The Extraction of Polyhydroxybutyrate from Methanotrophs using Switchable Solvents

by

Mark Douglas Lawley

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Abstract

One of the major challenges that humanity faces is the buildup of plastic waste in the environment. As a biodegradable plastic with similar physical properties to consumer plastics, poly(3-hydroxybutyrate) (PHB) is an excellent option for their replacement. PHB is produced inside bacterial cells, typically induced by periods of nutrient limitation, and can be produced from a variety of low-cost feedstocks. However, the challenges associated with the extraction of PHB from bacterial cells remain significant. In this thesis, switchable hydrophilicity solvents (SHSs) – which can be toggled between a hydrophobic and hydrophilic form – were examined as recyclable solvents for the extraction and processing of PHB.

Through a theoretical screening using group-contribution parameters followed by experimental validation, it was found that two SHSs were good for recovering PHB: N,N-dimethylbenzylamine (DMBA) and N,N-dimethylcyclohexylamine (DMCHA). Both of these showed gelation of porous PHB at room temperature and dissolution at high temperatures. It was shown that both DMBA and DMCHA could dissolve PHB and precipitate it through the addition of water and carbon dioxide (switching the solvent into its hydrophilic form), and the solvent could afterwards be recovered by switching back to the hydrophobic form and recycled to perform subsequent dissolutions and recoveries. However, the molecular weights of the PHB were reduced due to the high temperatures and long exposure times needed, and the molecular weight of the final product was further affected by longer PHB chains being more prone to gelation while filtering the PHB-rich solvent.

When PHB was dissolved in DMBA to high concentrations at elevated temperatures, a gel was formed upon cooling. It was observed that the solvent in these gels could still be switched through the addition of carbon dioxide and water. This “switched gel” still had a large portion of its mass as liquid, and could be formed into shapes and dried. Similar to the precipitation of dissolved PHB, it was shown that

DMBA could be switched back from its hydrophilic form and recovered for subsequent dissolution-gelation cycles, although with lower recycle rates due to the amount of solvent retained in the gel.

Using the knowledge gained from previous experiments, extraction procedures from PHB-containing methanotrophic bacteria were developed using the SHSs DMBA and DMCHA. The focus of extraction experiments was to determine whether certain steps (dewatering, mechanical lysis before treatment, separation by centrifugation, heating) were necessary for PHB extraction, with the aim of informing a simple and efficient extraction protocol. Two primary methods carried out at room temperature were found to be effective separations. In the first, concentrated PHB-containing biomass was vortexed with water and SHS, the mixture was centrifuged, the interface layer was separated, and the solvent form was switched through the addition of water and carbon dioxide. In a second method, unconcentrated PHB-containing bioreactor effluent was stirred in the presence of a SHS, separated by pipet, and the solvent was then switched to recover PHB. A third method was tested where dried or concentrated PHB-containing biomass was held at high temperature, as in the dissolution experiments, but it was found that this did not effectively separate PHB from biomass. For the room-temperature protocol from unconcentrated biomass, it was demonstrated that the SHS could be recovered and recycled for subsequent extractions.

The two successful PHB separations have significant advantages over many published extraction protocols in that they were performed at room temperature with minimal process steps and a recyclable extraction agent, which gives them the potential as low-cost PHB extraction solvents with minimal material and energy use.

Preface

Part of this thesis has been previously published, and other chapters will be submitted for publication.

Chapter 3 was published as Lawley, M. D., Boon, D., Stein, L. Y., & Sauvageau, D. (2022). Switchable solvents for the reversible dissolution of poly(3-hydroxybutyrate). *ACS Sustainable Chemistry & Engineering*, *10*(8), 2602–2608. https://doi.org/10.1021/acssuschemeng.1c06377. I was the primary author, and was responsible for conceptualizing, designing, performing, and analyzing the results of experiments, and writing the manuscript. David Boon was an undergraduate researcher who was responsible for performing the visualization of PHB in switchable hydrophilicity solvents at room temperature and conducted a series of experiments that led to the conclusion there was no significant PHB dissolution at room temperature. Dr. Dominic Sauvageau and Dr. Lisa Stein were supervisory authors, who contributed to conceptualization and manuscript composition, and provided advice to the design and analysis of experiments.

Chapter 4 was published as Lawley, M. D., Stein, L. Y., & Sauvageau, D. (2024). Dissolution and recovery of poly(3-hydroxybutyrate) in switchable solvents and the formation of a switchable gel. *Current Research in Green and Sustainable Chemistry*, 9, 100421. https://doi.org/https://doi.org/10.1016/j.crgsc.2024.100421. I was the primary author, and was responsible for conceptualizing, designing, performing, and analyzing the results of experiments, and writing the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Stein were supervisory authors, who contributed to conceptualization and manuscript composition, and provided advice to the design and analysis of experiments. Dr. Violeta Toader from the Quebec Centre for Advanced Materials (QCAM/CQMF) performed the GPC analyses for this study.

Chapter 5 will be submitted for publication as “Extraction of poly(3-hydroxybutyrate) from *Methylocystis* sp. Rockwell using switchable hydrophilicity solvents”. I was the primary author, and was responsible for conceptualizing, designing, performing, and analyzing the results of experiments, and writing the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Stein were supervisory authors, who contributed to conceptualization and manuscript composition, and provided advice to the design and analysis of experiments. Dr. Hem Sharma, Dr. Marina Lazic, Rachel Rieberger, and Nina Deschner all contributed by growing the biomass used in the experiments.

Dedication

To whom it may concern:

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Table of Contents

Abstract ii
Preface iv
Dedication v
Acknowledgements vi
Table of Contents vii
List of Tables x
List of Figures xi
List of Abbreviations xii
List of Symbols xiii
1 Introduction 1
1.1 Motivation: Improving Production of Biodegradable Plastics 1
1.2 Thesis Scope 2
1.3 Thesis Structure 2
1.4 References 3
2 Literature Review 5
2.1 Polyhydroxybutyrate (PHB) 5
2.2 Extraction of PHB from Biomass 11
2.3 Switchable Solvents 31
2.4 Polymer Dissolution 34
2.5 Methanotrophic microorganisms 37
2.6 Conclusion 42
2.7 References 42
3 Switchable Solvents for the Reversible Dissolution of Poly(3-hydroxybutyrate) 60
3.1 Abstract 60
3.2 Introduction 60
3.3 Materials and Methods 62

3.4 Results 65
3.5 Discussion 69
3.6 Conclusion 71
3.7 Acknowledgements 72
3.8 References 72
4 Dissolution and Recovery of Poly(3-hydroxybutyrate) in Switchable Solvents and the Formation of a Switchable Gel 76
4.1 Abstract 76
4.2 Introduction 76
4.3 Materials and Methods 78
4.4 Results 81
4.5 Discussion 91
4.6 Acknowledgements 95
4.7 References 95
5 Extraction of Poly(3-hydroxybutyrate) from *Methylocystis* sp. Rockwell Using Switchable Hydrophilicity Solvents 99
5.1 Abstract 99
5.2 Introduction 99
5.3 Materials and Methods 101
5.4 Results 107
5.5 Discussion 112
5.6 Conclusion 114
5.7 Acknowledgements 114
5.8 References 114
6 Conclusions and Future Direction 119
6.1 Summary of the Work 119

6.2 Avenues for Further Research 120

6.3 References 121

Unified Bibliography 122

A Appendix 145

List of Tables

Table 2.1 Material properties of PHA copolymers and common petrochemical plastics. 6
Table 2.2: Recently developed PHB extractions from biomass by solvent extraction. 15
Table 2.3: PHA extraction from biomass by digestion-dissolution 16
Table 2.4: PHA Extraction from biomass by chemical NPCM digestion 19
Table 2.5: PHA extraction from biomass by enzymatic digestion 21
Table 2.6: PHA Extraction from biomass by mechanical separation 22
Table 2.7: Unique PHA extraction methods. 24
Table 2.8: PHA extraction processes with variations in pre-treatment steps. 26
Table 2.9: PHA extraction processes with variations in purification steps. 28
Table 3.1: Properties of investigated non-protonated SHSs 66
Table 3.2: Solubilized PHB in SHSs at various conditions..... 68
Table 5.1: Solvent recovery rates after each cycle of the extraction process 110

List of Figures

Figure 2.1: Molecular structure of common PHAs 6
Figure 2.2: Bread packaged in a PHB films. 8
Figure 2.3: PHB in bacterial cells. 8
Figure 2.4 PHB film degradation. 11
Figure 2.5 An overview of the typical steps in a PHA extraction process. 12
Figure 2.6: Switchable hydrophilicity solvent structure and mechanism 31
Figure 2.7: A schematic of a process using a SHS to extract biofuels or PHB from bacterial cells. 32
Figure 2.8: The mechanism of polymer dissolution 36
Figure 2.9: Metabolic pathway for the conversion of methane to PHB in alphaproteobacterial methanotrophs, such as *Methylobacterium extorquens* AM1 and *Methylocystis* sp. Rockwell. 39
Figure 3.1: Visual demonstration of PHB solubility in SHSs 67
Figure 3.2: PHB solubilization in SHSs 69
Figure 4.1: Procedure for dissolving and precipitating PHB with SHS and recycling SHS for re-use 79
Figure 4.2: PHB recovery with DMBA 82
Figure 4.3: DMBA recycling after PHB dissolution 84
Figure 4.4: PHB recovery with DMCHA 85
Figure 4.5: Impact of water on PHB dissolution in DMBA and DMCHA 87
Figure 4.6: Effect of dissolution and recovery on PHB molecular weight 89
Figure 4.7: Forms of PHB-DMBA gels 90
Figure 4.8: FTIR analysis of PHB-DMBA gel and recovered liquids 91
Figure 5.1: Procedure for the extraction of PHB from unconcentrated biomass. 103
Figure 5.2: PHB partitioning in water-solvent system 107
Figure 5.3: Effect of hydrophobic DMBA on *Methylocystis* sp. Rockwell. 109
Figure 5.4: SHS PHB extraction from bioreactor harvested *Methylocystis* sp. Rockwell 110
Figure 5.5: SHS PHB extraction from concentrated *Methylocystis* sp. Rockwell cultures. 111
Supplementary Figure A.1: The effects of hydrophobic DMBA on *Methylocystis* sp. Rockwell. 145
Supplementary Figure A.2: The effects of hydrophilic DMBA on *Methylocystis* sp. Rockwell. 145
Supplementary Figure A.3: FTIR analysis of PHB-DMBA gel and associated fractions. 146

List of Abbreviations

AOS	Sodium alpha olefin sulfonate
ATCC	American Type Culture Collection
AU	Anson Units
CAS	Chemical Abstracts Service
CDW	Cell dry weight
DCM	Dichloromethane
DMBA	N,N-dimethylbenzylamine
DMCHA	N,N-dimethylcyclohyexylamine
DBAE	2-(dibutylamino)ethanol
DIPAE	2-(diisopropylamino)ethanol
EDTA	Ethylenediaminetetraacetic acid
EP	N-ethylpiperidine
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatograph
GPC	Gel permeation chromatography
HPH	High-pressure homogenization
ID	Internal diameter
LAS	Linear alkylbenzene sulfonic acid
LD_{50}	Lethal dose, 50%
mcl-PHA	Medium-chain-length polyhydroxyalkanoate
NPCM	Non-polyhydroxyalkanoate cell mass
PHA	Polyhydroxyalkanoate
PHB, P(3HB)	Poly(3-hydroxybutyrate)
P(4HB)	Poly(4-hydroxybutyrate)
P(3HHx)	Poly(3-hydroxyhexanoate)
PHBV	Poly(hydroxybutyrate-co-valerate)
PHV, P(3HV)	Poly(3-hydroxyvalerate)
PTFE	Polytetrafluoroethylene
RT	Room temperature
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscopy

List of Symbols

ΔG^M	Free energy of mixing
M_N	Number-average molecular weight
M_W	Weight-average molecular weight
R	Ideal gas constant
R_A	Hansen solubility radius (distance between solute and solvent)
R_M	Radius of solubility sphere (maximum distance for solubility)
r	Correction factor (ratio of size of polymer to size of solvent)
T	Temperature
v	Molar volume of the solvent
x_i	Molar fraction of component *i*
δ_d	Hansen solubility parameter for dispersion forces
δ_p	Hansen solubility parameter for polar forces
δ_{hb}	Hansen solubility parameter for hydrogen bonding forces
φ_i	Volume fraction of component *i*
χ_{12} or χ	Flory Huggins parameter for a polymer-solvent pair

1 Introduction

1.1 Motivation: Improving Production of Biodegradable Plastics

Plastic products, with their low cost and broad range of properties, are used in a wide variety of applications. These materials gained prominence in the 1950s and have since become ubiquitous in our society. However, the use of plastics for disposable items and their resistance to degradation in natural settings have revealed that the true cost of their usage is paid at the environmental scale. It is estimated that 4.9 billion tonnes of plastics were discarded to landfills or the natural environment between 1950 and 2015, including approximately 200 million tonnes of waste discarded each year from 2010-2015 (Geyer et al., 2017).

Currently, there are efforts from governments in Canada and around the world to phase out single-use plastics, such as packaging materials, in favour of materials which will not accumulate in the environment (United Nation Environment Programme, 2022). There has been some reluctance to phasing out and replacing these goods with paper or cardboard alternatives, as seen with the replacement of single-use straws and grocery bags, due to their inferior physical properties (Thurton, 2023). Biodegradable plastics would greatly improve this situation and allow for the same convenience of normal plastics while mitigating environmental effects.

A core motivation for this project was to improve extraction processes for the biodegradable plastic poly(3-hydroxybutyrate) (PHB), which are a key limitation to its widespread production. Microbes which produce and degrade PHB are ubiquitous in the natural environment (Mergaert et al., 1993; Braunegg et al., 2004; Roohi et al., 2018) giving PHB the potential to be a biodegradable substitute for many consumer plastics, especially those used for disposable goods (Koller & Mukherjee, 2022). PHB is a microbial storage molecule which is accumulated intracellularly under certain conditions and can be the product of bioconversion from a variety of feedstocks (Page & Cornish, 1993; Cavalheiro et al., 2009; Fernández-Dacosta et al., 2015; Carillo et al., 2019). The use of methane-consuming bacteria for PHB production adds an additional environmental benefit in that methane, a potent greenhouse gas, which might otherwise be released to the atmosphere, can be converted into this biodegradable plastic. The bioproduction of PHB has seen many improvements over recent years, especially from methane feedstocks thanks to the work of my colleagues Hem Sharma (H. K. Sharma et al., 2022) and Marina Lazic (Lazic et al., 2022). However, since PHB is accumulated intracellularly, its extraction from bacteria remains one of the innovation bottlenecks due to the persisting high energy and material costs for this part of the process (Rostkowski et al., 2012).

Switchable hydrophilicity solvents (SHSs) are a relatively recent development in green chemistry which have the potential to improve extraction processes. They are able to be switched between a hydrophobic and a hydrophilic form, which gives the potential for easy recycling through an extraction process as the product can be dissolved in one form, precipitated in the other, and the solvent can then be returned to its original form (Jessop et al., 2012). SHSs have been demonstrated in the extraction of lipids and bio-oils from microorganisms (Phan et al., 2009; Boyd et al., 2012) and are capable of dissolving and precipitating polystyrene (Jessop et al., 2011). These solvents have potential to be used as a means to extract PHB from microorganisms and to then be subsequently recycled, which is the topic of this thesis.

1.2 Thesis Scope

The overall goal of this thesis was to determine if SHSs are an appropriate extraction platform for PHB from methanotrophic bacteria, and if possible, to demonstrate a successful extraction method. As solvents with good recyclability, such a process would significantly improve the extraction of PHB, a key part of the process.

The specific aims of this thesis include: 1) Identifying SHSs that are the best candidates for PHB dissolution using theoretical models and testing in the laboratory, 2) examining in an abiotic setting the interactions between SHSs and PHB to inform extraction processes and determine if SHSs are useful for other steps of PHB processing, and 3) extracting PHB from methanotrophic bacteria using SHSs and demonstrating that the solvents can be recycled and re-used in subsequent extractions.

1.3 Thesis Structure

Chapter 2 reviews the literature relevant to PHB as a material, its biological production, and offers a comparison of PHB extraction methodologies from microbial biomass. This chapter also reviews the literature regarding SHSs, polymer-solvent interactions, and methanotrophic bacteria.

Chapter 3 uses a group-contribution model from existing literature to predict the solubility of PHB in various SHSs and tests good candidates experimentally. This work validates the applicability of the model to SHSs and demonstrates the dissolution of PHB in multiple SHSs.

Chapter 4 examines the dissolution and recovery of PHB in SHSs and the recycling of the solvent in this process. The work also investigates switchable PHB-SHS gels which are formed under certain conditions, and the effects of water content within recycled solvents on PHB dissolution capability.

Chapter 5 studies the extraction of PHB from methanotrophic bacteria using SHSs in multiple process schemes, including the recycling of solvents.

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2 Literature Review

2.1 Polyhydroxybutyrate (PHB)

Poly(3-hydroxybutyrate) (PHB) is the most common type of bioproduced and biodegradable polyesters within the class of polyhydroxyalkanoates (PHAs). When biologically produced, PHB is produced exclusively as the (R) enantiomer poly[(R)-3-hydroxybutyrate]. PHAs are used as carbon and energy storage molecules by many microorganisms. They are generally produced when carbon is available but there is a shortage of other nutrients, most commonly nitrogen (Grage et al., 2009; Pieja et al., 2011; Tays et al., 2018; Zaldívar Carrillo et al., 2018). With over 250 known microorganisms from 69 genera producing PHAs (Braunegg et al., 2004; Roohi et al., 2018) and more than 295 microbial strains, including bacteria and molds, able to degrade PHAs (Mergaert et al., 1993), these polymers are commonly found in natural ecosystems. At the end of their life cycle, PHAs can be completely converted into energy, water, and either carbon dioxide or methane depending on the conditions under which they are biodegraded (Reddy et al., 2003; Martínez-Tobón et al., 2018).

PHB is the most common PHA in nature both in terms of its abundance and the fact that most PHA copolymers contain high proportions of PHB (Steinbüchel & Valentin, 1995). Many microorganisms only copolymerize other PHAs with PHB if they are fed specific precursor molecules (Steinbüchel & Lütke-Eversloh, 2003). As a result, PHB is the best-studied PHA in terms of bioproduction, application, and biodegradation. PHB homopolymer is thus the focus of this thesis rather than examining a wide range of PHAs, but it is expected that many of the findings will be applicable to other PHAs with some modifications.

2.1.1 Physical Properties of PHB

The PHB homopolymer is similar in properties to polypropylene, although it is stiffer and more brittle (Holmes, 1985; Sudesh et al., 2000). When extracted from bacterial cells, PHB typically has a crystallinity between 55-80% (Terada & Marchessault, 1999; Sudesh et al., 2000). It has a glass transition temperature of 4°C and a melting temperature of approximately 180°C (Terada & Marchessault, 1999; Sudesh et al., 2000). These properties are useful for some applications where stiffness is a benefit, but not as good for applications where the material needs to be flexible. However, there are more than 150 different PHA monomers which can be copolymerized biologically (Steinbüchel & Valentin, 1995; Steinbüchel & Lütke-Eversloh, 2003) and they often display better physical properties (e.g. flexibility) than PHB (Sudesh et al., 2000). Most common PHA monomers are distinguished by their side chain length although there can be variation in the backbone chain length, additional side chains, and, in rarer cases, PHA synthase is also able to copolymerize unsaturated monomers or those with additional functional groups (Hazer &

Steinbüchel, 2007; Steinbüchel & Lütke-Eversloh, 2003). The range of physical properties can be further improved and diversified by the creation of composite materials (Doyle et al., 1991; Degli Esposti et al., 2019; Lopera-Valle et al., 2019) or the use of different polymer processing techniques (Anbukarasu et al., 2015; Cal et al., 2019).

The molecular structures of PHB and other common PHAs are shown in Figure 2.1, and the physical properties of some PHB copolymers are shown in Table 2.1. Note that the physical properties can depend on the molecular weight, how the sample is processed and the arrangement of monomers in a copolymer.

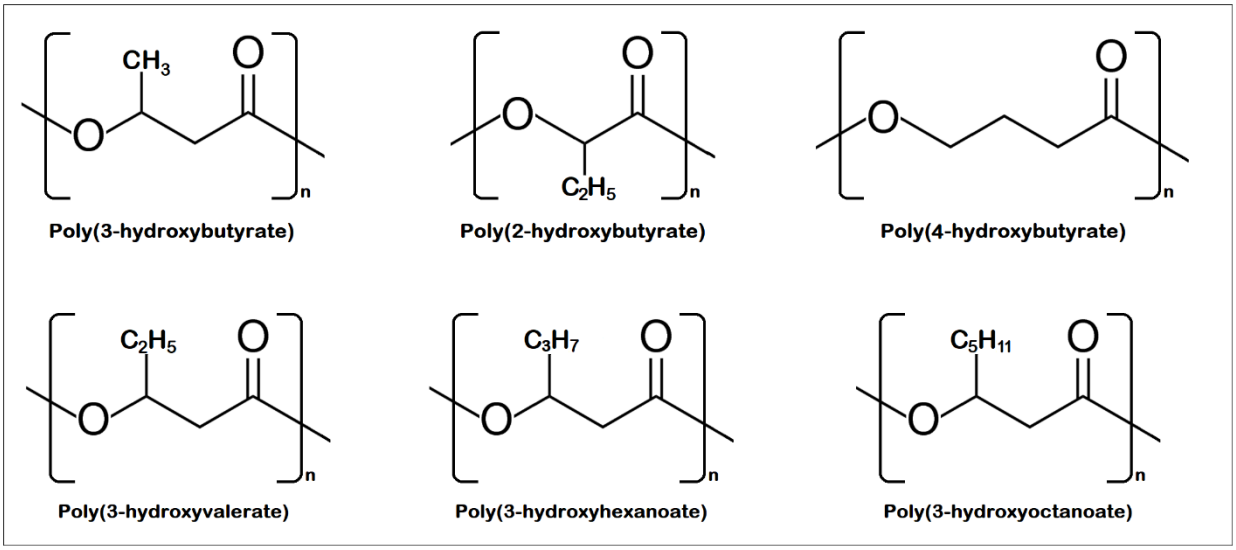

Figure 2.1: Molecular structure of common PHAs

Table 2.1 Material properties of PHA copolymers and common petrochemical plastics.

Polymer	T_m (°C)	T_g (°C)	Young's Modulus (GPa)	Tensile Strength (MPa)	Elongation to break (%)	Reference
P(3HB)	177 - 180	4	3.5	40 - 43	5	G.-Q. Chen & Wu, 2005; Jacquel et al., 2007; Sudesh et al., 2000
P(3HB-co-10%-3HV)	150	--	--	25	20	G.-Q. Chen & Wu, 2005
P(3HB-co-20%-3HV)	135 - 145	-1	0.8 - 1.2	20 - 32	50 - 100	G.-Q. Chen & Wu, 2005; Jacquel et al., 2007; Sudesh et al., 2000
P(3HB-co-10%-3HHx)	127	-1	--	21	400	G.-Q. Chen & Wu, 2005
P(3HB-co-6%-3HA)[a]	133	-8	0.2	17	680	Sudesh et al., 2000
P(2HB-co-12%-3HB)[b]	--	--	0.6 -1.0	31 - 38	202 - 393	Kageyama et al., 2021
P(4HB)	--	--	0.07	50	1000	Williams et al., 2013
Low-Density Polyethylene	130	-30	0.2	10	620	Sudesh et al., 2000
Polyethylene Terephthalate	262	--	2.2	56	7300	Jacquel et al., 2007
Polypropylene	170 - 176	-10	1.7	34 - 38	400	G.-Q. Chen & Wu, 2005; Jacquel et al., 2007; Sudesh et al., 2000

P(3HV): Poly(3-hydroxyvalerate), P(3HHx): Poly(3-hydroxyhexanoate), P(4HB): Poly(4-hydroxybutyrate)
a: 3HA units: 3-hydroxydecanoate (3mol%), 3-hydroxydodecanoate (3mol%), 3-hydroxyoctanoate (<1mol%), 3-hydroxy-cis-5-dodecenoate (<1mol%)
b: This is a block copolymer, with the ranges resulting from different treatments (solvent cast, annealed, and melt-quenched)

2.1.2 Applications of PHB and other PHAs

The biodegradable nature of PHB is one of its key attributes such that applications which make the best use of this trait are primary targets. The biocompatibility of PHB (Kai et al., 2003; G.-Q. Chen & Wu, 2005; Utsunomia et al., 2020) creates opportunities for multiple medical applications. The ability of PHB to biodegrade and be resorbed by the body is a key trait for applications such as tissue engineering wherein biodegradable implants can provide a scaffold for new tissue to grow (Sabir et al., 2009; Atala et al., 2012; Saska et al., 2018). In addition to PHB, a range of PHA copolymers have been investigated for such applications (Butt et al., 2018; G.-Q. Chen & Wu, 2005; Kai et al., 2003; Saska et al., 2018; Utsunomia et al., 2020; Williams et al., 2013). The breadth of mechanical properties has allowed PHB and other PHAs to be employed in a variety of other medical devices including wound dressings, stents, and drug delivery systems (Butt et al., 2018; G.-Q. Chen & Wu, 2005; Utsunomia et al., 2020; Williams et al., 2013).

A second key potential use for PHB and other PHAs is replacing non-biodegradable polymers in single-use plastics. Their biodegradability means that even when used as disposable products, they will not accumulate in the environment to the same extent as single-use plastics, and their physical properties allows them to replace current disposable plastic products without sacrificing the ease of use that comes with plastic disposables. PHB homopolymer has been tested for food packaging, as shown in Figure 2.2 (Bucci et al., 2007), and there has been significant research into using PHB as a plastic that is responsive to stimuli such as the presence of pathogens to improve food safety (Anbukarasu et al., 2017; S. Y. Chen et al., 2021; Elias et al., 2016). A significant amount of research has also focused on making composites with PHB. One common approach is blending PHB with poly(lactic acid) (Arrieta et al., 2014; Burgos, 2017). Other approaches have used PHB with reinforcement agents to improve packaging qualities or with nanocomposites aimed at giving the composite an antimicrobial effect (Iglesias-Montes et al., 2022; Mittal et al., 2023; R. Sharma et al., 2020).

PHB and PHAs were commercialized in the late 20th century, although interest waned before resurging in recent years. The British company “Imperial Chemical Industries” was the first to commercialize PHAs, creating a PHBV copolymer branded “Biopol” that was used as shampoo bottles (Liggat, n.d.). Commercial production of PHAs in the last decade includes cutlery, straws, food packaging, bags, and films (Koller & Mukherjee, 2022). However, PHA production in 2022 was only 87 kt (European Bioplastics, 2022) as compared with total world plastics production of 400 Mt in 2022 (Plastics Europe, 2023).

Figure 2.2: Bread packaged in a PHB films.
Left: A PHB film. **Right:** A PHB composite film with poly(ethylene glycol), clove essential oils, and nano-silica.
(Mittal et al., 2023. Reproduced with permission from Elsevier)

2.1.3 Biological Production of PHB

In microbes, PHB is generally accumulated as 5-10 granules per cell (Figure 2.3), each having a diameter between 50-700 nm, with 0.5%-2% of the granule being an outer layer composed of protein and lipids (de Koning & Lemstra, 1992; Grage et al., 2009). PHB crystallization is prevented in vivo by the protection of the granules from heterogenic nucleation by this protein-lipid outer layer. The risk of homogeneous nucleation is kept low by the small size of the granules (de Koning & Lemstra, 1992). The removal or disruption of the outer lipid layer, which can be done through complete drying, centrifugation, sonication, freezing and thawing, high heat exposure, or exposure to acetone or alkaline treatments, induces crystallization of the granules (Horowitz & Sanders, 1994).

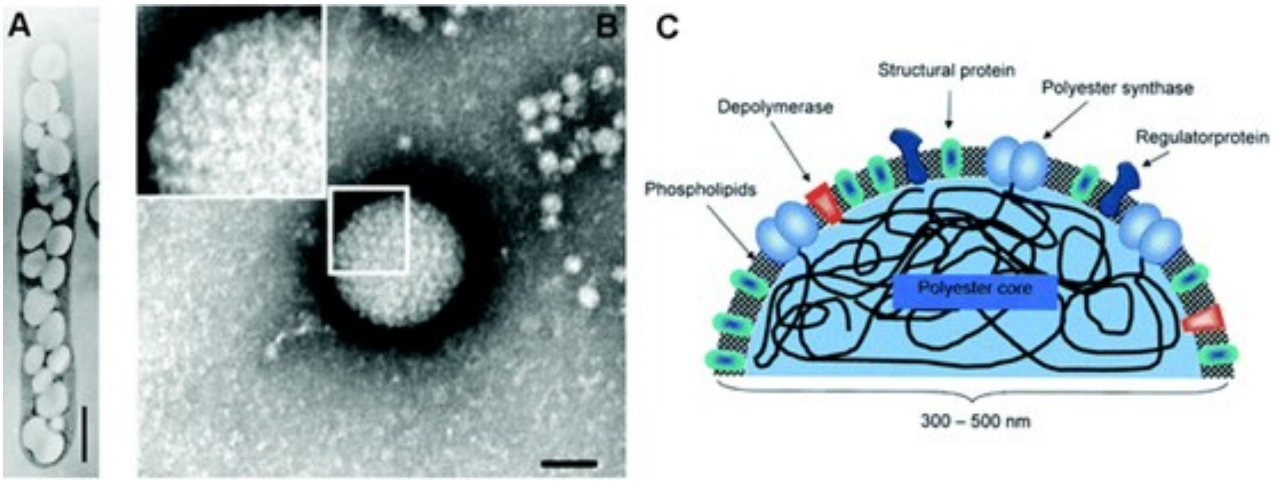

Figure 2.3: PHB in bacterial cells.
(A) TEM image of PHB in *Cupriavidus necator* (500 nm bar); **(B)** TEM image of a PHA granule from *Caryophanon latum* (50 nm bar) **(C)** Schematic of PHB granule showing granule associated proteins.
(Grage et al., 2009. Reproduced with permission from the American Chemical Society.)

Because the synthesis and accumulation of PHB generally requires a nutrient imbalance or limitation (Zaldívar Carrillo et al., 2018; Grage et al., 2009; Pieja et al., 2011; Tays et al., 2018), production processes often employ a two-stage fermentation system where the cells are grown in a first stage before being subjected to conditions which encourage high PHB accumulation. However, it has been shown that some organisms under the right conditions are able to accumulate PHB while in exponential growth phase (Zaldívar Carrillo et al., 2018; Lazic et al., 2022; Sharma et al., 2022).

As a biologically produced polymer, one of the attractive aspects of PHB as a replacement for petroleum-based polymers is the fact that it can be produced from renewable resources. Although PHB can be produced through microbial fermentation of sugars (Bonatto et al., 2004; García et al., 2014), the wide variety of PHB-producing microbes enables its bioproduction from an assortment of feedstocks. This flexibility enables the valorization of waste streams which further increases the environmental benefits of PHB while simultaneously improving its economic prospects. As PHB must be relatively competitive in price to displace petroleum-sourced polymers, much effort has been expended on improving its bioproduction from waste streams with the goal of driving down production costs. Some examples of these feedstocks include waste glycerol from biodiesel processing (Cavalheiro et al., 2009; Posada et al., 2011), cheese whey (Koller et al., 2013; Yellore & Desai, 1998), wastewater (Fernández-Dacosta et al., 2015), waste methane (Chidambarampadmavathy et al., 2017; Rostkowski et al., 2012), and methanol (Zhang et al., 2008; Carillo et al., 2019).

In natural ecosystems, PHB is by far the most common PHA, but copolymerization with a variety of PHAs in nature is observed, although at very low concentrations (Steinbüchel & Valentin, 1995). Research on natural occurrence in various aquatic ecosystems found that PHA variability across environments was mainly caused by variation of the total bacterial biomass. In one study, only PHB was found in water samples, but sedimentary microbial mats had both PHB and PHV (Pedrós-Alió et al., 1990). The range of possible PHAs is due to the variety of PHA synthase enzymes and their low substrate specificity, which can polymerize PHA from a range of available precursor molecules (Sagong et al., 2018; Steinbüchel & Lütke-Eversloh, 2003). Despite the broad range of PHA possibilities, the provision of those precursor molecules is often a challenge. PHAs other than PHB often require the feeding of structurally related precursors to the bacterial culture in order to be synthesized (Steinbüchel & Lütke-Eversloh, 2003), making many PHA copolymers quite expensive to create. For some cases this problem can be avoided, as PHA copolymers can alternatively be created in mixed cultures in activated sludge, where some of the organisms will create the necessary precursors for others to convert to PHA (Y. Jiang et al., 2012), or from feedstocks that can be converted into the necessary precursors, such as fatty acids with odd carbons that can be converted to PHBV (Steinbüchel & Lütke-Eversloh, 2003).

