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Scale-Up of the Downstream Process for Polyhydroxyalkanoate Copolymer P(HB-co-HHx) Extraction with Nonhalogenated Solvents

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Abstract. Biobased and biodegradable polyhydroxyalkanoates (PHAs) are promising alternatives to common plastics. Due to their high production costs, only a minimal share of global plastic production is composed of PHA. A major contributor to the high costs minimizing the potential to occupy a larger market share is the downstream process. To obtain high recovery yields and pure products, most approaches rely on large amounts of solvents. While short-chain-length PHA (*scl*-PHA) is poorly soluble in nonhalogenated solvents, medium-chain-length PHA (*mcl*-PHA) was shown to be soluble in nonhalogenated solvents. In this study, an approach to recover poly(hydroxybutyrate-*co*-hydroxyhexanoate) with acetone and 2-propanol was scaled up 30-fold to 300 g of lyophilized cells per recovery cycle. High PHA purities of 90–100 % were reached from extractions at moderate temperatures from 30–58 °C. In two-stage extractions, up to 100 % PHA was recovered, while the molecular weight was not reduced. Solvents were recovered by distillation in a concentration step and after precipitation. Furthermore, the material properties were analyzed. PHA recovered from the distillation bottom had an increased HHx content compared to the first and second extractions using recovered solvents and was of low purity, indicating efficient and pure precipitation of the recovered PHA during the 2-stage extractions.

Keywords: P(HB-*co*-HHx), *mcl*-PHA, nonhalogenated solvents, PHA recovery.

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Масштабирование процесса экстракции сополимера П(ГБ-со-ГГ) негалогенированными растворителями

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Аннотация. Синтезируемые микроорганизмами биоразлагаемые полигидроксиалканоаты (ПГА) являются многообещающей альтернативой обычным пластмассам. Однако высокая стоимость ПГА ограничивает объемы их мирового производства. Одной из основных причин высоких затрат, сводящих к минимуму возможность занять большую долю рынка, является сложный и дорогостоящий процесс извлечения полимеров из клеточной биомассы. Для получения высоких выходов извлечения и чистых образцов полимеров используются большие количества растворителей. При этом известно, что короткоцепочечные ПГА плохо растворяются в негалогенированных растворителях, в то время как среднецепочечные – растворяются. В этом исследовании процесс извлечения сополимера гидроксипропаноата и гидроксигексаноата с использованием ацетона и 2-пропанола масштабирован в 30 раз (до 300 г лиофилизированной клеточной массы на один цикл извлечения). Высокая чистота ПГА (90–100 %) достигнута при проведении экстракции при умеренных температурах от 30 до 58 °С. При двукратной экстракции полнота извлечения полимера достигала 100 % без снижения значений молекулярной массы. Растворители возвращали для повторных циклов перегонкой на стадии концентрирования и после осаждения извлеченного полимера. Проанализированы свойства полученных образцов ПГА. Полимер, извлеченный из остаточной от дистилляции фракции, по сравнению с полимером, полученным в результате двукратной экстракции с повторным использованием растворителей, имел повышенное содержание фракции мономеров гексаноата и низкую степень чистоты. Это свидетельствует об эффективности осаждения полимера, извлеченного в процессе двукратной экстракции.

Ключевые слова: П(ГБ-со-ГГ), среднецепочечные ПГА, негалогенированные растворители, извлечение ПГА.

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Introduction

Plastic pollution is a worldwide problem, yet global plastic production is still increasing and reached 359 million t per year in 2018, and with it, the need for environmentally friendly alternatives has increased. One of these alternatives is biobased and biodegradable polyhydroxyalkanoates (PHAs), which can be produced from low-cost feedstocks with microorganisms (Riedel and Brigham, 2019, 2020). During downstream processing, PHA is either extracted from the cells or the surrounding non-PHA cell material is removed, which was recently comprehensively reviewed (Koller et al., 2013b; Gahlawat et al., 2020). In this context, solvent-based PHA downstream processing often relies on large amounts of halogenated, mostly chlorinated, solvents, which cause a very negative ecological footprint (Pérez-Rivero et al., 2019; Saavedra del Oso et al., 2021).

