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Enzyme Recycling During Fed-Batch Hydrolysis of Cellulose Derived from Steam-Exploded *Eucalyptus viminalis*

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ABSTRACT

The recovery and recycling of cellulases during fed-batch hydrolysis of cellulosic substrates derived from SO_2-impregnated steam-treated *Eucalyptus viminalis* chips was examined. An initial enzyme loading of 10 FPU and 25 CBU g^{-1} cellulose was shown to be effectively recovered and recycled to hydrolyse seven consecutive batches of pretreated substrate at a 5% (w/w) cellulose concentration. Although the alkali-treated residue derived from steam-treated eucalyptus did not provide good cellulase recovery, subsequent alkaline peroxide treatment of this residue dramatically enhanced cellulase recycling efficiency. A glucose production rate of 26.1 ± 1.7 mg mL^{-1} d^{-1} was obtained for seven consecutive days, with an elapsed time of 24 h between each recycling step, without any requirement for further addition of cellulases after the first hydrolysis step. However, a considerable loss of both protein and enzymatic activity was observed throughout the experiment. In preliminary work, an excess of cellobiase activity was added at the beginning of every new hydrolysis step to avoid cellobiose accumulation in the reaction mixture. This was shown to be unnecessary when an additional step of ultrafiltration was used to recover and recycle the cellobiase activity present in the hydrolysates.

Index Entries: Steam-explosion; cellulose hydrolysis; enzyme recycling; readsorption of cellulases.

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INTRODUCTION

The cost of enzyme production and consumption has been recognized as one of the most critical bottlenecks hindering the economic viability of a biomass to ethanol process (*1*). As a result, there is a considerable amount of work underway to try to increase the specific activity of the cellulases by molecular manipulation, whereas other work has substantially increased the productivity and yields of various cellulolytic fungi. Another option is to try to determine how many times a commercial cellulase preparation such as Celluclast (Novo Industri, Bagsvaerd, Denmark) can be effectively recovered and recycled to hydrolyze several batches of substrate. This would have a direct impact on the cost effectiveness of the process, because it would allow a considerable increase in hydrolysis yields with a concomitant reduction in enzyme requirements.

Several strategies have been attempted to try to increase the yields of enzyme recovery and recycling during cellulose hydrolysis (*2–5*). These methods usually use ultrafiltration and/or readsorption techniques to recover the enzymes that had been desorbed after partial or complete hydrolysis of the substrate. Previously, we showed (*6*) that, after almost complete hydrolysis of the peroxide-treated fraction derived from steam-exploded *Eucalyptus viminalis* chips, most of the enzyme present in the supernatant of the hydrolysis reaction could be readsorbed and reused to hydrolyze fresh substrate. β-Glucosidase had to be supplemented at every recycling step to avoid cellobiose accumulation and subsequent endproduct inhibition of the cellulases. However, in this preliminary study, protein recovery was not assessed quantitatively and there was no attempt to identify which enzyme components were preferably desorbed from or retained within the residue.

The recovery of cellulases that are still adsorbed to the substrate has also been proposed by several authors as an effective method for enzyme recycling (*2,3,6*). The transfer of the adsorbed enzymes to fresh substrate could be achieved by simply adding the residual substrate to a fresh batch of the pretreated material. Previously, Eriksson and Vallander (*7,8*) developed a model to carry out a semicontinuous hydrolysis of steam-exploded wood. In this model, a partial recovery of the cellulases was obtained by recycling a fraction of the unhydrolysed residue that had been withdrawn from a reaction mixture at selected incubation times. β-Glucosidases and other loosely adsorbed components were recovered and recycled by ultrafiltration of the hydrolysis liquor (hydrolysate) obtained when the residue was harvested. It was found that an additional supplementation of about 30% of the enzymes added at the beginning of the recycling procedure was required to maintain good yields throughout the 4-d hydrolysis experiments.

Although the high lignin content of the pretreated substrate used was suggested as the main reason for a partial loss of enzyme components during the recycling stages (*4*), there is little evidence in the literature to

support the occurrence of nonspecific and/or irreversible adsorption of cellulases onto the lignin component of pretreated materials. A partial loss of β-glucosidase activity was reported by Sutcliffe and Saddler (*9*) when this enzyme was placed in contact with lignin-containing residues derived from steam-treated wood. It was observed that the adsorption pattern of β-glucosidases onto steam-exploded lignin increased with the severity of the pretreatment conditions. However, mechanisms such as the simple entrapment of these enzymes within the tridimensional matrix of the pretreated lignin could also have been involved.

