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Fermentation of Cellulosic Substrates in Batch and Continuous Culture by *Clostridium thermocellum*

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Fermentation of dilute-acid-pretreated mixed hardwood and Avicel by *Clostridium thermocellum* was compared in batch and continuous cultures. Maximum specific growth rates per hour obtained on cellulosic substrates were 0.1 in batch culture and >0.13 in continuous culture. Cell yields (grams of cells per gram of substrate) in batch culture were 0.17 for pretreated wood and 0.15 for Avicel. Ethanol and acetate were the main products observed under all conditions. Ethanol:acetate ratios (in grams) were approximately 1.8:1 in batch culture and generally slightly less than 1:1 in continuous culture. Utilization of cellulosic substrates was essentially complete in batch culture. A prolonged lag phase was initially observed in batch culture on pretreated wood; the length of the lag phase could be shortened by addition of cell-free spent medium. In continuous culture with ~5 g of glucose equivalent per liter in the feed, substrate conversion relative to theoretical ranged from 0.86 at a dilution rate (*D*) of 0.05/h to 0.48 at a *D* of 0.167/h for Avicel and from 0.75 at a *D* of 0.05/h to 0.43 at a *D* of 0.11/h for pretreated wood. At feed concentrations of <4.5 g of glucose equivalent per liter, conversion of pretreated wood was 80 to 90% at *D* = 0.083/h. Lower conversion was obtained at higher feed substrate concentrations, consistent with a limiting factor other than cellulose. Free Avicelase activities of 12 to 84 mU/ml were observed, with activity increasing in this order: batch cellobiose, batch pretreated wood < batch Avicel, continuous pretreated wood < continuous Avicel. Free cellulase activity was higher at increasing extents of substrate utilization for both pretreated wood and Avicel under all conditions tested. The results indicate that fermentation parameters, with the exception of free cellulase activity, are essentially the same for pretreated mixed hardwood and Avicel under a variety of conditions. Hydrolysis yields obtained with *C. thermocellum* cellulase acting either in vitro or in vivo were comparable to those previously reported for *Trichoderma reesei* on the same substrates.

Clostridium thermocellum, a thermophilic, anaerobic bacterium, has been considered as a catalyst for ethanol production from renewable lignocellulosic substrates (18, 19, 27, 31). This organism produces a very active cellulase complex (12) and ferments cellulose to ethanol (23). Lynd (19) recently concluded that in situ production of cellulase is the distinguishing feature of thermophilic bacteria which holds the greatest potential for positive economic impact relative to a yeast-based system for ethanol production from cellulose.

Several key issues must be addressed before the potential savings offered by using thermophiles for ethanol production from cellulose can be realized. These issues include (i) demonstration that thermophilic cellulases are active and achieve high substrate conversion on affordable cellulosic substrates and (ii) demonstration that thermophiles can grow and achieve high substrate conversion on affordable cellulosic substrates, particularly at high substrate and relatively high product concentrations.

Lynd and Grethlein (20) have demonstrated complete hydrolysis of Avicel and dilute-acid-pretreated mixed hardwood by using *C. thermocellum* cellulase. In this report, we present results from both batch and continuous cultures of *C. thermocellum* on Avicel and pretreated mixed hardwood. Hydrolysis of cellulosic substrates in the presence of cells is compared with in vitro hydrolysis with *C. thermocellum* cellulase and also with previously reported results with the cellulase of the aerobic mesophilic fungus, *Trichoderma reesei*. Preliminary results from continuous culture of *C. thermocellum* on cellulosic substrates have been presented previously (21).

MATERIALS AND METHODS

Sources of substrates and chemicals. Mixed hardwood (90% birch and 10% maple) was obtained as a 60-mesh (0.25-mm nominal particle size) sieved wood flour from Wilner Wood Products (Norway, Maine). Avicel type PH105, a purified microcrystalline cellulose with a particle size of 20 μm, was obtained from FMC Corp., Philadelphia, Pa. All chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted.

Source and strain of *C. thermocellum*. *C. thermocellum* ATCC 27405, kindly provided by Arnold Demain (Massachusetts Institute of Technology), was used for all growth experiments involving cellulose.

Stock cultures were maintained in GBG medium (described below) with an initial concentration of either cellobiose or cellulosic substrates of 0.5% (wt/vol).

Substrate pretreatment. Acid hydrolysis pretreatment of Wilner mixed hardwood was performed in the continuous plug flow reactor described by McParland et al. (22). Heating was by direct steam injection. Wood flour slurries at 10% (wt/vol) containing 14.6 g of 98% H_2SO_4 per liter, an amount to give 1% acid after steam dilution with the reactor run at 210°C, were used as feeds. The pretreated wood used as a fermentation substrate throughout this study was treated

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for 9 s at 220°C. After pretreatment, cooled slurries were neutralized with NaOH, and washed on a 520-B filter (Schleicher and Schuell, Inc., Keene N.H.) in a filtration apparatus with a 24-inch (1 in. = 2.54 cm)-diameter filtration apparatus (Bel-Art, Pequannock, N.J.) until the filtrate was only slightly colored. Acidified slurries were stored at room temperature for a period not exceeding 3 weeks. Neutralized washed solids were stored at 0 to 5°C. Solids were added to the growth medium prior to autoclaving as a never-dried filter cake containing about 75% moisture.

Medium composition and preparation. GBG medium used in batch experiments contained NaH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, MOPS (morpholinopropanesulfonic acid) buffer, resazurin (Eastman Kodak Co., Rochester, N.Y.), yeast extract (Difco Laboratories, Detroit, Mich.), $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and $FeSO_4$ at the same final concentrations reported previously (20). GBG medium was prepared in aluminum crimp-seal tubes or serum vials (Bellco Biotechnology, Vineland, N.J.) under N_2 gas prior to autoclaving. Cellulosic substrates were added to the culture vessels prior to autoclaving.

