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Effect of particle size on the rate of enzymatic hydrolysis of cellulose

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ABSTRACT

The effect of particle size on enzymatic hydrolysis of cellulose has been investigated. The average size of microcrystalline cotton cellulose has been reduced to submicron scale by using a media mill. The milled products were further subjected to hydrolysis using cellulase. High cellulose concentration (7%) appeared to retard the size reduction and resulted in greater particles and smaller specific surface areas than those at low concentration (3%) with the same milling time. Initial rate method was employed to explore the rate of enzymatic hydrolysis of cellulose. The production rate of cellobiose was increased at least 5-folds due to the size reduction. The yield of glucose was also significantly increased depending upon the ratio of enzyme to substrate. A high glucose yield (60%) was obtained in 10-h hydrolysis when the average particle size was in submicron scale.

1. Introduction

Being the most abundant polysaccharide on the earth, cellulose is generally utilized in food, fuel, biomaterial and energy (Gan, Allen, & Taylor, 2003). In food, cellulose is generally recognized as fiber, which is the most sought information on nutrition labels (Todd & Variyam, 2008) due to growing interest in its health benefits. In US, the entire fiber market is worth $192.8 millions in 2004. Insoluble fiber dominates the market with a market share of $176.2 millions (Heller, 2008). In addition to health benefits, cellulose can be converted to biofuel by a multistep process that includes pretreatment, enzymatic hydrolysis, and fermentation. Pretreatment is an important and necessary step that opens up the tightly structured cell wall, thereby, allowing carbohydrolytic enzymes access to cellulose (Zeng, Mosier, Huang, Sherman, & Ladisch, 2007). Owing to the refractory structure of cellulose, hydrolysis is the key process for the biological conversion of cellulosic materials. Thus, increasing the yield of glucose from cellulose is helpful for developing bioethanol without competing with agricultural crops.

Enzymatic hydrolysis of cellulosic biomass depends on many factors: physical properties of the substrate (composition, crystallinity, degree of polymerization, etc.), enzyme synergy (origin, composition, etc.), mass transfer (substrate adsorption, bulk and pore diffusion, etc.), and intrinsic kinetics (Zhang & Lynd, 2004a). The enzymatic kinetics of cellulose degradation has been studied intensively in recent 50 years. Nevertheless, kinetics of cellulose degradation is still poorly understood because of competing effects such as physical properties of the substrate, enzyme synergy, and mass transfer to the intrinsic kinetics (Peri, Karra, Lee, & Karim, 2007). The structural heterogeneity and complexity of cell-wall constituents such as microfibrils and matrix polymers are part of reasons causing the recalcitrance of cellulosic materials (Himmel et al., 2007). The cellulose-hydrolysing enzymes (i.e., cellulases) are divided into three major groups: endo-glucanases, cellobiohydrolases (exo-glucanases), and β-glucosidases. The endo-glucanases catalyze random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. The enzymes of β-glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from cellobiose (Kumar, Singh, & Singh, 2008). Therefore, glucose and cellobiose are two major products from enzymatic hydrolysis of cellulose by cellulase. Two major steps (including adsorption of enzymes onto surfaces of cellulose and breakage of β-1,4-glucosidic bond between glucose) are involved in enzymatic hydrolysis of cellulose (Peri et al., 2007). Langmuir isotherm was generally used to describe the adsorption of cellulase onto the surface of cellulose due to its simplicity and good fitting to experimental data. However, the necessity of detail characteristics of the adsorption phenomena constrained its applications. Michaelis–Menten equation (Bezerra & Dias, 2007; Michaelis & Menten, 1913) was the most widely used to describe enzymatic kinetics. The initial rate of hydrolysis (v_0) can be expressed as:

$$v_0 = \frac{V_{\max}[S]_0}{K_m + [S]_0}, \quad (1)$$

where $V_{\max}$ denotes the maximum rate of hydrolysis and $[S]_0$ is the initial concentration of substrate, K_m is the Michaelis–Menten constant and physically represents the concentration of substrate

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as the hydrolysis rate reaches $V_{max}/2$, and it is also considered as an index of affinity between substrate and enzyme. Either increasing V_{max} or decreasing K_m enhances the reaction rate.

In some enzymatic reactions, the products inhibit the reaction via three different ways: competitive, noncompetitive or uncompetitive. Some investigators (Bezerra & Dias, 2004; Gruno, Valjamae, Pettersson, & Johansson, 2004) have revealed the presence of product competitive inhibition in enzymatic hydrolysis of cellulose using cellulase prepared from *Trichoderma reesei*. With the introduction of competition inhibition, Eq. (1) can be modified as (Gusakov, Sinitsyn, & Klyosov, 1985):

$$v_0 = \frac{V_{max}[S]_0}{K_m\left(1+\frac{[P]}{K_i}\right)+[S]_0}, \quad (2)$$

where [P] is the product concentration, and K_i denotes the inhibition constant. Apparently, v_0 obtained from Eq. (2) is less than that obtained from Eq. (1). To understand the effect of inhibition on hydrolysis rate by using initial rate method, the product concentration can be considered as a constant for a short time period when a finite quantity of product is added. During this short time period, the formation of product from substrate is assumed negligible. The concentration of product ([P]) can be considered as a constant ($[P]_0$) in Eq. (2) which is rearranged as:

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_{mapp}}{V_{max}[S]_0}, \quad (3)$$

where

$$K_{mapp} = K_m\left(1+\frac{[P]_0}{K_i}\right). \quad (4)$$

In a plot of $1/v_0$ versus $1/[S]_0$, the intercept on ordinate is the reciprocal of V_{max} and the intercept on abscissa is negative reciprocal of K_{mapp}. The plot is known as the Lineweaver–Burk (L–B) plot (Lineweaver & Burk, 1934). In this study, L–B plot was employed to examine the product inhibition behavior by altering the concentration of $[P]_0$ during the hydrolysis of cotton fiber by cellulase prepared from *T. reesei*.

