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Enrichment of a mixed microbial culture of PHA-storing microorganisms by using fermented hardwood spent sulfite liquor

Joana Pereira [a], Diogo Queirós [a], Paulo C. Lemos [b], Simona Rossetti [c], Luísa S. Serafim [a,*]

[a] CICECO – Aveiro Institute of Materials, Chemistry Department, University of Aveiro, Campus Universitário de Santiago, P-3810-193 Aveiro, Portugal

[b] LAQV, REQUIMTE, Chemistry Department, Faculty of Science and Technology, University NOVA of Lisbon, 2829-516 Caparica, Portugal

[c] Water Research Institute C.N.R., Via Salaria Km 29,300, 00015 Monterotondo, Italy

*Correspondence to: Luísa S. Serafim, Departamento de Química/CICECO, Universidade de Aveiro, Campus Universitário de Santiago, P-3810-193 Aveiro, Portugal.

E-mail: luisa.serafim@ua.pt

Highlights

- Mixed microbial culture (MMC) selected with acidified pulp and paper waste.
- The MMC adapted to the imposed conditions, demonstrating its robustness.
- The MMC showed a stable polyhydroxyalkanoates (PHA) accumulation capacity.
- New generation sequencing analysis showed an increase in PHA producers in the SBR.
- *Acidovorax* and *Comamonas* were the dominant genera.

Abstract

Pulp and paper factories produce several residues that can be explored and valorized through polyhydroxyalkanoate (PHA) production via a three-step process. The objective of this work was focused on the selection step. Acidified hardwood spent sulfite liquor (HSSL), a fermented waste stream from a pulp and paper factory, was used to select a mixed microbial culture (MMC) in a sequencing batch reactor (SBR) operated for 156 days under different operational conditions. The MMC adapted to the imposed conditions, revealing its robustness whenever the operational parameters were changed. Feast-to-Famine ratio was kept below or equal to 0.2, with constant production of a copolymer of P(3HB-*co*-3HV), and with storage contents values over 30%. Changes in the operational conditions, namely cycle length, and organic load rate (OLR), successfully led to the selection of an MMC with a stable accumulation capacity and an increased biomass concentration. Next Generation Sequencing analysis was performed on samples collected during the SBR operational period. The analysis of the microbial composition of the MMC showed a rise in PHA-accumulating bacteria over time. *Acidovorax* and *Comamonas* species were found mainly to drive the PHA storage process during the first two periods of operation. After an increase in the OLR, in the last period, a shift towards *Comamonas* dominance occurred, suggesting a higher tolerance to the inhibitory compounds of the HSSL for this genus.

Keywords

Polyhydroxyalkanoates; Mixed Microbial Cultures; Hardwood Sulfite Spent Liquor; Aerobic Dynamic Feeding; Copolymers; Next Generation Sequencing analysis; Short-chain organic acids; three-step process

Abbreviations

ADF, aerobic dynamic feeding; COD, chemical oxygen demand; CSTR, continuous stirred-tank reactor; DO, dissolved oxygen; F/F, feast and famine; GB, glucose biopolymer; HPLC, high pressure liquid chromatography; HRT, hydraulic retention time; HSSL, hardwood spent sulfite liquor; LS, lignosulphonates; MMC, mixed microbial cultures; NGS, next generation sequencing; OLR, organic loading rate; OTU, operational taxonomic unit; P(3HB), poly(3-hydroxybutyrate); P(3HB-co-3HV), poly(3-

hydroxybutyrate-co-3-hydroxyvalerate); P(3HV), poly(3-hydroxyvalerate); PHA, polyhydroxyalkanoates; SBR, sequencing batch reactor; SCOA, short-chain organic acids; SRT, sludge retention time; VSS, volatile suspended solids

Introduction

The amount of waste produced daily and the resources required for its treatment before disposal are critical societal issues. Moreover, not all waste is treated and some is discharged directly into the environment. Persistence of plastics has a huge impact in all ecosystems. Alternatives to conventional plastics, particularly those which are completely biodegradable, have received considerable attention over the last 20 years. Among the biodegradable materials, polyhydroxyalkanoates (PHA) stand out due to their wide range of properties [1]. The main obstacle to the commercialization of PHA is the high production cost. So far, several bottlenecks were tackled to reduce PHA price, from new microbial isolates and the use of metabolic engineering for upgrading of bacterial strains to the development of more efficient fermentation processes [2]. Nevertheless, the price needs to be further reduced in order to make the process competitive with other materials. Alternatives include the use of waste or by-products and mixed microbial cultures (MMC) of undefined composition in a treatment/valorization strategy. However, most of these substrates are too complex to be converted directly into PHA and require a previous step of anaerobic fermentation to convert their organic content into short-chain organic acids (SCOA).

The percentage of each acid produced in the fermented feedstock determines the monomeric composition of the PHA obtained, which, in turn, determines the physical and mechanical properties of the final polymer [3–6]. For this reason, the key for a successful PHA production by MMC is the selection of a microbial community capable of storing PHA using different SCOA as carbon source [7]. When working with real and complex substrates supplied by the industry, they often vary in their characteristics over time. The enriched culture should be robust and resilient enough to adapt to those changes and still deliver good accumulation rates. Usually, the enrichment strategies rely on alternating anaerobic-aerobic or feast-famine conditions together with the manipulation of some operational parameters that include sludge and hydraulic retention time (SRT and HRT), pH, organic loading rate (OLR), and carbon to nitrogen ratio, among others [4].

