UTILIZING A LIPID BILAYER-BASED SOLVENT TO ENHANCE BUTANOL PRODUCTION FROM A CONTINUOUS FERMENTATION OF CLOSTRIDIA PASTEURIANUM

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UTILIZING A LIPID BILAYER-BASED SOLVENT TO ENHANCE
BUTANOL PRODUCTION FROM A CONTINUOUS FERMENTATION
OF CLOSTRIDIA PASTEURIANUM

BY

JOHN NUNES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN
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CHEMICAL ENGINEERING

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OF

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2018
Abstract

This work highlights the capabilities of a lipid bilayer-based solvent for butanol extraction. Previous work in our group has shown that lipid bilayers have a high affinity for butanol extraction. Here, we show higher butanol partition coefficients than previously seen in other solvents. Our partition coefficients were found using a quantitative NMR method to allow for in situ measurements to be taken which resulted in higher partition coefficients than previously found by HPLC methods. Two lipids were used for these experiments in order to examine the effect that lipid bilayer phase has on the butanol partition coefficient and we see that a mixed phase bilayer (DPPC/DOPC) resulted in the highest butanol partition coefficient. Additionally, butanol’s effect on bilayer size was examined by dynamic light scattering. DPPC vesicles showed the largest change in size when butanol was added due to the smaller spaces in between lipid head groups in a DPPC bilayer.

Working in collaboration with Dr. Carmen Scholz’s group at the University of Alabama Huntsville, a continuous fermentation was developed that used glycerol as a feedstock for Clostridium pasteurianum. We also utilized our lipid bilayer solvent in an extractive fermentation of these continuous cultures. Our results showed an increase in butanol production and yield at lower dilution rates, which can also be attributed to the extraction of butanol from the system.
Acknowledgments

First, I’d like to thank my parents for their support and love. I’d like to thank Dr. Bothun for his help and guidance throughout this process and also for giving me the great opportunity to work with him. I’d like to thank my family and friends for being so supportive of me with a special thank you to my lab mates at URI who have helped me and provided constructive feedback countless times. I’d like to thank the URI Chemical Engineering department for welcoming me and their assistance. Finally, I’d like to thank Becky for always being there when I needed her and pushing me to succeed.
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Chapter 1

INTRODUCTION

Growing energy demands for the world will require innovative options for energy production. Alternative fuel can be an important and viable option to expand on current energy choices (Dresselhaus and Thomas 2001). Ethanol is a common fuel additive used today in fuel to enhance the fuel's properties. However, when present in large amounts, ethanol can be corrosive to car engines. Due to the corrosive nature of ethanol, car engines would need to undergo modifications to combat this corrosion (Surisetty et al. 2011). In contrast, butanol offers fuel properties superior to those of ethanol and thus has the potential to be a more innovative fuel source (Nanda et al. 2017; Lee et al. 2008; Dürre 2007; MacLean and Lave 2003). The fuel properties of butanol are so similar to gasoline that butanol is considered a “drop-in” biofuel meaning it is capable of being used in car engines with limited or no modifications (Dürre 2007). In addition to its use as a biofuel, butanol has a number of other uses in the chemical industry such as in the production of paints, lacquers, and resins (Harvey and Meylemans 2011; Nanda et al. 2017). Batch fermentation utilizing the Clostridia strain has also shown promise when it comes to butanol production (Zheng et al. 2009; Jones and Woods 1986; Tomas et al. 2003; Lee et al. 2008).
Butanol production via fermentation does come with certain limitations, namely, that butanol is toxic to cells (Sardessai and Bhosle 2002). This toxicity can severely impact the production of butanol and life of the cells (Venkataramanan et al. 2014). In an effort to reduce the impact butanol can have on a fermentation, extractive fermentation has been utilized to remove butanol as it is formed in a fermentation setting. By removing the butanol, not only does this limit the toxicity that butanol exhibits which increases cell growth but it also drives the reaction to produce more butanol due to Le Chatelier’s principle. The effectiveness of an extractive fermentation is based on the solvent choice. While many solvents have been used to extract butanol, this work focuses on vesicles as the solvent of choice. Vesicles show excellent potential as a solvent for butanol extraction due to their biocompatibility and high preliminary partition coefficient results for butanol into vesicles (Kurniawan et al. 2012).

The overall goal of this work was to design and operate a continuous fermentation process coupled with extractive fermentation utilizing a vesicle solvent. In order to establish an extractive fermentation, details about the vesicle solvent needed to be found. Partition coefficients were found using a quantitative nuclear magnetic resonance (qNMR) approach. When butanol partitions into a bilayer, the bilayer expands to include the butanol (Löbbecke and Cevc 1995). This phenomenon was observed using dynamic light scattering during this work. A continuous fermentation of *Clostridia pasteurianum* was then established in collaboration with the University
of Alabama chemistry department. Following this, an extraction system was set up using vesicles as the solvent and a hollow fiber membrane to bring the solvent into contact with the fermentation broth.

Chapter 2 will provide a detailed background on relevant information in regards to this research. Chapter 3 contains a manuscript that is in preparation to be published which covers butanol partition coefficient results as well as butanol’s effect on vesicle size. Chapter 4 contains another manuscript format that is in its preliminary stages of editing. This chapter contains information regarding continuous fermentation and extractive fermentation along with our current results. Chapter 5 contains conclusions and potential ideas for future work on this project. The appendix will show NMR background and procedures used.

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Zheng, Y.; Li, L.; Xian, M.; Ma, Y.; Yang, J.; Xu, X.; He, D. (2009): Problems with the microbial production of butanol. In Journal of Industrial Microbiology & Biotechnology 36 (9), pp. 1127–1138. DOI: 10.1007/s10295-009-0609-9.
This chapter covers a variety of topics and provides an up-to-date review of each of them. First, butanol’s inhibitory effect on cell growth is discussed. Then, information about butanol’s effect on vesicle phase is presented. Finally, extractive fermentation of butanol is shown with a variety of solvents shown.

2.1 Butanol Toxicity and Cell Inhibition

The inherent toxicity of butanol becomes an issue in a fermentation setting as butanol will kill cells (Sardessai and Bhosle 2002). Seen in Figure 2.1 below where butanol is added after two days and a decreased optical density is measured, this implies that the cell growth was stunted after the addition of butanol (Venkataramanan et al. 2014). Butanol is known to partition between the headgroups of a lipid bilayer causing a cell membrane to fluidize, killing the cell (Bowles and Ellefson 1985; Vollherbst-Schneck et al., 1984). The toxicity of a solvent is related to the solvent’s log P. The log P is a value representing how well a solvent will partition in an equimolar octanol/water mixture (Sardessai and Bhosle 2002). A
log P lower than 4 means that the solvent will be highly toxic to cells; butanol shows a log P around 0.8 (Sardessai and Bhosle 2002).

