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Combined Sedimentation and Filtration Process for Cellulase Recovery During Hydrolysis of Lignocellulosic Biomass

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Abstract

A combined sedimentation and ultrafiltration process was investigated for recovering cellulase enzymes during the hydrolysis of lignocellulosic biomass. Lignocellulosic particles larger than approx 50 μm in length were first removed via sedimentation using an inclined settler. Ultrafiltration was then used to retain the remaining lignocellulosic particles and the cellulose enzymes, while transmitting fermentable sugars and other small molecules. The permeate flux from the ultrafiltration step for a feed consisting of 0.22 w/v% cellulase is 64 ± 5 L/m^2-h, while that for a feed consisting of the settler overflow from a mixture 0.22 w/v% cellulase and 10 wt% lignocellulose fed to the settler is 130 ± 20 L/m^2-h. The higher permeate flux in the latter case is presumably due to binding of a portion of the cellulase enzymes to the lignocellulosic particles during hydrolysis and filtration, preventing the enzymes from fouling the membrane. A filter paper activity assay shows little loss in enzymatic activity throughout the combined sedimentation/ultrafiltration separation process.

Introduction

Owing to depleting energy reserves, alternatives to nonrenewable, fossil-based fuels are being investigated. A liquid fuel that can be inexpensively produced from renewable resources is desired. Ethanol derived from agricultural or industrial lignocellulosic biomass is one such alternative fuel, and it has been investigated for decades *(1–3)*. Because of their expense, however, lignocellulosic biomass-to-ethanol processes are not currently utilized on a large scale. One of the most significant production expenses is the cellulase enzyme used to hydrolyze cellulose to fermentable sugars, estimated at approx 20% of the total cost of ethanol production

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(4). Because much of the cellulase remains active after hydrolysis, recovery and recycle of this enzyme could significantly reduce operating costs.

One method to recover cellulase enzymes from a lignocellulosic mixture is through the use of inclined sedimentation followed by membrane filtration *(5)*. Inclined sedimentation is used for primary solids removal of the larger lignocellulose (LC) particles, as they would otherwise clog the membrane filter *(6)*. Particles can be efficiently removed using sedimentation vessels with inclined walls *(7–9)*, and inclined settling has successfully been demonstrated for solid–liquid separations in several fermentation and other bioconversion applications *(10–13)*. This simple and inexpensive technique involves pumping feed into narrow, inclined channels with large surface area but small channel height. As the fluid moves up the channels, the particles settle due to gravity onto the upward-facing surfaces and then slide down to the bottom of the settler, where they can either be removed separately or returned to the feed material. Inclined vessels provide the advantages of short settling distances and large surface areas for sedimentation *(14)*. Following sedimentation, the overflow from the inclined settler is fed to an ultrafiltration (UF) unit, where the cellulase enzymes are retained by the UF membrane while water, sugars, and other small molecules pass through the membrane. The retained cellulase enzymes can be reused for further hydrolysis.

In addition to recovering active cellulase enzyme, the combined sedimentation/filtration separation strategy has the second advantage of providing for the possibility of separating the enzymatic hydrolysis and the fermentation reactions in the biomass-to-ethanol process, such that both reactions can be maintained at their ideal temperatures and other conditions. Recent engineered cellulases remain stable and active at temperatures as high as 81°C *(15)*. To take advantage of the faster reaction kinetics, it is important to run the hydrolysis reaction at a high temperature, typically 50°C or more *(16)*, which maximizes the enzyme activity. In contrast, these high temperatures are harmful to the microorganisms used to ferment sugars to ethanol, and so fermentation is typically operated at a temperature near 30°C *(4)*. Additionally, the hydrolysis reaction is known to exhibit end-product inhibition *(17–19)*. By removing sugars from the hydrolysis vessel, this inhibition can be substantially reduced *(20–24)*. Currently, most biomass-to-ethanol processes employ simultaneous hydrolysis and fermentation in the same vessel *(25)*, so that the inhibitory sugars are converted as they are formed. In contrast, the separation of sugars from cellulase enzymes by ultrafiltration or other means allows for hydrolysis and fermentation to be employed separately, and it creates a sugar stream that has a variety of uses.

