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Recovery of Cellulases After Hydrolysis by Adsorption on Steam-Pretreated Willow

Scientific Note

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Index Entries: Enzyme recovery; cellulases; adsorption; desorption; willow.

INTRODUCTION

Processes based on enzymatic saccharification for the production of ethanol from lignocellulosics are yet not economically feasible (*1*). One of the most crucial steps is the enzymatic hydrolysis. Enzyme recycling could improve the economics since the cost of enzymes constitutes a major part of the total cost of hydrolysis (*2*).

In a previous paper (*3*), the adsorption of enzymes on fresh substrate, steam pretreated willow, was studied focusing on the adsorption capacity of the substrate. It was shown that a large fraction of the enzymes in solution could be adsorbed. A major problem is the recovery of the enzymes that remain adsorbed on the hydrolysis residue (*4,5*). These account for the main part of the total amount of enzymes, at least when low enzyme loadings (below 20 FPU/g substrate) are used in the hydrolysis. Higher enzyme loadings will naturally result in a higher degree of recovery (*6*), but will not reduce the total amount of enzymes consumed. Sinitsyn et al. (*7*) have shown that the recovery of endoglucanases from the solid residue was enhanced by neutralizing the pH from 4.5, the optimum for hydrolysis. The recovery may also be enhanced by the addition of chemicals (*8*).

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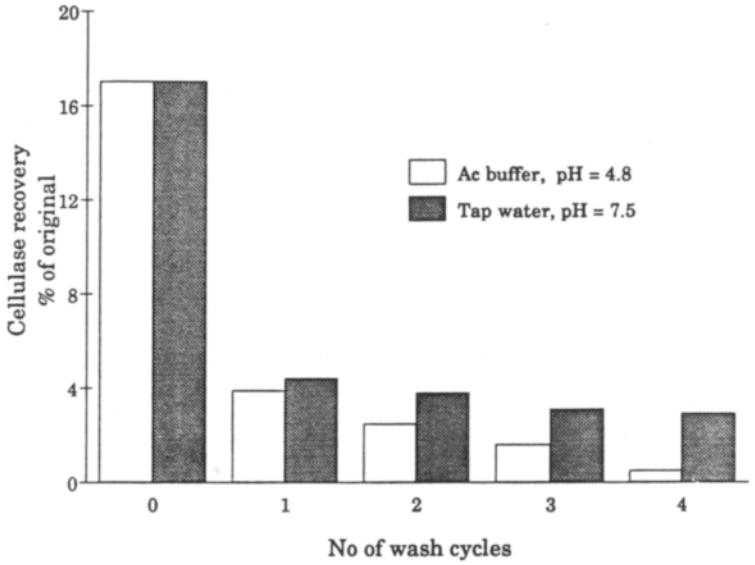

Fig. 1. Recovery of cellulases from residuals of hydrolyzed willow using batch washing—0 indicates hydrolysate.

The results from some preliminary experiments on the desorption of cellulases from the hydrolysis residue are shown in Fig. 1. The desorption was performed by batchwise washing using buffer solution or tap water. Although the elution power of common tap-water is low, some desorption occurs even after four washes. This encouraged us to further explore the desorption step.

The present study was concerned with the investigation of the effect of temperature and pH on the recycling of cellulases based on desorption from the hydrolysis residue and readsorption on fresh substrate (steam pretreated willow). The desorption–adsorption process is performed in a continuously working unit with a recirculating wash stream. The degree of cellulase recycling is assayed by hydrolysis.

MATERIALS AND METHODS

Substrate

A fast-growing species of willow, Salix Caprea Q082, was ground and fractioned. The willow was mixed with water to 30 wt% dryness and stored for 24 h at 8°C prior to steam pretreatment. The steaming was carried out at 220°C for 10 min. The pretreatment method is described in detail elsewhere (*9*). The composition of the pretreated substrate was 48% cellulose, 42% lignin, and 7% hemicellulose based on ODM (oven dry material). The substrate was stored wet at −18°C prior to hydrolysis.

