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Research Article

Biopolymers from Urban Organic Waste: Influence of the Solid Retention Time to Cycle Length Ratio in the Enrichment of a Mixed Microbial Culture (MMC)

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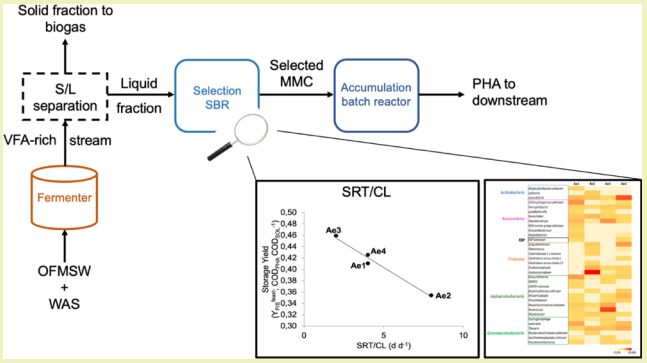
ABSTRACT: In this study, the performance of the selection process for polyhydroxyalkanoate (PHA) production from mixed microbial cultures (MMCs) at pilot scale was deeply investigated with the solid retention time (SRT) to cycle length (CL) ratio as main affecting parameter. Four different runs were tested by varying the SRT/CL ratio maintaining the same organic loading rate (OLR). The pilot-scale selection and accumulation reactors were fed with a fermented mixture of source-selected organic fraction of municipal solid waste (OFMSW) and waste activated sludge (WAS), refined with a centrifuge and membrane unit for the coarse solid removal. The selected biomass obtained in the most performing run was characterized by a specific storage rate of 375 mg $COD_P/g\ COD_{Xa}$ h and a storage yield of 0.46 COD_P/COD_{SOL}. Accumulations performed with the same biomass were characterized by a storage yield of 0.62 COD_P/COD_{VFA}. The microbiome composition was assessed. In the most performing run, putative PHA-storing bacteria affiliated with *Paracoccus* genus were found at high abundance (36.8%), in contrast to all other runs. An overall PHA yield of 110 g PHA/kg VS was estimated for the best scenario, revealing an interesting perspective for biorefinery technology chains based on the three-stage process for PHA production.



KEYWORDS: *mixed microbial cultures (MMCs), polyhydroxyalkanoates (PHAs), urban organic waste, solid retention time to cycle length ratio (SRT/CL), biorefinery*

INTRODUCTION

Background. Switching the primarily fossil resource-based economy to a biobased economy can contribute to the process of slowing down global warming and natural resource depletion.[1] Hence, global research focused on the development of many new functional materials and sustainable products obtainable from renewable resources, such as lignocellulosic biomass,[2−4] food waste,[5] corn and sugarcane biomasses,[6] etc. Polyhydroxyalkanoates (PHAs) are natural polyesters of hydroxyalkanoates (HAs) synthesized by microorganisms as intracellular granules for carbon and energy storage.[7] Generally, PHA is stored under nutrient-limited conditions coupled with an excess of carbon source by a wide variety of microorganisms.[8] For several years now, PHAs have been produced at the industrial scale mostly by means of pure cultures.[9] However, pure or recombinant cultures require sterile conditions and specific carbon sources followed by a targeted recovery process, resulting in 4−9 times higher production costs compared to conventional plastics.[10]

Processes for PHA Production from MMCs and Types of PHA Produced. To enhance the market competitivity, PHA production from mixed microbial cultures (MMCs) using waste streams or wastewaters as carbon sources has been exponentially investigated.[11] Typically, PHA production from MMCs is achieved with the application of discontinuous feeding regimes (feast and famine regime) and alternating redox conditions to enrich the presence of PHA-storing microorganisms in the activated sludge of wastewater treatment plants (WWTPs).[12] These conditions can be tuned based on the type and availability of the carbon source and on the desired process that needs to be applied. More recently, the three-stage process (divided into acidogenic fermentation of the organic feedstock for volatile fatty acid (VFA) production, PHA-storing microorganisms' selection from the MMC, and PHA accumulation maximization) has been implemented at a

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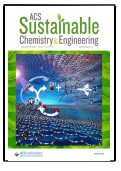

pilot scale for both wastewater and organic waste valorization in a new biorefinery technology chain perspective.[13] A wide variety of substrates have been used in previous studies, such as organic fraction of municipal solid waste (OFMSW),[14] cheese whey,[15,16] olive oil mill wastewater,[17] etc. However, this process can be quite challenging, mostly due to the intrinsic variability of nonsynthetic organic substrates used as carbon sources, that consequently translates into a variability of the selected microbial culture and of the obtained final polymer. The amount and quality of the produced polymer are tightly bound to many factors, especially the performance of the selection reactor, the carbon source quality, the accumulation strategy, and the microorganism type.[18] The most common of the class are poly(3-hydroxybutyrate), P(3HB), and poly(3-hydroxyvalerate), P(3HV), and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(3HB-co-3HV), which exhibit thermoplastic characteristics with mechanical properties comparable to polypropylene and polyethylene.[19] Previous studies demonstrated that the polymer composition changed with respect to the substrate used,[15] feedstock composition, and feeding strategies[20] and to the strategy adopted for the accumulation performance, including the important role played by environmental factors (temperature, pH, dissolved oxygen, byproduct inhibition, and nutrient concentrations).[21]

