High-Pressure, High-Temperature Bioreactor for Comparing Effects of Hyperbaric and Hydrostatic Pressure on Bacterial Growth

CHAD M. NELSON, MICHAEL R. SCHUPPENHAUER, AND DOUGLAS S. CLARK*
Department of Chemical Engineering, University of California, Berkeley, California 94720-9989

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We describe a high-pressure reactor system suitable for simultaneous hyperbaric and hydrostatic pressurization of bacterial cultures at elevated temperatures. For the deep-sea thermophile ES4, the growth rate at 500 atm (1 atm = 101.29 kPa) and 95°C under hydrostatic pressure was ca. three times the growth rate under hyperbaric pressure and ca. 46% higher than the growth rate at 35 atm.

Several different methods have been used to study the effects of pressure on the growth and metabolism of bacteria. Pressure has been applied hydrostatically (11, 18, 20) (liquid only), hyperbarically (2, 9, 10, 13) (both liquid and gas phases), and directly to solid medium (19). Hydrostatic pressurization offers the advantages of easy pressure control and safety, but the effectiveness of hydrostatic systems is limited when gaseous substrates and products are involved. Although it is possible to provide gaseous substrates for hydrostatic batch experiments (1), the partial pressure of the substrate (and therefore the liquid-phase concentration) varies with the total pressure until complete dissolution and there is little release of gaseous products from the liquid. However, if either the substrate concentration or the product concentration affects the growth or metabolism, it is desirable to control the concentration of substrates and minimize the concentration of inhibitors.

Hyperbaric systems provide some solutions to these problems, but they are not without difficulties of their own. The choice of pressurizing gas can be very important. Noble gases were shown to inhibit growth of both the fungus Neurospora crassa (5) and HeLa cells (4) in the order Xe > Kr > Ar > Ne and He, and Ar inhibited methane production by Methanococcus jannaschii at a partial pressure of 242 atm (1 atm = 101.29 kPa) (9). Particular care must be exercised when gaseous substrates or products are involved. For example, H₂, which is a substrate of methanogens and a product of Pyrococcus furiosus, severely inhibited the growth of M. jannaschii at a partial pressure of about 248 atm (9) and was inhibitory to P. furiosus when cultured in the absence of elemental sulfur (7).

The majority of evidence indicates that He does not inhibit growth, at least at pressures up to 245 atm (15). In fact, hyperbaric He pressures below ca. 75 atm stimulated the growth of Saccharomyces cerevisiae at 24°C (17), and methanogenesis and growth of M. jannaschii at both 86 and 90°C were accelerated by hyperbaric He pressures up to 750 atm (9). Furthermore, hydrogenase activity in crude extracts of M. jannaschii more than tripled as the He pressure increased from about 7.5 atm to 260 atm (8). High-pressure He also increased the rate of gas production by the deep-sea isolate ES4 in a stainless steel reactor and increased the maximum temperature at which gas was produced (10). In addition, in studies of He in binary inert gas mixtures, He reduced the anesthetic effect of other, more potent, inert gas narcotics (3). On the other hand, He enhanced the sensitivity to O₂ of Escherichia coli and S. cerevisiae (17).

Despite the different techniques available for high-pressure studies of biological functions, comparisons of hydrostatic and hyperbaric pressure effects on microorganisms are somewhat scarce (for a comprehensive review of such studies up to 1982, see reference 3). In cases where pressure inhibits growth, hyperbaric He can be less inhibitory than hydrostatic pressure (6, 14, 17). For example, Taylor found that the growth of the barotolerant marine organism EP-4 under an oxy-helium atmosphere at 500 atm was less inhibited than the growth of cultures incubated under 500 atm of hydrostatic pressure (14). Growth of the bacterium Acholoplasma laidlawii was also less inhibited by high-pressure He than by hydrostatic pressure (6).

The present work describes a bioreactor suitable for precise determination of growth rate as a function of hyperbaric and hydrostatic pressures, simultaneously. The effects of hyperbaric and hydrostatic pressures of up to 500 atm on the growth rate of ES4 were compared at 95, 100, and 105°C. Preliminary results indicate that the growth rate at elevated pressure can depend dramatically on the method of pressurization.

A high-pressure reactor system described elsewhere (10) was modified to allow hydrostatic studies in parallel with hyperbaric experiments (Fig. 1). A second high-pressure vessel (High Pressure Equipment Co., Erie, Pa.) was connected to the gas and liquid lines feeding the hyperbaric system. The temperature and pressure of each vessel can be controlled independently, or the pressure can be equilibrated between the two.

