APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1989, p. 2086–2088
0099-2240/89/082086-03$02.00/0

Vol. 55, No. 8

NOTES

Cultivation Techniques for Hyperthermophilic Archaebacteria: Continuous Culture of *Pyrococcus furiosus* at Temperatures near 100°C

S. H. BROWN AND R. M. KELLY*

Department of Chemical Engineering, The Johns Hopkins University, Baltimore, Maryland 21218, and Center of Marine Biotechnology, University of Maryland, Baltimore, Maryland 21202

Received 9 February 1989/Accepted 4 May 1989

A system which allows continuous cultivation of hyperthermophilic archaebacteria at temperatures approaching 100°C has been developed. Continuous cultivation of the hyperthermophile *Pyrococcus furiosus* was carried out with this system; the resulting dilution rate and gas production profiles are discussed.

The discovery of bacteria which are hyperthermophilic, i.e., which have optimal growth temperatures at or above 100°C, has generated considerable scientific interest (6, 11). However, the biotechnological potential of these organisms has yet to be realized, in large part because of a lack of understanding of their growth and metabolic characteristics. A key factor in the study of these bacteria will be the development of cultivation strategies which will allow exploration of metabolic behaviors and optimization of growth parameters and at the same time provide sufficient amounts of biomass for further studies. Continuous culture is one mode of operation which meets these requirements, and in fact it has been used previously in research involving more moderate thermophiles (2, 3, 9, 10). In this paper we report preliminary results concerning the design and operation of a system which allows continuous cultivation of hyperthermophilic bacteria at temperatures approaching 100°C.

Pyrococcus furiosus is a hyperthermophilic archaebacterium which grows optimally at 98 to 100°C. This organism was first isolated from shallow geothermal marine sediments by Fiala and Stetter (4). It is an obligately anaerobic heterotroph and grows both in the presence and in the absence of elemental sulfur. When sulfur is present, H_2S and CO_2 are produced as a consequence of growth, along with trace amounts of H_2. In the absence of sulfur, only CO_2 and H_2 are produced, and the H_2 eventually becomes inhibitory to cell growth. The relationship between the production of these gases, particularly H_2S, and the metabolism of *P. furiosus* has not yet been determined; however, a hydrogenase from *P. furiosus* has recently been characterized (2a). Examination of cell extracts has revealed that several proteins are synthesized during growth on elemental sulfur that are different from those produced in sulfur-free medium (6). *P. furiosus* can reach cell densities of over 10^8/ml, which is relatively high for this class of organism, making it an attractive candidate for further research.

P. furiosus DSM 3638 was obtained from the Deutsche Sammlung von Mikroorganismen, Federal Republic of Germany. The organism was grown in artificial seawater supplemented with 0.1% yeast extract and 0.5% tryptone (Difco Laboratories, Detroit, Mich.). The artificial seawater was modified from that described by Kester et al. (7) and was formulated as follows: solution A, 47.8 g of NaCl, 8.0 g of Na_2SO_4, 1.4 g of KCl, 0.4 g of $NaHCO_3$, 0.2 g of KBr, and 0.06 g of H_3BO_3 per liter; solution B, 21.6 g of $MgCl_2 \cdot 6H_2O$, 3.0 g of $CaCl_2 \cdot 2H_2O$, and 0.05 g of $SrCl_2 \cdot 6H_2O$ per liter; solution C, 12.5 g of NH_4Cl, 7.0 g of K_2HPO_4, and 50.0 g of CH_3CO_2Na per liter. Equal volumes of solutions A and B were mixed while being stirred, the yeast extract and tryptone were added, and the resultant solution was sterilized by autoclaving. Solution C was sterilized separately, and after cooling, 20 ml was added aseptically to 980 ml of the mixture described above. Anaerobic conditions were achieved by flushing the medium with prepurified N_2 (Linde Gases, Baltimore, Md.) and adding 0.5 g of Na_2S per liter (after autoclaving). Resazurin (1.0 mg/liter) was used as a redox indicator. The final pH of the medium after sulfide addition was ~6.8.

