COMPRESSED SOLVENT AND PRESSURE EFFECTS ON METABOLISM AND GROWTH IN WHOLE-CELL BIOPROCESSING

G.D. Bothun[†], B.L. Knutson[†,*], H.J. Strobel[‡], and S.E. Nokes[§]
[†]Department of Chemical and Materials Engineering, University of Kentucky, Lexington, KY 40506-0046
[‡]Department of Animal Sciences, University of Kentucky, Lexington, KY 40546-0215
[§]Department of Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY 40546-0276
[*]Corresponding author: bknutson@engr.uky.edu, (859) 323-1929

Supercritical and compressed fluid technologies have been successfully applied to bioprocessing applications, including enzymatic catalysis, product recovery and purification, and pharmaceutical formulation. These processes offer enhanced mass transfer relative to traditional liquids, tunable solvent strength, and ease of solvent and product recovery through system depressurization. In contrast, there has been limited application of supercritical fluid technology to whole-cell bioprocesses, which do not require expensive isolation steps and are capable of multistep biochemical processes.

We have recently demonstrated metabolic activity of a model anaerobic thermophilic bacterium, *Clostridium thermocellum,* while incubated in the presence of compressed and supercritical alkanes. Thermophilic bacteria are of interest for the production of fuels and chemicals based on their ability to utilize insoluble carbohydrates and their high growth rates. Pressurized investigations in batch systems reveal significant and unexpected effects of solvent choice, solvent phase, and hydrostatic pressure on biocompatibility and metabolic selectivity.

Although metabolic activity has been observed in batch incubations of resting cells in the presence of compressed fluids, the effect of solvent and pressure on growing cells has not been determined. Continuous culture provides a platform for determining metabolic selectivity and growth parameters with changes in operating conditions. This work examines the effect of compressed solvent and pressure on cellular processes using continuous culture experiments. Membrane fluidity is used to infer the interactions between dissolved solvent or product gases and the cellular membrane, which are a likely source of metabolic changes in the microorganism as a function of pressure and solvent. This work suggests that pressure is a significant variable for the tuning of metabolic processes of non-barophilic microorganisms and the *in situ* extraction of bio-based products.

INTRODUCTION

Compressed and supercritical fluid solvents can be employed in whole-cell bioprocessing to recover metabolic products and reduce end-product inhibition in fermentation broths [1-3]. Unlike traditional organic solvents, the solvent strength and selectivity of compressed fluids can be easily tuned with minor changes in temperature and pressure. Mass transfer is also enhanced due to low kinematic viscosities, high diffusivities, and buoyancy driven convection.

Furthermore, depressurization of compressed solvents that are gases at atmospheric conditions leads to a solvent-free product and aqueous processing stream, which is very important for bio-based separations where solvent toxicity and contamination are serious concerns. These properties have prompted interest in the use of compressed and supercritical fluid solvents in bioprocessing [4, 5].

Numerous studies have demonstrated the potential to extract a variety of aqueous post-fermentation products with compressed or supercritical fluids [1, 2, 6-8]. However, *in situ* extraction with supercritical CO_2 has resulted in significant cell inhibition or sterilization [9-13]. The potentially detrimental effect of compressed CO_2 on microorganisms support investigations on the use of compressed light hydrocarbons (i.e. ethane and propane) for *in situ* extraction. For example, phase behavior studies on compressed hydrocarbon/aqueous ethanol systems have shown that comparable distribution coefficients ($m_{ethanol} = y_{ethanol}/x_{ethanol}$) and greater ethanol selectivity ($S_{ethanol} = y_{ethanol}/y_{water}$) may be achieved at lower pressures relative to CO_2 [1, 14-17]. Compressed hydrocarbons may also be more appropriate for bioprocessing applications due to improved biocompatibility and reduced solubility in water relative to CO_2.

Our recent work, which demonstrates the metabolic activity of non-growing *Clostridium thermocellum* in the presence of compressed solvents, suggests the use of compressed hydrocarbons for *in situ* extractive fermentation [18, 19]. The anaerobe *Clostridium thermocellum* was employed as a model thermophilic bacterium for the conversion of cellobiose (a water-soluble cellulose monomer) to the major products ethanol, acetate, lactate and the gaseous products H_2, and CO_2. Although the cells were moderately inhibited relative to incubations at atmospheric pressure, they remained active in the presence of compressed ethane and propane at 70 bar and 333 K. Metabolic activity was negligible for incubations in the presence of compressed CO_2 at the same conditions. Improved ethanol selectivity relative to the products acetate and lactate was also observed in pressurized biphasic cultivation in the presence of ethane and propane [20]. Cell inhibition in the presence of compressed hydrocarbons was partially attributed to phase toxicity.

