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Continuous Fermentation of Cellulosic Biomass to Ethanol

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ABSTRACT

Experimental results are presented for continuous conversion of pretreated hardwood flour to ethanol. A simultaneous saccharification and fermentation (SSF) system comprised of *Trichoderma reesei* cellulase supplemented with additional β-glucosidase and fermentation by *Saccharomyces cerevisiae* was used for most experiments, with data also presented for a direct microbial conversion (DMC) system comprised of *Clostridium thermocellum*. Using a batch SSF system, dilute acid pretreatment of mixed hardwood at short residence time (10 s, 220°C, 1% H_2SO_4) was compared to poplar wood pretreated at longer residence time (20 min, 160°C, 0.45% H_2SO_4). The short residence time pretreatment resulted in a somewhat (10–20%) more reactive substrate, with the reactivity difference particularly notable at low enzyme loadings and/or low agitation. Based on a preliminary screening, inhibition of SSF by byproducts of short residence time pretreatment was measurable, but minor. Both SSF and DMC were carried out successfully in well-mixed continuous systems, with steady-state data obtained at residence times of 0.58–3 d for SSF as well as 0.5 and 0.75 d for DMC. The SSF system achieved substrate conversions varying from 31% at a 0.58-d residence time to 86% at a 2-d residence time. At comparable substrate concentrations (4–5 g/l) and residence times (0.5–0.58 d), substrate conversion in the DMC system (77%) was significantly higher than that in the SSF system (31%). Our results suggest that the substrate conversion in SSF carried out in CSTR is relatively insensitive to enzyme loading in the range 7–25 U/g cellulose and to substrate concentration in the range of 5–60 g/L cellulose in the feed.

Index Entries: SSF; DMC; continuous fermentation; lignocellulose.

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INTRODUCTION

Biologically mediated process steps have a dominant impact on process economics associated with the conversion of cellulosic biomass to ethanol. Reducing the costs of associated bioreactors is thus an important direction for R & D. Continuous bioreactors offer potential advantages, such as high cell concentrations, amenability to advanced configurations (e.g., substrate retention or product removal), and increased on-line time.

The simplest continuous reactor configuration is the well-mixed continuous stirred-tank reactor or CSTR. CSTRs are beginning to be used by the corn ethanol industry for fermentation of soluble substrates (*1*). Owing to the complicated kinetics involved, analytical models for conversion of insoluble substrates in a CSTR are largely unavailable. Experimental data on insoluble substrate utilization in CSTRs are also limited. Nonwell-mixed continuous processing, recently proposed and modeled for insoluble substrates (*2*), may allow significantly higher throughputs of cellulosic substrates than CSTR configurations because of factors, such as substrate retention, biocatalyst retention, and substrate stratification. Thus, in addition to being a candidate for large-scale processing *per se*, the study of conversion of such substrates in CSTR is of interest in that it establishes a point of reference for more advanced reactor systems.

This article reports initial results from an ongoing study of continuous conversion of pretreated hardwood lignocellulosics to ethanol. Two conversion systems are considered in this work. The first is a simultaneous saccharification and fermentation (SSF) system comprised of externally supplied *T. reesei* cellulase with fermentation by *Saccharomyces cerevisiae*. The second is a direct microbial conversion (DMC) system, with both cellulase production and fermentation mediated by *Clostridium thermocellum*.

MATERIALS AND METHODS

Organisms and Enzymes

Saccharomyces cerevisiae strain D_5A was used for all SSF fermentations. The organism was supplied by the National Renewable Energy Laboratory (NREL) and was stored at 4°C in YPD tubes. *Clostridium thermocellum* strain ATCC 27405 was used for DMC fermentations, with culture handling as previously described (*3*). Enzymes used in the SSF work were Genencor CL cellulase (Genencor Inc., San Francisco) and Novozyme 188 β-glucosidase (NOVO Laboratories Inc., Wilton CT).

Cellulase activity is reported as filter paper activity units (*4*), and β-glucosidase activity was measured using the nitrophenyl-β-glucosidase assay (*4*); both these activities were determined at 50°C. The addition of β-glucosidase to the cellulase increases the specific activity of the raw cellulase solution by reducing cellobiose inhibition; cellulase activities are reported in the presence of any additional β-glucosidase that was added.

