Efficient P(3HB) extraction from *Burkholderia sacchari* cells using safer, non-chlorinated solvents

Alessandro Rosengart [a,c,+], M. Teresa Cesário [a,·], M. Catarina M.D. de Almeida [a,b] Rodrigo S. Raposo[a], Ana Espert [d], Elena Diaz de Apodaca [e], M. Manuela R. da Fonseca[a]

[a] Bioengineering Department, iBB-Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

[b]CIIEM-Centro de Investigação Interdisciplinar Egas Moniz, ISCSEM, Campus Universitário, Quinta da Granja, 2829–511 Monte de Caparica, Portugal

[c]Department of Industrial Engineering, University of Padova, via Marzolo 9, 35131 Padova, Italy.

[d]AIMPLAS - Technological Institute of Plastics, Gustave Eiffel, 4 - 46980 - Paterna (Valencia), Spain

[e]TECNALIA - Research and Innovation, Health Division, Technological Park of Alava, Leonardo Da Vinci 11-01510 Miñano (Álava) Spain

Abstract

A technique for poly-3-hydroxybutyrate (P(3HB)) extraction with safer, non-chlorinated solvents, was developed, aiming to attain high recovery yields and purities. A wide range of solvents was selected from the GlaxoSmithKline guide as sustainable industrial solvents and the solubility of P(3HB) on those solvents calculated using predictive equations from literature. Anisole,

· Corresponding author: Tel.: +351 21 8419137; Fax: +351 21 8419062.

e-mail address: teresa.cesario@tecnico.ulisboa.pt

+ Current address: Politecnico di Milano, Dipartimento di Chimica Materiali e Ingegneria Chimica "Giulio Natta". Via Mancinelli, 7 - 20131 Milano MI

cyclohexanone and phenetole were used as extraction solvents and the relevant process variables (extraction temperature, extraction time and mass of cells/solvent volume ratio) were optimized. Polymer recovery yields of 97% and 93% were obtained with anisole and cyclohexanone, respectively, at 120-130°C using a cell/solvent ratio of 1.5% (w/v). Maximum polymer purities using these experimental conditions were 98% for both solvents. Recovery yields and polymer purity attained with chloroform (reference solvent) were 97 and 98%, respectively. Higher cell/solvent ratios of 6.0 % (w/v) showed slightly lower recovery yields and purities The average molecular weight and the thermal properties of the polymers extracted with the alternative solvents were comparable to those of the polymers obtained by chloroform extraction, showing that the applied conditions did not significantly alter the properties of the extracted P(3HB).

Keywords

Downstream processing, Cell disruption, Separation, Purification, Poly(3-hydroxybutyrate) recovery, Anisole, Cyclohexanone

1 Introduction

Due to its versatility, plastic is present in almost all human life sectors, generally enhancing the quality of life. However, the durability of most of the oil derived plastics has raised much concern for the environment: the large amount of plastic debris dispersed in the biosphere becomes a troublesome inheritance, with long lasting deleterious impact [1-2]. Biodegradable plastics could overcome the environmental problem. One family of thermoplastic polymers, the

polyhydroxyalkanoates (PHAs), produced by several species of bacteria, depict mechanical and thermal properties comparable to oil-derived plastics [3-7]. However, these bio-polyesters cannot commercially compete yet with petroleum-derived polymers, whose market price is currently lower than 1 €/kg. In the last decades, research has aimed at reducing the production cost of PHA, focusing on both the upstream process and on the downstream. In the first case the goal is to enhance the productivity while choosing low cost fermentation substrates [4, 8], in the second case the goal is to improve the technology of extraction, separation and purification of the produced polymer [9-10]. In particular, the downstream alone is responsible for 60-80% of the total cost [11]. Even though the price of poly-3-hydroxybutyrate lowered from ca. 15 euro/kg in the 90s [12] to 3.7-4.5 euro/kg in 2011 (http://www.icis.com/Articles/2011/02/15/9433445/pha-shows-great-promise-in-packaging-applcation.html), its value is still too high to compete with oil-derived plastics. PHA recovery is in fact a complex and costly issue due to the necessity to break and separate the cellular membranes without damaging the polymer [13]. Membrane rupture cannot be achieved by the classical mechanical methods used in biology and pharmacology (bead mills, high pressure homogenizers) since they are too expensive and difficult to scale up [14]. The most common separation/recovery approaches are mainly two: i) the extraction of P(3HB) with selective solvents and ii) the lysis of cellular membranes with aqueous solutions of NaOH, HClO or surfactants, as the P(3HB) granules are insoluble and can be separated [9, 15].

