THE USE OF HIGH PRESSURE CARBON DIOXIDE FOR *IN SITU* PRODUCT RECOVERY OF BUTYRIC ACID IN A TWO-PHASE PARTITIONING BIOREACTOR

by

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Abstract

The production of organic acids in solid-liquid two-phase partitioning bioreactors (TPPBs) is challenging, as acids absorb into amorphous polymers only in their undissociated form. Thus, at near-neutral fermentation *p*H values, where dissociated conjugate base species are dominant, partitioning is restricted. This thesis utilizes high pressure CO_2 to achieve temporary *p*H reductions during the production of butyric acid (pK_a=4.8) by *Clostridium tyrobutyricum*, permitting acid absorption and *in situ* product recovery (ISPR), reducing end-product inhibition and improving fermentative performance.

A growth medium with minimized buffering was developed which did not interfere with *p*H reductions, and using CO_2 sparging a *p*H of 4.8 was achieved, compared to 5.3 in the original medium, with no difference in cell growth. Buffering from the accumulation of butyric acid during fermentation was observed, however, ISPR of butyric acid was demonstrated using CO_2 at atmospheric pressure, yet this did not achieve improvements in reactor performance, and it was concluded that high pressure CO_2 may overcome buffering to improve recovery.

To determine what *p*H would need to be reached with CO_2, a first-principles study of *p*H-dependent partitioning was performed, identifying partition coefficient (PC), polymer fraction (F) and *p*H as variables for determining organic acid recovery. Through polymer screening based on thermodynamic affinity for butyric acid, an absorptive polymer was selected (Pebax®2533), and partitioning tests for both butyric acid (PC=4.2) and benzoic acid (PC=70, pK_a=4.2) validated *p*H-dependent partitioning models. 60 bar pCO_2 was shown to achieve up to 40% recovery of butyric acid, yielding a distribution coefficient (D) of 1.8, and 90% recovery of benzoic acid (D=24), demonstrating clear improvement over atmospheric pressures.

Finally, exposure of cell populations to 60 bar pCO_2 showed no adverse biological effect, and at this pressure medium buffering effects were substantially overcome. During fed-batch production of butyric acid the use of high pCO_2 provided the necessary *p*H reductions to

achieve substantial acid recovery during fed-batch production of butyric acid, as overall yields, titres, and volumetric productivities were increased by 35%, 60%, and 96%, respectively. High pCO_2 represents a novel method for achieving ISPR of butyric acid, which could be extended to the production of other organic acids.

Co-Authorship

Chapters 3 and 5 have been accepted or submitted to refereed journals and were co-authored by Dr. Andrew J. Daugulis, who provided editorial and technical advice. Chapter 4 was co-authored by Dr. Scott Parent and Dr. Andrew J. Daugulis, who provided technical and editorial advice, respectively.

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Finally, I dedicate this thesis to my wife, Maggi, who gave me the years and love I needed to achieve this herculean feat. You made my PhD richer and more meaningful, and I will fondly remember this period where we were destitute but happy. I can only look forward to the new chapters we will write together.

Statement of Originality

I hereby certify that all of the work described within this thesis is the original work of the author. Any published (or unpublished) ideas and/or techniques from the work of others are fully acknowledged in accordance with the standard referencing practices.

Eric Charles Peterson.

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List of Abbreviations

D: Distribution coefficient (unitless dimensions)

DNS: Dintrosalicylic acid assay

EPI: End product inhibition

F: Polymer fraction (g polymer phase/g aqueous phase)

GC: Gas chromatography

HPLC: High pressure liquid chromatography

ISPR: *In situ* product recovery

$\mathbf{m_{pol}}$: Mass of polymer phase (g)

$\mathbf{m_{aq}}$ Mass of aqueous phase (g)

$\boldsymbol{n_S^{pol}/n^{tot}}$: Mole fraction of a non-reactive solute in the aqueous phase

$\boldsymbol{n_S^{aq}/n^{tot}}$: Mole fraction of a non-reactive solute in the polymer phase

$\boldsymbol{n_{HA}^{pol}/n^{tot}}$: Mole fraction of acid in the polymer phase

$\boldsymbol{n_{HA}^{aq}/n^{tot}}$: Mole fraction of acid in the aqueous phase

$\boldsymbol{n_{tot}^{aq}/n^{tot}}$: Mole fraction of acid and conjugate base in the aqueous phase

$\boldsymbol{n_{A-}^{aq}/n^{tot}}$: Mole fraction of conjugate base in the aqueous phase

PC: Partition coefficient (unitless dimensions)

PBT: Poly(butylene teraphthalate)

PBO/PTMG: Poly(butylene oxide)/ Poly(tetramethylene glycol)

$\mathbf{pCO_2}$: Partial pressure of carbon dioxide (gauge pressure)

PEO: Poly(ethylene oxide)

RO: Reverse osmosis water

$\mathbf{T_g}$: Glass transition temperature (°C)

$\mathbf{T_m}$: Melting temperature (°C)

TPPB: Two-phase partitioning bioreactor

VVM: Ratio of volume of sparged gas to bioreactor working volume per minute

$\mathbf{Y_{P/S}}$: Product yield, g product produced per g glucose consumed

Chapter 1

Introduction

There is global growing interest in the biological production of chemical feedstocks as a substitute for petrochemically-derived sources. This approach may be able to provide some freedom from the growing price of oil, and also represents a paradigm shift to a more sustainable bio-based economy founded on biomass conversion rather than intensive resource extraction. While many different chemicals can be produced biologically, including fuels, and building block chemicals, the bioproduction of such molecules will only be competitive in the face of a thriving petrochemical industry, if improvements to process performance during fermentation can be made. Two-phase partitioning bioreactors (TPPBs) have been shown to improve volumetric productivities and yields, and this is achieved via inclusion of an absorptive phase, which acts to absorb end-products during fermentation through *in situ* product recovery (ISPR) (Freeman *et al.* 1993; Morrish and Daugulis 2008; Gao and Daugulis 2010; Khan and Daugulis 2010). Extractive fermentation utilizing solvent phases has been used for many years, but some drawbacks (i.e. emulsification, bioavailability) have been encountered. Recently, absorptive polymers have been employed in place of extractive solvents to achieve partitioning, which operate on the same basis of thermodynamic equilibria, and their physical properties confer good handling and low bioavailability. As a result of ISPR achieved with absorptive polymers, aqueous-phase product accumulation is reduced, diminishing end-product inhibition (EPI), and concentrations are maintained at sub-cytotoxic levels, which in turn benefits microbial activity and overall fermentative performance.

This thesis aims to demonstrate *in situ* product recovery of organic acids via polymeric absorption in two-phase partitioning bioreactors, using butyric acid as a model target molecule. Unlike all other solutes studied to date in TPPBs however, organic acids are unique due to their dissociative nature, and this can hinder absorption into polymer phases, as the charged

conjugate base of an acid will not partition, and this is the dominant species present under typical fermentation conditions (i.e. near neutral *p*H values). Thus, in this thesis, the use of CO_2 has been studied as a method for temporarily reducing *p*H values, which permits greater absorption into the sequestering phase, while avoiding ion accumulation and resultant osmotic stress that would otherwise occur using traditional methods for *p*H adjustment.

Of primary concern in this work is the weak nature of carbonic acid (H_2CO_3) arising from dissolved CO_2, which has a pK_a of 3.6, and thus cannot lower *p*H values below approximately 3 in water regardless of H_2CO_3 concentration. However, while *p*H reductions can be easily achieved through carbonic acid dissociation in water, buffering effects from medium components and conjugate base concentrations interfere with *p*H reductions. One method for increasing these *p*H reductions in the face of buffering is to apply elevated partial pressure of CO_2 (pCO_2), as this will increase solubility and provide more carbonic acid. The success of using high pCO_2 to facilitate acid recovery using absorptive polymers is determined by two major factors, namely *p*H and its effect on partitioning. While it is likely that elevating pCO_2 will improve *p*H reductions compared to atmospheric conditions, characterization of *p*H dependent partitioning is required to fully understand this phenomenon. Furthermore, the overall absorption achieved by this *p*H reduction is dependent on the partition coefficient that a polymer demonstrates for a specific acid, and thus careful polymer selection strategies need to be undertaken to identify a polymer exhibiting desirable partitioning.

Importantly, biological tolerance to increasing pressure is also a critical consideration, if such a high pCO_2 process for improving acid absorption in polymers is to be incorporated into an ISPR strategy for improved fermentation of butyric acid. Thus the effect of exposure to high pressure on cell populations must be vigorously inspected, to ensure a process can be developed that exerts no deleterious biological effects. Overall, if ISPR of an acid in a TPPB is to be achieved, the combination of all the above mentioned considerations must be made if high

pressure CO_2 *p*H reductions are to facilitate *p*H dependent partitioning in absorptive polymers. Provided no adverse effects on microbial activity arise from this exposure to high pressure, the potential improvements in acid absorption afforded through use of high pressure CO_2 could demonstrate increased yields and volumetric productivity, as a result of alleviation of end product inhibition.

1.1 Structure of thesis

This thesis progresses from a general review of ISPR of organic acids in Chapter 2, which addresses biological organic acid production, TPPBs, polymer selection strategies, and high pressure CO_2 *p*H reductions, as well as the effect of high pressure on cells. Chapter 3 focuses on experimental application of CO_2 sparging at atmospheric conditions to media and fermentations, and initial attempts at rational polymer selection for use in TPPBs. The effects of medium composition and acid concentration on *p*H reductions were investigated, and a medium was developed that showed increased *p*H reductions with no decrease in cell growth. Also, initial polymer selection strategies were employed to identify a polymer for use in a TPPB, which prompted a more thorough investigation of selection strategies, as shown in Appendix A. When CO_2 sparging was intermittently applied to a TPPB, butyric acid absorption into the polymer phase was observed, but the overall acid removal achieved under these conditions proved insufficient to provide substantial product gains.

With the strategy of increasing acid recovery, Chapter 4 focuses first on characterization of *p*H-dependent partitioning, to clearly identify what *p*H values need to be reached with CO_2 to increase polymeric absorption of acids. The ability of high pCO_2 to enhance *p*H reductions and facilitate improved uptake of acids with absorptive polymers was compared to CO_2 at atmospheric conditions, and it was found that a strong relationship between pCO_2 and absorption exists. Furthermore, it was also shown that the effect of acid buffering strength was overcome at 60 bar pCO_2 in tests performed in water. This chapter also highlights the importance of polymer

fractions in achieving effective acid recovery, and largely represents the only variable easily manipulated by an operator. Using experimental data from high pressure partitioning tests, % acid recovery was estimated at high pressure for a range of polymer fractions, which suggested that high pCO_2 *p*H reductions for improving acid absorption could be achieved with this technique.

Chapter 5 focuses on the application of high pCO_2 acid recovery techniques to fermentations first through investigation of the effect of the medium developed in Chapter 3 on high pressure *p*H reductions, and what effect exposure to high pressure has on cell populations. Ultimately, this chapter also focuses on integration of high pCO_2 *p*H reductions into a butyric acid fermentation to achieve ISPR, determining if productivities and yields could be improved. During this study, polymers were shown to be quickly regenerated and reused multiple times over the duration of the fermentation, demonstrating the ease for recovering acid from the polymer after absorption. Chapter 5 also highlights that increasing butyric acid concentrations result in a higher driving force, as although distribution coefficients and % recovery are reduced from buffering strength arising from acid production, the total amount of acid recovered was increased, demonstrating that substantial acid recovery can be achieved regardless of concentration at elevated pCO_2. Additionally, it was noted that the online recovery of acid resulted in reduced need for *p*H control (base addition), which would confer additional benefit to fermentations through reduced osmotic stress, along with alleviation of end-product inhibition.

Finally, Chapter 6 provides a conclusion, and proposes future directions for further investigation of aspects pertaining to the recovery of organic acids in the context of TPPBs.

1.2 Objectives

The overall objective of this work is to demonstrate that high pressure CO_2 *p*H reductions can facilitate ISPR of organic acids by increasing polymeric absorption in TPPBs to improve process performance using butyric acid as a model target molecule. An initial objective was the

development of a medium formulation capable of achieving improved *p*H reductions as a result of CO_2 sparging through minimization of medium buffering strength while not affecting cell performance. A second objective was to develop models to describe *p*H-dependent partitioning of organic acids in absorptive polymers, using a first principles approach to better understand the relationship between partition coefficient, polymer fraction, and *p*H, with experimental validation of these relationships. Subsequently, a critical objective was to demonstrate the extent to which increasing pCO_2 improves partitioning of butyric acid, while determining to what extent these elevated pressures can overcome buffering effects typically present in acid fermentation. The penultimate objective was the determination of cell tolerance to high pressure CO_2 and the development of method for achieving high pCO_2 *p*H reductions without adversely affecting cell populations. Lastly, the final objective was the demonstration of semi-continuous cyclical high pCO_2 *p*H reductions during production of butyric acid from *C. tyrobutyricum* to achieve ISPR, reduce end product inhibition, and demonstrate improved biological performance in terms of yields and volumetric productivity.

Chapter 2

Literature review

2.1 Bioproduction of organic acids

The biological production of organic acids can be viewed as a platform for providing chemical feedstocks for a diversity of applications (Jang *et al*. 2012). For instance, the bioproduction of certain organic acids (i.e. citric, lactic, and acetic acid) is well established, and these acids can be used as additives in the food and pharmaceutical industry, typically as acidulants, flavouring agents, and preservatives (Sauer *et al*. 2008; Soccol *et al*. 2008). Furthermore, novel applications are arising for biologically produced acids, which can be used as feedstocks for the production of polymers and textiles (Jang *et al.* 2012). For example, lactic and succinic acid represent chemical building blocks which enable the production of materials such as poly(lactic acid) (Sauer *et al.* 2008), poly(butylene succinate) (Beauprez *et al.* 2010), and succinic acid is also a precursor to a variety of chemical commodities such as 1,4-butanediol, tetrahydrofuran (Kurzrock and Weuster-Botz 2010), gamma-butyrolactones, pyrrolidinones (Werpy and Petersen 2004), adipic acid and more (Song and Lee 2006). Furthermore, succinic acid bioproduction plants have emerged in Japan and France (Beauprez *et al*. 2010; Song and Lee 2006), indicating a demand for this biological organic acid. Clearly, the development of a bio-based carboxylate platform to produce organic acids for use as chemical feedstocks and commodities represents a promising new growth market in a sustainable economy.

2.1.1 Butyric acid uses and bioproduction

Recently, the biological production of butyric acid has drawn attention as another chemical commodity which could be produced through the carboxylate platform. Butyric acid is a linear C4 monocarboxylic acid, which can be used to synthesize butyryl polymers in the

chemical industry (Dolejs *et al*. 2014). Furthermore, its use is widespread in the food and beverage industries (Zigova and Sturdik, 2000), and it is commonly used to enhance a butter-like note in food flavours (Dolejs *et al*. 2014), while esters of butyric acid are used both as additives for increasing fruit fragrance and aromatic compounds in perfumes (Jang *et al*. 2012). Interestingly, recent studies have focused on the use of butyric acid as a substrate for the biological production of butanol (Tashiro *et al*. 2004; Baba *et al*. 2012; Al-Shorgan *et al*. 2012; Ventura and Jahng 2013), which results in lower byproduct formation and higher yields, compared to conventional butanol production from dextrose (Tashiro *et al*. 2004). Thus, butyric acid may represent a valuable feedstock for producing butanol, which has recently attracted significant interest as a potential biofuel. Up to 200 000 metric tonnes of butyric acid is produced annually in the USA, and this acid is listed as a high production volume chemical (Dolejs *et al*. 2014). While butyric acid can be produced chemically through oxidation of butane and butyraldehyde (Jang 2012), significant research has been done to show this acid can be produced biologically.

The most typical organism studied for butyric acid production is *Clostridium tyrobutyricum*, which is a gram positive spore-forming strict anaerobe that also produces acetic acid, hydrogen, and carbon dioxide from a variety of carbohydrates (Dolejs *et al*. 2014). However, through metabolic engineering using gene-knockout techniques a butyric acid producing strain of *Clostridium acetobutylicum* has recently been generated (Jang *et al*. 2013), further demonstrating the increased interest in clostridial butyric acid production. As can be seen in Table 2-I, butyric acid titres of up to almost 80 g L^{-1} can be achieved through fed-batch cultures methods employing immobilized cell technologies such as fibrous bed bioreactors (FBB), with yields (g product/g substrate consumed) and productivities (g L^{-1} h^{-1}) ranging from 0.36-0.46 (theoretical yield 0.49) and 0.3-0.8 g L^{-1} h^{-1} respectively. Thus, a robust system for

biological production of butyric acid exists, and could potentially provide a commercial source of this organic acid.

Table 2-I Comparison of various butyric acid fermentation titres, yields, and productivities as reported in the literature

Organism	Culture method	g L^{-1}	$Y_{P/S}$	g L^{-1} h^{-1}	Reference
C. acetobutylicum HCEKW	Batch	31	0.35	0.72	Jang *et al.* 2013
C. tyrobutyricum CNRZ 596	Batch	44	0.38	0.59	Michel-Savin *et al.* 1990a
C. tyrobutyricum CNRZ 596	Fed batch	42.5	0.36	0.82	Michel-Savin *et al.* 1990b
C. tyrobutyricum 25755	Cyclical batch FBB	86.9	0.46	0.3	Jiang *et al.* 2011
C. tyrobutyricum 25755	Fed batch FBB with reactive extraction	n/a	0.45	0.48	Wu and Yang 2003

2.1.2 Recovery methods for organic acids

In 2004, a report by the US Department of Energy (DOE) identified nine organic acids amongst a list of 12 building block chemicals that could be produced from sugars, which could be subsequently converted to "high value, bio-based chemical and materials", such as solvents and polymers, relieving the chemical industry of a dependence on petroleum (Werpy and Petersen 2004). However, the report outlined technical barriers to actualizing the use of acids as alternative chemical feedstocks, citing the need to increase yields and productivities in these biotransformations, while reducing downstream recovery costs by reducing unwanted salts (e.g. $CaSO_4$) arising from traditional crystallization techniques. It has been generally emphasized that developing cost-saving and energy-effective downstream processes are important if organic acids are to be viewed as a commercially viable alternative to petrochemical-based production (Kurzrock and Weuster-Botz 2010; McKinlay *et al*. 2007). Even in the case of well establish production methods such as that for citric acid, improvement is required to reduce operating costs (Dhillon *et al*. 2011). Classical approaches to the recovery and purification of organic acids predominantly employ precipitation and crystallization (Soccol *et al*. 2008), and in some cases, recovery processes during biological organic acid production can account for 60% of total process costs (Kurzrock and Weuster-

Botz 2010). Regardless of application, all organic acid production therefore stands to benefit from improved recovery steps.

The conventional method of acid recovery is precipitation by addition of $Ca(OH)_2$, the major criticism of which is the generation of high amounts of waste gypsum. Other approaches are to use crystallization, electrodialysis, or ion-exchange. All these methods have their respective failings however, largely related to costs, energy demand, or waste generation, and have been thoroughly outlined in several reviews for multiple acids (Pazouki and Panda 1998; Schugerl 2005; Beauprez *et al.* 2010; Kurzrock and Weuster-Botz 2010) and will not be further considered here.

A promising alternative approach to organic acid recovery is liquid-liquid extraction, wherein a second immiscible phase can be added directly to fermentations for product recovery. Due to the largely hydrophilic nature of organic acids, extraction into relatively hydrophobic organic phases can show poor results, however. To overcome this many studies have examined reactive extraction, or the use of hydrophobic aliphatic amines for improved affinity of organic acids in immiscible solvents (Kertes and King 1986; Kurzrock and Weuster-Botz 2010; Pazouki and Panda 1998; Schugerl 2005). Through interfacial proton transfer from acid to amine ion-pairs are formed, and the resulting complexes partition into the solvent, thus achieving superior uptake. However, the majority of reactive extraction studies have been done with the intent of application as a downstream process, and operational considerations such as solvent toxicity are not considered, which would be of significant concern in bioprocesses. For such instances, non-toxic immiscible solvents have been employed, and the direct addition of a second partitioning phase during fermentation reduces end-product accumulation, which results in lower inhibition and better productivity, thus improving the bioprocess. In recent years, work has begun to focus on the direct addition of a solid polymer phase in fermentations, which has been shown to behave in a similar manner to that of

immiscible solvent phases, with notable operational benefits. Specifically, two-phase partitioning bioreactors (TPPBs) capitalize on such benefits, and will be discussed in detail.

2.2 Two-phase partitioning bioreactors

Over the past 25 years, more and more research has focused on the development of two-phase partitioning bioreactors (TPPBs). Such bioreactors differ from conventional reactors via inclusion of a second immiscible phase, with the goal of partitioning toxic compounds to or from the aqueous phase, thus reducing product inhibition, and increasing bioprocess performance. This reactor design emerged from a technique initially termed extractive fermentation (Kollerup and Daugulis 1985; Minier and Goma 1981; Minier and Goma 1982), and later *in situ* product removal (ISPR), which was defined as the fast removal of product from a cell, by not only a second phase, but by techniques such as pervaporation, membrane separation, and precipitation (Freeman *et al*. 1993).

Another major advantage to ISPR is reduced product recovery costs (Gyamerah and Glover 1996). In general, TPPBs present the possibility of closed-loop systems with respect to everything except compounds removed by the second phase. This is an advantage in that water is conserved and there is no wastage of substrate or nutrients. However, any by-products or additives not removed by the second phase would accumulate and eventually reach toxic levels (Murphy *et al*. 1982). Ideally, if such toxic levels could be avoided in an effective and economical method, TPPBs could present a powerful shift in biosynthetic operations, wherein substrate is added, and product is removed indefinitely in a concentrated form with no wastage of substrate, nutrients, or water.

As research in extractive fermentation continued, attempts to apply this technique to target molecules focused on butanol-acetone, and lactic, acetic, propionic and butyric acid fermentations, with growing interest in higher-added value, low volume products such as pharmaceuticals, as well as biologically produced food-additives (Freeman *et al*. 1993). This

provides economic incentive to develop improved techniques for bioproduction of food additives, as consumers are willing to pay more for “natural” compounds (Morrish and Daugulis 2008; Jain *et al*. 2010). Additionally, biological production can also result in higher product purity with respect to by-products and stereoselectivity (Khan and Daugulis 2010).

The term “Partitioning Bioreactor” appeared with the development of a new biodegradative application of two-phase systems, wherein instead of product removal, relatively toxic substrates were added to the secondary phase, and delivered to the aqueous phase in low concentrations. Rather than targeting products to be produced and removed, these biodegradative systems were developed for the treatment of industrial waste streams, where toxic bioproducts could be degraded by microbial activity. These toxic compounds quickly reach inhibitory concentrations, and thus the benefit of the partitioning phase was that it sequestered the majority of a toxic substrate, while allowing a small portion of compounds to equilibrate with the aqueous phase, allowing degradation to occur, until the toxic compound was completely consumed. Thus, TPPBs can be used to both remove products, and deliver substrates, without incurring microbial inhibition (Collins and Daugulis 1996; Daugulis *et al*. 2011).

2.2.1 Partitioning phase selection in TPPBs

Through the history of TPPBs, a range of materials have been used, and can be separated largely into three groups, namely liquid solvents, encapsulated solvents, and solid polymers. Initial TPPB investigations incorporated organic solvents as a second phase, using primarily silicone oil (Daugulis *et al*. 2011), although dodecanol was also commonly employed (Gyamerah and Glover 1996; Kollerup and Daugulis 1985; Minier and Goma 1981; Minier and Goma 1982), and several other types of solvent have been employed (Daugulis *et al*. 2011). While liquid-liquid TPPBs demonstrated that a second phase can act to relieve inhibition by reduction of concentration of toxic materials, several disadvantages were apparent.

Specifically, solvent biodegradation can occur if a solvent is used with mixed cultures (Amsden *et al*. 2003; Daugulis *et al*. 2011; Gao and Daugulis 2010). Furthermore, some solvents were toxic and retarded microbial activity (Freeman *et al*. 1993; Morrish and Daugulis 2008; Gao and Daugulis 2010; Khan and Daugulis 2010). Another drawback is that required mixing can result in emulsification, which makes separation of phases difficult (Gao and Daugulis 2010; Morrish and Daugulis 2008), although attempts were made to negate these problems by using immobilized cell bioreactors (Gyamerah and Glover 1996; Morrish and Daugulis 2008). In attempts to overcome these problems, solvents encapsulated in a polymer coating were used, obviating the negative effects of emulsification and both solvent degradation and toxicity (Whelehan *et a*l. 2010; Wyss *et al*. 2006; Zhao *et al*. 2010). While encapsulated solvents managed to overcome operational problems, commonly used solvents can be relatively expensive, (for example, 2-undecanone can cost upwards of 80$ per kg) (Amsden *et al*. 2003; Daugulis *et al*. 2011), and these costs can only be increased by using encapsulated solvents. Conversely, a much simpler and more economical approach to overcoming these operational challenges is the use of absorptive solid polymers, which are non-cytotoxic, affordable, easy to manufacture, far less susceptible to microbial degradation, and easily recoverable (Amsden *et al*. 2003; Morrish and Daugulis 2008; Daugulis *et al*. 2011). Additionally, polymers have the ability to be tailored to provide target molecule affinity, using techniques such as monomer selection, functionalization, co-polymerization and crosslinking (Prpich and Daugulis 2004). Furthermore, polymers have been shown to be readily reusable (Amsden *et al*. 2003; Prpich and Daugulis 2004), a substantial benefit for process costs and operability.

Thus, absorptive polymers pellets provided an attractive alternative to solvents in TPPBs, and were first employed for the partitioning of phenol as a substrate for biodegradation (Amsden *et al*. 2003). Since then, a wide range of polymers have been used for degradation of an assortment of xenobiotics such as biphenyl (Rehmann and Daugulis

2007), BTEX (Littlejohns *et al*. 2010), and phenanthrene (Isaza and Daugulis 2010), as well as biosynthesis of various value-added compounds, such as 3-methylcatechol (Prpich and Daugulis 2007), carvone (Morrish and Daugulis 2008), L-phenylacetylcarbinol (Khan and Daugulis 2010), benzaldehyde (Jain *et al.* 2010; Khan and Daugulis 2010), indandiol (Dafoe and Daugulis 2011), succinic acid (Hepburn and Daugulis 2012) , and vanillin (Ma and Daugulis, 2013). The use of polymers as a second phase in these systems has resulted in marked improvement of biosynthetic production (Jain *et al*. 2010; Khan and Daugulis 2010; Morrish and Daugulis 2008), and as can be seen in Table 2-II, both volumetric productivities and yields have been noticeably improved in TPPBs, compared to single phase bioreactors.

Table 2-II Comparison of volumetric productivities and yields of various biological products in conventional single phase bioreactors and TPPBs

	Single phase bioreactor		Solid-liquid TPPB		
Product	**mg L^{-1} h^{-1}**	**$Y_{P/S}$**	**mg L^{-1} h^{-1}**	**$Y_{P/S}$**	**Reference**
Methylcatechol	128	n/a	350	n/a	(Prpich and Daugulis 2007)
Carveol	31	n/a	106	n/a	(Morrish and Daugulis 2008)
2-Phenylethanol	50	0.6	380	0.93	(Gao and Daugulis 2009)
L-Phenylacetylcarbinol	0.71	0.34	0.85	0.41	(Khan and Daugulis 2010)
Indandiol	29	0.09	92	0.4	(Dafoe and Daugulis 2011)
Benzaldehyde	41	0.42	97	0.85	(Craig and Daugulis 2013)
Vanillin	268	n/a	450	n/a	(Ma and Daugulis 2013)

However, some disadvantages stem from the solid nature of these absorptive polymers. Specifically, equilibration between the aqueous phase and the solid is substantially slower than in TPPBs employing solvents, which is due to lower solute diffusivity in solid polymers (Amsden *et al*. 2003; Morrish and Daugulis 2008), and decreased surface area, compared to microdroplet formation formed when employing vigorous agitation of solvents (Morrish and Daugulis 2008). However, careful selection of lower polymer molecular weights could improve diffusivity, and reduced the need for increased surface area to achieve good absorption kinetics. Regardless, the operational benefits that polymers confer are simply too attractive

and outweigh these disadvantages, and thus polymers will exclusively be considered for further developments in TPPB techniques.

Numerous studies have focused on the use of adsorptive resins for these purposes, as they can show excellent uptake characteristics, but come at substantial costs. Specifically, the high surface areas necessary for adsorption make for very brittle material properties that would degrade under high shear conditions in a stirred tank. Additionally, commercially available resins are expensive in terms of industrial applications (Gao and Daugulis 2010; Nielsen and Prather 2009). Also, it has generally been observed that adsorptive resins do not fully desorb solutes, whether succinic acid (Davison *et al*. 2004) or butanol (Nielsen and Prather 2009). Furthermore, this incomplete desorption can require thermal treatment (Davison *et al*. 2004; Nielsen and Prather 2009) or treatment with acids, bases (Davison *et al*. 2004; Tung and King 1994), or solvents (Tung and King 1994), to achieve reasonable results. Finally, Davison *et al.* (2004) surveyed a wide body of available resins for succinic acid uptake, and this study was not able to identify a polymeric resin capable of sustainable regenerative capabilities (Davison *et al*. 2004). Thus, while adsorptive resins have been tailored to demonstrate the highest partitioning possible, these resins have serious flaws that would hinder practical use in TPPBs. Therefore, practical studies into the use of polymers should focus on optimization of absorptive polymers, as described above, and will be discussed in detail.

2.3 Rational polymer selection, and polymer-solute affinity

While polymers for application in TPPBs to date have largely been determined through heuristic means, it is important to develop a strategy for rationally selecting polymers as an effective partitioning phase for a given target molecule. Considerations of important polymer properties and potential approaches for prediction of suitable polymers have been studied in detail.

2.3.1 Glass transition temperature and crystallinity

Two important considerations in selection of polymers for the absorption of small molecular weight target molecules are the glass transition temperature (T_g) and crystallinity, if kinetic limitations to uptake are to be avoided. For diffusion to occur in polymers, polymer chain mobility is required to allow for transport of molecules through free space between polymer chains (Morrish and Daugulis 2008). The glass transition temperature is the point where the polymer undergoes a phase change from “glassy” to “rubbery” state, allowing for chain mobility, and this transition point can be determined using differential scanning calorimetry (Amsden *et al*. 2003). Uptake via diffusion, or absorption, is desirable as it allows for use of total polymer mass, whereas adsorption is a surface phenomenon, and is thus limited by surface area. Therefore, ensuring that a polymer is above its glass transition temperature is an important selection criterion for superior uptake in absorptive polymers. It is worth noting that plasticizers are commonly used to lower T_g (Krevelen 2009) and that uptake of solutes in TPPBs has the same effect (Amsden *et al*. 2003). More interestingly, water itself can act as a plasticizer on polymers (Hansen 2007), lowering T_g, permitting absorption to occur (Hansen 2007; Parent *et al*. 2012). Therefore, understanding the factors influencing T_g deserve important consideration.

Another important consideration is the degree of crystallinity of a polymer, as highly organized crystalline structures are densely packed, and again lack the free space afforded by chain mobility, and thus diffusion in crystalline polymers is negligible (Parent *et al*. 2012). This has been confirmed by experiments in liquid-solid partition tests, with increasing crystallinity resulting in decreased uptake (Gao and Daugulis 2010). What is desirable rather, are amorphous polymers, which lack the ordered structure that defines crystallinity. Thus, polymers demonstrating absorptive uptake of target solutes will be amorphous rubbery polymers. While important physical properties such as crystallinity and T_g are relatively well characterized, what presents more of a challenge is understanding what determines affinity

between a polymer and a solute. In an attempt to better understand what influences this affinity, an examination of the fundamental thermodynamic principles, applied approaches for predicting solubility, and observed trend from previous work has been undertaken.

