

H₂-CO₂ Recirculation and pH Control for Growth of Methanogens in Mass Culture

DAVID R. BOONE,* INDRA M. MATHRANI, AND ROBERT A. MAH

Division of Environmental and Occupational Health Sciences, School of Public Health, University of California, Los Angeles, California 90024

Received 6 November 1986/Accepted 27 January 1987

We modified a fermentor (10-liter liquid volume) for the growth of anaerobic, H₂-CO₂-catabolizing bacteria. Gas in the fermentor (ca. 10% CO₂, 50% H₂, 40% CH₄) was recirculated by a diaphragm pump. During growth, the gas composition was maintained by the addition of a mixture of 80% H₂ and 20% CO₂, and this addition was controlled by a pH auxostat. During gas addition, gas was discharged from the recirculating gas stream and was collected by the displacement of an acidified salt solution.

Bryant and co-workers (1) grew mass cultures of methanogenic bacteria by using a one-pass flowthrough of a mixture of H₂ and CO₂ to provide the substrate. The major disadvantage of this technique is that it requires the use of large amounts of gas. If the gas is added too slowly, the consumption of CO₂ by the methanogens lowers the concentration of the gas and causes the medium to become alkaline. If the gas is added more rapidly, most of it passes through the fermentor unused, with only small portions actually consumed for methanogenesis. Furthermore, large amounts of gas may strip sulfide from the solution, and frequent sulfide addition during growth may be necessary (unpublished data). Recirculation of gas was tried (1), but initiation of growth was erratic, and there were problems with water vapor condensation in the lines and with provision of sufficient gas flow.

Sowers et al. (2) showed that during growth of *Methanosarcina* spp., acetic acid addition with a pH auxostat allows growth to a high density. In this paper we describe a system which allows the growth of H₂-oxidizing methanogens to high cell densities.

MATERIALS AND METHODS

Organisms and culture techniques. Methanogenic bacteria were from our culture collection. We used anaerobic techniques and alpha medium (4) with 5 mM sodium acetate, 7:3 N₂-CO₂ gas phase, and 4.0 g of NaHCO₃ per liter (final pH, 7.0). Cultures were maintained and inocula were grown on medium in serum vials or sealed round-bottom flasks. After inoculation, these vessels were pressurized to 170 kPa with H₂, and during growth, vessels were frequently repressurized to 170 kPa with 3:1 H₂-CO₂. Inocula for the 10-liter fermentor were grown in 1 liter of medium in a 2-liter round-bottom flask. All cultures were grown at 37°C with shaking.

Fermentor. A 14-liter Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) was used. Medium (10 liters) was as described above, except that bicarbonate was omitted. Medium was prepared in the fermentor vessel open to the air and was adjusted to pH 7.0. The vessel containing medium was autoclaved for 2 h, and

the gas space was flushed with O₂-free N₂ while the medium cooled. Then 12.8 mM NaOH (to establish a bicarbonate buffer) and 2.5 g of cysteine were added from sterile stock solutions. Stock solutions were prepared in serum vials which were pressurized with O₂-free N₂ after autoclaving. The solutions were added to the fermentor through a septum by using a double-male Luer lock connector with two needles attached; one needle was inserted through the septum into the interior of the fermentor, and the other was inserted into the pressurized vial containing the stock solution. After the medium had cooled, the gas phase of the fermentor was replaced by H₂ and recirculated with a diaphragm pump (Fig. 1). The pump was sealed in an anaerobic jar. A sintered-steel sparger (New Brunswick Scientific) was used to maximize dissolution of gas. pH, monitored by an autoclavable probe, was brought to 7.0 by adding a 4:1 mixture of H₂ and CO₂ to the recirculating gas stream (Fig. 1). A water trap was used to collect condensed water vapor before the gas reentered the fermentor. The gas passed through sterile cotton filters when leaving or entering the fermentor; the filters were heated to prevent water condensation. These filters allowed the recirculation pathway outside the fermentor to be nonsterile. Sulfide was added from a stock solution 1 h before inoculation, and inoculum was added in the same manner as the solutions. During growth, sulfide concentration of the medium was monitored; when it fell below 0.3 mM sulfide solution, sulfide was added to a final concentration of 0.6 mM.

