PRESSURIZED SOLVENTS IN WHOLE-CELL BIOPROCESSING: METABOLIC AND STRUCTURAL PERTURBATIONS

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PRESSURIZED SOLVENTS IN WHOLE-CELL BIOPROCESSING:
METABOLIC AND STRUCTURAL PERTURBATIONS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Engineering at the University of Kentucky

By
Geoffrey D. Bothun
Lexington, Kentucky

Director: Dr. Barbara L. Knutson, Associate Professor of Chemical Engineering
Lexington, Kentucky
2004
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ABSTRACT OF DISSERTATION

PRESSURIZED SOLVENTS IN WHOLE-CELL BIOPROCESSING: METABOLIC AND STRUCTURAL PERTURBATIONS

Compressed and supercritical fluids, such as pressurized CO₂, ethane, or propane, provide a versatile and environmentally acceptable alternative to conventional liquid organic solvents in bioprocessing applications – specifically in the areas of product extraction, protein purification, microbial sterilization, and enzymatic and whole-cell biocatalysis. While their advantages have been well demonstrated, the effects of compressed and supercritical fluids on whole cells are largely unknown.

Metabolic and structural perturbations of whole cells by compressed and supercritical fluid solvents were examined. These perturbations exist as cell metabolism and membrane structure are influenced by pressure and the presence of a solvent phase. Continuous cultures of Clostridium thermocellum (a model ethanol-producing thermophilic bacterium) were conducted under elevated hydrostatic and hyperbaric pressure to elucidate pressure- and solvent-effects on metabolism and growth. Fluorescence anisotropy was employed to study liposome fluidization due to the presence of compressed and supercritical fluids and their partitioning/accumulation in the phospholipid bilayer.
Under elevated hydrostatic pressure (7.0 and 13.9 MPa; 333 K), significant changes in product selectivity (towards ethanol) and growth were observed in \textit{C. thermocellum} in conjunction with reduced maximum theoretical growth yields and increased maintenance requirements. Similarly, metabolism and growth were greatly influenced under hyperbaric pressure (1.8 and 7.0 MPa \textit{N}_2, ethane, and propane; 333 K); however, severe inhibition was observed in the presence of supercritical ethane and liquid propane. These changes were attributed to mass-action effects on metabolic pathways, alterations in membrane fluidity, and the dominant role of phase toxicity associated with compressed and supercritical fluids.

Fluorescence anisotropy revealed fluidization and melting point depression of dipalmitoylphosphatidylcholine liposomes in the presence of \textit{CO}_2, ethane, and propane (1.8 to 20.7 MPa; 295 to 333 K). The accumulation of these fluids within the bilayer upon pressurization and the ordering effects of pressure influenced liposome fluidity, the melting temperature, and the gel-fluid phase transition region. These results demonstrate the disordering effects of compressed and supercritical fluids on biological membranes and the ability to manipulate liposomes.

KEYWORDS: Bioprocessing, compressed solvent, supercritical fluid, liposome, fluorescence spectroscopy.

Geoffrey D. Bothun

April 9, 2004
PRESSURIZED SOLVENTS IN WHOLE-CELL BIOPROCESSING:
METABOLIC AND STRUCTURAL PERTURBATIONS

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April 9, 2004
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By
Geoffrey D. Bothun
Lexington, Kentucky

Director: Dr. Barbara L. Knutson, Associate Professor of Chemical Engineering
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Acknowledgements

I would like to acknowledge my family, friends, and colleagues for their continued support in my pursuit. Specifically, I would like to first thank Dr. Barbara Knutson for her guidance over the last four and a half years. Her advice and example not only allowed me to complete this project, but has prepared me for what I hope is a successful career in academia. Dr. Herbert Strobel has also been invaluable to this project, from helping me conduct experiments and analyzing my samples (thank you Eva) to editing manuscripts and preparing presentations. I would also like to acknowledge the following people for their contributions: Jason Berberich, Yeh Wei Kho, Dr. Mark Meier (Department of Chemistry, MacSpartan calculations), and Sue Nokes (Department of Biosystems and Agricultural Engineering, UK Bioprocessing Group). Thanks to my Ph.D. committee for their time and guidance, and the Department of Chemical and Materials Engineering at UK.

I am grateful to my family and friends, who have played a key role in this project. It was their support and inspiration that helped me through the tough times. My family has instilled in me the drive, and dare I say stubbornness, to see this project through, and I owe most of it to them. And finally, I am grateful and very lucky to have the love and support of my wife Amy, who has keep me grounded throughout this process.
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Chapter 1

Introduction

Utilization of enzyme and whole-cell biocatalysts are essential for the future production of high-value and commodity products (such as pharmaceuticals, cosmetics, and bulk chemicals) given the current need for processes with improved sustainability and reduced environmental impact (ACS Technology Vision 2020, 1996; DOE 2002). Enzymes provide enhanced selectivity and specificity relative to synthetic catalysts; however, high costs associated with enzyme isolation and purification have limited their use primarily to the production of high-value products. Enzymes are also very sensitive to the solvent environment, where small changes in the bulk solvent, such as pH, polarity, alkalinity, and temperature, can denature proteins (Shuler and Kargi 1992). In contrast, whole-cell biocatalysts provide for sequential enzymatic reactions and eliminate the need for expensive isolation steps and the addition of external cofactors. Despite these advantages, whole cells may be greatly perturbed or destroyed when exposed to harsh operating conditions and environmental stresses. The use of both whole cell and enzyme biocatalyst technologies for the production of commercial products requires the development of novel in situ separation processes, improved bioreactor designs/contacting schemes, and versatile reaction environments that promote selectivity and efficiency (ACS 1996; DOE 2002).

Pressurized solvents, such as compressed and supercritical fluids, are gaining interest in biotechnology and bioprocessing as an alternative to conventional organic solvents (Randolph 1990; Jarzebski and Malinowski 1995; Reverchon 1997). In addition to their general classification as “green” solvents, pressurized solvents that are gases at atmospheric conditions (such as CO₂ and light hydrocarbons) offer distinct advantages over liquid organic solvents, such as the ability to tune solvent strength with small changes in temperature and pressure, and enhanced mass transfer due to low kinematic viscosity (viscosity/density; McHugh and Krukonis 1994; Hyde et al. 2002). Product recovery and purification are greatly simplified with pressurized solvents as the solvent is completely removed upon depressurization. Obtaining a solvent-free product
is particularly important in biotechnologies such as biomaterials and pharmaceutical production. Fractional separation is also possible by manipulating pressure and/or temperature in a stepwise fashion (McHugh and Krukonis 1994). Ultimately, pressure provides an additional processing variable to control solvent strength and selectivity, which influences reaction rate/selectivity and extraction efficiency.

Pressurized solvent technologies have been extended to enzyme catalysis to reduce mass transfer limitations, and simplify enzyme and product separation. By manipulating solvent strength, the solubility of reactants and products can be optimized to reduce product inhibition and improve reaction efficiency. A number of investigations have demonstrated enzyme catalysis in compressed and supercritical fluids (Randolph et al. 1985; Kamat et al. 1995; Mesiano et al. 1999; Sarkari et al. 1999; Knez and Habulin 2002; Knez et al. 2003), with improved enantioselectivity, regioselectivity, and overall yield. While the benefits of pressurized solvent technologies in enzyme catalysis have been well demonstrated, little is known regarding the effects of pressurized solvents on whole cells or model cell membranes (liposomes). Unlike enzymes that are relatively insensitive to pressures less than 100-500 MPa (Gross and Jaenicke 1994), cellular processes such as membrane fluidity are influenced by only several atmospheres of pressure (Barkai et al. 1983). Therefore, concomitant pressure- and solvent-effects may influence whole-cell biocatalysis in the presence of pressurized solvents.

A promising new technology is the formation of liposomes (spherical phospholipid self-assemblies) using pressurized CO₂ (Frederiksen et al. 1997; Castor and Chu 1998; Otake et al. 2001). Liposomes are formed by two methods; spraying a CO₂ phase containing the phospholipid into water or through reverse phase evaporation where water is gradually metered into a CO₂/phospholipid phase. These methods are capable of producing unilamellar liposomes with controlled size distributions (Otake et al. 2001) while eliminating the need for organic solvents. In addition to their wide-spread use as model cell membranes, liposomes are currently used in pharmaceutical formulations (e.g. skin treatments, drug delivery, and gene therapy) (Sato and Sunamoto 1992; Castor and Chu 1998; Metselaar et al. 2002; Ulrich 2002) and as
microreactors for enzymatic reactions (Walde and Marzetta 1998; Oberholzer et al. 1999) and materials synthesis (Collier and Messersmith 2001).

The inhibitory effects of compressed and supercritical CO\textsubscript{2} on a variety of microorganisms (Haas et al. 1989; Isenschmid et al. 1992; Lin et al. 1992; Dillow et al. 1999; Knutson et al. 1999; Sims and Estigarribia 2002; Spilimbergo et al. 2002) suggests that \textit{in situ} processing with CO\textsubscript{2} is not feasible due to cell inactivation and sterilization. However, microbial sterilization by pressurized CO\textsubscript{2} treatments is gaining considerable attention (Spilimbergo et al. 2002). In contrast, there have been relatively few studies on the use of pressurized hydrocarbons (such as ethane and propane) in whole-cell bioprocessing, which exhibit improved biocompatibility relative to CO\textsubscript{2}.

Our research group has recently demonstrated the metabolic activity of non-growing \textit{Clostridium thermocellum}, a model ethanol-producing thermophilic bacterium, in batch cultures pressurized with ethane and propane (Knutson et al. 1999; Berberich et al. 2000a,b). Relative to atmospheric pressure controls, the metabolic activity of non-growing \textit{C. thermocellum} was inhibited by 60 and 80\% in the presence of ethane and propane at 7.0 MPa and 333 K, respectively (Berberich et al. 2000b). In addition, the presence of 7.0 MPa ethane and propane shifted product selectivity toward the ethanol (Berberich et al. 2000a). Further Investigations of batch cultures of non-growing \textit{C. thermocellum} demonstrated that incubations in the presence of pressurized ethane and propane exceeded the biocompatibility predicted by conventional measures of solvent toxicity in traditional liquid solvents (such as the octanol/water partition coefficient, $\log P_{o/w}$; Berberich et al. 2000b). These results demonstrated the ability to manipulate product selectivity in non-barophilic microorganisms with moderate pressures. However, the effects of hydrostatic and hyperbaric pressures on growing cells (e.g. bioenergetics, metabolic selectivity, and molecular/phase toxicity) are unknown.

\textbf{Dissertation Objectives}

Determining the effects of pressurized solvents on whole cells and modular cell membranes would advance emerging technologies in whole-cell biocatalysis, CO\textsubscript{2}-based sterilization, and materials synthesis. The critical link between the concomitant effects of pressure (which increases solvent and gaseous product solubility) and solvent
(which induces metabolic and structural perturbations) on whole-cell processes has not been made. In addition, the effect of pressurized solvents liposome structure is unknown. The goals of this research are to develop experimental techniques to quantify the effects of both pressure and solvent in whole-cell bioprocesses and characterize metabolic and structural perturbations in whole cells and liposomes induced by compressed solvents.

In Chapter 2 a review of whole-cell bioprocessing in continuous cultures under atmospheric and pressurized conditions and the mechanisms of solvent toxicity will be provided. This review will also focus on formulating the mathematical equations that describe the continuous bioreactor, previous investigations of metabolic activity in the presence of pressurized solvents, and describing solvent toxicity in terms of molecular and phase toxicity. In addition, the effects of pressurized solvents on non-growing \textit{C. thermocellum} will be discussed. Finally, a fluorescence technique will be presented that can be used to quantify changes in membrane fluidity due to the accumulation of dissolved solvent molecules (molecular toxicity).

Chapter 3 examines the effect of elevated hydrostatic pressure (up to 17.3 MPa) on the growth and metabolism of \textit{C. thermocellum}. This study demonstrates the utilization of continuous cultures to measure changes in microbial metabolism, growth, and bioenergetic parameters at high pressures and the potential for pressure-induced product selectivity in non-barophilic microorganisms.

Chapters 4 and 5 examine membrane perturbations by pressurized solvents, which are a likely cause for inhibition and metabolic selectivity in whole cells. Membrane fluidity in a model phospholipid membrane (liposome) will be measured in the presence of pressurized \textit{CO}_2 (Chapter 4) and pressurized light hydrocarbons (Chapter 5) using a fluorescing membrane probe molecule. Liposome fluidization is attributed to the accumulation of solvents within the bilayer disrupts the acyl region, which alter membrane fluidity and the phospholipid melting temperature. The effects of pressure and solvent (size, topology, aqueous/membrane concentrations) on liposome fluidization and the application of conventional melting point theory are demonstrated.
Chapter 6 presents the development of a novel high-pressure biphasic continuous bioreactor to study the effects of pressurized fluids and solvents on solvent toxicity. Metabolic selectivity of *C. thermocellum* in biphasic cultures suggests the ability to manipulate and reverse cell activity using pressurized solvents. The mechanism of solvent toxicity is elucidated by the degree of inhibition induced by different pressurized solvents. The reactor technology that has been developed facilitates investigations of complex pressure and dissolved gases effects on cell metabolism and bioenergetics.

References


Chapter 2

Background

This review will first focus on the concept of solvent toxicity in whole-cell bioprocesses, which naturally arises when considering biphasic incubations, particularly in the presence of pressurized solvents (compressed and supercritical fluids). Continuous cultures provide a simplified platform (steady-state) to study shifts in metabolism and bioenergetic parameters due to external perturbations, such as pressure- or solvent-effects. Bioreactor design for cultures at atmospheric pressure (single and biphasis) and under elevated hydrostatic pressure is presented. Attention will be given to *Clostridium thermocellum*, which has been recently used as a model thermophilic bacterium to investigate the effects of pressurized solvents in batch cultures (non-growing cells). This section will conclude by focusing on a fluorescence technique used to quantify changes in membrane fluidity due to molecular toxicity.

Whole-Cell Biocatalysis

Whole-cells as biocatalysts offer distinct advantages over enzymes, as (i) they do not require expensive isolation and purification biocatalysts, (ii) the addition of external cofactors (whole cells regenerate cofactors) is avoided, and (iii) they are capable of multi-step chemical reactions. However, whole cells are particularly susceptible to product inhibition, where the accumulation of product in the bulk media reduces the thermodynamic driving force for product formation (Herrero 1983; Shuler and Kargi 1992). In addition to product inhibition, the accumulation of end-products can also become toxic to whole cells. For example, product toxicity is observed in *Saccharomyces cerevisae* (yeast) when the ethanol concentration exceeds approximately 110 g/L (Erickson 1988). Therefore, the design of productive and continuous whole-cell bioprocesses requires removing of metabolic end-products, particularly for solvent-producing microorganisms.
The removal of end-products from aqueous fermentation media is frequently accomplished downstream (or *ex situ*), where the cell biomass is separated from the media before product removal (Table 2.1). Downstream product recovery, rather than *in situ* recovery, may require additional unit operations and lengthen operating time. Distillation is the most common method of separating and recovering volatile fermentation products, at the expense of high energy consumption and harsh thermal conditions. The productivity of whole cells requires optimal operating conditions (i.e. constant temperature and pH); therefore, the use of thermal separations cannot be employed *in situ*. In contrast, solvent extraction provides an effective way to separate volatile (and non-volatile) fermentation products from aqueous solution *in situ*. However, the effect of an aqueous-solvent interface due to an excess solvent phase (phase toxicity) and the presence of dissolved solvent in the aqueous phase (molecular toxicity) on cell viability and function must be determined.

### Table 2.1 Selected processes for recovering fermentation products.

<table>
<thead>
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<th>Process</th>
<th>Nature</th>
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<th>Pre-Processing</th>
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<td>Distillation (ex situ)</td>
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<td>cell removal – thermally labile</td>
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<tr>
<td>Pervaporation (ex situ)</td>
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<td>molecular toxicity</td>
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**Solvent-Effects on Whole Cells**

Solvent toxicity is conventionally expressed in terms of the natural logarithm of the octanol/water partition coefficient \( \log P_{o/w} \); molar or mole fraction basis), which is a measure of solvent hydrophobicity, or rather “lipophilicity” (Sardessai and Bhosle 2002). Solvent toxicity, as it relates to \( \log P_{o/w} \), is balanced by the degree of solvent partitioning...
into the membrane and the solubility of solvent in the aqueous phase. For instance, while lipophilic solvents (such as long chain alkanes) have high log $P_{o/w}$ values, cell toxicity is minor due to very low aqueous solubility. The low aqueous solubility prevents such solvents from reaching a high concentration within the membrane. In contrast, solvents with low log $P_{o/w}$ values are considered toxic due to high aqueous solubility, which leads to high membrane concentrations. Generally, log $P_{o/w}$ values less than 2 are considered toxic and values greater than 5 are considered to be biocompatible (molar basis). However, the exact degree of solvent toxicity is dependent upon the solvent, the operating conditions, and the microorganism employed (Vermuë et al. 1993).

Sikkema et al. (1994) have developed a widely accepted experimentally-based correlation that relates log $P_{o/w}$ to the partitioning behavior of a solvent between a phospholipid cell membrane and water ($log P_{m/w}$; Table 2.2). The following equation (2.1) has been used to estimate log $P_{m/w}$ for virtually every class of solvent (alcohols, amines, alkanes, aromatics, etc.; Sikkema et al. 1994; Weber and de Bont 1996):

$$log P_{m/w} = 0.97 \cdot log P_{o/w} - 0.64$$  \hspace{1cm} (2.1)

Although the mechanism for solvent toxicity in whole cells is unclear, it has been attributed to enzyme inactivation (due to denaturing or solvent-competition with active sites), membrane disruption, and the extraction of cellular components (Sikkema et al. 1995; Angelova and Schmauder 1999). The membrane is particularly susceptible to solvent toxicity due to the hydrophobic nature of the acyl-chain (phospholipid “tail”) region in the phospholipid bilayer, which allows for the accumulation of hydrophobic solvent molecules. It is this property that has allowed log $P_{o/w}$ to be a generally accepted measure of solvent toxicity. Compromises in membrane integrity inhibit the activity of membrane-bound enzymes for catalysis and transport.

Solvent-effects on whole cells can be classified by two categories; molecular and phase toxicity (Bar 1988; León et al. 1998). Molecular toxicity exists in a single phase, and is typically due to the dissolution of organic solvent molecules into the aqueous broth and the subsequent partitioning of solvent molecules into the cell and cell membrane (León et al. 1998). Molecular toxicity can occur due to enzyme-solvent interactions; however, it is primarily associated with fluidization and/or disruption of the
cell membrane. In contrast, phase toxicity is caused by the presence of an aqueous/solvent interface due to an excess solvent phase, which may lead to interfacial cell contact, the extraction of essential nutrients from the aqueous phase, the extraction of cellular or membrane components, and limited nutrient availability due to cell adsorption at the aqueous/solvent interface (Bar 1986).

Table 2.2 Examples of reported aqueous solubility and $\log P_{o/w}$.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Aqueous solubility $^a$ (mole fraction)</th>
<th>$\log P_{o/w}^b$ (molar)</th>
<th>$\log P_{m/w}^c,d$ (mole fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkanes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethane (1.8 MPa) $^f$</td>
<td>$4.1 \times 10^{-4}$</td>
<td>1.81</td>
<td>1.12</td>
</tr>
<tr>
<td>Ethane (7.0 MPa) $^f$</td>
<td>$8.9 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propane (1.8 MPa) $^f$</td>
<td>$1.5 \times 10^{-4}$</td>
<td>2.36</td>
<td>1.65</td>
</tr>
<tr>
<td>Propane (7.0 MPa) $^f$</td>
<td>$2.2 \times 10^{-4}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentane</td>
<td>$1.0 \times 10^{-5}$</td>
<td>3.39</td>
<td>2.65</td>
</tr>
<tr>
<td>Hexane</td>
<td>$2.2 \times 10^{-6}$</td>
<td>3.95</td>
<td>3.19</td>
</tr>
<tr>
<td>Heptane</td>
<td>$4.8 \times 10^{-7}$</td>
<td>4.50</td>
<td>3.73</td>
</tr>
<tr>
<td>Octane</td>
<td>$1.1 \times 10^{-7}$</td>
<td>5.15</td>
<td>4.36</td>
</tr>
<tr>
<td>Decane</td>
<td>$5.1 \times 10^{-9}$</td>
<td>6.34</td>
<td>5.51</td>
</tr>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>$3.1 \times 10^{-1}$</td>
<td>-0.31</td>
<td>-0.94</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>$1.8 \times 10^{-2}$</td>
<td>0.88</td>
<td>0.21</td>
</tr>
<tr>
<td>1-Hexanol</td>
<td>$1.2 \times 10^{-3}$</td>
<td>2.03 $^e$</td>
<td>1.33</td>
</tr>
<tr>
<td>Dodecanol</td>
<td>$3.4 \times 10^{-7}$</td>
<td>5.04 $^e$</td>
<td>4.25</td>
</tr>
</tbody>
</table>

$a$ calculated at 298 K by existing correlation (Marche et al. 2003) based on experimental data (Shaw 1989b, a)  
$b$ octanol-water partition coefficient obtained from (Sangster 1997)  
$c$ $\log P_{m/w}$ (mole fraction) = $42.4 \cdot \log P_{m/w}$ (molar) based on a DPPC bilayer  
$d$ calculated by equation 2.1  
$e$ octanol-water partition coefficient obtained from (Trudell and Bosterling 1983)  
$^f$ aqueous solubility calculated at 298 K (Appendix C)

Pressurized Solvents in Bioprocessing and Biotechnology

Compressed or supercritical fluid solvents are frequently described as “pressurized solvents.” A supercritical fluid is a fluid at or above its critical temperature $(T \geq T_c)$ and pressure $(P \geq P_c)$. A compressed fluid refers to a gas that has pressurized to yield a dense gas or liquid state (Figure 2.2). For a compressed fluid, either $T$ or $P$
(but not both) may be beyond the critical value. Critical properties for some common fluids are presented in Table 2.3.

Table 2.3  Critical properties of common fluids.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>( T_c ) (K)</th>
<th>( P_c ) (MPa)</th>
<th>( V_c ) (L/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CO}_2 )</td>
<td>304.2</td>
<td>7.4</td>
<td>( 9.4 \times 10^{-2} )</td>
</tr>
<tr>
<td>Ethane</td>
<td>305.3</td>
<td>4.9</td>
<td>( 1.5 \times 10^{-1} )</td>
</tr>
<tr>
<td>Propane</td>
<td>369.8</td>
<td>4.2</td>
<td>( 2.0 \times 10^{-1} )</td>
</tr>
<tr>
<td>Hexane</td>
<td>507.6</td>
<td>3.0</td>
<td>( 3.7 \times 10^{-1} )</td>
</tr>
<tr>
<td>Water</td>
<td>647.1</td>
<td>22.1</td>
<td>( 5.6 \times 10^{-2} )</td>
</tr>
</tbody>
</table>

*\( 1 \text{ MPa} = 10 \text{ bar} = 9.9 \text{ atm} = 145 \text{ psia} \) (absolute pressure)

Compressed and supercritical fluids (pressurized fluids) can be used to replace liquid organic solvents as a “green” alternative for materials synthesis and processing (Reverchon 1999; McLeod et al. 2003; Xiangrong and Wai 2003), biocatalysis (Randolph et al. 1985; Mesiano et al. 1999; Knez and Habulin 2002), and extractive separations (McHugh 1984; McHugh and Krukonis 1994; Reverchon 1997; Wai and Wang 1997). A compilation of high pressure phase equilibria reveals the interest in supercritical fluids for the extraction and processing of numerous solutes including alcohols, paraffins, halogens, fats, and organic acids (1988 to 1999; Dohrn and Brunner 1995, 2002). Moreover, pressurized solvents that are gases at atmospheric conditions (such as \( \text{CO}_2 \) and light hydrocarbons) offer distinct advantages over liquid organic solvents such as the ability to “tune” solvent strength with minor changes in temperature and pressure, and enhanced mass transfer due to low kinematic viscosity (Figures 2.1 and 2.2) (Randolph 1990; Jarzewska and Malinowski 1995). Low kinematic viscosity yields high diffusivity and reduced convective mass transfer resistance.

Product recovery and purification are greatly simplified with compressed and supercritical fluid solvents as the fluid is completely removed upon depressurization. Obtaining a solvent-free product is particularly important in biotechnologies such as the development of biomaterials and pharmaceutical formulations. One limitation has been the low solubility of hydrophilic molecules in most pressurized solvents; however, polar co-solvents or surfactants can be added to improve solubility. Fractional separation is
also possible by manipulating pressure and/or temperature in a stepwise fashion. Ultimately, pressure provides an additional processing variable to control solvent strength and selectivity, reaction rate, and the rate of material synthesis in pressurized solvent systems.

**Figure 2.1** Pure component pressure-temperature phase diagram.

The use of pressurized solvents as a media for enzyme catalysis has received considerable attention in the last decade, and is now gaining increasing interest based on the need to improve the performance and selectivity of bioprocesses (Randolph et al. 1985; Mesiano et al. 1999; Knez and Habulin 2002). The majority of studies have focused on lipase existing as a free or immobilized enzyme in compressed or supercritical light hydrocarbons and CO₂. Regio- and enantioselectivity are two major advantages to using lipase for oxidation, hydrolysis, and esterification in pressurized solvents (Knez and Habulin 2002). Enzyme kinetics, selectivity, activity, and stability in pressurized solvents have been described primarily in terms of water content, reaction activation volume, and mass transfer properties of the solvent.
Recently, Knez and coworkers (Knez and Habulin 2002) have made significant progress in this field, demonstrating enhanced enzyme catalysis relative to conventional solvents in both batch and continuous operation in the presence of compressed/supercritical fluids. Despite increasing work in the area of enzyme catalysis in compressed and supercritical fluids, the advantage of replacing organic solvents with pressurized solvents has not been fully demonstrated (Mesiano et al. 1999). Even more complex is the effect of these solvents on whole cells, which may be appreciably inhibited by pressurized hydrocarbons (Berberich et al. 2000b), or inactivated by pressurized CO₂ (Spilimbergo et al. 2002). The effects of pressurized solvents on a model thermophilic bacterium, *Clostridium thermocellum*, will be discussed in detail.

**Figure 2.2** Kinematic viscosity as a function of pressure at 298.15 K. Data obtained from National Institute of Standards and Technology (NIST).
**Clostridium thermocellum** as a Model Thermophile in Pressurized Cultures

*Clostridium thermocellum* is a non-barophilic, thermophilic bacterium capable of converting of cellobiose (a disaccharide derived from cellulose degradation) to the liquid products ethanol, acetate, and lactate and the gaseous products CO$_2$ and N$_2$. Some of the advantages to using thermophilic bacteria include the ability to operate at elevated temperatures and high specific growth rates (Ng et al. 1977). The ability to utilize cellullosic substrates (Wang 1979; Linden 1988; Lynd 1989) and the benefits inherent to thermophilic bacteria make *C. thermocellum* a potential candidate for ethanol production (Zeikus 1980; Lynd 1989). However, low ethanol tolerance of *C. thermocellum* suggests that continuous extraction be employed to enhance productivity. It has been shown (Herrero 1983) that the addition of each of the fermentation products (ethanol, acetate, and lactate) has led to a decrease in productivity of the added product and an increase in productivity of the other products. Despite the established effect of excess product concentrations on ethanol productivity in *C. thermocellum*, there have been no definitive studies on the influence of continuous extraction on fermentation (Lynd 1989), particularly for compressed solvents.

*C. thermocellum* utilizes cellullosic substrates (cellobiose) to produce ethanol, lactate, and acetate through anaerobic fermentation (Figure 2.3). Metabolic reactions are facilitated through oxidation/reduction using nicotinamide adenine dinucleotide (NAD) and ferrioxiden (FD), and energy is transduced in the form of adenosine triphosphate (ATP). ATP is primarily produced (ADP → ATP) within the cell by substrate level phosphorylation reactions of glycolysis. Energy is consumed within the cell (ATP → ADP) for cellular repair, mobility, and the excretion of internal molecules through active transport mechanisms.

The first catabolic process is glycolosis, or the conversion of the substrate(s) to pyruvate (2CH$_3$COCOOH), producing ATP and NADH (Figure 2.3, A). Pyruvate then reduces to lactate (CH$_3$COHCOOH, B) or condenses to acetyl CoA (C; coenzymeA, acyl carrier and condensing enzyme), producing hydrogen via FD. Further phosphorylation converts acetyl CoA into acetyl-P, and eventually the product acetate (CH$_3$COOH) and ATP generation (D). Acetyl CoA is also reduced to acetaldehyde (CH$_3$CHO) for the reductive formation of ethanol (CH$_3$CH$_2$OH, E).
Figure 2.3  Simplified metabolic pathways for C. thermocellum with specified reactions A-E. NADH act as hydrogen donors/acceptors for reduction or oxidation and ADP provides stored energy as it forms ATP. Adapted from Ng et al. (1977) and Zeikus (1980).

Recent investigations by our research group (Knutson et al. 1999; Berberich et al. 2000b, a; Berberich 2001) regarding the biocompatibility of supercritical and compressed solvents were performed to determine the feasibility of in situ extraction for the conversion of cellobiose to ethanol with non-growing C. thermocellum in batch fermentation. Non-growing cells were chosen to study the effects of pressure and
solvent on metabolism without the complications associated with cell growth, such as utilization of nutrients and substrate for growth.

