Bioconversion of Cellulose to Acetate with Pure Cultures of *Ruminococcus albus* and a Hydrogen-Using Acetogen

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Bioconversion of cellulose to acetate was accomplished with cocultures of two organisms. One was the cellulolytic species *Ruminococcus albus*. It ferments crystalline cellulose (Avicel) to acetate, ethanol, CO₂, and H₂. The other organism (HA) obtains energy for growth by using H₂ to reduce CO₂ to acetate. HA is a gram-negative coccobacillus that was isolated from horse feces. Coculture of *R. albus* with HA in batch or continuous culture alters the fermentation products formed from crystalline cellulose by the ruminococcus via interspecies H₂ transfer. The major product of the fermentation by *R. albus* and HA coculture is acetate. High concentrations of acetate (333 mM) were obtained when batch cocultures grown on 5% cellulose were neutralized with Ca(OH)₂. Continuous cocultures grown at retention times of 2 and 3.1 days produced 109 and 102 mM acetate, respectively, when fed 1% cellulose with utilization of 84% of the substrate.

An inexpensive source of acetate would be useful for the economical production of calcium magnesium acetate, a non-polluting, noncorrosive, deicing salt (11, 12). Fermentation of biomass, particularly cellulose, might provide inexpensive acetate. Although there are several single species of bacteria, both mesophilic and thermophilic, that stoichiometrically transform glucose to acetate, none of them possesses cellulase. However, pure cultures of individual species might be combined to effect the bioconversion of cellulose to glucose and stoichiometric conversion of the glucose subunits to acetate. One species must produce cellulase and the catabolic activities of the combined species must interact to accomplish a combined culture fermentation of cellulose to acetate. This was achieved with cocultures of *Clostridium thermocellum* and the H₂-using acetogen *Acetogenium kivui* at 65°C (4).

We used cocultures of *Ruminococcus albus* and *Methanobrevibacter smithii* to produce a fermentation of crystalline cellulose (Avicel) to acetate and methane. We showed that *R. albus* ferments cellulose to ethanol, acetate, H₂, and CO₂ (8, 9). The addition of the H₂-using methanogen, *M. smithii*, decreased ethanol formation and increased acetate formation, and methane was produced instead of hydrogen (10). In this study, we demonstrate that cocultures of *R. albus* and a H₂-using acetogen produce acetate as the major product of cellulose fermentation at 37°C.

MATERIALS AND METHODS

Organisms, media, and growth conditions. The cellulolytic species *R. albus* 7 was maintained in SMS medium with 0.1% each glucose, cellobiose, and maltose and 2% agar (7). The H₂-using species was an anaerobic, gram-negative coccobacillus (strain HA) isolated from horse feces after enrichment on a medium with bromoethanesulfonate and H₂ and CO₂ as the source of energy. It produces acetate as the sole product of growth and incorporates CO₂ into both carbons of acetate. It uses the following compounds as energy sources: arabinose, carbon monoxide, cellobiose, dextrin, esculin, formate, fructose, galactose, glucose, glycerol, H₂-CO₂, inositol, lactose, maltose, mannitol, melezitose, melibiose, pyruvic acid, raffinose, rhamnose, ribose, salicin, succinic acid, sucrose, trehalose, and xylose. It does not use cellulose (Avicel), carboxymethyl cellulose, dulcitol, lactic acid, methanol, peptone, starch, valine, and xylan.

