



This is an electronic reprint of the original article. This reprint may differ from the original in pagination and typographic detail.

# Scalable and Sustainable Processing of Intracellular Polyhydroxyalkanoates with Biobased Solvents

Jablonski, Piotr; Dinh, Ngoc phuoc; Lascu, Irina; Tănase, Ana-Maria; Christensen, Mikkel; Khokarale, Santosh govind; Sundman, Ola; Mikkola, Jyri-Pekka; Irgum, Knut

Published in: ACS Sustainable Chemistry and Engineering

DOI: 10.1021/acssuschemeng.3c05422

Published: 25/12/2023

Document Version Final published version

Document License CC BY

Link to publication

Please cite the original version:

Jablonski, P., Dinh, N. P., Lascu, I., Tănase, A.-M., Christensen, M., Khokarale, S. G., Sundman, O., Mikkola, J.-P., & Irgum, K. (2023). Scalable and Sustainable Processing of Intracellular Polyhydroxyalkanoates with Biobased Solvents. ACS Sustainable Chemistry and Engineering, 11(51), 17990-18000. https://doi.org/10.1021/acssuschemeng.3c05422

**General rights** 

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

#### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

**Research Article** 

# Scalable and Sustainable Processing of Intracellular Polyhydroxyalkanoates with Biobased Solvents

Piotr Jablonski,\* Ngoc Phuoc Dinh, Irina Lascu, Ana-Maria Tănase, Mikkel Christensen, Santosh Govind Khokarale, Ola Sundman, Jyri-Pekka Mikkola, and Knut Irgum



to assess the molecular weights of the extracted polymers. The examined ranges in the model were, respectively, 1.6-8.4% (w/v) of lyophilized cells content per 10 mL of solvent, 3-17 min extraction time, and temperatures from 116 to 144 °C. Time and temperature strongly affected the extraction yields and molecular weights of the obtained polymers while the concentration of bacterial biomass only effected the molecular weight. Several quadratic and interaction coefficients were significant in the well-fit partial least-squares regression models ( $R^2 > 0.8$ ,  $Q^2 > 0.6$ ) indicating that nonlinear effects and interacting parameter contributed to the optimization targets. The optimized extraction should be performed at 130 °C for 15 min with 2% loading of bacterial biomass. The predicted yield and molecular weight of the polymer matched the values obtained from the real experiment under the optimized conditions. The method setup provided similar yield and higher molecular weight in much shorter time compared to overnight Soxhlet extraction with CHCl<sub>3</sub>. The clean <sup>1</sup>H nuclear magnetic resonance spectra of polymers extracted from bacteria indicate that high purity materials can be obtained using an optimized extraction scheme. Additionally, the Cyrene solvent could be recycled at least five times and still performed the extraction equally well as the fresh solvent. Finally, the current method demonstrated a high potential for scalability using a HP4750 stirred filtration cell. Three different filtration conditions were tested, achieving up to 97.4% recovery at 80 °C using a 0.3  $\mu$ m glass fiber membrane, with a flux of 312.5 LMH.

KEYWORDS: Biobased polymers, Biodegradable polymers, Green solvents, Extraction, Experimental design

# INTRODUCTION

Plastics have become practically indispensable in every area of our life. Global annual plastic production currently exceeds 400 Mt, with most of the commonly used plastics being non biodegradable and mainly derived from fossil sources. As a consequence, the generated plastic waste accumulates in the environment.<sup>1</sup> In order to reverse this trend, there is a need to find sustainable alternatives to fossil raw materials that can be used to produce biodegradable plastics with performance on par with the most common plastics in use today. Biopolymers have emerged as important contenders, in particular, polyhydroxyalkanoates (PHAs), which are aliphatic biopolyesters produced by a variety of microorganisms and stored as energy reservoirs in the form of intracellular inclusion bodies.

refractive index and multiangle light scattering detectors was used

Production of PHAs can be optimized by feeding the cultures carbon substrates rich in energy but low in some essential elements (notably N or P) or in some microelements, or by cycling the feed in a "feast and famine" regime.<sup>2</sup> There are three classes of PHAs, depending on the number of carbon atoms in each monomer unit. Short chain length PHA (scl-PHA) composed of monomers with up to five carbon atoms,

Received:August 24, 2023Revised:November 19, 2023Accepted:November 21, 2023Published:December 13, 2023





the most abundant scl-PHA being poly(3-hydroxybutyrate) (PHB); medium chain length (mcl-PHA) with six to fourteen carbon atoms per monomer unit; and long chain length (lcl-PHA) with monomers containing 15 and more carbon atoms.<sup>3</sup> PHAs are comparable to high-volume synthetic thermoplastics such as polypropylene, with properties that strongly depend on several factors, such as monomer composition and molecular weight, as well as degree of crystallinity.<sup>4,5</sup>

One of the main disadvantages of biopolymers is their price (2.2 to 6.6  $\epsilon/kg$ ), which is significantly higher than fossil-based plastics (<1 €/kg).<sup>6,7</sup> Currently an interesting area of research is cost reduction in upstream processing by the utilization of waste materials to produce the feedstock. Vu et al. proposed utilization of short carboxylic acids from acidogenic fermentation of food waste, resulting in PHB accumulation of 10% cell dry weight (CDW).8 Similarly, Christensen et al. achieved high PHB production (72% CDW) in growth media supplemented with acetate species.<sup>9</sup> Recently, Acedos et al. proposed utilization of pretreated slaughterhouse waste in experiments scaled up to 15 L, resulting in 62% CDW of mcl-PHA.<sup>10</sup> Another promising alternative source of carbon is crude glycerol, a byproduct from biodiesel production, as proposed by Lascu et al., who achieved PHB yield of 50% CDW from a Photobacterium ganghwense C2.2 strain. Additionally, the harvested cells contained 12% CDW of industrially valuable fatty acids, recovered from the crude glycerol.<sup>1</sup>

Downstream processing of PHA contributes the other half of the production costs and comprises steps like biomass separation, biomass pretreatment in order to lyse the cell wall, and recovery, separation, and purification of PHA. Current research is mainly focused on optimization of recovery by sustainable and cost-efficient methods.<sup>6,12</sup> There are two approaches in PHA recovery, one of which is digestion or dissolution of the non-PHA cell mass (NCPM). Yang et al. studied NCPM digestion aided by several surfactants and found sodium dodecyl sulfate (SDS) and linear alkylbenzene sulfonate (LAS-99) to be most promising. The extractions were performed at 60-80 °C for 30 min and resulted in 90% and 81% recovery for SDS, and 86% purity with 87% recovery for LAS-99. The main limitations of this method are that the surfactant is nonreusable, a low purity of the recovered polymer, and a high polymer content required in the CDW for effective purification and recovery.<sup>13</sup> Villano et al. proposed NaClO (5% active Cl<sub>2</sub>) for NCPM digestion, with reaction times from 3 to 24 h at room temperature. The 24 h treatment is promising with 100% PHA recovery and 98% PHA content in suspended solids; however, there is a lack of precise analytical data for purity.<sup>14</sup> Moreover, inclusion of a chlorination step hardly qualifies it as a modern green process. Recently, Dubey et al. proposed a new polymer recovery approach based on selective dissolution of NCPM by the ionic liquid (IL) 1-ethyl-3-methylimidazolium diethyl phosphate. Samples were stirred at 60 °C for 24 h, followed by precipitation of the recovered polymer by adding methanol. The polymer recovery reached 60% at 86% purity, and 60% of the IL could be recovered after two cycles.<sup>15</sup> The current drawbacks of this approach are the modest polymer purity and recovery and the costs of the solvent and its limited reusability.