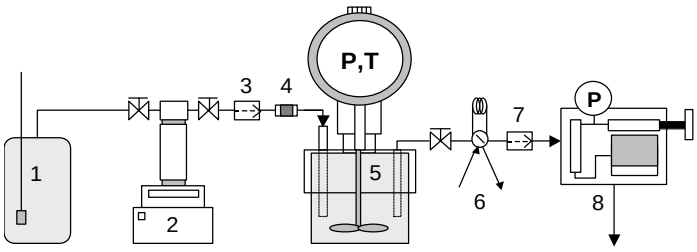
The application of compressed and supercritical fluid technologies in whole-cell bioprocessing requires knowledge of solvent and pressure effects on cellular metabolism and physiology. Hydrostatic pressure (in the absence of a gaseous or compressed solvent headspace) increases the solubility of the fermentation products, H_2 and CO_2. In the presence of these dissolved gases, the metabolic activity of the microorganism can be altered through changes in the thermodynamic driving force (mass action) of the individual reaction pathways and interactions of the gases with the cellular membrane. The presence of compressed solvents in biphasic fermentations (hyperbaric pressure) adds further complexity. Dissolved gas effects (molecular toxicity) and the presence of the fluid-fermentation broth interface (phase toxicity) may also alter metabolic activity. The current inability to describe dissolved gas effects, even in the absence of an extractive solvent, hinders the design of pressurized bioprocessing techniques, such as *in situ* extractive fermentation.

Pressure-effects on bacterial metabolism and growth can be determined through continuous culture experiments. Continuous culture results are more readily incorporated in metabolic models than batch cultures. We have developed a high-pressure bioreactor and investigated the continuous culture of *C. thermocellum* under elevated hydrostatic pressure (7.0 and 17.3 MPa at 333 K). The effect of pressure on product formation and selectivity, substrate

utilization, and growth was quantified. Membrane fluidity was determined using (fluorescent labeled) model dipalmitoyl phophatidylcholine (DPPC) vesicles and *C. thermocellum* in the presence of CO_2, H_2, ethane, and propane as a function of hyperbaric pressure. Pressurized continuous culture experiments and membrane fluidity studies suggest the combined effects of pressure and compressed solvent in whole-cell bioprocessing.

MATERIALS AND METHODS

Pressurized Continuous Culture. All procedures were performed in an anaerobic, sterile environment with basal media. An ISCO syringe pump fed aqueous media into a high-pressure Parr Minireactor (100 ml) and a DBR back-pressure regulator was used to maintain system pressure (Figure 1). The reactor was then inoculated with a cell suspension and pressurized over 30 minutes (7.0 and 17.3 MPa, 333 K). Media was then fed into the system (2 g/L cellobiose) at the desired dilution rate (D = volumetric flowrate/reactor volume, 0.05 to 0.3 h^{-1}). In steady-state continuous culture, the dilution rate (D) is equal to the growth rate of the cells (D = μ), and the rate of product formation and substrate consumption are constant. The reactor was then sampled through a sampling loop after > 98% cell turnover was achieved. Samples were analyzed for ethanol, acetate, lactate, glucose, cellobiose, and biomass concentrations.



1. Media resevior (feed, CO_2 sparge)
2. Syringe pump (feed)
3. Unidirectional flow valve (feed)
4. Filter frit (0.5 μm)
5. Continuous culture apparatus (100 mL)
6. Sample collection (1 mL, effluent)
7. Unidirection flow valve
8. Back-pressure regulator

Figure 1. High-pressure continuous culture apparatus.

Membrane Fluidity Measurements. Membrane fluidity was measured using a Varian Cary Eclipse Fluorescence Spectrophotometer with a custom designed high-pressure variable volume view cell fitted with optical windows. DPPC vesicles and *C. thermocellum* (isolated, washed, and resuspended in a saline solution) were labeled with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes), which is embedded within the hydrophobic region of the phospholipid bilayer [21]. The anisotropy of DPH describes the viscosity of the microenvironment surrounding the probe.

Before the aqueous vesicle/cell suspensions were loaded into the view cell it was first purged with the fluid of interest. The fluid (CO_2, H_2, ethane, or propane) was then introduced through an ISCO and the system was pressurized (0.8 to 20.7 MPa). Sufficient compressed fluid was added to maintain a two-phase (compressed fluid-aqueous) system. Anisotropy was measured by relating the excitation (λ_{ex} = 340 nm) and emission (λ_{em} = 452 nm) intensities of vertical and horizontal polarized light. The fluidity of DPPC vesicles was measured at 295 and 333 K, below (gel phase) and above (fluid phase) the melting temperature (T_m = 314 K).

