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Continuous Attrition Bioreactor with Enzyme Recycling for the Bioconversion of Cellulose

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ABSTRACT

A new type of reactor, the attrition bioreactor, has been developed to increase the rate of the enzymatic hydrolysis of cellulose and also to cut pretreatment costs. It was found that the attrition bioreactor could be operated continuously or semicontinuously in conjunction with a membrane filter to produce a high cellulose conversion rate and low enzyme consumption. The membrane filter served to contain the enzyme and cellulose within the reactor while allowing sugar to permeate as a product.

Index Entries: Attrition bioreactor; enzymatic hydrolysis; cellulose; enzyme recycling.

INTRODUCTION

The attrition bioreactor (ABR) is a new concept for the enzymatic hydrolysis of cellulose that combines the pretreatment and hydrolysis steps of the conventional method into a one-step process (*1–3*). The time required for the hydrolysis of newsprint or sawdust can be reduced from days to hours by using this process. This enhancement of the conversion of cellulose in an ABR is a result of a combination of factors, including the reduction in crystallinity and the increase in pore volume and surface area of the cellulose being converted, and the increased accessibility of glucosidic bond sites to the cellulase complex (*2*).

A major concern in the implementation of this combined process in the ABR was the possible deactivation of the cellulase enzyme caused by

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the shear introduced by the addition of milling media. Various enzymes have been reported to be susceptible to deactivation in a shear environment, possibly a result of the breakage or disturbance of their tertiary structures (*4–12*). Deeble and Lee (*2*) and Nielson et al. (*13*) observed the deactivation of cellulase during attrition milling. Although there was some enzyme deactivation under high shear, it is offset by the increased rate of hydrolysis in an ABR. The deactivation of cellulase caused by interfacial and shear effects combined has been found to be much more severe and extensive than that caused by the shear effect alone (*4*). A recent study by Jones and Lee (*3*) showed that the deactivation of cellulase in an ABR could be significantly reduced by eliminating the air-liquid interface in the reactor, which was accomplished by filling it full and sealing it.

The purpose of this study was to develop a continuous ABR with enzyme recycling for the enzymatic hydrolysis of cellulose.

MATERIALS AND METHODS

Enzyme

The source of the cellulase enzyme used was a dried commercial preparation of *Trichoderma reesei* (Cellulase TV Conc.) supplied by Miles Laboratories, Elkart, IN. The cellulase had a filter paper activity of approximately 125 FPU/g. The cellulase enzyme solution was prepared by mixing a weighed portion of the enzyme into a .1*M* sodium acetate (NaAc) buffer of pH 4.7. This solution was used in all experiments at various concentrations of cellulase enzyme.

The cellulase enzyme activity was measured by the filter paper method (*14*). An IU/mL or FPU/mL is μmol glucose/min-mL enzyme solution. An 8 g/L enzyme solution corresponds to approximately 1 FPU/mL.

Substrates

The substrate used in these experiments was a pure microcrystalline cellulose with an average particle size of 50 μm supplied by FMC Corp., Philadelphia, Penn. (Avicel PH 101)

Sugar Analysis

Total reducing sugars were measured by the 3,5-dinitrosalicylic acid (DNS) method as described by Miller (*15*).

Attrition Bioreactor

The attrition bioreactor (ABR) is shown in Fig. 1. The reactor consists of a jacketed stainless steel vessel, milling media, and an impeller. Mixing is provided by a variable speed experimental agitator (Chemineer