Assessment of the Selection Performances. Both PHA stages (selection and accumulation) have a major impact on the overall process yield. However, it has been demonstrated that in MMCs the core element that allows to achieve a high PHA storage performance in the accumulation stage (in terms of high PHA yields, high specific storage rate, and maximum intracellular content) consists in the efficiency of the culture-enrichment stage to select high-performing PHA-storing microorganisms.[22] The higher the selective pressure applied, the more the culture is enriched exclusively in PHA-storing microorganisms. Many studies report the feast to cycle length ratio (feast/CL)[23,24] or the feast to famine ratio (F/F)[14,25] as the most important indicator for a good and stable selection process. These two parameters are calculated from the duration of the feast phase, usually observed by the dissolved oxygen (DO) profile. Both proved to be useful to assess the enrichment of a MMC and values of F/F lower than 0.3−0.4 are required for the imposition of an effective internal limitation, achieved through a long enough famine phase, to induce the PHA storage.[26] Nevertheless, when dealing with real substrates, nonsterile conditions, and open cultures, many other factors can influence the selection performances. Parameters such as the solid retention time (SRT), hydraulic retention time (HRT), cycle length (CL), organic loading rate (OLR), DO, carbon source quality, and nutrient concentrations and ratios play a fundamental role, translating into a difficulty to properly assess the selection process.[11] Many authors also reported internal nitrogen (N) or phosphorous (P) limitation being triggering factors for the achievement of maximal storage capacity.[27,28] Indeed, the complexity of the process promoted the search for other indicators that could give useful information on the selection process. Previous studies also focused on the cycle length[29,30] and the number of cycles per SRT[31] to further investigate the selection performances.

In the present study, the ratio between SRT and CL is discussed and evaluated as the main parameter that gives deeper insights into the quality of the selective pressure applied in the selection reactor. The study was carried out at a pilot scale inside a full-scale WWTP, with a mixture of organic fraction of municipal solid waste (OFMSW) and waste activated sludge (WAS) as the carbon source for the PHA line.

EXPERIMENTAL SECTION (MATERIALS AND METHODS)

Pilot Plant Process Scheme and Reactors. The proposed pilot plant configuration illustrated in Figure 1 is the same as the one adopted in a previous study,[32] as well as the pilot reactors used.

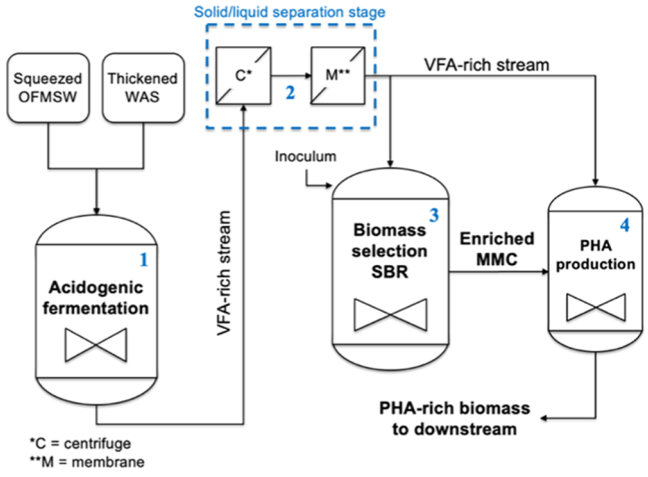

Figure 1. Pilot plant process configuration adopted in the present study.

The pilot plant platform was located inside the Treviso municipal WWTP (northeast Italy). The well-known three-stage process was applied for PHA selection and production. The first stage consisted of the acidogenic fermentation (unit 1) of a mixture composed of 30% v/v WAS and 70% v/v OFMSW for VFA production, followed by a solid/liquid separation stage for the refining of the fermented stream (unit 2). The refined fermented VFA-rich stream was then fed to the sequencing batch reactor (SBR, unit 3) for PHA selection and also to the PHA accumulation reactor (unit 4). Both reactors for selection and accumulation worked under a fully aerobic regime and were automatically operated through a programmable logic controller (PLC, MyRio Labview from National Instrument) that also acquired real-time signals from immersion probes.

The acidogenic fermentation was carried out in batch mode in a 400 L reactor equipped with a mechanical stirrer (HRT of 6 days; OLR 12−15 kg volatile solid (VS)/m^3 d). Roughly 8−10 batch fermentation runs were conducted during each SBR operation. The temperature was controlled by means of a thermostatic jacket and maintained at 72 °C for 48 h for the mixture thermal pretreatment, then set at 37 °C for 4 days. The thermal pretreatment was applied to foster substrate solubilization, according to the results obtained in previous studies.[32,33] The fermenter pH was left uncontrolled and maintained itself at around 5.0−5.5 for the whole duration of the study. The solid/liquid separation stage was divided first into a coaxial centrifuge equipped with a 5.0 μm porosity nylon filter bag and a subsequent ultrafiltration membrane with 0.2 μm porosity. The PHA selection from MMC was performed in a 100 L working volume reactor equipped with Bibus EL-S-250 linear membrane blowers (ensuring a continuous maximum DO level of 8.0 mg O_2/L and a complete stirring of the mixed liquor) and with immersion probes (DO; pH; temperature). The temperature was maintained between 25 and 28 °C with an immersion heater. Since no settling phase was programmed, the HRT was equal to the SRT in all runs. The SBR was operated and monitored under four different runs with an intermediate OLR (4.0 g COD/L d) with a real substrate at the pilot scale. Different settings of SRTs and CLs were tested, and process performances were assessed in each run after steady-state

