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# **Construction of a Structural Enzyme Adsorption/Kinetics Model to Elucidate Additives Associated Lignin-Cellulase Interactions in Complex Bioconversion System**

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27 Enzymatic hydrolysis is a rate-limiting process in lignocellulose biorefinery. The reaction  
28 involves complex enzyme-substrate and enzyme-lignin interactions in both liquid and solid  
29 phases, and has not been well characterized numerically. In this study, a kinetic model was  
30 developed to incorporate dynamic enzyme adsorption and product inhibition parameters into  
31 hydrolysis simulation. The enzyme adsorption coefficients obtained from Langmuir isotherm  
32 were fed dynamically into first order kinetics for simulating the equilibrium enzyme  
33 adsorption in hydrolysis. A fractal and product inhibition kinetics was introduced and  
34 successfully applied to improve the simulation accuracy on adsorbed enzyme and glucose  
35 concentrations at different enzyme loadings, lignin contents, and in the presence of Bovine  
36 Serum Albumin (BSA) and lysozyme. The model provided numerical proof quantifying the  
37 beneficial effects of both additives, which improved the hydrolysis rate by reducing the  
38 non-productive adsorption of enzyme on lignin. The hydrolysis rate coefficient and fractal  
39 exponent both increased with increasing enzyme loadings, and lignin inhibition exhibited  
40 with increasing fractal exponent. Compared to BSA, addition of lysozyme exhibited higher  
41 hydrolysis rates, which was reflected in the larger hydrolysis rate coefficients and smaller  
42 fractal exponents in the simulation. The model provides new insights to support process  
43 development, control, and optimization.

44

45

46 **Keywords:** cellulase, enzymatic hydrolysis, kinetic model, enzyme adsorption, biorefinery

## 47 **Nomenclature**

48	$E_B$	enzyme adsorption, mg/g
49	$E_B^C$	enzyme adsorption on cellulose, mg/g
50	$E_B^L$	enzyme adsorption on lignin, mg/g
51	$E_{eq}$	equilibrium enzyme adsorption, mg/g
52	$E_{eq}^i$	equilibrium enzyme adsorption at equilibrium, mg/L
53	$E_F$	free enzyme concentration in the solution, mg/L
54	$E_T$	total enzyme concentration, mg/g
55	$h$	fractal exponent
56	$k$	time-dependent hydrolysis rate constant, g/mg-h
57	$k_{ad}$	adsorption rate constant, 1/h
58	$k_d$	Langmuir equilibrium constant, mg/L
59	$k_h$	hydrolysis rate coefficient, g/mg-h
60	$K_{iG}$	glucose inhibition coefficient, g/L
61	$S_G$	glucose concentration, g/L
62	$X_{ad}$	additives
63	$X_C$	cellulose concentration, g/L
64	$X_H$	hemicellulose concentration, g/L
65	$X_L$	lignin concentration, g/L
66	$X_S$	solid concentration, g/L
67	$r_C$	rate of cellulose hydrolysis, g/L-h
68	$\delta_{Max}$	maximum adsorption capacity, mg/g

## 1. Introduction

Converting lignocellulosic biomass into carbonaceous polymers is a sustainable approach to reduce our reliance on fossil fuels. Lignocellulosic biomass is abundant and contains various building block chemicals for valorization. Application of the “wastes-derived biomass” can also avoid the competition between biofuel production and food supply. After tremendous efforts and breakthroughs in the key techniques, however, large scale application of lignocellulose biorefinery is still challenging, which may owe to both technical and non-technical issues. Enzymatic hydrolysis is a cost intensive process to release fermentable carbon (*e.g.*, glucose and xylose) from the polysaccharides (Humbird et al., 2011). Supplementation with commercial enzymes to enrich the activities of xylanase, pectinase and  $\beta$ -glucosidase might improve the cellulose hydrolysis efficiency (Berlin et al., 2007). Compared to chemical conversion, enzymatic hydrolysis has attracted significant attention due to its high conversion yield, minimal byproducts formation, and mild operation conditions, but this process also suffers from the significant biomass recalcitrance against bioconversion (dos Santos et al., 2018). Cellulose is entrapped in the complicated 3-D structure of hemicellulose and lignin, which is difficult to reach by cellulase (Leu & Zhu, 2013). Pretreatment is an essential process to break down the recalcitrant structure of the lignocellulosic biomass and improve the efficiency of enzymatic saccharification. The mechanism of delignification and performances of different pretreatment approaches have been reported outstandingly elsewhere (Mosier et al., 2005; Sathitsuksanoh et al., 2013). The beneficial or negative impacts of dissolved and residual lignin on the pretreated substrate have also been addressed in many publications (dos Santos et al., 2018; Liu et al., 2016). Phenolic compounds including lignin degradation products, hydroxynamic acid derivatives, tannis and gallic acids released during pretreatment might cause 20-80% deactivation of the major enzymes for cellulose hydrolysis (Ximenes et al., 2011). The existing work focuses on

the development of a robust numerical method to simulate the dynamic status of enzymatic hydrolysis of lignocellulosic substrate in a complex hydrolysate.

Extensive studies of cellulose hydrolysis kinetics have been conducted in the past several decades (Jeoh et al., 2017; Liao et al., 2008; Ye & Berson, 2011). Enzymatic hydrolysis has been characterized as a two-phase heterogeneous process, comprising solid and liquid phases, which is the dynamic interaction between enzyme adsorption on the surface of cellulose and release of reducing sugars. The empirical formula can be applied to quantify the effects of substrate properties, such as specific surface area, crystallinity and lignin content; and enzyme properties like enzyme concentration, and enzyme to solid ratio. Michaelis-Menten is the typical empirical model for homogeneous reactions and is applicable for the initial rate estimation of cellulose hydrolysis. However, the dynamic interaction and mechanism between cellulase and cellulose are not characterized in the empirical functions. Enzyme adsorption and hydrolysis product inhibition were commonly considered into the kinetic model of enzymatic hydrolysis of lignocellulosic biomass. However, most of the studies mainly focused on the effects of reducing sugar inhibition. Kadam et al. (2004) incorporated the inhibitions of cellobiose, glucose and xylose into the lignocellulose hydrolysis model. Shi et al. (2017) reported that surface morphology and structure of substrate were changed by the dynamic enzyme activities by examining Atomic Force Microscope in combination with Fourier-transform infrared and Raman spectroscopy. The altered surface features might greatly impact enzyme adsorption on the surface of cellulose and release of reducing sugars. Structural change in the lignocellulosic fibers and composition during the enzymatic hydrolysis make enzyme adsorption a dynamic process as the hydrolysis progresses. Liao et al. (2008) proposed a dynamic enzyme adsorption based on Langmuir isotherm that incorporated changes of adsorption constant as the cellulose hydrolysis progresses. Toyosawa et al. (2017) investigated the effects of non-catalytic protein addition on enzymatic hydrolysis

and found that application of lysozyme reduced the non-specific adsorption of cellulase on lignin and improved enzymatic hydrolysis. However, the kinetic study has not been conducted to describe the relationship between enzyme adsorption and hydrolysis of lignocellulosic substrate with the impacts of enzyme loadings and different additives.

