

Properties of absorbent polymer extractants for the selective removal of target molecules from fermentation systems

By

Julian Thomas Samper Dafoe

A thesis submitted to the Graduate Program in Chemical Engineering in conformity with the
degree of Doctor of Philosophy

Queen's University
Kingston, Ontario, Canada
January, 2014

Copyright © Julian Thomas Samper Dafoe, 2014

Abstract

This thesis investigated polymer properties for their application as extractants in two-phase partitioning bioreactors (TPPBs), which are intended to remove inhibitory fermentation products as they are produced. Three applications of polymer TPPB extractants were studied, followed by an investigation into poly(ether)-based polymers' affinity toward representative target molecules, to identify properties which confer improved extraction performance.

The first investigation aimed to replace a liquid extractant (silicone oil) using a block copolymer, Hytrel® 8206, in the biotransformation of indene to *cis*-(1S,2R)-indandiol, a chiral pharmaceutical intermediate, by *Pseudomonas putida* ATCC55687. The polymer simultaneously delivered substrate and removed the product and by-products to alleviate inhibition, improving operability and productivity relative to silicone oil, which could only deliver substrate. Subsequently, soft segment composition and proportion were varied in different block copolymers to selectively extract product or by-product(s) from the same biotransformation, altering the cells' production profile. This demonstrated selective polymer extraction to help direct substrate utilization toward the product rather than by-product(s) in complex biotransformations.

The next study was on absorptive extraction of a hydrophilic target molecule, 4-valerolactone, produced by recombinant *Pseudomonas putida* KT2440, featuring an equilibrium-limited final step. The aim was to demonstrate the first application of equilibrium-pulling using selective product absorption, improving production by 30%. Furthermore, this study showed that limited polymer water absorption is helpful to aid in extraction of hydrophilic target molecules, but high polymer water content compromises selectivity, diminishing the equilibrium-pulling effect.

Finally, the effects of soft block proportion, molecular weight, and chain-end composition on affinity toward representative target molecules, carveol and carveone, were studied using commercial block copolymers and their representative homopolymer components. Target molecule affinity

improved at low molecular weights in the absence of polar homopolymer end-groups. End-group polarity had an effect whose direction depended on the polarity of the target molecule, improving affinity toward a third, polar target molecule, 4-valerolactone, thereby providing a means to tailor selectivity. Crystallinity and hard segment proportion were both found to reduce uptake.

This work has provided insights into the selection of polymeric TPPB absorbents by identifying polymer properties which improve affinity and selectivity toward different fermentation target molecules, especially relatively hydrophilic ones. The future design of purpose-built polymer extractants will benefit from considering these findings.

Co-Authorship

Chapters 2, 3, 4, 5, and 6 have been submitted to refereed journals and were co-authored by Dr. Andrew J. Daugulis, who provided editorial and technical advice. Dr. J. Scott Parent provided technical and editorial advice for work presented in Chapter 6 and is listed as co-author.

Acknowledgements

I would like to thank my supervisor, Andrew Daugulis, for his mentorship and guidance. From initially accepting me into the Group and throughout my graduate studies, it has been the most enriching working relationship imaginable. His outlook has continually motivated me to persevere, from running bioreactors to water skiing every summer. I would also like to thank Scott Parent for teaching me everything I know about polymers, and especially for his help and thoughtful advice throughout, which has shaped the direction of this work.

Thanks to my original lab colleagues who taught me how to do *everything*, and thank you to those who have arrived (and left again!) for all of the discussions, struggles, and laughs together. My friends, who have visited me in Kingston, and who I've been fortunate to go visit, have been a source of encouragement and reward in celebrating the small victories along the way.

I thank my family for their continual support and encouragement, which I have felt from wherever they have been in the world. I am grateful to Chelsea's parents, Bill and Holly, for initially encouraging me to apply to grad school, and especially to Bill for his advice and perspective, which have been crucial.

Finally, I owe the most gratitude to my partner Chelsea, who graciously moved back to her home town for five or more years. I could not have accomplished this without her optimism, support, advice, and above all, patience.

Table of Contents

Abstract.....	ii
Co-Authorship.....	iv
Acknowledgements.....	v
Table of Contents.....	vi
List of Tables.....	ix
List of Figures.....	x
Chapter 1 Introduction	1
1.1 Absorbent polymers used as the auxiliary phase in two-phase partitioning bioreactors	1
1.1.1 Physico-chemical properties of absorbent extractants	1
1.1.2 Polymer interactions.....	2
1.2 Structure of thesis.....	2
1.3 Objectives.....	4
Chapter 2 Literature review: <i>In-situ</i> product removal in fermentation systems: Improved process performance and rational extractant selection	5
2.1 Abstract.....	6
2.2 Introduction	7
2.2.1 Scope.....	7
2.3 Liquid-liquid systems.....	11
2.3.1 Organic solvents.....	12
2.3.2 Ionic liquids	15
2.3.3 Aqueous two-phase systems	18
2.4 Solid-liquid systems.....	21
2.4.1 Adsorbents.....	22
2.4.2 Absorbents.....	25
2.5 Other systems	28
2.5.1 Reactive extraction	28
2.5.2 Combined systems.....	31
2.6 Conclusions and future directions	35
2.7 References	36

Chapter 3 Bioproduction of <i>cis</i> -(1S,2R)-indandiol, a chiral pharmaceutical intermediate, using a solid-liquid two-phase partitioning bioreactor (TPPB) for enhanced removal of inhibitors	42
3.1 Preface to Chapter 3	43
3.2 Abstract	44
3.3 Introduction	45
3.4 Experimental	48
3.4.1 Chemicals	48
3.4.2 Microorganism	49
3.4.3 Analytics	49
3.4.4 Indene biotransformation experiments	50
3.4.5 Single-phase operation	51
3.4.6 Liquid-liquid two-phase operation	51
3.4.7 Polymer selection	51
3.4.8 Solid-liquid two-phase operation	53
3.5 Results and Discussion	54
3.5.1 Polymer selection	54
3.5.2 Bioconversion experiments	56
3.6 Acknowledgements	61
3.7 References	62
Chapter 4 Manipulating the composition of absorbent polymers affects product and by-product concentration profiles in the biphasic biotransformation of indene to <i>cis</i> -(1S,2R)-indandiol	64
4.1 Preface to Chapter 4	65
4.2 Abstract	66
4.3 Introduction	67
4.4 Materials and Methods	70
4.4.1 Chemicals	70
4.4.2 Microorganism	70
4.4.3 Partition coefficient experiments	70
4.4.4 Biotransformations	71
4.4.5 Analytics	73
4.5 Results and Discussion	73
4.5.1 Partition coefficient experiments	73

4.5.2	Bioreactor experiments	78
4.6	Conclusion.....	86
4.7	Acknowledgements.....	87
4.8	References	87
Chapter 5 Production of 4-valerolactone by an equilibrium-limited transformation in a partitioning bioreactor: impact of absorptive polymer properties		
		90
5.1	Preface to Chapter 5	91
5.2	Abstract.....	93
5.3	Introduction	94
5.4	Materials and Methods.....	98
5.4.1	Chemicals	98
5.4.2	Microorganism.....	99
5.4.3	Polymer synthesis	99
5.4.4	Water uptake	100
5.4.5	Polymer partition coefficients	100
5.4.6	Biotransformations	101
5.4.7	Analytics.....	102
5.5	Results and Discussion	103
5.5.1	Polymer extractant characteristics	103
5.5.2	Water uptake experiments.....	103
5.5.3	Partition coefficient experiments	106
5.5.4	Biotransformations	107
5.5.5	Polymer selectivity.....	111
5.6	Conclusions	113
5.7	Acknowledgements.....	114
5.8	References	114
Chapter 6 Block copolymers as sequestering phases in two-phase biotransformations: Effect of constituent homopolymer properties on solute affinity		
		117
6.1	Preface to Chapter 6	118
6.2	Abstract.....	119
6.3	Introduction	120
6.4	Materials and Methods.....	122

6.4.1	Chemicals	122
6.4.2	THP-terminated poly(tetramethylene oxide) (THP-PTMO)	122
6.4.3	Partition coefficient experiments	123
6.4.4	Analytical methods	123
6.5	Results and Discussion	124
6.5.1	Pebax [®] series: effect of copolymer soft segment proportion	124
6.5.2	THP-PTMO series: effect of molecular weight on partition coefficients	126
6.5.3	Effect of degree of crystallinity on partition coefficients	130
6.5.4	Effect of polar end-group concentration on partition coefficients	131
6.5.5	Effect of end group polarity on target molecule selectivity	134
6.6	Conclusions	135
6.7	Acknowledgements.....	136
6.8	References	136
Chapter 7	Summary and conclusions	139
7.1	Future work.....	142
Appendix A:	UNIFAC predictions of partition coefficients.....	144
A.1	References	147
Appendix B:	Hansen Solubility Parameter estimations of relative polymer-solute affinity	148
B.1	References	155

List of Tables

Table 2-1:	Other applications of <i>in-situ</i> product removal.....	9
Table 3-1:	Properties of evaluated polymers.....	48
Table 3-2:	Comparison of single-phase, two-liquid, and solid-liquid productivity. Overall product titer calculated by dividing total product mass in both phases by total working volume.	57
Table 4-1:	Polymer properties	69
Table 4-2:	Major metabolite properties	70
Table 5-1:	Performance comparison of both polymer ISPR experiments	111

Table 6-1: Properties of Pebax® ‘33’ block copolymer series. Poly(amide-12) (PA-12) is the hard segment component, and poly(tetramethylene oxide) (PTMO) is the soft segment component	121
Table 6-2: Partition coefficients (measured directly, and normalized to the soft PTMO fraction given in Table 6-1) for carveol and carvone in the Pebax® series.....	125

List of Figures

Figure 2-1 Scanning electron micrographs of a) a macroporous adsorbent resin bead (Rohm & Haas Amberlite IRA938) (50 μm scale indicated) (Krug and Daugulis 1983); b) an absorbent polymer bead (Pebax 2533) (3 mm scale indicated); c) the smooth, monolithic surface of Pebax 2533 (100 μm scale indicated)	23
Figure 3-1: Indene bioconversion by <i>P. putida</i> TDO enzyme system showing indene conversion to detected products and by-products, adapted from Connors et al. (1997). The monooxygenation product, 1-indenol, is a result of “improper fit” of the non-natural substrate, indene, in the dioxygenase active site. ³ 1-indenol undergoes slow, spontaneous isomerization to 1-indanone.....	46
Figure 3-2: Photograph comparing swelling behavior in different grades of Hytrel® when exposed to indene: 3548 swelling and cracking on left, 8206 unaffected on right. Unexposed beads in foreground for comparison with paper clip for scale.	54
Figure 3-3: Polymer distribution coefficients for biotransformation products: 1-indanone (a), 1-indenol (b) and <i>cis</i> -(1S,2R)-indandiol (c). Distribution coefficient is expressed as the concentration in the polymer (g/kg) divided by the concentration in the aqueous phase (g/L). Error bars represent one standard deviation from the mean value of five samples.	55
Figure 3-4: Time course plot of overall product concentration (closed symbols, left axis) and substrate concentration in auxiliary phase (open symbols, right axis) under liquid-liquid (triangles) and solid-liquid (squares) operation. Single-phase, high substrate feed operation (circles) lacks an auxiliary phase.	56
Figure 4-1a) Partition coefficient values of <i>cis</i> -(1S,2R)-indandiol measured in high-purity water or fermentation medium in the three polymers. Error bars represent the standard deviation within the triplicate samples for each metabolite.....	76
Figure 4-1b) Partition coefficient values of 1-indanone measured in high-purity water or fermentation medium, and 1-indenol PC values measured in fermentation medium in the three polymers. Error bars represent the standard deviation within the triplicate samples for each metabolite.	76

Figure 4-2: Aqueous <i>cis</i> -(1S,2R)-indandiol concentration with the three polymer auxiliary phases. Symbols: ■, Hytrel® 8206; ♦, Hytrel® 3078; ▲, Pebax® 2533.	79
Figure 4-3: <i>cis</i> -(1S,2R)-indandiol concentration in the polymers. Symbols: ■, Hytrel® 8206; ♦, Hytrel® 3078; ▲, Pebax® 2533.	80
Figure 4-4: Overall <i>cis</i> -(1S,2R)-indandiol concentration in the reactor (aqueous and polymer phases combined). Symbols: ■, Hytrel® 8206; ♦, Hytrel® 3078; ▲, Pebax® 2533.	82
Figure 4-5: Aqueous 1-indenol concentration with the three polymer auxiliary phases. Symbols: ■, Hytrel® 8206; ♦, Hytrel® 3078; ▲, Pebax® 2533.	82
Figure 4-6: 1-indenol concentration in the polymers. (■) Hytrel® 8206 (♦) Hytrel® 3078 (▲) Pebax® 2533.	84
Figure 4-7: Overall 1-indenol concentration in the reactor (aqueous and polymer phases combined). (■) Hytrel® 8206 (♦) Hytrel® 3078 (▲) Pebax® 2533.	85
Figure 5-1: Water absorption (bars) and 4VL partition coefficient values (■) in (a) BMI-PEO or (b) Hytrel® 8206 and XLPEGDA.	105
Figure 5-2: Plot of biomass (●), 4HV accumulation (■) and 4VL (♦) during growth, 4HV accumulation, and 4VL accumulation phases. The dashed line indicates shifting pH to 6.0 to induce lactonization of 4HV to 4VL.	108
Figure 5-3: 4VL (♦) and 4HV (■) accumulation in polymers during ISPR (g/kg) in: a) BMI-PEO b) Hytrel® 8206.	112
Figure 5-4: Overall 4VL mass in the bioreactor vs. time normalized to onset of the pH shift. The first (dashed) line indicates the pH shift from 7.0 to 6.0, and the second (dotted) line indicates the addition of the polymer: a) 240 g BMI-PEO b) 930 g Hytrel® 8206.	113
Figure 6-1: Partition coefficients (measured directly) of carvone and carveol in PTMEG2900 (left axis) and PA-12 (right axis).	125
Figure 6-2: Partition coefficients of carvone and carveol in the THP-PTMO series with increasing molecular weight.	128
Figure 6-3: Partition coefficients of carvone and carveol in the PTMEG series with increasing molecular weight. Solid lines represent values normalized to amorphous portion measured by DSC. Dashed lines represent values measured directly.	131
Figure 6-4: Partition coefficient of 4-valerolactone in the PTMEG series with increasing molecular weight. Solid lines represent values normalized to amorphous portion measured by DSC. Dashed lines represent values measured directly.	133

Figure A-1: Partition coefficients of carveol and carvone estimated using the UNIFAC-vdW-FV model in THP-PTMO of varying molecular weight.....	145
Figure A-2: Partition coefficients of carveol and carvone estimated using the UNIFAC-vdW-FV model in PTMEG of varying molecular weight.....	146
Figure B-1: Hansen Solubility Parameter “overall” Ra distance between THP-PTMO and carveol/carvone	150
Figure B-2: Hansen Solubility Parameter component Ra distances between carveol and THP-PTMO	151
Figure B-3: Hansen Solubility Parameter component Ra distances between carveol and THP-PTMO	151
Figure B-4: Hansen Solubility Parameter “overall” Ra distance between PTMEG and carveol/carvone .	152
Figure B-5: Hansen Solubility Parameter component distances between PTMEG and carveol.....	153
Figure B-6: Hansen Solubility Parameter component distances between PTMEG and carvone.....	153
Figure B-7: Hansen Solubility Parameter component distances between PTMEG and 4-valerolactone .	154

Chapter 1 Introduction

1.1 Absorbent polymers used as the auxiliary phase in two-phase partitioning bioreactors

This thesis has examined the application of solid polymers as absorbent extractive phases, replacing liquid solvents which have been conventionally used in two-phase partitioning bioreactors (TPPBs). Polymers have been found to be nearly universally effective in improving bioprocess performance, and while the straightforward application of polymers to conventional two-liquid biphasic fermentations has recently gained popularity, there is little to no insight available which compares the properties of the selected polymers to those of the target molecules in the context of biphasic biotransformations.

There are several advantages that solid polymers provide over liquid solvents used as extractants. Polymers are widely biocompatible when in contact with microorganisms, whereas specific organisms must be evaluated for tolerance to individual liquid solvents. Similarly, most synthetic polymers are non-biodegradable, whereas liquid solvents must also be evaluated in this regard with respect to each specific biocatalyst. The requirements above greatly restrict the range of satisfactory solvents, while nearly all polymers meet these requirements. Finally, liquid solvents often become trapped in emulsions formed when in contact with the biocatalyst's hydrophobic cell membrane or extracellular products such as surfactants, while the phase stability of solid polymers allows their simple recovery from fermentation medium.

1.1.1 Physico-chemical properties of absorbent extractants

Absorbent polymers operate similarly to organic solvents, where solute molecules are dissolved within the solvent matrix. Permeation within polymers operates by solution and diffusion. Absorption of a solute by a polymer requires both favourable chemical affinity to promote dissolution of the target molecule within the polymer, as well as a soft polymer matrix (below its glass transition temperature),

allowing chains to move in order to accommodate sorbed molecules. Compared to the conventional organic solvents which have been utilized as extractant phases for their well-predicted thermodynamic behaviour, polymers have complex structure-property relationships. These aspects arise from polymers' macromolecular chemical structure and affect both physical and chemical properties.

1.1.2 Polymer interactions

Polymers can exhibit relatively strong intramolecular and intermolecular interactions, for example by dipole interactions or hydrogen bonding between adjacent polar or hydrogen-bonding donor/acceptor sites, which may hinder target molecule sorption by increasing the glass transition temperature or favouring crystallinity. If the effects of strong polar interactions contributing to a rigid polymer structure were reduced, these interactions may improve the sorption of relatively polar target molecules which engage in similar, polar interactions.

Because water is a polar molecule which participates strongly in hydrogen bonding, hydrophilic polymers which are in glassy or crystalline states when dry will swell upon absorbing water as it interacts with the polymer at available sites, and simultaneously interrupts the inter-chain interactions which had previously held the polymer in its rigid configuration. Water uptake by hydrophilic polymers causes a reduction in glass transition temperature by the relaxation of intra/intermolecular interactions, termed plasticization, or in the case of completely soluble polymers, dissolution may occur. The water-polymer complex no longer behaves as the pure polymer from the perspective of a sorbed molecule, because it contains a significant amount of internally-bound water. In this way, the absorption of polar, relatively hydrophilic molecules may be enhanced in a polar, aqueous polymer phase compared to a strictly hydrophobic, organic polymer phase.

1.2 Structure of thesis

This thesis progresses from a general review of *in situ* product removal (ISPR) strategies employed in the literature in Chapter Two, which discusses the use of polymer extractants and places

them in the context of alternatives such as liquid solvents, ionic liquids, aqueous two-phase systems, adsorbent resins, and other strategies. Chapter Three contains a preliminary investigation into a solid polymer effectively replacing a liquid solvent in the biocatalysis of a pharmaceutical intermediate, which also enabled the extraction of the by-products and product. The same biocatalytic system is investigated in the context of polymer composition in Chapter Four, where three polymers having different compositions, and consequently, affinities towards the product and by-products, were found to have distinct effects on the relative production of metabolites in the biotransformation. The potential for targeting relatively hydrophilic compounds was also investigated in this chapter, where a polymer exhibiting significant water uptake conferred enhanced affinity relative to non-water absorbing polymers towards the hydrophilic product, *cis*-(1S,2R)-indandiol, presumably by absorbed water providing a favourable, aqueous-like environment for sorption of the target molecule, and by the polarity of the polymer itself (indicated by its water uptake). The effect of varying amounts of water uptake among different polymers was accounted for to permit comparison of polymers on an equal basis, as previous reports had neglected this important feature.

The removal of hydrophilic target molecules from fermentation medium is a challenge, and this aspect was the main focus of Chapter Five, where 4-valerolactone (4VL) was selectively removed relative to its precursor, 4-hydroxyvalerate (4HV), from an equilibrium-limited biocatalytic system in order to shift the equilibrium towards greater 4VL production. The affinity of different poly(ethylene oxide)-containing polymers towards the water-miscible target molecule was found to improve with increasing water content in the polymers; however, selectivity towards 4VL relative to 4HV was compromised at extreme water content, such that block copolymer architecture was proposed to impart good affinity while controlling the extent water uptake to achieve selective product removal.

Chapter six examines in more detail the aspects of polymer selection which were brought to light in the previous chapters. By focusing on polymer-solute interactions in a systematic approach,

without the presence of a biocatalyst, the properties of absorbent polymers which were found to affect the biocatalytic systems could be more closely investigated. Aspects of polymer absorbents including their composition, architecture, molecular weight, end-group composition, and crystallinity were examined for their effects on polymer affinity towards a series of target molecules across a range of hydrophobicity.

Finally, Chapter seven provides a conclusion, and proposes future directions for further investigation of aspects pertaining to polymer-solute interactions in the context of TPPBs.

1.3 Objectives

It is the overall objective of this thesis to examine the selection of absorbent polymer extractants to improve affinity towards target molecules in biocatalytic systems, particularly those molecules which are relatively hydrophilic and represent a challenge in their separation from water. The initial objective was to expand the range of extractable target molecules by introducing polymers as an alternative to hydrophobic liquids. A subsequent objective was to examine the aspects of polymer composition and architecture, specifically, comparing the use of block copolymer architecture with other polymer types, which contributed towards water uptake and, in turn, improving or hampering target molecule affinity and selectivity. The final objective was to investigate the properties of block copolymers which impart favourable performance in TPPB applications by analyzing the homopolymer components of several commercial block copolymers; specifically, the nature of the poly(tetramethylene ether) soft segment with respect to molecular weight and relative polarity. These objectives aim to improve the state of knowledge of polymer selection for biphasic biotransformations.

Chapter 2 Literature review: *In-situ* product removal in fermentation systems: Improved process performance and rational extractant selection

Julian T. Dafoe, Andrew J. Daugulis

With minor changes to fulfill formatting requirements, this chapter is substantially as it appears in *Biotechnology Letters* DOI 10.1007/s10529-013-1380-6 (2013).

2.1 Abstract

The separation of inhibitory compounds as they are produced in biotransformation and fermentation systems is termed *in-situ* product removal (ISPR). This review examines recent ISPR strategies employing several classes of extractants including liquids, solids, gases, and combined extraction systems. Recent studies demonstrating improvement through the simple application of an auxiliary phase have been tabulated and summarized to indicate the breadth of recent ISPR activity. Studies within the past five years which have highlighted and have discussed “second phase” properties which have an effect on fermentation performance are particularly the focus of this review. ISPR, as a demonstrably effective processing strategy, continues to be widely adopted as more applications are explored; however, focus on the properties of extractants, and their rational selection based on first principle considerations, will likely be key to successfully applying ISPR to more challenging target molecules.

Keywords: absorption; adsorption; biocatalysis; extractive fermentation; ionic liquids; reactive extraction

2.2 Introduction

The incorporation of an extractant phase for *in-situ* product removal (ISPR) of inhibitory fermentation and biotransformation molecules is a powerful tool to alleviate the effect of high aqueous-phase concentrations of target molecules, improving bioreactor productivity. Such a processing configuration has been termed a “two-phase partitioning bioreactor” (TPPB) reflecting the presence of distinct aqueous and sequestering phases, as well as the fact that the target molecule will differentially partition between these two phases. In addition to reducing toxic product concentrations, ISPR can be applied to shift unfavourable reaction equilibria, reduce the number of downstream processing steps, favour the accumulation of an intermediate in multistep reaction systems, and prevent product losses due to degradation or volatility.

The affinity of a target molecule for the extractant phase is measured by the distribution (partition) coefficient at equilibrium, while selectivity is measured by the distribution coefficient towards the target molecule relative to another species, which can be another fermentation product molecule, or water itself. An auxiliary phase possessing a high distribution coefficient is desirable to minimize the amount of sequestering phase required, which has economic consequences throughout the design, extraction, recovery, and recycling aspects of a bioprocess. Selectivity towards the target molecule relative to water is also important for concentrating the target molecule in the extractant phase. The extractant phase may be an immiscible liquid, a solid, or a gas, and consideration of target molecule properties, in conjunction with those of the extractant phase and the particular biocatalytic system, largely determine the applicability of a particular extraction strategy. Nevertheless, several different types of extractant have been utilized for a few common target molecules, demonstrating the flexible applicability of ISPR as a generic processing strategy.

2.2.1 Scope

Although the operational configuration of the sequestering phase in contact with the aqueous fermentation medium can vary, the direct removal of target molecules from the fermentor during

operation, termed *in-situ* product removal (ISPR), is the focus of this review. To illustrate the breadth of recent work, reports over approximately the last five years of process improvements with the addition of an extractant phase, and reports which emphasize biological aspects of a biphasic transformation are tabulated in Table 2-1. Several authors have additionally provided insights into the characteristics and sorption mechanisms of the extractant phases, and have identified promising strategies for targeted extractant selection. These reports are of particular interest and form the basis of this review.

Studies investigating the pre-treatment of streams prior to fermentation have also been considered here, because these operations have been shown to have a direct effect on the subsequent fermentation and require similar considerations to true ISPR processes. Conversely, downstream processing operations which do not require any consideration of the bioreactor itself are not included. Protein, peptide, and antibiotic products are not included in this review due to their unique requirements for stability; such molecules are almost exclusively extracted by ISPR using aqueous two-phase systems (ATPS). Finally, TPPB systems in which the sequestering phase is used to *deliver* inhibitory substrates, rather than *remove* fermentation products are, although very similar in operation, also not considered here, and have recently been reviewed (Muñoz et al. 2012).

Table 2-1: Other applications of *in-situ* product removal

Target molecule	Extractant class	Remarks	Reference
p-hydroxystyrene	Organic solvent	Solvent selected from thirteen screened for product affinity and biocompatibility based on enzyme activity.	(Jung et al. 2013)
p-hydroxystyrene		1-decanol selected without rationale given. Solvent presence negatively affected product yield on substrate attributed to toxicity, yet productivity improved <i>via</i> extraction.	(Verhoef et al. 2009)
Isoprenoid and polyketide		<i>n</i> -dodecane selected based on a previous report as an extractant for a similar (hydrophobic) product, without further rationale.	(Boghigian et al. 2011)
(R)-epichlorohydrin		Cyclohexane selected based on screening ten solvents for product yield and enantiomeric excess. Attributed success to solvent hydrophobicity and toxicity to solvent hydrophilicity.	(Jin et al. 2013)
2,3-butanediol		Eleven solvents screened hierarchically for biocompatibility, bioavailability, and product capacity. Oleyl alcohol chosen based on miscibility.	(Pahlavanzadeh et al. 2009)
2,3-butanediol		Eleven solvents screened based on high log P (biocompatibility), high boiling point, high product partition coefficient, low water solubility, bioavailability, and product capacity. Oleyl alcohol chosen based on miscibility.	(Anvari et al. 2009)
Styrene oxide	Organic solvent blend	Solvent blend employed without providing rationale.	(Julsing et al. 2012)
Methane	Liquid polymer solvent (silicone oil)	Silicone oil used as hydrophobic phase for methane delivery from air stream without rationale provided, but speculated to improve substrate availability by reducing Henry's constant.	(Zúñiga et al. 2011)
Lactic acid	Natural adsorbent (activated carbon)	Activated carbon selected because of low cost, high surface area, and product affinity at low pH, prompting pH-uncontrolled mode of operation.	(Gao et al. 2011)
2-phenylethanol	Adsorbent resin	Non-polar macroporous resin selected from eight tested based on productivity enhancement; attributed to hydrophobicity and surface area.	(Mei et al. 2009)
2-phenylethanol		Non-polar macroporous resin selected from eight tested (rationale not shown) including ion-exchange, polar, and non-polar macroporous.	(Wang et al. 2011)
2-phenylethanol		Non-polar macroporous resin selected from eight tested based on product capacity and selectivity with respect to substrate.	(Hua et al. 2010)
2-phenylethanol		Resin selection criteria unclear. Semi-continuous operation by alternating adsorption/elution with two resin columns improved volumetric productivity.	(Wang et al. 2011)
2-phenylethanol		Non-polar macroporous resin selected from seven tested based on high adsorption ratio, good biocompatibility, and quick product recovery. No data shown.	(Rong et al. 2011)
Poly-L-lysine		One resin chosen from four tested based on adsorption capacity; no rationale provided.	(Liu et al. 2011)
Propionic acid and vitamin B12		Resin selected from nine tested based on adsorption capacity. Ranking of resin capacities in model solution differed from that in fermentation broth, affecting selection.	(Wang et al. 2012)
(S)-1-phenyl-(1S,2R)-ethanediol		Resin selected from six tested based on rate of adsorption/desorption and capacity. Compromise in affinity required because substrate affinity was too high to permit	(Hu et al. 2010)

		biotransformation.	
Epichlorohydrin		Resin selected from seven tested based on adsorption capacity and product selectivity relative to substrate; no rationale provided.	(Zou et al. 2013)
n-butanol		Adsorbent resin selected from five tested, including adsorbents and absorbents, based on capacity. Solid polymer extractants out-perform organic solvents.	(Choi et al. 2011)
Linear and branched 2C-5C alcohols	Adsorbents and absorbent	Compared solute extraction efficacy of five adsorbent resins and one absorbent polymer. Solute hydrophobicity improved extraction via hydrophobic interactions, allowing prediction of extraction extent based on solutes' relative hydrophobicity. Did not differentiate adsorption/absorption mechanisms.	(Nielsen et al. 2010)
Propionic acid	Anion-exchange resin	Anion-exchange resin chosen, no selection criteria stated. Compared different feeding strategies and modes of contact with ion-exchange resin. External fluidized bed found to provide greatest ISPR improvement due to resin regeneration step.	(Wang et al. 2012)
Succinic acid		Macroporous polystyrene anion-exchange resin used in two parallel columns for expanded bed adsorption and elution in fed-batch operation. No resin selection rationale provided.	(Li et al. 2011a)
Phenylacetylcarbinol	Absorbent polymer	Absorbent polymer chosen for product partitioning was saturated with substrate and used for substrate delivery and simultaneous product removal.	(Khan et al. 2010)
2-phylethanol		Absorbent polymer chosen based on product partitioning was used for selective product removal; hydrophilic substrate was not absorbed.	(Gao et al. 2009)
<i>cis</i> -(1S,2R)-indandiol		Absorbent polymer chosen based on product and by-product partitioning was loaded with substrate <i>via</i> solvent partitioning and used for simultaneous substrate delivery and product/by-product removal.	(Dafoe et al. 2011)
Benzaldehyde		Absorbent polymer selected based on product selectivity <i>via</i> maximizing the product/substrate partition coefficient ratio.	(Jain et al. 2010)
Hexanoic acid	Reactive extractant	Trioctylamine 10% (v/v) in oleyl alcohol was selected based on previous reports of its use extracting organic acids. Toxic effects on biocatalyst noted.	(Jeon et al. 2013)
Ethylene glycol and glycolic acid	Combined membrane / anion-exchange resin	Hollow-fiber membrane used to protect cells from non-biocompatible anion-exchange resin. Resin selected from three tested based on product capacity.	(Wei et al. 2009)

2.3 Liquid-liquid systems

Liquid extractants are typically water-immiscible organic solvents, immiscible liquid polymers, or liquid polymer-based aqueous two-phase systems (ATPS); however ionic liquids (ILs) have also recently gained attention as potential extractants, and have been used as both immiscible solvents and as ATPS components in TPPBs. Although Table 2-1 provides a complete list of ISPR/TPPB systems utilizing immiscible organic solvents, we report below on recent examples using liquid extraction where novel features of the extractants are identified and discussed, and which have a bearing on fermentation performance.

Water-immiscible solvents have been conventionally used to extract hydrophobic fermentation products. The extractant's $\log K_{o/w}$ value (octanol-water partition coefficient) must be sufficiently high to maintain a biphasic system with water; additionally, a solvent's $\log K_{o/w}$ value is also a measure of biocompatibility, as above an organism-specific critical $\log K_{o/w}$ value, all solvents are considered to be biocompatible (Garikipati et al. 2009). A third requirement mainly specific to liquid organic solvents is non-bioavailability, which is important for extractant stability and biocatalyst performance, ensuring that the intended carbon source, rather than the solvent, is exclusively metabolized. Generally, an organism's critical $\log K_{o/w}$, as well as a solvent's bioavailability, must be experimentally determined for each specific system.

Because ISPR solvent extractants are generally limited to hydrophobic liquids, their extraction potential is related to the target molecule's hydrophobicity and chemical affinity towards the extractant, generally described by the principle of "like dissolves like". The hydrophobic, liquid polymer, silicone oil (polydimethylsiloxane) has enjoyed widespread use for its nonvolatility, non-bioavailability, and high hydrophobicity providing good biocompatibility, which has led to its adoption as a default hydrophobic liquid extractant in TPPBs. However, it shares many of the same drawbacks as organic solvents including high cost, and emulsion formation which hampers re-use. Critically, its affinity is strictly towards

hydrophobic target molecules. To our knowledge, there are no reports providing insight into the properties of silicone oil for use as an extractant, likely because it is a single, defined material with the only possible variation being viscosity, which is a function of molecular weight. In light of this, we believe that new silicone oil ISPR applications are unlikely to emerge beyond their facile use for hydrophobic target molecules.

2.3.1 Organic solvents

In cases involving system-specific constraints, extractant phase selection requires considerations beyond the basic aspects of biocompatibility, non-bioavailability and target molecule affinity. Recent experimental studies discussed below have identified additional desirable/necessary extractant properties, which introduce considerations of ecological impacts arising from solvent selection, mass transfer rate, or impose restrictions on extractant properties to enable its incorporation into a final product. Additionally, advances in the understanding of extractant-target molecule interactions have enabled more effective rational extractant selection strategies using simulations requiring no experimental data, described below.

Organic solvents as extractant phases have been compared on the basis of ecological impacts, in addition to economic considerations, in order to minimize the environmental footprint of the biocatalytic epoxidation of styrene. Renewably-sourced ethyl oleate, a component of biodiesel, was found to have an ecological cost which is 9% lower than the conventional solvent derived from petroleum, bis(2-ethylhexyl)phthalate, determined by considering comprehensive environmental criteria of the two solvents including their production, handling risks, and environmental fate (Kuhn et al. 2012). Comparison of solvents on an ecological basis is an important consideration to ensure process sustainability, and this should be considered in parallel with economic and performance aspects, especially with the use of solvents which cannot be recycled indefinitely.

Garikipati and co-authors found that in extractant selection, the high viscosity of dioctyl phthalate imposed a mass transfer limitation during mixing, rationalizing the choice of lauryl acetate as a lower-viscosity solvent despite its having slightly lower affinity towards the product, 1-naphthol (Garikipati et al. 2009). In this case, the volumetric productivity was improved by increasing the mass transfer rate, rather than the extent, of extraction. An ideal extractant would provide both benefits simultaneously, however *a priori* predictions of such diverse properties are not currently available.

Solvent extraction ISPR has been effective in reducing end product toxicity in the fermentative production of transportation biofuels, and several groups have recognized the potential to use the extractant itself as a component of the fuel mixtures. The examples below show that hydrophobic products provide flexibility in selecting a liquid extractant which may satisfy additional criteria beyond the basic requirements of biocompatibility, non-bioavailability and affinity, sometimes at the expense of compromised performance. The inclusion of an auxiliary solvent phase which itself is a useful product may reduce or remove the requirement for downstream processing or product recovery, as demonstrated by the extraction of acetone, n-butanol, and ethanol from fermentation broth using biodiesel, resulting in improved diesel fuel properties (Li et al. 2010). Similarly, the use of farnesane as an extractant for microbially-produced limonene resulted in moderate alleviation of monoterpene toxicity relative to other solvents. In another study, farnesane was used to extract limonene, producing a solvent blend that had properties similar to jet fuel and could potentially be used without further processing (Brennan et al. 2012). Other researchers added an enzyme for the esterification of n-butanol (the inhibitory fermentation product) to butyl butyrate, a more hydrophobic species, and improved the partition coefficient in the auxiliary phase nearly 1000-fold. Its extensive extraction by hexadecane, a model diesel fuel compound, maintained a favorable equilibrium position towards esterification at fermentation pH while improving fuel quality (van den Berg et al. 2013). That is, in this approach, the improvement in product extraction concurrently increased its contribution to improved fuel properties

of the blended auxiliary phase. The above solvent extraction approaches share the similar objective of providing process simplification by incorporating the extracted product within the auxiliary phase as a solvent blend having desirable fuel properties, reducing the number of subsequent downstream separation steps.

Hydrophilic products require more careful consideration of extractant interactions to facilitate adequate extraction because favourable interactions with the extractant are required in order to overcome their affinity for water, and a high distribution coefficient is desirable to reduce process expense by decreasing the amount of extractant required in a TPPB configuration. In an attempt to improve extractant selection strategies for ethanol, a water-miscible product which is difficult to extract, Keasler *et al.* examined specific interactions between extractants and the solute using molecular simulation techniques. The extraction of ethanol from water by several similar 10-carbon alcohols was predicted using computationally-intensive Monte Carlo simulations, and a tradeoff was found between extractant capacity and selectivity relative to water. The ability of primary alcohols to form large hydrogen-bonded ethanol clusters also promoted the co-extraction of water, whereas branched alcohols had lower ethanol capacity and also had the highest ethanol selectivity relative to water (Keasler et al. 2013). Insights into the mechanistic differences between similar extractants improve our understanding of such interactions, and these findings may be applicable to new extractant materials. Detailed investigations of extractant-target molecule interactions such as this are necessary to understand subtle extractant behaviours and may be useful in fine-tuning processes for minor improvements in capacity/selectivity, but ultimately, primary selection criteria such as biocompatibility and non-bioavailability must remain at the forefront.

The above reports are advances in the application of liquid solvent extractants to ISPR that go beyond merely describing a new system, and offer novel insights. Other recent demonstrations of improvements using liquid solvent extractants, compiled in Table 2-1, show the breadth of applications,

and a recent patent application filed on the subject suggests that ISPR liquid solvent extraction may be approaching commercialization (Grady et al. 2010). While the use of organic solvent extractants in ISPR situations is now fairly mature, a focus on identifying and characterizing relevant solvent properties, from first principles, will likely be a source of inspiration for advances in future systems.

