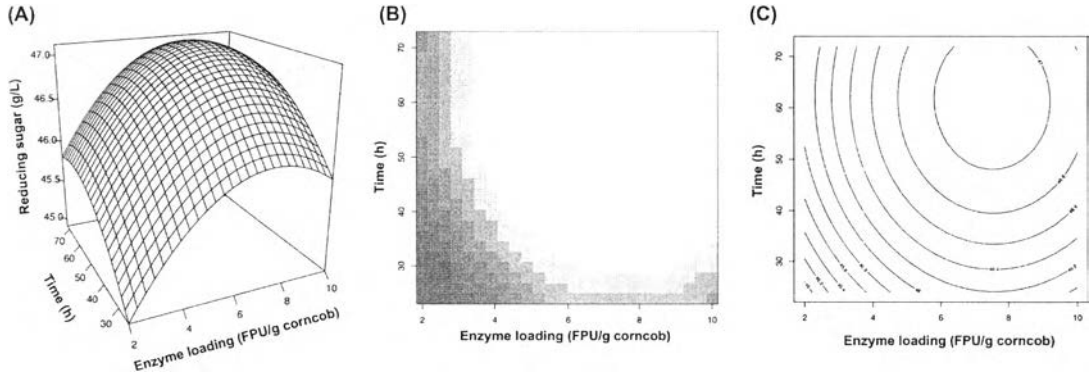

Figure 6.4 Sugar yield from acid pretreatment and enzymatic hydrolysis under the effect of enzyme loading and hydrolysis time shown in (A) response surface, and (B,C) contour plots.

As a result of the enzyme loading obtained from RSM, it was 8.29 FPU or 176.84 CMU per 1 g of corncob. This value was different compared to the loading of cellulase enzyme in other studies, with 22.8 FPU/g glucan [17], and 60

FPU/g glucan [18], 50 FPU/g cotton stalk [20], 30 FPU/g sugarcane bagasse [21]. Furthermore, the time spent for enzymatic hydrolysis (72 h) was almost 10 h higher when compared with the time obtained from the RSM in this study.

The confirmation experiments were conducted by using the optimum conditions with the Celluclast 1.5L and the house–blended enzyme (at the same loading), ARR2–7 cellulase enzyme, to insure that they can be applied to other enzymes. The result of the reducing sugar obtained from Celluclast 1.5L was 51.82 ± 1.83 g/L, with a 2.43% error from the predicted value. After applying to ARR2–7, the reducing sugar was 51.01 ± 0.94 g/L which was close to the result from Celluclast 1.5L at the same loading and time. It is supposed that this model could be used with other enzymes to obtain the same results.

6.4.5 Effect of Cellobiase Loading

It is well known that cellulolytic enzymes are inhibited by hydrolysis end products such as cellobiase [28]. The inhibitory effect of cellobiase can be alternated by adding cellobiase. Many works have used a combination of cellobiase with cellulase or even with xylanase enzyme to make a cocktail enzyme promising to completely utilize cellulose and hemicellulose [18, 19, 29]. It was reported that the cellobiase is added into hydrolysate to accelerate the rate of enzymatic hydrolysis and produce higher glucose concentration. In the present work, Novozymes 188, a cellobiase enzyme, was added to the Celluclast 1.5L enzyme coupled with the conditions obtained from RSM as a quarter of the ratio of the cellulase enzyme. The cellulase:cellobiase ratio was varied from 4:0 to 4:5. Figure 6.5 shows the effect of cellobiase on the sugar concentration and it can be seen that a higher reducing sugar concentration was obtained as the more cellobiase was added. The proper volume of cellobiase loading is 4:4 or an equal volume of cellulase and cellobiase, at this loading a glucose concentration of 23.06 g/L was obtained. Further increasing cellobiase loading did not markly enhance the reducing sugar. This 4:4 volume ratio, after being converted to FPU/CBU, is 1:12, compared with the optimum cellulase:cellobiase enzyme loading 1:4 that used to hydrolyse newspaper [30]. However, the optimum amount of cellobiase to corncob hydrolyse increase only 12% reducing sugar, which is much lower than the previous study [30-31]. Nevertheless, the addition of cellobi-

ase to cellulase did not always improve the hydrolysis of cellulose at the terminal digestibility as it was found in acid pretreated switchgrass [32]. From a practical viewpoint, the difference can be attributed to different cellobiase activities in the enzyme mixture. Thus, with a slight increase in reducing sugar and glucose in this study, cellobiase enzyme is not practical for enzymatic hydrolysis of acid pretreated corncob.

