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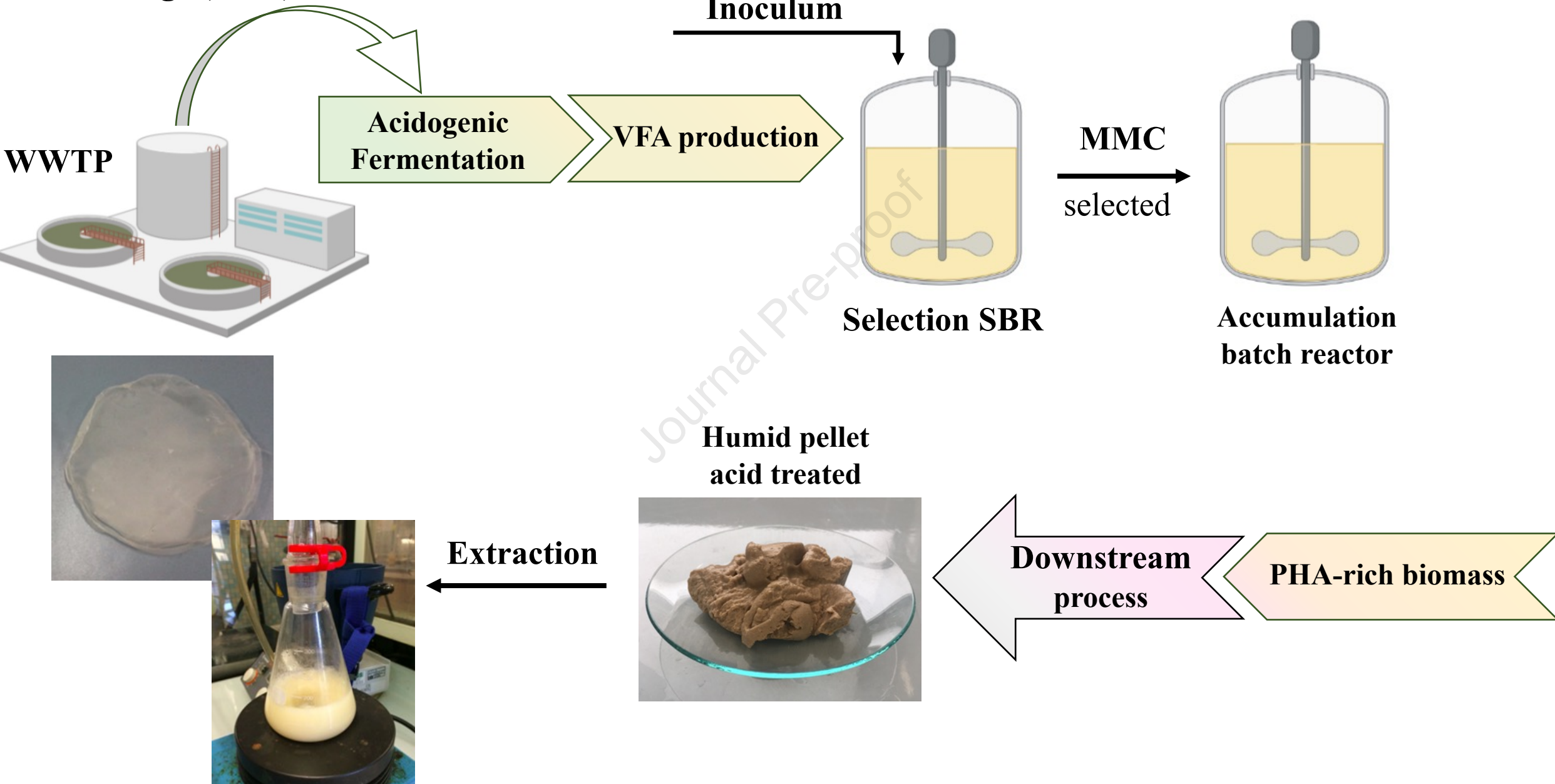
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Author contribution

Laura Lorini: Data curation, Formal analysis, Methodology, Writing - original draft; Writing - review & editing. **Gianluca Munarin:** Formal analysis, Investigation, Methodology. **Gaia Salvatori:** Data curation, Formal analysis, Writing - original draft. **Sara Alfano:** Data curation, Formal analysis, Writing - original draft. **Paolo Pavan:** Funding acquisition. **Mauro Majone:** Visualization. **Francesco Valentino:** Project administration, Resources, Conceptualization, Supervision, Validation, Writing - original draft, Writing - review & editing.

Thickened Waste Activated Sludge (WAS)



Sewage sludge as carbon source for polyhydroxyalkanoates: a holistic approach at pilot scale level

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Abstract

In the present study, polyhydroxyalkanoates (PHA) production by mixed microbial cultures (MMC) has been carried out using thermally pre-treated excess thickened waste activated sludge (WAS), applying the feast-famine approach at pilot scale. The preliminary results of WAS fermentation conducted both in mesophilic and thermophilic conditions, in combination with a thermal pre-treatment at 70 °C for 48 h, highlighted how the thermal hydrolysis has a crucial role for the solubilization of the chemical oxygen demand (COD), which allows a significant increase of the final volatile fatty acid (VFA) concentration (roughly 8.5 g COD_{VFA}/L). Since thermophilic fermentation after thermal pre-treatment was the best performing condition, it has been applied at pilot scale in order to routinely produce VFA as precursors for the following PHA synthesis. The selection and enrichment of PHA-producing biomass was successfully established and maintained in a Sequencing Batch Reactor (SBR) during the whole experimentation period, under short hydraulic retention time (HRT; 2 days) and medium-low organic loading rate (OLR; 2.0-2.2 g COD/L d). At the end of the production process an average PHA content of 53 ± 3 % w/w was achieved. The polymer was finally extracted and recovered from the biomass using traditional chloroform and sodium hypochlorite extraction and then characterized for the quantification of thermal properties ($T_m = 156.8 - 160.9$ °C) and molecular weight ($M_v = 396 - 405$ kDa). A final overall mass balance, usually poorly reported in the literature, has been also assessed, resulting in an overall yield of 56 g PHA per kg of volatile solids (VS).

Keywords: Polyhydroxyalkanoates; Waste activated sludge; Acidogenic Fermentation; Molecular weight; Biorefinery

Abbreviations

3HBp: 3-hydroxybutyrate precursors, 3HV: 3-hydroxyvalerate, 3HVp: 3-hydroxyvalerate precursors, BNR: biological nutrient removal, COD: chemical oxygen demand, CPS: cellulosic primary sludge, DO: dissolved oxygen, DSC: differential scanning calorimetry, FID: flame ionization detector, GC: gas chromatograph, HRT: hydraulic retention time, MMC: mixed microbial cultures, Viscosimetric molecular weight: M_v , NPCM: non-polymer cellular material, OFMSW: organic fraction of municipal solid waste, OLR: organic loading rate, PHA: polyhydroxyalkanoates, PHB: polyhydroxybutyrate, SBR: sequencing batch reactor, TGA: thermogravimetric analysis, TS: total solids, TSS: total suspended solids, VFA: volatile fatty acids, VS: volatile solids, VSS: volatile suspended solids, WAS: waste activated sludge, WWTP: wastewater treatment plant.

