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Optimization of Green Extraction and Purification of PHA Produced by Mixed Microbial Cultures from Sludge

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Abstract: Sludge from municipal wastewater treatment systems can be used as a source of mixed microbial cultures for the production of polyhydroxyalkanoates (PHA). Stored intracellularly, the PHA is accumulated by some species of bacteria as energy stockpile and can be extracted from the cells by reflux extraction. Dimethyl carbonate was tested as a solvent for the PHA extraction at different extraction times and biomass to solvent ratios, and 1-butanol was tested for purifying the obtained PHA at different purification times and PHA to solvent ratios. Overall, only a very small difference was observed in the different extraction scenarios. An average extraction amount of 30.7 ± 1.6 g of PHA per 100 g of biomass was achieved. After purification with 1-butanol, a visual difference was observed in the PHA between the tested scenarios, although the actual purity of the resulting samples did not present a significant difference. The overall purity increased from 91.2 ± 0.1% to 98.0 ± 0.1%.

Keywords: polyhydroxyalkanoates; PHA; PHBV; mixed microbial culture; green extraction; dimethyl carbonate; purification; 1-butanol; wastewater valorization

1. Introduction

The constant search for environmentally-friendly alternatives for fossil-based materials has been backed up lately by an increase of research in the field of bioplastics such as polyhydroxyalkanoates (PHAs). PHAs are biodegradable polymers synthesized by a variety of bacteria in intracellular granules that serve as energy storage [1,2]. Industrial PHA production has been made feasible by using selected strains of pure microbial cultures to ferment refined substrates [3]. However, a much more sustainable, and perhaps cheaper, option can be found in the use of industrial residue streams as a source of bacterial feed [4–6]. VFAs are organic acids with an aliphatic chain of less than five carbons which can be present in or derived from a large variety of residue streams. VFAs have been shown to be an interesting and very feasible feedstock in the PHA production process by both pure and mixed microbial cultures [7].

Applying a mixed microbial culture (MMC) in the process could furthermore reduce the costs of PHA production, because sterilization of the substrate and reactors is not needed. It has been observed that activated sludge of municipal wastewater treatment plants can be used as a source of MMC with a good PHA-accumulating potential [8,9]. Certain fermentation strategies can be used to explore this accumulating potential and generate a PHA-rich biomass [10–12]. A very useful method is a dynamic fed-batch fermentation with alternating repeated periods of feast and famine, which

can also be combined with an aerobic or anaerobic environment [13–15]. Through application of a pulsed VFA-feeding regime, it is possible to reduce the effects of too extreme pH variations caused by the addition of VFAs to the medium, as well as to favor the maintenance of the PHA-accumulating bacterial population over other non-accumulating species during the famine periods. This method also stimulates the PHA-accumulating bacteria to stockpile the biopolymer intracellularly throughout multiple feast and famine cycles, which highlights this feed-on-demand process amongst other approaches even on an industrial scale [8].

When studying the use of different feedstocks for PHA production, it is also important to understand the relationship between the feed composition and the monomeric proportions of the resulting polymer. When VFAs are the bacterial feed, acetic and propionic acid are the main precursors in a mechanism for the production of the monomers 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in PHA [16–19]. Acetic acid can be converted into 3HB via acetyl-CoA, while acetic acid together with the odd numbered propionic acid are used to form 3HV via the conversion to acetyl-CoA and propionyl-CoA [17,20]. The combined production of 3HB and 3HV as monomers leads to the synthesis of copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Figure 1).

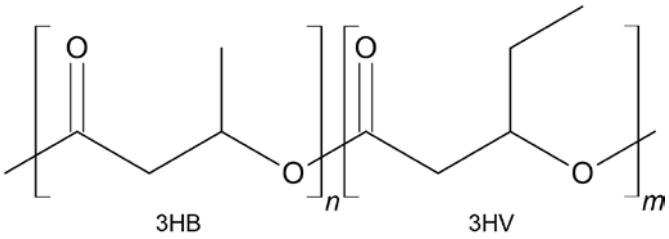

Figure 1. Representation of molecular structure of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Drawn on ACD/ChemSketch Freeware, version 2019.2.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2020).