The second approach to PHA recovery, in contrast to NCPM dissolution, is selective dissolution of PHA, usually followed by precipitation of PHA by an antisolvent. This method often requires an additional cell disruption step prior to dissolution in order to provide access for the solvent to the

polymer granules. The most common disruption techniques are thermal breakdown, mechanical homogenization or grinding, sonication, and lyophilization, alone or in combination with NCPM digestion.<sup>3</sup> Most of the previously reported PHA dissolution methods resulting in high yield and purity are based on halogenated solvents such as chloroform or dichloromethane (DCM).<sup>16</sup> Due to their toxicity, there is a need for nonhalogenated and sustainable solvent extraction methods. Koller et al. proposed an extraction scheme based on acetone at elevated temperature and pressure. Extractions at 120 °C for 20 min in a closed stainless-steel apparatus resulted in 91.6% yield at 98.4% purity with the molecular weight  $(M_{\rm w})$ and molecular mass dispersity  $(D_M)$  of the polymer intact. Fiorese et al. used 1,2-propylene carbonate to obtain 95% yield at 84% purity by extraction at 130  $^\circ C$  for 30 min, with a polymer precipitation period of 48 h.  $^{18}$  Recently, Jiang et al. successfully extracted PHB using cyclohexanone and  $\gamma$ butyrolactone for 3 min at 120 °C, achieving 95% yield with 99.5% purity for cyclohexanone and 50% yield with 97.2% purity for  $\gamma$ -butyrolactone. Both solvents required addition of methanol as antisolvent and the polymer was recovered with  $M_{\rm w}$  and  $D_{\rm M}$  largely unaffected.<sup>19</sup> Using dimethyl carbonate as the extraction solvent, de Souza Reis et al. found the extraction yields to be 20% lower than in the case of chloroform (no absolute value given) at a maximum PHB purity of 91.2%, which decreased with increasing biomass to solvent ratio. The purity could be increased to 98% by an additional purification step using 1-butanol, although the overall yield was never reported with the extra purification step taken into account.<sup>20</sup> Parodi et al. reported on a circular extraction concept, using as solvents methyl 3-hydroxybutyrate (MHB) derived from PHA as the methanolyzed monomeric methyl ester, and methyl 3methoxybutyrate (MMB), likewise prepared from PHA in a three-step process. Both solvents yielded high recoveries and purities when used to extract PHA at 130 °C for 10 min, achieving 96% yield at 96% purity for MHB and 98% at 98% purity for MMB. Extraction with MMB gave polymer with  $M_w$ similar to DCM extraction, whereas the  $M_w$  of polymers extracted with MHB were  $\approx$  50% lower. Judging from their size exclusion chromatograms, the polymers extracted by MMB and MHB appeared to have significantly higher  $D_{\rm M}$  than those extracted by DCM.<sup>21</sup>

Dihydrolevoglucosenone (Cyrene) is a biodegradable dipolar aprotic solvent, derived in two simple steps from renewable cellulose waste, that has recently emerged as a useful green solvent with properties similar to those of N-methyl-2pyrrolidone and N,N-dimethylformamide. It has a high boiling point (203 °C) and possesses a corresponding low vapor pressure and low toxicity. Recent reports state that the large quantity price is as low as low 2 €/kg, making it cost competitive compared to solvents derived from fossil resources, in particular to the common PHA solvent chloroform, which is recognized as an emerging air pollutant subject to regulatory actions to minimize its use. Although the bulk purchase cost in Europe is around 0.80 €/kg, chloroform is associated with a steep disposal cost (an estimate given by a large recycling player in Sweden is  $3.20 \notin kg$  at the ton scale), which must be added to the solvent  $\cos^{22-27}$  Spent Cyrene can, on the other hand, be used in the biorefinery as a clean and carbon dioxide neutral liquid fuel. Elhami and coworkers<sup>28</sup> reported that biomass rich in (3-hydroxybutyrateco-3-hydroxyvalerate) (PHBV) from mixed microbial cultures fed with organic waste could be extracted with Cyrene after the

pubs.acs.org/journal/ascecg

Experiment	Run Order	Time (min)	Temperature (°C)	Concentration (% w/v)	Yield (%)	$M_{\rm n}~({\rm kDa})$	$M_{\rm w}~({\rm kDa})$	$D_M$ (unitless)	
N1	1	5	120	2.5	44.9	309	406	1.31	
N2	15	15	120	2.5	51.1	340	404	1.19	
N3	16	5	140	2.5	69.0	212	322	1.52	
N4	2	15	140	2.5	79.0	256	388	1.52	
N5	9	5	120	7.5	43.3	267	381	1.43	
N6	11	15	120	7.5	49.7	255	359	1.41	
N7	13	5	140	7.5	65.6	227	331	1.46	
N8	8	15	140	7.5	80.9	243	386	1.59	
N9	5	3	130	5	52.1	237	371	1.57	
N10	4	17	130	5	68.6	257	446	1.74	
N11	12	10	116	5	35.9	287	372	1.30	
N12	10	10	144	5	79.2	170	298	1.75	
N13	6	10	130	1.6	66.9	305	490	1.61	
N14	17	10	130	8.4	65.1	241	427	1.77	
N15	14	10	130	5	68.9	269	487	1.81	
N16	3	10	130	5	65.6	247	487	1.97	
N17	7	10	130	5	63.0	243	405 <sup>a</sup>	N/A	
<sup>a</sup> This value is an outlier; Grubb's test $p < 2.2 \times 10^{-16}$ for the alternative hypothesis.									

Table 1. Extraction Parameters (Independent Variables) of Circumscribed Central Composite Design Presented alongside Dependent Variables Yield,  $M_n$ ,  $M_w$ , and  $D_M$  for Polymers Extracted in Each Experiment

biomass had been dewatered and subjected to prolonged drying at elevated temperature to intentionally reduce the molecular weight. Maximum extraction yield was found to be  $57 \pm 2\%$  at > 99% purity when extracted at 120 °C for 2 h. The ability of Cyrene to dissolve PHBV pellets was also demonstrated by Tomietto et al.,<sup>29</sup> who suuessfully prepared porous membranes using a variety of additives and porogens. This encouraged us to systematically investigate the possibilities of using Cyrene to solubilize bacterial PHA without a preceding thermal pretreatment to reduce the molecular weight. In this report, we hence demonstrate an optimized, highly efficient, and rapid method for extraction of PHB from bacterial biomass by using Cyrene as a solvent under mild extraction conditions. The experiments were performed based on Design of Experiment (DoE) coupled with data evaluation by multivariate analysis. The influence of key parameters such as the extraction time, temperature, and PHB concentration were examined and evaluated versus the molecular weight, as determined by SEC. The polymer content in bacteria was examined using gas chromatography coupled with flame ionization detection (GC-FID) as monomer methyl esters after depolymerization by catalytic methanolysis. The purity of extracts as well as reusability of the solvent were confirmed by NMR analysis.

# EXPERIMENTAL SECTION

Materials and Chemicals. Dihydrolevoglucosenone [(1R)-7,8-dioxabicyclo[3.2.1]octan-2-one, Cyrene; 99%) and poly(3-hydroxy-butyric acid-co-3-hydroxyvaleric acid) with 12 mol % of 3-hydroxyvaleric acid (PHBV) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol (99.8%), methanol (99.8%), and chloroform (99.9%) were obtained from VWR (Radnor, PA, USA), and sulfuric acid (95%–98%) and benzoic acid (99.9%) were from Merck (Darmstadt, Germany). All chemicals were used without further purification.