In this paper, we have looked at enzyme recovery and recycling during a fed-batch hydrolysis of steam-treated substrates derived from SO_2-impregnated steam-exploded *E. viminalis* chips. We determined how many times an enzyme preparation containing 10 FPU g^{-1} cellulose could be reused to effectively hydrolyze a 5% (w/v) suspension of this substrate. The recycling of β-glucosidases (cellobiases) by ultrafiltration techniques and the influence of the alkali-insoluble lignin on the effectiveness of recycling were also assessed.

MATERIALS AND METHODS

Substrate Preparation and Analysis

The cellulosic substrates used in this study were prepared from SO_2-impregnated *E. viminalis* chips that had been steam-exploded using the conditions described previously (*10,11*). The alkali-insoluble fraction was designated as SEE-WIA. The peroxide-treated fraction was abbreviated to SEE-WIA/H_2O_2.

Klason lignin (acid-insoluble lignin) was determined according to TAPPI Standard Method T222 os-74. The cellulose content was determined from the acid hydrolysate of a Klason lignin determination using HPLC, with an HPX-87H column (Bio-Rad, Richmond, CA) (*12*).

Enzymatic Hydrolysis

Enzymatic hydrolyses of pretreated substrates were carried out using the Celluclast cellulase preparation (Novo Nordisk, Denmark) supplemented with the Novozym β-glucosidase preparation (Novo). Both filter paper (FPU) and cellobiase (CBU) activities were determined as described previously and applied according to the actual cellulose content of the pretreated substrates (*13,14*).

Batch-Mode Hydrolysis of the Substrates

The batch hydrolysis of a 5% (w/v) suspension of the substrates was performed using an enzyme loading with a final filter paper activity of 10

FPU g^{-1} cellulose and cellobiase activity of 25 CBU g^{-1} cellulose. The substrates were hydrolyzed in duplicate at 45°C at a rotation of 150 rpm. The hydrolysis buffer used as 50 m*M* sodium acetate, pH 4.8, containing 6 μg mL^{-1} of tetracyclin was a preservative. The release of soluble sugars during hydrolysis was monitored by HPLC (*15*).

Fed-Batch Hydrolysis of the Substrates with β-Glucosidase Supplementation

During the semicontinuous hydrolysis of the substrates, an enzyme preparation containing 10 FPU g^{-1} cellulose and 25 CBU g^{-1} cellulose was added at the beginning of the first hydrolysis step and no further addition of Celluclast was made. The substrate concentration of 5% (w/v) was also used. Other hydrolysis conditions were those described in the previous section.

The method used for enzyme recycling was an adaptation of the method described earlier (*6*) (Fig. 1A). The substrates were hydrolyzed for 24 h and, after the hydrolysis mixture was cooled in an ice bath, the unhydrolyzed residue was harvested and washed with an excess of hydrolysis buffer. The washed residue was then resuspended in fresh hydrolysis buffer containing both cellobiase activity and enough fresh substrate to reconstitute the initial 5% (w/v) substrate consistency. The amount of fresh substrate that was added at the beginning of each new hydrolysis step was equivalent to the amount of glucose found in the hydrolyzate (allowing for the "anhydro" correction and the cellulose content of the substrate) obtained after a 24 h hydrolysis run using fresh enzyme (batch hydrolysis). An excess of Novozym was added to the reaction mixture at the initiation of each new hydrolysis step (i.e., each recycling step), because this enzyme was not expected to adsorb efficiently to the cellulosic residue. The mixture containing fresh substrate, added cellobiase, and the hydrolysis residue, was then incubated at 45°C for subsequent hydrolysis. This procedure was repeated several times until the glucose yield per hydrolysis step started to drop. Glucose yield was defined as, the percentage of available cellulose at time zero that is released as glucose, after the indicated incubation time.

The protein content of the hydrolysates obtained after each hydrolysis step was determined using the Bio-Rad assay and the immunoglobulin IgG as a standard. The enzymes present in each of these hydrolysates were collected by ultrafiltration (UF), using an Amicon membrane with a molecular weight (MW) cutoff of 10 kDa. After the sugar concentration of the hydrolysates was reduced by ultrafiltration to noninhibitory levels, the UF retentates were resuspended in hydrolysis buffer and assayed for their filter paper and cellobiase activity as described previously.

