Biotechnology and Bioengineering

Title: Recovery of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from *Ralstonia eutropha* cultures with non-halogenated solvents

Running title: Recovery of P(HB-*co*-HHx)

Sebastian L. Riedel[1,3], Christopher J. Brigham[1], Charles F. Budde[2], Johannes Bader[3], ChoKyun Rha[4], Ulf Stahl[3] Anthony J. Sinskey[1, 5, 6]*

Department of Biology[1], Department of Chemical Engineering[2], Biomaterials Science & Engineering Laboratory[4], Division of Health Sciences and Technology[5], Engineering Systems Division[6], Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

Department of Applied and Molecular Microbiology[3], Technische Universität Berlin, Seestraße 13, 13353 Berlin, Germany

* Corresponding author. Mailing address: Bldg. 68-370, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, USA. Phone: (617) 253-6721. Fax: (617) 253-8550. E-mail: asinskey@mit.edu

Keywords: Polymer recovery, non-halogenated solvents, polyhydroxyalkanoate, poly(hydroxybutyrate-*co*-hydroxyhexanoate), methyl isobutyl ketone

ABSTRACT

Reduced downstream costs, together with high purity recovery of polyhydroxyalkanoate (PHA), will accelerate the commercialization of high quality PHA-based products. In this work, a process was designed for effective recovery of the copolymer poly(hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-*co*-HHx)) containing high levels of HHx (>15 mol%) from *Ralstonia eutropha* biomass using non-halogenated solvents. Several non-halogenated solvents (methyl isobutyl ketone, methyl ethyl ketone, butyl acetate and ethyl acetate) were found to effectively dissolve the polymer. Isoamyl alcohol was found to be not suitable for extraction of polymer. All PHA extractions were performed from both dry and wet cells at volumes ranging from 2 mL to 3 L using a PHA to solvent ratio of 2% (w/v). Ethyl acetate showed both high recovery levels and high product purities (up to 99%) when using dry cells as starting material. Recovery from wet cells, however, eliminates a biomass drying step during the downstream process, potentially saving time and cost. When wet cells were used, methyl isobutyl ketone (MIBK) was shown to be the most favorable solvent for PHA recovery. Purities of up to 99% and total recovery yields of up to 84% from wet cells were reached. During polymer recovery with either MIBK or butyl acetate, fractionation of the extracted PHA occurred, based on the HHx content of the polymer. PHA with higher HHx content (17-30 mol%) remained completely in solution, while polymer with a lower HHx content (11-16 mol%) formed a gel-like phase. All PHA in solution could be precipitated by addition of 3-fold volumes of *n*-hexane or *n*-heptane to unfiltered PHA solutions. Effective recycling of the solvents in this system is predicted due to the large differences in the boiling points between solvent and precipitant. Our findings show that two non-halogenated solvents are good candidates to replace halogenated solvents like chloroform for recovery of high quality PHA.

INTRODUCTION

Polyhydroxyalkanoates (PHA), microbially produced polyesters, are of special interest because these biocompatible and biodegradable polymers offer processing properties that are similar to chemically synthesized plastics. Depending on their composition, they can be used for fabrication of a wide range of products including packaging material (Chen 2009), household products (Philip et al. 2007), up to medical scaffolding (Shum-Tim et al. 1999; Sodian et al. 1999; Williams et al. 2002), sutures (Shishatskaya and Volova 2004; Shishatskaya et al. 2002) and other applications.

Efficient recovery and purification of PHA from cells is required for its cost efficient industrial production. Comprehensive reviews of published recovery strategies are presented by Kunasundari and Sudesh (2011) and Jacquel *et al*., (2008). For example, different chemical-based digestion methods have been developed One critical consideration of these processes, is that harsh chemical treatment to achieve high purities may lead to a reduction of the molecular weight of the polymer (Ramsay et al. 1994). Yang et al. (2011) developed an strategy for poly(hydroxybutyrate-*co*-hydroxyvalerate) (P(HB-*co*-HV)) recovery using linear alkylbenzene sulfonic acid LAS-99 as an alternative to the commonly used sodium dodecyl sulfate. In this method, only 20% of the surfactant was required, compared to previous SDS-based methods. The main disadvantages of these chemical based strategies are the large amount of salt produced as a by-product and the amount of surfactant-containing wastewater generated from the process, potentially resulting in high costs for wastewater treatment.

Digestion-based recovery strategies utilize enzymatic treatment of cellular components to release PHA. Kapritchkoff and coworkers (2006) investigated the utilization of different enzymes for the recovery of PHA. Compared to solvent-extracted PHA, the molecular weight may be lower following enzymatic recovery methods despite the mild reaction conditions (Kathiraser et al. 2007).

Mechanical methods have been combined with chemical treatments for cell disruption during PHB recovery, including the use of bead mills or high pressure homogenization, along with sodium hypochlorite treatment (Tamer et al. 1998). After disruption of the cells, a separation of PHA from cell debris must still be achieved. Such separation methods include centrifugation, filtration, floatation or aqueous two-phase systems (ATPS). However, currently ATPS systems are not often industrially applied due to their tremendous complexity (Bensch et al. 2007).

Recovery of PHA from bacterial cells using organic solvents is often applied in industrial processes due to the recovery efficiency of the process, polymer purity obtained, and the possible removal of endotoxins from the recovered polymer, which is important for medical applications (Lee et al. 1999; Sevastianov et al. 2003). In a first step, the PHA is extracted from biomass with a suitable solvent (*e.g.* chloroform) and then separated from the residual biomass, *e.g.* through centrifugation and filtration. Polymer precipitation is then conducted with the addition of a non-PHA-dissolving solvent (*e.g.* methanol) or the polymer is recovered through cooling the solution or by solvent evaporation. Chloroform was the first solvent used to extract PHB from cells (Lemoigne 1927) followed by other halogenated solvents, including dichloromethane (Baptist 1962a). For the recovery of medium chain length (MCL) PHAs, a variety of ketones, esters, and alcohols are potentially usable (Kinoshida et al. 2006; Noda 1998; Van Walsem et al. 2007).

In this work, we examine the recovery of poly(hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-*co*-HHx)) polymer from wet and dry biomass using non-halogenated solvents. All of the PHA recovered was produced using high cell density palm oil fermentations, comprised of >139 g/L of biomass with a PHA content of 74% and an average HHx monomer content of 19 mol% (Riedel et al. 2012). Another success factor considered in this work was the separation of residual oil or fatty acids from the PHA. Lastly, some representative solvents used in this

work were found to promote separation of different polymers based on monomer content. These observations suggest the development of a unique polymer purification and separation procedure.

MATERIALS AND METHODS

Production of cell material for P(HB-*co*-HHx) recovery

Ralstonia eutropha Re2058/pCB113, a strain engineered from the *R. eutropha* wild-type strain H16 (ATCC 17699) (Budde et al. 2011), was grown using fermentation conditions described previously (Riedel et al. 2012), with triacylglycerols and fatty acids from different plant oils as sole carbon sources, to produce biomass containing P(HB-*co*-HHx) with high levels of HHx (>15 mol%). Cells from fermentation broths were harvested, frozen at -80˚C, and processed as described below.

Test of solvents for PHA recovery

To test solvents for PHA recovery the polymer P(HB-*co*-20mol%HHx) with a purity of 99%, was used as starting material (each 30-60 mg). Equal volumes of the non-halogenated solvents methyl isobutyl ketone, methyl ethyl ketone, butyl acetate (MIBK, MEK, BA, Sigma-Aldrich, St. Louis, MO) , ethyl acetate (EA, Muskegon, MI) and isoamyl alcohol (IA, Mallinckrodt Chemicals, Phillipsburg, NJ), were added the PHA, in sealed screw top test tubes, to form 5% or 10% PHA/solvent-solutions. The PHA was dissolved by heating at 100ºC in a heat block for 4 h. After incubation, each solution was filtered through a 0.2 µm polytetrafluorethylene (PTFE) membrane filter. A defined aliquot was transferred into a pre-weighed borosilicate glass test tube. The glass tubes were incubated at temperatures 10°C below the boiling point of each solvent until dry. The samples were further dried under vacuum until they reached a constant weight.

