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An environmentally sustainable extraction protocol for polyhydroxyalkanoates from mixed culture biomass

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ABSTRACT

Polyhydroxyalkanoates (PHA) have recently been established as one of the main biopolymer alternatives to conventional petroleum-based plastic materials. Still, despite being easily produced, PHA extraction from sewage sludge has yet to be improved. The lack of an environmentally friendly and efficient extraction protocol is one of the main challenges hampering the spread of the process. In this context, this study presents promising results for a new extraction procedure of PHA using bio-based organic solvents for non-PHA cell mass (NPCM). PHA-rich sewage biomass was produced by adopting aerobic dynamic feeding as an enrichment strategy and controlled pulsate-feeding, automatically performed by homemade software adopting synthetic wastewater and real fermented sludge liquid. Extraction results highlighted the high efficiency of the bio-based solvents such as 2-methyl tetrahydrofuran (2-MeTHF), glycerol, glycerol formal and ethyl lactate. Especially, the adoption of 2-MeTHF resulted in higher PHA recovery yield (78.3 ± 11.9 %) and similar purity (93.2 ± 2.4 %) than chloroform (71.6 ± 14.5 % and 96.1 ± 1.3 %, respectively), known to be one of the best solvents to extract PHA. Furthermore, contrary to all the other solvents tested, 2-MeTHF showed good efficiency even without pretreating the biomass sample with NaClO, allowing a one-step extraction process that reduces the process cost. Around 96 % of 2-MeTHF was successfully recovered at the end of the extraction, showing similar purity compared to the commercial one. PHA thermal analysis showed that the polymer obtained had properties in line with commercial polymers. The overall protocol indicates a promising and consistent environmentally friendly procedure to recover PHA from sewage sludge biomass.

1. Introduction

The environmental waste caused by the accumulation of plastics and fossil fuel depletion has raised the research of valid sustainable alternatives such as biopolymers. Many biopolymers are biodegradable, thus reducing the accumulation of plastic waste in the environment. They are biosynthesized from renewable biological sources, often identified as wastes, thus reducing the carbon footprint of the production process [1,2]. In the last 30 years, polyhydroxyalkanoates (PHA) have emerged as a valid alternative. Currently, only the pure cultures-based PHA is in the market [3]. Their properties made them comparable to conventional polypropylene, especially for high melting point, good tensile strength and high elongation at break [4–6]. Being limited by the high production cost and strict conditions of pure culture production, research has focused on PHA production from mixed microbial cultures (MMCs) available in waste streams like sewage sludge. Since 1974, when Wallen and Rohwedder observed PHA production in WWTP for the first time, literature has started evaluating the possibility of resource recovery from waste feedstocks, laying the foundations of the circular bioeconomy approach [7–9].

Sewage sludge-based PHA production is usually performed in a three-step process: i) sewage sludge acidogenic fermentation/co-fermentation with other feedstocks to produce volatile fatty acids; ii) biomass enrichment to enrich the PHA producers' microorganisms population and/or PHA accumulation; iii) PHA extraction [10,11]. The PHA extraction cost and environmental impact are currently the limiting steps that hamper the process of diffusion and scale-up [5,12]. PHA extraction from microbial biomass can be carried out by (i) PHA dissolution with an organic solvent, (ii) whole cell lysis with surfactants or enzymes, and (iii) whole cell lysis with physical (i.e., mechanical cell

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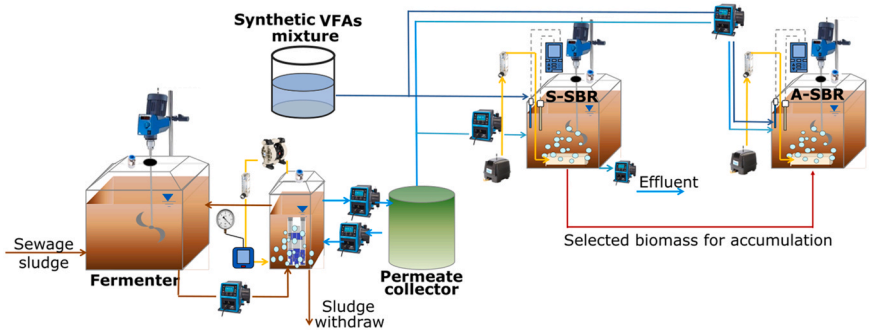

Fig. 1. Schematic representation of the pilot plant configuration adopted.

disruption) or chemical (i.e., NaClO, NaOH or H_2O_2) methods [13,14].

Despite being the most efficient solvents for PHA extraction [15], halogenated solvents are being replaced by green solvents because of their severe impacts on toxicity and environmental pollution [16–19]. Several green solvents, such as propylene carbonate, ethyl acetate, methyl isobutyl ketone, ionic liquids, dimethyl carbonate and 1,3-dioxolane, have already been tested [20–22]. However, the PHA recovery yields from these alternative solvents are still lower than the ones reached with halogenated ones [13,23]. A polymer purity comparable to that achieved with a halogenated solvent can be obtained using a non-ionic surfactant to dissolve the non-polymeric cellular mass and a green solvent for extraction [24,25]. PHA extraction can also be performed using a non-ionic surfactant to dissolve the non-polymeric cellular mass, followed by dimethyl carbonate extraction. Results showed increased recovery when the non-ionic surfactant pre-treatment was employed [26]. Nevertheless, despite the high polymer purity (>90 %), the recovery efficiency was less than 60 %, highlighting how challenging it is to perform an environmentally friendly PHA extraction without compromising the overall performance. Natural deep eutectic solvents (hydrophobic NADES) are a new category of green solvents. Synthesized by mixing different components, their popularity is increasing due to their cheapness, low toxicity and biodegradability. Mondal et al. (2023) [23] extracted PHA produced by MMC from samples with a concentration of around 0.49 g PHA/g Volatile Suspended Solids (VSS). The extraction adopted achieved a polymer purity of 99 % and a recovery yield of 42 %, while Didion et al. (2024) [27] achieved a slightly higher recovery yield (66 %) and lower purity (85 %). Despite their high potential value in terms of low environmental impact and low economic costs, green solvent-based extraction is still not as efficient as conventional halogenated solvents. Moreover, several steps may be required when performing the extraction with green solvents, thus increasing the overall complexity and carbon footprint by adopting high temperatures, sonication, biomass pre-treatments, etc.

