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Are cellulases slow? Kinetic and thermodynamic limitations for enzymatic breakdown of cellulose

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ABSTRACT

Cellulases are of paramount interest for upcoming biorefineries that utilize residue from agriculture and forestry to produce sustainable fuels and chemicals. Specifically, cellulases are used for the conversion of recalcitrant plant biomass to fermentable sugars in a so-called saccharification process. The vast literature on enzymatic saccharification frequently refers to low catalytic rates of cellulases as a main bottleneck for industrial implementation, but such statements are rarely supported by kinetic or thermodynamic considerations. In this perspective, we first discuss activation barriers and equilibrium conditions for the hydrolysis of cellulose and how these parameters influence enzymatic turnover. Next, we propose a simple framework for kinetic description of cellulolytic enzyme reactions and show how this can pave the way for comparative biochemical analyses of cellulases acting on their native, insoluble substrate. This latter analysis emphasizes that cellulases are characterized by extraordinarily low off-rate constants, while other kinetic parameters including specificity constants and rate constants for association and bond cleavage are quite like parameters reported for related enzymes acting on soluble substrates.

Introduction

Research in cellulolytic enzymes has grown strongly over the past decades, and this development has been driven primarily by interest in the deconstruction of lignocellulosic biomass. Hence, the abundance of lignocellulosic residue in agriculture and forestry makes an attractive feedstock for large-scale production of sustainable fuels and materials. The dominant upscaling approach within this area has been the so-called biochemical platform, where the enzymatic conversion of polysaccharides to fermentable sugars (saccharification) represents a key step. This process has been extensively investigated as described in literature ranging from fundamental studies of enzyme structure, -kinetics, and -inhibition to applied trials that screen the performance of complex enzyme cocktails on real biomass. All branches of this spectrum commonly state that *cellulases are slow*, and that this slowness makes up a major impediment for industrial saccharification. It is not difficult to find data to support this interpretation. Many works with an applied scope have used enzyme dosages of 10–20 mg protein/g glucan and reported final glucan conversions of some 60–80% [1–3]. If we assume an average molecular mass of the cellulases of 50 kDa, these numbers imply that each enzyme molecule has hydrolyzed about 10^4 glycosidic bonds during the whole saccharification process. This is what some enzymes accomplish in one second, but as the total time of saccharification was 3–7 days, we arrive at an apparent rate of a few hydrolytic events per minute. This estimate appears to justify the view that cellulases are slow enzymes. However, saccharification is a complicated process, which is influenced by numerous parameters including inhibition, synergy, enzyme instability and particularly a limited accessibility of the scissile bond. It is necessary to single out the importance of these and other parameters to qualify general discussions on cellulase

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slowness. In the following, we address this in a discussion of the rate of the basic enzymatic reaction whereas technological complications that slow down saccharification are beyond the current scope. Specifically, we will review data on the rate of different steps in the cellulolytic reaction and compare this to other hydrolytic enzymes. We will also discuss cellulose resilience towards hydrolysis from a thermodynamic perspective and propose some simple principles for comparative analyses of cellulase kinetics. We hope that this will elucidate whether cellulases are inherently slow enzymes, and what steps in the complex reaction pathway make up the most important limitations.

Cellulolytic enzymes

Lucid and comprehensive reviews on the different classes of cellulolytic enzymes and their application may be found elsewhere [4–9]. Here we provide a short overview that mainly serves to introduce the enzymes that will be used as examples below.

Some organisms produce cellulases as free enzymes that are either secreted as a single catalytic domain or covalently attached to one or more carbohydrate-binding modules [10]. The secretome of cellulose-degrading fungi, exemplified here by the archetype *Trichoderma reesei,* an anamorph of *Hypocrea jecorina*, consists of several enzymes that synergize to degrade cellulose (Fig. 1). The two cellobiohydrolases (CBHs) Cel6A and Cel7A use a processive mechanism (see Fig. 4) to release cellobiose from the non-reducing and reducing end of cellulose, respectively. Different endoglucanases (EGs), such as Cel7B, cleave cellulose chains primarily in amorphous regions with no or low processivity, while oxidative lytic polysaccharide monooxygenases (LPMOs) also attack glycosidic bonds in crystalline regions. Finally, β–glucosidases (BGs) cleave soluble saccharides (primarily cellobiose) into glucose in the aqueous bulk.

Fungal cellulases have attracted particular research attention. This is partly due to their role in natural carbon cycling, but also because fungal enzymes have been particularly valuable for industrial applications [5]. This is especially true for cellulases from thermophilic fungi, which show better conformational stability and hence often remain active in prolonged processes at higher temperatures. Here, we will mostly use data for fungal cellulases with particular focus on Cel7A, Cel6A and Cel7B from *Trichoderma reesei* which are among the most thoroughly studied cellulases.

The challenge faced by cellulases: stability of cellulose

Enzyme efficacy is optimized through evolution but curbed by physical restrictions for the reaction in question. The latter may be particularly important for cellulases, because the substrate appears to be stable and highly resistant to hydrolysis. In this section, we will discuss this so-called recalcitrance of cellulose by considering its kinetic- and thermodynamic stability.

Kinetic stability of cellulose

A biochemical reaction that progresses very slowly without a catalyst (*i.e.* has a high activation barrier) is difficult to accelerate to high turnover numbers by an enzyme. This correlation is quite intuitive if we assume that enzymes can lower activation barriers by a certain amount, and this was discussed in detail in seminal works by Wolfenden and coworkers [11,12]. They compared the rate of different biochemical reactions in the presence and absence of enzyme and found that the uncatalyzed hydrolysis of glycosidic bonds was particularly slow. This kinetic stability was illustrated by extrapolations from higher temperatures, which suggested room temperature half-times as high as 5 million years near neutral pH. As typical k_{cat} values for cellulases are around 1 s^{-1} at room temperature (see below), the enzyme appears to accelerate the hydrolytic reaction by some 10^{15} times, and this is in the high end of what is seen for enzyme catalysis [11,13,14]. This type of estimate has sometimes been used to support the conclusion that cellulases are in fact catalytically very proficient, but unable to attain high turnover rates because the substrate is kinetically stable. In other words, physical constraints of hydrolyzing cellulose are so severe that even a highly adapted enzyme can only achieve a moderate turnover. While this interpretation undoubtedly captures important aspects of the cellulolytic process, some caveats remain. For example, rates of uncatalyzed hydrolysis were assessed based on soluble model compounds (e.g. methyl O-glucopyranosides), and the result showed quite similar stability of respectively α– and β– glycosidic bonds [15]. This means that the catalytic proficiency of cellulases is not particularly high in comparison with other glycoside hydrolases. Amylases (that hydrolyze the α-glycosidic bond in starch) and β-glucosidases (BGs, see Fig. 1), may have turnover numbers in the hundreds or thousands per second [16], and if indeed the kinetic stabilities of these substrates are comparable,

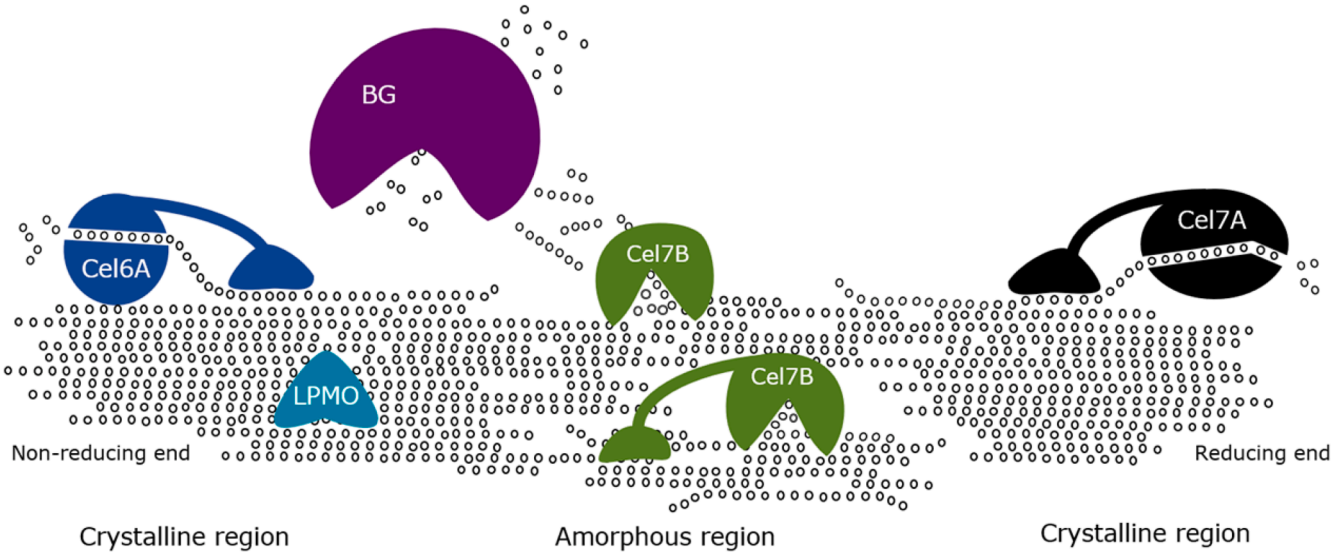

Fig. 1. Selected enzyme classes from the secretome of the cellulolytic fungus Trichoderma reesei. Processive cellobiohydrolases (CBHs, Cel6A and Cel7A) attack the ends of cellulose strands, while endoglucanases (EGs, Cel7B and Cel12A in this example) make endolytic attacks. Lytic polysaccharide monooxygenases (LPMOs) catalyze oxidative cleavage of glycosidic bonds, while beta-glucosidases (BGs) break down soluble, oligomeric saccharides to glucose. The following will focus on CBHs and EGs.

some amylases and BGs show a much higher degree of acceleration compared to cellulases. Moreover, the uncatalyzed half-time of the glycosidic bond in methyl O-glucopyranosides is strongly dependent on pH. Specifically, half-times dropped about three orders of magnitude between neutral and pH 5 [15], where most work on cellulases and industrial saccharification is performed. It follows, that the degree of acceleration for cellulases under relevant conditions is about 10^{12}, and this is quite average for enzyme reactions [13]. We conclude that the slowness of cellulases may in part reflect the high kinetic stability of the glycosidic bond. However, other glucosidases reach much higher turnover numbers on substrates that are equally stable, when judging from the half-life of soluble model compounds, and it appears that other factors than the intrinsic kinetic stability of the glycosidic bond curtail cellulolytic reactions. Estimated activation barriers for the enzyme-catalyzed and non-catalyzed hydrolysis of cellulose are illustrated later in Fig. 4.

Thermodynamic stability of cellulose

The recalcitrance of cellulose towards enzymatic breakdown also depends on its thermodynamic (or equilibrium) stability. The thermodynamic stability of glycosidic bonds has been assessed in many works, where the equilibrium between a reactant (with intact bond) and products (with hydrolyzed bond) is established through the addition of an enzyme (catalyzing the reaction in both directions). Measurements of the concentrations of reactants and products in the equilibrated system allow estimation of an apparent equilibrium constant, K_{app} and the associated standard free energy change, $\Delta G^{o}_{react} = -RTln\ K_{app}$, of the hydrolytic reaction. Goldberg and coworkers have assembled a large amount of such data [17], and curated different types of measurements with respect to standard state and concentration units. This allows direct comparisons of the otherwise disparate literature data, and in the following, we use these standardized values rather than the values from the original papers. The overall picture shows that the driving force for hydrolysis of a glycosidic bond is small. If we again use methyl O-glycopyranosides as our model substrate, K_{app} for the hydrolysis to glucose and methanol is only about 5 (using the molal standard state). This corresponds to $\Delta G^{o} = -4$kJ/mol (or −1 kcal/mol), and it has the interesting consequence that far from all glycosidic bonds are hydrolyzed at equilibrium. Rather, a dilute solution of β-methyl glycopyranoside will have an equilibrium distribution of about 25% reactant and 75% hydrolysate. Disaccharides including maltose, isomaltose, lactose, β-gentiobiose, α-melibiose and cellobiose have slightly more favorable standard free energies of hydrolysis between −6 and −16 kJ/mol (corresponding to an equilibrium condition with 85–99% monosaccharide). As in the case of kinetic stability, no systematic difference in the thermodynamic stability of α− and β-glycosidic forms could be detected in the compiled data [17].

