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Modeling the Enzymatic Hydrolysis of Dilute-Acid Pretreated Douglas Fir

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Abstract

Glucose yield from the enzymatic hydrolysis of cellulose was investigated as a function of cellulase enzyme loading (7–36 filter paper units [FPU]/g cellulose) and solids concentration (7–18% total solids) for up to 72 h on dilute sulfuric-acid pretreated Douglas Fir. The saccharification was performed on whole hydrolysate with no separation or washing of the solids. Enzyme loading had a significant effect on glucose yield; solids concentration had a much smaller effect even at higher glucose concentrations. The data were used to generate an empirical model for glucose yield, and to fit parameters of a cellulose hydrolysis kinetic model. Both models could be used for economic evaluation of a separate hydrolysis and fermentation process.

Index Entries: Cellulose hydrolysis; softwood; ethanol; enzyme kinetics; cellulase.

Introduction

The production of ethanol from lignocellulosic biomass has received considerable attention because of the potential of producing large quantities of ethanol for use as a transportation fuel *(1)*. Hemicellulose and cellulosic components of lignocellulosic biomass are hydrolyzed to their component sugars for subsequent conversion to ethanol by a fermentative process. Hemicellulose is usually hydrolyzed with a chemical process (e.g., by acid or caustic treatment commonly referred to as "pretreatment"); cellulose is hydrolyzed by chemical (acid) or biological (enzyme) attack. The economic success of ethanol production will depend on efficient conversion of cellulose and hemicellulose to their monomeric sugars and the efficiency of fermenting those sugars to ethanol, while also reducing capital and operating costs.

The enzymatic approach to hydrolyzing cellulose to glucose is promising because enzymes can achieve high yields, and do not catalyze glucose

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degradation reactions common to the dilute-acid process. However, the cellulose must be accessible to enzymatic attack, which depends on the severity of the pretreatment process. A greater degree of hemicellulose and/or lignin removal during pretreatment increases the accessibility of the cellulose and thus the efficacy of enzymatic cellulose hydrolysis.

Two processes often discussed for producing ethanol from cellulose are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In the SHF process, cellulose is hydrolyzed with cellulase enzyme at 45–50°C in a hydrolysis reactor to produce glucose. The process is performed at a temperature that favors rapid cellulose hydrolysis, without significantly degrading enzyme activity because of thermal degradation. The glucose is then fed to a separate vessel for ethanol production by fermentation.

When cellulose, enzyme, and the fermentative organism are added together in one vessel, the cellulose is hydrolyzed to glucose and simultaneously converted to ethanol by the microorganism (SSF). The SSF process is typically conducted at 35–38°C, which optimizes ethanol production, but is not the optimal temperature for either the enzyme or the microorganism. Because SSF is conducted at a lower temperature, the cellulose hydrolysis rates are reduced. A typical fermentation will take 5–7 d, depending on the accessibility of the cellulose and initial solids loading of the fermentation. The long residence time may make contamination control difficult in a continuous process, but may be manageable in a batch process. Additionally, the fermentation is conducted in the presence of solids, which requires more agitation power and attention to solids buildup and transport. The major advantage of SSF is that the immediate consumption of sugars by the microorganism produces low sugar concentrations in the fermentor, which significantly reduces enzyme inhibition compared to SHF.

The separation of hydrolysis and fermentation allows operation at the optimal temperature for each process. However, it is not clear that this separation results in any performance advantage for the SHF process, because inhibition of enzymatic activity by higher sugar concentrations reduces yields, or requires a lower solids concentration to achieve the same yield. Contamination may also be a concern because of the abundance of sugars available in the hydrolysis reactor.

Economic evaluation is necessary to determine the relative merits of SSF and SHF. This requires information on both SSF and cellulose hydrolysis performance as a function of solids concentration, residence time, and enzyme loading. The literature contains data on SSF of pure cellulose (e.g., Solka Floc or Sigmacell) *(2–4)* or washed pretreated substrates *(5–7)*, but separating the solids and liquids and washing the pretreated solids may not be the most economical route for cellulose conversion. The additional equipment and water added to the process significantly increases capital and operating costs. The literature contains few if any data on SSF performance with unwashed pretreated material. This material is usually

too toxic to ferment at higher solids concentrations without some type of detoxification, as has been proposed in the literature *(8)*.

