Production of PHA using Mixed Microbial Cultures and a Mixture of Complex Feedstocks

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Abstract

Nowadays, there is a huge demand for biomaterials, such as polyhydroxyalkanoates (PHA), produced from renewable sources. PHA production by mixed microbial cultures in sequential batch reactors with several waste feedstocks was already reported in literature. In this study, PHA production at pilot-scale (60 L) with two waste feedstocks, namely fermented fruit pulp waste (Feed-C) and the fermented organic fraction of municipal solid waste (Feed-N) was studied. The first is rich in carbon but limited in nutrients, while the latter has a low carbon content but a high nutrients titre. Three operational conditions were sequentially tested under the same organic loading rate of 5.8 gCOD L^{-1} d^{-1}(150C-mmol L^{-1} d^{-1}) and an SRT of 4 days. In the first condition, Feed-C was used as carbon source and synthetic ammonia solution as nitrogen source (uncoupled feeding). The PHA content achieved in the accumulation assays was 63.93 $\pm$ 8.70 %, (g_{PHA} $g_{VSS}{}^{-1}$). Further, in condition 2, the synthetic ammonia solution was replaced by Feed-N as a nitrogen source. A PHA content of 31.59 $\pm$ 2.46 %, (g_{PHA} $g_{VSS}{}^{-1}$)was achieved. In the third condition, the operation using both feedstocks under a lower HRT (0.66 days) was studied. In this case, PHA content of 53.44 $\pm$ 0.06 %, (g_{PHA} $g_{VSS}{}^{-1}$)was obtained. The global productivity achieved in the first condition was 0.62 gPHA L^{-1} h^{-1}, decreasing to 0.42 gPHA L^{-1} $h^{-1}$with the culture selected with both feedstocks (in condition 3). The accumulation stage was also performed with Feed-N. The results showed a significant decrease in PHA content (14.95 $\pm$ 1.02 %, (g_{PHA} $g_{VSS}{}^{-1}$)and 26.61 $\pm$ 2.37 %, (g_{PHA} $g_{VSS}{}^{-1}$), for condition 2 and 3, respectively) which was due to a predominant growth response of the culture. For the two feedstocks the selected culture was dominated by *Amaricoccus* and *Paracoccus* genera which are know as PHA storing microorganisms. The present work opens the possibility of using different low cost feedstocks as carbon and nitrogen source for PHA production, lowering the associated costs and contributing to a circular economy.

Keywords:*Mixed microbial cultures, polyhydroxyalkanoates, waste feedstocks, culture selection.*

Introduction

The plastic industry continues to growth and a life without plastic seems inconceivable. In 2018, global plastics production reached almost 360 million tonnes, and in Europe alone reached 62 million tonnes. Bio-based polymers such as polyhydroxyalkanoates (PHA) have the potential to replace petroleum-based polymers and help solve some of the most urgent problems caused by the overuse of petroleum-based polymers [1].

PHAs are biodegradable polyesters synthesized by numerous bacteria in the form of granules for carbon and energy storage [2], under nutrient - limiting conditions.Nowadays, the production at industrial scale is accomplished with pure cultures, but although high productivity can be achieved, this process is associated with high costs, namely the high price of refined feedstocks, production in batch or fed-batch modes and sterilization requirements [3–5]. Alternatively, the production with mixed microbial cultures (MMCs) is an advantageous over pure cultures since no sterile conditions are required, less expensive carbon sources can be used and fewer process controls are required. Additionally, since the variety of organisms working with complex substrates is higher, more diverse PHAs are formed [5–7].

MMC PHA production usually takes place in three separate stages [8–10]: (1) Acidogenic fermentation, where raw complex substrates are fermented in order to obtain readily biodegradable organic matter, the fermentation products; (2) selection stage: by applying transient conditions, usually feast and famine regimes, is possible to select an enriched-culture in PHA-storing organisms; (3) PHA accumulation: the enriched culture is fed with the fermentation products resulted in the first stage, aiming to maximize PHA production. MMC cultures prefere volatile fatty acids for PHA production, so any possible feedstock. Waste feedstocks are great candidates for PHA production since they are potentially economical substrates and turn the waste management easier for industries [10]. The organic fraction of municipal solid wastes (OFMSW) is an abundant type of food waste originates in households, restaurants, small businesses and garden wastes. OFMSW is mainly constituted by carbohydrates, proteins and lipids, having thus a high potential application as substrate in biotechnological processes [11, 12].

The selection stage aims to obtain a culture with high PHA storage capacity at the highest possible rate [3], since the richer the MMC in PHA-producers the higher the PHA production in the following step [13]. Under feast and famine (F/F) regimes, the substrate availability allows for competition, between the microorganisms in the culture and thus promotes selection. The feast phase is characterized by an abundance in C source whereas famine by a lack of substrate. Hence, microorganisms have to adapt to survive and growth under the lack of substrate and compete on the occasions where substrate is available [3, 7, 8, 14].

Usually, selection stage occurs in a selection batch reactor (SBR) and is influenced by several parameters, such as F/F ratio, organic loading rate (OLR), sludge retention times (SRT) and Carbon to Nitrogen (C/N) ratio. During the selection stage proper growth has to occur, so all nutri-

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ents required for that must be available, such as nitrogen and phosphorus. Generally, polymer storage ability of the biomass can be improved under dynamic conditions with nitrogen deficiency, when compared to a nutrient excess scenario [3, 15]. Low C/N ratios are beneficial for cell growth while higher ratios boost PHA accumulation [16]. Regarding feeding strategies, nitrogen can be added with the carbon source or uncoupled (in the famine phase). The latter showed improvements in PHA production [13] and brings the advantage of implementing higher OLRs without compromising selection efficiency [14]. Some waste streams, such as cheese whey, some activated wastewater sludge and OFMSW contain the necessary nutrient with acceptable C/N/P ratio, eliminating the associated costs of supplementation. As to accumulation stage, different feeding strategies are reported in literature, such as pulse fed-batch, continuous feeding and variations of these ones [8]. Regarding nutrient supply, literature is not yet consensual on the best strategy to apply, however nitrogen starvation has generally led to higher PHA content [17, 18].

The feasibility of PHA production by MMCs with waste feedstocks in pilot scale has been demonstrated. In this work, two feedstocks with complementary composition were studied namely (1) Fermented fruit pulp waste (Feed-C) and (2) Fermented food waste (Feed-N). Both feedstocks are rich in organic matter but have different nitrogen availability: Feed-C has no nitrogen content whereas Feed-N has a high nitrogen content. Taking advantage of the complementarity of the composition between the two wastes, an integrated process for PHA was developed. The main objective was to evaluate the possibility of replacing the synthetic nutrient solution by a low-cost feedstock rich in nitrogen, thus contributing for the reduction of the operational costs and ultimately to a more cost-effective process.