Based on the research gap described above, this study aimed to evaluate the impact of several organic solvents for non-PHA cell mass (NPCM) in the PHA extraction process. PHA-enriched biomass produced at the pilot scale was used to perform the extraction tests. Due to their source from sustainable biobased materials, the following solvents have been tested: 2-methyl tetrahydrofuran (2-Me-THF), glycerol, glycerol formal and ethyl lactate. The solvents were tested with PHA produced by MMC, adopting both a synthetic and real fermented sludge liquid. The extraction was carried out on biomass samples with different PHA concentrations to prove the solvents' efficiency [28]. These solvents were tested with the established PHA extraction protocol reported by Mannina et al., 2019 [29] by comparing PHA extraction yields and purity. The best extraction solvent, i.e., 2-Me THF, was recycled and further tested to prove its recyclability while the extracted polymer was analysed through Differential Scanning Calorimetry (DSC) and

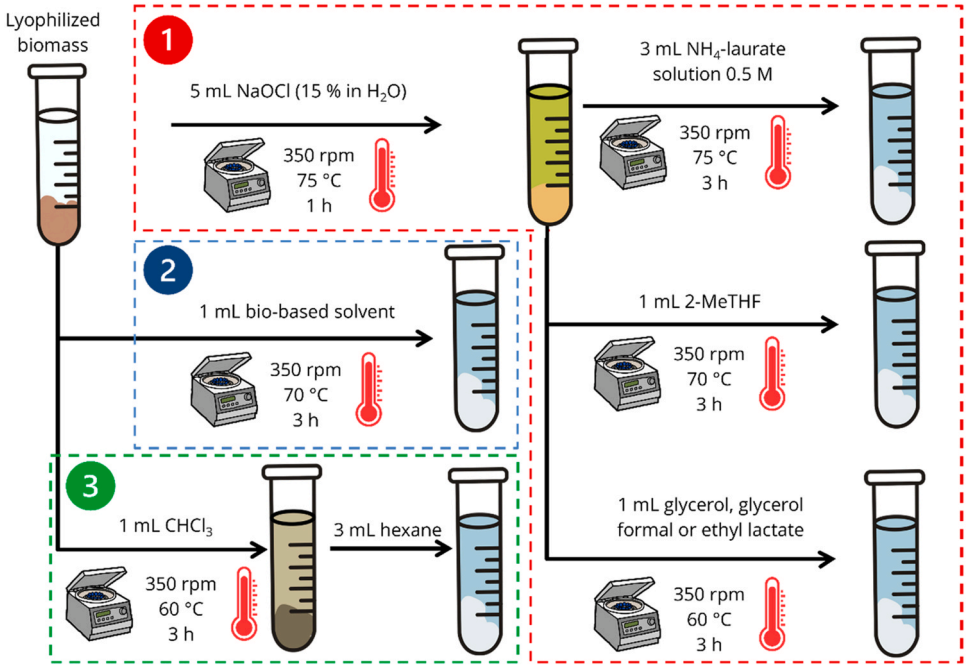

Fig. 2. Schematic representation of the three different extraction protocols tested.

Thermogravimetric Analysis (TGA). Finally, a preliminary economic assessment of the extraction process was carried out.

2. Materials and methods

2.1. Pilot plant configuration

The PHA-rich biomass samples were collected from the PHA production pilot plant at the Water Resource Recovery Facility (WRRF) of Palermo University [30]. The PHA production line adopts the aerobic dynamic feeding enrichment strategy, and it is composed of a fermenter coupled with an ultra-filtration unit equipped with a hollow fiber membrane and two sequencing batch reactors (SBR) devoted to PHA producers' enrichment (S-SBR) and PHA accumulation (A-SBR). At the end of the enrichment process, the selected biomass automatically accumulated PHA by tailored homemade software [29,30]. Wasted sewage sludge from the water treatment line of the WRRF was adopted as inoculum for the fermenter and S-SBR [31].

The pilot plant was operated with a synthetic substrate and real fermented sewage sludge liquid as a volatile fatty acids (VFAs) source (Fig. 1). The synthetic substrate was composed of acetic acid and propionic acid in a ratio of 70:30 [32], while the real substrate was composed, on average, of acetic acid, propionic acid, and butyric acid in a ratio of 65:10:25 [32].

2.2. PHA extraction protocols

Three protocols were followed based on previous literature studies varying in dependence on "sustainable" organic solvents for non-PHA cell mass (NPCM) or organic solvent for PHA (chloroform) used (Fig. 2).

1. Two-step extraction. The extraction protocol proposed by Mannina et al. (2019) [29] was adopted and performed in triplicate. The protocol is based on the first NaClO pre-treatment and the destruction of non-PHA cell mass (NPCM). Briefly, 20 mL of biomass sample was grabbed from the A-SBR in a falcon tube. 1 mL of formaldehyde was added to the falcon tube and then centrifuged (1694 RCF, 10 min) at room temperature. The residual biomass pellet was lyophilised, and 50 mg of lyophilised biomass was weighed in a glass tube. 5 mL of NaClO (15 % in H_2O) were added to the test tube, which was then mixed (350 rpm) at 75 ℃ for 1 h in an Eppendorf block heater (ThermoStat™). After centrifugation (1694 RCF, 10 min) and washing (5 mL of MilliQ Grade water, twice), the pre-treated biomass was mixed (350 rpm, 75 °C, 3 h) with an ammonium laurate solution prepared according to the protocol.

   Other solvents were tested in the pre-treated biomass purification as a substitute for ammonium laurate, adopting the operating conditions of the above-cited protocol: 1 mL of 2-MeTHF was added to the test tube which was then mixed (350 rpm) at 70 ℃ for 3 h; when glycerol, glycerol formal and ethyl lactate were used, 1 mL of solvent was added and the tube was mixed (350 rpm) at 60 ℃ for 3 h [28].

   After centrifugation (1694 RCF, 10 min), the pellet was washed twice with MilliQ Grade water (1 mL) for all solvents, with the exception of 2-MeTHF, which was used for the other two washings. The pellet obtained after the centrifugation was dried at 60 °C overnight.
2. One-step extraction. 1 mL of 2-MeTHF, glycerol, glycerol formal, or ethyl lactate was directly added to the lyophilized biomass and mixed (350 rpm) at 70 ℃ for 3 h. After centrifugation (1694 RCF, 10 min), the pellet was washed twice with 2-MeTHF (1 mL). The pellet obtained after the centrifugation was dried at 60 °C overnight.
3. Chloroform extraction method. The PHA extraction was performed directly without first washing with NaClO solution, using $CHCl_3$ as the extraction solvent, as reported in the literature [33]. 1 mL of $CHCl_3$ was added to 50 mg of lyophilised biomass in a glass tube and mixed for 3 h at 60 ℃. After centrifugation, the organic solution was added to 3 mL of hexane to favor PHA precipitation [32]. The organic PHA was removed, and the solid residue was dried at 60 ℃ overnight.