The *Clostridia* species of bacteria has shown an increased ability to produce butanol. *Clostridium acetobutylicum* has been shown to produce butanol around 10 g/L (Monot et al., 1982). Additionally, *Clostridium pasteurianum* can produce butanol well and also shows superior resistance to butanol’s toxicity. *Clostridium pasteurianum* has been shown to grow even when using crude glycerol, which is a byproduct of conventional biodiesel production (Venkataramanan et al. 2012). The glycerol pathway for butanol production is seen in Figure 2.2.
2.2 Vesicle Characterization

Phospholipids were used in this study because they are normally present in a cell membrane. This is important because butanol has shown that in the presence of cell membranes, it gathers in the space between the lipid head groups along the lipid/water interface (Bowles and Ellefson 1985; Vollherbst-Schneck et al., 1984). In this study the phospholipids used were zwitterionic with varying degrees of unsaturation and can be seen in Table 2.1 below.
Table 2.1: Lipids used in this study with molecular weight and structure. Soy lecithin is a mixture; the structure shown is representative and the molecular weight is an average (Avanti Polar Lipids, Inc, Alabaster, AL).

| Lipid Name                                | Structure | Molecular Weight [g/mol] |
|-------------------------------------------|-----------|--------------------------|
| Dipalmitoylphosphatidylcholine (DPPC)     | ![DPPC Structure](image1) | 734.05                   |
| Dioleoylphosphatidylcholine (DOPC)        | ![DOPC Structure](image2) | 786.13                   |
| L-a-phosphatidylcholine (Soy Lecithin)    | ![Soy Lecithin Structure](image3) | 775.04                   |

Once amphiphilic, both hydrophobic and hydrophilic, lipids enter a water environment the hydrophilic head groups point towards the aqueous environment while the hydrophobic tails of the lipids interact with each other. This forms a lipid bilayer with an aqueous core. The lipid bilayer is normally represented by two phases and an intermediate phase. When below the melting temperature of the bilayer the phase is a gel like system. This changes to a fluid phase when above the melting temperature. The intermediate phase occurs when the bilayer approaches its melting temperature. When butanol is in the presence of a lipid bilayer a fourth phase has been shown to form known as the interdigitated phase which causes the lipid head
groups to space out and the tails to lie side by side instead of being end-to-end. Each of these phases can be viewed in Figure 2.3.

![Diagram of vesicle phases](image)

*Figure 2.3: The four phases that can be present in a vesicle. The interdigitated gel occurs once butanol is added to the system and returns to a fluid phase after the melting temperature ($T_m$) is reached (Kurniawan et al. 2012).*

### 2.3 Extractive Fermentation

Extractive fermentation is the process of removing an end product from a fermentation using a solvent to extract it. Removing a product then lowers the end products potential inhibition on the bacteria’s growth (Dhamole et al. 2012). When a product is removed, the fermentation should be pushed to produce more of the missing product, in this case butanol. Extractive fermentation can take two different forms: direct addition of a solvent to the fermentation (Dhamole et al. 2012) or passing the fermentation broth through an extraction unit to bring the media in contact with a solvent (Zhang et al. 2017; Roffler et al. 1988). Extractive fermentation’s effectiveness at increasing butanol yields has been demonstrated a
number of times. A liquid-liquid extraction using oleyl alcohol running counter currently across a plate column showed a 70% increase over a batch fermentation (Roffler et al. 1988). Long chain alcohols also provided high butanol partitioning with 2-Ethyl-1,3-hexanediol reported as having a mass partition coefficient of 8.1 (Barton and Daugulis 1992). While organic solvents have been shown to increase butanol production, these solvents tend to also be toxic to the bacteria. Thus, extractants with a biocompatibility towards butanol are becoming more relevant.

Zhang et al. uses mixtures of aliphatic fatty acids and oleyl alcohol to extract butanol from a fermentation and shows an improvement of 11% over using oleyl alcohol alone (Zhang et al. 2017; Dhamole et al. 2012). Surfactant micelles have been shown to increase butanol productivity by over 200% with a mass partition coefficient of 3.5. Ionic liquid solvents have also shown to be effective solvents for butanol (Cascon et al. 2011; Davis and Morton 2008). Tetrahexyammonium dihexylsulfosuccinate ([THA][DHSS]) showed a mass partition coefficient with a value of 7.99 presented (Cascon et al. 2011). 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([bmim][Tf₂N]) was shown to have a distribution coefficient of 14 (Davis and Morton 2008). Vesicles provide a highly biocompatible solvent with a high affinity towards butanol. In addition to the toxicity inferred from the low log P of butanol, the log P also highlights the ability for butanol to partition. It has already been stated that butanol has a fluidizing effect on a cell membrane due to its partitioning between the headgroups of the bilayer. Combining both of these concepts leads to the idea that
cell membranes could be used as a solvent to extract butanol. Some preliminary studies that use model cell membranes to extract butanol have shown high partition coefficients (Kurniawan et al. 2013; Kurniawan et al. 2012). The partition coefficient is the ratio of the concentration of a solute between two solvents. As of this writing vesicles have not been used to extract butanol from a fermentation.

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CHAPTER 3

BUTANOL PARTITIONING INTO A LIPID BILAYER AS EXAMINED BY NMR

This chapter has been prepared in manuscript format with the intent to publish in the area of colloidal science or bioenergy. The work done here represents a collaborative effort with Dr. Scholz’s group at the University of Alabama Huntsville.

3.1 Introduction

Growing energy demands world-wide require innovative options for sustainable energy production. Alternative energies that are naturally derived are an important and viable option to expand on current energy choices beyond fossil fuels (Dresselhaus and Thomas 2001). Biofuels such as ethanol are commonly used as a fuel additive (typically 10 to 15% by volume) to lower greenhouse gas emissions, however, ethanol is corrosive to car engines and has a lower energy density than gasoline (MacLean and Lave 2003; Surisetty et al. 2011). Due to the corrosive nature of ethanol, car engines require modification in order to use gasoline with higher ethanol content such as E85 or pure ethanol (Surisetty et al. 2011). Therefore, alternatives to ethanol are sought that are compatible with conventional engines and have higher energy densities similar to gasoline (Table 3.1).

Butanol can be produced by fermentation as a biofuel and the properties of butanol are similar to those of gasoline, allowing it to be used as a fuel without any further modifications to most engines (Surisetty et al. 2011; Dürre 2007; Harvey and
Meylemans 2011). Butanol can also be produced from glycerol, which is the primary by-product of biodiesel production – 10 kg of glycerol are produced for every 100 kg of biodiesel (Yazdani and Gonzalez 2007). Unless further refined, this crude glycerol by-product has limited uses and is often discarded as a waste product. Hence, producing butanol from glycerol achieves two goals of biorefining; creating a value-added alternative fuel from a bio-derived feedstock that is a by-product of biodiesel production.