2.3.2 Ionic liquids

A more recent class of materials with potential for use as liquid extractants is ionic liquids (ILs). ILs are salts which are liquid at room temperature and are comprised of a pair of counterions, opening a range of possible compositions and properties, making them potentially attractive as extractants. There is considerable flexibility in “constructing” the cation/anion pairs by selecting from imidazolium, pyrrolidinium, pyridinium, phosphonium, or ammonium-based cations, combined with an anion, providing the range of properties seen within the IL family. One of the more representative features of ILs is their very low volatility. Additionally, many ILs have been found to be toxic to biocatalysts, or perform poorly relative to organic solvents with respect to target molecule affinity and selectivity and, perhaps most importantly, ILs are generally very expensive, an order or more higher in price than are organic solvents. The lack of straightforward IL applications in Table 2-1 indicates that their use for ISPR requires more rigorous considerations than alternative ISPR extractants. Nevertheless, the reports reviewed below demonstrate recent advances in the use of ILs and, in some cases, suggest that ILs could surpass conventional solvents in extraction performance by providing a greater opportunity for fine-tuning the structure-property relationships towards the goals of biocompatibility, affinity, and selectivity.

In order to improve on the state of ionic liquid extractants for n-butanol recovery *via* ISPR, Garcia-Chavez *et al* reviewed previous investigations and found that anion carboxylate functionality improved n-butanol’s distribution coefficient, while its hydrogen-bonding ability played a significant role in water uptake, hampering selectivity. Using this information, the authors designed a new IL containing carboxylate functionality with two aromatic rings in the anion and long alkyl chains in the cation to

improve its hydrophobicity. The new task-specific IL (TSIL) out-performed the distribution coefficient of the benchmark solvent, oleyl alcohol, by a factor of six with a 30% improvement in selectivity. The entire process including recovery and re-use was simulated using various extractants, and it was found that for the new IL, a solvent : feed ratio of 0.071 would perform equivalently to a ratio of 0.456 for oleyl alcohol in terms of n-butanol recovery, representing a significant economic improvement in the energy required to heat the solvent for recovery and re-use (Garcia-Chavez et al. 2013). The reduction of solvent amounts is motivation to seek specialized materials, but their additional cost must be justified, and this report concedes that biocompatibility is an additional, important factor in their application which has not yet been investigated.

The application of ILs to fermentation systems certainly requires considering biocompatibility, and the lack of tabulated IL properties (e.g. Log $K_{o/w}$) makes standardized *a priori* prediction, as can be done for organic solvents, currently impossible. In the fungal hydroxylation of epoxyprogesterone, Mao *et al* screened seven ILs, only one of which demonstrated nearly-full biocompatibility, yet all ILs had some deleterious effect on cell growth. The inhibitory effect was attributed to the anion, while improvements in distribution coefficient were attributed to the cation structure, gained by increasing alkyl chain length and increasing the surfactant nature of the cation. The effect of ILs on substrate conversion mirrored their toxicity profiles, and only the biocompatible IL improved productivity relative to the control. The distribution coefficients of the substrate and product were both exceptionally high, an expected result based on their hydrophobicity, such that the primary effect of ILs on the biotransformation was IL toxicity. The authors suggest that a lower phase ratio could reduce harmful interfacial contact of the biocatalyst with the IL phase, and may be a strategy to optimize extraction and biocompatibility (Mao et al. 2012). Despite their toxicity, the high distribution coefficients of the ILs offered some protection from substrate inhibition in every case; however biocompatibility should be an absolute requirement of extractant phases.

ILs are valued for their solvation power and nonvolatility, which could substantially decrease energy costs of ethanol distillation if ILs were to be used as ISPR components in ethanol production. Neves *et al.* undertook a detailed study of seven phosphonium-based ionic liquids in ternary phase systems with ethanol and water, creating phase diagrams which were extremely well-predicted by the NRTL model. The COSMO-RS model was less accurate in predicting exact phase compositions, but was able to correctly rank the ILs without requiring experimental data, and was also used to estimate the performance of two additional ILs for which data are not available (Neves et al. 2011). The authors considered the cost and biocompatibility of the chosen ILs which compare favorably to imidazolium-based ILs, but note that by using tabulated EC_{50} values as an indication of biocompatibility, interactions with cell membranes are not represented and biocompatibility with whole cells would require additional experimentation. This report underscores the comprehensive nature of extractant selection requirements, where multiple, unrelated parameters must be considered simultaneously, and the properties of IL extractants, at this stage of our understanding, may prevent characterization to a similar degree as organic extractants.

The relatively high cost of ILs may be acceptable if their performance enhancements surpass the cost-effectiveness of alternative extractants, particularly if the value of the recovered product is high. However, the relative scarcity of literature discussing the application of ILs in ISPR suggests that the understanding of IL biocompatibility is heuristic at best, and progress must be made in that regard, as well as in reducing IL cost, to exploit their attractive performance as immiscible solvents. Overall, our general understanding of IL properties and their interactions with cells is somewhat limited, and an important future area of research, focusing on defining extractant properties, will be in addressing these aspects.

2.3.3 Aqueous two-phase systems

Aqueous two-phase systems (ATPS) are a subset of TPPBs, comprised of water-soluble polymers and/or salts in aqueous solutions at concentrations which invoke phase separation into two phases, either salt-rich or polymer-rich, consisting mostly of water. ATPS have traditionally been favored for the recovery of proteins and antibiotics, whose labile structures can be preserved in such an aqueous environment, however examples of small molecule recovery are more rare, and recent examples are described below. The reports reviewed below were selected as they demonstrate ATPS applications which provide insight into the effects of selected ATPS components (i.e. the composition of the two aqueous phases) and operating parameters on biocompatibility, product partitioning, and selectivity.

The recovery of extracellular cyanobacterial products, beta-carotene and lutein, was investigated as single-step ISPR using an ATPS rather than a downstream extraction process as had been previously proposed. The ATPS composition consisting of polyethylene glycol (PEG)/potassium salts was found to prevent cyanobacterial growth, while a PEG/dextran system allowed growth only at lower dextran molecular weights, attributed to the potential occlusion of aqueous nutrients in a dextran phase with a very high excluded volume at high molecular weight. This effect, arising solely from the molecular weight of an ATPS component, suggests that determining biocompatibility in these systems may be less systematic and more complicated than for solvent-based TPPBs because of the complex interactions with water occurring in both phases, which may cause more direct interactions with the biocatalyst, such that water activity may be an additional criterion in determining ATPS biocompatibility. Biomass partitioning is an important factor in ATPS design, and can provide preliminary separation of the product from the biocatalyst as a subsequent downstream operation. In this particular case, the biomass partitioned to the phase interface, but its presence in the PEG phase required centrifugation prior to product recovery with hexane (Chavez-Santoscoy et al. 2010). While this study extends the applicability of ATPS to valuable small molecule products, it is questionable whether an ATPS system would be

advantageous, given the complications of system tuning for biomass viability, product partitioning, and the requirement for ultimate solvent extraction, while the hydrophobic product would likely partition effectively into a biocompatible organic solvent.

ISPR using surfactant micelles is another ATPS configuration, termed a cloud-point system for its phase separation, which is induced at certain operating conditions (typically at an elevated temperature) rather than being inherently biphasic as with a TPPB. Five amphiphilic copolymers of polyethylene oxide (PEO) and polypropylene oxide (PPO) in varying proportions were investigated as non-ionic surfactants for the extractive fermentation and subsequent cloud-point extraction of n-butanol from model fermentation broth. The n-butanol capacity was very high for the hydrophobic surfactants, although a water-insoluble surfactant was unable to capture any of the n-butanol. Of the five surfactants studied, only the more hydrophobic ones proved to be biocompatible, attributed to the hydrophile-lipophile balance, with the more hydrophilic surfactants exhibiting toxicity. The combined effects of surfactant biocompatibility and n-butanol capacity resulted in only one surfactant improving the n-butanol titer. Micelle formation was ruled out, indicating that n-butanol was associating with free, dissolved surfactant molecules and this was sufficient to reduce its toxicity. n-Butanol was recovered by cloud-point separation of the surfactant at 70°C, demonstrating a n-butanol partition coefficient of 3.5, followed by n-butanol evaporation at elevated temperature (Dhamole et al. 2012). This partition coefficient value is not particularly high relative to other reports of n-butanol ISPR (Barton et al. 1992; Oudshoorn et al. 2009). While the application of a carefully-selected surfactant reduced n-butanol toxicity, it is conceivable that similar improvements (and similar complications) would arise from n-butanol ISPR using a biocompatible solvent having similar affinity, but would offer a simpler operation as an inherently biphasic system.

Two inhibitory molecules sufficiently different in polarity that they would not be effectively extracted together using a hydrophobic liquid prompted the investigation of cloud-point extraction of L-

phenylacetylcarbinol produced from benzaldehyde, an inhibitory substrate, using a hydrophilic nonionic surfactant. This required downstream solvent extraction with an equal volume of butyl acetate, followed by water extraction to separate the product from the surfactant, resulting in high recovery of components and alleviation of substrate/product inhibition (Wang et al. 2010). This configuration was efficient in terms of productivity, yet the requirement for subsequent extraction steps using large amounts of solvent represents an unattractive processing step and expense. Ideally, an extractant could be recovered directly, requiring only a single recovery step prior to re-use, potentially avoiding the direct contact of toxic solvent with the fermentation medium.

For the recovery of intracellular pigments from *Monascus* fungus, the use of an organic solvent (vegetable oil), an ionic liquid, and several surfactants were each compared for their extraction performance in a combined perstraction/cloud-point extraction approach. In this system, the extractant intentionally permeabilized the cell membrane to promote the secretion of intracellular pigments, while micelles formed in the aqueous phase partitioned and concentrated the products separate from the aqueous phase. The use of a surfactant was preferable to immiscible organic solvents, which must compromise extraction efficiency with biocompatibility, while amphiphilic surfactants could fulfill both roles. One surfactant was found to outperform the others in improving productivity by alleviating intracellular inhibition, while the IL failed to promote any pigment secretion. This result was presumed to be due to the IL's potential toxicity, as biocompatibility was evaluated solely based on production. Concerns about widespread toxicity of ILs discussed above are obvious here in comparison to other ISPR materials. Additionally, the production profile was skewed, attributed to the relative polarity of the various pigments affecting their extent of extraction and hence level of inhibition. At high surfactant concentrations the growth morphology became filamentous and productivity dropped, suggesting a possible biocompatibility problem (Hu et al. 2012). This system showed that intracellular products may require additional extractant features, such as a surfactant nature, in order to facilitate secretion and

subsequent extraction. This aspect must be considered simultaneously with the conventional requirements of biocompatibility and product affinity; furthermore, such features may be mutually exclusive, narrowing the array of candidate materials.

Aqueous two-phase systems have been applied to the extraction of small molecules where conventional liquid solvents may not satisfy both biocompatibility and product extraction requirements; however in some cases the motivation to adopt ATPS rather than alternative extraction schemes is not obvious, as simpler operation may be achieved using conventional solvents which are biocompatible. Phase separation at elevated temperature, typically requiring centrifugation of biomass prior to product recovery, is energy-intensive and imposes logistical constraints on process continuity. The relatively complicated nature of selecting the phase-forming components for this extraction approach to meet both general (target molecule affinity, biocompatibility) and system-specific requirements means that such systems may be more suited to the high-value products which are typically extracted using ATPS, rather than commodity products. This is likely why we have been unable to provide any ATPS reports in Table 2-1; similar to ILs, their complexity requires considerable characterization which generally makes ATPS less accessible as a simple ISPR strategy.

2.4 Solid-liquid systems

Solid extractants (particularly as polymers) provide more options for selecting ISPR materials because their complex chemistry and related structure-property relationships enable wider variation in thermodynamic properties, which have an effect on target molecule affinity (e.g. homopolymer/copolymer composition, degree of crystallinity, glass transition temperature, etc.) than liquid extractants while generally also being biocompatible, as well as being non-bioavailable. As solids, they are mechanically stable and do not dissolve in the fermentation medium, making them largely inert with respect to cellular toxicity, and immune to losses in TPPB configurations associated with water-solubility. The nature of solid extractants differs depending on systems' requirements for cost (more on cost later)

and performance, ranging from natural materials such as zeolites and activated charcoal to synthetic macroporous or gel-type resins, and, recently, soft amorphous polymers.

Porous resins are typically hard, glassy solids in order to maintain their porous configuration and surface morphology. Resins may be functionalized with surface chemistry to impart properties such as polarity, and acid / base functionality may be incorporated on the surface of porous materials, or within the bulk in the case of gel-type ion exchange resins.

2.4.1 Adsorbents

Adsorbent resins have become a popular choice of ISPR extractant, typically selected by screening many commercial materials using trial-and-error methods, and subsequently comparing product capacity and selectivity, or similar parameters, in order to select an extractant. That is, there does not appear to have been any attempt to this point, to predict which types of resin adsorbents would be effective from first principles' considerations; rather, observations tend to be made in hindsight. Nevertheless, their relative simplicity of implementation has led to adsorbent resins representing the majority of recent ISPR studies, shown in Table 2-1. Several studies of particular interest are discussed below for their initial insight into extractant selection strategies, which may be useful in guiding selection for future investigations. These reports consider additional process parameters, such as tiers of selectivity for multiple target molecules, sequential ion-exchange resins in series, product/substrate selectivity, or characteristics of the resins which introduce an effect on system operation and performance.

Several aspects of adsorbents, beyond their distinct mechanisms of sorption, distinguish them from absorbents. The performance of adsorbents in fermentation medium is generally lower than in model solutions due to the presence of other solutes which compete for the finite number of surface sorption sites (Mirata et al. 2009; Ranjan et al. 2009). Furthermore, the presence of abundant surface area on macroporous resins provides access for biofilm formation and fouling by cells (Mirata et al.

2009; Wang et al. 2012; Wei et al. 2009). The difference in surface morphology between a macroporous adsorbent resin and an adsorbent polymer is shown in Figure 2-1.

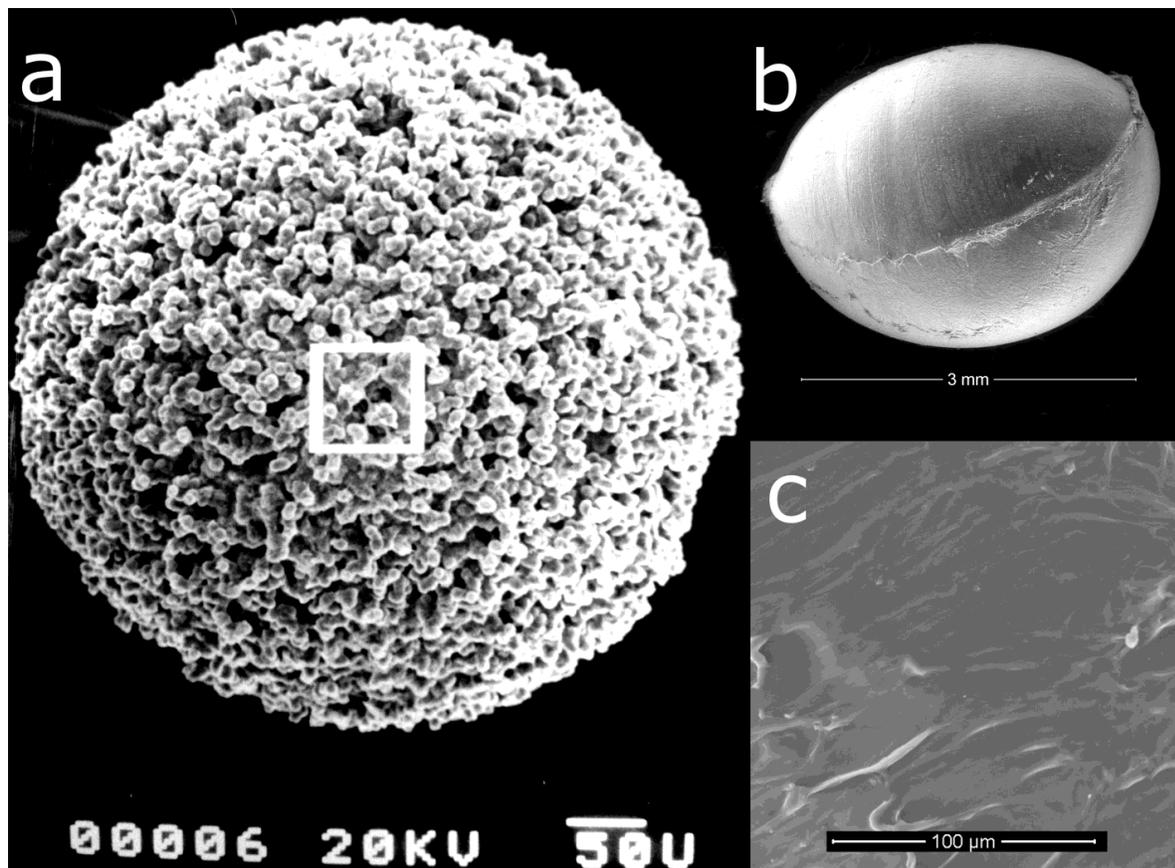


Figure 2-1 Scanning electron micrographs of a) a macroporous adsorbent resin bead (Rohm & Haas Amberlite IRA938) (50 μm scale indicated) (Krug and Daugulis 1983); b) an adsorbent polymer bead (Pebax 2533) (3 mm scale indicated); c) the smooth, monolithic surface of Pebax 2533 (100 μm scale indicated)

Neutral adsorbents such as zeolites, activated charcoal, and non-functionalized resins interact with target molecules through hydrophobic interactions, where affinity and selectivity arise from the solutes' relative affinity between the aqueous and adsorbent phases. Beyond relative hydrophobicity, other adsorbent characteristics can be used to provide selectivity. As an example, in a recent study three hydrophobic zeolites, having different pore sizes selected for the removal of three inhibitory hydrolysate components by size exclusion, enabled the selective, individual removal of three valuable co-products (hydroxymethylfurfural, furfural, and vanillin) which would inhibit ethanol fermentation, while

preserving fermentable sugars (Ranjan et al. 2009). Such selectivity among several small molecules, based on resin pore size being matched to molecular dimensions, is a promising tool for recovery of fermentation products as well.

The extractive fermentation of organic acids is gaining attention to improve process economics for commodity production, yet carboxylic acids typically carry a negative charge at fermentation pH due to their low pK_a values, and will not engage in hydrophobic interactions for product recovery using conventional hydrophobic resins. The extraction of acids in their ionic form may be accomplished using ion-exchange resins which engage in ionic bonding, but must be pre-equilibrated prior to and regenerated after use with salt solutions which pose a problem for waste generation.

The production of perillic acid by limonene oxidation is a suitable system for ion-exchange ISPR, where the carbon source, glycerol, was found to not adsorb, in contrast with alternative, ionic carbon sources, which would be expected to have similar affinity towards the resin as the product itself and could introduce a nutrient limitation. Furthermore, from seven anion-exchange resins which were screened, only two were found to not affect medium pH appreciably, while product affinity was the final resin selection criterion. However, the hydrophobic substrate, limonene, was adsorbed significantly by all resins, signifying a loss of yield on substrate, and necessitating a fed-batch feeding strategy using a limonene-saturated air stream and limonene-saturated resin (Mirata et al. 2009). In this case, the extractant was secondary to the selection of carbon source in terms of process viability, yet the substrate, which cannot be similarly substituted, imposed a limitation on yield by its extensive retention.

Facing a similar challenge, an ISPR strategy designed to overcome inhibition from (R)-(-)-mandelic acid sought a resin which had high product selectivity relative to the substrate, (R,S)-mandelonitrile, to ensure the substrate was supplied without limitation. The authors determined that strong base functionality provided the best affinity towards the acid product, while gel-type architecture provided greater selectivity than porous architectures (Xue et al. 2010). These insights, considering

properties of polymer architecture that relate to affinity, may help improve adsorbent affinity and selectivity in future studies.

The use of adsorbent and ion-exchange resins for product recovery is effective for hydrophobic compounds, and target molecules which carry a charge at fermentation pH, and these systems are well-represented in Table 2-1, indicating their straightforward implementation. However, recovery of strongly-adsorbed molecules often requires heating or concentrated salt solutions to regenerate the extractant, which introduce significant energy and waste stream demands. The literature indicates that trial-and-error is still widely employed in selection to characterize adsorbent properties, and a compromise among several selection criteria will usually be necessary. As noted, few attempts have been made to approach the rational selection of ISPR resins *via* first principles considerations, which will hamper progress in expanding their application.

2.4.2 Absorbents

A distinct class of materials, soft (having a low glass transition temperature), amorphous (lacking significant crystallinity) polymers, has been used as an alternative to the above-mentioned adsorbents, primarily in the authors' Group, as "solid solvents" because they operate by passive solution and diffusion of the target molecule within the polymer structure itself, a distinct mechanism from surface adsorption or strong ionic interactions seen with conventional resins. An important aspect of absorptive ISPR is the rate of target molecule extraction relative to the rate of biocatalytic production, which is governed by the diffusivity of the target molecule within the polymer, and should be sufficient to keep pace with the biotransformation rate. If mass transfer rate was limiting, the diffusive path length could be reduced by decreasing the size of absorbent polymer particles, thereby improving the overall mass transfer rate (Fam and Daugulis 2012). This kinetic aspect of absorbent ISPR has received less attention than polymer-target molecule affinity at equilibrium, possibly because it has only rarely been identified as a problem (Rehmann and Daugulis 2007); this aspect is a focus of ongoing work in our Group.

In contrast to surface-area-based adsorbents, the performance of absorbents in fermentation medium is generally higher than in model solutions due to the presence of additional solutes in the fermentation medium, thereby acting to increase the target molecule's activity in the aqueous phase, resulting in a higher proportion in the extractant at equilibrium, similar to what is seen with liquid extractants (Dafoe and Daugulis 2013b). The effect of competition for site occupation, as seen with adsorbents, does not occur because the absorption mechanism permits internal permeation and swelling of the polymer, giving a solute capacity which can vary depending on the solute's compatibility with the polymer (Parent et al. 2012). This results in typically constant distribution ratios across the concentration ranges of interest. Since the entire amount of amorphous polymer is used to sorb target molecules, it is the mass of the polymer, rather than the surface area available, that determines overall extent of uptake with these materials. This fundamental difference in uptake mechanism was confirmed by changing the specific surface area of fixed masses of absorbent polymer beads, and demonstrating that the absorption capacity remained unchanged, meaning that these materials' performance is mass, not surface area, dependent (Craig and Daugulis 2012). The lack of macroporous architecture in these materials also means that a relatively small surface area is exposed to the fermentation culture, such that cell attachment has not been observed, in contrast to many reports of resins and solvents becoming fouled by entrapped cells, preventing their re-use.

Although many earlier successful examples of soft-polymer ISPR systems have now been published (some recent examples are provided in Table 2-1), the selection of such polymers has historically been *via* trial-and-error, substantially as has been the case for hard resin testing and selection. Recently, however, the selection of soft polymers for absorptive extraction has been examined from a first-principles' perspective, where the polymers were treated as solvents in judging the relative affinity towards target molecules through the use of solubility parameters, a useful tool

which moves polymer selection from a heuristic approach towards rational selection (Parent et al. 2012).

Absorbent polymers have been utilized for selective product removal, and also for simultaneous substrate delivery and product removal, and recent examples demonstrating their utility are given in Table 2-1. Absorbed molecules are easily recovered by contacting the polymers with a solvent which typically has a much higher affinity for the target molecule than water, requiring a relatively small volume for complete recovery and regeneration of the polymer. Alternatively, volatile products could be recovered by heating the polymer, avoiding the use of solvents altogether and providing significant energy savings.

Absorbent polymers exhibit similar overall trends to other extractants with respect to hydrophilic target molecules partitioning modestly, because affinity for water decreases the extent of extraction. However, the availability of hydrophilic polymers can somewhat address this shortfall, while solvent extractants are necessarily strictly hydrophobic. Block copolymers containing hydrophilic chain segments may be stabilized against dissolving in water by incorporating hard, glassy or crystalline segments within the polymer chain. In this way, the biocompatibility requirement of the relatively water-soluble polymer extractant is circumvented because it remains in a separate, solid, phase. This feature of absorbent copolymers is currently the focus of more in-depth investigation in our Group as a potentially useful tool for target molecule selectivity.

Such hydrophilic block copolymers exhibit greater affinity towards relatively hydrophilic target molecules which would be impossible to extract using hydrophobic solvents, as was demonstrated for the simultaneous removal of a relatively polar pharmaceutical intermediate, *cis*-(1*S*,2*R*)-indandiol, and its toxic by-product, 1-indenol, both of which a hydrophobic liquid solvent (silicone oil) was unable to extract (Dafoe and Daugulis 2011). Additionally, less-polar target molecules had higher affinity towards the hydrophobic polymers, while the more polar product had preferential affinity towards a hydrophilic

polymer relative to hydrophobic ones, resulting in different biocatalytic production profiles as a result of preferential removal by each polymer (Dafoe and Daugulis 2013b). The extent of water uptake by hydrophilic polymers must be carefully considered from a selectivity standpoint, to avoid the co-extraction of other water-soluble medium components (Dafoe and Daugulis 2013a). Also, when comparing target molecule affinity, polymers with different water uptake levels must be analyzed on an equal mass basis, and should be in equilibrium with water prior to measuring target molecule partitioning to avoid simply transferring aqueous volume and skewing partition coefficients.

Being commercially-produced materials on a commodity scale, these absorbent polymers cost much less than either specialized resins, ionic liquids, or biocompatible solvents (Quijano et al. 2010), typically in the range of \$5-7 per kg. Despite their ease of use and good performance, polymer selection remains in a less-developed state than selection schemes for solvents due to the complex structure-property relationships with polymers. The advantages of soft polymers relative to other extractants has motivated a more thorough understanding of the interactions between the polymer and the target molecule, as well as other fermentation components such as water, to enable more effective polymer selection, and such thermodynamic approaches are currently underway (Poleo and Daugulis 2013). The number of reports demonstrating the straightforward application of soft absorptive polymer ISPR to diverse biocatalytic systems, shown in Table 2-1, indicates that this strategy is both effective and simple to implement.

2.5 Other systems

2.5.1 Reactive extraction

In order to overcome the often modest distribution coefficients which impede the effective extraction of relatively hydrophilic products, the inclusion of a reactive extractant, a species with functionality complementary to a target molecule's reactive group, typically as a component in an immiscible organic phase solution, can improve uptake by stoichiometrically binding the product in the

extractant phase, enhancing its solubility and providing a high driving force for removal. The reactive mechanism ensures that only compounds with complementary functionality will interact, providing a basis for selectivity among target molecules with different functionalities. However, the covalently-bound complex may subsequently require increased energy and material inputs for ultimate product recovery. The following reports demonstrate recent advances in reactive extraction, which include imparting reactive functionality to a solid-phase support, using a blended extraction mechanism for a wider target molecule profile, or using commercial materials to guide the synthesis of effective reactive extractant solvents. The system-specific considerations required in conceiving a reactive extraction scheme has limited the breadth of straightforward adoption; only a single application appears in Table 2-1.

A reversible binding process was sought for the removal and recovery of 3-hydroxypropionaldehyde (3-HPA), a toxic product which is non-volatile, hydrophilic, and heat-sensitive, and for which conventional extractants fail or perform poorly. By binding reactive sulfite ligands to a chloride-functionalized ion-exchange resin, a 3-HPA-sulfite adduct could be formed on the resin surface. The presence of the modified resin had a negative effect on cell growth and viability, however, as up to 35 mol% of bound 3-HPA leaked out of the resin as the adduct. Such cytotoxicity has often been reported for the use of reactive extractants as ISPR materials. Furthermore, the adduct was eluted rather than the free product and required several complex steps for product recovery. This represented significant additional processing expense (Ruetti et al. 2011). The modification of resins for reactive, reversible product binding is an example of designing complementary functionality towards target molecule into the extractant, however the viability suffers from adduct leakage from the reactive ligand.

For the detoxification of biomass hydrolysate intended for the production of ethanol, reactive extraction with trioctylamine was examined for the removal of five inhibitors (formic acid, acetic acid,

levulinic acid, 5-hydroxymethylfurfural, and tetrahydrofuran) from a model solution using solvents containing trioctylamine (Jeong et al. 2013). n-Octanol was the most appropriate diluent compared to alkanes of various chain lengths, as it was speculated that solvent polarity owing to its hydroxyl functionality would promote solubility of the polar amine complexes relative to the non-polar alkanes. For each target molecule, the extent of extraction depended on its relative affinity towards the aqueous phase, with only 2-4% of formic acid removed. The extraction of acids was found to occur via reactive extraction, while extraction of the small neutral species was found to occur via non-reactive solvent extraction in the organic phase, which was improved by the addition of 10% kerosene as a non-polar diluent. Despite being a pretreatment step rather than a classical example of ISPR, this strategy provides insight for ISPR applications which use reactive extraction for multiple target molecules, showing that a compromise in extraction performance across all targets must be reached by adjusting extractant composition.

As trioctylamine (TOA) has long been the benchmark extractant for carboxylic acids, there may be potential to improve on its binding capacity using rational means. A recent study examined several commercial functionalized silica compounds in order to design a strategy to synthesize novel liquid extractants for lactic acid (Leeman et al. 2013). Based on results of carboxylic acid affinity for the functionalized silicas, an extractant synthesized with dimethylaminopyridine functionality for extraction and tridecyl alkyl chains for hydrophobicity was found to significantly exceed the capacity of TOA at all temperatures and lactic acid concentrations; however, being a solid, its maximum loading in the diluent n-octanol was only 20%. Two other extractant candidates were found to increase pH and form emulsions in water upon complexation with lactic acid, suggesting that these complexes were soluble in the aqueous phase and were leaching adsorbed lactic acid. Although only one candidate met the process requirements and had less than optimal capacity due to its solid state, the use of readily-available commercial materials to guide synthesis of extractant candidates is a rapid and cost-effective

approach to extractant design and synthesis, and proved to be an effective strategy in outperforming the current benchmark extractant, trioctylamine.

Reactive extraction demonstrates its greatest potential in the removal of reactive, hydrophilic molecules which are difficult to extract using conventional means; however, reactive extractants are often non-biocompatible and additional steps must be taken for their implementation without harming the biocatalyst. The additional affinity towards the extractant makes the product equally more difficult to recover, which should also be considered in evaluating process viability.

2.5.2 Combined systems

The establishment of several distinct ISPR categories described above has led to recent examples combining different approaches in order to exploit the advantages, or to reduce the drawbacks, of individual methods. Consequently, these combined systems have increased complexity, and due to their additional, system-specific considerations, reports of straightforward applications do not appear Table 2-1. These strategies strive to improve on limitations inherent in established processes, such as mass-transfer rate, or to separate the potentially adverse interactions between extractant and cells in the fermentation medium. In all cases, continuous extraction is preserved through the use of membranes in conjunction with conventional solvent extraction, or by using alternative, biologically inert modes of extraction such as gas stripping or eletrodeionization.

To circumvent solvent toxicity, the use of hollow-fiber membranes to physically separate the aqueous and extractant phases reduced the requirement for strict biocompatibility, allowing cytotoxic pentane to be used for its volatility to aid in the recovery of 1-phenylethanol. This configuration required a compromise between extraction efficiency and biocompatibility. Less hydrophobic solvents which were non-biocompatible, but which possessed higher solute affinity, were prone to leak through the membrane into the aqueous phase, while more hydrophobic, biocompatible solvents with higher affinity could dissolve the membrane itself (Mihal et al. 2012). As noted, the chosen solvent (pentane)

was the compromise solvent balancing solute affinity with membrane compatibility. The addition of a membrane enabled the use of a non-biocompatible solvent, but introduced additional factors (i.e. membrane compatibility and leaching) which required specific considerations during process design. Nevertheless, this configuration performed well over multiple extractant re-use cycles, and was well-predicted by a mathematical model for future applications.

Using a porous poly(tetrafluoroethylene) (PTFE) membrane to separate the extractant from the fermentation medium enabled the use of 1-dodecanol, a solvent which was non-biocompatible towards the organism, *Clostridium saccharoperbutylacetonicum* strain N1-4, for the extraction of n-butanol. Dodecanol out-performed the benchmark biocompatible solvent, oleyl alcohol, in this configuration due to its higher partition coefficient. Furthermore, the porous PTFE membrane enabled 20-fold greater n-butanol productivity per specific membrane area than a previous report using a monolithic silicone membrane, indicating that the membrane surface area could be significantly reduced with this configuration (Tanaka et al. 2012). The remarkable improvement in specific productivity resulting from simply replacing the membrane material indicates that there is potential for subsequent improvements using a rational material selection approach, however the different mechanisms of membrane permeation, either sorption and diffusion or conduction through pores, means that a comprehensive selection strategy may not be available as with soft polymers, which all operate *via* absorption.

The use of a silicone membrane contacting the fermentation medium with a vacuum on one side was used to remove, and subsequently condense, volatile products from the acetone-n-butanol-ethanol fermentation. The mechanism of membrane permeation was found to follow the sorption and diffusion mechanism, enabling characterization with a mathematical model. n-Butanol mass-transfer coefficients across the membrane were found to decrease upon increasing solution complexity due to reduced solute activity, while fouling was apparently not a significant problem (Li et al. 2011b). This pervaporation configuration enabled higher and more rapid n-butanol production with continuous

extraction, without requiring solvent recycling; however, the amount of co-extracted water greatly exceeded the amount of n-butanol, producing a dilute extract. This demonstrates that the introduction of vacuum in combination with the membrane imposes an additional degree of complexity, and membrane material selection efforts under this constraint may be necessary to maximize product selectivity.

The use of a liquid membrane consisting of trioctylamine dissolved in dichloromethane at varying concentrations, held in a U-shaped cell separating the feed aqueous phase from a solution of NaOH for back-extraction, was investigated to improve the separation of succinic acid from model fermentation medium which additionally contained acetic and formic acid. Please refer to the article for further information on the apparatus employed here (Galaction et al. 2013). The acid co-products were extracted sequentially into TOA based on their relative acidity, and TOA concentrations beyond those which could stoichiometrically bind the smaller acids were required for succinic acid extraction. In addition to suffering from diffusion limitation through the liquid membrane, reactive extraction combined with membrane permeation also introduces the kinetic limitation of complex formation and subsequent re-extraction into the extractant phase. The results of this study suggest that combining reactive extraction with membrane permeation may be favorable to selectively remove other acid by-products, leaving succinic acid, with the most favorable selectivity in this regard occurring with the highest pH gradient between the feed and extractant phases and at a relatively low TOA concentration in dichloromethane (Galaction et al. 2013). A high extraction capacity was provided by the back-extraction step in this configuration; however a final separation step for recovery of succinic acid, the desired product, would be required.

The accumulation of n-butanol has been addressed with intermittent gas stripping in order to design an economical process with few steps. Relatively high aqueous n-butanol titers above 8 g L^{-1} were tolerated by an immobilized, solvent-tolerant *Clostridium* strain in repeated batch cultures, which

increased the driving force for mass transfer to the stripping gas and greatly improved n-butanol recovery. Collection and condensation of the gas stream yielded a 15% w/v n-butanol aqueous solution which spontaneously phase-separated into an organic phase containing >60% w/v n-butanol, 4% w/v acetone, 1% w/v ethanol, with the remainder presumably being water, a very effective first extraction step (Xue et al. 2012). The presence of free cells was found to negatively affect stripping efficiency, such that the immobilization technique may likely be necessary for both continuous cultivation and product recovery. This study has shown that improvements to biocatalyst solvent tolerance open new options for ISPR which can take advantage of more intensive production, but also impose new considerations for process configuration.

In a continuous adsorption/desorption process, a membrane bioreactor with ion-selective membranes was packed with a mixture of acid and base ion-exchange resins, and a 15V DC field was applied across the fermentation medium. This configuration continually removed adsorbed lactate from the resin which accumulated in a membrane-separated phase through electrodeionization (Boontawan et al. 2011). Cell deactivation by lactate exposure was reduced 5-fold, and fed-batch operation was maintained for ten days. The coupling of resin adsorption and electrodeionization permitted the use of a relatively small amount of resin which could be chosen mainly based on its selectivity, as capacity was not a limiting factor in this configuration.

A similar electroextractive configuration was used for the removal of inhibitory organic acids in the production of H₂ by separating the cell-containing vessel from an electrolyte solution using a combination of ion-selective membranes. Electrokinetic removal of organic acids successfully regulated culture pH and alleviated inhibition, while H₂ was measured in the off-gas. The culture continued for 25 days until ethanol accumulation was presumed to become inhibitory as it was not removed by electrodeionization (Redwood et al. 2012). The above examples of electrodeionization demonstrate efficient removal of charged species, at the expense of system complexity and accumulation of neutral

inhibitors, which may require additional, combined extraction approaches. Nevertheless, the use of a mild electric current for the removal of ions through selectively-permeable membranes is a noninvasive, continuous strategy which could be applied to many fermentation systems.

Careful consideration of a particular system's requirements is necessary when combining different ISPR strategies in order to achieve improvements, and these configurations are not "one-size-fits-all" solutions due to their complexity; therefore there are no examples of combined ISPR systems contained in Table 2-1. In the above examples, combined approaches were conceived in order to counteract a drawback arising from a property of a particular system (e.g. biocompatibility problems); however, additional complexity and drawbacks are inevitably introduced. The degree of complexity permissible depends on the value of the product, and development of combined ISPR processes may be worthwhile in cases where biocatalysis is an attractive route for production of a valuable product and conventional ISPR strategies are ineffective.

2.6 Conclusions and future directions

The shape of recent literature discussing ISPR indicates that the field is expanding in both breadth, through an increase in the number of applications, and depth, through advances in technical know-how and constantly improving benchmarks. Emphasis on improving extractant capacity and selectivity will intensify processes without requiring dramatic alterations to equipment or biocatalysts. Nevertheless, expected improvements to engineered biocatalysts in the areas of enzyme selectivity and tolerance to inhibitory molecules will also benefit from better extractant performance in more intense processes. While a highly-selective extractant with a low capacity would suit a continuous process, many bioprocesses operate in batch mode for logistical reasons, and a high capacity enables simpler batch operation with less frequent turnaround.

Cost is an important factor in extractant selection, especially for high-volume, low-cost products. The use of exotic, task-specific materials or complex process configurations must improve

performance sufficiently to justify the inevitable increase in cost. Consideration of all relevant extractant properties in order to arrive at a rational choice for a particular system remains a challenge; one must consider the basic requirements (affinity, biocompatibility, and non-bioavailability) simultaneously with system-specific ones (e.g. operability concerns or the ultimate fate of the product) and system complexity introduces additional considerations. Understanding the interactions which govern target molecule-extractant affinity is of utmost importance for any class of extractant to gain widespread adoption, and recent strides have been made in this regard in the case of absorbent polymers; although promising, ionic liquids currently appear to lack systematic approaches. With the above aspects considered, it is our opinion that soft, absorbent polymers are an attractive yet under-recognized class of ISPR materials having advantages over all alternatives in the areas discussed: biocompatibility, non-bioavailability, phase-stability, cost, simplicity of implementation, and affinity towards a wide range of target molecules. We further believe that these are the most promising materials for many future ISPR applications.

