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Adsorption of *Trichoderma reesei* cellulases on protein-extracted lucerne fibers

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SUMMARY

Protein-extracted lucerne fibers (PELF) had a higher adsorptive capacity for *Trichoderma reesei* cellulases than a variety of other cellulosic substrates compared on an equal carbohydrate basis. Adsorption at room temperature reached a maximum at about 5 min; desorption was directly proportional to the extent of carbohydrate solubilization. Cellulase binding conformed to a Langmuir isotherm; the maximum cellulase-binding capacity of PELF was 111 filter paper units per g dry weight. About 85% of the cellulase was recovered in the soluble fraction after PELF hydrolysis. Soluble carbohydrates in the hydrolysate inhibited cellulase adsorption to fresh substrate (50% inhibition at a hydrolysate concentration of 7% glucose equivalents). The effect of these carbohydrates on cellulase adsorption was a complex one composed of both enhancing and inhibitory influences. Artificial hydrolysates (known sugars in proportions identical to actual hydrolysates) inhibited adsorption, but glucose, cellobiose and xylose resulted in adsorption enhancement. Acid treatment of the hydrolysate to convert oligosaccharides to monomers increased reducing sugar concentrations and eliminated its capacity for adsorption inhibition.

INTRODUCTION

The extent of cellulase adsorption on the surface of cellulosic substrates is the major factor determining the rate of hydrolysis [16]. Cellulase adsorption also provides a promising mechanism for enzyme recovery/recycling in industrial-scale lignocellulosic biomass, since fresh addition of lignocellulose to the product stream is the cheapest and simplest vehicle for returning enzyme to the reactor [4]. A recent communication [15] described production of *Trichoderma reesei* C30 cellulases during growth on protein-extracted lucerne fibers (PELF). The adsorption of endoglucanase and cellobiohydrolase on PELF was severely inhibited by the PELF hydrolysate. Because this inhibition would decrease the feasibility of using PELF as an adsorptive vehicle in a cellulase recovery system, we made a detailed study of cellulase adsorption on PELF and the inhibition of that adsorption by PELF hydrolysate.

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MATERIALS AND METHODS

Preparation of PELF

The process for extraction of protein from lucerne and the major component composition of the resultant PELF may be briefly described as follows. Freshly harvested lucerne is mechanically pulped and pressed to yield a presscake and a green juice containing about 85% of the soluble protein. The juice is heated by steam injection to coagulate the protein which is recovered by centrifugation and dried. The approximate composition (percent of dry wt.) of the presscake fiber is 39% cellulose, 18% lignin, 14% hemicellulose, 14% pectin, 7% crude protein and 3% ash [17]. Some PELF samples were pre-treated with dilute alkali (1 g NaOH/7 g PELF) at 85°C for 30 min, washed repeatedly with distilled H_2O and adjusted to pH 5.0 with 0.5 N H_2SO_4. Excess moisture was expressed at 4000 p.s.i. in a piston press (Triton Electronics Ltd, Essex, U.K.). The partially dewatered cakes were stored at −18°C until use. The batch-to-batch variation in dry matter content varied from 20.1 to 33.7%. Alkali-treated PELF composition averaged 49% cellulose, 15% hemicellulose, 11% lignin, 7% pectin, 6% protein and 6% ash.

Cellulase source

The cellulase complex used in this study was produced by *T. reesei* in submerged culture and marketed by Novo Industri A/S (Bagsvaerd, Denmark) under the trade name Celluclast. This preparation had a specific cellulase activity equivalent to a filter paper activity (FPA) of 0.32 IU/mg protein at a concentration of 30.6 IU/ml. The low cellobiase (EC 3.2.1.21) activity of the Celluclast preparation was supplemented by the addition of Cellobiase (produced in submerged fermentation by *Aspergillus niger* and marketed by Novo Industri A/S). This supplemented Celluclast had a cellobiase activity of 36.7 IU/ml.

Assays

Cellulase was measured as FPA according to the procedure of Mandels et al. [9]. Average standard deviation between replicates was ± 7.65%. All samples were assayed in triplicate and averaged. Cellobiase activity was estimated by the β-glucosidase assay of Sternberg [14]. Assays were done in duplicate; average standard deviation was ± 4.9%. Reducing sugar and protein concentrations were estimated by the method of Bernfeld [2] and Lowry et al. [8], respectively. Average standard deviations for reducing sugars and proteins were ± 0.33% and ± 2.14%, respectively.

