ASSESSING THE BIOCOMPATIBILITY OF OLIGOMERS AND AMINE-FUNCTIONALIZED POLYMERS FOR USE IN TWO-PHASE PARTITIONING BIOREACTORS

by

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Abstract

Two-phase partitioning bioreactors (TPPBs) are a bioprocessing tool that limits inhibition of cell growth by toxic compounds in bioreactors by utilizing an immiscible secondary phase to sequester compounds that inhibit microbial cell growth or function. The earliest TPPBs used organic solvents as the sequestering phase despite challenges identifying solvents that were inexpensive and biocompatible, although recent efforts have utilized polymers as the sequestering phase in TPPBs, as most commodity polymers are inexpensive and biocompatible.

Research on the selection of polymers for use as the sequestering phase in TPPBs has focused on predicting solute uptake by first principles in order to maximize the partitioning coefficient of a target solute. Some research has shown that low molecular weight (MW) polymers have improved partitioning coefficients, although their biocompatibility is in need of further study; this thesis investigates the effect of polymer MW on microbial biocompatibility. Trends in biocompatibility were assessed for polypropylene glycol to *Saccharomyces cerevisiae* and *Pseudomonas putida*, which were selected due to their prior use in TPPBs. Given that log P has been previously used as an important physiochemical property for predicting biocompatibility, with higher log P values associated with improved biocompatibility, experiments were performed to determine the average log P of polymers. Polymer samples were also water-washed to shift the log P upwards by removing low MW polymer chains. Average log P determination experiments showed that as the polymer MW increased, the measured log P also increased. Biocompatibility, as measured by the change in optical density of the cultures after 24 hours of exposure to the polymers, also improved with increasing MW and log P, and that polypropylene glycols possessing MWs of 1000 and higher were all found to be biocompatible. It was also shown that water-washing noticeably improved biocompatibility by removing low MW polymer from samples.

Initial research was also undertaken to examine the use of amine-functionalized reactive polymers for extracting organic acids in TPPB in terms of their efficacy and their biocompatibility. An amine functionalized polyacrylate was synthesized which was stable under acidic conditions, and did not inhibit

cell growth over 24 hour exposure. It was also shown to extract 60-85% of organic acids from aqueous solution over 2 hours with polymer concentrations of 10 g/L, and acid concentrations of 2.5 g/L.

This work addresses two major areas. The first is improving the understanding of the biocompatibility of low molecular weight PPGs. Second is improving the understanding of the biocompatibility and acid extraction of amine-functionalized hydrogels, and investigates the potential for such materials for use in acid producing TPPBs.

Co-Authorship

Dr. Andrew Daugulis was a major contributor to all chapters of this thesis. His contributions were both technical and editorial in nature. Dr. Scott Parent also made substantial technical contributions to chapter 5.

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List of Abbreviations

ATCC – American Type Culture Collection

HPLC – High pressure liquid chromatography

IUPAC – International Union of Pure and Applied Chemists

OD – Optical density

OECD - Office of Economic Cooperation and Development

MW – Molecular weight

NAP – Non-aqueous phase

PAH – Polycyclic aromatic hydrocarbons

PC – Partitioning coefficient

PPG – Polypropylene glycol

TPPB – Two phase partitioning bioreactor

Chapter 1

Introduction

1.1 Background

It has been generally recognized that biotechnology will need to play an increasing role in industrial processes of society. Biotechnological processes have a positive public perception, potentially lower costs, and lower environmental impact relative to petrochemical processes. One common challenge in bioprocessing technologies is that elevated concentrations of substrate or product needed to achieve high process efficiency can inhibit cell growth or cell function, which reduces productivity. Researchers have attempted to develop strategies to overcome this obstacle and has developed the two-phase partitioning bioreactor (TPPB) technology platform. TPPBs utilize a secondary extracting phase, mixed with a cell-containing aqueous phase, to lower the effective concentration of toxic compounds in the bioreactor (Malinowski, 2001). Originally these sequestering phases had been immiscible organic solvents, although these have a number of drawbacks that have been difficult to overcome (Bruce and Daugulis, 1991). The most important challenge has generally been the identification of low-cost solvents that do not inhibit the growth of cells, as many biocompatible solvents tend to be too high in price to justify their use commercially.

As an alternative to simple organic solvents, recent efforts have turned to investigating the use of polymers as a partitioning phase, which has been shown to be a field rich with opportunity (Amsden et al., 2003; Dafoe and Daugulis, 2013). Polymers have been shown to effectively partition many toxic compounds, and tend to be low in cost, biocompatible, and easy to handle. Recent efforts in this area have focused on understanding the thermodynamic basis of solute partitioning in these solid-liquid TPPB, and this work has successfully identified a number of polymer properties that predict solute uptake (Parent et al., 2012; Poleo and Daugulis, 2014). One of the most important that has been identified is the molecular weight (MW) of the polymer, whereby lower MW has been shown to result in improved partitioning coefficients (Bacon et al., 2014). This finding suggests that the use of lower MW polymers will lead to

better reactor performance due to the enhanced solute affinity of the polymers, although there is concern that these polymers may begin to encounter reduced biocompatibility similar to that found for simple immiscible organic solvents. If a short oligomer inhibits growth, but a large polymer is biocompatible, it would be expected that some intermediate MW is the threshold for biocompatibility. What is the chain length at which a polymer becomes low enough to inhibit cell growth? What polymer qualities best allow for maximizing partitioning coefficients while limiting toxicity? The literature at present does not offer any answers to these questions.

Although the use of polymers in TPPBs has focused on absorptive uptake by amorphous non-reactive materials, recent research in the TPPB production of organic acids has suggested that such polymers may be limited in their ability to effectively partition such compounds. These acid products have great potential for a wide range of applications in the chemical industry, but biotechnological approaches have struggled to reach the level of efficiency necessary to compete effectively with petrochemical-based processes (Patel et al., 2006; Werpy et al., 2004). TPPB technology could be adapted to such fermentations through the use of reactive polymers such as amine-functionalized hydrogels. Initial results in this field suggest that these materials are potentially useful (Peterson et al., 2015). Although the application of such polymers to improve acid-producing fermentations is very appealing, a number of questions remain, such as the performance, stability and biocompatibility of such polymers.

1.2 Objectives

The ultimate objective in this work is to improve the ability to select polymers for the purpose of designing solid-liquid TPPBs. A rich body of literature is being built on this subject, focusing on subjects such as developing a fundamental understanding of partitioning behavior. This work attempts to add to the understanding of polymer biocompatibility across MWs of polymers. Given that the vast majority of typical polymers are entirely biocompatible, the focus here is on polymers with lower MW and reactive polymers, which have seen very little development in the context of TPPBs. In addition to such phenomenological experimentation on this topic, there is a need to build an understanding of the factors that influence polymer biocompatibility relative to existing knowledge regarding organic solvent

biocompatibility, which has been relatively well characterized. Polymeric materials are different than monomeric materials in many ways, including their higher MW, their polydisperse composition, diverse physical properties, and the possibility of structure customization. It is therefore necessary to adapt biocompatibility theories developed for organic solvents for use in understanding the biocompatibility of polymers. This thesis will therefore be discussing existing theories of biocompatibility relative to the findings presented here.

There is also very little information on the use of reactive polymers in TPPBs. This thesis aims to identify some materials that could potentially be used for reactive extraction in TPPBs. The qualities that will be focused on include biocompatibility, acid extraction, and polymer stability. A material that is able to successfully meet these criteria will have shown strong potential for use in TPPB systems.

Beyond this, several questions that remain open in this field need to be addressed. Are there ways to improve the biocompatibility of polymers by shifting their MW? How does one determine the log P of a polymeric substance? What factors affect the stability of reactive polymers? These, and many related questions will be addressed.

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Chapter 2

Literature Review

2.1 Two-Phase Partitioning Bioreactors

Different approaches to bioreactor design have been a natural response to the diverse synthetic and degradative roles that microbes can play, as well as to their morphological and physiological variety. One of the most common problems with bioprocessing technology relates to inhibition of cell growth by toxic chemicals. Some chemicals of industrial interest that are produced by microorganisms have a toxic effect on the producing organism, particularly as process strategies attempt to increase product titers. There are also many chemicals that can be degraded by microorganisms that inhibit growth, and this situation is exacerbated as biotreatment systems attempt to increase contaminant treatment throughputs. These phenomena have been noted as for many years, and have been approached in a variety of ways in order to improve process efficiency (Mattiasson and Larsson, 1985).

Two-phase partitioning bioreactors (TPPBs) are one such method, which use the physical/chemical properties of the product to partition it out of the aqueous reaction mixture and into a sequestering phase. Many of these toxic chemicals have hydrophobic qualities, and therefore will preferentially separate into an organic phase. By adding a non-aqueous phase (NAP) to the bioreactor the toxic chemical can be continuously extracted from the reaction mixture. By lowering the concentration of the inhibitory fermentative product in the aqueous phase the growth of the microorganism is improved, allowing for better productivity. These NAPs have traditionally been immiscible organic solvents, which can later be separated from the aqueous phase for product recovery.

TPPB systems has been investigated for many years for both biosynthetic and biodegradation applications. Research has led to a number of strategies with potential commercial uses. This includes a wide range of applications, such as improving biofuel fermentative rates, producing pharmaceutically relevant compounds, and synthesizing building blocks for higher value product synthesis (Dafoe and Daugulis, 2013). It is also possible to use TPPBs for the purpose of degrading xenobiotic pollutants.

There is much interest in developing low cost, and environmentally friendly methods to remove toxic products from wastewater and soil. There are many microbial strains that are able to biodegrade common pollutants in industrial waste, such as volatile organic compounds, BTEX compounds, as well as highly recalcitrant materials such as PAHs and PCBs (Darracq et al., 2012). By utilizing a NAP to sequester the toxic waste product, the microbes in the reactor are able to degrade the compound without experiencing inhibited growth, since the NAP reduces the toxic effects of high substrate concentrations. TPPBs can also be used for on-demand substrate delivery by adding NAP loaded with solute that will partition into the aqueous phase as solute is consumed in solution via equilibrium (Baumann et al., 2005; Daugulis et al., 2003).

This above collection of applications provides a number of examples of the utility of the TPPB biosynthetic and biodegradation strategies. Although such reactors have a wide range of applications, the design of such reactors is non-trivial. Often the most challenging aspect is the selection of an appropriate NAP for the bioprocess of interest, as it will often ultimately dictate the success of the process.

2.2 Selection of the Non-aqueous Phase

A wide range of desirable qualities has been described for TPPB systems. A number of attributes of a desirable NAP were originally defined by Bruce and Daugulis (1991). These include the following:

Favorable distribution coefficient of the product
High selectivity
Low emulsion forming tendencies
Low aqueous solubility
Chemical and thermal stability
Favorable properties for product recovery
Nonbiodegradability
Nonhazardous
Inexpensive
Available in bulk quantities
Biocompatability

This list of desirable traits has continued as the standard for NAP selection. More recent work by Kuhn et al. (2015) has advocated that factors such as environmental impact of the solvent should also be considered in addition to these criteria. It has also been found that systems that use viscous solvents may be mass transfer limited in their use, meaning that mass transfer rates might also need to be considered in solvent selection (Garikipati et al., 2009). These additional qualities, though, are a supplement to the core criteria listed above. In this section some of the most important properties of the NAP will be described.

2.2.1 Biocompatibility

If the selected NAP inhibits or stops the growth of the microbe this will act as a limitation on the productivity of the TPPB system. IUPAC defines materials as being biocompatible when they come into contact with a living system without producing an adverse response (Vert et al., 2012). The nature of an adverse response depends on the system under investigation, and in the case of microbial growth could involve changes in cell growth, nutrient consumption, or product formation. In this thesis 24 hour growth experiments are used to test biocompatibility, as it has previously been shown to be an appropriate length of time to see variation in cell growth between inhibitory and non-inhibitory conditions for fast growing organisms (Clemens et al., 1999; Fernandes et al., 2002; Vrionis et al., 2002; Sondi and Salopek-Sondi, 2004; Baumann et al., 2005; Reddy et al., 2007; Holtze et. al 2008). It is clear then that toxicity to the microbes must be limited in order to improve productivity, therefore solvents must be selected that do not impede microbial growth. Although factors such as cost are important considerations to determine the viability of a system on larger scales, if the NAP is toxic to cells, the process is simply impossible. This makes biocompatibility likely the most important criterion of NAP selection. Work has been done to understand the relationship between the properties of a solvent and biocompatibility, and the most important theory derived from this work focused on the log P (i.e. the octanol-water partitioning coefficient) of the solvent. Other theories have focused on the molecular weight (MW) of the organic solvents. These theories are discussed in more detail in section 2.3 of this review, as the connection between log P, MW and biocompatibility is a central focus of this thesis.

2.2.2 Nonbiodegradability

It is very desirable that the NAP of interest be nonbiodegradable, since if the NAP selected for a TPPB is biodegradable the metabolism of the microorganism can be disrupted. By using the NAP as a carbon source, for example, there is a possibility that that this will lead to competition with the metabolic pathway of interest (Darracq et al., 2012). This is particularly the case in applying TPPBs to substrate degradation, in which a relatively recalcitrant substrate is the target for degradation, and the NAP may be a preferred substrate, and the organisms employed are selected on the basis of their ability to degrade xenobiotic compounds. It has been shown that potential NAPs that are nonbiodegradable are rare, especially for liquid organic solvents, across all possible microbial systems including consortia. A recent review by Darracq et al. (2012) has summarized a range of different solvents that have been used in TPPB design, and has shown that the vast majority can be biodegraded by microorganisms.

2.2.3 Low Cost

Although cost is not a strict limitation on the possibility of using a particular NAP for a given system, if TPPB applications are to be adopted in industrial settings, they must be cost competitive with alternative methods. Developing systems that use a low cost NAP that is biocompatible with microorganisms has proven to be difficult. Most widely available low cost organic solvents have been shown to be extremely toxic to the microbial species to be used in such systems (Darracq et al., 2012). A small number of liquid compounds have been identified that are non-toxic to microbes, and do not biodegrade, but they tend to have prohibitive costs. An example of this might be FC 40 (a polyfluorinated compound), which has favorable microbial interactions (Césario et al., 1998), but can cost over $1,000 a liter. This clearly limits its application in TPPB strategies, as such costs are difficult to justify at large scales.

One of the most common solvents used in TPPB systems is silicone oil. Silicone oil was found to be one of the few liquids that can be used as the sequestering phase that does not lead to biodegradation, and did not seem to have any adverse interactions with the microbes (Darracq et al., 2012). It is also widely available, and is relatively non-hazardous. Despite all of these attractive qualities, silicone oil

costs hundreds of dollars a liter (Quijano et al., 2010). This makes silicone oil impractical for most industrial applications, and therefore greatly limits its use in TPPB reactors. There is therefore clearly still a need for low-cost, biocompatible, non-biodegradable NAPs.

2.3 Predicting biocompatibility

Biocompatibility is an essential consideration in NAP selection, though it is not obvious which solvents are biocompatible at first glance. Given that biocompatibility is species specific, and that there are also almost limitless possibilities in solvents, it is impossible to systematically look at each species-solvent pairing experimentally. Having prediction methods to estimate the biocompatibility to eliminate possibilities is essential to logical NAP selection. The two chemical properties that are most discussed in relation to biocompatibility are molecular weight and log P. The importance of each of these is discussed below.

2.3.1 Molecular Weight

A chemical property that has been related to biocompatibility that has been extensively discussed in the literature is MW (Brink and Tramper, 1985; Bruce and Daugulis, 1991; Mojaat et. al, 2008; Marques et. al, 2009). Initial work on the subject of biocompatibility focused on the molecular weight of organic solvents in two-phase systems (Cruz et. al, 2004). One study was able to show a sharp decline in the toxicity of solvents that exceeded 150 g/mol in weight in a species of eukaryotic algae (Mojaat et. al, 2008). This may present an important consideration in predicting toxicity, although this parameter is confounded with log P in such a way that it is difficult to differentiate the contribution of these two quantities in many instances (Mojaat, 2008). Other studies have shown clear violations of this trend, as certain molecules with high molecular weights were seen to be more toxic than molecules with much lower weights (Bruce and Daugulis, 1991). Later work has shown that log P is a superior measure for biocompatibility (Laane et. al, 1987).

2.3.2 Critical Log P

Log P, or log $K_{o/w}$ as it is also known, is a chemical property of molecules. It is used as a measure of the hydrophobicity of a compound, and quantifies the relative affinity of a chemical towards octanol versus water. The mathematical formulation of log P for the chemical "A" is shown in below:

$$\log P = \log_{10} \frac{[A]_{octanol}}{[A]_{water}} \qquad 2\text{-}1$$

where $[A]_{octanol}$ and $[A]_{water}$ are A's concentration in the given phase at equilibrium. Log P is a measure of the partitioning equilibrium of a chemical between octanol and water. Although this quantity may seem an arbitrary measure of hydrophobicity, it is a very useful metric for understanding the interactions of organic molecules with biological systems. Log P (and variants on log P) values are used to estimate uptake, distribution, and excretion patterns of pharmaceuticals in humans (Bhal et al., 2007; Lipinski et al., 2001; Tetko et al., 2001) and are used to predict the environmental fate of organic molecules (Verhaar et al., 1992; Pramanik and Roy, 2013). Log P has also been found to be one of the most important quantities in understanding toxicity of organic solvent molecules to microbial systems, as understood through the critical log P value.

Work done by Laane et al. (1987) suggested that the log P value of a compound was the most important quality to determine the toxicity of a compound to microbes, with low log P solvents inhibiting cell growth, and high log P compounds being biocompatible. This was further advanced by the description of organism specific biocompatibility trends, in which each organism has some log P value such that higher log P values are completely biocompatible and lower log P values are toxic (Inoue and Horikoshi, 1990). This was later designated as being the "critical log P" value of a particular cell, below which compounds inhibited growth, and above which the compound would be sufficiently hydrophobic to no longer interact with the microorganism. Essentially no microbial growth is observed in the presence of solvents below the critical log P, while growth above the critical log P is close to that of a control. This trend is demonstrated in Figure 2-1, which shows the growth of *P. putida* when exposed to various

solvents. The critical log P value is clearly in the range of 3-3.5 log P, as shown by the dramatic increase in cell growth and glucose consumption at log P values above this level.

