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Recovery of Cellulase from Lignaceous Hydrolysis Residue

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ABSTRACT

The adsorption of cellulase protein on lignaceous hydrolysis residue was studied over the range of initial cellulase concentrations: 20–200 FPU/g cellulose. The adsorbed protein (mg/g of substrate) was found to depend on the solids concentration as well as the concentration of protein in solution. Soluble reaction products did not affect the adsorption of cellulase on residue. Over the range of enzyme loadings studied, the residue did not saturate with protein. Activity could be recovered from the washed residue by mixing with fresh substrate.

Index Entries: Fungal cellulase hydrolysis; cellulase recycle; lignin; protein adsorption.

INTRODUCTION

Currently, ethanol cannot compete on a cost basis with gasoline in the transportation fuel market. Improvement of the biologically mediated process steps constitutes an opportunity to reduce production costs. The cost of fungal cellulase production in Simultaneous Saccharification and Fermentation (SSF) represents $0.13/gal of ethanol or about 7.3% of ethanol production cost (*1*). However, Hogsett et al. (*2*) note that when cellulase is "free," as in Direct Microbial Conversion (DMC), cost savings on the order of $0.50/gal are possible. A large portion of these savings are achieved by eliminating feedstock diversion for cellulase production and reducing bioreactor costs. Recycling enzyme in SSF could achieve many of the same results that cellulase production by thermophiles brings to DMC. Recycling cellulase may also be used to increase process yield, a sensitive cost parameter (*1*).

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After the enzymatic hydrolysis of lignocellulosic biomass, a portion of the cellulase returns to solution, whereas another fraction is adsorbed on the lignaceous residue. The fungal cellulase contains several individual enzymes, and each may demonstrate different adsorption characteristics on the lignin and cellulose components of lignocellulosic materials. Several efforts have been made to study recovery of cellulase following hydrolysis (*3–5,8–11*). Proposed methods of recovery include washing with buffer, neutralization, adsorption of enzymes in supernatant on fresh substrate, and contacting hydrolysis residue with fresh substrate.

Ooshima et al. (*3*) found that β-glucosidase was taken up slowly as lignin was exposed during batch hydrolysis, whereas other enzymes were released to solution as hydrolysis of the cellulose fraction progressed. By extrapolation of adsorption data at 80% conversion, Ooshima concluded that all of the enzyme components adsorb in the same proportion on the lignaceous residue. Sinitsyn et al. (*4*) similarly demonstrated that β-glucosidase adsorbs on hydrolysis residue. Fujishima et al. (*5*) have also shown that β-glucosidase preferentially adsorbs on hydrolysis residue, and hypothesized an electrostatic interaction between residue and enzyme since pH and ionic strength influence adsorption.

Although significant effort has been devoted to studying the desorption of cellulase from cellulose using buffers, solvents, and surfactants (*6,7*), far less work has been performed regarding the recovery of enzyme from hydrolysis residue. Sinitsyn et al. (*4*) found that neutralizing from pH 4.5 was more effective, as a method of recovering activity from partially hydrolyzed hardwood, than eluting with hydrolysis buffer, but were unable to achieve complete recovery of activity. This result agrees with Otter et al. (*7*), who found that increasing pH released the adsorbed Avicelase activity and also noted a reduction in cellulase stability above pH 9.0. Nutor and Converse (*8*) also investigated the use of hydrolysis buffer to desorb protein from hydrolysis residue and found the protein adsorbed to residue did not return to solution after prolonged washing. Fujishima (*5*) found that only 10% of adsorbed β-glucosidase was recovered in solution after washing with hydrolysis buffer for 24 h. They observed that the remaining fraction was stable over long periods and could be used as an immobilized catalyst.

Several investigators have demonstrated that cellulase activity can be recovered from the hydrolysate by mixing with fresh substrate (*9,10*). Using potato wastes, Singh demonstrated that approx 55% of the initial activity could be recovered from the hydrolysate by adsorption on fresh substrate after 1 d of hydrolysis with 50 FPU/g substrate. At an enzyme loading of 15 FPU/g solids, Mes-Hartree et al. (*11*) also found that 60% of filter paper activity could be recovered after a 2-d combined hydrolysis and fermentation process. However, Vallander and Eriksson (*9*) were unable to recover activity from solution after hydrolysis for 1 d at 10 FPU+ 10 IU β-glucosidase/g substrate. This unsuccessful result may have been owing to product inhibition by sugars in the hydrolysate.

