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Kinetics of Enzymatic High-Solid Hydrolysis of Lignocellulosic Biomass Studied by Calorimetry

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Abstract Enzymatic hydrolysis of high-solid biomass (>10% *w*/*w* dry mass) has become increasingly important as a key step in the production of second-generation bioethanol. To this end, development of quantitative real-time assays is desirable both for empirical optimization and for detailed kinetic analysis. In the current work, we have investigated the application of isothermal calorimetry to study the kinetics of enzymatic hydrolysis of two substrates (pretreated corn stover and Avicel) at high-solid contents (up to 29% *w*/*w*). It was found that the calorimetric heat flow provided a true measure of the hydrolysis rate with a detection limit of about 500 pmol glucose s^{-1}. Hence, calorimetry is shown to be a highly sensitive real-time method, applicable for high solids, and independent on the complexity of the substrate. Dose–response experiments with a typical cellulase cocktail enabled a multidimensional analysis of the interrelationships of enzyme load and the rate, time, and extent of the reaction. The results suggest that the hydrolysis rate of pretreated corn stover is limited initially by available attack points on the substrate surface (<10% conversion) but becomes proportional to enzyme dosage (excess of attack points) at later stages (>10% conversion). This kinetic profile is interpreted as an increase in polymer end concentration (substrate for CBH) as the hydrolysis progresses, probably due to EG activity in the enzyme cocktail. Finally, irreversible enzyme inactivation did not appear to be the source of reduced hydrolysis rate over time.

Keywords Cellulase kinetics · High solid · Isothermal calorimetry · Saccharification · Lignocellulose · Biomass

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Introduction

Cellulosic biomass is a promising feedstock for the production of fluid, CO_2-neutral fuels [1–3], for example, second-generation bioethanol, which is fermented from glucose after the cellulose has been enzymatically hydrolyzed [3, 4]. Industrially, as well as in nature, cellulose hydrolysis is catalyzed by the cellulase complex, which includes three main classes of *glucoside hydrolases*, namely cellobiohydrolases (CBH), *endoglucosidases* (EG), and beta-glucosidases (BG) [5]. These hydrolylases show a high degree of synergy due to the different attack points on the cellulose polymer, where CBH catalyzes hydrolysis from polymer ends producing cellobiose, and EG attacks internal glucosidic bonds producing smaller polymers, and hence, new attack points for the CBH [6–8]. Finally, BG catalyzes the hydrolysis of cellobiose to glucose, and hence, limits product inhibition of CBH and EG [9–13].

Hydrolysis of lignocellulosic biomass under industrial conditions (>10% solid) is a slow process which requires several days to reach high (>80%) yields of glucose from cellulose even at quite high enzyme loads [14–16]. A typical kinetic profile consists of an initial burst, where around 10–20% of the cellulose is hydrolyzed followed by a rapid decline in hydrolysis rate [17–20]. The molecular mechanism underlying this course remains to be fully elucidated, but may be a combination of several effects including substrate heterogeneity [18, 21], product inhibition [13], and processive enzyme action [17]. Currently, long hydrolysis time and high enzyme consumption pose some major challenges for commercial second-generation bioethanol, and hence, considerable efforts are directed to optimize cellulase performance [6].

An important limitation in cellulase research is the lack of real-time assays on the natural substrates [6]. Cellulase activity is inherently difficult to assay as the hydrolysis produces essentially no change in optical properties. Instead, the most common approach has been to measure changes in product concentration over time, e.g., by reducing end labeling (such as the DNS assay) [22], or by chromatography coupled to, e.g., refractive index (RI) or PA detection. These approaches all include an inactivation step followed by the addition of probes, purification [22, 23], and/or the extraction of sugars from insoluble material [24]. These procedures can be cumbersome, and if the data are to be fitted to rate equations, a relative large uncertainty might be introduced by the differentiation procedure required to convert the measured concentrations into reaction rates.

