# Enzyme Inactivation by Ethanol and Development of a Kinetic Model for Thermophilic Simultaneous Saccharification and Fermentation at 50 °C with *Thermoanaerobacterium saccharolyticum* ALK2

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**ABSTRACT:** Studies were undertaken to understand phenomena operative during simultaneous saccharification and fermentation (SSF) of a model cellulosic substrate (Avicel) at 50°C with enzymatic hydrolysis mediated by a commercial cellulase preparation (Spezyme CP) and fermentation by a thermophilic bacterium engineered to produce ethanol at high yield, *Thermoanaerobacterium saccharolyticum* ALK2. Thermal inactivation at 50°C, as shown by the loss of 50% of enzyme activity over 4 days in the absence of ethanol, was more severe than at 37°C, where only 25% of enzyme activity was lost. In addition, at 50°C ethanol more strongly influenced enzyme stability. Enzyme activity was moderately stabilized between ethanol concentrations of 0 and 40 g/L, but ethanol concentrations above 40 g/L accelerated enzyme inactivation, leading to 75% loss of enzymatic activity in 80 g/L ethanol after 4 days. At 37°C, ethanol did not show a strong effect on the rate of enzyme inactivation. Inhibition of cellulase activity by ethanol, measured at both temperatures, was relatively similar, with the relative rate of hydrolysis inhibited 50% at ethanol concentrations of 56.4 and 58.7 g/L at 50 and 37°C, respectively. A mathematical model was developed to test whether the measured phenomena were sufficient to quantitatively describe system behavior and was found to have good predictive capability at initial Avicel concentrations of 20 and 50 g/L. Biotechnol. Bioeng. 2011;108: 1268–1278.

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**KEYWORDS:** cellulase inactivation; thermophilic simultaneous saccharification and fermentation; ethanol; *T. saccharolyticum*

## Introduction

Cellulosic biofuels are potential renewable alternatives to petroleum-based fuels (Wyman, 2007). However, emergence of a cellulosic biofuels industry is impeded by the difficulty of converting cellulose into reactive intermediates (Himmel et al., 2007; Lynd et al., 2008). Both biological and non-biological approaches to overcoming this recalcitrance have been proposed and are actively under investigation (Lynd et al., 2002; Mohan et al., 2006). Biologically based processes for conversion of cellulosic biomass depend on cellulase enzymes, which may be either added to the fermentation system or produced by the fermenting microorganism.

Simultaneous saccharification and fermentation (SSF) combines hydrolysis of cellulose and the fermentation of hydrolysis products to ethanol within one reaction vessel (Takagi et al., 1977). In addition, the SSF configuration also reduces the accumulation of soluble sugars which can inhibit cellulose hydrolysis. However, due to the use of fermenting microorganisms with mesophilic temperature optima, many SSF systems operate at temperatures lower than the optima for commercial cellulase preparations (Olofsson et al., 2008). For example, commercial cellulase systems based on enzymes produced in *Trichoderma reesei* typically have optimal activity at 50°C, yet SSF using *Saccharomyces cerevisiae* is typically carried out between 30 and 37°C (Dien et al., 2003; Olofsson et al., 2008).

There have been a few reports of SSF using organisms with temperature and pH optima more closely matched to those of commercial cellulases. SSF at elevated temperatures has been reported for ethanol production by Spindler et al. (1988) using *Candida acidothermophilium* and *Saccharomyces uvarum* and Shaw et al. (2008) using an engineered strain of *Thermoanaerobacterium saccharolyticum*, and for lactic acid production by Patel et al. (2005) using *Bacillus* sp. strain 36D1 and Ou et al. (2009) using

*Bacillus coagulans.* In both the Ou et al. and Shaw et al. studies, elevated temperatures allowed for similar overall hydrolysis with lower cellulase loadings (e.g., mg or units of cellulase/$g_{cellulose}$). In particular, Shaw et al. (2008) observed a 2.5-fold reduction in the enzyme loading required to get equivalent hydrolysis for SSF at 50°C using *T. saccharolyticum* strain ALK2 compared to mesophilic SSF using *S. cerevisiae* at 37°C.

Mathematical models of mesophilic SSF have been developed to predict performance, compare bioprocessing configurations, and test whether understanding of operative phenomena (e.g., hydrolysis, fermentation, and inhibition) is sufficient to explain aggregated system behavior. Philippidis et al. (1993) described the activities of cellulase and β-glucosidase and reported the inhibition of glucose, cellobiose and ethanol on these enzymes. South et al. (1995) incorporated a conversion-dependent cellulose hydrolysis rate constant and Langmuir adsorption capable of describing saturation with respect to either substrate or enzyme. The South et al. model has subsequently been elaborated to accommodate intermittent feeding (Shao et al., 2009) and xylan hydrolysis and xylose co-fermentation (Zhang et al., 2009). Hydrolysis models have also been reported at 50°C (Fan and Lee 1983; Kadam et al., 2004). However a mathematical model combining hydrolysis and fermentation at thermophilic temperatures (tSSF) has not been previously reported. In particular, the long-term stability of mesophilic fungal cellulases at thermophilic temperatures and the combined effect of temperature and ethanol, as encountered during tSSF, have not been described.

In this study we evaluate the impact of ethanol on cellulase activity during SSF at 50°C and develop a mathematical model for tSSF of Avicel using a commercial cellulase preparation (Spezyme CP) and fermentation by *T. saccharolyticum* ALK2.

## Materials and Methods

### Enzymes

The *T. reesei* enzyme mixture, Spezyme CP (61 FPU/mL), was kindly provided by Genencor Intl. (Rochester, NY). β-glucosidase, Novozyme 188, was purchased from Sigma (St. Louis, MO). β-glucosidase activity measurements were made by standard techniques (Ghose 1987) with glucose concentrations measured using the Sigma glucose hexokinase assay kit.

### Enzymatic Hydrolyses

All enzymatic hydrolyses were performed in 50 mM citric acid buffer at pH 4.8. Cellulose concentrations and enzyme loadings are noted individually. Avicel (PH-105, FMC, Philadelphia, PA) and water were sterilized by autoclaving. Buffer, enzyme and ethanol solutions were filter sterilized using 0.22 μm Millipore Steriflip vacuum filters and added aseptically.

