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Efficient Dilute-Acid Hydrolysis of Cellulose Using Solvent Pretreatment

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ABSTRACT: Pretreatment with N-methylmorpholine-N-oxide (NMMO), phosphoric acid, and sodium hydroxide was evaluated for improvement of dilute-acid hydrolysis of cotton fiber, the most difficult to break down cellulose. The pretreatments improved the yield of glucose formation by acid hydrolysis. Compared to the other methods, phosphoric acid pretreatment resulted in higher glucose yields and minimal byproduct formations by hydrolysis under milder conditions. Furthermore, the solid residue of the hydrolysis was subjected to enzymatic hydrolysis in order to convert the remaining cellulose to glucose. Different combinations of parameters in dilute-acid and enzymatic hydrolysis were considered for obtaining a high glucose yield with minimal enzyme loading. A process involving phosphoric acid pretreatment, dilute-acid hydrolysis, and enzymatic hydrolysis using only 5 FPU/g cellulase and 10 IU/g β-glucosidase resulted in total glucose yield of 95.4%, and fermentation of the hydrolysates resulted in a yield of 458 g of ethanol/kg of initial cellulose (0.47 g ethanol/g glucose).

1. INTRODUCTION

Fermentable sugars for the production of renewable fuels can be obtained directly from sugar resources such as sugar cane or semidirectly from the hydrolysis of starchy resources such as corn and wheat. However, utilization of food supplies for energy production creates a global food versus energy conflict.[1] Cellulosic materials are alternative feedstocks, from which fermentable sugars can be obtained through a hydrolysis process. However, efficient and inexpensive hydrolysis is one of the main obstacles in the production of renewable fuels and chemicals from cellulosic materials, and their utilization has so far remained elusive.[2−5]

Cellulose, an unbranched polymer of a $\beta(1\rightarrow4)$ D-glucopyranosyl unit, has a strong tendency to form a highly ordered structure as a result of its chemical constitution and spatial conformation. A tight packing arrangement of cellulose fibrils in complex crystalline domains makes the conversion of native cellulose inefficient. In order to disrupt the crystalline structure and make the cellulose more amenable to conversion, several methods have been introduced for the pretreatment of cellulosic materials prior to enzymatic hydrolysis.[6,7]

Treatment with cellulose solvents is among the most desired methods for improvement of enzymatic hydrolysis, owing to the modest reaction conditions.[8] Treatment with NaOH is one of the oldest processes for pretreatment of cellulosic structures which has been used since 1919 for improving the digestibility of straws by ruminants.[9] Currently, alkaline processing is considered one of the most efficient pretreatments of cellulosic materials in which disintegration of the cellulose structure can occur.[10,11] On the other hand, a few cellulose solvents, e.g., concentrated phosphoric acid (85% w/w) and N-methylmorpholine-N-oxide (NMMO), have been recently developed for fractionation of lignocellulosic materials under modest reaction conditions and considered as third generation cellulose solvent-based technologies.[12] Pretreatment of cellulosic materials by phosphoric acid, a low cost cellulose solvent, has been suggested. The concern with acid recovery and reconcentration was addressed by combining dissolution of cellulose in phosphoric acid and regeneration of cellulose using a highly volatile organic solvent such as acetone.[13] Moreover, N-methylmorpholine-N-oxide (NMMO) is a direct solvent of cellulose used in modern and environmentally friendly fiber making industries via a process called Lyocell technology. Treatment with NMMO has recently been used both for the separation of cellulose from waste textiles[14] and for the pretreatment of lignocelluloses for the enhancement of their bioconversion.[15] NMMO is a direct cellulose solvent without derivatization, while almost complete recovery of NMMO and cellulose is possible in the treatment with NMMO.[15]

Treatment of cellulosic compounds with cellulose solvents facilitates their bioconversion by both the separation of cellulose from complex structures and the disruption of their highly ordered crystalline structure.[16] However, all of the mentioned pretreatments were conducted for the improvement of enzymatic hydrolysis of cellulosic materials, and to our knowledge, there is no publication on the evaluation of any type of pretreatment for the improvement of dilute-acid hydrolysis.

In past decades, low cost conversion of cellulosic feedstocks to monomer sugars by acid hydrolysis was widely studied and also used on an industrial scale. The main drawback of dilute-acid hydrolysis is a low yield of cellulose hydrolysis, while a yield close to 100% can be obtained by enzymatic hydrolysis after a pretreatment.[17] The inefficiency of dilute-acid hydrolysis is related to the high crystallinity of cellulose and difficulty in

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diffusion of the acid inside the high crystalline parts of cellulose.[18,19] To improve the low yield, several processes, e.g., two-stage hydrolysis, and application of special types of reactors, e.g., percolation and shrinking-bed reactors, have been developed.[20,21] Utilization of a pretreatment process for improvement of acid hydrolysis of lignocelluloses has also been suggested.[22] However, the yields from cellulose by the acid processes are still inadequate, and activities in the field of cellulose hydrolysis are nowadays mainly shifted to enzymatic hydrolysis. Nevertheless, in spite of a large amount of research and progress, the price of a hydrolytic enzyme is still high. On the other hand, a dilute acid process can be considered among the cost-effective hydrolysis methods if the yield of glucose formation through it can be improved.