Solvent extraction grants the best performances in terms of purity and quality of the polymer (chain length). The solvent acts in two ways: it modifies the cell membrane permeability, solving thereafter the released PHAs. The separation of P(3HB) from the solvent is performed by solvent evaporation or by precipitation with the addition of a non-solvent (anti-solvent) to the polymer-solvent solution. Addition of a non-solvent, i.e. a solvent where the polymer shows limited

solubility, alters the solvation potential of the solvent and lowers the solubility of the solute. Examples of non-solvents that have been used for P(3HB) precipitation are hexane and ethanol[16]. The most routinely used P(3HB) solvents in laboratory scale are chlorinated organic compounds, namely chloroform and dichloromethane [10, 17].However, large-scale application of these solvents causes environmental and health concern. Moreover, the process requires high volumes of solvent and anti-solvent for the precipitation [11], increasing the cost of extraction and subsequent solvent recovery. Some efforts have been done towards the selection of green solvents [18-19], although the definition of "green solvents" by itself is not simple and is a subject of study [20]. So far, no good alternatives to chlorinated solvents for P(3HB) extraction have yet been found. One of the reasons for this technological delay could lie in the approach usually followed, based on long and expensive experimental studies of new solvents, focusing more on the "green aspects", and less on efficacy and industrial feasibility.

In this work the approach has been changed, combining several solutions published earlier. The first one is based on the GSK Solvent Selection Guide [21-23] that helps in the adoption of an objective definition of 'green solvent'. The second one is the set of predictive equations developed for the solubility of P(3HB) in different organic solvents [24-25]. These equations allowed the screening of a large number of industrial solvents, leaving experimental analysis only to confirm the performances of few best solvents.

2 Materials and methods

2.1 Microorganism and cell culture

Burkholderia sacchari DSM 17165, a strain able to grow on different types of simple sugars and to accumulate PHAs, was used throughout this work. A fed-batch cultivation was carried out in a 2L stirred tank reactor and used glucose as carbon source. The accumulation of P(3HB) was triggered upon phosphate exhaustion in the cultivation medium. All the details on P(3HB) production have been described in a previous paper [26]. The lyophilized cells used contained 57.7% (w/w) of P(3HB).

2.2 Recovery method with chloroform

An amount of 0.6g of dry lyophilized cells was suspended in a volume of 40mL of chloroform (analytical reagent grade- Fisher Scientific) in a sealed flask with magnetic stirring (36 h, 4 ºC). The final suspension of disrupted cells was vacuum filtered to remove the cell debris, using cellulose filters (Rotilabo Rundfilter typ114A, diameter 90mm 3-5µm retention). The recovered solution of polymer was slowly poured into a 1 L Erlenmeyer containing 160mL (4 parts the volume of the solvent) of previously cooled 96% ethanol at 4°C as anti-solvent, under agitation. The precipitated polymer suspension was then filtered through a pre-weighed cellulose filter and dried to evaporate solvent residues. The P (3HB) extracted using this methodology served as standard to compare the extraction yield, the molecular weight and the thermal properties with their counterparts obtained with the other selected solvents, under various conditions.

2.3 Recovery method with alternative solvents

Anisole (99%), cyclohexanone (99.8%) and phenetole (99%), all purchased from Acros Organics were tested for P(3HB) extraction. A mass of 0.6g of lyophilized cells was suspended in 40 mL of the solvent in a 100mL glass balloon. The sealed balloon was immersed in an oil bath with temperature control and agitation was provided using Teflon covered magnetic stirrer. After a defined period of time, the hot liquid was filtered under vacuum using a pre-weighed cellulose filter (Rotilabo Rundfilter type114A, diameter 90 mm, 3-5µm retention) to remove the cell debris. The P(3HB) solution was poured into 160 mL ethanol at room temperature and the precipitated polymer was separated using a pre-weighed filter, then dried to evaporate solvent residues and weighed to calculate the amount of polymer recovered. This extraction protocol was tested at different temperatures and for different periods of time (one-factor-at-a-time approach) [27]. In an attempt to reduce the relative amount of solvent, the protocol was also performed for increased amounts of initial dry cells (up to 2.4g) in a single extraction batch (40mL). Replicates were carried out for each set of extraction conditions. The Q test (95% confidence level) was used for identification and rejection of outliers.

2.4 Determination of the purity of the recovered polymer

Purity was assessed for each extracted polymer dissolving first the polymer in chloroform at a concentration of 0.6% w/v. Then, 1.0mL of the polymer solution was mixed with 1.0mL of an acidic methanol solution containing the internal standard (97mL of methanol, 3mL of H_2SO_4 95–97% and 0.3g of hexanoic acid). After vortexing for 1min, the preparation was incubated for 5h at 100°C in a Memmert GmbH oven (Model 200). After cooling, the sample was neutralized with the addition of 1.0mL of a 60g/L solution of Na_2CO_3 and then vortexed again for 1min. The preparation was

subsequently centrifuged at 2800g for 5min. The organic phase was analyzed in a gas chromatograph (Agilent Technologies 5890 series II) equipped with a FID detector and a 7683B injector. The capillary column was a HP-5 from Agilent J&W Scientific, 30m in length and 0.32mm internal diameter. The oven, injector and detector temperatures were kept constant at 60°C, 120°C and 150°C, respectively. Data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and Shimadzu GC Solution software (Version 2.3), respectively. Peak identification and calibration curves were obtained using as standard 3-methyl hydroxybutyrate (Sigma). Replicates of the purity assays were carried out and the mean value considered. The Q test (95 % confidence level) was used for identification and rejection of outliers.