Fermentation activity in the presence of compressed CO$_2$ (6.9 MPa, 333 K) was greatly reduced, resulting in a decrease in cellobiose utilization and an 80% reduction in ethanol production (Knutson et al. 1999). This was expected as CO$_2$ has been shown to inactivate many microorganisms (Thibault et al. 1987; Haas et al. 1989; Isenschmid et al. 1992; Dillow et al. 1999). In the presence of compressed ethane and propane (70 bar, 333 K), ethanol production decreased approximately 60%, but it was clear that bioactivity was maintained. Although significant, the inhibition by compressed hydrocarbons was much less than that observed for incubations in the presence of excess liquid pentane, heptane, and hexane at atmospheric pressure, which rendered the cells completely inactive (Berberich et al. 2000b). The presence of compressed propane increased the selectivity of the fermentation to the product ethanol relative to the acid products, lactate and acetate. The observed solvent toxicity was attributed to the direct contact of the cells with the aqueous-hydrocarbon interface, or phase toxicity (Berberich et al. 2000a). Molecular toxicity may also have led to such shifts in productivity, as such gases can dissolve within the cell membrane.

Shifts in product selectivity for C. thermocellum in batch incubations have also been observed under hydrostatic pressures as low as 0.5 MPa. Relative to ambient pressure controls (0.1 MPa), acetate production was inhibited by approximately 75% after 10 hours under hydrostatic pressure, while ethanol production was unchanged (Berberich 2001). It was hypothesized that an increase in hydrostatic pressure resulted in a higher partial pressure of hydrogen and CO$_2$ produced by the bacteria, resulting in a mass-action affect that inhibited particular metabolic reaction rates. Hydrogen is produced in the formation of acetate, and in general, increasing hydrogen partial pressure has been linked to an increase in ethanol yield due to the inhibition of acetate production (Lynd 1989). This is further supported by Sundquist et al. (Lynd 1989), who have found that ethanol yields decreased when continuous ethanol extraction was performed in a reduced pressure flash-vessel.
**Bioprocessing in Continuous Culture**

Steady-state productivity and energy requirements for maintaining and/or repairing damaged cell components (maintenance energy) can be determined through continuous fermentation in a chemostat reactor. A chemostat is a continuous stirred tank reactor (CSTR) for biological systems where operating conditions such as temperature, pressure, and flowrate can be held constant. Chemostat operation can be performed at atmospheric pressure (with and without a gas headspace), and under elevated hydrostatic and hyperbaric pressure (Figure 2.4).

Most microbiological studies employ batch culture approaches. However, batch cultures are dynamic systems where product concentrations increase as substrates are consumed. Determining bioenergetic requirements in batch cultures is difficult because intracellular, extracellular, and membrane compositions are continuously changing with time. Analyzing metabolic activity and bioenergetics is simplified in chemostat cultures, as productivity and cell growth are constant.

![Diagram of fermentation under hydrostatic and hyperbaric pressure](image)

**Figure 2.4** Fermentation conducted under hydrostatic and hyperbaric pressure.
Product formation and cell growth is commonly expressed in terms of the yield coefficient, $Y$. Yield coefficients are stoichiometrically related parameters that relate the production or consumption of one material to that of another material (Shuler and Kargi 1992). For example, the amount of biomass formed due to cell growth (growth yield coefficient; g dry cell weight (DCW)/g substrate consumed) and the amount of product formed (product yield coefficient; mM product formed/mM substrate consumed) is related to substrate consumption as follows:

\[
Y_{X/S} = \frac{\Delta[X]}{\Delta[S]} 
\]

\[
Y_{P/S} = \frac{\Delta[P]}{\Delta[S]} 
\]

(2.2)

(2.3)

where $[X]$ and $[S]$ represent biomass and substrate concentration, respectively. Similarly, product formation(s) may also be related to substrate utilization. The overall cell biomass and substrate balances for chemostat operation are as follows:

\[
V_R \frac{dX}{dt} = F(X_0 - X) + V_R \mu X - V_R k_d X = 0 
\]

\[
V_R \frac{dS}{dt} = F(S_0 - S) + V_R \mu X \left( \frac{1}{Y_{X/S}^M} \right) - V_R q_p X \left( \frac{1}{Y_{P/S}} \right) = 0 
\]

(2.4)

(2.5)

where $V_R$ is the reactor volume, $F$ is the volumetric flowrate, $\mu$ is the specific growth rate (h$^{-1}$), $k_d$ is the rate of cell death (h$^{-1}$), $q_p$ is the rate of total product formation (mM product/g DCW/h), $Y_{X/S}^M$ is the maximum theoretical growth yield, and $Y_{P/S}$ is the product yield. Assuming that cell death is negligible compared to growth and no cells are being feed into the reactor ($X_0 = 0$), cell growth within a chemostat (equation 2.4) is constant at steady-state, and equal to the space time (inverse residence time) or dilution factor ($D = F/V_R$, h$^{-1}$). To prevent the cells from being washed out of the system, the dilution factor must be chosen as to not exceed the maximum cell growth rate ($\mu_{\text{max}}$), which varies as the environment within the reactor changes. Growth rates can therefore be selected by adjusting the flow of media (containing substrate and nutrients) into the reactor.

Cell growth rate in continuous culture is frequently described by the well-known Monod kinetic model (Bailey and Ollis 1986):
\[
\mu = \frac{\mu_{\text{max}}[S]}{[S] + K_s}
\]  
(2.6)

where \(\mu_{\text{max}}\) is the maximum specific growth rate \((\text{h}^{-1})\) and \(K_s\) is the saturation constant \((\text{mM})\), which corresponds to the substrate concentration at one-half of the maximum specific growth rate. The saturation constant is a measure of a microorganism’s affinity for a substrate, where small values of \(K_s\) represent high affinity for a substrate. At steady-state, it can be seen from equation 2.4 that the specific growth rate equals the dilution rate \((\mu = D, \text{h}^{-1})\).

When the extracellular product yield is negligible and the system is at steady-state, equation 2.5 can be rearranged to yield the following relationship:

\[
\frac{(S_o - S)}{X} = \frac{1}{Y_{X/S}} = \frac{1}{Y_{X/S}^M} + \frac{k_d}{Y_{X/S}^M D}
\]  
(2.7)

where \(Y_{X/S}\) is the apparent (experimentally observed) growth yield as defined in equation 2.2 and the term \(k_d / Y_{X/S}^M\) is defined as the maintenance coefficient \(m_S\) \((\text{g substrate/g DCW/h})\), which is independent of growth rate. Therefore, maintenance energy accounts for the energy required to maintain an energized and functional membrane (Pirt 1965; Pirt 1982). Harsh operating conditions (temperature, pressure, and/or organic solvents) can inhibit cell metabolism and function by inducing structural perturbations to the membrane, which in turn effects maintenance energy requirements.

**Continuous Culture under Elevated Hydrostatic Pressure**

Continuous cultures conducted under elevated hydrostatic pressure have been limited to marine barophilic and oligotrophic bacteria (up to 71 MPa) (Jannasch et al. 1996), and microorganisms for enhanced oil recovery (up to 200 MPa; Bubela et al. 1987). Bubela et al. (1987) developed the first experimental apparatus to study the transient effects of oil reservoir conditions on microorganisms. A hydraulic pump, high-pressure unidirectional flow valves, and a backpressure regulator were used to regulate media flow and maintain system pressure. The flow line was also fitted with a rupture disk as a safety measure to prevent system over-pressurization. Sterilization of the high-pressure system, including a 1 L reaction vessel, was achieved using 75% \((\text{v/v})\) alcohol. Jannasch et al. (1996) constructed a similar system to study productivity and
substrate consumption in continuous cultures of *Shewanella* under elevated hydrostatic pressure. In this case, an HPLC pump was used to feed media through the reactor and sterilization was achieved by autoclaving. At a dilution (specific growth) rate of 0.066 h\(^{-1}\), optimal cell growth of barophilic *Shewanella* was observed at 31 MPa. These studies focused specifically on cell growth of barophiles and excluded metabolic selectivity, bioenergetic parameters, and the effects of dissolved product gases.

**Continuous Culture in a Two-Phase System**

The few two-phase (organic-aqueous) chemostat studies that have been reported can be categorized by the following three objectives: (i) to improve biodegradation or digestion (Zhang and Noike 1991; Elaalam et al. 1993); (ii) to enhance aqueous fermentations where hydrophobic products are formed (Preusting et al. 1991; Prichanont et al. 1998; Schmid et al. 1998; Schmid et al. 1999); and (iii) to employ a second organic phase as the substrate source (van Ginkel et al. 1987; Favre-Bulle et al. 1993; Isken et al. 1999). Schmid et al. (1998; 1999) developed criteria for constructing a large-scale aqueous, aerobic bacterial bioprocess in the presence of bulk amounts of organic solvents. The focus of these studies were to answer questions regarding the feasibility of building a reactor capable of withstanding explosions, what downstream processing will involve, and how to safely operate the system. There have been no reported studies of a high-pressure two-phase chemostat (> 1 MPa) employing either liquid or compressed gases.

Favre-Bulle et al. (1993) studied the effects of *n*-octanol, a solvent phase, on octanoic acid production by *Escherichia coli*. It was reported that cells remained viable over the entire continuous culture (200 h) (Favre-Bulle et al. 1993). Similarly, propene and ethene has been used in the gas phase to determine growth parameters of gaseous alkene-utilizing bacteria at various dilution rates (van Ginkel et al. 1987). Isken et al. (1999) presented a study in which the effects of various organic solvents such as toluene, hexane, and cyclohexane on solvent-tolerant *Pseudomonas putida* S12 were observed. The effect of solvent toxicity was quantified by measuring changes in product yield and maintenance energy relative to a solvent-free control.
**Metabolic Flux Analysis**

Metabolic flux analysis can provide quantitative knowledge on the influence that particular metabolic pathways have with respect to energy consumption and cellular functions (Stephanopoulos et al. 1998). By experimentally determining the concentration of fermentation products (and potentially other intracellular metabolites), it is possible to construct a scheme for quantifying the effect of operating conditions on metabolism. Such a metabolic flux analysis can be developed for any type of reactor (batch, semi-batch, or continuous/chemostat), but using a chemostat simplifies the analysis as metabolism can be measured under steady-state conditions. The metabolic flux analysis reduces to a system of interrelated linear algebraic equations accounting for the various metabolic reactions:

\[ x_i = \sum_n v_i^n r_i^n \]  

(2.8)

where \( x_i \) represents the molar amount of species \( i \), \( v_i \) is the stoichiometric coefficient for reaction \( n \), and \( r_i \) is the rate of reaction. A detailed description of the metabolic flux analysis for *Clostridium thermocellum* grown in continuous cultures under elevated hydrostatic pressure is given in Appendix A.

**Membrane Fluidity via Fluorescence Anisotropy**

Molecular toxicity due to the accumulation of solvents within the cell membrane inhibits whole-cell bioprocesses by altering membrane fluidity. In the case of pressurized solvents, molecular toxicity is expected to be significant due to the high solubility of gases within the aqueous phase. Quantifying the effects of dissolved gases and solvents on membrane fluidization is required to improve whole-cell bioprocesses using pressurized solvents.

Changes in membrane fluidity can be measured quantitatively and *in vivo* through steady-state fluorescence spectroscopy by using a probe molecule to penetrate the lipid bilayer (Lentz et al. 1976; Jahng 1979; Parasassi et al. 1990). The most commonly used fluorescent probe molecule for measuring membrane fluidity in biological membranes is 1,6-diphenyl-1,3,5-hexatriene (DPH) (Parasassi et al. 1984). DPH is a cylindrically shaped molecule that freely rotates in water (unconfined) and
fluorescence is negligible. As DPH penetrates or is embedded-within the lipid bilayer (confined), the hydrophobic tails of the lipids inhibit the molecule's rotational motion (Figure 2.5) resulting in fluorescence emission (Parasassi et al. 1984).

![Figure 2.5](image)

**Figure 2.5** Schematic representation depicting the ability to measure changes in membrane fluidity with fluorescence spectroscopy. The rotational motion of the probe molecule DPH is inhibited within the DPPC bilayer ($T_m \approx 315$ K). Circled regions denote the hydrophilic “head.”

The fluorescence intensity of DPH is related to the degree in which the rotational motion is inhibited. This inhibition is in turn a function of the membrane fluidity. Fluorescence anisotropy ($<r>$) is defined as:

$$<r> = \frac{I_{VV} - I_{VH}}{I_{VV} + G \cdot I_{VH}}$$  \hspace{1cm} (2.9)
where $I$ represents the fluorescence emission intensity, $V$ and $H$ represent the vertical and horizontal orientation of the excitation and emission polarizers, and $G = (I_{HV})(I_{HH})^{-1}$ accounts for the sensitivity of the instrument towards vertically and horizontally polarized light. A past difficulty in using DPH was the uncertainty of depth to which it penetrated the lipid bilayer. However, Kaiser and London (1998) have shown that DPH is located deep within the hydrophobic “tails.” Model phospholipid vesicles can be synthesized for preliminary membrane fluidity measurement, although it is possible to use reconstituted vesicles from cellular membrane components. Changes in the fluorescence emission intensity of DPH correlate with changes in membrane fluidity, and subsequently the permeability of the phospholipid bilayer.

Membrane fluidity measurements based on DPH anisotropy have been used to characterize the effects of anesthetics and organic solvents on the main phase transition (gel-fluid) of phospholipid bilayers. Studies by Papahadjopoulos et al. (1975) and Vanderkool et al. (1977) demonstrated bilayer fluidization by anesthetizing solutes such as dibucaine, trilene, ethrane, halothane, ether, ethanol, and chloroform. These studies demonstrated a decrease in $T_m$ with increasing solute concentration, which shows that anesthetizing solutes disorder phospholipid bilayers. The main phase transition occurs at a temperature where the entropic contributions due to changes in bilayer order counterbalance the enthalpic contributions (cohesive energy) of mixing due to an expanding bilayer (Cevc and Marsh 1987).

Early investigations using fluorescence anisotropy focused on determining the aqueous/bilayer partition coefficient from melting point depression. The following equation was frequently used to estimate solute concentration in the bilayer (Papahadjopoulos et al. 1975; Vanderkool et al. 1977; Kamaya et al. 1981):

$$\Delta T_m = \frac{RT_m T_{m,o}}{\Delta H_{m,o}} x_i$$

(2.10)

where $\Delta T_m = T_m - T_{m,o}$ is the change in melting temperature, $R$ is the gas constant, $T_m$ is the phospholipid melting temperature (solute present), $T_{m,o}$ is the melting temperature (no solute present), $\Delta H_{m,o}$ is the phase transition enthalpy, and $x_i$ is the solute mole fraction within the bilayer. Cevc and Marsh (1987) and Evans and Wennerstrom (1999)
outline the principles of deriving equation 2.10, which is based on regular solution theory.

However, there are two main assumptions associated with the derivation of equation 2.10 that may yield inaccurate results. First, it is assumed that the accumulation of solute in the bilayer does not change $\Delta H_{m,o}$, which is very unlikely at high solute concentrations based on the enthalpic (mixing) and entropic contributions of disorder in “non-dilute” systems (Evans and Wennerstrom 1999). For instance, at the phase transition the chemical potential of the gel and fluid phases are equal; therefore, the phase transition enthalpy is equal to the phase transition entropy, $\Delta H_{m,o} = T \Delta S_{m,o}$ (Cevc and Marsh 1987). Increasing solute concentration disorders the bilayer and reduces the entropy, and thus the phase transition enthalpy. Second, it is assumed that the concentration of solute in the fluid phase is much greater than the concentration of solute in the gel phase ($x_i^{(fluid)} >> x_i^{(gel)}$) (Kamaya et al. 1981). This assumption has been proven incorrect for a number of systems such as halothane (Simon et al. 1979) and n-alkanes (McIntosh et al. 1980).

Using DPH anisotropy, it has been recently shown that aspirin (Ghosh et al. 1996b) and corticosteroids (a group of steroid hormones) (Ghosh et al. 1996a) perturb DPPC bilayers, leading to reductions in both $T_m$ and the phase transition enthalpy. Drug partitioning into DPPC bilayers, representing model cell membranes, increases membrane fluidity and influences the structure and function of different surface proteins and receptors (Lawrence and Gill 1975; Loh and Law 1980). The authors have demonstrated a correlation between the biochemical action of drugs and the nature of the drug-induced bilayer perturbation (Ghosh et al. 1996a).

Linear ($C_6$ to $C_{12}$) and branched ($C_8$ an $C_{10}$) alkanols were incorporated into gel phase DPPC bilayers (Aguilar et al. 1996) to study the solute partition coefficient, and the effect of solute topology on melting point depression. Based on DPH anisotropy, “pseudo-equilibrium” partition constants (K) were estimated for the alkanols. A linear relationship was established between log K and the number of carbon atoms in linear alkanols. Branched alkanols did not exhibit this relationship, demonstrating the dependence of membrane fluidity on solute structure (Aguilar et al. 1996). These
studies have demonstrated that bilayer fluidization is dependent upon solute size and structure, and the concentration within the bilayer.

References


Chapter 3

Metabolic Selectivity and Growth of *Clostridium thermocellum* in Continuous Culture under Elevated Hydrostatic Pressure

**Introduction**

The pervasive effect of dissolved gases on microbial product yield, product selectivity, and cell growth suggests the potential to optimize bio-based solvent production by manipulating the concentration of dissolved product gases (from fermentation) and/or exogenous gases (sweep or anesthetic gases) with total system pressure (Jones and Greenfield 1982). At near-atmospheric pressures, changes in metabolism with pressure are frequently related to the solubility of dissolved gases in fermentation media (Jones and Greenfield 1982), which increases with system pressure, or gas partial pressure. For example, partial pressures of CO₂ and H₂ above approximately 0.1 bar alter metabolism in a broad range of bacteria, fungi, and yeasts (Jones and Greenfield 1982; Eklund 1984; Lamad et al. 1988; Dixon and Kell 1989; McIntyre and McNeil 1998). Elevated levels of dissolved gases, particularly CO₂ and H₂, can result from fermentation activity, and are amplified by increased hydrostatic pressures (0.1 bar/m depth of H₂O) near the base of large-scale fermentors (McIntyre and McNeil 1998).

The effect of dissolved gas concentration on metabolic activity is attributed to changes in biochemical pathways and function of the plasma membrane (Jones and Greenfield 1982). The increase in dissolved gas concentration due to hydrostatic pressure (in the absence of a gas headspace) can change the thermodynamic driving force of individual reaction pathways by increasing the concentration of the product gas in the fermentation broth. Furthermore, the dissolution of gases (such as aqueous CO₂) within the cellular membrane creates disorder, altering membrane fluidity even at near-atmospheric pressures (Jones and Greenfield 1982). Changes in membrane fluidity, in turn, influence transmembrane and lateral permeability, and the function of membrane-bound enzymes for catalysis, substrate or product transport, and maintaining pH and ion gradients.
Investigations of moderate pressure (< 100 MPa) provide fundamental information on microbial processes (Abe et al. 1999; Bartlett 2002) that are directly relevant to industrial fermentation. Recent investigations of the effect of pressure on microorganisms focus on biohydrogenations (up to 3.6 MPa) (Schieche et al. 1997); the use of bacteria for enhanced oil recovery (up to 200 MPa) (Bubela et al. 1987); metabolic activity of extremophiles (typically > 50 MPa) (Jannasch et al. 1996; Abe and Horikoshi 2001); sterilization of microorganisms with high hydrostatic pressure (> 300 MPa) (Hauben et al. 1997; Smelt 1998; Lee et al. 2001); and the potential for in situ recovery of fermentation products using compressed or supercritical solvents (> 7 MPa) (Knutson et al. 1999; Berberich et al. 2000a, 2000b; Berberich 2001). The presence of compressed solvents in biphasic fermentations (hyperbaric pressure) adds further complexity. Dissolved solvent (molecular toxicity) and the presence of the fluid-fermentation broth interface (phase toxicity) may also alter metabolic activity and physiology (Bar 1988). The current inability to fully 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.

Fermentation in a controlled gas environment (with respect to total pressure and individual gas partial pressures) has a significant impact on the metabolic selectivity of the thermophilic bacterium Clostridium thermocellum (Lamad et al. 1988; Felix and Ljungdahl 1993; Berberich et al. 2000a). Its ability to degrade plant fiber makes it an attractive candidate for the efficient conversion of biomass to bio-fuel; however, the commercialization of biofuel production by C. thermocellum is limited by the coproduction of organic acids (acetate, and lactate); Figure 3.1 (Wiegel 1980; Herrero et al. 1985; Lynd 1989; Beguin and Aubert 1993). In addition to organic solvents, C. thermocellum produces gaseous end-products (H₂ and CO₂). Since this bacterium has industrial relevance and responds to changes in partial gas pressures, we have used it as a model thermophilic microorganism to study metabolism and growth in pressurized cultures.
Figure 3.1  Cellulose fermentation in *C. thermocellum*. (1a) cellulose degradation and (1b) extracellular glucose formation via cellulosome; (2) $H_2$ production via hydrogenase; (3) lactate formation via lactate dehydrogenase; (4) ethanol formation; (5) acetate formation via acetate kinase.

We have developed a high-pressure bioreactor to examine cellulose fermentation by *C. thermocellum* and to extend our previous observations regarding hydrostatic pressure effects in batch cultures. Continuous culture of microorganisms provides for the analysis of the effect of pressure independent from other time-dependent factors associated with batch fermentation (such as growth, substrate and product levels, and cell age) (Kuriyama et al. 1993). Although the effects of dissolved fermentation gases on metabolic activity has been well documented in the past 25 years, attempts to quantify metabolic effects in continuous culture are limited (Mollah and Stuckey 1992; Kuriyama et al. 1993; McIntyre and McNeil 1998). As reviewed by McIntyre and McNeil (McIntyre and McNeil 1998), the few continuous culture experiments investigating dissolved gas effects on growth and metabolic activity are substantially different than batch results.
Experiments involving *C. thermocellum* were conducted under elevated hydrostatic pressure (7.0 and 17.3 MPa at 333 K) to observe the effects of pressure on product formation and selectivity, growth, and substrate utilization. Growth yields and maintenance requirements in continuous cultures conducted at atmospheric pressure and under elevated hydrostatic pressure are compared. Analysis of continuous cultures under elevated pressure, particularly over the large range of pressures that can be explored in this bioreactor, provides insight into the complexities of dissolved gas effects on fermentation and complements existing results in batch cultures.

**Materials and Methods**

**Cell Culture Preparation**

*Clostridium thermocellum* ATCC 31549 was obtained from the American Type Culture Collection (Rockville, MD). Since this culture is actually a co-culture of *C. thermocellum* JW20 and *Thermoanaerobacter ethanolicus*, the clostridial strain was isolated as previously described (Erbeznik et al. 1997). The organism was grown in a basal medium that included (per liter): 0.61 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 0.5 g of (NH₄)₂SO₄, 0.5 g of NH₄Cl, 0.09 g of MgCl₂·6H₂O, 0.03 g of CaCl₂, 0.5 g of cysteine, 4 g of Na₂CO₃, 2 g of yeast extract, 10 mL of a vitamin mixture (Cotta and Russell 1982) and 5 mL of a microminerals mixture (Cotta and Russell 1982).

Cultures were grown on 11.7 mM cellobiose at 333 K and harvested during the later portion of exponential growth by centrifugation (10,000 x g for 20 min at 298 K). Cell pellets were then resuspended with basal media containing yeast extract at 2 g/L. Preliminary experiments demonstrated that the organism cannot grow in the absence of yeast extract (data not shown). Because *C. thermocellum* is sensitive to oxygen, cell manipulations were performed in sealed vials that had been purged with nitrogen.

**Continuous Culture at Atmospheric Pressure**

Continuous culture experiments at atmospheric pressure (0.1 MPa) were performed at 333 K with a New Brunswick MultiGen fermentor (345 ml working volume). After inoculation, the system was kept in batch mode for 24 h before initiating continuous media flow. Deoxygenated media containing 2 g/L cellobiose was fed into
the fermentor using a peristaltic pump (LKB Pharmacia) to achieve the desired dilution rate \((D = 0.05\) to \(0.32\) h\(^{-1}\)). Samples were taken from the reactor at steady-state, which was defined as 98% turnover of the culture vessel contents. The cell culture was continuously mixed using an impeller (300 rpm), and sparged with deoxygenated CO\(_2\) in conjunction with the bicarbonate buffering system. Product gases and sparging CO\(_2\) were vented to maintain atmospheric pressure. The results of the continuous culture at atmospheric pressure are based on multiple chemical analyses of duplicated experiments conducted independently.

**Continuous Culture under Elevated Hydrostatic Pressure**

Fermentation under elevated hydrostatic pressure (7.0 and 17.3 MPa) was carried out at 333 K in a continuously stirred (impeller, 300 rpm) 100 mL stainless steel Parr Mini Reactor (rated to 623 K and 20.7 MPa; Figure 3.2). All tubing and fittings were made of 304 stainless steel and inline unidirectional valves (Nupro model SS-53F2) were installed in the inlet and outlet streams to prevent backflow. A filter (0.5 \(\mu\)m; Nupro model SS-2F-7) located at the feed inlet prevented particulates and microbial contaminants from entering the bioreactor. Pressure was maintained throughout the system by a backpressure regulator (DB Robinson Design and Manufacturing LTD., model BPR-10-316) connected to the outlet stream of the bioreactor. A pressure sensor on the syringe pump and an Ashcroft pressure gauge were used to monitor system pressure, and a rupture disk prevented system over-pressurization.
Prior to experimentation, the feed pump (Isco syringe pump model 500D) and Parr Mini Reactor were sterilized by exposure to absolute ethanol for 24 h. To remove residual ethanol, the pump was rinsed with sterile deionized water, and the Parr reactor was heated to 373 K and continuously sparged with deoxygenated CO₂ for 2 h. After assembly, the entire system was re-sterilized with supercritical CO₂ (\(P_c = 7.4\) MPa; \(T_c = 304.4\) K) at 10 MPa and 308 K for 3 h. The inner cavity of the pump cylinder was depressurized to remove the supercritical CO₂. Media was then loaded into the pump by pulling a vacuum on the empty cavity.

To initiate the continuous cultures, the bioreactor was inoculated and pressurized to the selected operating pressure over 30 min. After pressurization, the culture was maintained in batch mode for 24 h before initiating continuous flow. Media containing 2 g/L cellobiose was fed into the bioreactor to achieve the desired dilution rate (\(D = 0.05\) to 0.3 h\(^{-1}\)). Samples were collected at steady-state (98% turnover) from the effluent stream using an inline sampling valve (Valco model C2-2006) fitted with a 1 ml sample loop. An outlet valve on the reactor was closed to maintain pressure within the reactor during sampling. Samples were collected by rapidly depressurizing the sample loop and flushing with sterile air. After collecting the sample, the loop was first re-pressurized by
slowly introducing media from the bioreactor. After the re-pressurization, media flow was resumed at the desired dilution rate.

**Sample Analysis**

Samples were withdrawn anaerobically from the culture vessels and centrifuged (10,000 x g, 10 min, 277 K) to separate the cells. The resulting supernatant was stored at 269 K until analysis. Bacterial dry cell weights (DCW) were determined using optical density measurement at 600 nm ($OD_{600}$); it was previously determined that one optical density unit corresponds to 0.464 g DCW per liter (data not shown). Samples collected from the high-pressure bioreactor were analyzed by light microscopy at atmospheric pressure immediately after collection to determine if any irreversible morphological changes occurred due to pressure.

Cellobiose, ethanol, glucose, and lactate in supernatant were measured by enzymatic methods as previously described (Bergmeyer 1963; Russell and Baldwin 1978). Acetate concentrations were determined in acidified samples by gas chromatography using a column (1.83 m x 4 mm) packed with Supelco SP-1000 (1% H$_3$PO$_4$, 100/120 mesh). Nitrogen was used as a carrier gas and the inlet and detector temperatures were 458 K and 463 K, respectively. The oven temperatures increased from 398 K to 408 K at a rate of 9°C/min after an initial isothermal period of 0.5 min.