Table 1
Details of the samples used for the extraction, determined by GC.

Sample	Substrate used in the accumulation	PHA [% wt VSS]	HB [% wt VSS]	HV [% wt VSS]
T5	Synthetic	12.5	8.5	4.0
T6	Synthetic	16.9	12.5	4.4
T7	Synthetic	18.8	15.3	3.5
T8	Synthetic	21.2	17.4	3.8
T17	Synthetic	31.6	18.5	13.1
T18	Synthetic	42.8	25.9	16.9
T19	Synthetic	55.7	30.8	24.9
T10	Real fermented sludge liquid	25.7	25.0	0.7

2.3. Samples adopted for the extraction protocols

The samples used to test the several extraction protocols were obtained during the accumulation process, performed either with a synthetic substrate or real fermented sludge liquid. The characteristics of the samples are summarized in Table 1. The T5, T6, T7, T8, T17, T18 and T19 samples were collected from the same accumulation process described in the literature [34]. The samples were collected at different times, meaning that the PHA concentration differs because of the increasing trend typical of the accumulation process. Specifically, T5 was collected after 5 substrate feedings, while T19 was collected after 19 feedings. The sample T10 was collected from an accumulation process with real fermented sludge liquid [32]. To test all the solvents and conditions adopted for all the samples would have required a real-scale plant PHA production rate. This paper collects samples from a pilot plant treating around 60 L of waste sludge per day, meaning that a limited amount of PHA is produced. In view of that, the first screening was carried out on samples T17-T19 with a higher PHA concentration. The efficiency of the best condition was then compared to the literature protocol and the reference extraction with $CHCl_3$, adopting samples T5-T8 because of their lower PHA concentration. The same approach was used to test it with T10, produced with VFAs produced from sludge fermentation [32].

2.4. Analytical methods

PHA concentration analysis was carried out according to the procedures of Werker et al. (2008) and Montiel-Jarillo et al. (2017) [35,36]. Briefly, lyophilised biomass samples were mixed with butanol and hydrochloric acid and incubated at 100 ℃ for 8–20 h. One hexane aliquot and two MilliQ grade water aliquots were used to extract the hydroxyalkanoate esters. The organic PHAe was filtered (0.22 μm) and stored in 2 mL vials. The sample analysis was carried out with an Agilent gas chromatographer (GC) (8860) equipped with a flame ionisation detector (FID) and a Restek Stabilwax column (30 m x 0.53 mm×1.00 μm film thickness) to determine the PHB and PHV monomer concentrations. The GC was calibrated by using Poly[(R)-3-hydroxybutyric acid] (PHB) (363502–10 G) and Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) (403105–10 G) standards from Merck - Sigma Aldrich, Germany. Total suspended solids (TSS) and Volatile suspended solids (VSS) were measured according to the standard methods [37].

2.5. Economic analysis

The economic analysis considered the chemical and equipment costs. According to the Sigma-Aldrich shopping website, the chemical cost was related to the solvent use, which was converted into euros €. When applicable, the solvent recovery was calculated, considering the euros €

saved by recovering 2-MeTHF by distillation. The solvent recovery term was subtracted from the solvent used to calculate the overall chemical cost. The equipment costs are divided into five shares related to the five instruments adopted: lyophilization (ScanVac, Cool Safe and Carpanelli, vacuum pump M50L4), mixing and heating (Eppendorf, block heater ThermoStat™), centrifugation (Hermle centrifuge, Z 327), drying (Velp Scientifica, ECO 16 Thermoreactor) and distillation (LLG, Unistirrer 3). The kWh due to power consumption was converted to euros € applying a conversion factor of 0.289 €/kWh, according to the average electricity prices (including taxes) for household consumers in the European Union in the first semester of 2024 [38].

2.6. Biopolymer characterisation

2.6.1. Nuclear magnetic resonance analysis

The biopolymer structure and percentage of each monomer were confirmed by ^{1}H Nuclear magnetic resonance (NMR) analysis performed on a Bruker magnetic resonance spectrometer operating at 400 MHz. Extracted biopolymers (10 mg) were dissolved in $CDCl_3$ for analysis. NMR spectra were recorded at 25 °C, and the solvent residual peak was used as a reference.

2.6.2. Thermal analysis

2.6.2.1. DSC analysis. DSC measurements were carried out with a TA Instruments mod. 2920 Differential Scanning Calorimeter (TA Instrument Inc., New Castle, DE, USA) equipped with a TA Instruments Refrigerated Cooling System. Samples (~4 mg) were weighed in aluminum TA Tzero Hermetic Pans, which were sealed with aluminum lids. The analysis was performed with heating and cooling rates of 10 °C min^{-1} under a N_2 atmosphere of 50 mL min^{-1}. Heating and cooling ramps were carried out in a temperature range between 0 ℃ and 180 ℃.

2.6.2.2. Thermogravimetric analysis (TGA). TGA was performed using the TGA/DSC SDT650 (TA Instruments) apparatus under nitrogen flow, which was 50 mL min^{-1}. The samples were weighed in open aluminum pans, with mass ranging from 1 to 4 mg. After a calibration of 20 min at 100 °C, samples were heated up to 500 °C with a scanning rate of 10 °C min^{-1}.

2.6.3. Molecular mass determination

Weight-average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (PDI; M_w/M_n) were determined by means of GPC-MDS performed on an HPLC-GPC Agilent 1260 Infinity series using a PL gel mixed C organic SEC column (5 µm with cut-off 200–2000000 Da), isocratic runs were performed for 13 min with 100 % THF as eluent and a flow rate of 1 mL/min. Biopolymers (1 mg) were dissolved in 1 mL of THF and filtered before injection.

2.6.4. Recycling of 2-MeTHF

2-MeTHF used for PHA extraction from several biomass samples were collected to obtain a total volume of ~10 mL. This volume was distilled using simple distillation glassware, heating the solvent at 70 ℃. The distilled solvent was analysed *via* gas chromatography-mass spectrometry (GC-MS), while the solid residue was analysed *via* ^{1}H NMR.