Word Count: 8401

1 Introduction

Large quantities of sewage sludge, produced inside wastewater treatment plants (WWTPs), require adequate and environmentally safe management and disposal. Indeed, sludge management is one of the most critical issues in WWTPs operation, and it can represent from 20% to 60% of the overall operating costs (Andreoli et al., 2007). During the last few decades, sewage sludge conversion into value-added products, such as bioenergy or biobased materials, became significantly attractive. This alternative has the advantage of limiting the disposed sludge quantity and can contribute to reduce the overall operating costs (Alloul et al., 2018). In this context, anaerobic digestion is a very useful and consolidated technology for sludge management, since it can convert the biodegradable organic carbon into biogas (Alloul et al., 2018). On the other hand, WWTPs and anaerobic digesters are often designed with spare capacity to cater for variation in the wastewater flow and future population growth (Morgan-Sagastume et al., 2016). However, recent progress in water conservation and slower population growth in many developed countries have left many wastewater treatment facilities with oversized digesters not utilized. In addition, volatile fatty acids (VFAs), as an important intermediate product of anaerobic digestion, are a promising substrate for many different bioprocesses (Luo et al., 2019), e.g., biopolymer production (Valentino et al., 2018) bioenergy generation (Lee et al., 2014), as well as biological nutrient removal (BNR) (Liu et al., 2018). Indeed, VFA-rich liquid has great potential to be used directly as an external carbon source in biological nutrient removal which can make WWTPs self-sufficient plants in terms of chemical use (Zhang et al., 2013). Therefore, in recent studies, the use of VFAs, produced from co-digestion of sewage sludge and external organic waste (Owusu-Agyeman et al., 2020) or from acidogenic fermentation of cellulosic primary sludge (CPS) (Da Ros et al., 2020), as a carbon source for BNR has been evaluated. In this view, Da Ros et al. (2020) developed an economical assessment of two scenarios for the implementation of cellulosic

primary sludge recovery considering the anaerobic digestion as reference scenario. The results showed that the production of bio-based VFAs from CPS as carbon source in BNR and/or as chemical precursors for biopolymers synthesis give higher net benefits instead of the only biogas production. Moreover, even though anaerobic digestion is a mature and sustainable technology for sewage sludge valorization, other recent studies indicated the viability of applying alternative technologies with the aim of producing or recovering higher-value products, such as polyhydroxyalkanoates (PHAs) (Alloul et al., 2018; Kleerebezem et al., 2015; Morgan-Sagastume et al., 2016). PHAs are biodegradable polyesters synthesized by several types of bacteria able to accumulate PHA as intracellular carbon and energy source, under nutrient-limited growth conditions (Lorini et al., 2020). Since they are completely biodegradable and they have thermoplastic properties comparable to traditional plastics, the interest on these biopolymers is progressively increasing, also considering the possibility to exploit organic fermentable wastes as substrates for their synthesis and to produce them from mixed microbial cultures (MMC) (Rodriguez-Perez et al., 2018). Sewage sludge is currently one of the most available waste within urban scenarios, however disposal problems are encountered and several legislations within European Union regulate sludge management (Li et al., 2018). More recently, Italian legislation included the production of biopolymers as one of the alternatives for energy and materials recovery from sludge (“Discipline for the management of waste consisting of sewage sludge and implementation of Directive 86/278/CEE concerning the protection of the environment, especially the soil, in the use of sewage sludge, 2019). Therefore, integration of PHAs production and wastewater treatment may help to both solve waste disposal issues and increase PHA competitiveness with the traditional fossil-based polymers for the plastics industry.

During the last few years, some studies described different methods for PHA production at pilot scale, to be integrated in existing WWTPs by using available resources: the organic fraction of municipal solid waste (OFMSW) (Valentino et al., 2018), mixture of OFMSW and waste activated sludge (WAS) (Moretto et al., 2020a), WAS and municipal wastewater (Morgan-Sagastume et al., 2015); primary sludge and municipal wastewater (Bengtsson et al., 2017); WAS and anaerobic

digestion rejected water (Frison et al., 2015). In the view of PHA production implementation, the quality of the polymer needs to be also considered. Previous studies demonstrated the possibility of producing biobased materials from waste-derived PHA at high viscosimetric molecular weight (M_v), obtained by electrospinning for packaging applications (Melendez-Rodriguez et al., 2020). On the other hand, PHA characterized by low M_v or even stabilized PHA-rich biomass stabilized may be exploited in different environmental application: both extracted PHA and raw PHA-rich biomass are an efficient slow-release carbon source in anaerobic conditions for enhancing the biological reductive dichlorination in the frame of groundwater remediation from chlorinated hydrocarbons (Amanat et al., 2021a; Amanat et al., 2021b). These results clearly suggested the possibility to directly use the PHA-rich biomass derived from waste, with no need of extraction procedures, bringing remarkable advantages from an economic and environmental point of view.

The present study focuses on PHA production by MMC using pre-treated excess thickened WAS, applying the feast-famine approach in a traditional three-step process, developed at pilot scale in Treviso municipality (northeast of Italy). Furthermore, the paper shows the preliminary results of the fermentation of WAS conducted both in mesophilic and thermophilic conditions, also evaluating the effects of a thermal pre-treatment. At the end of the production process, PHA synthesized from WAS has been extracted from the biomass and then characterized for the quantification of thermal properties and molecular weight. A final mass balance, usually poorly reported in the literature, has been also assessed.

2 Materials and methods

2.1. Organic substrate

The substrate used in this study was thickened WAS from the Treviso WWTP. The WAS has been collected from the static thickener of the full-scale plant. Thickened WAS had a TS contents of 30 ± 2 g TS/kg, with a volatile solid (VS) fraction of 77% VS/TS. The substrate had a soluble COD

(COD_{SOL}) = 0.9 ± 0.1 g/L and a low concentration of nutrients (ammonium N-NH₄⁺ = 0.16 ± 0.03 g/L; phosphate P-PO₄³⁻ = 0.32 ± 0.04 g/L).

2.2. Batch fermentation tests

Batch tests were performed to investigate the effects of temperature (T) on WAS fermentation. Mesophilic (37 °C) and thermophilic (55 °C) conditions were tested. In parallel, another thermophilic trial was performed after a thermal pre-treatment (70 °C for 48 h). Each test was performed in triplicate, in 1.0 L glass bottles sealed with a silicon plug and an aluminum seal (working volume 0.85 L). A volume of 50 mL of inoculum screw-pressed food waste was added (65 gTS/kg) since a sufficient amount of fermentative microorganisms was required. Batch fermentation tests were monitored up to ten days. Liquid samples (15.0 mL) were collected each day for VFA analysis and pH measurements as well as for N-NH₄⁺, P-PO₄³⁻ and COD_{SOL}. The slurry was manually mixed twice a day, one hour before and 6 h after each sampling. The bottles were opened for the sampling and then maintained under helium flux for 10 min to re-establish anaerobic conditions.

2.3. Pilot reactors

A process scheme of all the pilot units installed at the Treviso WWTP is presented in Figure 1. The acidogenic fermentation was carried out in an anaerobic batch reactor (V = 380 L). Several batch tests were conducted for 7 days, to produce a VFA-rich stream fermentation liquid (FL). The pH (5.0 – 5.5) was not controlled since the alkalinity of the feedstock ensured enough buffer capacity. The reactor was operated at 55 °C, with thermally pre-treated sludge. The thermal pre-treatment consisted in the application of high temperature (70 °C) for 48 h to the feedstock inside the fermentation reactor. After this time, the reactor temperature was decreased and maintained at 55 °C for 5 days. A centrifugation unit (C) was used for the first solids/liquid separation, necessary for obtaining a cleaner filtered fermented stream (C-FL), which was then ultrafiltered with a ultrafiltration ceramic membrane module (unit “UF”) as described elsewhere (Moretto et al. 2020a) for the production of

the ultrafiltered fermentation liquid (UF-FL). In the aerobic line, the PHA-MMC selection was conducted in a 100 L working volume sequencing batch reactor (SBR). The SBR stimulated the storage response of initial activated sludge (inoculum), selecting a PHA-accumulating consortium. The activated sludge was collected from the domestic full-scale WWTP of Treviso. The aerobic run was conducted at 2.0 days of HRT and a cycle length of 12 h. The reactor was aerated by means of linear membrane blowers (Bibus EL-S-250), which allowed stirring the system. The temperature (22–25 °C, maintained with immersion heater) and pH (8.5 – 9.0) were continuously measured but not controlled. The applied OLR was set at 2.0 - 2.2 g COD_{SOL}/L d. The PHA accumulation reactor was operated in fed-batch mode with the same equipment as the selection reactor, with a working volume ranging between 70 L and 90 L. The operation of reactors was automated and controlled via a programmable logic controller (myRio Labview by National Instrument) except for the transfer of biomass from the selection/enrichment step to accumulation, and the treatment of VFA-rich stream in the two solid/liquid separation units, which were manually conducted.

