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A generic model for glucose production from various cellulose sources by a commercial cellulase complex

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Abstract
The kinetics of cellulose hydrolysis by commercially available Cellubrix were described mathematically, with Avicel and wheat straw as substrates. It was demonstrated that hydrolysis could be described by three reactions: direct glucose formation and indirect glucose formation via cellobiose. Hydrolysis did not involve any soluble oligomers apart from low amounts of cellobiose. Phenomena included in the mathematical model were substrate limitation, adsorption of enzyme onto substrate, glucose inhibition, temperature dependency of reaction rates, and thermal enzyme inactivation. In addition, substrate heterogeneity was described by a recalcitrance constant. Model parameters refer to both enzyme characteristics and substrate-specific characteristics.

Quantitative model development was carried out on the basis of Avicel hydrolysis. In order to describe wheat straw hydrolysis, wheat straw specific parameter values were measured. Updating the pertinent parameters for wheat straw yielded a satisfactory description of wheat straw hydrolysis, thus underlining the generic potential of the model.

Keywords: Cellulose, glucose, model

Introduction
Oil is the major energy resource to the world, but its reserves are limited. Its usage has various negative impacts, such as pollution and an annual net production of CO₂, the number one contributor to the greenhouse effect. With this in mind, several nations, The Netherlands among them, have committed themselves to a decrease in the annual production of CO₂, and to partly substitute oil-based transport fuels by renewable biofuels, of which ethanol is an important example.

Ethanol can be produced through fermentation of a sugar source, such as sugar cane or glucose syrup, from starch. For the introduction of fuel ethanol to be successful, however, the price of ethanol should be competitive with the price of petrol. Ethanol from traditional sugar sources is already more expensive than petrol because of the relatively high sugar cost; if demand for ethanol rises, a dramatic increase in sugar price would be expected. Over recent years, therefore, research has focused on (ligno)cellulosic waste materials, such as grass or wheat straw. These materials can act as an abundant and cheap source of fermentable sugars (Wyman 1994).

Generally, production schemes for bio-ethanol from, for instance, wheat straw, consist of pretreatment, enzymatic hydrolysis, fermentation, and ethanol recovery (Sheehan & Himmel 1999). Pretreatment (either thermal, chemical, or mechanical) opens up the cellulose fibers and makes them more susceptible to subsequent hydrolysis, which can either be chemical (Jacobsen & Wyman 2000) or enzymatic; the latter is generally considered to be the favorable option (Wyman 1994). The sugars that are liberated by hydrolysis are then fermented to ethanol. Hydrolysis and fermentation may be operated in series (Separate Hydrolysis and Fermentation, SHF) or in parallel (Simultaneous Saccharification and Fermentation, SSF).
SSF (Wingren et al. 2003)). Diluted ethanol is finally concentrated by distillation or pervaporation (O’Brien et al. 2000).

Within this process, the enzymatic hydrolysis of the pretreated cellulose accounts for a large portion of the total costs. Previous research (Wooley et al. 1999) has indicated that this step needs optimization and cost reduction. Up to now, research on enzymatic cellulose hydrolysis has been carried out with various pretreatments and substrates (Gregg et al. 1998; Ortega et al. 2000). For these conditions and substrates, models have been developed to describe the hydrolysis result. Some of these are more mechanistic, linking to relevant process parameters, such as enzyme dosage (Philippidis & Wyman 1992); others feature more empirical equations (Markovic et al. 2001).

However, none of these models incorporate specific features of the substrate itself; as a consequence, these models are highly specific and not portable towards other substrates. Yet, it has been shown (Desai & Converse 1997) that substrate characteristics have a profound impact on the production rate of glucose from cellulose. Attempts to capture these characteristics in a modeling approach (South et al. 1995) have not specifically shown change in substrate reactivity while excluding other potential effects, such as possible thermal inactivation, and have limited themselves to fitting generalized type equations without specifically measuring changes in substrate reactivity.

