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

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2

3 Title: Recovery of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from *Ralstonia*

4 *eutropha* cultures with non-halogenated solvents

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

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20

21

22

23 **ABSTRACT**

24 Reduced downstream costs, together with high purity recovery of polyhydroxyalkanoate  
25 (PHA), will accelerate the commercialization of high quality PHA-based products. In this  
26 work, a process was designed for effective recovery of the copolymer poly(hydroxybutyrate-  
27 *co*-hydroxyhexanoate) (P(HB-*co*-HHx)) containing high levels of HHx (>15 mol%) from  
28 *Ralstonia eutropha* biomass using non-halogenated solvents. Several non-halogenated  
29 solvents (methyl isobutyl ketone, methyl ethyl ketone, butyl acetate and ethyl acetate) were  
30 found to effectively dissolve the polymer. Isoamyl alcohol was found to be not suitable for  
31 extraction of polymer. All PHA extractions were performed from both dry and wet cells at  
32 volumes ranging from 2 mL to 3 L using a PHA to solvent ratio of 2% (w/v). Ethyl acetate  
33 showed both high recovery levels and high product purities (up to 99%) when using dry cells  
34 as starting material. Recovery from wet cells, however, eliminates a biomass drying step  
35 during the downstream process, potentially saving time and cost. When wet cells were used,  
36 methyl isobutyl ketone (MIBK) was shown to be the most favorable solvent for PHA recovery.  
37 Purities of up to 99% and total recovery yields of up to 84% from wet cells were reached.  
38 During polymer recovery with either MIBK or butyl acetate, fractionation of the extracted  
39 PHA occurred, based on the HHx content of the polymer. PHA with higher HHx content (17-  
40 30 mol%) remained completely in solution, while polymer with a lower HHx content (11-16  
41 mol%) formed a gel-like phase. All PHA in solution could be precipitated by addition of 3-  
42 fold volumes of *n*-hexane or *n*-heptane to unfiltered PHA solutions. Effective recycling of the  
43 solvents in this system is predicted due to the large differences in the boiling points between  
44 solvent and precipitant. Our findings show that two non-halogenated solvents are good  
45 candidates to replace halogenated solvents like chloroform for recovery of high quality PHA.  
46

## 47 INTRODUCTION

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

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

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

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

79 Recovery of PHA from bacterial cells using organic solvents is often applied in industrial  
80 processes due to the recovery efficiency of the process, polymer purity obtained, and the  
81 possible removal of endotoxins from the recovered polymer, which is important for medical  
82 applications (Lee et al. 1999; Sevastianov et al. 2003). In a first step, the PHA is extracted  
83 from biomass with a suitable solvent (*e.g.* chloroform) and then separated from the residual  
84 biomass, *e.g.* through centrifugation and filtration. Polymer precipitation is then conducted  
85 with the addition of a non-PHA-dissolving solvent (*e.g.* methanol) or the polymer is recovered  
86 through cooling the solution or by solvent evaporation. Chloroform was the first solvent used  
87 to extract PHB from cells (Lemoigne 1927) followed by other halogenated solvents, including  
88 dichloromethane (Baptist 1962a). For the recovery of medium chain length (MCL) PHAs, a  
89 variety of ketones, esters, and alcohols are potentially usable (Kinoshida et al. 2006; Noda  
90 1998; Van Walsem et al. 2007).

91 In this work, we examine the recovery of poly(hydroxybutyrate-*co*-hydroxyhexanoate)  
92 (P(HB-*co*-HHx)) polymer from wet and dry biomass using non-halogenated solvents. All of  
93 the PHA recovered was produced using high cell density palm oil fermentations, comprised of  
94 >139 g/L of biomass with a PHA content of 74% and an average HHx monomer content of 19  
95 mol% (Riedel et al. 2012). Another success factor considered in this work was the separation  
96 of residual oil or fatty acids from the PHA. Lastly, some representative solvents used in this

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

100

## 101 **MATERIALS AND METHODS**

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

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

### 109 **Test of solvents for PHA recovery**

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

121 **Test of precipitants for recovery of PHA**

122 To test precipitants, 5% stock solutions of PHA in MIBK and BA were prepared in sealed  
123 vessels. For each precipitant tested, 1 mL of the 5% PHA solution was transferred into pre-  
124 weighed borosilicate glass test tubes. PHA was then precipitated by addition of 0.5-5 volumes  
125 of precipitant (*n*-hexane or *n*-heptane) at room temperature. The tubes were briefly vortexed  
126 and incubated at room temperature for 1 h. Following mixing, the tubes were centrifuged for  
127 10 min at  $2500 \times g$  and  $20^{\circ}\text{C}$ . The supernatant was discarded and the PHA pellet was initially  
128 dried in a heating block at  $50^{\circ}\text{C}$  and finally in a vacuum oven at  $80^{\circ}\text{C}$  until dry.

