TREATMENTS FOR MAINTAINING NATIVE GRANULE MORPHOLOGY DURING INDUSTRIAL PURIFICATION OF PHB-co-HHx

by

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(Under the Direction of JASON LOCKLIN)

ABSTRACT

Polyhydroxyalkanoates (PHAs) are a promising alternative to traditional petrochemical-based plastics, often compared to polypropylene in their mechanical properties while being fully biodegradable in a variety of environments. An unusual property of native PHA granules is their amorphous structure due to the biological nature of their synthesis. It is critical for the successful commercial production of these polymers that granule crystallization be well controlled during purification; otherwise, the granules coalesce into large masses during centrifugation that are unable to be adequately purified and may damage industrial centrifugal equipment. The research presented in this dissertation elucidates the challenges associated with granule coalescence as well as mechanisms responsible for initiating granule crystallization. Using infrared spectroscopy, the crystallinity of PHA granules in their native environment can be ascertained and various treatments of the granules can be evaluated for their effectiveness to initiate granule crystallization. By ensuring granules are semicrystalline prior to their centrifugation, they are maintained in their native morphology throughout their purification, leading to a higher standard of purity and a polymer that is more stable and suitable for a variety of consumer applications. Additionally, mitigating granule coalescence reduces equipment downtime and material losses. These benefits are relevant to any aqueous PHA purification process and are essential for successful commercialization of these polymers.

INDEX WORDS: Polyhydroxyalkanoates, purification, downstream processing, crystallization

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CHAPTER 1

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of polyesters that have gained attention for their biodegradability and physical properties similar to petroleum-based polymers. These emerging polymers are becoming increasingly relevant with the growing concern over the fate of petroleum-based plastics in the environment. An analysis by Geyer et al. has shown that of the 8300 million metric tons of plastic produced to date, only 9% has been recycled, while 79% has accumulated in landfills or the natural environment [2]. Without intervention, the current trend in plastic management will lead to 12,000 million metric tons of

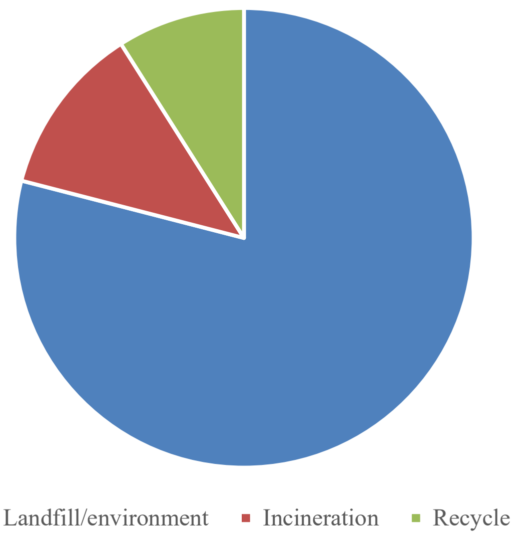

Figure 1.1: Fate of all plastic waste as of 2015 [2].

plastic disposed in landfills or the environment by 2050. One approach to limiting this accumulation of plastic waste is the introduction of biodegradable polymers, like PHAs. These polymers have the potential to be produced from waste feedstocks and support a circular economy wherein discarded plastics are composted for agricultural use.

PHAs are naturally occurring and are produced by microorganisms as an energy storage molecule, with polyhydroxybutyrate (PHB) being the most common polyester among PHAs[5-9]. PHB has a high crystallinity of up to 70% and a melting temperature that is close to its degradation temperature [10, 11]. As a result, it is often too brittle to be used alone and is difficult to process without degrading[12]. To overcome this limitation of PHB, organisms can be engineered to produce PHAs with a portion of 3-hydroxybutyrate monomers substituted with hydroxy acids containing longer side chains[13-15]. Examples of such monomers include 3-Hydroxyvalerate, 3-Hydroxyhexanoate, and 3-Hydroxyoctanoate (Figure 1). The side chains of these monomers serve to interrupt the crystal

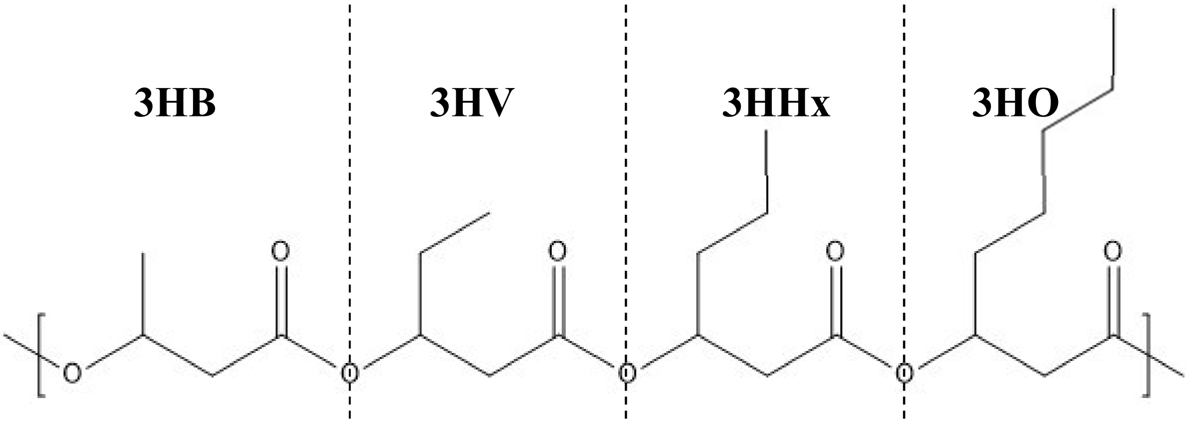

Figure 1.2: Examples of PHA Monomers. 3HB: 3-Hydroxybutyrate; 3HV: 3-Hydroxyvalerate; 3HHx: 3-Hydroxyhexanoate; 3HO: 3-Hydroxyoctanoate

structure of the polymer, resulting in a polymer with lower crystallinity and melting point. This modification provides flexibility and elongation of the polymer as well as the ability to process the polymer well below the degradation temperature [16].

As the production of PHAs has grown in scale, purification of the polymer has been the most significant challenge [17]. Early extraction methods involved dissolution of PHA from wet or lyophilized biomass using chloroform, followed by filtration and then precipitation in ethanol or other poor solvent [18]. Solvent extraction is impractical on a commercial scale for several reasons. First, the relatively low solubility of PHAs in solvents requires solvent-to-polymer ratios of more than 20-to-1 [19]. Furthermore, a large quantity of non-solvent, such as ethanol, must then be added at a rate of 3 or more times that of the polymer solution. These two steps result in at least 60 liters of hazardous solvent waste generated per kilogram of PHA, which must then be recovered and separated by distillation. Finally, the use of hazardous and flammable solvents requires the installation of costly safety systems and specialized equipment that can tolerate the solvents.

The use of aqueous, solvent-free cleaning processes has since become the focus of research for developing industrially viable processing of PHAs [20]. Various methods involve the use of detergents [21], enzymes [22], alkaline digestion [23], mechanical disruption [24], and a combination thereof. With these methods, the cell material surrounding the amorphous PHA granule is disintegrated, and the PHA can be purified and concentrated via sedimentation, filtration, floatation, or other means of separation while the solubilized biomass is removed.

SELECTION OF SEPARATION TECHNOLOGIES FOR PHA

Concentration by centrifugation is the favored separation method for practical scale-up to industrial production of PHAs due to the ability to handle large solids loading, the rejection of lightweight biological contaminants while capturing the relatively dense PHA granules, and the high volumetric throughput that can be achieved. While filtration, especially tangential flow filtration, is commonly used in separation processes in the production of biomolecules, PHA differs substantially from most products traditionally produced in the biotechnology industry. Firstly, the vast majority of bioproducts are either small molecules or soluble proteins. Even in the case of proteins produced as insoluble inclusion bodies, the early steps in purification involve the solubilization of the inclusion bodies for further processing, enabling them to be more readily purified using filtration. In contrast, PHA granules are recovered as spherical granules and remain so for the entire aqueous process. Secondly, PHA granules are produced in very high concentrations, approaching 200g/L, whereas other biological macromolecules produced industrially are produced on the order of milligrams to a few grams per liter.

Tangential flow filtration of PHA granules may be possible at a high dilution rate, but this would require copious quantities of water to both initially dilute the granules and then continually replace the eluted lysis broth with fresh water. Thirdly, and most significantly, budgetary limitations on PHA is much more limited than many other industrial bioproducts, with recombinant human insulin, for example, costing \$24,750 per kilogram [25] and many other therapeutic proteins

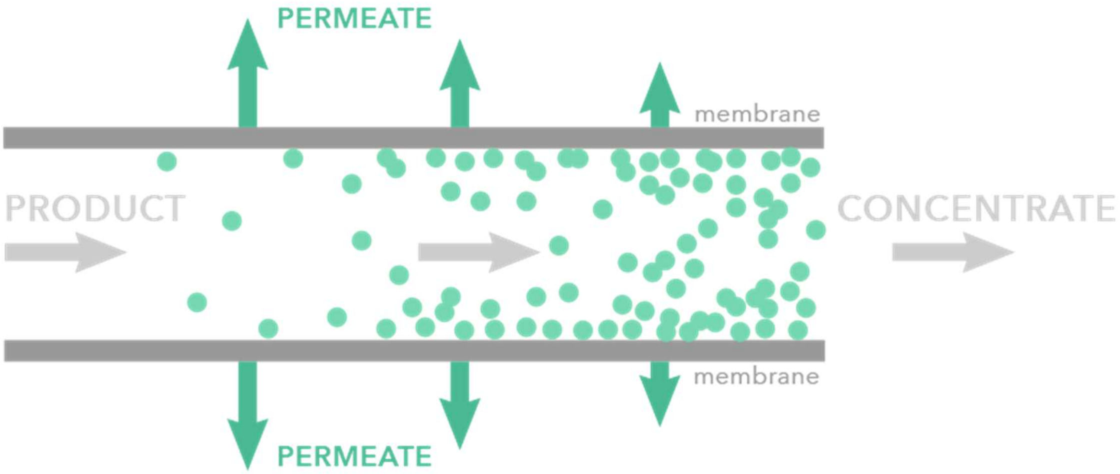

Figure 1.3: Schematic representing tangential flow filtration [1]. While more resistant to membrane blinding than traditional filtration, TFF loses efficiency with increasing solids content.

costing millions, or even billions, of dollars per kilogram [26]. In stark contrast, current price estimates for PHA are as low as $3.83 per kilogram [27], and traditional polyolefin commodity resins are only $0.82-$1.52 per kilogram [28]. This narrow profit margin of PHA production requires separation technologies that minimize operational costs while maintaining a high throughput to manage the high production rates of a commodity polymer. While there are other separation processes that have been explored for the collection of purified PHA, such as dissolved air flotation [29] and even consumption and excretion by animals [30-32], these methods are limited in their ability to generate purified polymer at the necessary throughput and purity for industrial application. For these reasons, centrifugation will be regarded as the most appropriate separation process for PHAs, and the research presented here will keep in mind the behavior of these polymers as they interact with the relevant industrial centrifuges available on the market.

There are two main designs of industrial high-throughput centrifuges available: disc-stack and decanter centrifuges [33-35]. In addition, there are many specialty centrifuges that combine aspects of these centrifuges with other technologies, such as incorporating filtration screens. Disc-stack centrifuges consist of a chamber, referred to as the bowl, equipped with a central shaft with a series of stacked discs, typically angled between 40 and 50 degrees and spaced 0.5-3.0 mm apart [36]. As the process fluid is introduced to the bowl, the fluid must travel through the spaces between the discs in order to exit the centrifuge. During operation, the discs rotate at several thousand RPMs to generate acceleration ranging from 4,000-14,000 x g [37]. The small spacing between the discs minimizes the distance suspended solids must travel before being captured, at which point they begin to migrate outward on the disc before accumulating on the

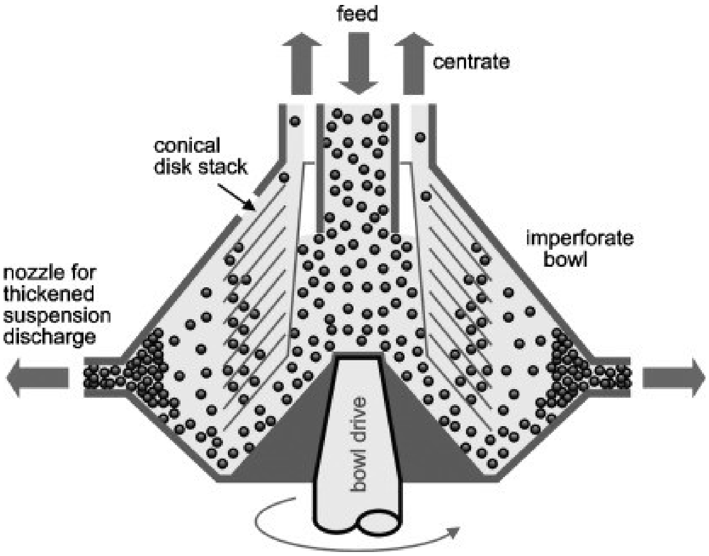

Figure 1.4: Schematic of a disc-stack centrifuge [4].

walls of the bowl, either to be intermittently discharged by automatic hydraulic opening of the bowl, or by continuous discharge through nozzles.

Decanter centrifuges consist of a hollow cylindroconical bowl that tapers to a cone on one end [3]. A screw pushes accumulated solids from the bowl and up into the cone section, where the solids are further dewatered before exiting the centrifuge into a chute. Meanwhile, the clarified liquid spills over an impeller on the open circular side of the bowl. Solids capture can be enhanced by raising the impeller and thereby increasing the holdup volume and retention time within the bowl, while solids dryness can be enhanced by lowering the impeller to lower the liquid volume and allow the solids to spend more time in the dewatering phase of the bowl.

The selection and sizing of a centrifuge is dependent on the characteristics of the particles to be separated and the surrounding fluid. The effect of these characteristics on settling velocity can be ascertained from Stokes' Law:

$$v = \frac{2}{9}\frac{\rho_p - \rho_f}{\mu} g R^2$$

Where:

ρ_p is the density of the particle

ρ_f is the density of the surrounding fluid

g is the gravitational field strength

μ is the dynamic viscosity

R is the particle radius

Disc-stack centrifuges are advantageous for particles of low settling velocity, since the distance the particle must travel is only the spacing between the discs, typically 0.5-3.0mm. It can be deduced, then, that disc-stack centrifuges may be used in instances where particles are small or have low density, or when the surrounding fluid is highly viscous. In PHA production, this is most applicable to concentration of intact cells, as the overall density of the cell is relatively low, and the fermentation broth is often quite viscous. However, disc-stack centrifuges are limited in their throughput, and are best suited for wet solids content of 10% or less. With packed-cell volumes of over 30%, PHA fermentations may require dilution or extended recirculation times to achieve effective separation and minimal process loss with this centrifuge.

The decanter centrifuge, on the other hand, is more robust in its ability to handle large solids loading and is frequently used in high volume throughput operations such as municipal wastewater sludge dewatering, petrochemical refining, and PVC resin production. Once cell lysis occurs, releasing the dense PHA granules, and the viscous fermentation broth is replaced with fresh wash water, the settling velocity of the granules becomes appropriate for separation in a decanter centrifuge.