Enzymes

The enzymes employed were two commercial enzymes from Novo Industries, Denmark. Celluclast 2L, a cellulase from *Trichoderma reesei* with an activity (*10*) of 90 FPU/mL and Novozym 188, a cellobiase from *Aspergillus Niger* with a β-glucosidase activity of 45 μmol *p*-nitrophenol/g min using *p*-nitrophenol-β-D-glucopyranoside as substrate (*11*). All enzyme loadings are given as percent of enzyme solution based on ODM substrate.

Analysis

The amount of recovered cellulases was assayed by hydrolysis of fresh substrate. The hydrolysis data obtained were compared with standard hydrolysis curves with known amounts of cellulases. The standard hydrolysis curves were obtained for Celluclast concentrations varying from 0 to 20%. The hydrolysis was performed at 40°C for 96 h in stirred vessels with a volume of 0.1 L. An amount corresponding to 5 g ODM substrate was immersed in 0.1*M* acetate-buffer solution (pH = 4.8) together with Celluclast and 5% Novozyme making a total weight of 50 g. Samples of about 1 g were withdrawn at 24, 48, 72, and 96 h, and analyzed for glucose with HPLC.

EXPERIMENTAL PROCEDURE

Production of Hydrolysis Residue

In order to produce the solid hydrolysis residue, an amount corresponding to 360 g ODM of substrate was hydrolyzed in six stirred vessels of 1 L with 20% Celluclast and 5% Novozym. The total weight was adjusted to 3600 g with the addition of 0.1*M* acetate buffer solution (pH = 4.8). The hydrolysis was performed under the same conditions as for the standard hydrolysis. After 96 h, the hydrolysis was interrupted, and the residue was filtered off using a glass fiber filter. The six solid residue fractions were thoroughly mixed, and both hydrolysate and residue were stored at −18°C prior to the desorption and adsorption studies.

Continuous Desorption and Adsorption

The batchwise washing, shown in Fig. 1, leads to very diluted solutions. This makes both the analysis and the testing of the hydrolysis capacity of the recycled enzymes very difficult. The continuous desorption–adsorption equipment, shown in Fig. 2, works with a limited amount of recirculating liquid. Dilution of the enzymes is thus minimized, and the recycled enzymes can be directly assayed by a subsequent hydrolysis

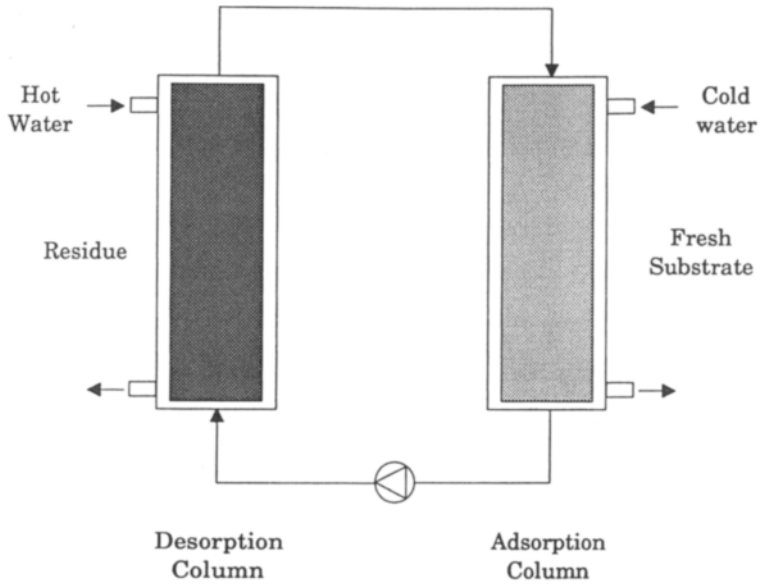

Fig. 2. Equipment for recovery of cellulases by continuous desorption–adsorption.

of the fresh substrate. The two double-mantled glass columns have an inner diameter of 30 mm and volumes of 68 and 107 mL, respectively. Including tubing, the total volume in the system is about 200 mL. A sample of the hydrolysis residue, equal to the amount obtained from hydrolysis of 5 g fresh substrate, was immersed in 50 mL rinsing liquid and placed in the larger column together with glass fiber filter aid. In parallel, a sample of 5 g of the fresh substrate was placed in the smaller column. The system was filled with rinsing liquid, and a steady circulating flow of about 40 mL/min was maintained for about 14 h.