**Results**

**Substrate Utilization and Cell Growth**

Results for atmospheric pressure and pressurized treatments are presented as the average (± standard deviation) between duplicated runs conducted independently (Table 3.1). No irreversible morphological changes were discernable in *C. thermocellum* (a rod-shaped bacterium) in continuous incubations at 7.0 or 17.3 MPa, as analyzed by light microscopy (data not shown). This result indicates that the measurement of cell concentration based on optical density following depressurization of the sample is valid.
Figure 3.3  Cellobiose consumption (A) and cell density (B) as a function of dilution rate ($D$, h$^{-1}$) in continuous cultures of *C. thermocellum* at 0.1 (▲), 7.0 (◇), and 17.3 MPa (×) hydrostatic pressure.
Table 3.1  *C. thermocellum* continuous cultures grown on cellobiose at 0.1, 7.0, and 17.3 MPa hydrostatic pressure. Standard deviation of duplicate runs given in (parenthesis).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dilution Rate (D, h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td><strong>0.1 MPa</strong></td>
<td></td>
</tr>
<tr>
<td>Extracellular Glucose (mM)</td>
<td>0.1 (&lt; 0.05)</td>
</tr>
<tr>
<td>Ethanol (E)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>Acetate (A)</td>
<td>20.2 (0.7)</td>
</tr>
<tr>
<td>Lactate (L)</td>
<td>1.0 (0.2)</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>89.8 (7.4)</td>
</tr>
<tr>
<td><strong>7.0 MPa</strong></td>
<td></td>
</tr>
<tr>
<td>Extracellular Glucose (mM)</td>
<td>0.3 (0.1)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>12.8 (2.0)</td>
</tr>
<tr>
<td>Acetate</td>
<td>8.5 (1.6)</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.0 (0.5)</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>80.6 (0.6)</td>
</tr>
<tr>
<td><strong>17.3 MPa</strong></td>
<td></td>
</tr>
<tr>
<td>Extracellular Glucose (mM)</td>
<td>0.2 (0.1)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>17.6 (3.5)</td>
</tr>
<tr>
<td>Acetate</td>
<td>7.4 (0.3)</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.9 (0.3)</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>81.5 (8.1)</td>
</tr>
</tbody>
</table>

* dilution rates within ± 0.01 h⁻¹
The formation of metabolic end-products (Table 3.1) and the consumption of cellobiose (Figure 3.3A) indicate that fermentation occurs at all pressures examined. In addition, microbial growth is evident in all treatments (Figure 3.3B). The residual cellobiose concentration is similar for cultures grown at 7.0 MPa hydrostatic pressure and atmospheric pressure; however, it increases steadily at dilution rates greater than 0.1 h\(^{-1}\) under 17.3 MPa (Figure 3.3A). While there is a slight increase in extracellular glucose in pressurized cultures, this increase is not statistically significant relative to atmospheric pressure (Table 3.1). Cell growth is reduced by approximately 40% at 7.0 MPa and 60% at 17.3 MPa hydrostatic pressure for the dilution rates investigated (Figure 3.3B).

**Bioenergetic Parameters**

The maximum theoretical growth yield (\(Y_{X/S}^M = \Delta X/\Delta S\), g DCW/g cellobiose consumed) and maintenance requirement (\(m_S\), g cellobiose consumed/g DCW/h) were determined from a Pirt transformation (Pirt 1965; Pirt 1982), which is a plot of the apparent (experimental) growth yield as a function of inverse dilution rate (graphs not shown). The amount of cellobiose converted to extracellular glucose was deducted from the apparent cellobiose consumed when calculating the theoretical growth yield. The maximum theoretical growth yield decreases markedly in pressurized treatments (as much as 2-fold at 17.3 MPa) relative to atmospheric pressure (Table 3.2). The decrease in \(Y_{X/S}^M\) at 17.3 MPa was accompanied by a 3-fold increase in the maintenance coefficient. Comparatively, the values for \(Y_{X/S}^M\) and \(m_S\) determined at atmospheric pressure (Table 3.2) are very similar to those reported by Strobel (Strobel 1995) for *C. thermocellum* LQRI grown on cellobiose (\(Y_{X/S}^M = 0.31\) g DCW/g cellobiose and \(m_S = 0.05\) g cellobiose/g DCW/h).

The growth yield (\(Y_{X/ATP}^M = \Delta X/\Delta AT\), g DCW/mole ATP formed) and maintenance requirement (\(m_{ATP}\), mmol ATP formed/g DCW/h) based on the calculated ATP formation were also determined. Unlike \(Y_{X/S}^M\) and \(m_S\), these parameters account for changes in both substrate consumption and product selectivity, and can be used to compare bioenergetics between different microorganisms. Similar to \(Y_{X/S}^M\), \(Y_{X/ATP}^M\) decreases 0.5-fold at 7.0 MPa and 1.6-fold at 17.3 MPa, relative to atmospheric
pressure (Table 3.2). The maintenance requirement increases approximately 0.4-fold in pressurized treatments (Table 3.2).

**Table 3.2** Growth yield and maintenance coefficients for continuous cultures of *C. thermocellum* at atmospheric pressure and under elevated hydrostatic pressure. Uncertainty associated with parametric analysis is expressed in terms of *(standard deviation)*.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hydrostatic Pressure (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Growth Yield</td>
<td></td>
</tr>
<tr>
<td>$Y_{X/S}^M$ a</td>
<td>0.28 (0.01)</td>
</tr>
<tr>
<td>$Y_{X/ATP}^M$ b</td>
<td>18.1 (1.5)</td>
</tr>
<tr>
<td>Maintenance</td>
<td></td>
</tr>
<tr>
<td>$m_S^c$</td>
<td>0.05 (0.01)</td>
</tr>
<tr>
<td>$m_{ATP}^d$</td>
<td>2.6 (0.5)</td>
</tr>
</tbody>
</table>

| a g DCW/g cellubiose consumed |
| b g DCW/mol ATP formed |
| c g cellubiose/g DCW/h |
| d mmol ATP/g DCW/h |

**End-Product Formation and Selectivity**

Carbon recovery, based on product formation and cellubiose consumption (calculated by equation 3.1), decreases with increasing dilution (growth) rate at atmospheric pressure (Table 3.1). In contrast, there is no clear trend in carbon recovery for pressurized treatments.

\[
\text{% Carbon Recovery} = \frac{\text{Carbon in raffinate}}{\text{Carbon in feed}}
\]

\[
3 \cdot (\text{ethanol (mM)} + \text{acetate (mM)} + \text{lactate (mM)})
12 \cdot (\text{cellubiose (feed, mM)})
+ 12 \cdot (\text{cellubiose (mM)}) - 6 \cdot (\text{glucose (mM)})
12 \cdot (\text{cellubiose (feed, mM)})
\]

Product yield, which is defined as product formation divided by substrate consumption ($Y_{P/S} = \Delta P/\Delta S$, mM/mM), is altered under elevated hydrostatic pressure (Figure 3.4). Ethanol yield is significantly increased in pressurized cultures relative to atmospheric pressure at all dilution rates (Figure 3.4A). For example, at $D = 0.05 \text{ h}^{-1}$
ethanol yield increases from 0.2 at atmospheric pressure to 2.3 at 7.0 MPa and 2.8 at 17.3 MPa. In addition, a decrease in the production of organic acids (acetate and lactate) is observed with pressure (Table 3.1). This inhibition is most significant for acetate yield (Figure 3.4B) at low dilution rates ($D < 0.15 \text{ h}^{-1}$). However, lactate yields are also reduced under elevated hydrostatic pressure at high dilution rates (Table 3.1). For example, at $D = 0.2 \text{ h}^{-1}$ lactate concentration was reduced from 1.2 mM at atmospheric pressure to 0.4 and 0.2 mM at 7.0 and 17.3 MPa hydrostatic pressure, respectively. While there are differences in product yield for pressurized and non-pressurized cultures, an increase in pressure from 7.0 to 17.3 MPa did not dramatically alter product yield.

Although $\text{H}_2$ and $\text{CO}_2$ formation are not determined experimentally, their concentrations in the fermentation broth were calculated (Figure 3.5) using a simplified metabolic flux analysis (Appendix A). Assuming equilibrium conditions in the mixed vessel, the hydrogen formed in continuous cultures at atmospheric pressure (15.6 to 38.3 mM as a function of dilution rate) primarily evolved into the headspace (Figure 3.5B). The aqueous solubility of $\text{H}_2$ at 333 K and atmospheric pressure is 0.7 mM (Harvey 1996). However, for incubations at 7.0 MPa ($[\text{H}_2]_{\text{aq}} = 15.3$ to 19.1 mM) and 17.3 MPa ($[\text{H}_2]_{\text{aq}} = 12.4$ to 16.2 mM) all of the $\text{H}_2$ produced is assumed to be soluble in the aqueous phase. At 7.0 MPa and 17.3 MPa, the aqueous solubility of $\text{H}_2$ is 50.9 and 130.4 mM (333 K), respectively (Harvey 1996).
The calculation of the aqueous CO₂ concentrations considered both the formation of CO₂ by *C. thermocellum* and the amount of CO₂ in the feed media (due to active sparging). The solubility of CO₂ in water under atmospheric pressure is 32.9 and 13.2 mM at 298 and 333 K, representing the sparging and the incubation conditions, respectively (Dean 1979). In this case, all of the CO₂ produced in cultures at atmospheric pressure (11.2 to 20.1 mM) and some of the CO₂ in the initially sparged
media should evolve into the headspace at 333 K. In contrast, all of the CO$_2$ produced during fermentation at 7.0 MPa (20.7 to 26.1 mM) and 17.3 MPa (17.4 to 25.5 mM) is well within the aqueous solubility limit; [CO$_2$]$_{aq} = 913.8$ and 2260.8 mM at 333 K, respectively (Dean 1979). The sparging of the feed media makes a significant contribution to the total [CO$_2$]$_{aq}$ at 7.0 and 17.3 MPa (Figure 3.5A).

![Figure 3.5](image)

**Figure 3.5** Aqueous CO$_2$ and H$_2$ concentration (mM) as a function of dilution rate (D, h$^{-1}$) in continuous cultures operated under atmospheric pressure (■), and 7.0 (○) and 17.3 MPa (×) hydrostatic pressure.

**Discussion**

The growth of *C. thermocellum* JW20, a non-barophilic microorganism, is demonstrated for the first time at elevated pressure in continuous cultures. Enhanced ethanol production and reduced acetate production are similar to results from previous batch fermentations conducted at elevated pressure with non-growing cells (Berberich 2001). Relative to atmospheric pressure controls, however, the increases in the ethanol to acetate ratios (E/A) observed in pressurized continuous cultures are much greater (nearly 60-fold at 7.0 MPa) than those observed in batch cultures with non-growing cells (5.7-fold at 7.0 MPa) (Berberich 2001). The ethanol concentration in pressurized batch cultures of non-growing cells (6 mM; 7.0 MPa) was similar to cultures at atmospheric pressure (5 mM). Thus, the increase in E/A in pressurized batch cultures (relative to
atmospheric pressure) was primarily due to inhibited acetate formation. In contrast, a large increase in broth ethanol concentration and a concomitant decrease in acetate concentration were observed in pressurized continuous cultures relative to incubations at atmospheric pressure (Table 3.1).

The difference in lactate production by pressurized and atmospheric pressure continuous cultures (Table 3.1) is not significant. However, lactate production is reduced in continuous cultures relative to batch cultures with non-growing cells (14 mM at 0.1 MPa; 18 mM at 7.0 MPa hydrostatic pressure) (Berberich 2001). Similarly, Tarhan (Tarhan 2001) observed reduced lactate production from approximately 23.0 mM lactate at 2.0 mM cellobiose/g DCW/h to 0.3 mM at 0.1 mM cellobiose/g DCW/h in fed-batch cultivation of non-growing C. thermocellum. These results suggest that controlling cellobiose feed rate in C. thermocellum through fed-batch or continuous cultivation reduces lactate formation.

The ability to manipulate product selectivity in Clostridia species by controlling the solubility of product gases has been demonstrated in biphasic batch incubation with low pressures of H₂ (pH₂ < 0.3 MPa). Increased ethanol to acetate ratios (E/A) have been noted for C. thermocellum (4 to 170%, (Sudha Rani et al. 1997); 9 to 57%, (Lamad et al. 1988); and 67 to 108%, (Lamad and Zeikus 1980)); and C. saccharolyticum (201%, (Murray and Khan 1983)) incubated in the presence of an H₂ headspace (approximately 0.5 to 2.5 bar) relative to an atmospheric headspace. Similarly, the concentration of dissolved gas can also be controlled by purging the headspace and by varying the rate of agitation. Mistry and Cooney (Mistry and Cooney 1989) observed a decrease in lactate formation and a 149% increase in ethanol production in C. thermosaccharolyticum when the N₂ purge rate was reduced ten-fold in continuous culture. Under non-pressurized conditions, Doremus et al. (Doremus et al. 1985) observed an increase in butanol productivity in batch cultures of C. acetobutylicum by introducing a 0.1 MPa N₂ headspace, and by reducing the agitation rate (rate of hydrogen evolution). Similarly for batch cultures of C. thermocellum, Lamed et al. observed a decrease in E/A with an increase in stirring from 0 to 200 rpm (Lamad et al. 1988). Adding 1.0 and 2.5 atm pH₂ in the headspace reversed the effects of agitation, thereby increasing E/A. Similar to our results, increases in E/A produced by C.
thermocellum grown on cellobiose in batch cultures (strains YS, AS-39, and LQRI) were a result of both an increase in ethanol formation and a decrease in acetate formation due to the accumulation of H₂ in the broth (Lamad et al. 1988).

Previous work in our laboratory demonstrated shifts in product selectivity of non-growing C. thermocellum metabolizing cellobiose at hydrostatic pressures as low as 0.5 MPa in batch culture (Berberich 2001). At 7.0 MPa, ethanol production increased 165% (to 5.8 mM) relative to atmospheric pressure (10 h incubation). In addition, acetate production was reduced 78% (to 1.7 mM), with essentially no change in lactate production. Surprisingly, acetate production in the presence of compressed H₂ at 7.0 MPa (26% decrease relative to atmospheric pressure) was much greater than that obtained under 7.0 MPa hydrostatic pressure or a 7.0 MPa N₂ headspace (40% decrease). Therefore, the degree of acetate inhibition in these pressurized batch incubations was not directly related to the concentration of dissolved H₂. These results suggest that metabolic activity at moderate pressure is not strictly dictated by dissolved gas concentration, but is also affected by the presence of an aqueous/pressurized gas interface, the method of pressurization, and/or by pressure alone.

Under the pressures employed in this study, all of the H₂ and CO₂ formed is soluble in the aqueous phase. Thus, metabolic pathways involving these dissolved product gases, particularly [H₂]ₐq (Lamad and Zeikus 1980; Lamad et al. 1988; Sudha Rani et al. 1997), may be altered under elevated hydrostatic pressure. Furthermore, since gases can partition into the membrane bilayer and cause a disordering effect (Chin et al. 1976; Jones and Greenfield 1982; Xiang and Anderson 1999), membrane-based perturbations are also possible. Based on the interaction between CO₂ and the bilayer, Jones and Greenfield (Jones and Greenfield 1982) and Kuriyama et al. (Kuriyama et al. 1993) suggested that the cell membrane is the likely target for growth inhibition under elevated CO₂. In addition, Chin et al. (Chin et al. 1976) showed significant disordering of phosphatidylcholine bilayers in aqueous suspension saturated with H₂ at 10.3 MPa and 293 K. Such interactions could contribute to the general depression of microbial growth observed at elevated hydrostatic pressures.
Substrate Utilization and Bioenergetic Parameters under Elevated Hydrostatic Pressure

Cellobiose utilization at atmospheric pressure and 7.0 MPa hydrostatic pressure is similar, as indicated by the residual cellobiose concentration (Figure 3.3A). However, decreased substrate utilization is observed at 17.3 MPa with increasing dilution rates (Table 3.2), and this observation is consistent with a reduced theoretical maximum growth rate. Extracellular glucose concentrations appear to increase in pressurized incubations; however, the increase is not statistically significant relative to atmospheric pressure. Increasing levels of extracellular glucose could be an indication that more cellobiose is being converted to glucose externally rather than being transported into the cell (Figure 3.1, path 1b) (Strobel 1995). Such an increase would be consistent with inhibition of the cellobiose membrane-transport system, possibly due to pressure and/or dissolved gas effects on the cellular membrane.

The maintenance coefficient represents the rate of energy consumption the cell requires to maintain an energized membrane, repair damaged cellular components, transfer components into and out of the cell, and for motility (Shuler and Kargi 1992). A comparison of maintenance energy as a function of hydrostatic pressure and solvent environment can be regarded as an indirect measure of the energy expenditures required to subsist under elevated pressure. Increases in \( m_S \) and \( m_{ATP} \) suggest that there may be detrimental changes in membrane fluidity in pressurized continuous cultures. Increasing hydrostatic pressure reduces the amount of microbial biomass produced per unit of cellobiose consumed, and increases the amount of cellobiose required to maintain proper cell function. In addition, a decrease in the growth of \( C. \) thermocellum was accompanied by an increase in the rate of ATP consumption for maintenance.

Initially, we hypothesized that the elevated solubility of \( H_2 \) and \( CO_2 \) within the fermentation broth due to pressure may lead to the accumulation of gases within the membrane, causing the decrease in growth yield and increase in maintenance. Kuriyama et al. (Kuriyama et al. 1993) demonstrated the relationship between \( CO_2 \) accumulation and growth yield, reporting a linear decrease in \( Y_{X/ATP}^M \) for aerobic yeast fermentation with sufficient oxygen supply under increased \( CO_2 \) partial pressure (pCO\(_2\).
However, based on the similar H₂ and CO₂ formation, there is not a significant difference between H₂ and CO₂ broth concentrations at 7.0 and 17.3 MPa (Figure 3.5). Despite the similarity in dissolved gas concentrations in the bulk aqueous phase, pressure may alter the partitioning of product gases into the membrane. In many instances pressure also acts to reverse the fluidizing effects of dissolved gases (Braur et al. 1982). Thus, we are not able to delineate the individual effects of pressure and dissolved gases due to the coupled nature of these variables and the competing effects of pressure and dissolved gases on biomembranes.

However, our observations demonstrate a near-linear relationship between hydrostatic pressure and the growth yield and maintenance requirement with respect to both cellobiose consumption and ATP formation; $Y_{X/S}^M$ and $Y_{X/ATP}^M$ decrease with increasing hydrostatic pressure, while $m_S$ and $m_{ATP}$ increase (Table 3.2). The relationships observed between the bioenergetic parameters and hydrostatic pressure are consistent with the findings of Tyurin-Kuz'min (Tyurin-Kuz'min 1997), that show a linear increase in the viscosity of a model phospholipid membrane (decrease in fluidity) with increasing hydrostatic pressure. Considering the low concentration of dissolved product gases in the broth relative to saturation (Figure 3.5), changes in growth yields and maintenance requirements may be influenced by the bilayer-ordering effects of pressure, which lead to an increase in membrane viscosity (Tyurin-Kuz'min 1997; Bartlett 2002).

As membrane fluidity changes, the cell adjusts the composition of the lipid bilayer to maintain an optimal viscosity (known as the homeoviscous response), which increases the maintenance requirement. This response can be to fluidize a rigid membrane (due to increasing hydrostatic pressure (Tyurin-Kuz'min 1997; Bartlett 2002)), or “stiffen” a fluid membrane (due to the accumulation of dissolved gases (Chin et al. 1976; Kuriyama et al. 1993)). Such self-regulating changes in membrane structure may require additional energy for lipid synthesis, leading to additional ATP consumption for maintenance. Thus, changes in metabolism in microbial cultures under elevated hydrostatic pressure may be partially due to the concomitant effects of pressure and increased dissolved gas concentration on the cell membrane.
**Product Selectivity under Elevated Hydrostatic Pressure**

Based on increased ethanol formation and decreased acetate formation (Figure 3.4), drastic increases in $E/A$ are observed in continuous cultures under elevated hydrostatic pressure relative to atmospheric pressure. At $D = 0.05 \text{ h}^{-1}$, $E/A$ increases from 0.04 at atmospheric pressure to 1.51 and 2.39 at 7.0 and 17.3 MPa, respectively. Comparatively, a higher dilution rate (0.2 $\text{ h}^{-1}$) leads to an increase from 0.24 at atmospheric pressure to 1.59 at 7.0 MPa, and 2.02 at 17.3 MPa.

The effect of dissolved $\text{H}_2$ on the metabolism of thermophilic bacteria is frequently described as a mass-action effect, which acts upon the regulation of reduced and oxidized electron carriers in the metabolic pathway (Lamad et al. 1988). Ultimately, changes in the redox balance influence acetate production and subsequently $E/A$. Comparatively, the formation of acetate from acetyl-CoA (Figure 3.1, path 5) is energetically favorable to ethanol production, as ATP is generated (Murray and Khan 1983). However, in order for *C. thermocellum* to produce a single acetate molecule (with a concomitant ATP molecule), two $\text{H}_2$ gas molecules must also be formed so that an oxidation/reduction balance is maintained. Increasing $[\text{H}_2]_{\text{aq}}$ shifts the flow of electrons from reduced ferrodoxin (FdH$_2$) to NAD$^+$, causing the reaction $\text{NADH} + \text{H}^+ \leftrightarrow \text{NAD}^+ + \text{H}_2$ to become thermodynamically unfavorable (Lamad and Zeikus 1980; Murray and Khan 1983; Doremus et al. 1985; Lamad et al. 1988). Elevated levels of $[\text{H}_2]_{\text{aq}}$ inhibit acetate production and increase the concentration of NADH. This increase provides more reducing equivalents that are further utilized for ethanol production (Figure 3.1, path 4; acetyl-CoA + 2 NADH $\leftrightarrow$ ethanol + 2 NAD$^+$) (Murray and Khan 1983). Increased ethanol formation in *Clostridia* under elevated $\text{H}_2$ concentrations has been demonstrated in batch (Murray and Khan 1983; Lamad et al. 1988) and continuous cultures (Mistry and Cooney 1989). It has also been shown that the membrane-bound hydrogenase enzyme is inhibited as $[\text{H}_2]_{\text{aq}}$ increases, which further reduces $\text{H}_2$ production (Figure 3.1, path 2) (Lamad and Zeikus 1980; Doremus et al. 1985).

Reduced pH due to the formation of carbonic acid from aqueous $\text{CO}_2$ may also impact individual reaction rates. At near-atmospheric partial pressures of $\text{CO}_2$, the relative concentrations of aqueous carbon dioxide and carbonic acid are adequately buffered inside the cell, suggesting that pH is not the predominant effect of $\text{CO}_2$ on
reaction rates (Jones and Greenfield 1982). Continuous cultures of *C. thermocellum* are buffered with 4 g/L Na$_2$CO$_3$, which maintains pH 6.7 in atmospheric cultures based on the CO$_2$ solubility at 333 K and 0.1 MPa. However, under elevated hydrostatic pressure, a reduction in pH occurs as all of the CO$_2$ present in the system (due to sparging and production) is soluble in the fermentation broth. Based on the concentration of CO$_2$ within the media at 7.0 and 17.3 MPa hydrostatic pressure (Figure 3.5), pH could be reduced to roughly 6.2 (based on the Henderson-Hasselbalch equation). Acidification may alter the dissociation of acid products, as well as H$_2$ formation via regulation of electron carriers (ferrodoxin), NADH concentration, and the reduction of pyruvate to acetyl-CoA.

The shift in product selectivity (Figure 3.4) from acetate (at atmospheric pressure) to ethanol (at elevated hydrostatic pressure) is expected on the basis of increased [H$_2$]$_{aq}$ in batch incubations of *C. thermocellum*. However, H$_2$ concentration is not solely responsible for the shifts in metabolism and bioenergetics under elevated pressure. Our previous investigation demonstrates that batch incubation in the presence of 7.0 MPa exogenous H$_2$ results in an acetate reduction of only 26% (relative to atmospheric pressure). This is comparable to the decreased acetate production at significantly lower H$_2$ concentrations in batch cultures, which were achieved by controlling the rate of agitation (Lamad et al. 1988) and by introducing a 0.1 MPa pH$_2$ headspace (Lamad and Zeikus 1980). Thus, inhibition of acetate production is not directly related to H$_2$ concentrations in elevated pressure incubations. This suggests that changes in metabolism and growth of *C. thermocellum* under elevated hydrostatic pressures are due to both mass-action effects and changes in membrane fluidity, which lead to product selectivity, reduced growth yield, and increased maintenance requirements.

**Conclusions**

Identifying the effects of pressure on microbial growth and product formation is important for the design of fermentation processes involving pressurized conditions such as supercritical fluid extraction, syngas conversion, and high-pressure cultivation of non-barophilic microorganisms. The development of pressurized continuous culture
techniques is needed to determine the influence of pressure on bioenergetic parameters. We have demonstrated that \textit{C. thermocellum} grows in continuous cultures under elevated hydrostatic pressure. The observed shifts in product selectivity (toward ethanol) with hydrostatic pressure were consistent with previous batch experiments at near-atmospheric partial pressures of hydrogen, as well as high-pressure batch experiments employing non-growing \textit{C. thermocellum}.

The mechanism of dissolved gas effects on metabolic activity is complex, with confounding microbe-dependent thermodynamic and mass action effects. The high ethanol to acetate ratios achieved in pressurized incubations of \textit{C. thermocellum} are primarily attributed to the mass action effect of the enhanced concentration of H$_2$ in the fermentation broth, although this effect does not describe the ability to form acetate under elevated hydrostatic pressure. Significant decreases in growth yield, and concomitant increases in maintenance requirement, in pressurized treatments suggest that alterations in growth and product selectivity are also related to changes in membrane fluidity. Based on the linearity between growth yields/maintenance and hydrostatic pressure and the small change in product gas formation from 7.0 to 17.3 MPa, it is evident that pressure alone is altering membrane order and the subsequent energy requirement for maintaining an optimal fluidity. However, changes in membrane fluidity may also be due the accumulation of dissolved H$_2$ and CO$_2$, which stems from increased gas solubility under pressure.

The ability to quantify pressure effects through continuous culture experiments can be used to achieve a balance between growth and yield as a function of pressure for non-barophilic microorganisms, assist in the selection of pressures and solvents for \textit{in situ} compressed or supercritical fluid extraction, and screen microorganisms that are adapted to growth under pressurized conditions.

**References**


Chapter 4

Liposome Fluidization and Melting Point Depression by Pressurized CO₂ Determined by Fluorescence Anisotropy

Introduction

Compressed and supercritical CO₂ has gained increasing interest as alternative solvents in bioprocessing and biotechnology due to (i) tunable solvent strength with small changes in temperature and pressure (ii) enhanced mass transfer relative to liquid solvents due to low kinematic viscosity, and (iii) the ability to completely separate solvent from product upon system depressurization (Randolph 1990; Jarzebski and Malinowski 1995). Pressurized CO₂ \( (T_c = 304.8 \text{ K}; P_c = 7.4 \text{ MPa}) \) has been utilized in pharmaceutical formulation (Kim 1998; Reverchon 1999), enzyme catalysis (Chrischoou 1996; Nakaya 2001), and bio-based extraction (van Eijs 1988; Beckman 1996; Reverchon 1997). In addition, pressurized CO₂ has been contacted with aqueous solutions of whole cells or model cellular membranes, a demonstrated technique for the inactivation of a broad range of microorganisms (Haas 1989; Lin 1992; Dillow 1999; Sims 2002; Spilimbergo et al. 2002) and the formation or processing of liposomes (Batzri and Korn 1973; Frederiksen et al. 1997; Castor and Chu 1998; Otake et al. 2001), which are commonly used for drug delivery applications and gene therapy (Metselaar et al. 2002; Ulrich 2002).

Despite numerous applied studies employing compressed and supercritical CO₂ for sterilization, the mechanism of whole cell inactivation remains unclear. Spilimbergo et al. (2002) recently summarized the proposed inactivation mechanisms in their investigation of the sterilization of \textit{Pseudomonas aeruginosa} and \textit{Bacillus subtilis}. It was suggested that inactivation is due to (i) diffusion of CO₂ into the cells leading to a decrease in pH and a subsequent loss of activity of key enzymes, and (ii) extraction of intracellular substances, including phospholipids, by CO₂. Further analysis determined a high solubility of CO₂ in model cell membrane phospholipids (based on a binary CO₂-phospholipid system), suggesting that (iii) the enhanced permeability of the membrane in the presence of CO₂ contributed to the inactivation of the cells. While cell membranes
are frequently described as being in a fluid state (Singer and Nicolson 1972), probing the fluidity of gel and fluid bilayers is of interest as they coexist in biological membranes during many cellular processes, such as cell division (Rapp et al. 2001). In addition, microbial sterilization and liposome formation can occur over a wide range of temperatures, which may span the gel of fluid phases.

The phospholipid bilayer membrane is susceptible to structural and functional changes due to the dissolution of solvent or gas molecules into the aqueous phase and their subsequent partitioning into the membrane. Disordering of biological membranes due to the presence of dissolved gases (particularly anesthetics) and liquid solvents has been well documented, as summarized by recent review articles (Brauer 1982; Weber and de Bont 1996; Frangopol and Mihaiescu 2001). The conflicting theories on the mechanism of anesthesia include the hypothesis that a critical volume expansion of the bilayer occurs due to solvent or gas partitioning into the hydrocarbon region of the cell membrane. This expansion “fluidizes” the membrane, reducing the viscosity of the bilayer and the function of integral membrane proteins. Alternatively, the release of surface-bound water due to anesthetic partitioning near the phospholipid headgroups has also been proposed as the primary cause of anesthetic action (Ueda 1999). Surface dehydration influences the phospholipid packing density by altering electrostatic interactions between polar heads. In both cases, bilayer permeability and stability are compromised.

The ability to significantly vary the aqueous solubility of solvents with temperature and pressure is unique to compressible and supercritical fluids. Variations in solubility could potentially lead to large changes in the concentration of the solvents within the lipid bilayer. To date, there have been no fundamental investigations of membrane fluidization by pressurized solvents, particularly CO₂, with a goal of manipulating liposome or cellular membrane properties. However, the anesthetic effect of CO₂ on living organisms is well documented at near-atmospheric pressure (see Wisoff 1928, Leake and Waters 1929, and Black 1935), and is likely dependent upon CO₂ concentration within the bilayer (Simon 1980), and the presence of [H⁺] or [HCO₃⁻] due to carbonic acid formation (Sears and Eisenberg 1961; Jones and Greenfield 1982). Figure 4.1 is a schematic depicting the location of DPH within the bilayer, and
fluidization of the membrane due to the partitioning of dissolved solutes and the presence of charged species.