2.3.2 Partitioning, chemical potential, and activity

Traditionally, partition coefficients (PCs) are the benchmark of successful uptake of a solute from an aqueous phase to any sequestering phase, including absorptive polymers, and is expressed as the ratio of solute concentration within the sequestering phase to the remaining aqueous concentration at equilibrium. The law of mass action states that chemical potential of solute *i* (μ_i) is equal in all phases in a system at equilibrium, and total chemical potential is expressed through Equation 2.1,

$$\mu = \mu_i^0 + RT \ln a_i \quad \textbf{(2.1)}$$

wherein μ^0_i represents chemical potential of a theoretical reference state of *i* exhibiting ideal behaviour, and a_i represents relative activity, which accounts for interaction between solutes and phase, accounting for non-ideal behaviour of a solute. Thus it can be seen that μ_i invariably depends on activity (a_i), and thus activity across phases is equal when the system is in equilibrium. Activity in turn is expressed as a product of the mole fraction of *i* (x_i) and the activity coefficient of *i* (γ_i), which expresses deviation from ideality, as shown in Equation 2.2 (Housecroft and Sharpe 2005).

$$\boldsymbol{a_i = \gamma_i x_i} \quad \textbf{(2.2)}$$

While activity coefficients vary with solute fraction, as systems approach infinite dilution (i.e. less than 0.1 M solute), this activity coefficient approaches a constant value. For the purposes of a given solute in a TPPB system, γ_i^{Aq} can be treated as a fixed property under dilute conditions, which is advantageous for polymer selection, but care must be taken if high titres are achieved. The amount of solute *i* can be expressed in several ways, but the implications of these relationships are that within a TPPB, to attain thermodynamic equilibrium

secondary phases presenting lower γ_i will demonstrate superior partitioning of solute *i*, and this effect will increase as the aqueous activity coefficient of *i* increases. As can be seen in Equation (2.3) a logical strategy for selection of polymers demonstrating elevated partition coefficients should focus on minimized polymer activity coefficients for a given solute *i.*

$$PC = \frac{\gamma_i^{aqueous}}{\gamma_i^{polymer}} = \frac{\Omega_i^{aqueous}}{\Omega_i^{polymer}} \quad \textbf{(2.3)}$$

2.3.3 Solubility parameters, uses and challenges.

While approaches minimizing activity coefficients in polymers for TPPBs has not previously been investigated, numerous studies have been performed for understanding and predicting solubility of solvents in polymers, which operate by the same thermodynamic principles. To predict miscibility of solvents in a given polymer, the commonly used Flory-Huggins model is often used to minimize a given solvent's activity coefficient in a polymer through use of the Flory-Huggins interaction parameter (χ_{12}), as shown Equation 2.4.

$$ln\gamma_{solute}^{polymer} = ln\frac{\Phi_1}{x_1} + \left(1 - \frac{1}{m}\right) + \chi_{12}\Phi_2^2 \quad \textbf{(2.4)}$$

As Φ and x represent volume and mole fractions respectively, and m represents the ratio of molar volumes of polymer and solvent, it can be seen that the activity coefficient is largely dependent on the interaction parameter. However, available interaction parameters for solvent-polymer combinations are limited, which restricts their use in a polymer selection strategy for TPPBs. Conveniently, this interaction parameter can be estimated by use of Hildebrand solubility parameters, as shown in Equation 2.5 (Blanks and Prauznitz 1964; Lindvig *et al*. 2002; Parent *et al*. 2012)

$$\chi_{12} = \chi_0 + \frac{v_1}{RT}(\delta_1 - \delta_2)^2 \quad \textbf{(2.5)}$$

Where v_1 represent molar volume and χ_0 is a constant value. Unlike interaction parameters which need to be determined for each combination, solubility parameters were determined to be the square root of a material's cohesive energy density, as calculated by its

enthalpy of vaporization divided by its molar volume, and is calculable independently of a specific combination. By selection of a solvent with similar solubility parameters to that of a polymer, interaction parameters can be minimized, resulting in lower activity coefficients. If the difference in solubility parameters between solvent and polymer is small enough, miscibility is achieved. Although such miscibility is not achieved in TPPBs, it can be argued that selection of polymers with solubility parameters closer to that of a target solute should theoretically provide more thermodynamically favorable conditions, resulting in higher uptake (Parent *et al*. 2012).

Among others, the most pressing flaws found with Hildebrand solubility parameters is that they are substantially less successful in predicting solubility of polymers in polar solvents and are largely only applicable to non-polar-solvents, as they do not take into account specific interactions such as hydrogen bonding, or polarity (Blanks and Prauznitz 1964). A generally accepted improved method of predicting miscibility in polar and non-polar solvents alike through the use of solubility parameters was outlined by Hansen, who divided the total or Hildebrand solubility parameter into three components, namely dispersive (D), polar (P) and hydrogen-bond donating forces, as outlined in Equation 2.6 (Hansen 2007).

$$\delta_{total}^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \qquad \textbf{(2.6)}$$

Just as total, or Hildebrand, solubility parameters predict solubility through the difference of respective solubility parameters, so too do Hansen solubility parameters (HSPs), wherein the sum of squares of the difference between respective solubility parameters is equal to the square of the total solubility parameter (Equation 2.7), which describes a spheroid zone of interaction.

$$R_a^2 = 4(\delta_{D1} - \delta_{D2})^2 + (\delta_{P1} - \delta_{P2})^2 + (\delta_{H1} - \delta_{H2})^2 \qquad \textbf{(2.7)}$$

The closer the respective HSPs then, the smaller this sphere, and the more likely that miscibility will be achieved. While this method goes further to state that miscibility will be

achieved if this sphere is smaller than a given value, this value is not clearly defined. Thus, considering the fact that miscibility is desirable for application in TPPB, the goal of achieving the smallest area (*vis* smallest difference in respective solubility parameters) would be acceptable as a criterion for selecting polymers demonstrating good solute affinity under this method. Another potential benefit of HSPs is the use of the respective parameters as a simplified language for describing the nature of a given solute. For instance, a molecule with a high hydrogen-bonding parameter or a polarity parameter would be likely to have a high affinity in water and demonstrate lower partitioning compared to a solute with no polar or hydrogen-bonding parameters. Generally speaking, there are two methods for determining HSPs for a given solute. The first method is by calculation through known chemical properties of a solute, with properties such as boiling point and dipole moment reflecting dispersion and polar components. However, this method produces a hydrogen-bonding parameter only as the difference between the other two parameters and that of a known total solubility parameter. The other method is by calculation through group contributions, which breaks molecules in functional groups, and calculates their relative contributions to a molecule's HSP components. Several methods have been developed with improving predictions with each new model, and software tools have been developed to calculate these contributions for a given molecule, as part of a larger software program named Hansen Solubility Parameters in Practice (HSPiP), which allows for relatively easy prediction of solvent-polymer solubility, and includes a large database of both experimentally determined and predicted data sets for a wide range of solvents. At the current time, this software presents the most accessible method for determination of a solvent that will achieve good solubility in polymers, and was a logical place to begin investigation into methods for rational selection of absorptive polymers.

However, application of this method to TPPB systems is difficult, if not impractical. Firstly, rather than selecting a good solute for a given polymer, a given solute needs a good

polymer. While a larger database exists for solvents, and good HSP predictions can be made for most solvents, polymer predictions are less reliable, as they do not account for crystallinity and T_g as discussed above. Furthermore, software such as HSPiP is designed to allow experimental determination of HSPs by exposure to a range of solvents to generate a sphere of interaction, and this method is not amenable to high throughput screening of a database of polymers for rational selection. Finally, and most importantly, these solubility parameter systems have been designed for binary systems, comprising the solvent and polymer only. In the case of TPPBs, a ternary system is represented instead, with a large aqueous phase that substantially dilutes a target solute. Hansen specifically states that water causes substantial interference when using solubility parameters, and is largely unpredictable. This correlates with results seen in our group, wherein use of HSPs to qualitatively predict partitioning resulted in inconsistent results, with poor trends. While such an approach was attractive due to the relative accessibility of pre-existing data, it has become clear to us that solubility parameters represent an oversimplification of a relatively complex ternary polymer-aqueous-solute system that would only yield qualitative results, and thus a different approach is needed.

2.3.4 Activity Coefficient and UNIFAC based models to predict partitioning

The underlying goal of Flory-Huggins and solubility parameters is to predict and minimize activity coefficients for solute *i*, thus permitting higher mass fraction with the same activity. As in TPPB systems the activity coefficient in water is fixed for a given solute *i*, direct prediction of activity coefficients for that solute would theoretically permit quantitative partitioning predictions by determining at which mass fractions solute activities would be equal in both phases. Thus, the polymer which yields the lower activity coefficient for a given solute should be the superior polymer for solute *i,* provided that physical criteria (i.e. crystallinity/T_g) are met. The universal functional activity coefficient (UNIFAC), first described by Fredenslund

et al. (1975), generates activity coefficients calculated from group contributions derived from a large dataset of binary vapor-liquid and liquid-liquid equilibria. Three years later, Oishi and Prausnitz (1978) published a model for calculation of activity coefficients of solvents in polymers, which adds a correction to the UNIFAC model to consider free volume, as polymer molecules are more tightly packed compare to solvents, and this can result in underestimation of activity coefficients.

Conveniently, freeware exists that will perform the necessary calculation of solvents in both aqueous (XLUNIFAC) and polymer (UNIFAC-vdW-FV) phases. By using both programs to calculate activity coefficients for a solvent in respective phases, an estimate for PC can be estimated as shown in Equation 2.3 and thus the above-mentioned activity coefficient models could potentially be used as tools for quantitative prediction of partitioning, which would be a substantial improvement towards rational selection of polymers.

2.3.5 Summary

While physical properties such as crystallinity and T_g provide basic criteria for rational selection of a polymer with good absorptive qualities for a given solute, prediction of polymer-solute affinity is substantially more complicated. A first-principles thermodynamic approach based on the law of mass action suggests that amorphous rubbery polymers with lower infinite dilution activity coefficients will demonstrate superior uptake. However, due to the ternary nature of TPPB systems, commonly used solubility parameters are not sufficient to describe these systems, and focus has been turned to the use of group-contribution based UNIFAC models for direct prediction of activity coefficients. In the case of highly hydrophilic solutes, which exhibit low activity coefficients, polymers demonstrating water uptake may present an approach to produce more favorable conditions for partitioning. However, regardless of polymer selection, no polymer-acid affinity will be observed unless the acid is present in its

undissociated state, and thus diminished extraction will be observed as *p*H values rise above the pK_a, which poses problems for ISPR.

2.4 Practical *p*H shifting for ISPR through use of CO_2

2.4.1 *p*H-dependent partitioning.

A critical consideration for uptake of organic acids is *p*H, as overwhelming evidence exists that only an undissociated acid demonstrates classical partitioning. With respect to organic acid uptake in a partitioning phase, the majority of research has focused on extraction into organic solvents, and it has been well demonstrated there is a clear relationship between *p*H and extraction with acid uptake in biphasic systems largely reliant on low *p*H values (Galaction *et al*. 2011; Kertes and King 1986; Yabannavar and Wang 1991). The most effective *p*H for extraction is that at which only the undissociated form is present (Kertes and King 1986; Nielsen *et al.* 2010; Nielsen *et al.* 1988; Vandak *et al*. 1997; Yang *et al*. 1991) and thus the dissociation constant (K_a), and the lowest pK_a are critical considerations in terms of organic acid uptake (Kertes and King 1986; Vandak *et al*. 1997). Vandak *et al.* (1997) have shown that the distribution coefficient increases with decreasing *p*H for butyric acid, with similar phenomena demonstrated for lactic (Thang and Novalin 2008), acetic, propionic, and butyric acid (Figure 2-1) (Yang *et al*. 1991), confirming that the degree of partitioning is based on the proportion of undissociated acid. A similar effect has been observed in polymeric resins (Thang and Novalin 2008), with lactic acid uptake dependent on *p*H, as well as in silica based sorbents (Jun *et al*. 2007), and current research in our research group has demonstrated that similar *p*H dependence exists for absorptive amorphous polymers as well (Hepburn and Daugulis, 2012).

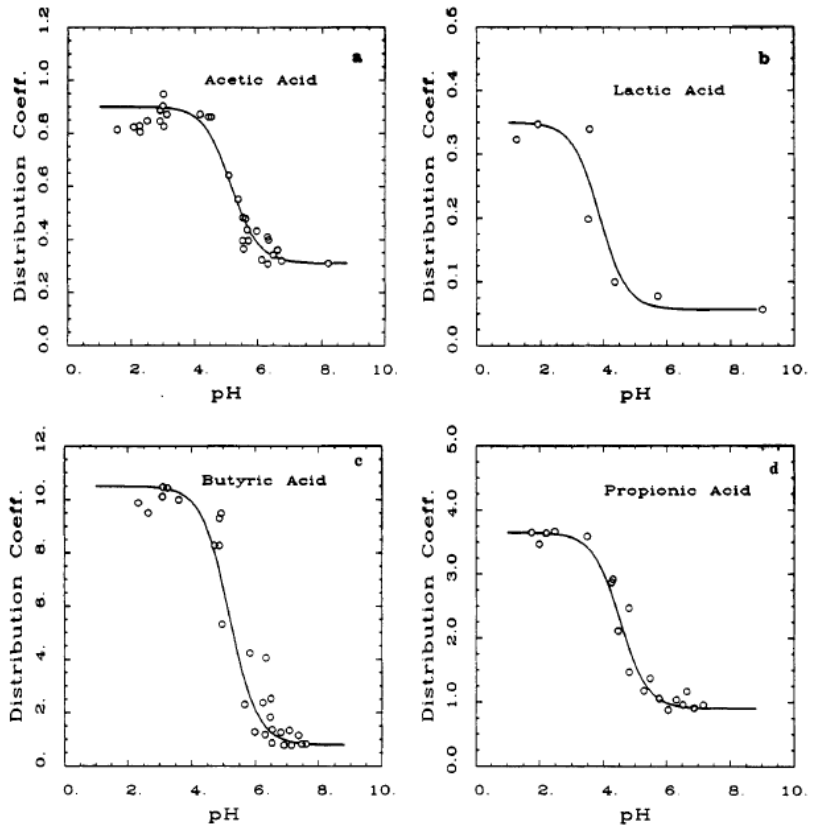

Figure 2-1 Effect of *p*H on distribution coefficients for carboxylic acid extractions from water with 50% quaternary amine salt Aliquat 336 in kerosene for (a) acetic acid (b) lactic acid (c) butyric acid (d) propionic acid (Reprinted (adapted) with permission from Yang *et al.* 1991. Copyright (1991) American Chemical Soceity)

This *p*H dependence can be a benefit however, as desorption can be facilitated by raising the *p*H. In fact, Yang *et al.* (1991) suggests using a *p*H swing to facilitate this desorption. Furthermore, if the pK_a values of two organic acids differ by more than one *p*H value, effective separation can be achieved through careful *p*H control (Kertes and King 1986; Schugerl 2005), showing another use of this *p*H dependence. Generally speaking, for organic acids there is a direct relationship between the non-dissociative state and uptake into a second phase, occurring independently of phase selection, and the dissociation constant and the pK_a are the critical points. The consequences of this are significant, as many fermentations proceed optimally at near neutral *p*H values, and thus production and extraction are likely to be mutually exclusive, and ISPR would not be achievable in the classical sense. If simultaneous production and extraction of organic acids is to occur, a method needs to be

developed that permits an acceptable swing between a *p*H optimal for microbial activity (near-neutral) and a *p*H where the undissociated form of a target organic acid can be present. Traditional *p*H changes in fermentation are achieved through addition of acids and bases, however this is undesirable, as large amounts would be repetitively required causing strain to the microbes from the accumulation of salts, bringing about osmotic stress and reducing performance (Fang *et al.* 2011). Additionally, reductions in use of acids and bases also represent reduced process costs. Thus, traditional methods of *p*H are not acceptable for effective fermentation and ISPR of organic acids in TPPB, and alternative methods need to be approached. The acidic nature of dissolved carbon dioxide presents an attractive option, as it leaves no salt. However, the extent of this *p*H swing is dependent on CO_2 solubility, which is a function of pressure, and thus high pressure studies are merited, and will be described subsequently.

2.4.2 CO_2 facilitated *p*H swings

It is well known that dissolution of CO_2 in H_2O induces a *p*H drop via carbonic acid formation, dissociating quickly to carbonate and bicarbonate (Bortoluzzi *et al*. 2011; Roosen *et al*. 2007), as shown in the following equilibria (Equations 2.8-2.10).

$$CO_{2aq} + H_2O \rightleftarrows H_2CO_3 \qquad K = 1.7\times10^{-3} \qquad (2.8)$$

$$H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad K_a = 4.5\times10^{-7} \qquad (2.9)$$

$$HCO_3^- \rightleftarrows CO_3^{-2} + H^+ \qquad K_a = 4.7\times10^{-11} \qquad (2.10)$$

The solubility of CO_2 is a critical factor in this process, and is positively influenced by pressure, and negatively by temperature (Dodds *et al*. 1956; Toews *et al*. 1995). In water, the *p*H is a function of the dissolved [CO_2], the dissociation constant K_i, and the ionic product of water K_w according to Equation 2.11 (Bortoluzzi *et al*. 2011).

$$pH = -\frac{1}{2}Log\left(K_i\left[CO_{2(aq)}\right]\right) + K_w \qquad (2.11)$$

Although CO_2 solubility rises with pressure, *p*H stabilizes relatively quickly as pressure increases, reaching *p*H 3.2 in water, and subsequent increases in pressure result in little

change in *p*H (Figure 2-2). We can see that in water, the majority of the *p*H change occurs before pressure reaches 1 MPa, or 10 bar. This is supported by the experimental data of Bortoluzzi *et al.* (2011), who showed similar *p*H stabilization over time at 5 bar CO_2 (Figure 2-3)

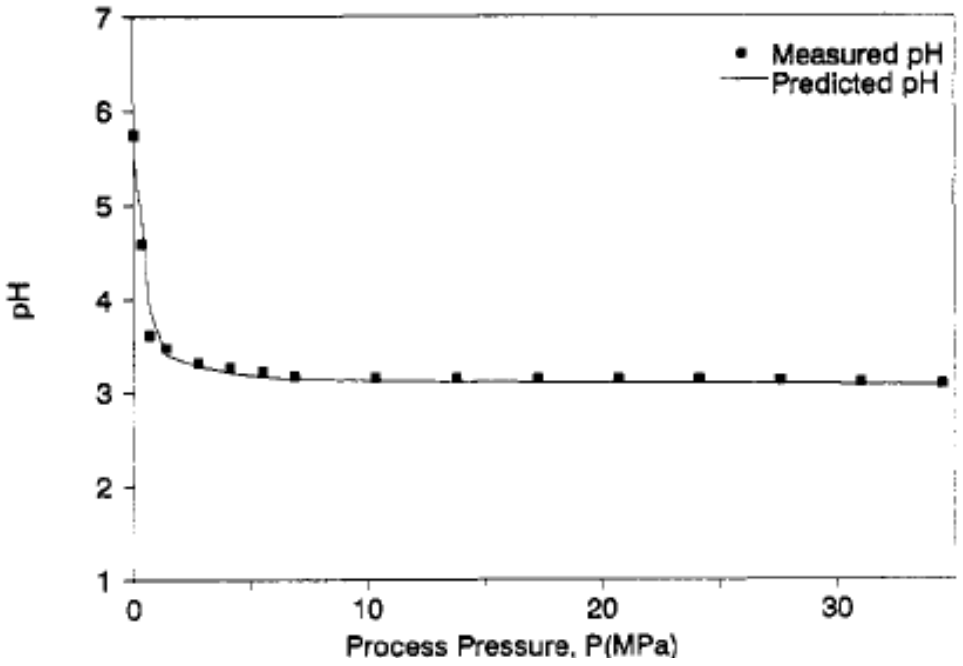

Figure 2-2 Measured and predicted *p*H of a pure water-CO_2 simulation system at pressures up to 34.48 MPa (Reprinted with permission from Meysammi *et al.* (1992))

This relationship between CO_2 and *p*H drop is less simple in dissolved solutions, as opposed to pure water. In a detailed study of *p*H prediction in CO_2 pressurized systems, Meysammi *et al.* (1992) found that pressure had a relatively weak effect on *p*H change, whereas *p*H was strongly dependent on system composition. Specifically, the presence of other acids had a strong effect on the degree of *p*H change, as shown in Figure 2-4.

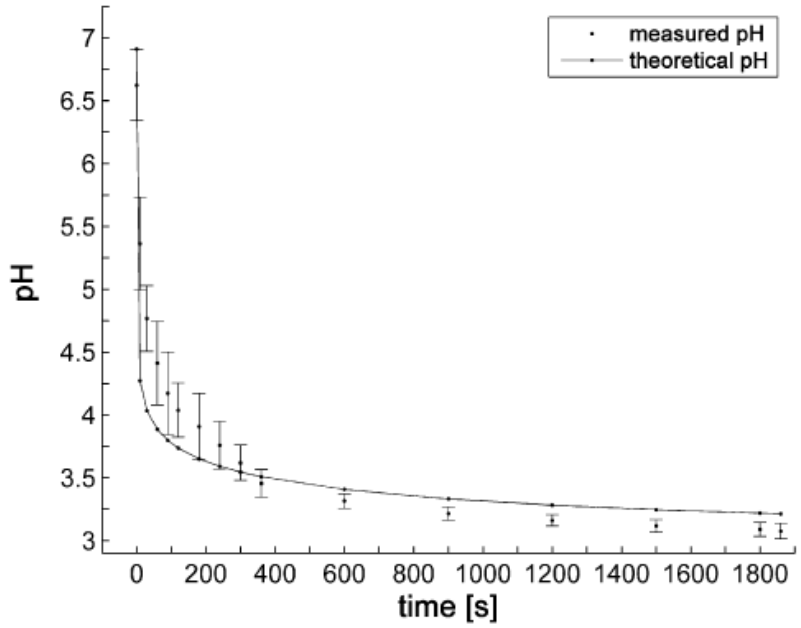

Figure 2-3 pH values in Ringer solution at 25 °C achieved as a function of time when exposed to 5 bar pCO_2 (Reprinted with permission from Bortoluzzi *et al.* (2011))

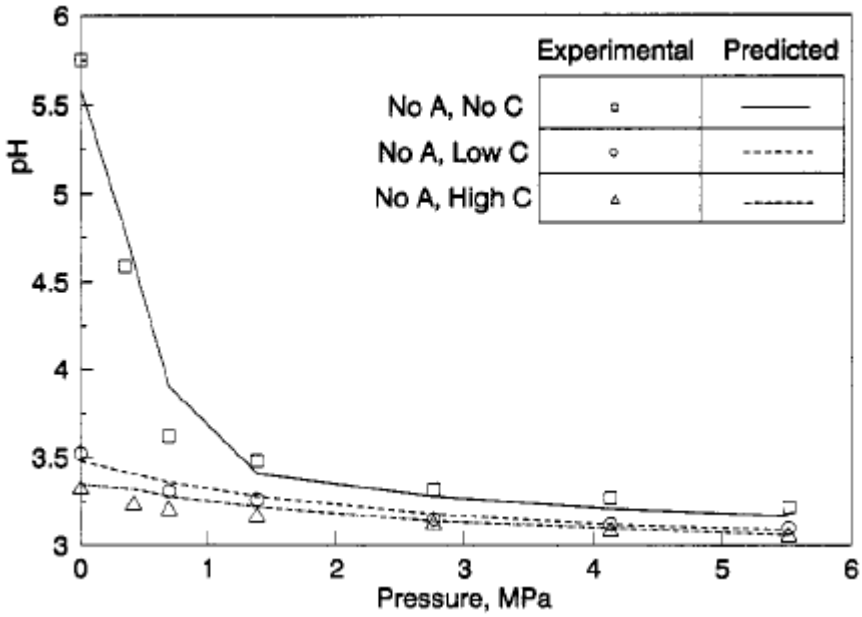

Figure 2-4 Measured and predicted *p*H of a citric acid-water-CO_2 system as a function of pCO_2. Concentrations: low C = 3.04×10^{-4} M citric acid; high C = 6.08×10^{-4} M citric acid. (Reprinted with permission from Meysammi *et al.* (1992))

Meysammi *et al.* (1992) showed that CO_2-*p*H drops are substantially less with orange juice, compared to water-citric/ascorbic acid systems, and they account for this by proposing the complex nature of orange juice provides buffering. Indeed, there is substantial evidence that the relationship between CO_2 concentration and *p*H depends less on the effect of

pressure and temperature, and more on the presence of buffers and other polar and ionic species (Bortoluzzi *et al*. 2011; Garcia-Gonzalez *et al*. 2010; Gill and Tan 1979; Jones and Greenfield 1982; Roosen *et al*. 2007). Buffers in particular can have a strong effect on final *p*H in pressurized systems (Ziegler *et al*. 2003), with increasing pressure having little effect on *p*H change at high concentrations (Holmes *et al*. 1999). For instance, while 150 mM phosphate buffers limited a *p*H drop to *p*H 4.75-5, higher buffer concentrations have been predicted to effectively prevent a change in *p*H from 7.5 ranging from 100 to 1000 bar (Jessop and Leitner 1999). Thus, special consideration must be taken on media composition to achieve acid *p*H values.

Roosen *et al.* (2007) studied the effect of bicarbonate concentrations on *p*H under pressure, and found that increasing concentrations of $NaHCO_3$ decreased final *p*H values, and increases in pressure could not overcome this restriction (Figures 2-5 and 2-6). As can be seen, even substantially low concentrations of $NaHCO_3$ (~0.5 g L^{-1}) impede the *p*H from dropping to beyond a value of 4 at high pressures (Roosen *et al*. 2007)

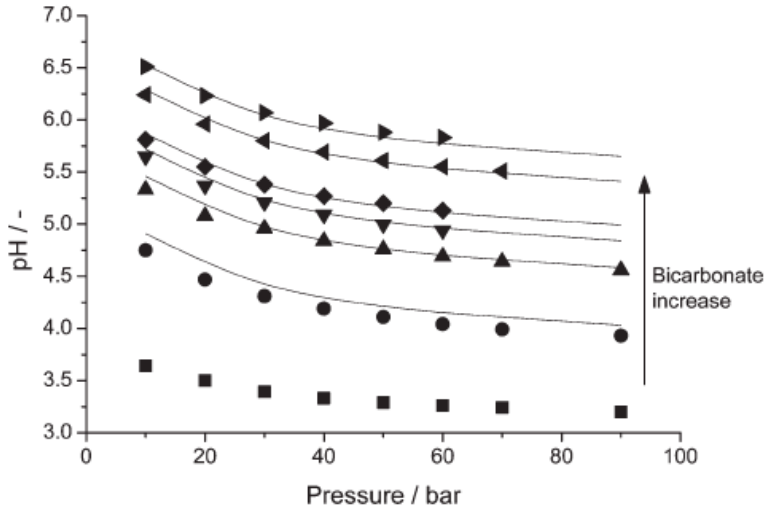

Figure 2-5 Determination of *p*H as a function of pCO_2 at 333 K. Dots are the measured values, the solid lines represent predicted values; (concentrations: 0, 0.005, 0.02, 0.04, 0.06, 0.2, 0.4 mol L^{-1} (Roosen *et al.* (2007)-reprinted with permission of the Royal Chemistry Society)

Bortoluzzi *et al.* (2011) found that at 60 bar CO_2, a system with 7 g L^{-1} of both monobasic and dibasic phosphates dropped to a *p*H of 4.5 vs. a *p*H approaching 3 in a non-buffered

media, and thus the effect of phosphate buffers on this *p*H is less severe, compared to Roosen *et al.* (2007), which is likely due to lessened effects on solubility, as phosphates do not tie directly into CO_2 equilibria, unlike $NaHCO_3$. Furthermore, complex medium components have been shown to restrict *p*H shifting at 13 bar CO_2 solely in the presence of yeast extract, peptone, and dextrose (YPD), and similar findings were found with brain heart infusion (BHI) media (Garcia-Gonzalez *et al*. 2010). In general, if a maximal *p*H drop is to be achieved by CO_2 pressurization in fermentation media and broth, a critical consideration is the system composition. Specifically, buffers and complex media components such as yeast extract show a strong effect on limiting *p*H change, and thus should be omitted or reduced as much as possible.

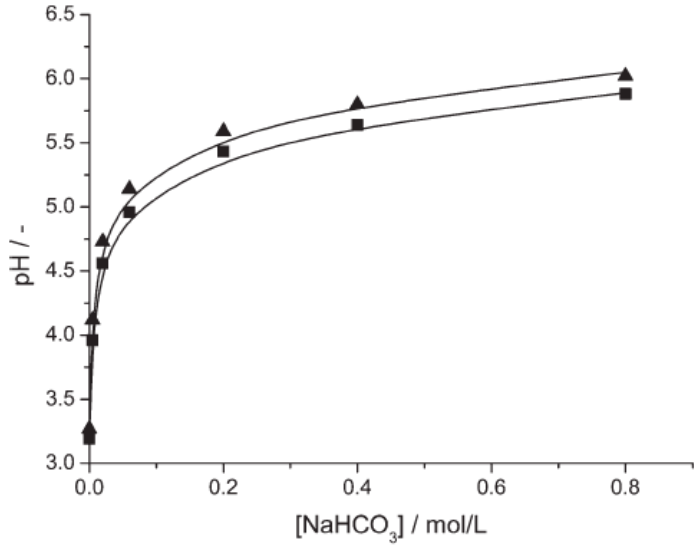

Figure 2-6 Determination of *p*H as a function of $NaHCO_3$ concentration at 295 K; points denote experimental values, the solid lines represents predicted *p*H (triangle 3.0 MPa, rectangle: 5.0 MPa) (Roosen *et al.* (2007)-reproduced with permission from the Royal Chemistry Society)

2.4.3 *p*H Colorimetry and fluorescence

Determination of *p*H in a pressurized CO_2 reactor is challenging, because *p*H is largely dependent on dissolved CO_2 concentration, which in turn is dependent on pressure, and any sampling for the purposes of traditional *p*H determination would invariably require depressurization, and thus the *p*H would not accurately reflect values in the reactor. While *p*H

as a function of CO_2 can be calculated in pure water using Henry's law, and pK_a values (Holmes *et al*. 1999), a more desirable approach is direct measurement. However, use of potentiometric *p*H probes in pressurized systems is limited, as specialized probes are required to withstand higher pressures. Current suppliers offer *p*H probes tolerating up to 6 MPa of pressure (Buchi, Corr Instruments), however these probes cost upwards of 4000$, and thus are prohibitively expensive for a fragile probe prone to breakage. Additionally, inclusion of *p*H probes in pressure studies necessitates custom-fitted pressure vessels (Roosen *et al*. 2007), which incurs further costs.

A simpler method has been well developed for pressurized CO_2 reactors by Toews *et al*. (1995) using a colorimetric method involving the *p*H indicator bromophenol blue, which was observed through a quartz cell window at various pressures and temperatures ranging from 70-200 atm and 25-70℃. The resultant colour change was detected using UV-Vis spectrometry, and compared to a six-point standard between *p*H 2.6-3.6, with the absorbance recorded for both the acidic and basic forms at respective wavelengths of 430 nm and 590 nm (Figure 2-7). The ratio of the absorbance (430 nm: 490 nm) was used to compensate for imprecision, resulting in good *p*H resolution.

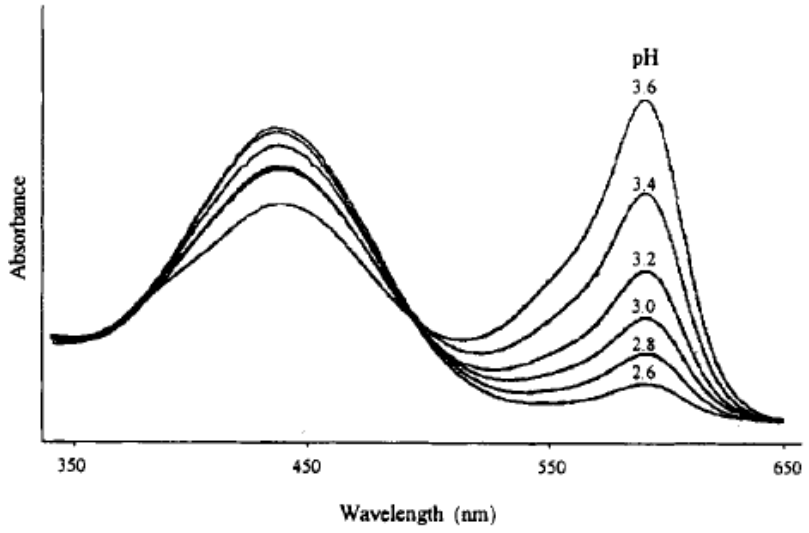

Figure 2-7 Absorption spectra of bromophenol blue (1.54 x 10^{-3} %) in citric acid buffers over the wavelength range 340-650 nm (Reprinted (adapted) with permission from Toews *et al.* (1995). Copyright (1995) American Chemical Society)

What is important to note is that bromophenol blue has a linear range of *p*H 3.0-4.6, and thus range of a *p*H indicator is an important design consideration in this method. This method has been used by other researchers, commonly using nitrophenylsulfonate (NPS) (Holmes *et al*. 1999; Ziegler *et al*. 2003), nitrophenyl, or bromophenol blue (Ziegler *et al*. 2003), bromocresol purple, and bromocresol green (Roosen *et al*. 2007) as *p*H indicators, each with different *p*H ranges.

Recently, novel methods for direct quantification of *p*H in larger volumes (300 mL) has been developed using fibre optics probes, which involved dual optic cables, either transmitting a halogen lamp or receiving reflected light, which is read by an optical spectrometer, which can be detected via either optical fibres to reactor view cells (Garcia-Gonzalez *et al*. 2010; Spilimbergo *et al*. 2010), or more directly through a probe directly inserted into the reactor itself (Bortoluzzi *et al.* 2011).

2.4.4 The effects of pressure and gas composition on microbial activity

A substantial concern regarding integration of high CO_2 pressures into fermentative processes is what effect this will have on microbial performance. Barophilic organisms are known to thrive in the marine benthic layer under high pressure (Prieur and Marteinsson 1998), and many studies have shown that elevated pressure can exert a benefit on aerobic processes due to increase pO_2 (Belo *et al*. 2003; Campelo and Belo 2004; Lopes *et al*. 2008; Lopes *et al*. 2009). Furthermore, industrial processes have been developed that capitalize on oxygen pressure under pressures up to 10 bar to improve process performance, such as the deep shaft process for wastewater treatment (Hait and Mazumder 2011). Thus in both nature and industry, microbes have been shown to withstand or adapt to elevated pressures, and can

even survive rapid depressurization without cell lysis (Isenschmid *et al*. 1995; Bertoloni *et al*. 2006; Spilimbergo *et al*. 2009).

Strong evidence suggests that rather than overall pressure, it is gas composition that exerts an influence on cell growth. Elevated oxygen concentrations only confer benefit to a certain point (pO_2 0.17-.21 MPa), after which further increases in pO_2 quickly became inhibitory (Belo *et al.* 2003; Campelo and Belo 2004), This relationship between pO_2 and inhibition was the same using both air and pure oxygen, demonstrating that composition, and not overall pressure was responsible for this phenomenon (Belo *et al.* 2003). These studies showed similar inhibitory behavior with pCO_2 (pCO_2 0.48 MPa) (Belo *et al.* 2003; Campelo and Belo 2004), which suggests differences in toxicity between gases. Thibault *et al.* (1982) investigated the effect of substantially higher pressures (up to 7 MPa) of CO_2, N_2, and air on ethanol production and growth in *Saccharomyces cerevisiae*. At the same pressures (7.0 MPa), a substantially higher inhibition on ethanol production was observed with pure CO_2 (25% of 1 atm air), compared to pure N_2 (60% of 1 atm air), demonstrating that the gaseous composition has a more significant effect on metabolism than absolute pressure, with CO_2 strongly inhibiting end-product formation (Thibault *et al*. 1987; Vezzù *et al*. 2009).