Strain HA was maintained in BACE medium with 202 kPa of 80% H₂:20% CO₂ (H₂:CO₂). BACE medium contained the following constituents per liter: NaHCO₃, 7.5 g; NH₄Cl, 1 g; K₂HPO₄, 0.3 g; KH₂PO₄, 0.3 g; (NH₄)₂SO₄, 0.3 g; NaCl, 0.61 g; MgSO₄·7H₂O, 0.15 g; CaCl₂·2H₂O, 81 mg; MnSO₄·H₂O, 4.5 mg; FeSO₄·7H₂O, 3.0 mg; CoSO₄·7H₂O, 1.8 mg; ZnSO₄·7H₂O, 1.8 mg; CuSO₄·5H₂O, 100 µg; AlK(SO₄)₂·12H₂O, 180 µg; H₃BO₃, 100 µg; Na₂MoO₄·2H₂O, 100 µg; Na₂SeO₄, 1.9 mg; NiCl₂·6H₂O, 92 µg; nitrilotriacetic acid, 15 mg; thiamine-HCl, 2 mg; D-pantothenic acid, 2 mg; nicotinamide, 2 mg; riboflavin, 2 mg; pyridoxine-HCl, 2 mg; biotin, 1 mg; cyanocobalamin, 20 µg; *p*-aminobenzoic acid, 100 µg; folic acid, 50 µg; cysteine·HCl·H₂O, 0.5 g; 15 ml of a cysteine-sulfide reducing solution containing 2.5% each cysteine-HCl·H₂O and Na₂S·9H₂O; resazurin, 1 mg; yeast extract, 2 g; and 100 ml of clarified rumen fluid.

Stock cultures were refrigerated and transferred weekly. *R. albus* and HA cocultures were established by simultaneous inoculation (10% [vol/vol] of each) into BACE medium containing 1% cellulose and 101 kPa of 80% N₂:20% CO₂ (N₂:CO₂). All incubations were at 37°C. Batch cocultures and HA cultures grown with H₂:CO₂ were mixed by rotation during incubation. Cellulose (Avicel, type PH-105; FMC Corp., Philadelphia, Pa.) or glucose was added to media prior to autoclaving at concentrations indicated in text, figures, and tables.

Details on the continuous culture apparatus design and computer-controlled operation were previously reported (8, 9). Culture contents were removed each hour, and an equivalent volume of nutrients was added to the fermentor. A series of solenoid valves and variable-speed peristaltic pumps controlled by a personal computer were used for sequential medium addition and culture wasting of the fermentors.

Analysis of substrate and fermentation products. Soluble fermentation products (i.e., formate, acetate, ethanol) were measured by high-pressure liquid chromatography with an Aminex ion exclusion column (HPX-87H; Bio-Rad Laboratories, Richmond, Calif.) according to the method of Ehrlich et al. (1). Liquid samples were removed from the fermentors and centrifuged at 16,000 × *g* for 15 min, and the supernatants were passed through a 0.2-µm-pore-size membrane filter and stored at -10°C in O-ring-sealed centrifuge tubes until they were analyzed. Total carbohydrates and reducing sugars were measured by the anthrone method (3) with glucose as the standard. H₂ was analyzed on a gas chromatography unit equipped with a thermal conductivity detector and columns (6 ft [ca. 200 cm] by 1/8 in. [ca. 0.3 cm]) packed with molecular sieve 5A and Porapak Q (Supelco, Inc., Bellefonte, Pa.).

Radioisotopic experiment. Batch cocultures (10 ml) of *R. albus* and HA were grown on 1% cellulose in BACE medium amended with ¹⁴C-labeled bicarbonate. The total HCO₃⁻-CO₂ concentration was 1,577 µmol, and the radiospecific activity was 2,790 dpm/µmol. Cocultures were grown for 48 h. After measurement for H₂, the coculture was acidified with 500 µl of 5 N H₂SO₄ and then analyzed for radioactive CO₂ and acetate. Methods for recovering and measuring the quantity and radioactivity of CO₂ and acetate and the radiospecific activities of the methyl and carboxyl groups of acetate were previously described (13).

