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Using ANOVA models to compare and optimize extraction protocols of P3HBHV from *Cupriavidus necator*

Beatrice Gutt[1]

Karl Kehl[1,2]

Qun Ren[1]*

Luciano F. Boesel[2]*

[1]Laboratory for Biointerfaces, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland

[2]Laboratory for Protection and Physiology, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland

* Corresponding authors

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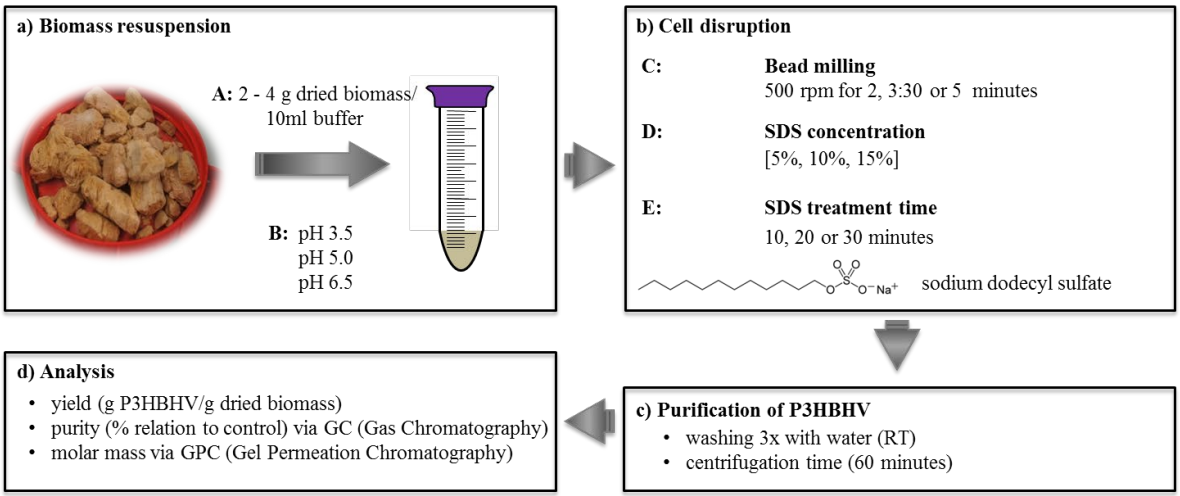
ABSTRACT

Polyhydroxyalkanoates (PHAs), produced by microorganisms, have attracted considerable attention owing to their biodegradability. However, the cost for the production of PHA is still too high to be competitive against petro-based polymers, greatly caused by the expensive downstream processing (DSP). The DSP, moreover, is often ecologically unfriendly as well due to usage of large amounts of highly volatile organic solvents. To overcome these limitations, we systematically compared for the first time 7 different extraction protocols reported previously using the same starting biomass of *Cupriavidus necator*. Design of experiments (DoE) and analysis of variance (ANOVA) models were applied to further improve two of the most promising protocols. Finally, we developed a method where a combination of mechanical disruption of cells via bead milling with an addition of sodium lauryl sulfate (SDS) was used. This method was optimized with a response surface methodology and allowed a fast PHA extraction within approximately 2 hours with a PHA recovery of almost 100% and PHA purity of 94%.



1. INTRODUCTION

Plastic litter has become a serious issue with a negative social and environmental impact. One way to tackle this problem is to use biodegradable polymers such as polyhydroxyalkanoates (PHAs) as an environmentally friendly solution for plastic bags, food packaging and other packaging products.

PHAs are natural polyesters produced by a large number of different bacteria as an intracellular storage compound. PHA is accumulated in the form of granules of approximately 0.2-0.5 µm inside bacterial cells[1]. These PHA granules contain amorphous polymer chains, covered by an outer monolayer of phospholipids and proteins[2]. In order to recover PHA, it is necessary to break bacterial cells and remove the monolayer. Commonly, PHAs are extracted using solvents such as chloroform and dichloromethane[3-6]. These methods result in pure products, for example with rather high purity levels of about 98%[5], but at the same time lead to high amounts of solvent waste byproduct and waste expenses. Additionally, the biodegradable polymer is not competitive to petrochemical based plastics due to high extraction costs. To overcome these limitations alternative extraction protocols have been developed and reviewed[7-8]. Among them, different solvents with low volatile organic compounds (1,2-propylene carbonate, ionic liquids, etc) have been tested and PHA with purity levels of about 84% and yield of 95% were obtained with 1,2-propylene carbonate[9]. Enzymatic and chemical digestions belong as well to the alternatively tested extraction methods. Kathiraser et al. for example combined enzymatic digestion applying alcalase with previous heat treatment (121°C for 1 minute) to release PHA out of cells[10]. They reached purity levels of 93% and nearly 90% PHA recovery with additional crossflow and continuous ultrafiltration[10]. PHA with a purity of about 90% was reached by using the enzymes

bromelain or pancreatin for the extraction of PHA out of *C. necator* biomass[11]. In addition, mechanical disruption (high pressure homogenization, bead milling and sonication) has been discussed for the scaling up and economic extraction of PHA[12-13]. The combination of mechanical disruption (high pressure homogenization) and surfactant treatment (sodium dodecyl sulfate (SDS)) was applied to obtain PHA with rather high purity of 95% and yield of 98%[14].

Although these protocols showed great prospects in obtaining PHAs of adequate quality, it is not clear which method is the most suitable one for scaling up under industrial surroundings with reasonable yield, purity and cost. Moreover, these protocols involved different biomass, feed source and/ or washing procedure, making direct comparison of all these methods practically impossible. Thus, it is necessary to systematically evaluate the reported methods with the same starting biomass under comparable conditions. In this study we compared the seven most promising protocols (as reported in literature) to extract pure P3HBHV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)) out of *C. necator* biomass. The best one which used bead milling in combination with SDS was further optimized by using CCD (central composite design) and analysis of variance (ANOVA) models. This optimized protocol resulted in PHA with 94% purity and 100% yield.

2. EXPERIMENTAL SECTION

All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless otherwise noted.

1 Bacterial growth and biopolymer production.

C. necator (DSM 428) was grown in continuous culture medium supplied with butyric acid (84%) and valeric acid (16%) under dual (carbon, nitrogen) limited growth conditions (C/N = 12.5 g g^{-1}) with a dilution rate of 0.1 h^{-1} according to Durner et al.[15].

2 Extraction protocols

Each purification method was performed in at least two independent experiments. After each method, purified P3HBHV was dried in a vacuum dryer (VTR 5036, Heraeus, Hanau, Germany) for at least 24 h at 30 °C and 30 mbar.

Solvent extraction with dichloromethane. The lyophilized cells were transferred into pure dichloromethane (1 g dried cell biomass (dBM) in 10 mL solvent). Afterwards the suspension was stirred at 55 °C for 120 min, the solution was centrifuged (30 min, 10000 g) and concentrated by distillation at 40 °C and 400 mbar in a rotary evaporator until the solution became viscous. The viscous solution was added dropwise under stirring to a 4-fold quantity of ice-cold methanol and P3HBHV was precipitated[3, 5-6].