### Ethanol Inhibition of Cellulase Activity, $k_{S/E}$, and Rate Constant for Cellulose Hydrolysis, $k_1$

The initial rate of cellulose hydrolysis was measured in the presence of added ethanol to determine the degree of inhibition. Two grams per liter Avicel supplemented with 4 FPU Spezyme CP/$g_{cellulose}$ and 40 IU β-glucosidase/$g_{cellulose}$ was incubated at 37 or 50°C for 2 h in the presence of ethanol concentrations ranging from 0 to 80 g/L. Glucose, cellobiose and ethanol concentrations were measured via HPLC (Bio-Rad Aminex HPX-87H). Measured cellobiose concentrations were below 0.02 g/L throughout these experiments. Glucose and cellobiose concentrations, were used to calculate conversion, *x*, which can be calculated either by products produced or substrate consumed, as shown in Equation (1)

$$x = \frac{0.9[G] + 0.95[Cb]}{[St]_0} = \frac{[St]_0 - [St]}{[St]_0} \qquad (1)$$

Relative activity at each ethanol concentration was calculated as the rate of sugar production normalized by the rate in the absence of ethanol. The ethanol concentration at which relative cellulase activity decreased 50% was defined as the ethanol inhibition parameter, $k_{S/E}$. The rate constant $k_1$ was determined by fitting 0 g/L ethanol conversion data to the model prediction.

### Determination of Cellobiose Inhibition Parameter, $k_{S/C}$

The cellobiose inhibition parameter was fit to the conversion data from the hydrolysis of 20 g/L Avicel in 50 mM citric acid buffer, pH 4.8 with Spezyme CP added at enzyme loadings of 2.5, 4, 6, 10, and 15 FPU/g cellulose. Samples were drawn at 0, 2 and 4 h and glucose and cellobiose concentrations, measured using HPLC, were used to determine conversion, *x*.

### Enzyme Incubation and Residual Activity, *A*

Enzyme preparations were incubated at 37 and 50°C in the presence of 50 g/L Avicel with 25 FPU Spezyme CP/$g_{cellulose}$ and ethanol concentrations ranging from 0 to 80 g/L. Samples were taken every 24 h and frozen until measured for residual activity and soluble products via HPLC.

Residual activity was measured by a modified Avicel assay (McBride et al., 2010). Samples were diluted 10-fold in 50 mM citrate buffer (pH 4.8). Three hundred microliter of diluted enzyme samples were added to 300 μL fresh Avicel substrate mixture (20 g/L Avicel, 0.02% sodium azide, 50 mM citrate buffer pH 4.8, 1 mL/L β-glucosidase) in a deep-well microtiter plate, for a final enzyme concentration of 5 FPU/g cellulose. The 96-well plate was shaken at 50°C for 4 h at 1,000 rpm. At 0 and 4 h, the solution was mixed with a pipette and 125 μL was removed to measure reducing sugar concentration.

Drawn samples were centrifuged in a 96-well PCR plate. Fifty microliter of supernatant was drawn off and incubated at 99°C for 5 min with 100 μL modified DNS solution (Miller et al., 1960) on a thermocycler (Eppendorf Mastercycler Gradient), after which the plate was cooled to 4°C. Absorbance was measured on a Spectramax 190 plate reader at 565 nm to determine reducing sugar concentration with a glucose standard. Relative residual activity (RRA) was calculated, as shown in Equation (2), as the change in reducing sugars (RS) over 4 h as a function of ethanol concentration compared to the 0 hr incubation sample with an initial ethanol concentration of 0 g/L

$$RRA_{i,T,t} = \frac{RS_{i,T,t=4} - RS_{i,T,t=0}}{RS_{i=0,0,t=4} - RS_{i=0,0,t=0}} \qquad (2)$$

where $i$ is the ethanol concentration, $T$ is the incubation time, and $t$ is the residual activity assay time. Residual activity as a function of ethanol concentration was used to determine the parameters for Equation (8).

## Thermophilic SSF

Thermophilic SSF experiments were run at 20 or 50 g/L Avicel in 1.5 L Sartorius Aplus bioreactors under anaerobic conditions at 50°C, as described by Shaw et al. (2008), with Spezyme CP supplemented at 4 FPU/g Avicel using a modified version of MTC media (Zhang and Lynd 2003). In this formulation, the vitamin and mineral solutions were excluded and the medium was supplemented with 5 g/L urea, 10 g/L yeast extract, and 5 g/L tryptone. The pH was adjusted to 5.0 and buffered by the media. Though no active pH control was used, the pH stayed at $5.0 \pm 0.05$ (as monitored by a pH probe during tSSF experiments) due to the fact that *T. saccharolyticum* ALK2 does not produce organic acids as significant fermentation products. All reagents were chemical grade purchased from Sigma or Fisher. Avicel, water and resazurin (0.2%, optional) were sterilized by autoclaving. The remaining medium components were added after separate sterilization by autoclaving and/or 0.22 μm filtration. The size of inoculum, drawn from a continuous culture fed with 10 g/L glucose and 10 g/L xylose with a 17 h residence time, was scaled according to the initial substrate concentration with 10% (v/v) for 20 g/L Avicel, and 25% (v/v) for 50 g/L Avicel. It may be noted that smaller inocula could have been used at high substrate concentrations if the inocula were also prepared at high substrate concentrations. Samples were withdrawn periodically for measurement of remaining solids concentration via quantitative saccharification (Lu et al., 2006) and soluble products by HPLC. Carbon mass balances were calculated for all experiments reported and the final carbon recovery was consistently greater than 90% (data not shown).

## Modeling

Equations (4)–(15), presented subsequently, were incorporated into a mathematical model programmed using Berkeley Madonna software (http://www.berkeleymadonna.com). Model parameters were fit to experimental data in an order chosen to minimize interference by other parameters, as shown in Figure 1. Prior to curve fitting, independently measured parameters were calculated and verified for fit. The curve fit algorithm in Berkeley Madonna, which minimizes the root mean square (RMS) between predicted and experimental data, was used to determine parameter values for $k_c$, $k_1$, $k_2$, $k_3$, $k_4$, $m$, and $c$. $k_c$ was fit to cellobiose hydrolysis rate data (not shown). The parameters describing declining specific activity of the enzyme–substrate complex with conversion ($m$ and $c$) were determined using data from tSSF experiments at 20 g/L Avicel with an enzyme concentration of 4 FPU/g$_{cellulose}$. Because $m$ and $c$ are dependent on $k_1$ as well as $k_{S/C}$ and vice versa, values were determined iteratively. Values of $m$ and $c$ were used in the determination of $k_1$, which was then updated to re-determine $m$ and $c$. $k_{S/C}$ was also updated iteratively and this process was repeated until the RMS values between predicted and experimental values were minimized for the data sets used to determine $m$ and $c$, $k_1$ and $k_{S/C}$. The entire model was solved in Berkeley Madonna using a fourth-order Runge–Kutta method. The sensitivity function within Berkeley Madonna was used to evaluate the sensitivity of parameter values to the conversion, $x$.