This study aims to construct a kinetic model to simulate the dynamic status and interactions of enzyme adsorption and cellulose hydrolysis with product inhibition and cellulose accessibility. As enzyme adsorption equilibrium was changed with time and during enzymatic hydrolysis, a pseudo first order adsorption kinetic was used to reflect the changing properties of enzyme-cellulose system. The specific objectives of the experimental and numerical works designed in this study are: (a) to understand the effects of different pretreatment methods and initial enzyme loadings on enzyme adsorption and hydrolysis, hence further determine the parameters that can best reflect the enzyme adsorption in the system; (b) to demonstrate the dynamic change of enzyme adsorption and enhancement of cellulose conversion in addition of Bovine Serum Albumin (BSA) and lysozyme during the entire cellulose hydrolysis process; and (c) to develop a mathematic model that incorporate the dynamic interaction among enzyme adsorption, cellulose hydrolysis, and product inhibitions for different lignocellulosic substrates.

## **2. Model development**

### *2.1 System boundary*

The proposed model structure is depicted in **Figure 1**. The model was validated under three designed conditions with specific order (from simple to complex) of experiments. In the first system (**Figure 1a**), cellulase was introduced to break down the cellulose ( $X_C$ ) into reducing glucose without the interference of lignin on the substrate. The enzyme adsorbed on the surface of cellulose ( $E_B^C$ ) is the active enzyme, which can be quantified by the total

enzyme deducted by the measured free protein concentration (mg/g) in the supernatant (free enzyme,  $E_F$ ). Further in the second system (**Figure 1b**), enzyme could be adsorbed by both cellulose and lignin ( $X_L$ ) with various tendencies. The enzyme adsorbed on the surface of lignin is defined as ineffective enzyme-substrate or enzyme-lignin complex ( $E_B^L$ ). In the third system (**Figure 1c**), additives ( $X_{ad}$ ) were introduced in the hydrolyzate, which may offer various forms of beneficial effects to saccharification, such as selectively attach on the surface of lignin, and/or prevent the non-productive adsorption of cellulase to lignin.

Several key assumptions have been made to simplify the model. The structure of substrate is assumed to be uniform for enzyme adsorption. The three major working enzymes involved in cellulose hydrolysis are considered as one enzyme with multiple functions as described by Liao et al. (2008). Although one of the major enzyme  $\beta$ -glucosidase which hydrolyze the cellobiose into glucose is subjected to both product and substrate inhibition (Hong et al., 1981; Zhao et al., 2004), cellobiose inhibition was considered insignificant in this study due to the high activity of  $\beta$ -glucosidase in the applied commercial enzyme. The measured concentrations of cellobiose were negligible during the experiments. The conversion of cellobiose to glucose is not a rate limiting factor to cellulose hydrolysis and is not described in the model. Also, since the xylose concentration was very low, inhibition caused by xylose was neglected. Thus, enzyme adsorption on cellulose and glucose inhibition were the only limiting factors considered for the enzymatic hydrolysis.

## 2.2 Selection of model principles

To construct a proper model structure to simulate the interested mechanisms during enzymatic hydrolysis, we reviewed the most commonly applied model structures in the literatures and summarized them in **Tables S1** and **S2**. The simulation models were composed of ordinary differential equations (ODEs) associated with different reaction kinetics and

sub-functions (to characterize/quantify the relationships among different parameters with physical meanings). The enzyme adsorption functions in the hydrolysis model can be classified into eight major categories (**Table S1**). The complexity of the model increased over time, of which the three adsorption functions published between 1983 and 2007 (Fan & Lee, 1983; Kadam et al., 2004; Liao et al., 2008; Xu & Ding, 2007) did not consider the dynamic changes of adsorbed enzyme on the substrates. Gan et al. (2003) applied separated first order adsorption and desorption kinetics to simulate the dynamic changes of adsorbed enzyme on the substrate, but the model cannot quantify the impacts of non-productive adsorption of enzyme to lignin. Wang et al. (2014) developed a model structure with the combination of the adsorption parameters and dynamic adsorption of enzyme on the substrate, but the simulation results did not fit well with the experimental data.

The enzyme adsorption kinetics were incorporated into five basic forms of reaction kinetics (**Table S2**). Most of the hydrolysis kinetics are constructed based on the first order function associated with inhibiting terms of concerned reagents, products, impurities, and/or additives (Kadam et al., 2004; Liao et al., 2008; Mutturi & Lidén, 2014). The model structure proposed by Gan et al. (2003) described the comprehensive relationships among various forms of celluloses with reasonable fits between the simulated and measured sugars, but the model structure need further development to characterize the hydrolysis of lignocellulosic substrate and the impacts of additives.

### *2.3 Model structure*

To quantify the impacts of lignin content to enzyme adsorption, a few modifications were made to combine the equilibrium enzyme adsorption functions with the hydrolysis kinetics. The simulation was first expressed by the adsorption isotherm under equilibrium condition (no hydrolysis, at 4°C) to obtain the equilibrium adsorption coefficients. The enzyme



adsorption followed by Langmuir isotherm with a first order reaction was described in Equation (1):

$$E_{eq}^i = \frac{\sigma_{Max} \cdot X_s^0 \cdot E_F}{k_d + E_F} \quad (1)$$

where  $E_{eq}^i$  is the concentration of enzyme adsorbed on the surface of substrate (mg/L) at equilibrium;  $\sigma_{Max}$  is maximum adsorption capacity (mg/g-substrate);  $k_d$  is Langmuir equilibrium constant, (mg/L);  $E_F$  is measured free enzyme concentration in the supernatant (mg/L); and  $X_s^0$  is the initial solid concentration (mg/L).

With the information of adsorption isotherm, the dynamic status of enzyme adsorption on the substrate can be described by pseudo first order kinetics as follows:

$$r_B = k_{ad} \cdot (E_{eq} - E_B) \quad (2)$$

$$E_{eq} = \frac{E^i}{X_s} \quad (3)$$

where  $k_{ad}$  is adsorption rate constant (1/h);  $E_B$  and  $E_{eq}$  are the actual and equilibrium enzyme concentration during enzymatic hydrolysis (mg/g).

The actual absorbed enzyme on cellulose ( $E_B^C$ ) and lignin ( $E_B^L$ ) can be deduced using the following equations:

$$E_B^C = E_B \cdot \frac{X_C}{X_s} \quad (4)$$

$$E_B^L = E_B \cdot \frac{X_L}{X_S} \quad (5)$$

217

218 The cellulose hydrolysis ( $r_c$ ) was modeled as a first order kinetic reaction in turn of  
 219 concentration of adsorbed enzyme on cellulose, cellulose concentration, and glucose  
 220 inhibition as:

$$r_C = \frac{k_h \cdot E_B^C \cdot X_C}{1 + S_G/K_{IG}} \quad (6)$$

222 where  $k_h$  is the hydrolysis rate coefficient (g cellulose/mg protein-h);  $K_{IG}$  is the glucose  
 223 inhibition constant (53.16 L/g) (Philippidis et al., 1993).

224

225 A fractal kinetic equation was used to describe the hydrolysis rate coefficient  $k_h$ , which  
 226 decays over time with a fractal exponent  $h$  as shown in Equation (7).