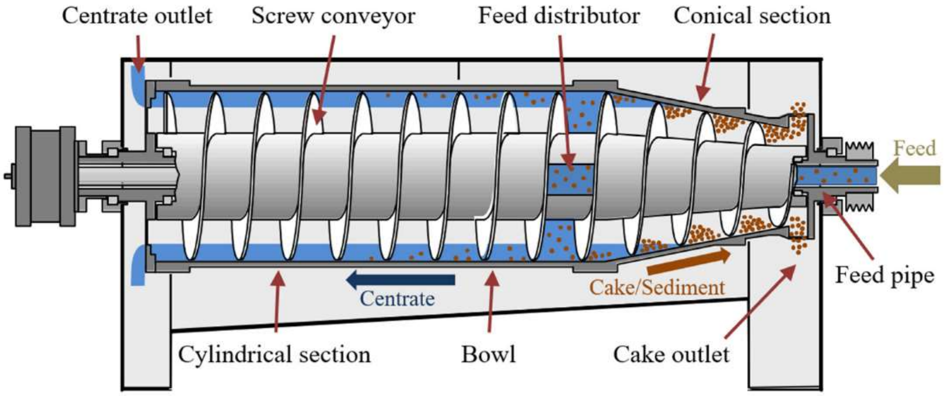

Figure 1.5: Schematic of a Decanter Centrifuge [3] .

As previously discussed, PHA granules in their native state are kept amorphous by the cell. As the granules are released from the cells and proteins associated with the granules become denatured, the granules begin to crystallize [38] [39]. However, if the granule remains coated with protein or is otherwise kept in an amorphous state prior to sedimentation, the granules will aggregate when forced into contact with one another and crystallize in large masses [39]. This leads to residual proteins and other biological components to be retained within these agglomerates, mitigating further purification of the polymer. Furthermore, practical handling issues arise if the aggregates are too large to be managed with conveying systems commonly used in downstream processing or for polymer extrusion. This aggregation phenomenon is a significant challenge when it occurs within the aforementioned industrial centrifuges. Amorphous granules will seize the discs in a disc-stack centrifuge, while the decanter centrifuge will form large agglomerates that clog pipes and pumps in subsequent purification (Figure 4).
In addition to the granule-associated proteins, research has suggested that the granules are plasticized by intragranular water [40, 41], although this is disputed by some [39]. Sedlacek et al. observed that PHA crystallized within cells when exposed to 200 g/L salt solution, whereas all other lysis methods required drying of the PHA before crystallization could be observed [41]. The authors attributed this observation to the removal of intragranular water through osmotic pressure. During industrial aqueous processing of PHA, the granules become compacted during centrifugation, during which coalescence occurs if they are still in an

amorphous state. After coalescing, the polymer becomes sufficiently dewatered

Figure 1.6: **Left:** Centrifuge operator displaying a seized disc-stack due to polymer crystallization during compaction. **Right:** Clogged pipe due to large agglomerates formed during separation in a decanter centrifuge**. Image credit: Bryan Scott, RWDC Industries**

by the compressive forces of the centrifuge to initiate crystallization at which point the polymer granules crystallize in large, fused masses.

These masses clog equipment, such as pumps and centrifuges, and can even result in permanent damage to costly machinery. Furthermore, the fused masses of polymer are unable to be further purified and become waste. The following chapters seek to illuminate the mechanism involved in initiating granule crystallization and to develop industrially relevant physical treatment methods to prevent these aggregates from forming.

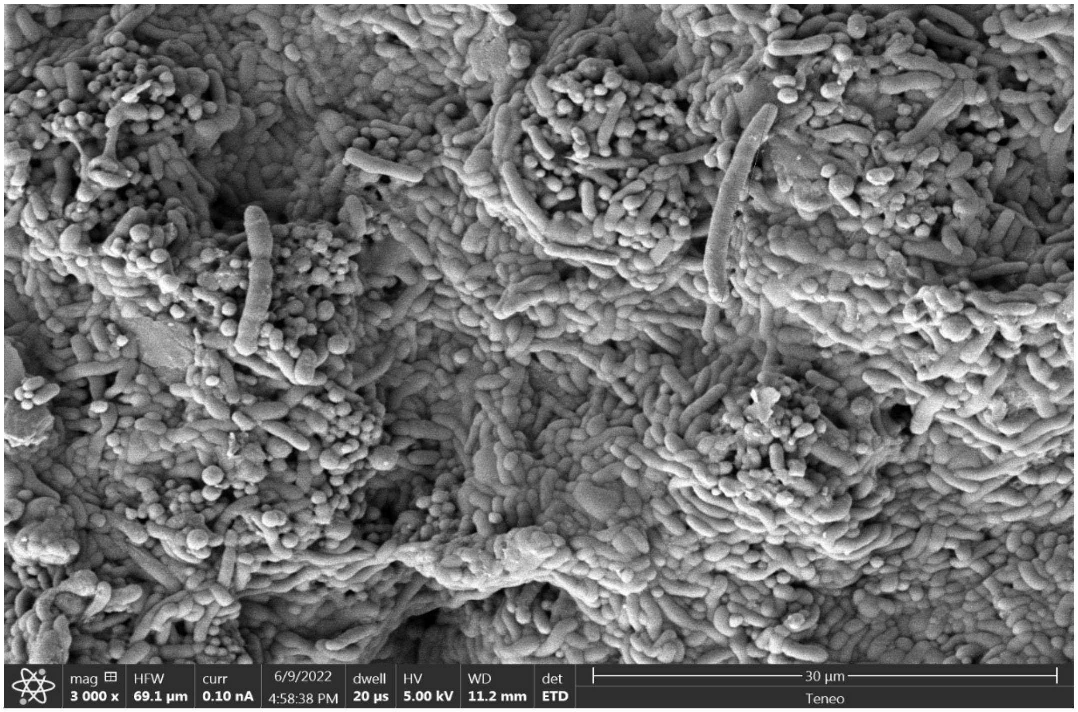

Figure 1.7: SEM image of coalesced granules after centrifugation.

CONSIDERATIONS FOR DESIGNING A MINIMAL AQUEOUS PURIFICATION PROCESS

Much research on the aqueous purification of PHAs has been dedicated to improving the cost-effectiveness, efficiency, and environmental friendliness of producing PHA with satisfactory properties. In contrast, the goal of the research presented herein is to address a specific challenge of aqueous purification, namely granule coalescence, which is relevant to any given aqueous process. As such, the chosen purification method used throughout this work is quite simple, comprising of only the following steps:

1. Biomass concentration through centrifugation.
2. Cell lysis by sodium dodecyl sulfate in a mild alkaline environment.
3. Rinsing of resulting granules to remove residual detergent and biomass.

4. Final rinsing of the granules with dilute sulfuric acid.

The simplicity of the protocol serves to minimize the confounding factors when comparing performance of polymers produced by a fermentation process that has inherent variability across batches. The first step, biomass concentration, removes the majority of residual fermentation byproducts and allows the biomass to be diluted to a constant concentration to maintain consistency evaluating polymers from separate fermentation processes. Cell lysis using sodium dodecyl sulfate in an alkaline environment is the one of the most ubiquitous procedures reported and can be found in a variety of publications and patent applications[42-45]. This lysis method is also indiscriminate in its ability to denature proteins and digest most biological components, unlike a more specialized procedure involving enzymes, for example. Finally, a dilute acid wash ensures trace residual contaminants, such as carboxylic acids, as well as crotonic acid and crotonate end-groups are fully protonated to preclude them from catalyzing degradation via the E1cb mechanism (Fig. 7).

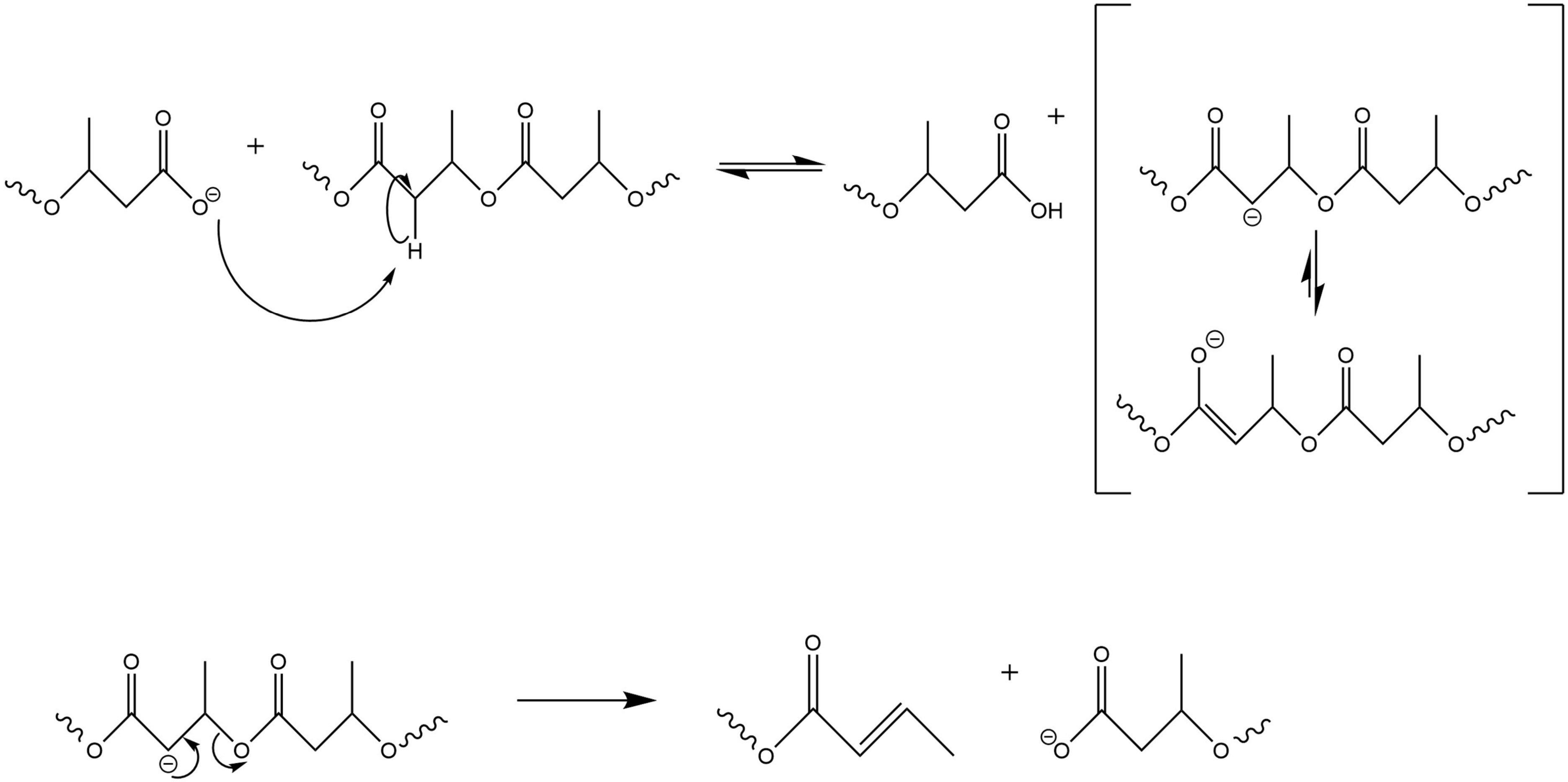

Figure 1.8: E1cb degradation mechanism for PHB

OBJECTIVES AND DISSERTATION OUTLINE

In chapter two, methodology for reproducing and observing granule coalescence at bench-scale will be developed. Currently, there is sparse, if any, literature documenting the challenges associated with PHA granule coalescence. Therefore, there are no reported bench purification methods that seek to observe and address granule coalescence. Most of the commonly used protocols are not representative of any practical industrial process. For example, many publications, seeking to rapidly isolate the purest PHA possible, utilize a hot chloroform extraction followed by ethanol precipitation. Of the purification methods that are aqueous-based, many involve an initial lyophilization step of the PHA-rich biomass. Others describe purification only of PHB homopolymer. The presented research will demonstrate the coalescing phenomenon that is particularly prevalent among PHA copolymers, as well as describe methodology to predict the occurrence of coalescence during aqueous industrial processing.

In chapter three, aqueous treatments of amorphous granules will be examined for the initiation of crystallization prior to sedimentation. These methods will include treatments that both denature the granule-associated proteins, as well as dewatering the granules through an applied osmotic stress. The performance of these treatments will be quantified in terms of particle size – indicating the preservation of native granules – as well as effect on purity, molecular weight, and thermal stability of the resulting polymer, if any.

Finally, chapter four will present concluding remarks about the crystallization of PHA copolymers during purification and offer an outlook for addressing this challenge in PHA processing.

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CHAPTER 2

BENCH PURIFICATION PROTOCOL FOR OBSERVING GRANULE COALESCENCE PHENOMENA[1]

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[1] Broich, Michael. To be submitted to *Industrial and Engineering Chemistry Research*

ABSTRACT

Polyhydroxyalkanoates (PHAs) are a class of biopolymers that have gained attention for their biodegradability and mechanical properties that make them potential candidates for replacing petrochemical-based plastics in consumer applications. Despite solvent extraction and precipitation being commonly reported as a rapid and effective means of purifying these polymers, the quantities of solvents required at a commercial scale are impractical for environmental, safety, and economic reasons. Instead, aqueous purification of the native granules is necessary, selectively digesting and removing the fermentation biomass while collecting purified polymer. One challenge of aqueous purification is the coalescence of amorphous granules during compaction in a centrifugation operation. This present research demonstrates this phenomenon in a variety of Polyhydroxybutyrate-co-Hydroxyhexanoate (PHB-co-HHx) with HHx content ranging from 0-17%. It is observed that PHB homopolymer crystallizes readily upon cell lysis and thereafter the granules are maintained in their native morphology. In contrast, PHAs containing HHx comonomers do not crystallize spontaneously, and the amorphous granules coalesce into large masses. Additionally, an infrared spectroscopy protocol is developed that can predict coalescence behavior based on the in-situ crystallinity of the granules. Finally, the importance of maintaining native granule morphology

is elucidated by the improved thermal stability, purity, and particle size of the resulting polymer.

INTRODUCTION

Industrial production of bio-based and biodegradable polymers is becoming increasingly relevant as legal and societal pressures increase to reduce persistent plastic waste, especially in the single-use consumer plastics market. Polyhydroxyalkanoates are one such alternative, being both bio-based and readily biodegradable in a variety of environments [1-3].

Polyhydroxyalkanoates are energy storage molecules accumulated by certain species of bacteria in the form of intracellular granules[4]. The industrial mass production of these polymers typically involves fermentation in a bioreactor, leading to a high-density cell culture with large accumulations of intracellular PHA, up to 90% by weight [5-8]. Once the accumulation of polymer has completed, it is necessary to release the granules from within the cell membranes and purify them from biological residues.

A remarkable feature of PHA granules is the ability of the bacteria to maintain them in an amorphous state in order for native polymerase and depolymerase enzymes to readily access the accumulated granules for metabolism [9-13]. It is believed that the bacteria maintain the polymer in this amorphous state through granule-associated proteins, called phasins, and intragranular water that behaves as a plasticizer [14-18].