Propylene carbonate method. 1 g of dBM was resuspended in 10 ml 1,2-propylene carbonate and stirred at temperatures from 55°C to 120°C for 15 to 60 minutes. After centrifugation for 60 minutes at 10000 g, the supernatant was dropped slowly in ice-cold methanol (ratio 1:4) to precipitate P3HBHV.

Ionic liquids method. 1 g dBM was dispersed in 10 ml 1-butyl-3-methylimidazolium acetate[16]. Cell lysis took place at 85°C while stirring for three hours. After that the samples were centrifuged for 30 minutes at 10000 g. The P3HBHV purification was performed either via

dropping the supernatant in ice-cold methanol (ratio 1:4) or by vortexing the supernatant with ice-cold methanol.

Enzymatic lysis method. The enzymatic cell lysis was tested without further mechanical assistance. 1 g dBM was dispersed in lysozyme solution (2 mg/ml). The dBM-lysozyme suspension was incubated for one hour at 37°C while shaking. Afterwards the samples were centrifuged for 30 minutes at 10000 g. The P3HBHV purification was performed by washing the cell pellet three times with methanol.

Sonication method. Cell disruption via sonication was performed with the Digital Sonifier® Cell Disruptor 450 by Branson with an amplitude of 80% for 2 minutes. The samples were kept on ice during sonication. 1 g of dBM was dispersed in 10 mL buffer prior to sonication (20 mM Tris-HCl (pH 7.5)). The suspension of the biomass was then sonicated. After sonication all samples were centrifuged for 30 minutes at 10000 g. The cell pellet containing P3HBHV was further extracted with dichloromethane or washed with methanol.

Bead milling. Mechanical cell disruption via bead milling was performed with the Planetary Mono Mill Pulverisette 6 from Fritsch. The cells were broken via collision of 300 metallic beads at 500 rpm for two minutes. 1 g of dBM was dispersed in 10 mL buffer (20 mM Tris-HCl (pH 7.5)) and was put into the bead mill beaker where cells were disrupted at 500 rpm for 2 minutes. Samples were centrifuged for 30 minutes at 10000 g and purification was performed via the traditional dichloromethane method or by washing the cell pellets three times with methanol. P3HBHV was precipitated and dried.

Surfactant method. To disrupt cells and purify P3HBHV 2.5% to 15% surfactant solutions were used. The applied surfactants were SDS, IGEPAL® CA-630 (Octylphenoxy poly(ethyleneoxy)ethanol and Trilon® M (MGDA, methylglycinediacetic acid, BASF SE, Ludwigshafen a. R., Germany). 1 g dBM was dispersed in 10 ml surfactant solution and stirred for 3 or 6 hours at 90°C. Alternatively, the cell-SDS solution was stirred for 24 h at room temperature. After that all samples were centrifuged for 30 minutes at 10000 g. P3HBHV purification was performed by washing the pellet three times with methanol or dH_2O.

Combination of methods (mechanical disruption plus surfactant). 1 - 4 g of dBM was dispersed in buffer (after McIlvaine pH 2.6, 3.5, 4.5, 5.5, 6.5 or 7.5) and was then put in the bead mill beaker where cells were disrupted at 500 rpm for 2 to 5 minutes. Afterwards, SDS solution was added to a final concentration of 1.6% to 18.4% and stirred at room temperature (22°C) for 3.2 to 36.8 minutes. All samples were centrifuged for 60 minutes at 10000 g. P3HBHV purification was performed by washing the pellet three times with water.

3 Material Characterization

Determination of the extraction yield. To assess the yield all reaction tubes were pre-weighted before use and after drying of the particular sample. The calculated weight difference shows the resulting yield after purification.

GC (gas chromatography). Approximately 10 mg of purified P3HBHV (or dBM) were used to determine the polymer purity. P3HBHV purified by traditional dichloromethane method[6, 17] was used to obtain standard curves. The samples were prepared according to the method reported previously[6, 17] and analyzed by gas chromatography (GC) (A200s, Trace GC 2000 series, Fisons

Instruments, Rodano, Italy) equipped with a polar fused silica capillary column (Supelcowax-10: length 30 m; inside diameter 0.31 mm; film thickness 0.5 μm; Supelco, Buchs, Switzerland).

DSC (Differential scanning calorimetry). Thermal properties were determined using a Mettler-Toledo DSC822e apparatus. The following 3-step program was applied to all specimens: first heating from -25 °C to 200 °C at 10 °C/min; cooling to -25 °C at a cooling rate of 10 °C/min; second heating to 200 °C at 10 °C/min. STARe software was used for the determination of the glass transition temperature (Tg, defined as the midpoint of the change in heat capacity of the sample), the melting temperature (Tm) and the enthalpy of fusion (ΔHm). The latter were obtained from both the first and the second heating runs while Tg was determined during the cooling run[18].

FTIR-ATR. Fourier-transformed infrared spectroscopy (FTIR) was performed with the Attenuated Total Reflectance (ATR) module (Bio-Rad, USA). Both specimens and background spectra were collected at a 2 cm^{-1} resolution by adding 64 spectra. The IR spectra were obtained on a FT-IR Spectrometer with an ATR unit[17].

TGA (Thermogravimetric analysis). TGA was performed with a TG 209 F1 (Netzsch, Germany) by using the following temperature program: from room temperature to 400°C at a rate of 20°C/min. Both degradation temperature as well as the amount of residual mass were obtained from the curves.

4 Analysis of variance (ANOVA)

For studying the effects of 5 factors on the yield and purity of the combined method (mechanical disintegration and surfactant), we used design of experiments methodology with a sequential experimentation approach. This methodology allows the optimization of processes by performing a reduced number of experiments, therefore not only optimizing the parameters under study, but also the resources needed to achieve the optimum performance[19-20]. We started with a screening step by using a 25-1 fractional factorial, consisting of 5 factors each at two levels (yielding 16 runs). These are depicted in Table 1b. In such kind of design, only half of all possible factor combinations were tested; as it has a resolution of V, meaning that each main effect is aliased with a single 4-factor combination, and each 2-factor combination is aliased with a single 3-factor combination [19]. As we were expecting our system to be driven by (some of) the main effects and eventually some of the two-way interactions (TWI), this design was considered to be the best choice, since each main effect is only aliased with a single 4-factor interaction. We added 3 replicates at the central point to check for curvature effects when using the linear model.

After the screening design, we selected the three factors with the most relevant effects for the next step, a central composite design (CCD), which is the most widely-used experimental design for fitting a second-order response surface to the data[19]. CCDs allow the experimenter to find the optimum location for a process while minimizing the number of experiments to be performed and still allowing a reasonable estimate of the variance to be found. In order to obtain a rotatable CCD (that is, a design where the variance does not depend on the direction from the central point), we choose a value of α equal to 1,682. α defines the axial points, which were added to the design points already obtained during the screening design. Six additional replicates at the central point were performed to make the CCD uniform-precision, that is, where the variance of the response at the origin is equal to the variance of the response at unit distance from it.

