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High-Pressure Equipment for Growing Methanogenic Microorganisms on Gaseous Substrates at High Temperature

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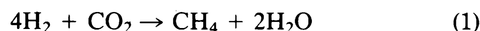
High-pressure, high-temperature investigations on thermophilic microorganisms that grow on hydrogen or other gaseous substrates require instrumentation which provides sufficient substrate for cell proliferation up to 2×10^8 to 3×10^8 cells per ml under isothermal and isobaric conditions. To minimize H_2 leakage and to optimize reproducibility at high pressure and high temperature, 10-ml nickel tubes with a liquid/gas ratio of 1:2 were used in a set of autoclaves connected in series. By applying a hydraulic pump and a 2.5-kW heating device, fast changes in temperature (up to 400°C) and pressure (up to 400 MPa) can be accomplished within less than 10 min. To quantify bacterial growth, determinations of cell numbers per unit volume yielded optimum accuracy. Preliminary experiments with the thermophilic, methanogenic archaebacterium *Methanococcus thermolithotrophicus* showed that bacterial growth depends on both temperature and pressure. At the optimum temperature, increased hydrostatic pressure up to 50 MPa enhanced the growth yield; at a pressure of >75 MPa, cell lysis dominated. Changes in cell proliferation were accompanied by changes in morphology.

The limits of viability in extreme environments of the biosphere are mainly determined by temperature and pressure, which cause metabolic dislocation or inhibit translation, cell division, or other life processes (6–8, 11).

A variety of techniques have been developed to monitor biochemical reactions under pressure (4) and to grow microorganisms at elevated temperature and pressure (20, 21). Upon extending the experiments to thermophilic (or barophilic) archaebacteria growing on gaseous substrates, such as H_2, H_2S, CO_2, etc., a number of technical problems arose which had not been successfully addressed. They refer mainly to the corrosion of the material used to build either the fermentors or the cuvettes required to follow cell proliferation as well as to specific biochemical reactions (18, 19). In the case of methanogenic microorganisms, the problem is complicated by the fact that the metabolic energy of these bacteria is essentially based on the reaction



$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

In connection with high-pressure, high-temperature experiments in aqueous media, this overall reaction implies (i) extreme volume changes, (ii) pH shifts due to H_2CO_3 dissociation, (iii) potential loss of substrate due to the high permeability of H_2, and (iv) reduction in mechanical strength of metal containers due to H_2 solubility in most metals (9, 12).

Some of these implications are biologically relevant in approaching the question of whether high pressure might alter the range of viability for thermophilic methanogenic bacteria. The large negative reaction volume of their metabolic net reaction (equation 1) may favor growth; on the other hand, hydrothermal decomposition of biomolecules may set a physicochemical limit to biochemical reactions in general. The latter problem has been addressed (2). In the present communication, we describe a simple device for growing methanogens on their gaseous substrates at extremes of temperature and pressure.

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MATERIALS AND METHODS

Equipment. A number of high-pressure autoclaves described previously (14) were put in series (Fig. 1) to gather reproducible data under identical pressure and temperature conditions. The temperature control of the autoclaves at ≤400°C was effected by a 2.5-kW heater covered by ceramic insulation and connected to a temperature regulation unit. Thermocouples (Thermocoax, A 4 S 500; Philips, Kassel, Federal Republic of Germany) were mounted within the core of the autoclaves. The temperature within the autoclaves was measured by an additional thermocouple close to the probes. The temperature was kept constant within ±1°C; no temperature gradient beyond this limit was detectable.

Pressure was generated by using a hydraulic pump (type 66 D-40; Dunze, Oberursel, Federal Republic of Germany) and petrol-ether-Diala oil (Shell) (1:1) or ethylene glycol as the pressure-transmitting medium. Bourdon gauges (class 0.1, 400 MPa; Wigand, Klingenberg/Main, Federal Republic of Germany) monitored pressure. The accessible pressure range was 0.1 to 400 MPa with a long-term accuracy of better than 1%.

