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ORIGINAL PAPER

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Production of caproic acid by cocultures of ruminal cellulolytic bacteria and *Clostridium kluyveri* grown on cellulose and ethanol

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Abstract Ruminal cellulolytic bacteria (*Fibrobacter succinogenes* S85 or *Ruminococcus flavefaciens* FD-1) were combined with the non-ruminal bacterium *Clostridium kluyveri* and grown together on cellulose and ethanol. Succinate and acetate produced by the cellulolytic organisms were converted to butyrate and caproate only when the culture medium was supplemented with ethanol. Ethanol (244 mM) and butyrate (30 mM at pH 6.8) did not inhibit cellulose digestion or product formation by S85 or FD-1; however caproate (30 mM at pH 6.8) was moderately inhibitory to FD-1. Succinate consumption and caproate production were sensitive to culture pH, with more caproic acid being produced when the culture was controlled at a pH near neutrality. In a representative experiment under conditions of controlled pH (at 6.8) 6.0 g cellulose l^{-1} and 4.4 g ethanol l^{-1} were converted to 2.6 g butyrate l^{-1} and 4.6 g caproate l^{-1}. The results suggest that bacteria that efficiently produce low levels of ethanol and acetate or succinate from cellulose should be useful in cocultures for the production of caproic acid, a potentially useful industrial chemical and bio-fuel precursor.

Mention of specific products is intended only to provide information and does not constitute an endorsement by the U.S. Department of Agriculture over other products not mentioned.

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Introduction

Caproic acid, a six-carbon straight-chain fatty acid, is found in trace amounts in natural fats and is thus produced only as a minor by-product of triglyceride hydrolysis (Sauer 1992). As a result, its commercial use is primarily as a precursor for the synthesis of fine chemicals (e.g., flavorants and other hexyl esters (Budavari 1989)). The market for caproic acid would be expanded greatly if it could be produced from inexpensive starting materials and if its known ability to be converted to alkane fuels by Kölbe electrolysis was exploited (Levy et al. 1980, 1981, 1983). One proposed source for caproic acid involves 'biorefining', an intentional interruption of the anaerobic digestion of biomass such that aliphatic acid intermediates, rather than methane and carbon dioxide, are the end-products of digestion (Levy et al. 1981).

An alternative route to caproic acid may involve direct fermentations by defined microbial cultures. The anaerobic bacterium *C. kluyveri* is unusual in that it can produce caproic acid from ethanol plus either acetate or succinate (Kenealy and Waselefsky 1985). These latter compounds are common products of the carbohydrate fermentations of a number of anaerobic microbes. Because caproic acid can be readily extracted from aqueous solutions by water-immiscible organic solvents (Levy et al. 1981, 1983), *C. kluyveri* may have the potential to upgrade certain biomass fermentations that produce end-products at concentrations too low for economic recovery.

Unfortunately, no information is available on the behavior of *C. kluyveri* in coculture with other fermentative organisms. We therefore undertook an examination of the coculture of *C. kluyveri* with two species of cellulolytic bacteria, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. These ruminal bacteria are among the most rapid cellulose fermenters known, but their major fermentation end-products (acetate and succinate) rarely accumulate above 2 g/l in monocultures (Shi and Weimer 1992; Weimer 1993). The purpose of these initial studies was not to develop an economically viable fermentation route to caproic acid, but to test the concept that cocultures of these two bacterial groups, supplied with cellulose and a dilute ethanol cosubstrate, can produce significant quantities of caproic acid.

Materials and methods

Bacterial strains and growth media

Clostridium kluyveri ATCC 8527 was routinely cultivated in CM-5 medium as described by Kenealy and Waselefsky (1985). *Ruminococcus flavefaciens* FD-1 and *Fibrobacter succinogenes* S85 (formerly *Bacteroides succinogenes*, Montgomery et al. 1988) were grown under a CO_2 gas phase in a modified Dehority medium (Weimer et al 1991) with Sigmacell 50 microcrystalline cellulose (Sigma, St. Louis, Mo, USA) as the cellulose substrate and substituting, in place of clarified rumen fluid, the vitamin mixture of Schaefer et al. (1980) supplemented with 0.13 mg tetrahydrofolic acid/l vitamin stock solution. Cocultures of *C. kluyveri* with the ruminal bacteria were grown in the same modified Dehority medium.

