Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by the Simultaneous Saccharification and Fermentation Process

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The biochemical conversion of cellulosic biomass to ethanol, a promising alternative fuel, can be carried out efficiently and economically using the simultaneous saccharification and fermentation (SSF) process. The SSF integrates the enzymatic hydrolysis of cellulose to glucose, catalyzed by the synergistic action of cellulase and β-glucosidase, with the fermentative synthesis of ethanol. Because the enzymatic step determines the availability of glucose to the ethanologenic fermentation, the kinetics of cellulose hydrolysis by cellulase and β-glucosidase and the susceptibility of the two enzymes to inhibition by hydrolysis and fermentation products are of significant importance to the SSF performance and were investigated under realistic SSF conditions. A previously developed SSF mathematical model was used to conceptualize the depolymerization of cellulose. The model was regressed to the collected data to determine the values of the enzyme parameters and was found to satisfactorily predict the kinetics of cellulose hydrolysis. Cellobiose and glucose were identified as the strongest inhibitors of cellulase and β-glucosidase, respectively. Experimental and modeling results are presented in light of the impact of enzymatic hydrolysis on fuel ethanol production.

Key words: enzymatic hydrolysis • cellulose • β-glucosidase • SSF • ethanol

INTRODUCTION

Cellulosic biomass is an abundant renewable resource that can serve as substrate for the production of alternative fuels, such as ethanol.[13] Extensive research has demonstrated that SSF, the simultaneous saccharification (hydrolysis) of cellulose to glucose and fermentation of glucose to ethanol, improves the kinetics[12] and economics[14] of biomass conversion by minimizing accumulation of hydrolysis products that are inhibitory to cellulase and β-glucosidase, minimizing the contamination risk because of the presence of ethanol, and reducing the capital equipment requirements. However, a recent economic analysis of ethanol production from biomass[7] still identifies the SSF unit operation as the major contributor to the cost of ethanol (>20%), thereby raising the need for optimization of the SSF performance.

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Analysis of the phenomena involved in the SSF process has identified four main factors that influence its kinetics: (1) the quality of the cellulosic substrate; (2) the quality of the cellulase and β-glucosidase enzyme system; (3) the mode of interaction between substrate and enzyme; and (4) the mode of interaction between enzyme and fermentative organism.[11] The susceptibility of cellulosic biomass to enzymatic degradation seems to be influenced by the structural characteristics of the substrate, whereas the quality of the enzyme complex determines the performance of its components, as they hydrolyze cellulose. The enzyme–substrate interaction regulates the extent of enzyme adsorption onto the substrate and, hence, the rate of this heterogeneous reaction. Finally, the microorganism–enzyme interaction affects the growth kinetics of the cells and their ethanol productivity, as well as potential inhibitory effects of hydrolysis products (cellobiose, glucose) and metabolites (ethanol) on enzyme and microbial activity.

It becomes obvious from the above discussion that the behaviors of the cellulase and β-glucosidase enzymes play a major role in the progress of the SSF. It is therefore of utmost importance to comprehend the kinetic characteristics of the hydrolytic enzymes before any attempt is made to optimize the performance of the SSF. Such an understanding can be aided by an SSF mathematical model previously formulated.[11] The model is based on expressions that conceptualize the rate at which each enzyme operates. It should be noted that although, in general, cellulase consists of 1,4-β-D-glucan glucanohydrolases (endoglucanases), 1,4-β-D-glucan cellobiohydrolases (exoglucanases), and β-D-glucoside glucohydrolase (β-glucosidase), in this study, for practical reasons, the enzyme complex is treated as two distinct entities: (a) cellulase that hydrolyzes cellulose to cellobiose with negligible formation of glucose through the cooperation of endo- and exoglucanases; and (b) β-glucosidase that hydrolyzes cellobiose to glucose. Here, we present a study of the kinetic properties of each of the two hydrolytic enzymes (cellulase, β-glucosidase) under realistic SSF conditions and the determination of their kinetic parameters through regression of the

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appropriate model equations to collected experimental data. It should be emphasized that, unlike previous studies which employed optimal enzyme activity conditions and did not distinguish between cellulase and β-glucosidase, the present investigation was carried out under conditions that simulated the SSF process and uncoupled the two different classes of enzymatic activity.