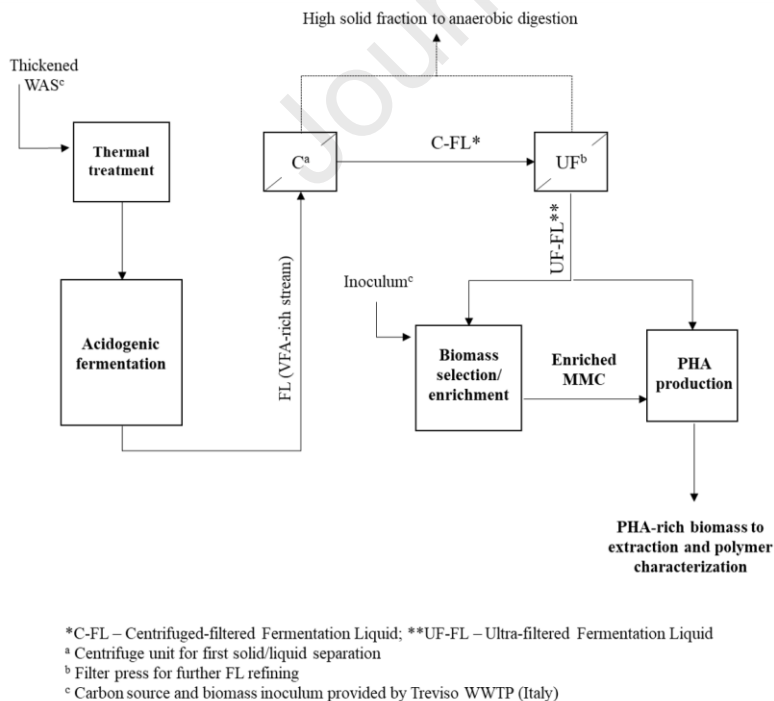


Figure 1. Process scheme developed at pilot scale for WAS valorization toward PHA production.

2.4 PHA-rich biomass stabilization and PHA extraction

At the end of the accumulation tests, PHA-rich biomass was collected, acidified until pH 2 by adding H₂SO₄ (Lorini et al., 2021a). Then, it was left settle under gravity and the thickened slurry was centrifuged in a Heraeus Megafuge 40 with a Swinging Bucket Rotor (maximum radius: 195 mm; minimum radius: 83 mm) from Thermo Fisher Scientific (Waltham, MA, United States) for 15 min at 4500 rpm. The wet pellet was stored in the fridge at 4 °C until extraction.

Acidified PHA-rich biomass has been treated by two standard methods for recovering PHA, such as solvent extraction with chloroform (benchmark) and the NaClO oxidation, following the procedures reported in a previous work (Lorini et al., 2021a).

2.5 PHA characterization

Capillary Viscosimetry

A volume of 20 mL of PHA solution in chloroform (0.6 % w/v) was transferred into the Ubbelohde capillary viscosimeter and the flow time was measured through the optical sensor. At least four dilutions were made directly into the viscosimeter by adding predetermined aliquots of solvent, a flow time of each solution was measured to determine the intrinsic viscosity [η] of polymer samples. The viscosity average molecular weight (M_v) was calculated according to a procedure described elsewhere (Lorini et al., 2021a). The gravimetric flow time measurement was performed by a SCHOTT AVS 350 viscosimeter equipped with a AVS/SHT sensor, a LAUDA CD15 thermostatic bath (working at 30 °C), and a SCHOTT GERÄTE Ubbelohde capillary viscosimeter (ID = 0.46 mm).

Thermogravimetric Analysis (TGA)

The thermal stability of the extracted PHA was evaluated by TGA using a Mettler TG50 thermobalance. About 4-8 mg of dried samples were weighted on the balance. The analysis was conducted in N₂ flow (20 mL min⁻¹) by heating the samples from 30 to 500 °C at 10 °C min⁻¹.

Differential Scanning Calorimetry (DSC)

The thermal properties of the PHA samples were determined by a differential scanning calorimeter Mettler Toledo DSC 822e. All the analysis were carried out under N₂ flux (30 mL min⁻¹) on about 4–8 mg of polymer, weighted in aluminum pans. The intrinsic thermal properties of samples were evaluated applying to the following temperature program: a) first heating scan at 10°C min⁻¹ from RT to 190°C; b) rapid cooling at 30°C min⁻¹ from 190 to -70°C; c) second heating up to 190°C at 10°C min⁻¹. The first heating is necessary to erase all previous sample thermal history. The sample crystallinity (χ_c) was evaluated as described elsewhere (Lorini et al., 2021a).

2.6 Analytical methods

Biomass from the selection SBR was collected twice per week for total suspended solids (TSS), volatile suspended solids (VSS), PHA, ammonium and phosphate characterization. All analyses were performed according to Standard Methods (APHA, 1998) except for VFA and PHA. VFAs were quantified by means of an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionization detector (FID) (T = 230 °C) and an Agilent J&W DB-FFAP fused silica capillary column (15 m length, 0.53 mm i.D. × 0.5 mm film) using hydrogen as carrier. The inlet (T = 220 °C) was working in split mode with a split ratio of 20:1. The instrument operated with a ramp temperature from 80 °C to 200 °C with 2- ethyl butyric acid used as internal standard. Sample preparation before GC analysis included centrifugation at 4500 rpm for 5 min, subsequent filtration of the supernatant through 0.2 mm acetate cellulose syringe filters (Whatman®) and acidification at pH 2 with orthophosphoric acid. PHA samples with 5.0 mL of unfiltered mixed liquor were treated with 1.0 mL NaClO solution (5% active Cl₂) and then stored at 4 °C for following analysis. The polymer was extracted, hydrolysed and esterified to 3- hydroxyacyl methyl esters and quantified by a GC method (Braunegg et al., 1978). 3-Hydroxybutyric (3HB) methyl ester and 3- hydroxyvaleric (3HV) methyl ester monomers were quantified using P (3HB-co-3HV) Sigma-Aldrich standard polymer at 5 wt% HV content as a reference.

2.7 Calculations

Fermentation yield (Y_{VFA}) were quantified in relation to the characteristics of initial unfermented feedstock, in particular the $VS_{(0)}$, as it follows: $Y_{VFA} = \Delta COD_{VFA}/VS_{(0)}$.

All the parameters characterizing SBR reactor performances were calculated after pseudo steady state was achieved. The pseudo steady state was recognized when the feast phase length remained approximately constant (within a 5% deviation from average) for at least one week. In each SBR cycle, the dissolved oxygen (DO) was continuously monitored in order to identify the period in which the readily biodegradable substrates (mainly VFA) were exhausted (end of feast phase), as indicated by a sudden increase of the O_2 concentration. The non-polymer biomass (or active biomass, X_A) was calculated as the difference between VSS and PHA concentrations: $X_A = VSS - PHA$. The biomass PHA content (g PHA/g VSS) was defined as the ratio between the PHA and VSS concentrations. For rates and yields calculation, the mass values of X_A , PHA and VFA were converted into COD units by using the relative conversion factor from oxidation stoichiometry. The storage yields were defined by the ratio between the produced PHA and the consumed VFA in the feast phase: $Y_{P/S}^{feast} = \Delta COD_{PHA}/\Delta COD_{VFA}$. The observed yield was calculated as it follows: $Y_{OBS}^{SBR} = X_A/(OLR \cdot HRT)$, considering the definitions of both HRT (ratio between reactor's volume "V" and influent's flow rate "Q"; $HRT = V/Q$) and OLR (ratio between influent's concentration "C" and HRT; $OLR = C/HRT$). In fed-batch accumulations, the final PHA content in the biomass was calculated at the end of each test as explained above.

