A culture apparatus for maintaining $H_2$ at sub-nanomolar concentrations

David L. Valentine*, William S. Reeburgh, Douglas C. Blanton

Department of Earth System Science, University of California at Irvine, 220 Rowland Hall, Irvine, CA 92697-3100, USA

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Abstract

We devised a microbial culture apparatus capable of maintaining sub-nanomolar $H_2$ concentrations. This apparatus provides a method for study of interspecies hydrogen transfer by externally fulfilling the thermodynamic requirement for low $H_2$ concentrations, thereby obviating the need for use of cocultures to study some forms of metabolism. The culture vessel is constructed of glass and operates by sparging a liquid culture with purified gases, thereby removing $H_2$ as it is produced. We used the culture apparatus to decouple a syntrophic association in an ethanol-consuming, methanogenic enrichment culture, allowing ethanol oxidation to dominate methane production. We also used the culture apparatus to grow pure cultures of the ethanol-oxidizing, proton-reducing Pelobacter acetylenicus (WoAcy 1), and to study the bioenergetics of growth.

Keywords: Ethanol oxidation; Hydrogen production; Interspecies hydrogen transfer; Secondary fermentation; Syntrophy

1. Introduction

The syntrophic degradation of organic material is an environmentally and economically important process which occurs during anaerobic digestion (Schink, 1997). Syntrophic degradation, also called secondary fermentation, involves the cooperation of two or more organisms to consume a single substrate; the substrate (organic acids, alcohols, amino acids, and aromatics) is generally a product of primary fermentation. Hydrogen ($H_2$) is thought to be the key intermediary in this process, transferring reducing equivalents from the organisms which degrade the organic substrates to respiring organisms. Due to thermodynamic constraints, the organic substrates can only be consumed in this fashion when the concentration of $H_2$ is low. Respiring organisms utilize the $H_2$, and maintain low concentrations so that the syntrophic oxidation of the organic substrates is sufficiently exergonic.

Many anaerobic microorganisms are capable of acting as syntrophic partners during the degradation of organic material. Many of these organisms are found within the Genus Syntrophomonas, though others include sulfate reducers, species of Pelobacter, benzoate degraders, and others. Many such organisms are available in pure culture as they are also capable of growth on substrates which do not require syntrophic coupling. Previous attempts to grow these organisms on ‘syntrophic’ substrates in the absence of partner organisms have met with only
limited success (Mountfort and Kaspar, 1986; Stams et al., 1993; Schink, 1997).

This study describes the design, construction, and use of a flow-through culture apparatus capable of growing monocultures of ‘syntrophs’ by externally maintaining the thermodynamic requirement for low H₂ concentration. We further describe the growth of both pure and enrichment cultures of H₂-producing ethanol oxidizers.

2. Materials and methods

2.1. Design considerations

The general principle of operation involves continuous stripping of a liquid culture with H₂-free gas. A set of mass flow controllers (MFCs) are used to control flows and mixing ratios of gases, which subsequently flow through a purifier to remove H₂, CO, and O₂. The gas then flows through a stirred glass culture vessel, where biologically produced H₂ is rapidly transferred from the liquid to the gas phase. The resultant H₂-containing gas flows through a series of traps to remove water and hydrogen sulfide. The analytical portion of the apparatus is located downstream and is used to measure the concentrations of gases entering and exiting the culture vessel. The location of the analytical portion of the apparatus allows for passive sampling of gas metabolism from the culture. A schematic diagram of the entire apparatus is shown in Fig. 1.

2.2. Culture vessel

Several precautions must be observed in the design and operation of the culture vessel: (1) dissolution and stripping of gases must be rapid, (2)
H₂ contamination of the vessel must be minimized, (3) strict anaerobic conditions must be maintained, and (4) all experiments must be performed aseptically.