CELLULOSE HYDROLYSIS MODEL

As mentioned earlier, a mathematical model has been developed for the SSF process to support further improvements in the current biomass conversion technology.[11] The portion of this model that describes the kinetics of cellulose hydrolysis to cellobiose and eventually to glucose consists of the following mass balance equations:

Cellulose:

$$\frac{dC}{dt} = -r_1 \tag{1}$$

Cellobiose:

$$\frac{dB}{dt} = 1.056r_1 - r_2 \tag{2}$$

Glucose:

$$\frac{dG}{dt} = 1.053r_2 \tag{3}$$

with the rates of the hydrolytic action of cellulase (e_c) and β-glucosidase (e_g), r_1, and r_2, respectively, being:

$$r_1 = \frac{k_1 \alpha_t \phi e_c}{(K_e + e_c)\left(1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}\right)} \frac{K_{1E}}{K_{1E} + E} \cdot (1 - K_{1L}L) \tag{4}$$

$$r_2 = \frac{k_2 B e_g}{K_m\left(1 + \frac{G}{K_{2G}}\right) + B\left(1 + \frac{B}{K_{2B}}\right)} \frac{K_{2E}}{K_{2E} + E} \cdot (1 - K_{2L}L) \tag{5}$$

where α_t is the surface area of cellulose available for cellulase adsorption (analogous to the number of adsorption active sites); ϕ is the reactivity coefficient of cellulose; k_1 and k_2 are the specific rates of cellulose and cellobiose hydrolysis, respectively; K_e is the equilibrium constant for cellulase adsorption to cellulose; K_m is the Michaelis constant for β-glucosidase; K_{1L} and K_{2L} are constants for cellulase and β-glucosidase adsorption to lignin, respectively; and K_B, K_G, and K_E are the inhibition constants for cellulase (subscript 1) and β-glucosidase (subscript 2) by cellobiose (B), glucose (G), and ethanol (E), respectively. Ethanol, along with carbon dioxide, is the major metabolic product of the fermentative organism. The model equations include noncompetitive inhibition of cellulase by cellobiose, glucose, and ethanol and of β-glucosidase by ethanol, competitive inhibition of β-glucosidase by glucose, as well as substrate inhibition by cellobiose. Enzyme deactivation was assumed negligible (i.e., e_c and e_g are constant) and lignin concentration (L) was considered constant because lignin is not degraded under the employed SSF conditions. The coefficients, 1.056 and 1.053 in Eqs. (2) and (3), respectively, account for the incorporation of water molecules during hydrolysis of cellulose and cellobiose.

According to the model, cellulase catalyzes the hydrolysis of cellulose molecules to cellobiose, which is then broken down to glucose by the catalytic action of β-glucosidase. The hydrolysis of cellulose is a heterogeneous reaction occurring at the solid–liquid interface of the biomass particles. In contrast, the subsequent breakdown of cellobiose to glucose is catalyzed in the liquid phase by β-glucosidase. The rate expressions (4) and (5) describe the kinetic behavior of the enzymes based on the values of their parameters, K_{1B}, K_{1G}, K_{1E}, K_m, K_{2B}, K_{2G}, and K_{2E}.

PARAMETER DETERMINATION PROCEDURE

Determination of the seven enzyme parameters was achieved by performing critical experiments, each one examining the effect of a specific component of the SSF system on the activity of the enzyme of interest. The major components of SSF are cellobiose, glucose, and ethanol; and therefore, their interaction with the hydrolytic enzymes is expected to have a significant effect on the progress of cellulose hydrolysis and, furthermore, on the performance of the SSF. The appropriate forms of expressions (4) and (5) were regressed to the collected kinetic data using nonlinear regression algorithms to determine the enzyme parameters and, at the same time, examine the predictive ability of the respective model equation.

The employed nonlinear regression algorithm is a fast converging hybrid of the Gauss–Newton and the steepest descent methods, which was developed based on the Levenberg–Marquardt least-squares minimization procedure.[10] Caution, however, needs to be exercised when convergence is achieved to ensure that the minimum approached is a global rather than a local one. This is accomplished by starting the algorithm from several different initial estimates of the parameter values in order to reach the smallest residual of squared deviations between model predictions and experimental data.

EXPERIMENTAL PROCEDURE

Cellulose (α-cellulose), cellobiose, and δ-gluconolactone (Sigma Chemical Co., St. Louis, MO) were employed in the cellulase and β-glucosidase assays. All studies were performed in YP medium (10 g/L yeast extract, 20 g/L peptone) of initial pH 5.0 at 38°C to simulate the SSF process. During the cellulase assays, in addition to cellobiose, the small amounts of glucose produced were also monitored and taken into account to calculate the equivalent cellobiose and thus assess the total amount of cellobiose released by cellulase. In the β-glucosidase assay, only the glucose formed was monitored. Cellobiose concentration was measured by HPLC (Model 1090 Series II/L,

Hewlett-Packard, Avondale, PA) using an organic acids column (HP87H from Biorad, Richmond, CA). Glucose concentration was measured with a YSI glucose analyzer (YSI, Yellow Springs, OH).