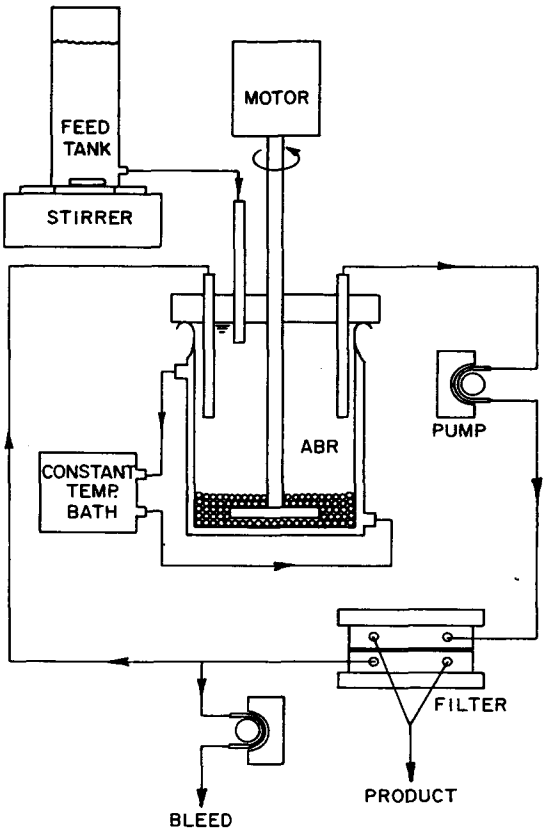

Fig. 1. Attrition bioreactor in continuous operation.

Model ELB Kit). The impeller (a curved-blade turbine impeller) was located just over one ball diameter above the bottom of the vessel. This allowed free movement of the balls under the impeller. The milling media was 3/16 inch 302 stainless steel ball bearings (Hoover Universal, Hartford, CT). The vessel specifications are listed in Table 1.

Table 1
Standard Operating Conditions for the ABR

Substrate	Avicel PH101
pH	4.7
Buffer	.1*M* NaAc
Enzyme	Cellulase of *T. reesei*
Enzyme concentration	1000 FPU/L
Ball size	3/16" 302 stainless steel
Impeller type	Curved-blade turbine
Impeller diameter	6.5 cm
Agitator speed	200 rpm
Reactor temperature	50°C
Height of milling media	2.4 cm
Internal diameter of ABR	10.3 cm

Continuous operation of the ABR employs a membrane filter to separate the enzyme and cellulose from soluble sugars. The membrane filter acts as a molecular sieve, allowing smaller molecules to permeate the membrane while large ones remain in the original solution. The advantage of membrane filtration in cellulose hydrolysis is in separating the desirable sugar product from the cellulose and enzyme. Membrane filtration effectively contains the enzyme and cellulose within the ABR while producing a clean product stream of glucose, cellobiose, and buffer.

Figure 1 also shows the schematic diagram of the ABR (during continuous operation) with its attendant equipment (*16*). The reactor was sealed with a cover and filled with liquid. The outlet stream was pumped by a peristaltic pump. Retained reactor solution (retentate) was returned to the reactor, whereas the permeate (filtrate), containing desired sugars, was a net product. The purpose of the bleeding stream was to prevent the accumulation of the substrate, which was difficult to hydrolyze. The ultrafiltration system is composed of a Pellicon Cassette System (Millipore, Bedford, MA) with a standard high volume Lucite cell, a polysulfone 10,000 mol wt filter, and a retentate linear channel.

To maintain a liquid-full ABR, a gravity feed tank above the reactor supplied cellulose solution as product solution was removed by the membrane filter. Solid settling in the feed tank was prevented by thorough mixing with a magnetic stirrer. The gravity feed tank eliminated the need for a reactor level controller or an additional pump. Only two peristaltic pumps were required to maintain process operations.

RESULTS AND DISCUSSION

Batch Operation

Figure 2 shows the effect of the initial substrate concentration on its conversion (grams of sugar produced per gram of substrate) in an ABR under standard conditions (*see* Table 1). The ratio of the height of the liquid to the vessel diameter (H/D) was .8. As the initial substrate concentration was increased, the conversion decreased, although the product concentration increased. This may be a result of several factors, including product inhibition, a change in the rheological properties, and the limited availability of cellulase enzyme. Increasing the substrate concentration beyond 160 g/L was not practical because the solution becomes too thick to be a liquid medium.

Figure 3 compares the performance of the ABR with that of a regular stirred reactor (SR) with no milling media, both with an initial substrate concentration of 80 g/L. The conversion in the ABR after six h was 55%, whereas the SR reached a conversion of only 25% in the same amount of time. This is an improvement of more than 100% over the SR. The improvement is even more dramatic when the time for the two reactors to reach a given level of conversion is compared. For example, it took 6 h in