PHAs are categorized based on the number of carbon atoms of their monomer building blocks. Monomers consisting of 3–5 carbon atoms are termed *short-chain-length* (*scl*), and monomers of 6–14 carbon atoms are termed *medium-chain-length* (*mcl*) (Steinbüchel et al., 1992). Various *scl-mcl*-copolymers can be produced to obtain PHAs with tailored properties, making them promising alternatives to conventional plastics for various applications (Chen, 2010). Specifically, the *scl-mcl*-copolymer poly(hydroxybutyrate-*co*-hydroxyhexanoate) (P(HB-*co*-HHx)) shows reduced crystallinity, reduced melting temperature and higher elasticity

compared to polyhydroxybutyrate (PHB) and thus makes it suitable for further processing (Noda et al., 2010). In seawater or soil, P(HB-*co*-HHx) degrades easily within months without pretreatment (Chen, 2012; Shang et al., 2012). Regarding downstream processing, *mcl*-PHA and copolymers thereof are more soluble in nonhalogenated solvents than *scl*-PHA (Jiang et al., 2006; Furrer et al., 2007; Cerrone et al., 2019). Methyl isobutyl ketone, methyl ethyl ketone, ethyl acetate and acetone were mainly reported in combinations with precipitation solvents such as ethanol, n-hexane or 2-propanol to precipitate the extracted PHA that was recovered from dried cells or wet biomass (Furrer et al., 2007; Riedel et al., 2013; Bartels et al., 2020; Saavedra del Oso et al., 2021).

Recently, a recovery method was developed by Bartels et al. using acetone and 2-propanol to achieve recovery yields up to 100% with the possibility of separating and recycling the solvents due to their zeotropic nature (Bartels et al., 2020). In this work, the approach by Bartels et al. (2020) was scaled up to process 300 g of dried cells, and the recycling of the solvents was assessed.

Material and Methods

Biomass for P(HB-co-HHx) recovery

Engineered *Ralstonia eutropha* Re2058/pCB113 cells (Budde et al., 2011) containing the copolymer P(HB-*co*-HHx) were produced in a 150 L bioreactor utilizing waste fats of porcine origin (ANiMOX GmbH, Berlin, Germany) as the main carbon source adapting the cultivation

strategy performed by Riedel et al. (Riedel et al., 2015).

Extraction and precipitation with acetone and 2-propanol

Bartels et al. (2020) recently identified acetone and 2-propanol as a suitable solvent and nonsolvent pair for the *scl-mcl*-copolymer P(HB-*co*-HHx). The established volume to weight ratio of 10:1 (acetone: freeze-dried cells) was scaled up to a larger volume (Fig. 1). Cells (300 g) and 3 L of solvent were added to a 5 L round-bottom flask and rotated in a water bath at 30–58 °C for 1 h in a rotary evaporator to extract the PHA. Separation of the extract from cell debris was performed by centrifugation (4000 rpm, 10 min, room temperature) and subsequent decantation. The separated extract was concentrated using a rotary evaporator and precipitated with 4 °C cold 2-propanol overnight. PHA was recovered by centrifugation at 4000 rpm for 10 min and dried either at 50 °C, in a vacuum pot or in a freeze dryer.

Two-stage extraction

Extraction was performed as described above. Residual cells were dried at 50 °C and pooled with similar residual cells, if necessary, prior to the next extraction. The remaining PHA from the residual cell material was extracted under the same conditions as the first extraction.

Extraction conditions

Different temperatures from 30 to 58 °C as well as different solvent evaporations (40–68 % of the initial volume of acetone) from the PHA extract were tested. Different 2-propanol concentrations occurring either due to the evaporation of acetone and subsequent addition of 6 L of 2-propanol or varying addition of 2-propanol were investigated. The final

concentrations of each solvent per recovery cycle are given for the respective results.