Cellulase enzyme under the commercial name Laminex, synthesized by a *Trichoderma reesei* strain, was purchased from Genencor (San Francisco, CA). The cellulase and β-glucosidase volumetric activities of the Laminex enzyme complex were found to be 84 IFPU/mL and 91 IU/mL, respectively, when measured according to the IUPAC methods.[4] The initial rate of each hydrolytic reaction was determined based on samples collected every 15 seconds during only the first minute of the assay to prevent substrate reactivity and enzyme deactivation from influencing the calculation of the initial hydrolysis rate. Before being analyzed, the samples were incubated in boiling water for 20 minutes to inactivate the enzymes by denaturation. The collected data were then regressed linearly to estimate the slope of the correlation between elapsed hydrolysis time and substrate concentration, which represented the initial hydrolysis rate. In all determinations, the correlation coefficient of these linear regressions exceeded 0.98.

CHARACTERIZATION OF ENZYME KINETICS

The cellulases synthesized by cellulase-producing organisms are mixtures of cellulase components (endo- and exoglucanases) and β-glucosidase rather than pure enzymes. Therefore, to carry out an evaluation of the kinetic behavior of each enzyme (cellulase, β-glucosidase), it is necessary to distinguish between the cellulolytic and cellobiolytic activities. It has been reported that δ-gluconolactone, a cellobiose analogue, selectively inhibits the activity of β-glucosidase, while having little effect on cellulase.[8] If this is valid for the *T. reesei* cellulase complex employed in this study, then δ-gluconolactone can be used to evaluate the kinetics of cellulase action on cellulose, uncoupled from the hydrolytic action of β-glucosidase. In parallel, the β-glucosidase activity of the cellulase complex can be investigated using cellobiose as substrate.

The goal of the first assay was to assess the effect of δ-gluconolactone on the individual activities of cellulase and β-glucosidase. Cellulose at 60 g/L was used as substrate for cellulase and cellobiose at 10 g/L for β-glucosidase, which represent realistic substrate levels during the SSF operation. Various concentrations of the inhibitor δ-gluconolactone were employed in the range 0 to 10 g/L. The results are depicted in Figure 1 after being normalized to the rate exhibited in the absence of the inhibitor for each enzyme. Practically no effect was observed on the activity of cellulase, although slight inhibition was noted above 3 g/L of δ-gluconolactone. In contrast, δ-gluconolactone had a significant impact on the activity of β-glucosidase. At concentrations higher than 3 g/L, the enzyme lost approximately 70% of its activity. Qualitatively similar results have been reported for other cellulase preparations.[8] Based on our findings,

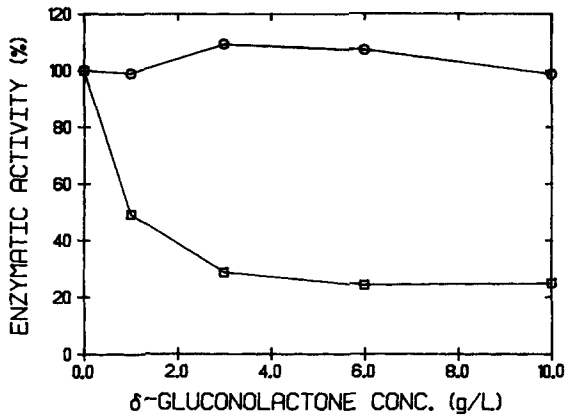

Figure 1. Effect of δ-gluconolactone concentration on the activities of cellulase (○) and β-glucosidase (□) in the presence of 60 g/L cellulose and 10 g/L cellobiose, respectively. The data have been normalized to the activity of the corresponding enzyme in the absence of the inhibitor.

a δ-gluconolactone concentration of 3 g/L was selected for all subsequent experimentation as the optimal level that inhibits β-glucosidase without significantly affecting cellulase.

When pure cellulose is used as substrate, as is the case in this study, the lignin-related terms can be dropped from Eqs. (4) and (5). Furthermore, because the quality and initial concentration of cellulose, cellulase, and β-glucosidase do not vary from experiment to experiment, then ϕ, e_c, and K_e in Eq. (4) and e_g in Eq. (5) can be assumed constant and lumped into the parameters k_1 and k_2, respectively. Hence, Eqs. (4) and (5) simplify to

$$r_1 = \frac{k_1' C}{1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}} \frac{K_{1E}}{K_{1E} + E} \tag{6}$$

$$r_2 = \frac{k_2' B}{K_m\left(1 + \frac{G}{K_{2G}}\right) + B\left(\frac{B}{K_{2G}}\right)} \frac{K_{2E}}{K_{2E} + E} \tag{7}$$

where the total substrate surface area, α_t, was considered proportional to the residual cellulose concentration.[3]