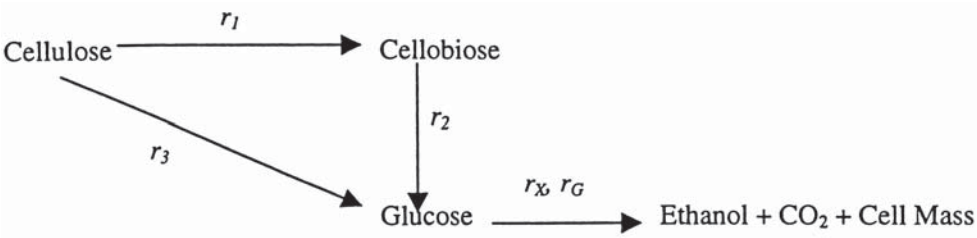
Enzymatic hydrolysis of cellulose has been studied for about 25 years, beginning with the pioneering work of Mandels *(9)*. However, most of the reports are on the enzymatic hydrolysis of washed pretreated material *(10–12)*. Comprehensive data were presented by Schwald et al. *(10)* on enzymatic hydrolysis of washed SO_2-catalyzed stream exploded aspen at substrate concentrations of 2–8% and enzyme loadings of 6–15 FPU/g of substrate. Other studies have used unwashed substrates *(13,14)*, but the data are limited; the information is usually inadequate for use in a good economic optimization of the process.

The goal of this work was to investigate the cellulose hydrolysis of pretreated Douglas Fir as a function of initial solids concentration (7.5–18%), enzyme loading (7–36 FPU/g of cellulose), and residence time (12–72 h). No alteration or detoxification was done to the hydrolysate (solids and liquor produced during pretreatment). The data were used to develop an empirical model for glucose yield. Kinetic expressions are also presented for the hydrolysis of cellulose, and experimental data were used to calculate the kinetic parameters.

Cellulose Hydrolysis Kinetics

A robust kinetic model would be capable of predicting cellulose conversion at multiple solids and enzyme loadings with a single set of kinetic parameters. This could be used to optimize economically either an SSF or SHF process. The kinetic model developed at the National Renewable Energy Laboratory (NREL) has previously been tested with pretreated biomass and multiple-enzyme loadings, but not with multiple-solid loadings. The development of this model has been described in detail elsewhere *(15–18)*. This study provided the opportunity to develop further the model for multiple-solid loadings.

The model's mechanism includes simultaneous cellulose hydrolysis by the endoglucanase and exoglucanase components of cellulase to cellobiose and glucose, conversion of cellobiose to glucose by β-glucosidase and metabolism of glucose to ethanol, carbon dioxide, and cell mass by an ethanologenic microorganism. The mechanism is shown figuratively below:



The fermentation rates (r_X, r_G) will not be discussed because the focus of our work was on the hydrolysis reactions.

The mass balance equations for the hydrolysis section of the model are:

$$\text{Cellulose: } \frac{d(C)}{dt} = -r_1 - r_3 \tag{1}$$

$$\text{Cellobiose: } \frac{d(B)}{dt} = 1.056r_1 - r_2 \tag{2}$$

$$\text{Glucose: } \frac{d(G)}{dt} = 1.053r_2 + 1.111r_3 \tag{3}$$

where *(C)*, *(B)*, and *(G)* are the cellulose, cellobiose, and glucose concentrations (g/L), respectively, t is time (h), and r_1, r_2, and r_3 are the reaction rates (g/(L–h)).

The hydrolysis reaction rates are given by:

$$r_i = \frac{k_i'(C)e^{-\lambda t}}{1 + \frac{(B)}{K_{1B}} + \frac{(G)}{K_{1G}}} \cdot \frac{K_{1E}}{K_{1E} + (E)} \quad i = 1,3 \tag{4}$$

$$r_2 = \frac{k_2'(B)}{K_m\left(1 + \frac{(G)}{K_{2G}}\right) + (B)} \tag{5}$$

where k_1', k_3' (h^{-1}), and k_2' (g/[L–h]) are the lumped specific rate constants. K_{1B}, K_{1G}, K_{1E}, and K_{2G} are inhibition constants (g/L) for inhibition of cellulase by cellobiose, cellulase by glucose, cellulase by ethanol, and β-glucosidase by glucose, respectively, K_m is the cellobiose saturation constant for β-glucosidase (g/L), *(E)* is ethanol concentration (g/L), and λ is a constant (h^{-1}) in the exponential expression that accounts for the rate of decrease in cellulose-specific surface area. This exponential expression attempts to describe an experimentally documented *(18–21)*, time-dependent decline in the cellulose hydrolysis rate. Because ethanol was not present, Eq. 4 can be reduced to the following simpler expression:

$$r_i = \frac{k_i'(C)e^{-\lambda t}}{1 + \frac{(B)}{K_{1B}} + \frac{(G)}{K_{1G}}} \quad i = 1,3 \tag{6}$$

The lumped specific rate constants k_1' and k_3' exhibit Michaelis-Menton dependence on the cellulose concentration as described in the following equation:

$$k_i' = \frac{k_i(e)_T\ e_c^*}{K_{eq} + (e)_T\ e_c^*} \quad i = 1,3 \tag{7}$$

where $(e)_T$ is the total (free and bound) concentration of the cellulase and β-glucosidase enzyme complex (g/L), e_c^* is the specific cellulase activity of

the enzyme preparation (FPU/g protein), k_1 and k_3 are the maximum specific rates (h^{-1}) of cellulose hydrolysis to cellobiose and glucose, respectively, and K_{eq} is the cellulase adsorption saturation constant (g/L).