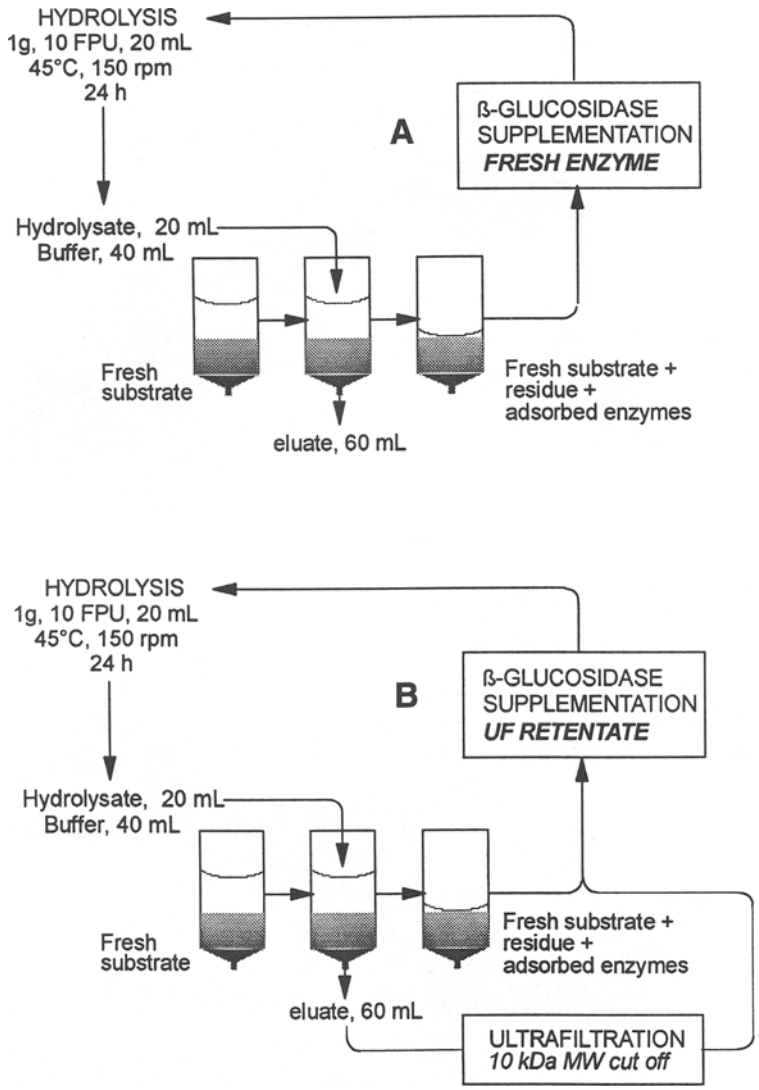

Fig. 1. Schematic representation of the methodology used for the semi-continuous (fed-batch) hydrolysis of pretreated cellulose. β-glucosidase supplementation was performed using (**A**) a fresh batch of Novozym or (**B**) the ultrafiltration retentate obtained from the preceding hydrolysis step.

Fed-Batch Hydrolysis of the Substrates Without any Further Enzyme Supplementation

The same fed-batch hydrolysis procedure described above was followed, except that it was the enzymes present in the UF retentate of a previous hydrolysis step that was used to supplement the next hydrolysis step, rather than using a fresh Novozym preparation containing an

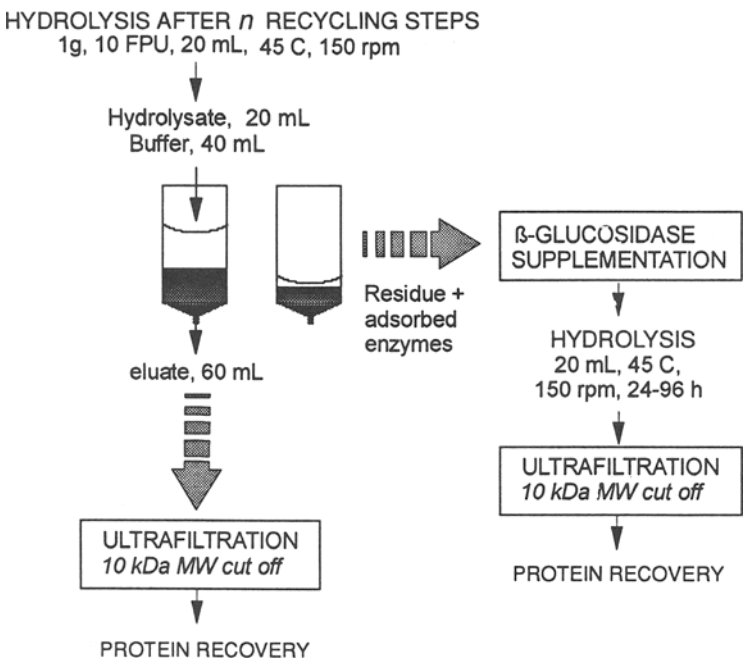

Fig. 2. Schematic representation of the methodology used to characterize both loosely and tightly adsorbed enzyme components of the Celluclast/Novozym preparation.

excess of cellobiase activity (Fig. 1B). These UF retentates were shown to contain a considerable amount of cellobiase activity and virtually no filter paper activity.