Test of precipitants for recovery of PHA

To test precipitants, 5% stock solutions of PHA in MIBK and BA were prepared in sealed vessels. For each precipitant tested, 1 mL of the 5% PHA solution was transferred into pre-weighed borosilicate glass test tubes. PHA was then precipitated by addition of 0.5-5 volumes of precipitant (*n*-hexane or *n*-heptane) at room temperature. The tubes were briefly vortexed and incubated at room temperature for 1 h. Following mixing, the tubes were centrifuged for 10 min at 2500 × *g* and 20ºC. The supernatant was discarded and the PHA pellet was initially dried in a heating block at 50ºC and finally in a vacuum oven at 80ºC until dry.

Test of lipid and fatty acid precipitation

Since PHA investigated in this work was produced by cultures grown on plant oils, it was necessary to determine if residual lipids from the palm oil culture broth could be precipitated by methods used for polymer precipitation. Solutions (5% w/v) of palm oil, oleic acid (C18:1), palmitic acid (C16:0), and lauric acid (C12:0) were prepared using BA or MIBK as the solvents in screw-capped tubes. Three volumes of *n*-hexane were added, and the solutions were observed for precipitation of triacylglycerols or fatty acids during overnight incubation at room temperature or 4ºC.

Recovery of PHA from dry cells in 2 mL scale

Sealed bottles containing 600 mL volumes of fermentation broth were thawed in warm water, and then centrifuged for 20 min at 7200 × *g*. The cell pellet was washed with a mixture of 500 mL water and 100 mL *n*-hexane (Mallinckrodt Chemicals, Phillipsburg, NJ) to remove any residual oil. The wet cell pellet was homogenized by mixing with a spatula, frozen at -80˚C and then freeze-dried. The PHA content of the freeze-dried cells was determined as described below. Equivalent masses of freeze-dried cells, containing 40 mg of PHA, were weighed in screw capped sealed glass tubes. In each tube, 2 mL solvent was added to form 2%

PHA/solvent mixtures. Chloroform, MIBK, MEK, BA, or EA were used as solvents for polymer recovery. PHA was extracted by incubating samples at 50˚C, 75˚C or 100˚C for 4 h and were mixed by briefly shaking tubes by hand every 30 min. Samples were cooled to room temperature and centrifuged at 2000 × *g* for 10 min at room temperature. In some cases, the formation of a gel-like phase between the residual cells and organic phase was observed. This gel-like phase will thus be referred to as PHA/solvent-gel, in contrast to the PHA/solvent-solution (*e.g*. PHA/MIBK-gel or PHA/BA-solution). A typical PHA/solvent-gel that formed during a MIBK extraction is shown in Fig. 1. PHA/solvent-solutions and PHA/solvent-gel, if present, were each transferred to individual, pre-weighed borosilicate glass tubes. PHA was then precipitated with 3 volumes of *n*-hexane, briefly vortexed at room temperature, centrifuged at 2000 × *g* and then washed twice with *n*-hexane. The washed polymer was dried overnight at 50˚C. Monomer composition of the P(HB-*co*-HHx) copolymer was determined by methanolysis, as described below.

Recovery of PHA from dry cells in 40 mL scale

Samples of freeze-dried biomass, containing 0.8 g PHA, were each extracted with 40 mL of non-halogenated solvents (EA, MIBK, MEK or BA) to form 2% PHA/solvent mixtures. Extraction occurred at 100˚C in 125 mL flat-bottom flasks under reflux cooling conditions for 4 h. The samples were cooled to room temperature and centrifuged at 6000 × *g* for 10 min in 50 mL polypropylene tubes. The flat-bottom flask was rinsed twice with 2.5 mL solvent and used to wash residual cell material. The PHA was precipitated with three volumes of *n*-hexane at room temperature and washed three times with *n*-hexane. The washed polymer was dried at 50˚C overnight. Both the monomer compositions of PHA polymer and residual cell material were determined as described below.

Larger scale recovery from dry cells with ethyl acetate (EA)

A 2% PHA solvent mixture was created by adding 1.78 L of EA to freeze-dried cells containing 35.5 g PHA. PHA was extracted in a 5 L round bottom flask for 4 h at 80-90˚C. The sample was centrifuged at 2200 × *g* for 20 min at room temperature**.** Aliquots of 1 L PHA/EA-solution were precipitated with 3 L *n*-hexane at room temperature in 4 L Erlenmeyer flasks with stirring. Supernatant was removed through decantation and the precipitated PHA was washed twice with *n*-hexane, manually crushed into smaller particles with a spatula, placed in a glass bowl, and then dried at 50˚C overnight.

Larger scale recovery from dry cells with methyl isobutyl ketone (MIBK)

A total volume of 1.35 L MIBK was added to freeze-dried cells containing 27 g PHA, to form a 2% PHA solvent mixture. The PHA mixture was then transferred to a 5 L round bottom flask. Polymer was extracted at 100˚C with stirring under reflux conditions for 4 h. The sample was cooled to room temperature overnight and centrifuged in glass centrifuge bottles at 2200 × *g* for 10 min at room temperature. Aliquots of 1 L PHA/solvent-solution were precipitated with 3 L *n*-hexane at room temperature in 4 L Erlenmeyer flasks with stirring. Supernatant was removed through decantation and the precipitated PHA was washed twice with *n*-hexane and then dried at 50˚C overnight. Before drying, the washed polymer pellet was manually crushed into smaller particles with a spatula and transferred into a flat-bottom glass bowl.

Larger scale recovery from wet cells

Equal volumes (400-750 mL) of fermentation broth (containing cells and PHA) were thawed in warm water and centrifuged for 20 min at 7200 × *g* at room temperature. The wet cell pellet was transferred into a 5 L round-bottom flask, and solvent was added to form a 2% PHA solvent mixtures (*e.g.* wet cells containing 60 g PHA in 3 L solvent). PHA was extracted at 100˚C with stirring under reflux conditions for 4 h. At the beginning of the extraction, 0.33 L of water per 1 L solvent was added to enhance mixing of the wet cell pellet with the solvent.

After extraction, the sample was cooled to room temperature overnight and centrifuged in glass centrifuge bottles at 2200 × g for 10 min at room temperature. Aliquots of 1 L PHA/solvent-solution were precipitated with 3 L *n*-hexane at room temperature in 4 L Erlenmeyer flasks under stirring. Supernatant was removed through decantation and the precipitated PHA was washed 3 times with *n*-hexane and dried at 50˚C overnight. Before drying, the washed polymer pellet was manually crushed into smaller particles using a spatula and transferred into a flat-bottom glass bowl.

After polymer extraction from wet cells using EA, residual cell material was further separated from residual PHA/solvent-solution by centrifugation at 6700 × g in polypropylene tubes. During centrifugation, residual cell material separated into two different fractions. Part of the residual cell material collected at the solvent/water interface, while the rest formed a pellet at the bottom of the tube. The interface between the organic and aqueous phases had a yellow colored top portion and a white bottom portion (Supp. Fig. 2). Three separate sections of the centrifuged material (residual cells/interface-top, residual cells/interface-bottom and residual cell pellet) from one polypropylene tube were transferred into different polypropylene tubes, washed three times with water, freeze-dried and analyzed to determine PHA content by methanolysis.

