Kinetic Modeling for Enzymatic Hydrolysis of Pretreated Creeping Wild Ryegrass

Yi Zheng, Zhongli Pan, Ruihong Zhang, Bryan M. Jenkins

ABSTRACT: A semimechanistic multi-reaction kinetic model was developed to describe the enzymatic hydrolysis of a lignocellulosic biomass, creeping wild ryegrass (CWR; Leymus triticoides). This model incorporated one homogeneous reaction of cellobiose-to-glucose and two heterogeneous reactions of cellulose-to-cellobiose and cellulose-to-glucose. Adsorption of cellulase onto pretreated CWR during enzymatic hydrolysis was modeled via a Langmuir adsorption isotherm. This is the first kinetic model which incorporated the negative role of lignin (nonproductive adsorption) using a Langmuir-type isotherm adsorption of cellulase onto lignin. The model also reflected the competitive inhibitions of cellulase by glucose and cellobiose. The Matlab optimization function of “lsqnonlin” was used to fit the model and estimate kinetic parameters based on experimental data generated under typical conditions (8% solid loading and 15 FPU/g-cellulose enzyme concentration without the addition of background sugars). The model showed high fidelity for predicting cellulose hydrolysis behavior over a broad range of solid loading (4–12%, w/w, dry basis), enzyme concentration (15–150 FPU/g-cellulose), sugar inhibition (glucose of 30 and 60 mg/mL and cellobiose of 10 mg/mL). In addition, sensitivity analysis showed that the incorporation of the nonproductive adsorption of cellulase onto lignin significantly improved the predictability of the kinetic model. Our model can serve as a robust tool for developing kinetic models for system optimization of enzymatic hydrolysis, hydrolysis reactor design, and/or other hydrolysis systems with different type of enzymes and substrates.

Introduction

Enzymatic hydrolysis of cellulose has advantages over chemical conversion techniques for its high sugar yield, low energy consumption, mild operating conditions (low temperature and low pressure), low cost of reactors, and minimal byproduct formation (Sun and Cheng, 2005; Wald et al., 1984). At present, the ethanol derived from lignocellulosic biomass with enzyme hydrolysis is more expensive than that produced with established techniques such as concentrated acid and two-stage dilute acid hydrolysis; however, it has potential in cost reductions in the future (Lynd et al., 1991; Ragauskas et al., 2006; Wyman, 2003).

The enzymatic hydrolysis of lignocellulosic biomass is still a critical cost center in the overall biocconversion process. Since generation and analysis of experimental data are labor-intensive, time-consuming, and analytically challenging (Kadam et al., 2004), it is usually difficult and may not be possible to conduct experiments to obtain experimental data for optimization and economic evaluation of the enzymatic hydrolysis process. A kinetic model of enzymatic hydrolysis could be a useful forecasting tool in this context.

Although many factors such as substrate structure morphology and structure (accessible area, crystallinity, degree of polymerization, lignin concentration and distribution, etc.) and cellulase system complex (multiple enzyme activities, synergism, adsorption, inhibition, etc.) are important factors to develop a powerful kinetic model to accurately predict the enzymatic hydrolysis behavior, it is hard even impossible to do so due to the difficulties in precisely measuring parameters such as crystallinity and degree of polymerization of lignocellulosic biomass. Therefore, a kinetic model based on observable, macroscopic properties of the overall system could be sufficiently useful in system design and economic analysis of processes for fermentable sugars and/or ethanol production. In previous efforts, parameters of substrate’s structural features and cellulase system complex had ever been incorporated into several models; however, the effect of lignin, one of the
primary components of the cell wall, was omitted in most models. Lignin has been implicated as a competitive adsorbent for hydrolyze cellulose by reducing the amount of cellulase available to hydrolyze cellulose (Bernardez et al., 1993; Berlin et al., 2005; Kristensen et al., 2007; Ooshima et al., 1990; Sutcliffe and Saddler, 1986; Yang and Wyman, 2006; Zheng et al., 2008). It has been proposed that lignin residue blocks the progress of cellulase in breaking down the cellulose chain (Eriksson et al., 2002; Mansfield et al., 1999). Additionally, the removal and/or redistribution of lignin were thought to have a significant effect on the rate of enzymatic hydrolysis (Cherboglazov et al., 1988; Converse, 1993; Lynd et al., 2002; Yang and Wyman, 2004). We believe that this research is the first to incorporate the negative role of lignin in the kinetic model for the enzyme hydrolysis of lignocellulose.

**Review of Mathematical Modeling of Enzymatic Hydrolysis**

Zhang and Lynd (2004) reviewed available models for enzymatic hydrolysis. Most models were developed based on the enzymatic hydrolysis reaction scheme shown in Figure 1, in which hydrolysis reactions of r1, r2, and/or r3 were involved. Some researchers thought r2 could be ignored (Philippidis et al., 1992). Based on structural features and functions, mathematical models were classified into four categories: (1) nonmechanistic model; (2) semimechanistic model; (3) functionally based model; (4) structurally based model. The detailed features, utilities, and limitations of various types of models were also summarized by Zhang and Lynd (2004). According to current knowledge of enzymatic hydrolysis and related analytical techniques, it is rather difficult even impossible to accurately determine some of substrate structural features and/or enzyme activities. Although both functionally and structurally based models can help deepen the understanding at the level of substrate structure and multiple enzyme activities, they can provide little information for practical applications such as system design and optimization. Therefore, just a few functionally and structurally based models had been proposed (Converse and Optekar, 1993; Fenske et al., 1999; Okazaki and Moo-Young, 1978; Suga et al., 1975; Zhang and Lynd, 2006).

