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# Empirical Evaluation of Inhibitory Product, Substrate, and Enzyme Effects During the Enzymatic Saccharification of Lignocellulosic Biomass

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**Abstract** The cellulose hydrolysis kinetics during batch enzymatic saccharification are typified by a rapid initial rate that subsequently decays, resulting in incomplete conversion. Previous studies suggest that changes associated with the solution, substrate, or enzymes may be responsible. In this work, kinetic experiments were conducted to determine the relative magnitude of these effects. Pretreated corn stover (PCS) was used as a lignocellulosic substrate likely to be found in a commercial saccharification process, while Avicel and Kraft lignin were used to create model substrates. Glucose inhibition was observed by spiking the reaction slurry with glucose during initial-rate experiments. Increasing the glucose concentration from 7 to 48 g/L reduced the cellulose conversion rate by 94%. When product sugars were removed using ultrafiltration with a 10 kDa membrane, the glucose-based conversion increased by 9.5%. Reductions in substrate reactivity with conversion were compared directly by saccharifying PCS and Avicel substrates that had been pre-reacted to different conversions. Reaction of substrate with a pre-conversion of 40% resulted in about 40% reduction in the initial rate of saccharification, relative to fresh substrate with identical cellulose concentration. Overall, glucose inhibition and reduced substrate reactivity appear to be dominant factors, whereas minimal reductions of enzyme activity were observed.

**Keywords** Saccharification · Cellulase · Kinetics · Inhibition · Cellulose · Corn stover · Membrane

## Introduction

The development of a fuel that is renewable, domestically-produced, and environmentally-sound is an ongoing effort. A process that converts widely-available lignocellulosic biomass to ethanol via enzymatic saccharification is a possible solution. However,

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economic analyses suggest that such a process would not be competitive without additional development and optimization in several areas [1–3]. Two predominant costs are associated with the biomass feedstock and the enzyme preparation [2].

There are several potential rate-reducing mechanisms, such as product inhibition, substrate accessibility, and unproductive enzyme binding. However, for a particular substrate, enzyme preparation, and reaction configuration, some of these mechanisms will be more significant. For example, if a membrane reactor is developed that can remove glucose while recycling active enzymes, reduction in substrate reactivity over time could become the dominant rate-reducing mechanism. While some researchers have aggregated several rate-reducing mechanisms using kinetic models [4–6], it is difficult to make accurate estimates of the rates of conversion across different substrates and cellulase mixtures [7].

The relative effects of plausible rate-reducing mechanisms are studied in this work using a battery of experiments that can be easily applied to specific enzyme preparations, substrates, and pretreatments. Specifically, changes occurring during the course of a reaction were examined to assess their relative importance and were classified into three main groups: (1) the reduction of enzyme activity, (2) reduction of substrate reactivity, and (3) accumulation of inhibitory glucose product in solution. Such kinetic information can be used to determine the dominant rate-reducing mechanisms and point to the most promising ways to optimize enzyme usage and substrate conversion throughout a hydrolysis reaction.

## Reduction of Enzyme Activity

Reduction of enzyme activity can occur in a variety of ways, including lignin binding [8] and thermal denaturation [9]. Moreover, due to the importance of enzyme synergism for rapid reaction [5, 10, 11], the loss of only one type of enzyme, such as exoglucanase, may result in a substantial drop in the overall reaction rate. Non-specific lignin binding of cellulases is common, although the strength and reversibility of the adsorption may vary depending on the cellulase as well as the biomass type and pretreatment [12]. In addition, thermal denaturation is always a concern, even with more thermo-stable enzymes, since an optimized enzymatic saccharification reaction will be run as warm as feasible, balancing enhanced kinetics with the increased denaturation rate. Reduction of enzyme activity could be observed experimentally by quenching the saccharification reaction and restarting with the original amount of enzyme. If the subsequent rate of reaction is significantly greater than for the unquenched rate of reaction, then, it would be concluded that enzyme loss is important.

## Reduction in Substrate Reactivity

Many changes to the substrate may occur during the enzymatic saccharification, nearly all of which could retard the reaction rate as conversion proceeds. Two primary effects are examined here, including (1) a reduction in cellulose reactivity due to changes in cellulose crystallinity and/or surface accessibility and (2) an increase in competitive lignin binding due to increased relative lignin content.

Native biomass exhibits a high degree of crystallinity with limited accessibility, and enzymatic saccharification proceeds very slowly when raw lignocellulosic biomass is mixed with enzymes. Consequently, pretreatment methodologies have been designed to act by reducing cellulose crystallinity and/or increasing substrate accessibility via removal of lignin and hemicellulose [13–15]. While it is known that pretreatments are critical to

increasing the initial reactivity of the substrate, there is no strong consensus on whether the reactivity of the substrate decreases with increased conversion. Some researchers argue that substrate reactivity does not appreciably change during the reaction [16, 17], while others maintain that such changes do occur [4, 18, 19]. The importance of substrate reactivity may vary greatly depending on the lignocellulose morphology caused by different pretreatments and substrates.

