Changes in the Enzymatic Hydrolysis Rate of Avicel Cellulose With Conversion

Bin Yang, Deidre M. Willies, Charles E. Wyman

Thayer School of Engineering, Dartmouth College, Hanover, NH 03755; telephone: (603) 646-3193; fax: (603) 646-2277; e-mail: Charles.Wyman@Dartmouth.edu

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Abstract: The slow down in enzymatic hydrolysis of cellulose with conversion has often been attributed to declining reactivity of the substrate as the more easily reacted material is thought to be consumed preferentially. To better understand the cause of this phenomenon, the enzymatic reaction of the nearly pure cellulose in Avicel was interrupted over the course of nearly complete hydrolysis. Then, the solids were treated with proteinase to degrade the cellulase enzymes remaining on the solid surface, followed by proteinase inhibitors to inactive the proteinase and successive washing with water, 1.0 M NaCl solution, and water. Next, fresh cellulase and buffer were added to the solids to restart hydrolysis. The rate of cellulose hydrolysis, expressed as a percent of substrate remaining at that time, was approximately constant over a wide range of conversions for restart experiments but declined continually with conversion for uninterrupted hydrolysis. Furthermore, the cellulose hydrolysis rate per adsorbed enzyme was approximately constant for the restart procedure but declined with conversion when enzymes were left to react. Thus, the drop off in reaction rate for uninterrupted cellulose digestion by enzymes could not be attributed to changes in substrate reactivity, suggesting that other effects such as enzymes getting ''stuck'' or otherwise slowing down may be responsible.

Keywords: cellulase; cellulose; enzymatic hydrolysis; restarted hydrolysis; substrate reactivity

INTRODUCTION

Cellulose can be hydrolyzed to glucose, a sugar that is easily fermented to ethanol and other compounds that can be sustainable substitutes for petroleum-derived products (Lynd et al., 1991). However, low conversion costs are essential to realize large scale applications for cellulose-rich feedstocks, and cellulase enzymes are attractive hydrolysis catalysts because they can achieve the nearly theoretical yields vital to economic success (Lynd et al., 1996; Wooley et al., 1999; Wyman, 1999). Typically, the rate of cellulose hydrolysis by enzymes decreases rapidly with conversion, leading to decreased yields, long processing times, and high enzyme usage; and the rate of soluble sugar formation per amount of adsorbed enzyme dramatically declines as hydrolysis progresses (Nutor and Converse, 1991; Wang and Converse, 1992). Many hypotheses have been presented to explain this observation, including thermal instability of cellulases (Caminal et al., 1985; Converse et al., 1988; Eriksson et al., 2002a,b; Gonzalez et al., 1989), hydrolysis product inhibition (Eriksson et al., 2002b; Gan et al., 2003; Gusakov and Sinitsyn, 1992; Holtzapple et al., 1990; Kadam et al., 2002; Todorovic et al., 1987), cellulase inactivation (Converse et al., 1988; Gusakov and Sinitsyn, 1992; Gusakov et al., 1987; Mukataka et al., 1983; Ooshima et al., 1990; Reese, 1982; Sinitsyn et al., 1986; Sutcliffe and Saddler, 1986), enzyme slowing down/stopping (Desai and Converse, 1997), substrate transformation into a less digestible form (Zhang et al., 1999), and/or the heterogeneous structure of the substrate (Nidetzky and Steiner, 1993; Zhang et al., 1999). ''Restart'' experiments have been used to identify factors that control the rate of cellulose hydrolysis (Desai and Converse, 1997; Gusakov et al., 1985; Nidetzky and Steiner, 1993; Ooshima et al., 1991; Valjamae et al., 1998; Zhang et al., 1999), and some results indicated that the drop in rate for continual hydrolysis of cellulose could be explained by declining substrate reactivity (Zhang et al., 1999). However, others concluded that substrate reactivity was not the principal cause for the long residence time required for good cellulose conversion (Desai and Converse, 1997). Thus, although a declining rate with time has been widely observed for continual hydrolysis of cellulose, the cause of this observation is still uncertain (Valjamae et al., 1998; Zhang et al., 1999).