The lumped specific rate constant k_2' is proportional to the β-glucosidase concentration with reduction owing to irreversible adsorption to lignin *(17)* and is described in the following expression:

$$k_2' = k_2\ (e)_T\ e_g^*[1 - K_L(L)] \quad (8)$$

where e_g^* is the specific β-glucosidase activity of the enzyme preparation (IU/g protein), k_2 is the maximum specific rates (g/[IU–h]) of cellobiose hydrolysis to glucose, K_L is the β-glucosidase adsorption to lignin factor (L/g), and *(L)* is the lignin concentration (g/L).

Materials and Methods

Feedstock Preparation and Pretreatment

Douglas Fir logs of 7–20 cm in diameter were selected and harvested by Colorado State Forest Service agents (Golden District) from a north-facing slope in Golden Gate Canyon near Golden, CO. After harvesting and debranching, the logs were manually debarked and chipped using a 65-hp (48.5-kw) Brush Bandit mobile knife chipper (Foremost, Inc., Remus, MI). The wood chips were milled in a rotary knife mill (Mitts and Merrill, model 10 × 12, Reduction Technology, Inc., Leeds, AL) equipped with a 3/8-in (9.5-cm) rejection screen, and blended. The final milled chips were packed in polyethylene-lined drums and stored at –20°C until needed.

Before pretreatment, the frozen chips were thawed and fines removed by screening through a 2-mm screen. The screened chips were soaked in 0.35% dilute sulfuric acid at 60°C for 6 h, drained overnight to approximately 40% solids, and air-dried to 55% solids before pretreatment. The chips were then pretreated in a steam-explosion apparatus *(22)* that was heated by direct steam injection to 215°C. After 140 s, the particles were explosively decompressed in a collection tank. The hydrolysate was collected and frozen for later use.

Analysis of Wood and Pretreated Solids

Total solids (insoluble + dissolved solids) concentration of the hydrolysate was determined by oven-drying a sample of the material at 105°C. Water-insoluble solids concentration of the hydrolysate was determined by extensively washing the solids and oven-drying them at 105°C. The composition of the wood and pretreated material was determined by methods previously described *(23)*.

Enzyme Hydrolysis Experiments

Enzymatic digestions were carried out on aliquots of hydrolysate. The digestions were conducted in shake flasks in a laboratory-shaking

Table 1
Number of Flasks Run at Each Factor Level
in the Enzymatic Hydrolysis Experiment

Enzyme loading (FPU/g cellulose)	Solids concentration (%)		
	7.5	12.8	18.0
7.0	1	1	1
21.5	2	2	2
36.0	1	1	1

incubator at 45°C and 200 rpm. A 2-factor (initial solids concentration and enzyme loading), 3-level design was used to generate a response surface of monomeric and total (monomeric + oligomeric) glucose yield. The factor levels and number of flasks run at each point are shown in Table 1. Each shake flask contained 150 g of solution with the amount of hydrolysate required to achieve the desired solids concentration. A 50-m*M* citrate buffer was used to maintain the pH at 4.8. Contamination was minimized using 40 µg/mL tetracycline and 30 µg/mL cycloheximide. The pH was checked and adjusted to 4.8 with NaOH. The solutions were warmed to 45°C, enzyme was added, and then distilled water was added to achieve the desired 150 g. The measured activities of the cellulase enzyme (Iogen Super Clean cellulase, lot #BRC 191095, Iogen Corp., Ottawa, Ontario, Canada) were 91 FPU/mL and 198 IU/mL β-glucosidase activity using p-nitrophenyl-β-D-glucopyranoside as the substrate. Samples were withdrawn from the flasks at 1-, 2-, 4-, 6-, 12-, 24-, 48-, and 72-h. Identically prepared flasks (without enzyme addition) were sampled to determine initial sugar concentrations. Glucose yields were calculated as glucose produced (correcting for initial glucose in the solutions) over potential glucose from the glucans in the pretreated material.

Analysis of Liquor Composition

Monomeric sugars after enzymatic hydrolysis were measured by high-performance liquid chromatography (HPLC) using a Biorad HPX-87P column (Biorad Laboratories, Hercules, CA) at 85°C with water at 0.6 mL/min as the mobile phase. Oligomeric sugars were converted to monomeric form after a 4% sulfuric acid hydrolysis at 121°C for 1 h, and then measuring the monomeric sugars using the HPX-87P column. Acetic acid, furfural, and hydroxymethylfurfural (HMF) were measured by HPLC using a Biorad HPX-87H column at 85°C with a 0.1 *N* sulfuric acid solution at 0.6 mL/min as the mobile phase.