The effect of partial pressure of CO_2 (pCO_2) on microbes generally has been well documented, and was thoroughly reviewed by Jones and Greenfield (1982), who show that carbon dioxide-mediated growth inhibition is fundamental to all organisms, although the inhibitory values changes with species (Jones and Greenfield 1982). In fact, numerous studies have employed high pressure CO_2 as a non-thermal sterilization technique, achieving good results at elevated pCO_2 levels (Bertoloni *et al.* 2006; Bortoluzzi *et al*. 2011; Debs-Louka *et al*. 1999; Spilimbergo *et al*. 2002). Substantial research into the mechanism of cell inactivation has led to the conclusion that high pCO_2 solubilizes cell membranes, disrupts cell function, and lowers intracellular *p*H (Bertoloni *et al.* 2006; Jones and Greenfield 1982; Spilimbergo *et*

al. 2009). However, while it is generally acknowledged that elevated CO_2 has a negative effect on microbial activity, interspecies variation exists (Jones and Greenfield 1982) which can be seen across the above mentioned studies, and this variation may arise due to different exposure conditions, as well as from adaptation to high CO_2 environments such as those fermentative or capnophilic organisms would be exposed to. Thus, for both given exposure conditions and specific organisms, the effect of elevated pCO_2 needs to be determined experimentally. It is especially interesting to note that L'italien *et al.* (1988) demonstrated that *S. cerevisiae* was capable of repeatedly tolerating 7 MPa for one hour, with an immediate return of ethanol production upon depressurization to 1 atm, and thus online cyclical pressurization with elevated pCO_2 is feasible in some cases.

2.4.5 Summary

As can be seen, substantial work has been done to show the extent to which high pressure CO_2 can change *p*H, highlighting the strong negative effect buffering exerts on *p*H swings, and colorimetric methods for *p*H determination have been developed, as *p*H probes are not suitable under these conditions. However, while research indicates that high pressure CO_2 can improve *p*H swings compared to atmospheric sparging, significant evidence exists that prolonged exposure to such conditions could exert a negative effect on cell membranes, and thus careful consideration of cell exposure to high CO_2 is important.

2.5 Advanced *p*H control by acid removal

Aside from the benefits mentioned above, if CO_2-mediated ISPR demonstrating good acid removal could be achieved, it is highly likely that such a system would need reduced, if any, *p*H control via base-addition. As end-products would be removed during extraction, the recirculated broth would be returned with a less acidic, if not neutral *p*H for further fermentation. The benefits of reduced base addition are obvious, for as discussed above, this results in salt accumulation leading to reduced productivity, while also incurring higher material

cost. An autonomous *p*H control system through ISPR has been demonstrated by Ataei and Vasheghani-Farahani (2008), who controlled *p*H during lactate fermentation using recirculation through an ion-exchange column, increasing productivity 500%, due to product removal and the obviation of *p*H control via base addition (Ataei and Vasheghani-Farahani 2008). Liquid-liquid extraction of an organic acid has also shown that *p*H control can be facilitated through acid removal, with no external *p*H control required, although it was not sufficient to maintain optimal *p*H (Wu and Yang 2003). Obviously this approach would be different if using absorptive polymers rather than ion-exchange resins or organic solvents, and would depend on the extractive capacity of a polymer to achieve acceptable results. Such an approach, though technically challenging, deserves consideration, as it demonstrates obvious advantages, and could contribute to a fundamental change in the bioproduction of organic acids.

2.6 Previous work for extraction of organic acids in TPPBs

Previous work in our research group has made good advances towards actualizing a process wherein succinic acid can be efficiently fermented and extracted using a polymer as a secondary partitioning phase via CO_2-mediated *p*H swings (Hepburn and Daugulis, 2012). Several factors need consideration, and the major contributions of previous work will be outlined below. Initial tests confirmed that 1 atmosphere pCO_2 can lower the *p*H of RO water sufficiently to reach a target *p*H of <4, which is to be expected from the literature and our understanding of the CO_2-HCO_3^-equilibrium. However, tests showed that medium components limited *p*H swings to unacceptable *p*H values (Figure 2-8).

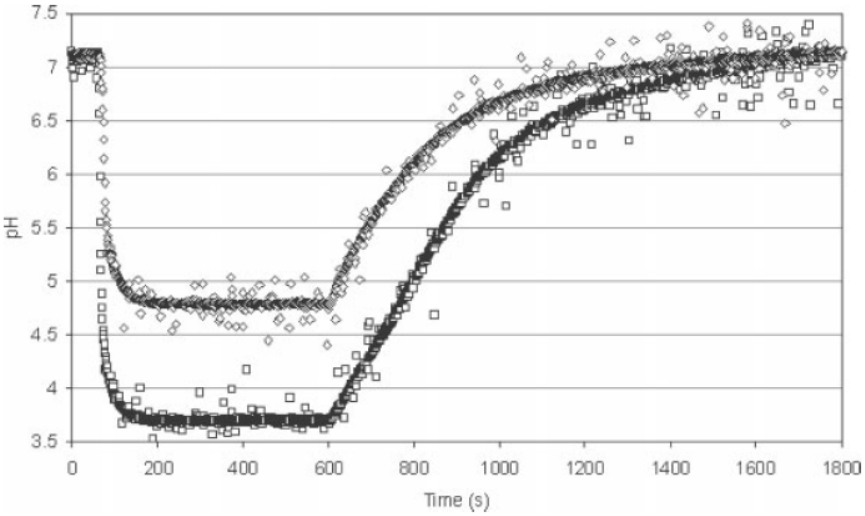

Figure 2-8. *p*H adjustment of RO water (open squares) and minimal medium (open diamonds) using carbon dioxide sparging to first reduce *p*H (100-200 s), after which nitrogen gas sparging was used to return *p*H to neutral values (600-1800 s) at atmospheric pressure, 20 °C and 500 rpm (Taken from Hepburn (2012))

Medium components were tested individually, and it was shown that both phosphate and yeast extract were responsible for this *p*H limitation (Figure 2-9). A second medium was developed with reduced component concentrations, and while good growth was still achieved, target *p*H values were not. Thus, a medium capable of achieving a desirable *p*H while allowing growth needs to be developed, and tests need to be conducted at higher pressures to determine if target *p*H can be achieved with this medium.

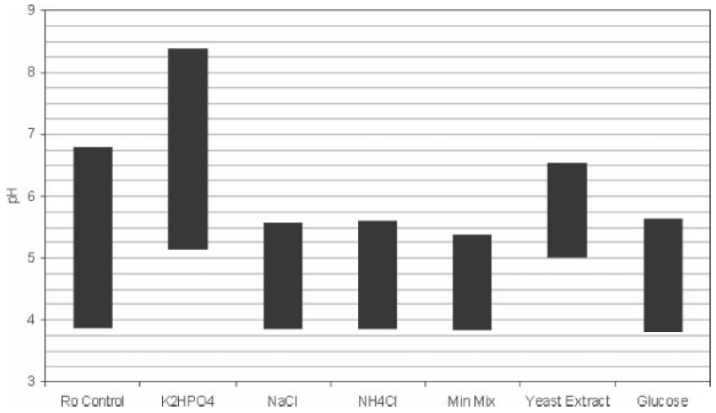

Figure 2-9. Changes in pH resulting from CO_2 sparging for individual medium components at original growth medium concentrations (Taken from Hepburn (2012))

Biologically, It was determined that cells could tolerate a sustained *p*H shock from H_2SO_4 at a *p*H of 4.2 for up to four hours, with a return of growth as measured by OD (Hepburn and Daugulis, 2012). However, exposure for over 15 minutes resulted in a substantially increased lag period of twelve hours, which is less than desirable with respective to volumetric productivities. Attempts to revive a cell population in a bioreactor after a H_2SO_4-KOH *p*H shift were unsuccessful, although succinic acid removal was achieved via uptake into Hytrel 8206. It is likely the inability to revive the bioreactor cell population was due from either cell lysis from H_2SO_4 addition or the accumulation of salts from the large additions of acid and base necessary to achieve *p*H shifts with the high concentrations of neutralized acid typically produced during fermentation, highlighting the importance of CO_2-mediated *p*H swings, which leave no salts.

In general, it has been shown that the components necessary for actualization of a CO_2-mediated ISPR process for organic acids demonstrate positive results, showing that a microbe can tolerate *p*H shocks, CO_2 can mediate *p*H swings, polymers exists with affinities for organic acids, and uptake is *p*H dependent. What now needs to be achieved is the integration of these various components, while improving *p*H swing effectiveness with pressurized CO_2 and polymer-solute affinity, but not at the expense of fermentative productivity.

2.7 References

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Chapter 3

Demonstration of *in situ* product recovery of butyric acid via CO_2-facilitated *p*H swings and medium development in two-phase partitioning bioreactors

With minor changes to fulfill formatting requirements, this chapter is substantially as it appears in: *Biotechnology and Bioengineering* **111 (**3): 537-544 (2014)

3.1 Preface to Chapter 3

An examination of the effectiveness of CO_2 *p*H reductions for achieving acid absorption under atmospheric pressure in fermentations is a logical place to start, if CO_2 is to be used to facilitate ISPR with absorptive polymers. Butyric acid was selected as a model organic acid for the study of partitioning achieved through *p*H reductions resulting from CO_2 use, as biological production of this acid is well investigated and ISPR has been demonstrated using reactive extraction (Wu and Yang, 2003). Furthermore, additional reactive extraction studies have demonstrated improved distribution coefficients for butyric acid compared to other common organic acids (Yang *et al.* 1991), and a similar effect may be observed with amorphous polymers, which operate passively through absorption. Through careful analysis of typical growth media for *Clostridium tyrobutyricum*, which anaerobically produces butyric acid (Wu and Yang, 2003), adaptations were made to minimize buffering capacity from medium components and maximize potential *p*H reductions. Concurrently, growth studies were conducted to ensure said modifications do not reduce cell growth or butyric acid production.

This chapter also puts forth initial investigations into rationally selecting absorptive polymers through use of solubility parameters, which led subsequently to a wide survey of polymers through prediction of thermodynamic affinity, which is extensively described in Appendix A. Using this minimized medium and a polymer demonstrating affinity for butyric acid (Hytrel® 3078 PC=3.0), fermentations were performed and subjected to intermittent CO_2 sparging to facilitate temporary *p*H reductions and achieve partitioning through acid uptake in an absorptive polymer.

It must be noted that after *p*H reduction and acid absorption, the partitioning phase needs physical separation from the reactor contents before *p*H is returned to near-neutral values, as otherwise desorption of butyric acid into the aqueous phase would occur. To prevent this desorption, fermentation broth was recycled through a polymer-packed column

during CO_2 induced *p*H reductions to provide easy separation of phases between temporary *p*H reductions. Overall, this chapter demonstrates that CO_2 sparging improves acid uptake in absorptive polymers, but also indicates that higher CO_2 solubilities afforded by elevated pressures could improve organic acid partitioning.

3.2 Abstract

Production of organic acids in solid-liquid two-phase partitioning bioreactors (TPPBs) is challenging, and highly *p*H-dependent, as cell growth occurs near neutral *p*H, while acid sorption occurs only at low *p*H conditions. CO_2 sparging was used to achieve acidic *p*H swings, facilitating undissociated organic acid uptake without generating osmotic stress inherent in traditional acid/base *p*H control. A modified cultivation medium was formulated to permit greater *p*H reduction by CO_2 sparging (*p*H 4.8) compared to typical media (*p*H 5.3), while still possessing adequate nutrients for extensive cell growth. *In situ* product recovery (ISPR) of butyric acid (pK_a = 4.8) produced by *Clostridium tyrobutyricum* was achieved through intermittent CO_2 sparging while recycling reactor contents through a column packed with absorptive polymer Hytrel® 3078. This polymer was selected on the basis of its composition as a polyether copolymer, and the use of solubility parameters for predicting solute polymer affinity, and was found to have a partition coefficient for butyric acid of 3. Total polymeric extraction of 3.2 g butyric acid (7.8% of total butyric acid produced) with no CO_2 mediated *p*H swings was increased to 4.5 g (10.7% of total butyric acid produced) via CO_2-facilitated *p*H shifting, despite the buffering capacity of butyric acid, which resists *p*H shifting. This work shows that CO_2-mediated *p*H swings have an observable positive effect on organic acid extraction, with improvements well over 150% under optimal conditions in early stage fermentation compared to CO_2-free controls, and this technique can be applied to other organic acid fermentations to achieve or improve ISPR.

Keywords: *Clostridium tyrobutyricum*, butyric acid, two-phase partitioning bioreactor (TPPB), *in situ* product recovery (ISPR), carbon dioxide

3.3 Introduction

Interest in biologically produced feedstocks as an alternative to petrochemicals has spurred substantial research into products of microbial fermentation, with the hope of improving process efficiency and reducing production costs, which would aid in overcoming the economic challenges required to attain industrial feasibility. In the case of organic acids, one of the most prominent costs incurred is product recovery, which can represent up to 60% of process costs, and typically employs techniques such as precipitation, ion-exchange, and reactive extraction (Kurzrock and Weuster-Botz 2010). However, these approaches cannot easily be performed during fermentation, and largely represent downstream separation processes only. Two-phase partitioning bioreactors (TPPBs) represent another technique wherein an immiscible phase is included in the fermentation vessel, effectively partitioning target molecules away from fermentation broth, achieving *in situ* product recovery (ISPR) (Daugulis *et al.* 2011). Recently, research has focused on the use of absorptive commodity polymers as the sequestering phase in TPPBs as an alternative to employing organic solvents (Amsden *et al.* 2003; Morrish and Daugulis 2008; Daugulis *et al*. 2011), as effective commodity polymers have been shown to be relatively more affordable (e.g. 5$ kg^{-1}) compared to absorptive resins (e.g. 170$ kg^{-1}) (Nielsen and Prather 2009) and easier to handle compare to other liquid extractants, which are substantially more expensive (e.g. >150$ kg^{-1}) (Quijano *et al.* 2010). To date polymer selection for use in TPPBs has been largely heuristic, which can restrict application of TPPBs to production of new molecules, as no straightforward method for predicting polymer-solute affinity has been available. Thus, development of a rational polymer selection strategy for use in TPPBs based on first principles thermodynamics is underway (Parent *et al.* 2012), and use of accessible polymer properties such as Hildebrand solubility parameters may provide methods of ranking polymers for solute affinity.

Regardless of phase selection, an important process concern in the extraction of organic acids is *p*H. It is well accepted that organic acids will partition into sequestering phases only as the undissociated species (Kertes and King 1986; Yang *et al.* 1991; Garcia 1999). As optimal uptake occurs below the lowest pK_a of an organic acid, extractive and bioproductive operations are therefore often exclusive due to differences in optimal *p*H ranges. This exclusion implies that product recovery of organic acids cannot be achieved in a manner similar to target molecules that partition independently of *p*H, wherein a second phase is simply added directly to a reactor. Online extraction of dissociable species thus requires both cyclic *p*H changes to alternate between bioproduction and extraction, and physical separation to ensure extraction efficiency.

An important operational consideration is how reversible *p*H swings can be achieved, especially if online extraction for ISPR is implemented as an alternative to downstream processing. Traditional acid/base addition for *p*H control results in elevated ion concentrations, increasing osmotic stress and hindering conventional batch fermentation (Liu *et al*. 2008), and this effect is exacerbated if this type of *p*H control is used to facilitate the necessary *p*H swings for extraction. An alternative is the use of carbon dioxide sparging, reducing the *p*H via carbonic acid dissociation. While it is well known that CO_2 lowers *p*H by formation and dissociation of carbonic acid, the use of CO_2 to lower *p*H for facilitating absorption represents a novel application of this well-known phenomenon. Previous work (Hepburn and Daugulis 2012) has shown that alternate sparging between CO_2 and N_2 permits *p*H shifting between 7 and 3.5 with no ion accumulation in reverse osmosis (RO) water. In the case of media however, the buffering capacity of medium components limited *p*H swings, and careful consideration of medium composition is required. This work focuses on the extraction of butyric acid produced by *Clostridium tyrobutyricum*, and the three fold objective of this study is to:

a) Test suitability of Hildebrand solubility parameters for prediction of polymer affinity for an organic acid,

b) Reformulate a medium for *C. tyrobutyricum* that minimizes buffering capacity, while not adversely affecting growth or butyric acid production, and

c) Investigate the use of intermittent CO_2-mediated *p*H swings to facilitate organic acid ISPR.

This approach would theoretically apply to a range of organic acids and extractive techniques, and could potentially be used for widespread application in organic acid fermentation, particularly through the use of elevated CO_2 pressures.

3.4 Materials and Methods

3.4.1 Organism, polymers, and chemicals

Clostridium tyrobutyricum (ATCC 25755) was initially grown on media described elsewhere (Wu and Yang 2003) and cryopreserved in 15% glycerol at -75 °C until needed. All polymers were kindly donated by Arkema and DuPont, and are listed in Table 3-I. All polymers were washed with agitation three times, first with hot tap water on a stir plate, twice with RO water, and allowed to air dry overnight to remove processing contaminants. All chemicals used in this study were purchased from Fisher Scientific Company, Ltd (Ottawa, ON).

Table 3-I Polymer properties for Pebax® and Hytrel® (PBT= Polybutylene terephthalate, PBO = Polybutylene oxide, PEO = Polyethylene oxide)

Polymer grade	Pebax® 2533[1]	Pebax® 1074[1]	Pebax® 1657[1]	Hytrel® 3078[2]
Hard segment	Nylon 12	Nylon 12	Nylon 6	PBT
% Hard segment	20	45	40	n/a.
Soft segment	PBO	PEO	PEO	PBO
% Soft segment	80	55	60	n/a
Soft segment T_g (°C)	-77	-55	-55	-77
% Water absorption	1.2	50	120	0.8

1=Yampolskii and Freeman (2010) and respective Arkema Inc. datasheets, 2=Dupont, personal correspondence and datasheet

3.4.2 Partition coefficient determination

Partition coefficients (PC) for butyric acid were determined using methods outlined elsewhere (Dafoe and Daugulis 2011), with the additional step of weighing polymers after partitioning tests to determine water absorption to allow for correction of aqueous volume at equilibrium. Hildebrand solubility parameters of the soft segments of all polymers (Brandrup *et al.* 1999) were used as a measure to compare predicted polymer affinity for butyric acid to observed partitioning.

3.4.3 Culture conditions and medium formulation

C. tyrobutyricum was cultured under anaerobic conditions in sealed 150 mL serum bottles, or in sealed 500 mL shake flasks with closable vents and a spargeline, which were sparged aseptically with N_2 for 20 minutes post and prior to autoclaving. All bottles, flasks, and reactors described herein were autoclaved for at least 20 minutes at 100 kPa gauge pressure and 121℃.

A medium formulation with minimized buffering capacity was developed capable of enhanced CO_2-*p*H swings. Medium A represents a typical medium found in the literature (Wu and Yang 2003), which consists of yeast extract, 5 g L^{-1}; $(NH_4)_2SO_4$, 3 g L^{-1}; K_2HPO_4, 1.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.6 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 0.03 g L^{-1}. Peptone was omitted from medium A to simplify the effect of a single complex medium component on *p*H swing. Medium B represents a modified formulation for improved CO_2-*p*H swing capacity wherein the above formulation remains unchanged except for a reduction of K_2HPO_4 to 0.3 g L^{-1}. All bottles and flasks contained 10 g L^{-1} glucose, and were incubated at 37℃ and 180 rpm. Growth and *p*H shifting studies were performed in serum bottles and in bioreactors, respectively, with decreasing K_2HPO_4 and yeast extract concentrations.

3.4.4 CO_2-N_2 sparging for *p*H shifting in media, with or without butyric acid

To determine CO_2-*p*H swing capacity for media, CO_2 sparging tests were performed in 5 L Bioflo III reactors (New Brunswick Scientific, Edison, NJ), with sparging performed at 1 VVM (Volume gas•Working Volume^{-1}•min^{-1}), 500 rpm, and 37 ℃ with no *p*H control. As optimal growth of *C. tyrobutyricum* occurs at *p*H of 6.0 (Wu and Yang 2003) this value was used as the initial *p*H in all sparging tests. CO_2 was sparged for 5 minutes, followed by 15 minutes of sparging with N_2 to return the *p*H to its starting level. To determine the effect of butyric acid on *p*H swings, sparging tests were also performed on medium B with either 5 or 10 g L^{-1} butyric acid and compared to medium B sparge tests in RO water as a control.

3.4.5 Batch reactor culture conditions

C. tyrobutyricum was grown in batch on medium B in 5 L BioFlo III reactors with a 2 L working volume under anaerobic conditions at 37 °C, 200 rpm agitation and 0.25 VVM N_2 sparging, while *p*H was controlled to 6.0 by addition of 3 M KOH and H_2SO_4. Inoculum was generated on medium B over 12 hours first in serum bottles, and then flasks as described above were added anaerobically to reactors (10% v/v). All batch reactors were grown on 60 g L^{-1} glucose.

3.4.6 CO_2-*p*H swing mediated online extraction of butyric acid

To demonstrate that *p*H dependent extraction of an organic acid can be facilitated by CO_2 during fermentation without the use of strong acid addition, batch reactors were prepared with modified medium and inoculated as described above. Once automatic *p*H control was initiated and 50mL 3M KOH were added, one reactor was sparged with 1VVM CO_2 at 500 rpm, with the resultant *p*H decrease recorded using TracerDAQ data acquisition software (MicroDAQ.com, Ltd, Contoocook, NH), while the second reactor was left as a CO_2-free control. After sparging, the contents of both reactors were then cycled anaerobically at 3 L h^{-1} for one hour through a 1 L glass column packed with previously unused 700 g of Hytrel 3078,

corresponding to a polymer fraction of 35% (w/v) within the total system. The packed column was autoclaved and sparged with N_2 to drive of excess oxygen prior to extraction. CO_2-mediated *p*H swing extractions were performed four times over the course of the fermentation, separated by three-hour intervals. To characterize extraction, fresh polymers were employed for each extraction event, and triplicate overnight desorptions in 2 L of 0.1 N KOH were performed on polymer masses post extraction to determine butyric acid absorbed at each point over the course of the fermentation. To determine what effect both extractive runs exerted on microbial activity, a batch reactor without extraction was run as a control.

3.4.7 Analytical methods

Aqueous samples were analyzed using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 × 7.7 mm) at 60 °C with a 10 mmol L^{-1} H_2SO_4 mobile phase at 0.7 mL min^{-1}, and a UV-Vis detector (Varian Prostar, PS325) at 220 nm. Cell concentration was measured using optical density at 600 nm. Glucose was measured using the dinitrosalicylic (DNS) assay (Miller 1959) at 540 nm.

3.5 Results and Discussion

3.5.1 Polymer selection and partition coefficient calculation

If polymers are to demonstrate good absorption of any target molecule, important polymer properties to consider are glass transition temperature (T_g), crystallinity, and polymer-solute affinity. Recent work has identified T_g and crystallinity as key initial determinants for selection of absorptive properties, citing the need for chain mobility and intermolecular free space to permit diffusion of the target molecule into a polymer matrix (Parent *et al*. 2012). While such physical polymer properties are relatively straightforward as criteria for polymer selection, determination of polymer-solute affinity is more challenging.

In terms of solute affinity, previous work has highlighted good partitioning of hydrophilic target molecules into polyether copolymers such as Pebax® (Arkema) and Hytrel® (DuPont),

both polyether block copolymers with polyamides and polybutylene terephthalate (PBT) as respective hard segments (Prpich and Daugulis 2004; Gao and Daugulis 2010; Dafoe and Daugulis 2011; Hepburn and Daugulis 2012). In this study, three grades of Pebax® (grades 2533, 1074, & 1657) were tested based on differences in their hard and soft segments, as well Hytrel® 3078. All selected hard segments (nylon-6, nylon 12, and PBT) are semicrystalline with glass transition temperatures higher than room temperature, and thus it is likely that any absorption will be achieved only by the polyether soft segment, whether polyethylene oxide (PEO) or polybutylene oxide (PBO). To try to explain this affinity, use of Hildebrand solubility parameters (Hildebrand and Scott 1962) has been suggested as a potential tool (Parent *et al*. 2012). Solubility parameters are commonly used terms widely available for most polymers and solvents, and can be used to predict binary polymer-solvent interactions, wherein the absolute difference between the parameters for two given materials can predict solubilization, and the minimization of this difference results in improved solubility. By comparing the difference between solubility parameters of respective soft segments and that of butyric acid, it may be possible to explain differences in partitioning across polymer grades.

Table 3-II Polymer soft segment solubility parameters (SS δ, $MPa^{1/2}$), respective difference to the solubility parameter of butyric acid (20.3 $MPa^{1/2}$, Brandrup *et al.* 1999) and partition coefficients (PC) of butyric acid, with additional reported PC values corrected for polymer water uptake (H_2O), soft segment fraction (%SS), or both (H_2O+%SS)

Polymer	SS δ	PC	PC_{H2O}	$PC_{\%SS}$	$PC_{H2O+\%SS}$
Pebax® 1074	18.5	3.5	4.0	6.4	7.3
Pebax® 1657	18.5	2.3	3.5	3.8	5.8
Pebax® 2533	18.1	4.1	4.1	5.1	5.1
Hytrel ® 3078	18.1	3	3	n/a	n/a

Table 3-II shows that the soft segment solubility parameter for PEO-bearing polymers Pebax® 1074 and 1657 (18.5 $MPa^{1/2}$) is closer to that of butyric acid (20.3 $MPa^{1/2}$), compared to the soft segment solubility parameter of PBO-bearing Pebax® 2533 and Hytrel® 3078 (18.1 $MPa^{1/2}$), and thus it would be expected that PEO-bearing grades would yield better partitioning. However, Pebax® 2533 and Hytrel® 3078 yield partition coefficients of 4.1 and 3

respectively, compared to that of PEO-bearing Pebax® 1074 (PC=3.5) and 1657 (PC=2.3), suggesting limitations in the use of solubility parameters, if conventional approaches to calculating partitioning are employed. In general partitioning is calculated through observation of aqueous concentrations after 24 hour exposure to the polymers, with mass balance calculations used to determine solute concentration in the polymer phase. However inaccuracies can exist, if mass balances assume only uptake of the solute, while ignoring other parameters such as water transport. Specifically, PEO-bearing grades have high moisture absorption (Table 3-I), and this water absorption must be taken into account when calculating partition coefficients, as the decrease in volume skews aqueous solute concentration to appear higher, resulting in underestimation of partitioning. An accurate aqueous phase volume is necessary to calculate mass balance, and is easily determined by observing changes in polymer mass. If volume decreases in the aqueous phase are accounted for, partition coefficients for water absorbing Pebax® 1074 and 1657 are increased to 4.0 and 3.5, respectively. Thus, if corrections are made to reflect water uptake, similar partitioning is observed between PBO-bearing 2533 and PEO bearing 1074, which is still not entirely consistent with solubility parameter predictions.

Another important consideration is polymer soft segment fraction, if it is assumed that only the soft segment plays a role in partitioning. The PBO fraction of Pebax® 2533 is substantially higher than that of PEO grades (Table 3-I), and thus presents more absorptive mass. If partitioning is normalized to reflect only absorptive mass, PEO-bearing Pebax® 1074 and 1657 show mixed improvements in uptake (PC=6.4, PC=3.8, respectively) to that of PBO-bearing Pebax® 2533 (PC=5.1), and thus does not support solubility parameter predictions. If correction for water uptake and soft segment fraction are combined, however, Pebax® 1074 and 1657 surpass 2533, yielding partition coefficients of 7.3 and 5.8, which fits with solubility parameter predictions. However, although both PEO grades contain similar soft segment

proportions, partitioning is significantly different, which brings into question the assumption that solely soft segment is responsible for uptake. A final consideration may be a second effect of water, which can exhibit a plasticizing effect on polymers, and it is possible that this permits uptake in the hard segments as well. Overall, through the use of solubility parameters to predict affinity, while also correcting for water uptake and soft segment amount, qualitative uptake prediction was achieved. The polymers selected using these approaches have shown a marked improvement in partitioning compared to previous studies with organic acids (Hepburn and Daugulis 2012), and further study of these strategies is merited. In the case of these experiments, as the low melting temperature of Pebax® 2533 (130 ℃, Arkema datasheet) could prove problematic during steam sterilization, Hytrel® 3078 was ultimately used for the ISPR studies as it shares the same soft segment (PBO), while possessing a higher melt temperature (177 ℃, DuPont datasheet) making it suitable for autoclaving and thus was used for all subsequent experiments.

3.5.2 Development of a *p*H-shiftable medium

To maximize the ability of CO_2 to decrease *p*H, minimization of buffering medium component concentrations is important; however any reduction in component concentrations cannot negatively affect cell growth and acid production. Previous studies in our group determined that yeast extract and dibasic phosphate were responsible for the majority of the buffering capacity observed in media used for organic acid production (Hepburn and Daugulis 2012). As shown in Figure 3-1, medium studies investigating the effect of yeast extract on growth and butyric acid production showed a direct relationship between yeast extract and microbial activity, with any reduction in yeast extract concentration showing a negative impact on both response variables. To reduce potential buffering contributed from excess dibasic phosphate, it was found that concentrations could be reduced from 1.5 g L^{-1} to 0.3 g L^{-1} with no

observable difference to original medium A. It is likely that any phosphate over this concentration is in excess of the cellular requirements of *C. tyrobutyricum*.

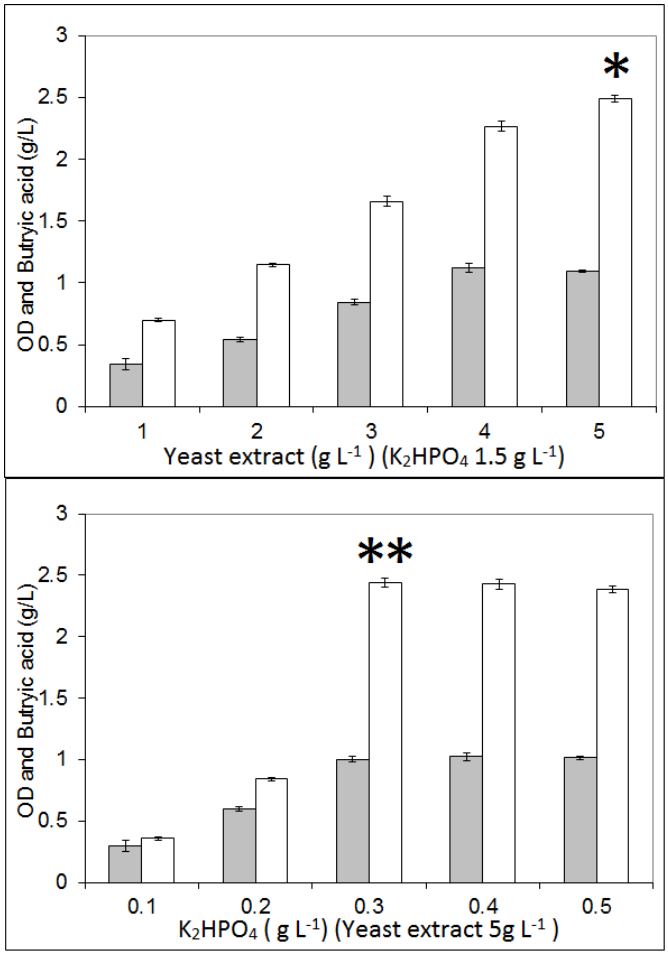

Figure 3-1 *C. tyrobutyricum* growth studies of decreasing dibasic potassium phosphate and yeast extract concentrations. "*" represents medium A, while ""represents modified medium B. Gray bars represent optical density mean values, white bars represent butyric acid concentration mean values. Error bars represent standard deviation (n=3).**

Thus Figure 3-1 shows that while no reduction in yeast extract was possible without decreasing growth, medium formulation B with reduced phosphate was determined to have no observable effect on growth or acid production.

Sparging tests were performed on both Media A and B to determine the extent to which phosphate reduction improved *p*H swing capacity. As can be seen in Figure 3-2, Medium B achieved a lowest *p*H value of 4.8 in sparging tests compared to medium A (*p*H 5.25), which represents a significant increase in *p*H swing capacity, especially considering the

logarithmic nature of the *p*H scale. Thus, a medium formulation for *C. tyrobutyricum* was developed which resulted in lower *p*H values when sparged with CO_2 while not adversely impacting growth and butyric acid production, satisfying both objectives for medium suitability in this application.

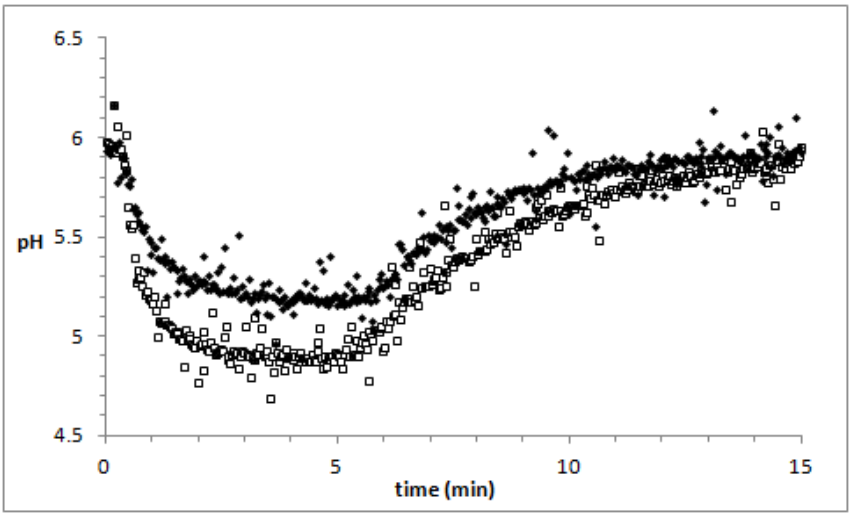

Figure 3-2 *p*H adjustment of media tested using CO_2-N_2 sparging tests at atmospheric pressure. Closed diamonds represents an original medium A, and open squares represents improved *p*H swingable medium B.