2.5 P(3HB) characterization

2.5.1 Molecular weight determination

The average number molecular weight (*Mn*) and the average weight molecular weight (*Mw*) of the extracted polymers were determined by Size Exclusion Chromatography (SEC). The polymer sample (15mg) was dissolved in 3mL of chloroform during 15h at room temperature under agitation in a 15ml glass vial with PE caps. The solutions were then filtered using a Teflon filter with a pore diameter of 0.2µm and introduced in a SEC system (Waters Millennium) composed of three columns assembled in series (PLgel 5µm Guard, Polymer Laboratories, 50 x 7.5mm; PLgel 5 µm 104Å, Polymer Laboratories, 300 x 7.5 mm,; PLgel 5 µm 500Å, Polymer Laboratories, 300 x 7.5mm). Elution was achieved at 30°C, at a flow rate of 1mL/min and using chloroform under degassed helium as mobile phase. The refractive index was used for detection (Waters 2410).

2.5.2 Thermal properties

The thermal properties of the polymer were analysed using Differential Scanning Calorimetry (DSC) (DSC Diamond from Perkin Elmer) and the degradation temperature was determined by Thermogravimetric Analysis (TGA) (TGA Q5000 IR from TA Instruments). The DSC experiments were carried out in nitrogen. Samples were first heated from 20°C to 200°C at a heating rate of 20°C/min and subsequently cooled to 20°C at a cooling rate of 20°C/min. Finally, a second heating took place between 20°C and 200°C, again at 20°C/min. Crystallization temperature (Tc) and melting temperature (Tm) were taken at the peak maximum of the exotherm and endotherm, respectively. TGA experiments were conducted in air. Samples were heated from 50°C to 800°C at a heating rate of 20°C/min.

2.6 Solvent selection and solubility prediction

2.6.1 Solvent selection

The main purpose of this work is to replace standard P(3HB) extraction methods based on toxic chlorinated solvents with “greener” and safer solvents . As the definition of “green” is neither rigorous nor strict, an objective criterion of choice among the universe of industrial solvents was needed. The *Solvent Selection Guide* [21] offers a strong improvement to the green performance assessment of solvents and results from the long-standing experience of GlaxoSmithKline Beecham, the pharmaceutical corporation. The company has created a tool-kit for engineers and chemists to select the best solvents, with the purpose of reducing human health impacts, multiple environment impacts and process safety risks. The solvent evaluation procedure, particularly in the second released version, is based on the risk evaluation reports of each substance and on the modern approaches of Life Cycle Assessment (LCA) and Environmental Foot-print Analysis [22].

These complex assessment techniques grant the highest level of objectivity and completeness, offering a reliable statement over the environmental behavior of the 110 solvents in the guide. The solvents are evaluated according to nine different categories, namely: waste problems, environmental impact, health, flammability and explosion, reactivity and stability, life cycle score, legislation flag (if the chemical has been banned by international agreements) , EHS flag (in case of environmental and health safety issues), boiling point and melting point. A value from 1 (very bad) to 10 (very good) is assigned to the solvents for each category. The solvents with scores lower than 4, receive a red label in the respective category. The "green" solvents are hence the ones with the best performances in the categories "environmental impact", "Waste" and "Life cycle". In the present work, for P(3HB) extraction purposes, it was determined that besides being "green", the solvents must score higher than 5 in the "Health" category to be considered good substitutes of chlorinated solvents.

2.6.2 Predictive model of solubility

The model applied in this work to predict the P(3HB) solubility in the various solvents was published by Jacquel et al. (2007). These authors adapted to the homopolymer P(3HB) the equations based on Hansen's solubility parameters which are widely used in plastic and pigment technology. Hansen developed a refined model of solubility [28], anchored on the definition of three parameters to describe the atomic interactions between solvent and solute: δ_d, called dispersion component; $\delta_{p,}$, related to polar interaction; and δ_h, specific for hydrogen bonding. These parameters allow for a representation of the behavior of two miscible substances and, along the years, they have been estimated for a large number of industrial solvents [29]. The Hansen parameters for P(3HB) were calculated for the first time using a fully predictive group contribution method [24]. Jacquel et al (2007), however, conferred more reliability to the model

by calculating them from experimental data. For the purpose, they used a P(3HB) homopolymer with a *Mw* of 690000 Da at 50°C, obtaining δ_d = 19.3 $MPa^{0.5}$, δ_p = 5.3 $MPa^{0.5}$ and δ_h = 6.3 $MPa^{0.5}$. A new empirical equation for the solubility of P(3HB) was developed as a function of the solvent type, the temperature and the chain length of the polymer [25]:

$$s\ [g/L] = \left[141.7 + 0.454 \cdot \exp(0.063 \cdot T) + 292.8 \cdot \exp\left(-0.210 \cdot 10^{-4} \cdot Mw\right)\right] \cdot \exp(-0692 \cdot r) \quad (1)$$

In this equation, *s* stands for the solubility (g/L); *T* is the temperature (°C); *Mw* is the weight average molecular weight (Da); *r* is the so called solubility radius.