Hydrolysate production

Alkali-treated PELF (5% w/v) was suspended in 1 liter of 0.05 M acetate buffer pH 5.0 containing 0.02% NaN_3 and cellulase complex (30 FPU and 36 cellobiase units/g PELF). The thick slurry was mechanically stirred at 45°C. At about 12 h intervals, PELF was added at 5% increments to a total of 20%. After hydrolysis, insoluble material was removed by centrifugation (12 200 × *g*, 2°C, 30 min). Residual cellulase activity was removed from hydrolysates by two-step ultrafiltration in a stirred Amicon Model 202 cell pressurized at 500 kPa with oxygen-free nitrogen at room temperature. The viscous hydrolysate was first passed through a GR 61PP membrane (Danski Sukkerfabrikker, Denmark) having a MW exclusion of 2×10^4, then through an Amicon UM10 membrane (MW exclusion of 10^4). This method reduced residual FPA in the filtrate to below 0.04 IU/ml. The ultrafiltration method was superior to heat denaturation for removal of cellulases due to lower protein background and potential for side reactions caused by heating.

Characteristics of PELF hydrolysate

Hydrolysate sugars were identified on a Hewlett Packard Model 5880A gas chromatograph with autosampler and flame ionization detector as previously described [15]. Hydrolysate sugars were also analyzed on a high performance liquid chromatography system consisting of an Isco Model 314 pump, a Bio-Rad HPX-87P column heated to 85°C, and an Erma Model ERC-7510 refractive index detector. Flow rate was 0.67 ml distilled, degassed water per min. Oligosaccharide standards were prepared by HCl hydrolysis of Whatman CF11 cellu-

lose powder by the method of Miller et al. [10]. Other sugar standards were purchased from Sigma Chemical Co. Sugars were quantitated by peak area on an LDC/Milton Roy C1-10 plotter integrator.

Adsorption reactions

Adsorption experiments were routinely run at room temperature (22–25°C) to eliminate artifacts from temperature variation during sampling and to duplicate conditions easily obtainable on an industrial scale. At this temperature, the cellulase reaction rate was only about one-third that of the maximum at 50°C. Reactions (100 ml volumes) were started by adding appropriate volumes of Celluclast to PELF slurries in 0.05 M acetate buffer pH 5.0 to yield initial free fluid enzyme concentrations of 1.58 FPU and 1.90 cellobiase IU/ml. Clarified reaction fluid samples were taken at intervals up to 2 h by filtration through Whatman type GF/A glass fiber pads under pressure in Millipore model 42-025010 ultrafiltration cells. Residual soluble cellulase components were precipitated by addition of 3 volumes acetone at room temperature to separate them from interference by hydrolysate sugars during assay. The FPA of these re-dissolved samples was compared to similarly treated zero-time samples having the same initial Celluclast concentration but no PELF. Percent cellulase adsorption was calculated as:

$$\frac{\text{initial} - \text{residual}}{\text{initial}} \times 100 = \text{percent adsorbed}$$

RESULTS

Influence of PELF pre-treatment on cellulase adsorption

PELF had a higher cellulase adsorptive capacity than a variety of other cellulosic substrates (Table 1). NaOH treatment of PELF doubled the fibers' ability to bind cellulases and quadrupled its initial rate of hydrolysis by the *T. reesei* cellulases. Because of the marked improvement in adsorptive hydrolysis rate and capacity, we used NaOH-treated PELF in all further experiments.

PELF hydrolysis rate and products

At a temperature of 45°C (which may be employed under industrial conditions as a compromise between the maximum reaction rate and enzyme stability), a conversion equivalent to 43–50% of the treated PELF dry matter was attained within 24 h. The addition of 5% (w/v) increments of PELF resulted in hydrolysates having reducing sugar concentrations (as glucose equivalents) of about 9.4%. The concentration range of identified sugars in PELF hydrolysates is shown in Table 2. Some components of the hydrolysate could not be identified.

