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# An Anaerobic Chemostat That Permits the Collection and Measurement of Fermentation Gases

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A chemostat was designed to allow anaerobic growth in the culture vessel in the absence of a continuous stream of $O_2$-free gas. Produced gases were collected within the culture and collection vessels, and pressure build-up was prevented by allowing gases to expand into a collapsed football bladder. The culture overflow was collected in a flask, held at 0 C, that was emptied by applying a positive $CO_2$ pressure to the system. *Ruminococcus albus*, a $H_2$ and $CO_2$-producing anaerobe, was used to test the operation of the apparatus. $H_2$ production was measured by sampling the various gas spaces of known volume and measuring $H_2$ concentration by gas chromatography. Measurement of accumulated fermentation gases and the effects of the accumulation on fermentations can be studied with the apparatus.

All continuous culture units have certain features in common: a reservoir of sterile medium; a controllable medium delivery system (usually a pump); a growth vessel with provisions for mixing, aeration, temperature regulation, and volume control; and a culture collection vessel (1). With aerobic organisms, the culture vessel is sparged with air at a rate rapid enough to provide adequate mixing and to assure that oxygen is not a limiting nutrient. In most, if not all, continuous culture studies of anaerobes a stream of $O_2$-free gas is substituted for air, and anaerobiosis is maintained by keeping the culture vessel under positive gas pressure (2, 3, 5). Though this method is effective, it has a number of drawbacks. Measurement of gaseous metabolic products in large quantities of effluent gas is difficult. In addition, sparging removes gaseous metabolic products from the culture and this may cause alterations in metabolic patterns by shifting the equilibria of reactions that produce gaseous products. To study the effects of hydrogen-utilizing anaerobes on the metabolism of hydrogen-producing fermentative anaerobes, we constructed a continuous culture device that does not require gas flow to maintain anaerobiosis and, furthermore, that permits the quantitative estimation of gaseous metabolic products.

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## MATERIALS AND METHODS

A diagram of the continuous culture system is shown in Fig. 1. The system was completely closed to the atmosphere during continuous culture. Except for the period when the collecting flask was emptied, no gas entered or left the system. Anaerobiosis was initially established by gassing ($CO_2$) the sterile growth vessel and collection flask for 12 to 24 h and during the filling of the culture vessel with medium. The gas line to the growth vessel was then pinched off and the collection exit was closed. Anaerobiosis was maintained during continuous culture by keeping the system sealed from the atmosphere. A rubber football bladder was included in the system to serve as an expandable volume to collect gases produced by organisms and displaced by inflowing medium. Small samples of the culture were removed by clamping off the rubber tubing between the overflow arm and the collecting flask and inserting a hypodermic syringe fitted with a 25-gauge needle. The tubing was wetted with isopropanol and flamed both before insertion and after removal of the needle. When it was necessary to empty the collecting flask, the line leading to the bladder was clamped off, and $CO_2$ was blown into

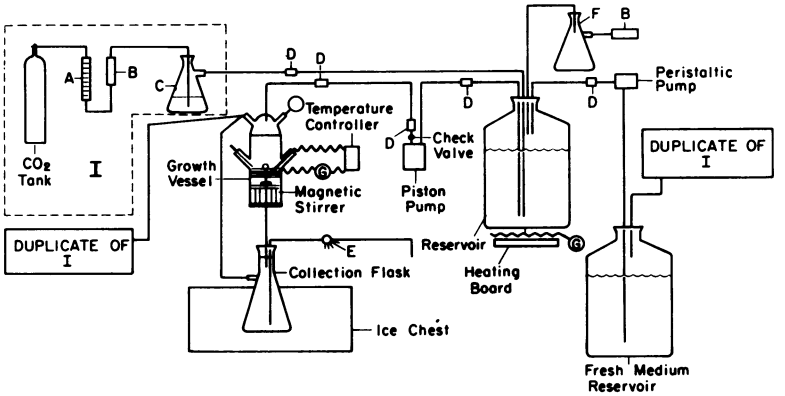


FIG. 1. *Continuous culture system. A, Hot copper column; B, cotton filter; C, gas wetting flask; D, 14/35 standard taper connections; E, 3-way stopcock; F, water vapor trap; G, rheostat; (—), rubber tubing; and (∿), electrical wire.*

the top of the system (above the level of the culture) at 2 to 4 lb/in². The collecting flask exit tube was opened and the collected culture was forced out. When collection was complete, the system was resealed and an evacuated bladder was attached. The terminal part of the culture exit line was sterilized by isopropanol applied through the three-way stopcock. Any residual $CO_2$ pressure was relieved by closing the gas inlet before the gas exit, or through the bladder line before attachment of the evacuated bladder. The bladders were never allowed to fill to the point where expansion of the rubber exerted a back pressure.

The medium in the reservoir was kept anaerobic by continually passing a stream of $O_2$-free $CO_2$ through a cotton filter and a flask of sterile water, and then into the reservoir. Because the medium we used was buffered with bicarbonate, it was necessary to maintain the reservoir at approximately 37 C. The reservoir was heated by a coil of insulated nichrome wire mounted on a wooden board. If this precaution was not observed, bubbles of $CO_2$ formed in the pump and flow was stopped. The reservoir was refilled by pumping in sterile, anaerobic media.

Culture inoculation and other additions were done by removing the cap from a hypodermic needle inserted into one of the growth vessel's side arms (Fig. 2) and attaching a syringe containing the material to be added.

