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Cellulase kinetics as a function of cellulose pretreatment

Andreas S. Bommarius [a,b,*], Adrian Katona [a], Sean E. Cheben [a], Arpit S. Patel [a], Arthur J. Ragauskas [c], Kristina Knudson [c], Yunqiao Pu [d]

[a] School of Chemical and Biomolecular Engineering, Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, GA 30332-0363, USA
[b] School of Chemistry and Biochemistry, Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, GA 30332-0363, USA
[c] School of Chemistry and Biochemistry, Institute of Paper Science and Technology, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, GA 30332-0400, USA
[d] Institute of Paper Science and Technology, Georgia Institute of Technology, 500 10th Street, Atlanta, GA 30332-0620, USA

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ABSTRACT

Microcrystalline cellulose (Avicel) was subjected to three different pretreatments (acid, alkaline, and organosolv) before exposure to a mixture of cellulases (Celluclast). Addition of β-glucosidase, to avoid the well-known inhibition of cellulase by cellobiose, markedly accelerated cellulose hydrolysis up to a ratio of activity units (β-glucosidase/cellulase) of 20. All pretreatment protocols of Avicel were found to slightly increase its degree of crystallinity in comparison with the untreated control. Adsorption of both cellulase and β-glucosidase on cellulose is significant and also strongly depends on the wall material of the reactor. The conversion–time behavior of all four states of Avicel was found to be very similar. Jamming of adjacent cellulase enzymes when adsorbed on microcrystalline cellulose surface is evident at higher concentrations of enzyme, beyond 400 U/L cellulase/8 kU/L β-glucosidase. Jamming explains the observed and well-known dramatically slowing rate of cellulose hydrolysis at high degrees of conversion. In contrast to the enzyme concentration, neither the method of pretreatment nor the presence or absence of presumed fractal kinetics has an effect on the calculated jamming parameter for cellulose hydrolysis.

1. Introduction

While currently almost all ethanol from renewable carbon sources is obtained either from starch, most notably from corn, or from sucrose residues, shortage of capacity and competition from requirements for food will drive the need for lignocellulosics, such as corn stover, grasses, or wood, to serve as carbon sources for biofuels for future large-scale applications. The current typical biological processes for utilizing lignocellulosics are based either on the separate hydrolysis and fermentation (SHF) or the simultaneous saccharification and fermentation (SSF) formats. Both require four essential processing steps: (i) pretreatment of the lignocellulosic raw material, (ii) enzymatic hydrolysis of cellulose to cellobiose and then to glucose with the help of cellulases and β-glucosidases, (iii) mostly anaerobic fermentation of glucose to ethanol (or other fuel alcohols), and (iv) concentration and purification of the fermentation broth to pure ethanol (or other fuel alcohol).

Both pretreatment and enzymatic hydrolysis have proven to be the most difficult steps in this sequence. Pretreatment is non-trivial owing to the heterogeneity of the lignocellulosic source and the tight three-dimensional structure of lignocellulosics due to the network of lignin, cellulose, and hemicellulose. Consequently, several pretreatment processes exist, based on the addition of acid or base, either in water or in partially organic media. Enzymatic hydrolysis is a hurdle because of the heterogeneous nature of the cellulose substrate and the fact that degradation of the cellulose chain progresses in only one direction for each cellobiohydrolase, effectively reducing the reaction to a one-dimensional process. Cellulose is known to consist of relatively easily accessible amorphous regions with few lateral interactions between the cellulose chains as well as of crystalline domains that are much harder to hydrolyze. In addition, the hydrolysis product of the cellulase reaction, cellobiose (glucosyl β-1→4 glucose) (Scheme 1), is well known to severely inhibit cellulase. Therefore, β-glucosidase is added to the hydrolysis process to cleave cellobiose to glucose. As the lignocellulosic substrate contains non-hydrolysable lignin besides cellulose and hemicellulose, cellulase cannot be recovered after hydrolysis. All these factors lead to high required enzyme load and thus to current price contributions of hydrolysis enzymes that are much too high for large-scale use.

* Corresponding author at: School of Chemical and Biomolecular Engineering, Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, GA 30332-0363, USA. Fax: +1 404 894 2291.
E-mail address: andreas.bommarius@chbe.gatech.edu (A.S. Bommarius).

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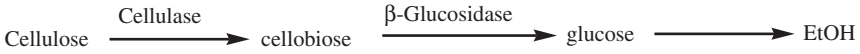


Scheme 1. Sequence from cellulose to biofuels.

As access of cellulase to the cellulose network is a key issue for hydrolysis, it is not surprising that success of hydrolysis depends on the quality of pretreatment. While one important factor contributing to the complex nature of cellulose hydrolysis is the presence of lignin, pretreatment even is believed to influence the extent of cellulose hydrolysis in the absence of lignin. As stated above, the degree of crystallinity should influence the rate of hydrolysis. Also, unless the ratio of cellulase and β-glucosidase is optimized, inhibition of cellulase by cellobiose causes inefficient hydrolysis.

1.1. Scope

In this work, we revisit the issue of the influence of pretreatment on the enzymatic hydrolysis of cellulose. Microcrystalline cellulose (Avicel) serves as the substrate, as we deliberately focus on the influence of pretreatment on the cellulose and not on the other components, i.e. hemicellulose and lignin. We will apply three kinds of pretreatment to Avicel, acidic, alkaline, and a combination of organic solvent and acid treatment, the 'Organosolv' process, check for changes in crystallinity, and optimize rates with respect to both total enzyme loading and relative amounts of cellulase and β-glucosidase. We will fit two models to the kinetic data, fractal kinetics, caused by the heterogeneous nature of the cellulose substrate, and jamming kinetics, occurring when cellulase concentration becomes so high that the enzyme molecules interfere with each other on the crystalline parts of the cellulose surface.

1.2. Substrates

Lignocellulosics from woody biomass contain 40–50% cellulose, 24–40% hemicellulose, and 18–25% lignin in the case of hardwoods, or respectively 40–50% cellulose, 25–35% hemicellulose, and 25–35% lignin in the case of softwoods (Malherbe and Cloete, 2002). The heterogeneity of lignocellulosics is cause for many discrepancies between findings in the literature. To focus on the influence of cellulase kinetics, we selected Avicel as our cellulosic model substrate. Avicel is a commercially available microcrystalline cellulose, produced by acidic hydrolysis of wood pulp and spray drying of the resulting colloidal dispersion of cellulose fibers (Zhang et al., 2006). Avicel is rated to be a very good model for exoglucanases, such as exocellulases (CBH I and II). It is characterized by a low degree of polymerization (DP) but limited accessibility to enzyme, as it contains still a major amount of crystalline cellulose (Zhang et al., 2006).

1.3. Biocatalysts

We will employ cellulase from *Trichoderma reesei*, the best developed cellulase at this point (Galbe and Zacchi, 2002), and it is commercially available in a preparation known as Celluclast®. Cellulase is a mixture of several enzymes, the exoglucanases cellobiohydrolase I and II (CBH I and II, more recently termed Cel7A and Cel6A, respectively) as well as endoglucanases. CBH I and II cleave bonds from the reducing and non-reducing end of cellulose, respectively, to yield mainly cellobiose, the β-1→4-bound dimer of glucose and the repeating unit of the cellulose polymer chain (Nidetzky et al., 1994).

Standard enzyme kinetics, as reflected in the Michaelis–Menten kinetics, accounts for substrate binding and catalysis. The kinetics of the reaction of cellulose to cellobiose and then on to glucose is expected to be impacted by at least four phenomena beyond standard binding and catalysis: (i) inhibition of cellulase by cellobiose; (ii) adsorption of cellulase on cellulose; (iii) restriction of dimensionality of the kinetics by the three-dimensional cellulose network; and (iv) interference of cellulase action by crowding on proximate cellulose fibers ('jamming').

1.4. Product inhibition by cellobiose

While cellobiose is the predominant hydrolysis product of CBH I, it is also a strong inhibitor of CBH I (Holtzapple et al., 1984, 1990). For this reason, a sufficiently low level of cellobiose is crucial for both an acceptable overall reaction rate as well for a high yield of glucose. This objective is achieved by adding a sufficient amount of β-glucosidase to the system of cellulose and cellulase.