227

$$k_h = k \cdot t^{-h} \quad (7)$$

229

230 The overall mass balance for the cellulose (solid) and glucose (dissolved) can be  
 231 expressed as follows:

232

233 Enzyme adsorption:

$$\frac{dE_B}{dt} = r_B \quad (8)$$

235 Cellulose:

$$\frac{dX_C}{dt} = -r_C \quad (9)$$

237 Glucose:

$$\frac{dS_G}{dt} = 1.11 \cdot r_C \quad (10)$$

239 Total solid:

$$X_S = X_C + X_L + X_H \quad (11)$$

241 Total enzyme:

$$242 \quad E_T = \frac{E_F}{X_S} + E_B \quad (12)$$

243 where 1.11 is the conversion constant of cellulose to glucose.

244

#### 245 *2.4 Validation of parameters*

246 The model was coded by Matlab R2013a (MathWorks). The dynamic functions were  
247 solved by using the ‘ODE45’ function (Runge-Kutta formula). The simulation results were  
248 fitted with the experimental data obtained at designed conditions, chemicals, and substrates as  
249 detailed in the next section. The optimization function ‘lsqnonlin’ developed upon  
250 Levenberg-Marquardt algorithm was adopted for the non-linear least square (SSD)  
251 minimization over the residual between model values ( $X_{pi}$ ) and the corresponding experiment  
252 data ( $X_{expi}$ ). The objective function to estimate the kinetic parameters is as follows:

253

$$254 \quad SSD = \min \sum_{i=1}^n \sqrt{(X_{pi} - X_{expi})^2} \quad (12)$$

255

256 In addition to parameter optimization, sensitivity analysis was also conducted to visualize and  
257 identify the importance of different parameters that significantly affect the reaction.

258

### 259 **3. Experiments**

260 The basic concept of experimental design, measured parameters, and determined kinetics  
261 have been illustrated in **Figure 2**. Based on the designed experiment conditions, control  
262 parameters and substrate properties were gradually applied in the model to simulate the  
263 output parameters with increasing complexity. The proposed kinetic coefficients for

adsorption isotherm and reaction kinetics were then validated from the basic to more complex systems.

### *3.1 Materials/pretreatment of lignocellulosic materials*

Three lignocellulosic substrates were selected in this study to investigate the impacts of lignin contents to enzyme hydrolysis. The tested Kraft pulps were produced from Australian *Eucalyptus globules* wood chips by using a M/K digester (Peabody, MA, USA), with a liquid-to-solid ratio of 4 (w/v). The liquid involved NaOH (25% sulfidity) and Na<sub>2</sub>S (17% active alkali). The digester was heated to 160°C at a rate of 1.5°C per min, and after that the temperature was maintained isothermally for 90 minutes (total cooking time is approximately 180 mins). The resulted unbleached Kraft pulps (UEK) were used as substrate for the experiments of cellulase adsorption and enzymatic hydrolysis. Bleached Kraft pulps (BEK) were prepared from oxygen bleached pulps by commercial DEDD bleaching sequence to completely remove lignin (Ko et al., 2011). Steam explosion pulps (SEP) were prepared from 1 kg dried rice straw in 1.5% H<sub>2</sub>SO<sub>4</sub> solution and saturated steam at the temperature of 190°C for 10 to 20 minutes. The solid to liquid ratio is 1:7. All the sample sizes ranged from 0.075 to 0.15 mm were selected for the further experiments by fiber size fractionation with 100-mesh screen. Chemical composition analysis of the samples were performed according to the National Renewable Energy Laboratories (NREL) standard procedures (Sluiter et al., 2006; Sluiter et al., 2008). The enzyme used in this study is C-Tec (Novozyme, China) with a protein concentration of 50 g/L.

### *3.2 Adsorption of cellulase*

Adsorption of cellulase on various pretreated substrates was conducted at 4 and 40°C in sodium acetate buffer (pH 6.0). The solid loading was 0.1% (w/v) and the working volume

was 15ml. The lower solid loading was used to avoid mass-transfer limitation that otherwise might confound the major findings. The enzyme dosages were 5, 10, 20, 30, 40, 50 and 60 mg/g solid for the adsorption equilibrium experiment. Samples were centrifuged at 5,000 rpm at 4°C for 20 minutes after the solution achieving adsorption equilibrium (well mixed in shaking flask (150 rpm) for 1 h). The remaining free enzyme concentration in the supernatant was determined by Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976). Samples were mixed with Coomassie blue dye and then were measured by a UV spectrometer (PerkinElmer LAMBDA 25) at 590 nm. The absorbed enzyme was calculated from the difference between initial enzyme and measured free enzyme concentration. The pretreated substrates underwent hydrolysis when the adsorption was conducted at 40°C. Thus, enzyme adsorption was determined by accounting the content of solid cellulose remaining in the system at each sampling point.

### *3.3 Enzymatic hydrolysis of lignocellulose biomass*

Enzymatic hydrolysis of various pretreated substrate was carried out after similar approaches as stated in Ko et al. (2011). The enzyme loadings were 0.5, 5, and 50 mg/g solid. The pH was set at 6.0 and the temperatures were 4 and 40°C to investigate the adsorption and hydrolysis processes, respectively. The pH 6.0 was defined to facilitate the process of simultaneous scarification and fermentation (SSF) as the optimal pH for most of the fermenting microorganisms was natural. Aliquot samples of 0.5 ml were taken at designed time intervals (1, 2, 4, 8, 12, 24 and 48h) from the mixtures and were centrifuged at 5,000 rpm at 4°C for 20 mins. Reducing sugar concentrations were measured by the dinitrosalicylic acid (DNSA) assay (Sengupta et al., 2000). Similarly, reducing sugar concentrations were determined by accounting the content of solid cellulose remaining in the system at each

sampling point. Enzyme activity, protein concentration, reducing sugar concentration, reaction rate, and substrate concentration were measured at each sampling point.

### *3.4 Adsorption and hydrolysis with additives*

The three pretreated substrates were mixed with excessive dosages of BSA and lysozyme for 24 hours until saturation adsorption, and then the saturated adsorbed substrates were washed twice by sodium acetate and DI water. The substrates were then resuspended in sodium acetate buffer (pH 6) for cellulase adsorption and hydrolysis as described in the section 3.2 and 3.3.

## **4. Results and discussion**

### *4.1 Lignin induced influence on adsorption isotherm*

The chemical compositions of the three selected substrates for enzymatic hydrolysis experiments and simulations are presented in **Table 1**. The key control parameters defined from the selected substrates is the lignin contents, of which increased from 0, 3.8, to 18.9% for BEK, UEK, and SEP, respectively. The Langmuir adsorption parameters for different pretreated lignocellulosic biomass were determined by nonlinear regression as illustrated in **Figure S1**, and the measured adsorption data were shown in **Table S3**. The amount of protein adsorbed on the substrates varied significantly with the types of tested substrates, pretreatment methods and additives. BEK samples showed the highest enzyme maximum adsorption capacity of 36.38 mg/g-substrate, followed by UEK sample (25.86 mg/g-substrate). SEP samples showed the lowest maximum adsorption capacity of 19.07 mg/g-substrate. This trend is consistent with the amount of lignin available in the composition of the pretreated substrates (**Figure 3**). SEP samples with the lowest enzyme adsorption capacity contained the highest amount of lignin, while the lignin-free sample BEK