In the present work, we introduce a real-time calorimetric method to measure the rate of enzymatic hydrolysis of two different cellulosic substrates (Avicel and pretreated corn stover (PCS)) at high-solid contents (up to 29% *w*/*w*). Calorimetry has recently proved useful for measurements of enzymatic hydrolysis of low solid samples (<2% solid *w*/*w*) [19], and the present work aims towards further development of this technology for high-solid samples. Implementation of a new assay technology for high solids is motivated by the industrial relevance [1–4] and growing interest in high-solid hydrolysis [14–16, 24–26]. The general principles behind the calorimetric method will be presented together with a discussion of advantages and limitations. Furthermore, a detailed kinetic analysis is performed based on dose–response curves in order to obtain insight of the rate-limiting factors of high solid hydrolysis.

Material and Methods

Materials

Enzymes and Substrates

The commercial product Celluclast™ (Novozymes A/S Denmark) was used in different dosages ranging from 5 to 83 filter paper units per gram cellulose (FPU g^{-1}). Celluclast is based on the hydrolytic enzymes from *Trichoderma reesei* and is approximately 60% CBH I, 20% CBH II, and 12% EG II [5]. The activity of beta glucosidase (BG) in Celluclast is quite low, and we therefore added 25% (*v*/*v*) Novozym188® (Novozymes A/S Denmark), which contains *Aspergillus niger* BG (specific activity of 250 cellobiose units ml^{-1}) to the Celluclast stock. The specific activity of Celluclast stock was determined to be 82 FPU ml^{-1} by standard methods [22]. The buffer was 50 mM $NaC_2H_3O_2$ and 2 mM $CaCl_2$, and pH, 5.

Pretreated corn stover and the partially crystalline cellulose Avicel® (Fluka, Steinheim GE) were used as substrates. Dry senescent corn stover was pretreated by the National Renewable Energy Laboratory (NREL) by addition of sulfuric acid to 1.2% *w*/*w* and heated to 192 °C in a Sunds reactor for 3 min. The material was treated with washing and ground/sieving procedures described elsewhere [19]. The composition of the PCS was determined by NREL Laboratory Analytical Procedures to be 57.5% cellulose, 7.0% xylan, and 27.2% lignin (*w*/*w*). Avicel suspensions were homogenized in a Turrax T homogenizer (IKA Labortechnik, Uppsala, Sweden) for 10 min on ice.

Methods

Isothermal Calorimetry

All calorimetric measurements were conducted on a Thermal Activity Monitor (TAM 2277, TA Instruments, New Castle, De, USA) at a temperature of 25 °C. The reaction vessels were 4-ml stainless steel cylinders closed with tight lids. An experiment was started by thorough manual mixing of enzymes and substrate in 20-ml beakers on ice. The mass of the solution (enzyme + buffer) added to a given mass of substrate was adjusted to give a constant solid content at all enzyme dosages. Approximately 2 g of the paste-like enzyme/substrate mixture was weighed precisely in the calorimetric vessel and immediately mounted in the calorimeter for 40 min thermal pre-equilibration. Then, the cell was placed in the calorimetric detector and allowed to equilibrate for an additional 30 min before the data acquisition started. The enzymatic hydrolysis proceeded for 18 h (70 min equilibration time +16.8 h calorimetric monitoring), at which point the sample was removed from the calorimeter and prepared for high-performance liquid chromatography (HPLC) as described below. Baselines (heat flow from samples without added enzyme) were conducted 1–2 times a week and subtracted from hydrolysis experiments. Due to the high viscosity of high solid samples, it is not possible to apply stirring, which may result in mass transfer limitations and hence reduce hydrolysis rate. This was tested by a parallel experiment measuring the sugar production (by HPLC) with and without agitation (samples were mounted on a rotational mixer which mimics industrial gravitational mixing). The result showed no effect of continuous agitation, and hence, indicate that hydrolysis in calorimetric

experiment is not limited by lack of stirring (data not shown). The calorimeter was chemically calibrated by titrating 10% propan-1-ol to water according to the procedure outlined by Briggner and Wadsö [27].