The present paper presents a mechanistic model for cellulose degradation by a commercially available cellulase. The model relates specific substrate characteristics on the one hand, and the glucose production rate on the other hand. In addition, relevant phenomena, such as product inhibition, temperature dependency, and the effect of enzyme dose, are included. In this way, a more generic model for the enzymatic conversion of cellulose to glucose is obtained. It should be a basis for the rational economical evaluation and optimization of bio-ethanol production.

Materials and methods

**Enzyme and activity assay**

Cellubrix (Novozymes Corp., Denmark) was used as cellulase preparation. Its cellulolytic activity was determined as reducing sugars with the standard filter-paper assay (Ghose 1987), and was found to be 56 filter paper units (FPU) per mL of solution.

The β-glucosidase activity in 1.0 mL of enzyme preparation was determined by the addition of 1.0 mL of a 15-mM solution of cellobiose in a 0.05-M citrate buffer (pH 4.8), and incubation for 30 min at 50°C. The enzyme was then inactivated (5 min at 100°C) after which glucose was measured. The β-glucosidase activity was found to be 204 U mL⁻¹ of Cellubrix (1 U giving formation of 1 μmol glucose per minute, so a maximum turnover rate of 0.0108 g glucose U⁻¹ h⁻¹).

The concentration of protein (Bradford 1976) per mL of Cellubrix was found to be 32.3 mg mL⁻¹.

For several experiments, Novozymes 188, a commercially available β-glucosidase (Novozymes Corp.) was used. Cellulase activity was determined to be 0.14 U mL⁻¹ and a β-glucosidase activity of 2033 U mL⁻¹ was found. Protein concentration (Bradford 1976) was found to be 85.2 mg mL⁻¹.

For determination of cellulase activity, Celluclast (Novozymes Corp.) was used. Celluclast is known to have a cellulase activity of 61 FPU mL⁻¹ (Nieves et al. 1998).

All experiments were conducted at pH 4.8 (citrate buffer). Most cellulase preparations have a pH optimum of approximately 4.8 (Srinivas & Panda 1998); in addition, this is also a very acceptable pH for ethanol fermentation by yeast (Oliero et al. 1982). Therefore, no attempts were made to optimize the pH.

Glucose was measured with a glucose-specific glucose oxidase/peroxidase enzyme kit (GOPOD; art. nr. 1448676, Roche Diagnostics Nederland BV).

**Substrates**

Measurements were performed both with model celluloses (Avicel and Whatman, No. 1001090 filter paper) and with pretreated wheat straw, representing an industrially usable type of biomass. Avicel was supplied by Merck (order nr. 1.02330.0500).

Wheat straw was purchased locally in The Netherlands (province of Groningen). For pretreatment, it was first soaked at room temperature for 16 h in 4 M KOH in a kettle with impeller. Subsequently, the supernatant was removed by centrifugation (10 min at 9000) and the pellet was again soaked for 16 h in 4 M KOH. After removal of the supernatant by centrifugation (9000 rpm for 10 min), the pellet was washed with distilled water over a 250-μm sieve until pH 7 was reached; the pellet mass was then dried at 50°C for 12 h. The composition of the pretreated wheat straw was found to be (% w/w): rhamnose 0.7, arabinose 2.1, xylose 6.8, mannose 0.4, galactose 0.8, glucose 55.2, and non-sugar components 34.0.
**Initial glucose production rates**

During incubation of cellulose with enzyme in buffer, initial glucose production rates were determined by measuring glucose concentrations every 15 min over 1 h. Initial glucose production rates were calculated as the slope of a linear fit to these data points.

**Temperature dependence**

To determine the temperature dependence of the initial Avicel hydrolysis rate, initial glucose production rates (see above) were determined at 25, 30, 35, 40, and 50°C. At each temperature, 12 g L⁻¹ Avicel was incubated in buffer with 0.59 mL Cellubrix (equaling 17.6 FPU g⁻¹ cellulose).

To determine the temperature dependence of enzyme inactivation, a series of enzyme solutions (4.0 mL Cellubrix in 100 mL of citrate buffer) were incubated for various periods of time at several incubation temperatures, Avicel was added (12 g L⁻¹), so enzyme loading would be 18.7 FPU g⁻¹, and the residual enzyme activity was measured at 32°C.