3 Results and discussions

3.1 Batch fermentation tests

The fermentation process converts bio-degradable organic compounds (represented by carbohydrates, proteins, and lipids) to volatile fatty acids, carbon dioxide (CO_2) and hydrogen (H_2), in the absence of elemental oxygen (Ramos-Suarez et al., 2021). The conversion pathways of the substrate occur in

three stages: hydrolysis, acidogenesis and acetogenesis. Three sets of batch tests were conducted at different temperature starting from the same sample of thickened WAS. Hence, the initial solids as well as the macronutrients (nitrogen and phosphorus) content were similar in all trials. VFA concentrations were monitored daily for each batch test. Figure 2 shows the average VFA concentrations in the thermophilic (A), pre-treated thermophilic (B) and mesophilic (C) batch tests, which have been performed in triplicate for 7 – 10 days. In terms of VFA production rates and yields, the pretreated thermophilic condition was better performing. Some literature studies report that thermophilic temperature ranges are preferable to mesophilic ones for sludge (primary or WAS) fermentation process (Ramos-Suarez et al., 2021). However, in each condition tested in this study, with or without thermal pre-treatment, VFA production started immediately. Overall, VFA concentrations obtained under condition A (7.7 ± 0.3 g COD_{VFA}/L) and B (8.8 ± 0.1 g COD_{VFA}/L) are higher than those obtained under condition C (5.2 ± 0.3 g COD_{VFA}/L), as literature studies suggest for WAS fermentation (Garcia-Aguirre et al., 2017; Ramos-Suarez et al., 2021). Indeed, thermal pre-treatment and thermophilic temperatures favored the organic matter solubilization, making solubilized COD more easily and efficiently converted into VFA. Moreover, VFA production was significantly improved after the organic matter pre-treatment at 72 °C: approximately 9.0 g COD_{VFA}/L were achieved in 8 days, while 7.7 g COD_{VFA}/L were obtained after 9 days from the thermophilic (A) tests and only 5.2 g COD_{VFA}/L were reached in 8 days in mesophilic tests (C). As expected, thermophilic temperatures enhance the hydrolysis process in which complex molecules are solubilized and become more available for the conversion into VFAs (Ramos-Suarez et al., 2021; Zhang et al., 2019). Indeed, in these tests an even higher maximum VFA concentration was achieved (9.1 g COD_{VFA}/L), in comparison with that reported by (Zhang et al., 2019) in which sewage sludge was fermented at 55 °C after thermal pre-treatment (110°C, 1 h) and a maximum VFA concentration of 8.4 ± 0.1 g COD_{VFA}/L was obtained. Overall, the maximum VFA concentration reached in condition B was higher than those reported in the literature regarding sludge fermentation (0.9 – 7.7 g COD_{VFA}/L) (Ramos-Suarez et al., 2021).

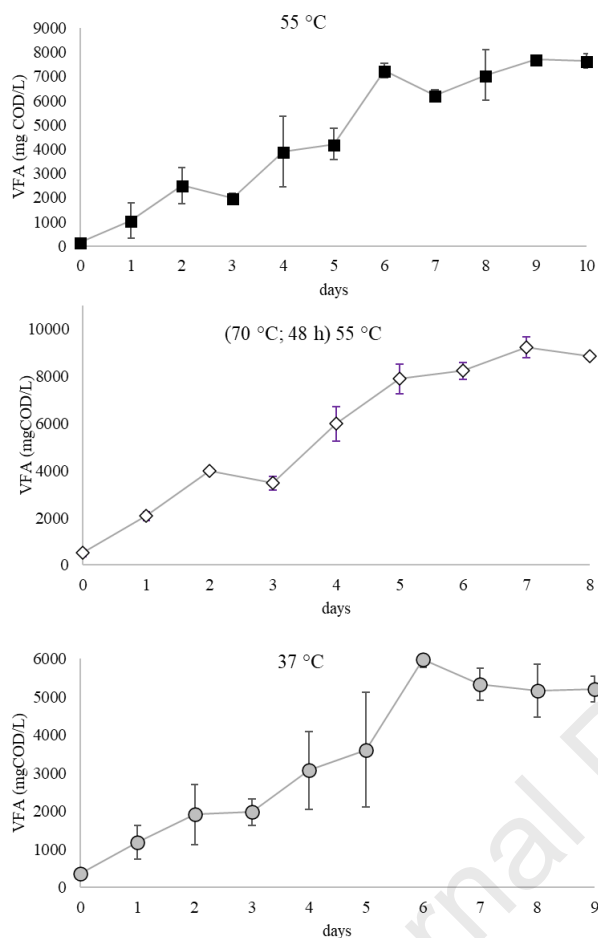


Figure 2. Average VFA concentration monitored during the three replicates of batch tests in each condition: thermophilic (A); thermophilic with thermal pre-treatment at 70 °C for 48 h (B); mesophilic (C).

3.1.3. VFA distribution

The distribution of VFA in the fermented feedstock is an important aspect of the process, especially if the production needs to be addressed to a specific group of VFA as feedstock for MMC-PHA production. Indeed, the content of even numbered C-atoms VFA (3-hydroxybutyrate precursors; 3HBp) and the odd numbered C-atoms VFA (3-hydroxyvalerate precursors; 3HVp) affects monomers synthesis and consequently the PHA composition (Estevez-Alonso et al., 2021). In the tests conducted in the present study, the qualitative VFA composition was not dependent from the fermentation conditions, since a decreasing dominance of acetic, butyric, propionic and valeric acid was observed in all trials. As shown in Figure 3, acetic, butyric and propionic acids were the most abundantly

observed in the thermophilic WAS fermentation (respectively 55 %, 22 % and 18 % COD basis; condition A) and in the thermophilic fermentation of thermally pre-treated WAS (58 %, 19% and 15% COD basis; condition B), according to previous literature investigation (Garcia-Aguirre et al., 2017). In both thermophilic conditions, valeric acid was present in a low percentage (8 and 5 %, for condition B and A respectively). The average acetic, propionic and butyric concentration achieved in thermophilic tests were quite similar independently from the thermal pretreatment (4.14, 1.35 and 1.66 g COD/L in condition A; 5.20, 1.35 and 1.71 g COD/L in condition B). Contrary, average valeric acid concentration doubled when the thermal pretreatment at 70 °C was applied (0.38 g COD/L in condition A; 0.72 g COD/L in condition B). Overall, the relative VFA percentage was affected by the temperature more than thermal pretreatment. In particular, acetic and butyric acid showed a 50% decrease (roughly), with a final concentration of 2.27 and 0.97 g COD/L respectively (series C). On the other hand, valeric acid double its concentration up to 0.81 g COD/L (series C) compared to thermophilic value (0.38 g COD/L; series A); and propionic acid remained almost constant in all trials. As a consequence, in the three conditions tested, the molar fraction of acids containing an odd number of carbon atoms (i.e. propionic and valeric) with respect to total VFA $[3HVp/(3HBp+3HVp)_{VFA}]$ was always oriented to the predominance of acids with even number of C-atoms, but more strongly in the two series (A-B) conducted at thermophilic temperature. In fact, mesophilic tests showed higher $3HVp/(3HBp+3HVp)_{VFA}$ molar ratio (i.e. 0.28 mol/mol) than those obtained in thermophilic ones (0.16 and 0.14 mol/mol, respectively for A and B sets). It is well known that this parameter can be used to characterize complex VFA mixtures and to predict polymer composition in terms of monomers stoichiometry (Estevez-Alonso et al., 2021).

