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Extraction of short-chain-length poly-[(*R*)-hydroxyalkanoates] (*scl*-PHA) by the "anti-solvent" acetone under elevated temperature and pressure

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Abstract A novel method was developed for extraction of short-chain-length poly(hydroxyalkanoates) (*scl*-PHA) from microbial biomass by the well-known "*scl*-PHA anti-solvent" acetone at elevated temperature and pressure in a closed system combining components for extraction, filtration, and product work-up. Recovery of *scl*-PHA using this new approach was compared with established methods using chloroform at ambient pressure. The new method performs similar regarding product purity (98.4 vs. 97.7 %) and extraction yield (96.8 % by both methods), and is by far faster than established chloroform extraction (20 min vs. 12 h). Separation of the polymer from acetone is simply achieved by cooling down the acetone solution of *scl*-PHA, thus allows for a nearly quantitative recovery of the solvent that conveniently can be reused. Characterization of *scl*-PHA extracted by both methods does not reveal any significant difference in terms of molar mass and thermo analytical parameters.

Keywords Acetone · Anti-solvents · Biopolymer extraction · Chloroform · Short-chain-length poly(hydroxyalkanoates) (*scl*-PHA)

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Introduction

Poly(hydroxyalkanoates) (PHA) are biodegradable polyesters and potential alternatives to petrol based polymers used to produce plastics (Chen 2010; Koller et al. 2010). PHA are accumulated by numerous prokaryotes as reserves under unfavorable conditions like ample carbon supply and suboptimal availability of compounds for cellular reproduction (Zinn et al. 2001). Items made of PHA are amenable to complete degradation into water and CO_2 (Koller et al. 2011).

As intracellular products, PHAs have to be separated from non-PHA cell mass (NPCM). Direct extraction of PHA from biomass represents the best-established method; here, high throughput of hazardous solvents and enormous energy demand constitute the state-of-the-art, antagonizing sustainability and economics. Economics of PHA extraction depends on

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equipment, energy and chemicals needed, on product recovery yields and the possibility to reutilize the applied chemicals (Jacquel et al. 2008).

Typical halogenated extraction solvents like chloroform show excellent performance in isolation of thermoplastic short chain length (*scl*)-and elastomeric medium chain length (*mcl*)-PHA; the toxicity especially of chloroform constitutes the main drawback of this method (Ramsay et al. 1994). After polyester extraction, its solubility is reduced by adding a PHA anti-solvent, resulting in precipitation of highly pure PHA (Baptist 1962). Asrar et al. (2000) emphasize the low or nonexistent solubility of *scl*-PHA in most non-halogenated solvents, including acetone, under conditions of industrial scale extraction. For this intensively time-demanding process (classically 12 h), extraction solvents are needed up to the 20-fold quantity of the biomass. For the subsequent precipitation, about 10 l of the PHA anti-solvent has to be added per liter of PHA solution.

By screening methods for *scl*-PHA extraction that do without halogenated solvents, a new strategy was developed. A broad range of volatile liquids, mainly low molecular ketones and alcohols that are reported as "*scl*-PHA anti-solvents" were used for solubility and extraction experiments at elevated pressure (ca. 7 bar) and temperatures (ca. 120 °C) well above the solvent's normal boiling point. Therefore, special equipment, consisting of two closed vessels and a filtration part, was developed to extract PHA under elevated pressure. During solvent screening, acetone turned out as an especially promising alternative due to the minor impact on molar mass, its recyclability, price, and its suitability both as degreasing and as extraction solvent. A comparison was done between the adopted extraction method based on acetone under elevated pressure and that based on chloroform at atmospheric pressure. The parameters comparatively monitored were extraction yield, product purity and molar mass. No major difference was detected for the investigated parameters between the two extraction methods.

Materials and methods

Production of PHA-rich biomass

Biomass containing 87.5 % of the PHA-terpolyester poly(3-hydroxybutyrate-*co*-21.8 %-3-hydroxyvalerate-*co*-5.14 %-4-hydroxybutyrate) (PHB4HBHV) was used for PHA extraction via different methods; the production of this terpolyester was described previously (Koller et al. 2007).