The equation of the solubility radius is reported as equation 2 where *k* and *j* identify the two different species:

$$^{kj}r = \sqrt{\left[4\left(^{k}\delta_d - ^{j}\delta_d\right)^2 + \left(^{k}\delta_p - ^{j}\delta_p\right)^2 + \left(^{k}\delta_h - ^{j}\delta_h\right)^2\right]} \quad (2)$$

The numerical parameters of the equation were calibrated by 175 experimental points of measured solubility, obtained with polymer-solvent systems at temperatures ranging from 30°C to 70°C and *Mw* from 4500 Da to 1300000 Da, using 20 different solvents [25]. The ranges of temperature and chain length suggest the limitations of this method: if these boundaries are crossed, the reliability of the prediction strongly decreases. It should also be noted that the experimental points used to propose the solubility equation were obtained in the low solubility area [25]. Thus, for the current purpose, the solubility values generated by equation (1) were used only as mere indicators to enable a first evaluation of the solvents previously selected using the GSK Solvent selection Guide.

3 Results and discussion

3.1 Solvent selection and solubility prediction

Ten solvents with the highest scores concerning human health impacts, multiple environment impacts and process safety risks were chosen from the GSK list and the P(3HB) solubilities in those solvents and in chloroform and water (references) predicted (Table 1). Some of these solvents namely: ethyl acetate, butyl acetate, isoamyl alcohol [19], propylene carbonate [18] and glycerol [10] have been reported for PHA extraction purposes. Table 1 also includes some recently proposed green solvents such as the biomass-derived ethyl lactate [20].

All the reported values of the Hansen parameters were obtained from Hansen's handbook (2007), except for phenetole [31]. Since none of the solvents selected from the GSK Guide was used for the original calibration of equation (1), this equation has been applied in the current work in a merely predictive mode. However, the distinction between bad solvents (< 1 g/L) and good solvents (> 10g/L) was extremely clear, which conferred confidence to the results.

Based on these results, the solvents further investigated were anisole, phenetole and cyclohexanone.

Table 2 summarizes the physical properties and the hazard statements for these solvents.

Although cyclohexanone is classified as a harmful chemical (if inhaled, swallowed or in contact with eyes) and it is flammable, it scores, to what concerns occupational safety (PEL: 50 ppm TWA-8h and REL: 25 ppm), higher than chloroform (PEL: 50 ppm ceiling limit and REL: 2 ppm). Besides other health hazards, EPA has classified chloroform as a Group B2, probable human carcinogen (http://www.epa.gov/ ttnatw01/hlthef/chlorofo.html), while cyclohexanone is a non-halogenated non-carcinogenic solvent (http://toxnet.nlm.nih.gov/cpdb/chempages/CYCLOHEXANONE.html).

Health risks related to cyclohexanone are evidently minor if compared to chloroform ones, and also flammability is one class lower than ethanol, used as anti-solvent. For an industrial application of these solvents all the necessary safety measures would be required, but these safety issues are, however, common in all the solvent-based industries (e.g. for solvent recovery by distillation) and do not raise any new technical problem.

3.2 P(3HB) extraction

Anisole, cyclohexanone and phenetole were experimentally tested to optimize the extraction process. The assays focused on the extraction temperature, on the duration of the extraction and on the cell mass to solvent volume ratio.

The raw recovery yields, defined as in equation 3 and reported in Table 3, were calculated from the raw mass of polymer recovered in the different assays after mass balance evaluation

$$Y = \frac{m_{rec}}{x \cdot m_{cell}} \times 100 \quad (3)$$

where m_{rec} is the amount of raw P(3HB) recovered after the extraction, x is the polymer mass fraction in the lyophilized cells (0.577, *c.f.* section 2.1) and m_{cell} is the mass of cells being extracted. The recovery yield of the pure polymer was calculated by multiplying the raw recovery yield value by the purity of the polymer. The relative recovery was defined as $Y_i / Y_{CHCl3,}$ where Y_i is the recovery yield using solvent *i* and Y_{CHCl3} is the recovery yield obtained by the standard chloroform extraction at 4°C (95.6%, *c.f.* Table 3).

The extraction of P(3HB) with the proposed green solvents was initially carried out at 60°C for two hours (samples A1, P1 and C1) and at 120°C for one hour (samples A5, P4 and C6) using a cell/solvent ratio of 1.5% (w/v). The three solvents showed very poor extraction performances at

60°C, while the extraction at 120°C substantially enhanced the P(3HB) recovery (Table 3). In fact, after thermolysis of the cell membrane, the granules of P(3HB) are released and solubilized, as described for other methods in the literature [32] Differently, chloroform is an aggressive chemical that does not need heating to cause cellular disruption [12] and this is why the P(3HB) standard extraction method can be performed at low temperatures. Nonetheless, it should be noted that the P(3HB) extraction requires, besides refrigeration at 4°C to preserve chain length [33], at least 36 h of contact time between chloroform and the bacterial cells.