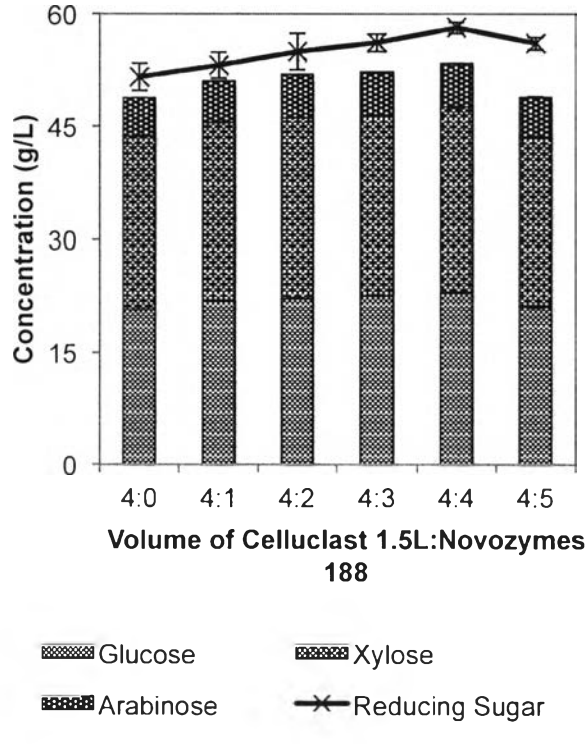

Figure 6.5 Concentrations of the sugars at different loading ratios of Celluclast 1.5L:Novozymes 188.

6.4.6 Fermentation of H_3PO_4 Pretreated and Enzymatic Hydrolysis of Corncobs with Different Conditions

Four different experiments were applied to the H_3PO_4 pretreated and enzymatic hydrolysis of corncobs (CHP2G, CHP2, CHP2A, and CHCP2). The results of ABE fermentation with *Clostridium beijerinckii* TISTR 1461 are given in Figure 6.6(A–C). The addition of 10 g/L synthetic glucose to hydrolysate was investigated to see whether the addition was sufficient (excess 40 g/L total sugars) or

not (CHP2G compared to CHP2 which had no added sugars). The other 2 experiments included the addition of ammonium acetate to 70 mM (CHP2A) [33, 34] and the hydrolysate obtained from the enzyme cocktail (Celluclast 1.5L and Novozymes 188, CHCP2). The phosphoric control was phosphoric acid pretreatment and enzymatic hydrolysis of corncobs (CH). The control experiment was composed of the mixture of glucose and xylose (20 g/L each, 40 g/L total) and supplied with P2 medium nutrients containing yeast extract, buffer, minerals, and vitamins (P2). All experiments contained 40 g/L initial total sugars except CHP2G which further added with 10 g/L glucose added. From Figure 6.6, the products from fermentation were found in all experiments excluding CH. There were only remaining acid from hydrolysis and small amount of utilized sugar detected. Thus, the nutrients contained in corncob hydrolysate were not sufficient for cell growth compared to the hydrolysate from degermed corn [8] which has a slightly lower yield in ABE compared to its control experiment. In addition, a constant value of acids present in CH indicated the initial value of acids from hydrolysate while other hydrolysates have a higher value due to the P2 supplement which contained ammonium acetate. This is the reason why CHP2A has the highest initial acetic acid, 6.03 g/L (data not shown). However, acetic acid did not inhibit the ABE fermentation but it depressed its formation [35]. The experiment that has total ABE concentration closer to P2 that contained mock sugars (11.80 ± 0.25 g/L) is CHP2G (11.64 ± 0.12 g/L). In Figure 6.6(B), the total sugars consumed in CHP2G were also similar to P2 where ABE yields were roughly the same. From this point of view, there were insufficient sugars since the addition of 10 g/L glucose gave a better result. But the remaining sugars in the broths were higher than 10 g/L of xylose and arabinose, implying that the lack of sugar is not the limited factor. Nevertheless, it cannot be concluded that the ABE fermentation was inhibited by the C5 sugars left in the broths since P2 which composed of mixed sugars utilized xylose up to 13.39 ± 0.40 g/L. This is due to an excess amount of acetic acid during the fermentation that consequently affected the shift in metabolism between the acidogenic and solventogenic phase since the hydrolysate have initial acetic acid higher than P2 [36]. The ABE yields in Figure 6.6(C) from all experiments were similar in range of 0.33–0.36 except the lowest one was 0.28 obtained from the hydrolysate from the enzyme cocktail (cellulase and

cellobiase). The ABE productivity in Figure 6.6(C) of P2 and CHP2G has almost the same value of 0.24. The results of ABE yield and productivity were comparable with other works using other hydrolysate from various biomass [6, 12, 37].