**Validation experiments on wheat straw**

To determine the dependence of the initial wheat straw hydrolysis rates on enzyme dosage, initial glucose production rates (see above) were measured at 40°C with 0.14 g of pretreated wheat straw and various amounts of enzyme in a total working volume of 6 mL (buffer + enzyme solution).

The recalcitrance constant for wheat straw was measured with 0.5 g pretreated wheat straw and 170 μL Cellubrix (equivalent to 19 FPU g⁻¹ wheat straw, or 38 FPU g⁻¹ cellulose) in 10 mL of citrate buffer. After incubation for 30 min at 40°C in a vertical test-tube rotator, solids were removed by centrifugation (10 min, 10 000 rpm) and glucose was determined in the supernatant. The pellet was washed twice with deionized water, and re-incubated with fresh buffer and a constant amount of fresh enzyme to determine restart rates on partially converted substrate. Differences in restart rates, thus, depended on the fractional substrate conversion, and were not affected by effects, such as loss of catalytic activity by sugar inhibition or enzyme inactivation.

**Numerical procedure**

For modeling purposes and solving the set of differential equations, a first-order Euler forward algorithm was used.

**Results and discussion**

**Outline reaction mechanism**

Over time, various mechanisms have been proposed for cellulose hydrolysis by cellulose degrading enzymes, including Michaelis–Menten-like kinetics (Beltrame et al. 1984; Gonzalez et al. 1989), models that consider enzyme adsorption onto cellulose (Scheiding et al. 1984; Philippidis et al. 1992b; Nidetzky et al. 1993), and semi-empirical models (Aguado et al. 1993). These models, however, sometimes do not consider effects occurring at increased conversions and/or prolonged reaction times (e.g. product inhibition and enzyme inactivation), and in any case, do not incorporate the specifics of a chosen substrate into their model. Generally, three types of enzymes are considered, i.e. (1) endo-glucanase or β-1,4-glucan glucanohydrolase, (2) exo-glucanase or β-1,4-glucan cellobiohydrolase, and (3) β-glucosidase, or cellobiase.

The first two enzymes each consist of a variety of sub-types which together work on the degradation of cellulose, producing both monomeric glucose and dimeric celllobiose (Beldman 1986).

Their combined action can roughly be described as follows: endo-glucanase acts on the cellulose chains, and cleaves cellulose to shorter-length polysaccharide chains. Exo-glucanase can split celllobiose and glucose from these chains. However, concentrations of these intermediate, insoluble macromolecules cannot be determined experimentally. Besides that, the mix of endo-glucanases and exo-glucanases is very complex and highly variable between enzyme preparations, and it is, therefore, very difficult to specifically determine and account for all types of endo-glucanase and exo-glucanase activity. In the present paper, therefore, the reaction scheme is simplified to take into account the direct formation of glucose and celllobiose from cellulose through the combined action of both endo-glucanase and exo-glucanase. This combined activity of both endo-glucanases and exo-glucanases will be referred to as cellulase activity.

The third enzyme is a single enzyme that acts upon a homogeneous and soluble substrate and which can be adequately described using Michaelis–Menten kinetics. The specific kinetics of the Novozymes β-glucosidase (the commercial preparation Novozymes 188, which is also used in preparing Cellubrix) have been investigated (Dekker 1986). Present results will also show that β-glucosidase in Cellubrix is in excess compared to endo-glucanase and exo-glucanase activity. As a result, celllobiose is formed in negligibly small concentrations, even at high glucose concentrations that are inhibitory to β-glucosidase in particular (Von Lieb &
Luca 1999). Consequently, the kinetic characteristics of β-glucosidase were not investigated separately in the present paper.

In summary, the following simplified reaction mechanism is assumed:

\[
\begin{align*}
cellulose & \xrightarrow{\nu_1} \text{celllobiose} \\
\text{celllobiose} & \xrightarrow{\nu_2} \text{glucose} \\
\text{cellulose} & \xrightarrow{\nu_3} \text{glucose}
\end{align*}
\]

(1)
in which \(\nu_1\), \(\nu_2\), and \(\nu_3\) are reaction rates by volume and depend on enzyme concentration.