RESULTS

Batch growth of *R. albus* and HA with cellulose. Figure 1 compares the fermentation products formed after 5 days of

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FIG. 1. Comparison of products formed from 1% cellulose after 5 days of batch growth of *R. albus* alone (open bars) and a coculture of *R. albus* and HA (closed bars). AC, acetate; F, formate; ET, ethanol.

incubation of *R. albus* without and with the H₂-using acetogen, HA, with cellulose (62 mM hexose equivalent [C₆eq]). Addition of HA diminished formation of H₂, formate, and ethanol. There was a large increase in the production of acetate. The final ratios of ethanol to acetate were 1.05 for *R. albus* alone and 0.22 for the coculture. The carbon in the products of the coculture, including calculated amounts of CO₂, indicated that 70% of the cellulose was fermented with an O/R for the fermentation of 1.1. The values with *R. albus* alone were 53% and 1.2. *R. albus* forms approximately 1 mol each of ethanol and acetate and 2 mol each of H₂ and CO₂ from each mole of hexose fermented (6, 8, 9). HA could increase acetate yields by using H₂ to reduce CO₂ without altering the yield of ethanol. Since it forms 1 mol of acetate from 4 mol of H₂ and 2 mol of CO₂, the stoichiometry is C₆H₁₂O₆ → 1.5 CH₃COOH plus CH₃CH₂OH plus CO₂, and the final ratio of ethanol to acetate is 0.67. However, H₂ utilization can also eliminate ethanol and increase acetate production by *R. albus*. The partial pressure of H₂ must be reduced enough to allow *R. albus* to produce H₂ from NADH rather than oxidizing NADH by reducing acetyl ~S-conenzyme A to ethanol. If no ethanol is formed, the stoichiometry is C₆H₁₂O₆ → 3 CH₃COOH and the final ratio of ethanol to acetate is 0. Figure 2 shows the percentage of hexose that is used by each pathway at ethanol/acetate ratios of 0 to 0.67. The ratio of 0.22 obtained in the batch coculture indicates that approximately 50% of the hexose was fermented to three acetates. No correction was made for the small amount of H₂ that accumulated that was not used to reduce CO₂ to acetate.

The effect of pH on the batch coculture fermentation was examined. Three concentrations of bicarbonate were used with 101 kPa of N₂:CO₂ to produce initial pHs of 6.6, 6.8, and 7.2. The best utilization of cellulose and the lowest ratio of ethanol to acetate were obtained when the initial pH was 7.2 (Fig. 3). The ethanol-to-acetate ratios were 0.29, 0.28, and 0.20 at pHs 6.6, 6.8, and 7.2, respectively, which represent ca. 45% homoacetate fermentation at pHs 6.6 and 6.8 and 54% at pH 7.2. The final pH in all cases was 5.4. Neither H₂ nor formate was formed in the cocultures.

¹⁴CO₂ incorporation into acetate. The fermentation of cellulose (62 mM C₆eq) by batch cocultures of the two species was examined with added ¹⁴CO₂. After fermentation, the acetate concentration was 54 mM and the ethanol concentration was 27 mM (ethanol/acetate = 0.5; homoacetate fermentation =

FIG. 2. The relationship between the ethanol/acetate ratio to the percentage of hexose used by the pathways C₆H₁₂O₆ → 3 CH₃COOH (closed squares) and C₆H₁₂O₆ → 1.5 CH₃COOH plus CH₃CH₂OH plus CO₂ (open squares).

15%). The radiospecific activity of the acetate and its methyl and carboxyl groups was 1,798, 935, and 863 dpm/μmol, respectively. The methyl and carboxyl groups of acetate contained equivalent amounts of radioactive carbon (52 and 48%, respectively). Calculations based on the specific activity of CO₂ showed that 31% of the acetate produced from glucose was formed by reduction of ¹⁴CO₂. The results are consistent with coculture fermentation of cellulose to acetate by the Wood-Ljungdahl pathway (5, 14).

pH control. Increased concentrations of acetate by cocultures in a batch medium would decrease pH values without pH control. We tested the sensitivity of *R. albus* to Na⁺, K⁺, and NH₄⁺ to assess the possibility of using NaOH, KOH, or NH₄OH addition to cultures to neutralize acids. The inhibitory concentrations were too low to consider use of the bases to neutralize acid production. For the least toxic cations, the maximum concentration that could be added without inhibiting growth was only about 200 mM. Separate experiments with sulfate salts instead of chlorides showed that the inhibition was due to the cations and not to chloride ion.