One of the main obstacles in bioconversion of lignocellulosic materials is the recalcitrance of crystalline cellulose to hydrolysis. The current study aimed at the improvement of dilute-acid hydrolysis of the most recalcitrant type of cellulose, i.e., cotton cellulose, with solvent pretreatment. Treatment with NMMO and concentrated phosphoric acid, as the third generation cellulose solvent-based technologies,[12] and NaOH, as one of the oldest and the most efficient pretreatments methods, was employed. The pretreated cellulose was then subjected to dilute-acid hydrolysis for glucose production. Furthermore, the residual solid from the acid hydrolysis was enzymatically hydrolyzed. Thus, a process for complete conversion of cellulose with lowest amounts of hydrolytic enzyme consumption was followed. The resulting hydrolysates were also evaluated for ethanol production by fermentation.

2. MATERIALS AND METHODS

2.1. Materials. Defatted cotton fiber with a cellulose content of over 99 wt % was obtained from Soof and Sateen Ltd. (Iran) and used as a substrate in all experiments. N-methylmorpholine-N-oxide (NMMO) 50% (w/w) (BASF, Ludwigshafen, Germany) was concentrated by vacuum evaporation to 85% (w/w).[23] In addition, sodium hydroxide (99%) and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany).

Commercial cellulase (Celluclast 1.5L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark) were used for the enzymatic hydrolysis. Celluclast 1.5L showed activity of 52.5 FPU/mL measured according to the procedure presented by Adney and Baker,[24] and it contained 42 mg/mL of protein, as measured by Bradford assay.[25] β-glucosidase activity was 205 IU/mL measured according to Ximenes et al.'s[26] method.

2.2. Phosphoric Acid Pretreatment. A cellulose solution was prepared by mixing 1 g of cellulose with 19 g of 85% phosphoric acid in a 50 °C water bath for 60 min. After dissolution, 60 mL of precold acetone was added and mixed well for quenching the solution. The suspension was then filtered on a filter paper (Whatman No. 1) to collect the precipitated cellulose and washed thoroughly with distilled water until pH 7 was reached.[13]

2.3. Pretreatment with NMMO. The cellulose was pretreated by mixing 1 g of cellulose with 19 g of 85% NMMO in a 120 °C oil bath for 3 h. After the treatment, 60 mL of deionized water was rapidly added to the suspension and manually mixed. The precipitated cellulose was then separated using a filter paper (Whatman No.1), and the solid was washed with extensive amounts of boiling water to achieve a clear water.[15]

2.4. Pretreatment with NaOH. One gram of the cellulose was soaked in a 19 g solution of 7% w/v NaOH and mixed at 100 rpm for 10 min. The slurry was then cooled to −20 °C overnight. Then, the temperature was increased to room temperature, and 120 mL of deionized water was added to precipitate the dissolved cellulose.[10] Afterward, the mixture was filtered on a filter paper (Whatman No.1) to collect the pretreated cellulose and washed thoroughly with 150 mL of deionized water.

2.5. Dilute-Acid Hydrolysis. The hydrolysis parameters, i.e., solid loading, acid concentration, temperature, and residence time, were selected considering the previous studies[27] and preliminary investigation. Pretreated and untreated celluloses, using 2, 5, and 7.5% solid loadings, were subjected to acid hydrolysis with 0.5 and 1 wt % sulfuric acid at 150 and 180 °C for 30 and 60 min. Depending on the solid loading, a specified amount of treated or untreated materials was soaked in dilute acid overnight prior to the hydrolysis. The acid hydrolysis experiments were conducted using a high-pressure stainless steel reactor with a 500 mL working volume which was equipped with a pressure gage and a thermometer and placed in an oil bath equipped with a temperature controller.[28]

2.6. Enzymatic Hydrolysis. The enzymatic hydrolysis was performed in 118 mL glass bottles (717561, Pajuhesh Setayesh Sepahan, Isfahan, Iran) at 45 °C and 120 rpm for 72 h using 50 mL of 0.05 M sodium citrate buffer. Solid loading of 2% (based on the dry weight) for untreated and pretreated materials was used, unless otherwise mentioned. The suspensions with an adjusted pH of 4.8 were then supplemented with 0.5 g/L sodium azide as an antibacterial agent except for suspensions considered for fermentation after hydrolysis, which was autoclaved at 121 °C for 20 min for sterilization. The enzyme loading for the hydrolysis varied from 5 to 15 FPU cellulase (4 to 12 mg protein) and 10 to 30 IU β-glucosidase per gram of cellulose. Liquid samples were periodically taken for sugar content analysis.

Enzymatic hydrolysis of the remaining solid after the dilute-acid hydrolysis was also conducted with or without separation of the clear supernatant (by decantation for 10 min) under the same conditions and enzyme loading mentioned above. The pH of the slurry obtained after dilute acid hydrolysis was adjusted to 4.8 using 10 M NaOH or concentrated HCl and directly used for enzymatic hydrolysis without washing of the pretreated cellulose.