The subsequent experiments at 120-130°C aimed at further reducing the extraction time to 30 and 15 min and to increase the cell/solvent volume ratio to 6% (w/v). Recovery yields remained high with lower contact times and, in the case of anisole (sample A3) and cyclohexanone (sample C2), the attained recovery yield was similar to the extraction using chloroform (the relative recovery yield is 1.0 and 0.98, respectively). In the assays with a higher cell load, the recovery yields using both solvents decreased slightly compared to the standard method with chloroform (0.87 -0.90 relative recovery yield for cyclohexanone (C3 and C5) and anisole (A2 and A4)). These results strongly suggest that both anisole and cyclohexanone offer adequate alternatives to chlorinated solvents. Phenetole proved to be a good extraction solvent at 120-130°C only for a low cell load (1.5% w/v) (Table 3). For higher cell masses, the solution became too viscous and much of the sample was lost in the filter, yielding very low raw recovery yields (39% and 32.2% for 15 and 30 min, respectively). Consequently, phenetole will not be as suitable as cyclohexanone or anisole to replace chloroform in a large-scale plant. Cyclohexanone has already been mentioned in the literature, but it was considered to be a “bad solvent” for P(3HB) [34]. This erroneous statement derives from the use of Terada and Marchessault's equation (1999) for solubility calculations, which is a predictive equation based on group contribution and not on experimental analysis. A

PHA extraction process using cyclohexanone was patented by Metabolix [34]. However, the technique here reported is different from the one described in the patent in that no azeotropes are required between the first and the second solvent extraction.

The purity of the recovered polymers was assessed, as cellular impurities such as proteins [35] [36] and traces of solvent can be present, even though solvent remains were minimized by carefully drying the samples. Table 3 reports the calculated purity of each sample. Replicates were carried out for each extraction and the mean value was calculated (Q test, 95% of confidence). The results indicate that the purity of the polymers recovered with the tested green solvents is, in all assays, of at least 91.2%. The high purities of 98.2 ±1.6% and 98.3 ±0.6% were reached with cyclohexanone and anisole, respectively: these values are comparable to the purity of the polymer extracted with chloroform (98.2 ±2.5 %). The good extraction performances of both anisole and cyclohexanone and the high purity of the extracted polymers make these solvents interesting alternatives to chloroform.

The choice to perform the separation at a temperature of 120°C could raise some questions about the industrial economic feasibility of the process. Even though, a proper quantitative assessment should be carried out by means of process scale-up and simulation, some preliminary qualitative considerations can support the cause of a higher temperature process. The industrial-scale process will reasonably require a solvent-recovery facility, which is typically a distillation unit. Hence, the distillation itself will provide both solvent and anti-solvent at temperatures close to their boiling point, without extra heating costs before extraction. In addition, the boiling point of the solvents is higher (154°C for anisole and 155°C for cyclohexanone) than the temperature range for extraction (120-130°C) proposed in this study. This opens the possibility for the industrial scale

process to be performed even under more severe conditions which would probably allow higher solubilities and/or a lower extraction time. In spite of the higher temperatures to be reached during distillation, the entire process would benefit from the greatly reduced amount of solvents required, which would result in lower energy consumption and a decrease in equipment sizing. Finally, an industrial application of the proposed method will require further optimization of some variables left out for the small scale study, but essential at higher scale. For instance, the possibility of reducing the anti-solvent volume - used in large excess at lab scale - and the possibility of using wet cells instead of lyophilized ones could provide significant economic advantages.

3.3 Extracted polymer characterization

The physical properties of the polymer samples extracted using anisole, cyclohexanone, phenetole and chloroform (as reference) were characterized. The results of the chain length determination by SEC are reported on Table 3. The polymer extracted with chloroform had a molecular weight (Mw) of 5.6 $x10^5$Da and a polydispersity (PI) of 2.3. These values are in the range of the values reported in the literature for P(3HB): $1.0x10^4$ - $3.0x10^6$ and 2.0, respectively [37]. The polydispersity reflects the degree of heterogeneity of the polymer's chain lengths.

The *Mw* (Da) and PI of the polymers extracted with the tested solvents and applied conditions varied in the range 4.5 x 10^5 - 6.8 x 10^5 Da (Mw) and 1.8 -2.3 (PI) for anisole, 4.8 x 10^5 – 5.6 x 10^5 Da (Mw) and 1.9-2.2 (PI) for phenetole and 6.0 x 10^5 - 8.0 x 10^5 Da (Mw) and 1.5-2.4 (PI) for cyclohexanone. These values are comparable (or higher in the case of cyclohexanone) to those of the polymer extracted with chloroform showing that the conditions used in the extraction procedure (temperature and time) did not adversely affect the average polymer chain length. In

the case of cyclohexanone, the molecular weight of the polymer was even higher with an extraction time of 15 min (both using 1.5 and 6.0% (w/v) cells/solvent ratio), confirming the efficiency of the extraction using cyclohexanone under these conditions. A slight decrease of the polymer's molecular weight with increasing contact time (from 15 min to 60 min) is observed specially with cyclohexanone. This tendency has also been reported by other authors and reflects the effect of the length of the thermal treatment applied during the extraction procedure on the average size of the polymer chains [18].