Table 1

Adsorptive capacities and conversion rates of PELF compared to known cellulosic substrates

Substrate[a]	Cellulase adsorbed[d] (%)	Protein adsorbed [d] (%)	Initial rate of carbohydrate conversion (%/h)
Cotton fibers	14.7	5.1	2.3
Cellulose powder[b]	2.6	3.8	2.2
Microcrystalline cellulose[c]	26.3	33.7	14.4
PELF	26.8	–[e]	20.7
PELF (NaOH-treated)	65.2	54.0	80.7

[a] Concentration adjusted to provide 14 mg total carbohydrate per FPU.
[b] Whatman CF11.
[c] Sigmacel, type 100.
[d] Maximum percentages during 2 h reaction.
[e] PELF protein precluded measurement of adsorbed protein.

The sum of identified sugars as calculated from GC measurements averaged about 24% lower than total reducing sugars estimates.

Desorption of cellulose from PELF during hydrolysis

After enzymatic hydrolysis of NaOH-treated PELF for 24 h at 45°C, an average of 87.7% of total carbohydrate was converted to soluble sugars. The hydrolysate contained 88.2% of the original FPA, indicating that most of the cellulases were released as their substrate was solubilized. There was a consistent correlation between the percent of carbohydrate conversion and the release of FPA from PELF. During routine experiments, maximum FPA adsorption (65%) was observed at 5 min, at which time residual carbohydrate was still 90% of the zero-time level. If both values were set arbitrarily to 1.0, at 20 min carbohydrate conversion would average 0.11 and cellulase desorption would average 0.12 as fractions of their respective observed maxima. At 120 min, the values for carbohydrate conversion and cellulase desorption would be 0.27 and 0.28, respectively. This consistent ratio of cellulase desorption to carbohydrate solubilization indicated that carbohydrate was the major PELF component which bound cellulase and that the rate of carbohydrate conversion determined the rate of cellulase desorption.

The binding of *T. reesei* cellulase on purified cellulosic substrates has been found to obey the Langmuir isotherm [1,11]. It was therefore of interest to ascertain whether the cellulase binding to PELF also conformed to the isotherm. Fig. 1 shows cellulase adsorbed per gram of PELF as a function of free cellulase at equilibrium. The adsorption data conformed well to a Langmuir isotherm: a double-reciprocal transformation of these data had a linear regression correlation coefficient of 0.99 and yielded a value for maximum cellulase binding capacity of 111 FPU/g dry PELF.

Inhibition of cellulase adsorption by hydrolysate

Fig. 2 shows the inhibition of cellulase adsorption on PELF as a function of hydrolysate concentration. This relationship took the form typical of a saturation curve. The data of the hyperbolic curve were subjected to a linear transformation in a double-reciprocal plot. The hydrolysate concentration required for 50% inhibition of cellulase adsorption was calculated to be 7.25% w/v expressed as glucose equivalents.

We wanted to determine whether this inhibitory

Table 2

Sugar composition of hydrolysates

Values are given as ranges obtained from three hydrolysates by GC analysis.

Sugar	Percent of total identified sugars
Arabinose	3.6– 4.3
Cellobiose	1.4– 1.7
Fructose	0.2– 0.4
Galactose + mannose	4.5– 6.3
Glucose	69.7–72.8
Rhamnose	3.6– 3.8
Ribose	2.8– 4.9
Xylose	15.2–18.9
Total pentoses	19.4–24.1
Total hexoses	74.2–79.2

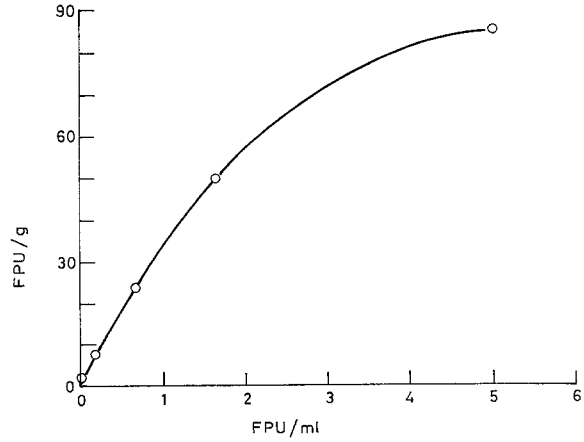

Fig. 1. Cellulase adsorption (FPU per g PELF dry wt.) as a function of free cellulase concentration at equilibrium. Each point shows the average of three values for maximal adsorption observed during a 2 h incubation at room temperature for each cellulase/PELF ratio.