Recovery yield determination

The recovery yield was calculated according to Equation 1:

$$\text{RecoveryYield}[\%] = \frac{\text{extractedmaterial}[\text{g}]}{\text{lyophilizedcells}[\text{g}] \times \text{PHAcontent}} \times 100 \quad (1)$$

Molecular weight characteristics

Molecular weight characteristics were determined by gel permeation chromatography (GPC) equipped with a differential refractive index detector (Merck-Hitachi, RI-Detector L-7490) using two sequentially coupled columns (Agilent PLgel 5 µm MIXED-C300 × 7.5 mm, 50 × 7.5 mm Guard). Polystyrene standards in the range of 9.6–3187 kDa were used for calibration (Agilent Polystyrol PS-1). PHA samples were

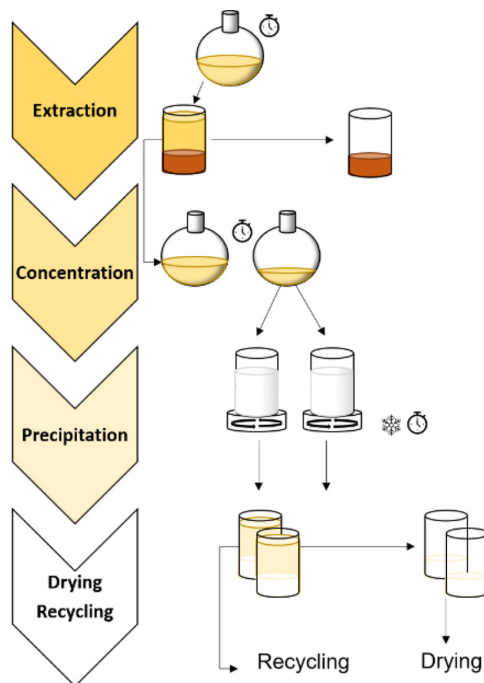


Fig. 1. Schematic description of the workflow to recover *scl-co-mcl*-PHA from lyophilized cells using acetone and 2-propanol

weighed to obtain a concentration of 5 mg mL⁻¹ in 2 mL of chloroform containing 2-butanone at a concentration of 1 mg mL⁻¹ in a borosilicate tube (Borosilicate Screw Thread Culture Tube with PTFE-Faced Rubber Lined Caps, Ø16 mm x 100 mm, KIMAX®) and incubated at 55 °C for 24 h (Hettich Lab Technology). After cooling to room temperature, the samples were filtered into an amber HPLC vial through 0.2 µm PTFE syringe filters. Analysis of 15 µL samples was performed at 30 °C and a flow rate of 0.8 mL min⁻¹ chloroform. Signal data points (n_i) and respective MW (M_i) calculated by the calibration data were used to calculate the number average (M_n), weight average (M_w), and dispersity values (\mathcal{D}) according to Equations 2–4 (Bartels et al., 2020):

$$M_n = \frac{\sum_{i=1}^k (n_i \cdot M_i)}{\sum_{i=1}^k n_i} \quad (2)$$

$$M_w = \frac{\sum (n_i \cdot M_i^2)}{\sum (n_i \cdot M_i)} \quad (3)$$

$$\mathcal{D} = \frac{M_w}{M_n} \quad (4)$$

Determination of PHA content, molar HHx content and purity

The determination of PHA content by gas chromatography (GC) was previously described by Bartels et al. (2020) and performed accordingly.

Dry substance and ash content determination

To check whether the PHA was fully dried after precipitation and to ensure that no residual solvent might interfere with further processing or impact the melting temperature, dry substance was calculated by balancing the weight of the sample (0.6 g) that was dried as described in this procedure and the weight after drying in an

oven at 105 °C. Samples were weighed every 2 h until a constant weight was reached. Ashing was performed by heating recovered PHA samples in a muffle furnace (Phoenix Black Muffelofen, CEM) for 5 h at 550 °C. After cooling to room temperature, the ash content was determined by weighing.