Data Analysis

The basic principle of the TAM is passive heat conduction [28], where exchange of heat between the sample and surroundings (heat sink) is measured as a function of time. The primary experimental observable is heat flow $\left(\frac{dq}{dt}\right)$, measured in μW ($\mu J s^{-1}$). If the formation of a reaction product, P, is associated with a molar enthalpy change, ΔH, and occurs on a time scale which is slow compared to the time resolution of the instrument, the rate of reaction, $\frac{dP}{dt}$, is directly reflected in the heat flow.

$$\frac{dP}{dt} = \frac{dq}{dt} \cdot \frac{1}{\Delta H} \tag{1}$$

For simple reactions in dilute solutions, Eq. 1 is immediately applicable, but for complex systems like biomass, the constancy of ΔH through the course of the experiment must be validated against a separate method. Thus, side reactions and contributions from dilution, mutarotation, or the dissolution of cellulose crystals may couple to the hydrolysis of the β-1,4 glucosidic bond and contribute to the measured heat flow [19]. Therefore, the heat output was tested for correlation with the production of soluble sugars measured by HPLC. The cumulative heat, Q, for a trial is the area under the calorimetric trace

$$Q = \int_0^{t=18h} \frac{dq}{dt} dt \tag{2}$$

As discussed above, the thermal equilibration precluded data acquisition for the first 70 min. To account for the sugar produced in this period, the calorimetric signal was extrapolated to $t=0$ by a function which is the sum of two exponentials, $\frac{dq}{dt}(t) = ae^{-k_1 t} + be^{-k_2 t}$. This function accounted very well for the experimental data from 70 min to 18 h (see Fig. 1), and it has previously been shown to represent well the rate vs. time both for pure cellulases, enzyme cocktails, and for different substrates [18, 19, 29]. If the value of Q scales proportionally with the amount of sugar, it is acceptable to apply Eq. (1) and to use the slope as an apparent enthalpy change ΔH_{app} even if the different contributions to ΔH_{app} have not been identified specifically.

Having determined ΔH_{app}, the extent of reaction (ζ; or the degree of conversion) can be calculated as

$$\zeta = \frac{QM_w}{\Delta H_{app} m_{cel}} 100\% \tag{3}$$

where M_W is the molecular weight of one glucopyranose unit (162 g/mol) and m_{cel} the mass of cellulose in the sample. Unlike methods that rely on extraction [30], this approach is independent on changes in liquid and solid fractions of the sample through the course of the reaction.

HPLC

The concentration of soluble sugars in the liquid phase was analyzed by HPLC coupled to RI detection (Agilent 1100 RI, CA, USA) using an Aminex HPX-87H column (Bio-Rad,