To ensure that gas exchange between phases occurs rapidly, the bottom of the culture vessel contains a glass frit which produces fine bubbles (estimated size 10–100 μm) which give the solution a milky white appearance. The glass frit allows for an even distribution of bubbles, though when the impeller is not used organisms may accumulate near the surface of the frit. The vessel is also equipped with a glass stirrer to mix the liquid medium and maintain uniform conditions. The screw-shaped stirrer was fashioned from a piece of pyrex plate (6" × 1 1/2" × 1/8"); the top and bottom halves are threaded in opposite senses, minimizing vortex formation, shearing, and disruption of cells. The stirrer is driven by a variable speed power head (model RZR-1, Caframo Ltd., Wiarton, Ontario, Canada), and is generally operated between 200 and 600 rpm. The rod of the stirrer is fitted to a bore in the Teflon™ plug, and is lubricated with a small amount of grease (Krytox™, Dupont, Deepwater, NJ). The snug fit of the glass rod through the hole in the Teflon™ plug, coupled with the use of grease, is sufficient to create a seal under slight positive pressures. We have not observed biofilm formation during experiments.

Because metal surfaces are known to produce H₂ in the presence of water, metal has been eliminated completely from portions of the vessel which contact water. Though the vessel consists primarily of glass, minor amounts of Teflon™, PFA (perfluoroalkoxy), and Teflon™-coated rubber are also present. Portions of the vessel constructed using PFA are the Swagelok™ fittings and the tubing leading from the vessel, while the plug located on the top of the vessel is made of Teflon™. The only components made from Teflon™-coated rubber are O-rings. Though Teflon™ and PFA are slightly permeable to H₂ (≈ 10⁻¹⁰ cm²s⁻¹ cm Hg⁻¹), the contact areas are minimized so that outside gases are flushed from the system and are not detectable.

Because trace quantities of O₂ can kill cultures of strict anaerobes, it is important to maintain strict anaerobic conditions within the culture vessel. Six precautions are employed to ensure anaerobicity: (1) the vessel is purged for at least 24 h prior to addition of medium, (2) the vessel is kept under positive pressure to prevent air contamination, (3) a reducing agent, generally cysteine, is added in minor quantities to maintain reduced conditions, (4) minor amounts of resazurin are added to the medium as a visible redox indicator, (5) all gases are of the highest purity available, and (6) gases flow through a heated column which removes traces of O₂ in addition to H₂ and CO (see Section 2.3.). In addition to cysteine, sulfide can also be used as a reducing agent, though sulfide is lost as gaseous hydrogen sulfide at a rate which is pH dependent. Other reducing agents, including thiosulfate, are also compatible with the culture apparatus.

To ensure that no contamination is introduced to the culture vessel the entire vessel is cleaned and autoclaved prior to use (30 min, 121°C), sterile plugs consisting of glass wool are located directly upstream and downstream of the vessel, sterile technique is used in handling any components of the vessel, and sampling ports located on top of the vessel are sterilized before each use.

2.3. Gases

Mass flow controllers (model 8100, Unit Instruments, Yorba Linda, CA) are employed to precisely control the flow-rate and mixing ratios of gases. The MFCs are controlled by a digital power supply (model DX-5, Unit Instruments, Yorba Linda, CA) which is capable of simultaneously controlling several channels. Each tank of gas is connected to an individual MFC, and flow-rates are confirmed by use of a bubble flow meter (The Gilibrator, Gilian Instruments Corp., W. Caldwell, NJ). The following gases have been used with the culture apparatus: (1) UHP N₂ (So-Cal Airgas, Lakewood, CA), (2) Anaerobe Quality CO₂ (Matheson Gas Products), (3) 214 ppm CH₄ in UHP N₂ (So-Cal Airgas), and (4) UHP CH₄ (Scott Specialty Gas).