**Culture Conditions.** Photobacterium ganghwense strain C2.2 was repeatedly streaked on solid medium MP<sup>30</sup> at 20 °C, in order to obtain isolated colonies. A single colony was inoculated in 250 mL of liquid MP and cultivated at 20 °C with stirring at 150 rpm, for 20 h, until the culture reached an OD<sub>600</sub> of 5.5. Biomass was produced using a BIOSTAT Plus bioreactor (Sartorius, Germany) starting with

an inoculum of 10% v/v of cell culture having an initial  $OD_{600} = 0.5$  in 2 L PHA production medium.<sup>31</sup> Proper aeration of the cultures was ensured by a 0.2 vvm aeration rate and agitation at 300 rpm using a Rushton turbine. Cultivation media were supplemented with 2% (w/ v) glycerol as the carbon source and 0.25% (w/v) urea as the nitrogen source. The 20 °C set temperature was maintained by connecting the double-jacketed bioreactor vessel to a Grant (Shepreth, UK) LT ecocool 100 refrigerated circulating bath. During fermentation, the pH was left unadjusted since initial bioreactor experiments using this bacterial strain (not included in this paper) where pH was adjusted to 7.0 throughout the entire cultivation resulted in poor yields of both biomass and PHA, whereas twice the yield was reached in a batch cultivation experiment without any pH adjustment or control. The culture was monitored for growth and formation of PHA granules during the process, by measuring OD<sub>600</sub> and by preparing wet-mount slides from 0.5 mL culture, staining with 20  $\mu$ L Nile Red stock solution and examining the cells by a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Cells were harvested by centrifugation of 40 mL culture portions in preweighed tubes at 18,500 rcf for 15 min using an Hermle Z 326 K centrifuge from Hermle Labortechnik (Wehingen, Germany). The recovered pellets were lyophilized at -55 °C for 24 h by a Martin Christ (Osterode am Harz, Germany) Alpha 1-2 LD plus freeze-dryer using a target pressure in the vacuum chamber of 3.6 Pa.

Quantitative Analysis of PHA by GC-FID. Lyophilized bacteria pellets were disintegrated by an Andersson CEG 1.0 Blade Grinder from NetOnNet (Borås, Sweden). Approximately 10-20 mg of lyophilized cells was weighed and transferred into screw capped culture tubes from Duran borosilicate glass (DWK Life Sciences, Mainz, Germany). Here, 1.5 mL of a mixture of 85:15 (v/v) sulfuric acid and methanol containing 400 ppm of benzoic acid as the internal standard was added into the vials, followed by addition of 1.5 mL of chloroform. Bacterial and polymer standard samples were then heated at 100 °C for 2 h. After cooling to room temperature, 0.75 mL of water was added to each sample vial followed by vigorous shaking for 30 s to ensure an efficient liquid-liquid extraction. After phase separation, the organic phase was carefully transferred by Pasteur pipet into GC vials for further analysis. The samples were subjected to analysis by an Agilent 7820A GC-FID system (Agilent Technologies, Santa Clara, CA, USA), equipped with a 30 m long by 0.32 mm i.d. DB-5MS column from Agilent with a 0.25  $\mu$ m film thickness. The injector temperature was set to 250 °C in splitless mode with an injection volume of 1  $\mu$ L. The temperature program was set to 50 °C with hold for 5 min and then a linear increase to 250 °C at a rate of 15

 $^{\circ}$ C/min, followed by an increase to 310  $^{\circ}$ C at a rate of 30  $^{\circ}$ C/min. Hydrogen was used as the carrier gas at a flow rate of 0.8 mL/min with a column head overpressure of 37 kPa. Polymer content was quantified based on methanolysed PHBV as the external standard via a calibration curve. Two technical replicates were used for each methanolysis sample.

**Viscosity Measurements.** Viscosities of dissolved polymer in Cyrene, with the concentration based on optimized conditions and the highest concentrations used in the experiments (0.54 and 2 wt-%, respectively), were measured using a Brookfield RV DV1 viscometer (AMETEK Brookfield, Middleboro, MA, USA) with a cone and plate arrangement, at temperatures of 60 and 80 °C maintained by a Julabo (Seelbach, Germany) F12 heating circulator. The viscosity ( $\eta$ ) values of the dissolved polymers were obtained according to the given equation:

$$\eta = (100/\text{RPM})(\text{TK})(\text{torque})(\text{SMC})$$
(1)

where TK, RPM ,and SMC are the viscometer torque constant (0.09373), the rotational speed, and the spindle multiplier constant (0.327), respectively.

**Differential Scanning Calorimetry.** A TA Instruments DSC Q1000 system (Waters, New Castle, DE, USA) was used with a heating rate of 10 °C min<sup>-1</sup> and a nitrogen atmosphere. For swelling DSC, 2 mg of PHB sample was added to a crucible followed by addition of 10  $\mu$ L of solvent and sealing by a universal crimper press. A sealed crucible containing 10  $\mu$ L of solvent only was employed as reference.

Design of Experiments (DoE) for Polymer Extraction from Bacteria. Three key attraction parameters, including time, temperature, and concentration of bacteria in solvent, were studied. A circumscribed central composite (CCC) design with seventeen experiments at varying extraction conditions was carried out in a randomized order as shown in Table 1. The center point was run in triplicate (N15-N17) to evaluate the repeatability of the extraction method. For each experiment, an appropriate amount (0.16-0.84 g)of lyophilized and homogenized bacteria samples were weighed and transferred into 25 mL screw cap vials to obtain the desired polymer concentration. Here, 10 mL of Cyrene was added to each sample vial, which after capping were fixed to a custom-built agitation unit placed in the oven of a HP 5890 gas chromatograph and heated to the desired temperature under continuous end-overend tumbling at  $\approx 15$ rpm. At the end of the extraction time, the vials were rapidly cooled down in ice-cold water for about 20 s followed by transfer of the extracted bacteria suspensions into 25 mL Nylon centrifugal filters of 0.22  $\mu$ m pore size from ThermoFisher Scientific (Waltham, MA, USA), preheated to 60 °C. Forced filtration took place by inserting the centrifugal filters into 50 mL Falcon centrifuge tubes and subjecting the assemblies to centrifugation in an Eppendorf (Hamburg, Germany) 5810R centrifuge at 40 °C and 2500g for 20 min. After the centrifugation, the filter inserts were removed from the Falcon tubes, and 30 mL of ice-cold ethanol was added to the filtrates in each tube in order to precipitate the extracted polymers. This was followed by vigorous shaking, after which the tubes were left for 30 min at room temperature to allow complete precipitation. The precipitated polymer was recovered by vacuum filtering on preweighed 25 mm diameter Whatman Nylaflo Nylon membrane filters with 0.2  $\mu$ m pore size from Cytiva (Marlborough, MA, USA) and additionally washed with approximately 10 mL of fresh ethanol. The filters with the polymer precipitate were dried overnight in an OVA03100 vacuum oven from Gallenkamp (Cambridge, UK) under partial vacuum (≈0.1 Pa) at 40 °C. Recovery yield was determined as

Recovery (%) = 
$$\frac{\text{Mass of extracted PHA (g) } \times 100}{\text{Total mass of PHA in lyophilized cells (g)}}$$
(2)

Subsequently, for comparison, 5 wt % of bacteria pellet was placed in a Soxhlet apparatus and extracted overnight with 80 mL of chloroform. The polymer was recovered from the chloroform extract by addition of 240 mL of ethanol, followed by precipitation and filtering, using the same procedure as above. The principal component analysis was carried out in **R** version 4.2.2 using the *princomp* and *biplot* functions on all the data in Table 1, after scaling and centering using the *scale* function.<sup>32</sup>

**Nuclear Magnetic Resonance Spectroscopy.** The purity of reused solvent and extracted polymer was examined by <sup>1</sup>H NMR. Spectra were acquired at 298 K with a Bruker (Billerica, MA, USA) DRX-400 spectrometer with a 9.4 T magnet, corresponding to a Larmor precession frequency of 400.2 MHz for <sup>1</sup>H. Samples ( $\approx 0.25$  mg) were dissolved in 0.5 mL CDCl<sub>3</sub> (99.8 atom % D, Acros, Geel, Belgium), and data were further processed with the TopSpin 4.0.7 software from Bruker.