Through introducing fresh enzyme to partially converted cellulose, ''restart'' experiments provide a valuable tool to assess whether substrate reactivity changes with conversion. However, a key to this approach is complete enzyme removal from the solid cellulose before introduction of new cellulase for continued hydrolysis, and this study presents ''restart'' results based on application of a new technique to assure removal of old enzyme from Avicel cellulose. High enzyme loadings were used to give virtually complete coverage of accessible cellulose and assure that the hydrolysis rates

Correspondence to: C.E. Wyman
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reflected the reactivity of cellulose. These results provide a new perspective on the change in cellulose reactivity with conversion during hydrolysis.

MATERIALS AND METHODS

Substrates

Avicel PH101 (Sigma, St. Louis, MO), a microcrystalline cellulose containing more than 97% cellulose and less than 0.16% water soluble materials, was used as the substrate for all experiments reported here.

Protein Measurement

The nitrogen content of all samples, including enzyme, Avicel, and residual solids following enzymatic hydrolysis of Avicel, were determined by a Flash EA^{TM} 112 N/Protein plus CHNS/O Analyzer (CE Elantech, Lakewood, NJ). The protein content in percent by dry weight was then calculated from these nitrogen readings using a procedure published by the National Renewable Energy Laboratory (NREL) to estimate the amount of enzyme or protein (Hames et al., 2004). Our initial data showed that measuring protein in the solids provided more consistent results than analyzing the liquid portion by the Bio-Rad assay (data not presented).

Enzymes

Hydrolysis experiments were performed using the complete *Trichoderma reesei* cellulase system Spezyme CP (Genencor, Palo Alto, CA) from a single batch maintained by NREL that had a titer of 28 FPU/mL and a protein content of 95.4 mg protein/mL. The commercial β-glucosidase preparation Novozym 188 (Sigma) with a titer of 450 CBU/mL and protein content of 103 mg protein/mL was added to some experiments to reduce end-product inhibition due to cellobiose accumulation. Protein contents of both enzymes were measured as described above, and activity measurements were as described elsewhere (Ghose, 1987).

Avicel Solubilization

Enzymatically hydrolyzed cellulose was filtered through a Steriflip filter unit (0.22 μm Millipore Express PLUS PES membrane, Millipore, Bedford, MA), and the residual solids were dried in a Precision oven (Precision, Winchester, VA) at $105 \pm 3^{\circ}$C until a constant weight was achieved. Samples were cooled in a desiccator, and cellulose solubilization was determined from the loss in dry weight.

Enzymatic Hydrolysis System

Batch hydrolysis was carried out in 50 mL polypropylene tubes (Millipore) containing 2% cellulose (w/v) in 40 mL of 50 mM acetate buffer (pH 4.8). The reaction tubes were inserted in a ''Roto-Torque'' fixed speed rotator (model 7637-20, Cole-Parmer Instrument Company, Vernon Hills, IL), and the entire system was placed in an incubator (model 1540, VWR International, Cleveland, OH) at $50 \pm 1^{\circ}$C. The rotation speed was 68 rpm. Substrates were pre-incubated at 50°C for sufficient time (about 15 min) to reach reaction temperature prior to the addition of enzyme. The solid residue was recovered by filtration with the Steriflip filter unit, and the solubilization of Avicel was measured as described above.

