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High rate selection of PHA accumulating mixed cultures in sequencing batch reactors with uncoupled carbon and nitrogen feeding

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ABSTRACT

The selection and enrichment of a mixed microbial culture (MMC) for polyhydroxyalkanoates (PHA) production is a well-known technology, typically carried out in sequencing batch reactors (SBR) operated under a feast-famine regime. With a nitrogen-deficient carbon source to be used as feedstock for PHA synthesis, a nutrient supply in the SBR is required for efficient microbial growth. In this study, an uncoupled carbon (C) and nitrogen (N) feeding strategy was adopted by dosing the C-source at the beginning of the feast and the N-source at the beginning of the famine, at a fixed C/N ratio of 33.4 g COD/g N and 12 h cycle length. The applied organic loading rate (OLR) was increased from 4.25 to 8.5 and finally to 12.725 g COD/L d. A more efficient selective pressure was maintained at lower and intermediate OLR, where the feast phase length was shorter (around 20 % of the whole cycle length). However, at the higher OLR investigated, the PHA content in the biomass reached a value of 0.53 g PHA/g VSS at the end of the feast phase, as a consequence of the increased C-source loaded per cycle. Moreover, 2nd stage PHA productivity was 2.4 g PHA/L d, 1.5 and 3.0-fold higher than those obtained at lower OLR. The results highlight the possibility of simplifying the process by withdrawing the biomass at the end of the feast phase directly to downstream processing, without a need for the intermediate accumulation step.

Introduction

In recent years, the production of fully biodegradable biopolymers from renewable resources has become widespread [1–3]. Polyhydroxyalkanoates (PHA) are polyesters of hydroxyalkanoic acids, naturally produced as storage carbon (C) sources by different species of PHA-producing microorganisms [4]. They are completely biodegradable and can be produced from renewable resources and waste material, showing elastomeric and thermoplastic properties comparable with traditional plastics [5]. Mixed microbial cultures (MMCs) have been proposed as a cost-effective means of producing PHA from renewable resources (i.e. activated sludge and organic wastes) through the selection of PHA-storing microorganisms, obtained applying alternate dynamic feeding conditions [6–8]. High selective pressure for the PHA-storing bacteria in activated sludge has been obtained by setting periodic alternating feast (C feeding) and famine (absence of C sources) conditions [9–13]. Establishing these conditions enables a physiological adaptation of microbial species, leading to the selection of microorganisms which produce and accumulate PHA as intracellular C source.

As extensively reported previously [14,15], the typical parameter for a good selection of PHA-storing biomass is the ratio between the length of the feast phase and the whole cycle length, which should be lower than 20 %. A sequencing batch reactor (SBR) is generally used for the selection of the PHA-accumulating biomass, as it is possible to apply the required dynamic feeding strategy [16]. The step following is the PHA production, usually conducted in an accumulation batch reactor inoculated with the selected PHA-producing biomass. The use of renewable and fermentable feedstock can lead to a significant reduction of costs in the overall process [17–20]. Hence, VFA-rich streams are typically used as feedstock for MMC-PHA accumulation processes. These streams may contain varying levels of nutrients (N and P) that can affect process performance. Nutrient deficient waste streams as feedstock for both the selection and accumulation steps may be used even though nutrient limitations can cause an unstable growth of PHA-producing microorganisms in the SBR [21–24]. On the other hand, it has been demonstrated that N limitation during the accumulation step can substantially increase the production performances in terms of PHA

Abbreviations: COD, chemical oxygen demand; DO, dissolved oxygen; FID, flame ionization detector; fOMW, fermented olive mill wastewater; HB, 3-hydroxybutyrate; HRT, hydraulic retention time; HV, 3-hydroxyvalerate; MMC, mixed microbial cultures; OLR, organic loading rate; OUR, oxygen uptake rate; PHA, polyhydroxyalkanoates; SBR, sequencing batch reactor; SRT, sludge retention time; SS, suspended solids; VFA, volatile fatty acids; VSS, volatile suspended solids

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storage yield and PHA final content [12,21–25].

A focus of current research has been the optimization of N supply [17]. A particular application of the uncoupled feeding strategy has been reported to show how the latter allows using specific feedstock (e.g. 1,3-propanediol), which was found to be unsuitable for PHA production from MMC under coupled C and N feeding conditions [26] whereas, in previous studies [27,28], N and C feeding have been uncoupled, stimulating a PHA storage response during the feast phase (in the absence of N) and microbial growth in the famine phase (adding nitrogen). The impact of regulation of N feeding on the process has been evaluated firstly in a laboratory-scale SBR with a cycle length of 6 h under an applied organic load rate (OLR) equal to 8.5 g COD/L d (Chemical Oxygen Demand, COD) [27]. Two SBR runs were performed with the C source fed at the beginning of the SBR cycle simultaneously with the N source (coupled feeding strategy) or with the latter fed at the end of the feast phase (uncoupled feeding strategy). As a main result, it was found that PHA content at the end of the feast phase was doubled with uncoupled feeding. Accordingly, in the present study the effect of the applied OLR has been investigated maintaining the uncoupled C and N feeding strategy, with a fixed C/N ratio. The SBR was operated with a 12 h cycle length and at three OLRs (4.25, 8.5 and 12.75 g COD/L d), employing a synthetic mixture of acetic and propionic acids as C feeding solution. The OLR was explored up to 12.75 g COD/L d, a level rarely considered in the literature, and was varied in order to evaluate its effect on both PHA storage properties and process productivity.