The effect of cellobiose on cellulase activity was examined in the concentration range of 0 to 60 g/L in the presence of 3 g/L δ-gluconolactone and 60 g/L α-cellulose and at an enzyme loading of 25 IU/g cellulose. Cellobiose is the direct product of cellulase action, and as such it has a strong inhibitory impact on the activity of the enzyme (Fig. 2). At a cellobiose concentration as low as 6 g/L, the activity of cellulase was reduced by 60%. Equation (6) describes the rate of cellulose hydrolysis to cellobiose; for the cellobiose inhibition assay extrapolated to time zero ($G = E = 0$, $C = 60$ g/L), it simplifies to

$$(r_1)_{t=0} = \frac{\text{constant}}{1 + \frac{B}{K_{1B}}} \tag{8}$$

because the initial cellobiose concentration is the only variable in this assay. Fitting this equation to the measured

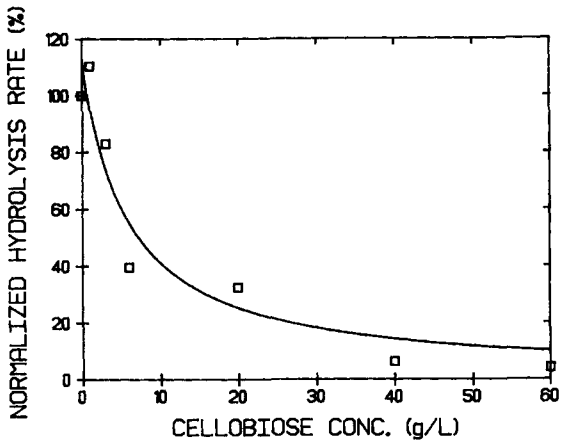

Figure 2. Activity of cellulase at a cellulose concentration of 60 g/L, and an enzyme loading of 25 IU/g cellulose with varying cellobiose concentration.

initial rates of cellulase with the developed nonlinear regression algorithm, we obtained $K_{1B} = 5.85$ g/L. Figure 2 verifies that Eq. (8) can successfully describe the initial cellulose hydrolysis rate at various cellobiose concentrations.

For β-glucosidase, cellobiose serves as substrate. The kinetics of cellobiose hydrolysis was investigated by varying the substrate concentration in the range of 0 to 70 g/L and the enzyme concentration from 5 to 40 IU/g equivalent cellulose. The results of the study are depicted in Figure 3. The measured rates are shown by the symbols, whereas the continuous lines represent the optimal model predictions. More specifically, Eq. (7), when extrapolated to time zero ($G = E = 0$) for a fixed β-glucosidase concentration, simplifies to

$$(r_2)_{t=0} = \frac{(\text{constant})B}{K_m + B + \dfrac{B^2}{K_{2B}}} \tag{9}$$

Equation (9) was fitted to the data. Figure 3 demonstrates that, at each enzyme concentration, the hydrolysis kinetics follow Michaelis–Menten instead of substrate-inhibition kinetics (K_{2B} very large). Therefore, there is no evidence of substrate inhibition on β-glucosidase activity, at least up to 70 g/L of cellobiose, which is well beyond the typical cellobiose levels (<10 g/L) encountered in the SSF process.[11] The average value of the Michaelis constant at all different enzyme loadings was $K_m = 10.56$ g/L with a standard deviation of 2.04 g/L. As expected from Eq. (7), the maximal rate of cellobiose hydrolysis k_2' $(=k_2 e_g)$ exhibited a linear dependence on the concentration of β-glucosidase (Fig. 4).

Similarly, the effect of glucose on enzyme activity was studied in the range of 0 to 80 g/L, which again covers the typical glucose levels of no more than 8 g/L observed during the SSF process. In the case of cellulase, the substrate was α-cellulose (60 g/L), the enzyme loading was 25 IU/g cellulose, and the assays were conducted in the presence of 3 g/L δ-gluconolactone to prevent β-glucosidase activity. The inhibition of cellulase by glucose was less than 20% below 20 g/L and about 60% at 80 g/L, as seen in Figure 5. Once again, Eq. (6), simplified for initial rate measurements ($B = E = 0$, $C = 60$ g/L) to the form

$$(r_1)_{t=0} = \frac{\text{constant}}{1 + \dfrac{G}{K_{1G}}} \tag{10}$$

was fitted to the experimental data. Figure 5 indicates that Eq. (10) can satisfactorily predict the rate of cellulose hydrolysis over a wide range of glucose concentrations. The nonlinear regression algorithm yielded $K_{1G} = 53.16$ g/L as the optimal value of the inhibition constant.

