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On Improved Mechanistic Modeling for Enzymatic Hydrolysis of Cellulose

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Abstract

An improved model for enzymatic hydrolysis of cellulose is developed that considers oligomer reactions with beta-glucanases, inhibition of oligomers to cellulases and enzyme decay processes during hydrolysis. Our oligomer reactions with beta-glucanases are modeled based on the enzymatic glucan chain fragmentation kinetics to describe the further fragmentation of oligomers in solution after being solubilized from the insoluble glucan chains. The inhibition effects on all cellulases by different types of cello-oligomers are then taken into account by competitive adsorption of cello-oligomers to the active site of cellulases, which is a critical factor contributing to the decrease in the rate of enzymatic hydrolysis of cellulose. As another factor affecting the kinetics of cellulose hydrolysis process, enzyme decay factor is incorporated into the model as the typical first order decay process. We consider two different processes for cellulases losing activity during hydrolysis in order to better understand the impact of enzyme decay on hydrolysis. Numerical simulation results are presented to investigate the phenomenon of hydrolysis rate slow-down commonly observed in experiments. Improvement of the predictive capability of the new model over previous one is clearly demonstrated by comparing the simulations with experimental data. By considering all the possible hydrolysis rate slow-down factors, the simulation results can agree with the experimental data very well, showing that our model is capable to fully capture the rate decrease of cellulose hydrolysis.

Keywords: Modeling; Enzymatic hydrolysis; Cellulose; Site formalism; Enzyme decay; Inhibition

Nomenclature

D_κ: Decay factor of type-κ enzymes

$I_\kappa(l)$: Oligomer adsorption coefficient for (κ, l) EO complexes (1/mM)

k, k ': number of glucose units contained in a glucan chain

k: chain length, equal to the number of glucose units contained in a glucan chain

l_S: minimum insoluble chain length for glucan chains, =7

$L_{\kappa,\mu}$: substrate adsorption coefficient for (κ, μ) ES complexes (1/mM)

$R_S(l)$: changing rate of soluble oligomers in solution contained l glucose units

$R_{S,\sigma}(l)$: production rate of soluble oligomers contained l glucose units dissolved from glucan chains exposed on class-σ SAC surfaces (mM/min)

$T_{1/2k}$: half-life of type-κ enzymes (min)

u_κ: total concentration of type-κ enzymes (mM)

v_κ: concentration of free type-κ enzymes in solution (mM)]

$x_{S,\sigma}$: total concentration of glucose units dissolved from glucan chains exposed on class-σ SAC surfaces (mM)

$x_S(l)$: concentration of oligomers dissolved from glucan chains containing l glucose units

$y_{\mu,\sigma}$: concentration of free type-μ sites on glucan chains exposed on class-σ SAC surfaces (mM)

$y_S(l)$: concentration of free oligomers dissolved from glucan chains containing l glucose units

$z_{\kappa,\mu,\sigma}$: concentration of (κ, μ) ES complexes exposed on class-σ SAC surfaces (mM)

$z_\kappa(l)$:concentration of (κ, l) EO complexes formed by type-κ enzymes and oligomers containing l glucose units (mM)

κ: index of enzyme types

$\gamma_{\kappa,\mu}$: cutting rate coefficient for (κ, μ) ES complexes (cuts per (κ, μ) ES complex per time)

μ, μ': index of site types

σ: index of SAC classes

Introduction

The enzymatic hydrolysis of cellulose into soluble and fermentable oligomers (e.g., glucose and cellobiose) has been under intensive investigation due to the potential utilization of lignocellulosic biomass to produce sustainable biofuel and replace the non-renewable fossil transportation fuel. In order to optimize the design of reactors and the biofuel production process, it is critical to have a mechanistic model describing the hydrolysis kinetics of solid cellulosic substrates being solubilized by all kinds of cellulases in detail. Unlike non-mechanistic and semi-mechanistic models which usually include less than two substrate and/or enzyme variables and are used to fit experimental data [1], mechanistic models involve multiple substrate and enzyme variables and can provide insights on the complex chemical and physical properties of both enzyme and substrate and all the enzymatic and material transformations occurring during hydrolysis.

Over the past 10 years, many advanced mechanistic models have

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been developed in full generality to describe the enzymatic hydrolysis process of cellulose based on more realistic representations of cellulosic substrate [2-5]. For example, Zhou et al. [2] developed a detailed mechanistic model for enzymatic hydrolysis of cellulose considering the substrate morphologies and their coupling with morphology-depended substrate hydrolysis kinetics. As described in the model, the whole process of cellulose hydrolysis could be viewed as the process of fragmentation and solubilization of glucan chains and lead to the evolution of cellulosic substrate morphology, that is, the organization of glucan chains, which, in turn, significantly influenced the cellulose hydrolysis kinetics. The concept of smallest accessible compartment (SAC) was first proposed, defined as a minimal volume by external and internal surfaces exposed to enzyme-accessible hydrated interior voids of the solid cellulosic substrate material, and used as minimal time-evolving structural unit to keep track of the cellulosic substrate morphology. In addition, a site representation formalism of enzyme hydrolytic fragmentation coupled with morphology evolution was also introduced in the model. The formalism considered all the β-(1, 4)-glycosidic bonds on glucan chains as six types of bond sites based on their locations and reactions with different kinds of cellulases that mainly act on the solid substrate. Basically, these cellulases can be categorized into two broad classes, which are endo-glucanases and exo-glucanases. Endo-glucanases usually adsorb onto the glucan chains of insoluble part of substrate and randomly cut the internal β-(1,4)-glycosidic bonds. Unlike endo-glucanases, exo-glucanases only cut the terminal β-(1,4)-glycosidic bonds at the ends of each glucan chain. Since the two ends of each glucan chain are chemically distinct from each other, exo-glucanase can be divided into two groups, which are cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II). CBH IIs usually cut the terminal bonds from the non-reducing end of each glucan chain, while CBH Is cut the terminal bonds from the reducing end of each glucan chain. These cellulases can be produced in nature by many different cellulolytic fungi species. The most commonly used species in industry is *Trichoderma* species, especially *Trichoderma reesei* (*T. reesei*). Thus *T. reesei* EG1 (Cel7B), *T. reesei* CBH II (Cel6B) and *T. reesei* CBH I (Cel7A) are the three major cellulases that often used in model simulation work to analyze the process of enzymatic hydrolysis of cellulosic substrate. The site representation formalism was proposed to not only take into account all kinds of solid-substrate-acting cellulases but also change the view of solid cellulosic substrate from a bundle of glucan chains with different lengths into a composite of six types of bond sites, so that the total number of ordinary differential equations (ODEs) can be reduced at magnitude of two orders and solved much more efficiently.