**Cellulase activity**

Glucose and celllobiose production during Avicel hydrolysis by Cellubrix were determined. Glucose and total reducing sugars in solution (i.e. a degree of polymerization of 6 and below) were measured before and after treatment of samples with an excess of Novozymes 188 by which any celllobiose was converted to glucose. The celllobiose concentration was calculated from the increase in glucose concentration upon Novozymes 188 treatment. Results are presented in Figure 1.

Figure 1 shows that the total amount of reducing sugars, expressed in glucose equivalents, equals the sum of reducing glucose equivalents from celllobiose and glucose. The small amount of sugars at \(t=0\) represents the stabilizing sugars in the enzyme preparation itself. All reducing sugars in solution, therefore, were either monomers or dimers. It is concluded that Cellubrix forms celllobiose and glucose from Avicel, without appreciable amounts of soluble higher oligomers (trimers, tetramers and pentamers). This is in agreement with earlier data (Philippidis et al. 1992a). A reaction mechanism that accounts for celllobiose as the only intermediate (Equation 1) therefore seems a realistic representation of Avicel hydrolysis by Cellubrix. This is in contrast to enzymatic starch hydrolysis, in which a variety of oligomers is found (Marchal 1999; Paolucci-Jeanjean et al. 2000).

Second, the graph shows only a small initial increase in the concentration of celllobiose, which after a few hours drops to almost zero. Apparently, the capacity for celllobiose hydrolysis exceeds the formation rate of celllobiose, even at glucose concentrations that are strongly inhibitory to the Novozymes β-glucosidase (Dekker 1986).

The ratio between direct glucose formation from cellulose (production rate \(\nu_3\); Equation 1) and indirect glucose formation through celllobiose (production rate \(\nu_1\); Equation 1) was experimentally established with Celluclast. This is a Novozymes preparation with an endo-glucanase and exo-glucanase activities (i.e. “cellulase”), but with negligible β-glucosidase activity (Nieves et al. 1998). In effect, Cellubrix is formed from Celluclast by celllobiase addition. Initial rate measurements of glucose and celllobiose production by Celluclast hydrolysis were determined using linear regression; results are presented in Figure 2.

Figure 2 shows that the Celluclast preparation can directly form glucose from cellulose, or produce...
cellobiose, which agrees with the reaction mechanism proposed. The ratio between cellobiose and glucose production rates was found to be \( v_1 : v_3 = 0.166/0.122 = 1.33 \). Since Celluclast is a Cellubrix constituent, it is assumed that this ratio will hold for Cellubrix as well.

**Cellulase kinetics**

As has been previously mentioned, several types of kinetic model have been proposed in the literature. If cellulose hydrolysis obeyed Michaelis–Menten kinetics, the initial enzymatic turnover rate should linearly increase with the amount of enzyme used. Such a linear dependency would not be expected, however, if hydrolytic activity was determined by adsorption of the enzyme onto the substrate. Therefore, the impact of increasing amounts of enzyme or of substrate on the glucose production rate was determined. Figure 3 shows the glucose production rate to be first order in Avicel concentration.

The turnover rates by the cellulase (\( v_1 \) and \( v_3 \) in Equation 1) thus obey:

\[
v_i = k_i C_C
\]

with \( k_i \) the reaction rate constant \((i = 1 \text{ or } 3)\), which depends on enzyme dosage, and \( C_C \) the cellulose concentration (i.e. Avicel).

Quantification of \( k_i \) and determination of the effect of the enzyme dose on the glucose production rate was carried out at four enzyme concentrations. Figure 4 shows the glucose production rates (linear regression with \( R^2 \geq 0.92 \)) per gram of cellulose (i.e. the reaction rate constant \( k_i = v_i/C_C \)). Results suggest a Langmuir-like dependence between glucose production rate and enzyme dosage, which is analogous to the type of dependence found by others (Philippidis et al. 1993) and can be described by:

\[
k_i = \frac{k_{\text{max},i} \cdot C_E}{K_L + C_E}
\]

in which \( k_i \) (\( i = 1 \text{ or } 3 \) for the relevant reaction) is the rate constant, \( k_{\text{max},i} \) its maximum value at full saturation of the substrate with enzyme, \( C_E \) the enzyme concentration, and \( K_L \) the Langmuir adsorption constant for Cellubrix on Avicel.