338 had the highest adsorption capability. The different enzyme adsorption could be due to the  
339 higher affinity of the cellulase to the holocellulose than lignin (Nakagame et al., 2010). Ko et  
340 al. (2015) reported that the maximum cellulase adsorption capacities of isolated lignins of  
341 difference severities from liquid hot water pretreated hardwood were 36.6-44.8 mg/g lignin,  
342 while showed a significant difference in the maximum adsorption capacity of lignin isolated  
343 from different pretreatment strategies. Isolated lignin from sulfur dioxide pretreatment had  
344 the highest adsorption capacity (67.5 mg/g lignin) and ammonia fiber expansion (AFEX) had  
345 the lowest adsorption capacity (38.7 mg/g lignin). Steam explosion of acid-impregnated  
346 biomass resulted in high removal of the hemicellulose but not the lignin. Besides, bleaching  
347 using a strong oxidizing agent accelerates the ring opening of the phenolic structures and the  
348 alkaline condition of the reaction medium assists in the extraction of the fragmented lignin.  
349 Using such an efficient alkaline-based oxidative delignification process, lignin is eliminated  
350 and cellulose is well-exposed to enzyme, leading to higher adsorption of cellulase on the  
351 surface of cellulose. Kumar et al. (2012) showed the lignin restricted cellulose hydrolysis  
352 through unproductive adsorption of cellulase inhibition of swelling of cellulose and therefore  
353 the cellulose accessibility. The cellulose accessibility and hydrolysis yield increased with the  
354 increasing lignin removal. Li et al. (2016) reported that addition of lignin caused reduction in  
355 maximum adsorption capacity of enzyme on pretreated corn stover. Lignin adsorbed onto the  
356 cellulose and sequentially reduced the surface area for enzyme adsorption. Selig et al. (2007)  
357 reported that lignin droplets produced from corn stover during pretreatment can deposit back  
358 onto the surface of celluloses, which potentially imposed adverse effects on enzyme  
359 adsorption and the efficiency of cellulose conversion. The lignin redeposited on the surface of  
360 pretreated biomass with a more condensed, hydrophobic, syringyl-deficient form of lignin  
361 after high temperature and severity pretreatment, which adsorbed more enzyme protein (Dos  
362 Santos et al., 2019). Compared to the low operation temperature, the adsorption kinetic of

lignin was slower and imposed minimal impact on enzyme activity at high and optimal operational temperature (Zheng et al., 2013). The enzyme adsorption on cellulose reached the adsorption peak within 0.25 hour, while adsorption on lignin took more than 12 hours to reach equilibrium at 50°C. The increasing enzyme loadings might result in enzyme crowding or competition for binding sites among the three working enzymes, impurity and other proteins that limit enzyme adsorption to cellulose.

#### *4.2 Effects of enzyme loading*

The effects of different enzyme loadings on enzymatic hydrolysis of the three pretreated substrates were investigated. The time course of enzyme adsorption and cellulose hydrolysis of three pretreated lignocellulosic biomass with initial enzyme loading of 0.5, 5 and 50 mg/g solid are plotted over time as shown in **Figure 4**. The experimental data of adsorbed enzyme and releasing sugar were fitted with the proposed model and the kinetic parameters were listed in **Table S4**. The enzyme absorption and glucose yield increased with the increasing enzyme loadings. The hydrolysis efficiency increased more than 16 times for BEK and UEK, and increased 11 times for SEP at the higher enzyme loading (50 mg/g). A higher enzyme to substrate ratio effectively enhanced the hydrolysis performance as reported in many other studies (Chandra et al., 2011; Gregg & Saddler, 1996; Tengborg et al., 2001). The enzymatic hydrolysis rate was retarded with the prolonged experimental time. The hydrolysis rate coefficient and the fractal exponent increased with the increasing enzyme loading for all the three pretreated substrates. The hydrolysis rate coefficient remained the same with a further increase in the enzyme loading, which might due to saturated adsorption of enzyme to cellulose (Wang et al., 2011). The increase in fractal exponent with increasing enzyme loading might be caused by overcrowding of the enzyme, which limited the enzyme diffusion on the surface of substrate at increasing enzyme loading (Xu & Ding, 2007).



The hydrolysis rate coefficients were similar among three pretreated substrates, while SEP showed a higher impact on the fractal exponent, which might be ascribed to the high lignin content of SEP substrate. The fractal exponent of the cellulose hydrolysis of BEK and UEK were relatively low compared to SPE, which confirmed that fractal exponent is related to the nature of the substrate. Wang et al. (2011) had performed enzymatic hydrolysis of Avicel with various loadings of isolated lignin and simulated with the fractal kinetics. The lignin inhibition on the enzymatic hydrolysis was exhibited with the increased fractal exponent while the hydrolysis rate coefficient was unchanged. The hydrolysis performance of substrate with high lignin could be improved by increasing of enzyme loading.

#### *4.3 Effects of additives*

Enhancement of enzymatic hydrolysis by addition of surfactants and polymers had been studies extensively. Yang and Wyman (2006) reported that only a little amount of BSA was absorbed on pure cellulose while lignin containing substrate adsorbed a significant amount of this additive. Toyosawa et al. (2017) showed that the lysozyme adsorbed only to lignin containing substrate and prevent nonproductive adsorption of cellulase by covering the surface of lignin. To improve the efficiency of cellulose conversion, BSA and lysozyme were added in attempt to block protein adsorption site of lignin and therefore reduce the nonproductive adsorption of enzyme. Kim et al. (2015) reported that the addition of BSA reduced the enzyme loading from 15 FPU/g solid to 1.3 FPU/g solid while achieving 80% of hydrolysis conversion. Ko et al. (2015) reported that a significant improvement of enzymatic hydrolysis from 17% to 72% for the liquid hot water pretreated hardwood chips with pre-incubation of 50mg/g BSA at low enzyme loading of 8 mg/g solid.

The effects of BSA and lysozyme addition on the enzyme adsorption and cellulose hydrolysis of BEK, UEK and SEP samples were investigated. The time course of enzyme

adsorption and cellulose hydrolysis of three pretreated lignocellulosic biomass with and without addition of BSA and lysozyme at 5 mg/g enzyme loading are plotted over time in **Figure 5**. The results of BSA and lysozyme addition on the adsorption isotherms of cellulose onto various pretreated substrates at 4°C are showed in **Figure S1**. With the addition of saturated BSA, the maximum adsorption capacity of the three pretreated substrates decreased; and the maximum adsorption capacity of BEK and UEK decreased while SEP increased with the addition of lysozyme (**Figure 3** and **Table S3**). The enzyme adsorption showed the same pattern with and without BSA addition reaching equilibrium at 4.5 mg/g and 4.0 mg/g for BEK and UEK hydrolysis; while only slightly decrease of enzyme adsorption with BSA addition for SEP hydrolysis. In contrast, the enzyme adsorption reduced with the lysozyme addition for all three samples. The equilibrium enzyme adsorption was reached at approximately 1.0 mg/g for UEK and SEP samples.