For continuous culture experiments, GBG medium was also used, but the final concentration of MOPS was 2.5 g/liter and the final concentration of yeast extract was 2 g/liter. Medium was prepared as follows: a solution containing 15 to 17 liters of distilled water, NaH_2PO_4, K_2HPO_4, MOPS, yeast extract, resazurin, and insoluble substrates was autoclaved in a 20-liter glass carboy (VWR, Boston, Mass.). Carboys were equipped with a glass nipple at the bottom. Avicel or washed, pretreated wood was added prior to autoclaving at concentrations given in the text. Medium carboys were fitted with #12 two-hole stoppers wired down to the mouth of the carboy. The stopper was fitted with two 316 stainless steel tubes (1/4-inch outside diameter), one longer tube with attached baffles extended nearly to the bottom of the carboy and a second shorter tube extended into the headspace (Fig. 1). Each tube was connected to a tubing T with amber latex tubing (1/4-inch inside diameter) having a 3/16-inch wall, one branch of the T leading to a gas filter (Acro-50 type, 0.2-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.) and the other branch leading to a length of amber latex tubing equipped with a stainless steel luer-lock fitting (Microgroup, Medway, Mass.). All branches leading from the tubing T's were clamped shut during autoclaving with the exception of the latex tubing with the luer-lock fitting connected to the shorter stainless steel tube, which was used as a vent.

A solution containing $MgCl_2$, $CaCl_2$, cystine hydrochloride, and $FeSO_4$ in 300 ml of distilled water was prepared in a 1-liter vacuum flask. The vacuum flask was equipped with a tubing T connected to the sidearm by amber latex tubing. One branch of the T led to a length of amber latex tubing with a gas filter at the end, and the other branch led to a vent which was left open during autoclaving. A 316 stainless steel tube (1/4-inch outside diameter) passed through a butyl rubber stopper wired to the top of the flask and extended to the bottom of the flask. This tube was connected to a length of amber latex tubing equipped with a luer-lock fitting. After autoclaving for 45 min and cooling, the flask headspace was evacuated and flushed with N_2 gas and the contents were aseptically transferred to a separately autoclaved carboy prepared as described above.

An additional solution containing 33% $(NH_4)_2SO_4$ was prepared in 158-ml serum vials. The ammonium sulfate solution was evacuated and flushed with N_2 gas, autoclaved, sealed for 45 min, allowed to cool, and aseptically transferred to a separately autoclaved carboy containing substrate, phosphates, yeast extract, MOPS, and resazurin.

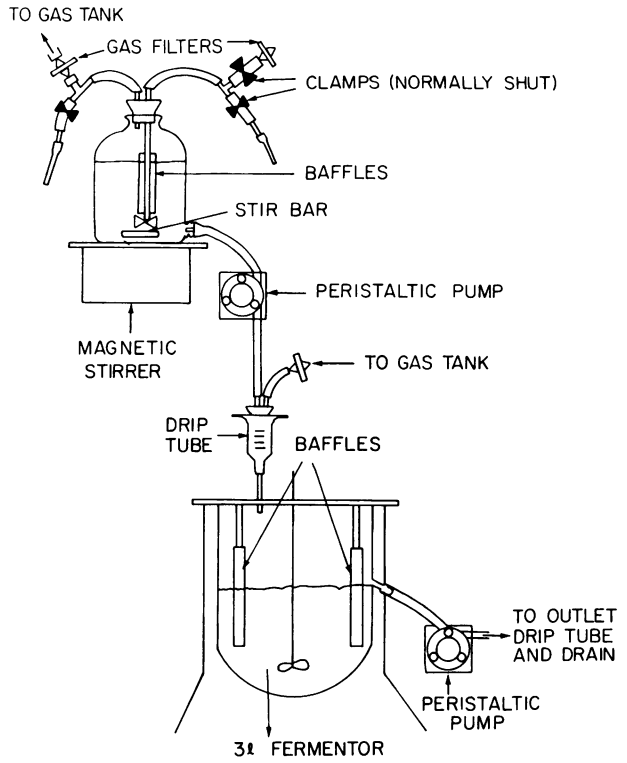

FIG. 1. Apparatus for continuous fermentation of cellulosic substrates showing the path of medium to and from the fermentor. Relative heights are as in the apparatus although not to scale.

Quantification of substrates, cells, and fermentation products. Ethanol, acetic acid, lactic acid, glucose, and cellobiose in samples taken directly from the fermentor were analyzed by high-pressure liquid chromatography on a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Richmond, Calif.) operated at 60°C with a 0.1% H_2SO_4 running buffer and a refractive index detector. Samples of 0.9 ml were acidified with 0.1 ml of 10% (wt/wt) H_2SO_4, and centrifuged in a Fisher microcentrifuge for 5 min at 7,000 × *g*. The apparatus used for high-pressure liquid chromatography analysis has been described previously (4). Samples were stored frozen prior to analysis. Analysis of glucose and cellobiose in samples from continuous cellulose-grown cultures was verified with a Bio-Rad HPX-85 column operated at 80°C with a deionized water running buffer. This column is more sensitive to sugars in the presence of growth medium than is the HPX-87H column; the detection limits for sugar analysis in growth medium with the HPX-85 column were 0.15 g/liter for cellobiose and 0.05 g/liter for glucose.