Materials and Methods

Experimental Procedures

Acidogenic Fermentation The acidogenic fermentation stage took place in a 100-L acrylic Uflow Anaerobic Sludge Blanket (UASB) reactor, where fruit pulp waste provided by Sumol+Compal S.A. was used to produce a stream rich in fermentation products. The feedstock was stored at -20°C to prevent degradation and the UASB feed solution was prepared twice a week in a 500 L refrigerating tank. The fruit waste was diluted with tap water down to the desired substrate concentration and the feed solution was supplemented with a nutrient solution, composed of: ammonium chloride, NH_4Cl, and potassium dihydrogen phosphate, KH_2PO_4 , in order to establish a COD:N:P ratio of 100:0.5:0.1. To maintain granules consistency, cellular structure and activity, calcium ($CaCl_2$: 480 mg L^{-1}), magnesium, ($MgSO_4.7H_2O$: 120 mg L^{-1}) and iron ($FeCl_3$: 80 mg L^{-1}) were also added to the medium.

The reactor was operated continuously with a working volume of 60L and a recirculation flow of 1.45 L min^{-1}. A MMC culture from the brewery industry (Super Bock Group, S.A.) of an anaerobic process was used to inoculate the reactor. Feeding and recirculation stream are located in the bottom part of the reactor, keeping the biomass granules in suspension, and the fermentation products outlet stream, in the upper part. During this operation an OLR of 20 gCOD L^{-1} $d^{-1}$was applied. The outlet stream suffered a settling by gravity, for solids removal, and after was kept in a refrigerator container ready to be used in the selection stage.

Culture Selection in SBR The culture selection stage was conducted in a SBR with a total volume of 60 L. Due to the global pandemic COVID-19 and for safety reasons, a purge from another SBR in the pilot plant, operating in similar conditions, (GLOPACK project), was used as inoculum. The reactor is made of stainless steel. Aeration was provided by a compressor and measured by a flow meter (1 vvm) and a mechanical impeller allowed for stirring, ensuring aerobic conditions. Dissolved oxygen (DO) levels were monitored through an immersion probe (ISM ®, Mettler Toledo) as well as pH. Temperature as control and kept between 18-22 °C using a thermostatic jacket. A computer software (BioCTR 59m) was used to acquire data from the probes, which allowed to monitor the different phases of the cycle, kept for 12 hours. The SBR was operated with nitrogen supply uncoupled from the main carbon source in three different conditions, with an OLR of 150 C-mmol L^{-1} d^{-1}, corresponding to 6 gCOD L^{-1} d^{-1}.

In condition 1, the reactor was operated with a carbon rich feed, namely fermented fruit waste (Feed-C, for simplicity), and synthetic ammonia solution. A working volume of 53L was used. The cycle had the following sequence: a feed phase where carbon (7.5L) and mineral medium (15.2L) were fed, a reaction phase where the carbon was consumed, a famine phase where only nitrogen feed was supplied (approximately 2 hours after carbon entrance, 0.3L), settling of the biomass and withdrawal phase. By the end of the night cycle, a biomass purge of 13.25 L was carried out and after a 45 minutes settling, a 9.75 L of supernatant was removed whereas in the morning cycle, the volume discarded was 23 L (supernatant). Overall, this results in an SRT of 4 days and a HRT of 1 day. The reactor was under stirring and agitation during the entire cycle, except during the settling phase and supernatant withdrawn. Feed-C concentration was 600 Cmmol $L^{-1}$and the pH 5. The mineral medium added had the following composition (mg L^{-1}): $FeCl_3{\cdot}6H_2O$: 12; H_3BO_3: 1.2; $CoCl_2{\cdot}6H_2O$: 1.2; $MnCl_2{\cdot}4H_2O$: 1;$ZnSO_4{\cdot}7H_2O$: 1; $Na_2MoO_4{\cdot}2H_2O$: 0.47; $CuSO_4{\cdot}5H_2O$: 0.24; KI: 0.24;EDTA: 3395;$MgSO_4{\cdot}7H_2O$: 2370; $CaCl_2{\cdot}2H_2O$: 277. To enable bacterial growth, nitrogen feed was a synthetic solution of ammonium chloride and potassium phosphate, with a ratio of 100:6.5:1 Cmmol:Nmmol:Pmmol.

In condition 2, the SBR was operated with Feed-C, derived from the UASB reactor, and a feedstock provided by *Valorsul*, abbreviated to Feed-N. The working volume was changed to 40L which lead to a change in withdrawal, purge and feed volumes. To meet the desired ratio, C:N:P - 100:7:1 Cmmol:Nmmol:Pmmol the Feed-N was supplemented with monopotassium phosphate (KH_2PO_4). pH control took place, being a maximum value of 8.5, regulated by a pH pump connected to the computer software, for H_2SO_4 (1.5M) addition whenever necessary. Condition 3 is similar to condition 2, regarding the use of Feed-C and Feed-N. However, mineral media was fed simultaneously to Feed-C in the SBR. Since the SRT was kept constant but HRT was changed to 0.66 day, the frame of the cycle was changed and settling phases of one hour each took place. The SBR operation was followed by cycle monitoring, at least two times in each condition. Substrate and nitrogen consumption, biomass growth and PHA production were assessed. Additional analysis were performed in order to characterize the feedstocks used in the process.

PHA Accumulation To assess the PHA accumulation capacity of the culture selected under each condition, batch tests were performed in a separated 1L bioreactor (BIOSTAT ® plus). Aeration was controlled by a flowmeter at 4 vvm, and a mechanical impeller allowed stirring, ensuring once again aerobic conditions. These tests occurred when the reactor was considered stable, in each condition. A biomass purge from the SBR was harvested

and used to inoculate the reactor for each accumulation test (between 0.4 to 0.6L). DO levels were acquired through a DO probe (ISM ®, Mettler Toledo) as well as pH. A pulse-wise strategy was implemented, controlled by DO concentration. Temperature was controlled with a thermostatic jacket and kept at 20 °C.

Analytical Methods

Biomass concentration (X) was estimated by determination of the volatile suspended solids (VSS), according to standard procedure described in [19]. Protein content of Feed-N was determined spectrophotometrically at 750 nm as described by Lowry et al. [20] and total sugar content were measured by the Dubois method described in [21]. High performance liquid chromatography was used to measure the concentration of fermentation products, such as ethanol and organic acids (lactic, acetic, propionic, butyric, valeric, caproic, heptanoic and ocatanoic and iso forms). A VWR Hitachi Chromaster equipped with a RI detector 5450 and Diode Array Detector 5430, a Biorad Aminex HPX-87H 300x7.8MM column and a Biorad 125-0129 30x4.6mm pre-column was used for the current analysis. Sulphuric acid, H_2SO_4 0.01 N, was applied as eluent at a flow rate of 0.6 mL/min and temperature of 60°C. The concentration of the various fermentation products was estimated based on a standard calibration curve ranging between 4-1000 mg/L. Before HPLC analysis samples were previously filtered through a 0.2 µm pore size filter Ammonium and phosphate, NH_4^+ and PO_4^{2-} concentration were determined by a colorimetric method in a flow segmented analyzer (Skalar San ++, Skalar Analytical, Netherlands). A calibration curve of 4-20 mg $L^{-1}$of ammonium cloride, NH_4Cl 99%, Sigma, and orthophosphoric acid, H_3PO_4 85%, Panreac, allowed the estimation of nutrients concentration. Samples were diluted with mili-Q water and doubled filtered, when necessary.

