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Testing Alternative Kinetic Models for Utilization of Crystalline Cellulose (Avicel) by Batch Cultures of *Clostridium thermocellum*

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ABSTRACT: Descriptive kinetics of batch cellulose (Avicel) and cellobiose fermentation by *Clostridium thermocellum* were examined with residual substrate and biosynthate concentrations inferred based on elemental analysis. Biosynthate was formed in constant proportion to substrate consumption until substrate was exhausted for cellobiose fermentation, and until near the point of substrate exhaustion for cellulose fermentation. Cell yields (g pellet biosynthate carbon/g substrate carbon) of 0.214 and 0.200 were obtained for cellulose and cellobiose, respectively. For cellulose fermentation a sigmoidal curve fit was applied to substrate and biosynthate concentrations over time, which was then differentiated to calculate instantaneous rates of growth and substrate consumption. Three models were tested to describe the kinetics of Avicel utilization by *C. thermocellum*: (A) first order in cells, (B) first order in substrate, and (C) first order in cells and substrate, and second order overall. Models (A) and (B) have been proposed in the literature to describe cultures of cellulolytic microorganisms, whereas model (C) has not. Of the three models tested, model (c) provided by far the best fit to batch culture data. A second order rate constant equal to 0.735 $L\,g\,C^{-1}\,h^{-1}$ was found for utilization of Avicel by *C. thermocellum*. Adding an endogenous metabolism term improved the descriptive quality of the model as substrate exhaustion was approached. Such rate constants may in the future find utility for describing and comparing cellulose fermentation involving other microbes and other substrates.

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KEYWORDS: *Clostridium thermocellum*; anaerobic cellulose fermentation; growth rate; cell yield; kinetic model

Introduction

For microorganisms growing on cellulosic biomass, the literature contains frequent reference to exponential growth rates (Lynd et al., 2002). First-order substrate utilization kinetics have also been suggested, and have been shown to fit data obtained in continuous culture under some conditions (Hogsett, 1995; Jensen et al., 2009; Weimer et al., 1990). An exponential increase in the concentration of cells occurs when the growth rate is proportional to—that is, first order with respect to—the concentration of cells present. This is only reasonable to expect when substrate is present in excess and is kinetically unimportant. First-order kinetics with respect to substrate are only reasonable to expect when cells (and their enzymes) are present in excess and kinetically unimportant. The two common representations of the kinetics of microbial cellulose utilization are thus at best limiting cases applicable to mutually exclusive situations.

The uncertain and at times contradictory state of the field with respect to the kinetics of microbial cellulose utilization is at least in part a result of methodological difficulties. In particular, measurements of biosynthesis and substrate consumption-central to the study of microbial physiology are not straightforward for microorganisms growing on insoluble substrates such as cellulosic biomass.

In a companion paper (Holwerda et al., 2013), we developed and evaluated methods to infer "biosynthate" using *Clostridium thermocellum*, an extensively studied cellulolytic obligate anaerobe (Bayer et al., 2004; Demain et al., 2005; Lynd et al., 2002). A data set based on carbon and nitrogen analysis was generated in this prior study for an Avicel fermentation, which is of sufficient quality to estimate instantaneous rates of biosynthesis and substrate consumption in batch cultures of a cellulose-grown microbial culture. In this article, we test alternative kinetic models for microbial cellulose utilization.

Materials and Methods

Organism, Culturing Conditions and Media Composition

The organism, composition of the media (LC, low carbon medium) and the specific culturing conditions in bioreactors are as described in Holwerda et al. (2012). Data used in this paper were generated from cultures of *C. thermocellum* ATCC 27405 grown on either 5 $g\,L^{-1}$ crystalline cellulose (Avicel PH105) or 5 $g\,L^{-1}$ cellobiose in LC medium.

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Analytical Methods

The amounts of nitrogen and carbon in sample pellets taken from fermentations in bioreactors was determined using a Shimadzu TOC-Vcph Total Organic Carbon analyzer with added Total Nitrogen unit and ASI-V autosampler (Shimadzu Scientific Instruments, Columbia MD), with an acidified glycine standard for nitrogen determination, and a serial dilution of an Avicel suspension as standard for carbon determination (Holwerda et al., 2012).