![Diagram of fluidization of a phospholipid bilayer labeled with the fluorescent probe 1,6-diphenylhexatriene (DPH) due to (i) the partitioning of dissolved solutes into the bilayer and (ii) interactions between charged species and the hydrophilic phospholipid headgroups.]

**Figure 4.1** Fluidization of a phospholipid bilayer labeled with the fluorescent probe 1,6-diphenylhexatriene (DPH) due to (i) the partitioning of dissolved solutes into the bilayer and (ii) interactions between charged species and the hydrophilic phospholipid headgroups.

Steady-state fluorescence polarization has been used to determine the fluidizing effects of anesthetic gases and liquids on phospholipid liposomes under atmospheric (Vanderkool et al. 1977) and elevated pressure (halothane, 0.1 to 50 MPa (Kamaya et al. 1979)), and bilayer ordering by elevated hydrostatic pressure (0.1 to 60.8 MPa) (Tyurin-Kuz'min 1997). A common membrane probe is the cylindrical molecule 1,6-diphenyl-1,3,5-hexatriene (DPH), which is positioned parallel to the hydrocarbon tails deep within the bilayer (Kaiser 1998). The anisotropic nature of the embedded fluorescent probe reflects the local bilayer viscosity, and is related to the configurational entropy (degree of chain ordering) in the hydrocarbon core (Lakowicz 1979).
Membrane fluidity of DPH-labeled dipalmitoyl phosphatidylcholine (DPPC, $T_m \approx 315 \text{ K}$) vesicles was measured by steady-state fluorescence anisotropy as a function of pressure and temperature in the presence of excess CO$_2$ (two-phase system). The measurements of membrane fluidity were used to determine the effect of CO$_2$ pressurization on liposome fluidization at 295 and 333 K (isothermal; 3.5 to 13.9 MPa $P_{CO_2}$) and melting point depression at 1.8, 7.0, and 13.9 MPa $P_{CO_2}$ (isobaric; 295 to 323 K). The role of decreasing pH and increasing carbonic acid concentration with increasing CO$_2$ pressure was examined through isobaric measurements of the melting temperature in partially buffered liposome solutions (100, 500, and 1000 mM Na$_2$CO$_3$). Pressure-induced ordering within the bilayer was determined directly from isothermal and isobaric anisotropy measurements in the absence of CO$_2$. The technique is extended to a thermophilic bacterium, *Clostridium thermocellum*, where fluidization at 333 K is compared to DPPC liposomes. DPPC bilayer fluidization by excess pressurized CO$_2$ was analyzed by conventional anesthetic melting point depression theory and related to the concentration of CO$_2$ in the gel and fluid phase.

**Materials and Methods**

**Chemicals**

L-α-dipalmitoylphosphatidylcholine (DPPC, > 99%) was purchased from Sigma Chemical Company. The membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH, > 99%), was purchased from Molecular Probes (Eugene, OR). Ultra high purity CO$_2$ (> 99.99%) and N$_2$ (> 99.99%) were obtained from Scott Gross Co. Chloroform (> 99.9%) and tetrahydrafuran (> 99.9%) were purchased from Fisher Scientific and Mallinckrodt Chemicals, respectively. KH$_2$PO$_4$-NaOH (50 mM) pH 7 buffer from Fisher Scientific was filtered (Whatman filter #1, 11 μm) before use. Deionized ultra-filtered water (DIUF, Fisher Scientific) was used without further purification. Sodium carbonate (Na$_2$CO$_3$) was obtained as an anhydrous powder from Matheson Company, Inc.
Unilamellar Liposome Preparation

Aqueous stock solutions of 1 mM DPPC labeled with DPH (1:500 molar ratio probe to lipid) were prepared following the procedure of Bingham et al. (1965). Briefly, solutions of DPPC dissolved in chloroform (~ 1:50 wt. ratio DPPC to chloroform) and DPH dissolved in tetrahydrafuran (0.1 mM stock) were mixed and co-evaporated under a gentle stream of N₂ until a dry DPPC/DPH film remained. Vacuum was used (~ 15 min) to remove residual solvent from the film. The film was then hydrated with either pure DIUF water, KH₂PO₄-NaOH pH 7 buffer, or aqueous Na₂CO₃ solutions (100, 500, and 1000 mM) and maintained above the melting temperature (T > 315 K) in a water bath for 1 hour before shaking. The DPPC/DPH film was suspended as multilamellar liposomes by vigorously shaking for approximately 1 hour. The liposomes were sonicated before use for 20 min at T > 315 K, which has been previously shown to yield unilamellar liposomes (Vemuri and Rhodes 1995). To obtain a working solution, the stock was diluted tenfold with DIUF water or pH 7 buffer.

Preparation of DPH-Labeled Clostridium thermocellum

Clostridium thermocellum were labeled following the procedure of Baut et al. (1994). Cells were grown as previously described (Chapter 3) to a cell density of approximately 0.464 g dry cell weight/L and centrifuged (5 min, 4000 rpm) to obtain a cell pellet. Cell density was determined from optical density at 600 nm (Chapter 3). The cells were washed by repeatedly suspending the pellet with a 0.9 wt% saline solution (distilled water) followed by centrifugation. The pellet was resuspended with 100 mL of the saline solution and a 2 mL aliquot of the tetrahydrafuran/DPH (0.1 mM) solution was added. The suspension was shaken by hand, incubated for 20 min, and re-centrifuged to remove residual tetrahydrafuran. DPH-labeled C. thermocellum were obtained in a suspension containing 0.56 g dry cell weight/L in 100 mL saline solution. Assuming all available DPH was embedded within the cell membrane; the ratio of DPH to cell protein (mg-basis) was 1:9577.
Fluorescence Anisotropy Measurements

High pressure anisotropy of DPH in DPPC vesicles was measured using a custom designed stainless steel variable volume view cell (10-25 ml working volume, rated to 20.7 MPa) from Thar Technologies (Pittsburgh, PA) mounted within the sample compartment of a Varian Cary Eclipse fluorescence spectrophotometer (Walnut Creek, CA; Figure 4.2). The spectrophotometer was fitted with a Varian manual polarizer for both excitation and emission. A xenon pulse lamp was used as the light source, with an operational wavelength range of 200-900 nm (±1.5 nm accuracy; ±0.2 nm reproducibility). The spectroscopic cell was temperature controlled using an Omega controller (model CN9000A) with heating tape and was continuously mixed with a magnetic stirrer. Steady-state DPH anisotropy within the DPPC bilayer was determined at $\lambda_{ex} = 350$ nm and $\lambda_{em} = 452$ nm with an excitation slit width of 5 nm, an emission slit width of 10 nm, and a one second average sampling time.

Figure 4.2 Apparatus for high-pressure fluorescence anisotropy measurements.

Experiments at isothermal conditions were conducted by adjusting the temperature of the liposome-loaded cell to 295 or 333 K. Under elevated hydrostatic pressure (in the absence of CO$_2$), experiments were performed in a single aqueous phase (containing liposomes) exposed to successive increases in pressure (3.5 to 20.7 MPa).
MPa). Pressure increases were achieved in the variable volume spectroscopic cell by applying back-pressure to a floating piston using a hand operated hydraulic pump. DPH anisotropy was measured after 5 minutes of equilibration under hydrostatic pressure. For isothermal measurements in the presence of CO₂, the view cell was sparged and loaded with a working DPPC solution (15 mL). The headspace (~ 5 ml) was then filled with pressurized CO₂ supplied using an Isco syringe pump (Lincoln, NE, model 500D). The total pressure within the variable volume high-pressure cell was maintained by supplying pressurized CO₂ at constant pressure using the syringe pump. The initial pressure was adjusted to 3.5 MPa, and increased successively to 7.0, 10.3, and 13.9 MPa. At each pressure increment, the system was equilibrated for a minimum of 15 minutes at constant temperature and pressure before the anisotropy was measured.

The determination of DPH anisotropy at isobaric conditions was conducted by sparging the variable volume view cell, loading it with a working DPPC solution (15 mL), and pressurizing with excess CO₂ (1.8, 7.0, and 13.9 MPa). The view cell was then heated to 323 K and equilibrated for 15 minutes. DPH anisotropy was measured at 1 or 2 K increments as the cell was cooled to ~ 295 K over a period of 3 hours at constant CO₂ pressure.

Fluorescence anisotropy \(<r>\) is defined as:

\[
< r > = \frac{I_{VV} - I_{VH}}{(I_{VV} + G \cdot I_{VH})}
\]  \hspace{1cm} (4.1)

where \(I\) represents the fluorescence emission intensity, \(V\) and \(H\) represent the vertical and horizontal orientation of the excitation and emission polarizers, and \(G = (I_{HV})/(I_{HH})^\dagger\) accounts for the sensitivity of the instrument towards vertically and horizontally polarized light (Lakowicz 1999). In the presence of pressurized CO₂, absolute anisotropy \(<r>_{CO₂ pressure}\) includes the combined effects of both dissolved CO₂ and pressure. The reported anisotropy values for experiments conducted under isothermal conditions in the presence of pressurized CO₂ were normalized with respect to hydrostatic pressure to account for the effect of increased bilayer ordering on DPH anisotropy:

\[
< r >_{\text{norm}} = \frac{< r >_{CO₂ pressure}}{< r >_{\text{hydrostatic pressure}}}
\]  \hspace{1cm} (4.2)
where a value of $<r>_{norm} = 1$ indicates that there was no change in anisotropy due to the presence of pressurized CO$_2$ relative to the equivalent hydrostatic pressure. Results for anisotropy are the average of five consecutive measurements for a single solution at each experimental condition. Experimental uncertainty of DPH anisotropy measurements were less than 5%.

The DPPC chain melting temperature ($T_m$) was determined from plots of absolute anisotropy ($<r>$) as a function of temperature under isobaric conditions. Above and below $T_m$, the hydrocarbon core of the bilayer exists as a liquid-crystalline lamellar (or fluid) phase or as an ordered gel phase, respectively. DPH anisotropy values in a fluid DPPC phase are significantly lower than in a gel phase; therefore, upon cooling the fluid-gel phase transition is identified by an abrupt increase in anisotropy. The temperature at the midpoint of this transition is taken as $T_m$, and $\Delta T$ is the temperature range associated with the phase transition.

**Analysis of Aqueous and Bilayer Phase Compositions**

The concentration of CO$_2$ in the aqueous phase and the aqueous/bilayer CO$_2$ partitioning coefficient were needed to determine the amount of CO$_2$ in the bilayer, and the role of CO$_2$ concentration on membrane fluidity and melting point depression. Aqueous solubilities of CO$_2$ ($x_{CO_2}^{aq}$) was determined by the Krichevsky-Kasarnovskiy equation for gas solubility (Krichevsky and Kasarnovskiy 1935) using reported CO$_2$/water Henry’s constant (Harvey 1996) and the partial molar volume of CO$_2$ in water calculated according to Lyckman et al. (Lyckman et al. 1965). The fugacity coefficient of pure CO$_2$ as a function of pressure was determined by the Peng-Robinson equation of state (Reid et al. 1977). Calculated aqueous CO$_2$ solubilities were in excellent agreement (> 99%) when extended to conditions of available CO$_2$/H$_2$O phase equilibria (298.15 K; 7.6 to 20.3 MPa (King et al. 1992)).

The solubility of CO$_2$ in phospholipid bilayers has not been measured as a function of pressure. Therefore, CO$_2$ solubility within the fluid-phase bilayer ($x_{CO_2}^{f}$) was estimated from $\log P_{aw}$ (the natural logarithm of the $n$-octanol/water partition coefficient) predicted on a mole fraction basis using a group contribution equation of state (GCA-
The following equation was used to approximate the molar quantities \((\log P_{o/w})\) from mole fraction quantities (Berberich et al. 2000):

\[
\log \left( \frac{M'_o}{M'w} \right) = \log \left( \frac{x'_o}{x'w} \right) - 0.82
\]

(4.3)

where \(x_i\) is the mole fraction and \(M_i\) is the molarity of the solute in the octanol-rich (o) or water-rich (w) phase. The following empirical correlation was used to determine the partition coefficient of \(\text{CO}_2\) between the bilayer and water, \(\log P_{m/w}\) (Sikkema et al. 1994):

\[
\log P_{m/w} = 0.97 \cdot \log P_{o/w} - 0.64
\]

(4.4)

The calculated \(\log P_{m/w}\) was essentially constant over the range of temperatures and pressures investigated in this work (i.e., \(\log P_{m/w} \approx -0.25\) molar basis; 1.38 mole fraction basis).

Carbonic acid formation due to dissolved \(\text{CO}_2\) was calculated by conventional methods (Harris 1948):

\[
\begin{align*}
\text{CO}_2(aq) + H_2O & \rightleftharpoons H_2\text{CO}_3(s) \iff H^+ + H\text{CO}_3^- \\
H^+ + H\text{CO}_3^- & \rightleftharpoons H^+ + \text{CO}_3^{2-}
\end{align*}
\]

where \(K\) is the ratio \([\text{H}_2\text{CO}_3][\text{CO}_2(aq)]\)^{-1}, and \(K_{a1}\) and \(K_{a2}\) are the first and second acid dissociation constants, respectively. The carbonate concentration, \([\text{CO}_3^{2-}]\), is neglected as \(K_{a1} \gg K_{a2}\). Based on \([\text{CO}_2(aq)] >> [\text{H}_2\text{CO}_3]\) (Harris 1948), the aqueous pH in the presence of pressurized \(\text{CO}_2\) was determined using the Henderson-Hasselbalch equation:

\[
pH = -\log K_{a1} + \log \left( \frac{[\text{HCO}_3^-]}{[\text{CO}_2(aq)]} \right)
\]

(4.5)

where \([\text{HCO}_3^-]\) equals \([H^+]\) in an unbuffered system, or the concentration \([\text{Na}_2\text{CO}_3 + H^+]\) in a buffered system, and \(K_{a1} = 4.45 \times 10^{-7}\) (Harned and Davis 1943). The ionic activity coefficients were assumed constant at unity.

Equation 4.5 was tested against a detailed model presented by Holmes et al. (Holmes et al. 1999), which was also developed to calculate pH in pressurized \(\text{CO}_2\) systems with aqueous \(\text{Na}_2\text{CO}_3\) buffering. This model utilizes experimental solubility data.
and incorporates the activity of ionic species. At 308.15 K and 34.5 MPa $P_{CO_2}$, pH was predicted at approximately 5.2, 5.7, and 6.1 with 100, 500, and 1000 mM Na$_2$CO$_3$ buffering of a bulk aqueous phase, respectively (Holmes et al. 1999). Based on the approach presented herein, pH calculated under the same conditions (pH = 5.2, 5.9, and 6.2, respectively) are similar to those predicted by Holmes et al. (Holmes et al. 1999). The calculated aqueous/fluid-bilayer CO$_2$ solubility, pH, and results for melting point depression for liposomes suspended in pure water at 1.8, 7.0, and 13.9 MPa $P_{CO_2}$ are summarized in Table 4.1.

Table 4.1  Bilayer fluidization of DPPC liposomes in pure water by pressurized CO$_2$ (in excess).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$P_{CO_2}$ (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>Aqueous phase</td>
<td></td>
</tr>
<tr>
<td>$x_{CO_2}^{aq}$ (x10$^{-2}$)</td>
<td>0.7</td>
</tr>
<tr>
<td>pH (at $T_m$) b</td>
<td>3.4</td>
</tr>
<tr>
<td>DPPC bilayer</td>
<td></td>
</tr>
<tr>
<td>$x_{CO_2}^{f}$ (x10$^{-1}$)</td>
<td>1.8</td>
</tr>
<tr>
<td>$x_{CO_2}^{g}$ (x10$^{-1}$)</td>
<td>0.1</td>
</tr>
<tr>
<td>$T_m$ (K)</td>
<td>310.2</td>
</tr>
<tr>
<td>$\Delta T_m$ (K) f</td>
<td>-4.8</td>
</tr>
<tr>
<td>$\Delta H_{VH}$ (KJ/mol) g</td>
<td>1167</td>
</tr>
</tbody>
</table>

a aqueous solubility at $T_m$ (mole fraction)
b calculated using equation 4.5
c fluid bilayer solubility at $T_m$ (mole fraction)
d based on Log $P_{o/w}$ (GCA-EOS (Gros et al. 1996)) and equation 4.4 (Sikkema et al. 1994)]
e gel bilayer solubility at $T_m$ based on $\Delta H_m$ for large unilamellar liposomes (equation 4.7, mole fraction)
f incorporates pressure-ordering on $T_m$ (increases by 0.22 K/MPa)
g van’t Hoff enthalpy, $\Delta H_{VH} = 2049$ KJ/mol at 0.1 MPa (no CO$_2$ present)

Results and Discussion

Isothermal Liposome Fluidization with Pressurized CO$_2$

Bilayer fluidization was measured under isothermal conditions at 295 and 333 K as a function of $P_{CO_2}$ in liposome suspensions prepared with pure water as well as pH 7 phosphate buffer (Figure 4.3). The effects of solvent and pressure on fluidity were
differentiated by normalizing DPH anisotropy measurements in the presence of CO$_2$ with respect to measurements at the equivalent hydrostatic pressures ($<r>_{\text{norm}}$). At 295 K the absolute DPH anisotropy ($<r>$) in fully hydrated DPPC liposomes existing in the gel phase (no CO$_2$ present) did not change with increasing hydrostatic pressure (up to 20.9 MPa). However, at 333 K $<r>$ increased slightly with hydrostatic pressure, suggesting a linear decrease in the fluid phase fluidity with increasing pressure ($2.8 \times 10^{-4} <r>_{\text{norm}}$/MPa; data not shown) over the pressure range investigated (0.1 to 20.9 MPa).

**Liposome Hydration with Pure Water**

For liposomes prepared with pure water, normalized anisotropy decreases with increasing $P_{CO_2}$ (3.5 to 13.9 MPa) from $<r>_{\text{norm}} = 1.02$ to 0.60 and $<r>_{\text{norm}} = 0.93$ to 0.80 at 295 and 333 K, respectively (Figure 4.3). In addition, the sudden decrease in $<r>_{\text{norm}}$ between 3.5 and 7.0 MPa at 295 K appears to indicate CO$_2$-induced gel-fluid phase transition. This decrease in $<r>_{\text{norm}}$ corresponds to a reduction in absolute anisotropy from 0.2 to 0.1, where $<r> = 0.1$ is indicative of a fluid DPPC phase at 7.0 MPa $P_{CO_2}$ (depicted in Figure 4.4). Fluidization at both 295 and 333 K is reversible with system depressurization, as CO$_2$ is removed from the aqueous and bilayer phases. For example, depressurization (degassing) from 13.9 to 0.1 MPa at 295 K for 6 h resulted in a post-treatment $<r>_{\text{norm}}$ that deviated from the pure hydrated DPPC by only 1.4% (data not shown). For the pressure range 3.5 to 13.9 MPa, aqueous CO$_2$ solubility increases from $x_{CO_2}^{aq} = 1.8 \times 10^{-2}$ to $2.7 \times 10^{-2}$ at 295 K and $8.8 \times 10^{-3}$ to $1.9 \times 10^{-2}$ at 333 K. Bulk phase pH is similar at 295 and 333 K for the entire pressure range (pH = 3.1 to 3.3; equation 4.5), suggesting that increased adsorption of CO$_2$ in the bilayer with pressure is the primary cause of fluidization. Furthermore, a minimum $<r>_{\text{norm}}$ is observed at 295 K at approximately 7.0 MPa $P_{CO_2}$, which is consistent with pressure reversal of anesthesia. In this case, elevated pressure increases the order of the acyl chains and reverses the fluidizing effects of CO$_2$. At this point it is unclear why a minimum is not evident at 333 K.
Liposome Hydration with pH 7 Phosphate Buffer

DPH anisotropy within liposomes prepared with pH 7 phosphate buffer decreases from \(<r>_{\text{norm}} = 0.98\) to 0.81 and \(<r>_{\text{norm}} = 0.98\) to 0.90 as \(P_{CO2}\) increases from 3.5 to 13.9 MPa at 295 and 333 K, respectively (Figure 4.3). The conditions of the pH 7 buffered solution (50 mM KH\(_2\)PO\(_4\)-NaOH pH 7) correspond to a bulk pH of approximately 4.9 at 295 K and 5.1 at 333 K between 3.5 and 13.9 MPa \(P_{CO2}\). Comparatively, there is a large reduction in pH in the non-buffered liposome suspensions in the presence of CO\(_2\) (pH 3.1 to 3.3), where a more fluid bilayer is observed (Figure 4.3). Thus, the presence of a buffering component appears to reduce membrane fluidity and stabilize the liposome. The high degree of membrane fluidization observed at 295 K in non-buffered systems is consistent with high concentrations of HCO\(_3^-\) (at low pH). HCO\(_3^-\) may bind to the choline headgroups and yield a lateral expansion of the bilayer due to electrostatic repulsion between neighboring heads. Such an expansion due to anion binding would produce a more disordered bilayer (Tatulyan et al. 1992).
**Isobaric Melting Point Depression by Pressurized CO₂**

Melting point depression in the presence of dissolved CO₂ was determined from DPH anisotropy in aqueous DPPC liposomes suspended with a typical commercially-available pH 7 phosphate buffer (50 mM KH₂PO₄-NaOH) under isobaric conditions at $P_{CO₂} = 1.8$, 7.0, and 13.9 MPa (Figure 4.4). To account for the ordering effects of pressure on the acyl region of the bilayer and the resulting melting temperature ($T_m$), experiments were first conducted under elevated hydrostatic pressure. At atmospheric hydrostatic pressure (0.1 MPa), the gel-fluid phase transition occurs at $T_m = 314.7$ K, and increases approximately 0.22 K/MPa (data not shown) in agreement with previous results (Kamaya et al. 1979; Barkai 1983).

![Figure 4.4](image_url)

**Figure 4.4.** Melting point depression in aqueous DPPC liposomes (50 mM KH₂PO₄-NaOH pH 7 buffer) indicated by DPH fluorescence anisotropy ($\langle r \rangle$). Anisotropy is expressed as a function of temperature (K) under 0.1 MPa hydrostatic pressure and hyperbaric CO₂ pressure ($P_{CO₂} = 1.8$, 7.0, and 13.9).
Accumulation of CO₂ within the bilayer at 1.8 MPa $P_{CO_2}$ yields a DPPC melting temperature of 310.2 K (Figure 4.4), corresponding to an aqueous and fluid-bilayer solubility of $x_{CO_2}^{aq} = 0.007$ and $x_{CO_2}^f = 0.18$, respectively, as determined from equation 4 (Table 4.1). Comparatively, Kamaya et al. (Kamaya et al. 1981) have reported $T_m = 310.2$ for DPPC in the presence of halothane at $x_f = 0.17$, which is very similar to the CO₂ concentration at 1.8 MPa $P_{CO_2}$. As $P_{CO_2}$ is increased from 1.8 MPa to 7.0 and 13.9 MPa the resulting melting temperature converges to 299.2 K, which is a significant reduction relative to both atmospheric pressure and 1.8 MPa $P_{CO_2}$. The reduction in $T_m$ with increasing $P_{CO_2}$, is due to the accumulation of CO₂ within the fluid bilayer (Table 4.1).

The observed change in melting temperature includes both the increase in $T_m$ due to elevated hydrostatic pressure and the reduction in $T_m$ due to the accumulation of CO₂ within the membrane under hyperbaric pressure. To look at the pure solubility effect, we define the melting point depression as the observed melting temperature due to the accumulation of CO₂ minus the melting temperature due to hydrostatic pressure (314.7 K + 0.22 $P_{hydrostatic}$) where $P_{CO_2} = P_{hydrostatic}$. Removing the ordering-effects of hydrostatic pressure at 1.8, 7.0, and 13.9 MPa $P_{CO_2}$ results in $\Delta T_m = -4.9$, -17.0, and -18.6 K, respectively.

In addition to reducing $T_m$ to near-ambient temperature, the presence of CO₂ at 7.0 and 13.9 MPa broadened (or extended) the two-phase gel-fluid phase transition region ($\Delta T_r = 6$ K) relative to atmospheric pressure ($\Delta T_r = 3$ K), 1.8 MPa $P_{CO_2}$ ($\Delta T_r = 4$ K), and elevated hydrostatic pressure (data not shown). This broadening is indicated by the decrease in slope near $T_m$ (Figure 4.4). The results for melting point depression in the presence of excess pressurized CO₂ at 1.8, 7.0, and 13.9 MPa, for liposomes suspended in pH 7 phosphate buffer demonstrate that the bilayer was in a stable gel and fluid state at 295 and 333 K. In addition, fluidization of both the gel and fluid phase is observed in the isobaric fluorescence spectra and is related to the $P_{CO_2}$, consistent with isothermal measurements (Figure 4.3). Significant fluidization at conditions below $T_m$ further suggests that CO₂ is appreciably soluble in the DPPC gel phase.
Effect of Na$_2$CO$_3$ concentration on liposome fluidization

The influence of carbonic acid formation on bilayer fluidization and $T_m$ in the presence of 7.0 MPa $P_{CO_2}$ was determined as a function of Na$_2$CO$_3$ concentration (Figure 4.5). Sodium carbonate is commonly used in fermentation processes and was chosen as a model buffer based in part due to the simple relationship between Na$_2$CO$_3$ concentration, the concentration of dissolved CO$_2$ as a function of pressure, and pH (where Na$_2$CO$_3$ buffers via H$_2$CO$_3$ formation and CO$_2$ evolution). For aqueous suspensions containing 100, 500, and 1000 mM Na$_2$CO$_3$, the DPPC melting temperature is $T_m = 299.2$, 299.7, and 300.2 K at 7.0 MPa $P_{CO_2}$, respectively (Figure 4.5). Under these conditions, bulk phase pH is approximately 5.3, 6.0, and 6.3 when buffered with 100, 500, and 1000 mM Na$_2$CO$_3$ (equation 4.5). Greater anisotropy observed at 1000 mM Na$_2$CO$_3$ over the entire temperature range relative to 100 and 500 mM Na$_2$CO$_3$ indicates a less fluid bilayer with increased buffering.

![Figure 4.5](image)

**Figure 4.5** Melting point depression in aqueous DPPC liposomes indicated by DPH fluorescence anisotropy ($<r>$). Anisotropy is expressed as a function of temperature (K) and Na$_2$CO$_3$ concentration (mM) at 7.0 MPa.
The addition of Na$_2$CO$_3$ affects two solution properties that can influence $T_m$; an increase in ion concentration and a reduction in acidity. Previous work summarized by Koynova and Caffrey (1998) suggests that the melting temperature of DPPC is relatively unaffected above pH 3. Therefore, the pressure-dependent, CO$_2$-induced, pH reduction in the presence of these buffered systems is not expected to influence $T_m$ directly. With respect to ion concentration, it has been reported that $T_m$ is insensitive to monovalent salt concentrations less than 500 mM, and only mildly sensitive to concentrations of 1000 mM (Jacobson and Papahadjopoulos 1975). Comparatively, we have shown that increasing the concentration of Na$_2$CO$_3$ from 100 to 1000 mM to partially buffer carbonic acid formation has virtually no effect on the DPPC melting temperature or the width of the phase transition region (Figure 4.5).

Despite the fact that $T_m$ of DPPC liposomes is insensitive to ion concentration and pH, increasing Na$_2$CO$_3$ concentration significantly reduces membrane fluidity in the gel phase (Figure 4.5). A reduction in membrane fluidity was also observed at 295 K for DPPC liposomes suspended with 50 mM pH 7 phosphate relative to pure water (Figure 4.3). These results are consistent with previous work that suggests stronger cation-dipole (headgroup) interactions occur in the DPPC gel state (Izumitani 1994). However, at this point we do not know whether the reduction in gel phase fluidity with the addition of Na$_2$CO$_3$ can be attributed to changes in ion concentration or pH.

**Membrane fluidization of Clostridium thermocellum**

CO$_2$-induced membrane fluidization was examined in a model thermophilic bacterium, *C. thermocellum*, and compared to DPPC liposomes at 333 K. This temperature corresponds to the optimal growth rate of *C. thermocellum* and the operating temperature of our previous pressurized fermentations in batch (Knutson et al. 1999; Berberich et al. 2000a,b) and continuous cultures (Chapter 3). DPH anisotropy ($<r>$) decreases with increasing $P_{CO_2}$, indicating fluidization of the *C. thermocellum* membrane. Based on absolute anisotropy, *C. thermocellum* membrane is less susceptible to CO$_2$-fluidization than model cell membranes (DPPC liposomes). This resistance may be due to the presence of rigid membrane components, such as carbohydrates, proteins, or cholesterol, which strengthen the bilayer and minimize
structural perturbations (Singer and Nicolson 1972). Investigations such as this, which directly probe fluidization of bacterial membranes, may provide insight into the role of dissolved product gas concentrations on metabolic selectivity and the mechanism of bacterial sterilization with pressurized CO₂.

**Figure 4.6** DPH fluorescence anisotropy (<r>) in DPPC liposomes and labeled *Clostridium thermocellum* as a function of P<sub>CO₂</sub> at 333 K.