1.5. Adsorption of cellulase on cellulose

Before the enzyme can act on the substrate, enzyme molecules must be adsorbed from the bulk of the liquid phase onto the surface of the cellulose. Once on the surface, the enzyme molecule must diffuse across the surface to the reaction site.

1.6. Fractal kinetics

Cellulose is a three-dimensional network of fibers with openings that are often on the length scale of the size of a cellobiohydrolase molecule. Therefore, mobility of CBH molecules is restricted in space and its diffusion cannot be modeled by Fick's law (Rothschild, 1998). As dissolved CBH molecules adsorb on the surface of cellulose and must diffuse to the reaction site, significant (two-dimensional) surface diffusion exists. In addition, the movement of a catalytically active CBH I molecule along the cellulose chain is caused by processivity and renders that portion of the transport one-dimensional. To compensate, the volume that hosts diffusion, collision, and reaction is considered to have fractional dimension and thus cellulase kinetics is modeled with non-integer order with respect to the concentrations of reaction partners (Xu and Ding, 2007). Fractal theory was developed to describe irregularities throughout mathematics, physics, biology, and chemistry that result from spacial restrictions of rate processes (Mandelbrot, 1983). The theory of fractals has been applied successfully to the kinetics of cellulose conversion to cellobiose and glucose (Xu and Ding, 2007).

1.7. Jamming effects

In the crystalline part, the cellulose fibers are oriented in parallel to each other with a characteristic lateral distance that is on the same order as the diameter of CBH I and II, which are both large enzymes (65 and 58 kDa, respectively; Wohlfahrt et al., 2003). On the surface of the crystalline part of cellulose fibers, the movement of CBH molecules is already restricted to one dimension along the fiber. At high enzyme concentrations, and therefore several enzyme molecules binding adjacent to one another, the CBH molecules crowd onto the same small surface

area of cellulose and tend to impede one another as not all bound enzymes proceed at the same rate (Xu and Ding, 2007). This effect is termed jamming and results in a reduced overall reaction rate. Also, active enzymes can get lodged between enzymes that are bound but inactive (Väljamäe et al., 1998).

2. Materials and methods

2.1. Materials

As substrate for enzymatic hydrolysis, Avicel PH-101 from Fluka (St. Louis, MO, USA) was used throughout. As enzymes for hydrolysis of cellulose to glucose, cellulase from *T. reesei* ATCC 26921 (viscous liquid) (Celluclast®, C2730) from Sigma (St. Louis, MO, USA), and β-glucosidase from almonds (lyophilized powder) from Sigma (G0395) were used. 1,4-Dinitrosalicilic acid, phenol, KNa tartrate, sodium sulfite, and sodium hydroxide were from Sigma and used as received. Deionized water from a Barnstead nanopure Diamond™ Ultrapure unit (Barnstead Intl, Dubuque, IA, USA) was used throughout. For the *Bradford assay*, Bradford reagent from Pierce (Rockford, IL, USA) was employed.

2.2. Enzymatic assays

2.2.1. Assay for cellulase from T. reesei

1 U corresponds to the amount of enzyme which liberates 1 μmol glucose from carboxymethylcellulose per minute at pH 5.0 and 37 °C (see Supplemental Information). Protein content, density, and specific activity were found to be 3.6 wt% (determined by Bradford assay), $\rho = 1.2$ g/mL (25 °C), and 0.7 U/mg, respectively.

2.2.2. Assay for β-glucosidase from almonds

The enzyme was available as a lyophilized powder. 1 U will liberate 1.0 μmole of glucose from salicin per min at pH 5.0 at 37 °C (see Supplemental Information). The specific activity was founds to be 5.2 U/mg.

2.2.3. Enzymatic hydrolysis of cellulose assay

In a 30 mL round bottom flask were added 100 mg/mL (10%, w/v) cellulose, the corresponding amounts of cellulase and β-glucosidase, and 50 mM sodium acetate buffer, pH 5.0 (thermostated before at 50 °C) until the 8 mL final volume. The pH value was adjusted to pH 5.0 using 4% NaOH, if necessary. The reaction mixture was placed in an incubator at 50 °C and mixed using a magnetic stirrer. At designated time points, aliquot samples were taken and reducing sugar concentrations measured using the DNS assay.

2.2.4. Sample loading

At each relevant time point, 200 μL of sample was pipeted, then centrifuged at 12,000 × *g* for 5 min at room temperature, and the supernatant kept for analysis of reducing sugar concentration by DNS assay.

2.3. Protein determination assay

2.3.1. Bradford assay (assay for protein concentration determination)

Two calibration curves for BSA (bovine serum albumin) in two different ranges were constructed: 125–2000 μg/mL (standard Bradford assay) and 5–250 μg/mL (micro Bradford assay).

2.3.2. Standard Bradford assay

Sample: 1000 μL Bradford reagent+20 μL protein sample; blank solution: 1000 μL Bradford reagent+20 μL buffer.

2.3.3. Micro Bradford assay

Sample: 450 μL Bradford reagent+50 μL protein sample; blank solution: 450 μL Bradford reagent+50 μL buffer.

Each solution was collected in a 1.5 mL Eppendorf tube and then mixed gently for 5 min avoiding foam formation. The absorbance at 595 nm was recorded using an Eppendorf BioPhotometer and then the protein concentration was determined using the calibration curves.

2.4. Reducing sugars determination assay

2.4.1. DNS assay (assay for reducing sugars)

DNS (1,4-dinitrosalisilic acid) reagent of 150 μL was added to 150 μL reducing sugar (cellobiose; glucose) in a 1.5 mL Eppendorf tube. Then the mixture was heated at 90 °C for 15 min using an Eppendorf Thermomixer R to develop the red-brown color. Subsequently, 50 μL of a 40% potassium sodium tartrate (Rochelle salt) solution was added to this mixture to stabilize the color. After cooling to room temperature using an Eppendorf Thermomixer, the absorbance was recorded with a spectrophotometer at 575 nm. Calibration curves were used to determine the reducing sugars concentration. The sample was diluted properly, as the measured absorbance to be integrated in the linear range. (Preparation of DNS reagent: 1,4-dinitrosalicylic acid: 1 g, phenol: 0.2 g, sodium sulfite: 0.05 g, sodium hydroxide: 1 g, add water: 100 mL.)

2.5. Pretreatments applied to Avicel MCC

Three different pretreatments were separately applied to Avicel microcrystalline cellulose (MCC 94.35% dry matter). The conditions for each treatment are summarized in Table 1. The sulfuric acid and organosolv pretreatments were performed in mechanically stirred, thermostatically controlled Parr pressure reactors with a water/ice cooled condenser attached.

The first treatment was sulfuric acid pretreatment (Soderstrom et al., 2003). A single stage sulfuric acid treatment was applied because Avicel does not contain hemicelluloses or 5-carbon sugars that interfere with glucose fermentation. The Avicel was pre-impregnated with 1% sulfuric acid (g/g) by mixing the Avicel with the liquid phase and soaking overnight at room temperature. The cellulose slurry was then heated in a sealed 1-gal Parr pressure reactor to 200 °C (225 psi chamber pressure). After 2 min at reaction temperature, the reaction was rapidly cooled by allowing the steam to exit through the condenser.

The second pretreatment method tested was organosolv pulping (Pan et al., 2007). Avicel was mixed with the ethanol/sulfuric acid/water liquor and heated in a 250 mL Parr reactor to 170 °C. After 60 min at reaction temperature, the chamber was vented through the condenser. The pretreated solids were recovered by centrifugation, then washed three times with warm 65% ethanol in water followed by three washes with warm distilled water. To obtain sufficient pretreated solids, two batches of 15 g of Avicel were treated with organosolv pulping.

The third pretreatment was an ammonia steeping process (Dominguez et al., 1997). Avicel was allowed to react with 5.6 M ammonium hydroxide in sealed Erlenmeyer flasks (three flasks, each with 30 g Avicel) in a temperature-controlled shaking incubator (50 °C) for 2 days. The pretreated solids were recovered by centrifugation and later washed with distilled water until the

solution was at pH 6 and no ammonium odor was detectable. 98% of the starting material was recovered as insoluble solids.