PHA quantification and characterization (monomer identification) was carried out by gas chromatography (GC), using a protocol adapted from [22]. Briefly, lyophilised biomass was subjacted to methanolysis in 2 mL acidic methanol (20% H_2SO_4) and extracted with chloroform (2 mL). To improve accuracy and reduce error, heptadecanoate (HD), at around 1 g L^{-1}, was added to the chloroform solution, acting as a internal standard. The mixture was digested at 100 °C for 4 h. After the digestion step, the organic phase (where monomers are dissolved in chloroform) was extracted and injected (2 µL) into a gas chromatograph equipped with a flame ionization detector (Bruker 430- GC) and a Resyek column (60 m, 0.53 mm internal diameter, 1 mm film thickness, Bruker, USA),using helium as carrier gas at 1 mL/min at constant pressure of 14.50 psi. Calibration standards were made using an Aldrich copolymer of P(3HB-co-3HV) containing 12%(HV) and 88% (HB) (%(w/w)) with concentrations between 0 and 6.3 g L^{-1}. Standards were prepared the same way as samples.

Microbial Assessment For the analysis of the dynamic of the bacterial community and to study of the abundance of PHA accumulating microorganisms in the SBR, Fluorescence *in situ* Hybridization (FISH) was performed as previously described by [23]. In every condition, samples were collected from the SBR at the end of the feast phase and fixed with 4% paraformaldehyde (PFA, Gram-negative bacteria), according to [24]. This fixation begins with the addition of 1 mL of PFA to 0.5 mL of sample, incubation at 4°C, for 4 hours, centrifugation at 12782 x g for 3 min, supernatant discarding, and wash the remaining pellet with 1 mL of phosphate-buffered saline (PBS). This washing step was repeated once more, and lastly, the cells were resuspend in 0.5 mL of absolute ethanol and stored in -20°C. After, samples were applied to the wells of specific glass-slides for FISH analysis,identified and placed in an oven at 46 °C for 10 min. Next, samples suffered a dehydration in a grading series of ethanol solutions (50%, 80% and 98%) and were dried under compressed air. Hybridization buffer containing 2M NaCl, 10% sodium dodecyl sulphate (SDS), 1M Tris-HCl, formamide and miliQ water, pH 8, was applied to each well (8 µL). The amount of formamide in the buffer was adjusted accordingly to the probes used and miliQ water was also adjusted to make a final volume of 2 mL. Then 0.7 µL of EUBMIX and 0.7 µL of the specific probe were added to each well. To end this step, each slide was placed in a falcon tube containing a moisturizing tissue with the remaining hybridization buffer and incubated in the oven at 46 °C for 1.5-3 hours. Later, a washing step took place. The washing buffer (2M NaCl, 10% SDS, 1M Tris-HCl, 0.5M EDTA and miliQ water) was prepared in a 50 mL falcon tube and pre-heated to 48 °C in a thermostatic bath. The slides were then place in the buffer tube for 10-15 min, washed with MiliQ water at 4 °C and dried with compressed air. In the end, vectahield mounting media was added to the dried slides (enough to cover all the wells). The slides were visualized using an epifluorescent microscope Zeiss Imager D2 at 1000 X. The EUBMIX probe is a mixture of EUB338 [25], EUB338II, EUB338III [26]. This probe was labelled with fluorescein isothiocyanate (FitC), which emits in green light, and specific probes, were labelled with Cyanine 3 (Cy3), which emits in red light when excited. The probes used in this study and the targeted group are referenced in Table 1.

Table 1: FISH probes used in this study.

Probe Name	Targeted Group	Reference
AMAR839	Amaricoccus	[27]
LGC0355	Firmicutes	[28]
Azo644	Azoarcus	[29]
THAU832	Thauera	[30]
LAMP444	Lampropedia	[31]
CF319a	Flavobacteria	[32]
Zra23a	Zoogloea	[33]
Meg938/1028	Meganema	[34]
UCB823	Plasticicumulans acidivorans	[35]
PAR651	Paracoccus	[36]
HGC69A	Actinobacteria	[37]
ARC915	Archaea	[38]

Calculations Feast/famine ratio (F/F, h h^{-1}) was determined as the ratio between the lengths of the feast phase and the famine. In condition 2 and 3, feast phase length is given by the sum of lengths of Feed-C and Feed-N. Famine phase length is calculated by subtracting feast time to total cycle time. The OLR is determined by multiplying the concentration of fermentation products, in C-mmol L^{-1} d^{-1}or gCOD L^{-1} d^{-1}, by the flow (Q, Ld^{-1}) divided by unit of volume (V, L).

PHA content was calculated as a percentage of VSS on a mass basis, considering that VSS was constituted by cells (X) and PHA. The generic formula of $C_5H_7NO_2$ was considered [39], and a factor of 1.42 gCOD $X^{-1}$was considered.

$$PHA(\%, g_{PHA}g_{VSS}{}^{-1}) = 100 \cdot \frac{PHA}{VSS} \quad (1)$$

For PHA the sum of the respective monomers was considered, considering the following conversion factors for HB, HV and HHx, respectively: 1.47 $gCOD_{PHA}$ $g_{PHA}{}^{-1}$, 1.92 $gCOD_{PHA}$ $g_{PHA}{}^{-1}$ and 2.11$gCOD_{PHA}$ $g_{PHA}{}^{-1}$, based on the oxidation stoichiometry. The change in PHA content (Δ PHA) was determined as the PHA content in the end of an accumulation assay minus the PHA content at the beginning of the cycle. The specific substrate uptake rate, -q_s in $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$was determined from the linear regression of experimental concentrations plotted through time, dividing by the X value in the beginning of the cycle. The

same principle for specific ammonium uptake rate, -q_N in mgN $gCOD_X^{-1}$ h^{-1}, but the X value considered was the one in end of feast 1. For accumulations assays only the maximum values of these parameters were presented. For specific PHA storage rate the maximum value obtained was considered, q_{PHA} in $gCOD_{PHA}$ $gCOD_X^{-1}$ h^{-1}. Storage yield, $Y_{PHA/S}$, in $gCOD_{PHA}$ $gCOD_{FP}^{-1}$, was calculated by dividing the amount of PHA formed by the amount of fermentation products consumed. Similarly, growth yield, $Y_{X/S}$ in $gCOD_X$ $gCOD_{FP}^{-1}$was estimated with biomass concentration diving by the fermentation products consumed. Global productivity, gPHA L^{-1} h^{-1}considered the final point of accumulations assays.