Most of the proposed hydrolysis models for the design of industrial systems fall into the category of semimechanistic model. Semimechanistic models are very useful where minimal structural information is necessary for descriptive purposes, especially reactor design, although they may not yield much insight into the detailed effect of substrate features (surface area, crystallinity, degree of polymerization, etc.) and multiple enzyme activities on enzymatic hydrolysis (Zhang and Lynd, 2004). The utility in design also makes the semimechanistic model attractive for assessing system performance and optimization. Therefore, the literature review in this paper and our modeling research focused on the semimechanistic model.

The most relevant modeling efforts in reported research were summarized below. As the first attempt to develop a kinetic model using rice straw as a substrate, Wald et al. (1984) presented their kinetic model by involving two substrate systems and a multi-enzyme system. Two substrate systems included a shrinking cellulose sphere with an amorphous shell (easily hydrolyzed by enzymes) and a shrinking crystalline core (more resistant to enzymatic hydrolysis). A multi-enzyme system was quantitatively represented by two activity measurements such as filter paper and cellobiose activities. The enzyme adsorption and product inhibition were also considered. The substrate surface properties were included in this model to address the enzyme adsorption, which was expressed as a function of available sorption sites and of accessible surface area via the Langmuir adsorption isotherm. This model was capable of simulating enzymatic saccharification of rice straw at substrate and enzyme concentrations up to 333 g/L and 9.2 FPU/mL, respectively. In earlier works, enzyme adsorption was largely ignored by researchers in developing their kinetic models (Gusakov et al., 1985a,b), who generally used Michaelis-Menten (M-M) kinetics to describe enzymatic hydrolysis of cellulose. The proposed model satisfactorily predicted glucose and cellobiose accumulation in a batch reactor up to 70--80% substrate conversion in terms of substrate concentration from 5 to 100 g/L and enzyme concentration from 5 to 60 g/L. The incorporation of the M-M kinetics into kinetic models may be applicable when substrate concentration is not close to maximum loading.

Philippidis et al. (1993) developed a kinetic model to simulate enzymatic hydrolysis of pure cellulose. In this model, values of kinetic parameters were determined by nonlinear regressions through the collected data. Because

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**Figure 1.** Reaction scheme for modeling cellulose hydrolysis. [Enzymes in r1 include endo-β-1,4-glucanase (EG) and exo-β-1,4-cellobiohydrolase (CBH). Enzymes in r2 include CBH and exo-β-1,4-glucanase. And an enzyme involved in r3 is β-glucosidase. Solid arrows represent the reaction routes and the dashed arrows show the inhibition of sugars on corresponding enzymes.] Modified from Kadam et al. (2004).

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this model did not consider the enzyme adsorption, enzyme inactivation, or substrate complexity, modeling results were only fairly good for the first 24 h of enzymatic hydrolysis. Cellobiose and cellulose concentrations were overestimated and underestimated, respectively, after 24 h. The most recent kinetic model of enzymatic hydrolysis of lignocellulosic biomass was proposed by Kadam et al. (2004), who developed and validated a kinetic model for batch enzymatic hydrolysis of dilute acid pretreated corn stover in shake flasks and stirred-tank reactors. This model differs from previous models because it incorporated xylose inhibition in addition to glucose and cellobiose inhibitions, and the effects of temperature and substrate reactivity (SR) were also considered in this model. This model could potentially be useful for predicting the hydrolysis performance of cellulase, which has different levels of β-glucosidase supplementation. It also predicted the inhibition of sugars well at certain initial glucose (≤50 g/kg), xylose (≤40 g/kg), and cellobiose (≤30 g/kg) concentrations.

Simultaneous saccharification fermentation (SSF) is believed to be currently the most effective configuration for cellulosic ethanol production and enzymatic saccharification is one of the most critical processes within the SSF; therefore, the enzymatic saccharification model was further advanced into the SSF process (Ljunggren, 2005; Philippidis and Hatzis, 1997; Philippidis et al., 1992; South et al., 1995). The model developed by South et al. (1995) was applied to batch and continuous reactor systems to simulate the SSF for ethanol production from lignocellulosic substrates. It was reported to be successful over a fourfold range of enzyme concentration and a 12.8-fold range of substrate concentration. In the SSF-based models developed by Philippidis et al. (1992) and Philippidis and Hatzis (1997), the enzyme–substrate adsorption equilibriums were not included and the cellobiose-to-glucose conversion was assumed to be independent of enzyme concentration and activity. The time-dependent decline in the rate of cellulose hydrolysis was thought to be the result of reduced enzyme mobility. The decline rate was described by an exponential decay term and was considered proportional to the specific effective surface area of cellulose rather than the time. Such assumptions on the decline rate made the model less general. Caminal et al. (1985) and Sadana (1992) modeled the enzyme inactivation as a first-order kinetic with an exponential decay. They believed that enzyme inactivation was another reason causing the decline of cellulose hydrolysis rate. The inactivation was related to the duration of enzyme exposure to the temperature and stirring. Recently, Ljunggren (2005) improved the model by incorporating a substrate dependent parameter, $C_{dp}$ (crystallinity and degree of polymerization) accounting for different substrate susceptibilities to enzymatic hydrolysis. The simulation results of this model showed that modeling of enzymatic hydrolysis and SSF processes could be performed successfully using a rather complex model for the enzymatic hydrolysis process and a simple model for the fermentation process.