2.6. Solid-state NMR analyses for Avicel samples

The solid-state cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR experiments were performed on a Bruker Avance/DMX-400 spectrometer operating at frequencies of 100.59 MHz for ^{13}C following literature methods (Pu et al., 2006). All the experiments were carried out at ambient temperature using a Bruker 4-mm MAS probe. The samples (≈35% moisture content) were packed in 4 mm zirconium dioxide rotors and spun at 8 kHz. Acquisition was carried out with a CP pulse sequence using 4.5 μs pulse, 2.0 ms contact pulse, and 5.0 s delay between repetitions. 8192 scans were accumulated for each sample.

The CP/MAS ^{13}C NMR spectra of the Avicel samples are shown in Fig. 4. The most informative region in the NMR spectrum of cellulose sample is the signal cluster with a chemical shift distribution between δ 79 and 92 ppm. The fairly sharp signals from δ 86 to 92 ppm correspond to C-4 carbons from crystalline forms, whereas the broader upfield resonance line from 79 to 86 ppm is assigned to the amorphous domains. The degree of crystallinity was determined as a crystallinity index (CrI) (Eq. (1)) from the areas of the crystalline and amorphous C4 signals:

$$\mathrm{CrI} = \frac{A_{86-92\ \mathrm{ppm}}}{A_{79-86\ \mathrm{ppm}} + A_{86-92\ \mathrm{ppm}}} \times 100\%. \quad (1)$$

2.7. Analysis of pretreated samples and determination of degree of conversion

The pretreated samples and the control were analyzed for moisture to allow an accurate calculation of the initial concentration of glucose units as well as determination of the degree of conversion from the DNS assay results for glucose. The results are listed in Table 2.

2.8. Determination of cellulase adsorption on cellulose

Cellulose hydrolysis was carried out in a 15 mL glass test tube with 100 mg/mL (10%, w/v) of substrate and enzyme concentration ranging from 1 to 150 mg/mL. The solution was diluted to 5.0 mL using a 50 mM Na-acetate buffer (pH 5.0). The reactions were run for 5 min at 50 °C and subsequently a 1 mL sample was taken and centrifuged at 10,000 × *g* for 5 min. The resulting supernatant was analyzed using the Bradford protein assay to determine free enzyme concentration. From the free enzyme concentration, the amount of bound enzyme could be determined. The enzyme adsorption was assumed to follow a Langmuir-type isotherm, as in Eq. (2) (Kadam et al., 2004):

$$E_B = \frac{E_{max} K_{ad} E_F S}{1 + K_{ad} E_F}, \quad (2)$$

where E_B is the bound enzyme concentration, E_F is the free enzyme concentration, S is substrate concentration, E_{max} is maximum enzyme adsorption in g enzyme/g cellulase, and K_{ad} is the adsorption coefficient. To determine K_{ad} and E_{max}, a linearized form of Eq. (2) was used:

$$\frac{S}{E_B} = \frac{1}{E_{max} K_{ad}} \frac{1}{E_F} + \frac{1}{E_{max}}. \quad (3)$$

Once the data was plotted, with S/E_B on the y-axis and $1/E_F$ on the x-axis, a linear regression was carried out using Microsoft Excel. From the equation for this line, K_{ad} and E_{max} were calculated (Table 4).

2.9. Calculation of fractal kinetic and jamming kinetics

Eq. (4) was used to determine the fractal parameter (Xu and Ding, 2007):

$$\frac{\log([E]_i/[E]_j)}{\log(t_j/t_i)} = 1 - f, \quad (4)$$

where E_i and E_j are initial enzyme concentrations in runs i and j, t_i and t_j are the reaction times in runs i and j until 20% conversion to glucose is reached, and f is the fractal parameter. The fractal parameter f is used to determine the jamming parameter j using the following equation (Eq. (5)) (Xu and Ding, 2007):

$$\left(1 - \frac{[E]_i}{j[S]}\right)[E]_i t_i^{1-f} = \left(1 - \frac{[E]_j}{j[S]}\right)[E]_j t_j^{1-f}, \quad (5)$$

with S being the substrate concentration of 100 mg/mL. The data from the reactions with varying enzyme concentrations provide the time at which 20% conversion to glucose had occurred.

3. Results

3.1. Adsorption of cellulase and β-glucosidase on Avicel

At a constant concentration of Avicel (10%, w/v), we determined the amount of soluble cellulase and β-glucosidase (in contrast to adsorbed enzyme) when contacted with pretreated Avicel (Fig. 1). The results for cellulase on untreated and pretreated Avicel as well as for β-glucosidase are plotted in Fig. 2a and b, respectively. We found that adsorption not only depends on the state of pretreatment of Avicel but also on the wall material of the vessel the experiments are conducted in. We found that the polypropylene walls of Eppendorf tubes adsorb so much cellulase that the determination of adsorption on Avicel could not be performed. Thus, we only considered data obtained in glass containers for interpretation. The adsorption onto glass was quantified by performing the same adsorption experiments, including magnetic stirrers, but without Avicel. We found that 24% of cellulase and 42% of β-glucosidase were lost to the glass tube walls. Fig. 3 shows a plot of free enzyme versus bound enzyme for both experiments. A linear regression of the data provided the percentages of enzyme bound to the glass walls.

Table 1
Summary of pretreatment conditions

Pretreatment	Time	Temperature (°C)	Liquor:Avicel (v/oven-dry wt)	Liquor
Sulfuric acid	2 min	200	8:1	1.0% H_2SO_4 (g/g)
Organosolv	60 min	170	7:1	1.1% H_2SO_4 (g/g)
Ammonia	2 days	50	10:1	5.6 M NH_4OH

Table 2
Moisture content and concentration of reducing ends at 20% and 100% conversion

Substrate	Dried content (%)	Moisture content (%)	100% conversion (mM) glucose	20% conversion (mM) glucose
Avicel	96.45	3.55	540.1	108
Acid	94.23	5.77	527.7	105.5
Alkaline	83.39	6.61	467.0	93.4
Organosolv	94.68	5.32	530.2	106

Table 3
Enzyme loading for conversion–time experiment

Cellulose: non-pretreated (Avicel), acid-, alkaline- and organosolv-pretreated			
0.1 U cellulase 2 U β-glucosidase 100 mg/mL cellulose	0.5 U cellulase 10 U β-glucosidase 100 mg/mL cellulose	1.5 U cellulase 30 U β-glucosidase 100 mg/mL cellulose	3 U cellulase 60 U β-glucosidase 100 mg/mL cellulose
4 mg cellulase 0.38 mg β-glucosidase 800 mg cellulose	20 mg cellulase 1.92 mg β-glucosidase 800 mg cellulose	60 mg cellulase 5.77 mg β-glucosidase 800 mg cellulose	120 mg cellulase 11.54 mg β-glucosidase 800 mg cellulose
5 U cellulase 100 U β-glucosidase 100 mg/mL cellulose	15 U cellulase 300 U β-glucosidase 100 mg/mL cellulose	50 U cellulase 1000 U β-glucosidase 100 mg/mL cellulose	*Assay procedure*: $V = 8$ mL; 50 °C; 50 mM sodium acetate buffer, pH 5.0
200 mg cellulase 19.2 mg β-glucosidase 800 mg cellulose	600 mg cellulase 57.7 mg β-glucosidase 800 mg cellulose	2000 mg cellulase 192.3 mg β-glucosidase 800 mg cellulose	

Alternatively, the binding of cellulase on glass can be prevented all together by adding a small amount of bovine serum albumin (Xu and Ding, 2007). Bovine serum albumin will not affect cellulase adsorption or activity on cellulose (Peters and Feldhoff, 1975).

We then proceeded to fit the data to the Langmuir adsorption model (Eq. (3)) and obtained very good fits; the values for maximal free enzyme concentration E_{max} and adsorption constant K_{ad} are listed in Table 4.