A schematic of the experimental system is shown in Fig. 1. The culture vessel was a five-neck round-bottom flask (Lab Glass Inc., Vineland, N.J.) with a total volume of 2 liters. A gas inlet tube was used to sparge the vessel, and the gas stream exiting the reactor was passed through a Graham condenser to reduce water losses and then through a gas washing bottle containing 3.0 N NaOH to remove H_2S. Samples for gas analysis were taken through a rubber septum mounted on the condenser outlet. The temperature in the culture vessel was maintained at 98°C by using a heating mantle, proportional temperature controller, and a type J thermocouple (Cole-Parmer Instrument Co., Chicago, Ill.). Although *P. furiosus* grows optimally at 100°C, operation slightly below this optimum prevents boiling while supporting growth rates close to the maximum.

Medium for continuous culture experiments was added aseptically to sterile polycarbonate or polypropylene carboys (Nalge Co., Rochester, N.Y.) and maintained under anaerobic conditions by purging with prepurified N_2 that was filtered through a 0.2-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). This culture medium was added to the reactor by using a Masterflex peristaltic pump (Cole-Parmer)

* Corresponding author.

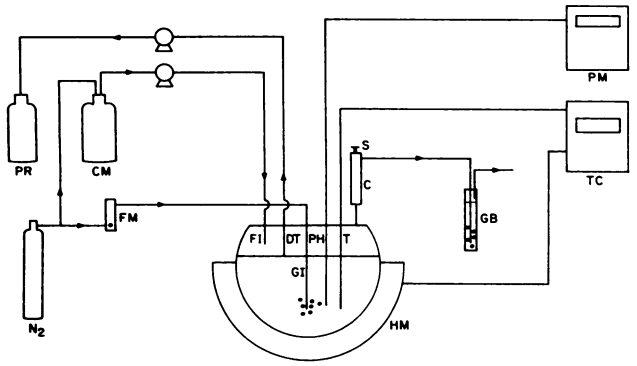

FIG. 1. Schematic of continuous culture system. CM, Culture medium storage; PR, product reservoir; N_2, nitrogen cylinder; FM, flowmeter; FI, feed inlet; DT, dip tube; GI, gas inlet tube; PH, pH probe; T, thermocouple; C, condenser; S, septum for gas sampling; GB, gas washing bottle; HM, heating mantle; TC, temperature controller; and PM, pH monitor.

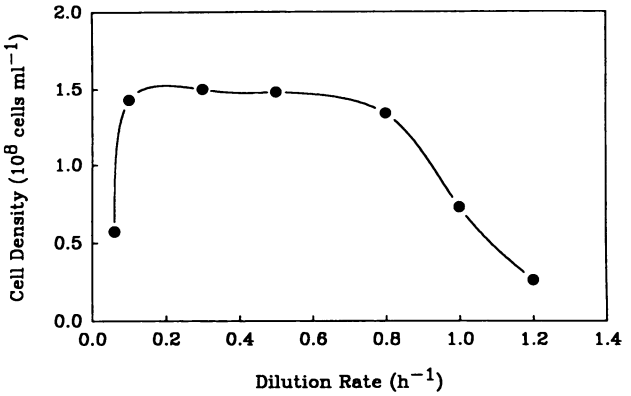

FIG. 2. Effect of dilution rate on cell density of *P. furiosus* grown in continuous culture at 98°C.

with a size 14 pump head. A constant reactor volume was maintained by using a dip tube and a size 16 pump head connected in parallel with the inlet pump. Teflon perfluoroalkoyl tubing (Cole-Parmer) was used between the feed reservoir and the reactor, except for a short section of silicone tubing in the pump head itself. Inlet tubing was autoclaved and aseptically connected to the medium reservoir and the reactor with Luer Lock fittings (Popper & Sons, New Hyde Park, N.Y.). A miniature double-junction pH electrode (Phoenix Electrodes, Houston, Tex.) and a Chemcadet pH controller (Cole-Parmer) were used to monitor pH in the reactor. Teflon thermometer adapters with Viton O-rings (Cole-Parmer) were used to hold the thermocouple, inlet and outlet tubes, and pH probe in the 24/40 joints of the culture flask.