Thermal characterization of the extracted polymers was carried out by DSC, while their degradation temperature was determined by TGA. Table 4 reports the melting and the crystallization temperature (Tm; Tc), the melting and crystallization enthalpy (ΔHm; ΔHc), the crystallinity degree and the degradation temperature of the P(3HB) extracted under different experimental conditions. Concerning the DSC determinations, all values were retrieved from the second heating melting curves. This second heating gives more consistent information about the polymer melting and crystallization behavior than the first heating, since the polymer is melted from a crystallized form which is achieved during the previous controlled cooling. The melting temperature (Tm) and crystallinity degree of the P(3HB) extracted with chloroform, was 171.7°C and 65%, respectively. These values are very similar with the ones reported in literature, namely a melting temperature for P(3HB) of 174°C and a crystallinity degree in the range of 55 to 80% [38] These values of crystallinity indicate that the polymer is a rigid and brittle material, which limits its use in some applications [38].

The average melting and crystallization temperatures of the polymers extracted with the three assayed solvents were similar. In fact the average melting temperature was 171.0°C, 168.8°C and 170.1°C for anisole, phenetole and cyclohexanone, respectively, and the crystallization

temperature 59.7°C, 56.4°C and 61.0°C, respectively. The Tm values are similar to the ones obtained using chloroform (Tm_{CHCl3} = 171.7°C), while the Tc values are slightly higher (Tc_{CHCl3} = 50.8°C). The average melting enthalpy for each solvent is slightly lower compared to ΔHm_{CHCl3} (94.6J/g), suggesting a lower crystallinity degree (Table 4). This value of crystallinity was calculated (Table 4) based on the melting enthalpy of the sample and the melting enthalpy of a 100% crystalline PHB (ΔHm_0 = 146 J/g) [39], and applying a simple rule: % crystallinity = (ΔHm / ΔHm_0) x 100 [39] [40]).

The crystallinity degree of the samples extracted with the tested alternative solvents is in general similar (60-66%) to the crystallinity degree of the polymer extracted with chloroform (65%), except for samples C5 (55%) and C6 (31%). This might be related to the plasticizing effect of solvent residues in these particular polymer samples, due to poor solvent evaporation. According to the literature, a good plasticizer induces a decrease of the thermal parameters Tm and ΔHm as happens with these samples [41]. Choi and Park (2004) claim that plasticization requires a good compatibility between the plasticizer and the polymer and thus similar solubility parameters as already calculated using the Hansen parameters (Table 1).

Crystallinity affects rheological properties of the polymer, especially the melting behaviour of the polymer during processing. In general, polymers with low crystallinity degrees show a wider processing window in terms of melting temperature, whilst polymers with higher crystallization degrees typically show a sharper melting range.

As for the TGA curves, the temperature of degradation (T_{deg}) corresponds to the temperature at the point where the weight loss starts. The T_{deg} values of the polymer extracted with the solvents under study were found to be similar to the one obtained with chloroform (T_{deg} = 278.92°C).

The values in Table 4 are within the range reported in the literature. Namely, the TGA curves determined by Aoyagi et al (2002) show a range of T_{deg} from ca. 230°C to 270°C depending on the heating rate (3, 5, 7, 10°C/min) [42]. Kopinke et al. (1996) report a thermal decomposition of pure PHB samples within a 235-305°C temperature range and show that biomass impurities originate P(3HB) samples with lower thermal stabilities [43]. In the present work, thermogravimetric analyses were conducted under an air flow and not under an inert atmosphere as most works report. The T_{deg} determined in these conditions has, to our knowledge, only been reported once by Villano et al, 2014 for medium-chain PHA [44]. The reason for choosing air flow in the TGA assays was to simulate the conditions during polymer processing at large scale, such as in extrusion or injection moulding, where an open hopper and screw movement permit the presence of oxygen into the barrel where the polymer is melted.

From polymer characterization it is possible to conclude that the P(3HB) from the novel extraction protocols has a quality similar to the chloroform reference, confirming the suitability of the new extraction methods.

4 Conclusions

The goal of this work was to find an effective and environmentally/occupationally friendly solvent for P(3HB) extraction capable of replacing the routinely used chlorinated solvents . Remarkably, at laboratory-scale, results similar to chloroform were obtained with anisole and cyclohexanone. Along with the short time of extraction (15 min only), the unraveled beneficial features of anisole and cyclohexanone confer great potential to these solvents for P(3HB) extraction. Based on the

aforementioned arguments and results, the substitution of the standard halogenated solvents by either anisole or cyclohexanone is thus proposed.