One promising method to produce butanol is by fermentation using the *Clostridia* strain of bacteria (Nanda et al. 2017; Jones and Woods 1986; Monot et al., 1982). Fermentation by *Clostridia* species produces butanol, acetic acid, butyric acid, acetone and ethanol (Nanda et al. 2017). Various *Clostridia* strains have shown promising results for butanol production with yields of 10 g/L (*C. beijerinckii*) (Zhang

![](image)

### Table 3.1: Table of NHOC, AKI fuel properties of ethanol, butanol, gasoline. NHOC – Net heat of combustion, AKI – Anti-knock index (Harvey and Meylemans 2011).

| Fuel       | NHOC | Density (g/mL) | AKI |
|------------|------|----------------|-----|
| Gasoline   | 32.3 | 0.74           | 87  |
| Ethanol    | 21.1 | 0.79           | 113 |
| n-Butanol  | 26.8 | 0.81           | 87  |
et al. 2017), 9 g/L (C. pasteurianum) (Jensen et al. 2012) and 17 g/L after being metabolically engineered (C. acetobutylicum) (Lee et al. 2008). Furthermore, crude glycerol, despite containing impurities such as salts, methanol, and free fatty acids from biodiesel production, has been shown to be a capable feedstock for the fermentation of C. pasteurianum to produce butanol (Venkataramanan et al. 2012).

Efforts are currently underway to increase butanol production by Clostridia via metabolic engineering, fermentation optimization, and/or extractive fermentation.

A limitation to any fermentation process is product inhibition of cellular activity as high product concentrations are toxic to bacteria. Butanol toxicity is responsible for the low butanol concentrations achieved by fermentation. Butanol is a small amphiphilic molecule that partitions into the lipid bilayer of bacterial membrane leading to cell growth inhibition due to membrane fluidization (Sardessai and Bhosle 2002; Bowles and Ellefson 1985; Kurniawan et al. 2012; Vollherbst-Schneck et al., 1984). Membrane fluidization reduces lipid ordering within the membranes, which in turn can make the membrane more permeable and reduce the function of membrane-bound proteins.

In order to minimize butanol toxicity during fermentation, butanol can be removed as it is being produced via extractive fermentation. In addition to reducing membrane fluidization, removing butanol from the fermentation broth drives the metabolic reaction to produce more butanol. Several solvents have been shown to be effective
at removing butanol from cell cultures by extractive fermentation. For example, oleyl alcohol is a commonly used solvent that has been shown to increase butanol productivity by 20% with *C. beijerinckii* (Zhang et al. 2017) and by 24% with *C. acetobutylicum* (Roffler et al. 1988). However, organic solvents themselves can be toxic to cells and limit butanol production (Lemos et al. 2017). A variety of non-organic solvents have been studied with the intent of finding a solvent capable of extracting butanol without inhibiting cellular activity.

One novel approach to extractive fermentation is to use molecular self-assemblies in aqueous phases, such as surfactant micelles, which exhibit a high affinity for butanol partitioning. For example, a 225% increase in butanol and acetone production was observed when non-ionic Pluronic surfactant L62 was added as micelles directly to a fermentation (Dhamole et al. 2012). Dhamole et al. investigated a wide range of surfactants in addition to L62 and found that the micelles assisted in the ‘capture’ of butanol (Dhamole et al. 2015), presumably into the amphiphilic and/or hydrophobic regions of the self-assembly. This capability has also been reported for lipid bilayer vesicles, which would provide a sustainable alternative to synthetic surfactants given that lipids can be isolated from natural or biological resources. Though scarcely investigated, lipid bilayer-based solvents show promise as they provide high butanol partition coefficients (K<sub>P</sub>) and are biocompatible (Kurniawan et al. 2012; Kurniawan et al. 2013).
This study examines the solvent characteristics of lipid bilayer vesicles; specifically, vesicle structure (swelling) and partitioning as a function of butanol concentration and lipid composition (ratio of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)). DPPC and DOPC were chosen because they have been previously investigated by our group and they offer different chemical structures, which have been shown to influence butanol partitioning (Kurniawan et al. 2012). DPPC and DOPC are similar molecules with a phosphocholine headgroup, but DPPC has saturated C_{16} tails and DOPC contains a double bond on each of its C_{18} tails (Table 3.2).
Table 3.2: DPPC and DOPC were the lipids used in this study with molecular weight and structure shown.

| Lipid Name                                      | Structure | M.W. (g/Mol) |
|------------------------------------------------|-----------|--------------|
| 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) | ![Structure Image](image1) | 734.05       |
| 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)  | ![Structure Image](image2) | 786.13       |

When DOPC forms a vesicle, the presence of the double bond creates more space between the lipid head groups than DPPC, which has been shown to increase butanol partitioning (Kurniawan et al. 2012; Kurniawan et al. 2013). Mixtures of DOPC and DPPC have been shown to have high butanol partition coefficients due to the inclusion of DOPC, which forms fluid phases in the bilayer along with the gel-like DPPC phases (Kurniawan et al. 2012). As butanol is extracted by a vesicle, the bilayers expand and the vesicle size becomes larger (Tierney et al. 2005; Löhbecke and Cevc 1995). The degree of expansion and its relation to butanol partitioning, which are critical to evaluating the solvent capacity of lipid vesicles, have not been examined. In
this work, vesicle size was examined by dynamic light scattering (DLS) and an in situ quantitative nuclear magnetic resonance (qNMR) approach was developed to determine the amount of butanol in the lipid phase. By using this approach, the butanol partition coefficients could be found without requiring the separation of the lipids as high performance liquid chromatography (HPLC) would require (Zhang et al. 2004; Kitamura 1999).

3.2 Methods/Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, >99% purity) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, >99% purity) were purchased from Avanti Polar Lipids (Alabaster, AL) dissolved in chloroform and were used without further purification. Deuterium oxide (99.9 atom % D) was purchased from Sigma-Aldrich. Deionized (DI) ultrafiltered water was obtained from a Millipore Direct Q-3 purifier. Butanol, ethanol, 1,3 propanediol, butyric acid and acetic acid were purchased from Sigma Aldrich.

3.2.1 Vesicle Preparation

Vesicles were prepared using the Bangham method (Bangham et al. 1965). In short, lipids previously dissolved in chloroform were placed under a flow of nitrogen gas until the chloroform evaporated and a thin-film of lipids remained. The lipids were dried further by placing them in a vacuum chamber for 30 min. DI water or deuterium oxide was added before the lipid/water solution was placed in a heat bath
to form vesicles. This allowed for nuclear magnetic resonance experiments. Following this, they were sonicated for 10 min at 42 °C.