2.7. Fermentation. Fermentations were carried out in 118 mL anaerobic bottles (717561, Pajuhesh Setayesh Sepahan, Isfahan, Iran) using a flocculating yeast strain of *Saccharomyces cerevisiae* CCUG 53310 (Culture Collection University of Gothenburg, Sweden). The microorganism maintenance and propagation was conducted as previously reported by Goshadrou et al.[29] No sodium azide was added in the enzymatic hydrolysis step when the hydrolysates were supposed to be fermented. The hydrolysates were supplemented with all necessary nutrients,[30] inoculated with 1.0 ± 0.1 g L^{-1} yeast, and cultivated at 32 °C for 48 h under anaerobic conditions. The yield of ethanol (g/kg) was defined as the ratio of produced ethanol (g) over the initial cellulose loading (kg).

2.8. Analytical Methods. All samples were analyzed by high-performance liquid chromatography (HPLC), equipped with a RI detector (Agilent Technologies 1260 Infinity, Germany). Glucose was monitored on an Aminex HPX-87P column (Bio-Rad, CA, USA) at 80 °C. Deionized water was used as the mobile phase at a flow rate of 0.6 mL/min. Ethanol

Table 1. The Yield of Glucose Production from Untreated and Pretreated High Crystalline Cellulose by Dilute-Acid Hydrolysis

hydrolysis conditions			glucose yield by dilute-acid hydrolysis (%)			
temperature (°C)	acid concentration (%)	time (min)	H_3PO_4	NMMO	NaOH	untreated
150	0.5	30	10.9	4.5	7.8	3.9
150	0.5	60	21.2	13.6	12.6	7.9
150	1	30	15.1	11.6	9.1	8.6
150	1	60	30.9	18.3	19.7	13.8
180	0.5	30	43.0	15.2	26.1	13.2
180	0.5	60	31.9	28.2	28.5	23.9
180	1	30	18.7	38.7	30.4	28.6
180	1	60	15.0	20.3	19.8	21.3

was analyzed on an Aminex HPX-87H column (Bio-Rad, CA, USA) at 60 °C with 0.6 mL/min eluent of 0.005 M sulfuric acid.

In order to measure the swelling capacity of the samples, 0.1 ± 0.01 g of each sample was put in a small nonwoven bag.[23] The bag was consequently immersed in a 1 wt % sulfuric acid solution for 1 h. The swelling capacity was calculated as $(W_2 - W_1)/W_1$, where W_1 is the initial weight of the dry cellulose and W_2 is the final weight of the swollen cellulose.[23]

All experiments were performed in duplicate, and the results are presented as averages. The average standard deviation of the results was less than 4.0%.

3. RESULTS AND DISCUSSION

3.1. Effects of Solvent Pretreatments on Dilute-Acid Hydrolysis. Dilute-acid hydrolysis of cellulose results in a low glucose yield, while it is possible to obtain a very high glucose yield (nearly 100%) with acid hydrolysis of starch, an amorphous polymer of alpha-glucose.[31] The recalcitrant structure of cellulose is believed to be responsible for the low hydrolysis yield. In this study, it is suggested to modify the cellulose structure to increase the yield. To evaluate this idea, three different cellulose pretreatments were investigated for the improvement of glucose production from high crystalline cellulose with acid hydrolysis.

The yields of glucose production with hydrolysis at 150 and 180 °C using 0.5 and 1% sulfuric acid for 30 and 60 min from untreated and pretreated cellulose are presented in Table 1. The results showed that the yield of glucose formations was improved by the pretreatments depending on the hydrolysis conditions.

In comparison to other applied pretreatments, H_3PO_4 pretreatment resulted in a higher yield of glucose production by acid hydrolysis, even under mild conditions (Table 1). A maximum glucose yield of 43% was obtained by hydrolysis with 0.5% acid at 180 °C for 30 min from H_3PO_4 pretreated cellulose. Comparable improvement was also observed for the improvement of enzymatic hydrolysis of cotton by Kuo and Lee[16] using four different cellulose dissolution agents, NaOH/urea solution, NMMO, 1-butyl-3-methylimidazolium chloride ionic liquid, and 85% phosphoric acid. They obtained the highest glucose yield through the H_3PO_4 pretreatment and explained the improvement by the formation of small particle size with a low degree of polymerization (DP)[16] as a result of the pretreatment. Increasing the reaction time from 30 to 60 min under the same conditions resulted in a 26% lower glucose yield. Furthermore, increasing the acid concentration from 0.5 to 1% resulted in a 56% reduction in the glucose yield. Increasing the acid concentration for hydrolysis of NMMO and NaOH pretreated cellulose resulted in a higher yield after 30 min but a lower yield after 60 min of hydrolysis. Pretreatment of cellulose with NMMO and NaOH resulted in respective glucose yields of 39 and 30% by hydrolysis with 1% acid at 180 °C for 30 min. Severe acid hydrolysis conditions, i.e., high acid concentration and high temperature for a long reaction time, can increase the rate of formation of glucose from cotton. On the other hand, the rate of degradation of formed glucose will also be increased by applying the severe conditions.[32] A higher rate of glucose degradation could be the reason for the lower glucose yield observed at a high acid concentration for a longer reaction time.