Substrates

Two different pretreated hardwood lignocellulosic feedstocks were used in this investigation. Substrate 1 was dilute-acid pretreated mixed hardwood flour prepared in the continuous-plug flow reactor described by McParland (*5*). The hardwood flour (Louis O. Beede Co., Lowell, MA) was approx 90% birch milled to 100% passing a #60 standard sieve. Substrate 1 was pretreated using a 7–10 wt% wood slurry direct steam-heated to 220°C for 9–11 s in the presence of sufficient concentrated H_2SO_4 to yield a 0.8–1.0% solution after dilution by steam. The resulting wood slurry was filtered and then washed with an equal volume of water to remove the majority of soluble pretreatment byproducts. Substrate 2 was dilute-acid pretreated poplar chips prepared by NREL. The pretreatment of the popular chips consisted of heating to 160°C for 20 min in the presence of 0.45% acid. Using quantitative saccharification, both substrates 1 and 2 were found to be comprised of approx 60 wt% cellulose and essentially devoid of any noncellulose carbohydrate components. Typical particle sizes for the two feedstocks after pretreatment were approx 0.05 mm for substrate 1, and 2 mm for substrate 2.

Experiments undertaken using time/temperature sterilization monitors (Diack Inc., Behlah, MI) indicated it was necessary to autoclave the 20-L quantities of 5 wt% cellulose wood slurry used for in excess of 9 h. At the concentrations used, the slurry settles to form a dense bed of solids, which we presume reduces convection within the carboy, resulting in an increase in required autoclaving times. The wood slurries used in these studies were sterilized by autoclaving at 121°C for a period of 12 h. Washed and filtered wood was stored without neutralization at 4°C until use, up to a maximum of 14 d. Other medium components were sterilized separately and aseptically added after the wood slurry had cooled, to give the desired final substrate concentration.

The medium composition for both the batch and continuous SSF work was as reported previously by NREL (*6*). In addition to pretreated wood at concentrations specified in the text, SSF medium included yeast extract (10 g/L), peptone (20 g/L), penicillin (10,000 U/L), and streptomycin (10 mg/L). As evidenced by microscopic inspection, neither reactors nor feed carboys showed biological contamination in the presence of the antibiotics. Medium for the inhibition experiment was formulated using nondiluted filtrate from a typical wood pretreatment at conditions used to pretreat substrate 1. Filtrate was pH adjusted to 4.5 with NaOH, supplemented with 10 g/L yeast extract, 20 g/L peptone, and glucose to 20 g/L and filter-sterilized using a 0.2-μm Acrocap filter (Gelman). Table 1 presents HPLC analysis of the pretreatment filtrate. For SSF carried out at dilute (5 g/L cellulose) concentration, the medium components were reduced to 2 g/L yeast extract and 4 g/L peptone.

Medium for the DMC studies contained: 0.50 g/L yeast extract, 2.00 g/L MOPS sodium salt, 0.1 g/L $(NH_4)_2SO_4$, 1.00 g/L citric acid·H_2O, 0.6

Table 1
Major Carbohydrate-Derived Products Found in Hydrolyzate of a Typical Pretreatment of Substrate 1 (Mixed Hardwood Pretreated for 10 s at 220°C in the Presence of 1% H_2SO_4), as Determined by HPLC

	Concentration, g/L
Glucose	2.6
Acetic acid	1.0
Hydroxymethyl furfural (HMF)	ND[a]
Xylose	8.7
Furfural	0.1

[a]Not detected using the HPLC analysis as previously described.