Results

In this study, *scl-co-mcl*-PHA recovery using acetone as a solvent and 2-propanol as a nonsolvent was scaled up by a factor of 30 to 300 g of lyophilized cells. The nonhalogenated solvents were separated and recovered by distillation and utilized for further recovery cycles to increase the sustainability of the process by reducing the overall solvent usage. Recovery was performed on different lyophilized cell materials (Batch 1 and Batch 2). The batches from different cultivations had different PHA contents of 76.1 or 81.1 % with either 16.1 or 19.2 mol% HHx content, respectively.

P(HB-co-HHx) extraction at moderate temperatures and impact of solubilized PHA concentration and variation of 2-propanol concentration

As shorter extraction times were reported to result in lower molecular weight reduction at 21 and 70 °C (Bartels et al., 2020), PHA was extracted for 1 h at 30–58 °C with different subsequent extract concentrations and precipitation conditions (Fig. 2A). The solubility is temperature dependent. At lower temperatures of 30 °C, the recovery yield was 49 %, while an extraction temperature of 58 °C resulted in a 1.5-fold higher recovery yield of 78 %. The largest increase in recovery yield was observed in a temperature range from 30–40 °C (Fig. 2B). After extraction at 40 °C, the PHA was concentrated by 40 to 68 % of the initial volume of the extract (Fig. 2C). When the extract was concentrated by