Pretreatment for removal of lipids (decreasing step)

For removal of lipids, the biomass was dried by lyophilization and degreased by overnight Soxhlet-extraction with acetone. This biomass was subjected to three different PHA-extraction processes:

(a) "Classical" chloroform extraction (overnight stirring with chloroform)
(b) Overnight extraction with chloroform under reflux (Soxhlet)
(c) High-pressure extraction with acetone above the solvents boiling point

"Classical" chloroform extraction

Twenty-one gram of biomass were continuously stirred overnight (12 h) in 500 ml chloroform at ambient temperature. The system was kept in the dark by covering with aluminum foil to prevent free radical formation from chloroform.

Chloroform extraction under reflux

Twenty-one gram of biomass were extracted overnight (12 h) under chloroform reflux using a Soxhlet apparatus. Again, the system was light-protected.

Novel method using acetone under pressure

Apparatus design

The equipment consists of a cylindrical "extraction unit" (*EU*), 130 mm high, 165 mm diameter, a "filtration unit" (*FU*) 380 mm diameter, a cylindrical "precipitation unit" (*PU*), 130 mm high, 165 mm diameter and connecting pipes (Fig. 1). High grade (A4, acid resistant) steel is used in the pipes connecting the *EU* vessel to the *FU*, and the cover plates of the *EU* and *PU* vessels; aluminum is used in the core part of the *EU* and the *PU* vessels. In order to withstand the high pressure during the process, the equipment is tightly closed by using *O*-ring seals consisting of acetone-proof material. *EU* and *PU*

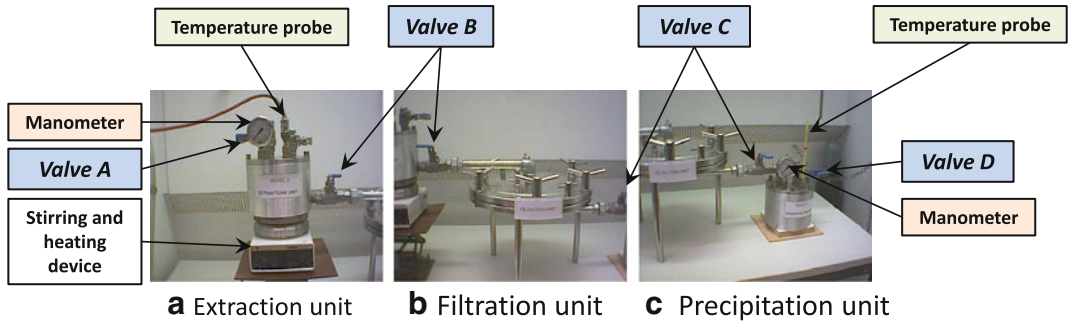

Fig. 1 Experimental set-up for extraction of *scl*-PHA under high temperature and pressure conditions using the "*scl*-PHA anti-solvent" acetone. The apparatus consists of an extraction unit (**a**), a filtration unit (**b**) and a precipitation unit (**c**). *Valve A* serves for inlet of nitrogen to generate an oxygen-free atmosphere before heating, *Valve B* connects the "Extraction unit" (*EU*) with the "Filtration unit" (*FU*), *Valve C* connects the *FU* with the "Precipitation unit" (*PU*); *Valve D* serves for pressure equilibration and removal of oxygen residues by flooding with nitrogen gas. The *A*–*D* valves are of spherical type

vessels are equipped with manometers and temperature probes.

Extraction

The new process uses 21 g lyophilized, powdered biomass loaded in *EU* vessel containing 700 ml acetone heated at 120 °C in for 20 min under continuous stirring (Fig. 1a). *EU* is equipped with a temperature probe and a manometer to monitor the extraction conditions. Under the apparatus assembled as above described and a temperature of 120 °C and a pressure of 7 bar is reached, PHA is completely dissolved in acetone. Oxygen has to be removed from acetone by flushing the entire system with nitrogen gas before heating (inlet of nitrogen gas in *Valve A*, with outlet via *Valve D* (Fig. 1). This apparatus set-up is needed because, under extreme conditions, acetone and oxygen might create an explosive mixture. The gas leaving the system via *Valve D* is analyzed in an oxygen measuring device (*O_2-analyzer PMA 12, M&C Products Analysentechnik GmbH, Germany*) in order to guarantee complete oxygen removal.

