ARTICLE

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Kinetic Modeling of Cellulosic Biomass to Ethanol Via Simultaneous Saccharification and Fermentation: Part II. Experimental Validation Using Waste Paper Sludge and Anticipation of CFD Analysis

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ABSTRACT: A kinetic model of cellulosic biomass conversion to ethanol via simultaneous saccharification and fermentation (SSF) developed previously was validated experimentally using paper sludge as the substrate. Adsorption parameters were evaluated based on the data obtained at various values for fractional cellulose conversion. The adsorption model was then combined with batch SSF data to evaluate the cellulose hydrolysis parameters. With the parameters evaluated for the specific substrate, the discrete model was able to predict SSF successfully both with discrete addition of cellulase only and with discrete feeding of substrate, cellulase, and media. The model tested in this study extends the capability of previous SSF models to semi-continuous feeding configurations, and invites a mechanistic interpretation of the recently observed trend of increasing conversion with decreasing feeding frequency [Fan et al. (2007a) Bioprocess Biosyst Eng 30(1):27–34]. Our results also support the feasibility and utility of determining adsorption parameters based on data obtained at several conversions, particularly when the model is to be applied to extended reaction times rather than only initial hydrolysis rates. The revised model is considerably more computationally efficient than earlier models, and appears for many conditions to be within the capability of simulation using computational fluid dynamics.

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KEYWORDS: cellulose; ethanol; model validation

Introduction

Conversion of cellulosic biomass to ethanol and other liquid fuels is of interest in light of the possibility of accessing feedstocks available at large scale, low cost, and with positive environmental attributes together with increasing impetus to find substitutes for petroleum (Greene et al., 2004). Waste feedstocks available at a centralized location on a year-round basis minimize feedstocks logistics challenges and are thus attractive points of entry for the nascent cellulosic biofuels industry. Paper sludge offers some particular advantages as a feedstock for ethanol production, with many sites offering some of all of the following features: negative feedstock cost, no requirement for pretreatment to make the material accessible to biological hydrolysis, and incorporation into an existing infrastructure (Lynd et al., 2001). About half of paper sludge produced in the U.S. is disposed via landfill, with the remainder burned, land applied, or employed for various beneficial uses (e.g., aggregate, animal bedding; NCASI, 1999). Alternatives to landfill are specified as a research priority in Agenda 2020 for America's Forest, Wood, and Paper Industry (AF&PA, 1994).

Processing paper sludge pursuant to biofuel production has been investigated in flask studies with respect to enzymatic hydrolysis (Duff et al., 1995), separate hydrolysis and fermentation (Jeffries and Schartman, 1999), and simultaneous saccharification and fermentation (SSF; Lark et al., 1997; Lynd et al., 2001). Fan et al. (2003) and Fan and Lynd (2007a,b) reported conversion of paper sludge to ethanol at concentrations >4 wt% in an intermittently fed laboratory system capable of aseptic, metered feeding of paper sludge that was operated for periods of up to 50 days. It was observed that decreasing feeding frequencies (feedstock additions per residence time) were accompanied by

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increased fractional cellulose hydrolysis (Fan and Lynd, 2007a).

The experience of the authors (Wyman and Lynd) and others, in efforts aimed at commercializing processes for biological production of commodity products, indicates that scale-up is a significant bottleneck. The state of the art approach for systematic scale-up analysis is to establish concentration and temperature profiles using computational fluid dynamics (CFD) models in conjunction with kinetic models that describe the rate of reaction as a function of local concentrations. Combined application of CFD and kinetic models has been reported for bioreactors featuring soluble substrate (Enfors et al., 2001; Moilanen et al., 2005; Vrábel et al., 2001) but not for insoluble substrates such as cellulosic biomass.

This study was undertaken to experimentally determine parameter values for the SSF model developed in a companion paper (Paper I) using paper sludge as the substrate, and to test the predictive power of the model under conditions different from those used to obtain parameters. After experimental validation of the model, we addressed the issue of reducing computational intensity in order to make feasible implementation in a CFD framework.