Results and Discussion

The present study aimed at the production of PHA, by mixed microbial cultures, using fruit pulp waste (Feed-C) as nitrogen deficient feedstock and food waste (Feed-N) with high nitrogen content. First, a proper characterization of each feedstock is presented, summarizing its composition and potential functions in the established process. After, the selection of a mixed culture able to produce PHA was evaluated under 3 different operational conditions described in section Materials and Methods (Experimental Set-up).

Feedstock Characterization

In Table 2 the compoisiton of the two substrates used in the SBR, Feed-N and Feed-C, as well as TSS, protein, total carbohydrates and COD are given.

Table 2: Food waste (Feed-N) and pulp fruit waste (Feed-C) characterization.

Parameter	Feed-N	Feed-C
TSS (gTSS L^{-1})	18.2 ± 1.0	0.5
Protein (gCOD L^{-1})	12.7 ± 0.4	0
Carbohydrates (gCOD L^{-1})	3.3 ± 0.1	—
Fermentation Products (gCOD L^{-1})	29.4 ± 1.1	13.0
COD_{SOL} (gCOD L^{-1})	28.9 ± 0.4	14.2
COD_{FP}/COD_{SOL} (%)	97.9	91.5
COD_{TOT} (gCOD L^{-1})	56.0 ± 1.4	14.8
COD_{FP}/COD_{TOT} (%)	52.6	87.8
Ammonia (gN L^{-1})	2.1 ± 0.8	0
Phosphate (gP L^{-1})	0.3 ± 0.1	0

Comparing the two feedstock a lot of differences stand out. The amount of solids present in Feed-C is almost negligible, which suggests that fermented fruit pulp can be seen as a "clean" substrate. Another factor that contributes for this classification is the fact that fermentation products are roughly 88% of the total organic matter, indicating that acidogenesis stage was successfully performed. Taking these values into account, Feed-C presents as a good feedstock for PHA production. Due to the fact that no nitrogen nor phosphate is present, in a reactor, bacterial growth would only occur if a synthetic solution of ammonia or another nitrogen rich feedstock was added.

In contrast, Feed-N presents more solids, and a higher percentage of non-fermented matter, since only 53% of total COD is composed by fermentation products. The concentration in fermentation products and the ratio between it and soluble COD is high, therefore this feed is source of fermentation products. In opposition to Feed-C, Feed-N can act as a nitrogen source and allow bacterial growth. OFMSW is composed of carbohydrate, protein, starch, fat, cellulose and lipids [12] and the proportion between these components is a function of origin and seasonality of waste collection. Since the sum, in gCOD L^{-1}, of proteins, carbohydrates and fermentation products doesn't justify total COD, it's possible that other components, such as lipids are present. Apart from the mentioned components, the presence of mineral salts is also considered.

In relation to fermentation products (FP) analysis, Feed-N has octanoic, heptanoic acids and a higher percentage of acetic and propionic acids. The results obtained also show that the concentration of FP and the variability (% of each organic acid) are not homogeneous. Results vary from batch to batch however, Feed-N is mostly composed by butyric, caproic and valeric acid. Regarding Feed-C, this analysis demonstrates a homogeneous feedstock mainly constituted by butyric and caproic acids.

SBR Performance

In order to achieve a proper selection of PHA-accumulating bacteria with two waste feedstocks 3 operational conditions were tested: Condition 1, 2 and 3. As previously mentioned, a SRT of 4 days and an HRT of 1 day were defined for Condition 1 and 2. For Condition 3, HRT was changed to 0.66 days. The OLR of the process was 143.9 ± 6.19 C-mmol L^{-1} d^{-1}, around 5.8 gCOD L^{-1} d^{-1}. Adding up to the described parameters, that influence the selective pressure in favor of PHA-storing organisms, feast to famine length ratio (F/F) presents as one of the main factors, since low F/F favours PHA storage. Figure 1 shows the feast to famine ratio during the 130 days of SBR operation. Marked in green are the days where conditions were changed. With the addition of Feed-N to the process, feast phase length is given by the sum of the two feast phases (feast 1 for Feed-C and feast 2 for Feed-N).

For a good enrichment of a MMC during selection stage, values of F/F lower then 0.2-0.3 (h h^{-1}) [7, 14] are required for inducing PHA storage, since an effective internal limitation is achieved through a long famine phase. In condition 1, these values were reached and the SBR is considered stable Condition 2* and 3* are transient states that lead to changes in operation, resulting in Condition 2 and 3, respectively. In the end of Condition 2, from the 67^{th} day of operation forward, F/F presents values between 0.3 and 0.4, however when compared with Condition 1, data reproducibility is not achieved. Condition 3 presents a similar behaviour, and after the 120^{th} day of operation the values are within the same range (0.10-0.25 h h^{-1}). Since the biggest difference between condition 1 and the others is the source of nitrogen it can be hypothesized that the values of feast N caused the instabilities in F/F values.

Condition 1 In this condition, SBR was operated with Feed-C and a chemically-defined solution of nutrients to enable growth, in uncoupled regime, as literature proves for a faster selection and more efficient culture.

As mentioned, each condition of operation was characterized by monitoring the cycle and accumulation tests, when the reactor was considered stable, at least after 3 SRT. The F/F ratio are very low for both days (the 15^{th} and the 21^{th}

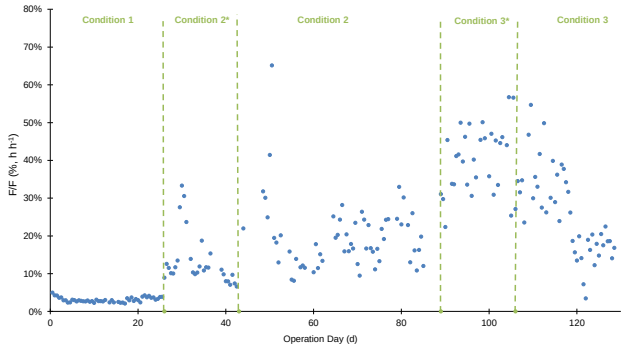


Figure 1: east to famine ratio (F/F, % h h^{-1}) over the operation days of the SBR, during the 3 studied conditions.