Figure 2 is a close-up view of the second (hydrostatic) high-pressure vessel. The vessel is heated by grounded heating tape (catalog no. 11-463-44; Fisher Scientific, Pittsburgh, Pa.) and wrapped in fiberglass insulation. The temperature is controlled by a CN9121 microprocessor temperature controller (Omega Engineering, Stamford, Conn.). Two type J thermocouples (one internal [TC2005; Thermo- electric, Los Angeles, Calif.] and one external [SA1- fast response TC; Omega Engineering, Stamford, Conn.], at positions indicated in the figure) can be used as the input source for the controller. By using the external thermocouple, the temperature can be controlled to within 1°C. The time lag after a 5°C step change in set-point is less than 10 min. The vessel can be heated from room temperature to 100°C in less than 30 min.

Although He is used as a pressurizing gas, a Teflon piston equipped with Viton O-rings provides a barrier against direct
liquid-gas contact. A ferromagnetic steel ball is included to provide magnetic mixing of the liquid on demand and to mark the location of the piston. The ball can be magnetically lifted to promote mixing prior to sampling, or a stack of ceramic ring magnets can be oscillated up and down by a motor-driven crank and cable if continuous mixing is desired.

The hydrostatic vessel has an internal volume of ca. 58 ml, including the volume of the piston and mixing ball. The routine working volume is 50 ml. The geometry of the system allows subsampling of nearly all the liquid. The reactor consists of an 18-in.-long, 1-in. outer-diameter tube (1/2-in. inside diameter) of 316 stainless steel (High Pressure Equipment Co.) (1 in. = 2.54 cm). The maximum working pressure of the system is 15,000 lb/in². The interior surface was honed and polished to allow free motion of the piston for the entire length, and the interior metal surface was plated with 24-karat gold to reduce corrosion and the likelihood of heavy metals leaching from the steel. Initial attempts to electroplate the gold directly to the steel produced an inferior coating that flaked and peeled with the application of pressure and mechanical stress. The surface was replated with an undercoat of electroless nickel, which enabled better adhesion of the gold and increased the mechanical strength of the coating. The interior of the hyperbaric system was treated in a similar fashion to minimize differences between the two systems. A glass liner can also be used with the hyperbaric system (10), but the liner does not completely eliminate liquid-steel contact. All wetted thermocouples and the steel ball were also gold plated.

To minimize erosion of the gold due to piston motion, sub-sized O rings were used on the piston. The nominal outer diameter of the O rings was 3/8 in., but they were stretched to provide a seal. Since the pressure drop across the piston was not large (less than 1 atm when static, up to 10 atm during sampling), little leakage across the piston was detected. It is also possible to use standard 1/2-in. O rings with deeper-than-standard grooves in the piston to minimize compression of the O ring. This approach has the advantage of longer O ring life but could reduce the lifetime of the piston at high pressure. Vertical alignment of the vessel facilitates removal of trapped gases and containment of sulfur when required. When elemental sulfur is required, it is provided prior to sealing and evacuation of the reactor system.

A pure culture of ES4 was obtained from Robert M. Kelly (North Carolina State University, Raleigh). ES4 is an extremely thermophilic chemoheterotroph isolated from a deep-sea hydrothermal vent environment (12). Artificial seawater medium was prepared as described elsewhere (10). Prior to each series of growth measurements, all reactor surfaces and tubing were sterilized with 70% ethyl alcohol before 30 g of S⁰ (rhombic, unsterilized) per liter was added to each vessel. The piston for the hydrostatic vessel was positioned as close to the top as possible to still allow containment of the sulfur. After the system was sealed, we raised the temperature to 95°C (or 100°C) and purged the system several times with He before adding sterile, reduced medium. Any gas present initially in the hydrostatic reactor was removed through the sample line. The reactor was then left overnight to reach thermal equilibrium. Preincubation with sterile medium scavenges trace amounts of O₂ that might be present and serves as a control prior to inoculation.

We started each growth measurement by aseptically transferring 1.0 ml of a cell suspension containing ca. 1 × 10⁸ cells per ml to 250 ml of preheated medium. The inoculated medium was then placed in an anaerobic glove chamber for subsequent transfer to the reactor system. Fifty milliliters was used to purge liquid inlet lines and the high-pressure liquid compressor. The remaining liquid in the reactor sys-
tem was vented prior to partitioning the new inoculum between the two reactors, with 100 ml going into the larger, hyperbaric vessel and 50 ml going into the hydrostatic vessel. The pressure was then slowly increased to the desired level by adding He. The final 50 ml of medium was incubated at 90°C and ambient pressure as a control.

At pressures greater than 50 atm, liquid samples were removed at regular intervals from both systems and decompressed as described previously (10). The loss in pressure due to sampling was made up by periodically adding He. For each liquid sample, ca. 1 ml was removed to purge the lines and sampling syringe and 2 ml was removed for cell counts. When the total system pressure was less than 50 atm, liquid samples were removed directly without decompression, including 1 ml to purge sample lines. The pressure drop associated with each sample was less than 3%, and the pressure was not increased before a total drop of 10%.