Materials and Methods

Materials

Waste paper sludge used in this study was obtained from the Fraser Mill, Gorham, NH and stored in ~1 L aliquots in a large freezer at −23°C. Hydrolysis rates for the sludge were shown to be the same before and after freezing (data not shown). Sludge composition is shown in Table I. Spezyme CP cellulase was kindly provided by Genencor International, Inc. Novozyme 188 β-glucosidase was obtained from Sigma–Aldrich (St. Louis, MO). The activities of the cellulase and the β-glucosidase were 57 FPU per mL and 11,00 IU per mL, respectively determined using the protocols reported by Ghose (1987). Cellulase was supplemented by β-glucosidase with an activity ratio of 1:3 for SSF experiments. *Saccharomyces cerevisiae*, strain D5A (NREL), prepared in YPD media (Sigma Y1375) was used for SSF inoculation. The KN medium, developed by Kadam and Newman (1997) and consisting of 0.3% (v/v) Corn steep liquor supplemented by 5 mM $MgSO_4$, was used in all SSF experiments. The DC protein assay from Bio-rad (Hercules, CA) was used to measure protein concentrations. The concentrations of SSF products were obtained using HPLC with an Aminex HPX-87H column at 65°C. The concentrations of enzymatic hydrolysis products were obtained using HPLC with a HPX-87P column at 80°C. All the data points reported in this work are the average of two replicate reactions with samples analyzed in duplicate using HPLC.

Table I. Composition of the paper sludge by dry weight.

Cellulose	48.2%
Xylan	13.9%
Mannan	1.7%
Ash	32.6%
Total	96.4%

Cellulase Adsorption

Filter sterilized cellulase was added to 21.7 g/L of cellulose previously sterilized in 250 mL Erlenmeyer flasks to reach a range of protein concentrations from 0.125 to 4.89 g/L. Each flask was mixed intensively with a magnetic stirrer bar and kept at 37°C in a rotary shaker. Samples were drawn after reacting for 0.5, 3, 9, and 55 h. The samples were centrifuged, and the concentrations of hydrolysis products and protein in the supernatant were measured. Cellulose conversion, x was calculated by equation (1) using the concentrations of cellobiose, [Cb] (g/L) and glucose, [Glu] (g/L) produced during hydrolysis

$$x = \frac{0.9[\mathrm{Glu}] + 0.95[\mathrm{Cb}]}{[\mathrm{C}]_0} \tag{1}$$

where $[C]_0$ is the concentration of cellulose at the start of the experiment.

Free cellulase protein concentration, $[E_f]$ (g/L) was calculated using equation (2) by subtracting the background absorbance in the paper sludge control, $[E_c]$ (g/L) from the measured total free protein concentration, $[E_{tm}]$ (g/L). The adsorbed cellulase protein concentration, $[E_a]$ (g/L) was calculated using equation (3) by subtracting the $[E_f]$ from the total cellulase protein concentration, $[E]_0$ (g/L) added at the start of the experiment.

$$[\mathrm{E_f}] = [\mathrm{E_{tm}}] - [\mathrm{E_c}] \tag{2}$$

$$[\mathrm{E_a}] = [\mathrm{E}]_0 - [\mathrm{E_f}] \tag{3}$$

As reviewed in Paper I, a cellulase adsorption model and experimentally determined parameters were reported by Ooshima et al. (1990) for pretreated wood using data obtained at very low cellulose conversion. In this work, we modified their approach by taking into account the cellulose conversion during adsorption for the purpose of obtaining parameter values applicable to a range of conversion values. The equation for cellulase adsorption adapted from Equation (1) and (3) in Paper I for a single particle population at equilibrium is

$$[\mathrm{CE}] = [\mathrm{E_a}]\frac{1+\sigma_\mathrm{C}}{\sigma_\mathrm{C}} = \frac{K_\mathrm{C}[\mathrm{E_f}](1+\sigma_\mathrm{C})[\mathrm{C}](1-x)}{1+K_\mathrm{C}[\mathrm{E_f}]} \tag{4}$$

The data for $[E_f]$, $[E_a]$, and x were used to evaluate the equilibrium adsorption constant, K_C (L/g) and the adsorption capacity, σ_C (g/g) using Polymath (polymath-software.com).