Use of elemental analysis is described in (Holwerda et al., 2013). To obtain residual substrate concentration from elemental analysis we used the following equation:

$$C_{Ts} = C_{Tp} - C_{Tb} = C_{Tp} - N_{Tp}\frac{R_{Cpb}}{N_{pb}} \quad (1)$$

where C_{Ts} is the total pellet substrate carbon ($g\,L^{-1}$), C_{Tp} is the total amount of carbon in pellet ($g\,L^{-1}$), C_{Tb} is the total pellet biosynthate carbon ($g\,L^{-1}$), N_{Tp} is the total amount of nitrogen in pellet ($g\,L^{-1}$), $R_{Cpb/Npb}$ is the ratio between carbon and nitrogen from pellet biosynthate.

Biosynthate

Biosynthate was defined as the aggregate result of biosynthesis (Holwerda et al., 2013). Soluble or supernatant protein was measured with a Biorad protein assay, and the carbon content of the measured supernatant protein was estimated on basis of the measured carbon content for Bovine Serum Albumin (BSA) by elemental analysis.

Curve Fitting and Rate Data

We have applied a sigmoidal fit curve to biosynthate data based on a five-parameter version of Richards curve (Richards, 1959). Curves generated using these parameters were then differentiated to calculate the specific rates of growth and substrate consumption by dividing the differential by its parent-curve, see also (Holwerda et al., 2013).

Individual biosynthate data points from Avicel grown cultures obtained by elemental analysis were used to calculate the specific growth rate in the following fashion:

$$dx/dt/x(t) = [x_1 - x_0]/[t_1 - t_0]/[[x_1 + x_0]/2] \quad (2)$$

where x_1 is the biosynthate concentration at time 1 ($g\,L^{-1}$), x_0 is the biosynthate concentration at time 0 ($g\,L^{-1}$), t_1 is the time point 1 (h), t_0 is the time point 0 (h).

Solid Substrate Models

Equations for dS/dt and dX/dt were integrated using Berkeley–Madonna software (www.berkeleymadonna.com). A Runge Kutta (RK4) ODE solver was used to solve for X and S. The model was improved by curve fitting to fermentation data and adding the parameters k (reaction rate constant), k_e (endogenous metabolism constant) and $Y_{X/S}$ (yield), these terms are explained more in the Results section.

Yield Calculations

The yield calculations are based on gram carbon/gram carbon using (inferred) pellet carbon and pellet nitrogen values, soluble protein values, and HPLC values for cellobiose. The yield is presented in both pellet biosynthate carbon versus substrate carbon and dry weight (equivalent for Avicel) versus substrate, for equations and values see Holwerda et al. (2013).

Results

Cell Yield

Inferred biosynthate and cellulose carbon measurements using Equation (1) together with the previously described validation experiments (Holwerda et al., 2013) were used to estimate values for the cell yield, $Y_{X/S}$ (dimensionless, calculated on a carbon basis).

As shown in Figure 1, the cell yield is approximately constant until at (cellobiose) or near (Avicel) substrate exhaustion. The carbon-based pellet biosynthate yield is rather similar for both substrates at 0.200 g biosynthate carbon/g substrate carbon for cellobiose and 0.214 g biosynthate carbon/g substrate carbon for Avicel. Taking into account the carbon contents of measured soluble protein (see Fig. 1A and B) these values correspond to yields of 0.213 and 0.222 on a carbon mass basis (g pellet & supernatant carbon/g substrate carbon) for cellobiose and Avicel, respectively.

Testing Alternative Kinetic Models

Three models were tested to describe the kinetics of Avicel utilization by *C. thermocellum*: (A) cell growth first order in cells, (B) substrate consumption first order in substrate, and (C) substrate consumption first order in cells, first order in substrate, and second order overall. Rates of substrate consumption and cell growth were assumed to be related by the cell yield. For each model, best-fit curves are compared to experimental data using both differential and integral plots (Fogler, 2005).

Hypothesis 1: Constant Specific Growth Rate (First Order in Cells)

The rate of cell growth in batch cultures may be assumed to be proportional to the concentration of cells present (X, $g\,L^{-1}$), with the proportionality constant equal to the specific growth rate, μ (h^{-1}):

$$\frac{dX}{dt} = \mu X \quad (3)$$

where dX/dt is the rate of biosynthate formation in $g\,L^{-1}\,h^{-1}$, μ is the specific growth rate ($g\,g^{-1}\,h^{-1}$), X is the biosynthate concentration in $g\,L^{-1}$.