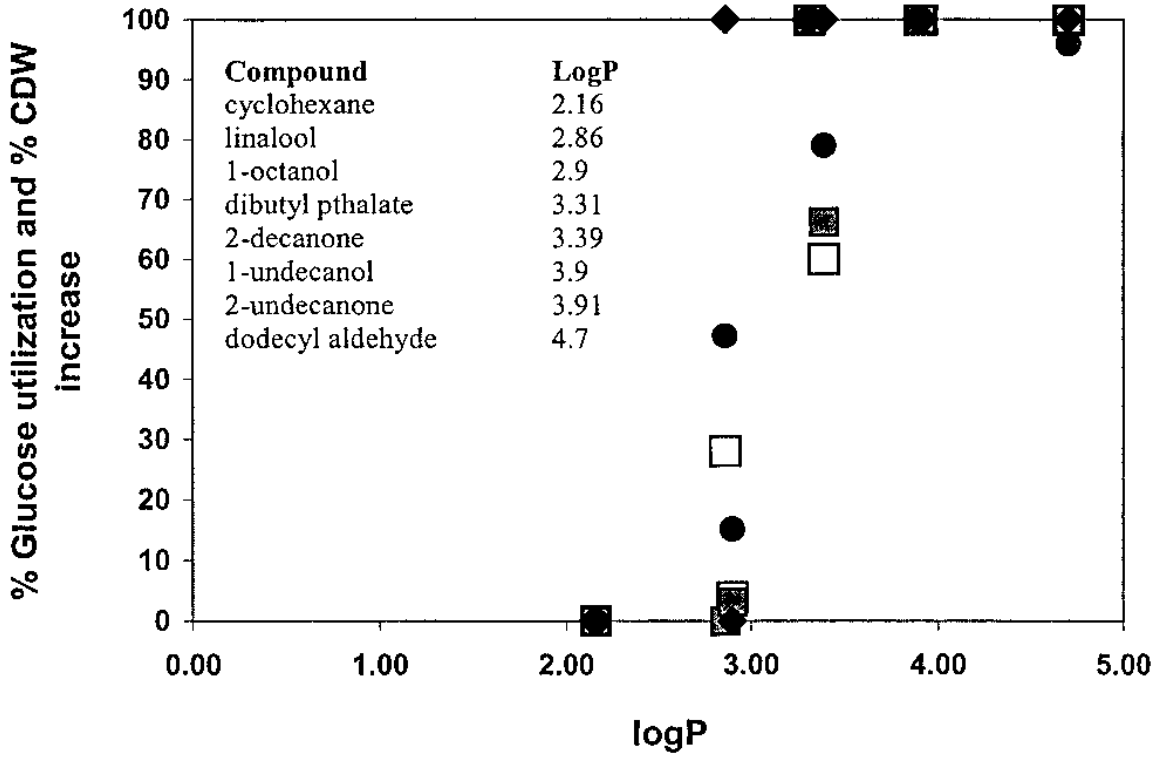

Figure 2-1: Graph demonstrating critical log P phenomenon. Results show glucose utilization and change in Optical Density (cell growth) (%) by *Pseudomonas putida* in the presence of various organic solvents. Graph taken from Vrionis et al. 2002.

A number of critical log P values have been determined for different organisms, a selection of which is summarized above in Table 2-1. The theoretical underpinnings of critical log P are not well understood, as solvent toxicity appears to be a complex phenomenon. It is clear that when the polarity of a compound is sufficiently low it will not interact with the organism in any capacity because of its low solubility and its inability to penetrate the cell membrane, and will therefore not be toxic. Analysis of microbes that have been exposed to solvents possessing low log P values are seen to have an extremely disorganized membrane structure (Osborne et al., 1990; Sikkema et al., 1995), suggesting some interference with membrane function. Membrane chemistry has long been considered to play an important role in solvent resistance. A simple example of this is seen in Table 2-1, as low critical log P values are generally seen in Gram-negative bacteria (Inoue and Horikoshi, 1995), although there are some

extremophillic Gram-positive bacteria that have been reported to have extremely low critical log P values (Na et al., 2005; Zahir et al., 2006).

Table 2-1: Critical log P of a selection of microbial species

Gram-negative bacteria	**Critical Log P value**	**Reference**
Burkholderia cepacia	3.0	Zahir et al., 2006
Pseudomonas aeruginosa	3.4	Inoue and Horikoshi, 1991
Pseudomonas putida	3.1	Inoue and Horikoshi, 1991
Escherichia coli	3.8	Inoue and Horikoshi, 1991
Aeromonas hydrophilia	4.5	Inoue and Horikoshi, 1991
Proteus vulgaris	4.2	Inoue and Horikoshi, 1991
Flavobacterium suaveolens	5.1	Inoue and Horikoshi, 1991
Gram-positive bacteria	**Critical Log P value**	**Reference**
Rhodococcus opacus	2.1	Na et al., 2005
Bacillus cereus	2.3	Zahir et al., 2006
Bacillus subtilis	4.9	Inoue and Horikoshi, 1991
Micrococcus luteus	4.8	Inoue and Horikoshi, 1991
Staphylococcus aureus	4.8	Inoue and Horikoshi, 1991
Clostridium acetobutylicum	~5.0	Barton and Daugulis, 1992
Streptococcus faecalis	5.1	Inoue and Horikoshi, 1991
Lactobacillus casei	5.1	Inoue and Horikoshi, 1991
Corynebacterium herculus	6.0	Inoue and Horikoshi, 1991
Eukaryotic organisms	**Critical Log P value**	**Reference**
Dunaliella salina	3.5	Mojaat et al., 2008
Candida utilis	4.8	Khan and Daugulis, 2010
Saccharomyces cerevisea	5.0	Bruce and Daugulis, 1991

Other forms of cell damage have been seen in microorganisms exposed to organic solvents, such as protein denaturation and DNA damage (Sikkema et al., 1995). This suggests that solvent toxicity is a phenomenon that involves a range of cell components beyond integration into the plasma membrane. Work is currently being done to further the understanding of solvent tolerance in order to engineer cell lines with increased resistance to organic solvents for biotechnology applications (Nicolaou et al., 2010) This research has shown that complex interactions ultimately dictate solvent tolerance, involving genes such as those related to ATPases and Heat Shock Proteins, as well as changes in the plasma membrane composition (Isken and de Bont, 1996; Bernal et. al, 2007; Heipieper et al., 2007). Solvent tolerance in yeast has been linked to over 250 genes, emphasizing the complexity of this phenotype (Hu et al., 2007). It is not yet clear how this complex set of genetic factors leads to the critical log P value that is observed in cells, and therefore this value must be determined experimentally.

General mechanisms of solvent toxicity have been divided into two categories: dissolved toxicity and contact toxicity, although there appears to have been little definitive work in recent years to resolve or integrate these mechanisms (Bar, 1986; Bar, 1987; Hocknull and Lilly, 1988; Cho and Schuler, 1986). Dissolved toxicity, or chemical toxicity, can be understood as being a result of the interaction of solvent molecules with some cellular structure, such as the plasma membrane, or genetic material. These chemical associations take place as a result of the water-soluble fraction of the solvent molecules, ultimately causing interference with some cellular component (Bar, 1986; Bar, 1987) Contact toxicity, or phase toxicity, results from nutrient deprivation, either through partitioning essential nutrients out of the aqueous phase, or forming films over cells to stop the diffusion of nutrients (Bar, 1986; Bar, 1987; Cho and Schuler, 1986). It is interesting to note that it was the same authors, almost 30 years ago, who sought explanations for the toxicity of organic solvents to cells. These mechanisms of toxicity are not mutually exclusive, but rather can occur simultaneously. It is possible to discriminate between their effect through various considerations of experimental set-up, such as utilizing immobilized cell reactors, or through using solvent concentrations that are below saturation, to avoid forming a secondary phase (Bar, 1987; Bar, 1988; Cho and Schuler, 1986). In this regard, the critical log P framework of biocompatibility focuses on the phenomenon of dissolved toxicity, as it assumes toxicity will be observed at sub-saturation concentrations. This implies that increases in solvent beyond the concentration necessary to saturate the aqueous phase will have no effect on cell growth.

Although the complexity of solvent tolerance is not yet fully understood, it is clear that log P has been extremely useful in phenomenologically (rather than mechanistically) predicting the applicability of a given NAP to a certain TPPB strategy. There are important limits to this theory though. Most studies done to this point when determining the microbial toxicity of NAP have focused on low MW compounds in the form of pure organic solvents, rather than polymers (Bruce and Daugulis, 1990; Inoue and Horikoshi, 1990; Barton and Daugulis, 1992; Khan and Daugulis, 2010). It has not yet been verified that the critical log P theory of toxicity extends from simple organic molecules to more complex molecules

such as oligomers and polymers. This question is of special relevance given the recent advances in TPPB research using polymers as a NAP.

2.4 Polymers in Two Phase Partitioning Bioreactors

An area of interest in TPPB research in recent years has been the use of polymers as the immiscible sequestering phase in TPPBs. Polymers are defined as chemicals that use a repeating subunit (a monomer) to create a larger molecule (the polymer). There is great flexibility that is possible in both chain length and monomer composition in a polymer, allowing for a wide range of chemical and physical qualities. Hydrophobic organic molecules are able to partition into many polymer systems in a similar manner to what is observed in liquid solvents. This implies that polymers have the potential to act as an alternative to organic solvents when selecting the secondary phase in TPPB.

Polymers have a number of properties that make them extremely attractive as an alternative to using immiscible organic solvents as the partitioning phase in a TPPB. Most synthetic polymers are biologically inert solids that are immiscible in water, and under these circumstances the polymer has minimal interaction with microbes in the aqueous phase, and will therefore not influence its metabolism in any way. Solid synthetic polymers are then both inherently biocompatible and nonbiodegradable (Amsden et al., 2003). This is a very important advantage that is imparted by polymers compared to traditional NAP.

Most polymers that have been used in TPPBs are also extremely low in cost in comparison to organic solvents, as they have tended to be bulk, commercial polymers. General-use polymers can be utilized for TPPB applications, which often cost under $5 per kilogram, and are available in bulk quantities. For some applications waste polymers, such as used automobile tires, have also been utilized, which have a near-zero cost (Poleo and Daugulis, 2013; Tomei et al., 2012). This obviously compares very favorably with many solvents, and makes polymers an attractive option for industrial scale applications.

Although not all polymers are solid at room temperature, many commodity materials exist that range in physical properties from viscous liquids to rubbery solids to hard plastics. Solid materials have many advantages over liquids in terms of handling, such as ease of transferring material, and separating polymer from fermentation media. This leads to simplified recovery protocols. There is also the potential to recycle polymers that are used in certain TPPBs in order to further reduce costs. It is possible to recycle liquid solvents, though this tends to be more difficult than polymer beads. Polymers are also generally non-toxic to humans and non-volatile, meaning that danger to those operating the reactors is minimized. All of these properties have increased interest in examining the use of solid polymers in TPPB.

There have already been a number of TPPB systems designed to utilize polymers. These include TPPBs that are able to enhance the production of commercially relevant fragrance and flavor molecules such as carvone (Morrish and Daugulis, 2008) and benzaldehyde (Craig and Daugulis, 2012). High value chemicals such as the pharmaceutical precursor *cis*-(1S, 2R)-indandiol have also been produced using polymer based TPPB systems (Dafoe and Daugulis, 2011). Polymers have also been used in TPPBs dedicated to hazardous chemical degradation, with the objective of applying such strategies to waste water treatment (Darracq et al., 2012(b); Tomei et al., 2011). This approach can also be adapted to be used in combination with other technologies, such as cell immobilization (Zhao et al., 2009). This range of applications clearly illustrates the potential of polymer based TPPB strategies.

Seeing this vast potential in the use of solid polymers in TPPB provides many new areas of investigation. The degree of variability that can exist in polymer molecules is virtually unlimited, so identifying a polymer that is suitable for a given application cannot be done randomly. Efforts are currently being made to understand the thermodynamics of polymer-substrate interactions in TPPBs (Parent et al., 2012; Poleo and Daugulis, 2013). Much of this work focuses chiefly on the chemical and physical properties that lead to the desired partitioning coefficients for a given TPPB strategy to develop suitable selection criteria for polymers. Progress in this area of research is described below.

2.4.1 Polymer selection in TPPB: partitioning coefficient of the target molecule

Most high molecular weight polymer molecules have many of the desired properties of a NAP that were described in section 2.2, as they are generally biologically inert and very low in cost. An area of more fundamental interest in polymer TPPB selection has been understanding and predicting the properties of polymers that are most important in terms of their affinity for the solutes when used as a NAP in a TPPB. Predicting affinity between pure organic solvents and solutes is relatively simple, although the use of organic solvent blends has been reported (Bruce and Daugulis, 1991; Jusling et al., 2012). Predictive methods for identifying organic solvents that are compatible with a given target molecule have also been available for many years (Kollerup and Daugulis, 1985). Research has therefore generally focused on identifying solvents that are both biocompatible and inexpensive rather than solvent-solute partitioning, as this has generally been the major obstacle for TPPBs using an organic solvent as a NAP.

Polymers, in contrast to organic solvents, are much more complex in terms of composition and structure. There is a need to test and adapt prediction theories developed in solvent-aqueous TPPBs for use in polymer-aqueous TPPBs. The manufacturer can dictate the number of monomers in a polymer chain, with different chain length leading to different physical and chemical properties. There is also potential of integrating a mixture of different monomer subunits within a given polymer chain (known as a copolymer), which again influences the physical properties of the final product. Given only these two parameters there is effectively limitless variability in the molecular structure of polymers, while there are still more complexities that can be introduced by introducing cross-linking, branch-chains, or in the preparation of blends. In the case of TPPBs it is therefore important to investigate which set of polymer properties best meets the needs of the strategy under development. A rational selection process is needed, given that it is impossible to exhaustively test polymers in order to determine a “best” material for a given process.

As has been stated above, biocompatibility in polymer-TPPBs has rarely been a concern. A more pressing issue is the partitioning behavior of the target molecule. The mathematical expression of a partitioning coefficient for "A" in a polymer-water two-phase system is given in equation 2-2:

$$PC = \frac{[A]_{Polymer}}{[A]_{Water}} \qquad 2\text{-}2$$

Where PC is the partitioning coefficient, $[A]_{polymer}$ is the concentration of the "A" in polymer and $[A]_{water}$ is the concentration "A" in water. The partitioning coefficient is therefore a metric of the ability of a polymer to absorb a solute relative to the ability of water, with a higher partitioning coefficient indicating higher polymer-solute affinity.

It is in the interest of the experimenter to be able to adjust the partitioning coefficient of the NAP for the target molecule. As the partitioning coefficient of the polymer increases the solute concentration in the fermentation medium experienced by the microbe will then drop if the same amount of polymer is present. This can allow concentrations of the inhibitory molecule to be sufficiently low such that they do not interfere with the metabolism of the microbe. This is very desirable in systems where the microbes are producing a growth-inhibiting product. In cases where the NAP is sequestering a toxic substrate (as is the case in TPPB used in waste water treatment) there must also be enough substrate available such that the desired biotransformation can proceed at the required rate (Rehmann and Daugulis, 2008). Given this, it is important then that operators are able to predict partitioning coefficient for the target molecule with relative reliability in order to choose appropriate polymers for a given system.

Studies have been performed investigating the trends in partitioning coefficients in solid polymers, and have divided the associated trends into two major categories: accessibility and affinity (Parent et al., 2012). Accessibility can be roughly described as the degree to which the bulk of a polymer is available for the association with solute molecules. If the polymer has a crystalline, or "hard" segment a lowered partitioning coefficient is observed between the solute and the polymer. A crystalline polymer phase is rigid, and the grouping of chains does not allow solute molecules to easily penetrate (Parent et al., 2012). The crystalline segment of a polymer is then virtually inaccessible to solvent molecules,

leading to polymers with a crystalline segment having a lowered partitioning coefficient (Parent et al., 2012). This has been shown through the use of polymers with similar molecular properties, but differing proportions of crystallinity, where it has been seen that an increased crystalline segment leads to a lower partitioning coefficient (Gao and Daugulis, 2010; Parent et al., 2012).

Similarly, the glass transition temperature is another important physical property of a polymer for estimating the partitioning of a solvent. A glassy state is defined as being firm and brittle rather than being malleable. Polymers will enter a glass state if the temperature falls below the glass transition temperature. Materials in this glassy state then allow for less movement at the molecular level. This lack of movement compromises the diffusivity of solvent molecules, meaning the partitioning coefficient is reduced (Parent et al., 2012).

The metric of affinity can be defined as the thermodynamic compatibility of a polymer for a given solute (Parent et al., 2012). Unlike crystallinity or glass transition temperature affinity is specific to a solute-polymer pair. A number of approaches to understand and predict affinity between solutes and polymers have been proposed, and many have been examined in the context of TPPBs (Poleo and Daugulis, 2013). One such system that shows promise in its predictive potential is the Hansen solubility parameter (HSP). This framework uses the atomic dispersion forces, molecular dipole interactions, and the molecular hydrogen bonding in order to estimate the thermodynamic affinity between two chemicals (Hansen, 2007). The mathematical expression for this quantity is shown in equation 2-3

$$(Ra)^2 = 4(\delta_{D2} - \delta_{D1})^2 + (\delta_{P2} - \delta_{P1})^2 + (\delta_{H2} - \delta_{H1})^2 \qquad 2\text{-}3$$

Ra is defined as the HSP "distance" between two chemicals, δ_D is a measure of the atomic dispersion forces of a given chemical, δ_P is a measure of the molecular dipole and δ_H is a measure of the hydrogen bonding. A small Ra value indicates that two compounds are similar in their thermodynamic properties. This should be reflected in high affinity between the two compounds, and therefore high mutual solubility. When applying this framework to TPPB strategies it has been found that the predictive powers are promising when examining binary systems (Bacon et al., 2015; Poleo and Daugulis, 2013).

Although initial results using the HSP model in conjunction with glass transition temperatures and crystallinity are encouraging, there is room for improvement. Further research will hopefully provide more precise models for the selection of polymers for TPPB system.

2.4.2 Effect of molecular weight on the physical properties of polymers

One observation that has recently come to light in the study of polymer-solute interactions is evidence of a connection between decreased molecular weight of a polymer and an increase in the partitioning coefficient (Bacon et al., 2014). Dibutyl adipate, a compound with a molecular weight of 258 g/mol, has molecular qualities that are similar to polymer poly(*n* –butyl acrylate). When comparing the partitioning coefficients of the polymer at a molecular weight of 100,000 to the dibutyl adipate monomer, it was found that the partitioning coefficient for benzene, butyl acetate, and n-butanol were 85%, 156% and 160% higher, respectively (Bacon et al., 2014). Similar effects have been noted in early work using liquid polymers such as poly(propylene glycol), where a decrease of weight from 4000 g/mol to 1000 g/mol corresponded to a 160% increase in partitioning of butanol (Barton and Daugulis, 1992).