Extraction is the next step in the production process of PHA, which is done by solubilizing the intracellular PHA followed by separation of the extracted residual biomass and isolation of PHA from the solvent. Reflux and Soxhlet extractions, with and without biomass pretreatments, have been described in literature for many different solvents [21–24]. Non-halogenated solvents have been the focus of many researches for their reduced toxicity, although the chlorinated ones, such as chloroform and dichloromethane, are still considered reference solvents because of their high efficiency [22,25,26]. The non-halogenated solvent dimethyl carbonate performs much better than a range of solvents such as diethyl carbonate, propylene carbonate and ethyl acetate and it achieved satisfactory yields of PHA recovery when compared to dichloromethane [27,28]. Furthermore, dimethyl carbonate (DMC) is considered to be a green solvent for its low toxicity [29] when compared to chloroform and dichloromethane [30]. Therefore, DMC was chosen as the solvent for PHA extraction in the present work. 1-Butanol has also been shown to be efficient as a solvent for PHA extraction [9], with the advantage of leading to a simple separation process through gelation of the polymer when cooling down the mixture [31]. Due to this easiness in separating the solvent from the solid PHA, 1-butanol was chosen as a purification agent in this study.

Total PHA content in biomass and purity evaluation of the obtained polymer can both be done with various simple analytical techniques. Thermal gravimetric analysis (TGA) [32] can be applied for a quick overview of these parameters when focusing on the degradation temperature of the produced PHA. Gas chromatography combined with mass spectrometry (GC-MS) is a more accurate technique that allows the investigation of the monomer concentration, composition and purity of the product [33]. Some studies made a parallel in between these techniques, showing that, even though different PHA content values were obtained by each method, there is a direct correlation between them [34]. For the GC-MS analysis, a pre-treatment step has to be added for the PHA to be able to be analyzed.

In the present work, the aim was to optimize a green extraction and purification of PHA from a mixed microbial culture, using dimethyl carbonate and 1-butanol, respectively. The biomass to solvent ratio or PHA to solvent ratio and the extraction or purification time were the parameters to be optimized.

2. Material and Methods

2.1. Fermentation Process for PHA Production

The fermentation process was adapted from the work of Valentino (2015) and the patent of Werker (2013) [8,12]. The accumulation of PHA was done in two identical 2.5 L bioreactors (Infors™ MINI-2.5-BACT, Bottmingen, Switzerland) set at 25 °C and 200 rpm and aerated with 1.5 L min^{-1} of air. Thickened secondary sludge from the wastewater treatment plant of water board Brabantse Delta in Bath, the Netherlands, was used as a source of PHA-accumulating bacteria. A synthetic feed was made with a solution of acetic and propionic acid in a molar proportion of 3:1, respectively, and a total chemical oxygen demand (COD) of 20 g L^{-1}. This feed composition guarantees that the PHA produced is the co-polymer PHBV in an expected monomer distribution of 50% of each [17]. To start the process, 500 mL of sludge with volatile suspended solids (VSS) concentration of about 8 g L^{-1} was mixed with 500 mL of tap water in the reactor. The mixture was then left for 2 h without any feed under the fermentation conditions in order to stabilize the biomass. A single feed pulse of 10 mL was then given, followed by the first starvation period of 1 hour. By the end of this period, the dissolved oxygen (DO) level was then considered to be the maximum achieved. The feed-on-demand was then automatically controlled by a DO value corresponding to 80% of the maximum as the set point as the condition for the next pulse feed. The feed pulses corresponded to a volume of 10 mL given over 1 min every time the mentioned condition was met. The whole process was set to a total of 22 h. The process was automatically stopped by dosing hydrochloric acid until a pH of at least 2 was reached in order to cease the bacterial metabolism. At that point, stirring and air inlet were shut down.

2.2. Homogenization of Biomass

The mixture inside the bioreactors was left for a few minutes to settle and the PHA-rich biomass was centrifuged (5810 series, Eppendorf™, Nijmegen, The Netherlands) and washed twice with tap water. The biomass was then freeze-dried (CHRIST Alpha 1-4 LD Plus) overnight. The dried biomass resulted from 20 runs (10 in each bioreactor) was mixed and made homogeneous using a mortar and pestle.