Uninterrupted and ''Restart'' Cellulose Hydrolysis

A 2% w/v concentration of cellulose was hydrolyzed at enzyme loadings of 60 and 240 FPU/g cellulose for 1, 2, 3, 4, 5, 15, and 16 h using the equipment noted previously, and the extent of hydrolysis was measured to establish the reaction rate for a typical uninterrupted batch hydrolysis operation. However, for ''restart'' experiments, solids produced at these times were recovered by filtering the hydrolyzate through the Steriflip filter unit. The solid residue was then mixed with 40 mL phosphate buffer (pH 7.4) and Pronase E (one unit will hydrolyze casein to produce color equivalent to 1.0 mole (181 g) of tyrosine per min at pH 7.5 at 37°C), a mixture of non-specific endo- and exo-proteinases (P5147, Sigma), at a final concentration of 10 mg/mL, and the mixture was rotated at 68 rpm at 37°C overnight to complete cellulase proteolysis. Next, a general use proteinase inhibitor cocktail mixture (P2714, Sigma) was added at a ratio of 1 mL of cocktail solution/mg Pronase and incubated at 37°C with a rotational speed of 68 rpm for 2 h to stop cellulase proteolysis. Then the mixture was filtered through the Steriflip unit, and the solid residue was washed with distilled water, 1.0 M NaCl, and distilled water again, in that order. The solid residue was mixed with fresh 50 mM acetate buffer (pH 4.8) to achieve 2% cellulose (w/v), and enzyme was added to achieve a loading of 60 FPU/g cellulose. Enzymatic hydrolysis was performed for 1 h by the same protocol described above, and the extent of cellulose hydrolysis was measured.

Hydrolysis With β-Glucosidase Addition

Avicel was hydrolyzed in two parallel hydrolysis experiments with a Spezyme CP loading of 60 FPU/g cellulose that was supplemented with Novozyme 188 β-glucosidase at a loading of 120 CBU/g cellulose. After 1 h of hydrolysis at 50°C, the reaction vials were immediately chilled on ice to stop the reaction, and the contents were filtered through the Steriflip unit. The solid residue was then cleaned by the ''restart'' procedure using proteinase, proteinase inhibitor, and washing, as described above. Material from one set of these experiments was used to determine Avicel solubilization, and that from the other was mixed with fresh buffer (i.e., 40 mL) and the same amount of fresh enzyme as employed initially, that is, 60 FPU/g cellulose supplemented with Novozyme 188 β-glucosidase at 120 CBU/g cellulose, to hydrolyze the residual cellulose for 1 h at 50°C. This

procedure was repeated four times with Avicel solubilization determined for each.

As a control, Avicel was hydrolyzed with Spezyme CP at a loading equal to the total from all of the above experiments, 240 FPU/g cellulose supplemented with Novozyme 188 β-glucosidase at a loading of 480 CBU/g cellulose. After hydrolysis for 4 h, the mixture was filtered as described before, and the solid residue was washed with distilled water in the Steriflip filter unit to determine Avicel solubilization. All samples were run in duplicate or triplicate.

Calculation of Maximum Cellulase Adsorption

Adsorption measurements were conducted with 2% cellulose (w/v) in 0.05 M Na acetate buffer (pH 4.8) to which was added Spezyme CP at final protein loadings ranging from 1 mg/mL to 20 mg/mL. Cellulose substrate was prepared by enzymatic treatment of Avicel followed by proteinase treatment and washing as described above. Enzyme adsorption was equilibrated while the sample was mixed by turning end over end for 2 h at 4°C to prevent hydrolysis. The solid was then collected by centrifugation at 14,000 rpm for 8 min in a Allega^{TM} 6R Centrifuge (Beckman Coulter, Fullerton, CA) and dried in the Precision oven at 105°C prior to determination of the amount of adsorbed enzyme by the nitrogen method described above. Free cellulase was calculated as the difference between bound protein and the total protein initially added to the reaction medium, and all measurements were made in duplicate or triplicate. The maximum enzyme adsorption capacity $[A]_{max}$ in units of mg cellulase/mg cellulose was then estimated using the Langmuir isotherm (Beldman et al., 1987):

$$[E]_{ad} = \frac{[A]_{max} \times [S]_{total} \times [E]_{free}}{K_d + [E]_{free}} \quad (1)$$

in which $[E]_{ad}$ is the concentration of adsorbed protein (mg cellulase/mL); $[E]_{free}$ is the concentration of cellulase in solution (mg cellulase/mL); $[S]_{total}$ is the total substrate concentration (mg cellulose/mL); and K_d is an equilibrium constant (mg/mL) that was obtained along with $[A]_{max}$ by fitting the data. The maximum adsorption capacity calculated by this method was judged to better account for the total amount of cellulose accessible to cellulase and compensate for enzyme adsorbing on itself than simply applying $[E]_{ad}$. Although the maximum adsorption capacity accessible to enzyme was calculated from adsorption data at 4°C, it was not judged to change significantly at the hydrolysis temperature and should provide a useful indication of cellulose accessibility in any event.