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Table 1. Operating Parameters Applied in the Four Runs Tested in the Selection Process

run	HRT (d)	SRT (d)	OLR (g COD/L d)	CL (d)	SRT/CL (d/d)	feeding frequency (d^{-1})	operation length (d)	load per cycle (g COD/L)
Ae1	1	1	4	0.25	4	4	44	1.0
Ae2	2	2	4	0.25	8	4	48	1.0
Ae3	1	1	4	0.5	2	2	45	2.0
Ae4	2	2	4	0.5	4	2	76	2.0

conditions were reached. As a consequence, different SRT/CL ratios of 2 (run Ae3), 4 (runs Ae1 and Ae4), and 8 (run Ae2) were investigated. Maintaining the same carbon source and OLR in all four runs allowed to focus only on the effects of SRT and CL. The experiments started from a 0.25 day cycle length with the SRT varying from 1 day (run Ae1) to 2 days (run Ae2) up to a 0.50 day cycle length again with the SRT varying from 1 day (run Ae3) to 2 days (run Ae4). In all four runs, the end of the feast phase was identified by the DO profile acquired by the PLC. The DO trends were analyzed for feast−famine regime evaluation as widely described in the literature.[12] In each run performed, a stable feast−famine regime was observed approximately after three SRTs. The feast phase length was recorded in real time by the PLC during the whole duration of the research. Feast phase length to CL ratios were calculated once per day taking into account one representative feast phase duration for each day and for each SBR run: 44 days for run Ae1, 48 days for run Ae2, 45 days for run Ae3, and 76 days for run Ae4. The operating conditions tested in the selection process are reported in Table 1.

PHA accumulation batches were conducted in fed-batch mode with selected biomass (X) collected at the end of the feast phase. The reactor was provided with the same equipment as the selection reactor. The same permeate fed in the selection reactor was also used in the accumulations. The carbon source was progressively dosed in spikes to reach 3 g COD/L concentration in the accumulation reactor immediately after each addition. When the DO level recorded by the PLC started to increase, indicating that the carbon source was consumed, another spike was added. Each accumulation lasted about 6−8 h on average, and after that time the biomass reached its maximum PHA intracellular content and the accumulation was stopped. Accumulation batches started with an initial volume of 70−80 L and ended when the reactor working volume reached approximately 120 L. From 8 to 15 accumulation batches per SBR run were performed, for a total of 50 accumulation batches approximately.

Organic Substrates. The characteristics of the mixture used in the acidogenic fermentation of the fermented stream and of the permeate from the S/L separation, which was used for the PHA selection and accumulation reactors, are listed in Table 2.

All of the parameters refer to a monitoring period of 2 years approximately. The permeate fed to the PHA line showed a predominance of acetic acid (24−34%) and propionic acid (10−20%), along with butyric acid (30−40%) and caproic acid (25−35%) expressed on a COD basis. The values given in parenthesis represent a range of variation from the minimum to the maximum value over the whole operation period. The OFMSW was collected door-to-door in 50 districts of the Treviso province and sent to a dedicated plant, where a mechanical pretreatment by means of a screw-press was applied. The solid fraction was sent to composting and the liquid fraction (ca. 15% total solid basis) was sent to the Treviso WWTP for full-scale anaerobic co-digestion with biological sludge. The WAS was collected from the static thickener after the biological nutrient removal (BNR) process was applied in the water line of the full-scale WWTP. The same activated sludge was also used as inoculum for the PHA selection.

Table 2. Organic Substrate Characterization

parameter	organic waste mixture	fermented stream	permeate (after S/L separation)
TS (g TS/kg)	64 ± 2	46 ± 2	
VS (g VS/kg)	51 ± 2	35 ± 2	
VS/TS (%)	80 ± 1	76 ± 1	
COD_{SOL} (g COD/L)	22 ± 1	37 ± 3	36 ± 2
VFA (g COD/L)	2.8 ± 0.5	32 ± 3	31 ± 2
VFA/COD_{SOL}	0.13 ± 0.05	0.86 ± 0.05	0.86 ± 0.05
ammonium (mg N-NH_4^+/L)	380 ± 28	691 ± 15	689 ± 15
phosphate (mg P-PO_4^{3-}/L)	121 ± 7	221 ± 6	220 ± 6
TKN (g N/kg TS)	29 ± 3	28 ± 3	28 ± 3
P (g P/kg TS)	2.3 ± 0.1	2.9 ± 0.5	2.9 ± 3

Analytical Methods. The organic substrates were weekly collected and characterized. Samples from the selection SBR were collected daily. TS, VS, total suspended solids (TSSs), volatile suspended solids (VSSs), soluble chemical oxygen demand (COD_{SOL}), ammonium, phosphate, total Kjeldahl nitrogen (TKN), and total phosphorus (P) were determined according to Standard Methods.[34] VFAs were analyzed using a gas chromatograph (GC), the sample was centrifuged at 4500 rpm for 5 min, and the supernatant was then filtered with a 0.2 μm acetate cellulose filter (Whatman) and acidified at pH 2 with ortophosphoric acid. The GC used was an Agilent 6890 N equipped with an inlet set at 220 °C in split mode with a split ratio of 20:1, a flame ionization detector (FID) set at 230 °C, and an Agilent J&D DB-FFAP fused silica capillary column (15 m length, 0.53 mm i.d., 0.55 mm film). The run consisted of a ramped temperature from 80 to 200 °C and the analytes were determined with an internal standard (2-ethyl butyric acid). Regarding PHA analyses, samples were prepared by adding 1 mL of NaClO (5% active Cl_2) to 5 mL of an unfiltered fresh sample and the solution was stored at −4 °C for subsequent extraction, hydrolyzation, and esterification. Then, 3HB methyl ester and 3HV methyl ester monomers were quantified with a GC using P(3HB-co-3HV) standard polymer (Sigma-Aldrich) at 5 wt % HV content as reference.[35]

Calculations. Performances of the selection SBR were assessed after reaching steady-state conditions, identified with the achievement of a constant feast phase length for at least two SRTs. All parameters were calculated taking into account the actual carbon source (permeate after S/L separation) fed into the selection and accumulation reactors.