SSF

SSF experiments were carried out in 250 mL serum vials (Bellco, Vineland, NJ). Before sterilization by autoclaving at 121°C, the vials were purged with carbon dioxide. The temperature of SSF experiments was maintained at 37°C in an air bath shaker. pH remained nearly constant at 5.8 due to carbonates present in the sludge. Three SSF experiments (I, II, III) were performed with different reacting conditions. The data obtained from experiment I were used for evaluating hydrolysis parameters using curve fits (Berkeley Madonna, berkeleymadonna.com). Experiments II and III were performed to gather data for comparison with model predictions.

Experiment I, batch SSF was carried out with cellulase loadings of 5, 10, 15, and 20 U/g cellulose and a substrate concentration of 31.9 g/L of cellulose. Samples were drawn at 6, 12, 24, 48, 72, and 120 h. Concentrations for cellobiose, glucose, and ethanol, [Eth] (g/L) in the supernatant were measured. These product concentrations were used to calculate cellulose conversion using Equation (5)

$$x = \frac{0.9[\mathrm{Glu}] + 0.95[\mathrm{Cb}] + \frac{0.9}{Y_{\mathrm{Eth/G}}}[\mathrm{Eth}]}{[\mathrm{C}]_0} \qquad (5)$$

In experiment II, batch SSF with discrete changes in the cellulase concentration, was carried out with similar conditions to experiment I except that the cellulase concentration was 5 U/g cellulose initially and cellulase was added discretely to reach 7.5, 10, and 12.5 U/g cellulose at 24, 48, and 72 h, respectively. In experiment III, a transient discretely fed SSF with a feeding frequency of four and a residence time of 96 h, was carried out starting as a batch SSF with a cellulase concentration of 12 U/g cellulose and a cellulose concentration of 31.9 g/L. Every 24 h, three quarters of the total volume of the partially reacted slurry was transferred to another serum bottle with paper sludge and KN media sterilized in advance. Cellulase was added after the transfer to maintain the same concentration: 12 U/g cellulose.

Results

Adsorption and Hydrolysis Parameters

Adsorption of Spezyme CP cellulase to paper sludge was evaluated after hydrolysis was allowed to proceed for various times (0.5, 3, 9, and 55 h) resulting in various values for fractional cellulose conversion up to 65% as shown in Figure 1. Adsorption parameters K_C and σ_C (Table II) were then fit to data from all conversions by minimizing the sum of squares for the observed and the predicted data. As may be seen from Figure 1, there is very good agreement (rms = 0.013 g/L) between predicted and observed values.

The new adsorption parameters together with the conversion data from Experiment I were used to fit the parameters k, e, and c in the cellulose rate equation in Paper I. Figure 2 shows the batch SSF experimental conversion data versus curve fit in 120 h for the four different cellulase loadings. The values for the parameters