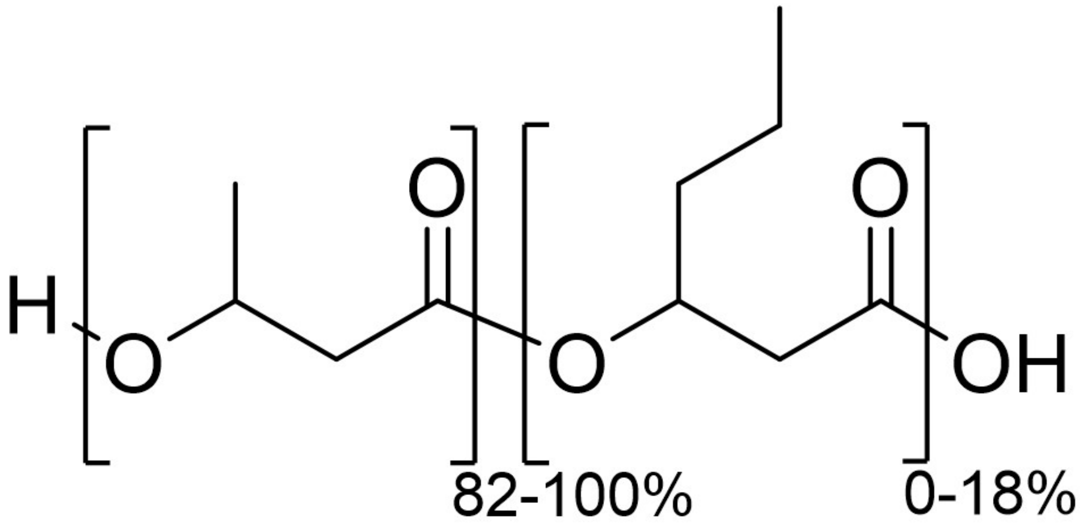


Figure 2.1: Composition of PHAs used in this study.

Freeze-fractured native granules show PHB granules remain elastic even at extremely cold temperatures (-100°C), despite the polymer being incredibly brittle when crystallized [14, 19, 20]. Once the granule-associated proteins are denatured and the plasticizing water is removed, the granules readily crystallize [21]. If the amorphous granules are subjected to compaction through centrifugation or other means once released from the cell, they will coalesce into large masses and subsequently crystallize as the intragranular water is expelled due to the compaction force.

The issue of PHA granule coalescence is of particular interest for industrial, large-scale purification of PHAs. The coalescence and irreversible fusion of granules leads to the failure of centrifugation equipment and pumps, in addition to generating large agglomerates of PHA that must be discarded. Due to the high operation costs and cost of discarded polymer, it is critical to understand and prevent this phenomenon of granule coalescence prior to large-scale separation processes in order to prevent equipment damage and downtime. Therefore, it is necessary to develop purification methods at the bench scale that allow for the reliable recreation of granule coalescence in order to evaluate processing conditions that mitigate granule fusion.

Currently, there is sparse, if any, literature documenting the challenges associated with granule coalescence in an industrial context. Therefore, there are no reported bench purification methods that seek to observe and address granule coalescence. Most of the commonly used protocols are not representative of any practical industrial process. For example, much of the prior work, seeking to isolate the purest PHA possible without concern for industrial scalability, utilize a hot chloroform extraction followed by ethanol precipitation [22-25]. Without a cell lysis step followed by sedimentation of amorphous granules, solvent-based methods do not provide an opportunity to observe compaction and coalescence of native granules.

Many of the reported aqueous-based methods similarly use industrially impractical techniques for purification. For example, many protocols will begin by lyophilizing the biomass prior to purification[26, 27]. Since intragranular water is believed to play a critical role in maintaining granules in an amorphous state, this initial step precludes the observation of coalescence during compaction since the plasticizing water will have already been removed. Additionally, reported aqueous methods often use intense treatments, such as concentrated sodium hypochlorite, that would not be viable for an industrial process due to reagent costs, equipment incompatibilities and detrimental effects on the resulting polymer.

Finally, a limitation of previously reported purification techniques is that many are focused on the purification of PHB homopolymer[28]. Many of the most relevant PHAs with suitable mechanical properties contain at least one comonomer, with proportions ranging from 3-20% or higher. The incorporation of comonomers in PHAs reduces the crystallinity of the polymer and can impact the rate and aptitude of the copolymer granules to crystallize. This means that, while a purification protocol may be suitable for maintaining discrete granules of PHB homopolymer, the difference in crystallization behavior may render the protocol ineffective for PHA copolymers.

The present research will seek to demonstrate and characterize granule coalescence phenomenon during PHA purification. Additionally, the research presented in this chapter will present a purification protocol to clearly differentiate whether a sample of PHA granules will coalesce in a centrifugation process, allowing for the evaluation of industrially relevant techniques for mitigating this occurrence.

MATERIALS AND METHODS

Polymers Tested

Polyhydroxybutyrate-co-hydroxyhexanoate of varying comonomer content and molecular weights were acquired from the New Materials Institute

Biopolymer Center. The properties of these polymers are summarized in the table below.

Table 2.1: Properties of Polymers Tested

	Hydroxyhexanoate Content (%)	Molecular Weight (Mw, Da)
1	0	837,000
2	7	872,000
3	12	1,001,000
4	17	772,000

Purification Protocol

The purification protocol was performed as follows:

1. At the conclusion of fermentation, the culture is heated to 60°C to inactivate the bacteria.
2. The fermentation broth is then centrifuged at 10,000 x g for 20 minutes to capture the PHA-rich biomass.
3. The biomass is suspended again using filtered water.
4. The suspension is subjected to cell lysis using 20 g/L sodium dodecyl sulfate (SDS) at a pH of 9.0.
5. The granules are centrifuged at 10,000 x g for 20 minutes and the compacted PHA is evaluated for granule fusion.

Crystallinity by FTIR-ATR

Fourier-Transform Infrared Spectroscopy (FTIR) was used to evaluate the crystallinity of PHA granules prior to and after lysis treatment and compaction. This was done by placing approximately 5µL of lysis broth on the Attenuated Total Reflectance (ATR) module and drying the sample on the crystal using a stream of nitrogen. The spectrum was then captured and evaluated for the presence of a peak at wavenumbers 1735 and 1723cm^{-1}, which are indicative of amorphous and crystalline PHA, respectively. In addition, wavenumbers 1184cm^{-1} and 1226cm^{-1} were evaluated for a similar shift. Due to an overlap between these peaks, the spectra were subjected to deconvolution using a Gaussian fit in Origin.

Crystallinity by Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed on several powder and film PHA samples. The samples were heated from 20°C to 200°C at 10°C/min. The melting endotherm of the resulting thermogram was integrated and, using an enthalpy of fusion of 146 g/J for 100% crystalline PHA, the crystallinity was determined.

Particle Size Analysis

Particle size analysis was completed using a Malvern Mastersizer 3000 with Hydro MV dispersion unit. PHA granules were suspended in 10% sodium dodecyl sulfate solution and sonicated for full dispersion of the granules. The dispersed granules were then added to the sample cell of the dispersion unit and particle size was measured in triplicate. Results were recorded as the mean and 90th percentile by volume.

Comonomer Content Analysis by Gas Chromatography

The hydroxyhexanoate content of each polymer was determined by gas chromatography. For each sample, approximately 10mg of lyophilized PHA-rich biomass was dissolved in 1mL chloroform within an 8mL reaction tube and subjected to acidic methanolysis at 100°C for 150 minutes in order to hydrolyze the polymer into its monomeric units and convert the monomers into their respective methyl esters. 1mL of water and 1mL of 0.2% toluene in chloroform were added to form two phases, a lower chloroform phase containing the derivatized methyl esters and an upper aqueous phase containing sulfuric acid. The toluene was present to serve as an internal standard across all samples. In addition to the polymer samples, pure 3-hydroxybutyrate and 3-hydroxyhexanoate were subjected to the same derivatization and used to generate standard curves to determine the detector response to each component.

Quantification of Residual Biomass

Residual protein in purified PHA granules was quantified using a modified Lowry assay (BioRad DC assay kit). Oven dried samples were collected and washed with 10% SDS solution with vigorous mixing for 30 seconds. The samples were then centrifuged and the supernatant was collected and directly pipetted into the assay kit. Samples were placed into a 96-well plate and measured at 750nm. Results were quantified in reference to a bovine serum albumin standard.

Residual lipids were quantified using a sulfo-phospho-vanillin assay. Oven dried samples were collected and washed with hexanes with vigorous mixing for 30 seconds. The samples were then centrifuged and the supernatant was pipetted into vials and placed on a 100°C heating block for 1 minute to evaporate the hexane. Then, 200μL sulfuric acid was added and the sample was incubated at 100°C for 10 minutes. The samples were then placed on ice and a solution of

15mM vanillin in 50% phosphoric acid was added to each sample. The samples incubated at 37°C for 15 minutes before loading into a 96-well plate. The presence of unsaturated fatty acids resulted in a color development that was quantified at 540nm in reference to canola oil as a standard.

Molecular Weight Determination

The molecular weight of purified polymers was determined using a Malvern Omnisec GPC equipped with triple detection: viscometer, refractive index detector, and light scattering detector. Two HMJ HM-MBMMW2010 columns were installed in the instrument. The instrument was calibrated with narrow 105 kDa and wide 245 kDa polystyrene standards. Polymer samples were dissolved in chloroform at a concentration of 1 mg/mL and filtered through a 0.2 µm PTFE filter. 100 µL was then injected into the instrument and elution was performed using chloroform at 35°C and 1 mL/min for 40 minutes.

Thermogravimetric Analysis

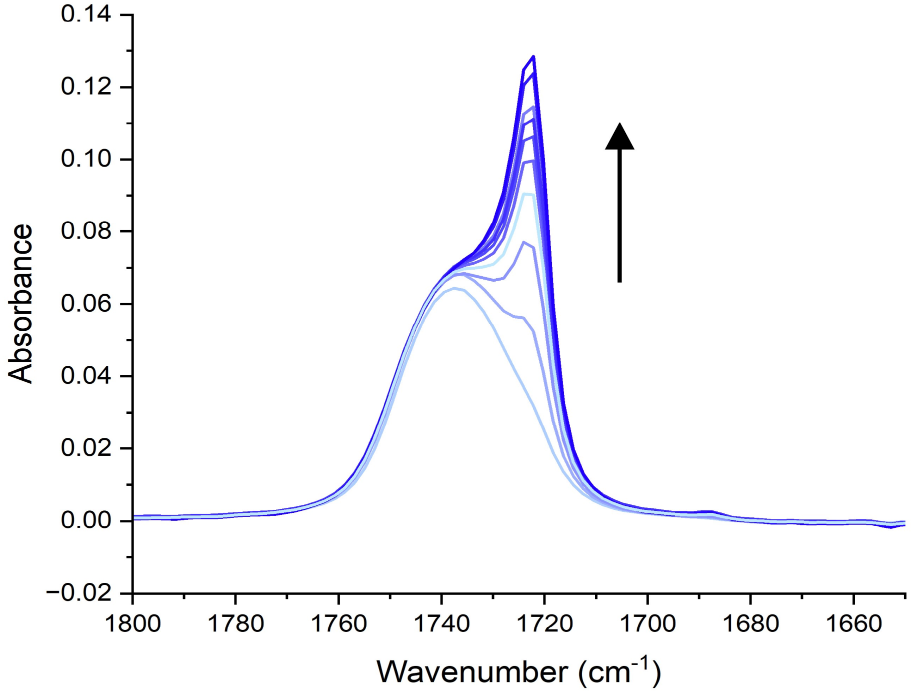
TGA analysis was conducted on TA Instruments Discovery TGA. 7-12 mg samples were loaded on platinum TGA pans and heated from 40C to 600C at 20C/min under an Air (or Nitrogen) purge of 25 mL/min. The data generated was analyzed using the TA Instruments TRIOS software to obtain the onset point of thermal degradation.

RESULTS

Determination of Crystallinity by FTIR

For determination of polymer crystallinity, the traditionally employed methods are differential scanning calorimetry and x-ray diffraction. These methods, however, have the requirement that the polymer must be dry and relatively pure. For the purpose of monitoring crystallinity of granules in an aqueous environment, it is necessary to measure crystallinity without drying the polymer, since doing so will induce crystallinity that is not representative of the aqueous state of the granule. Additionally, the broth surrounding the granule is highly contaminated with biological components from the cell lysis process. For these reasons, FTIR was used to estimate the crystallinity of the granules in their native aqueous environment.

To validate the use of FTIR for this purpose, a variety of clean, dry PHA samples with varying degrees of crystallinity were selected and placed on the ATR crystal. Peaks were integrated at wavenumbers 1723 and 1735, and the ratio of these peaks were taken as a crystallinity index, denoted as FTIR crystallinity index 1. In addition, the ratio of peaks at wavenumbers 1220 and 1180 were similarly taken as another crystallinity index, denoted as FTIR crystallinity index 2. The crystallinities of these same samples were then measured using DSC and a linear fit produced a relationship between the crystallinity index and the percent crystallinity (Figure 3). With this correlation in place, the crystallinity of aqueous granules as measured by FTIR can be estimated and compared to DSC data.



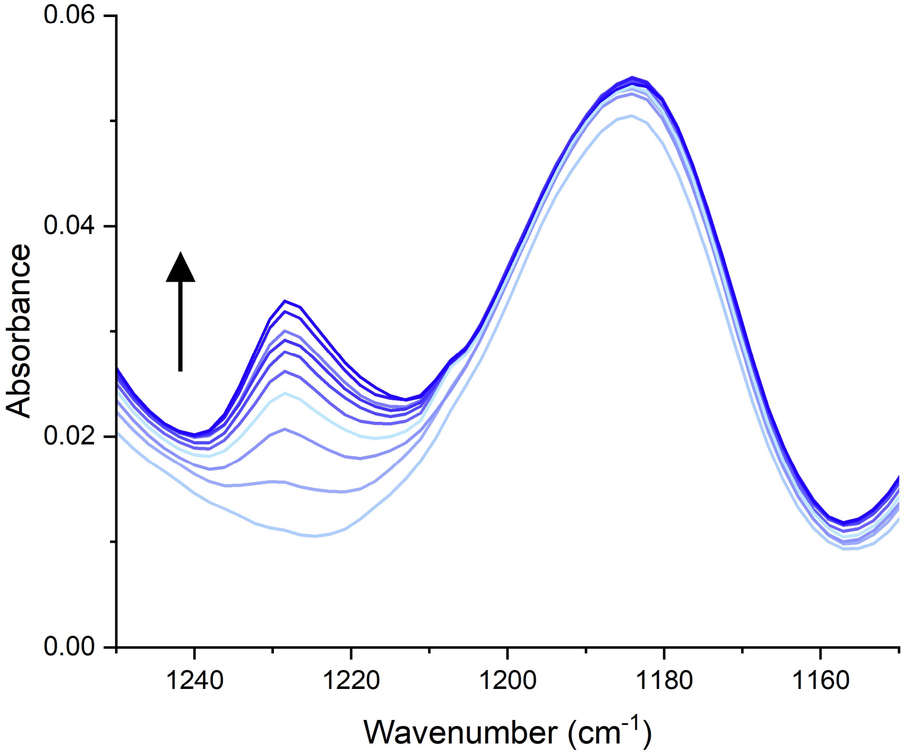


Figure 2.2: Top: Crystallization of solvent-cast PHA film on FTIR-ATR. As the polymer crystallizes, the peak at wavenumber 1740 shifts to 1720. Bottom: As the film crystallizes, the peak at 1226 increases relative to 1184.