Investigators have also studied the recovery of cellulase from lignin-rich residue by mixing with fresh substrate (*9,10*). Singh recovered 33% of initial activity from residue by mixing partially converted material with fresh substrate. Vallander and Eriksson also showed that enzyme bound to partially hydrolyzed residue could be used to degrade fresh substrate.

These results suggest that β-glucosidase is adsorbed strongly on hydrolysis residue and that other enzymes are also adsorbed to a lesser extent. However, these results are often based on data from partially hydrolyzed residue containing a significant fraction of cellulose (*4,9,10,11*). Conclusions regarding the extent of desorption at the level of conversion anticipated in a process cannot be made using existing data, since the degree of conversion may strongly influence the amount of protein adsorbed to the residual material (*3*).

In this work, the adsorption characteristics of *Trichoderma* cellulases on residue prepared by essentially complete enzymatic hydrolysis are described. Isotherms for protein adsorption on residue are presented at several initial solids concentrations. The effect of initial enzyme loading on protein recovery is quantified. The activity that can be recovered from residue by mixing with fresh substrate is determined. The purpose of this work is to provide data that could be used to design and evaluate a process that includes enzyme recycle.

MATERIALS AND METHODS

Substrate Pretreatment/Composition

Ground mixed harwood (Wilner) was pretreated in a plug-flow explosion reactor as described previously (*12*). Wood was pretreated by heating a 10% solids slurry for 10 s at 220°C. Acid concentration after steam injection was 1% H_2SO_4. The pretreated wood was neutralized with concentrated NaOH, rinsed with tap water, and then filtered to a solids concentration of 12–15%. This material (PTW220) was stored at 4°C before use in enzymatic hydrolysis. PTW220 contained 56% potential glucose as measured by quantitative saccharification. Residue after complete enzymatic hydrolysis represented 32–38% of initial unreacted wood dry mass.

Assay Techniques

Glucose determination (*13*): Glucose, total sugar, and total potential sugar in solids were determined as described earlier (*8,13*). Protein in solution: The concentration of protein in solution was determined as described before (*8*).

Sample Preparation

Samples for sugar determination were acidified to 1% H_2SO_4 immediately to halt any further conversion. Samples are then microfuged and refrigerated until assayed. To prepare a sample for protein determination, the sample is microfuged immediately; the supernatant is then transferred to a new vessel and refrigerated until assayed.

Composition of Cytolase CL

The protein content of the crude cellulase concentrate was 55.9 ± 2.7 mg/mL with activity of 68 FPU/mL yielding a specific activity of 1.22 FPU/mg protein. The sugar content of the crude concentrate was 2 mg/mL. Thus, when diluted for hydrolysis, initial sugar did not influence hydrolysis rates.

Hydrolysis Procedure/Preparation of Hydrolysis Residue

Batch hydrolysis was performed at 37°C on an orbital shaker at 150 rpm in 250-mL stoppered flasks. Hydrolysis buffer consisted of 50 m*M* sodium acetate, pH 4.8, supplemented with 0.5% sodium azide. Pretreated wood was exposed to Cytolase CL (Genencor) at several initial solids concentrations (1.0, 2.0, 3.5% dry solids) and enzyme loadings (9, 18, 27, 36, 54, and 90 mg protein/g dry solids). Conversion was determined by measurement of sugars in solution as well as determination of potential glucose in remaining solid fraction. Residue was produced when the conversion of solids was no longer increasing. This usually was achieved after 4 d of reaction.

Adsorption Calculations

Protein adsorbed to the hydrolysis residue has been expressed as mg protein/g residue, and was calculated as the difference between initial concentration and amount measured in solution. Samples of residue were filtered on 0.22 μm filters and dried at 105°C to determine the solids fraction of the mixture.

Effect of Glucose on Adsorption of Cellulase

A series of residue samples prepared, as described above, at 1.0% initial PTW220 were used. Tubes containing well-mixed samples of hydrolysis solution were supplemented with glucose. Protein in solution was measured after 10 and 36 h.