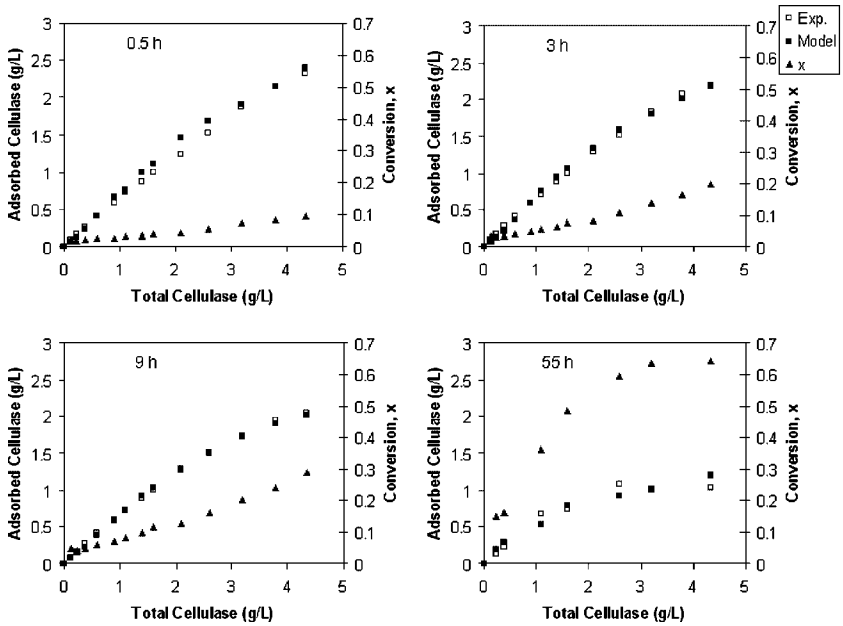

Figure 1. Adsorbed cellulase concentration and conversion data versus model fit for an initial cellulose concentration of 21.7 g/L after 0.5, 3, 9, and 55 h.

Table II. Parameter values for SSF of paper sludge.

K_c	0.414 L/g
σ_C	0.267
k	0.778 h^{-1}
e	0.466
c	0 h^{-1}

are given in Table II. Experimental data are well represented by the model for the four different cases tested.

Predictive Application of the Model

The model was first applied to test the situation with discrete increase of cellulase concentration during SSF (Experiment II, material and methods). Figure 3 shows the experimental conversion data versus predictions for batch SSF with cellulase added discretely at 24, 48, and 72 h. The predictions are well represented by the data, suggesting the robustness of the adsorption model with respect to addition of fresh cellulase during the course of hydrolysis.

The model was also applied to discrete feeding of paper sludge, cellulase, and media (Experiment III). Figure 4 shows good agreement between experimental conversion data and predictions for the discretely fed SSF experiment over a period of 96 h. This suggests that the particle population approach presented in Paper I is able to correctly account for the reactivities of different particles fed at various times.

Anticipation of CFD Analysis

Available CFD analysis software such as FLUENT is currently limited to about 50 equations per element. For the model described in Paper I, the number of equations that has to be solved depends on the number of particle populations tracked. As shown in Table III, to track n discrete particle populations, there will be a total of $2n+8$ equations, which implies that the maximum number of particle populations that can be handled is around 20.

Although we have reduced the number of particle populations significant by developing a discrete model (from 100,000 to less than 70 for a single reactor), the number of particle populations tracked can be further reduced by tracking only those particle populations that are reactive (eliminating particle populations with high particle conversion). The fractional error incurred by limiting the analysis to n particle populations compared to results without such elimination is defined in Equation (6)

$$\varepsilon(n) = 1 - \frac{x(n)}{x(>> n)} \tag{6}$$

where $x(n)$ and $x(\gg n)$ are steady state end-of-cycle conversions with and without eliminating particle populations, respectively. For staged reactors, the number of equations solved will be too large to be incorporated into CFD for the exhaustive method (Paper I), while the equations will be much less for the average reaction constant method (Paper I). Table IV summarizes the total number of equations (N) solved for one-stage and two-stage reactor configurations for different residence times and feeding frequencies with one percent fractional error. For the two cases using the average constant approach (single inlet particle population for one-stage reactor), most scenarios full within the limit, while for the second reactor in two-