2.1.4 Biodegradation of PHB

One of the primary advantages of PHB over petroleum-based plastics and even some bioproduced plastics is their ability to be degraded in natural ecosystems, as seen in Figure 2.4. While organisms that use PHB as a storage molecule require the ability to depolymerize it to use the stored carbon and energy, a separate set of enzymes gives microbes the ability to depolymerize extracellular PHB (Holmes, 1985; Martínez-Tobón et al., 2018; Mergaert et al., 1993; Tanio et al., 1982; Volova et al., 2010). The organisms which depolymerize extracellular PHB are benefactors of PHB-producing organisms that die and release their stored PHB, and they are also the organisms biodegrading anthropogenic PHB-based products released into the environment. As a result of the commonality of PHB in microbial life, PHB-degrading organisms exist in diverse ecosystems including sewage sludge (Jendrossek & Handrick, 2002; Mergaert et al., 1993; Morse et al., 2011; Tanio et al., 1982), compost heaps (Dilkes-Hoffman et al., 2019; Jendrossek & Handrick, 2002), a variety of soil types (Dilkes-Hoffman et al., 2019; Fernandes et al., 2020; Jendrossek & Handrick, 2002; Mergaert et al., 1993), and both marine (Dilkes-Hoffman et al., 2019; Jendrossek & Handrick, 2002; Mergaert et al., 1993; Tanadchangsaeng & Pattanasupong, 2022) and freshwater environments (Jendrossek & Handrick, 2002; Mergaert et al., 1993).

The end result of PHB biodegradation is carbon dioxide and water under oxic conditions, and carbon dioxide and methane under anoxic conditions (Reddy et al., 2003). Combining this with production strategies that utilize methane or carbon dioxide leads to very low environmental impacts for PHB-based products over their whole life cycle. The production-degradation cycle could even provide opportunities for PHB products to be fed to anaerobic digesters at the end of their life cycle to create a new batch of PHB products.

The degradation rate of PHA-based products is dependent on several factors including the degradation environment, the PHA(s) used and their copolymerization ratio, and the physical characteristics of the object. The biodegradation rate of PHAs in marine environments is lower than in soil, both of which are lower than the biodegradation rate in compost or anaerobic digesters (Dilkes-Hoffman et al., 2019). In a marine environment, an object such as a plastic bag will biodegrade in 1-3 months, a water bottle 1-4 years, and a piece of cutlery up to 6 years (Dilkes-Hoffman et al., 2019). The enzymatic nature of PHA degradation means that characteristics such as monomer content will have varying effects depending on which PHA depolymerase enzymes are present in the degradation environment. Some PHA depolymerase enzymes, for example, are specific to PHB, while other enzymes show varying activities between PHB and PHV (Guérin et al., 2010).

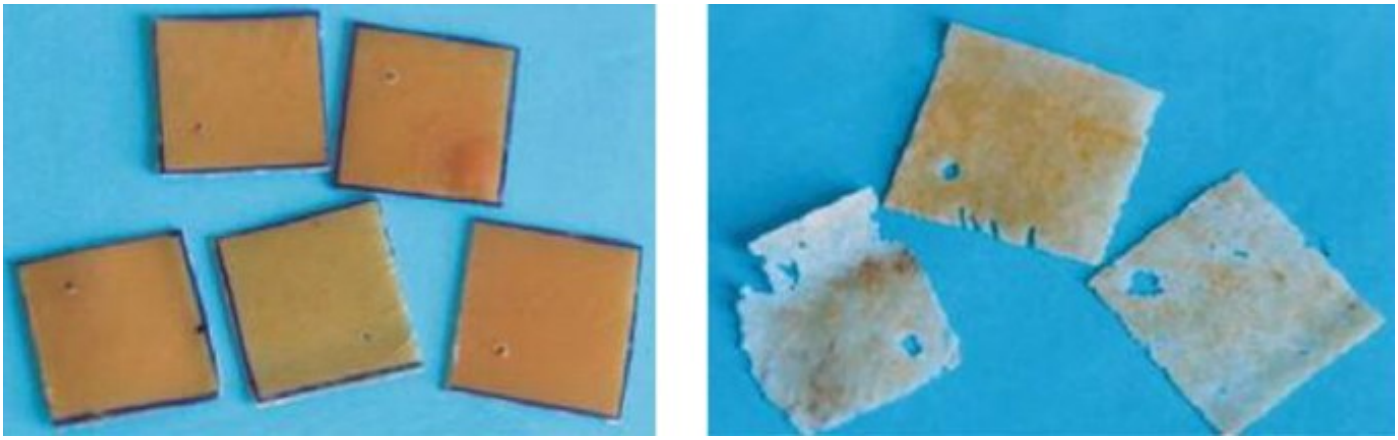

Figure 2.4 PHB film degradation.
PHB films (30 mm x 30 mm, 1.24 mm thick) before (left) and after (right) 60 days of exposure to an anaerobic medium in a septic tank.
(Bucci et al., 2007. Reproduced with permission from Elsevier.)

2.2 Extraction of PHB from Biomass

While PHB production from fermentation has improved through greater yields, simplified growth patterns and lower-cost feedstocks (Chidambarampadmavathy et al., 2017; Sharma et al., 2022), downstream processing, including PHB recovery, represents an increasingly large percentage of the total production cost. In addition to limiting the economic prospects of PHB, the current state of downstream processing weakens the environmental case for their immediate use. For example, the energy needed to produce PHB using current downstream processing methods generates more carbon emissions than the production of petrochemical polymers such as polyethylene terephthalate, with a significant portion coming from the extraction stage (Amabile et al., 2024, Fernández-Dacosta et al., 2015; Rostkowski et al., 2012, Rueda et al., 2023). The environmental benefit of biodegradable plastics may outweigh some degree of increase in global warming potential, but for a product that is not yet competitive on price, achieving an unquestionable environmental upside is important.

Strategies for PHB extraction from biomass can generally be grouped into three categories. One category is solvent extractions, using a solvent that dissolves PHB but not the non-PHB cell material (NPCM). A second type of strategy is the enzymatic or chemical dissolution of the NPCM, leaving PHB granules to be recovered by centrifugation. A third approach is the use of mechanical stress to lyse the cells and a physical separation of the PHB granules from the NPCM. These methods are sometimes used in tandem, such as a physical process as a pre-treatment for dissolution, or methods that use simultaneous NPCM digestion and PHB dissolution.

2.2.1 General Extraction Process

The general process for extraction of PHB from microbial biomass can be subdivided into six steps (Jacquel et al., 2008; Pérez-Rivero et al., 2019; Saavedra del Oso et al., 2021). As illustrated in Figure 2.5, the first two steps consist of pre-treatment stages, which include dewatering of the biomass and any additional pre-treatment steps. These are not necessarily done in order, as for example some processes will heat-treat liquid cultures before removing the bacterial biomass from the culture medium (Kapritchkoff et al., 2006; Yasotha et al., 2006). The middle two steps, which typically consist of the extraction of the product from bacterial cells and the separation of the polymer from the extraction agents, represent the main extraction process. The final two steps are post-processing stages. There is typically an additional purification stage, where the PHB product extracted from bacteria is washed with a compound intended to remove remaining biomass or extraction agents or dried to remove residual solvent. Finally, some processes include methods for recycling extraction agents, or the valorization of solvent waste.

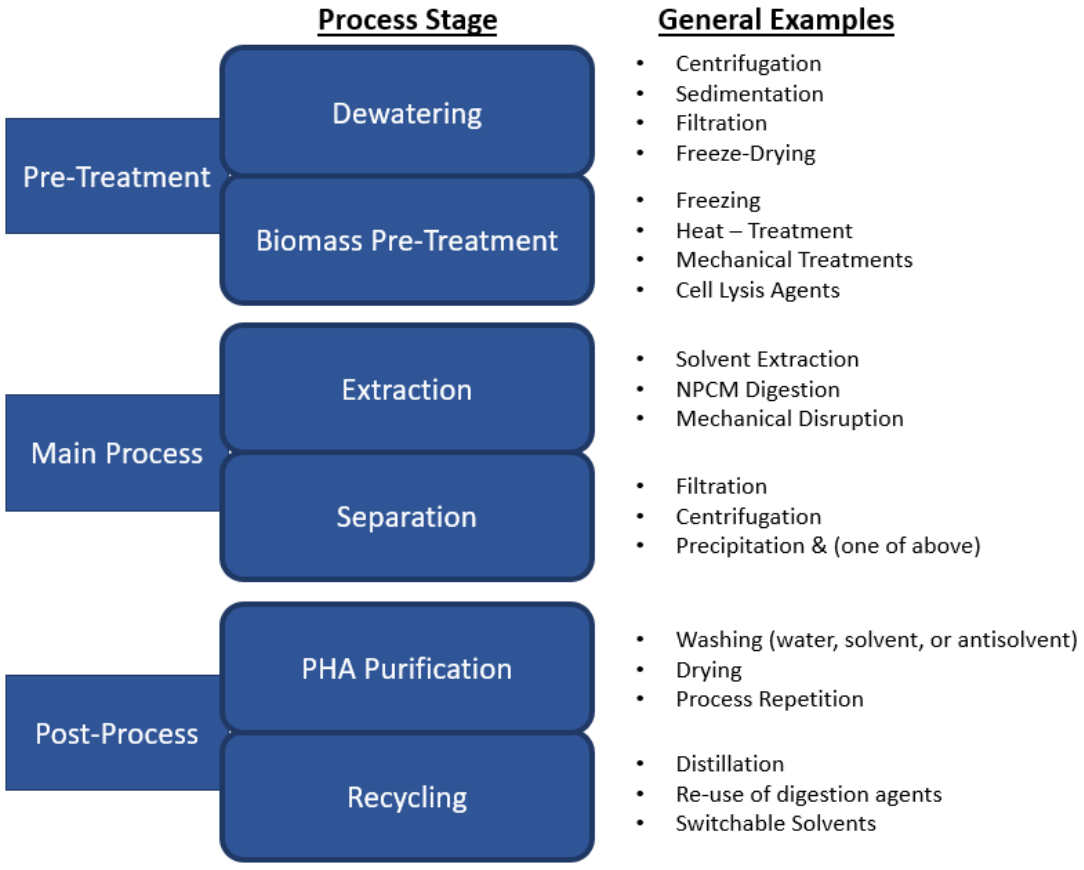

Figure 2.5 An overview of the typical steps in a PHA extraction process.
(Jacquel et al., 2008, modified from original. Reproduced with permission from Elsevier.)

The comparisons of various extraction processes are often difficult due to the broad range of decisions and approaches taken by researchers and engineers that affect PHB recovery rate and purity. These include the selection of pre-treatment and purification steps, temperature, time, solvent:biomass ratio, original PHB content in the culture, and even the microorganisms used to produce PHB.

Tables 2.2 - 2.7 detail the PHB extraction procedures reported in literature. Studies which reported the purity and recovery of PHB from bacterial biomass were selected to allow for better comparison, but in all cases the procedures for the processes are given in detail to provide context for the numbers. A process with higher recovery or yield than another process is not necessarily "better", and in fact, many new studies demonstrated lower recovery or yield than existing methods because the investigations were aimed towards improving other concerns, such as lower material and energy requirements or a simplified process. Most of the papers cited in the tables tested several processes, and the processes shown are those that were highlighted by the original authors for providing the highest recovery and purity or that had high performance with a simplified process. The "Recycling" step was omitted from these tables because few studies examined it, which is discussed in section 2.3.7 of this thesis.

2.2.2 PHB Extraction by Solvent Extraction

Solvent extraction of PHB from biomass is a very well-established method, typically with relatively high recovery rates and high polymer purities (Ramsay et al., 1994). The most common solvents used as reference are chlorinated hydrocarbons, typically chloroform and dichloromethane, with common antisolvents used to precipitate the polymer out of solution being methanol, ethanol, or water. Chloroform-methanol is commonly used at lab scale, while dichloromethane-water has been used industrially in the 20th century (Jacquel et al., 2008).

There has been significant research effort devoted to finding solvents which are able to replicate the success of chlorinated solvents but overcome some of their drawbacks. Chlorinated hydrocarbons and methanol are harmful chemicals, and the large volumes required for PHB extraction at industrial scale create significant amounts of chemical waste. These can typically be recovered to some degree through distillation, but that is an energy-intensive and expensive process; in some cases, the energy for distillation can account for 25% of the polymer production cost (Byrom, 1987; Fernández-Dacosta et al., 2015; Rostkowski et al., 2012). The ability of solvents to extract PHB in the presence of water is another focus point for improvement, as completely drying the biomass before extraction is also energy intensive (López-Abelairas et al., 2015).

Table 2.2 details various PHB extraction protocols which have been developed to improve upon the chloroform-methanol extraction method. Some of the extraction methods described were performed similarly to chloroform extraction methods but using safer or more sustainable solvents (Gnaim et al., 2022; G. Jiang et al., 2018; Rosengart et al., 2015). Other processes improved on the process by extracting PHB from wet concentrated biomass (García et al., 2019; Mongili et al., 2021; Samorì et al., 2015; Wongmoon & Napathorn, 2022) or from unconcentrated bioreactor effluents (Dubey et al., 2018; Parodi et al., 2021). Typically, these methods of alternative solvent extraction still require the use of antisolvents, but in some cases the antisolvent precipitation was replaced by cooling (for solvents being used near or above their boiling point).

Because solvent-only extractions require the solvent to both penetrate the cell membrane and dissolve the polymer, some other solvent extraction approaches feature a combination digestion-dissolution step, or a NPCM digestion step, followed by polymer dissolution. This is often used to improve yields, especially since some microorganisms are not as easily penetrated by solvents. (Holmes et al., 1980). The combination of digestion-dissolution treatments allows for the use of solvents at typically lower temperatures or using less solvent than solvent-only methods. A comparison of these methods are shown in Table 2.3.

Table 2.2: Recently developed PHB extractions from biomass by solvent extraction.

Bacteria	PHA	Culture PHA %	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Cupriavidus necator A-04	PHB	70.3	Centrifuged	Washed with 0.85% sodium chloride (x2)	1,3-dioxolane, 5% w/v wet biomass in 40mL, 30°C, 36h	3 volumes water added, centrifuged	Washed twice with water, dried	93.5	97.7	Wongmoon & Napathorn, 2022
Cobetia apmhilecti	PHB	76.3	Dried	--	Methyl levulinate 2% w/v cells 140°C, 1h	Cooled to 50°C, centrifuged, 3 volumes cold methanol, centrifuged	Dried at 70°C	96.5	89.6	Gnaim et al., 2022
Escherichia coli	PHB	54	Centrifuged	--	Dimethyl carbonate (2.5% w/v biomass) 90C, 1.5h	Centrifuged, Precipitated with 3 volumes of ethanol	Air Drying	67.2	86.1	Mongili et al., 2021
Mixed Culture	PHB	39	--	--	Methyl 3-methoxybutyrate 1mL with 300mg slurry (79% water content) 130°C, 10min	Centrifuged	Solvent removed by distillation	92	98	Parodi et al., 2021
Mixed Culture	PHB	39	--	--	Methyl 3-hydroxybutyrate 1mL with 300mg slurry (79% water content) 130°C, 10min	Centrifuged	Solvent removed by distillation	77	96	Parodi et al., 2021
Azotobacter vinelandii OPNA	PHB	89.5	Centrifuged	--	Ethanol, 0.3g wet biomass in 10mL. Stirred, 77°C (boiling point) 30min	Centrifuged. PHB precipitates	Resuspended in 10mL acetone, centrifuged, dried at RT	85	95	García et al., 2019
Cuprivadius necator H16	PHB	82.3	Centrifuged, freeze-dried	Acetone (20:1 vol:mass) overnight, RT	Cyclohexanone 0.3g dry biomass in 3mL 120°C, 3min	Precipitation in 30mL methanol	--	99	99.5	G. Jiang et al., 2018
Halomonas hydrothermalis	PHB	74	Centrifuged	--	Ionic liquid $[C_2mim][(C_2)_2OPO_3]$, [A] 60°C 24h	Methanol antisolvent, filtration	--	60	86	Dubey et al., 2018
Cupriavidus necator	PHB	71	[Wet biomass, 61% moisture content]	Sonicated 10min	200mg dry biomass in 5mL acetone/ethanol/propylene carbonate (1:1:1 v:v). 120°C, 1h	Filtered, precipitation by cooling to room temperature 48h.	--	83	90	T. Fei et al., 2016
Burkholderia sacchari	PHB	57.7	Freeze-dried	--	Anisole 0.6g biomass in 40mL, 120-130°C, 30mins	Filtration, precipitation (160mL ethanol)	Air-dried	98.4	96.7	Rosengart et al., 2015
Burkholderia sacchari	PHB	57.7	Freeze-dried	--	Cyclohexane 0.6g biomass in 40mL, 120-130°C, 15mins	Filtration, precipitation (160mL ethanol)	Air-dried	98.2	93.4	Rosengart et al., 2015
Cupriavidus necator DSM545	PHB	74.2	Centrifuged to 50-100g/L biomass;	--	Dimethyl carbonate 1mL slurry in 2mL solvent 90°C 1h	Centrifuged, filtered, ethanol precipitation	Dried at 60°C under vacuum	92	96	Samorì et al., 2015

A: The name of the ionic liquid is 1-Ethyl-3-methylimidazolium diethyl phosphate.

RT: Room Temperature

Note: *Ralstonia eutropha* is an earlier synonym for *Cupriavidus necator.* (Vandamme & Coenye, 2004)

Table 2.3: PHA extraction from biomass by digestion-dissolution

Bacteria	PHA	Culture PHA %	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Cupriavidus necator	PHB	81	Centrifuged	10% NaOCl 1g wet biomass in 10mL 37°C 1h	Chloroform 0.1% w/v wet pellet, 100°C, 60min	Precipitated with one volume ice-cold ethanol	Washed with water, dried at RT	96	98	Aramvash et al., 2017
Cupriavidus necator	PHB	81	Centrifuged	10% NaOCl 1g wet biomass in 10mL 37°C 1h	Ethylene carbonate 0.1% w/v wet pellet, 150°C, 60min	Centrifuged, one volume ice-cold ethanol	Washed with water, dried at RT	98.6	98	Aramvash et al., 2017
Cupriavidus necator	PHB	81	Centrifuged	10% NaOCl 1g wet biomass in 10mL 37°C 1h	Dimethylsulfoxide 0.1% w/v wet pellet, 150°C, 60min	Centrifuged, one volume ice-cold ethanol	Washed with water, dried at RT	60.6	95	Aramvash et al., 2017
Cupriavidus necator	PHB	81	Centrifuged	10% NaOCl 1g wet biomass in 10mL 37°C 1h	Propanol 0.1% w/v wet pellet, 100°C, 60min	Centrifuged, one volume ice-cold ethanol	Washed with water, dried at RT	28.5	97	Aramvash et al., 2017
Cupriavidus necator	PHB	81	Centrifuged	10% NaOCl 1g wet biomass in 10mL 37°C 1h	Methanol 0.1% w/v wet pellet, 50°C, 60min	Centrifuged, one volume ice-cold ethanol	Washed with water, dried at RT	81.2	99	Aramvash et al., 2017
Cupriavidus necator	PHB	65	Centrifuged, freeze-dried	Blended	NaOCl 13% v/v + DCM 1:1 v/v; 4h, 37°C 500rpm; 2.5% w/v biomass	10 volumes ethanol	Washed with water (x2), ethanol, freeze - dried	89	99	López-Abelairas et al., 2015
Cupriavidus necator DSM 545	PHBV	75	Centrifuged	Autoclaved cells (121°C for 15min) were treated at pH 4 and 60°C with Celumax BC enzyme in acetate buffer for 1h.	4mL hydrolysate mixed with 1mL chloroform	Centrifuged	Evaporation of solvent	93.2	94	Neves et al., 2012
Cupriavidus necator	PHB	71	Centrifuged (after pre-treatment)	pH 9 (1M NH_4OH), 60°C for 5min, then pH to 4.0 (1M HCl)	1,2-propylene carbonate. 150mL for 11.5g wet biomass. 130°C, 30min	Filtered hot, let stand 24h, two volumes acetone added, let stand 24h, filtered	Washed with acetone and air-dried	95	88	Fiorese et al., 2009
Sinorhizobium meliloti	PHA	50	--	80°C for 10min. *Microbispora* sp. were then cultivated on PHA-containing *S. melitoti* for 24h, then filtered to remove pelleted *Microbispora* sp.	Chloroform (4 volumes broth to 1 volume chloroform), RT for 10min	Bottom phase of chloroform layer was separated and dried	--	98	90	Lakshman et al., 2006
Methylobacterium sp. V49	PHB	70	Centrifuged x2	Acetone	NaOCl (30%) + chloroform (1:1), 40°C 90min	Separatory funnel, centrifuged	Chloroform and hexane	95	97	Ghatnekar et al., 2002
Alcaligenes eutrophus	PHB	70	Centrifuged, washed, freeze-dried	--	8g cell powder added to 100mL chloroform and 100mL 30% NaOCl; 30°C for 90min	Centrifuged, precipitation with nonsolvent (70% methanol 30% water), filtered		90	97	Hahn et al., 1994

RT: Room temperature
DCM : Dichloromethane
Note: *Alcaligenes eutrophus* is an earlier synonym for *Cupriavidus necator* (Vandamme & Coenye, 2004)

2.2.3 PHB Extraction by Digestion

Studies examining the extraction of PHAs using chemical digestion methods are presented in Table 2.4. In these cases, PHA specificity is not as significant a factor since only the NPCM is targeted by the extractive agents. For this reason, extraction studies including PHAs other than PHB have been included for comparison, since it is likely that the methods and conditions could be adapted to PHB extraction.

A variety of commercial detergents have been investigated as separation agents in digestion processes and some of them have proven to be quite effective (Yang et al., 2011; Park et al., 2021). Sodium hydroxide and sodium hypochlorite are the most common digestion agents. Their relatively low cost make their use promising. There has been great interest in testing their application to different biomass extraction conditions, such as mixed cultures and wet extractions, and in optimizing their use.

The digestion of NPCM and the subsequent recovery of PHB granules (typically by centrifugation) is an approach that has advantages over solvent extraction, such as reduced operating temperature and a greatly simplified process. The separation step is reduced to only centrifugation without the need for antisolvent precipitation, and the purification step typically consists of a wash with water or ethanol, eliminating the need for solvent evaporation. Although the process does require addition of material – since some is lost through the process – the omission of a solvent recovery step helps to lower costs (Fernández-Dacosta et al., 2015; Rostkowski et al., 2012).

On the other hand, one common drawback of chemical digestion methods is the tendency to reduce the molecular weight of the polymer and increase its dispersity, which may reduce product quality (López-Abelairas et al., 2015; Mannina et al., 2019; Villano et al., 2014). Another significant limitation of many digestion processes that were recently tested is the inclusion of freeze-drying as the first step in the process. The energy requirements for freeze-drying are extremely high, making it difficult to implement at large scale (Jacquel et al., 2008). Freeze-drying is not an absolute necessity for digestion methods; however, in the case of sodium hydroxide digestion, the polymer purity is significantly affected by the omission of a pretreatment step (Rodrigues et al., 2022; Villano et al., 2014).

Some studies testing the switchable anionic surfactant, ammonium laurate, as a digestion agent have achieved PHB purities approaching 100% from freeze-dried biomass (Mannina et al., 2019; Samorì et al., 2015). This switchable surfactant has the added advantage that it can be recovered and recycled after the extraction process, potentially leading to reduced chemical use and waste generation.

Table 2.5 contains a list of enzymatic NPCM digestion methods, some of which also include chemical digestion agents featured in Table 2.3. Like chemical digestion methods, enzymatic digestion consists of fairly simple processes, although the inclusion of heat- and mechanical treatments prior to enzymatic

treatment is typical in order to kill the PHB-containing cells and weaken their structure. The enzymatic digestion of NPCM has been employed commercially (Holmes & Lim, 1990), however in recent years the focus of NPCM digestion research has shifted towards chemical digestion due to the cost associated with enzyme production (Kapritchkoff et al., 2006).

Table 2.4: PHA Extraction from biomass by chemical NPCM digestion

Bacteria	PHA	Culture PHA %	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Mixed Culture (Activated sludge)	PHA	44	Centrifuged	Washed twice with deionized water, centrifuged	100mg activated sludge, NaClO 25% v/v, 30min	SDS (2:1 SDS:biomass dry weight) 30min.	10mg of extract treated with 5mL methanol, 15min	86	67	Xiong et al., 2023
Mixed Culture	PHBV	44	--	Treated with sulfuric acid	20 g/L bioimass 0.3 M NaOH 4.8 h	Centrifuged	Washed with 2 volumes of water	88	57	Rodrigues et al., 2022
Mixed Culture	PHBV	44	--	Treated with sulfuric acid	20 g/L bioimass 9% NaClO 3.4 h	1 volume water added, Centrifuged	Washed with 2 volumes of water	91	83	Rodrigues et al., 2022
Mixed Culture	PHBV	70	Freeze-dried (after pre-treatment)	Treated with sulfuric acid	100 g/L dry bioimass 0.3 M NaOH 4.8 h	Centrifuged	Washed with 2 volumes of water	98	95	Rodrigues et al., 2022
Mixed Culture	PHBV	70	Freeze-dried (after pre-treatment)	Treated with sulfuric acid	100 g/L dry bioimass 9% NaClO 3.4 h	1 volume water added, Centrifuged	Washed with 2 volumes of water	98	90	Rodrigues et al., 2022
Halomonas sp. YLGW01	PHB	67.4	Freeze-dried	--	5% Tween®20 , 2.5:1 w/w detergent:cells, 60°C, 3h.	Centrifuged	Washed 4 times, freeze-dried	94.8	99.1	Park et al., 2021
Halomonas sp. YLGW01	PHB	67.4	Freeze-dried	--	5% SDS , 2.5:1 w/w detergent:cells, 60°C, 3h.	Centrifuged	Washed 4 times, freeze-dried	79	92	Park et al., 2021
Mixed Culture (Simulated Wastewater)	PHB	52	Freeze-dried	5 g biomass, 5mL NaOCl (4.7% Cl2), 75°C 1h; Centrifuged, washed 2x (water), resuspended 1mL	Ammonium laurate [75 mmol NH_4OH, 50mmol (10g) Lauric acid in 300mL H2O]; 3h 75°C	Centrifuged	Washed once with 0.1M NH_4OH solution	77	100	Mannina et al., 2019
Cupriavidus necator DSM545	PHB	74	Freeze-dried	--	Ammonium laurate [0.25M NH_4OH, 0.17M lauric acid] 50mg biomass in 3mL H2O pH 10, 90°C, 3h	Centrifuged	Washed with 0.1N NH_4OH, water, ethanol (x2), dried at 60°C under vacuum	100	98	Samorì et al., 2015
Cupriavidus necator	PHB	65	Centrifuged, freeze-dried	Blended	NaOH 0.5 N, 4h 37°C 500RPM, 2.5% w/v biomass	Centrifuged	Washed with water (x2) then with ethanol, freeze-dried	78	92	López-Abelairas et al., 2015
Cupriavidus necator	PHB	65	Centrifuged, freeze-dried	Blended	NaOCl 13% v/v, 4h, 37°C 500RPM; 2.5% w/v biomass	Centrifuged	Washed with water (x2) then with ethanol, freeze-dried	81	98	López-Abelairas et al., 2015
Cupriavidus necator	PHB	65	Centrifuged, freeze-dried	Blended	H_2SO_4 3.5% v/v, 6h, 80°C, 5% w/v biomass	pH adjusted with 0.5N NaOH, washed with water	3% NaOCl for 1h	80	98	López-Abelairas et al., 2015
Mixed Culture	PHBV	46	--	--	NaClO (5% Cl), stirred at RT 24h. 6:1 v:v bioreactor effulent :extraction solution. 4g/L biomass in effluent	Centrifuged	--	100	98	Villano et al., 2014

Mixed Culture	PHBV	46	--	--	NaOH (1M), stirred at RT 3h 6:1 v:v bioreactor effluent : extraction solution. 4g/L biomass in effluent	Centrifuged	--	87	54	Villano et al., 2014
Ralstonia eutropha H16	PHB	65.2	Freeze-dried	Grinded	30g/L cells in 13% v/v NaOCl. pH 12.3, 1h	Half initial volume of water added, allowed to sediment for 8h	Washed + centrifuged with water (x2) and isopropanol. Freeze - dried	69.1	99.4	Heinrich et al., 2012
Cupriavidus necator	PHB	50	Freeze-dried	--	20g/L of cells, 0.05M NaOH, 3h, 4°C	Centrifuged	1% v/v ethanol, 200rpm 30°C 3h; centrifuged, water washed	96.9	96.6	Mohammadi et al., 2012
Ralstonia eutropha H16	PHBV	82	Centrifuged, freeze-dried	--	5% AOS-40 detergent] (Cells : Detergents = 1:2.5) for 3h at 60°C	Centrifuged	3 washes with water	87	91	Yang et al., 2011
Ralstonia eutropha H16	PHBV	82	Centrifuged, freeze-dried	--	5% Brij®58 (Cells : Detergents = 1:2.5) for 3h at 60°C	Centrifuged	3 washes with water	99	83	Yang et al., 2011
Ralstonia eutropha H16	PHBV	82	Centrifuged, freeze-dried	--	LAS-9 detergent (Cells : Detergents = 1:0.5) for 3h at 60°C	Centrifuged	3 washes with water	87	86	Yang et al., 2011
Alcaligenes eutrophus	PHB	60	Freeze-dried	--	2g dry cell powder treated with 100mL water containing 0.28g betaine surfactant and EDTA disodium salt at pH 13 and 50°C for 10min	Centrifuged, rinsed with water, cfuge	4 volumes acetone added, filtered	93.3	98.7	G.-Q. Chen et al., 2001

RT: Room Temperature
SDS: Sodium Dodecyl Sulfate
EDTA: Ethylenediaminetetraacetic acid
AOS: Sodium alpha olefin sulfonate
LAS: Linear alkylbenzene sulfonic acid
Note: *Ralstonia eutropha* and *Alcaligenes eutrophus* are earlier synonyms for *Cupriavidus necator* (Vandamme & Coenye, 2004)

Table 2.5: PHA extraction from biomass by enzymatic digestion

Bacteria	PHA	Culture PHA %	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Cupriavidus necator DSM 428	PHB	37	Centrifuged	--	Na_2HPO_4 buffer (pH 8.3); Alcalase 0.3 AU/g, SDS 0.3 g/g, EDTA 0.01 g/g [all measured per gram dry cell weight]. Biomass at 5%w/v	Centrifuged	--	Not given	94	Martino et al., 2014
Sinorhizobium meliloti	PHA	50	--	80°C 10min, cooled. Culture then treated with the lytic supernatant of *Microbispora* sp. (5mg biomass per mL supernatant)	Hydrolysate broth was then treated with 0.6% Triton X 100 and 0.06% EDTA. pH 6-7, 50°C, 10min.	Centrifuged	Dried at 50°C	94	92	Lakshman & Shamala, 2006
Ralstonia eutropha DSM 545	PHB	73.4	Centrifuged, freeze-dried (after heat)	Heat Treatment 85°C for 15min, blended, sieved	Trypsin, 2% w/w (biomass). 50°C, pH 9, 1h	Centrifuged	Washed twice with 0.85% saline	Not given	87.7	Kapritchkoff et al., 2006
Ralstonia eutropha DSM 545	PHB	73.4	Centrifuged, freeze-dried (after heat)	Heat Treatment 85°C for 15min, blended, sieved	Pancreatin, 2% w/w (biomass). 50°C, pH 8, 8h	Centrifuged	Washed twice with 0.85% saline	Not given	90.3	Kapritchkoff et al., 2006
Ralstonia eutropha DSM 545	PHB	73.4	Centrifuged, freeze-dried (after heat)	Heat Treatment 85°C for 15min , blended, sieved	Bromelain, 2% w/w (biomass). 50°C, pH 9, 1h	Centrifuged	Washed twice with 0.85% saline	Not given	88.8	Kapritchkoff et al., 2006
Pseudomonas putida	mcl-PHA	18	Centrifuged, resuspension in water	Autoclaved 121°C for 1min	Alcalase (0.3 AU/g CDW) and SDS (0.08g/g CDW) for 40min; EDTA (0.4 g/g CDW) and Lysozyme (0.005g/g CDW) for 15min	Crossflow ultrafiltration	Diafiltration	90	92.6	Yasotha et al., 2006

AU: Anson Units (Enzyme activity)
SDS: Sodium Dodecyl Sulfate
EDTA: Ethylenediaminetetraacetic acid
CDW: Cell dry weight
mcl-PHA: Medium chain length PHA (6-12 carbon atoms)
Note: *Ralstonia eutropha* is an earlier synonym for *Cupriavidus necator* (Vandamme & Coenye, 2004)

2.2.4 PHB Extraction by Mechanical Treatment

Mechanical PHB extraction typically requires physical disruption to lyse the PHB-containing biomass and allow for the separation of intact PHB granules through centrifugation. Typical mechanical separation methods include bead-milling and high-pressure homogenization. They typically have lower material requirements than other methods, with some mechanical treatments requiring no addition of chemicals. Mechanical extraction methods generally start with concentrated wet cells and require no pretreatment. There has been relatively little research into mechanical PHB extraction, however ultrasonic disruption has been tested by several authors to improve digestion or dissolution methods.