day of operation), 2.4% and 3.3%, respectively, being favorable for PHA storage in feast phase. Figure 2a presents TSS/VSS and biomass (X) in g L^{-1}, NH_4^+ concentration in mgN L^{-1}, concentration of fermentation products (FP) in gCOD $L^{-1}$and PHA content (%, (g_{PHA} $g_{VSS}{}^{-1}$)) during the cycle of the 20th day of operation. VSS trend follows the expected: increase during the feast phase, due to the production of PHA, then decrease until the nitrogen is fed into the reactor, when this happens, biomass grows, increasing VSS. After ammonium consumption, during famine phase, VSS start to decrease, since PHA reserves serve as carbon and energy for cells. Regarding fermentation products, butyric acid followed by caproic acid are preferential, since they present the higher substrate uptake reate, -q_S, 1.78 $gCOD_{But}$ $gCOD_X{}^{-1}$ $h^{-1}$and 0.87$gCOD_{Cap}$ $gCOD_X{}^{-1}$ h^{-1}, respectively. Table 3 sums up the variation of several parameters for selection and accumulation performance of each condition. It was obtained a storage yield ($Y_{PHA/S}$) of 0.45$gCOD_{PHA}$ $gCOD_{FP}{}^{-1}$and a polymer that contains HB, HV and HHx in its composition. Butyrate and acetate can be used for the production of HB whereas propionate and valerate can be used for HV production. Since Feed-C is composed mainly by the referred acids the monomer composition presented is expected. The presence of 3-hydroxyhexanoate it's a reflection of the presence of caproate which is the precursor of this monomer.

After the selection of PHA-accumulating bacteria in SBR, accumulation tests were performed, on the 16th day of operation. Generally, the accumulation tests performed lasted for about 5h to 7h, in fed-batch mode being the substrate fed pulse-wise (in this case, 8 pulses were applied). Once the substrate is consumed, DO starts to increase and a new pulse is fed to the reactor. A food to microorganism ratio was calculated (9.6 C-mmol $gVSS^{-1}$) and kept constant during all the assay. Figure 3 shows DO and pH variation, during the accumulation test, the global consumption of fermentation products (gCOD L^{-1}) and PHA content (%, (g_{PHA} $g_{VSS}{}^{-1}$)).

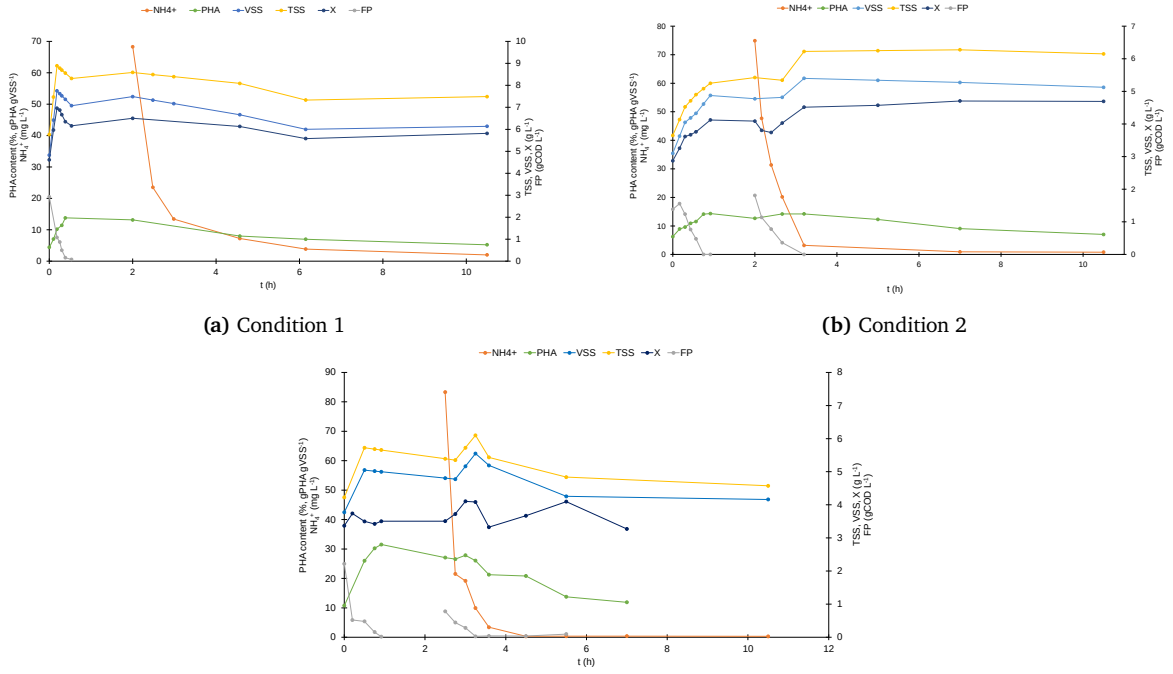
As expected, when the reactor is fed, DO drops, which corresponds to the consumption of fermentation products as can be seen in the Figure 3. Since the biomass went through a long famine period and consumed part of the PHA stored intracellular, the first and second pulse will probably present the highest consumption rate of fermentation products. Fermented consumption rates decrease, from -4.7 $gCOD_{FP}$ h^{-1} to -4.4 $gCOD_{FP}$ h^{-1} to -4.0 $gCOD_{FP}$ h^{-1} in the first three pulses, and similar in the next. Maximum substrate uptake rate of 1.37 $\pm$ 0.09 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$was achieved in the first pulse. PHA trend presented in figure 3, follows the expected: increasing over time. However in the last three pulses there's a sightly decrease, explained by the fact that PHA-accumulating organisms reached the maximum storage capacity, and eventually started to consume the polymer. The highest PHA content of 75.33 (%, (g_{PHA} $g_{VSS}{}^{-1}$)) was achieved after 4.85 hours of accumulation. For the assay performed in parallel, the maximum PHA content was obtained after 5.63 hours of accumulation and is roughly 55.92 %, (g_{PHA} $g_{VSS}{}^{-1}$). Table 3 shows an overview of the most important kinetics parameters that characterize condition 1.

From the values obtained for content is possible to concluded that the culture was really subjected to the selective pressure of feast and famine regime and a culture enriched in PHA-accumulating organism was obtained. The selected biomass presents good storing capacity and productivitiy.

Condition 2 In this condition, Feed-N replaced the synthetic ammonia solution, also contributing to the organic loading rate. As reported in Table 2 Feed-N presents a more complex composition than Feed-C. since the amount of solids, proteins and other components are potential obstacles to biomass acclimatization. During condition 2* the reactor foamed a lot, resulting in biomass loss. Although an anti-foam strategy was applied, it was not enough to improve the reactor stability, in this period. Therefore in condition 2, the working volume was decreased to 40L, to improve reactor stability. Though the foam was not eliminated, the biomass was not washed out from the reactor. Figure 1 shows that biomass acclimatization took several days, but the performance of the reactor was improved comparing with condition 2*.