Almost all of the mechanistic models have recognized that the enzyme accessibility of substrate, or the enzyme-accessible substrate surface area, is a critical rate-limiting factor during the process of enzymatic hydrolysis [2-4]. In order to increase the kinetics of hydrolysis and obtain more soluble oligomers, it will be helpful to increase the amount of accessible glucose units (β-(1,4)-glycosidic bonds) exposed on substrate surface by pretreatments before hydrolysis proceeding. However, most of the simulation results from the mechanistic models still could not agree with the experimental data completely, especially not be capable to capture the full extent of the hydrolysis rate slow-down, probably due to the reason that these models did not consider the complex inhibition relationships between substrate and oligomers except glucose and cellobiose, and possible enzyme decay and inactive absorption. Some of the models also did not consider the oligomer reactions which happen in liquid phase with the beta-glucanases. Unlike endo- and exo-glucanases, beta-glucanases mainly act on soluble cello-oligomers in solution which often contains no more than 7 glucose units. The most commonly used cellulolytic fungi species to created beta-glucanases in industry is *Aspergillus* species, especially *Aspergillus niger*

(*A. niger*) because *Trichoderma* species do not produce significant amount of beta-glucanases compared to other cellulases. *A. niger* beta-glucosidase (BG) is the major beta-glucanase and often used with other cellulases to increase their efficiency during enzymatic hydrolysis of cellulose, because dissolved oligomers could cause strong inhibition effects by adsorbing the free cellulase molecules. BGs can hydrolyze soluble oligomers into smaller ones mainly by releasing glucose from them and avoid the strong inhibitions between cellulases and long-chain oligomers. It is clear that the oligomer reactions with beta-glucanase will affect hydrolysis kinetics and change inhibition equilibrium. Ignoring the kinetics slow-down factors will also hinder the precise prediction of final conversion level of cellulosic substrate after a long time-scale of enzymatic hydrolysis process.

In this study, we improve the model described by Zhou et al. [2] by incorporating (1) the reactions involving beta-glucanases digesting soluble oligomer sugars in solution, (2) the comprehensive competitive inhibition effects of oligomers from glucose (G1) to cellohexaose (G6) on enzymatic hydrolysis, and (3) the enzymatic thermal deactivation and inactive adsorption. Here, we consider enzymatic thermal deactivation as the process that the hydrolytic enzymes lose the ability of binding to substrates, and consider enzymatic inactive adsorption as the process that the hydrolytic enzymes lose the ability to continue the subsequent catalytic reactions although they are able to bind to substrate. We carry out numerical analysis to investigate the impacts of aforementioned factors on the kinetics of enzymatic hydrolysis of cellulose.

Materials and Methods

Oligomer reactions with beta-glucanases

The basic part of the model we used in this study was from the mechanistic model of enzymatic hydrolysis of cellulose presented by Zhou et al. [2]. In their work, the concept of smallest accessible compartment (SAC) was first proposed to describe the geometrical construction of pretreated pure cellulosic substrate material. An SAC unit was defined as a minimal volume that is delimited by external surfaces and by internal surfaces at the internal surfaces of the voids exposed to all kinds of cellulases. SACs initially had a random distribution of sizes, and to represent the distribution, the parameter of SAC geometry class was used and labeled by "σ" with σ=1,2... MMD where MMD was the population size of SAC geometry classes. During the process of enzymatic hydrolysis, the size of each SAC would shrink due to the enzymatic ablation of glucose units from the SAC surfaces. And glucose units which were originally blocked would gradually become exposed on the SAC surfaces. In addition, Zhou et al. [2] proposed in their work an innovative surface site concentration formalism which treated each SAC as a composite of six different types of β-(1,4)-glycosidic bond sites. These site types were labeled by index "μ" and referred to as N-, O-, X-, Y-, L-, and R-sites. N-site bonds could only adsorb, and only be cut by endo-glucanases. O-site bonds could not adsorb any enzyme molecule due to the obstruction. X-site bonds could adsorb and be cut by either endo-glucanases or cellobiohydrolase IIs, which were located a distance of k_X glucose units from the non-reducing ends of glucan chains. Y-site bonds could adsorb and be cut by either endo-glucanases or cellobiohydrolase Is, which were located a distance of k_Y glucose units from the reducing ends of glucan chains.

L- and R-site bonds represented the non-reducing and reducing ends of glucan chains respectively. They were both broken bonds and could not adsorb any enzyme molecule. Then the production rate of glucose units dissolved from glucan chains of class-σ SACs was given by

$$\dot{x}_{S,\sigma} = \sum_{l=1}^{l_s-1} l \cdot R_{S,\sigma}(l) \quad (1)$$

where $R_{S,\sigma}(l)$ is the production rate of oligomers containing l glucose units ($1 \le l < l_S = 7$) dissolved from insoluble glucan chains exposed on class-σ SAC surface caused by the enzymatic cuts on β-(1,4)-glycosidic bonds. l_S is the minimal length of insoluble glucan chains, which could be varied into any length if needed. In the mode, we considered cellohexaose (G6) as the longest cello-oligomer in solution based on the typical industrial applications.