Separation of NPCM

The hot solution obtained in the *EU* vessel is filtrated to eliminate residual cell-biomass pellets (*FU*, Figs. 1b, 2) by opening the two valves connecting on the one hand the *EU* and the *FU*, and, on the other hand, the *FU* and the *PU*. The pressure release caused by this opening allows the content of the *EU* to pass

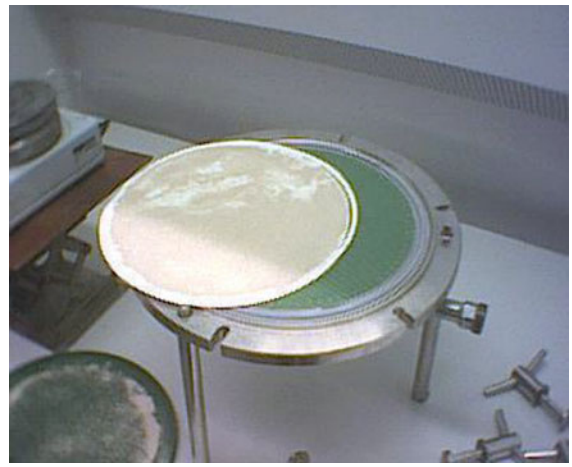

Fig. 2 Internal of the "Filtration unit" *FU* after reassembling. Retained NPCM is visible on the filter paper

through the *FU* (consisting of a paper filter, *Pall Corporation, USA*, 297 mm diameter, and 1 µm pore size). Now, NPCM is retained in the *FU* (Fig. 2), whereas the polymer solution flows into the *PU*.

Separation of PHA

Upon the filtration step is over, the product is precipitated from the solvent simply by cooling down the *PU* to ca. 4 °C. Cooling is performed by putting the *PU* in a vessel containing crushed ice. After precipitation, the biopolyester can be easily removed by the vacuum-assisted deposit of the filter; the filtrate (acetone) can be reused.

Determination of polymer purity

Samples of the isolated biopolyester after drying under vacuum were submitted to cleaving transesterification with methanol under acidic conditions for further GC-analysis as described before elsewhere (Braunegg et al. 1978).

Molar mass determination of polymer samples

Molar mass data were obtained from measurements on a Jasco PU-1580 HPLC connected to Jasco 830-RI detector and equipped with two PLgel 5 µm mixed-C columns. Chloroform was used as solvent at a flow rate of 1.0 ml min^{-1}. Monodisperse polystyrene standards were used for calibration.

Thermal characterization of polymers

Thermal analysis characterization was performed on a Mettler TA 4000 System instrument consisting of DSC-30 Differential Scanning Calorimeter, TGA-50 furnace with M3 microbalance, and TA72 GraphWare software. Measurement protocol: first, second and third heating from −30 to 200 °C at 10 °C min^{-1}; first cooling (quenching after the first heating) from 200 to −30 °C at 100 °C min^{-1} and the second cooling from 200 to −30 °C at 10 °C min^{-1} at 80 ml min^{-1} nitrogen flow.

Results and discussion

The degreasing of PHA-containing biomass by acetone in a Soxhlet extractor leads to co-extraction of a small amount (ca. 1 %) of biopolyester with lipids. The extracted polymer fraction was characterized by rather low molar mass (ca. 200 kDa).

The remaining biomass containing the major part of the biopolyester (>99 %) was separated and extracted according to the methods described in the experimental part. As expected, both chloroform extraction methods resulted in products of high purities (Soxhlet: 99.0 %; batch: 97.7 %) and more or less quantitative extraction yields. The material that was isolated with acetone under pressure and at 120 °C also showed extremely high purity (98.4 %).

Cooling down the polymer solution in acetone below 4 °C did not quantitatively precipitate the polymer. When the precipitated polymer was separated from acetone by filtration, it was possible to precipitate the remaining polyester fraction by drop wise addition of cold water to the filtrate (acetone containing residual dissolved polymer). This polymer (only negligible amounts) was characterized by minor purity (83 %) and lower molar mass.

Regarding the extraction yields, the highest value was obtained by using the Soxhlet extraction with chloroform (98.9 %), followed by the chloroform extraction in batch mode (96.8 %). The yield obtained with the procedure based on the use of acetone under pressure was 96.8 % including also the 5.2 % of the low molar mass polymer fraction that was precipitated with water addition.