2.7 References

- Anvari M, Khayati G (2009) In situ recovery of 2, 3-butanediol from fermentation by liquid–liquid extraction. *J Ind Microbiol Biotechnol* 36:313-317
- Barton WE, Daugulis AJ (1992) Evaluation of solvents for extractive n-butanol fermentation with *Clostridium acetobutylicum* and the use of poly (propylene glycol) 1200. *Appl Microbiol Biotechnol* 36:632-639
- Boghigian BA, Myint M, Wu J, Pfeifer BA (2011) Simultaneous production and partitioning of heterologous polyketide and isoprenoid natural products in an *Escherichia coli* two-phase bioprocess. *J Ind Microbiol Biotechnol* 38:1809-1820
- Boontawan P, Kanchanathawee S, Boontawan A (2011) Extractive fermentation of l-()-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. *Biochem Eng J* 54:192-199
- Brennan TC, Turner CD, Krömer JO, Nielsen LK (2012) Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 109:2513-2522

- Chavez-Santoscoy A, Benavides J, Vermaas W, Rito-Palomares M (2010) Application of Aqueous Two-Phase Systems for the Potential Extractive Fermentation of Cyanobacterial Products. *Chem Eng Technol* 33:177-182
- Choi JM, Yeom SH (2011) Simulation study of biobutanol production in a polymer-loaded two-phase partitioning bioreactor (PL-TPPB): Model development. *Korean J Chem Eng* 28:2170-2177
- Craig T, Daugulis AJ (2012) Polymer characterization and optimization of conditions for the enhanced bioproduction of benzaldehyde by *Pichia pastoris* in a two-; phase partitioning bioreactor. *Biotechnol Bioeng* 110:1098-1105
- Dafoe JTS, Daugulis AJ (2011) Bioproduction of *cis*-(1S, 2R)-indandiol, a chiral pharmaceutical intermediate, using a solid–liquid two-phase partitioning bioreactor for enhanced removal of inhibitors. *J Chem Tehcnol Biot* 86:1379-1385
- Dafoe JT, Daugulis AJ (2013a) Production of 4-valerolactone by an equilibrium-limited transformation in a partitioning bioreactor: impact of absorptive polymer properties. *Bioproc Biosyst Eng* doi: 10.1007/s00449-013-1020-5
- Dafoe JT, Daugulis AJ (2013b) Manipulating the Composition of Absorbent Polymers Affects Product and By-product Concentration Profiles in the Biphasic Biotransformation of Indene to *cis*-1, 2-indandiol. *Biochem Eng J* 77:7-14
- Dhamole PB, Wang Z, Liu Y, Wang B, Feng H (2012) Extractive fermentation with non-ionic surfactants to enhance n-butanol production. *Biomass Bioenergy* 40:112-119
- Galaction A, Poștaru M, Cașcaval D, Kloetzer L (2013) Selective Separation of Carboxylic Acids Obtained by Succinic Acid Fermentation Using Facilitated Pertraction. *Solvent Extr Ion Exch* 31:171-183
- Gao F, Daugulis AJ (2009) Bioproduction of the aroma compound 2-Phenylethanol in a solid–liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. *Biotechnol Bioeng* 104:332-339
- Gao MT, Shimamura T, Ishida N, Takahashi H (2011) pH-uncontrolled lactic acid fermentation with activated carbon as an adsorbent. *Enzyme Microb Technol* 48:526-530
- Garcia-Chavez LY, Garsia CM, Schuur B, de Haan AB (2013) Biobutanol Recovery Using Non-Fluorinated Task Specific Ionic Liquids (TSILs). *Ind Eng Chem Res* 52:4902-4910
- Garikipati SJ, McIver AM, Peeples TL (2009) Whole-cell biocatalysis for 1-naphthol production in liquid-liquid biphasic systems. *Appl Environ Microbiol* 75:6545-6552
- Grady MC, Jahic M, Patnaik R (2010) Method for producing n-butanol using two-phase extractive fermentation. Patent application US 20100221802 A1
- Hu Z, Zhang X, Wu Z, Qi H, Wang Z (2012) Perstraction of intracellular pigments by submerged cultivation of *Monascus* in nonionic surfactant micelle aqueous solution. *Appl Microbiol Biotechnol* 94:81-89

- Hu Q, Xu Y, Nie Y (2010) Enhancement of *Candida parapsilosis* catalyzing deracemization of (R, S)-1-phenyl-1, 2-ethanediol to its (S)-enantiomer by a highly productive “two-in-one” resin-based in situ product removal strategy. *Bioresour Technol* 101:8461-8463
- Hua D, Lin S, Li Y, Chen H, Zhang Z, Du Y, Zhang X, Xu P (2010) Enhanced 2-phenylethanol production from L-phenylalanine via in situ product adsorption. *Biocatalysis Biotransformation* 28:259-266
- Jain AN, Khan TR, Daugulis AJ (2010) Bioproduction of benzaldehyde in a solid–liquid two-phase partitioning bioreactor using *Pichia pastoris*. *Biotechnol Lett* 32:1649-1654
- Jeon BS, Moon C, Kim B, Kim H, Um Y, Sang B (2013) In-situ extractive fermentation for the production of hexanoic acid from galactitol by *Clostridium* sp. BS-1. *Enzyme Microb Technol*
- Jeong G, Kim S, Park D (2013) Detoxification of hydrolysate by reactive-extraction for generating biofuels. 18:88-93
- Jin H, Liu Z, Hu Z, Zheng Y (2013) Production of (R)-epichlorohydrin from 1, 3-dichloro-2-propanol by two-step biocatalysis using haloalcohol dehalogenase and epoxide hydrolase in two-phase system. *Biochem Eng J* 74:1-7
- Julsing MK, Kuhn D, Schmid A, Bühler B (2012) Resting cells of recombinant *E. coli* show high epoxidation yields on energy source and high sensitivity to product inhibition. *Biotechnol Bioeng* 109:1109-1119
- Jung D, Choi W, Choi K, Jung E, Yun H, Kazlauskas RJ, Kim B (2013) Bioconversion of p-coumaric acid to p-hydroxystyrene using phenolic acid decarboxylase from *B. amyloliquefaciens* in biphasic reaction system. *Appl Microbiol Biotechnol* 97:1501-1511
- Keasler SJ, Lewin JL, Schultz NE, Ross RB, Gryska NM, Nakamura M, Siepmann JI (2013) Molecular insights for the optimization of solvent-based selective extraction of ethanol from fermentation broths. *AIChE J* 59:3065-3070
- Khan TR, Daugulis AJ (2010) Application of solid–liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. *Biotechnol Bioeng* 107:633-641
- Leeman M, Vosseveld F, Visser TJ, Schuur B, de Haan AB (2013) Novel extractants for the recovery of fermentation derived lactic acid. *Sep Purif Technol* 111:82-89
- Li Q, Cai H, Hao B, Zhang C, Yu Z, Zhou S, Chenjuan L (2010) Enhancing Clostridial Acetone-butanol-Ethanol (ABE) Production and Improving Fuel Properties of ABE-enriched Biodiesel by Extractive Fermentation with Biodiesel. *Appl Biochem Biotechnol* 162:2381-2386
- Li Q, Wang D, Hu G, Xing J, Su Z (2011a) Integrated bioprocess for high-efficiency production of succinic acid in an expanded-bed adsorption system. *Biochem Eng J* 56:150-157
- Li SY, Srivastava R, Parnas RS (2011b) Study of in situ 1-butanol pervaporation from A-B-E fermentation using a PDMS composite membrane: Validity of solution-diffusion model for pervaporative A-B-E fermentation. *Biotechnol Prog* 27:111-120

- Liu S, Wu Q, Zhang J, Mo S (2011) Production of epsilon-poly-L-lysine by *Streptomyces* sp using resin-based, *in situ* product removal. *Biotechnol Lett* 33:1581-1585
- Mao S, Hua B, Wang N, HU X, Ge Z, Li Y, Liu S, Lu F (2012) 11 α hydroxylation of 16 α , 17-epoxyprogesterone in biphasic ionic liquid/water system by *Aspergillus ochraceus*. *J Chem Technol Biot* 88:287-292
- Mei J, Min H, Lü Z (2009) Enhanced biotransformation of L-phenylalanine to 2-phenylethanol using an *in situ* product adsorption technique. *Process Biochem* 44:886-890
- Mihal M, Markoš J, Annus J, Štefuca V (2012) Intensification of 1-phenylethanol production by periodical membrane extraction of the product from fermentation broth. *J Chem Technol Biot* 87:1017-1026
- Mirata MA, Heerd D, Schrader J (2009) Integrated bioprocess for the oxidation of limonene to perillic acid with *Pseudomonas putida* DSM 12264. *Process Biochem* 44:764-771
- Muñoz R, Daugulis AJ, Hernández M, Quijano G (2012) Recent advances in two-phase partitioning bioreactors for the treatment of volatile organic compounds. *Biotechnol Adv* 30:1707-1720
- Neves CMSS, Granjo JFO, Freire MG, Robertson A, Oliveira NMC, Coutinho JAP (2011) Separation of ethanol–water mixtures by liquid–liquid extraction using phosphonium-based ionic liquids. *Green Chem* 13:1517-1526
- Nielsen DR, Amarasiriwardena GS, Prather KLJ (2010) Predicting the adsorption of second-generation biofuels by polymeric resins with applications for *in-situ* product recovery (ISPR). *Bioresource Technol* 101:2762-2769
- Oudshoorn A, van der Wielen LAM, Straathof AJJ (2009) Assessment of Options for Selective 1-butanol Recovery from Aqueous Solution. *Ind Eng Chem Res* 48:7325-7336
- Pahlavanzadeh H, Khayati G, Vasheghani-Farahani E, Ghaemi N (2009) Extractive Capacity of Oleyl Alcohol on 2, 3-Butanediol Production in Fermentation Process with Use of *Klebsiella pneumoniae* PTCC 1290. *Iran J Chem Chem Eng -Int Engl Ed* 28:103-109
- Parent JS, Capela M, Dafoe JT, Daugulis AJ (2012) A first principles approach to identifying polymers for use in two-phase partitioning bioreactors. *J Chem Technol Biot* 87:1059-1065
- Poleo EE, Daugulis AJ (2013) A comparison of three first principles methods for predicting solute–polymer affinity, and the simultaneous biodegradation of phenol and butyl acetate in a two-phase partitioning bioreactor. *J Chem Technol Biot* doi:10.1002/jctb.4116
- Quijano G, Hernandez M, Villaverde S, Thalasso F, Muñoz R (2010) A step-forward in the characterization and potential applications of solid and liquid oxygen transfer vectors. *Appl Microbiol Biotechnol* 85:543-551
- Ranjan R, Thust S, Gounaris CE, Woo M, Floudas CA, Keitz Mv, Valentas KJ, Wei J, Tsapatsis M (2009) Adsorption of fermentation inhibitors from lignocellulosic biomass hydrolyzates for improved ethanol yield and value-added product recovery. *Micropor Mesopor Mat* 122:143-148

- Redwood MD, Orozco RL, Majewski AJ, Macaskie LE (2012) Electro-extractive fermentation for efficient biohydrogen production. *Bioresour Technol* 107:166-174
- Rong S, Ding B, Zhang X, Zheng X, Wang Y (2011) Enhanced Biotransformation of 2-Phenylethanol with Ethanol Oxidation in a Solid-Liquid Two-Phase System by Active Dry Yeast. *Curr Microbiol* 63:503-509
- Ruetti DP, Lacroix C, Jeremic T, Mathis M, Die A, Vollenweider S (2011) Development of a reversible binding process for in situ removal of 3-hydroxypropionaldehyde during biotechnological conversion of glycerol. *Biochem Eng J* 55:176-184
- Tanaka S, Tashiro Y, Kobayashi G, Ikegami T, Negishi H, Sakaki K (2012) Membrane-assisted extractive butanol fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 with 1-dodecanol as the extractant. *Bioresour Technol* 116:448-452
- van den Berg C, Heeres AS, van der Wielen LA, Straathof AJ (2013) Simultaneous clostridial fermentation, lipase-catalyzed esterification, and ester extraction to enrich diesel with butyl butyrate. *Biotechnol Bioeng* 110:137-142
- Verhoef S, Wierckx N, Westerhof R, De Winde JH, Ruijsenaars HJ (2009) Bioproduction of p-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. *Appl Environ Microbiol* 75:931-936
- Wang H, Dong Q, Guan A, Meng C, Shi X, Guo Y (2011) Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. *J Biosci Bioeng* 112:26-31
- Wang P, Wang Y, Su Z (2012) Microbial Production of Propionic Acid with *Propionibacterium freudenreichii* Using an Anion Exchanger-Based In Situ Product Recovery (ISPR) Process with Direct and Indirect Contact of Cells. *Appl Biochem Biotechnol* 166:1-13
- Wang H, Dong Q, Meng C, Guo Y (2011) A continuous and adsorptive bioprocess for efficient production of the natural aroma chemical 2-phenylethanol with yeast. *Enzyme Microb Technol* 48:404-407
- Wang P, Wang Y, Liu Y, Shi H, Su Z (2012) Novel in situ product removal technique for simultaneous production of propionic acid and vitamin B12 by expanded bed adsorption bioreactor. *Bioresour Technol* 104:652-659
- Wang Z, Liang R, Xu J, Liu Y, Qi H (2010) A Closed Concept of Extractive Whole Cell Microbial Transformation of Benzaldehyde into l-Phenylacetylcarbinol by *Saccharomyces cerevisiae* in Novel Polyethylene-Glycol-Induced Cloud-Point System. *Appl Biochem Biotechnol* 160:1865-1877
- Wei G, Yang X, Zhou W, Lin J, Wei D (2009) Adsorptive bioconversion of ethylene glycol to glycolic acid by *Gluconobacter oxydans* DSM 2003. *Biochem Eng J* 47:127-131
- Xue C, Zhao J, Lu C, Yang S, Bai F, Tang I (2012) High-titer n-butanol production by *Clostridium acetobutylicum* JB200 in fed-batch fermentation with intermittent gas stripping. *Biotechnol Bioeng* 109:2746-2756

- Xue Y, Liu Z, Xu M, Wang Y, Zheng Y, Shen Y (2010) Enhanced biotransformation of (R, S)-mandelonitrile to (R)-(-)-mandelic acid with in situ production removal by addition of resin. *Biochem Eng J* 53:143-149
- Zou S, Du E, Hu Z, Zheng Y (2013) Enhanced biotransformation of 1, 3-dichloro-2-propanol to epichlorohydrin via resin-based in situ product removal process. *Biotechnol Lett* 1-6
- Zúñiga C, Morales M, Le Borgne S, Revah S (2011) Production of poly- β -hydroxybutyrate (PHB) by *Methylobacterium organophilum* isolated from a methanotrophic consortium in a two-phase partition bioreactor. *J Hazard Mater* 190:876-882

Chapter 3 Bioproduction of *cis*-(1S,2R)-indandiol, a chiral pharmaceutical intermediate, using a solid-liquid two-phase partitioning bioreactor (TPPB) for enhanced removal of inhibitors

Julian T. Dafoe, Andrew J. Daugulis

With minor changes to fulfill formatting requirements, this chapter is substantially as it appears in:
Journal of Chemical Technology and Biotechnology 85(11):1379-1385 (2011).

3.1 Preface to Chapter 3

This chapter is a preliminary investigation into the application of an absorbent polymer sequestering phase for the simultaneous supply of substrate and the removal of inhibitory compounds from the biotransformation of indene to *cis*-(1*S*,2*R*)-indandiol, a chiral pharmaceutical intermediate in the production of the HIV drug, Crixivan® by Merck & Co. Extraction using polymers is a novel and advantageous approach compared to the original extraction process using liquid silicone oil, which not only imposed operational and analytical problems due to emulsion, but was only able to supply the substrate, and exhibited no uptake of either the major inhibitor, 1-indenol, nor the desired product, *cis*-(1*S*,2*R*)-indandiol. The use of an absorbent polymer, Hytrel® 8206, selected by screening several polymers for target molecule affinity, alleviated operational problems while providing slightly higher overall productivity than silicone oil, likely due its superior removal of inhibitors. The different extents of extraction of the various fermentation products between single phase, two-liquid, and solid-liquid operation resulted in different metabolic profiles, indicating that polymer-target molecule affinity is an important aspect affecting the outcome of the fermentation. Finally, the polymer's affinity towards the desired product enabled preliminary separation for partial recovery, which may be improved using a polymer with a higher partition coefficient.

3.2 Abstract

BACKGROUND: A solid-liquid two-phase partitioning bioreactor (TPPB) was used in the biotransformation of indene to *cis*-(1*S*,2*R*)-indandiol by *Pseudomonas putida* 421-5 (ATCC 55687). Metered substrate feeding in single-phase operation, or delivery from an immiscible liquid, have been previously employed to regulate the exposure of the biocatalyst to inhibitory concentrations of the substrate. In contrast, the solid-liquid platform provided in-situ substrate addition (ISSA) as well as simultaneous in-situ product removal (ISPR) as a means of overcoming substrate and product toxicity. Three different modes of operation were compared for their effects on the performance of this biotransformation: Single-phase, fed-batch operation was carried out as a benchmark in 2.75 L aqueous medium, and subsequently with the inclusion of either 700 g liquid silicone oil or 700 g solid polymer beads.

RESULTS: Biphasic modes achieved a 3-fold productivity improvement with respect to single-phase (30 to 90 mg/L/h), and solid-liquid productivity was similar to liquid-liquid operation while achieving more extensive removal of inhibitory compounds resulting in a slightly higher product titer (1.29 vs. 1.16 g/L). The operability of the reactor was improved by the phase stability of the solid polymer beads relative to immiscible organic solvents, preventing emulsion formation and facilitating analytics.

CONCLUSION: Solid polymer beads replaced the immiscible liquid auxiliary phase for substrate delivery while performing simultaneous inhibitory molecule sequestration.

Keywords: indandiol; indene bioconversion; two-phase partitioning bioreactor; polymer beads; *Pseudomonas putida*

3.3 Introduction

Applications of whole cell-based biotransformations are increasingly targeting the production of high-value compounds, and often impart a specific chirality to their products due to the inherent “handedness” of many biological processes.¹ An example of the bioproduction of a chiral pharmaceutical intermediate is the conversion of indene to *cis*-(1*S*,2*R*)-indandiol, which possesses the stereochemical configuration required in the synthesis of Crixivan® (indinavir sulfate), an HIV protease inhibitor from Merck and Co. Inc. The biological process is a potentially viable alternative to the synthetic approach, in which chiral resolution of the required enantiomer represents the most difficult step in the pathway.²

Pseudomonas putida F1 is among several organisms known to express toluene dioxygenase (TDO), which oxygenates toluene to *cis*-toluene dihydriol, and can also asymmetrically convert indene in the presence of toluene to *cis*-(1*S*,2*R*)-indandiol favouring the *cis*-(1*S*,2*R*) stereochemical configuration. Mutagenesis and screening studies by Merck researchers produced an isolate, *P. putida* 421-5 (ATCC 55687), which expresses TDO inducible with indene alone.³ The biocatalytic system shown in Figure 3-1 illustrates the reactions, with the significant accumulation of an inhibitory by-product, 1-indenol, adding complications to achieving high product titers.³

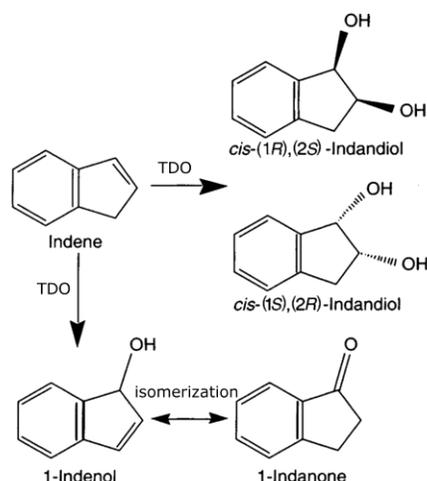


Figure 3-1: Indene bioconversion by *P. putida* TDO enzyme system showing indene conversion to detected products and by-products, adapted from Connors et al. (1997). The monooxygenation product, 1-indenol, is a result of “improper fit” of the non-natural substrate, indene, in the dioxygenase active site.³ 1-indenol undergoes slow, spontaneous isomerization to 1-indanone.

P. putida 421-5 (ATCC55687) produces the desired enantiomer in a 2:1 ratio, corresponding to an enantiomeric excess of approximately 30%, defined as the percentage of the desired enantiomer among the sum of both enantiomers.⁴ The “ee” is fixed by the enzyme system in operation, such that “ee” is not a performance metric appropriate for evaluating a bioproduction platform, but one that could be used when comparing cells.⁵ Instead, yield, overall product concentration, specific productivity and overall volumetric productivity are more representative metrics of a production platform’s performance. Low product yields reported for this system in the range of 0.2 are a result of by-product formation and kinetic resolution of the desired enantiomer through selective degradation of the (1*R*,2*S*) enantiomer.^{2,3} Up to 97% of indene mass has been accounted for including evaporation losses in another study investigating feeding strategies.⁶

The bioconversion of indene has previously been accomplished by controlled substrate feeding or by employing a two-phase partitioning bioreactor (TPPB) platform, in which an immiscible carrier liquid such as silicone oil or soybean oil sequesters the hydrophobic compound, maintaining sub-inhibitory aqueous concentrations as a result of the distribution coefficient.⁷ Biocatalytic consumption of

substrate from the aqueous phase causes additional substrate to diffuse from the carrier phase, maintaining the concentration equilibrium between phases, and accomplishing *in-situ* substrate addition (ISSA) at the rate of biocatalytic demand.⁸ This process, driven thermodynamically by the concentration gradient, is bi-directional, and can be similarly exploited to remove inhibitory products as they are formed as in *in-situ* product removal (ISPR).⁹

The substrate and desired product, indene and *cis*-(1S,2R)-indandiol, are known to be inhibitory to the biocatalyst at concentrations of 5 and 15 g/L, respectively. The by-products 1-indenol and its isomer, 1-indanone are the most inhibitory compounds (toxic at 1.5 and 3 g/L, respectively) with only 1-indenol accumulating significantly in this system.⁷ The use of a hydrophobic carrier liquid for improving this biotransformation has focused exclusively on substrate delivery until this point; however, the by-product 1-indenol is more inhibitory than indene with a toxic concentration of 3 g/L, and could be proactively targeted to reduce biocatalyst inhibition and improve performance.¹⁰ The extent of extraction is determined by the partition coefficient toward a target molecule and the phase ratio. While hydrophobic target molecules require a small auxiliary phase ratio to achieve observable effects, a 25% phase ratio was chosen to provide adequate removal of the desired product, *cis*-(1S,2R)-indandiol.

Two-liquid-phase TPPBs have commonly used a rationally-selected immiscible liquid phase with affinity for the substrate and compatibility towards the biocatalyst.¹¹ The simultaneous requirements of biocompatibility, non-bioavailability and good partitioning, with biocompatibility being an absolute requirement, often results in a compromise in partitioning performance.¹² Recently, absorbent polymer beads have replaced immiscible liquids acting as the sequestering phase in otherwise identically-operated systems, with reports of significant improvements.^{13,14} Solid polymer beads have been shown to significantly improve the operability of TPPBs compared to immiscible liquids because they are generally biocompatible, non-bioavailable in pure cultures, and do not suffer from emulsion formation in the presence of biomass.¹⁵ Association of the cell membrane with a dispersed immiscible liquid phase

results in an emulsion which traps a significant portion of the biomass away from the aqueous phase, negatively impacting productivity as well as analytical procedures.¹⁵⁻¹⁷ The presence of silicone oil complicates the quantification of products distributed between the two phases, which has been identified as a major hurdle in this bioproduction system.⁵

The bioconversion under investigation suffers inhibition from both substrate and products.⁷ Using solid polymer beads having affinity towards the biotransformation products in place of silicone oil permits simultaneous *in-situ* substrate addition (ISSA) and *in-situ* product removal (ISPR), and is expected to improve performance by removing inhibitory products from the aqueous phase which would otherwise inhibit the biotransformation.

3.4 Experimental

3.4.1 Chemicals

Unless otherwise stated, all compounds were purchased from Sigma-Aldrich or Fisher Scientific Canada. Indene used for analytical standards was 98% pure, and >90% pure for bioconversions. *cis*-(1S,2R)-indandiol was purchased from Wako Chemicals, Richmond, VA. Polymers used are described in Table 3-1.

Table 3-1: Properties of evaluated polymers

Name	Composition	Glass transition temperature (°C) ^{reference}	Supplier
Hytre[®] 8206	poly(butylene terephthalate)-polyether	-59 ¹⁸	DuPont
Hytre[®] 3548	poly(butylene terephthalate)-polyether	-40 ^a	DuPont
Elvax[®] 40W	ethylene/vinyl acetate	-17 ¹⁹	DuPont
Kraton[®] D4150K	styrene/butadiene block copolymer	-80 ^b	Kraton Performance Polymers Inc.
PEBAX[®] 4033	polyether block amide	-65 ^b	Arkema Canada Inc.
Silicone rubber	poly(dimethylsiloxane)	N/A	Mastercraft
Silicone oil (5 cSt)	poly(dimethylsiloxane)	N/A	Sigma-Aldrich

a) E. I. du Pont de Nemours and Company

http://www2.dupont.com/Plastics/en_US/assets/downloads/processing/H81091.pdf

b) Automated Creations Inc. <http://www.matweb.com/>

3.4.2 Microorganism

The indene-converting organism, *P. putida* 421-5 (ATCC 55687), isolated by Merck researchers, was purchased from ATCC and used for all biotransformations. The employed organism converts indene to indandiol during its growth phase, unlike several indandiol-producing strains used in other studies which convert indene during the stationary phase. The choice of this organism was based on its commercial availability and its high specific productivity, which was thought to be a challenge for the solid-liquid two-phase partitioning bioreactor system from a system performance standpoint: a high rate of production requires an equally rapid mass flux rate between the two phases in order to meet the biocatalyst's metabolic demands. The chiral distribution of the product is assumed to have no impact on the diffusion processes operating in the two-phase systems because the polymers lack orderly chiral character.

3.4.3 Analytics

Cell concentrations were determined by measuring optical density at 600 nm with a Biochrom Ultrospec 3000 UV/Vis spectrophotometer using a calibration curve relating optical density to cell dry weight (CDW). It was not possible to quantify biomass in the silicone oil run due to emulsion formation affecting optical density measurements.

HPLC analysis of bioconversion was performed by separation on a Varian Pursuit C8 5 μ 4.6 x 250 mm column and detection on a Varian ProStar 325 UV/Vis at 220 nm, as described by Connors et al. (1997)³ and using an injection volume of 20 μ L. Under these conditions, retention times for *cis*-(1S,2R)-indandiol, 1-indenol, 1-indanone, and indene were 9.6, 13.9, 14.9, and 21.6 minutes, respectively. Concentrations were determined by area count calibrations generated with known analytical standards with the exception of 1-indenol which is unavailable commercially. The initial consumption of glucose was monitored using the DNS assay²⁰ and refractive index HPLC, but a biotransformation product interfered with these methods at later times. Other studies on this system have used more sophisticated methods to measure glucose.¹⁰

3.4.3.1 1-indenol analysis

A large HPLC peak was produced in accordance with literature reports of 1-indenol's presumed formation as a monooxygenation by-product, and eluted one minute before its close relative 1-indanone.³ The identity of 1-indenol in this system was confirmed in another study by NMR and Mass Spectrometry.⁴ To further substantiate the identity of this peak in our study, the octanol-water distribution coefficient (LogP) values of *cis*-(1S,2R)-indandiol and 1-indanone were predicted by the group contribution method with the Molspiration miLogP 2.2 tool as 0.47 and 1.72, respectively. (Ertl P. (<http://www.molinspiration.com/cgi-bin/properties>)) Indene's LogP of 2.92 was obtained from published data.²¹ The LogP values were correlated to their observed retention times under identical reversed-phase HPLC analysis conditions.²² The predicted LogP value for 1-indenol of 1.39 was found to fit this linear trend ($R^2 = 0.99$). Area counts, expressed throughout as mAu*min/L for injection volumes of 20 μ L, were used for quantification of 1-indenol concentration because this compound is not commercially available.

3.4.4 Indene biotransformation experiments

All experiments were conducted using a 5 L BioFlo III bioreactor (New Brunswick Scientific, Edison, NJ) filled with 2.4 L of medium K as described by Buckland et al. (1999).² The bioreactor was inoculated with a 24-hour culture of *P. putida* 421-5 (ATCC 55687) grown in 6 x 125 mL Erlenmeyer flasks each containing 40 mL TSB incubated at 180 RPM and 30°C. The vessel was aerated at 0.6 vvm and agitated at 500 – 600 RPM with two Rushton turbines. Dissolved oxygen was monitored with a Broadley-James D100 series OxyProbe and remained above 40% air saturation. pH was maintained at 7.0 with 5M KOH using a Broadley-James FermProbe. Antifoam 204 was added dropwise as required. A sterile 50% glucose feed was initiated prior to glucose depletion at 55 mL/hr (10 g/L-h) as described by Buckland et al. (1999)² using an Imed Gemini® gravity-fed intravenous pump.

3.4.5 Single-phase operation

As a benchmark for comparison to two-phase experiments, two single-phase, fed-batch biotransformations were performed using a medium amended for higher cell density to compensate for biocatalytic inactivation in an attempt to reduce indene toxicity by reducing the specific loading of inhibitors which preferentially associate with the cells, as reported by Amanullah et al. (2002b).¹⁰ Sterile-filtered indene was fed hourly at a rate of 0.1 – 0.2 g/L-h to avoid its excessive accumulation or loss by air stripping, resulting in the cumulative addition of 5.6 g and 10 g of indene after 12 h for each experiment, respectively. Samples were passed through a 0.2 μm filter prior to HPLC analysis.

3.4.6 Liquid-liquid two-phase operation

To reproduce the optimized two-liquid-phase operation described by Buckland et al. (1999) as a basis for two-phase comparison, the bioreactor was run with the addition 700 mL of silicone oil ($\rho = 0.99$, $\eta = 5$ cSt) with the addition of 30 g/L indene, giving a phase fraction of 25% (w/v). Bioreactor samples were extracted with 2 volumes of isopropanol by shaking at 180 RPM and 30°C for 20 minutes. Samples were centrifuged to remove the cells and oil from the aqueous/alcohol phase and the supernatant was analyzed by HPLC.

3.4.7 Polymer selection

To compare different polymers' abilities to partition the various components of this biotransformation under realistic conditions, seven commercially-available polymers, including liquid silicone oil ($\eta = 5$ cSt), were evaluated for their affinity for the biotransformation products in the aqueous phase. All polymers evaluated occur as small spherical or oval beads approximately 1 mm in diameter, and have a glass transition temperature (T_g) well below the operating temperature of the biotransformation. This property is thought to be important for polymer chain mobility, ensuring that the polymeric matrix permits the diffusion of large molecules for which a rigid glassy structure would be impermeable.¹⁵

The by-product, 1-indenol, has been identified as one of the most inhibitory compounds and is also the major bioconversion product in this system.⁷ For this reason, 1-indenol affinity was chosen as the basis for polymer selection. Because 1-indenol is not commercially available, spent biotransformation medium containing *cis*-(1S,2R)-indandiol (250 mg/L), 1-indenol (450 mAu*min/L), and 1-indanone (50 mg/L) was centrifuged and passed through a 0.2 µm filter to be used in evaluating the polymers. Aliquots of 10 mL were placed in 20 mL scintillation vials, containing varying masses of each polymer from 0.5 to 2.5 g in 0.5 g increments. The vials were agitated in a shaker at 30°C and 180 RPM overnight to establish equilibrium conditions. Concentrations were measured by HPLC at equilibrium and compared to a control lacking polymer beads, and concentrations in the polymers were calculated by mass balance. The ratio of the concentration in the polymer (g/kg) to aqueous concentration (g/L) at equilibrium gave the distribution coefficient of each compound for each polymer in the concentration range.

Prior to the 1-indenol affinity evaluation, seven commodity polymers had been previously evaluated for affinity towards 1-indanone due to its structural similarity to 1-indenol and its commercial availability; however, 1-indenol was determined to be a more appropriate test compound for reasons given above.

Because the delivery of indene from a polymer first requires its loading into the polymer phase, the partitioning behaviour of HytreI® for indene was investigated. To determine the polymers' stability when in prolonged contact with the aromatic solvent, 5 g of polymer beads were immersed in 5 mL of indene for several days.

3.4.7.1 Polymer Loading and Washing

The beads were immersed in 700 mL isopropanol with the addition of 50 g indene and equilibrated overnight to give an estimated indene concentration of 50 g/Kg in the polymer beads using the equation:²³

$$S_{\text{polymer}} = \frac{M_{\text{indene}}}{M_{\text{polymer}} + M_{\text{liquid}} / K_{S/L}} \quad (1)$$

Where S_{polymer} is the concentration of indene in the polymer (g/kg), M_{indene} is the mass of indene (g), M_{liquid} is the mass of isopropanol in the system (kg), and $K_{S/L}$ is the distribution coefficient for indene between the solid polymer and the liquid solvent.

The loading solution was decanted from the beads through a sterilized stainless steel screen and washed with five volumes of sterile water because residual isopropanol within the beads was found to strongly inhibit the culture. A target aqueous isopropanol concentration of below 1% (v/v) in the reactor medium was found to not adversely affect the culture and was confirmed using refractive index HPLC. Indene removal as a result of the washing procedure was found to be approximately 25%, resulting in an indene concentration in the polymer of approximately 33 g/L based on Hytrel®'s density of 1.16 g/kg.

3.4.8 Solid-liquid two-phase operation

The bioreactor was run with the addition of 700 g Hytrel® 8206 ($\rho = 1.16$) polymer beads loaded with 33 g/L indene, giving a solid phase fraction of 25% (w/v). Bioreactor medium samples were periodically withdrawn and filtered for HPLC analysis or extracted with 2 volumes of isopropanol prior to centrifugation and HPLC analysis of the supernatant. Contents of the polymer beads in the reactor were analyzed by removing 20 beads (approximately 0.4 g), briefly rinsing their surface with water, then placing them in a 20 mL scintillation vial containing 10 mL isopropanol and allowed to equilibrate at 180 RPM and 30°C for at least 4 hours before HPLC analysis. It was found that >90% of sorbed material is recovered in the first round of extraction and >90% of the remainder upon a second extraction. A negligible amount of material was extracted in the third round of extraction, demonstrating the simplicity of product recovery from absorbent polymer beads. Other studies have demonstrated the reusability of the polymer beads after this extraction procedure.^{13,14,24}

Concentrations in the polymer samples were calculated by mass balance of the extracted compounds assuming negligible losses due to volatility. Overall concentrations in the reactor were determined by the summation of polymer-sorbed and aqueous-phase masses, then dividing by the entire working volume represented by the combined aqueous and polymer volumes.

3.5 Results and Discussion

3.5.1 Polymer selection

Both grades of Hytrel® were found to have a distribution coefficient for indene in isopropanol of approximately 2. Excessive swelling and cracking of the 3548 grade of Hytrel® and absence of swelling in 8206 with prolonged contact with indene, illustrated in Figure 3-2, led to the selection of 8206 for biotransformation experiments despite its slightly lower affinity for the biotransformation products (Figure 3-3). Communication with DuPont indicated that the 8206 grade, lacking a crystallinity disruptor, may make it more solvent resistant than 3548 which contains a disruptor to increase softness.



Figure 3-2: Photograph comparing swelling behavior in different grades of Hytrel® when exposed to indene: 3548 swelling and cracking on left, 8206 unaffected on right. Unexposed beads in foreground for comparison with paper clip for scale.

Both grades of Hytrel® showed significantly higher affinity towards 1-indanone than the other polymers (Figure 3-3a). The distribution coefficient of Hytrel® for 1-indenol was found to be approximately 65-fold that of silicone oil (Figure 3-3b), indicating that there is potential for improving

by-product removal from this biotransformation by selecting from a range of commodity polymers. Commodity polymers cost much less than silicone oil at approximately \$5/kg²⁵ compared to \$300/kg (Sigma-Aldrich Co. (<http://www.sigmaaldrich.com>)), greatly reducing the cost of an otherwise identical system.

The partitioning behavior of *cis*-(1*S*,2*R*)-indandiol between the polymer and aqueous phases was much less extensive than with the other compounds, likely a result of its relative hydrophilicity due to the presence of two hydroxyl groups. There was no detectable uptake of *cis*-(1*S*,2*R*)-indandiol by any polymer except Hytre[®], which had a distribution coefficient of approximately 2 for each grade (Figure 3-3c). This suggests that the desired product remains almost entirely in the aqueous phase when silicone oil is employed as the auxiliary phase.

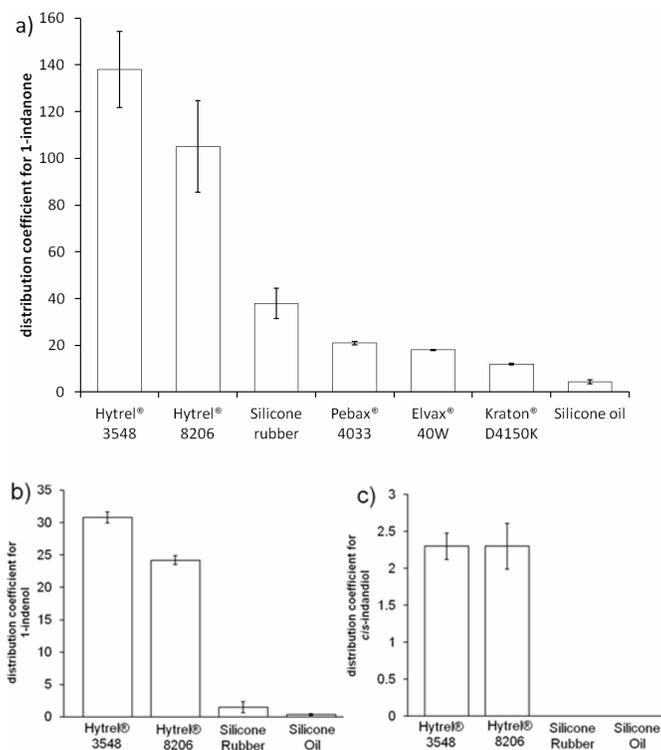


Figure 3-3: Polymer distribution coefficients for biotransformation products: 1-indanone (a), 1-indenol (b) and *cis*-(1*S*,2*R*)-indandiol (c). Distribution coefficient is expressed as the concentration in the polymer (g/kg) divided by the concentration in the aqueous phase (g/L). Error bars represent one standard deviation from the mean value of five samples.

