



Downstream processing and characterization of polyhydroxyalkanoates (PHAs) produced by mixed microbial culture (MMC) and organic urban waste as substrate

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Abstract

The utilization of food waste and sewage sludge as organic substrate from urban context for the synthesis of microbial polyhydroxyalkanoates (PHAs) has been only recently investigated at pilot scale. Within this context, two stabilization methods have been found for preserving the amount of PHA intracellularly produced by open mixed microbial culture (MMC): thermal drying and wet acidification of the biomass at the end of PHA accumulation process. The extracted PHA from the two differently stabilized biomasses was then characterized with regard to chemical composition, molecular weight, and thermal properties. The polymer contained two types of monomers, namely 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) at a relative percentage of 93.0–79.8 and 7.0–20.2 w/w, respectively. PHA extracted from wet-acidified biomass had higher average molecular weights (M_w) of 370–424 kDa while PHA recovered from thermally stabilized dried biomass had a 3-fold lower M_w (on average). The PHA decomposition temperatures $T_d^{10\%}$ and T_d^{\max} were in the range 260–268 °C and 269–303 °C, respectively, not dependent on the monomeric composition or molecular weight. Thermal properties such as melting temperature (T_m^1 132–150 °C; T_m^2 155–167 °C) and melting enthalpy (26–70 J/g) were quantified in a relatively broad range according to the different stabilization-extraction method and obtained composition.

Keywords Polyhydroxyalkanoates (PHAs) · Mixed microbial culture (MMC) · Downstream processing · Organic fraction of municipal solid waste (OFMSW) · Molecular weight (M_w)

1 Introduction

Polyhydroxyalkanoates (PHAs) are one of the most interesting biopolymers due to their mechanical properties and biodegradability. Their chemomechanical properties have been extensively discussed [1], but the same attention has not been dedicated to biodegradability aspects. The marketed product derived from pure strain; its biodegradation has been related to those microorganisms that could secrete extracellular PHA depolymerase even though other factors such as the environmental condition and/or PHA properties (e.g., composition,

crystallinity, additives) could affect the PHA degradation [2]. As an example, the effect of chemical structure on PHA biodegradability has been investigated by Weng et al. [3]: according to ISO 14855-1, the controlled composting conditions showed that higher 3-hydroxyvalerate (3HV) content corresponded to higher biodegradability. In the form of composites (together with waste wood sawdust fibers), the ASTM D5338-98 and ISO 20200-2004 standard methods revealed that the presence of fibers favored the physical disintegration of PHA, increasing the biodegradation rate of the polymeric matrix [4]. To the best knowledge of the authors, there are no biodegradability data in the literature for PHA synthesized by mixed microbial culture (MMC). The approach of MMC technology consists in the exploitation of organic wastes as feedstock [5, 6]. In addition, MMC technology involves the use of waste-activated sludge (always available in municipal wastewater treatment plants, WWTPs) as inoculum that needs to be enriched in microorganisms with high polymer accumulation capacity. PHA-storing organisms can grow by applying

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aerobic dynamic feeding conditions (ADF) through the well-known feast-famine regime [7]. In the last decade, MMC-PHA production using different types of waste has been the topic of several applied researches; moreover, recent investigation has been conducted at pilot scale to demonstrate the technical feasibility of the technology [8, 9]. In particular, PHA production from the organic fraction of municipal solid waste (OFMSW) has been assessed in order to quantify the process PHA productivity (g PHA/L day) potentially achievable and final polymer quality, also in consideration of a possible integration in the existing wastewater WWTP [10–12]. These are fundamental aspects for MMC-derived PHA commercialization and relative market scenario, which do not exist yet. In the view of a larger-scale MMC-based process, PHA recovery and, more in general, the whole downstream processing from PHA-rich biomass stabilization to PHA extraction are still the bottlenecks of the entire production chain. A recent review summarizes the sustainable approaches and latest developments for the downstream processing [13], emphasizing the environmental unsuitability of chlorinated solvents. With particular attention for the MMC PHA-rich biomass, several non-chlorinated solvents have been firstly exploited [14] together with a biomass stabilization protocol, where a temperature range of 120–160 °C was adopted in order to preserve the polymer quality. The latter might be also improved by optimization of time, temperature, and concentrations of reagents in the purification steps as a function of PHA composition [15]. Moreover, PHA content in the biomass at the end of the accumulation step has to be preserved to maximize the efficiency and the productivity of the process. In addition, recovery methods need to be flexible towards conditions that may be readily tuned to a range of polymer types [14]. For application and realization of bioplastic products, it is necessary to obtain a polymer with specific chemical, physical, and mechanical properties. The mechanical properties of PHA are good when the average molecular weight (M_w) is higher than 400 kDa, and specifically thermoplastic applications may require M_w higher than 600 kDa. Molecular weights typically range between 200 and 3000 kDa, and the distribution can be affected by the method of accumulating PHA in the biomass, the method for PHA recovering, and PHA processing into end-user products [15]. The few studies on chemomechanical and thermal characterization of MMC-PHA have been reviewed by Laycock and coworkers [15]. It has been noted that there is often a higher melting temperature (T_m) and a lower enthalpy of melting for MMC-PHA than those of pure culture-derived PHA [16]. This is probably due to the formation of block copolymers or polymer blends which in turn is a consequence of the feeding strategies adopted in MMC systems. The pulse feeding causes frequent variability in substrate concentrations that leads to a block formation depending on the substrate availability (e.g., 3-hydroxyvalerate synthesis in the presence of propionate)