Batch cultures, where only end-point data were gathered, were incubated in sealed 60-ml serum bottles at 39°C for the times indicated. Batch cultures that include data collected throughout the growth of the culture were carried out in 2-l reactors with or without pH control as indicated.

Analyses

Culture samples were centrifuged for 5 min at 13 000 g in a microcentrifuge (Beckman Inst., Fullerton, Calif., USA). The supernatants were treated with $Ca(OH)_2$ and $CuSO_4$ (Siegfried et al. 1984), and were analyzed for organic acids and alcohols by HPLC and refractive-index detection (Weimer et al. 1991). Standards run in this HPLC system included (compound/elution time in min): succinate/10.8, formate/12.2, acetate/13.3, ethanol/16.3, butyrate/19.8, crotonate (internal standard) 24.3, and caproate/43.8.

Cell pellets from the above centrifugation were washed twice with 0.9% (W/V) NaCl and analyzed for nitrogen content with a Carlo Erba NA1500 nitrogen analyzer (Fisons Instruments, Saddle Brook, N.J., USA) as described previously (Weimer et al. 1991). The amount of cell mass was estimated from the nitrogen data by the method of Pavlostathis et al. (1988b).

The hydrogen content of gas headspace samples, removed by syringes equipped with Mininert syringe valves (Supleco, Bellefonte, Pa., USA), was determined by gas chromatography using a Shimadzu GC-8A unit fitted with a 1.9-m-long × 3.2-mm-diameter stainless-steel column packed with 60/80 Carbosieve SII (Supleco). Chromatographic conditions were: injector temperature 150°C, detector (thermal conductivity) temperature 150°C, oven temperature 100°C, carrier gas, nitrogen, and detector current 80 mA.

Residual cellulose was determined gravimetrically by a modified neutral detergent treatment of culture samples (Weimer et al. 1990).

Results

Fermentation of cellulose to succinate and acetate

The fermentation of cellulose by *F. succinogenes* was compared under conditions with pH control (pH 6.7–7.0 by the addition of NaOH) and without pH control (Fig 1). The rate and extent of cellulose disappearance, and the extent of succinate and acetate production were marginally improved by controlling pH. Cellulose did not appear to be limiting the fermentations because in both cases there was significant cellulose remaining at the end of these fermentations. Moreover, because the total (undissociated plus ionized) concentrations of succinate and acetate were comparable under conditions presented in Fig. 1, the large difference in pH between the cultures suggest that accumulation of undissociated acids was not the cause of cessation of growth and cellulose consumption. Doubling the concentrations of either the vitamins or trace elements did not increase growth or the extent of cellulose hydrolysis by *F. succinogenes*; addition of 40 mM sodium succinate had a small (less than 10%) inhibitory effect on the consumption of cellulose but little effect on other culture parameters (data not shown).

Coculture of *C. kluyveri* and rumen cellulolytic organisms

In coculture experiments we tested the effect of ethanol addition (up to 244 mM) and the presence or absence of *C. kluyveri* on the disappearance of cellulose by *F. succinogenes* or *R. flavefaciens*. Table 1 shows that neither monoculture of the cellulolytic bacteria produced butyrate or caproate, and that ethanol slightly inhibited cellulose consumption by *R. flavefaciens* (but not *F. succinogenes*). When the incubations included *C. kluyveri* there was no production of butyrate or caproate unless ethanol, cellulose and the cellulolytic organisms were all present. The presence of ethanol and *C. kluyveri* further limited cellulose consumption by the rumen organisms. The lower consumption of cellulose along with lower levels of succinate and the production of formate by *R. flavefaciens* made further work with this strain less desirable.

Time-course analysis of cocultures of *C. kluyveri* and *F. succinogenes*

Combinations of *C. kluyveri* and either *F. succinogenes* or *R. flavefaciens* in cellulose-containing medium lacking