To minimize H_2 leakage, nickel tubes were used instead of the common silicon or Teflon containers (2, 14). Elastic hydraweld nickel tubes (Witzenmann, Pforzheim, Federal Republic of Germany) with an outer diameter of 12 mm, a wall thickness of 0.15 to 0.20 mm, and a length of 110 mm were creased and hard soldered at one end and closed at the other end by a hard-soldered Ni cylinder with a filling hole (Fig. 2A). To provide tightness at high temperature and pressure, a 60° copper cone was pressed into the filling hole with a 5-mm screw. Tubes with 0.15-mm thickness could be used 10 to 15 times; 0.20-mm tubes could only be used once.

Absence of leaks at 0.5 to 0.6 MPa of H_2 was tested in 500-ml Pyrex flasks with silicon stoppers and screw lids, filled with nitrogen for 24 h at 100°C. H_2 analysis was

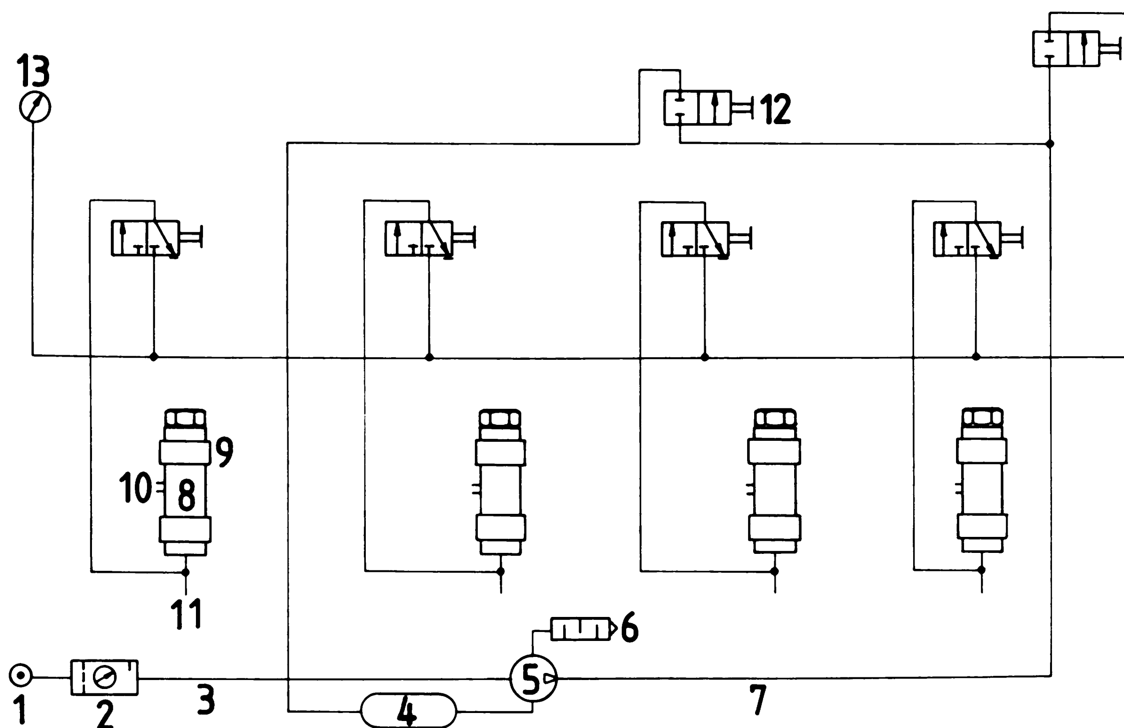

FIG. 1. Schematic representation of the high-pressure apparatus consisting of four autoclaves in series. Each can be loaded or unloaded separately while the others are kept under constant temperature and pressure conditions. Key: 1, compressed air inlet; 2, maintenance unit FRC-1/2-S-B (Festo); 3, compressed air line; 4, reservoir for pressure transmitting medium; 5, membrane pump, type 66D-40 (Dunze); 6, muffler; 7, high-pressure line; 8, pressure vessels; 9, heating element; 10, thermistor for temperature regulation; 11, thermocouple, for temperature control; 12, high-pressure valve; 13, Bourdon gauge (400 MPa).

performed by the method of Fleissner (3). The tightness of the tubes at high pressure and high temperature was tested by using the fluorescence of either Diala oil or dyes added to the pressure-transmitting medium (15).