One of the main advantages of the solvent pretreatments is high cellulose recovery.[6] In the current work, almost complete cellulose recovery (99 ± 1%) was observed in NaOH and NMMO pretreatments, and it was 92 ± 3% for the H_3PO_4 pretreatment. A cellulose recovery of more than 97% for different modes of NMMO pretreatment has previously been reported.[23]

3.2. Effects of Pretreatments on Swelling Capacity. Water swelling capacity is among the structural features of cellulose which indicates the likely ease of enzyme accessibility and eventual hydrolysis effectiveness.[23] Similarly, in dilute-acid hydrolysis, accessibility of cellulosic structure by acid solution is one of the main effective parameters.[33] Thus, in this study, a modified method was applied for measuring the acid swelling capacity of the cellulosic structures.[23] The results showed that swelling capacity was increased from 0.75 ± 0.10 g/g for untreated cellulose to 3.36 ± 0.39, 3.23 ± 0.25, and 1.47 ± 0.26 g/g for H_3PO_4, NMMO, and NaOH pretreated celluloses, respectively.

Cellulose microfibrils contain high crystalline (around 67% of the total cellulose) and amorphous regions. Aqueous solutions are mostly absorbed in the amorphous parts, making them soft and flexible.[34] In dilute-acid treatments, the acid, which is a catalyst for the hydrolysis, penetrates into the amorphous region of cellulose, while the high crystalline region remains unattacked. Thus, changing the crystallinity through the solvent pretreatments in the current work might be one of the responsible factors for increasing the swelling capacity of the pretreated cellulose structures.

Considering dilute-acid hydrolysis of pretreated materials (section 3.1), improvement of the hydrolysis yield may be correlated with the swelling capacity of pretreated materials. Dilute-acid hydrolysis (180 °C, 0.5% acid, and 30 min) of H_3PO_4 pretreated cellulose, which showed the highest swelling capacity, resulted in the highest glucose yield of 43.0% (cf. Table 1). Therefore, the higher accessibility of this pretreated cellulose by acid, as a result of higher swelling capacity, might be responsible for the higher yield of dilute-acid hydrolysis in comparison to other pretreatments.

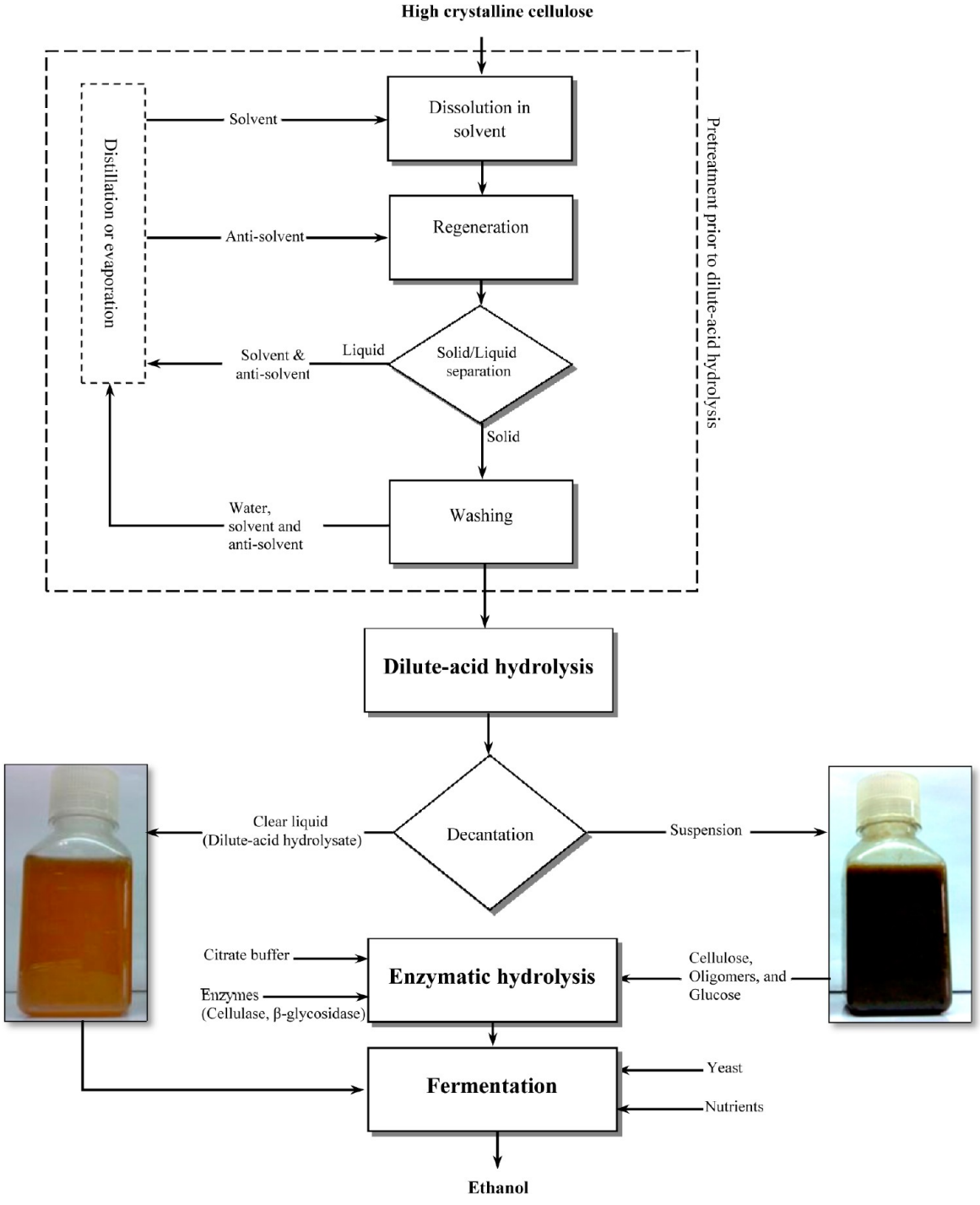

Figure 1. Developed process for complete conversion of cellulose to ethanol with minimal enzymes consumption.