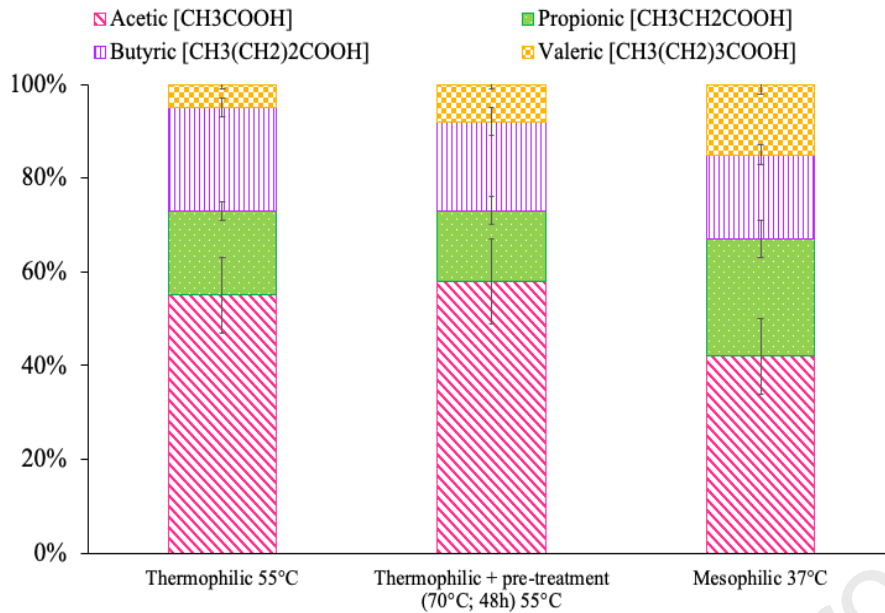


Figure 3. VFA distribution obtained from the three tested conditions.

3.2 Fermentation of waste activated sludge for PHA production

The best condition in terms of maximal VFA concentration and VFA/COD_{SOL} ratio was replicated at pilot scale, to produce the UF-FL for the enrichment of a PHA-producing bacteria and PHA accumulations in the aerobic PHA line. Table 1 summarizes the main features of the UF-FL, related to the thermophilic fermentation of the thickened and thermally pre-treated sludge. As recently reviewed by Ramos-Suarez et al. (2021), significantly lower VFA concentrations can be obtained from sludge fermentation in comparison with other carbon sources such as fruit waste, OFMSW or OFMSW-WAS mixture (Moretto et al., 2020a). However, a higher average VFA concentration (8.4 ± 0.3 g COD_{VFA}/L) compared to those reported in literature for WAS fermentation (Ramos-Suarez et al., 2021) has been reached in this study. Indeed, thermal pre-treatment led to a significant COD and VS solubilisation, as explained previously in batch trials, together with an increase in nutrient concentration (0.76 ± 0.05 g N-NH₄⁺/L and 0.55 ± 0.03 g P-PO₄³⁻/L). Therefore, high COD and VS conversion into VFA were observed, leading to a similarly high fermentation yield (Y_{VFA}) of 0.38 ± 0.04 kg COD_{VFA}/kg VS₀, and COD_{VFA}/COD_{SOL} ratio of 0.74 ± 0.01 . The latter is recognized as a crucial parameter for an efficient enrichment of a PHA-accumulating consortia (Morgan-Sagastume

et al., 2015). Similar considerations were also discussed in a previous study (Moretto et al., 2020a) where WAS and OFMWS mixture were fermented in thermophilic condition after thermal pre-treatment, showing higher Y_{VFA} (0.65 kg COD_{VFA}/kg VS₀) and COD_{VFA}/COD_{SOL} ratio (0.86), compared to the results obtained without thermal pre-treatment (Y_{VFA} 0.40 kg COD_{VFA}/kg VS₀, COD_{VFA}/COD_{SOL} ratio 0.75). In this study, the thermal pre-treatment was applied to the sole WAS, more difficult to be fermented compared to the OFMSW; therefore, such pre-treatment was a strictly required step to enhance the solubilization of the organic solids and facilitate the acidogenic fermentation process. Hence, even though applied to the sole WAS, the thermal pre-treatment promoted the fermentation performances, as also suggested by the literature (Y_{VFA} 0.19 kg COD_{VFA}/kg VS₀; COD_{VFA}/COD_{SOL} ratio 0.45, Zhang et al., 2019). In terms of composition, acetic acid was predominant as in the batch tests (46 % of total VFA, COD basis), followed by butyric (31 %), propionic (17 %) and valeric (6 %). The average $3HVp/(3HBp+3HVp)_{VFA}$ ratio was equal to 0.20 ± 0.01 mol/mol. In terms of routine process operation, the numerous batch fermentation showed low fluctuations in the product quality (VFA content and composition). This is a fundamental aspect, extremely important for the final biopolymer quality and characteristics, among others the stability of the monomeric composition.

As mentioned above, the fermentation effluent was subjected to a first centrifuge filter and then to an ultrafiltration membrane module prior to be used in the aerobic PHA line. Soluble COD and VFA were not affected by the filtration at all as well as for soluble nutrient level.

Table 1. Parameters of thickened WAS fermentation liquid after ultrafiltration section (UF-FL) in thermophilic trials conducted at pilot scale.

Parameter	Unit	Average	Dev. Std.
COD _{SOL}	g/L	11.4	0.5
COD _{VFA}	g/L	8.4	0.3
N-NH ₄ ⁺	g/L	0.76	0.05
P-PO ₄ ³⁻	g/L	0.55	0.03
Acetic	gCOD/L	3.9	0.3

Propionic	gCOD/L	1.4	0.2
Butyric	gCOD/L	2.6	0.2
Valeric	gCOD/L	0.52	0.04
3HVp/(3HBp+3HVp) _{VFA}	mol/mol	0.21	0.01

3.3 Aerobic line: Biomass Selection and PHA production

The selection and enrichment of PHA-accumulating biomass was carried out in a pilot-scale SBR for about 120 days. The inoculum was subjected to dynamic feeding strategy, and it was regularly fed with the UF-FL coming from the two solid/liquid separation units after the fermentation step. As reported in Figure 4 A, a marked difference between PHA concentration at the end of the feast phase and at the end of the famine phase (i.e. the end of the cycle) was well established and stable for all the run. Despite the not negligible fluctuation in PHA concentration at the end of the feast (464 ± 12 mg PHA/L), the one measured at the end of the cycle was more stable (87 ± 6 mg PHA/L), meaning that the cellular growth was constant and efficient during the famine phase. This aspect was crucial for the establishment of a good selective pressure on the microbial consortium, leading to an effective PHA-storing microorganisms' enrichment. In addition, the ratio "feast phase/cycle length" was regularly measured and used to monitor the biomass adaptation and process stability. The average feast phase percentage was 6.6 ± 0.4 %, significantly below the threshold value (20%) for a good selection (Estevez-Alonso et al., 2021). This indicated that the process was technically feasible for long-term operation and it reflected also on a high storage yield ($Y_{P/S}^{\text{feast}} = 0.55 \pm 0.02$ COD/COD). In comparison with a previous study, in which PHA production from MMC at pilot scale was carried out exploiting fermented mixture of OFMSW and WAS (Valentino et al., 2019), the process herein discussed was conducted at lower OLR, equal to 2.0-2.2 g COD/L d. Under this relatively low OLR, the feast-famine approach was more robust and easier to be maintained if compared to higher OLR processes, where high biomass productivities can be reached but significant fluctuations were usually observed in the efficiency of biomass selection (Villano et al., 2010). Apart from this consideration, the operation at such OLR was dependent from the feedstock availability and, most important, from

the VFA concentration achieved in the dark fermentation process (8.4 ± 0.3 g COD_{VFA}/L, as discussed in section 3.2). The stability of the process had positive effects also on the monomeric composition of the polymer, indeed, with the exception of the first week in which a higher 3HV content was detected (24 % w/w), a stable 9.5 % w/w of 3HV content was maintained during the whole run, given to higher level of 3HB precursors in the feedstock (i.e., acetic and butyric acids), in line with data reported in literature (Valentino et al., 2019) (Figure 4 B).