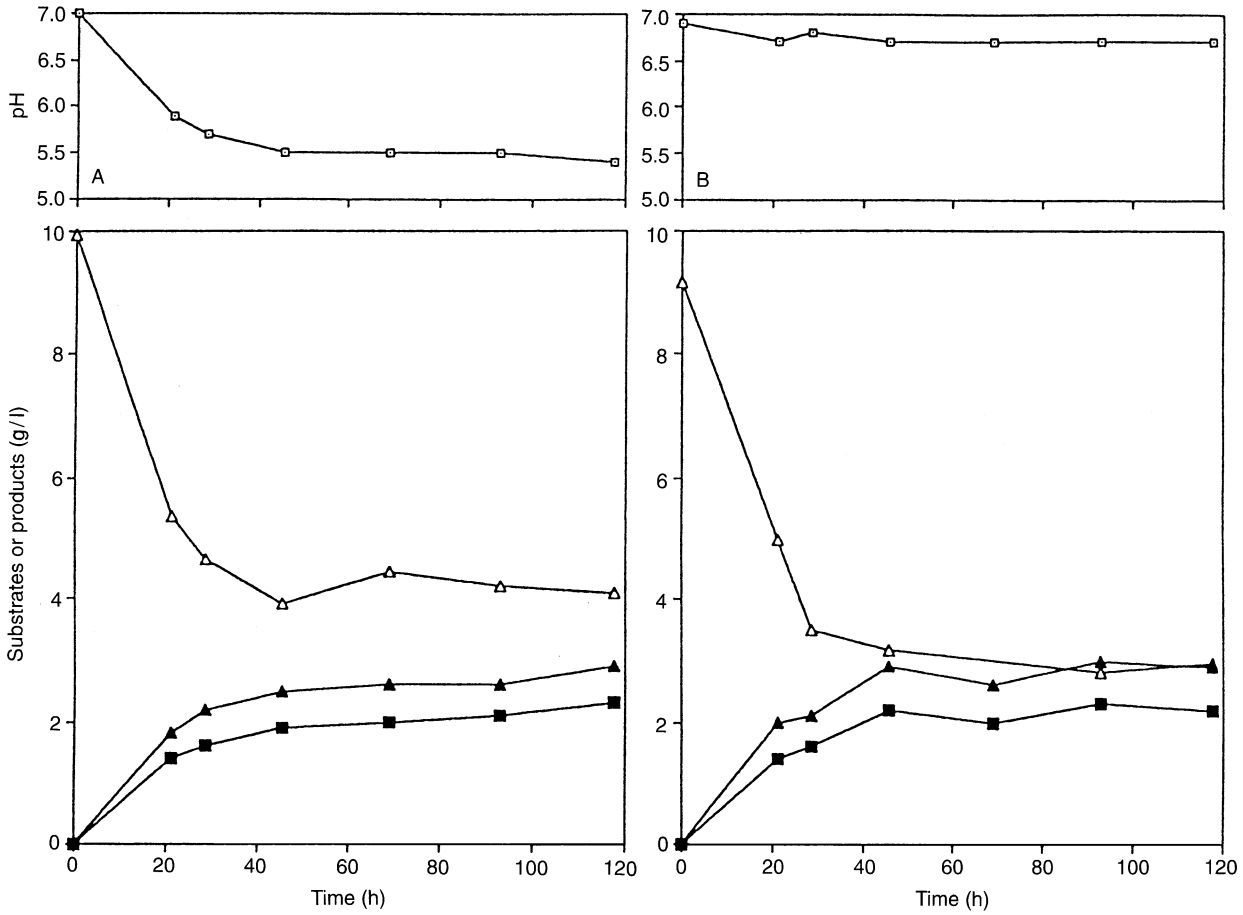

Fig. 1A, B The effect of pH on the fermentation of cellulose into succinate and acetate by *F. succinogenes*. The data represent the average of duplicate determinations and are representative of several experiments. **A** Without pH control; **B** pH controlled with sodium hydroxide. △ cellulose; ▲ succinate; ■ acetate

ethanol gave results similar to those of the cellulolytic monocultures (Table 1). When ethanol was added to the coculture it was consumed with the concomitant formation of butyrate and caproate. The effect of pH on the coculture containing *C. kluyveri* and *F. succinogenes* was examined by comparing conditions where the pH was controlled at 6.8 by NaOH addition (Fig. 2B) to those where the medium components provided the only buffering capacity (Fig 2A). The coculture proceeded from an initial stage where cellulose was consumed and both succinate and acetate accumulated, to a later stage where ethanol, succinate and

Table 1 Effect of ethanol and *C. kluyveri* on the consumption of cellulose and ethanol and on the accumulation of fermentation acids by ruminal cellulolytic bacteria. Cultures were incubated for 7 days at 39°C. Data represent average of duplicate tubes and are representative of several experiments. Where indicated the medium was inoculated with *C. kluyveri* (C.k.), *F. succinogenes* (S85) or *R. flavefaciens* (FD-1). Where indicated ethanol was added at 244 mM initial concentration. Cellulose values show the amount of anhydroglucose equivalents removed (mM)