3.3. Enzymatic Hydrolysis of Solid Residue of Dilute-Acid Hydrolysis. A part of cellulose was kept unconverted in the dilute-acid hydrolysis process. Since the best results of dilute-acid hydrolysis were obtained from H_3PO_4 pretreated cellulose, the solid residue of acid hydrolysis of the phosphoric acid treated cellulose was further hydrolyzed with a hydrolytic enzyme. It is worth mentioning that the residual solid was in the form of fine cellulose particles in H_3PO_4 pretreatment, while it was in the form of compacted pellets in the NMMO and NaOH pretreatments. One may expect high glucose yields from this solid residue by enzymatic hydrolysis, since it was pretreated once with concentrated H_3PO_4 and then with dilute sulfuric acid. However, addition of the enzymes to the mixture of the hydrolysate and solid residue resulted in a low enzymatic hydrolysis yield (*cf.* section 3.5), most probably due to the inhibitory effect of produced glucose and other inhibitors available in the liquid on the hydrolytic enzymes actions. Thus, the supernatant was separated by decantation, and the residual solid was subjected to the enzymatic hydrolysis (Figure 1).

Total glucose yields were calculated by addition of the glucose produced in dilute-acid hydrolysis to the glucose produced by the subsequent enzymatic hydrolysis (Figure 2). The acid pretreatment operating conditions highly affected the total glucose yields. The yield after 50 h of enzymatic hydrolysis

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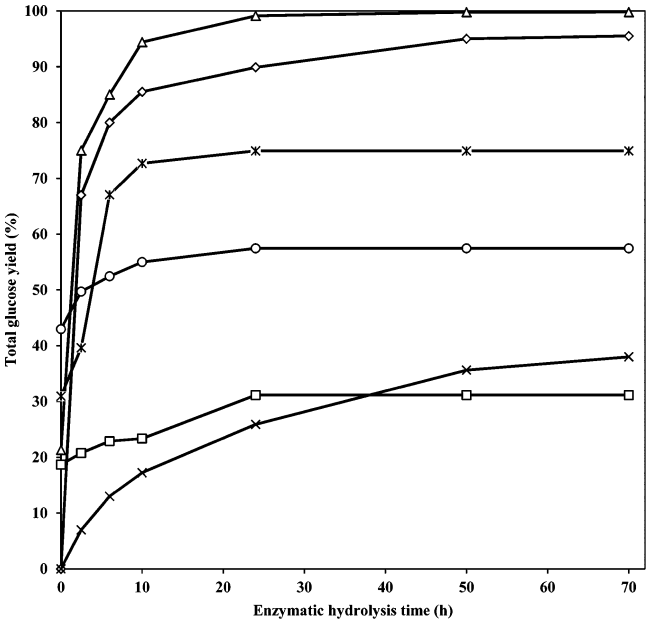

Figure 2. Glucose production yield by enzymatic hydrolysis of untreated (×) and H_3PO_4 treated cellulose (◊) as well as total yield of glucose produced from H_3PO_4 treated cellulose by both dilute-acid hydrolysis and subsequent enzymatic hydrolysis of residual suspension (*cf.* Figure 1). Dilute-acid hydrolysis was performed with 0.5% acid at 150 °C for 1 h (Δ), 1% acid at 150 °C for 1 h (*), 0.5% acid at 180 °C for 0.5 h (○), and 1% acid at 180 °C for 0.5 h (□). The enzymatic hydrolysis was performed with 15 FPU cellulase and 30 IU *β*-glucosidase per gram of treated cellulose. All results were averages of duplicates with an average standard deviation of less than 4.0%.

varied from 31.1 to 99.8%. A maximum glucose yield of 99.8% was obtained through a process containing the phosphoric acid pretreatment, dilute-acid hydrolysis with 0.5% acid at 150 °C for 1 h, separation of solid residue, and enzymatic hydrolysis of the solid residue using 15 FPU cellulase (12 mg protein) and 30 IU *β*-glucosidase per gram of the treated cellulose. In this process, dilute-acid treatment can be considered both as a glucose producing step with a 21.2% glucose yield and a pretreatment for the subsequent enzymatic hydrolysis. However, using enzymatic hydrolysis without acid hydrolysis, a glucose yield of 35.6% was obtained. In addition, enzymatic hydrolysis of H_3PO_4 pretreated cotton resulted in a yield of 95.0%.