**Interpretation of Melting Point Depression by Pressurized CO₂**

The following equation, which is derived from regular solution theory, describes changes in phospholipid melting temperature due to the presence of an impurity (Cevc and Marsh 1987; Evans and Wennerstrom 1999):

\[
\ln \left( \frac{1-x^f_i}{1-x^g_i} \right) + w^f x^f_i - w^g x^g_i = \left[ T_m - T_{m,o} \right] \frac{\Delta H_m}{RT_m T_{m,o}} \tag{4.6}
\]

where x<sup>f</sup><sub>i</sub> and x<sup>g</sup><sub>i</sub> are the mole fractions of solute i in the fluid (f) and gel (g) phase, T<sub>m,o</sub> is the melting temperature of DPPC under hydrostatic pressure, ΔH<sub>m</sub> is the DPPC phase transition enthalpy, P is the system pressure (MPa), and R is the ideal gas constant. The terms w<sup>f</sup> and w<sup>g</sup> represent the solute/acyl-chain interaction energy (due to mixing) with the fluid and gel phase. Calculating melting point depression is typically performed
based on the assumption that the entropic mixing contribution is much greater than the enthalpic contribution (i.e., $w^f$ and $w^g$ are negligible) (Evans and Wennerstrom 1999).

Increases in $T_{m,o}$ due to hydrostatic pressure (no CO$_2$ present) were incorporated into our analysis of melting point depression in the presence of CO$_2$ by modifying equation 4.6. The ordering effects of hydrostatic pressure yield an experimentally observed 0.22 K/MPa increase in $T_{m,o}$. The form of this modification, as shown in equation 7, was based in part on the results of Wann and MacDonald (Wann and MacDonald, 1988) who reported that the effects of inert gases and hydrostatic pressure on lipid bilayer fluidity and melting temperature are additive.

$$\ln\left(\frac{1 - x^f}{1 - x^g}\right) = \left[T_m - (T_{m,o} + 0.22(P - 0.1))]\right]\frac{\Delta H_m}{RT_m T_{m,o}}$$

(4.7)

With calculated values for $x^f$, equation 4.7 can be used to determine the gel phase CO$_2$ concentration ($x^g$) based on the observed melting point depression.

The gel phase CO$_2$ concentration in DPPC bilayers was determined from equation 4.7 by incorporating the observed melting point depression. $\Delta H_m$ does not depend on the nature of the phase transition (Marky and Breslauer 1987) and is therefore assumed constant despite a significant broadening of this region by pressurized CO$_2$ (Figure 4.3). Based on large unilamellar liposomes ($\Delta H_m = 31.4$ KJ/mol (Koynova and Caffrey 1998)), 1.8, 7.0, and 13.9 MPa CO$_2$ pressure (in excess) yield $x^g = 0.01$, 0.13, and 0.20, respectively. CO$_2$ concentration in the gel phase represents roughly 6, 23, and 32% of the fluid phase with increasing $P_{CO2}$. Similarly, basing our analysis on multilamellar liposomes ($\Delta H_m = 24.7$ KJ/mol Koynova and Caffrey 1998)), $x^g = 0.05$, 0.26, and 0.32 at 1.8, 7.0, and 13.9 MPa $P_{CO2}$. The presence of CO$_2$ in the gel phase demonstrate the ability for liposome fluidization over a wide range of temperatures, and are consistent with previous investigations of anesthetic gel phase solubility. For instance, Simon et al. (Simon et al. 1979) have shown that halothane adsorption in the gel phase is near 30% of that in the fluid phase.

Broad phase transition regions observed for DPPC liposomes (hydrated with 50 mM KH$_2$PO$_4$-NaOH pH 7 buffer) in the presence of pressurized CO$_2$ are consistent with a continuous phase transition, where melting is slow and intermediate gel and fluid
phase coexist. The van’t Hoff enthalpy can be used to qualitatively describe the cooperativity of the phase transition region (Marky and Breslauer 1987):

$$\Delta H_{VH} = 4RT_m^2 \left( \frac{\partial \alpha}{\partial T} \right)_{T_m}$$  \hspace{1cm} (4.8)

$$\alpha = \frac{<r>_T}{\Delta <r>_T}$$  \hspace{1cm} (4.9)

where $\alpha$ is the relative fraction of DPPC molecules in the fluid ($\alpha$) or gel ($\alpha - 1$) state and the term $(\partial \alpha / \partial T)$ represents the slope at $T_m$ ($\alpha = 0.5$). CO$_2$ present at 1.8 and 7.0 MPa leads to a decrease in $\Delta H_{VH}$ from 2049 KJ/mol at 0.1 MPa (no CO$_2$ present) to 1167 and 826 KJ/mol, respectively (Table 1). Increasing CO$_2$ pressure reduces the van’t Hoff phase transition enthalpy, denoting a decrease in the fraction of the bilayer structure that melts cooperatively (Marky and Breslauer 1987). A decrease in the van’t Hoff enthalpy is consistent with bilayer disordering.

Based on the negligible effects of buffering on $T_m$, the inclusion of CO$_2$ within the bilayer appears to be the primary cause of liposome fluidization. CO$_2$ has been shown to be miscible with a large range of organic solvents and hydrocarbons (Dohrn and Brunner 1995; Dohrn and Brunner 2002), suggesting that CO$_2$ may accumulate within the hydrophobic acyl region. However, the potential for hydrogen bonding and Lewis acid-Lewis base (LA-LB) interactions between CO$_2$ and the two carbonyl-linkages connecting the choline head with the two C$_{16}$ acyl tails suggests that CO$_2$ may also reside near the head region. In a study of the solvation of CO$_2$ using ab initio calculations, Raveendran and Wallen (Raveendran and Wallen 2002) demonstrate that the interaction between CO$_2$ and a carbonyl functional group is cooperative. Thus, a single CO$_2$ molecule may interact strongly with the choline headgroup of DPPC through (i) hydrogen bonding at the $\alpha$-carbon in the carbonyl ($^{\alpha}\text{C-H} \cdots \text{O}$), and (ii) LA–LB interactions between CO$_2$ and the $\alpha$-carbon ($\text{H}^{\alpha}\text{C} \cdots \text{O}$) and (the carbonyl oxygen ($>\text{C}=\text{O}$) (Meredith et al. 1996).

Adsorption of anesthetic molecules near the bilayer interface can displace surface-bound water molecules that preferentially bind to the phosphate moiety (Ueda 1999). Dehydration enhances the hydrophobic character of the bilayer surface, stressing the
bilayer as the acyl tails are exposed (Ueda 1999). Headgroup dehydration also disrupts localized electrostatic interactions, and the ionic linkage between the negative phosphate moiety and the positive choline head of neighboring phospholipids (Ueda 1999). Therefore, bilayer dehydration produces a less structured, or more fluid membrane. Brockerhoff (1982) indirectly referred to this as a restructuring of the “hydrogen belt,” where the presence of anesthetics redirects the hydrogen-bonding network in the headgroup region. Interfacial-dehydration may contribute to CO₂-induced fluidization by altering headgroup interactions, surface packing density, and in turn the local viscosity near the DPH probe.

The fluidity (viscosity), thickness, and water content of liposomes influence diffusion of solutes within the bilayer. These physical properties can be used to control the rate of trans-bilayer permeation for the incorporation/release of hydrophobic or hydrophilic species. Thus, pressurization in the presence of CO₂ provides for significant and reversible changes in membrane fluidity, which have potential application for controlling liposome formation and pharmaceutical loading (Sato and Sunamoto 1992), the permeability of liposome enzyme-microreactors (Walde and Marzetta 1998), and the rate of formation and morphology in materials synthesis (Collier and Messersmith 2001). In addition to fluidizing the bilayer, CO₂ also broadens the gel-fluid phase transition at near ambient temperature. Within this transition bilayer permeability is greatest due to interfaces between coexisting gel and fluid phases (Collier and Messersmith 2001). Processing at ambient temperatures is particularly beneficial for thermally labile compounds. In these applications, CO₂ may be used as an environmentally benign alternative to detergents (Oberholzer et al. 1999) or organic solvents (Felix 1991) for the control of liposome permeability.

Conclusions

Through pressurized fluorescence anisotropy experiments, we have demonstrated that DPPC bilayers exhibit significant fluidization and melting point depression in the presence of pressurized CO₂. Liposome fluidity by CO₂ is attributed to the dissolution and accumulation of CO₂ within the bilayer, the formation of carbonic acid (H⁺ or HCO₃⁻), and the ordering effects of pressure on the phospholipid acyl chains.
Based on the observed melting point depression, CO$_2$ fluidization occurred within both a gel and fluid DPPC bilayer with significant CO$_2$ absorption in the gel phase. Greater fluidization of the gel phase suggests that electrostatic headgroup interactions, which are sensitive to pH and ion concentration, appear to be more influential in the gel phase (relative to the fluid phase).

Significant melting point depression by pressurized CO$_2$ facilitates a broad gel-fluid phase transition region at near ambient temperatures that can be used to control, and greatly enhance bilayer permeability. Knowledge of membrane fluidization by CO$_2$ can be applied to liposome technologies to understand and manipulate CO$_2$-induced perturbations, and provide a pressure-tunable and reversible method to control the formation, permeability, and morphology of liposomes. Furthermore, this investigation suggests strategies to manipulate membrane fluidization by CO$_2$, with a goal of optimizing CO$_2$ technologies for microbial sterilization.

References


Chapter 5

Liposome Fluidization and Melting Point Depression by Compressed Gas and Liquid n-Alkanes

Introduction

Phospholipid bilayers are susceptible to large structural changes upon the incorporation of solutes molecules, which alter the configurational entropy of the phospholipid acyl chain region and result in a change in the microviscosity (or fluidity) of the bilayer (Weber and de Bont 1996; Beney 2001). The dissolution of solute molecules, and subsequent disordering of the bilayer, is related to the partitioning between the aqueous phase and the bilayer, and the molecular structure of the solute (molecular volume/area, axial dimensions, and topology). For instance, numerous investigations have demonstrated the effects of solute concentration and structure on the ordering of phospholipids bilayers using homologous series of n-alkanes (Haydon et al. 1977; Gruen and Haydon 1980; McIntosh 1980; Pope et al. 1984), and both branched and n-alkanols (Kamaya et al. 1981; Pope et al. 1984; Iiyama 1992). Such studies have provided insight into the influence of solute properties on anesthetic action and the general solvent properties of the bilayer with respect to hydrophobic solutes, such as pharmaceuticals (White 1976; Lemmich 1996).

Short chain n-alkanes (up to decane) thicken and disorder phospholipid bilayers; however, the inclusion of longer chain alkanes such as hexadecane yield no significant change (Gruen and Haydon 1980; McIntosh 1980). While hexadecane (C\textsubscript{16}) favorably partitions from the aqueous phase into the hydrocarbon core of the bilayer, low water solubility and the alignment of hexadecane within the bilayer (parallel to the phospholipid acyl chains) prevents disordering. In contrast, short chain n-alkanes (butane to decane) can organize more randomly within the bilayer, frequently accumulating in the geometric center and aligning perpendicular to the acyl chains (Haydon et al. 1977; Gruen and Haydon 1980; McIntosh 1980; Pope et al. 1984). In addition to improving our understanding of anesthetic theory (e.g. the "cut-off" effect) (Pope et al. 1989), characterizing alkane-phospholipid interactions is directly relevant to
the formation and structure of monolayers (McIntosh 1980), black planar membranes (BLMs) (Haydon et al. 1977), unilamellar liposomes (Pautot et al. 2003), and non-lamellar lipid phases (Gawrisch and Holte 1996). These assemblies have applications in areas such as biosensing, biomaterials, whole-cell biocatalysis, and drug delivery (Weber and de Bont 1996; Beney 2001; Collier and Messersmith 2001; Ti Tien and Ottova 2001; Metselaar et al. 2002).

Although the effects of short and long chain n-alkanes on the structure of phospholipid bilayers have been well characterized at atmospheric pressure, the interaction of compressible alkanes (i.e., ethane and propane under pressure) and lipid bilayers has not been examined. Compressed and supercritical ethane and propane are of immediate interest in whole-cell bioprocessing (Knutson et al. 1999; Berberich et al. 2000), enzyme catalysis (Mesiano et al. 1999; Knez and Habulin 2002) and materials synthesis (Cason and Roberts 2000; Shah et al. 2002; Ye and Wai 2003). Compressed and supercritical fluids offer distinct advantages over conventional solvents, including tunable solvent strength, high diffusivity, and low surface tension. In addition, the ability to remove and recover compressed or supercritical gases from processes through depressurization eliminates residual solvent residue. These properties provide a versatile environment for extraction, reaction, and materials synthesis (Randolph 1990; Reverchon 1997; Eckert and Chandler 1998; Xiangrong and Wai 2003).

Compressible solvents are unique in that the aqueous solubility, and therefore the expected concentration in the bilayer, vary significantly with pressure and temperature. A description of the combined effects of solute partitioning and pressure is needed to characterize the processing of phospholipids using compressed and supercritical solvents. Knowledge of the processing-property relationship of phospholipid bilayers in pressurized hydrocarbons could lead to improved liposome and whole cell technologies, such as the replacement of liquid n-alkanes with readily recoverable and tunable compressible fluids.

We have recently presented the first evidence of DPPC liposome fluidization by pressurized CO₂ (Bothun et al. 2003; Chapter 4), which is directly relevant to bacterial sterilization (Spilimbergo et al. 2002) and liposome formation (Frederikson et al. 1997) using compressed and supercritical CO₂. Both the gel (295 K, \( T < T_m \)) and fluid (333 K,
$T > T_m$) phase of DPPC bilayers were significantly fluidized with increasing CO$_2$ pressure (3.5 to 13.9 MPa), and thus the concentration of CO$_2$ within the bilayer. Increasing CO$_2$ pressure reduced the melting temperature to near ambient temperatures and dramatically broadened the phase transition region, leading to a continuous phase transition. For instance, at 7.0 MPa $P_{CO_2}$ ($x_{CO_2} = 0.57$ within a fluid bilayer) $T_m$ was reduced to 299 K with a broad phase transition region ($\Delta T_r = 7$ K). Trans-bilayer permeability is greatest within this region at the interface between coexisting gel and fluid states.

This work examines the fluidity, or bilayer viscosity, of fully hydrated dipalmitoylphosphatidylcholine (DPPC, $T_m \approx 315$ K) liposomes in the presence of pressurized ethane and propane using the fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Steady-state DPH anisotropy was used to investigate the effect of these pressurized alkanes on liposome fluidization at 295 and 333 K as a function of solvent strength (isothermal; 3.5 to 13.9 MPa). These temperatures represent stable gel (295 K) or fluid (333 K) DPPC phases at atmospheric pressure. The melting temperature ($T_m$), which denotes the main phase transition between the gel ($< T_m$) and fluid ($> T_m$) phase was determined via DPH anisotropy in the presence of ethane and propane at 0.8, 1.8, and 7.0 MPa. Pressure-induced ordering within the bilayer was evaluated directly from isothermal anisotropy measurements in the absence of pressurized solvents. The effect of molecular surface area on fluidization of gel and fluid phases provides a link between liquid, compressed, and supercritical alkanes.

**Materials and Methods**

**Chemicals**

L-α-dipalmitoylphosphatidylcholine (DPPC, >99%) was purchased from Sigma Chemical Co. The membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH, >99%), was purchased from Molecular Probes (Eugene, OR). Chloroform (>99.9%) and tetrahydrafuran (>99.9%) were purchased from Fisher Scientific and Mallinckrodt Chemicals, respectively. KH$_2$PO$_4$-NaOH (50 mM) pH 7 buffer from Fisher Scientific was filtered (11 µm, Whatman 1) before use. Deionized ultra filtered (DIUF, Fisher Scientific) was used without further purification. Pentane, hexane, heptane, octane, and decane
(n-alkanes, >99%) were purchased from Sigma Chemical Co. Ethane and propane (>99%) were obtained from Scott Gross Co.

**Unilamellar Liposome Preparation**

Aqueous stock solutions of 1 mM DPPC labeled with DPH (1:500 molar ratio probe to lipid) were prepared following the procedure of Bingham et al. (Bangham et al. 1965). Solutions of DPPC dissolved in chloroform (~ 1:50 wt. ratio DPPC to chloroform) and DPH dissolved in tetrahydrafuran (0.1 mM stock) were mixed and co-evaporated under a gentle stream of N₂ until a dry DPPC/DPH film remained. Vacuum was used (~15 min) to remove residual solvent from the film. The film was then hydrated with KH₂PO₄-NaOH pH 7 buffer and maintained above the melting temperature (\( T > 315 \) K) in a water bath for 1 hour before shaking. The DPPC/DPH film was suspended as multilamellar liposomes by vigorously shaking for approximately 1 hour. The liposomes were then sonicated for 20 min at \( T > 315 \) K, which has been previously shown to yield unilamellar liposomes (Vemuri and Rhodes 1995). To obtain a working solution, the stock was diluted tenfold with pH 7 buffer.

**Fluorescence Anisotropy Measurements**

High pressure anisotropy of DPH in DPPC vesicles was measured using a custom designed stainless steel variable volume view cell (10-25 ml working volume, rated to 20.7 MPa) from Thar Technologies (Pittsburgh, PA) mounted within the sample compartment of a Varian Cary Eclipse fluorescence spectrophotometer (Walnut Creek, CA). A detailed description of the equipment and techniques has been previously reported (Bothun et al. 2003). Steady-state DPH anisotropy within the DPPC bilayer was determined at \( \lambda_{ex} = 350 \) nm and \( \lambda_{em} = 452 \) nm with a one second average sampling time.

Experiments at isothermal conditions in the presence of compressed or liquid alkanes and under hydrostatic pressure were conducted by adjusting the temperature of the liposome-loaded cell to 295 or 333 K. Under elevated hydrostatic pressure (in the absence of alkane), experiments were performed in a single aqueous phase (containing liposomes) exposed to successive increases in pressure (up to 20.7 MPa). Pressure
increases were achieved in the variable volume spectroscopic cell by applying back-pressure to a floating piston using a hand operated hydraulic pump. DPH anisotropy was measured after 5 minutes of equilibration under hydrostatic pressure.

In the presence of an alkane solvent, the view cell was first sparged with the appropriate alkane and then loaded with a working solution of DPH labeled liposomes (15 ml). After adjusting the temperature, the headspace (~ 8 ml) was filled with either a liquid alkane or pressurized gas, which was supplied by a high-pressure Isco syringe pump (Lincoln, NE, model 500D). For the pressurized gas experiments, the total pressure within the variable volume high-pressure cell was maintained by supplying pressurized ethane or propane at constant pressure. The initial pressure was adjusted to 3.5 MPa, and increased successively to 13.9 MPa. For the gas and liquid alkanes, DPH anisotropy was measured periodically at 295 or 333 K until the anisotropy measurement remained constant to insure equilibrium (typically > 60 min). Fluidization by pentane at 333 K was not investigated due to its low boiling temperature (309.2 K).

To determine the melting temperature under isobaric conditions the view cell was sparged with gas, loaded with working DPPC solution (15 mL), and pressurized with ethane or propane (0.8, 1.8, and 7.0 MPa). The view cell was then heated to 323 K and equilibrated for 15 minutes. DPH anisotropy was measured at 1 or 2 K increments as the cell was cooled to approximately 295 K over a period of 3 hours at constant pressure.

Fluorescence anisotropy \(<r>\) is defined as:

\[
< r > = \frac{(I_{VV} - I_{VH})}{(I_{VV} + G \cdot I_{VH})}
\]

where \(I\) represent the fluorescence emission intensity, \(V\) and \(H\) represent the vertical and horizontal orientation of the excitation and emission polarizers, and \(G = (I_{HV} \cdot (I_{HH})^{-1}\)

accounts for the sensitivity of the instrument towards vertically and horizontally polarized light (Lakowicz 1999). In the presence of pressurized ethane and propane, anisotropy includes the combined effects of both dissolved ethane/propane and pressure. The anisotropy values for experiments conducted under isothermal conditions in the presence of pressurized ethane and propane were normalized with respect to
hydrostatic pressure to account for the effect of increased bilayer ordering on DPH anisotropy:

\[
<r>_{\text{norm}} = \frac{<r>}{<r>_{\text{hydrostatic pressure}}}
\]

where a value of \(<r>_{\text{norm}} = 1\) indicates that there was no change in anisotropy due to the presence of pressurized ethane or propane relative to the equivalent hydrostatic pressure. For the liquid alkanes (C₆ to C₁₀), anisotropy was normalized with respect to DPPC (no alkane present) at 0.1 MPa. Results for anisotropy are the average of five consecutive measurements for a single solution at each experimental condition. Experimental uncertainty of DPH anisotropy measurements was less than 5%.

The DPPC chain melting temperature \((T_m)\) was determined from plots of absolute anisotropy \((<r>)\) as a function of temperature under isobaric conditions. Above and below \(T_m\) the hydrocarbon core of the bilayer exists as a liquid-crystalline lamellar (or fluid) phase or as an ordered gel phase, respectively. DPH anisotropy values in a fluid DPPC phase are significantly lower than in a gel phase; therefore, upon cooling the fluid-gel phase transition is identified by an abrupt increase in anisotropy. The temperature at the midpoint of this transition is taken as \(T_m\), and \(\Delta T\) is the temperature range associated with the phase transition.

**Determining Aqueous and Bilayer Solubility of Ethane and Propane**

The concentration of alkane within the aqueous phase and phospholipid bilayer are required to determine the role of alkane concentration on membrane fluidity. Aqueous solubility of ethane and propane \((x_{i}^{aq})\) were determined by the Krichevsky-Kasarnovsky equation for gas solubility (Krichevsky and Kasarnovski 1935) using reported ethane/water and propane/water Henry’s constant (Harvey 1996; Dhima et al. 1999) and the partial molar volume of ethane and propane in water calculated according to Lyckman et al. (Lyckman 1965). The fugacity coefficient of pure ethane and propane was determined by the Peng-Robinson equation of state. Calculated values for ethane and propane solubility in binary alkane/water systems are within 90% of reported experimental values (Culberson and McKetta 1950; Kobayashi 1953).
Within the fluid phase of the bilayer, ethane and propane solubility ($x_f$) was estimated from the natural logarithm of the n-octanol/water partition coefficient ($\log P_{o/w} = 1.81$, ethane; $\log P_{o/w} = 2.36$, propane (Sangster 1997)). The $\log P_{o/w}$ values (Sangster 1997) were also used to calculate the bilayer solubility of the liquid alkanes. $\log P_{o/w}$ values for alkanes, including ethane and propane, are not significantly affected by temperature and pressure over the range of conditions investigated (0.8 to 20.7 MPa; 295 to 333 K) (Sangster 1997), and were assumed constant. The following empirical correlation was used to determine the partition coefficient of ethane and propane between the bilayer and water, $\log P_{m/w}$ (Sikkema et al. 1994):

$$\log P_{m/w} = 0.97 \cdot \log P_{o/w} - 0.64 \quad (5.3)$$

Aqueous/fluid-bilayer alkane solubility and results for melting point depression by compressed ethane and propane are compared to reported values for liquid n-alkanes (Table 5.1).

### Table 5.1  DPPC melting point depression by pressurized gas and liquid n-alkanes.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Pressure (MPa)</th>
<th>Concentration$^a$</th>
<th>$T_m$ (K)</th>
<th>$\Delta T_m$ (K)</th>
<th>$\Delta H_{vH}$ (KJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$x_{aq,b}^f$</td>
<td>$x_f^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethane</td>
<td>1.8</td>
<td>$4.1 \times 10^{-4}$</td>
<td>0.23</td>
<td>308</td>
<td>-7.1</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>$8.9 \times 10^{-4}$</td>
<td>0.50</td>
<td>300</td>
<td>-16.2</td>
</tr>
<tr>
<td>propane</td>
<td>0.8</td>
<td>$1.2 \times 10^{-4}$</td>
<td>0.24</td>
<td>305</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>$1.5 \times 10^{-4}$</td>
<td>0.29</td>
<td>299</td>
<td>-16.1</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>$2.2 \times 10^{-4}$</td>
<td>0.41</td>
<td>300</td>
<td>-16.2</td>
</tr>
<tr>
<td>hexane$^f$</td>
<td>0.1</td>
<td>$2.2 \times 10^{-6}$</td>
<td>0.15</td>
<td>302</td>
<td>-12.0</td>
</tr>
<tr>
<td>octane$^f$</td>
<td>0.1</td>
<td>$1.1 \times 10^{-7}$</td>
<td>0.10</td>
<td>306</td>
<td>-8.5</td>
</tr>
<tr>
<td>decane$^f$</td>
<td>0.1</td>
<td>$5.1 \times 10^{-9}$</td>
<td>0.07</td>
<td>309</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

$^a$ concentrations at $T_m$ (mole fraction)

$^b$ Krichevsky-Kasarnovski equation for gas solubility (Krichevsky and Kasarnovski 1935)

$^c$ based on reported $\log P_{o/w}$ (Sangster 1997) and equation 5.3 (Sikkema et al. 1994)

$^d$ accounts for increase in $T_m$ due to pressure (0.22 K/MPa)

$^e$ van't Hoff enthalpy, $\Delta H_{vH} = 2049$ KJ/mol at 0.1 MPa (no CO$_2$ present)

$^f$ based on reported aqueous solubility (Marche et al. 2003)
Results

Isothermal Bilayer Fluidization by Compressed Gas and Liquid n-Alkanes

Fluidization of fully-hydrated DPPC liposomes was measured under isothermal conditions at 295 and 333 K in the presence of excess pressurized ethane and propane (0.9 to 20.9 MPa), and in the presence of excess pentane, hexane, heptane, octane, and decane at atmospheric pressure (0.1 MPa). As previously stated, fluidization by pentane at atmospheric pressure was not investigated at 333 K due to its low boiling temperature. The effects of solvent and pressure on fluidity in the presence of excess pressurized ethane or propane were differentiated by normalizing these DPH anisotropy measurements ($<r>_{norm}$) against measurements at the equivalent hydrostatic pressure.

At 295 K absolute DPH anisotropy ($<r>$) in fully hydrated DPPC liposomes existing in the gel phase (no pressurized gas present) did not change with increasing hydrostatic pressure (up to 20.9 MPa) (Bothun et al. 2003). In contrast, $<r>$ increased $2.8 \times 10^{-4}$/MPa at 333 K with hydrostatic pressure, demonstrating a linear decrease in the fluidity of the fluid phase with increasing pressure.

Compressed Ethane and Propane

At 295 K, normalized DPH anisotropy decreases from $<r>_{norm} = 1$ to a minimum value of 0.62 and 0.66 at 7.0 MPa by pressurized propane and ethane, respectively, suggesting maximum bilayer fluidization at 7.0 MPa (Figure 5.1A). An increase in anisotropy above 7.0 MPa demonstrates a pressure-reversal of the anesthetic (fluidizing) action of ethane and propane at 295 K. Similarly, at 333 K DPH anisotropy decreases to a minimum value of $<r>_{norm} = 0.82$ and 0.91 at 10.4 MPa propane and ethane, respectively (Figure 5.1A). Anisotropy increases substantially at 13.9 MPa, demonstrating a dramatic pressure-reversal in a fluid state (333 K) that, in the presence of dissolved ethane, exceeds the effect of hydrostatic pressure on membrane ordering.
Figure 5.1 Normalized DPH anisotropy ($<r>_{\text{norm}}$) in DPPC liposomes at 295 and 333 K as a function of (a) hyperbaric ethane and propane pressure and (b) the number of carbon atoms in liquid alkanes.

A greater disruption in membrane fluidity is observed at 295 K than in the fluid phase (333 K) in the presence of pressurized ethane and propane, as determined from the normalized anisotropy values. Evaluation of the melting point depression is required to determine if a solvent-induced phase transition occurs at approximately 7 MPa and 295 K in the presence of ethane and propane. However, the magnitude of the reduction in $<r>$ (from approximately 0.2 to 0.1) is consistent with a phase transition (Figure 5.2).
Fluidization by ethane and propane was also reversible (> 97% as indicated by $<r>_{\text{norm}}$) upon depressurization at 295 and 333 K (data not shown).

**Liquid Alkanes - C₅ to C₁₀**

Significant bilayer fluidization is observed in the presence of pentane, hexane, heptane, octane, and decane at 295 K and atmospheric pressure (0.1 MPa) (Figure 5.1B). An increase in DPH anisotropy is observed with increasing chain length (Figure 5.1B). Previous investigations using differential scanning calorimetry suggest that a gel phase is present at 295 K in the presence of an excess of these n-alkanes (McIntosh 1980). An increase in $<r>_{\text{norm}}$ (decrease in fluidity) is also observed with increasing chain length at conditions of a fluid phase bilayer (333K) (Figure 5.1B). Similar to pressurized ethane and propane, changes in $<r>_{\text{norm}}$ indicate a greater disruption in the gel phase (295 K) than in the fluid phase (333 K), particularly in the lower carbon number n-alkanes.