## RESULTS

### Model Development

Enzymatic hydrolysis of cellulose is a complex, multistep process, and our knowledge of the many facets is incomplete. Models of cellulose hydrolysis help to assess our understanding of the system and the contribution of various known system properties, while also identifying bottlenecks and opportunities for improvements. Several models have been proposed in the literature, ranging from non-mechanistic, empirical models to very detailed functionally or structurally based models (Bansal et al., 2009). However, for many design purposes, a semi-mechanistic model, which can describe behavior using a minimal number of descriptive parameters can be sufficient. Such a model has been described (South et al., 1995) for mesophilic SSF and here is adapted to fit thermophilic SSF.

To understand the relative effects of ethanol within the tSSF system, the mesophilic SSF model proposed by South et al. was modified to accommodate temperature and ethanol dependent enzyme inactivation. In addition, the model was tailored to incorporate growth characteristics of *T. saccharolyticum* ALK2. Model equations are presented in this section with cellulose hydrolysis equations presented first, followed by microbial equations. Data used to determine parameters for the model are presented in the next section.
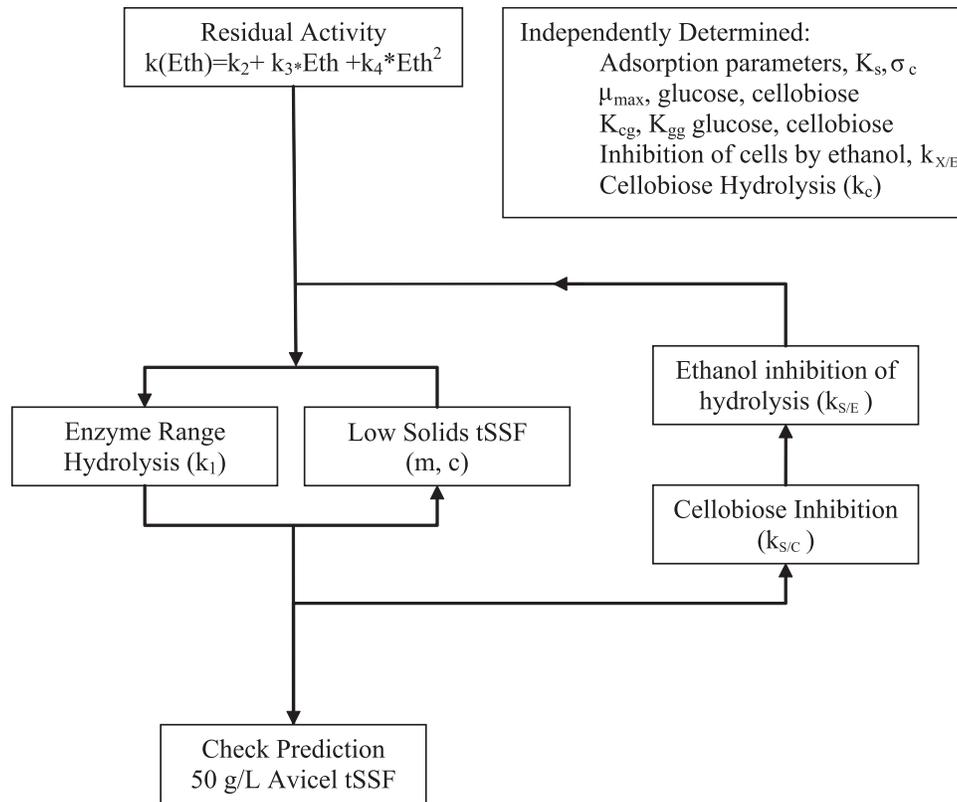
**Figure 1.** Flow diagram to determine model parameters. Parameter values were determined in an order to minimize interference by other parameter values. Adsorption, cell growth and cellobiose hydrolysis parameters were determined independently. Subsequently, enzyme inactivation was fit to experimental data. As the remaining parameters, $k_1$, $m$, $c$, $k_{S/E}$, and $k_{S/C}$, are interdependent, they were fit iteratively as shown.

## Cellulose Hydrolysis With a Conversion Dependent Rate Constant

As originally proposed by South et al., and used in several subsequent studies (Shao et al., 2009; Velkovska et al., 1997; Zhang et al., 2009) the rate of cellulose hydrolysis, $r_{St}$ ($g_{cellulose}\,L^{-1}\,h^{-1}$) is modeled in terms of the concentration of the enzyme–substrate complex, CE (g/L), with inhibition by cellobiose, Cb (g/L), glucose, G (g/L) and ethanol, Eth (g/L), and a conversion-dependent rate constant $k(x)$ (Equations 3 and 4)

$$r_{St} = \frac{dSt}{dt}$$
$$= -k(x)\left[\frac{CE}{1+\sigma_c}\right]\left[\frac{1}{1+\frac{Cb}{k_{S/C}}+\frac{G}{k_{S/G}}}\right]\left[\frac{k_{S/E}}{k_{S/E}+Eth}\right] \quad (3)$$

$$k(x) = k_1(1-x)^m + c \quad (4)$$

where St is the cellulose (g/L), $\sigma_c$ is the adsorption capacity of cellulase onto cellulose ($g_{enzyme}/g_{cellulose}$), $k_{S/C}$ is the cellulase inhibition constants for cellobiose (g/L), $k_{S/G}$ is the cellulase inhibition constant for glucose (g/L), $k_{S/E}$ is the

cellulase inhibition constants for ethanol (g/L), $k_1$ is the rate constant for cellulose hydrolysis ($h^{-1}$), $m$ is the constant of declining substrate-enzyme reactivity (dimensionless), and $c$ is the constant of declining substrate-enzyme reactivity ($h^{-1}$). Conversion, the fraction of substrate used, is calculated with substrate concentrations using Equation (1).