To guarantee anaerobic exchange of the gaseous substrates, the tubes were mounted in the device depicted in Fig. 2B. After mounting, the tube was opened from the outside; repetitive evacuation and filling with the desired gas mixture allowed a well-defined dosage of the gaseous substrates and subsequent tight closure of the vessel. The standard filling of the tubes was 3 ml of solution (bacterial suspension) plus 7 ml of H_2-CO_2 (4:1 at 0.4 MPa). Bacteria were incubated under sterile conditions in an anaerobe chamber (Coy Laboratory Products, Ann Arbor, Mich.).

Bacterial cultures, substances, and media. Inoculation of a 5% suspension of *Methanococcus thermolithotrophicus* in 20 ml of medium was performed at 65°C in serum flasks containing a gas mixture of 80% H_2 and 20% CO_2-N_2 (1:4) at 0.2 MPa (5). The following culture medium (MG^-) was used: 0.34 g of KCl, 4.30 g of $MgCl_2 \cdot 6H_2O$, 3.46 g of $MgSO_4 \cdot 7H_2O$, 0.26 g of NH_4Cl, 0.14 g of $CaCl_2 \cdot 2H_2O$, 0.14 g of K_2HPO_4, 18.0 g of NaCl, 5.0 g of $NaHCO_3$, 10 ml of mineral salts plus 10 ml of vitamins as described by Balch et al. (1), 1.0 ml of $Fe(NH_4)_2(SO_4)_2$ (0.2%), 1.0 ml of Resazurin (0.1%), 0.50 g of $Na_2S \cdot 9H_2O$, 0.5 g of cysteine hydrochloride, 1.0 g of sodium acetate, and water to 1,000 ml. To minimize pH changes upon pressurization at high temperature, the medium was supplemented with 120 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (G. Bernhardt, Ph.D. thesis, Regensburg University, Regensburg, Federal Republic of Germany, 1986).

Reagents (grade A purity) were purchased from Sigma (Heidelberg, Federal Republic of Germany), Fluka (Basel, Switzerland), and E. Merck AG (Darmstadt, Federal Republic of Germany). Quartz-double-distilled water was used throughout.

RESULTS AND DISCUSSION

Preliminary experiments with instrumentation previously developed for solution studies without gaseous substrates (13, 14) clearly proved that the problem of gas leakage cannot be overcome, especially for H_2. Minimum permeability or solution of H_2 in materials has been reported for gold, iron, and nickel. Corrosion of iron in the presence of sulfides and H_2 may cause embrittlement, eventually resulting in the explosion of autoclaves (1 ppm of hydrogen dissolved in high-strength steel has been shown to be sufficient to cause embrittlement [12]). Nickel may be assumed to be most suitable especially because Ni^{2+} has been shown to be essential in the metabolism of methanogenic bacteria (16, 17). For this reason, and because of the high elasticity of hydraweld nickel sheet metal, nickel tubes were used in the following high-pressure, high-temperature experiments. Since higher concentrations of metal ions generally inhibit bacterial growth, the amount of Ni and Cu after long incubation under conditions of high temperature and pressure (250°C, 26 MPa) compared with controls (20°C, 0.1 MPa) was determined by atomic absorption. Even under hydrothermal conditions, the concentrations of the metal ions are still below the inhibitory concentrations by at least 1 order of magnitude (0.088 ± 0.008 μg of Cu per ml versus 0.017 ± 0.008 μg/ml for the control; 7.80 ± 0.01 μg of Ni per ml versus 0.23 ± 0.01 for the control). Therefore, at 65°C,

perturbations due to Ni^{2+} or Cu^{1+}-Cu^{2+} cannot be effective in the present experiments. Similarly, dissolving the gaseous substrates (and subsequent ionization of H_2CO_3) does not affect the solution parameters significantly, since at the given temperature, the ΔpH/100 MPa in the presence of 120 mM HEPES does not exceed −0.07 (G. Bernhardt, A. Distèche, B. Koch, R. Jaenicke, and H.-D. Lüdemann, manuscript in preparation).