g/L NH_4Cl, 0.80 g/L $K_2HPO_4 \cdot 3H_2O$, 0.60 g/L $NaH_2PO_4 \cdot H_2O$, 0.60 g/L $MgCl_2 \cdot 6H_2O$, 0.30 g/L $CalCl_2 \cdot 2H_2O$, 0.15 g/L $FeSO_4 \cdot 7H_2O$, 0.5 mg/L $MnCl_2 \cdot 4H_2O$, 0.5 mg/L $CoCl_2 \cdot 6H_2O$, 0.5 mg/L $ZnSO_4 \cdot 7H_2O$, 0.05 mg/L $CuSO_4 \cdot 5H_2O$, 0.05 mg/L H_3BO_3, 0.05 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg/L $NiCl_2 \cdot 6H_2O$, 1 mg/L rezazurin, 10.0 mg/L, *d*-biotin, 0.60 g/L, L-cytseine·HCl, 100 mg/L pyridoxamine·2HCl, 20.0 mg/L, *p*-aminobenzoic acid, 0.5 ml/L, and 10.0 mg/L vitamin B_{12}. A more detailed report of the DMC medium formulation is forthcoming.

Feed and effluent solids content were measured by filtering 5–25 mL of material through a 0.4-μm Nucleopore filter (Gelman), rinsing the solids on the filter with 50 mL water to remove any soluble components, and drying overnight in a 72°C oven. Cellulose solubilization was measured by quantitative saccharification of the feed and effluent material (*7*). Hydrolysis and fermentation products were analyzed by high-pressure liquid chromatography as described previously (*7*); the detection limit of this analysis as performed is 0.1 g/L for compounds analyzed.

All steady-state data exhibited a variation around the mean of <5%, which corresponds to the reproducibility of the analytical techniques used. Carbon recovery is calculated assuming the stoichiometry of CO_2 production, and accounting for the carbon sources present in both the feed and the enzyme solution fed to the reactor. Conversion was calculated by two methods, as follows:

Method 1: $$\text{Conversion} = [(M_c)\text{in} - (M_c)\text{out} / (M_c)\text{in}] \quad (1)$$

where M_c = mass flow rate of cellulose carbon.

Method 2: $$\text{Conversion} = [(M_p)\text{out}^* (1 + Y_x) / (M_c)\text{in}] \quad (2)$$

where M_p = mass flow rate of product carbon out, with CO_2 calculated assuming 1 mol produced/mol ethanol.

$$Y_x = \text{Cell yield (g cell C/ g product C), assumed equal to 0.1} \quad (3)$$

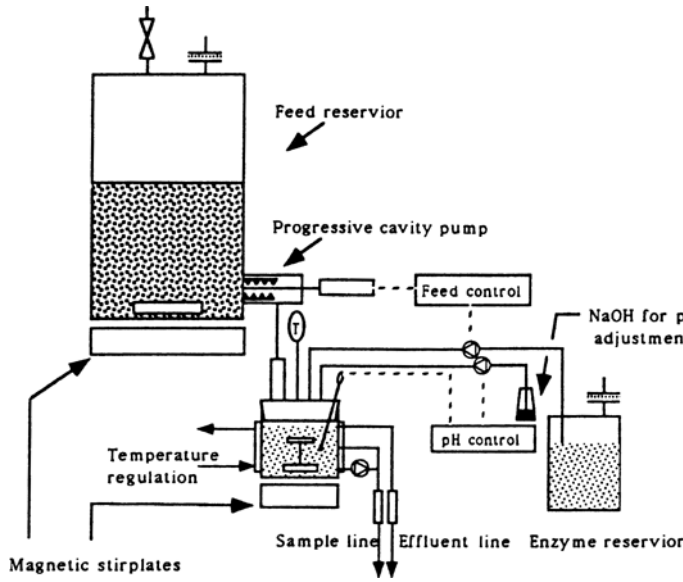

Fig. 1. Schematic arrangement of the SSF feed delivery system and SSF reactor.

Reported conversion are the averages of the conversion calculated by these two methods for SSF. Method 1 was used for DMC.

Experimental Conditions and Apparatus

SSF and inhibition experiments were carried out at 37°C with pH regulated at 4.5. DMC experiments were at 60°C with the pH regulated at 7.0. Temperature was maintained using water-jacketed reactors. Control of pH was by an ADI 1020 process controller (Applikon Dependable Instruments, Sheidam, Holland).

