

## Growth of Acetotrophic, Methane-producing Bacteria in a pH Auxostat

Kevin R. Sowers, Michael J. Nelson, and James G. Ferry

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia USA

**Abstract.** Three acetotrophic *Methanosarcina* species, which included marine, nonmarine, and thermophilic strains, were grown on acetate in a 10-liter pH auxostat. Specific growth rates and molar growth yields were constant throughout growth. Cell yields were up to 18-fold greater than previously reported. These properties of the pH auxostat indicate that it is a preferred culture method for the biochemical study of methanogenesis from acetate.

The biochemical study of methanogenesis from acetate requires suitable methods for the large-scale culture of methanogenic bacteria on acetate. Batch culture methods presently used have several disadvantages: (a) the pH and acetate concentration fluctuate widely because large amounts of substrate are consumed [5, 8], (b) several weeks (3–4) are required to obtain maximum cell yields [1, 4, 6, 7, 9, 20], (c) final cell yields are low [1, 4, 6, 9, 20], and (d) enzyme activities of cell extracts among cultures are variable [1]. The present study describes a modification of a continuously fed batch culture, the pH auxostat [11], that produces high yields of acetate-grown methanogenic bacteria in minimum time.

### Materials and Methods

Strain sources for *Methanosarcina barkeri*, "*Methanosarcina thermophila*" (*Methanosarcina* sp. strain TM-1), and *Methanosarcina acetivorans* are described [16, 17]. Media were prepared anaerobically under N<sub>2</sub> by a modification of the Hungate technique [3]. *Methanosarcina barkeri* and "*Methanosarcina thermophila*" were grown in DPB medium that contained the following constituents in demineralized water at the indicated final percent concentrations (wt/vol): NH<sub>4</sub>Cl, 0.14; K<sub>2</sub>HPO<sub>4</sub>, 0.13; KH<sub>2</sub>PO<sub>4</sub>, 0.13; NaCl, 0.05; MgSO<sub>4</sub>, 0.05; Na<sub>2</sub>S · 9H<sub>2</sub>O, 0.027; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.006; Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.001; cysteine-HCl · H<sub>2</sub>O, 0.027; yeast extract (Difco, Detroit, MI), 0.01; trypticase (BBL, Cockeysville, MD), 0.01; sodium acetate, 0.41. In addition, 1% (vol/vol) each of vitamin and trace element solutions [21] and Antifoam-C (Sigma, St. Louis, MO) were added. *Methanosarcina acetivorans* was grown on M-C2 medium as previously described [16], except that Na<sub>2</sub>CO<sub>3</sub> was omitted. Growth studies were conducted in 14-liter fermenters (model MP-114, New Brunswick Scientific) that contained 10 liters of medium. The media were prepared without the addition of Na<sub>2</sub>S · 9H<sub>2</sub>O, and the pH was adjusted to 6.8 before autoclav-

ing at 121°C for 50 min. After cooling the media to the appropriate growth temperatures under N<sub>2</sub>, 5 ml of a sterile Na<sub>2</sub>S · 9H<sub>2</sub>O solution (50% wt/vol) was added and the same amount added every 48 h thereafter during growth. Na<sub>2</sub>S · 9H<sub>2</sub>O was sterilized by autoclaving in a serum vial containing N<sub>2</sub>. "*Methanosarcina thermophila*" was grown at 50°C; all other species were grown at 35°C. The medium was sparged with N<sub>2</sub> at 600 ml/min and stirred at 400 rpm. The fermenters were inoculated with 1-liter batch cultures or by transferring 1 liter of culture from another fermenter. The fermenter was modified to operate as a pH auxostat by installing autoclavable reference and pH electrodes connected to a pH controller as previously described for the growth of formate-utilizing methanogenic bacteria [12]. Acetic acid (10 N) was delivered from a calibrated reservoir through Teflon tubing (I.D. 1.5 mm, Bethesda Research Laboratories, Bethesda, MD). As acetate was used by the culture, the pH controller compensated for the increase in pH by adding acetic acid, thereby maintaining a constant pH and substrate concentration. Late exponential phase cultures were harvested anaerobically with a continuous flow centrifuge [12] and stored in liquid N<sub>2</sub>. Acetate [8] and CH<sub>4</sub> [16] were assayed with a gas chromatograph as described.

### Results and Discussion

The growth characteristics of each strain are shown in Fig. 1. After an initial lag, exponential growth coincided with exponential acetate consumption and CH<sub>4</sub> formation, which indicates that catabolism and anabolism were coupled uniformly. The ratios of CH<sub>4</sub> produced and acetate consumed (Fig. 1) approached the expected ratio of 1:1 [5, 13, 14, 22]. The slightly higher ratio of CH<sub>4</sub> to acetate toward the end of the growth curves may be attributed to errors in the determination of CH<sub>4</sub>. Each culture was maintained at pH 6.8 ± 0.2. Acetate concentra-