**Isobaric Bilayer Fluidization by Pressurized Ethane and Propane**

Fluidization was also determined isobarically as a function of temperature (295 to 323 K) in the presence of excess ethane (1.8 and 7.0 MPa) and propane (0.8, 1.8, and 7.0 MPa). Significant DPPC melting point depression is observed in the presence of ethane and propane with increasing pressure. In addition, dissolution of ethane and propane within the bilayer increases membrane fluidity in both the gel ($<T_m$) and fluid ($>T_m$) phases and broadens the phase transition region ($\Delta T_r$) relative to atmospheric pressure (Figure 5.2). For example, $T_m$ is reduced from 314.7 MPa at atmospheric pressure to 305 and 308 K at 0.8 MPa propane and 1.8 MPa ethane, respectively (Figure 5.2). The reductions in $T_m$ associated with 0.8 MPa propane and 1.8 MPa ethane are accompanied by broadening of the phase transition region from $\Delta T_r = 3$ K to 8 and 4 K, respectively. An increase in pressure to 7.0 MPa ethane or propane further reduces $T_m$ to 300 K. Based on the anisotropies presented in Figure 5.2, isothermal measurements taken at 295 K (Figure 5.1A) were within the phase transition region at 7.0 MPa ethane and propane, very close to gel phase.
The aqueous solubility of ethane and propane and the fluid bilayer concentration increases with increasing pressure. However, increasing the propane pressure from 1.8 to 7.0 MPa does not reduce $T_m$ despite a considerable increase in bilayer concentration (Table 5.1). Therefore, pressure-reversal influences $T_m$ by ordering the acyl tails (Koynova and Caffrey 1998) and counteracting the fluidizing effects of increasing bilayer concentration (Wann and Macdonald 1988). Furthermore, 7.0 MPa ethane and propane yield the same reduction in $T_m$ even though the concentration of ethane within the bilayer is greater than propane at these conditions (Table 5.1). In this case the difference in chain length of compressed alkanes ($C_2$ or $C_3$) also appears to influence $T_m$. These results demonstrate the concomitant influences of pressure, bilayer solubility, and possibly molecular structure of the solute molecule on DPPC bilayer fluidity.

Figure 5.2 Melting point depression in aqueous DPPC liposomes indicated by DPH fluorescence anisotropy ($\langle r \rangle$). Anisotropy is expressed as a function of temperature (K) under atmospheric pressure (0.1 MPa hydrostatic, ■) and in the presence of excess ethane (1.8 MPa, × and 7.0 MPa, ◆) and propane (0.8, △; 1.8 MPa, ○; and 7.0 MPa, ⋆).
Discussion

Analysis of the DPPC Phase Transition Induced by Pressurized Ethane and Propane

Although short chain liquid n-alkanes (C$_6$ to C$_{10}$) are generally not classified as anesthetics, they have been shown to disorder and fluidize phospholipid membranes (De Young 1990; Maxwell 1995; Gruen and Haydon 1980; McIntosh 1980). These n-alkanes do not partition randomly throughout phospholipid bilayers, instead favoring the center of the bilayer and aligning perpendicular to the acyl chains (McIntosh 1980). The accumulation of C$_6$ to C$_{10}$ n-alkanes within the center of the bilayer produces greater disorder than a solute that is randomly distributed throughout the acyl region by yielding a thicker bilayer and reducing the phospholipid packing factor (Gruen and Haydon 1980; McIntosh 1980). In contrast, statistical mechanics experiments suggest that butane (C$_4$) distributes in a random orientation in the bilayer center, as opposed to aligning perpendicular to the chains [5]. Subsequently, butane is expected to penetrate into the acyl region and intercalate between the tails to a greater extent than the C$_6$ to C$_{10}$ n-alkanes (Gruen and Haydon 1980).

The smaller n-alkanes investigated in this study (ethane and propane) are expected to exhibit partitioning behavior similar to butane, accumulating within the center of the bilayer. Therefore, melting point theory, which is based on a homogenous bilayer phase (regular solution theory), did not adequately describe the measured effect of C$_6$ to C$_{10}$ n-alkanes or pressurized ethane and propane on the DPPC phase transition (results not shown). For example, this theory predicts gel phase bilayer concentrations less than or equal to zero ($x_i^g \leq 0$) for both gaseous and liquid n-alkanes. In contrast, the application of conventional melting point theory to the depression of the DPPC liposome melting point in the presence of pressurized CO$_2$ yields self-consistent results (Bothun et al. 2003; Chapter 4), suggesting that CO$_2$ is more randomly distributed in the bilayer.

Pressurized ethane and propane induced broad phase transition regions in DPPC liposomes, consistent with a continuous phase transition. Within this phase transition intermediate phases exist that contain both gel and fluid domains. The calorimetric phase transition enthalpy is a state-function and cannot be used to describe the nature of the phase transition (a broad or narrow transition) (Marky and Breslauer...
However, the van’t Hoff enthalpy is dependent upon the nature of the phase transition and can be used to analyze cooperativity, or the fraction of DPPC molecules within the bilayer that melt as a single unit, in the transition region (Marky and Breslauer 1987). The van’t Hoff enthalpy ($\Delta H_{\text{vh}}$) measures the sharpness of the phase transition:

$$\Delta H_{\text{vh}} = 4RT_m^2 \left( \frac{\partial \alpha}{\partial T} \right)_{T_m}$$  \hspace{1cm} (5.4)

$$\alpha = \frac{<r>T_r}{\Delta <r>_{\Delta T_r}}$$  \hspace{1cm} (5.5)

where $\alpha$ is the fraction of DPPC molecules in the fluid ($\alpha$) or gel ($\alpha - 1$) state, the term ($\partial \alpha/\partial T$) represents the slope at $T_m$ ($\alpha = 0.5$), and $R$ is the ideal gas constant. Low values of $\Delta H_{\text{vh}}$ denote a broad phase transition and small melting units (low cooperativity), while high values of $\Delta H_{\text{vh}}$ denote a sharp transition and large melting units (high cooperativity) (Marky and Breslauer 1987). Equations 5.4 and 5.5 were used to quantify the effects of pressurized ethane and propane on $\Delta H_{\text{vh}}$ of the DPPC phase transition using the normalized anisotropy measurements. Ethane and propane at 7.0 MPa lead to a decrease from $\Delta H_{\text{vh}} = 2050 \text{ KJ/mol}$ at 0.1 MPa (no gas present) to 510 and 430 KJ/mol, respectively (Table 5.1). Decreases in the van’t Hoff enthalpy with increasing ethane and propane pressure signify reduced melting cooperativity in DPPC bilayers (Figure 5.2).

**Comparison of Liposome Fluidization by Compressed Gas and Liquid n-Alkanes**

Solute molecular size affects the extent of molecular disordering, solute partitioning behavior, and solute location within the bilayer. The effect of C$_2$ to C$_{10}$ n-alkanes on liposome fluidity, examined across phases (gaseous, liquid, and supercritical), provides a link between these solute properties. DPH anisotropy with saturated liquid n-alkane solutions is approximately proportional to n-alkane chain length at 295 and 333 K (Figure 5.1b). Chain length is in turn inversely proportional to bulk phase density and n-alkane concentration within the bilayer (Table 5.1). Previous works have shown that C$_6$ to C$_{10}$ n-alkanes exist within gel and fluid phospholipid bilayers as separate regions (Gruen and Haydon 1980; McIntosh 1980). In contrast,
correlations between liposome fluidity and bulk density or concentration in the bilayer did not exist for pressurized ethane and propane, which were present as gaseous, supercritical, or liquid phases.

The solute-bilayer system can be strongly influenced by pressure-ordering, as suggested by the significant pressure reversal in anisotropy observed above 7.0 MPa at 295 K and 10.3 MPa at 333 K. Pressure-ordering is described as a hydraulic compression that decreases membrane fluidity and reverses the disordering effects of solutes that have accumulated within the bilayer (Wann and Macdonald 1988). For instance, Kaneshina et al. (1995) have demonstrated pressure-reversal of inhalation anesthetics (e.g. halothane) in fluid DPPC bilayers as indicated by a measurable reduction in the anesthetic partition coefficient at high pressures. In this case, the anesthetic was “squeezed out” of the bilayer with increasing pressure. Similarly, high anisotropy values at 333 K indicate pressure-reversal in a fluid bilayer at 13.9 and 20.7 MPa ethane. Substantial pressure-reversal observed in this system at these high pressures suggests that the acyl chains experience greater reordering due to the presence of ethane within the bilayer. Increasing pressure may also force ethane and propane from the bilayer and reduce the partition coefficient, an effect that is not accounted for in the calculated partition coefficients in Table 5.1. Thus, the increase in $r_{\text{norm}}$ at pressures $> 7.0$ MPa (295 K) and $> 10.3$ MPa (333 K) may be due to a decrease in the concentration of ethane and propane within the bilayer.

The term $(1 - r_{\text{norm}})/x_i$ is introduced to further analyze bilayer fluidization at 333 K by compressed gas and liquid $n$-alkanes. Our results indicate that $C_6$ to $C_{10}$ $n$-alkanes produce greater bilayer disorder in the fluid phase than pressurized ethane or propane, as described by the magnitude of $(1 - r_{\text{norm}})/x_i$ (Table 5.2). An increase in $(1 - r_{\text{norm}})/x_i$ is observed with increasing chain number from $C_6$ to $C_{10}$, denoting a greater reduction in bilayer order with large $n$-alkane molecules (Table 5.2). In the presence of compressed ethane, $(1 - r_{\text{norm}})/x_i$ varies slightly between 3.6 and 10.4 MPa and becomes negative at 13.9 and 20.8 MPa (Table 2), consistent with pressure-reversal of fluidization (Figure 1a). For compressed propane, $(1 - r_{\text{norm}})/x_i$ is essentially unchanged at 3.6 and 7.0 MPa (Table 5.2). Slight changes in $(1 - r_{\text{norm}})/x_i$ in the presence of ethane (up to 10.4
MPa) and propane (up to 7.0 MPa) demonstrate that pressure-effects on bilayer fluidity and solute partitioning are minimal at these conditions.

Table 5.2  Reduction in DPH anisotropy by pressurized gas and liquid n-alkanes under isothermal conditions.

<table>
<thead>
<tr>
<th>n-alkane</th>
<th>solute (MPa)</th>
<th>(1-&lt;r&gt;norm)/x_i</th>
<th>333 K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethane</td>
<td>3.6</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.9</td>
<td>-0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.8</td>
<td>-0.35</td>
<td></td>
</tr>
<tr>
<td>propane</td>
<td>3.6</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.9</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.8</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>hexane</td>
<td>0.1</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>heptane</td>
<td>0.1</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>octane</td>
<td>0.1</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>decane</td>
<td>0.1</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>a</th>
<th>change (reduction) in DPH anisotropy per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>solubility (x_i, Table 1) assumed constant at 295 and 333 K</td>
</tr>
</tbody>
</table>

Bilayer fluidization by compressed propane increases with pressure and reaches a maximum at 10.4 MPa, (1-<r>norm)/x_i = 0.57 (Table 5.2). This dramatic increase in (1-<r>norm)/x_i may indicate a pressure-induced reorganization of propane molecules within the bilayer. As a critical pressure is reached, propane is forced from the acyl chain region (chain intercalation) to the center of the bilayer (between chains). Similar to the C_6 to C_10 n-alkanes, accumulation of propane in the center of the bilayer would produce maximum fluidization. Further pressurization to 13.9 and 20.8 MPa by compressed propane reduces (1-<r>norm)/x_i, again consistent with the pressure-reversal effect (Figure 5.1a).

On the basis of DPPC melting behavior in the presence of compressed and liquid n-alkanes, 295 and 333 K represent gel and fluid bilayer phases, respectively.
Significant fluidization was observed in both phases (Figure 5.1), suggesting appreciable n-alkane solubility within gel and fluid bilayers. Given the difficulty in measuring phase behavior in assembled phospholipid bilayers, the following analysis is based on the assumption that n-alkane concentrations in the gel and fluid phase are equal.

Recent molecular dynamics simulations have demonstrated that partitioning into ordered bilayers is highly dependent on solute size (Marrink and Berendsen 1996; Xiang and Anderson 1999) and free surface area (Xiang and Anderson 1998). These results suggest that a correlation may exist between fluidity ($<r>$$_{norm}$) and total surface area of adsorbed n-alkane (a product of the bilayer solubility and the elliptical surface area of the molecule). The elliptical molecular surface area of compressed gas and liquid n-alkanes were determined in their relaxed state ($A_e$, C$_2$ to C$_{10}$; calculated by MacSpartan Plus 1.1.9, Wavefunction, Inc). At 295 K changes in $<r>$$_{norm}$ are related to the total surface area of adsorbed C$_2$ to C$_{10}$ n-alkanes present as gaseous or liquid phases ($A_e$$\cdot$$x_f$; Figure 5.3, $R^2 = 0.86$). Compressed ethane and propane are appreciably soluble in the bilayer (Table 5.1), resulting in large values of $A_e$$\cdot$$x_f$ and significant disorder relative to C$_6$ to C$_{10}$ n-alkanes. Disordering by pentane is comparable to ethane and propane at $A_e$$\cdot$$x_f$ = 71 Å$^2$. At pressures greater than 7.0 MPa, fluidization by ethane and propane was reversed, as denoted by a slight increase in $<r>$$_{norm}$ with $A_e$$\cdot$$x_f$. This correlation provides a link between bilayer fluidization by liquid and pressurized n-alkane solvents (C$_2$ to C$_{10}$) based on the total molecular surface area of inserted n-alkanes.
Significant bilayer fluidization in the presence of ethane, propane, and CO₂ (Bothun et al. 2003; Chapter 4) demonstrates that pressurized fluids may be used to control the permeability of the bilayer in both the gel and fluid state by manipulating the solubility with minor changes in pressure (as low as 0.8 MPa propane). In addition, pressurized CO₂, ethane, and propane reduce the melting temperature of DPPC from 315 K to near-ambient temperatures, with a considerable broadening of the phase transition region. Trans-bilayer permeability is greatest within this region at the interface between coexisting gel and fluid states. Hence, the temperature range in which permeability is greatest (ΔTᵢ) can be greatly extended through the addition of pressurized solvents. Manipulating the melting temperature and transition region is critical for controlling solute permeability in liposome-based materials synthesis and biocatalysis. Perhaps the greatest advantage is the ability to remove and recover these solvents through depressurization, leading to a reversible fluidization process. A pressure-induced reversible “phase switch” can therefore be accomplished using pressurized solvents as fluidizing agents.
Conclusions

Fluidization and melting point depression in DPPC liposomes were studied in the presence of a homologous series of compressed and liquid n-alkanes (C\textsubscript{2} to C\textsubscript{10}). Over the conditions investigated, maximum bilayer fluidization occurred at 7.0 MPa and 10.4 MPa ethane and propane within the gel and fluid phase, respectively. Above these pressures, fluidization was decreased as pressure-reordering countered the disordering due to solute accumulation. In addition, anisotropy was dependent on the total molecular surface area of the inserted compressed gas and liquid n-alkane, demonstrating the influence of solute size and bilayer concentration on fluidization and providing a link between liquid and compressed gas n-alkanes.

Significant melting point depression was observed due to the accumulation of ethane and propane (present as an excess compressed headspace) within the bilayer. A large broadening of the phase transition region was evident, with a reduction in the cooperativity of the transition as indicated by the van’t Hoff enthalpy. The broad transition suggests that the region of maximum bilayer permeability can be extended through the addition of pressurized ethane and propane and reversed by depressurization. Knowledge of pressure- and solvent-effects on the physicochemical properties of bilayers could be applied to liposome formation and materials synthesis in amphiphilic assemblies.

References


Chapter 6

Molecular and Phase Toxicity of Compressed and Supercritical Fluids in Biphasic Continuous Cultures of Clostridium thermocellum

Introduction

The productivity of aqueous-phase whole-cell bioprocesses can be enhanced through the continuous extraction of hydrophobic products using organic solvents. However, the applicability of solvent extraction in situ is limited by the degree of solvent biocompatibility, aqueous/solvent partitioning behavior of the product, and ease of downstream solvent separation and product recovery. Compressed and supercritical fluids (e.g., CO₂, ethane, and propane) have gained considerable attention as alternative solvents in biotechnology (Randolph 1990; Jarzebski and Malinowski 1995) and biocatalysis (Mesiano et al. 1999; Knez and Habulin 2002). This is based in part on the ability to completely separate these solvents from the product and processing stream through depressurization.

Additional advantages of employing compressed and supercritical fluid (SCF) solvents include the ability to tune solvent strength with minor changes in temperature and pressure and enhanced mass transfer relative to liquid solvents due to low kinematic viscosity (McHugh and Krukonis 1994). Numerous investigations have examined the phase equilibria of compressed and supercritical fluid/organic solute/water systems representative of in situ extraction in whole-cell processes (Dohrn and Brunner 1995; Chistov and Dohrn 2002). However, very little is known regarding the biocompatibility of compressed and SCF solvents and their effect on whole cell metabolism and growth. We have previously shown that pressurized solvents significantly alter product formation and substrate consumption in batch cultures of Clostridium thermocellum, which was used as a model thermophilic bacterium (Knutson et al. 1999; Berberich et al. 2000b, a). However, these investigations have shown that conventional measures of solvent toxicity do not describe the biocompatibility of these solvents (Berberich et al. 2000b, a).
Determining the biocompatibility of compressed and SCF solvents for aqueous whole-cell bioprocesses requires characterization of both molecular and phase toxicity (Bar 1986; León et al. 1998). Molecular toxicity, which is frequently correlated with the natural logarithm of the octanol/water partition coefficient \( \log P_{o/w} \) (Laane et al. 1987; Inoue and Horikoshi 1991), is due to the dissolution of solvent molecules into the aqueous phase followed by solvent partitioning into the cell membrane (Osborne et al. 1990; León et al. 1998). The solvent can then interact with the lipid membrane and reduce the degree of order within the hydrophobic core. Membrane disordering can inhibit the activity of membrane-bound enzymes and alter lateral and transmembrane permeability, which are essential for regulating the internal cell environment, metabolism, and growth. In contrast, phase toxicity is caused by the presence of an aqueous/solvent interface, leading to interfacial cell contact and adsorption, the partitioning of essential nutrients from the aqueous phase, extraction of cellular or membrane components, and limited nutrient availability (Bar 1986; León et al. 1998).

The application of compressed and SCF in whole-cell bioprocesses is limited by their complex solvent properties and the lack of knowledge concerning the effects of these solvents on cell function and stability. Solvent concentrations in the aqueous phase and cell membrane are sensitive to minor changes in temperature and pressure; therefore, molecular toxicity by compressed and SCF solvents can vary significantly with operating conditions. The magnitude of phase toxicity is likely coupled with molecular toxicity, as the density of the solvent phase and the aqueous/solvent interfacial tension are also functions of temperature and pressure. Currently, our understanding of the effects of compressed and SCF fluids on whole cells is limited to the (i) sterilization and disruption of microorganisms by pressurized CO\(_2\) (Lin et al. 1992; Dillow et al. 1999; Spilimbergo et al. 2002), (ii) viability of freeze dried *Psudomonas Aeruginosa* in the presence of CO\(_2\) (Shah 2003), and (iii) metabolic activity of non-growing *Clostridium thermocellum* (Wiegel 1980; Lynd 1989) in biphasic batch cultures using pressurized N\(_2\), CO\(_2\), ethane, and propane (Knutson et al. 1999; Berberich et al. 2000b, a).
The goal of this work is to develop techniques to characterize the effects of dissolved gases on whole cells and to elucidate the mechanism of toxicity by pressurized solvents using biphasic continuous cultures of *C. thermocellum*, a non-barophilic bacterium. *C. thermocellum* was chosen as a model ethanol-producing thermophilic bacterium based on its ability to convert cellulosic materials to the liquid products ethanol, acetate, lactate and the gaseous products H₂ and CO₂. A novel high-pressure continuous biphasic bioreactor was constructed by modifying our existing method for single phase operation under elevated hydrostatic pressure (Bothun et al. 2004). This platform allows for the delineation of pressure- and solvent-effects by comparing cultures conducted under elevated hydrostatic and hyperbaric (biphasic) pressures. Continuous cultures were conducted in the presence of compressed N₂, ethane, and propane at 333 K as a function of pressure (1.8 and 7.0 MPa) at a single dilution rate ($D = 0.167 \text{ h}^{-1}$). The ability to describe the effects of these fluids on the metabolism and growth of *C. thermocellum* based on the following solvent physiochemical properties was examined: aqueous solubility, $log P_{o/w}$, bulk solvent density, and membrane solubility. Optical density measurements were performed in the presence of 1.8 and 7.0 MPa propane to determine the role of solvent phase (gas or liquid) on interfacial cell adsorption and the contribution of adsorption to phase toxicity.

**Materials and Methods**

**Cell Culture Preparation**

*C. thermocellum* ATCC 31549 was obtained from the American Type Culture Collection (Rockville, MD). Since this culture is actually a co-culture of *C. thermocellum* JW20 and *Thermoanaerobacter ethanolicus*, the clostridial strain was isolated as previously described (Erbeznik et al. 1997). The organism was grown in a basal medium that included (per L): 0.61 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 0.5 g of (NH₄)₂SO₄, 0.5 g of NH₄Cl, 0.09 g of MgCl₂·6H₂O, 0.03 g of CaCl₂, 0.5 g of cysteine, 4 g of Na₂CO₃, 2 g of yeast extract, 10 ml of a vitamin mixture (Cotta and Russell 1982) and 5 ml of a microminerals mixture (Cotta and Russell 1982). Cultures were grown on 11.7 mM cellobiose at 333 K and harvested during the later portion of exponential growth by centrifugation (10,000 x g for 20 min at 298 K). Cell pellets were then
resuspended with basal media containing yeast extract at 2 g/L. Preliminary experiments demonstrated that the organism does not grow in the absence of yeast extract (data not shown). Because *C. thermocellum* is sensitive to oxygen, cell manipulations were performed in sealed vessels that had been purged with nitrogen.

**Continuous Culture under Elevated Hydrostatic and Hyperbaric Pressure**

Pressurized continuous culture experiments were performed at 333 K in a continuously stirred (impeller, 300 rpm) 100 ml stainless steel Parr Mini Reactor (rated to 623 K and 20.7 MPa). A detailed description of the high-pressure continuous bioreactor for a single phase system is presented in Bothun et al. (2004) (Chapter 3). This system was modified to accommodate the continuous flow of two immiscible phases; the fermentation broth and the compressed or supercritical fluid (Figure 6.1). Modifications include a syringe pump to receive the raffinate, and an additional syringe pump and back pressure regulator to control the compressed gas phase. The batch and continuous cultures under elevated hydrostatic pressure, and the biphasic continuous cultures under elevated hyperbaric pressure, were conducted consecutively within the same bioreactor.

After inoculation into the sterilized bioreactor (as described in (Bothun et al. 2004)), the bioreactor was pressurized using an Isco 500D syringe pump (Lincoln, NE) to the selected operating pressure (1.8 or 7.0 MPa hydrostatic pressure) over 30 min, at which point the culture was maintained in batch mode under pressure for 24 h before initiating continuous flow. Deoxygenated sterile media containing 2 g/L cellobiose (5.8 mM) was fed into the reactor at 16.7 ml/h to achieve the desired dilution rate (*D* = 0.167 h⁻¹). The reactor was maintained under hydrostatic pressure (in the absence of a gaseous or solvent headspace) using a second syringe pump (model 500D) as an effluent receiver until the continuous culture achieved steady-state (98% turnover of the culture vessel contents) under hydrostatic pressure. Based on *D* = 0.167 h⁻¹ and 98% turnover, steady-state is reached at 24 h. An effluent sample was collected at steady-state using a high-pressure sampling valve fitted with a 1 ml sample loop that was flushed with deoxygenated CO₂ to prevent oxygen from entering the system.
Pressurized biphasic continuous cultures were initiated in the hydrostatic pressure culture by introducing a headspace (approximately 25 ml) of compressed or SCF. A third Isco syringe pump (model 260 D) was used to supply the fluid at constant flow (4.2 ml/h). The media flow rate was adjusted to 12.5 ml/h to maintain a dilution rate of $D = 0.167 \text{ h}^{-1}$, while accounting for the reduced aqueous volume in the reactor with the addition of a compressed fluid headspace. In the biphasic continuous cultures pressure was maintained using a backpressure regulator (DB Robinson LTD, Edmonton, Alberta; model BPR-10-316) connected to the outlet stream of the compressed or SCF headspace.

Biphasic continuous cultures were conducted at 333 K in the presence of compressed $\text{N}_2$, supercritical ethane, and liquid propane at 7.0 MPa; and gaseous propane at 1.8 MPa. The point at which the reactor switches from steady-state cultures...
under elevated hydrostatic pressure to biphasic cultures represents \( t = 0 \) h. Effluent samples were taken periodically beginning 6 h after the compressed phase was introduced.

The ability to reverse the effects of biphasic incubations on cell growth and selectivity was demonstrated in cultures with compressed propane by (i) pressurizing with 7.0 MPa propane until steady-state was achieved, followed by \textit{depressurization} to 1.8 MPa propane and (ii) pressurizing with 1.8 MPa propane until steady-state followed by \textit{pressurization} to 7.0 MPa propane. In these experiments, each biphasic culture was preceded by a culture conducted under 7.0 and 1.8 MPa hydrostatic pressure, respectively.

\textbf{Sample Analysis}

Samples were withdrawn anaerobically from the culture vessel and centrifuged (10,000 x g, 10 min, 277 K) to separate the cells. The resulting supernatant was stored at 269 K until analysis. Bacterial dry cell weights (DCW) were determined using optical density measurement at 600 nm (OD\textsubscript{600}); it was previously determined that one optical density unit corresponds to 0.464 g DCW per liter (data not shown). Samples collected from the high-pressure bioreactor were analyzed by light microscopy at atmospheric pressure immediately after collection to insure that the system was not contaminated by foreign microbes.

Cellobiose, ethanol, glucose, and lactate in supernatant were measured by enzymatic methods as previously described (Bergmeyer 1963; Russell and Baldwin 1978). Acetate concentrations were determined in acidified samples by gas chromatography using a column (1.83 m x 4 mm) packed with Supelco SP-1000 (1% H\textsubscript{3}PO\textsubscript{4}, 100/120 mesh). Nitrogen was used as a carrier gas and the inlet and detector temperatures were 458 K and 463 K, respectively. The oven temperatures increased from 398 K to 408 K at a rate of 9°C/min after an initial isothermal period of 0.5 min.

Since the introduction of a compressed or SCF headspace in biphasic incubations can be considered a perturbation to cultures growing under elevated hydrostatic pressure, it may cause the system to deviate from steady-state. When such a perturbation occurs, the measured values for cell, substrate, and product concentrations
become time dependent and do not directly represent the amounts due to fermentation by the cells present at that specific time. Specifically, if cell growth and metabolism are severely inhibited by the introduction of a compressed or supercritical fluid phase, transient changes in these concentrations would be attributed to the dilution of components from the previous condition or sample.

**Cell Density in the Presence of Compressed Propane**

Adsorption of cells at the interface reduces the cell density in the bulk phase and may be a driving force for the observed phase toxicity in biphasic incubations. Cell density in biphasic aqueous/compressed propane systems was measured by optical density using a Hewlett-Packard UV spectrometer. The UV-vis spectrometer was fitted with a high-pressure variable volume spectroscopic cell (rated to 20.7 MPa). The spectroscopic cell has a working volume of 10-25 ml, a light path of 2.5 cm, and is capable of temperature control (Omega model CN9000A) and continuous mixing using a magnetic stirrer. Experiments were initiated by loading the spectroscopic cell with a cell suspension that contained no substrate and heating to 333 K. Substrate and yeast extract was excluded from the suspension to prevent cell growth, eliminating the need to quantify the effects of growth on interfacial adsorption.

The point at which the system reached 333 K was taken at $t=0$ h. Compressed propane at 1.8 or 7.0 MPa was then quickly loaded into the headspace and supplied at constant pressure using an Isco syringe pump. Cell density was determined at OD$_{600}$, which was measured a function of time and pressure. Results are expressed as % reduction in cell density, which accounts for slight variations in initial cell density between the two different cell suspensions. The effects of compressed propane on cell density are compared to the time-dependent cell density of a control experiment conducted under the same conditions at atmospheric pressure (in the absence of propane). The results at atmospheric pressure and 1.8 MPa propane represent single experiments without duplication. At 7.0 MPa propane standard deviations are associated with duplicate experiments from 0 to 6 h.
Results

Substrate Consumption and Growth

The initial condition, \( t = 0 \) h, represented the time at which elevated hydrostatic pressure (single-phase) cultures achieved steady-state. This point also corresponded to the introduction of a pressurized fluid phase and the starting point for the biphasic cultures. Results for cell density and cellobiose concentration at \( t = 0 \) h were consistent across all experimental runs at 7.0 MPa, indicating that the hydrostatic incubations reached similar steady state prior to initiating the biphasic experiments. In addition, the magnitude of the cell density, cellobiose concentration at \( t = 0 \) h were consistent with our previous results for hydrostatic continuous cultures of \( C. \) thermocellum (Bothun et al. 2004a; Chapter 3).

Continuous biphasic incubation in the presence of 7.0 MPa N\(_2\) (\( T_c = 126.2 \) K, \( P_c = 3.4 \) MPa) had little effect on cell density or residual cellobiose concentration when compared to the hydrostatic culture (\( t = 0 \) h, Figure 6.2A). This result demonstrated that compressed N\(_2\) did not influence cell growth or substrate consumption relative to 7.0 MPa hydrostatic pressure.