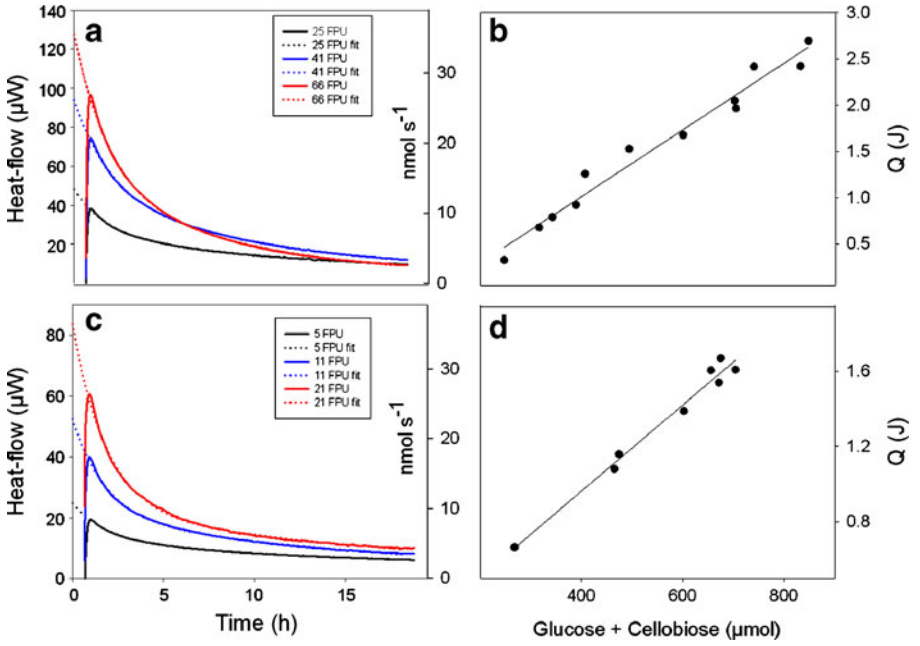

Fig. 1 Examples of calorimetric raw data, double exponential fits, and calibration curves: representative calorimetric data from the hydrolysis of 29% (*w*/*w*) PCS (*panel* **a**) and 19% Avicel (*panel* **c**). *Solid lines* represent calorimetric data and *dotted lines* fits of double exponential decay, extrapolated to time zero. The cumulated heat, *Q*, is plotted as a function of soluble sugars (glucose + cellobiose) in *panels* **b** (PCS) and **d** (Avicel)

Copenhagen, DK). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mlmin^{-1} at 60 °C. Calibration curves were performed on glucose, cellobiose, xylose, and arabinose, and fucose was used as internal standard. Samples from calorimetric experiments were transferred to 50-ml Falcon tubes with 8 ml 80 °C buffer and immediately heated to 98 °C for 20 min to inactivate the enzymes. The samples were vigorously shaken to enhance the extraction of sugars. The liquid and solid phase was separated by centrifugation, and the extraction step was repeated twice with 50% ethanol. This latter step, including ethanol, was introduced as it was found to improve extraction efficiency with up to 20% and generally reduce experimental scatter compared to extraction protocols with buffer or 5% H_2SO_4. The pooled supernatants were weighed and reduced to approximately 12 ml (quantified gravimetrically) by evaporation at 80 °C. The final solution was filtered through 0.45 μm syringe filters, mixed with internal standard, and mounted in a HPLC auto sampler.

Results and Discussion

Evaluation of the Calorimetric Assay

Calorimetry has been previously proven successful to characterize the activity of enzymes in dilute aqueous solutions [31–33], in complex crowded systems [34, 35], and water-rich suspensions of insoluble subtrates [36, 37] including hydrolysis of lignocellulose [19]. In

all of these studies, calorimetry has been applied in the "titration mode", where enzyme and substrate are mixed by injections under continuous stirring. This approach is not possible in the current study due to the high viscosity of high-solid samples which precludes stirring and injections during the calorimetric trial. Instead, substrate and enzymes were mixed in calorimetric vessels outside the calorimeter followed by calorimetric detection for 18 h. The validity of this approach for the current high-solid samples is confirmed in panels B and D of Fig. 1 which show that the heat Q (Eq. 2) increases proportionally to the amount of soluble sugar determined by HPLC (r^2=0.97 and 0.98 for PCS and Avicel, respectively). The figure also shows selected raw calorimetric data for 29% PCS (panel A) and 19% Avicel (panel C). The dashed lines represent the best fit of a sum of two exponentials and the extrapolation to t=0 as described in the "Material and Methods".

The slopes in Fig. 1b and d suggest that ΔH_{app} is −3.6 kJ/mol and −2.3 kJ/mol for PCS and Avicel, respectively. This is close to the enthalpy change of hydrolysis for the β-1-4 glucosidic bond in cellobiose [38], and contributions to the measured heat flow from side reactions and intermolecular interactions therefore appear to be small even in a multi-component and water-poor PCS sample. The reproducibility of calorimetric trials was satisfactory with an average variance (standard deviation/average of the total heat production over 18 h) of 6.8% (based on triplicate samples at ten different dosages). This was close to the variance for the HPLC data (6.4%). In contrast to HPLC, calorimetry is a "differential method" where the sugar content is derived from integration over time. This results in a time-dependent variance, since the signal/noise ratio decreases as the hydrolysis progresses. Thus, the reported variance is an average over 18 h, and in general, calorimetry is more sensitive than HPLC early in the hydrolysis, whereas calorimetry has limitations when the heat flow is integrated over prolonged time intervals, especially when the heat flow approaches baseline level. Based on the results presented in Fig. 1, we conclude that calorimetry is applicable for real-time measurements of enzymatic hydrolysis in high-solid samples, with accuracy in the same range as HPLC over extended hydrolysis times, and higher accuracy at lower hydrolysis times. The most important advantage of calorimetry is the real-time, differential nature of the measurement, which permits derivation of detailed kinetic information along the course of reaction (see "Kinetic Analysis" section). Typical assays such as HPLC or reducing ends are "integral techniques" which measures the cumulative effect over time, and hence, requires a high number of time points to get a fairly detailed progression curve. Furthermore, if rates are to be derived from integral methods, a relatively large error may be introduced when the slope is calculated.