One possible drawback of cellulase separations using UF membranes is the potential for the cellulase to become deactivated. Several studies have reported losses in cellulase activity due to shear inactivation *(26–29)*. If cellulase is inactivated to a large degree, then the advantage of concentrating and recycling the enzyme would be compromised. Encouragingly,

activity measurements during UF concentration of cellulase enzymes were taken by Roseiro *(30)*, which showed no decrease in the specific enzymatic activity, indicating that shear deactivation did not occur under the conditions employed.

In the current work, we report on a combined sedimentation/filtration process for the recovery of cellulase enzymes in a lignocellulosic biomass conversion process. A mixture of lignocellulosic (LC) particles and cellulase enzymes (CE) is fed into a laboratory-scale inclined settler, where the larger particles and bound enzyme are retained while the smaller particles and soluble enzyme are carried out the settler overflow. The settler overflow is then fed to a membrane ultrafilter, which retains the remaining LC and CE while passing small molecules.

Materials and Methods

Lignocellulosic Particles and Cellulase Enzyme

The lignocellulose (LC) particles are composed of ground yellow poplar, obtained from the National Renewable Energy Laboratory (NREL) after pretreatment in its pilot plant in Golden, CO. These particles contain approx 40% lignin and 60% cellulose, plus acetic acid and other residual components. They are oblong, with a typical length-to-diameter ratio of 2:1, and range in length from 1 to 1000 μm. The cellulase enzyme used in these experiments is a commercial preparation made specifically for NREL by Iogen Corporation (Ottawa, Ontario, Canada). It is supplied as a liquid solution, which is reported to contain 205 g/L total soluble protein (TP), of which 158 g/L represents high-molecular-weight proteins (HMWP) that are retained by an ultrafiltration membrane with 30 kDa a molecular-weight cutoff (MWCO) *(31)*. Unless indicated otherwise, enzyme concentrations are reported as the total protein in solution [e.g., 0.2 w/v% (TP)].

Sedimentation

A diagram of the inclined settler is shown in Fig. 1. A rectangular glass channel was used as the settler, which has length $L = 34$ cm, width $w = 4.5$ cm, and spacing $b = 1.5$ cm between the inclined walls. A peristaltic pump (Watson–Marlow) was used to draw fluid from the feed tank at a volumetric flow rate of $Q_0 = 0.2$ cm^3/s. The angle of inclination from the vertical was fixed at $\theta = 30°$. The larger particles settled onto the upward-facing inclined wall and slid down to the bottom to the settler (which was open so that the unreacted LC particles and bound enzyme are recycled directly to the feed tank or hydrolysis reactor). For concentrated feed streams, the LC particles formed a cake on the upward-facing inclined wall that eventually bridged across the channel; in this case, gentle shaking or mechanical scraping is needed to return the settled solids to the tank.

Prior to use, all LC samples were washed once with tap water and centrifuged, and the supernatant was discarded. This procedure helped to

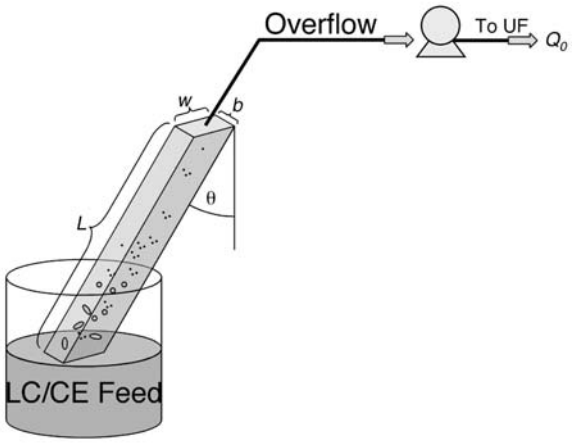

Fig. 1. Schematic of the inclined settler used to remove lignocellulosic particles from the feed prior to ultrafiltration.