[17]. Thermogravimetric analysis on MMC-PHA samples showed a similar thermal stability (with decomposition temperatures of about 270 °C) to the commercial poly-3-hydroxybutyrate [P(3HB)] and poly-3-hydroxybutyrate-co-3-hydroxyvalerate [P(3HB-co-3HV) (10 mol% HV)] [18]. Besides, the crystallization rate slows down as 3HV content increases [1]. Arcos-Hernández et al. have conducted a study in which PHA produced with different 3HV content were characterized, evaluating the possible effects on polymer properties [19]. In terms of mechanical properties, it has been demonstrated that the MMC-PHA Young's modulus (779–2893 MPa) increased significantly when the 3HV content was lower than 40 mol%; furthermore, the elongation to break of all but one of the samples was in the range of 3–6% which is similar to that expected for P(3HB) (brittle and stiff) [19]. In general, the progressive incorporation of 3HV in PHA from pure culture, mainly referred to commercial polymer, brings about a constant increase of ϵ_b from about 2.5 mol% at 3HB equal to 100 mol%, to 44 mol% at 3HB equal to 60 mol%. The elongation to break of samples obtained from MMC remains constant to about 3HB 65 mol% ($\epsilon_b \approx 3\%$) and shows a small increase from 54 to 28 mol% 3HB ($\epsilon_b \approx 5\%$) [20].

Hence, it is important to demonstrate that the quality of MMC-derived PHA can consistently meet the standards required for use in commercially interesting plastic applications. Thinking of an implementation at industrial scale, it is worthwhile to identify a method for biomass stabilization that allows to enhance and preserve the thermal and mechanical properties of the PHA and to conduct the recovery step at a later time and/or in a different site. In this regard, a biomass stabilization method has been conducted and optimized at pilot scale, in the context of a MMC-PHA production and fermented mixture of OFMSW and thickened waste-activated sludge (WAS) as urban waste feedstock [11]. Then, the stabilized biomass was subjected to downstream processing. The recovered polymer, entirely produced from renewable resources, was extensively characterized for thermal and chemical properties as well as M_w determination.

2 Materials and methods

Within the pilot platform of Treviso (northeast Italy), the PHA was produced from a feedstock composed by a mixture of the liquid slurry coming from squeezing of the OFMSW and waste activated sludge (WAS) from treatment of urban wastewater. The main process setup (extensively described in Valentino et al. [11]) was a first anaerobic fermentation reactor (380 L) for PHA precursor production (volatile fatty acid, VFA), a second aerobic reactor (sequencing batch reactor, SBR; 100 L) for biomass cultivation, and a third fed-batch aerobic reactor (70–90 L) for PHA accumulation within cellular wall (40–50 wt%). The following approach for PHA

maintenance within cellular wall (or biomass stabilization), extraction, and final characterization is explained in the following paragraph and depicted in Fig. 1.

2.1 PHA maintenance and biomass stabilization tests

The maintenance of PHA within cellular walls has been assessed in the biomass stabilization tests carried out with PHA-rich biomass taken at the end of accumulation step. Each trial was carried out in 0.2-L reactor, filled with biomass from pilot-scale plant. These tests were performed in order to find the best strategy for maintaining constant the PHA level in the cells, or close to the concentration quantified at the end of accumulation step, before the downstream processing. Each test was performed for a relatively long period of 48 h. The PHA concentration was always related to the initial one, determined at the end of accumulation. Five anaerobic conditions were explored:

- Anaerobic starvation by stopping the aeration system.
- Alkaline treatment by adding NaOH 0.2 M solution (up to pH 12.0).
- Acidic treatment by adding H₂SO₄ down to pH 2.0.
- Oxidative treatment by adding 0.1 v/v NaClO solution (5% active Cl₂).
- Thermal treatment at 145 °C for 30 min followed by overnight drying at 70 °C.

In the first four conditions (a–d), the biomass was maintained under mixing for the whole test period. In the last condition (e), the biomass was centrifuged at the end of accumulation, before the starting of thermal stabilization procedure. The determination of PHA concentration was performed by

taking the humid pellet at the end of 145 °C pretreatment and the dried material (final sample), both re-suspended at the same initial total suspended solid (TSS) concentration. Each stabilization test was performed in triplicate.

2.2 PHA extraction and recovery

Five different batches (B1-a, B2-a, B3-a, B4-a, B5-a), with PHA content more than 40% w/w on dry weight, have been selected to perform polymer extraction and characterization. Chloroform extraction and NaClO oxidation have been applied as standard methods. Both extractions have been conducted to recover PHA from dry PHA-rich biomass obtained after thermal stabilization. The NaClO treatment has been also carried out on acid-stabilized wet biomass (B2-a, B2-b).

2.2.1 Chloroform extraction

A 2–5-g sample of dried PHA-rich biomass (thermally stabilized) was put into a glass fiber extraction thimble placed inside of a 50-mL borosilicate glass Soxhlet extractor, equipped with a 100-mL borosilicate glass flask, filled with 70 mL of chloroform, a bubble condenser, and a boiler, in order to keep the solvent at boiling temperature for 24 h. The PHA solution was cooled down at room temperature and then transferred into a crystallizer of Ø = 8 cm, in order to obtain a polymer film by solvent evaporation under fume hood.