A schematic arrangement of the experimental apparatus used for the SSF work is shown in Fig. 1. The substrate was delivered from a magnetically stirred modified 20-L glass feed carboy by a progressing cavity pump (Pumpenfabrik type V4a, Pumpenfabrik Wangen GMBH, Wangen, Germany) to deliver the lignocellulosic substrate to the SSF reactor. The pump arrangement allowed the stator to penetrate the feed carboy, helping ensure that the pump drew material from a well-mixed area of the carboy. Representative feed delivery was verified by withdrawing samples from the feed carboy. Such samples had a constant solids concentration with respect to both sampling depth and time. Custom glass SSF reactors (NDS Technology Inc., Vineland, NJ) had a working vol of 1.25 L and were magnetically stirred at 200 rpm using modified two-tier stir bars. Enzyme was filter-sterilized into a sterile 4-L carboy and delivered to the reactor by a peristaltic feed pump. To achieve the desired feed flow rates,

it was necessary to run both feed slurry and enzyme pumps intermittently, typically on for 10 s and off for 60 s. Hyperlon stators performed adequately with insoluble feeds after autoclaving for 45 min, although a decrease in the pump's closed head discharge pressure was seen. Other stator materials (Viton, EPDM, Butyl-nitrile rubber) proved less satisfactory.

A transfer system was utilized to introduce material to the pump-equipped feed carboy. Feed was autoclaved in 20-L carboys, and transferred using a combination of pressure and gravity feeding, with mixing of the transfer vessel provided by nitrogen sparging. The feed system was successful in delivering substrate 1; however, the particle size of substrate 2 was such that it matted at the entrance to the pump stator and prevented representative feed delivery. No continuous fermentation of this material is reported.

Batch SSF experiments were carried out in 250-mL Erlenmeyer flasks in a temperature-controlled room. A 5% inoculum was taken from a continuous fermenter growing on a 20-g glucose/L feedstock at a 24-h residence time. Aseptic samples were obtained by forcing broth through sterile sample tubes using filter-sterilized air. Two separate studies were undertaken using different modes of stirring, one using a magnetically driven stir bar at approx 200 rpm, and the other agitating with a shaker table at 250 rpm.

The reactor used for DMC studies was a 1.5-L working volume glass bioreactor (Applikon Dependable Instruments, Sheidam, Holland) custom jacketed and modified to have gravity overflow level control. Dilute pretreated wood slurry was delivered to this reactor using a peristaltic pump feeding from a magnetically stirred 20-L carboy. The experimental setup is essentially as described previously (*8*).

Inhibition experiments were carried out using custom 250-mL working vol water-jacketed reactors (NDS Technology Inc., Vineland, NJ) fitted with #12 neoprene stoppers, pH electrodes (Cole-Parmer, Chicago, IL), and ports for inoculation, feeding, sampling, pH control, temperature measurement, and overflow. Strirring was by magnetically driven stir bars.

RESULTS

Substrate and Pretreatment Characterization

Much of the previous study of batch SSF has been reported by NREL for substrate 2 (poplar, 20 min, 160°C, 0.45% H_2SO_4), which differs substantially from substrate 1 (mixed hardwood, 10 s, 220°C, 1% H_2SO_4), used in previous studies at Dartmouth. To quantify the impact on hydrolysis of the different substrates, pretreatment regimes, and particles sizes, batch experiments were carried out at 35-g cellulose/L substrate concentration, using cellulase supplemented with β-glucosidase at 6 U β-glucosi-