3.2.2 Nuclear Magnetic Resonance (NMR)

NMR was used to determine the lipid/water butanol partitioning coefficient. During all quantitative NMR (qNMR) experiments the protocol described in Pauli et al. 2007 was followed using a 400 MHz NMR (Bruker Biospin AG, Magnet System 400'54 Ascend). NMR experiments were carried out with a non-spinning sample and $^{13}\text{C}$ decoupling to prevent side satellites from forming in the spectra, which can interfere with primary peaks when analyzing spectra. Sixteen scans and two mock scans were used to capture the NMR spectra. The MestReNova software was used to analyze data collected via NMR. After ensuring the spectrum baseline was properly phased, the integral of each peak associated with an analyte of interest was taken. To ensure consistency when measuring, the integral lengths and starting points were recorded and duplicated for each sample.

A calibration curve was constructed relating the concentration of butanol to the area under the peaks of butanol. A concentration range of 0.9 to 15 g/L of butanol was prepared for NMR analysis. Each sample prepared was 500 µL in volume. A spectrum was obtained for each sample and analysis was done by integrating each butanol signal with reference to the water peak. The area under the peaks were recorded for each of the concentrations via integration in the MestReNova program. A plot of
concentration versus area under the peak allowed for a relationship to be found between the NMR data and quantitative results. A quadratic polynomial fit was used to match this data and fit through concentration = 0 with an $R^2$ of 0.996.

Partition coefficients were found using an in-situ method where butanol and vesicles were present during testing. When butanol molecules are captured by a vesicle, the NMR signal associated with the captured butanol is hidden. On this basis, the number of moles of butanol in the water phase ($n_{b,w}$) were measured. The partition coefficient of butanol in lipid was measured for each lipid composition and calculated using equation (1):

$$K_P = \frac{n_{b,L}}{n_{b,L} + n_L} \frac{n_{b,w}}{n_{b,w} + n_w} \tag{1}$$

Where $K_P$ is the mole fraction lipid-water partition coefficient of butanol in lipid, $n_{b,L}$ is the moles of butanol in the lipid phase, $n_L$ is the total moles of lipid, $n_{b,w}$ is the moles of butanol in the water phase, and $n_w$ is the total moles of water (Kurniawan et al. 2012). The number of moles of butanol in the lipid phase ($n_{b,L}$) was calculated as $n_{b,L} = n_b - n_{b,w}$. Partition coefficients were measured at lipid concentrations ranging from 0.4 g/L to 4 g/L (2.5 – 5 mM) and a butanol concentration of 5 g/L was used following preliminary fermentation results in our group. It should be noted that 5 g/L butanol is well below the water solubility limit (73 g/L at 25 °C), therefore, the experiment results were not influenced by butanol phase separation. Each NMR sample was 500 µL in volume and control experiments were conducted with only
butanol and D$_2$O present, and then additional samples that contained a mixture of butanol, lipid and D$_2$O. One butanol solution was prepared and used for the control and lipid samples. The original butanol concentration was measured in the butanol/D$_2$O sample and then used to find the moles of butanol in the lipid phase ($n_{b,L}$).

3.2.3 Dynamic Light Scattering

As butanol molecules partition into a lipid bilayer the space between the head groups expands to accommodate the butanol (Tierney et al. 2005; Löbbecke and Cevc 1995). Bilayer expansion can be examined by measuring the hydrodynamic diameter of the vesicles via light scattering while adding butanol. A Malvern Zetasizer Nano ZA was used for DLS experiments.

First, the swelling effect of butanol alone was studied. Different liposome solutions (10mM DPPC, 10mM DOPC, 10mM 50/50 DOPC/DPPC) were prepared and modified by vesicle extrusion using a polycarbonate track-etched 100 nm membrane (Whatman). An initial DLS test was completed to identify the size of the liposomes with no butanol present. Butanol was then added in small amounts to the vesicles and the hydrodynamic diameter of the liposomes was measured after each butanol addition until a butanol concentration of 20 g/L was reached. This was chosen to match and exceed the higher butanol yields that have been reported (Roffler et al. 1988). Ethanol, 1,3 propanediol and acetic acid were also studied following the same
procedure. In these studies, only DPPC was examined because DPPC showed the largest change in size during previous experiments.

3.3 Results and Discussion

3.3.1 Partition coefficient with increasing lipid concentrations

Figure 3.1 shows two NMR spectra of butanol overlaid on each other; a spectrum of butanol without vesicles (red) and a spectrum of butanol in the presence of vesicles (green). When vesicles are present, the NMR shows butanol peaks at lower intensity compared to when vesicles are not present. Comparing the spectra provides direct evidence that the butanol extracted into the vesicles is shielded from NMR and that in situ measurements can be used to determine partitioning. This was further validated by centrifuging a butanol+vesicle sample, removing the vesicles with captured butanol, and analyzing the residual butanol in the supernatant by qNMR. These results were comparable to direct calculations in situ (results not shown).
Figure 3.1: NMR spectra of butanol without vesicles present (red) and in the presence of vesicles (green). The y-axis shows a relative intensity and the x-axis shows chemical peak shift. This difference is measurable and can then be used to find butanol/lipid partition coefficients.

Butanol partition coefficients were determined using qNMR at a butanol concentration of 5 g/L and three different lipid compositions: DPPC, DOPC and an equimolar mixture. For all lipid compositions $K_P$ increased with lipid concentration. The equimolar mixture showed the highest butanol partition coefficient, consistent with previous work using Langmuir monolayers (Kurniawan et al. 2013), while DOPC had the second highest and DPPC showed the lowest (Figure 3.2 A-C). The number of butanol molecules per each lipid composition was calculated based on the $K_P$ results.
The results showed that the equimolar composition provided the highest butanol to lipid ratio with a value of 7.1 moles butanol per mole of lipid while DPPC had the lowest with 4.5 moles of butanol per mole of lipid.

Figure 3.2: Partition coefficients of butanol in DPPC (A), DOPC (B) and 50:50 DPPC:DOPC (C). All partition coefficient experiments were conducted at 25 °C and at a butanol concentration of 5 g/L. A “*” represents p < 0.05, while “n.s.” represents p > 0.05. This basis is used throughout the results presented. (D) The number of butanol molecules per lipid molecule based on Kp results at a butanol concentration of 5 g/L and lipid concentrations of 4 g/L.

The partition coefficients measured here follow a similar trend to those found previously, where DPPC showed the lowest partition coefficient and a mixture of
DPPC:DOPC showed a higher $K_v$ value (Kurniawan et al. 2012). To our knowledge, butanol partition coefficients for DOPC alone have not yet been shown but the trend presented in Kurniawan et al. 2012 showed that with increasing DOPC present, the partition coefficient rose. We found while that DOPC alone provided an increased $K_v$ compared to DPPC, the equimolar mixture still resulted in the highest $K_v$. This suggests that the multiphase bilayer (gel and fluid) would be a more suitable solvent for butanol extraction. Butanol partition coefficients have been reported for other solvents. In most instances the partition coefficient reported is a mass-based partition coefficient and in order to effectively compare these to our own partition coefficients we needed to convert our mole fraction partition coefficients (Table 3.3). These converted values can be seen in the table below.