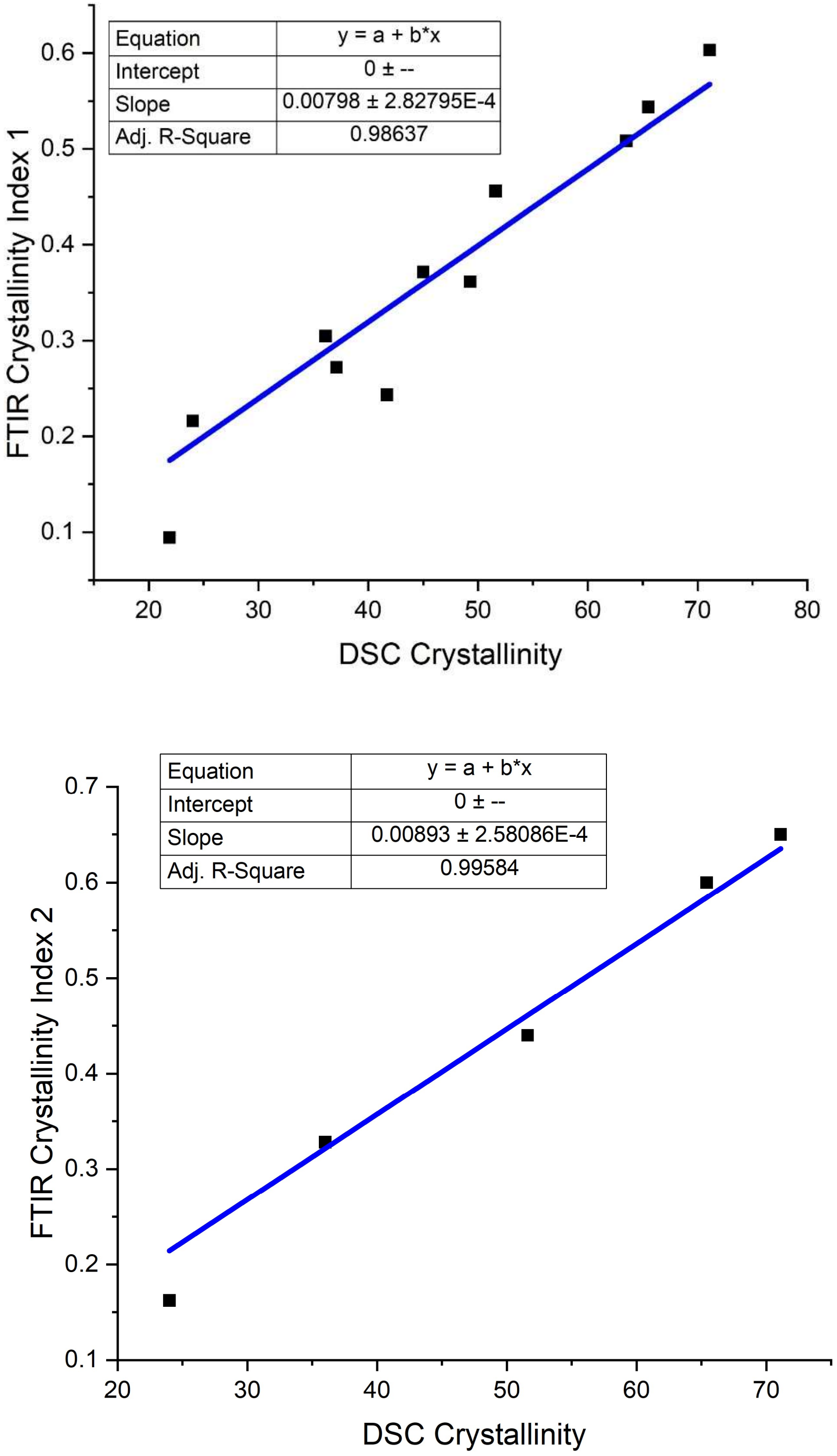


Figure 2.3: Linear fit between FTIR crystallinity indices and DSC crystallinity measurements.

Crystallization of PHB homopolymer

As reported previously in literature, PHB homopolymer crystallizes readily upon lysis and denaturation of the granule-associated proteins. Figure 4 shows the FTIR spectra of the homopolymer before and after the addition of sodium dodecyl sulfate to induce lysis. The broad peak around wavenumber 1180 cm^{-1} is attributed to the amorphous PHB, while the peak at 1224 cm^{-1} indicates the formation of crystalline PHB. The crystallinity of the granules post-lysis was determined to be 7% using the correlation for FTIR crystallinity index 2. This extent of crystallization is quite small compared to the expected crystallinity of PHB homopolymer, with a typical value of 60% crystallinity or higher reported.

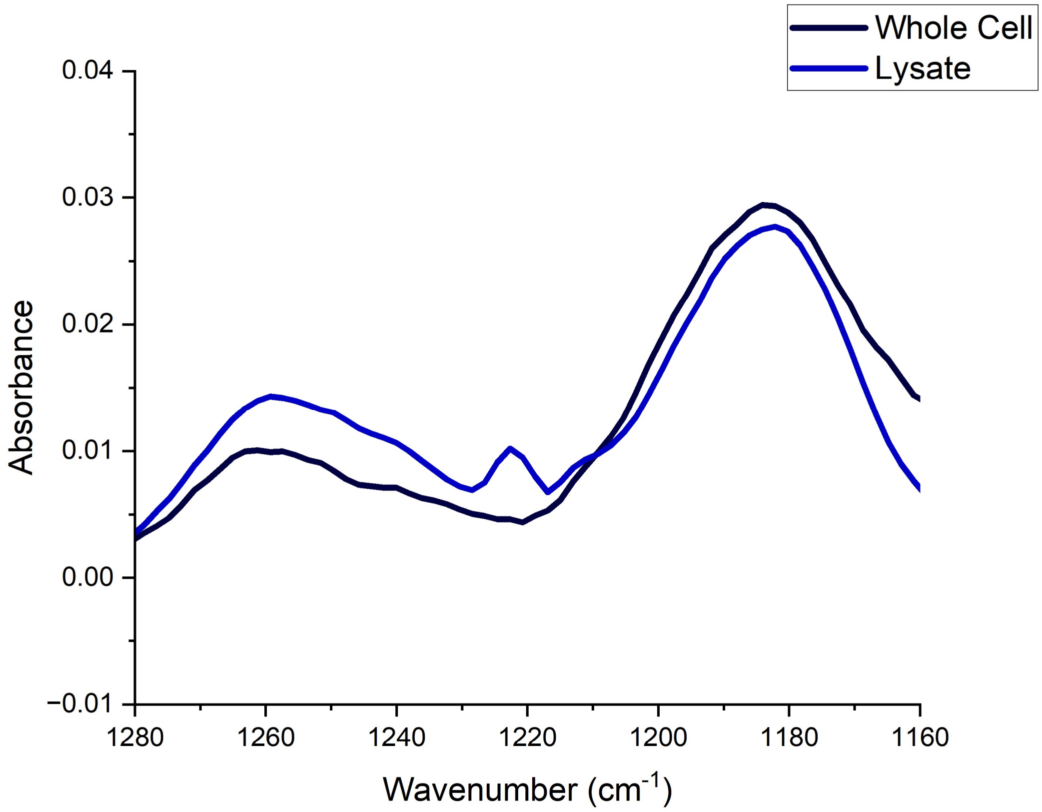


Figure 2.4: FTIR spectra of PHB homopolymer before and after cell lysis.

Due to the small degree of crystallinity, no shift could be detected from wavenumber 1735 cm^{-1} to 1724 cm^{-1} due to the peaks' close proximity. Furthermore, repeated centrifugation and washing of the granules in water did not increase the crystallinity of the granules. Only by drying the isolated granules did the crystallinity increase to its typical value expected of PHB.

Particle size of PHB homopolymer

Particle size analysis of the resulting purified granules revealed a monomodal distribution of particles in the range of 600nm to 9μm, with a mean of 1.4μm and with 90% of the granules having diameter less than 2.4μm. This measurement is consistent with SEM imaging, which revealed discrete, roughly spherical granules less than 3μm in diameter.

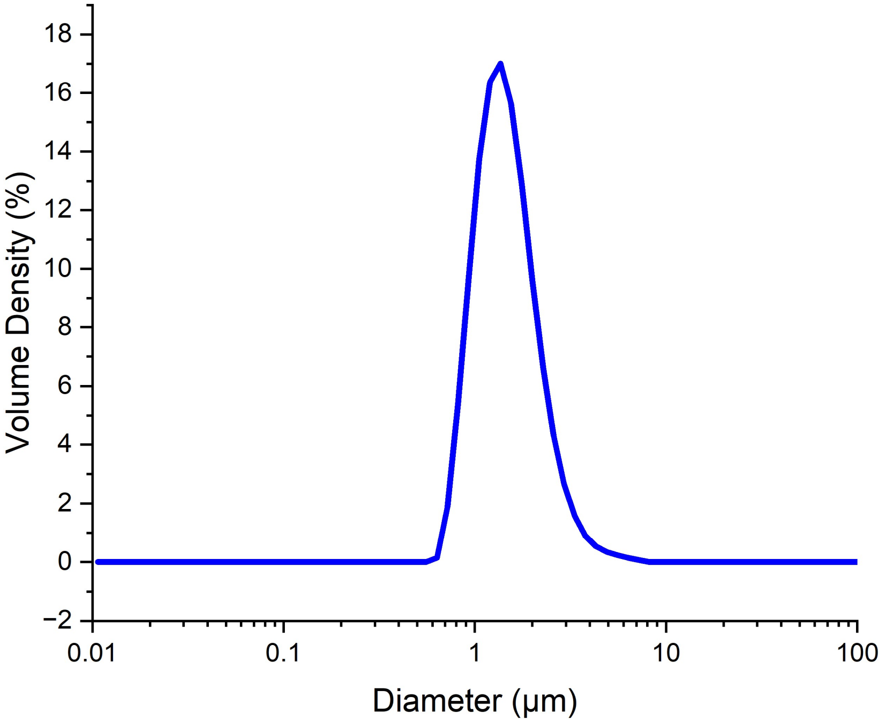


Figure 2.5: Volumetric particle size distribution of PHB homopolymer after lysis, centrifugation, and subsequent dispersion.

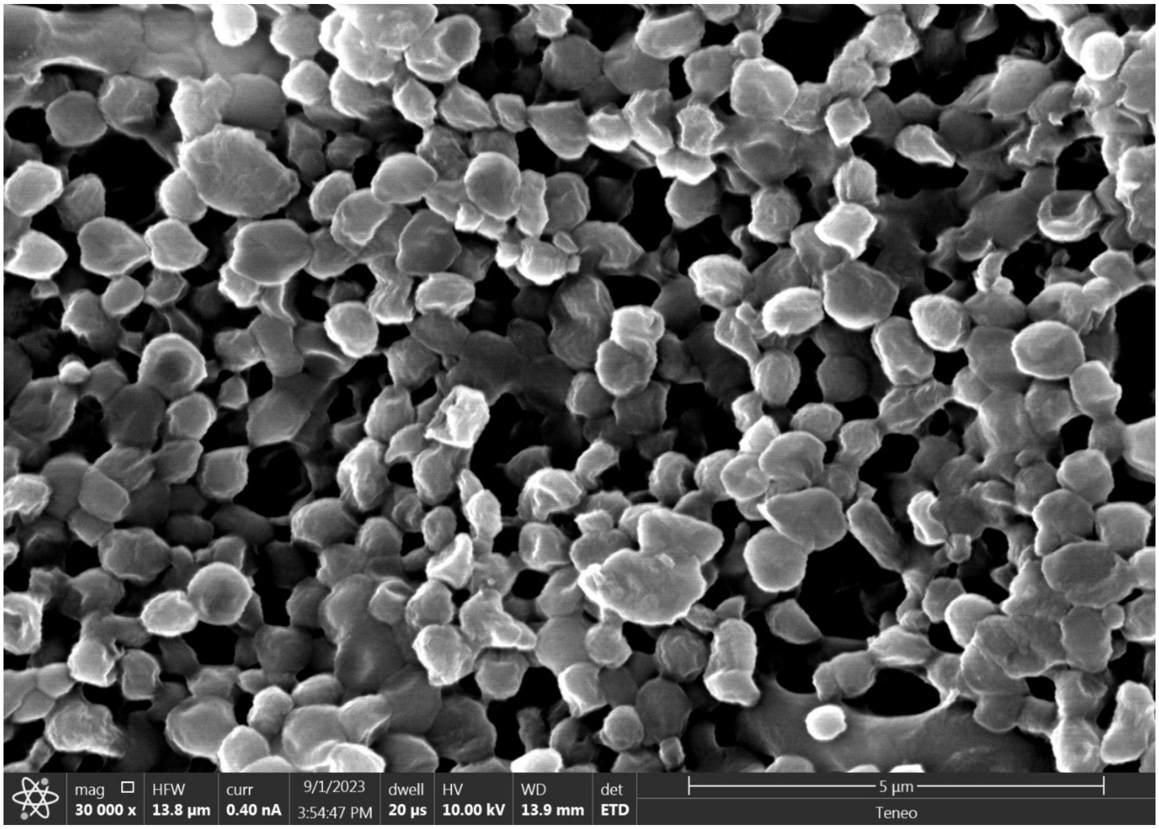


Figure 2.6: SEM image of Purified PHB homopolymer.

Crystallization of Polyhydroxybutyrate-co-Hydroxyhexanoate Granules

For the PHAs containing HHx comonomer, cell lysis did not initiate detectable crystallinity on FTIR. Figure 7 shows the FTIR spectra for PHA with 17% HHx and indicates no development of a signal at wavenumber 1224 cm^{-1} upon cell lysis. Similarly, no shift is detected at wavenumber 1735 cm^{-1}. Upon compaction of these polymers during centrifugation, the recovered granules were irreversibly fused into a single rubbery mass that progressively became more rigid.

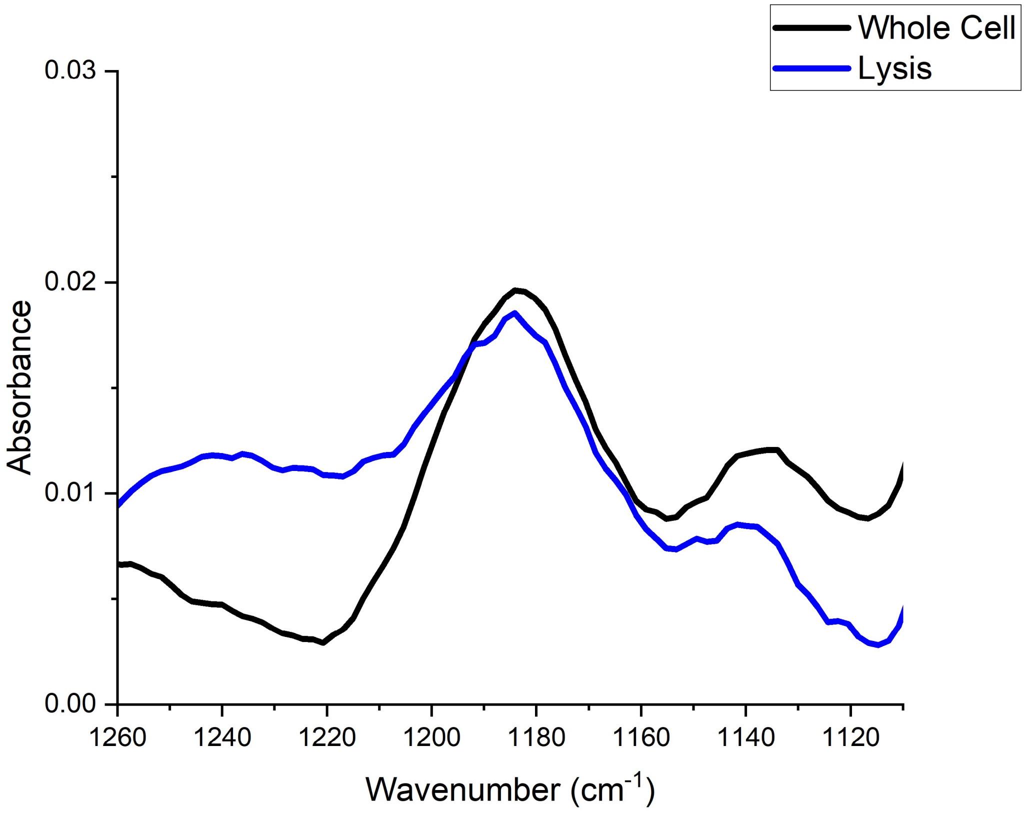


Figure 2.7: FTIR showing no development of crystallinity after lysis in 17% HHx PHA granules.