Kinetic Analysis

Enzymatic hydrolysis of high-solid suspensions cannot be rationalized by kinetic approaches resting on collision theory as coined in, e.g., the Michaelis–Menten framework. Thus, the substrate is insoluble and heterogeneous with a limited number of attack points for the cellulases. Moreover, the number and quality of such attack points is modified as the surface is eroded through the hydrolysis process [18]. Inactivation of enzymes by, e.g., adsorption to non-cellulosic residues such as lignin may also significantly influence hydrolysis kinetics [39]. In the present work, we attempt to shed some light on the importance of these parameters through a coherent analysis of the enzyme dosage, rate, time, and extent of the hydrolysis. Figure 2 shows calorimetric data of dose–response experiments using PCS (panels A and B) and Avicel (panels C and D) as substrates. The raw data in panel A and C is converted to the extent of cellulose conversion, ζ, calculated according to Eq. 3 in panels B and D. For PCS, the curves representing dosages of 66–

83 FPUg^{-1} cellulose are superimposed, which suggest saturation of enzyme on available substrate binding sites during the whole course of reaction. For Avicel, an analogous saturation is reached at about 30 FPUg^{-1} cellulose. These values are in line with dose–response experiments for diluted samples (2% PCS), where enzyme saturation has been observed at approximately 50 FPUg^{-1} cellulose [11]. The sugar yield was higher for PCS (29%) than for Avicel (20%), and this may be ascribed to higher accessibility in the pretreated material [40]; also in accord with the higher concentration of enzymes required for saturation (~66 FPUg^{-1} for PCS and ~32 FPUg^{-1} for Avicel). The time course of all curves exhibits "non-linear" or "burst-phase" behavior in the sense that the hydrolysis rate decreases ~5–10-fold within 18 h, even though only 8–29% of the cellulose had been hydrolyzed. Burst-phase kinetics seems to be a generic property of cellulase kinetics, also observed under diluted condition both for pure cellulose substrates and for corn stover [17–19, 29].

Figure 2 suggests that the initial burst and subsequent decline in reaction rate do not rely on irreversible adsorption to lignin or other non-cellulose components as the course of the rate curves are similar for PCS (57.5% cellulose and 27.2% lignin) and Avicel (96% cellulose). Also, time-dependent inactivation such as irreversible adsorption, denaturation, or aggregation of cellulases is not likely to play a dominant role (under the current experimental conditions) due to the complete overlay of curves for high dosages (>66 FPU for PCS and >30 FPU for Avicel). This suggests saturation of enzymes on substrate binding sites during the entire time course. If the declining rate was a result of irreversible