A Hopcalite™ (Callery Chemical, Pittsburgh, PA) column is used to remove trace levels of H₂ and CO from the gas stream (Haruta and Sano, 1981). Hopcalite™ is a mixture of metal oxides (CuO, MnO₂) which oxidizes H₂ and CO at 100°C, but does not significantly react with methane. The Hopcalite™ column (1/2" × 18" stainless steel) is
located downstream of the MFCs and is maintained at 100°C. Because H₂ and CO are typically found below 1 ppm in the source gases, their oxidation products, H₂O and CO₂, do not significantly influence the bulk composition of the gas flowing through the apparatus. In addition, the low levels of H₂O produced do not form measurable quantities of H₂ when in contact with the aluminum tubing feeding the culture vessel. The Hopcalite™ column adsorbs CO₂ from the gas stream until it becomes saturated and a steady state is achieved. Steady state is generally achieved within 30 min and does not influence experiments as the entire system is generally run at experimental conditions for 24 h prior to inoculation.

2.4. Analytical determinations

After gas flows through the culture vessel it is necessary to remove both water vapor and hydrogen sulfide before the gas contacts metal. Water vapor is removed by a reflux condenser followed by an anhydrous CaSO₄ trap. Hydrogen sulfide is quantitatively removed by flowing the dry gas through a trap consisting of copper sulfate-treated Chromosorb (Johns-Manville, Denver, CO). Metal tubing is used downstream of the final trap.

Hydrogen is quantified with a reducing gas analyzer (RGA-Trace Analytical, Menlo Park, CA) on samples collected downstream from the culture vessel; sampling does not perturb the culture. A digital valve sequence programmer (Valco Instruments Co. Inc., Houston, TX) is used to automate sampling. Samples with H₂ concentrations too high for detection with the RGA (>500 ppm) are collected in syringes, diluted, and measured with the RGA. Methane is detected in a similar fashion by use of a gas chromatograph equipped with a flame ionization detector. The sampling port located at the top of the vessel allows for removal of discrete liquid samples for other analyses.

2.5. Operation

The culture vessel is sterilized, assembled, and purged with H₂-free gas beginning more than 1 day before inoculation. Upstream and downstream H₂ measurements are generally made both before and after addition of medium. The sterilized medium is added to the culture vessel from a 1-liter crimp-top serum bottle. The serum bottle is fitted with a butyl-rubber stopper and is pressurized to about 2 × 10⁵ Pa with inert gas. One end of a sterilized, sharpened canula is threaded through the top port of the culture vessel, then the other end is pierced through the butyl rubber stopper of the inverted serum bottle. The bottle headspace pressure rapidly expels the medium into the culture vessel. Inoculation can be performed into the serum vial before transfer, or directly in the culture vessel through the top port, using a sterilized syringe.

2.6. Growth of ethanol oxidizers

A pure culture of Pelobacter acetylenicus strain WoAcY 1 (Schink, 1985) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ #3246). Cultures were grown to completion in media containing 1.0 g l⁻¹ acetoin (3-hydroxy-2-butanone) in anaerobic culture tubes using a modified Hungate technique (Hungate, 1969). Cultures were then inoculated into the culture apparatus containing a defined mineral salts medium with 20 mM ethanol. All media contained the following ingredients (per liter): 0.2 g KH₂PO₄, 0.25 g NH₄Cl, 1 g NaCl, 0.4 g MgCl₂·6H₂O, 0.5 g KCl, 0.15 g CaCl₂·2H₂O, 1 mg resazurin, 2.5 g NaHCO₃, 0.36 g Na₂S·9H₂O, 5.0 mg sodium EDTA dihydrate, 1.5 mg CoCl₂·6H₂O, 1.0 mg MnCl₂·4H₂O, 1.0 mg FeSO₄·7H₂O, 1.0 mg ZnCl₂, 0.4 mg AlCl₃·6H₂O, 0.3 mg Na₂WO₄·2H₂O, 0.2 mg CuCl₂·2H₂O, 0.2 mg NiSO₄·6H₂O, 0.1 mg H₂SeO₄, 0.01 mg H₃BO₃, and 0.1 mg Na₂MoO₄·2H₂O.