Plotting the reciprocal glucose production rate per gram of Avicel versus the reciprocal enzyme dosage gives a linear relation with \( 1/k_{\text{max}} \) as intercept and \( K_L/k_{\text{max}} \) as slope. From this linear fit \((R^2 = 0.998)\), \( k_{\text{max}} = 0.140 \) h\(^{-1}\) and \( K_L = 18.2 \) FPU L\(^{-1}\) were obtained. Figure 4 shows the model fit based on these values.

The value \( k_{\text{max}} = 0.140 \) h\(^{-1}\), however, is the total reaction constant for the production rate of glucose by the enzyme preparation, and, therefore, involves all three reactions previously mentioned. Since it has been shown that the production rate of glucose through the cellobiose pathway is limited by the cellobiose production rate by the cellulase, \( k_{\text{max}} \) can be seen as an overall reaction rate constant with \( k_{\text{max}} = k_{\text{max},1} + k_{\text{max},3} \). Since \( v_1 : v_3 = 4:3 \), for each 3 g of glucose formed through pathway 3, 4 g of cellobiose are formed through pathway 1, which can be turned into \( 4 \cdot 380/36 = 4.21 \) g of glucose.
through pathway 2. From $V_1://V_3 = k_{max,1}:k_{max,3}$ (Equation 3), $k_{max,1}$ and $k_{max,3}$ were calculated as:

$$k_{max,1} = 0.140 \cdot 4.21/(4.21 + 3) = 0.081 \text{ h}^{-1}$$

$$k_{max,3} = 0.140 \cdot 3/(3 + 4.21) = 0.058 \text{ h}^{-1}$$

These rate constants can, in combination with Equations (2) and (3), therefore describe the initial reaction rate of the endo-glucanase and exo-glucanase components of the enzyme preparation, i.e., the cellulase activity.

**Glucose inhibition**

It has been shown that cellulase is inhibited by its products glucose and cellobiose, although a large variety in inhibition constants is reported (Beltrame et al. 1984; Philippidis & Hatzis 1997). Since the present Avicel preparation, due to its high β-glucosidase content, hardly accumulates any cellobiose (cf. Figure 1), inhibition of cellulase activity by cellobiose was disregarded in the present research. Likewise, no attempt was made to quantify inhibition of β-glucosidase activity by glucose; instead, a literature value was used (Dekker 1986). Research focused on glucose inhibition of reactions 1 and 3 (Equation 1), while both reactions were assumed to be inhibited equally. To quantify glucose inhibition, initial production rates of glucose were measured at various starting concentrations of glucose (Figure 5). Values for $R^2$ for individual rate measurements were 0.97 and above.

Regression with a general type of inhibition equation (with $C_G$ the glucose concentration):

$$v(C_G) = v(0) \frac{K_i}{K_i + C_G}$$

yielded an inhibition constant $K_i = 6.3 \text{ g L}^{-1}$. The model fit based on Equation 5 and $K_i = 6.3 \text{ g L}^{-1}$ is given in Figure 5.

**Effect of temperature on initial turnover rate**

A generally accepted model for the increase of enzymatic turnover rate at increasing temperatures is the Arrhenius equation (Cornish-Bowden 2001):

$$v = A \cdot e^{-E_a/RT}$$

For determination of the activation energy, initial glucose production rates were determined at various temperatures (Figure 6). In all cases, values for $R^2$ exceeded 0.99.

From the data in Figure 6, $E_a = 29.8 \text{ kJ mol}^{-1}$ and $A = 99.0 \cdot 10^3 \text{ g L}^{-1} \text{ h}^{-1}$ were obtained.

**Effect of temperature on enzyme inactivation**

When trying to understand and model the degradation of cellulose through cellulase action for extended periods of time, one needs to take into account the possible effects of thermal inactivation on the enzyme complex, especially given the relative ease with which cellulases are thermally inactivated (Demerdash & Attia 1992).