*Table 3.3: Butanol/Lipid mass and mole fraction partition coefficients at a lipid concentration of 4 g/L and a butanol concentration of 5 g/L. Our results show that the equimolar mixture of DPPC and DOPC provide the highest butanol partition coefficient.*

| Lipid          | Butanol Partition coefficient | Mass basis | Mole fraction basis |
|----------------|-------------------------------|------------|--------------------|
| DPPC           | 22 ± 5                        | 900 ± 190  |
| DPPC:DOPC (50:50) | 33 ± 10                      | 1370 ± 365 |
| DOPC           | 28 ± 8                        | 1220 ± 480 |
The surfactant L62 used by Dhamole et al. yielded a mass partition coefficient of 3.5 and showed an increase in butanol productivity of over 200%. For organic solvents, a number of long chain alcohols were examined with the highest partition coefficient amongst them obtained with 2-ethyl-1,3-hexanediol with a mass partition coefficient of 8.1 reported (Barton, Daugulis 1992). Ionic liquid solvents have also shown to be effective solvents for butanol (Cascon et al. 2011; Davis and Morton 2008).

Tetrahexyammonium dihexylsulfosuccinate ([THA][DHSS]) yielded a mass partition coefficient of 7.99 (Cascon et al. 2011) and 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([bmim][Tf₂N]) a mass partition coefficient of 14 (Davis, Morton 2008). Each lipid composition provided a higher mass partition coefficient than partition coefficients examined.

While the trend reported here for butanol partitioning and lipid composition is a similar trend to previous results reported by our group, the values we found using NMR showed higher butanol lipid partition coefficients and higher butanol-lipid molecular ratios. One significant difference between this study and our previous work was this study analyzed each sample using NMR which allows for *in situ* measurements. While HPLC was used previously which requires the sample to be separated before being analyzed. This separation could be the cause for such a distinct difference between both the partition coefficients and the butanol/lipid ratios.
3.3.2 Liposome Swelling

DPPC, DOPC and an equimolar mixture of DPPC and DOPC had an increase in size as butanol was added as seen in Figure 3.3A. A linear fit was applied to each data series plotted through change in size = 0. The slope of this line with units of $\Delta$(diameter)/$\Delta$(g/L butanol) allowed for a quantitative comparison between lipid compositions (tabulated in Figure 3.3). DPPC showed the largest change in size per butanol added with a slope of 1.4. DOPC and an equimolar mixture of DPPC and DOPC showed smaller slopes of 0.8 and 1.1, respectively. The polydispersity index of the samples, which is a measure of size homogeneity and used to identify aggregation or sample destabilization, was less than 0.3 for these experiments. This indicates that the changes in size were not due to vesicle aggregation or butanol phase separation (into lipid-stabilized butanol droplets in water – i.e. an emulsion).

Acetic acid, 1,3 propanediol and ethanol were tested in the same way as butanol and the changes in vesicle size can be seen in Figure 3.3B. Acetic acid, ethanol and 1,3 propanediol showed a change in vesicle size (slope) of 0.3, 0.2 and 0.5, respectively. The vesicles used were 10 mM DPPC and, when compared with the butanol results, the changes in size for acetic acid, 1,3 propanediol, and ethanol were two to five times lower than when butanol was present.
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Change in vesicle size ($\Delta$ (diameter)/$\Delta$ (g/L butanol))

| Lipid             | Butanol | Acetic acid | 1,3 Propanediol | Ethanol |
|-------------------|---------|-------------|-----------------|---------|
| DPPC              | 1.42    | 0.33        | 0.58            | 0.25    |
| DPPC/DOPC (50:50) | 1.06    |             |                 |         |
| DOPC              | 0.81    |             |                 |         |

Figure 3.3: Change in vesicle size with (A) increasing butanol concentration and (B) increasing acetic acid, 1,3 propanediol and ethanol concentration (DPPC only). A table containing the slopes of linear fits through the intercept.
To our knowledge, the use of vesicles for in situ extractive butanol fermentation and the effect of butanol and lipid composition on vesicle solvent characteristics have not been previously studied. Comparing butanol partitioning and swelling results provides insight into the interdependence of these phenomena. qNMR was conducted at lipid:butanol mass ratios $\geq 0.73$ (Figure 3.2), which corresponds to swelling conditions $> 10$ g/L butanol where the lipid concentration was 10 mM or 7.34 g/L (Figure 3.3A). This provides direct evidence that butanol partitioning led to swelling. As butanol swells the vesicles, more space is created in the vesicles to accommodate more butanol. This was observed for qNMR measurements at low lipid concentrations (0.4 g/L) where the molar ratio of captured butanol to lipid was extremely high with ratios of 14, 24 and 24 for DPPC, DOPC and the equimolar mixture respectively.

While butanol has not been studied extensively, ethanol is known to increase vesicle size at higher concentrations due to bilayer expansion and lipid interdigitation (Löbbecke, L. and Cevc, G. 1995). Butanol also leads to bilayer expansion and our previous results showed that DPPC and DPPC:DOPC vesicles entered an interdigitated phase at butanol concentrations above 10 g/L (Kurniawan et al. 2012). According to this work, vesicle size increased linearly before and after butanol caused lipid interdigitation. This indicates that size of the vesicle does not reflect the effects of interdigitation, which is known to expand lipid bilayers (Löbbecke and Cevc 1995), at the conditions examined.
With respect to lipid composition, all vesicles swelled in the presence of butanol, but swelling was not correlated to the butanol partition coefficient, meaning a greater increase in size does not reflect a higher partition coefficient. This can be seen for DPPC, which showed the greatest change in size and the lowest partition coefficient. In turn, DOPC showed the smallest change in size with a high partition coefficient. This observation is consistent with the concept of free-space provided by unsaturated lipids with double bonds (DOPC). The free-space allows the bilayers to accommodate butanol with less expansion compared to saturated lipids (DPPC), which are tightly packed and must “unpack” to accommodate butanol.

3.4 Conclusions

The vesicle swelling tests revealed two specific traits about the effect of butanol on lipid vesicle solvents. The first is that butanol creates a larger swelling effect on vesicles when compared to other fermentation products. This can be attributed to the vesicles ability to preferentially extract butanol over other products. This is due to the greater hydrophobicity of butanol compared to acetic acid, 1,3-propanediol, and ethanol. Secondly, the butanol partition coefficient of vesicles does not correlate with the change in vesicle size with added butanol. After examining the partition coefficient results it is clear that the equimolar mixture of DOPC and DPPC is the most effective at extracting butanol. Not only did this composition provide the highest partition coefficient, but it also showed the highest
butanol-to-lipid ratio of the three compositions. Our group has previously shown that two phase bilayers create favorable partitioning conditions for butanol and these results support that idea as well. NMR was also shown as a method for measuring butanol/lipid partition coefficients. Initial results show that this method results in higher partition coefficients being found, possibly due the experiments being conducted without a separation of the solvent phase.