2.3. Extraction Process

The PHA was extracted from the biomass via reflux by adding 25 mL of dimethyl carbonate (DMC) as a solvent to different amounts of biomass in a round-bottom flask connected to a cooling column. Different biomass to solvent ratios, 0.01 g mL^{-1}, 0.025 g mL^{-1}, 0.05 g mL^{-1}, and 0.1 g mL^{-1}, were tested in duplicate by adjusting the amount of biomass. These biomass to solvent ratios will be further referred as 1%, 2.5%, 5%, and 10%, respectively. The round-bottom flask was immersed in a pan filled with glycerin, previously heated up to the boiling point of the solvent (90 °C for the DMC). Different times of extraction were tested (0.5 h, 1 h, 1.5 h, and 2 h). After the extraction, the pan was removed to allow the flask to naturally cool down to room temperature. A vacuum filtration with Whatman™ paper filters was then used to separate the biomass from the solution. A rotary evaporator (Hei-VAP Value, Heidolph^{TM}, Schwabach, Germany) was used to recover the solvent and to separate the PHA in the form of a film attached to the wall of the flask. The obtained PHA was left to dry overnight and then weighed. The experimental data obtained under the different extraction conditions was compared using first an F-test for variances and then the adequate t-test for the p values. Chloroform and dichloromethane were used as reference extraction solvents [26–28], for which a biomass to solvent ratio of 1% and an extraction time of 1 h were used.

2.4. Purification Process

The extracted PHA was purified with 1-butanol (≥99%, Sigma-Aldrich™, Zwijndrecht, The Netherlands) via reflux. 20 mL of the solvent was added to different amounts of the polymer in a round-bottom flask which was connected to a cooling column. The different PHA to solvent ratios tested in duplicate were 0.01 g mL^{-1}, 0.02 g mL^{-1}, and 0.04 g mL^{-1} (1, 2 and 4%). The flask was immersed in a pan with glycerin and heated up to the boiling point of 1-butanol (117.7 °C). The purification times tested were 0.5 h, 1 h, and 2 h. The round-bottom flask was then taken from the pan, closed with a cap and allowed to cool down at room temperature overnight. The solvent was then separated from the jelly-like polymer by physically pressing it out [31] with a piece of cloth. The PHA was allowed to dry at room temperature overnight. The 1-butanol was separated from the solubilized contaminants and recycled with a rotary evaporator (Hei-VAP Value, Heidolph™, Schwabach, Germany). The experimental data obtained under the different purification conditions was compared using first an F-test for variances and then the adequate t-test for the p values.

2.5. Analytical Methods

2.5.1. Thermal Gravimetric Analysis (TGA)

Samples of produced biomass were analyzed in duplicate for PHA content and every sample of extracted and purified PHA was analyzed for purity with TGA (TGA 500Q, TA instruments, Etten-Leur, The Netherlands). Because of the feed composition, all PHA produced was considered to be the co-polymer PHBV. A mass of around 10 mg was placed into platinum pans and a ramp mode of 5 °C per minute until 600 °C was set under nitrogen atmosphere, with flow rates of 40 mL min^{-1} on the balance, 60 mL min^{-1} on the sample (adapted from Hahn and Chang, 1995) [34]. The mass of PHA in each sample was determined as the mass loss in the temperature range between 250 °C and 270 °C. A commercial sample of PHBV was used to establish this range.