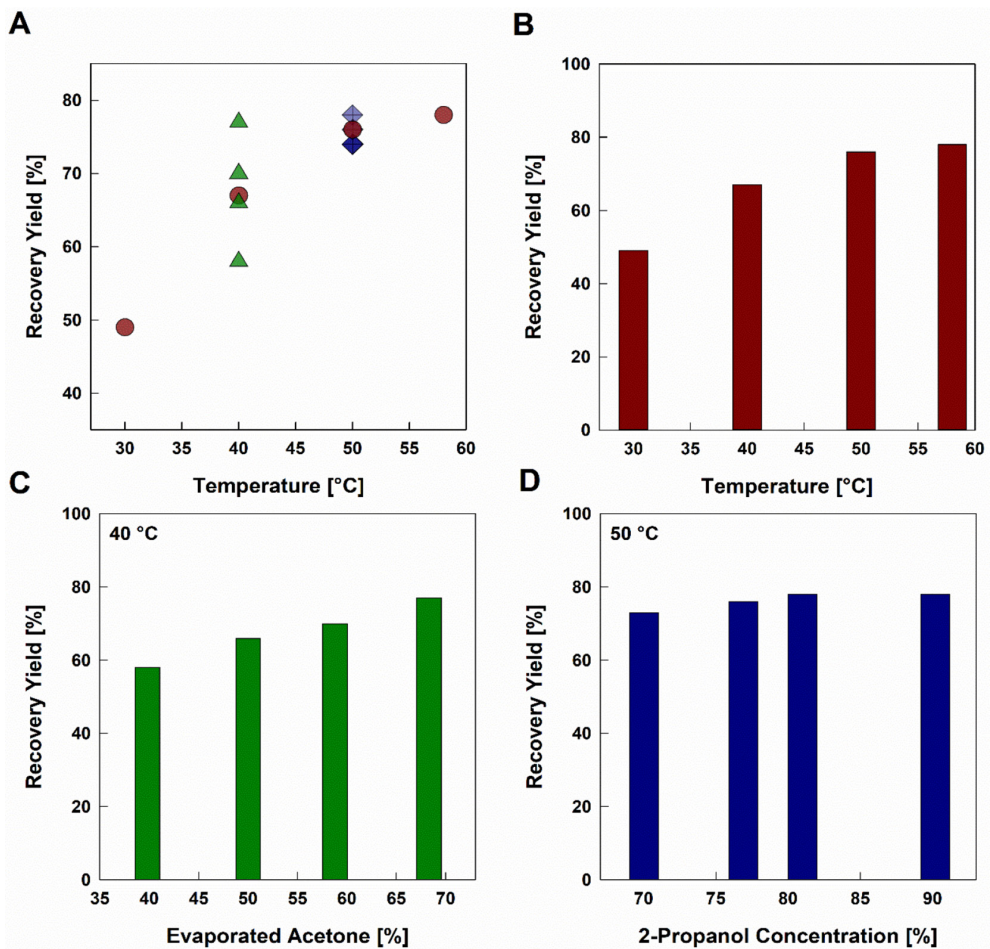


Fig. 2. P(HB-*co*-HHx) extraction at moderate temperatures (A). Recovery yields depending on different extraction, concentration and precipitation conditions; (B) extraction at 30–58 °C for 1 h; concentration by evaporation of 58.3 ± 0.6 % acetone and precipitation with 2-propanol at 4 °C (82.1 ± 1.3 % 2-propanol content in the solvent mixture); (C) extraction at 40 °C for 1 h, increasing the concentration of solubilized P(HB-*co*-HHx) in acetone by evaporation of 40–68 % acetone and precipitation with 58–77 % 2-propanol; (D) extraction at 50 °C for 1 h with evaporation of 50 ± 0.6 % acetone and varying 2-propanol concentration from 70–90 %

40 %, less 2-propanol was added for precipitation (17 % less, approximately 5 L instead of 6 L, resulting in a concentration of 74 %). The most concentrated extract was supplied with more 2-propanol (17 % more, 88 % (i. e., approximately 7 L)). Due to the higher concentration of the PHA extract, precipitation becomes more efficient. By realizing higher concentrations of PHA extract (68 %), the recovery yields at 40 °C increased up to a recovery yield of 77 %, which is comparable to yields at 50 or 58 °C, where the PHA extract was concentrated by approximately 58 %. Moreover,

by concentration of the solution in a rotary evaporator, pure acetone is recovered before the precipitation step, leading to a lower demand of 2-propanol to achieve a large volumetric excess. To investigate whether the extracted PHA was successfully precipitated, less acetone (50 %) was evaporated after extraction at 50 °C with subsequent addition of cold 2-propanol to achieve 70–90 % acetone in the mixture (Fig. 2D). With increasing concentrations of 2-propanol, only slightly improved recovery yields by 5 % were achieved. The recovery yields were comparable

to those of the extraction at 50 °C in the previous section.

Two-stage extraction

Two to three batches of residual cells from extractions at the same temperature were pooled for a second extraction cycle. The results were analyzed regarding purity, HHx content, recovery yield and molecular weight characteristics.

PHA purity and HHx content

High PHA purities in the range of 87.9–100 % were reached in all experiments (Fig. 3A, 3C, Supplementary Table 1). No drastic changes regarding the HHx content of the recycled material in the first or second extraction cycle were observed, nor were they observed in the residual cells (Fig. 3B). On the other hand, a slight decrease in the molecular weight was noticeable (Table 1). This might be due to the longer duration of PHA exposure to higher

temperatures and solvents or the lower solubility of higher molecular weight polymers.

Recovery yield from two-stage extraction at different temperatures

The recovery yields were obtained from 62 % at 30 °C up to 100 % at 50 or 55 °C (Fig. 3D).

Generally, Batch 2 (19.2 % HHx; 81.1 % PHA; higher MW) gave lower total recovery yields after the second extraction compared to Batch 1. This might be due to the temperature dependency shown in the previous section and to a smaller part the different starting material properties, as the differences are minor. The recovered product was clustered in two different ranges of molecular weights depending on the batch from which they were recovered. The molecular weight and dispersity index were shown to depend on the amount of PHA synthase (Sim et al., 1997) and are therefore cultivation- and strain-dependent.

