Title
A culture apparatus for maintaining H2 at sub-nanomolar concentrations

Journal
Journal of Microbiological Methods, 39(3)

ISSN
0167-7012

Authors
Valentine, David L
Reeburgh, William S
Blanton, Douglas C

Publication Date
2000-02-01

DOI
10.1016/s0167-7012(99)00125-6

ELSEVIER

Journal of Microbiological Methods 39 (2000) 243–251

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

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

Received 12 June 1999; accepted 18 October 1999

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

*Corresponding author. Tel.: +1-949-824-4081; fax: +1-949-824-3256.

E-mail address: dvalenti@uci.edu (D.L. Valentine)

0167-7012/00/$ – see front matter
PII: S0167-7012(99)00125-6

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 H_2 concentration. We further describe the growth of both pure and enrichment cultures of H_2-producing ethanol oxidizers.

2. Materials and methods

2.1. Design considerations

The general principle of operation involves continuous stripping of a liquid culture with H_2-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 H_2, CO, and O_2. The gas then flows through a stirred glass culture vessel, where biologically produced H_2 is rapidly transferred from the liquid to the gas phase. The resultant H_2-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)

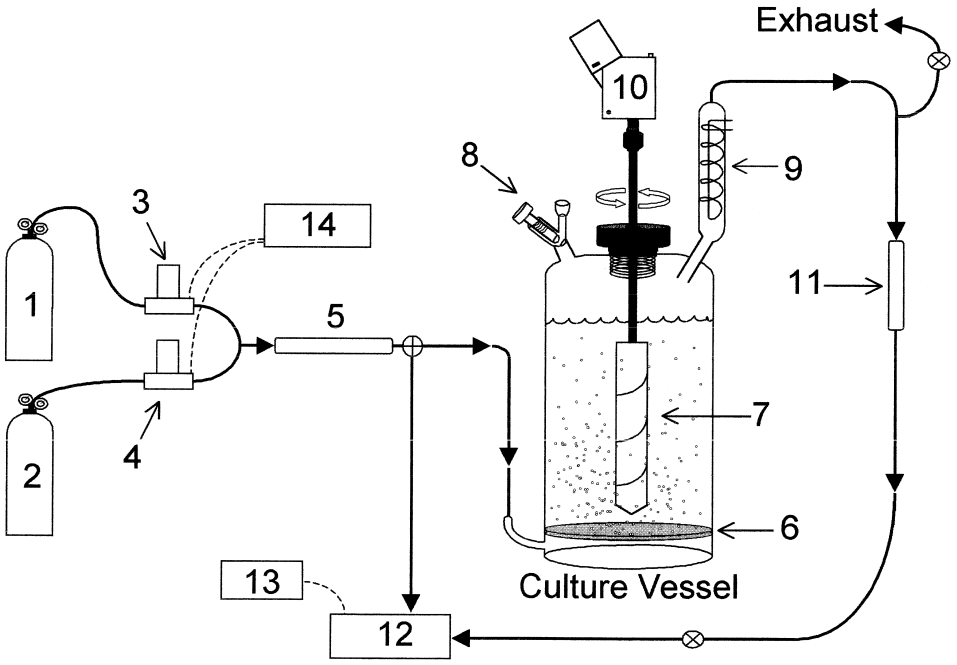

Fig. 1. Schematic diagram of the flow-through culture apparatus with emphasis on the culture vessel. Key to numbering: (1) high-pressure tank of UHP N_2, (2) high-pressure tank of anaerobe quality CO_2, (3) 100 sccm (standard cubic centimeters per minute) MFC, (4) 50 sccm MFC, (5) 18″×1/2″ stainless steel column containing Hopcalite™ catalyst, (6) fritted glass disk, (7) screw-type glass stirrer (see Section 2.2.), (8) sampling port, (9) water-jacketed reflux condenser, (10) Caframo stirrer, (11) water/hydrogen sulfide trap, (12) gas chromatograph equipped with a reducing gas analyzer (H_2) and/or a flame ionization detector (CH_4), (13) digital valve sequence programmer, (14) digital power supply for MFCs. Not pictured: water bath which controls the temperature of the culture vessel, as well as the tube furnace which encloses the Hopcalite™ column. Other symbols: two-way valve (⊗), and three-way valve (⊕).