Materials and methods

Sequencing batch reactor for MMC selection/enrichment

The selection and enrichment of PHA-accumulating biomass was performed in a fully aerobic SBR (1.0 L working volume), inoculated with an activated sludge from “Roma Nord” full-scale wastewater treatment plant (Rome, Italy). A mechanical impeller was used for mixing of the culture medium with O_2 provided through air pumps connected to ceramic diffusors. The operating cycle length was set at 12 h, in all three SBR runs. The cycle structure was composed follows: initial phase of carbon (C) source feeding (10 min; 0.42 L), a first reaction phase in which the C-source was consumed (150 min), a withdrawal phase for the discharge of the culture medium (3 min; 0.50 L), a nitrogen (N) source feeding phase (5 min; 0.08 L), and a second reaction phase (552 min) where the PHA was consumed as the only C-source of the medium. The temperature was maintained at 25 °C using a thermostatic jacket. The hydraulic retention time (HRT) was equal to 1.0 day, similar to the sludge retention time (SRT) since no settling phase was provided. A synthetic mixture of acetic acid (85 % on a COD basis) and propionic acid (15 %) was used as C-source, at a total concentration of 5.060 g COD/L (Run A), 10.119 g COD/L (Run B) and 15.179 g COD/L (Run C) after dilution with a mineral medium, composition reported elsewhere [27]. Based on a C-source flow rate of 0.84 L/d in each run, the applied organic load rates (OLR) were 4.25 g COD/L d (Run A), 8.5 g COD/L d (Run B), and 12.725 g COD/L d (Run C) respectively. The pH of the C-source was adjusted to 6.0–7.0, by addition of 3.0 M NaOH. Finally, the C-source was maintained at 4 °C in a refrigerated container for the whole period of operation. In the three runs, mass flows of both C- and N-sources were set in order to establish a C/N ratio of 33.4 g COD/g N (or 14.3 C-mol/N-mol) as the preferable value favoring of an increased PHA storage response, as reported previously [27]. The N-source solution was composed of $(NH_4)_2SO_4$ at a concentration of 3.66, 7.33, and 11.00 g/L, respectively for Runs A, B and C, which corresponded to a mass flow of 123.7, 247.4 and 371.1 g N/d. The reactor was controlled by digital timers connected to each peristaltic pump for flow rate management according to the cycle structure. Computer software was used to record the dissolved oxygen (DO) concentration and to detect the time required for C-source consumption (end of the feast phase) [27]. Volatile fatty acids (VFA), ammonia, PHA and suspended solids (SS) concentrations were monitored as previously described [27].

Batch accumulation tests

The storage performance of the biomass selected in Runs A and B was exploited by performing aerobic batch accumulation tests in a 0.5 L working volume reactor, at the same temperature and pH as the SBR. The reactor was mixed by magnetic stirrer and maintained under bubbling air (at DO concentration of 7.0–8.0 mg O_2/L). The Oxygen Uptake Rate (OUR) measurements were made over the course of each accumulation and before each was started, as previously described [13]. The tests were conducted under N-limiting conditions without ammonia addition. In this way the growth response was completely hindered. At the beginning of each test, a small volume of a synthetic solution at high VFA concentration (50 g COD/L) was added to reach an initial VFA/biomass ratio of approximately 2.0 g COD_{VFA}/g VSS [13]. The following VFA additions (each hour) were made in order to maintain an excess of C-source. The mixed liquor was sampled every hour to quantify VFA and PHA concentrations. The VSS concentration was determined at the beginning and at the end of each test.

Analytical methods

Ammonia and VFA quantifications were carried out after the filtration of the liquid samples through 0.45 μm porosity filters. Ammonia was quantified by the Nessler spectrophotometric method: the absorbance of reacted samples was measured at 420 nm wavelength (SHIMADZU Spectrophotometer UV-1800) [29]. The VFAs were measured after injection of 1.0 μL of filtered sample into a gas-chromatograph (Dani-Master, Milan, Italy) equipped with a flame ionization detector (FID). The concentrations of the single organic acids were converted into COD based on the oxidation stoichiometry as 1.067 g COD/g acetic acid and 1.51 g COD/g propionic acid.

Analytical determination of PHA was made on 5.0 mL of mixed liquor (without filtration). Each sample was immediately treated with 1.0 mL of a NaClO solution (5 % active Cl_2) in order to stop possible PHA microbial consumption, and then stored at −20 °C for the following analysis. Esterification into 3-hydroxyacyl methyl esters was necessary for the PHA determination by gas-chromatography (GC-FID Perkin Elmer 8410) [30]. The abundance of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) monomers was obtained using a commercial P(HB/HV) copolymer at 5 % w/w HV content (Sigma–Aldrich, Milan, Italy). The stoichiometry conversion numbers used in order to express PHA concentration in terms of COD were 1.67 g COD/g HB and 1.92 g COD/g HV.

Calculations

In the SBR, the amount of stored PHA (ΔPHA) was calculated as the difference between the maximum (end of feast) and minimum (end of cycle) PHA concentration. The non-polymer biomass or active biomass (X_A) was the difference between VSS and PHA (at the same cycle time): X_A = VSS - PHA. The specific PHA production rate was the ratio of the stored PHA to the feast phase length (t) per unit of X_A: qP^{feast} = ΔPHA/(t • X_A); both PHA and X_A were expressed on a COD basis. The specific substrate uptake rate was the ratio of VFA fed per cycle (ΔS) to the time required for its depletion (t, feast phase length) multiplied by X_A: $(-qS^{feast})$ = ΔS/(t • X_A); both VFA and X_A were expressed on a COD basis. The storage yield in the feast phase was the ratio between ΔPHA and ΔS: $Y_{P/S}^{feast}$ = ΔPHA/ΔS (COD basis). The observed yield was quantified at the end of the feast phase as the ratio between the VSS and the ΔS, as given by: Y_{OBS}^{SBR} = VSS/ΔS (COD basis). The polymer content in the biomass was the ratio between PHA and VSS concentration (at the same cycle time): %PHA = PHA/VSS = PHA/(X_A + PHA).