It should be noted that cotton is a suitable substrate as an indication of the potential benefits of different pretreatments and hydrolysis processes. However, lignocellulose substrates, including wood, grasses, agricultural residues, and purposely grown energy crops, are more complicated, and cotton cannot be a representative of some of their properties. In addition to high crystallinity, the formation of different inhibitors during pretreatment and hydrolysis can profoundly affect the enzyme activity in the case of lignocelluloses. Through some pretreatments, e.g., dilute acid, pentosans are partially converted to furfural, which also rehydrate to formic acid under severe conditions. These byproducts of pretreatment of lignocelluloses together with some phenolic compounds produced from lignin showed inhibitory effects on enzymatic hydrolysis by cellulase.[35−37] Using pure cellulose as a substrate, hydroxymethyl furfural (HMF), the product of dehydration of glucose,[28] is the only potential inhibitor. However, under the acidic conditions applied in the current study, no HMF was detected.

3.4. Change in Morphological Features of Cellulose. FTIR analysis in the range of 850−1500 cm^{-1} is used to characterize the polymorphs of the high crystalline cellulose.[38] Change in the crystallinity, another effective parameter on dilute-acid hydrolysis yield, through H_3PO_4 regeneration was studied by FTIR analysis. As shown in Figure 3, the FTIR

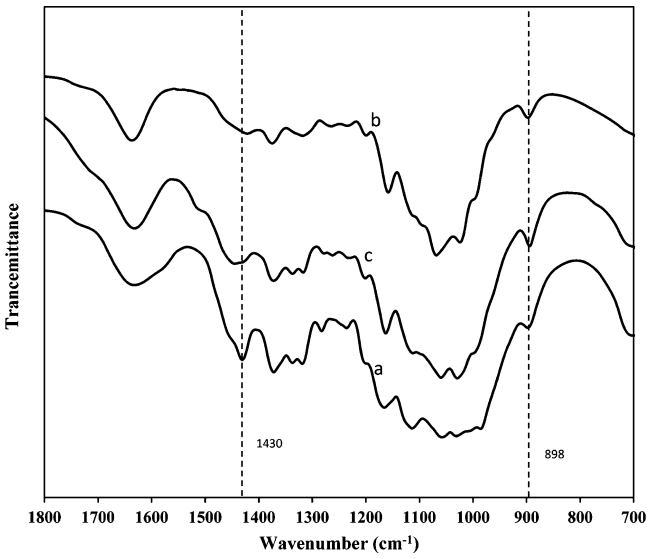

Figure 3. FTIR spectra for (a) untreated cellulose, (b) H_3PO_4 pretreated cellulose, and (c) residual cellulose after dilute-acid hydrolysis (at 150 °C using 0.5% acid for 60 min) of H_3PO_4 treated cellulose.

absorption band at 1430 cm^{-1}, which is assigned to the CH_2 scissoring motion in cellulose I,[16] became weak. In addition, the absorption band at 898 cm^{-1}, which is assigned as C−O−C stretching at the *β*-(1→4)-glycosidic linkage in cellulose II and amorphous cellulose,[16] became strong and sharp through the pretreatment. These two changes in FTIR spectra showed major changes in the crystallinity of cellulose from the highest crystalline form (cellulose I) to less crystalline and easily digestible forms (cellulose II) as well as amorphous cellulose.[16] Therefore, the untreated cellulose, similar to almost all native celluloses in the higher plants, contained cellulose I, and the regenerated cellulose was mainly in the form of cellulose II and amorphous cellulose. The changes in crystallinity could be another reason for the observed improvement in dilute-acid hydrolysis by the pretreatment.

SEM was used to study the morphological features and surface characteristics of H_3PO_4 pretreated cellulose before and after dilute-acid hydrolysis compared with the untreated cellulose. SEM analysis revealed extreme changes of the cellulose external surface area by the treatments (Figure 4). The highly ordered fibrous structure of cellulose, which has been shown to be composed of microfibrils (Figure 4a), was entirely changed to sponge-like structures through the H_3PO_4 pretreatment (Figure 4c and d). Furthermore, a disintegrated, disordered, and highly porous cellulose structure appeared after the dilute-acid hydrolysis (Figure 4f).

3.5. Effects of Pretreatments on the Hydrolytic Enzymes Requirement. The process of H_3PO_4 pretreatment, dilute-acid hydrolysis at 150 °C using 0.5% acid for 60 min, and enzymatic hydrolysis of the solid residue which resulted in the