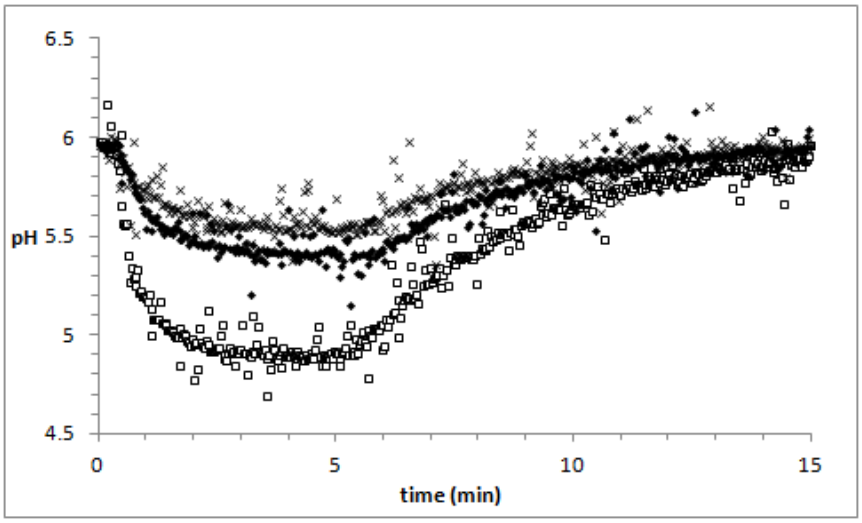

Figure 3-3 *p*H adjustment of modified medium B containing varying amounts of butyric acid using CO_2-N_2 sparging tests at atmospheric pressure. Crosses represent medium with 10 g L^{-1}, Closed diamonds represent medium with 5 g L^{-1} butyric acid, and open squares represents medium B

3.5.3 Buffering effect of organic acids on *p*H swing

Butyric acid, like many other organic acids, is a weak acid with a relatively high pK_a (4.8), and thus is a good buffer itself. It is likely that the combination of *p*H control using base and butyric acid production would result in an increased buffering capacity as the fermentation proceeds, thus decreasing *p*H swing capacity and ultimately reducing extraction. It was found that butyrate has a negative effect on total *p*H swing (Figure 3-3), with 5 g L^{-1} of butyric acid limiting *p*H swing in medium sparging tests to *p*H 5.4, while 10 g L^{-1} limited *p*H swing even further and only achieved a *p*H of 5.6, compared to a *p*H of 4.8, achieved on butyrate free medium B. At a *p*H of 5.6 only 14% butyric acid would be protonated compared to a 39% at a *p*H of 5.0, which is much closer to the pK_a for butyric acid (4.8). As only the neutral protonated species will partition, early extraction would result in better uptake, preferably below or near a concentration of 5 g L^{-1}, in the case of butyrate. However, in the case of heterofermentations, by-product acids (e.g. acetic acid, pK_a 4.7, PC=0.25 on Pebax® 2533) would also have a similar buffering effect, and consideration of total acid production is important.

3.5.4 CO_2-mediated *p*H swings for online extraction during fermentation

In order to determine what effect CO_2-mediated extraction has on batch performance, a comparison was made between a conventional batch run (Figure 3-4A), a CO_2-free extractive control run (Figure 3-4B), and an extractive run with CO_2 sparging (Figure 3-4C). While observable butyric acid recovery was achieved for the CO_2 sparged treatment, no significant improvements were observed in titre or yield compared to conventional or CO_2-free runs (Table 3-III), with approximately 40 g butyric acid produced and yields of 0.33-0.34 grams of product per gram of glucose added achieved in all runs.

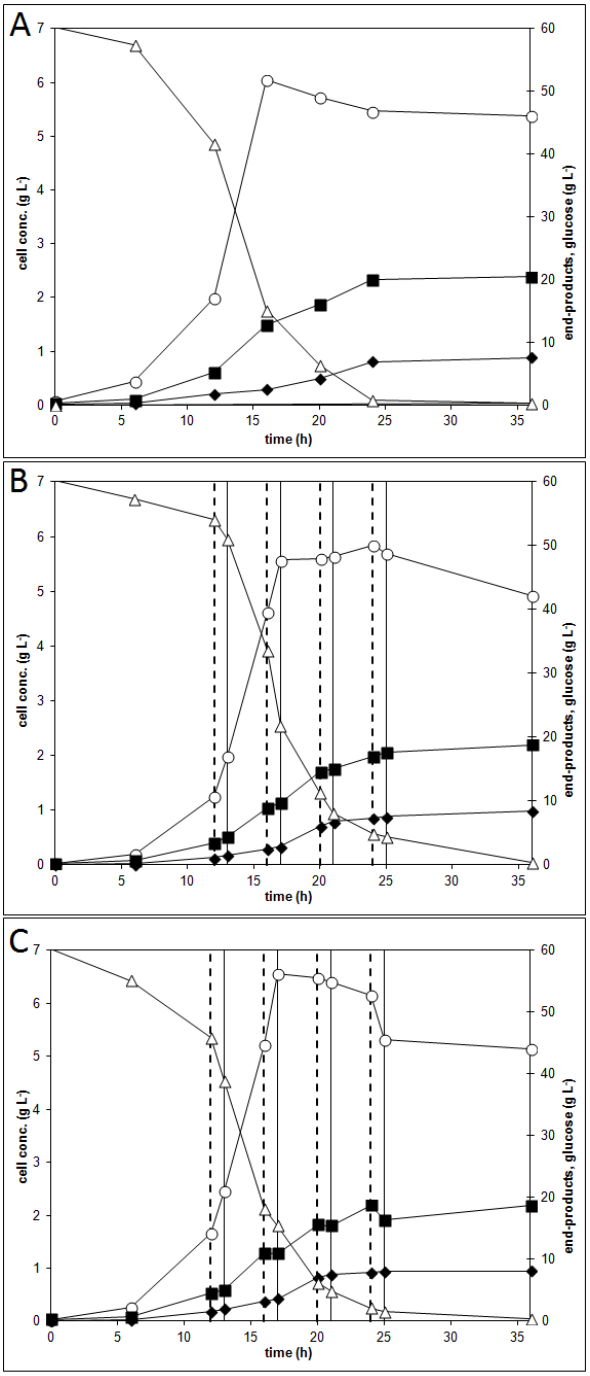

Figure 3-4 Batch fermentation runs of *C. tyrobutyricum* on modified medium B for a conventional reactor (A) or reactors with recycle through a polymer-packed column in the absence (B) or presence (C) of CO_2 sparging. Open circles represent OD, solid squares represent butyric acid, solid diamonds represent acetic acid, open triangles represent glucose. Dashed vertical lines indicate CO_2 sparging initiation and solid vertical lines represent CO_2 sparging termination.

While benefits from extraction might be expected due to the alleviation of end-product inhibition as butyric acid is known to be a strong non-competitive inhibitor of cell growth

(Vandak *et al.* 1997; Zhu and Yang 2004), the extraction achieved under these conditions proved insufficient to provide substantial product gains in terms of yields or productivity. However, both extractive runs demonstrated increased maximum cell growth (6.4-6.6 g L^{-1} vs. 6.0 g L^{-1}), possibly as a result of butyric acid removal.

Table 3-III Comparison of process parameters between conventional and extractive batch reactor runs with or without CO_2 sparging performed during production of butyric acid with *C. tyrobutyricum*

Parameter	Conventional batch	CO_2 free extraction	CO_2 extraction
Aqueous butyric acid (g L^{-1})	20.45	18.9	18.75
Extracted butyric acid (g)	-	3.22	4.51
Total butyric acid (g)	40.9	41.1	42.0
Butyric acid yield (g/g)	0.34	0.34	0.35
Butyric:Total acid ratio	0.73	0.71	0.72
Maximum cell conc. (g L^{-1})	6.0	6.4	6.6
Base added (moles)	0.78	0.81	0.82

As direct quantification of butyric acid uptake was masked by simultaneous production of additional acid, butyric acid desorbed from the polymers was used to indicate the extent of extraction achieved. Table 3-IV displays desorption values for each extraction, and CO_2-sparged extractions yielded 0.86, 1.20, 1.06, and 1.38 g butyric acid over the course of each extraction period respectively, for a total of 4.51 g butyric acid recovered over the total fermentation compared to CO_2-free extractions, which yielded 0.30, 0.90, 0.82, and 1.2 g butyric acid for each respective extraction, resulting in a total recovery of 3.22 g butyric acid. Thus, CO_2 sparging improved butyric acid recovery by 186%, 33%, 29% and 15% for each respective extraction, resulting in an overall improvement of 40%, clearly demonstrating CO_2-mediated *p*H swings exert a positive influence on extraction. However, as can be seen in Table 3-IV, it is apparent that these improvements decrease during the course of the fermentation, as do the lowest *p*H values achieved by CO_2 sparging. This is likely due to the accumulation of neutralized butyrate salts present as a result of *p*H control, and this supports abiotic sparge tests demonstrating that increased butyrate has a significant negative effect on

CO_2-*p*H swings due to increased buffering capacity, suggesting again that early extraction to maintain low butyrate concentrations would be beneficial.

Table 3-IV Comparison of extraction parameters between extractions with or without CO_2 sparging performed during butyric acid production with *C. tyrobutyricum*

Extraction	Butyric acid (g)		% improvement	Initial extractive *p*H		Final extractive *p*H	
-	CO_2 free	CO_2		CO_2 free	CO_2	CO_2 free	CO_2
1 (t=12h)	0.30	0.86	186	6	5.5	5.5	5
2 (t=16h)	0.90	1.20	33	6	5.6	5.3	5.2
3 (t=20h)	0.82	1.06	29	6	5.7	5.7	5.6
4 (t=24h)	1.2	1.38	15	6	5.7	5.8	5.6
total	3.22	4.51	40				

As seen in Figure 3-5, in early extractions CO_2 was able to reduce the *p*H to 5.5 quickly, with a slower reduction to a *p*H of 5.0 following over the span of the extraction. As no similar secondary decrease in *p*H during abiotic sparging tests was observed, it is likely that this drop is a result of further acid production during extraction, facilitating a lower final *p*H value and further extraction. Use of acid production to achieve *p*H values necessary for extraction has been demonstrated in other works (Engel *et al.* 2011), but the use of CO_2 as an initial effector for decrease of *p*H reduces the time required to achieve a useful *p*H drop.

Figure 3-5 also displays that N_2 sparging was not capable of returning the *p*H to original values, unlike sparge tests under abiotic conditions. It is likely the additional protonated acid produced during extraction buffers the system further, thus masking the stripping effects of N_2. However, as can be seen in Table 3-III, no additional base was necessary to return *p*H values to fermentative conditions. Interestingly, no change in the ratio of acids was observed between conventional and sparged reactors, with the butyric:total acid ratio constant near 0.7 (Table 3.III), indicating that short *p*H shifts do not result in metabolic shifts to by-product formation, which occurs with prolonged exposure to lowers *p*H values (Wu and Yang 2003; Zhu and Yang 2004).

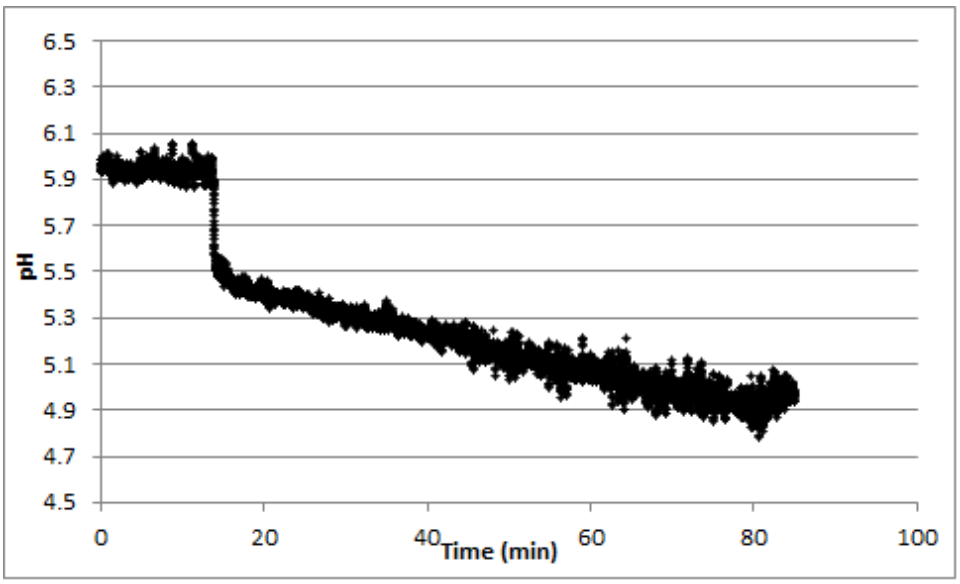

Figure 3-5 *p*H profile during CO_2 *p*H adjustment and simultaneous production (*C. tyrobutyricum*) and extraction of butyric acid from fermentation broth after 12 hours using CO_2-N_2 sparging at atmospheric pressure.

It is important to note that the butyric acid extracted here reflects partitioning afforded by Hytrel® 3078, which shows a partition coefficient of 3. If a sequestering phase with a partition coefficient an order of magnitude higher was employed, and CO_2 sparging afforded a 40% increase in partitioning, this could make a marked improvement in extraction. ISPR of butyric acid has been demonstrated elsewhere (Wu and Yang 2003), with similar studies proposed for succinic acid (Hepburn and Daugulis 2012) and lactic acid (Krzyzaniak *et al.* 2013) and it is possible that the use of CO_2-N_2 sparging could generally improve ISPR of organic acids by facilitating further *p*H-dependent uptake. Thus, improvements in yield and titre could be achieved through higher partitioning by both selecting polymers with increased polymer-solute affinity and improvement of *p*H swings through the use of elevated CO_2 pressure. Regardless, carbon dioxide sparging as described above presents a novel technique for facilitating ISPR of organic acids by allowing for rapid drops in *p*H to improve uptake, while avoiding osmotic stress and protracted exposure to low *p*H.

3.6 Conclusion

This work demonstrates CO_2-mediated *p*H swings result in improved organic acid ISPR, while modifying a medium to increase this effect at no expense to microbial activity, and this technique could be applied to other extractive processes to improve efficiency. This work further illustrates the ability of CO_2 to initiate rapid drops in *p*H during fermentation, significantly contributing to the *p*H drop achieved through acid production over the course of an extraction, while highlighting the importance of early extraction to mitigate buffering. The results shown here demonstrate initial attempts at CO_2-mediated ISPR, and require further steps before maximum capabilities of organic acid ISPR in TPPBs can be fulfilled. While this study demonstrates that Hildebrand solubility parameters qualitatively predict polymer-solute affinity with the use of corrections for soft segment and water uptake, Hansen solubility parameters and group-contribution activity coefficient models such as UNIFAC will be investigated with the goal of achieving superior partitioning. Importantly, preliminary research in our group has shown that elevated CO_2 pressures result in more effective *p*H swings and significant improvements in partitioning compared to atmospheric sparging, and thus future work will focus on use of pressurized vessels, rational polymer selection and equilibrium considerations to achieve effective ISPR for organic acids.

3.7 References

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Chapter 4

Characterization of organic acid uptake by absorptive polymers and high pressure CO_2-mediated acid recovery

4.1 Preface to Chapter 4

It is important to understand what *p*H values need to be achieved to meet recovery targets in the case of *p*H-dependent partitioning, and it is equally important to determine what partial pressures of CO_2 (pCO_2) are required to achieve this desired *p*H. Using a first principles approach, models were developed to describe partitioning as a function of *p*H, polymer fraction (F) and partition coefficient (PC), allowing for analysis of % acid recovery, and these models were validated using both butyric acid and benzoic acid partitioning into Pebax® 2533. This absorptive block copolymer was selected as it is comprised of a soft segment of 80% poly(tetramethylene glycol), as this homopolymer demonstrated the highest affinity for butyric acid from a wide screening of polymers based on predicted thermodynamic affinity as seen in Appendix A. Pebax® 2533 was selected over poly(tetramethylene glycol) due to improved handling properties arising from a 20% polyamide hard segment.

While *p*H determines organic acid partitioning, it is important to note that partitioning can influence *p*H as well. Specifically, through removal of acid from the aqueous phase by absorption, changes in *p*H can be observed, and partitioning tests were performed with both butyric and benzoic acid while measuring changes in equilibrium *p*H at varied polymer fractions. Thus, if acid recovery is sufficient for limiting *p*H reductions, this could potentially be used to maintain *p*H during fermentation. Therefore, studying these changes at a range of acid concentrations is important as well, if they are to be demonstrated during the acid production. Furthermore, partitioning tests were performed at a range of *p*H values as well, and characterize the relationship between equilibrium *p*H and both distribution coefficients and % recovery. This allows for analysis into how small decreases in *p*H achieved through high pCO_2 could yield substantial improvement in acid absorption during fermentation.

To determine how increasing pressure can improve *p*H reductions and subsequent absorption, partitioning tests were performed from 1 to 60 bar pCO_2 for both butyric acid and benzoic acid. Furthermore, additional partitioning tests at 60 bar pCO_2 were performed at a

range of butyric acid concentrations reflecting typical titres achieved in fermentations, to determine if acid buffering reduces *p*H reductions and subsequent polymeric absorption at high pCO_2. The resulting distribution coefficients were used to estimate *p*H values achieved under pressure, from which % recovery at various polymer fractions (F) was predicted. The goal of this was to examine if elevated polymer fractions could potentially improve acid recovery in scenarios where absorption is reduced due to buffering resulting from acid concentrations. Overall, this work provides a clear framework for understanding acid partitioning, and clearly demonstrates elevated pCO_2 improves polymeric absorption of organic acids.

4.2 Abstract

Application of two-phase partitioning bioreactors (TPPBs) to the biological production of organic acids is challenging due the *p*H-dependent nature of partitioning of these solutes, and the fact that near-neutral *p*H values typical in fermentations may exclude effective uptake. This study develops models for characterizing *p*H-dependent partitioning by an absorptive polymer phase, identifying crucial factors influencing uptake including partition coefficient (PC) and polymer fraction (F). Partitioning tests with Pebax® 2533 for both butyric acid (PC=4.2, pK_a 4.8) and benzoic acid (PC=70, pK_a=4.2) demonstrated that acid absorption resulted in increases to equilibrium *p*H values by as much as 0.6 and 1.8 *p*H units for each acid respectively when F=0.5. This effect was observed over a range of acid concentrations, and suggests that recovery through partitioning could potentially result in reduced need for *p*H control in the bioproduction of organic acids. Partitioning studies at a range of *p*H values showed that at their respective pK_a values, distribution coefficients (D) for butyric acid (D=2.0) and benzoic acid (D=38) were both 50% of PC, demonstrating that *p*H dependent partitioning is dictated solely by the availability of protonated species, and not influenced by PC. The use of high pressure CO_2 to reduce *p*H from near-neutral values and improve polymer absorption was investigated, and butyric acid distribution coefficients and percent recovery were shown to increase from D=0.14 and 3% at atmospheric conditions to D=3.0 and 40% after one hour at 60 bar CO_2. Under the same conditions, benzoic acid showed an increase from D=0.69 and 1% acid absorption to D=24 and 80% uptake, and thus for both acids the use of high pCO_2 substantially improved uptake. However, tests performed with increased butyric acid concentration reduced distribution coefficients due to elevated buffering capacity, however these reductions in absorption at higher concentrations plateaued beyond butyric acid concentrations of 20 g L^{-1}. Overall, this work validates predictive models for estimating partitioning for non-reactive and ionizable solutes, and demonstrates high pressure CO_2 *p*H-reductions as an effective option for increasing acid recovery

4.3 Introduction

Over the past two decades, two-phase partitioning bioreactors (TPPBs) have been demonstrated to confer benefits to a large number of biological processes, and this has most recently been achieved through direct inclusion of an absorptive polymer phase, which partitions solutes and reduces toxicity, whether from accumulated fermentation products (Jain *et al*. 2010; Morrish and Daugulis 2008) or inhibitory substrates earmarked for biological degradation (Daugulis *et al.* 2011). Here, it is critically important to recognize that the uptake of solutes by soft, amorphous polymers is by ***absorption*** (into the polymer matrix itself, analogous to equilibrium-based solvent extraction), in contrast to surface-area controlled ***adsorption*** as has been attempted through the use materials such as activated carbon or ion exchange resins. The main focus in the selection of absorptive polymers for TPPB applications has been on materials which possess high affinity for a given solute (Parent *et al.* 2012; Bacon *et al.* 2014), as this plays a key role in the success of a polymer as a sequestering phase. Partition coefficients have generally been used to express this affinity for a target molecule, as seen in Equation (4.1), where *n* and *m* represent moles of solute and the mass of the phase in which it is found, whether in the aqueous (aq) or polymer (pol) phases.

$$PC = \frac{[S]^{pol}}{[S]^{aq}} = \frac{n_S^{pol}/m_{pol}}{n_S^{aq}/m_{aq}} \tag{4.1}$$

Many previous TPPB studies have benefited from high PC values, ranging from >3000 in biodegradative studies of highly hydrophobic substrates (Rehmann and Daugulis 2007) to values in the range of 50-100 in research focusing on the production of biomolecules such as 2-phenylethanol (Gao and Daugulis 2010), carveol (Morrish and Daugulis 2008), and benzaldehyde (Craig and Daugulis 2013; Jain *et al.* 2010). In such cases, due to the high affinity of the polymer phase for the target molecule and the solute's hydrophobicity, a relatively low polymer fraction (F, Equation (4.2)) was generally required to achieve substantial uptake, with F=0.1 representing a typical value.

$$F = \frac{m_{pol}}{m_{aq}} \quad (4.2)$$

However, in recent years focus has shifted to the production of more hydrophilic biomolecules, such as butanol (Gao and Daugulis 2010), cis-indandiol (Dafoe and Daugulis 2011; Dafoe and Daugulis 2013), and succinic acid (Hepburn and Daugulis 2012), which demonstrate partition coefficients substantially lower than those mentioned above (i.e. PC < 5). In such cases, due to the diminished capacity of polymers to absorb these hydrophilic molecules, increases in F are necessary if appreciable product removal is to be achieved. Increasing F can come at a cost, however, and care must also be taken to ensure that elevated polymer fractions do not restrict mixing and cause other operational problems. Thus, in the case of hydrophilic solutes with relatively low PC values, careful specification of F will ensure that a desired extent of product recovery is achieved. To this end, Equations (4.3) and (4.4) present means to predict the distribution of a solute between the two phases as mole fractions of a target molecule in the polymer and the aqueous phases. Thus, for a given PC value, target aqueous fractions can be achieved through manipulation of F, allowing for aqueous concentrations to be maintained at sub-cytotoxic levels as determined by an operator.

$$\frac{n_S^{pol}}{n^{tot}} = \frac{F \cdot PC}{F \cdot PC + 1} \quad (4.3)$$

$$\frac{n_S^{aq}}{n^{tot}} = \frac{1}{F \cdot PC + 1} \quad (4.4)$$

In the more complicated case of ionizable solutes such as organic acids, however, prediction of distribution of mole fractions between phases becomes more challenging, as dissociation of aqueous acids into their respective conjugate bases must be accounted for. This is a critical consideration, as it is widely recognized that a dissociated acid does not participate in absorption (Kertes and King 1986), and thus as *p*H and in turn conjugate base fractions increase, uptake will decrease; in this way partitioning of organic acids is dependent on *p*H. Therefore, in such instances, partition coefficients can be determined only under very acidic concentrations where negligible amounts of conjugate base are present. At *p*H values that

result in a proportion of the acid present as a conjugate base, less uptake by an absorptive polymer phase is achieved compared to more acidic conditions, and these reductions in PC are represented as distribution coefficients (D), as expressed in Equation (4.5), where HA and A^- represent an acid and its conjugate base, respectively.

$$D = \frac{[HA]^{pol}}{[HA]^{aq}+[A-]^{aq}} = \frac{n_{HA}^{pol}/m_{pol}}{(n_{HA}^{aq}+n_{A-}^{aq})/m_{aq}} \quad (4.5)$$

Thus, the prediction of organic acid partitioning is substantially more complicated than that of non-reactive solutes due to the fraction of conjugate base which can be present, and the distribution of the various species between phases is related to *p*H according to Equations (4.6), (4.7), and (4.8), respectively.

$$\frac{n_{HA}^{pol}}{n^{tot}} = \frac{F \cdot PC}{F \cdot PC+\left(1+10^{(pH-pK_a)}\right)} \quad (4.6)$$

$$\frac{n_{HA}^{aq}}{n^{tot}} = \frac{1}{F \cdot PC+\left(1+10^{(pH-pK_a)}\right)} \quad (4.7)$$

$$\frac{n_{A-}^{aq}}{n^{tot}} \cdot = \frac{10^{(pH-pK_a)}}{F \cdot PC+\left(1+10^{(pH-pK_a)}\right)} \quad (4.8)$$

It is clear from the above equations that to accurately predict the mole fractions of the species arising from absorptive organic acid uptake in a TPPB, the *p*H must also be known along with PC. This would enable a suitable F to be selected to achieve a desired extent of inhibitory solute removal from the aqueous phase or, alternatively, as the percentage of acid recovered through polymer absorption (i.e. % recovery),

Additionally, through combination of Equations 4.6-4.8, it is possible to estimate a distribution coefficient at any given *p*H value as shown in Equation 4.9. Unlike PC values, which report partitioning of acids under fully acidified conditions, D accounts for the presence of conjugate base and provides a singular value for comparison to PC, to quantify how effective overall polymer uptake is at a given *p*H.

$$D = \frac{F \cdot PC/m_{pol}}{\left(1+10^{(pH-pK_a)}\right)/m_{aq}} \quad (4.9)$$

Given the high degree of sensitivity of distribution coefficients to *p*H, application of TPPB techniques to the production of organic acids becomes problematical, as typical fermentation *p*H values are often near-neutral and favour conjugate base formation, which would result in limited partitioning into an absorptive polymer phase. Previous work on the production of organic acids in TPPBs has focused on the use of carbon dioxide sparging to temporarily reduce *p*H values and increase acid uptake (Hepburn and Daugulis 2012; Peterson and Daugulis 2014), with some degree of success. Increased partial pressures of CO_2 (pCO_2) have been shown to achieve lower *p*H values by virtue of increased CO_2 solubility (Meyssami *et al*. 1992), and it is likely that acid partitioning achieved by elevating pressure could be substantially increased compared to atmospheric conditions, potentially providing a method to adjust *p*H values to favour temporary periods of product removal.

Thus, the overall objective of this study was to experimentally confirm the rigorous theoretical framework outlined above for predicting the proportions of non-reactive and ionizable solutes in both aqueous and polymer phases at equilibrium, while taking into account the presence of conjugate base fractions and the relationship between partitioning and *p*H. To ensure thorough validation, although focusing on butyric acid, *n*-butanol and benzoic acid were also tested, to include a non-reactive solute and a more hydrophobic organic acid, respectively. This work further demonstrates a simple method for reducing *p*H to improve organic acid partitioning through the use of increased partial pressures of carbon dioxide, while using the above-mentioned framework to estimate organic acid uptake. In these ways, this study provides users with tools to effectively predict partitioning of ionizable species regardless of *p*H, thereby allowing the selection of appropriate polymer fractions, and facilitating *p*H-dependent absorptive partitioning of organic acids in TPPBs.

4.4 Materials and Methods

4.4.1 Polymers and materials

Pebax® 2533, an absorptive polyether-co-amide block copolymer, was selected for use as a partitioning phase in this study as it has been shown to possess high affinity for butyric acid (see Appendix A) and *n*-butanol (Gao and Daugulis 2010), and was kindly donated by Arkema Inc.. All other chemicals used in this study were purchased from Fisher Scientific Company, Ltd (Ottawa, Canada).

4.4.2 Partition coefficient determination

Partition coefficients of Pebax® 2533 for *n*-butanol, butyric acid, and benzoic acid were all determined through contact of triplicate 1 g polymer samples in 10 mL aliquots of acidic solutions. Partition coefficients were determined for *n*-butanol and butyric acid at a concentration of 20 g L^{-1}. However, as the solubility of benzoic acid is relatively low (2.5 g L^{-1}), and this solute is substantially more hydrophobic, substantial uptake by the polymer could occur, which could alter the *p*H sufficiently to underreport PC. Thus, a 2.5 g L^{-1} concentration of benzoic acid was acidified via addition of 5% v/v 1 M H_2SO_4, when determining PC. After a 24-hour period to allow equilibration with the polymer, aqueous solute concentrations were determined with either HPLC or GC, and compared to a polymer-free control to calculate polymer solute concentration via mass balance, from which partition coefficients were determined as shown in Equation (4.1).

4.4.3 The effect of polymer fraction and acid concentration on equilibrium *p*H

To determine the effect of polymer fraction (F) on species distribution and equilibrium *p*H as a result of solute uptake, partitioning tests were performed via the addition of 10 mL of 2.5 g L^{-1} *n*-butanol, butyric acid, or benzoic acid with no *p*H adjustment to 0.5, 1.0, 1.5, 2.0, 2.5, 3.5, and 5.0 g of Pebax® 2533, along with polymer free controls. *n*-Butanol was included in these tests despite the fact it does not dissociate in order to experimentally validate Equations 4.3 and 4.4, which describe partitioning of non-reactive solutes. Tests were performed in triplicate and allowed to equilibrate for 24 hours, after which aqueous concentrations were

measured and partitioning was calculated as described by the above equations. Equilibrium *p*H values were also determined for all aqueous samples and used to determine respective concentrations of aqueous acid and conjugate base using classical *p*H theory (see Supplemental), from which mole fractions of the various species contained in the 2 phases (i.e. n_{HA}^{pol}/n^{tot} , n_{HA}^{aq}/n^{tot}, and n_{A-}^{aq}/n^{tot}) were calculated. To determine the effect of varying acid concentrations on partitioning and equilibrium *p*H, similar 24 hour partitioning tests were performed with Pebax ® 2533 (F=0.5) in the presence of 2.5, 5, 10, 15, 20, 25, 35, and 50 g L^{-1} butyric acid without *p*H adjustment in triplicate, followed by *p*H determination and calculation of partitioning and phase mole fractions as described above.

4.4.4 Distribution coefficients as a function of *p*H

As typical fermentation conditions for an organic acid such as butyric acid permit only a narrow *p*H range, often near neutral values, characterization of the variation in species distribution as a function of *p*H is an important consideration to determine the extent and nature of partitioning at a defined *p*H. Thus, solutions of 2.5 g L^{-1} of either butyric or benzoic acid were prepared and *p*H-adjusted using 3 M KOH or H_2SO_4 to yield a range of *p*H values from 2 to 7. Aliquots of these solutions (10 mL) were then used to conduct triplicate partitioning tests with 1 g of Pebax® 2533, which were allowed to equilibrate for 24 hours, after which equilibrium *p*H values were determined for all samples, and mole fractions of the various species distributed between the 2 phases were calculated.

4.4.5 The use of high pressure CO_2 to facilitate *p*H reductions and increased partitioning

To demonstrate the extent to which increased partial pressure of CO_2 (pCO_2) can enhance solute partitioning into an absorptive polymer through *p*H reductions, 500 mL of either 2.5 g L^{-1} butyric acid or benzoic acid in RO water were *p*H-adjusted to 6.0 using 3M KOH, as this *p*H value reflects the *p*H maintained in butyric acid fermentation by *C.*

tyrobutyricum (Wu and Yang 2003). 100 g of Pebax® 2533 was added to the solution, which was then transferred to a 1 L Parr pressure vessel equipped with an internally threaded sampling tube (Parr Instrument Company, Moline, IL, USA). The vessel was sealed and pressurized to 15, 30, 45 and 60 bar pCO_2 (gauge pressure) and mixed at 500 rpm for one hour, which was selected as being representative of an appropriate time interval for facilitating multiple cyclic extractions during the course of a fermentation. After one hour, the vessel was drained under pressure to ensure *p*H values remained constant. Aqueous concentrations of either butyric or benzoic acid were then measured and compared to respective non-pressurized, polymer free controls to determine acid uptake by the polymer through mass balance. As the *p*H of the solutions could not be determined within the pressure vessel, measured aqueous acid and conjugate base mole fractions could not be differentiated and were reported as total aqueous solute concentration (n_{tot}^{aq}/n^{tot}) alongside the mole fraction of acid absorbed by the polymer (n_{HA}^{pol}/n^{tot}). A similar experiment was performed to determine the extent to which increasing butyric acid concentrations affected polymer extraction at high pressure as a consequence of elevated buffering strength by the weak acid. In this case, partitioning tests were performed at 60 bar pCO_2 at butyric acid concentrations of 5, 10, 15, 20, 35, and 50 g L^{-1} in RO water with all solutions adjusted to a *p*H of 6.0 with 3 M KOH, and D and the mole fractions of all species in both phases (i.e. n_{tot}^{aq}/n^{tot} and n_{HA}^{pol}/n^{tot}) were quantified and reported as described above.