129 **Test of lipid and fatty acid precipitation**

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

137 **Recovery of PHA from dry cells in 2 mL scale**

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

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

#### 158 **Recovery of PHA from dry cells in 40 mL scale**

159 Samples of freeze-dried biomass, containing 0.8 g PHA, were each extracted with 40 mL of  
160 non-halogenated solvents (EA, MIBK, MEK or BA) to form 2% PHA/solvent mixtures.  
161 Extraction occurred at 100°C in 125 mL flat-bottom flasks under reflux cooling conditions for  
162 4 h. The samples were cooled to room temperature and centrifuged at  $6000 \times g$  for 10 min in  
163 50 mL polypropylene tubes. The flat-bottom flask was rinsed twice with 2.5 mL solvent and  
164 used to wash residual cell material. The PHA was precipitated with three volumes of *n*-hexane  
165 at room temperature and washed three times with *n*-hexane. The washed polymer was dried at  
166 50°C overnight. Both the monomer compositions of PHA polymer and residual cell material  
167 were determined as described below.

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



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

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

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

#### 187 **Larger scale recovery from wet cells**

188 Equal volumes (400-750 mL) of fermentation broth (containing cells and PHA) were thawed  
189 in warm water and centrifuged for 20 min at  $7200 \times g$  at room temperature. The wet cell pellet  
190 was transferred into a 5 L round-bottom flask, and solvent was added to form a 2% PHA  
191 solvent mixtures (*e.g.* wet cells containing 60 g PHA in 3 L solvent). PHA was extracted at  
192 100°C with stirring under reflux conditions for 4 h. At the beginning of the extraction, 0.33 L  
193 of water per 1 L solvent was added to enhance mixing of the wet cell pellet with the solvent.

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

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

## 211 **Analytical methods**

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

$$217 \text{ Recovery yield (RY, \%)} = \frac{\text{mass PHA recovered (g)} * \text{Purity (\%)}}{\text{mass PHA in cells used in recovery batch (g)}}$$

## 218 **RESULTS**

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

### 233 **Testing PHA solubility in chosen solvents**

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

### 239 **Test of precipitants for recovery of PHA**

240 The precipitants *n*-hexane and *n*-heptane were tested for PHA precipitation. PHA was  
241 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 4°C (Supp. Fig. 1). After addition of *n*-hexane,  
251 no lipid precipitation was observed at room temperature, but when solutions were incubated at  
252 4°C, 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 ( $\geq 98\%$ ) 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.

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

276 Interestingly, in the recovery processes where a gel phase was observed, the monomer  
277 compositions of polymer recovered from PHA/solvent-solution and PHA/solvent-gel were  
278 different from each other. Polymer recovered from PHA/solvent-solution had a notably higher  
279 HHx level (17 mol%) compared to the polymer recovered from the PHA/solvent-gel (14-15  
280 mol%) or compared to the total polymer recovered using chloroform as the solvent (15 mol%).  
281 The polymer recovered from the PHA/MIBK-solution or PHA/BA-solution at an extraction  
282 temperature of 50°C had an even higher HHx concentration at 21 mol% or 19 mol%,  
283 respectively. PHA recovery using MEK or EA exhibited a high recovery yield ( $\geq 95\%$ ) at  
284 temperatures of 75°C and 100°C. However, at 50°C, the recovery yield decreased to 87% or  
285 76%, respectively, concomitant with a slight increase in HHx content to ~17 mol%. No  
286 PHA/solvent-gel formation was observed during recovery with MEK and EA.