If the substrate reactivity does decrease with increased conversion, it may be due to the enzymatic hydrolysis of cellulose having a fractal nature [18]. The fractal nature of the cellulose could be caused by the pretreatment process. During pretreatment, hemicellulose and lignin may be solubilized, while cellulose may be swollen to varying degrees. If the native lignocellulose is sufficiently close-packed, the pretreatment will first affect the outer regions of the lignocellulosic particles and then progress inward with increased pretreatment time. The resulting outer regions would be more accessible to enzymes and amenable to saccharification than the inner regions, resulting in a reduction of substrate reactivity with increased conversion.

Because lignin is not reacted, its concentration relative to other solids increases as the reaction proceeds. In previous research, authors have cited the significance of enzyme-lignin binding and its effect on conversion [20–24]. Because lignin binding can be competitive with cellulose binding, an increase in the relative lignin-to-cellulose content could result in decreased cellulose binding and reduced enzymatic activity.

A change in substrate reactivity over the course of an experiment could be measured by quenching the reaction at various conversions and then comparing subsequent restart rates at varying degrees of pre-conversion. To make an appropriate comparison, several factors must also be investigated. The quenching and washing protocol must be evaluated to confirm that the substrate is not changed by boiling, that product sugars are removed, that enzymes are inactivated, and that any enzymes residually-bound to lignin or cellulose are removed or at least shown to have negligible effect. If the substrates are then reacted with identical masses of buffer, enzymes, and the cellulose of different conversions, then the effect of conversion can be directly observed. If the substrates reacted at different rates, reduced substrate reactivity would have been shown to be an important effect.

## Product Inhibition

The reduction in saccharification kinetics due to inhibition by products such as cellobiose and glucose is a well-known phenomenon [4, 25–29]. Product sugar inhibition can most easily be quantified by measuring reaction rates during initial-rate experiments in the presence of spiked sugar, a technique that minimizes the potentially confounding effects of enzyme inactivation and substrate reactivity. To best understand the inhibitory mechanisms, the effect of each sugar would be measured separately on each of the cellulolytic and beta-glucosidic enzymes. Then, the aggregate inhibitory effects on the saccharification could be modeled. However, because conversion rates appear to be most limited at high conversions, a simplified approach would evaluate the inhibitory accumulation of the final product in the pathway, which is glucose. In this way, the importance of product inhibition could be compared relative to other rate-reducing mechanisms for a particular enzyme preparation and substrate.

The inhibition of product sugars can be minimized by selectively removing the product sugars while retaining enzymes. Enhanced conversion and reaction rates have been achieved in a number of studies by minimizing product sugar inhibition, although prior experiments have typically been performed at low insolubles concentrations with model substrates [26, 30, 31]. Reduction of product sugar inhibition could be achieved using an ultrafiltration membrane

system capable of retaining enzymes while reducing product sugar concentration. However, it is possible that soluble intermediate sugars, such as cellobiose, could also be lost in the permeate. If the rate of reaction of intermediate sugars to glucose is fast relative to the hydraulic retention time of the membrane system, then these losses would be minimal.

## Materials and Methods

Either pretreated corn stover (PCS) or Avicel PH-101 was used as the cellulosic substrate, the latter containing nearly pure cellulose. PCS was provided by the National Renewable Energy Laboratory and was pretreated in the presence of 0.050 g sulfuric acid per dry gram biomass at 190°C and 25% insoluble solids concentration with an approximate residence time between 45 and 75 s. Additional information concerning the pretreatment conditions and reactor are described elsewhere [32, 33]. The PCS contained 54% (*w*/*w*) cellulose on a dry solids basis, with the balance comprised of lignin, xylan, protein, and ash. To remove inhibitory compounds introduced during pretreatment, PCS was washed in six cycles consisting of a 2-fold dilution with deionized water, centrifugation for 8 min at 1,700×g (Beckman, GPR Centrifuge), and decantation of the supernatant.

CPN cellulase (Iogen Corp., Ottawa, ON, Canada) was used as the complete cellulase enzyme preparation. It contained an activity of 55 Filter Paper Units (FPU)/mL solution, as measured by the IUPAC filter paper assay [34], and a protein concentration of 41 mg/mL, determined using the Bradford assay [35]. It was stored refrigerated at approximately 1°C. The reaction slurry was buffered to pH 4.9 using 50 mM citrate buffer, and 0.1% (*w*/*v*) sodium azide was added as a biocide. Saccharification reactions were performed at an initial insoluble solids concentration of 10% (*w*/*w*) on a dry solids basis. Slurries were incubated at 45°C in 50 mL centrifuge tubes on a shaker table operating at 400 rpm. CPN was loaded to a specific activity of 20 FPU/g cellulose.