2.5.2. Gas Chromatography/Mass Spectrometry (GC-MS)

Sample Preparation

Following the procedure in Lo et al. (2009) [33], an acidic methanolysis reaction was used to hydrolyze the polymeric chain and to convert the monomers into their methylated ester form. A PHA sample of each experiment was weighed for a mass between 2 and 5 mg and brought into a 5 mL reaction vial. 1 mL of chloroform (99%, Sigma-Aldrich™, Zwijndrecht, The Netherlands), 0.95 mL of methanol (Technical grade, BOOM™, Meppel, The Netherlands) and 0.05 mL of sulfuric acid (95%–98%, Sigma-Aldrich™, Zwijndrecht, The Netherlands) were added into the reaction vial which was closed tightly and shaken. The reaction vial was placed into a heating block (Techne Dri-block™, Staffordshire, England, UK) at 100 °C for 6 h and shaken every 1 h. The vial was then left to cool down to room temperature. The solution was transferred from the vial to a centrifuge tube and 1 mL of 1 M NaCl (Extra pure, BOOM™, Meppel, The Netherlands) solution was added. The tube was shaken and centrifuged (5810 series, Eppendorf™, Nijmegen, The Netherlands) for 3 minutes at 4000 rpm. The aqueous layer was discarded and another 1 mL of the NaCl solution was added. The tube was shaken and centrifuged again. The organic phase was then taken from the tube with a needle attached to a 1 mL syringe. The solution volume was measured with the syringe graduation, filtered (0.45 µm × 13 mm PTFE filter) and transferred to a vial.

GC-MS Settings

A calibration curve was made using standards of methyl-(R)-3-hydroxybutyrate and methyl-(R)-3-hydroxyvalerate (>98%, Sigma-Aldrich™, Zwijndrecht, The Netherlands) using chloroform as a solvent to achieve different dilutions. The GC-MS used was a 7820A GC System/5977E MSD Agilent Technologies™ with a HP-5MS capillary GC column (30 m × 0.25 mm, 0.25 µm), flow of

2 mL min^{-1} helium, sample injection of 1 µL, temperature of 250 °C at the injector and detector with a heating rate of 10 °C min^{-1}. The software NIST MS Search Program was used to identify the samples components through their mass spectrum.

3. Results and Discussion

3.1. PHA Accumulation

Although there were slight variations throughout the runs, the fermentation process was consistent, with an initial volatile suspended solids concentration of about 4 g L^{-1}. A range of 23 to 25 pulse feeds were given during each of the 20 runs. Each fermentation run resulted in around 5.5 g of dry biomass. It was observed that the biomass easily settled to the bottom of the vessel.

The TGA analysis of the dry biomass revealed a content of around 40% of PHA in mass, represented by a degradation peak at the temperature range of 265 to 277 °C, which was proved to be the right degradation temperature by comparing the analysis with a commercial sample of PHBV. Hahn and Chang (1995) [34] discovered a correlation between the PHA content measured through TGA and the PHA content measure through GC analysis, where the result from GC analysis are considered to be more accurate. This correlation is expressed as a linear model:

$$y = 1.16x - 15.27 \quad (1)$$

where x is the PHA content by TGA and y the real content. Using this correlation, the total PHA content is around 32%. This result is slightly lower, but still close, to the ones mentioned in the PHARIO report [9], for which the same MMC source was used and the PHA accumulation resulted in values around 39 g of PHA per 100 g of VSS. A difference, however, that might explain the higher production result in that work is the nitrogen and phosphorous supplement in the feed composition and the extra feeds during acclimation process of the biomass.

3.2. Extraction

The results of PHA extraction in all studied conditions were around 31 g of PHA per 100 g of biomass, with slightly higher extraction values at lower biomass to solvent ratios and longer extraction times (Table 1). This result indicates a very high polymer recovery, which contrasts with what is discussed in Samorì (2015) [27], where only about half of all the PHA inside the MMC biomass could be extracted with dimethyl carbonate without any cell pretreatment. It is important to mention, however, that the MMC used in that work for PHA accumulation has a different source and it was submitted to an extensive process of bacterial selection over time, which might affect the general composition of the biomass and, perhaps, the efficiency of the DMC as a solvent for PHA extraction.

Table 1. Extraction results of PHA with dimethyl carbonate. Freeze dried biomass after polyhydroxyalkanoates (PHA) accumulation was extracted in 25 mL dimethyl carbonate at its boiling point at different biomass to solvent ratios for different extraction times. The biomass to solvent ratio is expressed as a percentage of grams of biomass per 100 ml of solvent. Values represent averages ± standard deviation of duplicates in g of PHA per 100 g of biomass.