Prior research focused on the influence of SCOA composition on the selection of PHA-accumulating consortia and the type of PHA produced. Studies using synthetic acids revealed different behaviors by MMC when acclimatized to a specific SCOA, and in batch tests fed with a different one [5,6,8]. For instance, when using acetate or propionate as the sole carbon source, two distinct populations were selected and behaved differently when the acid fed was shifted [5]. This behavior was confirmed when an MMC acclimatized to a certain SCOA exhibited better PHA production in terms of kinetics and stoichiometry with the substrate used for the selection [6].

Moreover, mixed-carbon-acclimated MMC performed better in terms of PHA production and with faster kinetics [6].

A few of the published studies on the selection of PHA-storing MMC also carried out an in-depth view of the microbial aspect of the process and how variations in the process impacted the microbial community. As an example, three feast and famine systems operated with different fermented wastes, each enriched in a different SCOA (acetic, propionic and butyric), showed dominance of *Paracoccus* after the first days of selection [9]. A shift in the dominant species was then observed in all cases. After 100 days, the reactor fed with a propionic acid-rich stream showed a prevalence of the genus *Thauera*. In the final stage of operation, in both reactors fed with acetic and butyric acid, the dominant operational taxonomic unit (OTU) belonged to the *Comamonadaceae* family [9]. Fermented molasses enriched in SCOA enabled selection of an MMC dominated by genera *Azoarcus*, *Thauera,* and *Paracoccus* and able to produce a copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-*co*-3HV) [10]. The composition of microbial populations was correlated with substrate uptake. *Azoarcus* and *Thauera* primarily consumed acetate and butyrate, respectively, while *Paracoccus* consumed a broader range of substrates [10]. Fermented dairy manure liquor with diverse SCOA profiles was also used to select a P(3HB-*co*-3HV)-producing MMC, with *Meganema* as the most abundant genus [11]. Finally, hardwood spent sulfite liquor (HSSL), supplemented with SCOA, to simulate a fermented stream, supplied during 70 days was used to select PHA-storing microorganisms. The enriched MMC produced a P(3HB-*co*-3HV) polymer, with *Acidovorax* as the dominant genus [12].

The objective of the present work was the enrichment in PHA-storing microorganisms of an MMC which was able to consume SCOA resulting from acidogenic fermentation of HSSL, as described in [13]. After the conversion of HSSL carbohydrates to SCOA, the fermented stream was fed to a selection reactor operated under different conditions in order to optimize the process. The main challenge was to obtain a culture adapted to the complex matrix of fermented HSSL and capable of using the different SCOA to produce PHA at high production rates and yields in a further accumulation step. The impact of changing some operational parameters on the microbial composition of the enriched MMC was also evaluated.

Material and Methods

Culture

The seed MMC was collected from an aerobic tank of the wastewater treatment plant SIMRia - Aveiro Sul, Portugal.

Culture Medium

The culture medium used resulted from the biological acidification of HSSL supplied by Caima – Indústria de Celulose SA (Constância, Portugal). HSSL was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. A preliminary pretreatment to remove part of lignosulphonates (LS) of HSSL was performed, as described elsewhere [12]. HSSL was diluted (1:12 v:v) with a mineral solution comprising (per L distilled H_2O): 160 mg $MgSO_4 \cdot 7H_2O$, 80 mg $CaSO_4 \cdot 2H_2O$, 160 mg $FeSO_4$, 80 mg $Na_2MoO_4 \cdot 2H2O$, and 160 mg NH_4Cl. After adjusting the pH to 7.0, the medium was autoclaved for 20 min at 121 °C. Under sterile conditions, KH_2PO_4 (160 mg/L) and K_2HPO_4 (64 mg/L) were added. Finally, the HSSL was acidified in a continuous stirred-tank reactor (CSTR) as described previously [12]. The acidified effluent was centrifuged for 1 h at 2800 rcf and filtered using a 1 µm glass microfiber filter. The SCOA concentration was determined by high pressure liquid chromatography (HPLC) and the fermented stream was diluted with the mineral solution described above to achieve the desired OLR. The fermented stream was then autoclaved for 20 min at 121 °C before being supplied to the selection reactor.

Selection Reactor

The selection reactor was operated as a sequencing batch reactor (SBR) and worked for 156 days under an aerobic dynamic feeding (ADF) strategy, during which alternating feast and famine phases were imposed, in order to enrich the MMC in PHA-storing organisms. The reactor working volume was 1.5 L and it was operated under three different conditions. For the first 25 days, the cycle length was 24h, comprising 22.5 hours of aerobiosis, 1 h of settling, 0.5 h of withdrawing (half of the reactor volume was removed), and, finally, 15 min for volume replacement with fresh medium. This resulted in an HRT of 2 d and an OLR of 2.2 gCOD/L.d. Thereafter, the cycle length was reduced to 12h for 64 days, with 10.5 h of aerobiosis, resulting in an HRT of 1 d and an OLR of 4.5 gCOD/L.d. Finally, on day 90, the OLR was increased to 7.0 gCOD/L.d without changing the other parameters.