The PHA-storing capacity of the selected consortium was tested in fed-batch mode; the biomass was taken at the end of the feast phase from SBR and supplied with UF-FL by multi-spike strategy. In total, six accumulation tests have been carried out and the maximum PHA content achieved was in the range 0.47-0.62 gPHA/gVSS (on average 0.53 ± 0.03 gPHA/gVSS), higher than performances obtained by Valentino et al. using fermented OFMSW-WAS mixture (0.46 ± 0.05 gPHA/gVSS) (Valentino et al., 2019). To the authors' best knowledge, this result is the highest achieved in a pilot scale PHA production from fermented sewage sludge, indeed, as summarized in Table 2, other studies reported lower PHA content obtained at the end of the accumulation fed with fermented sewage sludge (0.19 ± 0.02 gPHA/gVSS, Frison et al., 2015; 0.27-0.38 gPHA/gVSS, Morgan-Sagastume et al., 2015), with fermented WAS pre-treated with high-pressure thermal hydrolysis (0.19 ± 0.02 gPHA/gTSS, Morgan-Sagastume et al., 2010), or thermally hydrolyzed fermented WAS (0.26 – 0.35 gPHA/gTSS, Zhang et al., 2019). It is also noteworthy that the fully aerobic condition and the VFA-rich stream utilisation in the SBR favour the PHA accumulation performances (rate and yield) in the aerobic fed-batch reactor, in the face of high energy and VFA consumption. Process configurations were different in Frison et al. (2015), where the biomass selection was performed under aerobic-anoxic condition; and in Morgan-Sagastume et al. (2015), where unfermented municipal wastewater was utilised for biomass selection instead of VFA-rich stream. In the other previous studies (Morgan-Sagastume et al., 2011; Zhang et al., 2019), process configuration was similar to that adopted in this study; however, the lower PHA-storing ability of the selected biomass could be due to the thermal hydrolysis pre-treatment applied to the sludge and the consequent high level of nutrients (especially

N-NH₄⁺ and P-PO₄³⁻) released in the VFA-rich stream. It is general knowledge that high level of nutrients negatively impacts on storage response (or PHA accumulation), in favour of concurrent growth-response in long-term accumulation tests (Estevez-Alonso et al., 2021).

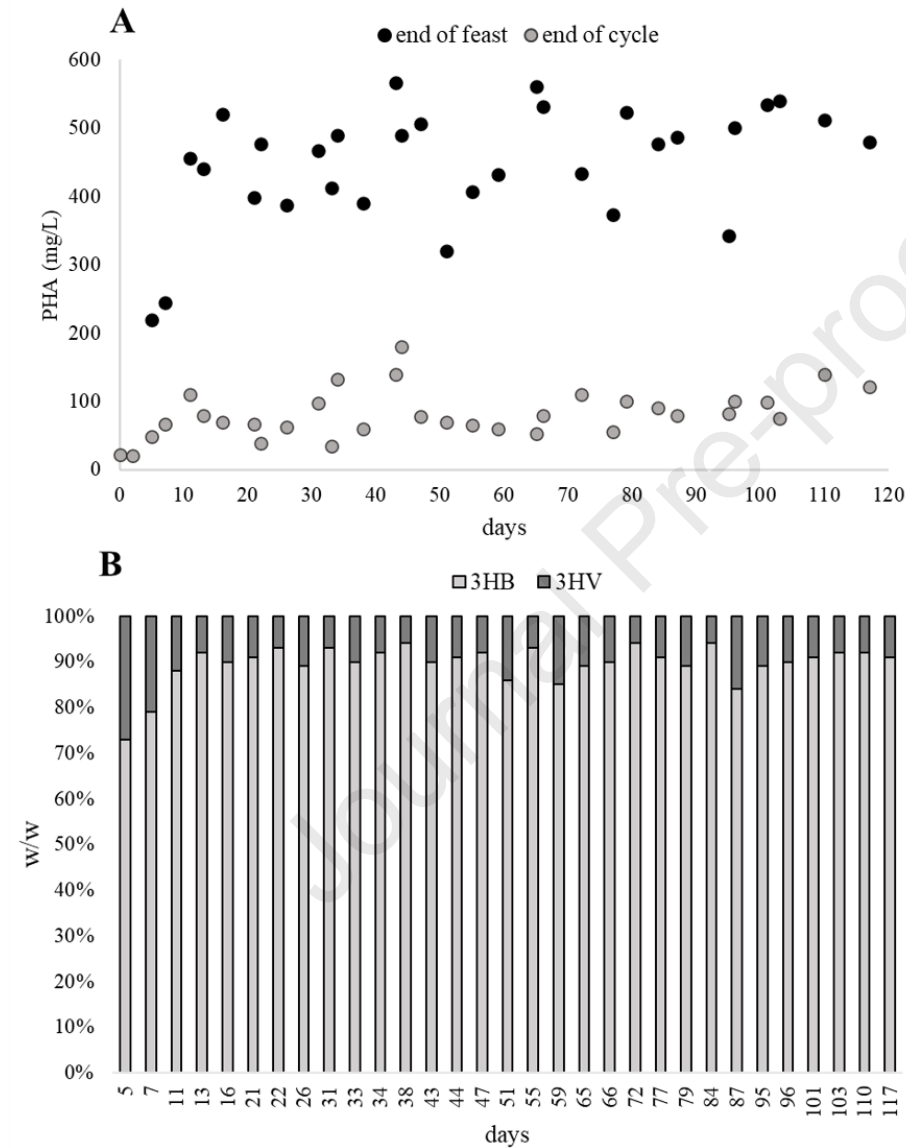


Figure 4. PHA concentration measured at the end of the feast and at the end of the cycle during the whole SBR run (A); monomeric composition of the PHA produced during the whole run (B).

Table 2. Main parameters (average values and standard deviations) monitored in the SBR and batch reactors

References		This study	Valentino et al., 2019	Moretto et al., 2020b	Zhang et al., 2019	Frison et al., 2015	Morgan Sagastume et al., 2015
Feedstock		Fermented WAS	Fermented OFMSW + WAS	Fermented OFMSW + WAS	thermally hydrolized fermented WAS	Fermented WAS	Fermented WAS
Parameter	Unit						
$Y_{P/S}^{feast}$	COD _{PHA} /COD _{SOL}	0.55 ± 0.02	0.22 ± 0.05 - 0.36 ± 0.04	0.35 ± 0.07 - 0.46 ± 0.02	-	0.21 ± 0.13 - 0.42 ± 0.03	-
OLR	gCOD/L d	2.0 - 2.2	4.0	4.0	3.5 – 5.0	0.72 ± 0.03 – 1.4 ± 0.1	0.7 ± 0.2 - 0 ± 0.8
VSS	g VSS/L	2.0 ± 0.3	1.5 ± 0.6 – 2.1 ± 0.9	-	0.9 – 1.3	-	1.5 – 2.3
Feast phase/cycle lenght ratio	h/h	0.07 ± 0.01	0.10 ± 0.02 - 0.14 ± 0.05	0.07 ± 0.02 – 0.12 ± 0.02	0.17	0.13 – 0.2	0.13 ± 0.01
PHA content	gPHA/gVSS	0.53 ± 0.03	0.43 ± 0.01 - 0.46 ± 0.05	0.40 ± 0.03 - 0.59 ± 0.03	26.4 – 36.4	0.19 ± 0.02 – 0.21 ± 0.02	0.27 – 0.28
PHA composition	wt % HB/HV	90.5/9.5	87/13 - 90/10	-	70.1/29.9 – 78.4/21.6	57/41 – 56/42	66/34 – 74/26