Analytical methods

PHA concentration per cell dry weight (CDW), purity of the recovered PHA, and HHx content of the copolymers were determined using a methanolysis protocol described previously (Budde et al. 2011). In this procedure, pure standards of poly-3-hydroxybutyrate and methyl 3-hydroxyhexanoate (Sigma-Aldrich, St. Louis, MO) were used to generate calibration curves. Recovery yield (RY) was defined as:

$$Recovery\ yield\ (RY, \%) = \frac{\text{mass PHA recovered (g)} * \text{Purity (\%)}}{\text{mass PHA in cells used in recovery batch (g)}}$$

RESULTS

Many non-halogenated solvents that can serve as alternatives to chloroform for PHA recovery have been identified in the academic and patent literature (Kinoshida et al. 2006; Noda 1998; Noda et al. 2005; Van Walsem et al. 2007). We chose to investigate MIBK, MEK, BA, EA, and IA as potential solvents for P(HB-*co*-HHx) produced from palm oil (Riedel et al. 2012). Physical properties and safety characteristics of the chemicals used in this study are compiled in Table 1. These properties would determine how effective a solvent would be in an industrial recovery process. Isolation of PHA from bacterial cells requires extraction, separation, and washing steps. All non-halogenated solvents used in this study have lower densities than water, which allowed for simple decantation of PHA solutions after extraction and centrifugation. Also, the residual biomass remained in the aqueous phase, separated from the polymer solution. This phenomenon is a process advantage over chloroform, which has higher density than water. Thus, PHA-chloroform solutions will form the bottom phase along with the residual cell material. A general flow diagram of the recovery studies performed in this work is presented in Fig. 2.

Testing PHA solubility in chosen solvents

To evaluate which solvents were capable of dissolving our PHA copolymer, previously-extracted P(HB-*co*-20mol%HHx), with a purity of 99% was used as the starting material. With the exception of IA, all solvents tested were able to dissolve the copolymer (Fig. 3), resulting in a workable PHA/solvent solution. Recovery yields of up to 99% were achieved from the 5% PHA solutions.

Test of precipitants for recovery of PHA

The precipitants *n*-hexane and *n*-heptane were tested for PHA precipitation. PHA was precipitated from 5 wt% solutions at room temperature**.** The various combinations of solvents

242 and precipitants gave similar results. A threefold volume of either precipitant (per volume of
243 PHA solution) was sufficient to recover almost 100% of the PHA (Fig. 4).

244 **Test of lipid precipitation by *n*-hexane**

245 In order to determine if residual lipids from the palm oil culture broth would also come out of
246 solution upon addition of *n*-hexane, attempts were made to precipitate oil or fatty acids from
247 different 5% lipid solutions in solvent (MIBK, BA). These test solutions contained a
248 significantly higher concentration of lipids than one would expect to co-extract with PHA. All
249 lipids went into solution in MIBK or BA at room temperature, although with palmitic acid,
250 the fatty acids precipitated when incubated at 4ºC (Supp. Fig. 1). After addition of *n*-hexane,
251 no lipid precipitation was observed at room temperature, but when solutions were incubated at
252 4ºC, lipids in the palmitic acid solution once again came out of solution (Supp. Fig. 1). These
253 findings indicate that substantial co-precipitation of oil and fatty acids during PHA
254 precipitation is unlikely using methods described in this work. However, precipitated PHA
255 must still be washed with additional volumes of precipitant in order to remove the residual
256 solvent, which can contain residual lipids, to ensure that lipid contamination of the polymer
257 does not persist upon drying.

258 **Copolymer recovery from *R. eutropha* cells at 2 mL scale**

259 In order to screen solvents for PHA recovery from biomass, P(HB-*co*-HHx) was extracted
260 from dry cells containing 76% PHA of CDW with an HHx concentration of 15 mol%.
261 Extractions were performed at various temperatures (50˚C, 75˚C, and 100˚C) with a PHA to
262 solvent ratio targeting 2% PHA solutions at the 2 mL scale (Table 2). Chloroform was used as
263 a control solvent and was able to recover almost all PHA present in cells (≥ 98%) at 75˚C or
264 100˚C. At 50˚C, the recovery yield from chloroform solutions was slightly lower at 95%, and
265 the HHx content of the recovered polymer also increased slightly as compared to samples
266 incubated at higher temperatures.

Along with the typical PHA/solvent-solutions, PHA/solvent-gel formation was observed at the bottom of the organic phase during polymer extraction with MIBK or BA (Fig.1). The PHA/solvent-gel formation was observed with these solvents only at temperatures of 100˚C and 75˚C. The final yield of polymer from PHA/MIBK-solution or PHA/BA-solution decreased with a decrease in recovery temperature, whereas the amount of polymer in the PHA/MIBK-gel was higher at the lower temperature. The recovery yield from PHA/BA-gel did not change as temperature decreased. The total recovery yield, taking into account PHA in solution and in the gel phase, decreased for both MIBK (79% to 72%) and BA (74% to 60%) extractions at 75°C compared to 100°C.

Interestingly, in the recovery processes where a gel phase was observed, the monomer compositions of polymer recovered from PHA/solvent-solution and PHA/solvent-gel were different from each other. Polymer recovered from PHA/solvent-solution had a notably higher HHx level (17 mol%) compared to the polymer recovered from the PHA/solvent-gel (14-15 mol%) or compared to the total polymer recovered using chloroform as the solvent (15 mol%). The polymer recovered from the PHA/MIBK-solution or PHA/BA-solution at an extraction temperature of 50˚C had an even higher HHx concentration at 21 mol% or 19 mol%, respectively. PHA recovery using MEK or EA exhibited a high recovery yield (≥ 95%) at temperatures of 75˚C and 100˚C. However, at 50˚C, the recovery yield decreased to 87% or 76%, respectively, concomitant with a slight increase in HHx content to ~17 mol%. No PHA/solvent-gel formation was observed during recovery with MEK and EA.

Copolymer recovery from dried *R. eutropha* cells at 40 mL scale

At the 2 mL scale, the extractions at 100˚C yielded the best results (see recovery yield and purity, Table 2). Larger volume (40 mL) extractions were performed from dry cells, containing 62% PHA with 22 mol% HHx. The purities of all recovered polymer samples from PHA/solvent-solutions were ≥ 95 %.

The data from 40 mL scale recovery experiments are shown in Table 3. A PHA/solvent-gel was observed using the solvents MIBK and BA, similar to results seen during the 2 mL extractions. The purities of the recovered polymer samples from the PHA/solvent-gel were lower relative to polymer from solution, with purities of 73% or 62%, using MIBK or BA, respectively. The HHx concentrations of polymer recovered from either PHA/MIBK-solution or PHA/BA-solution were, at 24 mol%, slightly higher compared to the polymer present in cells (22 mol% HHx). Furthermore, the HHx content of the polymer recovered from the PHA/MIBK-gel or the PHA/BA-gel was much lower, at 12 or 11 mol%, respectively. Extraction with either EA or MEK gave a high polymer recovery yield of ≥ 94%. The HHx content of polymer from the PHA/EA-solution or PHA/MEK-solution was ~21 mol%, which was similar to the HHx content measured in whole cells. Analysis of the residual cell mass from extractions using MEK or EA showed that only minor amounts of unrecovered PHA (2 wt% of cell mass) were present. However, the PHA content of residual cell mass from cells treated with either MIBK or BA was much higher, at 13% or 24%, respectively. The HHx monomer content of PHA not extracted from the cells with these solvents was 11-12 mol%. The total recovery yield from MIBK extraction was 84% (71% from PHA/MIBK-solution and 13% from PHA/MIBK-gel). The total recovery yield was lowest with BA, reaching only 76% (68% from PHA/BA-solution and 8% from PHA/BA-gel).