2.2.2 Hypochlorite oxidation

Dried PHA-rich biomass (thermally stabilized) or acid-stabilized wet biomass was suspended in a NaClO solution

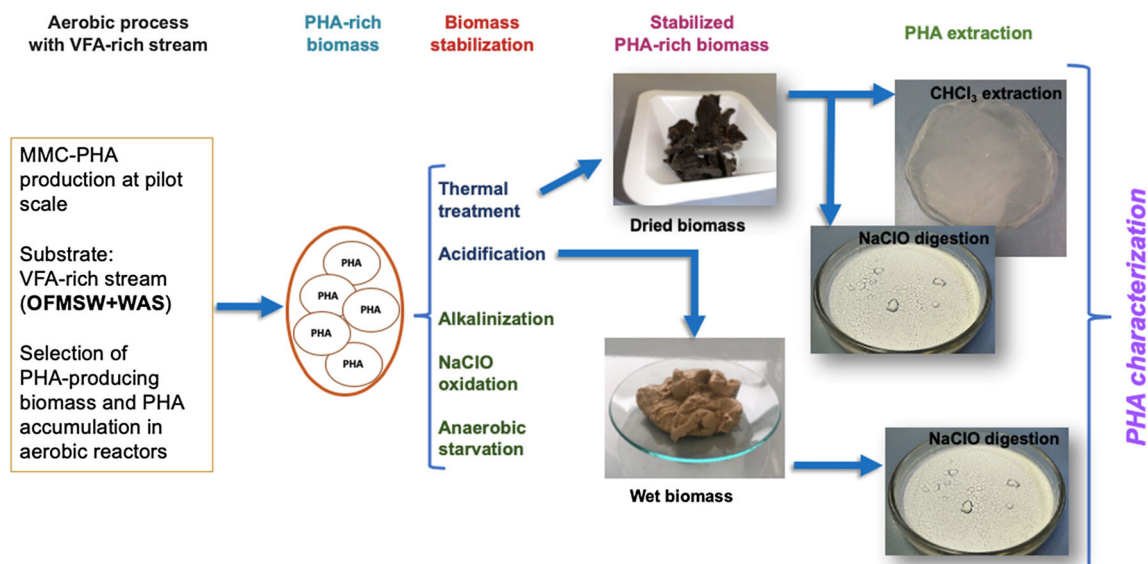


Fig. 1 Methodology applied to PHA-rich biomass for its stabilization (or PHA maintenance within cellular wall), PHA extraction, and final characterization

(1.5% active Cl_2). The suspension was kept under magnetic stirring overnight, centrifuged at 8500 rpm for 20 min, and, then, washed 3 times with distilled water. Wet pellet was finally transferred into a crystallizer and oven-dried at 70 °C.

2.3 PHA characterization

2.3.1 PHA content and composition determination (GC-FID)

Approximately 3.5 mg of dried biomass or 5.0 mL of re-suspended samples (wet and dried thermally treated biomasses) was suspended in 2 mL of acidified methanol solution (at 3% v/v H_2SO_4) containing benzoic acid (at 0.005% w/v) as internal standard and 1 mL of chloroform in a screw-capped test tube. Then, an acid-catalyzed methanolysis of the PHA occurred, and the released methyl esters were quantified by gas chromatography (GC-FID Perkin Elmer 8410) according to the method described in Braunegg et al. [21]. The relative abundance of 3HB and 3HV monomers was determined using a commercial P(3HB-co-3HV) copolymer with a 3HV content of 5 wt% (Sigma-Aldrich, Milan, Italy) as reference standard. The 3HV content in PHA was calculated as the ratio of 3HV and (3HB + 3HV) monomers (as wt%).

2.3.2 Capillary viscosimetry

Two hundred milligrams of dried polymer, extracted with NaClO or solubilized by chloroform, was suspended into a 50-mL borosilicate glass flask, filled with 50 mL of chloroform and kept at boiling temperature for 24 h. Possible non-soluble residues were filtered with GF/D Whatman® microfiber glass filters, and the PHA solution was poured into a crystallizer of $\varnothing = 8$ cm, in order to obtain a polymer film by solvent evaporation under fume hood. A volume of 25 mL of PHA solution at the concentration of 0.5% w/v in chloroform were prepared for all the samples of extracted polymer.

The equipment for the measure of the gravimetric flow time of each solution was composed by a SCHOTT AVS 350 viscosimeter with AVS/SHT sensor, a LAUDA CD15 thermostatic bath (working at 30 °C), and a SCHOTT GERÄTE Ubbelohde capillary viscosimeter (ID = 0.46 mm).

For each sample, 15 mL of solution 0.5% w/v was transferred in the Ubbelohde capillary viscosimeter, and the flow time was measured through the optical sensor. Then, at least 4 dilutions were made directly in the viscosimeter by adding predetermined aliquots of solvent. From the flow time of each solution, the intrinsic viscosity $[\eta]$ of polymer samples was determined. The viscometry average molecular weight (M_v) was calculated according to the Mark-Houwink equation:

$$M_v \rightarrow [\eta] = K \times M_v^\alpha \quad (1)$$

with $K = 7.7 \times 10^{-5}$ and $\alpha = 0.82$ [22]

2.3.3 Thermogravimetric analysis

The thermal stability of the samples was evaluated by thermogravimetric analysis (TGA) using a Mettler TG50 thermobalance. Approximately 5 mg of dried samples was weighted on the balance. The analysis was conducted in nitrogen flow (20 ml/min) by heating the samples from 30 to 500 °C at 10 °C/min.