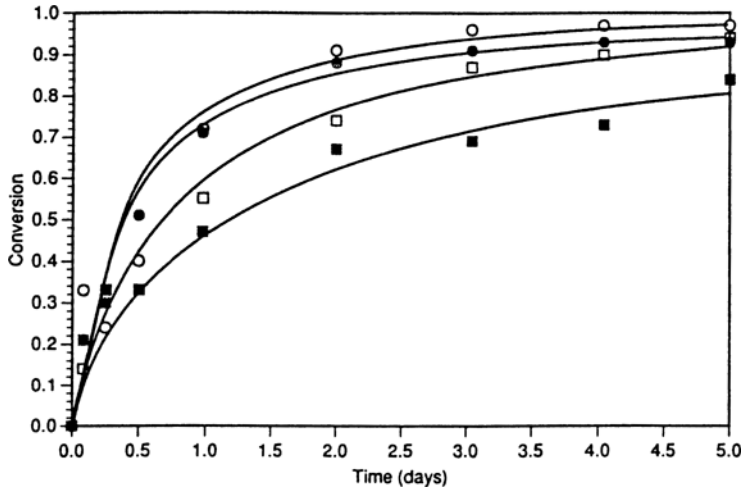

Fig. 2. Comparison of substrate 1 (mixed hardwood pretreated for 10 s at 220°C in the presence of 1% H_2SO_4) and substrate 2 (poplar wood pretreated for 20 min at 160°C in the presence of 0.45% H_2SO_4) in batch SSFs. Relative cellulose conversion using reactors with agitation by magnetic stirring—substrate concentration was 35 g cellulose/L, with cellulase loadings as shown. Cellulase enzyme was supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity. □ Substrate 1, 10 U cellulase/g cellulose; ○ substrate 1, 20 U cellulase/g cellulose; ■ substrate 2, 10 U cellulase/g cellulose; ● substrate 2, 20 U cellulase/g cellulose.

dase/U cellulase. Conversion based on cellulose solubilization in batch SSFs at different enzyme loadings is shown in Fig. 2. Results in Fig. 2 indicate that substrate 1 is marginally more reactive than substrate 2, with the difference more pronounced at lower enzyme loadings. Although differences are observed, the relatively similar behavior is notable in light of the very different raw materials and pretreatment conditions used.

In the magnetially stirred flasks, substrate 2 was visibly reduced in size as compared to both the initial material and material reacted in shake flasks. This size reduction was found to correlate with increased reaction rate, as shown in Table 2. After 3 d of reaction, SSF of substrate 1 showed essentially no sensitivity to the mode of mixing employed (conversion is within 3% after 3 d of reaction under the same substrate and enzyme conditions). For substrate 2, conversion increases significantly when SSFs are undertaken in mechanically stirred reactors (for both enzyme loadings, considered conversion was 20% higher after 3 d compared to the shaken flasks). The shake flask data for these experiments are in agreement with others who have used similar substate/enzyme combinations (*6*).

Dilute acid pretreatment produces a variety of different byproducts that may inhibit biological systems. To obtain a preliminary indication of potential inhibition, supplemented filtrate from a typical pretreatment run for substrate 1 was used as feed to *S. cerevisiae* growing in a CSTR at a

Table 2
Comparison of Cellulose Conversion of Substrate 1 (Mixed Hardwood Pretreated for 10 s at 220°C in the Presence of 1% H_2SO_4) and Substrate 2 (Popular Wood Pretreated for 20 min at 160°C in the Presence of 0.45% H_2SO_4) after 3 d SSF in Batch Reactors with Different Methods of Stirring[a]

	3-d cellulose conversion, %			
	Substrate 2		Substrate 1	
Enzyme loading (U/g cellulose)	10	20	10	20
Agitation				
Shaken flask	50	73	84	92
Stirred flask	69	91	87	96

[a]Cellulase enzyme for SSF was supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity and added at the cellulase loading indicated.

24-h residence time that was previously fed with a 20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone medium. In continuous cultivation, the steady-state outlet substrate concentration went from 0.13 g/L glucose without filtrate to 0.28 g/L glucose with filtrate. The steady-state substrate concentration in a CSTR is an indication of the impact of inhibition on growth rate (*9*). These data indicate measureable, but minor inhibition of the SSF system by pretreatment byproducts.

Continuous SSF and DMC

Washout curves for continuous cellulose conversion using the SSF system are presented with (Fig 3A) and without (Fig. 3B) added β-glucosidase. Initial SSF experiments in CSTRs, performed with a substrate 1 feed concentration of 50–62 g cellulose/L and a cellulase loading of 50 U/g cellulose, resulted in the accumulation of cellobiose as shown in Fig. 3A. Later studies of SSF were completed using substrate concentrations of 5–60 g cellulose/L, with cellulase supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity.