3.5.2 Bioconversion experiments

Product and by-product accumulation began 3 - 4 hours post-inoculation and lasted for approximately 8 - 9 hours in every run (Figure 3-4). Preliminary data showed reproducible productivity. The lag in product accumulation was likely a period of enzyme induction upon exposure to indene in the bioreactor, as the TDO system would not have been induced in the inoculation flasks lacking indene. Accumulated products appeared as chromatogram peaks corresponding to the conversion of indene to 1-indenol, *cis*-(1S,2R)-indandiol, and 1-indanone, illustrated in Figure 3-1. Degradation products, 1,2-indenediol, and keto-hydroxy indan, resulting from the kinetic enrichment of the *cis*-(1S,2R)-indandiol enantiomer, were not detected during the time course of the experiments. Indene concentrations in the aqueous phase remained very low throughout the production period in each experiment, demonstrating its higher affinity for the auxiliary phase and the biocatalyst than for the aqueous phase. The difference in productivity between single-phase and two-phase experiments despite similar substrate availability highlights the benefits of an auxiliary phase in alleviating inhibition arising from the use of an extremely hydrophobic substrate.

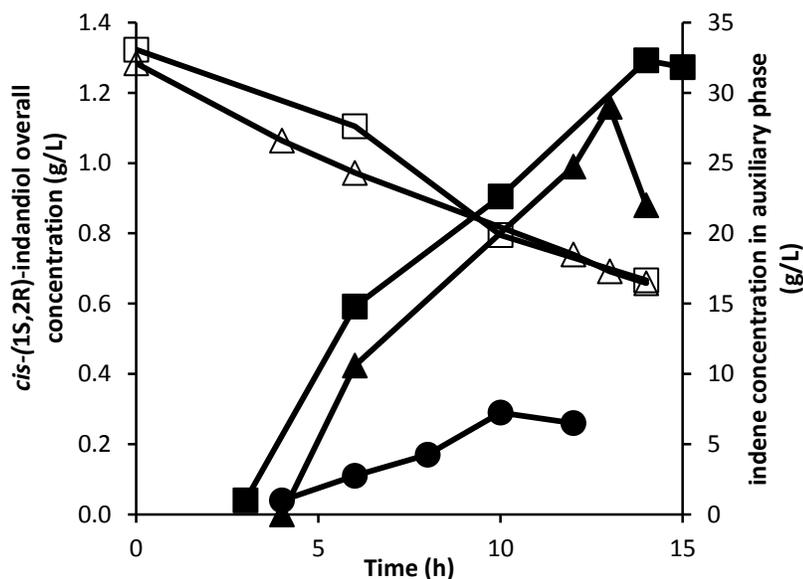


Figure 3-4: Time course plot of overall product concentration (closed symbols, left axis) and substrate concentration in auxiliary phase (open symbols, right axis) under liquid-liquid (triangles) and solid-liquid (squares) operation. Single-phase, high substrate feed operation (circles) lacks an auxiliary phase.

The earlier end point in the two-liquid experiment may be a result of biocatalyst inactivation with the accumulation of higher aqueous concentrations of 1-indenol, which was more suppressed when using the solid polymer. Indene and *cis*-(1*S*,2*R*)-indandiol did not accumulate to inhibitory concentrations in the medium. A nutrient deficiency was suspected but it was found that additional nutrient supplementation provided no change in growth or productivity in other experiments (data not shown). The extent and duration of each bioconversion used to calculate the performance metrics presented in Table 3-2 were based on the point of maximum product titer, as enzymatic degradation of the undesired enantiomer occurs rapidly with a consequent decrease in overall product titer.²

Table 3-2: Comparison of single-phase, two-liquid, and solid-liquid productivity. Overall product titer calculated by dividing total product mass in both phases by total working volume.

Performance metric	Operational mode (Auxiliary phase volume) Total volume			
	Single-phase Low indene feed 2.75 L	Single-phase High indene feed 2.75 L	Liquid-liquid (0.7 L) 3.45 L	Solid- liquid (0.6 L) 3.36 L
Substrate consumed (g) (overall)	5.6	10.0	9.4	13.5
Final product titer (g/L) (overall)	0.14	0.29	1.16	1.29
Time to completion (h)	13	12	13	14
Biomass concentration (g/L) (aqueous)	3.8	2.5	Biomass not quantifiable	2.7
Molar yield (indandiol / indene)	0.08	0.09	0.51	0.40
Specific productivity (mg _{product} / g _{cells} · h)	1.0	4.2	Biomass not quantifiable	11.8
Overall volumetric productivity (mg _{product} / L · h)	10	29	89	92

3.5.2.1 Single-phase operation

The single-phase, fed-batch runs were the least productive mode of operation evaluated (Table 3-2). This was likely a result of indene's rapid and direct exposure to the biocatalyst causing inhibition,

with higher feed rates resulting in lower biomass concentration. Without an auxiliary phase to sequester indene, it preferentially associates with the hydrophobic cell membrane resulting in cellular stress response and eventual membrane failure.⁷ A brown pigmentation was more intense in the experiment with the highest cumulative indene addition than with either lower indene feeding or the more productive biphasic experiments. The inhibitory by-product 1-indenol did not accumulate to inhibitory concentrations in single-phase experiments.

3.5.2.2 *Liquid-liquid operation*

The reactor operated using silicone oil achieved a similar final titer and volumetric productivity to that using polymer beads (Table 3-2). These are in agreement with reported volumetric productivities for other indene-converting systems; however, there is no reported specific productivity for a system using this biocatalyst in the presence of silicone oil. Although product titer has been reported to reach 2 g/L at the 23 L scale, the lack of biomass quantification makes comparisons of performance difficult.²

The presence of silicone oil complicated analytical procedures, requiring a phase separation step after whole-broth extraction, eliminating the possibility of determining relative concentrations between the two phases. However, maximum 1-indenol and 1-indanone concentration in the medium were estimated at 1720 mAu*min/L and 300 mg/L, respectively based on the concentration in the whole-broth extract. Optical density measurements were also confounded by the emulsion formed during agitation and aeration, so it was not possible to quantify biomass despite efforts to resuspend the cells in fresh medium.

3.5.2.3 *Solid-liquid operation*

The reactor operated using polymer beads achieved a similar and slightly higher overall product concentration than that of the silicone oil system (Table 3-2). Operation of the bioreactor using polymer beads simplified analytical procedures: direct aqueous sampling as well as the extraction of polymer-sorbed compounds enabled the separate analysis of each phase. Biomass concentration was quantified

without complications and enabled the determination of specific productivity, an important performance metric. Polymer beads were examined microscopically and found to have no microbial attachment to their surface. Electron microscopy of bead surfaces has also previously confirmed the absence of biofilm on polymer beads.²⁴

The polymer phase successfully removed 1-indenol, maintaining concentrations below 864 mAu*min/L in the medium throughout the experiment while sequestering approximately 12460 mAu*min/L. Similarly, the polymer phase absorbed a total of 689 mg of 1-indanone by the end of the experiment, and maintained very low aqueous concentrations of below 50 mg/L throughout. It is not possible to directly compare aqueous concentrations to the silicone oil experiment for reasons previously mentioned.

The mass distribution of *cis*-(1S,2R)-indandiol at the end of the solid-liquid runs was found to be approximately two-thirds remaining in the aqueous phase and one-third absorbed by the solid polymer due to the auxiliary phase volume ratio of 25%, corresponding to the observed distribution coefficient in which the product concentration in the polymer (2 g/kg) was twice that in the aqueous phase (1 g/L) of the solid-liquid system, resulting in an overall concentration of 1.29 g/L. While not an ideal case with respect to product separation and recovery, this is a demonstration of ISPR operating simultaneously with ISSA from a single auxiliary phase, and there is potential to improve product absorption using a second type of polymer.

3.5.2.4 Two-phase comparison

The molar yield was higher in each two-phase experiment than in single-phase (Table 3-2). Yield was calculated from the observed change in indene in the auxiliary phase for the two-phase runs, and the total amount of indene added for the single-phase runs because of its absence from the aqueous phase and near-complete association with the biomass. The substrate concentration in the auxiliary phase was similar but not identical between the liquid-liquid and solid-liquid systems, and productivity

was similar. Indene was oversupplied and incompletely consumed in both two-phase experiments such that substrate availability does not appear to be a limiting factor in these systems.

While yield values are higher than literature reports, they represent the combination of both enantiomers, with literature reports specifying *cis*-(1S,2R)-indandiol.² If the enantiomeric excess is assumed to be approximately 30% for this organism as reported in literature, the corresponding yield of the specific enantiomer would indeed be lower and similar to reported literature values of 0.2.

Substrate loss due to evaporation was likely more severe under single-phase operation than two-phase operation², and may negatively affect single-phase yield coefficients.

The slightly higher product yield in the two-liquid system relative to the solid-liquid system may be the result of enhanced removal of 1-indenol with respect to *cis*-(1S,2R)-indandiol by the polymer phase. Buckland et al. (1999) investigated the presence of each product inhibiting its own formation, and found that excess 1-indenol shunted production towards relatively more *cis*-(1S,2R)-indandiol and vice-versa. A reduced 1-indenol concentration in the aqueous phase could negatively affect the *cis*-(1S,2R)-indandiol yield coefficient as more 1-indenol is produced in response to lower feedback inhibition.

Alternatively, the difference in yield may be a feature of the solid polymer's higher affinity towards the product: as kinetic resolution of the undesired enantiomer occurs rapidly towards the end of the biotransformation, the portion of substrate retained in the solid auxiliary phase would be protected and yield would remain higher than with a liquid auxiliary phase having poor affinity and leaving the vast majority available for degradation. This hypothesis is supported by the apparently large difference in degradation rate at the end of the two-phase experiments. While not ideal for this system in terms of the desired enantiomer, this feature would be very useful where product degradation was a significant barrier.

The production kinetics are very similar between the two biphasic systems, suggesting that there was sufficient interfacial area available for substrate mass transport between the solid and aqueous phases to enable similar productivity to a liquid-liquid system having a larger interfacial area permitting near-instantaneous equilibrium.²³ If interfacial mass transfer were found to be a limiting factor with respect to product removal, the specific surface area of the polymer beads could easily be increased by size reduction, reducing the diffusion path length.²⁵ Additionally, in such situations, a criterion for the selection of solid polymer phases may be diffusivity: the rate at which a target molecule permeates and diffuses through the polymeric matrix.

Using solid polymer beads in place of silicone oil for indene delivery accomplished *in-situ* substrate addition with simultaneous product removal, and provided significant operational and cost benefits. Volumetric productivity under solid-liquid operation was similar to liquid-liquid operation, presumably due to the polymer's affinity for the major inhibitory by-product of this biotransformation.

Potential improvements to this biotransformation may arise from opposite ends of process development: biocatalyst modifications to improve yield, volumetric productivity, and "ee" through enzyme modification, by manipulating gene expression for constitutive production, or knocking out product degradation pathways would be useful, but would require considerable effort and expense. Identifying materials with superior affinity and selectivity for the desired product and inhibitory by-products could also significantly enhance performance by allowing more complete conversion of substrate to product by alleviating inhibition and achieving preliminary separation and product recovery. An improved biocatalyst would also benefit from advances in ISPR materials.

3.6 Acknowledgements

The financial support of Queen's University, National Sciences and Engineering Research Council of Canada, and DuPont is gratefully acknowledged.

3.7 References

- 1 Straathof AJJ, Panke S and Schmid A, The production of fine chemicals by biotransformations. *Curr.Opin.Biotechnol.* **13**:548-556 (2002).
- 2 Buckland BC, Drew SW, Connors NC, Chartrain MM, Lee C, Salmon PM, Gbewonyo K, Zhou W, Gailliot P, Singhvi R, Olewinski Jr RC, Sun W, Reddy J, Zhang J, Jackey BA, Taylor C, Goklen KE, Junker B and Greasham RL, Microbial Conversion of Indene to Indandiol: A Key Intermediate in the Synthesis of CRIVAN. *Metab.Eng.* **1**:63-74 (1999).
- 3 Connors N, Prevoznak R, Chartrain M, Reddy J, Singhvi R, Patel Z, Olewinski R, Salmon P, Wilson J and Greasham R, Conversion of indene to *cis*-(1S),(2R)-indandiol by mutants of *Pseudomonas putida* F1. *J.Ind.Microbiol.Biotechnol.* **18**:353-359 (1997).
- 4 Wackett LP, Kwart LD and Gibson DT, Benzylic Monooxygenation Catalyzed by Toluene Dioxygenase from *Pseudomonas-Putida*. *Biochemistry* **27**:1360-1367 (1988).
- 5 Stafford DE, Yanagimachi KS and Stephanopoulos G, Metabolic engineering of indene bioconversion in *Rhodococcus* sp. *Adv.Biochem.Eng.Biotechnol.* **73**:85-102 (2001).
- 6 Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW and Woodley JM, Fed-batch bioconversion of indene to *cis*-(1S,2R)-indandiol. *Enzyme Microb.Technol.* **31**:954-967 (2002).
- 7 Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW and Woodley JM, Measurement of strain-dependent toxicity in the indene bioconversion using multiparameter flow cytometry. *Biotechnol.Bioeng.* **81**:405-420 (2003).
- 8 Collins L, Daugulis A, Benzene/toluene/p-xylene degradation. Part I. Solvent selection and toluene degradation in a two-phase partitioning bioreactor. *Appl.Microbiol.Biotechnol.* **52**:354-359 (1999).
- 9 Freeman A, Woodley JM and Lilly MD, In situ product removal as a tool for bioprocessing. *Nat.Biotechnol.* **11**:1007-1012 (1993).
- 10 Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW and Woodley JM, Application of multi-parameter flow cytometry using fluorescent probes to study substrate toxicity in the indene bioconversion. *Biotechnol.Bioeng.* **80**:239-249 (2002).
- 11 Malinowski JJ, Two-phase partitioning bioreactors in fermentation technology. *Biotechnol.Adv.* **19**:525-538 (2001).
- 12 Bruce LJ, Daugulis AJ, Solvent selection strategies for extractive biocatalysis. *Biotechnol.Prog.* **7**:116-124 (1991).
- 13 Prpich GP, Daugulis AJ, A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. *Biotechnol.Bioeng.* **98**:1008-1016 (2007).
- 14 Gao F, Daugulis AJ, Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by *Kluyveromyces marxianus* . *Biotechnol Bioeng.* **104**:332-339 (2009).

- 15 Morrish JLE, Daugulis AJ, Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid–liquid two-phase partitioning bioreactor. *Biotechnol.Bioeng.* **101**:946-956 (2008).
- 16 Aldric J, Gillet S, Delvigne F and Thonart P, Development of an original approach to evaluate effects of surfactants, biomass and pollutants on the scaling-up of a two-phase partitioning bioreactor. *J.Chem.Technol.Biotechnol.* **85**:356-363 (2010).
- 17 Tomei M, Annesini M, Rita S and Daugulis A, Biodegradation of 4-nitrophenol in a two-phase sequencing batch reactor: concept demonstration, kinetics and modelling. *Appl.Microbiol.Biotechnol.* **80**:1105-1112 (2008).
- 18 Prpich GP, Daugulis AJ, Polymer Development for Enhanced Delivery of Phenol in a Solid-Liquid Two-Phase Partitioning Bioreactor. *Biotechnol.Prog.* **20**:1725-1732 (2004).
- 19 D'Orazio L, Mancarella C, Martuscelli E, Casale A, Filippi A and Speroni F, Polyamide 6/ethylene-co-vinyl acetate blends: Morphology-properties relationships in injection moulded samples. *J.Mater.Sci.* **22**:429-437 (1987).
- 20 Miller G, Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal.Chem.* **31**:426-428 (1959).
- 21 Klopman G, Li JY, Wang S and Dimayuga M, Computer automated log P calculations based on an extended group contribution approach. *J.Chem.Inf.Comput.Sci.* **34**:752-781 (1994).
- 22 Griffin S, Wyllie SG and Markham J, Determination of octanol-water partition coefficient for terpenoids using reversed-phase high-performance liquid chromatography. *J.Chromatogr.A.* **864**:221-228 (1999).
- 23 Rehmann L, Daugulis AJ, Biodegradation of biphenyl in a solid-liquid two-phase partitioning bioreactor. *Biochem.Eng.J.* **36**:195-201 (2007).
- 24 Amsden BG, Bochanysz J and Daugulis AJ, Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. *Biotechnol.Bioeng.* **84**:399-405 (2003).
- 25 Rehmann L, Sun B and Daugulis AJ, Polymer Selection for Biphenyl Degradation in a Solid-Liquid Two-Phase Partitioning Bioreactor. *Biotechnol.Prog.* **23**:814-819 (2007).

Chapter 4 Manipulating the composition of absorbent polymers affects product and by-product concentration profiles in the biphasic biotransformation of indene to *cis*-(1S,2R)-indandiol

Julian T. Dafoe, Andrew J. Daugulis

With minor changes to fulfill formatting requirements, this chapter is substantially as it appears in:
Biochemical Engineering Journal 77:7-14 (2013).

4.1 Preface to Chapter 4

Further to the previous chapter's investigation of a solid polymer extractant replacing an immiscible liquid, this chapter examines the effect of different absorbent polymer compositions on the indene biotransformation by altering the polymers' relative affinity towards the polar, non-inhibitory product, *cis*-(1S,2R)-indandiol, and the strongly inhibitory, non-polar by-products, 1-indenol and 1-indanone. Three block copolymers, polymers which differ in their amount and composition of soft blocks, and had varying affinities towards the non-inhibitory product and inhibitory by-products, which resulted in very different time course profiles due to the metabolites' relative abundance or scarcity in combination with their inhibitory effects. The action of polymer extractants on aqueous phase inhibitors can elicit a reaction from the biocatalyst to produce metabolites in different relative amounts in response to their removal, thereby providing a means to externally manipulate metabolic fluxes. Additionally, in comparing affinity among these polymers, it was noted that water uptake by the polymers would affect their measured partition coefficients if significant water uptake was not accounted for. This led to a recalculation of partition coefficients of polymers on a wet-mass basis, using pre-soaked polymers which were subsequently not affected by further water uptake while attaining equilibrium.

4.2 Abstract

The biotransformation of indene to the pharmaceutical intermediate, *cis*-(1*S*,2*R*)-indandiol, by *P. putida* 421-5 (ATCC 55687) produces large amounts of the inhibitory by-product, 1-indenol, as well as several different minor degradation products. Three segmented block copolymers, HytreI® 8206, HytreI® 3078, and Pebax® 2533, containing varying types and amounts of soft segment components, exhibited different affinity towards the product and by-product. The polymers were each used as the sequestering phase for metabolite removal from this transformation in a two-phase partitioning bioreactor (TPPB). The polymer with the highest affinity for each metabolite enhanced its production via more extensive partitioning into the polymer phase. This work reports the first use of polymers whose differing compositions are attributed to target molecule affinity and the different production profiles seen during two-phase biocatalysis. The uniquely high water content of 30 wt% in HytreI® 8206 emphasized the importance of accounting for water uptake in partition calculations, and potentially conferred enhanced affinity and diffusivity by providing an expanded polymer network, significantly improving biotransformation completion time compared to the low-water-absorbing polymers.

Keywords: Biotransformations; Absorption; Separation; Bioprocess Design; Extractive Fermentation; Polymers

4.3 Introduction

Biotransformations are subject to limitations arising from concentrations of substrate, product, or by-product(s) which have an inhibitory or toxic effect towards the microbial biocatalyst [1]. The addition of a dispersed absorbent polymer phase, having affinity towards target molecules, has been shown to improve productivity by providing a reservoir, separate from the cell-containing aqueous phase, into which compounds can partition [2-5]. The ratio of a target molecule's concentration in the polymer phase relative to the aqueous phase is the partition coefficient (PC), a measure of polymer-solute affinity, which determines the extent of target molecule uptake by the auxiliary phase at a given aqueous concentration.

The biphasic biotransformation of indene to the pharmaceutical intermediate *cis*-(1S,2R)-indandiol by *P. putida* 421-5 (ATCC 55687) was first investigated at Merck & Co. using silicone oil as a reservoir for delivering the hydrophobic substrate, indene [6]. The metabolic pathways of indene biotransformation are complex, with two primary products and five total possible products [6]. The initial selection of a hydrophobic liquid was rational considering the substrate's hydrophobicity; however, silicone oil has almost no affinity towards 1-indenol, the major by-product, and the dominant inhibitor of this process [7]. That is, the work by Merck was able to gradually deliver the hydrophobic substrate to the cells, but silicone oil did not alleviate the inhibition caused by the by-product, 1-indenol. Our initial investigation into this system replaced the liquid solvent with a polymer, Hytrel® 8206, having affinity towards both the major inhibitory by-product, 1-indenol, and the desired product, *cis*-(1S,2R)-indandiol, which is not inhibitory to this biotransformation (Table 2-1). The use of the polymer was found to out-perform the silicone oil partitioning phase and provide operational advantages due to its phase stability [7]. While hydrophobic target molecules require a small auxiliary phase ratio to achieve observable effects, a 25% phase ratio was chosen to provide adequate removal of the desired product, *cis*-(1S,2R)-indandiol.

Polymers which have been used as absorbent auxiliary phases in biotransformations all feature a soft, amorphous polymer chain network above its glass transition temperature (T_g), permitting chain mobility for permeation of target molecules [8]. This feature is distinct from adsorbent resins, where the material is held in its porous architecture by rigid polymer chains which are below their T_g , such that target molecule interactions occur at the material's surface, and diffusion occurs only within the bulk fluid filling the pore spaces rather than within the material itself [9, 10].

Our motivation for investigating soft, absorbent polymers, in contrast to glassy, macroporous adsorbent resins, applied as *in-situ* auxiliary phases in two-phase partitioning bioreactors (TPPBs) is several-fold. Adsorbent resins are already well-studied and characterized for many biotransformation applications [11], and suffer from mechanical resilience and fouling problems [12]. In contrast to adsorbents, absorbent polymers act similarly to immiscible liquid solvents, operating passively by diffusion governed thermodynamically by concentration gradients, resulting in partition coefficients based on linear sorption isotherms in the concentration ranges of interest rather than competitive site occupation as seen in porous adsorbent isotherms [2]. Finally, the attractive cost of commodity polymers relative to specialized adsorbent resins provides an economic incentive in process development [12].

The majority of polymers used in TPPBs are in the family of poly(ether)-containing block copolymers [4, 13-16], where the soft poly(ether) segments available for solute permeation are structurally supported by domains of a hard, glassy segments such as a poly(ester) (eg. Hytrel®), or poly(amide) (eg. Pebax®). The soft segment's poly(ether) composition determines affinity towards a target molecule, while the hard segment is thought to have a negligible contribution towards target molecule absorption due to glassy polymers' lack of chain mobility. Furthermore, segmented block copolymers containing poly(ether) segments which are highly polar will absorb significant amounts of water, while less-polar poly(ethers) will absorb almost no water. Until recently, this difference in water

uptake among different poly(ether)-based block copolymers has been neglected in the literature comparing polymers for use in TPPBs, but this property affects measurements of target molecule affinity, which are based on mass balance calculations assuming constant phase volumes.

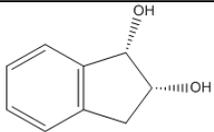
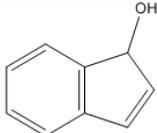
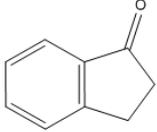
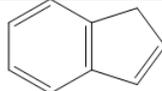
By adjusting the polarity of the soft segment of block copolymers via changing the poly(ether) monomer length, it was hypothesized that segmented block copolymers having different soft segment compositions would produce unique distributions of metabolites under identical conditions, and could have multiple, compound effects on this model complex biotransformation. Our aim was to manipulate the outcome of this biotransformation using three different commercial poly(ether)-based segmented block copolymers, having different soft segment compositions and amounts, described in Table 4-1, which impart differential affinity towards the toxic, hydrophobic major by-product and the relatively non-toxic, hydrophilic minor desired product, described in Table 4-2. This is the first report to attribute block copolymer composition to differences in relative target molecule affinity, which produced different outcomes in this model biotransformation.

Table 4-1: Polymer properties

Polymer name	% Hard segment	% Soft segment	T _g (°C)	Water absorption at equilibrium (%)	Density (kg/m ³)
HytreI® 3078	~50% poly(butylene terephthalate) ^[24]	~50% N/A ^a	-60 ^[34]	0.8 ^[31]	1060 ^[31]
HytreI® 8206	~50% poly(butylene terephthalate) ^[24]	~50% N/A ^a	-59 ^[29]	30 ^[32]	1170 ^[24]
Pebax® 2533	20% poly(amide)-12 ^[28]	80% poly(tetramethylene oxide) ^[28]	-65 ^[34]	1.2 ^[33]	1000 ^[33]

^a) Composition information not available

Table 4-2: Major metabolite properties

Name	Role	Toxic concentration (g/L) ^[30]	Structure	K _{o/w} ^a
(1S,2R)-indandiol	Desired product	15		5
1-indenol	Major inhibitory by-product	3		16
1-indanone	Minor isomerization product of 1-indenol	1.5		25
indene	Biotransformation substrate	5		1000

^{a)} Logarithmic values from [35] converted to linear values for PC comparison

4.4 Materials and Methods

4.4.1 Chemicals

All chemicals were purchased from either Sigma-Aldrich (Canada) or Fisher Scientific (Canada) except *cis*-(1S,2R)-indandiol, which was purchased from Wako Chemicals (USA). Hytrel[®] was provided by DuPont (Canada), and Pebax[®] was purchased from Foster Corporation (Putnam, CT).

4.4.2 Microorganism

P. putida 421-5 (ATCC 55687) was purchased from ATCC, grown for 24 h at 30^oC in TSB at 180 RPM and stored at -80^oC as 1 mL aliquots in 10% glycerol. The same growth conditions were used to generate inoculum flasks from frozen stocks.

4.4.3 Partition coefficient experiments

To measure polymer affinity towards the biotransformation metabolites, high-purity water, or spent medium from a previous experiment, was used to provide a measurement under both “ideal” and realistic conditions, respectively. The medium contained realistic ion concentrations, which may affect partition coefficients via water activity (A_w) modification. The medium was harvested and centrifuged for 1 h at 3600 RPM, and 10 mL of the supernatant was placed in sealed vials with polymer bead

samples of 2, 3, and 4 g (wet mass, surface dried by blotting on absorbent paper) for 24 h shaking at 180 RPM to reach equilibrium, providing triplicate measurements of the PC. Concentrations in the polymer-containing vials were measured by HPLC and compared to a control vial without polymer, and the change in concentration was used to calculate the partition coefficient via mass balance, assuming negligible losses. *cis*-(1S,2R)-indandiol and 1-indanone PCs were measured in both fluids, while 1-indenol was only measured in spent medium because it is not commercially available as an analytical standard.

4.4.4 Biotransformations

4.4.4.1 Polymer loading with substrate

Seven hundred grams (wet mass, surface dried) of the appropriate polymer beads (Hytrel® 8206, Hytrel® 3078, or Pebax® 2533) were split evenly into two 1 L Erlenmeyer flasks and 500 mL water was added to each. The polymer was sterilized in the flasks by autoclaving (Hytrel®) or by boiling for 15 minutes with agitation to avoid gelation due to a lower melting point (Pebax®). Indene was loaded into the polymers by filtering 14 mL into each flask through a 0.2 µm nylon syringe filter, sealing with a sterile rubber stopper, and equilibration on a shaker for 24 hours at approximately 250 RPM. The water was then decanted and the polymer beads were added directly from the flasks to the bioreactor 30 minutes prior to inoculation. This method effectively loaded indene into the polymer at 40 g/kg for each experiment because its low aqueous solubility (ca. 100 mg/L) avoided a significant remainder in the aqueous phase upon reaching equilibrium. A high loading was chosen to ensure a relatively constant driving force for substrate delivery regardless of consumption differences between experiments.

4.4.4.2 Bioreactor operation

The bioreactor (5L BioFloIII, New Brunswick Scientific, Edison, NJ) was filled with 2.64 L of medium, containing (g/L): K₂HPO₄ (2.0); (NH₄)₂SO₄ (2.0); MgSO₄·7H₂O (0.4); FeSO₄·7H₂O (0.02); yeast extract (3.0); tryptone-peptone (3.0); glycerol (20.0); and 3 mL trace element solution, containing

(mg/L): H_3BO_3 (300); ZnCl_2 (50); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (30); CoCl_2 (200); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (10); $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (20); and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (30) and autoclaved.

Glucose was autoclaved separately as a 50% (w/v) solution and 120 mL was added to obtain 20 g/L. pH was automatically maintained at 7.0 using 6M KOH. Dissolved oxygen was monitored and maintained above 30% saturation by adjusting agitation between 500 and 700 RPM. Anti-foam was added dropwise as required.

Each biotransformation was initiated by adding six 125 mL shake flasks containing 40 mL of a 24-hour late-exponential phase culture of *P. putida* 421-5 (ATCC 55687) in tryptic soy broth to 2.76 L of medium containing 700 g (wet mass) polymer beads in the bioreactor. At 3.5 hours after inoculation, a 50 mL bolus containing 6 g $(\text{NH}_4)_2\text{SO}_4$ and 6 g yeast extract was added aseptically, and a 50% (w/v) glucose feed was initiated at 60 mL/h for 12 h using an Imed® Gemini intravenous pump. The aqueous volume of the reactor was maintained near 3 L by removing a volume equivalent to the aqueous substrate additions at each time point.

Aqueous samples were periodically removed, centrifuged at 16,000 G for 5 minutes, and passed through a 0.2 μm syringe filter for HPLC analysis. Polymer samples (40 beads, ca. 1 g) were also periodically removed using a small sieve, their surface rinsed for 3 seconds with sterile water, then immersed in 10 mL isopropanol (IPA) for 12 h at 180 RPM to establish equilibrium. The polymer samples were equilibrated in fresh IPA twice more to ensure full extraction of metabolites. The IPA fractions were then analyzed by the same HPLC method to determine metabolite concentrations in the polymer. No metabolites were detectable in the third extraction except for indene, which was present in a large amount. Overall (aqueous + polymer) concentrations of *cis*-(1S,2R)-indandiol and 1-indenol were calculated by adding the mass present in both phases and dividing by the total reactor volume. 1-indanone was not considered to be a reliable indicator of biocatalyst performance because it is an

isomerization product of 1-indenol rather than a biocatalytic product, and is present in exceedingly small concentrations < 100 mg/L.

4.4.5 Analytics

Biomass was measured by relating optical density at 600 nm to a calibration line giving cell dry weight (g/L). Major metabolites, *cis*-(1S,2R)-indandiol, 1-indenol, 1-indanone, indene, with retention times of 6.8, 9.6, 10.5, and 16 minutes, respectively, were separated on a Varian Polaris C-18A 4.6x150 mm HPLC column at 1 mL/min using gradient elution from 15:85 to 85:15 acetonitrile:water over 15 minutes, and detection was at 220 nm on a Varian ProStar 325 UV/Vis detector. Concentrations were determined using standard calibrations with the exception of 1-indenol which is commercially unavailable. This compound, being the major product, produced the largest peak, and was identified based on previous reports of relative retention time and M-S confirmation in similar biocatalytic systems [6, 17]; 1-indenol was quantified using peak area only assuming a linear detector response. Minor other metabolites appeared as small peaks on the chromatogram emerging at later times in the biotransformation, however, being the desired product, maximum aqueous *cis*-(1S,2R)-indandiol concentration dictated the completion of each biotransformation.

4.5 Results and Discussion

4.5.1 Partition coefficient experiments

4.5.1.1 *Effect of water absorption by polymers on partition coefficient calculations*

The use of wet polymer mass (with the surface dried) in both the partition coefficient and bioreactor experiments was intended to avoid the inevitable transfer of water into the hydrophilic polymer as much as practically possible, which would lower the aqueous volume in the two-phase system as the polymer phase became swollen with water. Although the introduction of water within the polymer has the potential to slightly dilute the medium to which it is added, by introducing the polymer

as a pre-swollen water-polymer complex, volumes are held relatively constant and mass balance calculations reflect the system composition at equilibrium.

The significant uptake of water by hydrophilic polymers has not been previously reported in the literature during TPPB polymer selection, despite moderately water-absorbing polymers being investigated and applied in over a dozen publications to date, for example: [2, 14, 18, 19]. This effect most strongly affects PC values for compounds having modest partition coefficients in polymers with high water uptake by skewing phase volumes if water uptake is unaccounted for. An alternative point of view would be that absorbed water remains separate from the polymer and continues to be part of the bulk aqueous phase; however, the entire swollen polymer volume and its contents are indeed separated from the cells' perspective in the aqueous phase. In reality, there is likely both "bound" water associating directly with the polymer chains and "bulk" water existing as an aqueous phase somewhat entrapped in the polymer chain network [20]. The amount of each type of water would depend on many factors including hard and soft segment composition and polymer microphase morphology. This property would be an exceedingly important parameter to determine if thermodynamic predictions for TPPB polymer selection are to be attempted, as the presence of water in the polymer alters the chemical environment encountered by absorbed solutes. Unfortunately, predictive thermodynamic tools are currently unable to account for both types of water present in the polymer phase [21], and this aspect is a focus of ongoing research in our group. Accounting for water uptake permits the comparison of polymers that exhibit different extents of water absorption on an equal basis, such that partition coefficient values can be realistically compared.

4.5.1.2 Effect of medium salts on partition coefficients

It was not possible to compare 1-indenol partitioning in high-purity water to bioreactor medium because this compound is not commercially available. Instead, 1-indanone, a minor degradation product of 1-indenol, was used as a second target molecule, representative of a hydrophobic metabolite. The PC

values measured for the various polymers in biotransformation medium were significantly higher, by approximately two-fold, for each compound than those measured in high-purity water, shown in Figure 4-1. This is likely a result of medium components such as dissolved salt ions competing for hydration, thereby lowering the water activity (A_w) and increasing solute activity in the aqueous phase, driving more into the polymer at equilibrium.

Salt effects have been noted as being of potential importance in TPPB systems based on abiotic experiments containing high salt concentrations exceeding 100 g/L, corresponding to relatively low A_w values of approximately 0.95 [22], at which point PC values towards relatively hydrophobic target molecules begin to deviate [23]; however, the salt concentrations at these levels are unrealistic for biocatalytic systems due to osmotic stress on the cells. The influence on PC values seen here in actual bioreactor medium, due to much lower salt concentrations of approximately 20 g/L by the endpoint, is a particularly noteworthy phenomenon. Note that the relative differences in PC values shown in Figure 4-1 among the metabolites across all polymers correspond to their relative lack of affinity towards the aqueous phase, exemplified by their $K_{O/W}$ values (Table 4-2), which is directly related to their relative affinity for the polymer (Figure 4-1). That is, the more hydrophobic a solute is, indicated by a higher $K_{O/W}$ value, the more affinity it exhibits towards the polymer phase. In each case, the additional osmotic pressure from the aqueous phase salts is constant, and the differences in affinity among the polymers towards the target molecules are due to the different compositions of the polymers.

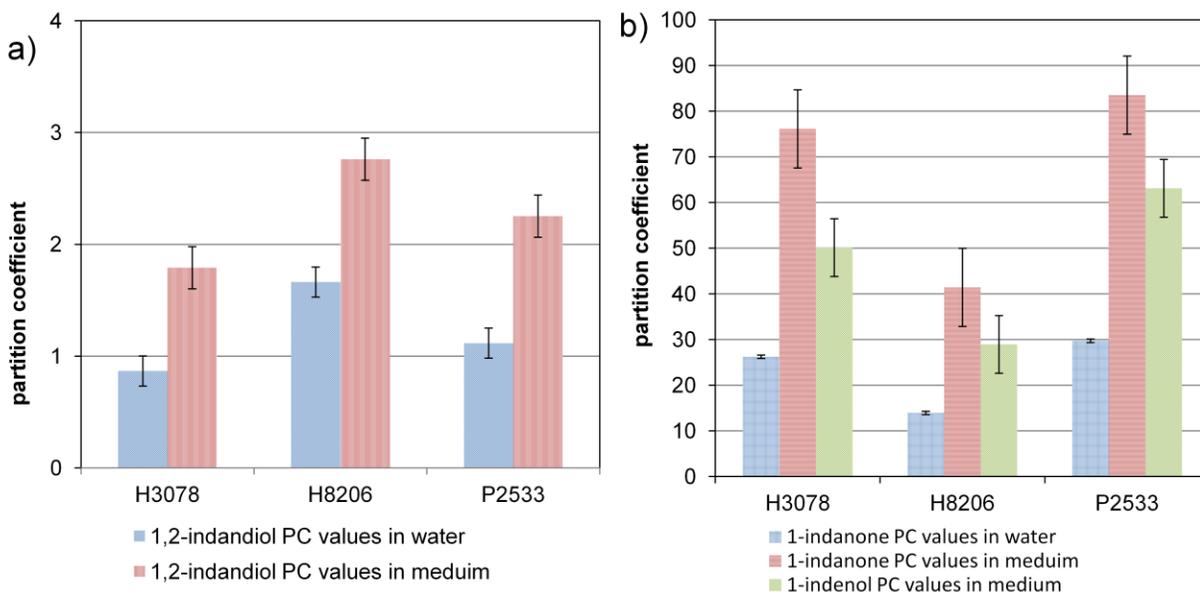


Figure 4-1a) Partition coefficient values of *cis*-(1*S*,2*R*)-indandiol measured in high-purity water or fermentation medium in the three polymers. Error bars represent the standard deviation within the triplicate samples for each metabolite.

Figure 4-1b) Partition coefficient values of 1-indanone measured in high-purity water or fermentation medium, and 1-indenol PC values measured in fermentation medium in the three polymers. Error bars represent the standard deviation within the triplicate samples for each metabolite.

4.5.1.3 Effect of soft segment proportion on partition coefficients

As discussed previously, the polymers employed in this investigation share segmented block architecture, containing soft, amorphous segments which allow solute absorption and permeation within the bulk polymer mass. This mechanism is distinct from the well-established use of glassy, styrene-divinylbenzene-based adsorbent resins upon which functionality may be grafted, and which engage solutes by surface interaction rather than dissolution and permeation [11]. This distinction is important in understanding the performance of these materials, and the following sections discuss the effects of this feature.