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References

[1] L.K. Blight, A.E. Burger, Occurrence of plastic particles in seabirds from the eastern North Pacific, Marine Pollution Bulletin, 34 (1997) 323-325.
[2] J.G.B. Derraik, The pollution of the marine environment by plastic debris: a review, Marine Pollution Bulletin, 44 (2002) 842-852.
[3] S. Khanna, A.K. Srivastava, Recent advances in microbial polyhydroxyalkanoates, Process Biochemistry, 40 (2005) 607-619.
[4] S.Y. Lee, Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria, Trends in Biotechnology, 14 (1996) 431-438.
[5] S.Y. Lee, Bacterial polyhydroxyalkanoates, Biotechnology and Bioengineering, 49 (1996) 1-14.
[6] M. Zinn, B. Witholt, T. Egli, Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate, Advanced Drug Delivery Reviews, 53 (2001) 5-21.
[7] C. Nawrath, Y. Poirier, C. Somerville, Plant polymers for biodegradable plastics: Cellulose, starch and polyhydroxyalkanoates, Molecular Breeding, 1 (1995) 105-122.
[8] S. Yup Lee, H. Nam Chang, Production of poly(hydroxyalkanoic acid), in: Microbial and Enzymatic Bioproducts, Springer Berlin Heidelberg, 1995, pp. 27-58.
[9] D. Heinrich, M. Madkour, M. Al-Ghamdi, I. Shabbaj, A. Steinbuchel, Large scale extraction of poly(3-hydroxybutyrate) from Ralstonia eutropha H16 using sodium hypochlorite, AMB Express, 2 (2012) 59.
[10] M.H. Madkour, D. Heinrich, M.A. Alghamdi, I.I. Shabbaj, A. Steinbüchel, PHA Recovery from Biomass, Biomacromolecules, 14 (2013) 2963-2972.
[11] N. Jacquel, C.-W. Lo, Y.-H. Wei, H.-S. Wu, S.S. Wang, Isolation and purification of bacterial poly(3-hydroxyalkanoates), Biochemical Engineering Journal, 39 (2008) 15-27.
[12] J.-i. Choi, S.Y. Lee, Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation, Bioprocess Engineering, 17 (1997) 335-342.
[13] B. Kunasundari, K. Sudesh, Isolation and recovery of microbial polyhydroxyalkanoates, Exp Poly Lett, 5 (2011) 620-634.

[14] I.M. Tamer, M. Moo-Young, Y. Chisti, Disruption of *Alcaligenes latus* for Recovery of Poly(β-hydroxybutyric acid): Comparison of High-Pressure Homogenization, Bead Milling, and Chemically Induced Lysis, Industrial & Engineering Chemistry Research, 37 (1998) 1807-1814.
[15] J.A. Ramsay, E. Berger, B.A. Ramsay, C. Chavarie, Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment, Biotechnology Techniques, 4 (1990) 221-226.
[16] J. Baptist, Process for preparing poly-B-hydroxybutyric acid, in: US Patent US, 1962.
[17] J.A. Ramsay, E. Berger, R. Voyer, C. Chavarie, B.A. Ramsay, Extraction of poly-3-hydroxybutyrate using chlorinated solvents, Biotechnology Techniques, 8 (1994) 589-594.
[18] M.L. Fiorese, F. Freitas, J. Pais, A.M. Ramos, G.M.F. de Aragão, M.A.M. Reis, Recovery of polyhydroxybutyrate (PHB) from *Cupriavidus necator* biomass by solvent extraction with 1,2-propylene carbonate, Engineering in Life Sciences, 9 (2009) 454-461.
[19] S.L. Riedel, C.J. Brigham, C.F. Budde, J. Bader, C. Rha, U. Stahl, A.J. Sinskey, Recovery of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from *Ralstonia eutropha* cultures with non-halogenated solvents, Biotechnology and Bioengineering, 110 (2013) 461-470.
[20] P.G. Jessop, Searching for green solvents, Green Chem., 13 (2011) 1391-1398.
[21] A.D. Curzons, D.C. Constable, V.L. Cunningham, Solvent selection guide: a guide to the integration of environmental, health and safety criteria into the selection of solvents, Clean Products and Processes, 1 (1999) 82-90.
[22] R.K. Henderson, C. Jiménez-González, D.J. Constable, S.R. Alston, G.G. Inglis, G. Fisher, J. Sherwood, S.P. Binks, A.D. Curzons, Expanding GSK's solvent selection guide–embedding sustainability into solvent selection starting at medicinal chemistry, Green Chemistry, 13 (2011) 854-862.
[23] C. Jiménez-González, A. Curzons, D.C. Constable, V. Cunningham, Expanding GSK's Solvent Selection Guide—application of life cycle assessment to enhance solvent selections, Clean Technologies and Environmental Policy, 7 (2004) 42-50.
[24] M. Terada, R.H. Marchessault, Determination of solubility parameters for poly(3-hydroxyalkanoates), International Journal of Biological Macromolecules, 25 (1999) 207-215.
[25] N. Jacquel, C.-W. Lo, H.-S. Wu, Y.-H. Wei, S.S. Wang, Solubility of polyhydroxyalkanoates by experiment and thermodynamic correlations, AIChE Journal, 53 (2007) 2704-2714.
[26] M.T. Cesário, R.S. Raposo, M.C. Almeida, F. van Keulen, B.S. Ferreira, M.M.R. da Fonseca, Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates, New Biotechnology, 31 (2014) 104-113.
[27] M. Friedman, L.J. Savage, Planning experiments seeking maxima, in: C. Eisenhart, M. Hastay, W. Wallis (Eds.) Techniques of statistical analysis, McGraw-Hill, New York, 1949, pp. 365-372.
[28] C.M. Hansen, The three-dimensional solubility parameter-key to paint component affinities: solvents, plasticizers, polymers, and resins. II. Dyes, emulsifiers, mutual solubility and compatibility, and pigments. III. Independent cal-culation of the parameter components, Journal of paint technology, 39 (1967) 505-510.
[29] C.M. Hansen, Hansen solubility parameters: a user's handbook, CRC PressI Llc, 2007.
[30] H. Traussnig, E. Kloimstein, H. Kroath, R. Estermann, Extracting agents for poly-d(-)-3-hydroxybutyric acid, in: U.S.Patent U.S. , 1990.
[31] D.L. Williams, K.D. Kuklenz, A Determination of the Hansen Solubility Parameters of Hexanitrostilbene (HNS), Propellants, Explosives, Pyrotechnics, 34 (2009) 452-457.
[32] Jiang X., J.A. Ramsay, B.A. Ramsay, Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440, Journal of Microbiological Methods, 67 (2006) 212-219.
[33] J. Ramsay, E. Berger, R. Voyer, C. Chavarie, B. Ramsay, Extraction of poly-3-hydroxybutyrate using chlorinated solvents, Biotechnology Techniques, 8 (1994) 589-594.
[34] Van Walsem J., E. Anderson, J. Licata, Single solvent polymer extraction methods, in: U.S. Patent and Trademark Office, Washington DC, U.S., 2009.
[35] T.G. Volova, A.N. Boyandin, A.D. Vasiliev, V.A. Karpov, S.V. Prudnikova, O.V. Mishukova, U.A. Boyarskikh, M.L. Filipenko, V.P. Rudnev, B. Bá Xuân, V. Việt Dũng, I.I. Gitelson, Biodegradation of