Thermal inactivation of enzymes is generally a first-order type of process according to:

$$\frac{dC_E}{dt} = -k_D \cdot C_E$$

in which $C_E$ equals the amount of cellulase activity present (FPU L$^{-1}$) and $k_D$ is the thermal
deactivation constant, whose dependence on temperature can also be described by an Arrhenius-type relationship (Cornish-Bowden 2001):

\[ k_D = A_D \cdot e^{-\Delta H / RT} \]  

The deactivation constant \( k_D \) was determined at various temperatures. Figure 7 shows a linear relation between \( \ln(k_d) \) and \( 1/T \) with \( \Delta H = 148 \text{ kJ mol}^{-1} \) and \( A_D = 3.64 \times 10^{18} \text{ h}^{-1} \).

**Effect of substrate conversion on turnover rate: substrate recalcitrance**

Previously, it has been shown that the glucose production rate falls off sharply as the hydrolysis proceeds, which can only partially be attributed to product inhibition or enzyme inactivation. Experiments, however, in which both phenomena were excluded (Desai & Converse 1997) by re-incubation of partially hydrolyzed cellulose with fresh buffer and fresh enzyme, still show a decrease of glucose production rate as function of fractional conversion. In this paper, this phenomenon will be referred to as **substrate recalcitrance**, the decrease in susceptibility of the substrate towards the enzyme due to the phenomenon that easily hydrolysable cellulose is digested first, and increasingly difficult, more recalcitrant cellulose remains. Two explanations are given for this recalcitrance. Some researchers report that it is caused by a lower specific turnover rate by the fresh enzyme, still show a decrease of glucose production (glucose production rate after re-incubation with fresh buffer and enzyme) at increasing conversion rate (and constant enzyme: substrate ratio) could be attributed to a combination of both mechanisms.

To account for substrate recalcitrance, the following equation was used:

\[ v = v(0) \cdot e^{-K_{rec}(1 - C_G(t)/C_G(0))} \]  

In this equation, \( v \) is the cellulase activity (so the equation relates to both \( v_1 \) and \( v_3 \) since both describe enzymatic action on the heterogeneous polymeric material), \( v(0) \) is the initial enzymatic turnover rate on native Avicel, which can be calculated from Equations 2 and 3. \( K_{rec} \) is the recalcitrance constant, a measure for the decrease in turnover rate at increasing conversion fractions. One can also look upon this constant as a measure for the heterogeneity of the substrate, since a relatively large \( K_{rec} \) for a given substrate means that it consists of parts which are very easily hydrolysable, and parts which are very resistant to cellulase preparations. In turn, a \( K_{rec} \) of zero would mean that every fraction of the substrate would be turned into glucose at the same rate, so it would effectively be a very homogenous substrate. The ratio \( 1 - [C_G(t)/C_G(0)] \) is the fraction of cellulose that has already been converted to glucose.

**Overall model**

Previously described experiments and mathematical relations (Equations 2–6, 8 and 9) can, combined with the kinetics of Novozymes 188 which has already been the subject of a previous study (Dekker 1986), be put together in a mathematical model describing the breakdown of cellulose by Cellubrix. The three turnover rates in Equation 1 can now be calculated with:

\[ v_1(t, T) = \frac{k_{max,1} \cdot C_E \cdot e^{-E_i/(RT)}}{K_L + C_E \cdot e^{-E_i/(R \cdot 313)}} \cdot \frac{e^{-K_0(T \cdot 313) \cdot C_G(t)}}{K_i + C_G(T)} \cdot \frac{K_i}{K_i + C_G(T)} \cdot \frac{K_i}{K_i + C_G(T)} \cdot \frac{1}{1 + C_G(t)/K_{i,2} + C_b} \]  

\[ v_2(t, T) = k_{max,2} \cdot e^{C_{total}} \cdot e^{-E_i/(R \cdot 313)} \cdot \frac{e^{-K_0(T \cdot 313) \cdot C_G(t)}}{K_i + C_G(T)} \cdot \frac{K_i}{K_i + C_G(T)} \cdot \frac{1}{1 + C_G(t)/K_{i,2} + C_b} \]  