Using 10 g/L of cellobiose as substrate and the same cellulase enzyme concentration as in the previous experiments (1500 IU/L), a very strong inhibitory effect of glucose was observed on β-glucosidase activity (Fig. 5). At a level of only 3 g/L, well within the range of glucose concentrations observed during the SSF, 75% of the enzyme activity was lost. At 20 g/L of glucose, the enzyme was practically inactive. The extreme sensitivity of β-glucosidase

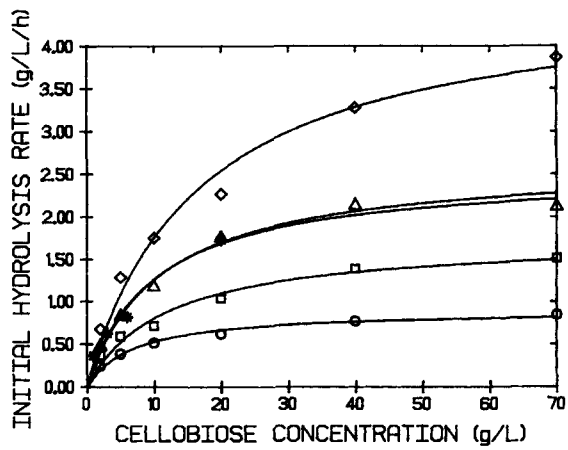

Figure 3. Impact of cellobiose concentration on the activity of β-glucosidase at various loadings of the enzyme: (○), 5 IU/g; (□), 10 IU/g; (△), 20 IU/g, (*), 25 IU/g; (◇), 40 IU/g (all enzyme concentrations are expressed per gram of equivalent cellulose).

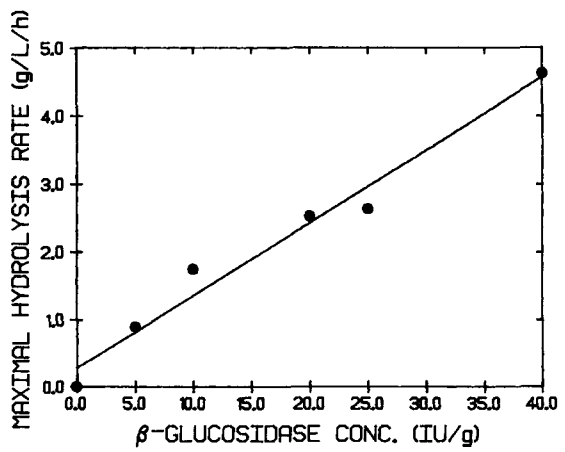

Figure 4. Variation in the maximal cellobiose hydrolysis rate [term $k_2 e_g$ of Eq. (5)] with β-glucosidase concentration.

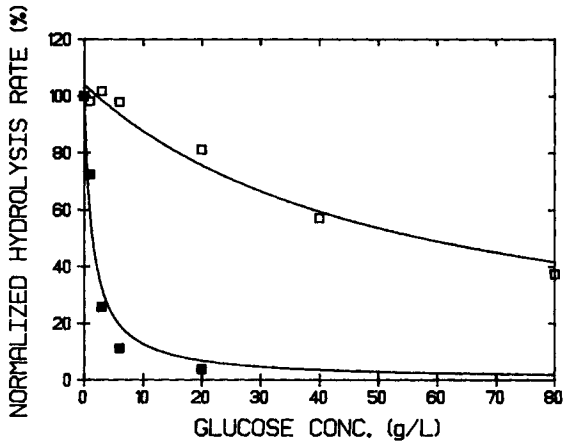

Figure 5. Dependence of the activities of cellulase (□) and β-glucosidase (■) on glucose concentration in the presence of 60 g/L cellulose and 10 g/L cellobiose, respectively, and at an enzyme loading of 25 IU/g cellulose in both cases.

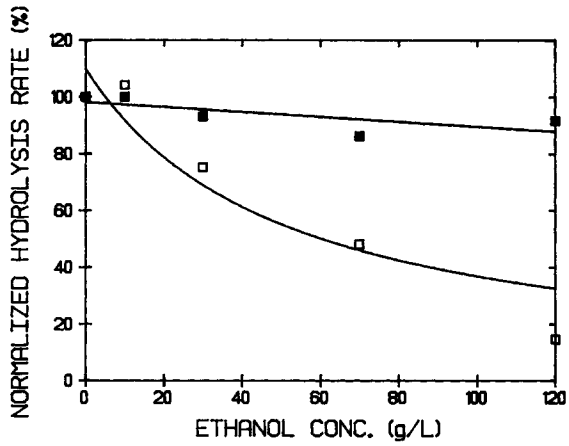

Figure 6. Effect of ethanol concentration on the activities of cellulase (□) and β-glucosidase (■) in the presence of 60 g/L cellulose and 10 g/L cellobiose, respectively, and at an enzyme loading of 25 IU/g cellulose in both cases.