With such a noticeable increase in partitioning, there is clearly an incentive to investigate how low the molecular weight can be decreased while still maintaining acceptable TPPB performance. Would polymers with lower MW inhibit cell growth? Given that the chief advantage of polymer NAPs is the near universal biocompatibility, there is a concern that lower MW polymers would inhibit growth. In addition to the concerns with immiscible organic solvents discussed before, some limited work was reported by Barton and Daugulis (1992) on the subject of polymers and biocompatibility. This work investigated butanol production of *Clostridium acetobutylicum* exposed to range of organic compounds, including a variety of polypropylene glycols. The results showed that polypropylene glycol with MW below 1000 inhibited the bacterial growth and butanol production, while higher MW polypropylene glycol did not (Barton and Daugulis, 1991). This result suggests that other polymers with lower MW could similarly inhibit growth.

Aside from research done in the area of silicone oils, the characteristics and behavior of short chain polymers, or oligomers, is an area that has yet to be explored in TPPB research (Aldric and Thonart,

2008). Given that these molecules are larger than simple organic solvents, but much smaller than polymers typically used in TPPBs, it is unclear what will determine biocompatibility. The research being proposed here aims to investigate this area by assessing the trends in oligomer biocompatibility. This will be done by utilizing a range of oligomers, and determining the degree of toxicity that each exerts on two different organisms. From this it is hoped that a theory of solvent toxicity can be expanded to include interactions between oligomer molecules and microbes. This will involve investigating if the critical log P theory of toxicity holds for such compounds, or if there are some additional considerations that must be taken into account when predicting toxicity of larger molecules. A major requirement of undertaking this study will therefore be quantifying the log P of short chain polymer molecules, in order to determine if there are trends relating log P with toxicity in short chain polymers.

2.5 Methods to determine log P

Given that log P has been a common measure of hydrophobicity over 30 years, it is unsurprising that a number of methods exist in order to measure or predict the log P of a compound. This includes experimental approaches as well as computer simulation techniques. Such methods have largely been developed for small organic molecules, so it is worth analyzing different strategies to assess their potential use for oligomer log P estimation. Software-based techniques will be compared with experimental methods, the latter possessing an equilibrium component and an analytical component. The equilibrium establishing methods of the shake flask, the slow stir, and high-pressure liquid chromatography (HPLC) method will be discussed to evaluate their use for the study of oligomers. Finally, methods of analysis to be used in log P experiments will be examined. By evaluating these options, one or more can be identified for use in this investigation.

2.5.1 Estimation of log P through software

Performing experiments in order to find the log P of a compound, in order to determine its biocompatibility, is a time consuming process. For a number of years many researchers have instead favored log P estimation software. Given the range of use for log P values, it is natural that a large number of different software applications have been developed in order estimate log P. Software

estimation techniques are particularly useful as a method of screening potential pharmaceutical compounds, as log P values are an important physical property in pharmacokinetics (Lipinski et al., 2001). Applications include free-to-use programs, and pay-to-use programs, many of which approach log P prediction using different computational methodologies (Tetko et al., 2001; Manhold et al., 2009).

Although these applications have high accuracy when analyzing small simple organic compounds, it has been found there may be a major limitation in these software platforms when analyzing large molecules (Manhold et al., 2009). It has been found that the predictive validity of log P prediction software is largely a function of the complexity of the molecule being analyzed, and is most closely linked to the number of non-hydrogen atoms. Most software prediction methods perform best when the number of non-hydrogen atoms is between 17 and 20, and struggle at giving reliable results if the number is greater than 35 (Manhold et al., 2009). This is a serious limitation for the application of such methods when analyzing oligomers and polymers, as many of these compounds of interest can have a non-hydrogen atom count that far exceeds 35. In addition, polymers typically exist in a polydisperse mixture; that is they contain a mixture of chain lengths, it would be impossible for a program to estimate an average log P value. This suggests that log P prediction software will not be sufficiently accurate for determining the log P values for the polymers of interest used in this study. Experiments will then need to be conducted in order to determine the log P values.

2.5.2 Shake flask method

This section, and the following sections, discuss the most common experimental methods for experimental methods to determine log P of organic compounds. This includes the shake flask, the slow stir, and the HPLC methods, which have been accepted by the Office of Economic Cooperation and Development (OECD).

The shake flask method is described in OECD method number 107 (OECD, 1995). It involves adding a pure sample of the compound of interest into a mixture of n-octanol and water. The concentration of the chemical sample should not exceed .01 mol/L in either phase. This mixture is then shaken for 5 minutes at room temperature (20-25 ^{o}C). The samples should then be centrifuged at high

speed in order to ensure proper phase separation. These phases are then analyzed using whatever method is available in order to determine the concentration of the analyte in solution. Samples from the octanol or aqueous phase can also be concentrated after they have been separated in order to assist in analysis. Concentrating the analyte is especially useful if the concentrations are very low, which occurs with extreme log P values.

This method has been found to be accurate for compounds with log P values between -2 and 4 (although it can give accurate readings for log P values as high as 5) (OECD, 1995). Although long chain polymers may have very high log P values, the monomer subunits of some polymers, or short polymer chains, may have log P values that are very low. This ability to estimate low log P values is an advantage over other methods, as these other methods struggle with giving accurate log P estimates for extremely hydrophilic compounds (OECD, 2004; OECD 2006). This suggests that the shake flask method may have some noticeable advantages over other methods when working with compounds that are relatively hydrophilic.

It should be noted that the shake flask method has its own limitations, as that maximum log P values appropriate for this method is 4 (OECD, 1995). This is due to the fact that stable microdroplets tend to form in the aqueous phase upon shaking which do not separate from the aqueous phase, even upon prolonged centrifugation. This may lead to results where the log P value is artificially lowered, as the concentration of solute in water is heightened by these microdroplets (OECD 2006). In response to this concern, alternative methods should be used for generating octanol-water-solute equilibrium for molecules with high log P values.

2.5.3 Slow-stir method

Given that the shake flask method is not suggested for use with molecules with log P values exceeding 4, alternative strategies have been developed to investigate the log P values of highly hydrophobic compounds. The slow-stirring method, which is described by the OECD, is a protocol that has been shown to give accurate results for log P values up to 8.2 (OECD, 2006; De Bruijin et al., 1989). The design of this procedure has attempted to limit the formation of stable octanol microdroplets in the

aqueous phase by minimizing disturbance, thereby improving the accuracy of results for compounds with very low affinity for water.

In the slow stir method 20-50 mL of n-octanol and 950-980 mL of water are added to a 1 litre stirring vessel (OECD, 2006). The chemical of interested is added such that the concentration does not exceed .01 moles/L in either phase. A magnetic stirrer is added, and the rate of stirring is adjusted so that a vortex that is between .5-2.5 cm is created. The length of the equilibration should be adjusted to suit the compound that is being analyzed, with more hydrophobic compounds requiring an extended time. The time is generally between a day and a week. After equilibrium has been established a sample should be taken of the aqueous phase for analysis. Extracting the analyte from the aqueous phase with organic solvents is often needed, as the chemical of interest will be extremely dilute, and may be below the limit of detection for the analytical method of choice. After the equilibrium has been established the quantity of analyte in the aqueous phase can be quantified, and the log P value can then be determined.

2.5.4 High Pressure Liquid Chromatography method

A final methodology for establishing water-solute-octanol equilibrium involves the use of high-pressure liquid chromatography (HPLC) (Eadsforth and Moser, 1983). This is described in OECD method 117 (OECD, 2004). This method has been suggested for use with compounds with log P values between 0-6, although it can be adjusted for use with compounds with very high log P values. A hydrophobic column is used along with a hydrophilic mobile phase, and samples of the analyte are injected into the column. As the analyte moves through the column an equilibrium is established between the analyte, the mobile phase, and the stationary phase, such that hydrophobic compounds will spend more time in the stationary phase and hydrophilic compounds will spend more time in the mobile phase. Hydrophobicity will then be proportional to retention time in the column. This allows for the calculation of a k value, as shown in equation 2-4:

$$k = \frac{t_r - t_o}{t_o} \qquad 2\text{-}4$$

Where t_R is the retention time (the time needed for the compound of interest to pass through the column) and t_0 is the dead time (volume of the system divided by the flow rate). This k value is then used in the following equation in order to determine the log P value.

$$\log P = A + B \bullet \log_{10} k \qquad 2\text{-}5$$

Where A and B are linear regression coefficients. These terms are specific to any given HPLC system, and are determined through the use of standard compounds. By determining the retention time of a number of compounds with known log P values in an HPLC system the A and B terms can be estimated. With this information the log P value of any compound can be computed using the retention time. One of the major advantages of this method is that it blends the equilibrium component of the log P experiment with the analytical component of the experiment. By determining the time that the compound takes to move through the column there is no need to then measure the concentrations of the analyte in a given phase afterwards. This lowers the amount of time needed to perform the experiments.

Great effort has been put into adapting HPLC based methods for a wide range of compounds. It has been shown to give reliable results for organic chemicals with high log P values, high molecular weights, and for compounds with halogen, organometallic, acidic or basic functionalities (Schon et al., 1998; Yamaguchi et al., 2012; Payen et al., 2008; Asami and Saitoh, 2006). This broad spectrum of application, and the relative ease of use, and speed of this method have made it very common within the research community.

The HPLC method may have difficulty in determining the log P values of oligomers and polymers because of polydispersity. Most polymers, even those with relatively small chain length, are not present as one simple compound, but rather are a mixture of compounds of slightly different sizes. This phenomenon is referred to as polydispersity. If the different compounds have slightly different retention times when analyzed by HPLC it will be difficult to determine which substance to base the retention time calculations from. Mathematical strategies have been developed to overcome this issue, through the use of a weight average log P, as shown in equation 2-6 (OECD, 2004).

$$weighted\,average\,\log P = \frac{\sum_i (\log P_i)(area\%_i)}{total\ area_i} \qquad 2\text{-}6$$

Where the weighted log P_i is the calculated log P of a given peak, and area $\%_i$ is the percent of the total area this given peak occupies. This expression allows for calculating the average log P of a series of peaks, in order to give a "weighted average" value. It should be noted that the usefulness of this method for polymer samples has yet to be investigated.

2.6 Reactive Extraction of Organic Acids by use of Reactive Polymers

One of the limitations of current TPPB technology is its use in acid-producing systems. There is a major demand for organic acids in the chemical, and food industry, and the use of reactive polymers is a possible approach that can overcome some of the challenges of acid-producing TPPBs. Reactive polymers include reactive centers integrated into the polymer chain, allowing them to extract target solutes of interest through chemical bonds, rather than through weaker interactions as is the case in absorptive polymers. Given that current TPPB research has struggled to identify absorptive polymers that have sufficiently high affinity for organic acids to properly extract products, reactive polymers are an alternative that may be able to overcome this issue. One component of this thesis will therefore be to synthesize reactive polymers appropriate for use as an *in situ* product removal strategies and examine the biocompatibility, stability, and extracting capacity of this material. As an introduction to this topic, the importance of organic acids, and the challenges related to their fermentative production are described in section 2.6.1. This is followed by a summary of the phenomenon of reactive extraction in section 2.6.2, and a description of reactive polymers in section 2.6.3.

2.6.1 Organic acid production

In 2004 the US Department of Energy created a list of 12 classes of chemicals that it deemed as being the "Top Value Added Chemicals from Biomass" (Werpy et al., 2004). This list is seen below:

Table 2-2: Top Value Added Chemicals from Biomass (Werpy et al., 2004)

Top Value Added Chemicals from Biomass
Succinic, fumaric, and malic acids
Furan dicarboxylic acid
3 Hydroxy propionic acid
Aspartic acid
Glutamic acid
Itaconic acid
Levulinic acid
3-Hydroxybutyrolactone
Glycerol
Sorbitol
Xylitol/arabinitol

Two-thirds of the compounds on this list are acids, implying that this entire class of compounds has potential as materials for the chemical industry. These are primarily "building block" compounds, which can be incorporated into existing products (Werpy et al., 2004). Often these can act as an alternative to existing petrochemicals, and can offer improvements in environmental impact, cost, and public perception over traditional production routes (Werpy et al., 2004).

Despite their benefits, there are some barriers to wide scale adoption of biosynthetic production of many organic acids. Processes exist to fermentatively produce a wide range of organic acids, but are not cost-competitive with their petroleum-based competitors (Sauer et al., 2008). Succinic acid is a classic example of this. In 2006 the annual biological production was approximately 16 000 tons per year, while its petroleum-based competitor had a demand over 10 time that (213 000 tons) (Patel et al., 2006). The biosynthetic method is simply too costly. Purification is responsible for between 50-70% of the costs associated with succinate production, and involves the use of large quantities of $Ca(OH)_2$ and CaO (Datta, 1992; Cheng et al., 2012). Improvements in purification and production of succinic acid could greatly enhance its position in the marketplace, as well as creating less waste. Such challenges are not uncommon in the fermentative production of organic acids, as purification is a major expense in many of the common biosynthetic processes, such as lactic and citric acid (López-Garzón and Straathof, 2014). Improvements in the area of purification of organic acids could therefore lead to substantial cost reduction. Reactive extraction is a method that is currently being explored for this purpose.

2.6.2 Reactive extraction

Reactive extraction is a method of product recovery, which utilizes an extractant that chemically interacts with the target compound, such as through acid-base reactions. This extractant is typically a basic organic compound dissolved in a secondary phase known as the diluent (which is typically an aliphatic hydrocarbon). This allows the target molecule to partition into the secondary phase with the extractant, away from a complex reaction mixture. This method is extremely useful for the separation of fermentation products, and has been developed for range of target acids (López-Garzón and Straathof, 2014).

Early applications of reactive extraction are described in the patent literature in the 1970's (Grinstead, 1974; Grinstead, 1976), which was followed by academic works investigating the use of reactive extraction for the removal of organic acids from aqueous media (Kertes and King, 1986). There are many organic acids that are of interest to the food, pharmaceutical and chemical industry, such as lactic, succinic, and citric acid. These compounds tend to be difficult to separate from an aqueous reaction mixture, as their hydrophilicity creates difficulty for traditional extraction procedures. Reactive extraction is able to make use of the carboxylic acid functionality to assist with separation. The most common extractants for these applications have been long-chain aliphatic tertiary amines such as tri-octyl amine or organophosphorous compounds, such as tri-octyl phosphine oxide (Shan et al., 2006). These classes of compounds have been shown to partition organic acids more rapidly than other organic compounds (Wasewar et al., 2004). Mathematical models have also been developed in order to predict the partitioning behavior of a given acid-extractant-diluent system, which has been shown to have good predictive value (Kyuchoukov and Yankov, 2012; Lux and Siebenhofer, 2012; Shan et al., 2006; Uslu, 2006)

Lactic acid has been a model molecule for developing such procedures. This simple organic acid has a large global market for both food products, and applications within the plastics industry, though its bioproduction is limited by the constraints described earlier (Wasewar et al., 2004). Furthermore, lactic acid partitions very poorly into traditional organic solvents (Wasewar et al., 2006). Through both

experimental and theoretical work, diluent-extractant combinations have been identified that aim to maximize lactic acid recovery from aqueous mixtures (Kyuchoukov and Yankov, 2012; Lux and Siebenhofer, 2012). Although lactic acid has been the focus of much research, work have been done on the reactive extraction of a range of other acids, including succinic, citric, hexanoic, and propionic. A recent review of the techniques for purifying carboxylic acids from fermentation media has summarized well the methods that have been developed for the reactive extraction of various acids (López-Garzón and Straathof, 2014).

Cytotoxicity of extractant and diluent molecules have generally been found to be one of the limiting factors in the development of reactive extraction bioreactors. If an acid extracting phase can be integrated directly into a bioreactor, the product can be removed as the fermentation takes place, which can lead to gains in productivity and reduced costs. *In situ* product recovery also grants a number of other advantages, such as eliminating feedback inhibition, thereby improving growth kinetics. The difficulty is that often the diluents or extractants that leads to rapid partitioning are not biocompatible with the microorganisms (Keshav et al., 2008), therefore, solvents with low log P values are unusable in applications that require direct contact with microorganisms. A variety of strategies have been developed to alleviate the problem of cytotoxicity in reactive extraction system, such as immobilized cell and membrane bioreactors (Yabannavar and Wang, 1991). Although there are some improvements in such systems, they are not without their own challenges, such as increased set up costs, fouling, and limited biocompatibility.

There has been some progress made in the use of reactive extraction systems as a means for *in situ* product removal. Jeon et al. reported in 2013 that they were able to enhance the production of hexanoic acid through the use reactive extraction in *Clostridium sp.* BS-1 (Jeon et al., 2013). Through the use of a 10% (v/v) alamine 336 in oleyl alcohol extractant the yield of hexanoic acid was raised from 6.96 g/L to 32 g/L. Zigová et al. (1999) reported enhancements in the production of butyric acid produced by *C. butyicum* using 20% Hostarex A327 in olyel alcohol and achieved a 20 g/L concentration of butyric acid, which exceeds the 7.3 g/L reported for a reference system. Wu and Yang (2003) then enhanced

these efficiency gains by use of *C. tyrobutyricum* in an immobilized cell bioreactor with the use of an Alamine 336 and oleyl alcohol phase raising the final concentration of butyric acid to 301 g/L. The use of an anionic exchange resin in *in situ* product recovery has also been reported for 3-hydroxypropionaldehyde extraction (Rütti et al., 2011).

In addition to such applications for use in bioreactors, work has also been done on the reactive extraction of a number of acids from chemical mixtures, including acetic acid (Mahfud et al., 2008; Rasrendra et al., 2011), benzoic acid (Agrahari et al., 2014; Mei et al., 2002), formic acid (Galaction et al., 2011) and succinic acid (Hong and Hong, 2000; Kurzrock and Wuester-Botz, 2011). These various areas of research show promise, but they are chiefly using similar compounds as the extractants and diluents described above that are not biocompatible. There are major advantages that could be gained through the use of reactive polymer as an extractant. This class of materials is described below.

2.6.3 Reactive polymers

One of the appeals of traditional polymers for many TPPB applications is that they are essentially biologically and reactively inert. Reactive polymers are specialty polymers, which contain some functional group that is chemically active. These reactive groups can be incorporated into the polymer backbone, or can be a grafted onto the structure after it has been synthesized (Kenawy et al., 2007; Singh et al., 2012). These materials are not being prepared for use as commodity polymers, but rather are formulated for specific applications, the most important of which is in creating anti-microbial materials, coatings and fabrics (Kenawy et al., 2007, Münoz-Bonilla and Fernández-Garcia, 2012). Utilizing antimicrobial polymers in hospitals, medical supply manufacturing facilities, and other at-risk locations can lower the chance of the infectious disease spreading to vulnerable populations. Polymers developed to date include those with an incorporated quaternary nitrogen group (Tiller et al., 2002), organometallic polymers (Al-Muaiki et al., 2000), incorporating antimicrobial agents into conventional polymers (Lin et al., 2001), and many others (Münoz-Bonilla and Fernández-Garcia, 2012). Beyond the work done in antimicrobial coatings and textiles, there are some addition applications that have been investigated for reactive polymers such as medical imaging (Zhu et al., 2012) and drug delivery (Kim et al., 2009).