Most previous models can predict the enzymatic hydrolysis and/or SSF process under certain conditions. However, they ignored naturally important nonproductive adsorptions of enzyme onto lignin so that the predictability of these models was limited. In our study, the negative effect of lignin on enzymatic hydrolysis was included in the kinetic model in terms of enzymatic adsorption, and the developed model was validated over a broader range of solid loading, enzyme concentration, and background sugar. All conditions used for model validation were beyond the typical conditions which were employed to estimate the kinetic parameters. Sensitivity analysis was conducted by including and excluding the lignin adsorption for demonstrating the importance of lignin adsorption in the kinetic model.

Objectives

This research was aimed at developing a semimechanistic kinetic model that is sophisticated enough to describe the complexities of the enzymatic hydrolysis of lignocellulosic substrate without requiring extensive structural analysis. The specific objectives of this research were to, (1) develop a kinetic model that incorporated SR, enzyme adsorption, end-product inhibition, and lignin’s nonproductive adsorption of enzymes to predict performance of the enzymatic hydrolysis of a model lignocellulosic biomass, dilute acid pretreated creeping wild ryegrass (CWR); (2) estimate kinetic parameters based on the results obtained under typical experimental conditions; (3) validate performance of the developed kinetic model over various substrate, enzyme, and background sugar (glucose and cellobiose) concentrations beyond typical experimental conditions; and (4) analyze the sensitivity of lignin’s nonproductive adsorption in the developed kinetic model.

Model Description

The model was developed based on the biochemistry of enzymatic hydrolysis. It incorporated end-product inhibition, SR (transformation), and adsorption of cellulase components onto and desorption from substrates. Kinetic parameters were estimated based on macroscopic observable phenomena.

Enzyme Adsorption

Most of the earlier kinetic models were based on the M-M enzyme kinetics. However, the M-M kinetics is only valid when the substrate is present in excess relative to the enzyme (Lynd et al., 2002). Models based on a Langmuir adsorption did not implicitly assume excess in either enzyme or substrate, and thus have a considerably broader range of potential application than M-M based models. The
Langmuir adsorption has been used for describing enzyme adsorption in the heterogeneous cellulose hydrolysis system. It should be noted that the underlying assumptions for the Langmuir adsorption, including uniform binding sites and no interaction between the adsorbing molecules, are not necessarily valid for cellulase adsorption onto lignocellulosic substrates (Kadam et al., 2004). The Langmuir formulation remains useful for mathematically reflecting the phenomenon of enzyme adsorption. It has been used in this study to describe the adsorption behavior of cellulase. This consideration will make our model more general than previous models in which nonproductive adsorption of lignin was not included.

End-Product Inhibition

It is well known that cellulolytic enzymes are inhibited by cellobiose and glucose. Most of the time, the xylose inhibition can be omitted due to trace xylose in the completely washed dilute acid pretreated lignocellulosic biomass. The patterns of enzyme inhibition by glucose and cellobiose have been the subject of research for a long time, with many different views on the nature of these inhibitions. Gusakov and Sinitsyn (1992), Holtzapple et al. (1990), and Zhang and Lynd (2004) offered reviews about the inhibition of various microorganism cellulases by both glucose and cellobiose. It was suggested that competitive inhibition is dominant (Atkinson and Mavituna, 1991; Ghose and Das, 1971; Gregg and Saddler, 1996; Kadam et al., 2004). Competitive inhibition is the most common mechanism in the literature, although other uncompetitive and noncompetitive mechanisms have been proposed. In this study, the competitive inhibition pattern was adopted to represent all the inhibitions of glucose and cellobiose on cellulase.

Substrate Reactivity

SR is a general concept to represent substrate structural features, including variations in crystallinity, degree of polymerization, substrate accessibility, etc. As reviewed by Zheng (2007), two contradictory conclusions were made to address the effect of SR on the nonlinearity of enzymatic hydrolysis of lignocellulosic substrate. Some researchers thought the SR is an important factor affecting the enzymatic hydrolysis of cellulose substrate (Desai and Converse, 1997; Gama et al., 1994; Klyosov, 1990; Lynd et al., 2002; Weimer et al., 1991). They found cellulose hydrolysis rate mediated by fungal cellulase and ruminal bacteria were typically 3–30 times faster for amorphous cellulose as compared to crystalline cellulose. However, several other researchers obtained equivocal results or found no substantial changes in crystallinity as the saccharification progresses (Lenz et al., 1990; Ohmine et al., 1983; Puls and Wood, 1991). Normally, it is rather difficult to measure the lignocellulosic substrate structural features based on current knowledge (Zhang and Lynd, 2004). Also, it is not necessary to distinguish the distinct enzymes adsorbed onto amorphous or crystalline cellulose regions for developing an effective kinetic model. Thus, the change of intrinsic SR may be a more practical way to model the “biphasic behavior than resorting to an assumed dichotomy between amorphous and crystalline regions” (Kadam et al., 2004). Therefore, all the transformations of substrate during the enzymatic hydrolysis were lumped into one parameter, SR, which was the normalized initial hydrolysis rate during the secondary hydrolysis of residual substrates (see the Experimental Design Section) and correlated with the normalized substrate concentration ($S/S_0$) as Equation (1) (Kadam et al., 2004)

$$SR = \alpha \frac{S}{S_0} \quad (1)$$

Proposed Kinetic Model

As shown in Figure 1, simplified enzymatic hydrolysis of cellulose into glucose consists of three distinct reactions, including $r_1$, $r_2$, and $r_3$. These reactions form the basis of our kinetic model. Each enzymatic reaction is potentially inhibited by the product sugars (glucose and/or cellobiose) and/or reactant sugar (cellobiose). Based on the sugar compounds detected in our enzymatic hydrolyzates, only glucose and cellobiose inhibitions were considered in our model.