R. albus and HA were grown in BACE medium with cellu-

FIG. 3. Effect of incubation of cocultures of *R. albus* and HA at different initial pHs on cellulose disappearance (open bars) and acetate (crosshatched bars) and ethanol (closed bars) formation. Incubations were for 5 days.

TABLE 1. Substrate used and products formed in continuous cocultures at different RT^a

RT (days)	Substrate hexose (mM)	Quantity of substrate or product						
		Substrate			Product			
		Acetate (mM)	Ethanol (mM)	Hydrogen (mM)	Ethanol/acetate	C ₆ →3 CH ₃ COOH (%)	C recovery ^b (%)	O/R ^b
1.3	30.64	66.65	0.18	ND ^c	0.00	99.00	73.52	0.99
2.0	51.94	109.45	11.16	1.01	0.10	74.00	80.91	1.04
3.1	52.67	102.27	17.30	8.21	0.17	60.00	81.57	1.24

^a Formate or other products were not detected by high-pressure liquid chromatography analysis. The results are the means of analyses performed during 48, 12, and 10 days of continuous culture at RT of 1.3, 2.0, and 3.1 days, respectively.

^b CO₂ calculated as equal to ethanol plus 0.5 × H₂.

^c ND, not done because total gas production was not measurable.

lose (62 mM C₆eq) with 0.6 and 0.3% Ca(OH)₂ and Mg(OH)₂, respectively. Cultures with Mg(OH)₂ were completely inhibited. Ca(OH)₂ did not inhibit growth of the organisms or their fermentation. There was a slight increase in the yield of acetate and a higher final pH than in the absence of Ca(OH)₂.

Ca(OH)₂ was then added to a batch coculture fitted with a pH electrode and controller that automatically added base to maintain a pH of 6.2 to 6.3. A total of 61.7 mmol of C₆eq as cellulose in 200 ml of BACE medium was inoculated with 20 ml of a coculture taken from a continuous coculture. The Ca(OH)₂ (10.5%) used to neutralize the culture was sterilized by autoclaving under N₂ in BACE medium without cellulose, minerals, bicarbonate, and cysteine. After 7 days, the amounts of products were as follows (total millimoles): acetate (143.5), ethanol (29.7), H₂ (4.1), and CO₂ (31.8). CO₂ was estimated by assuming equivalence between CO₂ and the sum of ethanol and H₂. All the cellulose was used. Carbon recovery was 102%, and the O/R ratio was 1.0. Assuming that ethanol fermentation represents the *R. albus* fermentation without interspecies interaction, 30 mmol of C₆eq was fermented to 30 mmol each of acetate and ethanol and 60 mmol of H₂ and CO₂. After subtracting the residual H₂, an estimated 14 mmol of acetate was formed from H₂ and CO₂ by strain HA and the remaining 100 mmol of acetate was formed from 33.3 mmol of C₆eq of cellulose by the C₆H₁₂O₆→3 CH₃COOH pathway. This pathway accounted for fermentation of 53% of the hexose. The estimate based on the ethanol/acetate ratio of 0.21 (Fig. 2), which does not correct for the small amount of unused H₂, gave essentially the same result.

The Ca(OH)₂ settled in the tubing of the pump line from the suspension to the culture. During the course of the experiment, 200 ml of suspension was added. If the suspension was homogeneous, 21 g of Ca(OH)₂ would have been added, but the actual amount was 5.5 g (74 mmol) based on dry weight disappearance from the Ca(OH)₂ reservoir. The 74 mmol added contained 148 meq of OH⁻. The total amount of acetate formed was 144 mmol. This suggests that Ca(OH)₂ stoichiometrically neutralized the acetate produced by the coculture. On the basis of the volume of base added, the fermentation was essentially complete in 4 days.