At 333 K and pressures ranging from 1.8 to 7.0 MPa, ethane can exist as a gas, a supercritical fluid, or a liquid, while propane can exist as a gas or a liquid. Therefore, biphasic incubations in the presence of compressed ethane (\( T_c = 305.4 \) K, \( P_c = 4.9 \) MPa) or propane (\( T_c = 369.8 \) K, \( P_c = 4.2 \) MPa) were conducted to observe the effects of gaseous (1.8 MPa) and condensed (7.0 MPa) solvent phases on \( C. \) thermocellum metabolism. In addition, these experiments were designed to examine the reversibility of cell inhibition or metabolic activity. Supercritical ethane at 7.0 MPa significantly reduced both cell density and cellobiose consumption within 6 h after cultures were switched from the hydrostatic pressure mode (Figure 6.3A). However, decreasing the ethane pressure to 1.8 MPa after 24 h (Figure 6.3A) restored cell density and residual cellobiose concentration to the levels observed under hydrostatic pressure. This result showed the ability to reverse the toxicity of supercritical ethane by depressurization to the gaseous state.
Figure 6.2  Cell density and residual cellobiose concentration (A), and product formation (B) for biphasic continuous cultures of *C. thermocellum* in the presence of compressed nitrogen at 333 K. Time 0 h indicates the point at which the continuous culture was at steady-state under hydrostatic pressure of 7.0 MPa and the time at which pressurized nitrogen was introduced.

Cell density and cellobiose consumption were significantly reduced following the introduction of compressed propane at 7.0 MPa (Figure 6.4A). The decrease in cell density observed at 6 and 24 h was similar to that observed in the presence of ethane at 7.0 MPa at 6 and 24 h (Figure 6.3A). Decreasing pressure to 1.8 MPa (Figure 6.4A) again restored cell density and residual cellobiose concentration to the levels observed
under hydrostatic pressure. Similar to supercritical ethane, this result showed the ability to reverse the toxicity of liquid propane by depressurization to the gaseous state.

Figure 6.3 Cell density and residual cellobiose concentration (A), and product formation (B) for biphasic continuous cultures of *C. thermocellum* in the presence of compressed ethane at 333 K. Time 0 h indicates the point at which the continuous culture was at steady-state under hydrostatic pressure of 7.0 MPa and the time at which pressurized ethane was introduced.

Biphasic incubations were also performed with gaseous propane at 1.8 MPa followed by pressurization to the liquid state at 7.0 MPa (Figure 6.4B). Cell density was reduced by 28% in the presence of 1.8 MPa propane (relative to the value at *t* = 0 h).
with little apparent change in residual cellobiose concentration from 0 to 24 h. Therefore, essentially all cellobiose fed into the reactor was consumed at 1.8 MPa despite a decrease in cell density. Such a decrease in cell mass without a reduction in cellobiose consumption suggested that the bacterial maintenance coefficient (i.e. the amount of cellobiose required to maintain cell function) increased due to the presence of gaseous propane. Pressurization to 7.0 MPa resulted in a 9-fold reduction in cell density and increase in residual cellobiose consumption to levels in the substrate feed, indicating that metabolism and growth had virtually ceased (Figure 6.4B).

Figure 6.4 Cell density (A, B), residual cellobiose concentration (A, B), and product formation (C, D) for biphasic continuous cultures of *C. thermocellum* in the presence of propane at 1.8 (gaseous) and 7.0 MPa (liquid). Time 0 h indicates the point at which the continuous culture was at steady-state under hydrostatic pressure at 1.8 or 7.0 MPa and the time at which pressurized propane was introduced.
**Product Selectivity**

The levels of acetate and lactate were similar for all continuous cultures conducted under 7.0 MPa hydrostatic pressure (t = 0 h, Figures 6.2B, 6.3C, and 6.4C,D). In addition, the low levels of lactate production were consistent with previous investigations of hydrostatic pressures effects in continuous cultures of C. thermocellum (Bothun et al. 2004a; Chapter 3). Ethanol concentrations were similar at t = 0 h for cultures conducted in the presence of N₂, ethane, and propane (Figures 6.2B, 6.3B, and 6.4C,D). The observed ethanol concentrations were comparable to previous investigations (12.8 ± 2.0 to 17.4 ± 0.2 mM from D = 0.05 to 0.3 h⁻¹ at 7.0 MPa; Bothun et al. 2004a; Chapter 3).

In addition to cell density and substrate consumption, product formation by C. thermocellum was altered in biphasic incubations with compressed ethane and propane relative to elevated hydrostatic pressure (Figures 6.3B and 6.4C,D). Although the introduction of compressed N₂ led to a slight increase ethanol concentrations, product formation was not notable altered relative to 7.0 MPa hydrostatic pressure. At this point it is unknown why ethanol concentrations increased by 6 and 24 h after N₂ was introduced. Ethanol and acetate production were reduced more than 80% by 24 h after the introduction of supercritical ethane at 7.0 MPa, relative to 7.0 MPa hydrostatic pressure (Figure 6.3B). This significant reduction in product formation, in conjunction with a decrease in cell density and substrate consumption, showed that C. thermocellum was inactivated in the presence of supercritical ethane. Reversal of metabolic inhibition was observed within 3 h after depressurization from a supercritical (7.0 MPa) to a gaseous (1.8 MPa) ethane headspace (Figure 6.3B).

Similar to supercritical ethane, C. thermocellum was inactivated by liquid propane. The introduction of a liquid propane (7.0 MPa) headspace into the culture at 7.0 MPa hydrostatic pressure (Figure 6.4C) resulted in reductions of ethanol (28%) and acetate (83%) production by 24 h. However, the cause for the notable increase in ethanol concentration by 6 h (31%) is unknown. Depressurization to gaseous propane (1.8 MPa) did partially reverse these inhibitory effects (Figure 6.4C). Biphasic treatments beginning with 1.8 MPa propane exhibited reduced ethanol production up to 24 h with very little change in acetate production (Figure 6.4D). Subsequent
pressurization to the liquid state resulted in a marked reduction in both ethanol and acetate production. Based on the levels of cell growth and metabolism after 24 and 48 h at 1.8 (Figure 6.4B,D) or 7.0 MPa (Figure 6.4A,C), inhibition of \textit{C. thermocellum} by compressed propane is both reversible and independent of path (1.8 $\rightarrow$ 7.0 MPa or 7.0 $\rightarrow$ 1.8 MPa).

\textbf{Cell Adsorption at the Compressed Propane/Water Interface}

Cell adsorption at the liquid propane interface may contribute to diminished cell densities observed in the bulk phase (Figure 6.4A,B). Time-dependent reductions in the density of non-growing \textit{C. thermocellum} observed in an atmospheric pressure control (no propane) and in the presence of 1.8 MPa propane were virtually identical, suggesting that gaseous propane did not influence the bulk phase density of \textit{C. thermocellum} through cell adsorption at the propane/water interface (Figure 6.5). In contrast, there was a significant reduction in cell density in the presence of liquid propane (7.0 MPa) within 6 h relative to gaseous propane.

Rapid depressurization from 7.0 MPa propane after 6 h restored the cell density to within 15\% of the value observed at atmospheric pressure (data not shown). The large reduction in cell density in the presence of liquid propane and the ability to reverse this reduction by depressurization suggests that (i) cell were not irreversibly damaged or lysed in the presence of liquid propane and (ii) adsorption of cells at the liquid-propane/water interface contributes to the decrease in bulk cell density observed in pressurized biphasic incubations at 7.0 MPa.
Figure 6.5  Cell density (%) as a function of time under atmospheric pressure (control, ○), and in the presence of propane at 1.8 (gaseous, ■) and 7.0 MPa (liquid, ♦). Error bars represent the standard deviation associated with duplicate experiments at 7.0 MPa from 0 to 6 h. Cell density of 100% represents OD_{600} at t = 0 h.

Discussion
The development of a continuous biphasic high-pressure bioreactor facilitates the investigation of dissolved gas effects (product gases or compressed fluid) and the presence of an aqueous/fluid interface on microbial activity. Previous investigations of dissolved gases have been limited to batch cultures and did not provide the level of information necessary to understand, model, and manipulate dissolved gas effects (Jones and Greenfield 1982; Lamed et al. 1988; McIntyre and McNeil 1998). The few continuous culture experiments investigating the effects of dissolved CO_2 on growth and metabolic activity are substantially different than batch culture results and suggest that the inhibitory effect of CO_2 are greatly exaggerated in batch systems (Dixon and Kell 1989; McIntyre and McNeil 1998). The ability to manipulate the metabolism of growing cells in biphasic, pressurized incubations had not been previously demonstrated. Our
system permits investigations of dissolved gas effects (product or compressed fluid) on metabolism and bioenergetics in whole cells.

We recently showed that elevated hydrostatic pressure (7.0 and 17.3 MPa) has significant impacts on the metabolism and growth of \textit{C. thermocellum} (Bothun et al. 2004a; Chapter 3). Relative to atmospheric pressure, increasing hydrostatic pressure in continuous cultures shifted product selectivity towards ethanol at the expense of acetate production. Similarly, high ethanol and low acetate concentrations were also observed at $t = 0$ h (steady state hydrostatic pressure conditions) and 7.0 MPa, prior to the introduction of the compressed solvents (Figures 6.2, 6.3, and 6.4). The inhibition of acetate production at high pressures can be partially attributed to the increased aqueous solubility of H$_2$ with pressure; production of an acetate molecule usually leads to the production of two H$_2$ molecules (Lamed et al. 1988). Therefore, an increase in H$_2$ concentration in the broth produces a mass-action effect that reduces the thermodynamic driving force for acetate production.

Elevated hydrostatic pressure also reduced theoretical growth yield while increasing the maintenance coefficient (Bothun et al. 2004a; Chapter 3). This increased maintenance coefficient suggested that detrimental changes in membrane fluidity were occurring due to ordering-effects of pressure on phospholipid bilayers or disordering by the accumulation of dissolved product gases (H$_2$ and CO$_2$). Similarly, at 1.8 MPa propane a reduction in cell density without a change in cellobiose consumption was consistent with an increase in the maintenance coefficient. Further investigations in biphasic pressurized cultures are needed to determine bioenergetic parameters by varying the dilution rate. The contribution of pressure- and solvent-effects on cell function could be estimated by comparing these parameters under hydrostatic and hyperbaric pressures.

\textbf{Molecular Toxicity of Compressed and Supercritical Fluids}

Solvent toxicity is frequently correlated with the octanol/water partition coefficient ($log P_{o/w}$), where $log P_{o/w}$ values approximately $< 2$ are considered toxic and those approximately $> 4$ are considered biocompatible (Inoue and Horikoshi 1991; Laane et al. 1987). The $log P_{o/w}$ is a measure of solvent hydrophobicity, typically reported at 0.1
MPa and 298 K, which qualitatively describes the partitioning behavior of a solvent into a lipid cell membrane. A correlation has been developed that relates $\log P_{o/w}$ to the membrane/water partition coefficient ($log P_{m/w}$) on a molar basis (Sikkema et al. 1994):

$$log P_{m/w} = 0.97 \log P_{o/w} - 0.64$$  \hspace{1cm} (6.1)

Traditional measures of solvent toxicity, such as $\log P_{o/w}$, are frequently described in terms of molecular toxicity due to solvent-membrane interactions (membrane fluidization). The $\log P_{o/w}$ values for nitrogen, ethane, and propane (1.5 to 13.9 MPa, 298 and 333 K) reported by Berberich et al. (2000b) were used to determine $\log P_{m/w}$ using equation 6.1. The values for $\log P_{o/w}$ were shown to be insensitive to pressure, indicating that the membrane partition coefficient for propane is also constant at 1.8 and 7.0 MPa.

Solvent concentration within the membrane is expected to be a better measure of molecular toxicity via membrane fluidization. The concentration of pressurized solvents within the membrane ($x_m^i$) can be determined from $P_{m/w}$ if the aqueous solubility ($x_a^i$) is known.

$$P_{m/w} = \frac{x_m^i}{x_a^i}$$  \hspace{1cm} (6.2)

The solubility of ethane and propane in water was calculated by the Krichevsky-Kasarnovsky equation (Krichevsky and Kasarnovsky 1935) based on experimental Henry’s constants (Dhima et al. 1999; Harvey 1996). At the conditions of this investigation, the aqueous solubility and membrane/water partition coefficient of propane were insensitive to pressure, leading to similar calculated concentrations of propane within the membrane at 1.8 and 7.0 MPa (Table 6.1).

Based on the concentration of propane within the bilayer (Table 6.1), molecular toxicity may play a role in inhibiting $C.\ thermocellum$ in the presence of 1.8 MPa propane relative to hydrostatic pressure. This inhibition was made evident by the transient decrease in cell density after the introduction of gaseous propane (Figure 6.4B). However, molecular toxicity did not describe the changes in cell activity upon pressurization from gaseous to liquid propane. Although the concentration of propane within the cell membrane is the same in the presence of gaseous (1.8 MPa; 823 mM) and liquid (7.0 MPa; 834 mM) propane, cell growth, substrate consumption, and product
formation were greatly inhibited at 7.0 MPa relative 1.8 MPa. Therefore, log $P_{o/w}$ is not an adequate measure of propane toxicity. In addition, the inhibition of *C. thermocellum* does not correlate with dissolved propane concentrations in the cell membrane and is inconsistent with molecular toxicity. Comparatively, Berberich et al. (2000a,b) also demonstrated that log $P_{o/w}$ does not describe the toxicity of compressed ethane and propane toward non-growing *C. thermocellum* (metabolic inhibition) in batch incubation. The toxicity of ethane and propane in batch cultures of *C. thermocellum* was consistent with the inhibition and possible disruption of membrane transport processes (Berberich et al. 2000a).

Table 6.1  Compressed solvent properties and aqueous/membrane concentrations at 333 K.

<table>
<thead>
<tr>
<th></th>
<th>Pressure (MPa)</th>
<th>Density (mol/L)</th>
<th>Phase</th>
<th>Aqueous$^a$ (mM)</th>
<th>Membrane$^{b,c}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitrogen</td>
<td>7.0</td>
<td>2.5</td>
<td>gaseous</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>ethane</td>
<td>7.0</td>
<td>5.3</td>
<td>supercritical</td>
<td>36</td>
<td>475</td>
</tr>
<tr>
<td>propane</td>
<td>1.8</td>
<td>0.9</td>
<td>gaseous</td>
<td>22</td>
<td>823</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>10.3</td>
<td>liquid</td>
<td>22</td>
<td>834</td>
</tr>
</tbody>
</table>

$^a$ calculated from Krichevsky-Kasarnovsky (1953) equation.
$^b$ calculated by equations 6.1 and 6.2.
$^c$ based on reported log $P_{o/w}$ values (Berberich et al. 2000b).

Furthermore, we have recently used high-pressure fluorescence anisotropy with a membrane probe molecule to determine the effects of propane (up to 20.8 MPa) on the membrane fluidity of fully-hydrated dipalmitoylphosphatidylcholine liposomes (DPPC, $T_m \approx 315$ K) (Bothun et al. 2004b). DPPC bilayers were employed as model cell membranes. Minimal disruption of the fluid bilayer at 333 K and a similar depression of the melting temperature at 1.8 and 7.0 MPa suggest that molecular toxicity does not vary above 1.8 MPa propane. However, at pressures > 7.0 MPa moderate disruption of the bilayer was reversed due to the pressure-reordering of the acyl region. Based on these results, fluidization of cell membranes is expected to be similar in the presence of gaseous and liquid propane. Hence, the large differences in cell activity observed at 1.8 and 7.0 MPa propane are attributed to phase toxicity.
Phase Toxicity by Compressed and Supercritical Fluids

A primary motivation for utilizing compressed and supercritical fluid solvents is the ability to manipulate fluid density, or solvent strength, with minor changes in pressure and temperature. In biphasic aqueous systems, changes in fluid density significantly influence interfacial tension and the attractive forces between colloids and the fluid interface. For example, increasing propane pressure at 298 K reduces the aqueous/fluid interfacial tension from 59 to 46 dynes/com at 0.8 and 10.0 MPa, respectively (Wiegand and Franck 1994). In addition, an increase in the attractive interaction of aqueous DPPC vesicles at the propane interface has been calculated with increasing pressure (Berberich et al. 2001). Therefore, alterations in solvent density are likely related to phase toxicity by compressed and supercritical fluids.

In the presence of pressurized solvents, the effect of interfacial tension on C. thermocellum inhibition was difficult to describe due to the lack of aqueous/fluid tension measurements. Comparatively, inhibition of C. thermocellum in batch incubations with liquid n-alkanes (pentane through dodecane; C5 to C12, respectively) does not correlate directly with interfacial tension. Berberich et al. (2000b) showed partial metabolic activity of non-growing C. thermocellum in the presence of alkane solvents > C10 over 24 h, and complete inactivation for solvents < C7 after just 2 h. In contrast, the interfacial tension is relatively independent of carbon number for these liquid n-alkanes (Janczuk et al. 1993).

While phase toxicity does not appear to be directly dependent upon interfacial tension, a relationship between C. thermocellum activity in continuous cultures and solvent density was observed for gaseous, liquid, and supercritical fluids. In biphasic batch incubations with non-growing cells (Berberich et al. 2000b) solvents with greater densities ($\geq 6.5$ mol/L; C5 to C7) resulted in inactivation, while solvents with lower densities ($\leq 4.9$; C10 and C12) only partially inhibited activity. Comparatively, we observed significant inhibition in the presence of ethane (SCF, 5.3 mol/L) and propane (liquid, 10.3 mol/L) at 7.0 MPa, while 1.8 MPa propane (gaseous, 0.9 mol/L), 1.8 MPa ethane (gaseous, 0.7 mol/L), and 7.0 MPa N2 (SCF, 2.5 mol/L) resulted in minimal or no inhibition (Table 5.1, Figures 6.2, 6.3, and 6.4).
Based on the inability to adequately describe cell inhibition by molecular toxicity, phase toxicity via interfacial cell contact is likely the primary cause of toxicity for compressed and SCF ethane and propane. The ability to induce or reverse propane toxicity by changing pressure further suggests that the reduction in cell density observed in the presence of 7.0 MPa ethane or propane may be partially attributed to increased interfacial cell adsorption at high solvent densities (Figures 6.3 and 6.4). For example, an increase in attractive interactions for aqueous DPPC vesicles at the aqueous/solvent interface is predicted with increasing propane pressure (Berberich et al. 2001).

The production of metabolic end-products in the presence of supercritical ethane and liquid propane indicates that metabolism remains partially active despite interfacial adsorption. This apparent activity may be due to destabilization and/or disruption of the cell membrane. Alternatively, a thick cell layer may be adsorbing at the interface due to strong attractive forces (Bar 1988; Bouchez-Naitali et al. 2001), resulting in partial activity of the cells. If a cell layers formed at the fluid interface, the cells directly contacting the interface would be susceptible to membrane destabilization, while the remaining cell layers would remain active, but the uptake of nutrients and substrate would be limited by mass transfer in the biofilm.

Conclusions

Low-density phases of compressed N$_2$ at 7.0 MPa, and gaseous ethane and propane at 1.8 MPa, yielded minimal inhibition of *C. thermocellum*. In contrast high-density compressed phases (supercritical ethane and liquid propane at 7.0 MPa) resulted in significant reduction in cell density, cellobiose consumption, and product formation; consistent with our previous investigations with liquid alkanes. Inhibition by supercritical ethane and liquid propane was rapidly reversed upon depressurization to gaseous phases, indicating the importance cell adsorption at the fluid interface.

Inactivation of *C. thermocellum* is consistent with phase toxicity, which is exacerbated by increased cell adsorption at the aqueous/solvent interface. Molecular toxicity due to membrane fluidization is present, but its role in solvent toxicity varies greatly with increasing pressure and is secondary to phase toxicity. Identifying the
mechanism of solvent toxicity in compressed and supercritical fluids, which exhibit unique and beneficial solvent properties, allows for the determination of appropriate solvent systems to manipulate product extraction and metabolic activity in situ. These results demonstrate the effect of solvent phase (gaseous and liquid propane) in a single solvent system and the ability to manipulate metabolic activity with pressure.

References


Chapter 7

Conclusions and Future Work

While compressed and supercritical fluids offer distinct advantages as processing solvents relative to conventional organic liquids, their use in whole-cell biotechnologies and bioprocesses has been hindered by the unknown combined effects of pressure and solvent on the integrity and function of the cell. The influence of compressed and supercritical fluid solvents on metabolic and structural perturbations in whole-cell bioprocessing was examined using a model thermophilic bacterium, Clostridium thermocellum, and unilamellar liposomes as model cell membranes. Experimental techniques were developed and implemented to quantify pressure- and solvent-induced perturbations on microbial inhibition.

Continuous Culture under Hydrostatic Pressure

Changes in metabolic selectivity, growth, and bioenergetic parameters can be easily deduced using a continuous culture bioreactor. Continuous cultures of C. thermocellum conducted at 333 K under elevated hydrostatic pressure revealed that pressure alone (7.0 and 13.9 MPa) inhibited growth and shifts product selectivity towards ethanol (Chapter 3). Shifts in product selectivity were partially attributed to a reduction in acetate formation due to increased aqueous H₂ solubility with pressure. In addition, increasing hydrostatic pressure resulted in a significant reduction in the theoretical maximum growth yield and an increase in the maintenance coefficient; this result implies that under elevated pressure more energy is required to maintain proper cell function and an energized membrane. Changes in membrane structure under elevated hydrostatic pressure were attributed to the ordering effects of pressure on phospholipid bilayers and the disordering effects of dissolved product gases (H₂ and CO₂) due to enhanced aqueous solubility with increasing pressure. This study demonstrates the ability to manipulate non-barophilic bacterium with moderated pressures.
Liposome Fluidization by Pressurized Solvents

Advancing the application of pressurized solvents in whole-cell bioprocessing requires knowledge of the solvent-effects on biomembranes and their role in cell inhibition. High-pressure fluorescence anisotropy revealed significant bilayer fluidization ($\propto$ viscosity$^{-1}$) and melting point depression in unilamellar liposomes composed of dipalmitoylphosphatidylcholine (DPPC, $T_m \approx 315$ K) in the presence of pressurized CO$_2$ (Chapter 4), ethane, and propane (Chapter 5). Pressurization with a 7.0 MPa CO$_2$, propane, or ethane headspace fluidized both the gel ($T < T_m$) and fluid ($T > T_m$) phase of the bilayer, and reduced $T_m$ to ambient temperature. Carbonic acid formation due to dissolved CO$_2$ influenced bilayer fluidity, demonstrating the concomitant effects of solvent-fluidization and pH associated with pressurized CO$_2$. Conventional melting point theory was used to calculate the concentration of CO$_2$, ethane, and propane within the gel phase, taking into account the effect of hydrostatic pressure on $T_m$ and the reduction in the phase transition enthalpy due to solvent accumulation with pressure. These results demonstrated that compressed and supercritical fluids perturbed the structure of model cell membranes. Investigations of bilayer fluidization by pressurized solvents are also directly relevant to liposome formation, which are extensively used as a medium for pharmaceutical formulation and materials synthesis, and CO$_2$-based microbial sterilization.

Biphasic Continuous Culture under Hyperbaric Pressure

A novel high-pressure continuous bioreactor was developed to study biphasic incubations in the presence of pressurized solvents. Continuous cultures of C. thermocellum conducted at 333 K in the presence of pressurized N$_2$, ethane, and propane further demonstrate the inability of log $P_{o/w}$ to characterize solvent toxicity (Chapter 6). Cell density, substrate (cellobiose) consumption, and product formation were minimally affected by supercritical N$_2$ (7.0 MPa) and gaseous propane (1.8 MPa) despite low Log $P_{o/w}$ values. In contrast, supercritical ethane and liquid propane at 7.0 MPa, which have significantly higher densities (mol/L), completely inhibited cellobiose consumption, and dramatically reduced cell density and product formation. Comparing these results with our previous observations of metabolic activity with non-growing C.
thermocellum in the presence of liquid alkanes (pentane to dodecane, C$_5$ to C$_{12}$) suggest that the density of the bulk solvent phase is a good indicator of solvent toxicity regardless of the phase (liquid or supercritical). It was demonstrated that phase toxicity is exacerbated by the increased cell adsorption of cell at the aqueous/solvent interface.

Ultimately, this work provides an initial basis for determining the effects of dissolved gases and pressurized solvents in terms of both metabolic and structural perturbations due to molecular and phase toxicity. In addition, techniques were developed and demonstrated for determining the influence of pressure- and solvent-effects on whole-cells due to the presence of a pressurized solvent phase (hyperbaric pressure). The results presented herein are applicable to in situ extraction schemes, liposome processing and material synthesis, and bacterial sterilization with compressed and supercritical fluids. Furthermore, this work represents the first scientific study that directly characterizes metabolic and structural perturbations of whole cells by compressed and supercritical fluid solvents.

Future Work

Unilamellar liposomes composed of a mixture of different phospholipids, fatty acids, and surfactants can be employed to better simulate the cell membrane and improve our understanding of bilayer perturbations in a more realistic system. This may include the incorporation of cholesterol, which comprises up to 30% in the membrane and can reduce the fluidizing effects of solvents and anesthetics (Lemmich et al. 1996). In addition, self-assembled templates for material synthesis and the biomaterial formation may also involve the incorporation of multiple surface active agents to manipulate structure and morphology. In addition to methods that involve the use of an external fluorescent probe molecule (such as DPH) embedded with the bilayer, membrane-bound proteins can be labeled with a fluorophore (such as tryptophan) to directly study their activity within the bilayer when subjected to solvent-fluidization (Chong et al. 1985; Tauc et al. 2002). X-ray diffraction and nuclear magnetic resonance can also be employed to quantitatively characterize the bilayer - determining bilayer thickness, headgroup spacing, and chain packing (Brady 1977; Ueda and Yoshida 1999).
Modeling membrane perturbations can provide theoretical evidence on the location of pressurized solvents within the bilayer and the nature of the adsorption site. Due to their complexity, modeling cell membranes is typically performed using molecular dynamics simulation (Xiang and Anderson 1994, 1999). However, previous studies have demonstrated the ability to obtain information on liposome swelling and solvent accumulation using Flory-Huggins (Maxwell and Kurja 1995) and lattice-based theories (Nagle 1980; O'Leary 1982; Xiang and Anderson 1994). Predictions of bilayer disorder can be compared to our fluorescence anisotropy studies, which qualitatively describe alterations in configurational entropy via membrane fluidity. Modeling the behavior of liposomes in the presence of compressed and supercritical fluids may allow for the prediction of biocompatible solvents based on solvent hydrophobicity and topology.

Pressurized solvents may be used to manipulate transmembrane diffusion (permeability) cellular products or components. These components may include solvents, fuels, biosurfactants, or intracellular proteins. Specifically, product formation and the extraction of extracellular components can be manipulated in permeabilized cells (Felix 1991; Flores et al. 1994), cells in which the bilayer permeability is controlled with the addition of organic solvents. However, the solvents typically employed are lipophilic (hydrocarbons or detergents), which means that solvent separation and product recovery must be accomplished with an additional unit operation (i.e. filtration followed by distillation). Employing compressed or supercritical fluids would provide a way to reversible alter bilayer permeability and completely remove the solvent from the system through depressurization.

Similarly, the ability to control liposome permeability is very important in the areas of liposome formation, such as the development of controlled release devices for pharmaceutical applications. The formation of liposomes using pressurized CO$_2$ has been demonstrated previously. Specific benefits of this process and include the ability to control morphology and narrow particle size distributions (Frederiksen et al. 1997; Otake et al. 2001). Altering gel or fluid phase fluidity and the melting temperature of liposomes using pressurized CO$_2$ could prove useful in predicting and controlling morphology. In addition, direct knowledge of bilayer fluidity in the presence of CO$_2$ could
be used to tailor post-formation loading and extraction techniques. Based on our results, compressed and supercritical ethane or propane may also be used to control liposome formation in this manner.

We have obtained preliminary results that suggest interfacial tension measurements and UV spectroscopy can be used to describe aqueous phospholipid systems in the presence of excess compressed and supercritical CO\textsubscript{2}. Isothermal equilibrium and kinetic interfacial tension measurements can be used to obtain the phospholipid melting temperature and the rate of interfacial liposome adsorption as a function of CO\textsubscript{2} pressure, respectively. From these measurements the degree of liposome destabilization at the CO\textsubscript{2}/H\textsubscript{2}O interface can be determined. Similarly, UV spectroscopy can also be used to determine the melting temperature of liposomes as solvent-fluidization leads to hydration of the acyl region and a change in the refractive index of the bilayer (Yi and MacDonald 1973; Gugliotti et al. 1998). By accounting for phase changes within the bilayer (refractive), UV spectroscopy may be used to determine changes in optical density (turbidity) due to a change in liposome size distribution as a function of temperature, CO\textsubscript{2} pressure, or ion/buffer concentration. Interfacial tension, UV spectroscopy, and fluorescence anisotropy can be combined to provide a complete description of the bilayer interior, the bulk phase, and the aqueous/solvent interface.

Finally, the knowledge gained from this investigation and the techniques developed to analyze extreme processing conditions and variable solvent toxicity have potential utility in other biological systems such as solvent-utilizing bacteria, bioremediation, and life in extreme environments. For instance, characterizing the general affect of solvents on biological systems (membrane fluidity and biphasic cultures) may lead to improved bioremediation processes by providing microbial “screening “ and determining bioenergetic parameters in harsh environments (sub-surface solvent remediation, oil reservoir conditions, geothermal/volcanic sites, etc.). Biphasic incubations in the presence of excess pressurized gases, such as light hydrocarbons, H\textsubscript{2}, and He, which are abundant in our universe, may be used to explore the effects of other planetary environments on earth’s microbial life.
References


Appendix A

Development of a Metabolic Flux Analysis for Cellobiose Fermentation by
Clostridium thermocellum in Continuous Culture

A simplified procedure for developing a metabolic flux analysis (MFA) for continuous cultures is presented in the Background section (Chapter 2). Appendix A is a supplement to Chapter 3, and provides a detailed description of the metabolic flux analysis applied to continuous cultures of C. thermocellum performed at atmospheric pressure and under elevated hydrostatic pressure. H2 and CO2 concentrations presented in Chapter 3 were calculated by MFA, which is based on the amount of substrate consumed and product formation. Table A.1 lists the 11 metabolic reactions, and the respective enzymes, that describe product formation in C. thermocellum.