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Chapter 4

LIPID VESICLE-BASED IN-SITU EXTRACTION FERMENTATION OF BIOBUTANOL PRODUCED BY

CLOSTRIDIUM PASTEURIANUM

This chapter is written in manuscript format with the intent to submit once additional results are obtained. The work presented here represents a collaborative effort with Dr. Carmen Scholz’s group at the University of Alabama in Huntsville.

4.1 Introduction

Butanol is becoming a highly sought after fuel source as it has similar fuel properties to that of gasoline and could even be used in a mixture of diesel (Atabani et al. 2012; Surisetty et al. 2011). While current butanol synthesis is usually undertaken by the conversion of fossil fuels (Surisetty et al. 2011), butanol can also be produced via fermentation. Fermentation can offer a biofriendly, renewable process that could potentially replace fossil fuels as the industrial source of butanol (Nanda et al. 2017). Even crude glycerol, an unrefined by-product of biodiesel, can be effectively utilized for butanol production (Venkataramanan et al. 2012).

Batch fermentations using Clostridia strain of bacteria have shown promising results of butanol yields (Kubiak et al. 2012; Lee et al. 2008; Groot et al. 1989), however, in order for fermentation to become a viable butanol production option, a more
industrial approach is needed. Continuous fermentation eliminates several negatives that batch production would have in an industrial setting, such as long down-times required for cleaning and sterilizing the equipment in between batches (Lee et al. 2008). Continuous cultures of Clostridium acetobutylicum and C. beijerinckii achieved butanol yields of 0.42 g/g (Huang et al. 2004) and 0.38 g/g (Qureshi and Blaschek 2000). Groot, et al. compared batch and continuous fermentations with a product recovery process integrated and found the yields of both to be comparable to each other (Groot et al. 1989).

Product removal is an important process that increases butanol production. Extractive fermentation removes product as it is formed to both limit the toxic effects of butanol (Sardessai and Bhosle 2002) and drive the fermentation to produce more butanol. Recent work showed butanol yields of 0.36 g/g using a mixture of oleyl alcohol and dodecanoic acid as a solvent (Zhang et al. 2017). Another study combined continuous fermentation using metabolically engineered C. acetobutylicum with ex situ recovery and found butanol yields of 0.18 g/g and 0.3 g/g (Lee et al. 2016). While organic solvents have been shown to be effective, biocompatible self-assembly solvents have recently been shown to be effective solvents (Dhamole et al. 2012; Vian et al. 2017).
This work showcases the potential of the *C. pasteurianum* strain of bacteria for butanol production. Additionally, a continuous fermentation process was used with *ex situ* recovery and to our knowledge this is the first time *C. pasteurianum* has been used in a continuous fermentation. Finally, a novel lipid bilayer self-assembled solvent was used due to the high affinity that lipid bilayers show towards butanol extraction (Kurniawan et al. 2012; Kurniawan et al. 2013).

4.2 Materials and Methods

L-α-phosphatidylcholine (95%) (Soy-Lecithin) was purchased from Avanti Polar Lipids (Alabaster, AL) in granule form and was used without further purification. Deuterium oxide (99.9 atom % D) and maleic acid (traceCERT, qNMR standard) were purchased from Sigma-Aldrich. *Clostridia pasteurianum* ATCC 6013 strain was used for these experiments. Deionized (DI) ultrafiltered water was obtained from a Millipore Direct Q-3 purifier. A 400 MHz NMR (Bruker Biospin AG, Magnet System 400’54 Ascend) was used for the NMR analysis.

4.2.1 Vesicle Preparation

Soy-Lecithin was added to preheated DI water at a temperature over 40°C. The lipids were heated and stirred until dissolved. Then the vesicle solution was autoclaved at a temperature of 120 °C before being placed in the glove box. The experiments here used 2 g/L of lipid.
4.2.2 Fermentation Media

The media used for this fermentation was a glycerol-based media. The compositions used for each volume can be seen in Table 4.1. The CaCO₃ solution was added immediately before inoculation of the bacteria after everything was mixed and autoclaved.

Table 4.1: Compositions of the glycerol media used during the fermentation.

|          | 500 ml | 1000 ml |
|----------|--------|---------|
| K₂HPO₄   | 1.87 g | 3.74 g  |
| KH₂PO₄   | 0.715 g| 1.43 g  |
| (NH₄)₂SO₄| 1.1 g  | 2.2 g   |
| Yeast extract | 0.55 g | 1.1 g   |
| MgSO₄/FeSO₄ solution | 5 ml | 10 ml |
| Trace metal solution | 1 ml | 2 ml |
| Glycerol | 12.5 g | 25 g    |
| CaCO₃ solution | 2.5 ml | 5.0 ml |

CaCO₃ solution is prepared by slowly adding 22g of CaCO₃ to 36.3 ml of HCl, then slowly adding HCl until the solution turns clear. Trace metal solution SL7 is composed of 10mL of 25% HCl solution per liter, 1.5 g/L FeCl₂·4H₂O, 190 mg/L CoCl₂·6H₂O, 100 mg/L MnCl₂·4H₂O, 70 mg/L ZnCl₂, 62 mg/L H₃BO₃, 36 mg/L Na₂MoO₄·2H₂O, 24 mg/L NiCl₂·6H₂O, 17 mg/L CuCl₂·2H₂O.

4.2.3 Bioreactor Schematic and Operation

A schematic of the bioreactor system is shown in Figure 4.1. Prior to butanol extraction with vesicles, bacteria were first grown in batch culture until an optical
density (O.D.) of 0.7 and a pH of 5 was obtained, signifying that the conditions were appropriate for butanol production. A dilution rate (DR) was chosen, more on that below, and the continuous fermentation was then started. Fresh media was pumped into the bioreactor while simultaneously removing media from the bioreactor to prevent accumulation. The fermentation continued in this way until 98% turnover of the bioreactor volume had been achieved which was considered reaching steady state. However, to ensure we did reach steady state an additional hour was spent and the O.D., pH and composition were checked at the start and end of that hour and compared to each other. Following this the extraction process was started. A hollow fiber membrane (SpectrumLabs, 300kD, 20 cm EL, modified polyether sulfone or mPES) was used to contact the vesicle solvent with the fermentation broth. Analysis was completed on samples taken from the bioreactor (B) and vesicle solvent container (D).
Figure 4.1: Schematic of the continuous fermentation with vesicle extraction process. P1-P4 are the pumps. (A) Fresh media inlet being fed into (B) the bioreactor with the C. Pasteurianum fermentation. (C) was the waste collection. (D) housed the vesicle solution being sent through (E) the hollow fiber membrane used to extract butanol.