4.4.6 Analytical methods

Aqueous samples were analyzed for butyric and benzoic acid using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 × 7.7 mm) at 60 °C with a 10 mM H_2SO_4 mobile phase at 0.7 mL min^{-1} used for butyric acid, and a Varian Pursuit C8 5 µm 4.6 × 250 mm column, with a mobile phase of 20 mM H_3PO_4 with 50:50 water/acetonitrile at 1 mL min^{-1} for benzoic acid, while both acids were quantified with a UV-Vis detector (Varian

Prostar, PS325) at 220 nm. *n*-Butanol was determined with a Varian 450-GC gas chromatograph equipped with a CP-8410 AutoInjector, VF-5ms 30 m column and FID detector.

4.5 Results and Discussion

4.5.1 Effect of polymer fraction and partition coefficient on solute distribution and equilibrium *p*H

To demonstrate how the polymer fraction F can influence species distribution in a non-reactive system, while also validating Equations (4.3) and (4.4), partitioning tests were performed using *n*-butanol as a model solute, as this molecule has been the focus of some interest for use in TPPBs (Barton and Daugulis 1992 ; Gao and Daugulis 2010). Figure 4-1 displays the fraction of *n*-butanol contained in the polymer at equilibrium (n_S^{pol}/n^{tot}) as a function of polymer fraction F, and the results show an increase in fractional uptake from 0.10 at F=0.05 to 0.49 at F=0.5, with a corresponding decreases in the aqueous phase (n_S^{aq}/n^{tot}) from 0.90 to 0.51. Although this result was not unanticipated, all mole fraction proportions for both phases were well predicted by Equations (4.3) and (4.4), as shown by the open symbols in Figure 4-1, which suggests that a targeted extent of solute removal can be achieved through careful selection of polymer fraction.

Of course a more complex scenario is represented in the case of ionizable species such as organic acids, wherein three fractions need to be accounted for, namely the undissociated acid in both the polymer (n_{HA}^{pol}/n^{tot}) and aqueous phases (n_{HA}^{aq}/n^{tot}), as well as the conjugate base present in the aqueous phase ($n_{A^-}^{aq}/n^{tot}$), with all three of these fractions being predicted by Equations (4.6), (4.7), and (4.8), respectively. In examining these formulae, it can be seen that the PC plays an important role in the distribution of species between phases, and thus a more pronounced effect should be seen with organic acids which possess higher PC values. Accordingly, along with butyric acid, benzoic acid uptake was studied, as

the PC for benzoic acid at 2.5 g L^{-1} with Pebax® 2533 was found to be 70 ±1, which is substantially higher than that of both butyric acid and *n*-butanol, which yield PC values of 4.2 ± 0.1 and 2.0±0.2 for Pebax® 2533, respectively.

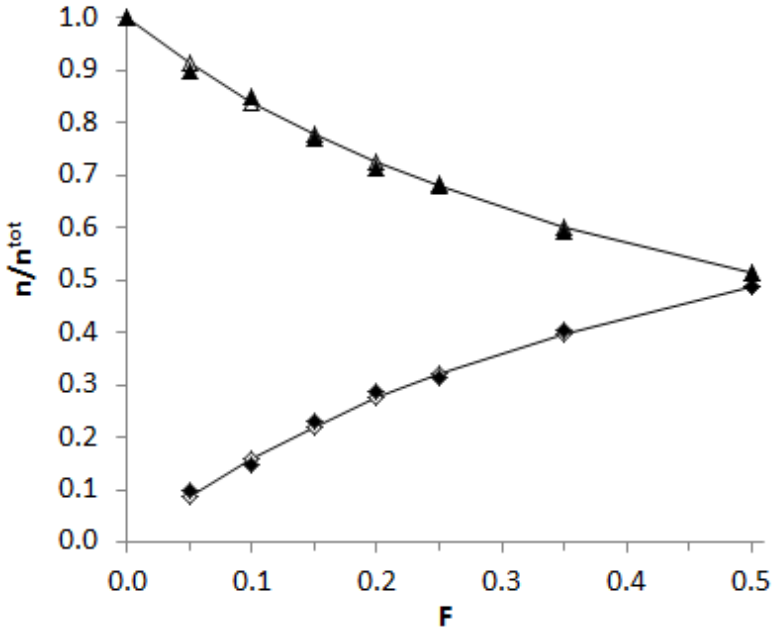


Figure 4-1 Measured and predicted mole fractions of *n*-butanol in the presence of Pebax ® 2533 (PC=2.0) as a function of polymer fraction (F) in both aqueous and polymer phases with closed and open symbols representing measured and predicted values. Initial n-butanol concentration was 2.5 g L^{-1}, and diamonds and triangles represent mole fractions in the polymer and aqueous phase respectively

As can be seen in Figure 4-2A, in the case of butyric acid as the polymer fraction F was increased the proportion of acid absorbed into the polymer showed almost linear improvement, ranging from 0.17 at F=0.05 to 0.67 at F=0.5, with the aqueous mole fraction decreasing from 0.79 to 0.28, and the aqueous conjugate base fraction rising slightly from 0.03 to 0.05. Thus, this demonstrates that effective butyric acid absorption and lowered aqueous acid concentrations achieved through use of higher polymer fractions can be effectively predicted. Stronger partitioning was observed for benzoic acid (Figure 4-2B), with an acid fraction of 0.79 in the polymer at F=0.05 with aqueous acid and conjugate base fractions of 0.17 and 0.04, respectively. In this case, increasing F improves partitioning only

slightly, with the acid fraction in the polymer rising to 0.9 at F=0.5, with low aqueous acid fraction of 0.01, and a conjugate base fraction of 0.08.

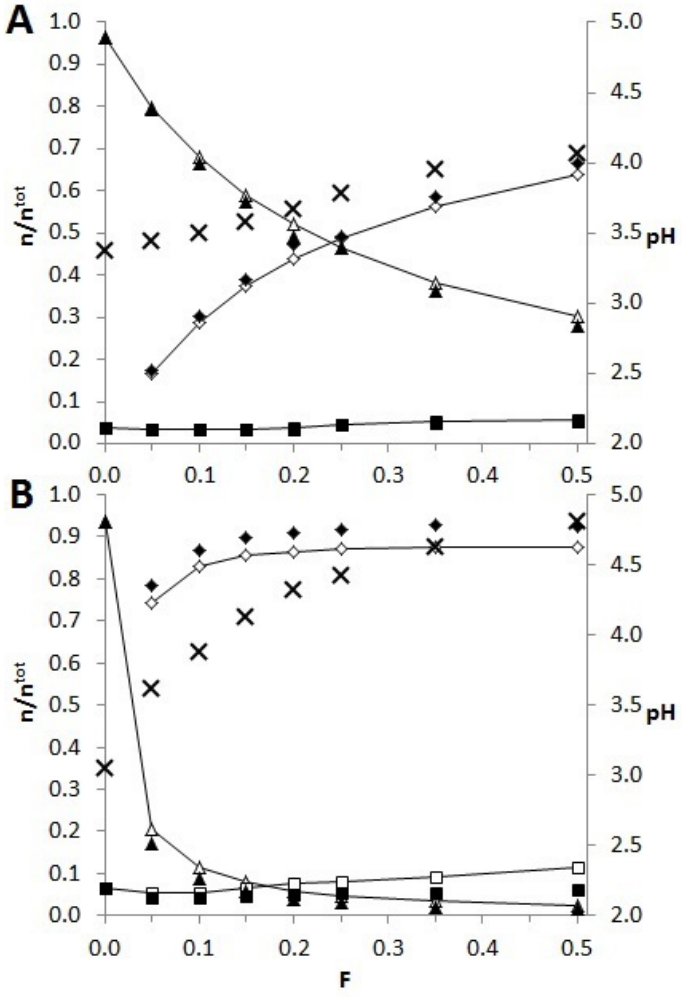


Figure 4-2 Measured and predicted species mole fractions in both aqueous and polymer phases as a function of polymer fraction (F) for either A) butyric acid or B) benzoic acid in the presence of Pebax® 2533, and closed and open symbols represent measured and predicted values. Initial concentrations of both butyric and benzoic acid were 2.5 g L^{-1} and diamonds, triangles and squares represent n_{HA}^{pol}/n^{tot}, n_{HA}^{aq}/n^{tot}, and n_{A-}^{aq}/n^{tot}, respectively, while X symbols represent measured equilibrium *p*H values.

These results clearly demonstrate that benzoic acid is sorbed to a much greater extent by Pebax® compared to butyric acid, with the polymer achieving nearly 80% recovery even at the lowest fraction tested (0.05), and this recovery only slightly increased with the use of additional polymer. This suggests that in the case of a relatively hydrophobic organic acid, elevated polymer fractions deliver diminishing returns and are unnecessary for achieving

effective acid removal. However, in contrast to benzoic acid recovery, which does not noticeably increase with F, butyric acid enjoys a substantial increase in partitioning as F rises, with % recovery values of 17%, 30%, and 50% at polymer fractions of F=0.05, 0.1, and 0.25, respectively. Therefore while solutes demonstrating high partition coefficients receive only diminishing returns as F increase, in the case of solutes demonstrating lower PCs, the extent of solute uptake by an absorptive polymer can be improved through careful selection of polymer fraction. In addition, as can be seen in Figure 4-2, Equations (4.6), (4.7), and (4.8) were shown to effectively predict the fraction of the various solute species in the 2 phases, and present useful tools for estimating acid uptake and species distribution. Figure 4-2 also shows a slight increase for both acids in the aqueous conjugate base fraction as the polymer fraction F was increased, which resulted in a concurrent increase of equilibrium *p*H values, as *p*H noticeably rose with an increase in polymer fraction as a result of increased acid uptake. In the case of butyric acid, the *p*H at equilibrium increased from 3.4 in the absence of polymer to 4.1 at F=0.5, while benzoic acid tests showed a rise in *p*H from 3.0 at F=0 to 4.8 with F=0.5. These results show that by selective removal of only the undissociated organic acid, the equilibrium *p*H values increased with higher acid uptake, demonstrating the direct effect that partitioning may exert on *p*H in TPPBs. That is, if sufficient acid recovery was achieved in a TPPB, it may be possible to prevent *p*H decreases resulting from biological acid production. However, the elevated acid concentrations typically achieved during fermentation could potentially mitigate these *p*H changes through increased buffering strength.

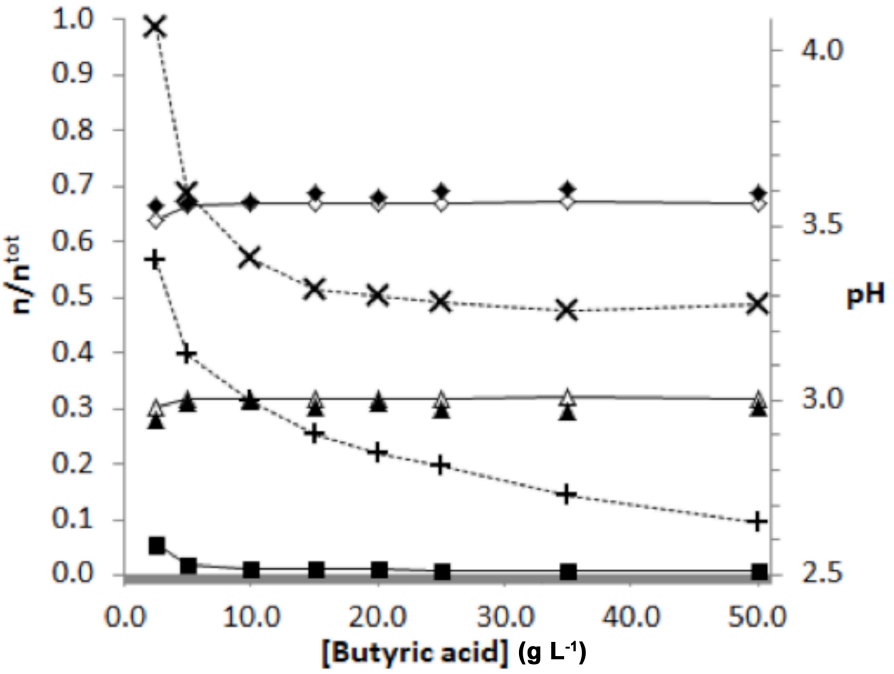


Figure 4-3 Measured and predicted species mole fractions in both aqueous and polymer phases as a function of initial butyric acid concentration for butyric acid in the presence of Pebax® 2533 (F=0.5), and closed and open symbols represent measured and predicted values. Diamonds, triangles and squares represent n_{HA}^{pol}/n^{tot}, n_{HA}^{aq}/n^{tot}, and n_{A-}^{aq}/n^{tot}, respectively, while + and X symbols with dashed lines represent measured initial and equilibrium *p*H values.

To examine the potential impact of acid concentration on solute fraction predictions and equilibrium *p*H, partitioning tests were performed at a fixed polymer fraction (F=0.5) for a range of butyric acid concentrations, and Figure 4-3 shows that the equations for predicting distribution coefficients and the solute fractions in the both phases accurately describe respective measured values regardless of initial acid concentration. Moreover, Figure 4-3 shows *p*H values both initially (i.e. prior to partitioning) and at equilibrium (i.e. once partitioning was complete). It can be seen that differences between initial and equilibrium *p*H values occur at all concentrations, and at an initial butyric acid concentration of 2.5 g L^{-1} the *p*H value increases from 3.4 to 4.1 due to absorption of the acid into the polymer, which represents a change of 0.7 *p*H units. As butyric acid concentrations are increased, a similar change in *p*H was measured, ranging from 0.5-0.7 *p*H units across all concentrations. Thus it is clear that *p*H increases occur as a result of acid absorption regardless of initial acid concentration, and these changes are not substantially mitigated by weak acid buffering. It is anticipated that

similar *p*H changes could be expected to arise from acid partitioning into absorptive polymers during TPPB operation, and this could have direct benefits in terms of TPPB operability. Specifically, if polymer absorption was used to reduce the accumulation of organic acids during biological production, this could potentially lessen the need for *p*H control and ease the resultant osmotic stress arising from base addition (Liu *et al.* 2008). Online organic acid removal has been shown to effectively regulate *p*H using adsorptive ion-exchange resins (Ataei and Vasheghani-Farahani 2008) and reactive extraction (Wu and Yang 2003), but such an approach has not been considered for TPPBs using absorptive polymers to date. Overall, the experimental results here demonstrate that solute mole fractions in both polymer and aqueous phases can be accurately predicted using PC, and F, as well as *p*H in the case of ionizable solutes, and through manipulation of these variables, both desired removal in TPPBs and *p*H regulation through acid recovery could be achieved.

4.5.2 The effect of *p*H dependence on species distribution in two-phase aqueous-polymer systems

If solute partitioning into an absorptive polymer is to be used for product recovery of organic acids during fermentations, characterization of species distribution as a function of *p*H is essential to determine the extent of removal under defined conditions, as fermentations are generally confined to relatively narrow *p*H ranges. To this end, partitioning tests were performed over a range of *p*H values for both butyric and benzoic acid, and Figure 4-4 shows the experimental and predicted distribution coefficients and species fractions for butyric and benzoic acids in both phases as a function of *p*H. It is clear from this figure that at higher *p*H values, distribution coefficients and undissociated acid fractions in both the polymer and aqueous phases are reduced, while the aqueous conjugate base fraction increases, and this behaviour was well predicted, using Equations (4.6), (4.7), (4.8) and (4.9).

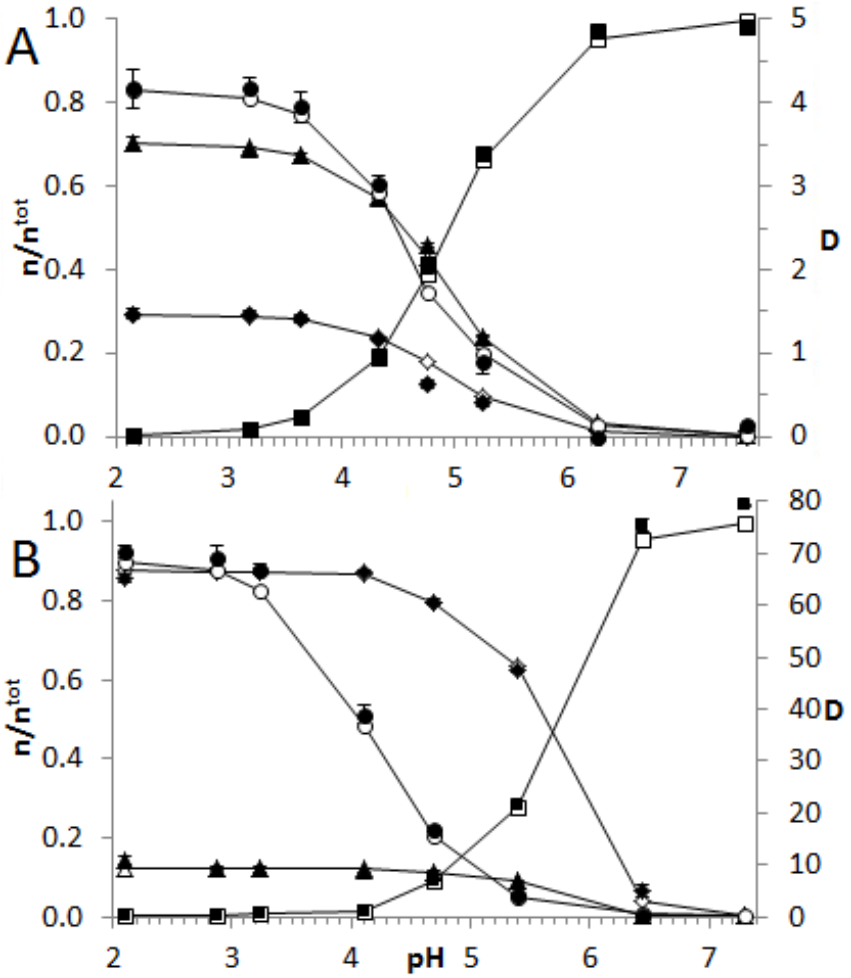


Figure 4-4 Predicted and measured species mole fractions in both aqueous and polymer phases and distribution coefficients as a function of equilibrium *p*H for either A) butyric acid or B) benzoic acid in the presence of Pebax ® 2533 (F=0.1), and closed and open symbols represent observed and predicted values. Initial concentrations of both butyric and benzoic acid were 2.5 g L^{-1} and diamonds, triangles and squares represent n_{HA}^{pol}/n^{tot} , n_{HA}^{aq}/n^{tot}, and $n_{A^-}^{aq}/n^{tot}$ respectively, while circles represent distribution coefficients.

Furthermore, when *p*H values approached the pK_a for both butyric and benzoic acid (4.8 and 4.2, respectively), D was found to be one-half of the PC value, with butyric acid demonstrating a D of 2.1 at *p*H 4.8 (PC = 4.2), while benzoic acid showed a D of 38 at *p*H 4.1 (PC = 70), as would be expected if only half of acid was available for partitioning when acid and conjugate base are present in equal amounts at the pK_a. The significance of this is that although it has been shown in Figure 4-2 that PC can influence acid-base equilibria through acid recovery and elevation of equilibrium *p*H values, the extent to which partitioning occurs is dictated by *p*H and pK_a at equilibrium, and increasing PC does not change this effect. Thus,

the absorptive partitioning of acids is dominated by *p*H, and should be a primary consideration for achieving successful absorptive recovery of acids in TPPBs, with the models outlined here providing a means of estimating this recovery. In other words, through the use of the equations described here, careful selection of F can be used to achieve a target removal value, in combination with the identification of polymers demonstrating increased PC values and the *p*H adjustment to improve absorption.

As can be seen in Appendix A, Pebax ® 2533 represents an absorptive polymer demonstrating the highest measured affinity for butyric acid after a thorough screening of a wide array of polymers based on thermodynamic affinity, and thus the further identification of polymers demonstrating improved PC values represents a challenge. However, it may be possible to adjust *p*H to improve acid recovery without adversely affecting biological performance. While it is clear that both butyric and benzoic acid demonstrate negligible partitioning above *p*H 6, by examining the undissociated acid fraction found in the polymer phase, it is apparent that with small changes from near neutral values to more acidic *p*H conditions a substantial increase in % recovery can be seen, and further decreases in *p*H below respective pK_a values (i.e. 4.8, 4.2) deliver diminishing returns in terms of acid removal. This is particularly evident in the case of benzoic acid (Figure 4-4B); at a *p*H of 5.4 D is reduced to 4.0, representing an almost 20-fold decrease from a PC of 70 at *p*H 3, yet 60% of the total amount of acid in the system is still contained in the polymer phase. In contrast, only 26% of the total butyric acid is present in the polymer phase at the same *p*H (Figure 4-4A), despite butyric acid possessing a higher pK_a value. Thus, although D is diminished in the case of both acids, appreciable product uptake can still be achieved through moderate *p*H reductions. At elevated PC values, as in the case of benzoic acid, these modest *p*H reductions result in substantial recovery, underlining the value of research currently being undertaken to identify polymers demonstrating improved partitioning through rational polymer selection

strategies (Bacon *et al.* 2014). In general however, these results show that while partitioning is negligible as *p*H approaches neutral values, improvements to uptake can still be achieved by small reductions in *p*H even above the pK_a value of a given acid, and high polymer fractions can capitalize on these small decreases in *p*H values.

4.5.3 High pCO_2 *p*H reductions for improving polymer absorption of acids

To achieve reductions in *p*H and thereby improve the uptake of acids by absorptive polymers in TPPBs, previous work (Hepburn and Daugulis 2012; Peterson and Daugulis 2014) investigated the use of CO_2 sparging at atmospheric conditions, and demonstrated reversible *p*H adjustments, which can be applied temporarily to facilitate acid uptake, after which the *p*H can be easily returned to fermentative values. To determine if increased partial pressures of CO_2 can provide further acid uptake through heightened CO_2 solubility, which would permit the formation of additional carbonic acid and more extensive *p*H reductions, partitioning tests for both butyric and benzoic acid were performed under elevated pressures of CO_2 for one hour. While it must be acknowledged that after one hour it is unlikely that equilibrium was achieved across phases, substantial absorption was achieved, and the reported high pCO_2 distribution coefficients and % recoveries reflect absorption achieved over one hour and thus, sorption kinetics are an important consideration as well when implementing absorptive extraction techniques. These distribution coefficients and mole fractions of the solutes in both Pebax ® 2533 and the aqueous phase were measured and are reported in Figure 4-5. Figure 4-5A shows that from an initial *p*H of 6.0, in the case of butyric acid D increased from 0.14 at atmospheric pressure to 3.0 at 60 bar pCO_2, resulting in an increase in % recovery in the polymer of from 3% to 40% as reflected by acid fraction in the polymer phase. For benzoic acid (Figure 4-5B) D rose from 0.69 to 24 under similar conditions, with a concurrent rise in % recovery of from 1 to 80%. These results demonstrate that increasing pCO_2 has a strong positive effect on solute partitioning into absorptive polymers and this is likely a result of

increased CO_2 solubility and associated *p*H reductions, which have been described previously (Meyssami *et al.* 1992).

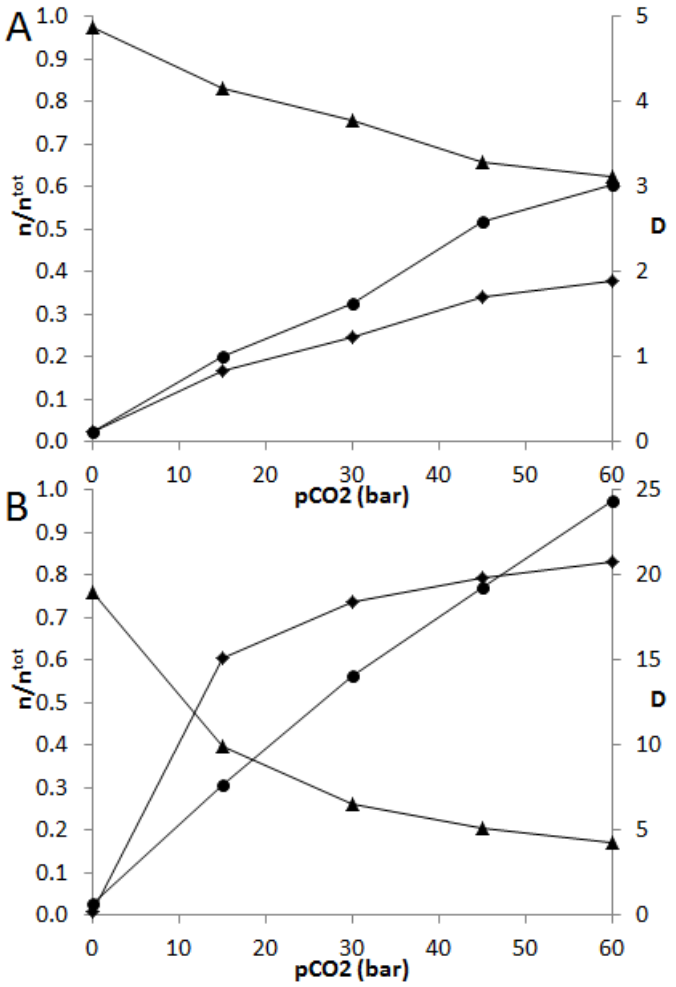


Figure 4-5 Measured species mole fractions and distribution coefficients of butyric acid in Pebax® 2533 (F=0.2) as a function of CO_2 partial pressure for A) butyric acid and B) benzoic acid after one hour. Initial concentrations of both butyric and benzoic acid were 2.5 g L^{-1} at *p*H 6, and diamonds and triangles represent n_{HA}^{pol}/n^{tot} and n_{tot}^{aq}/n^{tot} while circles represent distribution coefficients.

Similar to distribution profiles as discussed above, it is obvious from Figure 4-5 that in the case of benzoic acid, the largest gains in % recovery were made at lower pressures, rising to 60% at 15 bar pCO_2, after which diminishing returns were observed. In the case of butyric acid, % recovery was shown to benefit more generally from each increase of pressure, likely as a result of a lower PC in Pebax ® 2533. With regard to recovery of both acids however,

these results further demonstrate that small changes in *p*H result in a larger increase in partitioning, and these *p*H reductions can be achieved using high pressure CO_2.

Due to the weak acidic nature of butyric acid, it is likely that elevated acid concentrations can contribute some buffering strength against *p*H reductions afforded by CO_2 by partially hindering the extent of extraction achieved, and thus careful examination of the effect of initial acid concentration on achieved partitioning under high pressure is important. Figure 4-6 shows the results of partitioning tests conducted at 60 bar pCO_2 for increasing concentrations of butyric acid, and as can be seen, elevated butyric acid concentrations decrease both % recovery and distribution coefficients from 40% and 3 at 2.5 g L^{-1} to 30% and 1.9 at 50 g L^{-1}, respectively. While this decrease in uptake likely arises from increased buffering at higher acid concentrations, it is possible that this reduction is also a result of increases in *p*H resulting from acid absorption into the polymer, as described in Figure 4-3, as these *p*H changes were shown to increase at elevated butyric acid concentrations. However, while Figure 4-6 shows that some reduction in extraction occurs at typical butyric acid concentrations achieved during fermentation, substantial recovery into the polymer is still achieved, and it is interesting to note that at butyric acid concentrations higher than 20 g L^{-1}, no further reductions to % recovery are observed.

Overall, these results suggest that high pCO_2 mediated extraction may be effective in reducing *p*H and enhancing organic acid uptake by absorptive polymers regardless of acid concentration. As the equations for predicting distribution coefficients and acid species fractions in both phases (Equations 4. 6-4.9) were shown above to accurately describe measured respective values, these equations were used to predict the % recovery of butyric acid at 60 bar CO_2 across a range of polymer fractions and acid concentrations. For example, to calculate the % recovery after high pressure absorption at F=0.5 and an initial butyric acid concentration of 2.5 g L^{-1}, Equation (9) was manipulated to estimate that a *p*H of 4.4 was

achieved, if a distribution coefficient of 3.0 was observed after one hour at 60 bar pCO_2 (F=0.2). Figure 4-2 shows that at F=0.2 and F=0.5 changes in *p*H arising from absorption were 0.29 and 0.69 *p*H units respectively, and thus the estimated *p*H was adjusted from 4.4 to 4.8 to reflect this difference in F. Through Equation 4.6, this adjusted *p*H value was used to estimate that 52% of acid was absorbed into the polymer.

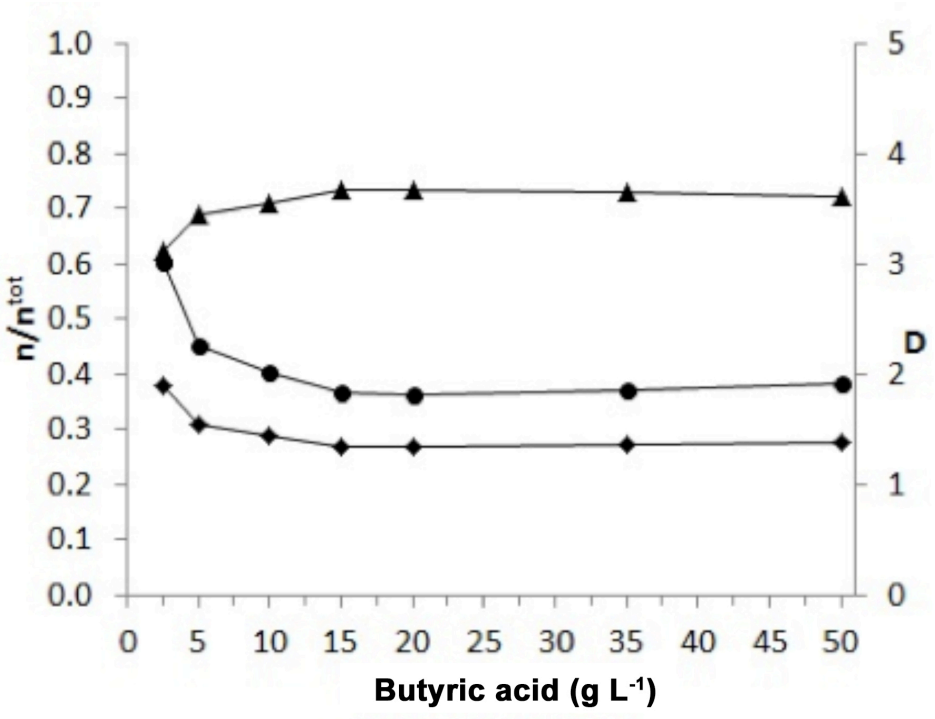
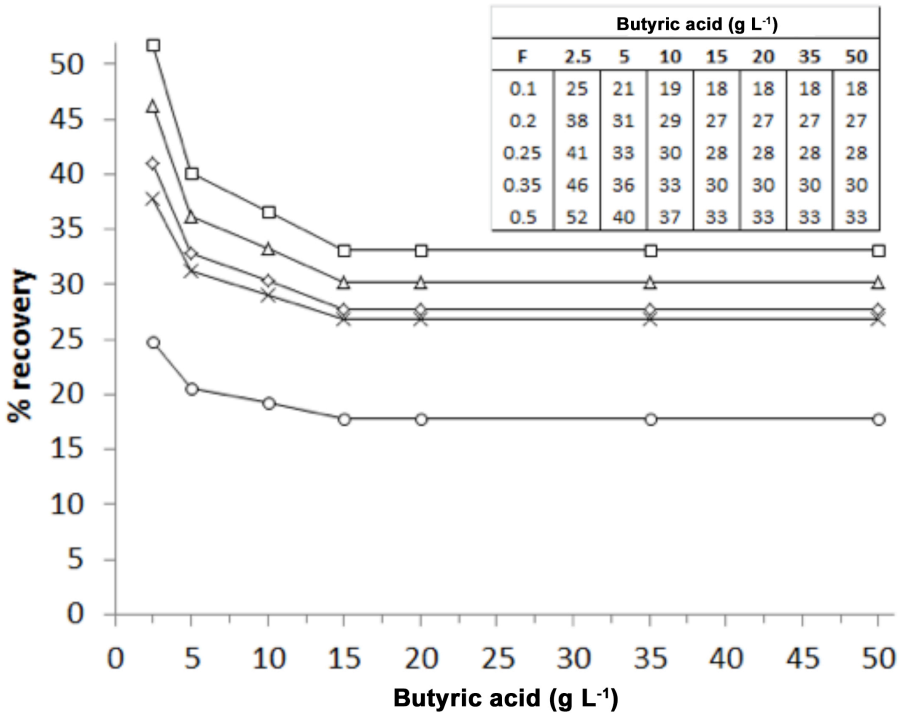


Figure 4-6 Measured species mole fractions and distribution coefficients of butyric acid in Pebax® 2533 (F=0.2) arising from *p*H reductions from a one hour exposure to 60 bar pCO_2 as a function of initial butyric acid concentration. Initial *p*H was 6.0, and diamonds and triangles represent n_{HA}^{pol}/n^{tot} and n_{tot}^{aq}/n^{tot} while circles represent distribution coefficients.

As shown in Figure 4-7, % recoveries across a range of polymer fractions were estimated in this manner using experimental high pressure partitioning data at different initial butyric acid concentrations. It can be seen that high pCO_2 butyric acid recovery using Pebax® 2533 could potentially be as high as 52% at F=0.5 at low butyric acid concentrations (2.5 g L^{-1}), with 33% recovery at 20 g L^{-1} and higher.

Furthermore, at 2.5 g L^{-1} butyric acid the % recovery is estimated to more than double, from 25% to 52%, as F is increased from F=0.1 to F=0.5. Thus, it is clear that increasing the

polymer fraction directly benefits % recovery regardless of butyric acid concentration and that F represents an important operating consideration when designing a process for achieving acid recovery through use of high pressure CO_2 and absorptive polymers. In general, considering the efficacy demonstrated here for reducing *p*H and improving partitioning, the use of high pCO_2 presents an attractive option for facilitating organic acid production in TPPBs, and future work will focus on its use during fermentations.