Table A.1 Metabolic reactions for cellobiose fermentation by C. thermocellum used in metabolic flux analysis (Erickson and Fung, 1988).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cellobiose ↔ 2 glucose</td>
<td>cellulase</td>
</tr>
<tr>
<td>2 glucose ↔ 2 pyruvate + 2 ATP + 2 NADH</td>
<td>phosphoenolpyruvate, phosphotransferase system, and EMP</td>
</tr>
<tr>
<td>3 pyruvate + Fd + CoA ↔ acetyl-CoA + CO2 + FdH2</td>
<td>pyruvate ferredoxin oxidoreductase</td>
</tr>
<tr>
<td>4 pyruvate + NADH + H+ ↔ lactate + NAD</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>5 Fdox + NADH ↔ Fdred + NAD</td>
<td>NADH-ferredoxin oxidoreductase</td>
</tr>
<tr>
<td>6 Fdred + 2H+ ↔ Fdox + H2</td>
<td>Hydrogenase</td>
</tr>
<tr>
<td>7 Acetyl-CoA + NADH ↔ acetaldehyde + CoA + NAD</td>
<td>acetaldehyde dehydrogenase</td>
</tr>
<tr>
<td>8 acetaldehyde + NADH + H+ ↔ ethanol + NAD</td>
<td>ethanol dehydrogenase</td>
</tr>
<tr>
<td>9 acetyl-CoA + Pi ↔ acetyl-P + CoA</td>
<td>phosphotransacetylase</td>
</tr>
<tr>
<td>10 acetyl-P + ADP ↔ acetate + ATP</td>
<td>acetate kinase</td>
</tr>
<tr>
<td>11 glucose + 0.873 NADH + 156 ATP/YX/ATP ↔ biomass</td>
<td></td>
</tr>
</tbody>
</table>

The coupled equations presented in Table A.1 were solved simultaneously based on a mass balance using Maple (Mathsoft™) with the input variables listed in Table A.2. These variables represent both measured and calculated values. For each dilution rate, the metabolite concentrations and relative reaction rates are calculated by Maple using
matrix algebra. The concentration of any metabolite within the system of reaction pathways can be easily determined by mass balance.

Table A.2 Parameter (variable) inputs for *C. thermocellum* MFA.

<table>
<thead>
<tr>
<th>Measured</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>celllobiose (mM)</td>
<td>ATP (mM)</td>
</tr>
<tr>
<td>glucose (mM)</td>
<td>([ATP] = [ethanol] + 2[acetate] + [lactate])</td>
</tr>
<tr>
<td>ethanol (mM)</td>
<td>(Y_{X/ATP})</td>
</tr>
<tr>
<td>acetate (mM)</td>
<td>graphically (described in Chapter 3)</td>
</tr>
<tr>
<td>lactate (mM)</td>
<td></td>
</tr>
<tr>
<td>biomass (g DCW/L)</td>
<td></td>
</tr>
</tbody>
</table>

References

Appendix B

Measuring Aqueous Phase pH due to Carbonic Acid Formation in the Presence of Pressurized CO₂

The information presented in this section is a supplement to the model presented in Chapter 4 for predicting aqueous phase pH due to the dissolution of CO₂. Additional preliminary experimental results are presented where both UV and fluorescence spectroscopy techniques were employed to determine pH *in situ* as a function of temperature, pressure, and sodium carbonate buffering (Na₂CO₃) using water-soluble probe molecules. The technique presented herein is the first reported study measuring pH in the presence of compressed and supercritical CO₂ using fluorescence spectroscopy. These results are presented to further demonstrate the accuracy of the calculation method and to depict the relationship between aqueous CO₂ solubility, bilayer CO₂ solubility, and aqueous pH.

Under pressurized conditions, the solubility of CO₂ increases dramatically, significantly altering pH due to the formation of carbonic acid. To summarize, the following equations describe the formation of carbonic acid due to dissolved CO₂.

\[
CO₂(aq) \rightleftharpoons H₂CO₃ \rightleftharpoons H^+ + HCO₃^-
\]

\[
H^+ + HCO₃^- \rightleftharpoons H^+ + CO₃^{2-}
\]

The Henderson-Hasselbalch equation is then employed to calculate pH (Lahiri and Forster 2003; Spilimberger 2002).

\[
pH = -\log K_{a1} + \log \frac{[HCO₃^-]}{[CO₂(aq)]}\tag{B.1}
\]

where \([HCO₃^-]\) equals \([H^+]\) in an unbuffered system, and the ionic activity coefficients were assumed constant at unity. Calculating aqueous phase CO₂ concentrations, CO₂ (aq), is presented in Appendix C. Comparisons between model predictions are discussed in Chapter 4.
Measuring Aqueous pH by Fluorescence Spectroscopy

LysoSensor Yellow/Blue DND-160 (Molecular Probes, Eugene, OR) is a dual adsorption (329, 384 nm) and dual emission (440, 540 nm) probe molecule ($pK_a = 4.2$) capable of measuring aqueous pH under acidic conditions (pH 3.5 to 6). Calibration was performed by dissolving the LysoSensor probe in colorless buffer solutions (pH 3.0 to 6.5, Fisher Scientific) at a concentration of approximately 1 $\mu$m. Similarly, aqueous solutions were prepared by dissolving the probe in deionized water. Fluorescence measurements were conducted at a fixed excitation wavelength ($\lambda_{ex} = 360$ nm) by monitoring the emission intensity ratio at $\lambda_{em} = 440$ and 540 nm (540/440). Calibration was fitted to the following equation:

$$\text{pH} = pK_a + \log \left[ \frac{I_{B(540 \text{ nm})}}{I_{A(540 \text{ nm})}} \times \frac{R - R_B}{R_A - R} \right]$$

(B.2)

where pH is associated with the buffer, $I_{B(540 \text{ nm})}$ and $I_{A(540 \text{ nm})}$ represent the endpoint basic ($B$) and acidic ($A$) intensities at $\lambda_{em} = 540$ nm, $R_B$ and $R_A$ represent the ratio of 540/440 at the basic and acidic endpoints, and $R$ is the ratio of 540/440 for the given pH. Plotting pH as a function of $R$ yields a straight line with $pK_a$ as the intercept. Based on the calibration performed, $pK_a = 4.6$ was determined at both $\lambda_{ex} = 360$ and 384 nm. Deviations between $pK_a$ observed experimentally and reported in the company’s product information may be due to the presence of 50 mM ion concentration in the buffers.

The equipment used to conduct the high-pressure fluorescence measurements is described in Chapter 4. Aqueous pH was measured in a two-phase water-CO$_2$ system as a function of CO$_2$ pressure (0.2 to 17.3 MPa) and temperature (298 and 333 K). Based on fluorescence measurements, increasing CO$_2$ pressures reduces the pH to 3.6 at 17.3 MPa. Results are compared with model predictions (Chapter 4) in Figure B.1. Discrepancies between measured and predicted pH are unknown; however, they may be related to differences in ion concentration (present in buffer calibration).
Figure B.1  Aqueous pH measured by fluorescence spectroscopy (LysoSensor probe) and model predictions (Chapter 4) at 298 and 333 K as a function of CO₂ pressure.

Figure B.2  Aqueous pH measured by fluorescence spectroscopy (LysoSensor probe) at 298 K with Na₂CO₃ buffering. Predictions represent systems with and without buffering.
The addition of 4 g/L Na₂CO₃ (37.7 mM) increases the pH significantly at demonstrated by both prediction and fluorescence measurements (Figure B.2). While this study demonstrates a new technique for evaluating aqueous pH in pressurized CO₂ systems, additional work is required to determine the properties of the LysoSensor probe in a pressurized environment.

![Figure B.3](image-url) **Figure B.3** Aqueous pH measured by UV spectroscopy (bromophenyl blue) at 298 and 333 K. Results are compared to model predictions (Chapter 4) and previous experimental results (Toews et al. 1995).

**Measuring Aqueous pH by UV Spectroscopy**

Bromophenyl blue, a colorimetric pH sensitive probe molecule, has been used to measure aqueous pH in the presence of pressurized CO₂ as a function of temperature and pressure (Toews et al. 1995). Calibration was achieved in a high-pressure variable volume view cell at 298 and 333 K in colorless pH 2.8, 3.2, 3.6, 4.0, and 4.4 buffer solutions using 0.01 mM bromophenyl blue. Samples were prepared using deionized water at 0.01 mM bromophenyl blue. Scans were performed from λ = 300 to 900 nm, depicting two peaks centered on 439 and 593 nm, corresponding to the electronic transition of the acidic (clear) and basic (blue) forms, respectively. The ratio of
absorbance at 439 and 593 nm (439/593) is directly proportional to ratio of acidic to basic indicator concentrations. The ratio of acidic/basic bromophenyl blue concentrations depends upon [H+]'. Our results are very similar to model predictions at 298 and 333 K, which accurately predict aqueous CO2 concentration (Figure B.3).

References


Appendix C

Calculating the Solubility of Pressurized Solvents in Aqueous Media and Within Model Phospholipid Bilayers

Determining the concentration of pressurized gaseous solvents within phospholipid bilayer membranes is essential in estimating the degree of solvent biocompatibility (i.e. solvent toxicity). As discussed, and demonstrated, in the preceding sections (chapters 3 and 4), the accumulation of solutes within the bilayer produces a disordering effect wherein the membrane becomes fluidized, denoting a change in the configurational entropy of the acyl region in the bilayer. Toxicity arises due to membrane fluidization, which changes membrane permeability and the activity of membrane-bound enzymes that are essential for regulating the intracellular environment.

The accumulation of solutes (or solvents) within a phospholipid bilayer occurs in two steps: (i) dissolution of solvent within the aqueous phase and (ii) partitioning of solvent from the aqueous phase into the bilayer. Therefore, the solubility of solvent in the aqueous phase (water) and the membrane/water solvent partition coefficient ($P_{m/w}$) must be known in order to calculate the concentration of solvent within the bilayer. While the aqueous solubility of numerous compounds has been reported there is little information on bilayer solubility, which can be very difficult to measure experimentally due in part to the inability to directly sample the bilayer phase without disrupting it. Based on this difficulty, general solvent compatibility is frequently estimated from the 1-octanol/water partition coefficient ($log \text{ } P_{o/w}$). If $log \text{ } P_{o/w}$ is known, the concentration of solvent within the bilayer can be estimated from existing correlations that demonstrate a linear relationship between $log \text{ } P_{o/w}$ and $log \text{ } P_{m/w}$. Two such correlations were reported Sikkema et al. (Sikkema et al. 1994) (equation C.1) and Osborne et al. (Osborne et al. 1990) (equation C.2).

$$log \text{ } P_{m/w} = 0.97 \log \text{ } P_{o/w} - 0.64 \quad \text{(C.1)}$$
$$log \text{ } P_{m/w} = 0.98 \log \text{ } P_{o/w} - 0.73 \quad \text{(C.2)}$$
Calculating the Aqueous Solubility of Pressurized Solvents

The aqueous solubility of gaseous solvents at elevated pressures was determined by the Krichevsky-Kasarnovskiy (K-K) equation (Krichevsky and Kasarnovski 1935):

$$ f_i^w = H_{iw} \exp \left[ \frac{v_i^\infty (P - P_{s,w})}{RT} \right] $$

(C.3)

where $f_i^w$ is the fugacity of species $i$ in the water ($w$), $x_i^w$ is the mole fraction, $H_{iw}$ is the Henry’s constant, $v_i^\infty$ is the solute partial molar volume at infinite dilution, $T$ is the temperature, $P$ is the absolute pressure, and $P_{s,w}$ is the aqueous phase saturation pressure. In the K-K equation it is assumed that the activity coefficient of species $i$ in water is equal to 1. Henry’s constants for ethane and propane in water were obtained from literature (Dhima et al. 1999; Harvey 1996). The partial molar volumes of ethane and propane dissolved in water ($v_i^\infty$) were calculated according to Lyckman et al. (Lyckman et al. 1965):

$$ v_i^\infty \left( \frac{P_{c,i}}{RT_{c,i}} \right) = f \left( \frac{P_{c,i}T}{c_w T_{c,i}} \right) $$

(C.4)

where $P_{c,i}$ and $T_{c,i}$ are the critical pressure and temperature of species $i$, respectively, $c_w$ is the cohesive energy density of water, and $f$ is a universal solvent function (Lyckman et al. 1965).

$$ f = 2.4 + 0.09 \left( \frac{c_w T_{c,i}}{P_{c,i}T} \right) $$

(C.5)

In a two-phase system containing a single gaseous component ($i$) and a pure water phase, the fugacity can be estimated by $f_i^w = \phi_i P$ where $\phi_i$ is the pure component fugacity coefficient. This fugacity coefficient can be calculated using the Peng-Robinson equation of state (PR-EOS). Equation C.3 can be used to calculate $x_i^w$, the mole fraction concentration of species $i$ in the aqueous phase. Comparison between experimental literature values and calculated model values are provided for the binary systems CO$_2$/water (Figure C.1), ethane/water, and propane/water (Figure C.2).
Figure C.1 Aqueous CO$_2$ solubility as a function of temperature and pressure determined by the K-K equation and obtained in literature (King et al., 1992 (King et al. 1992) and Wiebe, 1942 (Wiebe 1941)).

Figure C.2 Aqueous ethane and propane solubility as a function of temperature and pressure determined by the K-K equation and obtained in literature (Anthony and McKetta, 1967 and Kobayashi and Katz, 1953).
Calculating $\log P_{o/w}$ for Pressurized Solvents

Modeling the phase behavior of systems containing aqueous solutions and near-critical fluids can be difficult when dealing with associating chemicals. The lack of a term to account for additional attractive (associating) force due to hydrogen bonding, as in the case with aqueous alcohols, renders simple equations of state unreliable. The group contribution association equation of state (GCA-EOS) has been developed to predict the phase equilibria of systems containing near- and supercritical components and aqueous oxychemicals (Gros et al. 1996). The GCA-EOS has proven to be a reliable when compared to existing experimentally observed phase equilibrium (Diaz et al. 2000; Horizoe et al. 1993).

The GCA-EOS is based on the concept of residual thermodynamic properties (Gros et al. 1996):

$$A^R = A^{hs} + A^{disp} + A^{assoc}$$  \hspace{1cm} (C.6)

where the residual Helmholtz energy ($A^R$) is related to the repulsive hard sphere energy ($A^{hs}$), the attractive dispersive mean field energy ($A^{disp}$), and the associating energy ($A^{assoc}$) that accounts for attractive specific interactions such hydrogen bonding.

The repulsive force ($A^{hs}$) is based on an expression given by Carnahan-Starling for hard sphere mixtures (Skjold-Jørgensen 1984):

$$\frac{A^{hs}}{RT} = 3 \left( \frac{\lambda_2}{\lambda_3} \right) \cdot (Y - 1) + \left[ (\lambda_2)^2 \cdot (\lambda_3)^2 \right] \cdot (-Y + Y^2 - \ln Y) + n \ln Y$$  \hspace{1cm} (C.7)

where,

$$Y = \left( 1 - \frac{\pi \lambda_3}{6V} \right)^{-1}$$  \hspace{1cm} (C.8)

$$\lambda_k = \sum_{j=1}^{NC} n_j \cdot (d_j)^k$$  \hspace{1cm} (C.9)

In these equations $T$ is the temperature, $n$ is the number moles, $NC$ is the number of components, and $V$ is the total system volume. $A^{hs}$ is dependent upon a single component property, the hard sphere diameter ($d$).

$$d = 1.06565 \cdot d_c \left[ 1 - 0.12 \cdot \exp \left( \frac{-0.667 \cdot T_c}{T} \right) \right]$$  \hspace{1cm} (C.10)
The hard sphere diameter is expressed as a function of the pure component diameter \((d_c)\) and the critical temperature \((T_c)\). The pure component diameter can be accurately determined from vapor pressure equilibrium data (Skjold-Jørgensen 1984).

The dispersive term \(A_{\text{disp}}\) is based on a group contribution version of a density dependent local composition expression (Skjold-Jørgensen 1984), similar to the NRTL model (Prausnitz et al. 1999).

\[
\frac{A_{\text{disp}}}{RT} = -\left(\frac{z}{2}\right) \cdot \sum_{j=1}^{NC} (n_j \cdot q_j) \cdot \sum_{j=1}^{NC} \theta_j \cdot \left(\frac{g_{ji} \cdot q}{RTV}\right) \cdot \left[\frac{\tau_{ji}}{\sum_{k=1}^{NC} \theta_k \cdot \tau_{kj}}\right]
\]  
(C.11)

where,

\[
q = \sum_{i=1}^{NC} n_i \cdot \sum_{j=1}^{NC} (v_j) \cdot q_j
\]  
(C.12)

\[
\tau_{ji} = \exp\left(\frac{\alpha_{ij} \Delta g_{ij} q}{RTV}\right)
\]  
(C.13)

In equation C.11 \(z\) is the number of nearest neighbors to any segment, \(q_i\) is the group surface parameter for group \(i\), \(\theta_j\) or \(k\) is the surface fraction of group \(j\) or \(k\), and \(V\) is the total volume. Expressing the total number or surface segments \((q;\) equation C.12), \(n\) is the number of moles of component \(i\), \(v_j\) is the molar volume of group \(j\), and \(q_j\) is the number of surface segments for group \(j\). In equation C.13 \(\alpha_{ij}\) is a non-randomness parameter and \(\Delta g_{ij}\) represents a change in pure group parameter with \(T\). The dispersive energy term may be approximated by the pure group parameters for like groups \((ii)\) and unlike groups \((ij)\) as follows:

\[
g_{ii} = g_{ii}^* \cdot \left[1 + g_{ii} \left(\frac{T}{T_i} - 1\right) + g_{ii}^{**} \ln\left(\frac{T}{T_i}\right)\right]
\]  
(C.14)

\[
g_{ij} = k_{ij} (g_{ii} g_{jj})^{1/2}
\]  
(C.15)

\[
g_{ij} = k_{ij} \left[1 + k_{ij} \ln\left(\frac{T}{T_i}\right)\right]
\]  
(C.16)

\[
T_{ij}^* = 0.5 \left(T_i^* + T_j^*\right)
\]  
(C.17)
In this set of interaction parameters the term $g$ refers to pure group energy parameters (attractive), $k$ refers to the binary interaction parameters, and $T$ and $T^*$ refer to the system and reference temperatures, respectively.

The association energy ($A^{\text{assoc}}$) is based on an expression used in the statistical associating fluid theory (SAFT) equation (Chapman et al. 1990). Site-site interactions define the basis for this expression for associating groups.

$$
\frac{A^{\text{assoc}}}{RT} = \sum_{i=1}^{NGA} n_i \left[ \sum_{k=1}^{M_i} \left( \ln \chi^{(k,i)} - \frac{\chi^{(k,i)}}{2} \right) + \frac{M_i}{2} \right] 
$$

The term $NGA$ refers to the total number of associating groups, $n_i$ is the number of moles of associating group $i$, $M_i$ is the number of association sites assigned to group $i$, and $\chi^{(k,i)}$ represents the mole fraction of group $i$ bonded at site $k$. The number of moles of the associating group may be determined by:

$$
n_i = \sum_{m=1}^{NC} \gamma_{\text{assoc}}^{(i,m)} \cdot n_m
$$

where $NC$ is the number of components, $(\gamma_{\text{assoc}}^{(i,m)})$ is the number of associating groups $i$ in molecule $m$, and $n_m$ is the number of moles of molecule $m$. The mole fraction of group $i$ bonded at site $k$ is expressed as:

$$
\chi^{(k,i)} = \left[ 1 + \sum_{j=1}^{NGA} \sum_{l=1}^{M_j} \rho_j \chi^{(k,i)} \Delta^{(k,i,l,j)} \right]^{-1}
$$

where the molar density of group $j$ is simply $\rho_j = n_j/V$. The association strength between site $k$ of group $i$ and side $l$ of group $j$ is expressed as:

$$
\Delta^{(k,i,l,j)} = \kappa^{(k,i,l,j)} \left[ \exp \left( \frac{e^{(k,i,l,j)}}{kT} \right) - 1 \right]
$$

where $e$ is the association energy, $\kappa$ is the association volume, and $k$ is the Stephan-Boltzman constant. The group interaction parameters, association energies, and association volumes has been previously determined and are available in the literature (Diaz et al. 2000; Gros et al. 1996).

The solute equilibrium distribution coefficient for solute $i$ ($K_i$) can be determined by relating $A^K$ to the solute fugacity coefficients ($\phi$). The distribution coefficient is expressed as:

$$
K_i = \left( \frac{\phi_A}{\phi_B} \right)
$$
\[ K_i = \left( \frac{y_i}{x_i} \right) = \left( \frac{\phi_i^L}{\phi_i^V} \right) \]  \hspace{1cm} (C.22)

where \( y_i \) is the mole fraction of component \( i \) in the solvent (1-octanol) phase, \( x_i \) is the mole fraction of component \( i \) in the aqueous phase (water), and \( \phi_i \) represent the fugacity coefficient of component \( i \) in the liquid (L) and vapor (V) phase. The fugacity coefficient of each component can be calculated by taking the partial derivative of \( A^R \) with respect to the number of moles. The compressibility factor can also be also be determined by taking the partial derivative of \( A^R \) with respect to volume, \( V \).

Equation C.1 or C.2 can be used to calculate the solubility of pressurized fluids in the phospholipid bilayer based on the pressurized fluid 1-octanol/water partition coefficient (GCA-EOS) and the aqueous solubility (K-K). Results presented herein are based on equation C.1:

\[ P_{o/w} = K_i = \left( \frac{y_i}{x_i} \right) = \left( \frac{x_i^o}{x_i^w} \right) \]  \hspace{1cm} (C.23)

\[ \log P_{m/w} = 0.97 \log P_{o/w} - 0.64 \]  \hspace{1cm} (C.1)

\[ P_{m/w} = \left( \frac{x_i^m}{x_i^w} \right) \]  \hspace{1cm} (C.24)

References


Appendix D

List of Symbols

- **C**: concentration (specified units)
- **$c_w$**: cohesive energy density of water (J/m$^3$)
- **[$\text{CO}_2$]$_{\text{aq}}$**: aqueous carbon dioxide concentration (mM)
- **$D$**: dilution rate (flowrate/reactor volume; h$^{-1}$)
- **$DCW$**: dry cell weight (g)
- **$D_{AB}$**: diffusivity of component A through fluid B (cm$^2$/s)
- **$E/A$**: ethanol to acetate ratio (mM ethanol /mM acetate)
- **$f$**: universal solvent function
- **$f_i^w$**: the fugacity of species $i$ in water (MPa)
- **$F$**: media flowrate (mL/h)
- **GCA-EOS**: group contribution association equation of state
- **[$\text{H}_2$]$_{\text{aq}}$**: aqueous hydrogen concentration (mM)
- **$H_{iw}$**: Henry’s constant for species $i$ in water
- **$I$**: fluorescence intensity (absorbance units)
- **$k_d$**: cell death rate (h$^{-1}$)
- **$K_{a1}$**: first acid dissociation constant
- **$K_S$**: saturation constant (mM)
- **$\log P_{o/w}$**: octanol/water partition coefficient (M or mole fraction basis)
- **$\log P_{m/w}$**: membrane/water partition coefficient (M or mole fraction basis)
- **$m_S$**: maintenance requirement based on cellobiose consumption (g cellobiose consumed/g DCW/h)
- **$m_{ATP}$**: maintenance requirement based on ATP formation ($m_{ATP}$, mmoles ATP formed/g DCW/h)
- **$OD_{600}$**: optical density at 600 nm (absorbance)
- **$p\text{CO}_2$**: carbon dioxide partial pressure (MPa)
- **$p\text{H}_2$**: hydrogen partial pressure (MPa)
$P$ pressure (MPa) or product concentration (mM)
$P_c$ critical pressure (MPa)
$P_{sw}$ aqueous phase saturation pressure (MPa)
$q_p$ rate of product formation (mM product/g DCW/h)
$\langle r \rangle$ fluorescence anisotropy
$\langle r \rangle_{norm}$ normalized fluorescence anisotropy ($\langle r \rangle_{norm} = \langle r \rangle_{hydrostatic pressure}$)
$S$ substrate concentration (cellobiose; mM)
SCF supercritical fluid
$t$ time (h)
$T$ temperature (K)
$T_c$ critical temperature (K)
$T_m$ phospholipid acyl-chain melting temperature (K)
$v_i^{\infty}$ partial molar volume of species $i$ at infinite dilution (L/mol)
$V_c$ critical volume (L/mol)
$V_R$ reactor volume (mL)
$V_{R,a}$ aqueous volume within reactor (mL)
$Y_{P/S}$ product yield ($Y_{P/S} = \Delta P/\Delta S$, mM/mM)
$Y_{X/ATP}^M$ maximum theoretical growth yield based on ATP formation ($Y_{X/ATP} = \Delta X/\Delta ATP$, g DCW/mole ATP formed)
$Y_{X/S}^M$ maximum theoretical growth yield based on cellobiose consumption ($Y_{X/S} = \Delta X/\Delta S$; g DCW/g cellobiose consumed)
$X$ cell concentration (mM)
$x_i^w$ mole fraction of species $i$ in water

**Greek Symbols**

$\lambda_{em}$ fluorescence emission wavelength (nm)
$\lambda_{ex}$ fluorescence excitation wavelength (nm)
$\mu$ specific growth rate (h$^{-1}$)
$\mu_{max}$ maximum specific growth rate (h$^{-1}$)
$\nu$ kinematic viscosity (cm$^2$/s)
$\rho$ density (g/ml)
$\phi_i$  

pure component fugacity coefficient

*Abbreviations for Chemical Species*

- acetyl CoA: acetyl coenzyme A
- ATP: adenosine triphosphate
- $C_n$: alkane chain length ($n =$ number of carbon molecules)
- CO$_2$: carbon dioxide
- DPH: 1,6-diphenyl-1,3,5-hexatriene
- DPPC: dipalmitoylphosphatidylcholine
- FD: ferrioxiden
- H$_2$: hydrogen
- H$_2$CO$_3$: carbonic acid
- HCO$_3^-$: bicarbonate
- N$_2$: nitrogen
- Na$_2$CO$_3$: sodium carbonate
- NAD: nicotinamide adenine dinucleotide
References


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EDUCATION

M.S. Chemical Engineering, University of Kentucky (July, 2001)
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RESEARCH EXPERIENCE

• **M.S. Thesis:** “Application of Compressed Solvents to Polymeric Fractionation and Membrane-based Extraction,” University of Kentucky, 1999–2001. Advisor: Dr. Barbara Knutson
• **Undergraduate Research:** Patented lanthanum oxide ion exchange process for removing heavy metals from aqueous mine drainage, University of Nevada, Reno, 1996-1997. Advisor: Dr. Manoranjan Misra
• **Research Mentoring**
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• Laboratory instructor for high school Engineering Summer Program, University of Kentucky (Summer 2003)
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• Teaching assistant for CME 450: Process Control, University of Kentucky (Spring 2001)
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• Volunteer presenter on “What is Chemical Engineering?” for local elementary schools, University of Nevada, Reno (Spring 1996/Fall 1997)

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8/98 – 4/99 Chemical Engineer, Geobiotics, Inc., Lakewood, CO
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AWARDS AND ACHIEVEMENTS

• Graduate Research Assistantship, University of Kentucky (Fall 1999 – current)
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• Team leader for senior design project at the Waste Education and Research Consortium international design competition (1997)
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PROFESSIONAL AND ACADEMIC AFFILIATIONS

• Associate member, American Institute of Chemical Engineers (AIChE)
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OTHER


PUBLICATIONS

REFEREED JOURNALS

Bothun, G.D, Knutson, B.L., Strobel, H.J., Nokes, S.E. ‘Molecular and phase toxicity of compressed and supercritical fluids in biphasic continuous cultures of Clostridium thermocellum,’ submitted, Biotechnology & Bioengineering (April 2004).


**CONFERENCE PROCEEDINGS**


Bothun, G.D., Knutson, B.L. ‘Gas Antisolvent Fractionation of Poly (lactic acid) using Compressed CO$_2$,’ *AIChE annual meeting*, Los Angeles, CA (November 2000).

**INVITED PRESENTATIONS**

Bothun, G.D., Knutson, B.L. ‘Pressurized solvents in whole-cell bioprocessing: Metabolic and structural perturbations,’ Seminar, Chemical and Materials Engineering, University of Kentucky, Lexington, KY (March 2004).

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Bothun, G.D., Knutson, B.L. ‘Gas Antisolvent Fractionation of Poly (lactic acid) using Compressed CO₂,’ AIChE annual meeting, Los Angeles, CA (November 2000).
Bothun, G.D., Knutson, B.L. ‘Gas Antisolvent Fractionation of Poly (lactic acid) using Compressed CO₂,’ *Chemical Engineering Graduate Student Symposium*, Morgentown, WV (September 2000).