Larger scale PHA recovery

To demonstrate the scalability of a PHA recovery process, polymer was recovered from cell biomass using up to 3 L volumes of solvent (Table 4). Wet cells were used, instead of dry cells, to avoid an energy and time consuming drying step for these large quantities of cell material. For comparison, representative gel forming and non-gel forming solvents were used in this laboratory scale up process. MIBK was chosen over BA for the PHA/solvent-gel forming solvent, due to better yields in the previous experiments (Table 3). EA was chosen

over MEK, because of its lower solubility in water, which would enhance separation of wastewater and solvent following the PHA extraction step of a potential industrial process.

In recovery with MIBK at the 3 L scale from wet cells, PHA separation based on the HHx content was observed, with polymer from PHA/MIBK-solution at ~20 mol% HHx and polymer from PHA/MIBK-gel at ~15 mol% HHx (average HHx content of total PHA before recovery was 20 mol%). The purity of the polymer from PHA/MIBK-solution was observed to increase to >99% with efficient washing of the recovered polymer with *n*-hexane. The overall recovery yield from both PHA/MIBK-solution and PHA/MIBK-gel was 84%.

PHA recovery with EA from wet cells in a 1.5 L scale showed no PHA/EA-gel formation, as expected. The recovered polymer had a purity of 98% with an HHx content of 21 mol%. During centrifugation of the extraction mixture, a separation of the residual cell material into three distinct phases was observed, as described in Materials and Methods (Supp. Fig. 2). The PHA content from the residual cells/interface-top (the upper phase) had a PHA content of 58%, the residual cells/interface-bottom (the middle phase) had a PHA content of 31% and the residual cell pellet (bottom phase) a PHA content of 27%. We hypothesize that, during recovery of PHA from wet cells using EA as the solvent, a mixture of EA, water, and residual cell debris formed, resulting in a significant portion of the polymer remaining with the wet cell mass. All three phases had an average HHx content of 22 mol% (Supp. Fig. 2). The recovery yield from the PHA/EA-solution from wet cells was only 71%, whereas PHA recovery from dry cells in a 1.8 L extraction gave a recovery yield of 93% with a purity of 95%.

DISCUSSION

There are several requirements that must be met for a PHA production process to be sustainable and economically viable. High yield PHA production must be reached from a

341 readily available carbon source (*e.g.* palm oil; (Riedel et al. 2012)). Also, there must be an
342 efficient recovery process that allows for consistent isolation of high purity polymer (Jacquel
343 et al. 2008). The use of chlorinated solvents such as chloroform, methylene chloride or 1,2-
344 dichlorethane has been shown to lead to high purity levels during PHB recovery (Ramsay et al.
345 1994). Use of non-halogenated solvents will reduce the hazards for the operators and for the
346 environment. In this study, we designed a process for the recovery of P(HB-*co*->15mol%HHx)
347 from bacterial biomass. Based on their physical properties and safety characteristics (Table 1),
348 which are important for industrial scale-up process (*e.g.* energy to pump, energy to heat or
349 cool, solvent separation from wastewater) and use of recovered bioplastics for different
350 applications (*e.g.* food service, household, and medical products), respectively, the following
351 solvents were chosen for evaluation of PHA recovery: MIBK, BA, EA and IA. All solvents,
352 with the exception of IA, were able to effectively dissolve this polymer. We demonstrated
353 PHA extraction from dry and wet cells at different scales, from 2 mL up to 3 L. We decided to
354 focus on BA and MIBK, due to their lower miscibilities with water as compared to EA and
355 MEK (Table 1), resulting in a better separation of the organic phase from the aqueous phase
356 during PHA recovery from wet cells. Recovery from wet cells eliminates a biomass drying
357 step from the downstream process, saving time and cost. With MIBK, we were able to recover
358 PHA from wet cells with the same efficiency (recovery yield 84%) as from dry cells with
359 purities reaching 99% (Tables 3 and 4). With EA, the recovery yield with wet cells was 71%
360 (Table 4), which was significantly lower compared to the recovery yield from dry cells (93-
361 99%) (Tables 2-4).

362 During PHA recovery with BA and MIBK, a separation of the copolymer occurred based on
363 HHx content. PHA with a higher fraction of HHx monomer (17-30 mol%) was observed in
364 the PHA/solvent-solution, whereas polymer with lower HHx fraction formed a PHA/solvent-
365 gel (11-16 mol%) located between residual cell material and the PHA/solvent-solution. Also,

small amounts of PHA containing low levels of HHx (11-12 mol%) remained in the residual cell material. This indicates that higher HHx content makes the polymer more soluble, as has been observed previously (Noda et al. 2005). It is unclear whether the gel was present throughout the extraction, or only appeared as the solution cooled during centrifugation. The fractionation of PHA during recovery confirms our previous finding that the strain used in this study makes PHA with varying HHx content during fermentation on palm oil (Budde et al. 2011).

The recovery yield from the MIBK and BA PHA/solvent-solutions were observed to be much lower in 2 mL extractions compared to the other extractions (Tables 2-4). These results can be explained through a better separation of the PHA/solvent-solution from the PHA/solvent-gel due to greater force (higher RPM) during the centrifugation step of the larger scale extractions, as compared to the 2 mL extractions. Overall, MIBK had the capacity to recover more PHA from cells than BA in our studies (Tables 2 and 3). With non-gel forming solvents (MEK and EA) high recovery yields from 93 to 99% could be reached using dry cells (Tables 2-4).

Chen et al., (Chen et al. 2001) demonstrated the recovery of P(HB-*co*-11mol%HHx) from dry cells at an industrial scale using EA. In the aforementioned study, 5,000 L of EA was added to 200 – 500 kg dry cells, with a PHA content of 50%, to form 2-5% PHA solvent mixtures. Polymer was then precipitated with 3 volumes of *n*-hexane or *n*-heptane. Recovery yield or purity data from these extractions are not available. However, direct recovery from wet biomass would eliminate a drying step of the cells, potentially saving time and cost. In our study, the recovery yield from the EA extraction using wet cells as starting material in 1.5 L scale was 71%, much lower than the 93% recovery yield observed from the 1.8 L extraction using dry cells as starting material. The solubility of EA in water is 4 fold higher than that of MIBK (Table 1). The intermixture of the PHA/EA-solution with water and the wet residual cell material may explain the lower recovery yield from PHA/EA-solution using wet cells

391 compared to dry cells. The residual cell mass from the EA extraction using wet cells showed a
392 high PHA concentration (Suppl. Fig. 2), which may have resulted from PHA solution
393 becoming trapped in the biomass, whereas the PHA content of the residual cell mass from the
394 dry 40 mL extraction was negligible (Table 3). Another possibility is that the presence of
395 water simply reduced the solvating power of the EA, leaving some polymer unextracted. The
396 3 L scale up with MIBK using wet biomass exhibited a recovery yield up to 84%, which is the
397 same recovery yield observed using dry cells in 40 mL extractions.

398 The purity of polymer from the 3 L MIBK extractions from wet cells improved to 99% by
399 extra washing with *n*-hexane. The purity of the PHA recovered from wet cells with EA was
400 slightly lower at 95%, although the same *n*-hexane wash was performed. The higher PHA
401 purity reached with MIBK could be explained by the PHA/MIBK-gel formation, which
402 covers the residual cell mass, separating it from the PHA/MIBK-solution. We did not filter
403 PHA/solvent-solutions before the polymer precipitation in extractions of greater than 2 mL
404 volumes. Therefore, the slight contamination seen in the EA extraction probably comes from
405 residual cell material during the separation of the residual biomass from the PHA/EA-solvent-
406 solution prior to PHA precipitation. All PHA extractions in our studies were performed using
407 a PHA solvent ratio of 2% (w/v). All solvents used were shown to be capable of dissolving
408 P(HB-*co*-20mol%HHx) to concentrations of 5-10% (w/v). Higher PHA concentrations would
409 reduce the amount of solvent used, but would also result in more viscous PHA solutions (Van
410 Walsem et al. 2007), which are more difficult to pump, centrifuge, or filter during
411 downstream processing. The viscosity of polymer solutions is dependent on polymer structure,
412 polymer molecular weight, concentration, solvent type and temperature (Flory 1953).