The thermodynamic stability of insoluble cellulose cannot be readily investigated in a similar fashion because it is difficult (if not impossible) to establish an equilibrium between crystalline cellulose and soluble glucose in an experiment. In lieu of direct measurements several works have combined different data and used thermodynamic cycles to elucidate the equilibrium stability of the glycosidic bond in cellulose [18–20]. One particularly thorough study [21] reported thermodynamic parameters for both amorphous cellulose and several cellulose crystal allomorphs, and hence provided key information on both recalcitrance *per se* and the role of the crystal structure. In accordance with the results for soluble model compounds, this work found a very weak driving force for hydrolysis. If, for example, we consider the conversion of moist amorphous cellulose to solid (crystalline) glucose, ΔG° was about +2 kJ/mol [21]. In other words, hydrolysis in the moist, solid state of the glycosidic bond in amorphous cellulose is endergonic as cellulose is thermodynamically favored over glucose, see Fig. 2. Analogous estimates suggested that hydrolysis of crystalline cellulose was even more uphill in a thermodynamic sense. This was expected due to additional

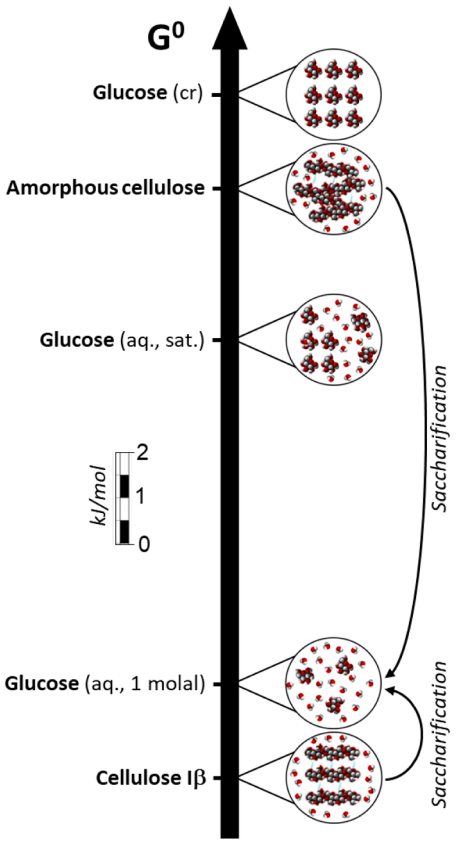

Fig. 2. Estimated standard free energies for selected states of reactants and products in a saccharification process based on literature data [21,29]. It appears that the free energy changes of bond breakage in the solid state are small and unfavorable, and that the driving force of the hydrolytic reaction (cellulose→ glucose (aq)) mostly comes from dissolution of glucose. The reaction, crystalline cellulose Iβ→ glucose (aq., 1 molal), for example, has small, positive ΔG^{0}, and this means that cellulose Iβ equilibrates with aqueous glucose at a concentration of only a few hundred mmolal. For amorphous cellulose, ΔG^{0} is larger and negative, and this corresponds to an equilibrium concentration of several molal.

stabilization of cellulose in the crystal lattice. For the crystal allomorph cellulose Iβ, which is the most common in higher plants [22], conversion to crystalline glucose was distinctly endergonic with $\Delta G^{0} = +15$ kJ/(mol glycosidic bond) [21]. We conclude that hydrolysis in the solid state is endergonic, and that the overall reaction only becomes spontaneous (and hence amenable to enzyme catalysis) due to a favorable free energy change of product dissolution. This observation may have some interesting corollaries for cellulase activity. It may suggest, for example, that the low activity of EGs on crystalline cellulose [23] may not only be a matter of low substrate accessibility, but also the absence of a driving force for endolytic hydrolysis, if little or no soluble product is released. As discussed above, this thermodynamic impediment is less severe for amorphous cellulose, which is readily hydrolyzed by EGs. The dependence of the thermodynamic driving force on product dissolution may also play a role in the so-called non-linear kinetics, which is observed for essentially any cellulolytic reaction (*c.f.* Fig. 3). Non-linear kinetics implies that reaction rates taper off gradually even if the substrate appears plentiful and no apparent inhibition can be identified [24]. The phenomenon obviously influences the cost of industrial saccharification because it creates a demand for long reaction times and high enzyme dosages. Origins of the slowdown have been studied extensively and both enzyme- and substrate related limitations have been highlighted [24–28]. The very weak thermodynamic driving force discussed above, suggests that the slowdown might also reflect that the reaction is

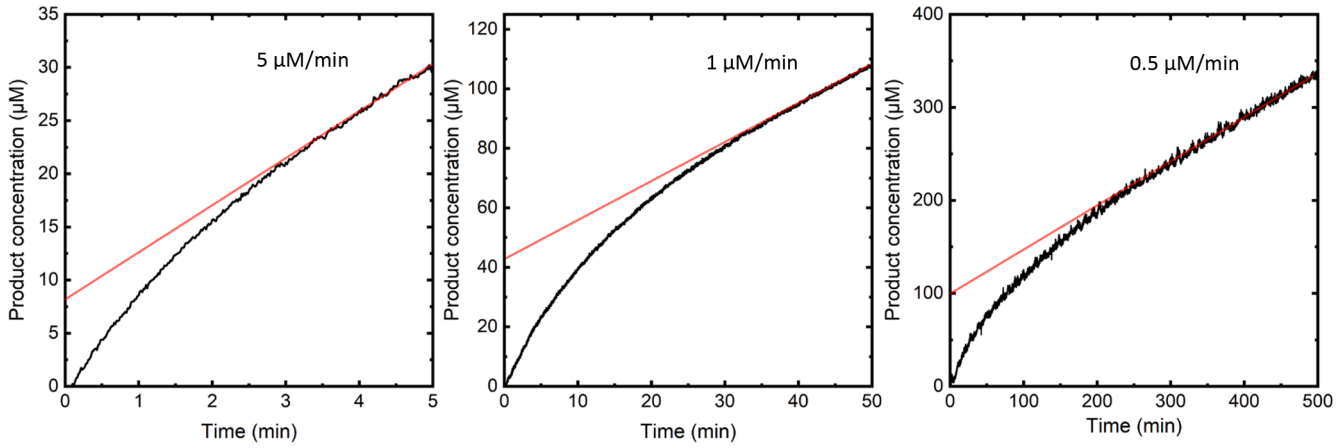

Fig. 3. Real-time progress curve for a cellulase at different timescales. In this example, 60 g/L microcrystalline cellulose (Avicel) was degraded using 100 nM of the enzyme Cel12A (EG3) from T. reesei at 25 °C and pH 5. The measurement was done using an amperometric biosensor with pyranose dehydrongease (PDH) from Agaricus meleagris immobilized on the surface of a carbon paste electrode as previously described [37]. In all cases, curve levels off towards a "near linear" course (slopes are given in the figure). However, when expanding the experimental timescale by an order of magnitude, we consistently observed a gradual slowdown.

approaching equilibrium [29]. This latter work suggested that the equilibrium concentration of glucose for the hydrolysis of amorphous cellulose was several molar at room temperature. This seems to be high enough to neglect equilibrium considerations for amorphous substrate. For the more stable (crystalline) cellulose Iβ, the equilibrium concentration at room temperature was only a few hundred mM glucose, and although this value was about twice as high at 50 °C (a typical temperature for technical saccharification) the equilibrium concentration is in the same range as final yields in industrial processes [3]. These results suggest that slow conversion towards the end of saccharification of crystalline substrate may at least in part reflect that the process becomes devoid of a driving force as it approaches equilibrium. This aspect might be of interest for the so-called high-solid effect, which states that the conversion scales inversely with the initial dry matter load [30]. Higher loads of biomass (i.e. less water) are inextricably linked with higher glucose concentrations, and if indeed the cellulose-glucose equilibrium influences the process, this (negative) effect would be more pronounced the higher the initial substrate load.

To close the thermodynamic discussion, we emphasize that while the parameters reviewed above highlight important aspects of cellulose stability, they cannot be directly transferred to industrial conditions. For example, the equilibrium condition depends critically on the glucose activity coefficient in the relevant solvent [29], and this parameter remains to be assessed in complex biomass suspensions. Moreover, it is unclear whether the conditions under the enzyme catalyzed process approach a simple equilibrium with equal reaction rates in both directions (hydrolysis and formation of cellulose). In particular, the reverse reaction where glucose is linked to the cellulose surface remains unexplored. It has been shown that some cellulases with a retaining mechanism may catalyze transglycosylation [31–33] and hence the formation of glycosidic bonds, but it remains unclear how this would happen on the surface of insoluble cellulose. In particular, cellulolytic secretomes contain BGs, which rapidly convert cellobiose produced by CHBs to glucose (c.f. Fig. 1), and there is no enzyme that efficiently catalyzes the direct interconversion between glucose and cellulose. This pathway with two independent steps might help obtain a kinetically controlled build-up of glucose beyond its equilibrium concentration. Specifically, the absence of a direct enzyme reaction can establish a kinetic barrier that impedes the step of cellulose synthesis from glucose. Consequently, BGs may have an additional role beyond alleviating product inhibition of cellulases. While this interplay remains to be further investigated, it appears safe to say that the driving force for hydrolysis of cellulose is very weak (particularly for crystalline cellulose), and this is a relevant parameter for the rate of high-solid biomass saccharification.