Reduction in particle size of cellulose could enhance the affinity between cellulose and enzyme and thus increase the hydrolysis rate. The hydrolysis rate has been doubled in 10-h reaction when the average size was reduced from 82 to 38 μm (Gan et al., 2003). The size reduction also enhances the production of glucose or reducing sugars. Reducing size from 590 to 33 μm resulted in 55% increase in glucose production in 72-h hydrolysis of cellulose (Dasari & Berson, 2007). It appears that size reduction is an attractive method to increase the yield of hydrolysates from cellulose. However, literatures concerned with the effect of reduction of size to submicron scale on the hydrolysis of cellulose are limited. This study was attempted to explore the effect of size reduction of cellulose on hydrolysis rate, kinetic parameters and yield of glucose. The change in crystallinity associated with size reduction was also discussed.

2. Materials and methods

2.1. Materials

Microcrystalline cotton cellulose (designated as unmilled cellulose, UC) (Sigma Cellulose, Type 20), cellulase (EC 3.2.1.4 prepared from *T. reesei* ATCC 26921, lyophilized powder, 6.0 unit/mg solid as labeled. One unit will liberate 1.0 μmol of glucose from cellulose in one hour at pH of 5.0 at 37 °C.), glucose and cellobiose were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Distilled deionized water (DDW) was used in the preparation of suspension.

2.2. Media milling

A semi-batch type media mill (MiniPur, Netzsch-Feinmahltechnik GmbH, Germany) with a driving motor of 0.94 kW was utilized to prepare samples. Media (yttria-stabilized tetragonal zirconia, YTZ) of 0.3 mm were placed at 70% v/v filling ratio in the milling chamber (200 mL). UC (15 or 35 g) was blended with 500 mL DDW to be a suspension. The temperature of suspension was maintained below 30 °C. The agitation speed was set at 3600 rpm. Milling was continued for 120 min and samples at 0, 15, 60 and 120 min were taken for further hydrolysis and analyses. The milled cellulose was designated as MC-a-b for the sample of concentration a% and milling time of b minutes, in which a was 3 or 7.

2.3. Particle size distribution (PSD)

The particle size distributions of samples were determined by using a laser diffraction particle size analyzer (LS 230, Beckman Coulter, CA, USA) with detecting range of 0.04–2000 μm. The instrument was calibrated with deionized water. All the samples were diluted 10 times, subjected to mild stirring and then degassed by sonication (5 min in a Branson 3510R-DTH, Branson Ultrasonic Corp., USA, run at 100 W and 42 kHz). Average diameters (in volume) of particles were obtained using the software with LS 230. All the measurements were done in triplicates and the average data were reported.

2.4. Crystallinity index (CrI)

The X-ray diffractograms were obtained by the X'Pert PRO (PANalytical, Netherlands) X-ray diffractometer with nickel-filtered Cu Kα radiation. The diffraction intensity was measured between Bragg angles (2θ) of 5°–50°. The crystallinity indices were calculated by using the empirical formula postulated by Segal, Creely, Martin, and Conrad (1959)

$$CrI\% = \left(1-\frac{I_{AM}}{I_{200}}\right)\times 100, \quad (5)$$

where I_{200} represents the maximum intensity of the 200 lattice diffractions at $2\theta = 22.7°$, and I_{AM} denotes the intensity of diffraction at $2\theta = 18°$.

2.5. Kinetics of enzymatic hydrolysis

As mentioned previously, cellobiose and glucose are two major products from the hydrolysis of cellulose using cellulase prepared from *T. reesei* (Kumar et al., 2008). To simplify the situation, enzymatic hydrolysis of cellulose was divided into two stages

$$\text{Cellulose} \overset{V_{max}(1),K_m(1),K_i(1)}{\rightarrow} \text{Cellobiose} \overset{V_{max}(2,K_m(2),K_i(2)}{\rightarrow} \text{Glucose}. \quad (6)$$

The first stage is the hydrolysis of cellulose to cellobiose, and the second one is the hydrolysis of cellobiose to glucose. Initial rate method was employed to explore the effect of size on the rate of hydrolysis using Eq. (2). For the first-stage hydrolysis, cellulose (including UC and MC) at designated quantity (0, 0.25, 0.5, 1.0 g) was considered as the substrate and cellobiose at designated quantity (0, 0.02, 0.06, 0.1 g) was considered as the competitive product. According to Gan, Allen, and Taylor (2002), substrate and competitive product were mixed in 100 mL sodium acetate buffer solution (10 mM, pH 4.7) for the hydrolysis in a jacket reactor at 40 °C maintained by a water bath. Reactions were initiated by adding cellulase (20 mg) to the mixture at a stirring rate of 400 rpm. The enzymatic hydrolysis was conducted for 0, 2.5, 5, 7.5, 10, 15, 20 or 30 min and then sample (about 15 mL) was taken and immediately immersed

in a hot water bath (90 °C) for 20 min to terminate the reactions for further analysis. For the second-stage, cellobiose at designated quantity (0, 0.02, 0.06, 0.1 g) was the substrate and glucose (0, 0.05, 0.1 g) was the competitive product. The enzymatic reaction was conducted by the procedures above. The reaction rates were determined by measuring the production of cellobiose and glucose for stage 1 and 2, respectively, using HPLC. The data collected in 15 min were used to estimate kinetic parameters (V_{max}, K_m, K_i) in Eq. (2) for both stages. The parameters were obtained by using non-linear regression with least squared method using Mathematica software (Wolfram Research Inc., Campaign, IL, USA), which was also used for statistical analysis in this study. Lineweaver–Burk (L–B) plot was used to examine the product inhibition behavior.

