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Short Communication

Factors affecting cellulose hydrolysis based on inactivation of adsorbed enzymes

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HIGHLIGHTS

- Factors affecting hydrolysis were examined based on an enzyme inactivation model.
- The activation energy for inactivation was within 10% of that for hydrolysis.
- Increasing temperature is effective only to improve the *initial* hydrolysis rate.
- Increasing the surface binding area of substrate can improve the hydrolysis rate.

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ABSTRACT

The rate of enzymatic hydrolysis of cellulose reaction is known to decrease significantly as the reaction proceeds. Factors such as reaction temperature, time, and surface area of substrate that affect cellulose conversion were analyzed relative to their role in a mechanistic model based on first order inactivation of adsorbed cellulases. The activation energies for the hydrolytic step and inactivation step were very close in magnitude: 16.3 kcal mol^{-1} for hydrolysis and 18.0 kcal mol^{-1} for inactivation, respectively. Therefore, increasing reaction temperature would cause a significant increase in the inactivation rate in addition to the catalytic reaction rate. $V_{max,app}$ was only 20% or less of the value at 72 h compared to at 2 h as a result of inactivation of adsorbed cellulases, suggesting prolonged hydrolysis is not an efficient way to improve cellulose hydrolysis. Hydrolysis rate increased with corresponding increases in available substrate surface binding area.

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1. Introduction

Enzymatic conversion of cellulose substrate is slow and presents one of the key bottlenecks that hamper the industrial development of ethanol from biomass. There have been numerous studies that analyzed factors affecting the hydrolysis rate. For example, Ferreira et al. (2009) used Response Surface Methodology to optimize reaction conditions, which was based on empirical models. Some other studies have focused on addition of surfactant or protein such as BSA to improve cellulose hydrolysis (Brethauer et al., 2011; Ouyang et al., 2010). These, however, do not provide insight into mechanistic details of the process. It would be more valuable to investigate how the hydrolysis is affected by intrinsic rate limiting steps, such as inactivation of adsorbed enzyme (Ye and Berson, 2011), or slow dissociation of inactive enzyme from substrate (Cruys-Bagger et al., 2012).

We previously developed a mechanistic kinetic model that accurately accounts for cellulose hydrolysis when considering first order inactivation of adsorbed cellulases (Ye and Berson, 2011). The inactivation as proposed in that model is supported by the demonstration of "traffic jams" of cellobiohydrolase on cellulose strips using AFM imaging (Igarashi et al., 2011). The effects of incubating time, reaction temperature and accessible surface area of substrate on hydrolysis, as they relate to adsorbed cellulase inactivation, are explored here.

2. Methods

2.1. Cellulose substrate and enzyme

Cellulose substrates used in these experiments were: Sigmacell Type 20 from Sigma–Aldrich St Louis, MO; microcrystalline cellulose, from Alfa Aesar Ward Hill, MA; and dewaxed cotton from

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Johnson & Johnson New Brunswick, NJ. All substrates were used to study the effect of surface area on cellulose hydrolysis. Sigmacell was used to study the effect of reaction time and reaction temperature on enzymatic hydrolysis. The substrates were hydrolyzed using Spezyme CP cellulase enzyme from Genencor International, Inc. (Rochester, NY, USA) [lot # 3016295230].

2.2. Cellulose hydrolysis and glucose measurements

Cellulose was hydrolyzed as reported previously (Ye and Berson, 2011), at 150 rpm in 250 mL flasks in an Innova 4230 incubator shaker. The pH of each flask was adjusted to 4.8 with citrate buffer. To prevent bacterial growth, 3 μL/mL of cycloheximide and 4 μL/mL of tetracycline was added to the slurry. The total operating volume of each test was 100 mL. 0.1, 0.2, 0.4, 1.2, and 2 g of cellulose substrate and 0.6 mL of Spezyme CP cellulases (50 FPU (filter paper unit)/mL cellulases activity) were incubated for up to 3 days at three temperatures 20, 35 and 50 °C. Spezyme CP contained sufficient β-glucosidase activity, which can convert 106 μM cellobiose per min using 1 mg protein (Ximenes et al., 2011). Furthermore, substrate concentration was intentionally kept below levels known to cause product inhibition and oligomer sugar accumulation. 1.5 mL samples were removed to determine the glucose concentration at incubation times of 2, 4, 8, 16, 24, 48, and 72 h. Experiments were performed with four samples and duplicate measurements were recorded for each sample at each time point. Released glucose was assayed as reported previously (Ye and Berson, 2011).