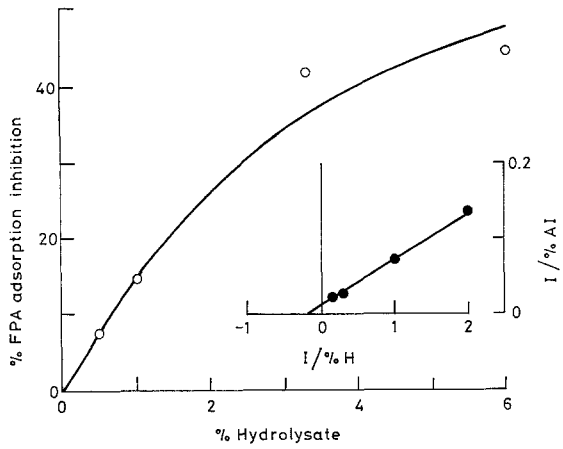

Fig. 2. Relationship of hydrolysate concentration (w/v as glucose equivalents) to extent of cellulase (FPA) adsorption inhibition. Each point was obtained by running a time course (2–120 min) for adsorption and subtracting values at each time from control values. Inset is a double-reciprocal plot of the data in which H and AI represent hydrolysate and adsorption inhibition, respectively.

effect would be exerted only by hydrolysate obtained from NaOH-treated PELF. To provide a basis for comparison, we did a similar experiment using PELF treated in the ammonia freeze-explosion (AFEX) process developed by Dale and Moreira [5]. AFEX-pre-treated PELF, hydrolyzed under our standard conditions (30 FPU/g dry fiber, pH 5.0, 45°C) yielded about the same conversion rate (25% dry matter conversion after 5 h) as did the alkali-treated PELF described earlier. The time course of AFEX-treated PELF hydrolysis is shown in Fig. 3a. However, the AFEX-treated PELF had only about one-third of the cellulase-adsorptive capacity, and this capacity was completely abolished by the presence of hydrolysate equivalent to 6% glucose (w/v) obtained from the AFEX-treated fibers (Fig. 3b).

In the absence of enzyme, NaOH-treated PELF released only very small amounts of protein and reducing sugar on prolonged incubation [15]. Therefore, the inhibition appeared to be caused by products released from the fibers during cellulolytic hydrolysis. The sugars were tested for inhibitory properties by preparing an artificial hydrolysate, the sugar composition of which was based on the average values of the data in Table 2. This artificial hydrolysate was used at a final concentration equivalent to 6% glucose in an adsorption reaction mixture. Results are shown in Fig. 4. Adsorption of cellulase was reduced only to about one-half the extent observed with the 6% enzymatic hydrolysate. The major identified components of the enzymatic hydrolysate were glucose and xylose. These sugars altered the shape of the adsorption time course, but did not cause inhibition of adsorption, while addition of cellobiose (the major product of cellulose hydrolysis through exoglucanase activity)

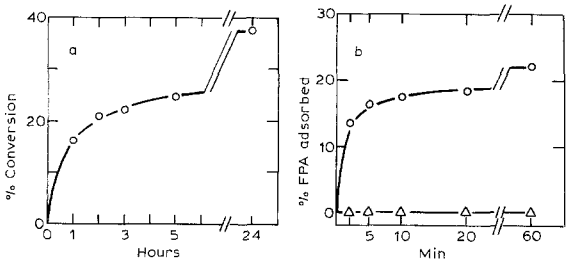

Fig. 3. (a) Conversion of AFEX-treated PELF to soluble sugars by 30 FPU/g dry fiber at 45°C, pH 5. (b) Inhibition of cellulase (FPA) adsorption on AFEX-treated PELF by hydrolysate from reaction depicted in (a); reducing sugar concentration of hydrolysate was adjusted to the equivalent of 6% (w/v) glucose. Symbols: control (no hydrolysate, ○), with 6% hydrolysate (△).

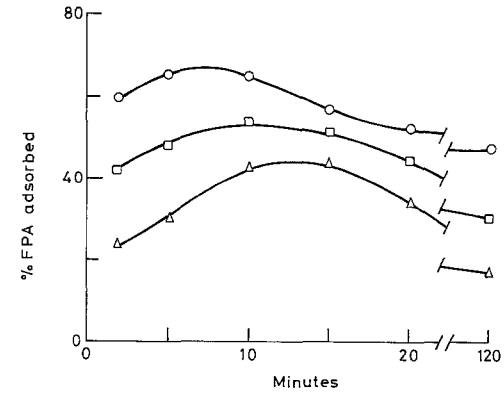

Fig. 4. Cellulase (FPA) adsorption/desorption patterns in the presence of 6% enzymatic hydrolysate (△) and 6% artificial hydrolysate (□) compared to control mixture prepared with no hydrolysate (○).