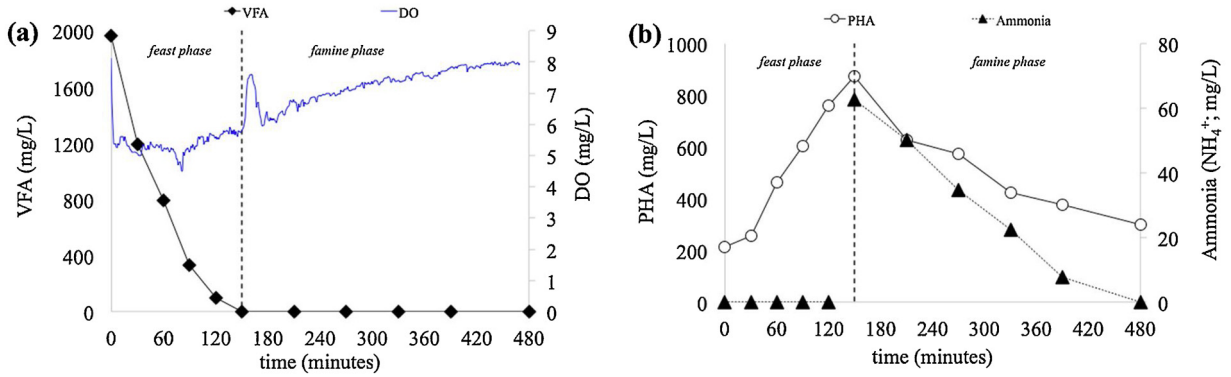

Fig. 1. Typical profiles of volatile fatty acids (VFA) and dissolved oxygen (DO) (a); ammonia and PHA (b) in a representative SBR cycle during Run A (OLR 4.25 g COD/L d).

In batch tests, the specific storage rate (qP^{batch}) was calculated by linear regression of the data versus time, by considering the period at constant production rate. This period lasted for a maximum of 4 h. The initial X_A concentration was taken into account, since growth response was considered negligible. The maximum polymer content in the biomass was also defined based on the PHA profiles; the storage yield ($Y_{P/S}^{batch}$) was calculated at the specific time that the maximum PHA content was achieved.

Results and discussion

Selection and enrichment of the mixed microbial culture in SBR: the effect of applied OLR

Three runs were carried out following the uncoupled C- and N-sources feeding strategy in order to maximize the storage yield in the feast phase and optimize the selective pressure on the culture. Therefore, the N-source was fed at the beginning of the famine (after complete VFA depletion) and the ammonia was used for the growth of PHA-storing organisms. The trend of the main parameters during a typical SBR cycle was reported in Figs. 1–3 for the three OLRs adopted. In both Figs. 1a and b, the profiles of VFA, dissolved oxygen (DO), PHA and ammonia in a typical cycle of Run A are shown. For as long as VFAs were available (feast phase), they were consumed along with oxygen; this led to a decrease of DO level (roughly from 8.0–5.0 mg/L). At the time of C-source depletion, the DO concentration showed a rapid inversion of its trend. Accordingly, PHA concentration reached its peak value (875 mg PHA/L), whereas the lowest concentration was measured at the end of the famine phase (190 mg PHA/L), as typically observed in a feast-famine regime [31]. Consumption of the PHA took place simultaneously with ammonia uptake, clearly suggesting the growth of the biomass on the stored PHA throughout the course of the famine. Similar trends were observed in the following Run B, where the applied OLR was duplicated compared to Run A. The VFA were removed in similar time (Fig. 2a), meaning that the selective pressure was efficiently maintained. The PHA concentration achieved higher value (2199 mg/L), according to the higher applied OLR. In the famine phase, ammonia was consumed concomitant with PHA uptake (Fig. 2b) and totally removed before the end of the cycle. This means that in the following feast phase, the selective pressure was optimal since the growth of non-PHA-storing organisms was prevented. The further rise in the OLR led to an increase of the feast phase length (Figs. 3a-b). Also in this run, ammonia was totally depleted before the end of the cycle. Hence, oxidation and storage were the two main mechanisms for the VFA uptake in the feast phase [13].

In the three runs, the storage yield ($Y_{P/S}^{feast}$) was high enough to consider the applied OLR range as technically feasible for the culture selection. Runs A and B exhibited the higher $Y_{P/S}^{feast}$ values (0.56 ± 0.02 and 0.57 ± 0.02 COD/COD respectively); in Run C (12.725 g COD/L d), the $Y_{P/S}^{feast}$ decreased to 0.53 ± 0.03 COD/COD. A maximal OLR of 40.8 g COD/L d is reported in the literature [32]; however, it has been demonstrated that the OLR increase may cause a progressive loss of selective pressure [31]. If this consideration is true for the coupled C- and N-source supply, it must still be demonstrated for