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

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

292 The data from 40 mL scale recovery experiments are shown in Table 3. A PHA/solvent-gel  
293 was observed using the solvents MIBK and BA, similar to results seen during the 2 mL  
294 extractions. The purities of the recovered polymer samples from the PHA/solvent-gel were  
295 lower relative to polymer from solution, with purities of 73% or 62%, using MIBK or BA,  
296 respectively. The HHx concentrations of polymer recovered from either PHA/MIBK-solution  
297 or PHA/BA-solution were, at 24 mol%, slightly higher compared to the polymer present in  
298 cells (22 mol% HHx). Furthermore, the HHx content of the polymer recovered from the  
299 PHA/MIBK-gel or the PHA/BA-gel was much lower, at 12 or 11 mol%, respectively.  
300 Extraction with either EA or MEK gave a high polymer recovery yield of  $\geq 94\%$ . The HHx  
301 content of polymer from the PHA/EA-solution or PHA/MEK-solution was  $\sim 21$  mol%, which  
302 was similar to the HHx content measured in whole cells. Analysis of the residual cell mass  
303 from extractions using MEK or EA showed that only minor amounts of unrecovered PHA (2  
304 wt% of cell mass) were present. However, the PHA content of residual cell mass from cells  
305 treated with either MIBK or BA was much higher, at 13% or 24%, respectively. The HHx  
306 monomer content of PHA not extracted from the cells with these solvents was 11-12 mol%.  
307 The total recovery yield from MIBK extraction was 84% (71% from PHA/MIBK-solution and  
308 13% from PHA/MIBK-gel). The total recovery yield was lowest with BA, reaching only 76%  
309 (68% from PHA/BA-solution and 8% from PHA/BA-gel).

### 310 **Larger scale PHA recovery**

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

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

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

325 PHA recovery with EA from wet cells in a 1.5 L scale showed no PHA/EA-gel formation, as  
326 expected. The recovered polymer had a purity of 98% with an HHx content of 21 mol%.  
327 During centrifugation of the extraction mixture, a separation of the residual cell material into  
328 three distinct phases was observed, as described in Materials and Methods (Supp. Fig. 2). The  
329 PHA content from the residual cells/interface-top (the upper phase) had a PHA content of  
330 58%, the residual cells/interface-bottom (the middle phase) had a PHA content of 31% and the  
331 residual cell pellet (bottom phase) a PHA content of 27%. We hypothesize that, during  
332 recovery of PHA from wet cells using EA as the solvent, a mixture of EA, water, and residual  
333 cell debris formed, resulting in a significant portion of the polymer remaining with the wet  
334 cell mass. All three phases had an average HHx content of 22 mol% (Supp. Fig. 2). The  
335 recovery yield from the PHA/EA-solution from wet cells was only 71%, whereas PHA  
336 recovery from dry cells in a 1.8 L extraction gave a recovery yield of 93% with a purity of  
337 95%.

## 338 **DISCUSSION**

339 There are several requirements that must be met for a PHA production process to be  
340 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,



366 small amounts of PHA containing low levels of HHx (11-12 mol%) remained in the residual  
367 cell material. This indicates that higher HHx content makes the polymer more soluble, as has  
368 been observed previously (Noda et al. 2005). It is unclear whether the gel was present  
369 throughout the extraction, or only appeared as the solution cooled during centrifugation. The  
370 fractionation of PHA during recovery confirms our previous finding that the strain used in this  
371 study makes PHA with varying HHx content during fermentation on palm oil (Budde et al.  
372 2011).

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

380 Chen et al., (Chen et al. 2001) demonstrated the recovery of P(HB-co-11mol%HHx) from dry  
381 cells at an industrial scale using EA. In the aforementioned study, 5,000 L of EA was added to  
382 200 – 500 kg dry cells, with a PHA content of 50%, to form 2-5% PHA solvent mixtures.  
383 Polymer was then precipitated with 3 volumes of *n*-hexane or *n*-heptane. Recovery yield or  
384 purity data from these extractions are not available. However, direct recovery from wet  
385 biomass would eliminate a drying step of the cells, potentially saving time and cost. In our  
386 study, the recovery yield from the EA extraction using wet cells as starting material in 1.5 L  
387 scale was 71%, much lower than the 93% recovery yield observed from the 1.8 L extraction  
388 using dry cells as starting material. The solubility of EA in water is 4 fold higher than that of  
389 MIBK (Table 1). The intermixture of the PHA/EA-solution with water and the wet residual  
390 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

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

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

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

440 2005), which is clearly a concern given that in a sustainable process, solvent would be  
441 continuously recycled.