Size Exclusion Chromatography (SEC). Extracted polymer samples were redissolved in chloroform at  $\approx$  5 mg/mL concentration, transferred to autosampler vials, and capped. The molecular weight distribution of the polymers was estimated by size exclusion chromatography in a system consisting of an Agilent G1311A pump and a Viscotek SEC-MALS 20 MALS detector equipped with RI and viscosity detection options from Malvern Panalytical (Malvern, UK). The detector was calibrated with polystyrene standards PSS-ps200k and PSS-psb310k. Separations were carried out on two series-connected 300  $\times$  8 mm i.d. Malvern T6000 M columns with chloroform as eluent. The temperatures of the autosampler, columns, and detector were 35 °C, and the eluent flow rate was set to 1 mL/min. The MALS data were evaluated by the Malvern Omnisec software version 11.20, using the Debye model with first-degree polynomial.

**Data Analysis of DOE.** The effect of each extraction parameter on the yield and on the  $M_n$  and  $M_w$  of the obtained polymers was optimized using MODDE 13 software (Sartorious Stedim Data Analytics AB, Sweden) to select experimental parameters by a Circumscribed Central Composite (CCC) design model, evaluating the experimental outcomes by PLS regression. The raw models were then optimized by removing insignificant coefficients using the autotune function in the software. Optimal conditions were predicted based on the tuned models and thereafter verified experimentally.

Polymer Extraction Upscaling Experiments. Filtration upscaling experiments were performed using a HP4750 Stirred Cell high pressure filtration unit from Sterlitech (Auburn, WA, USA), based on the optimized conditions from the model. Here, 2 g of lyophilized cells was added to 100 mL of Cyrene preheated to 130 °C in a borosilicate glass bottle and subjected to extraction for 15 min. After the extraction was continued, the mixture was immediately transferred into the filtration unit thermostatted to 60 or 80 °C. Filtration was performed using an aluminum oxide membrane filter (Sterlitech, 0.02  $\mu$ m, 47 mm) with propulsion by nitrogen gas at a constant overpressure of 300 kPa. As the permeate flow ceased, the filtration unit was washed with a fresh 30 mL aliquot of preheated (60 or 80 °C) Cyrene. The thusly obtained permeates were allowed to cool to room temperature and mixed with three times the excess volume of ethanol to precipitate the polymer. The solid polymers were then recovered by vacuum filtration on preweighed 47 mm diameter 0.22 µm pore size Nylon membrane filters from MerckMillipore (Burlington, MA, USA), followed by a wash with  $\approx 10$  mL ethanol. Additional filtration experiments were performed for comparison at 80 °C, using a 47 mm GF75 binder-free glass microfiber filter with 0.3  $\mu$ m pore size from Advantec (Dublin, CA, USA), following the same recovery steps as above. The permeate flux (LMH) for each membrane and temperature was calculated as

Permeate Flux (LMH) = 
$$\frac{V}{A \times t}$$
 (3)

where *V* is the volume of permeate collected at time *t* (h), and *A* is the active membrane area (0.00146 m<sup>2</sup>).

#### RESULTS AND DISCUSSION

Study of Polymer Extraction and Dissolution by Differential Scanning Calorimetry. Phase transitions of the biopolyesters during the extraction were investigated by DSC of whole cells and extracted polymer, with and without the presence of solvent (Figure 1). Lyophilized cells (9.8 wt %) in Cyrene showed a broad endothermic peak with maximum at



Figure 1. Differential scanning calorimetry of bacteria and polymer with and without Cyrene as added solvent.

113 °C, which is close to the extraction temperature in the glass vial experiments. Broadening of the peak is most likely associated with proteins in the cells.<sup>30,31</sup> Heating of a polymer-solvent mixture produced two endothermic peaks in the thermogram, where the first peak at 75 °C is interpreted as swelling of the polymer,  $T_{\text{swell}}$ , followed by the main dissolution temperature peak,  $T_{\text{dis}}$ , at 84 °C. The lower dissolution temperature of the polymer reference preparation compared to that of the intact "pristine" polymer in the dry whole cells is indicative of a lower fraction of crystalline phases in the dissolved and precipitated polymer. The calculated enthalpies of  $T_{swell}$  and  $T_{dis}$  were equal to 6.4 and 51.5 J/g, respectively. At a heating rate of 10 °C/min, swelling and dissolution peaks were partly overlapping; therefore, an additional thermogram was recorded at 2 °C/min heating rate to verify the presence of two separate transitions. By comparing polymer dissolution thermogram to the polymer melting, the main dissolution peak was about 100 °C lower than the melting peak,  $T_{melt}$ , of the extracted polymer at 176 °C, with 98.7 J/g melting enthalpy. This corresponds to an estimated degree of crystallinity of 68%, using 146 J/g as the enthalpy of fusion for 100% crystalline PHB.33 Lyophilized whole cells also showed a single endothermic peak at 176 °C, indicating that the extraction procedures did not alter the melting points of the polymers.

**Effects of Extraction Parameters.** In this study, we explored the effects of extraction time, temperature, and the bacterial biomass loading in the Cyrene solvent on the extraction yield and the molecular weight of the recovered polymers. Experimental design by the Circumscribed Central Composite (CCC) model was used to maximize the information in the minimum number of experiments. The CCC model includes a center point and a group of "star points" and was selected for its ability to assess the quadratic term of variables and produce regression models that can better estimate the curvature of the response and predict the optimal experimental parameters more accurately. In addition,

with the CCC design, larger ranges of experimental parameters can be studied with the same number of experiments compared to other central composite designs like face-centered or inscribed central composite design.

According to the GC analysis, the polymer content in the lyophilized cells used for the whole experimental setup was 27 wt % of the cell dry weight (CDW). The yields of all the extraction experiments performed on lyophilized bacterial biomass in this study are calculated based on this value.

Based on our preliminary experiments, the extraction using  $CHCl_3$  with a Soxhlet setup resulted in a polymer with  $M_n$  of 272 kDa and  $M_{\rm w}$  of 417 kDa, at 71% yield. The yields,  $M_{\rm p}$ , and  $M_{\rm w}$  of polymers at different extraction conditions are listed in Table 1. In comparison with CHCl<sub>3</sub>, the results obtained with Cyrene varied over rather wide ranges, with performances that were poorer in some cases and better in other. The high variations indicate that the extraction parameter changes in the tested experimental range had a significant effect on the extraction. It is worth noting that the three extraction replicates at the center condition of the design showed a relatively good repeatability, where the variations (RSD) of yield,  $M_{\rm p}$ , and  $M_{\rm w}$ were 4.5%, 5.5%, and 10.3%, respectively. The PLS regression models showed very good fits  $(R^2 > 0.85)$  accompanied by good predictabilities  $(Q^2 > 0.6)$ . The three center point experiments (N15-N17) gave repeatable results for yield and  $M_{\rm nv}$  whereas the  $M_{\rm w}$  in experiment N17 was found to be an outlier (Grubb's test  $p < 2.2 \times 10^{-16}$ ) for unclear reasons.