Based on the catalytic characteristics of enzymes, the cellulose-adsorbed cellulolytic enzymes were considered to be effective catalytic enzymes for hydrolyzing cellulose into glucose and cellobiose, and the free β-glucosidase is effective to convert cellobiose-to-glucose. Enzyme adsorptions were expressed by Langmuir isothermal equations.

The assumptions for rate equation derivation include, (1) enzyme adsorption follows a Langmuir isothermal adsorption with the first-order reactions ($r_1$ and $r_2$) occurring on the cellulose surface; (2) cellulose matrix is uniform in terms of its susceptibility to enzymatic attack (i.e., no distinction between amorphous and crystalline cellulose fractions); (3) little enzyme inactivation by thermal and mechanical effects; (4) conversion of cellobiose into glucose occurs by homogeneous catalysis in solution and follows the classical M-M kinetics; (5) the ratio of the lignin content exposed to enzyme to the total amount of lignin content contained in the pretreated CWR, $\lambda$, is equal to 1, that is, cellulose does not block the adsorption of enzymes onto lignin (Ooshima et al., 1990); and (6) β-glucosidase adsorbs onto lignin but not cellulose.

Based on these assumptions, the following six equations were derived, including three enzyme adsorption equations (Eqs. 2–4) and three reaction rate equations (Eqs. 5–7).
Enzyme Adsorption Equations

(1) The Langmuir isotherm for endoglucanase/cellobiohydrolase (EG/CBH) adsorption onto the pretreated CWR which contains cellulose and lignin

\[ \frac{E_{ib}}{S} = \frac{E_{imax}K_{lad}E_{if}}{1 + K_{lad}E_{if}} \]  

(2) Since EG/CBH was proved to be adsorbed not only on cellulose, but also on lignin, and \( \beta \)-glucosidase is adsorbed only to lignin (Zheng, 2007), the Langmuir adsorption of EG/CBH and \( \beta \)-glucosidase onto lignin was expressed

\[ E_{ibl} = \frac{E_{imax}K_{lad}E_{ifl}}{1 + K_{lad}E_{ifl}} \]  

\( i = 1, \) EG/CBH; \( i = 2, \) \( \beta \)-glucosidase

(3) Due to the adsorption of EG/CBH on both cellulose and lignin, the amount of EG/CBH adsorbed on cellulose, \( E_{ibc} \), was calculated using Equation (4)

\[ E_{ibc} = E_{ib} - E_{ibl} \times \lambda \]  

where, \( \lambda \) was assumed to be equal to 1 according to the assumption no. 5 proposed above.

The reactions in the model scheme shown in Figure 1 proceed at the rates described by Equations (5)–(7):

(1) Cellulose-to-cellobiose reaction with competitive glucose and cellobiose inhibition

\[ r_1 = \frac{k_{1r} \times E_{ibc} \times SR \times C}{1 + (G_2/K_{1G2}) + (G/K_{1G})} \]  

(2) Cellulose-to-glucose reaction with competitive glucose and cellobiose inhibition

\[ r_2 = \frac{k_{2r} \times E_{ibc} \times SR \times C}{1 + (G_2/K_{2G2}) + (G/K_{2G})} \]  

(3) Cellobiose-to-glucose reaction with competitive glucose inhibition

\[ r_3 = \frac{k_{3r} \times E_{ibl} \times G_2}{K_{3M}[1 + (G/K_{3G})]} + G_2 \]  

The numerators in equations for \( r_1, r_2, \) and \( r_3 \) included enzyme concentrations corrected by the lignin’s nonproductive adsorption. The substrate concentrations in the numerators in the equations of \( r_1 \) and \( r_2 \) were corrected by the transformation of substrate during enzymatic hydrolysis using SR. The denominators in the equations for \( r_1, r_2, \) and \( r_3 \) expressed competitive inhibitions of enzymes by end-products.

Mass balances of substrate (cellulose, products (glucose and cellobiose), and enzymes (EG/CBH and \( \beta \)-glucosidase) were established as follows:

Cellulose

\[ \frac{dC}{dt} = -r_1 - r_2 \]  

Glucose

\[ \frac{dG}{dt} = 1.1116r_2 + 1.053r_3 \]  

Cellulose

\[ E_{1T} = E_{1H} + E_{ib} \]  

\( \beta \)-Glucosidase

\[ E_{2T} = E_{2H} + E_{2b} \]  

Estimation of Kinetic Parameters in the Reaction Rate Equations

The proposed kinetic model was solved using the Matlab programming environment (Matlab 2007a, the MathWorks, Natick, MA). The Matlab optimization function “lsqnonlin” was used to simultaneously estimate various kinetic parameters. The parameters characterizing the Langmuir adsorption and SR correlations were determined independently by Zheng (2007) (see the Experimental Design Section) and used to serve as inputs.