2.3.4 Differential scanning calorimetry

The thermal properties of the PHA samples were characterized by a differential scanning calorimeter Mettler Toledo differential scanning calorimetry (DSC) 822e. All the experiments were carried out under N_2 flux (30 ml/min) on about 3–6 mg of polymer, weighted in aluminum pans. Unfortunately, because of the possible presence of chlorine traces, the samples treated with NaClO were not analyzed by DSC. The instrument furnace, in fact, has silver components which could be damaged by halogens.

Then, in order to evaluate the intrinsic thermal properties of the dry PHA obtained by solubilization in chloroform, unaffected by the previous sample treatments and thermal history, the following temperature program was selected:

1. A heating scan at 10 °C/min from RT to 190 °C.
2. A rapid cooling at 30 °C/min from 190 to 70 °C and annealing at 70 °C for 30 min.
3. Cooling from 70 to 30 °C and second heating up to 190 °C at 10 °C/min.

The first heating is necessary to erase all the previous sample history. Then, in the isothermal annealing, all the analyzed samples crystallized and reached the maximum crystallinity, independently from chemical composition or molecular weight. In fact, in the last heating ramp, no cold crystallization occurred. The sample crystallinity (X_c) was evaluated from the equation:

$$X_c = 100 \times \Delta H_m / (\Delta H_m^0 \times \text{purity}) \quad (2)$$

where ΔH_m is the melting enthalpy obtained from the first or second DSC heating scan and $\Delta H_m^0 = 146$ J/g is the enthalpy of fusion of 100% crystalline PHB sample [23].

3 Results and discussion

3.1 PHA trends and its maintenance within cellular walls

The stabilization tests showed interesting results during the 48 h of monitoring. Indeed, the comparison of PHA content

data collected for each inhibition test shows that there was not a significant decrease of the polymer for 48 h following the addition of H_2SO_4 (97 wt% of initial content) (Fig. 2a), demonstrating that the agent is suitable for microbial activity quenching and PHA stabilization. The treatment with NaClO could be considered also acceptable with a reduction of 19 wt% of initial polymer content. Shorter time can be also taken into account since the decrease of the PHA content was lower: no PHA loss for H_2SO_4 treatment, around 10 wt% of PHA loss for the oxidative NaClO treatment within the first 4 h.

Then, adding H_2SO_4 up to pH 2.0 did not affect the PHA content, as the literature suggests [24], although the possible effects on the molecular weight were investigated, as shown later in further discussion. On the contrary, it is important to consider that, by adding a strong oxidizing agent (NaClO), besides of a slight decrease of initial content, a reduction of molecular weight could occur. The anaerobic treatment and the addition of NaOH were not effective, showing a PHA loss higher than 40% from 18 h. Even in these cases, shorter stabilization time (up to 4 h) corresponded to lower PHA loss. However, compared with H_2SO_4 and NaClO treatment, alkaline and anaerobic stabilization methods were less effective. The polymer consumption under anaerobic conditions was not unexpected since dynamic conditions, such as those

caused by alternate redox conditions (anaerobic/anoxic/aerobic), have been used to sustain the enrichment of PHA-storing microorganisms [6]. Even though the present biomass was selected under fully aerobic environment, it maintained the capability of polymer degradation under anaerobic conditions. In the case of NaOH, the obtained result could be expected, because the base-catalyzed hydrolysis of polyester chain may possibly occur [25]. Thermal stability has been also reported to be negatively affected by NaOH solution, even in comparison with oxidative biomass treatment (NaClO) [26].

The results of the thermal treatments are reported in Fig. 2b. It is possible to highlight that PHA content is completely preserved until the end of the drying step, in agreement with the observations of Bengtsson et al. [8]. Therefore, during the first period of this work, thermal treatment (including thermal shock at 145 °C for 30 min) has been applied at pilot scale at the end of several batch accumulation tests (including B1-a, B2-a, B3-a, B4-a, B5-a), in order to produce PHA for further extraction and characterization. Moreover, during a second period of the study, the acidification has been applied and the PHA from two batches (B1-b, B2-b) has been extracted and characterized, in order to compare the results of both stabilization methods.

3.2 Extraction performances

Chlorinated compounds [17, 27, 28] or a mixture of chlorinated and non-chlorinated solvents [29] are consolidated extractive agents for PHAs from MMCs. In particular, chloroform is always used in benchmark protocols because it does not damage polymer structure and molecular weight, solubilizing the PHA inside the cellular membranes at low temperature [30]. In previous studies [26, 31], disruption of non-PHA cell material (NPCM) was obtained by using a NaClO solution in different conditions (concentration and retention time) with good recovery and purity level (higher than 90 wt%). In this study, both chloroform extraction and NaClO digestion have been used as consolidated methods for PHA recovery. The first set of extraction tests has been carried out on dry PHA-rich biomass, thermally stabilized at the end of the accumulation step, and the NaClO digestion has been applied also on an acid-stabilized wet biomass. In this case, the extraction has been conducted on the wet centrifuged biomass, in order to skip the drying step and compare the results with those from thermally stabilized biomass, evaluating possible differences and effects on the polymer properties.