We find that pretreatment of Avicel increases adsorption of cellulase two- to six-fold over untreated material in the sequence acid > alkaline ≈ organosolv. As cellulase is known to exert its catalytic action when adsorbed on cellulose, this finding might be important for the prediction of the effectiveness of cellulase action of the pretreated Avicel material. We also find that β-glucosidase adsorbs much less on cellulose than cellulase, as expected.

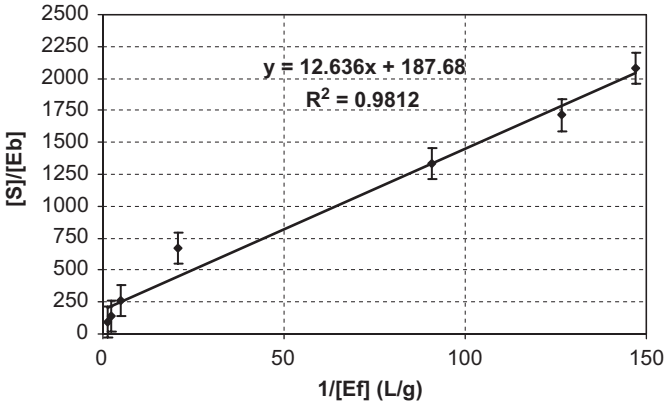


Fig. 1. Adsorption of cellulase on untreated Avicel.

3.2. Solid-state NMR analyses for Avicel samples

We measured the degree of crystallinity via solid-state ^{13}C NMR (Pu et al., 2006); the results are tabulated in Table 5 and the spectra for the first run with each pretreatment are depicted in Fig. 4.

We find that pretreatment of Avicel with any of the three methods increases the degree of crystallinity, in accordance with Liitiä et al. (2003). The increase is five to eight percentage points and does not differ significantly between pretreatment methods. Although one of the main reasons for pretreatment of lignocellulosics is the opening of the three-dimensional structure of the network of lignin and cellulose, pretreatment of Avicel, which in itself is a product from woody lignocellulosics that has been acid-washed, might not open up the crystalline areas of the cellulose network but instead shorten the cellulose fibers in the amorphous environment.

3.3. Conversion–time plots for non-pretreated and pretreated cellulose

The main hydrolysis product of cellulose by cellobiohydrolase I and II (CBH I and II), cellobiose, is a well-known strong competitive inhibitor of CBH I. We employed β-glucosidase to decrease the steady-state cellobiose concentration to a minimum level and thus to alleviate the inhibition on CBH I (see Fig. 5). We found that no gain in enzymatic rate could be reached beyond a [β-glucosidase]:[cellulase] ratio (expressed as ratios of units) of 20; so for all further investigations we employed this ratio to minimize the inhibitory effect of cellobiose on cellulase.

Next, we ran experiments with different cellulase concentration, 187.5, 1875, and 3750 U/L (Table 3), with non-pretreated and acid-pretreated Avicel, and plotted degree of conversion over time (Fig. 10).

Expectedly, increased cellulase concentration leads to an enhanced rate at specified degree of conversion (Table 9); also expectedly, rates dramatically decreased with rising conversion, corroborating the findings in Yang et al. (2006). No significant difference in conversion–time behavior is observed between acid-pretreated and non-pretreated Avicel (Fig. 10). Investigation of the conversion–time plot up to 96 h for non-pretreated Avicel revealed three or two phases for the enzymatic hydrolysis rate at high cellulose concentration (>1500 U/L) and low concentration (<1000 U/L), respectively (Fig. 11): over that time period, 100% conversion was reached for high but only 69% for low enzyme concentration (data in Table 9).

At high cellulose concentration (3750 U/L), phase I from 0% to 32% conversion with a high rate 94 mM/h of glucose formation is finished after 2 h, followed by phase II within 12 h from 32% to 50% conversion for six-fold lower rate. The reaction from 50% to complete conversion (phase III) takes an additional 83 h, reflecting a rate of 1 mM/h glucose, almost two orders of magnitude lower (!) than during phase I.

At half the enzyme concentration, 1875 U/L, the trends are similar to those for the first case. The rate of glucose formation is about two-fold lower in phases I and II (Table 9) than for phase I

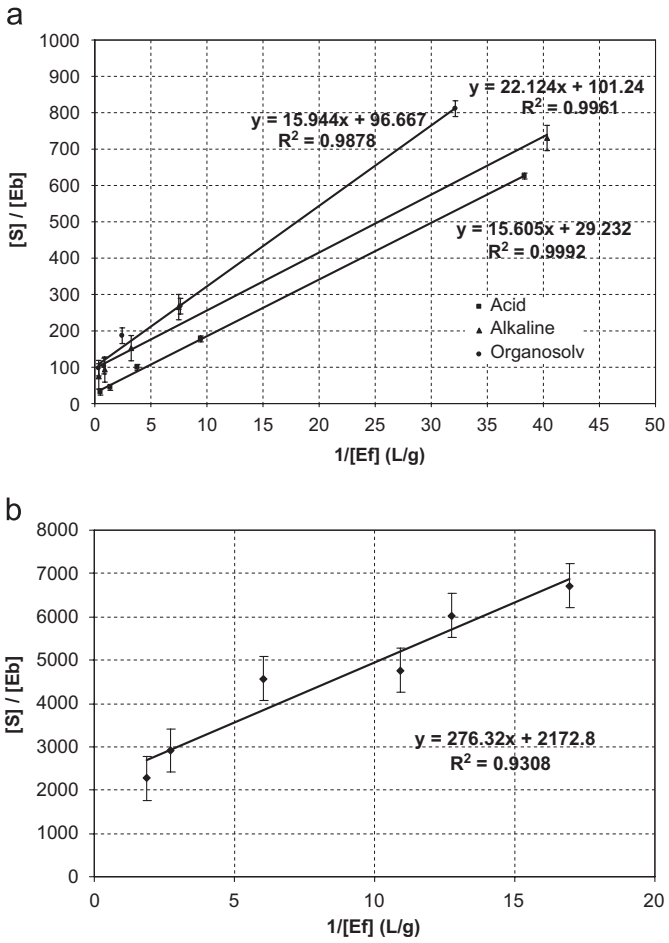


Fig. 2. (a) Adsorption of (a) cellulase on pretreated Avicel and (b) β-glucosidase on untreated Avicel; wall material of vessel: glass.

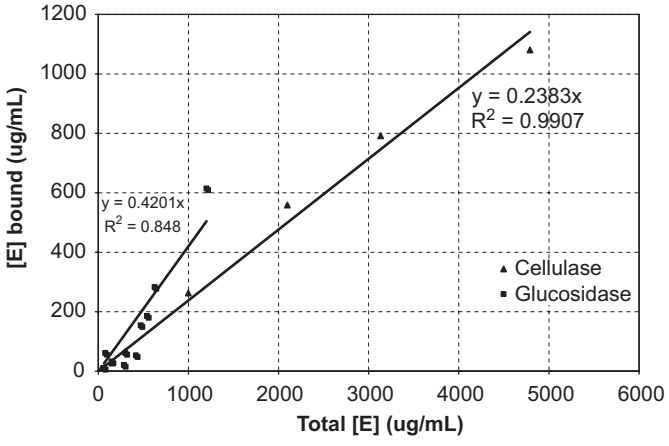


Fig. 3. Adsorption of cellulase and β-glucosidase on glass.

Table 4
Adsorption parameters of cellulase on pretreated Avicel fitted to the Langmuir model; wall material of vessel: glass

Pretreatment	E_{max} (g cellulase/g cellulose)	% Error	K_{ad} (L/g)	% Error
Acid	0.0342 ± 0.0053	15.6	1.87 ± 0.03	1.64
Alkaline	0.0103 ± 0.0020	19.5	6.06 ± 0.39	6.43
Organosolv	0.0099 ± 0.0012	17.9	4.58 ± 0.17	3.63
Untreated	0.0053 ± 0.0012	23.1	14.85 ± 0.92	6.19
β-Glucosidase	0.0005 ± 0.0001	17.8	7.86 ± 1.07	13.64

Table 5
Degree of crystallinity for differently pretreated Avicel

Sample	CrI (%)
Avicel PH-101 from Fluka	55.6
Ammonia-pretreated Avicel	60.2, 59.9
Organosolv-pretreated Avicel	62.0, 61.3
Acid-pretreated Avicel	61.4, 61.1

but similar in phase III. Also, the transition between the phases seems to occur at similar degrees of conversion, around 30–35% (1→2) and 50–55% conversion (2→3). The significance of this finding will be discussed here.