During the operational time, the SRT was kept at 5 d by purging 300 mL at the end of the aerobic period. Reactor stirring (400 rpm), aeration, feeding, and withdrawal pumps were controlled with timers. The SBR worked without pH control at room temperature. Dissolved oxygen (DO), temperature (Oxygen Meter Transmitter M300, Mettler-Toledo Thornton, Inc., Spain) and pH were continuously monitored. In order to prevent foam formation, diluted silicone anti-foam (1:20) was manually added when an excessive foam was observed.

Sampling

Several samples were collected periodically from the SBR in order to monitor the overall performance during the cycles. For each cycle, a sample was collected before

the beginning of the feeding (t = - 0.1 h), immediately after the stop of feeding pump (t = 0 h), with a 0.5 h interval until t = 3 h, and after that at intervals of 1 h until t = 8 h. At the time of the sample collection, values for pH, temperature, and DO were registered. Samples were centrifuged at 8500 rcf for 10 min. The solid component was separated from the supernatant and both frozen at -16 °C. The first was used for PHA and glucose biopolymer (GB) determination and the latter for pH, SCOA, LS, ammonium, and chemical oxygen demand (COD) determination.

Samples for the analysis of microbial community composition were also collected at the beginning of the operation and whenever the reactor performance was considered stable after a change on the operational conditions and stored at -16 °C.

Analytical Methods

Determination of PHA cell content was performed using gas chromatography according to [14]. HPLC was used to determine the concentration of SCOA. Samples were filtered with 0.2 µm pore size filters and then injected (Auto-sampler Hitachi L-2200, Hitachi, Japan) into an anion exchange column (Rezex™ ROA-Organic Acid H+ (8%), Phenomenex, USA) connected to a refraction index detector (Hitachi RI L-2490, Hitachi, Japan). The column was at 65 °C in an external oven (Oven Gecko-2000, CIL Cluzeau, France) and the eluent used was 0.01 N H_2SO_4, prepared with Milli-Q water. The eluent flow rate was 0.5 mL/min (Hitachi L-2130, Hitachi, Japan). The monitorization of LS content was performed as described elsewhere [15]. COD and volatile suspended solids (VSS) were quantified according to Standard Methods [16]. The ammonium concentration was followed using an Ion Selective Electrode (Thermo Scientific, USA), after adding 20 µL of Ionic Strength Adjuster (5 M NaOH, 0.05 M EDTA, 10 % methanol) to 1 mL of sample. Calibration was performed by resorting to a standard curve of NH_4Cl. GB was determined after being extracted from lyophilized cells through acidic digestion (1 mL HCl 0.6 mol/L, 2 h, 100°C). Digested samples were filtered, and the liquid fraction was analyzed by HPLC using an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA), at 60°C, and a refractive index detector (Merck, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL/min).

DNA extraction and Next Generation Sequencing (NGS)

DNA extraction for NGS analysis was performed on samples collected for microbial determination with a Power Soil DNA extraction kit (MoBio, Italy) following the manufacturer's instructions. DNA extracted from each sample was eluted in 100 µL sterile Milli-Q water and its concentration and purity were analyzed with a Nanodrop 3300 (Thermo Scientific, Italy). Finally, 10 ng of extracted DNA were used for the NGS analysis following the procedures described in the Supplementary Material.

Calculations

PHA content was calculated as a percentage of VSS on a mass basis (% of cell dry weight – cdw):

$$\% \text{ PHA} = \text{g HA/g VSS} \times 100 \quad (1)$$

Feast to famine ratio (F/F) was calculated by dividing the time needed to the consumption of SCOA by the remaining time of the cycle [17].

Total SCOA and acetic, propionic, and butyric acids volumetric (-r) and specific consumption rates (-q), PHA, poly(3-hydroxybutyrate) (P(3HB)), and poly(3-hydroxyvalerate) (P(3HV)) specific production rates were determined by adjusting linear functions to the experimental data for each variable concentration over time and calculating the first derivative at time zero. In the case of specific rates, each variable was divided by the biomass concentration at that point.

PHA production yield on substrate ($Y_{PHA/S}$) was calculated by dividing the amount of PHA by the total substrate consumed. PHA specific productivity was calculated by dividing the amount of produced PHA by biomass and time and the volumetric productivity dividing the amount of produced PHA by volume and time.

The Shannon Index (*H'*) was calculated according to the expression

$$H' = -\sum p_i \ln(p_i) \quad (2)$$

where p_i is the proportion of clones in the *i*th OTU (estimated using n_i/N).

Results and Discussion

Selection Step

HSSL has already been tested to acclimatize a PHA-storing MMC, but instability dominated the operational periods most of the times [18] or low PHA accumulation was achieved with a maximum of 6.6% cdw [17]. Supplementation of HSSL with SCOA allowed a stable process to be obtained after 25 days with better accumulation, reaching a maximum of 34.6% cdw [12]. In the present work, the SBR was operated for 156 days and the culture selection was achieved using fermented HSSL as substrate under ADF conditions. The fermented HSSL was obtained by acidogenic fermentation performed in an anaerobic CSTR operated without pH control, at 30 °C with retention time of 2.34 days. The performance of the CSTR in the acidogenic fermentation of HSSL is described elsewhere, with the main organic acids obtained being acetic, propionic, butyric, valeric, and lactic [13].