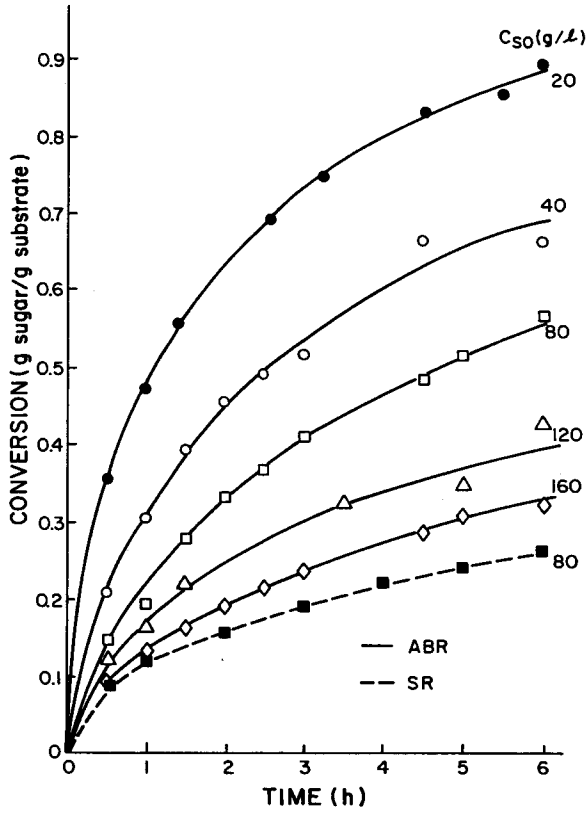

Fig. 2. The effect of substrate concentration on the conversion in an ABR and a regular stirred reactor (H/D = .8, V = .5 L, Other conditions: Table 1).

an ABR to convert 55%, whereas it took 50 h in a regular stirred reactor, which is more than 8 times longer (*see* Table 2).

To understand the magnitude of enzyme deactivation in the ABR, the enzyme solution was stirred in a regular stirred reactor (SR) and in an ABR in batch mode, and their relative enzyme activities were compared over time, as shown in Fig. 4. The enzyme deactivation in the ABR was more pronounced than that in the SR. About 30% of the initial enzyme activity was lost in the SR after 100 h of stirring, whereas about 60% was lost in the ABR. However, since it takes only about one eighth of the time to reach 50% conversion in an ABR compared to an SR (*see* Fig. 3), the ABR is still a significant improvement over the SR with regard to enzyme usage.

Semicontinuous Operation

The ABR was operated in a semicontinuous mode by making a series of batch runs and filtering the reaction solution after each one. Using a crossflow membrane filter with a polysulfone 10,000 mol wt filter, sugars and other soluble components with mol wt less than 10,000 were re-

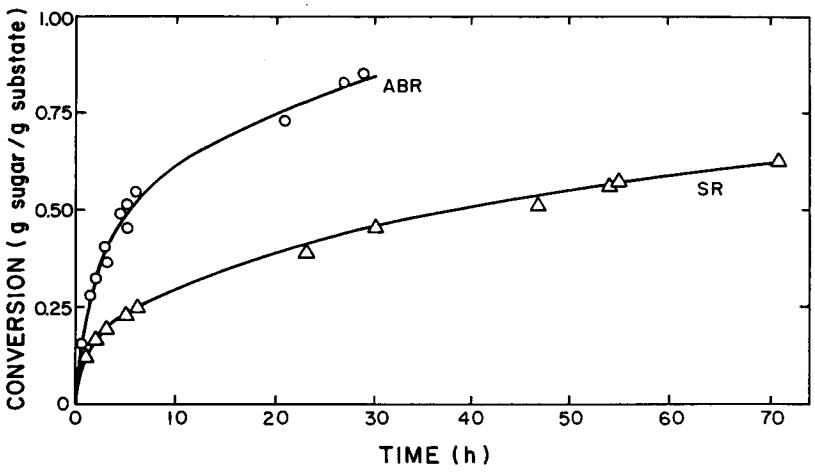

Fig. 3. Comparison of the ABR with a regular stirred reactor (SR) (H/D = .8, V = .5 L, C_{S0} = 80 g/L, Other conditions: Table 1).

moved as filtrate, whereas the unreacted substrate and the cellulase enzyme were recycled back into the reactor. Figure 5 shows the change with respect to time of the total sugar concentration, the amount of cellulose remaining in the reactor, and the amount of total sugars produced in a semicontinuous run. The initial substrate and enzyme concentrations were 76 and 7.6 g/L, respectively. The reaction solution was stored overnight between batch runs in a refrigerator (5°C) and was cycled through the membrane filter the next morning. The next batch run was then begun after replacing the product solution that was taken out as filtrate with fresh buffer solution. About 40 g of cellulose was also added three times during the run, as indicated by the arrow signs in Fig. 5.