A summary of experimental data on SSF experiments undertaken is presented in Table 3. Conversion appears to benefit slightly from increased enzyme loading. Similar hydraulic and solids residence times were calculated indicating that the reactors were well mixed.

Table 4 gives a comparison of the conversion of SSFs carried out in batch and CSTR culture after 1, 2, and 3 d at a nominal cellulase loading of 15 U/g cellulase. The batch system conversion is 8–13% above that in the CSTR over the 1- to 3-d range of residence times.

Continuous cultivation of *C. thermocellum* with complete feed utilization (at carbohydrate concentration above 10 g/L) has not been reported to date. To facilitate a direct comparison while working within this constraint, DMC and SSF studies were carried out in CSTRs using substrate

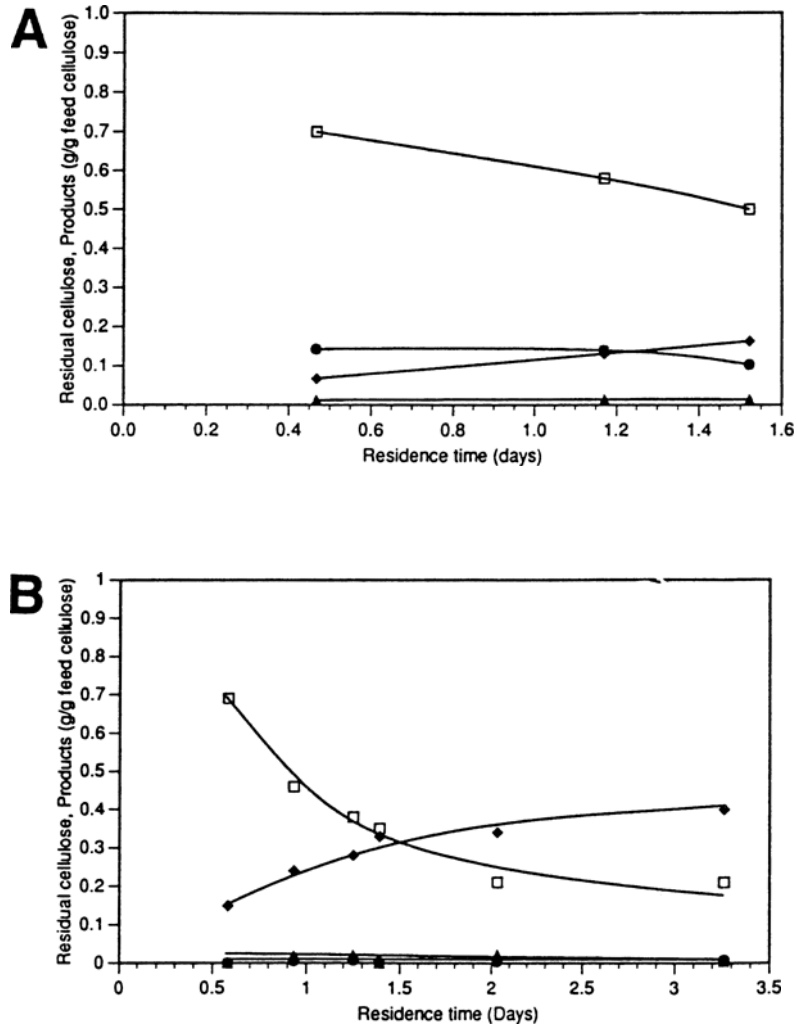

Fig. 3. A. Normalized product concentrations and residual substrate in CSTR by SSF. A variant of substrate 1 was used (mixed hardwood pretreated for 7.5 s at 220°C in the presence of 1% H_2SO_4) at between 50 and 62 g/L cellulose content (this has been normalized to g/g cellulose feed to allow for comparison). Cellulase loading was 50 U/g cellulose. □ Residual cellulose; ● cellobiose; ▲ glucose; ◆ ethanol. B. Normalized product concentrations and residual substrate in CSTR by SSF. Cellulase enzyme was supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity. Substrate 1 was used (mixed hardwood pretreated for 10 s at 220°C in the presence of 1% H_2SO_4) at between 35 and 60 g cellulose/L (this has been normalized to g/g cellulose feed to allow for comparison). Cellulase enzyme for SSF was supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity and added at 15 U/g cellulose. □ Residual cellulose; ● cellobiose; ▲ glucose; ◆ ethanol.