Glucose concentrations were measured using a YSI Analyzer (Yellow Springs Instruments, Yellow Spring, OH) with a glucose-detection membrane. Glucose concentrations measured with the YSI Analyzer were validated with standard solutions containing glucose at concentrations up to 5 g/L. The presence of up to 10 g/L cellobiose did not interfere with glucose measurements.

For certain experiments, reaction quenching was required. In these cases, the reaction was quenched by placing the reaction slurry in boiling water for 10 min. Enzyme inactivation was confirmed by the lack of glucose production after an additional 24 h of incubation. To ensure that boiling did not have a measurable effect on substrate accessibility, a simple saccharification experiment was conducted in which the reactivity of fresh, unboiled PCS was compared to fresh PCS that had been boiled for 10 min. The null hypothesis that the boiling had no effect could not be rejected using a *t*-test ($p=0.20$). Furthermore, the sample size and experimental standard deviation indicate that even a 5.5% change in reactivity relative to the unboiled PCS should have been detectable with 95% probability. Based on the literature, there is some concern that a small amount of the inactivated enzymes may remain bound to the cellulose or lignin [19], which could affect fresh cellulase binding. Potential effects of residual binding are discussed later in the Results and Discussion.

### Quantification of Cellulose Conversion

The conversion of cellulose was estimated from the change in glucose concentrations using roughly the same methodology described by Hodge et al. [36]. Conversion to glucose is the ratio of the total liberated glucose to the maximum amount of glucose at 100% conversion.

The coefficient of 1.111 is the stoichiometric ratio of molecular weights of glucose (MW 180.16) to the glucan monomer unit in cellulose (MW 162.14) (Eq. 1). The total initial cellulose mass is the product of the initial mass of cellulosic material, the initial insoluble solids fraction, and the initial cellulose fraction of the insoluble solids (Eq. 2). The initial insoluble solids fraction is quantified as the ratio of dried mass at 100°C to the fresh (wet) mass of washed cellulosic material (Eq. 3). As the reaction proceeds, the mass of cellulose decreases while the mass of inerts remains constant. Hence, the aqueous mass is the difference between the slurry mass and the remaining insolubles mass (Eq. 4). The total liberated mass of glucose is determined from the aqueous mass, density, and glucose concentration (Eq. 5). The density conversion assumes that any density change is due to aqueous glucose [37].

$$X = \frac{m_{glucose}}{1.111 m_{cell,o}} \tag{1}$$

$$m_{c,o} = m_{sl,o} f_{ins/sl,o} f_{cell/ins,o} \tag{2}$$

$$f_{ins/sl,o} = \frac{\text{dried mass}}{\text{fresh mass}} \tag{3}$$

$$m_{aq} = m_{sl} - (m_{cell} + m_{inert}) = m_{sl} - \left(m_{sl,o} f_{ins/sl,o} - X m_{cell,o}\right) \tag{4}$$

$$m_{glucose} = C_{glucose} \frac{m_{aq}}{\rho} \tag{5}$$

Where:

| | |
|---|---|
| $X$ | cellulose conversion |
| $m_{cell}$ | total cellulose mass |
| $m_{sl}$ | total slurry mass |
| $f_{ins/sl,o}$ | initial fraction of insoluble solids to slurry mass |
| $f_{cell/ins,o}$ | fraction of cellulose mass to insoluble solids mass |
| $C_{glucose}$ | glucose concentration |
| $m_{glucose}$ | total glucose mass |
| $m_{cell,o}$ | total initial cellulose mass |
| $m_{sl,o}$ | total initial slurry mass |
| $m_{aq}$ | total aqueous mass |
| $m_{inert}$ | total inerts mass |
| $\rho$ | aqueous density |

## Reduction of Enzyme Activity

### *Fresh Enzyme Replacement*

To test for the reduction of enzyme activity, the active enzymes were quenched at two times over a week-long batch reaction and replaced with fresh enzyme. Three reaction tubes

served as the experimental control and were reacted without quenching and sampled daily. After sampling at 24 h, reactions in three different flasks were quenched via boiling in water for 10 min. They were subsequently re-inoculated with the original amount of enzyme. This quenching and restart process was repeated after three days of cumulative reaction time. By comparing the conversions of the control flasks against the conversions of the quenched flasks with fresh enzyme addition, it was possible to determine if significant enzyme activity had been lost.

*Thermal Inactivation*

Thermal inactivation was evaluated as a potential cause of enzyme inactivation. The enzyme preparation was incubated for 5 and 10 days at 45°C. Three sets of three replicate flasks using PCS substrate at 10% (*w*/*w*) insolubles were then inoculated with either fresh enzymes as a control or the enzymes pre-incubated for 5 or 10 days. These flasks were sampled at 0.25, 1, 3, and 5 days. Glucose productions over time in each flask were compared to determine if significant loss of enzyme activity had occurred with prolonged incubation.