To provide well-defined conditions regarding temperature and pressure as well as the initial time in monitoring growth curves, the shift in growth conditions has to be sufficiently reproducible and fast compared with the growth rate. Altering the temperature and pressure in the range 90 to 250°C and 0.1 to 100 MPa, the heating and cooling period was of the order of 10 and 35 min, respectively; the final pressure can be set within 2 min. Upon heating and cooling, pressure changes may be easily compensated by hand (accuracy of approximately 3%).

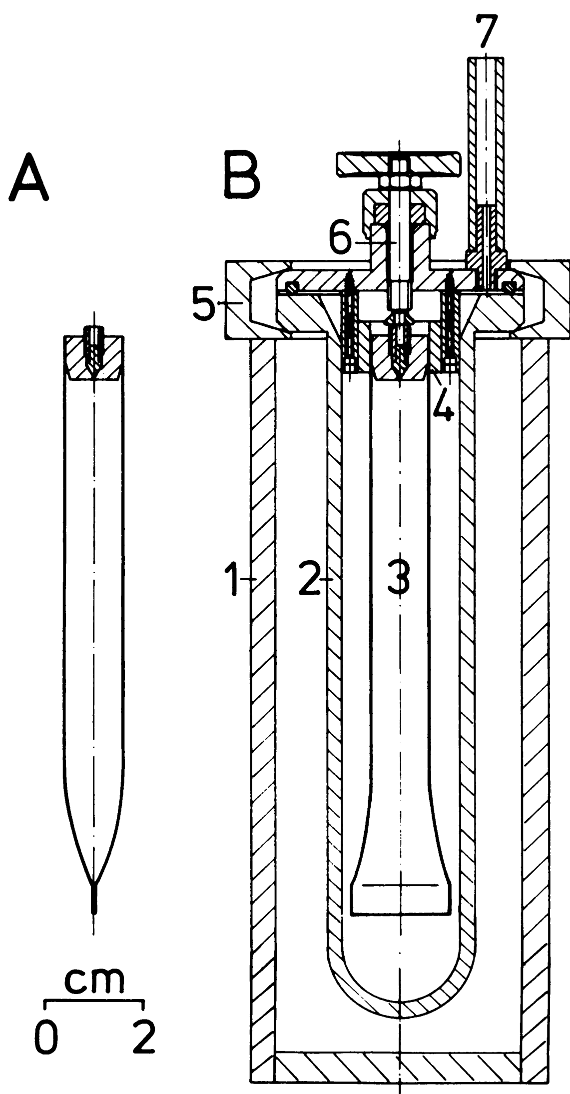

FIG. 2. Flexible nickel tube and filling apparatus. (A) Nickel tube (side view), sealed with a 60° copper cone and a steel screw as the culture vessel for microorganisms requiring gaseous substrates. (B) Device to allow gas exchange in nickel tubes under anaerobic conditions. Key: 1, plexiglass burst shield; 2, flanged glass vessel; 3, nickel tube (front view); 4, clamp for holding the nickel tube; 5, mounting attachment; 6, gas-tight rotating hex key handle; 7, gas inlet.

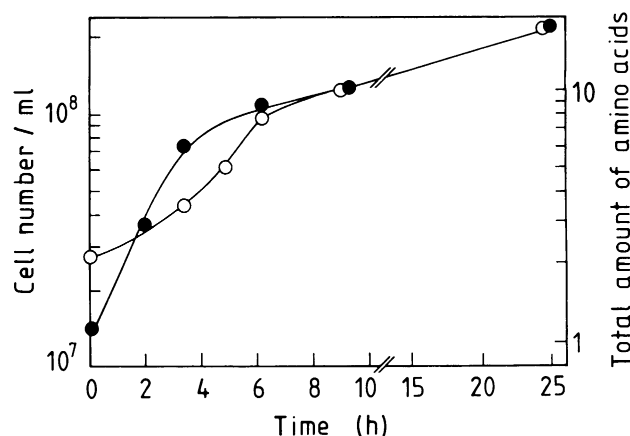

FIG. 3. Quantification of the growth of *M. thermolithotrophicus* SN1 in MG⁻ medium with HEPES buffer (120 mM) at atmospheric pressure. Symbols: (○) cell counting (number of cells per ml) in Neubauer chamber with Zeiss phase-contrast microscope; (●) total amount of amino acids (μg/ml). Cells were spun down by centrifugation; after hydrolysis and derivatization, the amino acid content was monitored by high-performance liquid chromatography.