Since microbial growth and PHA production are processes that compete for the carbon source [19], the selection step should be a compromise between them [20–23]. In order to balance both processes, bearing in mind that OLR plays a key role, the selection reactor was operated under different conditions, in three distinct periods (**Table 1**). Until day 26 (P1), OLR was kept low, 2.2 gCOD/L.d, allowing MMC adaptation to the fermented stream to occur. Even after the preliminary acidification step, the fermented HSSL still contained several recalcitrant compounds that could be toxic for

the MMC [13,24]. Operating at a low OLR would guarantee a true feast and famine condition. In this way, the selective pressure required to increase the number of PHA-storing bacteria was imposed. When the MMC reached an apparent stationary phase, the OLR was increased to 4.5 gCOD/L.d by decreasing the cycle length to 12 h. In this way, an increase in biomass concentration without losing the PHA production ability was expected. The SBR was operated under these conditions from days 26 to 90, corresponding to period P2. Finally, the OLR was increased to 7.0 gCOD/L.d by increasing the COD concentration in the feeding stream and the SBR worked from days 91 to 156 (P3).

During the operational period of the selection SBR, several parameters were monitored: SCOA uptake, PHA production. biomass concentration, F/F ratio, COD and ammonium uptake. These allowed evaluation of the adaptation of the MMC to the selective pressure imposed in the SBR reactor conditions and its capacity to accumulate PHA. **Figure 1** shows some of the parameters obtained throughout the operational period of the selection reactor.

From the start and during the first stage of operation (P1), F/F quickly stabilized with an average value of 0.18 ± 0.04. This was a good indicator of the selection of PHA-storing organisms since, according to the literature, there is a correlation between F/F ratio and the PHA accumulation ability of the MMC [20,22]. Several studies reported that F/F ratios below 0.20–0.25 were associated with good PHA storage capacity. In contrast, when higher values were observed, PHA storage played a negligible role and the substrate was diverted mainly towards growth [20,22]. Several reports using ADF as a selection strategy demonstrated the ability of PHA accumulation behavior for F/F under 0.2. The effect of the OLR on the performance of an SBR fed with a mixture of acetic, propionic, and lactic acids was tested and F/F ratios under 0.25 for periods with a good accumulation capacity with an average storage content of 29.2 ± 4.0% cdw were described [25]. Using acetic acid as sole carbon source, very low F/F ratios (0.1) were obtained when the selective pressure imposed was maximized and resulted in a PHA accumulation of 53% cdw in the SBR cycle [26]. The selected MMC was able to store up to 89% cdw during fed-batch accumulation assays [26]. In other works, when using municipal wastewater F/F ratios of 0.21 were achieved [27], while F/F ratios of around 0.15 were obtained for an SBR fed with fermented brewery wastewater [28].

When the cycle length was shortened to 12 h, a period of instability was observed, with F/F values ranging from 0.09 to 0.85. From day 42 of operation until day 89, F/F values decreased and remained at 0.16 ± 0.03 (Figure 1). This value showed that the change in operational conditions by increasing the amount of substrate supplied to the MMC did not affect its ability to consume it nor the capacity to store PHA. Moreover, the new F/F ratio was slightly lower than that observed under the previous operational

conditions. On day 90, the OLR was increased to 7.0 gCOD/L.d, which led to a new stage of adaptation to this condition. During this last period, the F/F ratio was kept at 0.21 ± 0.09. Once again, the MMC was able to adapt to the increase in the amount of substrate supplied. It is worth noting that all adaption periods required no longer than 4.5 SRT, confirming the MMC dynamics and capacity to adapt towards the new conditions.

Biomass concentration in the selection reactor increased throughout the operational time. A clear decrease in biomass concentration was observed after the inoculation of the reactor. The value for period P1 corresponded to an average biomass concentration of 1.5 ± 0.4 gVSS/L, followed by an increase to 2.4 ± 0.4 gVSS/L during P2. Finally another increase was observed for P3 to an average concentration of 3.4 ± 0.7 gVSS/L. This increase in biomass with the gradual increases of OLR occurred even with successive higher concentrations of toxic compounds present in the HSSL. Similar values were reported for selection reactors fed with brewery wastewater [28] or fermented sugarcane molasses [21,22]. The maximum concentration of biomass in a selection reactor fed with fermented sugarcane molasses was reported as 8.0 gVSS/L, after controlling pH at 8.0 as a strategy to increase biomass growth rate while maintaining the specificity of the enrichment for PHA-producing organisms [21].

In all periods, after the modification of an operational parameter, erratic accumulations were observed which eventually stabilized. In P1, PHA accumulation did not stabilize, despite the stabilizations observed for F/F ratio and biomass concentration on day 6. PHA accumulation reached a maximum value of 44.5 % on day 13 and, then decreased until the change of conditions. The average PHA content during this period was 21.4 ± 15.8%. PHA accumulation followed the same tendency observed for F/F ratio during P2 and P3. For P2, the average PHA storage content was 16.8 ± 8.7% cdw and during P3, 21.8 ± 15.0% cdw. Several authors have also reported a similar polymer content and analogous behavior of the MMC [29,30]. An average concentration of 15% was reported during the period of stable operation of an SBR reactor fed with an acetic and propionic acids mixture [29]. Others, using bio-oil as substrate, obtained a PHA content in the selection SBR of 9.2% [14], being a similar value reported with HSSL [17]. Lower contents were also observed in a pilot-scale reactor integrated into a municipal wastewater treatment plant that selected an MMC with PHA contents below 4% [27]. On the other hand, an average PHA content of 25% was observed when supplying fermented molasses as carbon source [22], and PHA content between 9.55 and 30.75% were reported in four SBRs operated at different SRT fed with fermented sugarcane wastewater [31].