The results of both the check for curvature as well as the CCD were analyzed with ANOVA. Data analysis was performed with the "R" program and the packages "R-commander" and "RcmdrPlugin.DoE"[21-23].

3. RESULTS AND DISCUSSION

The objectives of this study were two-fold: 1) to compare and evaluate extraction techniques for P3HBHV in order to identify the most suitable one in terms of purity and yield of the obtained polymer and, 2) to optimize the selected method using ANOVA models to improve purity and yield. Biomass used in this study was derived from *C. necator* grown on butyric (84%) and valeric acid (16%), and contained 41% P3HBHV (w/w) with a monomer weight composition of 69% (w/w) P3HB and 31% (w/w) P3HV.

Comparison of different methods for PHA purification.

Solvent extraction is designated as a widely used standard technique to obtain pure PHA out of dried biomass[3-6, 17]. We compared different extraction methods including the classical solvent treatment with dichloromethane, alternative solvents such as propylene carbonate and ionic liquids, enzymatic lysis, mechanical disintegration (bead milling and sonication), and the use of surfactants.

For the purification of PHA with the aid of the solvent 1,2-propylene carbonate, dried biomass was dispersed and treated with temperatures ranging from 55 to 145°C for 15 to 60 minutes. Best results were obtained by treating the biomass for 30 minutes at 145°C (33 % yield and 82%

purity; Figure 1, technique number 1). To our knowledge only two publications describe the use of propylene carbonate as solvent for PHA extraction[9, 24]. McChalicher et al. showed the use of 120°C for 15 minutes as the most favorable recovery technique for obtaining PHA out of unspecified bacterial biomass with a yield of 75%[24]. Applying these conditions, we reached values for recovered PHA of 57% yield (with a PHA content of 41% in the tested biomass) and only 50% purity. Higher values in yield were found to be caused by the impurities within the extracted PHA. Fiorese et al. treated *C. necator* biomass within a temperature range of 100°C up to 145°C for 15 to 45 minutes and showed the best performance with 130°C for 30 minutes, with a polymer yield of 95% and polymer purity of 84%[9]. Repeating these conditions in our lab, we could only reach 24% yield and 68% purity.

Ionic liquid 1-butyl-3-methylimidazolium acetate was applied for the recovery of PHA. A recovery yield of 33% and a purity of only 1% were obtained (Figure 1, technique number 2). The PHA extraction via ionic liquids is a relatively unknown and was mentioned in an US patent where ionic liquids were deployed for the extraction of biopolymers (chitin, chitosan, elastin, collagen, keratin and PHA) out of all kinds of biomasses[16]. Nevertheless, the insufficient values for purity (1%) disqualified this technique as an option for P3HBHV extraction. Furthermore, it was difficult to handle the samples due to the high viscosity of 1-butyl-3-methylimidazolium acetate and a dark brown sticky product was obtained.

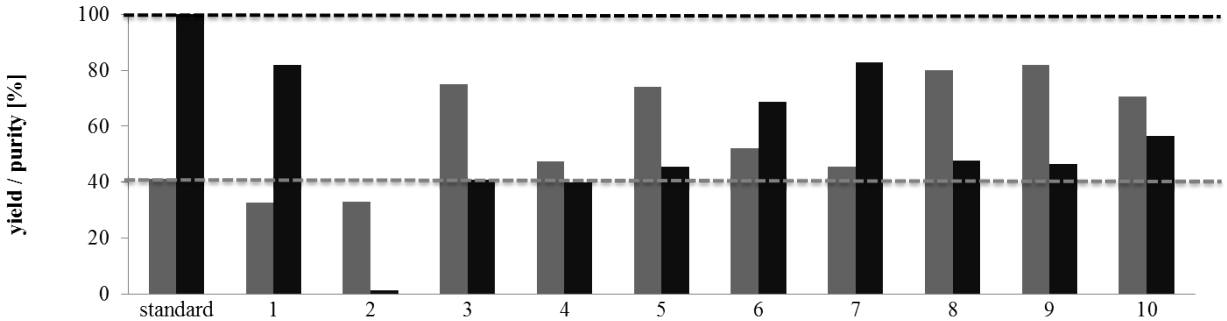
Enzymatic digestion of bacterial cells is a widely used method to break up cell membranes. We tested the use of Lysozyme (2 mg/ml) for one hour at 37°C for cell disruption and the subsequent purification of P3HBHV out of the pellet with methanol. Application of these conditions led to poor purity levels of 41% (Figure 1, technique number 3), which could be due to contamination

of other cell components such as proteins and membrane lipids. Adding dichloromethane to the protocol, which helps to separate P3HBHV from the rest of the biomass, purity levels of around 100% could be reached but a very low yield was obtained with a content of only around 1%. Applying enzymes for the extraction of PHA has been deployed previously and PHA purities of between 89% and 94% were reported[10-11, 25-26]. However, those previously reported processes were performed in combination with other treatments. Kathiraser et al for example described a first heat treatment of the biomass (121°C for 1 minute) before enzymatic treatment with Alcalase[10]. Afterwards SDS, EDTA (ethylenediaminetetraacetic acid) and Lysozyme were added and a final recovery via cross flow ultrafiltration and continuous diafiltration took place[10]. Applying only enzyme treatment without any additional chemical or mechanical treatment in our case is neither effective for purity nor for PHA recovery.

Harsh mechanical disintegration such as sonication and bead milling were tested as well for their ability to obtain high purity P3HBHV. Bacterial biomass was dispersed in 20 mM Tris buffer (pH 7.5) and treated via sonication or bead milling. Separation of pure P3HBHV was performed by washing the broken biomass three times with methanol. The purity levels (40% and 46% for sonication and bead milling, respectively (Figure 1, technique number 4 and 5)) obtained by these techniques were rather low. Combining these cell-disrupting techniques with the use of dichloromethane high purities of around 94% could be reached but with low PHA recovery yield of around 3% (data not shown). In literature these mechanical disintegration techniques were evaluated for their cell disrupting capabilities mostly to recover cell enclosed proteins. Mechanical disruption is favored due to mild damages to the products, an economical advantage in high scale pharmaceutical and biotechnological production[12]. These techniques could also be

suitable for our aim in obtaining pure P3HBV at low economic and environmental costs, if purity could be increased to at least 90%.