\[
DF = \frac{Q_M}{V_R}
\]

(4.1)

Dilution rate was found by dividing the flow rate of the media in to the reactor \((Q_M)\) by the initial volume of the reactor \((V_R)\). Two dilution rates were used in these experiments, 0.18 hr\(^{-1}\) and 0.09 hr\(^{-1}\). The flow rates remained consistent throughout the experiments. The reactor’s initial volume was changed in order to change the dilution rate.
4.2.4 Quantitative NMR

qNMR was performed to analyze butanol concentration and determine extraction performance. Maleic acid (Sigma, standard for quantitative NMR, TraceCERT) was used as an internal calibrant as its NMR shift is unique compared to butanol. Samples were prepared with a 90/10 split of H₂O/D₂O in pre-weighed NMR tubes. The volume of each sample was the same with 450 µL of a sample and 50 µL of internal calibrant solution. The NMR spectrum was collected for each sample using the same acquisition parameters as previously used in chapter 3, with the exception of the delay time. The delay time was extended from 20 s to 30 s to accommodate the longer relaxation time of maleic acid. The following equation was used to analyze this data and convert the intensity of a butanol peak to purity or mass fraction of butanol. Figure 4.2 shows an NMR spectrum of a sample from the vesicle solution after extraction. The maleic acid peak located at 6.25 ppm is compared to the available butanol peaks using Equation 4.2 (Malz and Jancke 2005).
Figure 4.2: NMR spectrum of a sample from the continuous fermentation. The maleic acid peak (6.25 ppm) is compared to the visible butanol peaks (1.4, 1.25, 0.8 ppm) in order to find how much butanol is in solution.

\[ P_x = \frac{I_x}{I_{std}} \frac{N_{std}}{N_x} \frac{M_x}{M_{std}} \frac{m_{std}}{m_{total}} P_{std} \]  

(4.2)

Once the purity is known, it can be converted to a concentration of butanol by using the partition coefficient and the molecular weights. This tells us the concentration of butanol outside of the vesicles. The butanol partition coefficient of soy-lecithin was then found and used to calculate the number of moles of butanol captured by the vesicles.
4.2.5 HPLC Analysis

Samples taken from the bioreactor were centrifuged immediately to separate any bacteria. The supernatant was analyzed for butanol concentration using an HPLC as described previously in Kurniawan et al. 2012. Each sample was measured at least in duplicate. The HPLC used was a Varian ProStar pump system with a Varian Star 800 Module Interface.

4.3 Results and Discussion

4.3.1 Validating qNMR for determining butanol concentration

Samples were taken at random time points from the bioreactor and butanol concentration was measured by both HPLC and NMR (Figure 4.3). This comparison was done to validate the qNMR approach, which is the basis for determining butanol partitioning into, and extraction by, lipid vesicles in situ. While HPLC is commonly used to determine fermentation product concentrations, it is not suitable for these in situ measurements – the vesicles would have needed to have been separated from the vesicle solvent phase prior to analysis. Good agreement was observed between qNMR and HPLC.
4.3.2 Butanol extracted by vesicle solvent

The mass of butanol extracted by the vesicle solvent was found by measuring the amount of butanol outside of the vesicles by qNMR, and then calculating the amount of butanol within the vesicle bilayers using previously measured partition coefficients for soy-lecithin. At butanol concentrations of 1 g/L, around the concentration found in our dilution rate 0.18 hr\(^{-1}\), the lecithin/water mole fraction partition coefficient was approximately 200. As seen in figure 4.4, the amount of butanol extracted increased over time, and similar results were observed at 60 min for the two dilution rates. This was expected given that the solvent flow was unchanged between dilution...
rates. The difference in dilution rate results can instead be seen in the total butanol produced as discussed below.

![Butanol Production Graph](image)

**Figure 4.4:** Amount of butanol (g) extracted by the vesicle solvent as a function of time at two dilution rates (A 0.18 h^−1 and B 0.09 h^−1). The blue bar represents the total butanol measured by qNMR therefore outside of the vesicles. The orange bar represents the butanol found by calculation using the partition coefficients from previous work. 60 min is highlighted here as that was the final time point both trials had in common.

4.3.3 Total butanol production

The total butanol produced during continuous culture with vesicle-based solvent extraction is shown in figure 4.5. The lower dilution rate, DR = 0.09 h^−1, produced more butanol compared to the higher dilution rate with a productivity of 1.11 g/h and a yield of 0.12 g butanol/g glycerol. For comparison, Roffler et al. conducted a
fed-batch extractive fermentation of butanol with oleyl alcohol and produced over
500 g of butanol after 50 hours with a yield of 0.18 g butanol/g glucose. More
recently, a batch extractive fermentation using oleyl alcohol exhibited a yield of 0.28
g/g (Zhang et al. 2017). Continuous cultures of Clostridium acetobutylicum and C.
beijerinckii achieved butanol yields of 0.42 g/g (Huang et al. 2004) and 0.38 g/g
(Qureshi and Blaschek 2000) without extraction present. The total time of these
experiments was longer than our time in each case. While our time did not exceed 2
hours, in all of these studies, butanol production increases significantly after 10 hours
of extractive fermentation.

Both dilution rates showed that over time the total butanol produced increased with
time. This is attributed to the vesicle extraction system which drove the reaction to
form more butanol by removing the product. The butanol production was increased
by 32% and 94% for DR of 0.18 h⁻¹ and 0.09 h⁻¹ respectively, higher than previously
reported values for oleyl alcohol solvents. Oleyl alcohol has shown butanol
productivity increases of 20% (Zhang et al. 2017) and 24% (Roffler et al. 1988). When
the percent increase of butanol is calculated at 60 minutes for the DR of 0.09 h⁻¹, a
value of 61% is found which is almost double the value for DR 0.18 h⁻¹ at the same
timepoint. At the lower dilution rate, we see that the amount of butanol in the
reactor rises even after the extraction process is underway. This is in agreement with
another continuous fermentation result that showed lower dilution rates produced
more butanol (Huang et al. 2004). This highlights the importance of lower dilution
rates as the bacteria are given more time to grow and produce butanol. Initial results also show the vesicle solvent extracts more butanol when in the presence of higher amounts of butanol.

![Graph A](image1.jpg)

![Graph B](image2.jpg)

| Dilution Rate (hr⁻¹) | Butanol Production Rate (g/hr) | Increased Butanol Production | Butanol Yield (g butanol / g glycerol) |
|----------------------|-------------------------------|------------------------------|----------------------------------------|
| 0.09                 | 1.11                          | 94%                          | 0.12                                   |
| 0.18                 | 0.29                          | 32%                          | 0.07                                   |

*Figure 4.5: Total butanol produced (g) over time in the reactor, in the solvent and in total for two dilution rates (A - 0.18 hr⁻¹, B - 0.09 hr⁻¹). The DR of 0.09 hr⁻¹ showed almost triple the total butanol production of the DR 0.18 hr⁻¹. A table showing the production of butanol per hour, the percent increase of butanol totals and the butanol yields.*
Molar ratios of butanol to lipid for each experiment were calculated and are presented in Figure 4.6. The ratios here are consistent with our previous results, which showed butanol/lipid ratios from 4 to 7 as seen in Chapter 3.