Results obtained by GC-FID analysis conducted on extracted samples (Table 1) show that both the extraction methods allowed to reach satisfying performances in terms of purity, with an average value of $93.5 \pm 0.8\%$ w/w and $88.1 \pm 4.9\%$ w/w for CHCl_3 and NaClO, respectively, and recovery yield of $86.8 \pm 3.9\%$ w/w and $78.2 \pm 7\%$ w/w for CHCl_3 and NaClO, respectively. The variability of the HV

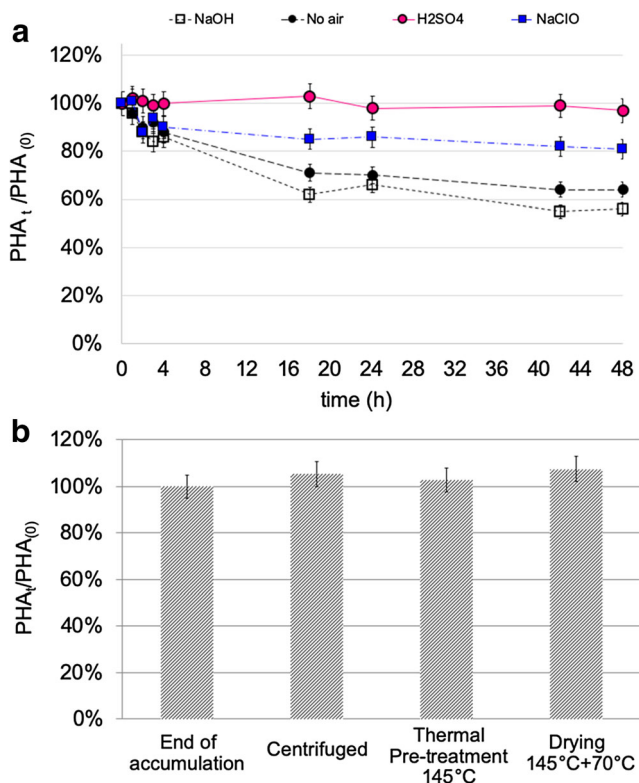


Fig. 2 Relative PHA content variation measured during 48 h after the beginning of the stabilization tests (a). Relative PHA content variation measured during thermal treatments, both with and without thermal shock (b)

Table 1 Extraction performances of thermally stabilized and acid-stabilized samples determined by GC-FID analysis

Sample	PHA content in raw biomass (% w/w)	HV content of PHA in raw biomass (% w/w)	Pre-treatment	Extraction	Purity (% w/w)	Recovery (% w/w)	HV content (% w/w)
B 1-a	56.1 ± 4.4	18.6 ± 0.1	Thermal drying	CHCl ₃	92.9 ± 6.1	80.5 ± 5.3	19.2 ± 0.1
				NaClO	97.2 ± 0.7	75.4 ± 0.5	17.7 ± 0.2
B 2-a	45.8 ± 2.3	7.7 ± 0.4		CHCl ₃	95.2 ± 1.1	97.9 ± 1.1	7.7 ± 0.2
				NaClO	87.0 ± 2.1	83.5 ± 2.0	7.0 ± 0.1
B 3-a	39.6 ± 4.4	13.0 ± 0.7		CHCl ₃	91.5 ± 1.4	89.6 ± 1.1	14.9 ± 0.4
				NaClO	75.2 ± 2.6	52.8 ± 2.2	13.9 ± 0.1
B 4-a	38.7 ± 4.8	13.4 ± 0.6		CHCl ₃	92.4 ± 3.0	75.4 ± 2.5	13.6 ± 0.2
				NaClO	80.3 ± 4.1	85.6 ± 4.4	14.0 ± 0.3
B 5-a	42.3 ± 1.2	22.8 ± 0.1		CHCl ₃	95.4 ± 9.4	90.4 ± 8.9	20.2 ± 0.3
				NaClO	100.9 ± 4.1	93.9 ± 3.8	19.7 ± 0.3
B 1-b	40.1 ± 1.3	13.4 ± 0.3	Wet acidification	NaClO	95.8 ± 2.1	90.0 ± 1.9	13.0 ± 0.1
B 2-b	45.4 ± 0.5	13.7 ± 0.1		NaClO	97.5 ± 1.4	91.8 ± 2.5	13.6 ± 0.1

^a Acid-stabilized wet biomass

content among the samples is probably a consequence of the wide period of time in which the biomass was collected; in fact, the different batches have been produced during 8 months of operation, so that the seasonality effect on the substrate composition and on the temperature has to be taken into account. On the other hand, both the recovery methods preserved the same 3HV content after the extraction tests. According to the extraction performances, there is the evidence that the stabilization method used at the end of the accumulation step did not affect the PHA recovery process from the biomass. In fact, similar PHA purity and recovery have been obtained for each biomass treatment.

3.3 Thermogravimetric analysis

Thermogravimetric analysis allowed to determine important characteristics of the polymers, such as decomposition temperatures and related thermal stability, as well as to confirm GC-FID data on polymer purity. Indeed, according to Hahn and Chang [32], it is possible to estimate the polymer content in the samples from the main weight loss occurring between 250 and 320 °C [32]. The results of the TGA experiments, including the PHA content, the temperatures at 10% decomposition ($T_d^{10\%}$), and the temperature at maximum decomposition rate (T_d^{\max}), are reported in Table 2. Moreover, the TGA thermograms showed that all the impurities were consisted in inorganic material, since residual weight remained at the highest temperature.