H_2 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 ($6'' \times 1\,1/2'' \times 1/8''$); 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 H_2 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 H_2 ($\cong 10^{-10}$ $cm^2\,s^{-1}\,cm\ Hg^{-1}$), the contact areas are minimized so that outside gases are flushed from the system and are not detectable.

Because trace quantities of O_2 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 O_2 in addition to H_2 and CO (see Section 2.3.). In addition to cysteine, sufide 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 N_2 (So-Cal Airgas, Lakewood, CA), (2) Anaerobe Quality CO_2 (Matheson Gas Products), (3) 214 ppm CH_4 in UHP N_2 (So-Cal Airgas), and (4) UHP CH_4 (Scott Specialty Gas).

A Hopcalite™ (Callery Chemical, Pittsburgh, PA) column is used to remove trace levels of H_2 and CO from the gas stream (Haruta and Sano, 1981). Hopcalite™ is a mixture of metal oxides (CuO, MnO_2) which oxidizes H_2 and CO at 100°C, but does not significantly react with methane. The Hopcalite™ column ($1/2'' \times 18''$ stainless steel) is

located downstream of the MFCs and is maintained at 100°C. Because H_2 and CO are typically found below 1 ppm in the source gases, their oxidation products, H_2O and CO_2, do not significantly influence the bulk composition of the gas flowing through the apparatus. In addition, the low levels of H_2O produced do not form measurable quantities of H_2 when in contact with the aluminum tubing feeding the culture vessel. The Hopcalite™ column adsorbs CO_2 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 $CaSO_4$ 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 H_2 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 H_2-free gas beginning more than 1 day before inoculation. Upstream and downstream H_2 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 2×10^5 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 KH_2PO_4, 0.25 g NH_4Cl, 1 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.5 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 1 mg resazurin, 2.5 g $NaHCO_3$, 0.36 g $Na_2S \cdot 9H_2O$, 5.0 mg sodium EDTA dihydrate, 1.5 mg $CoCl \cdot 6H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 1.0 mg $FeSO_2 \cdot 7H_2O$, 1.0 mg $ZnCl_2$, 0.4 mg $AlCl_3 \cdot 6H_2O$, 0.3 mg $Na_2WO_4 \cdot 2H_2O$, 0.2 mg $CuCl_2 \cdot 2H_2O$, 0.2 mg $NiSO_4 \cdot 6H_2O$, 0.1 mg H_2SeO_3, 0.1 mg H_3BO_3, and 0.1 mg $Na_2MoO_4 \cdot 2H_2O$.

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 ($4000 \times g$ 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 H_2SO_4 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 CH_3COO^- and pH were interpolated from measured concentrations, while H_2 and temperature were measured for each calculation. Values for ethanol were calculated from initial conditions by subtracting CH_3COO^- 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 $\Delta G^{\circ\prime}$ ($-T\Delta S$) as well as its effect on the deviation from equilibrium ($RT \times \ln\{Q\}$). The pH is important through its effect on $\Delta G^{\circ\prime}$ as well as through its effect on the speciation of CH_3COO^-/CH_3COOH. We assumed that all CH_3COOH was in the form of CH_3COO^- ($pK_a = 4.75$). In addition to the values already given, the following thermodynamic values were utilized: ΔS–$H_2 = 130.7$ J K^{-1} mol^{-1}, ΔS–$CH_3COO^- = 86.6$ J K^{-1} mol^{-1}, ΔS–$CH_3CH_2OH = 160.7$ J K^{-1} mol^{-1}, $R = 8.314$ J K^{-1} mol^{-1}, and $\Delta G^{\circ\prime}$–$H^+ = -5.69$ kJ per pH unit (0 at pH 0). The measured concentrations of H_2, H^+, and CH_3COO^- 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 H_2 levels below our analytical detection limit (10^{-3} Pa), corresponding to an equilibrium concentration below 10 picomolar in the liquid phase. Fig. 2 demonstrates the flushing of H_2 from an empty culture vessel which is given an initial pulse of 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 H_2 is about half as long because the total volume of gas in the system is about half as large. Because H_2 is relatively insoluble and the culture is constantly being sparged, the residence time of H_2 in the liquid phase is very short.