Discrete liquid samples were taken during growth for analysis of acetate, pH, and growth yield. Growth yields were determined in duplicate by harvesting cells at the end of the experiment, centrifuging 35 ml of the culture (4000×g for 1 h), desiccating the pellet and measuring the resulting mass. Acetate was measured with an HPLC using an organic acids column (Alltech, IOA-1000) and a UV/VIS detector set at 210 nm (0.5 mM H₂SO₄ mobile phase set at 0.6 ml min⁻¹).
2.7. Calculation of $\Delta G'$

Free energy yields ($\Delta G'$) were calculated using standard thermodynamic equations. Values for $\text{CH}_3\text{COO}^-$ and pH were interpolated from measured concentrations, while $\text{H}_2$ and temperature were measured for each calculation. Values for ethanol were calculated from initial conditions by subtracting $\text{CH}_3\text{COO}^-$ production; assimilation of ethanol derived carbon into cell mass was not considered, and is not likely to be significant for thermodynamic calculations. Several factors are involved in calculating $\Delta G'$. Temperature is important through the effect of entropy on $\Delta G''$ ($-T\Delta S$) as well as its effect on the deviation from equilibrium ($RT\ln(Q)$). The pH is important through its effect on $\Delta G''$ as well as through its effect on the speciation of $\text{CH}_3\text{COO}^- / \text{CH}_3\text{COOH}$. We assumed that all $\text{CH}_3\text{COOH}$ was in the form of $\text{CH}_3\text{COO}^-$ (pK$_a$ = 4.75). In addition to the values already given, the following thermodynamic values were utilized: $\Delta S-\text{H}_2 = 130.7 \text{ J K}^{-1} \text{ mol}^{-1}$, $\Delta S-\text{CH}_3\text{COO}^- = 86.6 \text{ J K}^{-1} \text{ mol}^{-1}$, $\Delta S-\text{CH}_3\text{CH}_2\text{OH} = 160.7 \text{ J K}^{-1} \text{ mol}^{-1}$, $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$, and $\Delta G''-\text{H}^+ = -5.69 \text{ kJ per pH unit (0 at pH 0)}$. The measured concentrations of $\text{H}_2$, $\text{H}^+$, and $\text{CH}_3\text{COO}^-$ were assumed to be equal to the concentrations apparent to the organism, and all activities were assumed to equal 1.

3. Results

The culture vessel is capable of achieving gas phase $\text{H}_2$ levels below our analytical detection limit ($10^{-3} \text{ Pa}$), corresponding to an equilibrium concentration below 10 picomolar in the liquid phase. Fig. 2 demonstrates the flushing of $\text{H}_2$ from an empty culture vessel which is given an initial pulse of $\text{H}_2$. The residence time calculated from Fig. 2 (15.6 min) closely matches the expected residence time (16 min) based on calculations using flow-rate and total volume. When the vessel contains liquid, the residence time of $\text{H}_2$ is about half as long because the total volume of gas in the system is about half as large. Because $\text{H}_2$ is relatively insoluble and the culture is constantly being sparged, the residence time of $\text{H}_2$ in the liquid phase is very short.

The $\text{H}_2$-stripping culture system has been used to analyze $\text{H}_2$ production from several different cultures including pure cultures of *Methanobacterium* strain Marburg, *Methanoseta thermophila* strain CALS-1, *P. acetylenicus* strain WoAcY 1, and ethanol-oxidizing methanogenic enrichment cultures similar to the classical ‘*Methanobacillus omelianskii*’ (Bryant et al., 1967). Fig. 3 demonstrates the net production of $\text{H}_2$, $\text{CH}_4$ and acetate in an ethanol utilizing methanogenic enrichment culture grown in the culture vessel with a defined mineral salts medium containing 20 mM ethanol. The metabolic activity of the $\text{H}_2$-producing organisms far exceeded the methanogenic activity when $\text{H}_2$ was held low, thereby uncoupling the ‘syntrophic’ association with the methane producers (Fig. 3).