Butyric acid (g L^{-1})							
F	2.5	5	10	15	20	35	50
0.1	25	21	19	18	18	18	18
0.2	38	31	29	27	27	27	27
0.25	41	33	30	28	28	28	28
0.35	46	36	33	30	30	30	30
0.5	52	40	37	33	33	33	33

Figure 4-7 Estimated % recovery of butyric acid in Pebax ® 2533 at varied polymer fractions (F) through use of one hour exposures at 60 bar pCO_2. Circles, crosses, diamonds, triangles, and squares represent F= 0.1, 0.2, 0.25, 0.35, 0.50, respectively. Inset table reports numerical values for estimated product recovery at varied butyric acid concentrations and F values.

4.6 Conclusion

The work in this study has provided a framework for rigorously characterizing and predicting partitioning for all solutes in TPPBs that use absorptive polymers, and the concepts outlined here are intended to show particular applicability to ionizable solutes such as organic acids, which demonstrate much more complex partitioning behaviour than that of non-reactive solutes. Partitioning phenomena have been explored through investigation of organic acids

demonstrating both high and modest PC values across a range of solute concentrations and polymer fractions. It is clear that while absorptive uptake of organic acids can increase equilibrium *p*H values, absorption overall is strictly dictated by *p*H and pK_a, and this constraint is not influenced by the degree of partitioning achieved. In the case of both benzoic and butyric acid, it was shown that small reductions in *p*H from neutral values yield large returns in terms of recovery, and by careful consideration of PC and polymer fraction, conditions resulting in maximized partitioning at a given *p*H can be estimated.

High pCO_2 extraction enables such *p*H changes, and has been demonstrated to achieve improved partitioning through *p*H reduction, with PC and F also playing key roles in influencing acid recovery. This high pressure technique shows promise for applying TPPB technology to organic acid production by providing a method of alternating between fermentative *p*H values and those suitable for achieving partitioning, as long as cells can tolerate high pCO_2. Overall, this study provides not only a framework for understanding organic acid partitioning but also a roadmap for application of high pressure CO_2 to overcome *p*H-dependence limitations based on both first-principles acid partitioning theory and experimental results, and could be extended to the production of other acids.

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4.8 Supplemental

S.1 Derivations for predicting mole fractions and distribution coefficients at equilibrium for non-reactive solutes in two-phase partitioning bioreactors

Legend:

n_{tot} = total moles of solute
n_S^{aq} = moles of solute in the aqueous phase
n_S^{pol} = moles of solute in the polymer phase
PC = partitioncoefficient
m_{aq} = aqueous mass
m_{pol} = polymer mass

$$n^{tot} = n_S^{aq} + n_S^{pol} \quad (1)$$

$$PC = \frac{[S]^{pol}}{[S]^{aq}} = \frac{n_S^{pol}/m_{pol}}{n_S^{aq}/m_{aq}} \quad (2)$$

$$n_S^{aq} = \frac{n_S^{pol} \cdot m_{aq}}{PC \cdot m_{pol}} \cdot \quad (2a)$$

Substitute (2a) into (1)

$$n^{tot} = \frac{n_S^{pol} \cdot m_{aq}}{PC \cdot m_{pol}} + n_S^{pol}$$

$$n^{tot} = n_S^{pol} \cdot \left[1 + \frac{m_{aq}}{PC \cdot m_{pol}}\right]$$

$$n^{tot} = n_S^{pol} \cdot \left[\frac{PC \cdot m_{pol} + m_{aq}}{PC \cdot m_{pol}}\right]$$

$$n_S^{pol} = n^{tot} \cdot \frac{PC \cdot m_{pol}}{PC \cdot m_{pol} + m_{aq}} \quad (3)$$

Substitute (3) into (2a)

$$n_S^{aq} = n^{tot} \cdot \frac{m_{aq}}{PC \cdot m_{pol} + m_{aq}} \quad (4)$$

if

$$F = \frac{m_{pol}}{m_{aq}} \quad (5)$$

then

$$\frac{n_S^{pol}}{n^{tot}} = \frac{F \cdot PC}{F \cdot PC + 1} \quad (4a)$$

$$\frac{n_S^{aq}}{n^{tot}} = \frac{1}{F \cdot PC + 1} \quad (5a)$$

S.2 Derivations for predicting species mole fractions and distribution coefficients at equilibrium for organic acids in two-phase partitioning bioreactors

Legend:

n^{tot} = total moles acid
n_{HA}^{aq} = moles acid in aqueous phase
n_{HA}^{pol} = moles acid in polymer phase
n_{A-}^{aq} = moles conjugate base in aqueous
PC = partitioncoefficient
D = distribution coefficient
m_{aq} = aqueous mass
m_{pol} = polymer mass

$$n^{tot} = n_{HA}^{aq} + n_{HA}^{pol} + n_{A-}^{aq} \quad (1)$$

$$PC = \frac{[HA]^{pol}}{[HA]^{aq}} = \frac{n_{HA}^{pol}/m_{pol}}{n_{HA}^{aq}/m_{aq}} \quad (2)$$

$$n_{HA}^{aq} = \frac{n_{HA}^{pol} \cdot m_{aq}}{PC \cdot m_{pol}}. \quad (2a)$$

$$D = \frac{[HA]^{pol}}{[HA]^{aq}+[A-]^{aq}} = \frac{n_{HA}^{pol}/m_{pol}}{(n_{HA}^{aq}+n_{A-}^{aq})/m_{aq}} \quad (3)$$

$$K_a = \frac{[A^-]^{aq} \cdot [H^+]^{aq}}{[HA]^{aq}} \quad (4)$$

$$pH = pK_a + \log\frac{[A^-]^{aq}}{[HA]^{aq}} \quad (4a)$$

$$\frac{[A^-]^{aq}}{[HA]^{aq}} = 10^{(pH-pK_a)} = \frac{n_{A-}^{aq}}{n_{HA}^{aq}} \quad (4b)$$

$$n_{A-}^{aq} = n_{HA}^{aq} \cdot 10^{(pH-pK_a)} \quad (4c)$$

Substitute (4c) in (1)

$$n^{tot} = n_{HA}^{aq} + n_{HA}^{pol} + n_{HA}^{aq} \cdot 10^{(pH-pK_a)} \quad (5)$$

$$n^{tot} = n_{HA}^{aq}\left[1 + 10^{(pH-pK_a)}\right] + n_{HA}^{pol} \quad (5a)$$

Substitute (2a) into (5a)

$$n^{tot} = \frac{n_{HA}^{pol} \cdot m_{aq} * \left[1+10^{(pH-pK_a)}\right]}{PC \cdot m_{pol}} + n_{HA}^{pol}$$

$$n^{tot} = n_{HA}^{pol} \cdot \left[1 + \frac{m_{aq} \cdot \left[1+10^{(pH-pK_a)}\right]}{PC \cdot m_{pol}}\right]$$

$$n^{tot} = n_{HA}^{pol} \cdot \left[\frac{PC \cdot m_{pol} + m_{aq} \cdot \left[1+10^{(pH-pK_a)}\right]}{PC \cdot m_{pol}}\right]$$

$$n_{HA}^{pol} = n_{tot} \cdot \frac{PC \cdot m_{pol}}{PC \cdot m_{pol} + m_{aq} \cdot \left[1+10^{(pH-pK_a)}\right]} \quad (6)$$

Substitute (6) into (2a)

$$n_{HA}^{aq} = n^{tot} \cdot \frac{m_{aq}}{PC \cdot m_{pol} + m_{aq} \cdot \left[1+10^{(pH-pK_a)}\right]} \quad (7)$$

Substitute (7) into (4c)

$$n_{A-}^{aq} = n^{tot} \cdot \frac{m_{aq} \cdot 10^{(pH-pK_a)}}{PC \cdot m_{pol} + m_{aq} \cdot \left[1+10^{(pH-pK_a)}\right]} \quad (8)$$

if

$$F = \frac{m_{pol}}{m_{aq}} \quad (9)$$

then

$$\frac{n_{HA}^{pol}}{n^{tot}} = \frac{F \cdot PC}{F \cdot PC + \left[1+10^{(pH-pK_a)}\right]} \quad (6a)$$

$$\frac{n_{HA}^{aq}}{n^{tot}} = \frac{1}{F \cdot PC + \left[1+10^{(pH-pK_a)}\right]} \quad (7a)$$

$$\frac{n_{A-}^{aq}}{n^{tot}} \cdot = \frac{10^{(pH-pK_a)}}{F \cdot PC + \left[1+10^{(pH-pK_a)}\right]} \quad (8a)$$

Substitute (6), (7), (8) into (3)

$$D = \frac{F \cdot PC / m_{pol}}{\left(1+10^{(pH-pK_a)}\right)/m_{aq}} \quad (10)$$

Chapter 5

The use of high pressure CO_2-facilitated *p*H swings to enhance *in situ* product recovery of butyric acid in a two-phase partitioning bioreactor

With minor changes to fulfill formatting requirements, this chapter is substantially as submitted to *Biotechnology and Bioengineering*

5.1 Preface to chapter 5

Chapter 5 represents work done toward integrating high pCO_2 *p*H reductions into the biological production of butyric acid to achieve ISPR with absorptive polymers and demonstrate any possible benefits as a result of this recovery, such as improved yields and productivities. As a first step, this work compares *p*H values achieved in the presence of medium components at both atmospheric conditions and 60 bar CO_2, to determine if the restrictions these components place on *p*H reductions can be overcome through use of elevated pressure. This work also determines the relationships between high pCO_2 *p*H reductions and partitioning in medium with or without the additional buffering strength of butyric acid, as previous partitioning tests were performed in water. Importantly, this chapter investigates biological tolerance to high pCO_2, and examines what effect 60 bar pCO_2 exerts on cell growth, while also determining the effect of exposure time on cell viability as well.

Subsequently, this chapter outlines approaches for incorporating high pCO_2 *p*H reduction methods into a TPPB, and application to butyric acid fermentations. This resulted in improved overall process performance in terms of yield and productivity, as well as a reduced need for *p*H control, and all of this resulted from butyric acid removal. It must be noted that polymeric absorption of butyric acid was performed in a separate high pressure vessel, rather than via direct inclusion of polymers into the bioreactor, as a return to near neutral *p*H values would result in desorption of recovered acid. However, an indirect advantage of this separation is the ease with which polymer exchanges can be performed. Importantly, rapid polymer regeneration using alkali desorptions was achieved, demonstrating the ease with which desorption is performed, and thus the approaches outlined in this chapter represents a robust method for the recovery of butyric acid from fermentations. Reactors could be operated with this technique for substantially longer periods at high productivity through combination of high substrate loading fed-batch methods and semi-continuous acid removal. Thus, this chapter

clearly outlines an operational roadmap for the use of high pCO_2 to achieve improved absorption during biological production of organic acids.

5.2 Abstract

Through the use of high partial pressures of CO_2 (pCO_2) to facilitate temporary *p*H reductions in two-phase partitioning bioreactors (TPPBs), improved *p*H dependent partitioning of butyric acid was observed which achieved *in situ* product recovery (ISPR), alleviating end-product inhibition (EPI) during the production of butyric acid by *Clostridium tyrobutyricum* (ATCC 25755). Through high pressure pCO_2 studies, media buffering effects were shown to be substantially overcome at 60 bar pCO_2, resulting in effective extraction of the organic acid by the absorptive polymer Pebax® 2533, yielding a distribution coefficient (D) of 2.4 ± 0.1 after one hour of contact at this pressure. Importantly, it was also found that *C. tyrobutyricum* cultures were able to withstand 60 bar pCO_2 for one hour with no decrease in growth ability when returned to atmospheric pressure in batch reactors after several extraction cycles. A 2 L fed-batch reactor with cyclic high pCO_2 polymer extraction recovered 92 g of butyric acid to produce a total of 213 g compared to 121 g generated in a 2 L control reactor. This recovery reduced EPI in the TPPB, resulting in both higher productivity (0.65g L^{-1} h^{-1} vs. 0.33 g L^{-1} h^{-1}) and yield (0.54 vs 0.40). Fortuitously, it was also found that repeated high pCO_2-facilitated polymer extractions of butyric acid during batch growth of *C. tyrobutyricum* lessened the need for *p*H control, and reduced base requirements by approximately 50%. Thus, high pCO_2-mediated absorptive polymer extraction presents a novel method for improving process performance in butyric acid fermentation, and this technique could be applied to the bioproduction of other organic acids as well.

Keywords: *Clostridium tyrobutyricum*, butyric acid, two-phase partitioning bioreactor (TPPB), *in situ* product recovery (ISPR), carbon dioxide, high pressure

5.3 Introduction

The biological production of organic acids as commodity chemical feedstocks represents a sustainable alternative to the use of petrochemicals, and industrial bioproduction is on the increase, with interest in a range of organic acids (Sauer *et al*. 2008; Weusthuis *et al*. 2011). To be competitive with petrochemically-derived organic acids, cost reduction is a critical consideration (Kurzrock and Weuster-Botz 2010), and both increased biological performance and improved acid recovery would generate significant benefits, whether through increased productivity, or reduced separation costs. Solid-liquid two-phase partitioning bioreactors (TPPBs) (Daugulis *et al*. 2011) can be used to achieve both these goals simultaneously, by absorbing an end-product into an inexpensive, easy-to-handle absorptive polymer phase, which is typically added directly to the bioreactor, achieving *in situ* product recovery (ISPR) (Prpich and Daugulis 2004; Morrish and Daugulis 2008; Khan and Daugulis 2010; Dafoe and Daugulis 2011; Craig and Daugulis 2013; Peterson and Daugulis 2014). Aside from the ease of recovery afforded by physical separation of solid polymers from fermentation broth, the reduction of end-product concentrations reduces end-product inhibition (EPI) during a fermentation, which in turn can improve productivities, yields and titres.

In the case of organic acids, however, absorptive removal by polymers is particularly challenging. Partitioning will occur only with protonated species of a given acid (Kertes and King 1986), and thus partitioning improves significantly at lower *p*H values, which can be problematic for fermentations, as they often require near-neutral *p*H values during operation. To overcome these mutually exclusive *p*H values, previous work (Peterson and Daugulis 2014) focused on the use of CO_2 sparging during butyric acid fermentations with *Clostridium tyrobutyricum* to lower *p*H values temporarily while recycling reactor contents through a column packed with an absorptive polymer, after which the *p*H could be readily returned to near neutral values. Unlike reactive extraction techniques, which employ toxic extractants to recover organic acids, extraction through CO_2-mediated *p*H swings does not result in cell toxicity (Peterson and Daugulis 2014) and these swings are easily and quickly reversible

(Hepburn and Daugulis 2012). While previous work has shown that CO_2 at atmospheric pressure makes a significant improvement in polymer extraction over CO_2 free controls, relatively modest extraction was achieved (Peterson and Daugulis 2014), resulting in no substantial improvement in process performance. This was because while CO_2 at atmospheric pressure can quickly achieve low *p*H values in water, medium components and butyric acid both increased buffering, which in turn prevented the desired *p*H drop and thus limited subsequent extraction. However, it may be possible that the increased solubility of CO_2 afforded by increased pressures (Dodds *et al.* 1956) may be able to overcome such buffering from fermentation broth.

High pCO_2-faciliated extraction presents a promising method for improving extraction of organic acids by lowering *p*H substantially more than is possible under atmospheric conditions due to increases in CO_2 solubility. The relationship between *p*H and CO_2 partial pressures is well documented (Meyssami *et al.* 1992), and elevated pressures have been shown to lower *p*H values to as low as 3.2 in water. However, medium components have been shown to restrict *p*H swings under high pCO_2 similar to tests under atmospheric conditions, as buffering effects from phosphate (Bortoluzzi *et al.* 2011) and complex medium additives (Garcia-Gonzalez *et al*. 2010) have been observed, and minimization of such components has shown increased *p*H swings (Peterson and Daugulis, 2014). Additionally, the presence of an organic acid has also been shown to restrict *p*H drops under high pCO_2 (Meyssami *et al*. 1992). Therefore, if high pCO_2 extractions are to be applied to fermentations, it is important to determine to what extent high pressure can improve *p*H depression in the presence of medium components, while also determining the effectiveness of such pressures to facilitate butyric acid extraction in a given medium under increasing acid concentrations.

Importantly, additional concerns need to be addressed if ISPR of butyric acid is to be achieved through high pCO_2. Specifically, as the goal of ISPR is to improve process performance, it is essential that any extraction techniques applied do not incur adverse effects on microbial growth or activity. Numerous studies have documented the use of high pressure

and supercritical CO_2 for inactivation of various microorganisms (Bertoloni *et a*l. 2006; Debs-Louka *et al.* 1999; Spilimbergo *et al.* 2002; Spilimbergo *et al*. 2009), highlighting the importance of such considerations. However, it has been shown that tolerance to high pCO_2 varies between organisms (Jones and Greenfield 1982), and also that microbial inactivation requires exposure for defined periods of time to be effective (Debs-Louka *et al.* 1999). Thus, for a given microorganism it may be possible to pressurize fermentation broth for short periods without inflicting serious harm on cells. This is supported by the work of L'italien *et al.* (1989), who demonstrated that *S. cerevisiae* was capable of repeatedly tolerating 70 bar pCO_2 for one hour, with an immediate return to ethanol production upon depressurization to atmospheric conditions, suggesting online cyclical pressurization with elevated pCO_2 is feasible in some cases. Overall, if high pCO_2 is to be used as an agent for achieving ISPR, it is paramount that extractive conditions be identified that do no harm to cell growth or process performance.

The objective of this study was to investigate the use of elevated partial pressures of CO_2 during fermentation to overcome buffering and permit improved ISPR of butyric acid. This was achieved through study of the effect of medium components on *p*H swings, extraction afforded by increasing pressures in medium with or without butyric acid, and cell tolerance to such pressures, followed by integration of high pressure extraction into batch and fed-batch reactors for ISPR demonstration. The techniques outlined herein represent a novel, non-toxic extractive approach to improving fermentation of butyric acid, and may be applied to the production of other organic acids.

5.4 Materials and Methods

5.4.1 Organism, medium, and materials

C. tyrobutyricum (ATCC 25755) was initially grown on medium described elsewhere (Wu and Yang 2003) and cryopreserved in 15% glycerol at -75 °C until needed. All further tests utilizing medium employed a formula comprised of yeast extract, 5 g L^{-1}; $(NH_4)_2SO_4$, 3 g L^{-1}; $MgSO_4$ 7·H_2O, 0.6 g L^{-1}; K_2PO_4, 0.3 g L^{-1}; $FeSO_4$·7·H_2O, 0.03 g L^{-1}. Pebax® 2533, an

absorptive polymer, was kindly donated by Arkema Inc.. All chemicals used in this study were purchased from Fisher Scientific Company, Ltd (Ottawa, Canada)

5.4.2 Polymer Selection

Pebax ® 2533 (Arkema Inc.), a polyether block amide copolymer, was selected as an absorbent phase on the basis of both its chemical affinity for butyric acid as well as its handling properties, as previous work (Peterson and Daugulis 2014) identified several polyether copolymers demonstrating good affinity for butyric acid. As Pebax ® 2533 demonstrates improved physical properties (i.e. structurally withstands rigorous mixing and handling required for use in TPPBs), this copolymer was chosen over a PBO homopolymer. Pebax ® 2533, which recovers butyric acid through absorption rather than adsorption, yields a partition coefficient of 4.1 for butyric acid (Peterson and Daugulis 2014). It must be noted that distribution coefficients (D), which are widely discussed in this work, represent *p*H-dependent partitioning at a given *p*H. Thus, distribution coefficients decrease with increasing *p*H and are dynamic, and cannot exceed the partition coefficient, which is constant and represents partitioning under fully protonated conditions.

5.4.3 *p*H shifting under high pressure and atmospheric conditions

To determine what effect high pCO_2 can have on *p*H reduction, changes to *p*H were studied in the presence of dibasic phosphate and yeast extract, as previous work had demonstrated the buffering effects of these medium components (Hepburn and Daugulis 2012; Peterson and Daugulis 2014). Direct *p*H determination at high pCO_2 was achieved by observing the spectra of samples loaded with Bromophenol Blue (10 mg L^{-1}) in a 10 mL stainless steel high-pressure view cell (Parr Instrument Company, Moline, IL, USA) with sapphire cell windows equipped for use in a UV-VIS spectrometer (Toews *et al.* 1995), wherein the ratio of the absorbance of the acidic form (430 nm) and the basic form (590 nm) of Bromophenol blue can accurately quantify *p*H colorimetrically within *p*H values 3.0-4.6. Initial tests were performed on RO water to determine maximum *p*H reduction, to be used as a

reference. A maximum pressure of 60 bar pCO_2 was selected as the upper end of test pressure, as this was determined to be the upper limit of CO_2 solubility before reaching critical conditions under moderate temperatures (i.e. 25-37 °C) (Dodds *et al.* 1956), thus providing a maximum theoretical potential for *p*H reduction. 10 mL samples were pressurized for one hour, with stirring provided by a magnetic stirplate. Concentrations of 0.3 g L^{-1} potassium dibasic phosphate and 5 g L^{-1} yeast extract, which reflect medium composition levels used for fermentations, were tested in RO water to determine achievable *p*H reductions. CO_2 at atmospheric pressure sparging tests were also performed to provide comparison to high pressure experiments in 5 L Bioflo III reactors with a 2 L working volume (New Brunswick Scientific, Edison, NJ) at 1 VVM CO_2, 200 rpm, and 25 °C without *p*H control to provide similar conditions to that in the pressure vessel for comparison.

5.4.4 High pCO_2-mediated extraction of butyric acid

To determine the impact of increased pCO_2 and butyric acid extraction from medium, 100 g of Pebax® 2533 was added to a 1 L Parr pressure vessel equipped with an internally threaded sampling tube (Parr Instrument Company, Moline, IL, USA) along with 500 mL of medium as described above, which also contained 5 g L^{-1} butyric acid. 3 M KOH was used to adjust *p*H to 6.0, as this value reflects optimal *p*H values maintained in butyric acid fermentation by *C. tyrobutyricum*. The vessel was then sealed and pressurized to 15, 30, 45 and 60 bar pCO_2 and mixed at 500 rpm for one hour, after which the vessel was drained under pressure, and the final aqueous concentration of butyric acid was determined to ascertain achieved extraction. A similar experiment was performed to determine the extent to which increasing butyric acid concentrations reduced extraction through elevated buffering strength. In this case, extraction at 60 bar pCO_2 at butyric acid concentrations of 5, 10, 15 and 20 g L^{-1} in medium *p*H-adjusted to 6.0 was tested as described above. All tests were performed in triplicate and compared to untested medium samples as controls.

5.4.5 Culture conditions

Unless otherwise stated, tests involving *C. tyrobutyricum* were performed with 100 g L^{-1} glucose in 5 L BioFlo III reactors with a 2 L working volume under anaerobic conditions at 37 °C, 200 rpm agitation and 0.25 VVM N_2 sparging, while *p*H was controlled to 6.0 by addition of 3 M KOH and H_2SO_4. All reactors were inoculated with cells grown on 10 g L^{-1} glucose over 18 hours first in 150 mL anaerobic serum bottles, then in sealed anaerobic 500 mL shake flasks with closable vents and a spargeline, after which the inoculum was added anaerobically to reactors (10% v/v). For all reactors, when the butyric acid concentrations were determined to remain constant for at least 12 hours the earliest time point at this concentration was considered to be the fermentation endpoint.

5.4.6 Cell tolerance to high pCO_2

To determine tolerance of *C. tyrobutyricum* to 60 bar pCO_2 exposure, a batch reactor was prepared as described above. Once the cells had achieved stationary phase, 750 mL of fermentation broth was anaerobically transferred to an autoclaved 1 L Parr vessel, and pressurized to 60 bar pCO_2. 100 mL aliquots were drawn off under pressure after 1, 2 and 3 hour intervals into aseptic anaerobic flasks. These aliquots were then used as inocula (10% v/v) for 24 hour serum bottle growth studies using 10 g L^{-1} glucose in medium, and the difference in growth between treatments was used to ascertain how long cells could withstand exposure without affecting cell viability.

To more definitively test the effect that a pressurized extraction regime could exert on the cells, a batch reactor was prepared as described above. Once acid production was observed to be well under way, as indicated by consumption of 250 mL of 3 M KOH, 500 mL of fermentation broth was transferred to a sterilized pressure vessel in the absence of a polymer phase and exposed to 60 bar pCO_2 for one hour with mixing at 500 rpm. After one hour the contents of the high pressure vessel were returned to the fermenter and after a rest period of three hours, the process was repeated, up to a total of 4 times after which the fermentation proceeded without further disruption. To determine what effect the cyclical pressurization

regime exerted on process performance, a batch reactor was ran under identical conditions without pressurization to act as a control.

5.4.7 Batch mode high pCO_2 online extraction of butyric acid

To demonstrate butyric acid extraction with an absorptive polymer through use of high pCO_2 cycling during fermentation, a batch reactor coupled to a cyclical high pressure extraction procedure was performed, wherein 500 mL of fermentation broth was transferred to a sterilized pressure vessel loaded with 300 g Pebax® 2533, and pressurized to 60 bar pCO_2 and mixed at 500 rpm for one hour, after which the liquid contents were returned to the bioreactor. After this extraction, a rest period of three hours was allowed, and the extractive procedure was repeated for a total of four extraction cycles. Extractions were commenced once substantial acid production was observed, as indicated by the consumption of 250 mL 3 M KOH for *p*H control. Within the timeframe in which extractions were performed, *p*H control was turned off, and the *p*H of the system was monitored. As Pebax® 2533 cannot withstand steam sterilization, to ensure adequate polymer sterility, prior to extraction the absorptive polymer phase utilized in these experiments was soaked overnight in 70% methanol at a polymer fraction (F) of 0.3 (g polymer/g solvent), followed by three overnight desorptions in RO water at the same F value, after which the polymer was spread to dry in a laminar flow hood under aseptic conditions. Polymers masses employed in extractions were desorbed sequentially three times for 12 hours in 0.25 M KOH under shaking (F=0.3), and butyric acid concentrations at the end of each desorption step were recorded.

5.4.8 Fed batch pCO_2 online extraction of butyric acid and polymer regeneration

To demonstrate that high pCO_2 extraction can be repetitively performed over the span of extended fermentations, a fed-batch reactor was employed. The reactor was operated in batch mode until glucose values were observed to be less than 10 g L^{-1}, at which point a 500 mL bolus consisting of glucose and medium components was added to yield a working volume of 2.5 L at original glucose and medium concentrations. Cyclical extraction events as described

above were initiated once 250 mL of 3 M KOH had been consumed, and three one-hour extractions separated by three-hour resting periods were performed, for a combined extraction window of 9 hours. After the initial three extractions were completed, the fermentation proceeded uninterrupted for 15 hours, excluding sampling. At this point (i.e. 24 hours from commencement of extraction), the same regime of three extractions was repeated, with identical resting times. This 24 hour extraction procedure was performed twice more for a total of 4 24-hour cycles, and represents 12 one-hour extractions over the span of the fermentation. After each extraction, the polymer mass was removed from the pressure vessel, and soaked aseptically in 0.25 N KOH (F=0.3) on a shaker for 12 hours, after which the alkali desorption solution was sampled and quantified to determine extracted butyric acid. Following this, the polymer mass was rinsed with sterile RO water twice (F=0.3) and dried aseptically in a laminar flow hood, after which it was reused for a second round of extractions. Thus, the polymer masses from the first and second extraction windows were reused for the third and fourth extraction windows, respectively. To provide a reference for comparison of process performance, a fed-batch reactor was performed under identical conditions without a high pCO_2 extraction regime to act as a control.

5.4.9 Analytical methods

Aqueous samples were analyzed for glucose, butyric and acetic acid using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 × 7.7 mm) at 60 °C with a 10 mM H_2SO_4 mobile phase at 0.7 mL min^{-1}, with either a refractive index detector (PS 356) for glucose quantification, or a UV-Vis detector (Varian Prostar, PS325) at 220 nm for quantification of butyric and acetic acid. Cell concentration was measured using optical density at 600 nm, and translated to cell dry weight via a predetermined calibration curve.

5.5 Results and Discussion

5.5.1 High pCO_2 extraction and the buffering effect of medium components

If higher pCO_2 levels are to be employed for increased polymer extraction, to ensure that *p*H values are less or equal to the pK_a of butyric acid (4.8) it is important to first characterize how low the *p*H can be reduced at elevated pressures, compared to reductions achieved through atmospheric gas sparging. As yeast extract and phosphate were earlier identified to be the primary sources of buffering in the medium used here, concentrations of these two components reflecting medium composition were tested at 60 bar. As can be seen in Figure 5-1, the *p*H values achieved for both yeast extract (*p*H 3.8) and phosphate (*p*H 3.2) under 60 bar pCO_2 are significantly lower than respective values of 5.2 and 4.7 under atmospheric pressure, demonstrating that elevated pCO_2 improves the extent of *p*H swings, which indicates that the use of high partial pressures of CO_2 could improve *p*H-dependent extraction. This *p*H dependency is determined by pK_a, and only *p*H values near or below this value will demonstrate appreciable uptake by an absorptive polymer. Thus, as the pK_a of butyric acid is 4.8, significant partitioning could be expected in the presence of 5 g L^{-1} yeast extract and 0.3 g L^{-1} phosphate.

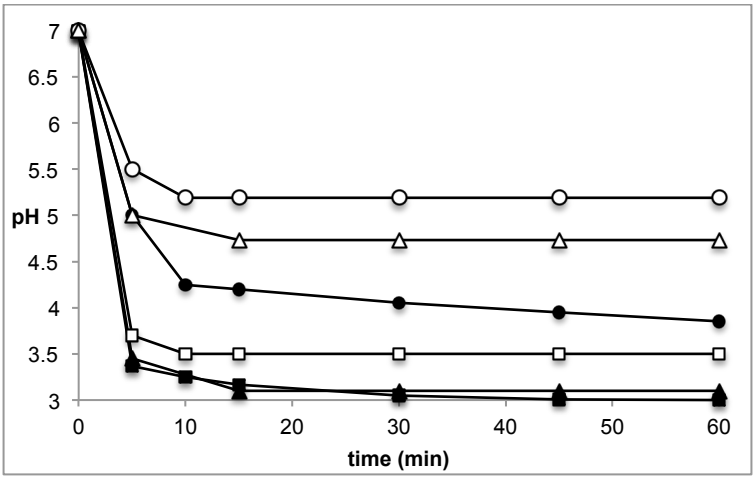

Figure 5-1 *p*H values achieved with pCO_2 in water (squares), 5 g L^{-1} yeast extract (circles) or 0.3 g L^{-1} phosphate (triangles) at through pressurization to 60 bar pCO_2 (closed symbols) or through sparging at atmospheric pressures (open symbols)

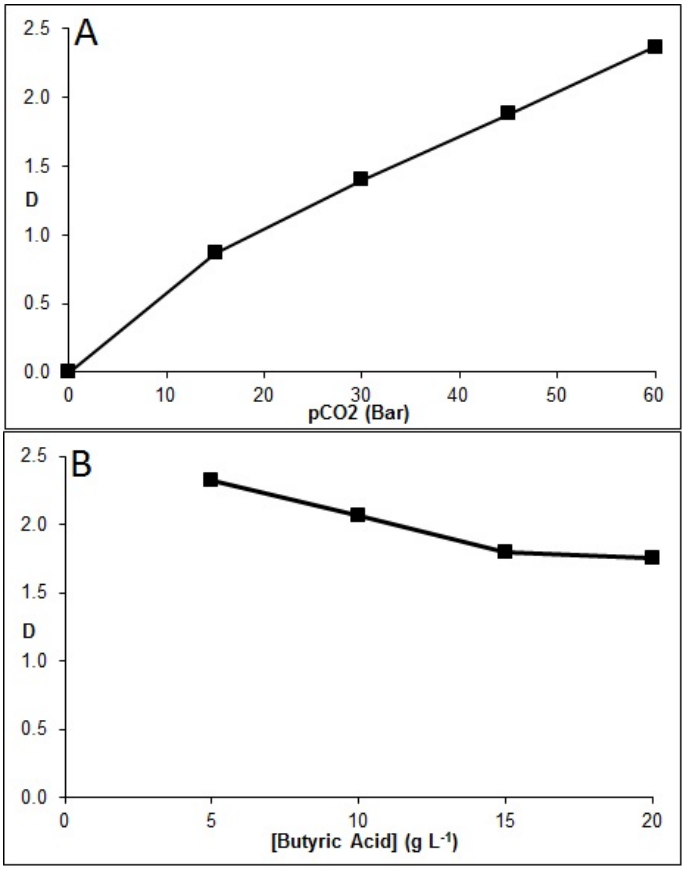

Figure 5-2 The effect of A) pCO_2 on distribution coefficients for 5 g L^{-1} butyric acid B) butyric acid concentration on distribution coefficients for butyric acid at 60 bar pCO_2. All tests were performed with Pebax ® 2533 as the absorptive polymer phase.

To quantify the relationship between pressure and polymer absorption, tests were performed at increasing pCO_2 values as shown in Figure 5-2a, which plots distribution coefficients achieved after one hour against increasing pressure conditions, and it is apparent that a direct relationship exists between pCO_2 and uptake, with results rising to a maximum value at 60 bar pCO_2 (D= 2.4 ± 0.1). Thus, as 60 bar pCO_2 provided the highest possible polymer extraction without risk of reaching undesirable supercritical conditions, this level of pCO2 was selected for online butyric acid extraction.