to glucose, its direct product, is demonstrated by the low value of the inhibition constant, K_{2G} = 0.62 g/L, which was determined by nonlinear regression of Eq. (7), as it is applicable to initial rate measurements (E = 0, B = 10 g/L)

$$(r_2)_{t=0} = \frac{\text{constant}}{1.95 + \dfrac{G}{K_{2G}}} \tag{11}$$

to the experimentally determined rates, where the previously calculated value of K_m was also taken into consideration. An almost identical K_{2G} value (0.59 g/L) has been reported for β-glucosidase from *Aspergillus niger* assayed in acetate buffer (pH 4.8) at 50°C[2]; however, substrate inhibition was reported for that enzyme (K_{2B} = 14.7 g/L).

Finally, the impact of ethanol, the major metabolite and final product of the SSF process, on enzyme activity was analyzed using ethanol concentrations from 0 to 120 g/L. The results, as depicted in Figure 6, indicate that cellulase was moderately inhibited by ethanol; at 30 g/L, the enzyme activity was reduced by 25% and, at 70 g/L, by more than 50%. When Eq. (6), reduced to the form

$$(r_1)_{t=0} = \frac{\text{constant}}{K_{1E} + E} \tag{12}$$

for initial rate measurements (G = B = 0, C = 60 g/L), was fitted to the measured cellulose hydrolysis rates, the optimal value of the inhibition parameter was found to be K_{1E} = 50.35 g/L. Surprisingly, β-glucosidase did not seem to be inhibited by ethanol to any significant extent, even at concentrations as high as 120 g/L (Fig. 6).

In summary, cellulase and β-glucosidase exhibit diverse kinetic behaviors and are affected to a considerably different extent by the presence of cellobiose, glucose, and ethanol. Table I presents an overview of the interaction of the enzymes with the three major components of the SSF process. At a statistical confidence level of 95%, the standard deviations did not exceed 10% and 8% of the values shown in Table I for cellulase and β-glucosidase activity, respectively. Caution should be exercised when comparing these enzyme parameter values, which were obtained under realistic SSF conditions, with previous reports,[2,5,8] where the assays were conducted under conditions optimal for enzymatic activity, usually in 0.05 *M* citric buffer of pH 4.5 to 5.0 and at temperatures of 45° to 50°C. Moreover, enzyme properties seem to vary widely among cellulases produced by different organisms[8] and may also depend on the purity of the enzyme preparation. For example, the inhibition constants of a *T. longibrachiatum* cellulase were found to be 13 and 4.2 g/L for glucose and cellobiose, respectively,[5] significantly different from the values of 319.5 and 54.3 g/L reported for a *T. reesei* cellulase preparation.[8] In both of the cited studies,[5,8] dyed cellulose was used as substrate under optimal enzyme activity conditions, and the progress of the reaction was followed by monitoring the release of dye instead of cellulose degradation; furthermore, no differentiation was

Table I. Kinetic characteristics of cellulase and β-glucosidase with regard to their interaction with cellobiose, glucose, and ethanol.

Enzyme component	Kinetic parameter value		
	Cellobiose	Glucose	Ethanol
Cellulase	K_{1B} = 5.85 g/L (strong inhibition)	K_{1G} = 53.16 g/L (weak inhibition)	K_{1E} = 50.35 g/L (moderate inhibition)
β-Glucosidase	K_m = 10.56 g/L (substrate)	K_{2G} = 0.62 g/L (very strong inhibition)	Insignificant inhibition

made between the synergistic cellulolytic and cellobiolytic activities of the employed cellulases.

The divergent behaviors of cellulase and β-glucosidase (Table I) may, to some extent, reflect the different natures of the two catalytic actions: cellulase catalyzes a heterogeneous reaction based on the synergism of multiple components, while β-glucosidase acts in a homogeneous environment. Although the enzyme parameters do not help elucidate the mechanism of either enzymatic action, they serve the purpose of allowing the formulation of appropriate mathematical models that can predict the progress of the SSF process and thereby can be used for process optimization.