\[ v_3(t, T) = \frac{k_{max,3} \cdot C_E \cdot e^{-E_i/(RT)}}{K_L + C_E \cdot e^{-E_i/(R \cdot 313)}} \cdot \frac{e^{-K_0(T \cdot 313) \cdot C_G(t)}}{K_i + C_G(T)} \cdot \frac{K_i}{K_i + C_G(T)} \cdot \frac{1}{1 + C_G(t)/K_{i,2} + C_b} \]
Assuming an ideally mixed batch reactor, the change in glucose, cellobiose and cellulose concentrations can thus be described with:

\[
\frac{dC_G}{dt} = -(v_1(t) + v_2(t)) \\
\frac{dC_{Cb}}{dt} = 1.053 \cdot v_1(t) - v_2(t) \\
\frac{dC_G}{dt} = 1.111 \cdot v_2(t) + 1.111 \cdot v_3(t)
\]

In these equations, the factors 1.053 and 1.111 account for hydrolysis water. The value used for \( k_{\text{max},2} \) was 0.0108 g U\(^{-1}\) h\(^{-1}\) (see Materials and methods).

For the Michaelis constant \( K_m \) and the inhibition constant \( K_{i,2} \) of glucose for \( \beta \)-glucosidase, \( K_m = 1.92 \) g L\(^{-1}\) and \( K_{i,2} = 0.54 \) g L\(^{-1}\) were used (Dekker 1986). As has been shown previously, the kinetics of the \( \beta \)-glucosidase are relatively unimportant, since the capacity for cellobiose production by Cellubrix is far smaller than the capacity of glucose production from cellobiose.

The model above was used to describe the progress of cellulose hydrolysis in time, with \( K_{\text{rec}} \) as a fit parameter. Two experiments were conducted using 40 g L\(^{-1}\) Avicel and enzyme/temperature combinations of 9.2 FPU g\(^{-1}\) cellulose/37°C and 18.3 FPU g\(^{-1}\) cellulose/50°C.

Figure 8 shows that, for a value of \( K_{\text{rec}} = 2.8 \), the model adequately describes glucose production at various temperatures and enzyme loadings, and for conversions up to approximately 60% (25 g L\(^{-1}\) of glucose from 40 g L\(^{-1}\) of Avicel).

Wheat straw as a substrate

As has been previously shown, three substrate characteristics describe the production rate of glucose from cellulose by Cellubrix, namely \( k_{\text{max}} \) values for reactions 1 and 3, \( K_L \) and \( K_{\text{rec}} \). In order to demonstrate the portability of the model towards other substrates, these three parameters values were determined for a cellulose extract of wheat straw.

Experiments for determination of \( k_{\text{max}} \) and \( K_L \) were conducted as previously mentioned. For all enzyme:substrate ratios, linear regression for determination of the initial glucose production rate yielded values for \( R^2 \) of 0.97 and above. A plot of the reciprocal glucose production rate versus the reciprocal enzyme dosage (\( R^2 = 0.999 \) ) yielded \( k_{\text{max}} = 0.58 \) h\(^{-1}\) and \( K_L = 93.5 \) FPU L\(^{-1}\). Using the previously established ratio of 1:1.33, \( k_1 \) and \( k_3 \) were calculated to be 0.34 and 0.24 h\(^{-1}\), respectively. Figure 9 shows both the experimental glucose production rate as a function of enzyme dosage and a model fit using these \( k_{\text{max}} \) and \( K_L \) values.

Figure 10 shows the restart rates of glucose production (i.e. excluding effects of potentially inhibitory product by washing away any formed glucose and excluding effects of potential inactivation of the enzyme by re-incubating with fresh enzyme preparation), relative to the initial rate of glucose production, as a function of the conversion rate. Using a first-order fit, a recalcitrance constant \( K_{\text{rec}} \) 5.2 was found for wheat straw.
Using the values of these three parameters should give an accurate model representation of glucose production from pretreated wheat straw. Figure 11 shows the results with 0.7 g L\(^{-1}\) of wheat straw cellulose at 40\(\degree\)C and 1 mL Cellubrix per liter (equaling an enzyme loading of 80 FPU g\(^{-1}\)). This high value of enzyme dosage was chosen due to the far higher \(K_L\)-value of Cellubrix on wheat straw. The individual points show the measurements, the line shows the model prediction. As can be seen, the model accurately predicts glucose production for a given substrate, when the values for \(K_L\), \(k_{\text{max},1}\), \(k_{\text{max},3}\) and \(K_{\text{rec}}\) (which are the parameters describing the substrate/enzyme interaction) are known. This conclusion was found to hold for wheat straw up to a cellulose conversion of approximately 60% (Figure 11).