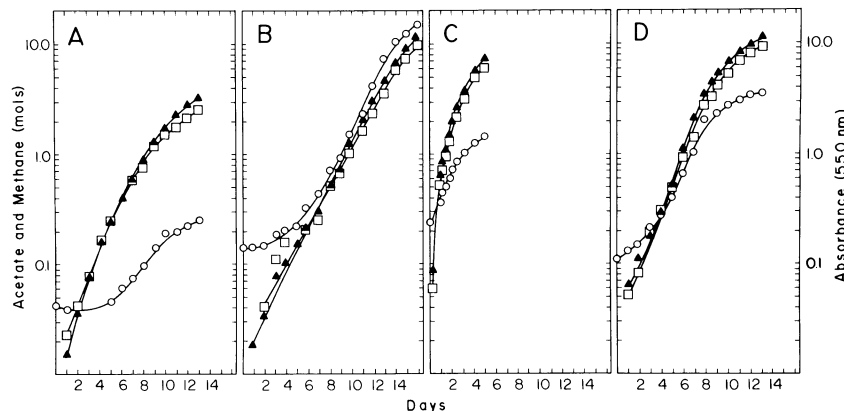


Fig. 1. Growth of *Methanosarcina barkeri* strain MS (A), *M. barkeri* strain 227 (B), "*M. thermophila*" (C), and *M. acetivorans* (D) in a pH auxostat. Absorbance (○) was determined at 550 nm in a 1-cm light path with a Hitachi spectrophotometer (model 100-60). Total acetate utilized (□) was determined from depletion of 10 N acetic acid in the reservoir. Total methane produced (▲) was determined by the equation  $Y_{t_2} - Y_{t_1}/2.303 m$ , where  $Y$  is  $\mu\text{mol CH}_4/\text{min}$  at the time points  $t_1$  and  $t_2$ , and  $m$  is the slope [12].

Table 1. Growth of acetotrophic methane-producing bacteria on acetate with a pH auxostat

<i>Methanosarcina</i> species	Specific growth rate ( $\text{h}^{-1}$ ) <sup>a</sup>	Final yield		Molar growth yield (g dry wt/mol acetate) <sup>c</sup>
		(g wet wt/10 l)	(g dry wt/10 l) <sup>b</sup>	
<i>M. barkeri</i> strain MS	0.028	27	3	1.2
<i>M. barkeri</i> strain 227	0.029	163	20	2.7
" <i>M. thermophila</i> "	0.059	59	10	1.7
<i>M. acetivorans</i>	0.033	35	12	2.4

<sup>a</sup> Growth rates are mean values calculated from the exponential growth phase (Fig. 1).

<sup>b</sup> 10 ml of culture from a pH auxostat was passed through a predried filter (0.2- $\mu\text{m}$  pore size, Millipore Corp., Bedford, MA). The cells and filter were washed in demineralized water, then dried overnight at 90°C and reweighed. The dry weight of osmotically sensitive *M. acetivorans* was determined as previously described [16].

<sup>c</sup> The molar growth yields were determined from absorbance and acetate consumption in exponential growth (Fig. 1). One optical density unit corresponded to the following dry weights (g/liter): *M. barkeri* strain MS, 0.79; *M. barkeri* strain 227, 0.13; "*M. thermophila*," 0.54; *M. acetivorans*, 0.32.

tion in each culture was maintained at  $50 \pm 5 \text{ mM}$ , which does not limit the growth of *M. barkeri* and "*M. thermophila*" [14, 20, 22]. Furthermore, acetate was maintained below inhibiting concentrations for *M. barkeri* [14].

The pH auxostat has several advantages compared to previous methods for large-scale cultivation of acetate-grown methanogenic bacteria [11]. Optimal pH and acetate concentration were maintained throughout growth, which resulted in maximum growth rates over a longer time. The molar growth yields from the pH auxostat (Table 1) were equivalent to maximum values reported from batch cultures [14, 15, 20, 22], but the total cell yields were higher. The final dry and wet weight yields of cells (Table 1) were from 3.6- to 17-fold greater than yields reported for *M. barkeri* strain MS [20] and strain 227 [9, 20]. Wet weight yields of 0.21 g/day/liter and 1.0 g/day/liter for *M. barkeri* strains MS and 227, respectively, were obtained in the pH auxostat compared with reported values of 0.05 g/day/liter [6] and 0.04–0.05 g/day/liter [1] for the

same strains in noncontinuously fed batch cultures. Since only a small amount of free energy is available from the conversion of acetate to methane ( $\Delta G^\circ = -31.0 \text{ kJ/mol}$  of  $\text{CH}_4$  produced) [18], a large amount of substrate is required. The pH auxostat provides a constant concentration of acetate, even at high cell density, which results in a high yield of metabolically active cells. Cell extracts of *M. barkeri* strain 227 grown in the pH auxostat contained methyl coenzyme M methylreductase activity threefold greater (unpublished results) than activities reported from extracts of cells grown in noncontinuously fed batch cultures [1]. Alternative substrates, such as  $\text{H}_2\text{-CO}_2$ , methanol, and methylated amines, provide high yields of acetotrophic methanogenic bacteria [1, 5, 19]; however, acetate metabolism by these cells appears to be repressed [2, 10, 14, 20] (S. H. Zinder, personal communication). The pH auxostat is an ideal source of cells for the biochemical study of methanogenesis from acetate because of the simplicity of this culture

method and the high yields of acetate-grown cells provided in a minimum time.

#### ACKNOWLEDGMENTS

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