Time and temperature both had positive effects on yield; in addition, temperature had a negative quadratic term of smaller magnitude (Figure 2). This means that within the investigated range, an increased temperature will initially increase the yield, but this increase will eventually level off and start to decrease at excessively high temperatures. The biomass loading showed a



**Figure 2.** Coefficient contributions to the optimized PLS models for each of the response factors. Yield (n = 17, df = 13;  $R^2 = 0.96$ ),  $M_n$  (n = 17, df = 10;  $R^2 = 0.91$ ), and  $M_n$  (n = 17, df = 9;  $R^2 = 0.88$ ).

limited effect on the yield in the study range. No interaction terms were found to be significant in the regression model for yield, meaning that the effects of extraction parameters were independent. Coefficients of a few interaction terms in the  $M_n$  and  $M_w$  regression model are significant, hinting at synergetic effects between the extraction parameters, contributing to the curvatures of the response surfaces for yield,  $M_n$  and  $M_w$ .

A principal component analysis of the results in Table 1 was carried out to further clarify the correlations between the independent (time, temperature, and concentration) and dependent variables  $(M_n, M_w, \text{ yield, and } D_M)$ . A biplot of the loadings and scores is shown in Figure 3, with the two first



**Figure 3.** Biplot of a principal component analysis of the data in Table 2. A scree plot is inserted in the lower right corner of the main figure.

components explaining 43% and 25%, of the total variance, respectively. The plot can be interpreted by examining the magnitude and directions of the loading arrows for the independent and dependent variables, which are correlated if pointing in the same direction, and vice versa for pairs pointing in opposite directions. Experiments located near the center of the plot have had little influence on the two first principal components of the model, unlike experiments in the extremes of the plot, which are found next to the variable loading vectors that they have contributing most to in the model. Along the abscissa with the most significant first principal component PC1, the most obvious result is that temperature is strongest among the independent variables, positively correlated with yield and inversely correlated with  $M_{\rm n}$ . In the orthogonal PC2 dimension, time is positively correlated with  $M_{w}$ , and to a lesser extent with  $M_{\rm p}$  and yield. The increase of both  $M_{\rm p}$  and

 $M_{\rm w}$  with extraction time, coupled with their negative correlation with temperature, is interpreted as higher molecular weight polymers requiring longer time to solubilize, whereas higher temperatures led to higher risk of scission of polymer molecules with correspondingly lower molecular weights.<sup>34-</sup> This is further corroborated by the negative correlation of concentration with both  $M_{\rm n}$  and  $M_{\rm w}$ , which can be explained by the loss of some higher  $M_{\rm w}$  polymer in the filter cake formed by NCPM high biomass loadings. Correlations of  $D_M$ with yield and temperature can also be inferred from the biplot, which can be explained by dissolution of more high molecular weight polymers as the yield increases, possibly also by thermal scission at elevated temperatures leading to values of  $D_{\rm M}$  approaching the value of 2 according to Carother's equation for step growth polymers, which also applies to polymer breakdown by random scission.<sup>34–37</sup>

**Optimization of the Extraction Parameters.** In the extraction process, it is desirable to achieve a high yield, while the polymer is preserved from degradation. Therefore, we have set the condition of optimization to maximize for all response parameters (Table 2) and use the *Max* values for *Predicted* to set for *Target* values. Since we expected, based on the abovementioned experiment, that the extraction using Cyrene should be equal to, or better than, Soxhlet extraction with CHCl<sub>3</sub>, the *Min* values were therefore set using rounded values obtained from the Soxhlet extraction.

Based on the setting conditions mentioned above, a "sweet spot plot" has been calculated and is shown in Figure 4, with



**Figure 4.** Sweet plot calculated based on the optimized PLS model based on data from the MODDE experiment series, with preset objectives and targets (Table 3) and a bacterial biomass concentration of 2.5%. The predicted yield at the sweet spot is 70% with  $M_{\rm w}$  = 420,000 and  $M_{\rm n}$  = 270,000.

T	at	le	2		0	bjective	and	Target	for	the	Optimization	Calcu	lation
---	----	----	---	--	---	----------	-----	--------	-----	-----	--------------	-------	--------

						Predicted	
Name	Units	Condition	Objective	Min	Target	min	max
Yield	%	Required	Maximize	70	88	37.1	80.8
$M_{ m n}$	kDa	Required	Maximize	270	360	195	359
$M_{ m w}$	kDa	Required	Maximize	420	480	273	479

the green area representing the optimal area where the extraction performance best met all the criteria including yield,  $M_{\rm n}$  and  $M_{\rm w}$ . All optimization was achieved only at low concentration (2.5%, w/v) of bacterial biomass in Cyrene. The temperature range of the green zone was around 130–136 °C (Figure 4), which lies well in the temperature range of the first negative peak (90–140 °C) in the DSC curve of the bacteria in Cyrene and toward the higher temperature side of the apex (113 °C).

The optimal conditions calculated using the Optimizer tool in the MODDE software with the objective settings mentioned above were extraction for 15 min at 130 °C with a 2% loading of bacterial biomass in Cyrene. At those conditions, the predicted yield,  $M_{\rm p}$  and  $M_{\rm w}$  were 70%, 306 kDa and 466 kDa, respectively. The experiment performed at the optimal extraction condition mentioned above resulted in a yield of 71.7%, a  $M_{\rm n}$  of 300 kDa and a  $M_{\rm w}$  of 428 kDa. The yield and  $M_{\rm n}$  were in very good agreement with the values predicted by the models and confirmed the high predictability of the model. On the other hand, the  $M_w$  value was lower than predicted value, yet the difference is less than 12%, and therefore, the real values are still considered to be aligned with the predicted values calculated using the model. The optimized extraction model resulted in a polymer of very high purity, as confirmed by <sup>1</sup>H NMR spectroscopy (Figure 5).



Figure 5. <sup>1</sup>H NMR spectrum of extracted polymer based on optimized conditions, with expanded scale insert.

**Reusability of Extraction Solvent.** The solvent recycling potential was investigated by running five consecutive extractions of fresh bacterial biomass while reusing the solvent. The series started with fresh Cyrene, which was recycled in consecutive runs after removal of ethanol used as antisolvent from the filtrates from the precipitation steps by rotary evaporation, with small additions of fresh Cyrene needed to prepare extraction mixtures of identical composition, based on optimized conditions given by the DoE model (Table 2). The results in Figure 6 show that the polymer recovery was 70.2 ± 1.8% with stable and reproducible molecular weights with  $M_n$  at 303 ± 8.2 kDa and  $M_w$  at 419 ± 17.6 kDa, resulting in  $D_M$  of



**Figure 6.** Yields and molecular weights from five repeated extractions of polymers using recycled Cyrene. The  $M_w$  value in Experiment 5 is an outlier (Grubb's test p = 0.001264 for the alternate hypothesis).

 $1.38 \pm 0.046$ , all given as means  $\pm 95\%$  confidence intervals. The recovered Cyrene was analyzed by NMR after each step with spectra presented in Figure 7. As seen in Figure 8, the



Figure 7. <sup>1</sup>H NMR spectra of recovered solvent after each cycle.

molecular weights of the polymers extracted remained stable over five extraction cycles, demonstrating high solvent recyclability (92%-96%) and the purity of the polymer produced.