We improved the mechanistic model to not only consider the processes that endo- or exo-glucanases cut the β-(1,4)-glycosidic bonds of insoluble glucan chains to produce oligomers, but also consider explicitly the reactions of oligomers after being released from solid substrates that involve beta-glucanases in solution. The production rates of soluble oligomers in solution are given by

$$x_S(l) = \Sigma R_{S,\sigma}(l) + R_S(l) \quad (2)$$

where $R_s(l)$ is the production rate of the oligomers containing l monomer units after being dissolved from glucan chains, which describes the reactions between oligomers and beta-glucanases. $R_S(l)$ did not exist in the model of Zhou et al. [2] where all the oligomers kept increasing in solution by being hydrolyzed from solid substrate.

Based on the study of sub-site structure of *A. niger* beta-glucosidase [6], the reaction mechanism between oligomers and beta-glucanases in solution can be considered as: an oligomer which contains l glucose units can adsorb and be hydrolyzed by an beta-glucanase molecule into a glucose unit and an oligomer containing $l-1$ glucose unit(s). So the expression of $R_s(l)$

$$\begin{cases} R_{Sk}(l) = \gamma_\kappa(2) \cdot z(2)_\kappa + \sum_{2}^{l_s-1} \gamma_\kappa(l) \cdot z(1) \\ R_{Sk}(l) = -\gamma_\kappa(l) \cdot z(l_\kappa) + \gamma(l+1) \cdot z(l+1)_S \quad 2 \le l \le l\text{-}2 \\ R_{Sk}(l_{kS} - {}_S\,1) = -\gamma_\kappa(l_S - 1) \cdot z(l-1) \end{cases} \quad (3)$$

where $\gamma_\kappa(l)$ is the oligomer cutting rate coefficient, $z_\kappa(l)$ is the concentration of (κ, l) enzyme-oligomer (EO) complexes where the oligomers contain l glucose units adsorb the type-κ beta-glucosidase enzyme molecules. The expression of EO complexes $z_\kappa(l)$ can be written, based on the enzyme adsorption equilibrium, as

$$Z_\kappa(l) = I_\kappa(l).v_\kappa.y_S(l) \quad (4)$$

with $l \le l < l_S = 7$. Here, $I_\kappa(l)$ is the oligomer adsorption coefficient; $y_S(l)$ is the concentration of free oligomers containing l glucose units dissolved from glucan chains, and v_κ is the concentrations of type-κ free enzyme molecules in solution which could be any type of cellulases.

Inhibition effects

Cellulase inhibition describes the process that inhibitors limit the activity of cellulase molecules. Based on the impacts on cellulase, cellulase inhibitors can be classified into reversible and irreversible inhibitors in which the oligomer products of cellulose hydrolysis (e.g. glucose and cellobiose) are reversible inhibitors [7]. Reversible inhibitors can be classified into three types, which are competitive, uncompetitive and mix (or non-competitive) inhibitors. Most of the inhibition processes between the cello-oligomer inhibitors and cellulases are competitive inhibitions [7] where inhibitors can only bind free cellulase molecules in solution, which can also be described by adsorption Equation (4), where v_κ is now the concentration of type-κ cellulose (both endo- and exo- glucanases) free enzyme molecules in solution, and $I_\kappa(l)$ is the oligomer adsorption coefficient specifically for different cellulases.

If the EO complexes are formed by oligomers and endo- (or exo-) glucanases by oligomers binding onto active sites of binding domain of cellulases, and since we assume that catalytic domain cannot act on soluble oligomers, their corresponding values of $\gamma_\kappa(l)$, oligomer cutting rate coefficient, will be 0. Similarly, we could consider the inhibition of glucose (G1) to the beta-glucanases hydrolysis, where glucose binds onto the active site of beta-glucanase and no further catalytic action can be taken. Thus for beta-glucanases, if they are adsorbed by glucose units, $\gamma_\kappa(l)$ will also be 0 in that glucose units are the finial products of oligomer reactions and cannot be further dissolved. In Equation (4), v_κ and $y_S(l)$ are related to their corresponding total concentrations u_κ and $x_S(l)$. The relationships are written as

$$u_\kappa = v_\kappa + \sum_{\mu,\sigma} z_{\kappa,\mu,\sigma} + \sum_l z_\kappa(l) \quad (5)$$

$$x_{s\kappa}(l) = y_s(l) + \sum_\kappa z(l) \quad (6)$$

$z_{\kappa,\mu,\sigma}$ is the concentration of enzyme-substrate complexes formed by type-μ bond sites and type-κ enzyme molecules on class-σ SAC surfaces. Equations (5) and (6) can be used to find the expressions of v_κ and $y_S(l)$ as functions of u_κ and $x_S(l)$ respectively, which are written as

$$v_k = \frac{u_k}{1 + \sum_{\mu,\sigma} L_{k,\mu} \cdot y_{\mu,\sigma} + \sum_l I_k(l) \cdot y_s(l)} \quad (7)$$

$$y_s(l) = \frac{x_s(l)}{1 + \sum_k I_k(l) \cdot v_k} \quad (8)$$

where $y_{\mu,\sigma}$ is the concentration of free type-μ sites on glucan chains exposed on class-σ SAC surfaces, $L_{\kappa,\mu}$ is the adsorption coefficient between type-κ cellulase molecules and type-μ sites on glucan chains. Then the more detailed expressions of $R_S(l)$ can be obtained by combining Equations (3), (4), (7) and (8).