Table 2.6: PHA Extraction from biomass by mechanical separation

Bacteria	PHA	Culture PHA%	Dewatering	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Mixed Culture (Wastewater)	PHB	50	Sedimented to 3 g/L	Ultrasonic Disruption in NaOH 10min 1.3 kW/L, 0.2M NaOH	Centrifuged	30% NaClO 1min, Centrifuged, Washed with deionized water twice and dried at 60C	67.9	71.9	Zou et al., 2023
Mixed Culture (Wastewater)	PHB	50	Sedimented to 3 g/L	Ultrasonic Disruption in NaOH 30min 2.6 kW/L, 0.2M NaOH	Centrifuged	30% NaClO 1min, Centrifuged, Washed with deionized water twice and dried at 60°C	73.7	81.7	Zou et al., 2023
Cupriavidus. necator DSM454	PHB	Not Given	20g cell pellet from 1L culture	HPH "maximum pressure" 3 passes ice-cold Tris-HCl EDTA, 5% SDS (10min/L)	Washed 3 times in 20% ethanol by centrifuge	Washed with ethanol	Not Given	85	Etxabide et al., 2022
Methylobacterium sp. V49	PHB	70	Centrifuged x2	HPH 500 kg/cm2; 2 passes	Centrifuged	Rinse with distilled water, centrifuged	95	80	Ghatnekar et al., 2002
Methylobacterium sp. V49	PHB	70	Centrifuged x2	HPH 400 kg/cm2; 2 passes with 5% SDS	Centrifuged	Rinse with distilled water, centrifuged	98	95	Ghatnekar et al., 2002

HPH: High-pressure Homogenization
EDTA: Ethylenediaminetetraacetic acid
SDS: Sodium dodecyl sulfate

2.2.5 Unique PHB Extraction Methods

There are many PHB extraction protocols which do not fit neatly into any of the categories detailed above. Many of these are attempts to bypass the significant and persistent drawbacks of typical PHB extraction methods through a complete reimagining of key process steps.

The use of biomass-consuming animals as a method of NPCM digestion is a very unique approach which may allow for greatly decreased material use, as the extraction platform is grown from biomass and can be repurposed as protein in animal feed after use (Zainab-L et al., 2022). However, secondary purification is essential to remove PHB from other contents when concentrating it in fecal pellets. This could be promising if the process can be developed such that the need for freeze-drying is avoided and the need for secondary purification is minimized.

The use of a two-phase aqueous detergent-based extraction is another unique approach that was recently investigated in a continuous system (Murugesan et al., 2021) as opposed to batch systems for most PHB extraction studies. This process achieved a high purity (86.0%) and a higher recovery (85.5%) than batch operation of the same process. This and another liquid-liquid extraction method (Murugesan & Iyyasamy, 2017) rely on detergents to lyse the cells, and based on the properties of the two phases, PHB and the NPCM will preferentially associate with one of the two liquid phases which forms the basis for their separation. The studies on these extractions have been performed at low temperatures and with minimal pretreatment and the demonstrated continuous operation of the extraction is another useful attribute.

In another study, the use of distilled water alone for extraction of PHB from the halophilic *Halmonas* sp. by osmotic shock has the potential to greatly decrease energy and material requirements of extraction (Rathi et al., 2013).

Table 2.7: Unique PHA extraction methods.

Bacteria	PHA	Culture %PHA	De-watering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Mixed Culture	PHBV	66	Broth was acidified, neutralized, washed, then freeze-dried		Subcritical water: Extraction vessel filled with water. 80bar, 150°C, 30min	Dried	0.2g in 5mL NaOCl (5% v/v) RT, 3h. Dried	81	84	Meneses et al., 2022
Cupriavidus necator	PHA (PHBV)	85	--	Fermentation broth was diluted 20 times (Water Added)	Fermentation broth mixed with 4.5wt% TX114 + 0.5% TMN6 detergent solution at pH 3 with 0.1M NH_4Cl in a modified rotating disc contactor at 40°C	Liquid-liquid phase separation in contactor; PHAs accumulate in detergent effluent, biomass in broth effluent	--	84.4	92.5	Murugesan et al., 2021
Cuprivadius necator H16	PHB	70	Freeze-dried	Washed with water	Biomass fed to mealworms (30 days old, starved 24h)	Fecal pellets were sieved	Dried at 50°C	99.7	82	Zainab-L & Sudesh, 2019
Escherichia coli	PHB		Microwaved 10min (700W, 2450 MHz) in 10mM EDTA. Centrifuged, washed with water, ethanol.		Pellet was suspended in boiling chloroform.	Filtered through glass wool	Solvent evaporation	93.8	97.2	Balakrishna Pillai et al., 2018
Cupriavidus necator H16	PHB	39.0	Freeze-dried	--	Freeze-dried biomass fed to rats, fecal pellets collected and dried at 60°C	Pellets treated with 2% SDS (4:1 solution:pellet) at RT for 24h. Centrifuged	Resuspended in distilled water and centrifuged. (3x)	not given	97	Kunasundari et al., 2017
Cupriavidus necator	PHBV	84.9	--	--	Aqueous cloud point extraction: 3wt% TX100 + 2wt% TX114 surfactant, pH 5, 0.1M ammonium chloride.	Phase separation created by heating from RT to 36°C. Mixture was ultrasonicated (6kHz, 6min, 2s pulses) Centrifuged	Drying pellet at 1h 100°C	84.4	94.3	Murugesan & Iyyasamy, 2017
Cupriavidus necator H16	PHA		--	Ultrasonic cell disruption 30kHz 15min	Thermoseparating Aqueous Two-Phase Extraction: EOPO 3900 / Ammonium Sulfate 14wt% each, pH 6	--	--	72.2	59.6	Leong et al., 2017
Cupriavidus necator Re2058/pCB113	PHB-co-HHx	54	Freeze-dried	--	Fed to Mealworms (50g mealworms fed 40g of culture over 16 days)	Water with 1% SDS (5:1 solution: mealworm fecal pellet) 250rpm 10h;	Washed with 0.001N HCl, dried at 60°C	<72	100	Murugan et al., 2016
Cupriavidus necator		50	Freeze-dried	--	Water, 1h, 30°C	Centrifuged	1% v/v ethanol, 200rpm 30°C 3h; centrifuged, washed with water	96.1	80.6	Mohammadi et al., 2012
Halomonas sp. SK5	PHB	48	Freeze-dried	--	Stirring at 30°C for 18h in distilled water.	Centrifuged	Washing with water, oven-dried	98	94	Rathi et al., 2013
Halomonas sp. SK5	PHB	48	Centrifuged	--	Stirring at 30°C for 1h in distilled water with 0.1% SDS	Centrifuged	Washing with water, oven-dried	98	96	Rathi et al., 2013
Sinorhizobium meliloti	PHA	50	--	Heated to 80°C for 10min and cooled.	*Microbispora* sp. cultivated on PHA-rich *S. melitoti* , 24h. Hydrolysate treated with chloroform 10min, RT.	Dried at 50°C	Dried at 50°C	98	90	Lakshman & Shamala, 2006

EDTA: Ethylenediaminetetraacetic acid
TX100, TX114, TMN6: Commercially available detergents (Triton X 100, Triton X 114, Tertigol 6)
EOPO 3900: Poly(ethylene glycol-ran-propylene glycol)monobutyl ether ($EO_{50}PO_{50}$, Mn ~ 3900 g/mol)
SDS: Sodium dodecyl sulfate
RT: Room Temperature

2.2.6 Impact of pre-treatment and purification steps

Since the various extraction studies detailed in Tables 2.2-2.6 were all part of differing process schemes, direct comparisons between process types are difficult to establish. To provide some additional context, papers that have duplicated the core process while varying pre-treatment or post-treatment steps have been included in Tables 2.7 and 2.8. This provides the opportunity to showcase some approaches which had better performance using freeze-dried biomass compared to wet biomass. It also provides an opportunity to examine the differences in process performance created by changes in starting material. Surprisingly, dried biomass does not always lead to a strict improvement in extraction yield and purity. In fact, there are even different outcomes for different processes within the same category. Some digestion extraction studies showed that additional drying can provide purity increases of around 10% (Rodrigues et al., 2022; G.-Q. Chen et al., 2001), and some solvent extraction studies showed that drying improved the recovery rate (Parodi et al., 2021; Wongmoon et al., 2022). However, there are also digestion and solvent extraction investigations for which drying did not improve performance metrics, and in some cases even led to a slight decrease in purity or yield. (Rodrigues et al., 2022; Samorì et al., 2015). These contrasting patterns indicate that there cannot be a simple numerical adjustment to estimate the differences between a process that has only been tested from freeze-dried biomass versus a process that has used centrifuged biomass, since the impact is unique to the system. Generally, drying the biomass before treatment tends to primarily affect PHB purity in digestion methods and PHB yield in dissolution methods, but that is not always the case.

Many studies which compared identical processes with the addition of a post-treatment step included additional NPCM digestion steps. Two studies compared the effect of additional washing steps (Mannina et al., 2019; Extabide et al., 2022). In every case seen in Table 2.8, the addition of a purification step improved the purity of the polymer product but reduced its recovery rate.

Since authors typically use the same microorganism throughout their study, there are limited opportunities for a direct comparison of PHB extraction processes between organisms. In one case, two strains of *Cupriavidus necator* were tested and found to have no difference in PHB extraction by use of the solvent 1,3-dioxolane (Wongmoon et al., 2022). Some comparisons can be made between similar extraction methods used in different studies. For example, ammonium laurate digestion performed much better when extracting PHB from *Cupriavidus necator* than from a mixed microbial culture (Samorì et al., 2015; Mannina et al., 2019, Mannina et al., 2020).

Table 2.8: PHA extraction processes with variations in pre-treatment steps.

Bacteria	PHA	Culture %PHA	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Cupriavidus necator H16	PHB	70.3	Centrifuged	Washed with 0.85% sodium chloride (x2)	1,3-dioxolane, 5% w/v dry biomass in 2mL, 80°C, 6h. Vortexed every 30min	3 volumes water added, centrifuged	Washed twice with water, dried	86.8	98	Wongmoon et al., 2022
			Centrifuged, dried (after pre-treatment)					90.4	97.2	
Cupriavidus necator A-04		67.2	Centrifuged					86.1	97.9	
			Centrifuged, dried (after pre-treatment)					91.6	97.9	
Mixed Culture	PHBV	44	--	Treated with sulfuric acid	20 g/L biomass 0.3 M NaOH 4.8 h	Centrifuged	Washed with 2 volumes of water	88	57	Rodrigues et al., 2022
			Dried at 60°C (after pre-treatment)					89	78	
			Freeze-dried (after pre-treatment)					92	66	
			--		20 g/L biomass 9% NaClO 3.4 h	1 volume water added, Centrifuged		91	83	
			Dried at 60°C (after pre-treatment)					95	73	
			Freeze-dried (after pre-treatment)					90	80	
Mixed Culture	PHB	39	--	--	Methyl 3-methoxybutyrate 1mL with 300mg slurry (79% water content) 130°C, 10min	Centrifuged	Solvent removed by distillation	92	98	Parodi et al., 2021
			Freeze-dried		Methyl 3-methoxybutyrate 1mL, 67mg biomass 130°C, 10min			98	97	
			--		Methyl 3-hydroxybutyrate 1mL with 300mg slurry (79% water content) 130°C, 10min			77	96	
			Freeze-dried		Methyl 3-hydroxybutyrate 1mL, 67mg biomass 130°C, 10min			96	94	
Escherichia coli	PHB	54	Centrifuged	--	Dimethyl carbonate (2.5% w/v biomass) 90°C, 1.5 h	Centrifuged, Precipitated with 3 volumes of ethanol	Air-dried	67.2	86.1	Mongili et al., 2021
				Dried 95°C overnight				68.7	81.1	

Cupriavidus necator H16	PHB	68 70	Freeze-dried	-- Washed with water	30-day-old mealworms starved for 24, then fed prepared biomass	Fecal pellets were sieved	Dried at 50°C	100 99.7	72 82	Zainban-L et al., 2019
Cupriavidus necator DSM545	PHB	74.2	Centrifuged to 100g/L biomass; Freeze-dried	--	Dimethyl Carbonate 1mL slurry in 2mL solvent 90°C 1h Dimethyl Carbonate 50mg biomass in 2mL 90°C 1h	Centrifuge, filtration, ethanol precipitation	Dried at 60°C under vacuum	92 87	96 95	Samori et al, 2015
Cupriavidus necator	PHB	71	Centrifuged Centrifuged (after pre-treatment)	-- pH 9 (1M NH_4OH), 60°C for 5min, then pH to 4.0 (1M HCl)	1,2-propylene carbonate. 150mL for 11.5g wet biomass. 130°C, 30min	Hot solution was filtered, let stand for 24h, mixed with two volumes acetone and allowed to stand for 24h more, then filtered again	Washed with acetone and air-dried	95 95	84 88	Fiorese et al, 2009
Alcaligenes eutrophus	PHB	60	Centrifuged Dried at 60°C Freeze-dried	--	2g dry cell powder treated with 100mL water containing 0.28g betaine surfactant and EDTA disodium salt at pH 13 and 50°C for 10min	Centrifuged, rinsed with water, centrifuged	4 volumes acetone added, filtered	not given 85.8 93.3	87.4 94.3 98.7	G.-Q. Chen et al, 2001

EDTA: Ethylenediaminetetraacetic acid

Table 2.9: PHA extraction processes with variations in purification steps.

Bacteria	PHA	Culture %PHA	Dewatering	Pre-Treatment	Extraction	Separation	Purification	Recovery (%)	Purity (%)	Reference
Mixed Culture (Activated Sludge)	PHA	44	Centrifuged	Washed twice with water, centrifuged	100mg activated sludge, NaClO 25% v/v, 30min	SDS (2:1 SDS:biomass dry weight), 30min	-- 10mg of extract treated with 5mL methanol, 15min	81 86	44 67	Xiong et al., 2023
Cupriavidus necator DSM454	PHB	not given	20g cell pellet from 1L culture	--	Resuspended in Tris-HCl EDTA 5% SDS, 3 passes ice-cold HPH "maximum pressure" (10min/L)	Washed 3 times in 20% ethanol by centrifuge (8000 x g, 20min, 10°C)	-- Washed with ethanol Washed with ethanol, dissolved in chloroform, precipitated in methanol, filtered	not given not given not given	66 85 95	Etxabide et al., 2022
Mixed Culture	PHBV	66	Broth was acidified, neutralized, washed, then freeze-dried		Subcritical water: Extraction vessel filled with water. 80bar, 150°C, 30min	-- Dried, 0.2g in 5mL NaOCl 5% (v/v) RT, 3 h	Samples dried at 60°C until constant weight	88 81	77 84	Meneses et al., 2022
Mixed Culture (Simulated Wastewater)	PHB	52	Freeze-Dried	-- 50mg biomass, 5mL NaOCl (4.7% Cl_2), 85°C 1h; Centrifuged, washed with water x2	Ammonium laurate (0.75 mmol NH_4OH, 0.5mmol lauric acid in 3mL H_2O); 3h 75°C	Centrifuged	-- Washed once with 0.1M NH_4OH solution Washed once with 0.1M NH_4OH, twice with ethanol -- Washed once with 0.1M NH_4OH solution Washed once with 0.1M NH_4OH, twice with ethanol	96 91 85 74 67 60	57 62 67 102 104 108	Mannina et al., 2019
Cuprivadius necator H16	PHB	39.0	Freeze-dried	--	Freeze-dried biomass fed to rats. Fecal pellets collected, dried at 60°C	-- Pellets treated with 2% SDS (4:1 solution:pellet) at RT for 24h. Centrifuged	Pellet resuspended in distilled water and centrifuged again. (3x)	not given not given	89 97	Kunasundari et al., 2017
Pseudomonas putida KT2440	mcl-PHA	65.6	Centrifuged, washed with water, freeze-dried		0.2N NaOH, 22°C, 2h, centrifuged, then 0.1N NaOH, 80°C, 15min	Centrifuged	Washed with distilled water, dried Lysozyme 0.01g/(g biomass) in 10mM Tris-HCl (pH 8), 37°C, 1h, centrifuged. Washed with water, dried	88.7 83.6	94.7 98.9	X.J. Jiang et al., 2014

SDS: Sodium dodecyl sulfate
RT: Room Temperature
EDTA: Ethylenediaminetetraacetic acid
HPH: High-pressure homogenization

2.2.7 Recycling PHB Extractive Agents

Due to the cost associated with solvent recovery by distillation and the material requirements from ineffective recovery of digestive agents (López-Abelairas et al., 2015), a significant opportunity for process improvement comes from better handling of the extractive agents after the PHB has been extracted. Although this is typically excluded from studies focusing on extraction, some work has focused on recycling PHB extractive agents.

Sodium hydroxide and sodium hypochlorite digestion solutions were tested for their reusability as part of an economic assessment of extraction methods (López-Abelairas et al., 2015). This study found that sodium hydroxide and sodium hypochlorite could be re-used with no drop in performance at a 40% replacement rate, and sulfuric acid could be re-used at a 20% replacement rate with only a 2% drop in performance.

The use of the switchable anionic surfactant ammonium laurate for digestion (described in Table 2.3) is promising with regards to reducing material use. One study on this method included a partial recycling step to lauric acid (Samorì et al., 2015), with the ammonium carbonate formed to be used as a microbial protein source.

Some extraction methods with promise for recyclability are those that are a significant departure from typical methods. A thermoseparating aqueous two-phase extraction was tested for reusability and achieved 60% PHB yield and 47% PHB purity on the third run, down from 72% yield and 60% purity initially (Leong et al., 2017).

This method still needs to be improved but clearly has potential. Similarly, the extraction of PHB by biological digestion of NPCM has potential as a recyclable digestion platform, in fact possibly creating more of the extraction agents through the process, with excess being able to be converted into animal feed if necessary.

Some of the solvent extraction methods shown in Table 2.2 have utilized nontoxic or less environmentally harmful solvents, but the re-use of these solvents still requires distillation, the energy costs for which may remain prohibitive. One unique approach to reducing material costs is the creation of solvents for PHB extraction from waste PHB (Parodi et al., 2021). The re-use of 1,3-dioxolane without any separation from the water antisolvent has been tested but was effective only at a 66% replacement rate, with significant decreases in yield and purity, highlighting the need for further development (Wongmoon & Napathorn, 2022).

Due to the fact that extraction from biomass is a significant portion of the cost of PHB production, and a significant portion of that cost comes from material requirements (in typical digestion) or distillation (with

solvent extraction), there is both an economic and an environmental drive to improve PHB extraction with recyclable extractive agents that are able to replicate the performance of current extraction methods but with lower material or energy requirements.

2.3 Switchable Solvents

Switchable solvents are a category of solvents which can have their polarity and/or hydrophilicity toggled between two states through the addition or removal of a trigger, such as carbon dioxide (Jessop et al., 2005, 2010, 2012; Vanderveen et al., 2014). Of particular interest are switchable hydrophilicity solvents (SHSs), which are able to be switched between a hydrophobic and a hydrophilic form by adding or removing carbon dioxide in the presence of water (Durelle et al., 2015; Jessop et al., 2010, 2012; Vanderveen et al., 2014).

2.3.1 Mechanism of Switchable Hydrophilicity Solvents

Many SHSs are tertiary amines (as seen in Figure 2.6a) and are nonpolar solvents that can be protonated under certain conditions to switch them into a polar solvent. As shown in Figure 2.6b, when carbon dioxide is introduced into water in the presence of these solvents, the amine becomes protonated to a polar compound as negatively charged bicarbonate ions are formed in the solution (Durelle et al., 2015; Vanderveen et al., 2014). This increased polarity makes the solvents miscible with water, and similarly can change their interactions with other compounds; compounds may be soluble or miscible with one form of a SHS but not the other. The solution can be switched back by heating and/or sparging with an inert gas such as nitrogen. These measures will cause the reverse reaction: the release of carbon dioxide gas and the reversion of the solvent to its nonpolar state.

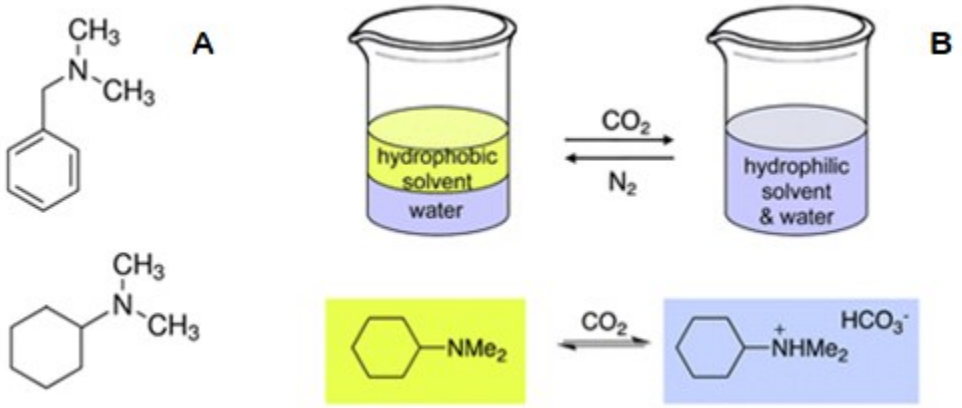

Figure 2.6: Switchable hydrophilicity solvent structure and mechanism
(A) The structure of two tertiary amine SHSs, and **(B)** the mechanism of solvent switching using carbon dioxide and water, shown alongside the change in water-solvent miscibility.
(**(B)** Jessop et al., 2012, modified from original Reproduced with permission from the Royal Society of Chemistry.)

2.3.2 Applications of Switchable Hydrophilicity Solvents

The ability to toggle the solubility or miscibility of a target compound in a solvent provides a unique approach for extraction processes in which the product can be easily uptaken by and then separated from the solvent, leading to a simple solvent recycling procedure (Figure 2.7) (Boyd et al., 2012; Jessop et al., 2012). This approach has the potential to decrease the material and energy costs of solvent extraction processes, which often rely on distillation or antisolvents to separate the product from the extractive solvent. In this way, SHS extractions could improve both the environmental impact and economics of bioproducts.

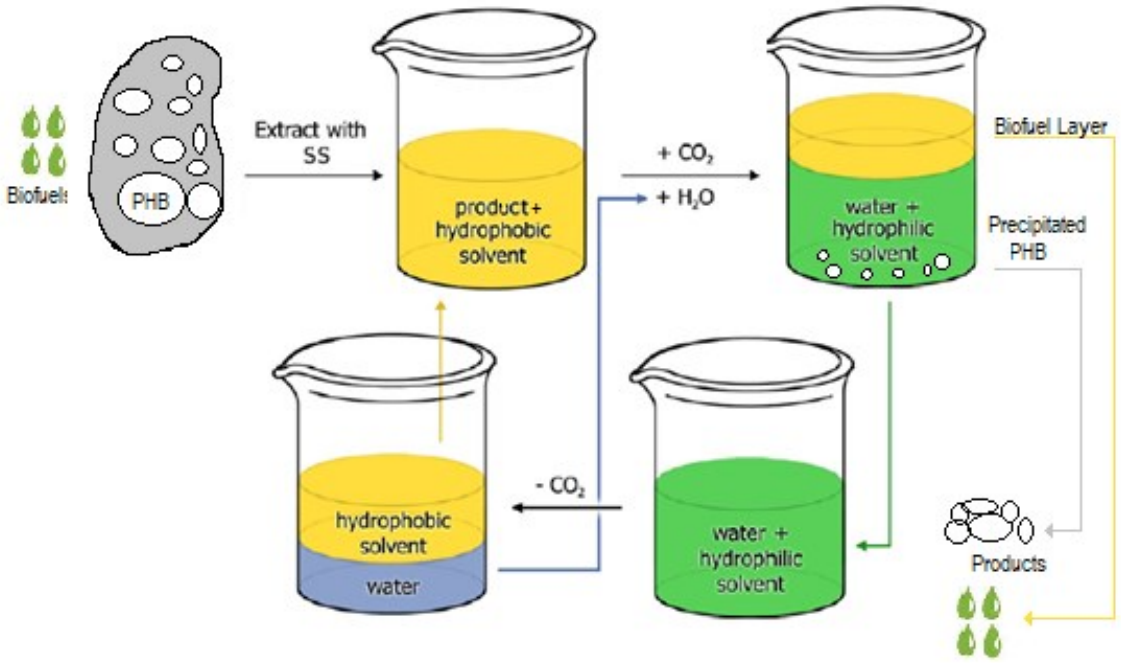

Figure 2.7: A schematic of a process using a SHS to extract biofuels or PHB from bacterial cells.
(Boyd et al., 2012, modified from original. Reproduced with permission from Elsevier.)

SHSs have been used at the lab scale in a variety of processes, including the separation of oil from soybean flakes (Phan et al., 2009), the extraction of lipids and astaxanthin from algae (Al-Ameri & Al-Zuhair, 2019; Boyd et al., 2012; Du et al., 2015; Guo et al., 2022; Huang et al., 2018; Samorì et al., 2013), and other extractions such as bitumen from sand (Holland et al., 2012) and the separation of phenols from pyrolysis oils (Fu et al., 2014). SHSs have also been used in analytical chemistry applications to efficiently remove trace compounds from food products for quantification (Wang et al., 2018; Yilmaz & Soylak, 2015).

For biological extraction, SHSs have been shown in some cases to disrupt algal cell walls (Al-Ameri & Al-Zuhair, 2019; Cicci et al., 2018; Guo et al., 2022; Huang et al., 2018; Samorì et al., 2013). This is a useful feature which potentially removes the need for a pre-treatment step, as extraction procedures often

rely on mechanical forces or detergents to disrupt cell membranes prior to the main extraction process (López-Abelairas et al., 2015; Aramvash et al., 2017; Leong et al., 2017). However, this ability may vary between organisms, as different microbes will have different properties for outer cell membranes or cell walls (Guo et al., 2022). The ability of these solvents to be used in the presence of water is another benefit which could lead to additional efficiency, since removing water is an energy-intensive process and a process which can handle wet biomass would lower that energy requirement (Du et al., 2015; Huang et al., 2018).

A small number of studies report the use of SHSs with polymers. Some SHSs have been shown to dissolve polystyrene and polyethylene and precipitate them by switching to the polar form (Jessop et al., 2011; Samorì et al., 2017). SHSs have also been used in some cases as reaction media for polymers, using the switchable hydrophilicity to precipitate the product (Su et al., 2018). One application of a similar class of molecules to PHB was the use of a switchable surfactant (ammonium laurate) to digest freeze-dried *Cupriavidus necator* biomass and release PHB granules, using carbon dioxide to create ammonium carbonate with the recovery of lauric acid (Samorì et al., 2015). (Note that as proposed in that paper, this is not a completely closed loop, as lauric acid would be combined with fresh ammonium hydroxide and the resulting ammonium carbonate would be used as a nitrogen source for bacterial growth.) Ammonium laurate has also been tested for PHB extraction from a freeze-dried mixed microbial culture grown on wastewater (Mannina et al., 2019). The use of SHSs for biopolymer recovery may have advantages over switchable surfactants since the SHSs can be recovered to their hydrophobic form without the material cost of regenerating the switchable surfactant.

2.3.3 Alternative Applications of Switchable Hydrophilicity Solvents

While the extraction process shown in Figure 2.7 is an effective way of using SHSs, recent advances may further improve the efficiency of these solvents as an extractive method.

A possible secondary benefit of SHSs as an extraction platform is the possibility that the solvent could be used in either the hydrophobic or hydrophilic form. Because a specific target product may only be dissolved by one form of the solvent, the other form can be used as a pretreatment step by extracting secondary products that are soluble in the opposite hydrophilicity from the primary product (Cicci et al., 2018). The use of each form of the SHSs to extract from a given matrix means that, with similar equipment and materials, there are effectively two solvents in one.

Another alternative approach in the use of SHSs is adding the solvent to the original matrix in the hydrophilic form, then switching to the hydrophobic form to extract a nonpolar product. This is common in

the microextraction of trace compounds with the goal of extracting and concentrating the target compound for quantitative analysis (Wang et al., 2018; Yilmaz & Soylak, 2015). The ability of SHSs to be mixed (and therefore dispersed) through the sample medium before being switched to the other phase removes the need for an additional dispersive solvent as is often used in classical solvent extraction (Wang et al., 2018; Yilmaz & Soylak, 2015). Microextractions commonly use sodium hydroxide to facilitate the phase switch, which is less desirable from a chemical use standpoint than heating or nitrogen sparging. However, introducing the solvent in its hydrophilic form before switching to the hydrophobic form to achieve better contact between the solvent and the product may have benefits beyond quantitative microextractions, and could be done with heating or nitrogen sparging.

While SHSs do have toxic effects, their potential recyclability could translate into little or no solvent being released into the environment during processing. The potential benefits of reduced material and energy costs makes SHSs good candidates for improved extraction processes that warrants their study for use with PHB, although attention will have to be placed on limiting the residual solvent in any polymer product. Additionally, there have been recent investigations into amine-free SHSs, which may offer the same advantages while being safer (Cunha et al., 2022).

2.4 Polymer Dissolution

2.4.1 Thermodynamics of Polymer Dissolution

For solutions to occur spontaneously, it is necessary that the free energy of mixing be negative. The free energy of mixing for polymer solutions is given by Stefanis & Panayiotou (2012):

$$\frac{\Delta G^M}{RT} = x_1 \ln(\varphi_1) + x_2 \ln(\varphi_2) + x_1 \varphi_2 \chi_{12} \tag{2.1}$$

where R is the universal gas constant, T is the absolute temperature, ΔG^M is the free energy of mixing, x_i and φ_i are respectively the molar and volume fractions of component i, component 1 is the solvent, and component 2 is the polymer. The Flory-Huggins parameter, χ_{12}, is the only parameter involved in the determination of the free energy of mixing that is a function of the properties of the compounds being mixed.

The thermodynamic favourability of the dissolution process can be predicted by the polymer-solvent Hansen Solubility Radius, R_A, and the comparison of that distance to the radius of the Solubility Sphere, R_M, which is expected to contain all good solvents of a given polymer. Although the concept of "good solvent" is murky, it has been used in many studies to indicate a solvent which is capable of gelling or dissolving a polymer. This terminology is used in both The Hansen Solubility Parameters Handbook (Hansen, 2007) and an extensive review on polymer dissolution (Miller-Chou & Koenig, 2003), and the phrase "thermodynamic 'goodness'" is used in the same context in an older paper (Cooper et al., 1986).

Of these, only the Hansen Solubility Parameters Handbook provides a definition, describing another study which had "arbitrarily set" for a good solvent to be one that resulted in more than 0.5% weight gain of the polymer (Hansen, 2007).

The Hansen Solubility radius is calculated according to Hansen (2007) as:

$$R_A^2 = (\delta_{d1} - \delta_{d2})^2 + .25 * \left(\delta_{p1} - \delta_{p2}\right)^2 + .25 * (\delta_{hb1} - \delta_{hb2})^2 \quad (2.2)$$

where δ_d, δ_p, and δ_{hb} are the Hansen Solubility parameters for the dispersion, polar, and hydrogen bonding forces present in the solvent (1) or polymer (2). The maximum Solubility Parameter difference that will still allow for solubility, R_M, defines the radius of the solubility sphere, and is calculated by (Hansen, 2007):

$$R_M^2 = 0.5 * \left(1 + \frac{1}{\sqrt{r}}\right) * \frac{R * T}{\upsilon} \quad (2.3)$$

where υ is the molar volume of the solvent, and r is a correction factor that is the ratio of the polymer size to the solvent size, often approximated as the degree of polymerization of the solvent.

The radius R_A can be compared to the radius of the solubility sphere, so R_A/R_M will be zero if the solubility parameters of the solute and solvent match perfectly, and it will be equal to one at the edge of the solubility sphere. A lower R_A/R_M ratio predicts better solubility, so a R_A/R_M of zero – equivalent to putting the solvent in the centre of the sphere – indicates the best possible solvent, while a solvent on the edge of the solubility sphere would be described as "the worst possible good solvent". This comparison can be related to the Flory-Huggins parameter by the equation (Hansen, 2007):

$$\chi_{12} = 0.5 * \left(\frac{R_A^2}{R_M^2}\right) \quad (2.4)$$

Thus, any good solvent would be expected to have a χ_{12} parameter of 0.5 or lower. Because the term including χ_{12} is the only positive term of the equation for the estimation of ΔG^M, and since the other terms on the right-hand side of Equation (2.1), x_1*ln(φ_1) + x_2*ln(φ_2) are all based on the fractions of molecules present, the χ_{12} parameter determines at which concentrations the polymer will be dissolved. A lower χ_{12} means dissolution occurs over a wider range of polymer to solvent ratios, with a zero χ_{12} term theoretically allowing any concentration of polymer to be dissolved in the solvent.

The Hansen Solubility Parameters Handbook (Hansen, 2007) stipulates that polymer crystallinity will cause special effects that may not be accounted for by the solubility parameters. This is an important factor to consider in PHB extraction because PHB granules are highly amorphous in vivo but become more crystalline when extracted using the most common methods (de Koning & Lemstra, 1992).

Crystalline polymers do not take up solvent as easily as amorphous polymers, and they have higher solubility parameters than amorphous regions; however, they are generally more easily penetrated by smaller solvent molecules (Hansen, 2007). Small non-solvent molecules with high diffusivity have been

used to increase the rate of dissolution of polymers in solvents (Miller-Chou & Koenig, 2003) – that behaviour may also help deal with diffusion into crystalline structures. Another way to potentially overcome the solubility limitations created by crystallinity is to perform the solubilization at higher temperatures. There is some evidence that crystalline polymers behave as predicted by solubility parameters when heated near their melting temperature (Terada & Marchessault, 1999). This is consistent with the radius of the solubility sphere increasing with temperature, as described by Equation (2.3); however, the solubility parameters of a given solvent will also change as the temperature increases (Hansen, 2007).

