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Impact of Organic Acids Supplementation to Hardwood Spent Sulfite Liquor as Substrate for the Selection of Polyhydroxyalkanoates-Producing Organisms

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Abstract: The effectiveness of polyhydroxyalkanoates (PHAs) production process from a waste stream is determined by the selection of a suitable mixed microbial culture (MMC). In this work, a feedstock from the paper industry, hardwood spent sulfite liquor (HSSL), supplemented with short-chain organic acids (SCOAs) to simulate a fermented effluent, was used as substrate to enrich a MMC in PHA-storing microorganisms. A stable culture was quickly established, and during the accumulation step the selected MMC reached a maximum PHA content of 34.6% (3HB:3HV-76:24). The bacterial community was analyzed through FISH analysis. Bacteria belonging to the four main classes were identified: *Betaproteobacteria* (44.7 ± 2.7%), *Alphaproteobacteria* (13.6 ± 1.3%) and *Gammaproteobacteria* (2.40 ± 1.1%) and *Bacteroidetes* (9.20 ± 3.8%). Inside the *Betaproteobacteria* class, *Acidovorax* (71%) was the dominant genus.

Keywords: polyhydroxyalkanoates; mixed microbial cultures; hardwood spent sulfite liquor; short-chain organic acids; Fluorescence *in situ* Hybridization (FISH)

1. Introduction

The Confederation of European Paper Industries (CEPI) on its Preliminary Statistics of 2015 on Paper and board production states that the production of the member countries fell slightly, by around 0.3% in 2015 after a decrease of 0.2% in 2014 [1]. In Portugal, the tendency was the opposite, with exportations increasing together with investments. The Navigator Company invested 159 million euros to dominate the European market in tissue paper production and ALTRI group invested in the manufacture of soluble paste-used in the textile industry for the manufacture of viscose, with increasing demand projected from China. This represents a massive side production of wastes and residues that could be valorized in a lignocellulosic-based biorefinery [2,3].

Part of residues of pulp and paper industry is usually burnt for energy production. Also, lignin and lignosulphonates are already used as additives in oil well drilling and concrete as dyestuff dispersants, agricultural chemicals, animal feed, and other industrial binders [4]. However, a significant fraction of these residues, namely sulfite spent liquors (SSL), can be converted into value-added

products. SSL, a by-product of acidic sulfite wood pulping process, are rich in C5 and C6 sugars that can be used for the production of succinic acid [5], short-chain-organic acids (SCOAs) [6] and biofuels such as bioethanol [7]. SSL also contains sugar hydrolysis products such as acetic acid that can be used for polyhydroxyalkanoates (PHAs) production [8,9].

The environmental hazards brought by conventional plastics represent a major societal concern. Biodegradable plastics as PHAs can help to tackle this problem, especially when produced from waste or from by-products, such hardwood spent sulfite liquor (HSSL), and when using mixed microbial cultures (MMC) [10]. Such a strategy allows for a cost reduction, since PHAs industrial production, so far, relies on pure cultures and high-cost synthetic substrates [10]. In the last years, efforts are being made to develop alternatives for PHAs production processes based on the use of industrial wastes and by-products as a substrate with the objective of reducing production costs [9,11–14]. However, researchers are still struggling with the low values of PHAs contents and volumetric productivities achieved in MMC processes when compared with the pure cultures. To overcome such a problem, the three-step process was designed [11]. In this process, a MMC enriched in PHAs-storing bacteria is achieved after submitting the microbial population to feast and famine conditions. By alternating periods of excess with periods of lack of carbon source, a selective pressure favorable to PHAs-storing organisms is imposed. This procedure, also known as aerobic dynamic feeding (ADF), is the key to the success of the process [15]. ADF can be tuned by manipulating several parameters such as organic loading rate (OLR) and cycle length. Higher OLRs usually result on the increase of volumetric productivity of PHA production by MMC, since a larger quantity of carbon supplied could result in higher cellular concentrations [12,16]. The shortening of cycle lengths usually leads to higher numbers of SBR's cycles per day and, consequently, increases the influent flow rate, with the specific substrate uptake rate expected to remain constant. Higher biomass concentration with similar substrate uptake and PHAs production specific rates would result in a superior volumetric rate without alteration on the feast/famine ratio (F/F) [12,17]. In the last years, researchers were able to increase the PHAs content in MMC process by applying ADF conditions. Johnson et al. [18] selected a MMC using acetic acid as a substrate that reached a PHAs content of 88% and, later, Jiang et al. [13] observed a PHAs content of 77% using fermented paper mill wastewater as a substrate.

SCOAs are the main precursors for PHAs production by MMC. MMC were reported preferentially to store polysaccharides instead of PHAs from sugar-based compounds when submitted to feast and famine conditions [19]. Queirós et al. [9,15] observed that notwithstanding being able to select a MCC with HSSL under ADF conditions, presenting feast-to-famine ratios indicative of PHAs storage, low accumulations values were obtained. Moreover, Queirós et al. [9] observed the accumulation of a glucose polymer that was probably produced by a side population able to uptake sugars present in HSSL.

As reviewed by Queirós et al. [15], in recent years, several works dedicated to study the composition of MMC populations selected in PHAs producing processes, revealed a broad phylogenetic diversity. However, a link between operational conditions and the selection of a specific taxon over others, as well as the impact of the amount and type of accumulating organisms on the overall PHAs production, remains unclear [20,21]. This is one of the key aspects of PHAs production by MMCs among the influence of the selected population on the polymer quality and the difficulties of the downstream processing.

The objectives of this work were to investigate the viability of enriching an MMC in PHAs-storing organisms with HSSL supplemented with SCOAs (acetic, propionic and butyric acids) and to follow the microbial community evolution through biomolecular analysis. The goal of the supplementation was to decrease the adaptation period and infer if a pre-fermentation of HSSL step would be justified. Moreover, the effect of manipulating the OLR and cycle length on the MMC selection was also evaluated.

2. Material and Methods

2.1. Culture Medium

Hardwood spent sulfite liquor (HSSL) *Eucalyptus globulus* was supplied by Caima-Indústria de Celulose SA (Constância, Portugal). HSSL, a by-product of magnesium-based acidic sulfite pulping, was collected from an inlet evaporator of a set of multiple-effect evaporators to avoid the presence of free SO_2. HSSL was submitted to a preliminary pretreatment to remove part of lignosulphonates, as described by Queirós et al. [8]. Total chemical oxygen demand (COD) of pretreated HSSL was determined, 205 gCOD L^{-1}, as well as the amounts of the main constituents: Lignosulphonates (LS), 120-160 g L^{-1}, xylose, 50 g L^{-1}, and acetic acid, 18 g L^{-1}. No phosphates and ammonia were detected in HSSL.