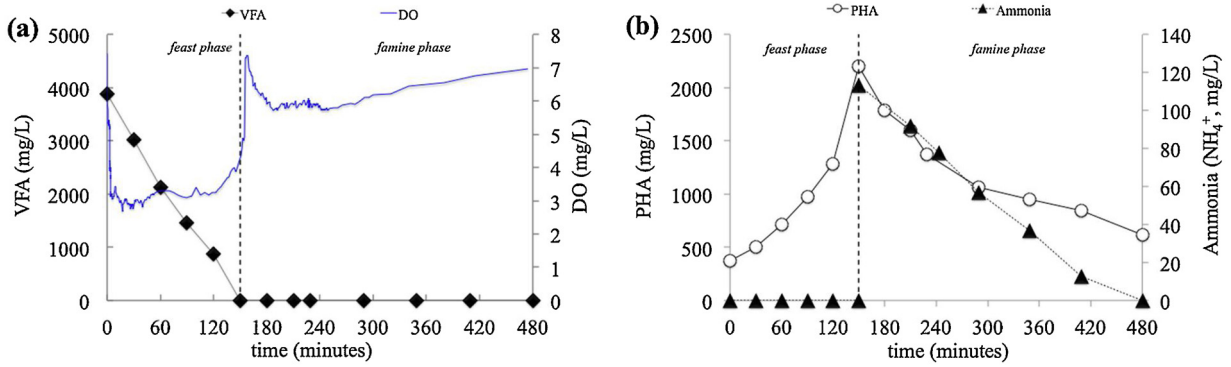

Fig. 2. Typical profiles of volatile fatty acids (VFA) and dissolved oxygen (DO) (a); ammonia and PHA (b) in a representative SBR cycle during Run B (OLR 8.5 g COD/L d).

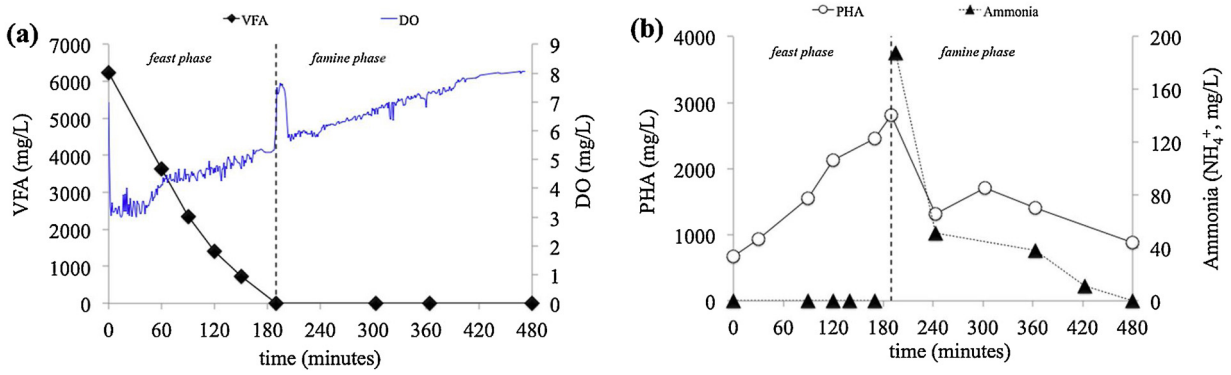

Fig. 3. Typical profiles of volatile fatty acids (VFA) and dissolved oxygen (DO) (a); ammonia and PHA (b) in a representative SBR cycle during Run C (OLR 12.725 g COD/L d).

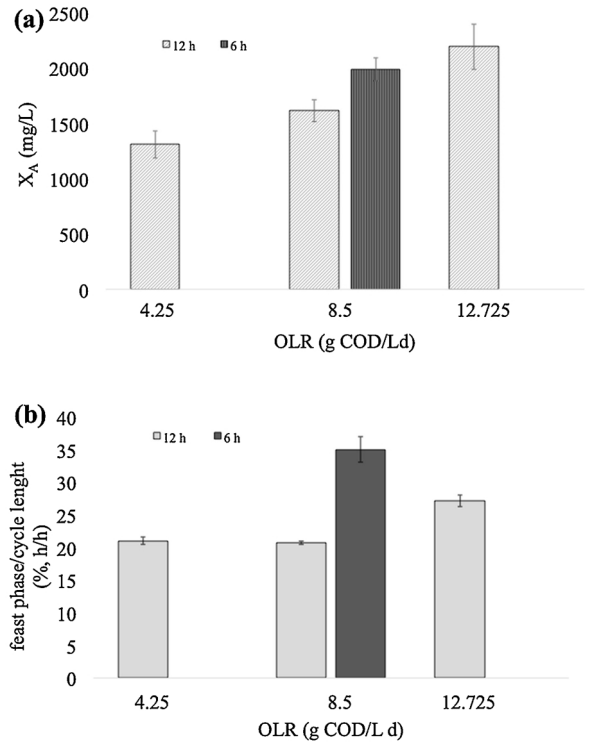

Fig. 4. Active biomass concentration (X_A) (a) and feast phase/cycle length ratio (b) as a function of applied OLR and overall cycle length (12 h and 6 h).