A successful solvent-based extraction process for PHB recovery will require the ability to extract PHB of varying crystallinities and/or will need to access the PHB before it undergoes a significant increase in crystallinity.

2.4.2 Kinetics of Polymer Dissolution

The dissolution of a polymer in a solvent consists of two main steps (Figure 2.8): the diffusion of solvent into the polymer – often forming a rubbery gel, followed by the disentanglement of polymer chains from the network (Miller-Chou & Koenig, 2003). The thickness of the resulting gel layer is determined by how fast the solvent is able to penetrate through the polymer, and how quickly the gelled polymer chains dissolve into the bulk solvent. At a certain point, when the solvent has penetrated to the centre of the polymer, the entire polymer phase is part of the gel, which then continues to dissolve into the bulk solution at the gel interface.

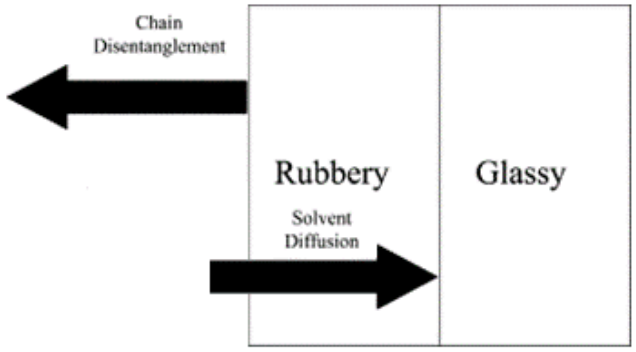

Figure 2.8: The mechanism of polymer dissolution
The rubbery gel layer forms on the surface of the polymer, and its expansion into the glassy region is determined by the rate of solvent diffusion into the polymer. The gel layer's outer interface is dissolving into the bulk solvent as polymer chains disentangle from each other and are released.
(Miller-Chou & Koenig, 2003, modified from original. Reproduced with permission from Elsevier.)

The rate at which the solvent can penetrate the polymer, and the rate at which the polymer-solvent gel dissolves into the bulk solvent, are the controlling parameters of dissolution. Solvent penetration is generally faster for smaller solvent molecules (Hansen, 2007; Miller-Chou & Koenig, 2003), and chain disentanglement is faster for smaller polymer chains (Miller-Chou & Koenig, 2003; Narasimhan & Peppas, 1996). Both rates increase with increasing temperature and agitation (Cooper et al., 1986; Miller-Chou & Koenig, 2003; Narasimhan & Peppas, 1996).

Due to the impact of solvent penetration on dissolution kinetics, the addition of small non-solvent molecules may increase the dissolution rate of polymers (Cooper et al., 1986.; Hansen, 2007; Miller-Chou & Koenig, 2003). Despite decreasing the thermodynamic favourability of the dissolution, these molecules can increase the effective diffusivity coefficient of the solvent molecules, leading to a net increase in polymer dissolution rate (Cooper et al., 1986; Miller-Chou & Koenig, 2003).

Multiple models attempt to explain experimental results of polymer dissolution kinetics, as reviewed by Miller-Chou and Koenig (2003) and Narasimhan (2001). However, most of these models focus on dissolution of amorphous polymers. In fact, there have been few attempts to develop predictive models for the dissolution of semicrystalline polymers. Two models have been proposed in this area, both of which posit that as the solvent penetrates into the polymer, crystalline regions are converted into amorphous regions before continuing with dissolution as amorphous polymer gel (Ghasemi et al., 2017; Mallapragada & Peppas, 1997).

2.5 Methanotrophic microorganisms

Methanotrophic microorganisms – microorganisms that can use methane as their sole carbon and energy source – are an important part of the global carbon cycle as they consume a great deal of the methane created by methanogenic microorganisms before it is released to the wider environment (Carere et al., 2017; Ghashghavi et al., 2017; Yun et al., 2021). Methanotrophic microorganisms are ubiquitous in natural environments (Ghashghavi et al., 2017; Saggar et al., 2008; Stein et al., 2011), although they are more common in habitats where methane is generated, including marine sediments, aquatic oxic-anoxic interfaces, wetlands, peatlands, and landfills (Ghashghavi et al., 2017; Yun et al., 2021). Methanotrophic archaea anaerobically oxidize methane (Carere et al., 2017; W.-L. Li et al., 2020), and methanotrophic bacteria (which will be referred to as “methanotrophs”) are primarily aerobic organisms (Carere et al., 2017; Guggenheim et al., 2019; Tays et al., 2018).

2.5.1 Methanotrophs

In addition to methane consumption, many methanotrophs are capable of directly using methanol as a sole substrate (Tays et al., 2018; Zhang et al., 2008). This is possible since the first reaction in the consumption of methane is its conversion to methanol catalyzed by methane monooxygenase enzymes (Guggenheim et al., 2019; Tays et al., 2018). The ability to use methane or methanol as feedstock gives methanotrophs a niche as bioconversion organisms to valorize low-value carbon sources unavailable to other microbes (Cantera, Muñoz, et al., 2018; Gęsicka et al., 2021).

The most significant differences between different types of methanotrophs are the pathway used for the assimilation of the metabolic intermediate formaldehyde and their ability to produce PHB. Methanotrophs from the class Alphaproteobacteria assimilate carbon from formaldehyde using the serine cycle, which allows them to produce PHB. Methanotrophs of the class Gammaproteobacteria use the ribulose monophosphate pathway, (in some cases, also having some of the enzymes from the serine or Calvin cycles) and are not able to produce PHB (Trotsenko & Murrell, 2008). There are also methanotrophs of the phylum Verrucomicrobia which are able to oxidize both methane and hydrogen through different pathways: oxidizing methanol directly to formate and fixing carbon dioxide into biomass using the Calvin cycle (Carere et al., 2017). The pathways for the conversion of methane to PHB in alphaproteobacterial methanotrophs such as *Methylocystis* sp. Rockwell are shown in Figure 2.10. PHB production and consumption can be seen as a storage mechanism for other pathways. Unlike other PHB-producing bacteria that are able to use PHB as a carbon source under carbon-limiting conditions, alphaproteobacterial methanotrophs only use PHB as a source of reducing power when carbon and nitrogen are both available (Pieja, Sundstrom, et al., 2011).

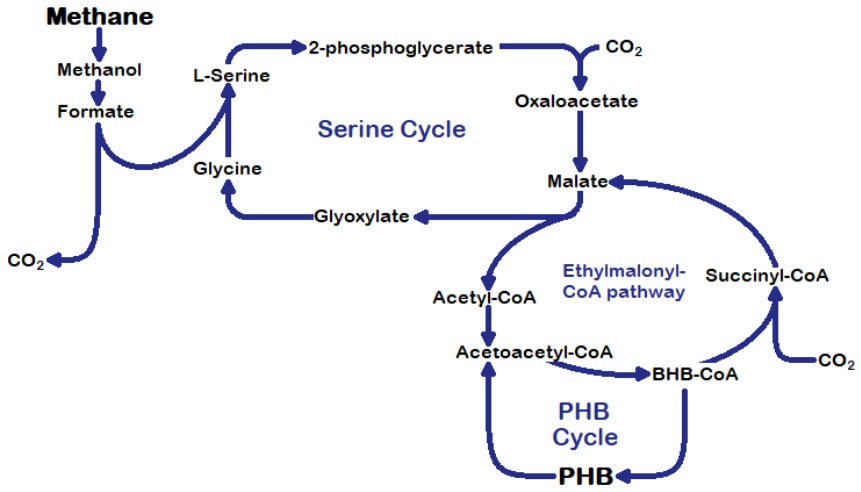

Figure 2.9: Metabolic pathway for the conversion of methane to PHB in alphaproteobacterial methanotrophs, such as *Methylobacterium extorquens* AM1 and *Methylocystis* sp. Rockwell.
(Zhu et al., 2016, modified from original. Reproduced with permission from Springer Nature.)

In general, gammaproteobacterial methanotrophs are able to grow faster than alphaproteobacterial methanotrophs, however alphaproteobacterial methanotrophs survive better and are able to outcompete gammaproteobacterial methanotrophs under oxygen or nitrogen limitation (Vecherskaya et al., 2001).

2.5.2 Methanotrophs as a Bioconversion Platform

Due to its significant global warming potential, the use of methane as a feedstock for bioconversion represents a significant environmental benefit. There is also an economic benefit to performing bioconversion using common industrial by-products, such as waste methane or methanol, as these low-cost feedstocks greatly reduce the costs of production. Feedstock costs can range from 40-80% of the total cost depending on which products are being made (Cho & Park, 2018; M. Li & Wilkins, 2020).

Methane is an abundant greenhouse gas (GHG) 27 times as potent as carbon dioxide over a 100-year period (Forster et al., 2021), and is the second most emitted GHG representing 14% of Canada's emissions in 2020 as determined by carbon dioxide equivalents (Environment and Climate Change Canada, 2023). Methanotrophs have great potential as a bioconversion platform to mitigate GHG emissions while generating valuable products. Major sources of methane include the fossil fuels industry, agriculture, and waste disposal (Duren et al., 2019; Environment and Climate Change Canada, 2023; Nisbet et al., 2020). In some cases, methane is flared off, creating carbon dioxide (although many flares run inefficiently, with the average flare allowing 9% of methane to escape unreacted) (Plant et al., 2022) while in other cases the methane is released directly into the environment. While some methane sources

are distributed and difficult to capture and valorize (such as enteric fermentation in cattle), a study of methane emissions in California in 2016 found that 34-46% of methane emissions in that state were from point-source emissions – sources less than 10 m in diameter emitting plumes of highly concentrated methane (Duren et al., 2019). Assuming a similar relationship holds in Canada, about 5% of GHG emissions are caused by methane point sources, as shown in Figure 2.10. In Canada in 2020, approximately one third of methane emitted from landfills was recovered, but less than half of the recovered methane was utilized, with the remainder being flared or oxidized (Environment and Climate Change Canada, 2023). Point sources of methane are the most likely avenues for the efficient valorization of waste methane through methanotroph bioconversion, especially those for which infrastructure exists to collect the methane but it is not utilized.

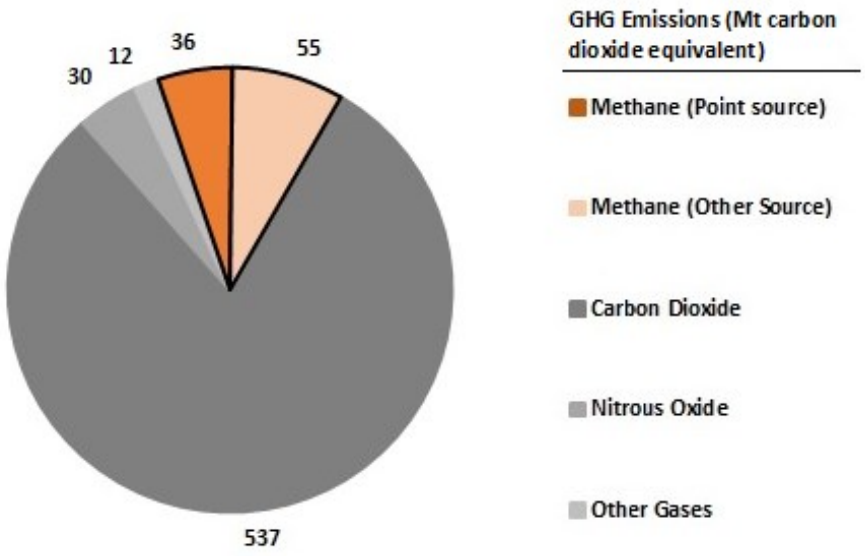

Figure 2.10: Methane point sources in the context of Canadian GHG emissions
Canadian GHG emissions in 2021 in Mt of carbon dioxide equivalent, showing the estimated fraction of methane emissions that are from point sources (and therefore could be more easily supplied to a bioconversion process).
GHG data from Environment and Climate Change Canada, 2023; point source frequency estimated from Duren et al., 2019.

Methane and methanol bioconversion of non-waste streams (e.g., natural gas and pure methanol) would require more efficient processes to be economically viable (in addition to being less environmentally beneficial) but could have benefits due to the scale of the resources (Q. Fei et al., 2014).

Methanotrophic bioconversion has been studied for the bioconversion of methane into a wide variety of products such as single cell protein production, (D'Mello, 1973; Øverland et al., 2010), PHB production (Zaldívar Carrillo et al., 2018; Lazic et al., 2022; Sharma et al., 2022), ectoine and hydroxyectoine (Cantera et al., 2018), and fatty acids for biodiesel production (Demidenko at al., 2017). Methanotrophs have also been genetically engineered to produce isoprenoids (Hwang et al., 2018; Sharpe

et al., 2007; Sonntag et al., 2015; Tao et al., 2007; Zhu et al., 2016), amino acids (Sirirote et al., 1986), specific proteins (Bélanger et al., 2004; Fitzgerald & Lidstrom, 2003), and organic acids (Sonntag et al., 2014). Methanotrophs have even been tested for use as a biocatalyst for the simple conversion of methane to methanol, due to the high conversion efficiency (Hur et al., 2017; Hwang et al., 2018). But of all current industrial applications, the most important are the production of single cell protein and PHB.

As mentioned above, PHB is produced and stored by alphaproteobacterial methanotrophs typically when cultivated under nutrient limitations (e.g., nitrogen or of other nutrients with excess carbon) (Helm et al., 2008; Pieja, Rostkowski, et al., 2011; Whittenbury et al., 1970). Gammaproteobacterial methanotrophs do not have the necessary enzymes for PHB synthesis (Pieja, Rostkowski, et al., 2011). Alternatively, the inhibition of the tricarboxylic acid cycle by the addition of citric acid to the culture medium has also been shown to promote PHB accumulation in alphaproteobacterial methanotrophs (Zhang et al., 2008). Depending on the optimized parameter, PHB accumulation can be as high as 85% of cell dry mass (in *Methylocystis hirsuta*) (Ghoddosi et al., 2019), in concentrations of up to 21 g/L (in *Methylocystis* sp. GB 25 DSM 7674) (Wendlandt et al., 2005), or with a mass-average molecular weight of up to 3.1 MDa (mixed culture with a dominant species *Methylocystis* sp. GB 25 DSM 7674) (Helm et al., 2008).

In all cases, the extraction of bioproducts from cells or from culture broths is an important aspect in the efficiency and economic viability of bioconversion processes.

2.5.3 Methanotrophic Organisms Used in Experiments

Methylocystis sp. Rockwell is an alphaproteobacterial methanotroph which was isolated from an aquifer in southern California (Stein et al., 2011). It has been shown to produce PHB while growing exponentially (maintaining 53.1% of cell dry weight as PHB), which is a significant advantage as the nutrient restrictions typically needed to stimulate PHB accumulation prevent simultaneous culture growth (Sharma et al., 2022; Lazic et al., 2022). The nitrogen in the culture still needs to be restricted, but there are certain conditions for which both growth and PHB accumulation can be achieved.

Methylomicrobium album BG8, a gammaproteobacterial methanotroph, was originally isolated as part of an experiment in which mud, water, and soil samples were collected from the Americas, Europe, and North and East Africa (Whittenbury et al., 1970). Previous names for this organism include *Methylobacter albus, Methylomonas albus,* and *Methylomonas alba* (Kits et al., 2013). Its physiology has been studied extensively and it has been tested as a protein source for young chicks (D'Mello, 1973). As a gammaproteobacterial methanotroph it cannot accumulate PHB, and so is used as a negative control for the PHB extraction experiments in this thesis.

2.6 Conclusion

The bioconversion of the greenhouse gas methane into the biodegradable plastic PHB would have significant advantages if deployed at scale. The extraction of PHB from bacterial cells remains a key challenge in this, as well as in all other PHB bioproduction processes. There is a wide variety of different PHB extraction methods in literature, most of which suffer from unrecoverable extraction agents or high energy costs for solvent recycle. Switchable hydrophilicity solvents have great potential as easily recyclable solvents for PHB extraction, which is the focus of the experiments presented in this thesis.

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3 Switchable Solvents for the Reversible Dissolution of Poly(3-hydroxybutyrate)

3.1 Abstract

The biopolymer poly(3-hydroxybutyrate) (PHB) is an excellent candidate to replace many petroleum-sourced polymers for a wide range of applications. Improving PHB recovery and processing methods remains an important step towards expanding its implementation and economic viability. Switchable hydrophilicity solvents (SHSs) are a class of molecules which can be toggled between hydrophobic and hydrophilic forms through the addition of carbon dioxide and water, which makes them promising candidates as recyclable solvents for the recovery of bioproducts such as PHB. Here, we used Hansen Solubility Parameters and the Stefanis-Panayiotou group contribution method to select candidate SHSs for processing PHB. We evaluated their ability to dissolve PHB over a range of temperatures and found that the theoretical methods accurately predicted interactions between PHB and the solvents below 100°C. Above 100°C PHB was dissolved in all candidate solvents and kinetic factors became significant in determining the extent of PHB dissolution during fixed-time experiments, with N,N-dimethylcyclohexylamine dissolving as much as 25.86 g/L PHB in 25 h. These results show that the solubility parameter model is valid for SHSs and that these solvents exhibit a reversible interaction with PHB.

3.2 Introduction

Plastics are ubiquitous in modern life, mostly due to their low cost and wide range of physical properties. However, these factors have led to the production of enormous quantities of plastic waste, much of which eventually finds its way to the environment (Geyer et al., 2017). In recent years, there has been increased focus on reducing the impact of plastics on natural ecosystems, with one of the main approaches involving a transition to biodegradable plastics for disposable products (Lambert & Wagner, 2017).

Poly(3-hydroxybutyrate) (PHB) is a polyhydroxyalkanoate (PHA), a class of polyesters notable for their biological origin and the fact that they can be completely degraded and assimilated by microbes in a wide range of environments (Kosseva & Rusbandi, 2018; Martínez-Tobón et al., 2018; Sudesh et al., 2000). This is an important distinction from many other bio-produced polymers which are seen merely as compostable (Lambert & Wagner, 2017). PHB has similar mechanical properties to polypropylene, a plastic commonly used in packaging, although PHB is stiffer and more brittle (G.-Q. Chen & Wu, 2005;

Holmes, 1985; Ray & Bousmina, 2005; Sudesh et al., 2000). PHB can be blended with other PHAs in order to form more ductile polymers, similar in properties to low-density polyethylene (G.-Q. Chen & Wu, 2005; Kageyama et al., 2021; Sudesh et al., 2000). PHB also has excellent barrier properties against water and oxygen (Cyras et al., 2007; Jost, 2018). These qualities make PHB and its PHA copolymers promising candidates as biodegradable replacements to petroleum-sourced polymers in applications such as packaging, (Bucci et al., 2007; Dietrich et al., 2017) disposable products (Holmes, 1985), and medical devices (G.-Q. Chen & Wu, 2005; Misra et al., 2006). However, PHB is currently not economically competitive with petroleum-based polymers (Fernández-Dacosta et al., 2015; Kosseva & Rusbandi, 2018), mostly due to costs associated with feedstock and product recovery. Many efforts are thus underway to explore low-cost substrates for microbial PHB production (Kosseva & Rusbandi, 2018) and to improve the energetic and material requirements for extraction of PHB from microbial cells used for its production. As cheaper fermentation substrates have been developed, the extraction costs represent a greater portion of the overall processing costs (Fernández-Dacosta et al., 2015), hence the drive for further improvements.

One of the most efficient methods used as part of the PHB recovery process is solvent extraction. Solvents used thus far range from the common (chloroform) (Kosseva & Rusbandi, 2018) to the exotic (acetic acid at high temperature, ionic liquids, and others) (Anbukarasu et al., 2015; Aramvash et al., 2018; Fiorese et al., 2009; Ramos et al., 2020; Sequeira et al., 2020). While effective, solvent extraction using chloroform has many disadvantages including high costs of recovery and significant potential negative environmental impact, due mostly to the requirement of counter-solvents and/or distillation for the recovery of PHB and solvent (Fernández-Dacosta et al., 2015). By investigating solvents that can be recovered more efficiently and/or that are more environmentally friendly, PHB extraction can become more economical and effective.

SHSs are a group of molecules that have the ability to transition between hydrophilic and hydrophobic forms through protonation/deprotonation, which can be performed by the addition/removal of carbon dioxide to/from aqueous solutions (Jessop et al., 2005). This transition changes the solvent properties, including the molecules it can dissolve (Jessop et al., 2012), and can be used to facilitate extraction in one phase (e.g. hydrophobic) followed by a product recovery/solvent recycling step in a different phase (e.g. hydrophilic) without requiring distillation or the use of a counter-solvent. This has been demonstrated for the extraction of lipids from microalgae (Boyd et al., 2012), astaxanthin from algae (Huang et al., 2018), and oil from oilsands (Holland et al., 2012). The recyclability of these solvents significantly decreases materials costs and energy requirements. To date, no SHSs have been shown to dissolve PHB. Establishing whether these solvents can be used in its extraction and recovery would open

the door to new, more sustainable opportunities for the biopolymer industry and to better understanding of SHS-biopolymer interactions. Specifically, it would be important to understand whether solubility models apply to these compounds.

In this study, a variety of SHSs were evaluated, based on theoretical and experimental results, for their interactions with PHB under a range of conditions. The purpose of this work is to establish whether SHSs display affinity towards PHB and whether they could serve as the basis for novel PHB extraction processes. Additionally, this study also provides experimental data demonstrating that theoretical models used to predict polymer solubility in traditional solvents can be applied to SHSs.

3.3 Materials and Methods

3.3.1 Theoretical Solubility Model

A theoretical solubility model was used to perform an initial screening of prospective SHSs, selected from an existing study (Vanderveen et al., 2014), compatible with PHB. The Hansen Solubility Parameters (Hansen, 2007) of the SHSs were calculated using the Stefanis-Panayiotou 3-parameter group contribution method (Stefanis & Panayiotou, 2008), with the use of clarifications found in Appendix A.3 of their subsequent study (Stefanis & Panayiotou, 2012). The three-parameter model was selected to allow for the use of experimentally determined solubility parameters for PHB (Jacquel et al., 2007).

For dissolution to occur spontaneously, the free energy of mixing – given by Equation (3.1) for polymer solutions (Stefanis & Panayiotou, 2012) – must be negative,

$$\frac{\Delta G^M}{RT} = x_1 \ln(\varphi_1) + x_2 \ln(\varphi_2) + x_1 \varphi_2 \chi_{12} \tag{3.1}$$

where R is the ideal gas constant, T is the absolute temperature, ΔG^M is the free energy of mixing, and x_i and φ_i are, respectively, the molar and volume fractions of component i (component 1 being the solvent, and component 2 being the polymer). The Flory-Huggins parameter, χ_{12}, is the only parameter involved in the determination of the free energy of mixing that is a function of both chemicals being mixed.

The thermodynamic favourability of the dissolution process can be predicted by the Hansen solubility radius, R_A, and the comparison of that radius to the radius of the solubility sphere, R_M, which is expected to contain all good solvents for a given molecule.

The Hansen solubility radius is calculated by (Hansen, 2007)

$$R_A^2 = (\delta_{d1} - \delta_{d2})^2 + 0.25 * \left(\delta_{p1} - \delta_{p2}\right)^2 + 0.25 * (\delta_{hb1} - \delta_{hb2})^2 \tag{3.2}$$

where δ_d, δ_p, and δ_{hb} are the Hansen solubility parameters for the dispersion, polar, and hydrogen bonding forces present in the solvent or solute, respectively. The maximum solubility parameter difference that will still allow for solubility, R_M, defines the radius of the solubility sphere, and is calculated as (Hansen, 2007)

$$R_M^2 = 0.5 * \left(1 + \frac{1}{\sqrt{r}}\right) * \frac{R * T}{v} \tag{3.3}$$

where v is the molar volume of the solvent, and r is the correction factor that is the ratio of the polymer size to the solvent size, often approximated as the degree of polymerization of the solvent.

The radius R_A can be compared to the radius of the solubility sphere. R_A/R_M will be zero if the solubility parameters of the solute and solvent match perfectly – putting the solvent at the center of the sphere, indicating the best possible solvent for a molecule – and it will be equal to 1 at the edge of the solubility sphere – indicating "the worst possible good solvent". On the other hand, a R_A/R_M ratio above 1 predicts insolubility. This comparison can be related to the Flory-Huggins parameter by the equation (Hansen, 2007)

$$\chi_{12} = 0.5 * \left(\frac{R_A^2}{R_M^2}\right) \tag{3.4}$$

Considering this, any good solvent would be expected to have a χ_{12} parameter of 0.5 or lower. Because the term including χ_{12} is the only positive term in Equation (3.1) for the estimation of ΔG^M, and since the other terms on the right-hand side of the equation are all based on the fractions of molecules present, the χ_{12} parameter determines the range of concentrations at which the polymer will be dissolved. A lower χ_{12} means a wider range of polymer to solvent ratios will allow dissolution; a χ_{12} term equal to zero would theoretically mean any concentration of polymer could be dissolved in the solvent.

3.3.2 Chemicals

The SHSs in the experimental work of this study – N,N-dimethylbenzylamine (DMBA; ≥99%; CAS 103-83-3), N-ethylpiperidine (EP; 99%; CAS 766-09-6), N,N-dimethylcyclohexylamine (DMCHA; 99%; CAS 98-94-2), 2-(dibutlyamino)ethanol (DBAE; 99%; CAS 102-81-8), 2-(diisopropylamino)ethanol (DIPAE; ≥99%; CAS 96-80-0) – were used as supplied by Sigma-Aldrich (Canada). All were 99% purity or greater. PHB pellets, 98-99% wt % PHB with ~1 wt % Si impurities,(Anbukarasu et al., 2015) were used as received (BRS Bulk Bio-pellets, Bulk Reef Supply, Golden Valley, USA). The pellets had a number averaged molecular weight (M_n) of 79,000 ± 1,230 Da, as determined by gel permeation chromatography.

3.3.3 Porous PHB Preparation

Porous PHB was prepared as follows: 0.5 g of PHB pellets were dissolved in 20 mL acetic acid near its boiling point, then allowed to cool overnight. The solution was re-heated to re-dissolve the PHB. 1 mL of solution was then sampled using a micropipette and forcefully ejected into a tray of warm water causing the PHB to precipitate into a porous solid.

3.3.4 Dissolution Experiments

Two types of dissolution experiments were performed: a preliminary visualization of solubilized porous PHB and a quantitative assessment of the solubilization of PHB pellets. Porous PHB was selected for the visualization experiments due to its greater surface area per volume, which facilitates and decreases the time needed for the observation of interactions with the solvents.

In the visualization of solubilized porous PHB, 3 mL of a solvent of interest was added to a test tube containing 5.4-6.1 mg of porous PHB; the test tube was capped and the solution was allowed to sit at room temperature for 48 h before being examined visually for gel formation, observed by an increase in transparency of the PHB. This amount of porous PHB was selected based on preliminary experiments which showed it was appropriate for the observation of polymer-solvent interactions when submerged in 3 mL of solvent. For the second stage of the visualization experiments, 3 mL of de-ionized water was added to each test tube (except for DMBA, to which 10.5 mL of water was added, since DMBA requires a higher water:solvent ratio to switch forms (Durelle et al., 2015), and the solvent was switched to its hydrophilic form by sparging carbon dioxide through a gas dispersion tube. The PHB was then examined visually once more.

For the quantitative solubilization assessment, 2 mL of solvent was added to 5.5-mL test tubes each containing a single pellet of PHB (37.5 ± 7.0 mg). The volume of solvent was chosen based on preliminary experiments showing that it was sufficient to detect dissolution but not enough to fully dissolve a single pellet. The samples were heated using a heating block (Canlab Temp-Blok Module heater, 100 W) and thermometer to control the temperature. The experiments were conducted in a vessel with a constant flow of nitrogen gas to avoid potential oxidation reactions. The vessel and test tubes were purged with nitrogen before being heated to the experimental temperature, where they were held for 5 or 25 h, depending on the experimental condition tested. Normal heating times were approximately 8 min to stabilize at 75°C and 24 min for 127°C. Fluctuations in temperature were ≤ 2°C over the course of the experiments.

After this treatment, the liquid phase was drawn from the test tubes via a glass pipette and passed through a 0.2-µm PFTE syringe filter (Basix, Fisher Scientific) into pre-weighed aluminum weighing dishes. The final volume of solvent was recorded, and the dishes were dried under nitrogen flow at room temperature until the mass of the samples stabilized. The amount of PHB dissolved in the solvent was determined by comparing the additional mass present after drying experimental samples to solvent-only controls to account for any potential impurities present in the solvents. Negative calculated values are due to experimental error and are statistically equivalent to 0 g/L. Each experimental condition was performed in quintuplicate.

3.3.5 Statistical Analysis

For the quantitative assessment of PHB solubilization, Tukey's Honestly Significant Difference test was used to determine if pairs of treatments were solubilizing significantly different amounts of PHB (α=0.05). Treatments were then sorted into nonexclusive groups which had no internal significant differences. Error ranges reported in tables and graphs are ± one standard deviation.

3.4 Results

3.4.1 Theoretical PHB-Solvent Interactions

Flory-Huggins parameters (χ) were calculated for PHB and a group of potentially suitable SHSs in their non-protonated form at 25°C. The Hansen Solubility Parameter model was calculated for each potential solvent using the Stefanis-Panayiotou group contribution method (Stefanis & Panayiotou, 2012) and PHB solubility parameters based on both the Busamante formula ($\chi(1)$) and the barycentric method ($\chi(2)$) from (Jacquel et al., 2007). The Hansen Solubility Parameter model predicts that an χ value at or below 0.5 is indicative of a good solvent (Hansen, 2007); however, it is important to note that this approach has not been previously used for the prediction of interactions with SHSs. Table 3.1 shows the model-predicted ranking of solvent effectiveness in ascending order of $\chi(1)$ and $\chi(2)$ values at 25°C. Toxicity, boiling point, and flash point information are also given.

Table 3.1: Properties of investigated non-protonated SHSs

Switchable Hydrophilicity Solvent	χ(1)[a]	χ(2)[b]	LD50[c] (mg/kg)	Boiling Point[d] (°C)	Flash Point[d] (°C)
N,N-Dimethylbenzylamine (DMBA)*	0.064	0.014	265	183	53
N,N-Dimethylphenethylamine	0.065	0.017	300	210	71
N-Ethylpiperidine (EP)*	0.096	0.152	280	131[e]	17
N-Butylpyrrolidine	0.100	0.170	51^	156	35
N,N-Dimethylcyclohexylamine (DMCHA)*	0.282	0.266	348	159	43
N-Methyldipropylamine	0.397	0.524	267	117	-3
Dipropylamine	0.539	0.682	460	108	17
Triethylamine	0.682	0.894	460	89	-9
N,N-Dimethylbutylamine	0.736	0.897	188	95	-5
N,N-Dimethylhexylamine	0.850	1.128	500	148	34
Ethyl 4-(diethylamino)butanoate	0.863	1.220	7000	220	77
2-(Dibutylamino)ethanol (DBAE)*	1.613	1.569	1070	230	95
2-(Diisopropylamino)ethanol (DIPAE)*	1.882	1.866	940	190	64

[a] χ (1) values with PHB at 25°C were calculated based on the Busamante formula (Jacquel et al., 2007)
[b] χ (2) values with PHB at 25°C were calculated based on the barycentric method (Jacquel et al., 2007)
[c] LD_{50} values from oral administration on rats (Vanderveen et al., 2014)
[d] (Vanderveen et al., 2014)
[e] value as given by supplier
* indicates solvents selected for experimental work
^ indicates LD_{50} value for oral administration in mice

While the values of $\chi(1)$ and $\chi(2)$ differed slightly for all solvents (ranging from 0.064 to 1.882 and from 0.014 to 1.886, respectively), this only impacted the evaluation of one solvent, N-methyldipropylamine, which had an $\chi(1)$ of 0.397 but an $\chi(2)$ of 0.524. Other solvents had both χ values above or below 0.5 and the ranking of the molecules did not differ regardless of which basis was used to calculate χ values. Of the five molecules with both χ values below 0.5, two pairs showed very similar structures: DMBA and N,N-dimethylphenylthylamine, as well as EP and N-butylpyrrolidine. Because of these structural similarities, only one molecule from each pair was selected for further experimental work. DMCHA was the third molecule selected based on its theoretical parameters predicting good solubility and its unique chemical structure. Also included in experimental work were two molecules predicted to be poor PHB solvents: DBAE (with $\chi(1)$ of 1.613 and $\chi(2)$ of 1.569) and DIPAE (with $\chi(1)$ of 1.882 and $\chi(2)$ of 1.866). These were selected to confirm whether predicted poor solvents actually had poor interactions with PHB.

3.4.2 PHB Solubilization

Preliminary solubility experiments were performed using porous PHB exposed to the five candidate SHSs, chloroform (as positive control) and water (as negative control). After 48 h of exposure to the solvents at room temperature, clear differences could be observed by visual inspection (Figure 3.1a). As expected, PHB completely dissolved in chloroform. In DMBA at room temperature, the PHB formed a hazy and translucent gel. In EP and DMCHA, a portion of the PHB became translucent and the remaining PHB solids retained their whitish colour. PHB immersed in DBAE, DIPAE and water was completely opaque and showed minimal, if any, interaction with the solvents. The change in appearance of PHB observed with DMBA, EP and DMCHA was due to the formation of a gel. In each case, the gelation of PHB was reverted when the SHS was switched back to its hydrophilic form, returning the PHB to its original state as an opaque white solid (Figure 3.1b). Aside from the case of chloroform, for which dissolution occurred almost immediately after addition to the PHB sample, no PHB was detected (by assessment of mass) in the liquid phase of the solvents tested.