Materials and Methods

Preparations of Pretreated CWR and Enzymes

Raw CWR was initially pretreated with dilute sulfuric acid (1.4%, w/w) in a 1-L reactor (Carpenter 20 Cb-3, Parr, Co., Moline, IL) at 165°C for 8 min. The pretreated CWR slurry was washed to remove the solubilized contents, and then the solid fraction was used in this study as a model substrate which was composed of 53% (w/w, dry basis) cellulose and 38% lignin (w/w, dry basis) contents. The details of pretreatment processes were described by Zheng et al. (2007, 2008). The enzymes used for hydrolysis included cellulase (NOVO Celluclast 1.5L, available from Sigma, Cat. No. C2730) and \( \beta \)-glucosidase (Novo188, available from Sigma, Cat. No. C6105). They were provided by Novozymes, Inc. (Davis, CA) as gifts. Cellulase and \( \beta \)-glucosidase had respective activities of 90 FPU/mL and 490 CBU/mL, corresponding to 54 and 65 mg protein/mL, respectively.
Experimental Design

The overall procedures followed in this study included: (1) running independent experiments to determine the parameters of Langmuir-type enzyme adsorption and substrate activity; (2) obtaining experimental data of enzymatic hydrolysis under typical conditions; (3) fitting kinetic parameters using the hydrolysis experimental data and parameters obtained from step (1); and (4) validating the model’s predictability over a broader range of hydrolysis conditions beyond typical experimental conditions, which were employed for kinetic parameter estimation; (5) performing sensitivity analysis on the lignin’s nonproductive adsorption adopted in our model. Table I shows the detailed experimental conditions for steps (2–5).

Determination of Langmuir Adsorption Constants

To determine Langmuir adsorption constants, a series of enzyme adsorption experiments were conducted. Eight percent (w/w, dry basis) pretreated CWR or 3% (w/w, dry basis) lignin (prepared by complete enzymatic hydrolysis of pretreated CWR with excessive enzyme concentration) was mixed with the enzyme solution which contained a series of different enzyme concentrations. The mixture was buffered to pH 4.8 and incubated for 1 h (for pretreated CWR) or 5 h (for lignin) at 50°C with gentle shaking. Since cellulase activity is proportional to the protein concentration in the reaction solution, enzymatic hydrolysis rate can be correlated with the amount of absorbed enzyme (Ooshima et al., 1990). Therefore, free enzyme was measured as the protein concentration in the supernatant using the Bradford assay using Coomassie blue dye (Bio-Rad, Hercules, CA). Adsorbed enzyme was calculated by subtracting the free enzyme concentration from the initial enzyme concentration loaded to each reactor. In order to calculate the amount of enzyme adsorbed per unit amount of remaining solid, the dry weight of the solid residue was measured. Finally, bound enzyme concentration was correlated with free enzyme concentration using the Langmuir equilibrium isotherm and nonlinear regression to obtain Langmuir adsorption constants, which were shown in Table II.

Determination of the Substrate Reactivity

The SR was researched using washed pretreated CWR and Cellucalst 1.5 L cellulase. During enzymatic hydrolysis, replicate reactors were harvested at each time point (30 min, 1, 4, 8, 24, 48, 72, and 168 h after hydrolysis), and the residual substrate was thoroughly washed with deionized distilled water to remove the end-product inhibition and enzymes to the maximum extent possible. The washed residual substrate was then subjected to a secondary enzymatic hydrolysis for additional 1 h with the addition of fresh enzyme at a constant enzyme/substrate ratio. The cellulose content in the washed residual solid was estimated from net glucose and cellobiose produced during the previous hydrolysis at a given time. The normalized initial hydrolysis rate during the secondary enzymatic hydrolysis was calculated as the ratio of the initial hydrolysis rate of the secondary enzymatic hydrolysis and the original enzymatic hydrolysis. It was correlated with the normalized substrate concentration \( \frac{S}{S_0} \) by linear regression (data not shown). The slope, \( \alpha \) was determined to be 1.007 with a correlation coefficient, \( r^2 = 0.9884 \) (Table II) (Zheng, 2007).

Typical Experiments for Kinetic Parameter Estimation

The experimental data for kinetic parameter estimation were generated under typical enzymatic hydrolysis conditions, that is, total working weight of 50 g [8% solid (w/w, dry basis) corresponding to 4% (w/w, dry basis) cellulose] charged to 125-mL flasks; enzyme concentration of 15 FPU/g-cellulose supplemented with extra \( \beta \)-glucosidase of 15 CBU/g-cellulose, equivalent to about 9 and 2 mg protein/g cellulose; incubation temperature \( = 50°C \); shaking speed \( = 150 \text{ rpm} \); initial pH \( = 5.0 \); total incubation time \( = 168 \text{ h} \); and temperature, pH, and hydrolysis time were kept constants at 50°C, 5.0, and 168 h, respectively for all the experiments.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Typical experimental conditions for kinetic parameter estimation</th>
<th>Enzyme concentration (FPU/g-cellulose)</th>
<th>15</th>
<th>5, 15, 30, 60, 100, and 150</th>
<th>Solid loading (% solid, dry basis)</th>
<th>8</th>
<th>8</th>
<th>4, 6, 8, 10, and 12</th>
<th>Background glucose (mg/mL)</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>30 and 60</th>
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<tr>
<td>Enzyme concentration</td>
<td></td>
<td></td>
<td>15</td>
<td>15</td>
<td>Solid loading (w/w%, dry basis)</td>
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<td>4, 6, 8, 10, and 12</td>
<td>Background glucose (mg/mL)</td>
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<td>30 and 60</td>
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<tr>
<td>Solid loading</td>
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<td>Background cellulose (mg/mL)</td>
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</table>

*Temperature, pH, and hydrolysis time were kept constants at 50°C, 5.0, and 168 h, respectively for all the experiments.
and sampling time = 0, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, and 168 h after hydrolysis. The glucose and cellobiose concentrations in supernatant of liquid samples were analyzed on a high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu). The protein concentration in the solution was measured using the Bradford protein assay with BSA as a standard (Bio-Rad).

### Experiments for Model Validation

Experiments for model validation were conducted under conditions outside the range of the typical conditions with different enzyme concentrations [5–150 (FPU + CBU)/g-cellulose], dry solid loadings (4–12%, w/w, dry basis) and background sugar concentrations. For validation of the effect of background sugars, both glucose (30 and 60 mg/mL) and cellobiose (10 mg/mL) were used as background sugars. The kinetic concentration in the solution was measured using the Bradford protein assay with BSA as a standard (Bio-Rad).