Biomass to Solvent Ratio (%)	0.25 h	0.5 h	1 h	1.5 h	2 h
1%	31.4 ± 1.0^{abc}	32.2 ± 0.1^{b}	31.7 ± 0.2^{bd}	32.9 ± 0.2^{b}	
2.5%	30.6 ± 0.4^{cd}	30.4 ± 0.3^{cd}	31.5 ± 0.1^{ad}	32.3 ± 0.4^{bd}	
5%		30.5 ± 0.1^{c}	30.0 ± 1.0^{abc}	30.8 ± 0.7^{abc}	30.4 ± 1.3^{abc}
10%			29.3 ± 1.1^{abc}	27.8 ± 0.4^{a}	28.2 ± 0.5^{ac}

^{abcd} Average values not sharing a common superscript were significantly different ($p < 0.05$).

The scenarios with a 10% biomass to solvent ratio presented some practical issues because of the relatively high amount of biomass that settled in the bottom part of the extraction flask in direct contact with the heating source and with low or no contact to the solvent.

The PHA extraction process with DMC was compared with chloroform [23] and dichloromethane [28] as the reference solvents (Table 2).

Table 2. Comparative extraction results for the different solvents. The reflux extraction of PHA with dimethyl carbonate at 1% biomass to solvent ratio for 1 h was compared with the reflux extraction with chloroform and with dichloromethane at the same solvent ratio and time. Values represent averages ± standard deviation of duplicates of g of PHA per 100 g of biomass.

Solvent	g PHA/100 g Biomass
Dimethyl carbonate	31.7 ± 0.2
Chloroform	37.5 ± 0.2
Dichloromethane	39.0 ± 0.2

Although the amount of extracted PHA seems higher with chloroform or dichloromethane compared with dimethyl carbonate as a solvent, when manually stretched, the polymer films produced with chloroform and dichloromethane were both very brittle and not much elastic. They would immediately break apart when pulled. The brittleness was caused by a higher percentage of impurities in the PHA extracted with chloroform and dichloromethane, as it is further discussed in the results obtained with TGA. The PHA plastic films produced in the process with dimethyl carbonate, on the other hand, had a much higher plastic deformation capability, similar to a common strong plastic bag. However, regardless of the solvent used, the resulting solid PHA had a green/brown color after the solvent recovery in all the produced samples (Figure 2).

Figure 2. A sample of PHA film produced directly after the solvent recovery. The dark green color was common to all of the samples produced.

A TGA of PHA samples obtained directly by extraction with dimethyl carbonate reveals a purity of 91.2 ± 0.1% versus a purity of 82.5 ± 3.3% for the extraction with chloroform and of 86.4% ± 3.7% with dichloromethane. These results could explain the higher yields obtained for the extraction process with chloroform and dichloromethane meaning that these reference solvents are solubilizing not only the PHA, but also higher amounts of other compounds present in the biomass, which results in lower overall purity in these samples.

3.3. Purification

Purification of the extracted PHA with 1-butanol revealed that the whitest product was obtained with a PHA to solvent ratio of 1% and after 0.5 h of purification time (Figure 3).