Reduction in Substrate Reactivity

*Lignocellulosic Biomass*

PCS was saccharified to cellulose conversions of 40%, 48%, and 63% (conversions achieved over 14, 25, and 75 h, respectively), and then it was quenched. The harvested PCS was washed until glucose concentrations were less than 1 g/L. Subsequently, the PCS substrates at different conversions and fresh PCS (0% conversion) were compared by reacting each substrate on a constant cellulose-to-aqueous mass basis, so all slurries contained 1.2 g cellulose and 19.8 g buffer, with varying amounts of lignin. For the fresh PCS, the combined cellulose and lignin result in a 10% (*w*/*w*) initial insolubles concentration. However, because the lignin-to-cellulose content increases with conversion, the initial insolubles fraction increases with pre-conversion. Three replicate experiments were performed for each pre-conversion, and sampling was performed at 7.5, 15, 35, and 82 h. New conversions at 7.5 h were then compared to determine the effect of pre-conversion on the PCS conversion rate, although these experiments were not designed to attribute these effects to substrate crystallinity/accessibility or to increased lignin-to-cellulose content.

*Microcrystalline Cellulose*

To isolate the effects of crystallinity/accessibility from lignin, Avicel was used as a pure, microcrystalline cellulose substrate. Avicel was chosen due to its similar crystallinity index and fractional accessibility relative to other pretreated cellulose substrates [7].

Analogous to the experiments with PCS, Avicel was partially-reacted to conversions of 16%, 29% and 44%, quenched, and washed. Fresh Avicel was also washed to remove any soluble solids that may be present. Four different slurries were prepared. The first three contained partially-converted Avicel at the different conversions, while the fourth contained fresh Avicel as a control. Insoluble solids concentrations in all four slurries were 10% (*w*/*w*), consisting of 2 g cellulose and 18 mL buffer in each reaction tube. Three replicates were performed at each pre-conversion, and sampling was performed at 1, 3, and 5 d. New conversions after 1 d were

compared to determine whether a significant reduction in reactivity occurred due to increased pre-conversion.

### *Lignin Content*

Conversion of cellulose in a lignocellulosic substrate results in an increased lignin-to-cellulose content. To evaluate the effect of lignin content, Kraft lignin was used to approximate the binding effects of native lignin. Three model slurries were prepared, each with 3 g Avicel and 27 mL buffer. The first two slurries contained an additional 1 g and 2 g of Kraft lignin, respectively, and the third contained no lignin as a control. Both the Kraft lignin and Avicel were pre-washed to remove any residual soluble solids. Three replicate experiments at each lignin concentration were conducted and sampled at 1, 3, and 5 d. Conversions after 1 d were compared to determine whether a significant reduction in reactivity occurred due to an increase in lignin content.

## Product Inhibition

### *Quantifying Product Inhibition*

During a batch saccharification reaction, a reduction in rate is correlated to an increase in glucose concentration; however, quantification of glucose inhibition is confounded by possible losses in enzyme activity and substrate reactivity. To isolate the effect of glucose concentration on the rate of conversion, initial-rate batch reactions with varying concentrations of spiked glucose were compared to long-time batch reactions with no spiked glucose. Initial-rate batch reactions were conducted for 4 h, a duration selected to achieve measurable and reproducible glucose concentrations, even with initially-spiked, high glucose concentrations. Saccharification of 10% (*w*/*w*) PCS was performed with initial glucose concentrations of approximately 0, 10, 20, 30, 40, and 50 g/L, with three replicates at each initial concentration. Conversely, a set of long-time batch reactions at 10% (*w*/*w*) PCS was performed with three replicates and sampled at 4, 12, 24, 51, 95, and 119 h. In the long-time batch reactions, the change in glucose concentration is due only to glucose liberation from cellulose hydrolysis. By comparing the average conversion rates and glucose concentrations for the initial-rate batch reactions and long-time batch reactions, it was possible to determine the effect of glucose inhibition.