RESULTS AND DISCUSSION

Effect of Hydrostatic Pressure on Continuous Culture. Cultivations performed under elevated hydrostatic pressure are compared to cultivation at atmospheric pressure. Cell growth is inhibited by 40 and 60% at 7.0 and 17.3 MPa, relative to atmospheric pressure incubation. While substrate consumption is not significantly inhibited at pressure, there is a drastic shift in product selectivity. Figure 2 depicts the effect of hydrostatic pressure and dilution rate on the ratio of ethanol to organic acid (acetate + lactate) production (E/O).

Elevated hydrostatic pressure decreased the maximum theoretical growth rate, and the overall growth yield with respect to cellobiose consumption and ATP formation. In addition, the amount of energy required to maintain cellular function increased ~100%. The change in metabolism of *C. thermocellum* under pressure are likely due to the increased solubility of gaseous products (CO_2 and H_2) and changes in membrane fluidity due to both pressure and the dissolution of gases.

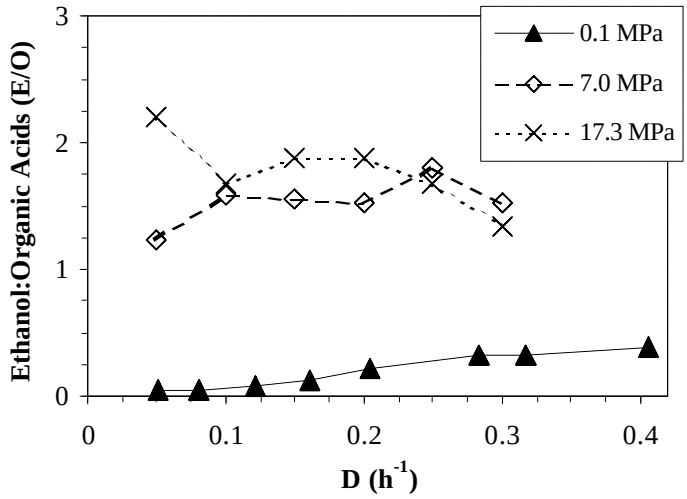


Figure 2. Ratio of ethanol to organic acid production as a function of pressure and dilution rate.

Effect of Compressed Fluids on Membrane Fluidity. Results of membrane fluidity measurements are expressed in terms of the normalized DPH anisotropy, which is the ratio of the anisotropy in the presence of a compressed fluid to the anisotropy at the corresponding hydrostatic pressure ($\langle r \rangle_{norm,\ P} = \langle r \rangle_{compressed\ solvent,\ P} / \langle r \rangle_{hydrostatic\ pressure,\ P}$). A value of $\langle r \rangle_{norm} = 1$ indicates that the compressed fluid had no effect on membrane fluidity. In the presence of compressed fluids, membrane fluidity is a function of the aqueous solubility of the gas, the gas partitioning between the aqueous phase and the membrane, and the physical properties of the gas upon compression. CO_2, H_2, ethane, and propane were chosen because they represent either a gaseous product or

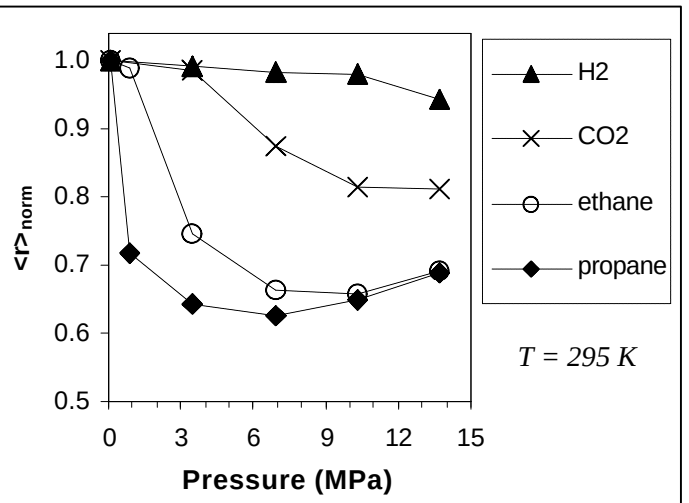


Figure 3. Normalized anisotropy of DPPC vesicle-bound DPH in the presence of compressed fluids.

a potential compressed extractive solvent. Preliminary results indicate that hydrostatic pressure and the presence of compressed CO_2, H_2, ethane, and propane all influence the fluidity of small unilamellar DPPC vesicles in both the gel and fluid phase (Figure 3).