Enzymatic thermal deactivation and inactive adsorption

Many experiments illustrated that even after alleviating the inhibition effects mostly, the rate of enzymatic hydrolysis of cellulose still decreases during hydrolysis, meaning that inhibition effect is not the only rate-limiting factor [4,8]. Many studies considered the process of cellulases losing activities as a contributing factor to the hydrolysis rate slow-down, which was called enzyme decay process and often modeled as a first order process [4,8]. Enzyme decay process naturally happen to all hydrolytic enzymes based on experimental observations [9,10]. In principle, the enzyme decay will make the enzymes lose their hydrolytic capability through two possible meanings according to the specific structure of enzymes. Most of the hydrolytic enzymes contain a carbohydrate binding domain and a catalytic domain. Typically, these two domains are away from each other and connected by a long segment of linker sequence. Some enzymes, however, only contain a catalytic core domain, such as *T. reesei* EG III (Cel12A), which bind to substrate through the substrate-binding sites in the catalytic core domain. So if the carbohydrate binding domains or the substrate-binding sites are deactivated, hydrolytic enzymes will lose the capability of binding to the cellulosic substrate chain sites. On the other hand, if the catalytic domain is deactivated, hydrolytic enzymes are still able to bind to substrate chain sites, but will not carry out the subsequent catalytic reactions.

We incorporated into the model these two different mechanisms, which are referred to as the model the enzymatic thermal deactivation and the enzymatic inactive adsorption, respectively. We defined the enzymatic thermal deactivation in the model as the process that the binding domains or sites of cellulase molecules kept losing the ability to adsorb on glucan chains during hydrolysis. By contrast, we defined enzymatic inactive adsorption in the model as the process that the catalytic domains of all cellulases kept losing the reactivity to cut glucan chains after adsorbing on the substrate. For a single cellulase molecule, the binding and catalyzing abilities could keep losing simultaneously. The decay processes of cellulase may be caused by the affection of temperature during hydrolysis, since each type of enzymes has its own optimal temperature to work efficiently for certain period of time.

The enzyme decay factor D_κ is set to $D_\kappa = (0.5)^{t\ /T1/2,\kappa}$ in the model for type-κ enzyme molecules where $T_{1/2,\kappa}$ is the half-life for type-κ enzyme molecules, which represent the time for an enzyme lost half of its enzymatic activity. We apply the decay factor D_κ to parameters u_κ and $z_{\kappa,\ \mu,\ \sigma}$ to represent the processes of enzymatic thermal deactivation and enzymatic inactive adsorption respectively and test the impact range of enzyme decay on the cellulose hydrolysis kinetics.

Model parameters

The specific activity and adsorption equilibrium coefficients of Endo-glucanase (EG1) and Exo-glucanases including Cellobiohydrolase I and II (CBH I and II) are from the work by Zhang and lynd [3]. For beta-glucanase (BG), the values of adsorption and kinetics parameters describing the reactions with oligomers are from the literature [6,11]. In the model, the values κ=1,2,3 and 4 represent EG1 CBH II CBH I and BG. The values of inhibition parameters are from various literature sources [4,12,13] due to the fact that we considered several different inhibition effects involving 4 different types of cellulases (EG1, CBHI, CBHII and BG) and 6 types of oligomers from glucose (G1) to cellohexaose (G6). The half-life of all kinds of cellulases was set to 42.5h as reported in the literature [14] (Table 1).

Results and Discussion

Testing cellulosic substrate accessibility

In this section, we investigate the effect of enzymatic accessibility of cellulosic substrates. In the work of Zhou et al. [15], the initial value of F_a for Avicel, that is, the ratio of enzyme-accessible glucose units to the total number of glucose units in Avicel was set to 0.00620, which was originally described in the literature [1]. Not until recently, Hong et al. [9] determined that the value of F_a for Avicel should be 0.00232 from the experiments testing the adsorption of fluorescent cellulase-like molecules on the substrate surface. We test some new values of F_a and compare the new simulation results with the original ones from the work of Zhou et al. [16]. The 12 experimental data [9,10] was used in the work of Zhou et al. [16] to compare their simulation results, and will be adopted in this work.

Figure 1 clearly shows that increasing the enzymatic accessibility for substrate prior to hydrolysis could allow more enzyme molecules attack the bonds on glucan chains and thus increase the hydrolysis rate and finial conversion level of substrate. Substrate accessibility in hydrolysis is believed to govern the entire hydrolysis process and act as a critical rate-affecting factor during hydrolysis. The previous initial value 0.00620 for F_a was obtained based on the nitrogen BET measurements. In the process of such adsorption-based measurements, nitrogen was usually used to test the initial accessible surface area of substrate. However, since nitrogen molecules are much smaller than enzyme molecules, and the substrate need to be in dry conditions, the value is probably overestimated and thus not accurate. Hong et al. [9], determined the accessible surface area of cellulosic substrate by testing the adsorption of molecules containing fluorescent proteins and cellulose-binding modules (CBMs), which had similar size as cellulases could be quantitatively tested. However, the value 0.00232 may be underestimated due to the fact that only one type of cellulose-binding module was used to create cellulase-like molecules. Recently, Levin et al. [4] pointed out in their work that the initial value of F_a from the work of Hong et al. [9] might be underestimated Based on their estimation by using the Random Sequential Adsorption (RSA) simulation process, the initial accessible surface area of Avicel should be at least 3.5 m^2g^{-1}, meaning that initial value of F_a should be 0.00341 for Avicel. We believe that the initial value of F_a should be set between 0.00232 and 0.00620 so that 0.00341 is more accurate and will be used in next sections. The parameter of cellulosic substrate accessibility is critical and reflects the cellulase-accessible surface area. However, it was estimated that during the early stage of hydrolysis the substrate accessibility could either decrease or increase if using different substrate morphologies [15], remaining the impact of substrate accessibility on the hydrolysis rate unclear. Most of the experiments only tested the value of substrate accessibility at the beginning and the end of hydrolysis and did not keep track of its changing through the entire process. So in order to better understand the role of substrate accessibility, it is highly recommended to test substrate accessibility at different time points during hydrolysis. By using smaller initial values for F_a, the new hydrolysis rates are a little slower than the original ones but still could not capture the full extent of the hydrolysis rate decreases shown in the experimental data. The enormous differences between the simulation results and experimental data also indicate that only considering substrate morphology as hydrolysis rate-limiting factor is not enough to reproduce the kinetics