For cellulose fermentation, dry weights of fermentor solids and pretreated wood in the fermentor feed were determined by filtering 10-ml samples through polycarbonate membranes (pore size, 0.4 μm; diameter, 47 mm; Nucleopore, Pleasanton, Calif.) and drying overnight in a 100°C oven. Feed dry weights for Avicel were determined by optical density in 1.38-cm tubes at 660 nm in a Hach DR3 spectrophotometer (Hach Company, Loveland, Colo.). It was found that the optical density:dry weight relationship for Avicel changes somewhat after autoclaving and prolonged stirring; a factor of 1.53 g/liter per optical density at 660 nm

was used in studies with autoclaved growth medium containing Avicel.

The composition of solids from cellulose-fed cultures was analyzed by both quantitative saccharification (5) and also by nitrogen analysis. Quantification of the nitrogen content of solids and determination of the cell nitrogen content were performed as follows: 1-ml samples of broth were centrifuged for 5 min at 7,000 × *g*. The supernatant was discarded, and the pellet was washed three times by suspending in distilled water followed by centrifugation. This washing procedure brought the measured nitrogen content of the pellet to a constant level when cellulase-containing broth was mixed with Avicel or pretreated wood; this level was essentially the same as controls never exposed to broth. Pellets were placed in preweighed aluminum foil boats, and dried at 100°C for at least 2 days. Sample dry weights were determined, and the nitrogen content of each sample was determined with a Carlo-Erba NA 1500 automatic carbon and nitrogen analyzer.

The mean nitrogen content of *C. thermocellum* cells (plus or minus standard deviation) was 0.106 ± 0.013 g of nitrogen per gram of cells based on five independent measurements. During growth on cellobiose, the cell nitrogen content was proportional to optical density. The measured nitrogen content of wood was 0.002263 per gram of wood, and remained constant throughout enzymatic hydrolysis. By using the nitrogen contents of wood and cells, which differed by a factor of ~50, the proportion of cells and wood in samples was calculated. Nitrogen could not be detected in Avicel samples. The glucose content of solids from Avicel fermentation was measured by quantitative saccharification and also by nitrogen analysis by using the following equation: $G_e = 180/162 \times DW \times (1 - f_s/f_c)$ where G_e is the glucose equivalent concentration, DW is the dry weight of the sample, f_s is the mass fraction of nitrogen in the sample, and f_c is mass fraction of nitrogen in cells. Results from these two techniques for determining the glucose content of solids were in excellent agreement.

Quantitative saccharification of cellobiose-grown *C. thermocellum* cells resulted in a peak measured as glucose from which an apparent cell glucose content of 0.18 g of glucose per gram of cells was calculated. This glucose content may have a small (≤3%) effect on measured extents of utilization for in vivo utilization of cellulosic substrates.

The carbon recovery was calculated as an index reflecting the extent to which consumed substrate was accounted for in fermentation products. Carbon recovery was calculated as a function of the concentrations (moles per liter) of ethanol (E), acetic acid (A), lactic acid (L), feed or initial substrate (S_o), and effluent or final substrate (S_e) by using the following equation: carbon recovery = $[3 \times (E + A + L)]/[6 \times (S_o - S_e)]$, which assumes stoichiometric production of CO_2 with acetic acid and ethanol. Soluble glucose equivalent values are used for S_o and S_e.

Analysis of free cellulase activity. Free cellulase activity of fermentation broths was determined at 60°C by measuring the decrease in turbidity of an Avicel suspension as described by Johnson et al. (7). Turbidity was measured in Hungate tubes (inside diameter, 1.38 cm; Bellco Glass, Vineland, N.J.) at 660 nm with a Hach DR3 spectrophotometer.

Samples for determination of free cellulase activity were withdrawn from the fermentor or batch culture vessel and processed immediately. Solids were removed either by filtration using a polycarbonate membrane (pore size, 0.4 μm), with the clarified liquid entering an evacuated and N_2-flushed test tube, or by centrifugation of 1-ml samples for 5 min at 7,000 × *g* in microcentrifuge tubes with 2 drops of 0.5 M dithiothreitol. These two techniques gave equivalent results. Measurements of cellulase activity were usually performed by preparing a series of assay tubes with broth dilutions spanning at least a factor of 10 and with duplicate tubes at each dilution. This approach ensured that broth samples were diluted to the point where the rate of Avicel hydrolysis was proportional to the amount of broth added. Cellulase activity is only reported for samples which were at steady state with respect to other measured variables. Generally, one dilution series was prepared for each residence time. Activity was calculated from the change in optical density over a period extending from 1 h of incubation to 7 to 10 h of incubation. The fraction of cellulose hydrolyzed during this time was typically 20 to 30%. A factor of 1.72 mg/ml per optical density at 660 nm was used to convert changes in optical density to changes in Avicel concentration for in vitro cellulase experiments. Cellulase activity (in milliunits per milliliter) was calculated according to this formula:

$$\text{Activity} = (\text{dilution factor}) \times \frac{\Delta OD_{660}}{\Delta t\ (\text{min})} \times \frac{1.72\ \text{mg of Avicel}}{\text{ml/OD}_{660}} \times \frac{1\ \mu\text{mol of glucose}}{0.162\ \text{mg of Avicel}}$$

where ΔOD_{660} is the change in optical density at 660 nm and Δt (min) is the change in time (in minutes). One unit of enzyme activity is 1 μmol of glucose equivalent produced per min.

Continuous fermentation of cellulosic substrates. Continuous culture on cellulosic substrates was carried out in a 3-liter round-bottomed fermentor (Applikon Dependable Instruments, Austin, Tex.), with a 1.7-liter working volume maintained by overflow through a sidearm in the fermentor jar. Temperature was controlled at 60°C by an Omega 4201 temperature controller (Omega Engineering, Stamford, Conn.) receiving a signal from a thermocouple in an oil-filled well in contact with the fermentor broth and controlling the power to a waterbath. pH was maintained at 7.0 by an Applikon E565-1 controller and amplifier receiving a signal from a gel-filled electrode (model SA73792-300-DL; Phoenix Electrodes, Houston, Tex.) and controlling the power to a Masterflex peristaltic pump (model 7543-20; Cole-Parmer, Chicago, Ill.) connected to a flask of 4 M NaOH by size 14 C-flex tubing (Cole-Parmer). The feed carboy and fermentor were maintained at a slight positive pressure by filter-sterilized nitrogen gas. The system was closed with respect to gas flow except for gas pumped out with the fermentor effluent.