In general, we observed that the membrane fluidity of DPPC vesicles in the presence of the compressed fluids decreases with increasing pressure. The membrane fluidity of DPPC vesicles decreased linearly with CO_2 and H_2 density over the conditions investigated. Similarly, *C. thermocellum* exhibited a decrease in fluidity with increasing CO_2 density (30% decrease at 13.7 MPa). CO_2 could affect the membrane fluidity through both dissolution in the membrane bilayer as well as the reduction of pH. Compressed propane and ethane had a large affect on the membrane fluidity of both model DPPC membrane vesicles and suspended *C. thermocellum* likely due to their preferential partitioning into the phospholipid bilayer. The minima associated with membrane fluidity in the presence of ethane and propane as a function of pressure indicates a reversal of this anaesthetic effect. Changes in membrane fluidity in the presence of compressed CO_2, H_2, ethane, and propane demonstrate how the accumulation of product gases and compressed solvents within the media may affect membrane integrity and function.

CONCLUSION

In order to capitalize on the benefits of supercritical and compressed fluid technologies for whole cell bio-based production, the effect of dissolved product gases and dissolved compressed solvents on metabolism must be quantified. We have developed techniques to describe both the biochemical and physiological effect of pressurized on whole cell metabolism. These techniques provide complementary information on the underlying source of dissolved gas effects on metabolism, which should aid in the design of compressed fluid based technologies for the recovery of fermentation products.

REFERENCES

[1] Del Re, G., Di Giacomo, G., Brandani, V., Martinez de la Ossa, E. *Liquid and supercritical fluid extraction of fermentation broths*, in *ISEC*, **1996**. Melbourne, Australia.

[2] van Eijs, A.M.M., Worke, J.M.P., Ten Brink, B. *Preconc. Drying of Food Mat.*, **1988**.

[3] Thibault, J., LeDuy, A., Cote, F. *Biotechnol. Bioeng.*, 30, **1987,** 74.

[4] Jarzebski, A.B., Malinowski, J.J. *Process Biochem.*, 30(4), **1995,** 343.

[5] Randolph, T.W. *TIBTECH*, 8, **1990,** 78.

[6] Budich, M., Brunner, G. *J. Supercritical Fluids*, 25, **2003,** 45.

[7] de Filippi, R.P., Moses, J.M. *Biotechnol. Bioeng. Symp.*, 12, **1982,** 205.

[8] Knez, Z., Posel, F., Krmelj, I., *High pressure extraction of organics from water*, in *Supercritical processing of foods and biomaterials*. **1991**, Blackie and Academic Professional: New York, NY. p. 181.

[9] Jones, R.P., Greenfield, P.F. *Enz. Microb. Technol.*, 4, **1982,** 210.

[10] Haas, G.J., Prescott, H.E., Dudley, E., Dik, R., Hintlian, C., Keane, L. *J. Food Safety*, 9, **1989,** 253.

[11] Isenschmid, A., Marison, I.W., von Stockar, U., *Effect of a near-critical and supercritical fluidn on the viability ratio of microbial cells*, in *Biocatalysis in Non-Conventional Media*, J. Tamper, et al., Editor. **1992**, Elsevier Science. p. 407-415.
[12] Dillow, A.K., Dehghani, F., Hrkach, J.S., Foster, N.R., Langer, R. *Proc. Nat. Acad. Sci.*, 96, **1999,** 10344.
[13] Spilimbergo, S., Elvassore, N., Bertucco, A. *J. Supercritical Fluids*, 22, **2002,** 55.
[14] Brignole, E.A., Skjold-Jorgensen, S., Fredenslund, Aa. *Ber. Bunsenges. Phys. Chem.*, 88, **1984,** 801.
[15] McHugh, M.A., Mallett, M.W., Kohn, J.P., *High pressure fluid phase equilibria of alcohol-water-supercritical solvent mixtures*, in *Chemical engineering at supercritical fluid conditions*, M.E. Paulaitis, Penninger, J.M.L., Gray, R.D., Davidson, P. (Eds.), Editor. **1986**, Ann Arbor Science: Ann Arbor, MI. p. 113.
[16] Horizoe, H., Tanimoto, T., Yamamoto, I., Kano, Y. *Fluid Phase Equilib*., 84, **1993,** 297.
[17] Zabaloy, M.S., Mabe, G.D.B., Bottini, S.B., Brignole E.A. *Fluid Phase Equilib*., 83, **1993,** 159.
[18] Knutson, B.L., Strobel, H.J., Nokes, S.E., Dawson, K.A., Berberich, J.A., Jones, C.R. *J. Supercritical Fluids*, 16, **1999,** 149.
[19] Berberich, J.A., Knutson, B.L., Strobel, H.J., Tarhan, S., Nokes, S.E., Dawson, K.A. *Biotechnol. Bioeng.*, 70(5), **2000,** 491.
[20] Berberich, J.A., Knutson, B.L., Strobel, H.J., Tarhan, S., Nokes, S.E., Dawson, K.A. *Ind. Eng. Chem. Res*., 39(12), **2000,** 4500.
[21] Kaiser, R.D., London, E. *Biochemistry*, 37, **1998**, 8180.