Conversion and Rates

Cumulative conversion was calculated as the total loss in cellulose dry mass to a particular time divided by the mass at time zero before reaction of any of the cellulose. Incremental conversion was determined as the loss in dry mass from the beginning to the end of a period divided by the dry mass of the material at the start of that time period and was reported for the start time. The 1 h hydrolysis rate was determined by dividing the amount of cellulose hydrolyzed in 1 h following addition of enzyme by the total amount of cellulose available at the start of that period and was reported at the start time. The specific hydrolysis rate was defined as the 1 h rate divided by the maximum enzyme adsorption capacity determined from the Langmuir isotherm (4°C) for a sample collected at the corresponding start time.

RESULTS

Solids were produced by Avicel hydrolysis at a loading of 60 FPU/g cellulose for 2 h to evaluate the effectiveness of five different techniques for removing protein. As shown in Figure 1, the nitrogen content in the solids was measured to be about 2.2% when the solids were boiled for 10 min and then washed with water, 1.0 M sodium chloride solution, and water again (Desai and Converse, 1997). Furthermore, it dropped considerably to about 1.2% when the solids were washed with DI water at room temperature. However, this value was still about three times the $0.40 \pm 0.02\%$ value for Avicel prior to treatment. Application of proteinase followed by washing the solids with 1.0 M NaCl and then water dropped nitrogen levels to $0.60 \pm 0.07\%$, much closer to that for Avicel prior to adding any enzyme. Introduction of proteinase inhibitors into this sequence following proteinase treatment dropped the nitrogen content to about $0.50 \pm 0.05\%$. However, values virtually equal to those for the original Avicel resulted when the sequence of adding proteinase and then proteinase inhibitors was followed by

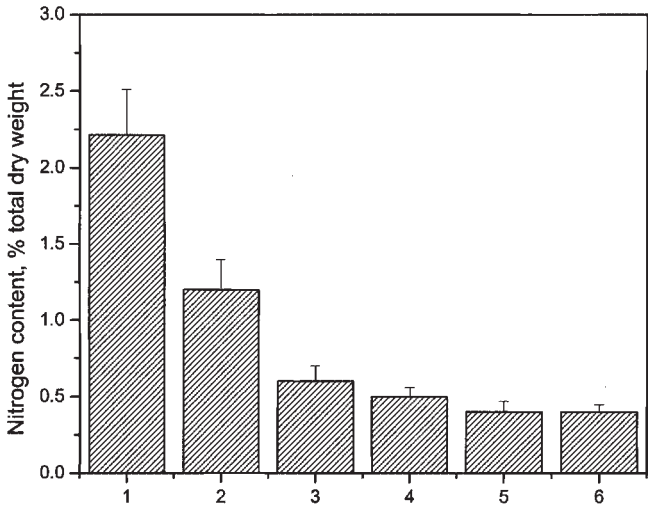

Figure 1. Nitrogen content in solid residues produced after 2 h of enzymatic hydrolysis and then application of one of the following treatments: (1) Boil for 10 min, and then wash with water, 1.0 M NaCl solution, and water, in that order. (2) Wash with water. (3) Add proteinase, and then wash with 1.0 M NaCl and water, in that order. (4) Add proteinase followed by proteinase inhibitor, and wash with 1.0 M NaCl and water, in that order. (5) Add proteinase followed by proteinase inhibitor, and wash with water, 1.0 M NaCl, and water in order. (6) Avicel only.

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Table I. One-hour hydrolysis rate and relative mass remaining in the solids for uninterrupted and restarted hydrolysis of Avicel cellulose.