Another area of interest is the use of reactive polymers for the purpose of extracting organic acids from chemical mixtures through the use of reactive extraction (Aşçi and Hasdemir, 2008; González-Sáiz et al., 1997). These methods could potentially be adapted to *in situ* product removal systems. By using a reactive polymer, it is anticipated that the growth of acid-producing microbes will be uninhibited, while still allowing for product removal, in addition to improved handling over extractant-diluent mixtures typically used in reactive extraction. This can reduce end-product inhibition, as well as improving down-stream processing as a result of the concentrating effect of the extracting polymer. TPPB systems using absorptive polymers have been largely unable to meet the PC values necessary to limit end-product inhibition in the production of organic acids (Hepburn and Daugulis, 2012; Peterson et al., 2015). Early results using polyesters with pendant tertiary amine groups have also shown potential in effectively extracting organic acids, and balancing pH (Peterson et al., 2015). Although this may be a useful method for *in situ* product removal, there is still a potential for the growth of the microbes to be compromised. As discussed above, one of the primary areas of investigation for reactive polymers is for use in anti-microbial surfaces, implying that there is a possibility of growth inhibition. There are also potential concerns about the stability of the polyester species that have so far been investigated (McCool and Senolges, 1989; Peterson et al., 2015). Breakdown of the polymers in TPPB is expected to inhibit cell growth and limit the ability to reuse the polymers. Polymers will need to be synthesized that do not inhibit the growth of cells, while reacting rapidly with acids in solution, and remaining stable.

2.7 Microorganisms

The investigation that is being proposed will require a number of tests to examine the biocompatibility of short chain and reactive polymers. Suitable organisms should be chosen which have well defined critical log P values and are of general interest to the biotechnology community. The microbes should also be relatively robust, and have short generation times in order to simplify testing. Given these criteria, the microorganisms that have been selected are the bacterium *Psuedomonas putida,* as well as the eukaryotic *Saccharomyces cerevisiae*.

2.7.1 *Pseudomonas putida*

Psuedomonas putida is an organism that that has been actively used in biotechnology for many years ago, although primarily not in the context of production, but rather to aid in the degradation of xenobiotics. *P. putida* has been found to efficiently degrade benzene, toluene, ethyl benzene xylene mixtures, as well as phenol and other compounds (Hill and Robinson, 1975; Kulkarni and Chaudhari, 2006; Kumar et al., 2005; Shim and Yang, 1999; Wackett and Gibson, 1988). Cell lines have also been engineered for biosynthetic applications (Poblete-Castro et al., 2012). *P. putida* is well suited for many biotechnology applications because of its tolerance to a wide range of conditions, as well as being able to metabolize a range of carbon sources (Poblete-Castro et al., 2012; Prpich and Daugulis, 2007). It also has a history of use in TPPB applications (Collins and Daugulis, 1997; Vrionis and Daugulis, 2002). This Gram-negative bacterium grows under aerobic conditions, and reliable methods have been developed for culturing and growth of the ATCC 11172 strain. The critical log P of this species has also been previously been reported, which has been found to be approximately 3.5 (Inoue and Horikoshi, 1991). It is therefore a strong candidate for use in studies of tolerance to various polymers.

2.7.2 *Saccharomyces cerevisiae*

This eukaryotic organism is an extremely important species in cellular biology in general, and is used in a number of biotechnology applications (Hong and Nielsen, 2012; Malinowski, 2001). One of the many applications is the fermentative production of ethanol, which has already been examined through the use of TPPB (Kollerup and Daugulis, 1985). Established methods for working with *Saccharomyces cerivisiae* in solid-liquid TPPB (Amsden et al., 2003) as well as a previously established critical log P value (found to be approximately 5) are available (Bruce and Daugulis, 1991). It is also hoped that by using a model eukaryotic microbe, the results of these experiments can then be generalized to a number of different eukaryotic microorganisms.

2.8 Scope of Thesis

The overarching objective of this project will be to further the development of the polymer selection criteria in polymer containing TPPB. Any complete polymer selection criteria will need to take

into consideration both solute partitioning and biocompatibility. Progress in understanding the relationships between polymers and solutes has suggested utilizing polymers with the lowest possible MW for improved partitioning. These advances in understanding physiochemical properties must now be complemented with an understanding of biocompatibility. This will be achieved by systematically determining the log P and biocompatibility of polypropylene glycols. The results from these experiments will be used to evaluate the application of the critical log P theory of biocompatibility.

In addition, initial work will be done to assess the viability of amine-functionalized hydrogels for the purpose of reactive extraction in TPPB systems. There are no prior results that examine the use of such materials in TPPB systems. Research must be done to assess their acid extraction capacity, and their biocompatibility. This will be done by synthesizing polymers, extracting organic acids such as acetic, succinic and butyric acid, and exposing *S. cerevisea* and *P. putida* to these materials. If this class of materials is found to be biocompatible, stable, and acid extracting, they will have potential as a partitioning phase in acid-fermenting TPPB.

Overall, this thesis aims at improving the understanding of biocompatibility of polymeric materials. By understanding the trends in polymer biocompatibility, TPPB design can be enhanced to use polymeric materials with improved partitioning. Only by studying polymers with lower MW or reactive centers, can the limits of TPPB technology be advanced.

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Biocompatibility of Low Molecular Weight Polymers For Two-Phase Partitioning Bioreactors

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3.1 Preface

One of the most pressing questions that this work attempts to address is the following: can the cytotoxicity of a polymer be predicted? If a monomer of a given compound completely inhibits growth, and a high molecular weight (MW) polymer is entirely biocompatible, at what MW is biocompatibility achieved? This chapter begins to address this question by focusing on polypropylene glycol (PPG). By focusing on a single polymer relationships between MW, log P and biocompatibility can be identified that can later be tested against other polymer types.

Initial experiments repeated work previously done to determine the critical log P of *Saccharomyces cerevisiae* and *Pseudomonades putida*. These two microorganisms were chosen for use in biocompatibility experiments because of 1) their prior use in two-phase partitioning bioreactors (TPPBs), 2) are of general interest in biotechnology, and 3) they represent two separate groups of organisms (prokaryote and eukaryote). Results showed that *S. cerevisiae* and *P. putida* have critical log P values of 5.0 and 3.5, which agree with previously reported values. These experiments were followed by tests determining the biocompatibility of PPG from 400-4000 MW. It was found that the data did not divide neatly into "biocompatible" and "not biocompatible" as would be typical using traditional monomeric organic solvents. There was also a negative correlation between the volume of PPG added to microbial broth and the growth of the microorganism, which is contrary to observations reported for organic solvents, where the microbial growth is independent of the amount of solvent added.

To assist in developing a better understanding of polymer biocompatibility, this paper develops two methods that are instrumental for conducting research in this area. The first method was the determination of the log P value of polymeric materials by use of an HPLC. This method has typically been used for monomeric samples of organic compounds. There are no prior reports of any method being used to determine the log P of a polymer. This value is necessary for comparing the growth of microbes exposed to polymeric materials relative to the critical log P theory of biocompatibility.

A second method that was developed was water washing of polymers for the purpose of removing low MW fractions. This involved mixing a volume of polymer with an equal volume of water

for 2 minutes for the purpose of removing the most water-soluble fraction from the polymer samples. Analysis of polymer samples by HPLC showed that water washing of PPG removed low log P fractions from polymers samples. Previous results had shown that low log P solvents strongly inhibited cell growth. By removing low log P polymer fractions from polymer samples, it may therefore be possible to improve biocompatibility. This chapter looks both at the effect of water washing polymers on their log P, as well as on changes to biocompatibility.

The results of these experiments showed that the low MW polymer fractions in polydisperse polymers are a major cause of inhibiting cell growth. Microbes exposed to water washed PPG had a higher cell density after 24 hours than those exposed to PPG that was not washed. Log P of washed polymer fractions were increased by up to .8 log P units. These results suggested that the low MW polymer chains that are found in polymers samples were an important consideration for understanding the biocompatibility of polymers, and were chiefly responsible for inhibiting microbial growth. In conclusion, the work described here shows the use of HPLC for log P determination, the importance of low MW polymer chains in microbial biocompatibility, and the influence of water-washing on cell growth.

3.2 Abstract

Two-phase partitioning bioreactors (TPPBs) improve the efficiency of fermentative processes by limiting the exposure of microorganisms to toxic solutes by sequestering them into a non-aqueous phase (NAP). A potential limitation of this technology, when using immiscible organic solvents as the NAP, is the cytoxicity that these materials may exert on the microbes. An improved TPPB configuration is one in which polymeric NAPs are used to replace organic solvents in order to take advantage of their low cost, improved handling qualities, and biocompatibility. A recent study has shown that low molecular weight polymers may confer improved solute uptake relative to high molecular weight polymers (i.e. have higher partition coefficients), but it is unknown whether sufficiently low molecular weight polymers may inhibit cell growth. This study has investigated the biocompatibility of a range of low molecular weight polymers, and compared trends in biocompatibility to the well-established "critical log P" concept. This was achieved by determining the biocompatibility of polypropylene glycol polymers over a molecular weight (MW) range of 425-4000 to *Saccharomyces cerevisiae* and *Pseudomonas putida*, two organisms which have been previously used in TPPB systems. The lower MW polymers were shown to have lower average log P values, and showed more cytotoxicity than polymers of the same structure but with higher molecular weight. Since polymers are generally polydisperse (i.e. polymer samples contain a distribution of MWs), removal of the lower MW fractions via water washing was found to result in improved polymer biocompatibility. These results suggest that the critical log P concept remains useful for describing the toxicity of polymeric substances of different MWs, although it is complicated by the presence of the low MW fractions in the polymers arising from polydispersity.

Keywords: Two-phase partitioning bioreactors, bioreactor design, bioengineering

3.3 Introduction

In situ product removal is a strategy used in bioreactor design to limit the accumulation of toxic fermentation products, thereby increasing process efficiency. One of the most successful approaches to *in situ* product removal has been the use of two phase partitioning bioreactors (TPPB) (Dafoe and Daugulis, 2014). This approach uses a non-aqueous phase (NAP) in order to extract product as it is being produced by a microbial population, thereby reducing cytotoxicity. Work in the field of TPPBs has largely focused on the use of organic solvents as the extracting phase and has been shown to enhance efficiency in a number of biosynthetic processes utilizing both prokaryotic and eukaryotic cells (Bruce and Daugulis, 1991; Barton and Daugulis, 1992; Vrionis et al., 2002; Newman et al., 2006; Heipieper et al. 2007; Yang et al. 2009, Brennan et al. 2012). TPPBs utilizing an organic solvent NAP have also been shown to be effective in promoting the efficient degradation of various toxic substrates (Yeom and Daugulis, 2011; Quijano et al., 2009; Darracq et al, 2012). The major challenge that has limited progress in the field of TPPB research has been selecting liquid partitioning NAPs which are non-toxic to the microorganisms involved. A clear relationship has been established between the logarithm of the octanol-water partitioning coefficient (log P, or log K_{OW}) of an organic solvent and the toxicity of that solvent to microorganisms (Bruce and Daugulis, 1991; Inoue and Horikoshi, 1991) solvents with low log P values tended to be toxic, while compounds with higher log P tended to be biocompatibile. The log P value at which solvents are biocompatible to a given microorganism has been termed the "critical log P", and this phenomenon has been experimentally demonstrated for a number of cell types (Bruce and Daugulis, 1991; Barton and Daugulis 1992; Inoue and Horikoshi, 1991; Khan and Daugulis, 2010). Biocompatible alternative NAPs that have been investigated include silicone oils (El Aalam et al., 1993) or ionic liquid extractants (Quijano et al., 2010a,b), which have tended to be prohibitively expensive. A challenge in selecting liquid NAPs for TPPBs has therefore been in identifying a material that is low in cost, rapidly uptakes the target compound, and has a log P value which is above the critical log P of the producing organism (Bruce and Daugulis, 1991).

Recent efforts of our group have focused on using polymers as NAPs in TPPB applications. Polymers tend to be non-toxic to organisms, non-volatile, low in cost, non-biodegradable, and have superior handling qualities. Materials such as DuPont's Hytrel® and styrene/butadiene rubber have been shown to be effective in improving productivity of a number of bioreactor systems via *in situ* product removal (Khan and Daugulis, 2010; Amsden et al., 2003; Morrish and Daugulis, 2008). Recycled automobile tires, which have a near-zero cost, have also been shown to enhance productivity in certain TPPB applications (Tomei et al., 2012). Given the demonstrated efficacy of polymeric NAPs, we have recently developed theoretical models to assist with rational polymer selection (Bacon et al., 2014, Parent et al., 2012, Poleo and Daugulis 2014). One outcome of this work was the demonstration that low molecular weight (MW) polymers tend to have higher solute uptake (i.e. have higher solute affinity) than an equivalent polymer of a higher MW (Bacon et al., 2014) and these results suggest that polymers with lower MW should be further explored as NAPs for bioreactor systems. Experimental work has shown that reducing the MW of a poly(n-butyl acrylate) from an average of 100,000 to 258 improved the partitioning coefficient of n-butyl acetate and benzene by over 150%, which clearly demonstrates that the gains in solute uptake by using polymers with the lowest possible MW are often substantial (Bacon et al., 2014)

A common explanation for a polymer's biocompatibility and non-bioavailability is that it can be considered to be a very large molecular weight organic solvent, and hence be effectively inert to microorganisms. It is important to recognize, however, that a spectrum exists that spans low MW monomeric liquid organic solvents at one end, and their higher MW polymeric counterparts at the other end. That is, polymers can exist over a vast range of monomeric ("mer") units and corresponding molecular weights, and that physically they can exist as liquids in the case of low molecular weight (small "mer") polymers or very hard, large molecular weight materials. Given that as MW decreases polymers begin to become more monomer-like (which may not be biocompatible), there is a potential concern that arises in the use of short-chain oligomers in TPPB applications. Limited work has been done to investigate the use of polymers as NAPs (Barton and Daugulis, 1992) over a range of MWs, and these findings have suggested that polymers with MW below 1000 Daltons could be toxic to microbes, even

though they have improved solute uptake, as noted earlier (Bacon et al., 2014). To the best of our knowledge, however, there has been no examination of the properties of the polymers that led to toxicity, nor any effort to correlate the toxicity of these polymers to the critical log P concept. The objective of this work was to improve the understanding of the patterns of toxicity over a range of polymer MWs by determining the relationship between the log P of the polymers and polymer MW to microbial biocompatibility. Specifically, trends in the toxicity of a range of relatively low MW polypropylene glycols (PPG) to *Saccharomyces cerevisiae* and *Pseudomonads putida* (a eukaryotic and a prokaryotic organism used in TPPB applications) were examined, and methods to reduce toxicity of polymers were explored.

3.4 Materials and Methods

3.4.1 Log P Determination of PPGs of Different MWs

The log P of the various polymer samples was estimated in order to assess the validity of the critical log P concept by determining the log P of the polymer samples and comparing this with the trends in biocompatibility. Accurate estimation of organic molecules with greater than 30 non-hydrogen atoms utilizing log P prediction software is challenging (Mannhold et al, 2009) There is also a range of molecular weights within each polymer sample as a result of the polydispersity of the polymers, making log P calculations impossible. A method that makes use of high pressure liquid chromatography (HPLC) for log P estimation of organic compounds has been previously developed (Eadsforth and Moser 1983) which first determines the retention time of a series of organic solvents of known log P, in order to create calibration equation, from which the log P of an unknown compound can be determined. The Organization for Economic Co-operation and Development (OECD) later adopted this method (OECD, 2004). A method for the calculation of the average log P of samples with multiple peaks has also been developed (Vik et al., 1998), and was also adopted by the OECD (OECD, 2004). The method finds the log P of each peak in the chromatogram, and calculates the weighted average log P through the following equation, which was also used in this work:

$$weighted\,average\,\log P = \frac{\sum_i (\log P_i)(area\%_i)}{total\ area_i} \qquad 3\text{-}1$$

To estimate the log P of the various PPG samples a Varian HPLC with Prostar 210 UV detector measuring at 200 nm, and a Polaris 5 C-18-A column (150 mm x 4.6 mm) were employed. The mobile phase was a 1:1 mixture of acetonitrile and water at a pumping rate of 1 ml/min.

3.4.2 Cultures and Media

Saccharomyces cerevisiae, an industrial ethanol-producing yeast strain obtained from Alltech (Nicholasville Kentucky USA) was cultivated in a medium (Doran and Bailey, 1986) containing 20 g/L glucose, 5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L yeast extract and 0.1 g/L $CaCl_2$. *Pseudomonas putida* ATCC 11172 was grown in medium described by Vrionis et al (2002), using 5 g/L glucose, 6 g/L K_2HPO_4, 4 g/L KH_2PO_4 and 2g/L $(NH_4)_2SO_4$, augmented with a divalent salt solution (0.66 g/L MgCl2 and 0.25 g/L $CaCl_2$) and 0.5% (vol/vol) trace element solution (0.3 g/L H_3BO_3, 0.089 g/L $ZnSO_4 \cdot 7H_2O$, 0.024 g/L $NiSO_4$, 0.018 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.003 g/L $CuSO_4 \cdot 5H2O$, 0.050 g/L $MnSO_4$, and 0.190 g/L $CoCl_2$); 0.0006% $FeCl_3$. Cultures were grown for 24 hours in 50 mL, of medium in 125 mL Erlenmeyer flasks at 30 ^oC and 180 RPM. All reagents used in the medium formulations were obtained from Fisher Canada.

3.4.3 Critical Log P Determination

The critical log P values of *S. cerevisiae* and *P. putida* were determined by utilizing a 1 mL inoculum in 50 mL of the corresponding medium described above, and 5 mL of an organic solvent (or no solvent as the control). The list of solvents used, along with supplier information and reported log P values can be found in Table 3-1. Cultures were allowed to grow for 24 hours at 30 ^oC and 180 RPM.