### Sensitivity Analysis on the Lignin’s Nonproductive Adsorption

The sensitivity of the lignin’s nonproductive adsorption was analyzed by comparisons between the experimental and the predicted results with and without lignin adsorption under typical conditions. The predicted results were obtained by fitting the kinetic parameters based on the experimental data with and without the lignin’s nonproductive adsorption. When the lignin’s adsorption was ignored, the $E_{1bC}$ in Equations (4)–(6) and the $E_{2fL}$ in Equation (7) were changed to $E_{1b}$ and $E_{2f}$ (i.e., $E_{2fL}=E_{2T}$ and $E_{2bL}=0$), respectively.

### Results and Discussion

#### Kinetic Parameters of the Reaction Rates

The kinetic parameters in reaction rate equations were derived via Matlab best-fit regression of hydrolysis data obtained under typical hydrolysis conditions (Table III). The predicted results of enzymatic hydrolysis of pretreated CWR under typical conditions are shown in Figure 2. Overall, the proposed model predicted the enzymatic hydrolysis behavior with reasonable accuracy. Comparisons were attempted between our kinetic parameters and those reported in previous publications (Kadam et al., 2004; Philippidis et al., 1993). Kadam et al. (2004) accounted xylene inhibition, but did not consider the lignin’s nonproductive adsorption into their model. Their kinetic parameters were close to ours, although the experimental conditions and model schemes were different from each other. The glucose inhibitory inhibition they considered might be balanced by the lignin’s nonproductive adsorption in our model. Philippidis et al. (1993) ran independent experiments using pure cellulose (Sigma α-cellulose) and T. reesei enzyme from Genencor to obtain their kinetic parameters, including $K_{1T} = 5.85$ mg/mL, $K_{1G} = 53.16$ mg/mL, $K_{2G} = 0.62$ mg/mL, and $k_{2G} = 10.56$ mg/mL. The lower inhibition they found for reaction $r_1$ might be balanced by the higher inhibition they reported for reaction $r_3$. Philippidis et al. (1993) did not model the direct conversion of cellulose-to-glucose, which could be one of the reasons for the difference between their results and ours. However, their conclusions about the strength of inhibition of sugars on enzymes were similar. These findings indicate that different reaction schemes and model structures make direct comparisons of different models difficult. The difficulties are primarily caused by the unclear mechanism of enzymatic hydrolysis of cellulose by the complicated cellulase system. Also, the comparisons are further confounded by different researchers using different experimental and operation conditions, including enzyme mixtures, loading range of enzyme and substrate, hydrolysis temperature, reactor system, etc.

### Table II. Parameters of the Langmuir adsorption and substrate reactivity.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>$E_{1\text{max}}$ (mg protein/g substrate)</td>
<td>42.55</td>
</tr>
<tr>
<td>$E_{2\text{max}}$ (mg protein/g substrate)</td>
<td>86.07</td>
</tr>
<tr>
<td>$E_{3\text{max}}$ (mg protein/g substrate)</td>
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<tr>
<td>$K_{1\text{ad}}$ (mL/mg protein)</td>
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<tr>
<td>$K_{2\text{ad}}$ (mL/mg protein)</td>
<td>0.51</td>
</tr>
<tr>
<td>$K_{3\text{ad}}$ (mL/mg protein)</td>
<td>0.75</td>
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<tr>
<td>SR (dimensionless) = $a/S_0$</td>
<td>$\alpha = 1.007$</td>
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</table>

### Table III. Estimated kinetic parameters of the reaction rate equations.

<table>
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<tr>
<th>Parameters obtained by regression of saccharification data</th>
<th>Values obtained in this study</th>
<th>Kadam et al. (2004)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1T}$ (mL/mg h)</td>
<td>16.5 (mL/mg h)</td>
<td>22.3 (g/mg h)</td>
</tr>
<tr>
<td>$K_{1G}$ (mg/mL)</td>
<td>0.04 (mg/mL)</td>
<td>0.02 (g/kg)</td>
</tr>
<tr>
<td>$K_{1G}$ (mg/mL)</td>
<td>0.1 (mg/mL)</td>
<td>0.1 (g/kg)</td>
</tr>
<tr>
<td>$K_{2G}$ (mg/mL)</td>
<td>7.1 (mg/mL)</td>
<td>7.2 (g/mg h)</td>
</tr>
<tr>
<td>$K_{2G}$ (mg/mL)</td>
<td>132.5 (mg/mL)</td>
<td>132.0 (g/kg)</td>
</tr>
<tr>
<td>$K_{2G}$ (mg/mL)</td>
<td>0.01 (mg/mL)</td>
<td>0.04 (g/kg)</td>
</tr>
<tr>
<td>$K_{3T}$ (mg/mL)</td>
<td>267.6 (h$^{-1}$)</td>
<td>285.5 (h$^{-1}$)</td>
</tr>
<tr>
<td>$K_{3G}$ (mg/mL)</td>
<td>25.5 (mg/mL)</td>
<td>24.3 (g/kg)</td>
</tr>
<tr>
<td>$K_{3G}$ (mg/mL)</td>
<td>2.1 (mg/mL)</td>
<td>3.9 (g/kg)</td>
</tr>
</tbody>
</table>

*The density of pretreated CWR slurry is approximately 1. Therefore, the data units of Kadam et al. (2004) are exchangeable with ours.
Model Validation

Although the model was shown to have high fidelity, it needs to be further evaluated under a wider range of experimental conditions beyond the typical conditions. Model validations were conducted at various enzyme concentrations, substrate loadings and background glucose and cellobiose and reported in the next section.