A framework for kinetic description of cellulases

The question whether cellulases are slow is most directly addressed from the vantage point of comparative biochemistry. This implies contrasting kinetic parameters for different cellulases as well as comparing parameters for cellulases with those from other hydrolytic enzymes. While this approach makes up a cornerstone in conventional enzymology, it is rarely used for cellulases. Rather, activity studies are typically reported as progress curves or end-point measurements. This type of data is useful for example in comparisons of enzymes, substrates or experimental circumstances, but they are difficult to use outside the conditions under which they are measured. The scarcity of general parameters for cellulase activity relies on challenges in the formal description of the complex interfacial reaction. The current level of understanding and volume of experimental data for cellulases does not allow detailed analyses with many intermediates akin to those made for many bulk enzymes, but we propose that clarification of three key aspects may set the stage for some comparative discussion of cellulases. These aspects are

i) Defining the timescale of the experiments.
ii) Defining a realistic, yet simple micro-kinetic reaction scheme.
iii) Defining an apparent molar concentration of substrate.

i) As already mentioned, progress curves of cellulases are not linear but show a ubiquitous slowdown, which is manifested on different time scales. If, for example, a cellulolytic reaction is monitored over seconds or minutes it often shows an initial burst followed by a near-linear region as exemplified by the data in the left panel of Fig. 3. However, longer experiments (center and right panel in Fig. 3) typically reveal that the slowdown continues, even if this is not readily detectable on the shorter time-scale. Therefore, comparisons of rates defined by a "near linear" part of a progress curve are deceptive unless the timescale is specified. These problems have occasionally been used as an argument to reject conventional enzyme kinetics and quasi-steady-state approaches altogether, but this may not be necessarily. Rather, we suggest that quasi-steady-state approaches may be reasonable and useful if applied within a well-defined time range - particularly early in the reaction process. Thus, as argued elsewhere [34–36], the reaction is likely to be close to steady-state (nearly constant concentration of enzyme-substrate complexes) when the usual prerequisite of large substrate excess is fulfilled. This interpretation can be reasonable even if reaction rates

drop at a later stage as a result of changes in substrate accessibility or other limitations.

ii) The reaction mechanism of cellulases is complex and includes numerous putative intermediates [38–41]. While detailed considerations of these intermediates have proven valuable in some studies [42], it is currently not realistic to resolve the associated number of parameters in kinetic modeling. Therefore, we will limit the current discussion to a quite coarse simplification that only distinguishes three steps: complexation, catalysis and de-complexation (see Fig. 4). The advantage of this simplified description is that it is realistic to resolve kinetic parameters from available experimental data. The limitation, on the other hand, is that we obtain so-called composite rate constants, each of which reflects the passage time between multiple putative intermediates. The use of composite rate constants precludes any learning on the rate of more subtle sub-steps, but it still provides useful information of the overall kinetics. The scheme in Fig. 4A defines the rate constants, k_{on}, k_{hyd} and k_{off}, as well as a processivity number (n). The latter reflects the average number of consecutive hydrolytic cycles the enzyme will conduct before it dissociates from the cellulose strand. For a truly non-processive cellulase, $n = 1$, but some work suggests that this behavior is rare and that many endoglucanases (EGs) show some degree of processivity [43,44].

iii) *Kinetic* studies of cellulases acting on suspended cellulose particles mostly use mass load (g cellulose per liter solvent) to quantify the amount of substrate, and as a result, some kinetic parameters have unconventional dimensions. Values of k_{on}, for example, may be reported in units of $(g/L)^{-1}\ s^{-1}$ [45,46]. Other works have used cellulose deposited on a fixed surface as substrates [47,48], and this also leads to k_{on}-values in units that are different from the conventional $M^{-1}s^{-1}$ for a second-order rate constant. On-rate constants (and other parameters) in these unconventional units may be useful in local comparisons, but they are not generally applicable in comparative discussions. One way to put different second-order rate constants on an equal footing is to introduce a parameter that enumerates the density of putative sites on the surface to which the enzyme can bind productively (attack sites). This idea has been applied occasionally in studies of enzymes that modify insoluble substrates [49,50], but it is well established within conventional (non-biochemical) heterogeneous catalysis [51]. In the current work, we will use the symbol Γ_{attack} (in units of mol/g cellulose) to denote the density of attack sites (see Fig. 5). We emphasize that adsorption sites and attack sites are not necessarily the same. Thus, some loci on the cellulose surface may be capable of adsorbing enzyme, but not forming a productive complex, and it follows that Γ_{attack} can only be determined from activity measurements (not from adsorption isotherms). This is illustrated in Fig. 5, which also shows that Γ_{attack} depends on both enzyme and substrate properties. Highly accessible substrates as well as enzymes that are efficient in abstracting a ligand from the solid surface, both give rise to high Γ_{attack}-values. It is also worth noting that Γ_{attack} may change as the reaction progresses. This was illustrated in a study that found a pronounced reduction in Γ_{attack} at medium and high degrees of cellulose conversion [52]. In short experiments with low conversion, it may be permissible to use a constant Γ_{attack} for a given system [34,36], and this strongly simplifies kinetic analyses.

Comparative biochemistry based on the conventional Michaelis Menten framework uses two parameters, k_{cat} and K_M, to characterize the enzymes. We propose that Γ_{attack} defined above, constitutes an additional parameter that enables comparative biochemical analyses of cellulases [35] and other enzymes that act on other insoluble substrates [53,54]. The higher complexity of an interfacial reaction requires one extra parameter, but when an experimental value of Γ_{attack} has been established [35,55], it allows us to define an apparent molar concentration of substrate (i.e. number of attack sites per liter of suspension). The total substrate concentration, $[S_0]$ for example, may be expressed

$$[S_0] = \Gamma_{attack}\ [S_0{}^*] \tag{1}$$

where $[S_0{}^*]$ is the (known) initial mass load of substrate (henceforth the asterisk will define mass load concentrations). It is also useful to define a molar concentration of unoccupied (free) attack sites [S], by introducing the mass balance

$$[S] = \Gamma_{attack}\left[S_0^*\right] - [ES] \tag{2}$$

In Eq. (2), [ES], is the (molar) concentration of the enzyme-substrate complex. The meaning of Γ_{attack} is further illustrated in Fig. 5.

Introducing Γ_{attack} allows the use of the law of mass action, which require an explicit stoichiometric relationship between enzyme and substrate. Applying the quasi steady-state assumption (d [ES]/dt = 0) for the enzyme-substrate complex and Γ_{attack} we can write

$$\frac{d\ [ES]}{dt} = k_{on}\ [E]\ \Gamma_{attack}\ [S^*] - \ [ES]\left(k_{off} + k_{cat}\right) = 0$$

This expression can be rewritten by using the two conserved quantities, $[E] = \ [E_0] - \ [ES]$ and $\Gamma_{attack}\ [S*] = \Gamma_{attack}\ [S_0*] - \ [ES]$ (Eq. (2)) to give Eq. (3)

$$\left(\ [E_0] - \ [ES]\right)\ \times\ \left(\Gamma_{attack}\ \left[S_0^*\right] - \ [ES]\right) = \ [ES]\ \times K_m \tag{3}$$

Where $K_m = \left(k_{off} + k_{cat}\right)/k_{on}$. The steady-state rate (V_{ss}) is given by $V_{ss} = k_{cat}\ [ES]$, where $[ES]$ is the steady-state concentration of productive enzyme-substrate complex at the solid-liquid interface of the insoluble substrate. By isolating $[ES]$ in Eq. (3) and multiplying with k_{cat}, we can find the steady-state rate equation in the two limiting cases of substrate excess ($E_0 << \Gamma\ [S_0*]$) or enzyme excess ($\Gamma\ [S_0*] << E_0$). These two rate equations are given in (4) and (5).

$${}^{conv}V_{ss} = \frac{{}^{conv}V_{max}\ \left[S_0^*\right]}{K_M^* + \ \left[S_0^*\right]},\ {}^{conv}V_{max} = k_{cat}\ [E_0],\ K_M^* = K_m \Big/ \Gamma_{attack} \tag{4}$$

$${}^{inv}V_{ss} = \frac{{}^{inv}V_{max}\ [E_0]}{K_m + \ [E_0]},\ {}^{inv}V_{max} = k_{cat}\Gamma_{attack}\ \left[S_0^*\right] \tag{5}$$

Eq. (4) is the well-known Michaelis-Menten equation which may be fitted to steady-state rates under conditions where the enzymes are gradually saturated with substrate. This is done by having a fixed (small) concentration of enzyme and vary the substrate load $\left[S_0^*\right]$ as shown in Fig. 6B. Note that the Michaelis-Menten parameter which is obtained from fit to such data will have units of g/L and we have therefore denoted this parameter K_M^*.

The lesser-known condition which Eq. (5) cover is the so-called inverse Michaelis-Menten approach [35]. In this case the maximal rate occurs when the surface of the insoluble substrate is saturated with enzyme. Fitting the inverse MM-equation (Eq. (5)) allow us to derive inverse MM-parameters ${}^{inv}V_{max}$ and K_M. Examples of such data is shown in Fig. 6C. Note that the x-axis under the inverse approach has molar units (mol/L) and hence the K_M obtained in this way will have the conventional units. If both conventional and inverse MM parameters are obtained, the attack-site density Γ_{attack}can be estimated. Either by the ratio of the normalized maximal rates or the ratio of the two MM-parameters as shown in eq. 6

$$\Gamma_{attack} = \frac{{}^{inv}V_{max}/\ \left[S_0^*\right]}{{}^{conv}V_{max}/\ [E_0]} \tag{6A}$$

$$\Gamma_{attack} = \frac{K_m}{K_M^*} \tag{6B}$$

Since K_M for both the conventional and inverse MM approach is independent on the concentration of respectively $[S_0{}^*]$ or $[E_0]$, Eq. (6a) and (6b) may also be used to test whether the criteria for applying the two equations is fulfilled. If the two approaches do not give similar

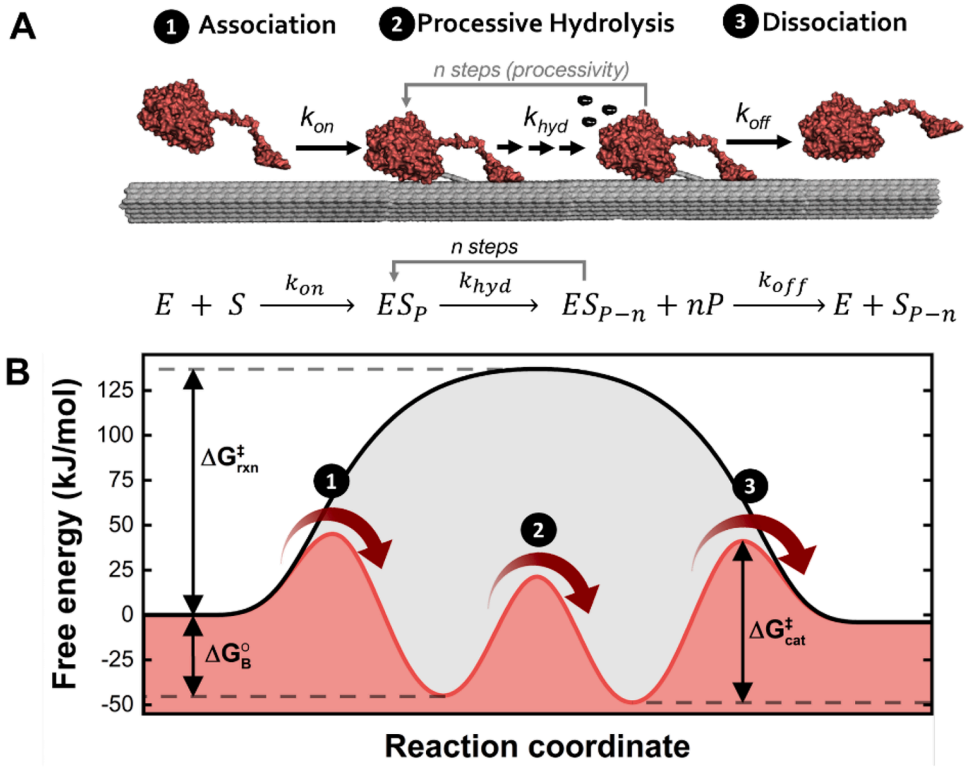

Fig. 4. A Simplified view on the microkinetic scheme, structures and energy landscaper for a processive cellulase. Panel A shows a reaction scheme for the enzymatic hydrolysis of cellulose. The scheme defines three rate constants, k_{on}, k_{hyd} and k_{off} and a processivity number, n, as discussed in the main text. Panel B shows a proposed energy landscape for the uncatalyzed (gray) and enzyme catalyzed (red) hydrolysis of the β−1,4 glycosidic bond in cellulose. The free energy values are best estimates based on several experimental and theoretical investigations as discussed in the main text. Under most conditions, step 3 makes up the largest free energy barrier and hence limits the turnover.