Chemical digestion of bacterial cells by detergents like sodium SDS functions by incorporation of the detergent into the lipid bilayer membrane and their disruption[4]. In addition to SDS two other surfactants were included in this study, namely IGEPAL® CA-630 and Trilon® M (Figure 1, techniques number 6 to 10). 1 g of dried biomass was dispersed in 10 ml surfactant solution (final concentration 5%). SDS was additionally tested with a concentration of 2.5% and all suspensions were stirred for 6 or 3 hours at 90°C. None of the alternatively tested detergents reached the purity levels as found with SDS treatment (Figure 1, techniques number 9 and 10). Treatment times of 3 hours at 90°C (Figure 1, technique 8) and of 24 hours at room temperature (yield: 41% and purity: 57%; data not shown) were tested as well. The most promising result was obtained by using 5% SDS for 6 hours at 90°C (purity 83% and yield 46%; Figure 1, technique number 6). Various detergents for the PHA purification were discussed earlier in different publications which described for example purities of P3HB extraction of around 99% by disrupting recombinant *E. coli* biomasses (77% PHA content) with 5% SDS for 1h at 37°C[27]. In addition to SDS various other detergents such as linear alkyl sulfonate (LAS-99) have been applied for 3h at 60°C for the extraction of P3HBHV from *C. necator* biomass with purities of around 88%[28]. From these previous two literature examples, we could see that if applying slightly different conditions such as the reduction of time and temperature (1h at 37°C), use of different bacterial biomasses (recombinant *E. coli*) or different detergents do make differences in the outcome of PHA purity. Therefore, to reduce extraction costs caused by energy consumption, human resources and time, it is necessary to investigate the conditions in detail.



Number	Technique	Biomass resuspension	Cell disruption	Purification agent
standard	dichloromethane	CH_2Cl_2	2 h @ 55°C	meOH
1	1,2-propylene carbonate	$C_4H_6O_3$	30 min @ 145°C	meOH
2	ionic liquids	1-Butyl-3-methylimidazolium actetate	3 h @ 85°C	meOH
3	enzymatic	lysozyme [2mg/ml]	1 h @ 37°C	meOH
4	sonication	buffer	2 min @ amplitude 80%	meOH
5	bead milling	buffer	2 min @ 500rpm	meOH
6	SDS	2.5% SDS solution	6 h @ 90°C	meOH
7	SDS	5% SDS solution	6 h @ 90°C	meOH
8	IGEPAL®	5% IGEPAL solution	6 h @ 90°C	meOH
9	Marlon A350	5% Marlon solution	6 h @ 90°C	meOH
10	Trilon® M	5% TrilonM solution	6 h @ 90°C	meOH

Figure 1. Dried *C. necator* biomass (41% P3HBHV content) was used to compare various PHA extraction protocols. The solvent extraction method using dichloromethane to separate PHA from the biomass is defined as standard. PHA extracted by this technique was considered as 100% pure and used as comparable factor for the GC result evaluation. High values for the yield occur due to the impurities found within certain extracted P3HBHV. The most promising technique is number 7: stirring biomass in 5% SDS solution for 6 hours at 90°C. Purity reaches 83 %.

Combination of methods.

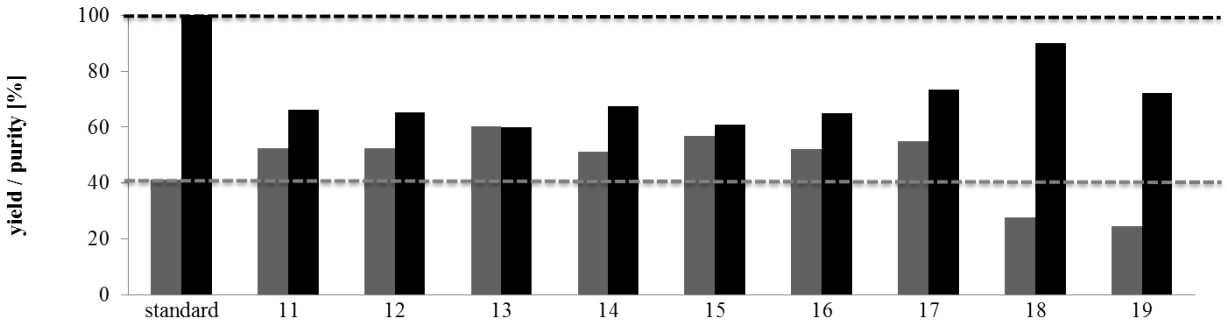
Considering only the quality of the obtained polymer, using the SDS method is the best option for purification of P3HBHV. Nevertheless, it is a time and energy consuming procedure, what would hamper its industrialization. We then decided to combine this method with another one, to improve the efficiency of the process. Cell disruption via mechanical bead milling was chosen as an energy efficient alternative for disrupting the biomass. In a first round dried biomass was

dispersed in 5% SDS solution and transferred to the bead mill which was operated for 2 minutes at 500 rpm (see technique number 5). Adding 5% SDS directly increased the purity from 46% (technique 5) to 66% (Figure 2, technique 11). Unfortunately, operating the bead mill with biomass resuspended in 5% SDS solution led to massive foam production and therefore hindered recovery of biomass suspension out of the bead mill. Yang et al. described a correlation of buffer pH and PHA recovery out of *C. necator* biomass using different detergents, with an optimal pH of 3.77 for the use of 1% LAS-99, a linear alkylebenzene sulfonic acid[28]. They reached a purity of 88% and a yield of 86% with a treatment of the biomass for 3 hours at 60°C. In this study, buffers in a range of pH 2.6 to pH 7.5 were tested to disperse biomass prior to bead milling to further enhance the yield and purity of P3HBHV. After the bead milling step SDS-treatment was initiated. SDS solution was added to a final concentration of 5% and the solution was stirred for 30, 60 and 120 minutes at room temperature (RT, 22°C). Figure 2 (techniques 12 to 17) shows the results of yield and purity of products obtained by the combination of methods with SDS treatment for 30 minutes. The highest purity (74%) was obtained using buffer pH 7.5. However, increasing the treatment time to 120 minutes decreases the purity to 61% (data not shown). Increasing the treatment time to 60 minutes and using with a buffer pH 2.6 increased the purity level to 76%; this value was maintained even if the treatment time was further increased to 120 minutes (data not shown). These preliminary data clearly showed correlations of SDS treatment time and buffer pH, which needed to be further analyzed.

Another factor, which is of concern, is the use of methanol for the final purification procedure. The product obtained by the combination of methods could not be completely dissolved in chloroform. Hence, for further evaluations dH_2O at room temperature and dH_2O heated up to 68°C were used as purification agent instead of methanol (Figure 2, techniques 18 and 19). For

these studies buffer pH 6.5 was chosen because of the most reproducible results in purity (data not shown). Purification was executed via 60 minutes centrifugation steps with dH_2O (RT, 22°C or 68°C). Both washing procedures showed higher purities than that using methanol. The best result was obtained when washing with dH_2O at RT (around 90%), which however decreased the yield (28%).

Combining mechanical disintegration with detergent treatment has been discussed earlier by Ghatnekar et al.[14]. The authors disrupted biomass of *Metylobacterium sp* V49 by high pressure homogenization by using 5% SDS solution to disperse dried biomass. They reach purities of 95% with a yield of 98%. However, the homogenization process (400 kg cm-2) alone takes 90 minutes in two cycles with an operating temperature of 40°C. This means really high energy consumption only for the mechanical disintegration compared to the bead milling procedure described by us, which takes 2 minutes at room temperature (around 22°C).