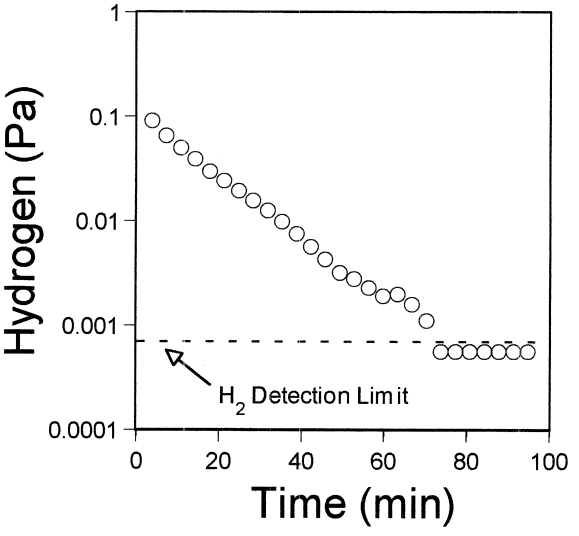

Fig. 2. Decay of an H_2 (○) pulse from an empty culture vessel as a function of time, measured downstream from the culture vessel. Flow rate of gas: 25 sccm, total volume of culture vessel: 400 ml.

The H_2-stripping culture system has been used to analyze H_2 production from several different cultures including pure cultures of *Methanobacterium* strain Marburg, *Methanosaeta 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 H_2, 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 H_2-producing organisms far exceeded the methanogenic activity when 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 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 H_2 production profile, while Fig. 5 demonstrates the net production of H_2 (calculated from a production profile) and acetate (which builds up in

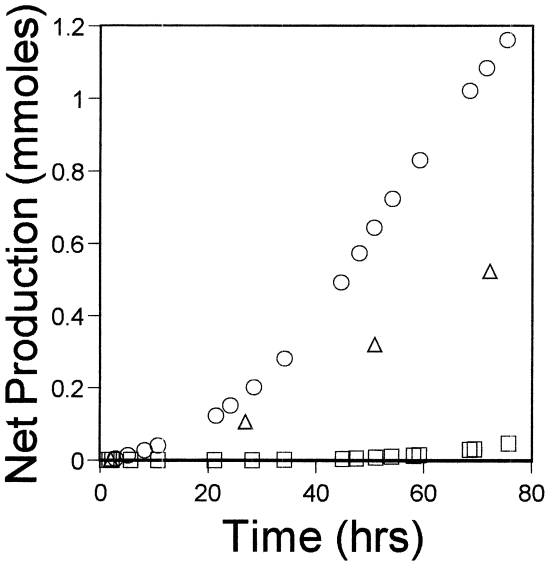

Fig. 3. Metabolic activity of an ethanol-consuming, methanogenic enrichment culture grown in the H_2-stripping culture vessel. The experiment was performed by inoculating 4 ml of an ethanol-consuming, methanogenic enrichment culture into 290 ml of a defined mineral salts medium with 20 mM ethanol. Acetate (△) 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 H_2SO_4 mobile phase set at 0.6 ml min^{-1}). Hydrogen (○) and CH_4 (□) were measured in the exhaust gas. 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 N_2 and 5% 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^{-3}) was again serially diluted and allowed to grow. This procedure was repeated three additional times until consistent growth was achieved.