The comparison of the results shows that PHA thermal stability was affected by the extraction agent used. Oxidative treatment produced samples with lower thermal stability than that from chloroform extraction for all thermally stabilized samples. In fact, the average $T_d^{10\%}$ and T_d^{\max} of CHCl₃-extracted samples were 272 ± 3 °C and 297 ± 3 °C while for

NaClO-extracted samples, the corresponding values were 259 ± 3 °C ($T_d^{10\%}$) and 278 ± 3 °C (T_d^{\max}), respectively. Compared with the thermally stabilized samples, the PHA samples extracted with oxidative NaClO treatment from wet-acidified biomass showed higher thermal stability, with an average $T_d^{10\%}$ and T_d^{\max} at 269 ± 1 °C and 291 ± 2 °C, respectively.

Overall, such values indicated the high thermal stability of the polymer, comparable or higher to those observed in other studies on MMC-PHA (Table 2) and on commercial P(3HB-co-3HV) (T_d^{\max} 256–290 °C [33]).

Regarding the effect of HV content on the thermal stability, the available data are contradictory, and it does not allow to enunciate a statement. The sample B2-a was characterized by lower HV content (7.7 wt%) compared with others, and it exhibited higher $T_d^{10\%}$ (278 °C) and T_d^{\max} (303 °C) values. However, literature data show that homopolymer P(3HB) (0 wt% HV content) may have a wide range of T_d^{\max} (268–290 °C), comparable with P(3HB-co-3HV) of this study with HV content roughly between 13 and 20 wt%.

3.4 Viscosimetry average molecular weight

The determination of viscosimetry average molecular weight (M_v) of each extracted sample was carried out to check possible polymer chain degradation due to the use of NaClO to disrupt NPCM. The results, reported in Fig. 3, were compared with that obtained from the PHA extracted from the biomass by chloroform, considered the benchmark sample, as discussed above.

The values reported in Fig. 3 showed that there is not a significant difference between the two applied extraction methods on thermally stabilized biomass samples.

Table 2 Comparison between PHA purity determined by GC-FID and TGA analysis; decomposition temperatures determined by TG thermograms

Sample	Feedstock	Pre-treatment	Extraction	TGA purity (% w/w)	$T_d^{10\%}$ (°C)	T_d^{\max} (°C)	References
B 1-a	OFMSW-WAS mixture	Thermal drying	CHCl ₃	100	265	291	This study
			NaClO	89	260	280	
B 2-a		Thermal drying	CHCl ₃	98	278	303	
			NaClO	90	265	284	
B 3-a		Thermal drying	CHCl ₃	97	277	301	
			NaClO	74	265	287	
B 4-a		Thermal drying	CHCl ₃	97	271	290	
			NaClO	75	254	269	
B 5-a		Thermal drying	CHCl ₃	83	267	298	
			NaClO	100	252	272	
B 1-b	OFMSW-WAS mixture	Wet acidification	NaClO	100	268	289	This study
B 2-b		Wet acidification	NaClO	99	270	293	
MMC-PHA	Synthetic VFA mix	–	NaClO	–	–	281–291	[26]
MMC-PHA	Synthetic VFA mix	–	HClO	–	–	267–273	[18]
MMC-PHA	WAS	Freeze-drying	CHCl ₃	–	–	275	[36]
MMC-PHA	WAS	Drying	CH ₃ CH ₂ CH(OH)CH ₃	–	–	291	[33]
MMC-PHA	Molasses	–	CHCl ₃	–	–	277–291	[17]

^a Acid-stabilized wet biomass

As a main result, NaClO digestion did not affect the molecular weight for thermally stabilized samples. In fact, the M_v values of all the five batches (B1-a, B2-a, B3-a, B4-a, B5-a) are comparable: 127 ± 16 and 132 ± 10 kDa for NaClO- and CHCl₃-extracted samples, respectively. Moreover, the M_v determination allowed to evidence any possible effects of the biomass pre-treatment on the PHA by the comparison between M_v of thermally stabilized and the acid-stabilized samples. Indeed, a NaClO digestion has been conducted on an acid-stabilized wet biomass in order to skip the drying step and better understand the differences between the two stabilization methods. As reported in Fig. 3, the two samples B1-b and B2-b show that after extraction, carried out following the same operative conditions of the previous samples, the polymer recovered from acid-stabilized biomass has a significantly

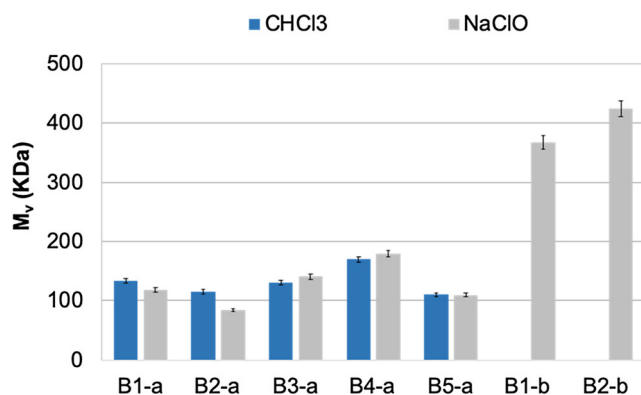


Fig. 3 Viscosimetry average molecular weight of extracted PHA (chloroform and hypochlorite) from thermally stabilized (series “a”) and acid-stabilized (series “b”) biomass