CELLULOSE HYDROLYSIS KINETICS

With the parameters K_{1B}, K_{1G}, K_{1E}, K_m, K_{2B}, K_{2G}, and K_{2E} determined, the model equations were used to determine the specific and maximal rates of cellulose and cellobiose hydrolysis, respectively, namely parameters k_1' and k_2' and, at the same time, verify the applicability of rate expressions (6) and (7). To test the validity of Eqs. (6) and (7), the progress of α-cellulose hydrolysis by the Laminex mixture of cellulase and β-glucosidase was followed. The concentrations of the substrate and enzyme were 60 g/L and 25 IU/g cellulose, respectively. The experiment was performed in YP medium (pH 5.0) at 38°C to resemble realistic SSF conditions. Samples were taken every 15 to 30 minutes during the first 6 hours of the hydrolysis and less frequently afterward, and the concentrations of cellulose, cellobiose, and glucose were determined (Fig. 7). The study was intentionally terminated after 103 hours. However, only the data of the first 6 hours were employed for parameter determination, whereas the data collected at later times were used to test the predictive ability of the model. Taking into consideration the fact that no ethanol is present in the system and no substrate inhibition was observed for β-glucosidase, Eqs. (6) and (7) simplify to

$$r_1 = \frac{k_1' C}{1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}} \tag{13}$$

$$r_2 = \frac{k_2' B}{K_m\left(1 + \frac{G}{K_{2G}}\right) + B} \tag{14}$$

Then, the mass balance equations for cellulose (C), cellobiose (B), and glucose (G) become

$$\frac{dC}{dt} = -\frac{k_1' C}{1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}} \tag{15}$$

$$\frac{dB}{dt} = -1.056\left(\frac{dC}{dt}\right) - \frac{k_2' B}{K_m\left(1 + \frac{G}{K_{2G}}\right) + B} \tag{16}$$

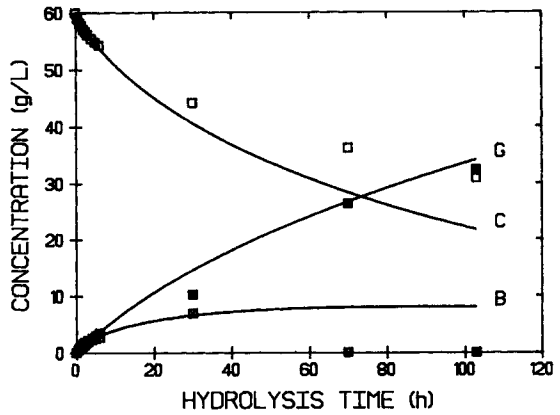

Figure 7. Experimental data (points) and model simulations (curve) of batch cellulose hydrolysis by cellulase and β-glucosidase (obtained from *T. reesei*) under SSF conditions: (□), cellulose (C); (⊠), cellobiose (B); (■), glucose (G).

$$\frac{dG}{dt} = -1.053\left[1.056\left(\frac{dC}{dt}\right) + \left(\frac{dB}{dt}\right)\right] \tag{17}$$

By employing the Levenberg–Marquardt method, convergence to a minimum weighted sum of squared residuals was achieved at the parameter values $k_1' = 0.025\ \text{h}^{-1}$ and $k_2' = 14.22\ \text{g L}^{-1}\ \text{h}^{-1}$. Figure 7 demonstrates the ability of the model to describe the progress of the enzymatic hydrolysis of cellulose. The fit between model prediction and experimental data was satisfactory during the first 6 hours of the reaction and remained reasonable even up to 103 hours, taking into consideration the relatively simplistic nature of Eqs. (15)–(17) (Fig. 7). The profile of cellobiose was overestimated by the model, whereas the cellulose profile was somewhat underestimated. These two types of deviation, when combined, point to a likely small loss of cellulase activity over time,[9] which has not yet been considered in the model equations. Such a gradual cellulase deactivation would slow down the rate of cellulose disappearance and therefore the accumulation of cellobiose.

To incorporate the concept of enzyme deactivation in the present mathematical model, an exponential decay term was introduced in Eq. (13) to account for a first-order loss of cellulase activity with respect to enzyme concentration:

$$r_1 = \frac{k_1' C e^{-\lambda t}}{1 + \frac{B}{K_{1B}} + \frac{G}{K_{1G}}} \tag{18}$$

where λ is the specific rate of cellulase deactivation. Such a term has been proposed in the past[1,6] in an effort to better fit experimental data to simple mathematical representations of cellulose hydrolysis kinetics. It should be emphasized that a detailed study of the enzyme kinetics is necessary to clarify whether an exponential decay is an actual phenomenon or the term simply compensates for deficiencies of mathematical models.