Reactor inocula were grown in sealed 125-ml serum bottles containing 50 ml of the medium described above along with 10 g of elemental sulfur per liter. These bottles were maintained under quiescent conditions at 98°C in a temperature bath (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) modified for high-temperature operation and containing silicone fluid (Dow Corning Corp., Midland, Mich.). Approximately 10 ml of a late-log-phase culture (~8 h old) was used to inoculate the reactor, which contained 750 ml of medium and 10 g of elemental sulfur per liter. The reactor was purged with prepurified N_2 at a rate of 50 ml/min to ensure anaerobic conditions and mix the vessel contents (no additional agitation was supplied). Continuous operation was initiated during late log phase, and the working volume of the reactor was maintained at 750 ml. Feed rate changes were made in the direction of increasing dilution rate, and a minimum of three reactor volume changes were allowed after each adjustment for the system to reach steady state. An additional 2.0 g of sulfur was added to the reactor after every other dilution rate increase to ensure that an excess of sulfur was always present. In general, the sulfur remained in the reactor and little, if any, was carried out in the effluent.

Bacterial growth was monitored by direct cell counts by using epifluorescence microscopy with acridine orange stain (5). The production of H_2S and CO_2 was measured with a gas chromatograph (model 3700; Varian, Sunnyvale, Calif.) with a HayeSep-N column (6 ft × 1/8 in. [ca. 183 × 0.318 cm]; Alltech Associates, Inc., Deerfield, Ill.) and a thermal conductivity detector. The production of H_2 could not be quantified because of interference from the N_2 purge. A standard 286/10 microcomputer (CompuAdd Corp., Houston, Tex.) with a DAS-16 A/D interface (Metrabyte Corp., Taunton, Mass.) was used for data acquisition and peak integration.

The cell density/dilution rate profile for a continuous culture experiment involving *P. furiosus* is shown in Fig. 2. Over this range of dilution rates, the pH in the reactor was between 6.4 and 6.8. If the growth-limiting substrate is an energy source, the decrease in cell density at the lowest dilution rate (0.06/h) could be an indication of a significant maintenance energy requirement. As the dilution rate is increased, the cell density increases, reaching a maximum of about 1.6×10^8 cells per ml. This maximum approaches those typically seen in batch experiments and is maintained up to a dilution rate of about 0.8/h, gradually declining as the dilution rate is increased further. The question of whether this gradual decrease in cell density is due to incomplete mixing in the reactor or is a manifestation of some metabolic change (for example, a shift to a different limiting substrate) has not yet been answered.

The specific production rates of H_2S and CO_2 from this experiment are shown in Fig. 3. These rates are based only on gas phase analysis and as such should be considered minimum values. However, under these conditions (N_2 purge, 98°C), the distribution of H_2S and CO_2 is shifted

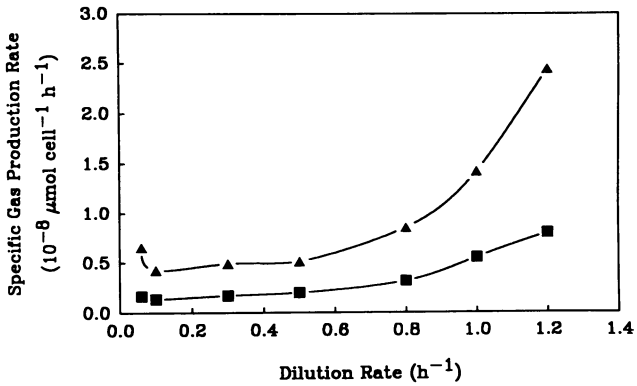

FIG. 3. Effect of dilution rate on the specific production rates of H_2S (▲) and CO_2 (■) by *P. furiosus* at 98°C.