At one-tenth the concentration of phase II, 187.5 U/L, only two phases were identified, with the onset of phase II occurring again at around 33% conversion. Both rates in phases I and II, 7.8 and 1.6 mM/h, respectively, were about 50% higher than expected from the proportional calculation based on phase I (Table 9; for significance, see the next section). Assuming a constant rate of hydrolysis of 1.6 mM/h in phase II, completion of conversion would be reached after a total of 150 h.

3.4. Check for fractal kinetics and jamming effects of cellulase on Avicel

The standard concentration of Avicel of 100 g/L was exposed to different amounts of cellulase (12.5–6250 U/L) and β-glucosidase (250–125,000 U/L) at a constant ratio of specific activities of 1:20.

At that ratio, cellobiose concentrations were deemed to be very low (see Section 3 above) and thus inhibition by cellobiose was deemed to be irrelevant. We proceeded to map out conversion over time for each of the three modes of pretreatment as well as the untreated control. The time was recorded at which 20% conversion was reached for each enzyme concentration. Table 6 lists the enzyme concentrations and their respective reaction times, while the conversion–time plots are depicted in Fig. 6.

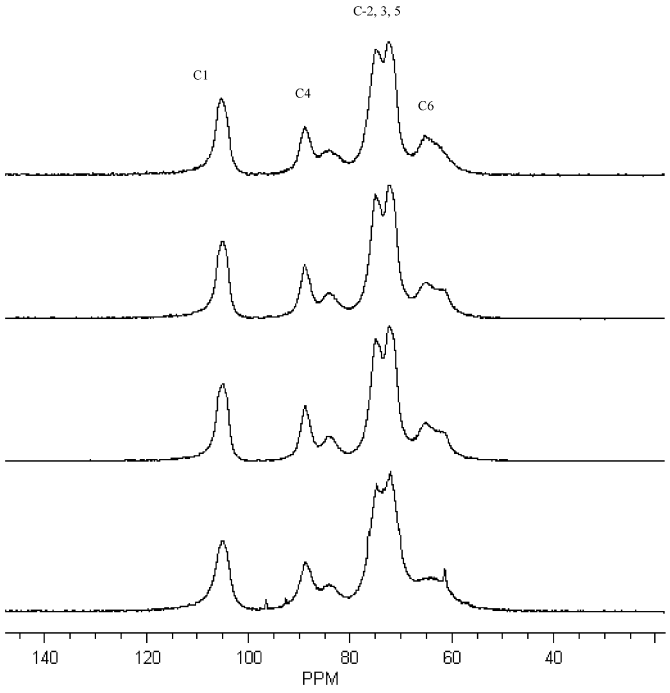


Fig. 4. CP/MAS ^{13}C NMR spectra of Avicel samples (from top to bottom: untreated, ammonia-treated, organosolv-treated, and acid-treated Avicel).

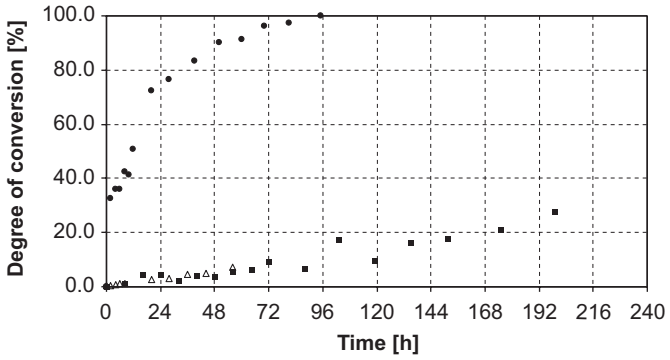


Fig. 5. Optimization of the [β-glucosidase]:[cellulase] ratio at fixed [cellulase]. Conversion: glucose formation from non-pretreated Avicel® at fixed amount of cellulase (1875 U/L) in 50 mM sodium acetate buffer, pH 5.0; 50 °C; $V = 8$ mL: (■) 0 U β-glucosidase; (●) 37,500 U/L β-glucosidase; (△) no enzyme (control).

It is observed that all preparations of Avicel behave similarly when exposed to varying amounts of cellulase and β-glucosidase: as expected, without exception, increasing enzyme concentrations led to lower reaction times to 20% conversion. However, with rising enzyme concentrations the decrease in reaction time diminished sharply beyond 400 U/L cellulase until, when increasing enzyme concentration from 1875 to 6250 U/L cellulase, only a minor decrease in reaction time from approximately 4 to 3 h is registered. We observed that at 6250 U cellulase/125,000 U β-glucosidase/L, the solution already was fairly viscous. Higher concentrations would require a mechanical stirrer for adequate mixing.

The ratios of enzyme concentrations $[E]_i/[E]_j$ were analyzed for the occurrence of fractal kinetics according to Eq. (4) and for jamming effects of cellulase on the surface Avicel by Eq. (5). The results are plotted in Fig. 7.

We found that the slope of the enzyme ratio, $-\log[E]_i/[E]_j$, over time ratio, $-\log t_j/t_i$, is larger than unity, in agreement with (Xu and Ding, 2007). A ratio larger than unity indicates that jamming effects are potentially important but that fractal kinetics cannot be discerned from Fig. 7.

As cellulase kinetics is found to be subject to jamming effects at higher concentrations of β-glucosidase/cellulase, jamming effects in cellulase kinetics dominate any potential fractal kinetic effects. However, other studies have found that the cellulose network of Avicel indeed leads to fractal kinetic effects: Ding and Xu found that the fractal dimension f of untreated Avicel equals 0.44. Therefore, we have calculated the jamming parameter j as a function of enzyme concentration (Table 8) and plotted the results (Fig. 9) in the same fashion as in the base case of $f = 0$.

Irrespective of the fractal kinetic order f, whether it is 0 (Table 7, Fig. 8) or 0.44 (Table 8, Fig. 9), the jamming parameter j increases from near zero (i.e. no jamming) at low enzyme concentration to values significantly above zero at high enzyme concentrations. At the highest enzyme concentration, 6250 U/L, which leaves the solution so viscous that this concentration can be regarded as the upper concentration limit, the jamming parameter j reaches a value around 0.09.

Interestingly, j does not at all seem to depend on the mode of pretreatment. While in the case of no assumed fractal kinetic contribution ($f = 0$), j for the untreated Avicel seems to be a bit smaller than for any of the treated cases, the four plots superimpose almost completely if $f = 0.44$.

In summary, the jamming parameter is found to depend just on the enzyme concentration, but neither on the fractal order of cellulase kinetics f nor on the mode of pretreatment. Up to a concentration of 375 U/L, the jamming parameter is less than

Table 6
Reaction times to 20% conversion[a] for different concentrations of β-glucosidase/cellulase

Treatment	Cellulase loading (0.7 U = 1 g)						
	0.1 U; 12.5 U/L	0.5 U; 62.5 U/L	1.5 U; 187.5 U/L	3 U; 375 U/L	5 U; 675 U/L	15 U; 1875 U/L	50 U; 6250 U/L
	20% conversion time (h)						
Acid	38.93	15.64	13.71	4.51	4.49	3.21	2.88
Alkaline	133.1	28.71	19.79	6.14	6.58	3.92	3.57
Organosolv	114.9	24.02	15.13	5.54	7.34	4.25	3.97
Untreated	146.5	13.85	11.56	5.20	4.26	3.08	3.54

[a] 20% conversion expressed as glucose concentration (see Table 2); ratio of activities β-glucosidase/cellulase: 20; $T = 50$ °C; pH 5.0; $V = 8$ mL.