For Condition 2 characterization, the cycles monitored on the 68th and 77th day of operation were chosen. The online DO profile obtained presents some differences when compared with the one for condition 1. Plateaus are formed until total carbon depletion, for Feed-C and Feed-N, indicating that the consumption rate of some fermentation products it's higher than others. In the first feast butyric acid and caproic acid are preferential, with a specific uptake rate of 0.23 $\pm$ 0.02 $gCOD_{But}$ $gCOD_X{}^{-1}$ $h^{-1}$and 0.16 $\pm$ 0.04 $gCOD_{Cap}$ $gCOD_X{}^{-1}$ h^{-1}, and in the second feast the order changes. Caproic acid presents a specific susbtrate uptake rate of 0.08 $\pm$ 0.01 $gCOD_{Cap}$ $gCOD_X{}^{-1}$ $h^{-1}$and butyric acid one of 0.08 $\pm$ 0.01 $gCOD_{But}$ $gCOD_X{}^{-1}$ h^{-1}.

Figure 2b presents the trends of the most relevant parameters for condition 2. The most considerable difference between condition 1 and this, is the fact that, Feed-N, besides being a major source of nitrogen, also contributes to the organic loading rate of the reactor. Since two feasts occur, it's expected to see a similar tendency between them. In the end of the first feast, VSS, TSS and PHA content follow the trend described in condition 1. Maximum PHA content, roughly 14% occurs in the end of the first feast. As expected, 2h after Feed-C another consumption of fermentation products occurs, together with ammonium con-



(a) Condition 1

(b) Condition 2

(c) Condition 3

Figure 2: Trend of total and volatile suspend solids and biomass (TSS, VSS, X g L^{-1}), fermentation products (FP, gCOD L^{-1}), ammonium concentration (NH_4^+ (mgN L^{-1})) and PHA content (PHA, %, (g_{PHA} $g_{VSS}{}^{-1}$)) during a cycle in SBR: condition 1, 2 and 3.

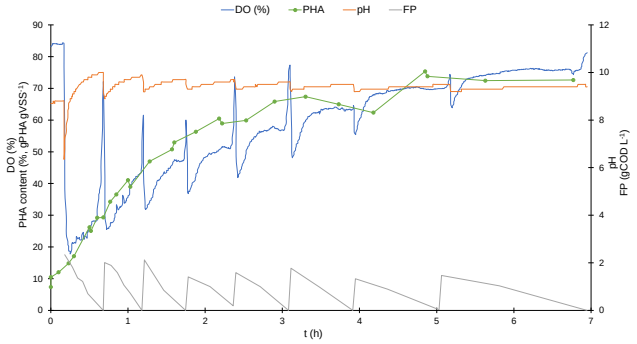


Figure 3: Trend of DO (%), pH, global fermentation products (gCOD L^{-1}) and PHA content (%, (g_{PHA} $g_{VSS}{}^{-1}$)) throughout kinetics test of condition 1.

sumption (grey and orange line of Figure 2b). After Feed-N feeding PHA content increases slightly and biomass grows, as indicated by the dark blue line. Regarding TSS and biomass, lower values were achieved, when compared with condition 1. Substrate uptake rate for feast 1 is higher than feast 2, but lower than for condition 1. In condition 2, it's achieve a lower PHA content, indicating that biomass lost some ability to storage PHA. In condition 1, biomass values were much higher, being F/F ratio more constant and the SBR stable. PHA composition is the biggest change when compared with condition 1, since the monomer HHx is no longer part of the polymer composition. Substrate composition is key for the type of polymers formed. Therefore, it is hypothesized that the culture selected under these conditions, doesn't present class II PHA synthase, which is responsible for HHx synthesis. Apart from this hypothesis, it can also be speculated that some compound present in Feed-N could influence monomer composition. With a similar substrate, in literature, only polymers composed by HB and HV were reported, which emphasizes the last option [40–43].

For condition 2 characterization, accumulations assays were performed on the 70 th and 75th day of operation, with Feed-C and Feed-N. For simplicity, tests performed with Feed-C will be denominated A and B, and with Feed-N, C and D. With the same pulse wise strategy, 5 pulses were fed to the reactor, over 8 hours. A F/M ratio of 14.6 C-mmol $gVSS^{-1}$was kept constant during all the assay. DO variation follows the same trend as the one presented in Figure 3. Similar to condition 1, PHA increases over time, decreasing in the last pulse. For test A, a maximum PHA content of 30.3% was obtained in the end of accumulation (7.93 h) and for test B the maximum achieved was 30.0% at 7.08 h. Maximum substrate uptake rate was 0.48 $\pm$ 0.05 $gCOD_{FP}$ $gCOD_X{}^{-1}$ h^{-1}, achieved in the first pulse. This value is much lower when compared with accumulation tests 1 and 2. As for condition 1, Table 3 sums up the most important parameters for tests A and B. In these tests, a lower initial cell density was used. The storage yield is very similar with condition 1, highlighting that the amount of substrate that is used for polymer storage is almost the same, besides the fact that condition was changed. A lower productivity and maximum specific PHA storage rate were achieved. Final PHA content was also lower when compared with condition 1. Since the biggest difference from one condition to the other is the fact that two real feedstocks are used in the selection stage, it can be hypothesized that, Feed-N induces instability due to it's complexity (more variability in fermentation products, more solids, more non-organic matter), lowering specific PHA storage values and global productivity. Furthermore, the lower productivity can also be explained by a less efficiency on the culture selection than in the condition 1.

Feed-N was also used in order to maximise PHA pro-

duction. Again, 6 pulses were fed to the reactor, over 6 hours with a F/M ratio constant of 15.0 C-mmol $gVSS^{-1}$. In these assays, C and D, global consumption of fermentation products occurred in the first pulse and a tendency for accumulate in the media took place in the rest. Longer acids, such as octanoic acid, heptanoic acid, caporoic acid and iso-valeric acid were consumed at a lower rate from the first pulse on, leading to some accumulation in the media. However, butyric and acetic acids are entirely consumed, which can be related to feed composition. The feed used was mainly composed by caproic, octanoic and butyric acid and a major difference, in % COD, between the preferential acid (butyric) occurs, from 52% in kinetic 1,2 to 16% in this assay. Since a total consumption of fermentation products didn't took place is not possible to ensure that PHA production was maximize. As in the previous accumulation assays, PHA trend follows the expected: increasing over time. For test C and D, a maximum PHA content was achieved at 5.13 hours, respectively, 15.0 and 16.8 %, (g_{PHA} $g_{VSS}{}^{-1}$). Maximum substrate uptake rate of 0.26± 0.03 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$was achieve in the first pulse, lower than condition 1 and test A and B. Table 3 presents the most relevant parameters for tests C and D. An ammonium uptake rate of -6.57 ± 0.42 mgN $gCOD_X{}^{-1}$ $h^{-1}$was obtained for the first pulse in kinetic C and D. Comparing with test A and B, storage yield is much lower and similar to growth yield, pinpointing a growth response over a storage one. Specific storage rate was lower than in tests A and B, but, global productivity is very similar to the one obtained in previous assays, indicating that due to microbial growth was possible to achieve comparable values.