The monomeric composition of PHA produced is an important factor in defining the final application of the polymer and therefore knowing how to manipulate it is highly

desirable [1]. The monomeric composition of PHA accumulated during the SBR operation was evaluated (**Figure 2** and **Table 2**) and showed that throughout the operational time a copolymer of P(3HB-*co*-3HV) was produced, which was expected since the carbon source had precursors for both monomers [5]. During P1, 3HB was the predominant monomer and its proportion in the polymer remained relatively constant, especially after stabilization (day 6), with an average of 65 ± 6.3%. The change in the cycle duration had no visible impact on PHA composition, but after stabilization at day 44, there was a small decrease in 3HB content to an average of 62%. After the change of the OLR on day 90, 3HB content increased from 62% to 78%, followed by a slight decrease to 72%. After that, it increased rapidly to previous values, reaching the highest proportion, 83%, on day 153.

Molar fractions of 3HB and 3HV remained stable during the three periods, which is advantageous for the desirable production of copolymers with a stable composition. The reason for typical variations in PHA composition is usually attributed, not to the microbial composition of the MMC, but to variations in the composition of SCOA fed to the SBR [5,6]. The acidogenic fermentation of HSSL resulted in SCOA profiles with slight variations over time [13]. As shown in Table 1, each period was fed with mixtures of SCOA having similar proportions of even (acetic and butyric acids) and odd (propionic and valeric) SCOA, the former being responsible for the production of 3HB, while the latter for 3HV [7]. Due to the constant dominance of acetic acid as the main fermentation product (plus butyric acid), 3HB was the predominant monomer. Previous studies with HSSL reported the accumulation of GB due to the high sugar fraction of the liquor [17], allowing a side population to develop and decrease PHA accumulation and process yield. Although some xylose was not converted into SCOA and consequently remained in the effluent from the acidogenic reactor, no GB production was observed.

P(3HB-*co*-3HV) production in selection SBRs using real feedstocks is widely documented [22,28,29,31,32]. Similar monomeric proportions to those of this study have been reported when using bio-oil [14], and with fermented molasses without pH control [21]. A copolymer of P(3HB-*co*-3HV) with 20% of 3HV was obtained using non-fermented HSSL [17], and using HSSL supplemented with synthetic-SCOA [12]. The accumulation of this copolymer was associated with the presence of odd SCOA [12]. The higher 3HV fraction obtained could be the result of the introduction of a pre-fermentation step, resulting in a wider variety of SCOA and a higher production of precursors for 3HV, such as propionic and valeric acids.

SBR cycles were monitored periodically to evaluate MMC performance. Several parameters were followed, namely the concentrations of SCOA, PHA, biomass, COD, ammonium, and LS, as well as DO percentage and pH. Average kinetic and

stoichiometric parameters of the three periods are shown in Table 2. In general, similar behavior was observed for all periods after reaching stabilization. SCOAs were consumed at different volumetric rates, with a clear preference for acetic over all the other acids in all periods. This preference could be explained by the metabolic pathways for the different acids [5,33]. Nevertheless, an increase in the preference for propionic acid consumption was observed after P1, probably as a result of a good MMC selection process (Table 2).

The MMC showed higher storage yields in P1, while the values decreased during P2 and P3, after an initial period of instability (Figure 1). Combination of long cycle lengths and low OLR led the MMC to direct the carbon to PHA accumulation instead of growth. Due to the long starvation period, the culture required more PHA to survive than in shorter cycles. The imposition of several cycles with these conditions steered the culture towards high PHA accumulation (Table 2). As the OLR increased, either through cycle manipulation (P2) or feed concentration (P3), the MMC started to develop cellular machinery to divert more carbon source for growth, leading to an increase in biomass concentration inside the reactor while PHA accumulated at lower rates (Table 2). Nonetheless, an increase in PHA accumulation rates was observed from P1 to P2 and P3 (Table 2), allowing an increase in volumetric productivity in the next step, with biomass again being formed. Even with the decrease in PHA production during the operation of the reactor, the specific productivity values increased throughout the operational period of the reactor.

Apparently SCOAs were the only carbon source to be consumed. Although other compounds such as LS and phenolics were likely to be consumed, their complexity and the reaction time made them difficult to metabolize. LS consumption was not observed during any cycle monitored, even though a slight use, possible from the microbial community with no accumulation capacity, was reported previously with HSSL [18].