While the scope of this project is to investigate the numerical relationship among the complex enzyme-cellulose-lignin interactions, the selected additives did show significant beneficial impacts to enzymatic hydrolysis at low enzyme loading (5 mg/g). With the addition of BSA and lysozyme, glucose yield increased from 29.3% to 36.0% and 58.4% for UEK; from 19.1% to 29.6% and 55.5% for BEK; and from 17.2% to 33.2% and 34.5% for SEP, respectively. Addition of lysozyme enhanced hydrolysis yield the most for UEK (36.4%), BEK (29.1%) and SEP (18.5%), in contrast to improvement of 6.6-17.1% with BSA supplement. The two additives BSA and lysozyme are oppositely charged at the designed pH (6.0) in this study, with acidic (4.7-5.3) and alkaline (10.7-11.3) isoelectric points, respectively (Mueller et al., 2010). Cai et al. (2018) reported that the isoelectric points of cellobiohydrolase I (CBH I) and CBH II were 3.4-4.7 and 5.3-5.6, respectively.  $\beta$ -glucosidase from *T. reesei* had a higher isoelectric points of 5.7 to 6.4 (Ko et al., 2015). The enzyme systems were negatively charged with and without addition of BSA in the buffer of 6.0, thus

an electrostatic repulsion was formed between the enzyme and cellulose surface. Zhan et al. (2019) observed that addition of lignin carboxylate with pI around the hydrolysis pH (4.8) showed significant enhancement of hydrolysis.

Apart from the traditional and expensive BSA and lysozyme, soybean protein was used as potential lignin-blocking additive to mitigate the adverse impact of lignin and improve the cellulose hydrolysis performance. Brondi et al. (2020) reported an increase of more than one quarter and overall conversion efficiency reached 80% in cellulose hydrolysis of sugarcane bagasse with addition of 12% (w/w) soybean concentration. Nitrogen gas was also used for displacing air to prevent enzyme deactivation by oxygen and hence improve hydrolysis performance especially at high solid loadings (Dos Santos et al., 2020).

#### *4.4 Adsorption capacity and hydrolysis*

Langmuir adsorption isotherm is widely used to describe and quantify enzyme adsorption (Kadam et al., 2004). However, Langmuir adsorption isotherm only described the enzyme adsorption at the equilibrium, while it is a dynamic process as it took over 12 hours to reach adsorption equilibrium (**Figure 4**). Especially at the high initial concentration of enzyme (50 mg/g), enzyme attached to the surface of cellulose rapidly with a peak adsorption of 15.7 mg/g, 11.7 mg/g and 9.5 mg/g for BEK, UEK and SEP within 1 hour, then followed by a desorption of enzyme caused by the initiation of hydrolysis, and eventually reached equilibrium. Enzyme adsorption on solid surface and their effectiveness are the two factors hypothesized to have a major impact on enzymatic hydrolysis. A linear relationship between enzyme adsorption and initial hydrolysis rate had been reported in previous studies (Nidetzky & Steiner, 1993).

The overall patterns of enzyme adsorption matched the adsorption on pure cellulose, with an absorption peak reached within 1 hour, followed by a bounce back release of adsorbed

cellulase to the solution, and eventually reached a stable adsorption (Zheng et al., 2013). The reaction rate of cellulose hydrolysis should be the same with and without lignin when the enzyme loading is above the  $k_d$  of the cellulase-cellulose adsorption. It is expected the hydrolysis rate reduced when the enzyme loading is below the value of  $k_d$ . The adsorption kinetic of cellulase regulates the distribution of cellulase between cellulose and lignin during the dynamic adsorption-reaction process. Porosity, adsorption kinetic, binding selectivity and chemical characteristic may all have significant impacts on enzyme adsorption on lignin containing biomass (Zheng et al., 2013). Although the enzymes were considered as an enzyme complex with multiple functions for the model development in this study, various enzyme components showed different degree of adsorption on lignin.  $\beta$ -glucosidase exhibited the strongest adsorption onto lignin (more than 80%) and indirectly lead inhibit the enzymatic hydrolysis of cellulose (Ko et al., 2015).

#### *4.5 Sensitivity and validation of kinetic coefficients*

Sensitivity analysis was performed to clarify the importance of the key reaction coefficients ( $k_{ad}$ ,  $k$ , or  $h$ ) to enzymatic hydrolysis reactions under different conditions. The optimal base values of all parameters were obtained by solving the ODEs and presented in **Table S4**. Using glucose concentration as an example, **Figure S2** presents the effects and significance of different kinetic parameters on the simulation results. The sensitivities of the simulation coefficients were presented in matrix form: the five experimental conditions were presented in columns for the enzyme loadings of (a) 0.5 mg/g, (b) 5 mg/g, (c) 50 mg/g, and with the addition of (d) BSA, and (e) lysozyme at the enzyme loading of 5 mg/g, respectively; and three substrates were presented in rows for (a) BEK, (f) UEK, and (k) SEP, respectively. The relative errors between simulation results and measured data were calculated by changing the kinetic coefficients from -40% to 40% of the standard deviation, when the other

parameters were fixed at the optimal values. The sensitivities of different kinetics coefficients showed no regular pattern among experimental conditions and substrates. Most of the simulation results were within  $\pm 20\%$  relative errors if the variation of selected coefficients were limited at  $\pm 10\%$  standard deviations of the optimal values. The adsorption coefficient  $k_{ad}$  showed a relatively small influence for all the operational conditions of the three pretreated substrates, while the fractal exponent coefficient  $h$  was the most significant parameter affecting the overall reaction.

The fractal kinetic parameters with the addition of BSA and lysozyme are summarized in **Figures 6a** and **6b**. The optimized fractal exponent coefficients  $h$  generally increased with the increased amount of lignin contents in the substrate, and increased with the increases of enzyme loadings. The value of this parameter was significantly reduced when additives were introduced in the processes, of which closer to 0.5 mg/g than 5 mg/g used, implying certain impacts of the additives to enzyme activities especially for high cellulose substrates (BEK and UEK). However, addition of BSA and lysozyme increased the hydrolysis rate coefficients (**Figure 6b**). Compared to the fractal exponent, the hydrolysis rate coefficient increased obviously in the presence of lysozyme and BSA, for which the value (0.012 and 0.016) at 5 mg/g enzyme loading performed nearly the same as those measured at 50 mg/g. Wang and Feng (2010) reported that the rate coefficient increased with the increases of the surfactant concentration until a saturated value was reached, while the fractal exponent decreased with the increase of the surfactant concentration. The addition of BSA and lysozyme were saturated before the enzymatic hydrolysis experiment in this study.

## 5. Conclusion

Key parameters of enzyme adsorption isotherm were integrated into cellulose hydrolysis kinetics, which facilitated the simulation of dynamic enzyme adsorption and sugar release

during enzymatic hydrolysis. The first order fractal model characterized quantitatively the cellulose hydrolysis rates and sugar yields at various enzyme dosages, lignin inhibition in different substrates, and the enhancement of cellulose conversion with additions of BSA and lysozyme. The two coefficients, *i.e.*, hydrolysis rate coefficient and fractal exponent coefficients, provided both numerical and physiochemical insights toward more effective and economic pretreatment, saccharification, and enzyme recycling process in lignocellulose biorefinery. The incorporation of dynamic enzyme adsorption into the cellulose hydrolysis model reveals the interrelationship of adsorbed enzyme and cellulose hydrolysis rate, providing new perspectives towards better understanding and future modification in the model structure.

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## **Data available statement**

The data that support the findings of this study are available from the corresponding author upon request.