2.6. Yield of enzymatic hydrolysis

To determine the production of cellobiose and glucose from cellulose in the long run, the reaction was conducted similarly to the previous procedures, but was extended to 120 h without adding competitive products. The initial concentration of cellulose was set as 0.25, 0.5, and 1 g and 20 mg cellulase was added for the hydrolysis. Thus, three ratios of enzyme to substrate (E/S) were used. Reaction was terminated at designated time and samples were taken for analysis of cellobiose and glucose concentration using HPLC. As the volume of reaction mixture was kept constant, the yields of glucose or cellobiose were calculated as:

$$\text{Yield}\,\% = \frac{\text{Concentration(g/L) of glucose(or cellobiose) at time of t}}{\text{Initial concentration(g/L) of cellulose}} \quad (7)$$

2.7. Analysis of cellobiose and glucose

The hydrolysates were filtered through a 45 μm membrane. The concentration of cellobiose and glucose in the filtrate was analyzed by using a HPLC (PU 2080, Jasco Co., Tokyo, Japan) equipped with a Hyperisil HS APS2 column (4.6 × 250 mm, 5 μm, England), a RI detector (RI-930, Jasco, Japan), and a SISC Chromatography data processor (SISC Co., Davis, California, USA). The mobile phase was acetonitrile/H_2O (70/30) at a flow rate of 1 mL/min. Sample injection volume was 20 μL. Reagent grade cellobiose and glucose were purchased from Sigma Co. (St. Louis, MO, USA) and were used to conduct qualitative analysis and to establish calibration curve for quantitative evaluation. Xylitol was used as an internal standard.

3. Results and discussion

3.1. Particle size distribution (PSD)

The PSD of UC exhibited a unimodal distribution ranged from 1.83 to 90.13 μm with a volume-averaged diameters of 25.52 μm. During the milling, large particles were broken into small ones; volume percentage of small particles was increased with milling time, in the meanwhile, the percentage of large particles was decreased. Thus, the unimodal distribution of UC would turn to milti-modal ones. For example, the milled cellulose (MC-3-120) exhibited bimodal PSD, which 74 vol.% percentage of particles were smaller than 1 μm and the average diameter was 0.78 μm. In general, the average diameter of MC decreased monotonically with the increase of milling time as illustrated in Fig. 1. The most remarkable size reduction occurred at the first 15 min. At that time period, the average particle size was reduced from 25.52 μm to about 5 μm. After 60-min milling, there existed a plateau in the average particle size. There was no significant difference between two concentrations (3% and 7%) in size reduction during the first 15-min milling. After 15-min milling, sample at higher concentration exhibited a greater particle size. Low concentration (3%) MC yielded a limited diameter of about 0.8 μm in 60 min. Nevertheless, high concentration (7%) MC led to a limited diameter of about 2.1 μm in 90 min. It was probably due to the increase in viscosity by the raise of concentration, which impeded the impact forces by media.

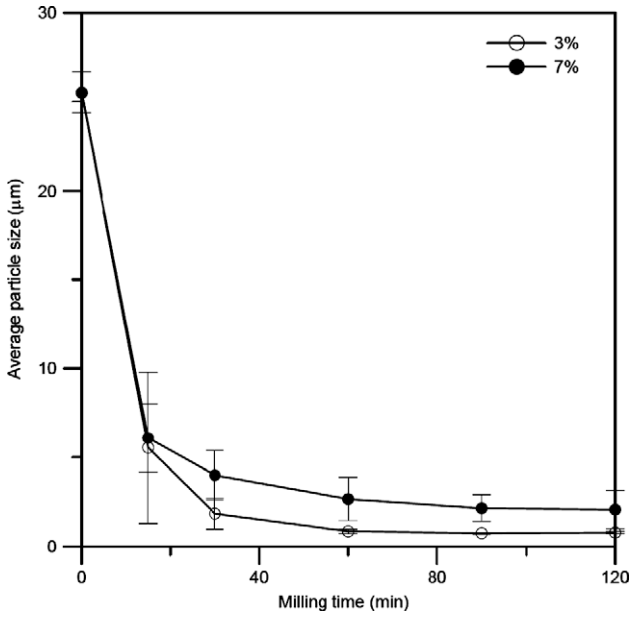

Fig. 1. Change in average particle size of cellulose along with the milling time.