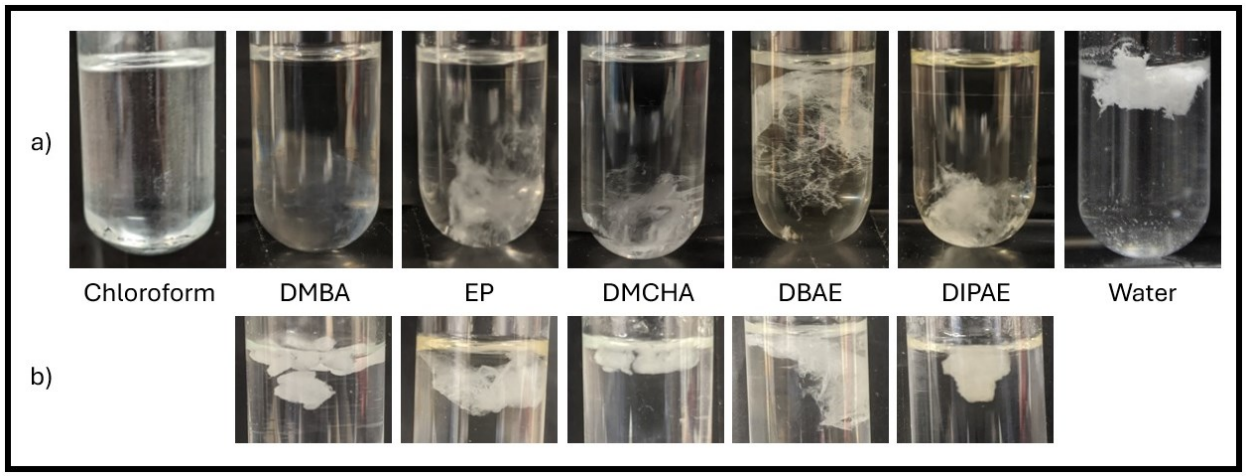

Figure 3.1: Visual demonstration of PHB solubility in SHSs
(a) PHB in chloroform, five different SHSs, and water after 2 days of exposure at room temperature. 5.4-6.1 mg PHB were placed in 3 mL of solvent. **(b)** PHB in the five SHSs after the solvents were reverted to their hydrophilic conformations.

In further assessing the solubilization of PHB, pellet dissolution was carried out at higher temperatures (75, 100 and 127°C) for 5 and 25 h. The solubilized quantity of PHB for each condition is reported in Table 3.2. It is important to note that these values do not represent the maximum solubility under these conditions, but rather the PHB solubilized over the exposure time based on the same initial amount of PHB added.

Table 3.2: Solubilized PHB in SHSs at various conditions.

	Solubilized PHB [mg/mL]				
	Condition 1	**Condition 2**	**Condition 3**	**Condition 4**	**Condition 5**
Temperature	75°C	75°C	100°C	100°C	127°C
Exposure Time	5h	25h	5h	25h	5h
Solvent					
DMBA	-0.07 ± 0.27^{de}	1.45 ± 1.15^{bcde}	-0.31 ± 0.62^{cde}	0.73 ± 1.55^{bcde}	8.29 ± 1.08^{a}
EP	0.26 ± 0.25^{de}	0.33 ± 0.46^{cde}	0.12 ± 0.34^{cde}	NT	4.32 ± 2.71^{abcde}
DMCHA	-0.03 ± 0.13^{de}	0.28 ± 0.08^{d}	0.95 ± 0.14^{bc}	3.14 ± 1.47^{bcde}	11.6 ± 2.31^{a}
DBAE	-0.33 ± 0.57^{cde}	-1.52 ± 2.97^{bcde}	NT	NT	8.12 ± 3.12^{ab}
DIPAE	-0.08 ± 0.12^{e}	0.02 ± 0.13^{de}	-0.03 ± 0.09^{e}	0.07 ± 0.13^{de}	8.55 ± 0.42^{a}

Values are given as average of 5 samples (n=5) ± one standard deviation.
Values sharing the same letter superscript are considered statistically equivalent based on Tukey test ($p<0.05$).

DMBA solubilized PHB to a concentration of 8.29 ± 1.08 mg/mL at 127°C after only 5 h, but significantly smaller amounts of PHB (between 0.73 ± 1.55 and 1.45 ± 1.15 mg/mL) were dissolved after 25 h of exposure at 100°C and 75°C, and no solubilization was observed after 5 h of exposure at these temperatures. In EP, PHB was solubilized after 5 h at 127°C to a concentration of 4.32 ± 2.71 mg/mL but limited solubilization was observed under the other conditions tested (the highest amount was 0.33 ± 0.46 mg/mL after 25 h of exposure at 75°C). Solubilization could not be assessed after 25 h at 100°C because most of the EP evaporated. Exposure to DMCHA for 5 h at 127°C led to the highest amount of solubilized PHB (11.6 ± 2.31 mg/mL). Exposure at 100°C led to 0.95 ± 0.14 mg/mL dissolved PHB after 5 h (the only solvent displaying significant solubilization under this condition) and 3.14 ± 1.47 mg/mL after 25 h. In fact, DMCHA was able to partially solubilize PHB under every condition tested except for 5 h exposure at 75°C. The only condition for which solubilized PHB was observed in DBAE and DIPAE was after 5 h at 127°C (8.12 ± 3.12 mg/mL and 8.55 ± 0.42 mg/mL, respectively). This was the only condition for which all solvents showed measurable levels of solubilized PHB. On the other hand, no appreciable dissolution of the PHB pellet was observed at 75°C, even after 25 h.

DMBA, DMCHA and DIPAE were selected for further testing (EP was discarded because of evaporation after long exposure at elevated temperatures). The solvents were exposed to various amounts of PHB (as pellets; corresponding to incremental increases in surface area) to assess whether the solubilization observed was limited by mass transfer or linked to thermodynamic equilibrium. Figure 2 shows the concentration of PHB solubilized when one to seven pellets were placed in solvents at 100°C for 25 h. As can be seen, DMCHA dissolved significantly more PHB than DMBA and DIPAE – between 1.3-

and 4.3-fold more solubilized PHB than the two other solvents over the range of conditions tested – reaching as much as 25.86 ± 2.57 mg/mL with seven pellets. The data for each solvent follow linear trends with the increasing number of pellets, which implies linear increases with respect to the surface area exposed to the solvents.

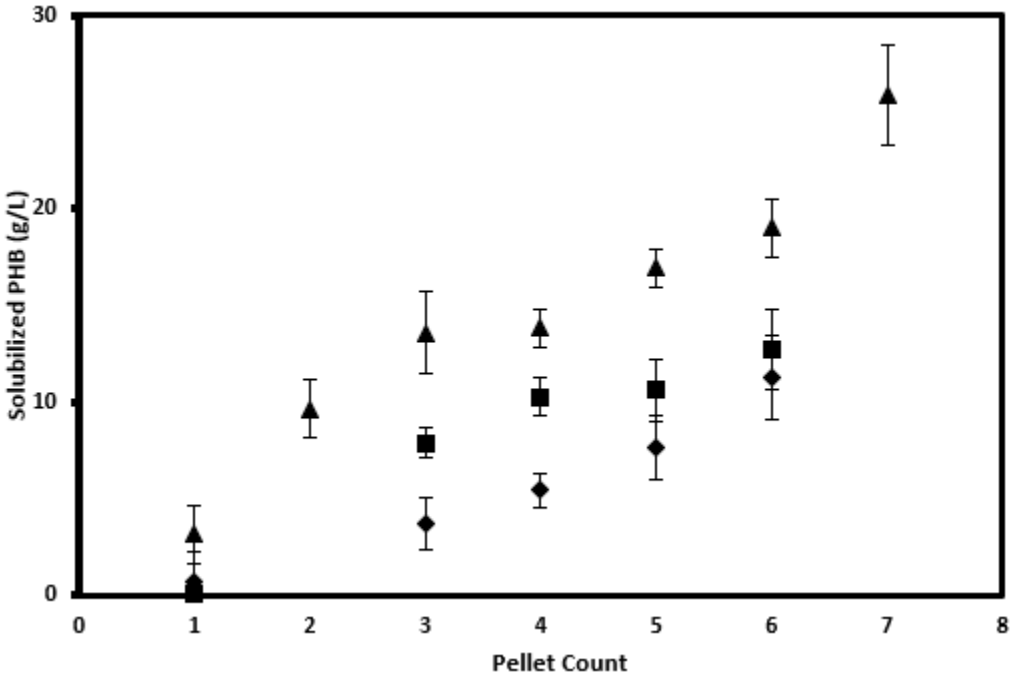

Figure 3.2: PHB solubilization in SHSs
Amount of PHB solubilized after 25 h of exposure at 100°C per initial volume of SHS as a function of the number of PHB pellets. Results are shown for PHB exposed to DMCHA (triangles), DIPAE (squares), and DMBA (diamonds). Error bars indicate ± one standard deviation (n=5).

3.5 Discussion

This work aimed to determine whether SHSs could be suitable for solubilization of PHB. It also aimed to validate if theoretical models developed and used in determining traditional solvent-polymer compatibility could be applied to SHS systems. This was done by selecting three SHSs predicted to be good solvents for PHB and two predicted to be non-solvents based on the results of two forms of the Hansen Solubility Parameter model and comparing their performance under a range of temperatures and exposure times.

The visual assessment of solvent-polymer interactions (Figure 3.1) follows the trend predicted by the theoretical model (Table 3.1). DMBA formed a gel with PHB; EP and DMCHA exhibited limited interaction; and DBAE and DIPAE showed no noticeable interaction. The formation of a gel layer is the first step of polymer dissolution (Miller-Chou & Koenig, 2003) and polymer swelling through solvent uptake is

often used for solubility parameter analysis (Hansen, 2007) The relative performance of EP compared to DMBA (inferior) and DMCHA (comparable) suggests that, of the two sets of χ values calculated for this solvent, the values obtained using the barycentric method ($\chi(2)$) (Jacquel et al., 2007) were better predictors of polymer-solvent interactions. With that in mind, solubility parameter models are generally developed on the basis of either being a good solvent or a non-solvent, and both sets of χ values (obtained using the Busamante or barycentric methods) accurately predicted the general behaviour of the solvents tested. It is interesting that these methods were applicable to the prediction of interactions between polymers and SHSs, considering their particular structures and properties.

The solubilization of PHB pellets at elevated temperatures (Table 3.2) generally followed theoretical predictions, but not as closely as with visual assessment. This could be at least partially attributed to the fact that these predictions were made for systems at 25°C, a much lower temperature than the range tested. DMCHA was the best performing solvent at every condition at or above 100°C, and both DBAE and DIPAE performed well only at 127°C. However, a difference in performance at high temperatures compared to room temperature is not surprising for two reasons. First, the solubilization experiments were time-limited instead of carrying on until saturation. The Hansen Solubility Parameters model is based on thermodynamics, not kinetics (Hansen, 2007), so there is the possibility that the observed superior performance of DMCHA was caused by favourable kinetics in relation to the other solvents. Second, the thermodynamics of solubility can become more favourable for some solvents as temperature increases. Solubility parameter values calculated at 25°C are often considered appropriate for higher temperatures; however, alcohols with a higher δ_{hb} than a polymer can be considered a special case for which increasing temperature can turn a non-solvent into a good solvent through the weakening of hydrogen bonds (Hansen, 2007). The alcohol SHSs (DBAE and DIPAE) both have significantly higher δ_{hb} than PHB, and this effect can explain why they performed well only at the higher temperatures tested.

In the multi-pellet experiments (Figure 3.2), the increasing relationship between the number of pellets added and the amount of PHB solubilized indicates that the solutions had not reached their PHB solubility limits at 100°C. This provides additional context to the efficacy of DMCHA at higher temperatures compared to the other solvents. The fact that saturation was not reached in these experiments indicates that differences in solubility seen at or above 100°C were primarily representative of the kinetics of dissolution – even if thermodynamics still determine if a polymer will dissolve in a given solvent under a specific set of conditions. Interpreting Figure 3.2 and Table 3.2 together suggests that elevated temperatures promoted the solubility of PHB in DBAE and DIPAE and that the cause of the superior performance for DMCHA was likely the kinetics of dissolution. The increasing trend in solubilized PHB

observed for the whole experimental range tested in Figure 3.2 for DMCHA indicates that the maximum solubility is likely greater than 25.86 mg/mL. It should be noted that this value is lower but approaching the solubility of PHB in chloroform, which has been measured from 40-80 mg/mL over a range of PHB molecular weights; however, chloroform dissolution only requires 3 h and a maximum temperature of 70°C (Jacquel et al., 2007). It is also lower than the solubility in acetic acid at 118°C, which has been established at 50 mg/mL after 1 h (Anbukarasu et al., 2016). The maximum solubility of PHB in these SHSs may be quite large at higher temperatures with sufficient time or stirring to improve the kinetics of dissolution. Anecdotal observations relevant to further application of these solvents for PHB processing are based on metastability and the effect of cooling on PHB solutions in DMBA and DMCHA. DMBA tended to form polymer–solvent gels upon cooling to ambient temperature, especially in cases where it was cooled before filtering to remove particles of PHB that were in suspension and not dissolved. In contrast, cooling of DMCHA solutions of PHB produced a precipitated polymer. These differences mean that each solvent may be useful for specific applications. The precipitation of PHB from DMCHA would lead to a simpler extraction protocol, but the gelation that occurs in DMBA could be useful if higher-molecular-weight polymer chains can concentrate in the gel (Tan et al., 1983; Koppe et al., 2009) or if gelation allows further separation from impurities present in the liquid solvent.

3.6 Conclusion

Multiple SHSs able to dissolve PHB over a range of temperatures have been identified. The interactions that these solvents exhibited with PHB could be reversed through switching the form of solvent by sparging of carbon dioxide in the presence of water. The Hansen solubility parameter theoretical model was applied to these molecules to successfully identify good solvents and nonsolvents of PHB at and below 100°C. However, the predictions collapsed at 127°C, where all of the solvents tested were capable of solubilizing PHB. DMBA was the solvent with the greatest uptake of PHB at room temperature, in line with the model's prediction of DMBA being the best solvent for PHB under those conditions; however, DMCHA dissolved the most PHB at high temperatures in time-limited experiments, likely due to faster kinetics. The ability of SHSs to dissolve PHB demonstrates that they have potential as a recyclable way of using solvent-processing methods for PHB. However, the high temperatures required for dissolution (above the flash point for each of the SHSs tested) remain a drawback of this system. Still, this study opens the door to a new class of promising PHB solvents and further work could lead to process development under safer conditions. These SHSs have the potential to significantly improve the economics of PHB production; this will rely on complete processes that minimize solvent losses and operate at lower temperatures, where possible.

3.7 Acknowledgements

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4 Dissolution and Recovery of Poly(3-hydroxybutyrate) in Switchable Solvents and the Formation of a Switchable Gel

4.1 Abstract

Poly(3-hydroxybutyrate) (PHB), a bio-produced and biodegradable polymer, has great potential as a replacement for petroleum-based polymers in many applications. However, strategies for the extraction and processing of PHB still require improvement. Switchable hydrophilicity solvents (SHSs), which can be toggled between hydrophobic and hydrophilic forms by the addition or removal of carbon dioxide in the presence of water, are easily recyclable and may improve PHB processing methods. Here, we have shown the ability to dissolve PHB in two SHSs (N,N-dimethylbenzylamine and N,N-dimethylcyclohexylamine), precipitate PHB by the addition of water and carbon dioxide, and recycle the solvent for subsequent dissolution and precipitation cycles. We have also demonstrated the ability for N,N-dimethylbenzylamine to form gels with PHB which maintain their water/solvent content as the solvent is switched to a hydrophilic form. These results demonstrate the usefulness of SHSs as a recyclable platform for PHB processing and their ability to create unique materials.

4.2 Introduction

As the world grapples with the ecological impacts of the buildup of plastics in the environment, biodegradable polymers have emerged as a potentially important part of the solution. Poly(3-hydroxybutyrate) (PHB), a member of a class of bio-produced and biodegradable polymers known as polyhydroxyalkanoates (PHAs), is one of the preeminent biodegradable polymers being developed for multiple applications. PHB is produced by microorganisms, typically when there is excess carbon but a limitation of other nutrients (Pieja et al., 2011; Carillo et al., 2018) as a storage molecule for carbon and energy. As a common natural polymer, PHB is biodegraded and assimilated by a variety of organisms across ecosystems, which is a significant environmental improvement over currently used plastics, including other bioplastics, many of which do not biodegrade as readily as PHB (Mergaert et al., 1993; Bucci et al., 2007; Meereboer et al., 2020; Solano et al., 2022). An important aspect of PHB production is its recovery from bacterial cells, a process that can be done through mechanical, chemical, or biological steps, or a combination thereof (Kosseva & Rusbandi, 2018; Mannina et al., 2020).

Solvent extraction, in particular, is a common approach (Kosseva & Rusbandi, 2018; Pagliano et al., 2021) However, the range of solvents which can dissolve PHB effectively is limited. While intracellular PHB is in an amorphous state, PHB becomes semicrystalline once it is removed from the bacterial cells (Grage et al., 2009). This semicrystalline structure limits the ability of solvents to penetrate into the bulk of the polymer and liberate polymer chains, especially for larger solvent molecules (Hansen, 2007). Thus, small, chlorinated hydrocarbons such as chloroform and dichloromethane are the most commonly used solvents for PHB dissolution (Jacquel et al., 2008). However, these solvents are harmful to the environment (Kosseva & Rusbandi, 2018) and require energy-intensive distillation to separate them from antisolvents in order to be re-used (Fernández-Dacosta, 2015). In attempts to overcome these drawbacks, a number of recyclable or less hazardous solvents have been studied to dissolve PHB for extraction or processing (Pagliano et al., 2021; Lawley et al., 2022; Z. Li et al., 2023).

Switchable solvents are a type of solvent that can be switched between two different forms through a trigger, such as carbon dioxide, temperature, or pH (Pollet et al, 2011; Jessop et al, 2012; Shih et al., 2015). One category of these is switchable hydrophilicity solvents (SHSs), which can be switched between hydrophobic and hydrophilic forms by adding or removing carbon dioxide in the presence of water (Jessop et al, 2012). These changing solvent properties have the unique advantage of allowing components to dissolve in one form and precipitate when the solvent is switched, removing the need for antisolvents or evaporation (Boyd et al, 2012). This has the potential to reduce the material and energy needs of PHB processing, since the solvent can be recovered and recycled without the need for distillation.

In a previous study, the ability of various SHSs to dissolve PHB was investigated (Lawley et al., 2022). Two switchable hydrophilicity solvents, N,N-dimethylbenzylamine (DMBA) and N,N-dimethylcyclohexylamine (DMCHA), were found to be particularly effective at dissolving PHB. However, PHB tended to form gels with DMBA, a potential complicating factor for processing. A previous study investigating PHB gels using the non-switchable solvents N,N-dimethylformamide and N-methyl-2-pyrrolidine found that they were shear-thinning and reversible (Fabri et al., 1998). PHB gels are typically formed when PHB was dissolved at high temperature at a concentration above its room-temperature solubility, then cooled back down (Fabri et al., 1998; Samorì et al., 2016; Lawley et al, 2022). PHB-(1,2-dichloroethane) and PHB-(γ-valerolactone) gels have been utilized as an intermediate step in high-temperature solvent extraction of PHB from biomass (Werker et al., 2015), and for cleaning paintings (Samorì et al., 2016). However, little is known of the interactions between PHB and DMBA or DMCHA in gels.

In this study, the circular process of PHB dissolution and recovery with the recycling of the SHSs and water was investigated. Additionally, the characteristics of the PHB gels with DMBA and the effects of solvent switching on them were studied.

4.3 Materials and Methods

4.3.1 Chemicals

The SHSs N,N-dimethylbenzylamine (DMBA; ≥99%; CAS 103-83-3) and N,N-dimethylcyclohexylamine (DMCHA; 99%; CAS 98-94-2) were used as supplied by Sigma-Aldrich (Canada). PHB pellets (98-99 wt% PHB with ~1wt% Si impurities) (Anbukarasu et al., 2015) (BRS Bulk Bio-pellets, Bulk Reef Supply, Golden Valley, USA) were used as received or ground (Cuisinart Spice and Nut Grinder) depending on the experiments. The PHB pellets had a weight-average molecular weight (M_w) of 145±6 kDa, as determined by gel permeation chromatography (GPC).

4.3.2 Dissolution and Recovery of PHB with Solvent Recycling

The process of PHB dissolution, recovery and solvent recycling is shown in Figure 1. Ground PHB pellet samples (0.20 g each) were added to test tubes each containing 10 mL of SHS and a PTFE-covered magnetic stir bar. The test tubes were capped, placed in a sand bath on a hot plate-magnetic stirrer (Corning, PC-420D), and held at 127±3°C for 12 h (Figure 4.1 , Step 1). The resulting solvent-PHB mixtures were then vacuum-filtered through a hydrophobic PTFE membrane (Fluoropore 1.0 µm hydrophobic PTFE) (Figure 4.1 , Step 2). The filter was air-dried and the filtrate was transferred to a beaker. Deionized water (50 mL for DMBA, 10 mL for DMCHA) was added to the filtrate and carbon dioxide (99.9%, Linde) was sparged through a gas dispersion tube for 2 h (Figure 4.1 , Step 3). This led to PHB precipitation as the switching process was completed. The mixture was then filtered through a hydrophilic PTFE membrane (Omnipore 0.45 µm hydrophilic PTFE), and the filter was left to air dry (Figure 4.1 , Step 4). The liquid fraction was then transferred to a round-bottom flask, with attached condenser and a sidearm for a gas dispersion tube and placed in a sand bath. This water-hydrophilic solvent mixture was then switched to the original form by heating to 60°C for 2 h while sparging with nitrogen (Figure 4.1 , Step 5). This led to the formation of two fractions (one hydrophobic phase and one hydrophilic phase), which were carefully separated by pipet. The respective recovered volumes were measured.

The above process was repeated three times (three dissolution and recovery cycles) (Figure 4.1, Step 6). Fresh distilled water and solvent were added at the start of each cycle to make up for losses from

the previous cycle, ensuring 10 mL of solvent and either 50 or 10 mL of water for DMBA and DMCHA, respectively, were present at the onset of each cycle.

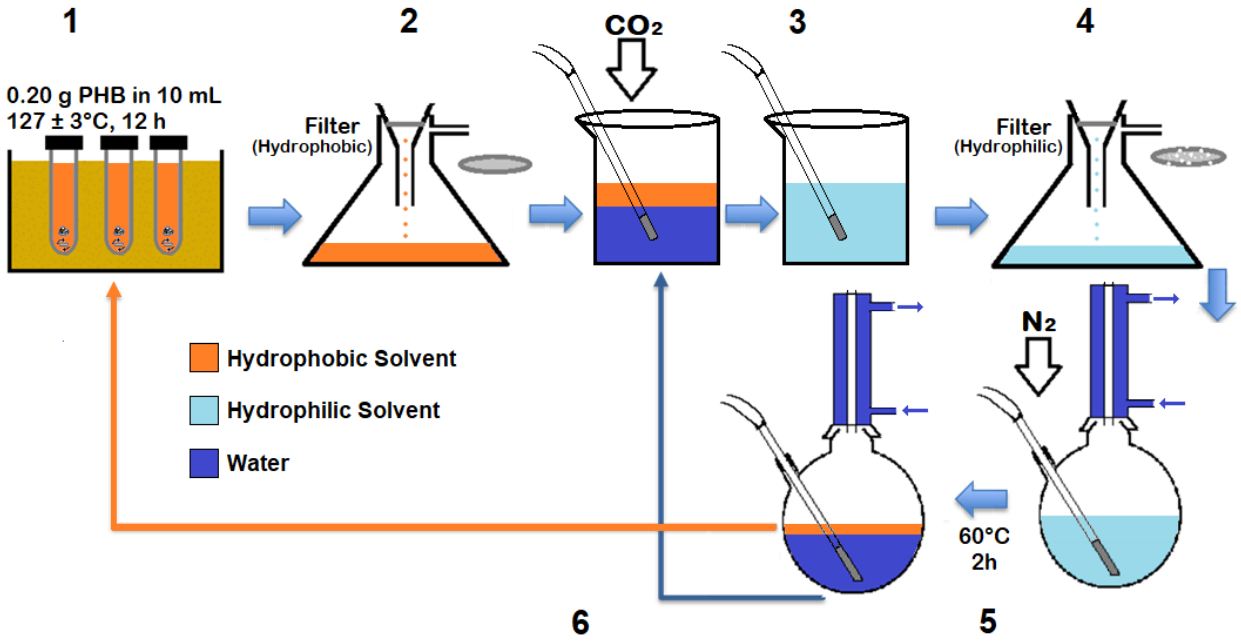

Figure 4.1: Procedure for dissolving and precipitating PHB with SHS and recycling SHS for re-use.
Step 1: PHB dissolution in SHS. **Step 2:** Filtering PHB-SHS solution through a hydrophobic PTFE filter. **Step 3:** Addition of water and carbon dioxide to switch the solvent to the hydrophilic form. **Step 4:** Filtration of hydrophilic SHS-PHB mixture through a hydrophilic PTFE filter. **Step 5:** Heating while sparging nitrogen to switch the solvent back to the hydrophobic form. **Step 6:** Separation of the SHS and water, and recycling back to steps 1 and 3 respectively.

To investigate the effect of the presence of water on the dissolution and recovery of PHB, a further experiment was conducted for a single stage of dissolution and recovery, in which four samples underwent the steps described above (0.20 g PHB in 10 mL solvent), and another four samples were treated with the same process but with an additional 0.25 mL of deionized water added to the solvent prior to dissolution.

Control experiments, in which only the SHSs underwent the three process cycles, were performed to account for any liquid losses during the experiments. All experiments were performed in triplicate, except the tests determining the effect of water, which were performed in quadruplicate. One process cycle replicate was determined to be an outlier based on the modified z-score method of Iglewicz and Hoaglin (1993).

An additional control experiment was performed to determine the pH of water present in the recycle stream. 0.25 mL of water was allowed to sit in a test tube with 10 mL of DMBA or DMCHA for at least 1 h. Afterwards, the water was extracted and its pH determined using a pH meter (Denver Instrument UB-10).

4.3.3 Gel Formation

PHB pellets were added to DMBA and magnetically stirred at 200 rpm while heating in a sand bath placed on top of a hot-plate magnetic stirrer (Corning, PC-420D and PC-620D), with a thermometer to monitor temperature. Temperatures and times for heating ranged from 100-140°C and 2-24 h, respectively. A typical treatment for gel formation was 7 h at 122±2°C at PHB concentrations ranging from 20 g/L to 200 g/L. Once pellets were dissolved (i.e. no longer visible), the solution was removed from the heat source and allowed to cool overnight for gel formation.

4.3.4 Gel Thermoreversibility

The thermoreversibility of the PHB gels was tested by slowly re-heating a gel in a sand bath until it returned to a clear liquid. The end points of the gel dissolution and re-formation were determined based on the absence and reappearance of turbidity in the solution.

4.3.5 Gel Hydrophilicity Switching

To switch the gel from hydrophobic to hydrophilic conditions, a gel sample was added to a beaker, taking care to minimize the shear stresses applied to the gel. Water was added to the gel sample based on the water:solvent ratio typically needed to switch the solvent in the gel (5:1 for DMBA). The gel was then mechanically disrupted using a stainless-steel scoopula while carbon dioxide was added to the mixture through a gas dispersion tube until the end of the switching reaction (indicated by increased bubbling at the surface of the mixture and by the precipitation of PHB).

4.3.6 Assessment of Recovered Products and Liquids

The PHB-solvent gels and liquids from switched gels were assessed by Fourier transform infrared spectroscopy (FTIR; Perkin-Elmer Frontier*,* run in attenuated total reflectance mode over the range 4000-650 cm^{-1}).

The molecular weight of PHB recovered from gels and from solvent solutions was determined by GPC: GPC analyses were run on a Shimadzu instrument equipped with a Shodex SHK-805L (300 x 8 mm,10 µm) analytical column and a SHK-G (10 x 4.6 mm, 8 µm) guard column. 2-mL samples were filtered using a syringe-filter type Puradisc 13 H-PTFE (0.2 µm, Whatman) when transferred into the autosampler vials. The mobile phase was high performance liquid chromatography-grade chloroform that was delivered at a flow rate of 1 mL/min using a LC-20AR pump and the signal of the refractive index detector (RID-20A) was analyzed and compared against a calibration curve. Injection volumes (30 µL for standards, 50 µL for

samples) were delivered with a SIL 20AC autosampler unit. Polystyrene standards with narrow molecular weight in the range from 266 to 1 x 10^6 g/mol were used for calibration. The temperature of the column oven was maintained at 40°C. Data analyses were performed with Lab Solution – GPC Postrun software.

In the case of the dissolution of PHB in DMCHA in the presence of water, the recovered liquid samples were analyzed for the presence of 3-hydroxybutyric acid. The liquid samples were subjected to methanolysis and analyzed with gas chromatography, as follows: Samples were added along with 2mL chloroform, 1mL methanol, and 1mL of acidified methanol (consisting of 24mL methanol, 1.5 mL sulfuric acid, and 1mL of a benzoic acid standard solution) to a screw-capped test tube using PTFE tape to ensure a tight seal. They were added to a boiling water bath for 5 h. Afterwards, the tubes were allowed to cool before 1 mL of water was added and the mixture was vortexed and allowed to settle for phase separation. The bottom (chloroform) phase was analyzed by gas chromatography using the peak area ratio of the methyl ester of the PHB monomer (methyl 3-hydroxybutyrate) and the internal standard (methyl benzoate). A HP 5890 gas chromatograph was used with a DB-5 column (30m x .25mm ID, 0.25µm coating), helium carrier gas at 1.5 mL/min, 250°C injection, 300°C flame ionization detector, oven temperature held at 80°C for 1 min, ramping 10°C/min to 120°C then 30°C/min to 270, held at 270°C for 5 min. The retention times were 2.8 minutes for methyl 3-hydroxybutyrate and 5.5 minutes for methyl benzoate.

4.4 Results

4.4.1 Dissolution and Recovery of PHB with Solvent Recycling: DMBA

The ability of DMBA to dissolve and precipitate PHB was evaluated by passing the hydrophobic and hydrophilic DMBA solutions through filters to separate any undissolved material at the different steps of the process. The hydrophobic filter collected PHB as a colourless PHB-DMBA gel, which was in part due to cooling of the solution as it was being filtered. After filtering, the solution remained clear until water and carbon dioxide were added. Dissolved PHB that passed through the hydrophobic filter was recovered by precipitation on the hydrophilic filter. Subsequent cycles used the recycled solvent (with added fresh solvent to restore the volume) to dissolve a new batch of PHB. It is also important to note that some of the 0.20 g of PHB used in each experiment was not dissolved and remained in the initial test tube.

Figure 4.2A shows the amount of PHB collected on each filter with DMBA as the solvent for the process performed over three cycles. The amount of PHB recovered by precipitation from 10 mL of DMBA (Figure 4.1 Step 4) ranged from 24±2 mg in the first cycle to 41±4 mg in the last cycle, and the amount collected on the first filter (Figure 4.1 Step 2) ranged from 7±3 mg to 95±48 mg, which was primarily PHB-DMBA gel but contained some undissolved granules. Figure 4.2B shows the total amount accounted for in

the precipitated PHB and undissolved PHB fractions for each cycle. The bulk of the PHB was undissolved (87±2% in the first cycle and 74±3% for cycles 2 and 3), either collecting on the hydrophobic filter or left undissolved in the test tube. The total amount of PHB accounted for ranged from 99±1% in the first cycle to 94±3% in the third cycle.

It should be noted that no visual difference was observed in the DMBA over the three processing cycles; it remained clear and colourless throughout.