the uncoupled feeding strategy. In this study, the progressive increase in OLR led to an almost linear increase in the active biomass (X_A) concentration from 1308 ± 122–2191 ± 205 mg/L (Fig. 4a). If this assumption appears obvious, the different feast phase length of Run C compared to Runs A and B is not. As shown in Fig. 4b, the feast phase/cycle length ratio (h/h) in Run C was 27.1 ± 0.9 %, higher than the values measured in Run A (21.0 ± 0.6 %) or B (20.7 ± 0.3 %). Even though the storage response was observed in the three runs, the MMC selection process suffered the increase of OLR from 8.5–12.725 g COD/L d. The specific substrate uptake rate ($-qS^{feast}$) rose from 641 ± 29–835 ± 31 mg COD_{VFA}/g COD_{Xa}/h as OLR increased up to 8.5 g COD/L d; the further increase in OLR caused a drop to 698 ± 65 mg COD_{VFA}/g COD_{Xa}/h. Hence, the biomass produced in Run C was kinetically slower as also indicated by the rate of PHA synthesis in the feast phase (qP^{feast}). In fact, qP^{feast} increased from 360 ± 21–481 ± 22 mg COD_{PHA}/g COD_{Xa}/h as OLR doubled from 4.25 to 8.5 g COD/L d, but it fell to 373 ± 43 mg COD_{PHA}/g COD_{Xa}/h at the maximum OLR investigated. Substrate inhibition phenomena may be excluded as explanation. In fact, at the end of C-source feeding phase, the relative substrate/biomass (S/X) ratio in Run C was close to 2.0 COD_{VFA}/COD_{Xa}, as observed in Run B. Hence, some alterations to cellular metabolism more likely occurred. The difference in polymer composition could confirm this hypothesis. Literature studies have shown how the modification of the C-source composition [33] or a change in the pH of the process [34,35] may impact on the HV content in the stored PHA. The uncoupled N-source feeding (compared to coupled C-N feeding) has also a significant effect on the microorganisms' metabolism [27]. In this study, despite the use of the same VFA mixture and uncoupled N-feeding, a polymer with a smaller percentage of HV monomers (0.14 ± 0.02 %, g HV/g PHA) was synthesized at the highest OLR investigated (Run C). In Run A and B, HV content was similar and equal to 0.25 ± 0.01 and 0.24 ± 0.01 % respectively.

Comparison with literature examples of the uncoupled N-source feeding

A wide margin in the understanding of process optimization and metabolism under uncoupled C- and N-sources feeding exists since this approach has been poorly investigated so far. Others have [26] assessed the conversion of 1,3-propanediol (1,3-PDO; produced from crude glycerol) into PHA: the authors assessed a yield of 0.24 C_{mol} PHA/C_{mol} 1,3-PDO only when limitation of N was applied. Hence, the uncoupled feeding strategy was required for PHA synthesis, since no net PHA production was quantified in a parallel process operating with simultaneous C-N supply. A similar comparison was performed in [28] by using fermented cheese whey (fCW) as substrate: the uncoupled feeding regime led to a remarkable increase of the storage response, which was quantified by a storage yield ($Y_{P/S}^{feast}$) of 0.72 C-mol_{PHA}/C-mol_S and rate (qP^{feast}) of 0.24 C-mol_{PHA}/C-mol_{Xa}/h; much higher than the respective values obtained with the coupled feeding regime.

Using a synthetic VFA mixture, feeding with coupled and uncoupled C- and N-sources was compared in another study, where a lab-scale SBR was set for MMC selection at a fixed OLR (8.5 g COD/L d) [27]. A doubled PHA production was quantified when C and N were fed separately. An optimal C/N ratio was defined at 33.4 g COD/g N, as used in the present work. The increased storage response compared to the traditional coupled feeding approach was explained by the lower growth rate of non-PHA-storing microorganisms during the feast phase. In the present study, the C/N ratio was kept fixed for all the SBR runs (33.4 g COD/g N), as well as the overall cycle length (12 h). The latter

Table 1
Main parameters with average data and standard deviations monitored and quantified in the SBR runs.

Parameters	This study			References						
	Run A	Run B	Run C	[27]	[28]	[28]	[13]	[13]	[24]	[24]
OLR (gCOD/L d)	4.25	8.5	12.725	8.5	8.5	8.5	8.5	8.5	4.7	6.0-8.4
C-source (VFA-rich stream)	VFA mixture (synth.)*			VFA mixture (synth.)*	fCW		VFA mixture (synth.)*		fOMW	
C-N feeding	uncoupled			uncoupled	uncoupled	coupled	coupled		coupled	
Cycle length (h)	12			6	12		6	2	6	
Feast phase/cycle length ratio (h/h, %)	21.0 ± 0.6	20.7 ± 0.3	27.1 ± 0.9	35 ± 2	20-60	20-80	20.0 ± 0.7	19.7 ± 0.5	5.6 ± 0.3	22-25
Active biomass (end of feast; mgX_A/L)	1308 ± 122	1614 ± 101	2191 ± 205	1940 ± 105	3300 ± 100	1300 ± 200	2050 ± 49	2181 ± 73	–	–
PHA concentration (end of cycle; mg/L)	235 ± 23	373 ± 30	658 ± 61	–	–	–	139 ± 13	73 ± 4	–	–
PHA concentration (end of feast; mg/L)	807 ± 58	1639 ± 40	2389 ± 145	–	–	–	523 ± 18	220 ± 8	–	–
PHA content (end of feast; gPHA/gVSS)	0.40 ± 0.02	0.52 ± 0.02	0.53 ± 0.03	0.28 ± 0.02	0.50	0.30	0.18 ± 0.05	0.09 ± 0.01	–	–
Storage Yield ($Y_{P/S}^{feast}$; COD/COD)	0.56 ± 0.02	0.57 ± 0.02	0.53 ± 0.03	–	0.72 ± 0.08 C-molP/C-molS	0.39 ± 0.06 C-molP/C-molS	0.26 ± 0.01	0.32 ± 0.01	0.56 ± 0.05	0.20-0.40
Observed Yield (Y_{OBS}^{SBR}; COD/COD)	0.63 ± 0.03	0.60 ± 0.01	0.57 ± 0.02	–	–	–	0.43 ± 0.01	0.41 ± 0.01	–	–
HV content (end of feast; gHV/gPHA)	0.25 ± 0.01	0.24 ± 0.01	0.14 ± 0.02	0.12 ± 0.03	–	–	–	–	–	–
Specific Substrate Uptake Rate (-qS^{feast}; mgCOD/gCOD$_{Xa}$/h)	641 ± 29	835 ± 21	698 ± 65	–	–	–	628 ± 45	648 ± 62	–	–
Specific Storage Rate (qP^{feast}; mgCOD$_P$/gCOD$_{Xa}$/h)	360 ± 21	481 ± 22	373 ± 43	–	0.24 ± 0.001 C-molP/C-molX/h	0.07-0.14 C-molP/C-molX/h	164 ± 10	190 ± 7	339 ± 48	25-60
PHA productivity (gPHA/L d; 2nd stage)	0.81 ± 0.05	1.64 ± 0.04	2.4 ± 0.1	0.78 ± 0.04	–	–	0.44 ± 0.01	0.18 ± 0.01	–	–