higher molecular weight (370 and 424 kDa, respectively) than that obtained from thermal treatment. This evidence is a confirmation of the effective capacity of acidification to stabilize PHA for downstream processing without affecting its molecular weight [24]. It is general knowledge that M_v depends on PHA chemical stability, which in turn may be strongly affected by chemical impurities [24]. The impurities may be organic (proteins, carbohydrates, and lipids) and/or inorganic (cations such as calcium, magnesium, sodium, and corresponding anions such as phosphate, sulfate, and chloride), and they are components found in the NPCM. The chemical stability of recovered PHA can be improved by removing the impurities; in particular, the acidification method allowed to solubilize ions associated with the biomass. Thereafter, dewatering and separating the ions from the PHA-rich biomass may have improved the PHA stability in the following extraction procedure. In addition, by decreasing the pH to 2.0, the microbial activity was immediately inhibited. This synergistic effect may have contributed to increase the chemical PHA stability, giving (after the extraction) a M_v roughly three times higher than M_v obtained with thermally stabilized biomass.

Contrary to what has been reported in previous studies [34, 35], there is no correlation between the molecular weight and the HV content. It is also true that recent examples of MMC-PHA produced from wastes and/or synthetic VFA mixture do not show a correlation between the M_w and the HV content in the copolymer [36, 37]. According to literature data, the commercial polymers from pure culture systems exhibit a wide range of molecular weight (M_w): 200–660 kDa (similar to the M_v of PHA from wet acid-stabilized biomass); in this

range, higher M_w values were related to the P-3HB homopolymer [33, 38]. On the contrary, it is often reported that MMC is able to produce PHA with higher M_w than these obtained in this study. Previous studies reported M_w of 340–540 kDa [26] and 440–630 kDa [15] for PHA produced from synthetic VFA mixture, 400–600 kDa [39] from crude glycerol fermentation liquid, 800 kDa [38] from percolates of the OFMSW, and 650 kDa [36] from WAS. There are also examples with similar or even lower M_w values compared with those ones obtained in this study: 110–250 kDa [37] from cheese whey and olive mill wastewater and 100–200 kDa [39] from crude glycerol fermentation liquid. All these data, however, have been collected from laboratory-scale equipment. Very few examples have reported PHA production and characterization from larger plant dedicated to waste management and services of wastewater treatment. In this context, PHA with M_w of 400–500 kDa has been produced from surplus full-scale activated sludge [9, 40]. There are no examples describing the pilot-scale MMC-PHA production and characterization from OFMSW-WAS mixture as carbon source.

3.5 DSC analysis

The DSC profiles of the first and second heating of B1-a sample is reported in Fig. 4.

The thermograms of the other samples do not remarkably change from that of B1-a.

The absence of the exothermic peak in the DSC thermograms of the second heating, due to cold crystallization, reveals that the sample reached the maximum crystallinity during the isothermal stage at 70 °C. In both thermograms, the main transition is the melting process occurring in the 130–175 °C temperature range. The double endothermic peak could be due to the melting of thin crystals, recrystallization, and remelting of the thickened crystals during the heating or to the presence of different crystal modification. However, the small but clear exothermic process between the two melting peaks in the first heating lets prevail the first possible

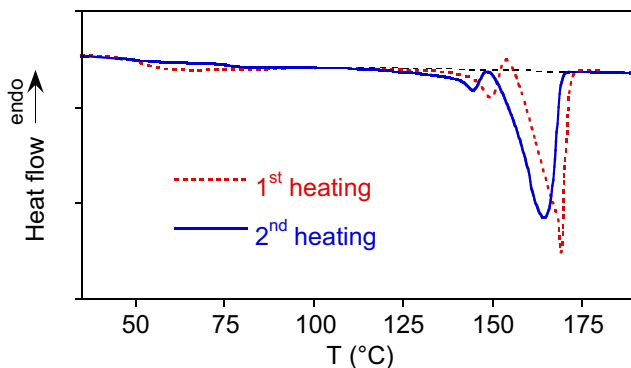


Fig. 4 First and second heating DSC thermograms recorded at 10 °C/min of the sample B1-a. The dashed line is the baseline used to evaluate the integral enthalpy of the melting process

mechanism. Analog interpretation has been given by de Koning et al. [41].

The low temperature transition at about 65 °C (first heating) and 75 °C (second heating) could be assigned to a small copolymer fraction characterized by a high 3HV content [42]. The values of the first (T_m^1) and second (T_m^2) melting peak as well as the enthalpy of fusion (ΔH_m) of each sample are reported in Table 3.

From the integrated melting enthalpy, the sample crystallinity was evaluated by Eq. 2 and reported in Fig. 5 as a function of the 3HB molar content.

The possible variation related to the process operation at pilot scale such as the unavoidable fluctuations of the operating conditions (e.g., organic loading rate, microbial storage response, etc.) could have brought to differences in the PHA synthesis and, as a consequence, in the scattering of the data obtained from the first DSC heating scan. On the other hand, the results of the second run clearly show that the crystallinity linearly increased with the 3HB content in the copolymer. Since the polymer was produced over a wide window time, possible variation of the selected microbial community could have brought to some differences in the copolymer microstructure and, as a consequence, to different thermal behavior. In fact, the PHA properties are not only determined by the monomer composition but also by the mechanisms of substrate consumption and polymer synthesis, which are strictly related to the type of selected microorganisms [17]. However, the results of the second run are good in agreement with the data reported in literature for 3HB concentration higher than about 50 mol% [17, 43]. This let infer that the PHA samples, obtained in different accumulation processes, besides the composition, did not differ in overall chemical structure. A comparison with previous MMC-PHA samples produced from synthetic VFA mixture and other waste streams shows the similarity of both melting temperatures and enthalpy with values of OFMSW-WAS-derived polymer (Table 3).