413 To recover dissolved polymer we chose to precipitate the polymer with alkanes, instead of
414 evaporating the solvent. Evaporation can be problematic in batch operations because the
415 polymer tends to coat the vessel after the solvent is removed. Additionally, any contaminants

that are also present in the solvent (e.g. residual lipids from plant oil fermentations) will co-purify with the PHA. We determined that adding a threefold volume of precipitant to PHA/solvent solution at room temperature precipitated the polymer sufficiently (Figure 4, Table 2). A smaller ratio may be possible at a lower precipitation temperature. The boiling point of *n*-hexane is lower than that of *n*-heptane. This suggests that *n*-hexane should be easier to separate from both BA and MIBK, making it a more promising precipitant for these solvents, due to lower cost during solvent recycling. However, *n*-heptane is rated as a class 3 chemical by the FDA, while *n*-hexane is class 2, and is therefore considered as less safe than *n*-heptane (http://www.fda.gov/RegulatoryInformation/Guidances/ucm128290.htm). If PHA is destined for biomedical applications, then *n*-heptane may be the preferred precipitant. If EA or MEK was chosen as the solvent, *n*-heptane or *n*-octane could be used as a precipitant due to the greater differences in their boiling points, as compared to *n*-hexane with the solvents.

It is possible that some residual palm oil and fatty acids may be associated with the biomass at the end of a high density fermentation. It was shown that these lipids dissolve in the solvents used in this work, but were not precipitated during the recovery process (Supp. Fig. 1). However, after precipitation, residual solvent can be removed from the polymer by washing with precipitant, to avoid contamination of PHA with residual lipids.

For a recovery process using wet cells as starting material, we recommend the solvent/precipitant pair of MIBK/*n*-hexane, based on the polymer recovery results obtained in this work, as well as the large differences in boiling points, which predicts effective recycling of solvent through distillation. BA could be used alternatively to MIBK because it is less miscible with water, has a higher boiling point, is less flammable, and has a higher PEL. However, the performed recovery studies showed higher recovery yields using MIBK. One potential issue with BA is that it can degrade by hydrolysis in the presence of water (Sakamuri

2005), which is clearly a concern given that in a sustainable process, solvent would be continuously recycled.

ACKNOWLEDGEMENTS

This work was funded by the Malaysia MIT Biotechnology Partnership Programme (MMBPP). The authors thank Mr. Tony DeBono for helpful discussions and ideas. We also thank Mr. John Quimby and Ms. Jingnan Lu for their critical reviewing of the manuscript. We thank our MMBPP collaborators for their continued support.

References

Baptist J; 1962a. May, 1962. Process for preparing poly-[beta]-hydroxybutyric acid. USA patent US3036959.

Baptist J; 1962b. Process for preparing poly-beta-hydroxy butyric acid. USA patent US3044942.

Bensch M, Selbach B, Hubbuch J. 2007. High throughput screening techniques in downstream processing: preparation, characterization, and optimization of aqueous two-phase systems. Chem Eng Sci 62:2011-2021.

Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ. 2011. Production of Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) from Plant Oil by Engineered *Ralstonia eutropha* Strains. Appl Environ Microbiol 77(9):2847-54.

Chen GQ. 2009. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38(8):2434-46.

Chen GQ, Zhang G, Park SJ, Lee SY. 2001. Industrial scale production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Appl Microbiol Biotechnol 57(1-2):50-5.

Flory PJ, ed. 1953. Principles of Polymer Chemistry. Ithaca, NY: Cornell University Press.

Jacquel N, Lo C-W, Wei Y-H, Wu H-S, Wang SS. 2008. Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochem Eng J 39:15-27.

Kapritchkoff FM, Viotti AP, Alli RC, Zuccolo M, Pradella JG, Maiorano AE, Miranda EA, Bonomi A. 2006. Enzymatic recovery and purification of polyhydroxybutyrate produced by *Ralstonia eutropha*. J Biotechnol 122(4):453-62.

Kathiraser Y, Aroua MK, Ramachandran KB, Tan IKP. 2007. Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. J Chem Technol Biotechnol 82(9):847-855.

Kinoshita K, Osakada Y, Ueda Y, Narasimhan K, Cearly AC, Yee K, Noda I; 2006 December 26, 2006. Method for producing polyhydroxyalkanoate crystal. USA patent. US 2005/0222373 A1.

Kunasundari B, Sudesh K. 2011. Isolation and recovery of polyhydroxyalkanoates. Express Polym Lett 5(7):620-634.

Lee SY, Choi J, Han K, Song JY. 1999. Removal of endotoxin during purification of poly(3-hydroxybutyrate) from gram-negative bacteria. Appl Environ Microbiol 65(6):2762-4.

Lemoigne M. 1927. Études sur l'autolyse microbienne origine de l'acide b-oxybutyrique formé par autolyse. Ann Inst Pasteur 41:148-165.

Noda I; 1998 October 13, 1998. Solvent extraction of polyhydroxy-alkanoates from biomass facilitated by the use of marginal nonsolvent. USA patent 5821299.

Noda I, Green PR, Satkowski MM, Schechtman LA. 2005. Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. Biomacromolecules 6(2):580-6.

Philip S, Keshavarz T, Roy I. 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. J Chem Technol Biotechnol 82:233-247.

Ramsay JA, Berger E, Voyer R, Chavarie C, Ramsay BA. 1994. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnol Techniques 8(8):589-594.

Riedel SL, Bader J, Brigham CJ, Budde CF, Yusof ZA, Rha C, Sinskey AJ. 2012. Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by *Ralstonia eutropha* in high cell density palm oil fermentations. Biotechnol Bioeng 109(1):74-83.

Sakamuri R. 2005. Organic Esters. In: Siedel A, editor. Kirk-Othmer Encyclopedia of Chemical Technology. Hoboken, NJ: John Wiley and Sons. p 497-526.

Sevastianov VI, Perova NV, Shishatskaya EI, Kalacheva GS, Volova TG. 2003. Production of purified polyhydroxyalkanoates (PHAs) for applications in contact with blood. J Biomater Sci Polym Ed 14(10):1029-42.

Shishatskaya EI, Volova TG. 2004. A comparative investigation of biodegradable polyhydroxyalkanoate films as matrices for in vitro cell cultures. J Mater Sci Mater Med 15(8):915-23.

Shishatskaya EI, Volova TG, Efremov SN, Puzyr' AP, Mogil'naya OA. 2002. Tissue response to biodegradable suture threads made of polyhydroxyalkanoates. Biomed Eng 36(4):210-217.

Shum-Tim D, Stock U, Hrkach J, Shinoka T, Lien J, Moses MA, Stamp A, Taylor G, Moran AM, Landis W and others. 1999. Tissue engineering of autologous aorta using a new biodegradable polymer. Ann Thorac Surg 68(6):2298- 2305.

Sodian R, Sperling JS, Martin DP, Stock U, Mayer JE, Jr., Vacanti JP. 1999. Tissue engineering of a trileaflet heart valve-early in vitro experiences with a combined polymer. Tissue Eng 5(5):489-94.

Tamer IM, Young MM, Chisti Y. 1998. Disruption of *Alcaligenes latus* for recovery of poly(ß-hydroxybutyric acid): Comparison of high-pressure homogenization, bead milling, and chemically induced lysis. Ind Eng Chem Res 37:1807-1814.

Van Walsem J, Zhong L, Shih S; 2007 August 7, 2007. Polymer extraction methods. USA patent 8003355.

Williams SF, Martin DP, Skraly FA; 2002. Medical devices and applications of polyhydroxyalkanoate polymers. USA patent US 2002/0173558 A1.