Care was taken to ensure and verify representative delivery of the feed slurry to the fermentor. A schematic diagram of the fermentor showing the path of slurries through the system is shown in Fig. 1. The feed carboy was stirred at ~250 rpm by a stir-plate (Bel-Art) driving a magnetic stir bar (1 by 6 in.) inside the carboy. Samples taken at various heights in the feed carboy showed no variation in the concentration of solids. The fermentor was stirred at 500 rpm, with stirring speed maintained by an Applikon motor and speed controller with magnetic coupling. Both the fermentor and feed carboy were equipped with baffles. The cellulose-containing slurry followed a downhill path from the feed carboy, through the drip tube to the fermentor, and out of the fermentor (Fig. 1). Adjustable-speed Masterflex peristaltic pumps were used to pump slurry to and from the

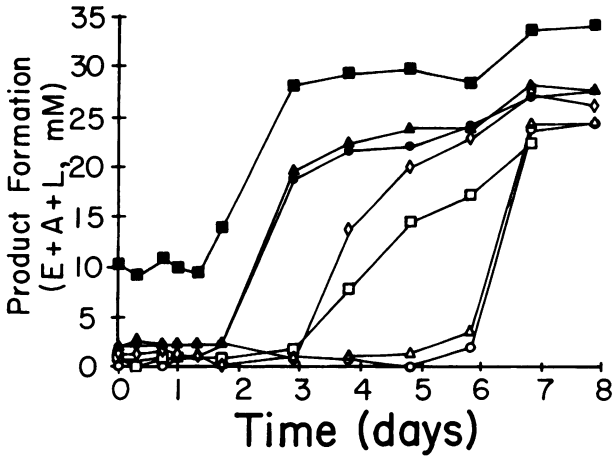

FIG. 2. Influence of addition of filter-sterilized cellulase-containing broth on the lag phase of *C. thermocellum* grown on pretreated wood. Symbols: ○, no broth added; △, 1% (vol/vol) broth added; □, 2% (vol/vol) broth added; ◇, 5% (vol/vol) broth added; ●, 10% (vol/vol) broth added; ▲, 15% (vol/vol) broth added; ■, 30% broth added. Cultures were grown in batch mode with 10 ml of GBG medium. E, A, and L refer to ethanol, acetic acid, and lactic acid, respectively.

fermentor (models 7523-10 and 7520-35, respectively; Cole Parmer). The speed of the outlet pump was maintained at approximately twice that of the inlet pump to remove gas formed during fermentation. Slurry was conveyed through amber latex tubing (1/8-in. inside diameter and 1/8-in. wall thickness) and size 16 C-flex tubing (0.123-in. inside diameter; Cole-Parmer) which was used in peristaltic pumps. There were no significant diameter changes in the tubing lines except for the drip tube. Drip tubes on the feed and effluent lines were fashioned from 20 or 30-ml syringes. Solids were prevented from accumulating in the bottom of the feed drip tube by the washing action of the falling drops and by a small flow of N_2 gas entering the fermentor through the drip tube. Medium bottles were repeatedly sampled to verify that the feed concentration of solids did not change as the bottles emptied.

Continuous cultures were started by filling the fermentor with reduced medium containing Avicel, inoculating with a culture grown overnight on Avicel, and turning on the feed pump once growth was evident. Continuous fermentation of pretreated wood was observed immediately after changing the feed from Avicel to pretreated wood at a 12-h residence time. A period of 3 residence times was generally found to be sufficient to achieve steady-state values of the parameters measured. At least two and generally three or more samples were taken for each set of steady-state data points reported, with samples separated by at least one residence time.

Results in continuous culture were obtained reproducibly with inoculum from stock cultures taken at different times.

RESULTS

Batch culture on cellulosic substrates. Avicel-grown cultures had a lag phase of about 6 days when inoculated at 2 to 5% (vol/vol) into medium containing washed pretreated wood as the growth substrate. This lag could be shortened or nearly eliminated by adding filter-sterilized, cellulase-containing broth (Fig. 2) or by increasing the size of the inoculum (data not shown). Controls with Avicel replacing

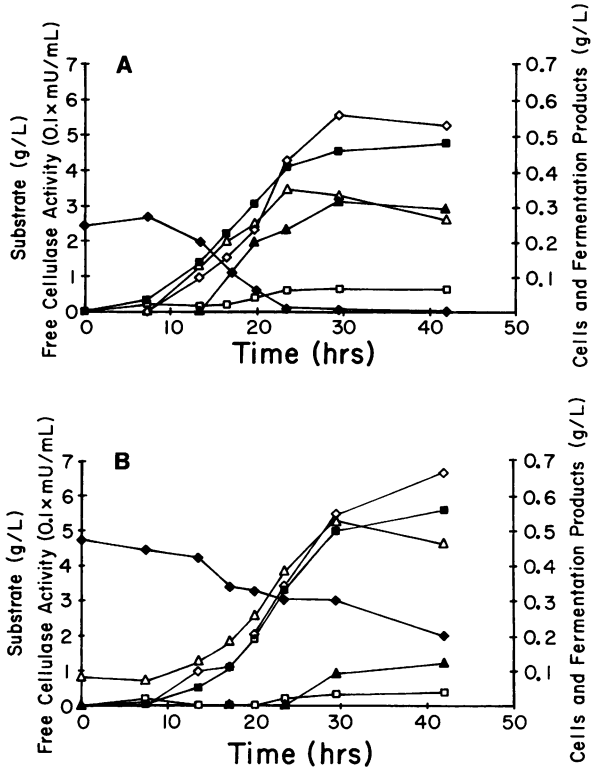

FIG. 3. Batch fermentation of cellulosic substrates by *C. thermocellum* on Avicel (A) or pretreated wood (B). Symbols: △, cells; ◆, substrate; ◇, ethanol; ■, acetic acid; □, lactic acid; ▲, Avicelase activity.