Parameter	Value	Units	Source
$^{L}1,N\,L1,X\ \ L1,Y$	3	1/mM	[2,3]
$^{L}2,X\ \ ^{L}3,Y$	4	1/mM	[2,3]
$^{L}3,N\ ,Glu\ ^{L}3,X\ ,Glu\ \ ^{L}2,N\ ,Glu\ \ ^{L}2,Y\ ,Glu$	0	1/mM	[2,3]
$^{\gamma}1,N\,\gamma1,X\ \ \gamma1,Y$	23.5	1/min	[2,3]
$^{\gamma}2,X$	10.6	1/min	[2,3]
$^{\gamma}3,Y$	6.2	1/min	[2,3]
$^{\gamma}2,N\ ^{\gamma}2,Y\ ^{\gamma}3,N\ ^{\gamma}3,X$	0	1/min	[2,3]
I_1 (1)	0.06	1/mM	[4]
I_2 (1) I_3 (1)	0.032	1/mM	[4]
I_1 (2) I2 (2) I3 (2)	0.13	1/mM	[12]
I_1 (3) I2 (3) I3 (3)	0.3	1/mM	[12,13]
I_1 (4) I2 (4) I3 (4)	0.37	1/mM	[12,13]
I_1 (5) I2 (5) I3 (5)	0.44	1/mM	[12,13]
I_1 (6) I2 (6) I3 (6)	0.51	1/mM	[12,13]
I_4 (1)	0.294	1/mM	[6]
I_4 (2)	1.136	1/mM	[6,11]
I_4 (3)	3.846	1/mM	[6,11]
I_4 (4)	4	1/mM	[6,11]
I_4 (5)	2.174	1/mM	[6,11]
I_4 (6)	1.449	1/mM	[6,11]
γ_4 (1)	0	1/min	
γ_4 (2)	1897	1/min	[6]
γ_4 (3)	1739	1/min	[6,11]
γ_4 (4)	1423	1/min	[6,11]
γ_4 (5)	895.8	1/min	[6,11]
γ_4 (6)	843.1	1/min	[6,11]

Table 1: Parameters used in the model.

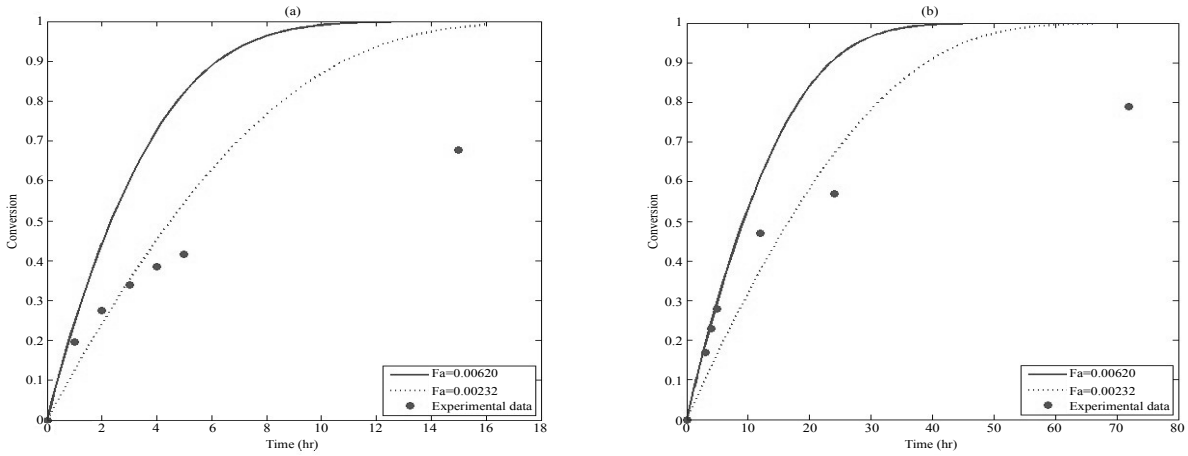

Figure 1: Simulation results using different initial values of Fa compared with experimental data. The substrate and enzyme have initial properties of (a) 20 g/L Avicel and 4.1 g/L Spezyme CP [9] and (b) 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10]. Commercial enzyme Spezyme CP consists EG1, CBHI and CBHII in a mass ratio of 0.17:0.24:0.13 (EG1/CBHI/CBHII) [17].

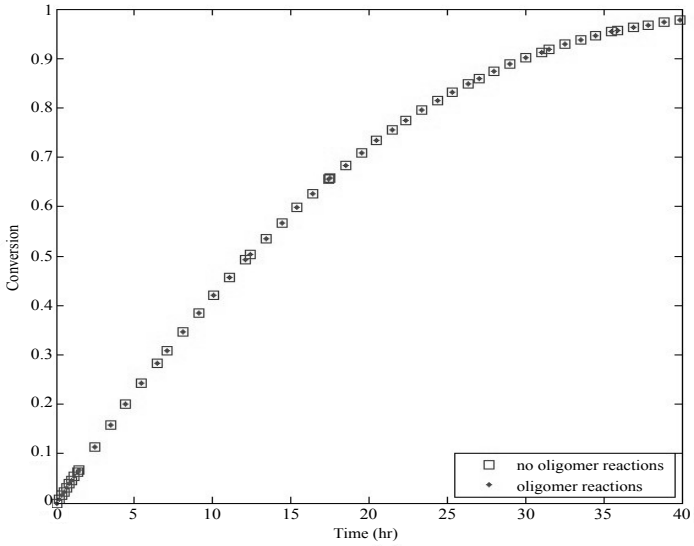

Figure 2: Simulation results of finial conversion level with and without consideration of oligomer reactions in solution. The substrate and enzyme have initial properties of 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10].

slow-down phenomenon during the process of enzymatic hydrolysis of cellulose. More hydrolysis rate-limiting factors, such as oligomer inhibition, should be considered when constructing a model of enzymatic hydrolysis of cellulose.