Yang YH, Brigham C, Willis L, Rha C, Sinskey A. 2011. Improved detergent-based recovery of polyhydroxyalkanoates (PHAs). Biotechnol Lett.

Yaws CL. 1999. Chemical Properties Handbook. New York: McGraw-Hill. 200 p.

FIGURE LEGENDS

Fig. 1.

Separation of PHA/MIBK-solution, PHA/MIBK-gel and residual cell mass, following PHA extraction from wet cells with MIBK. Polymer in solution was extracted for 4 h at 100°C under reflux conditions. The sample was cooled to room temperature and centrifuged for 10 min at 2200 × *g*.

Fig. 2. Flow sheet of a general PHA extraction process. P(HB-*co*-HHx) was extracted from wet or dry biomass using the non-halogenated solvents: EA, MEK, BA or MIBK. (*) The residual cell mass was washed twice with 2.5 mL solvent during the 40 mL extraction process from dry cells (**), or three times with water after the 1.5 L EA extraction from wet cells.

Fig. 3. Solubility of P(HB-*co*-HHx) polymer in various non-halogenated solvents. 5% and 10% PHA solutions were made using the MEK, BA, MIBK, IA, EA or chloroform. Data indicating recovery of the PHA polymer (% input) from polymer solutions are presented. Error bars indicate standard deviation from triplicate experiments. Asterisk (*) indicates that, after incubation with IA (10% PHA mixture), it was not possible to filter the solution and determine a recovery value.

Fig. 4. Examination of precipitants for P(HB-*co*-HHx) recovery. Using MIBK or BA as PHA solvents, 5% PHA solutions were made. The polymer was precipitated by addition of *n*- hexane or *n*-heptane to the solution at room temperature. Averages of two replicates are shown.

Table 1: Property data for chemicals that could potentially be used in a PHA recovery process. The top group of compounds consists of potential PHA solvents, with water included as a reference. The bottom three compounds (*n*-hexane, *n*-heptane, and *n*-octane) are used as PHA precipitants.

Compound	Boiling Point (°C)[a]	Density (g/cm^3)[a]	Viscosity (cP)[a]	Heat capacity ($J\ mol^{-1}\ K^{-1}$)[a]	Solubility in water (ppmw)[a]	PEL (ppm)[b]	FDA class[c]	Price ($US/ lb.)[d]
Water	100	1.03	0.91	76	N.D.	N.D.	safe	<0.01[e]
Chloroform	61	1.48	0.54	112	7.50e+03	50	2	0.26-0.47
Ethyl acetate	77	0.89	0.42	171	7.37e+04	400	3	0.48-0.59
Butyl acetate	126	0.88	0.68	228	6.80e+03	150	3	0.67-0.72
Methyl isobutyl ketone	117	0.80	0.60	212	1.90e+04	100	3	0.74-0.79
Methyl ethyl ketone	80	0.80	0.40	160	2.48e+05	200	3	0.32-0.34
Isoamyl alcohol	131	0.81	3.69	165	2.70e+04	100	3	na[d]
n-hexane	69	0.66	0.30	193	1.33e+01	500	2	1.15-1.19
n-heptane	98	0.68	0.39	230	2.24e+00	500	3	1.21-1.64
n-octane	126	0.70	0.51	255	4.31e-01	500	N.D.	na[d]

[a]Physical property data is from (Yaws 1999), measured at 25°C and 1 atm.

[b]PEL is the Permissible Exposure Limit established by the United States Occupational Safety and Health Administration (OSHA, standard number: 1910.1000 TABLE Z-1) .

[c]The FDA rates chemicals for use in manufacturing of biomedical products, where 1 is most toxic and 3 is least toxic, Q3C Feb 12 (http://www.fda.gov/RegulatoryInformation/Guidances/ucm128290.htm)

N.D. indicates no data was available.

[d]Price data from www.icis.com (2006-2008 prices); na = data not available.

[e]Average US pricing per gallon (US$1.50 per 1000 gallons). Source: American Water Works Association (www.awwa.org). Does not include price of wastewater treatment.

Table 2: P(HB-*co*-HHx) recovery from dry *R. eutropha* cells on a 2 mL scale. PHA was extracted for 4 h at 100˚C, 75˚C or 50˚C with the non-halogenated solvents (52.6 mg cells, 76% CDW of PHA, 2 mL solvent). Chloroform extractions were used as controls. In all cases, the extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature from PHA/solvent-solution (S) or PHA/solvent-gel (G) and dried at 50˚C. All values represent means from triplicate extractions with error bars indicating ± S.D.

Solvent (PHA/solvent-solution or gel)	Temperaure (˚C)	Recovery Yield (%)	Purity (%)	HHx (mol%)
Chlorofrom[c] (S[a])	100	99 ± 1	100 ± 1	15 ± 1
	75	98 ± 0	100 ± 0	15 ± 0
	50	95 ± 1	100 ± 1	16 ± 0
MIBK[b](S)	100	55 ± 2	99 ± 1	17 ± 0
	75	37 ± 2	99 ± 1	17 ± 0
	50	37 ± 2	100 ± 0	21 ± 0
MIBK(G[a])	100	24 ± 4	96 ± 2	14 ± 0
	75	35 ± 5	98 ± 2	15 ± 0
MEK[b,c] (S)	100	95 ± 1	100 ± 1	16 ± 0
	75	95 ± 1	99 ± 0	15 ± 0
	50	87 ± 3	100 ± 0	17 ± 0
BA[b](S)	100	42 ± 0	100 ± 1	17 ± 1
	75	27 ± 1	100 ± 1	17 ± 0
	50	41 ± 1	99 ± 2	19 ± 0
BA(G)	100	33 ± 5	100 ± 1	15 ± 0
	75	33 ± 3	95 ± 3	15 ± 0
EA[b,c] (S)	100	99 ± 0	100 ± 0	16 ± 0
	75	95 ± 0	97 ± 0	15 ± 0
	50	76 ± 0	100 ± 1	17 ± 1

[a](S) indicates PHA/solvent-solution, (G) indicates PHA/solvent-gel.
[b]MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate
[c]No PHA/solvent-gel formation was observed using MEK, EA or chloroform

Table 3: P(HB-*co*-HHx) recovery from dry *R. eutropha* cells (PHA content of 62% with HHx concentration of 22 mol%) at the 40 mL scale. PHA was extracted for 4 h at 100˚C using non-halogenated solvents. The extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature from PHA/solvent-solution or PHA/solvent-gel. PHA and the residual cell mass were dried at 50˚C. All values represent minimum and maximum data from duplicate extractions.

Solvent	**PHA/solvent-solution**		**RY[a](%)**	**Total RY (%)**	**Rcells[b]**	
***n*=2**	**purity (%)**	**HHx (mol%)**	(%)		**PHA content (%)**	**HHx (mol%)**
MIBK[c]	96 ± 1	24 ± 0	71 ± 0	84 ± 0	13 ± 4	11 ± 0
BA[c]	95 ± 2	24 ± 0	68 ± 0	76 ± 0	24 ± 1	12 ± 0
EA[c]	97 ± 1	21 ± 0	94 ± 0	94 ± 0	2 ± 0	20 ± 0
MEK[c]	97 ± 2	21 ± 0	95 ± 0	95 ± 0	2 ± 1	20 ± 0