Microbial community analysis

With the objective of evaluating the impact of the reactor conditions on the microbial community, high-throughput 16S rRNA gene sequencing was performed on MMC samples taken at the beginning of the operation and each time a stable period of the reactor performance was reached after changing the operational parameters. The bacterial community composition at phylum and genus level from each sample is represented in **Figure 3**. The initial inoculum mainly comprised *Bacteroidetes* (38.9% of all OTUs), *Chloroflexi* (28.4%) and *Proteobacteria* (18.5%) phyla (Figure 3a). Overall, 321 OTUs were present, decreasing to 129 when the last period was reached. This was a consequence of the selective pressure imposed by the SBR operating conditions. The establishment of a highly selected MMC was also reflected by the decreased Shannon

Index from 3.8 to 2.6, likely caused by the disappearance of microorganisms that failed to adapt to the complex matrix of the substrate (Table 3). Known PHA-accumulating microorganisms were found to be predominant in all samples after starting the operation. The relative abundance of the 25 most common OTUs in each sample collected can be found in Figure A of the Supplementary Material.

Proteobacteria was the main phylum found in the selection reactor, with a maximum of 94% of the total OTUs in P1. This was expected since many PHA accumulators belong to this phylum [34] as reported in previous works with the same substrate [12,18]. *Acidovorax* and *Alcaligenes*, both associated with PHA production [12,35], were the most abundant at 16.9% and 13.0%, respectively. Other known PHA-accumulators were also found at lower relative abundances, namely affiliated with *Paracoccus* [10], *Rhodobacter* [36], *Rhizobium* [37], and *Comamonas* [38] genera.

The change in the conditions seemed to favor *Acidovorax* since its prevalence increased to 31.8% in P2, indicating that the selection process was successful. The same genus was selected under similar conditions fed with HSSL, and the best results of PHA production were obtained during the phase dominated by members of this genus [12]. The reduction of cycle length and the increase in OLR seemed to result in a decreased proportion of *Alcaligenes* from 13.0% in P1 to 0.5% in P3. A different trend was reported for a PHA-producing microbial community fed with a synthetic mix of SCOA when studying the effect of the length of the cycle *Alcaligenes* dominated in shorter (2h) cycle lengths [39]. Since *Alcaligenes* is reported to tolerate high concentrations of synthetic acids [25], intolerance to other inhibitors present in the complex matrix of the HSSL could be a possible explanation for the differences found here.

On the other hand, *Acidovorax* severely decreased in P3 (0.2%), with the increase of the OLR. In this stage, *Comamonas* was the main genus present (43.3%), followed by *Rhizobium* (16.3%). The shift in the main PHA-accumulators could be explained by the OLR increase. An increased amount of acidified effluent in the culture medium increased not only the SCOA concentration but also that of other toxic compounds, namely LS and other phenolic components. The strain *Comamonas* sp.B-9 was reported to have the potential to degrade dark liquor from the pulp and paper industry [40], as it showed evidence of lignin degradation [41]. Since the LS concentration was 3.15 and 4.62 g/L (in P2 and P3 samples, respectively), higher tolerance to LS could explain *Comamonas* dominance in P3. The increase in the relative abundance of *Rhizobium* in the selection reactor with the increase of OLR could be attributed to the same phenomena. The capability of *Rhizobium* to metabolize lignin and lignin-like compounds has already been shown and *Rhizobium* sp. NCIM 5590 was used to treat paper mill effluent [42,43].

The results showed that *Acidovorax* was able to adapt to HSSL and dominate the culture, thus becoming the main PHA producer but only at lower OLRs, during P2 (4.5 gCOD/L.d) confirming the observations in previous studies with HSSL (4.7 gCOD/L.d [18] and 5 gCOD/L.d [12]). When the OLR increased to 7 gCOD/L.d, genera with more tolerance to inhibitors present in HSSL, such as *Comamonas* and *Rhizobium*, prevailed. Despite a shift in the main dominant bacteria from P2 to P3, the overall performance of the reactor was stable and significant accumulation rates were achieved, evidencing an advantage of working with MMC. This data, together with kinetic and stoichiometric parameters obtained in the SBR cycles and discussed before, suggest that the enrichment in PHA-producing microorganisms was successful.

Conclusion

This work explored the possibility of selecting an MMC to produce PHA from acidified HSSL, a pulp and paper waste stream. The selected MMC was able to accumulate PHA throughout the operation time, regardless of the slight variations in the substrate SCOAs composition. Several conditions were tested, resulting in three distinct periods of operation. Over the 156 days of operation, a copolymer of P(3HB-co-3HV) was produced with variations in the monomeric proportions. Low F/F ratios in all periods of operation and PHA accumulation indicated that the selection process was effective. An increased average biomass concentration from 1.5 ± 0.4 to 3.4 ± 0.7 g/L was achieved by increasing the OLR feed to the reactor, apparently without inhibitory effect. Finally, an assessment of the microbial culture dynamics over time showed an increase in PHA accumulators. *Acidovorax* and *Comamonas* species were primarily found to drive the PHA storage process under the conditions investigated. The increase of the applied OLR, and consequently of HSSL inhibitors, selected bacteria affiliated with the *Comamonas* genus suggesting a higher tolerance to those compounds. The MMC capacity to adjust to the operational conditions and variations of the process ensures its successful use in dynamic systems fed with complex substrates. This work has shown the potential for the development of a PHA 3-step production process in a biorefinery concept based on the pulp and paper industry that can be economically and ecologically sustainable.