Condition	Consumption of cellulose and ethanol or accumulation of acids (mM)							Final pH
	Cellulose	Ethanol	Succinate	Formate	Acetate	Butyrate	Caproate	
S85, no ethanol	26.9*1	—	21.3*1	0*1	9.3*1	0*1	0*1	6.0
S85 + ethanol	24.1*1*2	0*1	20.2*1	0*1	9.8*1	0*1	0*1	6.1
S85 + C.k., no ethanol	26.1*1	–	20.0*1	0*1	12.5*2	0*1	0*1	6.0
S85 + C.k. + ethanol	21.5*2	113.3*2	0*2	0*1	8.1*3	26.9*2	22.3*2	5.8
FD-1, no ethanol	22.9*2	–	13.6*3	20.7*2	18.9*4	0*1	0*1	5.9
FD-1 + ethanol	20.4*3	0*1	12.3*3	18.4*2	17.6*4	0*1	0*1	6.0
FD-1 + C.k., no ethanol	23.3*2	–	11.7*3	20.3*2	19.4*4	0*1	0*1	5.8
FD-1 + C.k. + ethanol	13.6*4	120.2*2	0*2	16.9*3	0*5	17.4*3	26.3*2	5.9
C.k. alone + ethanol	0*5	0*1	0*2	0*1	0*5	0*1	0*1	6.7

*1–*5 Values within same column having different superscripts differ significantly ($P < 0.05$)