The active biomass concentration (X_A, g/L) was calculated as the difference between VSS concentration (g/L) and PHA concentration (g/L) at the end of the cycle

$$X_A = \text{VSS} - \text{PHA} \tag{1}$$

PHA content in the biomass (g PHA/g VSS) was determined as the ratio between PHA concentration (g/L) and VSS concentration (g/L) both at the end of the feast phase

$$\text{PHA content} = \frac{\text{PHA}}{\text{VSS}} \tag{2}$$

The specific substrate uptake rate ($-qS^{feast}$), expressed in mg COD_{SOL}/g COD_{Xa} h, was assessed as the ratio between the substrate (soluble COD) consumed during the feast phase and the length of the feast phase per unit of X_A

$$-qS^{feast} = \Delta COD_{SOL}/t_{feast} \cdot X_A \tag{3}$$

The specific PHA consumption rate ($-qP^{famine}$, mg COD_P/g COD_{Xa} h) was calculated as the ratio between the PHA consumed during the famine phase and the length of the famine phase per unit of X_A

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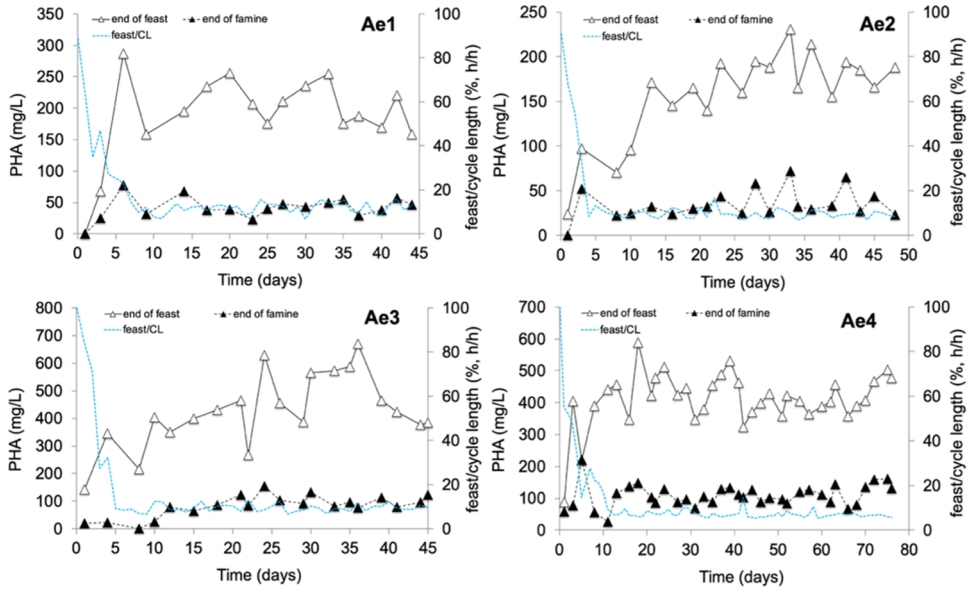

Figure 2. Trends of PHA concentrations (mg/L) at the end of the feast and famine phase and feast/CL ratio in all four runs.

$$-qP^{\text{famine}} = (\text{mg PHA}_{\text{feast}} - \text{mg PHA}_{\text{famine}})/t_{\text{famine}} \cdot X_A \quad (4)$$

The polymer specific storage rate during the feast phase (qP^{feast}) was determined as the ratio between the stored PHA and the length of the feast phase per unit of X_A, expressed as mg PHA/g X_A h

$$qP^{\text{feast}} = \Delta\text{PHA}/t \cdot X_A \quad (5)$$

The average observed growth yield of the selection reactor ($Y_{\text{OBS}}^{\text{SBR}}$) was calculated as the ratio between the active biomass (X_A) expressed as COD and the OLR and HRT applied to the reactor

$$Y_{\text{OBS}}^{\text{SBR}} = \frac{(X_A/(\text{OLR}\cdot\text{HRT}))}{0.93} \quad (6)$$

A correction factor of 0.93 was applied to the equation to refer the final results to the loaded COD_{SOL} instead of the consumed one since the consumed COD_{SOL} represents 93% of the loaded one.

The PHA production yield ($Y_{\text{P/S}}^{\text{feast}}$) was calculated as the ratio between the PHA produced and the COD_{SOL} consumed during the feast phase

$$Y_{\text{P/S}}^{\text{feast}} = \Delta\text{PHA}/\Delta\text{COD}_{\text{SOL}} \quad (7)$$

All PHA and X_A concentrations are referred to the COD basis. The factor used for biomass conversion to COD is equal to 1.42 g COD/g X_A, while the factor used for PHA monomer conversion to COD is based on the oxidation stoichiometry and is equal to 1.67 g COD/g HB monomer and to 1.92 g COD/g HV monomer for HB and HV monomers, respectively.