Table 2
Comparison of Productivity and Enzyme Usage for Batch or Semicontinuous Processes

Mode of operation	Enzyme recycling method	Duration of run, h	Productivity g sugar/L h	Enzyme usage FPU/g sugar	Ref.
Batch ABR	None	6	7.3	23	[b]
Batch SR[a]	None	50	0.73	23	[b]
Semicont. ABR	UF[c]	54	2.6	7	[b]
Semicont. SR	two-phase[d]	1678–2105	.36–.74	15.5–17.8	(*18*)
Semicont. SR	two-phase with UF[d]	1250	.12–.30	8.7	(*19*)

[a]Regular stirred reactor.
[b]This study.
[c]Ultrafiltration.
[d]Aqueous two-phase system based on crude dextran and polyethylene glycol (Substrate: Sloka Floc BW200; Enzyme: Celluclast 2.0 L Type X).

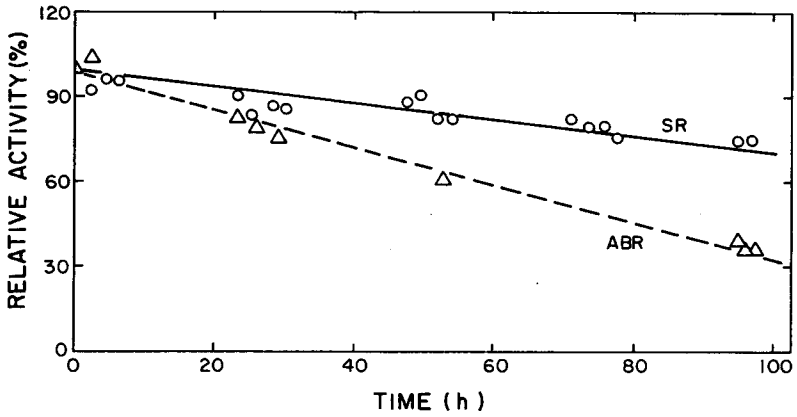

Fig. 4. Enzyme deactivation in the ABR and the SR. (H/D = 1.37, V = 1 L, C_{S0} = 0 g/L, Other conditions: Table 1).

In the first phase of this semicontinuous run, the conversion reached only 45%, which is about 10% less than that of the corresponding batch run of Fig. 2 (80 g/L initial substrate concentration). This is a result of the fact that the total amount of reaction solution for the semicontinuous run (1000 mL) was twice as much as that for the batch run of Fig. 2 (500 mL). Since the amount of milling media and the agitation speed were the same for the two runs, the amount of power input per unit reaction solution

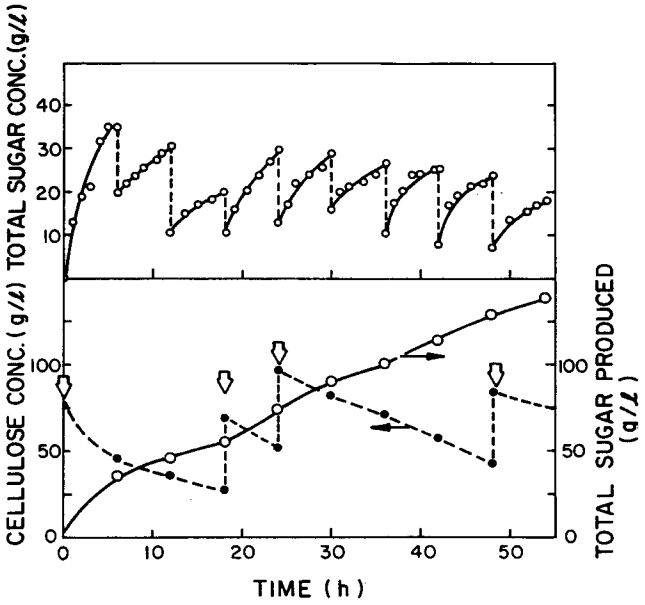

Fig. 5. Semicontinuous operation of the ABR with enzyme recycling. The vertical arrow indicates the substrate loading. The amount of cellulose in the reactor was estimated from the conversion data with the theoretical yield. (H/D = 1.37, V = 1 L, C_{S0} = 76 g/L, Other conditions: Table 1).

for the semicontinuous run was about half of that for the batch run. It has been shown in a previous study by Deeble and Lee (*2*) that conversion is a function of energy consumption, with conversion increasing rapidly with increased energy consumption, up to the maximum level.