Table 3-1: Information for solvents used in critical log P experiments

Solvent	Log P	Supplier
Gamma nonalactone	2.08	Givauden, United States
Ethyl benzenoate	2.64	Sigma Aldrich, Canada
Hexane	3.29	Fischer, Canada
Cyclohexane	3.44	OmniSolv, Canada
Octane	4.30	Acros, United States
Dodecanol	5.13	Acros, United States
Decane	5.0	Acros, United States
Dodecane	6.82	Fischer, United States
Oleyl Alcohol	7.56	Aldrich, United States
Hexadecane	8.86	Acros, United States

3.4.4 Polymers and Solvents

Bulk polypropylene glycol (PPG) with molecular weights of 425, 725, 1000, 2000 and 4000 with an unspecified polydispersity index, and PPG standards of average MW 470, 830, 1080, 2160, and 3100 with polydispersity indices (M_W/M_N) of 1.07, 1.06, 1.05, and 1.08 respectively were used in these experiments. All MW are given as the weight average (M_W). All polymers were purchased from Scientific Polymer Products. Solvents used to construct the standard curve for HPLC log P experiments were benzene, toluene, phenol, fluoranthene, dodecanol, dimethylphenol, pentanol, ethyl benzene and xylene (Log P values 1.5 - 5.2)

3.4.5 Biocompatibility of Polymers of Different MW

These experiments were undertaken to determine both the effect of polymer MW, and the effect of the amount of polymer addition, on cell toxicity. The toxicity of the bulk PPGs of various MWs was assessed by shake flask experiments, with each shake flask containing 1, 5, or 10 grams of the polymer, which are viscous liquids at room temperature. 1 mL of microbial inoculum and 50 mL of the corresponding medium (described above) was added to each 125 mL flask, which were incubated at 30 ^{o}C and 180 RPM for 24 hours. The addition of 5 g of polymer to 50 mL medium (10% NAP fraction) represents a "conventional" methodology when testing biocompatibility, as was also done above in determining the critical log P values of the 2 organisms. The use of lower (1 g) and higher (10 g) amounts of polymer NAP was intended to assess any volume dependency of toxicity. The critical log P model of toxicity is expected to be essentially volume independent, and in this study we aimed to assess if this was true in the case of polymeric NAPs. Two experiments utilizing liquid NAP (cyclohexane and dodecane, with log P values of 3.44 and 6.82 respectively) were also carried out in parallel for the purpose of comparison. These experiments were carried out in duplicate.

3.4.6 Effect of Removing Low MW Polymer Fractions

Since, as will be seen, the bulk PPG samples contained a range of MW fractions, polymers were water washed in order to remove the water soluble fraction of the polymer sample. The fraction of polymer samples that was miscible in water would be expected to have a relatively low log P value, and

therefore may contribute to toxicity of the polymer samples to microbes; by removing this fraction we wanted to test whether this low log P fraction had a strong influence on biocompatibility. Polymer sample washing was done by vortex mixing PPG with and an equal volume of RO water for 2 minutes. The phases were then separated by centrifugation at 3000 RPM for 5 minutes. NaCl (1 g) was added to assist in the phase separation of PPG 425 by increasing the ionic strength of the aqueous phase. Loss of mass after washing was determined gravimetrically, by measuring weight of the polymer fraction before and after the washing step. Washed bulk polymer was then used in the biocompatibility shake flask experiments as described above. These experiments utilized 1, 5, or 10 grams of washed PPG with a MW of 425, 725 or 1000 in 50 mL medium. This was done in order to determine the correlation between polymer MW and toxicity as well as the correlation between the polymer volume fraction and toxicity for the washed polymer samples compared to the unwashed polymers.

3.4.7 Analytical Methods

Cell growth was measured by optical density at 600 nm (OD_{600}) using an Ultraspec 3000 spectrophotometer (Biochrom). PPG with MW of 2000 or 4000 formed an immiscible mixture with the growth medium that interfered with the OD measurements. In such cases NaCl (1 gram) was added to assist in phase separation of 5 mL samples. If this was not sufficient, organic solvents such as hexane and isopropanol were added in sparing amounts, typically 1 mL of solvent to 5 mL of medium. This improved phase separation. Glucose was measured by HPLC (Varian Prostar, Mississauga, ON) with a PL Hi-Plex H column (300 mm x 7.7 mm) at 60 °C using a refractive index detector (PS 356, Varian Prostar) and a flow rate of 0.7 mL/min with a 10 mM H_2SO_4 mobile phase.

3.5 Results

3.5.1 Critical Log P

Figure 3-1 shows the cell density of *S. cerevisiae* and *P. putida* relative to control, grown in the presence of various organic solvents spanning a range of log P values, using optical density measurements and glucose residuals as metrics for cell growth. These experiments were conducted using a conventional ratio of solvent:aqueous phase volumes of 10%, or 5 mL solvent in 50 mL aqueous broth.

The results show an approximate critical log P of 5.0 and 3.5 for *S. cerevisiae,* and *P. putida,* respectively, and these values are similar to those previously reported for these organisms (Barton and Daugulis 1992, Vrionis et al., 2002). In practical terms, this suggests both that the presence of NAPs whose log P values are greater than 5.0 and 3.5, respectively, would be anticipated to be biocompatible, and also that *P. putida* is more tolerant to the presence of NAPs as its critical log P value is lower than that of *S. cerevisiae*.

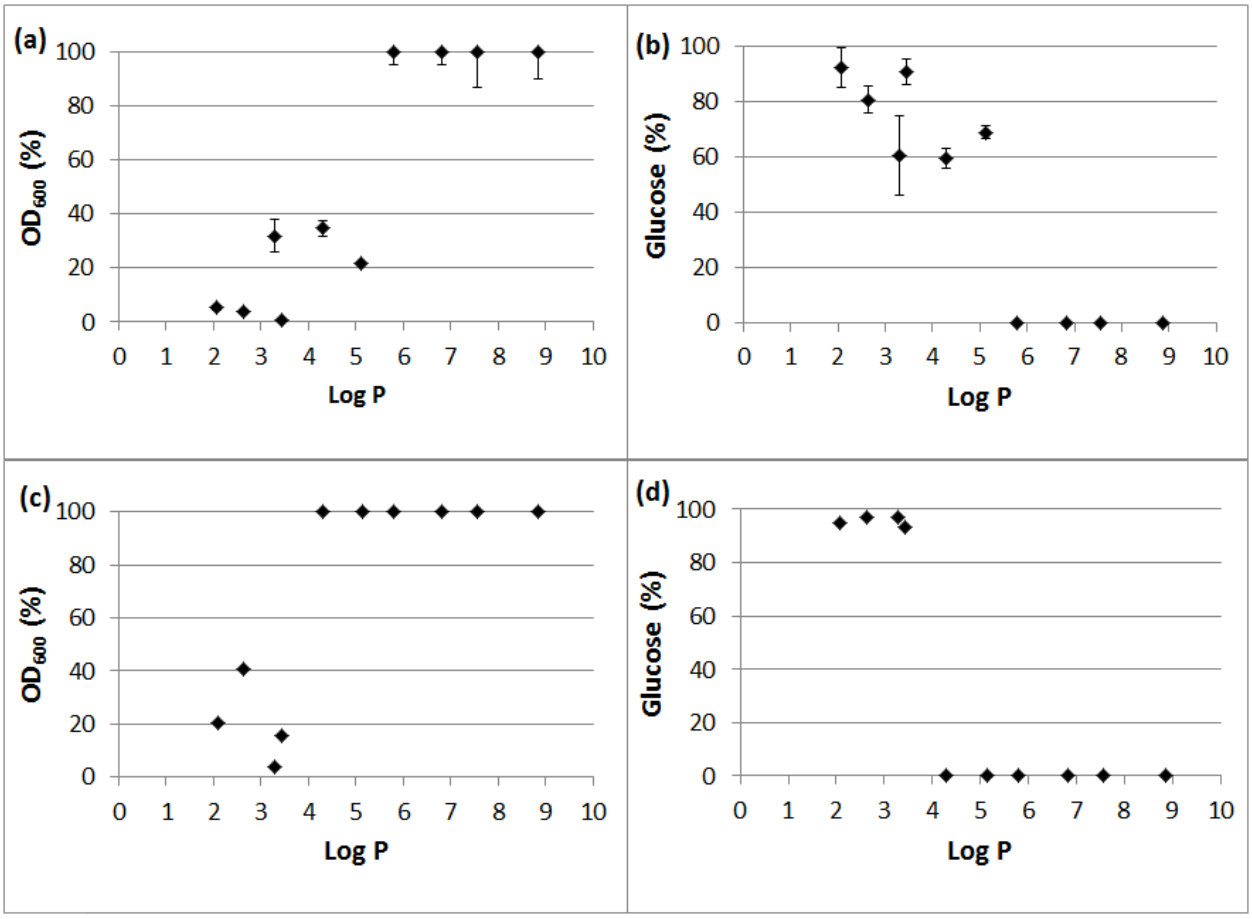

Figure 3-1: Biocompatibility versus log P of solvents after 24 hours of growth. a) Optical density at 600 nm of *S. cerevisiae* relative to control, b) Remaining glucose for *S. cerevisiae* relative to initial concentration c) Optical density at 600 nm of *P. putida* relative to control d) Remaining glucose for *P. putida* relative to initial concentration. Error bars indicate 1 standard deviation.

3.5.2 Log P of Different MW PPGs

Figure 3-2 shows the HPLC chromatogram for unwashed and washed polypropylene glycol (PPG) 1000 samples for the "bulk" grade of the polymer. Figure 3-2a distinctly demonstrates that PPG

1000 as received from the manufacturer is very clearly a mixture of different MW PPG polymers, as evidenced by the numerous peaks seen in the chromatogram. As noted earlier, even "pure" polymers will have a certain degree of polydispersity, or MW distribution about some average MW value, and this is in contrast to simple, pure solvents, which would be expected to have a single HPLC chromatogram peak. The data in Figure 3-2a also suggest that the various MW constituents in PPG 1000 would have different log P values, ranging from relatively low values (e.g. log P of 2.0 for the lower MW PPG species) to higher ones (and higher MW). The polymer fractions with lower log P values might be expected to inhibit cell growth, while higher log P polymers could be expected to be biocompatible according to the critical log P model of toxicity. Thus although PPG 1000 as received from the manufacturer can have on overall estimated log P value (see Table 3-2), it appears to have a range of components some of which would likely be biocompatible and some that would not.

Table 3-2: Calculated average log P values for PPG samples

MW of PPG samples	Average log P Unwashed	Average log P Washed	% Loss of mass after washing	MW of PPG samples Standards	Average log P Standards
425	1.19	1.29	20.0%	470	1.74
725	2.69	3.24	17.5%	830	2.45
1000	3.28	4.07	7.6%	1080	3.17
2000	3.39	-	<1%	2160	2.74
4000	4.05	-	<1%	3100	3.02

The chromatogram shown in Figure 3-2b is for a sample of PPG 1000 that had been water-washed to remove the more water soluble (lower MW) constituents, and a clear shift can be seen between the washed and unwashed samples, where many of the peaks with short retention time have become smaller or have disappeared completely. This suggests that the washing process has removed low log P compounds, which would be expected to lead to an increase in average log P for the treated PPG sample.

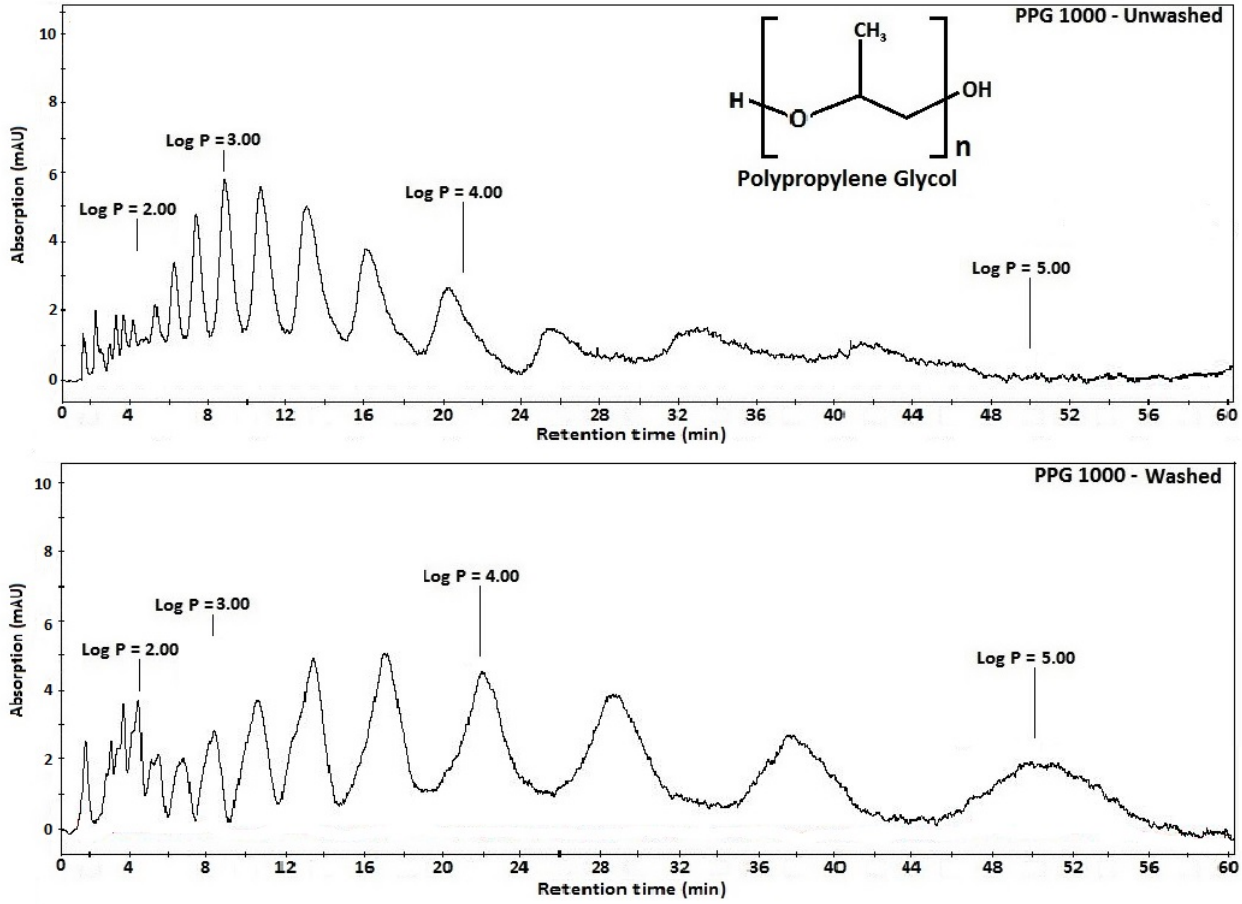

Figure 3-2: HPLC chromatograms of 1000 molecular weight polypropylene glycol (a) as received – unwashed and (b) washed

Figure 3-3a represents the chromatogram from the analysis of a PPG 1080 standard, with low polydispersity, and this chromatogram shows a tighter distribution of peaks, as would be expected in a sample with low polydispersity. Figure 3-3b is the chromatogram for a washed standard PPG 1080 sample, which shows that the relative intensity of the peaks does not shift when washing the standard samples. This suggests that a smaller fraction of the polymer sample is water soluble. This would be expected in a sample with very little low MW polymer content.

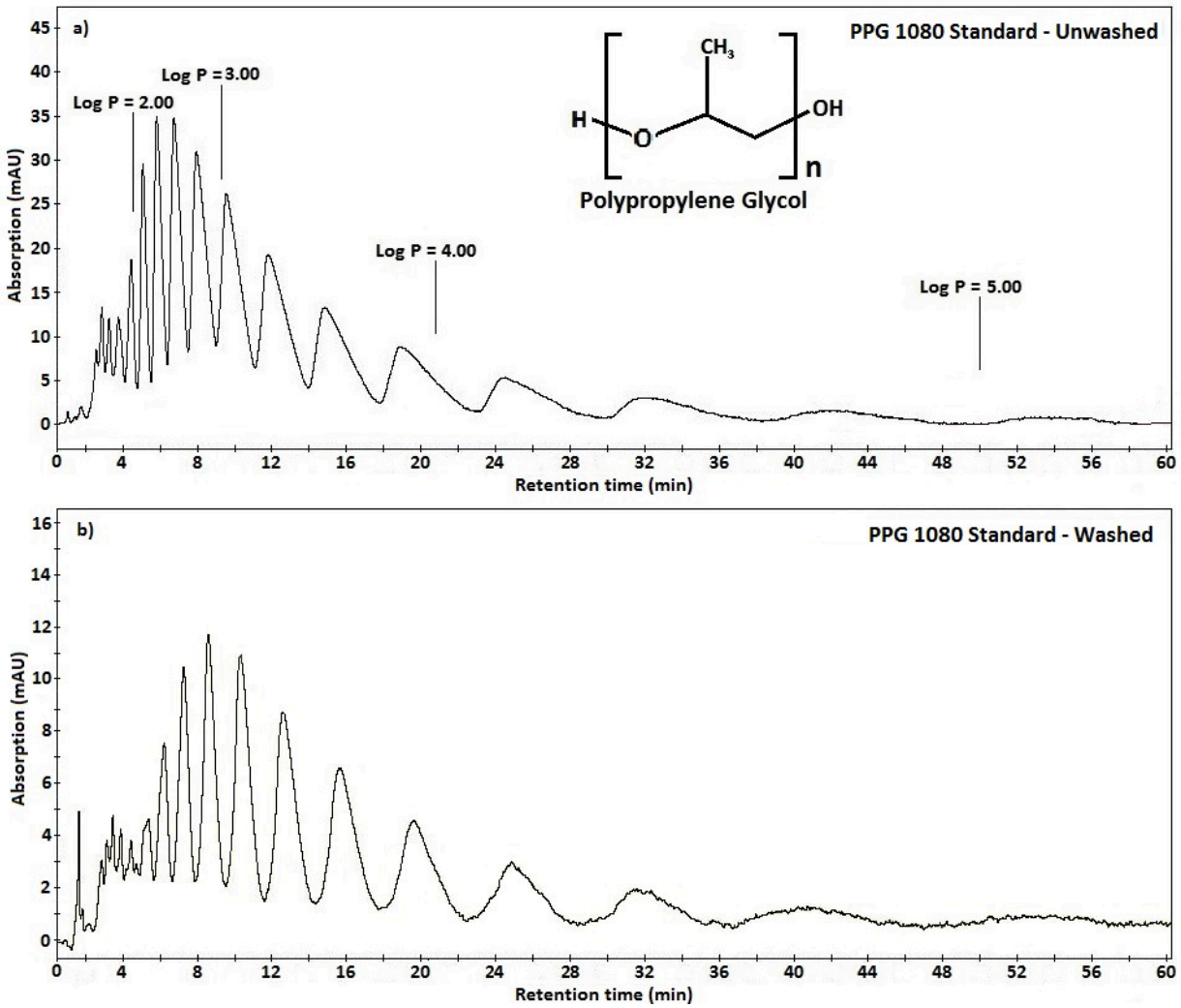

Figure 3-3: HPLC chromatograms of 1080 molecular weight polypropylene glycol standard (a) as received – unwashed and (b) washed

The shift in the polymer MW distribution seen in Figure 3-2 is shown quantitatively in the calculated average log P values given in Table 3-2. The log P values for the washed polymer samples are higher than unwashed samples, as a result of the low log P polymers being removed by washing, thus increasing the average log P. These shifts in log P are linked with a loss of mass, as seen in Table 2. Calculated average log P for PPG standards is given for comparison.