### *Mitigating Product Inhibition via Continuous Product Removal*

To identify the significance of product inhibition, batch reactions were performed in a stirred cell equipped to run with and without continuous buffer exchange. An off-the-shelf filtration cell (Millipore, Bellerica, MA) was modified for motorized stirring to function at PCS solids loading at 10% insolubles (*w*/*w*). A second motorized cell, used as the batch control, was machined with identical dimensions except that it was not designed for membrane filtration and permeate removal. Before inoculating the stirred cells, approximately 380 mL of 10% (*w*/*w*) solids PCS slurry was reacted in batch mode in a flask on a shaker table for 24 h to cause some liquefaction and reduction of particle size. This pre-reaction step was critical. If the slurry was not pre-reacted, the abrasive corn stover particles destroyed the membrane's integrity. After the 24 h pre-reaction, the slurry was divided by placing 180 mL slurry into each stirred cell. The impellors oscillated clockwise and counterclockwise at a frequency of 1 Hz to maintain an even distribution of settling

particles. For the stirred cell with permeate removal, enzyme retention and selective product removal were achieved with a 10 kDa molecular weight cutoff polyethersulfone (PES) membrane (Millipore). This pore size was chosen because it was known to retain the cellulases [38] and solids, yet it should be able to pass the much smaller glucose molecules (180 Da). During continuous buffer exchange, permeate was metered using a syringe pump and removed steadily at a rate of 18 mL/h. Sufficient trans-membrane pressure was ensured by pressurizing the cell with nitrogen at 30 psig. Samples of the cell slurry and permeate were collected throughout the reaction to determine the concentrations of glucose and total protein.

## Results and Discussion

### Reduction of Enzyme Activity

#### *Fresh Enzyme Replacement*

The addition of fresh enzymes, after inactivating the previously active enzymes, created a small but measureable increase in conversion over five days of reaction. As shown in Fig. 1, when the conversions with respect to effective time are compared, the flasks with quenching and fresh enzyme addition appear to reach slightly higher conversions than those flasks with no fresh enzymes. For ease of statistical comparison, the conversions were normalized at a given time point to the average conversion of the control. For example, if the conversions of the control and quench/restart experiments were 50% and 54%, respectively, the improvement would be 8% when the conversions are normalized by 50%. Within 48 h of the first quenching and fresh enzyme addition, corresponding to 72 h of cumulative reaction time, the conversions increased on average by 8%, with the 95% confidence interval between 0.2% and 15%. After 120 h of effective reaction time, the observed average of the fresh enzyme addition was increased by 6%±4% at 95% confidence. The slight improvement in conversion may be due to thermal inactivation of the enzymes in the control. In addition, it is possible that there is residual binding of inactivated enzymes on the lignin, which might reduce the nonspecific binding of the fresh enzymes.

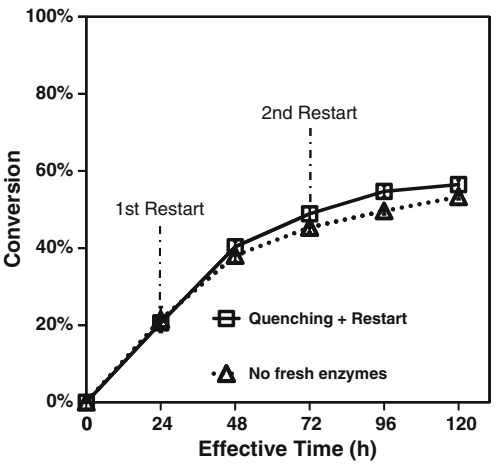


**Fig. 1** PCS conversion to glucose versus time is compared with and without enzyme replacement. The actual time was corrected to the effective reaction time by removing the quenching and monitoring time intervals. *Error bars* correspond to plus and minus one standard deviation for three flasks

*Thermal Inactivation*

Thermal inactivation may have been responsible for the slight increase in conversion when fresh enzyme was added. To assess this possibility, cellulose conversions using fresh and pre-incubated enzymes were compared. The cellulose conversion at each time point was normalized to the average conversion of the control replicates (i.e., if the conversions after 24 h were 25%, 20%, and 15% using enzyme pre-incubated for 0, 5, and 10 days, then the normalized conversions would be 100%, 80%, and 60%, respectively, when normalized by 25%). Two-way analysis of variance (ANOVA) showed that, while the duration of enzyme pre-incubation was significant ($p<0.001$), the time of sampling during the saccharification was not significant ($p=0.22$). Subsequently, data from all of the sampling times could be used to linearly regress the effect of the pre-incubation time on relative conversion rate. Per day of pre-incubation, a 0.9% reduction in relative conversion occurred, with a 95% confidence interval between 0.8% and 1.0%. The thermal inactivation results can be used to explain some of the enhancement in the previous experiment when the reaction was quenched and then restarted with fresh enzyme.