**Upscaling.** The most critical operation in upscaling is expected to be hot filtration of the extracted biomass. In order to test the scalability of the optimized model, we therefore performed filtration experiments using a Sterlitech HP4750 stirred cell. This forced flow dead end filtering unit, which uses nitrogen gas as feed propulsive agent and features a suspended magnetic stir bar to provide cross-flow filtration conditions, allows an elevated temperature to be used during filtration. This enables the determination of the industrially important





SEC Retention Volume (mL)

**Figure 8.** Expanded sections of size exclusion chromatograms from the solvent recycling experiments with overlaid  $M_w$  from MALS detector data. Chromatograms are based on the 90° signal from the MALS detector and plotted with normalized intensities. Data for polymer recovered from the pristine solvent experiment are plotted in black, with the four consecutive recycling experiment overlaid in shades of gray of increasing lightness.

filtration rate in liters per m<sup>2</sup> per hour (LMH) at a given feed pressure. Two radically different filter types were tested in this study: an aluminum oxide membrane filter with 0.02  $\mu m$  pore size and a binder-free glass microfiber depth filter with 0.3  $\mu$ m nominal pore size. Both filter types allow operation at high temperatures and have good chemical stability, allowing thermal and chemical regeneration. In order to maximize the recovery, an additional portion of fresh solvent, preheated to the filtration temperature, was added after each filtration. The filtration performed at 60 °C with the aluminum oxide membrane resulted in 81% yield, plus 5.3% in the wash filtrate for a total recovery of 86.4% at a flux of 127.2 LMH. Filtration with the same membrane at 80 °C resulted in 88.6% initial recovery and 5.6% in the additional wash for total of 94.3% with flux 169.1 LMH. For comparison, filtration at 80 °C using the glass microfiber filter gave an initial recovery of 91.2% plus 6.1% in the wash for total recovery of 97.4%, at a flux of 312.5 LMH.

Based on these results, we found clear correlations of temperature with filtration speed and recovery yield (Table 3), with higher filtration temperature resulting in faster flux and higher overall yields. These results are also in line with the temperature-dependent viscosity measurements shown in Table 4, where an increase in temperature from 60 to 80 °C resulted in  $\approx$  40% reduction in viscosity for PHA solutions in Cyrene at two different concentrations. The significantly faster flux of the glass microfiber depth filter compared to that of the aluminum oxide membrane filter can be ascribed to its larger pore size (0.3 vs 0.02  $\mu$ m), in spite of being rather thick (350

Table 4. Viscosities of Cyrene and PHB Dissolved in Cyrene at 60 and 80  $^\circ\mathrm{C}$ 

Sample	$\eta { m d/cP}$ at 60 $^{\circ}{ m C}$	$\eta { m d/cP}$ at 80 $^{\circ}{ m C}$
Cyrene	1.3	1.2
0.54 wt.% PHB in Cyrene	3.8	2.2
2 wt.% PHB in Cyrene	11.7	7.4

vs 60  $\mu$ m). A rough estimate of the hydrodynamic radius of a 400 kDa random coil polymer in a good solvent should be in the range of 20–25 nm, but there is no clear-cut relationship between pore size and molecular weight cutoff (MWCO) for ultrafiltration membranes.<sup>38</sup> In view of this, the higher recovery provided by the glass microfiber filter could potentially have been due to MWCO for the upper tail in the molecular weight range of PHA by the aluminum oxide membrane filter. This is, however, contradicted by the  $M_w$  data in Table 3 and the size exclusion chromatograms overlaid with  $M_w$  from the MALS detector in Figure 9. Other possible explanations to the higher



**Figure 9.** Expanded sections from size exclusion chromatograms for polymers recovered in the different filtration experiments with overlaid  $M_w$  from MALS detector data. Chromatograms are based on the 90° signal from the MALS detector and the peaks are plotted with normalized max intensities.

yield with the glass filter could be formation of polymer clusters in the feed or partial blockage of the pores of the aluminum oxide membrane filter by debris toward the end of the dead-end cross-flow ultrafiltration process. Polymer entanglement<sup>39</sup> should finally also be considered at high polymer concentrations.

The upscaling experiments showed significantly higher recovery compared with the DoE experiments. We attribute this promising observation to the higher filtration temperatures

Table 3. Yields,  $M_n$ ,  $M_w$ , and  $D_M$  of Extracted Polymer in Upscaled Extractions Filtered by HP4750 Stirred Cell

Filter type	Pore size $(\mu m)$	Filtration temperature (°C)	Flux (LMH)	Yield (%)	$M_{\rm n}~({\rm kDa})$	$M_{\rm w}~({\rm kDa})$	$D_M$ (unitless)
Aluminum oxide membrane	0.02	60	127.2	86.4	304	616	2.03
Aluminum oxide membrane	0.02	80	169.1	94.3	283	533	1.88
Binder-free glass fiber depth filter	0.3	80	312.5	97.4	282	532	1.89

and also to the suspended bacterial biomass by the magnetic stirrer, which establishes a tangential feed flow across the filter surface during the extraction, preventing the filter from clogging. Moreover, the extracted polymer had higher M<sub>w</sub> and  $M_n$  (Table 3) than in model experiments. That is likely due to the higher filtration temperature in the stirred cell 60-80 °C vs 40 °C, which is the upper limit set by most lab centrifuges including the Eppendorf 5810R used in this work. An additional explanation can be that the continuous stirring in the HP4750 tangential flow filtration cell provided a more homogeneous suspension throughout the entire filtration step, whereas the centrifuge filters will rapidly form a cake of suspended bacterial debris and prevent the highest  $M_w$  fraction from passing. On the other hand, by increasing the filtration temperature, we observed a decrease in  $M_w$  and  $M_n$  values, from 606 and 304 kDa to 544 and 283 kDa, respectively. Hence, finding an optimal filtration temperature in terms of  $M_{\rm w}$  and  $M_{\rm p}$  as well as optimizing flux are important parameters to consider for process scalability. The applied extraction method proved to be very efficient and scalable, in particular if tangential flow filtration was used.

## CONCLUSIONS

The extraction parameters for extraction of PHB from lyophilized bacteria were thoroughly investigated using a design of experiment approach. The PLS models that correlated the running conditions to the yield,  $M_{\rm n}$ , and  $M_{\rm w}$ showed high validity with excellent regression and high predictability. The extraction time and temperature were expected to have a high impact with nonlinear effects on the yield,  $M_{\rm n}$ , and  $M_{\rm w}$  of the obtained polymer. The verified optimal conditions are 130 °C for 15 min with a bacterial biomass concentration of 2%, resulting in a yield of 71.7%. This yield is in line with the predicted value of more than 70% for the first extraction cycle, producing a high molecular weight polymer ( $M_{\rm n}$  of  $\approx$  300 kDa and  $M_{\rm w}$  of  $\approx$  430 kDa) with high purity, as proven by NMR analysis. The upscaling of the extraction of the method was straightforward and showed results comparable to those of the small-scale setup, with successful recycling of the extraction solvent verified. The extraction of PHB from Photobacterium ganghwense with Cyrene has hence been proven to be quick, efficient, scalable, and sustainable.