Characterization of the Enzyme Profile During Fed-Batch Hydrolysis of Pretreated Substrates

We also wanted to determine which enzyme components remained tightly adsorbed to the hydrolysis residue throughout the recycling experiment. To accomplish this, the hydrolysis residue obtained at every new 24 h hydrolysis step was harvested and washed thoroughly with hydrolysis buffer (*see* Fig. 2). To this residue, hydrolysis buffer and an excess of cellobiase activity was added and the mixture was incubated until the substrate was almost completely solubilized. At this point, the enzymes that were originally adsorbed onto the 24 h hydrolysis residue were expected to be released back into solution. The final hydrolysate was centrifuged and the supernatant was concentrated using an Amicon apparatus with a 10 kDa MW cutoff to eliminate most of the soluble sugars. Both the protein content (Bio-Rad assay) and the enzyme activity against filter paper and cellobiose were monitored in these UF retentates.

Table 1
Chemical Composition and Enzymatic Hydrolysis of the Fractions Derived from SO_2-Impregnated Steam-Exploded Eucalyptus Chips

	Chemical composition,[b] percent			Cellulose
SO_2SEE[a]	Glucan[c]	Xylan[c]	TAP[d]	hydrolysis,[e] percent
Unextracted	41.7	14.1	31.0	1.1
SEE-WIA	86.7	1.2	7.5	50.1
SEE-H_2O_2	94.9	n.d.	0.6	59.8

[a] SO_2-SEE, SO_2-impregnated steam-exploded eucalyptus chips; WIA, water and alkali insoluble fraction; H_2O_2, alkaline peroxide-treated fraction.
[b] Calculated in relation to the substrate dry weight.
[c] Determined in the hydrolysates of a Klason lignin determination by HPLC (*12*).
[d] Klason lignin plus acid-soluble lignin.
[e] Extent of cellulose saccharification after 24 h of hydrolysis of the substrate at a 5% (w/v) consistency, using an enzyme loading of 9-10 FPU g^{-1} cellulose.

RESULTS AND DISCUSSION

Batch Hydrolysis of the Pretreated Substrates

The batch hydrolysis of both the alkali-washed and the peroxide-treated fractions derived from SO_2-impregnated steam-exploded eucalyptus chips was carried out at 5% (w/v) substrate concentration, using a commercial enzyme preparation (Celluclast plus Novozym, Novo Industri, Denmark) with a final activity profile of 10 FPU g^{-1} and 25 CBU g^{-1} cellulose.

Almost 50% of the cellulose component of the alkali-washed fraction was shown to be hydrolyzed to soluble sugars after 24 h (Table 1). The accessibility of this fraction was enhanced by further treating it with alkaline hydrogen peroxide (*11*). Under the same hydrolysis conditions, almost 60% of the peroxide-treated fraction was converted to soluble sugars. Owing to the high levels of β-glucosidase (cellobiase) added, glucose was the main hydrolysis product.

Fed-Batch Hydrolysis of the Alkali-Washed Fraction

The fed-batch hydrolysis of the alkali-washed fraction was carried out by collecting the residual substrate remaining after 24 h of hydrolysis and adding it to a flask containing 8 CBU g^{-1} total cellulose (residual + added) of cellobiase activity and enough fresh substrate to reconstitute the initial

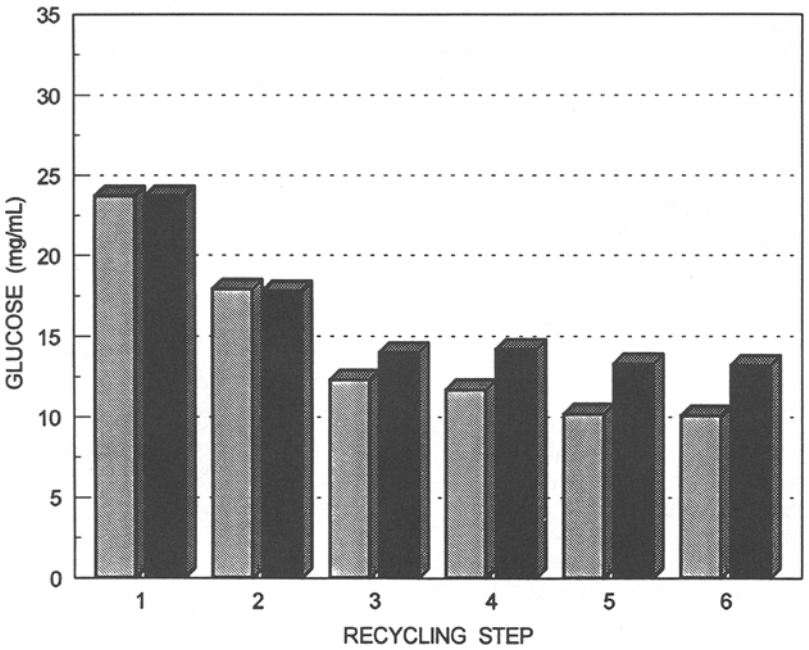

Fig. 3. Glucose yields obtained during a fed-batch hydrolysis of the alkali-washed residue derived from steam-treated eucalyptus. An enzyme loading, containing 10 FPU g^{-1} and 25 CBU g^{-1} cellulose, was added at the beginning of the first hydrolysis step and no further addition of cellulases was made. Fresh Novozym was supplemented to the mixture at the beginning of every new hydrolysis step. Left bar, 24 CBU g^{-1} cellulose; Right bar, 8 CBU g^{-1} cellulose.