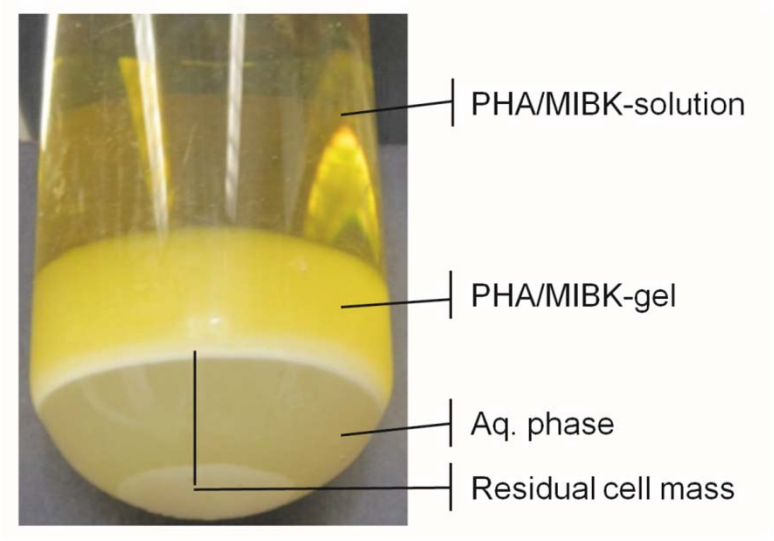
Solvent[d]	**PHA/solvent-gel**		**RY[a] (%)**
***n*=2**	**purity (%)**	**HHx (mol%)**	(%)
MIBK	73 ± 2	12 ± 0	13 ± 0
BA	62 ± 0	11 ± 0	8 ± 0

[a] PHA recovery yield (RY), total RY combines RY from PHA/solvent-solution and PHA/solvent-gel
[b]Residual cell mass
[c]MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate
[d]No PHA/solvent-gel formation was observed using: MEK and EA

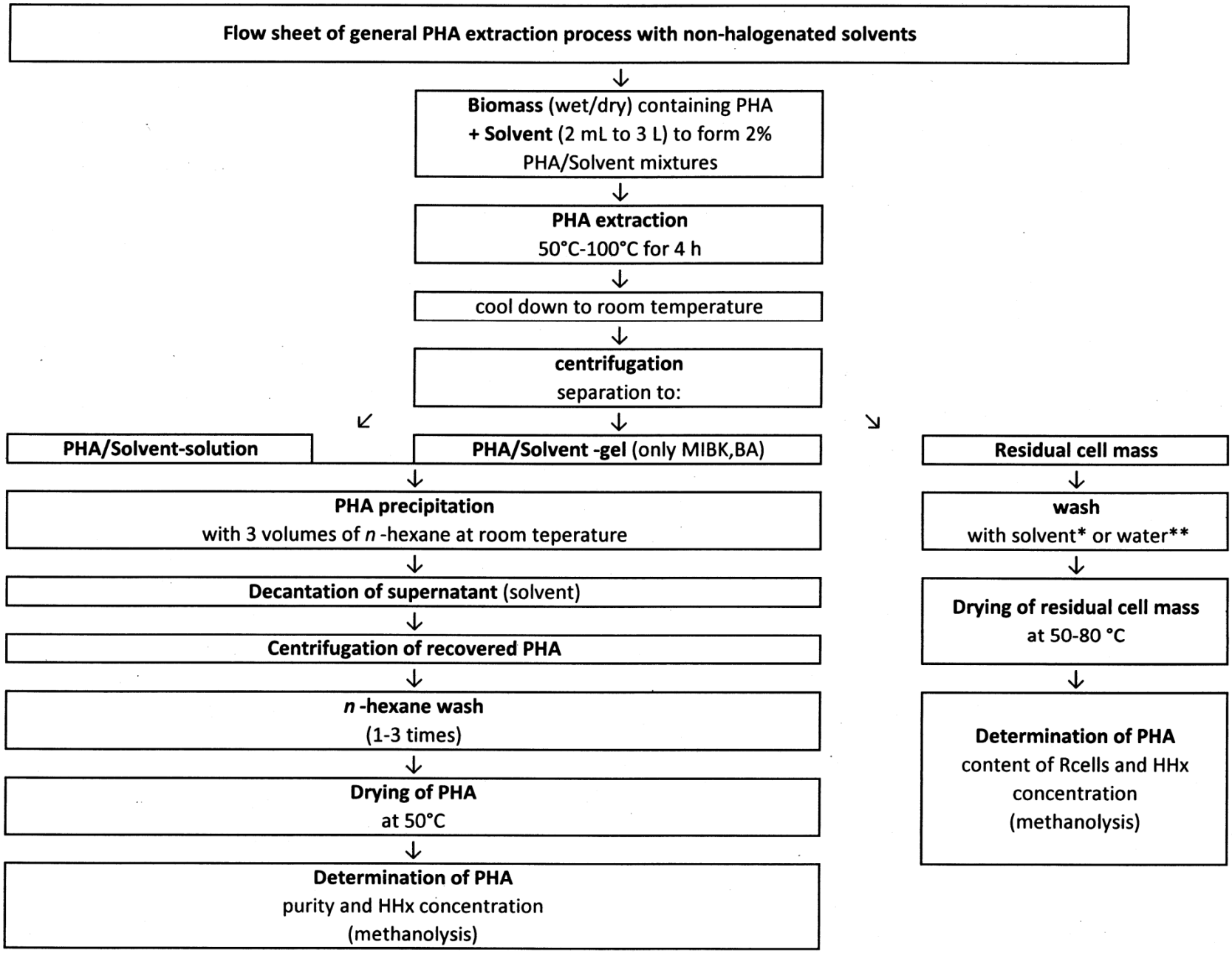
Table 4: Larger scale recovery of P(HB-*co*-HHx) from dry and wet cells. PHA was extracted for 4 h at 100˚C, with the non-halogenated solvents to form a 2% PHA mixture. The extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature and dried at 50˚C.

Solvent, Vol.	**Biomass (mol% HHx of PHA)**	**PHA recovered (g)**		**Purity (%)**		**mol% HHx of recovered PHA**		**Recovery yield (%)**		
		S[a]	G[a]	S	G	S	G	S	G	total
MIBK[b], 3 L	wet (20)	45	10	92	90	20	15	69	15	84
MIBK, 3 L	wet (20)	37	2	>99	80 - 99	21	14	61	3	64
MIBK, 1.35 L	dry (29)	20	2	>99	78 - 99	30	12 -18	74	5	79
EA[b], 1.5 L	wet (22)	21	nd[c]	95	nd[c]	21	nd[c]	71	nd[c]	71
EA, 1.78 L	dry(18)	33	nd	95	nd	17	nd	93		93

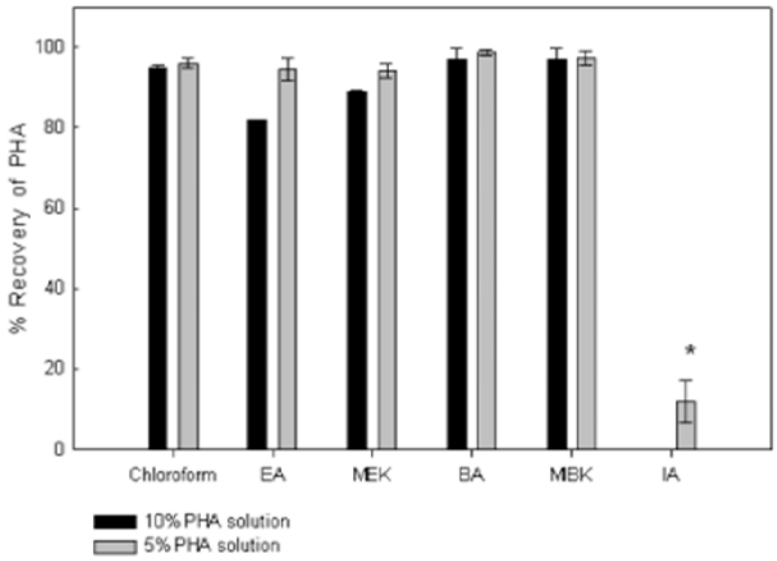
[a]S = PHA/solvent solution; G = PHA/solvent gel
[b]MIBK = methyl isobutyl ketone; EA = ethyl acetate
[c]No PHA/solvent gel was detected in EA-based recovery of PHA