In quantifying the bacterial growth at high temperature and high pressure, turbidity cannot be applied without ambiguity, because chemical reactions of the components of the medium, as well as physicochemical alterations (e.g., crystallization), rendered the absorbance irreproducible.

Growth could be monitored either by cell counting or total amino acid analysis (after centrifugation of samples and subsequent hydrolysis (Fig. 3). High-performance liquid chromatography analysis after derivatization with *o*-phthaldialdehyde by a modification of a previously described method (10; Bernhardt, Ph.D. thesis) reflected the logarithmic initial phase. Deviations from the result of cell counting in a Neubauer chamber with 0.0025-mm² area and 0.02-mm depth (with a Zeiss phase-contrast microscope) may be explained by the fact that the cellular amino acid pool and alterations of the metabolism due to changed conditions of growth, turnover, or both may shift the ratio of the number of cells and their protein content. This holds especially because high pressure is known to cause lysis as well as heterogeneity of the cell size. For the given reasons, counting the number of bacterial cells was taken to be the most reliable approach, especially because morphological alterations immediately become obvious upon collecting the data.

Preliminary results illustrating the combined effects of high temperature and high pressure on the growth yield of *M. thermolithotrophicus* are given in Fig. 4. First, the present simple instrumentation allowed us to gain insight into the growth properties of thermophilic microorganisms that depend in their metabolism on gaseous substrates, including hydrogen; cell proliferation was found to depend on both temperature and pressure. At the optimum temperature (65°C), increased hydrostatic pressure stimulated growth, which, beyond a limiting pressure (≈50 MPa), was overcompensated by cell lysis. Second, microscopic observation proved that transitions in shape from cocci to elongated large cells and "minicells" accompanied the pressure- and temperature-induced alterations in cell proliferation.

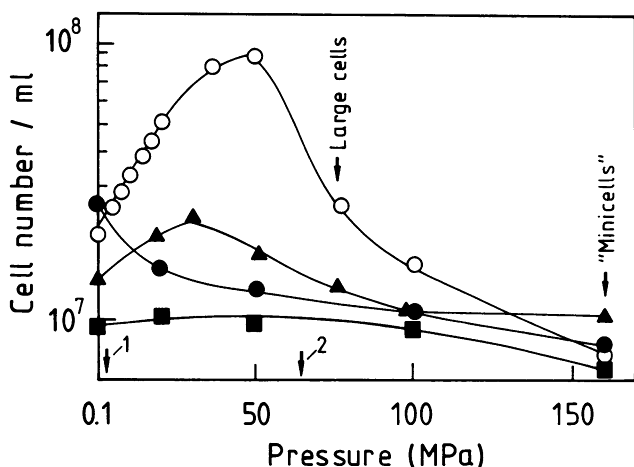

FIG. 4. Effects of hydrostatic pressure and temperature on the growth of *M. thermolithotrophicus* SN1 in MG^- medium with HEPES buffer (120 mM). H_2-CO_2 (4:1) total pressure was 0.4 MPa. Shown are cell numbers after 5.2 h of growth at varied pressure at (●) 56°C, (○) 65°C, (▲) 70°C, and (■) 75°C. "Large cells" and "minicells" refer to the pressure limits where anomalous cell shapes were observed. The lower limits of pressure where the total CO_2 (arrow 1) and H_2 (arrow 2) volumes are dissolved in the medium are indicated.

Since rapid short-time compression and decompression did not induce any morphological change, these alterations must reflect differences in growth behavior. Third, the tabulated solubilities of the gaseous substrates (H_2, CO_2) provide a clear correlation between supply of substrates and growth (cell mass), thus allowing us to separate the effects of the solvent parameters from possible nutritional limitations.

More detailed studies focusing on the limits of viability and the extent to which high hydrostatic pressure might be able to enhance the growth rate of methanogenic bacteria are in progress and will be reported in the near future (G. Bernhardt, H.-D. Lüdemann, K. O. Stetter and R. Jaenicke, manuscript in preparation).

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