* synthetic VFA mixture.

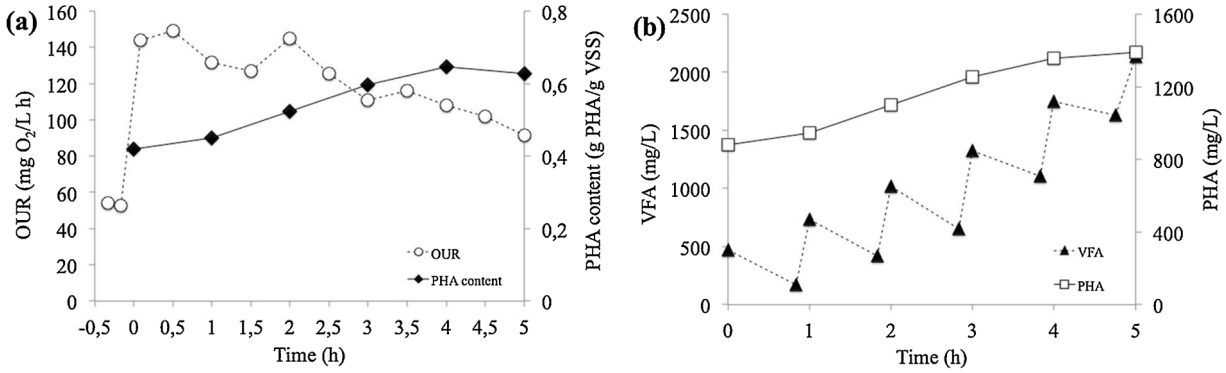

Fig. 5. Typical trends of the oxygen uptake rate (OUR) and PHA biomass content (a); volatile fatty acids (VFA) and PHA (b) in a representative accumulation trial conducted with biomass acclimated in Run A.

was twice that in [27] and this difference was particularly relevant for Run B (this study). Here, a longer cycle length was demonstrated to be more effective on the selective pressure and there was no residual ammonia at the end of the cycle. Hence, microorganisms that did not accumulate PHA in the feast faced more critical survival conditions. In consequence, the $Y_{P/S}^{feast}$ obtained in Run B (0.57 ± 0.02 COD/COD) was approximately 30 % higher than $Y_{P/S}^{feast}$ (close to 0.40 COD/COD) measured with lower cycle length [27]. In addition, higher cycle length caused a lower biomass concentration in the feast, allowing bacteria with higher specific uptake rate to thrive at the same oxygen transfer rate. Fig. 4 demonstrates this statement. As indicated in Fig. 4a, the two cases at similar OLR (8.5 g COD/L d) showed different X_A concentrations at the end of the feast: 1614 ± 101 and 1940 ± 105 mg X_A/L at cycle lengths of 12 h and 6 h respectively. Despite the higher biomass level, the length of the feast phase was 40 % greater: 20.7 ± 0.3 % h/h and 35 ± 2 % at cycle lengths of 12 h and 6 h respectively (Fig. 4b). Thus, a longer cycle length selected for a biomass with higher specific rates (for both VFA uptake and PHA synthesis) and storage yield.

Table 1 summarizes all the main parameters monitored over the course of the three SBR runs performed. Other data from previous reports have been also taken into account. Most concern MMC selection at relatively high OLR. In [28], the OLR and cycle length were similar to those of Run B; at the end of feast in the uncoupled feeding regime, the biomass accumulated up to 0.50 g PHA/g VSS, as obtained here. The much higher X_A concentration achieved (3300 mg/L) was particularly relevant in the perspective of the global polymer productivity. The higher X_A concentration was probably due to the choice of operating at higher SRT (4 d), which was not equal to the HRT (1 d), as here. In previous work, where the effect of the cycle length on the storage performances was investigated, a better PHA-accumulating biomass was selected with shorter cycle length (2 − 6 h) [13]. This useful knowledge in the fundamentals of MMC processes was related to the coupled feeding strategy and contrasts with the findings of this study, extensively explained in comparison to [27]. Moreover, even with better selection, the storage kinetic exhibited by the biomass cultivated under coupled feeding regime was considerably lower (sometimes < 50 %) compared to the specific rates in this work (164–190 vs 360 − 481 mg COD_{PHA}/g COD_{Xa}/h respectively). Instead, a similar rate (339 mg COD_{PHA}/g COD_{Xa}/h) and yield (0.56 COD/COD) were obtained in another study, where fermented olive oil mill wastewater (fOMW) was used as feedstock under coupled C-N feeding [24]. However, the OLR applied was much lower (4.7 g COD/L d) since the process suffered under higher OLR due to a possible inhibitory effect of fOMW on microbial activity. Even though lower OLRs facilitate MMC selection under a feast-famine regime, high OLRs are preferable since they allow higher cellular density to be obtained [24], which in turn positively affects polymer productivity [32]. In a following report [33], OLR was increased up to 7.0 g COD/L d using fOMW dosed with uncoupled N-C feeding strategy. The storage response was substantially enhanced and quantified as specific storage rate, equal to 496 mg COD_{PHA}/g COD_{Xa}/h, one of the highest reported.

