HIGH-PRESSURE CONTINUOUS CULTURING OF EXTREMOPHILES: PROTOCOLS. D. I. Foustoukos[1] and J. L. Houghton[2], [1]Earth & Planets Laboratory, Carnegie Institution of Washington, Washington DC 20015, USA (dfoustoukos@ciw.edu), [2]Department of Earth and Planetary Sciences, Washington University, St. Louis, USA (jhouhgton@levee.wustl.edu).

Introduction: Microorganisms adapted to high hydrostatic pressures at deep-sea hydrothermal vents (> 20 MPa, i.e., 200 bars) and within the subsurface of Earth's crust represent a phylogenetically diverse community thriving under extreme pressure, temperature, and nutrient availability conditions. These environments can be considered proxies for habitats that may be found on icy planetary bodies such as Enceladus and Europa [3-6]. The high-pressure adapted organisms ("piezophiles") exhibit a phylogenetic diversity that extends to all three domains of life [1].

We know very little about the function and physiological responses of piezophiles to extreme physical conditions. From the limited high-pressure cultures available, membrane cell and related membrane proteins, such as those involved in the bacterial secretion system, are probably the most pressure-sensitive biological structures [7, 8]. Piezophilic microorganisms have been shown to alter the viscosity of their membrane lipids to maintain the fluidity and permeability so that substrates and waste can quickly diffuse across cellular membranes [9, 10]. Energy production and central metabolism-related genes also appear to be overexpressed under high hydrostatic pressures, along with the upregulation of genes involved in amino acid/carbohydrate synthesis and respiratory functions [11-13]. Given the dearth of high-pressure cultivation experiments, integrating culture-based studies of the physiological and metabolic functions of piezophilic microorganisms with genomics is critical for revealing the microbial adaptation strategies in these extreme environments.

Chemostat Design: The pioneering work of Jannasch et al. [14] and Wirsen and Molyneaux [15] showed the critical attributes of continuous culturing to constrain the physiology functions and adaptation mechanisms of piezophilic organisms not only to fluctuating pressure conditions but also to growth substrate availability. These experimental setups allowed for the delivery of growth medium enriched in dissolved gases and soluble substrates while permitting periodic sampling of the incubated organisms. According to the fundamental concepts of continuous culturing [e.g., 16], adjusting flow rates of growth medium delivery controls microorganisms' growth inside the bioreactor. In this way, cultures can be maintained under a steady-state cell density to collect biomass samples large enough for quantitative molecular analysis.

Essential elements of the design are: i) an agitation mechanism to facilitate the homogenization of the medium solution and cultures, and ii) a backpressure-regulating valve that maintains the system under constant pressure and medium delivery rates. The bioreactor needs to be constructed with chemically inert materials that resist biofouling(e.g., Ti-alloys).

Culturing Protocols: The proposed protocols can be widely applied to high-pressure culturing systems.

Sterilization: For sterilization of high-pressure culturing setups, protocols need to consider the complexity of the systems. For example, autoclaving may only be feasible for the bioreactor itself. On the other hand, chemical sterilization can be applied to the other components (e.g., delivery pumps, pressure lines, sampling valves, backpressure regulator) ([17, 18]. Our proposed protocol is: (i) flushing a 10% (by weight) sodium hypochlorite aqueous solution (2 x volume of the reactor), ii) followed by a 50% (vol/vol) ethanol aqueous solution for at least 6 h (>10 x volume of the reactor), and (iii) rinsing and autoclaving the bioreactor at 150°C (vapor saturation pressure) with autoclaved deionized water to remove traces of sterilizing solutions (>10 x volume of the reactor). Inadequate rinsing with ethanol/H_2O solutions might leave residual sodium hypochlorite that can substantially influence the growth of the cultured community.