## Langmuir Adsorption of Cellulase

Also following South et al. as well as other studies (Bansal et al., 2009; Bothwell and Walker 1995; Zhang and Lynd 2004), adsorption of a single idealized cellulase activity to cellulose is modeled in terms of a Langmuir relationship:

$$[CE] = [E_a]\frac{1+\sigma_c}{\sigma_c} = \frac{K_s[E_f](1+\sigma_c)[St]}{1+K_s[E_f]} \quad (5)$$

$$E_t = E_a + E_f \quad (6)$$

where $E_t$ is total enzyme (g/L), $E_a$ is adsorbed enzyme (g/L), $E_f$ is free enzyme (g/L), and $K_s$ is the adsorption equilibrium constant (L/g).
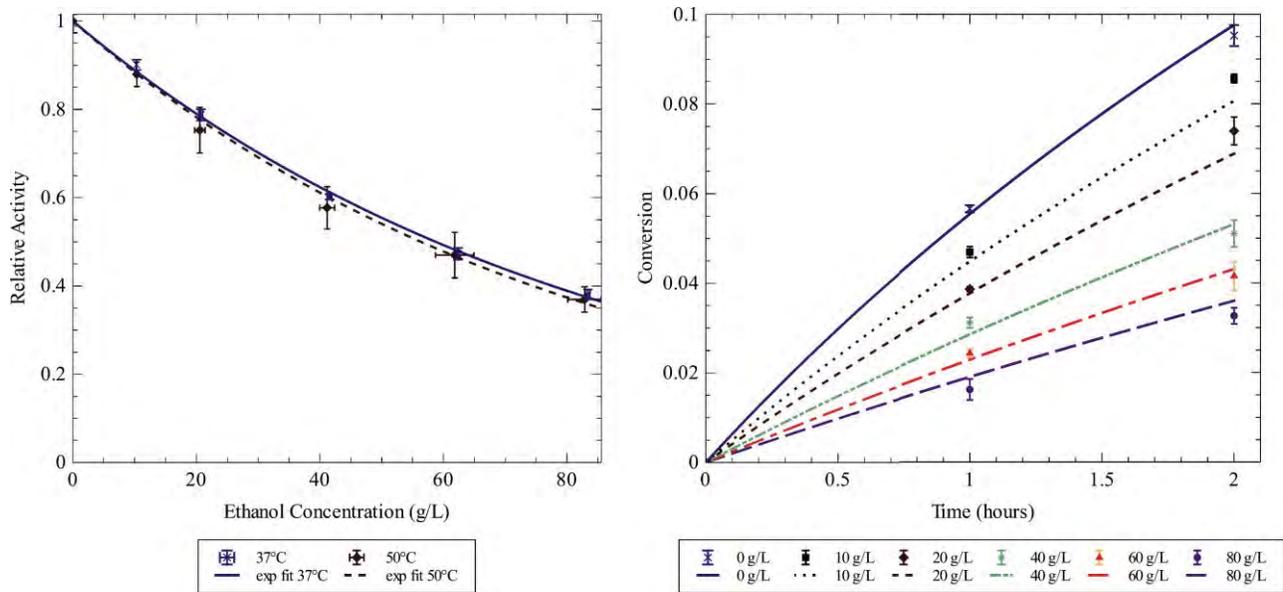
**Figure 2.** Inhibition of cellulase activity by ethanol: (**A**) Relative rate of hydrolysis as a function of initial ethanol concentration at both 37°C (x) and 50°C (diamonds). Data are representative of two independent experiments that showed similar results. Error bars show standard error from individual experiments. **B**: Measured (points) and model predicted (lines) conversion at given ethanol concentrations (0 g/L, x; 10 g/L, square; 20 g/L, diamond; 40 g/L, star; 60 g/L, triangle; 80 g/L, circle).

### Thermal and Ethanol Dependent Enzyme Inactivation

The models of South et al. and Philippidis et al., developed for temperatures of 37 and 38°C, respectively, assume a constant enzyme activity over the course of the fermentation. However, experimental data presented herein (Fig. 3) show that enzyme activity decreases over time due to the combined effects of thermal inactivation at 50°C and exposure to ethanol. In contrast to inhibition, which is reversible and already included in the South et al. model, inactivation is irreversible, thus reducing the effective enzyme concentration. To describe this inactivation, an additional variable, $A$ (dimensionless), representing residual enzymatic activity, was added to the model, as shown in Equation (7). Relative residual activity (RRS) was shown experimentally to follow first-order decay kinetics. The rate constant ($k(\text{Eth})$) was shown experimentally to be a function of ethanol concentration. Therefore, Equation (8) was constructed to match this behavior with the empirically determined constants $k_2$ ($\text{h}^{-1}$), $k_3$ ($\text{g}_{\text{ethanol}}\,\text{L}^{-1}\,\text{h}^{-1}$), and $k_4$ ($\text{g}_{\text{ethanol}}^2\,\text{L}^{-2}\,\text{h}^{-1}$). The resulting rate of cellulose hydrolysis in tSSF is represented by Equation (9)

$$r_{\text{A}} = \frac{dA}{dt} = k(\text{Eth})A \qquad (7)$$

$$k(\text{Eth}) = k_2 + k_3\,\text{Eth} + k_4\,\text{Eth}^2 \qquad (8)$$

$$r_{\text{St}} = \frac{d\text{St}}{dt}$$

$$= -k(x)\left[\frac{\text{CE}}{1+\sigma_{\text{c}}}\right]\left[\frac{1}{1+\frac{\text{Cb}}{k_{\text{S/C}}}+\frac{\text{G}}{k_{\text{S/G}}}}\right]\left[\frac{k_{\text{S/E}}}{k_{\text{S/E}}+\text{Eth}}\right]A \qquad (9)$$