3.5.3 Biocompatibility of PPG

Figure 3-4 shows the growth of *S. cerevisiae* and *P. putida* as measured by the percentage of optical density relative to a control, using 5 g of the various MW PPG polymers in 50 mL aqueous medium. A clear trend can be seen for both cell types: lower MW PPGs appear to inhibit growth, while

higher MW polymer are more often biocompatible. In both *S. cerevisiae* and *P. putida*, uninhibited growth is seen in samples with molecular weights exceeding 1000. This mirrors previously reported results seen for *Clostridium acetobutylicum*, which suggested that 1200 molecular weight PPG and higher were biocompatible (Barton, 1992). This work therefore shows that three separate organisms are biocompatible to 1000-1200 MW PPG and above. It is also of note that *P. putida* exhibits signs of NAP tolerance at lower molecular weights than does *S. cerevisiae*. This is consistent with the critical log P model of toxicity, as *P. putida* has been shown to have a lower critical log P, as shown in Figure 3-1, which suggests that the critical log P approach may also be applicable to polymeric NAPs.

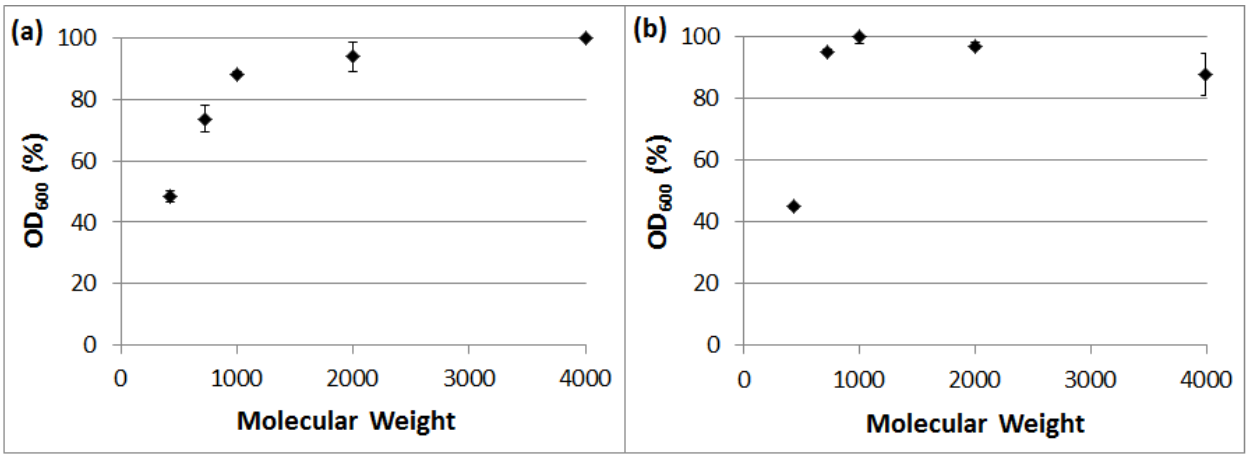

Figure 3-4: Growth of (a) *S. cerevisiae* and (b) *P. putida* relative to control in 50 mL culture broth with 5 grams unwashed PPG over a range of molecular weights.

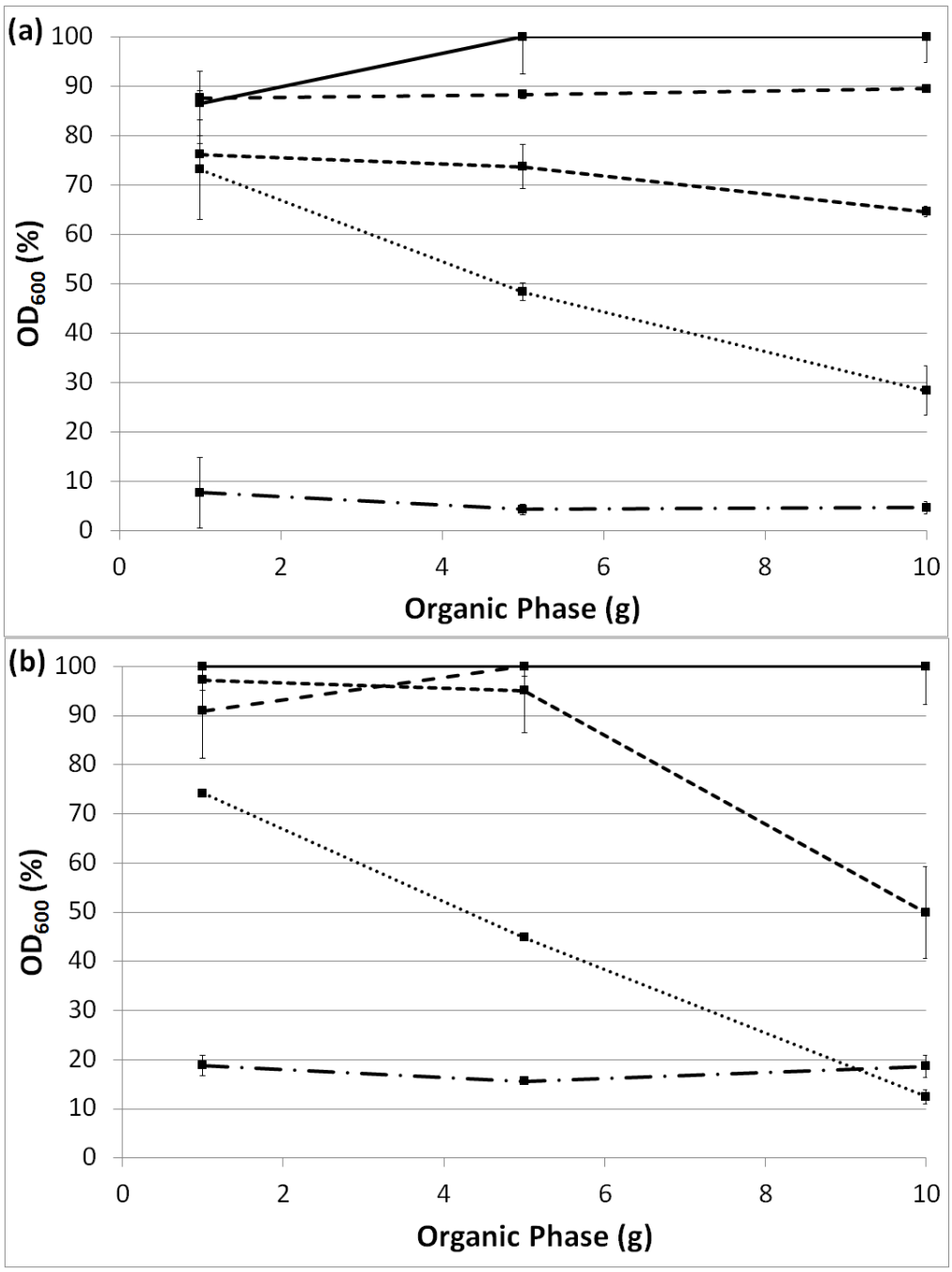

Figure 3-5: 24 hour growth relative to control over a range of volumes (a) *S. cerevisiae* (b) *P. putida*. (….) PPG 425 (‥) PPG 725 (– –) PPG 1000(▬) dodecane and (— .) cyclohexane. Error bars have been given to show 1 standard deviation.

Figure 3-5 shows the biocompatibility of *S. cerevisiae* and *P. putida* over a range of 1-10 grams of NAP addition using PPG 425, 725 and 1000, as well as dodecane and cyclohexane. As noted in the Materials section, the reason for using different amounts of NAP was to determine the polymer fraction

dependency of toxicity. That is, since low MW fractions are present in PPG (which may be cytotoxic), the addition of larger amounts of these polymers to the cultures could result in sufficient low MW material in order to cause toxicity. The PPGs tested clearly show a correlation between the amount of polymer added and cell biocompatibility, with higher polymer addition resulting in reduced cell growth. PPG samples with MWs above 1000 do not show this trend, and appear to be biocompatible regardless of the polymer fraction added. This experiment was repeated for PPG 2000 and 4000, although growth remained at approximately 100% of the controls under all conditions (data not shown). Trials were also preformed utilizing the organic solvents dodecane and cyclohexane. The log P of dodecane is approximately 6.8, which exceeds the critical log P of both *S. cerevisiae* and *P. putida*, suggesting that total biocompatibility would be expected at any volume, which is seen in these results. Cyclohexane, by contrast, has a reported log P of 3.4 and should therefore be expected to strongly inhibit growth, as the Log P value is below the critical Log P of both *S. cerevisiae* and *P. putida*. This is observed in these experiments at all levels of cyclohexane addition. The (expected) volume independence of organic solvents is a noticeable different relative to the volume dependence seen in the polymeric NAP experiments.

3.5.4 Washed PPG Experiments

Figure 3-6 compares the biocompatibility of washed and unwashed PPG samples. As noted earlier, PPG samples were washed with water in order to remove the low log P water soluble fraction. It is clearly seen in Figure 3-6 that the presence of washed polymer resulted in higher cell growth than in the case of unwashed polymer samples. This implies that the low molecular weight fraction that is removed as a result of washing was the chief cause of toxicity, and that washing polymer samples may be an effective means by which biocompatibility can be improved for relatively low MW polymers with high polydispersity.

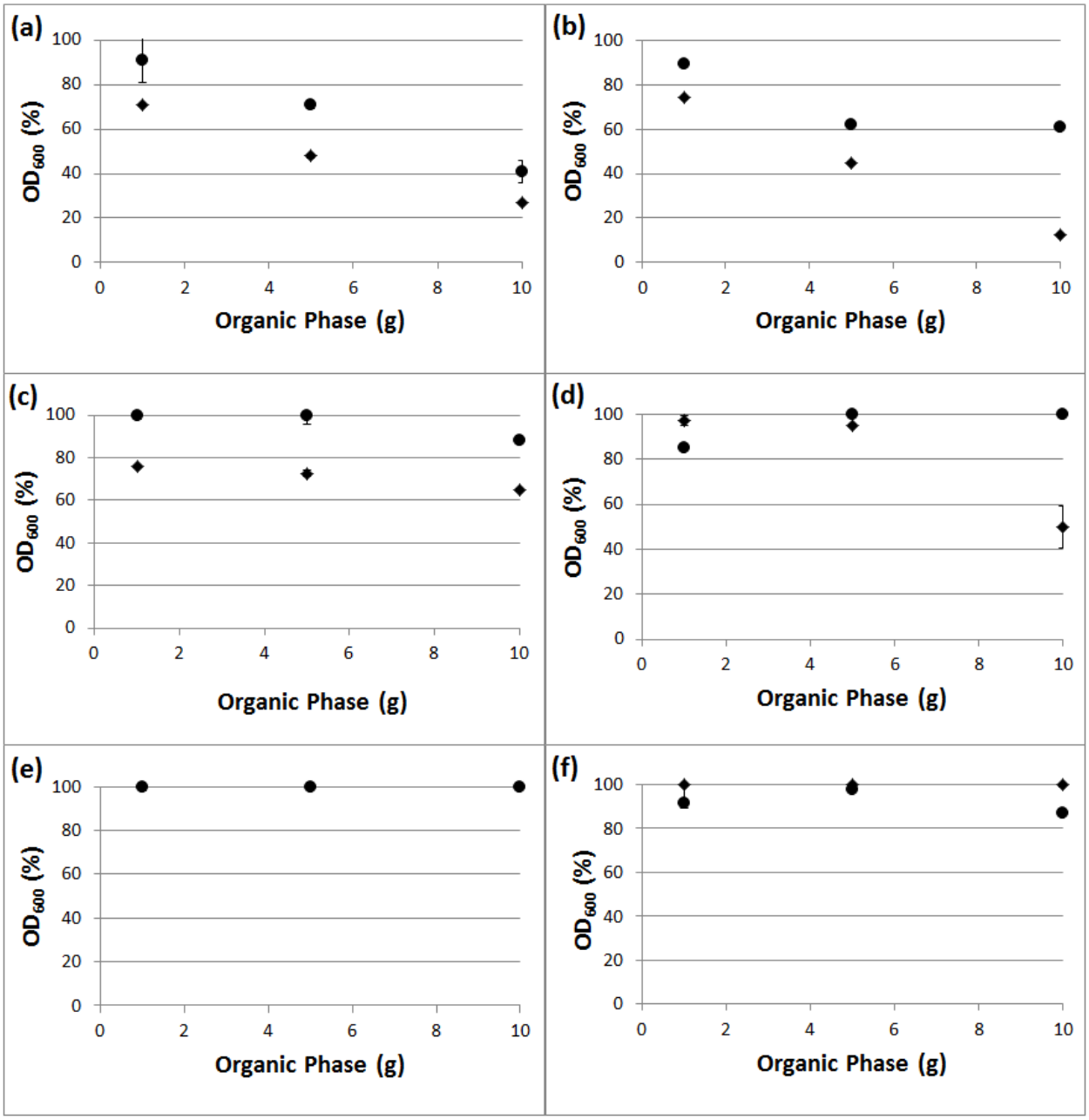

Figure 3-6: Cell growth after 24 hours in washed versus unwashed PPG. (a) *S. cerevisiae* with PPG 425 (b) *P. putida* with PPG 425 (c) *S. cerevisiae* with PPG 725 (d) *P. putida* with PPG 725 (e) *S. cerevesiae* with PPG 1000 (f) *P. putida* with PPG 1000. (•) washed polymer (⧫) unwashed polymer. Error bars have been given showing 1 standard deviation.

Figure 3-6 also shows that *P. putida* growth improved more than *S. cerevisiae* as a result of polymer washing. In panel (a) it can be seen that the average growth of *S. cerevesiae* when exposed to 10 grams of unwashed PPG 425 was 28% of the control, while the culture with washed PPG 425 was 40% of the control. In contrast, *P. putida*, had an average growth of 14% relative to the control when exposed to

10 grams of unwashed PPG 425, while growth when exposed to washed polymer was approximately 67% of control. This is seen again in the case of using 10 grams of washed PPG 725, where *S. cerevisiae* growth was 88% of the control, and *P. putida* growth was identical to the control. . It is of interest that *P. putida* shows more growth than *S. cerevisiae* in the presence of washed PPG, and therefore shows higher tolerance to polymeric NAP. This is as anticipated by the critical log P model of NAP tolerance. It should also be noted that many of the data points for both washed and unwashed samples in figures 6e and 6f lie at approximately 100%, which obscures many of the data points.

3.6 Discussion

The first insight to note from these results is that the connection between polymer molecular weight and cytotoxicity appears to follow the general trend that had been demonstrated in the past – lower MW polymers are less biocompatible than higher MW polymers (Barton and Daugulis, 1992). Importantly, this work establishes this trend in eukaryotic cells as well as prokaryotic cells, which has not been reported in the past.

Previous work had not identified the properties of polymer with lower MW which lead to toxicity, while here it was shown that polymers with lower MW have lower calculated log P values, and that the log P increases with molecular weight of the polymer. Log P has long been established as a key physical parameter for predicting toxicity (Inoue and Horikoshi, 1991) and these results show that polymeric compounds with low average log P are more toxic than are polymers with high average log P. In addition to this, by washing the polymers to remove the water soluble low MW constituents, the overall log P values increase, which corresponds to lowered toxicity. These results show that the critical log P model of NAP toxicity holds true to some degree in polymer systems.

Although log P is clearly still an important consideration in the toxicity of short chain polymers, these initial results suggest that there are some limitations to the critical log P model as it applies to polymer systems. Here it was found that PPG 1000, 2000 and 4000 are biocompatible for *S. cerevisiae*, although their weighted average log P values are below the critical log P of the organism. In addition,

there appeared to be a volume dependency of toxicity for certain polymeric NAPs, as shown in Figure 3-4. This effect has not been observed when using simple organic solvents, where small volumes of organic solvents with low log P values completely inhibit all cell growth, as is the case of cyclohexane shown in Figure 3-5. Therefore, although polymeric compounds seem to follow the general trend of toxicity being tied to decreasing log P, there are some additional factors that must be accounted for.

An important distinction when considering the difference between polymeric NAPs and monomeric NAPs is the phenomenon of polydispersity. In the case of a solvent such as cyclohexane, it is expected that >99% of the solvent is a single compound, with no important differences between solvent samples. When analyzing polymeric compounds, a distribution of molecular weights would be expected, as polymerization invariably leads to a range of molecular weights. This can be seen visually in Figure 3-2, as each of the peaks represents a slightly different log P. Although this phenomenon is to be expected, this work characterizes the polydisperse character through use of HPLC. The phenomenon of polydispersity has also been shown to be important to biocompatibility, as removing the low log P fraction of a polydisperse polymer sample improves biocompatibility. This explains the volume dependency seen in Figure 3-5 and 3-6 – the degree of toxicity is likely proportional to the amount of low log P polymer. Therefore, these results suggest that polymeric samples do not adhere strictly to the critical log P model as a result of the presence of a very low molecular weight fraction. Better understanding of this low molecular weight fraction could lead to further insights into polymeric NAP selection, and will be the object of future studies.

The simple washing procedure implemented in this study was effective at shifting PPG log P values as seen in Table 3-2, and the improving polymer NAP biocompatibility as seen in Figure 3-5. By examining the chromatograms of the washed polymers (Figure 3-2b), it is clear that this method is particularly useful for removing the low log P fraction from polymer samples. This also provides a relative increase in high log P fractions, as seen in the region around log P 5.00. It is also observed that large losses of mass are seen after washing samples with lower MW, as would be expected, as a result of a larger water soluble fraction. This is therefore a potential method for limiting the toxicity of lower MW

polymer samples in TPPB applications. By removing the very low log P fraction in a polymer sample it may be possible to improve the biocompatibility of short chain polymers, while retaining much of the gains in solute uptake (Bacon et al. 2014). Future work will aim to refine the method of washing polymer samples, and use it to examine the contents of the organic and aqueous phase after washing various polymers in order to add greater understanding to the models of polymer selection in TPPB systems.

This work reaffirms that toxicity of polymers decreases as the molecular weight increases (Barton and Daugulis, 1992) The MW of PPG at which biocompatibility was achieved for *S. cerevisiae* and *P. putida* was approximately 1000, with all samples exceeding 1000 being entirely biocompatible. The toxicity of polymers is connected to the log P of the polymer samples. Washing polymers by rinsing with water lead to an increase in log P and a decrease in toxicity. Although the biocompatibility results do not conform exactly to the critical log P model, there is a clear trend that low log P compounds tend to be more toxic than high log P compounds. Washing polymer was also shown to be an effective technique to reduce toxicity. This method will be more fully explored in future work, along with a more in-depth analysis of the low log P fraction of polymer samples, and further validation of this revised critical log P biocompatibility model using other polymer and other organisms.