Significant development in crystallinity was detected at both wavenumber 1224 cm^{-1} and 1724^{-1}, indicating that the amorphous granules had coalesced and subsequently crystallized together. This phenomenon of granules maintaining an amorphous state even after lysis, followed by substantial crystallization upon compaction during centrifugation was consistent among all polymers ranging from 6 to 17% HHx content.

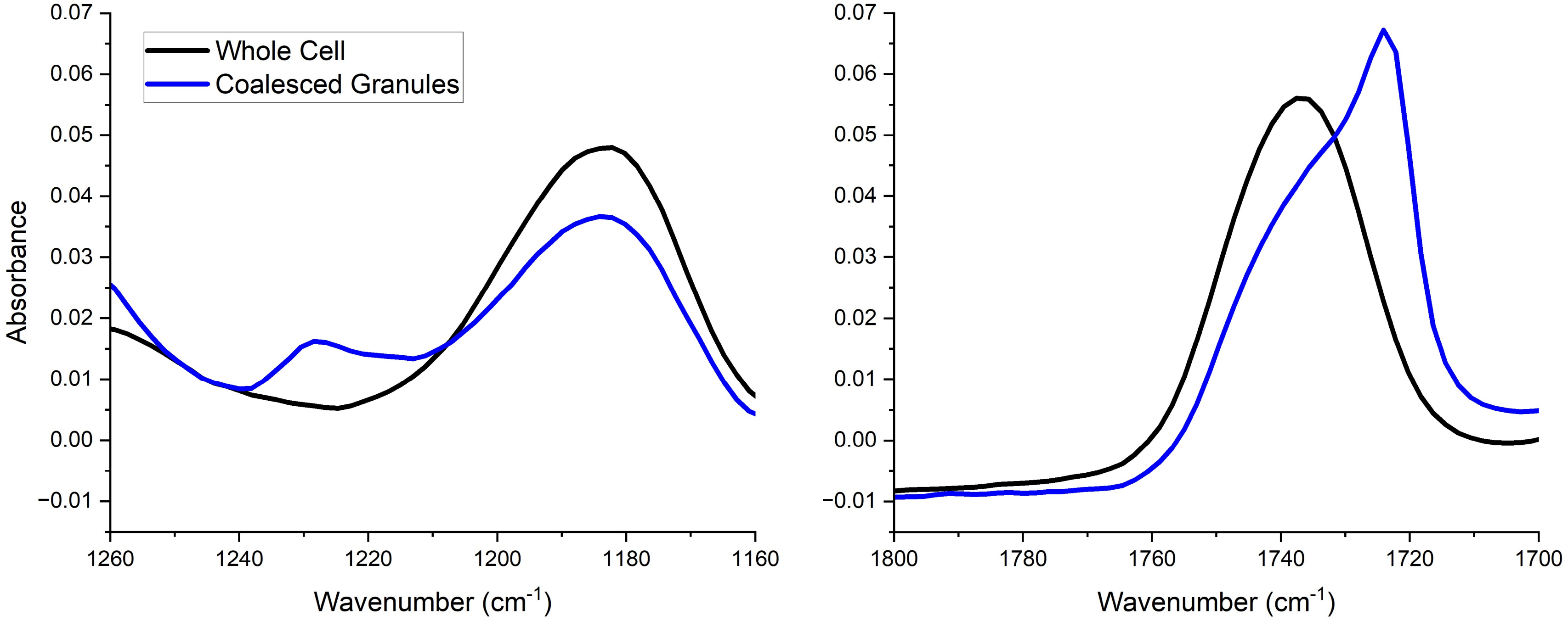


Figure 2.8: FTIR of PHA granules (7% HHx) in intact cells and in their final coalesced state.

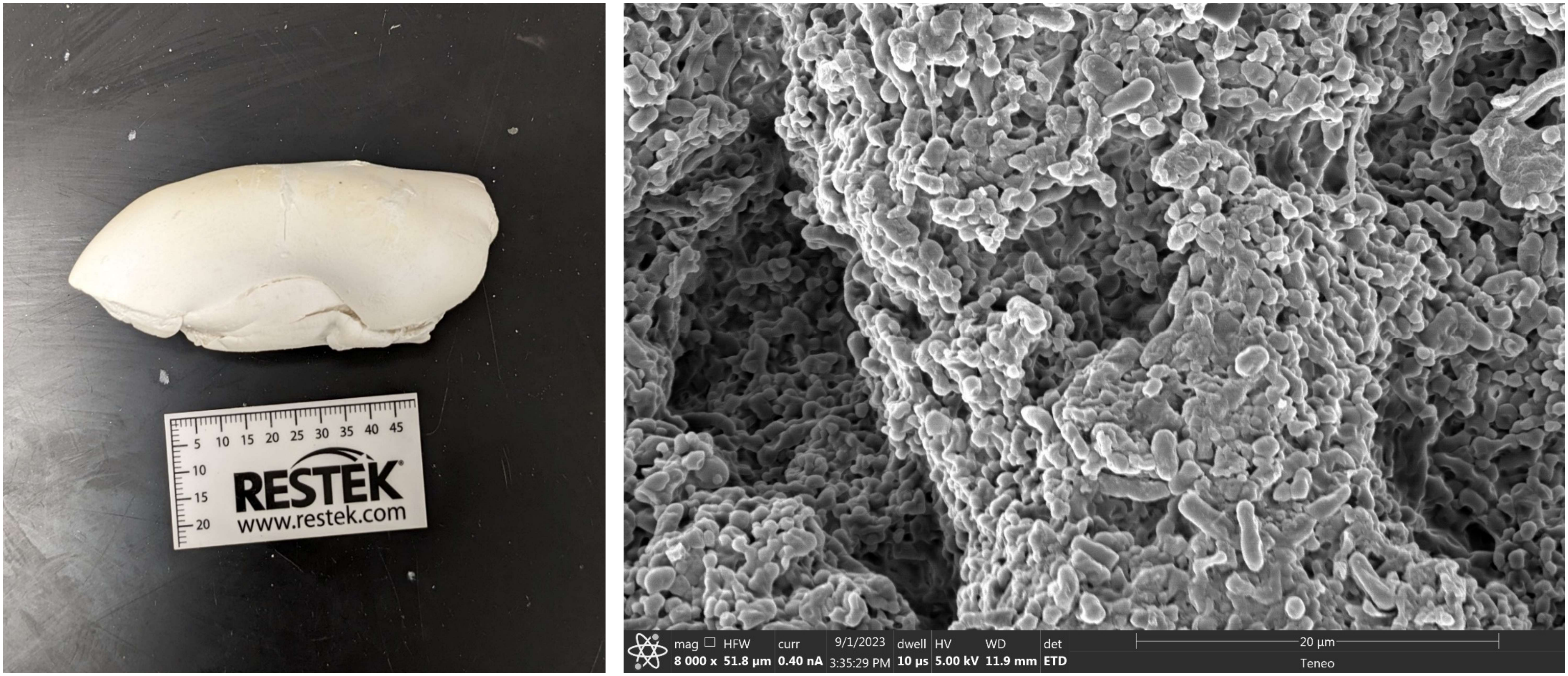


Figure 2.9: Coalesced PHA granules with HHx content of 12%. ***Left:*** *The granules coalesced immediately upon compaction during centrifugation and subsequently crystallized into a single mass.* ***Right:*** *SEM image of coalesced mass.*

Purity of homopolymer and copolymers

Residual lipid and protein content was quantified for both the purified PHB granules and the PHA agglomerates. Since the PHAs coalesced into singular large masses, they were first milled in a cryomill to obtain a powder that could be extracted. The PHAs that coalesced were found to have significantly higher amounts of residual biomass than the PHB homopolymer.

Table 2.2: Residual biomass components in purified granules

Hydroxyhexanoate Content (%)	Residual Lipid Content (mg/g)	Residual Protein Content (mg/g)	Purity
0	0.8	0.4	99.88%
7	14.2	12.7	97.31%
12	11.8	9.7	97.85%
17	15.1	12.0	97.29%

Thermogravimetric analysis also demonstrated lower thermal stability of coalesced granules compared to the PHB homopolymer, with onset of degradation occurring as much as 28°C sooner in the coalesced granules (figure 10).

Table 2.3: Onset of degradation for each polymer by thermogravimetric analysis.

Polymer HHx Content	Onset of degradation (°C)
PHB homopolymer	277
7% HHx	249
12% HHx	256
17% HHx	264

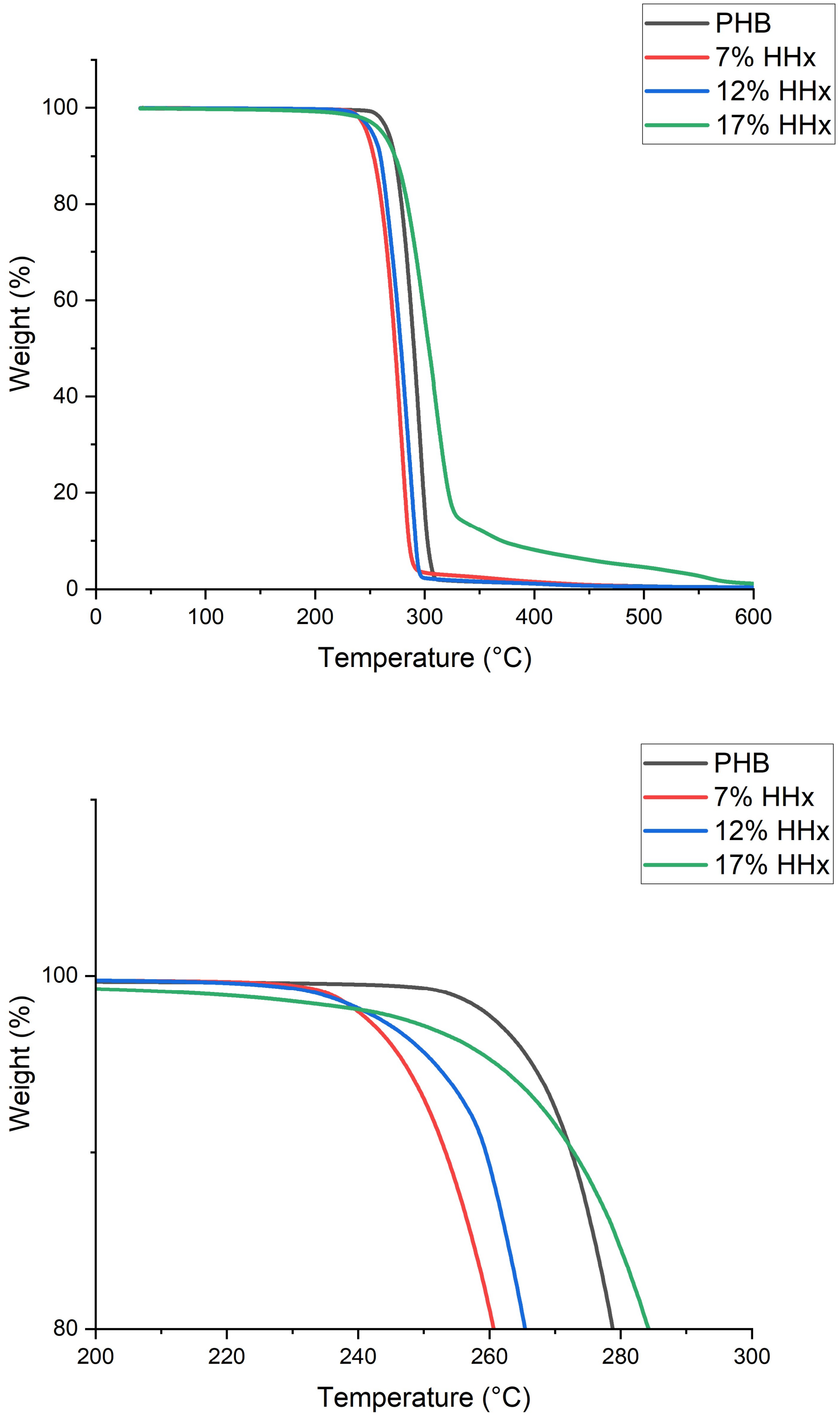


Figure 2.10: Thermogravimetric Analysis of PHB homopolymer and coalesced copolymers.

DISCUSSION

A distinct difference in granule crystallization behavior was observed between PHB homopolymer and PHAs containing 7 to 17% HHx content. Cell lysis alone was sufficient to initiate some crystallization in the homopolymer, while all polymers containing comonomer remained amorphous up until compaction during centrifugation. The resulting difference was stark; the PHB remained as individual, discrete granules, as evidenced by particle size analysis and SEM imaging, but the copolymers coalesced and crystallized into a single mass in the centrifuge.

The contrast in crystallization behavior between PHB homopolymer and the PHA HHx copolymers may be attributed to a synergistic effect of random branching interrupting crystallizable segments of polymer combined with the plasticizing effect of water. With PHB homopolymer, theoretically the entire polymer is eligible for crystal formation, with only entanglements preventing 100% crystallinity. In contrast, even a 5% comonomer content reduces the average crystallizable segment to only 19 units, as the branched HHx cannot be incorporated into the crystal structure and must be excluded into an amorphous region. Therefore, it may be that the more branched polymers have fewer opportunities for crystallization, and thus more plasticizing water must be removed than is required of the homopolymer.

Another distinct difference between the PHB homopolymer and the PHA copolymers was the resulting purity. Since the PHA granules coalesced immediately following lysis, further washing of the granules could not be accomplished. The agglomerates were discolored, and lipid and protein analyses revealed significant quantities of residual biomass remained entrained within the agglomerates. These residual biological contaminants contributed to significantly lower thermal stability of these polymers, with degradation occurring as much as 28°C sooner than the homopolymer. This is attributed to degradation via the E1cB mechanism, which entails abstraction of hydrogen adjacent to a carbonyl group[29]. The resulting anion cleaves a leaving group to result in an alkene, specifically crotonic acid in the case of PHAs. Biological materials contain many potentially basic components, such as carboxylates, that serve to catalyze degradation through this E1cB mechanism.

An unexpected result was the limited crystallization that occurred in the PHB homopolymer. While PHB is typically reported to have a crystallinity of 60% or higher, the FTIR analysis revealed that, after lysis, the granules only attained a crystallinity of 7%. The granules remained steady at this crystallinity despite repeated centrifugation and washing. Only upon fully dehydrating the granules did they crystallize to the expected extent.