Desorption with Buffer and the Effect of Products in Hydrolysate

Experiments to characterize the reversibility and extent of protein desorption from residue were conducted. A series of residue samples prepared by hydrolysis of 2.0% initial solids was used. These samples were paired into 30-mL portions and centrifuged for 10 min. Half the liquid portion of each tube was withdrawn. Then samples from paired tubes were combined and ultrafiltered using a PM-10 membrane (Amicon) MWCO 10,000. No protein leakage through membrane was detected.

Next, 15 mL of hydrolysate ultrafiltrate were returned to one of each pair of samples, whereas the corresponding samples received 15 mL of fresh buffer. Samples were then agitated on an orbital shaker at 37°C for 10 h. Protein adsorbed after washing with buffer or hydrolysate was calculated by comparing the concentration of protein anticipated if there were no desorption with the actual concentration measured. Dilution factors were determined by calculating the ratio of total soluble sugars in samples to which buffer had been added.

A second washing step was performed on each of the samples used above. After centrifugation, 15 mL of supernatant were withdrawn from each test tube and replaced with 35 mL of fresh hydrolysis buffer. Adsorbed protein and dilution factors were determined in the same manner as above.

Direct Recontacting

Constant Residue Added

Residue prepared by hydrolysis of 3.5% initial solids for 10 d was used in these experiments. Twenty-milliliter samples from each of the initial loading conditions were filtered on 0.22-μm disks. When the filter cake was no longer covered with liquid, an additional 10 mL, approx 40 times the interstitial volume, of fresh hydrolysis buffer were passed through the cake. The filter and residue were then placed in a flask, and 20 mL of 1% solids in hydrolysis buffer were added. Vessels were monitored for glucose and total sugar production.

Constant Protein Added

Residue prepared by hydrolysis of 3.5% initial solids for 10 d was also used in these experiments. Residue was recontacted with fresh substrate such that the enzyme-to-substrate loading was consistently 30 mg protein/g fresh substrate. The appropriate amounts of hydrolysis residue to add from each of the six initial loading conditions were calculated from the adsorption data presented in Fig. 1. Fresh substrate concentration in the vessel was approx 1.0% PTW220. Vessels were monitored for glucose and total sugar production concentrations.

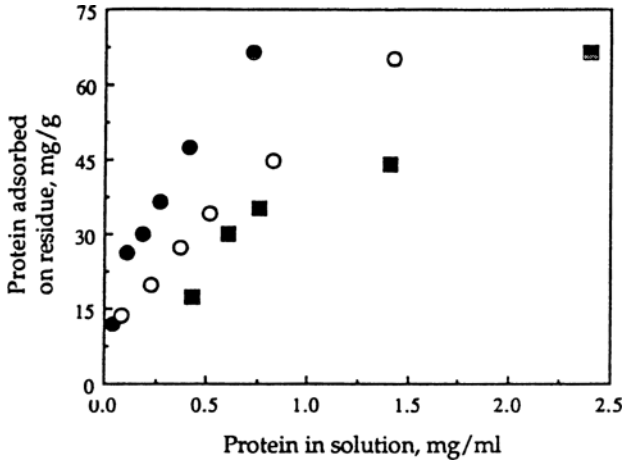

Fig. 1. Isotherms at 37°C of protein adsorbed to hydrolysis residue after 92±2% enzymatic hydrolysis of dilute-acid pretreated hardwood. Wood was hydrolyzed with cellulase from *T. longibrachiatum* (Cytolase CL, Genencor). A range of initial solids concentrations (● 1.0, ○2.0, ■ 3.5% dry wood) and enzyme loadings were used.

RESULTS AND DISCUSSION

Adsorption Isotherms

Isotherms of protein adsorbed on hydrolysis residue of dilute-acid pretreated hardwood for three initial solids concentrations are presented in Fig. 1. Protein measurements after 5, 6, and 7 d of hydrolysis were combined to develop isotherms for 1 and 2% initial solids. Values for 3.5% initial solids were based on data following 6 d of hydrolysis.

The data in Fig. 1, which relate protein adsorbed (mg/g) to protein concentration in solution (mg/mL), show that the isotherm is not independent of the substrate concentration. It can be shown that, in the case of competitive adsorption of two or more components for the same sites, Langmiur theory predicts this behavior (*14*). Hence, the data provide evidence of competitive adsorption on the residue, much as Ryu et al. found for adsorption on cellulose (*15*).

