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Anaerobic bioconversion of cellulose by *Ruminococcus albus*, *Methanobrevibacter smithii*, and *Methanosarcina barkeri*

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**Abstract** A system is described that combines the fermentation of cellulose to acetate, CH₄, and CO₂ by *Ruminococcus albus* and *Methanobrevibacter smithii* with the fermentation of acetate to CH₄ and CO₂ by *Methanosarcina barkeri* to convert cellulose to CH₄ and CO₂. A cellulose-containing medium was pumped into a co-culture of the cellulolytic *R. albus* and the H₂-using methanogen, *Mb. smithii*. The effluent was fed into a holding reservoir, adjusted to pH 4.5, and then pumped into a culture of *Ms. barkeri* maintained at constant volume by pumping out culture contents. Fermentation of 1% cellulose to CH₄ and CO₂ was accomplished during 132 days of operation with retention times (RTs) of the *Ms. barkeri* culture of 7.5–3.8 days. Rates of acetate utilization were 9.5–17.3 mmol l⁻¹ day⁻¹ and increased with decreasing RT. The *Kₐ* for acetate utilization was 6–8 mM. The two-stage system can be used as a model system for studying biological and physical parameters that influence the bioconversion process. Our results suggest that manipulating the different phases of cellulose fermentation separately can effectively balance the pH and ionic requirements of the acid-producing phase with the acid-using phase of the overall fermentation.

**Introduction**

Anaerobic bioconversion of organic matter to CH₄ and CO₂ is accomplished by complex microbial ecosystems in anoxic aquatic environments. Examples include man-made anaerobic waste decomposition systems and natural aquatic systems fed with large inputs of organic matter (e.g., swamps, rice paddies; Schink 1988; Zinder 1993). The microbial communities are mixtures of species that ferment natural organic substrates to acetate, H₂, and CO₂, and methanogens that form CH₄ from these products (Schink 1988; Zinder 1993). These systems have long retention times (RTs). Conventional anaerobic municipal sewage digesters are operated with RTs greater than 10 days. Short RTs disrupt the integration between the fermentative and methanogenic stages and lead to the accumulation of acid fermentation products and the cessation of CH₄ formation (Schink 1988; Zinder 1993).

Research on improved strategies for anaerobic digestion of wastes generally study the complex microbial populations that result from anaerobic enrichments of microorganisms from native habitats. This report describes a pure culture system that combines fermentation of cellulose to acetate, CH₄, and CO₂ with fermentation of acetate to CH₄ and CO₂. Khan (1980) reported conversion of cellulose to CH₄ with a batch co-culture of *Acetivibrio cellulolyticus* and *Methanosarcina barkeri*. *A. cellulolyticus* produces acetate, H₂, and CO₂; and *Ms. barkeri* produces CH₄ from its products. We previously showed fermentation of cellulose to acetate, CH₄, and CO₂ by co-cultures of the cellulolytic *Ruminococcus albus* and the H₂-using methanogen, *Methanobrevibacter smithii* (Pavlostathis et al. 1990). *R. albus* grown by itself ferments cellulose to acetate, ethanol, H₂, and CO₂. In co-culture, *Mb. smithii* uses H₂ and CO₂ to form CH₄, lowers the partial pressure of H₂, and favors the oxidation of NADH to H₂ by the *R. albus* NAD-linked hydrogenase. This eliminates NADH-dependent ethanol production from acetyl-SCoA, more acetyl-SCoA becomes available for acetate formation, and H₂ from NADH is used to form CH₄ (Pavlostathis et al. 1990).

As described in this report, we used a two-stage culture system to convert cellulose to CH₄ and CO₂. Fermentation of cellulose by a co-culture of *R. albus* and *Mb. smithii* produced acetate and CH₄. The acetate in the effluent was delivered to a culture of *Ms. barkeri* and was converted to CH₄ and CO₂. Fermentation of a 1%
cellulose feedstock to CH₄ and CO₂ was accomplished with a RT of the Ms. barkeri culture of 7.5–3.8 days.