Three different desorption temperatures, 40, 50, and 60°C, were evaluated. For each temperature, three different values of pH, 5, 7.5, and 10, were evaluated. The pH was adjusted by mixing the acetate buffer solution remaining in the hydrolysis residue with distilled water, 0.05*M* Na_2HPO_4 or 0.1*M* NaOH. The temperature in the adsorption column was kept at 8°C, avoiding hydrolysis.

The driving force in the desorption step was kept at a high level, since most of the cellulases desorbed from the residue would be adsorbed on the fresh substrate, thus maintaining the enzyme concentration in the circulating liquid at a low level. After the desorption–adsorption period, the fresh substrate, with recovered enzymes, was mixed with acetate buffer, and the pH was adjusted to 4.8. The substrate was finally hydrolyzed under standard hydrolysis conditions. Five percent of Novozyme was added, since β-glucosidase is not recovered by adsorption (*12*).

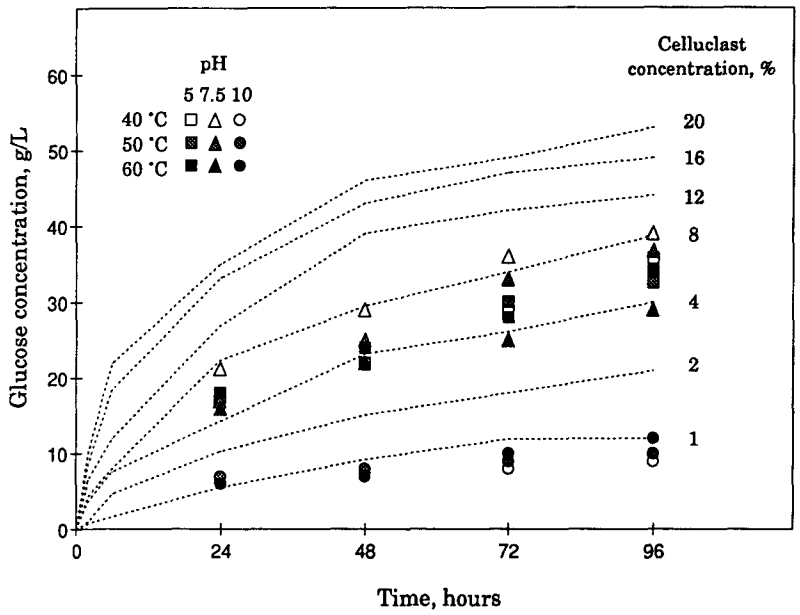

Fig. 3. Hydrolysis curves using cellulases recovered by continuous desorption–adsorption at various temperatures and pH. Dotted lines indicate standard hydrolysis curves.

RESULTS AND DISCUSSION

Recovery of Enzymes Adsorbed on Solid Residue

The glucose concentrations at 24, 48, 72, and 96 h obtained during the assay hydrolysis, with the cellulases recovered from the residue, are shown in Fig. 3. The data are corrected for the initial glucose concentration. The standard hydrolysis curves are included, as dotted lines, for comparison.

The degree of cellulase recovery was estimated from the data in Fig. 3. For each experimental point, the amount of recovered cellulases was assumed to be equal to the amount of cellulases required in the standard hydrolysis to obtain an equivalent glucose concentration. The degree of recovery for each desorption condition, calculated as the arithmetic mean of the values at 24, 48, 72, and 96 h hydrolysis, is shown in Fig. 4.