Another possible explanation for the substrate concentration effect could be that a soluble product of hydrolysis influences cellulase adsorption. With this in mind, the effect of glucose on adsorption was investigated. Samples with added glucose showed no difference in protein concentration after 10 and 36 h of agitation at 37°C.

The data in Fig. 1 have been replotted in Fig. 2, and they demonstrate a strong correlation between adsorbed enzyme and total enzyme loading. This plot demonstrates that the amount of protein adsorbed per amount of substrate is determined by the protein/residue ratio, independent of the protein or solids concentration in solution. Again this has been shown to be consistent with Langmiur theory in multicomponent adsorption (*14*).

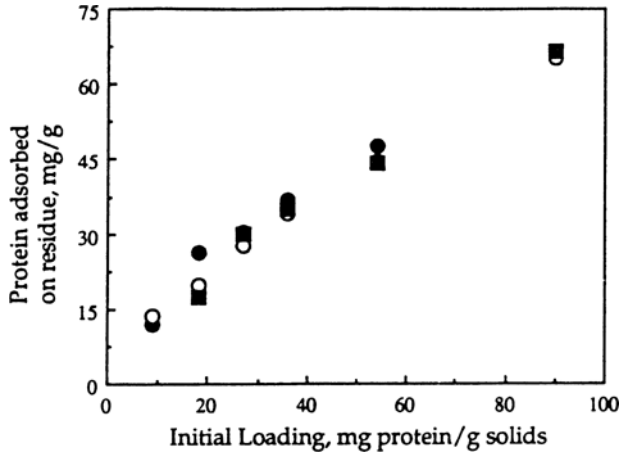

Fig. 2. Protein adsorbed to hydrolysis residue is replotted vs the initial enzyme loading. The correlation between adsorbed enzyme and initial enzyme loading is much stronger than the relation observed in Fig. 1. ● 1.0% Initial solids; ○ 2.0% initial solids; ■ 3.5% initial solids.

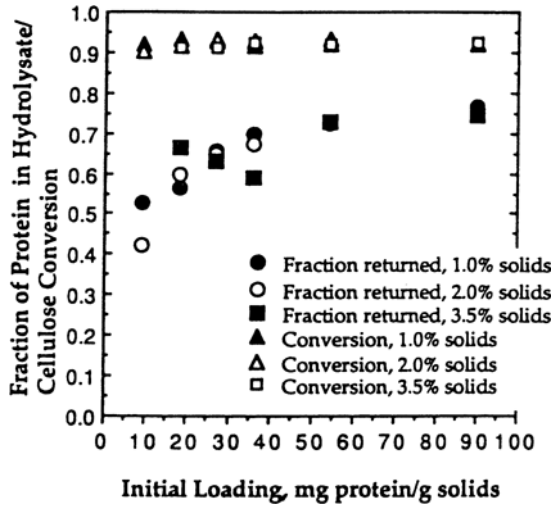

Fig. 3. Both the fractional conversion of potential glucose in the substrate and the fraction of protein remaining in solution at the end of hydrolysis are plotted vs the initial enzyme loading. Fractional conversion is consistently 92±2%. The fractional of protein in solution is lower at low initial enzyme loadings and appears to reach a maximum of 76% of initial protein when prepared at high-protein loadings.

Figure 3 presents the fraction of cellulase in solution after hydrolysis as a function of initial enzyme loading. The fraction recovered in solution at the end of hydrolysis increases with higher loading and reaches a maximum recovery of about 76% of initial protein added. Since conversion is very similar in all samples, the lower fractional recovery of protein at low

loadings cannot be the result of adsorption on excess unhydrolyzed cellulose. One possible mechanism to explain the lower recovery may be nonspecific association of cellulase enzymes with the residue when the residue surface is relatively unoccupied by other enzymes.

Another important observation regarding the hydrolysis residue gained from this work is that over the wide range of protein concentrations considered in this study, the residue does not saturate with protein. This finding disagrees with Ooshima et al. (*3*), who found that residue prepared at similar pretreatment conditions demonstrated an adsorption capacity of about 13 mg protein/g lignin. In Ooshima's study, however, the variation in protein concentration was obtained by adding varying amounts of fresh enzyme to the residue, whereas in this study, the initial enzyme loading was varied.