We assume that both Hytrel® grades are composed of approximately 50 wt% hard poly(ester) and 50 wt% soft poly(ether) [24], because hard segment proportion has been shown to have a dominant influence on physical and mechanical properties [25], and both grades share very similar glass transition

temperatures (Table 4-1). Based on their similar soft segment proportion, differences in target molecule affinity between the two Hytrel[®] grades were therefore due to the type of soft segments rather than the amount available. In contrast to both Hytrel[®] grades, Pebax[®] 2533 contains a much greater proportion (80 wt%) of relatively non-polar, hydrophobic soft segments, poly(tetramethylene oxide), PBO (Table 4-2), and also had higher affinity towards the more hydrophobic metabolite, 1-indenol, than either Hytrel[®] grade, shown in Figure 4-1b, suggesting that the greater proportion of soft segment provided additional absorptive material for solute uptake. The greater proportion of absorptive material in Pebax[®] 2533 did not provide the same advantage towards the hydrophilic metabolite *cis*-(1S,2R)-indandiol. The non-polar, hydrophobic PBO soft segment in Pebax[®] 2533 lacked the polarity to absorb *cis*-(1S,2R)-indandiol as extensively as the more hydrophilic poly(ether) in Hytrel[®] 8206, which was present at only 50 wt%, signifying the importance of soft segment composition in addition to its proportion within the copolymer.

4.5.1.4 *Effect of soft segment composition on partition coefficients*

The relative affinity towards each metabolite among the two grades of Hytrel[®], illustrated in Figure 4-1, showed differences due to the polymers' different soft segment compositions because both grades share an approximately equal soft segment proportion, and differ in polarity. Hytrel[®] 8206 absorbs significantly more water which indicates higher polarity, and had the highest affinity towards the more polar, hydrophilic, non-inhibitory product *cis*-(1S,2R)-indandiol. Hytrel[®] 3078 exhibits relatively low water absorption and thus has low polarity (Table 4-1), and exhibited higher affinity towards the less-polar, hydrophobic inhibitory by-product, 1-indenol. The polarity difference between the two grades of Hytrel[®], while having soft segments present in similar amounts, explains the dissimilar water uptake among the polymers, and also explains the different affinity towards the two target molecules of varying polarity, shown in Figure 4-1.

Additionally, a more polar, hydrophilic polymer may provide higher affinity towards relatively hydrophilic target molecules such as *cis*-(1S,2R)-indandiol due to the presence of water within the polymer. This effect has previously been systematically demonstrated for a polymer absorbing solutes under varying controlled moisture conditions, where absorption of hydrophilic solutes was preferentially enhanced at higher polymer water contents [26]. However, in a bioreactor, polymer composition is the only way to control water uptake.

There are two aspects of block copolymers to evaluate in order to achieve favorable partitioning of a particular target molecule: the soft segment present must be a relatively large proportion of the material, and it should be composed of a poly(ether) which has affinity for the target molecule.

4.5.2 Bioreactor experiments

4.5.2.1 Biomass

The *cis*-(1S,2R)-indandiol concentrations in these experiments were similar to our previous experiment comparing Hytrel® 8206 to a liquid extractant [7], yet the maximum biomass concentration was approximately 50% higher here at over 4 g/L compared to 2.7 g/L previously in the same growth period. Biomass growth curves were very similar between bioreactor experiments, and preliminary data showed reproducible productivity. The previous experiment's polymer loading method using indene dissolved in IPA, a good solvent for indene, resulted in the exposure of the culture to released IPA, which is water-miscible, upon addition of the polymer to the bioreactor. To avoid biocatalyst exposure to the toxic solvent, the present method using a dispersed indene phase in water with the polymer was developed, resulting in higher biomass levels due to the lack of IPA exposure. This procedure mimicked the direct loading of indene into hydrophobic silicone oil in the original Merck & Co. process while allowing good mixing of the polymer. The similar *cis*-(1S,2R)-indandiol concentrations despite significantly more biocatalyst suggest that the system was limited in production by the concentrations of

inhibitory metabolites in the aqueous phase, which was addressed in this work using polymers of different composition.

4.5.2.2 Relative polymer affinity

The aqueous concentrations of the non-inhibitory product *cis*-(1S,2R)-indandiol, and the inhibitory by-product 1-indenol, are shown in Figure 4-2 and 4-5, respectively. The bioreactor experiments produced similar aqueous concentrations in each run at early time points; while at later time points, the differences in aqueous product/by-product concentrations due to the polymers' differing compositions became evident: the 1-indenol aqueous concentration profile diverged at approximately 6 h among the different polymers used, while *cis*-(1S,2R)-indandiol reached fairly similar endpoint concentrations at different times.

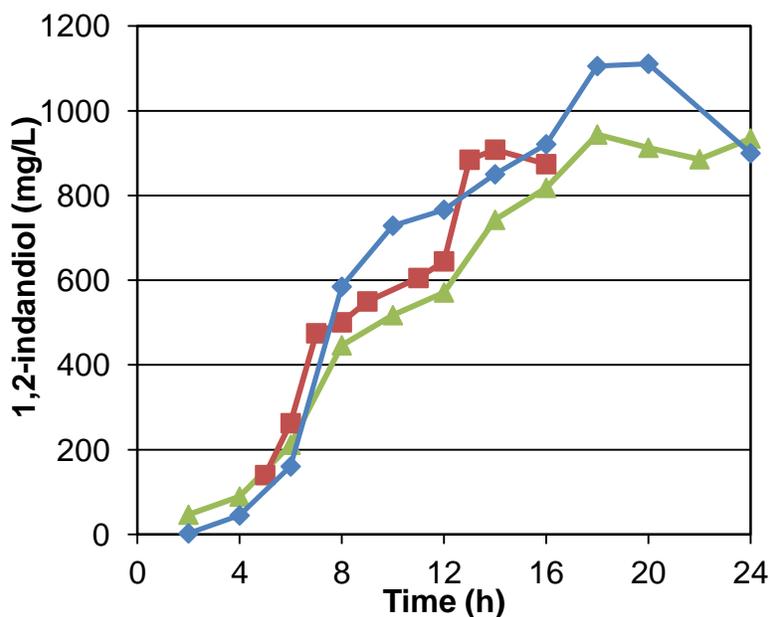


Figure 4-2: Aqueous *cis*-(1S,2R)-indandiol concentration with the three polymer auxiliary phases. Symbols: ■, Hytrel® 8206; ◆, Hytrel® 3078; ▲, Pebax® 2533.

The corresponding concentrations of *cis*-(1S,2R)-indandiol and 1-indenol in the polymer are shown in Figure 4-3 and 4-6, respectively, and the overall (aqueous + polymer) concentrations are shown in Figures 4-4 and 4-7, respectively. The polymer having the highest affinity for the product *cis*-

(1S,2R)-indandiol, Hytrel® 8206, sequestered the largest amount (Figure 4-3), resulting in the highest overall *cis*-(1S,2R)-indandiol concentration (Figure 4-4). Similarly, Pebax® 2533 sequestered the most by-product, 1-indenol (Figure 4-6), and that experiment produced significantly more of that metabolite overall (Figure 4-7). The bioreactor experiments have shown that the differences in affinity towards the target molecules, due to the polymers' distinct proportion and composition of soft segments, are responsible for manipulating the metabolite concentrations seen in the aqueous phase and encountered by the biocatalyst. This affected the biocatalyst's production and is responsible for the deviations in the time course plots and the outcomes of the biotransformations.

4.5.2.3 Metabolic flux vs. polymer flux

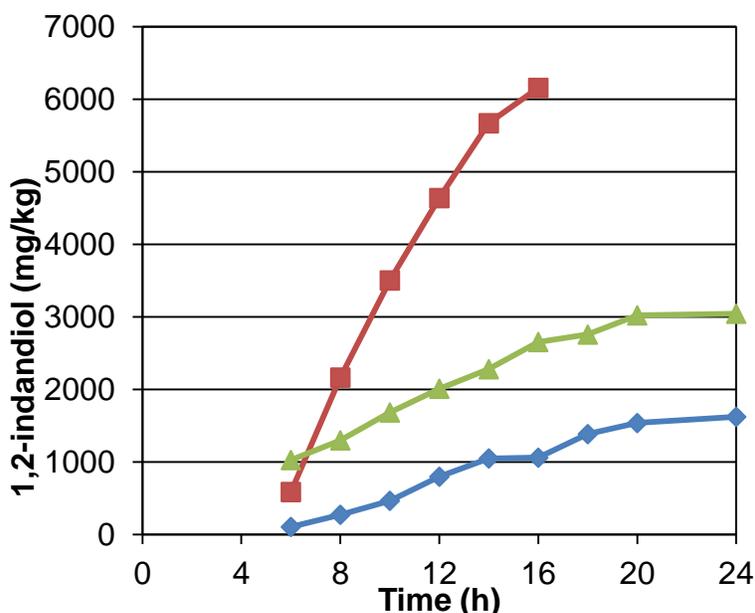


Figure 4-3: *cis*-(1S,2R)-indandiol concentration in the polymers. Symbols: ■, Hytrel® 8206; ♦, Hytrel® 3078; ▲, Pebax® 2533.

The overall (aqueous + polymer) concentration differences between bioreactor runs using different polymers would be expected as a result of the polymers' differential affinity towards the two major metabolites seen in the partition coefficient experiments (Figure 4-1). However, the extent of partitioning out of the aqueous phase in this complex system at equilibrium accounts for only part of the

observed effects occurring *in-situ*, because the metabolic rate of *production* also affects aqueous titre, and presumably depends on both biomass concentration and extent of inhibition. In other words, there are at least two dynamic processes occurring (absorptive removal and metabolic production) that affect the rates of changing concentrations, measured in each phase at any point in time. Furthermore, the relative rate of each process influences the other, resulting in a system with compounded effects. That is, the removal of 1-indenol may allow its further production by the biocatalyst, but the rate of production would depend on the level of biocatalyst inhibition imposed by the presence of aqueous 1-indenol. Because the rates of biocatalyst production and polymer absorption are not equal, the system is in a state of constant feedback, causing multiple, compounded effects.

Because this system features several possible routes for substrate bioconversion with additional minor degradation products, establishing a closed mass balance on the substrate is exceedingly difficult due to different fluxes possible through the various pathways, further affected by the polymers' differential action on aqueous phase concentrations. However, by monitoring the two major, primary products, a reasonable grasp of the relative flux of the substrate can be proposed, as described below. A detailed metabolic flux balance analysis would be required for a complete understanding; however, the influence of a polymer would still skew the expected outcome unless the entire system, including the polymer, could be accurately modeled as a dynamic system.

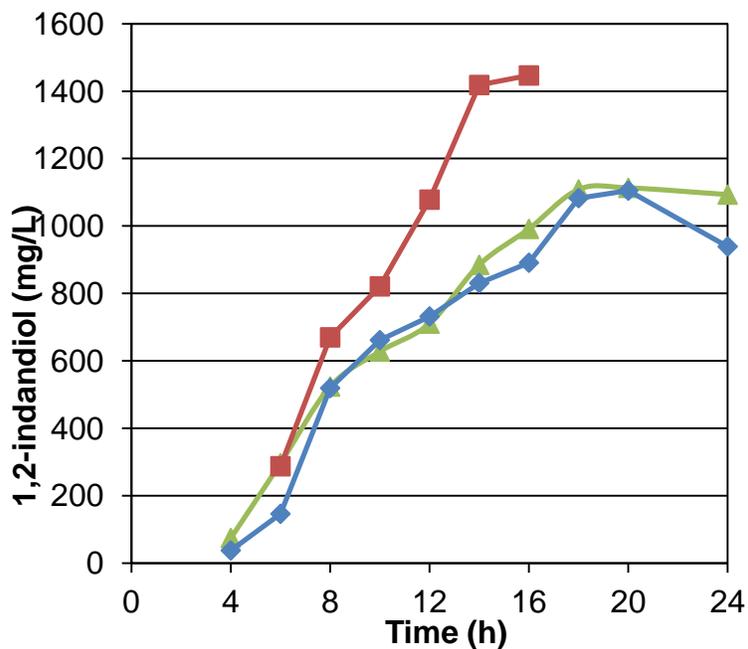


Figure 4-4: Overall *cis*-(1S,2R)-indandiol concentration in the reactor (aqueous and polymer phases combined). Symbols: ■, Hytrel® 8206; ◆, Hytrel® 3078; ▲, Pebax® 2533.

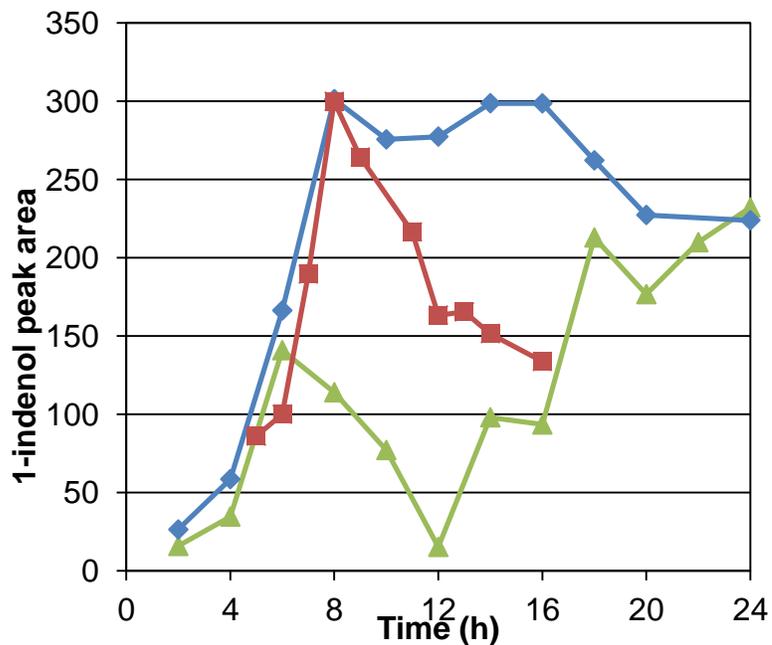


Figure 4-5: Aqueous 1-indenol concentration with the three polymer auxiliary phases. Symbols: ■, Hytrel® 8206; ◆, Hytrel® 3078; ▲, Pebax® 2533.

4.5.2.4 *cis*-(1*S*,2*R*)-indandiol

Being the desired product, a high overall concentration of *cis*-(1*S*,2*R*)-indandiol was the goal of this investigation; however, due to the toxicity of the by-product, 1-indenol, relative to *cis*-(1*S*,2*R*)-indandiol (Table 4-1), it was speculated whether a higher overall *cis*-(1*S*,2*R*)-indandiol concentration would be better achieved by sequestration of *cis*-(1*S*,2*R*)-indandiol itself; or, whether removing 1-indenol, the major inhibitory by-product, would allow a less-inhibited biocatalyst to produce more overall desired product. The accumulation of aqueous *cis*-(1*S*,2*R*)-indandiol in all three experiments, near 1000 mg/L, was similar to the titer obtained by Merck researchers employing a controlled indene feeding strategy in single phase fermentation using an optimized biocatalyst [17], and higher than titers reported for this specific strain [6, 27]. However, these lacked the benefit of additional products being sequestered in the auxiliary phase.

The reason for the continuous accumulation of *cis*-(1*S*,2*R*)-indandiol in both phases until the endpoint of the biotransformation, unlike 1-indenol, is likely due to the fact that it is not a strong inhibitor of the biotransformation, while 1-indenol is a strong inhibitor (Table 4-1). The cells would presumably be less prone to altering their production in response to aqueous concentrations, such that the overall differences in *cis*-(1*S*,2*R*)-indandiol production (Figure 4-4) are more obvious in its accumulation within the polymers (Figure 4-3) due to their differential affinity, despite similar aqueous concentrations (Figure 4-2). The apparently large difference between concentrations of *cis*-(1*S*,2*R*)-indandiol in Hytrel® 8206 and the aqueous phase suggest a greater PC value than was measured in medium. However, it is stressed that PC experiments reach thermodynamic equilibrium while the TPPB system is dynamic, constantly driving towards an ever-changing equilibrium endpoint. The discrepancy could be due to a steep drop in *cis*-(1*S*,2*R*)-indandiol aqueous concentration at the endpoint of the experiment, seen in Figure 4-2, which is impossible to attribute entirely to either a change in the biocatalyst's metabolism or the polymer's action.

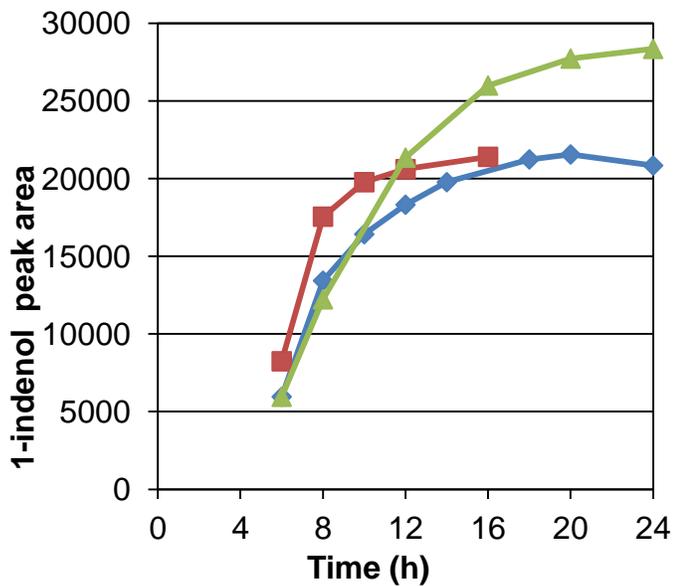


Figure 4-6: 1-indenol concentration in the polymers. (■) Hytrel® 8206 (◆) Hytrel® 3078 (▲) Pebax® 2533.

4.5.2.5 1-indenol

The accumulation of 1-indenol relative to *cis*-(1S,2R)-indandiol was similar to that reported in a previous study with this biocatalyst [6]. However, there is a distinct difference in the pattern of aqueous 1-indenol accumulation beyond relative differences in concentration; the concentration plateaued using Hytrel® 3078; peaked near the same concentration then declined using Hytrel® 8206; and peaked at a lower concentration then increased again using Pebax® 2533 (Figure 4-5). These dynamic differences could be caused by more extensive removal of 1-indenol by Pebax® 2533 compared to either grade of Hytrel®, causing a response by the cells to the lower concentration of toxic 1-indenol. While the aqueous 1-indenol concentration dropped dramatically using Pebax® 2533, Figure 4-6 shows that the concentration in the polymer steadily increased throughout the experiment, indicating that the biocatalyst was less inhibited by 1-indenol due to its sequestration by the polymer, and responded to its removal by producing additional amounts. The rate of production between 8 and 12 h was obscured by the faster rate of removal by the polymer, which was later overcome by biocatalyst production when 1-indenol began to accumulate in the aqueous phase again (Figure 4-5).

The 1-indenol profiles with both Hytrel® grades reached a similar, higher aqueous concentration at 6 h than with Pebax® 2533 (Figure 4-6), approximately corresponding to the polymers' relative partition coefficients towards 1-indenol (Figure 4-1). The deviation in aqueous 1-indenol concentrations between the Hytrel® grades, shown in Figure 4-5, was apparent in its accumulation in the polymer, shown in Figure 4-6, where Hytrel® 8206 absorbed 1-indenol faster than Hytrel® 3078, and again appears to have out-paced the rate of production by the biocatalyst, evident by 1-indenol's decline in the aqueous phase (Figure 4-5). Note that the overall 1-indenol plot (Figure 4-7) closely resembles the polymer 1-indenol plot. The major changes in 1-indenol concentration were occurring in the polymer phase due to the large partition coefficient towards this compound, which overshadows the deviations seen in the aqueous 1-indenol plot.

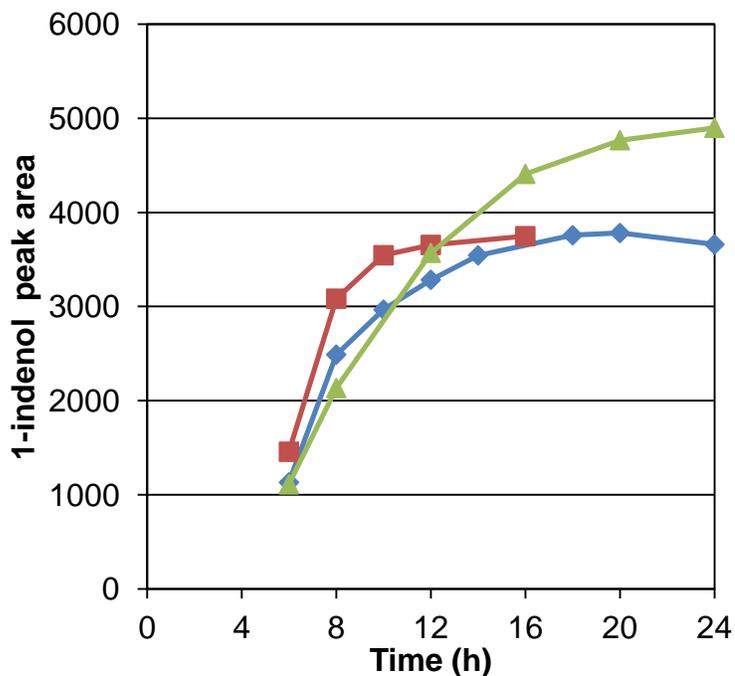


Figure 4-7: Overall 1-indenol concentration in the reactor (aqueous and polymer phases combined). (■) Hytrel® 8206 (◆) Hytrel® 3078 (▲) Pebax® 2533.

4.5.2.6 Rate of production

Among the different polymers, Hytrel® 8206 absorbed both *cis*-(1S,2R)-indandiol and 1-indenol at a faster rate than the hydrophobic polymers, shown in Figures 4-3 and 4-6, respectively. Because this

polymer absorbs a significant amount (30 wt%) of water within its chain network, it is reasonable to assume that water within the swollen network could provide an additional diffusive pathway to a dense polymer containing no water; not only would intermolecular chain interactions be loosened by bound water through plasticization, but the presence of bulk water between the chains could provide a continuous liquid path for solute diffusion which would be more rapid than diffusion through the amorphous solid network. This enhancement may have been equally effective in supplying the substrate to the aqueous phase, improving the biocatalytic rate, and was responsible for the experiment using HytreI® 8206 reaching completion approximately 8 h earlier than the experiments using the other polymers.

4.6 Conclusion

Three different commercial segmented block copolymers, varying in soft segment composition and proportion, exhibited different affinity towards the target molecules, extracting the hydrophilic, non-inhibitory product, and hydrophobic, inhibitory by-product to different extents owing to their different compositions. Water uptake by hydrophilic polymers was found to skew partition coefficient calculations and should be accounted for, and may also provide an expanded network with greater chain mobility, enhancing target molecule affinity and mass transfer to and from the aqueous phase. This study has shown that the addition of a polymer auxiliary phase having affinity towards a target metabolite can selectively enhance a system's productivity, where the polymer's relative affinity towards each metabolite is attributed to its soft segment composition and proportion. By using a polymer having affinity towards a certain metabolite, it was extracted from the aqueous phase, and more of that metabolite was produced by the cells in response to its removal. This metabolic response suggests that simply targeting an inhibitory by-product may not necessarily detoxify a system due to consequent responses in metabolic flux, and this would depend on the biocatalytic pathways' plasticity and ability to divert substrate. However, this also points towards targeting multiple, individual molecules

by using multiple, different polymers in amounts chosen based on the biotransformation's specific metabolic profile.

4.7 Acknowledgements

The financial support of the National Sciences and Engineering Research Council of Canada is gratefully acknowledged, as well as DuPont Canada's generous contribution of polymers.

4.8 References

1. Straathof AJJ (2003) Auxiliary Phase Guidelines for Microbial Biotransformations of Toxic Substrate into Toxic Product. *Biotechnol Prog* 19:755-762
2. Prpich GP, Daugulis AJ (2007) A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. *Biotechnol Bioeng* 98:1008-1016
3. Gao F, Daugulis AJ (2009) Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid Two-Phase Partitioning Bioreactor system by *Kluyveromyces marxianus*. *Biotechnol Bioeng* 104:332-339
4. Khan TR, Daugulis AJ (2010) Application of solid-liquid TPPBs to the production of L - phenylacetylcarbinol from benzaldehyde using *Candida utilis*. *Biotechnol Bioeng* 107:633
5. Jain AN, Khan TR, Daugulis AJ (2010) Bioproduction of benzaldehyde in a solid-liquid two-phase partitioning bioreactor using *Pichia pastoris*. *Biotechnol Lett* 1-6
6. Connors N, Prevoznak R, Chartrain M, Reddy J, Singhvi R, Patel Z, Olewinski R, Salmon P, Wilson J, Greasham R (1997) Conversion of indene to *cis*-(1S),(2R)-indandiol by mutants of *Pseudomonas putida* F1. *J Ind Microbiol Biotechnol* 18:353-359
7. Dafoe JTS, Daugulis AJ (2011) Bioproduction of *cis* - (1S, 2R) - indandiol, a chiral pharmaceutical intermediate, using a solid-liquid two - phase partitioning bioreactor for enhanced removal of inhibitors. *J Chem Technol Biot* 86:1379-1385
8. Parent JS, Capela M, Dafoe JT, Daugulis AJ (2012) A first principles approach to identifying polymers for use in two - phase partitioning bioreactors. *J Chem Technol Biot* 87:1059-1065
9. Huang J, Zhou Y, Huang K, Liu S, Luo Q, Xu M (2007) Adsorption behavior, thermodynamics, and mechanism of phenol on polymeric adsorbents with amide group in cyclohexane. *J Colloid Interface Sci* 316:10-18
10. Aiken G, Thurman E, Malcolm R, Walton HF (1979) Comparison of XAD macroporous resins for the concentration of fulvic acid from aqueous solution. *Anal Chem* 51:1799-1803
11. Phillips T, Chase M, Wagner S, Renzi C, Powell M, DeAngelo J, Michels P (2013) Use of in situ solid-phase adsorption in microbial natural product fermentation development. *J Ind Microbiol Biotechnol* 40:1-15

12. Nielsen DR, Prather KJ (2009) In situ product recovery of n-butanol using polymeric resins. *Biotechnol Bioeng* 102:811-821
13. Rehmann L, Sun B, Daugulis AJ (2007) Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. *Biotechnol Prog* 23:814-819
14. Gao F, Daugulis AJ (2010) Polymer-solute interactions in solid-liquid two-phase partitioning bioreactors. *J Chem Technol Biot* 85:302-306
15. Isaza PA, Daugulis AJ (2009) Ultrasonically enhanced delivery and degradation of PAHs in a polymer-liquid partitioning system by a microbial consortium. *Biotechnol Bioeng* 104:91-101
16. Rehmann L, Prpich GP, Daugulis AJ (2008) Remediation of PAH contaminated soils: Application of a solid-liquid two-phase partitioning bioreactor. *Chemosphere* 73:798-804
17. Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW, Woodley JM (2002) Fed-batch bioconversion of indene to *cis*-(1S,2R)-indandiol. *Enzyme Microb Technol* 31:954-967
18. Morrish JLE, Daugulis AJ (2008) Improved reactor performance and operability in the biotransformation of carveol to carveone using a solid-liquid two-phase partitioning bioreactor. *Biotechnol Bioeng* 101:946-956
19. Ouellette J, dos Santos SCC, Lépine F, Juteau P, Déziel E, Villemur R (2013) High absorption of endocrine disruptors by Hytrel: towards the development of a two - phase partitioning bioreactor. *J Chem Technol Biot* 88:119-125
20. Ping Z, Nguyen Q, Chen S, Zhou J, Ding Y (2001) States of water in different hydrophilic polymers—DSC and FTIR studies. *Polymer* 42:8461-8467
21. Kannan D, Duda J, Danner R (2005) A free-volume term based on the van der Waals partition function for the UNIFAC model. *Fluid Phase Equilib* 228:321-328
22. Malinowski JJ, Daugulis AJ (1994) Salt effects in extraction of ethanol, 1-butanol and acetone from aqueous solutions. *AIChE J* 40:1459-1465
23. Khan TR, Daugulis AJ (2011) Medium composition effects on solute partitioning in solid-liquid two-phase bioreactors. *J Chem Technol Biot* 86:157-160
24. Prpich GP, Daugulis AJ (2004) Polymer Development for Enhanced Delivery of Phenol in a Solid-Liquid Two-Phase Partitioning Bioreactor. *Biotechnol Prog* 20:1725-1732
25. Lilaonitkul A, West JC, Cooper SL (1976) Properties of poly (tetramethylene oxide)-poly (butylene terephthalate) block polymers. *J Macromol Sci Phys* 12:563-597
26. Aucejo S, Pozo MJ, Gavara R (1998) Effect of water presence on the sorption of organic compounds in ethylene-vinyl alcohol copolymers. *J Appl Polym Sci* 70:711-716
27. Buckland BC, Drew SW, Connors NC, Chartrain MM, Lee C, Salmon PM, Gbewonyo K, Zhou W, Gailliot P, Singhvi R, Olewinski Jr RC, Sun W, Reddy J, Zhang J, Jackey BA, Taylor C, Goklen KE, Junker B, Greasham RL (1999) Microbial Conversion of Indene to Indandiol: A Key Intermediate in the Synthesis of CRIXIVAN. *Metab Eng* 1:63-74

28. Trong Nguyen Q, Sublet J, Langevin D, Chappey C, Marais S, Valleton J, Poncin-Epaillard F CO₂ Permeation with Pebax[®]-based Membranes for Global Warming Reduction. In: B Freeman, Y Yampolskii, (Eds.) Membrane Gas Separation, 2010, Wiley. Hoboken NJ, pp 255-277
29. Tomei MC, Rita S, Angelucci DM, Annesini MC, Daugulis AJ (2011) Treatment of substituted phenol mixtures in single phase and two-phase solid–liquid partitioning bioreactors. J Hazard Mater 191:190-195
30. Amanullah A, Hewitt CJ, Nienow AW, Lee C, Chartrain M, Buckland BC, Drew SW, Woodley JM (2003) Measurement of strain-dependent toxicity in the indene bioconversion using multiparameter flow cytometry. Biotechnol Bioeng 81:405-420

Web References

- [31] <http://plastics.dupont.com/plastics/dsheets/hytre/GBHYTREL3078.pdf>; Last Accessed 28 Feb 2013
- [32] http://www2.dupont.com/Plastics/en_US/assets/downloads/processing/236359a.pdf; Last Accessed 28 Feb 2013
- [33] <http://www.pebax.com/export/sites/pebax/.content/medias/downloads/literature/tds-pebaxmed-2533sa01.pdf>; Last Accessed 28 Feb 2013
- [34] <http://www.matweb.com>; Last Accessed 28 Feb 2013
- [35] <http://www.chemspider.com>; Last Accessed 28 Feb 2013

Chapter 5 Production of 4-valerolactone by an equilibrium-limited transformation in a partitioning bioreactor: impact of absorptive polymer properties

Julian T. Dafoe, Andrew J. Daugulis

With minor changes to fulfill formatting requirements, this chapter is substantially as it appears in:
Biosystems and Bioprocess Engineering (2013) DOI: 10.1007/s00449-013-1020-5

5.1 Preface to Chapter 5

The previous chapters have focused on the potential for extraction of relatively polar, hydrophilic target molecules which demonstrate no affinity towards liquid solvents but do partition into some solid polymers. This approach was applied to an equilibrium-limited system producing an extremely hydrophilic, water-miscible target molecule, 4-valerolactone (4VL) which is produced via extracellular lactonization of 4-hydroxyvalerate (4HV).

The equilibrium-limiting feature of this system, in contrast to inhibition-limited systems, provides the opportunity to generate different equilibrium positions, depending on pH and relative product (4VL) and precursor (4HV) concentrations. The equilibrium position can be shifted *via* extraction of 4VL, thereby “pulling” the position further towards production as the equilibrium system immediately compensates by producing more 4VL. This is feasible because the system is not inhibited in the classical sense, and does not lose performance during the periods of equilibrium limitation. This demonstration of equilibrium-pulling *via* extraction with an absorbent polymer is the first such report in the literature.

This chapter also demonstrated that for extraction of 4VL using absorbent polymers, relatively polar functional groups having a sufficiently high Hildebrand solubility parameter comprising the absorptive, “soft” regions of a polymer can provide chemical affinity towards the polar target molecule. Such polar polymers are typically soluble in water, such that the nature of the polymer network providing resistance to extensive water uptake, contributed by the non-absorptive crosslinks or “hard” segments, is an important aspect. In this respect, several random copolymers and a commercial segmented block copolymer, all exhibiting affinity towards 4VL, were evaluated for water uptake. It was found that while water uptake promoted overall 4VL affinity, it also permitted nonselective solute uptake which hampered selectivity relative to 4HV. This phenomenon was demonstrated *in situ*, where

a highly water-absorbing polymer failed to pull the 4VL/4HV equilibrium appreciably, while a moderately water-absorbing one resulted in the production of 30% more 4VL.

5.2 Abstract

The biotransformation of levulinic acid to 4-valerolactone (4VL) is pH-dependent and equilibrium-limited, distinct from the more common irreversible biotransformations that are constrained by product toxicity or biocatalyst inhibition. Our processing strategy for this system was to selectively remove the product, 4VL, which is in equilibrium with its precursor, 4-hydroxyvalerate (4HV), in order to pull the reaction to a greater extent of conversion. 4VL is challenging to separate from the aqueous phase due to its water-miscibility, necessitating the use of water-absorbing polymers to provide affinity towards the hydrophilic product. Manipulating the composition of copolymers, thereby varying the architecture of polymer chains, conferred drastically different extents of water absorption and caused different biotransformation outcomes. A custom-synthesized random copolymer designed to maximize the proportion of material with affinity for the solute had high water uptake, which resulted in poor selectivity for the target molecule relative to its precursor. Conversely, a moderately water-absorbing commercial segmented block copolymer, Hytrel® 8206, demonstrated selectivity towards 4VL relative to its precursor, 4HV, and increased 4VL production by approximately 30% by shifting the equilibrium towards the product. This work has shown that water absorption is an important, previously-neglected criterion in evaluating polymer affinity and selectivity towards hydrophilic target molecules.

Keywords: polymers, ISPR, two-phase biocatalysis, equilibrium, extractive fermentation

5.3 Introduction

The production of 4-valerolactone (4VL) from renewable levulinic acid has received increasing attention as a potential “green” platform chemical and transportation fuel, yet investigations have focused almost exclusively on thermo-chemical processes requiring the use of solvents, harsh conditions and expensive catalysts [1-3]. Although engineered biocatalysts operating under mild aqueous conditions could provide alternative routes to such building blocks molecules, the yield and productivity of biological systems are generally lower than chemical catalysis due to substrate and/or product inhibition which can range from simple feedback inhibition to toxic cellular deactivation [4]. The bioproduction of 4VL is characterized by a different limiting feature, however, as it is an equilibrium-governed biotransformation whose performance could potentially be improved by selectively removing the product, 4VL, from the aqueous phase, thereby pulling the equilibrium reaction to greater conversion. Recently, a hybrid chemo-enzymatic approach using an ion-exchange resin catalyst, followed by two isolated enzyme-catalyzed steps in a solvent-based membrane reactor, rapidly produced enantiomerically-pure 4VL to a high yield on a small scale [5]. This approach, while extremely rapid and specific, suffers from additional costs of the specialized resin, isolated enzymes, cofactors, solvents and their distillation for re-use, which may be justifiable in the production of a high-value product, but limits applicability in processes intended to produce commodity products.

Aiming to minimize additional costs and maintain simple operation, in this study we investigate the effect of applying inexpensive polymer extractants to the whole-cell biotransformation of levulinic acid to 4-valerolactone by engineered *Pseudomonas putida* KT2440. This system operates via the action of two recombinant enzymes to first form 4-hydroxyvalerate (4HV), a hydroxyacid intermediate, which is subsequently converted to its lactone form in the extracellular medium by a paraoxonase (PON1) enzyme attached to the outer cell membrane surface [6]. The 4HV-4VL equilibrium reaction catalyzed by PON1 proceeds in either direction, governed by the relative concentrations of hydroxyacid and lactone

in the medium, based on their relative stability at a given pH [7, 8]. The equilibrium position favors 4HV at neutral pH, but shifts approximately 5-fold towards 4VL at pH 6 [6]. Because the PON1 enzyme is located on the membrane surface, the equilibrium position is adjustable by manipulating medium pH, whereas an intracellular enzyme would be inaccessible to pH manipulation [6]. While changing pH from 7 to 6 shifts the equilibrium position towards 4VL while preserving biocatalyst integrity, we are proposing to selectively remove 4VL from the aqueous phase relative to its hydroxyacid precursor, 4HV, which carries a negative charge, to further shift this equilibrium and bring about a greater extent of conversion. The necessary selectivity is dependent upon the ability of the auxiliary phase to absorb the neutral product, 4VL, while the ionized intermediate, 4HV, should remain largely unabsorbed in the aqueous phase if selective absorption is occurring rather than the nonselective uptake of fermentation medium.

The nature of a target molecule determines potential strategies for extraction. Hydrophobic, relatively apolar target molecules are easily extracted by immiscible solvents that are tolerated by the biocatalyst [9], or by adsorption on macroporous resins via hydrophobic interactions [10, 11]. Conversely, relatively hydrophilic target molecules have been separated using aqueous two-phase systems (ATPS), consisting of combinations of polymers, salts, ionic liquids, or surfactants which phase-separate under specific operating conditions, yielding a phase rich in the target molecule. These approaches require expensive components and are limited to biocompatible materials [12, 13].

A unique strategy, which we have adopted in this investigation, is the use of absorbent polymers featuring a soft, amorphous polymer network. These materials are phase-stable, inexpensive, and can range from hydrophobic rubbers to hydrophilic gels containing mostly water, depending on their composition. Using absorbent polymers obviates the requirement for biocompatibility screening, as the polymer phase is inert with respect to the biocatalyst, and the application is straightforward, requiring no process modifications aside from the addition of the polymer. The mechanism of solute uptake by

amorphous polymers above their glass transition temperature (T_g) is via absorption, identical to solvent extraction, with relative concentrations described by distribution coefficients and the equilibrium position not being surface area-dependent [14]. This is distinct from surface adsorption described by sorption isotherms which are commonly seen in the use of macroporous resins having high T_g s [15].