Validation of Enzyme Concentration

The predicted cellobiose concentration was more accurate than the predicted glucose and cellulose concentrations, when the enzyme concentrations were 5 FPU and 5 CBU/g-cellulose (Fig. 3). The model can closely simulate the glucose and cellulose concentrations during the first 24 h of enzymatic hydrolysis. However, the glucose and cellulose concentrations were overestimated and underestimated, respectively, after 24 h. There are three possible reasons for such deviations. The first reason could be that the enzyme concentration was too low to compensate the enzyme activity loss due to the mechanic mixing and heat inactivation, which were ignored in our model; the second reason might be that the degree of enzyme inactivation by lignin was underestimated; the third possible reason could be that the effect of end-product (glucose and cellobiose) inhibition was underestimated, especially when the enzyme concentrations were lower than 15 FPU and 15 CBU/g-cellulose. All these three reasons would lead to overestimating the available enzyme activity during hydrolysis. Therefore, the enzyme inactivation was more significant for low enzyme concentration than high enzyme concentration.

When enzyme concentrations between 15 FPU and 15 CBU/g-cellulose and 150 FPU and 150 CBU/g-cellulose were used, the model can accurately capture the trends of glucose, cellobiose, and cellulose concentrations during enzymatic hydrolysis over 168 h (the predicted results for the enzyme concentrations of 30, 60, and 100 FPU/CBU were not shown) (Fig. 4). The predictability of the model was much better for high enzyme concentration than low enzyme concentration. Since the enzyme concentration over 15 FPU and 15 CBU/g-cellulose could be high enough to compensate the enzyme activity loss due to the inactivation and/or end-product inhibitions, the fidelity of this model was increased. Therefore, the model could be recommended to be used for predicting the enzymatic hydrolysis of pretreated CWR with enzyme concentrations ranging from 15 FPU and 15 CBU/g-cellulose to 150 FPU and 150 CBU/g-cellulose. Enzyme concentrations higher than 15 FPU/g-cellulose might not be economically practical; however, the simulation results for high enzyme concentration can
show how good the model’s predictability is over a broad enzyme concentration range.

Validation of Substrate Loading

Experiments were conducted to investigate the effect of substrate loading on the enzymatic hydrolysis of pretreated CWR. The experimental data for glucose concentration were used to examine the validity of the kinetic model. The cellobiose and cellulose concentrations were not shown. The simulation results are shown in Figure 5, which indicates that the model performed very well when the substrate loading increased from 4 to 12% (w/w, dry basis). It was also found that the predictability of the model for lower substrate loading is a little better than that for high substrate loading. The relationship between substrate loading and inhibition could be one reason. Additional reason could be the limitation of heat and mass transfers due to the high solid loading. Exact reasons need to be further studied.

Validation of Sugar Inhibition

Since sugar inhibition is a critical negative factor to suppress the activity of enzyme, the model’s predictability was also assessed by examining if the model can correctly reflect sugar inhibition. Enzyme inhibition by each sugar is inversely proportional to its respective inhibition constant. The inhibition of reaction $r_1$ by cellobiose could be stronger than that by glucose because cellobiose is the reaction product of $r_1$. Conversely, the inhibition of reaction $r_2$ by glucose is more potent than that by cellobiose because glucose is the direct reaction product from cellulose. The comparisons between inhibition constants are consistent with these conclusions, that is, $K_{1G} \gg K_{1G2}$ and $K_{2G} \gg K_{2G2}$ (Table III).

In order to investigate the validity of the model for describing the cellobiose inhibition, 10 mg/mL cellobiose was charged into the hydrolysis reactor before the addition of hydrolytic enzymes. In our hydrolysis system, the cellobiose concentration typically peaks at low level from 2 to 10 mg/mL depending on the β-glucosidase concentration. In this study, the highest cellobiose concentration of 10 mg/mL was used for model validation. As shown in Figure 6, the cellobiose concentration decreased dramatically during the first 4 h of hydrolysis, which could indicated that the cellobiose derived from cellulose (reaction $r_1$) was inhibited by background cellobiose so that the increase of cellobiose concentration produced from cellulose was too low to compensate the consumption of cellobiose into glucose. Figure 6 also depicts that cellobiose inhibition can be modeled by the kinetic model with good accuracy.

As a primary sugar product, glucose has an important inhibition effect on both cellulase and β-glucosidase in theory. The inhibition characteristics of glucose on both enzymes were studied by adding different background glucose concentrations into the enzymatic hydrolysis reactor prior to the addition of enzymes. From Figure 7, it can be found that the glucose yield and enzymatic hydrolysis rate decreased with the increase of initial glucose concentrations (from 0 to 60 mg/mL) and the cellobiose concentration increased to peak and then decreased much more slowly compared with typical conditions (Fig. 2) with zero initial glucose concentration (data not shown). These phenomena indicate that glucose inhibited both cellulase in reaction $r_2$ and β-glucosidase in reaction $r_3$ because glucose is the end-product of both reactions. The model has showed good performance in predicting the inhibition of glucose on enzymes.
Sensitivity Analysis of the Lignin’s Nonproductive Adsorption