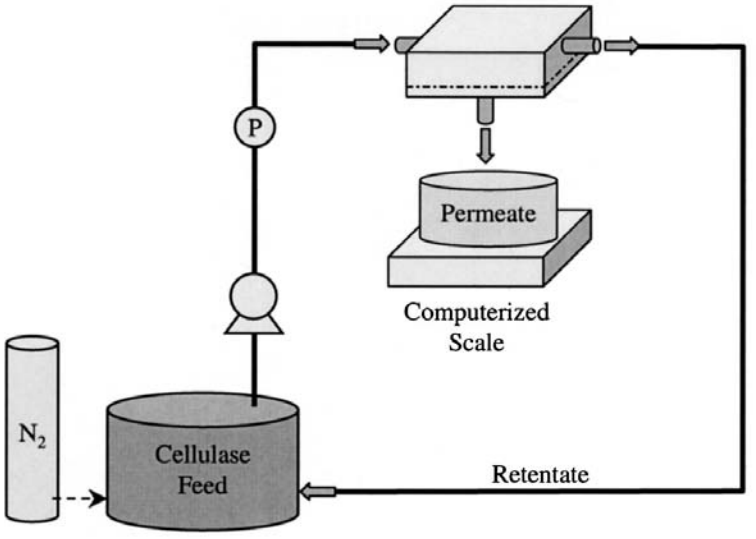

Fig. 2. Schematic of the crossflow ultrafiltration setup in the batch concentration mode to recover cellulase in the overflow stream from the inclined settler.

eliminate larger, buoyant particles from the feed, which otherwise tended to clog the overflow line from the inclined settler.

Crossflow Ultrafiltration

The overflow from the inclined settler is used as the feed to a crossflow ultrafiltration (UF) unit, shown in Fig. 2. The flat-sheet membrane apparatus was manufactured in-house and uses a small-scale filter channel of dimensions 3.8 cm long by 2.9 cm wide by 0.16 cm high; 50 kDa MWCO

polyethersulfone Biomax membranes from Millipore (cat. no. PBQK0MS10) were used, with the membrane replaced after each set of experiments. The permeate side has grooved channels to support the membrane, which has an effective filtration area of 6.4 cm^2. However, the total membrane area of 11 cm^2 (including the supports) is used in calculating the fluxes reported. Permeate mass is measured using an electronic balance (Denver Instrument XL-3100) interfaced with a personal computer. Nitrogen gas is used to provide transmembrane pressures up to 140 psi (9.65 bar). A geared pump (Cole–Parmer) is used to circulate the feed solution at a flow rate of $Q = 18.6$ cm^3/s. This flow rate corresponds to a channel Reynolds number of 640 (laminar flow) and a wall shear rate of 1500 s^{-1}.

Enzyme Assays

The total protein concentrations were measured using a BCA Protein Assay Kit (Pierce), calibrated with cellulase solutions with known enzyme concentrations. This assay is used for colorimetric detection and quantification of total protein. It measures the absorbance at 562 nm exhibited by the chelation of bichinconic acid (BCA) and cuprous ions.

The cellulase complex enzymatic activity was measured using the International Union of Pure and Applied Chemistry (IUPAC) guidelines. Activities are reported in terms of "filter paper units" (FPU) per milliliter of solution, based on the liberation of glucose from cellulosic filter paper after 1 h of incubation. The activity of the CE stock solution is about 55 FPU/mL. Samples were taken from the settler feed, the settler overflow, the membrane retentate, and the membrane permeate. Samples were pipetted into centrifuge tubes and centrifuged for 10 min to remove suspended solids before analysis. Controls were performed to account for the glucose in the samples from hydrolysis of the LC solids prior to sampling.

Results and Discussion

Crossflow Ultrafiltration of Enzyme Solutions

Ultrafiltration control experiments were performed first with aqueous solution of cellulase enzyme only, without lignocellulosic particles present. The initial experiments were operated with total recycle of both retentate and permeate, until steady state was achieved (typically within a few minutes). Figure 3 shows the steady permeate flux, J, reported in liters of permeate per m^2 of membrane area per hour (LMH), as a function of the transmembrane pressure (TMP) for several concentrations of cellulase enzyme. As expected, the results show a decrease in permeate flux with increasing cellulase concentration, approximately from 80 LMH at 0.056 w/v% TP in the feed to 35 LMH at 2.67 w/v% TP, at the highest TMP of 140 psi (9.65 bar). A weak increase in flux with increasing TMP is also observed.