HSSL was diluted with a mineral solution, 1:45 (*v*:*v*). The mineral solution was composed by (per liter of distilled water): 160 mg $MgSO_4 \cdot 7H_2O$, 80 mg $CaSO_4 \cdot 2H_2O$, 20 mg $FeCl_3$, 8 mg $Na_2MoO_4 \cdot 2H_2O$, 160 mg NH_4Cl. Then, the diluted HSSL was supplemented with acetic (12 Cmmol L^{-1}), propionic (8 Cmmol L^{-1}), and butyric (10 Cmmol L^{-1}) acids. The pH of the medium was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 °C. Finally, under sterile conditions, KH_2PO_4 (16 mg L^{-1}) and K_2HPO_4 (64 mg L^{-1}) were added to the medium. Thiourea (400 mg L^{-1}) was also added to inhibit nitrification.

2.2. Reactor Operation

Activated sludge was collected from the aerobic tank of the municipal wastewater treatment plant Aveiro Norte (SIMRia). Then, it was inoculated to a sequenced batch reactor (SBR) working under aerobic dynamic feeding (ADF) conditions with a working volume of 1.5 L and working in cycles of 24 h. Each cycle included 22.5 h of aerobiosis, with the addition of fresh medium the first 0.25 h, 1 h with no agitation nor aeration in order to promote the settling of biomass, and 0.5 h of withdrawing. The different periods of the cycle were controlled with timers connected to the reactor stirring (400 rpm), feeding and withdrawing pumps, and aeration pump. This cycle resulted in a hydraulic retention time (HRT) of 2 days. A sludge retention time (SRT) of 5 days was imposed by purging 300 mL at the end of the aerobic period. The cycle duration was changed to 12 h and comprised 10.5 h of aerobiosis, 1 h of settling and 0.5 h of withdrawing. The HRT and SRT were kept at 1 and 5 days, respectively. The OLR was 2.5 gCOD L^{-1} d^{-1} for 24 h cycles and 5 gCOD L^{-1} d^{-1} for 12 h cycles.

The SBR worked without pH control and at room temperature. Diluted silicone anti-foam (1:20) was manually added to prevent foam formation. The SBR was cleaned on a daily basis to prevent excessive biofilm formation. SBR cycles were monitored periodically by measuring dissolved oxygen, temperature (Oxygen Meter Transmitter M300, Mettler-Toledo Thornton, Inc., city, Spain), and pH, and by taking samples across the entire reaction period. Samples were centrifuged at 14,000 rpm and both supernatant and solid phase were stored for later quantification of SCOAs, LS, ammonium, COD and PHAs.

2.3. Accumulation Tests

Fed-batch assays to test the effect of no nutrient limitation, ammonium limitation, and phosphorus limitation were carried out in a bioreactor (BIOSTAT® A plus) without pH and temperature control, and sterile conditions. A respirometer with an oxygen probe was coupled to the bioreactor and recirculation of the medium was performed by a peristaltic pump. The stirring in the reactor and the respirometer was kept constant at 250 rpm, and the supply of air to the bioreactor was performed by an air pump (Boyu Air Pump 8 L min^{-1}, Guangdong Boyu Group Co., Ltd., Chaozhou, China).

In each test, 1 L of biomass was collected from the main bioreactor, after withdrawn and before the feeding period, and inoculated to the fed-batch reactor. Five pulses of 500 mL of feed were supplied to a final working volume of 3.5 L.

2.4. Analytical Methods

The biomass concentration was determined as volatile suspended solids (VSS) following the procedure described in Standard Methods [22]. Samples of 5.0 mL were periodically collected and filtered in pre-calcined and weighted microfiber membranes of 1.0 μm pore size. Membranes with biomass were dried at 100 °C in oven for 24 h and then weighted. Finally, the membranes were calcined for 30 min at 550 °C, and weighted after cooling in an exsiccator.

PHAs quantification and monomeric composition were determined based on the gas chromatography methodology described by Lemos et al. [23].

Glucose biopolymer (GB) was measured according to Moita et al. [14] after being extracted from lyophilized cells through digestion with 1 mL HCl 0.6 M for 2 h at 100 °C. Digested samples were filtered; the liquid fraction was analyzed by HPLC using an Aminex HPX-87 H column (Bio-Rad Laboratories, San Francisco,.CA, USA), at 60 °C, and a Refractive Index detector (Merck, Darmstadt, Germany), using H_2SO_4 0.01 N as eluent (0.5 mL min^{-1}).

Acetic, propionic and butyric acids were measured by HPLC according to Queirós et al. [8]. The HPLC was equipped with an ion exchange column Aminex HPX-87H at 40 °C (Oven Gecko-2000, CIL Cluzeau, France) with the eluent 0.01 N H_2SO_4 being pumped at a flow rate of 0.6 mL/min by a Hitachi L-2130 pump, and coupled to a refractive index detector (Hitachi L-2490, Hitachi, Japan). 650 μL of sample were filtered using centrifuge tube filters with cellulose acetate membranes of 0.2 μm pore size at 8000 rpm for 20 min. 20 μL of filtered samples were injected by an Auto-sampler (Hitachi L-2200, Hitachi, Japan). The concentrations of SCOAs in g/L were determined by using calibration curves obtained using standards of known concentrations of each analyzed compound. The standards concentrations were within the range of the expected concentrations of the analytes: 0.20 g/L to 4.00 g/L for propionate and butyrate and 0.25 g/L to 5.00 g/L for acetate.

LS were determined following the methodology of Restolho et al. [24] and considering a molar absorptivity of 7.41 g^{-1} cm^{-1} [25]. After a dilution of 1:200, the absorbance of samples was measured in a Spectrophotometer (Shimadzu UVmini-1240, Shimadzu, Tokyo, Japan) at 273 nm.

Chemical oxygen demand (COD) was quantified according to Standard Methods [22]. Each sample was diluted accordingly to the detection range of the method (100–900 mg/L), being substituted by 2.0 mL of distilled water in blank. After vigorous agitation, samples were digested 2 h at 150 °C. Then, samples were placed in the dark to cool down to room temperature. Finally, the absorbance was read at 600 nm with a colorimeter. A calibration curve was performed with glucose standards to calculate COD concentrations.

Ammonium concentration was followed using a Crison Ion Selective Electrode after adding 100 μL of 0.9 M $Al_2(SO_4)_3$ solution to 1 mL of sample and applying a calibration curve obtained with standard solutions of NH_4Cl.

2.5. Microbial Community Analysis

2.5.1. Nile Blue Staining

Nile blue staining was applied to fresh samples taken from the SBR at the end of the feast phase using the method of Ostle and Holt [26] to evaluate the PHAs accumulating capacity of the culture. Samples were observed using an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software, Matrix Optics, Kota Kinabalu, Malaysia).