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447

## 448 **References**

449

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

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

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

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

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

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

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

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

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

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

473 Kinoshita K, Osakada Y, Ueda Y, Narasimhan K, Cearly AC, Yee K, Noda I; 2006 December  
474 26, 2006. Method for producing polyhydroxyalkanoate crystal. USA patent. US  
475 2005/0222373 A1.

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

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

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

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

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

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

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

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

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

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

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

501 Shishatskaya EI, Volova TG, Efremov SN, Puzyr' AP, Mogil'naya OA. 2002. Tissue response  
502 to biodegradable suture threads made of polyhydroxyalkanoates. *Biomed Eng*  
503 36(4):210-217.

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

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

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

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

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

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

519 Yaws CL. 1999. *Chemical Properties Handbook*. New York: McGraw-Hill. 200 p.  
520  
521

## 1 FIGURE LEGENDS

### 2 Fig. 1.

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

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

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

16 **Fig. 4.** Examination of precipitants for P(HB-*co*-HHx) recovery. Using MIBK or BA as PHA solvents,  
17 5% PHA solutions were made. The polymer was precipitated by addition of *n*-hexane or *n*-heptane to  
18 the solution at room temperature. Averages of two replicates are shown.

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

Compound	Boiling Point (°C) <sup>a</sup>	Density (g/cm <sup>3</sup> ) <sup>a</sup>	Viscosity (cP) <sup>a</sup>	Heat capacity (J mol <sup>-1</sup> K <sup>-1</sup> ) <sup>a</sup>	Solubility in water (ppmw) <sup>a</sup>	PEL (ppm) <sup>b</sup>	FDA class <sup>c</sup>	Price (\$US/ lb.) <sup>d</sup>
Water	100	1.03	0.91	76	N.D.	N.D.	safe	<0.01 <sup>e</sup>
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 <sup>d</sup>
<i>n</i> -hexane	69	0.66	0.30	193	1.33e+01	500	2	1.15-1.19
<i>n</i> -heptane	98	0.68	0.39	230	2.24e+00	500	3	1.21-1.64
<i>n</i> -octane	126	0.70	0.51	255	4.31e-01	500	N.D.	na <sup>d</sup>

4  
 5 <sup>a</sup>Physical property data is from (Yaws 1999), measured at 25°C and 1 atm.

6 <sup>b</sup>PEL is the Permissible Exposure Limit established by the United States Occupational Safety  
 7 and Health Administration (OSHA, standard number: 1910.1000 TABLE Z-1 ).

8 <sup>c</sup>The FDA rates chemicals for use in manufacturing of biomedical products, where 1 is most  
 9 toxic and 3 is least toxic, Q3C Feb 12

10 (<http://www.fda.gov/RegulatoryInformation/Guidances/ucm128290.htm>)

11 N.D. indicates no data was available.

12 <sup>d</sup>Price data from [www.icis.com](http://www.icis.com) (2006-2008 prices); na = data not available.

13 <sup>e</sup>Average US pricing per gallon (US\$1.50 per 1000 gallons). Source: American Water Works  
 14 Association ([www.awwa.org](http://www.awwa.org)). Does not include price of wastewater treatment.



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

Solvent (PHA/solvent- solution or gel)	Temperature (°C)	Recovery Yield (%)	Purity (%)	HHx (mol%)
Chloroform <sup>c</sup> (S <sup>a</sup> )	100	99 $\pm$ 1	100 $\pm$ 1	15 $\pm$ 1
	75	98 $\pm$ 0	100 $\pm$ 0	15 $\pm$ 0
	50	95 $\pm$ 1	100 $\pm$ 1	16 $\pm$ 0
MIBK <sup>b</sup> (S)	100	55 $\pm$ 2	99 $\pm$ 1	17 $\pm$ 0
	75	37 $\pm$ 2	99 $\pm$ 1	17 $\pm$ 0
	50	37 $\pm$ 2	100 $\pm$ 0	21 $\pm$ 0
MIBK(G <sup>a</sup> )	100	24 $\pm$ 4	96 $\pm$ 2	14 $\pm$ 0
	75	35 $\pm$ 5	98 $\pm$ 2	15 $\pm$ 0
MEK <sup>b,c</sup> (S)	100	95 $\pm$ 1	100 $\pm$ 1	16 $\pm$ 0
	75	95 $\pm$ 1	99 $\pm$ 0	15 $\pm$ 0
	50	87 $\pm$ 3	100 $\pm$ 0	17 $\pm$ 0
BA <sup>b</sup> (S)	100	42 $\pm$ 0	100 $\pm$ 1	17 $\pm$ 1
	75	27 $\pm$ 1	100 $\pm$ 1	17 $\pm$ 0
	50	41 $\pm$ 1	99 $\pm$ 2	19 $\pm$ 0
BA(G)	100	33 $\pm$ 5	100 $\pm$ 1	15 $\pm$ 0
	75	33 $\pm$ 3	95 $\pm$ 3	15 $\pm$ 0
EA <sup>b,c</sup> (S)	100	99 $\pm$ 0	100 $\pm$ 0	16 $\pm$ 0
	75	95 $\pm$ 0	97 $\pm$ 0	15 $\pm$ 0
	50	76 $\pm$ 0	100 $\pm$ 1	17 $\pm$ 1