In Fig. 4, the permeate flux measured at the feed pressure of 140 psi (9.65 bar) is plotted as a function of HMWP concentration (note that

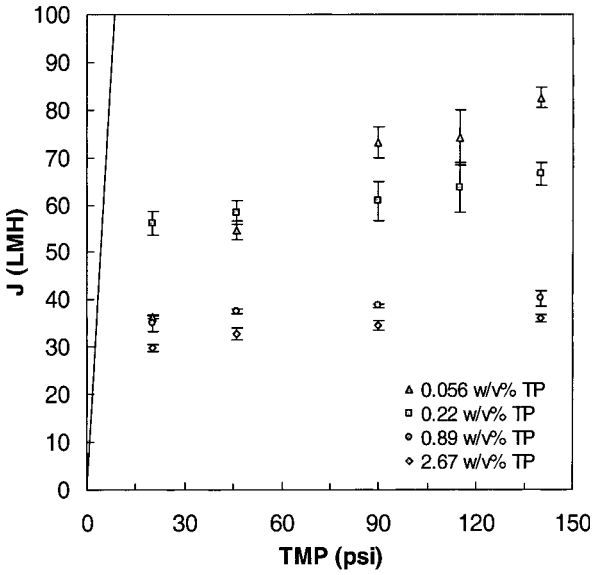

Fig. 3. Permeate flux as a function of transmembrane pressure (TMP) through the Biomax 50 kDa flat-sheet membrane using cellulase solutions at different concentrations of total protein (TP) as the feed at a wall shear rate of 1500 s^{-1}. The error bars represent plus and minus one standard deviation for three repeated measurements, and the solid line is the flux of pure water.

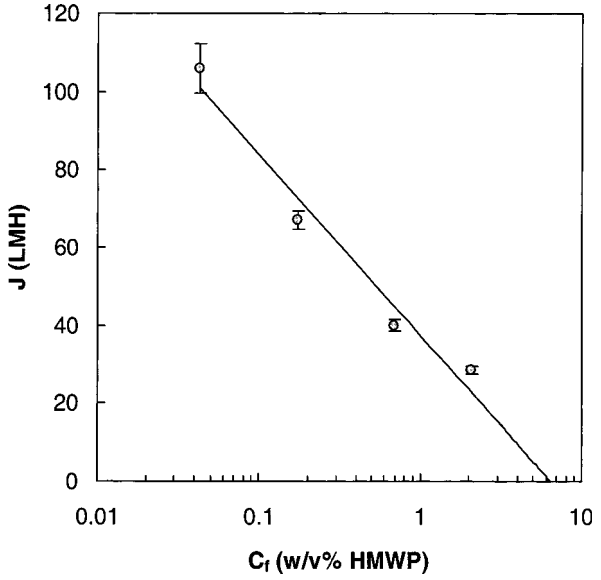

Fig. 4. The permeate flux as a function of cellulase feed concentration reported as high-molecular-weight protein (HMWP) at a transmembrane pressure of 140 psi and a wall shear rate of 1500 s^{-1}. The solid line is the best fit of the data by the gel-polarization model. The error bars represent plus and minus one standard deviation for three repeated measurements, and the solid line is the flux of pure water.