2.6.5. GC-MS analysis

0.1 mL of recycled 2-MeTHF was diluted in 1 mL of hexane for GC-MS analysis with triple quadrupole GC/MS Agilent 7010 and an Agilent 19091S-433UI column of 30 m x 250 µm x 0.25 µm. Measurements were performed using a ramp temperature of 10 °C/min from 40 °C to 270 °C and a split flow method of 20 mL/min. The GC–MS, mass spectrum data, were analysed using MassHunter Qualitative Analysis B.06.00, and the National Institute of Standard and Technology (NIST) database was used to interpret the data.

2.7. Calculations

PHA concentration was calculated as a weight ratio percentage based on the gram of PHA per gram of VSS inside the A-SBR as reported by Eq. 1:

$$PHA\ wt\% = \frac{gPHA}{gVSS} * 100 \quad (1)$$

The grams of PHA are calculated from the concentration analyzed with the GC-FID. In view of assessing the performance of the different extraction methods tested in this work, the PHA extraction, purity, recovery yield and an index of PHA ratio compared to the one of $CHCl_3$ were calculated as follows:

$$PHA\ extraction\ yield\% = \frac{Extracted\ polymer\ mass}{Lyophilized\ biomass\ sample\ mass} \quad (2)$$

$$PHA\ purity\% = \frac{Mass\ PHA}{Extracted\ polymer\ mass} = \frac{Area\ PHA}{Area\ PHA + Area\ impurity} \quad (3)$$

$$PHA\ recovery\ yield(\%) = \frac{PHA\ extraction\ yield * PHA\ purity}{PHA\ wt\%in\ lyophilized\ biomass} \quad (4)$$

Where the extracted polymer mass is obtained by weighing the solid residue after the extraction. PHA purity was initially determined by GC and ^{1}H NMR for samples with high PHA content, namely T17, T18 and T19. Since the results of the two methods are comparable [34,39,40], ^{1}H NMR analysis after extractions applying Eq. 3 was used to determine the purity of the other samples. In Eq. 3, the Area PHA is the sum of signal integrals corresponding to PHA and Area impurity is the sum of signal integrals not ascribable to PHA.

$$Y\ \frac{PHA(solvent)}{PHA(CHCl_3)} = \frac{PHA\ exctration\ yield(inaspecific\ solvent)}{PHA\ exctraction\ yield(in\ CHCl_3)} \quad (5)$$

Where Y PHA (solvent)/ PHA ($CHCl_3$) of Eq. 5 is the ratio between PHA extraction yield in all tested solvents and the corresponding one in chloroform since chloroform was used as a positive control.

The chemical cost required to extract 1 g of PHA was calculated as shown in Eq. 6:

$$CC_{PHA} = \frac{(CC_{SLUDGE}/VSS : TSS\ ratio)}{PHA\ recovery\ yield} \quad (6)$$

Where CC_{SLUDGE} is the chemical cost required to treat 1 g of sludge and the VSS:TSS ratio was equal to 0.75.

3. Results and discussion

The protocols adopted are based on a two-step solvent extraction: NaClO pre-treatment and NPCM destruction using an organic surfactant (ammonium laureate) or bio-based alternative solvents from renewable sources such as 2-Me-THF, glycerol, glycerol formal, and ethyl lactate. Extraction in $CHCl_3$, as a positive control, was also performed. A two-step procedure is also needed for $CHCl_3$ as the biomass is first dispersed in an organic solvent, and then the organic phase containing PHA is precipitated by hexane (Figure S1) [33]. A single-step procedure was then adopted for the biobased solvents in view of assessing the possibility of reducing the extraction steps, thus reducing the environmental and economic costs.

Table 2
PHA extraction yield, purity, recovery yield, HB and HV percentages in different extraction solvents for high PHA content samples.

Sample	Extraction solvent	PHA extraction yield (%)	PHA purity * (%)	PHA recovery yield (%)	HB* (wt %)	HV* (wt %)
T19	$CHCl_3$	45.9	97.5	63.9	23.6	22.4
	2-MeTHF	69.1	90.2	89.0	41.1	19.3
	NaClO + 2-MeTHF	68.1	89.3	86.8	46.3	15.7
	NaClO + NH_4-laurate	42.3	87.5** - 87.1	52.6	31.2	11.3
	$CHCl_3$	31.4	97.7	61.4	16.7	14.7
	Glycerol	75.2	69.3	69.7	34.3	40.6
T18	Glycerol formal	51.6	85.1	87.8	26.5	25.1
	NaClO + glycerol formal	39.1	91.1	71.1	13.3	12.6
	NaClO + NH_4-laurate	40.7	88.3** - 87.1	70.9	29.7	11.1
	$CHCl_3$	32.1	95.9	87.9	16.6	15.5
	NaClO + glycerol	28.2	97.1	54.4	9.2	9.2
T17	Ethyl lactate	59.6	87.5	80.6	15.7	15.7
	NaClO + Ethyl lactate	41.9	95.8	80.3	15.5	17.7
	$CHCl_3$ (only extraction)	35.1	75.2	75.3	16.4	18.7
	NaClO + NH_4-laurate	32.7	90.5** - 87.1	81.4	24.3	8.4

* Determined by ^{1}H NMR and expressed as weight percentage (wt%).
** Determined by GC analysis and expresses as weight percentage (wt%).

3.1. PHA accumulated by synthetic substrate

3.1.1. High PHA content samples

Table 2 and Figure S2 show PHA extraction and recovery yield, purity and percentage of monomers HB and HV obtained in the extraction solvents. The extraction was carried out on pre-treated biomass with NaClO or directly on lyophilized biomass samples obtained using a synthetic substrate. The samples tested had a PHA concentration higher than 30 % w/w (T17, T18 and T19).

PHA recovery yields were similar for $CHCl_3$ and ammonium laureate in all samples analysed. 2-MeTHF adoption resulted in a higher PHA recovery yield for T19 (64, 53 and 87 %, respectively, for $CHCl_3$, ammonium laureate and 2-MeTHF). In addition, the biomass pretreatment with NaClO did not show any positive effect on PHA recovery yield or purity when 2-MeTHF is used, indicating that the step can be bypassed by directly treating the lyophilized biomass.