Oligomer reactions

In this section, we demonstrate the simulation results after considering the oligomer reactions in solution without inhibition effects. In the work of Zhou et al. [2], the reactions involving beta-glucanases were not concerned. Although the main rate-limiting steps of the cellulose hydrolysis process is the reactions that endo- and exo-glucanases depolymerize insoluble long glucan chains into soluble oligomers, the changes of oligomers in solution could also affect the hydrolysis kinetics in turn and thus could not be ignored.

Figure 2 shows the comparison of final conversion level of cellulosic substrate between simulations considering and not considering oligomer reactions in solution, as we excepted, there is no difference of simulation results after incorporating oligomer reactions. Because the conversion level describe the ratio of the total amount of dissolved substrate to the initial amount of substrate. According to the mass balance, although the amount of each type of oligomer will be changed after considering oligomer reaction, the total amount of soluble substrate should be the same as that considers no oligomer reaction. In Figures 3 and 4, differences are illustrated for cello-oligomers during hydrolysis, indicating that if considering oligomer inhibition effects, simulation results taking into account the oligomer reactions will be more accurate.

Inhibition effects

In this section, we demonstrate the simulation results after considering the oligomer reactions in solution and all kinds of

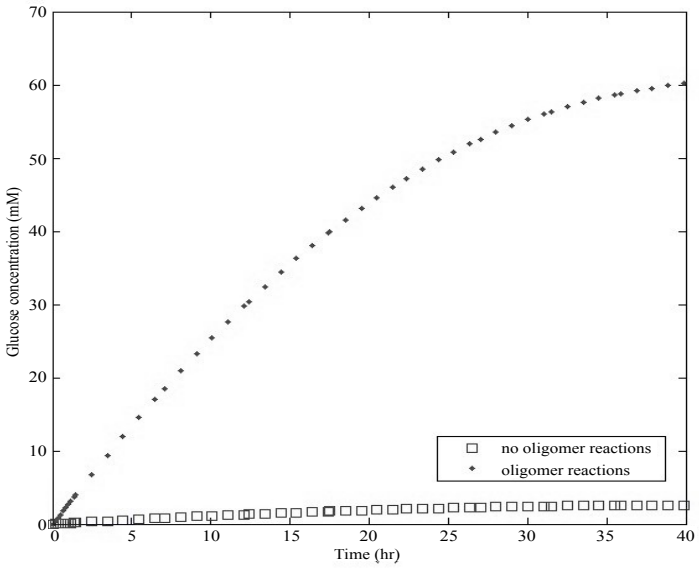

Figure 3: Simulation results of glucose concentration with and without consideration of oligomer reactions in solution. The substrate and enzyme have initial properties of 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10].

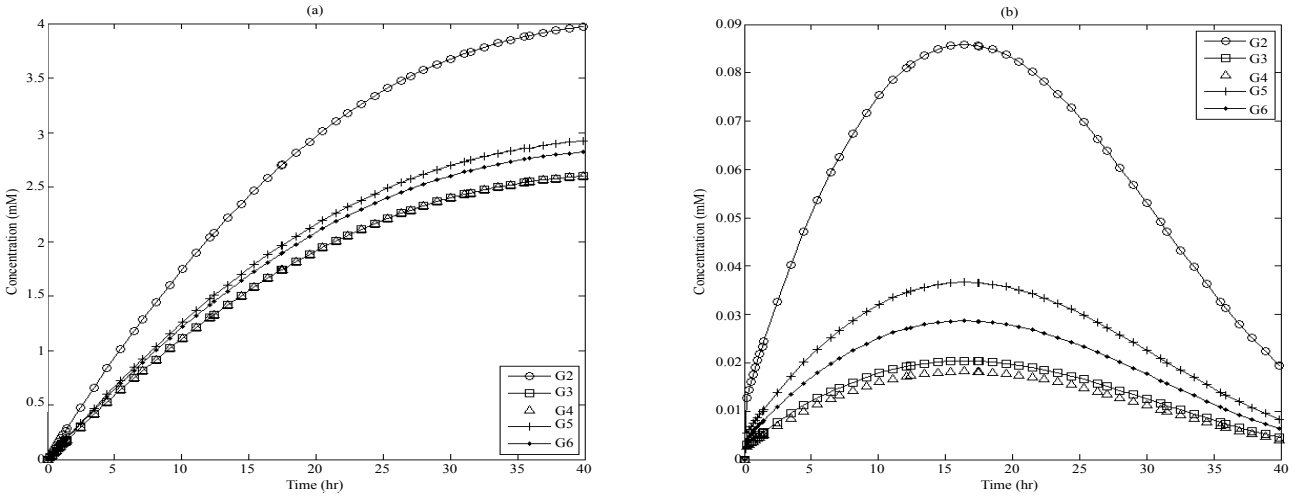

Figure 4: Simulation results of oligomer concentrations (a) without and (b) with consideration of oligomer reactions in solution. The substrate and enzyme have initial properties of 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10].

competitive inhibition effects between 4 types of cellulases and 6 types of cello-oligomers. As shown in Figure 5, after incorporating both the oligomer reactions and inhibitions, the simulation results can capture some extent of the decrease in the rate of cellulose hydrolysis often observed in experiments, showing that inhibition is one of the critical factors contributing to the phenomenon of hydrolysis rate slow-down.

However, the process of inhibition still remains to elucidate in more details. Although we considered all kinds of competitive inhibitions that could possibly happen in solution during enzymatic hydrolysis, it still might exist other types of inhibition relationships, such as uncompetitive inhibitions of cellobiose which can not only bind free cellulase molecules but also EO or ES complexes. Deeper understanding of the inhibitions during cellulose hydrolysis is needed to improve the kinetic models.