Table 1. Molecular weight characteristics of the recovered PHA after the first and second extraction

PHA	M_w [Da] $\times 10^5$	M_n [Da] $\times 10^5$	\bar{D} [-]	
B1.1	1.88 ± 0.02	0.79 ± 0.01	3.39 ± 0.07	1. Extraction
B1.2	2.06 ± 0.03	0.87 ± 0.03	2.36 ± 0.05	
B1.1+2	1.89 ± 0.01	0.94 ± 0.03	2.01 ± 0.05	2. Extraction
B1.3	1.90 ± 0.01	0.84 ± 0.01	2.26 ± 0.03	1. Extraction
B1.4	2.07 ± 0.01	0.93 ± 0.14	2.27 ± 0.26	
B1.3+4	1.79 ± 0.01	0.94 ± 0.03	1.91 ± 0.04	2. Extraction
B1.5	1.99 ± 0.001	0.89 ± 0.02	2.24 ± 0.06	1. Extraction
B1.6	2.08 ± 0.05	0.82 ± 0.03	2.54 ± 0.04	
B1.7	2.09 ± 0.02	0.87 ± 0.04	2.42 ± 0.08	
B1.5+6+7	1.75 ± 0.01	0.86 ± 0.005	2.04 ± 0.02	2. Extraction
B2.1	2.68 ± 0.04	1.12 ± 0.05	2.39 ± 0.07	1. Extraction
B2.2	2.70 ± 0.04	1.20 ± 0.01	2.25 ± 0.01	
B2.1+2	2.52 ± 0.03	1.08 ± 0.01	2.32 ± 0.01	2. Extraction
B2.3	2.77 ± 0.02	1.26 ± 0.02	2.19 ± 0.01	1. Extraction
B2.4	2.75 ± 0.01	1.14 ± 0.04	2.42 ± 0.07	
B2.5	2.70 ± 0.05	1.26 ± 0.02	2.15 ± 0.06	
B2.3+4+5	2.50 ± 0.07	1.34 ± 0.03	1.86 ± 0.10	2. Extraction