3.2. Crystallinity index, CrI%

X-ray diffractograms of UC (the one with milling time of zero in Fig. 2) illustrates that the maximum intensity of the 200 lattice diffraction is at $2\theta = 22°–23°$ and the intensity of the 110 lattice dif-

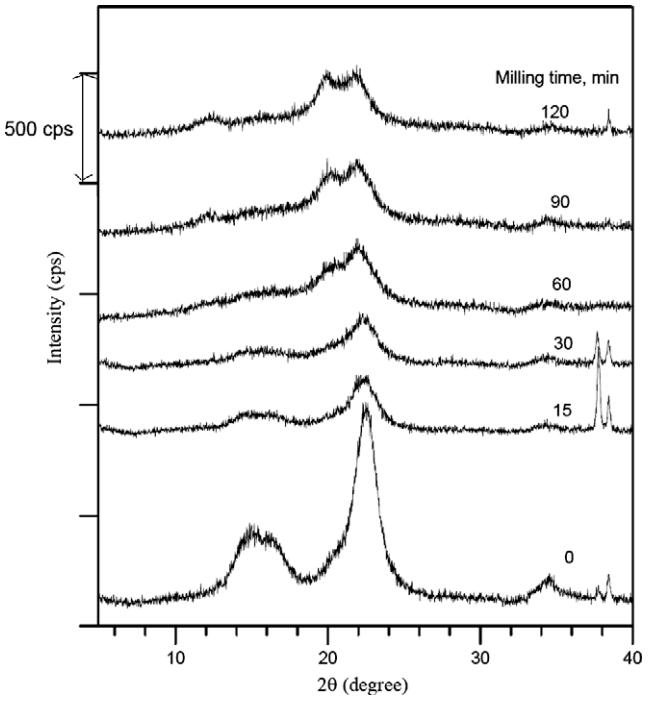

Fig. 2. X-ray diffractograms of unmilled cellulose (UC) and milled cellulose (MC) at 3%.

fraction is at $2\theta = 15°$, which is the typical crystal lattice for cellulose I (El-Sakhawy & Hassan, 2007). The intensity of these two peaks decreased as the milling was proceeded. Nevertheless, a new intensity was observed at $2\theta = 19°–20°$ when the milling time was longer than 60 min. This new peak indicated the formation of a new lattice diffraction due to recrystallization by the local high temperature occurred during the milling (Bhuiyan, Hirai, & Sobue, 2001; Yildiz & Gumuskaya, 2007). At 3% cellulose concentration, the reductions in both intensities at $2\theta = 15°$ and 22°–23° were more remarkable than those at 7% (data not shown). The data indicated that media milling also reduced the crystallinity of cellulose (Fig. 3). Milling for high concentration (7%) of MC resulted in a less reduction in *CrI*%. The value of *CrI*% reached the lowest value at 60 min, and then fluctuated slightly probably due to the occurrence of recrystallization. Reduction in the cellulose crystallinity upon mechanical pretreatment was reported to be a possible cause behind the improved hydrolysis (Chang & Holtzapple, 2000). The media milling reached a 20% reduction in *CrI*% in 120 min, which was greater than that (15% reduction) for hemp fiber by ball milling in 300 min (Ouajai & Shanks, 2006). The importance of crystallinity on the hydrolysis is not clear yet. The crystallinity of natural lignocellulosic has been considered as the major obstacle to produce fermentable sugar economically (Kumar et al., 2008). Nevertheless, several researchers (Caulfield & Moore, 1974; Fierobe et al., 2002; Howell & Stuck, 1975) have pointed out that the particle size was superior to the crystallinity on raising the reaction rate possibly due to the increase in the accessibility between cellulose and enzyme. This leads the need of further studies for differentiating the effect of size reduction and change in crystallinity on enzymatic hydrolysis.

3.3. Hydrolysis rate

Fig. 4 illustrates the example data for the production of cellobiose from UC and MC-7-60 in 30 min. Good linear relationship ($r^2 \geqslant 0.9$) between production of cellobiose and milling time was obtained using the data collected in 15 min. Obviously, the production rate of cellobiose from UC (Fig. 4a) was much slower than that for MC-7-60 (Fig. 4b) at three different initial substrate concentrations. As the enzyme activity was maintained constant, the rate of

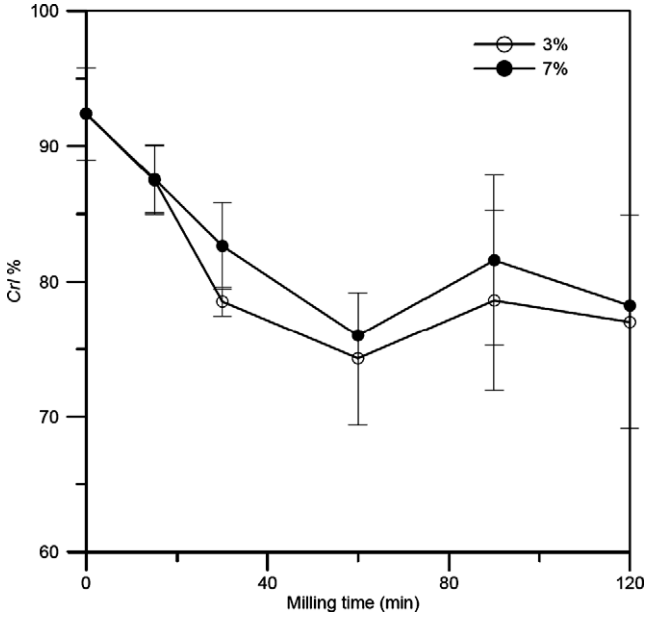

Fig. 3. Change in crystallinity (*CrI*%) during milling.