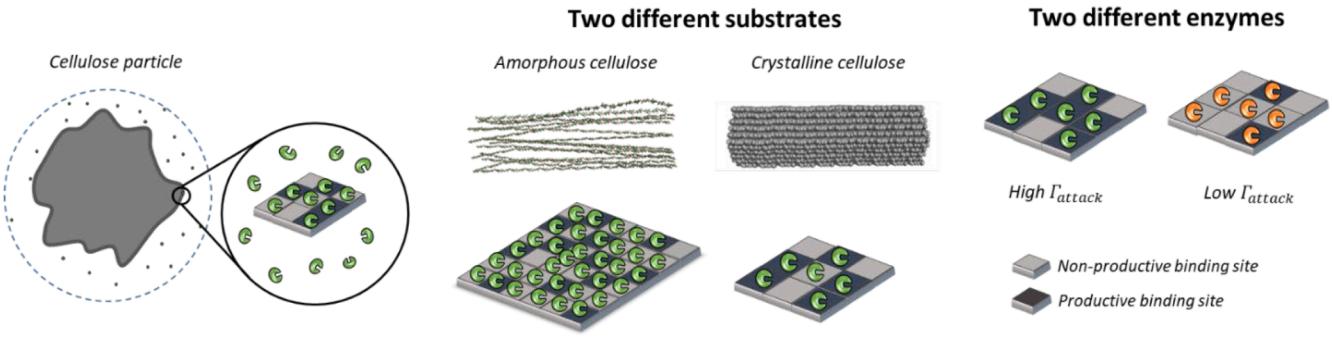

Fig. 5. Illustration of the parameter "attack site density", Γ_{attack}. The surface of the cellulose particle (left) has several sites to which the enzyme can bind. The enzyme can make a productive complex with some fraction of these sites. We call these sites attack sites (dark gray). Other adsorption loci that cannot form productive complexes are light grey. An accessible substrate such as amorphous cellulose has many sites per gram cellulose compared to more compact (e.g. crystalline) substrates (middle). If two enzymes attack the same substrate (right) the enzyme with better ligand binding will be able to form complexes with a larger fraction of the sites compared to an enzyme with less efficient binding. As a result, the former enzyme has a higher Γ_{attack}. This concept is exemplified by kinetic data in Fig. 6.

estimate of Γ_{attack} it may be because the criteria of large excess of either enzyme or substrate are not fulfilled.

As already stated, Eqs. (4)-(5) are only valid under the assumption of substrate- or enzyme excess. For both the conventional and inverse MM-equation it has been shown that this condition fulfilled if $E_0 << S_0 + K_M$ (^{conv}MM) [56] or $S_0 << E_0 + K_M$ (^{inv}MM) [57]. Adapting these inequalities to the conventional and inverse MM equation, we can express the validity of Eq. (4) and (5) as:

$$^{conv}MM: \quad [E_0] << \left([S_0^*] + K_M^* \right)\Gamma_{attack} \tag{7A}$$

$$^{inv}MM: \quad [S_0^*] << \left([E_0] + K_m \right)\frac{1}{\Gamma_{attack}} \tag{7B}$$

MM-based analysis of cellulases have been widely used [58–62], but its validity has been questioned, mainly with reference to a lack of substrate excess [63–65]. With the formal description presented above, this can be tested directly as exemplified in Fig. 6. Panel D of this figure shows contour plots for three cellulases, which identifies validity regions for ^{conv}MM (Eq. (4)) and ^{inv}MM (Eq. (5)). Outside the validity regions of respectively ^{conv}MM and ^{inv}MM, the kinetic parameters will become increasingly inaccurate. The highest mass load, which is possible to handle experimentally, is around 100 g/L for Avicel. As seen from Fig. 6D, this means that cellulases with low substrate accessibility (low Γ_{attack}) can only be adequately described by ^{conv}MM at extremely low enzyme concentrations. For such enzymes a more reliable approach is

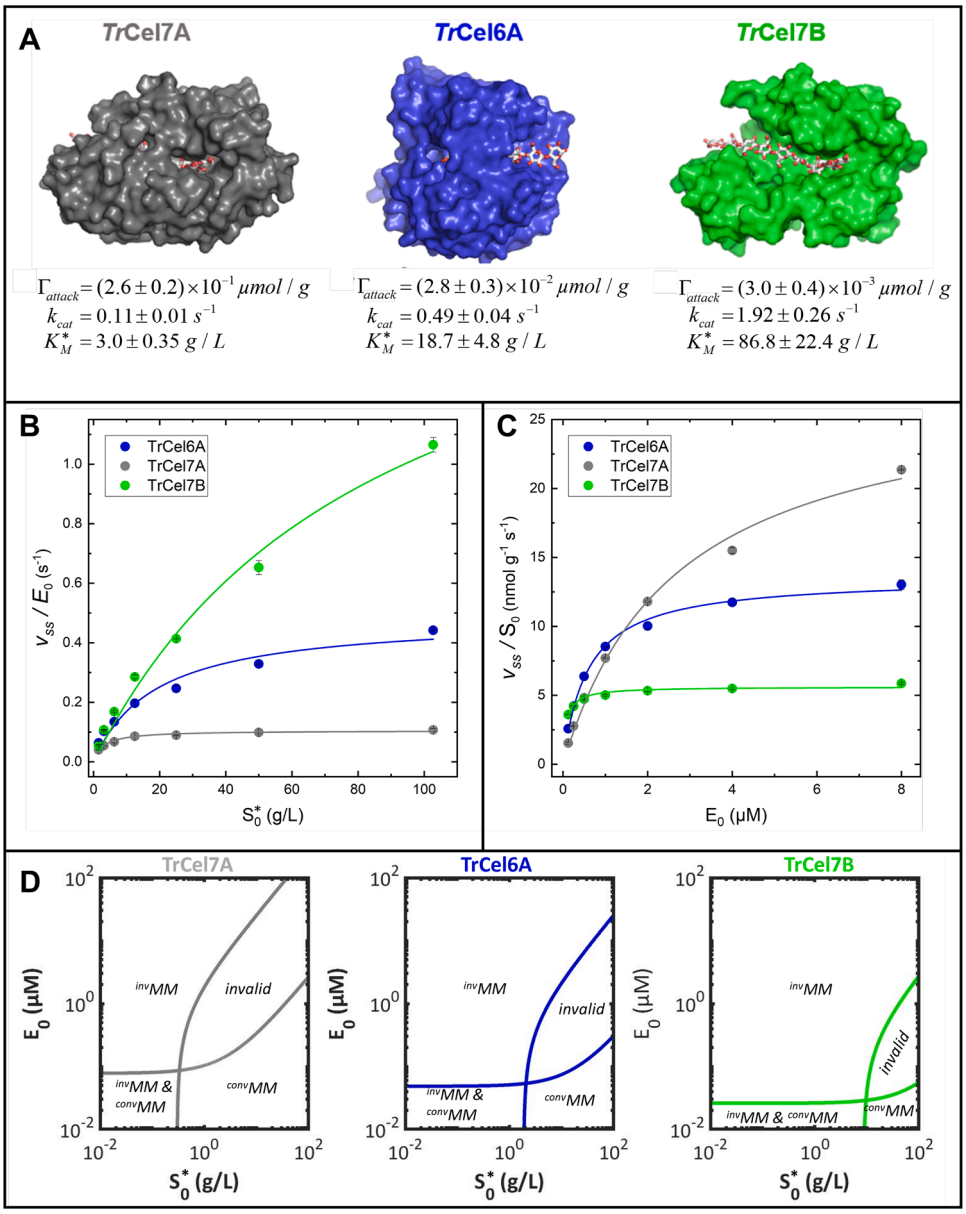

Fig. 6. Panel A. Structure of Michaelis complexes (based on PDB id 4C4C, 1CB2 and 1EG1) and steady state kinetic parameters for the hydrolysis of Avicel at 25 °C, pH 5 for respectively Cel7A, Cel6A and Cel7B [68,75,80,81]. Panels B and C illustrate two types of quasi-steady–state analysis of these three enzymes. Panel B shows conventional Michaelis Menten curves with the characteristic hyperbolic raise towards the maximal rate. It appears that $^{conv}V_{max}$ follows the sequence Cel7B>Cel6A>Cel7A. Panel C shows a dose-response curve for the same systems. As discussed in the main text, this may also represent a quasi-steady-state situation for a solid substrate, and it is sometimes called an inverse Michaelis-Menten plot [35,82]. Saturation in the inverse MM plot implies that all attack sites on the substrate surface are occupied, and it is interesting to note that the maximal rates in panel C follows the opposite sequence, Cel7A>Cel6A>Cel7B, compared to panel B. This is because the ability to "find" attack sites expressed as Γ_{attack}, falls dramatically through this series (see values in panel A). These results suggest that Cel7A has the ability to combine productively with many sites, but a low turnover. Conversely, Cel7B has a high turnover, but can only hydrolyze a small subset of sites. The ability to find attack sites (high Γ_{attack}) also have consequences for the validity domain of the conventional MM-equation (Eq. (4)) and inverse MM-equation (Eq. (5)). Panel D illustrates the enzyme concentration [E_0] and substrate load [S_0] where the two equations are valid or invalid for respectively Cel7A, Cel6A and Cel7B.

^{inv}MM since its validity spans a much larger [E_0]/ [S_0*] ratio.

Kinetic parameters for cellulases

Rate constants for hydrolysis (k_{hyd}) and dissociation (k_{off})

The maximal turnover number, k_{cat}, is probably the most extensively discussed kinetic parameter for any group of enzymes, but for cellulases, it has been associated with some ambiguity. In the traditional, uni-reactant Michaelis Menten model, k_{cat} describes the chemical conversion of the substrate, and the success of this approach has led to a common practice where k_{cat} is implicitly associated with the chemical step. However, this interpretation is not generally applicable to reaction schemes with several steps, because other processes than the chemical conversion may be slow, and hence govern maximal turnover. For complex reaction schemes, the maximal turnover, $k_{cat}=^{conv}V_{max}/E_0$, reflects the slowest first-order rate constant in the forward direction of the

process [66] .[6] In the following, we will stick to this definition and consistently use k_{cat} for the specific rate at substrate saturation, which can be readily read off the flat part of a Michaelis Menten curve as illustrated in Fig. 6. The two first-order rate constants defined in Fig. 4, k_{hyd} and k_{off}, will refer specifically to the chemical step (hydrolytic bond breakage) and dissociation respectively.

The best characterized cellulase is the cellobiohydrolases Cel7A, from *T. reesei*, and k_{hyd} data for this enzyme were compiled and critically assessed in a recent review [67]. This analysis suggested remarkably consistent values from different studies in the range 3–10 s^{-1} at room temperature. Other enzymes from *T. reesei* have shown moderately higher k_{hyd} values compared to Cel7A. For Cel6A, quenched flow analyses showed $k_{hyd} \sim 20$ s^{-1} on both microcrystalline- and amorphous cellulose [68]. This is a bit faster than the value 8 ± 5 s^{-1} derived from single-molecule tracking of Cel6A on crystalline fibers of cellulose I_α [69]. We are unaware of direct measurements of k_{hyd}-values for EGs, but activity measurements on short timescales have reported rapid turnover for Cel7B (20 s^{-1}) [70] and Cel5A (9 s^{-1}) [43] on amorphous cellulose. These numbers were derived from end-point measurements without kinetic modeling, and they hence represent minimum boundaries of k_{hyd} for these EGs.