#### AUTHOR INFORMATION

## **Corresponding Author**

Piotr Jablonski – Department of Chemistry, Umeå University, 90187 Umeå, Sweden; Present Address: Piotr Jablonski: CMC API Development, Novo Nordisk A/S, Smørmosevej 17, 2880 Bagsværd, Denmark; © orcid.org/ 0000-0002-3458-939X; Email: pija0009@student.umu.se

#### Authors

- Ngoc Phuoc Dinh Diduco AB, 90736 Umeå, Sweden; Present Address: Ngoc Phuoc Dinh: Klaria AB, Virdings Allé 2, 75450 Uppsala, Sweden.
- Irina Lascu Department of Genetics, Faculty of Biology, University of Bucharest, 050095 Bucharest, Romania; orcid.org/0000-0002-3287-9107
- Ana-Maria Tanase Department of Genetics, Faculty of Biology, University of Bucharest, 050095 Bucharest, Romania
- Mikkel Christensen Department of Chemistry, UiT-The Arctic University of Norway, 9037 Tromsø, Norway

- Santosh Govind Khokarale Wallenberg Wood Science Center, Department of Chemistry, Technical Chemistry, Chemical-Biological Center, Umeå University, 90187 Umeå, Sweden
- Ola Sundman Department of Chemistry, Umeå University, 90187 Umeå, Sweden
- Jyri-Pekka Mikkola Wallenberg Wood Science Center, Department of Chemistry, Technical Chemistry, Chemical-Biological Center, Umeå University, 90187 Umeå, Sweden; Industrial Chemistry & Reaction Engineering, Department of Chemical Engineering, Johan Gadolin Process Chemistry Centre, Åbo Akademi University, 20500 Åbo-Turku, Finland
- Knut Irgum Department of Chemistry, Umeå University, 90187 Umeå, Sweden

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.3c05422

#### Notes

The authors declare no competing financial interest.

# ACKNOWLEDGMENTS

This work was funded by grant from the Swedish Research Council for Sustainable Development (MISTRA, project 2016-02011, part of the ERA-NET Marine Biotechnology framework), by The Bio4Energy program, and by Romanian National Authority for Scientific Research and Innovation, CCCDI – UEFISCDI (Project 13/2017) within PNCDI III and FSS 4947/2023 by Ministry of Education. This work is also a part of the activities of the Johan Gadolin Process Chemistry Centre at Åbo Akademi University in Finland and the Wallenberg Wood Science Center under auspices of the Knut and Alice Wallenberg (KAW) Foundation. The Sterlitech HP4750 Stirred Cell high pressure filtration unit was purchased through a stipend from The Kempe Foundation.

# ■ LIST OF ABBREVIATIONS

CCC, Circumscribed Central Composite (Design); CDW, cell dry weight; DCM, dichloromethane;  $D_M$ , molecular mass dispersity; DoE, Design of Experiments; DSC, differential scanning calorimetry; FID, flame ionization detection; GC, gas chromatography; IL, ionic liquid; LMH, liters per m<sup>2</sup> and hour; MALS, multiangle light scattering; MHB, methyl 3-hydroxybutyrate; MMB, methyl 3-methoxybutyrate;  $M_n$ , number average molecular weight;  $M_w$ , weight average molecular weight; MWCO, molecular weight cutoff; NCPM, non-PHA cell mass; NMR, nuclear magnetic resonance; OD<sub>600</sub>, optical density at 600 nm; PHA, polyhydroxyalkanoates; PHB, poly(3hydroxy butyrate); PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PLS, partial least-squares (regression); RCF, relative centrifugal force; RI, refractive index; RPM, revolutions per minute; RSD, residual standard deviation; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; SMC, spindle multiplier constant; TK, torque constant

#### REFERENCES

(1) Geyer, R.; Jambeck, J. R.; Law, K. L. Production, Use, and Fate of All Plastics Ever Made. *Sci. Adv.* **201**7, *3*, No. e1700782.

<sup>(2)</sup> Gahlawat, G. Polyhydroxyalkanoates Biopolymers - Production Strategies; Springer: Cham, Switzerland, 2019. DOI: 10.1007/978-3-030-33897-8. ISBN: 978-3-030-33897-8.

<sup>(3)</sup> Al-Ashraf, A.; Ramachandran, H.; Huong, K-H.; Kannusamy, S. Microbial-Based Polyhydroxyalkanoates: Upstream and Downstream

Processing; Smithers Group Company: Shawbury, 2015. ISBN: 978-190903-061-9.

(4) Hahn, S. K.; Chang, Y. K.; Kim, B. S.; Chang, H. N. Optimization of Microbial Poly(3-hydroxybutyrate) Recovery Using Dispersions of Sodium Hypochlorite Solution and Chloroform. *Biotechnol. Bioeng.* **1994**, *44*, 256–261.

(5) Volova, T.; Kiselev, E.; Nemtsev, I.; Lukyanenko, A.; Sukovatyi, A.; Kuzmin, A.; Ryltseva, G.; Shishatskaya, E. Properties of Degradable Polyhydroxyalkanoates with Different Monomer Compositions. *Int. J. Biol. Macromol.* **2021**, *182*, 98–114.

(6) Saavedra del Oso, M.; Mauricio-Iglesias, M.; Hospido, A. Evaluation and Optimization of the Tnvironmental Performance of PHA Downstream Processing. *Chem. Eng. J.* **2021**, *412*, 127687.

(7) Kumar, M.; Rathour, R.; Singh, R.; Sun, Y.; Pandey, A.; Gnansounou, E.; Lin, K.-Y. A.; Tsang, D. C. W.; Thakur, I. S. Bacterial Polyhydroxyalkanoates: Opportunities, Challenges, and Prospects. *J. Clean. Prod.* **2020**, *263*, 121500.

(8) Vu, D. H.; Wainaina, S.; Taherzadeh, M. J.; Åkesson, D.; Ferreira, J. A. Production of Polyhydroxyalkanoates (PHAs) by *Bacillus megaterium* using Food Waste Acidogenic Fermentationderived Volatile Fatty Acids. *Bioengineered* **2021**, *12*, 2480–2498.

(9) Christensen, M.; Jablonski, P.; Altermark, B.; Irgum, K.; Hansen, H. High Natural PHA Production from Acetate in *Cobetia* sp. MC34 and *Cobetia marina* DSM 4741T and *in Silico* Analyses of the Genus Specific PhaC2 Polymerase Variant. *Microb. Cell Factories* **2021**, *20*, 225.

(10) Acedos, M. G.; Moreno-Cid, J.; Verdú, F.; Gonzalez, J. A.; Tena, S.; Lopez, J. C. Exploring the Potential of Slaughterhouse Waste Valorization: Development and Scale-up of a New Bioprocess for Medium-chain Length Polyhydroxyalkanoates Production. *Chemosphere* **2022**, 287, 132401.

(11) Lascu, I.; Tănase, A. M.; Jablonski, P.; Chiciudean, I.; Preda, M. I.; Avramescu, S.; Irgum, K.; Stoica, I. Revealing the Phenotypic and Genomic Background for PHA Production from Rapeseed-Biodiesel Crude Glycerol Using *Photobacterium ganghwense* C2.2. Int. *J. Mol. Sci.* **2022**, 23, 13754.

(12) Perez-Rivero, C.; Lopez-Gomez, J. P.; Roy, I. A Sustainable Approach for the Downstream Processing of Bacterial Polyhydroxyalkanoates: State-of-the-art and Latest Developments. *Biochem. Eng. J.* **2019**, *150*, 107283.

(13) Yang, Y-H.; Brigham, C.; Willis, L.; Rha, C.; Sinskey, A. Improved Detergent-based Recovery of Polyhydroxyalkanoates (PHAs). *Biotechnol. Lett.* **2011**, *33*, 937–942.