The pH seems to have a significant effect on the recovery. The lowest enzyme recovery was obtained at high pH. Otter and Munro (*8*) have investigated the effect of the desorption of cellulases from Avicel, and they found a maximum in the desorption at pH 10 in the presence of Triton X-100 or Tween 80. Our contradictory result is probably owing to a reduced adsorption capacity on fresh substrate at this pH, or perhaps the absence of Triton X-100 or Tween 80 in our experiments. It is not possible, from a

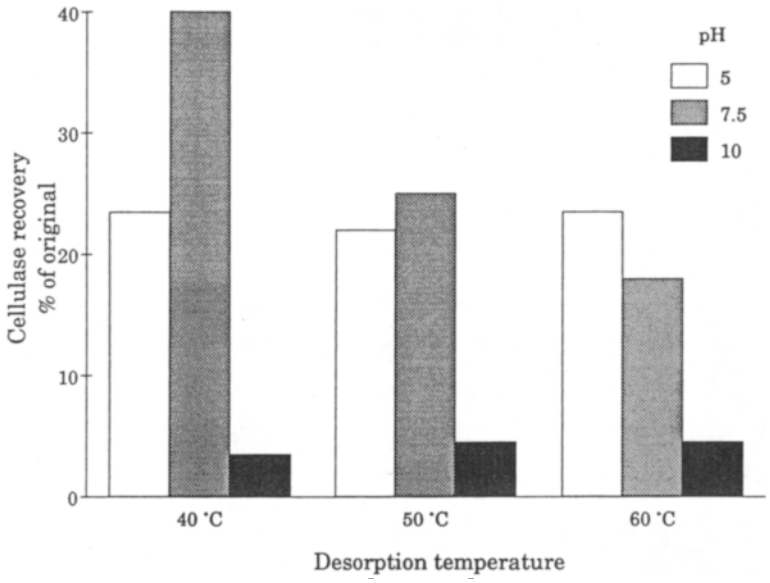

Fig. 4. Recovery of cellulases from residuals of hydrolyzed willow using continuous desorption–adsorption at various temperatures and pH.

practical point of view, to change the pH continuously between the two steps in the experimental setup. The difference between the recovery at pH 5 and pH 7.5 is in good agreement with the preliminary batch desorpton data in Fig. 1.

The temperature seems to have little effect at both low and high pH. For pH 7.5, an increase in temperature above 40°C gives a reduced recovery. This is probably because of enzyme deactivation (*13*). The optimum in cellulase recovery, 40%, was obtained at pH 7.5 and for a desorption temperature of 40°C.

Recovery of Cellulases from the Hydrolysate

As seen in Fig. 1, the hydrolysate contains about 20% of the original amount of cellulases (measured as CMC-ase activity), which must be recovered. To do this, an amount of the large batch hydrolysate, corresponding to 5 g substrate, was recirculated through 5 g of fresh substrate. The adsorption was performed at 8°C using the adsorption column. The substrate was then hydrolyzed according to the procedure described earlier to determine the hydrolysis capacity of the recovered cellulases. The resulting hydrolysis data are shown in Fig. 5. This corresponds to about 20% recovery of the total cellulases (calculated as described above).

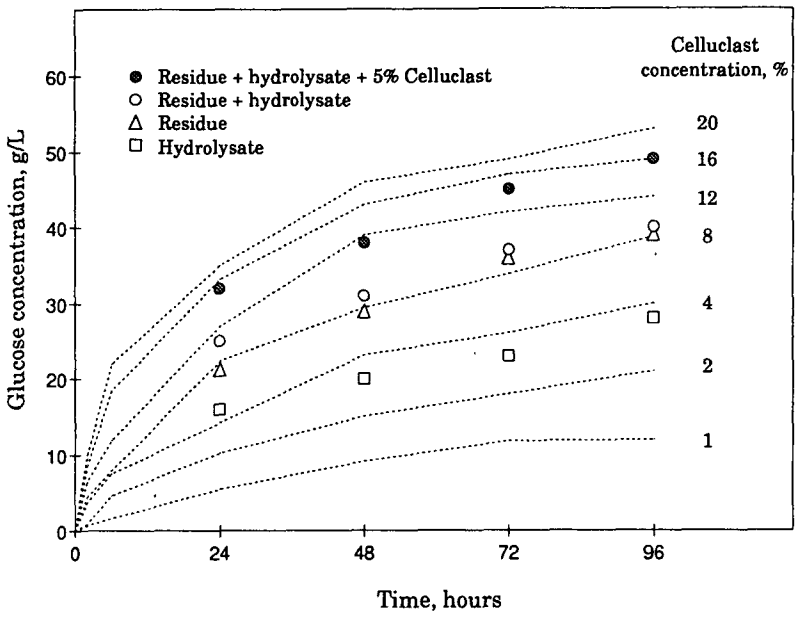

Fig. 5. Hydrolysis curves using cellulases recovered from solid residue and hydrolysate. Dotted lines indicate standard hydrolysis curves.