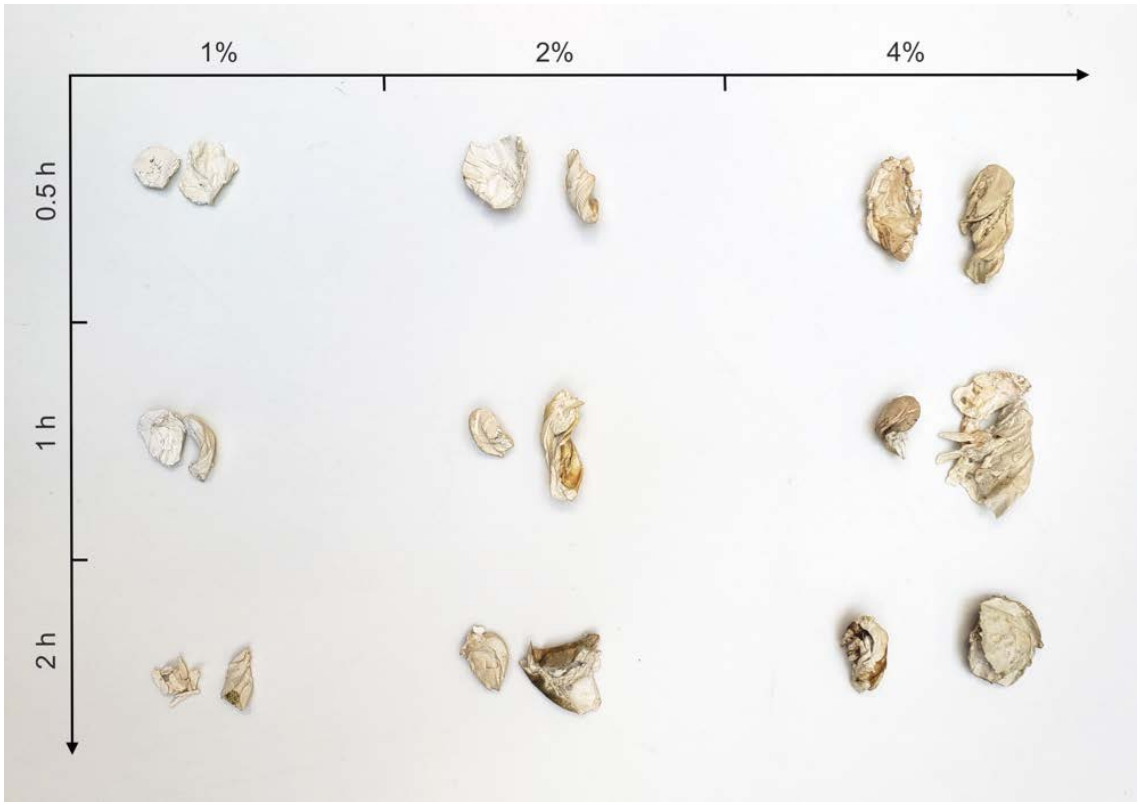

Figure 3. Comparison between the purified PHA at different experimental points.

It was expected that a lower PHA to solvent ratio led to PHA with less impurities. However, a longer purification time led to a darker-colored product, although not much difference was registered in the actual purity of the samples (Table 3).

Table 3. Purity results by TGA of the samples submitted to the purification process by 1-butanol. The PHA to solvent ratio is written as in grams of PHA per 100 ml of solvent Values represent averages ± standard deviation of triplicates of percentage of purity of purified PHA.

PHA to Solvent Ratio (%)	0.5 h	1 h	2 h
1%	98.3 ± 0.1^{acd}	98.5 ± 0.1^{a}	98.2 ± 0.2^{acd}
2%	98.4 ± 0.1^{bc}	98.9 ± 0.1^{ac}	97.9 ± 0.4^{acd}
4%	98.3 ± 0.3^{acd}	98.2 ± 0.1^{bd}	98.4 ± 0.3^{acd}

^{abcd} Average values not sharing a common superscript were significantly different ($p < 0.05$).

The evaluation of PHA by TGA revealed an increase in purity from 91.2 ± 0.1% to 98.0 ± 0.1% after purification with 1-butanol (Figure 4). The peak degradation temperature of the PHA was identified to be 253.4 ± 7.3 °C which is comparable to literature about different monomer compositions of the PHBV copolymer [35–37].