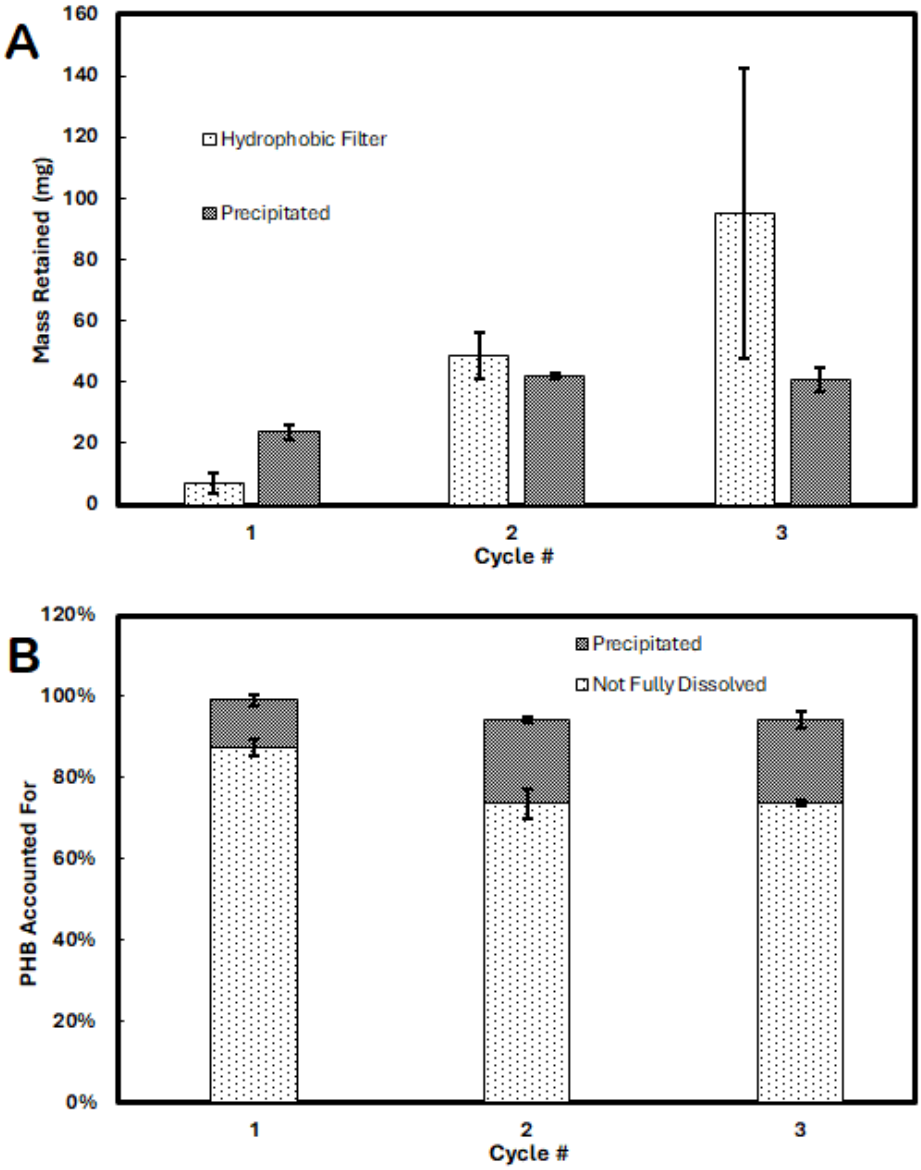

Figure 4.2: PHB recovery with DMBA
PHB recovery with DMBA over three cycles of PHB dissolution/PHB recovery/solvent recycling for the process described in Figure 4.1. **(A)** Amount of PHB collected on hydrophobic filter after dissolution in hydrophobic DMBA and as precipitate after switching to hydrophilic DMBA. **(B)** Amount of PHB accounted for in each cycle. This includes PHB collected on the two filters (Figure 4.1 Step 2 and Step 4), undissolved PHB that remained in the initial test tube (Figure 4.1 Step 1), and PHB rinsed from the glassware used. Error bars represent the standard deviations of the individual segments of the bar (n=3; n=2 for cycle 3 as one outlier is removed).

Figure 4.3A shows the amount of DMBA recovered from each cycle, and Figure 4.3B shows the correlation between the recovered solvent and the amount of PHB collected on the hydrophobic filter (Figure 4.1 Step 2). As can be observed, there is a decreasing amount of solvent recovered with each subsequent cycle, which correlated with an increase in PHB collection as a PHB-DMBA gel on the hydrophobic filter. This is likely due to the amount of solvent entrained in the PHB-DMBA gel that is collected and then dried on the hydrophobic filter. At the scale investigated, a significant portion of the solvent losses were due to the solvent remaining on the glassware, with the control experiment carried out with DMBA alone resulting in 8.9±0.7 mL of solvent recovered out of 10 mL initially added to the process.

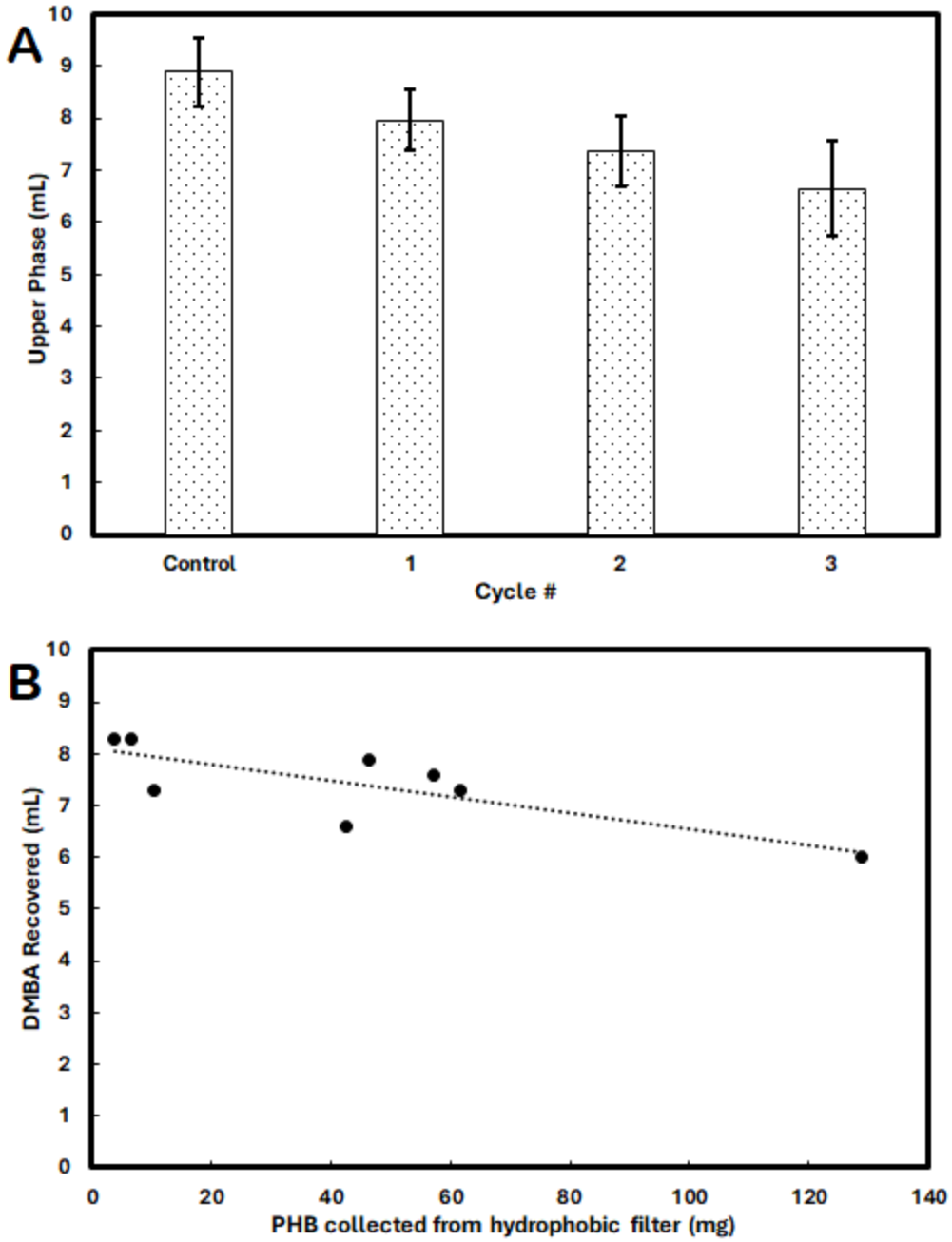

Figure 4.3: DMBA recycling after PHB dissolution
DMBA recovery for recycling over three cycles of PHB dissolution/PHB recovery/solvent recycling for the process described in Figure 4.1. **(A)** Amount of DMBA recovered at the end of each cycle. Note that fresh DMBA was added to the recovered DMBA at the beginning of each cycle to make up the starting volume to 10 mL. The control experiment consisted of conducting the experiment with DMBA alone. **(B)** Amount of DMBA recovered in each trial as compared to the amount of PHB collected from the first (hydrophobic) filter of that trial. The correlative line has an R^2 value of 0.67 with the equation y = -0.015x + 8.1.

4.4.2 Dissolution and Recovery of PHB with Solvent Recycling: DMCHA

Figure 4.4A shows the amount of PHB recovered on each filter when DMCHA was used as solvent over three process cycles, while Figure 4B shows the total accounted for as precipitated PHB and

undissolved PHB for each cycle. In the first cycle, most PHB was collected on the hydrophobic filter (Figure 4.1 Step 2). Based on visual observations, unlike in the case of PHB dissolved in DMBA, the PHB retained on the hydrophobic filter with DMCHA did not form a gel. Moreover, after filtration, the PHB precipitated while cooling to room temperature; another difference from the PHB-DMBA system. Furthermore, a very small portion of PHB was recovered from filtration in the second and third cycles. Visual observation showed that, after three cycles, the recovered DMCHA had a faint shade of orange.

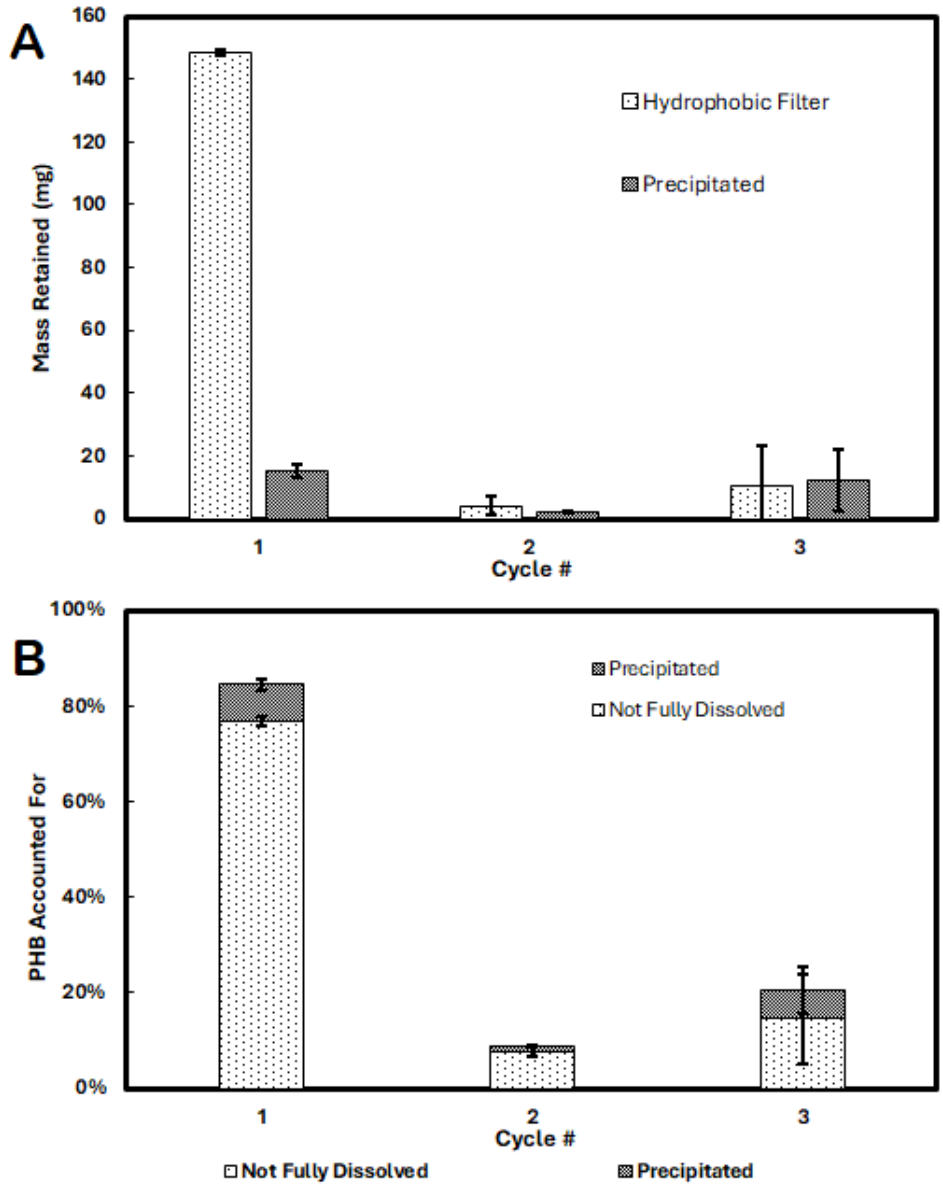

Figure 4.4: PHB recovery with DMCHA
PHB recovery with DMCHA over three cycles of PHB dissolution/PHB recovery/solvent recycling for the process described in Figure 4.1. **(A)** Amount of PHB collected on hydrophobic filter (Figure 4.1 Step 2) after dissolution in hydrophobic DMCHA and on hydrophilic filter (Figure 4.1 Step 4) after switching to hydrophilic DMCHA. **(B)** Amount of PHB accounted for in each cycle. This includes PHB collected on the two filters, undissolved PHB that remained in the initial test tube, and PHB rinsed from the glassware used. Error bars represent the standard deviations of the individual segments of the bar (n=3).

4.4.3 Impact of Water on PHB Dissolution

Figure 4.5 shows how the presence of water during PHB dissolution affected the process with fresh DMBA and DMCHA. With DMBA (Figure 4.5A), both the PHB retained on the hydrophobic filter (Figure 4.1 Step 2) and the PHB recovered on the hydrophilic filter, (Figure 4.1 Step 4) were significantly more abundant when PHB was dissolved in the presence of water. In the case of DMCHA (Figure 4.5B), the presence of water during dissolution greatly decreased the amount of PHB collected on the first (hydrophobic) filter but had a negligible effect on the amount of PHB recovered by the ensuing precipitation. The total amount of PHB accounted for in each case is shown in Figure 4.5C. The amount of PHB accounted for was lower in both solvents when water was present during dissolution, but this was especially true for DMCHA, where the amount of PHB was only 39% after dissolving with water present. With DMCHA there is far more PHB in the recovered water, likely due to polymer degradation into oligomers. No significant PHB was found in the recovered SHSs.

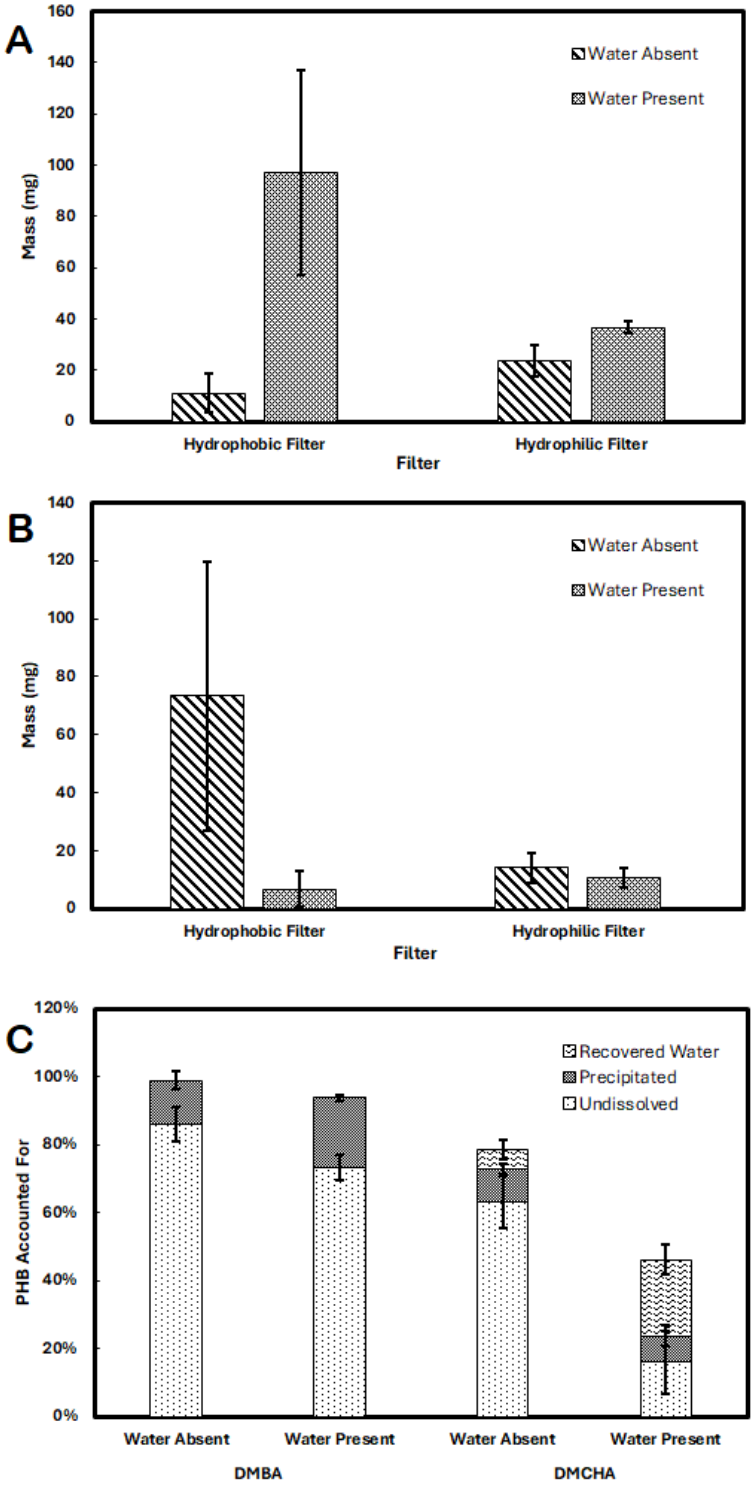

Figure 4.5: Impact of water on PHB dissolution in DMBA and DMCHA
(A) PHB recovered on each filter (Figure 4.1 Steps 2 and 4) with fresh DMBA in the presence and absence of water. **(B)** PHB recovered on each filter with fresh DMCHA in the presence and absence of water. **(C)** Amount of PHB accounted for after dissolution and recovery with DMBA and DMCHA in the presence and absence of water during dissolution (Figure 4.1 Step 1). This includes PHB collected on the two filters, undissolved PHB that remained in the initial test tube, and PHB rinsed from the glassware used. Error bars represent the standard deviations of the individual segments of the bar (n=4).

Figure 4.6 shows the change in the molecular weight of PHB through the dissolution and recovery process in DMBA and DMCHA. The molecular weight of the PHB recovered by precipitation was less than 10% that of the original PHB for all conditions tested. The PHB dissolved and precipitated from the SHSs was reduced to M_W of 10.4±0.7 kDa for DMBA, and 1.5±0.1 kDa for DMCHA, reductions of 93% and 99%, respectively. Degradation was greater when water was present during PHB dissolution, with the M_W of PHB recovered from DMBA in that process being only 2.8±0.3 kDa. There was no data for DMCHA when water was present during the dissolution stage because not enough PHB was recoverable to assess molecular weight. Also of note, PHB collected as a gel on the hydrophobic filter (Figure 4.1 Step 2) had a higher M_W than the PHB which was successfully filtered and precipitated. PHB which was left undissolved (Figure 4.1 Step 1) had an even higher M_W, although it was far less than the original material. A control with PHB dissolved in chloroform at its boiling point of 61°C and precipitated by evaporation (not filtered) showed no change in M_W.

The basic character of the SHSs may be a contributing factor to the polymer degradation observed. The pH of the process water was tested after being in contact with 10 mL of DMBA and DMCHA for 1 h, and was found to be 10.5 and 11.6, respectively. A control experiment, consisting of water with sodium hydroxide at pH 10 and 100°C for 12h, experienced an 18% decrease in M_W to 118.2±4.6 kDa.

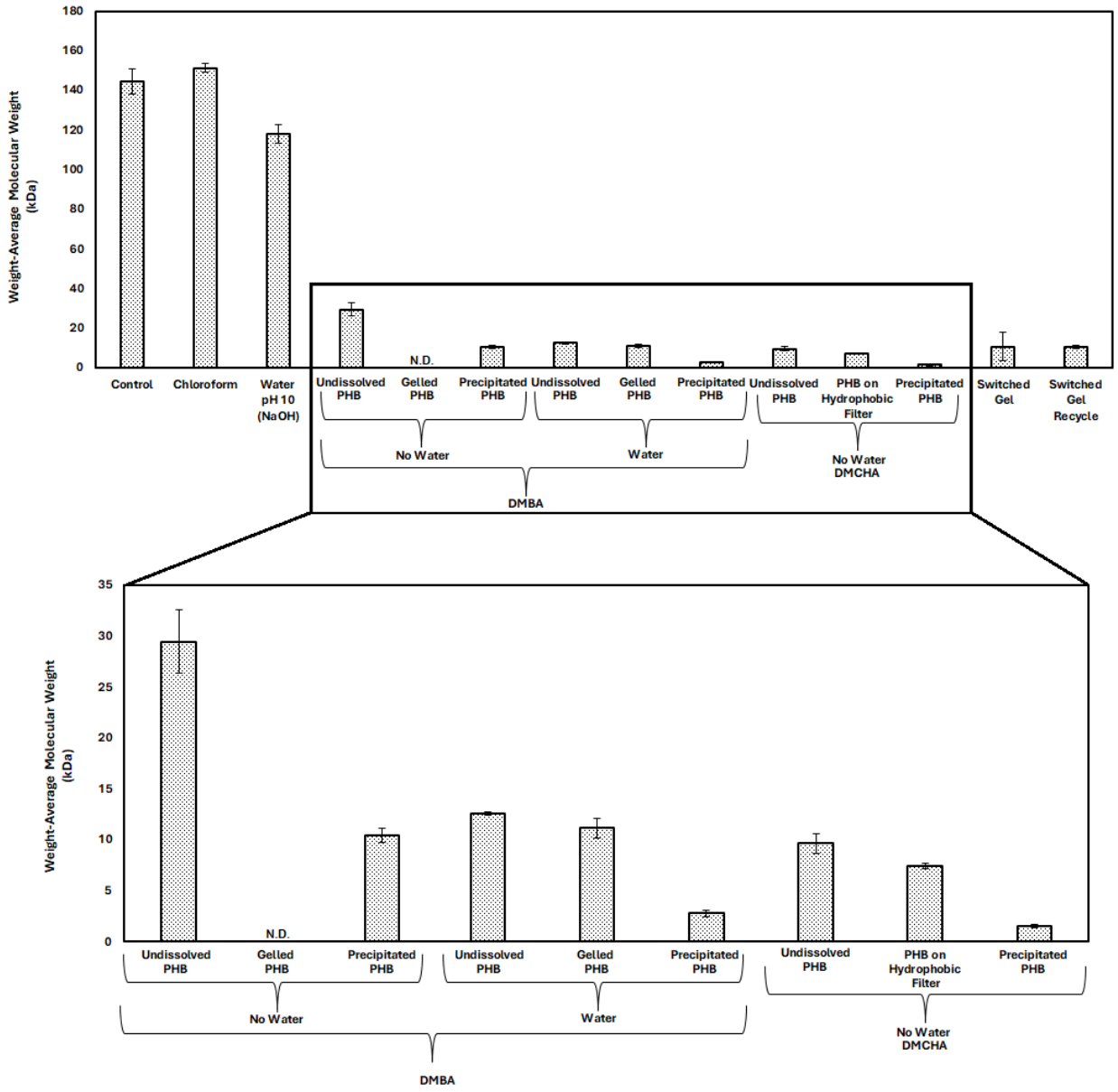

Figure 4.6: Effect of dissolution and recovery on PHB molecular weight
Weight-average molecular weight (M_W) of PHB recovered by dissolution and recovery with DMBA and DMCHA as solvents. The control consists of the starting PHB material dissolved in chloroform. No data is available for gelled PHB using DMBA without water present due to the low quantity available, or from any stage of the process after dissolution in DMCHA with water present.

4.4.4 The Formation of a Switchable Gel

PHB-DMBA gels were made by dissolving PHB in DMBA over a range of PHB concentrations and allowed to cool overnight. At lower concentrations (at or below 40 g/L) the gels typically formed a porous solid matrix, out of which liquid could be drained, and stirring the gel returned it to a liquid. At higher concentrations (100 g/L and 200g/L) the gels could be inverted to no effect and did not flow easily after vigorous stirring. It was found that the gels at 100 g/L were thermoreversible and could be returned to a

clear DMBA-PHB solution by heating up to 125°C (first becoming a turbid liquid at ~100°C), before setting back to a gel when allowed to cool to room temperature. These PHB-DMBA gels had a lower density than water. A typical PHB-DMBA gel is shown in Figure 4.7A.

It was found that the addition of carbon dioxide and water to a PHB-DMBA gel sample could switch the solvent back to its hydrophilic phase. This process could be observed as the PHB changed from a translucent gel (Figure 4.7A) to a white precipitate (Figure 4.7B). The monophasic liquid could then be subjected to the process of heating while sparging with nitrogen to recover DMBA to its hydrophobic form and water, which could be re-used to create further switchable gels. In the case of some larger PHB gel pieces, the outer layer would turn white, but the inner core would remain colourless. After switching the gel, the PHB "switched gel" would retain a great deal of liquid content until it was dried (averaging 82% of the gel by weight, based on drying tests) and was soft and malleable until dried. The switched gel could be formed into molds and dried, and the general form would be held as shown in Figure 4.7C. Once dried, the PHB would not re-uptake water or the hydrophilic switched solvent.

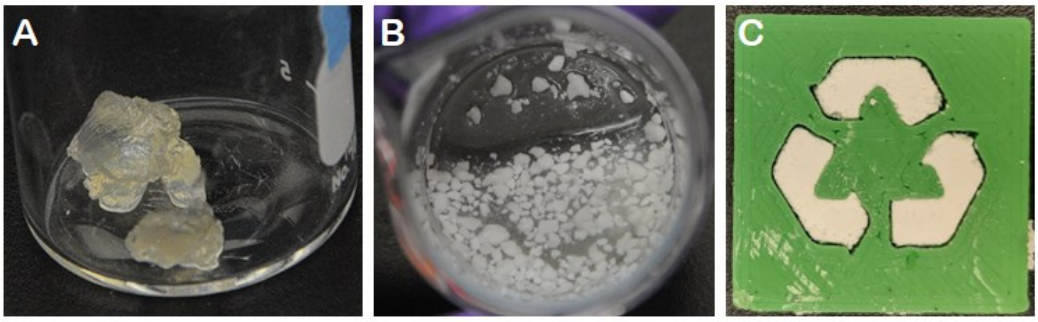

Figure 4.7: Forms of PHB-DMBA gels
(A): A PHB-DMBA gel with the solvent in the hydrophobic form. **(B)** A switched gel; PHB-DMBA gel after exposure to water and carbon dioxide converted the SHS to the hydrophilic form. **(C)** A switched PHB-DMBA gel which was put into a mold (green) and dried, retaining the molded shape.

4.4.5 FTIR Analysis of PHB-DMBA Gel, Switched Gel, and Recovered Liquids

FTIR was used to determine the presence of DMBA and PHB in the gel, in the hydrophilic switched gel, and in the recovered liquids after switching the solvent back to its hydrophilic form. Figure 4.8A shows the FTIR spectra of DMBA, PHB, PHB-DMBA gel and switched gel. A peak at 1750 cm^{-1} was characteristic of PHB while the peaks in the 2800-2900 cm^{-1} range were characteristic of DMBA. It is notable that these latter peaks were present in the PHB-DMBA gel but were barely visible in the spectrum of the switched gel. This suggests that while DMBA was an important component of the PHB-DMBA gels, only a small amount of DMBA remained in the switched gels. Figure 4.8B shows the spectra for recovered water and DMBA. The recovered water did not show any deviation from the typical water FTIR spectrum (Wallace, n.d.) but the recovered DMBA spectrum displayed a peak at 1750 cm^{-1}, which is indicative of the presence of PHB in

the switched gel and in the solvent after precipitation. Hence, some PHB partitioned into the DMBA after it was switched back to its hydrophobic form.

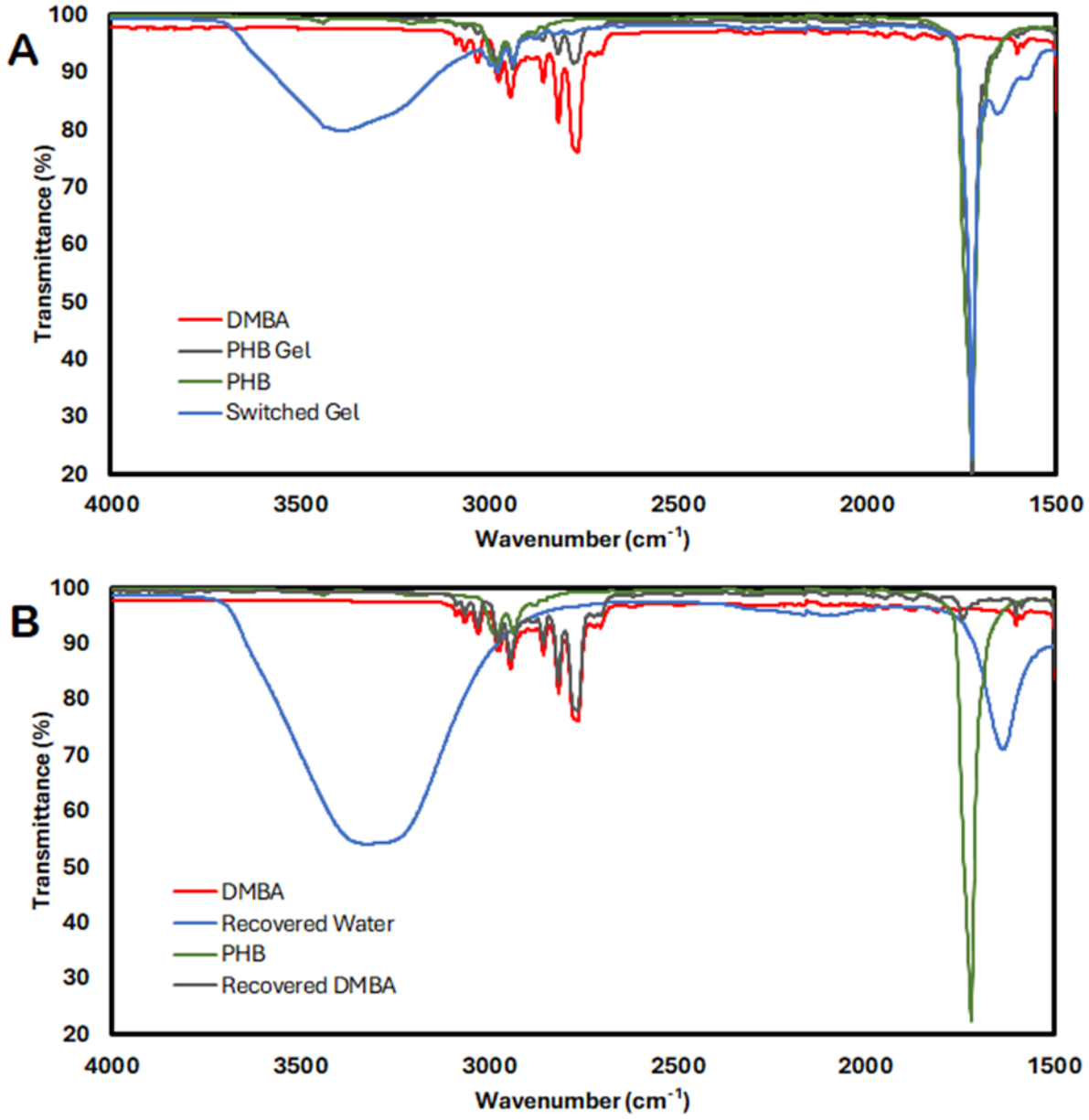

Figure 4.8: FTIR analysis of PHB-DMBA gel and recovered liquids
(A) Spectra of the PHB-DMBA gel and the switched gel overlain beside the spectra of DMBA and PHB. **(B)** Spectra of the recovered water and DMBA overlain beside the spectra of DMBA and PHB. FTIR spectra covering the range of 4000-650 cm^{-1} are available in Appendix A.

4.5 Discussion

4.5.1 Dissolution and Recovery of PHB with SHS Recycle

PHB was dissolved in DMBA in its hydrophobic form and recovered by precipitation through switching the solvent to its hydrophilic form, then the solvent was recovered and recycled by switching it

back to its hydrophobic form (Figure 4.1). The amount of PHB accounted for over three cycle of this process (Figure 4.2B) indicates that there was very little PHB retained in the hydrophilic form of the solvent after precipitation. This also indicates that subsequent increases in the amount of PHB recovered in each cycle was not due to carryover of PHB in the recovered solvent or water. The decrease in undissolved PHB when recycled solvent was used (Figure 4.2A) is surprising, but tests on fresh DMBA indicate that the presence of water in the recycled solvent (Figure 4.8B) could increase the amount of PHB dissolved per volume of solvent (Figure 4.5A). The absence of visual changes in the DMBA and the improved PHB dissolution in recycled DMBA indicate that it has potential as a recyclable solvent for PHB.

While PHB could be dissolved in DMCHA and be recovered by precipitation through switching the solvent (Figure 4.4A), not all PHB could be accounted for in the process (Figure 4.4B). This problem was exacerbated when using the recycled solvent in cycles 2 and 3, in which the presence of water was shown to impede PHB dissolution (Figure 4.5B, 4.5C). Since PHB degradation occurred during dissolution at high temperature (Figure 4.6), DMCHA was found to be unsuitable as a solvent for PHB using the tested process. However, it may still be suitable for lower temperatures where PHB degradation could be limited.

As SHSs rely on water to separate products and must then be recovered from the water to be recycled, carry-over of water within recycled solvent is inevitable, and the performance of the system with water present is integral to a cyclic process. Two key effects of the presence of water while dissolving PHB were observed: increased dissolution and degradation of PHB.