Kinetic Modeling Methodology

The cellulase system used in this work is similar to the one used by Philippidis et al. *(20)*; therefore, the same enzyme specific inhibition factors

were used in this work. These factors are: K_{1B} = 5.85 g/L, K_{1G} = 53.16 g/L, K_{2G} = 0.62 g/L, K_M = 10.56 g/L, e_c* = 410 FPU/g protein, and e_g*=820 IU/g protein. The other parameter values (k_1, k_2, k_3, K_{eq}, K_L, and λ) were determined by fitting experimental data to the kinetic equations.

The initial rates method was first used to calculate k_1' and k_3'; however, the method failed probably because of errors in the model and/or the data. As an alternative, *Scientist*, a commercial multivariate nonlinear least-squares fitting software package (MicroMath Scientific Software, Salt Lake City, UT), was used to calculate all kinetic parameters by minimizing the sum of the squares of the errors (differences between predicted and measured glucose and cellobiose concentrations) at all time points. To simplify the fitting process k_2 and K_L were combined into a single term:

$$k_2^* = k_2[1 - K_L(L)] \tag{9}$$

reducing Eq. 8 to:

$$k_2' = k_2^* (e)_T \; e_g^* \tag{10}$$

Attempts were made to calculate one set of the kinetic parameters k_1, k_2*, k_3, K_{eq}, and λ from all of the data at all solids loadings. However, a set of parameters that minimized the sum of the squares of the errors could not be found, so three sets of optimal kinetic parameters were developed—one at each solids loading. Multiple initial guesses were entered to reduce the chances of producing parameter sets with a local minimum error instead of a global minimum. The resulting three cellulase adsorption constants (K_{eq}) were averaged and the mean was used to recalculate k_1, k_2*, k_3, and λ with *Scientist*. The resulting rate constants k_1 and k_3 were averaged and the means of k_1, k_3, and K_{eq} were used to recalculate k_2* and λ. The new value of k_2* was used to calculate k_2 and K_L. Finally, λ was recalculated with *Scientist* for each of the three solids loadings and averaged to determine its final value.

Results

Pretreatment

The compositions of the raw feedstock (Douglas Fir) and the hydrolysate are shown in Table 2. Although not shown in the table, oligomeric sugar concentrations were low, usually less than 5% of the monomeric concentration. The only exception was mannose, whose oligomer concentration was 11% of the monomer concentration. The feedstock was effectively pretreated as indicated by the low amount of hemicellulosic polymers remaining in the pretreated solids. The pretreatment also solubilized a significant fraction of the cellulose, as shown by the large glucose concentration. A quarter of the glucose originated from glucomannan, assuming a mannan to glucan ratio of 3:1 typical of softwood glucomannans *(24)*.

Table 2
Composition of Feedstock (Douglas Fir) and Hydrolysate Fractions

Component	Feedstock[a] (%)	Hydrolysate	
		Solid[a] (%)	Liquor[b] (g/L)
Glucan/glucose	39.7	46.1	43.0
Xylan/xylose	5.3	0.0	15.6
Mannan/mannose	11.9	0.6	32.6
Galactan/galactose	3.3	0.1	10.3
Arabinan/arabinose	2.6	0.0	4.8
Lignin	29.8	45.1	
Acid-soluble lignin	0.4	0.3	
Ash	0.1	0.1	
Acetic acid			7.3
Furfural			2.0
HMF[c]			4.1
Total solids concentration		33.1	
Insoluble solids concentration		21.6	

[a]Values shown for sugars are for the polymeric constituents (e.g., glucan).
[b]Values shown are monomeric sugar concentrations only.
[c]HMF, hydroxymethylfurfural.

Hydrolysis Experiments

The response surfaces for monomeric glucose yield (i.e., conversion of cellulose to monomeric glucose) after 12, 24, 48, and 72 h are shown in Fig. 1A,B,C, and D, respectively. As expected, yield increases for increasing enzyme loading and decreases for increasing solid concentration, with a maximum monomeric and total glucose yield of 85 and 89%, respectively, achieved at 36 FPU/g and 7.5% solids. The results also show that enzyme loading has a much larger effect on yield than does solid concentration. Although, at the high-solids concentrations and high-enzyme loadings that produce more glucose, there is some inhibition of activity as shown by the curve of the contour lines. Monomeric glucose concentrations after 72 h ranged from 15 g/L at the low-solids concentration (7.5%) and low-enzyme loading (7.0 FPU/g cellulose) to 66 g/L at the high solids concentration (18%) and high-enzyme loading (36 FPU/g cellulose). Even at 66 g/L of glucose, the resulting ethanol concentration may still be too low to make the process economical.