2.5.2. Fluorescence in Situ Hybridization (FISH)

FISH was performed on paraformaldehyde-fixed biomass samples [27]. DAPI staining, for quantifying the total number of cells, and EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) were used with group specific probes (Table S1) in all the hybridizations. Probes containing FITC and Cy3 labels were purchased from MWG AG Biotech (Ebersberg, Germany).

Samples were observed under an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software, Matrix Optics, Malaysia).

2.5.3. 16S r.DNA Clone Library

DNA was extracted from an SBR sample collected during the pseudo-stationary phase of operation (on the 66th day, 12-h cycle). DNA extraction was performed using a PowerSoil® DNA Isolation Kit following the protocol described by the manufacturer. The primers used to amplify 16S rDNA were 27 forward (f) and 1492 reverse (r), (Table S2). The following PCR cycles were used: 98 °C for 2 min, followed by 35 cycles of 98 °C for 0.5 min, 58 °C for 0.5 min and 72 °C for 1 min and, finally, an extension at 72 °C for 10 min. After the last cycle, samples were cooled down to 4 °C and an agarose (1%) electrophoresis was performed to confirm the amplification. PCR products were purified using the QIAquick® PCR purification kit and quantified using the NanoDrop2000 Spectrophotometer.

The amplified 16S rDNA was ligated into pGEM®-T vector (Promega, Madison, WI, USA) and transformed into JM109 Competent Cells (Promega, Madison, WI, USA), following the manufacturer's instructions. The transformed cells were plated in LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C. Clones were screened for insertion of the correct size by PCR amplification using primers T7f and M13r. The following PCR cycles were used: 96 °C for 10 min and PCR amplification was performed as already above. DNA sequencing was carried out by BioFab (Rome, Italy) using the primers 530f, 926f and 907r, (Table S2). The complete sequences obtained were deposited in GenBank (accession numbers: KT262954, KT262955, KT262951, KT262956, KT262952, KT262957, KT262958, KT262959, KT262960, KT262961, KT262962, KT262953, KT262963, KT262964, KT2629).

3. Calculations

PHAs content was calculated as a percentage of TSS on a mass basis:

$$\%\,\mathrm{PHA} = \frac{\mathrm{gHA}}{\mathrm{gTSS}} \times 100 \quad (1)$$

Active biomass (X) was obtained by subtracting PHAs from VSS as (in g L^{-1}):

$$\mathrm{X} = \mathrm{VSS} - \mathrm{PHAs} \quad (2)$$

Feast to famine ratio (F/F) was calculated by dividing the time needed to consume all SCOAs by the remaining time of the cycle.

Active biomass elemental composition was determined by assuming the molecular formula $C_5H_7NO_2$, where 8 mg of active biomass were obtained from 1 mg of N [10]. Also, it was considered that the ammonia consumed was only used for growth since thiourea was supposed to fully inhibit nitrification.

The consumption rates of each acid fed were obtained by adjusting linear functions to the experimental data for each variable concentration, and calculating the first derivative at time zero (Acetic acid, $-r_{Acet}$; propionic acid, $-r_{Prop}$; butyric acid, $-r_{But}$). Yield of biomass production yield ($Y_{X/S}$) and PHAs production yield on substrate ($Y_{PHA/S}$) were calculated by dividing the amount of each parameter produced by the total amount of substrate consumed which corresponded to the sum of all SCOAs consumed.

4. Results and Discussion

4.1. SBR Operation

The SBR fed with HSSL supplemented with SCOAs worked for 70 days. The values of F/F ratio and SCOAs uptake rates were determined along the SBR operational period (Figure 1). The success of the MMC selection process can be evaluated using the F/F ratio. In general, low F/F

values, <0.2, ensure physiological adaptation of the microorganisms, favoring PHAs storage during the feast phase [17,28]. As shown in Figure 1, the F/F ratio stabilized at 0.091 around day 34, which was an indication of the enrichment in PHA-storing organisms. Based on the values of F/F, a pseudo-stationary state (PSS) was considered to be reached on day 34.

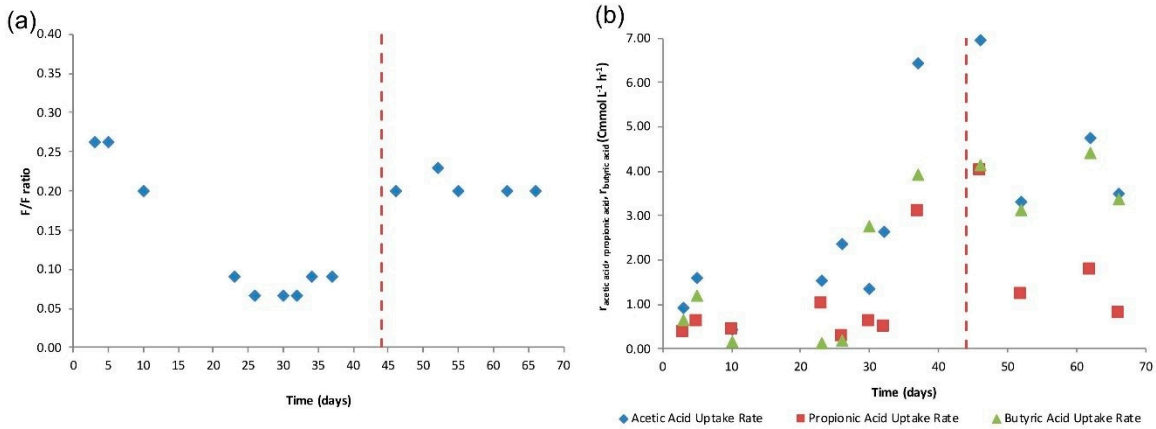

Figure 1. Evolution of F/F ratios (**a**) and uptake rates of acetic (blue diamonds), propionic (red squares) and butyric acids (green triangles) (**b**), along the SBR operational period. The red dashed line marked when cycle length and OLR were changed.

In the present work, the end of the feast phase was defined by a sudden increase of the dissolved oxygen (DO) concentration that corresponds to the depletion of most of the readily biodegradable COD substrates, in this case, SCOAs. In most of the cycles of the SBR, the sudden increase of DO concentration was usually followed by a slower and progressive increase that continued until the end of the cycle, suggesting the consumption of more complex carbon sources [29]. This consumption could be a result of the existence of a side population. The use of very complex substrates as HSSL with a high diversity of constituents can result on the development of undesired side populations, which can lead to a reduction of the overall production yield [9,30]. The success of the feast and famine system relays on the increase of the desirable carbon sources in order to obtain an enrichment in PHAs-storing organisms able to consume the desired external carbon fraction, and channel it to produce reserves, later used as substrate during the famine phase. By choosing the appropriated operational parameters, cycle length and OLR, together with the addition of SCOAs an enriched PHAs-accumulating MMC with reduced side-populations would be expected.