hydrolysis conditions. Celluclast was only added at the beginning of the first hydrolysis step. When this procedure was repeated several times, a considerable decrease in hydrolysis efficiency was observed after the second hydrolysis steps (Fig. 3). To determine whether we had added enough supplemental cellobiase activity, we increased the amount of Novozym added to each new hydrolysis step to 24 CBU g^{-1} cellulose. This increase in added cellobiase activity did not have any effect on the glucose yield obtained after each hydrolysis step.

When we determined the protein content of each of the hydrolysates obtained from each recycle step, a similar amount of protein was detected in solution after the second and subsequent recycling steps (Fig. 4). In both the experiments described above, the amount of protein detected was equivalent to the amount of β-glucosidase (Novozym) added at the beginning of each hydrolysis step. The activity profile of each of these hydrolysates was also determined after most of the soluble sugars had been removed by ultrafiltration. Although equivalent levels of cellobiase activity were detected, no filter paper activity could be detected. These results suggested that most of the cellulase components of the Celluclast

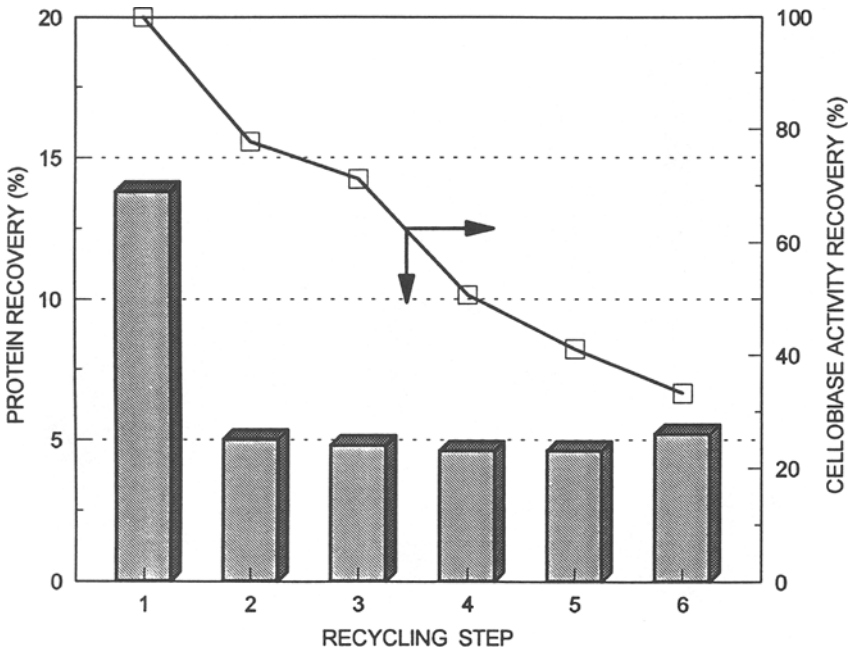

Fig. 4. Protein and cellobiase activity recovered in solution during a fed-batch hydrolysis of the alkali-washed residue derived from steam-treated eucalyptus. Fresh Novozym (8 CBU g^{-1} cellulose) was added to the mixture at the beginning of every new hydrolysis step.

preparation remained adsorbed onto the hydrolysis residue and were not eluted by a thorough washing with hydrolysis buffer. As the alkali-treated residue derived from steam treated eucalyptus did not provide good cellulase recovery, we next assessed whether a subsequent peroxide treatment of the substrate enhanced enzyme recovery.