Experimental data were fitted to a quadratic model with interaction terms using the three independent variables: solids concentration (S, %), enzyme loading (E, FPU/g cellulose), and time (t, h), which gives the following equations for monomeric (Y_m) and total (Y_t) glucose yields:

$$Y_m = -22.00 + 2.169E + 1.909S + 0.860t - 0.014E^2 - 0.066S^2 - 0.006t^2 - 0.027ES + 0.008ET - 0.014tS \quad (11)$$

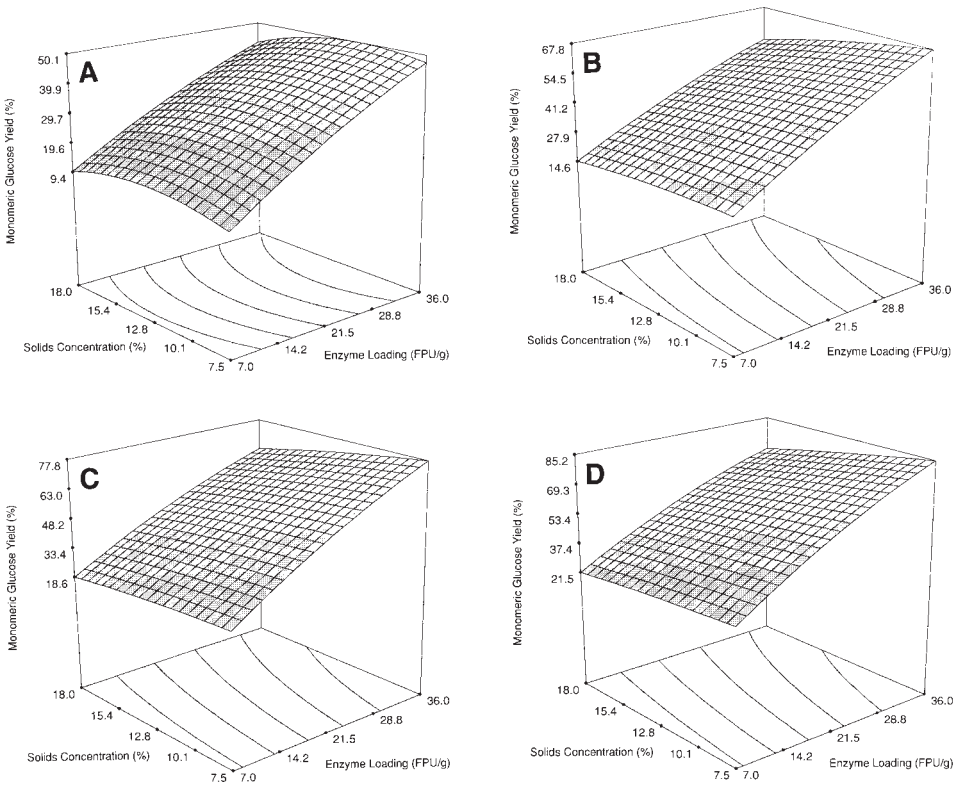


Fig. 1. Response surface of monomeric glucose yields after 12 h **(A)**, 24 h **(B)**, 48 h **(C)**, and 72 h **(D)** of enzymatic hydrolysis.

$$Y_t = -26.42 + 2.418E + 2.621S + 0.881t - 0.015E^2 - 0.072S^2 - 0.007t^2 - 0.033ES + 0.009ET - 0.016tS \quad (12)$$

Plots of predicted vs observed monomeric and total glucose yields are given in Fig. 2A,B, respectively. The plots show that yields are adequately predicted by the equations. All terms in the empirical model were significant at a 95% confidence level.

Kinetic Modeling Results

Using the above methodology, a single set of kinetic parameters was developed. The parameters were: $k_1 = 7.59\ h^{-1}$, $k_2 = 0.015$ g/(IU–h), $k_3 = 19.2\ h^{-1}$, $K_{eq} = 132000$ g/L, $K_L = 0.0073$ L/g, and $\lambda = 0.137\ h^{-1}$. This set of parameters produced a significant increase in the sum of the squares of the errors compared to parameters optimized for each solids loading. These increases were 313, 7, and 162% for the 7.5, 12.8, and 18% solids concentration, respectively. However, when all parameters except λ were set to the above values, λ was optimized to 0.068/h, 0.127/h, and 0.215/h for 7.5, 12.8, and 18% solids concentration, respectively. This procedure only