Lauzier et al. proposed a topotactic crystallization mechanism yielding granules with crystalline outer shells with amorphous cores [30]. In this model, the core of the granules remains amorphous due to plasticizing water trapped within the granule. Upon complete drying of the isolated granules, the crystallinity increases dramatically. Such a mechanism is consistent with the data presented here. As the protein coating on the granule is denatured by the lysis conditions and plasticizing water diffuses out of the surface layer of the granule, the outer surface crystallizes. As this crystalline shell forms, the remaining polymer inside the granule is kept amorphous due to the inability for water to further diffuse out of the granule. As these partially crystallized granules are compacted, their amorphous cores remain individually contained, with only crystalline-to-crystalline contact among the granules. This protection from coalescing provided by the crystalline shells results in the granules remaining in their native shape, as confirmed by particle size analysis and SEM imaging.

CONCLUSION

The preceding experiments investigated the crystallization of PHA granules upon release from the cell. Using FTIR, it was demonstrated that a small degree of crystallinity developed for the homopolymer prior to sedimentation, which corresponded to the preservation of granules in their native morphology. In contrast, the PHA copolymers demonstrated no crystallization prior to sedimentation, which corresponded to granule coalescence and subsequent crystallization. This correlation suggests that FTIR can be used as an indicator for successful crystallization of granules during polymer processing. Furthermore, aqueous treatments may be evaluated using this technique, allowing for rapid high-throughput confirmation of whether a given treatment has resulted in granule crystallization.

Prior research has suggested that PHA granules in aqueous suspension crystallize in a shell-core structure, with an amorphous core surrounded by a crystalline shell. An unintended consequence of the present research was to confirm this suspicion, based on the very limited degree of crystallinity developed by the granules while they remained in an aqueous environment. Fully dried granules developed nearly 10-fold crystallinity, suggesting that the plasticizing water in the core had remained until it could diffuse by evaporation.

Particle size analysis, purity assays, and thermal stability measurements all clearly indicate that granule coalescence is detrimental to PHA purification. Future research should focus on processing techniques to initiate granule crystallization to avoid granule coalescence.

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CHAPTER 3

CHEMICAL AND PHYSICAL TREATMENTS FOR THE CRYSTALLIZATION OF DISPERSE PHA GRANULES DURING AQUEOUS DOWNSTREAM PROCESSING[2]

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[2] Broich, Michael. To be submitted to *Journal of Biotechnology*

ABSTRACT

Interest in commercialization of Polyhydroxyalkanoates (PHAs) has persisted for decades due to their biodegradability and ability to provide mechanical properties comparable to existing petrochemical-based plastics. However, commercialization of these polymers has not yet been achieved, largely due to challenges associated with their purification. One such challenge is the amorphous nature of the polymer as it is synthesized as intracellular inclusion bodies. During purification, these amorphous granules coalesce into masses that are unsuitable for further processing and can damage centrifugation equipment. Previous research indicates that this coalescence does not occur if the granules develop even a small degree of crystallinity. The present work examines the underlying mechanisms that maintain the granule in an amorphous state as well as industrially-relevant treatments to initiate crystallization. It is demonstrated that, in polyhydroxybutyrate-co-hydroxyhexanoate (PHB-co-HHx), increasing HHx content leads to granules that are more resistant to crystallization. In all cases, crystallization can only be initiated if a certain minimum osmotic stress is applied to the granules, suggesting that intragranular water plays an integral role in maintaining granules in an amorphous state. Furthermore, analysis of purity, molecular weight, and particle size reveal successful preservation of the native granule morphology as compared to PHB homopolymer.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of biopolymers that are naturally produced by a variety of microorganisms as energy storage molecules[1, 2]. Because of their ubiquity in the natural environment, PHAs are widely recognized and consumed by many microorganisms that possess the necessary enzymes to digest them[3-6]. For this reason, PHAs are biodegradable in a wide variety of ambient environments, including marine environments[5-7]. While polyhydroxybutyrate (PHB) is the most ubiquitous polymer among PHAs, particular interest has grown for PHA copolymers, due to their improved toughness and flexibility compared to PHB homopolymer[8]. These copolymers typically contain a 3-hydroxybutyrate backbone with a small percentage of 3-hydroxyvalerate[9] or 3-hydroxyhexanoate (HHx)[10] incorporated randomly throughout, although branched comonomers of higher length have also been

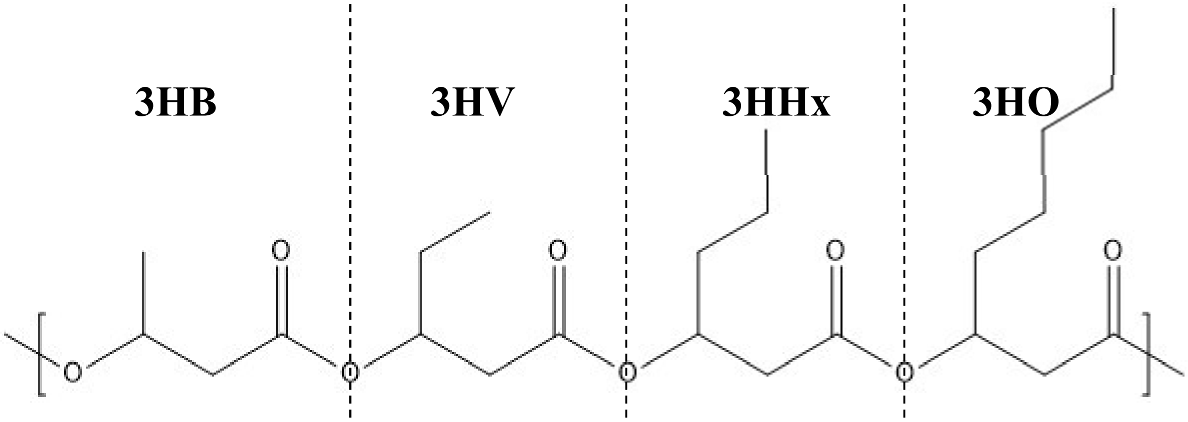


Figure 3.1: Examples of PHA Monomers. 3HB: 3-Hydroxybutyrate; 3HV: 3-Hydroxyvalerate; 3HHx: 3-Hydroxyhexanoate; 3HO: 3-Hydroxyoctanoate

investigated, such as 3-hydroxyoctanoate[11]. The structures of these monomers are depicted in figure 1.

For decades, research has sought to utilize these polymers to replace petrochemical-based plastics. This undertaking has been limited, as the production of PHAs poses a number of challenges due to the biological nature of the production process[12]. One such challenge is the unusual state of native PHA granules, which are kept amorphous by the cell in order for its native enzymes to interact with the granules for anabolism and catabolism[13]. Despite the fact that ambient conditions are above the glass transition temperature and well below the melting temperature of PHA, the cell maintains the granules in an amorphous state using granule-associated proteins and the plasticizing effect of intragranular water[14, 15]. Only after the denaturation of granule-associated proteins and the removal of intragranular water will the granules begin to crystallize. During industrial aqueous processing of PHA, the granules become compacted during centrifugation, during which coalescence occurs if they are still in an amorphous state. After coalescing, the polymer becomes sufficiently dewatered by the compressive forces of the centrifuge to initiate crystallization, at which point the polymer granules crystallize in large, fused masses. These masses clog equipment, such as pumps and centrifuges, and can result in permanent damage to costly machinery. Furthermore, the fused masses of polymer are unable to be further purified and become waste.

It has been previously documented that this phenomenon can be replicated in a bench purification process, making it is possible to examine various chemical and physical treatments that can serve to crystallize polymer granules while they remain individually dispersed in the aqueous suspension. As previously discussed, granule-associated proteins and intragranular water serve to maintain the granules in an amorphous state[13]. During the initial harvesting of intact whole-cell biomass, the granules remain within the cell, retaining their associated proteins and intragranular water[16, 17]. During the subsequent purification step, the cells are lysed, releasing the granules. The granule associated proteins are then easily denatured with detergents, acid or alkali, elevated temperature, or a combination thereof[12, 18, 19]. However, for PHA copolymers, the remaining intragranular water is sufficient to maintain the granule in an amorphous state. This is due to the fact that, upon crystallization, the branched units of the polymer must be excluded from the crystal structure. This requirement reduces the aptitude of the PHA granule to crystallize, requiring more intragranular water to be removed to initiate crystallization. Crystallization prior to sedimentation is critical to maintaining the native morphology of PHA granules and to prevent coalescence. The present research will build on previous techniques to probe this phenomenon and investigate the effects of various treatments to encourage the diffusion of this intragranular water out of the granule in order to initiate crystallization of the granule prior to sedimentation.

MATERIALS AND METHODS

Polymers Tested

Polyhydroxybutyrate-co-hydroxyhexanoate of varying comonomer content and molecular weights were acquired from the New Materials Institute

Biopolymer Center. The properties of these polymers are summarized in the table below.

Table 3.1: Properties of Polymers Tested

Hydroxyhexanoate Content (%)	Molecular Weight (Mw)
0	837,000
0.2	1,270,000
7	872,000
12	1,001,000
17	772,000

Purification Protocol

The purification protocol was performed as follows:

1. At the conclusion of fermentation, the culture is heated to 60C to inactivate the bacteria.
2. The fermentation broth is then centrifuged at 10,000 x g for 20 minutes to capture the PHA-rich biomass.
3. The broth is suspended back to the original volume using filtered water.
4. The broth is subjected to cell lysis using 20 g/L sodium dodecyl sulfate (SDS) at a pH of 9.0.
5. The broth is centrifuged at 10,000 x g for 20 minutes and the compacted PHA is evaluated for granule fusion.

Crystallinity by FTIR-ATR

Fourier-Transform Infrared Spectroscopy (FTIR) was used to evaluate the crystallinity of PHA granules prior to and after lysis treatment and compaction. This was done by placing approximately 2μL of lysis broth on the Attenuated

Total Reflectance (ATR) module and drying the sample on the crystal using a stream of nitrogen. The spectrum was then captured and evaluated for the presence of a peak at wavenumbers 1184cm^{-1} and 1226cm^{-1} , which are indicative of amorphous and crystalline PHA, respectively. In addition, wavenumbers 1735 and 1723cm^{-1} were evaluated for a similar shift. Due to an overlap between these peaks, the spectra were subjected to deconvolution using a Gaussian fit in Origin.

Crystallinity by Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed on several powder and film PHA samples. The samples were heated from 20°C to 200°C at 10°C/min. The melting endotherm of the resulting thermogram was integrated and, using an enthalpy of fusion of 146 g/J for 100% crystalline PHA, the crystallinity was determined.

Particle Size Analysis

Particle size analysis was completed using a Malvern Mastersizer 3000 with Hydro MV dispersion unit. PHA granules were suspended in 10% sodium dodecyl sulfate solution and sonicated for full dispersion of the granules. The dispersed granules were then added to the sample cell of the dispersion unit and particle size was measured in triplicate. Results were recorded as the mean, 10th and 90th percentile by volume.

Comonomer Content Analysis by Gas Chromatography

The hydroxyhexanoate content of each polymer was determined by gas chromatography. For each sample, approximately 10mg of lyophilized PHA-rich biomass was dissolved in 1mL chloroform within an 8mL reaction tube and subjected to acidic methanolysis at 100C for 150 minutes in order to hydrolyze the polymer into its monomeric units and convert the monomers into their respective methyl esters. 1mL of water and 1mL of 0.2% toluene in chloroform were added to form two phases, a lower chloroform phase containing the derivatized methyl esters and an upper aqueous phase containing sulfuric acid. The toluene was present to serve as an internal standard across all samples. In addition to the polymer samples, pure 3-hydroxybutyrate and 3-hydroxyhexanoate were subjected to the same derivatization and used to generate standard curves to determine the detector response to each component.

Quantification of Residual Biomass

Residual protein in purified PHA granules was quantified using a modified Lowry assay (BioRad DC assay kit). Oven dried samples were collected and washed with 10% SDS solution with vigorous mixing for 30 seconds. The samples were then centrifuged, and the supernatant was collected and directly pipetted into the assay kit. Samples were placed into a 96-well plate and measured at 750nm. Results were quantified in reference to a bovine serum albumin standard.

Residual lipids were quantified using a sulfo-phospho-vanillin assay. Oven dried samples were collected and washed with hexanes with vigorous mixing for 30 seconds. The samples were then centrifuged, and the supernatant was pipetted into vials and placed on a 100°C heating block for 1 minute to evaporate the hexane. Then, 200µL sulfuric acid was added and the sample was incubated at 100°C for 10 minutes. The samples were then placed on ice and a solution of 15mM vanillin in 50% phosphoric acid was added to each sample. The samples incubated at 37C for 15 minutes before loading into a 96-well plate. The presence of unsaturated fatty acids resulted in a color development that was quantified at 540nm in reference to canola oil as a standard.

Molecular Weight Determination

The molecular weight of purified polymers was determined using a Malvern Omnisec GPC equipped with triple detection: viscometer, refractive index detector, and light scattering detector. Two HMJ HM-MBMMW2010 columns were installed in the instrument. The instrument was calibrated with narrow 105 kDa and wide 245 kDa polystyrene standards. Polymer samples were dissolved in chloroform at a concentration of 1 mg/mL and filtered through a 0.2 µm PTFE filter. 100 µL was then injected into the instrument and elution was performed using chloroform at 35°C and 1 mL/min for 40 minutes.

RESULTS

Cell Lysis

Cell lysis was performed on the PHAs listed in table 1 according to the described purification protocol. Successful lysis was apparent by the drastic increase in viscosity upon the addition of SDS detergent, indicating significant release and denaturation of DNA. Ultrasonic mixing was applied to reduce the viscosity and further solubilize the lysed biomass. Over the course of the 1-hour purification, the lysis broth lightened in color significantly, confirming the release of PHA granules from within the cells. A two-microliter sample of lysis broth was analyzed using ATR-FTIR to confirm that the granules were indeed in an amorphous state prior to applying additional treatments.

Granule Crystallization by Osmotic Stress

Once cell lysis was completed, the resulting broth was comprised of a suspension of amorphous granules, along with digested cell biomass. At this point, 10 mL aliquots were collected and subjected to increasing amounts of osmotic stress, using sodium chloride as a solute, with the intent of drawing intragranular water out of the granules. Sodium chloride concentration was reported in osmoles, that is the total molarity of the dissociated ions:

$$NaCl \rightarrow Na^{+} + Cl^{-}$$

$$1M\ NaCl = 2\ OsM$$

The treated samples were then analyzed using ATR-FTIR to determine if any crystallinity had developed. For each polymer of given HHx content, a threshold osmolarity was observed below which the granules remained fully amorphous and above which significant crystallinity developed. Once this threshold was reached, increasing the osmolarity of the solution had no further effect.

With increasing HHx content, the osmolarity required to induce crystallization increased ranging from 0.7 OsM to induce crystallization of 0.3% HHx PHA, to 8.6 OsM for 17% HHx (Figure 2).