For sample T18, PHA recovery yield increases when glycerol or glycerol formal are used compared to $CHCl_3$, achieving 70, 88 and 61 % for glycerol, glycerol formal and $CHCl_3$, respectively. However, the purity of the obtained biopolymer significantly decreases, especially when adopting glycerol (Table 2). Despite the potentially promising role of glycerol as a substrate for PHA production [41,42] and solvent for PHA extraction, the results showed that the biomass pretreatment with NaClO is a mandatory step to achieve a high-quality polymer, despite the negative effect on the PHA extraction yield. Furthermore, glycerol-based solvents are quite viscous, meaning that more MilliQ-grade-water washing steps were necessary to remove the solvent after the extraction procedure. Regarding T17, ethyl lactate achieved similar results to $CHCl_3$ and ammonium laurate in terms of PHA recovery yield and purity.

Interestingly, a different HB:HV ratio is observed as a function of the extraction solvent. Nevertheless, the overall results are explained by dependence on PHA extraction or recovery yield, as reported for similar extraction studies [17,43,44]. To properly compare the data obtained by the different extraction solvents, an index related to the PHA extraction yield was calculated according to Eq. 5, where the PHA extraction yield in $CHCl_3$ was used as a positive control. As reported in Fig. 4, direct treatment of biomass with glycerol, glycerol formal, and ethyl lactate gives the best PHA index compared to $CHCl_3$ (2.4, 1.6 and 1.8, respectively). However, the low PHA purity achieved by these solvents suggested the mandatory need for biomass pre-treatment by NaClO. In all cases, ammonium laurate treatment is comparable to chloroform extraction, while using 2-MeTHF constantly achieves an index of 1.5, with or without the biomass pre-treatment (Fig. 3).

Preliminary results proved the convenience of using 2-MeTHF as increased PHA recovery yield with good purity was obtained by this solvent. Moreover, allowing the extraction in a single step with a single reagent will save time and energy by avoiding the biomass pretreatment and water, which will then be discarded as waste. 2-MeTHF proved to be an excellent alternative to the petroleum derivative

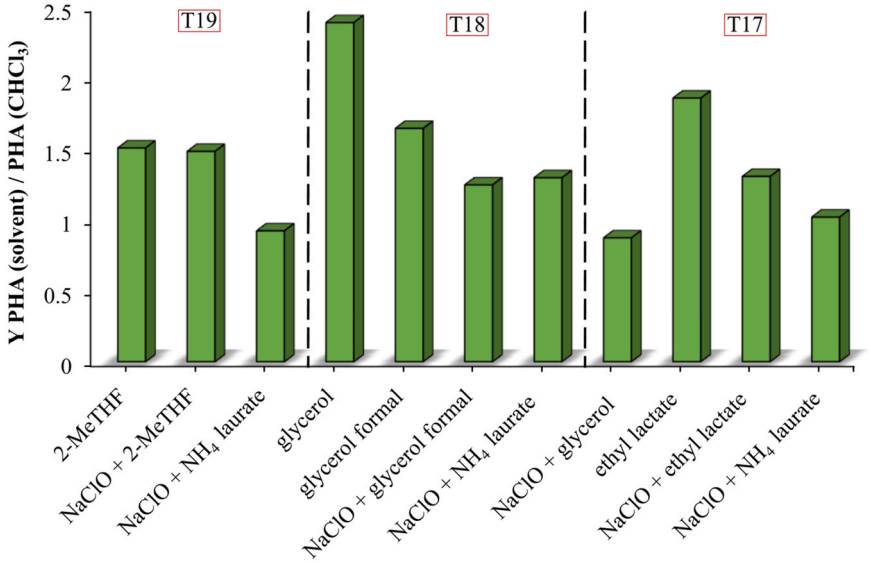

Fig. 3. The ratio of PHA extraction yield percentage in different solvents for high PHA content samples.

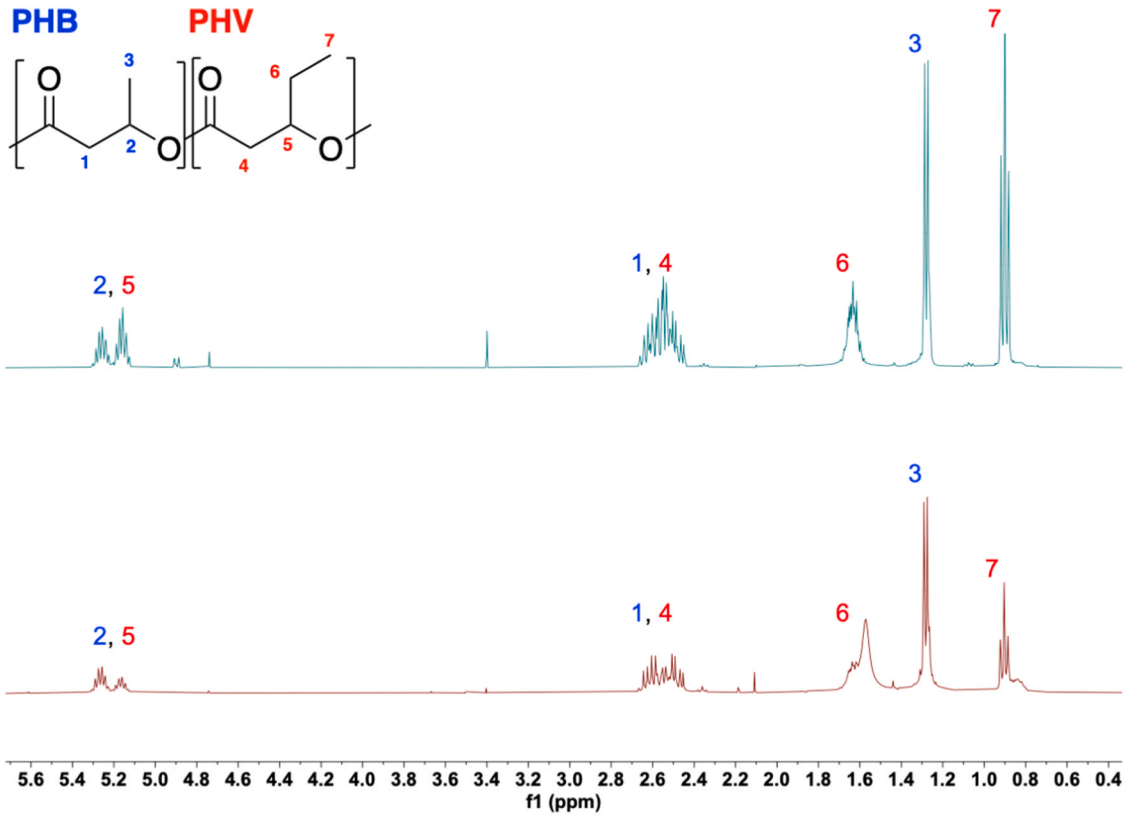

Fig. 4. ^{1}H NMR spectra in $CDCl_3$ of PHA extracted in 2-MeTHF (bottom in red) and $CHCl_3$ (upper in green). Representative PHA structure and peak assignment were reported in the inset.