7 <sup>a</sup>(S) indicates PHA/solvent-solution, (G) indicates PHA/solvent-gel.

8 <sup>b</sup>MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate

9 <sup>c</sup>No PHA/solvent-gel formation was observed using MEK, EA or chloroform

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

Solvent	PHA/solvent-solution		RY <sup>a</sup> (%)	Total RY (%)	Rcells <sup>b</sup>	
	<i>n</i> =2 purity (%)	HHx (mol%)			PHA content (%)	HHx (mol%)
MIBK <sup>c</sup>	96 ± 1	24 ± 0	71 ± 0	84 ± 0	13 ± 4	11 ± 0
BA <sup>c</sup>	95 ± 2	24 ± 0	68 ± 0	76 ± 0	24 ± 1	12 ± 0
EA <sup>c</sup>	97 ± 1	21 ± 0	94 ± 0	94 ± 0	2 ± 0	20 ± 0
MEK <sup>c</sup>	97 ± 2	21 ± 0	95 ± 0	95 ± 0	2 ± 1	20 ± 0
Solvent <sup>d</sup>	PHA/solvent-gel		RY <sup>a</sup> (%)			
<i>n</i> =2	purity (%)	HHx (mol%)	(%)			
MIBK	73 ± 2	12 ± 0	13 ± 0			
BA	62 ± 0	11 ± 0	8 ± 0			

6  
7 <sup>a</sup> PHA recovery yield (RY), total RY combines RY from PHA/solvent-solution and PHA/solvent-gel  
8 <sup>b</sup>Residual cell mass  
9 <sup>c</sup>MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate  
10 <sup>d</sup>No PHA/solvent-gel formation was observed using: MEK and EA

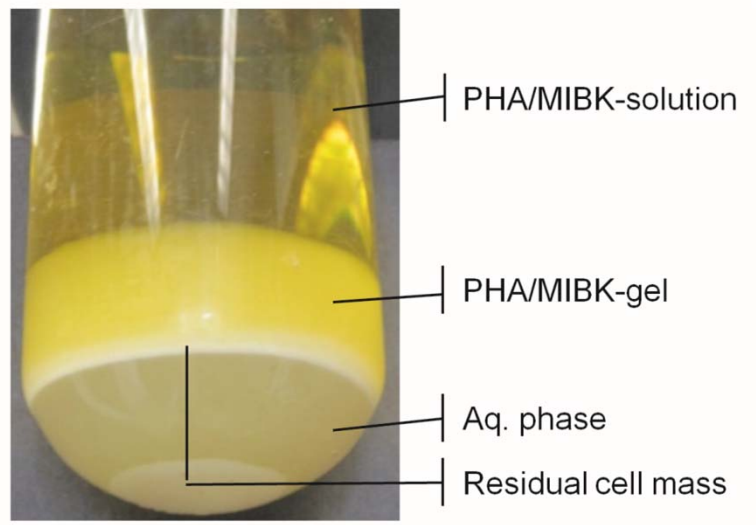
1 **Table 4:** Larger scale recovery of P(HB-*co*-HHx) from dry and wet cells. PHA was extracted for 4 h  
 2 at 100°C, with the non-halogenated solvents to form a 2% PHA mixture. The extracted polymer was  
 3 precipitated with 3 volumes of *n*-hexane at room temperature and dried at 50°C.