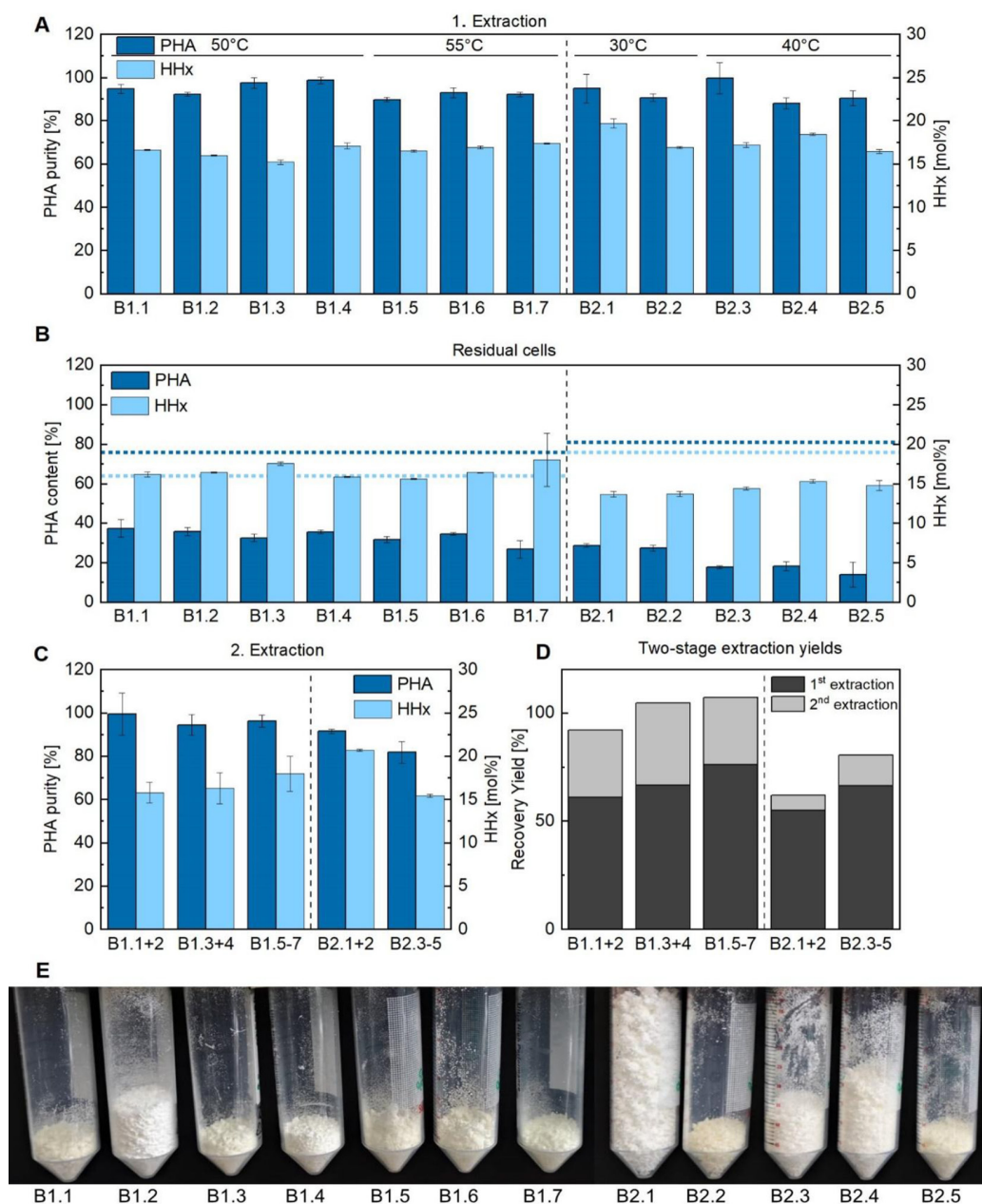


Fig. 3. Two-stage extraction of P(HB-co-HHx) from lyophilized cells at 50, 55, 30 and 40 °C. PHA purity and HHx content (Batch 1: 76.1 % PHA content, 16.1 mol% HHx; Batch 2: 81.1 % PHA content, 19.2 mol% HHx), Batch 1, 50 °C, B1.1, B1.2: 43.3 % evaporated acetone, final 2-propanol concentration: 77.9 %; Batch 1, 50 °C, B1.3, B1.4: 50 % evaporated acetone, final 2-propanol concentration: 80 %; Batch 1, 55 °C, B1.5, B1.6, B1.7: 49.7–50 % evaporated acetone, final 2-propanol concentration: 80–81.7 %; Batch 2, 30 °C, B2.1, B2.2: 54.5–60.7 % evaporated acetone, final 2-propanol concentration: 81.4–83.6 %; Batch 2, 40 °C, B2.3, B2.4, B2.5: 50–59 % evaporated acetone, final 2-propanol concentration: 81.4–83.6 % (A). PHA and HHx contents in the residual cells after extraction (B). PHA purity and HHx content after the second extraction (C). Recovery yields after two-stage extraction under different conditions (D). Properties of the recovered material (E). Error bars indicate technical triplicates of GC samples. Dashed lines indicate the initial PHA and HHx contents of the respective starting cell material

Coloration and bulk density

The bulk density, texture and coloration of the recovered polymer were investigated. Three grams of each recovery cycle (1 stage extractions) is shown (Fig. 3E). More granular material appeared more yellow in color and had a lower bulk density. The material properties seem to vary throughout all experiments without a connection to purity or solvents and their content in the mixture (Supplementary Tables 1, 2, and 3; Supplementary Figures 1, 2, and 3). Materials with lower bulk densities are preferable because they are more convenient to package, transport and further process.