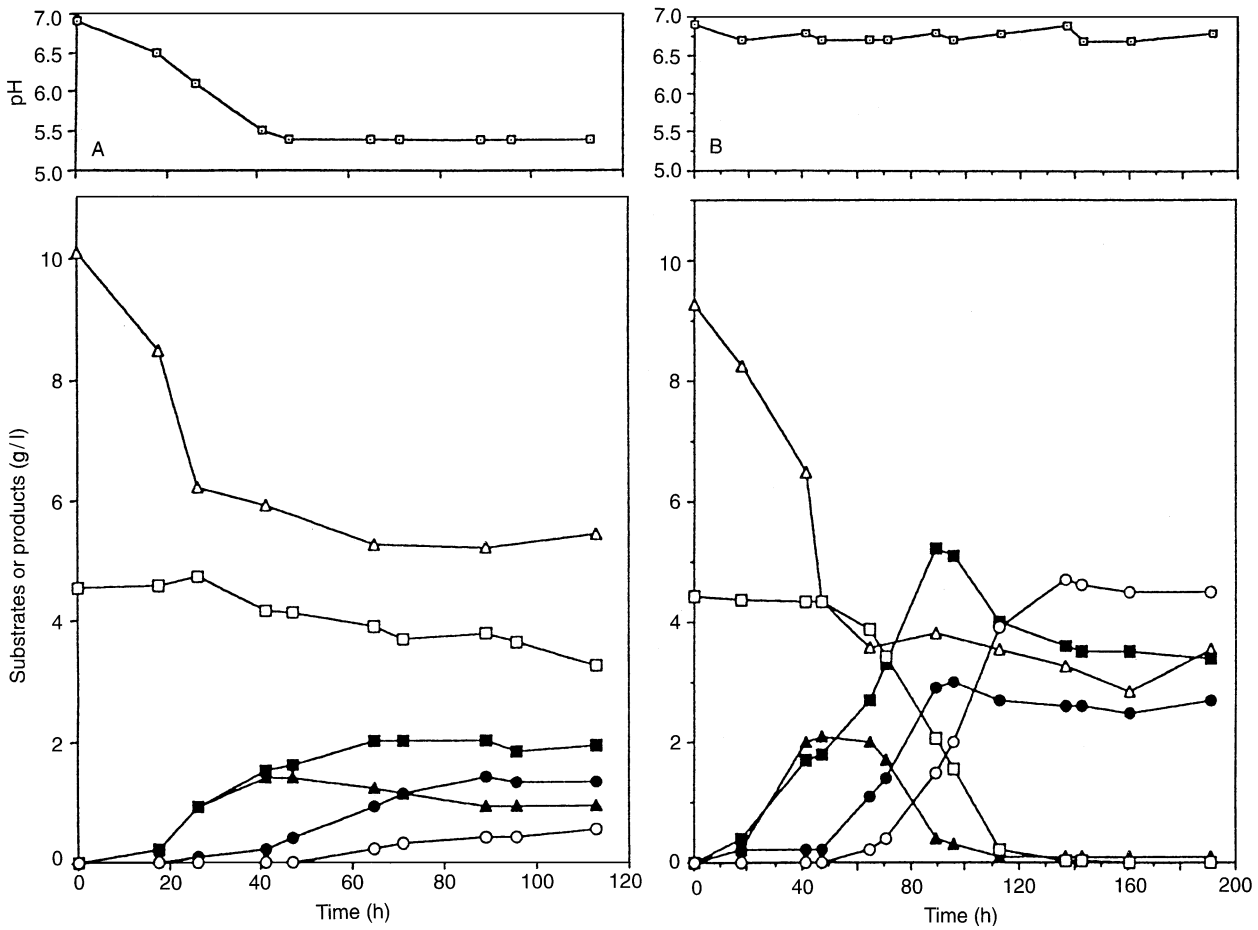

Fig. 2A, B The effect of pH on the fermentation of cellulose and ethanol by the coculture of *C. kluyveri* and *F. succinogenes*. The data represent the average of duplicate determinations and are representative of several experiments. **A** Without pH control; **B** pH controlled with sodium hydroxide. △ Cellulose, □ ethanol, ▲ succinate; ■ acetate, ● butyrate, ○ caproate. Note difference in scale of horizontal axis

acetate were consumed and butyrate and caproate were produced. Under the conditions employed in the experiments shown in Fig. 2A, a considerable decline in culture pH was observed before the major accumulation of butyrate and caproate had commenced. However, even without controlling the pH, small amounts of butyrate and caproate were formed toward the end of cellulose consumption.

Unlike the monoculture of *F. succinogenes*, the coculture was markedly affected by pH. Consumption of cellulose and ethanol was negatively affected by the low pH of the medium, as was the production of succinate, acetate, butyrate and caproate. Ethanol utilization was complete under conditions where the pH was held at 6.8 but was greatly reduced by allowing the pH to decline to 5.5. Likewise succinate utilization was incomplete when the pH was not controlled. Acetate was initially produced under both conditions and increased in concentration when butyrate and caproate were accumulating. Both the accumulation of butyrate as the succinate concentration declined, and the accumulation of caproate, which continued after the butyrate concentration reached a plateau value, are consistent with butyrate's role as an intermediate in the conversion of succinate to caproate (Kenealy and Waselefsky 1985).

The overall weight conversion (per liter) of 6 g cellulose and 4.4 g ethanol to 2.6 g butyrate and 4.6 g caproate was 69.2% (by weight) when the pH was controlled at 6.8 (Fig. 2B). When the pH was allowed to decline the coculture produced (per liter) 1.3 g butyrate and 0.4 g caproate from 1.0 g ethanol and 4.8 g cellulose for a weight conversion of 29.3% (Fig. 2A). It should be noted that in the pH-controlled reactor the conversions for cellulose into butyrate and caproate were 81.5% and 71.6%, respectively (calculated as a fraction by weight of the theoretical maximum on a carbon basis); for ethanol the conversions were 97.8% and 85.2% respectively.

Table 2 Effect of butyrate and caproate on the fermentation of cellulose by *F. succinogenes* S85 or *R. flavefaciens* FD-1 Cultures were incubated for 48 h at 39°C. Data represent the average of duplicate tubes and are representative of several experiments. Where indicated the cultures were inoculated with FD-1 (*R. flavefaciens*) or S85 (*F. succinogenes*). Cultures contained the indicated amount of added organic acid at pH 6.8 prior to inoculation. Cellulose values show the amount of anhydroglucose equivalents removed (mM)

Addition	Concentration changes (mM) of cellulose and acids				Final pH
	Cellulose	Succinate	Acetate	Formate	
S85	24.9*	21.1*	8.2*	0*	5.8
S85 + 30 mM butyrate	24.7*	19.8*,**	7.8*	0*	5.8
S85 + 30 mM caproate	22.0*	16.7**	7.3*	0*	5.8
FD-1	22.0*	12.5**	16.4**,***	16.3**	5.5
FD-1 + 30 mM butyrate	22.1*	13.6**	16.6**	16.4**	5.5
FD-1 + 30 mM caproate	15.0**	10.1**	13.9***	13.4**	5.7

*–*** Values in same column having different superscripts differ significantly ($P < 0.05$).