Condition 3 With Condition 2 it was proven that the enrichment of a PHA-accumulating culture is feasible using two complex feedstocks, but it could be enhanced. Condition 3 appears has an optimization of the previous one. Around 4.28 ± 2.45 g $L^{-1}$of biomass were being lost in withdrawal phase, based on weekly monitoring. Adding to this, an analysis resembling the settling rate test (SV30) was performed several times, being inconsistent, which lead to conclude that settling was not efficient. In this study, a decrease of HRT to 0.66 days was applied in order to improve the settling phase, and contribute to the reactor's stability. During condition 3* feast phase length increased significantly, see Figure 1, because biomass concentration decreased. Since two settling phases ocured, 10L of tap water were added after each phase. It was hypothesized that micronutrients fundamental for cellular activity, were being washed out. Hence, not being assimilated by the culture, taunting low cell density values. In order to overcome this limitation, a mineral media, was added.

As the previous ones, condition 3 was characterized through 2 cycles monitoring, from the 120th and 123rd days of operation. In Figure 2c, a typical trend for VSS, TSS, X, PHA, NH_4^+ and FP, in condition 3 is presented. Higher values of TSS, VSS and biomass were achieved, when compared with condition 2. A similar trend occurs in FP and ammonium consumption. Regarding PHA content, the value obtained is higher. A higher ammonium uptake rate was obtained, when compared to condition 2. Since the process is nitrogen limited [22] the competition is based on ammonium uptake rate, hence if in this condition the value is higher, a better selection is occurring. As for the other conditions, a higher production of PHA occurs in the first feast. Only after some consumption of PHA in the 2nd feast, the biomass presents some growth, suggesting that PHA producers are competing with other culture. Non-PHA producers likely uptake carbon from Feed-N, whereas PHA-accumulators consume PHA stored intracellularly in order to consume ammonium to grow, and fermentation products, presenting an advantage over the non-producers. Table 3 summarizes the most relevant parameters for condition 3 during a typical SBR cycle. Condition 3 accumulations tests were performed on the 125 th and 126 th day of operation, with Feed-C and Feed-N. For simplicity, tests performed with Feed-C will be denominated E and F, and with Feed-N, G and H. During 7 hours, 4 pulses of Feed-C were fed to the reactor, keeping a F/M ratio of 17.0 C-mmol $gVSS^{-1}$. DO profile is similar to the previous one, with a sharp increase seconds before another pulse is added, indicating depletion of fermentation products. Maximum PHA content of 53%, (g_{PHA} $g_{VSS}{}^{-1}$)was achieved in the end of the assay, 7.22 hours in both kinetics. Maximum substrate uptake rate, -q_S was 0.25± 0.02 $gCOD_{FP}$ $gCOD_X{}^{-1}$ $h^{-1}$and achieved in the first pulse. In assays G and H, a food to microorganism ratio of 17.0 C-mmol $gVSS^{-1}$was kept constant during 7.48 hours of accumulation, being 4 pulses fed. For test G, a maximum of 25.1%, (g_{PHA} $g_{VSS}{}^{-1}$)was produced at 4.97 hours, and for test H, this value was 29%, (g_{PHA} $g_{VSS}{}^{-1}$)at 7.48 hours. Maximum substrate uptake rate, -q_S was 0.25 ± 0.05 achieved in the first pulse. In these assays, contrary to tests C and D, a better consumption of longer acids is achieved in every pulse, being the accumulation in the media minimal. For the first pulse, an ammonium uptake rate, -q_N, of 9.89± 0.51 mgN $gCOD_X{}^{-1}$ $h^{-1}$was achieved. Similarly, Table 3 presents the values for the most important parameters of the accumulation tests performed with Feed-C and Feed-N. Comparing both assays, good storage yields were obtained, being demonstrated again the influence of nitrogen in accumulation assays, since it decreases PHA content due to microbial growth. From tests 1,2 and E,F an improvement on parameters occur, being related to the fact that lower cell density was achieved, increasing the parameters that rely on VSS. When compared to accumulation assays A and B an overall improvement is achieve, and since biomass concentration didn't suffer a lot of changes, a better selection of the biomass occurred.

Culture Dynamics

For the analysis of the dynamic of the bacterial community and study of the abundance of PHA accumulating microorganisms, FISH analysis was performed, for inoculum, condition 1, 2 and 3. Since microbial cultures can vary in terms of substrate, operation conditions and environmental factors, further information on microbial communities and PHA-accumulating microorganisms in mixed microbial cultures is still required [44].

For every condition an enriched culture was obtained, although no genera is dominating, the genera *Paracoccus* seems to be present in every condition. From *Alphapro-*

teobacteria, the genera *Amaricoccus* presented a shift in presence from condition 1 to the others. *Gammaproteobacteria*, identified with probes UCB823 and Meg938/1028 don't have a significant presence in any condition, the same happens for *Lampropedia* genera. From *Betaproteobacteria*, *Thauera* and *Zoogloea* genera were almost non-existent, contrary to *Azoarcus*, that showed a decrease presence from condition 1 to the others. As a result, the FISH analysis demonstrated the presence of several generas of bacterial population known for their ability to accumulate PHA [44]. A shift in the culture occurred from Condition 1 to the others and *Paracoccus* prevailed in every condition. Further studies are needed to conclude if change in culture is directly related to change in polymer composition. It can also be hypothesized that the selection imposed in this study allow the enrichment in a robust culture, that prevailed with the use of two different feedstocks, which can bring several advantages for the use of MMC in industrial scale with real and variable feedstocks.

Comparison

Condition 1 vs 2 Since two waste feedstocks were aimed to be used in the developed bioprocess, the first condition served to acclimatize biomass to its main carbon source Feed-C. From Figure 1 stability was reached after few cycles, given that the inoculum came from another reactor operated with the same feedstock and under similar conditions. A low F/F ratio was obtained, essential for an efficient enrichment. A good cell density was achieved for MMC, which is reflected in the low feast phase lengths presented. Feed-C is composed by a variety of fermentation products, which influences the type of polymer obtained, that in this case is composed by HB, HV and HHx. This highlights one of the advantages of using a complex feedstock: the possibility to generate new copolymers with a range of properties. Regarding the accumulation parameters presented, the culture selected under these conditions presents a good storing capacity, a good specific PHA storage rate, and final PHA content values similar to the ones presented in literature with MMC at pilot scale. The use of waste streams as feedstocks for PHA production allows to reduce operation costs but it is associated with low productivies.