![Figure 4.6: Ratio of moles of butanol to moles of lipid for two dilution rates.](image)

4.4 Conclusions

Initially, we are seeing that the addition of our lipid bilayer solvent increases the butanol production by 94% with a yield of 0.12 g/g. Of the dilution rates tested, the lower value provided higher butanol production due to the additional time that this allowed the bacteria to grow. More results are needed in order to determine how much of this increased butanol production is due to the extraction or due to the dilution rate’s influence. To our knowledge this is the first continuous fermentation
of *C. pasteurianum* completed and the first time a vesicle solvent has been used to extract butanol.

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Chapter 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In chapter three we examined butanol partitioning into DPPC and DOPC bilayers and the swelling effect of butanol on the bilayer. Butanol caused a larger swelling effect compared to the effect that the fermentation products caused (acetic acid, 1,3-propanediol, ethanol). This is due to the greater hydrophobicity of butanol compared to the others.

The butanol partition coefficients showed that the equimolar mixture of DOPC and DPPC are the most effective at extracting butanol (Figure 5.1). This is supported by previous work done in our group showing two phase bilayers create favorable partitioning conditions. We also found our butanol/lipid partition coefficients using NMR which resulted in higher partition coefficients being found compared to HPLC methods.
Our initial fermentation and extraction results show promising increases in butanol production. We also showed that a lower dilution rate produced more butanol (Figure 5.2), which is consistent with previous continuous fermentations in the literature. The inclusion of our lipid bilayer solvent resulted in an increase of 90% in butanol production from the start of the extraction.
Figure 5.2: A dilution rate of 0.09 provided an increase of 94% in butanol produced. We also see that the amount of butanol in the reactor rises once the extraction begins indicating an increase in production by the C. pasteurianum.

5.2 Future Work

The next step that this work should take would be to finish the continuous fermentation tests. This would then reveal how much of the increased butanol production is due to the continuous fermentation process or effectiveness of our vesicle solvent. Also, our future experiments in this area should take place over a longer time frame as some literature showed a rapid increase in butanol production after longer times.
Our current extraction method involves bringing the fermentation broth into contact with the vesicle solvent using a hollow fiber membrane. Another method could instead add the vesicles directly to the fermentation media.

Finally, a method should be established for the reclamation of butanol from the vesicles after the extraction takes place. A couple of different options exist for breaking apart vesicles. One option would be to dissolve the vesicles by using a surfactant like Triton x-100 or a detergent to release the butanol.
Appendix 1
NMR Background, Procedures and Calibration Curve

Nuclear magnetic resonance (NMR) is the phenomenon when nuclei absorb and then emit electromagnetic energy. A magnetic field is applied to the sample which causes the individual spins of the nuclei to align before the magnetic field is turned off and the molecule relaxes. NMR spectroscopy utilizes this phenomenon to collect data. The electromagnetic energy is released upon the molecule relaxing and this resonance is collected as a frequency which is then converted into chemical shift (Darbeau 2006).

NMR’s quantitative capabilities are based on the direct proportionality between the intensity generated, $I_x$, and the number of nuclei, $N_x$, that correspond to the resonance. There are multiple ways that this relationship can be utilized. One method, involves adding a known amount of a specific substance to a sample and comparing that known amount and its NMR signal to the unknown amount and the corresponding signal. This proportion is related by a spectrometer constant, $K_s$ (Malz, Jancke 2005). The spectrometer constant is dependent on the equipment and the acquisition parameters used. Therefore, to keep $K_s$ constant the acquisition parameters used must be the same for each sample (Bharti, Roy 2012; Malz, Jancke 2005).

$$I_x = K_s N_x$$
A molar ratio can be calculated based on the ratio of two different nmr signals. The spectrometer constant can be canceled out due to each signal appearing within the same spectrum so the parameters must be the same. This leads to the following:

\[
\frac{n_x}{n_y} = \frac{I_x N_y}{I_y N_x}
\]

Molar fractions can be calculated by adding a total summation term for \( n, I \) and \( N \).

Finally, if the purity of one substance is known, this can be used to calculate the purity associated with the resonance peak of a different substance which is shown here:

\[
P_x = \frac{I_x N_{std} M_x}{I_{std} N_x M_{std} m_{total}} P_{std}
\]

Where \( I \) is the intensity of the resonance peak, \( N \) is the number of nuclei associated with the resonance peak, \( M \) is the molecular weight, \( m \) is the mass of the sample, and \( P \) is the purity. Subscript \( x \) is related to the substance of unknown purity while \( std \) refers to the qNMR standard used (Malz, Jancke 2005; Bharti, Roy 2012). An example of a spectrum that has undergone this analysis can be seen in Figure A.1.

Adding in a standard proved useful for obtaining quantitative information in a setting where multiple chemicals were present in a solution however another approach was taken to quantitatively measure butanol alone in solution. When butanol was the only measurable solute in solution, the concentration of butanol was found by using a calibration curve. The calibration curve was based on the same principle as the previous technique where the intensity of the signal is related to the corresponding
protons. The curve was constructed by relating the integral of butanol (as related to the water peak) to a known concentration and fitted using a 3rd degree polynomial. Then, when an unknown butanol signal is measured the concentration could be found in this manner. A sample of a butanol spectrum used to make the calibration curve is included in Figure A.2 and the calibration curve can be seen in Figure A.3.

Quantitative NMR acquisition parameters were set according to this procedure (Pauli et al. 2007). In short, the parameters that needed to be adjusted were as followed: sample spin, $^{13}$C satellite removal, relaxation delay time, spectral window, transmitter position, pulse width, acquisition time, number of scans, receiver gain, dummy pulses, $^{13}$C decoupling. The goal of changing these parameters was to provide a clearer NMR signal and eliminate potential side peaks from appearing.
Figure A.1: Raw NMR data from an extractive fermentation test (Results seen in Chapter 4). This shows where the integrals were taken. Once taken, the absolute values were found in the MestReNova program “Table of Integrals”. This was using an internal calibrant maleic acid (seen at 6.3 ppm).
Figure A.2: One NMR spectrum used in the process of creating a calibration curve. The D$_2$O peak (4.7 ppm) is used to reference to the other butanol peaks (3.5, 1.5-1.2, 0.8 ppm).

Figure A.1: The calibration curve of butanol from NMR. Each butanol shift was recorded as a peak.

Peak 1 – 3.5 ppm, peak 2 – 1.5-1.2 ppm, peak 3 – 0.8 ppm.
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