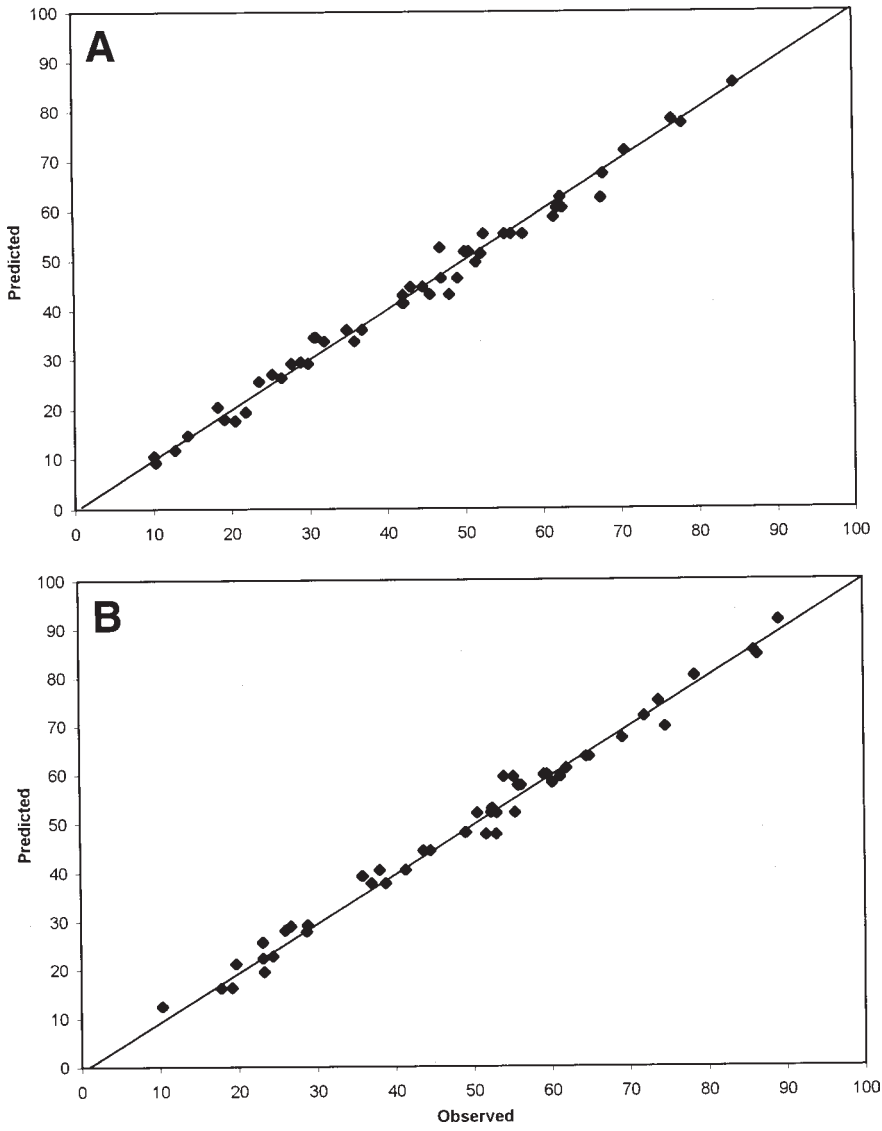


Fig. 2. Plots of predicted glucose yields generated from the three-variable equations vs observed monomeric **(A)** and total **(B)** glucose yields.

increased the sum of the squares of the errors by 10, 2, and 13%, respectively. Figures 3, 4, and 5 show these results graphically. Each graph shows measured glucose concentrations and glucose concentrations predicted by the model (solid lines). Figure 3A shows data for 7.5% solids concentration with its optimized λ (0.068/h); Figure 3B also shows the data for 7.5% solids concentration with the overall optimized λ (0.137/h). Figures 4 and 5 show the same information for 12.8% and 18% solids loading, respectively.