3.4 PHA extraction and characterization

3.4.1 Extraction efficiency

Solvent extraction presents some advantages in terms of efficiency (over others PHA extraction methods) such as a higher purity and a negligible degradation of the biopolymer, which maintains a high molecular weight (Mannina et al., 2020). The advantage of NaClO digestion applied on an acid-stabilized wet biomass is that contrary to halogenated solvents extraction, neither drying and neutralization steps are required, consequently time and cost of polymer extraction are reduced. The results obtained by GC-FID analysis (reported in Table 3) show that both the extraction methods allowed to obtain high degrees of purity (PHA %w/w) and PHA recovery yield (% w/w). It is also possible to observe that 3HV content remained approximately constant (13-14 % w/w), suggesting that both the extraction methods had no effects on the monomeric composition. In Table 3, PHA content (62 ± 0.3 % w/w) inside the neutralized dried biomass is also reported and it resulted higher of that quantified previously in the wet biomass (49 % w/w) at the end of the accumulation step and before the acidic stabilization. This PHA content increase in neutralized biomass might be due to the effects of the acidification, which may have favor the partial hydrolyzation of the non-polymer cellular material (NPCM) during the storage period in refrigerated conditions. Since the biomass was neutralized, several washes with distilled water were made, resulting in a loss of solubilized hydrolyzed NPCM and in a higher PHA weight percentage. It is noteworthy that the PHA produced solely from fermented sludge was characterized by a 3HV content (%w/w) comparable to that obtained by using a fermented mixture of OFMSW and WAS (Lorini et al., 2021b). This aspect results of notable interest thanks to the effective possibility of fermenting different feedstocks which may be converted in polymers having similar and adjustable composition.

Table 3. Extraction performances of acid-stabilized samples determined by GC-FID analysis

Sample	PHA (% w/w)	Recovery yield (% w/w)	3HV (% w/w)
Neutralized biomass	62 ± 0.3	-	12.9
CHCl ₃	100.9 ± 1.3	91.5	14.3
NaClO	99.8 ± 0.4	98.3	13.5

3.4.2 Viscosity average molecular weight (M_v)

Viscosimetric analysis of each extracted sample was conducted on PHA solutions in CHCl_3 . The polymer M_v obtained by chloroform extraction was chosen as a reference measure compared to NaClO treatment, because chloroform should not damage the polymer structure and molecular weight. Effectively, the solvent solubilizes PHA directly inside the cells even at low temperature. On the other hand, the use of NaClO for oxidation of the NPCM could cause a partial degradation of the polymer chains, resulting in a decrease of the molecular weight.

The quantified molecular weights, concerning extraction with chloroform and hypochlorite, were 405 ± 5 and 396 ± 2 kDa, respectively, indicating that there is no significant difference between the two extraction methods. In addition, the relatively high M_v confirms the effectiveness of the acid stabilization method as obtained in a previous work, where high values of M_v of samples stabilized by acidification were quantified (370 – 400 kDa) (Lorini et al., 2021b). A further observation in the previous work was comparing the acid stabilization with the thermal stabilization method (thermal treatment at 145 °C for 30 min followed by overnight drying at 70 °C), which caused a partial degradation of the polymer. In the present study, the high M_v of the sample extracted with chloroform (405 kDa) demonstrated that a preliminary drying step (oven at 60°C), following the acid stabilization and preparatory for solvent extraction, did not affect the molecular weight, which resulted comparable to that obtained from NaClO extraction (conducted without drying step).

3.4.3 Thermogravimetric analysis (TGA):

Thermogravimetric analysis permits to quantify important characteristics of the polymers, such as decomposition temperatures and related thermal stability. Figure 5 (A-B) shows typical thermograms, from TGA of extracted PHA samples, in which the weight percentage as a function of temperature is represented. This allows the quantitative determination of the weight loss, the temperature at the maximum rate of decomposition (T_d^{max}) (calculated from the minimum of the first derivative of the

weight function of temperature) and the temperature at 10 % decomposition ($T_d^{10\%}$). The sample extracted with chloroform showed a higher thermal stability than that recovered through NaClO oxidation, as evidence that thermal properties may depend on the drying process. The values of ($T_d^{10\%}$) and (T_d^{\max}) of the samples extracted by chloroform and NaClO are reported in the Table 4 (248.5 - 280 °C and 232.5 - 266 °C, respectively). The average values of T_d^{\max} are comparable to those present in the literature regarding the homopolymer Polyhydroxybutyrate (PHB) (250-290°C) (Balakrishna Pillai et al., 2017) and also copolymer P(3HB-co-3HV) produced from MMC and WAS (256 - 291 °C) (Morgan-Sagastume et al., 2015). Furthermore, the results obtained from a previous study (Lorini et al., 2021b) of PHA derived from WAS-OFMSW mixture, with 3HV content between 13-20 % w/w, showed average T_d^{\max} comparable with those obtained WAS derived PHA of this study.

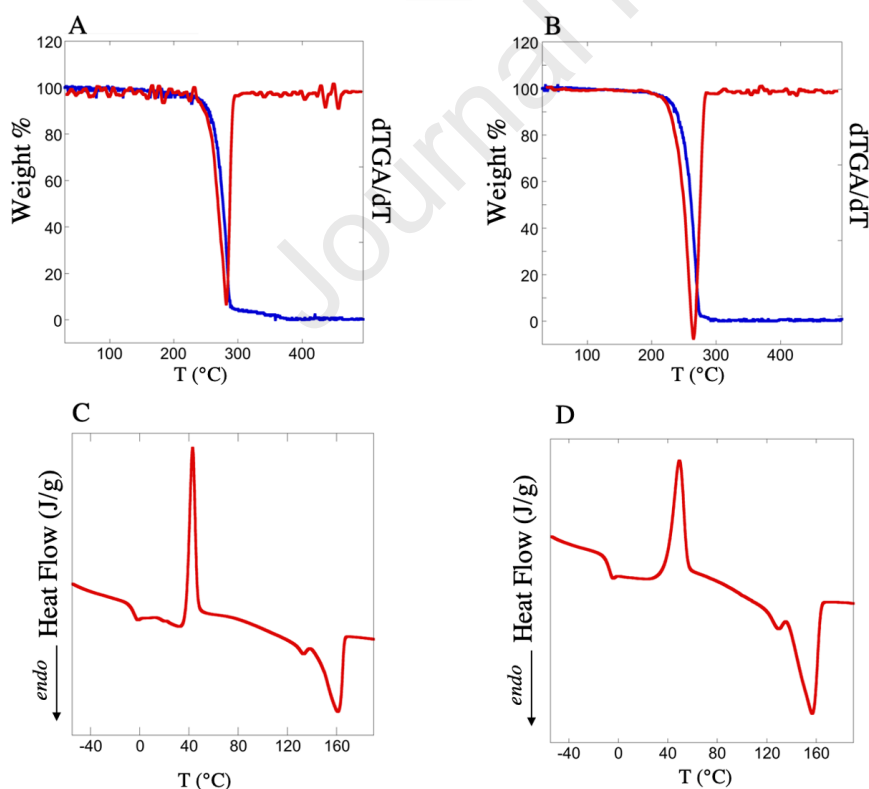


Figure 5. TGA graphs of the sample extracted with chloroform (A) and with NaClO (B); DSC thermograms of the sample extracted with chloroform (C) and with NaClO (D).