DNA Extraction. Aerobic sludge samples (10 mL) were taken over the SBR operation at four different sampling times during steady-state operation: day 21 for run Ae1, day 47 for run Ae2, day 23 for run Ae3, and day 24 for run Ae4. The samples were centrifuged at 15 000 rpm for 15 min and the resulting pellet was immediately stored at −20 °C until further processing. DNA extraction was performed using PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's instructions. Purified DNA from each sample was eluted in 100 μL sterile Milli-Q water and the quality of the extracted DNA ($1.6 < A_{260/280} < 1.8$ and $A_{260/230} > 2$) was analyzed with a Nanodrop 3300 (Thermo Scientific, Italy). DNA was stored at −20 °C in small aliquots.

High-Throughput 16S rRNA Gene Sequencing and Bioinformatic Processing. The extracted DNA was amplified in a first PCR with the primer pairs 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′) targeting the region V1−V3 of bacterial 16S rRNA gene according to the procedures described in a previous study.[36] Reactions were set up in 25 μL volumes containing 15 ng of DNA, 0.5 μM primers, and 1× Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). PCR settings were as follows: initial denaturation at 98 °C for 10 s, 30 cycle at 98 °C for 1 s, 60 °C for 5 s, 72 °C for 15 s, and final elongation at 72 °C for 1 min. The amplicon libraries were purified using the Agencourt AMpure XP bead protocol (Beckmann Coulter). Sequencing libraries were prepared from the purified amplicon libraries using a second PCR followed by purification and pooling in equimolar concentration (4 nM). The library concentration was measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). The samples were paired and sequenced (2 × 301 bp) on a MiSeq platform (Illumina) using a MiSeq Reagent Kit v3, 600 cycles (Illumina) following the standard guidelines for preparing and loading samples. Further, 10% Phix control library was added to avoid low complexity issues.

After checking read quality with fastqc, the sequences were processed and analyzed using QIIME2 v. 2018.2.[37] The reads were demultiplexed using a demux plugin (https://github.com/qiime2/q2-demux), and the primer sequences were removed using cutadapt plugin (https://github.com/qiime2/q2-cutadapt). The demultiplexed reads were denoised, dereplicated, and chimera-filtered using DADA2 algorithm.[38] Moreover, DADA2 resolved amplicon sequence variants (ASVs) differing by as little as one nucleotide.[39] Taxonomy was assigned to ASVs using a pretrained naive-Bayes classifier based on the 16S rRNA gene database at 99% similarity of the Silva132 release.[40] High-throughput sequencing of the region V1−V3 of the bacterial 16S rRNA gene yielded a total of 166 908 sequence reads after quality control and bioinformatic processing that resolved into 1517 ASVs. Sequencing results were used for the calculation of biodiversity indices for each sample (Dominance, Simpson, Shannon, Evenness) using PAST software (PALAEONTOLOGICAL STATISTICS, ver. 2.17).[41]

RESULTS AND DISCUSSION

Biomass Selection Performances Based on the SRT/CL Ratio. In every run tested, the feast to CL ratio was abundantly below 20%, widely recognized as the threshold for the establishment of a proper selection process.[42−44] Overall, the feast to CL ratio showed more differences in run Ae1 and Ae4, with values equal to 11.8 and 7.1%, respectively. In runs Ae2 and Ae3, this parameter was quite similar, 9.6 and 9.4%, respectively, even if both SRTs and CLs were different from

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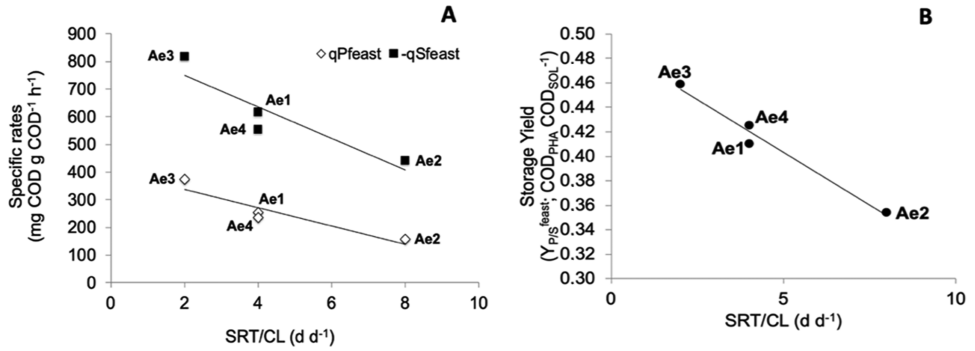

Figure 3. (A) Specific substrate uptake rate ($-qS^{feast}$) and specific storage rate (qP^{feast}) obtained in all four runs in relation to the SRT/CL ratio and (B) storage yield ($Y_{P/S}^{feast}$) trend related to the SRT/CL ratio in all four runs.

Table 3. Main Parameters and Performances Obtained in the Selection and Accumulation Processes of the Four Runs

		value (avg. ± S.D.)			
	unit	Ae1	Ae2	Ae3	Ae4
Parameter (Selection/Enrichment Stage)					
feast/CL	%	11.8 ± 0.2	9.6 ± 0.1	9.4 ± 0.1	7.1 ± 0.2
X_A (end of feast)	g/L	1.37 ± 0.04	2.36 ± 0.05	1.30 ± 0.01	2.54 ± 0.02
PHA content (end of feast)	g PHA/g VSS	0.13 ± 0.05	0.07 ± 0.04	0.20 ± 0.03	0.14 ± 0.02
F/M	g COD/g X_A d	2.92 ± 0.12	1.69 ± 0.13	3.08 ± 0.11	1.57 ± 0.12
specific storage rate (qP^{feast})	mg COD_P/g COD_{Xa} h	253 ± 29	156 ± 19	375 ± 36	235 ± 38
specific substrate uptake rate ($-qS^{feast}$)	mg COD_{SOL}/g COD_{Xa} h	617 ± 47	440 ± 32	816 ± 41	553 ± 44
storage yield ($Y_{P/S}^{feast}$)	COD_P/COD_{SOL}	0.41 ± 0.03	0.35 ± 0.07	0.46 ± 0.02	0.43 ± 0.03
observed yield (Y_{OBS}^{SBR})	COD_{Xa}/COD_{SOL}	0.56 ± 0.05	0.47 ± 0.04	0.59 ± 0.04	0.54 ± 0.03
Parameter (Accumulation Stage)					
PHA content	g PHA/g VSS	0.51 ± 0.07	0.40 ± 0.03	0.59 ± 0.03	0.49 ± 0.04
storage yield ($Y_{P/VFA}^{batch}$)	COD_P/COD_{VFA}	0.58 ± 0.09	0.47 ± 0.09	0.62 ± 0.05	0.55 ± 0.07

