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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