3.7 References

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Chapter 4

Organic Acid Extraction by Amine-functionalized Hydrogels for use in Two-Phase Partitioning Bioreactors

4.1 Preface

The final set of results that will be discussed in this thesis focuses on the design, synthesis and use of reactive polymers for the purpose of acid reactive extraction. Organic acids are a class of commercially important products, which have been difficult to produce biologically because of expensive and wasteful separation procedures. Absorptive polymers struggle to extract organic acids from fermentation broths at high partitioning coefficients. Amine functionalized hydrogels are able to extract acid from aqueous solution (Kertes and King, 1986), but they have not yet been tested for use in two-phase partitioning bioreactors (TPPB) for the purpose of *in situ* product removal. Although these have the potential to improve bioreactor efficiency and lower extraction costs, there are several questions that must be addressed, the first being their level of biocompatibility. Several reactive polymers have been reported in the literature for the purpose of inhibiting the growth of microbes as anti-microbial coatings and surfaces (Kenawy et al., 2007, Münoz-Bonilla and Fernández-Garcia, 2012), so it is possible that polymers used for the reactive extraction of acids would be similarly inhibitory. The second question concerns the stability of the polymers. Prior research has described amine-functionalized polyacrylates that are able to self-catalyze their own degradation (McCool and Senolges, 1989), though it is unclear if this is an issue for the materials used here. Stability issues will interfere with normal growth of the microorganism by introducing a possible toxin or alternative carbon source, reduce acid extraction as a result of losses in the extracting phase, and limit the ability to reuse the polymer. Finally, it is important to verify that these hydrogels uptake of a range of organic acids. It is expected that amine functionalized hydrogels will be able to effectively uptake carboxylic acids at rapid rates - a substantial advantage over absorptive polymers - although this must be demonstrated.

Experiments performed to address these matters showed that amine-functionalized hydrogels with either a polyacrylate and polyacrylamide backbone are able to effectively uptake benzoic and butyric acid from a 2.5 g/L solution over 2 hours. Between 60-85% of acid present was extracted when exposed to only 10 g/L of polymer. Although this is encouraging, additional experiments demonstrated that the polyacrylate hydrogel shows signs of substantial decomposition after 6 hours, while the polyacrylamide

remained stable. Results are then shown to demonstrate the acid extraction of a range of acids (including the dicarboxylic succinic acid). Finally, this polymer was shown to be biocompatible to *Saccharomyces cerevisiae* and *Pseudomonades putida*, suggesting that it does not strongly inhibit microbial growth.

This work simply aims to demonstrate the potential of the amine-functionalized hydrogel technology, and act as a stepping-stone to future research. These results are intended as an initial investigation on the subject of acid-extracting reactive polymers. A range of experiments are proposed to further examine the use of these reactive polymers, the most important of which will involve acid fermentation bioreactor trials.

4.2 Abstract

The fermentative production of organic acids is an area of great economic potential, although the use of biological production methods has been constrained by the expense and excessive waste of separation procedures currently in use for their recovery. Although reactive extraction of organic acids has be unsuccessful in enhancing downstream acid recovery from spent fermentation broth, it has not been adapted as an means of removing organic acids as they are produced as a means of mitigating the cytotoxic effects of acid build-up, that is, in a two phase partitioning bioreactor (TPPB) configuration. Typical methods of reactive extraction utilize extractants or diluents that have been found to inhibit cell growth, and are therefore not suitable for *in situ* applications such at two-phase partitioning bioreactor (TPPB) systems. This work is a preliminary investigation into the viability of using amine-functionalized hydrogels as the extracting phase for use in solid-liquid TPPB, and focuses on assessing the acid extracting capacity, stability, and biocompatibility of these materials. Two polymers were designed and synthesized, utilizing a polyacrylate and polyacrylamide structure and were compared for their ability to extract acid from a 2.5 g/L butyric and benzoic acid solution over 2 hours. Stability was also tested for each polymer by assessing the extraction of a 2.5 g/L solution of acetic acid (a typical organic acid) over 24 hours. To determine the capability of these polymers to extract a range additional acids, extraction tests were conducted with succinic, acetic, and formic acid using the polyacrylamide. Tests of acid extraction showed efficient uptake of all acids tested, although stability tests indicated that the polyacrylate structure is unstable under the conditions tested, while the polyacrylamide was stable. Initial results were also presented for the biocompatibility of the amine-functionalized polyacrylamide with *Saccharomyces cerevisiae* and *Pseudomonades putida*, showing no signs of growth inhibition. Further biocompatibility tests must be conducted for acid-fermenting microbes, or microbes that prefer acidic environments, to ensure this hydrogel is completely biocompatible. Future experiments are proposed to explore the use of amine-functionalized poylacrylamide for use in acid-fermenting solid-liquid TPPBs.

4.3 Introduction

In 2004, the United States Department of Energy published a list of bio-products with the most potential as "Value Added Chemicals from Biomass" to draw attention to opportunities in the field of biotechnology (Werpy et al., 2004). The majority of the chemicals listed were organic acids, including succinic, fumaric, aspartic and glutamic acids, suggesting that carboxylic acids, as a general class of chemical, have a large untapped potential for use in the chemical industry. Others have similarly highlighted the commercial opportunities of organic acids in areas such as food processing and polymer building-blocks (Patel et al., 2006; Sauer et al., 2008; Bozell and Peterson, 2010). Despite great promise, large scale bio-based production of only a limited number of organic acids has become a reality. Biotechnological methods have struggled to be cost competitive with petroleum-based products (Patel et al., 2006), and a major obstacle has been the challenges associated with economical product recovery. For example, the downstream processing of succinic acid requires the use of massive amounts of $Ca(OH)_2$ and CaO, as well as multiple purification steps. This downstream processing of succinic acid is estimated to cost between 50-70% of the total operating costs, and produces large amounts of waste (Bechtold et. al, 2008; Cheng et al., 2012). This is not entirely uncommon for the fermentation of organic acids, as other commodity chemicals such as lactic acid and citric acid have many of the same challenges (López-Garzón and Straathof, 2014). Substantial improvements in product recovery could greatly improve the efficiency and marketability of these products.

One approach to downstream processing that has been investigated is the use of reactive extraction of carboxylic acids from fermentation broths. Reactive extraction utilizes an organic base, often a tertiary amine (i.e. Alamine 336) or organophosphate (i.e. tributyl phosphate), to react with the acid contained in the fermentation broth. The reaction complexes will then partition into an inert diluent phase, which is often comprised of long chain aliphatic compounds (Kertes and King, 1986; Shan et al., 2006). Significant research has been done to understand the rate at which these reactions occur (Kyuchoukov and Yankov, 2012; Lux and Siebenhofer, 2012; Uslu, 2006). Although this research has been quite successful in predicting kinetics of the reactive extraction process, the implementation of this

technology has been limited as it does not greatly improve purification procedures. In order for substantial gains in efficiency to be achieved, fundamental changes to the fermentation process would be needed.

Two-phase partitioning bioreactors (TPPB) constitute a bioprocessing strategy that aims at reducing the toxic effect of fermentation products during fermentations via *in situ* product removal. This is done through the introduction of a sequestering phase that partitions growth-inhibiting compounds out of the fermentation broth (Malinowski, 2001), and also has the potential to simplify product purification. Attempts have been made to adapt reactive extraction for use in TPPBs, which has had success for the fermentation of hexanoic and butyric acid (Zigová et al., 1999; Wu and Yang, 2003; Jeon et al., 2013). Progress in this area has been limited by the cytotoxic effect of the extractants (e.g. tri-octyl amine or tri-butyl phosphate) and diluents needed for reactive extraction (Keshev et al., 2008). Clearly, a new approach must be taken in order to gain the benefits of reactive extraction, while limiting the toxic effects to microbes.

Efforts in the field of TPPBs have focused on the use of polymers as the partitioning phase (Amsden et al., 2003; Dafoe and Daugulis, 2013). Use of a solid polymeric sequestering phase has a number of advantages over immiscible organic solvents, such as being biocompatible, having improved handling properties, simplified reuse, and the possibility for modifying polymer structure to suit the needs of the system. The majority of polymers studied sequester target molecules on the basis of hydrophobic interactions (Parent et al., 2012; Poleo and Daugulis, 2013). Although this method of interaction is effective for many compounds, it has had only limited success for the partitioning of organic acids (Hepburn and Daugulis, 2012; Peterson and Daugulis 2014 a,b). Organic acids will only partition into absorptive polymers when in a non-ionic protonated form, which requires low pH, which interferes with microbial growth. Rather than relying on the relatively weak affinity, it is possible to utilize the benefits of reactive extraction, while eliminating the cytotoxic effects of traditional extractant and diluent molecules, through the use of amine-functionalized hydrogels. Research on the subject has shown that these materials are able to remove carboxylic acids from aqueous solutions, although they have not been

thoroughly tested for stability or biocompatibility (Aşçi and Hasdimir, 2008; González-Sáiz et al., 1997; Peterson et al., 2015).

This work discusses the synthesis of two amine-functionalized hydrogels and examines their ability to extract organic acid from aqueous solution, their stability under acidic conditions, and their biocompatibility. A polyacrylamide and a polyacrylate were synthesized and tested for their capacity to extract butyric and benzoic acids. These acids were selected on the basis of their use in prior acid-extraction experiments (Peterson et al., 2015). Further acid extraction experiments were conducted for polyacrylamide using succinic, formic and acetic. These acids were incorporated for the purpose of representing a diversity of structure. Stability was also tested by exposing polymers to 2.5 g/L solutions of acetic acid for 24 hours, and monitoring changes in acid retention. These experiments were preformed on the basis of previous reports suggesting the self-catalyzed decomposition of amine-functionalized hydrogels (McCool and Senolges, 1989). Unstable polymers are unsuitable for use in bioreactors, as their decomposition products may interfere with microbial growth, and will make polymer reuse impossible, greatly increasing costs.

Initial results assessing the biocompatibility of amine-functionalized polyacrylamides are also discussed. There are previous reports of anti-microbial reactive polymers, meaning that the biocompatibility of reactive polymers must be tested (Kenawy et al., 2007; Muñoz-Bonilla and Fernández-Garcia, 2012). *Saccharomyces cerevisiae* and *Pseudomonades putida* were used to assess biocompatibility on the basis of their use in previous biocompatibility experiments (Harris and Daugulis, 2015).

This Chapter represents a preliminary examination of the viability of amine-functionalized hydrogels for the purpose of use in TPPBs in the recovery of produced organic acids. Future work will be needed to investigate other elements of acid fermenting solid-liquid TPPBs design such as media formulation, microbe selection, pH control and so on. This work is simply an initial investigation of this topic to determine the potential of this technology for future research.

4.4 Materials and Methods

4.4.1 Polyacrylate Hydrogel preparation

The amine-functionalized polyacrylate hydrogel was prepared by solvent-free copolymerization of 2-(dimethylamino)ethyl acrylate (DMAEA, 4.25 g) with trimethylolpropane triacrylate (TMPTA, 0.75 g) at 70±1 °C for 24 hours using 2, 2'-azobis(2-methyl-propionitrile) (AIBN, 0.2 g) as a radical initiator. Soluble material within the resulting thermoset was removed by swelling the polymer with THF and recovering from excess acetone. The purified product was dried under vacuum at 23°C. Figure 5-1 shows the reaction scheme used to synthesize the polymer. All materials were purchased from Sigma-Aldrich.

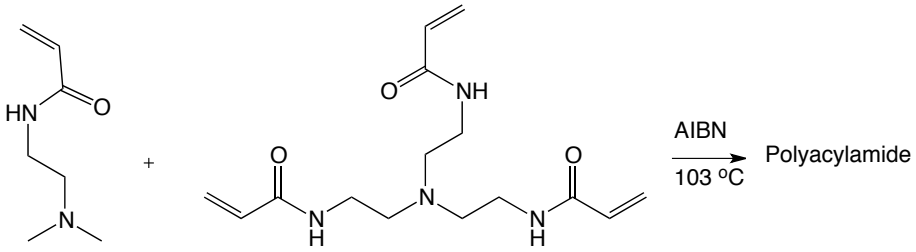


Figure 4-1: Synthesis of amine-functionalized polyacrylate hydrogel.

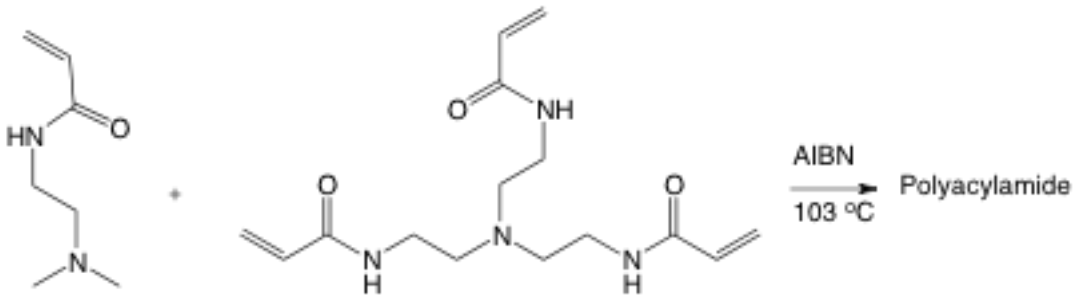
4.4.2 Polyacrylamide Hydrogel preparation

Monomer subunits of the functionalized polyacrylamide hydrogel were prepared by a method akin to that described by Chang and McCormick (1993). In a 250 mL three-neck round bottom flask methylene chloride (82 mL), N,N-dimethylethylenediamine (8.82 g) and 6 N sodium hydroxide (25 mL) were mixed and cooled via ice bath. A solution of acryloyl chloride (9.36 g) and methylene chloride (20 mL) was added dropwise over 1 hour. The mixture was removed from the ice bath, and was allowed to react overnight. The organic layer was then separated, washed with water and brine, and dried over anhydrous sulphate. The solvent was removed via rotary evaporation, leaving a yellow-orange oil (9.20 g, 63% yield).

The crosslinker used was synthesized following a method similar to that described by Patras et al. (2000). A solution of acryloyl chloride (6.78g) in dichloromethane (55ml) was added drop wise over 2 hours to Tris(2-aminoethyl)amine (3.65g) in dichloromethane (45ml) an 6 N NaOH (20 mL) whilst stirring vigorously on an ice bath, maintaining the temperature between 0-5°C, in a three necked round

bottom flask. The reaction mixture was then left to stir overnight at room temperature. The organic layer was then separated, washed with water and brine, and dried over anhydrous sulphate. The solvent was removed via rotary evaporation, leaving a white solid (2.44 g, 33% yield).

Polymer was synthesized by use of 0.900 grams monomer, 0.100 grams crosslinker, and 0.050 grams AIBN in a solvent free polymerization reaction. The mixture was heated to 103 ^{o}C for 30 minutes. The resulting solid was washed with water to remove unbound monomer, and was then filtered and dried by vacuum oven (60 ^{o}C). The scheme for polymerization reaction is given below in Figure 5-2.



Monomer Crosslinker

Figure 4-2: Synthesis of amine-functionalized polyacrylamide hydrogel.

4.4.3 Acid Extraction

Samples of each polyacrylamide and polyacrylate hydrogel (20 mg-100 mg) were added to 10 mL aliquots of 2.5 g/L either butyric or benzoic acid. These were allowed to equilibrate for 2 hours, with pH monitoring to ensure reaction completion (final pH 5.0-6.0). This was repeated with the polyacrylamide hydrogel for succinic, formic, and acetic acids. Levels of acid remaining in the aqueous phase were then analyzed to determine the extent of acid extraction.

4.4.4 Stability experiments

100 mg samples of polyacrylamide and polyacrylate were added to 20 mL of 2.5 g/L acetic acid. Reaction time was extended to 24 hours, with 100 µL aliquots removed for analysis at regular intervals. The level of acetic acid remaining in the aqueous phase was analyzed.

4.4.5 Cell Growth Experiments

Saccharomyces cerevisiae, an industrial ethanol-producing yeast strain obtained from Alltech (Nicholasville Kentucky USA) was cultivated in a medium (Doran and Bailey, 1986) containing 20 g/L glucose, 5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L yeast extract and 0.1 g/L $CaCl_2$. *Pseudomonas putida* ATCC 11172 was grown in medium described by Vrionis et al (2002), using 5 g/L glucose, 6 g/L K_2HPO_4, 4 g/L KH_2PO_4 and 2g/L $(NH_4)_2SO_4$, augmented with a divalent salt solution (0.66 g/L MgCl2 and 0.25 g/L $CaCl_2$) and 0.5% (vol/vol) trace element solution (0.3 g/L H_3BO_3, 0.089 g/L $ZnSO_4 \cdot 7H_2O$, 0.024 g/L $NiSO_4$, 0.018 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.003 g/L $CuSO_4 \cdot 5H2O$, 0.050 g/L $MnSO_4$, and 0.190 g/L $CoCl_2$); 0.0006% $FeCl_3$. Cultures were grown for 24 hours in 50 mL, of medium in 125 mL Erlenmeyer flasks at 30 ^oC and 180 RPM. All reagents used in the medium formulations were obtained from Fisher Canada. Amine-functionalized polyacrylamide (50, 100 or 200 mg) was added to flasks to observe difference in the density of growth.

4.4.6 Analytical methods

Aqueous samples were analyzed for acid concentration using an HPLC (Varian Prostar) equipped with a UV-Vis detector (Varian Prostar, PS325) operating at 220nm. Butyric, acetic, formic, and succinic acid separations employed a Varian Hi-Plex H column (300 × 7.7 mm) operating at 60 °C with a 10 mM H_2SO_4 mobile phase at 0.7 mL/min, while benzoic acid separations required a Varian Pursuit C8 5 μm column (250 x 4.6 mm), with a mobile phase of 20mM H_3PO_4 in 50:50 water/acetonitrile at 1 mL/min. Cell growth was measured by optical density at 600 nm (OD_{600}) using an Ultraspec 3000 spectrophotometer (Biochrom).

4.5 Results

4.5.1 Extraction of Butyric and Benzoic Acid

Figure 5-3 shows butyric and benzoic acid levels at varying levels of polymer addition, utilizing both the polyacrylamide and polyacrylate polymer species. These results clearly show that both polymer types are able to extract acid from aqueous solution, as between 60-85% of the acid is removed from

solution when using 10 g/L of polymer. This is a very encouraging result, and suggests that polymers of this type have potential for acid extraction.

Focusing on results between 2 g/L and 8 g/L, it can be seen that polyacrylate generally outperformed polyacrylamide. These gains are lost when examining the 10 g/L experiments, as the acid removal capacity of the polyacrylate polymer does not seem to improve beyond that achieved at 6 g/L. In the case of benzoic acid extracted by polyacrylate, there was a substantial loss in acid extraction. This is a cause for concern, as it is not immediately obvious how increased polymer content would lead to a decrease in acid extraction and may be a result of polymer degradation.