Table 3
PHA extraction yield, purity, recovery yield, HB and HV percentages in different extraction solvents tested with low PHA content biomass.

Sample	Extraction solvent	PHA extraction yield (%)	PHA purity * (%)	PHA recovery yield (%)	HB* (wt %)	HV* (wt %)
T8	2-Me-THF	24.1	94.7	69.7	16.3	7.8
	$CHCl_3$	32.6	94.5	91.7	15.5	18.7
	NaClO + NH_4 laurate	21.2	-	-	17.4	3.8
T7	2-Me-THF	49.4	91.9	82.5	39.7	29.7
	$CHCl_3$	40.9	95.9	71.3	19.1	21.9
	NaClO + NH_4 laurate	18.8	-	-	15.3	3.5
T6	2-Me-THF	54.8	96.9	88.5	34.3	20.6
	$CHCl_3$	28.9	96.8	46.6	12.7	16.2
	NaClO + NH_4 laurate	16.9	-	-	12.5	4.4
T5	2-Me-THF	38.1	91.7	63.4	34.5	3.4
	$CHCl_3$	43.3	94.6	74.5	35.2	8.1
	NaClO + NH_4 laurate	12.5	-	-	8.5	4.0

* determined by ^{1}H NMR and expressed as weight percentage (wt%).

tetrahydrofuran (THF), as it is derived from renewable resources such as corncobs and bagasse. In addition, it does not harm human health without irritating the eyes or respiratory system, as the THF [45].

However, it was scarcely applied as an extraction solvent of PHA due to the low solubility of the polymer in this solvent [33,46]. Indeed, both Yabueng et al. (2019) and Elhami et al. (2022) used the 2-MeTHF to solubilise the PHA with subsequent precipitation with another solvent

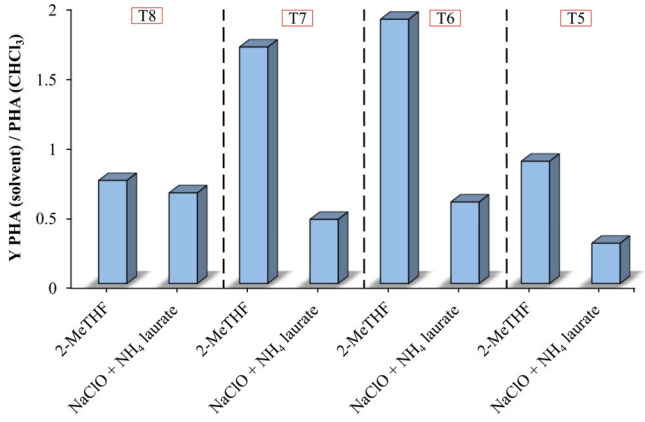

Fig. 5. PHA extraction yield index for low PHA content biomass samples.

[33,46]. As far as the authors are aware, this work is the first one to use 2-MeTHF as an NPCM solubilizer. Indeed, using it as a "washing" solvent to remove the NPCM of the biomass, as for ammonium laurate, glycerol, glycerol formal or ethyl lactate, improves its efficiency in extracting the PHA without using water, which will then be discarded as waste. The results obtained by 2-MeTHF in this work are comparable to those obtained by Samorì et al. (2014) with dimethyl carbonate (DMC) [43]. Still, DMC was used in pure cultures PHA extraction, which has several advantages compared to the mixed microbial cultures [42] and at higher operational temperature (90 °C), indicating the promising role of 2-MeTHF as an extraction solvent for mixed microbial cultures [47].

The presence and purity of PHA were confirmed by ^{1}H NMR measurements of polymers, as shown in Fig. 4. Signals corresponding to the protons of the PHB units are labelled as 1–3, while signals corresponding

Table 4
PHA extraction yield, purity, recovery yield, HB and HV percentages in different extraction solvents for PHA produced by real substrate.

Sample	Extraction solvent	PHA extraction yield (%)	PHA purity * (%)	PHA recovery yield (%)	HB* (wt %)	HV * (wt %)
T10	NaClO + 2-MeTHF	50.3	93.6	83.4	50.3	0
	$CHCl_3$	50.3	-	-	-	-
	NaClO + NH_4-laurate	25.7	-	-	24.9	0.7

* Determined by ^{1}H NMR and expressed as weight percentage (wt%).

to PHV units are labelled as 4–7. The assignment of NMR signals agrees with what has previously been reported in the literature and with that of commercial PHA [34,48].

3.1.2. Low PHA content in biomass samples

To test the solvent's consistency, the extraction procedure using 2-MeTHF was also performed on low PHA content biomass samples (T5, T6, T7 and T8) without the NaClO biomass pretreatment (Table 3). The extraction was also carried out with $CHCl_3$ and ammonium laurate to compare the different extraction methods (Fig. 5).

The Y index indicates a modest decrease in PHA extraction yield when 2-MeTHF is used in T8 and T5 compared to the positive control (Fig. 5). However, high index values of 1.7 and 1.9 were obtained in T7 and T6, respectively. Also, 2-MeTHF adoption resulted in a higher index than ammonium laurate in all the cases.

To test the reuse of 2-MeTHF, the exhaust solvent was collected and distilled after the sample was washed. 95.7 % of the 2-MeTHF used was recovered, and it showed comparable signals to commercial ones when analyzed by GC-MS, indicating that the solvent could be successfully reused (Figure S4). Moreover, only 0.3 % of PHA was found in the solid residue at the end of the distillation process, indicating an excellent extraction efficiency when using the bio-based organic solvent. The proven high efficiency and the more straightforward extraction method compared to those usually involved in literature make 2-MeTHF a possible candidate for successfully extracting PHA produced by mixed microbial cultures.