Solvent, Vol.	Biomass (mol% HHx of PHA)	PHA recovered (g)		Purity (%)		mol% HHx of recovered PHA		Recovery yield (%)		
		S <sup>a</sup>	G <sup>a</sup>	S	G	S	G	S	G	total
MIBK <sup>b</sup> , 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 <sup>b</sup> , 1.5 L	wet (22)	21	nd <sup>c</sup>	95	nd <sup>c</sup>	21	nd <sup>c</sup>	71	nd <sup>c</sup>	71
EA, 1.78 L	dry(18)	33	nd	95	nd	17	nd	93		93

4 <sup>a</sup>S = PHA/solvent solution; G = PHA/solvent gel

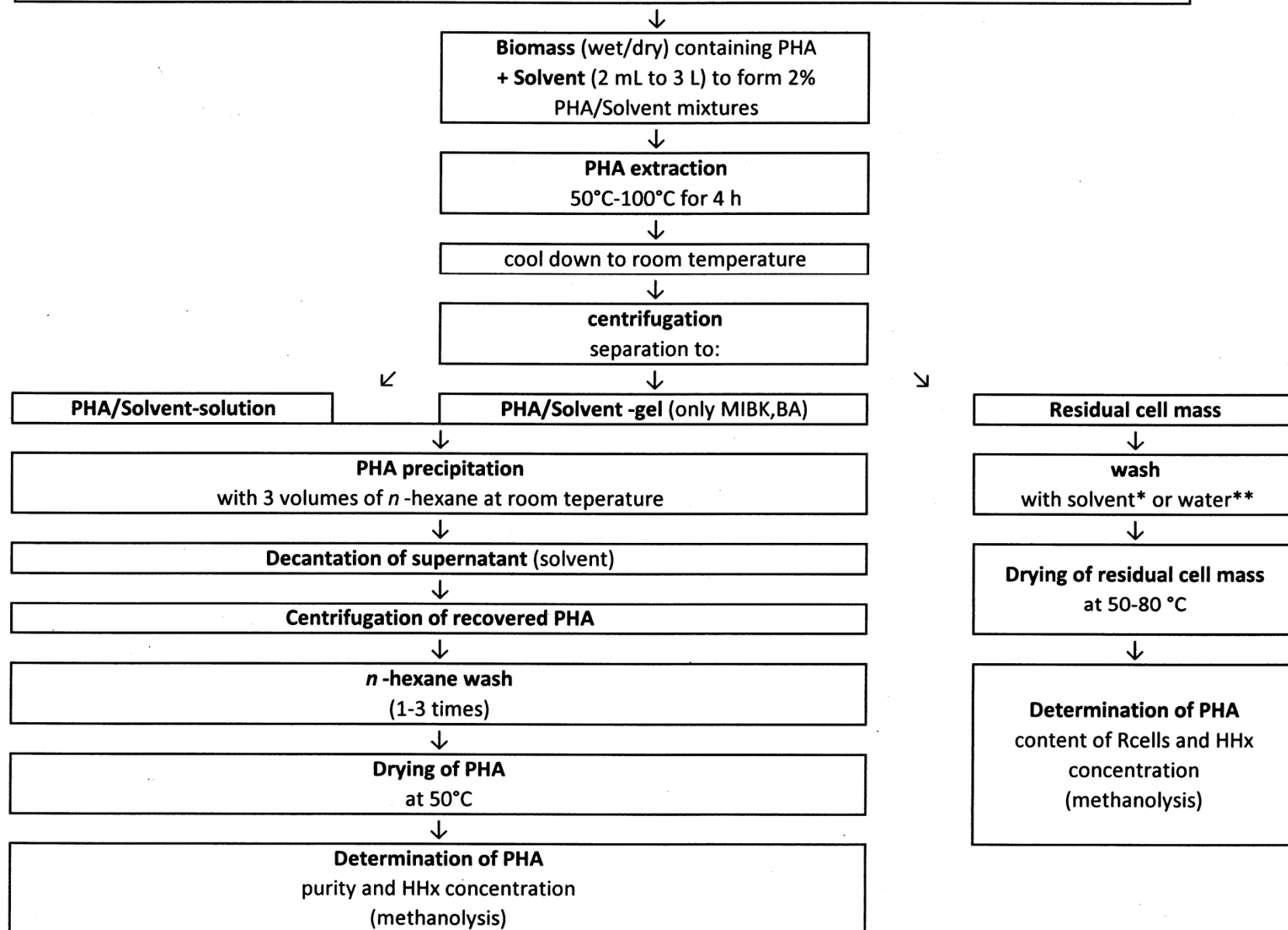
5 <sup>b</sup>MIBK = methyl isobutyl ketone; EA = ethyl acetate