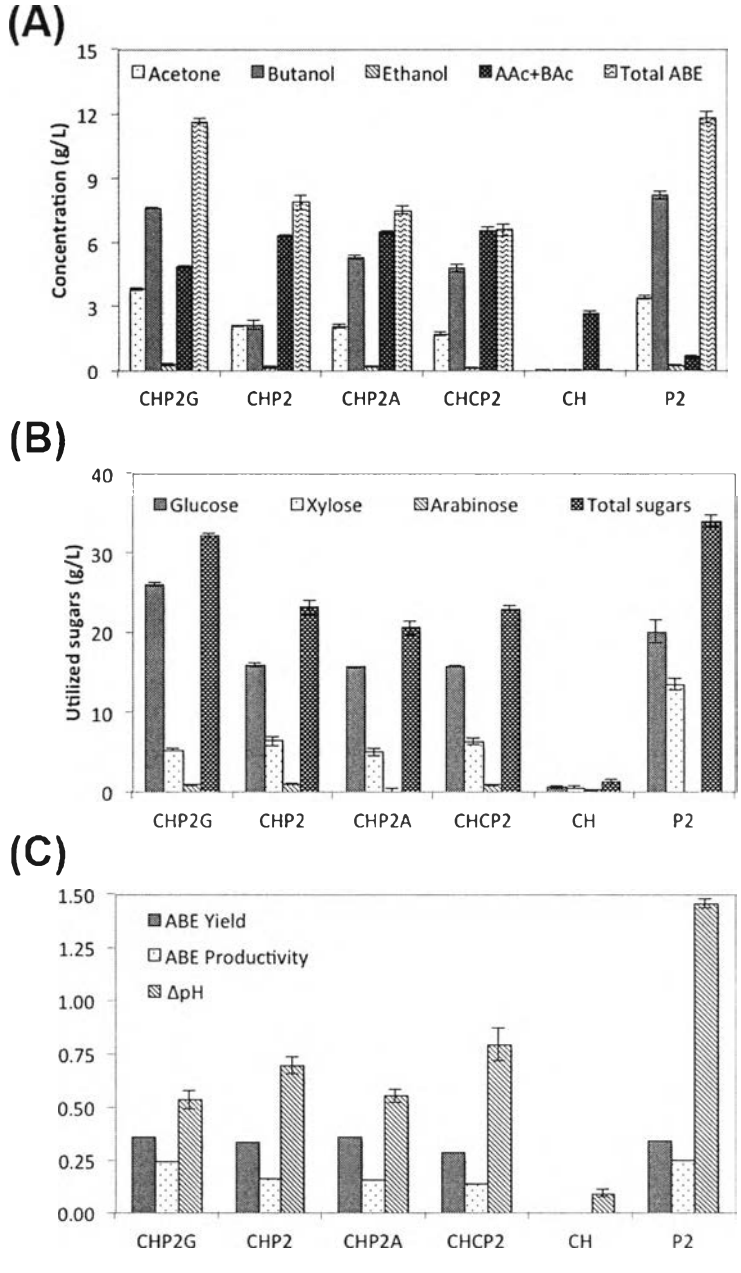

Figure 6.6 Concentration of solventogenic products and acids (A), utilized sugars (B) and ABE yield and productivity in the various conditions of fermentation broths at 48h (C).

6.5 Conclusions

ABE fermentation was performed using various acid pretreatments, H_2SO_4, HNO_3 and H_3PO_4, of corncobs to investigate the fermentability of *Clostridium beijerinckii* TISTR 1461. Among the three acids, H_2SO_4 pretreatment shows the best result in sugar yield in the combination with pretreatment and enzymatic hydrolysis but no potential for ABE fermentation. While the H_3PO_4 pretreatment, which has lower sugars, is the hydrolysate, used as a substrate, is comparable to synthetic sugars in the control experiment. Thus H_3PO_4 pretreatment shows promising results as a nontoxic substrate without having to add the process to remove inhibitors like overliming or XAD–4 resins which consumes time and is more costly.

6.6 Acknowledgements

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6.7 References

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