The enzyme levels considered in this work range from 9 to 90 FPU/g initial solids, or 20 to 200 FPU/g cellulose. The lower enzyme loadings considered in this study are similar to those that had been proposed in the Sequential Hydrolysis and Fermentation (SHF) process, and may also be similar to loadings made possible by efficient cellulase recycle. Seven FPU/g cellulose have been proposed as an optimal economic enzyme loading in the SSF process (*1*). At this level, about 50% of the protein is bound to residue even at maximum conversion. Thus, when using low enzyme loadings, recovering protein from the residue represents a major opportunity.

Desorption Studies

After 8 d of hydrolysis (92% conversion of initial potential glucose), samples of 2.0% initial solids were used to test the reversibility of adsorption. In the first of two desorption washing steps, the effect of soluble hydrolysis products was investigated. Volumes of hydrolysate were replaced with ultrafiltered hydrolysate and fresh hydrolysis buffer as described in the Methods section. Protein concentration after 10 h of contact was similar in both sets of flasks. This result indicates that soluble components released during hydrolysis do not influence adsorption of protein on the residue. Furthermore, it confirms the finding that glucose has no effect on adsorption (data not shown).

Data from both sets of samples (UF and Buffer washes) were combined to prepare Fig. 4. The method used to calculate the amount of protein adsorbed after washing is summarized in Table 1. A dilution factor is calculated from the ratio of total sugars in solution before and after washing. Using the calculated dilution factor and assuming no desorption, the anticipated protein concentration can be calculated. The difference between anticipated concentration and that measured is the amount desorbed. Using the solids concentration that was determined by drying residue at 105°C, we can then calculate the specific amount of protein ad-

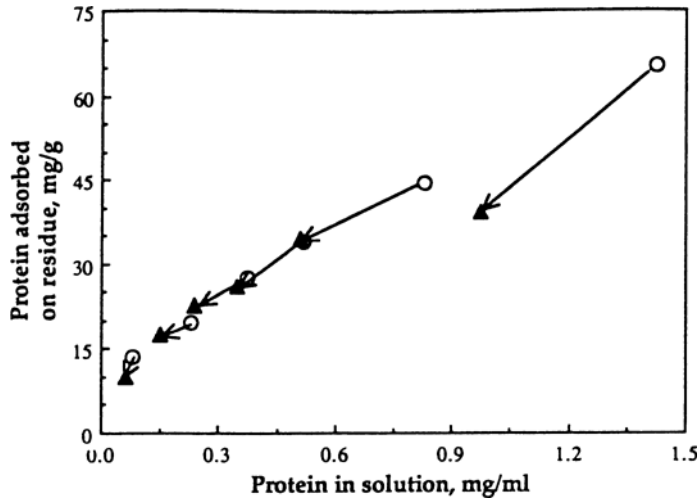

Fig. 4. Reversibility of adsorption of protein on hydrolysis residue. After washing residue with buffer and ultrafiltered hydrolysate, a fraction of the protein adsorbed to residue is recovered. Isotherm after washing agrees with the original isotherm. ○ Isotherm after hydrolysis, ▲ isotherm after wash #1.

Table 1
Protein Adsorbed on Residue

Sample	Protein added mg/mL	Protein in solution mg/mL	Protein adsorbed mg/mL	Solids content μg/mL	Protein adsorbed mg/g
2.9	0.191	0.081	0.110	8.10	13.58
2.18	0.382	0.229	0.153	7.78	19.67
2.27	0.573	0.373	0.200	7.30	27.40
2.36	0.764	0.516	0.248	7.30	33.97
2.54	1.146	0.829	0.317	7.10	44.65
2.90	1.910	1.425	0.485	7.44	65.19

sorbed per unit residue. Figure 4 shows that the isotherm after the first washing agrees quite well with the isotherm developed after hydrolysis. Arrows show which samples correspond to one another before and after washing. These results indicate that adsorption on the hydrolysis residue is reversible.

A second washing step was also conducted as described in the Methods section. It can be seen in Fig. 5 that much less desorption of protein from the residue was observed following the second wash. This result suggests that although a fraction of adsorbed protein can be recovered by washing with buffer, another fraction of adsorbed protein cannot be recovered.