pretreated wood exhibited a lag of <1 day for all amounts of broth added. After several transfers, no lag phase was observed on pretreated wood, even with 2 to 5% inocula. It may be noted that batch cultures started with inocula taken directly from a continuous Avicel-grown culture grew with little or no lag on pretreated wood. In addition, changing the feed to a continuous culture from Avicel to wood was accompanied by apparently uninterrupted growth (data not shown).

Batch growth curves for *C. thermocellum* ATCC 27405 on Avicel and mixed hardwood (90% birch, 10% maple) pretreated at 220°C, with 1% H_2SO_4, and for 9 s (referred to as pretreated wood) are presented in Fig. 3A and B, respectively. The data are similar with respect to both the time to reach stationary phase and product yields. Lactic acid production is very low for both substrates. The most significant differences between the two curves are the higher levels of free cellulase activity during growth on Avicel compared with that during growth on pretreated wood. Both growth curves show no further increase in cell nitrogen after 30 h. A soluble glucose equivalent content of 63% (wt/wt) was measured for the pretreated wood used in the experiment depicted in Fig. 3B. The final value for pretreated wood concentration in Fig. 3B corresponds to 58% reduction in the substrate dry weight. Mean cell yield values (grams of cells per gram of substrate) plus or minus standard deviation, calculated from the data in Fig. 3A and B, are 0.15 ± 0.02 for Avicel and 0.17 ± 0.03 for pretreated wood. Specific growth rates during exponential phase are 0.1/h on both substrates.

Continuous culture on cellulosic substrates as a function of dilution rate. Continuous culture of *C. thermocellum* at 60°C

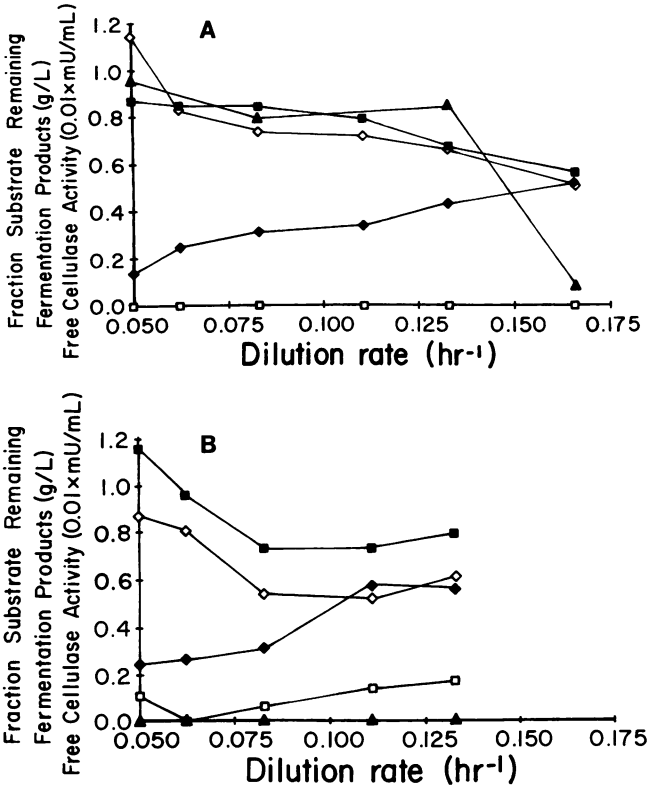

FIG. 4. Continuous fermentation of cellulosic substrates by *C. thermocellum* on Avicel (A) or pretreated wood (B). Symbols: ◆, fraction of degradable substrate remaining; ◇, ethanol; ■, acetic acid; □, lactic acid; ▲, Avicelase activity.

and pH 7.0 was carried out in a 3-liter Applikon fermentor with a 1.7-liter working volume. The medium reservoir and fermentor were equipped with baffles and stirred to maintain turbulent mixing, and the cellulose-containing slurry followed a downhill path from the medium reservoir to the medium outlet pump. Steady-state continuous fermentation was routinely achieved with both pretreated wood and with Avicel. Variation around the mean steady-state values was random when a period of 3 residence times was allowed between changing the dilution rate and taking the first point used in calculation of steady-state values.

Figure 4A and B present steady-state parameters for fermentation of Avicel and pretreated wood, respectively. Results are plotted with respect to the dilution rate, D, which is the ratio of the volumetric feed flow rate to the fermentor working volume. Concentrations of fermentation products are expressed in grams per liter, and free cellulase activity is expressed in milliunits per milliliter. The cellulose concentration in the feed medium was approximately 5 g of potential glucose per liter. However, the actual concentrations varied somewhat, in particular for pretreated wood, which was added as a filter cake with ~75% moisture. Neither glucose nor cellobiose were detected in continuous cultures during the fermentation of Avicel or pretreated wood.

Continuous fermentation of Avicel (Fig. 4A) is accompanied by production of ethanol and acetic acid and no measurable lactate. The ethanol:acetic acid ratio (in grams) varies between 0.88 and 1.31, with all values ≤0.98 except for a D of 0.05/h. The fraction of degradable substrate remaining varied from 0.14 (D = 0.05/h) to 0.52 (D = 0.167/h). Free cellulase activity was detected at all dilution rates, with the value nearly constant at about 90 mU/ml for all dilution rates except 0.167/h, which had much lower free cellulase activity.