	Uninterrupted hydrolysis		Restarted hydrolysis	
Time (h)	Relative mass remaining (%)	Incremental rate, %/h	Relative mass remaining (%)	Incremental rate, %/h
0	100	20 ± 1.1	100	20 ± 1.1
1	80.4 ± 3.7	10 ± 1.0	65.6 ± 2.1	18 ± 4.8
2	72.6 ± 2.4	8.3 ± 0.5	51.8 ± 1.3	21 ± 2.2
3	66.1 ± 3.6	7.6 ± 0.4	40.9 ± 3.8	21 ± 2.4
4	61.5 ± 1.9	4.9 ± 0.8	32.3 ± 4.6	21 ± 3.3
5	58.5 ± 2.4	—	25.5 ± 5.2	—
15	36.2 ± 1.8	1.9 ± 2.0	18.2 ± 3.2	28.4 ± 2.2

washing with water, 1.0 M NaCl, and then water. All wash volumes were 10 times the total volume of the original solution.

The new restart method was applied to Avicel for samples collected after total cellulose hydrolysis and compared to the results for uninterrupted hydrolysis at the same times. As shown in Table I, cumulative conversions for uninterrupted hydrolysis at 1, 2, 3, 4, 5, and 15 h were 20, 28, 34, 39, 42, and 64%, respectively. However, when incremental cellulose conversion for uninterrupted hydrolysis was calculated to facilitate comparison to restart data in Table I and Figure 2, the 1 h rate for uninterrupted hydrolysis dropped rapidly from 20% of available cellulose during the first hour to about 10% during the second. It then dropped further to about 8% during the third and fourth hours and then down to about 5% in the fifth and 2% at 15 h. By contrast, the 1 h incremental rates for the restart experiments dropped slightly from the same initial value 20% to about 18% in the first hour and then increased some to about 21% for the next three increments. It is also important to note that the 1 h hydrolysis rate for restarted hydrolysis rose some to about 28% during the period from 15 h to 16 h of digestion.

Incremental hydrolysis rates were calculated versus cumulative cellulose conversion for both uninterrupted and restart runs to better understand how the rate was affected by conversion. As shown in Figure 3, rates dropped rapidly with increasing conversion for uninterrupted hydrolysis from about 20%/h initially to a value of only about 1.9%/h when somewhat over 40% of the cellulose had reacted after 15 h. On the other hand, the rates for restart experiments were almost constant at about 20%/h until about 68% of the cellulose had reacted and then increased to close to 28%/h at 78% conversion after 15 h. Thus, cellulose remained very reactive when enzyme was completely removed over a wide range of conversions.

The surprisingly constant rate with conversion led us to evaluate whether the enzyme addition approach affected the results, and cellulose produced after 1 h of enzymatic hydrolysis was processed in four different manners. First, new buffer was simply added to the mixture of solids,

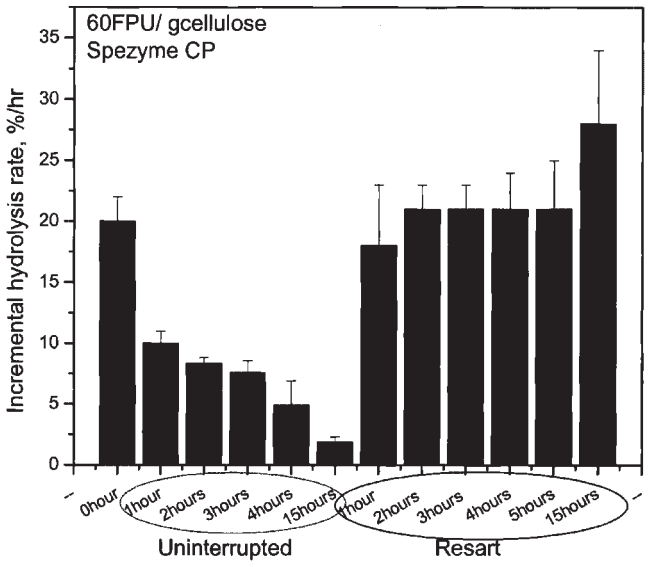

Figure 2. Comparison of cellulose conversion in a 1 h period for uninterrupted and restarted hydrolysis of Avicel cellulose beginning at each total cellulose reaction time shown.