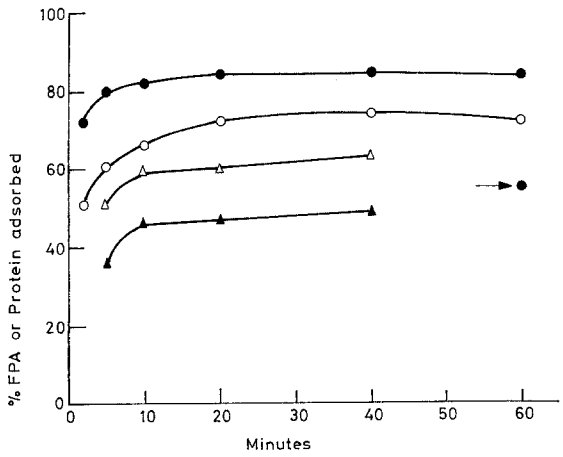

Fig. 5. Time courses of cellulase (FPA; open symbols) and protein (closed symbols) adsorption observed in the presence of 3% cellobiose (circles) or a mixture of 3% glucose, 1% xylose, and 0.3% cellobiose (triangles). Point indicated by arrow represents cellulase still adsorbed in control mixture (remainder of data from control time course not shown).

actually stimulated adsorption. At a 3% concentration, cellobiose raised the maximum cellulase adsorption from 65 to 75% and the maximum protein adsorption from 54 to 84% (Fig. 5). Furthermore, the desorption phase, characteristic of the control system (see control point indicated by arrow at 60 min), was absent in the cellobiose-containing adsorption mixture. This increased duration of maximum adsorption could not be attributed to inhibition of the cellulolytic hydrolysis rate by the high initial cellobiose concentration because the increase in reducing sugars during incubation was almost the same (6.78 mg/ml for the control versus 6.20 for the cellobiose-containing mixture).

HPLC analysis of hydrolysate composition revealed that about 4.5% of detected peak area was contributed by the oligosaccharides cellotriose and cellotetraose. Fig. 6 shows the relationship of glucose residue number to retention time for these oligosaccharides in the HPLC system. If these oligomers were inhibitory to the adsorption of cellulases on NaOH-treated PELF, acid hydrolysis would reduce their inhibitory effect. Enzymatic hydrolysate was treated with 1.0 N H_2SO_4 by refluxing with boiling chips for 1 h, then processed as if for GC analysis. This treatment to hydrolyze oligosaccharides to monomers caused an average 18% increase in GC-detectable sugars. The major increases were in glucose (average of 7.5%), in xylose

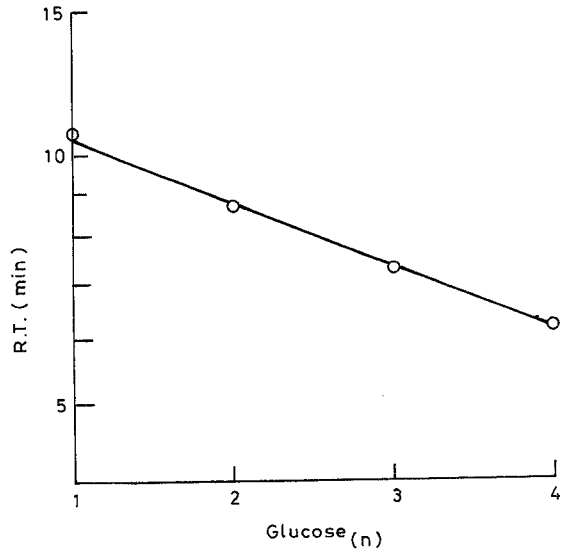

Fig. 6. Relationship between retention times (R.T.) and number of glucose residues in oligosaccharides from the hydrolysate of enzymatically digested PELF. These retention times, 6.24, 7.32, 8.68 and 10.74 min, were identical to those produced by cellotetraose, cellotriose, cellobiose and glucose standards, respectively.