2.3. Accessible surface area and pore size measurements

The accessible surface area and pore size of various cellulose substrates were measured by nitrogen gas adsorption and desorption isotherms as reported elsewhere (Choi et al., 2007) using an adsorption apparatus (Micromeritics Instrument Corporation, Tristar 3000). Measurement of accessible surface area by nitrogen gas adsorption has been applied in studies of enzyme adsorption and enzymatic hydrolysis of cellulose by many other researchers (Rahikainen et al., 2011; Lee et al., 2007; Choi et al., 2007). The operating conditions were as follows: sample mass was 0.14–0.21 g; temperature was 77.300 K; equilibration interval was 5 s. The ranges of specific surface area, pore size, and total pore volume for various cellulose substrates were determined from nitrogen adsorption and desorption isotherms, respectively, using the BJH model.

3. Kinetic modeling to determine activation energy

In order to examine effects of temperature on cellulose hydrolysis, activation energies of both the rate limiting step and enzyme inactivation need to be determined. The rate constants used in the Arrhenius equation,

$$\ln(k) = \frac{-E_a}{R}\frac{1}{T} + \ln(A) \tag{1}$$

were determined from the mechanism in Eq. (2). The mechanism represents cellulose hydrolysis as previously reported (Ye and Berson, 2011), and describes enzyme binding to substrate with association and dissociation rate constants k_1 and k_{-1}. Some active enzyme–substrate complexes produce product with an apparent hydrolysis rate, k_2, while others becomes inactive with an inactivation rate constant k_f and reactivation rate constant k_r.

(2)

Using the modeling procedure as previously reported (Ye and Berson, 2011), the rate equation of cellulose hydrolysis can be written as:

$$\frac{dP}{dt} = V_r = k_2 \times (E)_0 \times \frac{(S)}{K_m + (S)} \times \left\{ \frac{k_r}{k_f + k_r} + \frac{k_f}{k_f + k_r} \times \exp[-(k_f + k_r) \times t] \right\} \tag{3}$$

where, V_r is the real hydrolysis rate, k_2 is the rate of breakdown of the enzyme–substrate complex, $(E)_0$ is initial enzyme concentration, (S) is substrate concentration, k_f is the inactivation rate constant for adsorbed enzyme, k_r is the reactivation rate constant, t is reaction time, and K_m, which was derived from the Langmuir adsorption model, is defined in our previous model (Ye and Berson, 2011) as:

$$K_m = \frac{K_d + (E)_0}{A_{\max}} \tag{4}$$

$A_{\max}$ is the maximum adsorption sites per unit substrate (g/g). K_d (g/L) is the equilibrium constant of dissociation, which for a simple binding mechanism is given by k_1/k_{-1}.

Parameters in the model were determined using procedures as reported previously (Ye and Berson, 2011) for 50, 35 and 20 °C.

We compared this model with other models in a previous manuscript (Ye and Berson, 2011). While there are models that correlate enzyme deactivation to reaction temperatures, there is little in the current literature that incorporates activation energy into deactivation modeling. For example, Newman et al. (2013) developed an enzyme deactivation model to account for hydrolysis rate reduction as functions of incubating time and reaction temperatures. However, their model did not include activation energy associated with any step because it is a semi-mechanistic model, compared to our mechanistic model for describing the hydrolysis rate that is based on inactivation of adsorbed enzyme.

4. Results and discussion

4.1. Calculation of rate constants for determining activation energy

The Sigmacell substrate was hydrolyzed at three different temperatures 50, 35 and 20 °C. Rate constants were regressed from the experimental product–time (P–t) curves in Fig. 1 using the procedure reported in our previous modeling (Ye and Berson, 2011). The constants are listed in Table 1. Theoretically predicted (P–t) curves were generated from rate Eq. (3) using the parameters in Table 1, and are compared to the experimental results in Fig. 1. The theoretically predicted results were generally within one standard deviation of experimental results, implying that the theoretical modeling, with the determined parameters, can accurately account for hydrolysis at the three temperatures.

Activation energies for hydrolysis (k_2) and inactivation (k_f) were determined using Arrhenius plots (Fig. 2a and b). Activation energy is equal to (−slope × R), where R (1.985 cal K^{-1} mol^{-1}) is the gas constant. The activation energies for the k_2 and k_f steps were determined to be 16.3 and 18.0 kcal K^{-1} mol^{-1}, respectively. The activation energy for the hydrolysis step (k_2) is within the typical range of 4–20 kcal mol^{-1} for enzymatic reactions (Shuler and Kargi, 1992). The activation energy for the inactivation step (k_f) is close to that of the hydrolysis step (k_2), implying that increasing the reaction temperature may cause a corresponding increase in the inactivation rate. This is not unexpected; if jamming of cellobiohydrolases is the main reason for inactivation, the faster moving rate of cellobiohydrolases will correlate with higher frequency of inactivation.

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