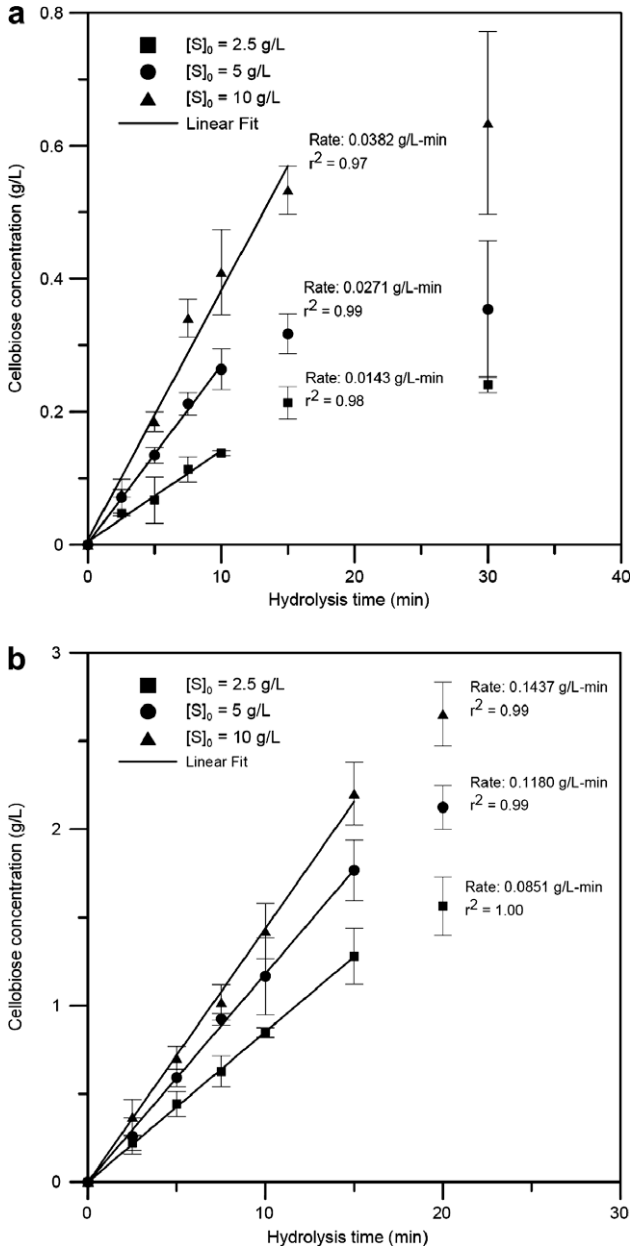

Fig. 4. Determination of initial rate of cellobiose produced from (a) UC, and (b) MC-7-60.

cellobiose production generally increased with the increase of initial substrate concentration ($[S]_o$) as illustrated in Fig. 5. The surface area and particle size of cellulose have been found as important substrate characteristics in determining the initial rates of hydrolysis (Eriksson, Borjesson, & Tjerneld, 2002). The total surface area was dramatically increased by media milling (from 0.24 m^2/g for UC to 9.60 m^2/g for MC-7-120 and to 25.50 m^2/g for MC-3-120), which resulted in significant increase in the hydrolytic rate of cellulose by enzymes. The effect of $[S]_0$ on the production rate of cellobiose from MC (average size smaller than 25.52 μm) was not as remarkable as that for UC (size of 25.52 μm). For example, when $[S]_0$ was increased from 2.5 to 10 g/L, the production rate of cellobiose from UC was increased about 2.7 times. Nevertheless, the production rate was increased only about 1.5 times for MC-3-120 (size of 0.78 μm). However, the size reduction significantly increased the production of cellobiose. At low $[S]_0$ (2.5 g/L), the production rate (0.1690 g/L-min) of

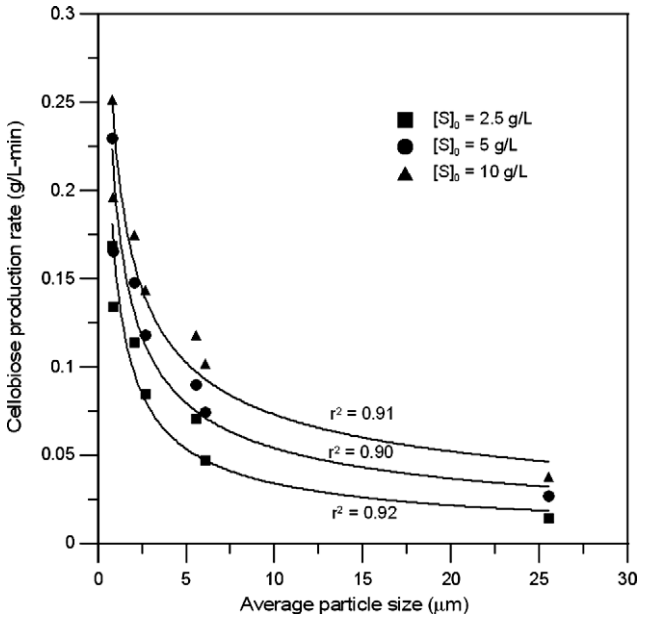

Fig. 5. Effect of average particle size on the rate of cellobiose production at three different initial substrate concentrations.