The other first-order rate constant in the forward direction of Fig. 4 is k_{off}. Again, most available data for this parameter pertains to *T. reesei* Cel7A, but unlike k_{hyd}, published k_{off}-values are divergent, and fall in a wide range between 10^{-1} s^{-1} and 10^{-6} s^{-1} [5,67,71]. We note that the lower range of this interval appears incompatible with measured reaction rates (a k_{off} of 10^{-6} s^{-1} corresponds to a half-life for enzyme dissociation of about one week), but for a critical discussion of different k_{off} measurements for Cel7A, we again refer to the review by Nill and Jeoh [67]. In the current context, it is important to note that all reported k_{off} values for Cel7A are lower than k_{hyd}. Most biochemical measurements have reported k_{off} between 10^{-2} s^{-1} and 10^{-3} s^{-1}, and thus suggest that decomplexation is hundred- or thousand-fold slower than bond breakage ($k_{hyd} = 3$ - 10 s^{-1}). This may not come as a surprise, because a processive reaction, as in the case of Cel7A, is only feasible when dissociation is slow [72]. Nevertheless, the observation that $k_{hyd} >> k_{off}$ has the interesting corollary that the maximal reaction rate at steady-state is governed by the off-rate. It also emphasizes the importance of the mentioned distinction between $k_{cat}={}^{conv}V_{max}/E_0$ and the rate of the chemical step. Rigorous analysis of the reaction scheme in Fig. 4 [73,74], shows that when $k_{hyd} >> k_{off}$ we may write ${}^{conv}V_{max} \sim nE_0k_{off}$, and insertion of $k_{cat} = {}^{conv}V_{max}/E_0$ yields $k_{cat} \sim nk_{off}$. In other words, the maximal turnover of Cel7A, which can be readily measured in experiments (*c.f.* Fig. 6), is proportional to the off-rate constant. This relationship is probably also valid for Cel6A [68], and it may also apply to some endoglucanases including Cel7B [74,75]. As illustrated in Fig. 6, k_{cat} of the dominant cellulases from *T. reesei* enzymes, scales as Cel7A < Cel6A < Cel7B, and according to the arguments above, this sequence reflects a gradual increase of k_{off} through the series. This interpretation appears reasonable from a structural perspective, as the binding cleft becomes more open with fewer ligand contacts [76–79] through the series (see Fig. 6).

One may argue that rate limitation by decomplexation is counter-intuitive as a processive enzyme could simply proceed forward (with high k_{hyd}) until the cellulose strand was fully degraded. However, both biochemical- and imaging studies suggest that the movement of processive cellulases is hampered or stopped by irregularities on the surface [43,45,83,84]. This leads to a population of complexed, but inactive enzymes that have to dissociate before they can be recruited for further catalysis [43]. One clear hallmark of this is so-called burst-phase kinetics exhibited for example by Cel7A and Cel6A [45,68]. This type of kinetics is well-known for (bulk) hydrolases [85], and it occurs when the second hydrolytic product is released very slowly. In this case, a fast release of the first hydrolytic product precedes steady-state, and hence gives rise to a biphasic progress curve with an initial burst. The very low k_{off} of Cel7A imparts a distinct burst phase as illustrated in Fig. 7. During this transient condition, each enzyme attacks a reducing end on the cellulose surface and performs one processive "run". In the early part of this stage, specific rates may approach the value of k_{hyd}, because the kinetics is unaffected by dissociation. However, as enzymes encounter obstacles (Fig. 4) that limit processive movement, the rate falls towards a slower steady state level as shown in Fig. 7B. Different investigations have indicated that the life time of the trapped state at the obstacle is in the order of minutes [43,74,86,87] and this means that at quasi-steady-state most enzymes will be temporarily captured in an inactive condition [68].

With respect to the question of whether cellulases are particularly slow, it is relevant to compare the k_{hyd} and k_{off} values discussed above with k_{cat} for other enzymes. To this end, we used two meta-analyses of kinetic databases for bulk enzyme reactions. Bar Evans et al. [89] reported an average k_{cat} of about 10 s^{-1} in a survey of thousands of enzymes acting on their native substrate. In a related study, Sousa et al. [90], surveyed data specifically on hydrolytic enzymes, and found an

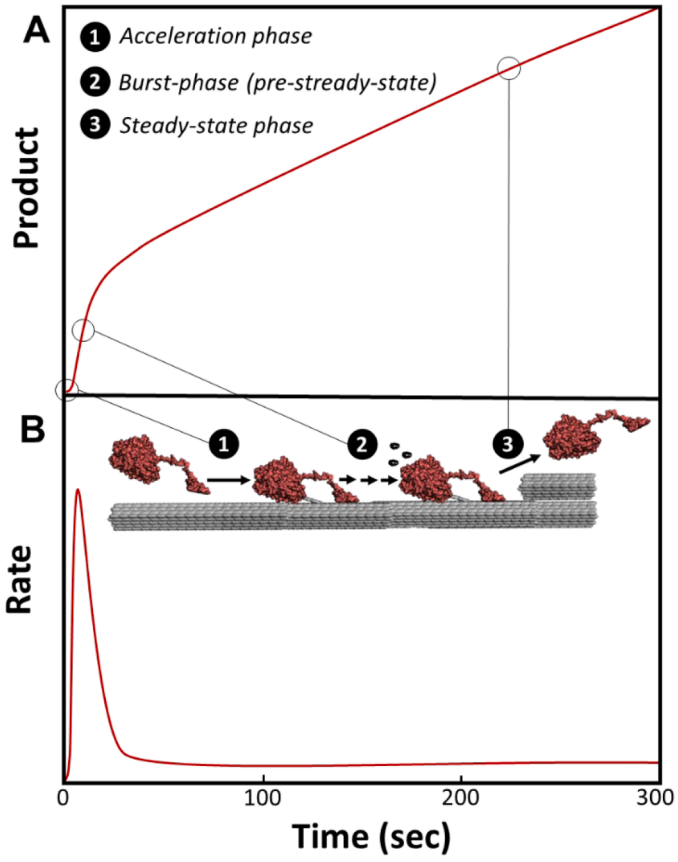

Fig. 7. Transient kinetics of Cel7A attacking Avicel. The enzyme molecules quickly combine with reducing ends (acceleration phase) and make the first hydrolytic run down a cellulose strand (burst phase) at a rate governed by k_{hyd} (c.f. Fig 4). This first run is terminated when the enzyme experiences some obstacle on the surface that prevents further processive movement, and subsequently (quasi steady-state phase) the rate is governed by the much lower k_{off}. Panel A shows product concentration measured in real time as described elsewhere [88], and panel B shows the reaction rate calculated as the slope in Panel A.

[6] If a multi-step reaction scheme has one first-order step, which is much slower than all other, k_{cat} will essentially be equal to the rate constant of this slow step. If, on the other hand, two first-order rate constants are of comparable size, they will both influence k_{cat}. A more exact way of defining k_{cat} is therefore that "no first order rate constant in the forward direction can be lower than k_{cat}", see [51].

overall average k_{cat} of about 4 s^{-1}. Sousa et al. found a similar average when considering only glucoside hydrolases, and further reported that over 80% of k_{cat} values for GHs fell between 0.4 and 60 s^{-1}. These numbers are close to the k_{hyd} values discussed above, and we conclude that cellulases conduct glycoside bond breakage at a rate that is quite typical. However, slow off-rates keep the maximal turnover for cellulases in the low end of the range that is typical for GHs. This is particularly true for the technically important Cel7A, which has strong ligand binding [91] and hence slow off-rates [92]. Other cellobiohydrolases and endoglucanases have weaker ligand binding, which leads to higher off-rates and hence higher maximal rates as exemplified in Fig. 6B

As a final consideration regarding k_{off}-values, it may be relevant to compare cellulases and enzymes acting on soluble substrates. Values tabulated by Fersht [66], indicates that k_{off} for enzymes and soluble ligands including amino acids, nucleotides and small metabolites typically fall in the 10^3–10^4 s^{-1} range. This is dramatically faster than k_{off} for Cel7A discussed above (the difference is some 6 orders of magnitude) and this reinforces the view that the most unusual kinetic characteristic of Cel7A, and probably cellulases in general, is an extraordinarily slow dissociation.

Rate of association

Formation of the Michaelis complex for a cellulase is an intricate process, which includes surface adsorption, recognition of an attack-site and transfer of a sizable piece of cellulose strand from the substrate surface to the enzyme's binding region [41]. For Cel7A, the piece of cellulose strand is at least nine pyranose units long [78] (and Fig. 6), and its removal from the cellulose surface is associated with a substantial unfavorable free energy change. Thus, both computational [93,94] and experimental [21] studies have suggested that abstraction of a ligand with nine pyranose units from a cellulose crystal comes with a free energy penalty of some 100–150 kJ/mol. This penalty is compensated by attractive enzyme-ligand interactions, but the transfer could be associated with a high activation barrier, and hence slow. Indeed, several works have suggested that very slow complexation could be the limiting factor in the overall rate of some cellulolytic reactions [95–98].

Most experimental evidence of on-rates for cellulases comes from adsorption measurements. This approach distinguishes whether the enzyme is in the aqueous bulk or associated with cellulose, but it does not discern adsorbed enzymes with respectively empty or filled binding site. Progress curves for adsorption have been quite divergent with some systems reaching apparent equilibrium within seconds, while others required tens of minutes to equilibrate [39,45,99–103]. More direct measurements of complexation kinetics have used kinetic modeling to quantify on-rate constants. While this has helped to understand the complexation process, different k_{on}-values are hard to compare because they rely on mass- or area units to specify the amount of cellulose [46,47,104]. As discussed above, this problem may be alleviated if the density of attack sites (Γ_{attack}) is known. Once an experimental value of Γ_{attack} has been established, we may use eqs. (1) and (2) to estimate an apparent molar substrate concentration. This idea was used in a study [92], where the complexation of Cel7A and amorphous cellulose was quantified in real-time based on intrinsic protein fluorescence. The on-rate constant was about 1×10^5 $M^{-1}s^{-1}$, and as k_{on} defined in this way takes the molar concentration of attack sites into account, it may be compared to values for bulk enzyme reactions. Typical literature values of k_{on} for enzymes and small soluble ligands fall in the range (1 - 50) x 10^6 $M^{-1}s^{-1}$ [66]; *i.e.* 1–2 orders of magnitude faster than for Cel7A. This shows that the complexation of Cel7A and its solid, polymeric ligand indeed proceeds slower than the binding of a small soluble ligand, but the difference is not large. In particular, the slowness of Cel7A in this respect is much less pronounced than for dissociation (k_{off} for Cel7A was 6 orders of magnitude slower compared to small ligands).

Another interesting aspect of k_{on} converted to apparent molar units is that it allows comparisons of the same enzyme on different substrates, and data for such comparisons have been published for Cel6A [68]. This work reported (mass-based) k_{on} values of 0.29 $(g/L)^{-1}s^{-1}$ for Cel6A attacking a semicrystalline substrate (Avicel) and a faster association (k_{on}= 0.93 $(g/L)^{-1}s^{-1}$) with amorphous cellulose. When these values were converted to molar units (using Γ_{attack} values measured independently) the two rate constants were almost identical, 5 - 7 $\times$ 10^6 $M^{-1}s^{-1}$. This suggests that the intrinsic rate of association is similar on these two substrates, and hence that observed differences primarily reflect variation in the accessibility of attack sites (i.e. the effective substrate concentration).