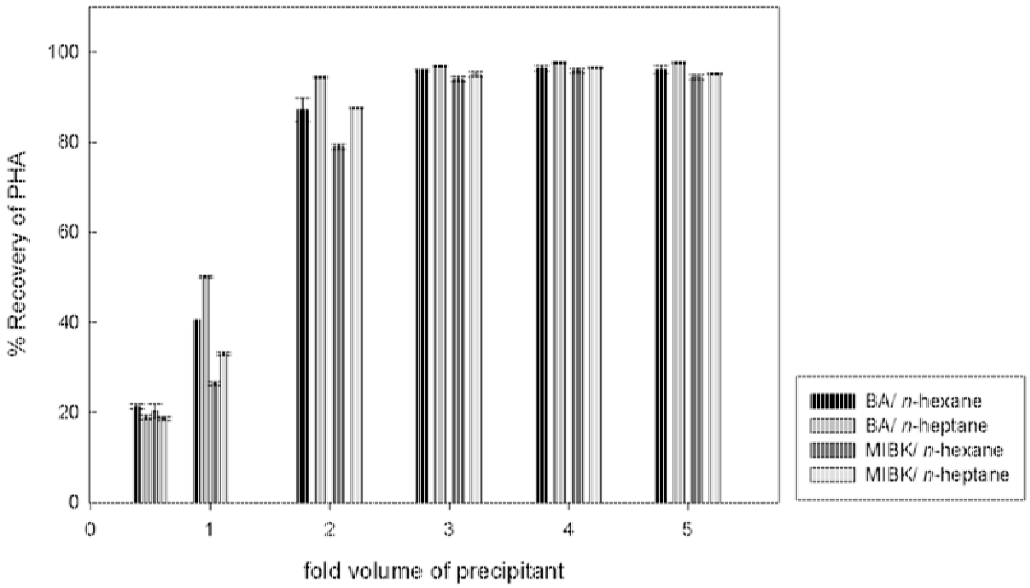
Riedel, et al, Figure 1



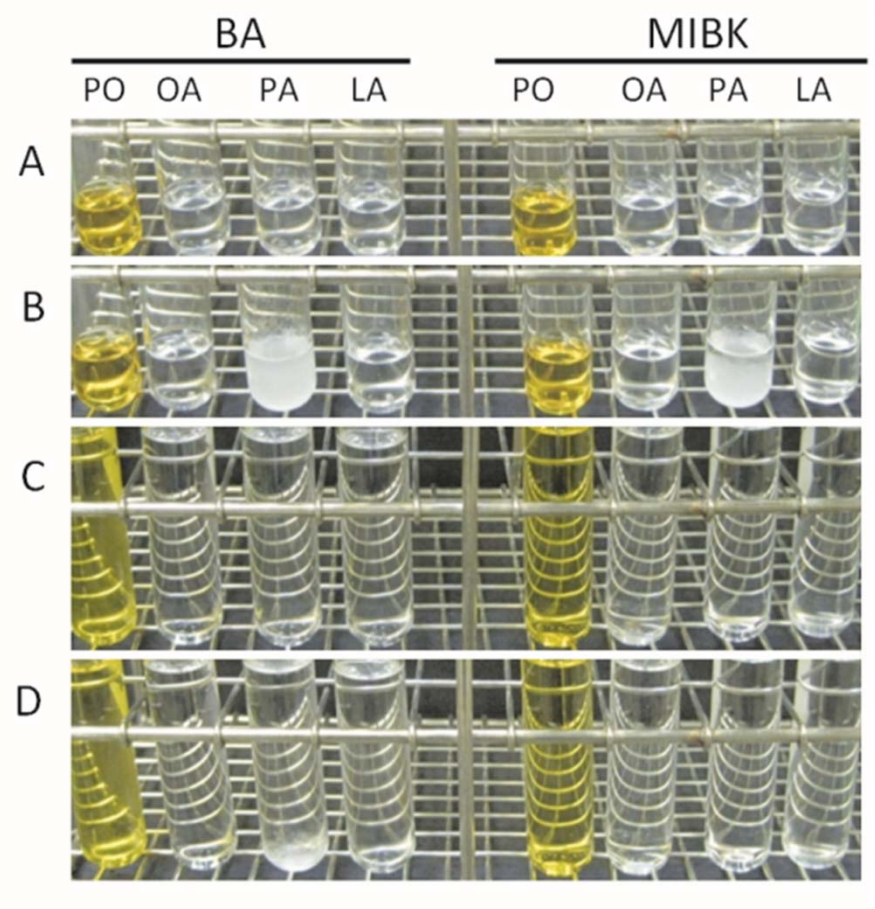
Riedel, et al, Figure 2



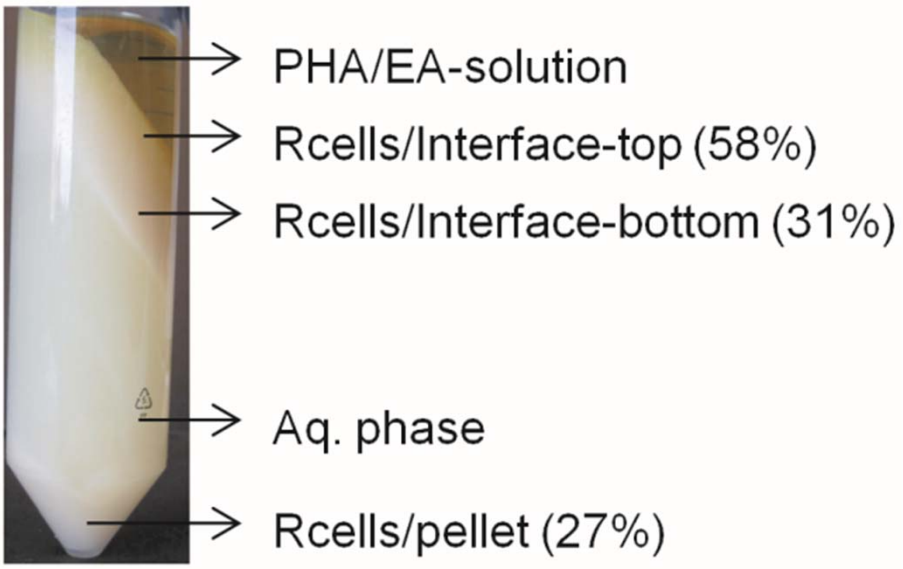
Riedel, et al, Figure 3



Riedel, et al, Figure 4



Riedel, et al, Suppl. Fig. 1



PHA content of Rcells fractions in brackets, HHx level was 22 mol% by all.

Riedel, et al, Suppl. Fig. 2

Supplemental Figure 1

Test of precipitation of lipids and fatty acids from solvents used in this work. Solutions of palm oil (PO), oleic acid (OA), palmitic acid (PA), and lauric acid (LA) were made (5% w/v) in BA and MIBK. The solutions were made at room temperature (A) and then incubated overnight at 4°C (B). The solutions were then warmed back to room temperature and three volumes of *n*-hexane were added to determine if precipitation occurred. The new solutions (with precipitant) were again incubated at room temperature (C) and overnight at 4°C (D). PA precipitates from BA and MIBK at 4°C, and from BA/*n*-hexane at 4°C.

Supplemental Figure 2

Separation of residual cell mass (Rcells) into phases following P(HB-*co*-HHx) extraction with EA. Cell debris was centrifuged after PHA extraction from wet cells. During the centrifugation step, an interface was observed to form between the PHA/EA-solution and the aqueous phase, as indicated in the figure. The interface had a yellow top portion (Rcells/Interface-top) and a white bottom portion (Rcells/Interface-bottom). The PHA concentrations of every Rcell fraction were observed to be different, but the mol% HHx content of polymer from each phase was the same.