polyhydroxyalkanoates (PHAs) in tropical coastal waters and identification of PHA-degrading bacteria, Polymer Degradation and Stability, 95 (2010) 2350-2359.
[36] T.U. Gerngross, P. Reilly, J. Stubbe, A.J. Sinskey, O.P. Peoples, Immunocytochemical Analysis of Poly-B-Hydroxybutyrate (PHB) Synthase in *Alcaligenes eutrophus* H16: Localization of the Synthase Enzyme at the Surface of PHB Granules, J. Bacteriology, 175 (1993) 5289-5293.
[37] S. Khanna, A.K. Srivastava, Recent advances in microbial polyhydroxyalkanoates, Process Biochem., 40 (2005) 607–619.
[38] A.J. Anderson, E.A. Dawes, Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates, Microbiological Reviews, 54 (1990) 450-472.
[39] P.J. Barham, A. Keller, E.L. Otun, P.A. Holmes, Crystallization and morphology of a bacterial thermoplastic: poly-3-hydroxybutyrate, J Mater Sci, 19 (1984) 2781-2794.
[40] S. Xu, R. Luo, L. Wu, K. Xu, G.-Q. Chen, Blending and Characterizations of Microbial Poly(3-hydroxybutyrate) with Dendrimers, Journal of Applied Polymer Science, 102 (2006) 3782–3790
[41] J.S. Choi, W.H. Park, Effect of biodegradable plasticizers on thermal and mechanical properties of poly(3-hydroxybutyrate), Polymer Testing 23 (2004) 455–460.
[42] Y. Aoyagi, K.a. Yamashita, Y. Doi, Thermal degradation of poly[(R)-3-hydroxybutyrate], poly[e-caprolactone] and poly[(S)-lactide], Polymer Degradation and Stability 76 (2002) 53-59.
[43] F.-D. Kopinke, M.a. Remmler, K. Mackenzie, Thermal decomposition of biodegradable polyesters -1: Poly (Βετα-hydroxybutyric acid) Polymer Degradation and Stability, 52 (1996) 25-38.
[44] M. Villano, F. Valentino, A. Barbetta, L. Martino, M. Scandola, M. Majone, Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process, New Biotechnology, 31 (2014) 289-296.

Captions to illustrations

Table 1: Hansen parameters and predicted P(3HB) solubility values on the selected solvents from the GSK list

Table 2: Physical-chemical properties and US health exposure limits of the chemicals used in the P(3HB) extraction process

Table 3: Recovery yield, purity and molecular weight (SEC) determination of the polymers extracted with different solvents and experimental conditions. In all assays lyophilized cells containing 57.7 % of P(3HB) were used. *Mn*: number average molecular weight, *Mw*: weight average molecular weight, PI: Polydispersity Index.

Table 4: Thermal properties (DSC and TGA) of P(3HB) samples after extraction: melting temperature (Tm), melting enthalpy (ΔHm), crystallization temperature (Tc), crystallization enthalpy (ΔHc), crystallinity degree (%) and temperature of degradation (Tdeg).