Number	Technique	Biomass resuspension	Cell disruption	SDS treatment	Purification agent
standard	dichloromethane	CH_2Cl_2	2 hrs @ 55°C		meOH
11	combination	5% SDS	2 mins @ 500rpm		meOH
12	combination	buffer pH 2.6	2 mins @ 500rpm		meOH
13	combination	buffer pH 3.5	2 mins @ 500rpm		meOH
14	combination	buffer pH 4.5	2 mins @ 500rpm		meOH
15	combination	buffer pH 5.5	2 mins @ 500rpm		meOH
16	combination	buffer pH 6.5	2 mins @ 500rpm		meOH
17	combination	buffer pH 7.5	2 mins @ 500rpm		meOH
18	combination	buffer pH 6.5	2 mins @ 500rpm	30 mins	dH_2O @ RT
19	combination	buffer pH 6.5	2 mins @ 500rpm	30 mins	dH_2O @ 68°C

Figure 2: PHBHV extraction by the combination of bead milling and detergent (SDS) treatment was evaluated.

Analysis of variance (ANOVA) for most promising extraction protocols.

Since the discovery of a correlation between detergent treatment time and buffer pH, we decided to use DoE/ANOVA to investigate other factors that could be significantly influencing the outcome of P3HBHV purity and yield. As mentioned in section 2.4, we decided to investigate 5 factors: A = initial weight of biomass (BM), B = pH values of buffers, C = bead milling time, D = concentration of detergent, and E = detergent treatment time (Table 1 and abstract figure).

Table 1. Levels of the 5 factors used for the screening design; factors B, D, and E were used for the CCD with extra levels α. α = 1,682.

Factor/code	Level				
	-α	**-1**	**0**	**1**	**+α**

A = BM (g)		2	3	4	
B = pH	2.48	3.5	5	6.5	7.52
C = milling time (min)		2	3,5	5	
D = [SDS] (%)	1.6	5	10	15	18.4
E = treatment time (min)	3.2	10	20	30	36.8

The results of the screening design (fractional factorial) are shown in Table 1b (Run 1-16). As this was an un-replicated design, we used normal probability plots to investigate the effect of each main factor and two-way interactions (TWI). In such plots, the effects that are negligible are normally distributed (lying along a line passing through the origin), whereas the large (or significant) effects will not lie along the straight line. The plots for both purity and yield are shown in Figure 3a. The x-axis shows the value for the effect of each factor or TWI. The following observations may be made: i) A, B, and to a lesser extent, E have large, possibly significant effects on yield; ii) no significant effects on purity, since the error is very high (given by the spread of the effects along the x-axis); factors B and D, as well as interactions involving it (A:D, B:C) had the largest effects; iii) C is irrelevant for both parameters; iv) none of the TWI had high or significant effects. Given that the effect of A (amount of biomass) was highly negative in the yield, and that our purpose was to increase both parameters, we decided to fix this factor at its lower level for the other tests. C (bead milling time), was discarded since it had no relevant effects. With that, we selected 3 factors that should be optimized: B, D, and E. As we used a simple linear model and un-replicated design to select the important factors, we performed 3 replicates at the central point (runs 17-19 in Table 2) to check for the curvature (lack of fit) of the model. ANOVA was performed on the linear model to determine if the deviations found at the central point were due to lack of fit of the model or could be explained by

it. The results for both parameters are shown in Tables S1 and S2: in both cases, the lack of fit was not significant, demonstrating the accuracy of the linear model for the range studied. Moreover, first order effects were only significant for the yield, corroborating the discussion above based on the normal probability plots. For purity, the high spreading of effects did not allow for any significant factors to be identified.

Table 2. Experiments (16 design points) for the screening design and in boldface experiments (8 design points, 6 axial points, 9 central points) for the CCD design.

Block	Run	A	B	C	D	E=ABCD	Yield [%]	Purity [%]
				Design Points				
1	**1**	**2**	**3.5**	**2**	**5**	**30**	**30**	**65**
1	2	4	3.5	2	5	10	15	55
1	**3**	**2**	**6.5**	**2**	**5**	**10**	**16**	**81**
1	4	4	6.5	2	5	30	13	47
1	**5**	**2**	**3.5**	**5**	**5**	**10**	**23**	**66**
1	6	4	3.5	5	5	30	19	61
1	**7**	**2**	**6.5**	**5**	**5**	**30**	**22**	**74**
1	8	4	6.5	5	5	10	14	72
1	**9**	**2**	**3.5**	**2**	**15**	**10**	**25**	**82**
1	10	4	3.5	2	15	30	19	84
1	**11**	**2**	**6.5**	**2**	**15**	**30**	**25**	**66**
1	12	4	6.5	2	15	10	13	83
1	**13**	**2**	**3.5**	**5**	**15**	**30**	**28**	**58**
1	14	4	3.5	5	15	10	18	71
1	**15**	**2**	**6.5**	**5**	**15**	**10**	**22**	**84**
1	16	4	6.5	5	15	30	16	89
				Axial Points				
2	**20**		**2.5**		**10**	**20**	**25**	**99**
2	**21**		**7.5**		**10**	**20**	**18**	**104**
2	**22**		**5**		**1.6**	**20**	**14**	**83**
2	**23**		**5**		**18.4**	**20**	**26**	**90**
2	**24**		**5**		**10**	**3.2**	**19**	**75**
2	**25**		**5**		**10**	**36.8**	**17**	**83**
				Central points				
1	**17**		**5**		**10**	**20**	**24**	**90**
1	**18**		**5**		**10**	**20**	**19**	**66**
1	**19**		**5**		**10**	**20**	**21**	**60**
2	**26**		**5**		**10**	**20**	**24**	**96**
2	**27**		**5**		**10**	**20**	**27**	**95**

2	**28**	**5**	**10**	**20**	**21**	**83**
2	**29**	**5**	**10**	**20**	**21**	**81**
2	**30**	**5**	**10**	**20**	**16**	**60**
2	**31**	**5**	**10**	**20**	**18**	**86**

We then designed a central composite design to optimize the values of the three selected factors. We added axial points with an α value of 1,682, besides performing additional six replicates at the central point (see also section 2.4). With the 8 design points and 3 central points from the screening design, that yielded a CCD with 23 runs (Table 2). The CCD was divided in two blocks, one for the 11 runs originating from the screening design, and the second block for the 12 new experiments. We used ANOVA to analyze and optimize the results of yield and purity.

We fitted a pure quadratic model to both responses as given in equation (1) below.

$$Y = \hat{\beta}_0 + \hat{\beta}_b B + \sum_{i=1}^{3} \hat{\beta}_i x_i + \sum_{i=1}^{3} \hat{\beta}_{ii} x_i^2 + \sum_{\substack{i=1 \\ i<j}}^{3} \sum_{j=1}^{3} \hat{\beta}_{ij} x_i x_j \qquad (1)$$

Where Y is the response (yield or purity), B is the blocking factor and the xi represent the three studied factors: x1 = B, x2 = D, x3 = E. The β are the coefficients for each term of the model and are given in Tables S3 and S4 (together with their respective errors) for each response.