In order to increase the OLR without increasing the concentration of inhibitors of HSSL fed to the SBR, on day 44, the cycle duration was changed from 24 h to 12 h. In this way the OLR increase resulted from the decrease of HRT and the concentration of all HSSL components remained constant in each cycle. According to Figure 1, the MMC stabilized quickly despite the expected increase in the F/F ratio to 0.2, but were still on the range that indicated a storage response [12,31]. Comparing the results obtained in previous works using HSSL as substrate, it is noteworthy that the supplementation of SCOAs led to a quicker stabilization of the MMC for both cycle durations: 20 days for cycles of 24 h and 5 days for 12 h. Previously, Queirós et al. [9] reported a period of more than 250 days to obtain a stable MMC fed with non-supplemented HSSL submitted to cycles of 8 h. Also, Queirós et al. [8] using non-supplemented HSSL and applying cycles of 12 h did not obtain a stable MMC.

As a result of the increase on cycle length, the volumetric substrate uptake rates of SCOAs were generally higher (Figure 1), as previously described in the literature [12]. Albuquerque et al. [12] observed that with the increase on OLR an increase of substrate uptake rates, biomass concentration, and volumetric productivities occurred alongside. In the present work, the increase on OLR affected in the same way the biomass concentration. After the increase SCOA consumption rates stabilized. The average acetic acid uptake rate, 2.99 $\pm$ 2.02 Cmmol L^{-1} h^{-1}, was higher than the average uptake

rates of propionic acid, 1.23 ± 1.13 Cmmol L^{-1} h^{-1}, and butyric acid, 2.19 ± 1.65 Cmmol L^{-1} h^{-1}. The values of SCOAs uptake rates demonstrated a preference of the MMC for acetic acid, then for butyric and propionic acids.

After the change on cycle length, since *F*/*F* values were still lower than 0.2, this could be a possible strategy to increase the overall productivity of PHAs production. Figure 2a shows the evolution of Δ%PHA, active biomass and PHA concentrations along the SBR operational time. The values of Δ%PHA and PHA concentration were unstable throughout the first 34 days of SBR operation and several peaks of production were detected. The average variation of Δ%PHA observed during the PSS defined for 24 h-cycles period, was 7.5 ± 2.4%. After the cycle length change, between the 46th and 66th days, the Δ%PHA was higher and PHA production became more stable, with a storage content in the range 9.07 ± 2.17%. Moreover, contrary to what Queirós et al. [9] observed, a second storage polymer, based on glucose, was not detected throughout the SBR operational time. Such fact could probably be a sign of the existence of a side population able to consume other carbon sources than SCOAs without the need to accumulate either polysaccharides or PHAs. A similar situation was observed by Marang et al. [30] when acclimatizing an MMC to acetate and methanol, allowing a side population to growth without PHA or polysaccharides accumulation due to methanol.

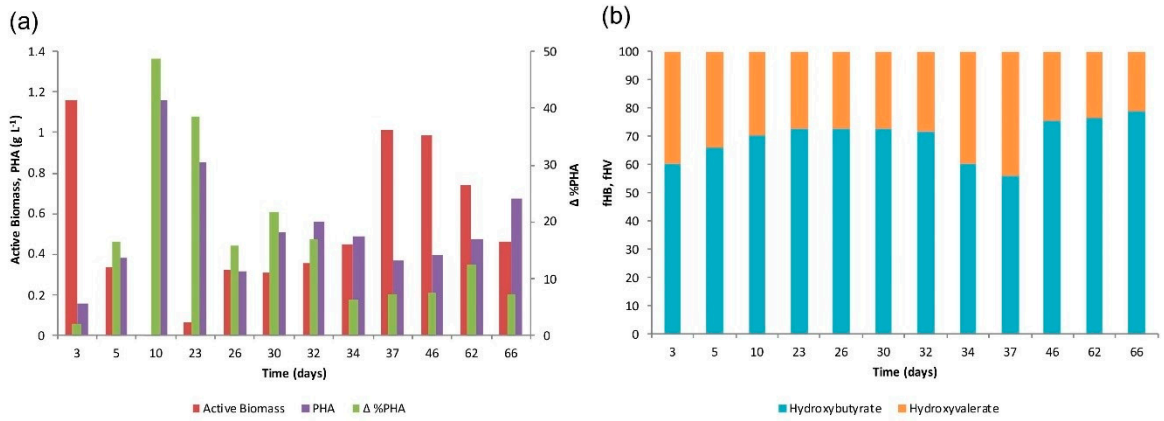

Figure 2. Evolution of Δ%PHA (green bar), Active Biomass (red bar), and PHA concentrations (purple bar) (**a**) and contents of 3HB (blue bar) and 3HV (orange bar) (**b**) throughout the SBR operational time.

Figure 2b shows the evolution of the produced monomers during the SBR operation. The formation of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P (3HB-co-3HV) with an average monomeric composition (on a molar basis) of 69:31 ± 7:7 (3HB:3HV) was observed throughout the SBR operational period. After changing the cycle duration on day 44, the copolymer composition became more stable, with a decrease in the 3HV content, 77:23 ± 1:1 (3HB:3HV). This represents an improvement in HV content relatively to the previous works with HSSL without SCOAs supplementation. Queirós et al. [8], using the same OLR and a 12-h cycle, observed the formation of poly(3-hydroxybutyrate), P(3HB), while Queirós et al. [9] reported the formation of P(3HB-*co*-3HV) with slightly lower amounts of 3HV, around 20%, with a high OLR (17 gCOD L^{-1} d^{-1}) and an 8-h cycle. Homopolymers, such as P (3HB), and copolymers, like P (3HB-*co*-3HV), can form different crystalline phases and present different physical properties. Consequently, the market application will be completely different [32]. Moreover, from the commercial point of view, P (3HB-co-3HV) is much more interesting than P (3HB), since it has a broader range of applications due to its improved physical and chemical properties [33]. Several works already showed that the monomeric distribution of microbial PHA produced by MMC can be controlled by manipulating the SCOAs composition in the feeding. A feeding with acetic acid as sole carbon source usually results in the formation of P (3HB) under ADF conditions [23]. When other SCOAs, such as propionic acid, and valeric acid are added, propionyl-CoA is formed as a precursor for PHA production, resulting in a copolymer containing 3HV

monomers [23,34]. Despite the small period of operation, the production of 3HV could be considered quite stable. The supplementation of propionic acid probably contributed to this stability. This is an important aspect since the stability of the PHA composition along reactors' operation time is also highly desirable [32].