### Cell Growth on Cellobiose and Catabolite Repression on Mixed Substrates

*T. saccharolyticum* ALK2 grows on both cellobiose and glucose, and the organism's metabolism thus appears in the rate equations for both sugars in the tSSF model. The accumulation of cellobiose, $r_{\text{Cb}}$, is determined by the rate of cellulose hydrolysis to cellobiose, cellobiose hydrolysis to glucose (Equation 10), and uptake of cellobiose by cells (Equation 11). The rate of glucose, $r_{\text{G}}$, accumulation is determined by cellobiose hydrolysis and consumption of glucose by cells (Equation 12). Uptake of both glucose and cellobiose is modeled using Monod equations, and the overall rate of cell formation was assumed to be the sum of cell formation due to glucose and cellobiose (Equation 13). Experimental data presented herein show glucose is utilized preferentially relative to cellobiose and the uptake rate of each substrate reduced by the presence of the other (Supplementary Material, Fig. S2c). The parameters $B_2$ and $B_3$ (L/g) in Equations (14) and (15) represent the inhibition of growth on cellobiose in the presence of glucose
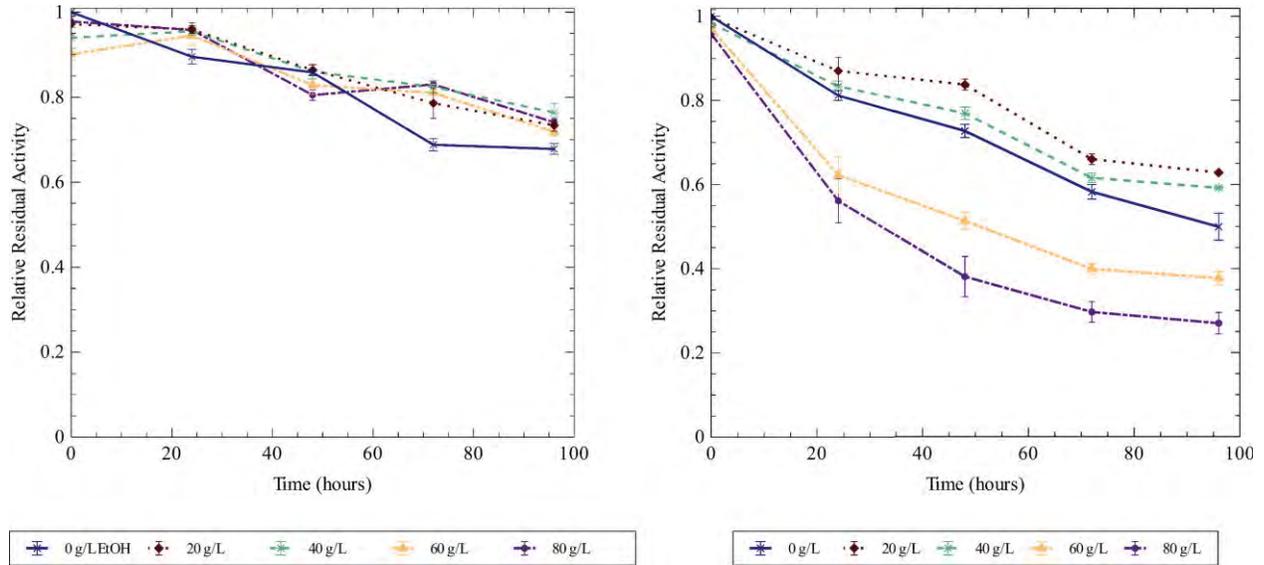
**Figure 3.** Relative residual enzyme activity as a function of incubation time and ethanol concentration: Residual activity of cellulase enzymes after incubation at (**A**) 37°C and (**B**) 50°C in the presence of ethanol at given ethanol concentrations (0 g/L, x; 20 g/L, diamond; 40 g/L, star; 60 g/L, triangle; 80 g/L, circle).

and that of glucose in the presence of cellobiose, respectively (Kwon and Engler 2005). Also shown in Equations (14) and (15), inhibition of cell growth by ethanol of cells grown on glucose or cellobiose is described by a threshold inhibition model, as previously described for inhibition of growth on glucose by South et al., where $k_{X/E}$ (g/L) represents the ethanol concentration above which growth is not detected. Since *T. saccharolyticum* ALK2 utilizes cellobiose, no additional β-glucosidase is added. However, Spezyme CP does have cellobiase activity and therefore in the tSSF model, BG (g/L) represents the β-glucosidase activity of the Spezyme CP cellulase mixture. The following equations represent the net rates of production of cellobiose, glucose and cells:

$$r_{CbG} = \frac{-k_c\, Cb\, BG}{K_m\left(1 + \frac{G}{k_{cg}}\right) + Cb} \quad (10)$$

$$r_{Cb} = \frac{dCb}{dt} = -1.056 r_{St} + r_{CbG} - \frac{r_{X_{cCb}}}{Y_{X/G}} \quad (11)$$

$$r_G = \frac{dG}{dt} = -1.053 r_{CbG} - \frac{r_{X_{cG}}}{Y_{X/G}} \quad (12)$$

$$r_{X_c} = \frac{dX_c}{dt} = r_{X_{cCb}} + r_{X_{cG}} \quad (13)$$

$$r_{X_{cCb}} = \frac{dX_{cCb}}{dt}$$
$$= X_c \frac{\mu_{max\,Cb}\, Cb}{Cb + K_{gc}}\left(1 - \frac{Eth}{k_{X/E}}\right)\left(\frac{1}{1 + (B_2 G)}\right) \quad (14)$$

$$r_{X_{cG}} = \frac{dX_{cG}}{dt}$$
$$= X_c \frac{\mu_{max\,g}\, G}{G + K_{gg}}\left(1 - \frac{Eth}{k_{X/E}}\right)\left(\frac{1}{1 + (B_3\, Cb)}\right) \quad (15)$$

$$r_{Eth} = \frac{dEth}{dt} = r_{X_c}\frac{Y_{P/G}}{Y_{X/G}} \quad (16)$$

where $k_c$ is the rate constant for cellobiose hydrolysis ($g_{cellobiose}/(g_{enzyme}\,h)$), $K_m$ the Michaelis constant for cellobiose hydrolysis (g/L), $k_{cg}$ the inhibition of cellobiose hydrolysis by glucose (g/L), $X_c$ the total cell concentration from cellobiose ($X_{cCb}$) or glucose ($X_{cG}$) (g/L), $\mu_{max}$ the maximum growth rate on cellobiose ($\mu_{maxCb}$) or glucose ($\mu_{maxG}$) ($h^{-1}$), $K_g$ the affinity of cells for cellobiose ($K_{gc}$) or glucose ($K_{gg}$) (g/L), $k_{X/E}$ the inhibition of growth by ethanol (g/L), $Y_{X/G}$ the cell yield ($g_{cells}/g_{glucose}$), and $Y_{P/G}$ the product yield ($g_{ethanol}/g_{glucose}$). Determination of these growth parameters is described in the Supplementary Material.