Fed-Batch Hydrolysis of the Peroxide-Treated Fraction

The peroxide-treated fraction derived from the steam-exploded *E. viminalis* that had been water and alkali-extracted was also hydrolyzed using the fed-batch conditions described above. Although the glucose yields obtained throughout seven consecutive hydrolysis runs (six recycling steps) were somewhat scattered, a production of 26.1 ± 1.7 mg mL^{-1} of glucose was maintained after the first hydrolysis step without any further addition of cellulase enzymes (Fig. 5). As a fixed amount of fresh substrate was added at the beginning of each new hydrolysis step, there was an apparent decrease in the net amount of cellulose hydrolyzed per hydrolysis step (Table 2). This was probably owing to the gradual decrease in

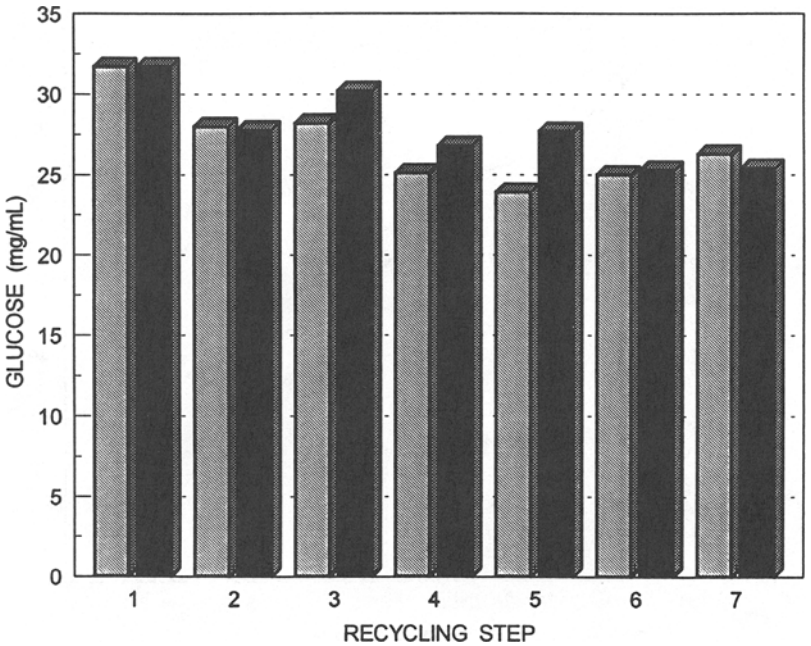

Fig. 5. Glucose yields obtained during a fed-batch hydrolysis of the peroxide-treated fraction derived from steam-treated eucalyptus. An enzyme loading, containing 10 FPU g^{-1} and 25 CBU g^{-1} cellulose, was added at the beginning of the first hydrolysis step and no further addition of cellulases was made. Left bar: fresh Novozym (10 CBU g^{-1} of cellulose) was supplemented to the mixture at the beginning of every new hydrolysis step. Right bar, cellobiase supplementation was performed using the ultrafiltration retentate derived from the hydrolysate of the previous hydrolysis step.

Table 2
The Progressive Reduction in Cellulose Saccharification During Fed-Batch Hydrolysis of Peroxide-Treated Eucalyptus and the Corresponding Increase in the Residual Substrate Concentration

Hydrolysis step[a]	Cellulose hydrolysis,[b] percent	Substrate concentration,[c] percent
1	59.8	4.9
2	53.4	5.0
3	49.6	5.4
4	41.2	5.7
5	34.5	6.3
6	33.6	7.1
7	32.3	7.7

[a]Number of consecutive hydrolysis steps.
[b]Defined as the percentage of available cellulose at time zero that was released as glucose after 24 h of hydrolysis.
[c]Calculated in relation to the amount of substrate that was hydrolyzed during the preceding hydrolysis step.

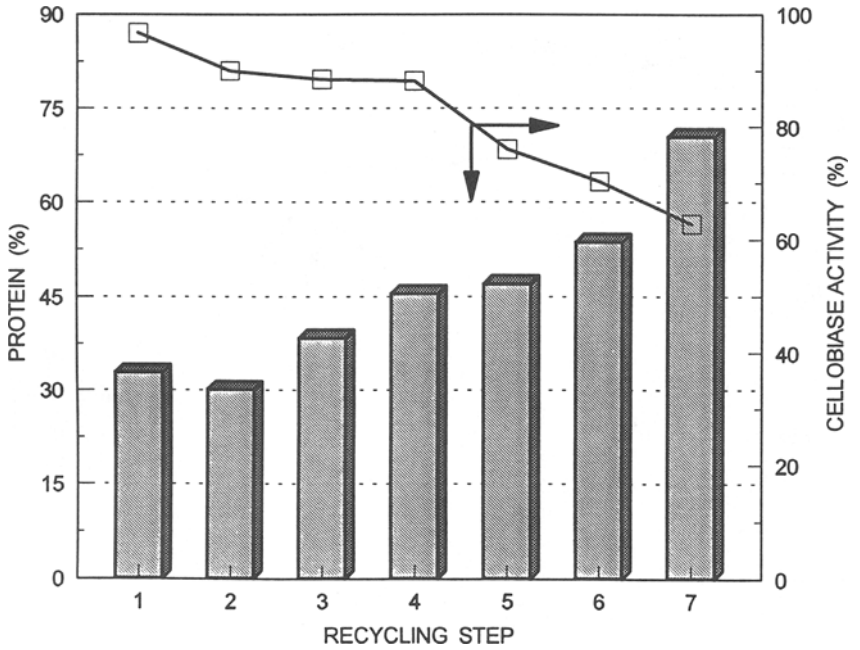

Fig. 6. Protein and cellobiase activity recovered in solution during a fed-batch hydrolysis (5%, w/w) of the peroxide-treated fraction derived from steam-treated eucalyptus. Fresh Novozym (24 CBU g^{-1} cellulose) was added to the mixture at the beginning of every new hydrolysis step.