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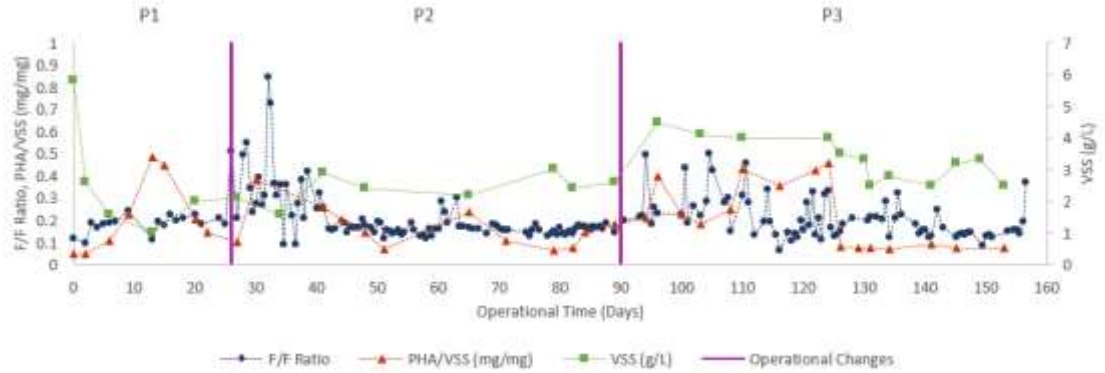

Figure 1. SBR Performance: Evolution of F/F ratio, biomass concentration and PHA content over the different periods of operation of the SBR: P1 with HRT of 2 d and OLR of 2.2 gCOD/L.d, P2 with 1 d and OLR of 4.5 gCOD/L.d, and P3 with 1 d and 7.0 gCOD/L.d

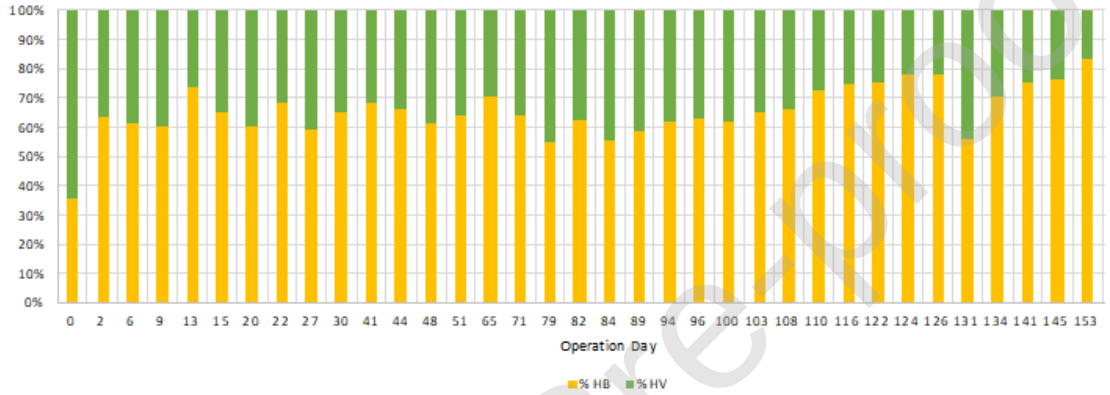

Figure 2. Molar monomeric composition of the P(3HB-*co*-3HV) produced over the different periods of operation of the SBR: P1 with HRT of 2 d and OLR of 2.2 gCOD/L.d, P2 with 1 d and OLR of 4.5 gCOD/L.d, and P3 with 1 d and 7.0 gCOD/L.d