Balancing association and dissociation

As discussed above, association and particularly dissociation of cellulases are slow, when compared to the chemical steps of hydrolysis, and it follows that these non-covalent processes govern the overall reaction rate. Under substrate saturation (at $^{conv}V_{max}$) all enzyme is in complex, and the rate of association becomes immaterial for the catalytic performance. Hence, the maximal turnover is governed by the off-rate as already argued. However, as association inevitably becomes slower at lowered substrate loads, it becomes rate-limiting at some critical (low) value of S_0. It follows that a balance between the magnitudes of k_{off} and S^*k_{on} determines whether the reaction is limited by association or dissociation at a given substrate load. This phenomenon is well-known from inorganic heterogeneous catalysis, where it is referred to as the Sabatier Principle [105]. This principle stipulates that the best catalytic performance is achieved when the catalyst binds the reactant with intermediate strength. Tighter binding will lead to the accumulation of catalyst-product complexes (dissociation limitation), while weaker binding is associated with a low concentration of complex (association limitation). The optimum binding strength is where these two effects are balanced. The Sabatier principle may be illustrated by so-called volcano curves, where the catalytic rate is plotted against catalyst-substrate binding strength at a fixed substrate load. As illustrated in Fig. 8, the principle may apply to cellulases [106] and other enzymes acting on

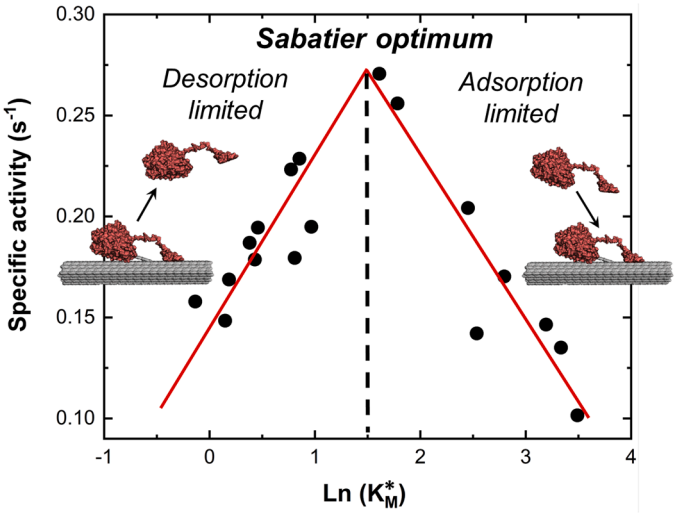

Fig. 8. Volcano plot for 18 cellulases. The graph shows the initial, steady-state rate for the hydrolysis of 10 g/l Avicel at 25 °C, pH 5 plotted as a function of the (conventional) Michaelis constant, K_M^* (c.f. Eq. (4)) for different cellulases. It appears that the reaction is slow for both strong substrate binding (low K_M^*) and weak substrate binding (high K_M^*), but faster at intermediate binding strengths. In the former case, the overall reaction rate is limited by slow desorption, while in the latter, the bottleneck is slow adsorption. This behavior is in line with the Sabatier principle. We emphasize that the location of the apex will change with experimental parameters such as temperature and pH, as well as the type and load of substrate. Data is from [106], and this reference also provides a list of the investigated enzymes.

solid substrates [107,108]. This emphasizes the importance of balancing association- and dissociation rates and the relationship may become useful for both mechanistic studies and enzyme engineering.

Specificity constant

Comparative biochemistry often uses the parameter $\eta = k_{cat}/K_M$ as a measure of the overall catalytic performance. This ratio reflects the slope of the (nearly linear) part of the Michaelis Menten curve for $[S_0] << K_M$, and it is denoted the specificity constant or kinetic efficiency. Apparent specificity constants for cellulases can be calculated whenever a Michaelis Menten analysis has been performed, but they are rarely discussed. This is at least in part because they suffer from the same limitations of mass-based concentration units as k_{on}. In analogy with k_{on}, however, η may be converted to apparent molar units using experimental Γ_{attack}- values and Eq. (2), and this opens for some comparative analysis. For the model cellulase, Cel7A, η has been reported to be about 3×10^5 $M^{-1}s^{-1}$ [75]. Interestingly, this value on insoluble substrate aligns with both the median value for bulk enzyme reactions [89] and η for the action of Cel7A on soluble oligosaccharides of a length that approximately match the size of the binding tunnel [109]. Other examples of apparent specificity constants for cellulases acting on insoluble cellulose include $\eta \sim 1 \times 10^6$ $M^{-1}s^{-1}$ for Cel6A [68], $\eta \sim 1 \times 10^7$ $M^{-1}s^{-1}$ for the endoglucanase Cel7B [75]. These comparisons suggest that molar specificity constants for cellulases acting on insoluble cellulose is similar or higher than for comparable bulk reactions. The discussion above further suggests that this is primarily due to low (molar) K_M values. Fig. 6, for example, showed a K_M for Cel7A about 0.5 μM, and this is much lower than the median value of 130 μM reported for bulk enzymes [89]. We propose that low K_M and the associated high η reflect enzyme adsorption on the substrate surface, which has the consequence that attack sites and enzymes in brought into proximity. This conclusion is supported by the observation of high η−values (in apparent molar units) for other enzymes acting on insoluble substrates [110,111], but further interpretation of specificity constants for interfacial enzyme reactions must await better theoretical descriptions of this type of reaction.

Conclusions

Slow enzymatic hydrolysis of cellulose is a key challenge for the implementation of biorefineries based on lignocellulosic feedstock. Whether the bottleneck for cellulose hydrolysis reactions is related to the substrate, or the enzyme has been heavily debated as substrate pretreatment or enzyme engineering are fundamentally different routes to optimize the saccharification. Here, we addressed this problem by investigating the question of whether cellulases are inherently "slow enzymes".

We found that rate constants for the chemical step of bond cleavage (k_{hyd}) in cellulases are comparable to those of other glycoside hydrolases. This indicates that cellulases are proficient catalysts for the hydrolysis of glycosidic bonds. The low steady-state turnover numbers are primarily due to extraordinarily slow off-rate constants (k_{off}), resulting from the strong binding of cellulases and their insoluble substrate. This tight binding is essential for the enzyme to effectively abstract a cellulose strand from the crystalline lattice, but it inevitably leads to slow dissociation that limits the rate of the overall catalytic process. Therefore, cellulases are not inherently slow biocatalysts, but they are limited by slow dissociation from the substrate. Does this mean that higher rates could be achieved by increasing the dissociation rate of cellulases by protein engineering? The short answer to this is no. Cellulases – both engineered variants and wild types - with relatively high dissociation rates indeed show high k_{cat}, but this comes at a price. Thus, higher dissociation rate is linked to lower substrate affinity. The trade-off between high speed (high k_{cat}) and low affinity (high K_m) is particularly delicate for cellulases as only a fraction of the total substrate is accessible for enzymatic attack (Γ_{attack}). This was exemplified in Fig. 6, which showed that Γ_{attack} and k_{cat} scaled inversely for selected cellulases. Under a given set of experimental conditions, the balance between speed and affinity can be rationalized using the Sabatier principles. A principle taken from inorganic heterogenous catalysis that states that the best catalyst is one that does not have too much or too little affinity for its substrate. Too much affinity gives slow turnover frequency, which originates from a low dissociation rate. Too low affinity is also ineffective as few enzymes are active (low concentration of ES complex). Although qualitative in nature, this principle could provide some guidelines for saccharification processes. It illustrates, for example, that the optimal binding strength (c.f. Fig. 8) depends on the substrate load, and it may also be useful as a tool in enzyme engineering as it indicates whether binding is too weak or too strong for optimal performance. More importantly for the current topic, if indeed the Sabatier principle is valid for cellulolytic enzymes, it helps us understand basic physical boundaries for the highly complex interfacial process, and thus in turn to assess whether cellulases are slow enzymes.

CRediT authorship contribution statement

Peter Westh: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jeppe Kari:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Silke Badino:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Trine Sørensen:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Stefan Christensen:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nanna Røjel:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Corinna Schiano-di-Cola:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Kim Borch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nanna sandager Rojel reports a relationship with Novonesis that includes: employment. Kim Borch reports a relationship with Novonesis that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

References

[1] N.D. Weiss, C. Felby, L.G. Thygesen, Enzymatic hydrolysis is limited by biomass-water interactions at high-solids: improved performance through substrate modifications, Biotechnol. Biofuels 12 (2019) 3.

[2] R. Kumar, C.E. Wyman, Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies, Biotech. Bioeng. 102 (2009) 457–467.