the enzyme:substrate ratio resulting from substrate accumulation. This gradual increase in the amount of substrate was shown to be inversely proportional to the effectiveness of cellulose hydrolysis, when this was calculated in terms of the amount of cellulose solubilized per gram of cellulose present in the reaction mixture.

Each of the hydrolysates obtained was then assayed for their protein content. A fixed amount of protein, equivalent to the amount of the β-glucosidase Novozym routinely added at the beginning of each new hydrolysis step, was shown to be recovered in each of the hydrolysates (Fig. 6). Although a considerable amount of cellobiase activity was detected in the retentates after ultrafiltration, no filter paper activity was detected. These results strengthened the probability that most of the cellulase components of the Celluclast preparation remained in close association with the unhydrolyzed residue. The slight loss in cellobiase activity that was observed did not seem to be followed by an equivalent loss in protein. Therefore, it seemed that part of the cellobiase activity was lost by inactivation or denaturation.

Previously we had shown that, when a batch hydrolysis of peroxide-treated eucalyptus at 6% (w/v) was stopped after 24 h and the hydrolysate was replaced with an equivalent volume of buffer containing only cellobiase activity, the residual cellulose could be completely solubilized

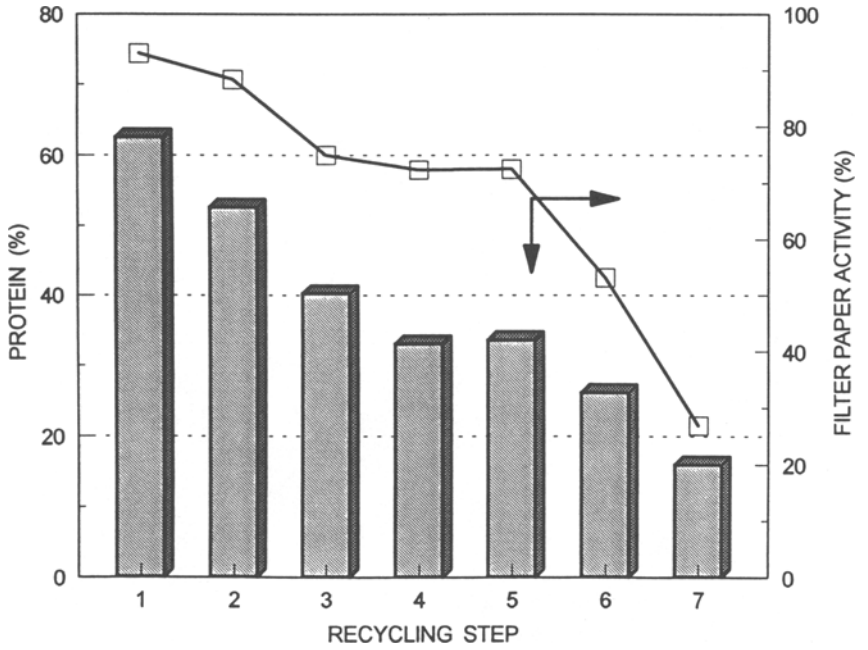

Fig. 7. Protein and filter paper activity recovered in solution after complete hydrolysis of the cellulosic residue obtained at the end of the previous hydrolysis step. Fresh Novozym (24 CBU g^{-1} cellulose) was added to assure complete hydrolysis of the residue.

to glucose without any further addition of cellulases (*6*). In the work reported here we used the same procedure to achieve complete hydrolysis of each of the residues recovered after a 24 h hydrolysis (Fig. 2). In this way we hoped to recover most of the enzymes that were adsorbed onto the hydrolysis residues and that would have been transferred to a fresh batch of substrate. The UF retentates, derived from each of these "final hydrolysates," were shown to contain an amount of protein many times higher than the average amount of protein usually detected in the corresponding hydrolysates (*see* Figs. 4 and 6). In fact, a considerable amount of both the original filter paper activity and protein was associated with these residual fractions (Fig. 7). However, after each subsequent recycle step there was a gradual decrease in both the protein content and the ability of these UF retentates to degrade filter paper (Fig. 7). This was unexpected since, despite this gradual loss of both protein and enzyme activity, the yields of soluble sugars (mostly glucose) per hydrolysis step remained relatively high, even in the latter recycling steps. This may be an indication that only those enzyme components that are essential for hydrolysis are closely associated with the substrate.