Reduction in Substrate Reactivity

*Lignocellulosic Biomass*

By reacting pre-saccharified PCS, it is evident from Fig. 2 that a large drop in substrate reactivity is associated with an increase in pre-conversion. The data at 7.5 h were normalized to the average conversion of the initially unreacted PCS (i.e., if the initially-unreacted PCS reached 25% conversion at 7.5 h, then the normalized conversions of slurries reaching 20% and 25% conversions would be 80% and 100%, respectively). Subsequently, the normalized conversion was regressed as a function of pre-conversion, as shown in Fig. 3. The regression indicates that, for every 10% increase in pre-conversion, there is an average 8.5%±0.6% reduction of the conversion rate at 95% confidence (up to 63% initial conversion). Kadam et al. [4] found similar results, and the change in reactivity was modeled as a 10% reduction in reactivity for every 10% increase in conversion. Note that the decreased reaction rate with increased pre-conversion, as shown in Figs. 2 and 3, is not due to reduce substrate concentrations, since all of the mixtures started with the same

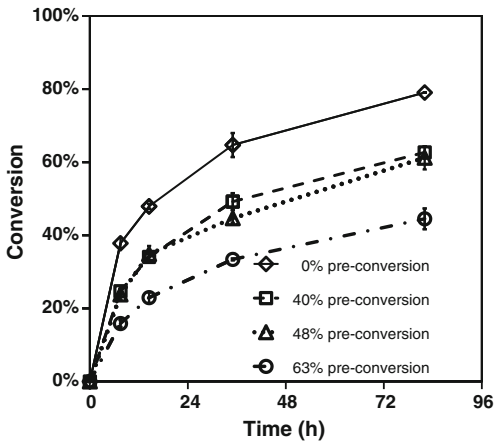


**Fig. 2** PCS conversion to glucose versus time is substantially reduced when using substrates at greater pre-conversion but at identical cellulose concentration. *Error bars* correspond to plus and minus one standard deviation of three replicate experiments

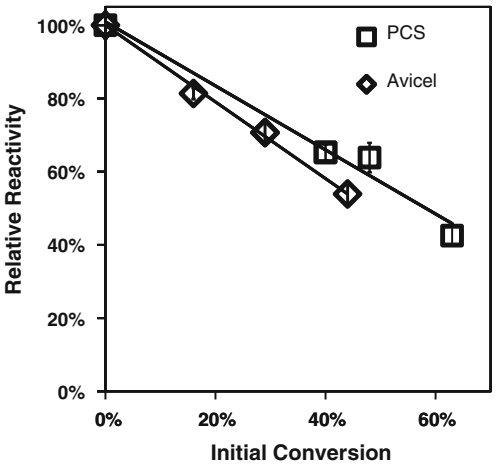


**Fig. 3** Normalized conversion rate data for PCS to glucose from the earliest time point (7.5 h) versus the amount of pre-conversion show a large, linear reduction in PCS reactivity relative to the unreacted PCS substrate with increased pre-conversion. Data for pure-cellulose Avicel show a similar reduction in reactivity as a function of conversion. *Error bars* correspond to plus and minus one standard deviation of three flasks

cellulose content. Instead, it is due to a decrease in the reactivity of the remaining substrate, perhaps due to a change in the cellulose structure or due to increased lignin binding.

*Microcrystalline Cellulose*

A large reduction in conversion rate due to increased pre-conversion was also measured using Avicel as a model substrate for the semi-crystalline cellulose present in PCS, as shown in Fig. 3. Because pre-saccharified and fresh Avicel are pure cellulose and were added at a constant cellulose basis, the effect of conversion is isolated from possible lignin effects. The Avicel, with initial cellulose conversions of 0%, 16%, 29% and 44%, when restarted at constant cellulose mass, reached new conversions of approximately 28%, 23%, 20% and 15%, respectively, after 24 h. The relative reactivities at different pre-conversions were calculated by normalizing the new conversions to the average conversion of the initially unreacted Avicel, analogous to the reactivity normalization for PCS. The regression for Avicel in Fig. 3 shows a drop in reactivity that is proportional to the degree of pre-conversion, with a proportionality constant of $-1.05\pm0.05$ at 95% confidence (i.e. the reactivity drops 10.5%±0.5% for every 10% increase in pre-conversion). This result is particularly interesting because it definitively shows a reduction in reactivity with pre-conversion for a pure-cellulose substrate. However, it does not indicate whether this effect is due to a change in crystallinity, accessibility, or some other mechanism.

*Lignin Content*

As shown in Fig. 4, a small reduction in conversion was obtained as the lignin-to-cellulose content was increased from 0.0 to 0.3 and 0.6. To quantify the effect of the lignin content, the conversions were normalized at each time to the average conversion without lignin. Using the data collected at 24 h, a linear regression of relative reactivity with respect to initial lignin-to-cellulose content was performed (not shown). For every 10% increase in the lignin-to-cellulose content, there was a corresponding 3%±1% decrease in relative conversion after 24 h at 95% confidence. Overall, the effect of the lignin-to-cellulose content was relatively small, corroborating the results of previous research [39]. However, only part of lignin's potential effects was observed here, since native lignin may also