strongly towards the gas phase. Therefore, rates based on gas phase analysis should be a fairly accurate measure of the true gas production rates. No correction has been made for abiotic production of either gas, but results from our laboratory (8; B. Malik et al., Biotechnol. Bioeng., in press) and another (1) have indicated that abiotic production rates under similar conditions are typically several orders of magnitude lower than those reported here. The specific production rates of both gases are seen to increase approximately linearly as the dilution rate is increased from 0.1 to about 0.6/h. Above this range, the relationship between specific gas production rate and dilution rate becomes nonlinear, particularly where H_2S is concerned. This result confirms earlier observations on the relationship between growth rate and gas production for *P. furiosus* grown in batch culture (W.-W. Su, M.S. Thesis, The Johns Hopkins University, 1987). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell extracts taken at various dilution rates did not reveal any profound differences in the protein profiles of cells growing at different rates (data not shown).

Interpretation of the results presented above, especially the gas production data, is contingent on a clearer understanding of the metabolism of *P. furiosus*. In particular, identification of the growth-limiting substrate has not yet been accomplished, although some progress has been made (I. I. Blumentals et al., Ann. N.Y. Acad. Sci., in press). At this point, relatively little information on the growth and metabolism of hyperthermophiles in general has been obtained. Culture systems such as that discussed above will play an important role in answering basic microbiological questions concerning these organisms. Perhaps the most significant result from this early work is that cell densities approaching batch maxima can be achieved at relatively high dilution rates. Considering that these maximal cell densities are low in comparison with those of most mesophiles, it is apparent that the most efficient strategy for generating large amounts of *P. furiosus* biomass for further studies will involve operating relatively small continuous reactors at high volumetric efficiencies.

This work was supported in part through National Science Foundation grant CBT-8813608 and the Office of Naval Research University Research Initiative (N0014-86-K-0696).

LITERATURE CITED

1. **Belkin, S., C. O. Wirsen, and H. W. Jannasch.** 1985. Biological and abiological sulfur reduction at high temperatures. Appl. Environ. Microbiol. **49**:1057–1061.
2. **Brannan, D. K., and D. E. Caldwell.** 1983. Growth kinetics and yield coefficients of the extreme thermophile *Thermothrix thiopara* in continuous culture. Appl. Environ. Microbiol. **45**: 169–173.

2a. **Bryant, F. O., and M. W. W. Adams.** 1989. Characterization of hydrogenase from the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. J. Biol. Chem. **264**:5070–5079.

3. **Cometta, S., B. Sonnleitner, and A. Fiechter.** 1982. The growth behavior of *Thermus aquaticus* in continuous cultivation. Eur. J. Appl. Microbiol. Biotechnol. **15**:69–74.
4. **Fiala, G., and K. O. Stetter.** 1986. *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch. Microbiol. **145**:56–61.
5. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
6. **Kelly, R. M., and J. W. Deming.** 1988. Extremely thermophilic archaebacteria: biological and engineering considerations. Chem. Eng. Prog. **4**:47–62.
7. **Kester, D. R., I. W. Duedall, D. N. Connors, and R. M. Pytkowicz.** 1967. Preparation of artificial seawater. Limnol. Oceanogr. **12**:176–178.
8. **Parameswaran, A. K., C. N. Provan, F. J. Sturm, and R. M. Kelly.** 1987. Sulfur reduction by the extremely thermophilic archaebacterium *Pyrodictium occultum*. Appl. Environ. Microbiol. **53**:1690–1693.
9. **Sonnleitner, B., S. Cometta, and A. Fiechter.** 1982. Growth kinetics of *Thermus thermophilus*. Eur. J. Appl. Microbiol. Biotechnol. **15**:75–82.
10. **Sonnleitner, B., A. Fiechter, and F. Giovannini.** 1984. Growth of *Thermoanaerobium brockii* in batch and continuous culture at supraoptimal temperatures. Appl. Microbiol. Biotechnol. **19**: 326–334.
11. **Wiegel, J., and L. G. Ljungdahl.** 1986. The importance of thermophilic bacteria in biotechnology. Crit. Rev. Microbiol. **3**:39–107.