If this model in biosynthate were correct, the quantity $(1/X(t))(dX/dt)$ should be constant or nearly so as a function of fractional substrate conversion χ. By differentiating the

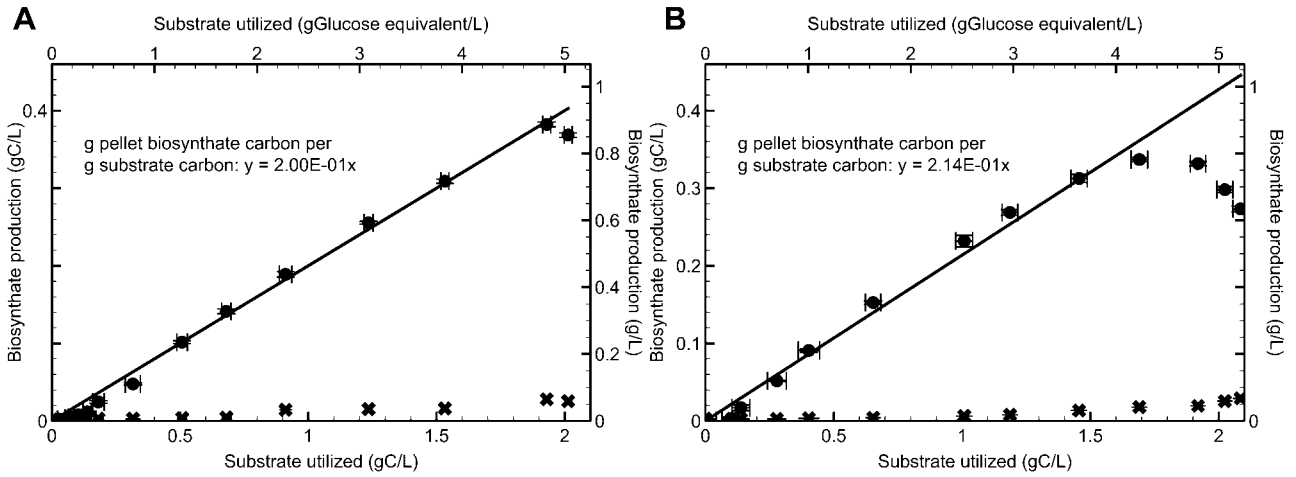

Figure 1. The biosynthate yield of cultures grown on cellobiose (**A**) and avicel PH105 (**B**) in black circles and the soluble/supernatant protein in gray crosses versus substrate utilized. Fit lines are based upon data points with increasing *y*-axis values.

sigmoidal fit curve and fermentation data points of biosynthate concentration versus time from Holwerda et al. (2013) values for the specific growth rate for an Avicel in LC medium grown culture were calculated. As shown in Figure 2A, $(1/X(t))(\mathrm{d}X/\mathrm{d}t)$ (i.e., mu) is in fact not constant, but instead reaches a maximum μ at approximately χ equal to 0.18 and declines thereafter, indicating that Equation (3) with constant growth rate is incorrect.

Figure 2B presents biosynthate and substrate concentrations as a function of time, obtained by integrating Equations (3) and (4).

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -1/Y_{X/S}\frac{\mathrm{d}X}{\mathrm{d}t} \tag{4}$$

where $\mathrm{d}S/\mathrm{d}t$ is the rate of substrate utilization in $\mathrm{g\,L^{-1}\,h^{-1}}$, $Y_{X/S}$ is the cell yield (g biosynthate/g substrate), S is the cellulose substrate concentration in $\mathrm{g\,L^{-1}}$.

It may be observed that the model fits experimental data well during the early stages of the fermentation when the substrate concentration is relatively high and the biosynthate concentration is relatively low, but not during the later stages of the fermentation.

Hypothesis 2: Substrate Utilization First Order in Substrate

First order substrate utilization in substrate for microbial utilization of cellulosic substrates corresponds to Equation (5):