Previously, commercially-available absorptive polymers were used for *in-situ* product removal (ISPR) of relatively hydrophobic target molecules from fermentation medium with excellent productivity enhancements [16-18]; however, many important target molecules are hydrophilic as judged by the octanol-water partition coefficient, limiting the extent to which they can be separated from water. The $\text{Log}K_{o/w}$ value for 4VL is calculated as being between -0.6 and 0.6, however it is clearly hydrophilic due to its miscibility with water. While hydrophobic target molecules require a small auxiliary phase ratio to achieve observable effects, a 30% phase ratio was chosen to provide adequate removal of the desired product, 4-valerolactone. The vast majority of absorptive polymers used in TPPB systems are segmented block copolymers containing amorphous poly(ether) soft segments with low T_g , which are mechanically stabilized by hard segments having high T_g or highly crystalline domains comprised of poly(ester), poly(amide), or poly(urethane). In this architecture, the hard segment, ostensibly inert with respect to target molecule absorption, is required only in the amount sufficient to mechanically stabilize the polymer, while the soft segment provides the necessary solute affinity for target molecule absorption [19]. It is therefore desirable to maximize the proportion of soft material available for absorption while incorporating the minimum amount of inert, hard material sufficient to preserve desirable physical properties.

The chemical composition of a copolymer's soft segment determines its affinity towards a target molecule. Hildebrand's solubility parameter (δ , $\text{MPa}^{1/2}$) describes the compatibility of chemical species according to regular solution theory, and materials with similar δ values will generally be miscible [19]. Solubility parameters are tabulated for many polymers and small molecules, and poly(ethylene oxide)

has the highest solubility parameter ($\delta \approx 20 \text{ MPa}^{1/2}$) within the poly(ether) series, is closest to that of 4VL ($\delta \approx 22 \text{ MPa}^{1/2}$) [20], and therefore should provide mutual affinity. Polymer δ values can be increased via higher polymer polarity, but this increases internal polar interactions at the expense of polymer chain mobility, and thereby reduces target molecule absorption by creating a tighter network [19]. To circumvent this, a polymer's polarity may be increased while maintaining flexible chain architecture through plasticization by absorbed water [19]. Water's exceptionally high solubility parameter ($\delta \approx 48 \text{ MPa}^{1/2}$), due to its strong hydrogen bonds, precludes meaningful comparisons of its solubility parameter value with those of other compounds, however, the incorporation of water in a polymer may increase its overall solubility parameter, providing a more favorable environment for hydrophilic molecules [19]. An early study on improving polymers for the extraction of relatively hydrophilic target molecules in TPPBs identified water absorption as an important characteristic in conferring affinity towards polar solutes, however, the target molecule, phenol, was relatively hydrophobic ($\text{Log}K_{O/W} = 1.5$), and water uptake in the polymers reached maxima of only about 10 wt% [21].

The effect of high water content within an absorbent polymer is not well understood in the context of TPPBs, as the presence of a continuous water phase within the material is expected to impose a tradeoff between target molecule affinity and selectivity [22]. The extent to which water exists as a polymer-associated, "bound" complex as opposed to a continuous "bulk" water phase depends on the polymer's composition and is not easily discerned [23]. Instead, water uptake is generally, and superficially, described as the percent of total mass gained upon swelling. Therefore, although water can improve affinity towards hydrophilic solutes, it may also cause poor selectivity relative to other hydrophilic solutes by providing an expanded network which is more available for permeation of all solutes.

In this work, the biotransformation of levulinic acid to 4VL serves as a model equilibrium-limited biocatalytic system for equilibrium pulling via product removal using absorbent polymers. We believe that this work reports the first attempt to use *absorbent* polymers to *pull* an equilibrium-governed biocatalytic system, despite demonstrations of equilibrium shifting by extraction using *adsorbent* porous resins or molecularly-imprinted polymers, which operate *via* surface interaction mechanisms, in contrast to the solution-diffusion mechanism occurring within absorbent materials [8, 24-25].

To achieve this objective, polymer composition, specifically the amount and type of stabilization provided to the polymer network, was tailored to provide different architectures, and the effect of these different architectures on water uptake was investigated in a range of polymers. One commercial, hydrophilic poly(ether)-poly(ester) segmented block copolymer, HytreI® 8206, was compared to two custom-made, random copolymers, crosslinked poly(ethylene glycol diacrylate) (XLPEGDA) and N,N'-m-phenylenedimaleimide-crosslinked poly(ethylene oxide) (BMI-PEO). These latter polymers are comprised of poly(ethylene oxide) (PEO) as the active material based on affinity (similar solubility parameter) towards the hydrophilic target molecule 4VL, and differ in their architecture (nature of network stabilization) that would affect physical properties and degree of water uptake.

5.4 Materials and Methods

5.4.1 Chemicals

All chemicals were from Sigma-Aldrich (Canada) or Fisher (Canada) with the exception of the polymers. HytreI® 8206, a segmented block copolymer of poly(ether) and poly(ester) of proprietary composition, with water uptake of 30%, was from DuPont (Canada). Poly(ethylene oxide) (PEO), 5×10^6 g/mol and polyethylene glycol diacrylate (PEGDA), 258 g/mol, the starting materials for the custom-synthesized polymers, BMI-PEO and XLPEGDA, were from Scientific Polymer Products (New York). Because it is not commercially available, the 4-hydroxyvalerate (4HV) analytical standard was prepared by saponification of a 100 mM 4VL solution assuming equimolar conversion [26].

5.4.2 Microorganism

P. putida KT2440 harboring the genes *tesB* and *PON1* for this biotransformation was kindly supplied by Dr. Kristala Prather of the Massachusetts Institute of Technology, Cambridge, MA. The culture was grown for 24 h in Lysogeny Broth (LB) supplemented with 10 mg/L tetracycline and 20 mg/L gentamycin for plasmid maintenance, and frozen in 1 mL aliquots containing 10% (v/v) glycerol at -80°C.

5.4.3 Polymer synthesis

5.4.3.1 XLPEGDA

This copolymer was synthesized to provide a PEO-containing network, providing similar 4VL affinity to the commercial Hytrel® 8206, while exhibiting a range of water uptake due to varying network stabilization, in order to compare its affect on affinity. XLPEGDA, comprised of 258 g/mol polyethylene glycol diacrylate, was crosslinked with 0.1 wt% 1-hydroxycyclohexyl phenyl ketone (HCPK) photoinitiator relative to prepolymer mass, by UV-irradiation in a covered glass mold (2 mm x 75 mm x 25 mm) with a mirror surface for 120 s at 5 mW/cm². Varying amounts of water (0, 20, or 40%) were included in the prepolymer solution to obtain different crosslink degrees while maintaining an identical chemical composition, giving a range of materials with different water uptake levels. The materials' designations corresponding to the weight percent of PEGDA present in the prepolymer solution [22].

5.4.3.2 BMI-PEO

A second copolymer was synthesized to provide a range of materials having the maximum feasible amount of "active", soft PEO content available for solute absorption (i.e. maximizing affinity), and minimizing the presence of inert material in the copolymer. BMI-PEO was synthesized using 250 g high MW poly(ethylene oxide) which was solution-coated with 0.04 wt% dicumyl peroxide dissolved in acetone and dried overnight. 1, 3, or 5% N,N'-m-phenylenedimaleimide (BMI) was added then blended in a slurry and cured at 160 °C for 30 minutes in a press. The polymer was cut into cubes of about 3 mm. The materials' designations correspond to the weight percent of BMI added to the prepolymer slurry.

5.4.4 Water uptake

As all polymers were expected to have moderate to considerable water uptake, triplicate samples of each polymer (ca. 1 g) were dried in an oven at 60°C for 24 hours and weighed on an analytical balance. The polymer samples were immersed in an excess volume of high-purity water of bioreactor medium as indicated in text, and shaken in 50 mL centrifuge tubes at 180 RPM at room temperature, periodically separated from the liquid, dried, and weighed to determine the extent of water uptake before being returned to the liquid until there was no significant change in mass at successive times. Water uptake was determined by equation 1:

$$\% \text{ water uptake} = \frac{M_{wet} - M_{dry}}{M_{dry}} * 100 \quad (1)$$

5.4.5 Polymer distribution coefficients

Polymer distribution coefficients towards the target molecule, 4VL, were determined in triplicate using a known mass of hydrated polymer, pre-swollen for 24 h in high-purity water, and added to a vial containing 10 mL of 3 g/L 4VL in high-purity water. Control vials without polymer were treated identically and the mass of absorbed 4VL was determined by mass balance calculation. The use of high-purity water eliminated potential osmotic effects on phase ratios due to solutes. The distribution coefficient of 4HV was not determined in high-purity water because its preparation required significant use of salts, which are known to skew distribution coefficient values [27], and would not provide results comparable to experiments performed using high-purity water.

Distribution coefficients for 4HV and 4VL were calculated in the bioreactor experiments using the concentration of the target molecule in the polymer, determined by solute desorption in high-purity water, and the concentration measured in the aqueous phase. The polymer mass was corrected by calculating its swollen mass in the reactor, and an aqueous phase density of 1000 kg/m³ was assumed to simplify comparisons with high-purity water.

5.4.6 Biotransformations

The biotransformation of levulinate to 4VL proceeded in four periods: growth period, 4HV accumulation period, 4VL production period, and ISPR period. The ISPR period is the focus of this investigation, and the experiments were conducted in the same manner except for the type of polymer that was added during the ISPR period.

Cells were grown from frozen stock for 24 h at 32^oC and 180 RPM in six 50mL shake flasks containing LB as described above. The bioreactor (BioFlo III, 5L vessel, New Brunswick Scientific, Edison NJ) contained 2.6 L Terrific Broth (TB): 12 g/L tryptone; 24 g/L yeast extract; 1.1 g/L KH₂PO₄; 4.7 g/L K₂HPO₄; 0.48 g/L MgSO₄·7H₂O, which was amended with antibiotics as above, and 1 mL/L of trace mineral solution, containing 22 g/L CaCl₂ and 0.1 g/L ferric ammonium citrate. Glucose was autoclaved separately as a 500 g/L solution and 120 mL was added prior to inoculation, giving 20 g/L. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM working concentration) was added for gene induction.

Aeration at 1.3 VVM during cell growth, was reduced to 0.3 VVM during stationary phase to minimize foaming. Dissolved oxygen (DO) was maintained above 20% of saturation. Antifoam 204 (Sigma-Aldrich) was added as required. pH was automatically controlled with 5M KOH and 5M H₂SO₄.

5.4.6.1 *Cell growth period and 4HV accumulation period*

The cells were grown at pH 7.0, 32 °C and 800 RPM during the first 8 hours until cell density peaked near 14 g/L CDW and DO rose rapidly, corresponding to the depletion of glucose. At the onset of stationary phase, agitation was reduced to 650 RPM and substrate feeds were initiated. Glucose (500 g/L) was fed at 1 g/L/h and levulinate (400 g/L, neutralized to pH 7 with 6M KOH and sterile-filtered) was fed at 3.5 g/L/h initially to obtain a high concentration and then adjusted according to the consumption rate to maintain a level between 10 and 20 g/L throughout the 4HV accumulation phase.

5.4.6.2 4VL accumulation period

When 4HV plateaued, the levulinate feed was stopped and the glucose feed rate was cut by half. The pH of the medium was adjusted to 6.0 using bioreactor control in order to shift the pH-dependent equilibrium further towards 4VL, and agitation was reduced in response to lower oxygen demand.

5.4.6.3 ISPR (polymer absorption) period

After 24 h of 4HV conversion to 4VL at pH 6.0, the rate of 4VL accumulation slowed as the system approached an equilibrium between the relative concentrations of 4HV and 4VL. The ISPR period was then initiated by adding a mass of polymer beads to the bioreactor to remove 4VL from the medium and renew the driving force for conversion for an additional 24 h. In the BMI-PEO experiment, 240 g of dry BMI-PEO pellets were added to the bioreactor. In the Hytrel® experiment, 930 g of dry Hytrel® 8206 pellets were added. The mass of dry polymer added was determined based on measured equilibrium water uptake in the bioreactor medium to provide a similar swollen mass of polymer in each experiment. Dry polymers were added because pre-swollen polymers of drastically different water contents would skew aqueous concentrations to varying degrees and introduce unacceptable errors.

5.4.7 Analytics

Biomass was monitored by optical density at 600 nm and correlated to cell dry weight (CDW, g/L). Glucose was monitored using the DNS assay [28]. Bioreactor medium samples were centrifuged at 16 KG for 5 minutes, and 100 µL of a 10 wt% H₂SO₄ solution was added to 900 µL supernatant and centrifuged again at 16 KG for 5 minutes to coagulate proteins and ensure protonation of all analytes. Polymer samples were periodically removed from the bioreactor after the initiation of ISPR, their surface rinsed briefly with high-purity water, then placed in 5 mL high-purity water for 24h to extract absorbed compounds. This desorption procedure was repeated twice for each polymer sample to ensure complete extraction, as there were no detectable desorbed compounds in the third desorption. Desorption samples were acidified prior to analysis in the same manner as the bioreactor medium samples. Liquid samples were passed through a 0.2 µm Nylon syringe filter prior to injecting

100 μL through a 20 μL sample loop. Bioreactor medium samples were run both undiluted and diluted 1 : 10 in high-purity water. The mobile phase was high-purity water with 0.1% H_3PO_4 and 5% acetonitrile at 1 mL/min. Separation was performed on an Agilent Zorbax SB-Aq 3.5 μm 4.6x150 mm column at 65°C, with detection using a Varian 356 Refractive Index detector at 35°C.

5.5 Results and Discussion

5.5.1 Polymer extractant characteristics

We chose to use inexpensive absorbent polymers as the sequestering phase because they can provide a range of water uptake capacities by means of different architectures and compositions. The mechanism of solute absorption, via diffusion among mobile chain segments, is distinct from fluid diffusion and surface interactions within the rigid fluid-filled pores of adsorbent resins [29], and similar to the polymer solution interactions occurring in aqueous two-phase systems (ATPS), where the polymer acts as a solvent. Absorbent polymers differ from ATPS in their phase stability as solids or gels, and do not undergo phase-forming transitions in response specific operating conditions, simplifying their implementation.

These characteristics of absorbent polymers place them in a unique, intermediate class between ATPS and adsorbent resins, offering a favorable combination of properties from both types of extractant, with the most attractive features being low cost [30], and ease of implementation. The term “hydrogel” is often used to describe polymers exhibiting significant water uptake, however this term lacks concrete boundaries and may include any polymer exhibiting water uptake [15].

5.5.2 Water uptake experiments

These experiments were conducted in order to compare the effect of the different copolymer architectures on water uptake, with the same active component in both synthesized polymers being poly(ethylene oxide) (PEO), chosen because of its affinity towards the water-miscible target molecule, 4VL based on their similar solubility parameters. The copolymers had either minimal random

crosslinking (BMI-PEO), extensive random crosslinking (XLPEGDA), or segmented block architecture (Hytrel®). The equilibrium water uptake by the synthesized and commercial polymers in high-purity water are shown by the bars in Figure 5-1 (a and b). The 10- to 20-fold difference in water uptake between the polymers is therefore due to the differences in their architecture, i.e. stabilization provided by the inert hard segments (Hytrel®) or crosslinks holding the network together (XLPEGDA and BMI-PEO), preventing the complete dissolution of the water-soluble PEO chains. The distance between crosslinks or hard domain regions has two effects: it determines the length of the poly(ether) chains which are able to move freely and absorb solutes, and in the same fashion also provides a “looser” network corresponding to higher water uptake. The two custom-synthesized copolymers were prepared by randomly crosslinking PEO-containing prepolymers, the difference being the relative amount of crosslinker, which determines the synthesized copolymers’ architecture, and therefore influences its water absorption. BMI-PEO, containing only 1 - 5 wt% inert material (BMI) forming the crosslinks between PEO chains, is highly available for water permeation, and caused this material to absorb the most water. Conversely, XLPEGDA contains terminal acrylate groups comprising approximately 75% of the prepolymer’s mass which become chemically crosslinked to a high extent with adjacent acrylate groups, resulting in a more constrained network that absorbs less water. The inclusion of increasing amounts of water in the prepolymer solution as a diluent increases the distance between adjacent acrylate groups, forcing some to react intramolecularly instead of intermolecularly, and creating “wasted crosslinks” which form a progressively looser network that is more amenable to water absorption [22].

The commercial copolymer, Hytrel® 8206, also contains significant hydrophilic poly(ether) content based on its 30% water absorption, which was the basis for its selection in this study [31]. Although its proprietary composition precludes direct comparison of component monomers with the prepared polymers, its segmented block copolymer architecture, consisting of approximately 50 wt%

inert, hard poly(ester) material, more extensively stabilizes this material against water infiltration than BMI-PEO, and is intermediate among the grades of XLPEGDA, shown in Figure 5-1b.

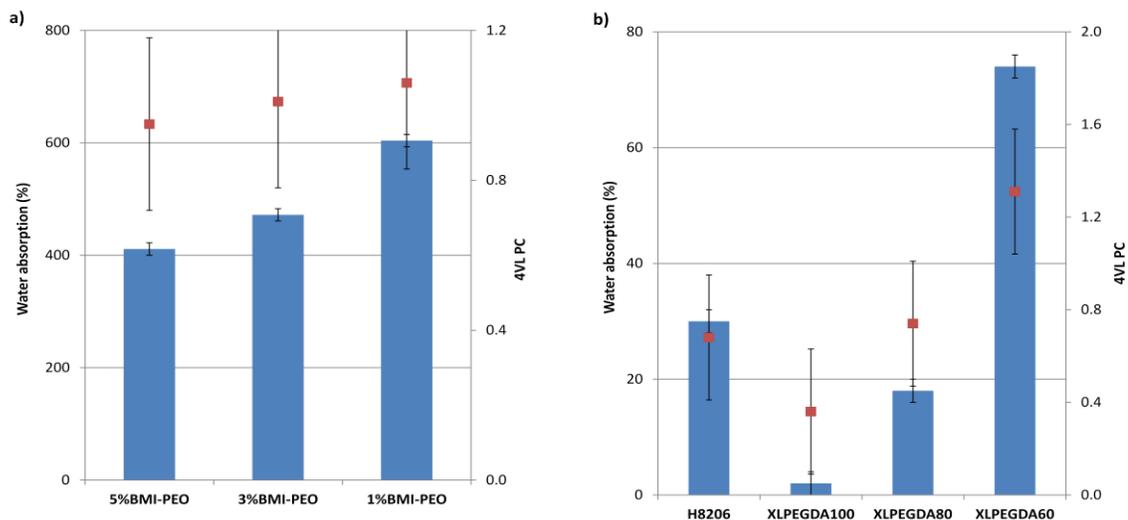


Figure 5-1: Water absorption (bars) and 4VL distribution coefficient values (■) in (a) BMI-PEO or (b) Hytrel® 8206 and XLPEGDA.

The architecture of a copolymer not only affects its ability to take up water, but also affects the physical properties of the swollen material. Of the three materials investigated, only BMI-PEO and Hytrel® had physical properties considered acceptable for use in the high-shear environment of a bioreactor. All grades of XLPEGDA were brittle solids when dry, but absorbed water to become a mucous-like material with poor physical integrity, likely a result of excessive intramolecular crosslinks rather than intermolecular ones, causing poor network integrity despite containing a large proportion of crosslinking material. Conversely, Hytrel® 8206, and all three grades of the BMI-PEO material despite having much higher water uptake, remained tough when swollen, due to their inert stabilizing components being largely intermolecularly bound, providing a more extensively stabilized network than XLPEGDA's significant intramolecular bonds. Physical toughness is an important consideration in sequestering phase material selection, as retrieval of the intact polymer is important for product recovery and polymer re-use. For this reason, only the 1%BMI-PEO, having the highest amount of active material for affinity (PEO), and Hytrel® 8206, were used in subsequent bioreactor experiments.

5.5.3 Distribution coefficient experiments

5.5.3.1 Polymer composition

The PC values of the lab-synthesized polymers, BMI-PEO and XLPEGDA, and the commercial polymer, HytreI® 8206, in high-purity water towards the target molecule, 4VL, are shown in Figure 5-1 (a and b). The swollen BMI-PEO polymers all had a distribution coefficient of approximately 1.0, indicating a nearly equal concentration of 4VL in the polymer and aqueous phases. This value is logical as the material's composition is mostly water when hydrated, such that PEO itself, which possesses affinity for 4VL, is a minor contributor to solute uptake because of its relatively small quantity in the swollen material. Higher 4VL PC values with ever-increasing water uptake within the XLPEGDA and BMI-PEO grades (Figure 5-1) suggests that water within the polymer expands the network, making it available for permeation and provides an aqueous environment for the hydrophilic target molecule, 4VL. Also, this trend shows that water is performing a substantial role in 4VL uptake because the PEO and crosslinker composition in XLPEGDA remains constant through the range aside from water content.

HytreI® 8206 had a lower PC and also lower water absorption than some of the synthesized polymers because this commercial block copolymer is composed of ~50 wt% hard, inert material, which restricts the polymer's ability to absorb water and is not available for solute uptake. Nevertheless, HytreI® 8206 absorbs 30% water which may also provide an expanded network for permeation of hydrophilic target molecules in a similar way to the trend seen within the synthesized polymers.

5.5.3.2 Effect of water uptake on distribution coefficients

The calculation of distribution coefficients using mass balance requires accurate accounting of each phase's mass *at equilibrium* (i.e. after water uptake). Virtually all prior studies evaluating polymers for TPPB applications have not considered, or accounted for, water uptake by polymers, and have unknowingly treated hydrophilic polymers the same as hydrophobic ones [32-34]. A sequestering phase which absorbs a large proportion of water affects such calculations and interpretations of distribution coefficients, with the calculated PC value being artificially higher when water uptake and consequent

changes in phase volumes are neglected. This effect is greatly exaggerated in highly water-absorbing polymers, but the effect is still significant even with modest amounts of water (eg. Hytrel 8206® at 30%).

By calculating the PC values using polymer samples which were pre-swollen for 24 h in high-purity water, potential changes between initial and equilibrium phase masses and volumes were eliminated. This approach assigns the entire bulk hydrated polymer mass as the sequestering phase, physically separated from the cell-containing aqueous phase, and the volumes of both phases remain unchanged throughout the experiment due to minimal water flux between the phases. The PC results using this approach are lower and more realistic than if only the dry polymer mass were incorrectly accounted for in the calculation; for example, the PC value of 1.0 calculated for 4VL in BMI-PEO would give a value of nearly 8.0 if water uptake were neglected. Because this new approach accounts for water uptake by the polymer, it enables a realistic comparison of polymers having widely different water absorptions which would otherwise give incorrect PC values if water uptake was neglected, and should be adopted in future investigations comparing polymer-target molecule affinity in aqueous systems to ensure comparison of polymers on an equal basis.

5.5.4 Biotransformations

Biotransformation experiments, employing two different polymers during the ISPR period, were performed to permit comparison on an equal basis, such that their affinity for 4VL and level of water absorption, due to their distinct composition and architecture, were responsible for the differences seen. Although biocatalytic systems are inherently variable, the experiments resulted in very similar time courses, divided into periods of cell growth, 4HV accumulation and conversion to 4VL at pH 6.0 for 24 h, followed by 4VL recovery using the polymers for an additional 24 h. Biomass growth curves were very similar between bioreactor experiments, and preliminary data showed reproducible productivity. It is during the final, ISPR period that our approach of absorbing 4VL to pull the equilibrium reaction

towards additional 4VL production was implemented, and allowed comparison of the polymers' affinity towards 4VL and water uptake for their impact on the biotransformation outcome.

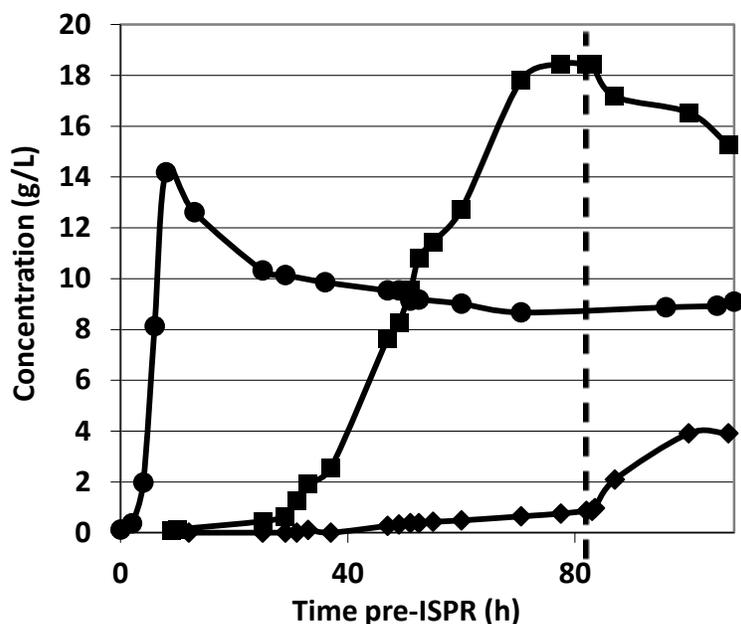


Figure 5-2: Plot of biomass (●), 4HV accumulation (■) and 4VL (◆) during growth, 4HV accumulation, and 4VL accumulation phases. The dashed line indicates shifting pH to 6.0 to induce lactonization of 4HV to 4VL.

Figure 5-2 shows a typical time course plot of biomass, 4HV and 4VL concentrations before and after pH shifting, prior to initiating ISPR. 4HV accumulation was approximately linear throughout each experiment until its plateau at approximately 18 g/L, indicating that controlling the levulinate feed rate maintained constant biocatalytic activity. As expected, conversion to 4VL was low prior to pH shifting, with 4% converted (4VL concentration divided by the sum of 4HV and 4VL concentrations) at pH 7.0. Conversion increased to approximately 27% at pH 6.0, with 4VL concentration rising to approximately 4 g/L. The difference in 4HV/4VL concentration ratios at pH 7.0 and 6.0 clearly demonstrates the pH-dependent nature of the reaction, with the equilibrium position, the feature of this model biotransformation, substantially favoring 4VL at lower pH. These results are similar to but slightly lower than the 33% conversion initially reported for this biocatalytic pathway; in our hands, this system was unable to produce equivalent titers of 27 g/L 4HV and 8 g/L 4VL [6].

ISPR was initiated after the lactonization reaction had occurred for 24 h at pH 6.0, when the system was approaching its final equilibrium 4HV/4VL ratio, in order to definitively demonstrate the differential action of the polymers. Alternatively, a polymer could be introduced either at the beginning of fed-batch operation, or upon pH shifting and the same effect would be achieved without the time required to reach equilibrium a second time.

Overall 4VL mass throughout the ISPR period was calculated using each polymer's swollen mass in the reactor at equilibrium, adding the change in volume to the polymer and subtracting it from the aqueous phase. Neglecting the polymer's dynamic swelling behavior has no effect on the calculated 4VL concentration at equilibrium. The polymers were added to the bioreactor dry, at a mass determined by the water uptake experiment performed in the bioreactor medium to give an equivalent swollen mass, in order to demonstrate the differential action of an equivalent wet mass of polymers. The addition of a pre-swollen polymer would dilute the bioreactor medium, skewing concentrations to different extents, potentially masking the effect of product absorption. This case is distinct from using pre-swollen polymers in the PC experiments, and medium uptake was accounted for prior to polymer addition.

5.5.4.1 BMI-PEO experiment

In the experiment with BMI-PEO, shown in Table 5-1, 240 g of dry polymer swelled by 375% in the bioreactor medium, less than the 600% in high-purity water. The difference in uptake between these two fluids is likely due to the presence of solutes in the medium, which decrease water activity and, consequently, its availability to hydrate the polymer chains. The swelling occurred at the expense of aqueous volume in the reactor, and increased the measured cell density, as cells were not absorbed by the polymer. However, aqueous concentrations of both 4HV and 4VL remained relatively stable, and the PC value of BMI-PEO towards 4VL in the reactor was similar to the value measured in high-purity water. The volumetric 4VL titer in the BMI-PEO experiment was lower relative to pre-ISPR because the additional volume occupied by the polymer was not offset by enhanced 4VL production. Synthesizing a

polymer with a composition having maximum available PEO content for solute absorption gave an architecture which permitted significant water absorption, yet hampered selectivity, as discussed below.

5.5.4.2 Hytrel® 8206 experiment

In the experiment using Hytrel® 8206, summarized in Table 5-1, 930 g of dry polymer swelled by 24% in the bioreactor. This modest water uptake also occurred at the expense of aqueous reactor volume; however, the selectivity towards 4VL relative to 4HV in this polymer was sufficient to shift the equilibrium by removing 4VL, due to its lower water uptake restricting the nonspecific permeation of all aqueous solutes. The mass of 4VL in the bioreactor using Hytrel® 8206 for ISPR increased by approximately 30%, and the overall concentration of 4VL increased by 12% on a volumetric basis, demonstrating that the polymer had a net beneficial effect which compensated for the additional volume it occupied. This represents the first successful report of equilibrium shifting of a biocatalytic reaction via product removal using an absorbent polymer.

Table 5-1: Performance comparison of both polymer ISPR experiments

Performance metric	1139 g BMI-PEO	1153 g Hytrel® 8206
Dry polymer mass (g)	240	930
Total reactor volume (3L aqueous + dry polymer)	3.2 L	3.8 L
4VL PC in reactor	0.91	1.50
4HV PC in reactor	0.48	0.12
4VL / 4HV selectivity	1.9	12.5
Volumetric 4VL improvement (g/L) from ISPR	- 6%	12%
Mass 4VL improvement (g) from ISPR	0%	30%

5.5.5 Polymer selectivity

The production of additional 4VL with the introduction of a sequestering phase requires selective absorption of 4VL relative to 4HV, calculated by the ratio of distribution coefficients observed in the reactor. Table 5-1 shows the variation in selectivity between the polymers which caused the observed differences. The vast majority of the hydroxyacid precursor, 4HV ($pK_a = 4.6$), is deprotonated at the pH of this system and carries a negative charge, such that any significant uptake of this charged compound would be a result of non-selective water absorption by the hydrated polymer and would cause a loss of driving force for lactonization. Non-aqueous, non-ionic sequestering phases are known to selectively absorb neutral chemical species and reject charged compounds, and affinity can be augmented by the presence of charged solutes in the aqueous phase via the “salt effect” [27]. While the proportion of soft PEO available for solute permeation was maximized in the composition of BMI-PEO in order to promote 4VL affinity based on similar solubility parameter values, this gave a loose architecture which allowed significant water uptake. The water uptake by BMI-PEO compromised selectivity by allowing diffusion of solutes (both charged and neutral) between both phases, shown by its relative uptake of both 4VL and 4HV in Figure 5-3a. Consequently, BMI-PEO did not appreciably shift the

equilibrium towards 4VL production, thereby hampering the effect of ISPR-driven equilibrium pulling, and produced no additional 4VL, shown in Figure 5-4a. In contrast, the segmented block architecture of Hytrel® 8206 provided more robust network stabilization, allowing only modest water absorption, and maintaining sufficient selectivity towards 4VL relative to 4HV, illustrated in Figure 5-3b. This selective removal of 4VL shifted the reaction equilibrium, producing 30% more 4VL, shown in Figure 5-4b. Furthermore, the distribution coefficient of Hytrel® 8206 towards 4VL in the reactor was found to increase significantly relative to the value measured in high-purity water, a result of solutes in the medium which decreased water activity and drove more 4VL into the polymer, demonstrating Hytrel's selectivity in contrast to BMI-PEO's nonselective absorption of all solutes. The results indicate that a tradeoff exists between affinity and selectivity due to water uptake by polymers, such that minimizing inert content in order to maximize affinity must be balanced against selectivity losses imposed by water uptake, a property determined by polymer architecture.

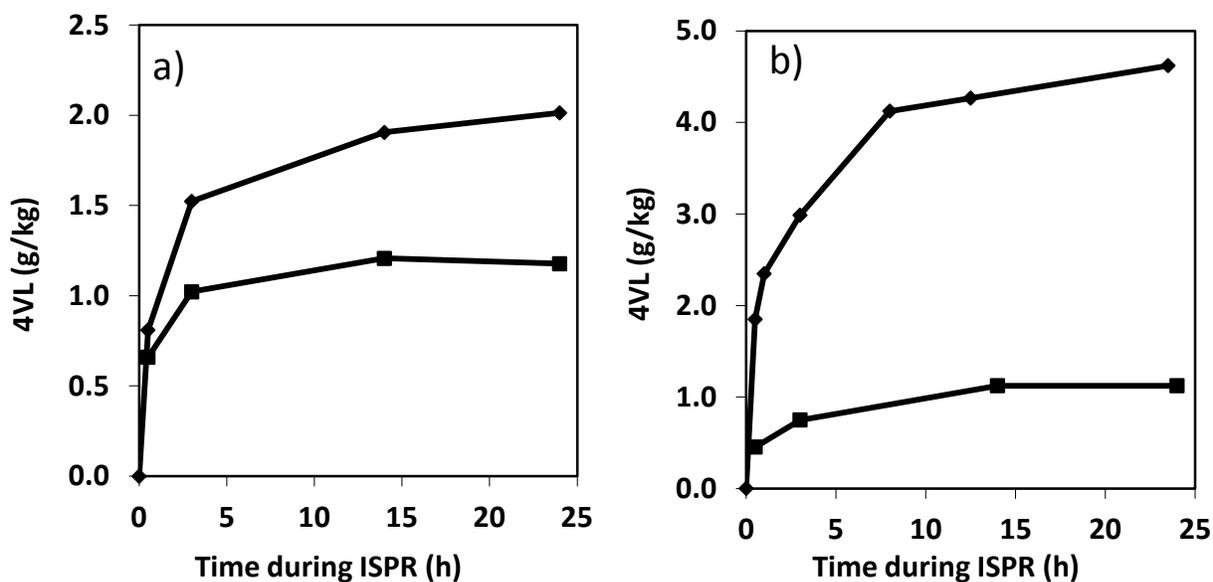


Figure 5-3: 4VL (◆) and 4HV (■) accumulation in polymers during ISPR (g/kg) in: a) BMI-PEO b) Hytrel® 8206.

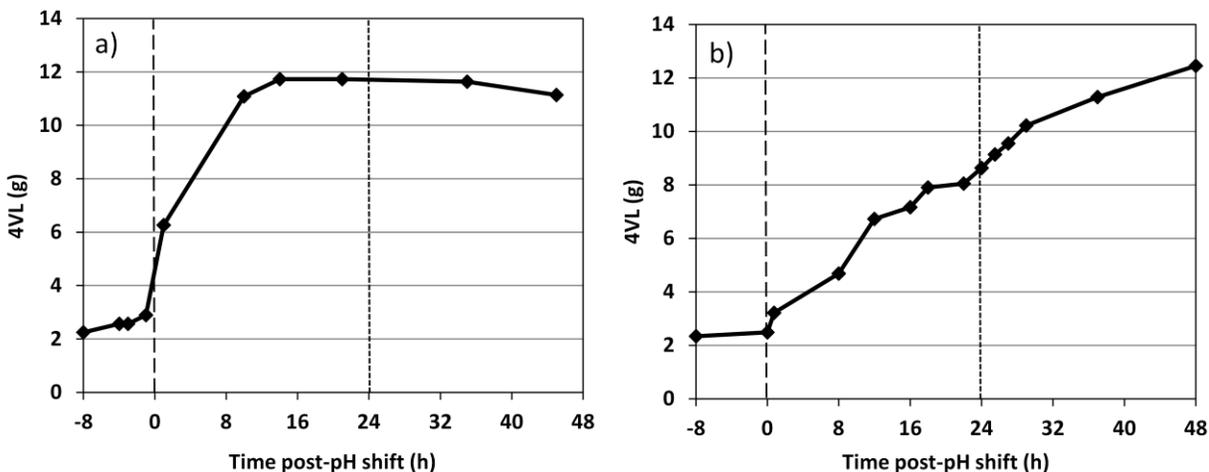


Figure 5-4: Overall 4VL mass in the bioreactor vs. time normalized to onset of the pH shift. The first (dashed) line indicates the pH shift from 7.0 to 6.0, and the second (dotted) line indicates the addition of the polymer: a) 240 g BMI-PEO b) 930 g Hytrel® 8206.

5.6 Conclusions

Although this model biotransformation system clearly presents a practical challenge in product extraction due to the target molecule's affinity for water, this investigation has shown for the first time that using a selective, *absorptive* polymer phase can pull a pH-dependent equilibrium biocatalytic reaction by removing the product, thereby increasing production. This approach is simpler, less expensive, and operates on a different mechanism than conventional separations employing adsorbents having high specific surface areas, or those using ATPS which require tuning of specific operating conditions to trigger phase separation. A relationship between increasing water uptake and higher target molecule affinity was observed in the range of synthesized polymers, but selectivity was hampered by this high water uptake. In contrast, a moderately water-absorbing, commercial segmented block copolymer shifted the equilibrium due to its greater selectivity towards 4VL and increased production by 30%. In TPPB systems involving hydrophilic target molecules such as this one, water content may be a necessary feature of selected polymers, but should be controlled through polymer architecture, for example by employing block copolymers for the advantages demonstrated in their physical properties and selectivity. Future work aims to characterize the effect of water, determining at

which point absorbed water begins counteracting the improvement to affinity by reducing selectivity in TPPBs.

5.7 Acknowledgements

The authors gratefully acknowledge Dr. Kristala Prather of Massachusetts Institute of Technology for providing the engineered biocatalyst, and Dr. Collin Martin (MIT) for his helpful discourse. The authors also thank Dr. Shailesh Doshi of DuPont Canada for providing polymers and for his stimulating discussions.