As shown in Figure 8a,b, the predictability of the kinetic model is very sensitive to the lignin’s nonproductive adsorption. The concentrations of glucose and cellulose were significantly overestimated and underestimated, respectively, without incorporating the lignin’s nonproductive adsorption into our kinetic model (Fig. 8a). It is expected that the actual functional cellulase concentration was highly exaggerated when the negative effect of lignin was not corrected so that more cellulose was hydrolyzed to release more glucose. Similarly, the cellobiose concentration was considerably underestimated during the entire hydrolysis process (Fig. 8b). β-Glucosidase has much higher affinity to lignin than cellulase so that β-glucosidase actually lost activity very quickly at the beginning due to the presence of lignin content (Eriksson et al., 2002; Yang and Wyman, 2006; Zheng et al., 2008). Therefore, much less actual effective β-glucosidase was available to convert cellobiose into glucose, as led to the accumulation of cellobiose so that the real cellobiose concentration with consideration of lignin’s adsorption was much higher than that without taking lignin’s adsorption into account. In addition, more available β-glucosidase could contribute to overestimated glucose by hydrolyzing more cellobiose into glucose without the lignin’s adsorption. As a result, the enzyme activity loss due to the lignin’s nonproductive and irreversible adsorption should be considered when developing more reliable and powerful kinetic models for enzymatic hydrolysis of lignocellulosic biomass.

Conclusions

A semimechanistic multi-reaction kinetic model was developed and validated for a batch enzymatic hydrolysis of dilute sulfuric acid pretreated CWR. This model incorporated several factors, including SR, structure of enzymes preparation, end-product (cellobiose and glucose) inhibition, and enzyme adsorption (productive and nonproductive adsorption of enzymes on cellulose and lignin, respectively). The kinetic parameters were estimated for an established set of typical hydrolysis conditions, and then the model’s predictability was validated outside this range. The model is capable of predicting hydrolysis performance beyond the typical design space with reasonable accuracy. In general, this model can satisfactorily simulate cellulose, glucose, and cellobiose concentrations over the solid loading range of 4–12% (w/w, dry basis) and the enzyme concentration range of 15–150 (FPU+CBU)/g-cellulose. In addition, the inhibition characteristics of glucose (30 and 60 mg/mL) and cellobiose (10 mg/mL) on hydrolytic
enzymes were well captured by the model. Our model differs from the previous models in that it incorporated both substrate transformation during enzymatic hydrolysis (i.e., SR) and the nonproductive adsorption of enzymes on lignin. The sensitivity analysis on the lignin’s nonproductive adsorption illustrated that the presence of lignin within the enzymatic hydrolysis system is an important factor needed to be considered when developing a more reliable and accurate kinetic model for enzymatic hydrolysis. Therefore, this model provides a solid foundation for future model refinements such as enzyme inactivation and a more precise model of the enzyme adsorption partitioning between cellulose and lignin. At present, our model can serve as a tool for partial optimization of enzymatic hydrolysis of lignocellulosic biomass for bioethanol production, and as a good reference for developing kinetic models for other enzymatic saccharification systems.

**Nomenclature**

- \( C \): cellulose concentration at a given time (mg/mL)
- \( E_{i,b} \): bound concentration of EG/CBH and \( \beta \)-glucosidase on pretreated CWR (mg protein/mL) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( E_{i,BC} \): bound concentration of EG/CBH on cellulose content in pretreated CWR (mg protein/mL)
- \( E_{i,bd} \): bound concentration of EG/CBH and \( \beta \)-glucosidase on lignin content (mg protein/mL) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( E_d \): concentration of free enzymes in solution when pretreated CWR was substrate (mg protein/mL) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( E_{i,b} \): concentration of free enzymes in solution when lignin is contained in substrate (mg protein/mL) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( E_{i,max} \): maximum mass of EG/CBH that can adsorb onto a unit mass of pretreated CWR (mg protein/g substrate)
- \( E_{i,max,d} \): maximum mass of EG/CBH and \( \beta \)-glucosidase that can adsorb onto a unit mass of lignin (mg protein/g lignin) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( E_I \): total enzyme concentration (mg protein/mL) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( G \): glucose concentration (mg/mL)
- \( G_i \): cellulose concentration (mg/mL)
- \( K_{i,ads} \): dissociation constant for EG/CBH adsorption/desorption reaction with pretreated CWR (mL/mg protein)
- \( K_{i,ads,d} \): dissociation constant for the EG/CBH and \( \beta \)-glucosidase adsorption/desorption reaction with lignin (mL/mg protein) (\( i = 1 \) for EG/CBH; \( i = 2 \) for \( \beta \)-glucosidase)
- \( k_o \): reaction rate constants (mL/mg·h) (\( i = 1 \) for cellulose-to-cellobiose; \( i = 2 \) for cellulose-to-glucose; \( i = 3 \) for cellobiose-to-glucose)
- \( K_{BG} \): inhibition constants of glucose on enzymes (mg/mL) (\( i = 1, 2, \) and 3. The \( i \) is the same as that in \( k_o \))
- \( K_{BG2} \): inhibition constants of cellobiose on enzymes (mg/mL) (\( i = 1 \) and 2. The \( i \) is the same as that in \( k_o \))
- \( K_{SM} \): substrate (cellobiose) saturation constants (mg/mL)
- \( L \): lignin concentration content (g/mL)
- \( r_i \): reaction rate (mg/mL/h) (\( i = 1, 2, \) and 3. The \( i \) is the same as that in \( k_o \))

**SR** substrate reactivity (dimensionless)

**S** substrate (cellulose) concentration at a given time (g/mL)

**S_0** initial substrate (cellulose) concentration (g/mL)

\( t \): elapse time during enzymatic hydrolysis (h)

\( \alpha \): a constant (dimensionless)

\( \lambda \): ratio of lignin content exposed to enzymes to the total amount of lignin content contained in pretreated CWR (dimensionless)

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**References**


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