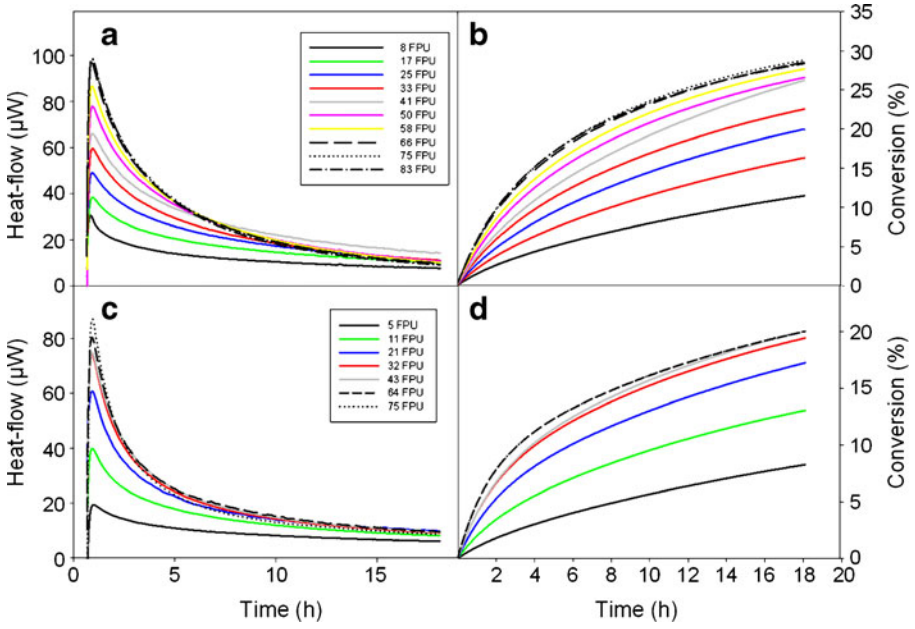

Fig. 2 Calorimetric dose–response experiments varying the enzyme loading from 5 to 83 FPUg^{-1} cellulose for the substrates PCS (*panels* **a** and **b**) and Avicel (*panels* **c** and **d**). *Panel* **a** Calorimetric raw data of PCS hydrolysis (heat flow as a function time) in ten experiments (each average of three) with enzyme dosage ranging from 8 to 83 FPUg^{-1} cellulose. *Panel* **b** Raw data in *panel* **a**, extrapolated to time zero and converted (by integration) to conversion (Eq. 3). *Panel* **c** Calorimetric raw data of Avicel hydrolysis in seven experiments with enzyme dosage ranging from 5 to 75 FPUg^{-1} cellulose. *Panel* **d** Raw data in *panel* **c** extrapolated to time zero and converted (by integration) to conversion (Eq. 3)

inactivation, increasing deviation between curves as a function of time would be expected as the pool of active enzyme declined. This interpretation is discussed further in Fig. 3.

The real-time information in Fig. 2 enables a multidimensional analysis of the hydrolysis process with respect to enzyme dosage and reaction rate, reaction time, and extent of hydrolysis. For example, we may express the reaction rate as a catalytic efficiency (CE), CE=rate/enzyme dosage, (also referred to as turnover number for monocomponent enzyme systems), and plot this against the extent of conversion (i.e., percent cellulose hydrolyzed, Eq. 3). This plot (Fig. 3) specifies the hydrolytic activity per unit enzyme (amount of Celluclast™, measured in FPU) in the sample under comparable substrate/product conditions. The advantage of such a plot is that it allows for a direct comparison of enzyme mixture performance at different dosages under identical substrate/product conditions along the course of hydrolysis.

The negative slope for all curves in Fig. 3 illustrates a general decline in efficiency as the hydrolysis progresses. More importantly, Fig. 3 also identifies some clear differences depending on substrate and the degree of conversion. At low conversion, CE decreases markedly with increasing enzyme dosage for both substrates. Thus, the initial value of CE in Fig. 3a at 8 FPU g^{-1} cellulose is about twice the value at 33 FPU g^{-1}, and this implies that the enzymes complete twice as many catalytic cycles per time unit at the lower dosage. This may suggests that the overall hydrolysis rate is limited by the availability of substrate. More specifically, the sites on the solid substrate that constitute good attack points for the cellulases become occupied even at the lower enzyme dosages. For PCS, this picture changes completely as hydrolysis progresses, and above ~10% conversion, the superposition of the curves in Fig. 3a shows that the enzymatic efficiency is independent on the dosage (up to 66 FPU g^{-1} cellulose). Hence, the hydrolysis rate is commensurate with the amount of enzyme (constant CE), and the availability of good attack points can no longer be the rate-limiting factor. As the applied enzyme cocktail contains 80% CBH and 12% EG [5], one interpretation of this shift from "substrate limitation" to "enzyme limitation" may be that at low conversion, there is a shortage of cellulose chain ends (i.e., substrates for the CBH), while at later stages, the action of endoglucanases has generated an excess of chain ends. In case of Avicel, CE decreased with enzyme dosage at all conversions (Fig. 3b), and hence, did not converge as hydrolysis progressed as seen with PCS. These results suggest that the accessibility of the substrate (number of attack points) is the rate-limiting factor