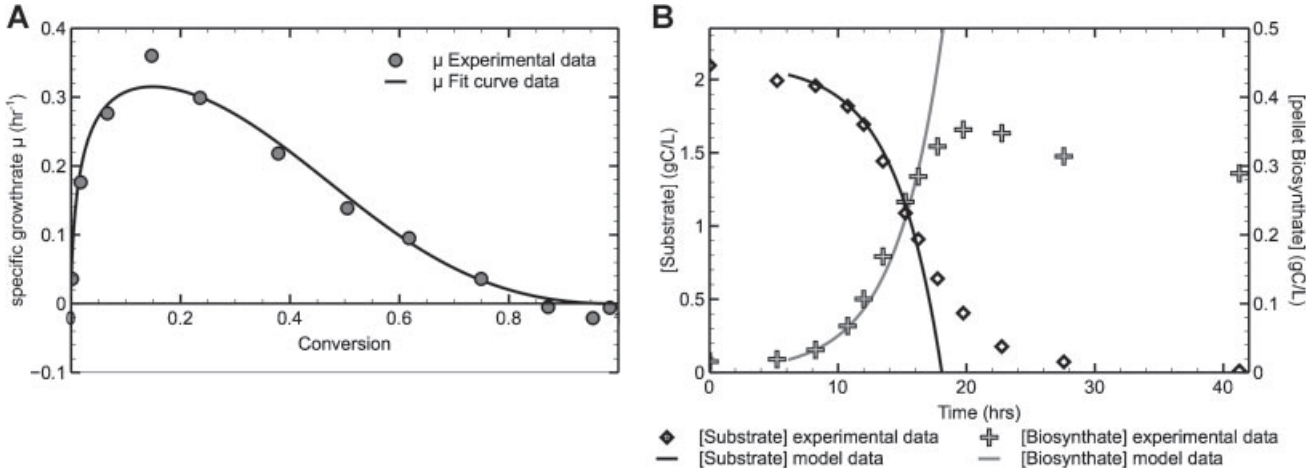

Figure 2. Testing the first order in biosynthate model; Differential plot (**A**) of the specific growth rate $\mathrm{d}X/\mathrm{d}t/X(t)$ versus conversion χ for cellulose fermentation. Integral plot (**B**) of substrate and biosynthate versus time.

$$\frac{dS}{dt} = -k_1 S \tag{5}$$

where k_1 is the reaction rate constant (h^{-1}).

If this model were correct, the quantity $(1/S(t))(dS/dt)$ (i.e., k_1) should be constant or nearly so as a function of fractional substrate conversion χ. As may be seen from Figure 3A, $(1/S(t))(dS/dt)$ is in fact not constant with conversion but instead increases markedly to a maximum at χ approximately equal to 0.9, indicating that Equation (5) with constant first order rate constant is incorrect.

Figure 3B presents biosynthate and substrate concentrations as a function of time, obtained by integrating Equations (4) and (5). Depending on initial conditions used, the model at best fits experimental data well during the later stages of the fermentation when the substrate concentration is relatively low and the biosynthate concentration is relatively high, but not during the earlier stages of the fermentation.

Hypothesis 3: Substrate Utilization First Order in Substrate and First Order in Cells, Second Order Overall

A second order rate law in the form of Equation (6) has to our knowledge not been proposed previously but seems logical to evaluate given the failure of the first order in cells and first order in substrate models. In particular we propose

$$\frac{dS}{dt} = k_2[S][X] \tag{6}$$

where k_2 is the reaction constant in L g biosynthate^{-1} h^{-1}.

If this model were correct, the quantity $(1/X(t))(1/S(t))(dS/dt)$ (i.e., k_2) should be constant or nearly so as a function of fractional substrate conversion χ. As may be seen from Figure 3A, this is in fact the case, indicating that Equation (6) is consistent with experimental data.

Figure 4B represents biosynthate and substrate concentrations as a function of time, obtained by integrating Equations (6) and (7).

$$\frac{dX}{dt} = -Y_{X/S}\frac{dS}{dt} = Y_{X/S}kS(t)X(t) \tag{7}$$

It may be observed that the model fits experimental data quite well except that the cell concentration is over-predicted as substrate exhaustion is approached. This discrepancy is largely corrected if the equation used to calculate the cell concentration includes an endogenous metabolism term (Pirt, 1975), corresponding to using Equation (8) instead of Equation (7)

$$\frac{dX}{dt} = -Y^{\text{true}}_{X/S}\frac{dS}{dt} - k_e X(t) \tag{8}$$

where k_e is the endogenous metabolism constant (h^{-1}), $Y^{\text{true}}_{X/S}$ is the yield based on biosynthate growth and substrate consumption excluding endogenous metabolism and/or maintenance (g g^{-1}).

The value of the second order rate constant, considering both models, was 0.735 L g C^{-1} h^{-1}. The yield parameter value for the model based on Equations (5) and (7) is $Y_{X/S} = 0.227$. Parameter values for the model based on Equations (7) and (8) are $k_e = 0.0568$ (h^{-1}), $Y^{\text{true}}_{X/S} = 0.294$.