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## Figure Captions

**Figure 1.** Schematic diagram of the dynamic model with concerned parameters for simulation of enzymatic hydrolysis of three scenarios: (a) pure cellulosic substrate; (b) lignocellulosic substrate; and (c) lignocellulose with additives.

**Figure 2.** Work flow for kinetic modeling of enzymatic hydrolysis.

**Figure 3.** Relationships of maximum adsorption capacity ( $\delta_{Max}$ ) and Langmuir equilibrium constant ( $k_d$ ) for three substrates at different hydrolysis conditions. Lignin and additives both play critical roles in enzymatic adsorption.

**Figure 4.** Simulation (dash lines) and experiment results (symbols) of dynamic enzyme adsorption and glucose release at 40°C with 1% (w/v) solid content for (a) & (d) BEK, (b) & (e)UEK and (c) & (f) SEP with enzyme loadings of 0.5 mg/g, 5 mg/g, and 50 mg/g.

**Figure 5.** Simulation (dash lines) and experiment results (symbols) of dynamic enzyme adsorption and glucose release at 40°C with 1% (w/v) solid content for (a) & (d) BEK, (b) & (e)UEK and (c) & (f) SEP with/without the addition of BSA/lysozyme at the enzyme loading of 5mg/g.

**Figure 6.** Relationship between substrate lignin contents and (a) fractal exponent and (b) hydrolysis rate coefficients.