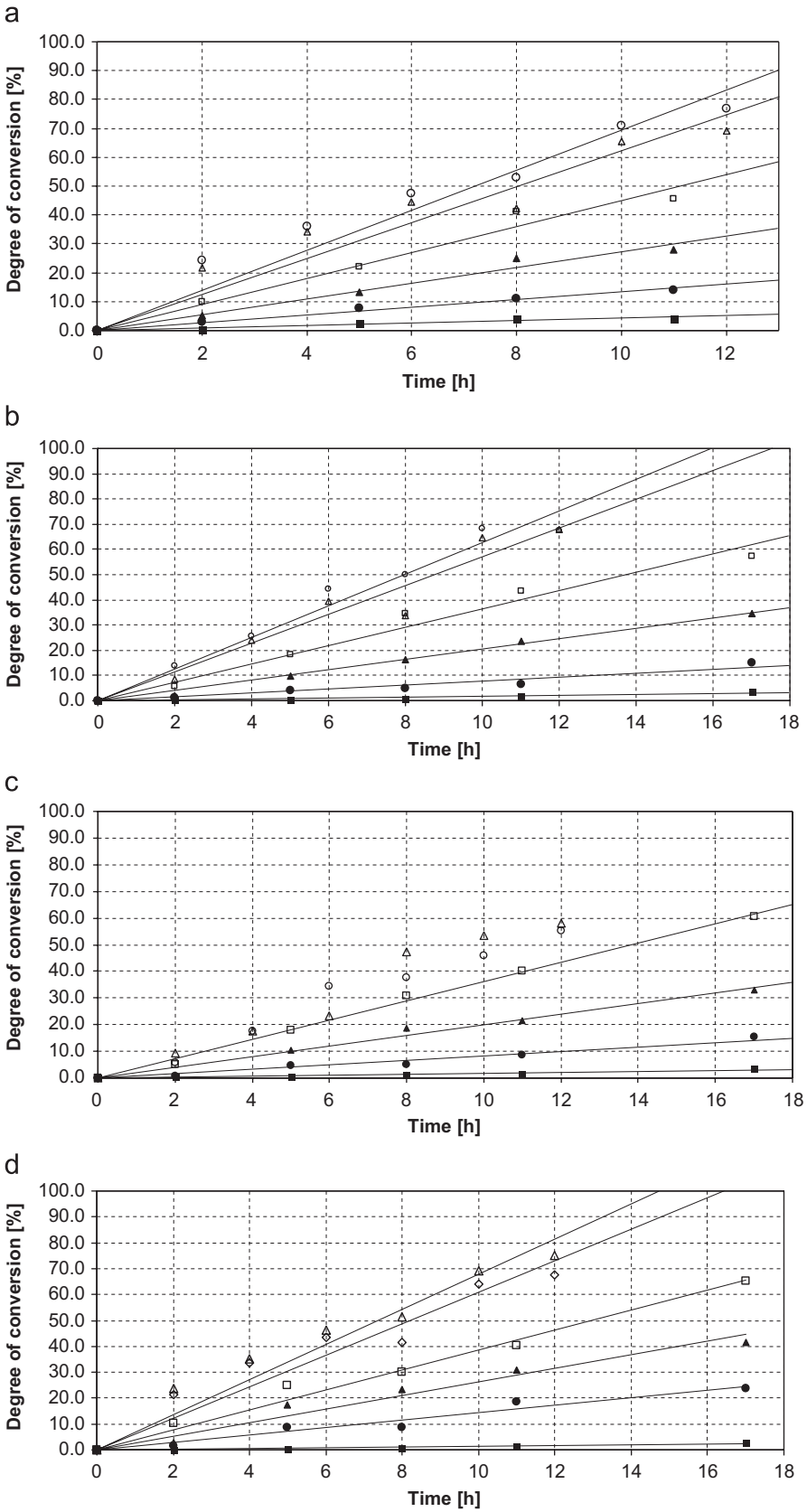


Fig. 6. Conversion–time behavior of pretreated Avicel up to intermediate degrees of conversion: (a) acid-pretreated, (b) alkali-pretreated, (c) organosolv-pretreated, and (d) untreated Avicel; $T = 50\,^{\circ}C$, pH 5.0, ratio of activities β-glucosidase/cellulase: 20; $V = 8$ mL: (■) 0.1 U cellulase+2 U β-glucosidase; (●) 0.5 U cellulase+10 U β-glucosidase (▲) 1 U cellulase+20 U β-glucosidase; (□) 3 U cellulase+60 U β-glucosidase (○) 15 U cellulase+300 U β-glucosidase; (△) 50 U cellulase+1000 U β-glucosidase.

0.005 and then rises exponentially to 0.09 at 6250 U/L, the upper limit at which the solution could be stirred (Table 9).

4. Discussion

All three pretreatments of Avicel employed in this work, ammonia, alkaline, and organosolv pretreatment, were found to slightly increase Avicel crystallinity, as measured by the crystallinity index, by a few percentage points. Consequently, the conversion–time behavior upon the treatment with cellulase was found not to differ significantly between the four states of pretreatment (including the control). The degree of crystallinity of cellulose in the samples investigated does not appear to impact cellulase activity, suggesting that cellulase activity is not confined to non-crystalline regions. Apparently, the mild acid wash of wood pulp to Avicel renders subsequent treatments ineffective or the methods applied in this work are too mild. In contrast, ammonia fiber explosion decrystallizes crystalline cellulose and deacetylates acetyl linkages (Gollapalli et al., 2002; Mitchell et al., 1990) and both of these effects increase the enzymatic hydrolysis of cellulose (Wyman et al., 2005).

The Langmuir parameters for cellulase adsorption on Avicel that we found are similar to those of other published studies. Previous investigations (Beldman et al., 1987; Kiriacou et al., 1988) of adsorption on crystalline cellulose used purified components of cellulase: six endoglucanases (Endo I-VI) and three exoglucanases (Exo I-III). Beldman et al. (1987) reported K_{ads} values ranging from 0.29 to 11.67 (L/g), and E_{max} values ranging from 0.0028 to 0.126 (g cellulase/g cellulose) using cellulase from *Trichoderma viride*. Kiriacou et al. (1988) determined E_{max} values ranging from 0.0047 to 0.0271 g cellulase/g cellulose using cellulase derived from *T. reesei*, which compare favorably with our own range from 0.0053 (untreated) to 0.0342 (acid-pretreated) g cellulase/g cellulose. Several other investigators have observed Langmuir adsorption behavior within this parameter range using Avicel as the substrate and cellulase from several strains of *Trichoderma* (Ooshima et al., 1983; Woodward et al., 1988; Stahlberg et al., 1991; Sethi et al., 1998).

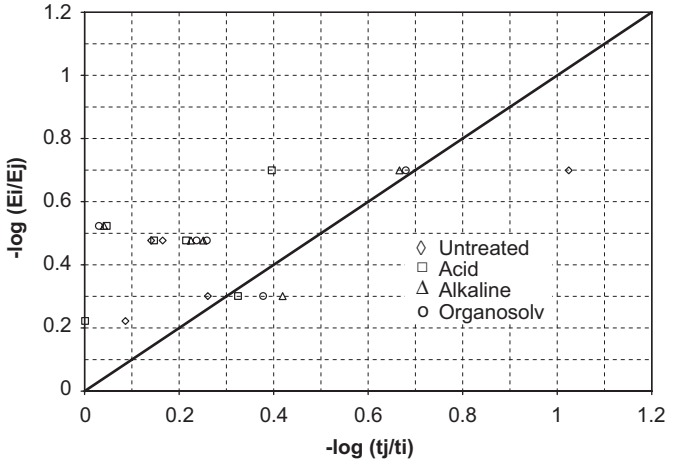


Fig. 7. Plot of enzyme ratio $-\log[E]_i/[E]_j$ over time ratio $-\log t_j/t_i$.

While organosolv- and alkaline-pretreated Avicel adsorb twice as much and acid-pretreated material six times as much maximum amount of cellulase, no consistent advantage of pretreated over untreated Avicel was apparent in the conversion–time plots (Figs. 6 and 10). Consistent with the low K_{ads} and high E_{max} values, acid-pretreated Avicel performs better than other forms at very low enzyme concentrations. However, this advantage is lost at moderate enzyme or high enzyme concentrations. At 375 U/L cellulase, just below the onset of jamming effects, the time to 20% conversion ranges from 4.5 h (acid) via 5.2 h (untreated) to 6.1 h (alkaline), i.e. pretreated Avicel differs less than 20% in reaction time from untreated material.