Table 3
Product Yields and Carbon Recovery for SSF in CSTR Being Fed with Substrate 1
(Mixed Hardwood Pretreated for 10 s at 220°C in the Presence of 1% H_2SO_4)[a]

Hydraulic residence time, d	Cellulose, g/L In	Cellulose, g/L Out	Cellulase loading, U/g	Product concentration, g/L Cellobiose	Glucose	Ethanol	Glycerol	Steady-state data points	Solids residence time,	Carbon recovery, %[b]
0.93	39.9	17.5	12.9	0.3	0.8	9.7	0.8	2	1.02	102
1.25	60.5	25.8	14.6	0.9	0.8	17.1	0.9	4	1.33	103
1.39	40.4	15.6	14.6	0.4	0.6	14.0	0.9	3	1.54	96
2.03	60.8	12.5	11.6	0.3	1.4	20.6	1.0	3	2.29	93
3.26	33.9	6.1	15.3	0.3	0.2	13.4	0.7	3	3.59	105
0.78	37.8	18.6	6.5	0.3	0.6	7.0	0.5	2	0.78	102
1.50	42.1	10.2	24.0	0.2	0.3	14.5	0.8	3	1.58	97
0.58	4.7	3.3	16.0	N.D.	N.D.	0.7	N.D.	3	0.61	103

[a]Data used in Fig. 3B.
[b]Carbon recovery is based on unreacted feed cellulose and recovered products, assuming stoichiometric CO_2 production rates.

Table 4
Comparision of Percent Conversion
in Batch and CSTR Reactor Systems
at Equal Average Residence Times[a]

Time, d	1	2	3
Batch reactor	68	84	91
CSTR	55	74	83

[a] Substrate 1 (mixed hardwood pretreated for 10 s at 220°C in the presence of 1% H_2SO_4) was used at concentrations between 35 and 60 g/L cellulose. Cellulase enzyme for SSF was supplemented with β-glucosidase at the rate of 6 U β-glucosidase activity/U cellulase activity and added at 15 U/g cellulose.

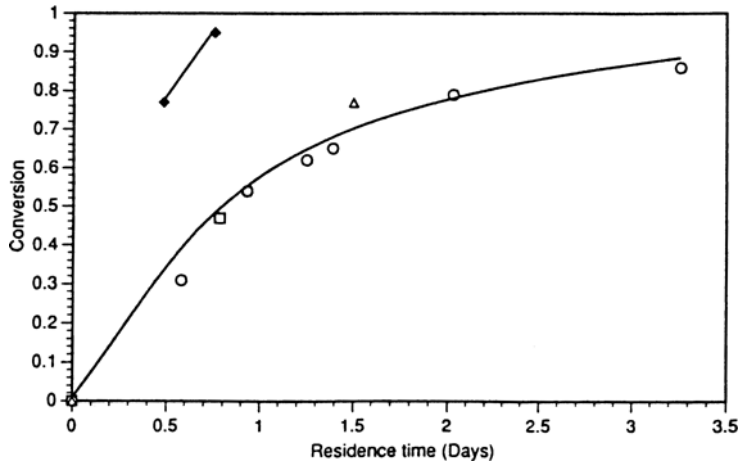

Fig. 4. Comparison of substrate utilization in CSTR by SSF and DMC. Substrate 1 (mixed hardwood pretreated for 10 s at 220°C in the presence of 1% H_2SO_4) was used at between 35 and 45 g cellulose/L for SSF and 5 g cellulose/L for DMC. Nominal cellulase loadings were as shown. □ 7 U/g cellulose; ○ 15 U/g cellulose; △ 24 U/g cellulose; ◆ DMC.