Table 3 shows the ANOVA results for both models. One important observation is the strong influence of blocking, which had a significant effect on both variables. Blocking was especially relevant for purity, since no other significant effect could be found in the pure quadratic model. Moreover, in both models, if blocking was removed, then the estimates of the coefficients had a lower significance than with blocking (data not shown), corroborating the strong importance of

blocking. The ANOVA table also showed that first order effects are significant, but only on the yield. Importantly, the lack of fit was not significant in either case, showing that the model was adequate to fit the obtained data. We also tried to fit simpler models (first order effects (FO) only, or FO +TWI), but in all cases the lack of fit was higher that with the pure quadratic models (not shown), demonstrating that this model is the most adequate one for our system.

Table 3. ANOVA for the yield and purity, showing the factors with significant effects in boldface.

Term (yield)	df[a)]	SS[b)]	MS[c)]	F value	Pr(>F)
Block.ccd	1	0.00448	0.00448	3.464	**0.087**
FO[d)]	3	0.01663	0.00554	4.285	**0.028**
TWI	3	0.00163	0.00054	0.421	0.741
PQ[e)]	3	0.00104	0.00035	0.267	0.848
Residuals	12	0.01552	0.00129		
Lack of fit	5	0.00666	0.00133	1.052	0.458
Pure error	7	0.00886	0.00127		

Term (purity)	df	SS	MS	F value	Pr(>F)
Block.ccd	1	1176	1176	8.46	**0.013**
FO	3	248	82.7	0.595	0.631
TWI	3	155	51.8	0.372	0.775
PQ	3	554	185	1.33	0.311
Residuals	12	1669	139		
Lack of fit	5	340	68	0.358	0.862
Pure error	7	1329	190		

Our objective was to find the best processing conditions for the extraction of P3HBHV, that is, those giving simultaneously the highest purity and yield. We performed an eingenanalysis on the

data to determine if invariant points exist and, if so, if they represent a maximum, a minimum, or a saddle in the values. The detailed results for the eigenanalysis of both models are shown in the supporting information. For both responses, the analysis indicated the existence of saddle points and no maximum in the curves. Therefore, we were not able to find an exact solution in analytical terms, and decided to investigate the response surface graphically.

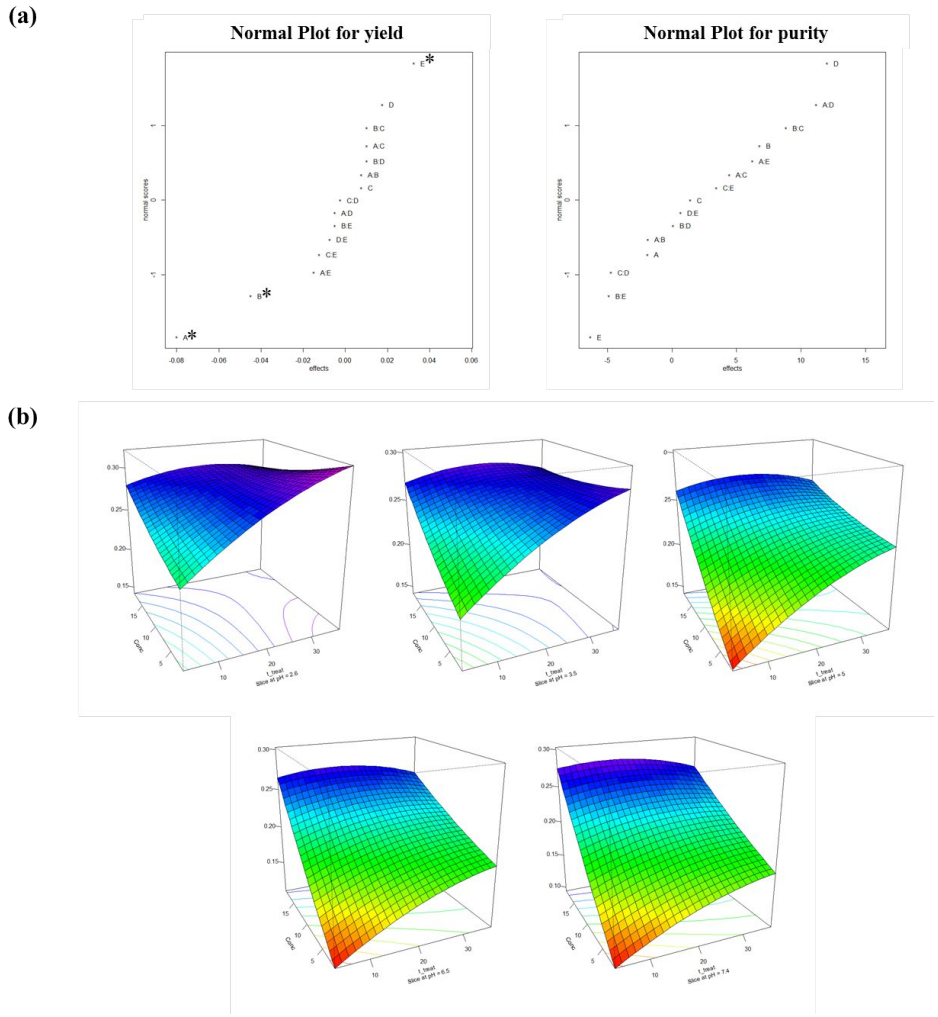
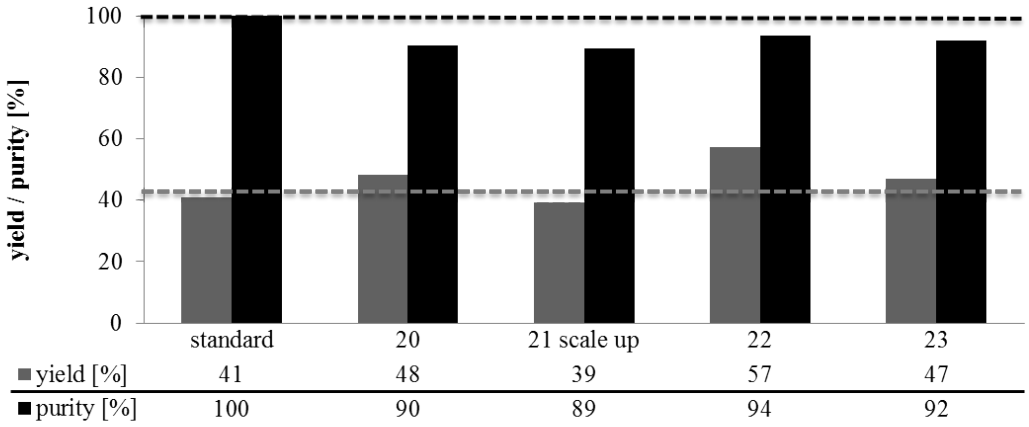


Figure 3. Results of DoE experiments of first and second round (axial points). (a) Evaluation of significant factors ($p < 0.1$) by normal probability plot. Significant factors are marked by asterisks. After evaluation we decided on factors B, D and E for defining the axial points. (b) Response surface plots for the yield, obtained by applying eq. (1) to the results of the CCD

design. Best values would be reached either using high SDS concentrations or a combination of low SDS concentrations, low buffer pH, and high SDS treatment time.