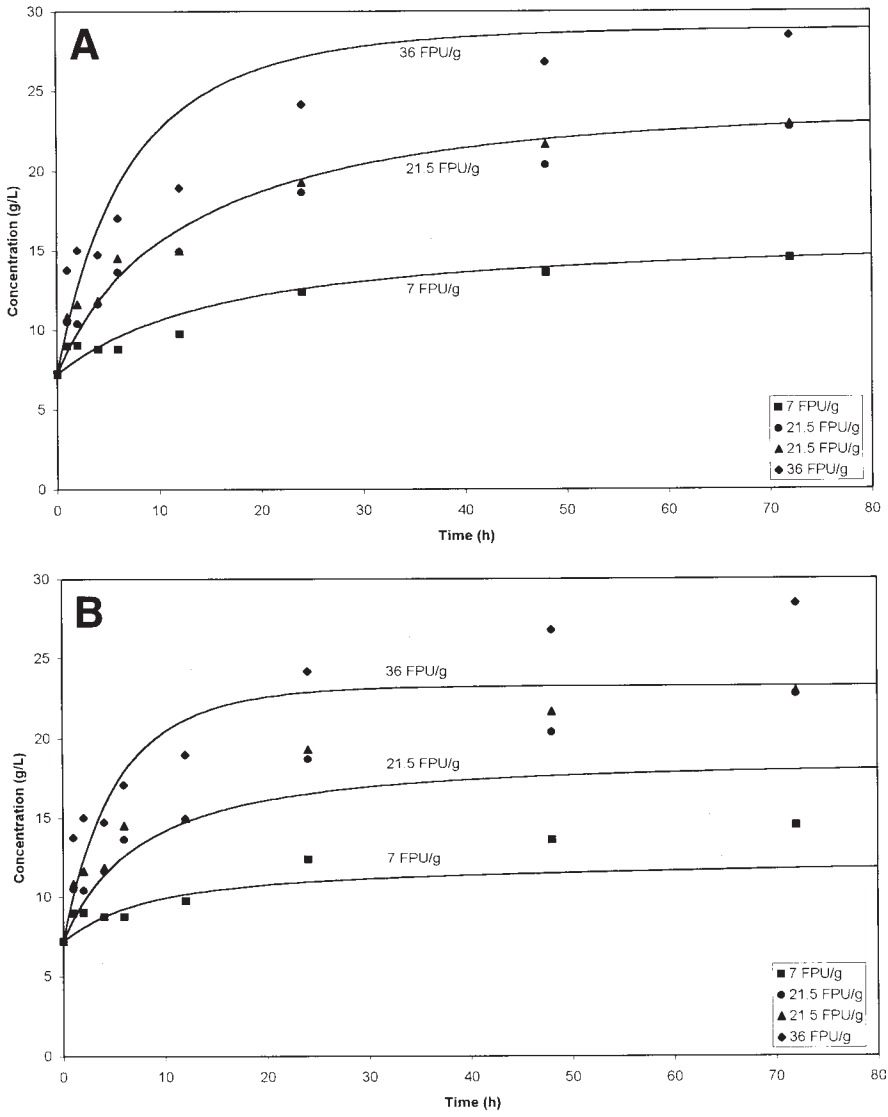


Fig. 3. Experimental data (points) and model simulations (solid lines) of 7.5% solids batch hydrolysis with λ optimized for this solids concentration **(A)** and λ average over all solids concentrations **(B)**.

The graphs show that the model fits the data reasonably well. The model overpredicts the initial hydrolysis rates at the high-solids concentration and low-enzyme loading. This may be caused by poor mixing because the low-enzyme level is inadequately liquefying the thick slurry, thus hindering complete distribution of the enzyme. The optimal λ is larger with higher solids concentration than with lower solids concentration indicating that the enzyme is not as active at higher solids concentration, even after compensating for inhibition and lignin adsorption. Although we do not know

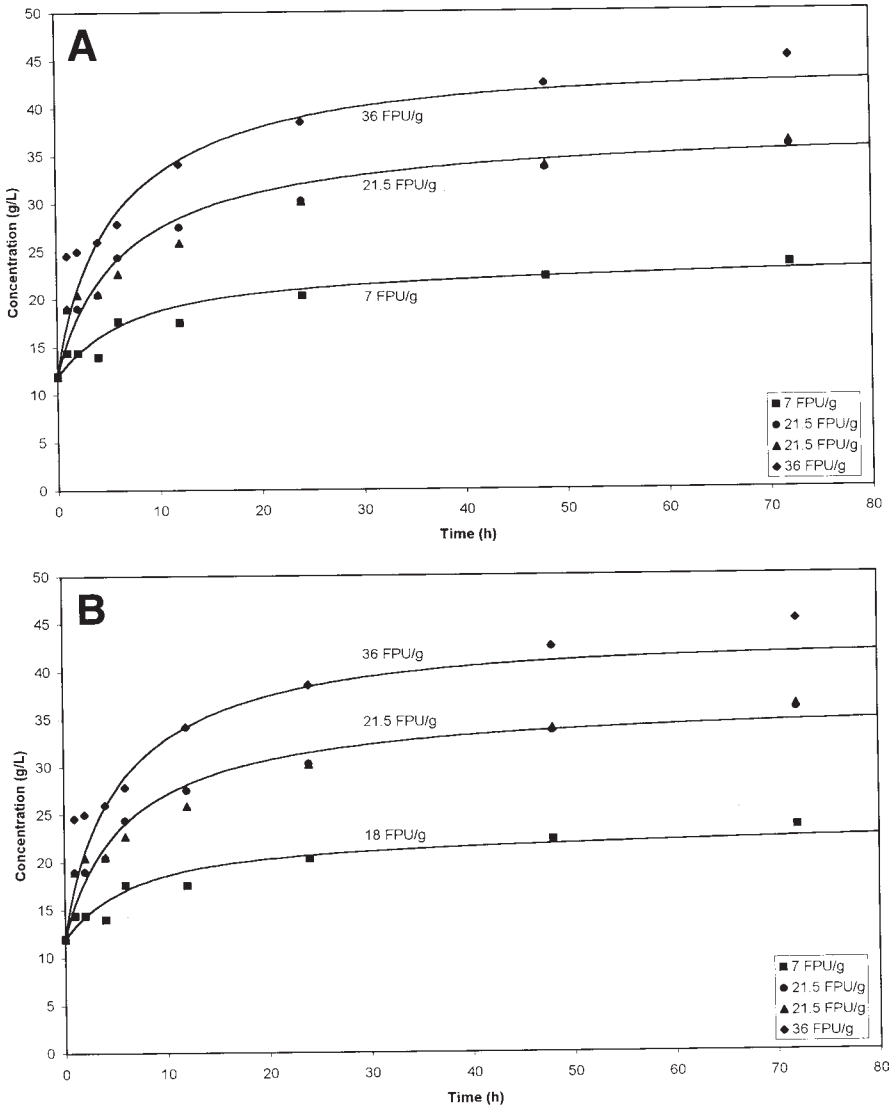


Fig. 4. Experimental data (points) and model simulations (solid lines) of 12.8% solids batch hydrolysis with λ optimized for this solids concentration **(A)** and λ average over all solids concentrations **(B)**.

the reason for the deactivation, adding an initial cellulose concentration to the cellulase deactivation term may account for this fact.