In the next two phases of the semicontinuous run there was no addition of fresh cellulose. The rate of sugar production, represented by the slopes of the curves, decreased with each succeeding phase, even though a large percentage of the product was removed and replaced with fresh buffer solution. This is probably mainly caused by the decrease in the amount of easily digestible substrate available. It is known that the cellulose structure is transformed into a less digestible form during hydrolysis (*17*). The nature of this less digestible form is not well known.

The addition of fresh cellulose at the beginning of the fourth phase revived the rate of conversion. However, the rate decreased slightly at the subsequent addition of cellulose as the unreacted cellulose accumulated. After 48 h of semicontinuous hydrolysis, the addition of cellulose did not increase the rate of reaction significantly. In addition to the buildup of unreacted cellulose, this can also be attributed in part to the deactivation of the cellulase in the reactor during attrition milling and hydrolysis.

The overall productivity of the semicontinuous run was 2.6 g sugar/L h, and the enzyme usage to produce 1 g of reducing sugar was 7 FPU/g sugar after 54 h of semicontinuous operation. The productivity of semicontinuous operation was lower than that of batch operation (7.5 g sugar/L h), but the enzyme usage was only one third of that for the batch run (22.2 FPU/g sugar). A comparison of these two runs, as well as a regular stirred reactor run and some other reported data, for productivity and enzyme usage is shown in Table 2. The ABR with enzyme recycling in this study required the lowest enzyme usage per gram of sugar produced. The two-phase system combined with ultrafiltration by Tjerneld, et al. (*19*) had a low enzyme usage (8.7 FPU/g sugar), but the productivity was less than one-twentieth of that of the ABR.

Continuous Operation

The flowchart for continuous operation of the ABR with enzyme recycling is shown in Fig. 6. The material balance for the substrate (cellulose) and product (total sugar) results in the following equations

$$FC_{SF} - BC_S + r_S V = V\, dC_S/dt \tag{1}$$

$$FC_P + r_P V = V\, dC_P/dt \tag{2}$$

Note that r_S and r_P are the rate of substrate consumption and product formation, respectively, due to the enzyme hydrolysis reaction. On the other hand, dC_s/dt and dC_P/dt are the change with respect to time of the concentration of the substrate and the product in the reactor, respec-

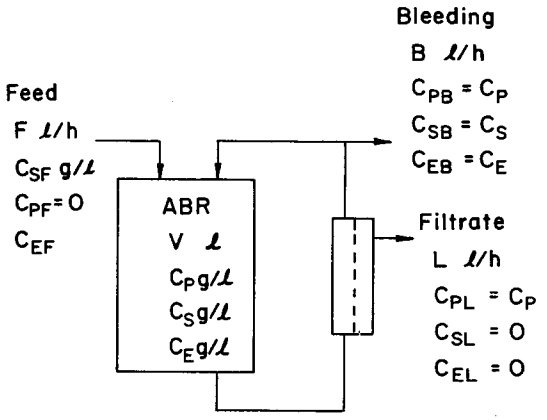

Fig. 6. Flow chart of the continuous operation of the ABR with enzyme recycling.

tively. The rates r_P and r_S can be related by using the yield constant ($Y_{P/S}$) as

$$r_P = -Y_{P/S} r_S \tag{3}$$

The theoretical yield for the hydrolysis of pure cellulose to glucose is 1.11. The combination of Eq. (1)–(3) gives the relationship

$$r_P = dC_P/dt + (F/V)\, C_P = -Y_{P/S}\, dC_S/dt + (Y_{P/S}/V)\,(FC_{SF} - BC_S) \tag{4}$$

If the reactor is operated at steady state and there is no bleeding, dC_P/dt, dC_S/dt, and B will be equal to zero and equation (4) can be simplified to

$$r_P = (F/V)\, C_P = (Y_{P/S}/V)\, FC_{SF} \tag{5}$$

From this equation, the product concentration and the inlet substrate concentration are related as

$$C_P = Y_{P/S}\, C_{SF} \tag{6}$$

The results of the three continuous runs are shown in Table 3. The operating parameters varied for the three runs were the flow rate and the inlet cellulose concentration. In each case, continuous operation was preceded by six hours of batch operation. The initial enzyme added was 8 g/L (about 1000 FPU/L) for all runs, and no more enzyme was added during continuous operation. The highest productivity obtained for these continuous runs was about 3 g/L h, with an enzyme usage of 7.7 FPU/g of reducing sugar produced, which is comparable to the result of the semicontinuous run (*see* Table 2).