Enzyme decay and the slow-down of enzymatic hydrolysis kinetics

In order to reproduce the phenomenon of hydrolysis rate slow-down, we first test the enzymatic decay factor in the model. It is clear that only considering the inhibition effect during hydrolysis is not enough to investigate the rate decrease of enzymatic hydrolysis of cellulose.

Similar view was given in the work of Levin et al. [4] where they tried to fit the data by using much shorter estimated half-life values

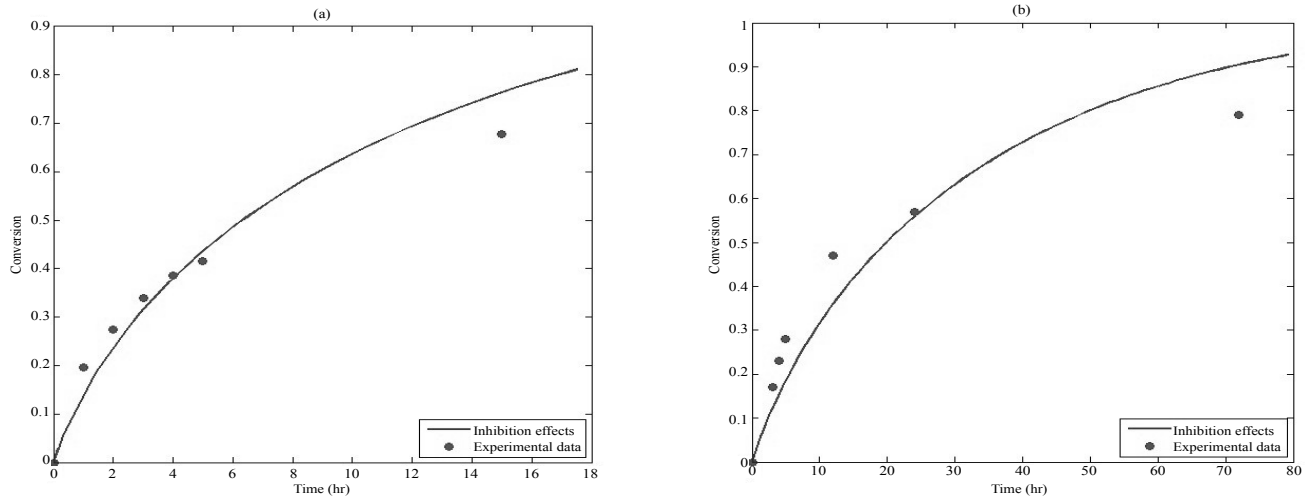

Figure 5: Simulation results considering oligomer reactions and inhibitions compared with experimental data. The substrate and enzyme have initial properties of (a) 20 g/L Avicel and 4.1 g/L Spezyme CP [9] and (b) 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10]. Commercial enzyme Spezyme CP consists EG1, CBHI and CBHII in a mass ratio of 0.17:0.24:0.13 (EG1/CBHI/CBHII) [17].

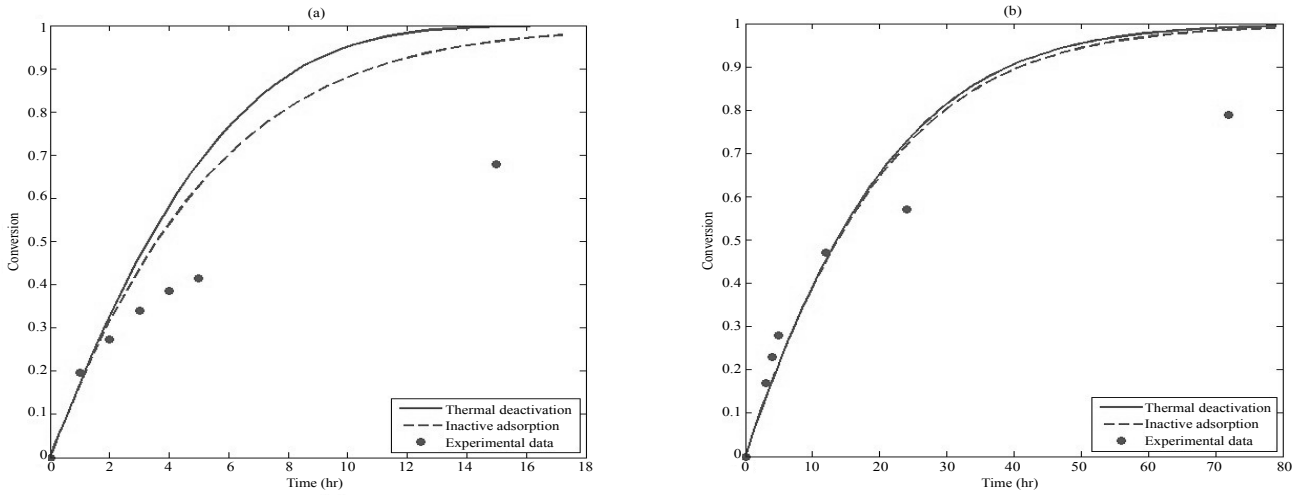

Figure 6: Simulation results considering different enzyme decay factors compared with experimental data. The substrate and enzyme have initial properties of (a) 20 g/L Avicel and 4.1 g/L Spezyme CP [9] and (b) 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10]. Commercial enzyme Spezyme CP consists EG1, CBHI and CBHII in a mass ratio of 0.17:0.24:0.13 (EG1/CBHI/CBHII) [17].