4.2. SBR Cycle

During the operational period, SCOAs consumption and PHA production in the SBR were characterized through monitoring of individual cycles. Figure 3 shows a representative 12-h cycle with an F/F ratio of 0.20 on the 66th day of operation, the most stable period of the reactor operation.

Along the cycle, acetic and butyric acids were consumed preferentially with uptake rates of 3.51 and 3.36 Cmmol L^{-1} h^{-1}, respectively. Propionic acid was consumed at 0.802 Cmmol L^{-1} h^{-1}. The three SCOAs were fully exhausted during the first two hours. The same tendencies of SCOAs consumption were observed in the 24 h cycles. During the first 2 h of the cycle, COD was consumed at a rate of 0.276 g L^{-1} h^{-1} while no LS consumption was observed, remaining their concentration around 2.8 g L^{-1}. Also, there was no consumption of xylose and glucose, the two major monosaccharides of HSSL, what could be due to the supplementation with SCOAs that allowed for selecting a culture unable to metabolize sugars.

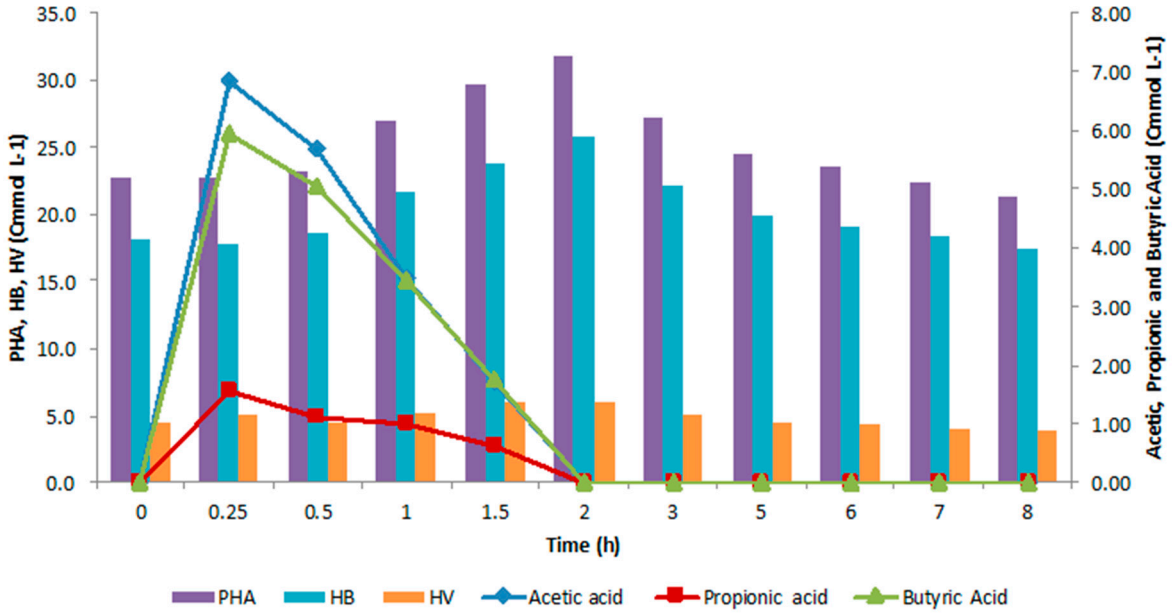

Figure 3. SBR representative cycle on the 66th day of operation: PHA, HB and HV content represented by purple, blue and orange bars, respectively, and acetic, propionic and butyric represented by blue, red and green lines.

This cycle started with biomass presenting a PHA content of 4.9%, meaning that a significant part of the stored PHA was never fully consumed. That was observed in most SBR cycles for both cycle durations studied. Despite the presence of intracellular PHAs at the beginning of the cycle, the MMC was able to store more PHA during the cycle. The maximum PHAs concentration, 31.8 Cmmol L^{-1}, was registered at 2 h, which corresponded to the end of the feast phase, when SCOAs were exhausted. In this cycle, the Δ%PHA was 7.3% and a $Y_{PHA/S}$ of 0.64 Cmmol PHA Cmmol S^{-1} was obtained. When the famine phase started, PHAs began to be consumed for growth and maintenance and their concentration decreased 33% between 2 h and 8 h. The monomeric composition of the produced P (3HB-co-3HV) was uniform throughout the cycle, being 79:21 (3HB:3HV) at the end of the famine phase. The polymeric composition during the cycle was more stable for 12 h-cycles than for 24 h-cycles.

Since pH was not being controlled, values along the cycle increased from 7.8 (after feeding) to 8.8 by the end of the cycle. This moderate pH variation probably resulted from the buffering capacity of HSSL previously reported by Queirós et al. [8]. Values of pH controlled above 8.0 were reported to improve PHAs content [35], the polymer yield per substrate [36] and PHAs volumetric productivity [37]. Leaving pH uncontrolled could improve PHAs content and polymer yield [36] but decrease the volumetric productivity [37]. Nevertheless, the buffering capacity of HSSL was an additional advantage of using it as substrate for PHA production since no pH control would be necessary for the large-scale application.

4.3. Accumulation Tests

Three accumulation tests were performed in fed-batch mode to study the MMC storage capacity: without nutrient limitations (A), under phosphorous limitation (B), and under nitrogen limitation (C). All tests were performed using biomass collected from the 12-h cycle period. In tests A and C, 5 feeding pulses were supplied with the same composition as the feed of the SBR, but in batch B only 3 pulses were given. Each pulse was added when a sudden increase in DO was detected, since it was usually associated with SCOAs depletion.

In test A, the maximum PHAs content obtained was 23.7% of cdw, after 7.75 h. The overall yield $Y_{PHA/S}$ obtained in this accumulation test was very low, 0.09 Cmmol PHA Cmmol S^{-1}. Such low value could be justified with the presence of nitrogen and phosphorous, nutrients required for cell growth being the carbons sources deviated from PHAs accumulation. The longer the culture was exposed to a medium without limitation, the more carbon is deviated from PHA storage to biomass growth [17]. Regarding the polymer stored, a slight decrease in 3HV proportion was observed when compared with the selection SBR, being produced a copolymer with a monomeric composition of 80:20 (3HB:3HV).