3.2. PHA accumulated by real substrate

To further test the extraction efficiency of 2-MeTHF, ammonium laurate and $CHCl_3$ were used to extract PHA produced by real fermented sludge liquid as a substrate (Fig. 1). The high concentration of impurities detected during the single-step extraction forced the adoption of the NaClO pre-treatment for 2-MeTHF (Table 4).

The PHA extraction yield was the same for $CHCl_3$ and 2-MeTHF, while it was halved for ammonium laurate. Notwithstanding the exact yield between conventional and green solvents, the purity was much higher in 2-MeTHF, as observed from the ^{1}H NMR spectra of the obtained polymer (Fig. 6). Chloroform extraction resulted in many impurities (green spectrum in Fig. 6), which did not allow the purity, recovery yield and monomer ratio to be correctly calculated.

Despite demanding a preliminary step with NaClO, 2-MeTHF proved

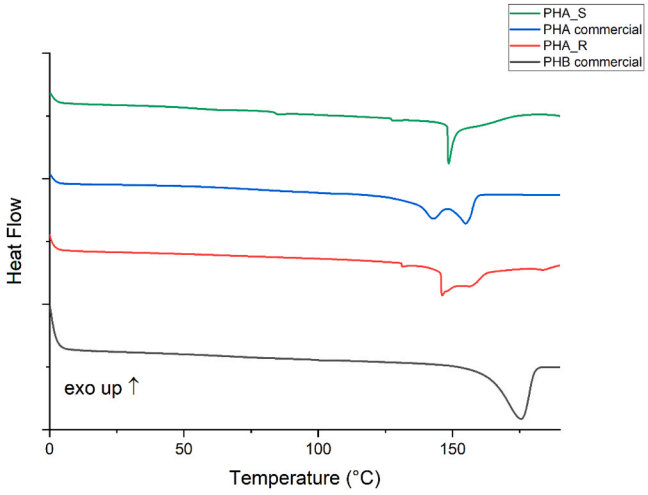

Fig. 7. DSC traces of commercial PHA and PHB extracted PHA from synthetic biomass (PHA _S) and real biomass (PHA _R). Exothermic peak upward.

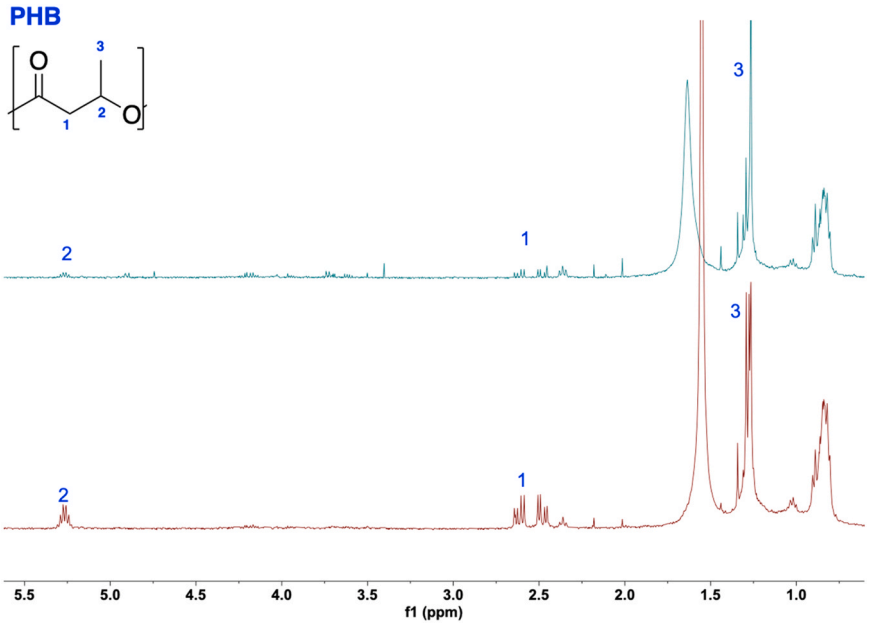

Fig. 6. ^{1}H NMR spectra in $CDCl_3$ of PHA extracted in 2-MeTHF (bottom in red) and in $CHCl_3$ (upper in green) from real biomass sample. Representative PHB structure and peak assignment were reported in the inset.

Table 5
Melting temperatures (T_m) and enthalpies (ΔH_m) of PHA samples obtained from DSC traces, degradation temperatures (T_d) determined from TGA traces, weight average molecular weight (M_w) and PDI determined by GPC-MDS.

	T_m (°C)	ΔH_m (J g^{-1})	T_d (°C)	M_w (g/mol)	PDI
PHA commercial	142.20 155.33	12.57 12.46	277.23		
PHB commercial	175.40	80.88	227.21		
PHA extracted from synthetic biomass (PHA _S)	148.52	11.80	219.84	576000	1.25
PHA extracted from real biomass (PHA _R)	146.17	25.87	232.41	315000	2.79

its efficiency also with PHA produced by real VFA stream, underlying its promising role as a novel environmentally friendly extraction solvent. Still, the mandatory NaClO pre-treatment to reduce the impurities highlights some limitations in adopting 2-MeTHF. This could be successfully overcome by testing the solvent with PHA produced by real fermented sludge's VFA stream in different conditions.

3.3. Characterization of PHA

DSC measurements have been used to perform thermal analysis of PHA recovery yielded from synthetic and real biomass samples using 2-MeTHF as an extraction solvent. PHBV and PHB, which are commercially available, have also been analysed for comparison.

DSC traces showing the melting process of the polymer are reported (Fig. 7). Commercial PHBV shows two endothermic peaks at 142.2 °C and 155.3 °C, probably indicating the presence of other additives such as plasticizers in the sample, while the commercial PHB shows one endothermic peak at 175.4 °C (Table 5).

Samples of PHA obtained after biomass treatment in 2-MeTHF show almost the same melting temperature obtained for commercial polymer, i.e., 148.5 °C and 146.2 °C, indicating that the polymer retains the same physical properties as the commercial one [44]. The achieved melting temperatures agree with those reported by the literature for similar cases [19,49,50]. Degradation temperatures determined as the maximum derivative of TGA traces agree with what was previously reported for PHA derived from biomass, falling in the range 220 – 280 °C (Table 5) [51].

Molecular weights were determined via GPC-MDS analysis for PHA extracted from real and synthetic biomass samples using as extraction solvent 2-MeTHF and $CHCl_3$ (Table S1). Large molecular weights

Table 6
Details of the economic cost to extract PHA from 1 g of wet sludge sample. The total cost is considered as the lyophilization cost, NaClO treatment (when applicable), and the 2-MeTHF recovery (when applicable).