cellobiose from MC-3-120 was almost 12 times of that (0.0143 g/L-min) from UC. At high $[S]_o$ (10 g/L), the percentage increase in production rate was reduced to about 700%. Among the MC, MC-3-120 exhibited the greatest production rate due to the smallest size (0.78 μm) and the largest specific surface area (25.50 m^2/g). The enhancement on the hydrolysis may be due to increased enzyme accessibility (Mansfield, Mooney, & Saddler, 1999). Sangseethong, Meunier-Goddik, Tantasucharit, Liaw, & Penner (1998) have reported that reducing particle size from 100 to 20 μm almost doubled the saccharification rate of microcrystalline cellulose (Avicel) at 0.1% w/v. Nevertheless, the same size reduction only resulted in 50% increase in the saccharification rate when the substrate concentration was high (2% w/v). Zeng et al. (2007) reported that reduction of size from 425–710 to 53–75 μm raised the initial glucose production rate from 0.1 to 0.18 g/L-h. With about fivefold size reduction, MC-3-15 (size of 5.54 μm) exhibited about 3–5 times of the hydrolysis rate for UC depending on substrate concentration. The data in this study illustrated that reduction of particle size into submicron scale significantly enhanced the hydrolysis rate of cellulose.

Increasing substrate concentration also raised the hydrolysis rate of cellobiose to glucose. The production rate of glucose was raised from 0.0066 to 0.0141 g/L-min when the substrate concentration was increased from 1 to 10 g/L. The production rate of glucose was comparably much slower than that of cellobiose (Fig. 5) probably due to low β-glucocidase activity (Wen, Liao, & Chen, 2004). For example, at same initial substrate concentration of 5 and 10 g/L, the production rate of glucose from cellobiose was 0.0116 and 0.0141 g/L-min, respectively. The production rate of cellobiose from UC was at least 2 times of those at the same substrate concentration. The significant increase in production rates of cellobiose from MC resulted in an increase in cellobiose concentration and thus raises the production of glucose.

3.4. Product inhibition

As discussed above, Eq. (1) is used for reactions without product inhibition and Eq. (2) is used for reactions with competitive product inhibition. Thus, it is necessary to examine the applicability of both equations (Bezerra & Dias, 2004; Gruno et al., 2004). Eq. (3) was used to verify the competitive inhibition exerted by the hydrolysates. The Lineweaver–Burk plots (which were ignored for conciseness) for the hydrolysis of cellulose (UC) and cellobiose demonstrated that the enzymatic hydrolysis was competitive inhibition exerted by products. Peri et al. (2007) pointed out that endo-glucanases and exo-glucanases were subjected to noncompetitive inhibition by soluble cello-oligosaccharides, cellobiose, and glucose, but the hydrolysis by β-glucosidase was solely inhibited by glucose through a competitive inhibition mechanism. The results concurred with the report for cotton fiber at 50 °C and pH of 4.5 (Gusakov et al., 1985) and confirmed the utilization of Eq. (2) in this study. Then, the Lineweaver–Burk plots were further employed to calculate the parameters (V_{max}, K_m and K_i) in the modified Michaelis–Menten equation (Eq. (2)) by non-linear regression.

Table 1 lists the regressed results for both the production of cellobiose from cellulose and glucose from cellobiose. Among the cellulose samples, UC yielded the lowest V_{max} and greatest K_m, which explained the low production rate of cellobiose from UC. Milling resulted in greater V_{max} and smaller K_m. For example, MC-7-15 having the greatest average particle size (6.08 μm) among MC exhibited a V_{max} as twice as that of UC and K_m as 2/3 of that of UC. Nevertheless, the milling did not significantly alter the value of K_i.. When the average particle size was smaller than one micron, the increase in V_{max} and reduction in K_m were even more remarkable for MC-3-60 and MC-3-120. In other words, milling resulted in an increase in the maximum initial reaction rate and the affinity between the substrate (cellulose) and enzyme (cellulase). Compared with the kinetic parameters obtained for cellulose, the hydrolysis of cellobiose exhibited lower value of V_{max} (0.016 g/L-min) and K_m (1.719 g/L), but similar K_i. Thus, the hydrolysis rate of cellobiose was slower than that for cellulose (Gusakov et al., 1985). It demonstrated that the activity of exo-glucanase was superior to that of β-glucosidase in the enzymatic hydrolysis, and the later (β-glucosidase) was strongly inhibited by the product. This agreed with the characteristics of cellulases prepared from *T. reesei* (Zhang & Lynd, 2004b). The results indicated that the size reduction of cellulose increased the hydrolysis rate by raising V_{max} and abating K_m. It appeared that K_i was not significantly affected by size reduction. Compared with data of Johnston, Shoemaker, Smith, and Whitaker (1998), this study showed much greater V_{max} and lower K_m probably due to high level of enzymes.

The analysis showed that the value of V_{max} increased linearly with the reduction in particle size with a correlation coefficient (r^2) of 0.68. The size reduction resulted in a linear decrease (r^2 = 0.89) in K_m. Obviously, the increase in V_{max} and decrease in K_m are desirable for enhancing the hydrolysis rate of cellulose. When the substrate concentration is very low and becomes negligible compared with K_m, the ratio of V_{max} to K_m would be an index of the production rate. The ratio V_{max}/K_m of MC-3-120 was about as 21-folds (calculated using the data in Table 1) as that for UC. How-

Table 1
Kinetic parameters (V_{max}, K_m, K_i) for the enzymatic hydrolysis of cellulose at different average particle sizes (d_m).