Gas composition in the fermentor. We used a pH auxostat to control pH by monitoring the CO₂ content of the gas. Because of this control we did not need a high buffer (CO₂-HCO₃⁻) concentration in the medium. The recirculating gas contained 10% CO₂, which was in equilibrium with ca. 12.8 mM HCO₃⁻ in the medium. The composition of the influent gas (the mixture in the tank) was 4:1 H₂-CO₂. With this mixture, the circulating gas was ca. 10% CO₂, 40% H₂, and 50% CH₄. As gas was added to the fermentor, gas was also displaced into the gas collector. However, because of the catabolism of the gas, the rate at which the gas left the fermentor (and entered the collector) was lower than the rate at which the gas entered the fermentor system. Assuming that the gas was catabolized so that 4 mol of H₂ and 1 mol of CO₂ yielded 1 mol of CH₄, the total number of moles of gas entering the recirculating gas stream was greater by 4 mol

* Corresponding author.

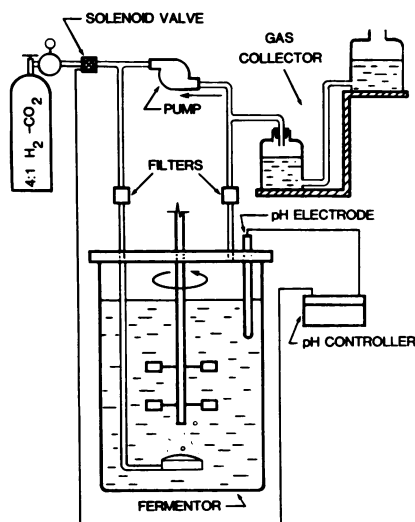


FIG. 1. Schematic diagram of the fermentor and system for gas control and circulation.

than the total (for each mole of CH_4 formed) number of moles leaving the recirculating stream and entering the gas collector, as expressed in the following equation: $G_O = G_I - G_I \cdot (4 \cdot F_R)$, where G_O is the rate of total gas leaving the recirculating stream (moles per hour), G_I is the rate of gas entering the recirculating stream (moles per hour), and F_R is the moles of CO_2 reduced to CH_4 per mole of gas.

The moles of CO_2 exiting the recirculating stream may be described by the following two equations: $\text{CO}_{2 \text{ out}} = F_I \cdot G_I - F_R \cdot G_I$ and $\text{CO}_{2 \text{ out}} = F_O \cdot (G_I - G_I \cdot 4 \cdot F_R)$, where $\text{CO}_{2 \text{ out}}$ is the rate of CO_2 leaving in the effluent (moles per hour), F_I is the fraction of CO_2 in the influent gas, and F_O is the fraction of CO_2 in the effluent gas. The first equation states that the rate of CO_2 leaving the fermentor is equal to the rate of CO_2 entering the fermentor minus the amount ($F_R \cdot G_I$) catabolized. The second equation states that the rate of CO_2 leaving the fermentor is equal to the fraction of CO_2 in the effluent gas multiplied by the exit rate of gas. Solving these simultaneous equations gives this relationship between the influent and effluent CO_2 contents and F_R : $F_R = (F_I - F_O)/(1 - 4F_O)$. For our fermentor, such calculations indicate that 83.3% of the influent gases was used for CH_4 production, and the produced (and recirculating) gas composition was 10% CO_2 , 40% H_2 , and 50% CH_4 .