## Determination of Cellulose Hydrolysis Parameters

### Adsorption

The adsorption equilibrium constant ($K_s$) and the adsorption capacity ($\sigma_c$) were found to be 1.24 L/g and 0.149 $g_{enzyme}/g_{cellulose}$ respectively as determined by fitting free and adsorbed enzyme concentrations to Equation (5), as described in the Supplementary Material (Fig. S1).

## Thermal and Ethanol Inactivation of Enzyme Activity

In tSSF, ethanol is produced in the same reactor as cellulose is hydrolyzed. While SSF alleviates hydrolysis inhibition by glucose and cellobiose, the ethanol produced in tSSF at 50°C may affect cellulase activity. Previous reports have described inhibition of cellulase activity by ethanol at 37°C for SSF conditions (Philippidis et al., 1993). In addition, ethanol has previously been shown to more strongly inhibit cellulase activity at higher temperatures (Wu and Lee 1997). To confirm this observation, inhibition of cellulase activity was measured at both 37 and 50°C by the reduction of initial hydrolysis rates measured in the presence of varying ethanol concentrations. Initial rates were used to minimize the effects of hydrolysis products and conversion. In contrast to previous results, little difference was seen between relative inhibition at 37 and 50°C (Fig. 2a). Based on the best fit exponential curve, the rate of cellulose hydrolysis will decrease 50% at an ethanol concentration of 56.4 g/L at 37°C and 58.7 g/L at 50°C. However, to account for effects in conversion, the ethanol inhibition parameter, $k_{S/E}$, was adjusted to fit conversion data directly and was found to be 37.6 g/L (Fig. 2b), which was used in the model.

In addition to the known inhibitory effect of ethanol, dilution experiments indicated irreversible inactivation of cellulase activity was occurring in tSSFs and a greater loss of activity was observed at higher ethanol concentrations (data not shown). To quantify the irreversible loss of activity and the effect of ethanol, cellulase enzymes were incubated at known ethanol concentrations and residual cellulase activity was determined over time. Enzyme inactivation was observed at both 37 and 50°C (Fig. 3). At 37°C roughly 30% activity was lost after 4 days. This inactivation was independent of the ethanol concentration, indicating that thermal inactivation predominates at 37°C. Heightened thermal inactivation was observed at 50°C in the absence of ethanol, where enzyme activity was reduced by 50% after 96 h. In contrast to 37°C, the rate of enzyme inactivation at 50°C was dependant upon the ethanol concentration. Moderate ethanol concentrations consistently mitigated the activity loss, while ethanol concentrations above 40 g/L accelerated the rate of activity loss. At 50°C with 80 g/L ethanol, 75% of activity was lost after 100 h. The constants $k_2$, $k_3$, and $k_4$ were fit to the residual activity data shown in Figure 3b and found to be $-0.0073$ $(h^{-1})$, $2.06E{-}4\,g_{ethanol}\,L^{-1}\,h^{-1}$, and $-3.85E{-}6\,g_{ethanol}\,L^{-2}\,h^{-2}$ respectively.

## Rate Constant for Cellulose Hydrolysis, $k_1$, and Inhibition by Cellobiose, $k_{S/C}$

The rate constant for cellulose hydrolysis, $k_1$, was determined by fitting 2 g/L Avicel hydrolysis data to Equation (9), and found to be 2.401 $h^{-1}$. The measured and predicted values are shown in Figure 4a. The cellobiose inhibition parameter for cellulase activity was also determined in a similar manner, fit to hydrolysis data on 20 g/L Avicel with enzyme loadings from 2 to 15 FPU/g cellulose, for a value of 0.322 g/L, shown in Figure 4b.
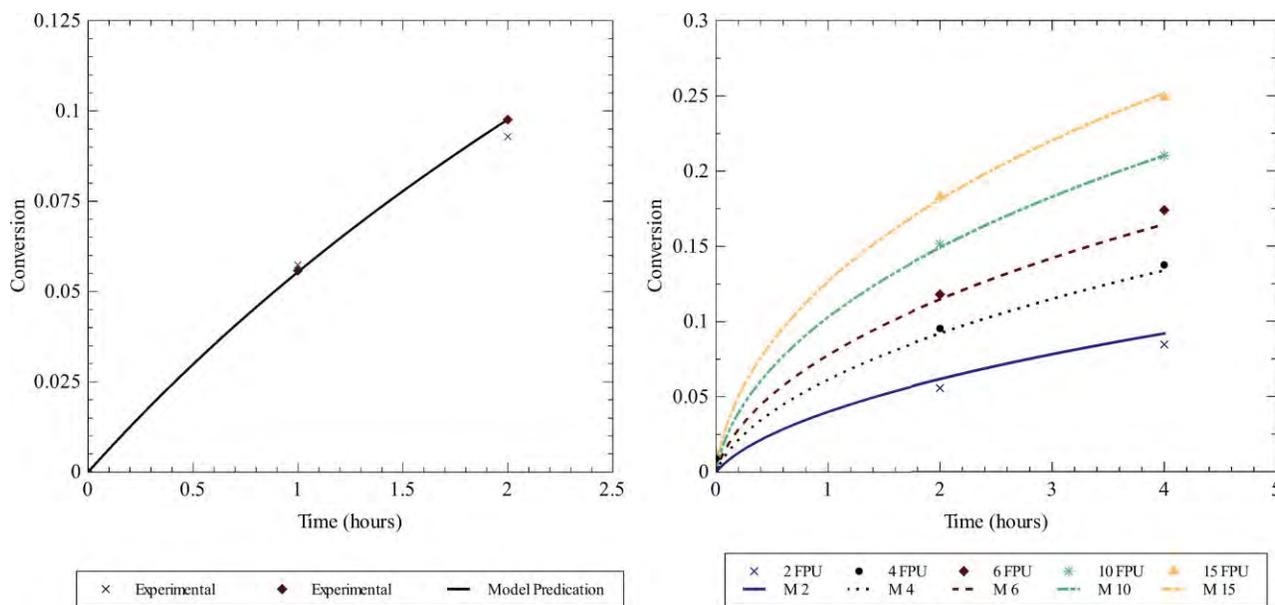


**Figure 4.** Initial rate of Avicel hydrolysis to determine rate constant, $k_1$ and cellobiose inhibition $k_{S/C}$. **A**: Experimental data from 2 g/L Avicel hydrolyzed for 2 h with 4 FPU/g cellulase and 40 IU/g beta-glucosidase was used to determine the rate constant for cellulose hydrolysis, $k$. **B**: Conversion data measured from 20 g/L Avicel hydrolyses supplemented with 2, 4, 6, 10, and 15 FPU/g cellulose was fit to determine $k_{S/C}$ (2 FPU, x; 4 FPU, circle; 6 FPU, diamond; 10 FPU, star; 15 FPU, triangle; M, model prediction at given FPU).