run Ae2 to run Ae3. From these results, it could be mistakenly interpreted that the most performing selection process in terms of specific storage rates and storage yields was run Ae4 since the duration of the feast phase was shorter than that of the other runs. The PHA concentrations (mg/L) at the end of the feast and famine phase and the feast/CL ratio obtained in each run are shown in Figure 2.

The SRT plays an important role in determining the reactor X_A concentration, indeed higher concentrations of biomass were obtained in runs Ae2 and Ae4 with 2 days of SRT (2.36 and 2.54 g/L, respectively) rather than in runs Ae1 and Ae3 with SRT equal to 1 day (1.37 and 1.30 g/L, respectively). However, the intracellular PHA content at the end of the feast phase did not reflect the increase in X_A concentration observed in run Ae2 and Ae4. Run Ae2 and Ae4 showed a PHA content equal to 0.07 and 0.14 g PHA/g VSS, respectively, while in run Ae1 and Ae3 the PHA content was 0.13 and 0.20 g PHA/g VSS, respectively. Hence, the storage response was differently affected and a combination of both parameters, SRT and CL, seemed to be more relevant.

Run Ae1 and Ae4 were more similar in terms of PHA level accumulated at the end of the feast phase but run Ae3 showed a visible increase with respect to all other runs. If the selective pressure applied was the same in all runs, the PHA content would have been expected to be similar. The observed differences may suggest that in run Ae3, where the CL was the highest (0.50 day) and the SRT the shortest (1 day), the culture is stimulated to store more PHA. A previous study has already shown how the SRT may affect the selective pressure,[45] however, deeper data analysis revealed that a combined effect of CL and SRT has to be taken into account for a comprehensive process evaluation.

Clear differences between the runs were also observed in terms of specific substrate uptake rate ($-qS^{feast}$) and specific storage rate (qP^{feast}). The obtained values for both parameters showed a decreasing trend when correlated with the SRT/CL ratio, as illustrated in Figure 3A.

Run Ae3, which had the lowest SRT/CL ratio (2 d/d), was characterized by the highest specific rates according to the highest PHA content at the end of the feast phase obtained: $-qS^{feast}$ equal to 816 mg COD_{SOL}/g COD_{Xa} h and qP^{feast} equal to 375 mg COD_P/g COD_{Xa} h. Even with different CLs and SRTs, run Ae1 and Ae4 exhibited similar kinetics as a consequence of the same SRT/CL ratio (4 d/d): $-qS^{feast}$ equal to 617 and 553 mg COD_{SOL}/g COD_{Xa} h and a qP^{feast} equal to 253 and 235 mg COD_P/g COD_{Xa} h, respectively. Run Ae2 showed the lowest rates, with a $-qS^{feast}$ equal to 440 mg COD_{SOL}/g COD_{Xa} h and a qP^{feast} equal to 156 mg COD_{PHA}/g COD_{Xa} h, at the highest SRT/CL ratio (8 d/d). From these results, it appears that lower SRT/CL ratios stimulate higher specific rates, both in terms of consumed substrates and of the stored polymer. The storage yield ($Y_{P/S}^{feast}$) correlated with the SRT/CL ratio showed a similar trend, with the highest values in run Ae3 (0.46 COD_P/COD_{SOL}) and lowest in run Ae2 (0.35 COD_P/COD_{SOL}). The $Y_{P/S}^{feast}$ trend of all runs is reported in Figure 3B.

Clearly, the storage yield is in agreement with the observed specific rates. Unexpectedly, run Ae4, where the lowest feast phase to CL ratio of 7.1% was observed, did not show the best performance in terms of specific rates and polymer storage

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yield. Indeed, Ae3 turned out to be the most performing in terms of PHA storage stimulation, compared to the other runs. From these results, it seems that the influence of the SRT/CL ratio is crucial for the evaluation of the selective pressure applied to the system. The main parameters and performances obtained in the selection and accumulation processes of all runs are summarized in Table 3. The F/M has also been calculated and was equal to 2.92, 1.69, 3.08, and 1.57 g COD/g X_A d, respectively, for run Ae1, Ae2, Ae3, and Ae4. Apparently, no correlation seemed evident, since the lowest performance (run Ae2) did not correspond to the lowest F/M value. This value can be assumed as a direct consequence of the operating conditions applied in the selection phase, especially the OLR, which was further discussed with respect to the SRT/CL.