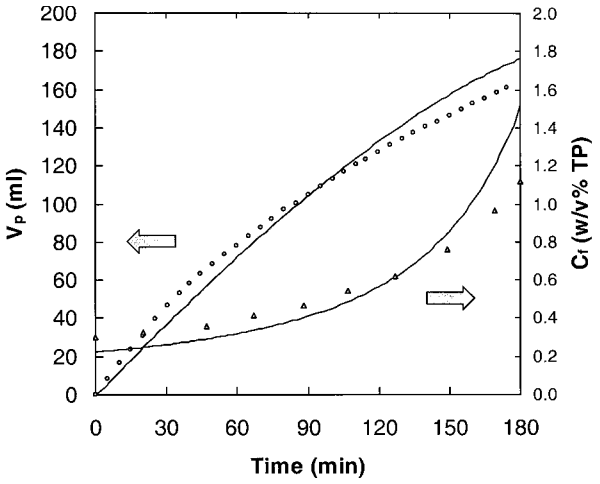

Fig. 5. Volume of permeate collected and total protein concentration in the feed during batch concentration of 200 mL of a 0.22 w/v% (TP) cellulase feed solution using the 50 kDa Biomax flat-sheet membrane at a transmembrane pressure of 140 psi and a wall shear rate of 1500 s^{-1}. Circles and triangles represent experimentally measured data points, while the lines are predicted by gel-polarization theory.

HMWP = 0.77 TP). The pressure-independent data should appear linear on a semilog plot, according to the gel-polarization model *(32)*:

$$J = k \ln\left(\frac{C_g}{C_f}\right)$$

in which J is the permeate flux, k is a mass transfer coefficient, C_f is the concentration of protein in the feed, and C_g is the concentration of protein at which a gel forms at the membrane surface. The best-fit line in Fig. 4 has parameter values of $k = 20$ LMH and $C_g = 6$ w/v% (HMWP). The differences between the model fit and the experimental data may be due, at least in part, to the fact that the maximum TMP of 140 psi (9.65 bar) is not enough to achieve pressure-independent fluxes.

Additional experiments were performed as batch concentration, in which the permeate was removed but the retentate was recycled so that the feed solution gradually decreased in volume and increased in enzyme concentration. Figure 5 shows the volume of permeate collected and feed concentration during a batch concentration using 200 mL of 0.22 w/v% (TP) cellulase as the initial feed. Over a 3-h period, approx 80% of the feed volume was removed as permeate, and the proteins in the feed solution were concentrated by almost four-fold. The average permeate flux during this period is approx 50 LMH, well in excess of the value of 14 LMH that was previously calculated *(5)* for the process to be economical. The protein concentration in the permeate remained approximately constant at 0.04 w/v% TP, or 20% of the initial feed concentration, representing the low-molecular-weight proteins which pass through the membrane.

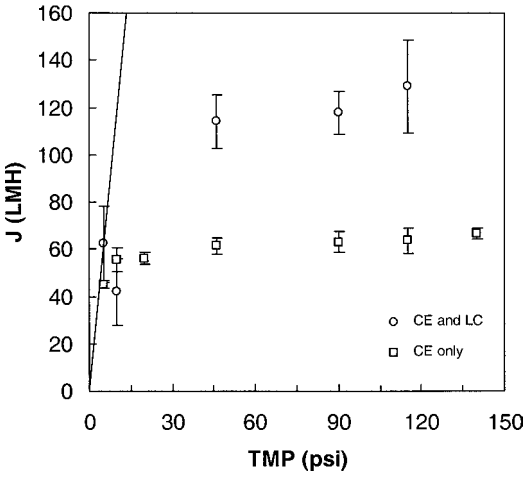

Fig. 6. Permeate fluxes using the Biomax 50 kDa MWCO membrane at a wall shear rate of 1500 s^{-1}. The circles represent fluxes using the overflow from the inclined settler as feed to the membrane filter. The feed to the inclined settler was 10 wt% LC and 0.22 wt% (TP) CE. The squares represent fluxes using a solution of 0.22 wt% (TP) CE as the feed to the membrane. The error bars represent plus and minus one standard deviation for three repeated measurements, and the solid line is the flux of pure water.

Inclined Sedimentation and Crossflow Ultrafiltration of Mixtures

In our previous work *(5)*, the flow rate through the settler and the angle of inclination were varied to give a desired concentration and size of particles in the clarified overflow stream. At the overflow rate of $Q_0 = 0.2$ cm^3/s and angle of inclination of $\theta = 30°$ used for experiments in this paper, over 90% by mass of the LC particles contained in the feed were removed in the overflow stream. The majority of particles by mass in the overflow stream were measured to be between 5 and 50 μm in length, while the feed solution to the settler contained mostly particles larger than 100 μm in length. The overflow from the settler was then fed to the crossflow ultrafilter.