Effects of butyrate and caproate on cellulose hydrolysis and metabolism of *F. succinogenes* and *R. flavefaciens*

Since cellulose consumption was reduced in the *C. kluyveri*/*F. succinogenes* coculture without pH control (Fig. 2A, B) and not in the *F. succinogenes* monoculture (Fig. 1A, B), butyrate and caproate were tested for effects on the metabolism and cellulose hydrolysis of *F. succinogenes* and *R. flavefaciens*. The latter species was once again considered in these experiments because a different sensitivity to the end products of *C. kluyveri* metabolism could alter the choice of organisms for a coculture designed to produce caproate.

The addition of 30 mM butyrate (Table 2) in the initial medium for the growth of *F. succinogenes* and *R. flavefaciens* had no effect on the amount of cellulose consumed, the final culture pH, or the production of organic acids by either organism. Substitution of caproate for butyrate resulted in a significant (32%) decline in the amount of cellulose consumed by *R. flavefaciens* (but not by *F. succinogenes*).

Mass balance of the *C. kluyveri*/*F. succinogenes* coculture

In preceding experiments the recovery of carbon from the fermentation was unexpectedly low, and the redox level of the products was more reduced than that of the substrates. We attributed this to the amount of cell material formed, which was not measured in these initial experiments. The rumen cellulolytics have a cell yield of 0.11–0.24 g cells/g cellulose consumed (Pavlostathis et al. 1988b; Shi and Weimer 1992; Weimer, 1993). *C. kluyveri* in general has a poor cell yield on ethanol and acetate (0.038–0.068 g cells/g ethanol consumed) but a higher yield on ethanol and succinate (0.056–0.256 g cells/g ethanol consumed) (Kenealy and Waselefsky 1985). Fermentation balances for the *F. succinogenes*/*C. kluyveri* (Table 3) coculture indicate that the overall carbon recovery was 81.7% with cells accounting for 3% of the substrate consumed. No effort was made to distinguish between *C. kluyveri* and *F. succinogenes* cell mass in this experiment.

Table 3 Fermentation balance of binary cultures of *C. kluyveri* and *F. succinogenes* grown on cellulose, ethanol and acetate. Cultures were incubated for 7 days at 39°C. Data in the table are the averages of triplicate cultures and are representative of many experiments. Potential products that were not detected by HPLC included: propionate, valerate, isobutyrate, isovalerate, malate, fumarate, methanol, C_3–C_5 alcohols, glycerol, acetone. Cellulose is reported in glucose equivalents (mM), hydrogen as equivalent values (mM), [i.e., total gas-phase hydrogen in the bottle (μmol) divided by volume of liquid in bottle (ml)]. Cells were determined by analysis of pellet nitrogen content and an assumed cell formula of $C_5H_7O_2N$ for the organic fraction of cell mass (Pavlostathis et al. 1988b). Redox values calculated using the convention of H = −0.5 and O = +1.0

Substrates consumed (mM)		Products formed (mM)	
Ethanol	112.3	Butyric acid	14.4
Cellulose	11.8	Caproic acid	30.8
Acetic acid	6.9	Hydrogen	16.6
		Cells	1.8
Redox substrates	−224.6	Redox products	−171.3
O/R (redox) index		0.762	
Carbon recovery		0.817	

Discussion

This research demonstrates that a fermentation can be designed that will produce caproic acid from mixtures of cellulose (available as a biomass residue), ethanol and carbon dioxide (both potentially available from fermentation of low-value waste streams, e.g., cheese whey). The present coculture experiments used the efficient cellulolytic ruminal anaerobes, *R. flavefaciens* and *F. succinogenes*, which produce succinate and acetate, together with *C. kluyveri* and exogenously added ethanol to generate caproate. Production of caproic acid was dependent on the cellulolytic organism to provide succinate or acetate (Weimer et al. 1991), and

on *C. kluyveri* to convert these acids plus added ethanol to caproate (Kenealy and Waselefsky 1985).

Considerable reduction in the amount of ethanol needed as a cosubstrate might be achievable through the use of ethanol-producing cellulolytic microbes (e.g., *R. albus*, a ruminal bacterium with cellulolytic capabilities similar to those of the ruminal species examined here; Pavlostathis et al. 1988a, b). Because caproic acid was inhibitory to cellulose conversion, particularly at lower pH (Fig. 2 and Table 2), the simultaneous extraction of caproic acid and recycling of organisms and caproic acid precursors into the reaction vessel might improve the productivity of the coculture and are thus worthy of further exploration.