5.8 References

1. Verevkin SP, Emel'yanenko VN (2012) Renewable platform-chemicals and materials: Thermochemical study of levulinic acid. *J Chem Thermodyn* 46:94-98
2. Chalid M, Heeres H, Broekhuis A (2012) Green polymer precursors from biomass-based levulinic acid. *Procedia Chem* 4:260-267
3. Horváth IT, Mehdi H, Fábos V, Boda L, Mika LT (2007) γ -Valerolactone—a sustainable liquid for energy and carbon-based chemicals. *Green Chem* 10:238-242
4. Straathof AJJ (2003) Auxiliary phase guidelines for microbial biotransformations of toxic substrate into toxic product. *Biotechnol Prog* 19:755-762
5. Götz K, Liese A, Ansorge-Schumacher M, Hilterhaus L (2013) A chemo-enzymatic route to synthesize (S)- γ -valerolactone from levulinic acid. *Appl Microbiol Biotechnol* 97(9):3865-3873
6. Martin CH, Wu D, Prather KLJ (2010) Integrated Bioprocessing for the pH-Dependent Production of 4-Valerolactone from Levulinate in *Pseudomonas putida* KT2440. *Appl Environ Microbiol* 76:417
7. Teiber JF, Draganov DI, Du BNL (2003) Lactonase and lactonizing activities of human serum paraoxonase (PON1) and rabbit serum PON3. *Biochem Pharmacol* 66:887-896
8. Efe C, Straathof AJJ, van der Wielen LAM (2007) Options for biochemical production of 4-hydroxybutyrate and its lactone as a substitute for petrochemical production. *Biotechnol Bioeng* 99:1392-1406
9. Malinowski JJ (2001) Two-phase partitioning bioreactors in fermentation technology. *Biotechnol Adv* 19:525-538
10. Li A, Zhang Q, Chen J, Fei Z, Long C, Li W (2001) Adsorption of phenolic compounds on Amberlite XAD-4 and its acetylated derivative MX-4. *React Funct Polym* 49:225-233
11. Phillips T, Chase M, Wagner S, Renzi C, Powell M, DeAngelo J, Michels P (2013) Use of in situ solid-phase adsorption in microbial natural product fermentation development. *J Ind Microbiol Biotechnol* 40:1-15

12. Nielsen DR, Prather KJ (2009) In situ product recovery of n-butanol using polymeric resins. *Biotechnol Bioeng* 102:811-821
13. Wang Z, Dai Z (2010) Extractive microbial fermentation in cloud point system. *Enzyme Microb Technol* 46:407-418
14. Craig T, Daugulis AJ (2013) Polymer characterization and optimization of conditions for the enhanced bioproduction of benzaldehyde by *Pichia pastoris* in a two-phase partitioning bioreactor. *Biotech Bioeng* 110:1098-1105
15. Hosaka S, Ozawa H, Tanzawa H (1979) Controlled release of drugs from hydrogel matrices. *J Appl Polym Sci* 23:2089-2098
16. Prpich GP, Daugulis AJ (2007) A novel solid–liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. *Biotechnol Bioeng* 98:1008-1016
17. Morrish JLE, Daugulis AJ (2008) Improved reactor performance and operability in the biotransformation of carveol to carveone using a solid–liquid two-phase partitioning bioreactor. *Biotechnol Bioeng* 101:946-956
18. Khan TR, Daugulis AJ (2010) Application of solid–liquid TPPBs to the production of L-phenylacetylcarbinol from benzaldehyde using *Candida utilis*. *Biotechnol Bioeng* 107:633
19. Parent JS, Capela M, Dafoe JT, Daugulis AJ (2012) A first principles approach to identifying polymers for use in two-phase partitioning bioreactors. *J Chem Technol Biot* 87:1059-1065
20. Various (1999) Solubility parameter values In: *Polymer Handbook*. Brandrup J, Immergut EH, Grulke EA (Eds), 4th edn. John Wiley & Sons, Hoboken, NJ, USA
21. Amsden B, Lau A (2008) Siloxane-based copolymers for use in two-phase partitioning bioreactors. *Can J Chem Eng* 86:1-5
22. Ju H, McCloskey BD, Sagle AC, Wu YH, Kusuma VA, Freeman BD (2008) Crosslinked poly (ethylene oxide) fouling resistant coating materials for oil/water separation. *J Membr Sci* 307:260-267
23. Ping Z, Nguyen Q, Chen S, Zhou J, Ding Y (2001) States of water in different hydrophilic polymers—DSC and FTIR studies. *Polymer* 42:8461-8467
24. Tufvesson P, Lima-Ramos J, Jensen JS, Al-Haque N, Neto W, Woodley JM (2011) Process considerations for the asymmetric synthesis of chiral amines using transaminases. *Biotechnol Bioeng* 108:1479-1493
25. Ramström O, Ye L, Krook M, Mosbach K (1998) Applications of molecularly imprinted materials as selective adsorbents: Emphasis on enzymatic equilibrium shifting and library screening. *Chromatographia* 47(7-8):465-469
26. Valentin HE, Schönebaum A, Steinbüchel A (1992) Identification of 4-hydroxyvaleric acid as a constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. *Appl Microbiol Biotechnol* 36:507-514
27. Malinowski JJ, Daugulis AJ (1994) Salt effects in extraction of ethanol, 1-butanol and acetone from aqueous solutions. *AIChE J* 40:1459-1465

28. Miller G (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31:426-428
29. Hu Q, Meng Y, Sun T, Mahmood Q, Wu D, Zhu J, Lu G (2011) Kinetics and equilibrium adsorption studies of dimethylamine (DMA) onto ion-exchange resin. *J Hazard Mater* 185:677-681
30. Nielsen DR, Prather KJ (2008) In situ product recovery of n-butanol using polymeric resins. *Biotechnol Bioeng* 102:811-821
31. Hytrel thermoplastic elastomer design guide - Module V
http://www2.dupont.com/Plastics/en_US/assets/downloads/design/H81098.pdf. Accessed April 13 2013
32. Rehmann L, Sun B, Daugulis AJ (2007) Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. *Biotechnol Prog* 23:814-819
33. Gao F, Daugulis AJ (2010) Polymer-solute interactions in solid-liquid two-phase partitioning bioreactors. *J Chem Technol Biot* 85:302-306
34. Rehmann L, Prpich GP, Daugulis AJ (2008) Remediation of PAH contaminated soils: Application of a solid-liquid two-phase partitioning bioreactor. *Chemosphere* 73:798-804

Chapter 6 Block copolymers as sequestering phases in two-phase biotransformations: Effect of constituent homopolymer properties on solute affinity

Julian T. Dafoe, J. Scott Parent and Andrew J. Daugulis

6.1 Preface to Chapter 6

This chapter addresses some questions which have arisen from the previous chapters' investigations into the application of absorbent polymers to improve different biocatalytic systems featuring diverse target molecules. The performance of polyether-containing block copolymers in solid-liquid biotransformations is remarkable, not only for their high affinity towards many compounds and their excellent physical properties, but particularly for the removal of relatively hydrophilic compounds, which are not appreciably absorbed by many/most other polymers. The complexity of commercial materials precludes directly extending these properties to block copolymers, yet it is useful to examine copolymer constituents as individual homopolymers, in which molecular weight and chain end-group effects can be controlled and their effects studied.

Furthermore, the affinity of the soft segment component of many poly(ether)-containing block copolymers, poly(tetramethylene ether), towards target molecules, shows an entropic contribution arising from reductions in molecular weight resulting in improved affinity in the absence of chain end-group effects. At low molecular weight, the nature of chain-ends can provide a significant enthalpic contribution to target molecule affinity or rejection.

This chapter demonstrates that chain end-group composition has a very strong effect on target molecule affinity, especially at low molecular weights where their contribution is relatively greater. The presence of polar end-groups in poly(tetramethylene ether glycol) was found to have an effect whose direction depended on the nature of the target molecule, where affinity towards a more polar solute was improved, while hydrophobic solutes demonstrated drastically poorer affinity. This behaviour, while intuitively an example of "like dissolves like", is a potential tool to improve affinity towards molecules suffering from poor extraction, and also to control selectivity among different target molecules; however, the extraction of hydrophilic molecules from aqueous solutions will always be limited by their strong affinity for water.

6.2 Abstract

BACKGROUND: Block copolymers can be used as extractants in solid-liquid two-phase partitioning bioreactors (TPPBs) and are usually selected on the basis of their partition coefficient toward target solutes and their thermo-mechanical properties. A series of Pebax® block copolymers containing varying proportions of soft poly(tetramethylene oxide) and hard poly(amide-12), was evaluated to determine the effect of hard segment proportion on solute affinity toward two biotransformation target molecules, carveol and carvone. Subsequently, representative homopolymers comprising the copolymers' soft segment were examined individually for the effects of molecular weight, crystallinity, and polymer end group polarity on affinity; the latter effect was also explored using a third biotransformation target molecule, 4-valerolactone.

RESULTS: Partition coefficients decreased with greater proportions of copolymer hard segments and homopolymer crystallinity, both of which act as non-absorptive domains, but which impart mechanical strength to the polymer. Partition coefficients increased with decreasing homopolymer molecular weight, which was ascribed to increased entropy of mixing. Hydroxyl vs. ether end group functionality had a variable effect on partitioning, providing a basis for selectivity.

CONCLUSION: Block copolymers can provide an attractive, low-cost option for polymeric sequestering phases in TPPBs, but commercial grades of these materials are not optimized for these applications. TPPB partitioning phase performance can be improved by selecting/fabricating polymers that minimize the molecular weight of the soft component, the proportion of hard segment, and by considering the effects of end group composition.

Keywords: crystallinity; extractive fermentation; partition coefficient; partitioning bioreactor; polymer properties; molecular weight

6.3 Introduction

Block copolymers comprised of discreet hard and soft “blocks” of substantially different composition are effective absorbents for *in-situ* product removal (ISPR) in two-phase partitioning bioreactors (TPPBs) for biotransformation applications, and can also be applied for substrate delivery in biodegradative applications. Block copolymers provide the soft, amorphous phase needed for solute absorption and the glassy, semi-crystalline “hard” phase that provides the mechanical integrity needed to withstand autoclaving and agitation in bioreactor vessels.¹⁻³ The materials of interest for TPPB applications have generally been thermoplastic elastomers, with the soft segment comprising the continuous phase, and existing as an amorphous liquid above its glass transition temperature (T_g) under service conditions.

Commercially-available block copolymers can be much less expensive than alternative sequestering phases such as specialized resins or liquid solvents,⁴ and the mechanism of solute absorption by these polymers has been shown to be identical to solvent extraction. While these materials are developed for their thermo-mechanical properties that are demanded by engineering applications, little is known about the structure-property relationships that govern their interactions with small molecules, and dictate their utility in TPPB applications.

The Pebax® series of poly(ether-*block*-amide) materials produced by Arkema are among the most thoroughly-characterized block copolymers, with reports of their mechanical and thermal properties⁵⁻⁹ being augmented with studies of their transport properties in membrane separations for the selective recovery of CO₂ and volatile organic compounds from gas streams,¹⁰⁻¹⁵ and in drug delivery systems.¹⁶⁻¹⁸ The Pebax® grades of present interest have soft blocks comprised of poly(tetramethylene oxide) (PTMO) that provide a glass transition (T_g) near -70°C and melting temperatures (T_m) less than 25°C. The hard blocks are comprised of poly(amide-12) (PA-12), that provide T_g s near 40°C and melting temperatures in excess of 130°C.^{5,10,11} The immiscibility of these blocks produces a phase-separated

morphology, wherein one block component forms the continuous phase, and the other resides in dispersed domains.⁷ Table 6-1 is a summary of the composition and thermo-mechanical properties of the Pebax® materials examined in this work. Note that of the four copolymers, only Pebax® 7033 appears to have a continuous polyamide phase, as indicated by shore D hardness.

Table 6-1: Properties of Pebax® ‘33’ block copolymer series. Poly(amide-12) (PA-12) is the hard segment component, and poly(tetramethylene oxide) (PTMO) is the soft segment component

Pebax® grade	2533	3533	4033	7033
PA-12 wt% ^{7,10}	12	13	27	73
PTMO wt% ^{7,10}	84	83	70	25
T _m (°C) (PA-12)*	142	149	165	175
T _g (°C) (PTMO) ¹⁰	-76	-72	-65	ND
T _m (°C) (PTMO)*	12	11	7	ND
Water absorption (%)	1.2 ³⁰	1.2 ³¹	1.2 ³²	1.1 ³³
Hardness (Shore D)	27 ³⁰	33 ³¹	42 ³²	70 ³³
Strain at break (%)	>750 ³⁰	>600 ³¹	>450 ³²	>350 ³³

ND: Not Determined

*: This study

These Pebax® grades were selected in this work for their different proportions of hard and soft blocks, in order to determine the effect of copolymer composition on the affinity toward two biotransformation molecules previously examined in a TPPB context, namely carveol and carvone.¹⁹ Our study begins with partition coefficient (PC) measurements to establish the sorption capacity of each copolymer domain. Subsequently, a series of tetrahydropyranyl ether-terminated poly(tetramethylene oxide) (THP-PTMO) materials of varying molar mass are prepared and investigated to isolate the effect of molecular weight on partition coefficients. Finally, a series of hydroxyl-terminated poly(tetramethylene ether glycol) (PTMEG) polymers are examined to explore the influence of end-group composition on the partitioning of carveol, carvone, and a third solute, 4-valerolactone, a water miscible target molecule that is difficult to extract from aqueous media.²⁰

The results of this study are useful to shape future TPPB polymer selection, or ultimately polymer design strategies, which are expected to transition towards the adoption of application-specific materials which may be synthesized to fulfill specific requirements. By incorporating the findings of this

study in terms of controlling properties such as molecular weight, end group composition, and crystallinity, the performance of TPPB sequestering phases should be improved.

6.4 Materials and Methods

6.4.1 Chemicals

(-)-Carveol (mixture of isomers), (R)-(-)-carvone, 4-valerolactone (4VL), p-toluenesulfonic acid monohydrate, and 3,4-dihydro-2H-pyran (DHP) were purchased from Sigma-Aldrich (Canada). Hydroxyl-terminated poly(tetramethylene ether glycol) (PTMEG) samples with average molecular weights of 250, 650, 1000, 2000, and 2900 g/mol, designated PTMEG followed by their molecular weight, were purchased from Scientific Polymer Products (Ontario, NY, USA). Pebax[®] grades 2533, 3533, 4033, and 7033, all medical grade and lacking additives, were used as received from Foster Corporation (Putnam, CT, USA), while poly(amide-12) was used as donated by DuPont Canada.

6.4.2 THP-terminated poly(tetramethylene oxide) (THP-PTMO)

Etherification of the hydroxyl end groups within PTMEG samples was accomplished by acid-catalyzed reaction with DHP in dilute solution. In a round-bottom flask, the amount of PTMEG of a certain molecular weight was chosen to provide an equivalent end group concentration across the molecular weight range. The polymer was dissolved in dichloromethane (18 mL), before adding DHP (2 g) and p-toluenesulfonic acid (9 mg). The resulting cement was stirred for 16 hours at room temperature, washed with a saturated NaHCO₃ solution (20 mL), and dried over anhydrous Na₂SO₄. Residual solvent was removed by rotary evaporation, followed by short-path Kugelrohr distillation under high vacuum (25°C) to give THP-PTMO samples as clear, pale yellow oils of varying viscosity. ¹H NMR analysis (CDCl₃): δ 4.52 (dd, -O-CH(O)-CH₂, 1H), δ 3.79 and 3.67 (dt, -O-CH₂(6-THP), 2H), δ 3.34 (m, THP-O-CH₂-CH₂-, 2H), δ 3.35-3.28 (m, -CH₂-O-CH₂-, 4(n-1)H), δ 1.80-1.43 (m, -CH₂-CH₂-CH₂(3,4,5-THP) and aliphatic -CH₂-CH₂-, (12+4n)H), N being the average number of monomer units present in the polymer, which was calculated as being between 3 and 4 for the sample derived from 250 g/mol PTMEG.

6.4.3 Partition coefficient experiments

Partition coefficients (PC) were determined by contacting a known mass of polymer with a stock solution containing solutes at a known concentration under agitation at 180 RPM. The contact time was 48 h to for standard partition coefficient experiments, while a contact time of two weeks was also used to compare the difference in absorption rate between the hard and soft components. As-received monolithic polymers were broken into small shavings to minimize transport limitations, while polymer pellets were used in their original form. While an ideal experimental system would use infinitely dilute solute concentrations in both phases to isolate polymer-solute interactions,²¹ the amount of target molecule used in the experiments depended on the polymer's affinity toward the solute, in order to obtain a reliable analytical measurement. For this reason, ca. 0.3 g of polymer was contacted with carveol/carvone solutions of approximately 500 mg/L each, while ca. 3 g of polymer was contacted with 4VL solutions of approximately 3 g/L. Aqueous samples were withdrawn and passed through a 0.2 µm nylon syringe filter prior to HPLC analysis. The concentration in the polymer phase was calculated by mass balance, comparing the concentration measured in a control vial without polymer to the experimental vials, assuming negligible losses. Partition coefficient experiments were conducted in triplicate, and reported values are the average with error bars representing one standard deviation from the mean. Reported values are specified in the Figures as being measured directly, or normalized to the PTMO fraction in Pebax® or the non-crystalline fraction in PTMEG using the following equation:

$$PC_{norm} = \frac{PC_{obs}}{1 - X_c}$$

Where PC_{norm} denotes the normalized partition coefficient, PC_{obs} denotes the value measured directly, and X_c denotes the fraction of the material which is composed of crystalline or hard domains:

6.4.4 Analytical methods

¹H NMR spectra were acquired in CDCl₃ with a Bruker AM-400 instrument, with chemical shifts referenced to the residual proton resonance of chloroform. DSC analysis was conducted using a TA

Instruments Q100 at a heating rate of 10^oC/min, with results reported for the first heating to assess samples as used in partition coefficient determinations. Degree of crystallinity was determined by dividing the integrated melting endotherm by the specific heat capacity of an ideal crystal; 172.2 J/g for PTMEG²²; and 245 J/g for poly(amide-12)²³.

Aqueous solute concentrations were measured by HPLC using a Varian Polaris C18-A 150x4.6mm 5µm column and a Varian ProStar 325 UV detector operating at 200nm. The mobile phase for carveol/carvone analysis was 50:50 acetonitrile:water at 1 mL/min, with both carveol isomers eluting at 5.5 minutes, and carvone at 7 minutes. For 4VL analysis, the mobile phase was 20:80 acetonitrile:water and the retention time was 4.2 minutes.

6.5 Results and Discussion

6.5.1 Pebax[®] series: effect of copolymer soft segment proportion

The proportion of hard and soft components, shown in Table 6-1, varied across the Pebax[®] '33' series from approximately a 12/86 distribution of hard/soft blocks in the softest grade, P2533, to 73/25 in the hardest grade, P7033. As discussed above, the hard and soft domains within these block copolymers exist in a microphase-separated morphology whose distribution depends on the relative abundance of each phase, among other factors. This complex morphology makes it difficult to attribute polymer properties to either homopolymer component.^{5,9}

Consider the carveol and carvone partition coefficient data listed in Table 6-2 for solute absorption experiments with polymer contact for a period of 48 hours. The data correlate strongly with copolymer composition, as increases in polyamide content from 12 wt% to 73 wt% reduce the observed partition coefficient by a factor of seven for both target molecules. The PC values normalized to the PTMO portion present in the Pebax[®] series which decrease at higher PA-12 content substantiate that PA-12 forms a continuous phase when present in a high proportion, preventing solute access to dispersed PTMO domains.

Table 6-2: Partition coefficients (measured directly, and normalized to the soft PTMO fraction given in Table 6-1) for carveol and carveone in the Pebax® series

Pebax® grade	Carveol PC	Carvone PC	Carveol PC PTMO-normalized	Carvone PC PTMO-normalized
2533	154.7 ± 3.9	157.0 ± 2.2	184	187
3533	152.1 ± 1.9	151.1 ± 0.5	182	180
4033	123.0 ± 1.2	108.9 ± 1.5	175	155
7033	22.2 ± 2.9	21.9 ± 2.9	89	88

To confirm that the hard component is relatively non-absorptive, PTMEG2900 and poly(amide-12) (PA-12) were studied as proxies for the PTMO and PA-12 blocks found in Pebax®. The difference in solute uptake between the soft and hard components is clearly demonstrated in Figure 6-1, which shows much lower partition coefficient values for PA-12 relative to PTMEG2900. These values were not normalized to their amorphous content, in order to represent the materials as they behave as a whole. In fact, the degree of crystallinity in PTMEG2900 is 3-fold higher at approximately 80% than in PA-12, at approximately 25%, indicating that it is the high T_g of PA-12, rather than an abundance of crystallinity, that is responsible for the difference seen in solute uptake rate.

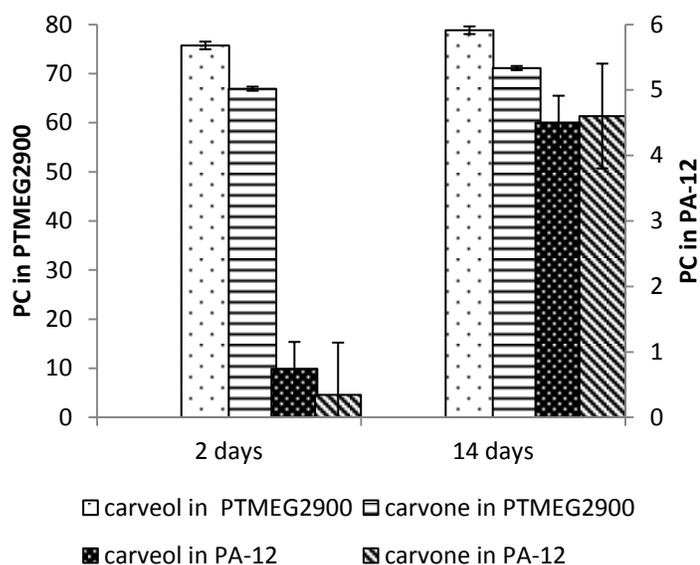


Figure 6-1: Partition coefficients (measured directly) of carveone and carveol in PTMEG2900 (left axis) and PA-12 (right axis).

Interestingly, the partition coefficients for carveol and carveone in the PA-12 homopolymer increase between two days and 14 days, while the PC in PTMEG2900 changes less than 10% over the same time period. The slow evolution of sorption capacity by PA-12 may be due to plasticization by solute and/or water to lower the polymer's T_g .²⁴ The improved partition coefficient value after two weeks indicates that PA-12 may have some thermodynamic affinity toward these solutes, but still much lower than that of PTMEG2900 which shows PC values that are an order of magnitude greater. However, the very slow mass transfer rate into PA-12 also suggests that this material is still glassy and practically impenetrable from a kinetic standpoint on the time scale of biotransformation experiments. In contrast, block copolymers are known to reach equilibrium quickly in the form of pellets, on the order of a few hours,^{25,26} which is attributable to rapid absorption by the soft segment, similar to what is seen here in PTMEG2900. Such rapid uptake would be an important feature to ensure kinetic rather than mass transfer limitations in TPPB systems.

These results confirm that the hard segment proportion acts as an impediment to solute uptake. While affinity may be improved by increasing the PTMO proportion, this inevitably comes at the expense of poorer mechanical properties which impart the materials' thermal resilience, indicated by the decreasing melting temperatures at lower PA-12 content shown in Table 6-1. The PTMO phase in this Pebax[®] series is in the molten state (i.e. is a liquid) at room temperature (Table 6-1), requiring mechanical stabilization by the hard segments to maintain a solid copolymer network. The hard segment should be present only in a sufficient amount to provide adequate mechanical and thermal properties to sustain repeated exposure to harsh conditions, such as sterilization by autoclaving, and long periods under agitation in bioreactor vessels.

6.5.2 THP-PTMO series: effect of molecular weight on partition coefficients

The hard and soft domains of segmented block copolymers form microphase-separated morphologies because they are unable to form solutions due to their different polarities, while being

covalently tied together.^{5,7} Looking at the absorptive soft segment individually can provide useful information by separating some entropic effects (due to molecular weight) from enthalpic ones (due to chemical composition) in their contributions to solute absorption. To clarify, entropic contributions to mixing arise from the number of possible conformations of the polymer and solute as they occupy a given space, which increase with a reduction in molecular weight. Enthalpic interactions arise from deviations from ideality which energetically favour or hinder polymer-solute mixing as a result of Van der Waals forces, described colloquially as “like dissolves like”.

The structure of PTMEG, the oligomeric prepolymer utilized in segmented polyether production, such as in the Pebax[®] copolymers, possesses polar glycol functionality to provide reactive end groups for polymerization. To remove the end group polarity and isolate the effect of polymer chain molecular weight, the hydroxyl-functional chain ends within PTMEG were converted to non-polar tetrahydropyranyl ether groups having identical functionality to the rest of the polymer chain. These THP-PTMO materials lack confounding properties arising from polar functionality or crystallinity. With this chemical modification, the materials can be directly compared on the basis of molecular weight to study its effect on target molecule affinity.

Figure 6-2 shows the partition coefficients of the THP-PTMO series towards carveol and carvone across an order of magnitude range in molecular weights. The partition coefficients are similar in magnitude to those seen with the PTMO-rich Pebax[®] grades, suggesting that THP-PTMO is an effective model of the PTMO component in Pebax[®]. The PC values are nearly constant above approximately 1000 g/mol and significantly increase by up to 50% at lower molecular weights, indicating that decreasing molecular weight is an effective way to improve partition coefficients. This is in agreement with Flory lattice thermodynamic theory, which states that the entropy of mixing should increase at lower molecular weights due to the greater number of available conformations of solute molecules and polymer chains in a lattice, thereby decreasing the overall Gibbs free energy of mixing and promoting

spontaneous sorption.^{27,28} This thermodynamic feature has also been confirmed experimentally and from first principles in our Group (Results in publication).

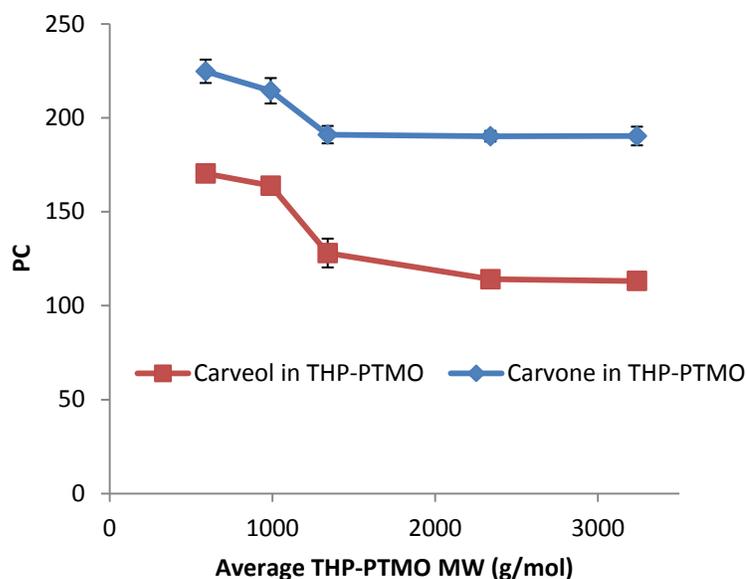


Figure 6-2: Partition coefficients of carvone and carveol in the THP-PTMO series with increasing molecular weight.

The improvement in PC values arising from reducing polymer chain length suggests that future efforts in the design or selection of TPPB extractants should consider low molecular weight materials. However, the physical state of a polymer affects its potential utility as an extractant. Solid polymers, characterized by high molecular weight, have been valued as TPPB extractants for their inertness with respect to many biocatalysts, while perhaps the main problem with using immiscible liquid solvents as extractants is their non-biocompatibility due to interactions with the biocatalyst in the aqueous phase.

As polymer molecular weight decreases, the boundary between oligomeric polymer chains and “monomeric” solvents blurs, and these materials no longer exist as solids, such that considerations of biocompatibility may again arise as the molecular weight continues to be reduced. This aspect of determining polymer biocompatibility at low molecular weights is the focus of ongoing work in our Group. Furthermore, solid extractants employed as small beads are convenient for handling and re-use, while liquid extractants existing as dispersed droplets tend to become partially entrained in the

fermentation medium by emulsion, hampering their recovery and re-use. On the other hand, a potential advantage in using low molecular weight polymers in the liquid state may be the enhancement of target molecule mass transfer by more rapid diffusion within the polymer, and also to/from the aqueous phase, driven by the extremely high interfacial surface area achieved by dispersing a liquid-liquid biphasic system.²⁶ The benefits of improved affinity and mass transfer from using a liquid polymeric extractant having lower molecular weight should be weighed against potential practical difficulties in handling and re-use.

In light of recent research in our Group, and the current study, we have come to believe that some performance benefits of liquid extractants in combination with favourable thermal and mechanical properties may be achieved using rational means, utilizing the insights arising from such work. For example, in the design of custom block copolymers, the incorporation of low molecular weight polymers chosen specifically for their affinity as soft blocks in block copolymers, while including a sufficient proportion of hard blocks to provide physical integrity, may improve the affinity of the soft segment relative to the commercial materials, which are currently designed primarily for their physical properties. The molecular weight effect in the block copolymer system would be somewhat hindered *in-situ* by the covalently tethered hard segment, increasing chain rigidity and effective molecular weight, such that this potential strategy of reducing soft segment molecular weight in a block copolymer would require further validation. Similarly, covalently crosslinking PTMO to build a larger network composed almost entirely of soft, absorbent polymer would increase the molecular weight toward an infinite value, but would be worthwhile to circumvent the previously-discussed restrictions imposed by hard segments. This strategy would maximize the available “active” material while also contributing good thermal properties, because a thermoset rubber lacks any melting point. Of course, a transition from commodity materials to tailor-made ones would increase cost.

6.5.3 Effect of degree of crystallinity on partition coefficients

The THP-PTMO series of polymers produced in this study were clear, liquid oils, indicating a lack of crystallinity, whose viscosity increased with molecular weight. Conversely, the PTMEG polymer series ranged from cloudy liquids of increasing viscosity at 250 and 650 g/mol, to white waxy solids of increasing hardness above 1000 g/mol. These physical features suggest increasing degrees of crystallinity as longer chains are able to pack uniformly to form larger crystallites, which are largely impenetrable to permeating molecules. The difference in crystallinity between the THP-PTMO and PTMEG series can be attributed to their different end group compositions, with THP-PTMO's relatively large ring ends preventing organized stacking of the polymer chains.

Because crystallites are unable to absorb solutes, the measured partition coefficients have been normalized by dividing measured PC values by the amorphous (non-crystalline) fraction of the material, as measured by DSC at the conclusion of each PC experiment.²⁴ The degrees of crystallinity in the PTMEG series after the PC experiments were similar but slightly lower than the as-received polymers, decreasing by less than 10% of the original values, whereas prolonged contact with only water produced almost no change in degree of crystallinity, suggesting that solute-polymer interactions are responsible for melting the crystallites in this hydrophobic polymer. Although these small changes in crystallinity suggest an additional route of sorption into crystallites, the magnitude of the effect is minor relative to absorption by the amorphous portion, similar to what is seen between PTMEG and PA-12 in Figure 6-1. This melting process is expected to be significantly slower than solute permeation through the amorphous portion of the polymer due to chain rigidity in crystallites, such that crystallinity remains a barrier to absorption which should be avoided in a potential extractant to the extent possible, in the same way that hard segments act as inert structural components of block copolymers. Nevertheless, this system-dependent change in crystallinity should be taken into account when comparing different semicrystalline materials on the basis of normalized partition coefficients, because the magnitude of changes in crystallinity may be very different across diverse materials.

6.5.4 Effect of polar end-group concentration on partition coefficients

The effect of polymer polarity contributed by the hydroxyl end groups of PTMEG is illustrated in Figure 6-3 as a dramatic decrease in polymer affinity towards the target molecules carveol and carveol at lower molecular weights, below 1000 g/mol. By normalizing to the amorphous fraction, shown by the solid lines in Figure 6-3, constant PC values are observed at high molecular weights, similar to what is seen in Figure 6-2, indicating that this effect of end group polarity arises only at low molecular weight. This phenomenon is contrary to and much stronger than the effect of molecular weight on partition coefficients shown in the THP-PTMO polymer series which was attributed to entropic effects in the absence of enthalpic end group effects. In the case of the hydroxyl-terminated PTMEG polymer series, while the same entropic effect at low molecular weights is presumably at play, it is clearly overshadowed by the effect of end group composition which acts to decrease the polymer's affinity towards the relatively hydrophobic target molecules, carveol and carveol, at low molecular weights.

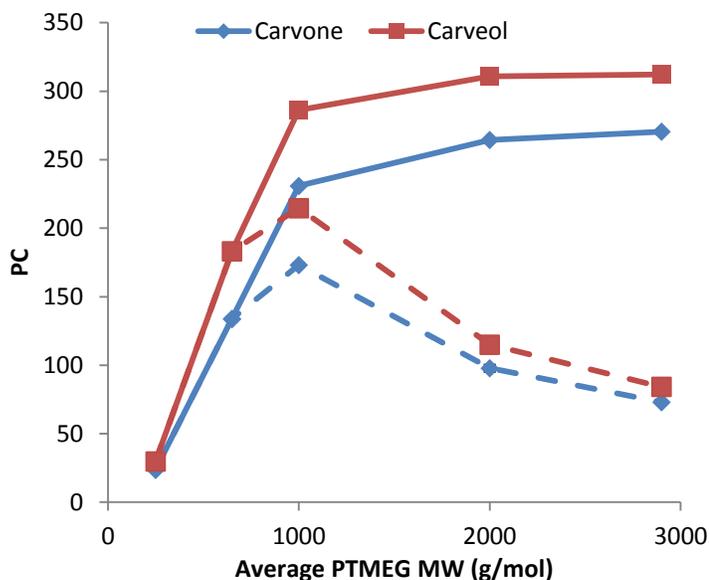


Figure 6-3: Partition coefficients of carveol and carveol in the PTMEG series with increasing molecular weight. Solid lines represent values normalized to amorphous portion measured by DSC. Dashed lines represent values measured directly.

The hydroxyl end group functionality present in the PTMEG series imparts significant polarity, and the magnitude of the contributed polarity depends on the amount of chain end groups relative to repeat units within the chain at various molecular weights; that is, the hydroxyl ends will contribute relatively more overall polarity to a very short PTMEG chain than to a longer one which is comprised mainly of relatively non-polar tetramethylene oxide functionality. This effect of end group composition and concentration can be intuitively described as an example of the phenomenon “like dissolves like”, where the relatively non-polar, hydrophobic target molecules, carveol and carvone, exhibit greater chemical affinity towards the relatively non-polar PTMEG grades at higher molecular weight.

It is postulated that reduced PC values at lower molecular weights could be due in part to the potential dissolution of the polymer within the aqueous phase at lower molecular weights, which could affect aqueous solute activity. The PTMEG polymers also absorb a small amount of water themselves (< 2 wt%)²⁹, a similar amount to the Pebax® series (Table 6-1). This low amount of water uptake is not sufficient to skew mass balance calculations, whereas highly water-absorbing polymers require explicit consideration of absorbed water in mass balance calculations.² However, the presence of even a small amount of water within the polymer could easily affect the polymer’s chemical composition with respect to target molecule affinity by reducing or increasing the solute’s activity in the polymer. The effect of water uptake on the polarity of the polymer may be partly responsible for the observed effect of end group polarity on solute affinity, but this can be treated as an additive effect arising from polymer polarity.

Because the increasing polarity of PTMEG at lower molecular weights hampers its affinity towards non-polar target molecules, it was hypothesized that this same end group polarity effect may provide an opposite, favourable trend in affinity towards a different more polar, hydrophilic target molecule, 4-valerolactone (4VL), a fermentation product that we have recently studied in a TPPB context.²⁰ Indeed, Figure 6-4 shows a trend of increasing PC by approximately 30% at lower PTMEG

molecular weights due to the favourable polarity imparted by the higher concentration of hydroxyl end groups, which is also a manifestation of “like dissolves like”. The correction for measured crystallinity shown as the solid line in Figure 6-4 indicates that there is a relatively high normalized PC for 4VL at higher molecular weights, but this may be an artifact due to 4VL’s very small PC values and the polymer being largely crystalline at high molecular weights. Nevertheless, Figure 6-4 demonstrates that for practical purposes, low molecular weight PTMEG is a more effective sequestering phase for the relatively polar, hydrophilic target molecule.

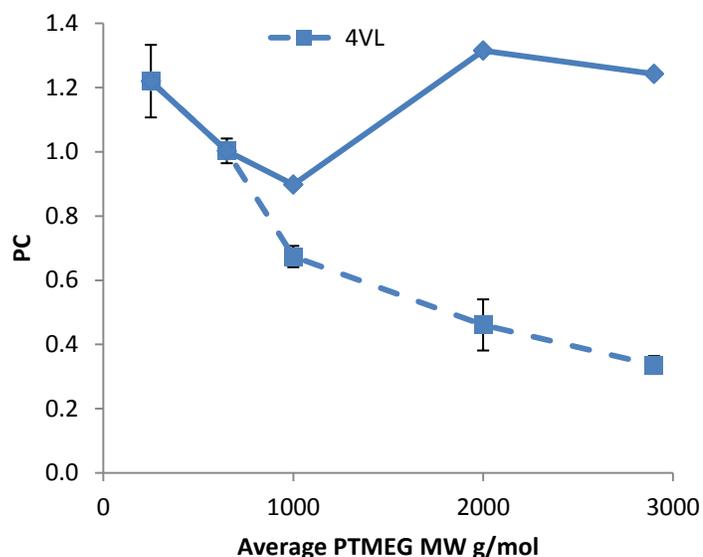


Figure 6-4: Partition coefficient of 4-valerolactone in the PTMEG series with increasing molecular weight. Solid lines represent values normalized to amorphous portion measured by DSC. Dashed lines represent values measured directly.

The effect of chain end group concentration, specifically its effect on polarity at low molecular weight, has a significant impact on target molecule affinity, the magnitude and direction of which depends on the polarity of the target molecule itself. This feature provides a strategy to improve affinity by selecting chain end functionality in commercial or custom-designed polymers, especially with low molecular weight polymers, and may also be useful in achieving selectivity among several target molecules.

6.5.5 Effect of end group polarity on target molecule selectivity

The end group polarity also has the effect of influencing target molecule selectivity, seen by the inversion of relative affinity towards carveol and carvone between the THP-PTMO and PTMEG series in Figures 6-2 and 6-3, respectively. This significant difference in relative PC values, which is constant across the tested molecular weight range, again illustrates the phenomenon of “like dissolves like”. Carveol’s greater polar surface area relative to carvone (20.23 \AA^2 vs. 17.07 \AA^2), owing to its hydroxyl functionality, potentially imparts favourable affinity towards the more polar PTMEG due to its similar end group functionality, while carvone exhibits greater affinity towards the non-polar THP-PTMO.

The logarithmic octanol/water partition coefficients ($\text{Log } K_{O/W}$) for carveol and carvone, 2.55 and 2.27 respectively, cannot provide any insight into the observed selectivity behaviour. This confirms that octanol-water partition coefficients, which assume a particular composition of the sequestering phase (octanol), do not provide specific approximations of polymer-solute affinity, because affinity is expected to deviate in either direction according to sequestering phase composition relative to the target molecule(s).²⁴ In contrast, polar surface area is a solute-specific property which appears to adequately describe the selectivity between similar target molecules, demonstrated here by manipulating polymer polarity. This means that solute-specific properties may be useful when comparing variations in affinity between similar solutes across different polymers as is done here, while $\text{Log } K_{O/W}$ is a binary metric which aggregates properties of the solute and solvents, and may be useful to roughly compare the affinity of very different solutes across similar polymers.