Role of SRT/CL Ratio in Relationship with the Applied OLR and Microbial Community. Few other authors investigated the performances of the selection process in terms of the CL or SRT/CL impact on PHA production and on the microbial composition of the selected culture, and the results are quite variable. A previous study[31] investigated the microbial competition in several SBRs fed with pure synthetic acetate (OLR 2.0 g COD/L d) at different temperatures (20 and 30 °C), with different CLs (1, 4, 12, 18 h), and SRT-HRT both fixed at 1 day. The authors found that the SRT/CL ratio had a strong impact on the PHA production but not on the variability of the microbial community. This study demonstrated that the lower the SRT/CL ratio is, the higher selective pressure the culture has to deal with since a higher intracellular polymer content needs to be achieved. The amount of substrate fed per cycle is increased, while biomass concentration at the beginning of the cycle decreased, resulting in a higher substrate to biomass ratio. Temperature instead was found to be the main parameter affecting the different types of microorganisms selected in the mixed culture, with a predominance of *Plasticicumulans acidivorans* at 30 °C. A maximum PHB intracellular content of 71.3 wt % was obtained at 30 °C with a CL of 18 h (SRT/CL equal to 1.33 d/d). Another study[30] investigated the impact of the CL on polymer production and on microbial community selection in a SBR operated at different CLs (1, 2, 4, 8 h) with SRT-HRT both equal to 1 day and high OLR (20.0 g COD/L d). The CL was recognized as the main parameter affecting both the storage and the growth response. This was due to the different extents of the famine and feast phase, which turned out to be crucial, especially in this high-load process. The highest storage yield of 0.46 COD/COD was obtained with a high-intermediate SRT/CL of 12 d/d. A strong influence of CL was assessed also on microbial composition, unlike in the other cited study,[31] probably due to the much higher applied OLR. A following work[46] confirmed the statement of Dionisi et al.[30] The authors tested four different runs with decreasing cycle lengths (8, 8, 6, and 2 h) coupled with different feeding frequencies of the carbon source. The results indicated that the lowest SRT/CL ratio values of the selection reactor did not correspond to the most performing runs. The runs with the lowest SRT/CL were indeed characterized by a higher concentration of nonpolymer biomass and lower storage response. The most performing results in terms of storage response were obtained at the highest SRT/CL ratio, which corresponded to the lowest cycle length investigated for 2 h. Moreover, the microbial community changed during the different runs investigated, showing an influence of the feeding frequency and of the cycle length on the culture selection. An explanation of the different results achieved in the present study might be identified in the applied OLR, which was more than doubled compared to the previous work (8.5 g COD/L d vs 4.0 g COD/L d). This theory can be also supported by the study of Dionisi et al.,[30] in which the OLR was even higher (20.0 g COD/L d) and the microbial community was found to be strongly affected. It may be deduced that with higher OLRs, the selective pressure applied on the biomass is more driven by high-intermediate SRT/CL, leading also to changes in the microbial community. On the contrary, in processes with lower or intermediate OLRs, the SRT/CL ratio needs to be maintained at low values to maximize the selective pressure applied to the system. The effect of SRT/CL on microbial community cultivation at low-intermediate OLRs is still not clear, since the outcomes from the work of Jiang et al.[31] are quite contradictory with respect to the microbial community differentiation obtained in this study. It has to be taken into account that the studies of all previously cited works were conducted with synthetic substrates and the applied OLRs were substantially different from the one applied in this study. Comparable results in terms of storage rate and storage yield, equal to 339 mg COD_P/g COD_{Xa} h and 0.56 COD/COD, respectively, were obtained with a similar OLR (4.7 g COD/L d) in a SBR fed with a real substrate (pretreated olive mill wastewater[47]) and SRT/CL of 4 d/d. The authors investigated the effect of the OLR on the selection performances by maintaining a fixed SRT/CL ratio. The length of the famine phase was recognized as the main selective pressure applied to the system, since when it was not long enough it did not ensure the required internal growth limitation, leading to less PHA accumulation and a higher growth response during the feast phase. Indeed, the feast to CL or to the famine phase ratio can give an immediate and direct indication of the effective management of the selection process. However, when evaluating more thoroughly the performances of the selection process and especially with complex and nutrient-rich real substrates, the SRT/CL ratio parameter can give better insights into how well the selection process actually takes place and how much the culture is enriched only in PHA-storing microorganisms.

Microbiome Composition. The majority of the bacterial taxa obtained from the samples taken under steady-state operating conditions was affiliated with phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. *Proteobacteria* was the most abundant phylum in almost all runs representing between 15.3 and 70.5% of total reads. Within this phylum, members of *Alphaproteobacteria* affiliated with *Brevundimonas* and *Mesorhizobium* were mainly found in runs Ae1 and Ae4 with relative abundances up to 15 and 8.4% (Figure S1). In run Ae3, 48.5% of total sequences were affiliated with the family *Rhodobacteraceae* with the predominance of genus *Paracoccus* (36.8%), in contrast to the low abundances observed in all other samples (<1%). Within *Gammaproteobacteria*, sequences affiliated with genera *Hydrogenophaga*, *Thauera*, and *Pseudoxanthomonas* were retrieved in all samples. Members of the genus *Lautropia* were only found in runs Ae1 and Ae4 (11.5 and 7.4% of total reads, respectively). *Bacteroidetes* represented between 6 and 38.6% of total reads and were affiliated with genera *Ferruginibacter*, *Leadbetterella*, *Persicitalea*, and *Empedobacter* in all samples and with *Flavobacterium* mostly in Ae3 run (16.3% of total reads). The occurrence of *Firmicutes* was mainly observed in run Ae2, in which sequences affiliated with the *Clostridiaceae* family reached up to 60.8% of total reads with the dominance of the genus *Acetoanaerobium* (50.6%).