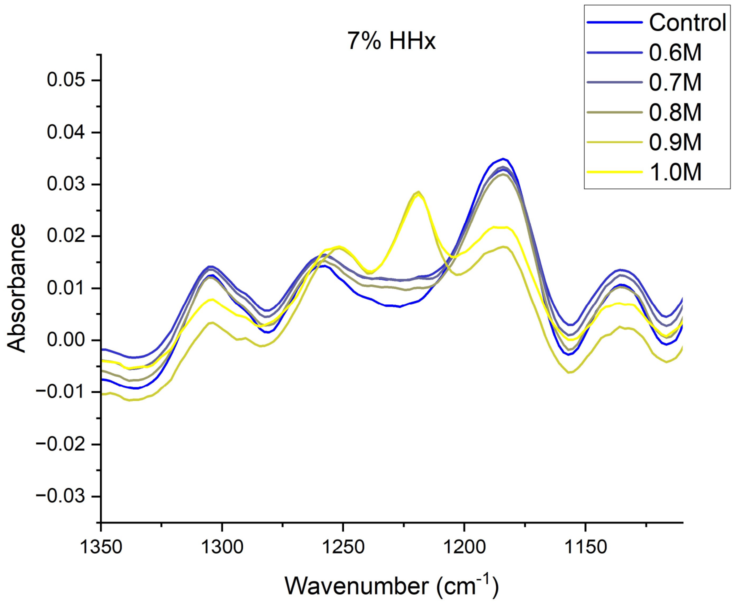
In addition to NaCl, osmotic stress experiments were repeated with divalent and trivalent chloride salts (Calcium chloride and iron chloride, respectively). Results with these salts were similar to sodium chloride, with slightly higher doses required, likely due to the tendency of divalent and trivalent cations to precipitate with residual phosphates.

Granule Crystallization by Solvent

To further explore the mechanism of granule crystallization via intragranular water removal, amorphous granules were similarly exposed to increasing amounts of ethanol to encourage the migration of intragranular water out of the granule. Ethanol was chosen due to its widespread availability and recognition for being relatively safe and non-toxic, though any water-miscible solvent is expected to provide an identical effect. Similar to osmotic stress using salt, a threshold was found for each sample, above which crystallization occurred and remained steady with increasing ethanol concentration, ranging from 5% for 0.3% HHx to 25% for 11.9% HHx. However, for higher comonomer content (17%), no amount of ethanol was capable of inducing crystallization.

Other Approaches for Initiating Granule Crystallization

In addition to osmotic stress and solvent dewatering, other approaches were explored in order to better understand the mechanism of granule crystallization. Freezing and boiling were explored to test whether a phase transition of the intragranular water would encourage crystallization. Neither approach resulted in any measurable crystallinity. Interestingly, autoclaving (121°C, 15PSI) successfully initiated granule crystallization.



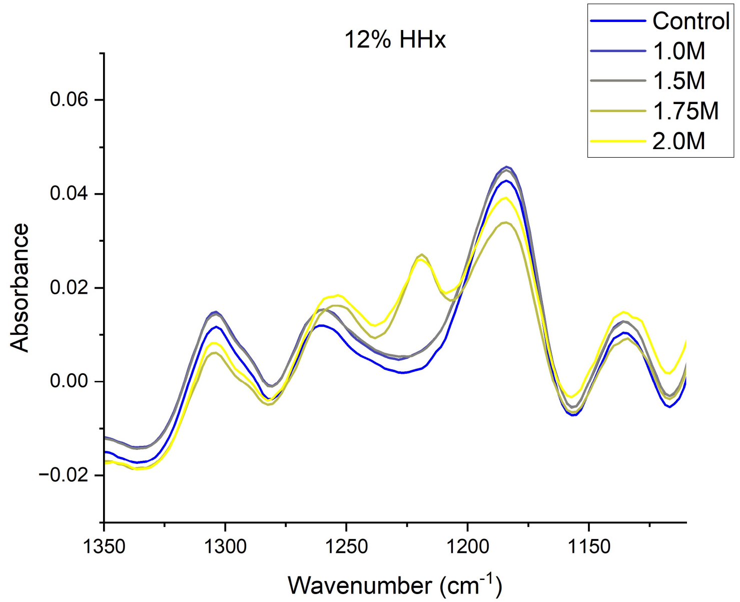
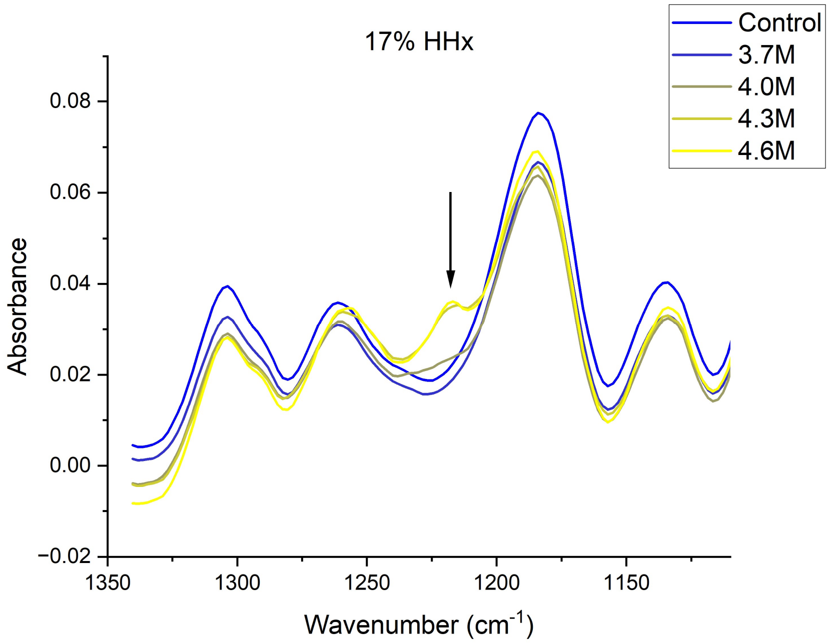


Figure 3.2: Osmotic stress of PHAs with increasing HHx content. Development of the band at 1224 cm^{-1} is indicative of crystallization as the necessary osmotic stress has been reached.



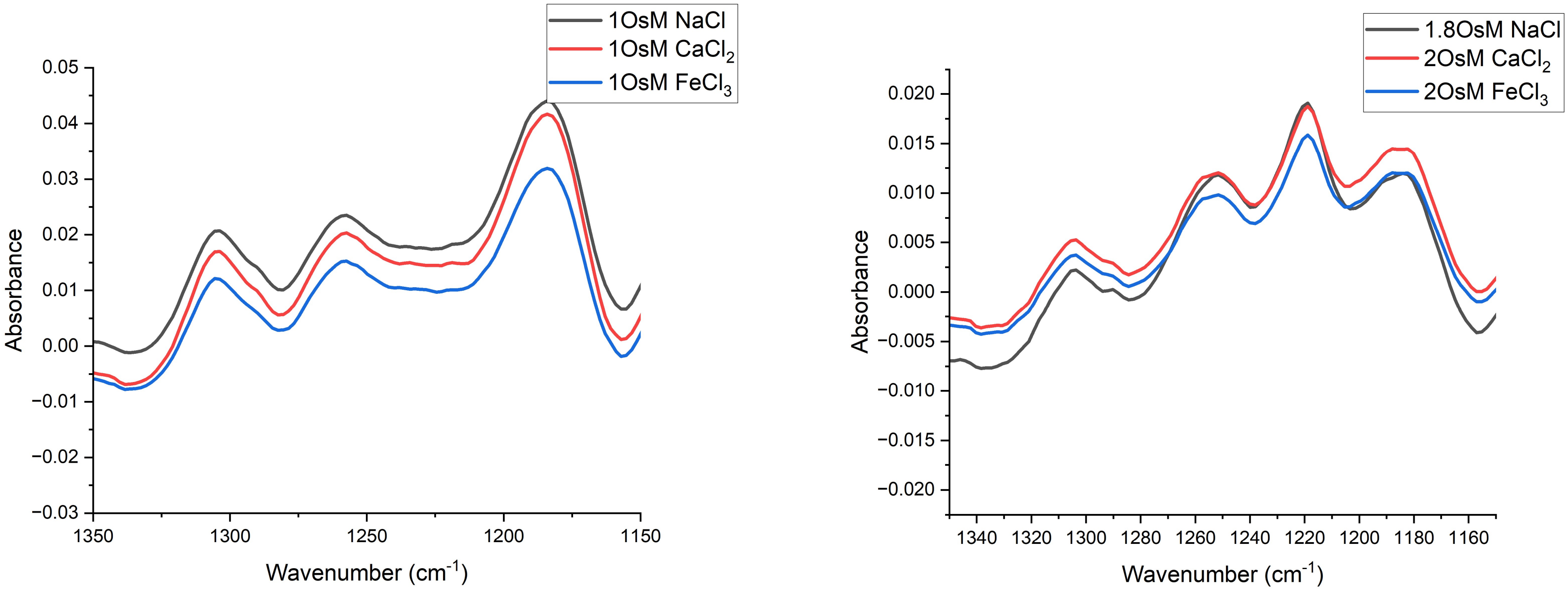


Figure 3.3: Osmotic stress with sodium, calcium, and iron chloride salts. ***Left:*** *Osmotic treatment below the threshold for 7% HHx.* ***Right:*** *Osmotic treatment at the threshold to achieve crystallinity.*

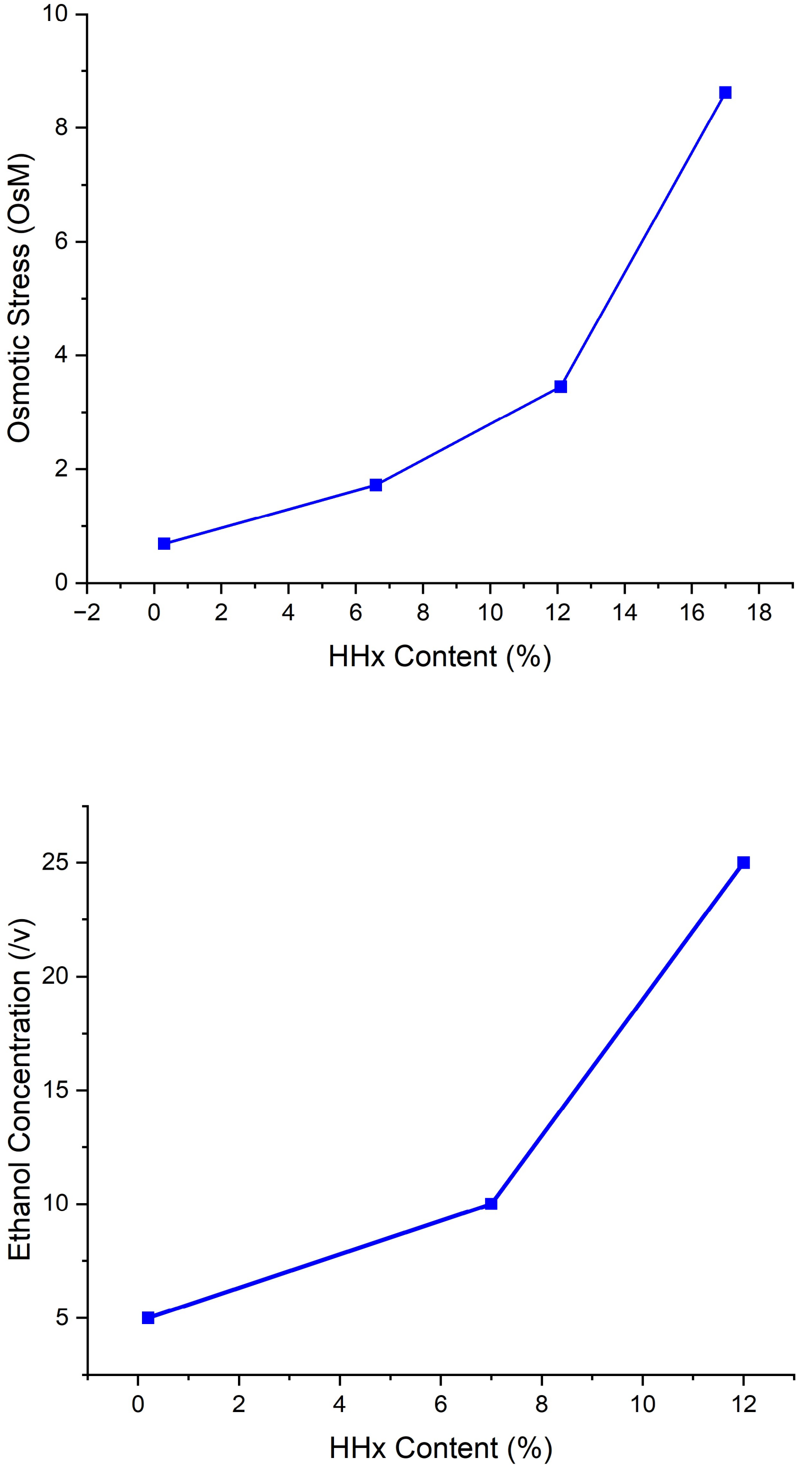


Figure 3.4: Minimum osmotic stress required to induce crystallization by HHx content. ***Top:*** *Osmotic stress induced by NaCl.* ***Bottom:*** *Osmotic stress induced by ethanol.*

To further explore the effect of pressure on the intragranular water, amorphous granules were subjected to 45,000PSI using a high-pressure homogenizer. The resulting granules remained amorphous.

Particle Size Analysis

Table 2 summarizes the particle size distribution of granules of each HHx content. All granules were subjected to the minimum osmotic stress to achieve crystallization during purification.

Table 3.2: Particle size distribution of purified granules.

HHx Content (%)	10th percentile (µm)	Mean (µm)	90th percentile (µm)
0	0.98	1.31	1.74
0.2	0.90	1.40	2.65
7	0.83	1.29	2.78
12	0.90	1.59	4.26
17	0.89	1.52	3.75

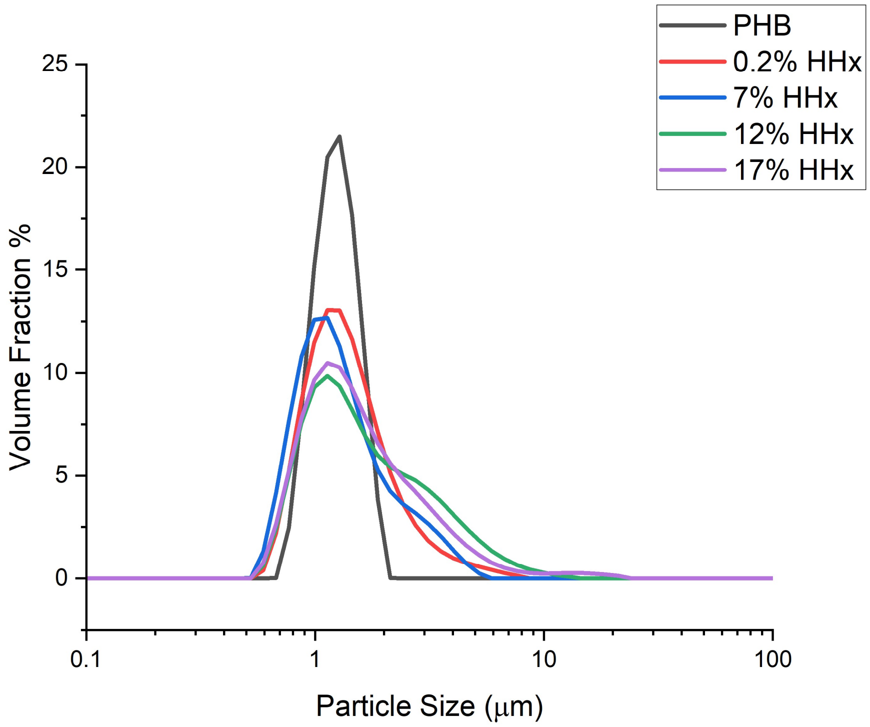


Figure 3.5: Particle size distributions of polymers crystallized with osmotic stress during cell lysis.