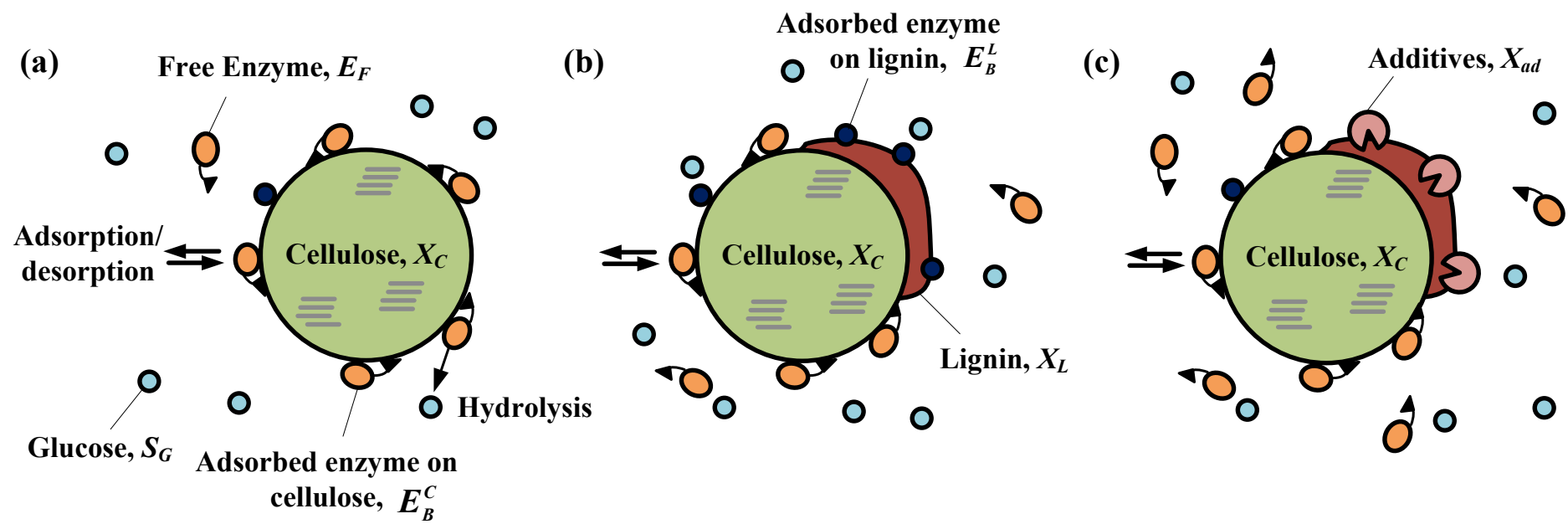


Figure 1

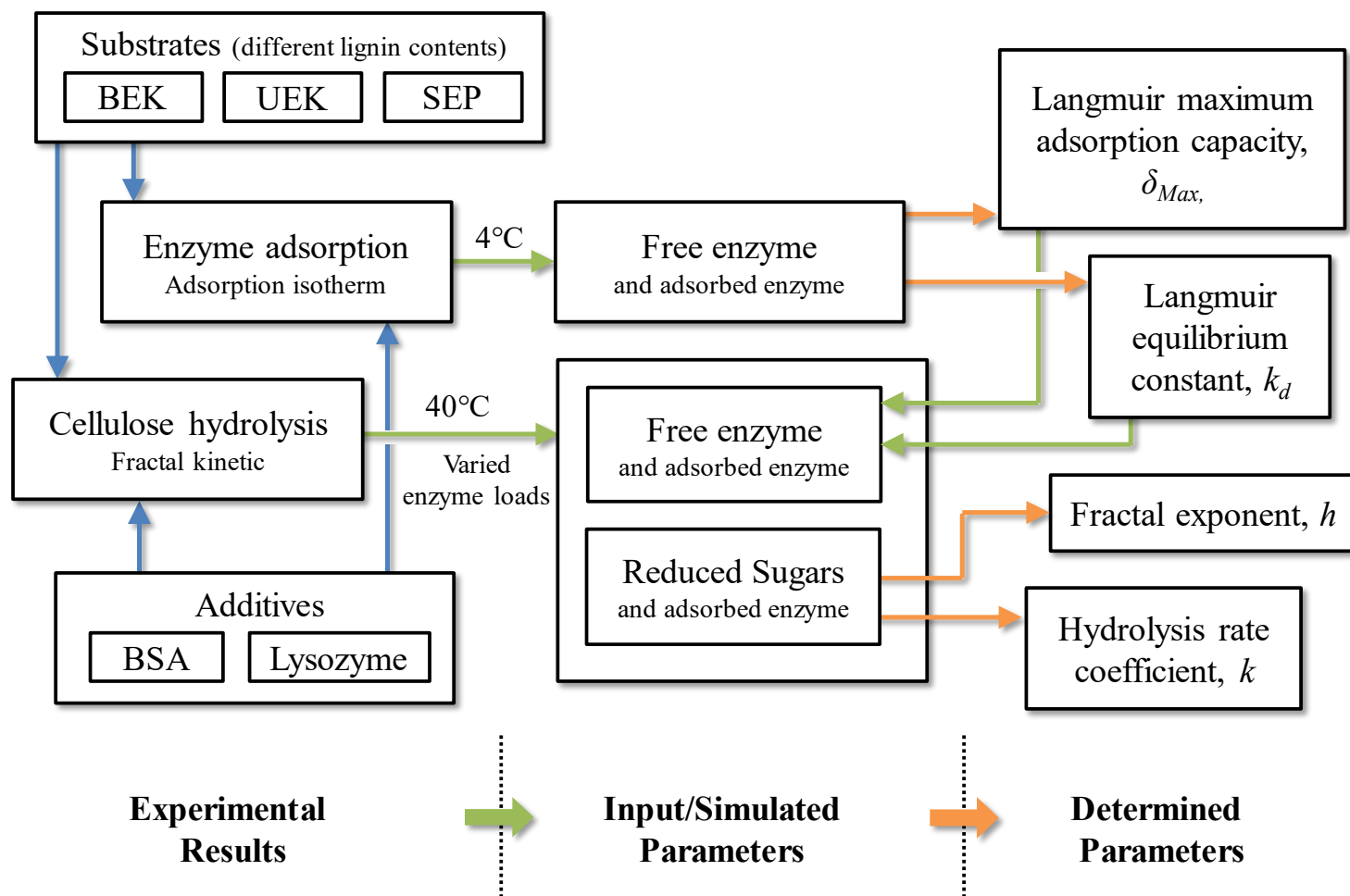


Figure 2

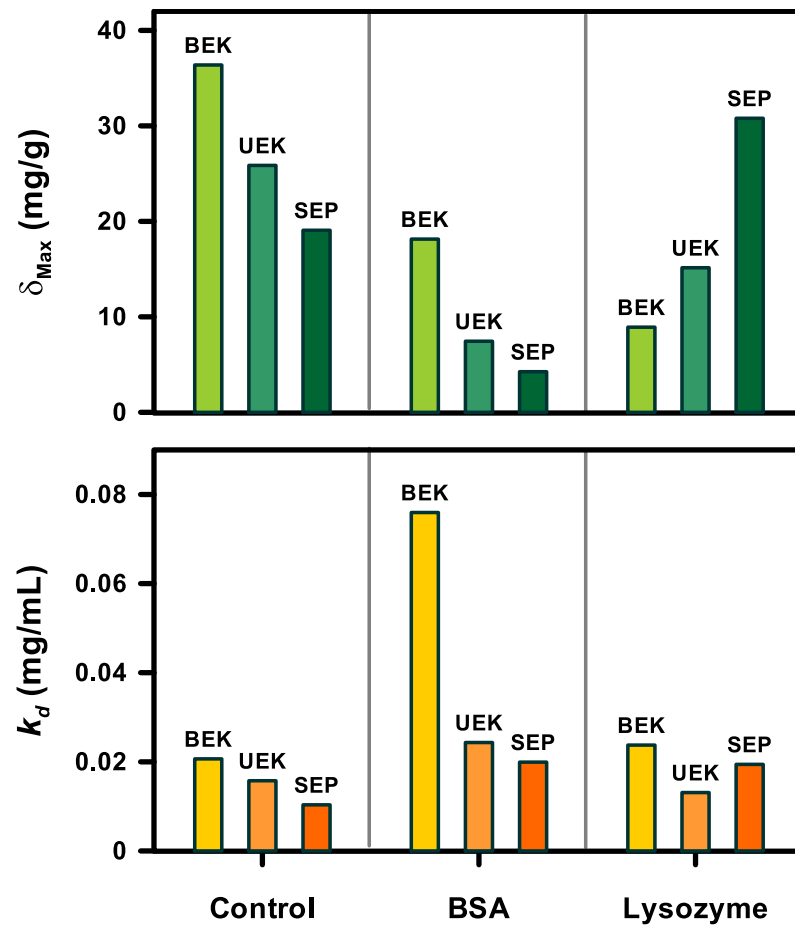


Figure 3

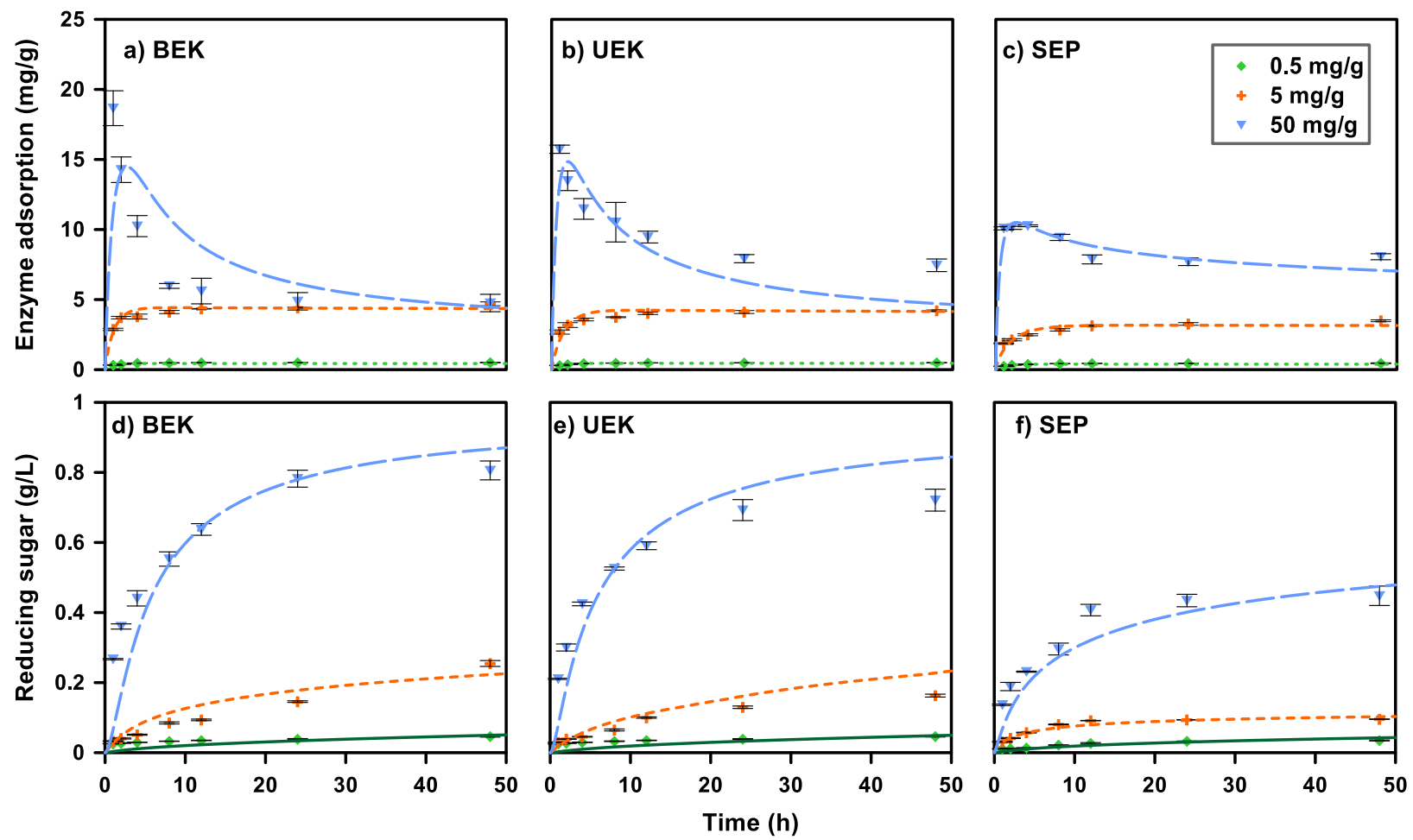


Figure 4

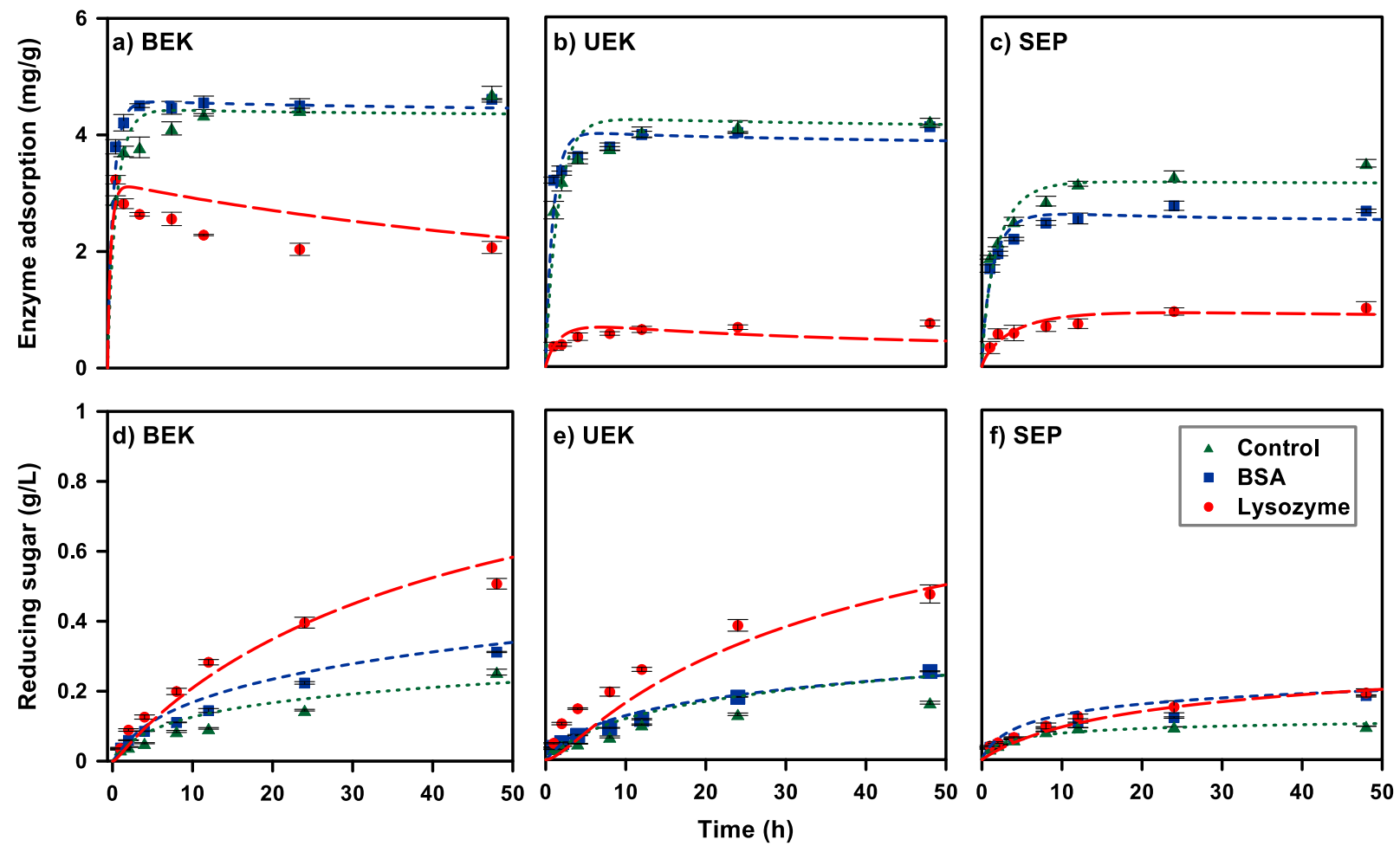


Figure 5