In a previous study (Lawley et al, 2022), it was found that DMBA was more thermodynamically compatible with PHB than DMCHA, but that the latter had better kinetics of dissolution. Here, the presence of water greatly increased the amount of PHB dissolved in DMBA, likely due to overcoming the kinetic limitations seen previously for this system. The thermodynamic compatibility of DMBA with PHB is demonstrated by the filtered solution being stable with dissolved PHB, whereas in DMCHA the solution continued to precipitate while it cooled after filtering at lower concentrations.

Partial PHB hydrolysis is likely the primary cause of increased PHB dissolution in the presence of water, as polymer solubility is inversely related to the length of a polymer chain (Jacquel et al, 2007). The observation of smaller chains being recovered as precipitate and larger chains remaining undissolved further supports this. There may also be an effect from the presence of water improving the performance of the larger SHS molecules. Because of its small molecular size, water is able to assist in the dissolution of polymers by penetrating into the polymer structure, softening the polymer and helping other molecules diffuse into it (Devotta & Mashelkar, 1997; Hansen, 2007). These factors may account for some of the difference observed between the amount of PHB dissolved when water is present or absent from the DMBA dissolution mixture (Figure 4.5).

Importantly, PHB underwent a significant level of hydrolysis, observed as a reduction in molecular weight, in the two different solvents, especially in the presence of water (Figure 4.6). While PHB could be recovered from DMBA, less than half of the starting PHB was recovered from DMCHA when dissolved in the presence of water. In the latter case, GC analysis (Figure 4.5C) confirmed that some portion of the uncollected PHB was retained in the water. This is likely due to PHB hydrolysis to oligomeric chains, which have higher water solubility. As hydrolysis of PHB can be catalyzed by acidic or alkaline conditions (Kučera et al., 2019; Momeni et al., 2023) , the basic environment created by the SHSs in residual water was the likely cause for the degradation observed, and the higher pH of water in DMCHA likely led to even higher degradation.

The molecular weight of the polymer chains had a significant impact on their fate in the process (Figure 4.6). In each condition, any PHB that was precipitated and collected on the hydrophilic filter (Figure 4.1 Step 4) had lower M_W than PHB which was left undissolved or collected on the hydrophobic filter (Figure 4.1 steps 1 and 2). Additionally, for DMCHA, PHB which had been left undissolved had a larger M_W than the PHB which was collected on the hydrophobic filter. The sharp decrease in M_W would have a significant negative effect on the mechanical properties of any PHB processed using these methods.

Since most products require high-molecular weight PHB, its extraction by DMBA or DMCHA would need to be improved to reduce degradation in order to be effective for most applications. However, low-molecular weight PHB has been shown to be useful as an additive to give more flexibility to PHB products (Hong et al., 2013) or in the preparation of block copolymers (Yu & Marchessault, 2000). For those applications the hydrolysis of PHB would not be a drawback, but further study would be useful in order to control the rate of degradation. It is likely that amorphous or lower crystallinity PHB, as is its native state in bacterial cells, would fully dissolve under less intense processing conditions (lower temperatures, shorter exposure times) and could therefore undergo less degradation. PHA copolymers, which typically have lower crystallinity even at small comonomer content (Chernozem et al., 2022), may also be able to be treated under less intense conditions, although they were not tested here. Since SHSs could extract PHB from wet bacterial biomass, bacterial growth media may also affect PHB extraction efficiency. In fact, media composition will likely impact hydrolysis due to the buffering capacity of many growth media and the effects of salinity on polymer hydrolysis.

4.5.2 Gel Characteristics and Switching

The PHB-DMBA gels which formed upon cooling of concentrated solutions were studied for their formation and the ability to switch hydrophilicity while maintaining gel structure. The primary differentiating

factor of whether a gel would form upon cooling and the character of that gel was the concentration of PHB in solution. The thermoreversibility, complete resistance to flow at low shear, and shear-thinning behaviour were similar properties to those of PHB-dimethylformamide gels examined by Fabri et al. (2007). Those authors concluded that the behaviour observed was caused by non-covalent intermolecular forces occurring within the polymer network. It is likely a similar behaviour would take place here.

A unique property of the PHB-solvent gels investigated in the present study is the fact that they were formed with a SHS, and the hydrophilicity switch could be trigger while in gel form without disrupting its structure. In some cases, water can act as a plasticizer and prevent crystallization of PHB (Grage et al., 2009) and it is possible that a plasticizer interaction with the water and switched solvent allowed the switched gels to maintain their soft malleable characteristics until being dried out. However, the fact that hydrophobic PHB-DMBA gel persisted at the core of larger particles shows that the solvent switching only occurred down to a certain depth in the gel particle. This implies the existence of mass-transfer limitations on the gel-switching process that could be caused by the carbon dioxide not penetrating into the gel particles when sparged through the solution, and a limited diffusion rate of polar SHS molecules into the gel. Determining the stability of the gel if the SHS was eventually replaced entirely by water would require further work. FTIR results indicated that some DMBA remained in the gel after switching and rinsing with carbonated water. If the DMBA in the switched gels can be completely replaced with water or another benign solvent while retaining its physical properties, then there is significant potential for the creation of useful PHB gels with this method.

The recovery and recycling of the DMBA from the gel indicates that the solvents can still be recycled in this process, although it may not be as useful due to the large amount of liquid retained in the gels. A process which aims to create gels and recycle the solvent would need further optimization of the solvent recovery from the gel. The presence of PHB in DMBA recovered from the gels (as determined by FTIR) also indicates some carry-over in the solvents, however in a closed system with solvent recycling that PHB would not be lost.

The moldability of the gels is a potentially useful characteristic that still requires refinement to overcome mass and volume loss while drying (Figure 4.7). It is possible that increased mechanical pressure on the gels could form it into shape while expelling excess liquid from the gel, reducing the effects of drying on the final shape. Similarly, even with the primitive methods for shape-forming tested herein, a partially dried gel may be able to conform in the mold as a way to limit the amount of mass and volume loss that occurs after the shape is cast.

4.5.3 Conclusion

Two SHSs, DMBA and DMCHA, were tested for their ability to dissolve PHB as well as the feasibility of recycling them for PHB extraction and recovery. It was found that DMBA was a good candidate for this process, however significant decreases in PHB molecular weight over the process represents a significant drawback. The quantity of polymer dissolved was improved by the presence of small amounts of water in the solvent, however this was at the cost of greatly decreased polymer molecular weight. Additionally, smaller polymer chains were more likely to be dissolved and pass through the first filter to be precipitated later. While DMCHA was also able to dissolve and precipitate PHB, the presence of water caused the PHB to hydrolyze extensively, making it unsuitable for this application. While studying the properties of DMBA-PHB gels that formed upon cooling, it was found that the solvent could be switched back to its hydrophilic form while maintaining the gel form, creating a hydrophilic gel. These gels were able to be molded into shape and dried. Taken together, these results indicate that DMBA has good potential as a recyclable solvent for processing PHB in multiple ways, but these processes should be studied in more detail with a focus on determining if the decrease in PHB molecular weight can be limited or accurately controlled.

4.6 Acknowledgements

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5 Extraction of Poly(3-hydroxybutyrate) from *Methylocystis* sp. Rockwell Using Switchable Hydrophilicity Solvents

5.1 Abstract

Poly(3-hydroxybutyrate) (PHB) is a bioproduced and biodegradable polymer with good properties as a potential replacement for many non-biodegradable plastics. It is a carbon and energy storage molecule produced by a wide range of microorganisms and can be biologically synthesized from a variety of feedstocks, including through the bioconversion of methane by methanotrophic bacteria. PHB is stored intracellularly and its extraction from bacterial biomass remains a costly challenge, both economically and in terms of energy and material use. Switchable hydrophilicity solvents (SHSs) are solvents which can be switched between a hydrophobic and a hydrophilic form, which allows for the separation of an extracted product without the need for antisolvents or evaporation. They have strong potential for use as recyclable solvents for biological extractions. In this study we investigated several approaches for the use of SHSs to extract PHB from methanotrophic bacteria and have demonstrated a process that allows for the room-temperature extraction of PHB from unconcentrated biomass directly harvested from bioreactors with recovery and re-use of the solvent. This extraction process removes the need for dewatering and the energy requirements of distillative solvent recovery that burden many other solvent-based PHB extraction procedures, opening the door to more environmentally friendly and cost-effective PHB recovery.

5.2 Introduction

The existence of consumer plastic products is a given in modern life, and the presence of cheap plastic products is a key factor of our quality of life. However, the widespread use of plastics leaves its mark on the environment. Used plastic products are constantly accumulating in ecosystems around the world, with recycling efforts still failing to address the majority of plastic waste generated (Geyer et al., 2017; Heller et al., 2020, Tumu et al., 2023). Biodegradable polymers can help alleviate this problem as they can act as replacements for current commodity plastics and, in some cases, would not accumulate in the environment.

One of the most promising biodegradable polymers is poly(3-hydroxybutyrate) (PHB), a polymer which is used as an energy and carbon storage molecule by a broad range of microorganisms. One of its

great advantages, from a sustainability perspective, is that it can be biodegraded and metabolized by many organisms in various ecosystems (Braunegg et al., 2004; Mergaert et al.,1993; Bucci et al., 2007). PHB can be produced by microbes from a range of possible feedstocks including sugar (Hanzlíkova et al, 1984; Unaha et al., 2023), fruit and lignocellulosic refuse (Sukruansuwan & Napathorn, 2018; Saratale et al., 2021; Tran et al., 2023) and, most significantly for economic and environmental purposes, from waste methane or methanol (Rostkowski et al., 2012; Carillo et al., 2019; Sharma et al., 2022). Much progress has been made in this area and the valorization of waste feedstocks into PHB has greatly reduced the cost of production (Pagliano et al., 2021; Li & Wilkins, 2020). However, the extraction of PHB from microbial biomass remains an expensive step in terms of material, energy, and cost; hence it is crucial to further improve the efficacy, cost and sustainability of this portion of the process (Pagliano et al., 2021; Saavedra del Oso et al., 2021).

Common methods for PHB recovery include solvent extraction, in which PHB is dissolved to remove it from the non-PHB cell material (NPCM); digestion extraction, in which the NPCM is digested to facilitate the separation of PHB; and mechanical extraction, in which methods such as bead mills or high-pressure homogenization are used to disrupt the bacterial cells and physically separate the PHB granules (Kosseva & Rusbandi, 2018). Typical solvent extraction methods use chlorinated hydrocarbons and require an antisolvent to precipitate the dissolved PHB (Kosseva & Rusbandi, 2018). The volumes required are often quite large, and the solvent and antisolvent must be separated by distillation to be reused, which requires a great deal of energy (Rostkowski et al., 2012; Fernández-Dacosta et al., 2015). Generally, sodium hydroxide, sodium hypochlorite, or sodium dodecyl sulfate are used in digestion extraction, which requires much less energy but doesn't enable the recovery all of digestion agents. Much effort has been made in recent years to develop solvents or digestion agents that lower energy and material requirements or that are less environmentally harmful if/when released (Pagliano et al., 2021). Mechanical disruption typically requires less material input but often encounters poor recovery, high capital costs, and large energy costs for cooling the machinery (Kosseva & Rusbandi, 2016, Mannina et al., 2020). These extraction methods have also been combined to improve extraction, such as by using digestion agents as pretreatment for solvent extraction or mechanical disruption preceding NPCM digestion (López-Abelairas et al., 2015; Aramvash et al., 2017; Zou et al., 2023). Many recent studies have investigated non-traditional extraction methods, such as the use of ionic liquids, liquid-liquid separation or the use of macroscopic organisms for NPCM digestion (Leong et al., 2017; Dubey et al, 2018; Zainab-L & Sudesh, 2019; Murugesan et al., 2021).

A technology that has the potential to significantly improve PHB extraction is switchable hydrophilicity solvents (SHSs). These solvents are normally hydrophobic but can be switched to a polar

hydrophilic form by the addition of carbon dioxide in the presence of water and return to their hydrophobic form through heating or nitrogen sparging (Jessop et al., 2012). Because switching of the solvent can cause a change in solubility of products, such as PHB, SHSs can act as their own antisolvent. The switching process thus allows for a simple solvent recovery step with greatly reduced energy requirements (Boyd et al, 2012). These lower energy requirements along with easy recyclability offer the potential for an economically and environmentally improved PHB extraction process. In fact, a switchable anionic surfactant has been used in a similar approach, in which it acted as a digestion agent for PHB recovery with partial recyclability (Samorì et al., 2015).

The present work explored methods for the direct extraction of PHB from the methanotrophic bacterium *Methylocystis* sp. Rockwell using SHSs. Various parameters were tested for their impact on the process including biomass concentration, water content of the biomass (wet or dry), temperature, choice of solvent, and the history of the solvent (fresh or recycled). The examined conditions ranged from high-temperature extraction from dried biomass to room temperature extraction from unconcentrated bioreactor effluent. Results show the impact of these parameters on PHB recovery and purity and provide a path for the development of more sustainable processes for PHB extraction.

5.3 Materials and Methods

5.3.1 Chemicals

The SHSs N,N-dimethylbenzylamine (DMBA; ≥99%; CAS 103-83- 3), N,N-dimethylcyclohexylamine (DMCHA; 99%; CAS 94-94-2), 2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene, (99%; CAS 7128-64-5) and reference PHB powder were used as supplied by Sigma-Aldrich (Canada). Chloroform, sulfuric acid, methanol, and benzoic acid were used as supplied by Thermo Fisher Scientific (Canada). $MgSO_4 \cdot 7H_2O$ and $MnCl_2 \cdot 4H_2O$ were supplied by Thermo Fisher Scientific (Canada); $CaCl_2 \cdot 2H_2O$, NH_4Cl, $NiCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 6H_2O$, KH_2PO_4, and Na_2HPO_4 were supplied by Sigma-Aldrich (Canada); $FeSO_4$ and $ZnSO_4 \cdot 7H_2O$ were supplied by Acros Organics (Canada); H_3BO_3 was supplied by EMD Chemicals (Canada), Na_2EDTA was supplied by Amresco (Canada); and $Na_2MoO_4 \cdot 6H_2O$ was supplied by Terochem Laboratories (Canada).

5.3.2 Microorganisms and Culture Conditions

The PHB-producing bacterium *Methylocystis* sp. Rockwell ATCC 49242 was grown under two sets of conditions. In the first condition (Biomass A), bacterial cultures were grown in 100 mL of modified ammonium mineral salts (AMS) medium in sterile 1-L septum-capped glass bottles with 4.8 mmol of

methane (>99.9%, Linde) and 1.2 mmol of methanol (Modified from Whittenbury et al., 1970). AMS medium contained, per liter: 1g $MgSO_4 \cdot 7H_2O$, 0.228 g $CaCl_2 \cdot 2H_2O$, 0.5 mL of 0.1%w/v Na_2MoO_4 solution, 1 mL of 3.8% w/v FeEDTA solution, 0.05 mL of 100 mM copper sulfate solution, 10 mL of trace element solution (per liter: 0.5 g $FeSO_4 \cdot 7H_2O$, 0.4 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $MnCl_2 \cdot 4H_2O$, 0.05 g $CoCl_2 \cdot 6H_2O$, 0.01 g $NiCl_2 \cdot 6H_2O$, 0.015 g H_3BO_3, 0.25 g Na_2EDTA), and Biomass A used 1 mmol/L NH_4Cl as the source of ammonium. The cultures were buffered to a pH of 6.8 with 1 mL of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) per 100 mL culture. The cultures were grown for 144 h in a 30°C incubator room on a shaker at 150 rpm (G10 Gyrotory Shaker, New Brunswick; Orbit Shaker, Lab-Line Instruments). Biomass A cultures had an average PHB content (see Quantification of PHB below) of 170 ±39 mg/L, corresponding to 43 ±11% of cell dry weight content. In the second condition (Biomass B), cultures were grown for 14 to 17 days in 3-L bioreactors (BioFlo/CelliGen 115, New Brunswick). 40 mL of pre-culture were inoculated in 2 L of AMS medium with 0.2 mmol methanol. Additional methanol was pulse-fed every 24 h, increasing in feeding from 2 mmol to 35 mmol over the growth of the culture. Methane was provided at a rate of 0.076 L/min (corrected to standard atmospheric temperature and pressure). The culture was stirred at 300 rpm and air was provided at a constant flow rate of 0.177 L/min (corrected to standard atmospheric temperature and pressure). The initial pH of the culture was 6.8 and dropped to 5.7 by the end of bioreactor growth. Every day a 20-mL sample was taken out for analysis, and 20 mL of AMS medium was added to keep the reactor volume constant. Biomass B entered the extraction process with an average PHB concentration of 674 ±111 mg/L, corresponding to 66 ±4 % of cell dry weight content.

As a control, the non-PHB producing methanotroph *Methylomicrobium album* BG8 ATCC 33003 was grown in 100 mL of nitrate mineral salts (NMS) medium in sterile 250-mL septum-capped glass bottles, with 2.5 mmol of methane. NMS medium has the same recipe as AMS medium except it contains 1 g/L KNO_3 as the nitrogen source (0.99 mmol nitrate per 100 mL).

To determine PHB content, 10 mL of culture was centrifuged for 30 min at 2,988 × g. The supernatant was decanted, and the pellet was assessed for PHB content through depolymerization/esterification/gas chromatography (see below). The cell dry weight was determined gravimetrically.

5.3.3 Room-Temperature Extraction from Unconcentrated Biomass

A schematic of the extraction process is shown in Figure 5.1. 100 mL of Biomass B (unconcentrated bioreactor effluent) and 20 mL SHS were added to a 250-mL round-bottom flask and stirred at 200 rpm for 1 h. The mixture was allowed to settle for at least 12 h, forming two layers. 80 mL of

the lower layer (aqueous) was removed by pipet. While the water was not replaced for DMCHA (1:1 water:solvent ratio), it was replaced with 80 mL of deionized water in the case of DMBA (5:1 water:solvent ratio). Carbon dioxide (99.9%, Linde) was bubbled through the mixture until it became monophasic. This mixture was vacuum-filtered through a pre-weighed 0.45-µm hydrophilic PTFE filter (Omnipore, MilliporeSigma), then the filter and product were rinsed with carbonated water. The product was then collected from the filter into a pre-weighed aluminum weighing dish and allowed to air dry until the mass was stable. The filtrate was heated to 60°C on a heating plate and sparged with nitrogen (>99.9%, Linde) for 2 h; and the resulting solvent and water fractions were collected. These solvent and water fractions were then recycled and used for a second extraction cycle, adding some fresh SHS to compensate for losses.

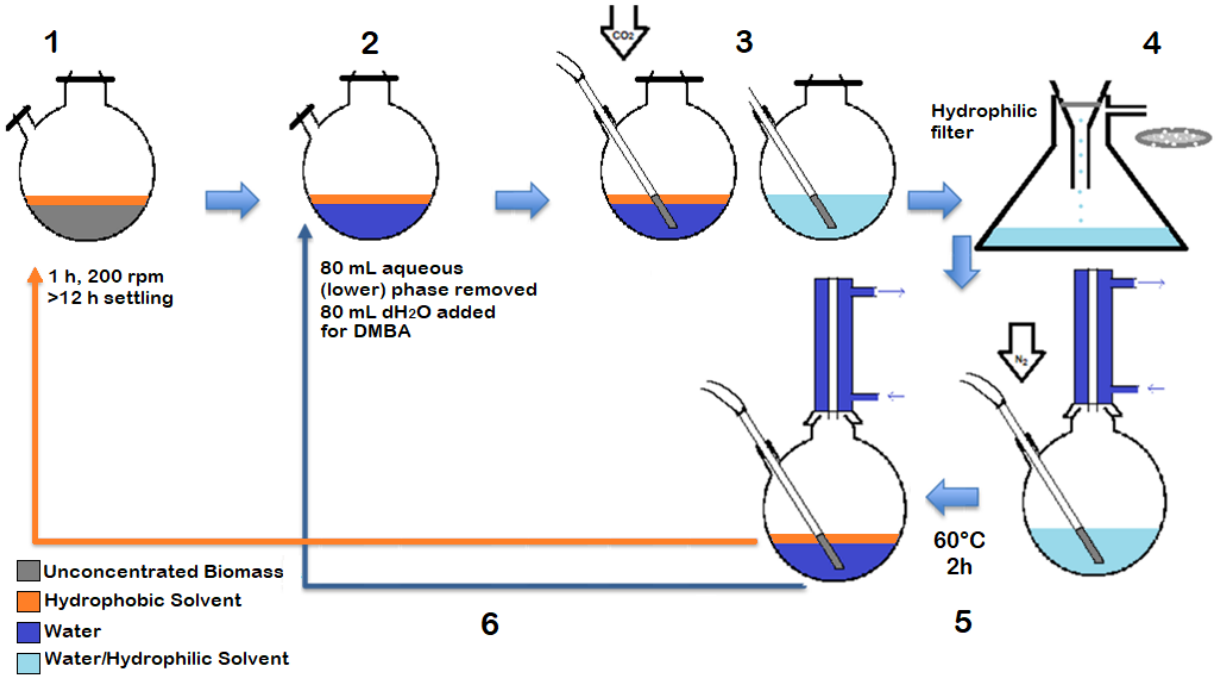

Figure 5.1: Procedure for the extraction of PHB from unconcentrated biomass.
Step 1: 100 mL of unconcentrated biomass (Biomass B) was stirred with 20 mL of SHS for 1 h, then left to settle at least 12 h. **Step 2:** 80 mL of the aqueous phase (lower phase) was removed and replaced by deionized water (80 mL) for DMBA, but not replaced for DMCHA. **Step 3:** The water-solvent mixture was sparged with carbon dioxide until it became monophasic, indicating the solvent had switched to its hydrophilic form. **Step 4:** Filtration of hydrophilic mixture to recover PHB. **Step 5:** Nitrogen sparging under heat was carried out to switch the solvent back to its hydrophobic form. **Step 6:** Separation of the resulting SHS (upper) and water (lower) phases, and recycling back to Steps 1 and 3, respectively.

5.3.4 Room-Temperature Extraction from Concentrated Biomass

The extractions from concentrated biomass were conducted according to a modified procedure of that shown in Figure 5.1. 240 mL of Biomass A was concentrated by centrifugation at 3,840 × g for 20 min (Beckman J2-HS centrifuge with Beckman-Coulter JA-14 rotor) and was either used as is (unlysed biomass) or subjected to lysis using a French press (lysed biomass). In both cases, the final volume of

concentrated culture used for extraction experiments was 10 mL. 10 mL of SHS (DMBA or DMCHA) was added to the concentrated biomass in a 50-mL Falcon tube, and vortexed for 30 s. The mixture was then centrifuged at 1,120 × g for 20 min (IEC Clinical Centrifuge, Damon). Typically, this resulted in the formation of a clear organic (upper) phase, a slightly turbid aqueous (lower) phase, and the accumulation of white biological material at the interface. After centrifugation, the upper and lower phases were removed by pipet and aliquoted. Deionized water (five times the SHS volume for DMBA or equal volume for DMCHA) was added to the remaining middle phase. Carbon dioxide was sparged through the mixture until it became monophasic. This resulting monophasic mixture was vacuum-filtered through a pre-weighed 0.45-µm hydrophilic PTFE filter (Omnipore, Millipore), then the filter and product were rinsed with carbonated water in a different flask. The product was then collected from the filter into a pre-weighed aluminum weighing dish and allowed to air dry until the mass was stable.

One set of experiments was conducted to determine whether a second extraction pass could improve recovery of material from the solvent and interface layers. The interfacial middle phase and 3 mL of the solvent (upper) phase from Step 2 (Figure 5.1) were taken and mixed by vortexing with an equal volume of water. This mixture was centrifuged a second time before collecting the interface and solvent layers and proceeding with the remainder of the extraction process, as described above.

5.3.5 Control Experiment: French Press Extraction

240 mL of Biomass A was concentrated by centrifugation at 3,840 × g for 20 min (Beckman J2-HS centrifuge with JA-14 rotor) and subjected to lysis using a French press (lysed biomass). 10 mL of concentrated culture was used for the extraction experiments. 10 mL of deionized water was added to the concentrated biomass in a 50-mL Falcon tube, and vortexed for 30 s. The mixture was then centrifuged for 20 min at 1120 × g (IEC Clinical Centrifuge, Damon). The supernatant was discarded. The pellet was resuspended in 10 mL of deionized water and the mixture was vacuum-filtered through a pre-weighed 0.45-µm hydrophilic PTFE filter (Omnipore, Millipore). The product was then collected from the filter into a pre-weighed aluminum weighing dish and allowed to air dry until the mass was stable.

5.3.6 High-temperature extraction from wet biomass

240 mL of Biomass A was concentrated by centrifuging, first at 3,840 × g for 20 min (Beckman J2-HS centrifuge with JA-14 rotor) then for 30 min at 2,988 × g (Evolution RC centrifuge with SS-34 rotor, Sorvall), in 12 mL screw-capped glass test tubes and decanting the supernatant. In a glass test tube, 10 mL of DMBA was added to the biomass pellet (approximately 100 mg) then heated to 127°C and stirred at

300 rpm for 12 h on a hot plate/stirrer (PC 420-D, Corning). The mixture was filtered while hot through a hydrophobic 1.0-µm PTFE filter (Fluoropore, Millipore). 50 mL deionized water was added and carbon dioxide was sparged through the mixture until it was monophasic (approximately 2 h). It was then filtered through a hydrophilic 0.45-µm PTFE filter (Omnipore, Millipore) and the retentate was kept as recovered product.

5.3.7 High-temperature extraction from dried biomass

240 mL of Biomass A was concentrated to 2 mL by centrifugation at 3,840 × g for 20 min (Beckman J2-HS centrifuge with JA-14 rotor), then for 30 min at 2,988 × g (Evolution RC centrifuge with SS-34 rotor, Sorvall). It was then dried overnight (more than 12 h) in an oven at 50°C (Isotemp 500 Series, Fisher), then crushed with a mortar and pestle. From this point, the same extraction procedure as for wet biomass (above) was used.

5.3.8 Assessment of PHB

PHB quantification was done through depolymerization/methyl esterification followed by gas chromatography (GC) (Modified from Braunegg et al., 1978). Shortly, product samples were weighed (Mettler-Toledo MX5), then added to screw-capped test tubes, along with 2 mL chloroform, 1 mL methanol and 1 mL of acidified methanol (consisting of 24 mL methanol, 1.5 mL sulfuric acid, and 1 mL of a benzoic acid as a standard). The test tubes were sealed using PTFE tape and placed in a water bath at 100°C for 5 h. The samples were allowed to cool before 1 mL of water was added, and the mixture was vortexed and allowed to settle for phase separation. The bottom phase (chloroform) was analyzed by GC, with the methyl ester of the PHB monomer (methyl (3-hydroxybutyrate)) as analyte and methyl benzoate as internal standard.

Two GCs were used with slightly different parameters. A HP 5890 GC with flame Ionization detector (FID) was used with a DB-5 column (30 m x .25 mm ID, 0.25 µm coating). Helium at 1.5 mL/min was used as carrier gas. The injection temperature was 250°C and the FID temperature was 300°C. The oven temperature was held at 80°C for 1 min after injection, ramping up 10°C/min to 120°C then 30°C/min to 270°C, and held at that temperature for 5 min. The retention times were 2.8 min for methyl(3-hydroxybutyrate) and 5.5 min for methyl benzoate. A Thermo Scientific Trace GC Ultra with FID was used with a TR-5 column (15 m x .25 mm ID, 0.25 µm coating). Helium was used as carrier gas at 1.5 mL/min. The injection volume was 1 µL, the injection temperature was 250°C and the FID temperature was 300°C. The oven temperature was held at 65°C for 1 min after injection, ramping up 10°C/min to 120°C then

30°C/min to 270°C and held for 3 min. The retention times were 1.8 min for methyl(3-hydroxybutyrate) and 4.4 min for methyl benzoate. Standard curves relating area ratio to methyl (3-hydroxybutyrate) concentration were prepared for each GC using PHB powder from Sigma-Aldrich (Canada) as standard. Similarly, to determine PHB cell content, 10-mL culture samples were centrifuged for 30 min at 2,988 × g (Evolution RC centrifuge with SS-34 rotor, Sorvall), the supernatant was discarded, and the pellet was treated (depolymerization/methyl esterification) and analyzed by GC as described above, being added in place of the weighed product sample. The PHB amount was divided by the cell dry weight to determine % dry cell weight content. PHB recovery and purity were determined by treating process samples as described above. Product purity was calculated as the PHB mass in a sample divided by the total mass of the sample assessed gravimetrically. Recovery was calculated as the % PHB recovered based on the initial PHB amount present in the biomass used for extraction.

5.3.9 Assessment of Cell Lysis with SHS

The Live/Dead *Bac*Light Bacterial Viability Kit was used as instructed by Invitrogen (Canada) on cell cultures with and without exposure to the SHS in order to determine the impact of the solvents on cell viability and integrity. *Methylocystis* sp. Rockwell was grown in 100 mL of NMS medium in sterile 250-mL septum-capped glass bottles, with 60 mL (2.5 mmol) of methane. 500-µL samples of culture were taken and centrifuged for 10 min at 15,000 × g (5424R microcentrifuge, Eppendorf) then resuspended in 500 µL of 0.85% NaCl buffer solution (as control) or a total volume of 500 µL of NaCl buffer solution and 20% v/v DMBA. These were vortexed twice, centrifuged, and resuspended in 500 µL of NaCl buffer solution. The cells were then centrifuged and resuspended in 50 µL NaCl buffer and 50 µL of the Live/Dead *Bac*Light dye solution was added. These samples were then examined under a fluorescence microscope (Leica DMRXA2 microscope) using a hemocytometer, and pictures were taken using a QImaging Retiga EX camera. For each sample, pictures were taken under green and red fluorescence. The images were processed with Fiji image analysis software (Schindelin et al., 2012) to remove background noise, align cells between the photos by overlaying the modified images, and count the cells. Cells which fluoresced red were counted as dead, and cells which fluoresced green with no overlapping red cells were counted as alive.

5.3.10 Statistical Analysis

Experiments were conducted in triplicate (with one outlier omitted for the recovery by the French Press control). Herberich et al. proposed a method for comparing multiple means under heteroscedasticity, which was used here to evaluate recovery and purity of each method of extraction from

concentrated biomass (Herberich et al., 2010). Tukey's test was also used to compare the recovery and purity of the extractions from unconcentrated biomass. For unconcentrated biomass, Welch's t-test was used to compare differences in results between experiments conducted with fresh and recycled solvent (6 samples pooling both solvents together) and the overall performances of DMBA and DMCHA (6 samples, pooling both fresh and recycled solvent together).

5.4 Results

In this work, different approaches to the extraction of PHB from methanotrophic bacteria using two different SHSs (DMBA and DMCHA) were investigated. Two general schemes were examined: extraction at room temperature based on the partitioning of PHB in water-hydrophobic SHS systems, and extraction at elevated temperatures through the dissolution of PHB in hydrophobic SHSs. This was done with biomass directly harvested from bioreactors (bioreactor-harvested biomass), lysed biomass, and concentrated biomass (Figure 5.2). The extraction from bioreactor-harvested biomass was additionally tested with recycled SHS.

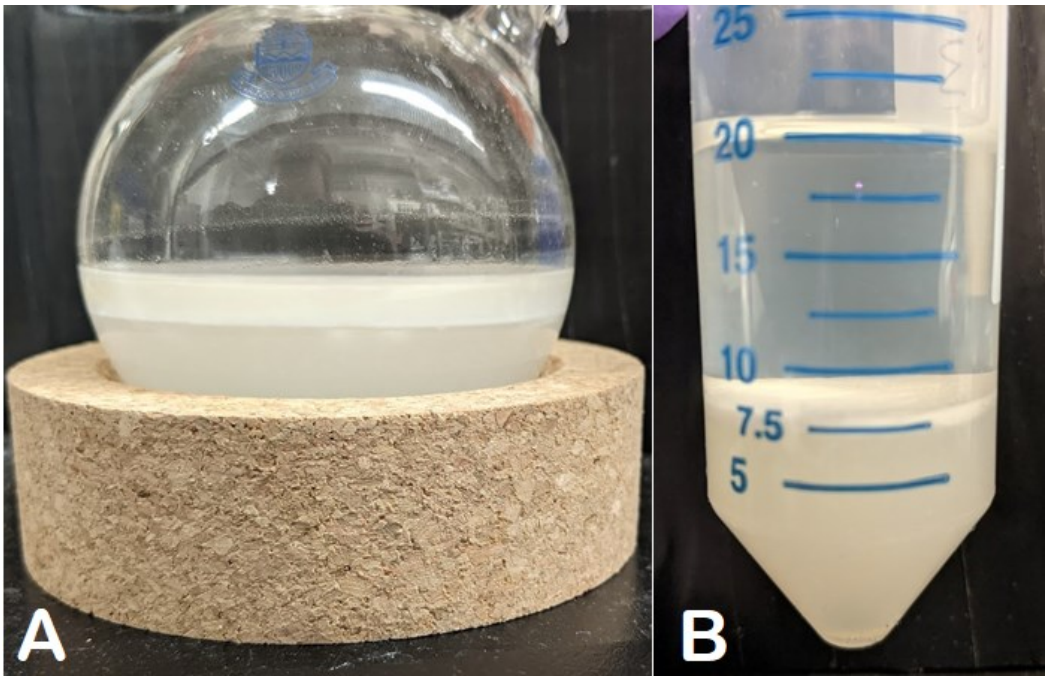

Figure 5.2: PHB partitioning in water-solvent system
(A) PHB partitioned into the upper phase of the water-solvent system from bioreactor-harvested biomass after stirring and settling (Figure 5.1 Step 1). **(B)** PHB concentrated at water-solvent interface after vortex mixing and centrifugation, from concentrated bacterial culture.