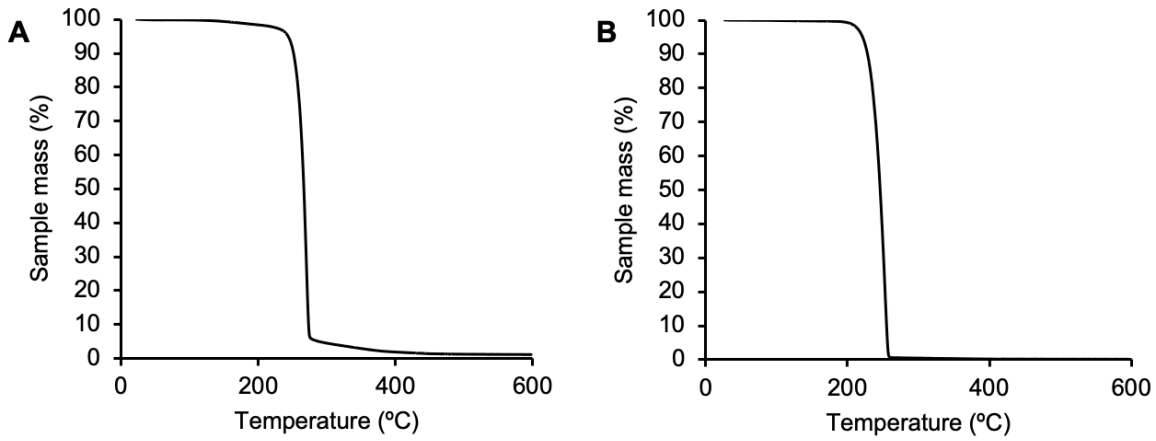

Figure 4. Thermal gravimetric analysis (TGA) of extracted PHA before (**A**) and after (**B**) purification with 1-butanol.

3.4. Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

Samples of the extracted PHA before and after purification were analyzed with GC-MS for its monomeric composition and identification of impurities (Figure 5). Besides 3-hydroxybutyrate and 3-hydroxyvalerate, the monomer 3-hydroxy-2-methylvalerate was also present in minor quantities in the samples. This monomer has been reported already as a common component of polymers synthesized by enriched cultures of glycogen-accumulating organisms (GAO) [35,38,39].

The GC-MS analysis revealed a monomer composition of 35.6 ± 2.5% 3-hydroxybutyrate and 64.4 ± 2.5% 3-hydroxyvalerate. Given the feed composition, a monomer distribution of the produced PHA of 50% 3-hydroxybutyrate and 50% 3-hydroxyvalerate was expected [17]. However, less energy is needed to metabolize propionic acid than acetic acid [40], which explains the higher percentage of 3-hydroxyvalerate in the PHA.

The non-purified PHA (Figure 5A) contained a bigger variety of impurities than the purified PHA (Figure 5B). Not much can be said about the absolute concentration of impurities before and after the purification process, as no calibration curves were made for the non-PHA related compounds. However, a reduction of 71.4%, 71.6%, and 63.7% in the areas of the impurities III, IV and V, respectively, was calculated, indicating a significant reduction in the overall concentration of such impurities.

Although the quantity of impurities was reduced after the 1-butanol treatments, hexadecanoic acid, octadecanoic acid and dehydroabietic acid were still found in all purified samples. Hexadecanoic and octadecanoic acids have been reported as storage compounds produced by mixed bacterial cultures [41]. The source of the dehydroabietic acid is unknown.