6 <sup>c</sup>No PHA/solvent gel was detected in EA-based recovery of PHA

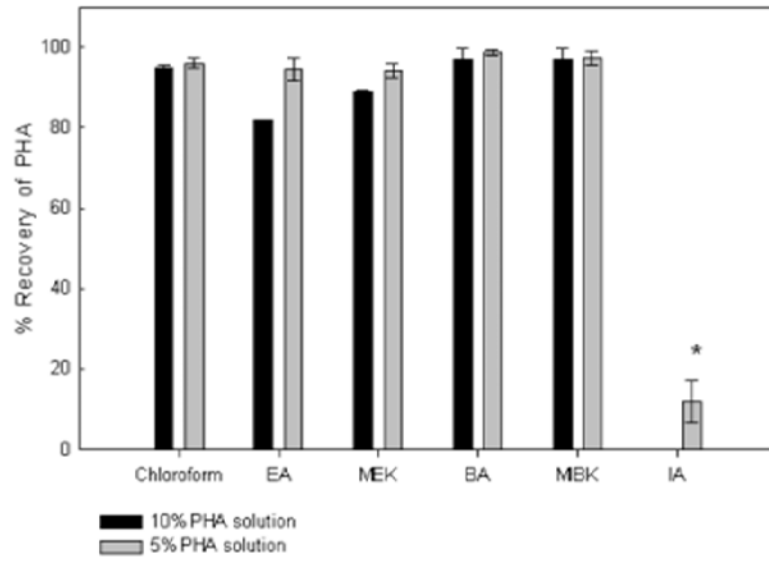


Riedel, et al, Figure 1

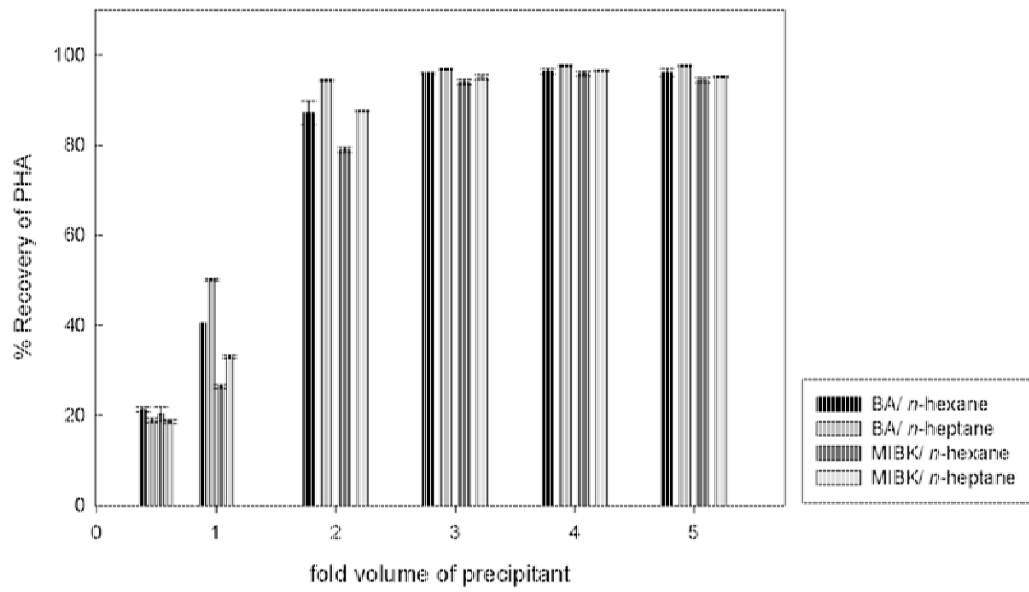
**Flow sheet of general PHA extraction process with non-halogenated solvents**