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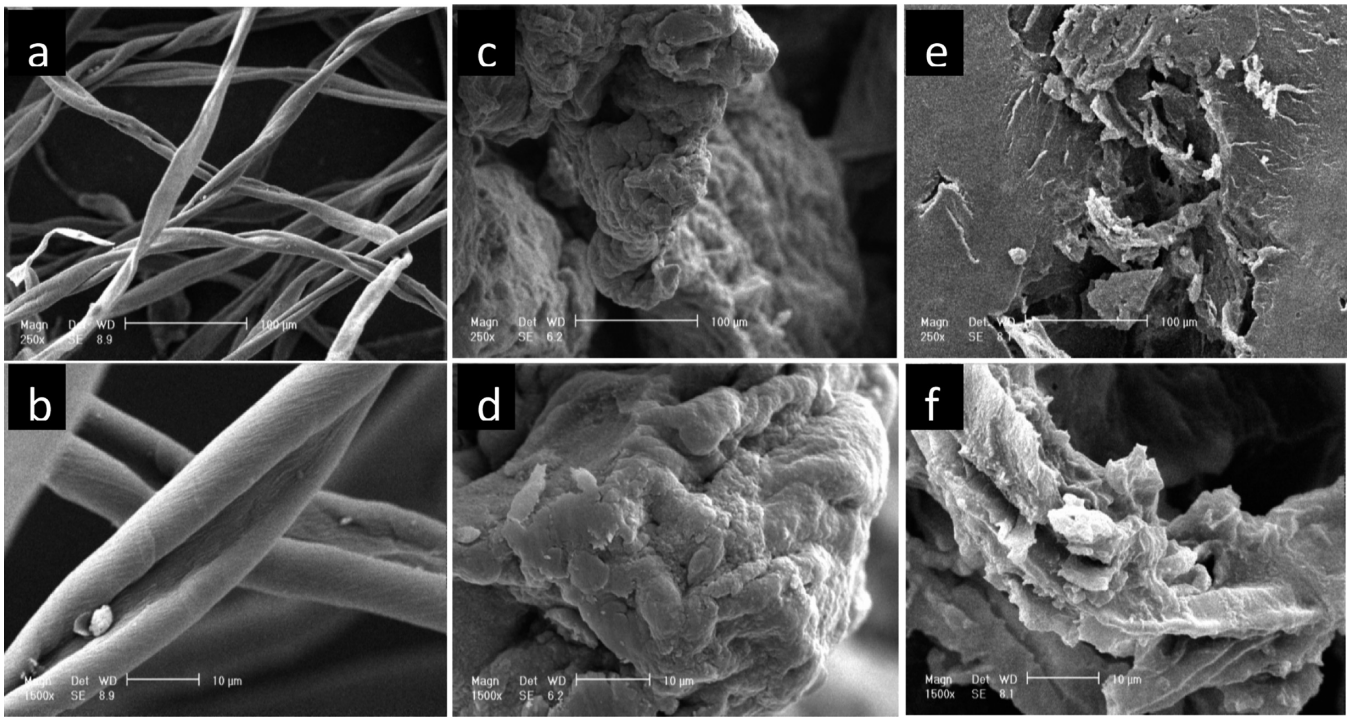

Figure 4. SEM images of untreated cellulose (a, b), H_3PO_4 treated cellulose (c, d), and residual cellulose after dilute-acid hydrolysis of H_3PO_4 treated cellulose (e, f) under different magnifications, 250× (a, c and e) and 1500× (b, d, and f).

highest glucose yield (*cf.* section 3.3) was conducted using different enzyme dosages (Figure 5). The results showed that glucose was produced with a yield of 95.4% through the process using only 5 FPU cellulase (4 mg protein) and 10 IU *β*-glucosidase per gram of cellulose. Increasing the enzyme loading from 5 to 15 FPU (4 to 12 mg protein) cellulase per gram of treated cellulose resulted in a minor increase in the yield of hydrolysis. In other words, even with a low enzyme loading of 5 FPU/g (4 mg protein/g), a relatively high glucose yield could be obtained by the process. Moreover, using 5 FPU/g cellulase (4 mg protein) and 10 IU/g *β*-glucosidase, the glucose production yield was only 27% in enzymatic hydrolysis of untreated crystalline cellulose and 85% in enzymatic hydrolysis of H_3PO_4 pretreated cellulose (Figure 5b and 5c), indicating the necessity of dilute-acid hydrolysis for obtaining a high glucose yield at these enzyme loadings.

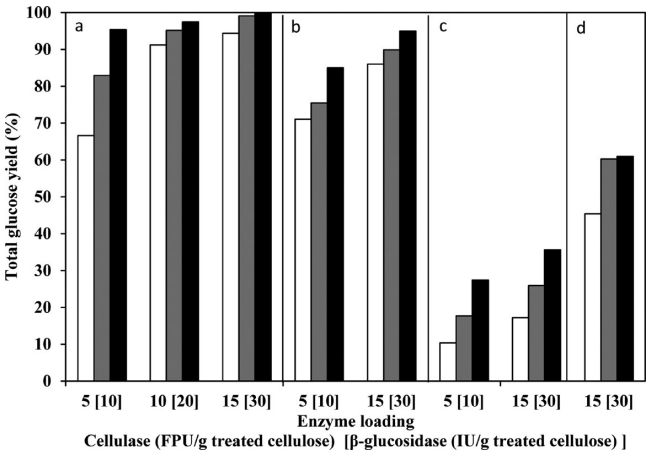

Figure 5. Total glucose yields from processes involving (a) H_3PO_4 pretreatment, dilute-acid hydrolysis (with 0.5% acid at 150 °C for 60 min), decantation of residual solid, and enzymatic hydrolysis of the solid residue (*cf.* Figure 1), (b) enzymatic hydrolysis of H_3PO_4 pretreated cellulose (without dilute-acid hydrolysis), (c) enzymatic hydrolysis of untreated cellulose, and (d) H_3PO_4 pretreatment, dilute-acid hydrolysis, and enzymatic hydrolysis of the whole acid hydrolysis slurry. The enzymatic hydrolysis was conducted for 12 (white), 24 (gray), and 48 h (black). All results were averages of two replications with an average standard deviation of less than 4.0%.