The observed strong decrease of the enzymatic hydrolysis rate of cellulose at higher degrees of conversion, leading either to lower yield or long processing times, has been observed before and explained with limited thermal stability of cellulase, cellobiose product inhibition, cellulase inactivation, enzyme slowing down/stopping, substrate transformation into a less digestible form, heterogeneous structure studies of the substrate (Väljamäe et al., 1998; Yang et al., 2006) and corroborated via fresh substrate addition (Väljamäe et al., 1998), and restart experiments (Yang et al., 2006).

We found β-glucosidase to exert a dramatic effect on Avicel hydrolysis: without added β-glucosidase, only a few percent Avicel are converted even after several days, whereas β-glucosidase accelerates glucose production up to an activity ratio of 20, at which point complete conversion of 10% (w/v) Avicel can be attained after less than 96 h (see Fig. 5).

In conversion–time experiments at a constant [β-glucosidase]:[cellulase] ratio of 20, we identified two to three phases in the conversion–time plots (Fig. 11). *Phase I*: During phase I at low degrees of conversion (in our case, from 0% to about 30% of conversion), by far the highest rate is observed. The substrate (cellulose) offers sufficiently many free binding sites for cellulase (and β-glucosidase) molecules to act independently of each other at maximum rate. Possibly, in addition, inhibition of cellulase by cellobiose is suppressed. Phase I corresponds to the 'initial stage' in Scheme 2. *Phase II*: With degrees of conversion rising (in our case, beyond about 30%), the processing and disappearance of cellulose leads to an ever-increasing ratio of [cellulase] to [free binding sites], as the cellulase concentration remains the same throughout the reaction. The increased ratio in turn causes competition for free binding sites and, increasingly, crowding of cellulase molecules, which decreases the enzymatic hydrolysis rate. This phenomenon, termed jamming, corresponds to the 'intermediate stage' in Scheme 2. *Phase III*: Within phase III, the jamming effect continually accelerates with increasing

Table 7
Jamming parameter j (Eq. (4)) for cellulase kinetics with the assumption of no fractal kinetics on cellulase ($f = 0$)

Treatment	Cellulase ratios (1 U = 1.4 mg)				
	0.1 U/0.5 U; 12.5/62.5 U/L	1.5 U/3.0 U; 187.5/375 U/L	3.0 U/5.0 U; 375/625 U/L	5.0 U/15 U; 625/1875 U/L	15 U/50 U; 1875/6250 U/L
	Jamming parameter				
Untreated	0.008	0.003	0.014	0.032	0.085
Acid	0.001	0.002	0.011	0.033	0.093
Alkaline	0.006	0.002	0.010	0.038	0.092
Organosolv	0.010	0.003	0.009	0.039	0.091

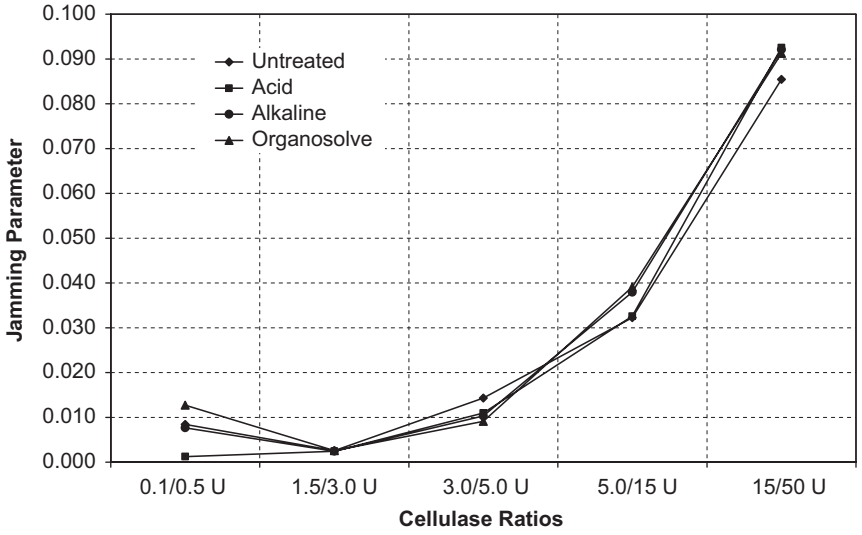


Fig. 8. Jamming parameter *j* as a function of enzyme concentration ratio of activities β-glucosidase/cellulase: 20; fractal kinetic order *f* assumed to be equal to 0).

Table 8
Jamming parameter *j* (Eq. (4)) for cellulase kinetics with fractal parameter *f* set to a value of 0.44 (Xu and Ding, 2007)

Treatment	Cellulase ratios (1 U = 1.4 mg)					
	0.1/0.5 U; 12.5/62.5 U/L	0.5/1.0 U; 62.5/125 U/L	1.0/3.0 U; 125/375 U/L	3.0/5.0 U; 375/625 U/L	5.0/15 U; 625/1875 U/L	15/50 U; 1875/6250 U/L
	Jamming parameter					
Untreated	0.002	0.003	0.003	0.012	0.030	0.087
Acid	0.001	0.004	0.003	0.011	0.030	0.091
Alkaline	0.001	0.006	0.003	0.011	0.032	0.091
Organosolv	0.001	0.004	0.003	0.010	0.032	0.090

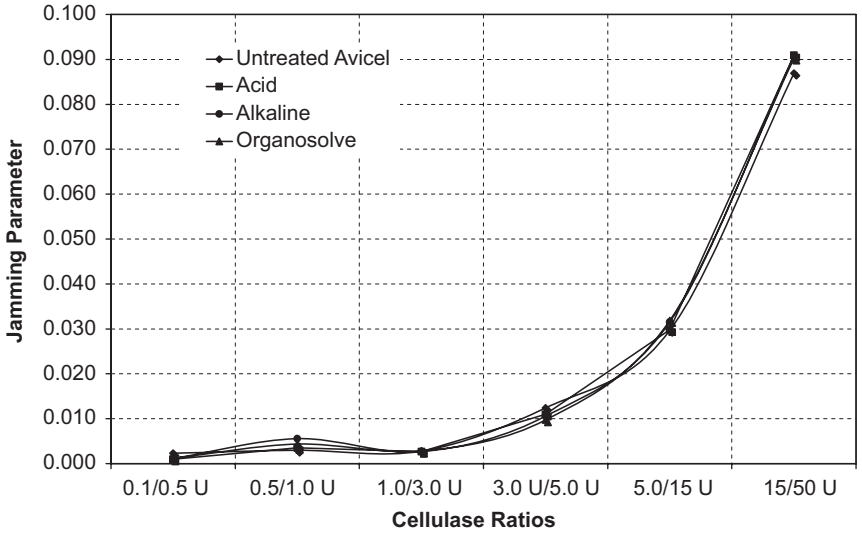


Fig. 9. Jamming parameter *j* as a function of enzyme concentration (ratio of activities β-glucosidase/cellulase: 20; fractal kinetic order $f = 0.44$ (assumed; Xu and Ding, 2007)).

conversion, as a constant number of cellulase molecules compete for ever fewer binding sites.

The decrease in reaction rate at high degree of conversion is not caused by product inhibition of cellobiose. The conversion–time plot with no β-glucosidase added to the same amounts of cellulose and cellulase displays a much-reduced rate from the start of the reaction (Fig. 5), attributed to inhibition by cellobiose. The decrease is not caused either by deactivation of cellulase. When fresh substrate (cellulose) was added to a system at intermediate conversion but with already markedly reduced

hydrolysis rate, the rate immediately jumped up almost to initial levels, demonstrating that no significant inactivation of cellulase had occurred (Väljamäe et al., 1998).