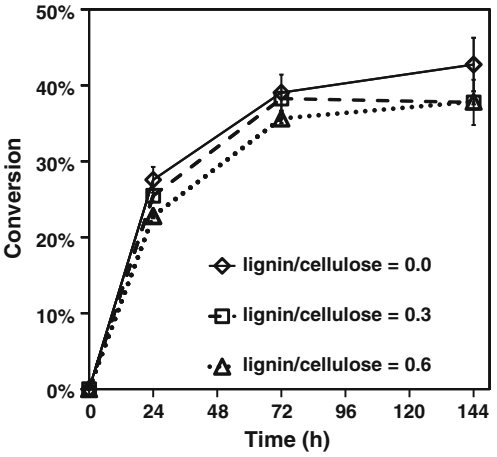


**Fig. 4** Conversion versus time data show that an increase in the initial lignin/cellulose ratio caused a small but significant reduction in conversion of Avicel to glucose throughout the reaction. *Error bars* correspond to plus and minus one standard deviation of three replicate experiments

physically obstruct the cellulose from cellulase binding and hydrolysis, an effect that cannot be observed in this type of experiment.

## Product Inhibition

### *Quantifying Product Inhibition*

By initially spiking the reaction mixture with glucose, it was possible to observe the isolated effect of glucose inhibition. Results indicate that an increase in glucose concentration from 7.5 g/L to 48 g/L reduced the conversion rate by 94%. Figure 5 provides a comparison of conversion rate versus glucose concentration for experiments with and without spiked glucose. Average conversion rates were calculated as the difference in conversions at the end and beginning of a sampling time interval divided by the duration of the time interval, while the average glucose concentration during this time interval was calculated as the mean of the glucose concentrations at the beginning and end. The initial-rate data with spiked glucose provide a direct measure of glucose inhibition. In contrast, the long-time batch data without glucose spiking may include other aggravating effects such as enzyme inactivation and decreased substrate reactivity.

To quantify the relative effect of glucose inhibition by comparing the two data sets in Fig. 5, it was necessary to fit the data points to smooth curves. Several fundamental models (zero-order and first-order in cellulose, Langmuir adsorption on cellulose, all with glucose inhibition) were attempted. However, residual analysis of these models showed that they are not adequate, likely because a single rate model cannot describe the aggregated kinetics of a complex, multiple enzyme reaction. At moderate and high glucose concentrations, the models under- and over-predicted the data, respectively (not shown). Instead, a simple quadratic expression relating the average conversion rate to glucose concentration was fitted to the data using least-squares regression, as shown in Fig. 6. The data from Fig. 5 are averaged over the multiple flasks and include error bars on both the conversion rates and glucose measurements. The conversion rates of the batch experiment without glucose addition are similar to the initial-rate experiments at low glucose concentrations; however, at high glucose concentrations, the conversion rates in the batch experiment are less than those in the initial-rate experiments.

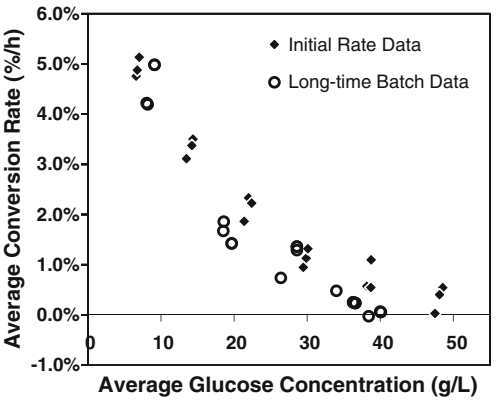


**Fig. 5** Average conversion rates of PCS to glucose over a time interval are plotted versus the average glucose concentrations during that interval for both long-time batch experiments and initial-rate experiments

An analysis of variances was then performed on the quadratic fits of the data to determine the local variances and confidence intervals on the conversion rate for the long-time batch and initial-rate data as functions of glucose concentration. Subsequently, a *t*-test on the differences in fitted rates at particular glucose concentrations was performed. At low average glucose concentration, the difference in rates was not found to be significant. However, the long-time batch data became significantly less than the initial-rate data at 95% confidence for glucose concentrations greater than 12.5 g/L. This result suggests that, although glucose inhibition may be a predominant factor, it is not the only significant factor causing the observed rate reduction during the saccharification of lignocellulosic biomass. As noted previously, the decline in substrate reactivity is also a significant factor.