Figure 3b shows the response surface of the model for the yield, with 5 “slices” being shown for different values of pH. The points of maximum response are found in two regions: either at high concentrations (D), or in a combination of low pH (B), low concentration (D), and high treatment times (E). For purity (not shown), the point of optimum was at different conditions: either at high pH, or in a combination of low pH and high concentration. As the optimum points for both responses were not coinciding, and as significant effects were only found for yield, we decided to base our choice on the results from the model for the yield. We chose low SDS concentrations (5%) with low buffer pH (pH 2.6) and high SDS treatment time (30 minutes) as the point with high values in yield while still keeping acceptable values for the purity. With technique number 20 we found the best possible factor combinations with P3HBHV purity of 90% (Figure 4). Even after a scale up and use of a 7.5 times bigger bead mill beaker and therefore 7.5 times higher amount of biomass we reached comparable results in purity (technique 21). The last step of the purification process is the washing, performed in 3 cycles of centrifugation and redispersion. This step is time, human resources and energy intensive, and contributes to the major fraction of the costs of purification. In order to make our process economically viable, we reduced washing steps to 2 cycles and only 1 cycle and found slightly higher values in the outcome of purity (technique 22: 94% and 23: 92%) when comparing to using 3 washing cycles (technique 20: 90%).



	Technique	Biomass resuspension	Cell disruption	SDS [conc]	SDS treatment	Purification agent
standard	dichloromethane	CH_2Cl_2	2 hrs @ 55°C	-	-	meOH
20	combination	buffer pH 2.6	2 mins @ 500rpm	5%	30 mins	3x dH_2O
21 scale up	combination	buffer pH 2.6	2 mins @ 500rpm	5%	30 mins	3x dH_2O
22	combination	buffer pH 2.6	2 mins @ 500rpm	5%	30 mins	2x dH_2O
23	combination	buffer pH 2.6	2 mins @ 500rpm	5%	30 mins	1x dH_2O

Figure 4. Evaluation of the best purification protocols defined by DoE. Results of yield and purity derived by GC measurements are shown.

Material characterization of the obtained polymer.

In order to confirm the suitability of our developed method as an extraction protocol of PHA, we compared the thermal and chemical properties of polymers obtained by the standard method with those obtained by the mechanical disintegration/surfactant method. Figures 5 and 6, and Table 4 summarize these results.

The thermal properties of the alternative materials are very close to those of the polymer extracted by the standard method (Fig. 5 and Table 4). One may observe a decrease of the Tg (both in cooling as well as heating) as well as in the melting temperature (2nd heating cycle). Sample 20, 22 and 23 showed a very weak endothermic peak at about 80 °C during the first heating. This effect could also be observed in sample 23, 2nd heating cycle, although not so

pronounced. We suspect that impurities that were not washed away with only one cycle, or not efficiently removed from the larger batches, were working as plasticizers for the polymer, decreasing its Tg and leading to the crystallization of P3HBHV in a second, less perfect crystal structure. This accounts for the second melting peak at lower temperatures.

Table 4. Material properties of extracted PHA

Technique	Tg (°C), cooling	Tg (°C), 2nd heating	Tm (°C), 1st heating	Tm (°C), 2nd heating	Td (°C)	Residual mass (%)
standard	-4.7	0.35	118	124	291	2.5
Nr. 20	-7.4	-2.2	117	122	292	9.4
Nr. 22	-9	-0.6	119	121	292	6.3
Nr. 23	-8	-1.3	119	121	289	7.3

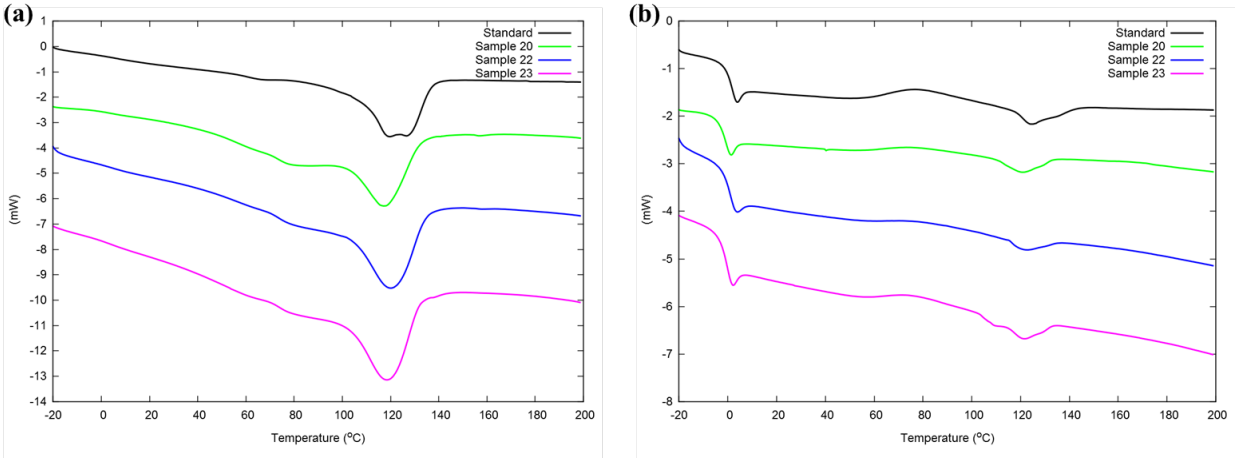


Figure 5: DSC curves for P3HBHV extracted using the standard method or the alternative method. (a) First heating curve; (b) second heating curve.

Regarding the TGA data (Table 4), we observed no change in the degradation temperature of the alternative materials compared to the standard one. Also the impurity level is very close, with about 6 – 9 % impurities in the alternative materials compared to 2 -3 % for the standard one.

This data moreover correlates very well with the impurity values obtained by GC (6 – 10 %, see Fig. 4).

Regarding to the FTIR curves (Figure 6), all materials present the bands characteristic of PHBV[29-30]. The strong band at 1720 cm^{-1} is due to the C=O stretching. Other important bands are the symmetric –C–O–C– stretching vibration (from 800 to 975 cm^{-1}), the antisymmetric–C–O–C– stretching (between 1060 and 1150 cm^{-1}), asymmetrical deformation of the C–H bond in CH2 and CH3 groups (bands at 1380 and 1309 cm^{-1}, respectively), and an aliphatic C–H stretching at 2935 cm^{-1} [29-30].

In general, the spectra of the samples extracted with the modified method are very similar to the one extracted with the standard methods, with all bands present at the correct position and relative intensities. Only two new bands appear in these samples with modified methods, at 1650 and 1540cm^{-1}. These bands corresponds to the amide I (stretching vibration of carbonyl group C=O) and amide II (bending absorption of N-H) bands, respectively. Such bands are usually present in proteins or polysaccharides[31] and indicate that not all cell wall substances were removed from the PHA, even after 3 washing cycles. Coincidently, these samples were also the ones with decreased Tg and Tm in DSC curves, indicating that such residual proteins and/or polysaccharides were acting as plasticizers or nucleation sites for crystals with lower perfection.