Continuous cocultures of *R. albus* and HA. Batch cocultures of *R. albus* and HA in 200 ml of BACE medium with 62 mM C₆eq of cellulose were established prior to continuous feeding of the same medium and continuous removal of culture contents. The retention times (RT) used were 1.3, 2.0, and 3.1 days. Most of the carbohydrate was degraded by the C₆H₁₂O₆→3 CH₃COOH pathway (Table 1). Some of the unrecovered carbon was probably in biomass which was not measured. In continuous cocultures of *R. albus* and *M. smithii* that produced acetate, CH₄, and CO₂ from cellulose (RT from 0.5

to 2.0 days), 10% of the cellulose carbon used was converted to biomass carbon (10). Table 1 shows that a small amount of H₂ was produced at the two longer RT with greater production at the RT of 3.1 days. Gas production was not sufficient at the RT of 1.3 days to accurately collect and measure gas produced by the coculture. The increase in H₂ was accompanied by an increase in the ethanol/acetate ratio and a decrease in the percentage of the C₆H₁₂O₆→3 CH₃COOH pathway. Increasing the RT from 1.3 to 3.1 days led to a decrease in the steady-state concentration of cellulose in the culture vessel and an increase in product concentrations. This is consistent with previous results that showed that the rate of cellulose decomposition by *R. albus* follows first-order kinetics and depends on the concentration of cellulose (8).

DISCUSSION

Batch culture fermentation of cellulose to acetate by the coculture of *R. albus* and HA with neutralization by Ca(OH)₂ produced 2% acetate (333 mM) and almost complete biodegradation of 5% cellulose to acetate. If the culture had not been diluted because of settling of Ca(OH)₂ in the feed lines, the final acetate concentration would have been 3.7% (616 mM). Klemp et al. (2) reported production of 3.75% acetate by batch cultures of the thermophilic homoacetogen *A. kivui* grown on glucose. Acetate yields and rates of conversion of glucose to acetate found by other investigators are reviewed by Wiegel (11).

Batch cocultures of *C. thermocellum* and *A. kivui* grown on cellulose at 65°C with pH control yielded 113 mM acetate with a starting concentration of 1% cellulose (4), and the maximum rate of fermentation of cellulose by the thermophilic coculture was 63 mg/liter/h. The minimum rate for the *R. albus*-HA coculture was 26 mg/liter/h. The calculation is based on total utilization of cellulose in 4 days and a final volume of 400 ml after dilution by the suspension of Ca(OH)₂.

It should be possible to increase the concentration of acetate formed by minimizing dilution and by increasing the concentration of cellulose. Additional studies are necessary to determine the maximum level of Ca(OH)₂ that the organisms can tolerate and to develop procedures for delivery of homogeneous, concentrated suspensions of Ca(OH)₂. Since bulk addition of Mg(OH)₂ to media prior to inoculation resulted in inhibition of batch cocultures of *R. albus* and HA, we did not pursue using Mg(OH)₂ to neutralize acetate. However, cocultures might tolerate addition of Mg(OH)₂ on demand as they grow and produce acid.

The rates of cellulose utilization by the continuous cocultures of *R. albus*-HA were 178, 195, and 127 mg/liter/h for RT of 1.3, 2.0, and 3.1 days, respectively. The RT of 2.0 days

provided the highest rate of cellulose decomposition and almost complete utilization of the cellulose fed to the cocultures. Ethanol accumulation at RT of 2 and 3.1 days is probably caused by the increasing accumulation of H_2 with increasing RT. The steady-state H_2 concentrations in the headspace were 0.3, 0.9, and 6.0 $\mu\text{mol/ml}$ of gas for RT of 1.3, 2.0, and 3.1 days, respectively. Although only small amounts of H_2 were formed (Table 1), the increase in the partial pressure of H_2 could have prevented some oxidation of NADH to H_2 by *R. albus* and elicited the use of NADH to produce ethanol. This probably could be avoided by gassing the headspace to prevent H_2 accumulation.

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