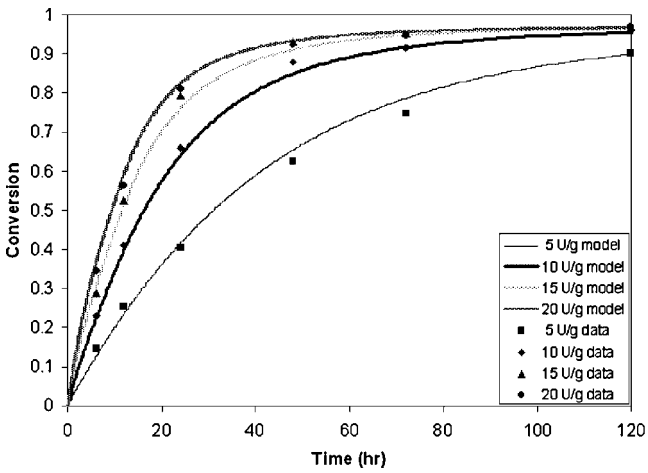

Figure 2. Batch SSF conversion data and curve fit for a cellulose concentration of 31.9 g/L with four different cellulase loadings in 5 days.

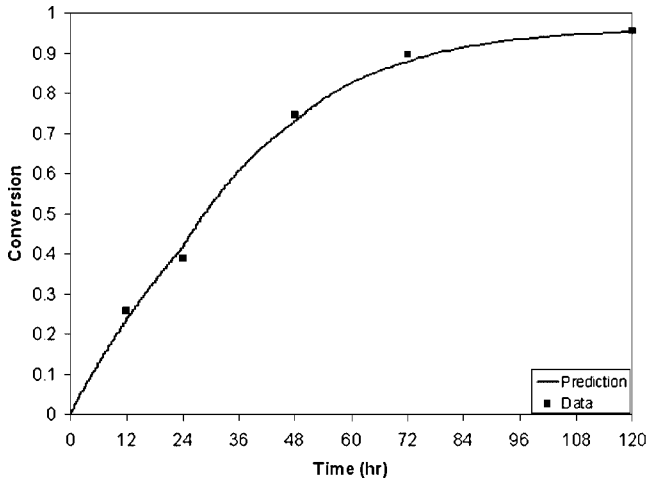

Figure 3. Added enzyme SSF conversion data and model prediction using the parameter values obtained from curve fitting to batch SSF data for a cellulose concentration of 31.9 g/L in 5 days with an initial cellulase loading of 5 U/g cellulose and cellulase added discretely to reach 7.5, 10, and 12.5 U/g cellulose at 24, 48, and 72 h, respectively.

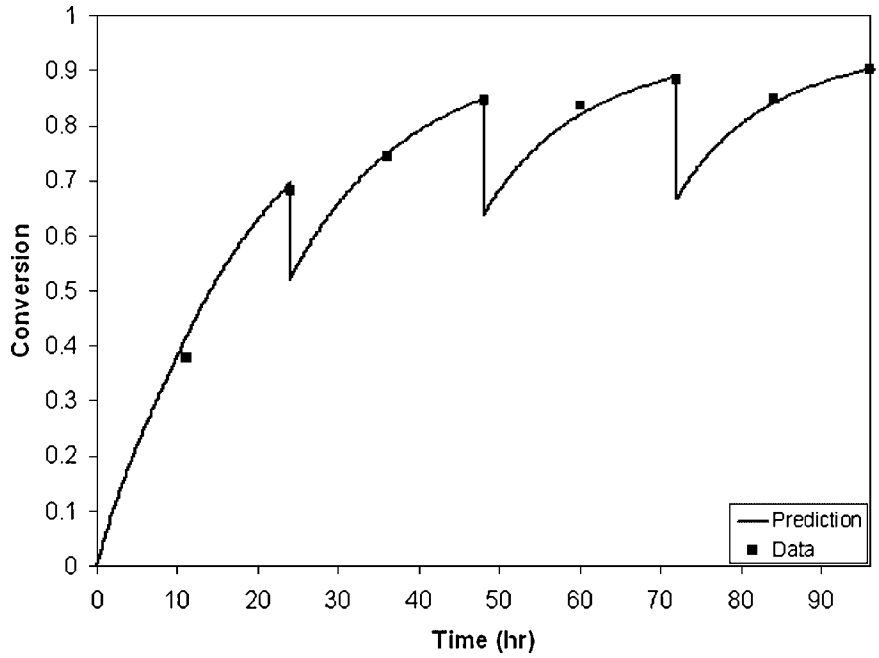

Figure 4. Transient discrete SSF conversion data and model prediction using the model presented in paper I together with updated values for parameters in Table II for a cellulose concentration of 31.9 g/L and a cellulase loading of 12 U/g cellulose.

stage reactor configuration, most scenarios are beyond the limit using the exhaustive method.