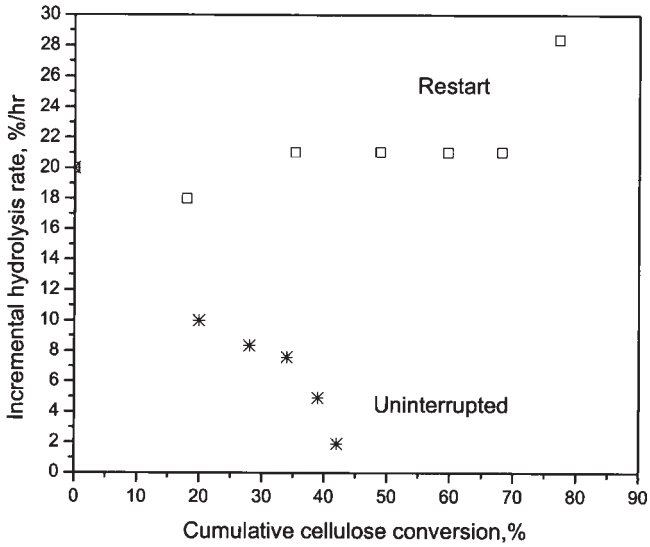

Figure 3. Incremental 1 h hydrolysis rates versus cumulative cellulose conversion for uninterrupted and restarted hydrolysis of Avicel cellulose.

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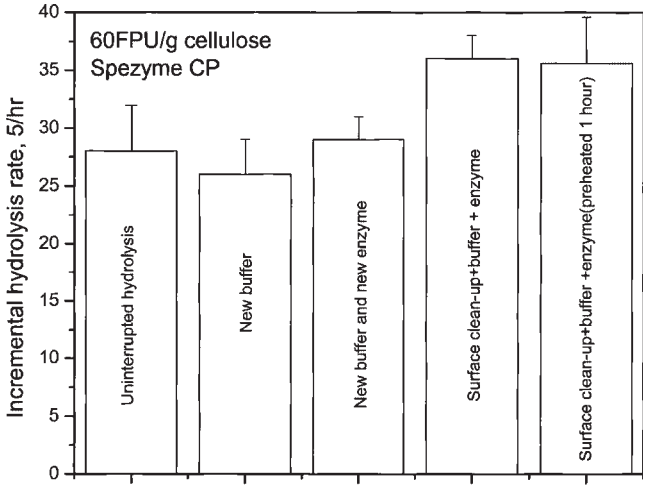

Figure 4. Effect of different treatments on 1 h hydrolysis rates of Avicel cellulose that had been enzymatic hydrolyzed for 1 h.

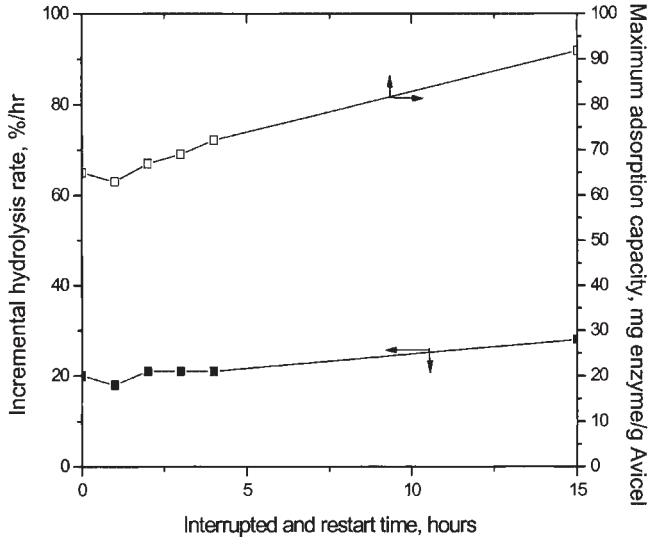

Figure 6. One-hour hydrolysis rates and corresponding maximum enzyme adsorption capacities at beginning of each period for restarted hydrolysis.

cellulase, and other components, but the results were about the same as when the reaction was continued for another hour, as shown in Figure 4. Adding fresh enzyme in addition to new buffer increased the rates slightly. However, applying the full sequence of proteinase, proteinase inhibitor, and wash with water, NaCl solution, and water again followed by fresh enzyme and new buffer increased the hydrolysis of cellulose from about 25% for continual hydrolysis to about 36%. A similar result was also observed if the system was heated for 1 h to bring it to the target digestion temperature prior to adding enzyme.