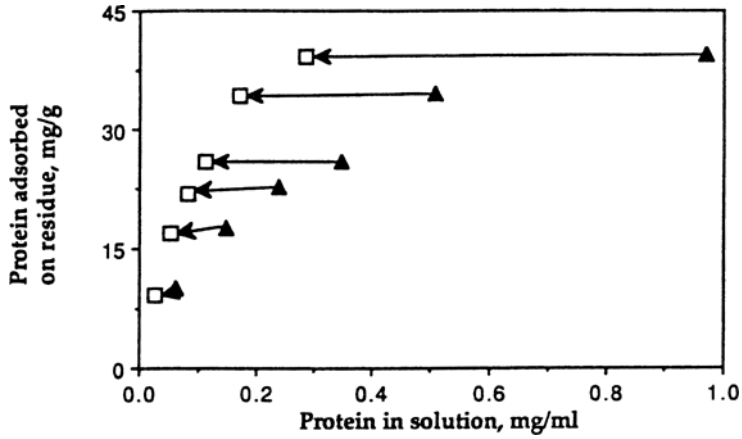

Fig. 5. Residue prepared at 2% initial solids was washed with buffer a second time. The amount of protein recovered from the residue was much less than in the first wash, indicating a degree of irreversible adsorption. ▲ Isotherm after wash #1; □ isotherm after wash #2.

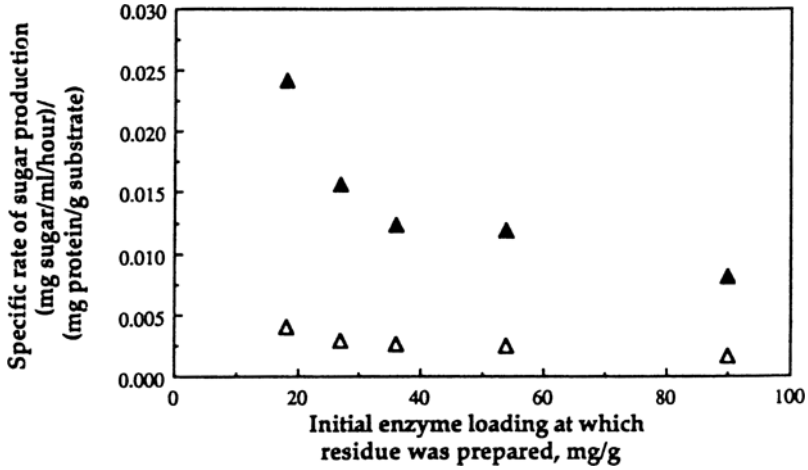

Fig. 6. Residue prepared at 3.5% initial solids and a range of initial protein concentrations was recontacted with fresh substrate. Filtered residue from 20 mL of hydrolysis reaction was added to 20 mL of 1% fresh substrate. Specific rates of glucose and total sugar production after 150 min are plotted against the enzyme loading at which the residue was prepared. △ Glucose; ▲ total potential glucose.

Direct Recontacting of Residue with Fresh Substrate

Constant Residue Added

The same amount of washed residue was collected from each flask and added to a fixed amount of fresh substrate. (See Methods section for details.) Rates of sugar production in all the samples were constant over

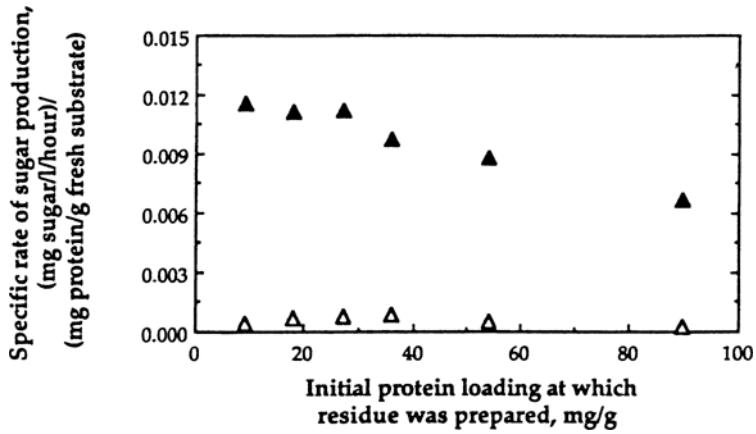

Fig. 7. Residue prepared at 3.5% initial solids and a range of initial protein concentrations was recontacted with fresh substrate. The amount of residue added to fresh substrate was chosen such that the protein loading was 30 mg/g in each flask. Specific rates of glucose and total sugar production after 150 min are plotted against the enzyme loading at which residue was initially prepared. ▲ Total potential glucose; △ glucose.

the first 150 min, but began to decline with longer incubation (data not shown). The average rate over the first 150 min was therefore used in preparing Fig. 6, in which the specific rate (i.e., the rate divided by the protein on the residue as calculated from the isotherms shown in Fig. 1) is presented vs the initial enzyme loading used in preparing the residue. The specific rate was used in this comparison, since the amount of protein on the recycled residue was not the same for all the samples.