		Chemical		Equipment					Total cost
		Solvent use	Solvent recovery	Mixing and heating	Centrifuge	Drying	Distillation	Lyophilization	
	U.M	Euros €							
Sample pretreatment		0.21	-					0.11	
Extraction 1	NaClO	0.29	-	0.17	0.04	0.48	-	-	
	NH_4^+ Laurate	0.01							1.32
	2-Me-THF	3.79	- 3.63				0.03		1.50
	Glycerol	0.25	-				-		1.56
	Glycerol formal	1.96							3.26
	Ethyl Lactate	0.37							1.68
Extraction 2	2-Me-THF	1.26	- 1.21	0.13	0.02	0.48	0.01		1.15
	Glycerol	0.25	-						1.34
	Glycerol formal	1.96							3.04
	Ethyl Lactate	0.37							1.46
	2-Me-THF (washing)	2.53	- 2.42				0.03		
Extraction 3	$CHCl_3$	0.18	-	0.13	0.02	0.48	-		1.60
	Hexane	0.47							

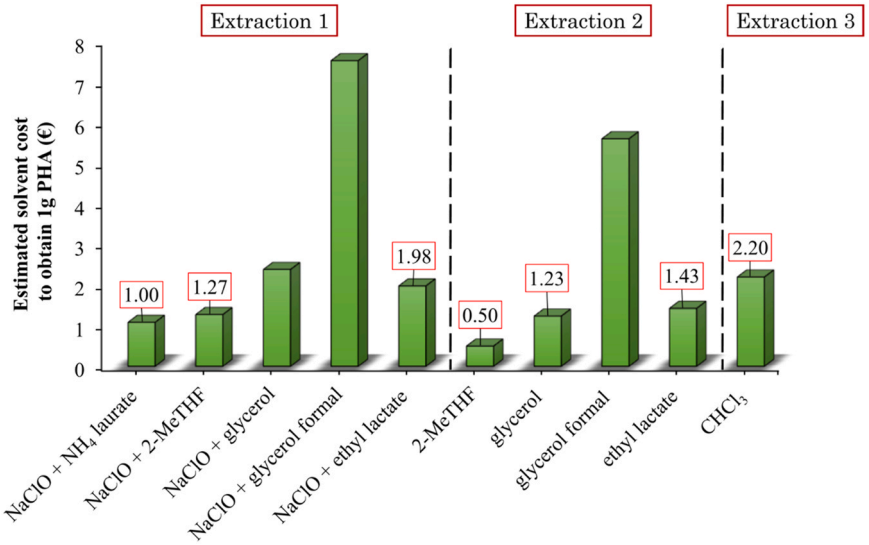

Fig. 8. Estimated chemical cost to obtain 1 g of PHA from sludge with average 56 wt% PHA content.

(~300000 or ~500000 g/mol), indicative of the polymer, were obtained in all cases. Molecular weights were comparable independently from solvent extraction used, with PDI values close to 1 for the polymer extracted in 2-MeTHF, indicating low polydispersity of the polymer in line with what was previously reported for PHA [52].

3.4. Practical-driven analysis of the economic cost

The results of the preliminary economic analysis are reported in Table 6. The total cost is related to the PHA extraction from 1 g of wet sludge sample. It can be observed that approximately 25–50 % of the extraction cost is due to the use of equipment. Since the instruments used are laboratory-scale, it can be expected that these costs will naturally decrease in the future scale-up. The highest percentage of the total extraction cost ($\geq$ 50 %) is related to using solvents. Another cost component, accounting for approximately 10–30 %, is sample pretreatment, specifically due to lyophilization. This is a preliminary step adopted in this study. Still, it is unnecessary to perform the extraction, as other studies have demonstrated good extraction efficiency even with fresh, non-lyophilized biomass samples [53]. Considering the solvent efficiency, Fig. 8 provides a preliminary estimate of the costs required to extract one gram of PHA from a sludge sample, assuming a 56 % g PHA/g VSS concentration, as sample T19. This calculation considers the average recovery yield of each solvent for the various samples used and assumes that these efficiencies remain the same in extracting this sludge sample. In this case, the most cost-effective solvent, given equal efficiency, is 2-MeTHF, mainly due to its ability to be recovered at approximately 96 %. Future studies should evaluate how many solvent recovery cycles can be performed per extraction, significantly reducing extraction costs on a larger scale. These results highlight that environmentally friendly extraction with green organic solvent shows slightly higher efficiency than conventional halogenated solvent extraction while reducing the cost by 77 %. However, these calculations are based on laboratory-scale conditions and are specific to this study. These costs are expected to be amortized in the future scale-up, but they still highlight the significant impact of PHA extraction from sludge.

4. Conclusions

Overall, all the bio-based solvents tested showed good results in terms of PHA recovery yield compared to the conventional $CHCl_3$ extraction or the two-step ammonium laurate protocol. The adoption of 2-MeTHF for low PHA concentration samples resulted in high purity (97.0 $\pm$ 0.9 %) and average PHA recovery yield (75.6 $\pm$ 11.9 %), which resulted in being comparable to $CHCl_3$ (71.0 $\pm$ 18.6 %). The promising role of 2-MeTHF as the replacement of halogenated solvents in PHA extraction is also sustained by the possibility of performing single-extraction processes using the same chemical, which will guarantee a reduction of the process' costs, increased operator safety and improved environmental protection. Indeed, this work successfully demonstrated the possibility of distilling and reusing the 2-MeTHF, recovering up to 95.7 % of the solvent used with purity similar to commercial ones. The results stand as pioneering in setting up a new environmentally friendly and sustainable protocol to recover PHA from sewage sludge biomass.

CRediT authorship contribution statement

Antonio Mineo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Giorgio Mannina:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation. **Antonio Palumbo Piccionello:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization. **Carla Rizzo:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sara Amata:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation.

Declaration of Competing Interest

Prof. Antonio Palumbo Piccionello on behalf of all the authors of the submission of the research paper entitled "**An environmentally sustainable extraction protocol for polyhydroxyalkanoates from mixed culture biomass"** by, Carla Rizzo, Antonio Mineo Sara Amata, Antonio Palumbo Piccionello and Giorgio Mannina declare that are not any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.117691.

Data availability

Data will be made available on request.

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