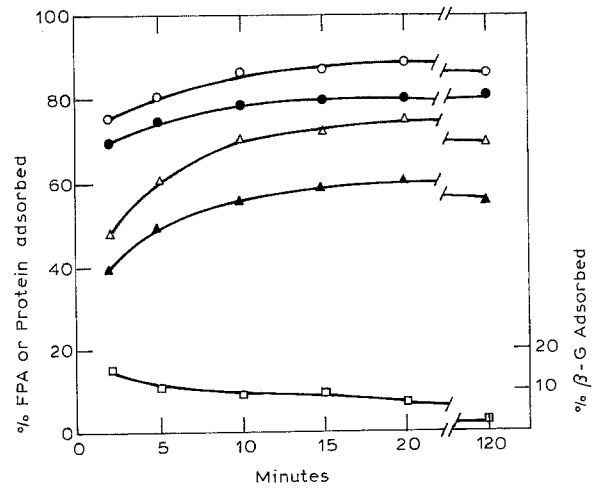

Fig. 7. Adsorption profiles at 2.5°C for cellulase (FPA; ○), protein (△) and β-glucosidase (B-G, □) in control reaction mixtures (no hydrolysate) compared to those in the presence of 6% hydrolysate (●▲). No adsorption of β-glucosidase was detected in the presence of hydrolysate.

(average of 36.5%), and in galactose/mannose (77.3%). The preparation was neutralized to pH 5.0 and the inhibitory effect on cellulase adsorption was tested, using it at a reducing sugars concentration equivalent to 6.0% glucose. Whereas the hydrolysate before acid treatment inhibited FPA adsorption by 44% when used in a 6% concentration, the acid-treated hydrolysate inhibited adsorption only 2.8% at the same concentration.

The inhibition which these hydrolysate components exerted on cellulase adsorption to PELF could be greatly reduced by lowering the temperature of the system. At the routine hydrolysis temperature of 45°C, binding of cellulases to PELF in the presence of hydrolysate was insignificant. Conversely, cooling of adsorption mixtures to 2°C enhanced cellulase and total protein adsorption both in the presence and absence of hydrolysate (Fig. 7). Additional benefits of cooling were prolongation of the adsorption maxima and detectable binding of β-glucosidase (cellobiase). However, the binding of this enzyme was only apparent in the absence of the hydrolysate.

DISCUSSION

The use of PELF as a substrate for enzymatic hydrolysis has not received the attention given to a variety of other lignocellulosic substrates such as newspaper [3], wood fibers [4], wheat straw [6], bagasse [7] and corn stover [12]. The hydrolysis yield from PELF, based on the percent of theoretical maximum reducing sugars liberated by enzymatic action, is about double that of other agricultural lignocellulosic substrates [17,18]. In addition to its potential value as a source of fermentable sugars, its capacity for binding cellulases merits study on its use as an enzyme-recycling vehicle, particularly in view of estimates that enzyme make-up will comprise over 50% of fixed capital cost in biomass conversions [19]. The ability of PELF to bind the cellulases of *T. reesei* apparently resides largely in its biodegradable carbohydrate components, since we have shown here that cellulase desorption is directly proportional to carbohydrate solubilization. The high recovery of cellulases (85–90%) after PELF hydrolysis contrasts with the lower enzyme recoveries from substrates which irreversibly bind 50–65% of the initial activity in a non-specific manner [3,4,6].

An earlier study [15] showed that the presence of PELF hydrolysate slowed cellulase adsorption rates and markedly decreased the extent of adsorption on fresh PELF. This inhibition is apparently not peculiar to hydrolysate from alkali-treated PELF since hydrolysate from freeze-exploded PELF produced hydrolysate which was even more inhibitory to cellulase adsorption on that substrate. Therefore it appears probable that the presence of hydrolysate will be a serious obstacle to the use of fresh substrate as a cellulase recovery/recycling vehicle during the bioconversion of PELF to fermentable sugars. The high enzyme costs would necessitate a substantial reduction in this inhibition if the process were to be economically feasible. Previous studies [11,13] have shown marked effects of temperature on cellulase adsorption to purified cellulosic substrates. Increased adsorption and prolongation of the adsorption maximum at low temperatures are probably attributable to the lower rate of hydrolysis of high-affinity binding sites on the cellulose polymer surface [13]. Whether cooling of adsorption mixtures for greater cellulase recovery during recycling in an industrial-scale PELF bioconversion would be economically feasible would require comparison of costs for cooling and re-heating versus those for cellulase production.

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