Figure 6 shows the steady-state permeate fluxes for ultrafiltration of solutions containing CE alone (0.22 w/v% TP) and of solutions from the settler overflow for a mixture of CE (0.22 w/v% TP) and LC (10 wt%) fed to the settler. The results indicate that the presence of LC roughly doubles the permeate flux. This effect is more dramatic in Fig. 7, which uses a lower concentration of CE (0.056 w/v% TP) and LC (2.5 wt%) fed to the settler. In this case, the permeate fluxes with the feed solution containing LC approach the flux of pure water, indicating negligible membrane fouling. Permeate fluxes with feed solutions containing only CE are much lower, showing that substantial membrane fouling occurred in this case. Apparently the presence of the lignocellulosic particles reduces membrane fouling by the cellulase enzymes, presumably due to binding of a portion of the cellulase enzymes to the lignocellulosic particles during hydrolysis *(33)* and/or due to the LC particles serving as a filter aid by forming a secondary

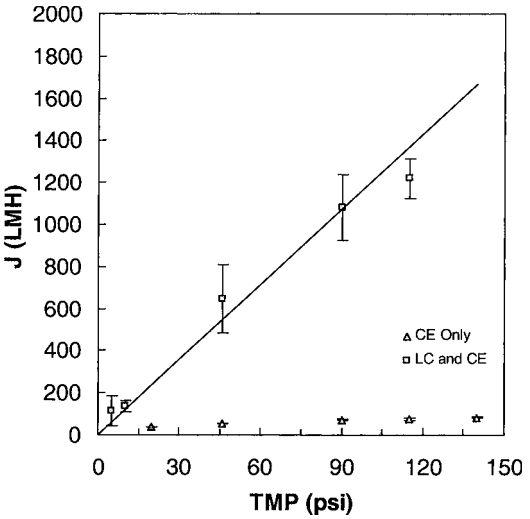

Fig. 7. Permeate fluxes using the Biomax 50 kDa MWCO membrane at a wall shear rate of 1500 s^{-1}. The squares represent fluxes using the overflow from the inclined settler as feed to the membrane filter. The feed to the inclined settler was 2.5 wt% LC and 0.056 w/v% (TP) CE. The triangles represent fluxes using a solution of 0.056 wt% (TP) CE as the feed to the membrane. The error bars represent plus and minus one standard deviation for three repeated measurements, and the solid line is the flux of pure water.

membrane, which sieves CE molecules and aggregates and prevents them from fouling the primary membrane *(34)*.

Shown in Fig. 8 are the permeate volumes collected over time for batch concentration of 100 mL of settler overflow containing CE alone (0.22 w/v% TP) and containing both CE (0.22 w/v% TP) and LC (10 wt%), where the concentrations in parenthesis are those in the feed to the inclined settler. Permeate was removed from the mixture containing both CE and LC at a faster rate than for the solution of CE alone, consistent with findings shown in Figs. 6 and 7. Only 43 min were required to filter 72% of the CE/LC feed (corresponding to an average flux of over 90 LMH), while 64 minutes were required to filter 72% of the CE feed.

Enzyme Activity

Figure 9 shows the enzymatic activities in the supernatant at three points along each of the batch concentrations shown in Fig. 8: the settler feed, the settler overflow, and the final membrane retentate. The settler overflow is the same as the initial 100 mL feed to the ultrafilter, whereas the final retentate represents the remaining 28 mL in the feed vessel and recirculation lines after 72 mL of permeate was removed. The enzymatic activity in the permeate was also tested, but it was found to be too low to measure and essentially negligible. The total activity of a 0.22 w/v% TP cellulase solution is expected to be about 0.6 FPU/mL (based on the reported activity