1 at a concentration of approx 5 g cellulose/L. As shown in Fig. 4, the DMC system achieved 77% conversion with a 0.5-d residence time and a 4 g/L cellulose feed, whereas the SSF system with 16 U/g cellulose achieved 31% cellulose conversion at a similar residence time (0.58 d) and substrate concentration (5 g cellulose/L). At 0.75 d, the DMC system converted 88% of the substrate.

DISCUSSION

Lab-scale experimentation of continuous fermentation of pretreated hardwood lignocellulosics, although operationally difficult, provides important insight into processing strategies potentially useful in large-scale ethanol production. Facilities adequate for the study of SSF and DMC systems fermenting very small lignocellulosic particles are presented here. Further development of the experimental apparatus is under way to allow investigation of feedstocks with larger particle sizes and to ensure long-term sterility of the slurry delivery system.

Batch results for substrate 2 were in general agreement with previous NREL data (*6*). The similarity between the reactivity of substrates 1 and 2 is more notable than the differences in light of the very different pretreatment conditions. The effect of stirring on the reaction rate of substrate 2 is consistent with either a mass-transfer limitation or a substrate-attrition effect for this substrate.

Conversion in CSTR SSFs is 8–13% less than that in batch reactors for reaction times in the range of 1–3 d (Table 4). Higher rates in batch reactors might be expected in light of the generally observed trend of decreasing reactivity with increasing conversion for hydrolysis of biomass.

Experimentation over a range of substrate concentrations (5–60 g/L) and enzyme loadings (7–24 U/g cellulose) showed SSF in a CSTR to be insensitive to these variables over this range. The insensitivity of conversion to substrate concentration indicates that the presence of substrate-concentration-related mass-transfer limitations and substrate or product inhibition is at most minor over the range examined. The insensitivity to differing enzyme loadings is somewhat surprising, particularly in light of the loading sensitivity observed in batch. Further work is to be undertaken to explore this effect fully.

A monotonic curve of g residual cellulose/g feed cellulose in relation to residence time (Fig. 4) was obtained at feed substrate concentrations varying by an order of magnitude. This result highlights the difference between conversion of insoluble substrates in a CSTR as compared to conversion of soluble substrates. Whereas we observed that the effluent cellulose concentration is roughly proportional to feed concentration at a given residence time, classical chemostat theory for soluble substrates (*10*) predicts an invariant effluent substrate concentration in relation to the feed concentration.

At comparable feed substrate concentrations and residence times, the DMC system conversion (77%) was 2.5-fold that of the SSF system (31%) at 16 U/g cellulase. Although the difference in feed concentrations in other experiments makes further comparison with SSF more indirect, it is notable that the time to 77% substrate conversion by DMC (0.5 d) is approx four times faster than in SSF at an SSF enzyme loading of 15 U/g cellulose. Further work on the DMC system, to establish rates at higher substrate concentrations is needed.

At the levels of furfural, HMF, and carbohydrate products measured in the filtrate (Table 1), significant inhibition of *S. cerevisiae* was not expected based on previous studies (*11*). Thus, our results indicate, by elimination, that lignacious byproducts from the pretreatment of substrate 1 are probable inhibitors of the fermentation.

The use of CSTRs for cellulose conversion has advantages over batch systems at high substrate concentrations when the substrate is physically difficult to handle. Since a CSTR operates at its effluent substrate concentration, the reactor has the potential to deal with more concentrated feeds in a well-mixed manner than the equivalent batch system. This difference may prove advantageous in the early stages of enzymatic hydrolysis with a concentrated feedstock. For DMC, continuous reactors have a further advantage over batch systems in that high-cell inventories allow high-cellulase production levels that would not be seen in a batch system.

Once work with SSF and DMC in CSTRs is completed, our data will allow comparison of these two conversion systems. In addition, CSTR data will provide a reference against which performance of alternative reactor designs can be evaluated. Future work is anticipated using novel solids reactor designs, including solids retaining and high-enzyme utilization reactors, such as the upflow solids retaining bioreactor (USRB) previously presented (*2*). In these nonwell-mixed reactor systems, the choice of ideal substrate is expected to be a function of both reactivity and physical properties, such as settling rates.

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