Pure cultures of *P. acetylenicus* were grown in a mineral salts medium containing 20 mM ethanol. The evolution of $\text{H}_2$ was monitored as a function of time in the exhaust gas of the culture apparatus during several experiments. Fig. 4 demonstrates a typical $\text{H}_2$ production profile, while Fig. 5 demonstrates the net production of $\text{H}_2$ (calculated from a production profile) and acetate (which builds up in...
Fig. 3. Metabolic activity of an ethanol-consuming, methanogenic enrichment culture grown in the \( \text{H}_2 \)-stripping culture vessel. The experiment was performed by inoculating 4 ml of an ethanol-consuming, methanogenic enrichment culture into 290 ml of a defined mineral salts medium with 20 mM ethanol. Acetate (△) concentrations in discrete samples removed from the culture vessel were measured with an HPLC using an organic acids column (Alltech, IOA-1000) and a UV/VIS detector set at 210 nm (0.5 mM \( \text{H}_2\text{SO}_4 \), mobile phase set at 0.6 ml min \(^{-1} \)). Hydrogen (○) and \( \text{CH}_4 \) (□) were measured in the exhaust gas. The experiment was performed at 28°C by inoculating 4 ml of an ethanol-consuming, methanogenic enrichment culture into 200 ml of a defined mineral salts medium initially containing 20 mM ethanol. The flow-rate of gas into the fermentor was set at 20 standard cubic centimeters per minute (sccm), 25% \( \text{CO}_2 \) and 75% \( \text{N}_2 \) (\( \text{H}_2 < 10^{-2} \) Pa). The pH of the culture changed from 6.9 to 6.6 over the course of the experiment, corresponding to the production of acetate. The experiment was performed at 28°C by inoculating 4 ml of an ethanol-consuming, methanogenic enrichment culture into 200 ml of a defined mineral salts medium initially containing 20 mM ethanol. The flow-rate of gas into the fermentor was set at 20 standard cubic centimeters per minute (sccm), consisting of 95% UHP \( \text{N}_2 \) and 5% \( \text{CO}_2 \). The inoculum was obtained by serially diluting sediment samples taken from a local marsh into a defined mineral salts medium with 20 mM ethanol. The lowest dilution to produce methane (10\(^{-5}\)) was again serially diluted into the same medium and allowed to grow. After 2 weeks, the lowest dilution to grow (10\(^{-5}\)) was again serially diluted and allowed to grow. This procedure was repeated three additional times until consistent growth was achieved.

The partial pressure of \( \text{H}_2 \) (○) as measured in the exhaust gas for a pure culture of \( \text{P. acetylenicus} \) grown by \( \text{H}_2 \) removal. The experiment was performed at 28°C by inoculating 200 ml of a defined mineral salts medium initially containing \( \text{H}_2 \) with 10 ml of an acetoin grown culture. The flow-rate of gas into the fermentor was set at 20 standard cubic centimeters per minute (sccm), 25% \( \text{CO}_2 \) and 75% \( \text{N}_2 \) (\( \text{H}_2 < 10^{-2} \) Pa). The pH of the culture changed from 6.9 to 6.6 over the course of the experiment, corresponding to the production of acetate.

The experiment was performed at 30°C, with an initial pH of 7.1. The gas flow-rate was 20 sccm, consisting of 95% UHP \( \text{N}_2 \) and 5% \( \text{CO}_2 \). The inoculum was obtained by serially diluting sediment samples taken from a local marsh into a defined mineral salts medium with 20 mM ethanol. The lowest dilution to produce methane (10\(^{-5}\)) was again serially diluted into the same medium and allowed to grow. After 2 weeks, the lowest dilution to grow (10\(^{-5}\)) was again serially diluted and allowed to grow. This procedure was repeated three additional times until consistent growth was achieved.