After introducing Eq. (18) into the mass balance equations of cellulose, cellobiose, and glucose [Eqs. (15)–(17)], the new model was fitted to the experimental hydrolysis data and the values of λ, k_1', and k_2' were optimized. The

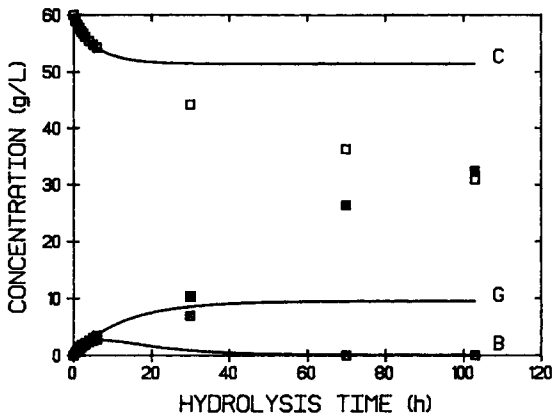

Figure 8. Experimental data (points) and model simulations (curve) of batch cellulose hydrolysis by cellulase and β-glucosidase (obtained from *T. reesei*) under SSF conditions. The model was modified to incorporate a cellulase deactivation term, as described by Eq. (18). (□), Cellulose (C); (⊠), cellobiose (B); (■), glucose (G).

algorithm yielded, for these three parameters, the values: $\lambda = 0.153\ h^{-1}$, $k_1' = 0.034\ h^{-1}$, and $k_2' = 12.98\ g\ L^{-1}\ h^{-1}$. The regression of this model to the data is demonstrated in Figure 8. It seems that inclusion of the first-order enzyme deactivation term improves the fit during the first 6 hours because the additional parameter, λ, gives more flexibility (degrees of freedom) to the model. However, the modified model fails to correctly predict the long-term profiles of cellulose and glucose by overestimating the loss of enzymatic activity (Fig. 8). As a result, the cellulose concentration is grossly overestimated and the glucose concentration is significantly underestimated because the availability of cellobiose diminishes according to the model. The good fit to the cellobiose profile is clearly superficial; the experimental data suggest that the low levels of cellobiose are due to the dynamically equal rates of cellobiose formation and disappearance, not to the cessation of cellulase activity, which is implied by Eq. (18) for the later times of the reaction. This failure of the modified model is a strong indication that additional information is needed about the physicochemical properties of the substrate and the enzyme–substrate interaction before developing more accurate and reliable mathematical expressions for the enzymatic hydrolysis of cellulosic biomass. Currently, in the absence of further information regarding the time-dependent variation in properties of the biomass substrate (surface area, reactivity, size distribution), a simple rate expression, such as Eq. (13), appears to provide a satisfactory description of the cellulose hydrolysis kinetics.

CONCLUSION

Experimental work was carefully designed first to uncouple the activity of cellulase from that of β-glucosidase, and then to assess the kinetics of each enzyme. Using nonlinear regression, equations developed previously as part of an SSF mathematical model were fitted to the collected kinetic data, and parameters regarding the hydrolytic activity of the enzymes and the kinetics of cellulose hydrolysis were determined. In general, the model was successful in depicting the progress of the enzymatic hydrolysis of cellulose to glucose. Improvements in its predictive ability will be based on better understanding of the role that the properties of the cellulosic substrate play during hydrolysis and the enzyme–substrate interaction. Following calibration of all parameters, the model will be a powerful tool for process design, optimization, and scale-up. It will help identify parameters that have a significant impact on the performance of the SSF process. Optimization of those parameters will then be the subject of research and development in order to enhance the ethanol productivity of the present bioconversion technology.

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NOMENCLATURE

α_t	available surface area of cellulose (m^2/L)
B	concentration of cellobiose (g/L)
C	concentration of cellulose (g/L)
E	concentration of ethanol (g/L)
e_c	concentration of cellulase in solution (g/L)
e_g	concentration of β-glucosidase (g/L)
G	concentration of glucose (g/L)
k_1, k_2	specific rate of cellulose and cellobiose hydrolysis, respectively ($g\ m^{-2}\ h^{-1}$ and h^{-1}, resp.)
k_1'	constant in Eq. (6) (h^{-1})
k_2'	constant in Eq. (7) ($g\ L^{-1}\ h^{-1}$)
K_e	equilibrium constant of cellulase adsorption to cellulose (g/L)
K_m	Michaelis constant of β-glucosidase for cellobiose (g/L)
K_{1B}, K_{2B}	inhibition constants of cellulase and β-glucosidase by cellobiose, respectively (g/L)
K_{1E}, K_{2E}	inhibition constants of cellulase and β-glucosidase by ethanol, respectively (g/L)
K_{1G}, K_{2G}	inhibition constants of cellulase and β-glucosidase by glucose, respectively (g/L)
K_{1L}, K_{2L}	constants for cellulase and β-glucosidase adsorption to lignin, respectively (L/g)
λ	specific rate of cellulose deactivation (h^{-1})
L	concentration of lignin (g/L)
r_1, r_2	volumetric rate of cellulose and cellobiose utilization, respectively ($g\ L^{-1}\ h^{-1}$)
t	time (h)
ϕ	cellulose reactivity coefficient (dimensionless)

Subscript

o	initial value

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