Materials and methods

Co-culture of R. albus and Mb. smithii

R. albus strain 7 (DSM 20455) and Mb. smithii strain PS (DSM 861) were used. The medium (which contained 1% cellulose (Avicel, type PH-105, FMC Corp., Philadelphia, Pa.), minerals, vitamins, cysteine-sulfide reducing solution, resazurin, bicarbonate, and clarified rumen fluid) had the same composition and was prepared as previously described (Pavlostathis et al. 1988a). All cultures were grown anaerobically. Details on the culture apparatus design, growth of the inocula, establishment of the co-culture, and computer-controlled operation were as previously reported (Pavlostathis et al. 1988a, b). The nutrient reservoir contents were continuously stirred and frequently recirculated by a variable-speed peristaltic pump. The suspension was dispensed to the fermentor after closing the circulation to the reservoir and opening the entry to the fermentor by computer-controlled operation of a pinch-type, two-way solenoid valve. Gas was collected in a football bladder. The temperature of the fermentors was kept at 37 ± 0.5 °C by water recirculation. The nutrient feed pump, the culture wastewater pumps, and the solenoid valves were controlled by a personal computer with C-P Net V 2.0 software (Cole Palmer, Vernon Hills, Ill.). Culture contents were removed each hour and an equivalent volume of nutrients was added to the fermentor.

Culture of Ms. barkeri

Anaerobic conditions and general procedures for cultivation were the same as for the co-culture except where indicated below. Ms. barkeri strain 227 (DSM 1538) was used. A 360-ml batch culture of Ms. barkeri, grown in the same medium as used for the co-culture, but without cellulose and with 0.5% sodium acetate and 0.001% FeSO₄·7H₂O, was transferred to a 1-L vessel. The vessel was preflushed with 80% N₂-20% CO₂. The medium fed to the culture was the same as the batch culture medium. The recirculation procedure for suspending cellulose prior to feeding was omitted. The RT was maintained at 5 days prior to establishment of the two-stage system. The computer control system for inflow and outflow pumps was the same as used for the co-culture.

Two-stage system

Anaerobic conditions and general procedures for cultivation were the same as for the co-culture except where indicated below. After establishment of the co-culture of R. albus and Mb. smithii and the culture of Ms. barkeri, the co-culture effluent was filtered through a sterile 0.45-μm capsule filter (Gelman Sciences, Ann Arbor, Mich.) into a holding reservoir (500 ml of liquid). The pH of the reservoir contents was adjusted and maintained at pH 4.5 by the addition of 1 N HCl by a pH controller (Chemenaut, Cole Palmer, Vernon Hills, Ill.). The contents were pumped from the reservoir to the Ms. barkeri culture. The volume of the co-culture was 500 ml and the volume of the Ms. barkeri culture was 400 ml. Flow rates of medium into and effluent out of the co-culture, reservoir, and Ms. barkeri culture were the same. Because of the larger volume of the co-culture, its RT was greater than the RT of the Ms. barkeri culture. When flow rates were changed during the study, the change was the same for all input and output streams. All RTs given are based on liquid measurements and do not consider the RT of solids.

Analytical procedures

The amount of gas collected was determined by measuring the volume of gas drawn into a syringe used to collapse the football bladder. A portion of the gas was analyzed for H₂ and CH₄ by gas chromatography (Miller and Wolin 1996). Soluble fermentation products were determined by high-performance liquid chromatography (Ehrlich et al. 1981) as described by Wolin et al. (1998). Cellulose was determined by the anthrone procedure (Koehler 1952).

Results

We integrated the fermentation of the acetic acid-using organism, Ms. barkeri, with the R. albus and Mb. smithii co-culture fermentation of cellulose to acetate and CH₄ by using a two-stage fermentation culture system. Integration of the three species together with or without nutrient feeding and removal of triculture contents did not yield efficient conversion of acetate to CH₄ and CO₂. After establishing the culture of Ms. barkeri with timed addition of the acetic acid-containing medium and removal of culture contents, the effluent from the co-culture was fed to a holding reservoir, the pH was adjusted to 4.5, and the reservoir contents were fed to the Ms. barkeri culture.

Figure 1 shows CH₄ and acetate production from cellulose by the co-culture for a period of 132 days. During this period, the RT was changed over the range 8.3–4.2 days as indicated in the figure legend. The average ratio of CH₄ to acetate in the co-culture for the 132-day period was 0.54 ± 0.08. This is close to the expected ratio of 0.5 for the production of 2 mol of acetate and 1 mol of CH₄ and CO₂ per hexose of cellulose (C₆H₁₂O₆ → 2 CH₃COOH + CH₄ + CO₂). Table 1 shows the amount of cellulose used and the amount of products formed for the entire period of two-

![Fig. 1 Methane and acetate production from cellulose by the Ruminococcus albus–Methanobrevibacter smithii co-culture. The retention times (RT) were changed during the experimental period. The RTs (days) were: 8.3 (1–17), 5.4 (18–36), 4.9 (37–69), 4.5 (70–84), and 4.2 (85–132)