Acetic acid and ethanol are the main products of continuous fermentation of pretreated wood (Fig. 4B) with slightly more acetic acid formed at all dilution rates. The ethanol: acetic acid ratio varies between 0.71 and 0.85. Lactic acid is also a significant product of pretreated wood fermentation but is produced at lower concentrations. The fraction of degradable substrate remaining varied from 0.25 (D = 0.05/h) to 0.57 (D = 0.11/h) (the fraction of substrate remaining at 0.133/h was slightly lower at 0.56). Free cellulase activity was undetectable at all dilution rates except 0.05/h, where it was still very low at 1.4 mU/ml.

Table 1 presents analyses of solids entering and leaving the fermentor for Avicel and pretreated wood. The standard deviation around the mean values are generally ≤±10% for fermentor solids measurements and also for product concentrations (product data not shown). The variations in feed concentrations of solids have some effect on the product concentrations presented in Fig. 4A and B. Carbon recover-

TABLE 1. Influent and effluent solids, effluent potential glucose, and carbon recovery for continuous fermentation of Avicel and pretreated wood by *C. thermocellum*

Substrate	D/h	n[a]	Influent solids (g/liter)[b]	Mean values (g/liter) ± SD for:		Carbon recovery (%)[c]
				Effluent solids	Effluent potential glucose	
Avicel	0.05	4	5.11 ± 0.08	1.16 ± 0.11	0.774 ± 0.1	72.7
	0.0625	3	4.61	1.52 ± 0.12	1.27 ± 0.03	75.4
	0.0833	3	4.79 ± 0.04	1.82 ± 0.12	1.66 ± 0.14	74.4
	0.111	3	4.72 ± 0.13	1.87 ± 0.03	1.78 ± 0.07	75.1
	0.133	4	4.53	2.41 ± 0.10	2.17 ± 0.12	80.3
	0.166	3	5.00	2.83 ± 0.22	2.88 ± 0.27	68.4
Pretreated wood	0.05	4	9.20	5.42 ± 0.10	1.49 ± 0.05	77.9
	0.0625	3	8.16	4.96 ± 0.44	1.42 ± 0.21	76.8
	0.833	3	7.65	4.54 ± 0.32	1.55 ± 0.09	74.0
	0.111	3	9.03	7.42 ± 0.26	3.40 ± 0.35	90.6
	0.133	4	9.39	7.76 ± 0.29	3.44 ± 0.16	93.1

[a] n is the number of steady-state data points.
[b] Values with no standard deviation were taken with a single feed carboy.
[c] A value of 0.657 is used for the mass fraction of potential glucose in washed pretreated wood.

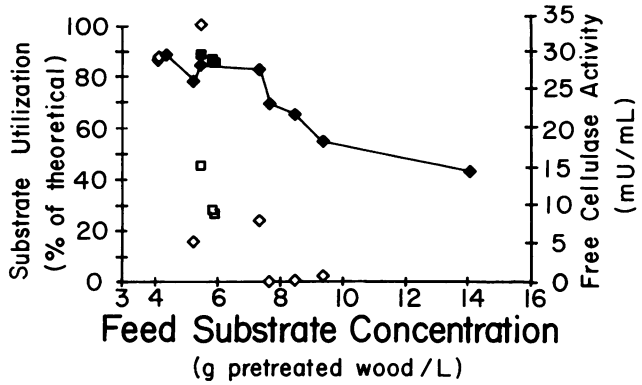

FIG. 5. The influence of feed substrate concentration on free cellulase activity and utilization of pretreated wood by *C. thermocellum* grown in continuous culture. Symbols: ◆, substrate utilization, D = 0.0833/h; ■, substrate utilization, D = 0.0625/h; ◇, free cellulase activity, D = 0.0833/h; □, free cellulase activity, D = 0.0625/h.

ies exclusive of cells and cellulase are generally 70 to 80% for both substrates.

Growth of *C. thermocellum* in GBG medium appeared to be totally dependent on the presence of the primary growth substrate. When medium with the primary growth substrate omitted was fed to a continuous culture formerly growing on pretreated wood at a 12-h residence time, the concentrations of fermentation products fell to unmeasurably low values.

Continuous culture on pretreated hardwood as a function of substrate concentration. Figure 5 presents substrate utilization and free cellulase data obtained for continuous culture at pretreated wood concentrations of 4.14 g/liter to 14.0 g/liter, and dilution rates per hour of either 0.0833 or 0.625. All points are averages of data taken over a period of at least 2 residence times after steady state was attained. When the pretreated wood concentration was ~8 g/liter, substrate conversion at a D of 0.0833/h was 69% and free cellulase activity was zero, as reported above. However, at feed substrate concentrations (S_o < 8 g/liter), substrate conversion was substantially higher, and when S_o was >8 g/liter, the conversion decreased with increasing substrate concentration. The substrate conversion at a D of 0.0833/h and when S_o was <7.5 g/liter was between 82.5 and 88% for all points except one which was 78%. Substantial free cellulase activity was measured for all points with S_o < 7.5 g/liter and for no points with S_o > 7.5 g/liter. Decreasing the dilution rate per hour from 0.0833 to 0.0625 had little effect on substrate conversion. Substrate utilization of 85.4 to 88.6% was obtained for three different steady-state levels at a D of 0.0625/h and when S_o was ~5.7 g/liter. The reported values for substrate conversion may be lower than the true conversion by ~3% because of the presence of the apparent glucose contained in cells which is measured as unreacted cellulose.