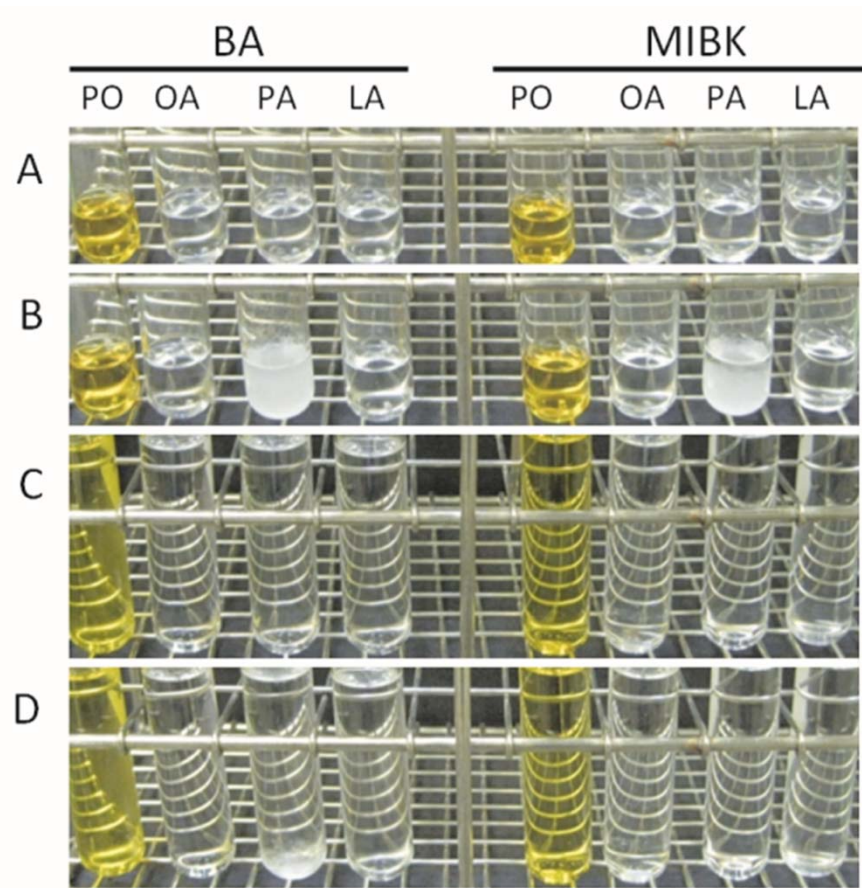
Riedel, et al, Figure 2



Riedel, et al, Figure 3

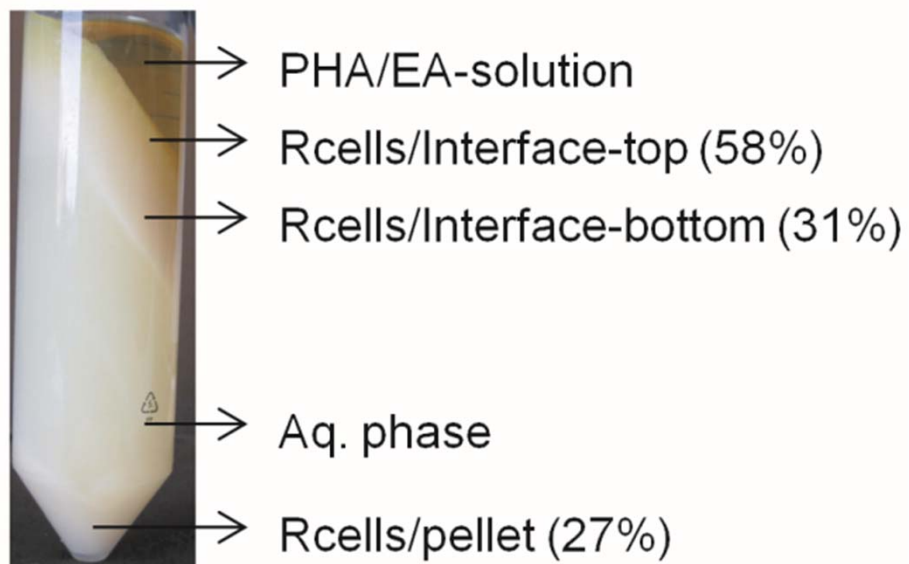


Riedel, et al, Figure 4



Riedel, et al, Suppl. Fig. 1





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

1 **Supplemental Figure 1**

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

9

10 **Supplemental Figure 2**

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

17