for cellulases. Here, we use the experimentally-determined half-life values for all the 4 types of cellulases in the model, and test the impact range between enzymatic thermal deactivation and enzymatic inactive adsorption on the hydrolysis kinetics. As shown in Figure 6 the impacts of enzymatic thermal deactivation and enzymatic inactive adsorption on the hydrolysis rate are very close to each other, probably because they are both reflecting the affection of temperature on the rate of hydrolysis. Also, both the impacts of enzymatic thermal deactivation and enzymatic inactive adsorption are weaker than the impact of the inhibition effects if comparing the results in Figures 5 and 6, indicating that inhibition is the prime factor contributing to the decrease in the rate of hydrolysis. After combining all the possible factor that cause the hydrolysis rate slow-down, the simulation results can agree with the experimental data very well in Figure 7, which indicate that considering inhibition and enzyme decay together in the model of enzymatic hydrolysis of cellulose can capture the full extent of the rate decrease phenomenon during the hydrolysis process. In order to enhance the hydrolysis rate and the conversion level of cellulosic substrate, a route is to increase the loading or improve the specific activities of cellulases. However, increasing the amount of cellulases will causes a huge waste of cellulase since only a small part of free cellulase molecules could adsorb onto the substrate and as hydrolysis proceeds all the cellulase molecules will decay and gradually lose the abilities of binding and catalyzing. Besides, since the oligomer inhibition effects impact the hydrolysis rate more than other rate-limiting factors as discussed before, highly efficient cellulases may increase the rate in the early stage of hydrolysis but will unavoidably end up with binding oligomers and could not enhance the conversion level too much. So a better approach

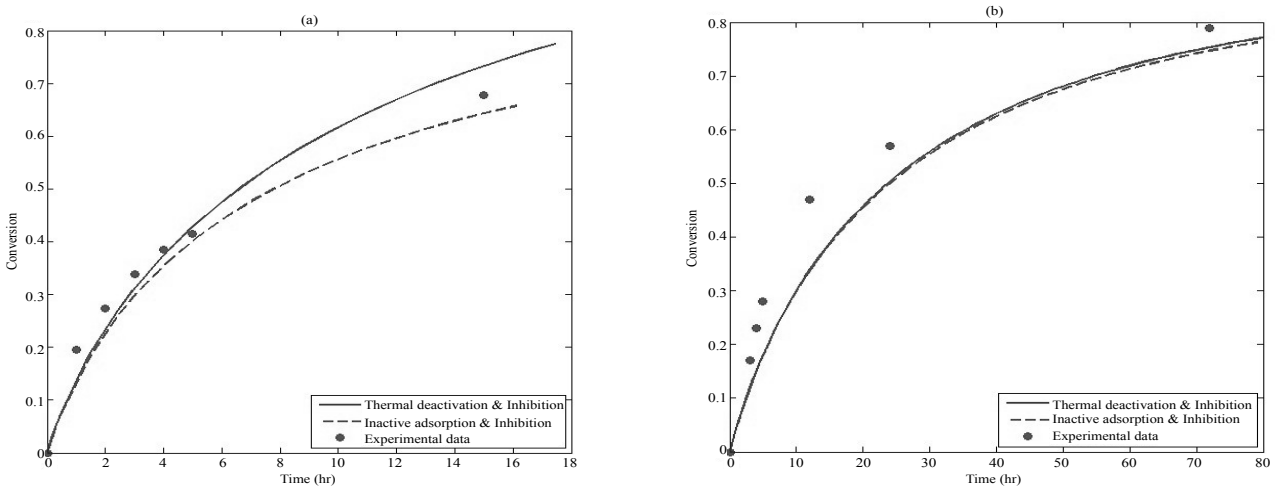

Figure 7: Simulation results considering all rate-limiting factors compared with experimental data. The substrate and enzyme have initial properties of (a) 20 g/L Avicel and 4.1 g/L Spezyme CP [9] and (b) 10 g/L Avicel and 0.51 g/L Spezyme CP together with 30 IU beta-glucanase/g Avicel (57.69 mg/L beta-glucanase) [10]. Commercial enzyme Spezyme CP consists EG1, CBHI and CBHII in a mass ratio of 0.17:0.24:0.13 (EG1/CBHI/CBHII) [17].

would be to add cellulases of small loading at different time points during hydrolysis with substrate filtered from the original solution into another reactor. This approach can theoretically reduce the impact of inhibition and keep the high efficiency of cellulases which need to be further tested by experiments.

Mechanistic modeling the entire process of enzymatic hydrolysis of cellulose is a challenging work. The differences between simulations and experiments probably come from two sources:

(1) The parameters used may not be accurate or may not be considered constants along the entire hydrolysis process (i.e. could be functions of time and operational conditions); and (2) there are some other factors that have not been taken into account, for example, lignin effect.

Conclusions

We have developed a detailed modeling framework for enzymatic hydrolysis of cellulose. The model for the first time not only consider the main hydrolysis step where long insoluble glucan chains are hydrolyzed by endo- and exo-glucanases on the surface of solid substrate, but also the reactions in solution which involve beta-glucanases and oligomers released from the conversion process of insoluble glucan chains. To investigate the phenomenon of hydrolysis rate slow-down often reported in the literature, the model considers the competitive inhibition effects of all possible cello-oligomers on cellulases and two theoretical enzyme decay processes, which are enzymatic thermal deactivation and the enzymatic inactive adsorption.

By using the model, we have presented analyses for the role of enzymatic accessibility of cellulosic substrate. As the enzyme-accessible surface area increases, both the hydrolysis rate and the finial conversion level of substrate could increase, showing that the enzymatic accessibility is a critical rate-limiting factor during the entire process of hydrolysis. After incorporating the reactions involving beta-glucanases and oligomers in solution and inhibition effects by oligomers, the simulation results can capture some extent of the decrease in the rate of cellulose hydrolysis often observed in experiments. We have tested the impacts of enzymatic thermal deactivation and the enzymatic inactive adsorption on the rate of hydrolysis and found the impacts of the two enzyme decay processes are very close to each other. We have presented detailed investigation of the phenomenon of hydrolysis rate slow-down. From the simulation results, we have found that inhibition effect of oligomer and enzyme decay are both critical factors contributing to the kinetics slow-down phenomenon during the process of enzymatic hydrolysis of cellulose.

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