Exploring biomass accumulation potential in the batch tests

In the most often applied three-stage scheme, the third stage is devoted to the increase of intracellular polymer content [31]. A large number of examples show that longer feast phase length in the SBR cycles causes lower performances in terms of PHA accumulation capacity [13,32,36,37]. Starting from this basic information, the batch accumulations here were performed with biomass produced over the course of Runs A and B, where the selective pressure on PHA-storing microorganism was more efficient. Fig. 5 shows the OUR and PHA biomass content (a), the VFA and PHA (b) in a representative example of accumulation test performed with biomass selected in Run A. The OUR increased after the first VFA addition and then exhibited a slowly decreasing trend until the end of the test, concurrent to the increase of the biomass PHA content (Fig. 5a). The evolution of PHA concentration reflected the trend of PHA content, since no cellular growth was allowed (Fig. 5b). The increase of PHA concentration was almost linear for up to 4 h, as well as the constant rate of VFA consumption. In the last hour of the test, microbial activity dropped remarkably since the polymer achieved a considerable intracellular content (0.64 g PHA/g VSS). Table 2 summarizes the parameters describing the biomass response in the batch tests (mean values and SD). Even though both biomasses were characterized by similar storage yields in the SBR, the specific storage rate (qP^{batch}) of biomass acclimated in Run A was 20 % higher than the Run B biomass: 302 ± 43 vs 241 ± 24 mg COD_{PHA}/g COD_{Xa} h respectively. This difference may be explained by the initial levels of PHA biomass content, which were closer to saturation for Run B biomass (0.52 ± 0.02, Table 1), slowing down the microorganisms' storage response. Essentially, all the batch accumulations performed with biomass selected in Run B may be considered productive up to the 3rd hour, since longer times did not correspond to higher PHA biomass content or PHA production. The biomass selected in Run A expressed its storage response for a longer time (up to 5 h), even though its accumulation potential was lower, for both yield and maximum PHA content (0.63 ± 0.03 COD_{PHA}/COD and 0.66 ± 0.02 g PHA/g VSS respectively), when compared to both parameters obtained with Run B biomass (0.74 ± 0.03 COD_{PHA}/COD and 0.70 ± 0.02 g PHA/g VSS respectively). Regarding the polymer composition, the two biomasses produced a polymer with similar composition (HV content around 30 % g HV/g PHA).

Table 2
Main parameters with average data and standard deviations monitored and quantified in the accumulation tests.

Parameters	This study		References							
	Run A	Run B	[13]	[24]	[24]	[28]	[28]	[36]	[36]	[42]
OLR (gCOD/L d)**	4.25	8.5	8.5	4.7	6.0-8.4	8.5	8.5	8.5		7.0
C-source (VFA-rich stream)	VFA mixture (synth.)*		VFA mixture (synth.)*	fOMW		fCW		VFA mixture (synth.)*		fOMW
C-N feeding**	uncoupled		coupled	coupled		uncoupled	coupled	coupled		coupled
Maximum PHA content (gPHA/gVSS)	0.63 ± 0.02	0.70 ± 0.03	0.45-0.46	0.32 ± 0.05	0.19-0.20	–	–	0.38-0.52		–
Specific Storage Rate (qPbatch; mgCODP/gCODXa/h)	302 ± 43	241 ± 24	196-216	125 ± 15	50-65	0.40 ± 0.03 C-molP/C-molX/h	0.25 ± 0.01 C-molP/C-molX/h	175-330		–
Storage Yield (YP/Sbatch; COD/COD)	0.66 ± 0.03	0.74 ± 0.02	0.45-0.53	–	–	0.96 ± 0.07 C-molP/C-molS	0.86 ± 0.07 C-molP/C-molS	0.26-0.48		–
HV content (gHV/gPHA)	0.29 ± 0.007	0.30 ± 0.02	–	0.10 ± 0.01	0.06 ± 0.01	–	–	–		0.19-0.22
PHA productivity (gPHA/L d; 2nd and 3rd stage)	2.24 ± 0.06	2.89 ± 0.05	1.25-1.70	1.50 ± 0.01	1.00-1.35	6.09	2.55	0.85		1.05

* synthetic VFA mixture.
** related to the biomass cultivation step (SBR).

Fig. 6. Volumetric PHA productivity of the 2nd stage and 2nd + 3rd stages as a function of the applied OLR.