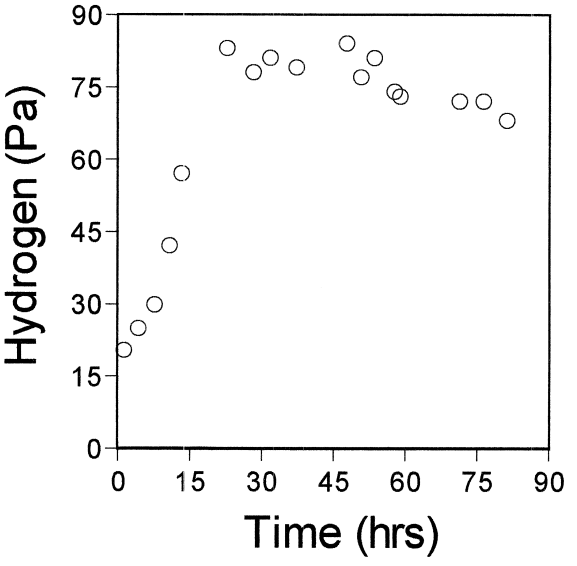

Fig. 4. The partial pressure of H_2 (○) as measured in the exhaust gas for a pure culture of *P. acetylenicus* grown by H_2 removal. The experiment was performed at 28°C by inoculating 200 ml of a defined mineral salts medium initially containing 20 mM ethanol with 10 ml of an acetoin grown culture. The flow-rate of gas into the fermentor was set at 20 standard cubic centimeters per minute (sccm), 25% CO_2 and 75% N_2 ($H_2 < 10^{-2}$ 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 liquid phase) during a separate experiment. The observed reaction stoichiometry shown in Eq. (1):

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad (1)$$

agrees well with the expected stoichiometry. Hydrogen production typically began within minutes of inoculation, and increased for several hours until stabilizing at a critical level corresponding to the minimum thermodynamic yield (Fig. 4). The partial pressure of H_2 in the exhaust gas of the culture vessel typically ranged from 30–85 Pa during this time, compared to 0.001 Pa for uninoculated control experiments and 0.01 Pa for killed controls. Long term changes in the partial pressure of H_2 evolved by the culture apparently act to maintain a consistent free energy yield for the catabolic pathway (Fig. 6), counteracting thermodynamic changes caused by changes in pH, temperature (Fig. 7), and in the relative proportions of ethanol and acetate.

Growth yields measured for *P. acetylenicus* (WoAcy1) 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,

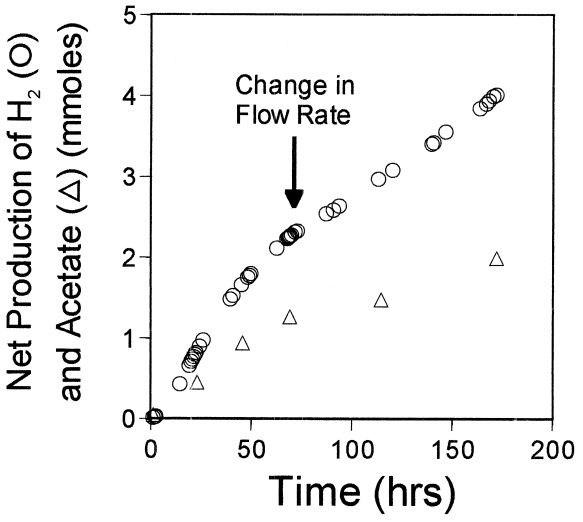

Fig. 5. Net production of H_2 (○) and acetate (△) by pure cultures of *P. acetylenicus*. The experiment was performed by inoculating 250 ml of a defined mineral salts medium (20 mM ethanol) with 5 ml of an acetoin grown culture. Hydrogen production was calculated from the measured mixing ratios of the exhaust gas flowing out of the culture vessel, while acetate was measured in discrete samples removed from the culture vessel. The flow-rate of gas through the fermentor was set at 40 sccm for the first 72 h and then at 20 sccm for the remainder of the experiment. The reaction stoichiometry demonstrates production of 2 mol H_2 per mol of acetate, as expected from ethanol oxidation (Eq. (1)).