Samples were aseptically transferred through a 21-gauge needle into sealed anaerobic vials that had been autoclaved previously. Cells were fixed in 10% formalin for a minimum of 1 h, stained with 0.5 μg of 4',6-diamidino-2-phenylindole per ml for a minimum of 10 min, filtered onto 0.22-μm-pore-size black polycarbonate filters, and counted with an epifluorescence microscope (Nikon Optiphote). Samples ranged in volume from 0.01 to 1.0 ml. Specific growth rates were based on cell number increase during exponential-phase growth. Growth rates were not reported when less than one doubling in cell number was observed.

ES4 was grown at 95°C and pressures of 35, 250, and 500 atm, pressurized both hyperbarically and hydrostatically. A minimum pressure of 35 atm is necessary to prevent sticking of the piston in the reactor. Results reported here are based on a minimum of two cell doublings during the exponential growth phase and three doublings during the entire growth experiment. Final cell densities were on the order of 10⁶ cells per ml. Stationary phase began 8 to 14 h after inoculation, depending on the growth rate (the stationary phase began earlier for higher growth rates). Specific growth rates were calculated from a minimum of three datum points collected during exponential growth. A minimum of four datum points were taken in each growth experiment. Growth curves measured at 500 atm and 95°C are shown in Fig. 3.

The growth experiments are summarized in Fig. 4. Also presented for comparison are previous growth rates measured in the hyperbaric system before plating with gold (10). Plating the stainless steel with gold eliminated the previously reported inhibition of growth by steel (10). All reported rates at hydrostatic pressure are mean values of at least two replicate runs, with error bars representing the range of measured values. The rates measured in the gold-plated hyperbaric system are within experimental error of the previous rates measured in the glass-lined vessel, and the growth rates obtained at 35 atm are within experimental error of growth rates measured at atmospheric pressure in glass bottles. In addition, the rates measured at 35 and 250 atm in the hydrostatic system were comparable to the rates measured in the hyperbaric system at the same pressure. At 500 atm, however, the growth rate measured in the hydrostatic system was significantly higher than the rates measured under hyperbaric pressure. Moreover, the rate was significantly higher than the rates at 35 and 250 atm, regardless of the pressurization method. When pressurized hydrostatically, increasing the pressure from 250 to 500 atm resulted in a 65% increase in growth rate.

Duplicate experiments were carried out at 105°C at all three pressures. Growth rates were first measured at 100°C, and then new medium was added and the temperature was increased to 105°C. The rates at 500 atm and 100°C are also presented in Fig. 4. Again, the hydrostatic rate is higher than the hyperbaric rate, although they differ by only about 60%. At 105°C, less than one doubling in cell number occurred during 12 h of incubation at 35 atm with either method of
pressurization. Previous experiments in the glass-lined hyperbaric system yielded the same results (10). In experiments at 105°C and at 250 and 500 atm, less than one doubling occurred in the hyperbaric system during 12 h of incubation. In the hydrostatic system, apparent growth was observed once at 250 atm and once at 500 atm (more than three doublings in both cases) but attempts to repeat the measurement resulted in less than one doubling in cell number at each pressure. Furthermore, cell counts for these conditions had standard deviations greater than 50%, and in some cases greater than 100%, due to irregularly shaped cells and debris.

The ability to compare hyperbaric and hydrostatic pressurization is important when one is trying to elucidate the effects of pressure on growth and metabolism of deep-sea bacteria. Although there is no gas phase present in situ, some factors are better mimicked in the laboratory with a hyperbaric system. In the deep ocean, concentrations of substrates and products remain relatively constant in the steady-state conditions of the sea. When volatile compounds (gases) are utilized or produced during growth, the liquid-phase activities will vary greatly in a hydrostatic system, whereas they can be held relatively constant in a hyperbaric system. On the other hand, the concentration of pressurizing gas in the liquid can be very high in a hyperbaric system. In either case, the potential effect of dissolved gases on growth cannot be ignored. Even relatively inert He group gases can affect growth and metabolism (3).

Hyperbaric He and hydrostatic pressures produced very different behavior in the thermophilic archaebacterium ES4. The system described is effective in comparing the effects of hyperbaric and hydrostatic pressure per se, with all other parameters (i.e., medium composition, bacterial strain, temperature, pressure, materials of construction, etc.) held constant. As has been demonstrated previously for other organisms (3, 6, 14, 16, 17), the method of pressurization can be very important in assessing the effects of pressure on the growth of deep-sea thermophiles.

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REFERENCES
15. Taylor, C. D. 1987. Solubility properties of oxygen and helium in hyperbaric systems and the influence of high pressure oxy-

**FIG. 4.** Specific growth rate of ES4 as a function of pressure in the glass-lined hyperbaric vessel, the gold-plated hyperbaric vessel, and the hydrostatic vessel. The first three groups are at 95°C, whereas the fourth group is at 100°C. Previous low-pressure experiments in the hyperbaric system were done at 8 atm.