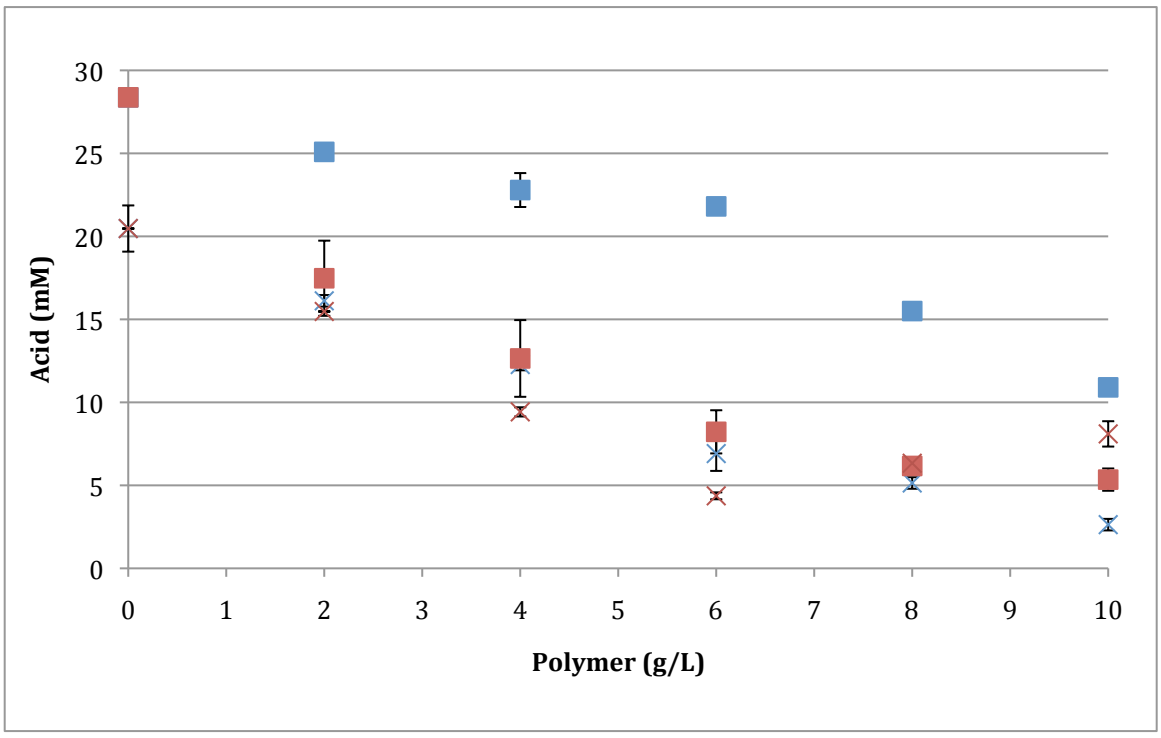


Figure 4-3: Acid levels versus amount of polymer added. ■ Butyric acid with polyacrylate polymer, × benzoic acid with polyacrylate polymer, ■ butyric acid with polyacrylamide polymer × benzoic acid with polyacrylamide polymer. Error bars indicate one standard deviation.

4.5.2 Stability Time Course

McCool and Senogles (1989) suggested that polydimethylamino ethyl acylate would be able to self-catalyze its degradation when exposed to acidic conditions. This breakdown would occur over the course of hours, and could be the cause of the unusual polyacrylate results observed in Figure 5-3. Figure

5-4 shows the extraction of a 41 mM solution of acetic acid over 24 hours using 5 g/L of polyacrylate and polyacrylamide polymers. The concentration of acetic acid in the case of polyacrylamide dropped rapidly, then maintained a constant level throughout the experiment. In contrast, acid concentration in the polyacrylate experiment dropped to very low concentrations within 2 hours, but by 6 hours the acid concentrations returned to higher concentrations. Acid concentrations continued to rise for the duration of the experiment. This strongly suggested that the polyacrylate polymer was degrading over the course of the experiment, releasing acetic acid that was once sequestered as the structure decomposes.

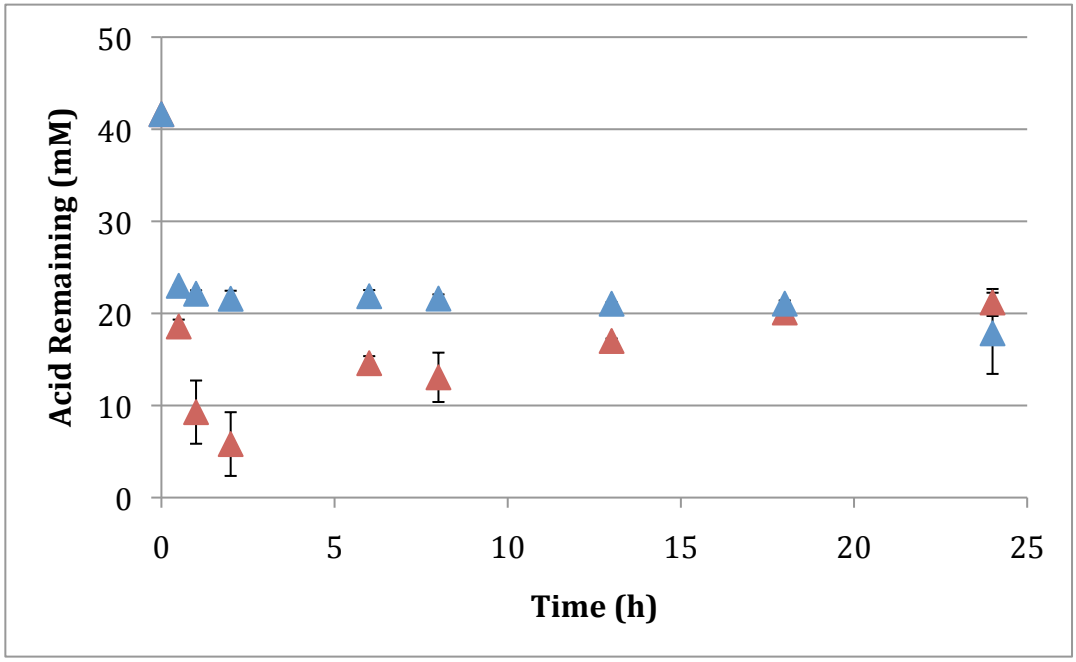


Figure 4-4: Extraction of a 41 mM acetic acid solution by 5 g/L polymer over 24 hours. ▲ polyacrylamide ▲ polyacrylate. Error bars indicate one standard deviation.

4.5.3 Extraction of Succinic, Acetic, and Formic acid

To ensure the efficacy of the polyacrylamide polymer in extracting a wide range of organic acids from solution, trials were conducted with succinic, acetic, and formic acid. The results of these experiments can be seen in Figure 5-5. The extractionwas most efficient for benzoic acid, with acetic, formic, butyric and succinic acid generally being very close in extents of extraction. Given that extractions were conducted at constant g/L basis, rather than mol/L basis, it is difficult to compare the

performance of acid uptake directly. It is worth noting that succinic acid, which is dicarboxylic, was extracted at roughly the same extent as other acids. This suggests that this method of acid-extraction is compatible with poly-acids, and is not limited to monocarboxylic acids, and that only one of the carboxyl functions needs to participate in the reactive extraction scheme.

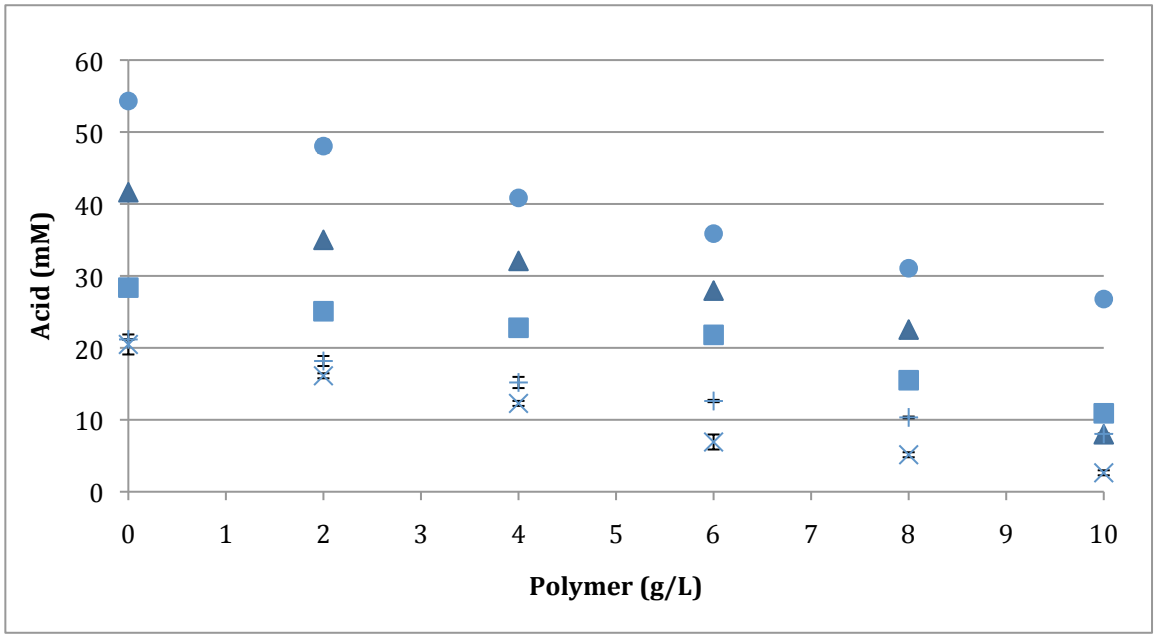


Figure 4-5: Levels of organic acid when extracted by varying levels of polyacrylamide. ■ Butyric acid, × benzoic acid, ▲ acetic acid, • formic acid, + succinic acid. Error bars indicate one standard deviation.

4.5.4 Biocompatibility

The focus of this experiment was to determine the potential for use of amine-functionalized polyacrylamide in TPPBs. If this polymer were substantially detrimental to cell growth, modifications of the compound would be necessary to determine a structure that is better suited for TPPB applications. Growth of both *S. cerevisiae* and *P. putida* were between 90-110% of control when exposed to concentrations as high as 4 g/L of amine-functionalized polyacrylamide. This suggests that this polymer is potentially compatible with a variety of microbes.

4.6 Discussion and Conclusions

Hydrolytic stability of polymer is essential for the proper functioning of a TPPB. The products of polymer breakdown could interfere with microbial growth, or the polymer could become ineffective for

solute removal as it loses mass. In addition to this, reusing polymer would be very difficult, resulting in costs that are higher than polymers that can be reused. The results shown in Figure 5-4 clearly show the amine-functionalized polyacrylate releasing acetic acid after 2 hours of equilibration. This strongly suggests that the polymer is degrading over time. Given these results, the amine-functionalized polyacrylate hydrogel described here would not appear to be a viable choice for *in situ* acid extraction.

The polyacrylamide used in these experiments, unlike the polyacrylate, appears to be stable in acidic solution. The polymer has also shown the ability to extract a range of acids, including low molecular weight acids (e.g formic) higher molecular weight acids (e.g. benzoic) and dicarboxylic acids (e.g. succinic acid). This suggests that this polymer has the capacity to extract a range of organic acid from aqueous solution. It should be noted that the experiments preformed here have focused on the extraction of acid under simplified conditions. In a fermentation vessel a complex, and changing mixture of sugars, buffers, and ions could easily affect the function and stability of this polymer. Further experiments are necessary to assess the extraction of acid from environments such as microbial media with added organic acid. Although current results cannot yet verify the efficacy of this polyacrylamide for the purpose of *in situ* product removal, the results reported here suggest it has that capacity.

The cell growth experiments were successful in demonstrating that amine-functionalized polyacrylamides show no signs of inhibiting microbial cell growth. This preliminary experiment was important in assessing the viability of this strategy, but does not yet provide much information about NAP selection for TPPBs utilizing the polymer described in these experiments. Biocompatibility is a microbe-specific phenomenon (Harris and Daugulis, 2015; Inoue and Horikoshi, 1991). Further experiments are necessary with a range of acid-producing microbes, focusing on cell growth rate and acid production. This additional series of experiments will provide the groundwork for TPPB fermentation experiments, and guide possible modification of the polymer molecule to better suit the needs of particular organisms.

It should be noted that there is some concern that the underlying principle of reactive extraction of acids by amine-functionalized polymers may not be possible for *in situ* product removal. Given that tertiary amine groups would become protonated under acidic conditions, it is possible that acid produced

through fermentation would not be able to bind to reactive centers in the polymer structure if acidic conditions are present. If this concern is avoided by using elevated pH, this may interfere with cell growth. Future work will need to investigate this concern, as it may be a fundamental barrier to TPPBs using reactive polymers to assist in producing organic acids. It may be possible to overcome this issue through use of operational mechanisms such as controlled polymer release, or using a polymer-containing external column. These measures would avoid the need for operating at high pH, and would not saturate the amine functionality at the outset of a fermentation.

The experiments reported in this work have not focused on modifying the structure of the polymer under discussion. In reality, there is significant room to customize its design. There is no specific reason to believe that the structure used was inadequate, but some alternative structure could offer improved performance in terms of acid extraction, stability, or biocompatibility. Possible modifications could include polymers with different ratios of cross-linker to monomer to vary physical properties, or using alternative alkyl groups to generate the tertiary amine structure, which might alter the rate of acid-base reaction. This alteration to the polymer structure is a broad area for investigation, although it is clear that ester-linked backbones should likely be avoided, given the instability in the polyacrylate structure described above.

In conclusion, amine-functionalized polyacrylamide hydogels appear to be a viable NAP for use in acid-fermenting TPPB. They are able to effectively extract a variety of acids from aqueous solution, are hydrolytically stable, and do not appear to inhibit cell growth. Further experiments will be necessary to develop TPPB protocols for acid-producing organism, and optimize the structure of the polymer.

4.8 References

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Chapter 5

Conclusions and Future Work

5.1 Conclusions

The results shown here have demonstrated a number of important concepts. In Chapter 3, it is shown that the phenomenon of polydispersity is very important in understanding the biocompatibility of polymers, unlike pure organic solvents that are monodisperse. Polydispersity must be considered when calculating the log P of PPG, suggesting this is an important consideration for all polymers. The OECD method for log P determination of organic compounds by HPLC was shown to be effective for estimating the log P of polypropylene glycols, and could be used for a variety of other MW polymers. A method was also developed for increasing the log P of polymer samples by water washing the polymers for periods as short as 2 minutes, which improved their biocompatibility. These results showed that lower MW polypropylene glycol polymers tend to be more inhibitory to microbial growth than higher MW polymers, and that the low MW fraction within a polymer sample is responsible for much of the inhibition of growth. It was also shown that log P is correlated with MW, with increased MW leading to increased log P.

Chapter 4 of this work examined a separate type of polymer – two versions of amine-functionalized hydrogels. These polymers were synthesized for the purpose of extracting organic acids through reactive extraction in order to lower the cost of purifying acids from fermentation media, and develop TPPB systems utilizing these new materials. Initially, two polymers were tested (polyacrylate and polyacrylamide), although the polyacrylate form was found to be hydrolytically unstable when exposed to a 41 mM solution of acetic acid over 24 hours. Since unstable polymer could interfere with microbial growth, and would be difficult to recycle, the polyacrylate was dropped from further tests. Results showed that amine-functionalized polyacrylamide hydrogel was able to extract 60-85% of a variety of organic acids from 2.5 g/L solutions over 2 hours using only 10 g/L of polymer, which represents an improvement over previously reported results. Experiments also showed the polyacrylamide was hydrolytically stable when exposed to 41 mM acetic acid over 24 hours, and does not inhibit the growth of *Saccharomyces cerevisea* nor *Pseudomonades putida* when they are exposed to polymer

concentrations as high as 4 g/L. It is therefore a material with strong potential for use in acid-producing TPPBs.

5.2 Future work

Chapter 3 of this thesis investigated the biocompatibility of polypropylene glycols to *Sacchromyces cerevisiae* and *Pseudomonades putida*. An area of research in need of further study is the biocompatibility of polymers of other types beyond polypropylene glycol. Polymer structure greatly influences the compounds that can be extracted from solution, therefore the biocompatibility of various polymers must be studied and compared to other properties such as log P and MW. In addition, work must be done to determine the PC values of partially water-soluble polymers. Results reported in the literature have shown that PC is higher in lower MW polymers of the same type, but these reports have been limited to polymers that are water insoluble. Polypropylene glycol and other hydrophilic polymers are often partially soluble (if not completely miscible) in water, which may complicate the partitioning behavior of these polymers. Previous work has also not tested the effect of water-washing on PC as this is irrelevant for insoluble polymers, though it may affect PC in partially water soluble polymers. Future investigations in this topic will determine if partially water-soluble polymers are useful for TPPB or if water-washed polymers should be used in TPPB applications. There also may be a benefit in assessing trends in biocompatibility for a variety of organisms, to determine if general patterns described for *S. cerevisiae* and *P. putida* hold for a species of interest for some particular application.

Experiments conducted using amine-functionalized hydrogels for the purpose of extracting acid that were reported in Chapter 5 were preliminary. There are many opportunities for future work that have yet to be investigated, the most important of which being the design of TPPB systems that use these new polymers. This will involve carefully considering organisms, selecting microbial media, and adjusting pH control strategies so that they are appropriate for TPPB containing amine functionalized polymer. There is also some concern that TPPB systems using an acid-extracting polymer may be fundamentally incompatible with microbial cell growth, as the pH needed for acid extraction may inhibit cell growth of acid producing microbes. It may be possible to overcome this issue with proper media composition and

pH control strategies, such as the use of an external column, or controlled polymer release. Work in this area could eventually lead to systems for the production of organic acid by microbes for use in industrial contexts.

It is also essential that methods be developed to recover acids that are extracted from solution, and regenerate the polymer without compromising its hydrolytic stability. It is economically impractical for the amine-functionalized polymers described in this work to be single use, meaning that methods must be developed to extract organic acid and recycle polymer. Other areas of investigation include analyzing acid uptake in the presence of more complex reaction mixtures, such as those anticipated after a fermentation experiment. There is also the possibility to further customize the polymer structure to improve acid partitioning behavior, or other desired properties (e.g. modifying distribution of cross-linker to monomer, changing alkyl groups bonded to tertiary amines, exploring linkages other than the ester and amide used in this work). Given the wide range of possible lines of inquiry, the usage of amine-functionalized hydrogels for the purpose of acid extraction is an area of experimentation that holds promise for future investigations.