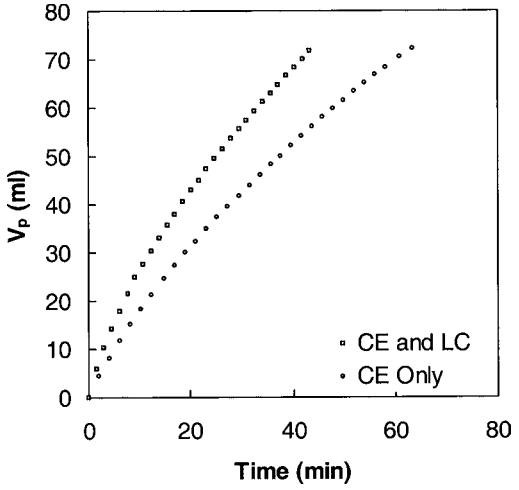

Fig. 8. Volume of permeate collected using the Biomax 50 kDa MWCO membrane at a wall shear rate of 1500 s^{-1}, with the overflow from the inclined settler as the membrane feed. The feed to the inclined settler contained either 10 wt% LC and 0.22 w/v% (TP) CE (squares), or 0.22 w/v% (TP) CE only (circles). Membrane feed volumes in both experiments were initially 100 mL and were concentrated to 28 mL.

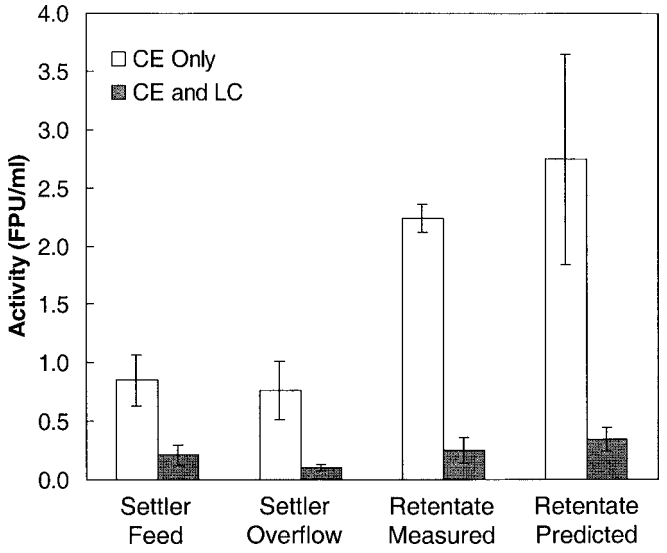

Fig. 9. Results of the filter paper activity assay. The dark bars represent activities of samples containing both LC and CE, while the light bars represent activities of samples containing CE only. All samples were centrifuged prior to measurement. The calculated retentate activity is based on the 3.6-fold reduction in feed volume shown in Fig. 8. The error bars represent plus and minus one standard deviation for three repeated measurements.

of 55 FPU/mL for the stock solution of 205 g/L TP). The activities of the CE-only solutions for the settler feed and overflow are slightly higher than this value but within the uncertainties of the measurements. At all sample points, however, the activities are lower for the mixture containing both CE and LC, indicating that much of the cellulase adsorbed onto the LC particles (and thus was easily recycled to the hydrolysis reactor with the LC particles in the settler underflow). Additionally, there appears to be little loss in activity during the combined sedimentation/filtration process for both solutions. The predicted retentate activity is based on the assumption that all enzyme present in the initial 100 mL feed remained in active form in the 28 mL final retentate. The small difference (approx 20%) between the predicted and measured enzymatic activity is within experimental uncertainty and more likely represents deposition of enzyme on the membrane surface than shear-induced deactivation.

Concluding Remarks

The combined sedimentation/filtration process is very effective in recovering cellulase enzymes during the hydrolysis of lignocellulosic biomass. The majority of enzyme binds to the lignocellulosic particles and is returned to the hydrolysis reactor with the settler underflow. The rest of the enzyme is recovered in primarily active form in the ultrafiltration retentate, which is recycled to the hydrolysis reactor, while sugars and other small molecules are recovered in the ultrafiltration permeate. After primary solids removal by the inclined settler, the small amount of residual solids in the settler overflow fed to the ultrafilter aids in the reduction of membrane fouling by the cellulase enzymes. The average flux obtained during batch concentration starting with a typical mixture of 0.22 w/v% cellulase and 10 wt% lignocellulose fed to the inclined settler is over 90 L/m2-h, which is more than six-fold greater than the average flux previously identified for the process to be economical *(5)*.

Acknowledgments

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