Total Recovery

To investigate the total recovery, the cellulases from both the residue and the hydrolysate was adsorbed on the same fresh substrate. First the desorption-adsorption process was performed under the optimal conditions, i.e., pH 7.5 and desorption temperature 40°C. Following this, the cellulases in the hydrolysate were adsorbed and the substrate was hydrolyzed after the addition of 5% Novozyme. The results are shown in Figs. 5 and 6.

The recovery procedure using both residue and hydrolysate was then repeated with a make-up of 5% Celluclast added in the assay hydrolysis. The results of this experiment are also shown in Figs. 5 and 6.

Discussion

The results are very promising. Figure 6 shows the degree of recovered cellulases measured as hydrolyzing capacity. Eighteen percent of the original cellulases was recovered from the hydrolysate and 40% from the solid residue (at pH 7.5 and 40°C) when recovered on separate substrates. This would give a total recovery of 58%, i.e., the total addition of Celluclast could be reduced from 18 FPU/g substrate to 7.6 FPU/g. The alternative with the cellulases from both the residue and the hydro-

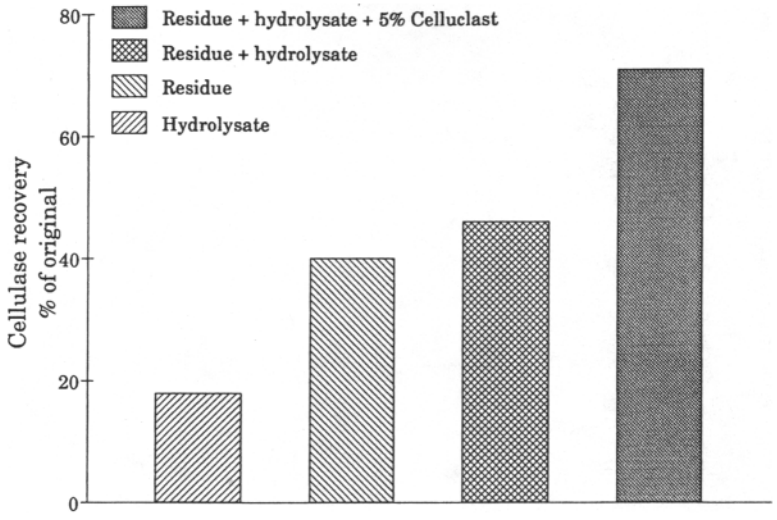

Fig. 6. Recovery of cellulases from solid residue and hydrolysate.

lysate being recovered on the same substrate resulted in a 46% recovery of the original cellulases which shows that the recovery yields were not additive. This could be because the adsorption capacity of the fresh substrate is limited. The experiment where make-up cellulases were added resulted in a cellulase concentration corresponding to 71%. The addition of cellulases seems to give an additive effect. This indicates that the enzymes from the hydrolysate probably should be recovered by addition of the hydrolysate to the fresh substrate, after removal of the sugars.

There are, however, many questions still to be answered by further experiments. We plan to investigate the following conditions:

Desorption temperatures below 40°C;
Variation of the pH around pH 7 and the influence of the ionic strength; and
Lower Celluclast concentration in the original hydrolysis.

The best conditions will be used to perform several repeated recovery and hydrolysis experiments in series, adding some make-up cellulases, to test the stability of the recovered cellulases. The recovery procedure will also be tested for other materials, such as delignified, steam-treated willow, with other desorption–adsorption characteristics (*14*).

ACKNOWLEDGMENT

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