5.4.1 Impact of SHS on bacterial cell integrity

Tests were conducted with *Methylocystis* sp. Rockwell in the presence of hydrophobic DMBA to determine if this SHS would reliably lyse bacterial cells. The tests served as an indication of the effects of the SHS on cell integrity and survival, assayed through a live/dead cell assay. Figure 5.3 shows the result of one such trial. An important decrease in cell count was observed after exposure to the SHS (reduction of 82±1%), indicative of substantial cell lysis. In addition, only 24% of the remaining cells were alive (76% dead cells), showing either toxicity or significant damage to the cell wall.

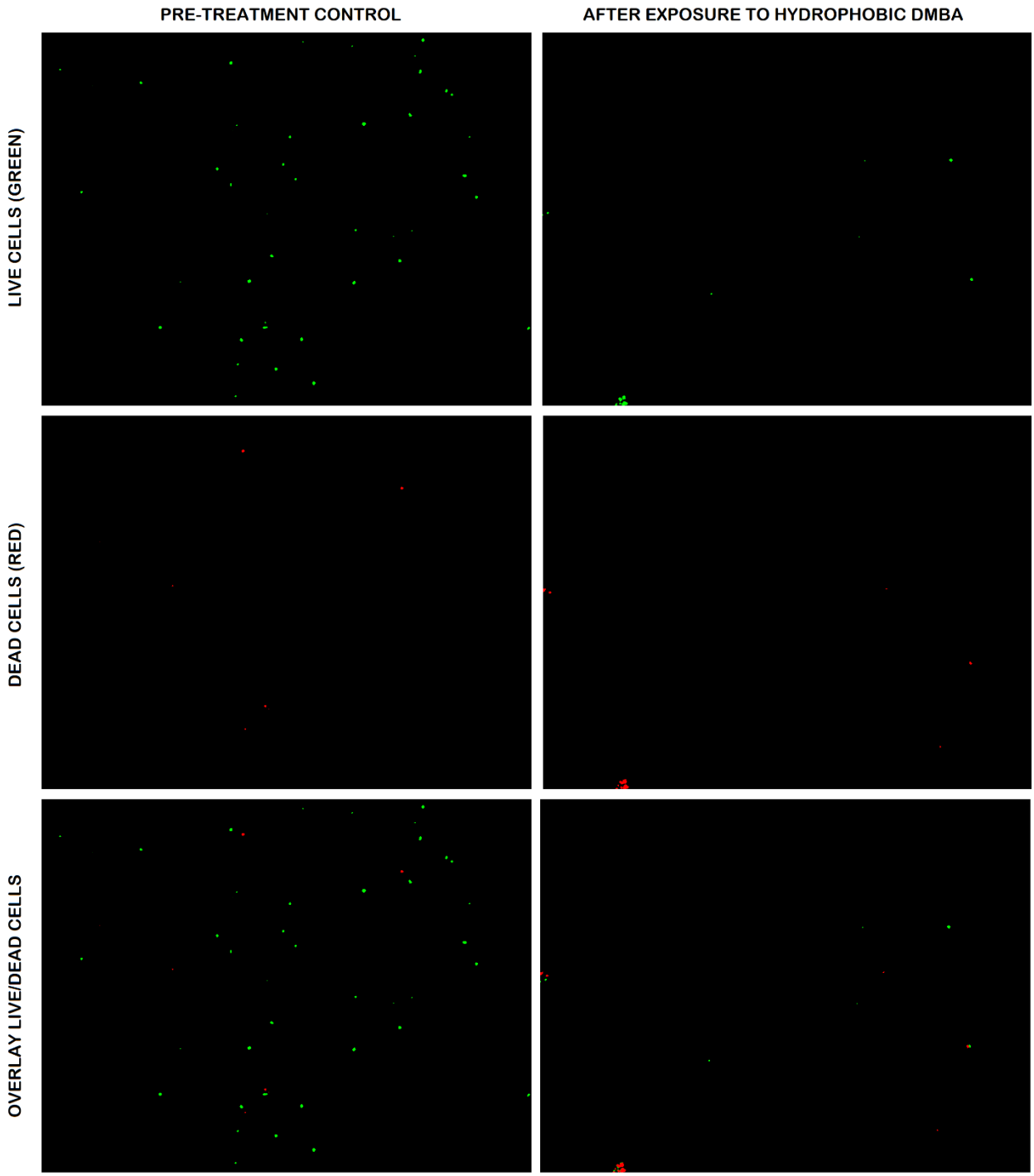

Figure 5.3: Effect of hydrophobic DMBA on *Methylocystis* sp. Rockwell.
Methylocystis sp. Rockwell cell culture was exposed for 2 h to an emulsion of 20% (v/v) DMBA in its hydrophobic state in water. **Left column**: Pre-treatment control. **Right column**: After exposure to DMBA solution for 2 h, centrifugation and recovery in the aqueous phase. **Row 1**: Live cells showing green fluorescence. **Row 2**: Dead cells showing red fluorescence. **Row 3**: Overlay image (live and dead cells).

5.4.2 Extractions from Bioreactor Harvested Biomass

Figure 5.4 shows the recovery and purity of PHB extracted with SHSs (either fresh or recycled from a previous extraction cycle) directly from bioreactor-harvested biomass at room temperature, using the process described in Figure 5.1. Comparing the PHB recovery with fresh or recycled solvent shows there were no statistical differences for a given SHS as determined using the method proposed by Herberich et al., 2010. Comparing results between solvents shows that DMCHA yielded a higher recovery (89±8%) than DMBA (70±11%). The purity of PHB recovered was similar for both solvents, at 83±8% for DMBA and 83±12% for DMCHA. The white PHB product was soft and malleable when wet but became hard when dried.

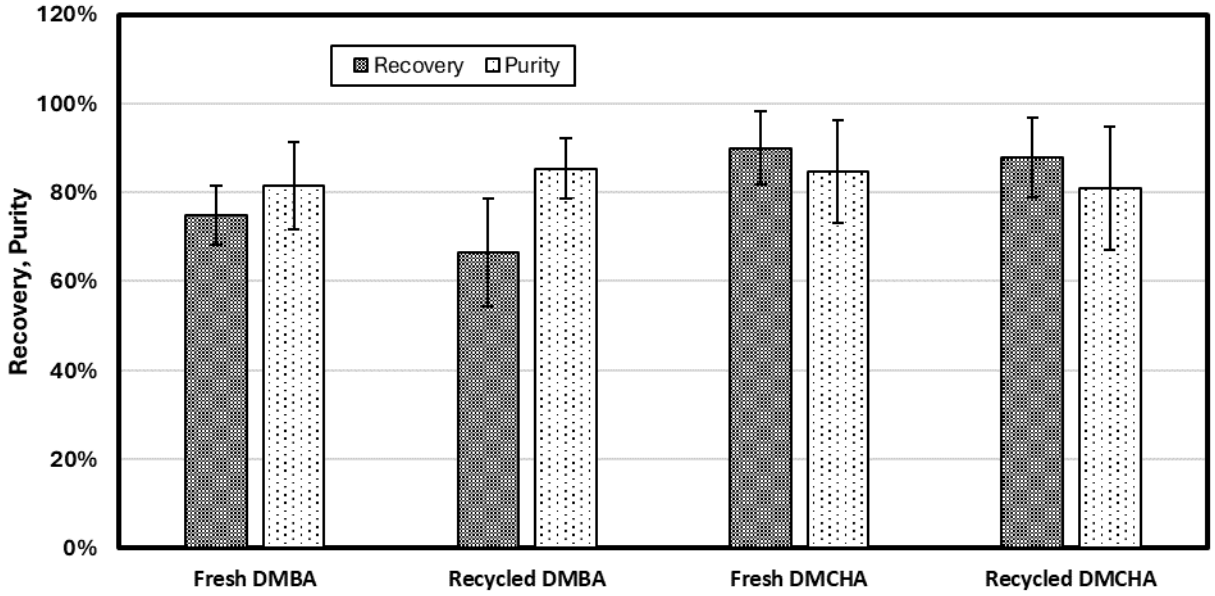

Figure 5.4: SHS PHB extraction from bioreactor harvested *Methylocystis* sp. Rockwell
Recovery and purity were determined for fresh and recycled DMBA and DMCHA extractions. PHB was extracted at Step 4 of the process shown in Figure 5.1. Experiments were performed with fresh SHS and with SHS recycled through Steps 5 and 6 in the process shown in Figure 5.1. Error bars show standard deviations (n=3).

Table 1 shows the solvent recovery for one-cycle and two-cycle extraction processes. The proportion of total solvent input which was recovered after the second extraction (two full extraction cycles and recovery) was 43±3% for DMBA and 28±1% for DMCHA. This does not include solvent which was retained in the water after switching the SHS back to its hydrophobic form.

Table 5.1: Solvent recovery rates after each cycle of the extraction process

Solvent	First cycle	Second cycle	Total (Two-cycle)*
DMBA	53±20%	64±9%	43±3%
DMCHA	59±4%	39±1%	28±1%

*Solvent retained by recovered water was not measured.

5.4.3 Extractions from Concentrated Biomass

Figure 5 shows the results of the treatments tested from concentrated biomass (5.9 times the concentration of bioreactor-harvested biomass) extracted at room temperature. Recovery and purity were evaluated separately and compared using Tukey's Honestly Significant Difference tests; the only statistically significant difference was observed between the recovery of the lysed filtered control and the two-pass DMCHA extraction ($p = 0.02$). Despite the lack of statistical differences in the data, some trends could be observed. The lysed cells control (French press, no solvents) led to the highest average recovery (87±4%) and a purity of 79±9%. Extraction from unlysed cells with fresh DMBA led to similar recovery (85±19%) and high purity (86±3%). Interestingly, extractions from unlysed cells using recycled SHSs led to lower, but not statistically different, recovery values, and the combinations of lysis (using a French press) and SHS extraction did not lead to greater recoveries or purities. High temperature extractions from wet biomass led to a recovery of 22±7% and purity of 80±3%. Extractions from dried biomass did not provide enough yield to quantify the recovery or product purity, and so these results are not shown. The character of the extracted PHB was the same as from the bioreactor-harvested biomass.

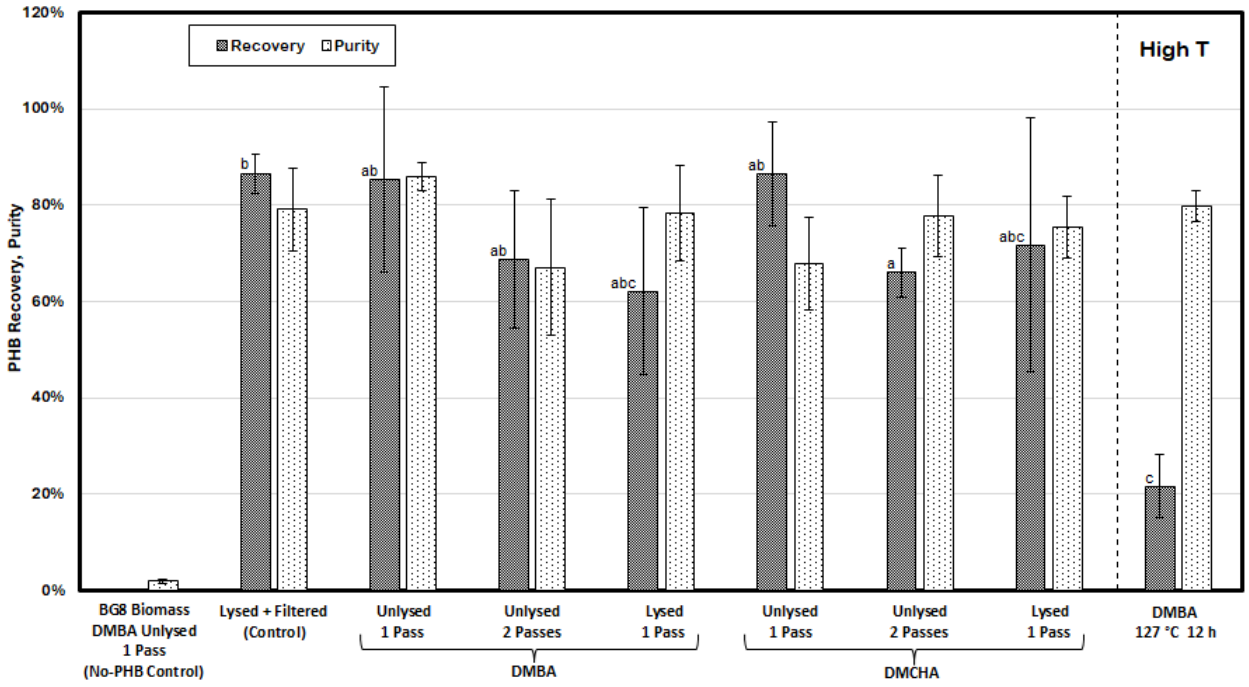

Figure 5.5: SHS PHB extraction from concentrated *Methylocystis* sp. Rockwell cultures.
Recovery and purity were determined for a range of extraction procedures. An extraction from *Methylomicrobium Album* BG8 is used as a no-PHB control. For DMBA and DMCHA, the 1-pass method refers to a single pass of the process shown in Figure 5.1; the 2-pass method refers to the biomass extract from a first pass undergoing a second pass of the separation procedure shown in Figure 5.1. Error bars show standard deviations (n=3; No-PHB control n=2; Lysed + Filtered control is n=2 for recovery and n=3 for purity). All values for purity are statistically equivalent except the no-PHB control. Recovery bars which share a letter have no statistically significant difference between them based on the method proposed by Herberich et al., 2010 ($p<0.05$).

5.5 Discussion

Although PHB does not fully dissolve in DMBA or DMCHA at room temperature, it still interacts with these SHSs (Lawley et al, 2022); for example, through the formation of a gel (Chapter 4). In this context, room-temperature methods were developed based on the PHB being associated with the solvent phase without necessarily being fully dissolved. These methods achieved high purity (> 80%) in all treatments tested, whether using fresh or recycled solvent (Figure 5.4), and the DMCHA extraction from unconcentrated biomass from the bioreactor had the highest average recovery of all methods tested in this study. The efficacy of this method is especially promising when considering that the extraction was performed at ambient conditions and without pre-treatment of the biomass. The addition of a French press pretreatment step did not improve recovery or purity of the polymer product. This is likely due to the fact that SHSs are able to lyse *Methylocystis* sp. Rockwell cells in the presence of water (Figure 5.3), allowing for efficient extraction even when the lysis pre-treatment step is omitted.

Hydrophobic DMBA and DMCHA have been shown to efficiently dissolve PHB in abiotic systems at elevated temperatures, although this also led to reductions in the polymer average molecular weight, which was likely due to hydrolysis in the presence of water (Lawley et al., 2022; Lawley et al., 2024). This being said, it was still relevant to investigate whether high-temperature extraction in DMBA would yield better recovery; high-temperature extraction with DMCHA was not tested due to the elevated level of PHB degradation observed with this SHS (Lawley et al, 2024). However, the high-temperature extraction from dried biomass tested in this study failed to provide sufficient PHB yield to be considered a viable extraction method, and the high-temperature extraction from concentrated wet biomass did not provide improvements in purity over the room-temperature extraction treatments.

Since the addition of pretreatment steps (including concentrating cells, drying cells and mechanical lysis) often improves the performance of bioproduct extraction processes (Heinrich et al., 2012, Mannina et al., 2019, Rodrigues et al., 2022, Wongmoon et al., 2022), the focus of some the experiments performed in this study was to determine if any such step provided a significant benefit to the extraction of PHB from methanotroph biomass. Additionally, in many systems a second stage or pass of the same separation method will increase product purity (Seader et al., 2011). However, the addition of a French press step for cell lysis before treatment or of a second pass of treatment did not increase the purity of the PHB recovered (Figure 5.5). This means that of the methods tested for extraction from concentrated biomass, the simplest extraction process (consisting of a single-pass extraction of concentrated biomass without lysis) was preferable. Cell lysis by French press followed by filtration was performed as a control for mechanical disruption and extraction (Kosseva 2018). This relatively simple procedure provided

effective PHB extraction (Figure 5.5). In fact, the PHB recovery and purity obtained through the use of SHSs did not significantly differ from those obtained using the French press control; although a full comparison of the performance and sustainability of the different methods tested would require accounting for the energy requirements of the mechanical method and the material and energy costs of SHS. It is also interesting to note that SHSs could be recovered after centrifugation (after Step 1 in Figure 5.1) and reused, and that only a small amount of solvent was entrained in the PHB product after switching phases to purify the product.

The unconcentrated (bioreactor-harvested) biomass protocol (Figure 5.4) was even simpler – omitting centrifugation steps for dewatering the culture and for facilitating phase separations that were present in the treatments with concentrated biomass – yet PHB recovery and purity remained high. This shows strong potential for SHS-based PHB extraction at room-temperature with relatively few processing steps/unit operations.

In addition, solvent recyclability without distillation is one of the major advantages of SHSs. In this study, the solvents were successfully recovered and re-used for further PHB extraction; although further processing improvement could further increase the recycling efficiency (on average, 54% of SHS was recovered after single extraction and recovery cycle). At the scale of the experiments conducted, a non-negligible portion of the solvents were simply lost to the glassware, and some solvent may be retained in the water after switching back to its hydrophobic form. A previous study on lipid extraction from microalgae using SHSs reported a recovery rate of 83% (Cicci et al., 2018). Other studies investigating the use of DMCHA to extract bitumen from oilsands showed that the recycled water contained 16 wt% DMCHA, a number which was consistent between cycles (Holland et al, 2012), and which could account for some of the unrecovered solvent in the present work. Another consideration in recycling SHSs comes from the carbon dioxide stripping step, in which the solvent is switched to its hydrophilic form. Complete reuse of process water and the use of a vacuum instead of nitrogen to remove/recover carbon dioxide from solution in industrial implementation of SHSs at larger scales would allow for efficient recovery and reuse of both solvent and carbon dioxide (Cicci et al., 2018, Jessop et al., 2018).

The extraction of PHB from unconcentrated bioreactor-harvested biomass addresses several aspects improving sustainability of the process, including the reduction of material and energy requirements. It also has many advantages compared to the other PHB extraction processes: it does not require extensive dewatering or energy-intensive distillation for solvent recovery (Fernández-Dacosta et al., 2015; Pagliano et al., 2021); and it allows for SHS recovery, unlike digestion agents, and limits the amounts of wastewater compared to digestion processes (Fernández-Dacosta et al., 2015; Pérez-Rivero, 2019; Saavedra del Oso, 2021). The process demonstrated here has the potential to efficiently extract PHB from

unconcentrated cultures of methanotrophic bacteria, with solvent recycling under minimal energy requirements.

5.6 Conclusion

Several different processes for extracting PHB from methanotrophic bacteria (*Methylocystis* sp. Rockwell) using SHSs (DMBA and DMCHA) were developed and tested. It was found that the most effective processes were those conducted at room temperature; despite the incomplete dissolution of PHB at those conditions, PHB recovery over 80% were reached. While the inclusion of additional processing steps (for cell lysis, culture concentration, culture drying, and multiple extraction passes) was also investigated, ultimately the processes based on single-pass extraction of unlysed, unconcentrated cell cultures performed as well or better than other considerations. The solvents used were able to be recycled and re-used without distillation. Since the process enable recycling of the solvent, low temperature extraction, and the omission of a dewatering step, it has great potential as a more efficient, sustainable approach to PHB recovery from bacterial cultures.

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6 Conclusions and Future Direction

6.1 Summary of the Work

In this thesis, switchable hydrophilicity solvents (SHS) were tested as recyclable solvents for the processing of poly(3-hydroxybutyrate) (PHB) and extraction from methanotrophic bacteria. Candidate SHS were screened based on theoretical models and their PHB solubility was tested experimentally. Multiple SHS were determined to be able to dissolve PHB, and two of them – N,N,dimethylbenzylamine (DMBA) and N,N-dimethylcyclohexylamine (DMCHA) – were selected for further study.

It was demonstrated that PHB could be dissolved in hydrophobic DMBA and DMCHA and then precipitated by switching the SHS to their hydrophilic forms through addition of water and carbon dioxide. The solvents were successfully returned to their original form and could be re-used multiple times in further dissolution/precipitation cycles. However, the molecular weight of the PHB was found to decrease by more than 85% in dissolution performed at high temperatures, and decreased even further when water was present during dissolution. It was also found that PHB-DMBA gels could be formed, and that DMBA in the gels could still be switched from its hydrophobic form to hydrophilic form using water and carbon dioxide. These switched gels could then be formed into shape and would retain that shape when dried.

The extraction of PHB from bacterial cells by DMBA and DMCHA was investigated under a variety of conditions. High-temperature extraction using similar protocols to abiotic PHB dissolution and recovery had very poor recovery. High recovery and purity were achieved using an extraction protocol at room temperature that relied on PHB partitioning into the solvent phase of a biphasic water-solvent system, with most of the non-PHB cell mass (NPCM) partitioning into the aqueous phase. This extraction protocol was tested on both biomass directly harvested from bioreactor cultures and concentrated biomass. High yields and product purities were achieved. In the case of biomass directly harvested from the bioreactor, it was demonstrated that the solvent could be recovered and recycled for a subsequent round of extraction. The demonstrated PHB extraction process from unconcentrated bioreactor effluent has excellent potential due to the elimination of a dewatering step, being conducted at ambient conditions, and the recyclability of the solvents.

Biodegradable polymers such as PHB are the world's best hope for the plastic waste problem, but their widespread deployment still has technological barriers. With product recovery being the key problem for PHB production, the extraction processes developed here offer a starting point for PHB extractions that could enable the widespread sustainable use of this remarkable biopolymer.

6.2 Avenues for Further Research

There are many ways in which this work could be built upon by future researchers. The most promising avenues for continued investigation are improvements to the room-temperature extraction process and the study of the switchable gels.

The application of SHSs to PHB extraction has significant potential, especially with regards to the demonstrated methods at ambient temperature. There is significant advantage in a process that does not require any heat and that is able to extract PHB from bioreactor effluent with no pretreatment. The measured solvent recovery rate was not as high as those observed in previous SHS extraction studies; however, discussions of SHSs use at the industrial scale generally consider that with proper care and some process changes (such as using low pressure instead of nitrogen purging to remove carbon dioxide from the hydrophilic solvent form) the rate of solvent losses will be greatly reduced (Cicci et al., 2018). It is most likely that the bulk of solvent losses would come from any SHS that remains dissolved in water after switching back to the hydrophobic form. These can be a significant factor, but the SHS retained in the water can be recovered using hexane (Du et al., 2015).

There is some solvent which will be retained in any water removed from the process, and it is possible that this significantly contributes to solvent losses. The water used to switch the solvent to its hydrophilic form is one source of loss which would be less significant at a larger scale, as that water is recycled so any solvent captured there is not lost. However, in the case of the unconcentrated biomass harvested from the bioreactor, there will be some solvent that remains with the discarded water. This may require further investigation into whether partial dewatering does bring improvements from a solvent retention standpoint, or if subsequent treatment to recover the solvent from this water is the least costly approach.

Other types of switchable solvents – such as multi-amine SHSs which exhibit greater polarity changes (Vanderveen et al., 2018) or amine-free SHSs which would improve process safety (Cunha et al., 2022) – could also be investigated for PHB extraction. Since these other switchable solvents offer advantages relative to the SHSs studied here, it would be pertinent to examine if any of these other solvents could be used in place of DMBA or DMCHA.

Two other areas of study with great potential are the extraction of other PHAs and copolymers from microbes and the extraction from mixed microbial communities. There is a great deal of interest in PHAs beyond PHB, and increasingly mixed microbial communities, such as activated sludge, are seen as more economically viable production methods for PHAs, so it is relevant to understand whether the demonstrated extraction process can be applied to these different PHA bioproduction processes.

The switchable PHB-DMBA gels that were formed are a unique phenomenon that warrants further study. These gels may have useful applications in the formation of biodegradable devices in the health industry, however it would be of high importance to ensure that there is no residual SHS before they can be used. Should an effective solvent exchange be demonstrated, the switched gels could likely be infused with other water-soluble compounds, which may be useful in either the wet or dried form of the switched gel.

The ability to shape the switched gels and have them dry in shape is promising, however this would require improvements from the current form to avoid easy crumbling of the formed shapes. Although it was not studied in detail, the PHB which was precipitated after extraction from biomass can be similarly shaped, and so improvements beyond the methods shown here could lead to a process wherein PHB is extracted and preliminarily processed in one operation.

The extraction and processing protocols demonstrated in this thesis show great potential for advancing the use of PHB. These avenues for further study – generalizing the extraction protocol to other PHAs, culture conditions and switchable solvents, determining how the process would be best adapted to larger scales, and a further investigation on the details or utility of the switchable gels – would be important next steps in bringing this technology towards application.

6.3 References

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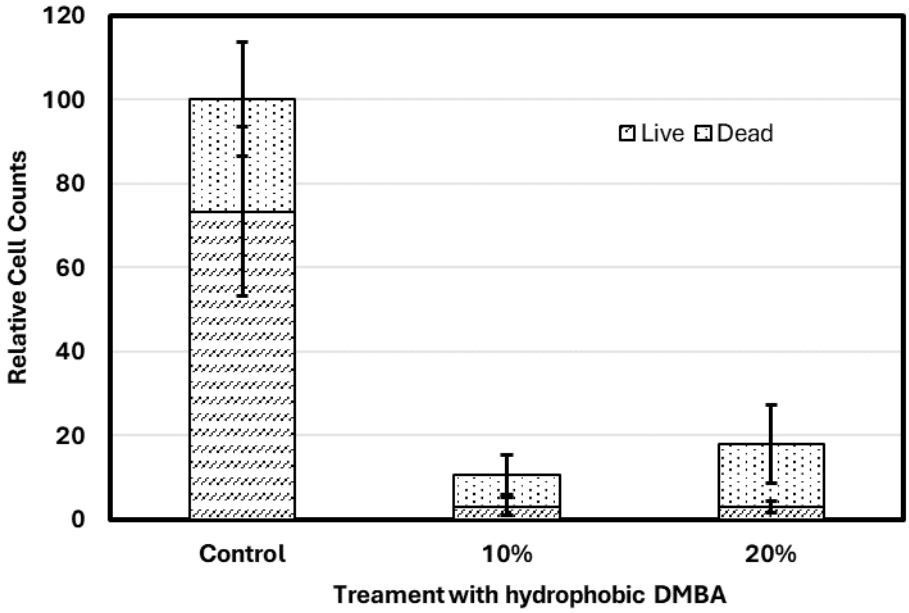
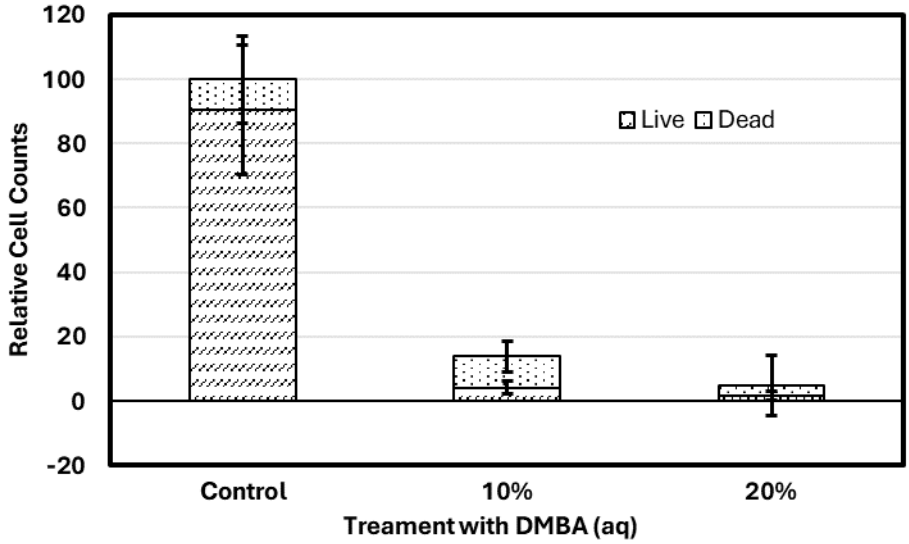
A Appendix

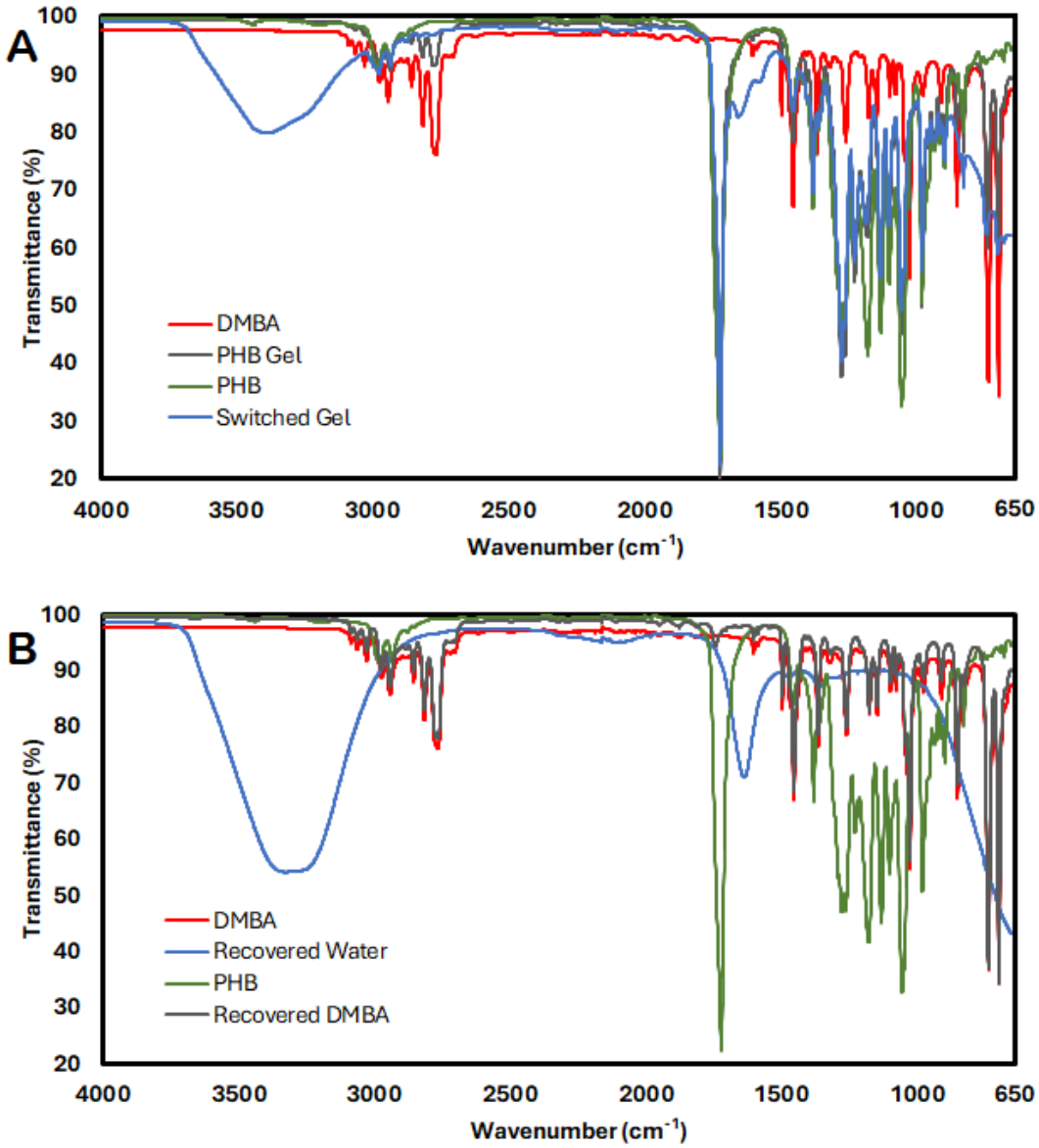
Effect of DMBA on *Methylocystis* sp. Rockwell



Supplementary Figure A.1: The effects of hydrophobic DMBA on *Methylocystis* sp. Rockwell.
Cultures were treated as per the methods in Section 5.3.9, with the addition of a 10% v:v treatment. Cell counts are normalized to the total number of cells in the control. Error bars are standard deviations (n=3 sample, average cells counted in controls: 87).



Supplementary Figure A.2: The effects of hydrophilic DMBA on *Methylocystis* sp. Rockwell.
Cultures were treated as per the methods in Section 5.3.9, with the addition of a 10% v:v treatment and the exception that instead of hydrophobic DMBA, hydrophilic DMBA was used. Hydrophilic DMBA was prepared at a 5:1 water:solvent ratio. Cell counts are normalized to the total number of cells in the control. Error bars are standard deviations (n=3 samples, n=2 control, average cells counted in controls: 868).



Supplementary Figure A.3: FTIR analysis of PHB-DMBA gel and associated fractions.
(A) Spectra of the PHB-DMBA gel and the switched gel overlain beside the spectra of DMBA and PHB. **(B)** Spectra of the recovered water and DMBA overlain beside the spectra of DMBA and PHB.