Figure 7 shows the change of the total sugar concentration with respect to time for runs number 1 and 3. In both cases, steady state was reached within 10 h and was maintained for about 15 h, after which the total sugar concentration began to drop gradually. This drop is not predicted by Eq. [4], which suggests that a decrease in product concentra-

Table 3
Summary of Continuous Runs

Run no.	Av. flow rate, F, L/h	Inlet sub. conc. C_{SF}, g/L	Av. prod. conc., C_P, g/L	Productivity, g/L h	h of cont. run	Enzyme usage,[a] FPU/g sugar
1	.052	30	27	1.4	36	13
2	.170	15	18	3.1	30	9
3	.145	20	20	2.9	46	7

[a]Enzyme added/(sugar in the total filtrate + sugar in the reactor at the end of the run).

tion would lead to an increase in the substrate concentration in the reactor, which would in turn result in an increased rate of reaction and a revival of steady state. The failure to regain steady state in actual practice was probably the result of several factors. The most significant of these were probably enzyme deactivation, the accumulation of the less digestible form of cellulose, and the decrease of the amount of easily digestible cellulose available for hydrolysis.

These factors were taken into consideration in the continuous run shown in Fig. 8. The enzyme was supplemented in the feed stream according to the expected rate of deactivation of cellulase in the ABR and the reactor contents were bled in order to prevent the accumulation of the less digestible cellulose. The operating conditions are listed in the figure caption. As expected, the product concentration reached a steady-

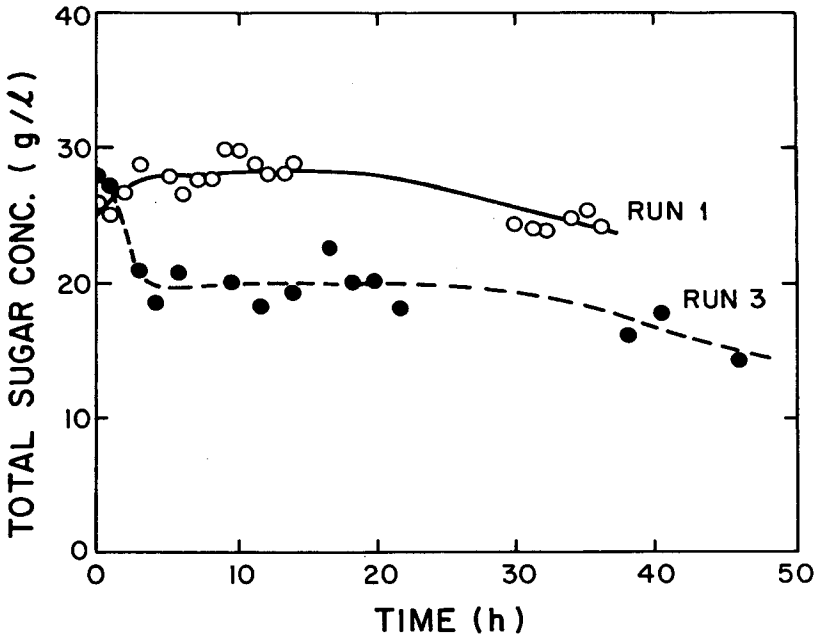

Fig. 7. The change of product concentration with time in continuous runs. (H/D = 1.37, V = 1 L, C_{S0} = 80 g/L, Other conditions: Tables 1 and 3).