3.6 Potential applications

Experiences of a consistent carbon source availability and MMC-PHA synthesis under conditions of routine production are a necessary step for defining the product applications. A wider PHA characterization and the development of bioplastic formulations require high amount of material. Even at kilograms order at pilot scale are still insufficient for compounders and/or product-market developers [6]. However, from the screening of data obtained in this study, a first evaluation can be made.

For commodity applications, three mechanical properties are generally considered [1]: the elongation at break, the Young's modulus, and tensile strength. The manipulation of this properties is generally required for reducing the material's brittleness, which has been a reported as obstacle for PHA

Table 3 Thermal properties of the samples measured in the first and second DSC heating scan

Sample	Feedstock	First heating			Second heating			References
		T_m^1 (°C)	T_m^2 (°C)	ΔH_m (J/g)	T_m^1 (°C)	T_m^2 (°C)	ΔH_m (J/g)	
B1-a	OFMSW-WAS	150	169	45	144	164	54	This study
B2-a	mixture	143	159	66	143	158	70	
B3-a		139	157	32	132	155	55	
B4-a		147	167	58	150	167	60	
B5-a		141	160	26	134	157	42	
MMC-PHA	WAS	136	144	24				[36]
MMC-PHA	Cheese whey	126–147	144–163					[37]
MMC-PHA	Olive mill wastewater	133	150					[37]
MMC-PHA	Molasses	129–154	144–165	5–17				[17]
MMC-PHA	Synthetic VFA mix		143–168	1–78				[15]
MMC-PHA	Synthetic VFA mix	89–174	150–172	20–82				[17]

application. At this stage, the available amount of PHA did not allow to set the required tests. However, the composition of synthesized PHA (roughly 7–20 wt% 3HV content) could mean good stiffness, improved toughness (and/or brittleness), and increased flexibility to pure P(3HB) usually produced by pure culture. It is also true that the PHA mechanical properties may deteriorate when the M_w is lower than 400 kDa, and for thermoplastic applications, the M_w should be higher or equal to 600 kDa [1]. Hence, the PHA extracted from thermally stabilized biomass is undoubtedly unsuitable for thermoplastic applications; on the contrary, the PHA derived from wet-acidified biomass deserves further investigation.

One of the market scenarios which could not require PHA at high M_w (or eventually at a level of purity lower than 98–100 wt%) is the groundwater remediation with permeable

reactive barriers (PRBs) where zero-valent iron (ZVI) is employed as reactive media for treating plumes polluted by chlorinated hydrocarbons [44]. The patented PRB-ZVI technology needs a long-lasting slow-release organic source (e.g., PHA) to enhance the degradation performance of ZVI barriers, stimulating the biological reductive dechlorination of the PRB [45].

The future commercial developments need to include fundamental advancement in the downstream processing, which in turn requires a demonstration production scale (in the order of tons of PHA produced per year). This is necessary for the material processing and definition of market applications.

4 Conclusions

The present work showed the importance of applying an adequate and efficient stabilization method on a PHA-rich biomass, in order to preserve the properties and the amount of the polymer before the downstream processing. At the end of the accumulation step conducted at pilot scale, thermal treatment and acidification lead to a complete biomass inhibition and stabilization of the PHA amount. However, only acidifying the PHA-rich biomass allowed to recover a high molecular weight polymer (370–424 kDa). Moreover, no correlation has been found among 3HV content and molecular weight, while the crystallinity of the PHA was significantly affected by the copolymer composition [1, 43]. Further investigation on extraction and characterization of PHA from acidified biomass is needed. More thorough analysis has to be carried out for the determination of thermal and mechanical properties of the more recently produced polymer.

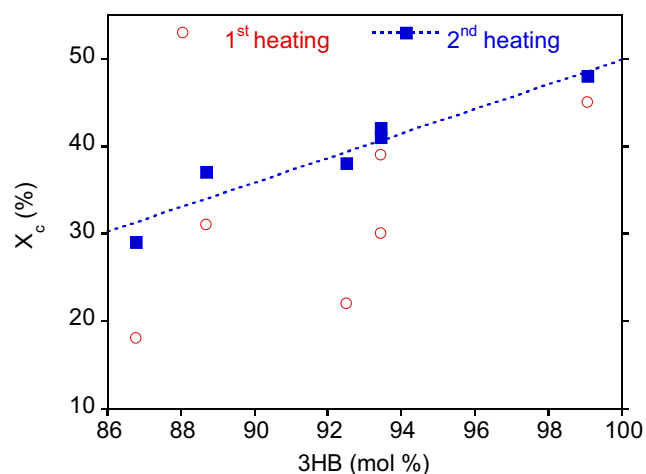


Fig. 5 Sample crystallinity measured in the first and second DSC heating scan as a function of the 3HB molar content in the copolymer samples. The dashed line is a guide for the eyes

In fact, up to now, studies have been focused on correlations between process parameters, feeding strategies, and final mechanical properties for mixed culture PHA, but researches on the characterization of PHA from OFMSW and WAS are still missing. Thanks to the very recent diffusion of pilot-scale processes involving solid wastes for PHA production, it is now important to demonstrate that the chemomechanical properties are comparable with those of pure culture PHA and can be modulated on the basis of future applications.

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