Table 1 presents the data for weight average molar mass (M_w) and polydispersity (P_i; P_i corresponds to the ratio of M_w to number average molar mass M_n) of the isolated polyesters as well as melting points (T_m) and values for the onset temperature of decomposition (T_d). In this context it is remarkable that the new acetone method resulted in a product exhibiting the same M_w than obtained with the classical chloroform method (more than 1 MDa; see Table 1). When chloroform extraction under reflux was performed overnight, the values were slightly lower due to the fact that the polyesters were dissolved in hot chloroform for quite a long time (Chodak 2002).

The polymer precipitated from acetone by addition of water showed a significantly lower molar mass (M_w app. 820 kDa; see Table 1). The polyester fraction that was isolated already during the degreasing step (acetone under reflux) was, compared with the other samples, of extremely low molar mass (app. 200 kDa). P_i values are nearby identical for all extraction methods (1.5; see Table 1). Only the low molar mass fraction (isolated during the degreasing step) had a higher polydispersity ($P_i = 2.55$).

Due to the small amounts of the polymer fractions precipitated with water or co-extracted with lipids, determination of melting points was not possible for these samples. DSC analysis of the major polymer fractions always traces two melting endotherms in the melting diagram (see Table 1), indicating that the 3HV and 4HB monomers in the 3HB matrix are not randomly distributed. The extraction method had no significant influence on the melting temperatures (1st melting point around 140 °C, 2nd melting point around 150 °C). These values are indeed promising

Table 1 Collected results for molar mass distribution and thermo analysis of poly-(3-hydroxybutyrate-*co*-21.81 %-3-hydroxyvalerate-*co*-5.14 %-4-hydroxybutyrate) isolated by different methods

Extraction method	Weight average molecular mass (M_w) (kDa)	Polydispersity (P_I)	First melting point (T_{m1}) (°C)	Second melting point (T_{m2}) (°C)	Onset of decomposition (T_d) (°C)	Extraction yield (%)	Purity (%)
Chloroform batch (room temperature)	1,032	1.5	142.8	154.8	236	96.8	97.7
Chloroform Soxhlet (reflux)	987	1.5	139.5	149.5	236	98.9	99.0
Acetone method (under pressure at 120 °C)	1,032	1.5	141.4	152.8	232	91.6	98.4
Residues precipitated from acetone with water	819	1.5	n.d.	n.d.	254	5.2	83.0
Extracted with acetone by Soxhlet	209	2.55	n.d.	n.d.	234	<1	37.2

when compared with literature data for pure homopolyester PHB produced by *Cupriavidus necator* for commercial application (around 180 °C).

T_d was noticed at similar temperatures for each polymer (230–240 °C); only the polyester precipitated from acetone with water showed higher thermostability (254 °C). This means that the isolated products exhibit a broad window of processability, corresponding to the enjoyable big difference between melting points and decomposition points as desired for processing towards marketable products.

In addition to the described experiments for extraction of PHB4HBHV from *Haloferax mediterranei* biomass, the new method was also tested by the authors of this work with different lots of PHA-rich biomass of *C. necator*, containing PHB or poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV). Also in these cases, the application of acetone under high pressure and temperature turned out to be comparable to the use of halogenated solvents in terms of extraction yield, product purity and low impact on M_w (data not shown).

Although the apparatus presented is designed for laboratory scale extraction for screening of novel PHA-solvents at different temperatures, resulting in <20 g of recovered PHA per extraction cycle, similar systems can easily be designed for commercial PHA recovery on a large scale. As a prerequisite, security precautions have to be taken into account considering the removal of oxygen from the overall system; in addition, high-quality materials for vessels and sealing are required.

Conclusions

In contrast to literature reports describing acetone as a "*scl*-PHA anti-solvent" even at elevated temperature up to 115 °C, especially regarding highly crystalline PHB, the herewith performed study demonstrates the successful extraction of *scl*-PHA from lyophilized biomass by acetone using a new technological approach. The system is based on an integrated unit process comprising:

(a) An extraction unit of the biopolyester with acetone under pressure (7 bar) at 120 °C.
(b) A filtration unit set on line for separation of the cell pellet from the solution of biopolyester.

(c) A recovery unit of the biopolyester and exhaust solvent
(d) A distillation unit for acetone recycling which is separated from the former units.

The method incorporates the desired feasible recyclability of the solvent, very limited time demand for extraction, and high product purity without negatively impacting the structural features of the biopolyester. Considering the even higher solubility of *mcl*-PHAs in acetone, the method can be applied for recovery of representatives of the entire PHA family.

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