However, as has been previously noted (Peterson and Daugulis 2014), butyric acid also contributes buffering strength to fermentation medium, potentially limiting the extent to which *p*H can be reduced by CO_2 sparging. Thus, as a fermentation proceeds and butyric acid accumulates, a decrease in distribution coefficients would be anticipated. Figure 5-2b

demonstrates distribution coefficients achieved at 60 bar pCO_2 as a function of butyric acid concentration, and it can be seen that D decreases with increasing acid concentration, and this effect was most pronounced with 20 g L^{-1} butyric acid, yielding a distribution coefficient of 1.8 ± 0.03. However, such reductions do not critically limit butyric acid removal, as D represents the ratio of butyric acid between polymer and aqueous phases, and thus at higher acid concentrations, although a reduced ratio of uptake is achieved, the higher amounts of butyric acid provides a higher concentration driving force for equilibrium-based partitioning. For example, in the above described experiments, while 60 bar pCO_2 in the presence of 5 g L^{-1} butyric acid removes 0.8 g acid (D=2.4), in the presence of 20 g L butyric acid 2.6 g L^{-1} (D=1.8) is absorbed. Thus, while proportionally less acid is recovered at increasing concentrations, the overall amount of butyric acid absorbed by the polymer is increased, demonstrating that the buffering effect of the acid is outweighed by the increased driving force provided by elevated concentrations. Therefore, high pCO_2 polymer extractions can be performed over a seemingly wide range of butyric acid concentrations, and will not lose effectiveness as butyric concentration rise toward the end of fermentations.

5.5.2 Cell tolerance to high pCO_2

While it has been demonstrated that elevated pCO_2 results in increased distribution coefficients, the impact of increased pressure on cell viability and performance are of critical importance, if such an extraction process is to be applied to achieve ISPR in fermentations. Figure 5-3 shows OD growth curves for serum bottles inoculated with cells exposed to 60 bar pCO_2 for 1, 2 or 3 hours and it can be seen that no difference was observed between a one hour exposure and a non-exposed control, while a two hour exposure showed a slight decrease in subsequent cell growth.

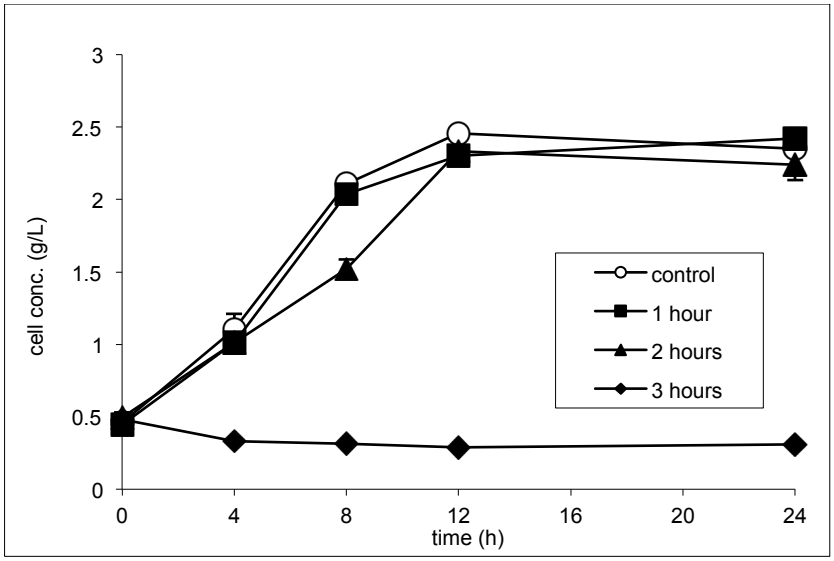

Figure 5-3 *C. tyrobutyricum* tolerance to 60 bar pCO_2, as determined by subsequent growth after one hour (squares), two hour (triangles) or three hour (diamonds) exposures, compared to a non-pressurized control (open circles).

A 3-hour exposure however failed to show signs of growth after 24 hours, indicating that *C. tyrobutyricum* cannot withstand 60 bar pCO_2 indefinitely. These results suggest that elevated pressure intervals of 1-2 hours could be performed during a fermentation without deleterious effect. Figure 5-4B shows a batch reactor which was subjected to repeated one hour pressurization cycles in the absence of polymer alongside a control run (Figure 5-4A) operated at atmospheric pressure, to investigate what effect such a cycling regime would exert on an actual fermentation. As can be seen in Table 5-I, which summarizes the performance shown in Figures 5-4A and 5-4B, no differences were observed between the batch control and the cyclically pressurized batch run in terms of butyric acid titre (68-72 g L^{-1} butyric acid), yield (0.35-0.37 g butyric acid produced/g glucose consumed) or productivity (0.50 g L^{-1} h^{-1}). However, the selectivity for butyric acid in the cyclically pressurized reactor was somewhat lower at 0.74 (g butyric acid/g total acid) compared to 0.84 in the control as a result of increased acetic acid by-product formation. This result is not unanticipated, as studies have shown selectivity is decreased at *p*H values lower than 6 (Zhu and Yang 2004).

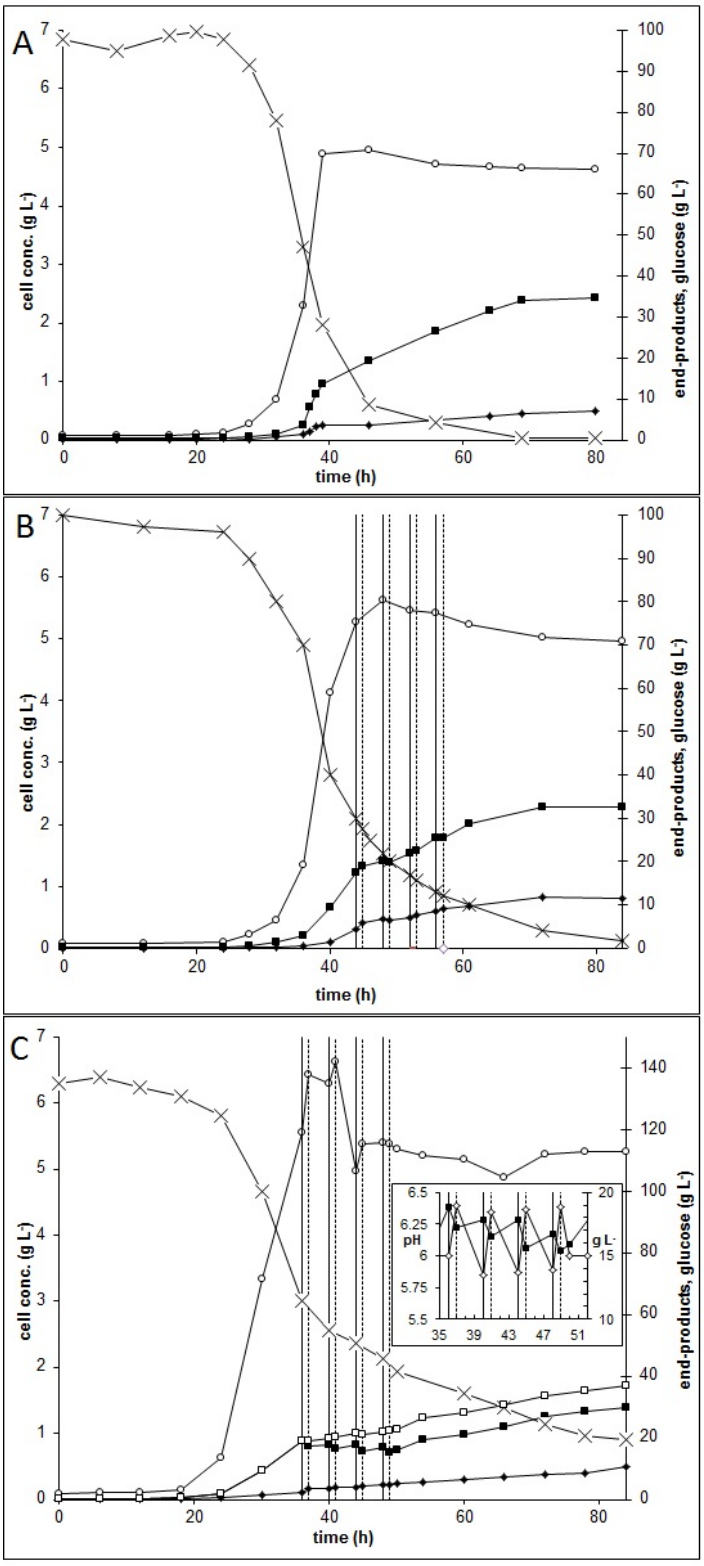

Figure 5-4 Batch fermentation of butyric acid from *C. tyrobutyricum* under A) conventional culture techniques, B) cyclical pressurization at 60 bar pCO_2, or C) cyclical pressurization and use of an absorptive polymer to achieve ISPR. Open circles represent OD, solid squares represent aqueous butyric acid, open squares represent butyric acid including extracted acid, solid diamonds represent acetic acid, crosses represent glucose, and open diamonds represent *p*H (inset). Dashed vertical lines indicate CO_2 sparging initiation and solid vertical lines represent CO_2 sparging termination.

However, if the extraction of butyric acid confers marked advantages, it is possible that the benefits of such a process could outweigh this disadvantage. Regardless, these results confirm that high pCO_2 extractions as outlined herein do not adversely affect cell viability, or process performance.

Table 5-I Parameters from batch and fed-batch fermentations for the production of butyric acid by *C. tyrobutyricum* compared to treatments with 60 bar pCO_2 pressure cycling and absorptive polymer extractions

Parameter	Batch			Fed-batch	
	Control	Pressure Only Polymer-free	Polymer ISPR	Control	Polymer ISPR
Aqueous butyric acid (g)	68.4	72.4	60	128	121
Butyric acid extracted (g)	-	-	14	-	92
Total butyric acid (g)	68.4	72.4	74	128	213
Acetic acid (g)	12.8	26	21	26	56
$Y_{P/S}$ (g butyric/g gluc.)	0.35	0.37	0.35	0.40	0.54
Productivity (g L^{-1} h^{-1})	0.50	0.50	0.44	0.33	0.65
Selectivity (g butyric/ g tot. acid)	0.84	0.74	0.78	0.83	0.79
g base/g butyric acid	0.86	0.79	0.44	0.67	0.62

5.5.3 Batch reactor extraction and *p*H effects

A batch reactor coupled with a polymer extraction regime facilitated by high pCO_2 *p*H swings was performed as shown in Figure 5-4c, and it is apparent that butyric acid concentrations in the reactor were decreased by extraction events at 36, 40, 44, and 48 hours (see inset Figure 5-4c). These decreases in butyric acid concentration coincide with the amount of butyric acid recovered from each fresh polymer mass (Table 5-II), which on average removed 3.5 ± 0.1 g butyric acid, for a total of 14.1 g butyric acid recovered, which combined with a final aqueous concentration of 30 g L^{-1} in 2 L yields 74 g of butyric acid. It can also be observed in Figure 5-4c that cell concentrations did not decrease as a result of extraction periods, and thus it is unlikely that any cell adhesion to the polymer mass took place. This is further supported by previous work that did not observe microbial attachment to polymers utilized in TPPBs (Amsden *et al*. 2003). It is interesting to note that during polymer desorptions approximately 90% of acid recovery was achieved in the first of three sequential 12 hour alkali soakings (Table 5-II), suggesting that a single stage desorption of the acid may be sufficient for polymer regeneration and reuse within the time frame of a fermentation. Furthermore,

medium components were not detected and acetic acid concentrations were negligible from desorptions. Thus, selective absorptive polymer extraction of butyric acid mediated through high pCO_2-mediate *p*H swings was clearly achieved during the batch fermentation shown in Figure 5-4c. However, total butyric acid produced was similar in both the control and the polymer-free pressurized runs, which produced 68.4 and 72.4 g butyric acid, and all three runs had yields of 0.35-0.37 g butyric acid/g glucose consumed. Also, a decrease in selectivity (0.78) was observed similar to polymer-free pressurized runs, but this decrease was diminished, likely as a result of butyric acid removal, which may affect end-product ratios.

Table 5-II Butyric acid recovery from three sequential 12 hour polymer desorptions in 0.25 M KOH after high pCO_2 polymer extractions during batch fermentation for the production of butyric acid from *C. tyrobutyricum*

Extraction #	t (h)	Desorption #1		Desorption #2		Desorption #3		Total butyric acid (g)
		Butyric (g)	% rec.	Butyric (g)	% rec.	Butyric (g)	% rec	
1	36	3.2	88.9	0.3	8.3	0.1	2.8	3.6
2	40	3.2	88.9	0.3	8.3	0.1	2.8	3.6
3	44	3.2	88.8	0.3	8.4	0.1	2.8	3.6
4	48	3.0	88.2	0.3	8.9	0.1	3.0	3.4

An additional interesting observation arising from this extractive fermentation was the effect that butyric acid extraction exerted on reactor *p*H values. Specifically, between the first and last extraction cycles shown in Figure 5-4c the *p*H control was shut off and the *p*H was maintained exclusively by butyric acid removal. As can be seen in the inset of Figure 5-4c, reactor *p*H values initially dropped to 5.8 due to ongoing acid production in the bioreactor during extraction, but increased to 6.3 after extracted broth was returned to the reactor as a result of decreased butyric acid concentration through polymer absorption. This phenomenon of *p*H control through acid recovery has been reported previously for both the production of butyric (Wu and Yang 2003) and lactic acid (Ataei and Vasheghani-Farahani 2008) through use of reactive extraction and ion-exchange resin techniques respectively, but to date this effect has not been demonstrated using absorptive polymers in TPPBs. The major benefit arising from acid extraction is apparent by examination of the total based added during the fermentations, as seen in Table 5-I, which shows that the extractive batch run utilized 0.44 g KOH per g butyric acid produced, compared to 0.86 g KOH per g butyric acid in the control,

which translates into an almost 50% reduction of base required. Furthermore, the intentional omission of *p*H control during the extractive batch run (Figure 5-4c) did not seem to affect process performance when compared to the pressurized polymer-free batch run (Figure 5-4b), which also employed conventional *p*H control. Aside from the cost savings from using less base for *p*H control, which can be significant in organic acid fermentation, this reduction would also decrease ion accumulation and the associated osmotic stress, and thus could potentially improve performance if optimized, especially over longer fermentations. Regardless, while overall this extractive regime achieved online extraction and lead to the simultaneous elimination of *p*H control, the lack of improvement in reactor performance indicates that such removal did not confer the benefits usually associated with ISPR. This is likely due to insufficient removal of product coupled with late stage extraction, as the alleviation of EPI achieved through this removal does not leave sufficient time for cells to benefit from reduced end-product toxicity. However, it is clear that extraction was achieved through the use of high pressure pCO_2 and absorptive polymers, and more intensive extractive regimes and culture techniques employing higher substrate loadings could be applied to potentially increase butyric acid removal and improve reactor performance.

5.5.4 Fed-batch reactor extraction

Extractive fed-batch techniques were subsequently employed to ensure that sufficient substrate and nutrients were available for extended fermentation after extraction, and as can be seen in Figure 5-5, a fed-batch reactor coupled with high pressure pCO_2 polymer extraction was performed alongside a fed-batch control reactor. This extraction regime was similar to that employed for the above mentioned extractive batch reactor, except that 12 extractions were performed, compared to 4 extractions performed in batch. These 12 extractions were distributed into four groups over the span of the fermentation, which was replenished with substrate and nutrients after 68 hours, and the results are displayed in Figure 5-5b. Also similar to the extractive batch reactors, extraction cycles demonstrated a clear reduction in aqueous butyric acid concentrations, as can be seen in Figure 5-5b at 36-44 h, 60-68 h, 84-92

h, and 108-116 h. Further confirming the successful extraction of butyric acid, post-extraction polymer desorptions yielded recovered butyric acid and distribution coefficients as reported in Table 5-III. As anticipated, due to the increased concentration driving force arising from higher accumulated levels of butyric acid, the butyric acid absorbed by the polymers increased during the course of the fermentation, with the averages of each group of three extractions yielding recovered butyric acid values of 5.9 ± 0.8, 6.8 ± 0.4, 8.0 ± 1.0, 10.1 ± 1.0 g. Thus, even though it has been demonstrated that higher butyric acid concentrations increase medium buffering and reduce the distribution coefficient for a given extraction, the elevated concentrations translate into a higher driving force, with more acid being extracted overall despite diminished distribution ratios. It is also interesting to note a single step alkali desorption was successfully employed for regeneration of polymer beads in a rapid manner, permitting their reuse for further extractions while demonstrating no obvious negative effects on extraction performance.

Table 5-III Total butyric acid recovered and estimated distribution coefficients (D) from single 12 hour desorptions in 0.25 M KOH after high pCO_2 polymer extraction intervals during fed-batch fermentations for the production of butyric acid from *C. tyrobutyricum.*

Extraction	Time of extraction (h)	Acid recovered (g)	D
1	36	4.9	1.8
2	40	6.4	1.9
3	44	6.3	1.9
4	60	7.2	1.5
5	64	7.0	1.6
6	68	6.4	1.5
7	84	7.0	1.5
8	88	8.0	1.2
9	92	9.0	1.2
10	108	10.7	1.2
11	112	10.6	1.4
12	116	8.9	1.0

As seen in Table 5-I, a total of 92 g butyric acid was extracted during the course of the fed-batch fermentation, which represented 43% of total acid produced, compared to 19% of total acid produced by the extractive batch run, clearly demonstrating the advantage of an increased number of extractions and higher acid concentrations afforded through fed-batch operation. Overall, the extractive fed batch system had superior performance to the control fed

batch reactor, whether in total acid produced (212 vs. 128 g), yield (0.54 vs 0.40 g butyric acid/g glucose consumed), or productivity (0.65 g L^{-1} h^{-1} vs 0.33 g L^{-1} h^{-1}). It must be noted however that the calculated yield exceeds the theoretical yield of butyric acid from glucose (0.49), and this is likely due to inaccuracies in glucose determination as a result of high substrate loadings. Regardless, the yields achieved utilizing the extractive methods outlined here are clearly superior to the fed-batch control. Furthermore, although as noted, the extractive batch run in this study was operated without *p*H control, the fed-batch run was performed using traditional *p*H control, to clearly demonstrate that any benefits are a result of EPI rather than reduced osmotic stress, and thus further improvements to this extractive fed-batch technique could be made through utilization of extraction to control *p*H. The approach used here represents a semi-continuous removal system for butyric acid, and could potentially extend fermentation times not only through batch feeding coupled with a regular extraction regime, but also through the reduction or elimination of *p*H control and subsequent ion accumulation, while also potentially reducing water use. The use of high pCO_2-mediated absorptive polymer extractions has been demonstrated to be an effective ISPR method that confers significant benefits on fermentation performance through alleviation of end-product inhibition.

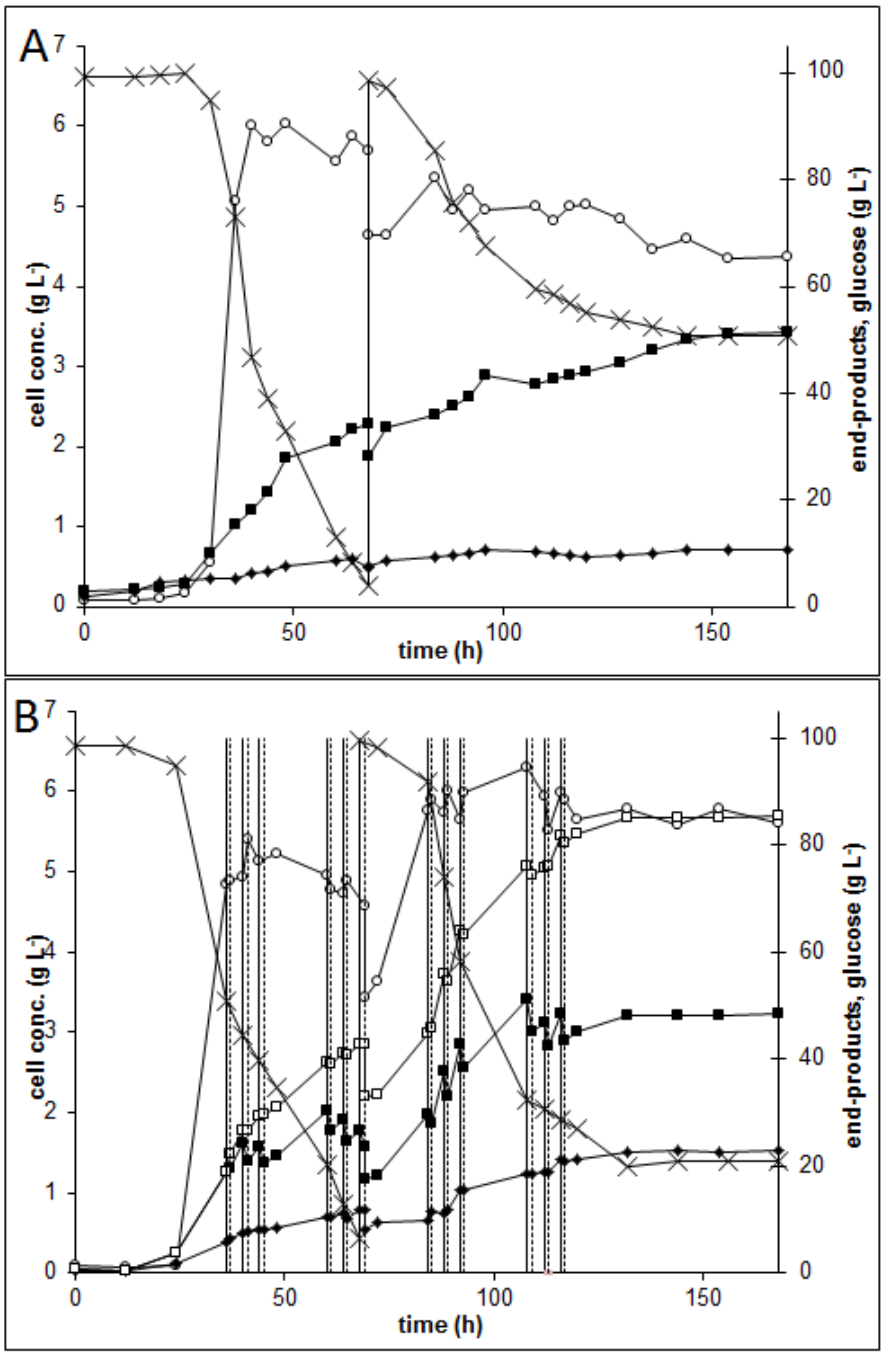

Figure 5-5 Fed-batch fermentation of butyric acid from *C. tyrobutyricum* under A) conventional culture techniques or B) cyclical pressurization and use of an absorptive polymer to achieve ISPR. Open circles represent OD, solid squares represent aqueous butyric acid, open squares represent butyric acid including extracted acid, solid diamonds represent acetic acid, and crosses represent glucose. Dashed vertical lines indicate CO_2 sparging initiation and solid vertical lines represent CO_2 sparging termination.

5.6 Conclusion

Elevated pCO_2 significantly increases the reversible *p*H drop of fermentation medium with subsequent improvements in *p*H-dependent partitioning of butyric acid, and a direct

relationship exists between pCO_2 and distribution coefficients up to 60 bar pCO_2. In contrast to atmospheric conditions, it has also been shown that medium components and typical end-products do not prevent CO_2- mediated extraction, and effective removal can be performed. Furthermore, it has been demonstrated that cultures of *C. tyrobutyricum* can tolerate repeated one hour cyclical exposures to 60 bar pCO_2. Multiple extractions and high substrate loadings achieved through fed-batch operation have been shown to significantly increase overall production, yields and titres of butyric acid generated by *C. tyrobutyricum*, and these benefits are clearly a result of alleviation of EPI. Through utilization of online extraction to control *p*H, it may also be possible to further increase productivity through reduced ion accumulation and osmotic stress over longer fermentations, and optimization studies of extraction-mediated *p*H control would be merited. Finally, for the first time a TPPB utilizing synchronous polymer regeneration and reutilization has been employed, and this work also points to the feasibility of the TPPB platform for producing other hydrophilic bioproducts such as organic acids.

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Chapter 6

Conclusions and Future work

6.1 Conclusions

The unifying goal of the work represented in this thesis was to develop a process that enabled ISPR of biologically produced organic acids, and this was ultimately achieved through *p*H reductions afforded through use of high pressure CO_2, which enabled acid uptake in absorptive polymers in a TPPB, representing a novel method for achieving organic acid partitioning. This high pCO_2 –mediated acid recovery has been demonstrated to be both effective and non-lethal to cells, achieving ISPR during fermentation with improved yields and volumetric productivities as a result, and contributes to the development of organic acids as bio-based chemical commodities. Successful demonstration of such a process was achieved using a multi-faceted approach considering medium effects, *p*H, high pressure, polymer selection and biological tolerance.

The first significant contribution to achieving this goal was the development of a growth medium amenable to *p*H reductions through use of CO_2 sparging, allowing improved reductions in *p*H. This was achieved through minimization of medium components shown to contribute buffering, while ensuring that modifications were made at no expense to microbial performance. It was also established that increasing butyric acid concentrations diminished *p*H reductions achieved with CO_2 as a result of increased buffering strength. Using this minimized medium, it was concluded that while use of CO_2 at atmospheric conditions achieved noticeable butyric acid recovery during *C. tyrobutyricum* fermentation, no significant gains were observed in terms of microbial performance, and this was due to relatively small *p*H reductions as a result of buffering from medium and butyric acid. However, increased *p*H reductions could be achieved through elevation of pCO_2, and high pressure methods for reducing *p*H were investigated as a means to overcome buffering from medium components and acid concentrations and thus achieve more significant *p*H reductions.

To fully determine to what extent partitioning was achieved as a result of *p*H reductions, *p*H-dependent partitioning models for organic acids were developed using a rigorous first-principles approach, contributing an accessible framework for understanding and predicting acid partitioning. These models were validated experimentally, and clearly demonstrated the relationship between partitioning and *p*H, polymer fraction, and PC, while providing insight that once a polymer is selected, manipulation of F represents the only available design option to improve acid recovery. Thus, as distribution coefficients increase, reductions in F can be made while still achieving recovery targets. Additional perspective on the effect of partition coefficients on *p*H-dependent partitioning has been added through investigation of solutes demonstrating both high and modest PC values, and it is clear that while acid removal can cause *p*H values to rise, partitioning overall is strictly dictated by *p*H and an acid's pK_a. By examining the relationship between *p*H and partitioning, it was concluded that small reductions in *p*H from neutral values produced large returns in terms of acid absorption, and by careful consideration of PC and polymer fraction, partitioning at a given *p*H can be maximized. Importantly, experimental tests concluded that high pCO_2 can achieve these small *p*H changes over a range of butyric acid concentrations, demonstrating improved partitioning through *p*H reduction as indicated in terms of distribution coefficients and % recovery, which both directly benefited from increasing pCO_2, indicating that such a process could provide effective online acid recovery.

The final and overall contribution of this thesis was the demonstration of the use of high pCO_2 as a method for achieving ISPR and EP. This was achieved by incorporating the cumulative research of previous thesis contributions, overcoming medium and acid buffering effects through the development of a high pressure method, while understanding *p*H-dependent partitioning and carefully designing a non-lethal system for use in fermentations. Unlike previous CO_2 *p*H reduction tests performed at atmospheric levels, buffering from medium components and increasing acid concentrations were overcome using 60 bar CO_2, demonstrating that absorption of organic acids can be achieved through the use of high pCO_2

*p*H reductions. Although D and % recovery for butyric acid were decreased at elevated butyric acid concentrations due to this buffering effect, the higher driving force afforded by these concentrations achieved absorption of a substantial amount of acid, demonstrating that acid recovery can be effectively achieved regardless of concentration. Importantly, it was clearly shown that cells of *C. tyrobutyricum* can withstand 60 bar pCO_2 for one hour with no negative effects, and that cyclical exposure of reactor contents to these conditions did not diminish reactor performance in terms of growth or acid production. However, it must be noted that microbial tolerance to this pressure was not indefinite, and exposure of three hours resulted in a total loss of cell viability. Thus, it was demonstrated that the use of high pCO_2 to achieve *p*H-dependent recovery of butyric acid is both non-lethal and effective under fermentation conditions. When this technique was applied to fermentation, multiple temporary *p*H reductions demonstrated substantial acid recovery, and through the use of high substrate loadings via fed-batch operation, overall yields, titres, and volumetric productivities of butyric acid generated by *C. tyrobutyricum* were increased by as much as 35%, 60%, and 96%, respectively, and these benefits are clearly a result of alleviation of EPI.

Additionally, ISPR of butyric acid was observed to reduce the need for conventional *p*H control methods through polymeric absorption and removal of produced acids, confirming that partitioning can modulate *p*H. This phenomenon could potentially be used as an alternative method of *p*H control, reducing ion accumulation and osmotic stress which would result from traditional *p*H control methods over prolonged fermentations. Overall, the work compiled in this thesis serves as definitive proof-of-concept that high pCO_2 can act as a non-toxic effective agent for facilitating organic acid recovery in a two-phase partitioning bioreactor.

6.2 Future work

While the work in this thesis serves as proof-of-concept for the applicability of high pCO_2 *p*H reductions for improving butyric acid absorption in TPPBs, further research into different aspects of this method could yield interesting results and additional improvements. For instance, while the semi-continuous recovery of butyric acid through cyclical *p*H reductions as

outlined in Chapter 5 was shown to be effective, further design and operational improvements could be made through use of semi-continuous recycling of reactor contents through a column packed with absorptive polymers under high pressure CO_2. However, the continuous cycling of fermentation broth through the high pressure pumps required for such a recycling technique would likely need effective filtration for cell removal. If such a method could be achieved, the alternating use of two extractive high pressure columns could provide an improved system for semi-continuous removal and product recovery using high pCO_2, with ready desorption and polymer regeneration through alkali desorption.

Furthermore, the use of high pCO_2 methods to achieve ISPR during the biological production of other organic acids such as propionic acid (Wang *et al.* 2012) or hexanoic acid (Agler *et al.* 2011) could also be demonstrated, potentially achieving similar results in terms of EPI and improved biological performance. In the case of multiprotic organic acids however, buffering effects from acid concentrations would only be exacerbated with an increasing number of carboxylate groups, and high pCO_2 attempts would likely prove successful only if improved sequestering phases can be developed. Thus, further work to improve the ability of polymers to recover organic acids is a crucial consideration if further improvements can be made. This can be achieved through two different approaches. The first approach is to identify polymers that exhibit higher partitioning coefficients through rigorous investigation and validation of thermodynamic polymer-solute affinity models. However, due to the highly hydrophilic nature of many organic acids (e.g. succinic acid, itaconic acid), PC values are inherently low, and the relative improvements to PC that may be afforded by improved selection methods may not yield substantial gains in acid recovery. The alternative approach is the use of reactive extraction, which as discussed above has been shown to be highly effective at recovering organic acids. However, typical liquid amine-based extractants present toxicity issues, and thus are not suitable for ISPR applications. Thus, research should be focused on the use of amine-based solid polymeric reactive extractants, such as poly(dimethylaminoethylmethacrylate) (PDMAEMA). It may be that the polymeric nature of

such an extractant would limit cytotoxicity by preventing transfer of amines to the aqueous phase while still achieving the high levels of extraction associated with reactive extraction techniques. To determine if such an approach is possible, work needs to be done to confirm that stable reactive amine-based extractive polymers can be synthesized which demonstrate high amounts of acid recovery, while also determining what effect these polymers exert on cell viability and performance. If high amounts of extraction can be achieved with such polymers without deleterious effect to cell populations, this development would represent a highly significant advance toward achieving ISPR and the associated benefits to cell performance in biological organic acid production, while also potentially improving downstream purification costs.

Additionally, the ability to control *p*H through the removal of acid via partitioning represents a promising direction for further work involving organic acid production in TPPBs. As discussed in Chapter 5, acid recovery was demonstrated to increase *p*H values in fermentations, and by careful consideration of frequency of high pCO_2 *p*H reductions and polymer fraction, it is likely that fermentative *p*H values could be maintained exclusively by acid absorption. However, the extent to which partitioning results in *p*H changes needs more characterization, and the findings presented in Chapter 4 represent a solid framework of experimental data from which a model for understanding absorption-induced *p*H changes could be developed, which could potentially predict *p*H changes arising from acid recovery. If high pCO_2 *p*H reductions were able to supplant traditional methods as a means for controlling *p*H, the resultant elimination of ion-accumulation and osmotic stress could result in significant gains in terms of biological performance, as acid titres typically achieved can result in significant base addition. Thus, along with overwhelming benefits of alleviation of EPI, further benefits to process performance through reduction of osmotic stress could be achieved through both high pCO_2 mediated polymeric absorption and reactive extraction of organic acids, and therefore further work to achieve this is merited.

6.3 References

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Appendix A

Polymer selection studies for application of TPPBs to butyric acid fermentation

A.1 Introduction

Butyric acid (pK_a 4.8), represents an ideal candidate for polymer selection strategies, because it is a miscible fermentative product capable of dissociation, while also being inhibitory to the cells that produce it, and previous studies have identified it as a molecule of interest for production in TPPBs (Peterson and Daugulis 2014). As has been the case for other more hydrophobic target molecules (Parent *et al.* 2012; Poleo and Daugulis 2013), several polymer-solute affinity prediction methods (i.e. Hildebrand solubility parameters, Hansen solubility parameters, and activity coefficient-based methods) were studied as described in section 1.5.