[3] D. Humbird, A. Mohagheghi, N. Dowe, D.J. Schell, Economic impact of total solids loading on enzymatic hydrolysis of dilute acid pretreated corn stover, Biotechnol. Prog. 26 (2010) 1245–1251.
[4] S.J. Horn, G. Vaaje-Kolstad, B. Westereng, V.G.H. Eijsink, Novel enzymes for the degradation of cellulose, Biotech. Biofuel 5 (2012) 45.
[5] C.M. Payne, B.C. Knott, H.B. Mayes, H. Hansson, M.E. Himmel, M. Sandgren, J. Stahlberg, G.T. Beckham, Fungal cellulases, Chem. Rev. 115 (2015) 1308–1448.
[6] Y.H.P. Zhang, L.R. Lynd, Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, Biotech. Bioeng. 88 (2004) 797–824.
[7] L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology, Microbiol. Mol. Biol. Rev. 66 (2002) 506–577.
[8] Y.B. Chaudhari, A. Várnai, M. Sørlie, S.J. Horn, V.G.H. Eijsink, Engineering cellulases for conversion of lignocellulosic biomass, Protein Eng. Des. Sel. 36 (2023).
[9] E.A. Bayer, R. Lamed, M.E. Himmel, The potential of cellulases and cellulosomes for cellulosic waste management, Curr. Opin. Biotech. 18 (2007) 237–245.
[10] V. Juturu, J.C. Wu, Microbial cellulases: engineering, production and applications, Renew. Sust. Energ. Rev. 33 (2014) 188–203.
[11] R. Wolfenden, Degrees of difficulty of water-consuming reactions in the absence of enzymes, Chem. Rev. 106 (2006) 3379–3396.
[12] R. Wolfenden, X.D. Lu, G. Young, Spontaneous hydrolysis of glycosides, J. Am. Chem. Soc. 120 (1998) 6814–6815.
[13] X. Zhang, K.N. Houk, Why enzymes are proficient catalysts: beyond the Pauling paradigm, Accounts Chem. Res. 38 (2005) 379–385.
[14] A. Radzicka, R. Wolfenden, A proficient enzyme, Science 267 (1995) 90–93.
[15] R. Wolfenden, X. Lu, G. Young, Spontaneous hydrolysis of glycosides, J. Am. Chem. Soc. 120 (1998) 6814–6815.
[16] L. Jeske, S. Placzek, I. Schomburg, A. Chang, D. Schomburg, BRENDA in 2019: a European ELIXIR core data resource, Nucleic Acids Res. 47 (2019) D542–D549.
[17] R.N. Goldberg, Y.B. Tewari, Thermodynamics of enzyme-catalyzed reactions .3. Hydrolases, J. Phys. Chem. Ref. Data 23 (1994) 1035–1103.
[18] B.E. Dale, G.T. Tsao, Crystallinity and heats of crystallization of cellulose - a micro-calorimetric investigation, J. App. Pol. Sci. 27 (1982) 1233–1241.
[19] M. Ioelovich, Study of thermodynamic properties of various allomorphs of cellulose, ChemXpress V. 9 (2016) 259–265.
[20] A.V. Blokhin, O.V. Voitkevich, G.J. Kabo, Y.U. Paulechka, M.V. Shishonok, A. G. Kabo, V.V. Simirsky, Thermodynamic properties of plant biomass components. Heat capacity, combustion energy, and gasification equilibria of cellulose, J. Chem. Eng. Data 56 (2011) 3523–3531.
[21] R.N. Goldberg, J. Schliesser, A. Mittal, S.R. Decker, A.F.L.O.M. Santos, V.L. S. Freitas, A. Urbas, B.E. Lang, C. Heiss, M.D.M.C. Ribeiro da Silva, B. F. Woodfield, R. Katahira, W. Wang, D.K. Johnson, A thermodynamic investigation of the cellulose allomorphs: cellulose(am), cellulose Iβ(cr), cellulose II(cr), and cellulose III(cr), J. Chem. Thermodyn. 81 (2015) 184–226.
[22] R.J. Vietor, R.H. Newman, M.A. Ha, D.C. Apperley, M.C. Jarvis, Conformational features of crystal-surface cellulose from higher plants, Plant J. 30 (2002) 721–731.
[23] Y.H. Zhang, L.R. Lynd, Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, Biotechnol. Bioeng. 88 (2004) 797–824.
[24] P. Bansal, M. Hall, M.J. Realff, J.H. Lee, A.S. Bommarius, Modeling cellulase kinetics on lignocellulosic substrates, Biotech. Adv. 27 (2009) 833–848.
[25] B. Yang, D.M. Willies, C.E. Wyman, Changes in the enzymatic hydrolysis rate of avicel cellulose with conversion, Biotech. Bioeng. 94 (2006) 1122–1128.
[26] S. Zhang, D.E. Wolfgang, D.B. Wilson, Substrate heterogeneity causes the nonlinear kinetics of insoluble cellulose hydrolysis, Biotech. Bioeng. 66 (1999) 35–41.
[27] V. Arantes, J.N. Saddler, Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates, Biotech. Biofuels 4 (2011).
[28] V. Novy, K. Aïssa, F. Nielsen, S.K. Straus, P. Ciesielski, C.G. Hunt, J. Saddler, Quantifying cellulose accessibility during enzyme-mediated deconstruction using 2 fluorescence-tagged carbohydrate-binding modules, Proc. Nat. Acad. Sci. 116 (2019) 22545–22551.
[29] M. Popovic, B.F. Woodfield, L.D. Hansen, Thermodynamics of hydrolysis of cellulose to glucose from 0 to 100 °C: cellulosic biofuel applications and climate change implications, J. Chem. Thermodyn. 128 (2019) 244–250.
[30] J.B. Kristensen, C. Felby, H. Jørgensen, Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose, Biotech. Biofuels 2 (2009) 11.
[31] P. Biely, M. Vrsanska, M. Claeyssens, The endo-1,4-beta-glucanase I from Trichoderma reesei. Action on beta-1,4-oligomers and polymers derived from D-glucose and D-xylose, Eur. J. Biochem. 200 (1991) 157–163.
[32] A.V. Gusakov, O.V. Protas, V.M. Chernoglazov, A.P. Sinitsyn, G.V. Kovalysheva, O.V. Shpanchenko, O.V. Ermolova, Transglycosylation activity of cellobiohydrolase I from Trichoderma longibrachiatum on synthetic and natural substrates, Biochim. Biophys. Acta 1073 (1991) 481–485.
[33] A.A. Klyosov, Trends in biochemistry and enzymology of cellulose degradation, Biochemistry 29 (47) (1990) 10577–10585.
[34] M. Andersen, J. Kari, K. Borch, P. Westh, Michaelis-Menten equation for degradation of insoluble substrate, Math. Biosci. 296 (2018) 93–97.
[35] J. Kari, M. Andersen, K. Borch, P. Westh, An Inverse Michaelis Menten approach for general description of interfacial enzyme kinetics, ACS Catal. 7 (2017) 4904–4914.
[36] J. Kari, S.J. Christensen, M. Andersen, S.S. Baiget, K. Borch, P. Westh, A practical approach to steady-state kinetic analysis of cellulases acting on their natural insoluble substrate, Anal. Biochem. 586 (2019) 113411.
[37] N. Cruys-Bagger, S.F. Badino, R. Tokin, M. Gontsarik, S. Fathalinejad, K. Jensen, M.D. Toscano, T.H. Sorensen, K. Borch, H. Tatsumi, P. Valjamae, P. Westh, A pyranose dehydrogenase-based biosensor for kinetic analysis of enzymatic hydrolysis of cellulose by cellulases, Enzyme Microb. Technol. 58-59 (2014) 68–74.
[38] S.P. Chundawat, G.T. Beckham, M.E. Himmel, B.E. Dale, Deconstruction of lignocellulosic biomass to fuels and chemicals, Annu Rev. Chem. Biomol. Eng. 2 (2011) 121–145.
[39] J. Jalak, M. Kurasin, H. Teugjas, P. Valjamae, Endo-exo synergism in cellulose hydrolysis revisited, Jbiol Chem. 287 (2012) 28802–28815.
[40] B.C. Knott, M.F. Crowley, M.E. Himmel, J. Stahlberg, G.T. Beckham, Carbohydrate-protein interactions that drive processive polysaccharide translocation in enzymes revealed from a computational study of cellobiohydrolase processivity, J. Am. Chem. Soc. 136 (2014) 8810–8819.
[41] T. Jeoh, M.J. Cardona, N. Karuna, A.R. Mudinoor, J. Nill, Mechanistic kinetic models of enzymatic cellulose hydrolysis a review, Biotech. Bioeng. 114 (2017) 1369–1385.
[42] B.C. Knott, M.H. Momeni, M.F. Crowley, L.F. Mackenzie, A.W. Gotz, M. Sandgren, S.G. Withers, J. Stahlberg, G.T. Beckham, The mechanism of cellulose hydrolysis by a two-step, retaining cellobiohydrolase elucidated by structural and transition path sampling studies, J. Am. Chem. Soc. 136 (2014) 321–329.
[43] M. Kurasin, P. Valjamae, Processivity of cellobiohydrolases is limited by the substrate, J. Biol. Chem. 286 (2011) 169–177.
[44] L. Murphy, N. Cruys-Bagger, H.D. Damgaard, M.J. Baumann, S.N. Olsen, K. Borch, S.F. Lassen, M. Sweeney, H. Tatsumi, P. Westh, Origin of initial burst in activity for trichoderma reesei endo-glucanases hydrolyzing insoluble cellulose, J. Biol. Chem. 287 (2012) 1252–1260.
[45] N. Cruys-Bagger, J. Elmerdahl, E. Praestgaard, H. Tatsumi, N. Spodsberg, K. Borch, P. Westh, Pre-steady state kinetics for the hydrolysis of insoluble cellulose by Trichoderma reesei Cel7A, J. Biol. Chem. 287 (2012) 18451–18458.
[46] N. Cruys-Bagger, H. Tatsumi, G.R. Ren, K. Borch, P. Westh, Transient kinetics and rate-limiting steps for the processive cellobiohydrolase Cel7A: effects of substrate structure and carbohydrate binding domain, Biochemistry 52 (2013) 8938–8948.
[47] S.A. Maurer, C.N. Bedbrook, C.J. Radke, Competitive sorption kinetics of inhibited endo- and exoglucanases on a model cellulose substrate, Langmuir 28 (2012) 14598–14608.
[48] A. Nakamura, H. Watanabe, T. Ishida, T. Uchihashi, M. Wada, T. Ando, K. Igarashi, M. Samejima, Trade-off between processivity and hydrolytic velocity of cellobiohydrolases at the surface of crystalline cellulose, J. Am. Chem. Soc. 136 (2014) 4584–4592.
[49] A.G. Marangoni, Enzyme Kinetics, a Modern Approach, John Wiley and Sons, Hoboken, New Jersey, 2003.
[50] O.G. Berg, M.K. Jain, Interfacial Enzyme Kinetics, John Wiley & Sons Ltd, West Sussex, 2002.
[51] J.A. Dumesic, D.F. Rudd, L.M. Aparicio, J.E. Rekoske, A.A. Trevino, The Microkinetics of Heterogenous Catalysis, ACS Professional Reference Books, American Chemical Society, Washington, DC, 1993.
[52] J. Nill, T. Jeoh, The role of evolving interfacial substrate properties on heterogeneous cellulose hydrolysis kinetics, ACS Sustainable Chem. Eng. 8 (2020) 6722–6733.
[53] Y. Tian, Y. Wang, X. Liu, K. Herburger, P. Westh, M.S. Møller, B. Svensson, Y. Zhong, A. Blennow, Interfacial enzyme kinetics reveals degradation mechanisms behind resistant starch, Food Hydrocoll. 140 (2023) 108621.
[54] E. Erickson, T.J. Shakespeare, F. Bratti, B.L. Buss, R. Graham, M.A. Hawkins, G. König, W.E. Michener, J. Miscall, K.J. Ramirez, N.A. Rorrer, M. Zahn, A. R. Pickford, J.E. McGeehan, G.T. Beckham, Comparative performance of PETase as a function of reaction conditions, substrate properties, and product accumulation, ChemSusChem. 15 (2022) e202101932.
[55] N. Karuna, T. Jeoh, The productive cellulase binding capacity of cellulosic substrates, Biotechnol. Bioeng. 114 (2017) 533–542.
[56] L.A. Segel, M. Slemrod, The quasi-steady-state assumption: a case study in perturbation, SIAM Rev. 31 (1989) 446–477.
[57] A.R. Tzafriri, Michaelis-Menten kinetics at high enzyme concentrations, Bul. Math. Biol. 65 (2003) 1111–1129.
[58] N. Cruys-Bagger, J. Elmerdahl, E. Praestgaard, K. Borch, P. Westh, A steady-state theory for processive cellulases, FEBS J. 280 (2013) 3952–3961.
[59] R.M.F. Bezerra, A.A. Dias, Discrimination among eight modified michaelis-menten kinetics models of cellulose hydrolysis with a large range of substrate/enzyme ratios: inhibition by cellobiose, Appl. Biochem. Biotechnol. 112 (2004) 173–184.
[60] R.F. Brown, M.T. Holtzapple, A comparison of the Michaelis-Menten and HCH-1 models, Biotech. Bioeng. 36 (1990) 1151–1154.
[61] B. Nidetzky, W. Steiner, M. Hayn, M. Claeyssens, Cellulose hydrolysis by the cellulases from Trichoderma reesei: a new model for synergistic interaction, Biochem. J. 298 (1994) 705–710.
[62] A. Amemura, G.J. Terui, J. Studies on fungal cellulases (II) kinetics of celluloytic action of Penicillium variabile cellulase ferment, Technol 43 (1965) 281.
[63] P. Bansal, M. Hall, M.J. Realff, J.H. Lee, A.S. Bommarius, Modeling cellulase kinetics on lignocellulosic substrates, Biotech. Adv. 27 (2009) 833–848.
[64] L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, Microbial cellulose utilization: fundamentals and biotechnology, Microbiol. Mol. Biol. Rev. 66 (2002) 506–577.