In test B, phosphorous limitation was imposed since it was previously reported to enforce less stress to the MMC than nitrogen [38]. Phosphorous limitation can decrease the ATP synthase activity, resulting in a restrained Krebs cycle, that promotes the conversion of excess carbon into PHAs [38]. In test B, growth rates obtained were lower than in the previous test, achieving part of the objective to limit cell growth. The MMC obtained a maximum PHA content of 22.8% of cdw, after 4 h and produced a P (3HB-co-3HV) with 29% of 3HV. The overall yield $Y_{PHA/S}$ obtained in this test was 0.15 Cmmol PHA Cmmol S^{-1}. Both PHA content and production yield were relatively low when comparing with other works using real complex substrates [14,39]. Despite the increasing PHAs content and overall $Y_{PHA/S}$, phosphorus limitation did not signify a robust strategy to maximize the PHAs accumulation.

In test C, the maximum PHAs content obtained was 34.6% of cdw at the end of the test (Figure 4). The overall yield $Y_{PHA/S}$ observed in this accumulation test was 0.78 Cmmol PHA Cmmol S^{-1}. As in previous tests, a copolymer P (3HB-co-3HV) was produced with an average monomeric composition of 76:24, near to the average composition obtained in the SBR at the same time period. The imposition of nitrogen limitation led to the best PHAs storage results. A considerable number of works already explored the strategy of nitrogen limitation to trigger and maximize the PHAs production [28,40,41]. The PHAs storage capacity in accumulation test C was lower than in others studies described in the literature. Dionisi et al. [41] reported a PHAs storage content of 55% using olive oil mill effluents, 54% by Bengtsson et al. [40] using paper mill effluents, and a range of 56–77% by Albuquerque et al. [28] from fermented molasses. Nevertheless, in test C, a high PHA yield on substrate was obtained, 0.78 Cmmol PHA Cmmol S^{-1}, comparing to the previous works, which ranged from 0.50 to 0.80 Cmmol PHA Cmmol $SCOA^{-1}$ [14,21,39].

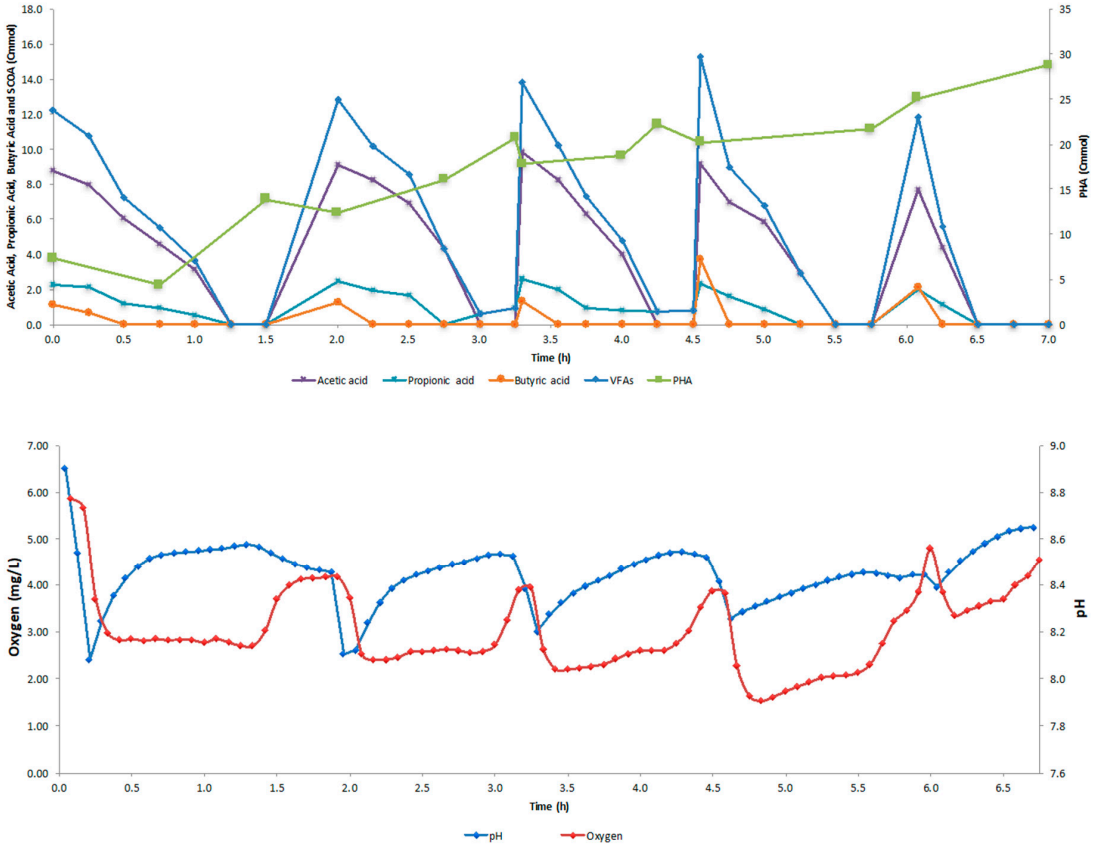

Figure 4. Evolution of pH (red line), DO (dark blue line), PHA (green line), Acetic Acid (purple line), Propionic Acid (light blue line), Butyric Acid (orange line) and SCOAs (blue line) concentrations in kinetic test performed with nitrogen limitation. Test performed on the 68th day of operation.

4.4. Microbial Community Characterization

The goal of the present study was also to establish the link between the microbial population composition and the PHAs-producing capacity of the MMC selected with HSSL supplemented with SCOAs. Nile Blue staining showed that almost all biomass could store PHAs. Such characterization can contribute towards the development of operational process strategies designed to optimize the structure of the microbial community [21]. Several works that address the microbial characterization of their enrichments already revealed diverse communities that probably resulted from the operational conditions applied and the type of substrate used [15].

FISH analysis was performed on original sludge and samples collected from the SBR on days 17, 32, 45, 51, 60 and 66 of operation. Initially, specific probes for the main phyla within Bacteria domain were applied. The microbial community was mostly composed of Bacteria since no Archaea were detected. Several FISH probes were used taking into consideration bacteria previously reported as PHA-accumulating organisms.

To identify the microbial community and to follow the MMC composition throughout the reactor operational period, specific probes for the main groups within Bacteria domain were applied: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Chloroflexi*, *Flavobacteria*, *Bacteroidetes*, *Sphingobacteria*, *Actinobacteria*, *Firmicutes*, *Planctomycetales*. Figure 5 shows the evolution of the composition during the reactor operation. Since the beginning of the process, *Betaproteobacteria* were the dominant group and their relative abundance only suffered slight fluctuations during the whole reactor operational time. Since some *Betaproteobacteria* were reported as PHA-producers, this result showed that the operational conditions applied to the SBR favored the maintenance of this bacterial group within the MMC. *Alphaproteobacteria* were the second most dominant group in the

bacterial community throughout the SBR operational time, contrary to what was described so far with HSSL [8]. This proves that a change in the substrate composition, the introduction of a different SCOAs profile, led to a different community enrichment presenting both PHAs storage capacities. When using solely HSSL as substrate at high OLR and shorter cycle lengths, 17 g COD L^{-1} d^{-1} and 8 h respectively, PHAs production was low, despite the presence of a considerable number of PHA-storing microorganisms and a glucose polymer was also produced [9]. For this case, the microbial community was dominated by *Alphaproteobacteria*.