Interestingly, the Pebax® series of copolymers do not exhibit such selectivity, with PC values for both carveol and carvone being approximately equal (Table 6-2). In the copolymer material, chain ends are tethered at either end to join the discreet blocks, and free ends may be terminated or degraded. Furthermore, the presence of relatively polar poly(amide) hard segments, which may become incorporated to some extent within the polyether phase,⁷ may affect the chemical composition, and thus overall polarity, of the material. The results indicate that the manipulation of chain end group

composition provides an additional, useful variable in TPPB extractant selection to adjust target molecule affinity and selectivity; however, the effect of chain-end composition has additional implications on polymer physical properties, as shown above with the degree of crystallinity.

6.6 Conclusions

Block copolymers containing a large proportion of soft, amorphous segments are effective absorbents for TPPB applications due to their favourable affinity conferred by the soft segments in combination with good mechanical and thermal properties conferred by the hard segments, which do not significantly absorb solute, and whose proportion in sequestering materials should be as small as practically possible.

The non-polar end group functionality in the THP-PTMO series demonstrates that a reduction in molecular weight improves solute affinity by increasing the entropy of mixing, while the presence of hydroxyl end groups in the PTMEG series demonstrates that polar end groups impart relatively more polarity at lower molecular weights, possibly increasing mutual polymer-water solubility, which can have either a beneficial or detrimental effect on solute affinity, depending on the nature of polymer interactions with the solute and with water.

The lack of bulky end groups in PTMEG allows the development of significant crystalline domains at higher molecular weight, which improve the mechanical resilience of the material, yet are inert with respect to solute absorption. The incorporation of hard blocks in copolymers may provide more effective control over the material's ultimate absorptive and mechanical properties than relying on varying degrees of spontaneous crystallinity as encountered in semicrystalline materials such as PTMEG. The normalization of partition coefficients is useful to compare the solute affinity of *absorptive* portions of different materials of varying crystallinity. It is also useful to consider the overall partition coefficient as observed, which allows practical comparison of whole materials as copolymers or semicrystalline materials.

This study has identified useful strategies for future extractant selection efforts, which are intended to improve on currently-available materials through modifications or the design of task-specific materials. Reductions in molecular weight, crystallinity, and hard segment proportion all act to improve solute partitioning at the expense of physical properties, while polymer polarity offers a tool for target molecule selectivity. Future investigations by our Group aim to characterize the potential consequences of the above-mentioned aspects on biocompatibility, and to elucidate the effects on mass transfer rates, two important considerations in bioprocess design.

6.7 Acknowledgements

The financial support of the National Sciences and Engineering Research Council of Canada is gratefully acknowledged. The authors thank Antonio Cillero Rodrigo for obtaining the NMR data.

6.8 References

- 1 Gao F, Daugulis AJ, Polymer-solute interactions in solid-liquid two-phase partitioning bioreactors. *J Chem Technol Biot* **85**:302-306 (2010).
- 2 Dafoe JT, Daugulis AJ, Manipulating the Composition of Absorbent Polymers Affects Product and By-product Concentration Profiles in the Biphasic Biotransformation of Indene to *cis*-1, 2-indandiol. *Biochem.Eng.J.* (2013).
- 3 Poleo EE, Daugulis AJ, A comparison of three first principles methods for predicting solute-polymer affinity, and the simultaneous biodegradation of phenol and butyl acetate in a two-phase partitioning bioreactor. *Journal of Chemical Technology and Biotechnology* (2013).
- 4 Quijano G, Hernandez M, Villaverde S, Thalasso F and Muñoz R, A step-forward in the characterization and potential applications of solid and liquid oxygen transfer vectors. *Appl.Microbiol.Biotechnol.* **85**:543-551 (2010).
- 5 Alberola N, Micromechanical properties of polyether block amide copolymers. *J Appl Polym Sci* **36**:787-804 (1988).
- 6 Bornschlegl E, Goldbach G, Meyer K. Structure and properties of segmented polyamides. *Frontiers in Polymer Science*: Springer; 1985. p. 119-124
- 7 Sheth JP, Xu J and Wilkes GL, Solid state structure-property behavior of semicrystalline poly (ether-block-amide) PEBAX[®] thermoplastic elastomers. *Polymer* **44**:743-756 (2003).
- 8 Faruque HS, Lacabanne C, A thermally stimulated current technique for measuring the molecular parameters of Pebax, a polyether-block amide copolymer. *J.Mater.Sci.* **22**:675-678 (1987).

- 9 Faruque HS, Lacabanne C, Study of multiple relaxations in PEBAX, polyether block amide (PA12 2135 block PTMG 2032), copolymer using the thermally stimulated current method. *Polymer* **27**:527-531 (1986).
- 10 Rezac ME, John T and Pfromm PH, Effect of copolymer composition on the solubility and diffusivity of water and methanol in a series of polyether amides. *J Appl Polym Sci* **65**:1983-1993 (1997).
- 11 Rezac ME, John T, Correlation of penetrant transport with polymer free volume: additional evidence from block copolymers. *Polymer* **39**:599-603 (1998).
- 12 Trong Nguyen Q, Sublet J, Langevin D, Chappey C, Marais S, Valleton J, Poncin-Epaillard F, CO₂ Permeation with Pebax®-based Membranes for Global Warming Reduction. In: B Freeman, Y Yampolskii, (Eds.) *Membrane Gas Separation*, 2010, Wiley. Hoboken NJ, pp 255-277
- 13 Armstrong S, Freeman B, Hiltner A and Baer E, Gas permeability of melt-processed poly (ether block amide) copolymers and the effects of orientation. *Polymer* **53**:1383-1392 (2012).
- 14 Bondar V, Freeman B and Pinnau I, Gas transport properties of poly (ether-b-amide) segmented block copolymers. *Journal of Polymer Science Part B: Polymer Physics* **38**:2051-2062 (2000).
- 15 Jonquieres A, Clément R and Lochon P, Permeability of block copolymers to vapors and liquids. *Progress in Polymer Science* **27**:1803-1877 (2002).
- 16 Lennox CD, inventor. Scrimed Life Systems I, assignee. Localized delivery of drug agents. USA patent 6280411B1. 2001 August 28, 2001
- 17 Cleary GW, Roy S, inventors. Cygnus Corporation, assignee. Transdermal drug-delivery composition. USA patent 4906463. 1990 March 6, 1990
- 18 Cleary GW, Roy S, inventors. Cygnus Corporation, assignee. Resilient transdermal drug delivery device. CA/USA patent 5,006,342. 1991 April 9, 1991
- 19 Morrish JLE, Daugulis AJ, Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. *Biotechnol. Bioeng.* **101**:946-956 (2008).
- 20 Dafoe JT, Daugulis AJ, Production of 4-valerolactone by an equilibrium-limited transformation in a partitioning bioreactor: impact of absorptive polymer properties. *Bioproc Biosyst Eng* DOI: 10.1007/s00449-013-1020-5 (2013).
- 21 Leo A, Hansch C and Elkins D, Partition coefficients and their uses. *Chem Rev* **71**:525-616 (1971).
- 22 Raftopoulos K, Janowski B, Apekis L, Pielichowski K and Pissis P, Molecular mobility and crystallinity in polytetramethylene ether glycol in the bulk and as soft component in polyurethanes. *Eur Polym J* **47**:2120-2133 (2011).
- 23 Blaine RL. TA Instruments Thermal Applications Note: Polymer Heats of Fusion. www.tainstruments.com/library_download.aspx?file=TN048.pdf
- 24 Parent JS, Capela M, Dafoe JT and Daugulis AJ, A first principles approach to identifying polymers for use in two-phase partitioning bioreactors. *J Chem Technol Biot* **87**:1059-1065 (2012).

- 25 Prpich GP, Daugulis AJ, A novel solid–liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. *Biotechnol Bioeng* **98**:1008-1016 (2007).
- 26 Fam H, Daugulis AJ, Substrate mass transport in two-phase partitioning bioreactors employing liquid and solid non-aqueous phases. *Bioproc Biosyst Eng* **35**:1367-1374 (2012).
- 27 Flory PJ, Thermodynamics of high polymer solutions. *J Chem Phys* **10**:51 (1942).
- 28 Patterson D, Polymer compatibility with and without a solvent. *Polym Eng Sci* **22**:64-73 (1982).
- 29 BASF. PolyTHF® - Soft Segment Intermediate.
<http://www.basf.com/group/corporate/en/brand/POLYTHF>
- 30 Arkema. PEBAX® 2533 SA 01 MED .
<http://www.pebax.com/export/sites/pebax/.content/medias/downloads/literature/tds-pebaxmed-2533sa01.pdf>
- 31 Arkema. PEBAX® 3533 SA 01 MED .
<http://www.pebax.com/export/sites/pebax/.content/medias/downloads/literature/tds-pebaxmed-3533sa01.pdf>
- 32 Arkema. PEBAX® 4033 SA 01 MED .
<http://www.pebax.com/export/sites/pebax/.content/medias/downloads/literature/tds-pebaxmed-4033sa01.pdf>
- 33 Arkema. Pebax 7033 SA01 MED.
<http://www.pebax.com/export/sites/pebax/.content/medias/downloads/literature/tds-pebaxmed-7033sa01.pdf>

Chapter 7 Summary and conclusions

The objective of this thesis was to explore the properties of absorbent polymers which impart favourable performance for their use as extractants in two-phase partitioning bioreactors (TPPBs). These materials have demonstrated remarkable improvements in performance and practicality over conventional immiscible liquid solvents, attributed to their affinity towards a wide range of inhibitory biotransformation molecules, and to their biocompatibility as inert solids which do not cross cell membranes. These important features make these materials widely applicable to many biotransformation systems.

The initial significant contribution derived from this thesis was the demonstration that a solid absorbent block copolymer, HytreI® 8206, could expand the range of extractable target molecules relative to silicone oil, a conventional liquid extractant, in the biotransformation of indene to *cis*-(1S,2R)-indandiol by *P. putida* 421-5 (ATCC55687). Using this polymer, the inhibitory by-products were preferentially extracted, with significant co-absorption of the desired product, neither of which had significant affinity for silicone oil. The superior extraction of inhibitors by the polymer led to greater productivity relative to silicone oil due to lower biocatalyst inhibition, and recovery of the solid polymer extractant was very simple, permitting its re-use. Importantly, the solid polymer completely avoided problems of emulsion and entrapment in the aqueous phase, greatly simplifying phase separation for analytics.

In the next contribution, the nature of copolymer composition was investigated for its effects on the indene biotransformation. The block copolymer used in the previous report, HytreI®8206, contained significant hydrophilic poly(ether) content, evidenced by its significant water absorption when compared to other poly(ether)-based block copolymers, which took up negligible amounts of water, indicating that their soft phases were relatively hydrophobic. This property affected their relative affinity towards the three biotransformation products, each exhibiting a different degree of hydrophobicity and

hence extraction potential. The less-polar inhibitory by-products were extracted to a greater extent using the hydrophobic copolymers, while the significantly polar product, *cis*-(1S,2R)-indandiol, demonstrated the highest affinity towards the hydrophilic copolymer, Hytrel® 8206. Additionally, a greater proportion of soft poly(ether) blocks within the hydrophobic block copolymers was found to improve affinity across all solutes, while the effect of relative hydrophobicity remained pronounced. The extraction selectivity had a significant effect on the outcome of the biotransformation, as the biocatalyst responded by producing additional amounts of those compounds which were most extensively extracted, providing an external means of manipulating metabolic fluxes. The partition coefficient towards all solutes was found to increase significantly when measured in bioreactor medium compared to high-purity water, highlighting a salt effect acting to increase solute activity in the aqueous phase, thereby increasing the driving force for partitioning into the polymer. An additional contribution of this work demonstrated that water uptake should be accounted for by pre-equilibrating the polymers in water when comparing solute affinity in polymers with varying water uptake levels, in order to avoid errors in mass balance calculations arising from unequal uptake of water between different polymers.

To further investigate the applicability of hydrophilic copolymers for extraction of hydrophilic target molecules, a new biocatalytic system was investigated which featured a water-miscible target molecule, 4-valerolactone (4VL), produced by the engineered biocatalyst, *P. putida* KT2440, in an equilibrium-limited reaction from the intermediate hydroxyacid, 4-hydroxyvalerate (4HV). To investigate the effects of polymer hydrophilicity, a custom-synthesized, hydrophilic rubber containing poly(ethylene oxide) as its major constituent, cross linked to varying extents with N,N'-m-phenylenedimaleimide (BMI-PEO), was compared to cross linked poly(ethylene glycol diacrylate) (XLPEGDA) networks containing varying amounts of water, and to the commercial hydrophilic block copolymer, Hytrel® 8206, for their affinity towards the target molecule, and subsequently for their performance as extractants in a TPPB. Mechanical integrity was a problem in the XLPEGDA series, while the other polymers had sufficient

resilience to be used in the TPPB. While the BMI-PEO polymer gave higher partition coefficients than HytreI® 8206 in water, its performance in the bioreactor was poor due to its lack of selectivity between the precursor, 4HV, and the product, 4VL. Additionally, the partition coefficient towards 4VL measured in the bioreactor medium was significantly improved relative to water for HytreI® 8206, whereas BMI-PEO demonstrated nearly identical values. This phenomenon arose due to the polymer's extreme degree of water uptake, where *all* aqueous solutes were absorbed together, eliminating the above-mentioned salt effect, and demonstrating that water uptake, which could be helpful at low amounts, becomes detrimental once selectivity is hampered. Conversely, the moderately water-absorbing commercial copolymer, HytreI® 8206, demonstrated adequate selectivity and target molecule affinity to pull the equilibrium reaction, improving production by 30%. This work represented the first application of polymer absorption to pull an equilibrium-governed reaction to greater extent of conversion.

In the final contribution derived from this thesis, the constituent homopolymers comprising the series of Pebax® commercial block copolymers, poly(tetramethylene oxide) (PTMO) and poly(amide-12) (PA-12), were first evaluated for their relative affinity, and it was established that the hard component of these block copolymers participates negligibly in solute uptake. Furthermore, as the proportion of hard segment was increased in the Pebax® series, partition coefficients which were normalized to the amorphous fraction of the material also decreased, suggesting a continuous PA-12 phase acted as a barrier to solute absorption by occluding portions of the soft poly(ether) phase. These findings confirm that hard segment proportion in the material should be minimized in polymer selection, while preserving adequate mechanical and thermal resilience. Subsequently, the soft PTMO component was examined in the absence of its hydroxyl-functional chain ends to isolate the effect of molecular weight on solute affinity, which was found to provide a significant improvement as molecular weight was reduced. However, in the glycol-functionalized PTMEG series, the polarity of end groups was observed to cause a drastic reduction in affinity towards relatively non-polar biotransformation target molecules,

carveol and carvone, while improving affinity at lower molecular weights towards the water-miscible target molecule, 4-valerolactone. This effect was apparent at low molecular weights, where the end-group concentration is relatively higher than at long chain lengths. This effect of chain end-group composition imparted selectivity between carveol and carvone which was reversed in the absence of end-group functionality. The presence of polar end-group functionality also hindered solute uptake by contributing to significant crystallinity, which acted as a barrier to absorption in a similar way that was seen with the hard segment in the copolymers, by contributing to mechanical resilience while impeding absorption. This suggests that both crystallinity and hard segment components should be minimized to the extent that is practically possible when selecting from available materials.

The combined contributions of this thesis have improved the understanding of the properties of polymer extractants influencing their performance, and have extended the application of polymers towards the extraction of target molecules which are difficult to separate from water due to their hydrophilicity.

7.1 Future work

It is conceivable that the best-performing, commercially-available materials are within the family of poly(ether)-based block copolymers containing a high proportion of the soft component. Emphasis on improving the extraction of relatively hydrophilic target molecules, many of which are emerging as important bioproducts, is a clear objective. Future efforts in extractant selection will likely transition towards polymer design using rational strategies, and will aim to circumvent the less-favourable aspects inherent in the commercially-available materials to strive for new benchmarks in extractant performance. For example, by imparting mechanical resilience through covalent cross links stabilizing a network composed almost entirely of the soft, absorptive component, the effect of an inert component can be minimized.

The biocompatibility of solid polymers is well-established, but if lower molecular-weight polymers improve affinity, they may approach a size at which biocompatibility is compromised. An investigation into the effect of reducing molecular weight from polymers to oligomers and monomeric solvents is currently underway in our Group. Finally, the property of diffusivity is one which has received less attention than polymer-solute affinity, but is nevertheless important to maintain a kinetically-limited biocatalytic system. Consideration of polymer properties which promote rapid diffusion, such as low glass transition temperature and degree of crystallinity, may be useful to improve selection criteria in this regard.

Appendix A: UNIFAC predictions of partition coefficients

This section contains estimates of partition coefficients for carveol and carvone, made using the UNIFAC-vdW-FV model¹ for solute activity in polymers across a range of polymer molecular weights. This aspect was investigated to attempt to provide a theoretical framework to describe the experimental results from Chapter 6, and which could also be useful in performing *a priori* estimates of partitioning. The outcome of this modeling effort produced results which generally captured some experimental trends, but failed to remain consistent across all systems studied. This suggests that the model may require some additional inputs, for example the inclusion of small amounts of water in a ternary system, which more closely mirrors the experimental conditions. However, the accurate quantification of water (and its effect on affinity) in polymers is complex, and should be the primary focus of a future investigation.

The UNIFAC-vdW-FV model is a modification of the standard UNIFAC model which includes a free-volume term for improved predictions with polymer systems. UNIFAC considers both entropic and enthalpic interactions contributed by constituent groups. Polymer structures were specified explicitly including end groups, and molecular weight was varied systematically by changing the number of repeat units within the chain. Because solute activity in each phase is equal at equilibrium, the partition coefficient is estimated as the ratio of activity coefficients at infinite dilution in the aqueous phase divided by the polymer phase, given by the following equation:²

$$PC = \frac{x_{\text{solute}}^{\text{organic}}}{x_{\text{solute}}^{\text{aq}}} = \frac{\gamma_{\text{solute}}^{\text{aq}}}{\gamma_{\text{solute}}^{\text{organic}}}$$

Aqueous solute activity coefficients were obtained from experimental reports,³ but could be estimated using an activity model if published data were unavailable. The predictions in Figure A-1 capture the improvement in partition coefficients at low molecular weights of THP-PTMO, and also show the plateau in PC values at higher molecular weights, but the magnitude of the effect is exaggerated

compared to what is seen experimentally in Figure 6-2. Similarly, the reduction in estimated partition coefficients with decreasing PTMEG molecular weight shown in Figure A-2 for carveol is superficially similar to the experimental results in Figure 6-3, yet carveol shows an opposite deviation which is not seen experimentally. Predictions for both solutes show a plateau in PC values at higher molecular weights, which was seen experimentally in Figure 6-3.

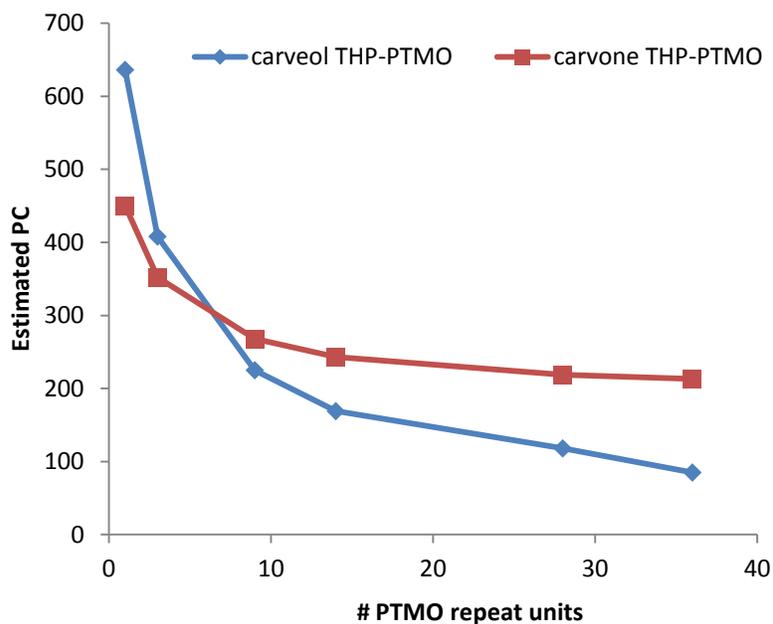


Figure A-1: Partition coefficients of carveol and carveone estimated using the UNIFAC-vdW-FV model in THP-PTMO of varying molecular weight

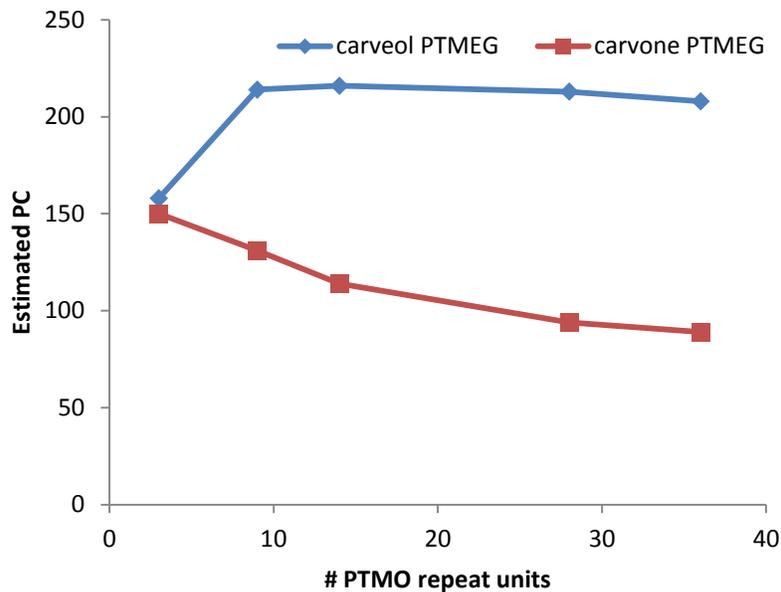


Figure A-2: Partition coefficients of carveol and carvone estimated using the UNIFAC-vdW-FV model in PTMEG of varying molecular weight.

The major shortcoming of these predictions appears to be that the predicted selectivity of each polymer toward carveol and carvone are not consistent with what was seen experimentally. Aside from the deviations discussed above, the predicted relative affinity of PTMEG toward each compound is inverted from what was seen experimentally, and what would be intuitively expected based on polymer and solute functionality. The predicted selectivity of THP-PTMO was consistent with experimental results at high molecular weight, but also became inverted at low molecular weight. The discrepancy in the PTMEG predictions may be partly a result of this polymer having mutual interactions with water, which is not included in these binary predictions. However, THP-PTMO is very hydrophobic and there was no evidence of water uptake or polymer dissolution, yet the predicted carveol/carvone selectivity does not correspond with the experimental results shown in Figure 6-2.

A.1 References

- 1 Kannan D, Duda J and Danner R, A free-volume term based on the van der Waals partition function for the UNIFAC model. *Fluid Phase Equilib.* **228**:321-328 (2005).
- 2 Parent JS, Capela M, Dafoe JT and Daugulis AJ, A first principles approach to identifying polymers for use in two-phase partitioning bioreactors. *J Chem Technol Biot* **87**:1059-1065 (2012).
- 3 Fichan I, Larroche C and Gros J, Water solubility, vapor pressure, and activity coefficients of terpenes and terpenoids. *J Chem Eng Data* **44**:56-62 (1999).

Appendix B: Hansen Solubility Parameter estimations of relative polymer-solute affinity

This section describes attempts at using a qualitative method to predict the relative affinity of polymers toward different solutes, using Hansen Solubility Parameters (HSP)¹. It was undertaken to provide an alternative theoretical framework to describe the experimental results from Chapter 6, and to discern the utility of HSP in judging polymer-solute affinity based on their individual properties, assuming constant solute affinity toward the aqueous phase. The predictions made using the HSP method appeared to capture the experimental results in relative polymer-solute affinity, yet the monotonic trends with increasing molecular weight are clearly artifacts, because the polymer is not expected to differ drastically at high molecular weights. This approach to polymer-solute affinity may be useful in future studies to provide a simple, accessible ranking of solute affinity across different polymers, in combination with a database of polymers' Hansen Solubility Parameters. However, due caution of the qualitative nature of HSP should be exercised, and it is not expected to provide the same level of detail as models which incorporate both enthalpic and entropic aspects of polymer-solute affinity.

The HSP method gives an idea of chemical compatibility (solubility) by comparing the similarity of dispersive (non-polar), polar, and hydrogen-bonding interactions between two molecules of interest, one being the solute and the other the polymer. The lumped "overall" solubility parameter is equivalent to Hildebrand's solubility parameter for regular solutions.² These properties are strictly enthalpic aspects of polymer-solute affinity, such that entropic effects of molecular weight are not captured by this method, and its extension to large molecular weights is not feasible. To estimate polymer-solute affinity, the component HSP values are compared by their mutual distance in a three-dimensional coordinate space, given by the following equation¹:

$$Ra = \sqrt{4(\delta_{d2} - \delta_{d1})^2 + (\delta_{p2} - \delta_{p1})^2 + (\delta_{hb2} - \delta_{hb1})^2}$$

Where δ_d are the dispersive HSP components of the solute and polymer, δ_p are the polar HSP components, and δ_{hb} are the hydrogen-bonding HSP components. A smaller overall Ra “distance” between polymer and solute suggests improved mutual solubility, such that polymers which minimize the Ra distance should exhibit the best solute affinity. However, the polymer’s physical properties must be favourable for sorption in the first place, requiring a low glass transition temperature and a low degree of crystallinity.

An advantage of HSP is its accessibility, with group-contribution predictions of parameter values available from polymer and solute structures using the Hansen Solubility Parameters in Practice software (HSPiP ver. 4.0.0.5). The HSP of carveol, carvone, and 4-valerolactone were computed using the HSPiP software from their respective SMILES structures. Polymer HSP were obtained using SMILES structures representing polymers of different chain lengths, with end groups given explicitly. This method circumvented HSPiP’s built-in database of polymer HSP values, which are somewhat arbitrarily assigned, and allowed the systematic analysis of end group effects which have a strong impact on polymer polarity.

The “overall” HSP Ra distances between THP-PTMO and carveol/carvone are given in Figure B-1. This plot shows that the Ra distance from THP-PTMO to carvone is lower than to carveol in the low molecular weight range, which corresponds to the selectivity shown experimentally in Figure 6-2.

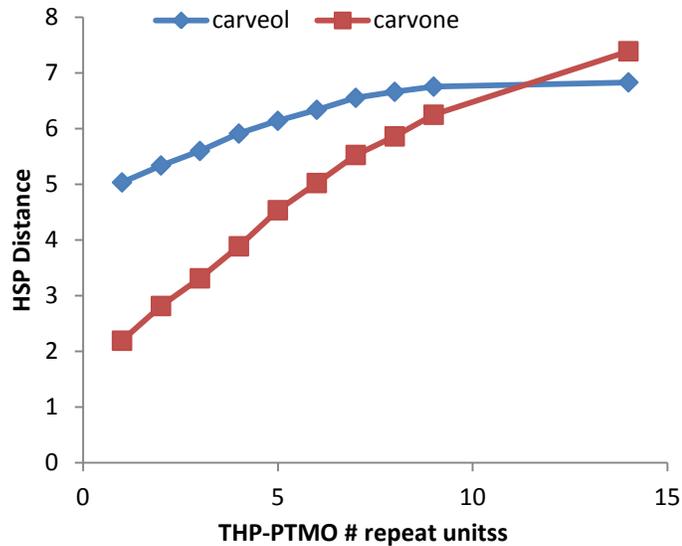


Figure B-1: Hansen Solubility Parameter “overall” Ra distance between THP-PTMO and carveol/carvone

Looking at the HSP component parameter distances between THP-PTMO and carveol and carvone by their difference, it is evident that the main contributors in carveol’s affinity (Figure B-1) are the polar and dispersive components at low molecular weights, shown in Figure B-2. For carvone, it appears that the dispersive and hydrogen bonding components are helping compatibility at lower molecular weights, while the polar component goes through a minimum and then increases at low molecular weight, shown in Figure B-3. Note that a small difference between solute and polymer HSP component values does not imply a high extent of that characteristic, only that their values are similar.

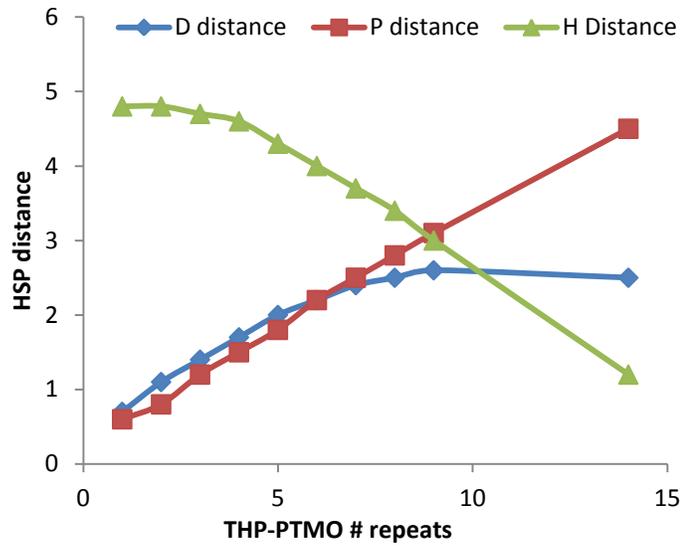


Figure B-2: Hansen Solubility Parameter component Ra distances between carveol and THP-PTMO

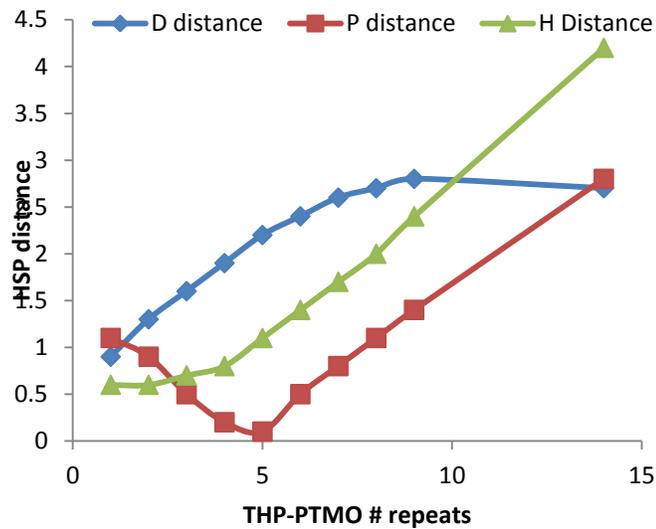


Figure B-3: Hansen Solubility Parameter component Ra distances between carveol and THP-PTMO

For the hydroxyl-terminated PTMEG polymer series, HSP would be expected to provide information about the compatibility of carveol and carvone with the polymer based on its changing polarity across the molecular weight range. Figure B-4 shows the overall HSP Ra distances between carveol/carvone and PTMEG, which correctly predicts the selectivity seen experimentally in Figure 6-3, and also superficially reflects the drastic decrease in affinity at lower molecular weights. The predictions

at high molecular weight show monotonically decreasing affinity, which was not seen experimentally, as the values plateaued at higher molecular weights in Figure 6-3.

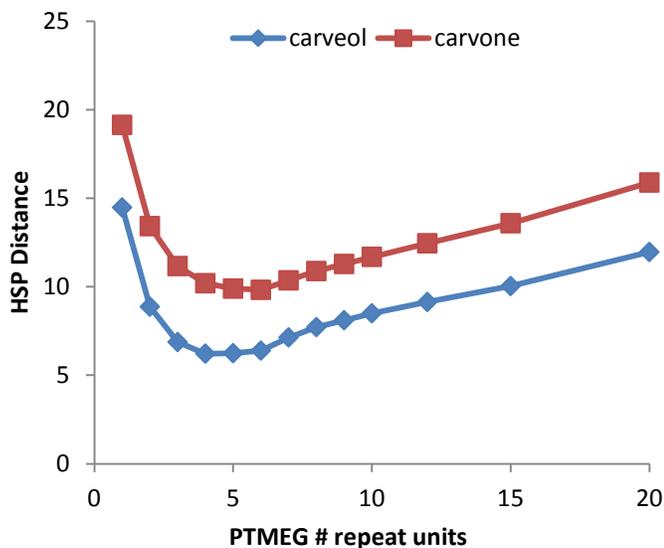


Figure B-4: Hansen Solubility Parameter “overall” Ra distance between PTMEG and carveol/carvone

The plots of component HSP distances from PTMEG for carveol and carvone, shown in Figures B-5 and B-6 respectively, are superficially similar, but the contribution from the hydrogen bonding component is stronger in carveol, which possesses a hydrogen bond donor, while carvone has a slightly stronger contribution from the polar component. Note that because the shapes of the overall plots in Figure B-1 and B-4 are a combination of the component plots, the strongest contributors from the component plots have the largest effect on the shape of the overall plot.

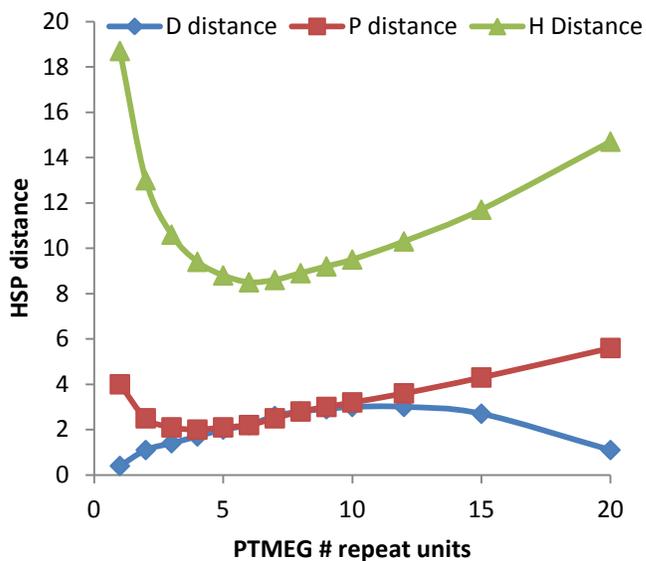


Figure B-5: Hansen Solubility Parameter component distances between PTMEG and carveol

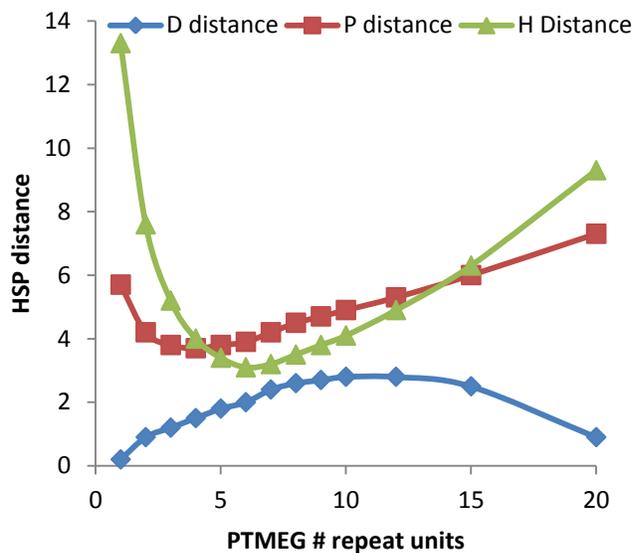


Figure B-6: Hansen Solubility Parameter component distances between PTMEG and carveone

Looking at the component plots for carveol and carveone, in B-5 and B-6 respectively, it is clear that the dispersive components show favourable affinity for both solutes, while the major differences in predicted affinity arise from the polar and hydrogen bonding components, which are similar in shape to the overall plots in Figure B-4. Carveol exhibits favourable hydrogen bonding affinity at a moderate

molecular weight, and while carvone has a similar shape, it is the polar component that lies closest to that of PTMEG.

Because the predicted affinity toward the relatively non-polar solutes, carveol and carvone, decreased at lower molecular weights with increasing PTMEG polarity, it was hypothesized that increasing PTMEG polarity may improve affinity toward a very polar solute, 4-valerolactone. Figure B-7 shows a complete inversion of HSP component contributions compared to the plots for carveol and carvone, with the dispersive component negatively contributing to affinity, while the polar and hydrogen-bonding components strongly favour affinity at low PTMEG molecular weights, where its polar character is strongest. This agrees with experimental results shown in Figure 6-4.

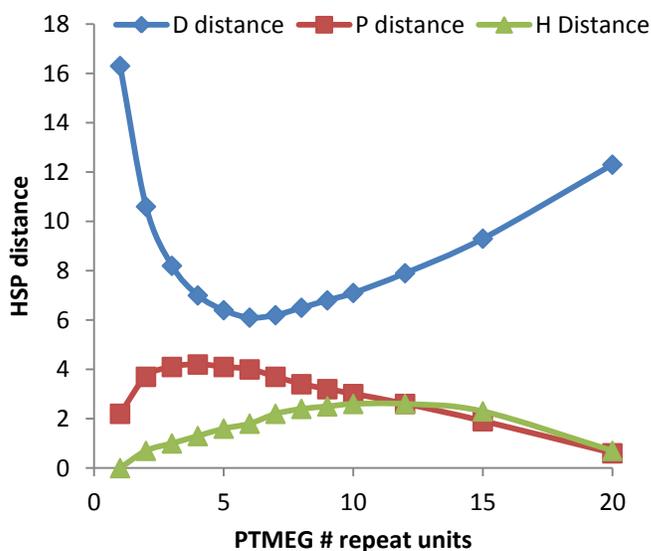


Figure B-7: Hansen Solubility Parameter component distances between PTMEG and 4-valerolactone

These analyses of polymer-solute affinity using Hansen Solubility Parameters give some insights into the potential mechanisms at play in the absorption of different solutes. Unfortunately, the qualitative nature of these predictions, and that they only consider polymer-solute interactions and neglect solute affinity for water, means that they cannot give estimates of relative magnitudes. However, the relative affinity of each target molecule toward different polymers appears to be

adequately captured for these systems. The HSP system could be useful to identify potentially promising polymers from a library when their physical properties are considered.

B.1 References

1 Hansen CM. Hansen solubility parameters: a user's handbook. CRC Press; 2007

2 Hildebrand JH. Solubility of non-electrolytes. Reinhardt Publishing Corporation; 1936