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Sequences belonging to *Exiguobacterium* were mainly retrieved in run Ae4 (14.5%). Lastly, *Actinobacteria* represented between 1.2 and 33.2% of total reads in all samples, with a large occurrence of *Leucobacter* in run Ae4 (32.9%). Overall, the analyses revealed distinct bacterial profiles over different runs. In particular, the relative abundance of putative PHA-storing bacteria selected under different runs varied and decreased with the increase of the SRT/CL ratio (Figure S1 and Table S1). The highest relative abundance of sequences affiliated with known putative PHA-storing bacteria was found in Ae3 (up to 64%). In contrast, only 17.9% of PHA-storing bacteria were found in Ae2 and around 28% in Ae1 and Ae4 (Figure 4).

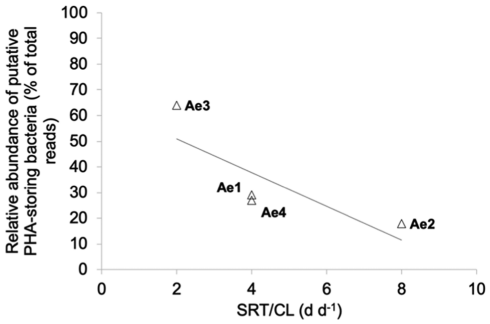

Figure 4. Relative abundance of sequences affiliated with putative PHA-storing bacteria out of total sequences in relation to the SRT/CL ratio.

This finding showed that the adoption of different SRT/CL ratios may have different selective pressures on the mixed microbial communities and a strong impact on the PHA storage process. In particular, *Flavobacterium* and *Paracoccus* were enriched in run Ae3, whereas *Brevundimonas*, *Exiguobacterium*, and *Thauera* were the main PHA-storing genera in runs Ae1 and Ae4. Lastly, run Ae2, characterized by the lowest PHA storage yield and rates, showed low abundances of PHA-storing bacteria mainly affiliated with *Leifsonia*, *Exiguobacterium*, *Rhodobacter*, and *Thauera* genera. In line with this finding, the highest selective pressure occurring in Ae3 was mirrored by the lowest evenness and biodiversity values (Table S2).

Global Process Yield. The overall yield was calculated for the most performing run (Ae3), in terms of storage yield and maximum PHA content achieved. The mass balance started from the amount of COD necessary for the production of 1 kg of PHA (equal to 1.7 kg of COD_{PHA}). The overall mass flow and process diagram is illustrated in Figure 5. Stages and performances adopted from a previous study[32] are indicated in a different color.

Taking into account an obtained storage yield in the accumulation reactor ($Y^{batch}_{P/VFA}$) equal to 0.62 COD_P/COD_{VFA} and an obtained growth yield in the selection reactor ($Y^{SBR}_{Xa/VFA}$) equal to 0.55 COD_{Xa}/COD_{VFA}, the COD needed is 2.74 kg of COD_{VFA} and 1.79 kg of COD_{VFA} for the accumulation and selection reactor, respectively, with a total of 4.54 kg of COD_{VFA} entering the aerobic line. The configuration adopted, reported in Figure 1, is equipped with a solid/liquid separation stage (centrifuge and membrane filtration system) for the clarification of the fermented stream entering the aerobic line. This stage causes a volume loss of the COD_{VFA} stream equal to 23%, meaning that the total COD entering the solid/liquid separation stage is equal to 5.89 kg of COD_{VFA}, with a loss of

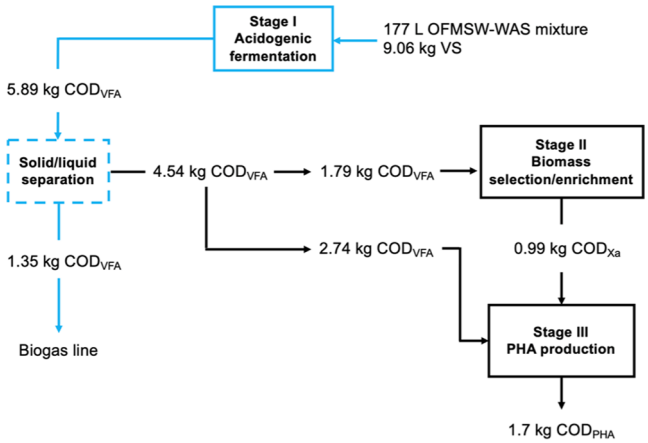

Figure 5. Overall mass balance and process diagram of the most performing run (Ae3). Stages and performances adopted from a previous study[32] are indicated in a different color.

1.35 kg of COD_{VFA} that is sent to the anaerobic co-digestion line for biogas production. Taking into account a fermentation yield (Y_{VFA}) based on the most performing condition proposed in Moretto et al.[32] of 0.65 kg COD_{VFA}/kg VS_{IN}, 9.06 kg of VS_{IN} is needed for the fermentation process to produce 5.89 kg of COD_{VFA}. The corresponding TS_{IN} needed for the fermentation process is equal to 11.33 kg of TS, considering that the OFMSW−WAS mixture is characterized by a VS/TS ratio of 80%. Since the TS concentration of the mixture is 64 kg TS/m^3, the volume of mixture needed for the fermentation process is equal to 177 L. This scenario leads to a calculated overall yield of 110 g PHA/kg VS, which is 45% more compared to the yield calculated in the cited previous work[32] adopting the same process configuration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c04980.

Frequency heatmap of bacterial communities at the genus level; relative abundance of bacterial taxa known for their capability to store PHA; biodiversity indexes; microbiological analysis (PDF)

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Notes

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