Table 9
Conversion and enzymatic hydrolysis rate data of Avicel; [β-glucosidase]:[cellulase] ratio: 20; 50 mM sodium acetate buffer, pH 5.0; 50 °C; $V = 8$ mL; 15 U (1880 U/L) cellulase

Phase	Time (h)	Conversion (%)	Rate (mM/h) glucose
3750 U/L cellulase			
I	0–2	0–35	94
II	2–10	35–52	14.5
III	10–95	52–100	1
1875 U/L cellulase			
I	0–2	0–32	52.4
II	2–29	32–50	6.5
III	29–95	50–100	0.8
187.5 U/L cellulase			
I	0–30	33	7.8
II	30–95	69	1.6
1880 U/L cellulase and 0 U β-glucosidase			
I	0–200	28	0.6

We found that jamming effects are important at high concentrations of cellulase beyond 400 U/L (Figs. 7–9) but not at lower cellulase concentrations, in contrast to (Xu and Ding, 2007), who fitted their experiments with a single jamming parameter at all concentrations. However, jamming is expected to be enzyme concentration-dependent. Remarkably, the jamming parameter j was found not to be depend neither on pretreatment nor on the fractal parameter f (Fig. 8 vs. 9): between a cellulase concentration of 400 and 600 U/L, j rises from 0.005 to 0.09, whether $f = 0$ or 0.44, in accordance with the literature, is assumed. Our finding of independence of j with pretreatment contrasts to Xu and Ding, 2007, who found marked differences between untreated and phosphoric acid-swollen cellulose (PASC).

A comparison was made with the jamming parameter data provided in Xu and Ding (2007), who used the same procedure and equations as in this work. Ding and Xu calculated a single value of 0.0004 for the jamming parameter j using a substrate concentration of 2 g/L and an enzyme concentration ranging from 0.02 to 2.4 μM. The jamming parameter values in our work range from 0.001 to 0.09 using a substrate concentration of 100 g/L and enzyme concentrations between 2.3 μM and 1.19 mM (based on enzyme molecular weight ≈61.5 kDa). With substrate and enzyme concentration 50 times and 115–500 times greater, respectively, than in the Ding and Xu study, so that the important

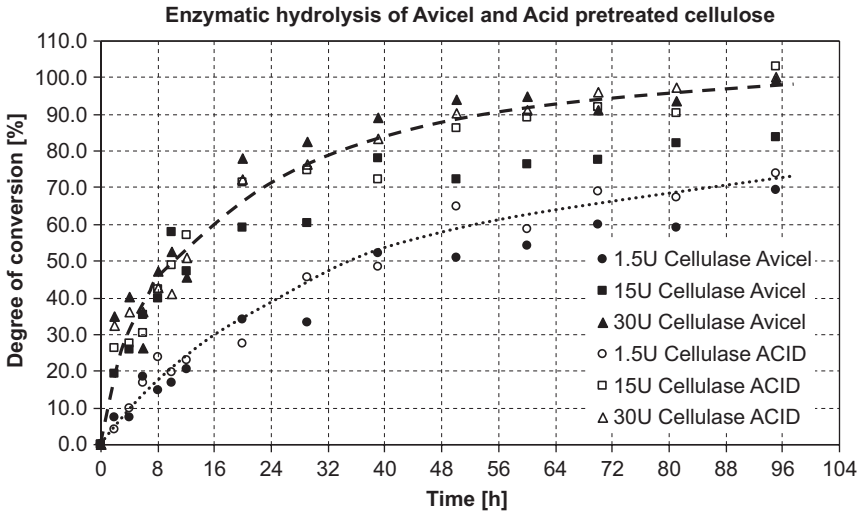


Fig. 10. Conversion–time behavior of non-pretreated and acid-pretreated Avicel at optimal ratio of activities β-glucosidase/cellulase: 20; $T = 50$ °C, pH 5.0; $V = 8$ mL. Non-pretreated: (■) 1.5 U cellulase, (●) 15 U cellulase, (▲) 30 U cellulase; acid-pretreated: (□) 1.5 U cellulase, (○) 15 U cellulase, (△) 30 U cellulase (1.5 U/8 mL = 187.5 U/L; 15 U/8 mL = 1875 U/L; 30 U/8 mL = 3750 U/L).

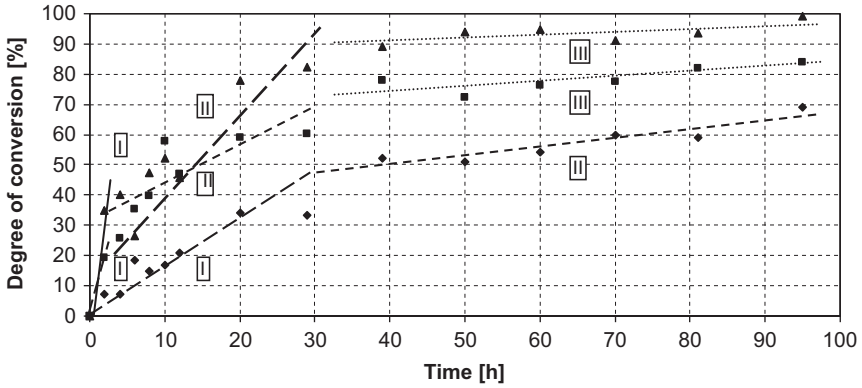
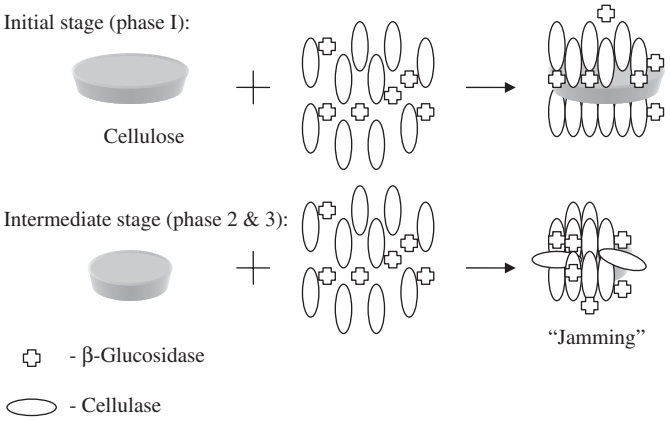


Fig. 11. Conversion–time behavior of non-pretreated Avicel at optimal ratio of activities β-glucosidase/cellulase: 20; $T = 50$ °C, pH 5.0; $V = 8$ mL: (●) 1.5 U cellulase; (■) 15 U cellulase; (▲) 30 U cellulase.



Scheme 2. Schematic model of enzyme adsorption on cellulose at initial and intermediate stage of enzymatic hydrolysis of cellulose.

enzyme:substrate ratio was two- to 10-fold higher, we found jamming parameter values two- to 250-fold higher. While the *j* values at low enzyme concentration match those of Ding and Xu, we observe the transition to higher *j* values at higher concentrations and thus to area- and/or space-limited conditions of adsorption in our study. Upon area limitations for adsorption to occur, the jamming parameter value is expected to rise, as was observed in our experiments.

5. Conclusions

Microcrystalline cellulose (Avicel) was subjected to three different pretreatments (acid, alkaline, and organosolv) before exposure to a mixture of cellulases (Celluclast®). Addition of β-glucosidase to avoid the well-known inhibition of cellulase by cellobiose markedly accelerated the cellulase reaction up to a ratio of activity units of 20. All pretreatment protocols of Avicel were found to slightly increase its degree of crystallinity in comparison with the untreated control. Adsorption of both cellulase and β-glucosidase on cellulose is significant and also strongly depends on the wall material of the reactor. The conversion–time behavior of all four states of Avicel was found to be very similar. The strong decrease in cellulose hydrolysis rate at high degrees of conversion, observed in accordance with the literature (Yang et al., 2006), is not caused by product inhibition by cellobiose or cellulase deactivation (Väljamäe et al., 1998) but by an increased ratio of cellulase molecules to free binding sites on cellulose and ensuing competition for binding sites, leading to an observed jamming effect among cellulase molecules at high cellulase concentrations and/or a high ratio of concentrations of cellulase and free cellulose binding sites. We have found that the jamming of adjacent cellulase enzymes upon one another when adsorbed on microcrystalline cellulose surface is evident, especially as the amount of enzyme in solution increases. Neither the method of pretreatment nor the presence or absence of presumed fractal kinetics has an effect on the extent of jamming of cellulase molecules.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.06.008.

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