Samples[a]	V_{max} (g/L-min)	K_m (g/L)	K_i (g/L)	d_m (μm)
UC	0.076	9.844	0.034	25.52
MC-3-15	0.152	3.055	0.026	5.54
MC-3-60	0.231	1.839	0.026	0.85
MC-3-120	0.304	1.877	0.016	0.78
MC-7-15	0.164	6.090	0.031	6.08
MC-7-60	0.187	2.960	0.026	2.66
MC-7-120	0.214	2.194	0.028	2.07
Cellobiose	0.016	1.719	0.028	–

[a] UC is the unmilled cellulose. MC-a-b is the cellulose milled at a concentration for b minutes.

ever, the production rate was only about 11 times as discussed previously (Fig. 5). This indicated that the presence of K_i and the substrate concentration was greater than K_i did reduce the hydrolysis rate. As discussed above, milling resulted in the decrease in both size and crystallinity of cellulose. Our analysis found that the three kinetic parameters were also dependent upon the crystallinity of cellulose. The decrease in crystallinity resulted in linear increase in V_{max} and decrease in K_m. Apparently, K_i was not significantly affected by reduction of crystallinity. The crystallinity of natural lignocellulosic has been considered as the major obstacle to produce fermentable sugar economically (Kumar et al., 2008). Both endo- and exo-glucanases are believed to hydrolyze the cellulose in the amorphous region. The hydrolysis in crystalline region was dominated by exo-glucanase. Thus the hydrolysis rate in crystalline region was much slower than that in amorphous region. Nevertheless, the crystallinity is not the only factor affecting the enzymatic hydrolysis of cellulosic materials (Laureano-Perez, Teymouri, Alizadeh, & Dale, 2005). Several literatures (Caulfield & Moore, 1974; Fierobe et al., 2002; Howell & Stuck, 1975) have pointed out that the particle size was superior to the crystallinity on raising the reaction rate possibly due to the increase in the accessibility between cellulose and enzyme. This leads the need of further studies for differentiating the effect of size reduction and change in crystallinity on enzymatic hydrolysis.

3.5. Yields of hydrolysis

As pointed out by Chundawat, Venkatesh, and Dale (2007), a three- to six-fold size reduction of untreated biomass is needed to see a measurable improvement in the glucan conversion. The media milling resulted in more than threefold size reduction and significantly increased the yield of glucose. Although Wen et al. (2004) pointed out that reducing particle to be smaller than 590–350 μm did not result in any increase in glucose yield, we did observe that particles in submicron scale exhibited greater yield than particles in micron scale. For example, at initial substrate concentration ($[S]_0$) of 2.5 g/L, the size reduction resulted in an increase in the yield of glucose during 120-h hydrolysis as illustrated in Fig. 6. In general, the yield of glucose increased rapidly as the hydrolysis proceeded at initial 40 h and then approached a plateau. At 10-h hydrolysis, two samples (MC-3-60 and MC-3-120) with average particle size smaller than one micron exhibited 60% glucose yield. Nevertheless, the glucose yield from UC was less than 30%. At 120-h hydrolysis, the final yield of MC-3-120 was 90%, which was about as twice as that (50%) for UC. Compared with the literatures, the glucose yield in this study was greater than the yield (62.4%) from mechanically micro/nanofibrilled Douglas fir added with ethylene glycol (Lee, Teramoto, & Endo, 2009) and the yield (67.2%) from pretreated stover (size of 53–75 μm) after 168-h hydrolysis (Zeng et al., 2007). Compared with the production (1031 mg) of reducing sugar from wet-milled (48 h) pure cellulose (2 g) with 3.5 mm glass beads (Kelsey & Shafizadeh, 1980), the media milling demonstrated significant improvement on glucose conversion. There existed about 40% increase in the glucose yield when the particle size was reduced from 25.52 to 6.08 μm, which was greater than the increase in digestibility (10–15%) by 10-fold particle size reduction (1000–100 μm) of corn stover (Chundawat, Balan, & Dale, 2008). The results were more significant than that (15–20% increase in glucan and xylan conversion) from PM corn stover being reduced from 850–500 to < 150 μm (Chundawat et al., 2007). Reducing size from 590–850 to 33–75 μm, the glucose production has been raised from about 9–14 g/L after 72-h hydrolysis using 10% initial solid concentration (Dasari & Berson, 2007). The present results indicated that the reduction of size to submicron scale enhanced remarkably the glucose yield by enzymatic hydrolysis. With the addition of surfactants, the glucose production has been increased from 120 to 180 mg from 1 g cellulose (Avicel) after 75 h hydrolysis (Mizutani, Sethumadhavan, Howley, & Bertoniere, 2002). It is worth of further study on the effect of adding surfactant in media milling on glucose yield.

Increasing the ratio of enzyme to substrate (E/S) generally resulted in an increase in glucose yield and decrease in cellobiose yield after 24-h hydrolysis (Table 2). The total yield (summation of glucose and cellobiose) was also increased by increasing E/S. This was probably due to the low hydrolysis rate of cellobiose as discussed above. In this study, the ratio of E/S was doubled from 0.02 to 0.04 and then from 0.04 to 0.08. It appeared that the first doubling in E/S resulted in greater percentage of increase in glu-