DISCUSSION

The data reported here are generally similar for Avicel and pretreated wood, including not only the fermentation rates and end product ratios but also cell yields and specific growth rates. Thus, it appears that the presence of insoluble lignin makes very little difference to *C. thermocellum* during growth on cellulosic substrates. This result is consistent with our previously reported in vitro cellulose hydrolysis experiments (20). It is not possible to make statements about soluble lignin, however, because pretreated wood was washed prior to fermentation.

The most significant differences between growth on pretreated wood and Avicel were the prolonged lag phase initially observed for pretreated wood but not for Avicel and the consistently higher levels of free cellulase during growth on Avicel. The higher cellulase activities observed in batch culture on Avicel relative to batch culture on pretreated wood, and also the earlier appearance of cellulase activity during growth on Avicel, are consistent with the higher amounts of enzyme necessary to saturate pretreated wood observed in vitro (20).

The values of ~0.1/h obtained for the specific growth rate of *C. thermocellum* ATCC 27405 on both Avicel and pretreated wood in batch culture may be compared with the value of 0.15/h for *C. thermocellum* LQRI growing on chromatography paper (30). The cell yields of ~0.15 g of cells per gram of substrate consumed are within the normal range for carbohydrate-fermenting anaerobes deriving energy via substrate-level phosphorylation (29). This does not support the widely held idea that thermophilic bacteria have higher cell maintenance requirements and lower cell yields (2, 27, 32) and is consistent with the results of Lacis and Lawford (11).

The data obtained in continuous culture establishes that *C. thermocellum* grows readily in continuous culture on not only Avicel, but also pretreated wood. In addition, this work indicates that continuous culture of thermophilic bacteria on cellulosic substrates can readily be studied on a small scale. Maximum dilution rates, and hence maximum growth rates since the reactor was well mixed, were >0.167/h for Avicel and >0.133/h for pretreated wood. In the only other study of continuous culture of *C. thermocellum* in pure culture on insoluble substrates known to the authors, Zertuche and Zall (33) report growth of *C. thermocellum* on Solkafloc in a fermentor with comparable working volume to the one used in our studies, maintained in a semicontinuous mode. Their results differ from those presented herein, in that steady-state fermentation was not obtained, and that the only feed flow rate used corresponded to a residence time of 55 h. Kleijntjens et al. (9) presented continuous culture data for a mixed culture which included *C. thermocellum*. Their results were all at residence times of >20 h.

Pavlostathis et al. (24, 25) have recently studied cellulose fermentation by the mesophilic bacterium *Ruminococcus albus* in a continuous well-mixed reactor. The studies of Pavlostathis et al. and the present study used the same Avicel substrate (PH-105; FMC Corp.) and similar substrate concentrations (~11 g/liter for Pavlostathis et al. and ~5 g/liter in the present study). Undefined sources of organic cofactors were present in both studies in the form of clarified rumen fluid (5% [vol/vol]) in the Pavlostathis studies and yeast extract (0.2% [wt/vol]) in the present study. Thus, the results from the present study and those of Pavlostathis et al. allow a reasonably direct comparison of the rate of cellulose fermentation by representative species of thermophilic and mesophilic cellulolytic bacteria. Pavlostathis et al. obtained cellulose utilization from 30 to 70% in hydraulic residence times of 0.5 to 2 days. In the present study, cellulose utilization from 48 to 86% was obtained in hydraulic residence times of 0.25 to 0.83 days. The residence times required to achieve the same extent of cellulose utilization are on average 3.9 times greater, and at least 2.7 times greater, for *R. albus* than for the *C. thermocellum*. Higher substrate utilization rates have been claimed to be a general

TABLE 2. Hydrolysis yields for *C. thermocellum* and *T. reesei* in vitro and in vivo

Source of cellulase	Substrate	Pretreatment conditions			Solids concn (g/liter)[a]	Enzyme loading (U/g)[b]	Reaction time[c] (h)	% Yield[d]	Reference
		Temp (°C)	$[H_2SO_4]$ (%)	Time (s)					
In vitro									
C. thermocellum	Hardwood	220	1.0	9.0	2.5	36.0	13	95.0	This work
T. reesei	Hardwood	220	1.22	7.2	14.0	24.3	24	85.6	4
C. thermocellum	White pine	220	1.0	10.0	0.52	7.0	47	43.0	This work
T. reesei	White pine	220	1.08	7.8	15.0	11.3	48	26.8	4
T. reesei	Poplar	200	0.5	7.9	18.14	27.6	24	100	11
In vivo									
C. thermocellum	Hardwood	220	1.0	9.0	4.14	7.0	12	86	This work
C. thermocellum	Hardwood	220	1.0	9.0	7.33	1.04	12	82.5	This work
C. thermocellum	Hardwood	220	1.0	9.0	5.48	2.74	16	88.6	This work

[a] Calculated from data on an unpretreated solids basis assuming all hemicellulose and 10% of lignin are solubilized.
[b] Calculated based on free cellulase activity for continuous cultures. Activity for *C. thermocellum* was measured on Avicel, and activity of *T. reesei* cellulase was measured on filter paper. However, the activity of both *C. thermocellum* and *T. reesei* cellulases are about equal on Avicel and filter paper (8).
[c] Time of experiment for batch experiments; residence time for continuous experiments.
[d] All yields are based on the glucose content of the solids before reaction.

feature of thermophilic organisms relative to mesophilic organisms (27, 31, 32). The comparison of *C. thermocellum* and *R. albus* discussed above is consistent with this claim for the case of cellulose fermentation. Batch culture studies (16, 28) have, however, reported similar rates for mesophilic and thermophilic fermentation.