Growth yields measured for \( \text{P. acetylenicus} \) (WoAcyl1) grown on ethanol are low, 2.2 ± 0.5 g mol \(^{-1}\) acetate-dry weight, corresponding to the low amount of free energy available for the entropically driven oxidation of ethanol. These yields are, however, similar to those estimated in coculture studies with the same organism (Seitz et al., 1990a).

4. Discussion

Anaerobic microorganisms, particularly those involved in terminal degradation of organic material,
are able to grow from very small quantities of energy. It is generally accepted that some anaerobic microorganisms are able to grow on a ‘biological energy quantum’ equivalent to the extrusion of one ion from the cytoplasm (Schink, 1997). Other anaerobes, like *P. acetylenicus*, are thought to conserve energy through substrate level phosphorylation even though the thermodynamic yield for the catabolic process is lower than the ~70 kJ mol\(^{-1}\) required for irreversible synthesis of ATP (Schink, 1997). Our calculations indicate that the amount of energy available to *P. acetylenicus* in these studies ranged from 26 to 33 kJ mol\(^{-1}\), equivalent to the irreversible formation of about one third of an ATP per mol of ethanol oxidized (Fig. 6); such an energy yield is near the absolute minimum for energy metabolism. Similar energetics and growth yields have been estimated in coculture experiments involving *P. acetylenicus* with various H\(_2\)-oxidizing syntrophic partners (Seitz et al., 1990a,b), though never during growth in pure culture. The key ability which allows *P. acetylenicus* to conserve energy presumably lies in its use of a transmembrane ion pump to drive the endergonic production of H\(_2\) from NADH (Hauschild, 1997). Recent estimates indicate that *P. acetylenicus* utilizes 2/3 of ATP production to drive an electrochemical gradient which in turn drives the endergonic production of H\(_2\) (Schink, 1997). The growth yield and free energy yields observed in the present pure culture study lend further support to this hypothesis.

Calculating thermodynamic yields from cultures grown in the apparatus assumes that equilibrium is rapidly achieved between the environment surrounding the cell, and the gas phase. The small size of the bubbles produced by the glass frit and the use of an impeller, help to facilitate rapid gas transfer. During growth, the cultures constantly produce H\(_2\), and therefore maintain an H\(_2\) flux from the cell into the surrounding liquid. Each cell is surrounded by a diffusive boundary layer in which diffusion is the
have a profound influence on the thermodynamics of H₂ production (Conrad and Wetter, 1990). Temperature affects H₂ production through its effect on entropy ($\Delta G^\circ = \Delta H - T\Delta S$), which influences the standard Gibbs free energy ($\Delta G^\circ$), as well as through its influence on the deviation from standard conditions ($\Delta G = \Delta G^\circ + RT \ln(Q)$). Results shown in Fig. 7 demonstrate the tightly coupled relationship between temperature, free energy yield, and H₂ production. The general result for H₂ producing reactions, holding all other factors constant, is that higher temperatures allow for higher H₂ concentrations. The converse is true for lower temperatures. Changes in pH can influence the free energy when there is a net production or consumption of protons during metabolism, as is often the case during syntrophic degradation. For example, acetic acid production caused the pH of the liquid culture shown in Fig. 4 to fall from 6.9 to 6.6 over the course of growth, causing a gradual decrease in headspace H₂. In general, H₂ concentrations will be lower at lower pH for proton liberating reactions. The magnitude of fluctuations in H₂ concentration is dependent on the specific reaction, but can easily change by a factor of four, for a 10 degree-change in temperature or a pH shift of one unit.

The culture apparatus described here shows potential for study of other forms of metabolism besides those already discussed. Suitable substrates may include additional alcohol, substituted aromatics, acetate, glycolate, and amino acids. The culture apparatus also shows potential for enrichment and isolation of other ‘syntrophs’. The advantage of a culture apparatus such as this is that it mimics natural conditions and fulfills the thermodynamic requirement for low H₂; this capability may obviate the need for use of cocultures in studying many forms of H₂ metabolism.

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