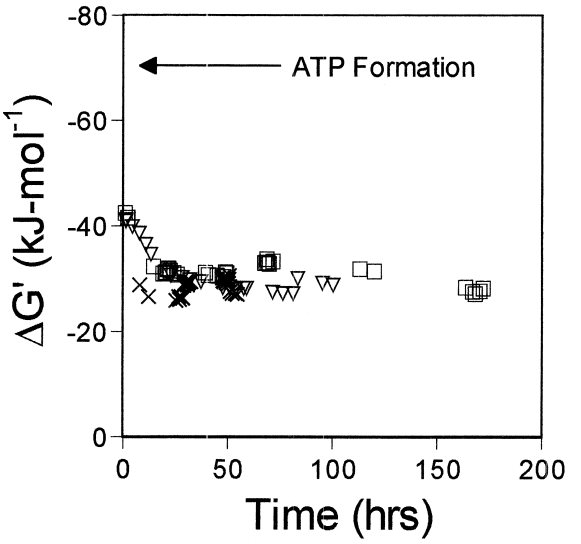

Fig. 6. Calculated free energy yield (per mol of ethanol catabolized) for the growth of *P. acetylenicus*. Each experiment was performed in the flow-through culture vessel using a defined mineral salts medium initially containing 20 mM ethanol. Results for three different experiments are shown (□, ×, ▽). Each experiment encompassed a range of pH values (6.27–7.03), flow-rates (20–40 sccm), and temperatures (297–317 K). The free energy necessary for irreversible synthesis of one ATP (Schink, 1997) is given for reference.

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

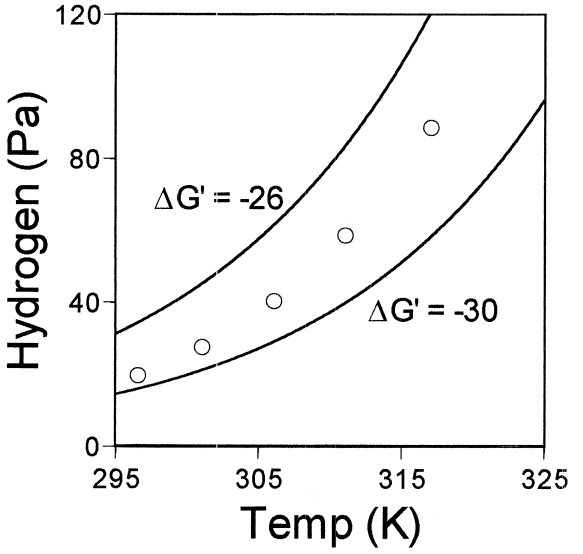

Fig. 7. Influence of temperature on the partial pressure of H_2 in the culture vessel (○) for growth of *P. acetylenicus* on ethanol in a defined mineral salts medium, as measured in the exhaust gas. Lines of constant free energy yield (26 and 30 kJ mol^{-1} ethanol) are given for reference. The trend of increasing H_2 partial pressure (and also production) with temperature is consistent with thermodynamic control by H_2. Other conditions including flow-rate, pH, and concentrations of reactants and products remained nearly constant, though slight deviations occurred and were accounted for in the lines of constant free energy.

dominant mixing process (Fenchel et al., 1998). Each cell experiences a microenvironment of higher localized H_2 concentrations so that use of gas phase H_2 concentrations to calculate thermodynamic yields consistently overestimates the actual energy available to the organism. The net effect is that an H_2 producing organism within the culture vessel is living from less energy than calculations indicate. Such factors may explain the small differences between free energy yields calculated with *P. acetylenicus*, and those calculated in coculture studies (Seitz et al., 1990b).