Discussion

Because of its potential for industrial application, waste paper sludge was chosen as substrate to evaluate the parameter values for the SSF model reported in Paper I. Kinetic parameters for adsorption and hydrolysis specific to the substrate were evaluated. Without adjusting parameter values evaluated from batch adsorption and SSF experiments, the model successfully predicts the results obtained with discrete addition of cellulase and the results obtained with discrete feeding of substrate, cellulase, and media. Anticipation of the model for CFD analysis with regard to reduction in computational requirements was analyzed.

Compared to dilute acid pretreated hardwood reported by South et al. (1995), the paper sludge used in this study has a lower reaction constant ($k = 2.8625$ h^{-1} for hardwood compared to 0.778 h^{-1} for paper sludge). However, the cellulase adsorption capacity of paper sludge is much higher than that of pretreated hardwood ($\sigma_C = 0.267$ for paper sludge compared to 0.0806 for pretreated hardwood). Furthermore, paper sludge has a much smaller exponent with respect to conversion ($e = 0.466$ for paper sludge compared to 5.3 for pretreated hardwood), indicative of a smaller decline of reaction rate constant with increasing

Table III. Number of equations that must be solved per CFD element for a single reactor (equations shown in Part I).

Equations for n discrete particle population	$2n$
	Cellulose concentration, ith population, [C(i)], Equation (8)
	Cellulose-enzyme complex concentration, ith population, [CE(i)], Equation (1)
Additional equations	8
	Lignin concentration, [L_f], Equation (4)
	Lignin-enzyme concentration, [LE], Equation (2)
	Cellulase enzyme concentration, [E_f], Equation (5)
	Cellobiose concentration, [CB], Equation (9)
	Cell concentration, [X_c], Equation (10)
	Ethanol concentration, [Eth], Equation (12)
	Glucose concentration, [G], Equation (11)
	Carbon dioxide concentration, [CD], not shown but needed in CFD
Number of equations (N)	$2n + 8$

Table IV. Total number of equations solved, enzyme loading 10 U/g, $\varepsilon = 1\%$.

		One-stage		Two-stage, 2nd reactor			
				Average constant		Exhaustive	
τ (days)	f	n	N	n	N	n	N
1	2	5	18	4	16	31	70
	4	11	30	8	24	110	228
	10	29	66	22	52	649	1,306
2	2	3	14	2	12	16	40
	4	7	22	4	16	44	96
	10	17	42	9	26	195	398
4	2	2	12	1	9	12	32
	4	3	14	1	9	24	56
	10	8	24	2	10	55	118

conversion when compared to pretreated hardwood as shown in Figure 5. The two substrates have about the same conversion after 5 days with the same cellulase loading due to compensatory differences in the values for the rate constant, adsorption capacity, and reaction exponent.

Prior efforts to model cellulase adsorption known to us have been based on data taken with fractional cellulose conversion at or near zero (Bothwell et al., 1997; Kim et al., 1998; Kim and Hong, 2000; Nidetzky and Claeyssens, 1994; Ooshima et al., 1990; Tomme et al., 1995). In this study, cellulose conversion was incorporated into the adsorption model and parameters were fit to data taken over a range of conversions from near zero to 65%. For paper sludge at least, it seems reasonable to assume a constant adsorption capacity normalized to the amount of cellulase remaining and that there is no reason to hypothesize changing adsorption affinity as a function of conversion. Although kinetic models used for biochemical studies often focus on initial reaction rates, models used for process designs featuring biomass hydrolysis are inevitably concerned with rates over the course of reaction. For the latter case, determining adsorption parameters using data from various conversions seems preferable as compared to only using data from unconverted substrate. Since lignin content was negligible in the paper sludge investigated in this study, cellulase adsorption to residual solids was not taken into account. Adsorption to lignin will, however, be an important consideration for most other biomass feedstocks.