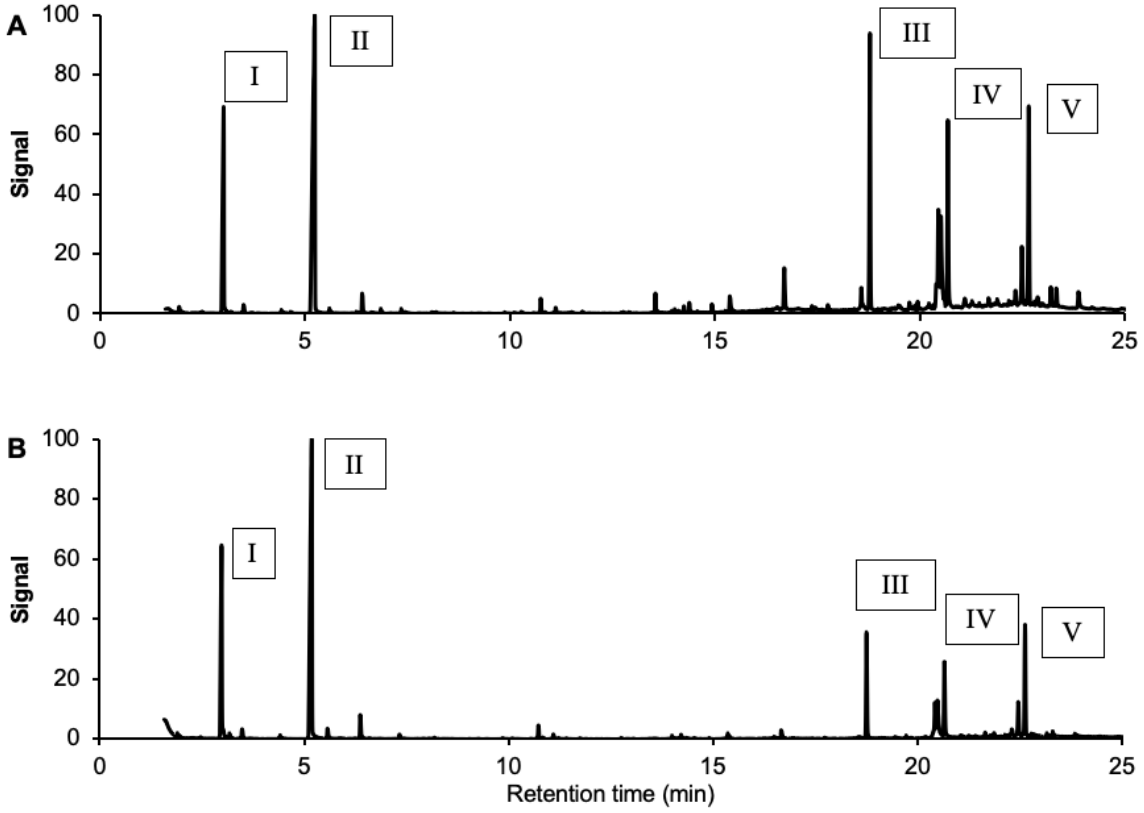

Figure 5. Chromatograms of PHA before (**A**) and after (**B**) purification. The identified compounds are: I—3-hydroxybutyric acid; II—3-hydroxyvaleric acid; III—Hexadecanoic acid; IV—Octadecanoic acid; V—Dehydroabietic acid.

3.5. Applicability

In this work, a mixed microbial culture was used for a PHA accumulation procedure followed by an extraction and purification with different solvents for obtaining a high purity final product. However, the extraction and purification are a two-step process that can be very costly when it comes to an industrial setting. For certain PHA applications where high purity is not a major factor, a single extraction with DMC could be enough for the commercial feasibility of the process. For applications where high purity PHA is required, the purification step can be added, although higher costs should be expected.

4. Conclusions

The extraction of PHA from mixed microbial cultures can be successfully done with dimethyl carbonate via reflux extraction. Overall, a very small variance of PHA yield was observed for different extraction times or biomass to solvent ratios. A ratio of 0.05 g ml^{-1} is considered to be ideal, as the use of higher amounts of biomass lead to practical difficulties. Although higher extraction yields can be obtained with chloroform or dichloromethane as solvents, that also leads to a decrease in the purity of PHA and a less sustainable extraction.

A purification of the extracted PHA with 1-butanol resulted in an increase in purity from 91.2 ± 0.1% to 98.0 ± 0.1%. Although the total purity is approximately the same for different purification times and PHA to solvent ratios, a 0.01 g mL^{-1} ratio for 0.5 h of purification time led to a whiter PHA.

Dimethyl carbonate is a great alternative to conventional hazardous solvents in the extraction process of PHA and 1-butanol can be used to increase the purity of PHA if necessary, leading to a more commercially attractive product, although this could lead to higher production costs. In terms

of a circular economy, the whole process opens new environmentally friendly possibilities for the bioplastic industry.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
COD	Chemical oxygen demand
DMC	Dimethyl carbonate
DO	Dissolved oxygen
GC-MS	Gas chromatography—mass spectrometry
MMC	Mixed Microbial Culture
PHAs	Polyhydroxyalkanoates
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
TGA	Thermal gravimetric analysis
VFAs	Volatile fatty acids
VSS	Volatile suspended solids

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