The free energy driving H_2 production under low H_2 concentrations is largely entropic rather than enthalpic and involves small changes in free energy from reactants and products with large free energies of formation (Thauer et al., 1977). Because of this thermodynamic situation, temperature and pH can have a profound influence on the thermodynamics of H_2 production (Conrad and Wetter, 1990). Temperature affects H_2 production through its effect on entropy ($\Delta G^{\circ\prime} = \Delta H - T\Delta S$), which influences the standard Gibbs free energy ($\Delta G^{\circ\prime}$), as well as through its influence on the deviation from standard conditions ($\Delta G' = \Delta G^{\circ\prime} + RT \ln\{Q\}$). Results shown in Fig. 7 demonstrate the tightly coupled relationship between temperature, free energy yield, and H_2 production. The general result for H_2 producing reactions, holding all other factors constant, is that higher temperatures allow for higher H_2 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 H_2. In general, H_2 concentrations will be lower at lower pH for proton liberating reactions. The magnitude of fluctuations in H_2 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 H_2; this capability may obviate the need for use of cocultures in studying many forms of H_2 metabolism.

Acknowledgements

J. Meyer constructed the culture vessel and devised the anti-vortex stirrer. R. Foulkes assisted in the laboratory. This project was funded by the NSF Life in Extreme Environments (LExEn) special competition (NSF grant MCB 9713967).

References

Bryant, M.P., Wolin, E.A., Wolin, M.J., Wolfe, R.S., 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. Arch. Mikrobiol. 59, 20–31.

Conrad, R., Wetter, B., 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Arch. Microbiol. 155, 94–98.

Fenchel, T., King, G., Blackburn, T.H. (Eds.), 1998. Bacterial Biogeochemistry: the Ecophysiology of Mineral Cycling, 2nd Edition, Academic Press, San Diego.

Haruta, M., Sano, H., 1981. Catalytic combustion of hydrogen. I. Its role in hydrogen utilization system and screening of catalyst materials. Int. J. Hydrogen Energy 6, 601–608.

Hauschild, E., 1997. Biochemie und energetik der syntrophen oxidation von ethanol durch *Pelobacter acetylenicus*. Ph.D. thesis, Universitat Konstanz, Germany.

Hungate, R.E., 1969. A roll tube method for the cultivation of strict anaerobes. In: Norris, J.R., Ribbons, D.W. (Eds.), Methods in Microbiology, Vol. 3B, Academic Press, New York, pp. 117–132.

Mountfort, D., Kaspar, H., 1986. Palladium-mediated hydrogenation of unsaturated hydrocarbons with hydrogen gas released during anaerobic cellulose degradation. Appl. Environ. Microbiol. 52, 744–750.

Schink, B., 1985. Fermentation of acetylene by an obligate anaerobe, *Pelobacter acetylenicus* sp. nov. Arch. Microbiol. 142, 295–301.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. MMBR 61, 262–280.

Seitz, H.J., Schink, B., Pfennig, N., Conrad, R., 1990a. Energetics of syntrophic ethanol oxidation in defined chemostat cocultures. 1. Energy requirement For H_2 production and H_2 oxidation. Arch. Microbiol. 155, 82–88.

Seitz, H.J., Schink, B., Pfennig, N., Conrad, R., 1990b. Energetics of syntrophic ethanol oxidation in defined chemostat cocultures. 2. Energy sharing in biomass production. Arch. Microbiol. 155, 89–93.

Stams, A.J.M., Vandijk, J.B., Dijkema, C., Plugge, C.M., 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl. Environ. Microbiol. 59, 1114–1119.

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Revs. 41, 100–180.