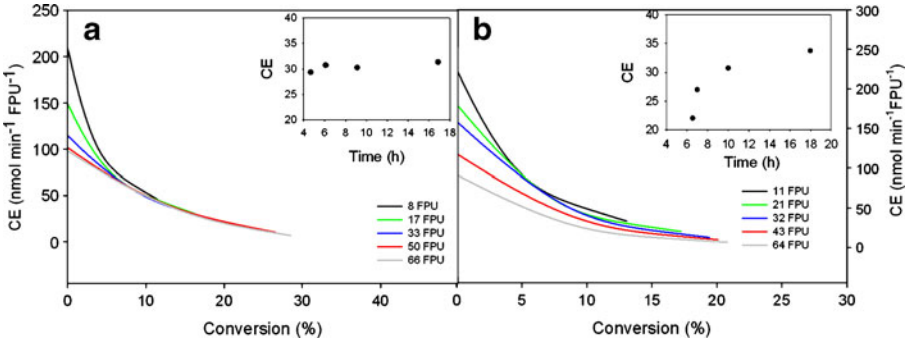

Fig. 3 Catalytic efficiency (CE) as a function of dosage, extent of reaction, and time. *Panel* **a** CE (rate/FPU) is plotted against conversion at different enzyme dosages hydrolyzing 29% PCS. *Insert* CE as a function of time required to reach 16% conversion for 17–66 FPU. *Panel* **b** CE (rate/FPU) is plotted against conversion at different enzyme dosages hydrolyzing 19% Avicel. *Insert* CE as a function of time required to reach 13% conversion for 11–43 FPU

during the entire course of hydrolysis. This difference between Avicel and PCS may be related to the higher crystallinity of Avicel compared to PCS, which is expected to decrease the accessibility of the substrate towards enzymatic hydrolysis.

The insert in panels A and B represent CE at a fixed conversion (16% and 13% for PCS and Avicel, respectively) plotted against the time required to reach the given conversion. Hence, each data point in the inserts represents a specific enzyme dosage (17, 33, 50, and 66 $FPU\,g^{-1}$ for PCS and 11, 21, 32, and 43 $FPU\,g^{-1}$ for Avicel). The advantage of such plots is that they allow comparison of CE under identical substrate (and product) conditions, but at different hydrolysis times, and hence, provide a way to distinguish time-dependent contributions to the slowdown in rate (e.g., irreversible enzyme inactivation) from time-independent contributions such as product inhibition and substrate erosion. In case of PCS (inset of Fig. 3a), CE was independent of the time required to reach 16% conversion. This substantiates the conclusion made earlier that time-dependent irreversible inactivation plays a minor role in these samples. Avicel did not show any sign of a time-dependent inactivation either (inset of Fig. 3b). In contrast to PCS, CE increased with time, and this was ascribed to enzyme saturation. Thus, the enzyme cocktail worked more efficiently at low dosage where the number of good attack points on Avicel was in excess. The lack of a time-dependent inactivation for PCS and Avicel suggests that the slowdown is governed by the degree of conversion (and build-up of products) and not on time. To summarize the analysis of Fig. 3, it is proposed that changes in substrate properties, such as accessibility or cellulose chain end concentration during hydrolysis, play an important role in cellulase kinetics. This is especially important for enzyme cocktails containing both CBH and EG. Furthermore, time-dependent inactivation did not appear to influence the observed hydrolysis rate under the experimental conditions investigated here.

In conclusion, calorimetry provides a sensitive real-time assay for enzymatic hydrolysis of high-solid lignocellulosic substrates. It provides some advantages. For example, it is a "differential" methodology in the sense that the primary observable scales with the rate of reaction, not the concentration of reactants or products. This enables kinetic analyses with respect to e.g., time, dosage, and extent of reaction as shown here, as well as other parameters such as solid content, temperature, and enzyme cocktail composition. Moreover, the method is continuous and does not require post-experiment procedures or corrections for the increase in the liquid fraction during hydrolysis. Promising future applications of this approach include studies of product inhibition since the sensitivity of the assays is independent on product concentration and screening trials for cellulase activity and the efficiency of different cellulase cocktails.

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