*Mitigating Product Inhibition via Continuous Product Removal*

In Fig. 7, we show that integrated reaction and ultrafiltration to continuously remove product sugars while retaining substrate and enzymes results in lowered glucose concentration in the reaction mixture. Because the rate of glucose production via saccharification is less than the rate of glucose permeation through the membrane, the glucose concentration begins to drop immediately after buffer exchange begins at 24 h. Cellulose conversion was then determined by measuring the cumulative mass of glucose generated, but it is noted that this technique

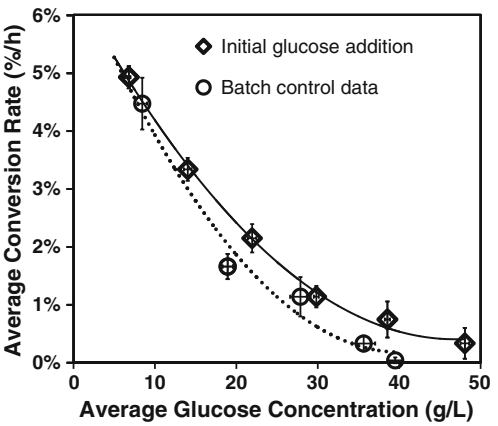


**Fig. 6** PCS conversion rate to glucose data from both the initial-rate experiments with initial glucose addition and the long-time batch experiments without glucose addition are plotted versus glucose concentration. Also shown are the least-squares quadratic fits. The *error bars* correspond to plus and minus one standard deviation for three flasks

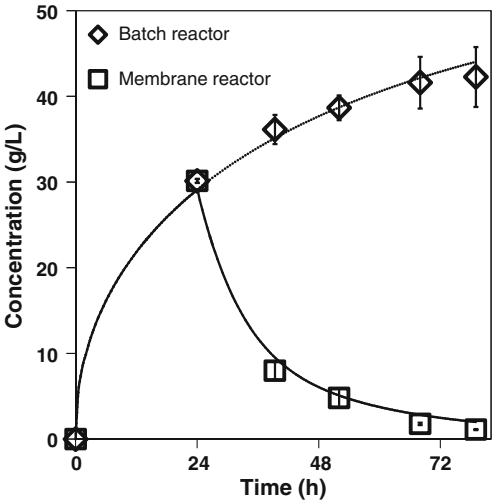


**Fig. 7** Glucose concentration versus time for a membrane reactor and a batch reactor. By continuously exchanging the reaction slurry's aqueous volume with fresh buffer, a reduction in the stirred cell's glucose concentration occurs. *Error bars* correspond to plus and minus one standard deviation of three replicate experiments. The *solid* and *dotted lines* correspond to model predictions of glucose concentrations

underestimates the overall hydrolysis by not including the production of intermediate sugars. As shown in Fig. 8, the reactor with continuous permeate removal resulted in enhanced conversion, with glucose-based conversions after 79 h of reaction being 9.5%±3.7% higher than the batch control, at 95% confidence using a paired *t*-test. Membrane integrity was maintained throughout the experiment, as measured by subsequent nitrogen bubble-point experiments. Models adapted from Kadam et al. [4] and fitted to batch glucose concentrations agree well with continuous glucose concentrations (Fig. 7) as well as glucose-based conversions (Fig. 8) for both the batch and continuous permeate experiments. Predicted overall cellulose conversions were expected to rise ~14% due to reduced glucose and cellobiose inhibition; however, the predicted gains of ~3% in glucose-based conversion are smaller. The large difference between cellulose-based and glucose-based conversions for the membrane-reactor model is due to the cellobiose intermediate and suggests significant cellobiose losses through the membrane. An optimized operating model for improving glucose-based conversions would need to account for cellobiose losses through the membrane.

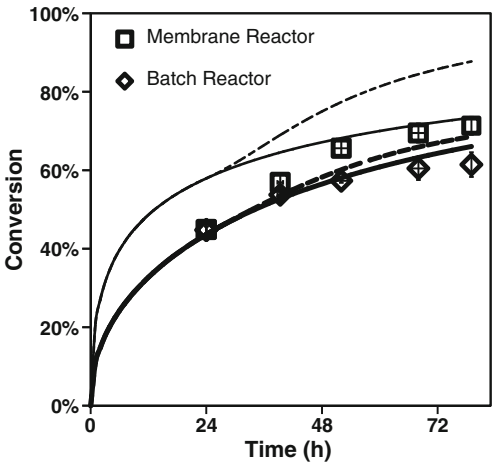


**Fig. 8** Conversion versus time for the membrane reactor and batch control. *Error bars* correspond to plus and minus one standard deviation of three replicate, paired experiments. The experimental data for glucose-based conversion are shown with model predictions of the membrane and batch reactors, depicted as *dashed* and *solid lines*, respectively, with both glucose- and cellulose-based conversions, depicted as *thick* and *thin lines*, respectively

## Conclusions

Several experiments were performed to elucidate the predominant sources of rate decay and inactivation during an enzymatic batch reaction to convert pretreated corn stover (PCS) to glucose. Various sources of inactivation were explored and categorized according to changes in the enzymes, the insoluble solids, and the solution. Overall, there appears to be minimal enzyme inactivation for the conditions used. However, large reductions in conversion rate were attributed to glucose inhibition as well as reduced substrate reactivity.

Experiments were also performed using a membrane reactor to continuously and selectively remove glucose while retaining enzymes. Although much lower glucose concentrations were achieved with the membrane reactor, the glucose-based conversions were only modestly improved.

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