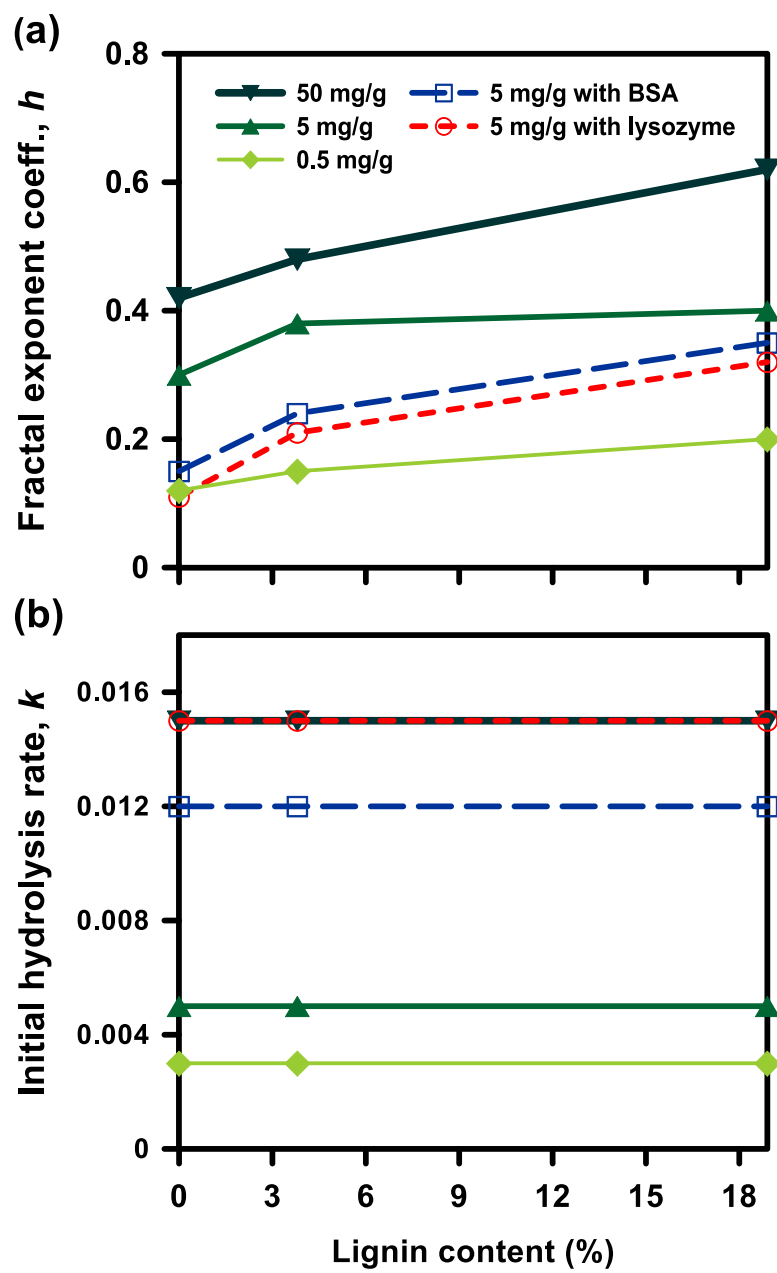


Figure 6



**Table 1.** Chemical compositions of pretreated substrates (wt.%)

Sample	Holocellulose	$\alpha$ -cellulose	Hemicellulose	Lignin	Ash
BEK	96.5	83.8	12.7	-	0.8
UEK	94.8	81.0	13.8	3.8	0.8
SEP	61.5	57.9	3.6	18.9	11.6