3.4.4 DSC analysis

The DSC profiles of the second heating scan of the samples extracted with chloroform and NaClO are reported in Figure 5 (C-D). Both thermograms present the exothermic-endothermic peaks of crystallization and melting. The values of enthalpies and temperatures of cold crystallization (ΔH_c , $T_{\text{cold crystallization}}$), melting (ΔH_m , T_m) and glass transition (T_g) are reported in the Table 4. The melting enthalpies and temperature (ΔH_m , T_m) can be compared with a previous study of PHA production from MMC, specifically T_m values were within the range of 160 - 175°C (Lorini et al., 2021b). Moreover, the T_m are similar with those found in the literature regarding the polyhydroxybutyrate (PHB) homopolymer (150-180 °C) (Domínguez-Díaz et al., 2015). The T_g values of the two standard extracted polymers were analogous, since the two extraction methods did not affect the monomeric composition of the recovered polymer (Table 1) and it was also possible to observe the value of T_g was comparable with a copolymer produced from dairy wastes with similar 3HV content (9 %w/w) (Colombo et al., 2020). In addition, melting ΔH_m , and consequently the relative crystallinities, were similar and consistent with those reported in literature for MMC-PHA with comparable monomeric composition (Lorini et al., 2021b).

Table 4. Thermal properties of the samples measured during the second heating scan by DSC.

Sample	$T_d^{10\%}$ (°C)	T_d^{max} (°C)	T_g (°C)	ΔH_m (J/g)	T_m (°C)	ΔH_c (J/g)	$T_{\text{cold crystallization}}$ (°C)	χ_c (%)
CHCl ₃	248.5	280	-5	67.1	160.9	37.7	43	46
NaClO	232.5	266	-7	63.6	156.8	32.3	49	44

3.5 Mass balance and overall PHA yield estimate

The quantification of the overall PHA yield is a pivotal point of the approach adopted in this study. This value affects the technology feasibility, especially from the economic point of view as already demonstrated in a previous study (Moretto et al., 2020a) where food waste and WAS were utilized as substrates. The overall yield depends on the biomass storage response (quantified as storage yield) and the maximum PHA content achieved. The mass balance has been assessed from a backward

calculation, starting from the COD amount (in form of VFA) necessary to produce 1 kg of PHA (or 1.7 kg of COD_{PHA}).

The average PHA biomass content obtained from the five performed accumulations was 0.53 ± 0.03 g PHA/g VSS; if 1.0 kg of polymer is considered, the corresponding active biomass (X_A) produced was 0.88 kg (or 1.23 kg COD_{Xa}). The observed yield in the selection reactor (Y_{OBS}^{SBR}) was 0.50 ± 0.02 COD_{Xa}/COD_{VFA}. Hence, 2.46 kg of COD_{VFA} is the necessary amount to sustain the X_A growth in SBR. In addition, given the average storage yield in the accumulations (0.57 ± 0.02 COD_{PHA}/COD_{VFA}), the required amount for 1.7 kg COD_{PHA} is 2.98 kg of COD_{VFA}. In total, 5.44 kg of COD_{VFA} needs to be entering the aerobic line for selection and accumulation reactors. The pilot scale system was equipped with two solid/liquid separation units (C and UF), necessary for the clarification of the VFA-rich. Both stages increased the required amount of VFA entering the aerobic PHA line, since around 20% of COD_{VFA} loss was counted; with a fair approximation, 6.80 kg of COD_{VFA} has been estimated. The minor part (1.36 kg COD_{VFA}) could be also valorized through other routes, such as the anaerobic digestion for biogas and compost recovery. Since the average fermentation yield (Y_{VFA}) of 0.38 ± 0.04 kg COD_{VFA}/kg VS₀ has been quantified, 17.9 kg of VS₀ are necessary to produce 6.8 kg of COD_{VFA} through the dark fermentation process. This scenario leads to a calculated overall yield of 56 g PHA/kg VS. According to the best authors' knowledge, this is the first of kind study reporting an overall yield in such process configuration, by using sewage sludge as carbon source. The literature provides partial PHA yields (0.11 kg COD_{PHA}/gCOD_{VFA}, Frison et al., 2015; 0.47 kg COD_{PHA}/gCOD_{VFA}, Tu et al., 2019) since the fermentation stage (VS conversion into VFA) is generally not considered. On the other hand, previous studies reveal higher yields, but in the same order of magnitude (76 g PHA/kg VS, Moretto et al., 2020a; 110 g PHA/kg VS, Moretto et al., 2020b; 114 g PHA/kg VS, Colombo et al., 2017). The reason behind such difference is related to the feedstock itself. The cited studies utilized different feedstock, easier to be converted into VFA compared to sewage sludge. Since PHA is synthesized from VFA as election substrates, a carbon source particularly putrescible can give high yields in terms of PHA production. This is the case of

Colombo et al. (2017), where only OFMSW was used; and the case of Moretto et al. (2020a; 2020b) where the OFMSW was utilized in a mixture with WAS. However, since the management of sewage sludge is an urgent issue, the global yield calculated in this study is an important piece of knowledge to be considered in future scale-up opportunities.

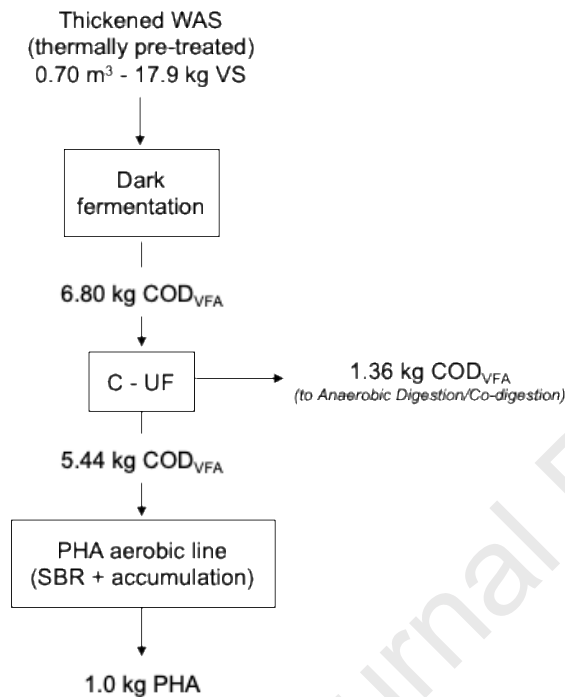


Figure 6. Mass balance of the multi-stage process.

4 Conclusions

Compared to a wide variety of organic wastes affected by seasonality effects (such as source sorted OFMWS), sludge originated from municipality is a fermentable substrate with stable physical-chemical features during a whole year. In turn, these fundamental characteristics may ensure stability in PHA production process, with a final product having constant and reproducible characteristics too. In particular, the chemical, mechanical and thermal properties of PHA are strictly dependent from the fermentative step and VFA distribution. Regarding this step, the methodology adopted in this study does not require chemicals addition or pH-control system, since the acidification process is balanced by sludge alkalinity. Stable pH, together with stable features of the feedstock are key aspects for the

success of the technology, whose final bio-product needs to be spread in the market after finding its application.

To set one or more potential applications, process performances and bio-products characteristics need to be consistent over time. Furthermore, to the best author's knowledge, this study is the first in literature reporting a PHA global yield related to a pilot scale process, based on sole waste activated sludge (at least in this process configuration). The results achieved in this study open to further discussions not only on the possible technical progress, but also on the economic aspects in an urban scenario, addressed to a circular economy and biorefinery approach. Until now, the sludge anaerobic digestion remains the dominant technology at full scale, and other methodologies (as this one proposed here) could change the paradigm of waste treatment plants into processes for the synthesis of bio-based products. Notwithstanding the application of green protocols for PHA extraction and recovery, to develop this approach, different aspects should be considering: the respective waste catchment area, the valorization of fermentation solid residues for biogas production (not evaluated in this study) and the potential PHA applications. Based on this holistic approach, a possible market price for sludge derived PHA may be defined together with the effective economic sustainability of the technology.

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Highlights

- Polyhydroxyalkanoates production from mixed microbial cultures and sewage sludge
- Sewage sludge fermentation in thermophilic conditions after thermal pre-treatment
- Characterization of polyhydroxyalkanoates derived from sewage sludge at pilot scale
- Overall yield of polyhydroxyalkanoates produced from sewage sludge at pilot scale

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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