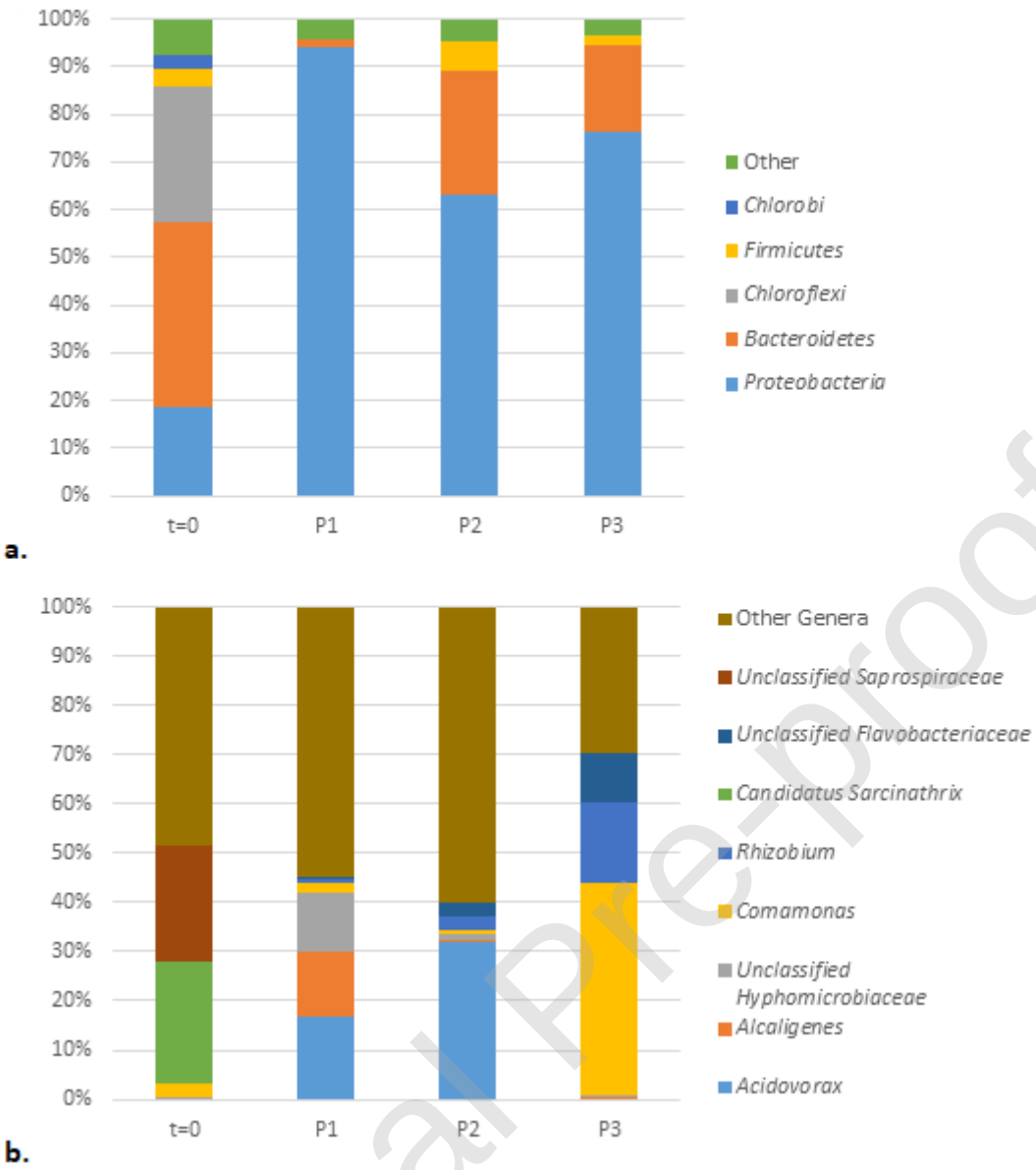

Figure 3. Relative abundances of OTUs at phylum (a) and genus (b) level in the SBR reactor at the beginning of the SBR operation (t=0) and at the end of each experimental phase. The relative abundance of each group, occurring at more than 2.5% (a) and 7.5% (b), is expressed out of total OTUs found in each sample.

Table 1. Conditions imposed on the SBR in each period of operation.

Period designation	Operation period (d)	Cycle length (h)	OLR (gCOD/L.d)	SCOA (Cmmol/L)	Average SCOA composition (%)*	SRT (d)	HRT (d)
P1	1-25	24	2.2	17.5	62:18:11:7:2	5	2
P2	26-89	12	4.5	35	58:18:13:10:1	5	1
P3	90-156	12	7.0	50	67:22:9:1:1	5	1

*acetic:propionic:butyric:lactic:valeric acids

Table 2. Average kinetic and stoichiometric parameters calculated for each period.

Period	P1	P2	P3
F/F	0.18 ± 0.04	0.21 ± 0.12	0.21 ± 0.09
X (g/L)	2.60 ± 1.68	2.40 ± 0.42	3.35 ± 0.66
r_{SCOA} (Cmmol/L.h)	2.32 ± 0.61	7.30 ± 2.72	3.74 ± 1.53
r_{Acet} (Cmmol/L.h)	1.45 ± 0.52	4.43 ± 1.84	2.57 ± 1.31
r_{Prop} (Cmmol/L.h)	0.44 ± 0.27	2.38 ± 1.72	1.16 ± 0.79
r_{But} (Cmmol/L.h)	0.44 ± 0.16	0.49 ± 1.03	n.a.
r_{PHA} (Cmmol/L.h)	0.90 ± 0.22	1.73 ± 0.23	1.57 ± 1.06
r_{HB} (Cmmol/L.h)	0.79 ± 0.14	1.16 ± 0.16	1.26 ± 0.81
r_{HV} (Cmmol/L.h)	0.14 ± 0.07	0.58 ± 0.08	0.32 ± 0.25
%PHA	0.21 ± 0.16	0.17 ± 0.09	0.22 ± 0.15
HB:HV (mol:mol)	61-39 ± 11	63-37 ± 5	68-32 ± 12
$Y_{PHA/S}$ (Cmmol/Cmmol)	0.39 ± 0.14	0.24 ± 0.14	0.42 ± 0.33
$Prod_{Esp}$ (gPHA/gX.h)	0.038 ± 0.026	0.078 ± 0.017	0.051 ± 0.036
$Prod_{Vol}$ (gPHA/L.h)	0.099 ± 0.024	0.188 ± 0.025	0.170 ± 0.115

n.a. – not applicable

Table 3. Period of operation, operational conditions, observed OTU and Shannon Index for each sample.

Period of Operation	Sample Day	Conditions	Observed OTU	Shannon Index
Start	1	24h Cycle; OLR of 2.2 gCOD/L.d	321	3.8
P1	20	24h Cycle; OLR of 2.2 gCOD/L.d	212	3.4
P2	78	12h Cycle; OLR of 4.5 gCOD/L.d	221	3.4
P3	149	12h Cycle; OLR of 7.0 gCOD/L.d	129	2.6