As it has been well-established that crystalline domains do not participate in absorption (Gao and Daugulis 2010; Parent *et al.* 2012), to potentially improve the relationship between predicted and observed polymer-solute affinity experimentally-determined partition coefficients have been corrected to account for the amount of the crystalline domain in a given polymer. This has been successfully applied qualitatively in one case (Parent *et al.* 2012) utilizing Equation (A-1), where PC represents experimental partition coefficient, α represents amorphous mass fraction of the polymer, and PC^* represents partitioning corrected to solely reflect the amorphous fraction.

$$PC = \alpha\, PC^* \qquad \text{(A-1)}$$

Although this relationship was found to be applicable to solutes such as polyaromatic hydrocarbons (PAHs) (Parent *et al.* 2012), this correction has yet to be extended to a range of polymers however, and requires validation before it can be more widely applied in polymer

selection strategies. As an added complication, “off the shelf” crystallinities may differ from *in situ* crystallinity in partitioning systems, as uptake of both water and solutes could have an effect on physical properties such as % crystallinity or melting temperature (T_m) of crystalline domains, and thus *in situ* physical polymer properties warrant investigation if such corrections are to be applied. Moreover, it is important to recognize that crystallinity is a dynamic property and is dependent on the thermal history of a given polymer. That is, the degree of crystallinity is determined through a polymer’s handling during production or subsequent processing steps, and thus % crystallinity is not commonly reported, limiting the use of this correction for practical purposes in general polymer screening.

Overall, this study seeks to compare experimental partitioning data for absorptive polymers to predicted polymer-solute affinities for butyric acid through use of Hildebrand solubility parameters, Hansen solubility parameters, and activity coefficient-based methods as calculated through UNIFAC. This study further investigates the relationship between crystalline properties and absorption, with the goal of improving affinity predictions. While this study focused on polymer selection for butyric acid in particular, the strategies outlined here could be applied more generally to other solutes, and these methods may be widely accessible for application to molecules produced in TPPBs.

A.2 Materials and Methods

A.2.1 Polymer selection

To allow for comparison between experimental partitioning data and predicted polymer-solute affinity, a list of candidate polymers with predicted affinity for butyric acid were generated for partitioning tests using Hansen Solubility Parameters in Practice (HSPiP) software 4.0 (http://www.hansen-solubility.com). Using solubility parameters provided by this software, over 650 polymers were ranked according to calculated differences in Hansen solubility parameters compared to butyric acid, wherein a smaller difference indicates higher polymer-solute affinity. The highest ranked 50 polymers according to differences in HSP

values were then sorted to omit polymers possessing T_g values higher than 30 °C a typical biological temperature, as these polymers would be glassy and would not appreciably absorb solutes (see Table A-I for a list of candidate polymers). Along with the prediction of polymer-solute affinities based on solubility parameters, activity coefficient (AC) values for butyric acid in these polymers were predicted using the UNIFAC-vdW-FV model (Kannan *et al.* 2005), which was based on later UNIFAC models (Oishi and Prausnitz 1978). To simplify calculation, AC values were calculated at infinite dilution, and thus represent estimated thermodynamic affinities only. These polymer AC values for butyric acid were compared to an experimental value for the aqueous infinite dilution AC of butyric acid (52.9) (Kojima *et al.* 1997) to predict PC, as shown in Equation (A-2) where γ and Ω reflect activity coefficients by mole fraction and weight fraction, respectively (Parent *et al.* 2012).

$$PC = \frac{{}^{aqueous}_{\gamma_{solute,}}}{{}^{polymer}_{\gamma_{solute}}} = \frac{{}^{aqueous}_{\Omega_{solute}}}{{}^{polymer}_{\Omega_{solute}}} \quad \text{(A-2)}$$

A.2.2 Partition coefficient determination for butyric acid

Polymer partitioning tests were performed with 0.5, 1, and 1.5 g polymer in 20 mL of 20 g L^{-1} butyric acid in reverse osmosis water. All polymers tested are shown in Table A-I, and were purchased from Scientific Polymers (Ontario, NY, USA), and 99% butyric acid was purchased from Sigma-Aldrich. To ensure that equilibrium had been reached, samples were shaken for seven days at 180 rpm and 30 °C. Butyric acid aqueous samples were analyzed using HPLC (Varian Prostar, Mississauga, ON) with a Varian Hi-Plex H column (300 × 7.7 mm) at 60 °C with a 10 mM H_2SO_4 mobile phase at 0.7 mL min^{-}, and a UV-Vis detector (Varian Prostar, PS325) at 220 nm. Partition coefficient (PC) was calculated via mass balance, as previously described (Dafoe and Daugulis, 2011).

A.2.3 Differential scanning calorimeter

If corrections for percent crystallinity are to be applied to PCs, it is imperative to accurately determine the degree of crystallinity of all polymers and differential scanning calorimetry (DSC) was performed using a TA instruments DSC Q100 operating at a heating rate of 10 K min^{-1} under a nitrogen purge. The melting endotherm recorded upon first sample heating was integrated and divided by the standard enthalpy of fusion ($^{\circ}\Delta H_f$) to calculate the degree crystallinity. First heating scans were chosen to represent degree of crystallinity, as this represents polymer samples as received, which were subjected to partitioning tests with no further handling. To determine the impact that water or solute may have on polymer crystallinity, polymer samples were placed in RO water or 20 g L^{-1} butyric acid for seven days and tested alongside dry polymer samples. Exposure of poly(tetramethylene glycol) (PTMG) to butyric acid resulted in substantial changes in polymer physical properties and thus the effect of increasing butyric acid concentrations on this polymer's melting temperature was also tested. To achieve this, 1 g samples of PTMG were exposed to 1, 2, 3 % butyric acid (w/w), which represent typical concentrations achieved during fermentations. Samples were then equilibrated, for 1 week before undergoing DSC analysis to determine melting endotherms. In this case, second heating scans were performed along with first heating scans to demonstrate the effect of butyric acid on PTMG physical properties under reproducible conditions, wherein samples were heated at a rate of 10 K min^{-1} to 373 K and then cooled to 173 K at the same rate before scanning to report defined thermal histories.

A.3 Results and Discussion

A.3.1 Analysis of predictive methods

Predicted polymer-solute affinities according to total (Hildebrand) solubility parameters, Hansen solubility parameters (HSPs), and AC-based predictions have been ranked and sorted according to experimental partition coefficients for butyric acid, as seen in Table A-I. Although smaller Hildebrand SP differences suggest higher solute-polymer affinity, no discernible trend was detected between observed PC and SP differences when displayed graphically (Figure A-

1a), and separation of polymers into amorphous (Figure A-1b) and semi-crystalline (Figure A-1c) groups also showed no trend.

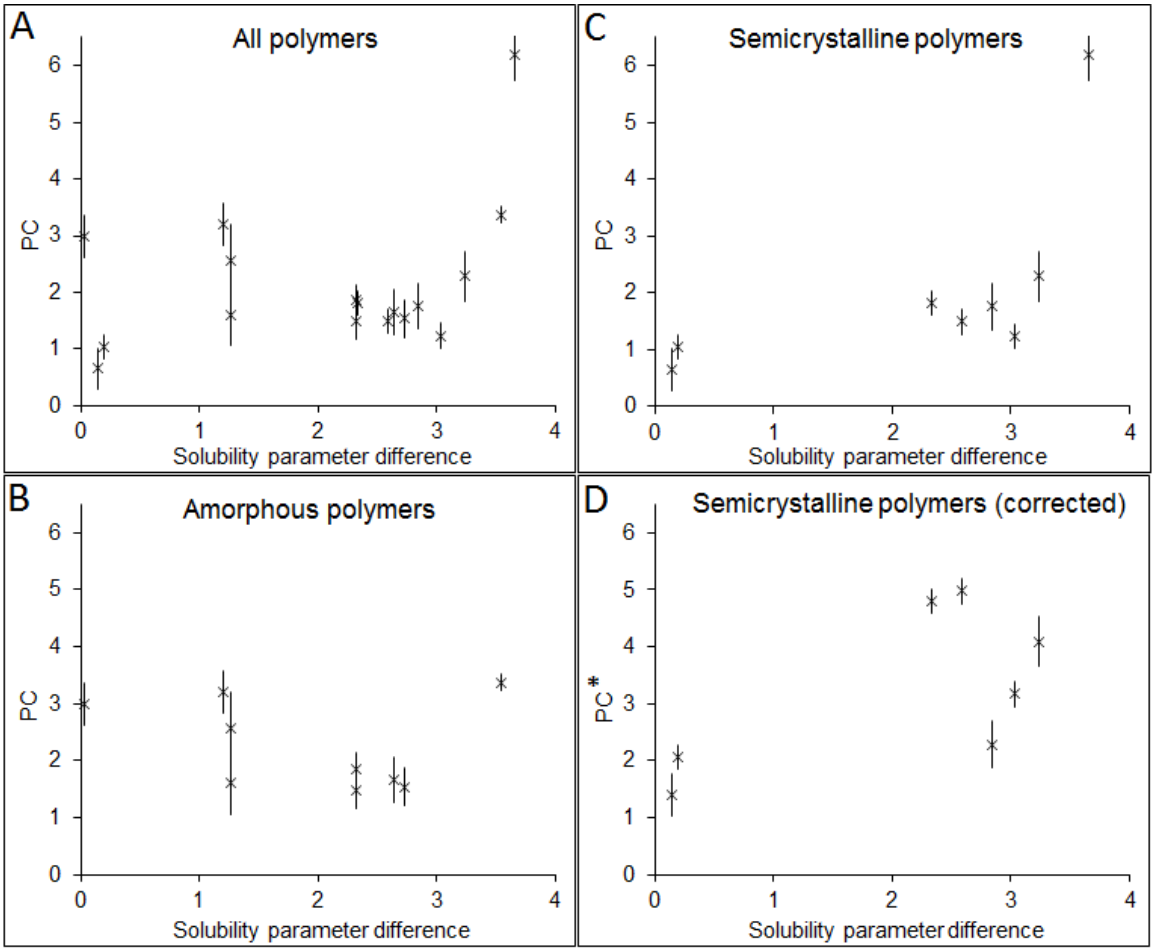

Figure A-1 Total solubility parameter predictive method assessment, displaying experimental partition coefficients for various polymers versus respective parameter difference to butyric acid. (Partition coefficient error is reported as standard deviation n=3)

Semicrystalline experimental partition coefficient values were corrected using Equation (A-1) to reflect PCs by the amorphous fraction only, using crystallinity values obtained from DSC (Table A-II), yet these corrections also had no effect on improving the predictions. Although not seemingly successful at identifying effective candidate polymers for butyric acid, the total solubility parameter approach has previously shown success for more hydrophobic solutes[13], suggesting that Hildebrand SPs may not be able to accommodate polar interactions or hydrogen bonding which would occur for hydrophilic solutes across polymers.

Table A-I Observed partition coefficients for butyric acid in absorptive polymers and predicted affinities, as ranked by Total solubility parameters (Hansen and Yamamoto 2013), Hansen solubility parameters (Hansen and Yamamoto 2013), and UNIFAC-based predictions (Kannan *et al.* 2005). (Partition coefficient error is reported as standard deviation n=3)

Polymer / Solute	PC at 20 g L^{-1}	Total		Hansen		UNIFAC		
		rank	δ -δ	rank	Ra	rank	$\Omega_{solute,x\rightarrow 0}$	pred. PC
Poly(tetramethylene glycol)	6.2 ±0.5	17	3.7	17	10.0	4	3.8	2.9
Poly(hydroxybutyl methacrylate)	3.4 ±0.2	16	3.5	3	3.6	3	2.9	3.8
Poly(hydroxyethyl methacrylate)	3.2 ±0.4	3	1.2	1	2.8	1	2.5	4.4
Poly(hydroxypropyl methacrylate)	3.0 ±0.4	3	1.2	2	3.2	2	2.7	4.1
Poly(vinyl acetate)	2.6 ±0.6	6	1.3	6	6.5	10	7.0	1.5
Poly(trimethylene adipate)	2.3 ±0.4	15	3.2	11	7.4	16	9.1	1.2
Poly(ethyl acrylate)	1.9 ±0.3	9	2.3	9	7.1	13	7.6	1.4
Poly(trimethylene succinate)	1.8 ±0.4	13	2.8	5	6.3	5	4.9	2.2
Poly(butylene adipate)	1.8 ±0.2	7	1.8	12	7.8	6	5.4	2.0
Poly(propyl acrylate)	1.7 ±0.4	11	2.6	14	7.9	17	10.2	1.1
Poly(methyl acrylate)	1.6 ±0.5	5	1.3	6	6.5	15	8.8	1.2
Poly(vinyl propionate)	1.5 ±0.3	8	2.3	9	7.1	7	5.6	1.9
Poly(caprolactone)	1.5 ±0.2	10	2.6	15	8.1	13	8.4	1.3
Poly(butyl acrylate)	1.5 ±0.3	12	2.7	15	8.1	14	8.8	1.2
Poly(ethylene adipate)	1.2 ±0.2	14	3.0	8	6.7	11	7.4	1.5
Poly(ethylene succinate)	1.0 ±0.2	2	0.2	4	5.7	8	6.0	1.8
Poly(oxymethylene)	0.7 ±0.4	1	0.1	12	7.8	9	6.4	1.7

Table A-II Crystallinities and melting temperature of semicrystalline polymers tested for butyric acid affinity, with samples equilibrated dry, in water, or 2% (w/w) butyric acid.

Polymer	Crystallinity			Tm (°C)		
	dry	wet	solute	dry	wet	solute
Poly(butylene adipate)	62	62	62	61	59	60
Poly(tetramethylene glycol)	58	59	53	44	42	26
Poly(caprolactone)	73	73	70	69	67	64
Poly(ethylene adipate)	58	61	61	54	53	50
Poly(ethylene succinate)	47	48	49	102	101	97
Poly(methylene oxide)	54	59	53	184	184	179
Poly(trimethylene succinate)	23	23	23	52	47	47
Poly(trimethylene adipate)	41	48	44	41	45	44

Unlike Hildebrand SPs, HSPs can potentially account for such polar interactions, and an overall correlation between predicted and observed partitioning was apparent when examining all polymers tested with the exception of one outlier (PTMG, Figure A-2a), where minimization of HSP distance (Ra) predicts high polymer-solute affinity. However, amorphous polymer partition coefficient values demonstrate a more clear relationship with HSP predictions, (Figure A-2b), with decreasing polymer-solute HSP distances resulting in higher partitioning, in contrast to semi-crystalline polymers (Figure A-2c), which demonstrated no apparent correlation, even when a correction was applied to omit crystalline polymer domains (Figure A-2d).

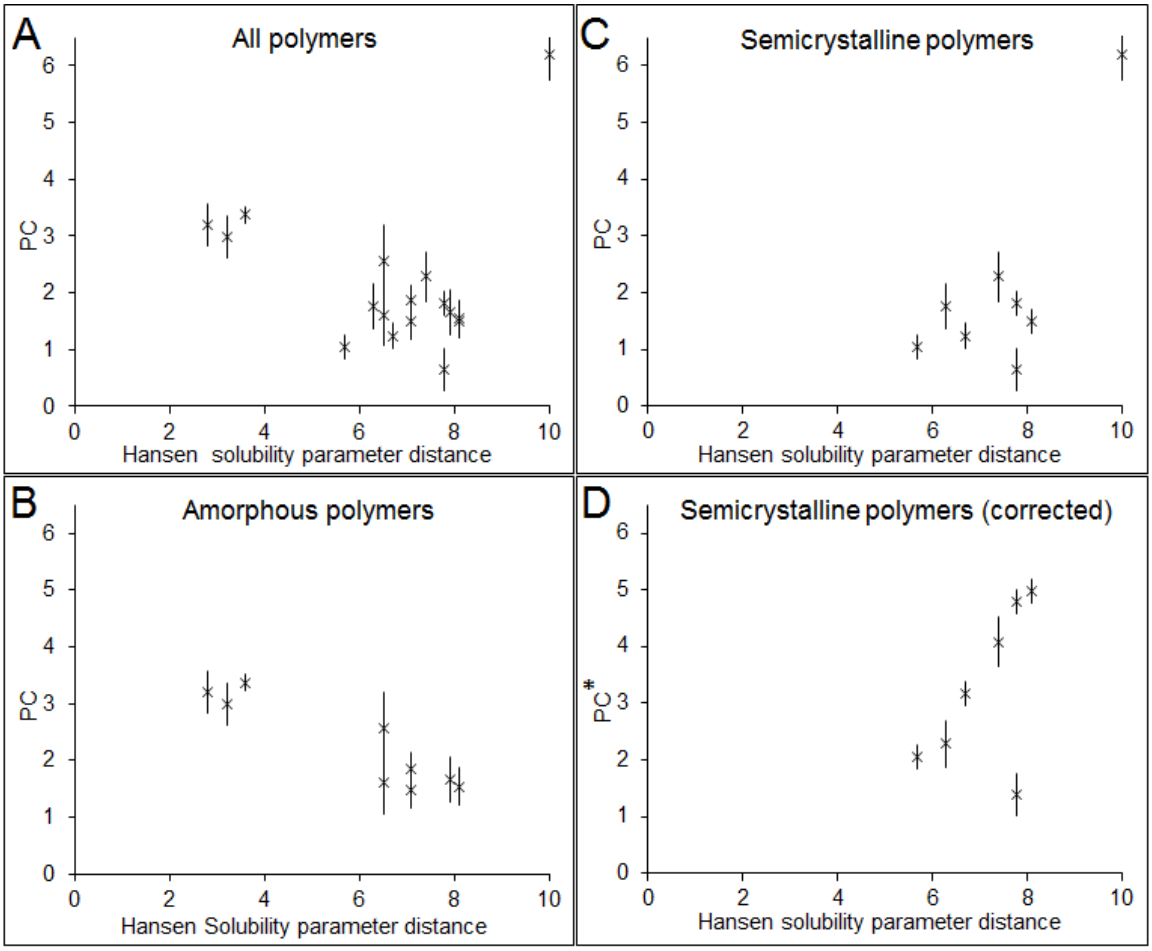

Figure A-2 Hansen solubility parameter predictive method assessment, displaying experimental partition coefficients for various polymers versus respective solubility parameter distance to butyric acid. (Partition coefficient error is reported as standard deviation n=3)

These results suggest that crystallinity may interfere with solute absorption, while also implying that currently-used crystallinity corrections are insufficient to explain what effect these crystalline domains actually may exert. Amorphous polymers (i.e. possessing no crystallinity) are obviously unaffected by such restrictions, concomitantly resulting in improved predictions (Figure A-2b). Notably, the use of HSPs successfully predicted high affinity for amorphous poly(hydroxyalkyl methacrylates) with Ra distances of 2.8-3.6, as represented by the three leftmost points in Figure A-2b and A-2c, while yielding partition coefficients of 3.0-3.4, as shown in Table A-I. These materials showed significant improvement in absorption over other polymers, with the exception of PTMG, which had a partition coefficient of 6.2 ±0.5. However, HSP predictions ranked PTMG last with a Ra distance of 10, and this prediction is inconsistent with observed partitioning, and cannot be explained at this time.

AC-based predicted PCs, calculated from aqueous and polymer activity coefficients for butyric acid, demonstrated behaviour similar to those made with HSPs, wherein correlation between predicted and observed PC for overall polymer values (Figure A-3a) was masked by semicrystalline polymer values. Amorphous values (Figure A-3b) again demonstrated a clear positive relationship between predictions and experimental values, compared to semi-crystalline values (Figure A-3c), which showed no observable trend. Incorporating the crystallinity correction in Equation A-1 (Figure A-3d) again did not rectify this predictive failure.

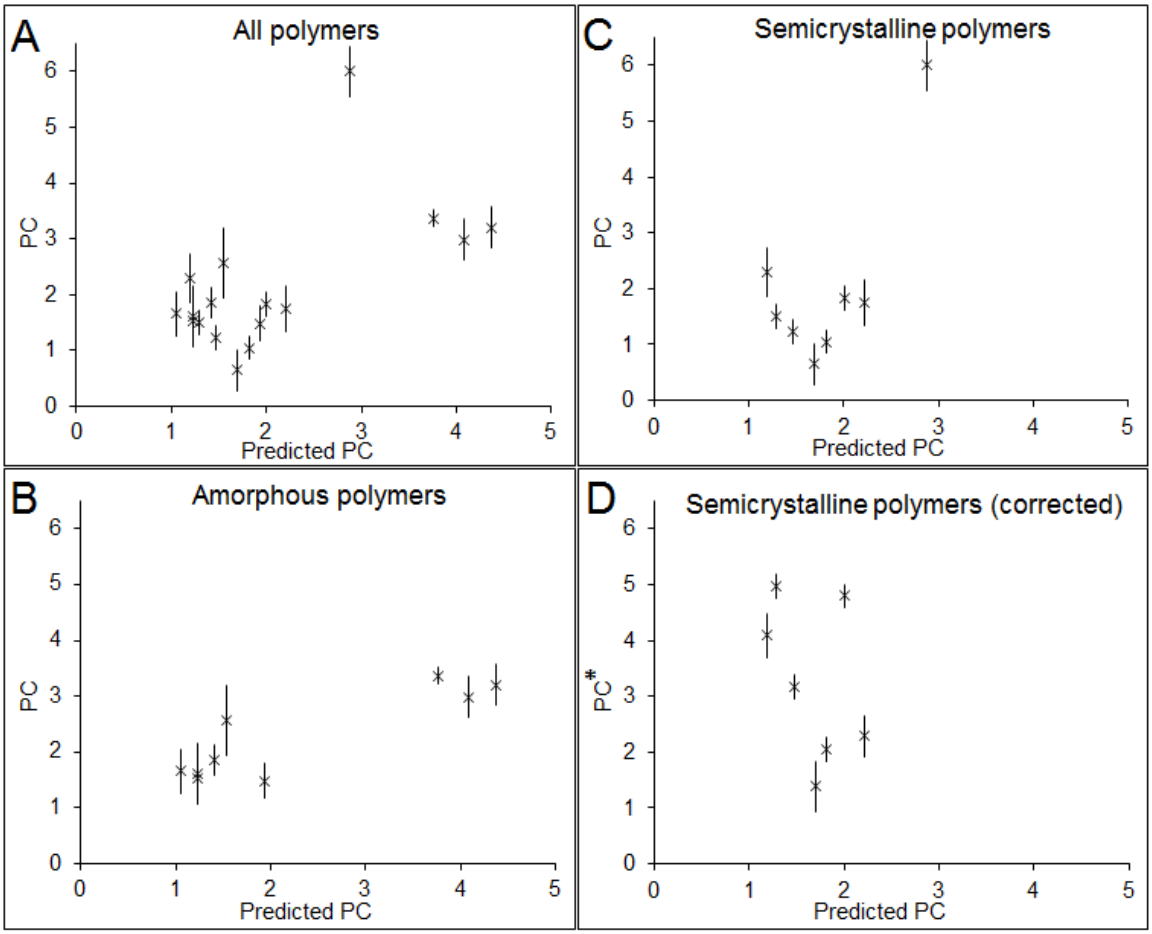

Figure A-3 UNIFAC predictive method assessment, displaying experimental partition coefficients for various polymers versus predicted partition coefficients. (Partition coefficient error is reported as standard deviation n=3)

Unlike HSP-based predictions, AC-based methods successfully predicted high affinity for PTMG, and the polymers predicted to have the highest affinities for butyric acid had the four highest observed partition coefficients (Table A-II), with predicted partition coefficients of 4.4, 4.1, 3.8 and 2.9 reflecting respective experimental values of 3.2 ± 0.4, 3.0 ± 0.4, 3.4 ± 0.2, and

6.2 ± 0.5, suggesting that AC-based predictions are superior in accuracy to HSP-based methods. However, it must be noted that 3 of these 4 polymers were identified through HSP predictions, underlining the useful value of both methods.

From this work it appears that absorptive polymers can, as a "first cut", be identified for hydrophilic solutes first by applying HSP screening, with candidates then subjected to the increasing rigour of an AC-based thermodynamic method to improve accuracy for predicting uptake for a target solute (i.e. butyric acid). It must be noted that unlike previous efforts in TPPB polymer selection, which compared predictions against polymers known to demonstrate good polymer-solute affinity, for the first time these predictive methods have been used to identify three new polymers expressing high affinity. Specifically, poly(hydroxyalkyl methacrylates) were shown to demonstrate significantly improved uptake for butyric acid compared to most other polymers tested. In general, HSP's ability to generate a solubility parameter for any polymer of a known composition makes this screening method very "user accessible", offering a high number of potential polymer candidates, from which polymer candidates can be subjected to more rigorous prediction using an AC-based method. However, it must also be noted that the AC based predictions here assumed infinite dilution conditions (i.e. <0.1 M), which does not accurately reflect experimental conditions (i.e. 0.23 M, or 20 g L^{-1}), and thus efforts should be undertaken to refine these predictions further to improve predictive accuracy.

A.3.2 Crystallinity

As a polymer's crystalline domains do not participate in absorption, it is important to characterize the behaviour of semi-crystalline polymers for potential TPPB applications. Specifically, the presence of water and solute under TPPB conditions could potentially interact with, and affect, crystalline domains, and these *in situ* effects could in turn affect uptake. Thus, to ensure that crystallinity corrections, as suggested above in Equation (A-1), accurately represent the *in situ* physical properties of the polymers tested, crystallinity was determined in

this work in the presence of both water and dilute levels of butyric acid. DSC results, which were used to determine percent crystallinity and the melting temperature of the polymer, as shown in Table A-II, indicate that % crystallinity did not markedly differ between dry polymers and those soaked in water or solute. However, when exposed to butyric acid, poly(tetramethylene glycol) (PTMG) samples, which exhibited the highest observed PC (6.2 ±2) in this work, showed decreased ***melting*** temperature (T_m) from 44 °C to 26 °C, as observed through DSC (Table A-II). Additionally PTMG was observed to undergo a visible phase change when exposed to 20 g L^{-1} butyric acid, demonstrating melt behaviour not observed when dry or soaked in water, as seen qualitatively in Figure A-4. PTMG samples exposed to butyric acid showed changes in physical properties, and more closely resembled a tacky amorphous material, compared to dry or water-soaked samples, which exhibited semi-crystalline behaviour at 30 °C. Furthermore, exposure of PTMG to pure butyric acid (data not shown) resulted in total solubilisation of the polymer and, in combination with a high observed PC, this suggests a strong affinity between these two materials which results in changes to polymer physical properties, likely through T_m depression.

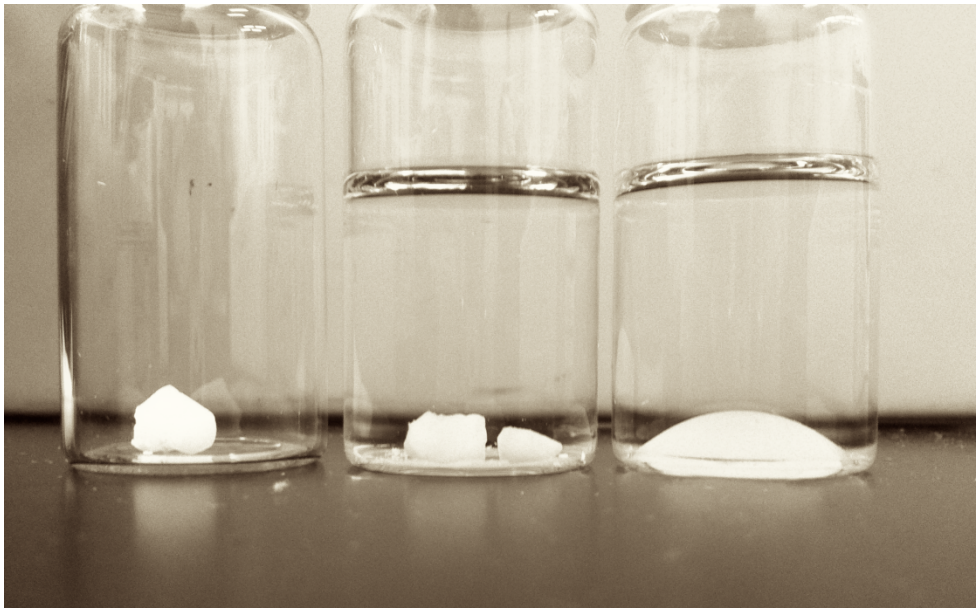

Figure A-4 Poly(tetramethylene glycol) after equilibration dry (left), in water (center), and 2% (w/w) butyric acid (right)

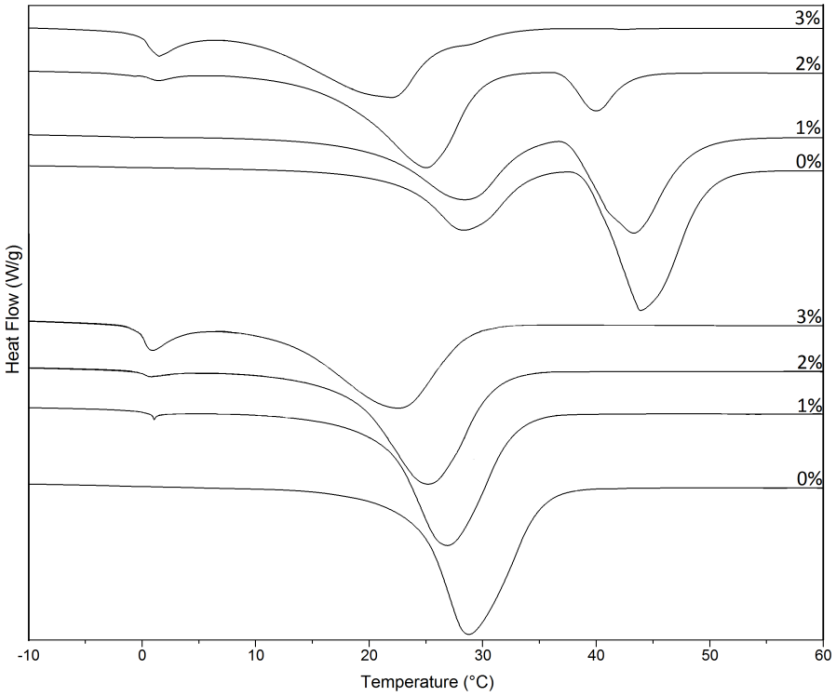

Figure A-5 DSC first (top) and second heating (bottom) scans of Poly(tetramethylene glycol) in the presence of 0 (dry), 1, 2 and 3% (w/w) butyric acid.

To further investigate T_m depression as a result of interaction between PTMG and butyric acid, PTMG polymer samples were exposed to increasing acid concentrations, which reduced melting temperatures (Figure A-5), as determined by DSC melting endotherms resulting from phase change from crystalline to melt state. Second heating scans, which yield repeatable results due to defined heating and cooling to erase a polymer's thermal history prior to scanning, showed that T_m decreased from 28°C to 22°C as exposure to butyric acid weight fraction rose from 0 to 3% (w/w). First heating scans however, which are performed without first erasing thermal history and thus more accurately represent "off the shelf" properties, show a more pronounced depression, with the major endotherm shifting from 44 °C to 26 °C at 2% (w/w). These differences in melting temperatures, as determined from 1st heating scans, would allow for PTMG to melt at experimental conditions in the presence of sufficient butyric acid, and is thus congruent with the physical change observed in these samples. Thus, in the presence of 2% (w/w) butyric acid at 30°C, although a DSC scan reports a crystallinity of 53% (Table A-II), this crystallinity largely exists only below the melting temperature, and PTMG crystallinity was decreased if not altogether eliminated through T_m

depression. Consequentially, this would improve the extent of solute absorption, which was limited by its original crystalline domains. This indicates that under typical fermentation temperatures (i.e. 30 °C), the polymer's physical properties in this case would be significantly different from dry conditions due to this observed reduced crystallinity.

As seen here, the *in situ* physical properties of the polymer used in a two-phase partitioning system may actually deviate significantly from typical "off the shelf" properties, and are an important consideration, especially as solute concentrations increase. Furthermore, it has been shown that the presence of crystallinity in a polymer significantly interferes with solute affinity predictions, as solubility parameters are based solely on minimization of the enthalpy of mixing, and do not account for the enthalpy of fusion that would also need to be overcome before a semicrystalline polymer would successfully mix (Barton 1983). In light of this, the solubility parameter approaches as outlined above appear to be insufficient for predicting uptake in semicrystalline materials, with or without crystallinity corrections. This does not necessarily preclude consideration of semicrystalline polymers from further studies, however, as it has been shown that block copolymerization in some cases can result in little or no crystallinity (Mondal and Hu 2006), and in fact copolymers bearing semicrystalline "soft segments" (e.g. Pebax 2533, a nylon-PTMG block copolymer) have demonstrated good partitioning and have been used previously in TPPBs (Gao and Daugulis 2010; Peterson and Daugulis 2014 ; Poleo and Daugulis 2013).

A.4 Conclusion

Due to low hydrophobicity, biologically produced organic acids represent one of the most challenging groups of solutes for production in TPPBs, requiring effective strategies for polymer phase selection. To address this, we are suggesting the use of a user-accessible screening method (i.e. HSPs) followed by rigorous activity coefficient calculations, representing useful tools for polymer selection. These predictive methods are especially accurate for amorphous polymers, which are unaffected by absorptive restrictions imposed by

semicrystalline regions. With respect to crystallinity, this work emphasizes that the relationship between degree of crystallinity and partitioning is complex, somewhat unclear, and not accounted for in current affinity predictions. In this work a semi-crystalline polymer exhibiting the highest affinity for butyric acid by a wide margin (e.g. PTMG) demonstrated T_m depression, which indicates that the presence of very high polymer-solute affinity may have a strong effect on crystallinity. This suggests that high affinity can potentially overcome crystalline effects, and thus semi-crystalline polymers should not be excluded from polymer selection strategies, due to possible *in situ* crystallinity reductions. Overall, this work represents an initial attempt to screen a wide array of available polymers on the basis of thermodynamic affinity, and has identified important considerations regarding the relationship between polymer-solute affinity, polymer physical properties and solute uptake.

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