(14) Villano, M.; Valentino, F.; Barbetta, A.; Martino, L.; Scandola, M.; Majone, M. Polyhydroxyalkanoates Production with Mixed Microbial Cultures from Culture Selection to Polymer Recovery in a High-rate Continuous Process. *New Biotechnol.* **2014**, *31*, 289–296. (15) Dubey, S.; Bharmoria, P.; Gehlot, P. S.; Agrawal, V.; Kumar, A.; Mishra, S. 1-Ethyl-3-methylimidazolium Diethylphosphate Based Extraction of Bioplastic "Polyhydroxyalkanoates" from Bacteria: Green and Sustainable Approach. *ACS Sustainable Chem. Eng.* **2018**, *6*, 766–773.

(16) Ramsay, J.; Berger, E.; Voyer, R.; Chavarie, C.; Ramsay, B. Extraction of Poly-3-hydroxybutyrate using Chlorinated Solvents. *Biotechnol. Tech.* **1994**, *8*, 589–594.

(17) Koller, M.; Bona, R.; Chiellini, E.; Braunegg, G. Extraction of Short-chain-length Poly-[(R)-hydroxyalkanoates] (scl-PHA) by the "Anti-solvent" Acetone under Elevated Temperature and Pressure. *Biotechnol. Lett.* **2013**, *35*, 1023–1028.

(18) Fiorese, M. L.; Freitas, F.; Pais, J.; Ramos, A. M; de Aragao, G. M. F.; Reis, M. A. M. Recovery of Polyhydroxybutyrate (PHB) from *Cupriavidus necator* Biomass by Solvent Extraction with 1,2-Propylene Carbonate. *Eng. Life Sci.* **2009**, *9*, 454–461.

(19) Jiang, G.; Johnston, B.; Townrow, D. E.; Radecka, I.; Koller, M.; Chaber, P.; Adamus, G.; Kowalczuk, M. Biomass Extraction Using Non-Chlorinated Solvents for Biocompatibility Improvement of Polyhydroxyalkanoates. *Polymers* **2018**, *10*, 731.

(20) de Souza Reis, G. A.; Michels, M. H. A.; Fajardo, G. L.; Lamot, I.; de Best, J. H. Optimization of Green Extraction and Purification of

PHA Produced by Mixed Microbial Cultures from Sludge. *Water* 2020, 12, 1185.

(21) Parodi, A.; D'Ambrosio, M.; Mazzocchetti, M.; Martinez, G. A.; Samorì, C.; Torri, C.; Galletti, P. Chemical Recycling of Polyhydroxybutyrate (PHB) into Bio-Based Solvents and Their Use in a Circular PHB Extraction. *ACS Sustainable Chem. Eng.* **2021**, *9*, 12575–12583.

(22) Sherwood, J.; De bruyn, M.; Constantinou, A.; Moity, L.; McElroy, C. R.; Farmer, T. J.; Duncan, T.; Raverty, W.; Hunt, A. J.; Clark, J. H. Dihydrolevoglucosenone (Cyrene) as a Bio-based Alternative for Dipolar Aprotic Solvents. *Chem. Commun.* **2014**, *50*, 9650–9652.

(23) Krishna, S. H.; Huang, K.; Barnett, K. J.; He, J.; Maravelias, C. T.; Dumesic, J. A.; Huber, G. W.; De bruyn, M.; Weckhuysen, B. M. Oxygenated Commodity Chemicals from Chemo-Catalytic Conversion of Biomass Derived Heterocycles. *AIChE J.* **2018**, *64*, 1910–1922.

(24) Sekar, A.; Varghese, G. K.; Varma, M. K. R. Chloroform - An Emerging Pollutant in the Air. In *New Trends in Emerging Environmental Contaminants*; Singh, S. P., Agarwal, A. K., Gupta, T., Maliyekkal, S. M., Eds.; Springer: Singapore, 2021, pp 101–129. DOI: 10.1007/978-981-16-8367-1 7.

(25) Jordan, A.; Stoy, P.; Sneddon, H. F. Chlorinated Solvents: Their Advantages, Disadvantages, and Alternatives in Organic and Medicinal Chemistry. *Chem. Rev.* **2021**, *121*, 1582–1622.

(26) Bryan, M. C.; Dunn, P. J.; Entwistle, D.; Gallou, F.; Koenig, S. G.; Hayler, J. D.; Hickey, M. R.; Hughes, S.; Kopach, M. E.; Moine, G.; Richardson, P.; Roschangar, F.; Steven, A.; Weiberth, F. J. Key Green Chemistry Research Areas from a Pharmaceutical Manufacturers' Perspective. *Green Chem.* **2018**, *20*, 5082–5103.

(27) *ChemAnalyst.* https://www.chemanalyst.com/Pricing-data/ chloroform-62 (accessed 2023-08-14).

(28) Elhami, V.; van de Beek, N.; Wang, L.; Picken, S. J.; Tamis, J.; Sousa, J. A.B.; Hempenius, M. A.; Schuur, B. Extraction of low molecular weight polyhydroxyalkanoates from mixed microbial cultures using bio-based solvents. *Sep. Purif. Technol.* **2022**, *299*, 121773.

(29) Tomietto, P.; Russo, F.; Galiano, F.; Loulergue, P.; Salerno, S.; Paugam, L.; Audic, J.-L.; De Bartolo, L.; Figoli, A. Sustainable fabrication and pervaporation application of bio-based membranes: Combining a polyhydroxyalkanoate (PHA) as biopolymer and Cyrene as green solvent. J. Membr. Sci. **2022**, 643, 120061.

(30) Lascu, I.; Mereuță, I.; Chiciudean, I.; Hansen, H.; Avramescu, S. M.; Tănase, A. M.; Stoica, I. Complete Genome Sequence of *Photobacterium ganghwense* C2.2: A New Polyhydroxyalkanoate Production Candidate. *MicrobiologyOpen* **2021**, *10*, No. e1182.

(31) Xiao, N.; Jiao, N. Formation of Polyhydroxyalkanoate in Aerobic Anoxygenic Phototrophic Bacteria and its Relationship to Carbon Source and Light Availability. *Appl. Environ. Microbiol.* **2011**, 77, 7445–7450.

(32) R Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, 2022. https://www.R-project.org.

(33) Barham, P. J.; Keller, A.; Otun, E. L.; Holmes, P. A. Crystallization and Morphology of a Bacterial Thermoplastic: Poly-3-hydroxybutyrate. *J. Mater. Sci.* **1984**, *19*, 2781–2794.

(34) Nguyen, S.; Yu, G.-e.; Marchessault, R. H. Thermal Degradation of Poly(3-hydroxyalkanoates): Preparation of Well-Defined Oligomers. *Biomacromolecules* **2002**, *3*, 219–224.

(35) Chen, Y.; Chou, I-N.; Tsai, Y.-H.; Wu, H.-S. Thermal Degradation of Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in Drying Treatment. J. Appl. Polym. Sci. **2013**, 130, 3659–3667.

(36) Aramvash, A.; Gholami-Banadkuki, N.; Seyedkarimi, M.-S. An Efficient Method for the Application of PHA-Poor Solvents to Extract Polyhydroxybutyrate from *Cupriavidus necator*. *AIChE Biotechnol*. *Prog.* **2016**, *32*, 1480–1486.

(37) Carothers, W. H. Polymers and Polyfunctionality. *Trans.* Faraday Soc. **1936**, 32, 39–49.

(38) Kim, K.J.; Fane, A.G.; Ben Aim, R.; Liu, M.G.; Jonsson, G.; Tessaro, I.C.; Broek, A.P.; Bargeman, D. A Comparative Study of Techniques Used for Porous Membrane Characterization: Pore Characterization. J. Membr. Sci. **1994**, *87*, 35–46.

(39) Wool, R. P. Polymer Entanglements. *Macromolecules* 1993, 26, 1564–1569.