Discussion

The equations provided in this paper can be used to predict glucose yields during the enzymatic hydrolysis of Douglas Fir, but may also be applicable to many softwood species. The equations are also specific to

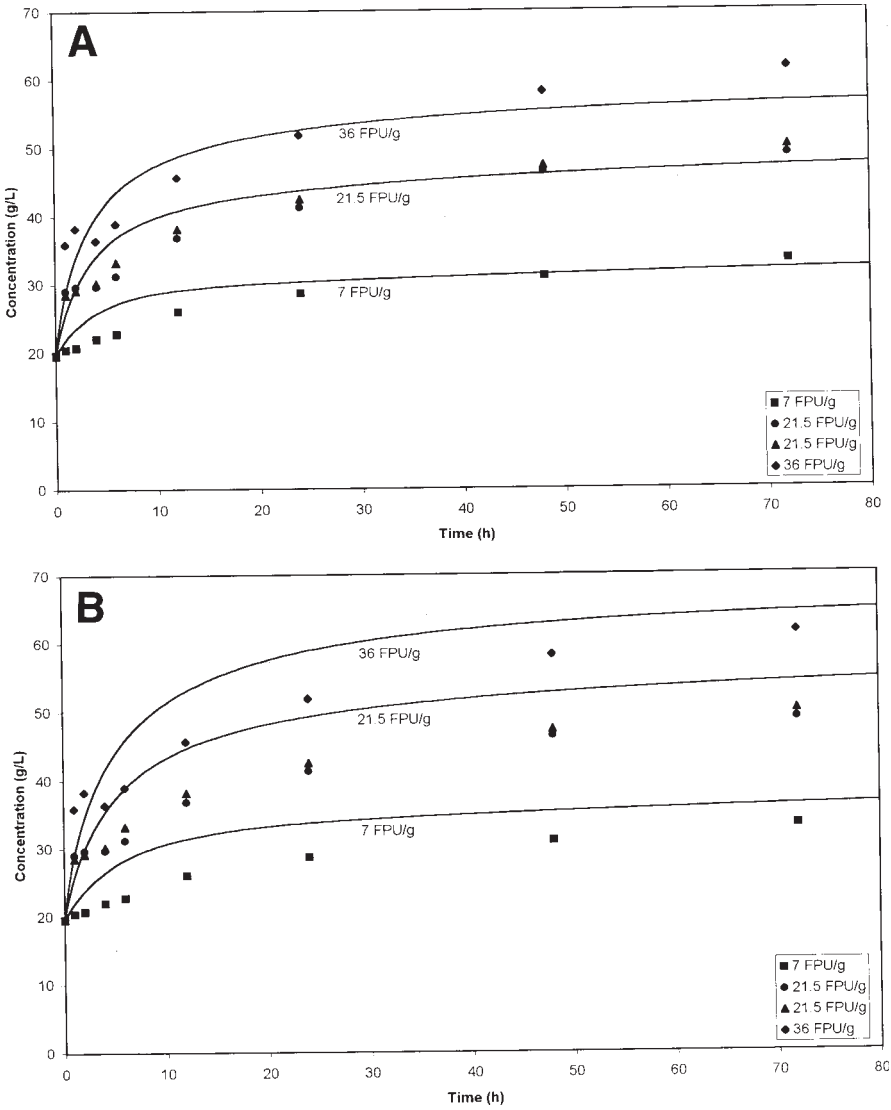


Fig. 5. Experimental data (points) and model simulations (solid lines) of 18.0% solids batch hydrolysis with λ optimized for this solids concentration **(A)** and λ average over all solids concentrations **(B)**.

the enzyme; in this case, an Iogen enzyme that has worked well in our laboratory on pretreated lignocellulosics. However, the results should still be representative of the current performance of cellulase enzymes.

Although beyond the scope of this work, the equations could be used for economic modeling of an SHF process. Previous work has concluded that it is cheaper to produce ethanol by the SSF process *(25)*. However, this work was based on SSF performance data for pure cellulosic feedstocks (e.g., Solka Floc), and not on the realities of handling and converting ligno-

cellulosic feedstocks. Work done during the last decade on conversion of wood and herbaceous feedstocks to ethanol has demonstrated the difficulties of converting nondetoxified hydrolysates to ethanol *(8)*. The work presented in this paper also clearly shows that separate hydrolysis still suffers from inhibitions and poor performance. At present, it is not clear how the relative economics of the two processes compare. Better enzymes would improve both processes; however, the SHF process would benefit significantly from improved thermal tolerance and reduction of end-product inhibition.

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