Molecular Weight Analysis of Purified Granules

Table 3 summarizes the molecular weight of granules before and after purification when treated with the necessary osmotic stress to induce crystallization. Size-exclusion chromatography overlays are presented in figure 6.

Table 3.3: Molecular weight of granules before and after purification

HHx Content	Whole Cell Mw (Da)	Purified Mw (Da)	Mw Loss (%)
0	837,000	830,000	< 1%
0.2	1,270,000	1,268,000	< 1%
7	872,000	864,000	< 1%
12	1,001,000	945,000	4.6%
17	772,000	450,000	41.7%

Table 4 summarizes the purity of granules after aqueous purification. Granules with HHx content from 0-12% had similar degrees of purity, but the 17% HHx polymer had significantly higher amounts of residual biomass.

Table 3.4: Resulting purity of granules collected after processing

HHx Content	Residual Protein (mg/g)	Residual Lipid (mg/g)	Overall Purity
0	0.4	0.8	99.88%
0.2	0.3	0.6	99.91%
7	0.4	0.8	99.88%
12	1.1	1.0	99.79%
17	7.1	1.9	99.10%

DISCUSSION

Treatment of amorphous granules with high osmolarity conditions was successful in inducing crystallization in all cases. Granules treated with the appropriate osmotic stress were maintained as discrete granules, as indicated by particle size analysis and the ability of the granules to be compacted by centrifugation and subsequently suspended in water. In contrast, granules compacted without adequate osmotic stress coalesced into a single mass during centrifugation. However, with increasing comonomer content, the osmolarity required to induce crystallization increases. At the highest comonomer content (17%), 4.3 molar, or 25 weight percent, sodium chloride solution is required to induce crystallization. This relationship between comonomer content and osmotic stress can be explained by the requirement that 3-hydroxyhexanaote comonomers be excluded from the PHB crystal lamella. As a result, these branched monomers are forced into the amorphous region between crystals. With increasing branching, overall crystallinity of a polymer decreases along with lamellar thickness and rate of crystal nucleation.

In the context of aqueous amorphous granules plasticized by water, higher comonomer content results in granules that are more resistant to crystallization. More of the plasticizing water must therefore be removed in order to create conditions that are favorable for granule crystallization, requiring a higher osmotic stress as a driving force to remove the water from the granule.

Similar to osmotic stress by addition of solute, ethanol was also effective at inducing granule crystallization by withdrawing intragranular water. However, significantly higher concentrations of ethanol were required, and at the highest comonomer content (17% HHx), no crystallization was observed. This reduced efficacy compared to salt is likely due to the ability of ethanol to solubilize PHAs with high HHx content.

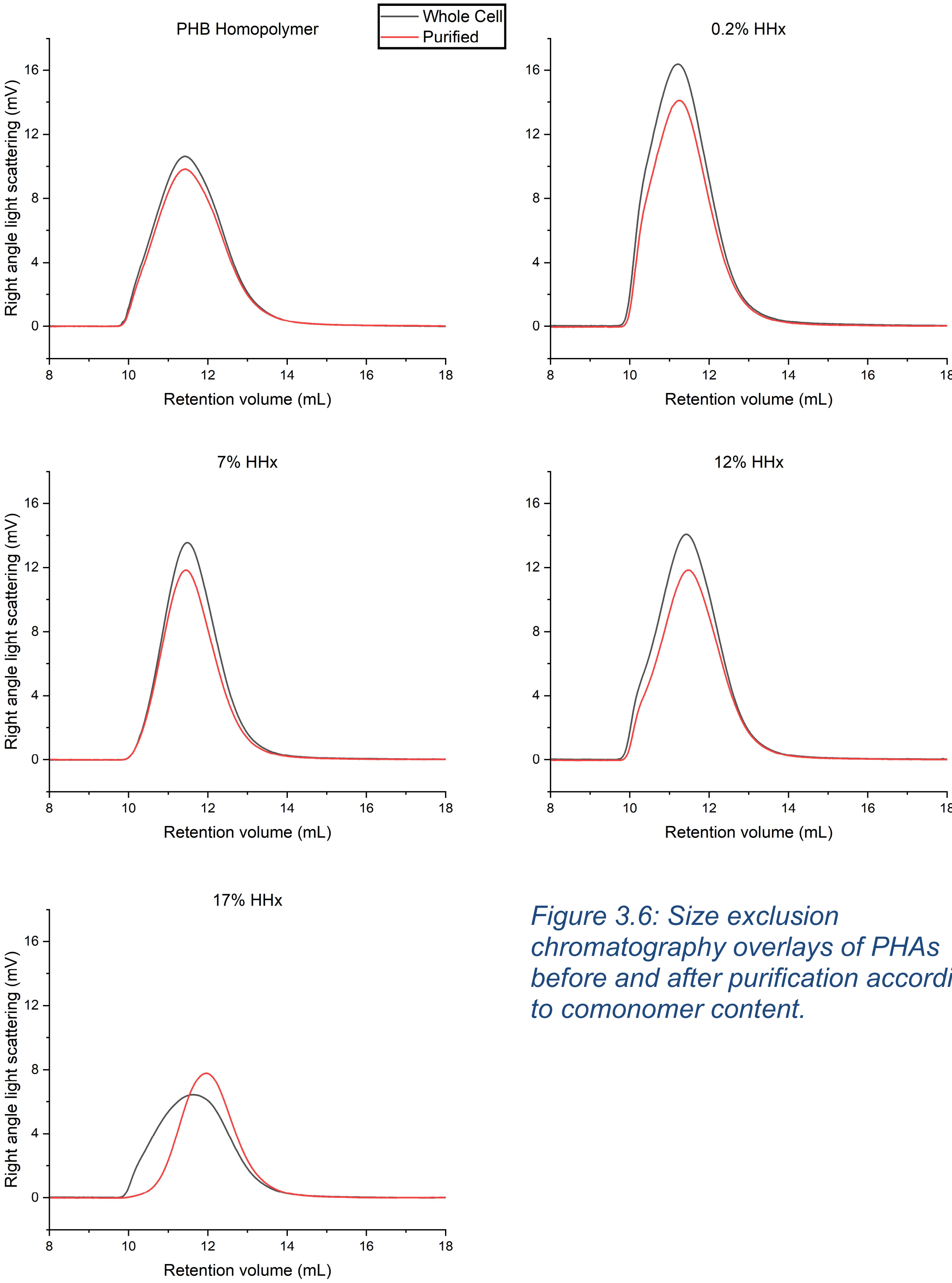


Figure 3.6: Size exclusion chromatography overlays of PHAs before and after purification according to comonomer content.

A critical parameter of any polymer is its molecular weight. Molecular weight is directly correlated to processing characteristics, such as viscosity and melt flow, as well as mechanical properties of the final plastic article. In the case of PHA, while adequate molecular weight must be achieved during its biosynthesis, it must additionally be maintained throughout the purification process. As such, any adjustment to purification methods should be evaluated to ensure that molecular weight isn't decreased significantly. In the case of PHB-co-HHx ranging in comonomer content from 0-12%, molecular weight was maintained greater than 95% of its original value. However, in the case of the 17% HHx polymer, molecular weight was reduced by nearly 42%.

The 17% HHx polymer similarly performed poorly when analyzed for purity, retaining 7.5-fold the amount of residual biological material than the PHB homopolymer. This is likely due to the extreme concentration of solute applied to induce crystallization, requiring 25 weight percent sodium chloride. At this concentration, a salting-out effect occurs, precipitating many of the proteins that had been solubilized by SDS. This precipitation results in the co-sedimentation of proteinaceous biomass with the PHA granules during centrifugation. Furthermore, excessive solute concentration leads to a decrease in the differential density between the PHA granules and surrounding water, resulting in poorer separation and reduced removal of lysis broth with centrifugation.

In addition to applying osmotic stress, the addition of ions in solution can contribute to a Debye screening effect. Debye screening occurs when a colloidal suspension of particles with some surface charge, such as the negatively charged PHA granules in this case, is disrupted through the addition of counter-ions. The counter-ions serve to mitigate the repulsive effect of the negatively charged particles from one another. At sufficient concentration of counter-ions, the repulsive effect of the particles' electrostatic forces is overcome by attractive Van der Waals forces, resulting in coagulation of the particles in suspension.

To deconvolute the effects of osmotic stress and Debye screening as the responsible mechanism to initiate crystallization of granules, divalent and trivalent calcium chloride and iron chloride were included in this study. These salts were chosen because, according to the Schulze-Hardy rule, critical coagulation concentration is inversely proportional to the valence of the counterion raised to the sixth power:

$$CCC = \frac{1}{z^6}$$

If Debye screening is the principal mechanism for granule crystallization during treatment with sodium chloride, it should be observed that divalent and trivalent cations are effective at 50-fold and 1000-fold lower concentrations, respectively. However, both calcium and iron cations were required at an osmolarity within a 10% margin of sodium chloride, indicating that screening of electrostatic forces is not a critical requirement for crystallization of amorphous PHA granules.

Furthermore, it is not reasonable to expect that a mechanism dependent on screening of electrostatic repulsion should require increasing intensity of treatment based on comonomer content, as found in the present research. Regardless of comonomer content, PHA granules have similar surface charges and should experience consistent critical coagulation concentrations.

Finally, in regard to electrostatic screening, the ability of ethanol to initiate crystallinity does not support an electrostatic mechanism, since ethanol does not contribute to Debye screening. Ethanol and other water-miscible solvents do, however, impart a driving force to withdraw intragranular water from the granule into the surrounding media.

A final consideration of applying osmotic stress during purification is the additional cost of purification as a result. As of 2023, bulk price of industrial-grade sodium chloride is approximately $0.10 per kilogram[20]. Depending on the comonomer content of the polymer being purified, the cost to treat 1000L of suspended PHA granules would range from $1.50 to $10.00. Assuming 1000L would contain 100kg PHA, this would result in an added cost of $0.015 to $0.10 per kilogram purified PHA. Even for the most optimistic market price for PHA of $3.83 per kilogram[21], incorporating this osmotic dewatering step into a purification process would add less than 3% to the overall materials cost for production.

CONCLUSIONS

For polyhydroxyalkanoates containing 0-12% hydroxyhexanoate, osmotic stress is an effective treatment to induce crystallization of individual granules in order to maintain them in their native morphology. With repeated centrifugation and resuspension, particle size and molecular weight of treated granules remained consistent with their original values. With increasing comonomer content, the granules are less prone to crystallization, and increasing amounts of osmotic stress are required to initiate crystallization after cell lysis. Eventually, at comonomer contents greater than 12%, the osmotic stress required becomes excessive, interfering with the ability to effectively purify the granules due to precipitation and coagulation of biological materials. Future research should be directed towards addressing purification of high comonomer content PHAs and identify approaches to removing intragranular water without the adverse effects of excessive salt. Alternatively, a successful purification process may be capable of maintaining native granule morphology without the requirement of crystallizing the granules, resulting in a suspension of purified, amorphous PHA.

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CHAPTER 4

CONCLUSIONS AND FUTURE OUTLOOK

In this dissertation, the phenomenon of coalescence of aqueous amorphous PHA granules was demonstrated and characterized through a variety of methods, including ATR-FTIR spectroscopy, particle size analysis, and SEM microscopy. It is clear that PHA granules are amorphous in their native state and that when they are subjected to centrifugation in this state, they coalesce, leading to difficulties in purification and handling in an industrial context.

Chapter 2 demonstrated that PHB homopolymer crystallizes readily once released from the cell, while PHAs with even modest amounts of branched comonomers remain amorphous. This, in part, explains the paucity of research describing the phenomenon of granule coalescence, since PHB homopolymer is by far the most studied and easily accessible PHA. Furthermore, the research presented in chapter 2 demonstrated that ATR-FTIR is a rapid, reliable technique for observing granule crystallinity in situ. Since intragranular water is a critical component of granule crystallinity, other methods of measuring crystallinity are inappropriate for this measurement. Differential scanning calorimetry and x-ray diffraction crystallography, for example, are methods commonly used for measuring polymer crystallinity but require dry polymer for analysis. The ATR-FTIR methodology requires only a couple microliters of sample and crystallinity can clearly be discerned from the 1224 cm^{-1} wavenumber. PHB homopolymer clearly developed a signal at this wavenumber, leading to granules with a crystalline shell prior to centrifugation, resulting in granules that maintain their native morphology. In contrast, PHB-co-HHx polymers ranging from 0.3-17% HHx content did not show any development of crystallinity according to the 1224 cm^{-1} wavenumber. This lack of signal correlated with the coalescence of the granules into large masses – limited in size only by the volume of the centrifugation container used. The ATR-FTIR technique allows for rapid and definitive determination of whether granule coalescence will occur during an industrial purification process.

Chapter 3 demonstrated that amorphous PHB-co-HHx granules could be crystallized by subjecting the granules to an osmotic stress. For each polymer tested, there was a clear threshold below which the granules remained fully amorphous. Once the threshold osmolarity was achieved, significant crystallinity was detected via ATR-FTIR, which remained constant with further increase in osmolarity. With increasing comonomer content, the threshold increased significantly, from a modest 0.7 OsM for 0.3% HHx polymer, to 8.6 OsM for 17% HHx PHA, with a sharp increase in solute required between 12% and 17% HHx content. The mechanism for crystallization was shown to be osmotic dewatering of the granules, rather than electrostatic Debye screening effects, due to the similar osmolarity required from mono-, di- and tri-valent salts to initiate crystallization. Furthermore, dilution with alcohol was similarly effective at initiating crystallization in polymers containing up to 12% HHx, although above this threshold of comonomer content, this treatment was not effective due to the increasing solubility of the polymer in alcohol. Once crystallization was successfully induced in the granules, they remained in their native morphology for the remainder of purification. With the exception of the highest, 17%, comonomer granules, the purified polymer was highly pure and stable, comparable to PHB homopolymer.

The ability to measure and induce PHA granule crystallization during purification is critical to the commercial-scale aqueous purification of these polymers. The previous chapters have established the framework for evaluating granule crystallization and illuminated the mechanism, namely intragranular water, that keeps them in an amorphous state. By subjecting the granules to treatments that withdraw the intragranular water, crystallization was reliably initiated, and the polymer could be successfully purified. While there is much work to be done to make commercialization of PHAs a reality, the presented research makes the aqueous purification of PHA copolymers more accessible by maintaining granules in their native morphology.

FUTURE OUTLOOK AND CONCLUDING REMARKS

While PHA of all comonomer contents could be successfully crystallized during lysis, thereby preserving the granules' native morphology throughout purification, the amount of solute required became excessive in the case of 17% comonomer. The resulting polymer was highly contaminated with residual biomass, and molecular weight of the collected granules was reduced nearly two-fold compared to the starting molecular weight. Even for the successfully purified polymer with 12% comonomer content the amount of salt required is excessive, requiring a concentration of approximately 10% by mass. While this approach of osmotic dewatering is feasible for low and moderate comonomer content PHAs, there is a necessity for alternate approaches for PHAs with higher comonomer content in order to maintain a purification process that is commercially cost-effective and scalable. Future work will focus on means of withdrawing intragranular water without excessive salt to avoid these pitfalls. Alternatively, procedures may be identified that can maintain individual granules despite being totally amorphous. As the diversity of PHAs continues to expand, there are some that may be incapable of crystallization at all. Such materials would necessitate a purification method that maintains discrete granules. The ability to do so without the use of solvent precipitation would significantly increase the capacity to produce commercial-scale quantities of these unique polymers.