### Declining Enzyme–Substrate Reactivity

Since inhibition by sugars is minimized in tSSF, conversion data from 20 g/L tSSF were used to determine parameters describing the declining reactivity of the enzyme–substrate complex (Fig. 5). Parameters m and c were determined to be 4.585 and 0.11, respectively.

### Growth Parameters for T. saccharolyticum ALK2

Growth parameters for *T. saccharolyticum* ALK2, including catabolite repression and inhibition of cell growth by ethanol were determined as described in the Supplementary Materials. Parameter values are reported in Table I.

### Model Validation

tSSFs were run at 20 and 50 g/L Avicel (data points, Fig. 5). At both initial solids loadings experimental data showed that glucose and cellobiose initially accumulated in media, indicating that cellulose hydrolysis rates were faster than soluble sugar uptake associated with fermentation during the first 8–12 h. After this initial period, the accumulated *T. saccharolyticum* ALK2 cells consumed soluble sugars faster than or equal to the rate of cellulose hydrolysis, thereby establishing and maintaining very low soluble sugar concentrations. Lactic acid and acetic acid remained below 0.7 g/L in all experiments. With all parameters determined (Table I), the model was solved and compared to this experimental data. The model accurately predicted cellulose, cellobiose, glucose and ethanol concentrations at 20 and 50 g/L Avicel (solid lines, Fig. 5).

A sensitivity analysis of the model has been performed using Berkeley Madonna, specifically analyzing the effect of each parameter on conversion (Supplementary Table SI). The model prediction is most sensitive to the parameters describing ethanol inactivation ($k_4$, $k_3$, and $k_2$) the declining enzyme–substrate reactivity ($c$) and parameter describing adsorption capacity of cellulase enzymes ($\sigma_c$). Perturbation of other parameters had little impact on the model (Supplementary Table SI).

## Discussion

In this study, ethanol was found to significantly decrease the half-life of *T. reesei* cellulases at 50°C. This ethanol-dependent thermal inactivation adds an important dimension to the tSSF model and improves our understanding of the performance trade-offs of running SSF at elevated temperatures.

While ethanol is known to inhibit cellulase activity (Ghosh et al., 1982; Philippidis et al., 1993; Takagi 1984; Wu and Lee 1997), the effect of temperature on ethanol inhibition of cellulase activity has been reported with conflicting results. In contrast the report of Wu and Lee, our results show that inhibition of cellulase activity by ethanol did not change with temperature. Temperature does, however, have an effect on the inactivation of cellulase activity by ethanol. Previous studies reported minimal enzyme inactivation in SSF at mesophilic temperatures (Philippidis et al., 1993) and in hydrolysis (Zhang et al., 1999). However, in tSSF cellulase enzymes are exposed to increasing ethanol concentrations over the course of several
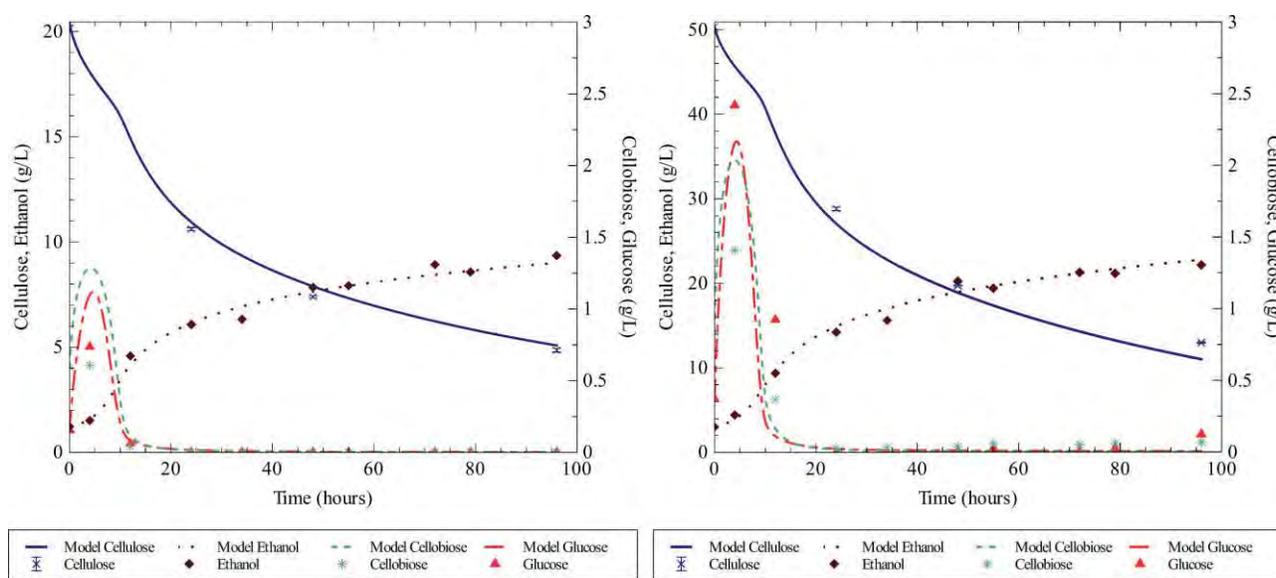


**Figure 5.** tSSF: Experimental data (data points) and model predictions (lines) for tSSF fermentations of (**A**) 20 g/L and (**B**) 50 g/L Avicel tSSF with Avicel (x, solid), ethanol (diamond, dot), cellobiose (star, dash) and glucose (triangle, dot dash) concentrations (cellulose (exp, model) x; solid, ethanol—diamond, dot; cellobiose—star, dash; glucose—triangle, dot dash).