Moisture content before drying

After extraction and precipitation of the PHA, the solvent mixture was decanted. The wet PHA was dried using a freeze dryer or a vacuum pot or was dried at 50 °C. The recovered PHA was regularly (approximately every 30 minutes) mixed and manually broken up into smaller pieces. The drying process was done for 6 hours. The solvent content of the wet mass was determined. All samples contained 81–92 % moisture before drying regardless of the extraction conditions (Supplementary Table 4).

Product recovery from the distillation bottom

After various recovery cycles, PHA was recovered from the distillation bottom. Interestingly, PHA showed a higher molar content of hydroxy hexanoate as well as a comparably high molecular weight (Table 2).

Dry substance and ash content

Solvent residues might hamper further processability of PHA. Because of the high moisture content prior to drying, dry substance and ash content were determined to ensure a pure and dry polymer. As the material is recovered from biological material, the inorganic content remaining after ashing should be near zero. The dry substance determination gave > 99 % dry substance and < 1 % ash content for all recovery cycles (Supplementary Table 4).

Discussion

The recovery method developed by Bartels et al. (2020) was scaled up by a factor of 30 from 10 to 300 g of lyophilized cells as the starting material. The concentration of the PHA extract and 2-propanol excess was further investigated. While it was previously reported that acetone is evaporated until the viscosity increases, the concentration of PHA in the extract impacts the efficiency of the precipitation step. However, increased temperatures or pressure contribute largely to higher recovery yields in short extraction times (Koller et al., 2013a; Bartels et al., 2020). However, high recovery yields might be obtained at moderate temperatures due to PHA extract concentration by acetone evaporation and recovery after extraction. Therefore, the energy demand is reduced, leading to a more sustainable process: heating the water bath and acetone to 55 or 58 °C has a 1.5- and 1.8-fold energy demand compared to extraction at 40 °C, respectively. A major drawback of solvent-based downstream processing is the large amounts of

Table 2. Properties of the material recovered from the distillation bottom

PHA	Purity [%]	HHx [mol%]	M _w [Da]	M _n [Da]	Đ [-]
Distillation Bottom	69.03±8.36	25.63±1.24	2.97±0.03×10 ⁵	1.1±0.001×10 ⁵	2.71±0.02

often halogenated solvents (Saavedra del Oso et al., 2021). Evaporation and recovery of acetone during the concentration step leads to a lower demand for 2-propanol to obtain a large nonsolvent excess to precipitate PHA. The concentration of the extract was also shown to result in higher recovery yields. Given the zeotropic nature of the solvent mixture, acetone and 2-propanol were separated and reused for multiple recovery cycles. Fractions of up to 30% 2-propanol in acetone were reported to still recover PHA adequately, and even pure 2-propanol was able to extract 14.5% of P(HB-co-HHx) (Bartels et al., 2020). Moreover, acetone extraction and 2-propanol precipitation are suitable to obtain PHA with low endotoxin content, making it utilizable for medical applications (Furrer et al., 2007).

In this study, no significant molecular weight or purity reduction depending on temperature was observed. Even though polymer solubility is expected to decrease with higher molecular weight (Terada and Marchessault, 1999) interaction between polar groups and hydrogen bonding. For polar polymers such as poly(3-hydroxyalkanoates), the minor differences in the

molecular weight of the different cell batches did not impact the recovery yield. The appearance of the recovered product varies regarding granularity and color as well as bulk volume, but for commercial use, a consistent product is desired. Most likely, the differences occur due to handling at the lab scale by mixing and breaking the polymer mass into smaller pieces while drying, which might be overcome with a less manual drying process. Losses of solvent due to drying on a lab scale can be easily avoided on larger scales by dryers that collect the withdrawn solvent.

Conclusion

P(HB-co-HHx) extraction from freeze-dried *R. eutropha* cells using a combination of acetone and 2-propanol was scaled up to 300 g of lyophilized cells as the starting material. The process was adapted to mild process conditions. The solvents were recycled over the course of the experiments, reducing the costs and making the process greener by using nonhalogenated solvents. This might contribute to further research on the PHA production process.

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