Next the effect of high levels of β-glucosidase supplementation was tested. As shown for the bar farthest to the right in Figure 5, the overall conversion for uninterrupted hydrolysis with a high cellulase loading of 240 FPU/g cellulose supplemented with 480 CBU/g cellulose of β-glucosidase was only about 40% after 4 h. However, applying our surface cleaning technique and restarting with fresh cellulase and β-glucosidase gave an approximately constant 1 h rate of 25% over an entire 5 h period even though only one-fourth of the amount of cellulase and β-glucosidase was in the system at any one time as for the uninterrupted hydrolysis. In addition, the cumulative conversion for these same restart experiments was 80% after 5 h, almost double that for continual hydrolysis.

When the maximum enzyme adsorption capacity calculated from Equation (1) was plotted for the cellulose produced at each reaction time, it followed a similar pattern to the 1 h hydrolysis rates for restarts, as shown in Figure 6. Furthermore, the specific 1 h hydrolysis rate was almost constant throughout the reaction period, as shown in Figure 7. However, the 1 h restart rate demonstrated a possibly slight

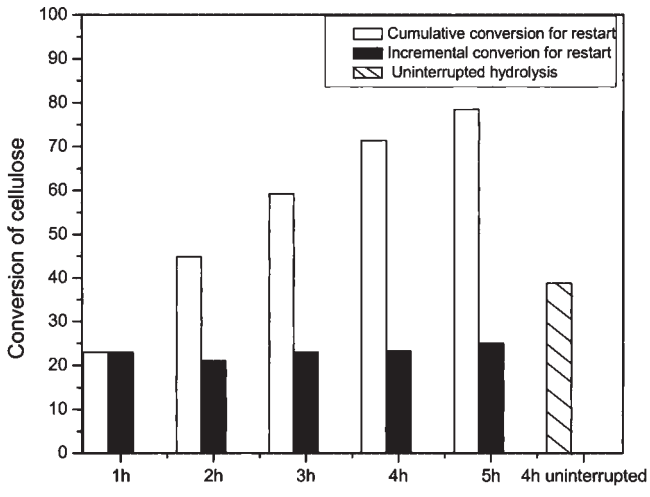

Figure 5. Incremental (1 h) and cumulative cellulose conversion for Avicel PH101 for restart experiments with β-glucosidase supplementation. The cumulative conversion of Avicel cellulose after 4 h of uninterrupted hydrolysis is shown on the far right.

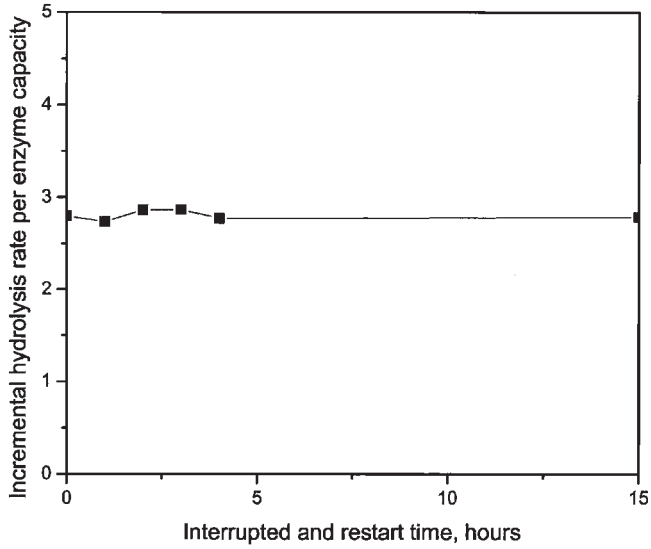

Figure 7. Specific hydrolysis rate of Avicel cellulose for restart experiments.