Prior SSF models incorporating decreasing substrate reactivity with increasing conversion have been reported for batch and/or continuous systems (Kadam et al., 2004; Kurakake et al., 1995; Phillippidis et al., 1992; South et al., 1995). At the same time, ''restart'' experiments in which cellulase is removed from the substrate followed by addition of new cellulase have caused some to question the interpretation that the widely observed phenomenon of decreasing hydrolysis rates with increasing conversion is in fact due to declining substrate reactivity (Desai and Converse, 1997; Gusakov and Sinitsyn, 1985; Ooshima et al., 1991; Yang et al., 2006). Although the experiments carried out in our study involved addition of cellulase after the hydrolysis reaction was initiated, we saw no indication of a need to accommodate a difference in the reactivity of new cellulase–cellulose encounters, as might be inferred from data from restart experiments.

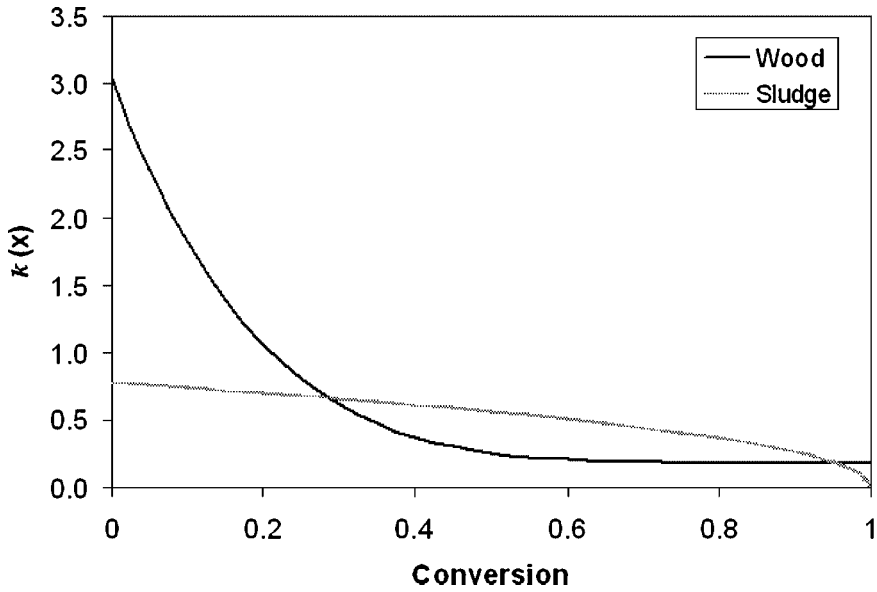

Figure 5. Comparison of conversion dependent rate constants for pretreated hardwood and paper sludge.

Nomenclature

[]	concentration of the symbol within (g/L)
$[C]_0$	initial cellulose concentration (g/L)
σ_C	adsorption capacity of enzyme on cellulose (g/g)
C	Total cellulose substrate
$\varepsilon(n)$	fractional error incurred by limiting the analysis to n particle populations
c	conversion independent component in rate function (h^{-1})
Cb	cellobiose
CE	cellulose enzyme complex
e	exponent of the declining substrate reactivity
E_a	cellulase protein adsorbed on cellulose
E_c	protein/background from paper sludge control with no cellulase added
E_f	cellulase enzyme not bound to cellulose
Eth	Ethanol
E_{tm}	measured total free protein
Glu	Glucose
k	hydrolysis rate constant (h^{-1})
K_C	cellulose adsorption constant (L/g)
x	reactor/cellulose conversion
$Y_{Eth/G}$	Ethanol yield per glucose consumed (g/g)

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