When the coculture containing *C. kluyveri* and *F. succinogenes* was examined over a pH-controlled time course (Fig. 2) the conversion of cellulose to succinate and acetate was observed prior to the production of butyrate and caproate. Although simultaneous production and consumption of succinate and acetate would be desirable for commercial production of caproic acid, it was not achieved in these experiments. In several attempts at establishing a coculture in a chemostat fed cellulose and ethanol we were occasionally able to generate a steady state where both cultures were growing and metabolizing, but were unable to maintain this steady state reproducibly (data not shown).

Even with pH control the maximum cellulose degradation by the rumen anaerobes was approximately 6 g l^{-1} (Fig. 1B). Factors limiting more complete digestion of cellulose were not immediately obvious. The fact that the rate of cellulose removal in the coculture (Fig. 2B) was not stimulated when succinate conversion to caproate and butyrate was maximal suggests that cellulose hydrolysis was not inhibited by succinate. The sensitivity of *F. succinogenes* to acetate anion at neutral pH was not tested in light of the high physiological concentrations of acetate (approx. 70 mM) normally encountered by these species in the rumen (Hungate 1966). Cellulose consumption does not appear to become uncoupled from growth as it did not increase upon prolonged incubation. The cessation of cellulose hydrolysis is thus probably due to the cessation of the growth of the cellulolytic bacterium. By contrast, the production of caproic acid by *C. kluyveri* can be uncoupled from growth and this metabolism can continue for a period of time even at pH 5.5 (see Fig. 2B).

Production of succinate by *R. flavefaciens* and *F. succinogenes* is accompanied by a fixation of CO_2 in amounts equal to and sometimes greater than the amounts produced by the organism from the acetate-producing pathway (Dehority 1971; Shi and Weimer 1992; Weimer 1993). *C. kluyveri* also fixes some CO_2 into cell material (Bornstein and Barker 1948). Together the fermentation should result in more carbon being present in the recoverable end-products compared to fermentations that generate CO_2. Using these CO_2-fixing organisms in coculture may offset some of the CO_2 generated when ethanol is formed by other sugar fermentations.

The fermentation balance had a carbon recovery of 82%, with substrates more reduced than products (oxidation/reduction, O/R index = 0.76). Since the fixation of CO_2 was not measured the redox level of the substrates is probably more oxidized. The CO_2 needed to balance the redox would be 26.6 mM, and this amount of CO_2 fixation would further reduce the carbon recovery to 75%. Because batch cultures of *F. succinogenes* can store considerable amounts of glycogen (Gaudet et al. 1992; Wells and Russell 1994) the cell mass could have been underestimated since the nitrogen content per cell would be lower than that assumed here for cell mass calculations, but it is unlikely that errors in cell mass estimations could account for much of the missing carbon. Although the data suggest that one or more fermentation products may not have been accounted for, a number of other common end-products of fermentation (Table 3) were not detected. Extracellular proteins (e.g., cellulases) are unlikely candidates for mass balancing, since in ruminal cellulolytic bacteria cellulases are primarily localized at the cell surface (Weimer 1992).

Producing chemicals and fuels from a renewable resource requires a system both to synthesize and to recover the chemicals. The concept of biorefining (Levy et al. 1980, 1981, 1983) involves the conversion of waste materials into organic acids, which could then be recovered and converted into useful chemicals or fuels by electrochemical processes. A limitation of the biorefining concept, as described in these reports, is the unstable nature of the biological process used to generate the acids (e.g., temporary inhibition of methanogens in an anaerobic digester). The use of the cocultures described here has obvious merit as an alternative to biorefining. The starting material, cellulose, is inexpensive. Acids and ethanol in low concentrations can be directly made from this material (Khan and Patel 1991) and the further fermentative conversion of these materials into caproic acid allows the extraction of almost all the fuel value that could be derived from the ethanol and acids without the costs of distilling or extracting these intermediates from dilute aqueous solution (Levy et al. 1983). Because caproate is a natural end-productof the coculture, the delicate process of manipulating the fermentation to encourage the accumulation of intermediates is obviated. Further work with natural lignocellulose biomass sources (e.g., agricultural and forestry residues), along with economic analysis of the fermentation conditions necessary to establish profitablility, should permit a determination of the feasibility of applying *C. kluyveri*-based cocultures to production of caproic acid and its derivatives.

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