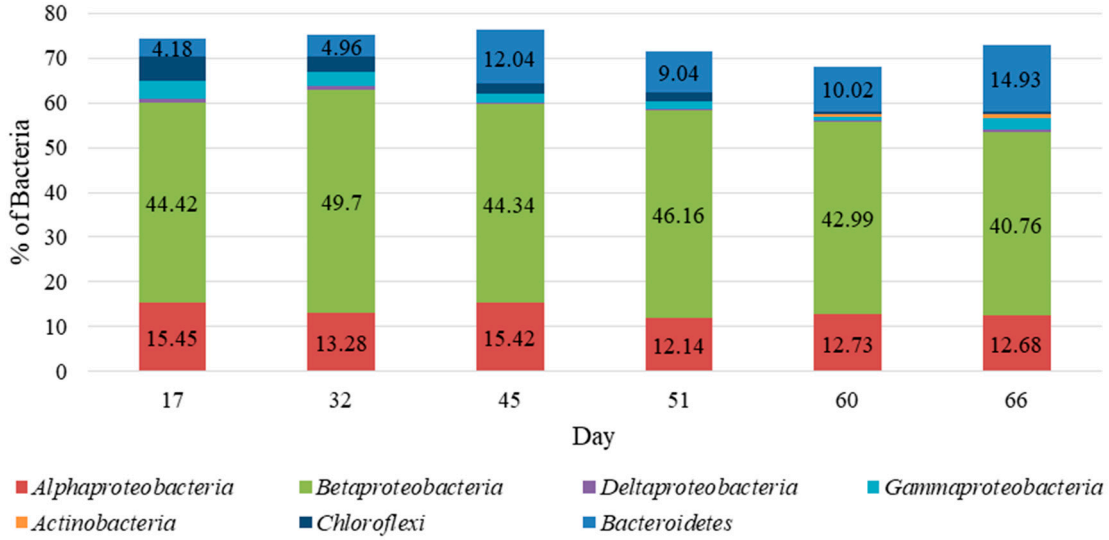

Figure 5. Evolution of bacterial community throughout the SBR operation.

As previously mentioned, the operational conditions were changed from 24 h to 12 h cycle on day 44 and the MMC needed 10 days to stabilize. By analyzing the bacterial composition selected for the 12 h cycles, it is possible to verify that the bacterial community did not suffer significant changes at the class level. Betaproteobacteria remained the dominant group, and their relative abundance was stable during this period (42.99 ± 0.38% on day 60 and 40.76 ± 2.15% on day 66). Results obtained in this study are in line with previous studies that identified PHAs-accumulating organisms belonging to *Alphaproteobacteria*, *Betaproteobacteria* or *Gammaproteobacteria* classes being selected under ADF [13,14,20,42].

Regarding *Alphaproteobacteria*, specific probes were applied for *Amaricoccus*, *Sphingomonas*, *Defluvicoccus* and *Defluvicoccus* related Tetrad-Forming Organism (TFO-DF), all already described as PHAs-accumulating organisms. Positive results were only obtained for TFO-DF (0.78 ± 0.38%), in far less extent than in others enrichments with HSSL [8].

Specific probes for *Thauera* and *Azoarcus* genera belonging to *Betaproteobacteria* and often detected in MMC processes as PHAs-accumulating organisms were also tested [15]. Positive results were obtained for both genera, but only in small amounts, *Thauera* corresponded to 0.72 ± 0.25%, and *Azoarcus* to 0.62 ± 0.02%, remaining most of *Betaproteobacteria* unidentified.

With the objective to identify the main bacteria responsible for PHAs accumulation in the selected MMC, a 16S rRNA gene clone library was constructed. The sample used to extract the DNA was collected from the SBR on the 66th day of operation. 60 clones were chosen for partial sequencing. The obtained 16S rDNA partial sequences were then analyzed by BLAST and grouped by genera (Table S3). After analyzing the results of the partial sequencing, 15 clones were chosen for complete sequencing and refinement of their taxonomic affiliation. The major part of the clones belonged to Betaproteobacteria. These results confirmed the observations of FISH analysis that showed the dominance of Betaproteobacteria in the bacterial community.

The representatives of the 15 most abundant clones are shown in Table S4 and their phylogenetic relations are presented in Figure 6. Almost all of them are known PHA producers previously found in MMC selection processes for PHA production [15].