Dissolved gases in growth medium: The delivery of volatile-enriched medium (e.g., H_2, CO_2, O_2) into the bioreactor is facilitated by i) gas-tight syringe pumps sampling the pressurized medium solution [17, 18]; ii) HPLC systems [19-21], or iii) direct delivery of growth medium through a piston accumulator [22]. These techniques allow for the delivery of volatile-enriched medium inside the bioreactor at pressures higher than the partial pressure of the gases dissolved in the media reservoir. To avoid degassing of the dissolved volatiles upon delivery, pressure conditions in the bioreactor need to exceed those of the media reservoir. In general, the gas dissolution is dictated by the thermodynamic equilibrium between the gas and liquid phase. For example, to attain dissolved H_2, O_2, and CH_4 concentrations commonly found in hydrothermal environments (e.g., > 10 mM), the medium reservoir needs to sustain pressures higher than 200 psi. In the case of CO_2, gas solubility also depends on pH (Fig. 1).

Inoculation Protocols: The inoculation protocol follows two stages. In the initial stage, an abiotic control experiment is conducted by prefilling the bioreac-

tor with a sterile growth medium and allowing over 24 h of pre-treatment at the pressure and temperature of culturing. Samples collected during this period assess contamination by living or dead environmental microorganisms**.** The second stage involves introducing a highly dense microbial population (preinoculum) in the bioreactor. In general, a volumetric ratio of 30-50 between preinoculum and bioreactor is adopted; however, inoculations may also be commenced at significantly lower ratios [e.g., ratio = 3, 23]. After bioreactor inoculation, the system remains in batch or very low fluid flow rate continuous culture (e.g., <1/2000 bioreactor volume (mL)/min) mode and under the pressure conditions of the growth medium reservoir until the microbial community attains cell densities comparable to those of the initial preinoculum.

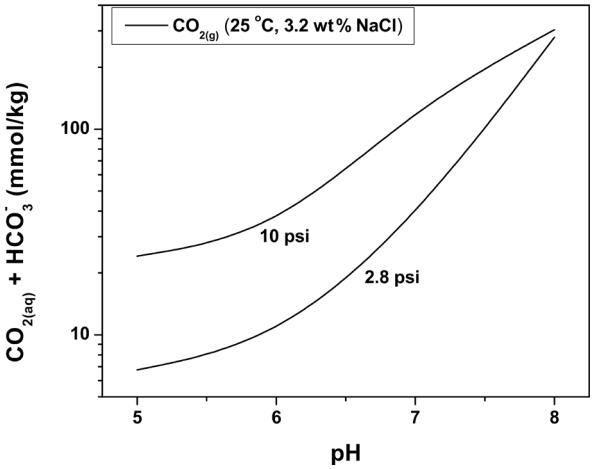

Figure 1. *The concentration of the bioavailable dissolved CO_2 ($CO_{2(aq)}$ + HCO_3^-) as function of $CO_{2(g)}$ partial pressure and pH.*

Mixing Protocols: For the internal mixing of cultures and medium at high-pressure conditions, the homogenization rates are expected to play a key role in the microbial response and adaptation to varying nutrient concentrations. For example, when transitioning between different growth media, the volume of influent required to replenish the media inside the reactor fully should be more than twice the bioreactor's volume, assuming conditions of continuous agitation. These volume constraints, along with the imposed dilution rates, would control the time needed to establish a new steady-state for the microorganisms cultured under constant pressure and temperature conditions. To this end, our established protocol for growth medium transfer requires i) effluent H_2O to be at a higher temperature than the influent medium, and ii) medium to be delivered at relatively modest flow rates (< 2 mL/min) in the absence of agitation to minimize disturbing the thermal and density gradients between the effluent and influent.

Sampling Protocols: Sampling under high-pressure conditions not only may induce decompression effects in the bioreactor but also result in cell lysis because of the large shear forces imposed by the decompression of collected biomass through narrow tubing and high-pressure valve stems. In short, samples are collected by allowing for undisturbed fluid flow through a wide-open high-pressure valve directly attached to the bioreactor. The sampling assembly involves a series of valves and sampling tubes. The assembly is prefilled with autoclaved deionized water to minimize depressurization in the sampling device when opening the valve to the bioreactor. Through a micrometering valve, fluid samples are collected in gas-tight syringes, while the cells trapped inside the medium-filled dead space of the tubing and valves represent intact microbial cells. This protocol minimizes cell lysis effects imposed by the decompression of the high-pressure culture and fluid flow through the stem of the outlet micrometering valve, while maintaining the integrity of chemical analyzes, particularly of dissolved gases.

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