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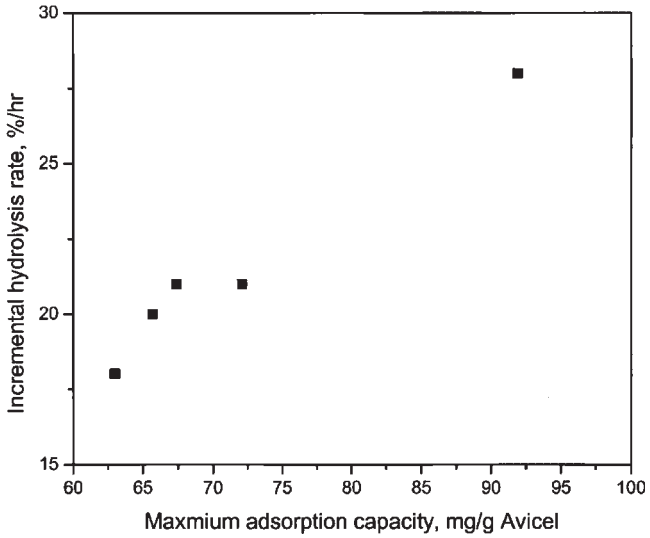

Figure 8. One-hour hydrolysis rates versus maximum enzyme adsorption capacities for restart experiments.

drop off with increasing maximum enzyme adsorption capacity, as shown in Figure 8.

DISCUSSION

The application of proteinase followed by proteinase inhibitors, followed by a wash sequence of water, 1.0 M NaCl, and water again was found to be effective in removing cellulase from cellulose as indicated by nitrogen content in the solids. Thus, we believe this approach clears the substrate of enzyme and provides a surface that is virtually free of bound protein that could otherwise interfere with subsequent protein adsorption and action.

Although the cellulose hydrolysis rate continually declined when the enzyme and solids were left together, the data clearly showed that the cellulose reaction rate did not drop with time or conversion when cellulose was cleared of enzyme. In fact, the rate increased slightly after the first 2 h and became even greater at the 15 h hold time that corresponded to reaction of about 78% of the cellulose. The relatively constant digestion rate with conversion could not be attributed to the addition of fresh enzyme or buffer, further reinforcing the idea that the substrate itself was at least as susceptible to enzyme attack after considerable reaction as it was initially. Adding β-glucosidase did not change the pattern of a nearly constant cellulose hydrolysis rate with conversion for restarts and a steady decline in hydrolysis rate with conversion for continual hydrolysis. In addition, the cellulose concentrations were too low for much sugar to accumulate. Thus, end product inhibition by cellobiose or glucose was not believed to account for the differences in rate with conversion between restarted and uninterrupted hydrolysis.

These results indicated that cellulose did not lose reactivity as it was converted over time and that the surface seemed to become more accessible later in the reaction. Furthermore, because we would expect enzyme to occupy most of the sites available on the cellulose surface at the high enzyme loadings used, the maximum enzyme-binding capacity should closely approximate how much enzyme is attached to cellulose. Thus, the close relationship between hydrolysis rates and maximum enzyme-binding capacities and the resulting nearly constant specific hydrolysis rates (i.e., the hydrolysis rate per maximum adsorption capacity) for restart experiments were consistent with hydrolysis being controlled by the amount of enzyme attached to the surface rather than by any changes in surface character. The possible gradual reduction in rate as maximum enzyme-binding capacity increased suggested that enzyme may interfere with itself as more is adsorbed on the surface, and steric hindrance of this nature would not be unexpected given the large size of cellulase enzymes. Overall, the results are consistent with the idea that the slow down in rate for uninterrupted hydrolysis is not due to a loss in cellulose reactivity but must be due to the action of the enzymes being slowed down by obstacles that interfere with their path or a loss in activity and/or processivity making them less effective (Breyer and Matthews, 2001; Zou et al., 1999).

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