The ratios of ethanol:acetic acid reported herein are not substantially affected by the dilution rate in continuous culture or by the growth substrate. Others have found fermentation product ratios of thermophilic bacteria to be influenced by the growth rate (1, 32). In addition, lower ethanol:organic acid ratios and growth inhibition of lignin-containing substrates have been reported (1, 3, 8).

Ethanol:acetic acid ratios of 0.61 to 1.87 have been reported (8, 26) in batch cultures of *C. thermocellum* grown on pretreated lignocellulosic substrates. The ethanol:acetic acid ratio depends on culture mode in our studies, with values for batch experiments approximately twice those obtained in continuous experiments. It may be noted that batch experiments were carried out in sealed serum vials with no stirring, whereas a small flow of gas and stirring (500 rpm) were present in continuous experiments. Lamed et al. (14) have noted a similar difference in ethanol:acetic acid ratios produced by batch cultures of *C. thermocellum*, with higher ratios produced in serum vials than in a 5-liter fermentor which was sparged and stirred. An inverse relationship between ethanol:acetic acid ratios and the degree of stirring or sparging (or both) has also been noted in the ethanol-producing thermophiles *Clostridium thermosaccharolyticum* (F. Mistry and C. L. Cooney, Abstr. 190th Am. Chem. Soc. Natl. Meet., 1985), and *Clostridium thermohydrosulfuricum* (J. A. Sundquist, H. W. Blanch, and C. R. Wilke, Abstr. 192nd Am. Chem. Soc. Natl. Meet., 1986). This effect has been attributed to the effect of stirring on the degree of H_2 supersaturation (14; F. Mistry and C. L. Cooney, Abstr. 190th Am. Chem. Soc. Natl. Meet., 1985, and J. A. Sundquist, H. W. Blanch, and C. R. Wilke, Abstr. 192nd Am. Chem. Soc. Natl. Meet., 1986).

The experiments in continuous culture at various pretreated wood concentrations establish that near 90% yields can be obtained in an in vivo system in 12 h. Essentially the same yields were obtained after 16 h, indicating that the last 10% or so of the substrate is much more recalcitrant than is the first 90%. In our previous in vitro work (21) and also subsequent experiments, we found roughly 5% of the substrate to be recalcitrant and the first 95% to be hydrolyzed relatively quickly. The difference between these results may be in part due to the apparent glucose content of cells, which would be measured in the in vivo case but not in the in vitro case.

Several investigators have reported that the equilibrium concentration of free cellulase is very low when the enzyme is not in excess relative to the number of binding sites on the substrate (13, 15, 17). Thus the presence of high free cellulase activity during continuous culture on Avicel at all dilution rates except the highest employed, $D = 0.167$/h, and on pretreated wood at $S_o < 7.5$ g/liter suggests that in these cases cellulase is present in excess relative to the number of available substrate binding sites. The absence of free cellulase during growth on pretreated wood at $S_o > 7.5$ g/liter is consistent with enzyme production at levels insufficient to saturate available binding sites. The trend of effluent substrate concentration increasing disproportionately with respect to increasing feed substrate concentration is contrary to expectation based on either Monod or first-order substrate consumption kinetics for the case where the system is substrate limited. Thus, it seems likely that some factor other than substrate is limiting the utilization of pretreated wood at $S_o > 7.5$ g/liter. The nature of this limitation is an important topic for future research.

Tables 2 and 3 present hydrolysis yields and cellulase activity data. Hydrolysis yields reported here and in our previous in vitro study (20) are compared with those reported for *T. reesei* (4, 10). Yields at comparable enzyme loadings and reaction times are somewhat higher for the *C. thermocellum* cellulase system than for *T. reesei* cellulase acting on both pretreated mixed hardwood and pretreated white pine. The substrate concentrations are, however,

TABLE 3. Free cellulase activity in *C. thermocellum* cultures[a]

Substrate	Substrate concn (g/liter)	Growth conditions	Free cellulase activity (mU/ml)	Reaction time (h)
Cellobiose	5.0	Batch	12	36
Avicel	2.4	Batch	32	42
Hardwood	4.75	Batch	12	42
Avicel	4.53	Continuous	84	7.5
Hardwood	4.14	Continuous	29	12

[a] Values were found in this study.

lower for the results with *C. thermocellum*. The results for hydrolysis of white pine suggest that *C. thermocellum* cellulase is substantially less effective against dilute-acid-pretreated softwoods than against similarly pretreated hardwoods. Similar conclusions have been drawn for the cellulase of *T. reesei* (4). Poplar hydrolysis by *T. reesei* cellulase is included in Table 2 to illustrate how much more easily this substrate is hydrolyzed by this system than is mixed hardwood. Hydrolysis of poplar with *C. thermocellum* cellulase has not been studied.

Yields from in vivo cellulase hydrolysis by *C. thermocellum* are slightly lower than in vitro yields using *C. thermocellum* cellulase, as already noted, and are essentially equal to the yields obtained with *T. reesei* cellulase in vitro. The lower apparent enzyme loading in the in vivo studies may be indicative of most of the enzyme being bound to cells or substrate, or a greater effectiveness of the enzyme in the presence of cells (or indicative of both).

The free cellulase activity of *C. thermocellum* culture broths increases in the order batch cellobiose, batch hardwood < batch Avicel, continuous hardwood < continuous Avicel. The activities obtained differ by a factor of 7 for enzyme produced with substrate concentrations which vary by less than a factor of 2, indicating the importance of the growth substrate and cultivation mode in determining the concentration of free cellulase. The low activity in cellobiose-grown broths is consistent with the report from Johnson et al. (6) that cellobiose is a potent inhibitor of cellulase synthesis. The relative cellulase syntheses during growth on wood and Avicel cannot be compared because the substrate- and cell-bound enzyme was not quantified.

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