The growth vessel (345-ml culture volume) was constructed for the present investigation (Fig. 2). All other pieces of glassware were standard items. Heavy-walled butyl rubber tubing and glass tubing were used for all connections. All glassware was sterilized by autoclaving. All $CO_2$ used was freed of $O_2$ by passage through a reduced, copper column.

A Milton Roy minipump (Milton Roy Co., St. Petersburg, Fla.) was used to deliver medium to the growth vessel. A stainless-steel one-way check valve with an opening pressure of 1 lb/in² (model 264-100, Nuclear Products Co., Cleveland, Ohio) was installed between the pump and the growth vessel. The pump as well as the tubing from the medium reservoir to the pump and from the pump to the growth vessel were sterilized with glutaraldehyde. A Yellow Springs In-

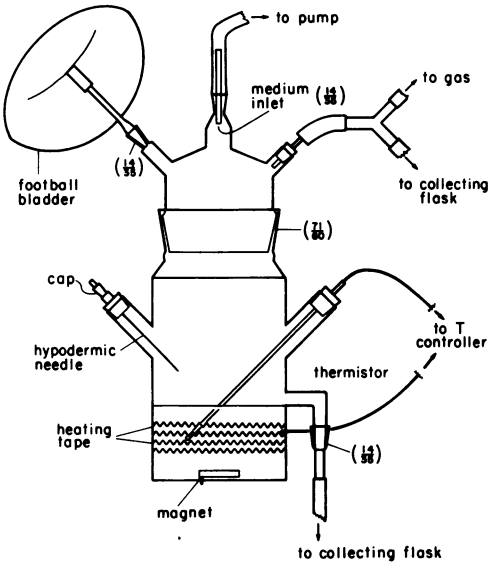


FIG. 2. *Continuous culture growth vessel. The side arm leading from the vessel to the collecting flask was moved 90° to the right of its true position to permit a clearer representation of the apparatus in the figure. Numbers in parentheses are the standard tapers used.*

strument Co. Thermistemp temperature controller, model 63 RC (Cole-Parmer Instrument Co., Chicago, Ill.), was used. The thermistor probe was sterilized with glutaraldehyde, and heat was supplied by a heating tape wrapped around the culture vessel. This arrangement was first observed in the laboratory of P. J. Van Demark at Cornell University.

The culture was mixed with a magnetic stirring bar. A layer of rigid plastic foam insulation was inserted between the bottom of the growth vessel and the magnetic stirrer. Because rapid and efficient mixing is essential for chemostat operation, and Powell (4) has pointed out that the subjective visual impression of violent stirring is not sufficient to guarantee adequate mixing, the efficiency of mixing

was determined by pulsing a steady-state continuous culture with isobutyrate-*1*-$^{14}C$ and by measuring the kinetics of washout of the radioactivity. Exponential washout with a rate constant (−0.12/h) equal in magnitude to the dilution rate (0.12/h) was obtained.

The volume of the culture was fixed by the level of the overflow side arm. The gas circulating line between the collecting flask and the growth vessel (Fig. 1) was essential in keeping the culture volume constant. If the circulating line was not included in the system, gas became trapped in the tubing leading from the growth vessel to the collecting flask, and the overflow line was effectively blocked with a resultant large increase in the volume of the culture.

For estimation of quantities of metabolic gases produced during continuous culture experiments, samples of the gas atmosphere were removed with a sterile hypodermic syringe inserted through rubber tubing. The gas in the rubber bladder was sampled after it was removed from the apparatus. A football needle attached to a small clamped, evacuated rubber tube was inserted. After opening a clamp close to the bladder, the bladder was squeezed several times to equilibrate the gas in the bladder and tube, a sample for gas analysis was withdrawn through the rubber tubing, and then the volume of the bladder was determined by displacement of liquid. The composition of the gas was determined by gas chromatography (6). To verify the gas analysis method, the growth vessel was filled with 4 N HCl, and 25 mmol of zinc metal was added. After $H_2$ evolution was complete, samples were taken at various points in the system. The hydrogen generated by the Zn-HCl reaction did not equilibrate throughout the system. By using appropriate sample points and the volumes of the particular parts of the apparatus, quantitative recovery of $H_2$ was obtained. The suitable sampling points were the bladder, the rubber tubing just below the overflow side arm, and the side arm on the collecting flask. The gas concentration at each point was multiplied by the volume of the gas space sampled (the bladder, growth vessel, and collecting flask), and the total amount of gas was determined by adding these three values. For analysis of cultures, gas was sampled just prior to emptying the collecting flask and changing the bladder. Since the system was flushed with $CO_2$ during the emptying procedure, the measured gases were produced by the culture in the interval between closing and sampling.

## RESULTS AND DISCUSSION

The apparatus was successfully used to cultivate the nonsporeforming, $H_2$-producing anaerobe, *Ruminococcus albus*, and mixed cultures of *R. albus* and a nonsporeforming, $H_2$-using anaerobe, *Vibrio succinogenes*. A limiting glucose medium was used and steady-state growth was achieved over a range of dilution rates of approximately 0.12 to 0.65/h. Resazurin in the medium remained reduced throughout the experiments. It was possible to measure fermentation products including $H_2$ and to study the influence of the $H_2$-using organism on the formation of fermentation products by *R. albus*. The details of these experiments are described in a separate publication (E. L. Iannotti et al., manuscript submitted for publication).

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