The fermentor may also be used at different pH values simply by changing the setting of the pH auxostat. However, at higher pH values (above 8.0), the CO_2 content of the recirculating gas may be very low, and it is important that the CO_2 content of the influent gas be at or below 20%; we ordinarily use 17:3 H_2 - CO_2 for growth of alkaliphilic methanogens at high pH. For growth at low pH values, a lower buffer concentration may be used (the CO_2 content in the recirculating gas must be kept lower than the influent gas concentration). Alternatively, an influent gas containing more than 25% CO_2 may be used, and the CO_2 content tends to increase during methanogenesis. Here, added gas with less CO_2 than the recirculating gas can control pH by lowering CO_2 pressure. For instance, an influent gas with 50% CO_2 may be used to control the pH by lowering the CO_2 content of a recirculating gas stream composed of 66.7% CO_2 , 16.7% CH_4 , and 16.7% H_2 .

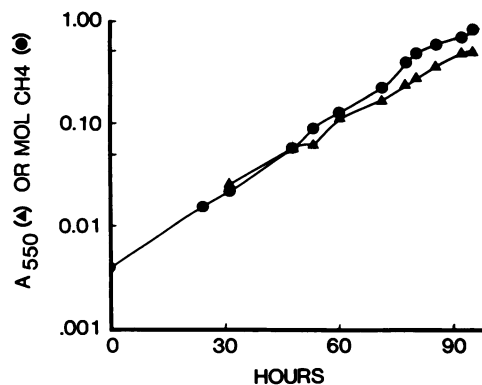


FIG. 2. Growth of *Methanospirillum hungatii* JF-1 in mass culture on H_2 - CO_2 .

Analytical techniques. Amounts of CH_4 , H_2 and CO_2 were determined by gas chromatography and thermal-conductivity detection. Sulfide content was determined by the methylene blue method (3). Optical density (at 600 nm) of 5-ml fermentor samples was determined in serum tubes (16-mm diameter).

RESULTS AND DISCUSSION

Figure 2 shows a growth curve for *Methanospirillum hungatii* JF-1 grown in H_2 - CO_2 in the fermentor. We obtained cell yields of about 3 g (dry weight) per liter of medium, similar to that obtained by Bryant et al. (1). The optical density and methane produced increased logarithmically until 92 h, when the increase in optical density stopped. Methane production continued, but the accumulation was linear rather than logarithmic (data not shown).

The CH_4 production rate of the culture could be monitored in several ways. The total volume of gas produced was proportional to the total volume of methane produced and was easier to monitor. Also, when the gas-metering valve was not adjusted during an experiment, the length of time the solenoid valve was open was proportional to the amount of gas added and thus to the amount of CH_4 produced; the time could be monitored by a clock powered by the output of the pH auxostat.

We have successfully used this auxostat technique to grow several strains of *Methanogenium* and *Methanobacterium* spp. When we grew *Methanogenium* sp., results were erratic at a high agitator speed, and we decreased the speed to about 80 rpm. When we grew *Methanobacterium alcaliphilum* (4) in the fermentor, we decreased the CO_2 content of our influent gas mixture to 15% and increased the pH setting of the pH auxostat to 8.3. During growth, the CO_2 content of the recirculating gas was about 2%.

ACKNOWLEDGMENTS

We thank Martin B. Bloes for excellent technical assistance and for preparing Fig. 1.

This work was supported by Gas Research Institute grants 5080-323-0423 and IFAS-GRI-FIA-MCS 2171.

LITERATURE CITED

1. Bryant, M. P., B. C. McBride, and R. S. Wolfe. 1968. Hydrogen-oxidizing methane bacteria. I. Cultivation and methanogenesis.

- J. Bacteriol. **95**:1118-1123.
2. Sowers, K. R., M. J. Nelson, and J. G. Ferry. 1984. Growth of acetotrophic, methane-producing bacteria in a pH auxostat. *Curr. Microbiol.* **11**:227-230.
 3. Trüper, H. G., and H. G. Schlegel. 1964. Sulfur metabolism in *Thiorhodaceae*. I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **30**:225-238.
 4. Worakit, S., D. R. Boone, R. A. Mah, M.-E. Abdel-Samie, and M. M. El-Halwagi. 1986. *Methanobacterium alcaliphilum* sp. nov., an H₂-utilizing methanogen that grows at high pH values. *Int. J. Syst. Bacteriol.* **36**:380-382.