[65] R. Sousa Jr., M.L. Carvalho, R.L.C. Giordano, R.C. Giordano, Recent trends in the modeling of cellulose hydrolysis, Brazil J. Chem. Eng. 28 (2011) 545–564.
[66] A. Fersht, Structure and Mechanism in Protein Science, 2nd ed., W.H. Freeman and Company, New York, 1998.
[67] J. Nill, N. Karuna, T. Jeoh, The impact of kinetic parameters on cellulose hydrolysis rates, Process Biochem. 74 (2018) 108–117.
[68] S.J. Christensen, J. Kari, S.F. Badino, K. Borch, P. Westh, Rate-limiting step and substrate accessibility of cellobiohydrolase Cel6A from Trichoderma reesei, FEBS J. 285 (2018) 4482–4493.
[69] A. Nakamura, T. Tasaki, D. Ishiwata, M. Yamamoto, Y. Okuni, A. Visootsat, M. Maximilien, H. Noji, T. Uchiyama, M. Samejima, K. Igarashi, R. Iino, Single-molecule imaging analysis of binding, processive movement, and dissociation of cellobiohydrolase Trichoderma reesei Cel6A and its domains on crystalline cellulose, J. Biol. Chem. 291 (2016) 22404–22413.
[70] M. Gruno, P. Valjamae, G. Pettersson, G. Johansson, Inhibition of the Trichoderma reesei cellulases by cellobiose is strongly dependent on the nature of the substrate, Biotech. Bioeng. 86 (2004) 503–511.
[71] M. Kurasin, S. Kuusk, P. Kuusk, M. Sørlie, P. Valjamae, Slow off-rates and strong product binding are required for processivity and efficient degradation of recalcitrant chitin by family 18 chitinases, J. Biol. Chem. 290 (2015) 29074–29085.
[72] A.L. Lucius, N.K. Maluf, C.J. Fischer, T.M. Lohman, General methods for analysis of sequential "n-step'' kinetic mechanisms: application to single turnover kinetics of helicase-catalyzed DNA unwinding, Biophys. J. 85 (2003) 2224–2239.
[73] N. Cruys-Bagger, J. Elmerdahl, E. Praestgaard, K. Borch, P. Westh, A steady-state theory for processive cellulases, FEBS J. 280 (2013) 3952–3961.
[74] J. Jalak, P. Valjamae, Mechanism of initial rapid rate retardation in cellobiohydrolase catalyzed cellulose hydrolysis, Biotech. Bioeng. 106 (2010) 871–883.
[75] C. Schiano-di-Cola, N. Rojel, K. Jensen, J. Kari, T.H. Sorensen, K. Borch, P. Westh, Systematic deletions in the cellobiohydrolase (CBH) Cel7A from the fungus Trichoderma reesei reveal flexible loops critical for CBH activity, J. Biol. Chem. 294 (2019) 1807–1815.
[76] M. Wu, W. Nerinckx, K. Piens, T. Ishida, H. Hansson, M. Sandgren, J. Stahlberg, Rational design, synthesis, evaluation and enzyme-substrate structures of improved fluorogenic substrates for family 6 glycoside hydrolases, FEBS J. 280 (2013) 184–198.
[77] M. Wu, L. Bu, T.V. Vuong, D.B. Wilson, M.F. Crowley, M. Sandgren, J. Ståhlberg, G.T. Beckham, H. Hansson, Loop motions important to product expulsion in the Thermobifida fusca glycoside hydrolase family 6 cellobiohydrolase from structural and computational studies, J. Biol. Chem. 288 (2013) 33107–33117.
[78] C. Divne, J. Stahlberg, T.T. Teeri, T.A. Jones, High-resolution crystal structures reveal how a cellulose chain is bound in the 50 angstrom long tunnel of cellobiohydrolase I from Trichoderma reesei, J. Mol. Biol. 275 (1998) 309–325.
[79] G.J. Kleywegt, J.Y. Zou, C. Divne, G.J. Davies, I. Sinning, J. Stahlberg, T. Reinikainen, M. Srisodsuk, T.T. Teeri, T.A. Jones, The crystal structure of the catalytic core domain of endoglucanase I from Trichoderma reesei at 3.6 angstrom resolution, and a comparison with related enzymes, J. Mol. Biol. 272 (1997) 383–397.
[80] S.J. Christensen, K. Krogh, N. Spodsberg, K. Borch, P. Westh, A biochemical comparison of fungal GH6 cellobiohydrolases, Biochem. J. 476 (2019) 2157–2172.
[81] S.F. Badino, J. Kari, S.J. Christensen, K. Borch, P. Westh, Direct kinetic comparison of the two cellobiohydrolases Cel6A and Cel7A from Hypocrea jecorina, Biochim. Biophys. Acta 1865 (2017) 1739–1745.
[82] C.J. Bailey, enzyme-kinetics of cellulose hydrolysis, Biochem. J. 262 (1989) 1001. -1001.
[83] K. Igarashi, T. Uchihashi, A. Koivula, M. Wada, S. Kimura, T. Okamoto, M. Penttila, T. Ando, M. Samejima, Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface, Science (1979) 333 (2011) 1279–1282.
[84] T. Eriksson, J. Karlsson, F. Tjerneld, A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of Trichoderma reesei, Appl. Biochem. Biotech. 101 (2002) 41–60.
[85] K. Brocklehurst, M. Resmini, C.M. Topham, Kinetic and titration methods for determination of active site contents of enzyme and catalytic antibody preparations, Methods 24 (2001) 153–167.
[86] N. Cruys-Bagger, K. Alasepp, M. Andersen, J. Ottesen, K. Borch, P. Westh, Rate of threading a cellulose chain into the binding tunnel of a cellulase, J. Phys. Chem. B 120 (2016) 5591–5600.
[87] J. Jung, A. Sethi, T. Gaiotto, J.J. Han, T. Jeoh, S. Gnanakaran, P.M. Goodwin, Binding and movement of individual Cel7A cellobiohydrolases on crystalline cellulose surfaces revealed by single-molecule fluorescence imaging, J. Biol. Chem. 288 (2013) 24164–24172.
[88] N. Cruys-Bagger, G.L. Ren, H. Tatsumi, M.J. Baumann, N. Spodsberg, H. D. Andersen, L. Gorton, K. Borch, P. Westh, An amperometric enzyme biosensor for real-time measurements of cellobiohydrolase activity on insoluble cellulose, Biotech Bioeng 109 (2012) 3199–3204.
[89] A. Bar-Even, E. Noor, Y. Savir, W. Liebermeister, D. Davidi, D.S. Tawfik, R. Milo, The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters, Biochemistry 50 (2011) 4402–4410.
[90] S.F. Sousa, M.J. Ramos, C. Lim, P.A. Fernandes, Relationship between enzyme/substrate properties and enzyme efficiency in hydrolases, ACS Catal. 5 (2015) 5877–5887.
[91] J. Jalak, P. Valjamae, Multi-Mode Binding of Cellobiohydrolase Cel7A from Trichoderma reesei to Cellulose, PLoS One 9 (2014) e108181.
[92] N. Røjel, J. Kari, T.H. Sørensen, S.F. Badino, J.P. Morth, K. Schaller, A. M. Cavaleiro, K. Borch, P. Westh, Substrate binding in the processive cellulase Cel7A: transition state of complexation and roles of conserved tryptophan residues, J. Biol. Chem. 295 (2020) 1454–1463.
[93] G.T. Beckham, J.F. Matthews, B. Peters, Y.J. Bomble, M.E. Himmel, M.F. Crowley, Molecular-level origins of biomass recalcitrance: decrystallization free energies for four common cellulose polymorphs, J. Phys. Chem. B 115 (2011) 4118–4127.
[94] M. Bergenstrahle, E. Thormann, N. Nordgren, L.A. Berglund, Force pulling of single cellulose chains at the crystalline cellulose-liquid interface: a molecular dynamics study, Langmuir 25 (2009) 4635–4642.
[95] D.B. Wilson, Cellulases and biofuels, Curr. Opin. Biotechnol. 20 (2009) 295–299.
[96] B.Z. Shang, R. Chang, J.W. Chu, Systems-level modeling with molecular resolution elucidates the rate-limiting mechanisms of cellulose decomposition by cellobiohydrolases, J. Biol. Chem. 288 (2013) 29081–29089.
[97] S.A. Maurer, C.N. Bedbrook, C.J. Radke, Cellulase adsorption and reactivity on a cellulose surface from flow ellipsometry, Ind. Eng. Chem. Res. 51 (2012) 11389–11400.
[98] J.M. Fox, S.E. Levine, D.S. Clark, H.W. Blanch, Initial- and processive-cut products reveal cellobiohydrolase rate limitations and the role of companion enzymes, Biochemistry 51 (2012) 442–452.
[99] H. Liu, J.Y. Zhu, X.S. Chai, In situ, rapid, and temporally resolved measurements of cellulase adsorption onto lignocellulosic substrates by UV-vis spectrophotometry, Langmuir 27 (2011) 272–278.
[100] P. Zhu, J.M. Moran-Mirabal, J.S. Luterbacher, L.P. Walker, H.G. Craighead, Observing Thermobifida fusca cellulase binding to pretreated wood particles using time-lapse confocal laser scanning microscopy, Cellulose 18 (2011) 749–758.
[101] J.N. Moran-Mirabal, N. Santhanam, S.C. Corgie, H.G. Craighead, L.P. Walker, Immobilization of cellulose fibrils on solid substrates for cellulase-binding studies through quantitative fluorescence microscopy, Biotech. Bioeng. 101 (2008) 1129–1141.
[102] W. Steiner, W. Sattler, H. Esterbauer, adsorption of Trichoderma-reesei cellulase on cellulose - experimental-data and their analysis by different equations, Biotech. Bioeng. 32 (1988) 853–865.
[103] B. Nidetzky, W. Steiner, M. Claeyssens, Cellulose hydrolysis by the cellulases from trichoderma-reesei - adsorptions of 2 cellobiohydrolases, 2 endocellulases and their core proteins on filter-paper and their relation to hydrolysis, Biochem. J. 303 (1994) 817–823.
[104] Y. Shibafuji, A. Nakamura, T. Uchihashi, N. Sugimoto, S. Fukuda, H. Watanabe, M. Samejima, T. Ando, H. Noji, A. Koivula, K. Igarashi, R. Iino, Single-molecule imaging analysis of elementary reaction steps of Trichoderma reesei Cellobiohydrolase I (Cel7A) hydrolyzing crystalline cellulose I (alpha) and III (I), J. Biol. Chem. 289 (2014) 14056–14065.
[105] J.K. Norskov, T. Bligaard, J. Rossmeisl, C.H. Christensen, Towards the computational design of solid catalysts, Nat. Chem. 1 (2009) 37–46.
[106] J. Kari, J.P. Olsen, K. Jensen, S.F. Badino, K.B.R.M. Krogh, K. Borch, P. Westh, Sabatier principle for interfacial (heterogeneous) enzyme catalysis, ACS Catal. 8 (2018) 11966–11972.
[107] J.A. Bååth, K. Jensen, K. Borch, P. Westh, J. Kari, Sabatier principle for rationalizing enzymatic hydrolysis of a synthetic polyester, JACS Au 2 (2022) 1223–1231.
[108] Y. Tian, Y. Wang, Y. Zhong, M.S. Møller, P. Westh, B. Svensson, A. Blennow, Interfacial catalysis during amylolytic degradation of starch granules: current understanding and kinetic approaches, Molecules 28 (2023) 3799.
[109] B. Nidetzky, W. Zachariae, G. Gercken, M. Hayn, W. Steiner, hydrolysis of cellooligosaccharides by trichoderma-reesei cellobiohydrolases - experimental-data and kinetic modeling, Enz. Microb. Technol. 16 (1994) 43–52.
[110] J.A. Bååth, K. Borch, K. Jensen, J. Brask, P. Westh, Comparative biochemistry of four polyester (PET) hydrolases, Chembiochem 22 (2021) 1627–1637.
[111] Y. Wang, Y. Tian, Y. Zhong, M.A. Suleiman, G. Feller, P. Westh, A. Blennow, M. S. Møller, B. Svensson, Improved hydrolysis of granular starches by a psychrophilic α-amylase starch binding domain-fusion, J. Agric. Food Chem. 71 (2023) 9040–9050.