**Conclusions**

The current description of cellulose hydrolysis kinetics was successful in describing the hydrolysis of both Avicel and wheat straw for conversions up to 60%. In addition, the portability of the model towards completely different substrates (i.e. pretreated straw versus Avicel) was clearly demonstrated by re-measurement of three distinct model parameters (\(k_{\text{max}}, K_L, K_{\text{rec}}\)). It was demonstrated that, irrespective of and taking into account product inhibition and possible inactivation of the enzyme, the reaction rate of the substrate drops as a function of increased conversion rate (i.e. the substrate becomes more recalcitrant). This drop in reaction rate can be described using first-order kinetics with a substrate-specific *recalitrance constant* (\(K_{\text{rec}}\)) as quantification of this effect.

**Notation**

- \(A\): Arrhenius constant for increase of initial rate at increasing temperatures (h\(^{-1}\))
- \(A_D\): Arrhenius constant for thermal enzyme deactivation (h\(^{-1}\))
- \(C_C\): cellobiose concentration (g L\(^{-1}\))
- \(C_{Cb}\): cellulose concentration (g L\(^{-1}\))
- \(C_{e}\): enzyme concentration (FPU L\(^{-1}\))
- \(C_G\): glucose concentration (g L\(^{-1}\))
- \(e_g\): \(\beta\)-glucosidase activity per gram of protein in the enzyme preparation (U g\(^{-1}\))
- \(e_{\text{total}}\): protein (enzyme) concentration per liter reaction volume (g L\(^{-1}\))
- \(E_a\): activation energy (J mol\(^{-1}\))
- \(h\): Planck's constant (6.626 \(\times\) 10\(^{-34}\) J s\(^{-1}\))
- \(k_i\): reaction rate constant \((i=1-3)\) (h\(^{-1}\))
- \(k_{D}\): thermal deactivation constant (h\(^{-1}\))
- \(k_{\text{max},i}\): maximum specific rate constant \((i=1\ or\ 3)\) (h\(^{-1}\))
- \(K_{i,2}\): inhibition constant of glucose on \(\beta\)-glucosidase (g L\(^{-1}\))
- \(K_{L}\): Langmuir adsorption constant (FPU L\(^{-1}\))
- \(K_{m}\): Michaelis constant for \(\beta\)-glucosidase (g L\(^{-1}\))

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Figure 10. Relative glucose production as a function of conversion fraction of the wheat straw, experimental data and model fit.

Figure 11. Glucose production on pretreated wheat straw, experimental data and model prediction. Final cellulose conversion approximately 60%; initial wheat straw cellulose concentration 0.7 g L\(^{-1}\).
\[ K_{\text{rec}} \] recalcitrance constant (–)

\[ N_A \] Avogadro’s number \((6.23 \times 10^{23})\) \((\text{mol}^{-1})\)

\[ R \] gas constant \((8.3142)\) \((\text{J mol}^{-1} \cdot \text{K}^{-1})\)

\[ t \] time (h)

\[ T \] temperature (K)

\[ v_1 \] production rate of cellobiose from cellulose by cellulase \((\text{mg L}^{-1} \cdot \text{h}^{-1})\)

\[ v_2 \] production rate of glucose from cellobiose by \(\beta\)-glucosidase \((\text{mg L}^{-1} \cdot \text{h}^{-1})\)

\[ v_3 \] production rate of glucose from cellulose by cellulase \((\text{mg L}^{-1} \cdot \text{h}^{-1})\)

\[ \Delta H \] deactivation enthalpy \((\text{J mol}^{-1})\)

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