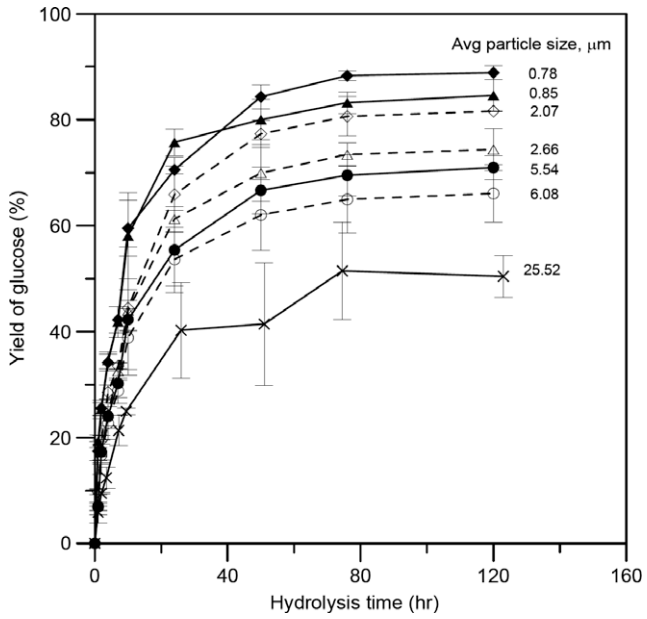

Fig. 6. The yield of glucose from cellulose at different particle sizes during hydrolysis.

Table 2
Yields of glucose and cellobiose from cellulose after 24-h hydrolysis.

Substrate[a]	E/S[b]	Yield[c] (%)	
		Glucose	Cellobiose
UC	0.02	14.22 ± 0.66	20.26 ± 1.23
	0.04	31.26 ± 0.78	19.95 ± 0.27
	0.08	40.29 ± 9.03	16.41 ± 1.13
MC-3-15	0.02	22.94 ± 0.97	15.86 ± 1.40
	0.04	38.64 ± 1.33	16.88 ± 1.16
	0.08	55.42 ± 8.11	14.23 ± 1.94
MC-3-60	0.02	35.89 ± 0.52	18.25 ± 2.69
	0.04	59.71 ± 5.61	20.66 ± 0.80
	0.08	75.69 ± 2.54	11.08 ± 2.52
MC-3-120	0.02	45.83 ± 1.57	20.92 ± 1.62
	0.04	56.03 ± 2.28	18.25 ± 1.58
	0.08	70.53 ± 0.74	8.59 ± 0.65
MC-7-15	0.02	23.48 ± 0.54	14.95 ± 0.60
	0.04	36.75 ± 2.11	17.06 ± 0.42
	0.08	53.63 ± 5.04	14.54 ± 3.64
MC-7-60	0.02	25.11 ± 2.25	16.76 ± 0.60
	0.04	41.71 ± 5.19	17.76 ± 1.96
	0.08	61.27 ± 1.61	13.77 ± 2.15
MC-7-120	0.02	33.51 ± 4.05	19.85 ± 0.50
	0.04	47.91 ± 5.08	15.16 ± 0.54
	0.02	65.89 ± 6.98	9.69 ± 1.01

[a] UC is the unmilled cellulose. MC-a-b is the cellulose milled at a concentration for b minutes.
[b] E/S is the ratio of enzyme to substrate.
[c] yield is calculated as: glucose (or cellobiose) (g/L)/initial cellulose (g/L).

cose yield than the second doubling in E/S, particularly for UC. That was probably due to no adequate substrate being acted by enzymes in the second doubling of E/S. As E/S was doubled from 0.02 to 0.04, there existed about 120% increase in glucose yield from UC, nevertheless, about only 22% increase in glucose yield from MC-3-120. The data indicated that the glucose yield from large particles with high E/S can be achieved by using small particles with low E/S. For example, the glucose yield (40.29%) from UC with E/S of 0.08 was achieved (45.83%) by using MC-3-120 with E/S of 0.02. At the same E/S (such as 0.02), the glucose yield (45.83%) from MC-3-120 was greater than 3-folds of that (14.22%) from UC. High production rate of glucose generally is desirable for further fermentation process. However, an economical analysis is required to select the optimum E/S ratio. Several investigators have explored the effect of size reduction on yield of reducing sugar (or glucose) after enzymatic hydrolysis. For example, when the particle was reduced from 74–105 to 38–46 μm, there existed 25% increase in yield of reducing sugars using E/S of 0.002 (Peters, Walker, Wilson, & Irwin, 1991). Gan et al. (2003) reported a 50% increase in the production of reducing sugar with E/S of 0.04 when particle size was reduced from 82 to 38 μm. The size reduction by media mill resulted in high glucose yield and could be an attractive method for pretreating cellulosic materials for enzymatic hydrolysis or fermentation.

4. Conclusions

The particle size of cellulose has been reduced to submicron scale by media milling. In the mean while, the crystallinity was also significantly reduced. In this study, the smallest average particle size (0.78 μm by volume) and the largest specific surface area (25.50 m^2/g) were obtained at low concentration (3%) after being milled for 120 min. At high concentration (7%), the particles were greater than those at low concentration at the same milling time. Media milling generally reduced the crystallinity of cellulose, nevertheless, induced the formation of crystal structure after 60-min milling. The product from high concentration had greater crystallinity than that at low concentration. As the particle size being reduced to submicron scale, the greatest increase (11.8-folds) in the production rate of cellobiose at low substrate concentration (2.5 g/L) was obtained. From the kinetic study, V_{max} was raised about 4 times and K_m was decreased to about fifth when the size of UC (25.52 μm) was reduced to 0.78 μm. The yield of glucose (80-h hydrolysis) was raised from 50% to 90%. The data have demonstrated that the media milling is an attractive method to enhance enzymatic hydrolysis of cellulose. However, media milling is an energy-intensive operation. Thus, detail energy balance is needed to evaluate the feasibility of utilizing media milling for mass production.

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