Taking into account the results of GC (Figure 4), TGA (Table 4), and FTIR (Figure 6), we may conclude that the majority of the impurities correspond to inorganic material (probably metal particles from the bead mill), which does not burn up to 400°C, with a small percentage of organic material (proteins and polysaccharides from the cell wall). Therefore, the new bands in

the FTIR spectra were of small intensity. Despite these impurities, the overall thermal properties as well as the melt processability of the polymer were maintained.

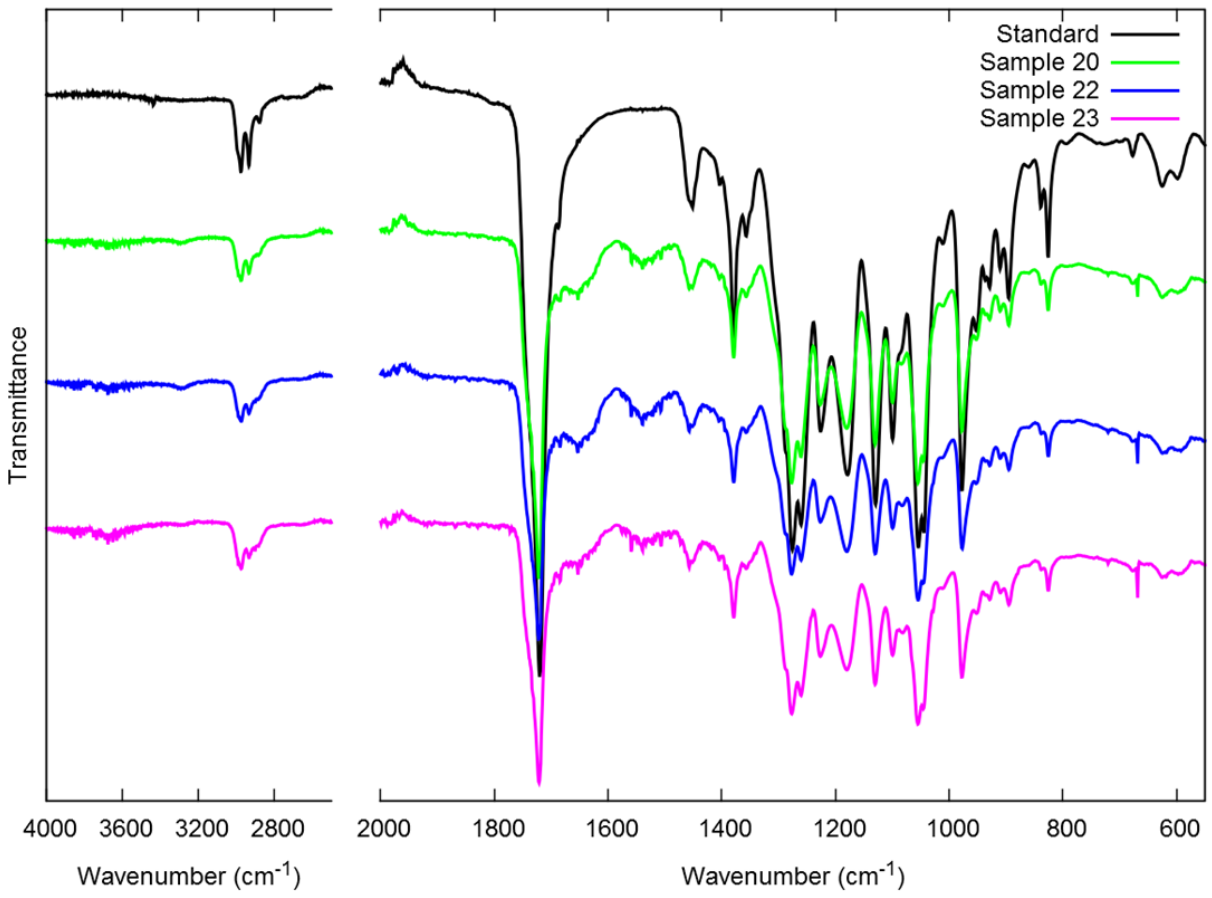


Figure 6: FTIR spectra for P3HBHV extracted using the standard method or the alternative method.

CONCLUSIONS

We developed an extraction and purification process of intracellular PHA which is economic (time and energy saving), fast, and ecologically acceptable. Several individual physical and chemical extraction methods were investigated; however, none of them led to results comparable with the standard chloroform extraction method. A combination of physical and chemical

techniques, namely mechanical disintegration and surfactant treatment, enabled to achieve improved results. DoE / ANOVA proved to be an invaluable tool in optimizing the process conditions, allowing us to obtain purities of up to 94% and PHA recovery of around 100%, higher than with the standard method. The process was also scalable and the obtained polymer had chemical as well as thermal properties similar to the standard one. The methods presented here could in future replace the time consuming and environmentally unfriendly PHA extraction protocol with chlorinated solvents. In future an even larger scale up could be evaluated to make this process industrially viable for the production of biodegradable plastics for packaging applications.

ASSOCIATED CONTENT

Table S1: Curvature check for the yield. No significant effect was found for the lack of fit.

Table S2: Curvature check for the purity. No significant effect was found for the lack of fit.

Table S3: Coefficients (β^_i) for the ANOVA model for yield

Table S4: Coefficients (β^_i) of the ANOVA model for purity.

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AUTHOR INFORMATION

Corresponding Author

* Dr. Luciano F. Boesel, Laboratory for Protection and Physiology, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland].

E-mail: luciano.boesel@empa.ch

* Dr. Qun Ren, Laboratory for Biointerfaces, Empa, Swiss Federal Laboratories for Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland.

E-mail: qun.ren@empa.ch

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ANOVA, analysis of variance; P3HBHV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PHAs, polyhydroxyalkanoates; DSP, downstream processing; DoE, design of experiments; SDS, sodium lauryl sulfate; CCD, central composite design; dBM, dried cell biomass; IGEPAL® CA-630, octylphenoxy poly(ethyleneoxy)ethanol; Trilon® M, MGDA, methylglycinediacetic acid; GC, gas chromatography; GPC, gel permeation chromatography; SEC, size exclusion

chromatography; PS, polystyrene; Mn, molar number; Mw , molar weight; PI, polydispersity index; DSC, differential scanning calorimetry; Tg, glass transition temperature; Tm, melting temperature; ΔHm, enthalpy of fusion; FTIR, fourier-transformed infrared spectroscopy; ATR, attenuated total reflectance; TWI, two-way interaction; P3HB, poly 3-hydroxybutyrate; P3HV, poly 3-hydroxyvalerate; EDTA, ethylenediaminetetraacetic acid; LAS-99, linear alkyl sulfonate 99; BM, biomass; Df, degrees of freedom; SS, sum of squares; MS, mean square; FO, first order effects; PQ, pure quadratic effect; Td, degradation temperature.

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