Even though the literature is rich in examples where the effect of nutrient level over the storage response stimulation is explained in detail [38,39], there are no data reporting how much polymer a biomass selected under C/N uncoupled feeding can accumulate. It is reasonable to suppose that 0.70 g PHA/g VSS achieved in this study represents an approximate threshold of polymer saturation (at least for high rate process). The comparison with [28], where the biomass was cultivated under similar conditions, would be not appropriate. In the short-time accumulations (up to 3.5 h) shown in [28], the biomass was probably unable to achieve saturation level (VFAs completely exhausted) and the PHA content was slightly > 0.30 g PHA/g VSS. However, a higher level was expected due the reported high storage yield ($Y_{P/S}^{batch}$ equal to 0.96 C-molP/C-molS). In previous high rate processes carried out at similar OLR with coupled C-N feeding strategy [13,24], the selected biomass often exhibited lower storage performance, in terms of yield ($Y_{P/S}^{batch}$; 0.45-0.53 COD/COD), rate (qP^{batch}; 50 − 216 mg COD_{PHA}/g COD_{Xa}/h) and polymer content (0.19-0.46 g PHA/g VSS) (Table 2). Other previous studies reported even higher PHA biomass content (0.83-0.89 g PHA/g VSS) with no necessity to operate under the uncoupled feeding strategy [40,41]. In these cases, the culture was highly enriched in *Plasticicumulans acidivorans* by operating the SBR at short SRT (1 day) and relatively high temperature (30 °C). The very short feast phase reported (7 − 50 min; 1.0–6.9 %) suggests a low applied OLR, far from those investigated in this study. This approach may facilitate the establishment of an efficient selective pressure. On the other hand, biomass productivity is negatively affected and, in turn, PHA productivity may be strongly limited [28,32].

Volumetric PHA process productivity

The performance of a PHA-accumulation process has often been related to the final PHA content in the biomass, which was required to be as high as possible to improve the economy in the downstream processing [40,42]. However, in order to consider the technology technically and economically viable, other important parameters must be taken into account such as the overall PHA storage yield and PHA productivity (g PHA/L d) [31].

PHA productivity was first evaluated by only considering the effluent from the 2nd step, when the PHA content in the mixed liquor was harvested at the end of the feast. Overall productivity, including the 2nd and 3rd stages, was then calculated by considering the PHA concentration at its maximum intracellular content. The increase of OLR in the SBR led to an expected increase in biomass concentration, as deduced from Fig. 4a. Biomass concentration strongly affected PHA productivity [16], which increased linearly from 0.81 ± 0.05–2.4 ± 0.1 g PHA/L d (Fig. 6). The lowest storage response of Run C biomass was

more than counterbalanced by the higher C-source amount fed per cycle. This was also observed in a previous report [13] where the highest productivity (0.53 g PHA/L d) was obtained at the highest C-source load per cycle. However, the process was not considered sustainable without the accumulation stage, due to the low PHA content at the end of the feast phase (< 0.20 g PHA/g VSS). In Run C the accumulation stage may not be strictly necessary since the PHA biomass content achieved at the end of the feast was > 0.50 g PHA/g VSS. The overall PHA productivity was calculated for Runs A and B, where the accumulation tests were performed with a better-selected biomass. In Run A, productivity from the 2nd and 3rd stages increased almost 3-fold (2.24 ± 0.06 g PHA/L d), but still remained lower when compared to the 2nd stage productivity of Run C. In Run B, the increase in productivity after the accumulation step was 1.8-fold, up to 2.89 ± 0.05 g PHA/L d.

This value was similar to that reached in previous work (2.81 g PHA/L d; [32]) where higher OLR was applied (25.5 g COD/L d). In that case the final biomass content was considerably lower (< 0.25 g PHA/g VSS) since the biomass storage response was not efficiently enhanced by coupled C-N feeding. Other studies developed with the same three-stage high rate process and coupled feeding strategy achieved lower PHA productivity (Table 2). Hence, this work demonstrated the possibility of maintaining high PHA productivity with similarly high PHA biomass content (up to 0.70 g PHA/g VSS). Compared to Run B, the study reported in [28] is particularly relevant. The authors confirmed that the uncoupled C-N feeding regime led to higher PHA productivity than that achieved with coupled feeding. In addition, a high global productivity of 6.09 g PHA/L d was quantified. This was probably ensured by the X_A level achieved in the second stage (e.g. biomass productivity), which was approximately twice that of Run B (3300 vs 1614 mg/L). This difference was not the result of the OLR (8.5 g COD/L d in both cases), but more likely related to the 4-fold higher SRT, maintained equal to 4 d with an appropriately short HRT (1 d). Hence, adjustment of certain process-related parameters may substantially affect polymer productivity, without interfering with the selective pressure efficiency.

Conclusions

This work has highlighted the possibility of significantly enhancing PHA production in an SBR operating with uncoupled C and N feeding by determining the optimal operating conditions. The OLR applied has been shown to have a significant impact on the aerobic MMC selection/enrichment, and, in consequence, on storage performances and productivity. An ideal selective pressure was maintained at up to 8.5 g COD/L d, and partially reduced at higher OLR. The relatively high PHA content achieved at the end of the feast phase (0.40 - 0.53 g PHA/g VSS) indicates the option of simplifying the process by withdrawing the biomass, at its maximum PHA content, from the SBR to downstream processing, with no need for the intermediate accumulation step. Perspectives for industrial technology competitiveness indicate that a PHA content slightly above 0.40 g PHA/g VSS is commercially viable [43] with a sustainable method for PHA extraction. PHA productivity could also be considerably increased after the 3rd stage, as well as the PHA content (2.89 g PHA/L d and 0.70 g PHA/g VSS, respectively), if relatively medium-high OLRs are applied (up to 8.5 g COD/L d). OLRs > 12.725 g COD/L d, together with higher SRT (freed from HRT) may be a possible solution for a further increase of PHA productivity, provided that an efficient selective pressure is maintained. Hence, a wide range of possibilities still exists, and, for process viability, each choice requires evaluation with respect to a specific N-deficient feedstock and economic analysis for the integration of MMC technology into a value chain feedstock valorization. The results obtained in this study also suggest that PHA properties (e.g. HV content) can be tuned by means of a shift in OLR, in spite of using the same C source.

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