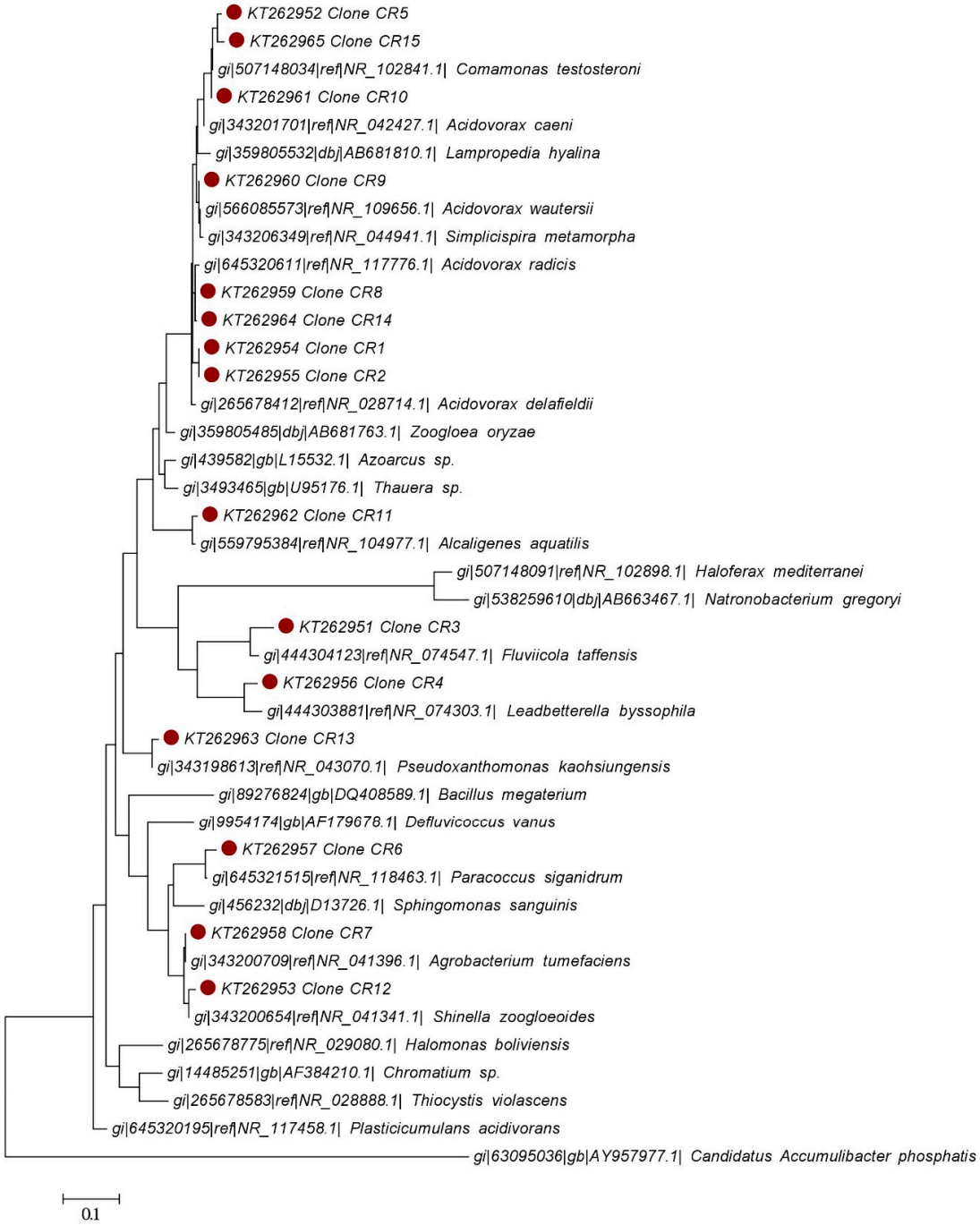

Figure 6. Phylogenetic tree based on full-length nucleotide sequences of 16S rRNA gene of clones CR1-15 (in the tree preceded by a filled red dot). Molecular phylogenetic analysis by maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Evolutionary analyses were conducted in MEGA6.

During the analysis of 16S rRNA gene sequences, six of the 15 sequences were related to *Acidovorax*, a genera previously shown to be able of PHAs storage [43]. Positive results for *Acidovorax* were obtained

for all the screened samples and its content in the microbial community increased from 2.62 ± 0.93% (in day 17) to 28.98 ± 2.51% (in day 66). During the period of the 12-h cycles, *Acidovorax* became the major population constituent of Betaproteobacteria, composing 71% of this class on day 66 (Figure 7).

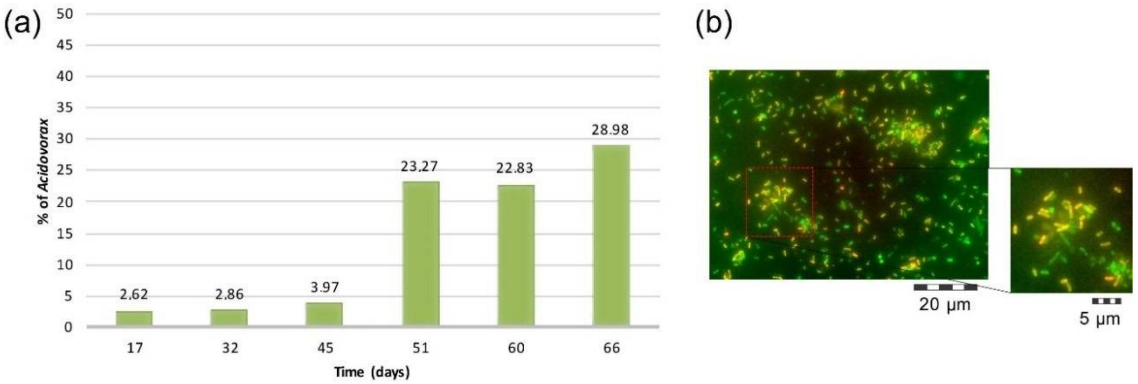

Figure 7. (**a**) *Acidovorax* content evolution throughout the reactor operation. Percentages based on the relative abundance of Betaproteobacteria. (**b**) FISH picture of *Acidovorax* on day 66. Green cells are hybridized with EUBmix and the yellow cells also hybridized with ACI145 probe..

Clones related to *Comamonas* spp. and *Novosphingobium* spp. were also detected. Ferreira et al. [42] isolated and characterized organisms able to store PHAs, from an MMC selected under the same conditions as those applied in this study (same OLR and cycle length) but using HSSL without SCOAs supplementation. Queirós et al. [8] verified that under these conditions, the culture was dominated by microorganisms belonging to Alphaproteobacteria. This shift on the dominant class of the MMC resulted from the simple addition of SCOAs, the known precursors of PHAs.

Several other microorganisms present in the clone library were already described as PHA-accumulating organisms in pure culture: *Comamonas acidovorans* using 1,4–butanediol and glucose [44], *Paracoccus denitrificans* consuming ethanol and n-pentanol [45], *Cupriavidus necator* using soya wastes from a soya milk dairy [46], and *Alcaligenes latus* using malt wastes from a beer brewery plant as substrate [47]. Sequences related to *Fluviicola taffensis*, *Leadbetterella byssophila*, *Paracoccus siganidrum*, *Agrobacterium tumefaciens*, *Alcaligenes aquatilis*, *Shinella zoogloeoides* and *Pseudoxanthomonas kaohsiungensis* were also present. Other clones had phylogenetic relationships with *Lampropedia hyalina*, *Azoarcus* sp., *Thauera* sp., *Bacillus megaterium*, *Plasticicumulans acidivorans*, *Thiocystis violacea*, and *Zoogloea* sp.

5. Conclusions

The supplementation of HSSL with a mix of SCOAs reduced the adaptation period of the culture greatly (34 days), while producing a copolymer of P (3HB-co-3HV). OLR and cycle length were shown to influence the kinetics of substrate consumption and the type of PHAs stored. Both substrate uptake and polymer production rates increased for a higher substrate concentration applied to the selection reactor. FISH analysis revealed that Betaproteobacteria class dominated the microbial population, with *Acidovorax*, a known PHAs producer as the major genus, when the OLR and cycle length were changed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/4/3/58/s1.

Author Contributions: D.Q. and L.S.S. conceived and designed the experiments; D.Q. and C.R. performed the experiments; P.L. assisted on PHA analyses and S.R. on microbial community study; D.Q. and C.R. wrote the paper.

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