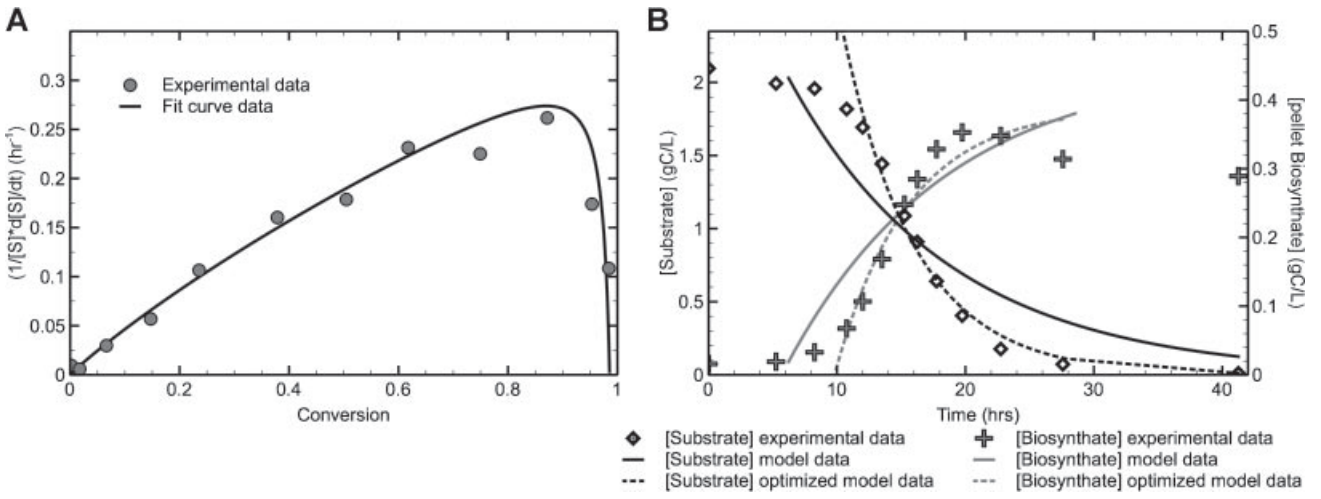

Figure 3. Testing the first order in substrate model; Differential plot (**A**) for the specific substrate utilization rate $dS/dt/S(t)$ versus conversion χ for cellulose fermentation. Integral plot (**B**) of substrate and biosynthate versus time. Solid curves assume initial conditions based on experimental data. Dashed curves are based on the best fit to experimental data with the initial conditions unconstrained.

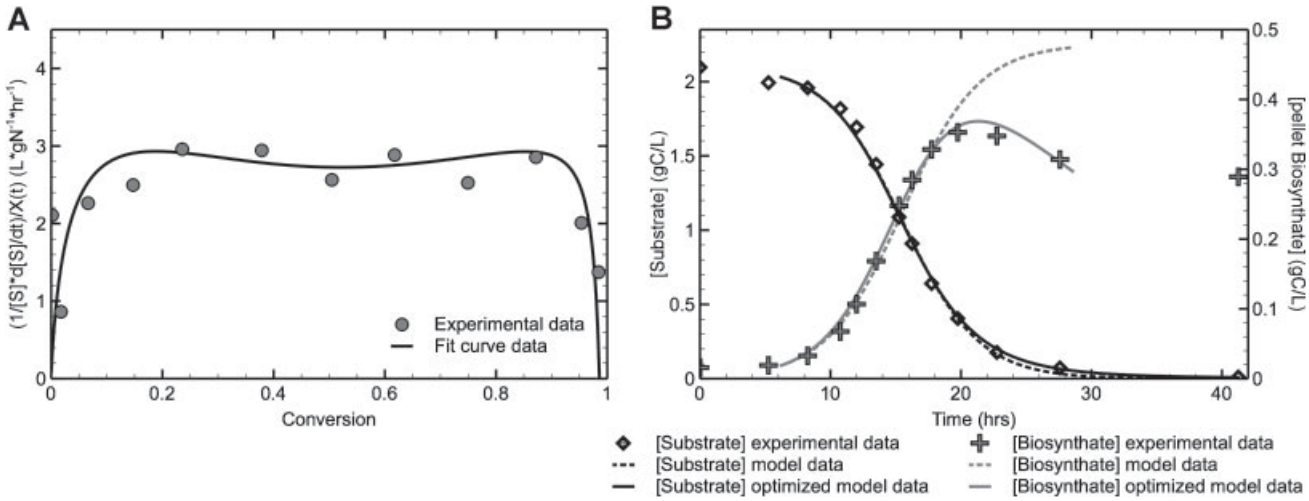

Figure 4. Testing the second order rate hypothesis; Differential plot (**A**) for the second rate order model $dS/dt/S(t)/X(t)$ versus conversion χ for cellulose fermentation. Integral plots (**B**) of substrate and biosynthate versus time. Dashed lines are for the model without endogenous metabolism, solid lines are for the model with endogenous metabolism.