In order to obtain a higher yield than 95% through a single enzymatic hydrolysis of H_3PO_4 pretreated cellulose, it was necessary to use a 3 times higher enzyme loading in comparison to the process containing both acidic and enzymatic hydrolysis (Figure 5b). Nevertheless, using a whole acid slurry in enzymatic hydrolysis without separation of the liquid hydrolysate resulted in a reduction of the total glucose yield to only 61% even with 15 FPU/g cellulase (12 mg protein/g cellulose) and 30 IU/g *β*-glucosidase (Figure 5d). Therefore, the process comprising dilute-acid hydrolysis, separation of liquid hydrolysate, and enzymatic hydrolysis of solid residue could be used for the production of glucose from high crystalline cellulose with a high yield of 95.4% using 3-fold lower enzyme requirements (5 FPU or 4 mg protein per gram cellulose). On the other hand, the glucose yield obtained through enzymatic hydrolysis of dilute-acid pretreated Avicel cellulose, using an enzyme loading of 5 mg protein/g cellulose, has been reported to be less than 65.2%.[39] Therefore, phosphoric acid pretreatment prior to dilute-acid and enzymatic hydrolysis of crystalline cellulose played an effective role in obtaining a high yield of glucose production (95.4%) from high crystalline cellulose.

Considering 100 g of H_3PO_4 treated cellulose, dilute-acid hydrolysis with 0.5% at 150 °C for 60 min resulted in the production of 21.2 g of glucose. Hydrolysis of the solid residue using 5 FPU cellulase (4 mg protein) and 10 IU *β*-glucosidase per gram of pretreated cellulose resulted in the production of

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an additional 74.2 g of glucose after 48 h of enzymatic hydrolysis

3.6. Overall Yield of Glucose and Ethanol Productions.

Glucose was produced through dilute-acid hydrolysis with 2, 5, and 7.5% solid loadings and subsequent enzymatic hydrolysis of the residual solid. The dilute-acid and enzymatic hydrolysates were then mixed and subjected to fermentation by *S. cerevisiae*. The overall yield of glucose and ethanol productions from the high crystalline cellulose are summarized in Table 2. The ethanol yield was in the range of 132 to 469 g of ethanol per kilogram of cellulose. The high ethanol yield of 458 g/kg (corresponding to production of 0.47 g ethanol per g consumed sugar) was obtained using the process involving H_3PO_4 pretreatment, dilute-acid hydrolysis at 150 °C with 0.5% acid for 60 min, decantation of the acid hydrolysate, and enzymatic hydrolysis of the residual solid using 5 FPU cellulase (4 mg protein) and 10 IU β-glucosidase loading per gram of cellulose.

Table 2. The Overall Yield of Glucose and Ethanol Production from High Crystalline Cellulose by the Process Involving H_3PO_4 Pretreatment, Dilute-Acid Hydrolysis, Enzymatic Hydrolysis of Residual Solid, and Fermentation (cf. Figure 1)[a]

solid loading (%)	cellulase (FPU/g)[b]	β-glucosidase (IU/g)[b]	g glucose/kg initial cellulose	g ethanol/kg initial cellulose
2	5	10	975 ± 6	458 ± 9
	15	30	1020 ± 9	469 ± 4
5	5	10	829 ± 3	373 ± 6
	15	30	940 ± 5	414 ± 5
7.5	5	10	775 ± 3	333 ± 4
	15	30	907 ± 5	381 ± 7
2[c]	5	10	869 ± 2	408 ± 7
2[c]	15	30	919 ± 3	423 ± 9
2[d]	5	10	280 ± 2	132 ± 3
2[d]	15	30	364 ± 4	167 ± 2

[a]A process involving H_3PO_4 pretreatment, dilute-acid hydrolysis at 150 °C with 0.5% acid for 1 h, decantation of liquid hydrolysate, and enzymatic hydrolysis of the solid residue at 45 °C for 48 h. [b]Enzyme loadings were based on grams of pretreated cellulose. [c]Enzymatic hydrolysis of H_3PO_4 pretreated cellulose (without dilute-acid hydrolysis). [d]Enzymatic hydrolysis of untreated cellulose.

4. CONCLUSIONS

The yield of glucose from high crystalline cellulose with dilute-acid hydrolysis can be significantly improved by pretreatment with a cellulose solvent. Increasing the swelling capacity, disintegrating the cellulose structure, and transformation of high crystalline cellulose to less crystalline cellulose could be responsible for the improvement. The residual solid after the hydrolysis can be hydrolyzed by minimal enzyme consumption. The result of the process is a hydrolysate with an overall glucose yield of 95.4% that can be fermented to ethanol with a high yield of 0.47 g/g. Overall, a process comprising pretreatment with phosphoric acid, dilute-acid hydrolysis, and enzymatic hydrolysis with only 5 FPU cellulase (4 mg protein) and 10 IU β-glucosidase per gram of solid and fermentation of the hydrolysates resulted in the production of 458 g of ethanol per kilogram of cellulose.

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Notes

The authors declare no competing financial interest.

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