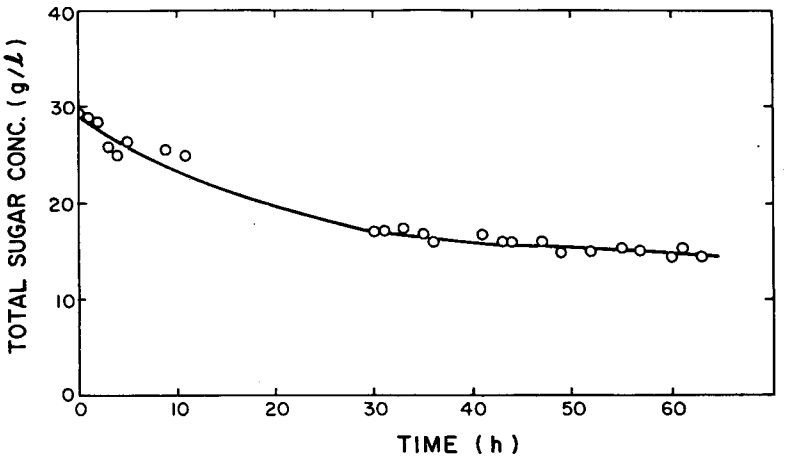

Fig. 8. The change of product concentration with time in a continuous run with bleeding stream. (H/D = 1.37, V = 1 L, C_{S0} = 80 g/L, C_{SF} = 20 g/L, C_{EF} = 2 g/L, F = .120 L/h, B = .035 L/h, Other conditions: Table 1).

state value that was maintained for about 30 h until the run was stopped. There was a very gradual decrease in the product concentration during this time that might be corrected by increasing the enzyme concentration of the feed and the flow rate of the bleeding stream. The average concentration of the product was 17.7 g/L and the overall productivity was 2.8 g/L h. The enzyme usage was 17 FPU/g sugar produced, which was greater than that of the continuous runs without the bleeding stream (*see* Table 3).

To confirm that the reactor had indeed reached steady state, the steady-state product concentration in this run was compared with that predicted from the material balance Eq. [4]. Setting dC_P/dt and dC_s/dt equal to zero in Eq. [4], we obtain

$$C_P = Y_{P/S}\left(C_{SF} - B/F\ C_S\right) \tag{7}$$

The substrate concentration of the bleeding stream (C_S in the above equation) can be estimated from the overall integral material balance of the run. The steady-state product concentration predicted by this equation was 15 g/L. This is very close to the experimental value of 16 g/L, which confirms that steady state had been reached.

CONCLUSIONS

The amount of cellulose converted in an ABR after six h of batch operation was over 100% greater than that in a regular stirred reactor, which took about eight times longer to reach the same level of conversion. Although 60% of the initial enzyme activity was lost in the ABR after 100 h, compared to 30% for the SR, this was more than made up for

by the 8-fold reduction of hydrolysis time in the ABR. The ABR proved to be much more efficient than the SR in the usage of enzyme.

It was demonstrated that the ABR can be operated in semicontinuous mode. The overall productivity of semicontinuous operation was 2.6 g sugar/L h, with an enzyme usage of 7 FPU/g sugar after 54 h of operation.

The ABR was also operated successfully in a continuous mode with enzyme recycling, with steady state being reached within 10 h. Continuous operation resulted in a productivity of about 3 g sugar/L h, with an enzyme usage of 8 FPU/g sugar produced. Steady state was maintained for a longer period of time by supplementing the enzyme according to its rate of deactivation in the ABR, and bleeding the reactor contents to prevent the accumulation of undigested substrate. The product concentration in this case reached a steady-state value and remained there until the run was stopped 30 h later. The average product concentration for the run was 17.7 g/L, and the productivity and enzyme usage were 2.8 g/L h and 17 FPU/g sugar produced, respectively.

ACKNOWLEDGMENT

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NOMENCLATURE

B	The flow rate of the bleeding stream, L/h.
C_E	Enzyme concentration, g/L.
C_P	Product sugar concentration, g/L.
C_S	Substrate concentration, g/L.
F	Medium flow rate, L/h.
L	The flow rate of the filtrate stream, L/h.
r_P	The rate of product formation by enzymatic hydrolysis, g/L h.
r_S	The rate of substrate consumption by enzymatic hydrolysis, g/L h.
t	Time, h.
V	The volume of the medium in the reactor, L.
$Y_{P/S}$	The yield constant, g of product formed/g of substrate consumed.

Subscripts

0	Initial value.
B	Bleeding stream.

F Feed stream.
L Filtrate stream.

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