The results in Fig. 6 show that the specific rate of total sugar production is greater for residue prepared at lower loadings. These results suggest that more of the enzyme on the hydrolysis residue prepared at low initial loadings possessed cellulolytic activity. This could possibly be because of CBH displacing EG at the higher loadings owing to competitive adsorption. Figure 6 also shows that the specific rate of glucose production is slightly greater for samples prepared at lower initial loadings. The proportion of sugars that are glucose ranges between 0.17 and 0.21, but shows no trend over the range of loadings considered.

Constant Protein Added

In order to check the above results, various amounts of residue were added to fresh substrate such that the protein loading was 30 mg/g fresh substrate regardless of the initial enzyme loading used to prepare the residue. In Fig. 7, the specific rate of sugar production, over the first 150 min, is plotted against the initial loading at which the residue was prepared. Except for the values at very low initial enzyme loading, the values of the specific hydrolysis rate agree well with the data in Fig. 6. For instance, in Fig. 6, residue prepared at 54 mg/g was recontacted with fresh substrate

Table 2
Sample Calculations for Protein Desorption from Residue

Sample 2% solids mg prot/g	Protein in solution[a] mg/mL	Dilution factor[b]	Protein anticipated[c] mg/mL	Protein observed wash #1	Protein desorbed mg/mL	Solids content μg/mL	Protein adsorbed[d] mg/g
2.9	0.081	1.930	0.042	0.062	0.020	8.10	11.11
2.18	0.229	1.730	0.132	0.148	0.016	7.78	17.66
2.27	0.373	1.810	0.206	0.239	0.033	7.30	22.89
2.36	0.516	1.800	0.287	0.346	0.059	7.30	25.84
2.54	0.829	1.890	0.439	0.508	0.069	7.10	34.88
2.90	1.425	1.750	0.814	0.972	0.158	7.44	43.99

[a] After complete hydrolysis of 2% solids.
[b] Calculated from ratio of total sugars in solution before and after washing.
[c] Protein in solution assuming no desorption from residue.
[d] After wash #1. Calculated from new value of protein adsorbed, mg/mL, and assumes constant solids content.

such that the new enzyme loading was 31.9 mg/g. The specific rate of hydrolysis associated with that sample was about 0.012. In Fig. 7, samples from a wide range of initial loadings were recontacted with fresh substrate at 30 mg/g. The specific rates of hydrolysis that resulted ranged from 0.007 to 0.012. As was seen in Fig. 6, the specific rate is higher for residue produced at lower initial loadings, although the effect is not as pronounced.

In both sets of recontacting experiments, the total conversion of substrate and the conversion of sugars to glucose by the recontacted protein were much lower than conversion observed when fresh enzyme was used for hydrolysis. Fresh crude cellulase achieved specific conversion rates on the order of 0.048, four to seven times greater than those observed for proteins recovered from residue. Presumably this was owing in large part to enzyme remaining on the residue. The degree of direct transfer of the enzyme from the solid residue to the solid substrate is currently under investigation.

Both the fresh cellulase and residue cellulase produced about 15% of all sugars as glucose. Thus, the activity recovered from residue does not seem to be enriched or deficient in β-glucosidase.

CONCLUSIONS

This study found that:

- Adsorption of cellulase on lignaceous hydrolysis residue, measured as mg protein/g substrate, depends on the substrate concentration as well as the concentration of free enzyme in solution. This is an indication of multicomponent competitive adsorption.
- Soluble products of hydrolysis do not effect the adsorption of cellulase on residue.

- Hydrolysis residue does not saturate with protein, even at very high initial enzyme loadings.
- Hydrolysis residue contains a recalcitrant fraction of cellulosic material, amounting to about 8% of the initial potential glucose, independent of enzyme loading or reaction time.
- Protein adsorbed on residue produced at higher enzyme loadings demonstrate lower activity per unit protein when recontacted with fresh substrate.

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