Discussion

Using techniques described elsewhere (Holwerda et al., 2013) for inferring substrate and biosynthate concentrations for Avicel-grown cultures of *C. thermocellum*, values for physiological parameters are estimated. Cell yield for growth of *C. thermocellum* under the soluble substrate conditions tested is 0.200 g inferred pellet biosynthate carbon/g cellobiose carbon. The corresponding value for crystalline cellulose (Avicel) is 0.214 g inferred pellet biosynthate carbon/g cellulose carbon. Cell yields are reported here in terms of pellet biosynthate since a single measurement is required and the contribution of supernatant protein was found to be insignificant (Fig. 1A and B). Cell yields were slightly (~6%) higher for cellulose than for cellobiose, which is directionally consistent with additional ATP being available from phosphorolytic cleavage of β-glucosidic bonds for Avicel (Zhang and Lynd, 2005). For cellobiose, no decline in cell yield is observed until the substrate is exhausted. However, for Avicel the cell yield is observed to decline before the substrate is fully exhausted. We speculate that this is because of the decreased substrate availability in the latter stages of cellulose fermentation when the cell:substrate ratio is high.

The literature contains reference to two mutually exclusive kinetic description of microbial cellulose utilization: (A) exponential increase in biosynthate with a constant value of the specific growth rate, μ, and (B) first-order substrate solubilization with a constant rate constant, k. We tested both of these descriptions and found that neither is applicable to batch fermentation of Avicel by *Clostridium thermocellum*. In particular, the instantaneous value of μ varies continuously as batch fermentation proceeds, first increasing (until a fractional conversion around 0.18), and then decreasing. When we determined the instantaneous value of $k\,(=dS/dt/S(t))$ we found the first order rate constant to progressively increase until most of the substrate is consumed (fractional conversion ~0.9). By using a simple second rate law incorporating pellet biosynthate concentration, we found $k\,(=dS/dt/S(t)/X(t))$ to be of constant value at $k = 0.735\ \mathrm{L\,g\,C^{-1}\,h^{-1}}$. This second order model fits the data very well, with inclusion of an endogenous metabolism term improving the agreement between predicted and measured biosynthate concentrations as substrate exhaustion is approached.

The second order model presented here is descriptive rather than mechanistic, as are all models that use single state variables to describe the state of both microorganisms (Monod, 1949) and cellulosic substrates (Zhang and Lynd, 2004). Recognizing this limitation, it is notable that the second order model represents a marked improvement over previously proposed models for microbial cellulose utilization, and it is relevant to consider this model in light of what is known about cellulose solubilization at a more mechanistic level. As addressed by Lynd et al. (2002), fundamental considerations as well as cell-free enzymatic studies establish that saturation of rate with respect to either substrate or enzyme, or a combination, is commonly encountered in biologically mediated solubilization of lignocellulose. Although substrate being in vast excess relative to biocatalyst is very common for enzymatic and microbial reactions involving soluble substrates, excess substrate concentration is often not achieved in systems involving saccharolytic organisms and enzymes acting on cellulosic substrates because only a small fraction of the total number of β-glucosidic bonds (typically about 1 in 10,000) are physically accessible. When substrate is not in excess, the derivation of the Michaelis–Menten equation (Johnson and Goody, 2011; Michaelis and Menten, 1913) is not valid because it cannot be assumed that the total concentration of substrate is equal to the concentration of non-adsorbed substrate. Furthermore, the apparent saturation constant in the Michaelis–Menten or

Monod equations is in fact not constant but instead exhibits a strong functional dependence on the amount of biocatalyst (cellulase or cellulolytic microorganism with surface-expressed cellulases) present such that the concentration of substrate necessary to achieve half maximal rate increases with increasing biocatalyst concentration.

More sophisticated kinetic models could include quantification of free and cell-bound cellulases. Development of such models is an interesting topic for future research, as is consideration of models that incorporate more extensive mechanistic information such as the concentration and location of cellulase enzymes and/or physical properties of the substrate. Application of methods such as those presented herein to kinetics for lignocellulosic substrates occurring in nature and/or industrial processes is also of considerable interest.

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