**Table I.** Constants for tSSF mathematical model.

| Parameter | Value | Source | Units | Description |
|---|---|---|---|---|
| $B_2$ | 6.839 | This work | g/L | Inhibition of cellobiose utilization by glucose |
| $B_3$ | 0.418 | This work | g/L | Inhibition of glucose utilization by cellobiose |
| $c$ | 0.12 | This work | $h^{-1}$ | Constant of decreasing enzyme substrate complex reactivity |
| $k_1$ | 2.38 | This work | $h^{-1}$ | Rate constant for cellulose hydrolysis |
| $k_2$ | $-0.0073$ | This work | $h^{-1}$ | Thermal inactivation constant |
| $k_3$ | 2.06E$^-$04 | This work | $g_{ethanol}\,L^{-1}\,h^{-1}$ | Ethanol inactivation constant |
| $k_4$ | $^-$3.85E$^-$06 | This work | $g_{ethanol}^2\,L^{-2}\,h^{-1}$ | Ethanol inactivation constant |
| $k_c$ | 7.27 | This work | $g_{cellobiose}/(g_{enz}\,h)$ | Rate constant for cellobiase activity |
| $k_{cg}$ | 0.0382 | Grous et al. (1985) | g/L | Inhibition of cellobiose hydrolysis by glucose |
| $K_{gc}$ | 0.722 | This work | g/L | Affinity of cells for cellobiose |
| $K_{gg}$ | 0.807 | This work | g/L | Affinity of cells for glucose |
| $K_m$ | 0.0524 | Grous et al. (1985) | g/L | Michaelis constant for cellobiose hydrolysis |
| $K_s$ | 1.241 | This work | L/g | Equilibrium adsorption constant |
| $k_{S/C}$ | 0.319 | This work | g/L | Inhibition of cellulase activity by cellobiose |
| $k_{S/E}$ | 37.6 | This work | g/L | Inhibition of cellulase activity by ethanol |
| $k_{S/G}$ | 53.16 | Philippidis et al. (1993) | g/L | Inhibition of cellulase activity by glucose |
| $k_{X/E}$ | 50.3 | This work | g/L | Inhibition of cell growth by ethanol |
| $m$ | 4.49 | This work | Unitless | Constant of decreasing substrate–enzyme complex reactivity |
| $Y_{P/G}$ | 0.46 | Shaw et al. (2008) | $g_{ethanol}/g_{glucose}$ | Yield of ethanol from glucose |
| $Y_{X/G}$ | 0.1 | South et al. (1995) | $g_{cells}/g_{glucose}$ | Yield of cells from glucose |
| $\mu_{maxCB}$ | 0.539 | This work | $h^{-1}$ | Maximum growth rate on cellobiose |
| $\mu_{maxG}$ | 0.596 | This work | $h^{-1}$ | Maximum growth rate on glucose |
| $\sigma_c$ | 1.149 | This work | $g_{enz}/g_{cellulose}$ | Adsorption capacity |
| Rate equations | | | | |
| $r_{St}$ | | | $g_{cellulose}/(L\,h)$ | Rate equation for accumulation of cellulose |
| $r_{Cb}$ | | | $g_{cellobiose}/(L\,h)$ | Rate equation for accumulation of cellobiose |
| $r_{CbG}$ | | | $g_{cellobiose}/(L\,h)$ | Rate of hydrolysis of cellobiose into glucose |
| $r_G$ | | | $g_{glucose}/(L\,h)$ | Rate equation for accumulation of glucose |
| $r_{Xc}$ | | | $g_{cells}/(L\,h)$ | Rate equation for total accumulation of cells |
| $r_{XcCb}$ | | | $g_{cells}/(L\,h)$ | Rate equation for the accumulation of cells from cellobiose |
| $r_{XcG}$ | | | $g_{cells}/(L\,h)$ | Rate equation for the accumulation of cells from glucose |
| $r_{Eth}$ | | | $g_{ethanol}/(L\,h)$ | Rate equation for accumulation of ethanol |

days. Under these conditions, we demonstrate that the stability of cellulase enzymes decreases significantly.

At ethanol concentrations seen in tSSF enzyme inactivation followed apparent first-order kinetics. The rate constant for thermal inactivation is comparable to that reported for *T. reesei* cellulase enzymes (Drissen et al., 2007). While second-order deactivation has also been reported for *T. reesei* component enzymes due to aggregation (Dominguez et al., 1992) and loss of synergy (Gusakov et al., 1982), the exponential decrease in residual activity measured here gave $R^2$ values greater than 0.9, demonstrating that a first-order approximation was sufficient for modeling total enzyme activity in this system.

While ethanol at high concentrations inactivates enzymes at 50°C, a modest stabilizing effect on the rate of enzyme inactivation was seen at lower ethanol concentrations. A similar stabilizing effect with moderate ethanol concentrations has been described for the yeast protein Yfh1 (Martin et al., 2008). This unexpected result might be explained by stabilization of hydrophobic interactions in the protein (Blanco-Torres et al., 2006), though this has not yet been investigated specifically in tSSF.

The fermenting organism *T. saccharolyticum* ALK2 can utilize both glucose and cellobiose, the products of cellulose hydrolysis. Uptake of these sugars was shown to be sequential, indicative of competitive uptake kinetics. In addition, this strain has been shown to tolerate ethanol concentrations up to 50 g/L, necessary for the economic recovery of ethanol (Zacchi and Axelsson 1989). As indicated by the minimal sugar concentrations for the majority of the tSSF, as in SSF, hydrolysis remains the rate-limiting step in tSSF. Since hydrolysis limits conversion for the majority of the tSSF, parameters affecting enzyme activity and stability are of great interest. Indeed, sensitivity analysis indicates that all the major factors influencing this tSSF model relate to the functional concentration of enzyme and enzyme activity. This suggests that areas for improvement of both the model and tSSF lie in optimizing the enzyme activity, specifically the stability of the enzymes in the presence of ethanol and high temperature.

We developed a model to describe tSSF that fits performance with *T. saccharolyticum* ALK2 and incorporates the observed enzyme inactivation in the presence of ethanol at 50°C, as well as catabolite repression of growth on a mixture of glucose and cellobiose for *T. saccharolyticum* ALK2. The fit between predicted and actual concentrations of cellulose, cellobiose, glucose and ethanol during tSSF at 20 and 50 g/L initial Avicel concentrations is quite good. These results are consistent with the proposed relationships and parameter values being adequate to describe system behavior.

At low initial solids concentration, the reduction in enzyme requirement justifies the use of tSSF. However, as shown by the rapid loss of activity at high ethanol concentrations at 50°C, enzyme stability will play an increasing role as initial solids concentrations are increased and higher ethanol concentrations are achieved. Such effects on enzyme stability must be accounted for when comparing the suitability of systems for the bioconversion of cellulose.

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