Recovery and Recycling of β-Glucosidases

β-Glucosidases are required to enhance glucose production by ensuring that the cellobiose concentration in the hydrolysis mixture is below inhibitory levels (*16*). These enzymes are also relatively expensive to produce. Because of the low affinity of these enzymes for cellulose, their recovery and recycling was carried out by ultrafiltration (UF) of the hydrolysates. The UF retentate, containing a significant amount of the original cellobiase activity, was then added to the next hydrolysis step instead of adding a fresh batch of β-glucosidase (Novozym). In this way we hoped to determine whether effective fed-batch hydrolysis could be obtained when no further addition of fresh enzyme was made after the first hydrolysis step.

We then compared the glucose yields obtained for each hydrolysis step, to those obtained when fresh Novozym was supplemented to the mixture at the beginning of every new hydrolysis step. The glucose yields obtained after seven consecutive hydrolysis steps were found to be quite comparable (Fig. 5, Table 3) to the values obtained in the earlier experiment. The amount of filter paper activity that was recovered after complete hydrolysis of the seventh hydrolysis residue (Fig. 2, $n = 7$), was also shown to be similar to the previous experiments. Approximately 41% of the original cellobiase activity added was recovered at the end of the recycle steps when UF retentates were used to supplement each hydrolysis step, compared to a 62.8% recovery when fresh Novozym was added to each step. Approximately 70% of the original protein, added to the first of the hydrolysis reactions utilizing recycle of the UF retentates, was recovered after the seventh hydrolysis step (Table 3). This did not correspond to the recovery of filter paper activity, with only 20% of the original activity obtained after the seventh recycle step for both cellobiase supplementation strategies (Table 3). Despite the considerable reduction in recovered filter paper activity, it was apparent from the hydrolysis yields in the previous experiments that the enzymes were still active on the steam exploded substrate. These results seem to confirm the previous observation that although a considerable amount of activity was lost during the recycle steps (*see* Fig. 1) the cellulase components that remain in close association with the residual substrate are able to carry out effective cellulose hydrolysis.

CONCLUSION

It is possible to carry out a semicontinuous hydrolysis of steam-treated *E. viminalis* wood chips, which have been alkali and peroxide-treated, without any further addition of enzymes (Celluclast and Novozym). This

Table 3
The Effect of β-Glucosidase Recycling on the Efficiency of Cellulose Saccharification During a Fed-Batch Hydrolysis of the Peroxide-Treated Fraction Derived from SO_2-Impregnated Steam-Exploded Eucalyptus Chips

	Cellobiase supplementation[a]	
Determination	Fresh Novozym[b]	UF retentate[c]
Number of hydrolysis steps	7	7
Glucose yield, mg mL^{-1} d^{-1}	26.1 ± 1.7	27.1 ± 1.8
Protein recovery, percent		
in partial hydrolysates[d]	70.4	—
after desorption[e]	20.1	70.2
Total	90.5	70.2
Activity recovery, percent		
Cellobiase activity[d]	62.8	41.0
Filter paper activity[e]	21.6	20.1

[a]A total amount of 9.6 mg of protein, containing 10 FPU g^{-1} and 25 CBU g^{-1} cellulose, was added at the beginning of the first hydrolysis step and no further addition of cellulases was made.

[b]At the beginning of every new hydrolysis step after recycling, 1.6 mg of a fresh preparation of Novozym was supplemented to the mixture.

[c]Obtained by ultrafiltration (UF) of the hydrolysate of the preceding hydrolysis step; this UF retentate was used for supplementation instead of a fresh batch of Novozym.

[d]Total amount of soluble protein recovered in all of the hydrolysates, calculated in relation to the total amount of protein added to the system.

[e]Total amount of protein (or filter paper activity) present in solution after complete hydrolysis of the residue of the preceding hydrolysis step, calculated in relation to the total amount of protein (or filter paper activity) added to the system.

should result in the more efficient production of soluble sugars from pretreated substrates, particularly if the time required for complete hydrolysis of a 5% (w/v) suspension of the substrate, is taken into account. A 96-h batch hydrolysis of a 5% (w/v) concentration of this substrate would result in a hydrolysis liquor containing 50 mg mL^{-1} of glucose (i.e., 12.5 mg mL^{-1} d^{-1} of glucose in 4 d). Our fed-batch process produced 27.2 ± 1.8 mg d^{-1} of glucose for seven consecutive days, using the same 10 FPU g^{-1} cellulose. This should result in substantial savings in terms of both enzyme requirements and higher bioconversion rates.

ACKNOWLEDGMENTS

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