Due to the complexity of Feed-N, high solid content, fluctuations in composition, variable values within the same batch and an high presence of non-organic matter, stability of the reactor took several days and forced changes in operation (40 L of working volume). From Figure 1 is not possible to assume that a "true stability" was achieved in condition 2, although F/F ratio is lower than 0.4 h h^{-1}. This instability caused lower biomass concentrations, hence a higher feast length and higher specific uptake rates. The culture derived from this enrichment was only capable of producing a PHA polymer constituted by HB and HV, losing from condition 1 the HHx monomer. It's possible to hypothesis that some compound, for example the non-fermented solids of Feed-N can influence polymer composition, being further investigation needed on this topic. Comparing condition 2 accumulation assays with Feed-C with condition 1, lower storage yield, specific PHA production rate and global productivity were achieved. It suggests that culture lost some accumulation capacity, which reflects the poor stability and selection of the culture. In the accumulation assays with Feed-N, a higher variability of fermentation products was present, which as mentioned lead to an accumulation in the media, not allowing the maximization of PHA production. Adding this reason to the fact that lower biomass concentrations were achieved, lower performance parameters were obtained. As previously mentioned, the role of nitrogen in the accumulation stage is still not consensual, however it is possible to conclude that without nitrogen supply higher PHA contents were obtained. In this accumulation stage, a storage as well as a growth response were observed, thus not benefiting PHA accumulation. When compared with studies using similar feedstocks, the PHA content and specific PHA storage rate obtained in this work with Feed-N is lower than the values reported, indicating that there is room for improvement.

Condition 2 vs 3 Due to biomass loss in condition 2 in withdrawal phase and poor settling, the HRT was lowered to 0.66 days with the aim of improving reactor's stability. This strategy also allowed the reduction of solid content. As can be seen in Figure 1, condition 3* provoked instability in the reactor, higher F/F ratios were obtained, which it was hypothesized as being related to micronutrients washout. Hence, instead of tap water, mineral media was added, which allowed a better stability in the reactor, when compared with condition 2. The F/F ratio values are not as close together as in condition 1, but close and low enough that stability can be considered, as can be seen in Figure 1. From condition 2 to 3, there's a slight change in biomass values and feast length is, as expected, approximately the same. Since higher PHA contents are obtained in the first feast, substrate uptake rate is lower in the second feast, and lower then condition 2. The drop in specific ammonium consumption can be related to the fact that a better selection was performed, since the PHA accumulator community began to consume ammonium quicker to gain a competitive advantage over non-producers. Regarding accumulation parameters, an improvement from condition 2 to 3 is noticeable and can be seen in Table 3, this can be justified by the fact that an lower HRT helps to eliminate a part of the non-fermented solids and clear potentially inhibitory compounds of the system, or non-soluble solids, introduced by Feed-N or secreted by bacteria, improving the settling capacity of the culture, resulting in a better selection. Comparing with condition 2, a higher storage yield, PHA content and specific PHA storage rate were obtained, in both accumulations. When compared with condition 1, overall parameters are lower, with exception to storage yield. It shows that although a better assimilation of carbon source to PHA is performed, lower PHA contents are obtained as well as a lower global productivity. Once again, in accumulations assays with Feed-N a lower PHA content was obtained and the growth response was similar to the storage one.

Conclusions

The results showed that both feedstocks are suitable for the bioprocess established due to high percentage of fermen-

Table 3: Average (n=2) performance parameters of enriched MMC in selection reactor and an overview of PHA-accumulation assays with the selected culture in each condition of operation. a: Considered at the end of the cycle.

	Condition 1	**Condition 2**		**Condition 3**	
Selection Performance Parameters	Feed-C	Feed-C	Feed-N	Feed-C	Feed-N
Initial VSS (g L^{-1})	5.80 ± 0.97	2.60 ± 0.50		3.37± 0.60	
Final X (g L^{-1})	6.74 ± 0.93	4.33 ± 0.37		3.38± 0.11	
Feast Length (h)	0.37 ± 0.11	1.01 ± 0.09	1.20 ± 0.00	1.02± 0.10	1.17± 0.09
-q_S ($gCOD_{FP}$ $gCOD_X^{-1}$ h^{-1})	0.67 ± 0.14	0.47 ± 0.0004	0.32 ± 0.08	0.50± 0.09	0.143± 0.001
-q_N (mgN $gCOD_X^{-1}$ h^{-1})	4.67 ± 1.67	—	15.70 ± 1.90	—	12.39± 0.57
PHA Composition [a] (%HB: %HV: %HHx)	58:23:19	76:24:0		78:22:0	
Accumulation Performance Parameters					
$Y_{PHA/S}$ ($gCOD_{PHA}$ $gCOD_{FP}^{-1}$)	0.59± 0.05	0.38± 0.02	0.17 ±0.007	0.80± 0.08	0.43± 0.03
$Y_{X/S}$ ($gCOD_X$ $gCOD_{FP}^{-1}$)	N.A	N.A	0.14 ± 0.02	N.A	0.48± 0.03
Final PHA content (%, (g_{PHA} g_{VSS}^{-1}))	63.93 ± 8.70	31.59 ± 2.46	14.95 ± 1.02	53.44 ± 0.06	26.61 ± 2.37
q_{PHA}($gCOD_{PHA}$ $gCOD_X^{-1}$ h^{-1})	0.12 ± 0.03	0.02± 0.002	0.02 ± 0.01	0.10± 0.01	0.06 ± 0.01
Global Productivity (gPHA L^{-1} h^{-1})	0.62 ± 0.05	0.43± 0.03	0.23± 0.01	0.41 ± 0.04	0.29± 0.01

tation products per soluble COD. By taking advantage of the complementarity of the composition between the two wastes, Feed-C and Feed-N, an integrated process for PHA was developed. The culture selection was performed using an SBR reactor operated at pilot scale under an OLR of 5.8 gCOD L^{-1} d^{-1}. The selection was achieved through a feast and famine regime, using Feed-C as the main carbon source and an uncoupled nitrogen source (synthetic or Feed-N). The results from the first condition, promoted biomass acclimatization, which coupled to a high OLR lead to an enriched culture in PHA accumulating microorganisms such as *Paracoccus* and *Azoarcus*. After replacing synthetic solution for Feed-N, several problems occurred although many changes were made in the operation, the system showed a poor stability, lower cell densities and a higher feast length. However, condition 2 was still characterized.

With condition 3, at a lower HRT, 0.66 days, the selection culture presented improved accumulation performance parameters under similar biomass concentrations. The abundance of *Amoricoccus* and *Paracoccus* genera in the culture effectively confirms a good selection with MMC in this condition. Feed-N influence in accumulation assays was also studied. Results show that Feed-N should not be used in the accumulation stage as the high nitrogen concentrations promoted a growth response over the PHA storage resulting in low PHA contents. In the future, the system should be operated under a lower OLR in order to achieve a more sustainable PHA production if higher cell densities and consequently higher productivities were reached.

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